Current Protocols in Cell Biology

Online ISBN: 9780471143031
DOI: 10.1002/0471143030

Table of Contents

32. Unit 3.30 Purification of Intact Chloroplasts from Arabidopsis and Spinach Leaves by Isopycnic Centrifugation
33. Unit 3.31 Isolation of Neuromelanin Granules
34. Unit 3.32 Isolation of Dense Core Secretory Vesicles from Pancreatic Endocrine Cells by Differential and Density Gradient Centrifugation
35. Unit 3.33 Isolation and Biochemical Characterization of Amyloid Plaques and Paired Helical Filaments
36. Unit 3.34 Isolation of Legionella-Containing Vacuoles by Immuno-Magnetic Separation
37. Unit 3.35 Isolation of Platelet Granules
38. Unit 3.36 Isolation of Nucleoli
39. Unit 3.37 Isolation of Cytotoxic T Cell and NK Granules and Purification of Their Effector Proteins
40. Unit 3.38 Isolation of Aggresomes and Other Large Aggregates
41. Unit 3.39 Isolation of Chromaffin Granules
42. Unit 3.40 Purification of Ribosomes from Human Cell Lines

6. Chapter 4 Microscopy

1. Introduction
2. Unit 4.1 Proper Alignment and Adjustment of the Light Microscope
3. Unit 4.2 Fluorescence Microscopy
4. Unit 4.3 Immunofluorescence Staining
5. Unit 4.4 Fluorescent Staining of Subcellular Organelles: ER, Golgi Complex, and Mitochondria
6. Unit 4.5 Basic Confocal Microscopy
7. Unit 4.6 Immunoperoxidase Methods for Localization of Antigens in Cultured Cells and Tissues
8. Unit 4.7 Cryo-Immunogold Electron Microscopy
9. Unit 4.8 Correlative Video Light/Electron Microscopy
10. Unit 4.9 Polarization Microscopy
11. Unit 4.10 Fluorescent Speckle Microscopy (FSM) of Microtubules and Actin in Living Cells
12. Unit 4.11 Two-Photon Excitation Microscopy for the Study of Living Cells and Tissues
13. Unit 4.12 Total Internal Reflection Fluorescence Microscopy for High-Resolution Imaging of Cell-Surface Events
14. Unit 4.13 Fluorescent Labeling of Yeast
15. Unit 4.14 Fluorescence Lifetime Imaging Microscopy
16. Unit 4.15 Biological Second and Third Harmonic Generation Microscopy
17. Unit 4.16 Analyzing Real-Time Video Microscopy: The Dynamics and Geometry of Vesicles and Tubules in Endocytosis
18. Unit 4.17 Scanning Electron Microscopy of Cell Surface Morphology
19. Unit 4.18 Fluorescence Imaging Techniques for Studying Drosophila Embryo Development
20. Unit 4.19 Quantitative Colocalization Analysis of Confocal Fluorescence Microscopy Images
21. Unit 4.20 Visualizing Protease Activity in Living Cells: From Two Dimensions to Four Dimensions
22. Unit 4.21 Photoactivated Localization Microscopy (PALM) of Adhesion Complexes
23. Unit 4.22 Culturing MDCK Cells in Three Dimensions for Analyzing Intracellular Dynamics
24. Unit 4.23 Interference Reflection Microscopy
25. Unit 4.24 Fluorescence Correlation Spectroscopy in Living Cells: A Practical Approach
26. Unit 4.25 Analysis of Mitochondrial Dynamics and Functions Using Imaging Approaches
27. Unit 4A Organelle Atlas: Appendix to Chapter 4

7. Chapter 5 Characterization of Cellular Proteins

1. Introduction
2. Unit 5.1 Overview of the Physical State of Proteins Within Cells
3. Unit 5.2 Determining the Topology of an Integral Membrane Protein
4. Unit 5.3 Determination of Molecular Size by Zonal Sedimentation Analysis on Sucrose Density Gradients
5. Unit 5.4 Analysis of the Association of Proteins with Membranes
6. Unit 5.5 Determination of Molecular Size by Size-Exclusion Chromatography (Gel Filtration)
7. Unit 5.6 Identification of Proteins in Complex Mixtures Using Liquid Chromatography and Mass Spectrometry
8. Unit 5.7 Determining Membrane Protein Topologies in Single Cells and High-Throughput Screening Applications

8. Chapter 6 Electrophoresis and Immunoblotting

1. Introduction
2. Unit 6.1 One-Dimensional SDS Gel Electrophoresis of Proteins
3. Unit 6.2 Immunoblotting and Immunodetection
4. Unit 6.3 Detection and Quantitation of Radiolabeled Proteins in Gels and Blots
5. Unit 6.4 Two-Dimensional Gel Electrophoresis
6. Unit 6.5 One-Dimensional Electrophoresis Using Nondenaturing Conditions
7. Unit 6.6 Staining Proteins in Gels
8. Unit 6.7 Agarose Gel Electrophoresis of Proteins

9. Unit 6.8 Fluorescence Detection of Glycoproteins in Gels and on Electroblots
10. Unit 6.9 Digital Electrophoresis Analysis
11. Unit 6.10 Two-Dimensional Blue Native Polyacrylamide Gel Electrophoresis
12. Unit 6.11 Measurement of Oxidatively-Induced Clustered DNA Lesions Using a Novel Adaptation of Single Cell Gel Electrophoresis (Comet Assay)

9. Chapter 7 Protein Labeling and Immunoprecipitation

1. Introduction
2. Unit 7.1 Metabolic Labeling with Amino Acids
3. Unit 7.2 Immunoprecipitation
4. Unit 7.3 Metabolic Labeling with Sulfate
5. Unit 7.4 Metabolic Labeling with Fatty Acids
6. Unit 7.5 Metabolic Labeling of Prenyl and Carboxyl-Methyl Groups
7. Unit 7.6 Metabolic Labeling and Immunoprecipitation of Yeast Proteins
8. Unit 7.7 Metabolic Labeling and Immunoprecipitation of Drosophila Proteins
9. Unit 7.8 Metabolic Labeling of Glycoproteins with Radioactive Sugars
10. Unit 7.9 Analysis of Oxidative Modification of Proteins
11. Unit 7.10 Radioiodination of Cellular Proteins

10. Chapter 8 Cell Cycle Analysis

1. Introduction
2. Unit 8.1 Overview of the Cell Cycle
3. Unit 8.2 Assays for CDK Activity and DNA Replication in the Cell Cycle
4. Unit 8.3 Methods for Synchronizing Cells at Specific Stages of the Cell Cycle
5. Unit 8.4 Determining Cell Cycle Stages by Flow Cytometry
6. Unit 8.5 Centrifugal Elutriation to Obtain Synchronous Populations of Cells
7. Unit 8.6 Dynamic Proliferation Assessment in Flow Cytometry

11. Chapter 9 Cell Adhesion

1. Introduction
2. Unit 9.1 Cell-Substrate Adhesion Assays
3. Unit 9.2 Quantitative Measurement of Cell Adhesion Using Centrifugal Force
4. Unit 9.3 Cadherin-Dependent Cell-Cell Adhesion
5. Unit 9.4 Analyzing Integrin-Dependent Adhesion
6. Unit 9.5 Analysis of Cell-Cell Contact Mediated by Ig Superfamily Cell Adhesion Molecules
7. Unit 9.6 Measurement of Adhesion Under Flow Conditions

12. Chapter 10 Extracellular Matrix

1. Introduction
2. Unit 10.1 Overview of Extracellular Matrix
3. Unit 10.2 Preparation of Basement Membrane Components from EHS Tumors
4. Unit 10.3 Preparation of Gelled Substrates
5. Unit 10.4 Preparation of Extracellular Matrices Produced by Cultured Corneal Endothelial and PF-HR9 Endodermal Cells
6. Unit 10.5 Purification of Fibronectin
7. Unit 10.6 Purification of Vitronectin
8. Unit 10.7 Proteoglycan Isolation and Analysis
9. Unit 10.8 Matrix Metalloproteinases
10. Unit 10.9 Preparation of Extracellular Matrices Produced by Cultured and Primary Fibroblasts
11. Unit 10.10 Purification and Analysis of Thrombospondin-1
12. Unit 10.11 Purification of SPARC/Osteonectin
13. Unit 10.12 Analysis of Fibronectin Matrix Assembly
14. Unit 10.13 Non-Radioactive Quantification of Fibronectin Matrix Assembly
15. Unit 10.14 Use of Hyaluronan-Derived Hydrogels for Three-Dimensional Cell Culture and Tumor Xenografts
16. Unit 10.15 Generation of Micropatterned Substrates Using Micro Photopatterning
17. Unit 10.16 Preparation of Hydrogel Substrates with Tunable Mechanical Properties
18. Unit 10.17 Engineering Three-Dimensional Collagen Matrices to Provide Contact Guidance during 3D Cell Migration
19. Unit 10.18 Imaging Cells in Three-Dimensional Collagen Matrix

13. Chapter 11 In Vitro Reconstitution

1. Introduction
2. Unit 11.1 Overview of Eukaryotic In Vitro Translation and Expression Systems
3. Unit 11.2 In Vitro Translation
4. Unit 11.3 In Vitro Analysis of Endoplasmic-Reticulum-to-Golgi Transport in Mammalian Cells

5. Unit 11.4 Cotranslational Translocation of Proteins into Canine Rough Microsomes
6. Unit 11.5 In Vitro Analysis of SV40 DNA Replication
7. Unit 11.6 In Vitro Transcription
8. Unit 11.7 Nuclear Import in Digitonin-Permeabilized Cells
9. Unit 11.8 In Vitro Translation Using HeLa Extract
10. Unit 11.9 Analysis of Eukaryotic Translation in Purified and Semipurified Systems
11. Unit 11.10 Preparation and Use of Interphase Xenopus Egg Extracts
12. Unit 11.11 Analysis of the Cell Cycle Using Xenopus Egg Extracts
13. Unit 11.12 Analysis of Apoptosis Using Xenopus Egg Extracts
14. Unit 11.13 Mitotic Spindle Assembly In Vitro
15. Unit 11.14 Analysis of RNA Export Using Xenopus Oocytes
16. Unit 11.15 In Vitro Analysis of Peroxisomal Protein Import
17. Unit 11.16 In Vitro Analysis of Chloroplast Protein Import
18. Unit 11.17 In Vitro RNA Splicing in Mammalian Cell Extracts
19. Unit 11.18 Endocytosis Assays in Intact and Permeabilized Cells
20. Unit 11.19 In Vitro Analysis of Yeast Mitochondrial Protein Import

14. Chapter 12 Cell Motility

1. Introduction
2. Unit 12.1 Chemotaxis Assays for Eukaryotic Cells
3. Unit 12.2 Invasion Assays
4. Unit 12.3 Cell Traction
5. Unit 12.4 Cell Wound Assays
6. Unit 12.5 Dictyostelium Cell Dynamics
7. Unit 12.6 Optical Microscopy–Based Migration Assay for Human Neutrophils
8. Unit 12.7 Actin-Based Motility Assay
9. Unit 12.8 In Vivo Marking of Single Cells in Chick Embryos Using Photoactivation of GFP

15. Chapter 13 Organelle Motility

1. Introduction
2. Unit 13.1 Microtubule/Organelle Motility Assays
3. Unit 13.2 In Vitro Motility Assay to Study Translocation of Actin by Myosin
4. Unit 13.3 Organelle Motility in Plant Cells: Imaging Golgi and ER Dynamics with GFP
5. Unit 13.4 Movement of Nuclei
6. Unit 13.5 Measuring Dynamics of Nuclear Proteins by Photobleaching
7. Unit 13.6 Functional Characterization of Proteins Regulating Actin Assembly

16. Chapter 14 Signal Transduction: Protein Phosphorylation

1. Introduction
2. Unit 14.1 Overview of Protein Phosphorylation
3. Unit 14.2 Immunological Detection of Phosphorylation
4. Unit 14.3 The Detection of MAPK Signaling
5. Unit 14.4 Labeling Cultured Cells with 32Pi and Preparing Cell Lysates for Immunoprecipitation
6. Unit 14.5 Phosphoamino Acid Analysis
7. Unit 14.6 Determination of Akt/PKB Signaling
8. Unit 14.7 Analyzing FAK and Pyk2 in Early Integrin Signaling Events
9. Unit 14.8 Rho GTPase Activation Assays
10. Unit 14.9 In Vitro GEF and GAP Assays
11. Unit 14.10 In Vivo Imaging of Signal Transduction Cascades with Probes Based on Förster Resonance Energy Transfer (FRET)
12. Unit 14.11 Biosensors for Characterizing the Dynamics of Rho Family GTPases in Living Cells
13. Unit 14.12 Analysis of Arf GTP-Binding Protein Function in Cells

17. Chapter 15 Protein Trafficking

1. Introduction
2. Unit 15.1 Overview of Protein Trafficking in the Secretory and Endocytic Pathways
3. Unit 15.2 Use of Glycosidases to Study Protein Trafficking
4. Unit 15.3 Endocytosis: Biochemical Analyses
5. Unit 15.4 Determining Protein Transport to the Plasma Membrane
6. Unit 15.5 Analysis of Membrane Traffic in Polarized Epithelial Cells
7. Unit 15.6 Analysis of Protein Folding and Oxidation in the Endoplasmic Reticulum
8. Unit 15.7 Measurements of Phagocytosis and Phagosomal Maturation
9. Unit 15.8 Analysis of Protein Transport to Lysosomes
10. Unit 15.9 Studies of the Ubiquitin Proteasome System
11. Unit 15.10 Measuring Retrograde Transport to the Trans-Golgi Network
12. Unit 15.11 Assays for Regulated Exocytosis of Mast Cell Granules

13. Unit 15.12 Analysis of Regulated Secretion Using PC12 Cells
14. Unit 15.13 Analysis of Endocytic Trafficking by Single-Cell Fluorescence Ratio Imaging
15. Unit 15.14 Quantitative Analysis of Endocytosis and Turnover of Epidermal Growth Factor (EGF) and EGF Receptor
16. Unit 15.15 Documenting GLUT4 Exocytosis and Endocytosis in Muscle Cell Monolayers

18. Chapter 16 Antibodies as Cell Biological Tools

1. Introduction
2. Unit 16.1 Production of Monoclonal Antibodies
3. Unit 16.2 Production of Polyclonal Antisera
4. Unit 16.3 Purification of Immunoglobulin G
5. Unit 16.4 Fragmentation of Immunoglobulin G
6. Unit 16.5 Antibody Conjugates for Cell Biology
7. Unit 16.6 Production of Antibodies That Recognize Specific Tyrosine-Phosphorylated Peptides

19. Chapter 17 Macromolecular Interactions in Cells

1. Introduction
2. Unit 17.1 Imaging Protein-Protein Interactions by Fluorescence Resonance Energy Transfer (FRET) Microscopy
3. Unit 17.2 Identification of Protein Interactions by Far Western Analysis
4. Unit 17.3 Interaction Trap/Two-Hybrid System to Identify Interacting Proteins
5. Unit 17.4 Mapping Protein-Protein Interactions with Phage-Displayed Combinatorial Peptide Libraries
6. Unit 17.5 Protein-Protein Interactions Identified by Pull-Down Experiments and Mass Spectrometry
7. Unit 17.6 Measuring Protein Interactions by Optical Biosensors
8. Unit 17.7 Chromatin Immunoprecipitation for Determining the Association of Proteins with Specific Genomic Sequences In Vivo
9. Unit 17.8 Isothermal Titration Calorimetry
10. Unit 17.9 Rational Design and Evaluation of FRET Experiments to Measure Protein Proximities in Cells
11. Unit 17.10 Identification and Analysis of Multiprotein Complexes Through Chemical Crosslinking
12. Unit 17.11 Visualization of RNA Using Fluorescence Complementation Triggered by Aptamer-Protein Interactions (RFAP) in Live Bacterial Cells

20. Chapter 18 Cellular Aging and Death

1. Introduction
2. Unit 18.1 Current Concepts in Cell Death
3. Unit 18.2 Analysis of Caspase Activation During Apoptosis
4. Unit 18.3 Assessment of Apoptosis and Necrosis by DNA Fragmentation and Morphological Criteria
5. Unit 18.4 Quantitative Fluorescence In Situ Hybridization (Q-FISH)
6. Unit 18.5 Analysis of Mitochondrial Dysfunction During Cell Death
7. Unit 18.6 Analysis of Telomeres and Telomerase
8. Unit 18.7 Nonisotopic Methods for Determination of Poly(ADP-Ribose) Levels and Detection of Poly(ADP-Ribose) Polymerase
9. Unit 18.8 Flow Cytometry of Apoptosis
10. Unit 18.9 Analysis of Cellular Senescence in Culture In Vivo: The Senescence-Associated β-Galactosidase Assay
11. Unit 18.10 High-Throughput Live Cell Imaging of Apoptosis

21. Chapter 19 Whole Organism and Tissue Analysis

1. Introduction
2. Unit 19.1 Overview of Metastasis Assays
3. Unit 19.2 Tail Vein Assay of Cancer Metastasis
4. Unit 19.3 Microanalysis of Gene Expression in Tissues Using T7-SAGE: Serial Analysis of Gene Expression After High-Fidelity T7-Based RNA Amplification
5. Unit 19.4 SAGE Analysis from 1 μg of Total RNA
6. Unit 19.5 The Chick Chorioallantoic Membrane as an In Vivo Angiogenesis Model
7. Unit 19.6 Experimental Metastasis Assays in the Chick Embryo
8. Unit 19.7 Imaging Tumor Cell Movement In Vivo
9. Unit 19.8 Embryonic Organ Culture
10. Unit 19.9 Three-Dimensional Tissue Models of Normal and Diseased Skin
11. Unit 19.10 Overview: Engineering Transgenic Constructs and Mice
12. Unit 19.11 Generation of Transgenic Mice
13. Unit 19.12 Overview: Generation of Gene Knockout Mice
14. Unit 19.13 Manipulation of Mouse Embryonic Stem Cells for Knockout Mouse Production
15. Unit 19.14 Generation of Gene Knockout Mice by ES Cell Microinjection

22. **Chapter 20 Expression and Introduction of Macromolecules into Cells**
    1. Introduction
    2. Unit 20.1 Direct Introduction of Molecules into Cells
    3. Unit 20.2 Protein Transduction: Generation of Full-Length Transducible Proteins Using the TAT System
    4. Unit 20.3 Calcium Phosphate Transfection
    5. Unit 20.4 Transfection Using DEAE-Dextran
    6. Unit 20.5 Transfection by Electroporation
    7. Unit 20.6 Transfection of Cultured Eukaryotic Cells Using Cationic Lipid Reagents
    8. Unit 20.7 Optimization of Transfection
    9. Unit 20.8 Inducible Gene Expression Using an Autoregulatory, Tetracycline-Controlled System
23. **Chapter 21 Fluorescent Protein Technology**
    1. Introduction
    2. Unit 21.1 Measuring Protein Mobility by Photobleaching GFP Chimeras in Living Cells
    3. Unit 21.2 Fluorescence Localization After Photobleaching (FLAP)
    4. Unit 21.3 Visualization of Protein Interactions in Living Cells Using Bimolecular Fluorescence Complementation (BiFC) Analysis
    5. Unit 21.4 Design and Use of Fluorescent Fusion Proteins in Cell Biology
    6. Unit 21.5 The Fluorescent Protein Color Palette
    7. Unit 21.6 Photoactivation and Imaging of Photoactivatable Fluorescent Proteins
24. **Chapter 22 Cell Biology of Chromosomes and Nuclei**
    1. Introduction
    2. Unit 22.1 Overview of Cytogenetic Chromosome Analysis
    3. Unit 22.2 Preparation of Cytogenetic Specimens from Tissue Samples
    4. Unit 22.3 Traditional Banding of Chromosomes for Cytogenetic Analysis
    5. Unit 22.4 Fluorescence In Situ Hybridization (FISH)
    6. Unit 22.5 Multi-Color FISH Techniques
    7. Unit 22.6 Comparative Genomic Hybridization
    8. Unit 22.7 Sister Chromatid Exchange
    9. Unit 22.8 Detection of Mitotic Figures and Components of the Mitotic Machinery
    10. Unit 22.9 Assembly and Micromanipulation of Xenopus In Vitro–Assembled Mitotic Chromosomes
    11. Unit 22.10 Replication Labeling with Halogenated Thymidine Analogs
    12. Unit 22.11 Assays for Ribosomal RNA Processing and Ribosome Assembly
    13. Unit 22.12 Visualization and Measurement of DNA Methyltransferase Activity in Living Cells
    14. Unit 22.13 Monitoring mRNA Export
    15. Unit 22.14 Analysis of DNA Replication in Saccharomyces cerevisiae by Two-Dimensional and Pulsed-Field Gel Electrophoresis
25. **Chapter 23 Stem Cells**
    1. Introduction
    2. Unit 23.1 Stem Cells: An Overview
    3. Unit 23.2 Mouse Embryonic Stem Cell Derivation, and Mouse and Human Embryonic Stem Cell Culture and Differentiation as Embryoid Bodies
    4. Unit 23.3 Maintenance and In Vitro Differentiation of Mouse Embryonic Stem Cells to Form Blood Vessels
    5. Unit 23.4 Differentiation of Mouse Embryonic Stem Cells and of Human Adult Stem Cells into Adipocytes
    6. Unit 23.5 Induction of ES Cell–Derived Cartilage Formation
    7. Unit 23.6 Hematoendothelial Differentiation of Human Embryonic Stem Cells
    8. Unit 23.7 Neural Differentiation of Human ES Cells
26. **Chapter 24 Lipids**
    1. Introduction
    2. Unit 24.1 Using Fluorescent Sphingolipid Analogs to Study Intracellular Lipid Trafficking
    3. Unit 24.2 Fluorescent Detection of Lipid Droplets and Associated Proteins
    4. Unit 24.3 Making Giant Unilamellar Vesicles via Hydration of a Lipid Film
    5. Unit 24.4 Visualization of Cellular Phosphoinositide Pools with GFP-Fused Protein-Domains
27. **Chapter 25 Nanotechnology**
    1. Introduction
    2. Unit 25.1 In Vivo Imaging Using Quantum Dot–Conjugated Probes
    3. Unit 25.2 Fabrication and Application of Nanofibrous Scaffolds in Tissue Engineering
28. **Chapter 26 Viruses**
    1. Introduction
    2. Unit 26.1 Production of Papillomavirus-Based Gene Transfer Vectors

3. Unit 26.2 BK Virus (BKV): Infection, Propagation, Quantitation, Purification, Labeling, and Analysis of Cell Entry
4. Unit 26.3 Methods Used to Study Respiratory Virus Infection
5. Unit 26.4 Compartmented Neuron Cultures for Directional Infection by Alpha Herpesviruses
6. Unit 26.5 HIV-1 Interactions with Cells: From Viral Binding to Cell-Cell Transmission

29. Chapter 26 Lipids

1. Unit 26.6 Methods for Monitoring Dynamics of Pulmonary RSV Replication by Viral Culture and by Real-Time Reverse Transcription–PCR In Vivo: Detection of Abortive Viral Replication

30. Chapter 27 RNA-Based Methods in Cell Biology

1. Introduction
2. Unit 27.1 Silencing of Gene Expression in Cultured Cells Using Small Interfering RNAs
3. Unit 27.2 Gene Down-Regulation with Short Hairpin RNAs and Validation of Specificity by Inducible Rescue in Mammalian Cells

31. Appendix 1 Useful Information and Data

1. 1A Useful Measurements and Data
2. 1B Compendium of Drugs Commonly Used in Cell Biology Research
3. 1C Identification of Motifs in Protein Sequences
4. 1D Safe Use of Radioisotopes
5. 1E Absorption and Emission Maxima for Common Fluorophores
6. 1F Importing Biological Materials
7. 1G Centrifuges and Rotors
8. 1H Internet Basics for Biologists

32. Appendix 2 Laboratory Stock Solutions and Equipment

1. 2A Common Stock Solutions, Buffers, and Media
2. 2B Medium Formulations
3. 2C Standard Laboratory Equipment

33. Appendix 3 Commonly Used Techniques

1. 3A Molecular Biology Techniques
2. 3B Spectrophotometric Determination of Protein Concentration
3. 3C Dialysis and Concentration of Protein Solutions
4. 3D Quantification of DNA and RNA with Absorption and Fluorescence Spectroscopy
5. 3E Silanizing Glassware
6. 3F Enzymatic Amplification of DNA by PCR: Standard Procedures and Optimization
7. 3G Micro RT-PCR
8. 3H The Colorimetric Detection and Quantitation of Total Protein

34. Appendix Suppliers

1. Selected Suppliers of Reagents and Equipment

PREFACE

The universe of cell biology is expanding. One way to sense this expansion is to attend a major meeting dedicated to the field, such as that of the American Society for Cell Biology. The poster sessions allow one to take a leisurely stroll through the exhibits and, even without stopping to look at any single poster, get a very good visceral feel for the scope of cell biology. For those who have taken such a stroll periodically over the past decade or two, the dynamic nature of cell biology and its explosive growth have been obvious—there is simply more and more about more and more. Once upon a time, poster sessions were dominated by images of cells fixed (literally) and captured in black and white by electron microscopists. While the electron microscope continues to contribute to our understanding of cell architecture, more recent poster sessions have witnessed the emergence of row upon row of television monitors where one can view computer-enhanced video images of living cells captured in the not-so-living colors of rhodamine red, fluorescein yellow, and the characteristic green that gives the now-famous fluorescent protein its name. Freeze-etching has been joined by freeze-frame. Furthermore, interspersed among the television sets and whirring VCRs are posters that document detailed molecular characterizations of a vast array of biochemical components that comprise the diverse cell types being investigated. Other posters record the latest genes to fall to the seemingly relentless onslaught of the cloners and sequencers, and still others offer functional insights obtained by knocking out these genes. Every point of the compass has its blot! Genetic studies in yeast, flies, and worms abound on the one hand, and on the other, a growing number of the presentations form bridges between basic research in cell biology and the practice of clinical medicine.

Clearly, the scientists who today identify themselves as cell biologists are a diverse community, and great value resides in that diversity. Borders that once separated research disciplines have faded, and cell biologists have come to appreciate that no single approach in isolation will open the profound mysteries of the cell. New techniques and new technologies come alongside the tried-and-true as the tools of cell biology expand along with the field. It is this changing face of cell biology and its methodologies that represented the greatest challenge in pulling together *Current Protocols in Cell Biology*. A foundational question addressed by the editors of this work involved where to draw the boundaries around the field of cell biology. Our decision has been to refuse to draw such boundaries—they are artificial at best and counterproductive at worst. Instead, we will attempt in this effort to match the diversity of our field. We will include "classic" methods that remain valuable tools for the modern cell biologist and will also provide protocols that we believe are destined to become tomorrow's classics.

There is no reason to suspect that the expanding universe of cell biology will cease expanding anytime soon. Indeed, part of the thrill of being a cell biologist is being constantly surprised by new innovations and discoveries. As a consequence, however, our community needs a reliable and user-friendly source of laboratory methods that is as expansive as the field itself. To begin to address this need, we have assembled a series of protocols that cover many aspects of cell biology. While this set of methods is incomplete, it can be considered a "starter toolbox" that includes many of the most versatile and essential instruments of our trade. This collection will expand as regular quarterly supplements are added to *Current Protocols in Cell Biology*. Through these supplements, the editors will endeavor to make the toolbox increasingly more useful over time. Because of the rapid pace of innovation and discovery in cell biology, we are expecting that our efforts will be met with some degree of very pleasant frustration as we strive to keep up with this fast-moving and exciting field.

Current Protocols in Cell Biology iii-vi, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cbprefs48

HOW TO USE THIS MANUAL

Format and Organization

This publication is available in both looseleaf and CD-ROM format. For looseleaf purchasers, a binder is provided to accommodate the growth of the manual via the quarterly update service. This format allows easy insertion of new pages, units, and chapters that are added. The index and table of contents are updated with each supplement. CD-ROM purchasers receive a completely new disc every quarter and should dispose of their outdated discs. The material covered in the two versions is identical.

Subjects in this manual are organized by chapters, and protocols are contained in units. Protocol units, which constitute the bulk of the book, generally describe a method and include one or more protocols with listings of materials, steps and annotations, recipes for unique reagents and solutions, and commentaries on the "hows" and "whys" of the method. Other units present more general information in the form of explanatory text with no protocols. Overview units contain theoretical discussions that lay the foundation for subsequent protocols. Other discussion units present more general information.

Page numbering in the looseleaf version reflects the modular arrangement by unit; for example, page 1.2.3 refers to Chapter 1 (Cell Culture), *UNIT 1.2* (Media for Culture of Mammalian Cells), page 3 of that particular unit.

Many reagents and procedures are employed repeatedly throughout the manual. Instead of duplicating this information, cross-references among units are used and recipes for common reagents are supplied in *APPENDIX 2A*. Cross-referencing helps to ensure that lengthy and complex protocols are not overburdened with steps describing auxiliary procedures needed to prepare raw materials and analyze results.

Introductory and Explanatory Information

Because this publication is first and foremost a compilation of laboratory techniques in cell biology, we have included explanatory information where required to help readers gain an intuitive grasp of the procedures. Some chapters begin with special overview units that describe the state of the art of the topic matter and provide a context for the procedures that follow. Chapter and unit introductions describe how the protocols that follow connect to one another, and annotations to the actual protocol steps describe what is happening as a procedure is carried out. Finally, the Commentary that closes each protocol unit describes background information regarding the historical and theoretical development of the method, as well as alternative approaches, critical parameters, troubleshooting guidelines, anticipated results, and time considerations. All units contain cited references and many indicate key references to inform users of particularly useful background reading, original descriptions, or applications of a technique.

Protocols

Many units in the manual contain groups of protocols, each presented with a series of steps. One or more *basic* protocols are presented first in each unit and generally cover the recommended or most universally applicable approaches. *Alternate* protocols are provided where different equipment or reagents can be employed to achieve similar ends, where the starting material requires a variation in approach, or where requirements for the end product differ from those in the basic protocol. *Support* protocols describe additional steps that are required to perform the basic or alternate protocols; these steps are separated from the core protocol because they might be applicable to other uses in the manual, or because they are performed in a time frame separate from the basic protocol steps.

Reagents and Solutions

Reagents required for a protocol are itemized in the materials list before the procedure begins. Many are common stock solutions, others are commonly used buffers or media, while others are solutions unique to a particular protocol. Recipes for the latter solutions are provided in each unit, following the protocols (and before the commentary) under the heading Reagents and Solutions. It is important to note that the *names* of some of these special solutions might be similar from unit to unit (e.g., RIPA buffer) while the *recipes* differ; thus, make certain that reagents are prepared from the proper recipes. On the other hand, recipes for commonly used stock solutions and buffers are provided once in *APPENDIX 2A*. These universal recipes are cross-referenced parenthetically in the materials lists rather than repeated with every usage.

Commercial Suppliers

Throughout the manual, we have recommended commercial suppliers of chemicals, biological materials, and equipment. In some cases, the noted brand has been found to be of superior quality or it is the only suitable product available in the marketplace. In other cases, the experience of the author of that protocol is limited to that brand. In the latter situation, recommendations are offered as an aid to the novice in obtaining the tools of the trade. Experienced investigators are therefore encouraged to experiment with substituting their own favorite brands.

Addresses, phone numbers, and facsimile numbers of all suppliers mentioned in this manual are provided in the *SUPPLIERS APPENDIX*.

Safety Considerations

Anyone carrying out these protocols may encounter the following hazardous or potentially hazardous materials: (1) radioactive substances, (2) toxic chemicals and carcinogenic or teratogenic reagents, and (3) pathogenic and infectious biological agents. Check the guidelines of your particular institution with regard to use and disposal of these hazardous materials. Although cautionary statements are included in the appropriate units, we emphasize that users must proceed with the prudence and precaution associated with good laboratory practice, and that all materials must be used in strict accordance with local and national regulations.

Animal Handling

Many protocols call for use of live animals (usually rats or mice) for experiments. Prior to conducting any laboratory procedures with live subjects, the experimental approach must be submitted in writing to the appropriate Institutional Animal Care and Use Committee (IACUC) or must conform to appropriate governmental regulations regarding the care and use of laboratory animals. Written approval from the IACUC (or equivalent) committee is absolutely required prior to undertaking any live-animal studies. Some specific animal care and handling guidelines are provided in the protocols where live subjects are used, but check with your IACUC or governmental guidelines to obtain more extensive information.

Reader Response

Most of the protocols included in this manual are used routinely in the authors' laboratories. These protocols work for them; to make them work for you they have annotated critical steps and included critical parameters and troubleshooting guides in the commentaries to most units. However, the successful evolution of this manual depends upon readers' observations and suggestions. We encourage readers to send their comments to *CurrentProtocols@wiley.com*, or to go to any specific unit on our website at

http://www.currentprotocols.com, where you can post comments, ask questions, and see author updates.

ACKNOWLEDGMENTS

This manual is the product of dedicated efforts by many of our scientific colleagues who are acknowledged in each unit and by the hard work by the Current Protocols editorial staff at John Wiley and Sons. We are extremely grateful for the critical contributions by Kathy Morgan (Series Editor) who kept the editors and the contributors on track and played a key role in bringing the entire project to completion. Other skilled members of the Current Protocols staff who contributed to the project include Joseph White, Janet Blair, Kathy Wisch, Michael Gates, Demetra Kagdis, Alice Ro, and Scott Holmes. The extensive copyediting required to produce an accurate protocols manual was ably handled by Rebecca Barr, Allen Ranz, Elizabeth Harkins, Lisa Christenson, Connie Parks, Karen Hopkin, Monte Kendrick, and Cathy Lundmark, and electronic illustrations were prepared by Gae Xavier Studios.

Recommended Background Reading

Alberts, B., Bray, D., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. 1997. Essential Cell Biology. Garland Publishing, New York.

A basic introductory cell biology text written by the authors of Molecular Biology of the Cell.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1994. Molecular Biology of the Cell, 3rd ed. Garland Publishing, New York.

Darnell, J., Lodish, H., and Baltimore, D. 1995. Molecular Cell Biology. Scientific American Books, New York.

Two comprehensive and lucid textbooks that convey effectively the synergistic convergence of biochemistry, genetics, structural biology, and traditional cell biology to form modern molecular and cell biology.

Juan S. Bonifacino, Mary Dasso, Joe B. Harford,
Jennifer Lippincott-Schwartz, and Kenneth M. Yamada

CHAPTER 1
Cell Culture

INTRODUCTION

Cell biology traces its roots to the introduction of the concept of "cells" by Robert Hooke in the second half of the 17th century. However, not until nearly halfway through the 20th century were techniques for the culture of cells developed. In fact, 1998 marked the golden anniversary of the first continuous mammalian cell line. Cell culture has become such an integral part of cell biology that it is somewhat difficult to imagine the field in the B.C. ("Before Culture") era. Cell culture also represents the primary way in which cell biology reaches into related disciplines, since the maintenance and propagation of cells has become an important component of biochemistry, biophysics, genetics, immunology, physiology, molecular biology, and neuroscience. Accordingly, it is altogether fitting that the first chapter of *Current Protocols in Cell Biology* should present methods related to the culture of cells.

The immediate aim of cell culture is to maintain or expand a population of cells, and the single most important consideration is cell viability. Determining the number of cells and their viability is important in standardizing culture and experimental conditions. As viable cells replicate in culture, passaging of the cells allows their number to be expanded to meet experimental needs. The ability to freeze, store, and recover cells provides an essential safeguard against losing a cell line to contamination, incubator malfunction, or an error on the part of the investigator. In addition to preserving the cells, maintenance of a frozen stock is desirable to avoid cellular senescence and genetic drift. Chapter 1 therefore begins with protocols for passaging cells, freezing and thawing cells, and determining cell number and viability (*UNIT 1.1*).

Success in cell culture is highly dependent on the choice of a medium. At minimum, a medium must provide the nutritional requirements of the cells as well as any required growth factors, and maintain pH and osmolarity compatible with survival. The historical development of a wide variety of culture media has influenced significantly the types of cells that can be studied experimentally, since cell lines that proliferate in a particular environment are always selected at the expense of those that do not. The second unit of Chapter 1 therefore focuses on media used in culturing cells and provides descriptions of standard, serum-free, and selective media, as well as the use of soft agar for anchorage-independent growth (*UNIT 1.2*).

The next three units of this chapter deal with microbial contamination of cell cultures. *UNIT 1.3* describes basic aseptic techniques and the laminar flow hoods that are the main weapons in the constant battle against contamination. *UNIT 1.4* provides protocols related to sterilization, namely filtration and heat sterilization (e.g., autoclaving), as well as the use of disinfectants. *UNIT 1.5* describes methods for detecting microbial contaminants (bacteria, fungi, and mycoplasmas). While the best way to deal with such contamination may well be to review the previous unit on autoclaving and faithfully apply its precepts, situations do arise where an attempt to salvage a contaminated culture is warranted. *UNIT 1.5* details the use of antibiotics for this purpose.

Of course there are cell biologists who do not see the growth of fungi as an annoying contamination of their mammalian cell cultures but as a desirable goal. For scientists who

Contributed by Joe B. Harford
Current Protocols in Cell Biology (2003) 1.0.1-1.0.2

wish to propagate yeast, *UNIT 1.6* provides recipes for media and descriptions of some basic culture methodologies.

UNIT 1.7 represents the first unit of Chapter 1 dealing with culture of plants cells, specifically the culture and transformation of BY-2 cells derived from tobacco. BY-2 cells have been described as the HeLa cell of higher plants.

Future units in Chapter 1 will cover specialized systems for cell culture (e.g., cell cloning, polarized cells, and three-dimensional cultures), as well as additional units on the propagation of plant cells, cells from other so-called simpler organisms, and viruses.

For additional information on mammalian cell culture, readers are directed to Freshney (1993).

LITERATURE CITED

Freshney, R.I. 1993. Culture of Animal Cells. A Manual of Basic Techniques, 3rd ed. Wiley-Liss, New York.

Joe B. Harford

Basic Techniques in Mammalian Cell Tissue Culture

Mary C. Phelan[1]

[1]Molecular Pathology Laboratory Network, Maryville, Tennessee

ABSTRACT

Cultured mammalian cells are used extensively in cell biology studies. It requires a number of special skills in order to be able to preserve the structure, function, behavior, and biology of the cells in culture. This unit describes the basic skills required to maintain and preserve cell cultures: maintaining aseptic technique, preparing media with the appropriate characteristics, passaging, freezing and storage, recovering frozen stocks, and counting viable cells. *Curr. Protoc. Cell Biol.* 36:1.1.1-1.1.18. © 2007 by John Wiley & Sons, Inc.

Keywords: mammalian cells • tissue culture • aseptic technique • medium • passaging cells • freezing cells

Tissue culture technology has found wide application in the field of cell biology. Cell cultures are utilized in cytogenetic, biochemical, and molecular laboratories for research as well as diagnostic studies. In most cases, cells or tissues must be grown in culture for days or weeks to obtain sufficient numbers of cells for analysis. Maintenance of cells in long-term culture requires strict adherence to aseptic technique to avoid contamination and potential loss of valuable cell lines (see *UNIT 1.3*).

An important factor influencing the growth of cells in culture is the choice of tissue culture medium. Many different recipes for tissue culture media are available and each laboratory must determine which medium best suits its needs. Individual laboratories may elect to use commercially prepared medium or prepare their own. Commercially available medium can be obtained as a sterile and ready-to-use liquid, in a concentrated liquid form, or in a powdered form. Besides providing nutrients for growing cells, medium is generally supplemented with antibiotics, fungicides, or both to inhibit contamination. Medium preparation is discussed in *UNIT 1.2*.

As cells reach confluency, they must be subcultured or passaged. Failure to subculture confluent cells results in reduced mitotic index and eventually in cell death. The first step in subculturing is to detach cells from the surface of the primary culture vessel by trypsinization or mechanical means. The resultant cell suspension is then subdivided, or reseeded, into fresh cultures. Secondary cultures are checked for growth and fed periodically, and may be subsequently subcultured to produce tertiary cultures, etc. The time between passaging of cells varies with the cell line and depends on the growth rate.

The Basic Protocol describes subculturing of a monolayer culture grown in petri plates or flasks. Support Protocols describe freezing of monolayer cells, thawing and recovery of cells, counting cells using a hemacytometer, and preparing cells for transport. Alternate Protocols 1 and 2 describe the passaging and freezing of cells in suspension culture.

CAUTION: When working with human blood cells, or infective agents, appropriate biosafety practices must be followed.

Current Protocols in Cell Biology 1.1.1-1.1.18, September 2007
Published online September 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0101s36

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

ASEPTIC TECHNIQUE

It is essential that aseptic technique be maintained when working with cell cultures. Aseptic technique involves a number of precautions to protect both the cultured cells and the laboratory worker from infection. The laboratory worker must realize that cells handled in the lab are potentially infectious and should be handled with caution. Protective apparel such as gloves, lab coats or aprons, and eyewear should be worn when appropriate (Knutsen, 1991). Care should be taken when handling sharp objects such as needles, scissors, scalpel blades, and glass that could puncture the skin. Sterile disposable plastic supplies may be used to avoid the risk of broken or splintered glass (Rooney and Czepulkowski, 2001).

Frequently, specimens received in the laboratory are not sterile, and cultures prepared from these specimens may become contaminated with bacteria, fungus, or yeast. The presence of microorganisms can inhibit growth, kill cell cultures, or lead to inconsistencies in test results. The contaminants deplete nutrients in the medium and may produce substances that are toxic to cells. Antibiotics and antimycotics can be used to combat potential contaminants (see Table 1.1.1). The solutions can be used to wash specimens prior to culture or to rinse contaminated cultures, and can be added to medium used for tissue culture. Antibiotics (penicillin, streptomycin, kanamycin, neomycin, or gentamycin) can be used individually or in combinations (i.e., penicillin/streptomycin/neomycin and penicillin/streptomycin/gentamycin). Likewise, fungicides such as amphotericin B (Fungizone) and mycostatin (Nystatin) can be used alone or as antibiotic/antimycotic solutions (i.e., penicillin/streptomycin/Fungizone). Particular care should be taken when using Fungizone, as it is typically very toxic to cell cultures, and adequate data are not available regarding potential adverse effects on the growth of human cells. Antibiotics and antimycotics are available from a number of vendors including Invitrogen, Sigma-Aldrich, and BioWhittaker.

All materials that come into direct contact with cultures must be sterile. Sterile disposable dishes, flasks, pipets, etc., can be obtained directly from manufacturers. Reusable glassware must be washed, rinsed thoroughly, then sterilized by autoclaving or by dry heat before reusing. With dry heat, glassware should be heated 1.5 to 2 hr at 160°C to ensure sterility. Materials that may be damaged by very high temperatures can be autoclaved

Table 1.1.1 Working Concentrations of Antibiotics and Fungicides for Mammalian Cell Culture

Additive	Final concentration
Penicillin	50–100 U/ml
Streptomycin sulfate	50–100 μg/ml
Kanamycin	100 μg/ml
Gentamycin	50 μg/ml
Neomycin	50 μg/ml
Mycostatin (Nystatin)	20 μg/ml
Amphotericin B (Fungizone)	0.25 μg/ml
Pen/strep/neomycin[a]	1×

[a]Usually supplied as 100× concentrated stock solution, which is diluted to 1× concentration in the medium.

20 min at 120°C and 15 psi. All media, reagents, and other solutions that come into contact with the cultures must also be sterile; media may be obtained as a sterile liquid from the manufacturer, autoclaved if not heat-sensitive, or filter sterilized. Supplements can be added to media prior to filtration, or they can be added aseptically after filtration. Filters with 0.20- to 0.22-μm pore size should be used to remove small gram-negative bacteria from culture media and solutions.

Contamination can occur at any step in handling cultured cells. Care should be taken to maintain the sterility of petri plates, pipets, and flasks that are used for tissue culture. The use of disposable, sterile culture supplies is convenient and has virtually eliminated the need to flame-sterilize instruments and vessels used for tissue culture. The initial cost of purchasing these single-use supplies outweighs the expense associated with the time and effort of washing, packaging, and sterilizing nondisposables. Good sterile technique must be followed. For example, if a sterile pipet tip should come into contact with the benchtop or other nonsterile surface, the pipet tip should be discarded and a fresh one obtained. If disposables are not an option, the necks of sterile containers, such as bottles and flasks, and the tips of pipets should be passed through a flame before the pipet is introduced into the container. After pipetting, the neck of the bottle or flask should again be flamed.

Certain instruments used for tissue culture (forceps, scissors, scalpels, and in some instances, glass bottles) may require autoclave sterilization prior to initial use (see *UNIT 1.4*). The autoclave relies on pressurized steam to destroy microorganisms. Instruments to be autoclaved should be thoroughly washed and dried, then packaged if necessary to ensure that sterility will be maintained after removal from the autoclave until use. Indicator tape or autoclave bags with indicator strips should be used to document that the items have been autoclaved. The indicator tape demonstrates that the item has been autoclaved, but does not ensure sterility.

Historically, if instruments such as forceps, tweezers, scissors, or scalpels were going to be re-used to handle several sequential specimens, they were rapidly sterilized between uses by dipping in 70% alcohol and flaming. Other methods of rapid decontamination are now available. For example, the Germinator 500 (Cellpoint Scientific) is a small benchtop unit [height, 6.76 in. (~17 cm) × width, 5.25 in. (~13 cm) × depth 5.25 in. (~13 cm)] used in tissue culture labs for decontamination of metal dissecting instruments. Instruments must be clean, dry, and free of debris prior to sterilization. The instruments are inserted into a stainless steel well [inside dimensions: diameter, 2 in. (~ 5 cm) × depth, 4 in (~10 cm)] filled with glass beads that are maintained at a constant temperature of 500°F (~260°C). Dry heat decontamination occurs through heat transfer from the glass beads to the instruments. The inserted parts of small instruments are dry sterilized within ~15 sec, while larger instruments may take as long as 1 min. The Germinator 500 eliminates the need for alcohol and open flames for sterilization. Due to the lack of a method for routine monitoring of sterilization by glass beads, the Germinator 500 operating manual recommends that this equipment be used for research purposes only.

Although tissue culture work can be done on an open bench if aseptic methods are strictly enforced, many labs prefer to perform tissue culture work in a room or low-traffic area reserved specifically for that purpose. At the very least, biological safety cabinets are recommended to protect the cultures as well as the laboratory worker. In a laminar flow hood, the flow of air protects the work area from dust and contamination and acts as a barrier between the work surface and the worker. Many different styles of safety hoods are available, and the laboratory should consider the types of samples being processed and the types of potential pathogenic exposure in making a selection. Manufacturer recommendations should be followed regarding routine maintenance checks on air flow

and filters. For day-to-day use, the cabinet should be turned on for at least 5 min prior to beginning work. All work surfaces both inside and outside of the hood should be kept clean and disinfected daily and after each use. A 10% household bleach solution, 70% alcohol, an iodophor, a quarternary ammonium compound, or commercially available liquid disinfectants can be used (*http://www.ehs.cornell.edu/bio/cabinets.htm*, last updated December, 2000).

Some safety cabinets are equipped with ultraviolet (UV) lights for decontamination of work surfaces. However, UV lamps are not required in biological safety cabinets, and their effectiveness has been questioned (*http://www.niehs.nih.gov/odhsb/biosafe/bsc/section6.htm*; *http://www.ehs.cornell.edu/bio/cabinets.htm*). UV lamps may produce a false sense of security as they maintain a visible blue glow long after their germicidal effectiveness is lost. Effectiveness diminishes over time as the glass tube gradually loses its ability to transmit short UV wavelengths, and may also be reduced by dust on the glass tube, distance from the work surface, temperature, and air movement. Even when the UV output is adequate, the rays must directly strike a microorganism in order to kill it; bacteria or mold spores hidden below the surface of a material or outside the direct path of the rays will not be destroyed. Another rule of thumb is that anything that can be seen cannot be killed by UV. UV lamps will only destroy microorganisms such as bacteria, viruses, and mold spores; they will not destroy insects or other large organisms. Aside from their general ineffectiveness, UV lights pose a safety hazard, as exposure can cause damage to the eyes and skin (*http://www.yale.edu/oehs/cad2_0.htm*). A more reliable approach to eliminate contamination is the use of well-practiced microbiological procedures, good aseptic techniques, and standard operational procedures for working in a biological safety cabinet, including thorough decontamination with an effective disinfectant before and after use of the biological safety cabinet. The current recommendation is that work surfaces be wiped down with ethanol instead of relying on UV lamps, although some labs use the lamps in addition to ethanol wipes to decontaminate work areas. If UV lamps are used as a secondary method of disinfecting the work surface, their radiation output should be tested with a UV meter during the annual certification of the biological safety cabinet to ensure that the proper intensity of light is reaching the work surface. The radiation output should be at least 40 $\mu W/cm^2$ at 254 nm when measured with a UV flux meter placed in the center of the work surface. The UV lamps should be replaced when they fall below the minimum requirements for protection (*http://www.ehs.cornell.edu/bio/cabinets.htm*).

Cultures should be visually assessed on a routine basis for evidence of contamination (see *UNIT 1.5*). Indicators in the tissue culture medium change color when contamination is present: for example, medium that contains phenol red changes to yellow because of increased acidity. Cloudiness and turbidity are also observed in contaminated cultures. Once contamination is confirmed with a microscope, infected cultures are generally discarded. Keeping contaminated cultures increases the risk of contaminating other cultures. Sometimes a contaminated cell line can be salvaged by treating it with various combinations of antibiotics and antimycotics in an attempt to eradicate the infection. In this procedure, the tissue culture medium is aspirated from the affected cultures and discarded. The cultures are rinsed with fresh "wash" medium that has been prepared by supplementing the routinely used tissue culture medium with the appropriate concentration of antibiotic and/or antimycotic (Table 1.1.1). The petri dish or flask should be gently swirled so that the cell surface is bathed in the clean medium. The wash medium can be left on the cultures for 1 to 5 min. Due to the toxicity of Fungizone and amphotericin, it is recommended that cell exposure to these agents be limited to less than 2 min. Aspirate the wash medium, replace with fresh medium and return the cultures to the incubator. Even under the best conditions, such treatment may adversely affect cell growth and it is often unsuccessful in ridding cultures of contaminants.

Mycoplasma are small (0.2 to 0.3 μm) intracellular bacteria that attach to the cell membrane, inhibit cell growth, and eventually lead to cell death. Because these parasites do not have cell walls, do not grow in colonies, and do not change the pH of the medium, they are difficult to detect visually in cultures. Mycoplasma can multiply to very high concentrations (10^7 to 10^8 organisms/ml) and adversely affect cultures by altering cell growth characteristics, inhibiting cell metabolism, disrupting nucleic acid synthesis, inducing chromosome aberrations, changing cell membrane antigenicity, and altering transfection rates and viral susceptibility (*http://www.unc.edu/depts/tcf/mycoplasma.htm*).

Mycoplasma are spread by cross-contamination from infected cultures through aerosolization during pipetting, or via the transfer of contaminated cells or contaminated reagents used in cell culture (*http://www.unc.edu/depts/tcf/mycoplasma.htm*). There are several methods of detecting mycoplasma in cell cultures and in cell culture reagents (see *UNIT 1.5*). In the direct method, both cultures and reagents can be plated on agar in order to grow and thereby reveal the presence of mycoplasma. Disadvantages of this method are that some strains of mycoplasma cannot be cultivated on agar and that the results can take from 2 to 4 weeks to obtain (*http://www.lerner.ccf.org/services/cell/cellculture.php*). Several indirect methods are available and should be selected based on the capabilities of the laboratory and the needs of the laboratory in terms of sensitivity, specificity, and time requirements. The DNA staining method uses Hoechst 33258 to highlight the A-T rich DNA of mycoplasma, so that mycoplasma appear as bright extranuclear spots in the cytoplasm. Results are available in 24 hr, but this method is not as sensitive as the direct method and can be difficult to interpret due to background bacterial/yeast/fungal contamination, excess debris, reduced or absent live cells, and broken nuclei from dead cells. The addition of an indicator such as Vero (Sigma-Aldrich) increases the sensitivity of this method. PCR primers that selectively amplify part of the mycoplasma DNA are also available. Kits include the ELISA mycoplasma detection kit from Roche Applied Science, the Mycoplasma Detection kit from Minerva Biolabs, the Myco Alert Mycoplasma Detection Assay by Cambrex (with results available in $<$20 min using the Cambrex instrument), and the VenorGem Mycoplasma PCR Detection Kit from Sigma-Aldrich.

Good aseptic technique and routine testing are the most effective methods for preventing mycoplasma contamination. Most standard antibiotics are not effective in treating mycoplasma contamination, and many laboratories prefer to dispose of contaminated cultures. In the event that the cultures are valuable and backup cultures are not available, it is imperitive to attempt to salvage the contaminated cells. Agents such as Mynox Mycoplasma Elimination Reagent and Mycoplasma Removal Agent (MRA) are commercially available from ICN Flow, and usually require a single treatment to decontaminate cultures (*http://www.unc.edu/depts/tcf/mycoplasma.htm*).

CULTURE MEDIUM PREPARATION

Choice of tissue culture medium comes from experience. An individual laboratory must select the medium that best suits the type of cells being cultured. Chemically defined media are available in liquid or powdered form from a number of suppliers. Sterile, ready-to-use medium has the advantage of being convenient, although it is more costly than other forms. Powdered medium must be reconstituted with tissue culture–grade water according to manufacturer's directions. Distilled or deionized water is not of sufficiently high quality for medium preparation; double- or triple-distilled water or commercially available tissue culture water should be used. The medium should be filter-sterilized and transferred to sterile bottles. Prepared medium can generally be stored $\leq$1 month in a 4°C refrigerator. Laboratories using large volumes of medium may choose to prepare their own medium from standard recipes. This may be an economical approach, but it is time-consuming and the savings may not offset the time required.

Basic media such as Eagle minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM; see recipe), Glasgow modified Eagle medium (GMEM), and RPMI 1640 and Ham F10 nutrient mixture (e.g., Invitrogen) are composed of amino acids, glucose, salts, vitamins, and other nutrients. A basic medium is supplemented by addition of L-glutamine, antibiotics (typically penicillin and streptomycin sulfate), and usually serum to formulate a "complete medium." Where serum is added, the amount is indicated as a percentage of fetal bovine serum (FBS) or other serum. Some media are also supplemented with antimycotics, nonessential amino acids, various growth factors, and/or drugs that provide selective growth conditions (see *UNIT 1.2*). Supplements should be added to medium prior to sterilization or filtration, or added aseptically just before use.

The optimum pH for most mammalian cell cultures is 7.2 to 7.4. Adjust pH of the medium as necessary after all supplements are added. Buffers such as bicarbonate and HEPES are routinely used in tissue culture medium to prevent fluctuations in pH that might adversely affect cell growth. HEPES is especially useful in solutions used for procedures that do not take place in a controlled CO_2 environment.

Fetal bovine serum (FBS; sometimes known as fetal calf serum, FCS) is the most frequently used serum supplement. Calf serum, horse serum, and human serum are also used; some cell lines are maintained in serum-free medium (Freshney, 2005). Complete medium is supplemented with 5% to 30% (v/v) serum, depending on the requirements of the particular cell type being cultured. Serum is obtained frozen, then is thawed, divided into smaller portions, and refrozen until needed.

There is considerable lot-to-lot variation in FBS. Most suppliers will provide a sample of a specific lot and reserve a supply of that lot while the serum is tested for its suitability. The suitability of a serum lot depends upon the use. Frequently the ability of serum to promote cell growth equivalent to a laboratory standard is used to evaluate a serum lot. Once an acceptable lot is identified, enough of that lot should be purchased to meet the culture needs of the laboratory for an extended period of time.

Although the use of heat-inactivated serum was once preferred, it is no longer standard, or may be standard in some labs simply because it is an established and routine practice (*http://www.unc.edu/depts/tcf/tech_tipsHI.htm*). Historically, heat inactivation was considered necessary to destroy complement protein due to its role in cell lysis. It has since been shown that the level of complement components in commercially available FCS was only 1% to 3% of adult levels, and that no significant cell lysis was detected with the use of commercially available FCS, even when undiluted (Triglia and Linscott, 1980). In addition, many researchers prewarm FBS to 37°C, which is enough to inactivate heat-labile complement components. Improvements in the filtration of serum products have also made the use of heat-inactivated serum nonessential for most cell types. In the past serum was filtered through 0.45-μm or 0.22-μm filters, raising concern that adventitious agents such as mycoplasma could persist. Today media suppliers use 0.1-μm or 0.04-μm porosity membranes to eliminate the need for heat inactivation (*http://www.unc.edu/depts/tcf/tech_tipsHI.htm*; Hyclone Labs, 1996). Not only is heat inactivation unnecessary for most cell types, it may sometimes be detrimental to cell growth. Heat inactivation not only destroys complement, it also degrades amino acids, vitamins, growth factors, and other nutrients that enhance cell growth. Furthermore, heat inactivation can increase the formation of precipitates that can be mistaken for microbial contamination (Hyclone Labs, 1996). Laboratories that continue the use of heat inactivation should conduct studies to determine whether this step is really indicated. Heat inactivation may be warranted when conducting immunologic studies or when culturing embryonic stem cells, insect cells, and smooth muscle cells. For cells obtained

from a commercial vendor such as ATCC, serum requirements are included in the cell descriptions.

Although FBS has historically been the serum of choice, many investigators are moving towards the use of alternative types of animal serum or to serum-free media. An increasing number of companies are offering serum-free media for a wide range of cell types. Focus on Alternatives (FOA) lists over 300 types of serum-free products along with their suppliers, applications, and the cell types supported for each product (*http://www.focusonalternatives.org.uk*, compiled in July, 2005). Although the term "serum-free" implies that a medium contains no serum, the medium may not be entirely free of serum-derived products. For example, bovine serum albumin may be used as the protein component of particular types of serum-free media (Newman, 2003). While some media are designed for culturing a particular cell type, others are general-purpose media that can support a variety of cell types.

Commercially prepared media containing L-glutamine are available, but many laboratories choose to obtain medium without L-glutamine, and then add it to a final concentration of 2 mM just before use. L-Glutamine is an unstable amino acid that, upon storage, converts to a form cells cannot use. Breakdown of L-glutamine is temperature- and pH-dependent. At 4°C, 80% of the L-glutamine remains after 3 weeks, but at near incubator temperature (35°C) only half remains after 9 days (Barch et al., 1991). To prevent degradation, 100× L-glutamine should be stored frozen in aliquots until needed.

Another option to prevent degradation is the use of a stabilized dipeptide form of L-glutamine such as GlutaMAX (Invitrogen), which is available both as a stand-alone medium and as a medium supplement. GlutaMAX media contain the dipeptide L-alanyl-L-glutamine, which is split by aminopeptidases in culture to release L-glutamime and L-alanine. This mechanism provides a controlled delivery of L-glutamine to the cells in culture. The GlutaMAX dipeptide is also available as a medium supplement to be used as a direct substitute for L-glutamine in cell culture medium preparations with minimal or no adaptation. Both GlutaMAX Media and Supplement are reported by the manufacturer to maximize cell performance, improve cell viability and growth, extend cell culture life, and minimize toxic ammonium build-up during cell culture (*http://www.invitrogen.com*).

In addition to practicing good aseptic technique, most laboratories add antimicrobial agents to medium to further reduce the risk of contamination. A combination of penicillin and streptomycin is the most commonly used antibiotic additive; kanamycin and gentamycin are used alone. Mycostatin and amphotericin B are the most commonly used fungicides (Rooney and Czepulkowski, 2001). Table 1.1.1 lists the final concentrations for the most commonly used antibiotics and antimycotics. Combining antibiotics in tissue culture media can be tricky, as some antibiotics are not compatible and one may inhibit the action of another. Furthermore, combined antibiotics may be cytotoxic at lower concentrations than the individual antibiotics. In addition, prolonged use of antibiotics may cause cell lines to develop antibiotic resistance. For this reason, some laboratories add antibiotics and/or fungicides to medium when initially establishing a culture but eliminate them from medium used in later subcultures.

Commercially available tissue culture media have been tested for sterility prior to release, and further testing within the research laboratory is generally not required. In clinical laboratories, sterility checks on tissue culture media are often performed as a quality-control monitor in compliance with requirements from the College of American Pathologists (CAP). A small aliquot from each lot of medium is incubated 48 hr at 37°C and monitored for evidence of contamination such as turbidity (infected medium will be cloudy) and color change (if phenol red is the indicator, infected medium will turn yellow). Any contaminated medium should be discarded.

BASIC PROTOCOL

TRYPSINIZING AND SUBCULTURING CELLS FROM A MONOLAYER

A primary culture is grown to confluency in a 60-mm petri plate or 25-cm^2 tissue culture flask containing 5 ml tissue culture medium. Cells are dispersed by trypsin treatment and then reseeded into secondary cultures. The process of removing cells from the primary culture and transferring them to secondary cultures constitutes a passage, or subculture.

Materials

Primary cultures of cells
HBSS (*APPENDIX 2A*) without Ca^{2+} and Mg^{2+}, 37°C
0.25% (w/v) trypsin/0.2% EDTA solution (see recipe), 37°C
Complete medium with serum: e.g., DMEM supplemented with 10% to 15% (v/v) fetal bovine serum (complete DMEM-10; see recipe), 37°C

Sterile Pasteur pipets
37°C warming tray or incubator
Tissue culture plasticware or glassware including pipets and 25-cm^2 flasks or 60-mm petri plates, sterile

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Remove all medium from primary culture with a sterile Pasteur pipet. Wash adhering cell monolayer once or twice with a small volume of 37°C HBSS without Ca^{2+} and Mg^{2+} to remove any residual FBS that may inhibit the action of trypsin.

   Use a buffered salt solution that is Ca^{2+}- and Mg^{2+}-free to wash cells. Ca^{2+} and Mg^{2+} in the salt solution can cause cells to stick together.

   If this is the first medium change, rather than discarding medium that is removed from primary culture, put it into a fresh dish or flask. The medium contains unattached cells that may attach and grow, thereby providing a backup culture.

2. Add enough 37°C trypsin/EDTA solution to culture to cover adhering cell layer.

3. Place plate on a 37°C warming tray 1 to 2 min. Tap bottom of plate on the countertop to dislodge cells. Check culture with an inverted microscope to be sure that cells are rounded up and detached from the surface.

   If cells are not sufficiently detached, return plate to warming tray for an additional minute or two.

4. Add 2 ml 37°C complete medium. Draw cell suspension into a Pasteur pipet and rinse cell layer two or three times to dissociate cells and to dislodge any remaining adherent cells. As soon as cells are detached, add serum or medium containing serum to inhibit further trypsin activity that might damage cells.

   If cultures are to be split 1/3 or 1/4 rather than 1/2, add sufficient medium such that 1 ml of cell suspension can be transferred into each fresh culture vessel.

5. Add an equal volume of cell suspension to fresh plates or flasks that have been appropriately labeled.

   Alternatively, cells can be counted using a hemacytometer (Support Protocol 3) or Coulter counter and diluted to the desired density so a specific number of cells can be added to each culture vessel. A final concentration of ~5×10^4 cells/ml is appropriate for most subcultures.

For primary cultures and early subcultures, 60-mm petri plates or 25-cm^2 flasks are generally used; larger petri plates or flasks (e.g., 150-mm plates or 75-cm^2 flasks) may be used for later subcultures.

Cultures dishes/flasks should be labeled with at least two unique identifiers, as well as date of culture and passage number.

6. Add 4 ml fresh medium to each new culture. Incubate in a humidified 37°C, 5% CO_2 incubator.

   If using 75-cm^2 culture flasks, add 9 ml medium per flask.

   Some labs now use incubators with 5% CO_2 and 4% O_2. The low oxygen concentration is thought to simulate the in vivo environment of cells and to enhance cell growth.

7. If necessary, feed subconfluent cultures after 3 or 4 days by removing old medium and adding fresh 37°C medium.

8. Passage secondary culture when it becomes confluent by repeating steps 1 to 7, and continue to passage as necessary.

PASSAGING CELLS IN SUSPENSION CULTURE

ALTERNATE PROTOCOL 1

Passaging of suspension cultures is somewhat less complicated than passaging of monolayer cultures. Because the cells are suspended in medium rather than attached to a surface, it is not necessary to disperse them enzymatically before passaging. However, before passaging, cells must be maintained in culture by feeding every 2 to 3 days until they reach confluency (i.e., until the cells clump together in the suspension and the medium appears turbid when the flask is swirled).

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

For materials, see Basic Protocol.

1. Feed cells as follows every 2 to 3 days until the cultures are confluent:

   a. Remove flask of suspension cells from incubator, taking care not to disturb those that have settled to the flask bottom.

   b. Aseptically remove and discard about one-third of the medium from the flask and replace with an equal volume of prewarmed (37°C) medium. If the cells are growing rapidly, add an additional 10% medium by volume in order to maintain an optimum concentration of 1×10^6 cells/ml. Gently swirl flask to resuspend cells.

   c. Return flask to incubator. If there is <15 ml of medium in the flask, incubate flask in horizontal position to enhance cell/medium contact.

      At higher volumes of medium, the flask can be incubated in the vertical position.

      If using a 25-cm^2 flask, there should be 20 to 30 ml of medium in the flask at confluency.

2. On the days cultures are not being fed, check by swirling flask to resuspend cells and observing color changes from pink to yellow/orange in the medium, which indicate active cell metabolism.

3. When cultures are confluent (~2.5×10^6 cells/ml), passage culture as follows:

   a. Remove flask from incubator and swirl flask so that cells are evenly distributed in the medium.

b. Aseptically remove half of the volume of cell suspension and place into a fresh flask, retaining the other half of the cell suspension in the original flask.

Alternatively, the entire cell suspension can be removed from the original flask and divided equally into two fresh flasks. The original flask can be discarded, or if there is concern about the need for additional cells, the original flask can be retained and fed in an attempt to salvage any residual cells.

c. Feed each flask with 7 to 10 ml prewarmed medium and return flasks to incubator.

Some labs prefer to split the cells 1/3 or 1/4, although increasing the split ratio will result in a longer interval before subcultures reach confluency.

SUPPORT PROTOCOL 1

FREEZING HUMAN CELLS GROWN IN MONOLAYER CULTURES

It is sometimes desirable to store cell lines for future study. To preserve cells, avoid senescence, reduce the risk of contamination, and minimize effects of genetic drift, cell lines may be frozen for long-term storage. Without the use of a cryoprotective agent, freezing would be lethal to the cells in most cases. Generally, a cryoprotective agent such as dimethylsulfoxide (DMSO) is used in conjunction with complete medium for preserving cells at −70°C or lower. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage.

Materials

Log-phase monolayer culture of cells in petri plate
Complete medium
Freezing medium: complete medium (e.g., DMEM or RPMI; see recipes) supplemented with 10% to 20% (v/v) FBS and 5% to 10% (v/v) DMSO, 4°C
Benchtop clinical centrifuge with 45° fixed-angle or swinging-bucket rotor

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Trypsinize cells from plate (see Basic Protocol, steps 1 to 4).

   It is best to use cells in log-phase growth for cryopreservation.

2. Transfer cell suspension to a sterile centrifuge tube and add 2 ml complete medium with serum. Centrifuge 5 min at 300 to 350 × *g* (~1500 rpm in Fisher Centrific rotor), room temperature.

   The benchtop centrifuge can be accessorized depending on the anticipated volume. A variety of brands are available, including Eppendorf, Thermo Electron, and Beckman, and can be obtained from Fisher Scientific, VWR, and other laboratory equipment vendors.

   Cells from three or more dishes from the same subculture can be combined in one tube.

3. Remove supernatant and add 1 ml of 4°C freezing medium. Resuspend pellet to obtain a density of 1×10^6 cells/ml.

4. Add 4 ml of 4°C freezing medium, mix cells thoroughly, and place on wet ice.

5. Count cells using a hemacytometer (see Support Protocol 3). Dilute with more freezing medium as necessary to get a final cell concentration of 10^6 or 10^7 cells/ml.

   To freeze cells from a nearly confluent 25-cm^2 flask, resuspend in roughly 3 ml freezing medium.

6. Pipet 1-ml aliquots of cell suspension into labeled 2-ml cryovials. Tighten caps on vials.

7. Place vials 1 hr to overnight in a −70°C freezer, then transfer to a liquid nitrogen storage freezer.

   Keep accurate records of the identity and location of cells stored in liquid nitrogen freezers. Cells may be stored for many years and proper information is imperative for locating a particular line for future use.

FREEZING CELLS GROWN IN SUSPENSION CULTURE

ALTERNATE PROTOCOL 2

Freezing cells from suspension culture is similar in principle to freezing cells from monolayer. The major difference is that suspension cultures need not be trypsinized.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Transfer cell suspension to a centrifuge tube and spin 10 min at 300 to 350 × *g* (−1500 rpm in Fisher Centrific centrifuge), room temperature.
2. Remove supernatant and resuspend pellet in 4°C freezing medium at a density of 10^6 to 10^7 cells/ml.

   Some laboratories freeze lymphoblastoid lines at the higher cell density because they plan to recover them in a larger volume of medium and because there may be a greater loss of cell viability upon recovery as compared to other types of cells (e.g., fibroblasts).

3. Transfer 1-ml aliquots of cell suspension into cryovials and freeze as for monolayer cultures.

THAWING AND RECOVERING HUMAN CELLS

SUPPORT PROTOCOL 2

When cryopreserved cells are needed for study, they should be thawed rapidly and plated at high density to optimize recovery.

CAUTION: Protective clothing, particularly insulated gloves and goggles, should be worn when removing frozen vials or ampules from the liquid nitrogen freezer. The room containing the liquid nitrogen freezer should be well-ventilated. Care should be taken not to spill liquid nitrogen on the skin.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Materials

Cryopreserved cells stored in liquid nitrogen freezer
70% (v/v) ethanol
Complete medium (e.g., DMEM or RPMI; see recipes) containing 10% to 20% FBS (see recipe), 37°C

1. Remove vial from liquid nitrogen freezer and immediately place it into a 37°C water bath. Agitate vial continuously until medium is thawed.

   The medium usually thaws in <60 sec. Cells should be thawed as quickly as possible to prevent formation of ice crystals that can cause cell lysis. Try to avoid getting water around the cap of the vial.

2. Wipe top of vial with 70% ethanol before opening.

   Some labs prefer to submerge the vial in 70% ethanol and air dry before opening.

3. Transfer thawed cell suspension into a sterile centrifuge tube containing 2 ml warm complete medium containing 20% FBS. Centrifuge 10 min at 150 to 200 × *g* (~1000 rpm in Fisher Centrific), room temperature. Discard supernatant.

Cells are washed with fresh medium to remove residual DMSO.

4. Gently resuspend cell pellet in small amount (~1 ml) of complete medium/20% FBS and transfer to properly labeled culture plate containing the appropriate amount of medium.

   Cultures are reestablished at a higher cell density than that used for original cultures because there is some cell death associated with freezing. Generally, 1 ml cell suspension is reseeded in 5 to 20 ml medium.

5. Check cultures after ~24 hr to ensure that cells have attached to the plate.

6. Change medium after 5 to 7 days or when pH indicator (e.g., phenol red) in medium changes color. Keep cultures in medium with 20% FBS until cell line is reestablished.

   If recovery rate is extremely low, only a subpopulation of the original culture may be growing; be extra careful of this when working with cell lines known to be mosaic.

SUPPORT PROTOCOL 3

DETERMINING CELL NUMBER AND VIABILITY WITH A HEMACYTOMETER AND TRYPAN BLUE STAINING

Determining the number of cells in culture is important in standardization of culture conditions and in performing accurate quantitation experiments. A hemacytometer is a thick glass slide with a central area designed as a counting chamber. Cell suspension is applied to a defined area and counted so cell density can be calculated.

The exact design of the hemacytometer may vary; the one described here is the Improved Neubauer from VWR (Fig. 1.1.1). The central portion of the slide is the counting platform, which is bordered by a 1-mm groove. The central platform is divided into two counting chambers by a transverse groove. Each counting chamber consists of a silver footplate on which is etched a 3 × 3–mm grid. This grid is divided into nine secondary squares, each 1 × 1 mm. The four corner squares and the central square are used for determining the cell count. The corner squares are further divided into 16 tertiary squares and the central square into 25 tertiary squares to aid in cell counting.

Accompanying the hemacytometer slide is a thick, even-surfaced coverslip. Ordinary coverslips may have uneven surfaces, which can introduce errors in cell counting; therefore, it is imperative that the coverslip provided with the hemacytometer be used in determining cell number.

Materials

70% (v/v) ethanol
Cell suspension
0.4% (w/v) trypan blue or 0.4% (w/v) nigrosin, prepared in HBSS (*APPENDIX 2A*)

Hemacytometer with coverslip (Improved Neubauer, Baxter Scientific)
Hand-held counter

NOTE: A disposable plastic hemacytometer, the C-Chip (DHC-N01), has exactly the same grid pattern as the Improved Neubauer. It is a single-use device available from INCYTO (*http://www.incyto.com*).

Prepare hemacytometer

1. Clean surface of hemacytometer slide and coverslip with 70% alcohol.

   Coverslip and slide should be clean, dry, and free from lint, fingerprints, and watermarks.

2. Wet edge of coverslip slightly with tap water and press over grooves on hemacytometer so the coverslip rests evenly over the silver counting area.

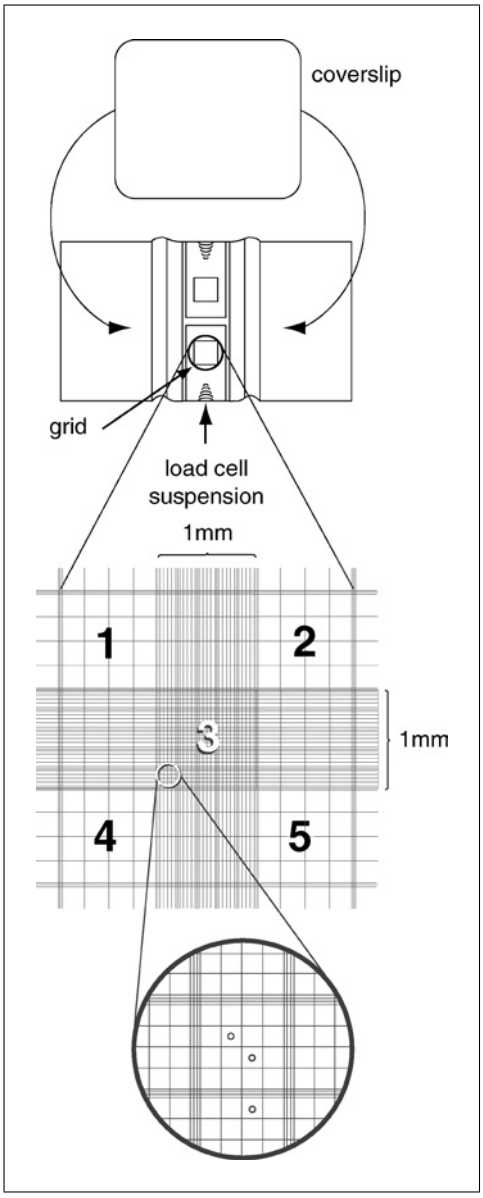


Figure 1.1.1 Hemacytometer slide (Improved Neubauer) and coverslip. Coverslip is applied to slide and cell suspension is added to counting chamber using a Pasteur pipet. Each counting chamber has a 3 × 3–mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added).

Prepare cell suspension

3. For cells grown in monolayer cultures, detach cells from surface of dish using trypsin (see Basic Protocol).

4. Dilute cells as needed to obtain a uniform suspension. Disperse any clumps.

   When using the hemacytometer, a maximum cell count of 20 to 50 cells per 1-mm square is recommended.

Load hemacytometer

5. Use a sterile Pasteur pipet to transfer cell suspension to edge of hemacytometer counting chamber. Hold tip of pipet under the coverslip and dispense one drop of suspension.

   Suspension will be drawn under the coverslip by capillary action.

   Hemacytometer should be considered nonsterile. If cell suspension is to be used for cultures, do not reuse the pipet and do not return any excess cell suspension in the pipet to the original suspension.

6. Fill second counting chamber.

Count cells

7. Allow cells to settle for a few minutes before beginning to count. Blot off excess liquid.

8. View slide on microscope with 100× magnification.

   A 10× ocular with a 10× objective = 100× magnification.

   Position slide to view the large central area of the grid (section 3 in Fig. 1.1.1); this area is bordered by a set of three parallel lines. The central area of the grid should almost fill the microscope field. Subdivisions within the large central area are also bordered by three parallel lines and each subdivision is divided into sixteen smaller squares by single lines. Cells within this area should be evenly distributed without clumping. If cells are not evenly distributed, wash and reload hemacytometer.

9. Use a hand-held counter to count cells in each of the four corner and central squares (Fig. 1.1.1, squares numbered 1 to 5). Repeat counts for other counting chamber.

   Five squares (four corner and one center) are counted from each of the two counting chambers for a total of ten squares counted.

   Count cells touching the middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines on the bottom or right side of the square.

Calculate cell number

10. Determine cells per ml by the following calculations:

    cells/ml = average count per square × dilution factor × 10^4
    total cells = cells/ml × total original volume of cell suspension from which sample was taken.

    The volume correction factor for the hemacytometer is 10^4: each square is 1 × 1 mm and the depth is 0.1 mm.

Stain cells with trypan blue to determine cell viability

11. Determine number of viable cells by adding 0.5 ml of 0.4% trypan blue, 0.3 ml HBSS, and 0.1 ml cell suspension to a small tube. Mix thoroughly and let stand 5 min before loading hemacytometer.

    Either 0.4% trypan blue or 0.4% nigrosin can be used to determine the viable cell number. Nonviable cells will take up the dye, whereas live cells will be impermeable to it.

12. Count total number of cells and total number of viable (unstained) cells. Calculate percent viable cells as follows:

$$\%\ \text{viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

13. Decontaminate coverslip and hemacytometer by rinsing with 70% ethanol and then deionized water. Air dry and store for future use.

SUPPORT PROTOCOL 4

PREPARING CELLS FOR TRANSPORT

Both monolayer and suspension cultures can easily be shipped in 25-cm^2 tissue culture flasks. Cells are grown to near confluency in a monolayer or to desired density in suspension. Medium is removed from monolayer cultures and the flask is filled with fresh medium. Fresh medium is added to suspension cultures to fill the flask. *It is essential that the flasks be completely filled with medium to protect cells from drying if flasks are inverted during transport*. It is also imperative that the flasks have nonvented caps. The

cap is tightened and taped securely in place. The flask is sealed in a leak-proof plastic bag or other leak-proof container designed to prevent spillage in the event that the flask should become damaged. The primary container is then placed in a secondary insulated container to protect it from extreme temperatures during transport. A biohazard label is affixed to the outside of the package. Generally, cultures are transported by same-day or overnight courier.

Cells can also be shipped frozen. The vial containing frozen cells is removed from the liquid nitrogen freezer and placed immediately on dry ice in an insulated container to prevent thawing during transport.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. Suitable suppliers for media and components include Invitrogen, Cambrex, Hyclone, and Sigma-Aldrich. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete DMEM

Dulbecco's modified Eagle medium, high-glucose formulation (e.g., Invitrogen), containing:

5%, 10%, or 20% (v/v) FBS (optional; see recipe)
1% (v/v) nonessential amino acids
2 mM L-glutamine (see recipe)
100 U/ml penicillin
100 μg/ml streptomycin sulfate
Filter sterilize and store ≤1 month at 4°C

Throughout this manual, the percentage of serum (usually fetal bovine serum) used in a protocol step is indicated by a numeral hyphenated to the base medium name. Thus, "complete DMEM-10" indicates that 10% FBS is used. Absence of a numeral indicates that no serum is used.

DMEM containing 4500 mg/liter D-glucose can be obtained from Invitrogen. DMEM is also known as Dulbecco's minimum essential medium.

Complete RPMI

RPMI 1640 medium (e.g., Invitrogen) containing:

2%, 5%, 10%, 15%, or 20% FBS (optional; see recipe)
2 mM L-glutamine (see recipe)
100 U/ml penicillin
100 μg/ml streptomycin sulfate
Filter sterilize and store ≤1 month at 4°C

FBS (fetal bovine serum)

Thaw purchased fetal bovine serum (shipped on dry ice and kept frozen at −20°C until needed). Store 3 to 4 weeks at 4°C. If FBS is not to be used within this time, aseptically divide into smaller aliquots and refreeze until used. Store ≤1 year at −20°C.

Repeated thawing and refreezing should be avoided as it may cause denaturation of the serum.

In some cases, heat inactivation may be warranted (see Culture Medium Preparation). To inactivate FBS, heat 30 to 60 min in a 56°C water bath. Alternatively, FBS may be inactivated through radiation treatment.

L-Glutamine, 0.2 M (100×)

Thaw frozen L-glutamine, aliquot aseptically into usable portions, then refreeze. For convenience, L-glutamine can be stored in 1-ml aliquots if 100-ml bottles of medium are used, and in 5-ml aliquots if 500-ml bottles are used. Store ≤1 year at −20°C.

Many laboratories supplement medium with 2 mM L-glutamine—1% (v/v) of 100× stock—just prior to use.

Trypsin/EDTA solution

Prepare in sterile HBSS (APPENDIX 2A) or 0.9% (w/v) NaCl:

0.25% (w/v) trypsin
0.2% (w/v) EDTA
Store ≤1 year (until needed) at −20°C

Most laboratories prefer to purchase trypsin/EDTA as a prepared solution, which is available from vendors including Sigma-Aldrich, Invitrogen, and Cambrex. This is a convenient and cost-effective alternative to preparing the solution within the laboratory. Trypsin/EDTA solution is available in various concentrations including 10×, 1×, and 0.25% (w/v). It is received frozen from the manufacturer and can be thawed and aseptically aliquotted into smaller volumes. Specific applications may require different concentrations of trypsin; the appropriate methods should be consulted for details.

EDTA (disodium ethylenediamine tetraacetic acid) is added as a chelating agent to bind Ca^{2+} and Mg^{2+} ions that can interfere with the action of trypsin.

COMMENTARY

Background Information

At its inception in the early twentieth century, tissue culture was applied to the study of tissue fragments in culture. New growth in culture was limited to cells that migrated out from the initial tissue fragment. Tissue culture techniques evolved rapidly, and since the 1950s culture methods have allowed the growth and study of dispersed cells in culture (Freshney, 2005). Cells dispersed from the original tissue can be grown in monolayers and passaged repeatedly to give rise to a relatively stable cell line.

Four distinct growth stages have been described for primary cells maintained in culture. First, cells adapt to the in vitro environment. Second, cells undergo an exponential growth phase lasting through ~30 passages. Third, the growth rate of cells slows, leading to a progressively longer generation time. Finally, after 40 or 50 passages, cells begin to senesce and die (Lee, 1991). It may be desirable to study a particular cell line over several months or years, so monolayer cultures can be preserved to retain the integrity of the cell line. Aliquots of early-passage cell suspensions are frozen, then thawed, and cultures reestablished as needed. Freezing monolayer cultures prevents changes due to genetic drift and avoids loss of cultures due to senescence or accidental contamination (Freshney, 2005).

Cell lines are commercially available from a number of sources, including the American Type Culture Collection (ATCC; *http://www.atcc.org*) and the Human Genetic Mutant Cell Repository at the Coriell Cell Repository (CCR; *http://www.coriell.org*). These cell repositories are a valuable resource for researchers who do not have access to suitable patient populations. An up-to-date listing of available cell lines will be provided upon request. The cell lines are preserved in liquid nitrogen, and information on the characteristics are supplied by the distributor. Immortalized cell lines can be maintained in culture indefinitely, while nontransformed cells have a limited life-span in vitro. For human fibroblasts, about 20 population doublings occur between the initial growth in the primary culture and the first subculture (Priest, 1997). About 50 to 60 population doublings is the standard rule for maintaining fibroblast cell lines in culture before risking the loss of cell lines through senescence, cytogenetic or biochemical changes, or contamination. If it is anticipated that these cells will be needed for future study, cells should be frozen at an early culture stage and retrieved for future use (Verma and Babu, 1995).

Critical Parameters

Use of aseptic technique is essential for successful tissue culture. Cell cultures can be contaminated at any time during handling, so precautions must be taken to minimize the chance of contamination. All supplies and reagents that come into contact with cultures must be sterile, and all work surfaces should be kept clean and free from clutter.

Cultures should be 75% to 100% confluent when selected for subculture. Growth in monolayer cultures will be adversely affected if cells are allowed to become overgrown. Passaging cells too early will result in a longer lag time before subcultures are established. Following dissociation of the monolayer by trypsinization, serum or medium containing serum should be added to the cell suspension to stop further action by trypsin that might be harmful to cells.

When subculturing cells, add a sufficient number of cells to give a final concentration of $\sim 5 \times 10^4$ cells/ml in each new culture of human cell lines. Optimal concentrations are cell-type specific. Package inserts provided with commercially available cell lines contain information on recommended cell density, culture media, and appropriate culture temperatures. Cells plated at too low a density may be inhibited or delayed in entry into growth stage. Cells plated at too high a density may reach confluence before the next scheduled subculturing; this could lead to cell loss and/or cessation of proliferation. The growth characteristics for different cell lines vary. A lower cell concentration (10^4 cells/ml) may be used to initiate subcultures of rapidly growing cells, and a higher cell concentration (10^5 cells/ml) may be used to initiate subcultures of more slowly growing cells. Adjusting the initial cell concentration permits establishment of a regular, convenient schedule for subculturing—e.g., once or twice a week (Freshney, 2005).

Cells in culture will undergo changes in growth, morphology, and genetic characteristics over time. Such changes can adversely affect reproducibility of laboratory results. Nontransformed cells will undergo senescence and eventual death if passaged indefinitely. The time of senescence will vary with cell line, but generally at between 40 and 50 population doublings fibroblast cell lines begin to senesce. Cryopreservation of cell lines will protect against these adverse changes and will avoid potential contamination.

Cultures selected for cryopreservation should be in log-phase growth and free from contamination. Cells should be frozen at a concentration of 10^6 to 10^7 cells/ml. Cells should be frozen gradually and thawed rapidly to prevent formation of ice crystals that may cause cells to lyse. Cell lines can be thawed and recovered after long-term storage in liquid nitrogen. The top of the freezing vial should be cleaned with 70% alcohol before opening to prevent introduction of contaminants. To aid in recovery of cultures, thawed cells should be reseeded at a higher concentration than that used for initiating primary cultures. Careful records regarding identity and characteristics of frozen cells as well as their location in the freezer should be maintained to allow for easy retrieval.

For accurate cell counting, the hemacytometer slide should be clean, dry, and free from lint, scratches, fingerprints, and watermarks. The coverslip supplied with the hemacytometer should always be used because it has an even surface and is specially designed for use with the counting chamber. Use of an ordinary coverslip may introduce errors in cell counting. If the cell suspension is too dense or the cells are clumped, inaccurate counts will be obtained. If the cell suspension is not evenly distributed over the counting chamber, the hemacytometer should be washed and reloaded.

Anticipated Results

Confluent cell lines can be successfully subcultured in the vast majority of cases. The yield of cells derived from monolayer culture is directly dependent on the available surface area of the culture vessel (Freshney, 2005). Overly confluent cultures or senescent cells may be difficult to trypsinize, but increasing the time of trypsin exposure will help dissociate resistant cells. Cell lines can be propagated to get sufficient cell populations for cytogenetic, biochemical, and molecular analyses.

It is well accepted that anyone can successfully freeze cultured cells; it is thawing and recovering the cultures that presents the problem. Cultures that are healthy and free from contamination can be frozen and stored indefinitely. Cells stored in liquid nitrogen can be successfully thawed and recovered in over 95% of cases. Several aliquots of each cell line should be stored to increase the chance of recovery. Cells should be frozen gradually, with a temperature drop of $\sim 1^\circ$C per minute, but thawed rapidly. Gradual freezing and rapid thawing prevents formation of ice crystals that might cause cell lysis.

Accurate cell counts can be obtained using the hemacytometer if cells are evenly dispersed in suspension and free from clumps. Determining the proportion of viable cells in a population will aid in standardization of experimental conditions.

Time Considerations

Establishment and maintenance of mammalian cell cultures require a regular routine for preparation of media and feeding and passaging cells. Cultures should be inspected regularly for signs of contamination and to determine if the culture needs feeding or passaging.

Literature Cited

Barch, M.J., Lawce, H.J., and Arsham, M.S. 1991. Peripheral Blood Culture. *In* The ACT Cytogenetic Laboratory Manual, 2nd ed. (M.J. Barch, ed.) pp. 17-30. Raven Press, New York.

Freshney, R.I. 2005. Culture of Animal Cells. A Manual of Basic Techniques, 5th ed. Wiley-Liss, New York.

Hyclone Labs. 1996. Heat inactivation: Are you wasting your time? *Art to Science* 15:1-5.

Knutsen, T. 1991. The ACT Cytogenetic Laboratory Manual, 2nd ed. (M.J. Barch, ed.) pp. 563-587. Raven Press, New York.

Lee, E.C. 1991. Cytogenetic Analysis of Continuous Cell Lines. *In* The ACT Cytogenetic Laboratory Manual, 2nd ed. (M.J. Barch, ed.) pp. 107-148. Raven Press, New York.

Newman, C. 2003. Serum-free cell culture—the ethical, scientific and economic choice. *The Biomedical Scientist*, pp. 941-942.

Priest, J.H. 1997. General cell culture principles and fibroblast culture. *In* The AGT Cytogenetics Laboratory Manual, 3rd ed. (M.J. Barch, T. Knutsen, and J.L. Spurbeck, eds.). Lippincott-Raven, Philadelphia.

Rooney, D.E. and Czepulskowski, B.H. (eds). 2001. Human Cytogenetics: Constitutional Analysis: A Practical Approach, 3rd ed. Oxford University Press, New York.

Triglia, R.P. and Linscott, W.D. 1980. Titers of nine complement components, conglutinin and C3b inactivator in adult and fetal bovine sera. *Mol. Immunol*. 17:741-748.

Verma, R.S. and Babu, A. 1995. Human Chromosomes: Principles and Techniques, 2nd ed. McGraw-Hill, New York.

Key Reference

Lee, 1991. See above.

Contains pertinent information on cell culture requirements, including medium preparation and sterility. Also discusses trypsinization, freezing and thawing, and cell counting.

Internet Resources

http://www.unc.edu/depts/tcf/info.html

This site contains troubleshooting information for treating tissue culture contamination, including discussion of contamination risks and recommendations.

http://www.cdc.gov/od/ohs.pddfiles/BCS-3.pdf

Primary Containment for Biohazards: Selection, Installation, and Use of Biological Safety Cabinets, 2nd ed. Sept 2001. U.S. Dept of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health, U.S. Government Printing Office, Washington.

http://www.yale.edu/oehs/PDG_files/cad_07_01.pdf

Clean Air Device (Primary Containment Device) Program Guide, July 2001, Yale University, Office of Environmental Health and Safety, 135 College Street, New Haven, Conn.

http://www.ehs.cornell.edu/bio/cabinets.htm

This site contains a comprehensive discussion of the use of biological safety cabinets, including operational procedures and the advantages and disadvantages of ultraviolet lights.

Media for Culture of Mammalian Cells

In culturing mammalian cells in vitro, one attempts to reproduce in a culture vessel the physiological environment and characteristic responses of individual cell types. This simple idea is not easily realized because the physiological environments of most cells are not yet completely characterized, and because the complex regulatory interactions between neighboring cells within tissues are still being elucidated. However, a body of knowledge about the survival and growth requirements of cells has been empirically derived over the past 90 years, making it possible to maintain and to propagate a variety of normal, immortalized, and transformed cell types in vitro. This received knowledge has directly influenced the compositions of culture media and culture substrata that are in use today, and it continues to evolve as the requirements of individual cell types become more explicitly defined. Part of this evolution may be founded on the ongoing discovery of novel growth factors, growth mediators, and survival factors that comes about as the range of specialized cells that can be propagated in vitro is extended. It may be further shaped by advances in understanding of the signaling mechanisms by which culture substrata and other external stimuli influence cells to proliferate, differentiate, become quiescent, or die.

At a minimum, the fluid medium in which cells are cultured must provide for their nutritional requirements, provide an energy source, maintain pH, and provide a level of osmolarity compatible with cell viability. In addition, the environment in which cultures are kept, typically a water-jacketed CO_2 incubator, must provide a constant temperature, humidity to prevent evaporation of medium, O_2 for respiration, and CO_2 for maintenance of the pH of bicarbonate-buffered medium. Culture media commonly used today consist of two parts: a basal nutrient medium and supplements. The basal nutrient medium, such as Dulbecco's modified Eagle's medium (DMEM; also known as Dulbecco's minimal

Table 1.2.1 Tissue Culture Products and Suggested Suppliers[a]

Supplier	Culture-related products
Bayer	Fatty acid–free BSA
Becton Dickinson Labware	Growth factors, hormones, Matrigel
Cell Systems	Specialized serum-free media
Chemicon International	Low-density lipoprotein
Clonetics	Specialized serum-free media
Corning Costar	Filter-sterilization apparatus, tissue culture vessles
Difco Laboratories	Agar
Gelman Sciences	Filter-sterilization apparatus
Life Technologies	Basal nutrient media, antibiotics
Hyclone Laboratories	Sera
J.T. Baker	Chemicals
Millipore	Water-purification system
Nalgene	Filter-sterilization apparatus
Research Organics	HEPES
Sigma Chemical	Inorganic and organic chemicals, BSA fraction V, FBS, sterols, antibiotics, growth factors, hormones, tissue culture vessels
Steraloids	Sterols
Upstate Biotechnology Inc. (UBI)	Insulin, transferrin, FBS, type I collagen, growth factors, hormones

[a]See *SUPPLIERS APPENDIX* for suppliers' addresses.

Contributed by J. Denry Sato and Mikio Kan
Current Protocols in Cell Biology (1998) 1.2.1-1.2.15

Eagle's medium), RPMI 1640, or Ham's F-12, is a buffered aqueous solution of inorganic salts, vitamins, amino acids and other anabolic precursors, energy sources such as glucose and glutamine, and trace elements. Supplements are either undefined, such as fetal bovine serum (FBS), tissue extracts, and conditioned medium, or defined, such as hormones and growth factors, transport proteins, and attachment factors. The compositions of basal nutrient media and medium supplements may vary considerably; however, both components of the complete medium are necessary for support of cell viability and proliferation.

The basic protocols in this unit describe the preparation of serum-supplemented medium (see Basic Protocol 1); serum-reduced or serum-free medium (see Basic Protocol 2); selective medium, such as HAT (see Basic Protocol 3) or cholesterol- and serum-free medium (see Alternate Protocol); and soft agar for anchorage-independent growth (see Basic Protocol 4). The support protocols discuss the use of pH buffers (see Support Protocol 1) and antibiotics (see Support Protocol 2) in culture media. Suppliers for culture products are listed in Table 1.2.1.

NOTE: For all solutions and media, use water purified using a reverse osmosis system (e.g., Milli-Q system from Millipore) and vessels that are thoroughly rinsed with water after use but never washed with detergent.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

BASIC PROTOCOL 1

PREPARATION OF SERUM-CONTAINING MEDIA

Many cell types, especially fibroblasts and transformed cells, survive and proliferate to some extent when cultured in a basal nutrient medium supplemented with 5% to 20% (v/v) serum. The degree of cell proliferation achieved under these nonoptimized conditions may be sufficient for the goals of the experiment as long as the cellular properties of interest are not lost. It is worth remembering that if tissue samples consisting of more than one cell type are cultured, this culture environment may confer a growth advantage to fibroblasts. In addition, the medium may select against the propagation of some types of cells: a number of epithelial cell types cease proliferating and differentiate in the "physiological" Ca^{2+} concentrations of commonly used serum-supplemented basal media. Finally, a serum-supplemented nutrient solution does not approximate a physiological environment for any cells except those in a healing wound.

Materials

Basal nutrient medium, such as DMEM, Ham's F-12, or RPMI 1640 (*APPENDIX 2B*; see Critical Parameters for discussion of medium selection)
HEPES (e.g., Research Organics)
Sodium bicarbonate (e.g., J.T. Baker)
Glutamine and pyruvic acid (e.g., Sigma)
Penicillin G and streptomycin sulfate (e.g., Sigma)
5 N NaOH (e.g., J.T. Baker)
Serum (e.g., Hyclone, Sigma, UBI; see Critical Parameters for discussion of fetal bovine serum)

Filter sterilization units (e.g., Nalge Nunc, Corning, Gelman)
0.2-µm-pore-size filters (e.g., Nalge Nunc, Corning, Gelman)

Additional reagents and equipment for culture of mammalian cells (*UNIT 1.1*)

1. Dissolve powdered medium with constant stirring in a 0.8× to 0.9× volume of water.

   If a commercially prepared liquid medium is being used, add penicillin and streptomycin from commercial stock solutions and proceed to step 8.

2. Add an amount of HEPES that yields a concentration of 15 mM in the final volume of medium.

   Omit this step if the powdered medium is formulated with HEPES.

3. Add the amount of sodium bicarbonate recommended by the medium supplier for use in a CO_2-controlled atmosphere (e.g., 14 to 36 mM in 5% CO_2 atmosphere).

   Omit this step if the powdered medium contains sodium bicarbonate.

4. Add glutamine to give a final concentration of 2 mM and pyruvic acid to give a final concentration of 0.01% (w/v).

5. Add penicillin G to give a final concentration of 100 IU/ml and streptomycin to give a final concentration of 50 µg/ml.

   Other antibacterial agents or antifungal agents should not be routinely included in culture medium. Gentamicin at a final concentration of 50 µg/ml or kanamycin at 100 µg/ml may be useful in eliminating gram-positive and gram-negative bacteria from primary cultures or from irreplaceable cultures, but it is best to discard any cultures that are contaminated with bacteria, yeast, or fungi.

6. Adjust the pH of the medium to 7.4 with 5 N NaOH, and add water to achieve the final (1×) volume. Readjust the pH of the medium to 7.4, if necessary.

7. Sterilize the medium by filtration through a 0.2-µm filter. Store the medium at 4°C in the dark.

   Vacuum-operated filtering units or bottle-top filters are useful for small volumes of medium (0.1 to 2 liters), whereas filter capsules (2 to 5 liters) or filter stands (>10 liters) that are used under positive pressure are more suitable for larger volumes.

8. Add serum to the desired final concentration at the time of use.

   Basal nutrient medium and the serum supplement should be stored individually at 4°C, and the complete medium should be made up at the time of use and only in the volume necessary. Working volumes of serum should be stored at 4°C and used within several weeks. Serum should not be subjected to repeated freezing and thawing, but it can be stored for at least 2 years at −20°C with little deterioration in growth-promoting activity. In this way, medium components are not wasted, and the chances of detecting, isolating, and eliminating contamination with minimal losses are increased.

PREPARING MEDIA FOR REDUCED-SERUM OR SERUM-FREE GROWTH

BASIC PROTOCOL 2

The most obvious advantages of serum-free cell culture are that it costs less and it simplifies the purification of cell products. However, it is in the increased knowledge of cell physiology that the real value of serum-free medium lies. Both reduced-serum and serum-free media are intermediates in a continuum between completely undefined mixtures of biological fluids and chemically defined, protein-free medium. Although mixtures of biological fluids and protein-free medium have useful applications, neither extreme provides a suitable environment for studies of cellular physiology. At the current time, the most physiologically relevant culture medium for an isolated cell type is a defined, protein-supplemented medium consisting of required components at optimal concentrations and extracellular matrix constituents. This set of conditions can be approached for an increasing number of cell types, but for some cell types, a reduced-serum medium is the best that can be achieved. This state of affairs reflects incomplete knowledge of cell growth requirements, and it suggests that there are novel mediators of cell proliferation and differentiation that remain to be discovered.

Table 1.2.2 Components of Reduced-Serum and Serum-Free Medium[a]

Component	Final concentration	Stock concentration	Suggested supplier(s)
Undefined supplements			
Sera[b]	5% to 20% (v/v)	100% (v/v)	Hyclone, Sigma, or UBI
Pituitary extract	5 to 100 µg/ml	1 to 2 mg/ml	UBI, Clonetics
Conditioned medium	5% to 50% (v/v)	100% (v/v)	Not commercially available[c]
Energy sources			
Glucose	1 to 4.5 g/liter	None	Sigma
Glutamine	1 to 2 mM	None	Sigma
Attachment factors			
Collagen type I	10 to 50 µg/ml	3 to 4 mg/ml	UBI, Sigma
Fibronectin	1 to 10 µg/ml	0.5 to 1 mg/ml	Sigma
Vitronectin	1 to 10 µg/ml	0.5 to 1 mg/ml	Sigma
Hormone			
Insulin	1 to 10 µg/ml	1 mg/ml	Sigma, UBI
Carrier proteins			
Transferrin	5 to 30 µg/ml	1 mg/ml	Sigma, UBI
BSA, fatty acid–free	0.5 to 4 mg/ml	50 mg/ml	Bayer
Trace element			
Selenium, sodium salt	1 to 20 nM	2 µM	Sigma
Lipids and lipid precursors			
Ethanolamine	1 to 20 µM	2 mM	Sigma
Fraction V BSA	0.05 to 5 mg/ml	50 mg/ml	Sigma
Unsaturated fatty acids	1 to 10 µg/ml	20 to 50 mg/ml	Sigma
Sterols	1 to 20 µg/ml	2 to 4 mg/ml	Steraloids, Sigma
Low-density lipoprotein	1 to 20 µg/ml	1 to 2 mg/ml	Chemicon International

[a]Nonsterile stock solutions should be sterilized by filtration. Add glucose and glutamine as dry powder (or frozen aliquots that have been thawed) to reconstituted powdered medium.

[b]See Critical Parameters for discussion of FBS.

[c]Conditioned medium must be made in the investigator's laboratory, and the choice of cells used depends on the investigator's purpose.

If all the growth requirements of the cells of interest can be satisfied, the undefined medium supplement can be completely eliminated. The limitation of this approach is that not all of the cell growth regulators operating within tissues have been discovered. As the variety of cell types that can be cultured in vito for extended periods expands, new growth activities may be identified and novel growth factors purified.

Materials

Basal nutrient medium, such as DMEM, Ham's F-12, or RPMI 1640 (*APPENDIX 2B*; see Critical Parameters for discussion of medium selection)

Nutrients: inorganic salts, amino acids, and vitamins (e.g., Sigma)

Trace elements (e.g., Sigma)

Supplements: growth factors and hormones (e.g., Sigma, UBI, Becton Dickinson Labware) and other assorted medium components (Table 1.2.2)

Additional reagents and equipment for culture of mammalian cells (*UNIT 1.1*)

Optimize nutrient medium

1. Starting with a complete medium empirically determined to best support the growth of the cells of interest, reduce the concentration of the undefined medium supplement until cell proliferation is suboptimal but cell viability remains high.

2. Vary the concentrations of individual components of the nutrient medium in a cell proliferation assay. Vary the concentrations of components in the following groupings, which have been found to be limiting for proliferation of at least one cell type:

   a. energy sources (glucose and glutamine);
   b. trace elements and electrolytes (Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, selenium, $H_2PO_4^-$, and HCO_3^-);
   c. amino acids (glutamine, cystine, cysteine, histidine);
   d. vitamins (biotin, vitamin B_{12});
   e. lipids and lipid precursors (oleic acid, linoleic acid, or cholesterol conjugated to fatty acid–free BSA; undefined lipids bound to fraction V BSA; low-density lipoprotein; ethanolamine or phosphoethanolamine).

   Determine the optimal concentration range for the most limiting factor, and fix its concentration in the center of that range. Repeat the growth assays to sequentially identify and optimize each limiting factor.

   Concentrations of 0.1×, 1×, and 10× those listed in the chosen medium formulation are initially useful to determine which components are limiting factors for proliferation.

3. Lower the concentration of the undefined supplement and repeat the optimization procedure. Continue optimizing the nutrient medium until reductions in the concentration of the undefined medium supplement can no longer be compensated for.

   Each new supplement concentration is tested on an individual culture for a finite period (3 to 10 days). The best concentration, supporting the most cell growth, is incorporated into the next round of testing on new cultures.

Optimize medium supplements

4. Optimize medium supplements such as growth factors, hormones, transport proteins, and attachment factors either independently of or in conjunction with the nutrient medium components.

   The set of defined supplements that are required for an individual cell type may be less complex in the presence of an optimized nutrient medium. If an optimized nutrient medium is not being used, then a nutrient-rich basal medium such as DMEM/F-12 (see recipe) is recommended for supplement optimization (see Critical Parameters). As with nutrient optimization, the strategy is to decrease the concentration of an undefined supplement to give suboptimal growth with high cell viability and then to restore proliferation with optimized concentrations of defined supplements.

PREPARATION OF SELECTIVE MEDIA: HAT MEDIUM

BASIC PROTOCOL 3

Culture medium can be used to intentionally select mammalian cells with desired properties from a mixed population of cells. This strategy is founded on the proven effectiveness of selective media in selecting strains of mutant microorganisms that, for example, cannot grow in minimal media but thrive in a medium supplemented with one particular nutrient. Although mammalian cells with desired characteristics proliferate in selective media, in this case selective pressure is applied to inhibit or, preferably, to kill cells that do not possess those characteristics. Thus, the efficacy of a selective medium depends on the efficiency with which it eliminates unwanted cells and on the degree to which the selected phenotypic trait is expressed. Because monoclonal antibodies are commonly used reagents in cell and molecular biology, the use of selective media that target differences in metabolic pathways is illustrated with two protocols for selecting hybridomas (see Background Information for further discussion). In both protocols, unfused spleen cells do not survive 7 to 10 days.

Materials

Spleen cell × myeloma fusion products (10:1)
RD medium (Life Technologies) with 10% FBS (Hyclone; see recipe)
4×10^{-5} M aminopterin (A solution; 100× stock in 0.1 N NaOH)
1×10^{-5} M hypoxanthine/1.6×10^{-3} M thymidine in water (HT solution; 100× stock)
HAT medium: RD/10% FBS/1× A solution/1× HT solution
96-well tissue culture plates

Additional reagents and equipment for culture of mammalian cells (*UNIT 1.1*)

1. Resuspend the fusion products in RD/10% FBS at a concentration of 1×10^6 myeloma cells per ml. Add 0.01× volumes of A and HT solutions to the cell suspension to make HAT selection medium.
2. Plate 0.1 ml of cell suspension per well into 96-well plates. Incubate the plates in a humidified 37°C, 5% CO_2 incubator.
3. Every 2 or 3 days, remove half the existing medium from the wells by aspiration and replace with fresh HAT medium.
4. After 21 days, screen hybridoma supernatants for the presence of the antibodies of interest.
5. Wean hybridomas stepwise from HAT medium by transferring them to HT-supplemented medium and then to RD/10% FBS over a 2-week period. Replace 50% of the medium with HT medium four times at 3-day intervals. Replace 50% of the medium with RD/10% FBS at 3-day intervals.

Aminopterin is toxic, so it is advisable to wean hybridomas from HAT medium as soon as possible.

ALTERNATE PROTOCOL

PREPARATION OF SELECTIVE MEDIA: CHOLESTEROL-FREE, SERUM-FREE MEDIUM

Most of the mouse myeloma cell lines survive in serum-supplemented medium, but they die in the absence of cholesterol (see Background Information for explanation). This conditional lethal defect has been exploited to create an alternative selection process for hybridomas using cholesterol-free medium. Because they are produced by fusion with spleen cells capable of producing cholesterol, NS-1 hybridomas survive in cholesterol-free medium, but the parent NS-1 myeloma cells are selected against. This selective medium allows for the outgrowth of up to 10 times as many hybridomas as HAT medium. This procedure can be used with any myeloma cell line that is unable to synthesize cholestrol.

Materials

Spleen cell × NS-1 myeloma fusion products (10:1)
RD medium with 5F supplement (see recipes)
100× BSA–oleic acid conjugate solution: fatty acid–free BSA (e.g., Bayer; 50 mg/ml) conjugated with oleic acid (e.g., Sigma; 500 μg/ml) in PBS
96-well tissue culture plates

Additional reagents and equipment for culture of mammalian cells (*UNIT 1.1*)

1. Make complete RD/5F medium by adding appropriate volumes of the stock solutions of insulin, transferrin, ethanolamine, 2-mercaptoethanol, and sodium selenite (e.g., Sigma) to RD medium. Supplement the medium with 1/100 vol of 100× BSA–oleic acid conjugate.

BSA–oleic acid acid conjugate is available from Sigma. For a protocol, see Kawamoto et al. (1983).

2. Resuspend fusion products at 2 or 3×10^5 NS-1 cells per milliliter of medium.
3. Plate 0.1 ml of cell suspension per well in 96-well plates. Incubate the plates in a humidified 37°C, 5% CO_2 incubator.
4. Add 0.5× volume of fresh medium to the wells every 2 or 3 days after removing half the existing medium by aspiration.
5. Screen hybridoma supernatants for antibodies of interest after 10 to 14 days. Maintain hybridomes in RD/SF medium supplemented with BSA–oleic acid.

GROWTH OF TRANSFORMED CELLS IN SOFT AGAR

BASIC PROTOCOL 4

Malignantly transformed cells can differ from their normal counterparts in a number of respects. Chief among these differences are a loss of contact-inhibited growth, the acquisition of an infinite life span, and the ability to form tumors in animal hosts. Freedman and Shin (1974) found that there was a general correlation between the tumorigenic potential of transformed cells in vivo and their ability to grow in an anchorage-independent manner in vitro. Although this generalization does not hold for every transformed cell, growth in soft agar can be used as a surrogate in vitro assay for transformation and tumorigenicity. The advantages of this method are that it is relatively easy, it may take much less time than an in vivo tumorigenesis assay, and it does not require the maintenance and care of experimental animals. However, if growth in soft agar is used as a measure of transformation of cells that have been manipulated in vitro, an in vivo tumorigenesis assay is required to determine whether the cells are malignantly transformed.

Materials

2% (w/v) agar (e.g., Difco; see recipe)
Basal nutrient medium, such as DMEM, Ham's F-12, or RPMI 1640 (*APPENDIX 2B*; see Critical Parameters for discussion of medium selection), with 24.6% and 20% FBS
Single-cell suspension
12-well culture plates (e.g., Corning Costar)
15-ml polycarbonate conical centrifuge tubes (e.g., Sarstadt), sterile

1. For each set of replicate wells, add 2.25 ml of 2% agar solution to 9.75 ml of medium containing 24.6% FBS to give a solution of 0.375% agar in 20% FBS. Dispense 2-ml aliquots into five sterile 15-ml polypropylene conical tubes and incubate at 45°C.
2. To separate tubes, add 50, 100, 200, 500, or 1000 cells to a final volume of 0.5 ml medium with 20% FBS. Prepare an additional set of tubes for each set of replicate cultures.
3. Add each cell suspension to an aliquot of agar solution, mix, and quickly pour into a well of a 12-well plate.
4. Incubate the plate at 37°C in a humid atmosphere of 5% CO_2 until cell colonies appear.
5. Count colonies >32 cells (five doublings) under phase contrast with an inverted microscope, and calculate colony formation efficiency (percentage of plated cells that formed colonies).

To prevent any cell attachment to the plastic substratum, cells in 0.3% agar can be overlaid on a preformed layer of 0.5 ml of 0.5% agar in medium supplemented with 20% FBS.

SUPPORT PROTOCOL 1

pH CONTROL IN MEDIA

Most cell lines proliferate in medium with a pH of 7.4, and they exhibit decreased viability and rates of proliferation as the medium becomes progressively more acidic or more basic (Eagle, 1973). Culture media must buffer the CO_2 and lactic acid produced as cells metabolize glucose and glutamine. Historically, bicarbonate, HCO_3^-, in conjunction with atmospheric CO_2, has been used as a buffering system. Each basal medium formulation has a recommended concentration for sodium bicarbonate, usually 20 to 40 mM, to maintain pH and osmolarity. Media that are to be incubated in an elevated CO_2 atmosphere contain higher concentrations of bicarbonate than those designed to be used at ambient CO_2 levels. Most cell culture media require an atmosphere of 5% CO_2 to maintain pH 7.4. However, certain media contain levels of bicarbonate that require different amounts of CO_2. For example, DMEM containing 3700 mg/liter of sodium bicarbonate equilibrates to ~pH 7.6 in a 5% CO_2 environment and requires 10% CO_2 to maintain pH 7.4. The low pK_a of bicarbonate (pK_a = 6.1) makes it a poor buffer around pH 7.4, and, in the absence of atmospheric CO_2, the breakdown of H_2CO_3 formed from bicarbonate releases CO_2 that comes out of solution, causing a rise in pH. With the development of Good buffers (Good et al., 1966), nontoxic buffering agents effective in the pH range of 6 to 8, such as PIPES (pK_a = 6.8), MOPS (pK_a = 7.2), TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; pK_a = 7.5), and HEPES (pK_a = 7.55), became available to the research community. HEPES in a concentration range of 10 mM to 25 mM has become a standard buffer in serum-free medium, but it is used in addition to and not in place of the bicarbonate and CO_2 system. Phenol red is an indicator dye that is commonly added to medium to provide a visual assessment of pH. Red at pH 7.4, it becomes orange (pH 7.0) and then yellow (pH 6.5) as the pH decreases; it turns violet (pH 7.6) and purple (pH 7.8) as the pH rises. Culture medium should generally be replaced as the phenol red changes from orange to yellow, which reflects the accumulation of lactic acid.

Materials

Powdered medium without $NaHCO_3$ or HEPES
HEPES (e.g., Research Organics)
$NaHCO_3$ (e.g., J.T. Baker)

1. Dissolve powdered medium in water with gentle stirring.
2. Add HEPES (mol. wt. 238.3) to give a final concentration of 15 mM, and stir until dissolved.
3. Add $NaHCO_3$ to the recommended concentration for the basal nutrient medium being used and stir.
4. Add other medium components, and adjust the pH to 7.4 (see Basic Protocol 1).
5. Filter sterilize the medium (see Basic Protocol 1, step 7).

   Filtering medium under vacuum may cause the pH to increase slightly. A small change in pH need not be compensated because other factors such as medium supplements, temperature, atmospheric pressure, and atmospheric CO_2 levels can also affect pH.

6. Check the pH of the complete medium after it has equilibrated with incubator temperature and CO_2 atmosphere.

USE OF ANTIBIOTICS IN MEDIA

Antibiotics can be added to culture media to eliminate microbial contaminants (Perlman, 1979). The most common contaminants encountered are bacteria, yeast, other fungi, and mycoplasma, and the most common routes of contamination are operator error and nonsterile medium components. Of the common microbial contaminants, yeast and other fungi are very difficult to eradicate, and it is recommended that all heavily contaminated cultures be discarded unless the cells cannot be replaced. Thus, it is wise to maintain duplicate cultures of important cells and to cryopreserve cell lines as soon as possible. Penicillin and streptomycin are broad-spectrum antibacterial agents that are often added to culture media (see Basic Protocol 1 and Table 1.2.3). However, the routine use of antibiotics in media is not recommended, because when used to compensate for poor aseptic technique, they may select for antibiotic-resistant strains of microorganisms. Antibiotics may be used to best effect in primary cultures of cells for which the sterility of the tissue samples is in doubt. The antifungal agent amphotericin B should be used sparingly, as it is toxic to mammalian cells (Perlman, 1979), and it may select for the property of cholesterol auxotrophy (Sato et al., 1987). Mycoplasma and viruses are too small to be retained by 0.2-µm sterilization filters; mycoplasma can be treated with gentamicin or kanamycin, but viruses cannot be treated with antibiotics. There is no reliable method for eliminating viral contaminants from cell cultures.

To be effective in culture, antibiotics must have the following characteristics: they must completely eliminate the microbial contaminant; they must not affect the viability or metabolism of mammalian cells; and they must be compatible with medium components in an aqueous environment. In addition, because the identity of a contaminating species is usually unknown, antibiotics should act on a broad spectrum of mircoorganisms. A list of antibiotics commonly used in culture media is provided in Table 1.2.3.

Materials

Antibiotic
Sterile solvent
0.2-µm-pore-size sterilizing filter

1. Dissolve the antibiotic at a 100× or greater concentration in an appropriate sterile solvent. If it is highly soluble in an aqueous solvent, use water or PBS.
2. If the antibiotic was not obtained in a sterile form, filter the solution through a 0.2-µm filter.
3. Store the antibiotic solution at 4°C prior to use or at −20°C for long-term storage.
4. Add the antibiotic to medium immediately prior to use.

Table 1.2.3 Some Antibiotics Used in Culture Media and Their Microbial Targets

Antibiotic	Concentration	Microbial targets
Amphotericin B	2.5 µg/ml	Yeast and other fungi
Ampicillin	100 µg/ml	Gram-positive and -negative bacteria
Chloramphenicol	5 µg/ml	Gram-negative bacteria
Gentamicin	50 µg/ml	Gram-positive and -negative bacteria, mycoplasma
Kanamycin	100 µg/ml	Gram-positive and -negative bacteria, mycoplasma
Penicillin G	100 IU/ml	Gram-positive bacteria
Streptomycin	100 µg/ml	Gram-positive and -negative bacteria
Tetracycline	10 µg/ml	Gram-positive and -negative bacteria, mycoplasma

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agar

Dissolve 2% (w/v) agar (e.g., Difco) in water by boiling it or heating it in a microwave. Maintain soft agar at 45°C.

DMEM/F12 medium, supplemented

Make 1:1 (v/v) mixture of DMEM and F-12. Filter sterilize the medium and store at 4°C. Add the appropriate volume of serum at the time of use.

Five factors (5F) supplement

Make the following 200× stock solutions:
2 mg/ml insulin in 10 mM HCl
2 mg/ml transferrin in PBS
2 mM ethanolamine in water
2 mM 2-mercaptoethanol in water
2 μM sodium selenite in water
Filter sterilize the stock solution and store at 4°C prior to use
Add 0.5 ml of each stock solution per 100 ml of medium

RD medium, supplemented

Make 1:1 (v/v) mixture of RPMI 1640 and DMEM. Add glutamine to 2 mM, penicillin G to 100 IU/ml, and streptomycin to 50 μg/ml. Filter sterilize the medium and store at 4°C. Add the appropriate volume of serum at the time of use.

COMMENTARY

Background Information

It is beyond the scope of this unit to describe all of the medium formulations that have been developed for mammalian cell culture. The following references, which provide surveys of media and conditions that have been used successfully to culture numerous cell lines and a variety of cell types, are recommended as sources of this information: Bottenstein et al. (1979); Jacoby and Pastan (1979); Barnes et al. (1984); Freshney (1987, 1992); Baserga (1989); Davis (1994); and Sato et al. (1994).

Because the immediate aim of the cell culturist is to maintain or expand a population of cells, culture media have been developed with an emphasis on maintaining cell viability and stimulating cell proliferation. These goals have necessarily led to culture media and culture conditions that select for cells that can proliferate at the expense of those that cannot. Thus, the choice of culture medium and the culture conditions used affect the properties and types of cells that can be studied in vitro. As an example of selection, normal rodent cells cultured on a plastic substratum in the presence of fetal bovine serum often go through a period of "crisis" in which a minority population of aneuploid cells survive and proliferate as immortalized but not completely transformed cells. Another example of unintentional selection through in vitro cell culture is the outgrowth of basal keratinocytes from cultured epidermal cells; this occurs because keratinocytes in the suprabasal layers of the epidermis differentiate and lose the ability to proliferate.

Conversely, for some applications, culture media have been developed to deliberately select for the survival of specific cell types of interest. Low-calcium, serum-free media developed for many epithelial cell types (Sato et al., 1994) are selective in that they promote the proliferation of epithelial cells but inhibit the outgrowth of fibroblasts in primary cultures; surviving fibroblasts are eliminated by dilution as the cultures are repeatedly passaged (*UNIT1.1*). Selective media are generally designed to exploit differences in susceptibility to metabolic inhibitors or differences in metabolic pathways. An example of a metabolic inhibitor as a selection agent is the use of the antibiotic G418 (geneticin) to kill nontransfected mammalian cells while allowing the growth of transfectants containing the bacterial gene for aminoglycoside phosphotransferase (*neo*^{r}; Southern and Berg, 1982).

Endogenous synthesis of purines and pyrimidines, which are essential components of nucleosides and nucleotides, can be blocked in mammalian cells by the folic acid analogue aminopterin (A). Such a block is circumvented in normal cells by salvage pathways that use hypoxanthine (H) and thymidine (T). However, cells deficient in the salvage pathway enzymes hypoxanthine-guanine phosphoribosyltransferase ($HGPRT^-$) and thymidine kinase (TK^-) cannot produce purines or pyrimidines by means of the salvage pathways in the presence of aminopterin, and die. Thus, HAT medium (Littlefield, 1964) selects for cells that contain functional HGPRT and TK enzymes, and it selects against cells that are $HGPRT^-$ or TK^-. This selection system has been made applicable to the selection of hybridomas by the generation of $HGPRT^-$ mouse myeloma cell lines. When these cells are fused with $HGPRT^+$ spleen cells from an immunized mouse, the resulting $HGPRT^+$ hybridomas survive in HAT medium, and the parental myeloma cells die.

Another example of the use of selective media takes advantage of cell line deficiencies. Most of the mouse myeloma cell lines that are commonly used to generate hybridomas (P3-X63-Ag8, NS-1-Ag4-1, X63-Ag8.653, and NS-0) are clonally derived from the MOPC21 tumor cell line P3 (Horibata and Harris, 1970), and they are all unable to synthesize cholesterol (Sato et al., 1984, 1987), which is an essential component of the plasma membrane. This trait is not a common characteristic of murine lymphoid cells or of mammalian cells in general, and it may have arisen from prolonged exposure to the antifungal agent amphotericin B (Sato et al., 1987). In all of these related myeloma cell lines, the defect in cholesterol biosynthesis has been traced to the enzyme 3-ketosteroid reductase (Sato et al., 1988). In cholesterol-free medium, NS-1 hybridomas are selected for while the cholesterol auxotrophic NS-1 parent cells are selected against (Myoken et al., 1989).

One must bear in mind that it is usually only after a population of cells has been expanded in culture that they can be characterized with respect to their physiological or differentiated properties. At one time it was thought that cells normally dedifferentiated in vitro and that culture techniques could not be used to study differentiated cellular functions. Subsequently, it was found that the dedifferentiation phenomenon resulted from the overgrowth of differentiated cells by contaminating fibroblasts. Once this problem was recognized, the first differentiated animal cell lines were established (Buonassisi et al., 1962; Yasumura et al., 1966). Thus, it is incumbent upon the cell culturist to choose culture media and culture conditions that not only support the viability and proliferation, if possible, of the cells of interest, but that allow those cells to manifest some or all of their differentiated properties in vitro.

From a historical viewpoint, it is of interest that early culture media consisted of undefined mixtures of biological fluids, tissue extracts, and simple salt solutions. The first continuous mammalian cell line, the mouse L cell fibroblast line (Sanford et al., 1948), was established from 20-methylcholanthrene-treated C3H mouse tissue explants grown in chicken plasma clots in 40% horse serum, 20% chick embryo extract, and 40% saline. The first continuous human cell line, the HeLa cervical adenocarcinoma line (Gey et al., 1952), was isolated in a mixture of chicken plasma, bovine embryo extract, and human placental cord serum. At about the same time, attempts were being made to make culture media more defined by creating synthetic nutrient media and by determining the nutritional requirements of cells in culture. An early synthetic nutrient medium, medium 199 (Morgan et al., 1950), was created to increase the longevity of primary chicken muscle cell cultures that were started as tissue explants in Earle's salt solution with 40% horse serum and 1% chick embryo extract. Medium 199 consisted of a salt solution with amino acids, vitamins, purines, pyrimidines, pentose sugars, adenosine triphosphate (ATP), adenylic acid, Tween 80 as a source of oleic acid, cholesterol, antioxidants, and iron in the form of ferric nitrate. Although medium 199 promoted the outgrowth of "large, flat and spindle-shaped" cells from the original tissue fragments, none of the medium components except glutamine clearly enhanced the life span of the cultures. The pioneering work of Eagle (Eagle, 1955) showed that L cells and HeLa cells had similar, demonstrable nutritional requirements for the thirteen essential amino acids, seven vitamins, glucose or other carbohydrates, and electrolytes. Strikingly, neither L cells nor HeLa cells would grow under these minimal essential conditions without the further addition of a small amount of dialyzed serum protein. Nonetheless, this research marked the beginning of concerted efforts to optimize basal nutrient media and to determine the growth requirements of cells in vitro.

Culture media for a number of normal, immortalized, and transformed cells have been

improved through the application of two complementary strategies. The approach of Ham and his colleagues has been to optimize the compositions of basal nutrient media for individual cell lines or cell types (Ham and McKeehan, 1979; Ham, 1984; Bettger and McKeehan, 1986) in the presence of ever-decreasing concentrations of dialyzed serum protein. These efforts gave rise to Ham's F-12 nutrient medium and the MCDB media, which are commonly used today. MCDB media are optimized basal media developed for specific call types by R. Ham and colleagues. In studying hormonally responsive cell lines in culture, Sato and his colleagues realized that a major role of serum in culture medium was to provide hormones and hormone-like growth factors that were required for cell proliferation and expression of differentiated functions (Bottenstein et al., 1979; Barnes and Sato, 1980; Barnes, 1987). This understanding led them to replace serum with purified hormones, growth factors, transport proteins, and attachment factors as supplements for preexisting nutrient media. In combination, these two experimental approaches demonstrated (1) that basal nutrient media could be optimized for individual cell types, but optimal cell proliferation required additional hormones or growth factors, transport proteins, and attachment mediators in the absence of serum, and (2) that the combinations of purified medium supplements for individual cell lines could be simplified when using an optimized basal medium.

General conclusions that can be drawn from the work of Ham and Sato are: (1) individual cell types require quantitatively balanced sets of nutrients, of which some are cell type specific; (2) cell proliferation and differentiated properties are regulated by hormones, growth factors, protein-bound nutrients, and attachment factors, of which many are present in serum and tissue extracts; (3) most cells in culture are growth stimulated by the serum components insulin and insulin-like growth factors, the iron-transporting protein transferrin, and unsaturated fatty acids or lipoproteins; and (4) because different cell types have similar but different growth requirements, it is unlikely that a single medium formulation will prove optimal for all cells.

Critical Parameters

When choosing or developing a cell culture medium, the single most important parameter is cell viability. This holds true whether the medium is used to maintain a population of differentiated cells, to stimulate cell proliferation, or to optimize the yield of a cellular product. An adequate serum-containing, serum-reduced, or serum-free medium formulation should promote a high degree of cell viability. Conversely, poor cell viability is a good indication that the culture medium or culture environment is deficient in one or more essential components. Suggestions for improving culture media are provided in Basic Protocols 1 and 2, and the reader is referred to *UNIT 1.1* for methods of assessing cell viability. On occasion, changes in culture conditions, such as a switch from serum-supplemented to serum-free medium, may cause the majority of cells in a culture to die, followed by the outgrowth of a surviving subpopulation of cells. Although this phenomenon has been referred to in the literature as adaptation or weaning, it is more likely to be a selective process in which the surviving cells differ phenotypically from the parental population. The investigator can reduce the chances of phenotypic changes becoming fixed in a population of cells by maintaining cells in culture medium that supports a high level of viability, by using low split ratios when passaging cells, and by periodically returning to cryopreserved stocks of low-passage-number cells.

The first choice for a basal nutrient medium should be one that other investigators have used successfully to culture the cells of interest and have reported in the literature. If for some reason that medium is not adequate for the purposes at hand, a number of basal media should be tested for the ability to support the proliferation of the cells of interest and to maintain their phenotypic properties. It is useful to start with basal media that have been used with similar or related cell types and in similar culture conditions (e.g., clonal or high-density cultures), but basal media developed for unrelated cell types may also yield good results (Ham, 1984). Commercially available basal media commonly used for continuous cell lines are DMEM; Ham's F-12 medium; a 1:1 mixture (v/v) of DMEM and Ham's F-12 medium (DMEM/F-12); and RPMI 1640, which was originally developed for lymphoid cells. The MCDB media were developed by Ham and his colleagues for individual types of normal cells, but they may also be effective on continuous cell lines. Most of these basal media are qualitatively similar but differ quantitatively. The basal medium selected based on empirical testing can be used as a starting point for further optimization. For excellent discussions on cel-

lular nutrition and procedures for optimizing basal nutrient medium see Ham and McKeehan, 1979; Ham, 1984; and Bettger and McKeehan, 1986.

The most commonly used serum supplement is fetal bovine serum (FBS). Less expensive alternatives to FBS are calf serum, newborn calf serum, calf serum fortified with transferrin or growth factors (available from Hyclone and Sigma), and horse serum. For normal human lymphocytes, the use of commercial human serum treated at 56°C for 30 min to inactivate complement may be appropriate. The most suitable serum supplement for the cells of interest should be determined empirically. Although variability in efficacy between batches of FBS owing to variations in composition is not as problematic as it once was, it is advisable to test batches of FBS at several concentrations for the ability to support the proliferation of cells of interest at low and high densities. Clonal growth assays are the most stringent tests of the efficacy of batches of serum, but acceptable batches of serum should also be able to support, at reasonable concentrations, the viability of high-density cultures.

A number of reduced-serum or serum-free media have been developed for continuous cell lines and nontransformed cells (Bottenstein et al., 1979; Jacoby and Pastan, 1979; Barnes et al., 1984; Freshney, 1987, 1992; Baserga, 1989; Davis, 1994; Sato et al., 1994), and most are optimized for a single cell line or cell type. However, the similarities between media developed for related cells are increasing the understanding of the nutritional and growth factor requirements of individual cell types, which in turn is making the development of serum-free medium a more rational process (Sato et al., 1994). As in choosing a basal nutrient medium for serum-supplemented medium, the best choice for a reduced-serum or serum-free medium is one that has been used by other investigators for the same or a related cell line or cell type. It is desirable to use a defined, serum-free medium whenever possible, as this affords the investigator the greatest degree of control over an in vitro culture–based experiment.

Proprietary serum-free media for specific cell types are commercially available from companies such as Clonetics and Cell Systems (see Table 1.2.1). If an optimized, preexisting serum-free medium is not available, then a serum-free or serum-supplemented medium that supports cell viability and suboptimal proliferation can be used as a starting point for further medium development. As described above (see Background Information), two complementary strategies for improving culture media are to optimize the components of the basal nutrient medium (Ham and McKeehan, 1979; Ham, 1984) and to replace serum or other undefined medium components with defined, purified protein and nonprotein supplements (Bottenstein et al., 1979; Barnes and Sato, 1980; Barnes, 1987; Sato et al., 1994). Under both strategies, concentrations of serum, dialyzed serum, or other undefined supplements are lowered stepwise to reduce the rate of cell proliferation, and the concentrations of defined medium components are individually manipulated until cell proliferation is restored. A completely defined culture medium or a much reduced serum-supplemented medium should be attained after a number of rounds of optimization. See Basic Protocol 2 for description of both these approaches.

Clues as to which defined supplements are likely to be growth stimulatory for the cells of interest are provided by the following sources: hormones or growth factors that act on the cell type of interest in vivo; autocrine factors that the cells have been found to produce in vitro; and defined supplements that have been included in serum-free media developed for similar or related continuous cell lines or cell types. Thus, the first place to search for potentially useful supplements is the literature. The following general suggestions are based on the serum-free media that have been developed over the past 20 years.

1. Most cells are growth stimulated by insulin or insulin-like growth factor I and require iron obtained by the iron-transporting protein transferrin.

2. Fatty acid–free BSA is a useful carrier protein for unsaturated fatty acids, sterols, and steroid hormones, which are insoluble in an aqueous solvent.

3. Cells of epithelial origin often respond to epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF or FGF-1), and dexamethasone or hydrocortisone.

4. Mesenchymal cells respond to EGF, aFGF, basic FGF (bFGF or FGF-2), and platelet-derived growth factor (PDGF).

5. In the absence of serum-derived attachment mediators, treating tissue culture plastic with attachment factors, such as type I collagen (*UNIT 10.3*), fibronectin, vitronectin, and laminin (*UNIT 10.2*), or with an incompletely defined, natural extracellular matrix (e.g., Matrigel, from Becton Dickinson Labware; *UNIT 10.2*) may

enhance the plating efficiencies and growth rates of adherent cells.

It is important to use highly pure water in preparing media and medium additives. Double-distilled water was a standard ingredient in medium for many years, but it has been superseded by purification systems that incorporate reverse osmosis. In the Milli-Q system, locally supplied water is subjected to deionization, reverse osmosis, and filtration through activated charcoal and a sterilizing filter. Use a vessel that is thoroughly rinsed with water after use but never washed with detergent.

Anticipated Results

As the survival and growth requirements of individual cell lines and cell types become better understood, the routine culture conditions for cells of interest become more defined. When all of the growth requirements of cells of interest are understood, any undefined medium supplements that were previously required can be completely eliminated. Defined culture conditions afford the investigator the greatest degree of control over in vitro culture experiments, and they provide more accurate insights into cellular physiology in vivo.

Time Considerations

Optimizing a basal nutrient medium or developing a serum-free medium formulation is not a trivial undertaking, and it can be very time-consuming with no guarantee of success. Thus, the investigator should carefully consider how important defined culture conditions are to the experimental goals before taking on either task. However, as optimized and defined media are developed for a wider array of cell types, it is becoming easier and less time-consuming to create defined media for additional types of cells. The amount of time required to mix and sterilize 5- to 20-liter batches of medium should not exceed 2 to 4 hours.

Literature Cited

Barnes, D.W. 1987. Serum-free animal cell culture. *BioTechniques* 5:534-541.

Barnes, D.W. and Sato. G.H. 1980. Serum-free cell culture: A unifying approach. *Cell* 22:649-655.

Barnes, D.W., Sirbasku, D.A., and Sato, G.H. (eds.) 1984. Cell Culture Methods for Cell Biology, Vols. 1-4. Alan R. Liss, New York.

Baserga, R. (ed.) 1989. Cell Growth and Division: A Practical Approach. Oxford University Press, Oxford.

Bettger, W.J. and McKeehan, W.L. 1986. Mechanisms of cellular nutrition. *Physiol. Rev.* 66:1-35.

Bottenstein, J., Hayashi, I., Hutchings, S., Masui, H., Mather, J., McClure, D.B., Ohasa, S., Rizzino, A., Sato, G., Serrero, G., Wolfe, R., and Wu, R. 1979. The growth of cells in serum-free hormone-supplemented media. *Methods Enzymol.* 58:94-109.

Buonassisi, V., Sato, G.H., and Cohen, A.I. 1962. Hormone-producing cultures of adrenal and pituitary tumor origin. *Proc. Natl. Acad. Sci. U.S.A.* 48:1184-1190.

Davis, J.M. (ed.) 1994. Basic Cell Culture: A Practical Approach. Oxford University Press, Oxford.

Eagle, H. 1955. Nutrition needs of mammalian cells in tissue culture. *Science* 122:501-504.

Eagle, H. 1973. The effect of environmental pH on the growth of normal and malignant cells. *J. Cell. Physiol.* 82:1-8.

Freedman, V.H. and Shin, S.-I. 1974. Cellular tumorigenicity in nude mice: Correlation with cell growth in semi-solid medium. *Cell* 3:355-359.

Freshney, R.I. 1987. Culture of Animal Cells: A Manual of Basic Technique, 2nd ed. Alan R. Liss, New York.

Freshney, R.I. (ed.) 1992. Culture of Epithelial Cells. Wiley-Liss, New York.

Gey, G.O., Coffman, W.D., and Kubicek, M.T. 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* 12:264-265 (Abstr).

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S., and Singh, R.M.M. 1966. Hydrogen ion buffers and biological research. *Biochemistry* 5:467-477.

Ham, R.G. 1984. Formulation of basal nutrient media. *In* Cell Culture Methods for Cell Biology, Vol. 1 (D.W. Barnes, D.A. Sirbasku, and G.H. Sato, eds.) pp. 3-21. Alan R. Liss, New York.

Ham, R.G. and McKeehan, W.L. 1979. Media and growth requirements. *Methods Enzymol.* 58:44-93.

Horibata, K. and Harris, A.W. 1970. Mouse myeloma and lymphomas in culture. *Exp. Cell Res.* 60:61-77.

Jacoby, W.B. and Pastan, I.H. (eds.) 1979. Cell Culture. *Methods Enzymol.*, Vol. 58.

Kawamoto, T., Sato, J.D., McClure, D.B., and Sato, G.H. 1983. Development of a serum-free medium of growth of NS-1 mouse mycloma cells and its explication to the isolation of NS-1 hybridomes. *Anal. Biochem.* 130:445-453.

Littlefield, J.W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* 145:709-710.

Morgan, J.F., Morton, H.J., and Parker, R.C. 1950. Nutrition of animal cells in tissue culture. 1. Initial studies on a synthetic medium. *Proc. Soc. Exp. Biol. Med.* 73:1-8.

Myoken, Y., Okamoto, T., Osaki, T., Yabumoto, M., Sato, G.H., Takada, K., and Sato, J.D. 1989. An alternative method for the isolation of NS-1 hy-

bridomas using cholesterol auxotrophy of NS-1 mouse myeloma cells. *In Vitro Cell Dev. Biol.* 25:477-480.

Perlman, D. 1979. Use of antibiotics in cell culture media. *Methods Enzymol.* 58:110-116.

Sanford, K.K., Earle, W., and Likely, G.D. 1948. The growth in vitro of single isolated tissue cells. *J. Natl. Cancer Inst.* 9:229-246.

Sato, J.D., Kawamoto, T., McClure, D.B., and Sato, G.H. 1984. Cholesterol requirement of NS-1 mouse myeloma cells for growth in serum-free medium. *Mol. Biol. Med.* 2:121-134.

Sato, J.D., Kawamoto, T., and Okamoto, T. 1987. Cholesterol requirement of P3-X63-Ag8 and X63-Ag8.653 myeloma cells for growth in vitro. *J. Exp. Med.* 165:1761-1766.

Sato, J.D., Cao, H.-T., Kayada, Y., Cabot, M.C., Sato, G.H., Okamoto, T., and Welsh, C.J. 1988. Effects of proximate cholesterol precursors and steriod hormones on mouse myeloma growth in serum-free medium. *In Vitro Cell Dev. Biol.* 24:1223-1228.

Sato, J.D., Hayashi, I., Hayashi, J., Hoshi, H., Kawamoto, T., McKeehan, W.L., Matsuda, R., Matsuzaki, K., Mills, K.H.G., Okamoto, T., Serrero, G., Sussman, D.J., and Kan, M. 1994. Specific cell types and their requirements. *In* Basic Cell Culture: A Practical Approach (J.M. Davis, ed.) pp. 181-222. Oxford University Press, Oxford.

Southern, P.J. and Berg, P. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327-341.

Yasumura, Y., Tashjian, A.H., Jr., and Sato, G.H. 1966. Establishment of four functional clonal strains of animal cells in culture. *Science* 154:1186-1189.

Contributed by J. Denry Sato
Adirondack Biomedical Research Institute
Lake Placid, New York

Mikio Kan
Texas A&M University
Houston, Texas

Aseptic Technique for Cell Culture

This unit describes some of the ways that a laboratory can deal with the constant threat of microbial contamination in cell cultures. Microorganisms are ubiquitous. Bacteria can be isolated from nearly any surface including inanimate objects and human skin. Fungal spores and bits of vegetative hyphae drift into a laboratory from air conditioning ducts and open doors. Mycoplasma infections most frequently originate from improperly sterilized media or serum. At the risk of eliciting paranoia in the novice cell culture user who has no training in microbiological techniques, the possibility for microbial contamination exists everywhere. Inherent with successful manipulation of cell cultures is the basic understanding that everything that comes into contact with the cells must be sterile or noncontaminating. This includes media, glassware, and instruments, as well as the environment to which the cultures are briefly exposed during transfer procedures. Because cleaning up a contaminated culture is too frequently a disheartening and unsuccessful experience, the best strategy is to employ procedures to prevent microbial contamination from occurring in the first place.

This unit begins with a protocol on aseptic technique (see Basic Protocol 1). This catch-all term universally appears in any set of instructions pertaining to procedures in which noncontaminating conditions must be maintained. In reality, aseptic technique cannot be presented in one easily outlined protocol, but rather encompasses all aspects of environmental control, personal hygiene, equipment and media sterilization, and associated quality control procedures needed to ensure that a procedure is, indeed, performed with aseptic, noncontaminating technique. Although cell culture can theoretically be carried out on an open bench in a low-traffic area, most cell culture work is carried out using a horizontal laminar-flow clean bench (see Basic Protocol 2) or a vertical laminar-flow biosafety cabinet (see Alternate Protocol). Subsequent units within this chapter address these diverse considerations—e.g., sterilization and disinfection, use of antibiotics, and quality control. Where applicable, use presterilized, disposable labware and other equipment. The wide availability and reliability of these products has simplified cell culture, particularly for small-scale laboratory needs.

ASEPTIC TECHNIQUE

BASIC PROTOCOL 1

This protocol describes basic procedures for aseptic technique for the novice in cell culture technology. One basic concern for successful aseptic technique is personal hygiene. The human skin harbors a naturally occurring and vigorous population of bacterial and fungal inhabitants that shed microscopically and ubiquitously. Most unfortunately for cell culture work, cell culture media and incubation conditions provide ideal growth environments for these potential microbial contaminants. This procedure outlines steps to prevent introduction of human skin flora during aseptic culture manipulations.

Every item that comes into contact with a culture must be sterile. This includes direct contact (e.g., a pipet used to transfer cells) as well as indirect contact (e.g., flasks or containers used to temporarily hold a sterile reagent prior to aliquoting the solution into sterile media). Single-use, sterile disposable plastic items such as test tubes, culture flasks, filters, and pipets are widely available and reliable alternatives to the laborious cleaning and sterilization methods needed for recycling equivalent glass items. However, make certain that sterility of plastic items distributed in multiunit packages is not compromised by inadequate storage conditions once the package has been opened.

Contributed by Rosalie J. Coté

Current Protocols in Cell Biology (1998) 1.3.1-1.3.10

Ideally, all aseptic work should be conducted in a laminar cabinet (see Basic Protocol 2 and Alternate Protocol). However, work space preparation is essentially the same for working at the bench. Flame sterilization is used as a direct, localized means of decontamination in aseptic work at the open bench. It is most often used (1) to eliminate potential contaminants from the exposed openings of media bottles, culture flasks, or test tubes during transfers, (2) to sterilize small instruments such as forceps, or (3) to sterilize wire inoculating loops and needles before and after transfers. Where possible, flame sterilization should be minimized in laminar-flow environments as the turbulence generated by the flame can significantly disturb the sterile air stream.

Materials

Antibacterial soap
70% ethanol or other appropriate disinfectant
95% ethanol

Clean, cuffed laboratory coats or gowns
Latex surgical gloves
Clean, quiet work area
Shallow discard pans containing disinfectant
Bunsen burner or pilot-activated burner (e.g., Touch-o-Matic, VWR)

Take personal precautions

1. Just prior to aseptic manipulations, tie long hair back behind head. Vigorously scrub hands and arms at least 2 min with an antibacterial soap.

   Superficial lathering is more prone to loosening than removing flaking skin and microbial contaminants. Loosely adhering skin flora easily dislodge and can potentially fall into sterile containers.

2. Gown appropriately. For nonhazardous sterile-fill applications, wear clean, cuffed laboratory coats and latex gloves.

   Greater stringencies may be necessary depending upon laboratory regulatory requirements. Work with potentially hazardous agents certainly mandates additional considerations for safety. Front-closing laboratory coats are not recommended for work with hazardous biological agents. Safety glasses should be worn by laboratory personnel when manipulating biological agents outside the confines of a biosafety cabinet.

3. Frequently disinfect gloved hands with 70% ethanol while doing aseptic work.

   Although the gloves may initially have been sterile when first worn, they will no doubt have contacted many nonsterile items while in use.

   Note that 70% ethanol may not be an appropriate agent for latex glove disinfection when working with cultures containing animal viruses, as studies have shown that ethanol increases latex permeability, reducing protection for the wearer in the event of exposure. In this case, quarternary ammonium compounds are more appropriate.

4. Dispose of gloves by autoclaving after use. Do not reuse. Bag and autoclave single-use laboratory coats after use. Bag, autoclave (if necessary), and wash other laboratory coats within the laboratory facility or send out for cleaning at a laundry certified for handling biologically contaminated linens.

   Never take laboratory clothing home for washing.

5. Thoroughly wash hands after removing protective gloves.

Prepare and maintain the work area

6. Perform all aseptic work in a clean work space, free from contaminating air currents and drafts. For optimal environmental control, work in a laminar-flow cabinet (see Basic Protocol 2 and Alternate Protocol).
7. Clear the work space of all items extraneous to the aseptic operation being performed.
8. Wipe down the work surface before and after use with 70% ethanol or other appropriate disinfectant.
9. Wherever feasible, wipe down items with disinfectant as they are introduced into the clean work space. Arrange necessary items in the work space in a logical pattern from clean to dirty to avoid passing contaminated material (e.g., a pipet used to transfer cultures) over clean items (e.g., flasks of sterile media).
10. Immediately dispose of any small contaminated items into a discard pan.
11. When the aseptic task has been completed, promptly remove any larger contaminated items or other material meant for disposal (e.g., old culture material, spent media, waste containers) from the work space and place in designated bags or pans for autoclaving. Disinfect the work space as in step 8.

Flame sterilize the opening of a vessel

12. For a right-handed person, hold the vessel in the left hand at ~45° angle (or as much as possible without spilling contents) and gently remove its closure. Do not permit any part of the closure that directly comes in contact with the contents of the vessel to touch any contaminating object (e.g., hands or work bench).

    Ideally, and with practice, one should be able to hold the closure in the crook of the little finger of the right hand while still being able to manipulate an inoculating loop or pipettor with the other fingers of the hand.

    Holding the vessel off the vertical while opening will prevent any airborne particulates from entering the container.

13. Slowly pass the opening of the vessel over the top of (rather than through) a Bunsen burner flame to burn off any contaminating matter.

    Be careful when flaming containers of infectious material. Any liquid lodged in the threads of a screw cap container will spatter as it is heated. Aerosols thus formed may actually disseminate entrapped biological agents before the heat of the flame is hot enough to inactivate them.

14. While still holding the vessel at a slant, use a sterile pipet and pipettor to slowly add or remove aliquots to avoid aerosol formation.
15. Flame-sterilize again as in step 13, allow the container to cool slightly, and carefully recap the vessel.

Flame sterilize small hand instruments

16. Dip critical areas of the instrument (i.e., those that come into contact with the material of concern) in 95% ethanol.

    Make certain that the alcohol is in a container heavy enough to support the instrument without tipping over.

    CAUTION: *95% ethanol is flammable; keep the container at a safe distance from any open flame.*

17. Remove the instrument from the alcohol, being careful not to touch the disinfected parts of the instrument. Allow excess ethanol to drain off into the container.

18. Pass the alcohol-treated part of the instrument through the flame of a Bunsen burner and allow residual alcohol to burn off.

19. Do not let the sterilized portion of the instrument contact any nonsterile material before use. Let the heated part of the instrument cool for ~10 sec before use.

20. After use, return the instrument to the alcohol disinfectant until needed again.

Flame sterilize inoculating loops and needles

21. Hold the inoculating wire by its handle and begin in the center of the wire to slowly heat the wire with the flame of a Bunsen burner. Proceed back and forth across the wire's full length until it glows orange.

22. While still holding the handle, allow the inoculating wire to cool back to room temperature (~10 sec) before attempting any transfer of material.

    If tranfers are made while the inoculating wire is hot, cells will be killed by the hot wire, and aerosols created from spattering material can disperse biological material throughout the work space.

23. After the transfer is made, reheat the inoculating wire as in step 21 to destroy any remaining biological material. Let cool to room temperature before putting aside for next use.

BASIC PROTOCOL 2

USE OF THE HORIZONTAL LAMINAR-FLOW CLEAN BENCH

Laminar-flow cabinets (hoods) are physical containment devices that act as primary barriers either to protect the material being manipulated within the hood from worker-generated or environmental sources of contamination, or to protect the laboratory worker and laboratory environment from exposure to infectious or other hazardous materials that are present within the hood. Cell culture applications utilize two types of laminar-flow hoods: (a) the horizontal-flow clean bench (described here) and (b) the biological safety cabinet (see Alternate Protocol). Both types of hoods use a high-efficiency particulate air (HEPA) filter and blowers that generate a nonmixing stream of air.

The horizontal laminar-flow clean bench is used to provide a near-sterile environment for the clean (i.e., noncontaminating) handling of nonhazardous material such as sterile media or equipment. Because the air stream pattern directs the flow of air within the hood directly back to the hood operator and the room (Fig. 1.3.1), horizontal flow hoods are never to be used with infectious agents or toxic chemicals.

Materials

70% ethanol or other disinfectant
Horizontal laminar-flow hood, certified for use
Swabs (e.g., cheesecloth, paper towels)
Pilot light–activated Bunsen burner (e.g., Touch-o-Matic, VWR)

1. Completely clear the bench of the laminar-flow hood and disinfect the bench working surface and the left and right sides of the hood with 70% ethanol or other disinfectant. Do not spray the back (gridded) wall where the HEPA filter is housed.

   Resist the urge to leave frequently used items (e.g., pipet canisters or a bag of disposable plastic tissue culture flasks) in the hood between uses. Their presence makes thorough disinfection of the work space difficult.

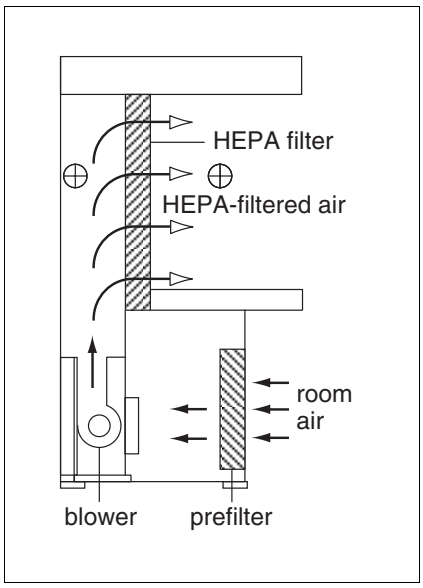


Figure 1.3.1 Horizontal laminar-flow clean cabinet. Solid arrows, dirty room air; open arrows, clean HEPA-filtered air; circled +, positive pressure with respect to room air.

2. Turn the hood blower and lights on and let the air circulate within the hood 10 min before use.
3. Place items needed for the specific procedure into the hood, wiping each item with 70% ethanol or other disinfectant just before introducing it into the laminar environment.

   Do not overcrowd the work space. For horizontal laminar-flow effectiveness, maintain a clear path between the work area and the back wall of the cabinet where the HEPA filter is located.

4. Wash hands well before working in the hood and wear a clean laboratory coat and surgical gloves to further protect the work from shedding of skin flora that can contaminant any product (see Basic Protocol 1).
5. While working in the hood, perform all work at least 4 in. back from the front opening, and avoid rapid movements that might disrupt the laminar air flow. Avoid moving materials or hands in and out of the cabinet as much as possible.
6. If flame sterilization is needed in the hood for a particular application, use a burner that can be activated by a pilot light when needed, rather than one that burns constantly.

   The open flame of a Bunsen burner causes turbulence that disrupts the unidirectional laminar air flow.

7. When work is completed, remove all material from the laminar work bench, clean any spills, and disinfect the bench working surface by wiping with 70% ethanol or other disinfectant.
8. Turn off hood blower and lights.

ALTERNATE PROTOCOL

USE OF THE VERTICAL LAMINAR-FLOW BIOSAFETY CABINET

Biological safety cabinets provide a clean, safe environment for both the worker and the product. The Class II, Type A biosafety cabinet (Fig. 1.3.2) is frequently encountered in cell culture laboratories, and this protocol describes the use of this type of barrier device. The Class IIA biosafety cabinet is suitable for work with low- to moderate-risk biological agents in the absence of toxic or radioactive chemicals.

Materials *(also see Basic Protocols 1 and 2)*

Class II, Type A Biosafety Cabinet (BSC), certified for use
Pilot light–activated Bunsen burner (e.g., Touch-o-Matic, VWR) or electronic incinerator (e.g., Bacti-Cinerator III, VWR)
Closed-front laboratory gowns (for personnel working with biological agents)

1. Turn the hood blower on and verify air flow by feeling (by hand) the current near the front grill of the work surface. Turn the germicidal UV light off if it is on. Turn the fluorescent light on.

   Before use, the cabinet should already be empty and clean from prior activity. The view window should be lowered to the proper operating height (normally 8 in.) or as specified by the cabinet manufacturer.

   UV light is effective only for decontaminating clean, solid surfaces with which it comes in contact. It is not effective in decontaminating the cabinet air flow. UV light is not effective against bacterial spores. UV germicidal light tubes should be replaced frequently (at least every 6 months for biosafety cabinets in use on a daily basis) to assure that they are emitting light at 254 nm and at an intensity appropriate for decontamination.

   CAUTION: *UV light is harmful to the eyes. Laboratory personnel should not be near the cabinet or looking at the UV light when it is in use.*

2. Wash and gown as required for the operation (see Basic Protocol 1, steps 1 to 5).
3. Wipe down the entire interior cabinet work surface area with 70% ethanol or other appropriate disinfectant.
4. Let blower run for 10 min to filter the cabinet air of any particulates.

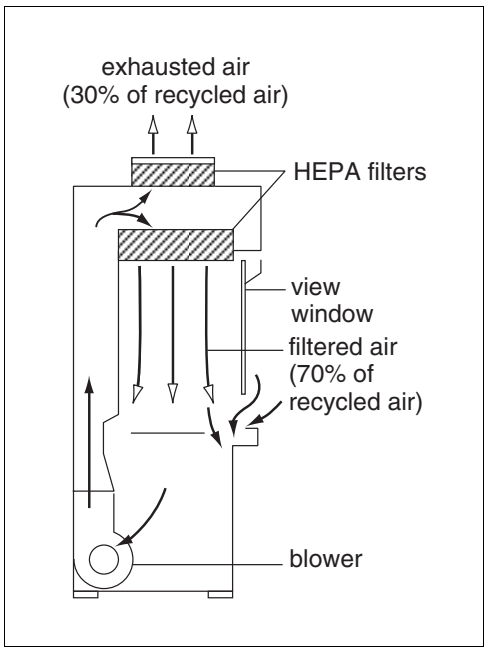


Figure 1.3.2 Biological safety cabinet, Class II, Type A. Note that filtered air is contaminated after passing through the work space, and is filtered again whether it is recycled to the workspace (70%) or exhausted (30%). Solid arrows, dirty (room/contaminated) air; open arrows, filtered air.

5. Raise the front view window as needed to bring necessary items into the cabinet. Wipe each item with 70% ethanol or other disinfectant as it is placed in the cabinet.

   Do not crowd the work space and make sure no air vents are blocked by supplies or equipment. Do not position material so that it obscures any of the air vents at the front edges of the laminar hood. One frequent source of air flow restriction in biosafety cabinets is "lost" paper towels that have been drawn into the air ducts at the back of the work surface.

6. Organize the work surface for a clean-to-dirty work flow. Place clean pipets, flasks, and sterile media bottles at one side of the cabinet; place discard pans, spent cultures, and other wastes on the other side.

7. Return the view window to the 8-in. operating level. Wait ~10 min for the blowers to filter the disturbed cabinet air before starting work.

8. While working, keep all material and perform work ≥4 in. back from the front opening of the cabinet, and minimize rapid movements or activity. Keep the view window opening as close to 8 in. as allows reasonable access to the work surface and equipment.

   These precautions assure that any drafts caused by arm movements will not disrupt air flow or churn room air currents into the clean work area.

9. If direct flame sterilization of items within the cabinet is necessary, use an electric burner or pilot light–activated flame burner located at the back of the work space.

   A constant open flame in the cabinet can disturb the laminar air flow.

10. At the end of the procedure, enclose all contaminated materials. Clean the cabinet work surface with 70% ethanol or other disinfectant, being especially careful to wipe any spills of culture suspensions or media that can serve as future contamination points. Clear all material from the cabinet.

11. Let the blower run for ≥10 min with no activity to remove any aerosols that were generated. During this period, turn off the fluorescent light and turn on the germicidal UV light. Allow the UV light to operate ≥30 min.

COMMENTARY

Background Information

Aseptic technique

The dictionary definition of asepsis simply implies freedom from pathogenic organisms. However, the practical definition of the term for cell biologists, as well as other biotechnologists working with pure cultures, has come to be synonymous with sterile or noncontaminating conditions. The successful manipulation of cell cultures under any circumstance inherently relies upon the ability to maintain rigorous aseptic (i.e., noncontaminating) working conditions. The concept of aseptic technique is simple in theory: prevention of sterile or uncontaminated material and objects from coming into contact with any nonsterile or contaminated material.

Practical application of the theory is often illusive for beginning students. However, breaches in aseptic technique can also cause significant problems for even well-experienced laboratories, particularly when the source of contamination is not readily evident. A single incident of culture contamination is frustrating in its own right, but repeated contamination (particularly by the same type of organism) invariably results in expensive losses and delays until the localization and source are identified.

The critical areas of concern with respect to successful aseptic technique include environmental conditions (laboratory or work space), source material (cell lines, media, and reagents), equipment (labware, instruments, and apparatuses), sterilization procedures and equipment (autoclave, dry heat, filtration), and human (laboratory personnel) considerations. Budgetary constraints aside, technological aids exist to greatly simplify the hardware needed

for aseptic work. Laminar-flow cabinets create clean working environments (see below); clean, certified cell lines are available from cell repositories; media manufacturers and biotechnology supply companies provide sterile media, sera and reagents; and presterilized disposable labware to satisfy most cell culture needs is available from any large distributor of scientific supplies.

Despite all the technological advances, the one weak link remaining in successful laboratory applications of aseptic technique is the human factor. Too frequently, contamination occurs because of the desire to work a little too quickly, the urge to eliminate an "unimportant" step, or lapses in concentration during mundane procedures. The only advice to offer as protection against the human factor is to work slowly and deliberately when performing procedures under aseptic conditions, don't eliminate procedural steps, and pay attention! Establishing a standard routine of procedures and of placement of materials can help prevent the omission of steps.

Laminar-flow cabinets

Laminar-flow cabinets or hoods have replaced the open laboratory bench for aseptic work in almost all cell culture and microbiology laboratories. Their effectiveness as physical barriers to contamination relies on a cabinet design incorporating high-efficiency particulate air (HEPA) filters to trap airborne contaminants, and blowers to move the filtered air at specified velocities and in a nonmixing (laminar) stream across a work surface.

As noted in each protocol for the particular type of laminar-flow application, the proper choice of cabinet is imperative. Horizontal laminar-flow cabinets are never used with biological or toxic chemical agents as they are not containment devices but rather serve to provide a strong stream of near-sterile air for particle-free working conditions. As this air is blown directly from the HEPA filter (at the back of the cabinet) across the work surface and out of the cabinet (directly into the operator's face and the room), the restricted use of the horizontal flow cabinet to nonhazardous material is obvious.

The Class IIA biosafety cabinet is a laminar containment device that (1) protects the material being manipulated within the cabinet by HEPA-filtered incoming air and (2) protects the operator and room environment from potentially hazardous material in the cabinet with an air curtain at the front of the cabinet (the view screen) and HEPA-filtered cabinet exhaust air. As Class IIA biosafety cabinets are not totally leak-proof, they cannot be used for high-risk biological agents (see current Center for Disease Control and NIH guidelines for the status of any biological material used in the laboratory; Richmond and McKinney, 1993). Because Class IIA cabinets operate with ~70% recirculated air within the cabinet (Fig. 1.3.2), the potential for accumulation of chemicals within the laminar work space limits use to low-level toxic or radioactive material.

Laminar-flow cabinets are not replacements for good microbiological aseptic technique and must be used in conjunction with standard concerns for asepsis if full efficiency of the equipment is expected. Similarly, there is a limit to the protection a laminar cabinet can provide if it is operated in an environment not conducive to clean work conditions. The cabinets should be installed and operated in a relatively clean, quiet laboratory environment. Laboratory doors should be kept closed while the cabinet is in use to minimize strong room air currents that could break the laminar air stream within the cabinet. The units should not be located directly near room air ducts or anywhere a strong environmental air flow exists. Additionally, air flow disturbance by personnel or equipment, particularly within a few feet in front of the cabinets, should be limited when the laminar device is in use.

Because of the critical nature of their function (particularly for the biosafety laminar cabinet), these devices must be certified at installation by professional laminar flow technicians in accordance with National Sanitation Foundation Standard No. 49 for Class II (laminar flow) Biohazard Cabinetry (NSF International, 1992) or other applicable regulatory and safety guidelines. As HEPA filters are brittle and will crack with normal usage of the unit, laminar cabinets must also be recertified annually or after 1000 hr use, and whenever they are moved.

Critical Parameters and Troubleshooting

Human sources of contamination

As noted above, bacterial shedding from human skin is a natural occurrence. However, under times of physiological or psychological stress, a human may shed so excessively that routine gowning procedures are inadequate. A clue to this condition can be the veteran technician who suddenly can't seem to transfer anything without contaminating it, especially when contamination is repeatedly bacterial and

by species of *Staphylococcus, Micrococcus*, or coryneforms.

Alleviation of the problem may be achieved by simply controlling the temperature of the laboratory. Gowned personnel sweat in 27°C (80°F) rooms, and people who sweat shed more than people who don't. Rigorous attention to gowning details as well as liberal washing of hands and arms with an antimicrobial soap just prior to aseptic work may alleviate the situation. If the problem involves psychological stress or physiological stress due to illness or medication, more rigorous gowning procedures may help. Use fresh, clean laboratory coats for each round of aseptic work and make sure laboratory coat sleeves are tucked inside gloves to prevent exposed wrists. Use disinfectants liberally. For worst-case incidences of excessive shedding, the only recourse may be to move the individual to nonaseptic procedures until the condition clears.

Decontamination of a laminar-flow cabinet

Any mechanical failure of a laminar cabinet must be evaluated by qualified, trained personnel. Increased incidences of microbial contamination (particularly by the same organism) could originate from (1) poor cleaning and disinfection of the cabinet work space, (2) a source of contamination lodged in the ducts within the cabinet (e.g., media or culture material spilled into the cabinet ducts), or (3) a crack in the HEPA filter.

Disinfect the catch basin if culture material has spilled through the vents in the work surface into the catch basin below. Use a strong disinfectant (such as 5% to 10% bleach in a sufficient volume to thoroughly contact the spilled material) and allow the disinfectant to stay in contact with the spill for 30 min. Drain the contents of the catch basin into a container suitable for final sterilization by autoclaving.

Visually inspect the working interior of the laminar cabinet for evidence of dried culture material or media, especially in the corners of the cabinet. Clean the interior of the cabinet with a laboratory detergent, rinse with water, dry, and treat the area with an appropriate disinfectant. Be very careful not to wet the exposed HEPA filter located on the back wall of horizontal flow cabinets, as this can compromise the filter integrity. Be careful not to let cleaning solutions enter any vents of the cabinet.

After thorough cleaning of the cabinet work surface, operate the cabinet (as detailed in Basic Protocol 2 or Alternate Protocol) using a control procedure for localizing the source of any remaining contamination. This can be achieved with a series of opened plates of trypticase soy agar and Emmons' modification of Sabouraud's agar systematically coded and placed across the work surface. Leave the media plates open and the cabinet operating for 30 min. Close the lids of the agar plates and incubate them at 26°C for 5 days. If significant microbial contamination appears in the plates, consult with a qualified laminar technician. The resolution to the problem will require either caulking leaks in the HEPA filter or sealing the cabinet for total interior decontamination of filter and ducts with formaldehyde gas.

A final source of frequent contamination in a laminar working condition can be the "sterile" equipment, labware, or solutions used. A poorly filter-sterilized phosphate-buffered saline solution can give rise to significant numbers of pseudomonad bacteria within weeks when stored at room temperature. Insufficiently processed autoclaved or dry heat–sterilized labware frequently results in contamination of cell culture material by spore-forming bacteria.

Anticipated Results

When proper aseptic techniques are used, it should be possible to maintain cell cultures without contamination.

Time Considerations

It takes ~1/2 hr to properly prepare oneself and the cell culture area for culture procedures and a similar amount of time to properly clean up afterward.

Literature Cited

Richmond, J.Y. and McKinney, R.W. (eds.) 1993. Biosafety in microbiological and biomedical laboratories, 3rd ed. U.S. Government Printing Office, Washington, D.C.

NSF (National Sanitation Foundation) International. 1992. Class II (laminar flow) biohazard cabinetry (NSF 49-1992). NSF International, Ann Arbor, Mich.

Key References

Barkley, W.E. and Richardson, J.H. 1994. Laboratory safety. *In* Methods for General and Molecular Bacteriology, 2nd ed. (P.E. Gerhardt, R.G.E. Murray, W.A. Wood, and N.R. Krieg, eds.) pp. 715-734. American Society for Microbiology, Washington, D.C.

Chapter provides an overview of general concerns for working with biological agents, from a classic publication on general methods in bacteriology that often overlaps to satisfy the technical needs of cell biologists.

Chatigny, M.A. 1986. Primary barriers. *In* Laboratory Safety: Principles and Practices (B.M. Miller, D.H.M. Gröschel, J.H. Richardson, D.Vesley, J.R. Songer, R.D. Housewright, and W.E. Barkley, eds.) pp. 144-163. American Society for Microbiology, Washington, D.C.

Offers detailed considerations on the types and uses of laminar-flow barrier technology. The main publication is well worth its price for anyone (staff, supervisors, administrators) responsible for safety in a biological laboratory.

Freshney, R.I. 1994. Culture of Animal Cells: A Manual of Basic Technique, 3rd ed., pp. 51-69. Wiley-Liss, New York.

Offers suggestions for maintaining aseptic conditions while working with cell cultures. A classic cell culture publication that surveys the field while providing enough detail for an individual with intermediate knowledge of microbiology and cell biology.

Contributed by Rosalie J. Coté
Becton Dickinson Microbiology Systems
Sparks, Maryland

Sterilization and Filtration

This unit describes conditions and procedures for the use of the autoclave and the convection or gravity oven for sterilization of heat-stable laboratory materials, for depyrogenation by heat, and for decontamination of biological waste. Sterilization is not an absolute but rather a probability function. Terminal sterilization processes such as autoclaving or dry heat should have a 10^{-6} or less probability that an organism will survive treatment. The proper choice of sterilization method, well-maintained equipment, validated procedures, and adherence to protocol are all necessary to keep the statistics in one's favor.

This unit includes protocols for a variety of sterilization and decontamination methods. Moist-heat or steam sterilization is used for liquids, dry goods, and decontamination of biological wastes. Dry heat or depyrogenation is used to sterilize laboratory glassware and equipment. The efficacy of sterilization using these methods should be monitored using biological indicators. Disinfectants such as ethanol, quaternary ammonium compounds, and sodium hypochlorite are used for decontamination of facilities and equipment, and for clean up of certain spills. Vacuum or positive-pressure filtration is an alternative method for sterilization of liquids that do not withstand steam sterilization.

AUTOCLAVING LIQUIDS

BASIC PROTOCOL 1

The autoclave is used for sterilization by moist heat. The standard conditions for moist-heat sterilization are exposure to saturated steam under pressure at 121°C for 15 min, although other temperature/time specifications can be utilized for specialized needs. In general, materials suitable for autoclaving as a nondestructive sterilization process must meet the following criteria: (1) stable to the temperature and time of the autoclave cycle, (2) unaffected by moisture, (3) packaged to permit exposure to steam, and (4) hydrophilic, if liquid. Materials are sterilized by autoclaving only if they are wetted with the steam; thus, sealing gaskets on certain types of laboratory equipment may not be effectively sterilized if tightened in place during the autoclave cycle. Materials can be decontaminated by autoclaving providing criteria (3) and (4) are met.

The autoclave cycle is based on the time it takes the material being sterilized to be in contact with saturated steam at 121°C for 15 min, and not the time the autoclave itself has been selected to run at that temperature. As autoclave efficiency is machine specific, and steam penetration is container and volume dependent, autoclave cycles and load configurations should ideally be validated to assure that sterilization conditions are achieved. For many small laboratories, the purchase expense of temperature-monitoring thermocouples might be prohibitive. However, frequent use of biological indicators to monitor autoclave conditions (see Support Protocol 1) is strongly recommended for all laboratories, particularly when the machine is used for decontamination of biological waste. Table 1.4.1 lists suggested autoclave times for load configurations in an autoclave with a $20 \times 20 \times 38$–in. ($51 \times 51 \times 97$–cm) chamber.

Materials

Heat-resistant containers and vessels (e.g., borosilicate glass, high-grade stainless steel, noncytotoxic plastic)
Liquid to be autoclaved
Moisture-resistant labels
Paper or aluminum foil
Autoclave indicator tape

Contributed by Rosalie J. Coté
Current Protocols in Cell Biology (1999) 1.4.1-1.4.21

Autoclave
Autoclavable discard pans

1. Use a heat-resistant vessel that can hold twice the volume of the liquid to be autoclaved to assure that boiling encountered during the heating and cooling periods of the autoclave cycle do not result in a boil-over of the vessel contents.

   The most frequent laboratory frustrations with autoclaves and sterilization of liquids are boil-over of material or blow-off of closures. When volume-to-container relationships are acceptable, and when the solution is not overly viscous, the problem most often occurs because the autoclave's slow exhaust or liquid cycle is not properly functioning and the machine is exhausting the chamber pressure too quickly. Adjustments or repairs to the unit should be made by qualified personnel.

2. Fill the vessel to desired volume with liquid to be autoclaved. Indicate the contents of the container with permanent ink and a moisture-resistant label.

3. Loosely cover any opening to the vessel. Do not overtighten screw-cap closures, as this prevents adequate pressure/steam exchange. Overwrap and secure cotton-plugged flasks with paper or aluminum foil to prevent plugs from blowing off if the autoclave is exhausted too rapidly.

4. Affix a piece of autoclave indicator tape to each item or package, as a visual reference (following the autoclave cycle) that the material has been processed.

   The color change of autoclave tape indicates only that the tape has been exposed to a 121°C temperature, and not how long it has been held at that temperature. Thus, it is not an indicator of successful sterilization.

5. Load the autoclave with vessels of similar size, volume, and configuration. Place all liquid-containing vessels inside autoclavable discard pans inside the autoclave. Close and lock autoclave door.

   The discard pans should be large enough to contain all fluid or glass in the event of boiling over or breakage during autoclaving.

6. Set autoclave controls for liquids or slow exhaust.

7. Select and set autoclave controls for appropriate sterilization time (see Table 1.4.1).

Table 1.4.1 Suggested Autoclave Run Times and Configurations for 121°C Sterilization in a 20 × 20 × 38–in. Autoclave[a]

Vessel	Size	Volume (ml)	Minimum time (min)[b]	Maximum time (min)[b]
Test tubes	13 × 100 mm	4-6	18	20
	16 × 125 mm	5-10	18	20
	20 × 150 mm	12-20	18	20
Flasks	100 ml	25-50	20	26
	250 ml	75-100	24	28
	500 ml	250	26	30
	1000 ml	500	28	32
	2000 ml	1000	30	32
Media bottles	125 ml	50	20	22
	500 ml	250-500	30	32
Empty glassware	All	All	35	90

[a]Chamber dimensions (metric equivalent, 51 × 51 × 97 cm).

[b]Sterilization times indicated are actual autoclave timer settings. Material within the vessels will be exposed to 121°C for 15 min using these process times. See text for further explanantion.

8. Start and run autoclave cycle to completion.

   Any interruption in the cycle (e.g., a sudden drop in chamber pressure) invalidates the run, and the sterilization cycle must be rerun to assure efficacy of the process. This is problematical if the liquid has limited stability to prolonged or repeated autoclaving, such as the microbiological media used for quality control in cell culture work. In such cases, it is best to start over with new media.

9. Open the autoclave door only when chamber pressure registers 0 lb/in^2 (100°C or less).

   CAUTION: *Never stand in the path of escaping steam when opening an autoclave.*

10. Remove flasks or containers only when all bubbling has stopped.

    CAUTION: *Superheated liquids can easily boil violently if even slightly jostled. Resulting boil-over can badly scald laboratory personnel.*

11. Cool vessels to ambient or other prescribed temperature in a relatively clean area not subject to excessive air currents.

    This helps avoid suctioning of heavily contaminated environmental air into the container, which can sometimes occur when a vacuum forms within the container as the liquid cools.

AUTOCLAVING DRY GOODS

ALTERNATE PROTOCOL 1

Heat-stable dry materials (including stainless steel instruments, glassware, fabrics, and plasticware) can be effectively sterilized by autoclaving, providing all surfaces of the dry material come in contact with the saturated steam at 121°C. This can become problematical for small items (such as forceps) that must be packaged in an outer container or wrapping that impedes the flow of steam, or for folded fabrics that tend to harbor pockets of cooler air. For this reason, autoclaving times for dry goods sterilization often rely on overkill, as these materials generally have much higher heat resistance. As noted above, validation studies should be done to determine the most effective times and configurations for a given autoclave. For further information on general autoclaving considerations, see Basic Protocol 1 introduction. For default times for an autoclave with a $20 \times 20 \times 38$–in. ($51 \times 51 \times 97$–cm) chamber, see Table 1.4.1.

Additional Materials *(also see Basic Protocol 1)*

Items to be autoclaved
Shallow heat-resistant container

1. Loosely arrange small items in a shallow, heat-resistant outer container. Loosely cover the outer container's opening with paper or aluminum foil. If container has its own lid, apply it loosely so that steam and pressure can penetrate. Cap larger items such as bottles or flasks, making sure that all closures (e.g., screw caps) are loose enough to permit penetration of pressurized steam.

   Small items can also be individually wrapped in paper or foil.

2. Code each item or package with permanent ink and a moisture-resistant label identifying its contents. Affix a piece of autoclave indicator tape to each item or package as a visual reference (following the autoclave cycle) that the material has been processed.

   The color change of autoclave tape indicates only that the tape has been exposed to a 121°C temperature, and not how long it has been held at that temperature. Thus, it is not an indicator of successful sterilization.

3. Where feasible, add a small amount of deionized or distilled water to the outer container or the individual items to assure adequate moisture for effective sterilization.

4. Arrange material in the autoclave to avoid dense overpacking that will impede effective sterilization. Where possible, arrange items to permit downward displacement of cooler, heavier air (e.g., place empty bottles or flasks on their sides rather than upright in the autoclave).

   This prevents pockets of cool air from being trapped in the bottom of the containers as the hot, pressurized steam flows into the vessels.

5. Set autoclave for a fast exhaust cycle.

   If the sterilizer is so equipped, a drying cycle that removes moisture from the dry goods under vacuum at the end of the timed sterilization run can also be used.

6. Select and set autoclave controls for appropriate sterilization time (see Table 1.4.1).

7. Start and run autoclave cycle to completion.

   Note that any interruption in the cycle (e.g., a sudden drop in chamber pressure) invalidates the run, and the sterilization cycle must be rerun to assure efficacy of the process.

8. Open the autoclave door to remove items only when chamber pressure registers 0 lb/in^2 (100°C or less).

   CAUTION: *Never stand in the path of escaping steam when opening an autoclave.*

ALTERNATE PROTOCOL 2

AUTOCLAVING FOR DECONTAMINATION OF BIOLOGICAL WASTE

Biological laboratory waste is most frequently decontaminated by autoclaving unless it contains hazardous chemical materials that can volatilize in the sterilization process. In many mid- to large-sized laboratories, biological waste includes varying combinations of spent media, discarded cultures, and solid material. An autoclave load size or configuration can vary dramatically with each run. For this reason, autoclave cycles for decontamination most often employ the overkill approach. Validation studies prior to actual-use procedures must be performed to assure that selected operation procedures are adequate to achieve the desired conditions for successful decontamination. For further information on general autoclaving considerations, see Basic Protocol 1 introduction.

CAUTION: Do not dispose of biological material containing hazardous chemicals or radioactive isotopes in the waste stream designated for autoclaving.

Additional Materials *(also see Basic Protocol 1)*

Items for decontamination
Plastic (polyethylene or polypropylene) autoclavable bags for biohazardous waste

1. Place items for decontamination into plastic autoclavable bags clearly labeled as containing biohazardous material.

   For greater ease with postautoclaving cleanup procedures, segregate plastic disposable material from reusable labware in separate bags.

   CAUTION: *For the safety of laboratory personnel who sort the autoclaved waste for washing or disposal, do not dispose of sharps or pipets as loose items in the bags. These items must be segregated in their own containers (containing a disinfectant, if appropriate).*

2. Add ~500 ml water to bags containing only dry items (such as empty glassware or contaminated lab coats) to supply sufficient moisture for steam generation.

3. Support bags by placing them in large, shallow, leak-proof, autoclave-resistant discard pans to prevent tearing of the bags and release of contents.

   For the safety of personnel carrying the discard pans, use a maximum weight limit of 25 lb per pan.

4. Securely seal each bag to prevent leakage of material. Transport biological waste only in closed containers.

5. Code each bag with permanent ink and a moisture-resistant label to permit general identification of its contents or source-lab should an accident occur (e.g., mycoplasma testing lab; QC lab).

6. Affix a piece of autoclave indicator tape to each item or package as a visual reference (following the autoclave cycle) that the material has been processed.

   The color change of autoclave tape indicates only that the tape has been exposed to a 121°C temperature, but does not indicate the time held at that temperature. Thus, it is not an indicator of successful sterilization.

7. Transport the waste using a sturdy laboratory cart or autoclave carriage and dolly.

8. Load the discard pans loosely into the autoclave to allow steam to flow over and around the material. While loading, slit each individual bag open in several spots to allow direct exposure of the contents of the bags to the steam from the autoclave.

   Do not stack pans directly on top of each other.

9. Close and lock autoclave door.

10. Run autoclave cycle for 90 min at 121°C on a fast-exhaust, gravity cycle.

    The cycle time listed here is for a large (24 × 36 × 48–in.; 61 × 91 × by 122–cm) autoclave. With small loads and smaller-chamber autoclaves, 45 min may be sufficient. Any interruption in the cycle (e.g., a spurious drop in autoclave steam pressure) invalidates the run.

11. Open the autoclave door to remove items only when chamber pressure registers 0 lb/in^2 (100°C or less).

    CAUTION: *Never stand in the path of escaping steam when opening an autoclave.*

USE OF BIOLOGICAL INDICATORS FOR MONITORING AUTOCLAVE PROCESSES

SUPPORT PROTOCOL 1

Biological indicators are used to effectively monitor the efficacy of moist- or dry-heat sterilization processes. The indicators contain standardized preparations and concentrations of resistant endospores of specific strains of bacteria that will survive suboptimal sterilization conditions, and proliferate when subsequently incubated under normal growth conditions. For greatest control of sterilized material, biological controls should be included with every load. Under general laboratory conditions, biological indicators should be used for validation studies in conjunction with thermocouple temperature-sensing probes, and they should be used for frequently scheduled monitoring of the performance of sterilization equipment and procedures. Bioindicator sources listed in this unit are examples only; other products by other manufacturers can work well. Specific manufacturer instructions for product use supercede general instructions described in this protocol.

This protocol describes the use of biological indicator ampules for monitoring sterilization. For monitoring dry-heat sterilization, see Support Protocol 2.

Additional Materials *(also see Basic Protocol 1; see Alternate Protocols 1 and 2)*

Biological indicator ampules: standardized concentration of *Bacillus stearothermophilus* (ATCC #7953) spores suspended in growth medium containing bromcresol purple as a pH indicator (e.g., Prospore from Raven Biological Laboratories)
55° to 60°C incubator

1. Label the desired number of biological indicator ampules with permanent ink to indicate location and autoclave run number.
2. Place one or more ampules in the most difficult locations to sterilize in the autoclave, including (1) near the front drain of the autoclave, located on the chamber floor at the door of the autoclave, and (2) suspended within the item being sterilized. For liquids, suspend the ampule in the container of liquid by a string tied around the ampule neck and secured around the opening of the container. For dry goods, tuck the ampule within the samples being autoclaved.

   Manipulating test ampules in loads containing biohazardous waste must be performed by personnel trained for dealing with the potential hazards of the material.
3. Run autoclave cycle at prescribed conditions (see Basic Protocol 1; see Alternate Protocols 1 and 2) and retrieve ampules.

   CAUTION: *After sterilization, handle ampules with care if still hot, as they are under pressure and might burst if strongly jostled.*

   Validation studies should never be performed in routine process cycles; unfortunately, they are too often used interchangeably in small laboratories. If validation and processing are used simultaneously, the sterilized material must be quarantined until the results of the sterilization monitoring tests are confirmed.
4. Place autoclaved test ampules and a labeled, unautoclaved positive control ampule in a vertical position in a 55° to 60°C incubator.
5. Incubate 48 hr.
6. Analyze results by noting the color of the test ampules and positive control ampule.

   Growth of the positive control confirms lack of sterilization. The positive-control ampule exhibits a color change from purple (prior to sterilization) to yellow (postincubation), with or without turbidity.

   Growth of the test sample indicates failed sterilization, and is seen as a color change from purple (prior to sterilization) to yellow (postincubation). Positive sterilization is indicated by a purple-colored test ampule (postincubation). An intermediate yellowish color is suspicious and necessitates additional testing of the autoclave parameters. An intermediate grayish color, without turbidity, usually indicates heat destruction of the bromcresol pH indicator, resulting from prolonged autoclaving conditions.

BASIC PROTOCOL 2

DRY-HEAT STERILIZATION AND DEPYROGENATION

Dry heat is used for components and materials that are resistant to the 140° to 180°C temperatures needed for effective dry sterilization; it is most often used for the sterilization of laboratory glassware and stainless steel instruments. It is also used for sterilization of nonaqueous, heat-stable liquids such as mineral oil. Depyrogenation of heat-tolerant materials is done with ovens capable of operating at the required processing temperatures of 220° to 350°C. As with autoclaving, standard dry-heat sterilization and depyrogenation times refer to the time the material is held at the prescribed temperature and not to the time the oven has been set to run. Dry-heat sterilization using gravity ovens generally requires a longer time than does sterilization with convection ovens, which evenly

distribute the heated air throughout the chamber with blowers. In all instances, process validation of any dry-heat sterilization protocol is required. The frequent use of bacterial spore strips (see Support Protocol 2) is advantageous for routine monitoring of the efficacy of an established sterilization process.

Materials

Items to be sterilized
Heat-resistant outer containers (borosilicate glass or stainless steel) for small items
Aluminum foil
Heat-resistant labels or tape
Dry-heat indicator tape
Laboratory oven (operating temperature of 140° to 180°C for sterilization; 220° to 350°C for depyrogenation)

1. Place small items to be sterilized into heat-resistant outer containers. Use aluminum foil to cover any openings to larger, individually sterilized items, or to cover any openings to large items that do not have their own closures.
2. Code each item or package as to its contents with permanent ink and a heat-resistant label.
3. Affix a piece of dry-heat indicator tape to each item or package as a visual reference (following the oven cycle) that the material has been processed.

   The color change of indicator tape shows only that the material has been exposed to a prescribed sterilization temperature, but does not indicate the time held at that temperature. It is not an indicator of successful sterilization.

4. Loosely arrange material in the oven. Do not overpack, as this prevents efficient heat penetration to all items.
5. Close and secure oven door.
6. Select operating temperature and time (see Table 1.4.2 for general guidelines).

   Note that times designated in Table 1.4.2 do not include temperature buildup time, as this is equipment specific. The actual sterilization time begins when the oven chamber reaches the prescribed temperature. Heating times are long for dry-heat sterilization, and can actually be longer than the sterilization time itself. Thus, a load of material might require 2 hr to reach 180°C, while needing only 0.5 hr at that temperature to be effectively sterilized.

7. Run dry-heat sterilization cycle to completion.

   Any interruption in the cycle (e.g., opening the door to add just one more item to the load) invalidates the run.

Table 1.4.2 Time-Temperature Relationships for Dry-Heat Sterilization

Oven temperature (°C)	Sterilization time (hr)[a]
180	0.5
170	1.0
160	2.0
150	2.5
140	3.0

[a]Sterilization time indicated is the amount of time for which material should be raised to a given temperature and does not include heating time.

8. Turn oven heating element off and allow material to cool to room temperature before removing items from the oven.

   This can take several hours for large loads in gravity ovens.

SUPPORT PROTOCOL 2

USE OF BIOLOGICAL INDICATORS FOR MONITORING DRY-HEAT STERILIZATION

Biological indicator strips are used to monitor dry-heat sterilization. For a general discussion of biological indicators, see Support Protocol 1.

Additional Materials *(also see Basic Protocol 2)*

Biological indicator strips containing standardized concentrations of *Bacills subtilis* (ATCC #9372) spores (e.g., Spore-O-Chex; PyMaH, or VWR)
Trypticase soy broth (see recipe)
30°C incubator

1. Label the appropriate number of biological indicator strips with location and cycle number or date.
2. Place one or more strips in the most difficult-to-sterilize areas of the load.
3. Run sterilization cycle at prescribed conditions (see Basic Protocol 2) and retrieve strips when cool.
4. Aseptically open the outer wrapping of the indicator, remove the spore strip, and insert it into an appropriately labeled tube of trypticase soy broth (6 to 10 ml per tube).
5. Prepare a positive control by aseptically inserting an unsterilized spore strip into a separate tube of trypticase soy broth. Prepare an uninoculated tube of trypticase soy broth as a negative control.
6. Incubate tubes at 30°C for 4 days.
7. Analyze results by noting turbidity of the broth. Compare test samples with positive and negative control tubes. Resterilize any material in loads with positive test samples.

   Bacterial growth in the positive control, indicated by cloudy medium, confirms lack of sterilization. Growth should be absent in the negative control; the medium should remain clear, with no precipitate.

BASIC PROTOCOL 3

USE OF DISINFECTANTS: 70% ETHANOL

Ethanol is widely used in many laboratories for benchtop or laminar-space disinfection. The antimicrobial activity of the alcoholic solution is very much dependent upon the working concentration of the solvent, proper preparation, storage, and conditions of its use. Ethanol is an effective disinfectant against vegetative bacterial and fungal cells, but is totally ineffective in germicidal activity against bacterial spores. Ethanol is suitable for spraying or swabbing, but is not recommended for large-volume applications. Ethanol is highly flammable, and spills near the flame of a Bunsen burner are always a possible safety hazard. Similarly, an elevated concentration of vaporized ethanol in a liberally disinfected biosafety cabinet could ignite in the presence of a flame or spark. Furthermore, 70% ethanol is not recommended for use in discard pans or for decontamination of biological spills in the catch basins of biological safety cabinets.

Materials

100% denatured ethanol
Ethanol-resistant spray-type storage container

1. Add 700 ml of 100% denatured ethanol to 300 ml deionized or distilled water. Mix well by stirring.

   Denatured ethanol is absolute ethyl alcohol to which small amounts of chemicals have been added to render it unsuitable for human consumption. This does not interfere with most industrial uses.

2. Store working solution in a tightly closed container to retard evaporation.

   Choose a spray bottle rather than a squirt bottle to retard the evaporation of solvent that occurs with the larger opening of a squirt bottle neck.

3. *For lab benches or laminar-flow cabinets:* Liberally spray the alcoholic solution in a crisscross pattern over the work surface, making certain that the entire area is wetted. Let disinfectant remain in contact with the surface for ≥10 min. Wipe away excess solution with absorbent towels

4. *For objects (e.g., media bottles, culture flasks):* Wet absorbent towels (cheesecloth or paper towels) with the alcoholic solution. Thoroughly swab the object, being careful not to introduce any of the liquid into the threads of screw caps or other container closures. Let disinfectant remain in contact with the object for 3 to 5 min. Wipe away excess solution with absorbent towels

USE OF DISINFECTANTS: QUATERNARY AMMONIUM COMPOUNDS

ALTERNATE PROTOCOL 3

The discovery of the antimicrobial activity of quaternary ammonium compounds during the early 20th century was a major advancement in the development of effective germicides. The inherent antimicrobial activity of these compounds was soon shown to be significantly improved by the addition of long-chain alkyl groups to the nitrogen moiety of the quaternary compound. The various quaternary ammonium compounds commercially used as disinfectants today are chemical modifications of this original concept. The mode of action of quaternary ammonium compounds is as cationic surface-active agents, although this chemical property does not fully explain the germicidal activity of the compounds. All have broad-based antimicrobial activity and have proven effectiveness against algae, gram-positive bacteria, some gram-negative bacteria, fungi, and certain viruses, when used at the manufacturer's recommended concentrations (0.1% to 2.0% active ingredient, or 200 to 700 ppm). They are relatively nontoxic to humans when used according to manufacturer's instructions and are not chemically destructive to equipment under normal use. They can be autoclaved without formation of toxic vapors and thus are frequently used as disinfectants in discard pans.

The limitations of quaternary ammonium compounds include lack of effectiveness at low concentrations against some commonly encountered gram-negative bacteria (e.g., *Pseudomonas* sp.). Like many other disinfectants, they are quickly inactivated by the presence of heavy organic burden.

Materials

Quaternary ammonium compound disinfectant of choice: e.g., Roccal (Sterling Winthrop), Micro-Quat (Ecolab), Zephirol (Bayer)
Tightly closed containers
Spray bottles
1-gallon jugs

1. Dilute concentrated quaternary ammonium compound according to manufacturer's instructions in deionized or distilled water and stir well to mix.

   Depending upon use, normal working concentrations of 235 ppm active ingredient are used for sanitization purposes such as floor mopping, whereas concentrations of 470 to 700 ppm active ingredient are used for disinfection of laminar-flow cabinet work surfaces or for use in discard pans.

2. Store working solution in tightly closed containers to retard evaporation.

   Prepare fresh working solutions frequently (e.g., once a month; follow manufacturer's instructions).

3. *For work surfaces:* Liberally spray the working solution in a crisscross pattern over the work surface, making certain that the entire area is wetted. Let disinfectant remain in contact with the surface for >10 min. Wipe away excess solution with absorbent towels.

4. *For use in discard pans:* Prepare a solution containing 700 ppm quaternary ammonium compound. Fill discard pan about half full with disinfectant solution. Carefully place used pipets into the pans to avoid splashing. Securely cover pan when moving it.

   Volume of disinfectant must be enough to fully cover the pipets placed into the pan, but not so full that spilling could occur when the pan is filled with pipets or when it is moved.

ALTERNATE PROTOCOL 4

USE OF DISINFECTANTS: SODIUM HYPOCHLORITE

Chlorine, in various forms, has a long history of use as a powerful disinfectant, yet the exact mode of germicidal action is unclear. Hypochlorites are the most widely used chlorine compounds for disinfection. Commercial liquid bleach products (e.g., Clorox) are solutions containing 5.25% (w/v) sodium hypochlorite. Sodium hypochlorite is effective against vegetative microbial cells, most spores, and many viruses. It has some residual effect after the treated surface dries. It can be used in sanitization procedures for laboratory floors and in laboratory coat washing. It is strongly germicidal and can be used to decontaminate small- to mid-volume spills of biological material.

Despite their germicidal effectiveness, chlorine solutions are limited in their use as laboratory disinfectants because of their corrosiveness to metals and their human toxicity. They should not be routinely used in discard pans or in any solutions that are autoclaved, as the chlorine fumes liberated are significant skin and respiratory irritants. Frequent autoclaving of chlorine solutions will corrode the chamber interior of the sterilizer. One exception to this autoclave ban is the need to sterilize any biological spill material in which bleach was used as a disinfectant during the cleanup process. A solution of 10% (v/v) household bleach is a strongly germicidal, containing ~0.52% (w/v) sodium hypochlorite. Excess hypochlorite is needed in mopping up spills of biological agents, to supply additional chlorine to replace that consumed by the large amount of organic matter associated with the spill.

Sodium hypochlorite solutions can be inactivated by organic matter (which consumes the available free chlorine that constitutes microbiocidal activity), by exposure to UV light, and by inorganic chemical reducing agents (such as ferrous or manganese cations and hydrogen sulfide). Hypochlorite solutions should be stored away from heat to avoid deterioration.

Materials

Household liquid bleach (e.g., Clorox, Dazzle)
5% (w/v) sodium thiosulfate solution

1. Add 100 ml household bleach to 900 ml water to give a 10% (v/v) solution. Mix well. Store in the dark away from heat.
2. *For cleanup of open spills:* Soak paper towels with bleach and gently cover the spill, being careful not to enlarge the area of the spill. Let bleach stay in contact with the spill for ≥20 min. During this time, decontaminate any nearby areas that may have been subject to spatters from the original spill by swabbing with additional 10% bleach. Collect used paper towels in a suitable container and place in an autoclave bag.
3. *For autoclaving bleach-containing waste:* Add ~1 vol of 5% sodium thiosulfate solution to the bleach solution to help neutralize the chlorine. Seal the bag and autoclave. Prominently label the autoclave room door to warn laboratory personnel of the potential for irritating vapors. When the autoclave cycle is finished, crack the autoclave door slightly to allow remaining chlorine fumes to dissipate before removing the bags.
4. *For sanitizing solution:* Add 14.8 ml household bleach to 3.78 liter water (0.4%). Mix well.

   Solutions containing 0.4% (v/v) household bleach (200 ppm available chlorine) are suitable for soaking lab coats without being so strong as to harm the fabric. The solution is acceptable for routine floor maintenance.
5. *For disinfection solution:* Add 44.4 ml household bleach to 3.78 liter water (1.2%). Mix well. Liberally apply to surface with clean absorbent towels and let stand for >10 min. Dry surface with a separate clean towel.

   This concentration of bleach (600 ppm available chlorine) is suitable for disinfecting biosafety cabinets during scheduled weekly maintenance. More frequent use on stainless steel may not be recommended because of the corrosiveness of the bleach.

FILTER STERILIZATION OF SOLUTIONS

All solutions that come in contact with cell cultures must be sterile in order to prevent microbial contamination. This includes non-nutritive preparations such as distilled/deionized water and reagents (e.g., dimethyl sulfoxide used as a cryoprotectant). Although heat-stable solutions can be sterilized by autoclaving, many solutions used in cell culture contain one or more heat-labile components (e.g., antibiotics), or are chemically formulated with ingredients that will form deleterious precipitates if subjected to steam sterilization temperatures (e.g., phosphate-buffered salines). Membrane filtration is the most common cold sterilization method for these types of solutions.

Filter membranes with 0.2-µm pore size are used for general sterilization purposes; however, some environmentally stressed bacteria (e.g., *Pseudomonas* sp.) as well as mycoplasma can pass through filters of this porosity. To provide a greater degree of assurance for complete removal of these common tissue culture contaminants, cell culture media and sera should be sterilized using 0.1-µm filter membranes. Filter manufacturers offer many different types of membranes. With respect to cell culture applications, membranes fabricated from cellulose acetate or cellulose nitrate are used for general purpose filtration of aqueous solutions such as media and buffers, but may need prewashing with hot distilled water to remove extractable substances that may be cytotoxic. Nylon membranes are very low in extractable substances such as surfactants or wetting agents; polyethersulfone membranes are low in extractables and have very low protein binding.

The availability of presterilized, ready-to-use, disposable filter systems has eliminated much of the labor and risk of failure inherent with earlier filtration methods. Filter units come in a wide variety of sizes to handle small (≤10-ml) to large (≥20-liter) volumes. Disposable systems are available for either vacuum or positive-pressure filtration. Many manufacturers have filter systems designed specifically for cell culture applications: the sterilization membranes, housings, and receiving vessels are certified noncytotoxic and nonpyrogenic.

This section outlines selection of filters and filter-sterilization procedures for various types of liquids encountered in cell culture laboratories. The most common small-volume filtration technique is positive pressure using a syringe to force the liquid through the filter membrane (see Basic Protocol 5). Volumes ranging from 50 ml to 1 liter are most efficiently processed with a vacuum (see Basic Protocol 4). Larger volumes should be filter sterilized with positive pressure (see Alternate Protocols 5 and 6). The primary use of membrane filtration is in the preparation of tissue culture media; this topic is treated in depth in *UNIT 1.2*. This unit focuses on problematic filtration needs that often appear in cell culture applications, such as the need to filter sterilize a hazy solution like the serum/yeast extract additives used in mycoplasma media, or the chemically aggressive reagent dimethyl sulfoxide (DMSO). The methods outlined in this section are equally adaptable for the preparation of tissue culture media or stock solutions of additives such as glutamine or puruvate. For media preparation, use noncytotoxic cellulose acetate/nitrate membranes, or similar membranes specific to the application, and food-grade silicon tubing. For further details on the use of vacuum versus positive-pressure filtration, see Background Information.

BASIC PROTOCOL 4

Vacuum Filtration

Solutions that are initially clean preparations, in that they are free of particulate debris and are not proteinaceous, can be directly filter sterilized with no difficulty. Solutions with high particle load require centrifugation and/or nonsterile prefiltration through depth filters (see Background Information) and larger-porosity membranes prior to sterile 0.2-µm filtration. This protocol uses 200-ml to 1-liter disposable systems designed for the final vacuum filtration of media, sera, and other aqueous solutions. The protocol also describes nonsterile prefiltration for particulate removal from 200-ml to 2-liter volumes of filtrate.

Materials

Solution to be filtered
47-mm funnel/support assembly (optional; e.g., Kontes, Millipore) attached to a 1- to 2-liter vacuum filtration flask (Fig. 1.4.1)
47-mm glass fiber depth filters (optional; Gelman, Millipore)
47-mm membrane filters (optional; 0.45-µm and 0.2-µm pore sizes)
Disposable, sterile filter unit (e.g., Corning, Nalgene) including:
  Filter funnel, housing an integrally sealed 0.2-µm filter membrane
  Funnel dust cover
  Removable receiver bottle and cap
  Barbed tubing adapter
  Nonsterile depth prefilters (included by most manufacturers)
Vacuum source

NOTE: Perform all procedures using aseptic technique (*UNIT 1.3*).

1. If the solution to be sterilized is a hazy suspension or has a noticeable precipitate, centrifuge 30 min at 10,000 × g to clarify. Alternatively, use a funnel/filter assembly

to process the solution through a series of nonsterile prefilters: depth filter, followed by 0.45-µm membrane, followed by 0.2-µm membrane.

Depending upon the particulate load, the filter membranes may have to be replaced if they clog before all the solution is processed.

If a water aspirator is used as the vacuum source, include an in-line catch flask or hydrophobic filter to prevent any water from accidentally being drawn into the vacuum port and into the filtration flask.

2. Remove a disposable, sterile filter unit, barbed tubing adapter, and individual wrapped sterile receiver cap from the plastic bag.
3. Check to make certain that the filter funnel is firmly attached to the receiver. Hand tighten if necessary.
4. Attach barbed tubing adapter to the side vacuum port on the neck of the filter funnel. Attach the vacuum tubing to the adapter.

   If a water aspirator is used, include an in-line catch flask (see step 1).

5. Set the filter upright and provide support to avoid tipping the unit when it is top-heavy with liquid in the funnel.
6. Remove the funnel dust cover and slowly add solution (centrifuged or prefiltered if necessary) to the funnel. Slowly apply a slight vacuum—5 pounds per square inch gravity (psig)—to prevent excessive foaming of proteinaceous solutions.
7. When filtration is complete, turn the vacuum source off. Carefully disconnect the filter unit from the vacuum tubing.

   If the tubing is pulled off while the vessel is still under full vacuum, the room air rushes into the receiving vessel at a much higher velocity.

8. Using aseptic technique, carefully remove the filter funnel from the receiver bottle and seal the bottle with the sterile screw-cap closure provided with the filter unit.

   If the total volume of solution to be filtered exceeds the capacity of the receiver bottle supplied with the filter unit, the initial volume of sterile filtrate can be aseptically transferred to a secondary sterile storage vessel, and the filter funnel can be reattached to the original receiver to process a second volume of solution.

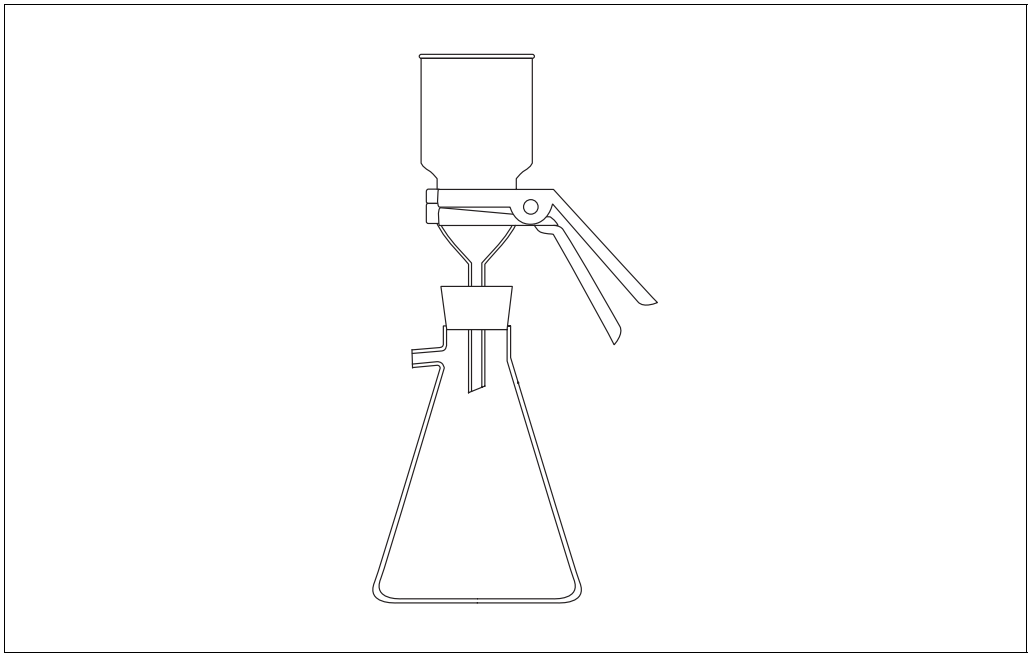


Figure 1.4.1 Funnel/support assembly for vacuum prefiltration.

BASIC PROTOCOL 5

Small-Volume Positive-Pressure Filtration of Nonaqueous Solutions

Dimethyl sulfoxide (DMSO) is used as the cryoprotectant for liquid-nitrogen preservation of cell cultures. The reagent is not stable to autoclaving conditions and must be filter sterilized. DMSO is an aggressive solvent that dissolves general-use filter membranes (such as cellulose acetate or cellulose nitrate) as well as the polystyrene filter units themselves. This protocol describes small-volume positive-pressure filtration using DMSO-resistant syringe-type filter units. For large volumes, see Alternate Protocols 5 and 6.

Materials

Dimethyl sulfoxide (DMSO)
Glass 25-ml syringe with Luer-lok tip
Sterile syringe filter: 25-mm-diameter nylon membrane, 0.2-µm pore size, polypropylene housing (Nalgene or equivalent)
Laminar-flow cabinet
Sterile amber glass storage vessels with polytetrafluoroethylene (Teflon, PTFE)–lined screw-cap closure

NOTE: Perform all procedures using aseptic technique (*UNIT 1.3*).

1. Load a glass 25-ml syringe with deionized or distilled water.
2. Aseptically remove a sterile syringe filter from its blister-package wrapper, being careful not to touch the outlet nipple.
3. Attach the inlet end of the filter to the syringe and finger tighten the Luer-lok connection.
4. Apply a firm, but not forceful, pressure to slowly discharge the water through the filter into a waste container.

   This initial step is necessary to wet the filter to permit flow of the DMSO through the nylon membrane.
5. Carefully remove the filter from the syringe, and rest the filter on the laminar-flow cabinet work surface, being careful to keep the outlet nipple facing up (i.e., not touching any surface).
6. Load the syringe with ~25 ml DMSO and replace the filter as in step 3.
7. Apply a firm, but not forceful, pressure to slowly discharge this first volume of DMSO into a waste container.

   This step clears any water remaining in the syringe and filter.
8. Reload the syringe with ~25 ml DMSO and discharge the filtrate into a suitable sterile amber glass storage vessel. Cap immediately with a PTFE-lined screw-cap closures. Store up to 6 to 9 months at room temperature.

ALTERNATE PROTOCOL 5

Large-Volume Positive-Pressure Filtration of Nonaqueous Solutions

This protocol uses a peristaltic pump to provide positive pressure for large-volume filtration. The setup is shown in Figure 1.4.2.

Materials

Dimethyl sulfoxide (DMSO)
Sterile filter capsule: 400-cm^2-surface-area nylon membrane, 0.2-µm pore size, polypropylene housing (Whatman Polycap 36AS or equivalent)
Glass 25-ml syringe with Luer-lok tip

Worm drive clamps
PTFE tubing: polytetrafluoroethylene (PTFE or Teflon) with 0.25-in. (6.4-mm) i.d., 0.06-in. (1.6-mm) wall thickness, 0.38-in. (9.5-mm) o.d. (Norton or equivalent)
Peristaltic pump assembly capable of providing an operating pressure of 15 to 20 lb/in.^{2}
Sterile amber glass storage vessels with PTFE-lined screw caps

NOTE: Perform all procedures using aseptic technique (*UNIT 1.3*).

1. Carefully remove a sterile capsule filter from its plastic bag.
2. Remove the nipple cover from the inlet barb.
3. Hold the filter over a waste container, and use a glass 25-ml syringe to carefully add water to the filter housing through the inlet barb. Fill the syringe as necessary and continue to flush the housing until water begins to drip from the sterile outlet side.

   This step wets the nylon membrane to allow the DMSO to pass through the filter.
4. Attach a piece of PTFE tubing to the inlet barb from the nonsterile DMSO reservoir. Using worm drive clamps, secure the tubing at all connections when working with positive pressure to prevent sudden blowing off of tubing in case of accidental overpressurization.
5. Secure the capsule filter to an upright support at a height with sufficient clearance to accept any receiving vessels.
6. Connect the tubing as shown in Figure 1.4.2, to the peristaltic pump head according to the pump manufacturer's instructions. Apply power to the pump and begin pumping.
7. Discharge the first 200 ml of DMSO filtrate to a waste container.

   This step clears any water remaining in the filter and tubing.
8. Collect sterile DMSO in suitable sterile amber glass storage vessels and cap immediately with PTFE-lined screw-cap closures. Store up to 6 to 9 months at room temperature.

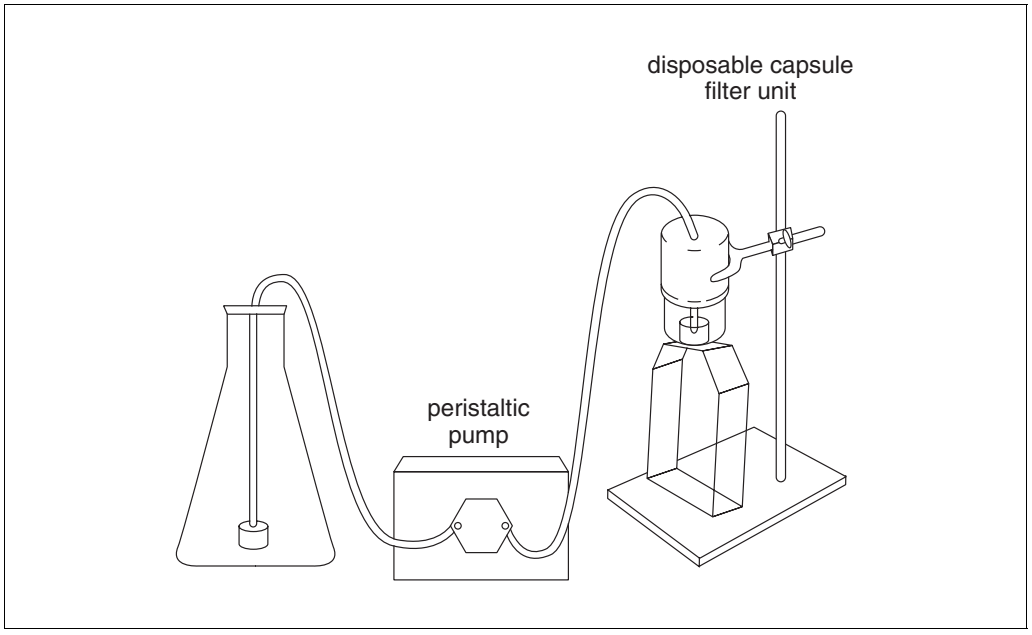


Figure 1.4.2 Positive-pressure filtration assembly for use with a peristaltic pump.

ALTERNATE PROTOCOL 6

Large-Volume Positive-Pressure Filtration Using Pressurized Nitrogen

This alternative method for large-volume filtration uses pressurized nitrogen to force the solvent through the filter membrane. It offers an advantage for filtration of DMSO in that it saturates the solvent with an oxygen-free gas phase that helps retard oxidation of the material during storage. The setup is shown in Figure 1.4.3.

Additional Materials *(also see Alternate Protocol 5)*

5-liter pressure vessel (or size to fit application; Gelman or Millipore)
Pressurized nitrogen tank
Additional tubing to fit pressure vessel and nitrogen tank

1. Prepare a capsule filter (see Alternate Protocol 5, steps 1 to 3).
2. Attach a piece of tubing to the filter inlet barb from the outlet barb of a 5-liter pressure vessel. Using worm drive clamps, secure the tubing at all connections when working with positive pressure to prevent sudden blowing off of tubing in case of accidental overpressurization.
3. Fill pressure vessel with DMSO. Close and secure pressure vessel lid. Open pressure relief valve on vessel.
4. Attach another piece of tubing from a pressurized nitrogen tank to the inlet barb on the pressure vessel and secure with a worm drive clamp.
5. Slowly open nitrogen feed valve and wait until gas can be heard escaping from the pressure relief valve.
6. Close pressure relief valve.

   Pressure will begin to rise in the vessel as indicated either on the pressure gauge included with the vessel, or by the gauges on the nitrogen tank regulator.

   To prevent bursting of the filter membrane, keep the operating pressure below the maximum rated pressure specified by the filter's manufacturer.
7. Collect sterile DMSO filtrate (see Alternate Protocol 5, steps 7 and 8).

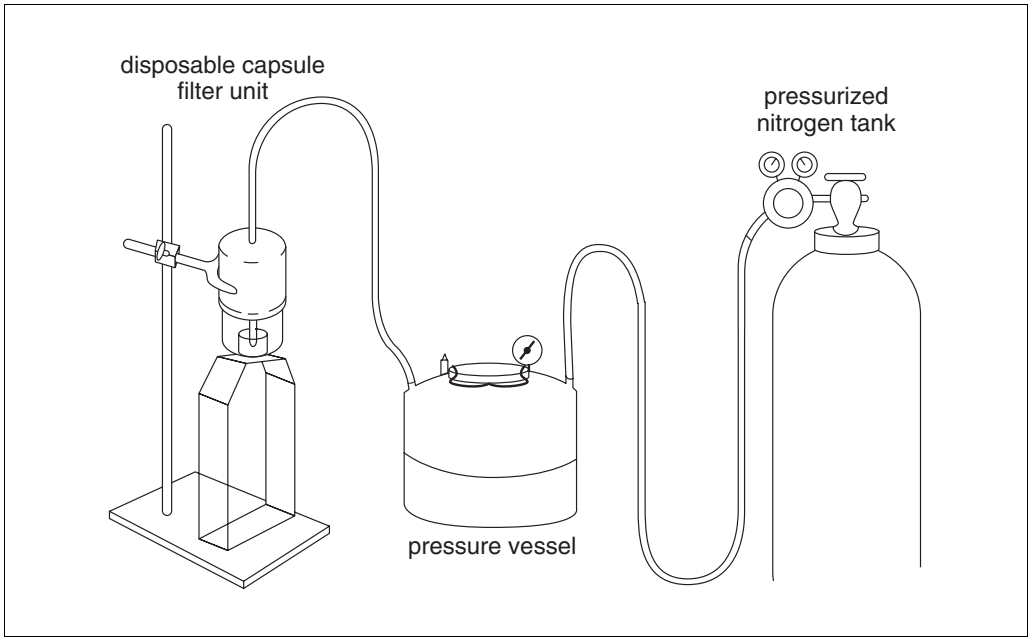


Figure 1.4.3 Positive-pressure filtration assembly for use with pressurized nitrogen.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Trypticase soy broth

Suspend 30.0 g trypticase soy broth powder (BBL) in 1.0 liter water and stir to dissolve. Dispense 6 to 10 ml per 16 × 125–mm tube and cap loosely. Sterilize by autoclaving at 121°C for 15 min. Store prepared tubes at 4° to 8°C for up to 6 to 9 months.

COMMENTARY

Background Information

Autoclaving

Steam (autoclave) and dry heat are destructive terminal sterilization processes in which the effectiveness of the method is characterized by the rate of the microbial killing. As the order of death in a terminal sterilization process is a logarithmic function, mathematical calculations will never result in a zero survival rate. Thus, theoretically, complete sterilization is impossible. Therefore, rather than expecting an absolute, the effectiveness of terminal sterilization processes is mathematically expressed in terms of the decimal reduction time (*D* value), which is the time required at a given temperature to destroy 90% of survivors. A corollary term used in discussions of terminal sterilization is the *Z* value, which is the temperature at which a survival curve decreases by one log. Factors influencing these values include the concentration and type of microbial contaminants initially in the material to be sterilized, the physical nature of the material undergoing sterilization, and the performance characteristics of the sterilization equipment.

Disinfectants

The use of disinfectants in the cell culture laboratory is directed both to issues of personal safety as well as quality control. In most instances the distinction between the two considerations blurs; however, this unit discusses the use of disinfectants primarily as a means for prevention of microbial contamination from the standpoint of quality control (i.e., maintenance of noncontaminating conditions for cell culture manipulations). Human safety considerations are limited to brief notations regarding potential effects of misuse of specific disinfectants (e.g., chemical incompatibilities, lack of effectiveness against certain biological agents) rather than a reiteration of the need for disinfectants as a means of personal defense against etiologic agents.

The list of common disinfectants is lengthy: alcohols (ethanol or isopropanol), chlorine compounds (bleach), hydrogen peroxide, phenolics, and iodophores (povidone-iodine), to name a few. Yet from this broad list, there is no universal disinfectant solution that can work effectively for all laboratory situations. Thus, the concern for any laboratory is deciding upon its different needs for disinfection (e.g., spill cleanup, work surface disinfection, discard pan disinfection, routine floor cleaning, lab coat laundering), and then selecting the appropriate disinfectant and concentration for each purpose. It is beyond the scope of this unit to provide a lengthy treatise on all the commercially available liquid germicides. Focus is directed, instead, to the three most commonly encountered disinfectants utilized in tissue culture: 70% ethanol, quaternary ammonium compounds, and sodium hypochlorite (bleach).

Filtration

Filtration as a method of sterilization has been in use for over one hundred years. Early filters were designed to trap contaminants within the depths of a thick, tortuous maze of filter material. Scintered glass filters and the asbestos (later cellulose) Seitz filters commonly used in the 1950s and 1960s worked on the entrapment principle. These depth filters have significant loading capacity and can retain much particulate matter before clogging. The limitation of depth filters is the structural nature of the filter matrix. At best, the loose matrix of a depth filter permits only a nominal designation of particle size retention. Because the pores of a depth filter are of random size and shape, there is a real probability that small-sized contaminants will successfully channel through the filter through interconnecting pores. In addition, the effects of moisture and pressure associated with autoclave sterilization and/or use of

depth filters tends to result in a shifting of the filter material (media migration) that compromises the integrity of the fibrous filter matrix.

By the mid 1970s membrane filters had essentially replaced depth filters for sterile applications. Membranes are classified as screen filters: they are thin and the pores are uniformly sized and spaced across the sheet. This structural consistency allows predictable retention characteristics. Thus, membrane filters can be rated according to the minimal diameter of the smallest particle they will retain (e.g., 1.2-µm, 0.45-µm, 0.2-µm). Membrane filters have limited loading capacity (i.e., they quickly clog). For this reason, they are most effectively used in tandem with depth filters and membrane filters of larger porosity to conserve the filter surface area on the final sterilization membrane.

The type of filtration system, as well as the type of membrane used in the system, depends upon the nature of the filterable material and the volume of material being filtered. A wide variety of configurations is available in both presterilized disposable units containing integrated filter membranes, as well as membrane discs that can be used in conjunction with reusable glass or stainless steel filter housings. In the past, positive pressure was the preferred filtration method for cell culture media because it reduced the foaming and concomitant protein denaturation associated with vacuum filtration systems. Unfortunately, positive-pressure filtration systems are efficient only for small (syringe filtration) or large (pressure vessel) volumes. With recent technological advances in new membrane matrices and improved membrane supports for filter housings that eliminate excessive foaming, vacuum filtration with presterilized disposable units is now the easiest and most effective sterilization method for intermediate volumes (0.1 to 2 liters) of tissue culture media.

Filter membranes are made from a number of different materials. The most common filters featured in presterilized, disposable filtration units are those fabricated from esters of cellulose acetate or cellulose nitrate. These general-purpose hydrophilic membranes are suitable for aqueous solutions such as tissue culture basal media and supplements. These membranes can, however, bind proteins and may be of concern for certain critical applications. With the huge cell culture market as a direct target, filter manufacturers have, in recent years, begun to offer presterilized filtration units customized for cell culture, featuring noncytotoxic, low-protein-binding membranes (nylon or polyethersulfone).

Not every solution utilized in cell culture applications is truly aqueous or hydrophilic. Nonpolar liquids, such as DMSO, or chemically aggressive solutions, such as concentrated acids or bases, demand special chemically resistant membrane filters. Quite often, filters resistant to nonpolar solvents will need pretreatment with an appropriate wetting agent to quickly render the membrane filterable to a particular nonpolar liquid. For example, PTFE (Teflon) membrane filters require prewashing with methanol prior to use with DMSO. This poses no problem providing all traces of the cytotoxic alcohol are removed by washing prior to collection of any sterile, unadulterated DMSO filtrate. Where residual toxic wetting agents are a concern, nylon filters may be a more appropriate consideration. Nylon membranes, when wetted with water, readily accept DMSO.

With wide recognition of the detrimental effects of insidious mycoplasma contamination and the concern about absolute removal of these contaminants from a major point of entry (i.e., serum) into a cell culture system, much emphasis is now placed on 0.1-µm filtration of cell culture media and sera. Unfortunately, this pore size is not widely available in disposable filtration units. Gelman Sciences does, however, offer presterilized filter units with 0.1-µm polyethersulfone (Supor) membranes for small- to large-volume cell culture media sterilization. Expect slower flow rates with 0.1-µm filtration.

When lacking presterilized filtration units, a laboratory can turn to individual membrane discs of specified porosity and membrane type, available from major filter manufacturers. These can be sterilized by autoclaving as part of an integrated unit in a small-volume filter assembly (e.g., Fig. 1.4.1), or for large-volume needs in a 142- or 293-mm filter holder (Gelman or Millipore). If this route for sterile filtration is chosen, follow the manufacturer's instructions precisely with respect to membrane sterilization times, and use a slow exhaust cycle to avoid the membrane cracking that can occur with rapid pressure changes.

This unit outlines procedures that are suitable for any number of variations. Large volumes of tissue culture media can be processed with the peristaltic pump or pressurized nitrogen procedures providing a hydrophilic filter is used. Similarly, a hydrophilic syringe filter can be used to sterilize small volumes of media. Sterilization of DMSO is, however, limited to

those filter membranes and filter unit housings fabricated of materials resistant to the reagent.

Critical Parameters

Autoclaving

Successful autoclave sterilization is dependent upon the contents of a load coming into full contact with saturated steam at 121°C for 15 min. Many operational factors tend to work against these criteria. With respect to the sterilizer itself, if an autoclave is improperly maintained, problems can occur with inadequate removal of air from the chamber or with excessive moisture buildup. Either condition compromises sterilization parameters. Most autoclaves have cool spots that can move about in the chamber like a current depending upon load pattern and configuration. The nature of the load content also influences the time necessary to reach the time-temperature relationship. Small volumes will heat to sterilization temperatures faster than large volumes. Agar solutions that have solidified prior to sterilization will take longer than those that are loaded into the autoclave while still molten. Dry materials take longer to sterilize than those that are moist.

For any laboratory investing in as expensive a pursuit as tissue culture, sterilization equipment and procedures should be validated to assure that conditions for sterilization are met. Ideally a laboratory should invest in a thermocouple to monitor temperatures within areas of autoclave loads, in order to determine exactly how long it takes the material of concern to reach sterilization temperature. In many cases, the lag between the time an autoclave temperature gauge indicates 121°C in the autoclave chamber and the time the contents of a large flask or discard pan within the chamber reaches the same temperature is sobering. Where thermocouples are not available, the use of biological indicators can yield useful information about autoclave procedures and machine performance, although the time needed for incubation and interpretation of results is a drawback.

A final, but most important, consideration for autoclaving is the source of steam generation. Autoclaves used in the preparation of media or for sterilization of materials that come into contact with cell cultures must be supplied with clean steam (i.e., steam generated from purified water). Steam generated directly from general building physical plant sources (e.g., the building heating system) is frequently produced from water treated with potentially cytotoxic boiler amines and/or chemical softening agents.

Dry-heat sterilization

Hot dry air is an inefficient means of sterilization and should be reserved for those materials that cannot effectively be exposed to saturated steam in an autoclaving process. Sterilization by dry heat can be accomplished with temperatures as low as 140°C, but 170° to 180°C are more routine operating temperatures because of the difficulty in controlling the rate of heat penetration into the load. The time-temperature relationships for dry-heat sterilization noted in Table 1.4.2 indicate a requirement for longer times and higher temperatures than for autoclaving, because dry heat is less efficient than moist heat. Lag time for conduction of heat into the materials to be sterilized can be significant, as can cool-down periods for large, dense objects. Dry-heat sterilization is applicable only for materials resistant to 140° to 180°C, and thus is unsuitable for paper, many plastics, or rubber. As noted for autoclaving, a dry-heat sterilization procedure should be validated with thermocouples and biological indicators.

Disinfectants

The term disinfection refers to the treatment of surfaces with chemical solutions to reduce microbial presence. Exposure to a disinfectant may result only in bacteriostatic or fungistatic rather than microbiocidal conditions. Disinfection does not imply sterilization, and it should never be used as an alternative to appropriate sterilization methods (e.g., autoclaving, dry heat, ethylene oxide, incineration). Similarly, disinfectants are not detergents and they should not be used as the sole method for cleaning solid surfaces. Indeed, many disinfectants are quickly inactivated when burdened with organic matter. Thus, routine disinfection of work surfaces in laminar-flow cabinets first requires that the surface be washed with a good detergent to remove dried media or other dirt before application of the germicide.

Ethanol. A solution of 70% ethanol is not germicidal against bacterial spores. While freshly prepared solutions are normally free of spores, the working solutions can contain bacterial spores from cross-contamination and poor aseptic technique. Once the spores are separated from the physical presence of the disinfectant (i.e., when the ethanol volatilizes from a surface, leaving the dried spores behind), they can germinate in suitable growth conditions. Thus, fresh working solutions of

70% ethanol should be prepared frequently (e.g., weekly).

The efficacy of ethanol as a disinfectant is highly concentration dependent. The mode of action of alcohol as a disinfectant is protein denaturation. Thus, 95% ethanol is a poor disinfectant because there is not enough water in the preparation to permit effective denaturation of contaminant proteins. As ethanolic concentration drops below 70%, there simply isn't enough of the solvent present to adequately react with large concentrations of proteinaceous matter. Because of the dilution effect, 70% ethanol is not effective in disinfecting large spills of culture material.

Quaternary ammonium compounds. Quaternary ammonium compounds are not effective against spores and, in this aspect, should be used with the same cautions noted for ethanol. Quaternary ammonium compounds are quickly inactivated by organic matter. They must not be used for disinfection of large spills of culture material, nor should they be used for kill pans (see below).

Bleach. Chlorine compounds, most frequently sodium hypochlorite, are strongly germicidal. Yet their potential for human toxicity and strong corrosiveness limits their use.

Biological spills. Strong disinfectant solutions are the first line of defense in decontaminating small to moderate spills of biological agents in both open areas and biosafety cabinets. The choice and concentration of disinfectant is particularly critical in this application. Use of 70% ethanol would be a poor choice as its germicidal activity is highly concentration dependent and dilution effects associated with spill cleanup would diminish its effectiveness (see above). Quaternary ammonium compounds are germicidal against a broad range of microbes, but they are quickly chemically overwhelmed and inactivated by organic matter (e.g., the culture material or medium in a spill). Chlorine compounds are strongly germicidal.

Kill pans. A final note of caution regarding disinfectants is their use in discard pans or pipet pans. Too many laboratories consider these containers kill pans and use them as a convenient way to dispose of excess liquid cultures or other contaminated solutions. Discard pans should never be used in such a manner. Most general disinfectants are inactivated by excess organic matter and/or exhibit diminished germicidal effects with dilution. Kill pans should contain 10% (v/v) household bleach (0.525% sodium hypochlorite).

Filtration

When using disposable filtration units, replace the filter funnel immediately with a permanent receiving vessel closure once filtration is complete. If the funnel is left on the receiver, the filter membrane will crack as soon as it dries, thereby compromising the sterility of the filtrate.

Be prepared and willing to prefilter any hazy or precipitated suspension. The additional steps will much repay the effort when balanced against the significant monetary expense, time, and frustration spent dealing with prematurely clogged sterilization filters. If one repeatedly filter sterilizes the same type of suspension, a prefiltration scheme can be tailored according to the nature of the particulates. For example, if particulates are retained only by 0.2-µm porosity filters, then omit prefiltration with depth filters and 0.45-µm filters.

Process large volumes of slow-filtering liquids (such as serum or other proteinaceous substances) in a cold room, if possible, to retard proliferation of microbial growth during the sometimes time-consuming, nonsterile prefiltration steps.

Anticipated Results

If sterilization and disinfection procedures are effective and proper aseptic technique is used, it should be possible to initiate and maintain cell cultures without any incidence of contamination.

Time Considerations

Preparation of materials for autoclaving and the autoclaving itself should take 1 to 2 hr; cooling solutions and equipment may require several hours. Dry-heat sterilization should take less than half a day plus cooling time. Monitoring the efficacy of sterilization requires several days to allow time for contaminants to grow. Disinfection of space and equipment requires a variable amount of time. The time required for filter sterilization can be a few minutes to hours depending on the solution.

Key References

Barkley, W.E. and Richardson, J.H. 1994. Laboratory safety. *In* Methods for General and Molecular Bacteriology (P. Gerhardt, R.G.E. Murray, W.A. Wood, and N.R. Krieg, eds.) pp. 715-734. American Society for Microbiology, Washington, D.C.

A well-written chapter covering all aspects of safety in any laboratory dealing with biological agents.

Block, S.S. 1983. Disinfection, Sterilization, and Preservation, 3rd ed. Lea & Febiger, Philadelphia.

A substantial text covering all aspects of the discipline. Contributors provide a great deal of information to the individual chapters.

Brock, T.D. 1983. Membrane Filtration: A User's Guide and Reference Manual. Science Tech, Inc., Madison, Wis.

Includes detailed information on the principles of membrane filtration, selection of filtration systems, and use of membranes for specific applications. Lacks information on the newer membrane types developed since its publication date, but still a worthwhile reference.

Perkins, J.J. 1976. Principles and Methods of Sterilization in Health Sciences. Thomas, Springfield, Ill.

A classic reference text that covers all aspects of dry-heat and steam sterilization.

U.S. Pharmacopeial Convention. 1995. The U.S. Pharmacopeia XXIII/The National Formulary XVIII. U.S. Pharmacopeial Convention, Rockville, MD.

The U.S. official standard for sterilization criteria and sterility testing. Describes methods for using biological indicators.

Vesley, D. and Lauer, J. 1986. Decontamination, sterilization, disinfection, and antisepsis in the microbiology laboratory. *In* Laboratory Safety: Principles and Practices (B.M. Miller, D.H.M. Gröschel, J.H. Richardson, D. Vesley, J.R. Songer, R.D. Housewright, and W.E. Barkley, eds.) pp. 182-198. American Society for Microbiology, Washington, D.C.

Presents substantial information on all aspects of decontamination concerns for the laboratory, including choice of disinfectants, spill containment and cleanup, and routine cleaning.

Millipore. 1993. Millipore Direct. Millipore, Bedford, MA.

This filter manufacturer's catalog and reference guide provides a wealth of background, practical, and technical information to assist a user in appropriate choices for filters and filtration equipment.

Contributed by Rosalie J. Coté
Becton Dickinson Microbiology Systems
Sparks, Maryland

Assessing and Controlling Microbial Contamination in Cell Cultures

This unit describes procedures for the detection of bacterial, fungal, and mycoplasmal contaminants in cell cultures. Bacterial and fungal contaminants are detected by direct culture under conditions that specifically favor bacteria, mycelia, and yeast (see Basic Protocol 1). The direct method for detecting mycoplasma contamination similarly involves screening with microbiological media designed to encourage proliferation of mycoplasma (see Basic Protocol 2). The two indirect methods presented are (1) a slight modification of Barile's adaptation on the use of the Hoechst stain to detect mycoplasma by DNA fluorescence (see Alternate Protocol 1), and (2) the use of polymerase chain reaction in conjunction with a commercially available mycoplasma detection kit (see Alternate Protocol 2 and Support Protocol 1). In addition, a procedure is described for controlling microbial contamination through the use of antibiotics (see Basic Protocol 3).

Testing for microbial contamination should be integrated into a cell culture program as part of routine quality control. Microbial and mycoplasma testing should be performed upon arrival of all incoming cell lines and on lot samples of ampules prepared for master or working cell banks and seed stocks. Testing for microbial contamination should also be done whenever contamination is suspected (e.g., unusually slow growth rates for a particular cell line, aberrant appearance of cells). Indirect mycoplasma screening methods should also be done on new lots of serum used in media preparation when first received by the laboratory. Testing for microbial contamination should be performed after the cells have been cultured in the absence of antibiotics for several weeks.

TESTING FOR BACTERIAL AND FUNGAL CONTAMINANTS

BASIC PROTOCOL 1

The media and methods described in this protocol are suitable for detection of most bacteria and fungi that would be expected to survive as contaminants in cell lines. Brain heart infusion and trypticase soy agar with sheep blood are used for the cultivation of nutritionally fastidious bacteria of clinical origin that may be present in primary tissue cultures or in material contaminated by bacterial flora from human skin and poor aseptic technique. Fluid thioglycollate supports the growth of bacteria that require reduced oxygen tension; these microaerophilic or slightly anaerobic contaminants are frequently spore formers that originate from inadequately autoclaved or heat-sterilized materials. Soybean/casein digest broth is a general-purpose medium that supports the growth of a wide range of bacteria of human or environmental origin. HEPES/trypticase/yeast extract (HTYE) broth is also a general bacterial growth medium, but has the advantage of supporting growth of nutritionally or physiologically stressed bacteria not easily culturable with other media. These general types of bacteria are primarily environmental in origin and can be found in distilled water carboys, in fouled deionization systems, or as air-borne contaminants. Emmons' modification of Sabouraud's agar and YM agar are used for detection of filamentous fungi (molds) and yeasts, respectively. Molds are frequently environmental contaminants that can thrive under a wide variety of conditions. They often take residence in air-handling ducts to generate a constant microscopic rain of spores into a laboratory. Similarly, molds can often be found as films colonizing the dispensing tubing from distilled water reservoirs. The types of yeasts normally found as cell culture contaminants are human in origin.

Many of the media listed below may be purchased in their final configurations as sterile plates or test tubes from microbiological media manufacturers such as BBL or Difco, as well as other suppliers. Be that as it may, the cell culture user should carefully evaluate

Contributed by Rosalie Coté
Current Protocols in Cell Biology (1999) 1.5.1-1.5.18

the configurations, performance, and cost of any prepared quality control medium before commencing full-scale use in a cell culture laboratory.

Materials

Medium for bacterial detection: e.g., brain heart infusion (BBL, Difco), fluid thioglycollate medium (BBL, Difco), HTYE broth (see recipe), soybean/casein digest broth USP (e.g., trypticase soy broth, BBL; tryptic soy broth, Difco), or trypticase soy agar (BBL)
Medium for mycelial and yeast fungal detection: e.g., Sabouraud's dextrose agar (Emmon's modified; BBL, Difco), or YM agar (Difco)
Sterile, defibrinated sheep blood (e.g., Colorado Serum, Waltz Farm)
Cell culture test samples
Antibiotic-free culture medium (optional)

Conductivity meter (Corning model 162 or equivalent), if not integrated with the laboratory water purification system
50°C water bath
16 × 125–mm borosilicate screw-cap test tubes with rubber-lined caps
100 × 15–mm sterile plastic disposable petri dishes
Semiautomated repeat-volume filling unit to accurately dispense 5- to 24-ml aliquots (optional)
Incubators: 26°C, 35° to 37°C, and 37°C with 5% (v/v) CO_2

NOTE: To avoid inadvertent contamination of clean cell lines, bacterial and fungal testing should be segregated to a laboratory not used for general cell culture work.

Prepare media

For liquid (broth) media:

1a. Reconstitute brain heart infusion, HTYE broth, soybean/casein broth, and fluid thioglycollate medium per manufacturer's instructions, or per specific recipe instructions, in 10-megaohm (or higher) distilled or deionized water. Heat to ~50°C with frequent stirring to dissolve components. Heat to boiling with frequent stirring to dissolve any medium containing even small amounts of agar (e.g., fluid thioglycollate).

2a. Dispense medium into 16 × 125–mm borosilicate screw-cap test tubes at 10 ml/tube for fluid thioglycollate medium, and a 5 ml/tube for all other media.

3a. Cap tubes loosely, threading caps securely enough to prevent them from blowing off during autoclaving, but loosely enough to permit pressure exchange within the tube head space during the sterilization process.

Because of the large (10-ml) volume required with fluid thioglycollate medium, one should anticipate significant tube blow-outs upon autoclaving, and should prepare ~25% more tubes than required to compensate for the rejected material.

4a. Sterilize tubes by autoclaving at 121°C for 15 min under slow exhaust or liquid cycle.

Autoclaving times indicate the time necessary to hold the medium at the 121°C temperature for 15 min, not the time selected to run an autoclave cycle (UNIT 1.4; Table 1.4.1). Autoclave efficiency is very much machine and maintenance specific. However, an autoclave cycle of 20 min for a single 6 × 12 test tube rack of bacteriological medium, and a 32-min cycle for a 2-liter flask containing 1 liter of bulk medium, provide general guidelines for achieving the 121°C for 15 min sterilization criteria for these medium/vessel configurations.

5a. Remove tubes of medium from autoclave immediately after sterilization cycle is completed and/or when autoclave gauges indicate atmospheric pressure in the auto-

clave chamber. Allow medium to cool to ambient room temperature in a location not subject to excessive air currents or temperature fluctuations.

Oversterilization or prolonged holding of bacteriological media at elevated temperatures will severely affect performance. Cooling of just-autoclaved media in laboratory areas with significant temperature fluctuations or personnel movement can cause environmental contamination of otherwise sterile media.

6a. When tubes of medium reach ambient temperature, fully tighten screw caps and store tubes at 4° to 8°C until further quality control checks or until use (up to 6 to 9 months).

Fluid thioglycollate is a medium formulated to detect slightly anaerobic bacteria and contains a small amount of agar to retard atmospheric oxygen diffusion into the medium, cysteine as a reducing agent, and methylene blue as an oxygen indicator. Freshly prepared fluid thioglycollate will have a very small zone of aerobiosis at the medium surface/head space interface, as indicated by a slight purple to orange band of oxidized methylene blue. As oxygen slowly continues to permeate with prolonged storage of the medium, the pigmented, oxidized band will enlarge. Do not use the medium if the color has changed to orange in greater than the top 25% of the medium. Oxidized fluid thioglycollate may be rejuvenated, but only once, by steaming the tubed medium in a boiling water bath to purge gaseous oxygen from the medium, and then cooling just prior to use. Other bacteriological media cited here have a shelf life of 6 to 9 months when stored in the dark at 4° to 8°C.

Any of the liquid media cited above may be adapted for agar plate or test tube slant use by the addition of agar as the solidification agent as noted below.

For bulk agar media for plates:

1b. Reconstitute trypticase soy agar, Sabouraud's dextrose agar, and YM agar per manufacturer's instructions in 10-megaohm (or higher) distilled or deionized water. Use an autoclavable container capable of holding at least twice the volume of the medium being prepared (e.g., use a 2-liter Erlenmeyer flask to autoclave 1 liter of medium) to avoid boiling over during the auotclave cycle.

2b. Sterilize the bulk medium by autoclaving at 121°C for 15 min under slow exhaust or liquid cycle (see step 4a annotation).

3b. Cool in a water bath to ~50°C.

4b. Add 50 ml/liter (5%) defibrinated sheep blood aseptically to trypticase soy agar.

It is critical that medium be cooled to 45° to 50°C before sheep blood is added.

5b. Dispense medium aseptically in 24-ml aliquots to 100 × 15–mm sterile plastic disposable petri dishes.

A semiautomated repeat-volume filling unit fitted with a weight on the inlet line to sink the tubing to the bottom of the flask of medium will greatly alleviate drawing of surface foam from the bulk flask to the petri dishes. Surface bubbles most often occur when postautoclaving agitation of the bulk flask is necessary to incorporate heat-labile additives such as sheep blood. A small number of media bubbles can be removed from the plates by lightly passing the flame of a Bunsen burner across the surface of the plated medium as soon as it is dispensed.

6b. Place plates in stacks of 10 to 20 and allow to cool and solidify overnight at room temperature. Store in vented plastic bags at 4° to 8°C until quality control checks or use (up to 12 weeks).

Cooling the plates in stacks of 10 to 20 retards formation of excessive condensation on the lids of the plates.

Prepare test samples

7a. *For lot sample preparation of cryopreserved ampules:* Use a 1-ml serological pipet to pool and mix the contents of ~5% of the cell culture ampules prepared from each freeze lot.

7b. *For cell culture vessels:* Examine cell culture vessels individually under low power, preferably with phase contrast, using an inverted microscope. Look for aberrant growth or appearance of the cells. Aseptically remove 5-ml aliquots from suspect cultures to use for further examination and testing. Quarantine any suspect cultures or containers to ensure that they will not be inadvertently mixed with and cross-contaminate clean cultures.

Unless the cultures are heavily contaminated, microbial growth will not be readily evident under low-power magnification. Mycelial fungal contamination sometimes is first noticed macroscopically by the appearance of small "cottony" or "lint-like" debris in the culture vessel.

8. Prepare wet mounts for microscopic evaluation of test samples and examine under oil immersion with high-power objectives (≥1000× magnification).

Bacterial contamination is recognized by the presence of small, uniformly sized spheres, rods, or spirals scattered throughout the field. The organisms may be individual, in clusters, or in chains. Rod-shaped bacteria may contain bright, refractile spores. The bacteria may be motile. Fungal yeast contamination appears as ovoid, fairly regularly sized nucleated cells scattered throughout the microscopic field. The yeast may be individual, in the process of budding off smaller daughter cells, or in short chains. Mycelial fungal contamination is characterized by the presence of long filaments or pieces of broken filaments in the microscopic field. Fungal spores, frequently appearing as spherical objects covered with spines or other protrusions, might be observed. Low-level contamination may not be detected under the microscope even by a trained microbiologist, particularly if the cell culture sample contains much debris.

9. If culture contains antibiotics, wash prior to inoculation of microbiological test media by centrifuging at 2000 × *g* for 20 min (at room temperature or 4° to 8°C), removing the supernatant, and resuspending the pellet in an equal volume of antibiotic-free medium. Repeat for a total of three washes to eliminate traces of antibiotics that might interfere with microbial cultivation.

Inoculate microbiological media with test samples

10. For each test sample, inoculate each of the following with 0.3-ml aliquots of cell suspension:

    2 tubes of brain heart infusion
    2 tubes of fluid thioglycollate medium
    2 tubes of HTYE broth
    2 tubes of soybean/casein digest broth
    2 plates of trypticase soy agar with 5% sheep blood
    2 plates of Sabouraud's dextrose agar, Emmon's modified
    2 plates of YM agar.

11. Incubate one plate of trypticase soy agar (with 5% sheep blood) at 37°C aerobically and the other at 37°C under 5% CO_2.

12. Incubate one sample each of the other media at 26°C and the other sample at 35° to 37°C.

13. Examine all inoculated media daily for 14 days.

Usually, visual evidence of bacterial growth appears within 72 hr; fungal growth within 96 hr. Low-level contamination, or proliferation of contaminants previously stressed by the presence of antibiotics or other adverse culture conditions, may take longer to appear.

Positive bacterial and yeast growth appears as turbidity or the formation of a precipitate in liquid media. In an undisturbed test tube, growth may be restricted to a pellicle of growth at the surface of the liquid. On solid media, these bacteria and yeast appear as distinct, slightly convex, discretely isolated circular or confluent areas of microbial colonial proliferation, most often off-white or yellow in color. Filamentous fungal colonial growth is characterized by the appearance of typical cottony, whitish-gray to green to black mold on plates.

14. Autoclave and discard any general-use cell culture preparations that are positive for contamination.

If a cell culture that has tested positive is critical to maintain, repeat the microbial evaluation. If still positive, autoclave and discard the culture. If it is necessary to attempt to clean a microbially contaminated critical cell culture, see Basic Protocol 3.

Reusable glassware from discarded contaminated cultures should be decontaminated by autoclaving, cleaned, and depyrogenated by dry heat (UNIT 1.4).

TESTING FOR MYCOPLASMA CONTAMINATION BY DIRECT CULTURE

BASIC PROTOCOL 2

This protocol describes the direct detection of mycoplasma contamination by screening with microbiological media designed to encourage proliferation of mycoplasma. Total incubation time for this method is ~35 days. This schedule is necessary to detect low levels of mycoplasma contamination that might otherwise be scored as false negatives.

Materials

Cell line for testing
Mycoplasma broth medium (see recipe): 6 ml medium in 16 × 125–mm screw-cap test tubes
Mycoplasma agar plates (see recipe): 10 ml solidified medium in 60 × 15–mm petri dishes

37°C incubators: one without CO_2 and one humidified with 5% (v/v) CO_2
Inverted microscope with 100 to 300× magnification

NOTE: To avoid inadvertent contamination of clean cell lines, mycoplasma testing should be segregated to a laboratory not used for general cell culture work.

1a. *For adherent cultures:* Select a cell culture that is near confluency and has not received a fluid renewal within the last 3 days. Remove and discard all but 3 to 5 ml of the culture medium. Scrape a portion of the cell monolayer into the remaining culture medium using a sterile disposable scraper.

1b. *For suspension cultures:* Take the test sample directly from a heavily concentrated culture that has not received a fresh medium supplement or renewal within the last 3 days.

Samples can also be taken directly from thawed ampules that have been stored frozen.

2. Inoculate 1.0 ml of the test cell culture suspension into 6 ml mycoplasma broth medium in a 16 × 125–mm screw-cap test tube. Also inoculate 0.1 ml of the test sample onto the center of a 60 × 15–mm mycoplasma agar plate.

3. Incubate the broth culture aerobically at 37°C. Incubate the agar plate in a humidified 37°C, 5% CO_2 incubator. Observe broth culture daily for development of turbidity and/or shift in pH (medium becomes redder for alkaline shift, yellower for acid shift).

As an alternative, a self-contained anaerobic system such as the GasPak equipment (Becton Dickinson Microbiology Systems) can be used in conjunction with a standard 37°C incubator to provide proper CO_2 levels.

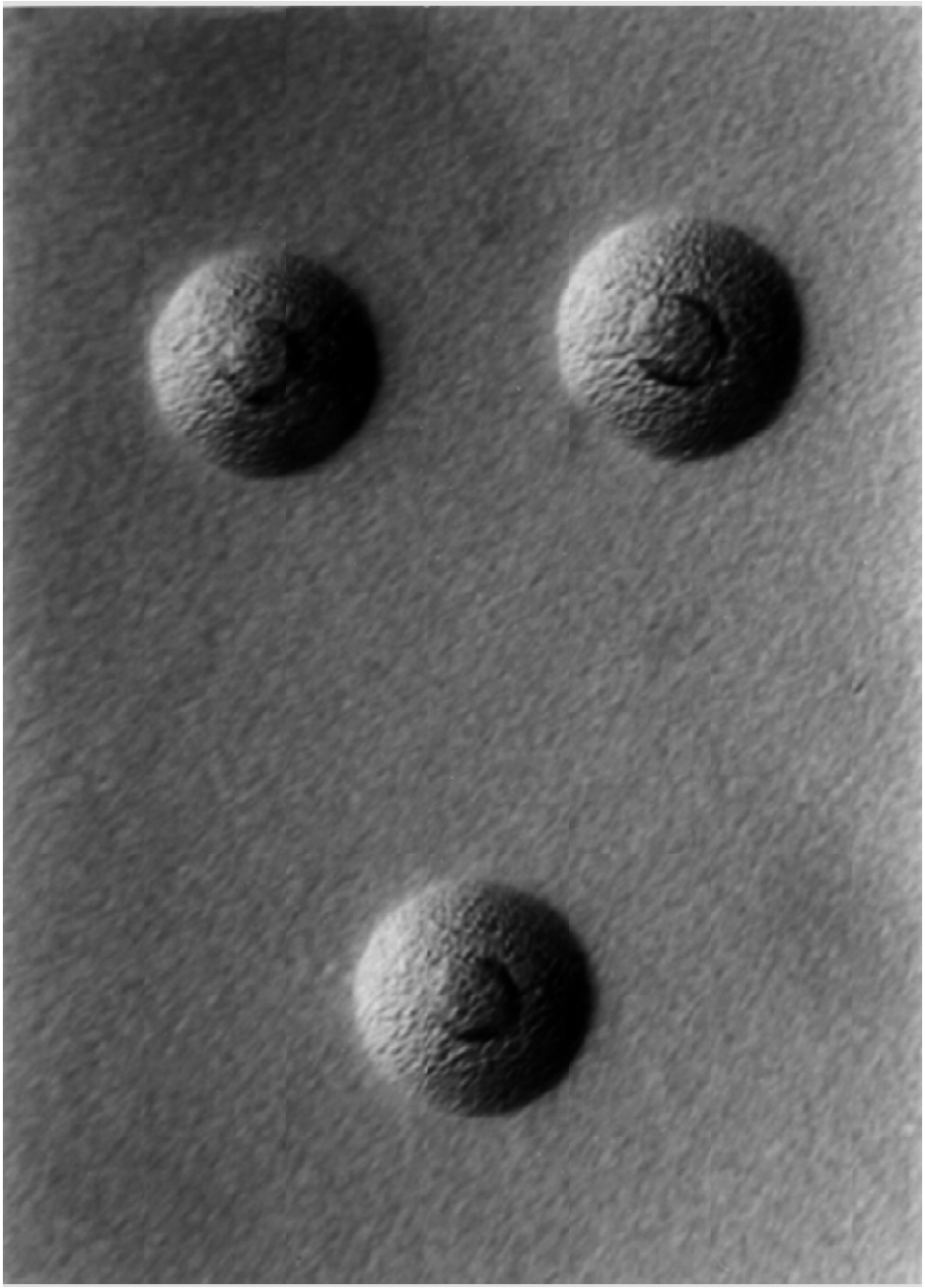


Figure 1.5.1 Mycoplasma colonies at 230× magnification. Figure provided by W. Siegel, Bio-Whittaker, Inc.

4. After 5 to 7 days of incubation and again after 10 to 14 days, remove a 0.1-ml sample from the broth culture and inoculate a fresh mycoplasma agar plate. Incubate these plates as in step 3.

5. Using an inverted microscope at 100 to 300× magnification, examine the agar plates weekly for at least 3 weeks for mycoplasma colony formation and growth.

   Mycoplasma colonies range from 10 to 55 μm in diameter and classically look like a fried egg, with the dense center of the colony embedded in the agar and the thinner outer edges of the colony on the surface of the substrate (Fig. 1.5.1). However, much variation in colony morphology occurs between species and culture conditions. Although colonies typically

appear within 4 days after inoculation, plates should be kept for the full incubation period before scoring them as negative.

6. To confirm presumptive mycoplasma colonies, subculture a small (~1 cm^2) section of the suspicious area of the agar plate into a tube of mycoplasma broth medium, incubate up to 14 days, and observe as in step 3.

   When other detection methods are not available, the ability to subculture presumptive mycoplasma colonies will help to differentiate authentic mycoplasma presence from artifacts such as air bubbles, tissue culture cells, or pseudocolonies.

INDIRECT TESTING FOR MYCOPLASMA BY STAINING FOR DNA

ALTERNATE PROTOCOL 1

The total time for this protocol, which includes the use of an indicator cell culture, is 6 days. The staining procedure itself takes ~1 hr. The use of an indicator cell culture provides a number of advantages. The indicator cell line supports the growth of more fastidious mycoplasma species. Both positive and negative controls are thus readily available for direct comparison with the culture samples being tested. Selection of a proper indicator cell is important to the success of this procedure. It must first have good viability. Transformed cell lines are not recommended as indicators as they produce significant nuclear background fluorescence, which interferes with interpretation of results. Cell lines that produce much debris, such as hybridomas, are also not recommended as indicator cells because of the amount of positive staining artifacts that confuse interpretation of results.

Materials

Complete EMEM-10: Eagle's minimum essential medium (EMEM) with Earle's salts (Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) bovine calf serum (see *UNIT 1.2* for media preparation methods)
Indicator cell line: e.g., African green monkey cell line Vero (ATCC #CCL81) or 3T6 murine cell line (ATCC #CCL96)
Cell culture for testing
Mycoplasma hyorhinis (ATCC #29052) or a known mycoplasma-infected cell line to use as a positive control, actively growing
Fixative: 3:1 (v/v) absolute methanol/glacial acetic acid
Hoechst stain (see recipe)
Mounting medium (see recipe)

60 × 15–mm culture dishes, sterile
No. 1 or no. 1½ coverslips, sterilized by autoclaving (*UNIT 1.4*)
37°C, 5% (v/v) CO_2/95% air incubator

NOTE: To avoid inadvertent contamination of clean cell lines, mycoplasma testing should be segregated to a laboratory not used for general cell culture work.

Prepare indicator cell cultures

1. Aseptically place a sterile glass no. 1 or 1½ coverslip into each sterile 60 × 15–mm culture dish.

   Use two culture dishes for the positive control, two dishes for the negative control, and two dishes for each test sample.

2. Aseptically dispense 3 ml complete EMEM-10 into each culture dish.

   Make certain that each coverslip is totally submerged and not floating on top of the medium.

3. Prepare a single-cell suspension of the indicator cell line in complete EMEM-10 at a concentration of 1.0×10^5 cells per ml.

4. Inoculate 1 ml indicator cell suspension into each culture dish.
5. Incubate overnight at 37°C in a 5% CO_2/95% air incubator.
6. Microscopically examine cultures to verify that the cells have attached to the glass coverslip. Code the top of each culture dish for identification purposes (to record the test samples to be inoculated).

Inoculate test samples

7. Add 0.5 ml/dish complete EMEM-10 to two culture dishes for negative controls.

8a. *For adherent cultures:* Select a test cell culture that is near confluency and has not received a fluid renewal within the last 3 days. Remove and discard all but 3 to 5 ml of the culture medium. Scrape a portion of the cell monolayer into the remaining culture medium using a sterile disposable scraper.

8b. *For suspension cultures:* Take the test sample from a heavily concentrated culture that has not received a fresh medium supplement or renewal within the last 3 days.

Samples can also be taken directly from thawed ampules from frozen stocks.

9. For each test sample, add 0.2 to 0.5 ml/dish test sample to two culture dishes.
10. Add 0.5 ml/dish actively growing *Mycoplasma hyorhinis* to two culture dishes for positive controls.

CAUTION: *To prevent spread of mycoplasma, infected strains should be destroyed and removed as quickly as possible. Benches and incubators should be cleaned. For safety of the investigator, BSL 2 laboratory conditions are appropriate.*

Alternatively, a known mycoplasma-infected cell line can be used.

11. Return the cultures to the CO_2 incubator and allow to incubate undisturbed for 6 days.

Cultures should be at 20% to 50% confluent. Confluence can interfere with microscopic examination for mycoplasma.

Fix, stain, and mount coverslips

12. Remove cultures from incubator. Aspirate medium and immediately add 5 ml fixative to each culture dish. Incubate for 5 min.

Do not allow the culture to dry between removal of the culture medium and addition of the fixative.

13. Aspirate fixative from each culture dish and repeat fixation for 10 min.

These fixing times are minimal. Additional fixation time will not harm the procedure and can be beneficial for some preparations.

14. Aspirate the fixative and let the cultures air dry.

Dry completely and store in a 60-mm petri or tissue culture dish if samples are to be accumulated at this stage for later staining.

15. Add 5 ml Hoechst stain to each culture dish, cover, and let stand at room temperature for 30 min.
16. Aspirate the stain and rinse each culture three times with 5 ml distilled water.
17. Aspirate well so that the glass coverslip is completely dry. Let air dry if necessary.
18. Place a drop of mounting medium on a clean glass microscope slide.
19. Use forceps to remove the glass coverslip containing the fixed cells from the culture dish and place face up on top of the mounting medium, being careful to eliminate air bubbles.

20. Add a second drop of mounting medium onto the top of the specimen coverslip and cover with a larger clean coverslip, being careful to eliminate air bubbles.
21. Label each slide to identify the specimen.
22. Observe each specimen by fluorescence microscopy at 500× using immersion oil. Use a blue glass excitation filter (330/380 nm) in combination with a 440-nm barrier filter (see *UNIT 4.2*). Compare test samples to positive and negative controls.

    The nuclei of the indicator cells appear as large (~20 μm), ovoid fluorescing bodies. Mycoplasma will appear as small fluorescing particles (0.1 to 1.0 μm) that are regular in shape and size in the cytoplasm or in intercellular spaces. If infection is heavy, the particles may be tightly clustered in some areas. With low-level contamination, not all cells will be infected. Thus, all of the slide should be examined.

INDIRECT TESTING FOR MYCOPLASMA BY PCR

ALTERNATE PROTOCOL 2

Kits are now available from a number of manufacturers for the detection of mycoplasma in cell cultures or other material using PCR. The kits, although expensive and requiring the expertise and equipment needed for molecular procedures, provide advantages with quick results (1 day) and the ability to speciate the contaminant and thus potentially identify its source. The PCR kits are also useful for detecting mycoplasma in cultures prone to forming artifact debris that often obscures definitive interpretations with staining detection methods. The procedure described here uses primers from a commercially available kit and a nested PCR assay that amplifies the spacer region between the 16S and 23S rRNA genes of mycoplasmas. This specific protocol details procedures using cell cultures; however, instructions provided with the kit also explain how to make modifications for analyzing test samples of serum or frozen cells. The novice practitioner of PCR methodologies is strongly urged to consult Sambrook et al. (1989) for specific details (also see *APPENDIX 3*).

Materials

Cells for testing
10× PCR buffer (usually provided with *Taq* polymerase)
2.5 mM 4dNTP mix: 2.5 mM each dGTP, dCTP, dTTP, and dATP
25 mM $MgCl_2$
5 U/μl *Taq* DNA polymerase
Mineral oil (if needed for thermal cycler)
Mycoplasma detection kit (ATCC), containing first- and second-stage primer mixtures (total 7 primers), as well as two positive control mycoplasma DNAs (*Mycoplasma pirum*, *Acholeplasms laidlawii*)

Thin-wall microcentrifuge tubes
Aerosol-preventive micropipettor tips, sterile
Positive-displacement micropipettors
Picofuge
Thermal cycler

Additional reagents and equipment for agarose gel electrophoresis (see Support Protocol)

NOTE: To avoid inadvertent contamination of clean cell lines, mycoplasma testing should be segregated to a laboratory not used for general cell culture work.

NOTE: To avoid amplification of contaminating DNA from laboratory workers, room contaminants, or previous mycoplasma DNA amplifications, all PCR should be performed using aseptic technique (also see special considerations for PCR experiments in *APPENDIX 2A*).

Prepare test sample

1a. *For confluent monolayers:* Detach cell monolayer from flask surface with a cell scraper. Gently agitate the flask to dispense the cells in the medium. Transfer 0.5 ml cell suspension (~5×10^4 cells/ml) to a sterile 1.5-ml microcentrifuge tube.

1b. *For suspension cultures:* Mix the suspension culture by gently pipetting to obtain an even dispersal of cells in the medium. Transfer 0.5 ml of the suspension (~5×10^4 cells/ml) to a sterile 1.5-ml microcentrifuge tube.

2. Centrifuge at 12,000 × *g* for 10 min at 4°C.

3. Discard 400 µl supernatant and use a pipet to resuspend the cell pellet in the remaining 100 µl medium.

Pipet gently to avoid formation of bubbles. The 100-µl cell suspension is ready for PCR.

Run first-stage PCR

4. Using a permanent ink marker, label each thin-wall microcentrifuge tube (reaction tube) with appropriate test sample or control DNA codes.

5. Prepare a master mix of PCR reagents in sufficient quantity for all samples to be tested (*n* + 1 or 2 reactions) plus a minimum of two positive DNA controls and one negative control. Use the following volumes per reaction:

5 µl 10× PCR buffer
1 µl first-stage primer mixture
1 µl 2.5 mM 4dNTP mix
1 µl 25 mM $MgCl_2$ (see annotation)
0.2 µl 5 U/µl *Taq* DNA polymerase
Deionized water to 45 µl.

Store the mix on ice until it is aliquotted.

The optimal reaction conditions for this procedure are 10 mM Tris·Cl (pH 8.3 to 8.8), 50 mM KCl, 2.0 mM $MgCl_2$, 50 µM of each dNTP, and 1 U Taq polymerase. Check for the inclusion and final concentration of $MgCl_2$ in the 10× PCR buffer supplied with the Taq polymerase and adjust the volume of $MgCl_2$ in the reagent mix, if necessary, to give a final concentration of 2.0 mM. Adjust the amount of water in the mix accordingly for a final reaction mix volume of 45 µl.

NOTE: *In this and all subsequent steps, use positive-displacement micropipettors and sterile aerosol-preventive micropipettor tips to prevent contamination of the amplification reaction.*

6. Pipet 45 µl reaction mix into each sample and control reaction tube.

7. If the thermal cycler used requires mineral oil to minimize sample evaporation, add 40 to 60 µl of mineral oil to each tube.

Keep the reaction tubes closed, except when aliquotting into them, to avoid possible cross-contamination.

8. Add 5 µl test sample (step 3) to the appropriate reaction tube containing reagent mix (final reaction volume 50 µl). If reaction tubes contain mineral oil, pipet the samples directly into the mix below the mineral oil layer (final tube volume 90 to 110 µl).

9. Add 5 µl of each positive control mycoplasma DNA into separate positive control tubes.

10. Add 5 µl sterile deionized water into the negative control tube.

11. Mix each tube thoroughly by flicking it with a finger, and centrifuge the tube briefly in a picofuge.

12. Place all the tubes into a thermal cycler and perform amplification using the following program:

Initial step:	30 sec	94°C	(denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	2 min	55°C	(annealing)
	2 min	72°C	(extension)
Final step:	5 min	72°C	(extension)
Chill:	indefinitely	4°C	(hold).

Store PCR products at 4°C or on ice until further use (2 to 3 weeks). For longer periods, store at −20°C.

Run second-stage PCR

13. Using a permanent ink marker, label each second-stage reaction tube with appropriate test sample or control DNA codes.

14. Prepare a master mix of reagents in sufficient quantity for all samples (n + 1 or 2 reactions), plus positive and negative controls, using the following volumes per reaction:

5 µl 10× PCR buffer
1 µl second-stage primer mixture
1 µl 2.5 mM 4dNTP mix
1 µl 25 mM $MgCl_2$ (see step 5 annotation)
0.2 µl 5 U/µl *Taq* DNA polymerase
Deionized water to 49 µl.

Store the mix on ice until it is aliquotted.

15. Pipet 49 µl reaction mix to each sample and control tube.

16. If the thermal cycler used requires mineral oil to minimize sample evaporation, add 40 to 60 µl of mineral oil to each tube.

Keep the reaction tubes closed, except when aliquotting into them, to avoid possible cross-contamination.

17. Carefully pipet 1 µl from the first-stage PCR reaction tube (step 12) into the second-stage reaction tube (final reaction volume 50 µl). If mineral oil was used, add the sample to the reagent mix below the oil layer (final tube volume 90 to 110 µl).

18. Mix each tube thoroughly by flicking it with a finger, and centrifuge the tube briefly in the picofuge.

19. Place all the tubes in the thermal cycler and run the program as above (step 12). Store PCR products at 4°C or on ice until further use (2 to 3 weeks). For longer periods, store at −20°C.

20. Analyze reaction products by agarose gel electrophoresis (see Support Protocol).

SUPPORT PROTOCOL

AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS

Agarose gel electrophoresis is used to analyze the products of the second-stage PCR for the presence of mycoplasma sequences. The protocol is written for one 10-lane gel, which can accommodate up to four unknown samples, two positive controls, one negative PCR control, one negative electrophoresis control, and two molecular weight marker lanes. For additional samples, the procedure can be scaled by using a comb with additional wells, or additional gels can be run.

Materials

Agarose (e.g., NuSieve, FMC Bioproducts)
1× TBE electrophoresis buffer (*APPENDIX 2A*)
10 mg/ml ethidium bromide solution
Second-stage PCR products from test samples and controls (see Alternate Protocol 2)
6× electrophoresis sample buffer (see recipe)
Molecular weight marker (100-bp DNA ladder)

Electrophoresis apparatus with a 10 × 14–in. gel tray and a 1-mm, 10-tooth comb
Power supply
UV light box

Prepare 2.5% agarose containing ethidium bromide

1. Seal a 10 × 14–in. gel tray from an electrophoresis apparatus according to manufacturer's instructions and place on a level surface.
2. Weigh 2.25 g agarose and place in a 250-ml Erlenmeyer flask.
3. Add 90 ml of 1× TBE electrophoresis buffer, swirl to mix, and heat to boiling to completely dissolve agarose.
4. Add 5.4 µl of 10 mg/ml ethidium bromide solution, swirl to mix, and cool to ~55°C.

   CAUTION: *Ethidium bromide is a mutagen and a potential carcinogen. Gloves should be worn and care should be taken when handling ethidium bromide solutions.*

Cast gel

5. Pipet 80 ml agarose solution into the center of the gel tray. Remove any bubbles.
6. Gently place a 1-mm, 10-tooth comb into the gel mold. Allow gel to harden until it becomes milky and opaque in appearance (~1 hr).
7. Remove tape or sealers from the gel mold. Place gel into electrophoresis tank.
8. Pour ~950 ml of 1× TBE electrophoresis buffer into the electrophoresis tank.

   Gel should be totally submerged in buffer, but not covered more than 1 cm.

9. Gently remove the gel comb.

Prepare sample

10. Add 10 µl of each second-stage PCR product to a separate microcentrifuge tube containing 2.0 µl of 6× electrophoresis sample buffer. Mix well.
11. Add 5 µl of 100-bp DNA ladder to 2 µl of 6× electrophoresis sample buffer. Mix well.

Load and run the gel

12. Add 7 µl DNA ladder to each of the first and last wells (lanes 1 and 10) of the gel.
13. Add 12 µl of 6× electrophoresis sample buffer to one lane as a negative control.

14. Add 12 µl unknown test samples and positive and negative PCR controls to the remaining wells.

15. Connect a power supply and run the gel at 75 V for ~1 hr and 40 min.

16. View the gel on a UV light box.

    CAUTION: *UV light is damaging to eyes and exposed skin. Protective eyewear should be worn at all times while using a UV light source.*

    With the primers used in Alternate Protocol 2, the mycoplasmas commonly encountered as cell culture contaminants should generate a second-stage PCR DNA amplicon that ranges in size from 236 to 365 bp. In contrast, the A. laidlawii positive control should generate two amplicons of 426 bp and 219 bp, and the M. pirum positive control should generate a single 323-bp amplicon. No discrete amplicon band should be seen in the negative controls. Fuzzy bands <100 bp are sometimes observed in control and test samples and are probably primer multimer artifacts.

USE OF ANTIBIOTICS TO CONTROL MICROBIAL CONTAMINATION

BASIC PROTOCOL 3

The best way to deal with a contaminated cell culture is to autoclave it. Be that as it may, situations will undoubtedly occur when there is a need to attempt to salvage a microbially contaminated cell culture. This brief protocol describes the use of antibiotics in cell culture and cautions against misuse of these potent antimicrobial agents.

Materials

Contaminated cell culture
Sterile antibiotic stock solution(s) as appropriate (Table 1.5.1)

Additional reagents and equipment for identifying microbial contamination (see Basic Protocols 1 and 2; see Alternate Protocols 1 and 2)

1. Immediately quarantine the contaminated culture to prevent possible cross-contamination with other cultures in the laboratory.

2. Identify the microbial contaminant as to type: bacterial, fungal, or mycoplasmal (see Basic Protocols 1 and 2; see Alternate Protocols 1 and 2).

   If bacteriological expertise is available, identification of a bacterial contaminant to genus can help narrow antibiotic selection to one that is particularly effective against the contaminant.

3a. *For an identified microbial contaminant:* Select an appropriate antibiotic from the Table 1.5.1, and prepare and filter sterilize a stock solution.

3b. *For an unknown bacterial contaminant:* Prepare and filter sterilize the following 10× antibiotic cocktail:

    2500 U/ml penicillin
    2.5 mg/ml streptomycin sulfate
    2.5 mg/ml neomycin
    25 U/ml bacitracin

4a. *For monolayers:* Remove contaminated medium by suction and add fresh medium containing antibiotics. Add specific antibiotic stock solutions (step 3a) to fresh medium to give the appropriate working concentration (Table 1.5.1). Alternatively, add 1 vol of 10× antibiotic cocktail (step 3b) to 9 vol fresh medium.

    Do not add greater than the recommended amount of antibiotic. All of these chemical reagents are cytotoxic in that they have essentially the same metabolic effect on cells as they do on microorganisms if the concentration is great enough.

Table 1.5.1 Antibiotics[a]

Organism	Antibiotic	Solvent	Stability (days at 37°C)	Working concentration
Bacteria (gram-positive only)	Ampicillin	Water	3	100 mg/liter
	Erythromycin	2 M HCl	3	100 mg/liter
	Gentamicin sulfate	Water	5	50 mg/liter
	Kanamycin sulfate	Water	5	100 mg/liter
	Neomycin sulfate	Water	5	50 mg/liter
	Penicillin-G, K^+ salt	Water	3	10^5 U/liter
	Streptomycin sulfate	Water	3	100 mg/liter
	Tetracycline HCl	Water	4	10 mg/liter
Fungi (molds and yeasts)	Amphotericin B	DMSO; DMF[b]	3	2.5 mg/liter
	Nystatin	DMF	3	2.5×10^6 U/liter
Mycoplasma	Gentamicin sulfate	Water	5	50.0 mg/liter

[a]All antibiotics must be filter sterilized in the solvent noted. Antibiotics are meant only for short-term use; if contamination is not cleared after 14 days of treatment or two subcultures, discard culture.

[b]Abbreviations: DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

4b. *For suspensions:* Centrifuge culture 10 min at 125 × *g*. Remove supernatant and resuspend cells in fresh medium containing antibiotics as described in step 4a.

5. Add additional antibiotic solution every 3 to 5 days to maintain the working concentration (note stability times at 37°C in Table 1.5.1). Continue treatment for 14 days.

   Change medium or passage cells as required during this time, being sure to maintain the antibiotic concentration.

6. Examine the contaminated culture microscopically during the treatment period; look for evidence of cytotoxicity as well as elimination of the contaminant.

7. If the antibiotic treatment is unsuccessful, destroy the culture by autoclaving. If culture appears to have been cleaned of the contaminant, inoculate into fresh, antibiotic-free medium.

   It is not recommended that cultures be maintained in media containing antibiotics. The chemicals may have adverse effects on cell characteristics and will also create the risk of selecting for a population of antibiotic-resistant contaminants that could pose a human health hazard.

8. Test for microbial and mycoplasma contamination (see Basic Protocols 1 and 2; see Alternate Protocols 1 and 2).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Dextrose/arginine supplement

50.0 g dextrose
10.0 g L-arginine HCl

Dissolve ingredients with stirring and heating at 37°C in ~800 ml deionized, distilled water. Bring final volume of solution to 1.0 liter with water. Filter sterilize and dispense in convenient aliquots (e.g., 100-ml aliquots for making 1 liter of mycoplasma agar or broth). Store frozen (at −20° to −70°C) until needed (up to 12 months).

Electrophoresis sample buffer, 6×

2.5 ml 1% (w/v) bromphenol blue
2.5 ml 1% (w/v) xylene cyanol
5.0 ml glycerol
Store up to 6 months at 4°C in glass container

Hoechst stain

Stock concentrate:

5.0 mg bisbenzamide fluorochrome stain (Calbiochem)
10.0 mg thimerosal (Sigma)
100 ml 1× HBSS (*APPENDIX 2A*) without sodium bicarbonate or phenol red

Prepare in a brown bottle wrapped in aluminum foil. Mix thoroughly 45 min at room temperature using a magnetic stirrer. Store 1-ml aliquots up to 1 year at −20°C in the dark.

The stock concentrate is heat and light sensitive.

The concentrate can support microbial growth and should be examined periodically for contamination. Filter sterilization of the concentrate is not recommended as it diminishes fluorescence.

Working solution: In a brown bottle, dilute 1.0 ml stock concentrate in 100.0 ml HBSS without sodium bicarbonate or phenol red. Mix thoroughly 20 to 30 min at room temperature using a magnetic stirrer. Prepare fresh immediately before use.

HTYE (HEPES/trypticase/yeast extract) broth

5.0 g trypticase peptone (BBL)
2.0 g yeast extract
4.0 g HEPES acid
1.0 liter H_2O
Adjust to pH 7.1 ± 0.1 with NaOH
Dispense as required
Autoclave at 121°C for 15 min
Store up to 6 to 9 months at 4° to 8°C

Mounting medium

22.2 ml 0.1 M citric acid
27.8 ml 0.2 M Na_2HPO_4
50.0 ml glycerol
Adjust to final pH 5.5
Store up to 6 months at 2° to 8°C

Periodically check pH of prepared mounting medium, as pH is critical for optimal fluorescence.

Mycoplasma agar plates

23.8 g mycoplasma agar base (BBL)
600 ml deionized, distilled H_2O

Heat to boiling with constant stirring to dissolve all ingredients. Sterilize by autoclaving at 121°C for 15 min (*UNIT 1.4*, Basic Protocol 1). Cool to 50°C and aseptically add the following sterile solutions which have been equilibrated to 37°C:
200 ml horse serum (Life Technologies)
100 ml fresh yeast extract solution (Life Technologies)
100 ml dextrose/arginine supplement (see recipe)

Aseptically adjust to pH 7.2 to 7.4 and dispense in 10-ml aliquots to sterile 60 × 15–mm petri dishes. Store up to 6 weeks at 4°C to 8°C.

continued

Solutions should be prewarmed to 37°C before adding to the agar solution to avoid immediate gelling.

The sterile fresh yeast extract solution used in the preparation of the mycoplasma broth and agar media frequently contains large amounts of sediment that must be removed before it is used in media preparation. Sediment can be removed by coarse filtration followed by filter sterilization (UNIT 1.4) with a 0.2-μm membrane.

The formation of bubbles in agar plates should be avoided as it can confuse examination. When preparing medium, add and mix heat-labile supplements carefully to avoid formation of bubbles. As most of the bubbles are at the surface of the medium, they can be avoided during dispensing by using a semiautomatic dispensing apparatus, such as a peristaltic pump with a sinker attached to the inlet tubing to assure that the medium is being drawn from below the surface of the flask. If a few bubbles form when the medium is dispensed into the plates, they may be removed by lightly passing the flame of a Bunsen burner across the agar surface before the agar has solidified.

Mycoplasma broth medium

14.7 g mycoplasma broth base (BBL)
20.0 mg phenol red
600.0 ml deionized, distilled H_2O

Mix well to dissolve all ingredients. Sterilize by autoclaving at 121°C for 15 min (*UNIT 1.4*, Basic Protocol 1). Cool to room temperature and aseptically add the following sterile solutions:
200 ml horse serum (Life Technologies)
100 ml fresh yeast extract solution (Life Technologies)
100 ml dextrose/arginine supplement (see recipe)

Aseptically adjust to pH 7.2 to 7.4 and dispense in 6-ml aliquots to sterile 16 × 125–mm test tubes. Store up to 12 weeks at 4° to 8°C.

The sterile fresh yeast extract solution used in the preparation of the mycoplasma broth and agar media frequently contains large amounts of sediment that must be removed before it is used in media preparation. Sediment can be removed by coarse filtration followed by filter sterilization (UNIT 1.4) with a 0.2-μm membrane.

COMMENTARY

Background Information

Bacterial and fungal contamination

Bacterial and fungal contamination of cell cultures can originate from many sources. Lapses in aseptic technique at any stage of culture manipulation can introduce low-level contamination that may take days or weeks to proliferate to noticeable levels. Mechanical or operational failures with air handling, primary barriers (e.g., biosafety cabinets), water purification and storage, or sterilization systems utilized by the laboratory can give rise to contamination in spite of adequate aseptic practices during culture transfers. Commonly encountered contaminants of human origin include *Escherichia coli, Micrococcus* and *Staphylococcus* sp., and the fungal yeast *Candida* sp. Common contaminants from environmental sources include *Bacillus* sp., *Escherichia coli*, *Pseudomonas* sp. and allied genera, and the ubiquitous filamentous fungi *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp. Testing procedures described in this unit detect most of the common organisms cited above as well as some less-frequently encountered microbial contaminants of cell cultures. None of the techniques listed, however, will detect all potential microbial cell culture contaminants. If microbial contamination is suspect but not culturable with these media, consultation with a reputable microbiology laboratory should be the course of action.

As an integral part of quality control in a cell culture lab, microbial testing should be performed on culture media, on incoming cultures, and on all seed stock and working cell banks.

Mycoplasma contamination

Mycoplasma contamination of cell cultures is a frequent occurrence that often goes unobserved because, unlike bacterial or fungal contamination, it is rarely visible to the naked eye. The presence of mycoplasma in cultures can

inhibit cell metabolism and growth, alter nucleic acid and protein syntheses, affect cell antigenicity, induce chromosomal alterations, interfere with virus replication, and mimic viral actions. Bovine serum (used in media preparation), laboratory personnel, original tissue samples, and other infected cell lines can be sources of mycoplasma contamination.

No one method, either direct or indirect, will detect all mycoplasma, particularly at low levels of infection. A good mycoplasma screening program will include both direct and indirect methods. Direct culture involves a long test period of over a month and may not detect mycoplasmal strains with nutritional requirements not satisfied by the growth medium. DNA fluorescence is an efficient method for screening mycoplasma contamination, but sometimes it takes experience to recognize the presence of contaminants amid background debris and artifacts. PCR is very fast and sensitive, but expensive in terms of both basic equipment and reagents.

Commercial mycoplasma testing services may be an option for laboratories without the experience or specialized equipment needed to fully screen a cell line for contamination with these microorganisms. A good testing service should be able to provide direct culture, indirect staining, and molecular (DNA/RNA hybridization or PCR) methods. Many of the larger reference culture collections with cell line holdings—e.g., American Type Culture Collection (ATCC), European Collection of Cell Cultures (ECACC), or German Collection of Microorganisms and Cell Culture (DSMZ)—offer mycoplasma testing services. Expect to pay approximately $100 U.S. per sample for direct culture methods and staining. PCR or other molecular techniques will increase the testing fee because of the cost of the reagents involved.

Mycoplasma testing, along with testing for bacteria and fungi, should be included in the routine quality control procedures for all cell culture laboratories. Tests should be performed on sera used in culture media, on incoming cultures, and on all seed stock and working cell banks.

Antibiotic treatment

Antibiotics are not substitutions for good aseptic technique and should not be routinely used as prophylactics against possible contamination. Antibiotics work by disrupting metabolic processes (e.g., protein synthesis) and the chemical mode of action does not always differentiate between microbial contaminants and cultured cells. Antibiotics can be cytotoxic, sometimes at working concentrations. The use of combined antibiotics can exert cytotoxic effects at lower concentrations than that of an individual antibiotic. Do not exceed the suggested working concentration for any antibiotic. As cytotoxicity is cell line dependent, it is necessary to monitor the effects of antibiotic use on cell characteristics as well as on the contaminant.

Critical Parameters

Because of the insidious nature of infection, mycoplasma testing should be segregated from rooms where routine cell culture work is conducted to avoid cross-contamination of clean cultures with mycoplasma-infected cell cultures or mycoplasma cultures being grown with the direct culture screening method. Any mycoplasma-infected material should be immediately destroyed by autoclaving.

It is important to note the stability of antibiotics at 37°C (Table 1.5.1). Antibiotic potency diminishes within days of incubation and additional antibiotic should be added during the 2-week treatment to ensure that working concentrations are maintained, in order to prevent development of antibiotic-resistant contaminants.

Anticipated Results

If contamination of cultures is suspected, careful application of these testing procedures should allow identification of most common microbial, fungal, and/or mycoplasma contaminants. Success rates for treatment of contaminated cultures cannot be generalized, as they depend on the type of contaminant and the severity of the infection.

Time Considerations

Testing for contamination can take a few minutes for visual inspection to several weeks for identification of contaminants such as mycoplasma. Treating contaminated cultures with antibiotics requires 14 days for treatment itself, plus additional days to weeks to verify that the culture is no longer contaminated.

Literature Cited

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Key References

Barnhart, E.R. 1990. Physicians' Desk Reference. Medical Economics Company, Oradell, N.J.

Provides invaluable information on the mode of action of, for instance, antibiotics and antagonisms.

Budavari, S., O'Neil, M.J., Smith, A., Heckelman, P.E., and Kinneary, J.F. (eds.) 1996. The Merck Index. Merck Research Laboratories Division of Merck & Co., Whitehouse Station, N.J.

A technical reference providing information on physical characteristics, solubilities, and stabilities of most chemicals (including antibiotics) used in a tissue culture laboratory.

Freshney, R.I. 1994. Contamination. *In* Culture of Animal Cells: A Manual of Basic Technique, 3rd ed., pp 243-252. Wiley-Liss, New York.

This chapter of the classic text on cell culture discusses various types of contamination and methods for detection and eradication.

Hay, R.J., Caputo, J., and Macy, M.L. 1992. ATCC Quality Control Methods for Cell Lines, 2nd ed. American Type Culture Collection, Rockville, Md.

A manual of procedures used in this cell culture collection's quality control program.

Sigma-Aldrich Co. 1998. Cell Culture Catalogue. Sigma-Aldrich Co., St. Louis, Mo.

Most specialty chemical companies feature a wealth of useful, technical information with their catalogues. This particular edition can be most helpful to the novice user of cell cultures.

Contributed by Rosalie Coté
Becton Dickinson Microbiology Systems
Sparks, Maryland

Media and Culture of Yeast

This unit describes basic aspects of preparing media for and growing *Saccharomyces cerevisiae* cells. It also covers some aspects of handling yeast strains, such as growth, storage, and shipment. *Saccharomyces* has been one of the most extensively studied organisms in the laboratory, and an extensive repertoire of genetic and molecular biology methods have been developed, as described in this volume and elsewhere (see Key References). A large number of yeast strains with useful characteristics have been documented, and many can be obtained for a nominal cost from public collections including the Yeast Genetics Stock Center (YGSC; Rebecca Contopoulou, Curator; e-mail: *ygsc305@violet.berkeley.edu*) or the American Type Culture Collection (ATCC). For contact information for these collections, see Internet Resources and *SUPPLIERS APPENDIX*.

Culturing *Saccharomyces* is relatively straightforward and can be accomplished with readily available and inexpensive materials. Wild-type yeast are prototrophic for most nutrients; that is, they are capable of synthesizing most metabolites from inorganic salts and a carbon source for energy. However most laboratory strains carry mutations that make them auxotrophic for one or more metabolites, such as amino acids or nucleotides, that must be supplied in the growth medium. The auxotrophic mutations, or *markers*, are used in the maintenance of plasmids and in other genetic experiments.

The first part of this unit will discuss the preparation of media. General aspects of media preparation will be described followed by specific recipes. The second part of the unit will discuss the growth and handling of yeast strains.

NOTE: All incubations of yeast cells are performed at 30°C unless otherwise noted.

PREPARATION OF MEDIA

Yeast media are referred to as either *liquid* or *solid*. Liquid medium is self-explanatory. A solid medium typically contains 2% (w/v) Bacto Agar. Other comparable gelling agents can be used in special circumstances, but they are not discussed in this unit.

Liquid Media

Liquid media are prepared in the same way as solid media (described below) except that agar is not added. As a result, liquid media can be sterilized either by autoclaving or by filter sterilizing with a 0.22-µm filter. Filter sterilization is faster and eliminates the risk of heat inactivation of any medium ingredients. Liquid medium is prepared in either bottles or growth flasks. In flasks, medium should never exceed one-fifth the flask volume, so that maximum aeration can be achieved during shaking.

Solid Media

Solid media can be prepared in Erlenmeyer or Fernbach flasks; however, the most convenient container for pouring plates is the Fleaker (Corning). Heat-stable ingredients are mixed in water until completely dissolved (with the exception of the agar, which is added but will not dissolve). It is convenient to add a magnetic stir bar to the flask or Fleaker prior to autoclaving. The medium is autoclaved at 121°C on the liquids or slow exhaust program/setting at 15 psi for 15 min, after which it is placed on a stir plate at room temperature and stirred as it is allowed to cool to 60° to 65°C. Alternatively, the flasks or Fleakers can be placed in a 55°C water bath. Filter-sterilized, heat-sensitive ingredients are added at this point, and the medium is mixed completely prior to plate pouring. At this point the agar should be fully suspended within the medium.

Contributed by Simon R. Green and Charles M. Moehle
Current Protocols in Cell Biology (1999) 1.6.1-1.6.12

Most yeast medium recipes, including those described in this unit, should result in a pH between 5.5 and 7.0. If a more acid medium is prepared, care should be taken to minimize or avoid autoclaving agar at pH ≤4.8, as the agar will hydrolyze and fail to gel when cooled.

A pouring area should be prepared in a quiet place with minimal dust and airflow, and the bench surface should be wiped with 70% ethanol or another sterilizing agent. The appropriate number of bags of petri dishes (typically 15 × 100–mm or 15 × 95–mm) should be opened by carefully cutting off the top of the bags. The bags are overturned, allowing the plates to slide out in a stack. The bags are saved for storing the filled plates. The bottom of the plates are labeled, and the plates overturned and divided into stacks of eight. When the medium has cooled to 55° to 60°C, it can be carefully poured into the plates. When pouring, the Fleaker or flask of medium should be held in one hand, allowing the other hand to grab the stack of plates. The top seven plates are lifted as a stack, the medium is poured into the eighth (bottom) plate, and the stack is lowered. This process is then repeated by picking up the top six plates, pouring the seventh plate, and so on. Although this system of pouring is awkward at first, it has the advantages of being faster and taking up less space than pouring plates in stacks of one.

One liter of medium should generate 40 to 50 plates. Sometimes bubbles will be formed while pouring medium. These can be removed by quickly passing a bunsen burner flame over the surface of the molten agar. The lid should be placed on the plates as soon as possible to minimize the risk of contamination. After the agar hardens (20 to 45 min), the plates should be flipped so that the lids are on the bottom. Plates should be allowed to dry at room temperature for 2 to 3 days. Because they are more prone to collecting condensation, media for yeast are left to dry at room temperature for a longer time than are media for bacteria. Once dried, the plates are placed in plastic bags, and the bags are taped closed and stored at room temperature or at 4°C for extended periods of time. Most media will be stable for ≥3 months; if excessive drying and contamination can be avoided, most media are stable for up to one year. Sometimes it is helpful to make small slits in the plastic bags to let them "breathe." This reduces condensation, which in turn leads to the appearance of fewer contaminating organisms.

Defined Versus Complex Media

Yeast media are often referred to as either *defined* or *complex*. A defined medium is made with chemically defined components, such as salts, sugars, and amino acids. A complex, or rich, medium includes the addition of a complex lysate or hydrolysate, such as yeast extract or peptone. For the purpose of these definitions, agar-containing medium may still be referred to as defined. The following sections provide details about materials for media, and give recipes for specific complex and defined media. A number of special-purpose media are detailed.

Materials

Media should be prepared using distilled or deionized water. Most materials for media preparation should be of high grade, although the specific vendor is usually not important. However, yeast extract, peptone, agar, and yeast nitrogen base (YNB) should all be Bacto brand, sold by Difco. Difco products are carried by several scientific distributors.

Best results are obtained when the sugar component of a yeast medium (e.g., dextrose, sucrose, raffinose, galactose) is not autoclaved with the other components of the medium. If dextrose is included with other ingredients during the autoclaving process, it can caramelize. The standard alternative method is to autoclave or filter sterilize a concentrated stock solution of the sugar and add it to the medium after the other components have been autoclaved. For dextrose, a sterilized 40% (w/v) stock solution is added at 50

ml/liter to the autoclaved mixture of other medium components. Other sugars are typically made as 20% (w/v) stock solutions and added at 100 ml/liter.

Complex Media

Yeast extract/peptone/dextrose (YPD or YEPD) medium

This complex medium is the one most commonly used for the routine growth of yeast when no auxotrophic selection is required.

10 g Bacto yeast extract (1% w/v final)
20 g Bacto Peptone (2% w/v final)
20 g agar (2% w/v final)
950 ml H_2O
Autoclave
Add 50 ml 40% (w/v) dextrose (2% final; sterilized separately by autoclaving or filtering)

Many researchers prefer to add adenine to YPD medium, especially when working with adenine auxotrophs. Red-colored strains of Saccharomyces almost always lack either ade1 or ade2 gene function. To make YPAD, add 20 mg/liter adenine sulfate to YPD prior to autoclaving.

Similar media can be made by substituting other sugars for dextrose. Generally, Saccharomyces grows best on dextrose, but specific experiments may require replacement of dextrose with another sugar. The most common substitutes are YPF (2% w/v fructose), YPGal (2% w/v galactose), YPSuc (2% w/v sucrose), YPRaf (2% w/v raffinose), or YPMal (2% w/v maltose). Except for fructose, not all yeast strains are capable of growing on these alternative sugars. Growth on raffinose is sometimes facilitated by adding 0.05% (w/v) dextrose.

Yeast extract/peptone/glycerol (YPG) medium

Nonfermentable glycerol is the only defined carbon source in this complex medium. Because there is no fermentable carbon source, YPG does not support the growth of *petite* yeast strains (mutant strains that lack functional mitochondria).

10 g Bacto yeast extract (1% w/v final)
20 g Bacto Peptone (2% w/v final)
50 ml glycerol (5% v/v final)
20 g agar (2% w/v final)
950 ml H_2O
Autoclave

Some recipes call for 3% glycerol, but 5% gives better growth. Glycerol can be autoclaved with the medium. Other nonfermentable carbon sources can be used, including ethanol, acetate, or lactate, or combinations thereof. Glycerol is the most commonly used nonfermentable carbon source.

Yeast extract/peptone/dextrose/glycerol (YPDG) medium

This complex medium is used to determine the proportions of wild-type and *petite* cells. The different cells form large and small colonies respectively.

10 g Bacto yeast extract (1% w/v final)
20 g Bacto peptone (2% w/v final)
30 ml glycerol (3% v/v final)
20 g agar (2% w/v final)
970 ml H_2O
Autoclave
Add 2.5 ml 40% (w/v) dextrose (0.1% final; sterilized separately by autoclaving or filtering)

Special-Purpose Complex Media

Galactose indicator medium

Several gene expression systems used in yeast research rely on a *GAL* (galactose) promoter for regulated expression. When such a system fails to work as expected, one source of trouble is the use of yeast strains that are unable to respond to galactose. Strains that respond to galactose will ferment it and release acid into the medium. Acidification causes the indicator dye to change color from blue to yellow.

10 g Bacto yeast extract (1% w/v final)
20 g Bacto Peptone (2% w/v final)
20 g agar (2% w/v final)
930 ml H_2O
Autoclave
Add 50 ml 40% (w/v) galactose (2% final; sterilized separately by autoclaving or filtering)
Add 20 ml 0.4% (w/v) bromthymol blue (0.08% final; sterilized separately by filtering)

Sporulation medium

Saccharomyces strains can be maintained as either haploids or diploids. Most diploid strains will sporulate when grown on a poor source of carbon and nitrogen.

10 g potassium acetate (1% w/v final)
1 g Bacto yeast extract (0.1% w/v final)
0.5 g dextrose (0.05% w/v final)
20 g agar (2% w/v final)
1 liter H_2O
Autoclave

For this recipe dextrose can be added prior to autoclaving.

Presporulation (PSP) medium

Sporulation of some *Saccharomyces* strains is facilitated by growth on a rich medium prior to plating on sporulation medium.

8 g Bacto yeast extract (0.8% w/v final)
3 g Bacto Peptone (0.3% w/v final)
20 g agar (2% w/v final)
750 ml H_2O
Autoclave
Add 250 ml warm 40% (w/v) dextrose (10% final; sterilized separately by autoclaving or filtering)

The dextrose solution should be warmed to ~40° to 55°C before it is added, so that the agar does not harden prematurely.

Dissection agar

Preparation of dissection agar is frequently tailored to the configuration of the dissection apparatus. In general, thin slabs of YPD medium (10 ml per standard 15 × 100–mm petri plate) are used. Best results are obtained with light-colored (most transparent) medium, which can be facilitated by minimizing the length of time it is heated and by autoclaving the dextrose separately.

Defined Media

It is generally best to add inositol to all synthetic media. Although wild-type yeast strains are prototrophic for inositol, some laboratory strains bear an *ino1* mutation. Unfortunately, this mutation is sometimes forgotten in reporting the genotype because yeast nitrogen base (YNB) contains enough inositol that the effect of the mutation is not easy to see. However, the amount of inositol in YNB is not enough to fully supplement the auxotrophy, and selection for compensating mutations can occur.

Salts and dextrose (SD) medium

This minimal medium contains enough nutrients to support growth of prototrophic yeast strains (strains having no nutritional requirements).

6.7 g Bacto yeast nitrogen base (YNB) without amino acids (0.67% w/v final)
20 g agar (2% w/v final)
950 ml H_2O
Autoclave
Add 50 ml 40% (w/v) dextrose (2% final; sterilized separately by autoclaving or filtering)

Similar media can be made by substituting other sugars for dextrose. Generally, Saccharomyces grows best on dextrose, but specific experiments may require replacement of dextrose with another sugar. The most common substitutes are SGal (2% galactose) and SRaf (2% raffinose). Not all yeast strains are capable of growing on these alternative sugars. Growth on raffinose is sometimes facilitated by adding 0.05% (w/v) dextrose.

Vendors carry several similar types of YNB, e.g., with or without amino acids or ammonium sulfate. YNB in this recipe includes ammonium sulfate. Although ammonium sulfate is the preferred nitrogen source, Saccharomyces will grow on other nitrogen sources, albeit more slowly. For these media, YNB without ammonium sulfate and without amino acids is used at 1.7 g/liter. Nitrogen sources include ammonium sulfate (0.5% w/v), arginine (0.1% w/v), asparagine (0.1% w/v), or proline (0.1% w/v).

Most yeast strains require one or more nutrients that are not included in SD medium. These nutrients can be added in various ways (see supplemented SD medium and SC medium).

Supplemented SD medium

Add supplements (see Table 1.6.1) individually or in groups to SD medium. Alternatively, add supplements to individual plates by spreading, and allow one day for supplement(s) to permeate the medium. Add most supplements before autoclaving; add tryptophan and histidine, which are sensitive to heat, after autoclaving. Store tryptophan in the dark, as it is also sensitive to prolonged exposure to light.

Synthetic complete (SC) medium

As an alternative to adding individual nutrients to SD medium, add a dry mixture of the most common supplements. Combine the dry reagents listed in Table 1.6.1 and mix thoroughly using a coffee grinder or a mortar and pestle. Then add the mixture before sterilizing the medium, without tryptophan and histidine, which should be added after autoclaving.

Alternative mixtures can be constructed (e.g., SC minus tryptophan) by omitting specific ingredients. These mixtures are sometimes referred to as "dropout" media—e.g., SC –Trp dropout medium.

5-Fluoroorotic acid (5-FOA) medium

Selection for *URA3* function is used for the stable maintenance of many yeast plasmids. For some experiments, however, loss of the *URA3* gene is desired. The *URA3* gene product converts 5-FOA into a toxin. Therefore, strains that have lost the *URA3* gene can be identified by their resistance to 5-FOA.

continued

Solution 1:
1 g 5-FOA (0.1% w/v final)
500 ml H_2O
6.7 g yeast nitrogen base (YNB) – amino acids (0.67% w/v final)
50 mg uracil (50 µg/ml final)
20 g dextrose (2% w/v final)
Appropriate supplements (see Table 1.6.1)

Add 5-FOA, H_2O, and a stir bar to a 1-liter flask. Autoclave or stir over a low to medium heat ~1 hr to dissolve completely. Add YNB, uracil, glucose, and supplements. Choose supplements from Table 1.6.1 based on the genotype of the strain, but always add uracil. If supplements are added in liquid form, be sure to adjust the volume of H_2O accordingly. Dissolve completely (warming to 55°C is useful) and filter sterilize.

Solution 2:
20 g agar (2% w/v final)
500 ml H_2O

Mix agar and H_2O in a 2-liter flask, autoclave, and cool to ≤80°C.

5-FOA medium:
Mix solution 1 into cooled solution 2
Cool to 55°C
Pour into petri dishes

Table 1.6.1 Common Supplements Used In Defined Media

Nutrient	Stock concentration (g/100 ml)	Stock volume per liter medium (ml)	Final concentration (mg/liter)[a]	Stock volume to spread on plate (ml)
Adenine sulfate[b]	0.2	10	20	0.2
L-Arginine·Cl	1	2	20	0.1
L-Aspartic acid	1	10	100	0.2
L-Glutamic acid	1	10	100	0.2
L-Histidine·Cl	1	2	20	0.1
L-Isoleucine	1	3	30	0.1
L-Leucine	1	3	30	0.1
L-Lysine·Cl	1	3	30	0.1
L-Methionine	1	2	20	0.1
Myo-inositol[c]	3.6	1	36	0.02
L-Phenylalanine	1	5	50	0.1
L-Serine	8	5	400	0.1
L-Threonine	4	5	200	0.1
L-Tryptophan	1	2	20	0.1
L-Tyrosine	0.2	15	30	0.2
Uracil	0.2	10	20	0.2
L-Valine	3	5	150	0.1

[a]Mixtures of supplements can be made in advance by weighing out individual components in the ratio indicated by this column; mixing the dry reagents in a coffee grinder, mill, or mortar and pestle; and then weighing out the appropriate amount of the mixture.

[b]When working with *ade1* or *ade2* mutants, the amount of adenine should be increased three fold to prevent accumulation of red color.

[c]The traditional recipe for SC medium does not include inositol. Inositol is added as a precaution because some common laboratory strains contain the *ino1* mutation, which is not always documented. YNB contains enough inositol for significant, albeit incomplete, supplementation, which makes recognizing the presence of an *ino1* mutation difficult.

Xgal medium

Wild-type *Saccharomyces* strains lack endogenous β-galactosidase activity, which facilitates use of the *E. coli lacZ*-encoded β-galactosidase enzyme as a reporter activity. This plate assay provides a semi-quantitative assay for activity.

6.7 g yeast nitrogen base (YNB) − amino acids (0.67% w/v final)
Appropriate supplements (see Table 1.6.1)
20 g agar (2% w/v final)
850 ml H_2O
Autoclave
Cool to 65°C
Add 100 ml 0.7 M potassium phosphate, pH 7.0 (70 mM final; best if warmed prior to adding to agar)
Add 50 ml 40% (w/v) dextrose (2% final; sterilized separately by autoclaving or filtering)
Add 2 ml 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) in 100% *N*,*N*-dimethylformamide (DMF)

Xgal can be prepared in advance, aliquoted, and stored as a frozen stock. Supplements are chosen from Table 1.6.1 based on the genotype of the strain. If supplements are added in liquid form, the volume of H_2O should be adjusted accordingly.

Xgal plates are frequently made as above, but without the substrate Xgal. Approximately 1 to 2 days prior to using the plates, 0.1 ml of 20 mg/ml Xgal stock is spread across the surface of the plate and allowed to diffuse through the agar.

GENERAL CONSIDERATIONS FOR CULTURING YEAST

For the most part, culturing *Saccharomyces* is fairly straightforward. Wild-type strains are typically grown at 30°C, although they are capable of growing at a wide range of temperatures. Many strains will grow to some extent at temperatures as low as 4°C and at least as high as 37°C. Liquid or solid media can be used. Liquid cultures grow better with aeration, but this is not required. For the most consistent results, it is important to maintain a reproducible level of aeration with each experiment by marking or taping the speed dial of the shaker.

Culturing Yeast in Liquid Media

Yeast strains are readily cultured in liquid media. For most experiments it is best to grow them with agitation, as this prevents them from settling to the bottom of the liquid. When cultures are grown in flasks, the liquid volume should be limited to 20% of the flask volume, although this guideline is sometimes exceeded. Another standard method is to grow 1- to 5-ml cultures in 18 × 150–mm glass tubes that are rotated on a tube roller (e.g., New Brunswick). Metal or plastic caps are readily available for these tubes. Many brands of tabletop or preparative centrifuges accommodate these tubes directly.

Yeast cells can be transferred from liquid or solid cultures to a new liquid culture by many methods. One popular method is to use sterile disposable 15-cm-long applicator sticks. The sticks can be autoclaved in capped culture tubes. Another popular method is to use nonflavored, noncolored wooden toothpicks, which can be autoclaved in their original boxes.

For any given set of conditions—namely, yeast strain, medium, temperature, and agitation—a generation time and saturation density can be readily determined. Most strains grown with agitation in YPD medium at 30°C will double every 80 to 100 min. An overnight culture seeded with ~3×10^4 to 1×10^5 cells or a ~1-mm colony will typically

grow to ~3×10^7 cells/ml. After two days the culture should reach ~5×10^8 cells/ml. When grown in SD or SC medium, the generation time will be on the order of 120 to 150 min. An overnight culture will typically grow to ~5×10^6 cells/ml.

The number of cells per milliliter of culture can be estimated using a spectrophotometer. With a standard 1-cm-path-length cuvette at 600 nm, an optical density (OD_{600}) of 1.0 is equal to ~2×10^6 cells/ml. Because this method relies on measuring light scattering and not true absorbance of the culture, this rule of thumb should be confirmed for each specific spectrophotometer. The conversion factor can vary widely depending on the configuration of the instrument. The spectrophotometer can be calibrated by comparing the OD_{600} reading with an accurate cell count obtained with a hemacytometer or by counting the number of colonies that can be grown from a diluted sample. Once an instrument is calibrated, however, the value should not change from day to day.

Culturing Yeast on Solid Media

Yeast strains are readily cultured on solid media. For most experiments it is best to use a 30°C incubator. While growth rates are hard to establish on solid media, colonies formed from single cells are generally visible to the unaided eye after 2 or 3 days and patches of cells are visible after one day. There are five common methods for transferring yeast cells to solid media: (1) spread plating a suspension of cells, (2) streaking cells with a toothpick or inoculating loop, (3) patching cells with a toothpick or inoculating loop, (4) replica plating from an existing plate, and (5) suspending cells in top agar and pouring them over an agar plate. Each of these is described below.

Spread plating cells

In this method, an appropriate number of yeast cells are calculated and suspended in a small volume of liquid, typically 0.05 to 0.25 ml. The appropriate cell number will depend on the nature of the experiment. It is generally desirable to produce 20 to 200 colonies per plate.

A glass rod can be fashioned into a spreader as follows. A roughly 30-cm length of 3-mm glass rod is cut and both ends are smoothed in a flame. Hold the rod over a bunsen burner flame ~5 cm from one end. When the glass is pliable, bend it ~45°. Hold the rod over the flame ~10 cm from the same end and bend it another 45°, so that one end has now formed a triangle. Optionally, the point at which the triangle section meets the shaft can be bent 30° from the plane of the triangle to make the spreader more ergonomic. Prior to use the spreader is sterilized by dipping the triangle end in 70% to 95% ethanol and igniting it in a flame. Before applying to a cell suspension, the spreader should be cooled by touching it onto the surface of the agar plate or onto the condensation that frequently collects on the lid of the plate. Alternatively, an unmodified sterile glass pipet can be used to spread cells, but this is more difficult. Plastic pipets are not recommended as they tear the agar.

Although not required, spread plating works better if an inoculating turntable (available from Fisher Scientific and other laboratory suppliers) is used. An inoculation turntable consists of a solid base and a freely rotating platform that is the approximate size of a petri plate.

The cells should be spread across the plate immediately after pipetting them onto the agar. If a turntable is used, give it a gentle push. If it is not used, the plate can be rotated frequently by hand. The flat part of the triangle is used to push the cells back and forth across the agar. The technique of spread plating yeast cells, especially with a turntable, differs from spread plating bacteria in one notable respect: yeast cells are more likely to be pushed to the periphery of the plate. To avoid this problem, gently push the "elbow" of the spreader into edge of the plate for a few rotations and then, while the plate is still

rotating, push the elbow back and forth across the plate in a chord that is roughly half way between the center and the edge of the plate.

Streaking cells

The streaking method is used for isolating single colonies of yeast. Single isolated colonies are desirable because, when the procedure is done properly, each colony is derived from a single cell, so that every cell in the colony is genetically identical.

In this method, the plate is divided into four pie-shaped sections and one sample of yeast cells is streaked for single colonies in each section. With practice and increasing skill, the plate can be divided into six or more sections. This method is best performed with sterile toothpicks. A minimal amount of yeast cells are picked with the toothpick and placed in a spot near the edge of the plate. A clean toothpick is used to smear the cells back and forth in a 4×10–mm patch with the long axis of the patch running roughly parallel to the lip of the plate. A third toothpick is drawn once across the short axis of the patch and for another ~15 mm towards the center of the plate. A fourth toothpick is dragged on the agar in an arc that parallels the plate edge and crosses the line made by the third toothpick; the toothpick is then drawn in a series of shorter and shorter arcs that parallel the plate edge and move consecutively towards the center of the plate. The plate is then incubated for 2 to 4 days until individual colonies are visible.

Patching cells

Once pure strains of yeast have been isolated, it is frequently useful to compile a set of the strains on a single agar plate, either for short-term storage or in preparation for testing them in parallel by replica plating. Between 30 and 100 patches can be arranged on a standard 100-mm petri plate.

Before patching yeast from one plate to another, an ordered grid is prepared on a piece of paper and is inserted in a clear plastic sleeve. A standard sheet of paper (A4 or 8×10–in.) can accommodate four identical grids for 100-mm petri plates. The agar plates are set on the grid with the lids facing up. The plates can be held in place with a piece of double-sided tape or with tape that has been rolled into a circle. Two pieces of tape are much more effective than one in holding the plates in place. Either sterile toothpicks or inoculating loops can be used for the patch technique, but toothpicks work better. The patches are made by barely touching a source colony with the end of the toothpick and then gently dragging the toothpick over a ~2-mm diameter spot on the recipient plate.

Replica plating cells

Replica plating is used for testing multiple strains or isolates in parallel. This technique requires three specialized pieces of equipment: a replica block, a locking ring, and velveteen cloth squares. All three are available commercially from Fisher Scientific, Cora Styles, and other suppliers. The diameter of the block must be designed for the diameter of the petri plates being used; 95-mm diameter plates will not work with a block designed for 100-mm plates. Having the correct type of cloth is also essential for replica plating to work.

Prior to the first use and after each subsequent use, the cloth squares are laundered in any standard washer and dryer with standard laundry detergent. If recombinant organisms are used, it is prudent to soak the contaminated squares in a disinfectant prior to laundering them. Care should be taken to monitor the lint screen during drying because a significant amount of lint is produced; failure to do this can damage the clothes dryer. After the squares are dried, excess lint can be removed with a standard lint brush, although this is not always necessary. The squares are sterilized by wrapping them in heavy-gage aluminum foil and autoclaving them. Depending on usage patterns, 5 to 50 squares can be stacked, soft-side down, on the center of a large piece of foil. The foil is then folded

around the stack to make a securely closed package that is held with a piece of tape. The package is then autoclaved on the dry cycle.

A *master* plate is made by spread plating, streaking, or patching cells. Typically, the master plate is incubated for 1 to 3 days before use. The master plate works best if the colonies are not extremely large and overgrown. Each good-quality master can be used to produce up to eight replicas.

The replica block and locking ring can be cleaned by squirting them with 70% ethanol and drying them with a paper towel. The package of velvets is carefully opened with the soft side of the cloth squares facing down. One velvet is picked up by the corner, turned over, and placed on the block. The package should then be partially closed to prevent contamination. The locking ring is pushed down so that the velvet is drawn tight. The master plate is gently but firmly pushed onto the surface of the velvet and then lifted up again. Each test plate is then pushed onto the velvet in the same manner.

Suspending cells in top agar

This is probably the least preferred method unless the yeast cell wall has been compromised and an osmotic support is needed. A stock of sterile Bacto agar is prepared in water or medium. The agar should be prepared so that the final concentration is between 0.5% and 1.0% after adding the yeast suspension. Once it has been sterilized, the agar stock solution can be melted any number of times in a boiling water bath. For a 100-mm plate, 3 to 10 ml of the melted agar is transferred to a sterile tube and cooled to 37°C. A small volume of yeast cells are added, and the suspension is mixed and poured over the surface of an agar plate. After a few minutes, the plates can be inverted and incubated for 2 to 4 days at 30°C.

Storage of Yeast Strains

Short-term storage of yeast strains

Most yeast strains will survive extended periods of storage at room temperature or 4°C; however, this type of storage should not be relied upon. Storage at these temperatures may result in loss of viability, or worse, selection of uncharacterized mutants that survive better under these conditions.

Long-term storage in glycerol

Two methods are commonly used for long-term storage of yeast strains: glycerol/water vials at −80°C and agar slants at room temperature or 4°C. The glycerol vials are the preferred method of storage.

Yeast strains can be stored indefinitely at −80°C in 15% (v/v) glycerol in water. Any glass vial may be used; however, 2-ml Wheaton vials (in a lab file) are an ideal size. These vials can be stored in standard freezer storage boxes (100 per box).

To prepare the vials, 1 ml of 15% glycerol solution is dispensed per vial, lids are placed loosely on the vials, and the vials are autoclaved. As soon as the vials are cool enough to handle, the lids are tightened. Prior to use, the vials may be stored at room temperature indefinitely.

To store the strain, the vials are labeled and a single colony is transferred using a sterile toothpick or inoculating loop from a fresh plate into each vial. Each vial is shaken until the colony is evenly distributed in the liquid. The vials are then transferred to a −80°C freezer. The best colonies are 1 to 3 days old, although older ones will work.

Labeling the vials can be difficult because some brands of label fall off at such cold temperatures and some inks run when they come in contact with freezer frost. One method

that works is to use good quality office labels and to cover the labels with one wrap of cellophane tape. The tape sticks to itself very well and protects the writing.

A sample of a liquid culture may be preserved by this method, although it is not recommended. If liquid cultures are to be preserved, the concentration of glycerol should be adjusted accordingly to allow for dilution by the liquid culture.

Long-term storage in YPAD slants

YPAD medium is prepared and the agar is dissolved into the medium in a boiling water bath. Portions of 1.5 ml are dispensed into 3-ml vials, screw cap lids are applied loosely, and the medium is autoclaved. After autoclaving, the vials are inclined so that the agar is just below the neck of the vial. After drying for 1 to 2 days, the lids are tightened.

A small sample of a fresh culture is transferred to the agar slant using a sterile toothpick or inoculating loop. The culture may be spread across the agar surface or it may be pushed into the agar. The screw-cap lid is tightened. Parafilm can be wrapped around the lid to improve the seal. The slants are stored in a cool (23°C) dark place. Viable yeasts can be recovered from the slants for ≥ 3 years.

Shipping Yeast Strains

Yeast strains can be shipped by any of several different methods. The shipped cultures may be dried, on agar, or in liquid as long as sterility is maintained. The simplest method is to use sterile swabs. Other methods generally involve more preparation time or are more vulnerable to damage in the mail.

Shipping yeast strains in sterile swabs

One convenient and safe method is to use sterile swabs for taking throat cultures, such as the Culturette system from Becton Dickinson. These systems contain a sterile swab and a saline solution housed in a leak-proof container that is resistant to rough handling. To use this system, the package is opened, a colony is swabbed from a fresh culture plate, the swab is inserted back into the package, and the package is labeled and taped or stapled shut. To revive the strain, the swab is removed and dabbed on a small area of a petri plate containing YPD or another appropriate medium. Toothpicks are then used to streak for single colonies and the plate is incubated.

Shipping yeast strains on filter paper

Yeast cultures can be dried onto filter paper and stored or transported. This method was developed by at the YGSC by John Bassel, and is best used for strains that are frequently sent out, because multiple filters may be prepared once and stored up to several years at 4°C. Sterile filter paper squares (Whatman no. 4, 1 × 1 cm) are prepared by packaging them in a sheet of heavy-duty aluminum foil (7 × 6 cm) and autoclaving the packages. The strain to be shipped is grown exponentially in YEPD and a 5-µl sample of culture is added to 0.2 ml evaporated milk. The milk is handled aseptically but is not sterilized. The filter paper squares are immersed in the milk suspension of cells and returned to the aluminum foil packet. The packets are folded only once and stored in a desiccator at 4°C for 2 to 3 weeks or until the liquid congeals into a hard, dry lump. Following desiccation, the packets are folded tightly and stored at 4°C in plastic boxes. To revive the strain, the filter paper is swiped on a petri plate of YPD or other appropriate medium and returned to its packet. Toothpicks are used to streak for single colonies and the plate is incubated.

Shipping yeast strains by other methods

Other methods include sending agar slants (described above) or petri plates. Care should be taken in packaging petri plates because they are easily broken in the mail.

KEY REFERENCES

Guthrie, C. and Fink, G.R. (eds.) 1991. Guide to yeast genetics and molecular biology. *Methods Enzymol.* vol. 194.

A compendium of technical methods in yeast cell culture, genetics, biochemistry, molecular biology, and cell biology.

Rose, M.D, Winston, F., and Hieter, P. 1990. Methods in Yeast Genetics, A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

The laboratory manual from a Cold Spring Harbor laboratory source on yeast molecular biology.

INTERNET RESOURCES

http://www.atcc.org/

Web site for the ATCC.

http://www.atcc.org/hilights/sc_info.html

Web site for the ATCC Saccharomyces page.

http://dgm2ibm.nihs.go.jp/ygsc.htm

This Web page provides access to YGSC catalogs, though it is not official.

http://genome-www.stanford.edu/Saccharomyces/

This Web site contains an extensive amount of information and links related to Saccharomyces.

http://www.tiac.net/users/cstyles/

This Web site contains specific information about Saccharomyces and is a source for some harder-to-find equipment.

Contributed by Simon R. Green
Fluorescience Ltd.
Leeds, United Kingdom

Charles M. Moehle
Genelabs Technologies, Inc.
Redwood City, California

BY-2 Cells: Culture and Transformation for Live Cell Imaging

This unit describes the production of stable transformants of tobacco Bright Yellow 2 (BY-2) cells with such fluorescent markers as the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* to study subcellular dynamics. These markers are increasingly used in plant cell biology, and the number of fluorescent proteins available for plant cell imaging and physiology is increasing almost exponentially (Brandizzi et al., 2002; Table 1.7.1).

BY-2 cells were initially established for mass production of raw material for cigarettes, but they have lately been described as the HeLa cell of the biology of higher plants (Nagata et al., 1992). This title is well deserved, as these cells are amenable to cell culture, transformation, and manipulation. BY-2 cells have a high synchronization efficiency and growth rate, and they are homogeneous. Because of their good size and the absence of autofluorescent chloroplasts, they are also easily transformed and imaged. When BY-2 cells are grown on solid medium they form calli. Calli are aggregated cells that arise from multiple divisions of a single cell—similar to a bacterial colony. The BY-2 calli are used as a way to keep a stock of cells that can be used to start suspension cultures as required.

Table 1.7.1 Examples of BY-2 Cellular Components Highlighted with Fluorescent Proteins[a]

Construct	Cellular location	Fluorescent protein	Reference
Korrigan (KOR1) and mutants	Golgi apparatus, growing cell plates	GFP	Zuo et al., 2000
Soybean α-1,2 mannosidase (Gm-Man1)	Golgi apparatus	GFP	Nebenführ et al., 2000
Rat sialyltransferase 1 (ST)	Golgi apparatus	GFP	Saint-Jore et al., 2002
N-acetylglucosaminyl transferase I (Nag)	Golgi apparatus and NE	RFP	Dixit and Cyr, 2002
ER-targeted GFP-HDEL	ER	GFP	Nebenführ et al., 2000
Arabidopsis thaliana RAN GTPase activating protein 1 (AtRanGAP1) and deletion clones	NE and other structures	GFP	Rose and Meier, 2001
Truncated lamin B receptor (LBR)	NE	GFP	Irons et al., 2003
Matrix attachment filament-like protein (MAF1)	Outer NE and other cellular structures	GFP	Rose and Meier, 2001
		CFP	Dixit and Cyr, 2002
Actin-binding domain of a mouse talin	Actin	GFP	Kost et al., 1998
Tubulin	Microtubules	GFP	Kumagai et al., 2001
Microtubule-binding domain (MBD)	Microtubule-binding domain	GFP	Granger and Cyr, 2000 Dixit and Cyr, 2002
Cyclin B1 and mutants	Cytoplasm and nucleus	GFP	Criqui et al., 2001

[a]Abbreviations: CFP, cyan fluorescent protein; ER, endoplasmic reticulum; GFP green fluorescent protein; NE, nuclear envelope; RFP, Red fluorescent protein.

Contributed by Federica Brandizzi, Sarah Irons, Anne Kearns, and Chris Hawes
Current Protocols in Cell Biology (2003) 1.7.1-1.7.16

Within a month a callus can grow to several centimeters in diameter, at which point a small piece (~2 to 5 mm^2 lump) should be transferred to fresh solid medium to keep the cells supplied with nutrients. If cells are already in suspension culture, stock plates can be made by plating 1 ml of suspension cells on solid medium. BY-2 calli are easily broken up by agitation in liquid medium. The establishment of a BY-2 suspension culture is described in Basic Protocol 1; Basic Protocol 2 describes their routine culturing.

Transient transformation of BY-2 cells may be achieved after protoplasting and electroporation (F.B. and C.H., unpub. observ.; *UNIT 20.5*) or directly by particle bombardment (An, 1985) and electroporation (Koscianska and Wypijewski, 2001; *UNIT 20.5*). Co-cultivation with *Agrobacterium tumefaciens* also easily leads to stable transformation.

BY-2 cells are readily susceptible to inducible expression systems such as the tetracycline repressor and, even more tightly, the glucocorticoid-inducible system, neither of which suffers from leakage of gene expression in the absence of the inducer (Criqui et al., 2000; David and Perrot-Rechenmann, 2001; Nishihama et al., 2001; Geelen et al., 2002). Besides chemical inducers, gene expression in the BY-2 cell system may be induced by heat shock. In this respect, the heat-sensitive promoter HSP18.2 may be used as an alternative to chemical inducers with fast kinetic responses (Yoshida et al., 1995; Shinmyo et al., 1998).

BY-2 cells can easily be used for following the cell cycle and for studying the effect of test compounds on its progression. As the authors describe in this unit, aphidicolin synchronization (Basic Protocol 3) may produce a good mitotic index. The current availability of cellular fluorescent markers opens the exciting possibility to follow the dynamics of organelles and the cytoskeleton during the cell cycle.

The transformation of BY-2 cells with *Agrobacterium* that contains a reporter construct in a suitable binary vector is an easy, quick, and very reliable method (Basic Protocol 4). In comparison with protoplast-based protocols, this method offers several advantages over transient transformation. These include working with a relatively unperturbed cellular environment, little variability of DNA expression among cells, longer imaging periods, amenability to synchronization of mitosis, and ease of handling. BY-2 cells can also be stably transformed with two constructs, each expressing a different fluorescent marker (Alternate Protocol).

When GFP is expressed as a chimeric protein either with selected peptide targeting sequences or with complete proteins that are resident in subcellular locations, expression of the chimeric constructs can be observed in living cells using conventional epifluorescence or, preferably, confocal laser scanning microscopes.

CAUTION: BY-2 cells are unlikely to survive outside the laboratory culture environment. It is, however, good laboratory practice to treat transformed BY-2 cells as biological hazards and therefore to dispose of them accordingly. The cells in culture should be killed by autoclaving.

NOTE: All procedures should be carried out under sterile conditions in a laminar flow hood sprayed with 70% (v/v) ethanol (and allowed to stand for 20 min before use). A heat bead sterilizer or flame should be used to sterilize metallic tools such as forceps and razor blades (tools must be cool before using on biological materials). All items placed in the hood should be sprayed with 70% (v/v) ethanol.

ESTABLISHING A BY-2 SUSPENSION CULTURE

This protocol describes the method to establish a BY-2 cell suspension from a BY-2 or another tobacco callus. Subculturing the cells is technically termed passaging (also see Basic Protocol 2). It is advisable to work in duplicate or even triplicate when establishing a suspension culture. This allows initiation of fresh suspensions of the same transformed cell line should any contamination occur.

Materials

Calli of wild-type and/or transformed BY-2 cells (available from various laboratories) grown on solid BY-2 medium (see recipe) in petri dish
50-ml conical flasks containing 20 ml liquid BY-2 medium (see recipe), covered with aluminum foil and autoclaved
Suitable filter-sterilized antibiotics, for culturing transformed BY-2 cells only

Razor blades, sterile
Packet of aluminum foil squares for covering flasks, sterile
Shaking incubator, 25°C

1. Cut a 2-cm^2 piece of wild-type or transformed BY-2 callus with a sterile razor blade directly on the petri dish on which the calli are growing.

2. Loosen aluminium foil from top of a sterile 50-ml conical flask containing 20 ml liquid BY-2 medium. Lightly flame neck of flask. Add suitable filter-sterilized antibiotics if needed. Open a sterile packet of aluminum foil squares so that they are readily accessible.

   This will aid the speed at which the items in the hood can be handled and hence will reduce the possibility of contamination.

   The flasks should be open for as short a time as possible to avoid contamination. Avoid passing anything (e.g., hands or sleeves) over the open flasks.

   The preparation and storage of antibiotics should be according to manufacturer's instructions. Antibiotics should be filter sterilized and handled as sterile material. To filter sterilize the antibiotic solution, a stock solution of the required concentration (usually 100×) is prepared. The antibiotic solution is aspirated into a sterile syringe without a needle. A 0.2-μm syringe filter is attached to the syringe, and the solution is pressed through the filter into a sterile tube.

3. Transfer the cut callus to the 50-ml flask with medium.

   When establishing suspension cultures it is best to begin in a small volume as the cells grow better. To encourage growth the authors have found that subculturing newly established suspensions 1 to 2 weeks after placing calli in liquid medium, the culture should be quite thick—like runny tomato ketchup. Passaging cultures at lower dilution ratio (1:10 instead of 1:20) can also encourage good suspension culture growth. Once the cells are growing well in the small volume they can be subcultured into larger volumes of medium.

4. Gently pipet the culturing medium up and down to break up callus.

5. Reseal the flask with a new square of aluminium foil.

6. Place cells in a shaking incubator set at 130 rpm and 25°C with illumination of choice.

   A flat-bed orbital platform in a 25°C culture room may also be used.

   BY-2 cell suspensions may be kept in the dark. An illumination regime of 16 hr light and 8 hr dark can also be used.

   Cells should be subcultured after 7 days (see Basic Protocol 2).

BASIC PROTOCOL 2

ROUTINE CULTURE OF BY-2 CELLS

This protocol describes the routine method to culture BY-2 cells and to maintain suspension and callus cultures. As described in Figure 1.7.1, the protocol requires liquid and solid media.

Materials

50-ml conical flasks containing 20 ml liquid BY-2 medium (see recipe), covered with aluminum foil and autoclaved
Suitable filter-sterilized antibiotics, for transformed BY-2 cells only
Wild-type or transformed stationary-phase BY-2 cells (i.e., 7-day-old cultures) grown in suspension (see Basic Protocol 1)

Packet of aluminum foil squares for covering flasks, sterile
Trimmed 1-ml pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile
Shaking incubator, 25°C

1. Loosen aluminium foil from top of a sterile 50-ml conical flask containing 20 ml liquid BY-2 medium. Lightly flame neck of flask. Add suitable filter-sterilized antibiotics if needed. Open a sterile packet of aluminum foil squares so that they are readily accessible.

   This will aid the speed at which the items in the hood can be handled and hence will reduce the possibility of contamination.

   The flasks should be open for as short a time as possible to avoid contamination. Avoid passing anything (e.g., hands or sleeves) over the open flasks.

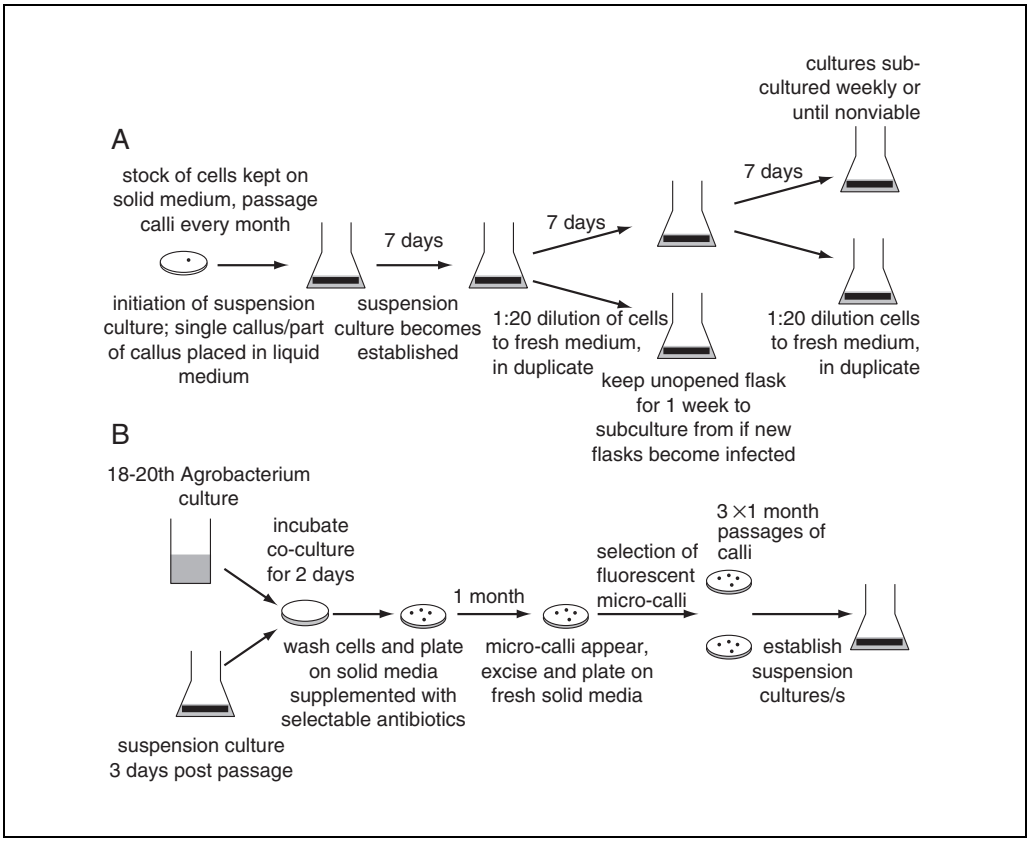


Figure 1.7.1 (**A**) Routine culture of BY-2 cells (see Basic Protocol 2). (**B**) Production of stable transformants (see Basic Protocol 4).

The preparation and storage of antibiotics should be according to manufacturer's instructions. Antibiotics should be filter sterilized and handled as sterile material. To filter sterilize the antibiotic solution, a stock solution of the required concentration (usually 100×) is prepared. The antibiotic solution is aspirated into a sterile syringe without a needle. A 0.2-μm syringe filter is attached to the syringe, and the solution is pressed through the filter into a sterile tube.

2. Use a trimmed 1-ml pipet tip to take a 1-ml aliquot of wild-type or transformed stationary-phase BY-2 cells from their flask.

   The ends of uncut tips will be too narrow to allow easy entry of cells. A razor blade can be used to cut the tips, and cut tips are autoclaved as normal.

   For larger volumes of cells, use 100 ml BY-2 medium in a 250-ml flask. For 100-ml suspension cultures, passage 10 ml cells into 100 ml fresh medium.

3. Remove foil from the new flask and pipet the cell aliquot directly into the fresh medium.

4. Cover the new flask with a new square of aluminum foil and seal firmly.

5. Place cells in a shaking incubator set at 130 rpm and 25°C with illumination of choice for 7 days.

   A flat-bed orbital platform in a 25°C culture room may also be used.

   BY-2 cell suspensions may be kept in the dark. An illumination regime of 16 hr light and 8 hr dark can also be used.

   After 7 days, cells should be passaged again.

6. Passage cells every 7 days.

APHIDICOLIN-MEDIATED SYNCHRONIZATION OF CELL CULTURES

BASIC PROTOCOL 3

Synchronization of cultures can be achieved by a variety of methods, which include mineral and hormone starvation, hydroxyurea treatment, heat shock, light-dark cycle control, or chemical inducers, such as aphidicolin. Aphidicolin is a toxin produced by the fungus *Cephalosporium aphidicolia*, and it acts as a specific and reversible inhibitor of DNA polymerase α (Ikegami et al., 1978). In the presence of this compound, cells are blocked in G1; when aphidicolin is washed out of the suspension, the cells are released from G1 and the cell cycle progresses to S phase (see Fig. 1.7.2; Menges and Murray, 2002).

The following describes how to synchronize BY-2 cells with aphidicolin. With this method it is possible to achieve a 40% mitotic index. Higher synchrony is possible using treatments such as propyzamide (1.6 mg/liter) 6 hr after aphidicolin release (Nagata et al., 1992). However, as this compound may perturb microtubule structure, its use may not be desirable if subcellular dynamics, such as the cell cycle, are being studied.

With the method described below, cells generally reach M phase 10 to 11 hr after the aphidicolin wash.

CAUTION: Aphidicolin is very toxic and should be handled with great care. Aphidicolin should be handled wearing gloves and in accordance with general good lab practice (i.e., with lab coat and eye protection). Inhalation of dust should be avoided. The chemical should be diluted in at least 1 liter of water and flushed down the drain.

Materials

50-ml conical flasks containing 20 ml liquid BY-2 medium (see recipe), covered with aluminum foil and autoclaved
5 mg/ml aphidicolin (Fisher) in dimethyl sulfoxide, store up to 1 yr at 4°C
Suitable filter-sterilized antibiotics, for transformed BY-2 cells only
Wild-type or transformed stationary-phase BY-2 cells (i.e., 7-day-old cultures) grown in suspension (see Basic Protocol 1)

Five 100-ml beakers, covered with aluminum foil and autoclaved
20-µm nylon filter mounted on a cut plastic autoclavable 50-ml beaker, sterile
Packet of aluminum foil squares for covering flasks, sterile
Trimmed 1-ml pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile
Shaking incubator, 25°C
1-liter liquid waste container

1. In a laminar flow hood, aliquot 80 ml BY-2 medium to each of five sterile 100-ml beakers.
2. Loosen aluminium foil from top of a sterile 50-ml conical flask containing 20 ml liquid BY-2 medium. Lightly flame neck of flask. Add 20 µl of 5 mg/ml aphidicolin and suitable filter-sterilized antibiotics, if needed. Open a sterile packet of aluminum foil squares so that they are readily accessible.

   This will aid the speed at which the items in the hood can be handled and hence will reduce the possibility of contamination.

   The flasks should be open for as short a time as possible to avoid contamination. Avoid passing anything (e.g., hands or sleeves) over the open flasks.

   The preparation and storage of antibiotics should be according to manufacturer's instructions. Antibiotics should be filter sterilized and handled as sterile material. To filter sterilize the antibiotic solution, a stock solution of the required concentration (usually 100×) is prepared. The antibiotic solution is aspirated into a sterile syringe without a needle. A 0.2-µm syringe filter is attached to the syringe, and the solution is pressed through the filter into a sterile tube.
3. Use a trimmed 1-ml pipet tip to take a 1-ml aliquot of wild-type or transformed stationary-phase BY-2 cells from their flask and transfer them to aphidicolin-supplemented medium.

   The ends of uncut tips will be too narrow to allow easy entry of cells. A razor blade can be used to cut the tips, and cut tips can be autoclaved as usual.
4. Cover the new flask with a new square of aluminium foil and seal firmly.
5. Place cells in a shaking incubator set at 130 rpm and 25°C and incubate 24 hr in the dark.
6. Hold a sterile 20-µm nylon filter mounted on a cut plastic 50-ml beaker over a sterile 1-liter liquid waste container and gently pour aphidicolin-treated cells onto the filter. Discard medium in liquid waste container.

   To construct the filter apparatus, the bottom end of a plastic beaker is cut off and discarded. A 2-cm section is cut from the bottom of the beaker. This ring is placed on top of the nylon filter, and then the filter and ring are inserted in the upper part of the beaker. In this way, the filter will be secured to the cut end of the beaker (see Fig. 1.7.2). The apparatus is wrapped in foil before autoclaving.
7. Quickly transfer filter with cells to a sterile 100-ml beaker containing 70 to 80 ml liquid BY-2 medium. Gently agitate cells in filter for 5 min.

   The transfers must be carried out quickly to prevent the cells from drying out.

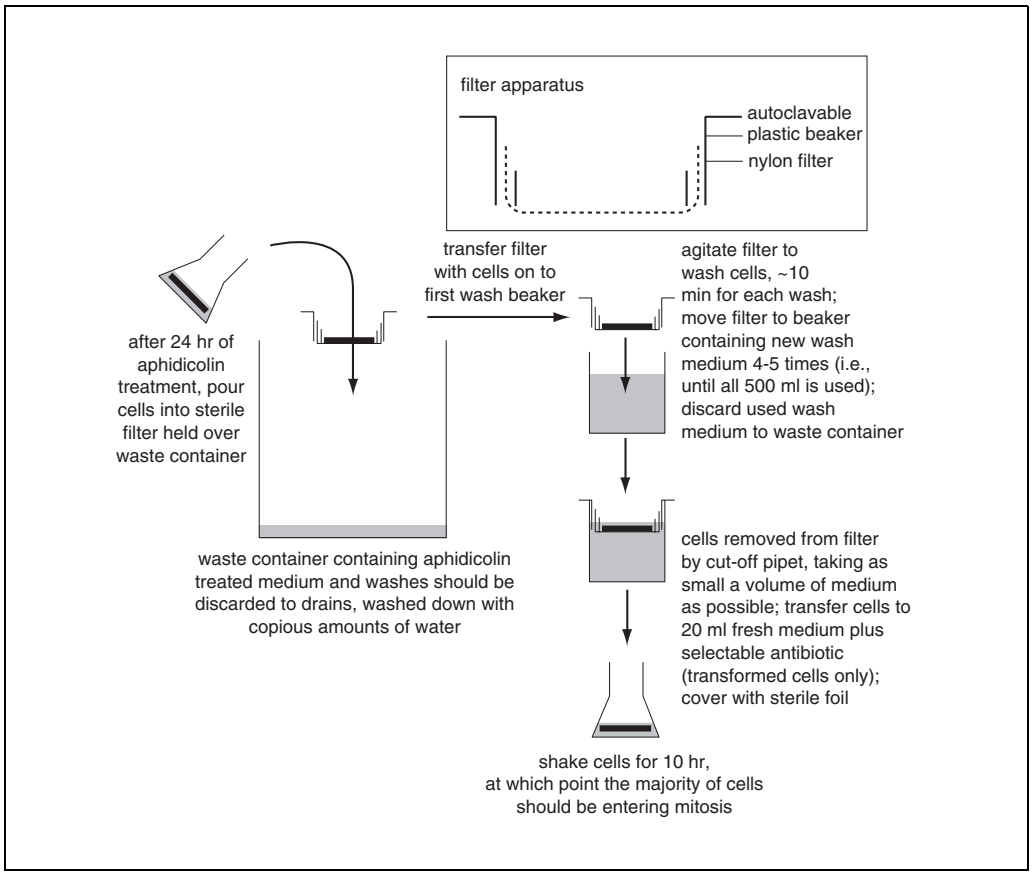


Figure 1.7.2 Aphidicolin synchronization of BY-2 suspension cultures.

8. Repeat transfer four more times, using a beaker with fresh medium each time. Discard used medium in liquid waste container.
9. Resuspend cells in new 50-ml flask containing 20 ml medium (with antibiotic if needed).
10. Shake cells 10 to 11 hr at 25°C.

At this point the majority of cells are entering into M phase.

STABLE TRANSFORMATION OF BY-2 CELLS MEDIATED BY *AGROBACTERIUM* FOR VISUALIZATION OF SUBCELLULAR ORGANELLES

BASIC PROTOCOL 4

This protocol involves the production of BY-2 lines that stably express GFP targeted to subcellular structures for subsequent live cell imaging (see Fig. 1.7.3). The procedures are suitable for other tobacco cell lines and may be modified for *Arabidopsis thaliana* suspension cells as well (Foreiter et al., 1997).

Stably transformed calli may be identified and selected directly on a petri dish with the aid of a portable UV lamp if the fluorescent protein used to transform cells has a UV peak excitation (such as the green fluorescent protein GFP5; Haseloff et al., 1997). Alternatively, a small portion of the callus may be analyzed with a conventional fluorescence microscope using the appropriate excitation-emission filter set to establish the positive transformation. In Figure 1.7.1B, a scheme of the production of stable transformants is given.

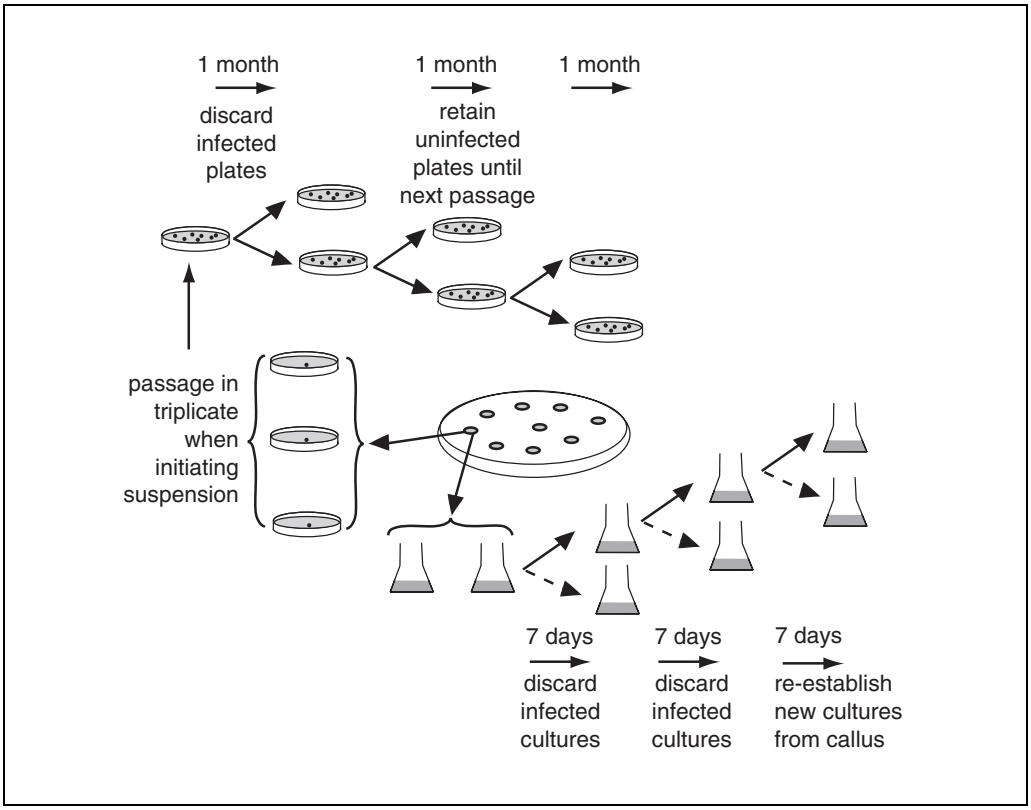


Figure 1.7.3 Passaging of stably transformed BY-2 cells.

NOTE: To transform BY-2 cells, a binary vector that can be transformed into agrobacteria is needed and can be the same as is used to transform tobacco and *Arabidopsis* plants. The binary vector, in which the fluorescent protein marker is subcloned, must carry two resistance markers (antibiotic resistance). One is a selectable marker for bacteria—to select positive transformants after bacterial transformation—and the other is a selectable marker for plants, which will allow the growth only of transformed plant cells. When working with these selection markers, it is very important to follow the manufacturer's instructions for the specific antibiotic in use. Factors such as light and pH, for example, may alter the properties of the antibiotics and thus affect the yield of stable transformants.

Materials

YEB medium (see recipe) containing appropriate filter-sterilized bacterial selection antibiotic

Agrobacterium tumefaciens transformed with vector containing appropriate GFP construct (e.g., strain GV3101::pMP90; Konez and Schell, 1986) transformed with another plasmid (e.g., pVKHI8En6, pBII21) which contains GFP and the insert of interest.

3-day-old wild-type BY-2 suspension culture (see Basic Protocol 1)

Liquid BY-2 medium (see recipe), sterile

Solid BY-2 medium (see recipe) plates with plant selectable antibiotic, 100 µg/ml carbenicillin (see recipe), and 20 µg/ml timentin (see recipe)

Shaking incubator, 25°C

Trimmed 1-ml pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile

5- and 10-cm petri dishes, sterile

1.5-ml microcentrifuge tubes, sterile

Forceps, sterile

Grow agrobacteria

1. Inoculate 5 ml YEB medium containing appropriate filter-sterilized bacterial selection antibiotic with a single colony of *Agrobacterium tumefaciens* transformed with a binary vector containing the appropriate GFP construct. Incubate 18 to 20 hr at 130 rpm and 25°C.

Transform BY-2 cells

2. Using a trimmed 1-ml pipet tip, transfer 1 ml of a 3-day-old wild-type BY-2 suspension culture to a sterile 5-cm petri dish.
3. Add 50 µl overnight *Agrobacterium* culture (step 1) and mix very gently with pipet tip.
4. Seal plate with Parafilm and incubate 2 days at 25°C in the dark without shaking.

   Alternatively, the plates may be wrapped in aluminium foil or placed in sealed boxes.

5. Add 1 ml fresh liquid BY-2 medium and collect the cells with a trimmed 1-ml pipet tip. Transfer cells to a sterile 1.5-ml microcentrifuge tube.

   Because the cell medium may have dried out during the 2-day incubation, 1 ml medium is added to facilitate collection of the cells.

   It may be necessary to divide cells between two tubes at this stage.

6. Allow BY-2 cells to settle to bottom of tube by gravity (2 min).
7. Remove excess medium and add 1 ml fresh liquid BY-2 medium. Resuspend cells by gently flicking the tube.
8. Repeat steps 6 and 7 two more times for a total of three washes.

Plate BY-2 cells

9. Resuspend cells with 1 ml fresh liquid BY-2 medium and use trimmed 1-ml pipet tip to transfer cells to solid BY-2 medium plates with plant selectable antibiotic, 100 µg/ml carbenicillin and 20 µg/ml Timentin.
10. Gently rotate plate to spread cells over the surface of the solid medium.
11. Seal plate with Parafilm and incubate in the dark at 25°C without shaking until microcalli appear (~1 month).
12. Use sterile forceps to excise individual microcalli and plate them onto fresh solid BY-2 medium plates with plant antibiotic. Place a maximum of nine calli per 10-cm plate.

    Calli can be screened, after excision and before passaging, for fluorescence using an UV lamp for constructs based on GFP5 (Haseloff et al., 1997); calli that have been successfully transformed will fluoresce.

13. Passage calli three times, allowing a 1-month interval between each passage, before establishing suspension cultures.

    Each passaging step except the first (when the microcalli are too small to divide) should be carried out in duplicate to ensure that, should one plate become contaminated, there is still another stock plate available. These passages are carried out to ensure that the cells are stably transformed.

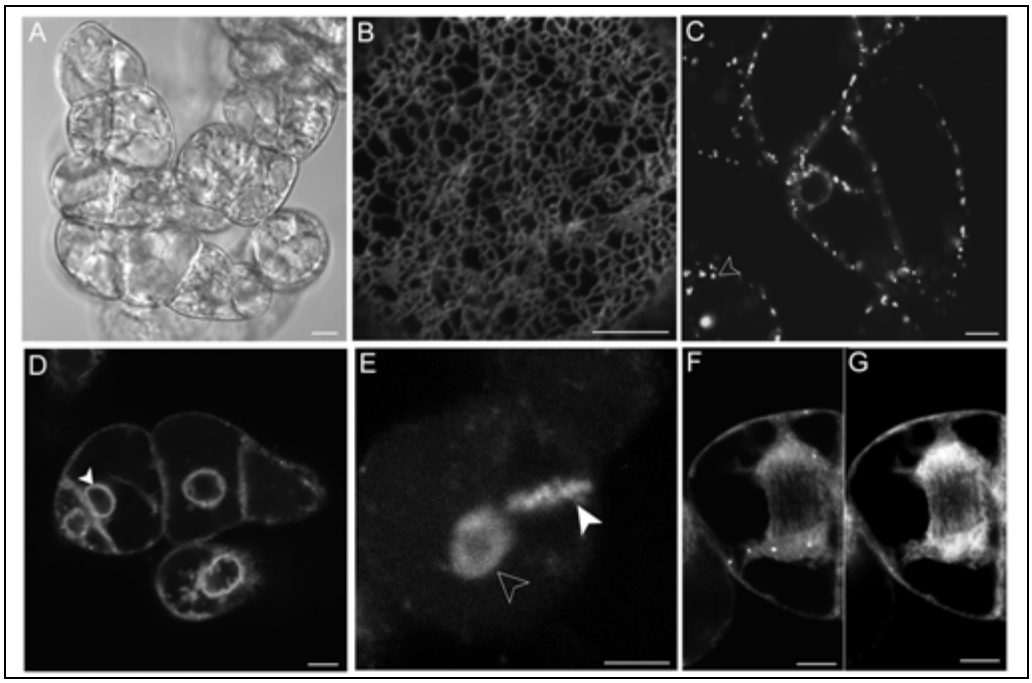


Figure 1.7.4 ((**A**) DIC image of BY-2 suspension culture. Membranous structures, nuclei, and vacuole are clearly visible. (**B**) Cortical endoplasmic reticulum (ER) visualized with yellow fluorescent protein (YFP) targeted and retained in a BY-2 cell stable transformant. This construct, SpYFP-HDEL, is composed of a sporamin signal peptide (to target the protein to the ER lumen), YFP, and the tetrapeptide HDEL to retain the protein in the lumen of the organelle (Irons et al., 2003). (**C**) To visualize the Golgi apparatus (arrowhead), the signal anchor of a rat sialyl transferase has been fused to green fluorescent protein (GFP) and expressed stably in BY-2 cells (Saint-Jore et al., 2002). (**D**) Nuclear envelope (arrowhead) of BY-2 cells stably expressing the amino terminus of the human lamin B receptor fused to GFP (LBR-GFP; Irons et al., 2003). (**E**) A histone-YFP (Boisnard-Lorig et al., 2001) construct highlights the chromatin in stably transformed cells. Cell in interphase (white arrowhead) and at metaphase (black arrowhead). (**F**,**G**) Stable BY-2 transformant co-expressing LBR-GFP (F) and SpYFR-HDEL (G) during cell division. Scale bars, 10 μm.

ALTERNATE PROTOCOL

STABLE TRANSFORMATION OF BY-2 CELLS WITH TWO CONSTRUCTS

BY-2 cells are also an amenable system for stably co-expressing two constructs (Fig. 1.7.4F, G). To achieve this, the fluorescent markers should be subcloned independently into a bacterial plasmid bearing the same antibiotic resistance. For the transformation, Basic Protocol 4 is carried out with the following changes. In step 1, two separate overnight cultures are grown for the two fluorescent marker constructs. In step 3, 50 μl of each bacterial population is added to 1 ml untransformed BY-2 cells. Following the transformation, calli are screened for co-expression of the two fluorescent markers using a fluorescent microscope with adequate excitation and emission filters to discriminate the two fluorochromes.

BASIC PROTOCOL 5

PREPARATION OF BY-2 CELLS FOR FLUORESCENCE MICROSCOPY

BY-2 cells are generally mounted in half-strength agarized medium to prevent excessive movement of the cells and are maintained in the culture medium to limit cellular stress. For longer observation (up to several days) of immobilized suspension culture cells, a chamber covered with the gas-permeable foil bioFOLIE (Vivascience) can be used as an alternative to the agar medium (J.W. Vos and A.M.C. Emons, Plant Cell Biology, Wageningen University; pers. comm.). BioFOILE allows gas exchanges at the junction of the gas and liquid phase and gently immobilizes cells.

Materials

Solid BY-2 medium (see recipe), for short-term observation only
Wild-type or transformed BY-2 suspension culture (see Basic Protocol 1)
70% (v/v) ethanol, for long-term observation only
Valap: 1:1:1 (w/w/w) Vaseline/lanolin/paraffin (*UNIT 13.1*)

Electrical tape, cut in 0.5-cm-wide strips, for short-term observation only
76 × 26–mm glass slides and 51 × 20–mm O thickness coverslips, for short-term observation only
Microwave oven, for short-term observation only
Trimmed 200-µl pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile
76 × 51–mm glass slides with 18-mm hole in the center and 24 × 24–mm O thickness coverslips, for long-term observation only
50°C oven, for long-term observation only
Double-sided tape, for long-term observation only
Scalpel, for long-term observation only
2.5 × 2.5–cm piece of bioFOLIE film (Vivascience), for long-term observation only
Sterile tissues, for long-term observation only

For short-term observation:

1a. Place two 0.5-cm-wide strips of electrical tape on a clean 76 × 26–mm glass slide perpendicular to the long sides of the slide. Place the strips of tape ~10 mm apart.

The electrical tape will prevent excessive compression of the cells.

2a. Warm a small amount of solid BY-2 medium in a microwave oven until melted but not boiling. Place 100 µl medium on the glass slide between the strips of tape and allow to cool.

3a. At the critical point at which the medium solidifies, use a trimmed 200-µl pipet tip to transfer 50 to 100 µl wild-type or transformed BY-2 suspension culture to the medium. Use the pipet tip to gently mix the cells and medium.

4a. Place a 51 × 20–mm O thickness coverslip over the sample prior to observing at the microscope.

The cells will be held in place by the pressure of the coverslip.

Cells should be observed within up to 4 hr. Discard after observing; autoclave the slide before discarding if the cells are transformed.

For long-term observation:

1b. Clean a 76 × 51–mm glass slide with an 18-mm hole in the center and a 24 × 24–mm coverslip with soap and hot tap water, rinse in distilled water, and dry in an oven at 50°C.

2b. Place two 3-cm-long strips of double-sided tape on the slide, to cover the surface of the slide, including the hole.

3b. Use a scalpel to remove the tape covering the hole.

4b. Clean a 2.5 × 2.5–cm piece of bioFOLIE film in 70% ethanol and allow to air dry.

5b. Mount the bioFOLIE film on the tape on the glass slide and remove excess tape and foil.

The bioFOLIE film is mounted on the underside of the glass slide.

6b. Clean with soap and water, rinse with 70% ethanol, and air dry in a laminar flow hood.

7b. Working under sterile conditions, use a trimmed 200-µl pipet tip to transfer 20 µl of a wild-type or transformed BY-2 suspension culture onto the bioFOLIE in the hole of the glass slide.

8b. Cover the hole with the coverslip. Blot excess fluid with a sterile tissue while gently pressing on the coverslip.

9b. Seal the slide with Valap to prevent drying and infection.

Cells should be observed within 2 to 3 days. Store the slides at 25°C during observations. Discard slides, autoclaving first if the cells are transformed.

REAGENTS AND SOLUTIONS

Use ultrapure distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Carbenicillin, 100 mg/ml

Dissolve 100 mg/ml carbenicillin disodium (Melford Laboratories) in ultrapure water. Filter sterilize with a 0.2-µm filter and store up to 6 to 8 weeks at −20°C.

To use at 100 µg/ml, add 100 µl to 100 ml medium.

2,4-Dichlorophenoxyacetic acid (2,4-D), 1 mg/ml

Dissolve 1 mg 2,4-D in 1 ml 70% (v/v) ethanol. Store up to 6 to 8 weeks at 4°C.

Liquid BY-2 medium

Place 750 ml water in a 1-liter beaker and add 30 g sucrose (AnalaR grade, BDH Chemicals). Stir until dissolved. Add 4.3 g Murashige and Skoog medium (without sucrose, indole acetic acid, kinetin, agar; ICN Biomedicals), 200 µl 1 mg/ml 2,4-D (see recipe), and 3.4 µl 100 mg/ml KH_2PO_4 (potassium dihydrogen orthophosphate; AnalaR grade, BDH Chemicals). Adjust pH to 5.8 with 0.1 M KOH (not NaOH), make up to 1000 ml with water, and mix well. Pour into flasks, cover with foil and autoclave 15 min at 121°C. Allow to cool and store up to 2 weeks at 4° to 8°C.

The final concentration of KH_2PO_4 in BY-2 medium is 540 mg/liter. BY-2 cells have a high phosphate usage, greater than that supplied in Murashige and Skoog medium alone (Nagata et al., 1992).

Solid BY-2 medium

Add 1% (w/v) low-melting-temperature agar (Bacto Agar; Difco) to liquid BY-2 medium (see recipe) in suitable flask. Autoclave 15 min at 121°C and allow to cool until flask can be held for pouring. Pour medium into sterile petri dishes (~25 ml/9-cm dish) in laminar flow hood. Allow plates to cool and store at 4° to 8°C.

If working with stable transformants, antibiotics should be added before pouring, and the flask should be swirled to mix them into the medium.

Timentin, 20 mg/ml

Dissolve 20 mg/ml Timentin (tricarcillin disodium salt/potassium clavunculate; Melford Laboratories) in ultrapure water. Filter sterilize with a 0.2-µm filter and store up to 6 to 8 weeks at −20°C.

An alternative to Timentin is ticarcillin/potassium clavulanate.

To use at 20 µg/ml, add 100 µl/100 ml medium.

YEB medium

In a 1-liter beaker dissolve 5 g beef extract (Difco), 1 g yeast extract (Merck), 5 g peptone (Difco), 5 g sucrose (BDH Laboratory), and 0.5 g $MgSO_4 \cdot 7H_2O$ in 0.8 liter water. Add water, while stirring continuously, up to 1 liter. Autoclave and store up to 4 to 6 weeks at room temperature.

The components may take some time to dissolve completely.

COMMENTARY

Background Information

The reader may find different protocols for BY-2 cell culture and transformation being used in different laboratories. This is because researchers tend to optimize a general protocol based on their experience with handling BY-2 cell lines.

The medium used for the same cell type sometimes differs between labs as a result of different working practices and preferences. Different suspension plant cell types have differing medium requirements. For example in the authors' experience carrot suspension cells grow optimally in M and S media supplemented with sucrose, coconut milk, zeatine, and 2,4-dichloro-phenoxyacetic acid (2,4-D), in comparison to BY-2 cells which require the addition of sucrose, KH_2PO_4, and 2,4-D only. In turn, *Arabidopsis* suspension cells proliferate in M and S supplemented with sucrose, NAA (naphthaleneacetic acid), and kinetin. The cells are all grown at the same temperature, with the same light and shaking regimes and are subcultured every 7 days.

There are several factors that have to be taken into consideration when working with transformed BY-2 cells. The expression of a fluorescent construct may vary among calli from the same transformation. Mistargeting of a protein may well be due to overexpression. Therefore, transgenic calli should be analyzed with a fluorescence microscope to ascertain the fidelity of the expression pattern. The authors have also found that levels of fluorescence of transformed BY-2 cells of a single line and patterns of expression may change after repeated passages. This may be due to aging of the culture, degradation of the fluorescent construct, and/or gene silencing.

Critical Parameters

The efficiency in obtaining transgenic calli may vary between experiments. This may be related to the quality of the BY-2 cells. In particular, cells that are in M and early G1 phase are more susceptible to transformation via *A. tumefaciens* than cells residing in G2 (Geelen and Inzé, 2001). Similarly, the strain of *Agrobacterium* used may affect the yield. For example, the *Agrobacterium* strain LBA4404, which constitutively expresses the *vir*G gene (van der Fits et al., 2000), has been found to be superior in generating transgenic calli (Geelen and Inzé, 2001).

Contamination of cultures may easily occur even if appropriate sterile procedures are followed. Therefore, to avoid wasted time and effort, it is preferable to work in duplicate or, in certain situations, in triplicate. This is to ensure that there is a backup stock of sterile material should infection occur at any point. The authors found that when routinely working with suspensions and calli (for the weekly and monthly passages, respectively), one should work in duplicate, but, when initiating a suspension culture, the calli should be passaged in triplicate. This is illustrated in Figure 1.7.3. When passaging calli and suspension cultures, the operator should discard infected plates. At each passage, new subcultures should be generated from one plate or flask only. It is advisable to retain the uninfected and unopened plates or flasks until the next passage to ensure a backup of material should contamination occur.

Once transformed lines are established, they can be passaged in a 7- to 14-day cycle. It is advisable, however, to maintain the line as calli on solid medium so that if expression levels decrease with time, a new suspension can be established. It is also the authors' experience that with some transformed lines, correct targeting of constructs is lost after three to four passages, and regular renewal from calli is then necessary (Fig. 1.7.3).

Troubleshooting

General rules for troubleshooting that apply in culturing and transforming of any plant cell line (Evans et al., 2003) apply for BY-2 cells.

In general, to enhance the possibility of a successful transformation, the operator should produce at least three initial plates of mixed agrobacteria and BY-2 cells.

There may be an absence of fluorescence in the transformed BY-2 cells. This may be due to several factors, including gene silencing, toxicity of the construct, mutation in the sequence of the fluorescent protein, degradation of gene product, or even misuse of the selectable markers. When cells are stably transformed via *Agrobacterium*, the DNA is integrated into the genome, hence resulting in an absence of a defined position effect. This may lead to silencing. The fidelity of a construct should always be checked by transient expression before attempting any stable transformation. Very often constructs may prove toxic to cells so that only lines with low levels of expression survive.

The gene product may not be tolerated by the cells and may be degraded. Nonspecific overproliferation of untransformed cells in calli may also occur. This may arise because of an impairment of antibiotic activity, for instance by temperature, light, or pH inactivation of the selective marker.

Anticipated Results

Transformation and culturing of BY-2 cells is a straightforward approach to set up an in vivo system to analyze cellular dynamics. This system is particularly well suited for investigation into cell cycle progression.

BY-2 cells are present in variable shapes from isodiametric to elongated and as single cells or concatamers (Fig. 1.7.4A). The large nucleus is suspended in a highly vacuolated cytoplasm. Numerous plastids are present in these cells, but they are not autofluorescent.

A variety of cellular organelles have been highlighted by in vivo fluorescent protein markers thanks to the ease of transformation of BY-2 cell and their ability to tolerate heterologous constructs based on green fluorescent protein (GFP) and its fluorescent derivatives (Table 1.7.1), including the actin cytoskeleton (Kost et al., 1998), microtubules (Granger and Cyr, 2000; Kumagai et al., 2001), and the Golgi (Nebenführ et al., 1999; Saint-Jore et al., 2002).

Figure 1.7.4B shows the endoplasmic reticulum (ER) of BY-2 cells stably expressing the yellow fluorescent protein targeted to the ER by an N-terminal sporamin signal peptide and retained by means of a C-terminal tetrapeptide, HDEL (SpYFP-HDEL). The Golgi apparatus can be visualized with a rat sialyl transferase membrane-anchoring domain GFP construct (Fig. 1.7.4C; Saint-Jore et al., 2002), and the nuclear envelope by the N-terminal domain of a mammalian lamin B receptor–GFP fusion (Fig. 1.7.4D; Irons et al., 2003). Chromatin can be visualized by a histone-YFP fusion (Fig. 1.7.4E, G; Boisnard-Lorig et al., 2001), which allows the visualization of chromosome dynamics in interphase and mitotic cells.

BY-2 cells are also an amenable system for stable co-expression of two constructs (Fig. 1.7.4F, G). Thus BY-2 cells may be used to express a double combination of fluorochromes for the analysis of organelle-organelle and protein-protein interactions.

Time Considerations

As shown in Figure 1.7.1B, production of stable cell lines of BY-2 cells may take about 4 months. Detection of fluorescence in calli may be possible after 1 month from transformation with agrobacteria. This may be useful to distinguish fluorescent calli from nonfluorescent ones and to propagate the former.

After inoculation of liquid medium with callus, it is advisable to let the culture establish for a minimum of 1 week before microscopy. This gives time for cells to divide and separate from clumps of callus.

Literature Cited

An, G. 1985. High-efficiency transformation of cultured tobacco cells. *Plant Physiol.* 79:568-570.

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, W., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J., and Berger, F. 2001. Dynamic analyses of the expression of the HISTONE::YFP fusion protein in arabidopsis show that syncytial endosperm is divided in mitotic domains. *Plant Cell* 13:495-509.

Brandizzi, F., Fricker, M., and Hawes, C. 2002. A greener world: The revolution of plant bioimaging. *Nature Mol. Cell Biol.* 3:520-530.

Criqui, M.C., Parmentier, Y., Derevier, A., Shen, W.H., Dong, A., and Genschik, P. 2000. Cell cycle-dependent proteolysis and ectopic overexpression of cyclin B1 in tobacco BY2 cells. *Plant J.* 24:763-773.

Criqui, M.C., Weingartner, M., Capron, A., Parmentier, Y., Shen, W.H., Heberle-Bors, E., Bogre, L., and Genschik, P. 2001. Sub-cellular localization of GFP-tagged tobacco mitotic cyclins during the cell cycle and after spindle checkpoint activation. *Plant J.* 28:569-581.

David, K.M. and Perrot-Rechenmann, C. 2001. Characterization of a tobacco Bright Yellow 2 cell line expressing the tetracycline repressor at a high level for strict regulation of transgene expression. *Plant Physiol.* 125:1548-1553.

Dixit, R. and Cyr, R.J. 2002. Spatio-temporal relationship between nuclear-envelope breakdown and preprophase band disapearance in cultured tobacco cells. *Protoplasma* 219:116-121.

Evans, D.E., Coleman, J., and Kearns, A. 2003. Plant Cell Culture—The Basics. BIOS Scientific Publishers, Oxford.

Forreiter, C., Kirschner, M., and Nover, L. 1997. Stable transformation of an arabidopsis cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity in vivo. *Plant Cell* 9:2171-2181.

Geelen, D.N.V. and Inzé, D.G. 2001. A bright future for the Bright Yellow-2 cell culture. *Plant Physiol*. 127:1375-1379.

Geelen, D., Leyman, B., Batoko, H., Di Sansebastiano, G.P., Moore, I., and Blatt, M.R. 2002. The abscisic acid-related SNARE homolog NtSyr1 contributes to secretion and growth: Evidence from competition with its cytosolic domain. *Plant Cell* 14:387-406.

Granger, C.L. and Cyr, R.J. 2000. Microtubule reorganization in tobacco BY-2 cells stably expressing GFP-MBD. *Planta* 210:502-509.

Haseloff, J., Siemering, K.R., Prasher, D.C., and Hodge, S. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. U.S.A.* 94: 2122-2127.

Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H., and Mano, Y. 1978. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-alpha. *Nature* 275: 458-460.

Irons, S., Evans, D.E., and Brandizzi, F. 2003. The first 238 amino acids of the human lamin B receptor are targeted to the nuclear envelope in plants. *J. Exp. Bot.* 54:943-950.

Koncz, C. and Schell, J. 1986. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of agrobacterium binary vector. *Mol. Gen. Genet.* 204:383-396.

Koscianska, E. and Wypijewski, K. 2001. Electroporated intact BY-2 tobacco culture cells as a model of transient expression study. *Acta Biochim. Pol*. 48:657-661.

Kost, B., Spielhofer, P., and Chua, N.H. 1998. A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes. *Plant J.* 16:393-401.

Kumagai, F., Yoneda, A., Tomida, T., Sano, T., Nagata, T., and Hasezawa, S. 2001. Fate of nascent microtubules organized at the M/G1 interface, as visualized by synchronized tobacco BY-2 cells stably expressing GFP-tubulin: Time-sequence observations of the reorganization of cortical microtubules in living plant cells. *Plant Cell Physiol.* 42:723-732.

Menges, M. and Murray, J.A.H. 2002. Synchronous *Arabidopsis* suspension cultures for analysis of cell-cycle gene activity. *Plant J.* 30:203-212.

Nagata, T., Nemoto, Y., and Hasezawa, S. 1992. Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. *Int. Rev. Cytol*. 132:1-30.

Nebenführ, A., Gallagher, L.A., Dunahay, T.G., Frohlick, J.A., Mazurkiewicz, A.M., Meehl, J.B., and Staehelin, L.A. 1999. Stop-and-go movements of plant Golgi stacks are mediated by the acto-myosin system. *Plant Physiol.* 121:1127-1141.

Nebenführ, A., Frohlick, J.A., and Staehelin, L.A. 2000. Redistribution of Golgi stacks and other organelles during mitosis and cytokinesis in plant cells. *Plant Physiol*. 124:135-151.

Nishihama, R., Ishikawa, M., Araki, S., Soyano, T., Asada, T., and Machida, Y. 2001. The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. *Genes Dev.* 15:352-363.

Rose, A. and Meier, I. 2001. A domain unique to plant RanGAP is responsible for its targeting to the plant nuclear rim. *Proc. Natl. Acad. Sci. U.S.A.* 98:15377-15382.

Saint-Jore, C.M., Evins, J., Brandizzi, F., Batoko, H., Moore, I., and Hawes, C. 2002. Redistribution of membrane proteins between the Golgi apparatus and the endoplasmic reticulum in plants is reversible and not dependent on cytoskeleton networks. *Plant J.* 29: 661-678.

Shinmyo, A., Shoji, T., Bando, E., Nagaya, S., Nakai, Y., Kato, K., Sekine, M., and Yoshida, K. 1998. Metabolic engineering of cultured tobacco cells. *Biotechnol. Bioeng.* 58:329-332.

van der Fits, L., Deakin, E.A., Hoge, J.H.C., and Memelink, J. 2000. The ternary transformation system: Constitutive virG on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* 43:495-502.

Yoshida, K., Kasai, T., Garcia, M.R.C., Sawada, S., Shoji, T., Shimizu, S., Yamazaki, K., Komeda, Y., and Shinmyo, A. 1995. Heat-inducible expression system for a foreign gene in cultured tobacco cells using the HSP18.2 promoter of *Arabidopsis thaliana*. *Appl. Microbiol. Biotechnol*. 44:466-472.

Zuo, J., Niu, Q.W., Nishizawa, N., Wu, Y., Kost, B., and Chua, N.H. 2000. KORRIGAN, an *Arabidopsis* endo-1,4-β-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. *Plant Cell* 12:1137-1152.

Key References

Geelen and Inze, 2001. See above.

Reviews the applications of BY-2 cells in plant cell biology studies. Includes a comparison of Arabidopsis and BY-2 cells as model plant cells.

Kodama and Komamine, 1995. Synchronization of cell cultures of higher plants. *Methods Cell Biol*. 49:315-329.

Summarizes the techniques used in synchronization in different plant cultures including determination

of cell number, mitotic index, and percentage of living cells.

Nagata et al., 1992. See above.

Comprehensively reviews the history, growth, characteristics, and applications of BY-2 cells.

Internet Resources

http://www.bch.msu.edu/pamgreen/bv2.htm

Describes useful hints for transforming BY-2 cells and culturing.

http://botany1.bio.utk.edu/cellbiol/default.htm

Illustrates GFP fluorescent BY-2 cells, with most of the images from BY-2 cells expressing a GmMan1-GFP fusion protein to label Golgi stacks. Also shows combinations with other fluorochromes to simultaneously highlight other cellular components in interphase and cell division and in the presence of different test compounds.

Contributed by Federica Brandizzi,
Sarah Irons, Anne Kearns, and
Chris Hawes
Oxford Brookes University
Oxford, United Kingdom

CHAPTER 2
Preparation and Isolation of Cells

INTRODUCTION

Chapter 1 describes the techniques of cell culture, but, of course, to culture cells one must have cells. Many cell biologists, if asked where cells come from, would probably give the American Type Culture Collection (ATCC) as their answer. As important as such repositories are as a resource, cells really come from organisms, and Chapter 2 deals with the preparation and isolation of cells from their true source.

As indicated in the introduction to Chapter 1, 1998 marked the golden anniversary of the first continuous mammalian cell line. That line was the L cell fibroblast line established from a mouse tissue explant by Sanford et al. in 1948 (*J. Natl. Cancer Inst.* 9:229-246). Fibroblast lines continue to be important now, half a century after the establishment of the L cell line. Fibroblasts can be obtained from humans without sacrificing the individual (human skin fibroblasts are indeed available from the ATCC), and fibroblast cultures established from patients with inherited disorders have often been used to identify genetic abnormalities. Skin fibroblasts are also instrumental in characterization of functional abnormalities in transgenic or knockout animals. The methodologies of preparing and maintaining skin fibroblasts are described in *UNIT 2.1*.

Blood represents a "renewable resource" for obtaining human cells. *UNIT 2.2* describes procedures for the isolation and maintenance of human lymphocytes from peripheral blood. Relatively large numbers of these cells (1 to 2 million per milliliter of blood) can be isolated by techniques that are moderately simple and inexpensive. In addition to the basic protocol for lymphocyte preparation by density-gradient centrifugation, protocols by which various subfractions of the lymphocyte population can be separated are provided. Monocytes/macrophages are isolated (or depleted) by utilizing their tendency to adhere to plastic. Monoclonal antibodies recognizing the specific surface molecules are utilized together with magnetic beads to select positively or negatively for populations of T cells or B cells. These cells can also be used to generate continuously growing B cell lines via transformation with Epstein Barr virus.

In *UNIT 2.3*, we move from blood to the blood vessels, from which endothelial cells can be isolated. Endothelial cells have been used extensively to explore cell-cell and cell-matrix interactions. Endothelial cell function (or dysfunction) participates in pathological processes as diverse as coronary artery disease and tumor invasion, as well as in the inflammatory response. The expression of surface proteins and secretion of soluble mediators by the endothelium controls vascular tone and permeability, regulates coagulation and thrombosis, and directs the passage of leukocytes into areas of inflammation. Endothelial cells play a central role in angiogenesis, the formation of new blood vessels that is necessary for tumor growth. As such, endothelial cells are being extensively studied in the quest for antiangiogenic agents that might be used in cancer therapy. A basic protocol for the preparation of endothelial cells from human umbilical vein is provided in *UNIT 2.3*, along with alternative protocols for isolation of endothelial cells from retroperitoneal adipose tissue, nasal mucosa, and human foreskins.

UNIT 2.4 provides guidance for preparing immortalized B cell lines via transformation by Epstein-Barr virus. This methodology allows for the establishment and maintenance of

Contributed by Joe B. Harford
Current Protocols in Cell Biology (2006) 2.0.1-2.0.2

cell lines derived from resting human B lymphocytes obtained from blood samples, including those obtained from individuals with genetic changes leading to disease conditions (e.g., sickle cell anemia and cystic fibrosis).

Animal tissues are heterogenous because they are composed of mixtures of cell types. Analysis of the functions of these cells necessitates obtaining pure samples of the cells of interest. One recent approach to the daunting task of isolating homogenous morphologically identified cell populations is laser capture microscopy (LCM). The technique as described in *UNIT 2.5* is based on selective adherence of visually identified cells within a tissue section to a plastic membrane following activation by low-energy infrared laser. Although initially developed at the National Cancer Institute for separation of malignant or premalignant cells from surrounding normal tissue, LCM is being applied to a wide variety of cell types. Hundreds of papers have already appeared in the literature wherein DNA, RNA, and/or proteins have been extracted and analyzed from laser-captured cells.

UNIT 2.6 describes a method for establishing cultures of human keratinocytes. Although the source for the protocol described is human newborn foreskin from circumcision, the method may also be used to prepare keratinocyte cultures from skin biopsies, from other surgical procedures, or from cadavers.

The interaction between neurons and glial cells represents an important but complex aspect of brain development and function. In *UNIT 2.7*, protocols are provided for the isolation and coculture of these cell types. The methods described include procedures for a coculture system (sandwich coculture) in which the cells are maintained in the same culture dish but are not in physical contact with one another. This arrangement allows diffusion between the cell types of soluble factors that mediate the interplay between the two cocultured cell types.

In future supplements to Chapter 2, protocols for the isolation of additional cell types will be provided.

Joe B. Harford

Establishment of Fibroblast Cultures

This unit describes methods for establishing fibroblast cultures from skin. Because fibroblasts can be expanded to relatively large numbers from a small skin sample, they have been widely used to study basic aspects of cell biology as well as genomic, biochemical, and/or functional abnormalities in human patients and in transgenic or knockout animals. Two protocols are described for the development of fibroblast lines from human and mouse skin samples; the same protocols are also applicable, with slight modification, to other animals including rats and rabbits. The first technique (see Basic Protocol) employs an "skin explant" culture system in which fibroblasts grow out of skin specimens. The second technique (see Alternate Protocol) employs a dissociated fibroblast culture system in which fibroblasts are first released from skin specimens by enzymatic digestion and then placed in culture. In general, the explant culture system is technically simpler, requiring almost no special experience or reagents, whereas the dissociated fibroblast culture is more suitable for obtaining relatively large numbers of fibroblasts in a short period.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly. Surgical equipment may be sterilized by simply soaking in 70% ethanol; however, it is important to rinse in PBS before use since ethanol will "fix" the tissue.

SKIN EXPLANT CULTURE

BASIC PROTOCOL

When skin specimens are "transplanted" onto culture plates, fibroblasts (in dermis) and keratinocytes (in epidermis) migrate over the plastic surfaces, as they do in an ordinary skin graft. Because fibroblasts will eventually overgrow keratinocytes in conventional culture media, relatively pure fibroblast cultures can be obtained by simply placing small pieces of skin on tissue culture dishes. On the other hand, the optional enzymatic separation of the epidermis will ensure the absence of epidermal components from the resulting fibroblast cultures; it will also allow investigators to establish keratinocyte cultures from the same skin samples.

Materials

Skin specimen (see Commentary)
Phosphate-buffered saline (PBS; see recipe)
0.5% (w/v) dispase II (Boehringer-Mannheim) in PBS (store up to 3 months at −20°C; optional)
0.3% (w/v) trypsin (from bovine pancreas; Sigma) in PBS (store up to 3 months at −20°C; optional)
Complete growth medium (DMEM or RPMI; see recipe)
Trypsin/EDTA solution (see recipe)
0.4% (w/v) trypan blue in PBS (store up to 6 months at room temperature)
Freezing medium: 10% DMSO/90% FBS or 10% DMSO/90% complete DMEM

15-ml and 50-ml polypropylene centrifuge tubes
Lids from 100-mm tissue culture dishes
Eye forceps (2 pairs)
Surgical scalpel with disposable no. 22 blade

Contributed by Akira Takashima
Current Protocols in Cell Biology (1998) 2.1.1-2.1.12

35-mm tissue culture dishes or 6-well plates
22-mm glass coverslips (wrap several coverslips in aluminum foil and sterilize by autoclaving)
Hemacytometer
25-cm^2 tissue culture flasks
1.5-ml cryotubes (e.g., Nunc)
Liquid nitrogen freezer

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: Use Milli-Q water or equivalent in all protocol steps and for preparing all solutions.

Prepare skin sample

1. Wash skin samples in PBS by gently shaking or agitating in a 50-ml polypropylene centrifuge tube.

   When handling skin samples from mice, rats, or rabbits, it is easier to remove hair before excision. This can be achieved by applying 70% ethanol on skin and shaving with a single-edged razor blade (e.g., Personna Prep).

2a. *For relatively large skin samples (e.g., foreskin, surgically removed samples, or cadaver skin):* Place sample on the lid of a 100-mm tissue culture dish and spread it out with the epidermal side down. Remove the subcutaneous tissue by scraping the dermal side using two pairs of eye forceps. Cut skin into strips of ~0.5-cm width using a surgical scalpel.

2b. *For smaller skin samples (e.g., punch-biopsy samples):* Place the sample on a 100-mm tissue culture dish and excise the subcutaneous tissue using a surgical scalpel and a pair of forceps.

Remove epidermis (optional)

3a. *For human skin samples:* Incubate 45 min to 4 hr with 0.5% dispase/PBS in a 37°C water bath, then separate as an intact sheet mechanically by gentle agitation for 10 sec or by using two pairs of forceps.

   The incubation time required for epidermal separation varies depending upon the thickness of the skin specimen and the completeness of the subcutaneous tissue removal.

3b. *For mouse, rat, and rabbit skin samples (optional):* Incubate with 0.3% trypsin/PBS for 30 to 60 min in a 37°C water bath or overnight at 4°C. Place the sample on the lid of a 100-mm tissue culture dish with the epidermal side up and scrape off the epidermis mechanically using two pairs of forceps.

   In general, trypsin works better than dispase for skin with deep hair follicles.

4. Wash the dermal sample in PBS by gently shaking or agitating in a 50-ml polypropylene centrifuge tube.

Culture fibroblasts

5. Place the dermal sample on the lid of a 100-mm tissue culture dish and cut it into small (2- to 3-mm) squares.

   Fibroblast outgrowth occurs only from sharply cut edges. Thus, it is crucial to use a new, fine surgical scalpel; disposable surgical blades (no. 22) can be used for this purpose.

6. Place 5 to 10 skin pieces in the center of a 35-mm tissue culture dish or 6-well plate.

   The 6-well plates are more convenient for handling multiple fibroblast cultures in parallel.

7. Place a sterile 22-mm glass coverslip gently over the skin specimens (Fig. 2.1.1).

   Skin specimens need to be attached physically to the plate. This can be achieved most effectively by making a sandwich using a coverslip (Fig. 2.1.1). The coverslip also assists the growth of fibroblasts by maintaining the microenvironment.

8. Add a few drops of 4°C complete growth medium (DMEM or RPMI) into the space below the coverslip (by applying at the edge of the coverslip so that it is drawn under), then add 1 to 2 ml of 4°C complete medium to the dish or well, gently so as to avoid disturbing the skin specimens.

   Either complete DMEM or complete RPMI can be used for growing fibroblasts.

9. Place the culture in a humidified 37°C, 5% CO_2 incubator. Check the fibroblast outgrowth every 3 to 4 days under an inverted phase-contrast microscope and change medium every 3 to 4 days, taking care not to agitate the coverslip.

   Initial outgrowth should be detectable within 3 to 4 days (Fig. 2.1.2B). The coverslip should be left on the skin specimens until the culture becomes confluent. The original skin specimens may come off the plate spontaneously during culture and will be removed in step 10.

10. Upon confluency, remove the coverslips and wash the dish or wells twice with 4°C PBS.

    Fibroblasts sometimes migrate over the surface of the coverslip instead of the tissue culture plate. If this is the case (as judged by focusing up and down with the microscope) transfer the coverslip into a new dish, wash it with PBS as in this step, then harvest cells by trypsin treatment as in step 11.

    Washing with PBS is essential because trypsin (see step 11) does not work effectively in the presence of the FBS present in the culture medium.

    In the absence of epidermal separation, the cultures are often contaminated with outgrowing keratinocytes, which are readily distinguishable by their cobblestone-like appearance. Contaminating keratinocytes can be removed selectively by incubating 10 to 30 min with 0.5% dispase/PBS, because keratinocytes are much more sensitive than fibroblasts to this treatment. Keratinocytes will be detached, leaving the majority of fibroblasts attached to the plate.

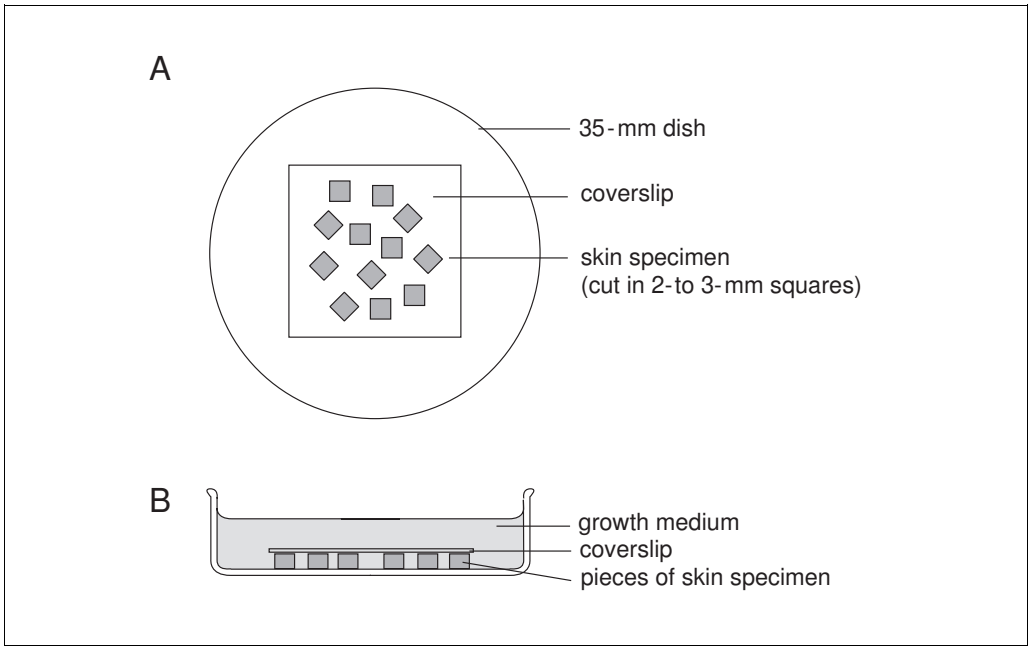


Figure 2.1.1 Skin explant culture: (**A**) top view; (**B**) side view.

11. Add 1 ml of 4°C trypsin/EDTA solution and incubate 3 to 5 min at room temperature while examining periodically under the inverted phase-contrast microscope. As soon as the fibroblasts round up, add 1 ml ice-cold complete growth medium (DMEM or complete RPMI) to inactivate trypsin and harvest cells by gentle pipetting.

    Avoid trypsinization for excessive periods of time and vigorous pipetting, which are the two common causes of the "low viability" problem (see Troubleshooting). Do not pipet the cells before the trypsin has been diluted by addition of complete culture medium.

    Harvest fibroblasts before they become overconfluent and form bundle-like alignments (Fig. 2.1.3B).

    The original skin specimens may be removed using forceps.

12. Collect fibroblast suspensions into a 15-ml polypropylene centrifuge tube and centrifuge 10 min at $150 \times g$, 4°C. Aspirate the supernatant, tap the pellet to dissociate the cells, and resuspend them in 100 to 200 µl of fresh 4°C complete growth medium.

13. Mix a 10- to 20-µl sample of the cell suspension with an equal volume of 0.4% trypan blue/PBS, then count total and viable cells under a microscope using a hemacytometer.

    The cell viability as measured by trypan blue exclusion should be >90%.

14. Plate $3–10 \times 10^4$ viable cells in 5 ml of fresh complete growth medium in a 25-cm^2 tissue culture flask.

    The cells will attach to a new flask within 2 to 3 hr and begin to exhibit the characteristic spindle shape in 24 hr.

    Contamination occurs less frequently in flasks than in dishes or multiwell plates

15. Change medium every 3 to 4 days until the culture becomes confluent. Harvest fibroblasts as described in steps 10 and 11.

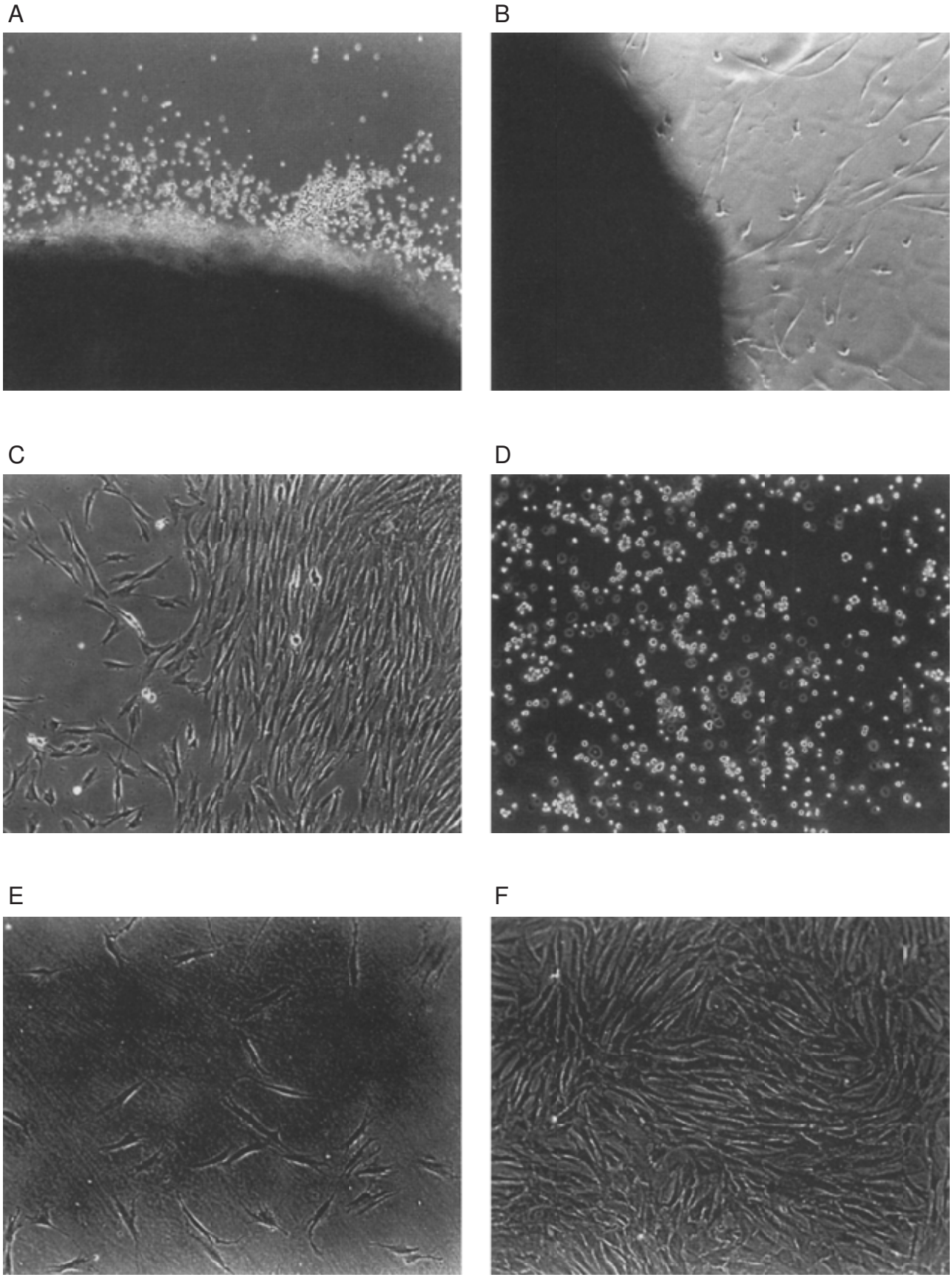
    See Figure 2.1.4 for the typical growth rate of human fibroblasts; their doubling time varies from 24 to 72 hr, depending upon the culture conditions. Human fibroblasts can be passaged up to 10 times without significant changes in morphology or growth rates. It is recommended, however, that frozen stocks be prepared after the second or third passage (see step 16).

    Maximal fibroblast growth requires 5% to 10% FBS (Fig. 2.1.5A). It is technically challenging to grow fibroblasts in the absence of added serum. On the other hand, there is minimal, if any, variation among FBS batches purchased from different vendors in their capacity to promote the growth of human fibroblasts (Fig. 2.1.5B). Human fibroblasts also grow well in the presence of heat-inactivated human serum, which may be used instead of FBS (Fig. 2.1.5C).

16. To freeze cells, resuspend in ice-cold 10% DMSO/90% FBS or 10% DMSO/90% complete DMEM at $0.3–1 \times 10^6$ cells/ml. Dispense into 1.5-ml cryotubes at 1 ml/tube and freeze first at −20°C, then move on to −80°C, and finally place in liquid nitrogen.

    The −20°C and −80°C freezing steps may be performed in a styrofoam box to promote a gradual drop in temperature. Commercially available cell-freezing instruments may also be used for this purpose.

Figure 2.1.2 *(at right)* Microscopic appearance (magnification, 40×) of fibroblast cultures established from a newborn foreskin sample using the skin explant culture system (panels A to C) or the cell dissociation culture system (panels D to F). (**A**) Skin explant, day 1; (**B**) skin explant, day 5; (**C**) skin explant, day 14. (**D**) Dissociation culture, day 1; (**E**) dissociation culture, day 5; (**F**) dissociation culture, day 14. Note that fibroblasts migrate out from the edge of a skin specimen (panel B) and become confluent, except for the area where the original skin specimen was located (panel C). In dissociated-cell cultures, fibroblasts attach (panel D), spread on culture plates (panel E), and become confluent (panel F).


A
B
C
D
E
F

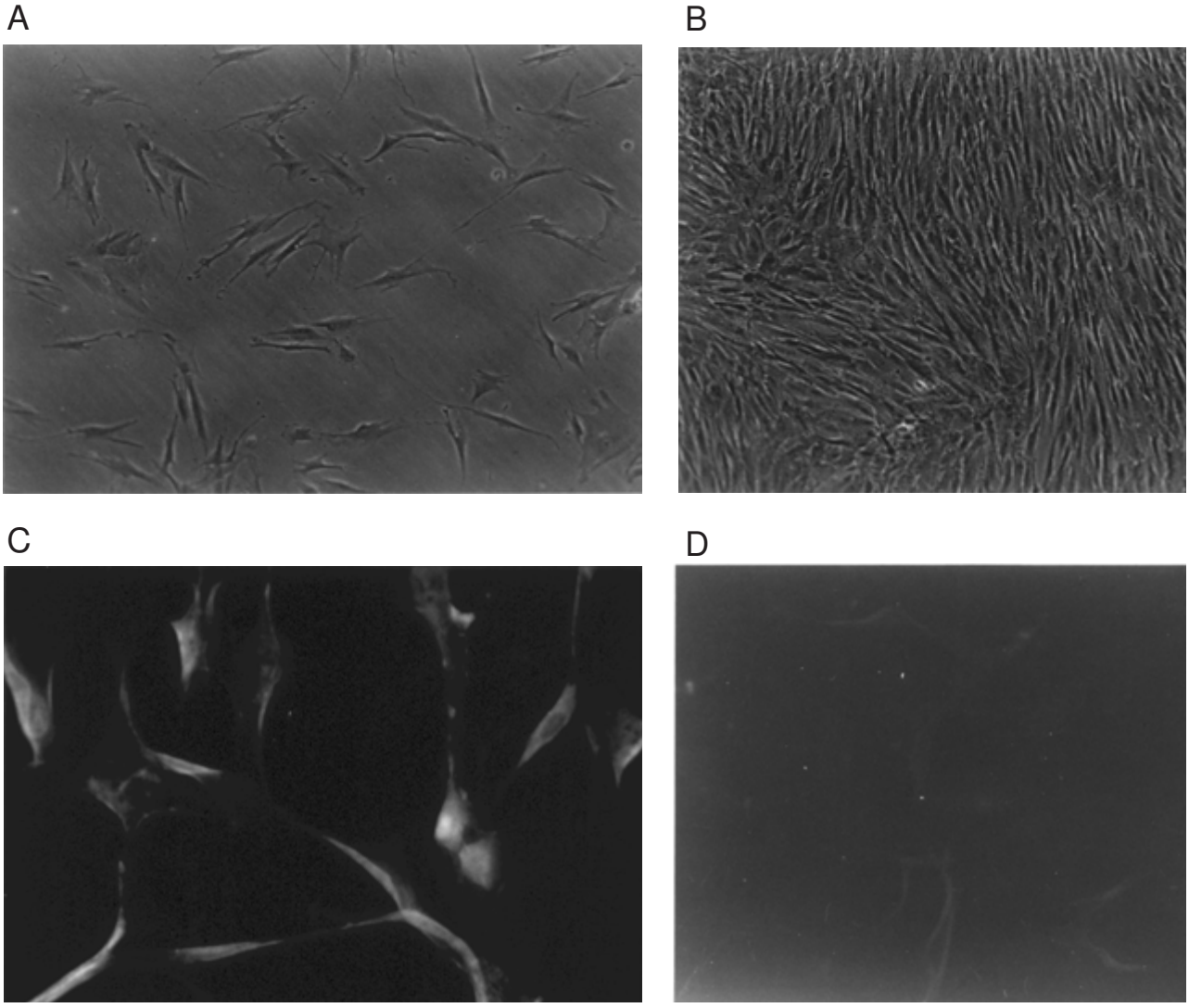


Figure 2.1.3 Identification of fibroblasts. Fibroblasts change their morphology depending upon the extent of confluency (or cell density). (**A**) Morphology at low density (10% to 20% confluence; magnification, 40×); (**B**) morphology at high density (100% confluence; magnification, 40×). (**C**) Indirect immunofluorescence staining of human fibroblast cultures with antibodies against type I collagen (magnification, 100×). (**D**) Indirect immunofluorescence staining of human fibroblast cultures with control antibodies (magnification, 100×). Briefly, fibroblasts were cultured for 2 days on LabTek chamber slides, fixed in 3% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and then subjected to immunofluorescence staining (*UNIT 4.3*) with rabbit anti-type I collagen (Chemicon), followed by labeling with FITC-conjugated anti-rabbit IgG (Jackson Immunoresearch).

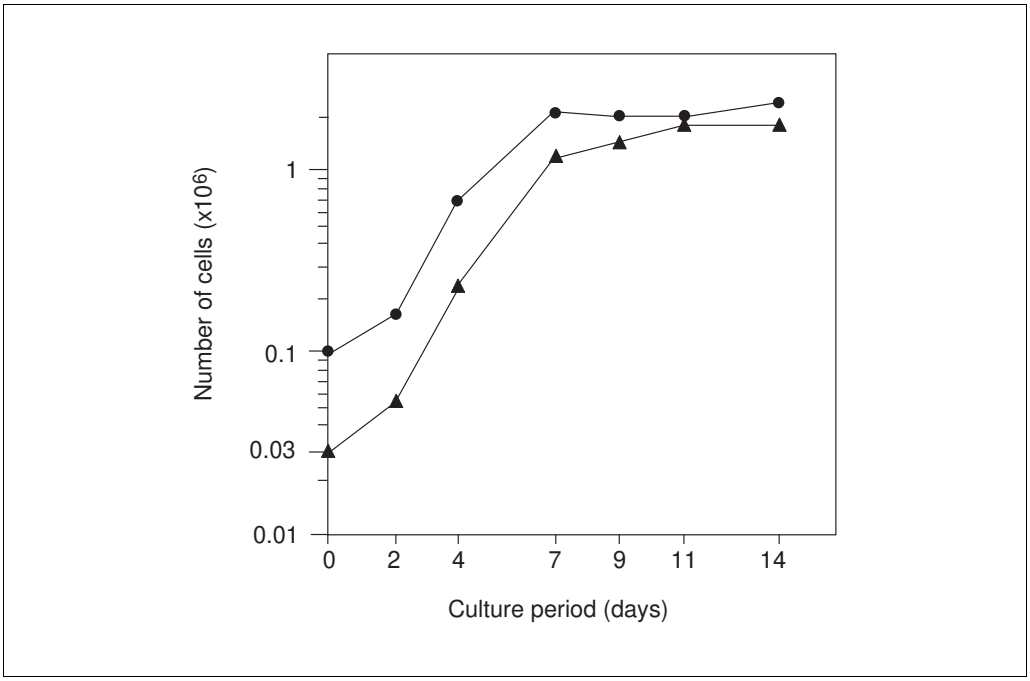


Figure 2.1.4 Fibroblast growth curve. A third passage of human fibroblast culture was plated on 35-mm dishes at either 30,000 (triangles) or 100,000 (circles) cells per dish, and cultured in complete RPMI. At the indicated time points, cultures were harvested by incubation with 0.3% trypsin/25 mM EDTA and counted to determine cell number. Note that cells grow relatively rapidly, with an approximate doubling time of 24 hr, and then stop dividing as they reach confluency.

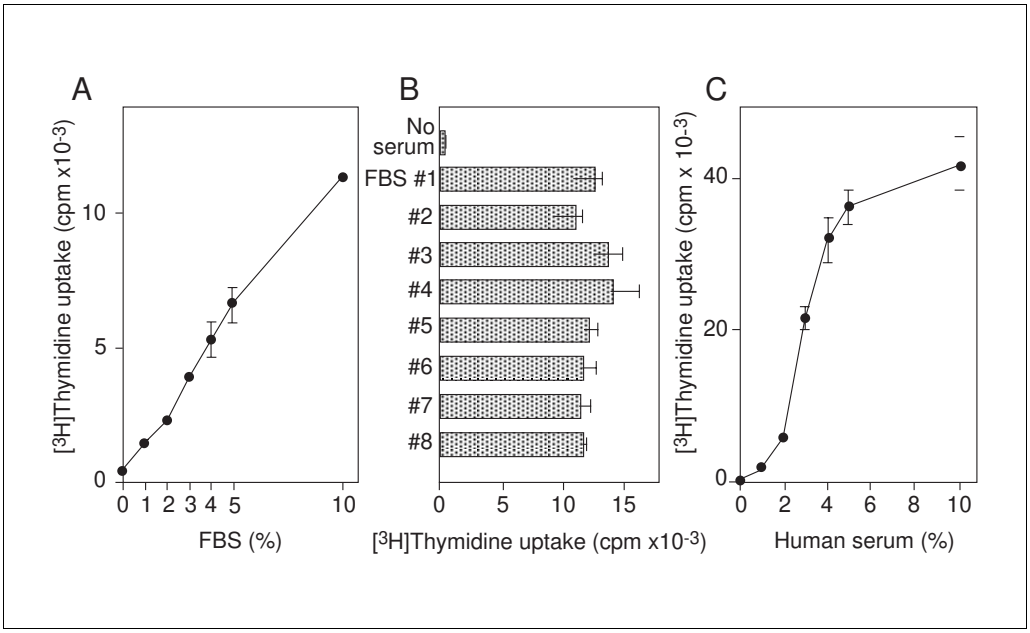


Figure 2.1.5 Serum requirement for fibroblast growth. A third passage of human fibroblasts (3000 cells/well) was cultured in flat-bottom 96-well plates, pulsed with [^{3}H]thymidine on day 3, and harvested on day 4 using an automated cell harvester. The culture media were supplemented with (**A**) different concentrations of FBS; (**B**) 10% FBS from different vendors; and (**C**) different concentrations of human serum. Data shown are the means and standard deviations from triplicate cultures.

ALTERNATE PROTOCOL

DISSOCIATED FIBROBLAST CULTURE

Although more complicated than the skin explant culture (see Basic Protocol), the dissociated fibroblast culture described in this protocol is more suitable for those experiments that require relatively large numbers of fibroblasts. After removal of the epidermis by dispase treatment, fibroblasts are released from the remaining dermis by enzymatic treatment with trypsin. The resulting dermal cells are then plated in suspension onto tissue culture plates. The most tricky step is the enzymatic digestion of dermal tissues. Investigators may need to compare various conditions (e.g., batches of trypsin, trypsin concentrations, and incubation periods) to maximize the cell yield while maintaining the cell viability. Alternatively, collagenase, which is less cytotoxic than trypsin, can be used for the same purpose.

Additional Materials *(also see Basic Protocol)*

1000 U/ml collagenase type IA in PBS (see recipe for PBS; store enzyme solution up to 3 months at −20°C)
Nylon mesh (85-µm mesh; Tetko; cut into 5-cm square, wrap in aluminum foil, and sterilize by autoclaving)

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: Use Milli-Q water or equivalent in all protocol steps and for preparing all solutions.

Prepare dissociated cell suspension

1. Wash skin samples in PBS, remove the subcutaneous tissues, remove the epidermis by enzymatic digestion, wash the dermal sample in PBS, and cut the sample into small squares (see Basic Protocol, steps 1 to 5).

   Since the trypsin that is used to digest the dermal connective tissue also dissociates epidermal cells, the epidermis must first be separated from dermal layer. Otherwise, the resulting cultures will be heavily contaminated by epidermal keratinocytes.

2. Place 10 to 20 dermal pieces in a 15-ml polypropylene tube with 3 ml of 0.3% trypsin/PBS and incubate 10 min in a 37°C water bath, inverting the tube several times every 2 to 3 min. Alternatively, incubate 10 to 20 dermal pieces 1 to 2 hr with 3 ml of 1000 U/ml collagenase at 37°C, agitating every 20 to 30 min.

3. Add 3 ml of ice-cold complete growth medium (DMEM or RPMI containing 10% FBS) to stop the reaction. Vortex the tube vigorously several times.

   Although fibroblasts detach from collagen fibers after treatment with trypsin or collagenase, mechanical agitation is required for releasing them into the solution. Do not vortex before the addition of complete medium.

4. Pass the fibroblast suspension through 85-µm nylon mesh (placed over the top of a tube) to remove dermal debris.

5. Centrifuge 10 min at 150 × *g*, 4°C. Aspirate the supernatant, then resuspend the pellet in 100 to 200 µl of complete growth medium.

6. Count total and viable cells (see Basic Protocol, step 13).

   The cell viability varies depending upon the conditions used for enzymatic digestion. Cutting skin sample into smaller sizes usually increases cell recovery as well as cell viability.

Culture fibroblasts

7. Plate 3–10 × 10^4 cells in 5 ml of complete growth medium in a 25-cm^2 tissue culture flask and begin incubation.

   Viable fibroblasts will attach to the flask within 24 hr and begin to exhibit the spindle-shape in 2 to 3 days (Fig. 2.1.2D and E).

8. Gently remove the medium containing nonadherent cells and add fresh medium on day 2.

   Because the presence of dead cells in culture affects the growth of viable fibroblasts, nonadherent, dead cells must be removed from the culture.

9. Change medium every 3 to 4 days until the culture becomes confluent.

   As fibroblasts become overconfluent, they appear as bundle clusters instead of spindle-shaped cells (Fig. 2.1.2 and Fig. 2.1.3). Harvest the cells before they reach this level.

10. Harvest fibroblasts by washing with PBS followed by incubation with trypsin/EDTA solution. Passage fibroblasts and prepare frozen stocks (see Basic Protocol, steps 10 to 16).

REAGENTS AND SOLUTIONS

Use Milli-Q water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete growth medium

500 ml DMEM or RPMI 1640 (Life Technologies or Sigma)
60 ml FBS (heat-inactivated 60 min at 56°C; *APPENDIX 2A*)
5 ml 1 M HEPES buffer solution (Life Technologies)
5 ml 100× nonessential amino acid mixture (Life Technologies)
5 ml 100× L-glutamine (Life Technologies)
5 ml 100× penicillin/streptomycin (Life Technologies)
5 ml 100× sodium pyruvate (Life Technologies)
Store up to 1 month at 4°C

Phosphate-buffered saline (PBS)

4 liters distilled water
32 g NaCl (140 mM final)
0.8 g KH_2PO_4 (1.5 mM final)
8.7 g $Na_2HPO_4 \cdot 7H_2O$ (8.1 mM final)
0.8 g KCl (2.7 mM final)
Adjust the pH to 7.4 with 1 N NaOH
Store indefinitely at room temperature

Trypsin/EDTA solution

Prepare the following stock solutions:
0.3% (w/v) trypsin (from bovine pancreas; Sigma) in PBS (see recipe for PBS)
1% (w/v) tetrasodium EDTA in PBS (see recipe for PBS)
Store stock solutions up to 3 months at −20°C

Combine 97.5 ml 0.3% trypsin/PBS and 2.5 ml 1% EDTA/PBS. Store trypsin/EDTA solution up to 1 week at 4°C.

COMMENTARY

Background Information

Fibroblasts are the major cellular component of connective tissues, where they play an important role in maintaining structural integrity. They produce and secrete a wide array of extracellular proteins, including proteinases, thereby regulating the biochemical composition and remodeling of tissues. Because biopsy samples can be easily obtained from skin, this tissue serves as a most convenient source of fibroblasts. Skin is composed of continually renewing multilayered squamous epithelium (the epidermis), connective tissue (the dermis), and subcutaneous (adipose) tissue. Human epidermis contains, in addition to keratinocytes (epithelial cells producing keratin intermediate filaments), relatively small numbers of Langerhans cells (antigen-presenting cells of the dendritic cell lineage) and melanocytes (which produce pigment granules called melanosomes). By contrast, mouse epidermis contains keratinocytes, Langerhans cells, and resident γδ T cells called "dendritic epidermal T cells." The dermis, in both human and mouse skin, is a fibrous and filamentous connective tissue that contains fibroblasts, endothelial cells, mast cells, macrophages, and occasionally other leukocyte populations. Despite the complexity of cellular composition, relatively pure fibroblast cultures can be obtained from skin specimens without sophisticated purification processes. This is primarily due to the fact that fibroblasts grow rapidly and continuously when cultured in the presence of serum, whereas other cell types require additional growth factors (e.g., epidermal growth factor or keratinocyte growth factor for keratinocytes), or show very little mitotic activity in vitro (Schuhmachers et al., 1995).

Morphological features—e.g., elongated cell bodies, oval nuclei, and linear or bundle-like alignment of cellular distribution—serve as conventional markers of fibroblasts in culture (Fig. 2.1.2 and Fig. 2.1.3). It is important to emphasize, however, that fibroblasts change their morphology dramatically depending upon the culture conditions, especially the extent of confluency (compare Fig. 2.1.3 panels A and B). Unfortunately, there is no antibody available that recognizes fibroblasts selectively. On the other hand, the absence of specific markers that are expressed by other dermal components (e.g., cytokeratin in keratinocytes, VCAM-1 on endothelial cells, IgE receptor on mast cells, and CD14 on macrophages) serves as a phenotypic marker of fibroblasts (Xu et al., 1995). Production of large amounts of type I collagen, as detected by immunofluorescence staining (Fig. 2.1.3C and D), can be used as a functional marker (Schuhmachers et al., 1995). Nevertheless, because the fibroblast cultures established by the standard protocols described in this unit are rarely "contaminated" by other cell types, especially after a few passages, it is generally accepted that they can be used as "fibroblasts" without further characterization.

Fibroblasts grow rapidly, with a doubling time of 24 to 72 hr (Fig. 2.1.4), and can be passaged successfully >10 times. Because of this outstanding mitotic potential, fibroblasts have been used for a variety of investigative purposes. For example, they serve as useful tools for studying the function and metabolism of extracellular matrix proteins as well as other fundamental aspects of cell biology. Fibroblast cultures established from patients with inherited disorders have often been used to identify genetic abnormalities. Moreover, autologous fibroblasts can be used as a "vector" in gene therapy to deliver transgenes into patients (Suhonen et al., 1996; Nolta and Kohn, 1997). Fibroblast lines generated from healthy human volunteers can be purchased from American Type Culture Collection (ATCC).

The protocols described in this unit are also applicable to other animal species. For example, in the author's laboratory, several fibroblast lines from rats and rabbits have been developed using the same protocols as described for mouse fibroblasts. After enzymatic separation of the dermal compartment, the remaining epidermal portion can be used to grow epidermal cells, such as keratinocytes and melanocytes.

As sources of human fibroblast cultures, newborn foreskin (obtained in circumcision), skin samples excised during surgical operations, cadaver skin (obtained from the transplantation unit), or skin biopsies are routinely used in the author's laboratory. Fibroblast cultures can be established from relatively small skin specimens; the author routinely uses 4-mm punch biopsies for this purpose (Pandya et al., 1995). It is also practical and feasible to establish fibroblast cultures without sacrificing experimental animals; mouse "ear punch" samples are used for this purpose by the author.

Critical Parameters

Because fibroblast outgrowth occurs predominantly from sharp edges of skin speci-

mens, it is crucial to use fine razor blades or surgical scalpels for cutting skin into small pieces. The author routinely uses disposable no. 22 surgical blades for this purpose. Drying of skin specimens is another common cause of poor fibroblast outgrowth. This can be avoided by adding a few drops of PBS while cutting skin specimens. If the surgical blades and forceps are to be soaked with 70% ethanol for sterilization, they should be rinsed well in PBS before use. When performed appropriately, outgrowing fibroblasts should become detectable within 3 to 4 days in skin explant cultures (Fig. 2.1.2B). In cell-dissociation cultures (see Alternate Protocol), the enzymatic digestion process is the most critical; if the viable cell count of resulting suspensions is <70% by trypan blue exclusion, this indicates overdigestion. When performed appropriately, the plated cells should attach firmly to culture plates within 24 hr and begin to spread in 2 to 3 days (Fig. 2.1.2D and E).

Fibroblasts can be frozen safely in 10% DMSO/90% FCS or 10% DMSO/complete DMEM and stored for >10 years in liquid nitrogen. Thus, it is suggested that several aliquots be frozen at a relatively early phase in culture (e.g., after the second or third passage). Because the original features of the cells may be altered during extended culture periods, it is not recommended that they be cultured continuously without experimental usage. If the cells suddenly stop dividing, or if the growth rate accelerates, the cultures need to be replaced. In the author's laboratory the original cultures are routinely discarded after the sixth passage and new cultures are started from a frozen stock.

Care should be taken in harvesting fibroblasts from culture plates. Although fibroblasts are more resistant to contact inhibition than other cell types (e.g., keratinocytes), it is suggested that cells be harvested during their exponential growth phase (Fig. 2.1.4). In the author's laboratory, after removal of culture medium, culture plates are routinely washed briefly with PBS, and then minimal amounts of 0.3% trypsin/25 mM EDTA are added. These plates can be incubated at room temperature under a microscope; as soon as the cells become rounded (before being released spontaneously from plates), the enzymatic reaction is stopped by the addition of ice-cold growth medium containing 10% FBS, and cells are harvested by tapping the culture vessel or gentle pipetting. These cells need to be centrifuged immediately to remove trypsin and EDTA. The cell viability should be >90% by trypan blue exclusion.

Troubleshooting

Bacterial or fungal contamination

Clean the skin well with 70% ethanol before taking a biopsy. Check all the culture media and reagents, including PBS, dispase, trypsin, collagenase, and complete growth medium. Sterilize surgical blades and forceps with 70% ethanol. Always keep the incubator clean.

Contamination by keratinocytes

Remove the epidermis before setting up the fibroblast cultures. Treat the contaminated cultures with 0.5% dispase for 10 to 30 min at 37°C to remove keratinocytes. Because keratinocytes usually require special growth factors for continuous growth, they will eventually disappear in the first or second passage.

Low cell yields or low cell viabilities after enzymatic digestion

Optimize the concentrations, batches of enzymes, and incubation periods. Cut skin into smaller pieces. Use freshly prepared trypsin (or collagenase) solutions; they will lose enzymatic activity gradually when kept at 4°C. Alternatively, prepare enzyme solutions in large quantities, divide them into 5- to 10-ml aliquots, and freeze them at –20°C.

Low cell viabilities during passage

Care must be taken not to overtrypsinize cultures. Wash the culture with PBS before trypsin treatment. Use freshly prepared trypsin solutions. Add ice-cold complete growth medium to stop the enzymatic reaction immediately after fibroblasts round up as determined by examination under a microscope. Avoid excessive pipetting.

Slow fibroblast growth

Increase FBS concentrations (up to 10%); most of the commercially available FBS batches work well for fibroblast cultures (Fig. 2.1.5). Check the temperature, CO_2 level, and humidity of the incubator. The growth rates of fibroblasts often slow down after >10 passages; thaw a frozen stock and start new cultures to avoid this.

Anticipated Results

In skin explant cultures, outgrowing fibroblasts become detectable within 3 to 4 days

and continue to grow thereafter. When skin pieces from one 4-mm punch-biopsy sample are plated in a 35-mm tissue culture dish, it usually takes 3 to 5 weeks to obtain a confluent culture. Starting from one newborn foreskin sample, one can obtain 5 to 10 confluent 35-mm dishes in 3 to 5 weeks. Approximately 1–2 $\times$ 10^6 cells can be harvested from a confluent 35-mm dish. Cell yields are usually higher in the dissociated fibroblast preparation (see Alternate Protocol); after enzymatic digestion with trypsin, ~1–3 $\times$ 10^5 cells can be harvested from a 4-mm punch-biopsy sample and 1–3 $\times$ 10^6 cells can be harvested from a newborn foreskin sample. The use of collagenase typically increases the yield up to 2-fold. When 1–2 $\times$ 10^5 cells are originally seeded in a 25-cm^2 flask, they will become confluent within 2 to 3 weeks, producing 2–3 $\times$ 10^6 cells. After the first passage to new culture plates, fibroblasts begin to grow much faster, with a typical doubling time of 24 to 72 hr (Fig. 2.1.4).

Time Considerations

Skin explant cultures can be set up in 30 to 60 min (without epidermal separation) or 2 to 4 hr (with epidermal separation). Dissociated fibroblast cultures take 3 to 4 hr, depending upon the extent of enzymatic digestion. Although it has been possible in the author's laboratory to establish fibroblast cultures from 1- to 2-day-old skin samples kept in complete growth medium at 4°C, it is highly recommended that cultures be set up immediately after taking biopsy samples. Subsequent passages can be made in 15 to 30 min.

Literature Cited

Nolta, J.A. and Kohn, J.B. 1997. Human hematopoietic cell culture, transduction, and analyses. *In* Current Protocols in Human Genetics (N.C. Dracopoli, J.L. Haines, B.R. Korf, D.T. Moir, C.C. Morton, C.E. Seidman, J.G. Seidman, and D.R. Smith, eds.) pp. 13.7.1-13.7.35. John Wiley & Sons, New York.

Pandya, A.G., Sontheimer, R.D., Cockerell, C.J., Takashima, A., and Piepkorn, M. 1995. Papulonodular mucinosis associated with systemic lupus erythematosis: Possible mechanisms of increased glycosaminoglycan accumulation. *J. Am. Acad. Dermatol.* 32:199-205.

Schuhmachers, G., Xu, S., Bergstresser, P.R., and Takashima, A. 1995. Identity and functional properties of novel skin-derived fibroblast lines (NS series) that support the growth of epidermal-derived dendritic cell lines. *J. Invest. Dermatol.* 105:225-230.

Suhonen, J., Ray, J., Blömer, U. and Gage, F.H. 1996. Ex vivo and in vivo gene delivery to the brain. *In* Current Protocols in Human Genetics (N.C. Dracopoli, J.L. Haines, B.R. Korf, D.T. Moir, C.C. Morton, C.E. Seidman, J.G. Seidman, and D.R. Smith, eds.) pp. 13.3.1-13.3.24. John Wiley & Sons, New York.

Xu, S., Ariizumi, K., Caceres-Dittmar, G., Edelbaum, D., Hashimoto, K., Bergstresser, P.R., and Takashima, A. 1995. Successive generation of antigen-presenting, dendritic cell lines from murine epidermis. *J. Immunol.* 154:2697-2705.

Contributed by Akira Takashima
University of Texas Southwest
Medical Center
Dallas, Texas

Preparation and Culture of Human Lymphocytes

This unit describes procedures for preparation and culture of human lymphocytes and lymphocyte subpopulations obtained from peripheral blood. Because of ease of access, peripheral blood is the primary source of human lymphocytes (mononuclear leukocytes) used in most studies of lymphocyte function. Peripheral blood is a mixture of cells including lymphocytes, granulocytes, erythrocytes, and platelets. Density gradient centrifugation (see Basic Protocol 1) has proven to be an easy and rapid method for separation of lymphocytes from these other peripheral blood cell populations. Lymphocytes and platelets can be separated from granulocytes and erythrocytes according to their lower densities—they will float on top of a density gradient of Ficoll-Hypaque, whereas granulocytes and erythrocytes will traverse this fluid and collect at the bottom of the tube (Fig. 2.2.1). Monocytes/macrophages can then be separated from the other lymphoid-cell populations by adherence to plastic tissue culture vessels (see Basic Protocol 2). The procedures described in this section can be applied to the isolation of peripheral blood lymphocyte populations obtained either from whole blood or via a leukapheresis procedure.

Human lymphocyte subpopulations can be purified based on their cell-surface display of specific distinguishing molecules that can be recognized by monoclonal antibodies. The physical basis for such separation procedures involves the coupling of antibody reagents to magnetic beads, which permit the rapid sequestration of cells that have been bound by the specific antibodies. T and B cells can be positively selected using monoclonal antibody–coated magnetic beads (see Basic Protocol 3) or by exposing the cells to monoclonal antibody and then purifying cells that have bound the antibody using magnetic beads coated with anti–immunoglobulin G (anti-IgG; see Alternate Protocol 1).

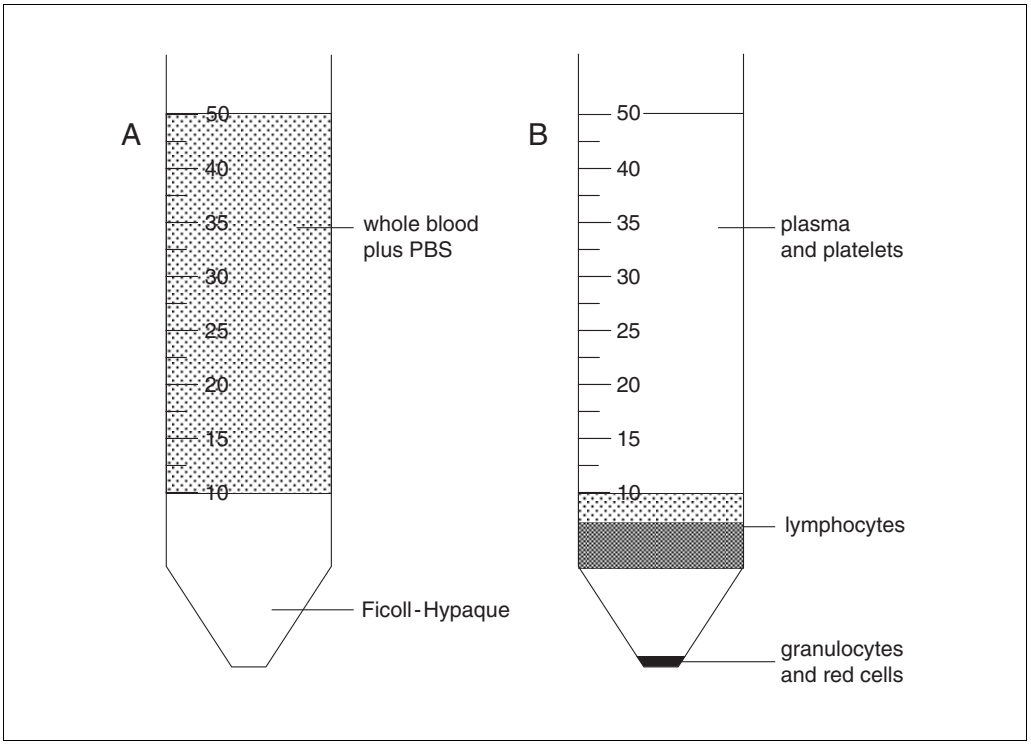


Figure 2.2.1 Isolation of human lymphocytes on a Ficoll-Hypaque gradient. (**A**) Before centrifugation; (**B**) after centrifugation.

Contributed by William E. Biddison
Current Protocols in Cell Biology (1998) 2.2.1-2.2.13

In addition, specific subpopulations can be isolated by negative selection, in which all unwanted subpopulations are removed using monoclonal antibodies and anti-IgG-coated magnetic beads (see Alternate Protocol 2).

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

BASIC PROTOCOL 1

PREPARATION OF LYMPHOCYTES BY FICOLL-HYPAQUE GRADIENT CENTRIFUGATION

In this procedure, whole blood or white blood cells from leukapheresis donors are centrifuged in the presence of a density gradient medium to separate lymphocytes from other peripheral blood cell populations.

Materials

Anticoagulated whole blood or white blood cells from leukapheresis donor
Phosphate-buffered saline (PBS), without calcium or magnesium (Bio-Whittaker), room temperature
Ficoll-Hypaque solution (see recipe), room temperature
Lymphocyte culture medium (LCM; see recipe), room temperature
Iscove's modified Dulbecco's medium (IMDM; Life Technologies) containing 20% heparinized human plasma
Freezing medium (see recipe)

50-ml conical centrifuge tubes
Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent)
1.5-ml cryotubes (e.g., Nunc)
Controlled-rate freezer (e.g., CryoMed from Forma Scientific)
Liquid nitrogen freezer

Additional reagents and equipment for counting cells and determining cell viability (*UNIT 1.1*) and flow cytometry (Robinson et al., 1998)

Prepare Ficoll-Hypaque gradient

For whole blood

1a. Pipet 15 ml of whole blood into a 50-ml conical centrifuge tube and add 25 ml room temperature PBS

2a. Using a 10-ml pipet, underlay with 10 ml room temperature Ficoll-Hypaque solution.

For leukapheresis preparation

1b. Pipet 10 ml of cell suspension from a leukapheresis preparation into a 50-ml conical centrifuge tube and add 30 ml room temperature PBS.

2b. Using a 10-ml pipet, underlay with 7.5 ml room temperature Ficoll-Hypaque solution.

Separate cells

3. Centrifuge 20 min at 800 × *g* (2000 rpm in H-1000 rotor), 20°C, with the brake off.

4. Aspirate most of the plasma- and platelet-containing supernatant above the interface band (granulocytes and erythrocytes will be in red pellet; Fig. 2.2.1). Aspirate the interface band (which includes the lymphocytes) along with no more than 5 ml of fluid above the pellet into a 10-ml pipet, then transfer to a new 50-ml conical centrifuge tube, combining the bands from 2 to 3 Ficoll-Hypaque gradients into one 50-ml tube. Add PBS to 50-ml mark.
5. Centrifuge 10 min at 600 × *g* (1500 rpm in H-1000 rotor), 20°C, with the brake on.
6. Aspirate supernatants and resuspend the pellet in each tube with 10 ml room temperature PBS. Combine resuspended pellets into as few 50-ml tubes as possible. Add PBS to 50-ml mark in each tube used.
7. Centrifuge 15 min 300 × *g* (750 rpm in H-1000 rotor), 20°C, with brake on.

   This low-speed centrifugation permits as many platelets as possible to remain above the pellet of lymphocytes.

Process cell pellet

8. Aspirate platelet-containing supernatant and resuspend lymphocyte pellet in room temperature LCM. Count cells and determine number of viable cells by trypan blue exclusion (*UNIT 1.1*).

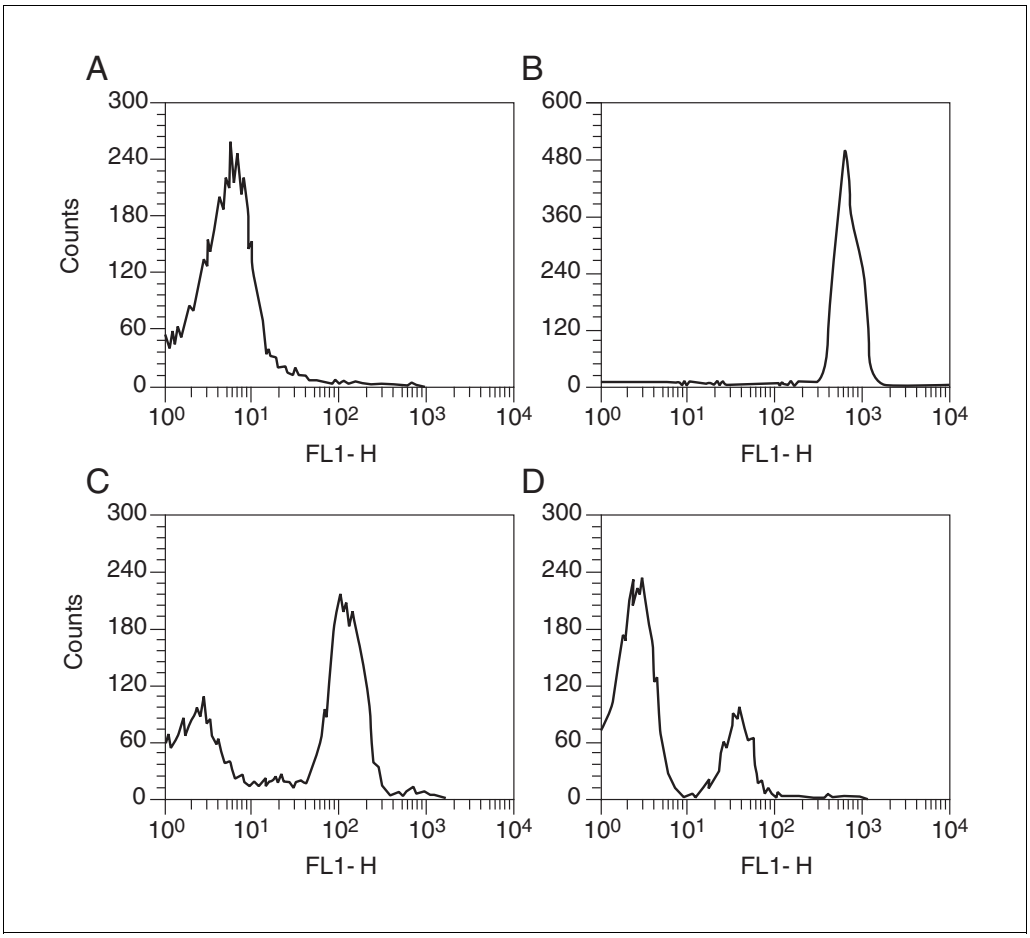


Figure 2.2.2 Flow cytometric analysis of human lymphocytes isolated by Ficoll-Hypaque density gradient centrifugation stained with (**A**) fluorochrome-labeled anti–mouse Ig antibody alone (negative control), (**B**) fluorochrome-labeled anti-CD45 monoclonal antibody, (**C**) fluorochrome-labeled anti-CD3 monoclonal antibody, and (**D**) fluorochrome-labeled anti-CD19 monoclonal antibodies. FL1-H refers to the pulse height for the fluorochrome.

9. Determine purity of lymphocyte preparation by flow cytometry (see, e.g., Robinson et al., 1998) using an anti-CD45 (anti–leukocyte common antigen) antibody (see Fig. 2.2.2 for typical results).

10. To cryopreserve cells, resuspend lymphocytes at twice the concentration desired in the freezing vials using MDM/20% human plasma. Add an equal volume of freezing medium, in increments, over a period of 1 to 2 min, mixing after each addition. Divide the cell suspension into 1-ml aliquots in 1.5-ml cryotubes, place cryotubes in a precooled 4°C controlled-rate freezer and freeze at −1°C/min until −50° or −60°C is attained. Finally, place cryotubes in the gaseous phase of a liquid nitrogen freezer.

BASIC PROTOCOL 2

PREPARATION OF MONOCYTES/MACROPHAGES AND "DENDRITIC-LIKE" CELLS FROM LYMPHOCYTE POPULATIONS

Monocytes/macrophages comprise 5% to 20% of the lymphocytes prepared by density gradient centrifugation (see Basic Protocol 1). To isolate this cell population, or to deplete these cells from the population of lymphocytes, the most expedient mechanism is to exploit the adherence property of monocytes/macrophages. By coating plastic tissue culture vessels with serum as a source of fibronectin and other components of the extracellular matrix, monocytes/macrophages, but not T and B cells, will adhere to these surfaces. This adherence can be significantly enhanced by the presence of recombinant human interleukin 3 (rhIL-3; Biddison et al., 1997). Cells with properties of dendritic cells and a markedly enhanced capacity for antigen processing and presentation can be generated from the monocyte/macrophage population by differentiation in the presence of rhIL-4 and granulocyte/macrophage colony stimulating factor (GM-CSF; Sallusto and Lanzavecchia, 1994).

Materials

Lymphocyte culture medium (LCM; see recipe), 37°C
Lymphocyte population (see Basic Protocol 1)
Recombinant human interleukin 3 (rhIL-3), interleukin 4 (rhIL-4), and GM-CSF (PeproTech)
5 mM tetrasodium EDTA in PBS (also available as Versene from Life Technologies), filter-sterilized using 0.22-µm Nalgene filter, prewarmed to 37°C
175-cm^2 tissue culture flasks
50-ml conical centrifuge tubes
Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent)
Additional reagents and equipment for counting cells and determining cell viability (*UNIT 1.1*) and flow cytometry (Robinson et al., 1998)

Allow monocyte/macrophage population to attach to plastic

1. Add 20 ml of 37°C LCM to 175-cm^2 tissue culture flasks. Incubate 30 min.

2. Add 500×10^6 lymphocytes in 10 ml LCM to each flask plus rhIL-3 to a final concentration of 200 U/ml.

3. Incubate 3 hr, gently rocking flasks every hour.

4. Remove nonadherent cells by aspirating the medium. Wash flasks twice, each time with 20 ml 37°C LCM.

Isolate macrophages/monocytes or "dendritic-like" cells

To isolate monocytes/macrophages

5a. Add 20 ml of 5 mM EDTA/PBS to each flask and incubate 20 min to release adherent monocytes/macrophages.

6a. Vigorously pipet medium up and down to detach adherent cells, then transfer to 50-ml centrifuge tube.

To generate and isolate "dendritic-like" cells

5b. Add 30 ml LCM containing 200 U/ml rhIL-4 and 200 U/ml GM-CSF to each flask and incubate 60 hr.

6b. Remove nonadherent cells and place in 50-ml centrifuge tubes, then add 20 ml of 5 mM EDTA/PBS to each flask and incubate 20 min to release adherent cells. Vigorously pipet medium up and down to detach adherent cells, then combine with nonadherent cells in 50-ml centrifuge tubes.

Separate and analyze isolated cells

7. Centrifuge 10 min at 600 × *g* (1500 rpm in Sorvall H-1000 rotor), room temperature. Aspirate supernatant and resuspend pellet in LCM.

8. Count cells and determine number of viable cells by trypan blue exclusion (*UNIT 1.1*).

9. Determine purity of the monocyte/macrophage or "dendritic-like" cell populations by flow cytometry (see, e.g., Robinson et al., 1998).

Monocytes/macrophages can be distinguished from T and B cells by the absence of cell-surface CD3 and CD19 and by the presence of CD14 or CD35. "Dendritic-like" cells can be distinguished from monocytes/macrophages by the presence of cell-surface CD1 molecules (Sallusto and Lanzavecchia, 1994).

POSITIVE SELECTION OF T AND B CELLS BY MONOCLONAL ANTIBODY–COATED MAGNETIC BEADS

BASIC PROTOCOL 3

In this procedure, T cells and B cells are positively selected based on their differential cell-surface expression of CD3 (T cells) or CD19 (B cells). Monoclonal anti-CD3 and anti-CD19 antibodies coupled to magnetic beads are commercially available. Aliquots of a lymphocyte population purified as described in Basic Protocol 1 are incubated in separate tubes with anti-CD3-coupled beads and anti-CD19-coupled beads. T cells and B cells will be bound by their specific antibody-coupled beads and are then physically separated from unbound cells by exposure of the tubes to a strong magnetic field. Unbound cells are removed and washed away, and the specifically bound cells are released by incubation with a soluble antiserum specific for mouse Fab fragments (Detachabead solution from Dynal) which competes with the bead-coupled monoclonal antibody that is bound to the surface of the cells and thus causes the cells to come off. The procedure here describes specific reagents for separation of T cells and B cells, but is directly applicable to separation of any lymphocyte subpopulation that can be distinguished by monoclonal antibody–coated beads—e.g., purification of $CD4^+$ and $CD8^+$ T cell populations with anti-CD4- and anti-CD8-coated magnetic beads.

Materials

Lymphocyte population (see Basic Protocol 1)
Anti-CD3 and anti-CD19 antibodies for flow cytometry (Becton-Dickinson or Coulter)
Anti-CD3- and anti-CD19-coated magnetic beads (Dynabeads M-450; Dynal)
PBS without calcium and magnesium (Bio-Whittaker)

PBS/HSA: PBS without calcium and magnesium (Bio-Whittaker) containing 0.5% (w/v) human serum albumin (American Red Cross Blood Services)

IMDM/HSA: Iscove's modified Dulbecco's medium (Life Technologies) containing 0.5% (w/v) human serum albumin

Polyclonal anti–mouse Fab antiserum (Detachabead; Dynal)

15-ml conical centrifuge tubes

Magnetic separation device (e.g., Dynal MPC-I or Advanced Magnetics Biomag Separator)

Platform rocker (e.g., Clay Adams Nutator, Becton Dickinson Primary Care Diagnostics)

Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent)

Additional reagents and equipment for flow cytometry (Robinson et al., 1998) and for counting cells and determining cell viability (*UNIT 1.1*)

Prepare and wash magnetic beads

1. Determine the approximate number of T cells and B cells in the starting population of lymphocytes by flow cytometry (or indirect immunofluorescence staining) using anti-CD3 and anti-CD19 antibodies, then determine the number of antibody-coated magnetic beads that will be required to purify the required number of T cells and B cells (5 to 10 beads will be needed for each specific target cell).
2. Based on the bead concentration supplied by the manufacturer, remove the required volume of anti-CD3- and anti-CD19-coated beads from the source vials and place each in a 15-ml conical centrifuge tube. Add PBS to the 14-ml mark and resuspend the beads.
3. Place tubes on magnetic separation device (vertical magnet) for 2 min, then gently aspirate supernatant, leaving beads clinging to one side of each tube.
4. Remove tubes from magnet. Add 10 ml PBS to each tube, resuspend beads, then place tubes on vertical magnet for 2 min. Aspirate PBS as in step 3.

Perform magnetic separation

5. Resuspend each tube of beads in 2 ml PBS/HSA. Put tubes on ice for 15 min.
6. Resuspend lymphocyte population ($\leq 200 \times 10^6$ cells) in 10 ml PBS/HSA. Place on ice for 15 min.
7. Add 5 ml of lymphocyte suspension to the tube with anti-CD3 beads and 5 ml to the tube with anti-CD19 beads.
8. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C.
9. Place tubes on vertical magnet for 2 min. Aspirate nonadherent cells, taking care not to disturb the beads that are clinging to one side of each tube.
10. Add 5 ml IMDM/HSA to each tube. Gently resuspend beads, then place tubes on vertical magnet for 2 min. Aspirate nonadherent cells as in step 9.
11. Add 3 ml IMDM/HSA to each tube. Gently resuspend beads, then place tubes with their conical bottoms on the horizontal magnet. Incubate 2 min at room temperature.

Release T cells and B cells from beads

12. Carefully remove 2.5 ml of supernatant. Tap tubes gently to resuspend beads.
13. Add 200 µl Detachabead solution. Incubate 30 min at room temperature with gentle resuspension every 5 min.

Smaller volumes of Detachabead solution may be used when starting with smaller numbers of lymphocytes; see manufacturer's instructions.

14. Add 3 ml PBS/HSA to each tube and resuspend beads. Place tubes on vertical magnet for 2 min, then aspirate and save supernatant containing detached cells, taking care not to disturb the beads clinging to the side of each tube. Repeat this step four times, saving and combining detached cell–containing supernatants from each separation.

15. Centrifuge detached cells 10 min at 600 × *g* (1500 rpm in H-1000 rotor), room temperature.

16. Aspirate supernatants and resuspend cells in IMDM/HSA. Count cells and determine number of viable cells by trypan blue exclusion (*UNIT 1.1*).

17. Determine purity of the T cell and B cell populations by flow cytometry using anti-CD3 and anti-CD19 antibodies (see, e.g., Robinson et al., 1998).

POSITIVE SELECTION OF T AND B CELLS BY MONOCLONAL ANTIBODIES AND ANTI-IgG-COATED MAGNETIC BEADS

ALTERNATE PROTOCOL 1

This procedure differs from Basic Protocol 3 in that it does not require the acquisition of separate magnetic beads coupled with individual monoclonal antibodies. The procedure is described for T and B cell separation, but can be applied to any lymphocyte subpopulation that can be distinguished by monoclonal antibodies. The principle of the technique is that a human mixed lymphocyte population is separately exposed to saturating amounts of either anti-CD3 or anti-CD19 mouse IgG monoclonal antibodies, unbound antibodies are washed away, then the cells that have bound these antibodies are physically separated from unbound cells by magnetic beads coated with goat anti-mouse IgG. The cells that are specifically bound to the goat anti-mouse IgG–coated beads are then detached by exposure to soluble antiserum against mouse Fab fragments.

Additional Materials *(also see Basic Protocol 3)*

Anti-CD3 and anti-CD19 IgG monoclonal antibodies (Becton Dickinson or Coulter)
Goat anti-mouse IgG–coated magnetic beads (Dynabeads M-450; Dynal)

Prepare cells and antibodies

1. Determine the approximate number of T cells and B cells in the starting population of lymphocytes by flow cytometry using anti-CD3 and anti-CD19 antibodies (e.g., Robinson et al., 1998).

2. Determine the saturating concentration of the anti-CD3 and anti-CD19 antibodies to be used by flow cytometry. Prepare a solution of each antibody in PBS/HSA at ten times (10×) the saturating concentration.

Treat cells with antibodies

3. Resuspend the lymphocyte population (≤200 × 10^6 cells) in 9 ml PBS/HSA and add 4.5 ml of the suspension to each of two 15-ml conical centrifuge tubes. Add 0.5 ml of 10× anti-CD3 antibody to one tube and 0.5 ml of 10× anti-CD19 antibody to the other tube.

4. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C.

5. Wash cells twice, each time by centrifuging 10 min at 600 × *g* (1500 rpm in H-1000 rotor), room temperature, removing the supernatant, resuspending in 10 ml PBS/HSA, and removing the supernatant. Finally, resuspend each tube in 5 ml PBS/HSA and put on ice for 15 min.

Prepare magnetic beads

6. Based on the number of T and B cells estimated in step 1, determine the number of goat anti-mouse IgG–coated magnetic beads that will be required to purify the required number of T and B cells (5 to 10 beads will be needed for each specific target cell).
7. Based on the bead concentration supplied by the manufacturer, remove two aliquots of the required volume of goat anti-mouse IgG–coated beads from the source vial and place in 15-ml conical centrifuge tubes. Add PBS to the 14-ml mark and resuspend the beads.
8. Wash beads (see Basic Protocol 3, steps 2 to 4).

Perform separation

9. Add 5 ml of anti-CD3-coated cells to one tube of washed anti-IgG-coated beads and 5 ml of anti-CD19-coated cell suspension to the other tube of anti-IgG-coated beads.
10. Perform magnetic separation to isolate T and B cells (see Basic Protocol 3, steps 8 to 17).

ALTERNATE PROTOCOL 2

ISOLATION OF T AND B CELL SUBPOPULATIONS BY NEGATIVE SELECTION

For certain experimental conditions, it is desirable to isolate lymphocyte subpopulations without antibody engagement of cell-surface molecules such as the immunoglobulin receptor on B cells and the CD3 complex on T cells. Therefore, negative selection procedures are followed that aim to maximize the elimination of all lymphocyte subpopulations except the desired one. For T cell isolation, the procedure involves elimination of B cells ($CD19^+$), monocytes/macrophages ($CD14^+$), and NK cells ($CD16^+$); for B cell isolation T cells ($CD3^+$), monocytes/macrophages, and NK cells are eliminated. The procedure is very similar to the above protocol for selection of T and B cells using anti-IgG–coated magnetic beads.

Additional Materials *(also see Basic Protocol 3)*

Anti-CD3, anti-CD14, anti-CD16, and anti-CD19 IgG monoclonal antibodies (Becton-Dickinson or Coulter)
Goat anti-mouse IgG–coated magnetic beads (Dynabeads M-450; Dynal)

Prepare cells and antibodies

1. Determine the approximate number of T cells, B cells, monocytes/macrophages, and NK cells in the starting population of lymphocytes by flow cytometry using anti-CD3, anti-CD19, anti-CD14, and anti-CD16 antibodies (e.g., Robinson et al., 1998).
2. Determine the saturating concentrations of the anti-CD3, anti-CD19, anti-CD14, and anti-CD16 antibodies to be used by flow cytometry. Prepare a solution of each antibody in PBS/HSA at ten times (10×) the saturating concentration.

Treat cells with antibodies

3. Resuspend the lymphocyte population ($\leq 200 \times 10^6$ cells) in 3.5 ml PBS/HSA in a 15-ml conical centrifuge tube.

4a. *For T cell isolation:* Add 0.5 ml each of 10× anti-CD19, 10× anti-CD14, and 10× anti-CD16 antibody preparations to the tube.

4b. *For B cell isolation:* Add 0.5 ml each of 10× anti-CD3, 10× anti-CD14, and 10× anti-CD16 antibody preparations to the tube.

5. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C.
6. Wash cells twice, each time by centrifuging 10 min at 600 × *g* (1500 rpm in H-1000 rotor), room temperature, removing the supernatant, resuspending in 10 ml PBS/HSA, and removing the supernatant. Finally, resuspend cells in 5 ml PBS/HSA and put on ice for 15 min.

Prepare magnetic beads

7. Based on the number of cells of each subpopulation estimated in step 1, determine the number of goat anti-mouse IgG–coated magnetic beads that will be required to bind the required number of antibody-coated lymphocytes (5 to 10 beads will be needed for each specific target cell).
8. Based on the bead concentration supplied by the manufacturer, remove the required volume of goat anti-mouse IgG–coated beads from the source vial and place in a 15-ml conical centrifuge tube. Add PBS to the 14-ml mark and resuspend the beads.
9. Wash beads (see Basic Protocol 3, steps 2 to 4).

Perform separation

10. Add 5 ml of washed antibody-coated cells (from step 6) to the tube with the washed anti-IgG-coated beads.
11. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C.
12. Place tube on vertical magnet for 2 min. Aspirate and save supernatant containing nonadherent cells, taking care not to disturb the beads clinging to the side of the tube.
13. Add 5 ml IMDM/HSA to tube. Gently resuspend beads.
14. Repeat steps 12 and 13 twice, saving and pooling nonadherent cells from each wash.
15. Centrifuge nonadherent cells 10 min at 600 × *g* (1500 rpm in H-1000 rotor), room temperature. Aspirate supernatant and resuspend cells in 5 ml IMDM/HSA. Count cells and determine number of viable cells by trypan blue exclusion (*UNIT 1.1*).
16. Determine purity of the T cell and B cell populations by flow cytometry using anti-CD3 and anti-CD19 antibodies (see, e.g., Robinson et al., 1998).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ficoll-Hypaque solution (density 1.07 to 1.08 g/ml)

Dissolve 6.2 g Ficoll (mol. wt. 400,000; e.g., Sigma) in 75 ml distilled water with slow stirring. Add 10.4 g sodium diatrizoate and stir until solution is clear. Add water to 100 ml. Filter-sterilize using 0.22-µm filter (Nalgene). Store up to 6 months at 4°C in the dark.

Alternatively, solutions can be purchased from commercial suppliers (e.g., Lymphocyte Separation Medium from Organon Teknika Cappel or equivalent product from Bio-Whittaker).

Freezing medium

Mix 20 ml Iscove's modified Dulbecco's medium (IMDM; Life Technologies) and 20 ml heparinized human plasma. Cool to 4°C. Slowly add 10 ml dimethylsulfoxide, mixing after each incremental addition. Cool to 4°C and filter sterilize using a 0.22-µm filter.

Lymphocyte culture medium (LCM)

Iscove's modified Dulbecco's medium (IMDM; Life Technologies) containing:
100 U/ml penicillin
100 µg/ml streptomycin
10% heparinized human plasma
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

Density-gradient separation of lymphocytes

Isolation of human lymphocytes from peripheral blood by density gradient centrifugation provides the following advantages: it is a simple technique, it is relatively inexpensive, it does not require special laboratory equipment, sterility is easily maintained, and high yields of lymphocytes are provided (Boyum, 1968). A typical yield of lymphocytes from whole blood of healthy adult donors is $1–2 \times 10^6$ cells/ml. Figure 2.2.2 shows a typical result from flow cytometric analysis of density gradient–purified lymphocytes in which 98% of cells are $CD45^+$ (positive for common leukocyte antigen), 61% are $CD3^+$ (T cells), and 21% are $CD19^+$ (B cells). The non-T non-B cell population contains monocytes/macrophages and natural killer (NK) cells.

Monocytes/macrophages can be easily purified from the lymphocyte population based on their differential adhesiveness to immobilized components of the the extracellular matrix contained in human serum. A typical yield of monocytes/macrophages from a lymphocyte population is 5% to 10%. Flow cytometric analysis shows that ≥95% of these cells are HLA class I^+, HLA-DR^+, and $CD35^+$, and ≤1% are $CD3^+$ T cells (Fig. 2.2.3). A subset of these monocytes/macrophages can be differentiated into immature antigen-presentation-competent "dendritic-like" cells by culture in IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). These "dendritic-like" cells have proven to be extremely useful antigen-presenting cells for the generation and cloning of rare self-reactive T cells (Biddison et al., 1997).

Magnetic-bead separation of lymphocytes

Cell-separation procedures based on magnetic-bead technology have the advantages of easy manipulation, safety from contamination with microorganisms, and avoidance of high shear forces that affect cell viability. The most direct procedures involve positive selection with specific monoclonal antibodies directly coupled to magnetic beads and the subsequent liberation of the bound cells by competing anti-Fab antibodies. This procedure also has the added advantage of providing a positively selected lymphocyte subpopulation free of any cell-bound selecting antibodies. A variation on this approach involves precoating the lymphocyte subpopulation of choice with a specific monoclonal IgG antibody and subsequent physical sequestration with anti-IgG-coated magnetic beads. In experimental situations in which specific antibody binding to cell-surface structures must be avoided, negative selection procedures are employed, wherein the lymphocyte subpopulations that are not desired are coated with specific IgG antibodies and are then removed by anti-IgG-coated magnetic beads. Each of these procedures has been used successfully to isolate human T cells, monocytes/macrophages, and B cells (Lea et al., 1986; Vartdal et al., 1987; Funderud et al., 1990).

Alternative procedures for positive selection are cell sorting of fluoresceinated antibody–coated cells with a flow cytometer and panning of antibody-coated cells on plastic surfaces containing immobilized anti-immunoglobulin reagents. This author's experiences with these techniques indicate that they present a much higher likelihood of contamination with microorganisms and subject the lymphocytes to conditions that can lead to more loss of viable cells than occurs with the magnetic-bead procedures. Cell sorting also has a major limitation in the number of cells that can be separated. Negative selection can also be provided by complement-fixing antibodies, but this approach involves the need to remove dead-cell debris before the remaining viable cells can be used for experimentation.

Critical Parameters and Troubleshooting

To maximize the yield and purity of lymphocytes using the density gradient protocol (see Basic Protocol 1), it is essential to harvest all of the cells at the interface between the

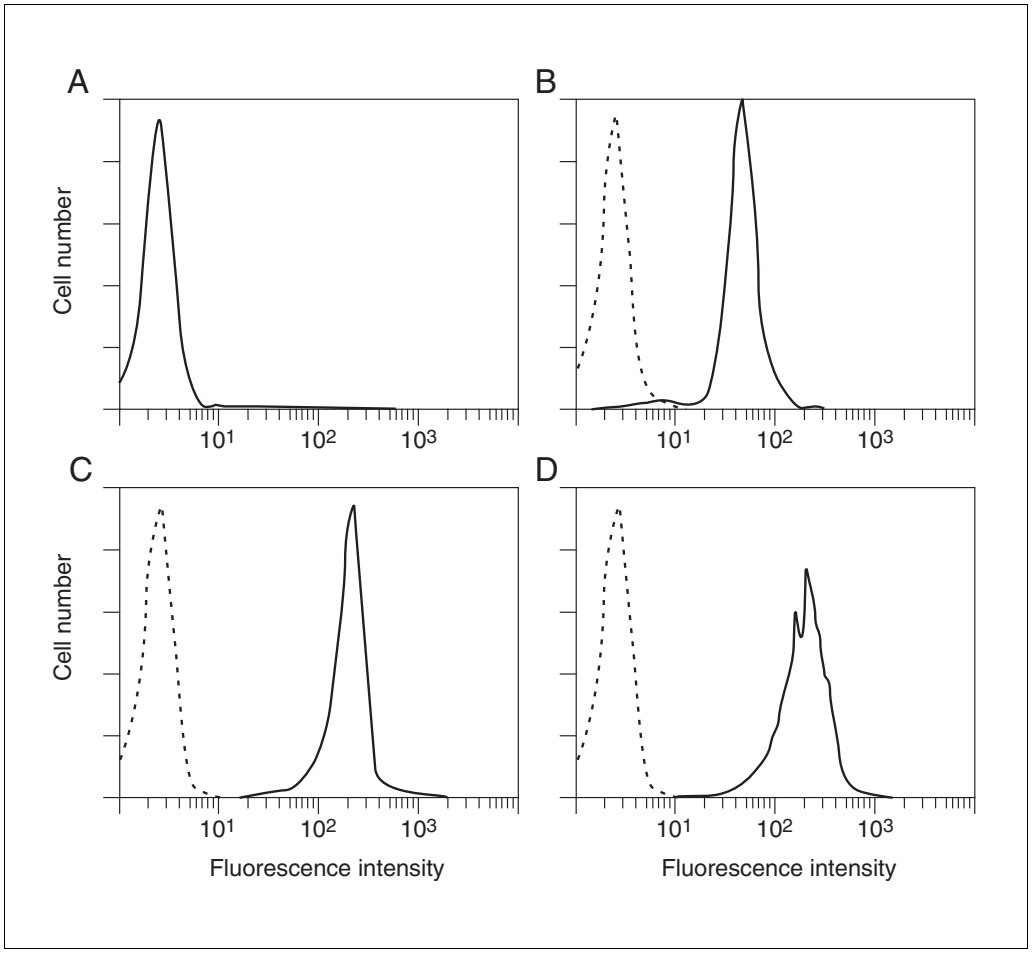


Figure 2.2.3 Flow cytometric analysis of isolated monocytes/macrophages. The solid lines represent cells stained with (**A**) fluorochrome-labled anti-CD3 monoclonal antibodies, (**B**) fluorochrome-labeled anti-CD35 monoclonal antibodies, (**C**) fluorochrome-labeled anti–HLA class I monoclonal antibodies, and (**D**) fluorochrome-labeled anti-HLA-DR monoclonal antibodies. The dotted lines in panels B, C, and D represent cells stained with fluorochrome-labeled anti–mouse IgG alone.

Ficoll-Hypaque solution and the plasma/platelet layer. This may require harvesting up to 5 ml of the underlying Ficoll-Hypaque solution. This is why it is recommended that at least 7.5 ml of this solution be used as an underlay for each tube. Great care should be taken not to disturb or harvest any of the pelleted granulocytes and erythrocytes. One common problem is the accumulation of platelets at the Ficoll-Hypaque interface, which produces clumping and contamination with platelets and erythrocytes. This problem can be partially avoided by further dilution of blood with PBS and by not centrifuging the cells at temperatures <20°C. The platelet-contamination problem can be alleviated in the subsequent washing steps. If significant cloudiness of the supernatant is observed (due to platelet contamination) following the 15-min centrifugation at 300 × *g* (see Basic Protocol 1, step 7), aspirate the supernatants, resuspend the cells in 50 ml PBS, and do another 15-min, 300 × *g* centrifugation to remove as many remaining platelets as possible.

Isolation of monocytes/macrophages by adherence is significantly enhanced by precoating the tissue culture flasks with serum-containing medium and including IL-3 during the adhesion incubation. No diminution in antigen-presenting function has been observed in the author's laboratory by cells cultured in IL-3. However, if the presence of IL-3 may add an unknown variable to other types of experiments, especially those that will utilize the nonadherent lymphocyte fraction, it is recommended that IL-3 not be included during the adherence step.

The specificity and avidity of the antibody reagents used in these cell-separation procedures are clearly of critical importance. Care must be taken to utilize appropriate concentrations of coating monoclonal antibodies to ensure that all specific target cells are coated with antibody. It is also crucial that, when precoating cells with soluble antibodies, all unbound antibodies are adequately removed during the washing steps. Another critical factor is the use of adequate numbers of antibody–coated magnetic beads to completely engage all appropriate available cells. The use of 5 to 10 beads per specific target cell helps to ensure a complete saturation of the available target cells. Certain pitfalls are relatively easy to avoid. Where called for in the protocols, be sure to do manipulations on cells that have been precooled and do appropriate incubations at 4°C. This will ensure that the selecting antibodies will not be removed by capping. Use of a sufficiently strong magnet will improve cell yields and purity and reduce the amount of time for each magnetic-selection step. This author's experience is that the Biomag Separator available from Advanced Magnetics is more than adequate for the task of selecting the Dynal Dynabeads. Also note that the protocols do not include use of fetal bovine serum (FBS) in any step. FBS and other heterologous sera should be avoided in all steps because they can produce unwanted activation of lymphocytes.

At the conclusion of each of the cell-separation procedures, the purity of the cell populations produced should be assessed by flow cytometry.

Anticipated Results

Whole blood from healthy adult donors will typically yield 1–2 $\times 10^6$ lymphocytes per ml of blood by the density gradient protocol. A typical leukapheresis in which lymphocytes are collected from 5 liters of blood will yield 4–6 $\times 10^9$ lymphocytes when processed by the density gradient protocol. Isolated lymphocytes should be ≥95% viable.

Isolation of monocytes/macrophages from 500×10^6 lymphocytes will usually yield 25–50 $\times 10^6$ cells with ≥95% viability. Generation of "dendritic-like" cells from the same number of starting lymphocytes will usually yield 5–10 $\times 10^6$ cells with ≥90% viability.

The lymphocyte subpopulations produced by the magnetic-bead techniques should be ≥95% pure and ≥95% viable by trypan blue dye exclusion. The overall yield of T and B cells from the starting lymphocyte preparation from peripheral blood should be 40% to 60%.

Time Considerations

Isolation of lymphocytes from peripheral blood (see Basic Protocol 1) usually takes 2 hr, depending on the volume of blood obtained or the number of lymphocytes collected by leukapheresis. Monocyte/macrophage isolation (see Basic Protocol 2) requires a subsequent 3.5-hr time period, and culture of the isolated monocytes/macrophages with IL-4 and GM-CSF requires an additional 60 hr of incubation time.

Positive selection using monoclonal antibody–coated magnetic beads (see Basic Protocol 3) requires 2 to 3 hr. The method using anti-IgG coated-beads (see Alternate Protocol 1) requires an additional hour for precoating of the cells with selecting antibodies.

Literature Cited

Biddison, W., Taub, D., Cruikshank, W., Center, D., Connor, E., and Honma, K. 1997. Chemokine and matrix metalloproteinase secretion by myelin proteolipid protein–specific $CD8^+$ T cells. *J. Immunol.* 158:3046-3053.

Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Invest.* 21(Suppl. 97):107-111.

Funderud, S., Erikstein, B., Asheim, H., Nustad, K., Stokke, T., Blomhoff, H., Holte, H., and Smeland, E. 1990. Functional properties of $CD19^+$ B lymphocytes positively selected from buffy coats by immunomagnetic separation. *Eur. J. Immunol.* 20:201-206.

Lea, T., Smeland, E., Funderud, S., Vartdal, F., Davies, C., Beiske, K., and Ugelstad, J. 1986. Characterization of human mononuclear cells after positive selection with immunomagnetic particles. *J. Immunol.* 23:509-519.

Robinson, J.P., Darzynkiewicz, Z., Dean, P.N., Orfao, A., Rabinovitch, P.S., Stewart, C.C., Tanke, H.J., and Wheeless, L.L. 1998. Current Protocols in Cytometry. John Wiley & Sons, New York.

Sallusto, F. and Lanzavecchia, A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and down-regulated by tumor necrosis factor alpha. *J. Exp. Med.* 179:1109-1118.

Vartdal, F., Gaudernack, G., Ugelstad, J., Kvalhelm, G., Lea, T., Bosnes, V., and Albrechtsen, D. 1987. Depletion of T lymphocytes from human bone marrow. Use of magnetic monosized polymer microspheres coated with T lymphocyte specific monoclonal antibodies. *Transplantation* 43:366-371.

Key References

Boyum, 1968. See above.

Comprehensive explanation of procedure for Ficoll-Hypaque gradient separation of human lymphocytes.

Funderud, S., Nustad, K., Lea, T., Vartdal, F., Gaudernack, G., Stensted, P., and Ugelstad, J. 1987. Fractionation of lymphocytes by immunomagnetic beads. *In* Lymphocytes: A Practical Approach (G.G.B. Klaus, ed.) pp. 55-61. Oxford University Press, New York.

Comprehensive description of magnetic bead technology for lymphocyte separations.

Contributed by William E. Biddison
National Institute of Neurological Disorders and Stroke/NIH
Bethesda, Maryland

Preparation of Endothelial Cells

This unit presents various approaches to obtaining endothelial cells from tissues for cell culture. The isolation of these cells from umbilical vein (see Basic Protocol) is particularly useful because the tissue source is readily available and the cells can be obtained in quantity. The isolated cells are generally pure and grow well. Although umbilical veins are the most frequently employed source, cells can be obtained from other vessels and from tissues—e.g., adipose tissue, nasal mucosa, and skin.

The isolation of human umbilical vein endothelial cells (HUVEC) is first described (see Basic Protocol). Additional protocols describe the isolation of microvascular endothelial cells from tissues with few contaminating cells (retroperitoneal adipose tissue and nasal mucosa; see Alternate Protocol 1) and from tissues with a significant number of contaminating cells (skin; see Alternate Protocol 2).

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations should be performed in a 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

PREPARATION OF ENDOTHELIAL CELLS FROM HUMAN UMBILICAL VEIN

BASIC PROTOCOL

This protocol describes the isolation and culture of human umbilical vein endothelial cells (HUVEC).

Materials

Fresh human umbilical cord
RPMI 1640 medium, 4° and 37°C
Phosphate-buffered saline (PBS; Life Technologies)
5 mg/ml collagenase CLSII (Worthington) in PBS (filter sterilized), 37°C
HUVEC medium (see recipe), 37°C

250-ml wide-mouthed tissue culture flasks
Sterile pads and gauze
Sterile scissors, razors, hemostats, and non-toothed forceps
Syringes and yellow 200-µl pipet tips
50-ml conical centrifuge tubes
Tabletop centrifuge
Nunc T-75 tissue culture flasks *or* 75-cm^2 gelatin-coated flasks (see recipe)
150-mm-diameter Nunc tissue culture dishes

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

1. Place the umbilical cord in a 250-ml wide-mouthed flask containing RPMI 1640 medium and store at 4°C for no more than 24 hr after delivery.

   Cords older than 24 hr should be discarded. It is easiest to drop off the medium in a wide-mouth flask at the hospital ahead of time so the staff can deposit the cord as soon as it is available.

Contributed by Hynda K. Kleinman and Maria C. Cid
Current Protocols in Cell Biology (1998) 2.3.1-2.3.6

2. Working in a sterile tissue culture hood with sterile pads on the hood counter top, wash the cord with PBS. Using sterile gauze to hold the cord, cut the ends with a razor to yield a 10- to 15-cm segment.

   Wear a lab coat and two pairs of gloves. The cord is human tissue and could be infectious or pathogenic.

3. Dilate vein using non-toothed forceps.

   Dilate carefully to avoid tears.

4. Using a syringe with a yellow 200-µl pipet tip attached, flush 5 to 10 ml 37°C RPMI 1640 through the vein.

5. Using a fresh syringe with a yellow 200-µl pipet tip, flush 5 to 10 ml of 5 mg/ml collagenase through the vein.

6. Clamp one end of the cord with a hemostat and squeeze ~10 ml more collagenase solution into the vein. Clamp top of cord with a second hemostat and invert the cord several times to spread the collagenase solution.

7. Place the clamped umbilical cord in a 250-ml wide-mouthed flask with warm RPMI 1640 and incubate 15 min at room temperature.

8. Open one end of the cord over a 50-ml conical centrifuge tube to catch the effluent from the vein. Flush vein with 15 ml of 37°C HUVEC medium and collect the effluent.

   The HUVEC medium reduces the collagenase activity and helps the cells to remain viable.

9. Add enough additional HUVEC medium to fill the tube and centrifuge 5 min at 170 × *g* in a tabletop centrifuge, room temperature.

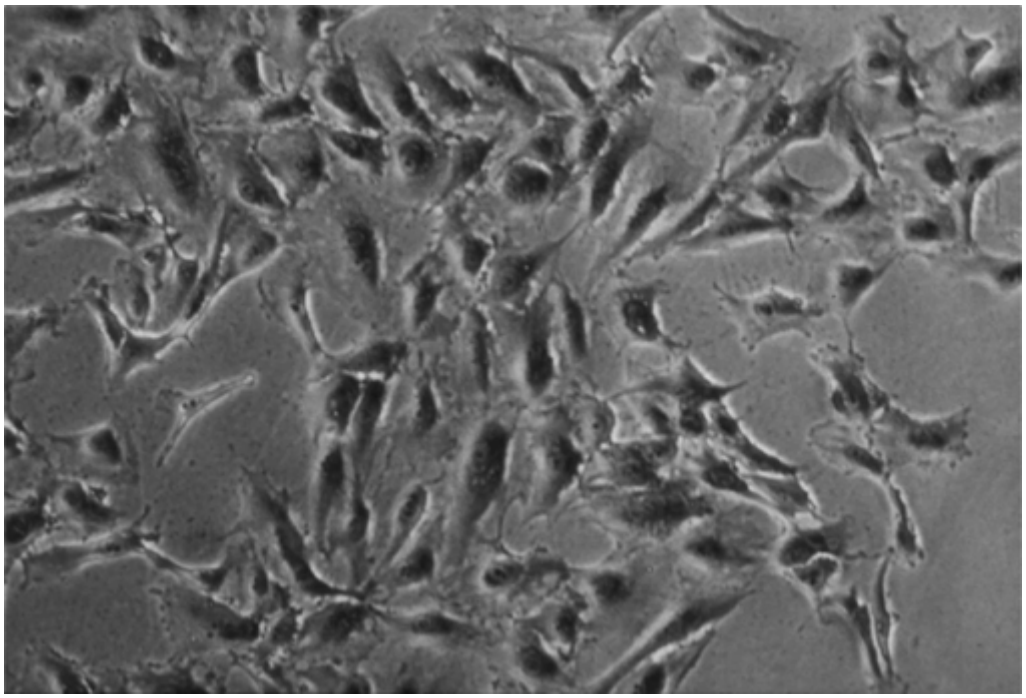


Figure 2.3.1 Culture of human umbilical vein endothelial cells 2 days after passaging. Passage 3 cells were at ~70% confluence and were fixed and stained, respectively, with Diff-Quik Fix and Solution II (Baxter). Cells were viewed using a Zeiss inverted microscope.

10. Decant and discard supernatant, tap tube to resuspend cell pellet, then add 15 ml 37°C HUVEC medium and transfer to two T-75 Nunc or 75-cm^2 gelatin-coated flasks. Incubate 1.5 hr.

    This is based on an average yield from a 10- to 15-cm cord.

11. After 1.5 hr, remove medium and add 15 ml fresh medium. Incubate 2 to 3 days (until confluent).

    The medium that is removed can be used to plate additional cells in another flask; after 1.5 hr, that medium should be removed and discarded, and fresh medium added. It is important with this second flask to be sure that there are not too many dead cells or fibroblasts (which can be detected microscopically on the basis of morphology).

12. When cells become confluent, split flask 1:4 into 150-mm Nunc tissue culture dishes.

    These cells are considered passage 1. Cells should not be used beyond passage 6. At passage 2, there should be 32 dishes. Some of the passage 2 cells can be frozen and stored in liquid nitrogen for future use (UNIT 1.1). Figure 2.3.1 shows a culture of endothelial cells with typical elongated morphology.

ISOLATION OF ENDOTHELIAL CELLS FROM RETROPERITONEAL ADIPOSE TISSUE OR NASAL MUCOSA

ALTERNATE PROTOCOL 1

It is well recognized that not all endothelial cells behave in a similar manner or have identical phenotypes. Preparations of endothelial cells from different tissue sources are often needed. Retroperitoneal adipose tissue or nasal mucosa can be obtained at the time of surgery. Usually, small pieces are provided from the normal edges of removed lesions such as polyps in the nasal mucosa.

Additional Materials *(also see Basic Protocol)*

Retroperitoneal adipose tissue or nasal mucosa
0.02% (w/v) collagenase A (Boehringer Mannheim) in PBS (filter sterilized)

Cell scraper
Laminin-coated 6-well tissue culture plates (see recipe)

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

1. Cut tissue into 2- to 4-mm-square pieces and wash with sterile PBS.
2. Incubate 3 to 5 tissue pieces for 30 min with enough 0.2% collagenase at 37°C to just cover the pieces.
3. Gently aspirate collagenase solution and wash the tissue once in PBS, discarding the supernatants.
4. Add 3 ml RPMI 1640 medium, squeeze tissue with a cell scraper, and collect cells in a 50-ml conical tube.
5. Filter through sterile gauze to remove tissue debris.
6. Centrifuge 5 min at 170 × *g* in a tabletop centrifuge, room temperature.
7. Discard supernatant, gently tap tube to resuspend cell pellet, and add 15 ml of room temperature HUVEC medium.
8. Plate 3 ml of cell suspension onto each well of a laminin-coated 6-well plate. Incubate 1 hr, then remove medium (along with unattached cells) and add 3 ml fresh medium.

    This step reduces the fibroblast contamination.

9. Incubate cells until confluent and passage as described above for HUVECs (see Basic Protocol).

ALTERNATE PROTOCOL 2

ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM DERMIS

Human microvascular endothelial cells can be isolated from neonatal foreskins (Kubota et al., 1988). These cells are relatively easy to prepare and are widely used.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Dermal tissue (e.g., neonatal foreskins)
Discontinuous Percoll gradient (see recipe)
5 mM tetrasodium EDTA in PBS (also available as Versene from Life Technologies), filter-sterilized using 0.22-μm Nalgene filter, prewarmed to 37°C

PBS containing 5% FBS, room temperature to 37°C (store up to 1 month at 4°C)
Laminin-coated 6-well tissue culture plates (see recipe)
Anti-CD31-coated petri dishes (see recipe)
Gelatin- or fibronectin-coated 6-well tissue culture plates (see recipes)

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

1. Prepare a cell suspension from dermal tissue as described for retroperitoneal adipose tissue or nasal mucosa (see Alternate Protocol 1, steps 1 to 6). Resuspend the pellet in 1 ml of RPMI 1640 medium.
2. Carefully layer 1 ml of the resuspended pellet on top of the discontinuous Percoll gradient. Centrifuge 20 min at 670 × *g* in a tabletop centrifuge, room temperature.
3. Collect each layer separately and transfer to individual tubes. Wash by centrifuging 5 min at 170 × *g*, room temperature, adding 10 ml RPMI 1640 medium, then centrifuging again and removing the supernatant.

   Endothelial cell fractions are usually in the 32% and 34% layers.

4. Resuspend pellets in HUVEC medium and culture each fraction in a well of a laminin-coated 6-well plate (see Alternate Protocol 1, steps 7 to 9).

   Usually the yield of cells is very low due to the small amounts of biopsy material available. All of the pellet obtained from each fraction should be placed in a well. The number of endothelial cell colonies will be small.

5. After 2 days, remove medium, wash cells with PBS, and detach cells by incubating ~5 min with 1.0 ml/well of 5 mM EDTA/PBS at room temperature.
6. Centrifuge 5 min at 170 × *g*, room temperature. Discard supernatant, tap tube to resuspend cell pellet, add 15 ml RPMI 1640 medium (prewarmed to 37°C), and incubate 1 to 3 hr on a CD31-coated dish at 37°C.
7. Gently aspirate the medium containing nonadherent cells and wash the dish twice gently with 15 ml of room temperature to 37°C PBS containing 5% FBS.
8. Wash dish gently with 15 ml of room temperature to 37°C PBS and release attached cells by trypsinization.

   The cells usually detach within a few minutes.

9. Centrifuge cells 5 min at 170 × *g*, room temperature. Discard the supernatant, tap the tube gently to resuspend the cell pellet, add HUVEC medium and culture on gelatin- or fibronectin-coated 6-well tissue culture plates (see Basic Protocol).

   Fibronectin is more expensive than gelatin.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Anti-CD31-coated petri dishes

Prepare 10 µg/ml anti-CD31 monoclonal antibody in 50 mM Tris·Cl, pH 9.5 (store according to supplier's instructions). Add 10 ml of this solution to a 10-cm-diameter petri dish and incubate 1.5 hr at 37°C. Remove the solution and use it to coat two more dishes in the same way. Wash each dish three times with 0.15 M NaCl, then add 10 ml of 1 mg/ml bovine serum albumin (BSA) to each dish and incubate overnight at 4°C. Remove the liquid before adding the cells.

The anti-CD31 is available from several suppliers. Preparations without BSA work better. The coated plates should be used immediately.

Fibronectin-coated 6-well tissue culture plates

Prepare 333 µg/ml fibronectin in PBS (Life Technologies) and filter sterilize (store up to 1 week at 4°C). Add 0.3 ml to each well and incubate 30 min at 37°C just prior to addition of cells. Remove the solutions containing unbound fibronectin prior to addition of the cells.

Gelatin-coated tissue culture flasks and plates

Prepare 0.1% (w/v) gelatin in PBS (Life Technologies) and filter sterilize (store up to 1 month at 4°C). Incubate flasks with the gelatin solution 30 min at 37°C, then remove gelatin and rinse with sterile PBS. Store coated flasks up to 1 day at 4°C or air dry and use within 1 week.

HUVEC medium

500 ml RPMI-1640 medium
100 ml defined iron-supplemented calf serum (HyClone)
100 mg endothelial cell growth supplement (ECGS; Collaborative Biomedical)
2500 U heparin
5 ml 100× glutamine
5 ml 100× penicillin/streptomycin
5 ml 100× Fungizone
0.5 ml 1000× gentamicin
Store up to 1 month at 4°C

Laminin-coated 6-well tissue culture plates

Prepare 333 µg/ml laminin in PBS (Life Technologies) and filter sterilize (store up to 1 year at −80°C). Add 0.3 ml to each well and incubate 30 min at 37°C just prior to addition of cells. Remove the solutions containing unbound laminin prior to addition of the cells.

Percoll gradient, discontinuous

Prepare 30%, 34%, and 38% (v/v) Percoll in PBS
Prepare 32%, 36%, and 40% (v/v) Percoll in RPMI-1640 medium (with phenol red)

Pipet 2 ml of the 40% Percoll solution into a 15-ml round-bottom Nalgene centrifuge tube. Carefully layer 2 ml of the 38% Percoll on top of this, followed successively by the 36%, 34%, 32%, and 30% Percoll.

The Percoll solutions are prepared in an alternating fashion with PBS and (phenol red containing) RPMI 1640 to make it possible to distinguish the layers easily.

COMMENTARY

Background Information

Endothelial cells line blood vessels and are attached to each other (cell-to-cell) and to the underlying basement membrane matrix (cell-to-matrix). Various methods have been developed to isolate these cells in quantity for cell culture. Human umbilical vein is an ideal source because it is easy to obtain, cells can be isolated in quantity, and the cells are generally pure and grow well (Gimbrone, 1976; Jaffe, 1980). A disadvantage is the expensive medium needed to propagate the cells. The ECGS (endothelial cell growth supplement) is costly but can be prepared by the investigator if very large quantities are needed (Gordon et al., 1983). Newer media are under development and appear to maintain the survival of the cells. Previously, tumor-conditioned medium had been used (Folkman et al., 1979), but that material was somewhat variable and the results were not as reproducible as those observed with the HUVEC medium used in this unit (see Reagents and Solutions). Another disadvantage of HUVEC is that the cells cannot be used beyond passage 6, due to changes in the phenotype.

Other blood vessel and tissue sources have yielded endothelial cells for culture. For example, endothelial cells have been prepared from nasal mucosa (Fukuda et al., 1989), bovine capillary, pancreatic islet, mouse hemangioendothelioma (Folkman et al., 1979; Voest et al., 1995), adrenal cortex, and human dermis (Kubota et al., 1988).

Critical Parameters and Troubleshooting

Once the cells are isolated, their identity as endothelial cells can be confirmed by their characteristic cobblestone morphology or by specific markers such as factor VIII or uptake of acetylated LDL (Kubota et al., 1988; Kibbey et al., 1992). The type of dish used to culture the cells is important and the protocols should be followed exactly.

Because the cells are of human origin, full precautions for infectious and viral diseases must be employed. Gloves (at least two pair) should be used even after the cells have been in culture for several passages.

As compared with other types of primary cells, HUVEC are more "delicate" and problems with growth are observed with slight changes in the carbon dioxide or the temperature.

Umbilical cord or biopsy tissue should be stored at 4°C in medium and should not be used if it is >24 hr old.

Anticipated Results

One umbilical vein should yield two confluent T-75 flasks in 3 to 5 days. By passage 2 there should be 32 confluent 150-mm dishes with ~1 $\times 10^7$ cells per dish. The yield from tissues is less than that from umbilical vein.

Time Considerations

Preparation of the cells for all three methods should not take more than half a day.

Literature Cited

Folkman, J., Haudenshild, C.C., and Zetter, B.R. 1979. Long term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 76:5217-5221.

Fukuda, K., Imamura, Y., Koshihara, Y., Ooyama, T., Hanamure, Y., and Ohyama, M. 1989. Establishment of human mucosal microvascular endothelial cells from inferior turbinate culture. *Am. J. Otolaryngol.* 10:85-91.

Gimbrone, M. 1976. Culture of vascular endothelium. *Prog. Hemostasis Thromb.* 3:1-28.

Gordon, P.B., Sussman, I.I., and Hatcher, V.B. 1983. Long-term culture of human endothelial cells. *In Vitro* 19:661-671.

Jaffe, E.A. 1980. Culture of human endothelial cells. *Transplant. Proc.* 12:49-53.

Kibbey, M.C., Grant, D.S., and Kleinman, H.K. 1992. Role of the SIKVAV site of laminin in promotion of angiogenesis and tumor growth: An in vivo Matrigel model. *J. Natl. Cancer Inst.* 84:1633-1638.

Kubota, Y., Kleinman, H.K., Martin, G.R., and Lawley, T.J. 1988. Role of laminin and basement membrane in morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* 107:1589-1598.

Voest, E.E., Kenyon, B.M., O'Reilly, M.S., Truit, G., D'Amato, R.J., and Folkman, J. 1995. Inhibition of angiogenesis in vivo by interleukin 12. *J. Natl. Cancer Inst.* 87:581-586.

Contributed by Hynda K. Kleinman
National Institute of Dental Research/NIH
Bethesda, Maryland

Maria C. Cid
Hospital Clinic i Provincia
Barcelona, Spain

Generation of Continuously Growing B Cell Lines by Epstein-Barr Virus Transformation

BASIC PROTOCOL

Epstein-Barr virus (EBV) has been widely used to transform human B cells in vitro and to produce continuously growing B cell lines for use in a variety of in vitro studies. Most human B cell lines that are transformed by EBV secrete little or no infectious viral particles. For this reason, a marmoset line transformed with the human Hawley strain of EBV, which secretes active infectious EBV into the culture supernatant, is used as a source of transforming virus (Miller and Lipman, 1973). This protocol explains how to prepare the marmoset-derived EBV and transform human B cells obtained from peripheral blood.

CAUTION: EBV is a known human pathogen. Appropriate biosafety practices must be followed.

Materials

Complete culture medium (see recipe), 37°C
B95-8 EBV-transformed marmoset cell line (ATCC #CRL 1612)
Anti-CD3 monoclonal antibody produced by OKT3 hybridoma (ATCC #CRL 8001)

25-cm^2 and 75-mm^2 tissue culture flasks
Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent refrigerated centrifuge and rotor) and 50-ml centrifuge tubes
0.45-µm sterile filter

Additional reagents and equipment for growing cells, determining cell viability by trypan blue exclusion, and cryopreservation of cells (*UNIT 1.1*) and preparation of peripheral blood lymphocytes (*UNIT 2.3*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Resuspend B95-8 cells in complete culture medium at 1×10^6 cells/ml and incubate in 75-mm^2 tissue culture flasks at 50 ml of culture per flask for 3 days (until ≥95% viable and in exponential growth phase; see *UNIT 1.1* for basic culture technique and determination of cell viability).

2. Transfer cultures to 50-ml centrifuge tubes. Centrifuge 10 min at $600 \times g$ (1500 rpm in H-1000 rotor), 4°C. Filter supernatant through 0.45-µm sterile filter, divide into 0.6-ml aliquots, and store at −70°C.

   The culture supernatants should contain 10^2 to 10^3 infectious units/ml (Miller and Lipman, 1973). Determination of EBV titers can be done by quantitative assessment of transformation of umbilical cord leukocytes (Miller and Lipman, 1973); however, because of the difficulty in obtaining such cells, this determination is usually omitted.

3. Prepare peripheral blood lymphocytes as described in *UNIT 2.3*. Resuspend lymphocytes in 37°C complete culture medium at 1×10^6 cells/ml, then place 5 ml of the lymphocyte suspension in an upright 25-cm^2 tissue culture flask.

4. Add anti-CD3 antibody to final concentration of 10 µg/ml. Incubate cells and anti-CD3 antibody for 1 hr with the flask in the upright position.

Contributed by William E. Biddison
Current Protocols in Cell Biology (1999) 2.4.1-2.4.3

5. Add 0.5 ml of the EBV-containing B95-8 supernatant (from step 2) to flask. Incubate with the flasks in the upright position for 1 to 2 weeks (until medium begins to turn orange/yellow and small clumps of cells become visible).
6. Add 5 ml of fresh 37°C complete medium, then incubate 2 to 3 days. After that period, remove 5 ml of the supernatant and add 5 ml of fresh 37°C complete medium and continue incubating. Repeat the feedings as described in this step until total cell number exceeds 5×10^6.
7. Transfer growing cells to 75-cm^2 flask in 50 ml of 37°C complete medium and incubate until cell concentration is $\geq 1 \times 10^6$/ml.
8. Cryopreserve aliquots of cells (*UNIT 1.1*) and maintain B cell line by splitting to 1×10^5 cells/ml in complete medium, incubating to 1×10^6 cells/ml, then splitting again.

   The cells can be maintained this way indefinitely in the absence of any contamination with microorganisms.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete culture medium

RPMI 1640 medium containing:
5% fetal bovine serum (FBS, heat-inactivated; *APPENDIX 2A*)
100 U/ml penicillin
100 µg/ml streptomycin
100 µg/ml gentamycin
2 mM L-glutamine
10 mM HEPES
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

EBV is able to transform a subset of human resting B cells from peripheral blood (Sugden and Mark, 1977; Aman et al., 1984). The outgrowth of EBV-transformed B cells is prevented by the presence of EBV-immune T cells contained within the peripheral blood lymphocyte population (Rickinson et al., 1979). For this reason, soluble anti-CD3 antibody is included in the transformation process to inhibit the ability of T cells to respond to EBV antigens presented by the transformed B cells. Other procedures to eliminate T cell reactivity that could be utilized include T cell depletion of the lymphocyte population (*UNIT 2.3*) and functional T cell inactivation by cyclosporin A (Tosato et al., 1982).

EBV-transformed B cell lines have proven useful in studies of the cell biology of antigen presentation (Roche and Cresswell, 1990) and in the production of large-scale protein preparations for characterization of MHC antigens (Orr et al., 1979) and MHC-bound peptides (Falk et al., 1991).

Critical Parameters

EBV-transformed B cells will not survive if there is an effective anti-EBV T cell response in the culture. It is essential that T cell functions be blocked with an adequate concentration of anti-CD3 antibody (10 µg/ml is sufficient). Also the number and viability of resting B cells in the culture must be sufficient to permit an efficient transformation by EBV (at least 5×10^6 peripheral blood lymphocytes are recommended). In addition, the titer of infectious EBV in the B95-8 culture fluid must be adequate for transformation. If transformation fails to occur, it will be necessary to obtain umbilical cord leukocytes to measure EBV infectivity (Miller and Lipman, 1973). Different batches of FBS have variable capacities for promoting the growth of EBV-transformed B cells. It is best to predetermine whether the available FBS can support the growth of existing EBV-trans-

formed B cell lines before using it for establishing new EBV-transformed lines. Great care should be taken to avoid contamination with mycoplasma (also see *UNIT 1.5*). Mycoplasma-containing EBV-transformed B cell lines will lose their capacity for continuous growth.

Anticipated Results

With the procedure described above, EBV-transformed B cell lines should be obtained which can maintain continuous growth indefinitely in the absence of contamination by microorganisms.

Time Considerations

Preparation of B95-8 culture supernatants requires a 3-day incubation. The establishment of the cultures for transformation takes 1 to 2 hr once the peripheral blood lymphocyte population has been obtained. Culture for 2 to 3 weeks is required to produce continuously growing EBV-transformed B cell lines.

Literature Cited

Aman, P., Ehlin-Henriksson, B., and Klein, G. 1984. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. *J. Exp. Med.* 159:208-220.

Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351:290-293.

Miller, G. and Lipman, M. 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc. Natl. Acad. Sci. U.S.A.* 70:190-194.

Orr, H., Lopez de Castro, J., Parham, P., Ploegh, H., and Strominger, J. 1979. Comparison of amino acid sequences of two human histocompatibility antigens: HLA-A2 and HLA-B7: Location of putative alloantigenic sites. *Proc. Natl. Acad. Sci. U.S.A.* 76:4395-4399.

Rickinson, A., Moss, D., and Pope, J. 1979. Long-term T cell–mediated immunity to Epstein-Barr virus in man. II. Components necessary for regression in virus-infected leukocyte cultures. *Int. J. Cancer* 23:610-617.

Roche, P. and Cresswell, P. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature* 345:615-618.

Sugden, B. and Mark, W. 1977. Clonal transformation of adult human leukocytes by Epstein-Barr virus. *J. Virol.* 23:503-508.

Tosato, G., Pike, S., Koski, I., and Blaese, R. 1982. Selective inhibition of immunoregulatory cell functions by Cyclosporin A. *J. Immunol.* 128:1986-1991.

Key Reference

Miller and Lipman, 1973. See above.

Presents detailed description of B cell transformation and determination of EBV titers using umbilical cord leukocytes.

Contributed by William E. Biddison
National Institute of Neurological Disorders and Stroke, NIH
Bethesda, Maryland

Laser Capture Microdissection

This unit describes a method for isolating pure populations of cells for biochemical and molecular analysis, called laser capture microdissection (LCM; see Basic Protocol). A second protocol outlines procedures for staining frozen or paraffin-embedded tissue samples for LCM (see Support Protocol).

ISOLATION OF A PURE CELL POPULATION FROM TISSUE SECTIONS

BASIC PROTOCOL

Laser capture microdissection (LCM) provides the scientific community with a rapid and reliable method for obtaining pure cell populations from tissue sections under direct microscopic visualization. It incorporates both an inverted light microscope (with or without a fluorescent module) and a near-infrared laser to facilitate the visualization and procurement of cells. Briefly, a stained slide is placed under a microscope, and a specific adherence cap with an ethylene vinyl acetate (EVA) film is placed onto the tissue. The user moves the slide until the area of interest lies in the center of the microscope's field of view. When the cells of interest are located, a near-infrared laser is fired, which melts the EVA film in the targeted area. The EVA film expands into the void of the stained tissue and solidifies within 200 msec as it rapidly cools. The targeted tissue bonds to the EVA film, retaining the exact cellular morphology, DNA, RNA, and proteins intact. Both frozen and fixed tissue samples are successfully microdissected using LCM. Recovered cells can be analyzed for DNA, RNA, and protein content, and used to construct cell-specific cDNA libraries (Emmert-Buck et al., 1996; Krizman et al., 1996; Simone et al., 1998, 2000; Banks et al., 1999). LCM offers a quick means of procuring pure cell populations; however, it is necessary to follow strict protocols pertaining to fixation, preparation, and handling of tissue samples to be microdissected.

Materials

Stained tissue samples, either frozen or formalin paraffin-embedded, cut into 2- to 10-µm sections, and mounted on plain, uncharged microscope slides
PixCell II Laser Capture Microdissection System (Arcturus Engineering)
CapSure transfer film (Arcturus Engineering)
Compressed gas duster
CapSure pads (Arcturus Engineering)
Cap removal tool (Arcturus Engineering)

Setup LCM apparatus

1. Load the CapSure cassette module with a CapSure cartridge.
2. Move joystick into vertical position to properly position the cap in relation to the capture zone. Place the microscope slide containing the prepared and stained tissue sample on microscope stage. When the target zone for microdissection appears in the viewing area, turn on Vacuum on front of controller to hold slide in place.
3. Slide CapSure cassette backward or forward to place cap at Load position. Slide transport arm over CapSure cap, then lift transfer film transport arm and place cap onto slide.
4. To enable the PixCell II laser, turn the keyswitch located on front of the controller and press the Laser Enable button.

Figure 2.5.1 shows components of the PixCell II.

Contributed by Lu Charboneau, Cloud P. Paweletz, and Lance A. Liotta
Current Protocols in Cell Biology (2001) 2.5.1-2.5.7

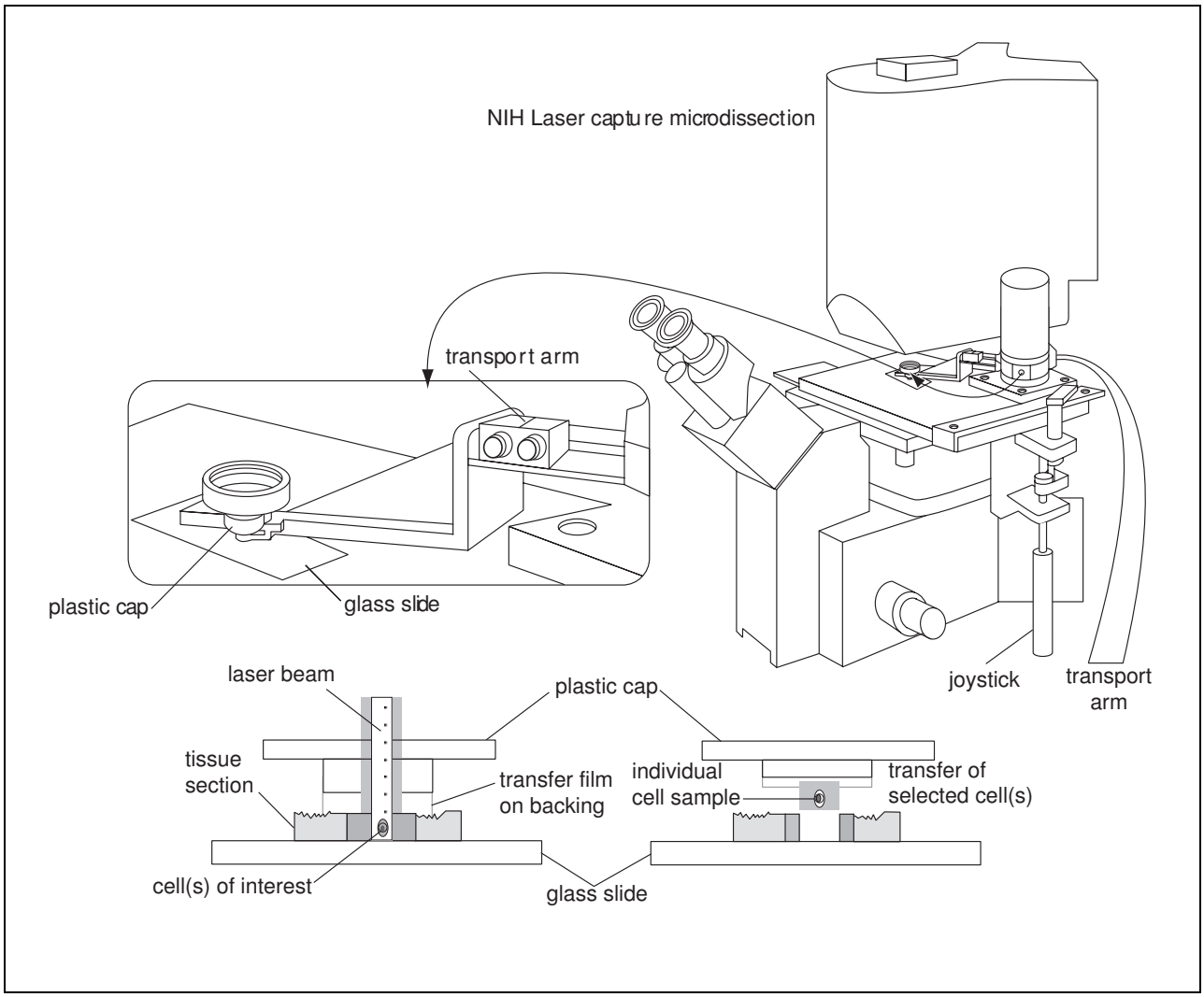


Figure 2.5.1 Schematic overview of the Laser Capture Microdissection Microscope. Through activation of a laser beam and under direct microscopic visualization, individual cells can be isolated for use in molecular analysis of any kind.

5. Verify that target beam is focused by selecting a spot size of 7.5 μm, using the Spot Size Adjust lever found on the left side of the microscope. Rotate microscope objectives until the 10× objective is in place. Reduce light intensity through the optics until field seen on the monitor appears almost dark and target beam is easily viewed. Using the Fine Focus Adjust located below the Spot Size Adjust lever, adjust target beam until it reaches the point of sharpest intensity and most concentrated light with little or no halo effect (Fig. 2.5.2).

   The laser is now focused for any of the three laser spot sizes.

6. Select the laser spot size suitable to perform the microdissection.

7. Adjust Power and Duration of the laser pulse on front of the controller to vary the diameter of the "capture zone", using target settings as a reference point. Adjust these settings up or down to customize the laser to the type and thickness of the tissue to be dissected. Suggested settings are listed in Table 2.5.1.

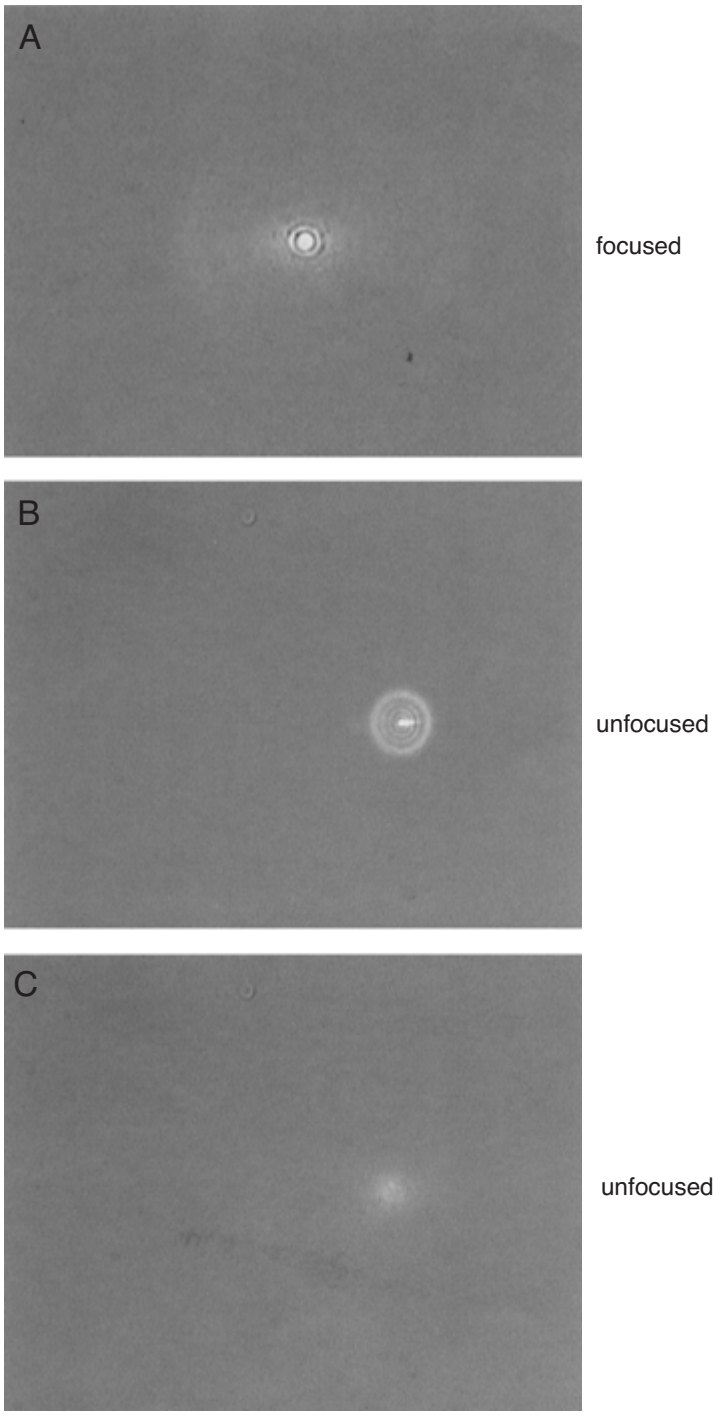


Figure 2.5.2 Focusing of the target beam. If transfer of cells to the cap is not adequate, improper focus may be the cause. Select a spot size of 7.5 μm using the Spot Size Adjust lever found on the left side of the microscope. Rotate the objectives of the microscope until the 10× objective is in place. Reduce the intensity of the light through the optics until the field viewed on the monitor is almost dark and the target beam is easily viewed. Compare the target beam with this figure. If the target is not focused, refocus the target beam as described (see Basic Protocol, step 2).

Table 2.5.1 Settings for LCM

Spot size	Power	Duration
7.5 µm	25 mW	3.0 msec
15 µm	30 mW	5.0 msec
30 µm	30 mW	8.0 msec

Perform LCM

8. Microdissect cells of interest. Guide microdissection with target beam and press Pendant switch for a single microdissection. To microdissect multiple shots, hold Pendant switch down.

   The tissue sample slide must contain no residual xylene, because it melts the transfer film, making microdissection impossible. Dry slide with a compressed gas duster before microdissection to remove residual liquid from staining procedure (see Support Protocol).

   To adjust the frequency interval, select Repeat on controller, then choose the desired time between laser pulses.

9. Observe wetting as the laser fires, maintaining a distinct clear circle surrounded by a dark ring (Fig. 2.5.3). If proper wetting is not observed, refocus the target beam (step 5).

Collect microdissected cells

10. After collecting the desired number of cells, lift cap from slide using the transport arm. Lift and rotate transport arm until cap reaches the Cap Removal Site. Lower transport arm, then rotate it back toward slide, leaving the cap in place at the removal site. Remove cap using the provided cap tool.
11. Blot polymer surface of CapSure transfer film with CapSure pads to remove nonspecific debris adhered to it.
12. Inspect slide and cap for successful microdissection (Fig. 2.5.3).
13. Insert polymer end of cap into top of a 500-µl microcentrifuge tube.

    The sample is ready for extraction of desired components or can be frozen at −80°C for later analysis.

SUPPORT PROTOCOL

HEMATOXYLIN AND EOSIN STAINING OF TISSUES FOR LCM

A reaction sequence for performing basic hematoxylin and eosin staining is outlined in Table 2.5.2. Although LCM is not limited to hematoxylin and eosin staining, it is probably the most versatile stain used. Staining can be done using Coplin jars or racks and dishes. DNA can be retrieved from frozen as well as paraffin-embedded tissue that has been fixed in formalin or ethanol. RNA and protein retrieval is best when frozen tissue is used or alternatively when ethanol-fixed paraffin-embedded tissue is used. RNA and protein are cross-linked when fixed in formalin. However, the morphology of formalin- or ethanol-fixed paraffin-embedded tissues is almost always better than that of frozen tissues, and this is often the deciding factor as to what tissue fixation to use.

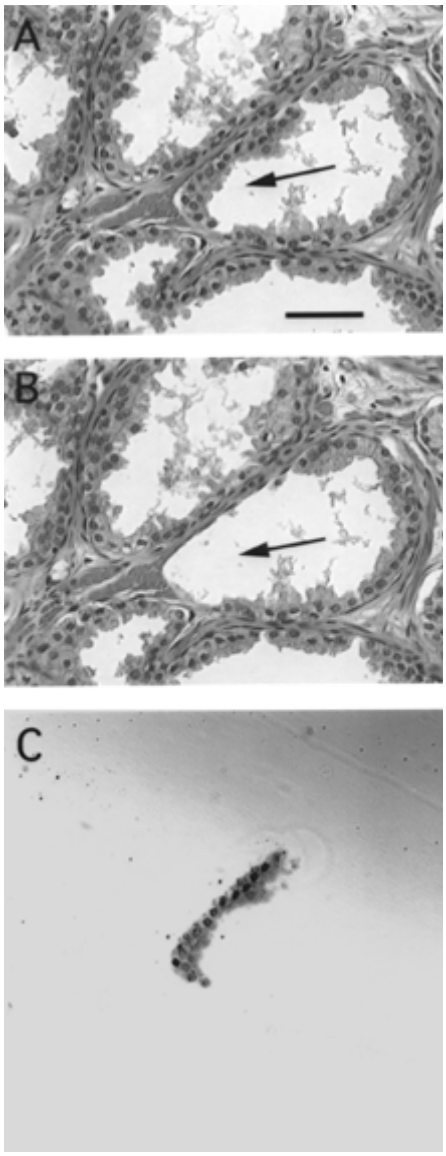


Figure 2.5.3 LCM capture of complex tissue structures. Successful transfer is achieved when nonspecific cells are absent on cap and when microdissection does not leave residual cells behind (see Critical Parameters and Troubleshooting). Example of good microdissection: (**A**) heterogeneous prostate tissue, (**B**) removal of normal epithelium, and (**C**) transfer to EVA film in <5 min.

COMMENTARY

Background Information

One of the problems encountered during the study of disease in actual pathologic lesions arises from the fact that cells in tissue do not exist in a vacuum, but rather interact with each other and neighboring cells in a complex environment. Components of this microenvironment—such as cell-to-cell contact, autocrine and paracrine growth factors, vascular and lymphatic circulation, among many others—influence growth and differentiation. Even more troublesome is the study of changes that occur during disease progression. Most solid tumors are comprised of a large variety of mixed cell populations. For example, a biopsy of breast tissue harboring a malignant tumor may also contain fat cells in the abundant adipose tissue surrounding the ducts, normal epithelium, myoepithelium in the branching ducts, fibroblasts and endothelial cells in the stroma and

Table 2.5.2 Reaction Sequence for Hematoxylin and Eosin Staining Frozen and Paraffin-Embedded Tissues for LCM

Reagent[a]	Frozen tissue	Paraffin-embedded tissue
Xylene	—	2 × 5 min
100% ethanol	—	30 sec
95% ethanol	—	30 sec
70% ethanol	5 sec	30 sec
Deionized water	10 sec	20 sec
Mayer's hematoxylin	30 sec	30 sec
Deionized water	10 sec	20 sec
Scott's tap water	10 sec	20 sec
70% ethanol	10 sec	20 sec
Eosin-Y	60 sec	60 sec
95% ethanol	2 × 10 sec	2 × 20 sec
100% ethanol	2 × 10 sec	2 × 20 sec
Xylene	30-60 sec	30-60 sec

[a]Mayer's hematoxylin and Eosin-Y are available from Sigma-Aldrich; Scott's tap water is available from Fisher.

blood vessels, premalignant carcinoma in situ lesions, and invasive carcinoma. Thus, one cannot simply grind up such tissue and hope to analyze molecular events taking place in premalignant lesions.

In the past, several approaches have been used to overcome this problem. The use of immunological techniques to visualize cells of interest or to deplete contaminating cells has been used to purify or at least enrich cell populations. Modern techniques, such as flow cytometry with cell sorting and affinity-labeled magnetic beads, allow separation of subpopulations from heterogeneous pools of single cells in suspension. However, these techniques are generally not practical when applied to tissue, as there is a requirement for the dissolution of intercellular adhesion and the formation of a suspension of individual cells. Alternatively, other approaches aim to imitate in vivo conditions such as cell cultures of specific cell populations. However, cultured cells may not accurately represent the molecular events taking place in the actual tissue they were derived from. Assuming methods are successful to isolate and grow the tissue cells of interest, the gene-expression pattern of the cultured cells is influenced by the culture environment and can be quite different from the genes expressed in the native tissue state. Another method of obtaining a pure or enriched cell population involves manual extraction of the cells from the tissue sections. This method has been widely used, but it requires a high level of manual dexterity and is often quite time consuming.

Laser capture microdissection (LCM) permits, under direct microscopic visualization, a quick and reliable means of procuring a selected cell population from a heterogeneous tissue, while maintaining the basic integrity of the RNA, DNA, and proteins within the cells (Emmert-Buck et al., 1996; Krizman et al., 1996; Simone et al., 1998, 2000; Banks et al., 1999).

Critical Parameters and Troubleshooting

Problems are rarely encountered during laser capture microdissection, but the most likely causes of difficulty are discussed below. The most probable cause for unsuccessful microdissection is improper handling of the tissue prior to microdissection. For best results, the use of plain uncharged and uncoated microscope slides is recommended. Although, some success has been achieved using positively charged slides, these are not recommended. Tissue sections should be flat and free of folds and wrinkles. The smallest of these imperfections in a prepared tissue section can prevent the proper seating of the polymer cap and limit effective cell capture.

Incomplete deparaffinization of embedded tissues also limits the amount of tissue transferred during microdissection. The times for deparaffinization listed in Table 2.5.2 are minimal and can be extended to allow for complete

removal of the paraffin from the sample, if necessary.

Incomplete dehydration of the tissue section prior to LCM will limit the operator's success in microdissection of the tissue. The xylene rinse, which is the final step of both staining protocols outlined in Table 2.5.2, is essential for the complete dehydration of the tissue on the slide. LCM is compatible with a wide range of immunohistochemical and fluorescent stains in addition to the outlined hematoxylin/eosin stain. It is necessary, however, for the final step of any staining protocol used to be a thorough dehydration step in xylene. If it is suspected that the slide is not completely dehydrated, allow it to sit in xylene for additional time.

Another cause for unsuccessful microdissection is improper focusing of the laser. To avoid this problem, the laser should be focused at the beginning of each LCM session, and whenever there is a change in the type of slide being used (see Basic Protocol).

Anticipated Results

Individual tissue cells can vary in their spacing density, shape, and volume. Therefore, the estimated cell yield is more precise when cells are collected using a greater number of laser pulses. Using the 30-µm laser spot size, the operator can expect to collect, on the average, five to six cells per laser pulse (Fig. 2.5.3).

Time Considerations

The output of LCM is the molecular information derived from analysis of the procured cells. Great care should be taken to ensure the stability of DNA, RNA, or protein molecules analyzed. Delays incurred during staining or while performing LCM can adversely affect molecule stability. DNA is by far the most stable of cellular components. The cellular material collected on the cap for DNA analysis is stable when refrigerated or stored at −20°C until testing is resumed. In contrast, samples microdissected for RNA or proteomic analysis are far less stable because of the endogenous RNases and proteinases. The amount of time that lapses between the start of the staining protocol and completion of microdissection should be closely monitored. Do not exceed 30 min from the time of thawing or deparaffinizing the tissue slide to refreezing of the final dissected sample at -80°C or immediate extraction.

Literature Cited

Banks, R.E., Dunn, M.J., Forbes, M.A., Stanley, A., Pappin, D., Naven, T., Gough, M., Harnden, P., and Selby, P.J. 1999. The potential use of laser capture microdissection to selectively obtain distinct populations of cells for proteomic analysis—preliminary findings. *Electrophoresis* 20:689-700.

Emmert-Buck, M.R., Bonner, R.F., Smith, P.D., Chuaqui, R.F., Zhuang, Z., Goldstein, S.R., Weiss, R.A., and Liotta, L.A. 1996. Laser capture microdissection. *Science* 274:998-1001.

Krizman, D.B., Chuaqui, R.F., Meltzer, P.S., Trent, J.M., Duray, P.H., Linehan, W.M., Liotta, L.A., and Emmert-Buck, M.R. 1996. Construction of a representative cDNA library from prostatic intraepithelial neoplasia. *Cancer Res.* 56:5380-5383.

Simone, N.L., Bonner, R.F., Gillespie, J.W., Emmert-Buck, M.R., and Liotta, L.A. 1998. Laser-capture microdissection: Opening the microscopic frontier to molecular analysis. *Trends Genet.* 14:272-276.

Simone, N.L., Remaley, A.T., Charboneau, L., Petricoin, E.F. III, Glickman, J.W., Emmert-Buck, M.R., Fleisher, T.A., and Liotta, L.A. 2000. Sensitive immunoassay of tissue cell proteins procured by laser capture microdissection. *Am. J. Pathol.* 156:445-452.

Contributed by Lu Charboneau,
Cloud P. Paweletz, and Lance A. Liotta
National Institutes of Health,
National Cancer Institute
Bethesda, Maryland

Preparation of Human Epidermal Keratinocyte Cultures

BASIC PROTOCOL

This unit describes a protocol for establishing keratinocyte cultures from human newborn foreskins. The foreskins are incubated with trypsin to dissociate the dermis and epidermis. Keratinocytes are obtained from the epidermis as described in the steps below, and fibroblasts can be obtained from the dermis as described in *UNIT 2.1*.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

Materials

Human newborn foreskin (for sources, see Background Information)
Betadine (Purdue Frederick) or 5% (v/v) Wescodyne (STERIS Corporation)
HEPES-buffered saline (see recipe)
0.25% (w/v) trypsin in HEPES-buffered saline
Keratinocyte primary culture medium (see recipe)
Hanks' balanced salt solution (HBSS; *APPENDIX 2A*)
Keratinocyte growth medium (either Keratinocyte-SFM from Invitrogen or KGM from Cambrex)
Trypsin/EDTA solution (Invitrogen) diluted 1:1 with Hanks' balanced salt solution (final 0.025% w/v trypsin, 0.26 mM EDTA)
10 mg/ml soybean trypsin inhibitor (Sigma) in Hanks' balanced salt solution
Freezing medium: 10% (v/v) DMSO/10% (v/v) heat-inactivated fetal bovine serum/80% (v/v) keratinocyte growth medium

100-mm sterile tissue culture dishes
Small curved forceps (4 pairs), sterile
Small sharp straight or curved scissors, sterile
Coarse filter such as a tea strainer or several layers of cheesecloth taped over the top of a 50-ml centrifuge tube, sterile
15- and 50-ml sterile disposable centrifuge tubes
75-cm^2 sterile tissue culture flasks
1-ml sterile cryotubes
Liquid nitrogen freezer

Additional reagents and equipment for cell culture (*UNIT 1.1*)

CAUTION: When working with human tissue, appropriate biosafety practices must be followed.

Prepare foreskin

1. Incubate foreskin in Betadine or 5% Wescodyne for 1 min.
2. Wash foreskin four times, each time by incubating for 30 sec in HEPES-buffered saline at room temperature, to remove the disinfectant.
3. Place foreskin in a 100-mm tissue culture dish and trim away as much subcutaneous tissue as possible by snipping it away with a small pair of sharp scissors. Cut the foreskin into 4- to 6-mm pieces, and float them, epidermis-side-up, in 5 ml of 0.25% trypsin in a 100-mm tissue culture dish overnight at 4°C.

   IMPORTANT NOTE: *The scissors used in this step must be sharp.*

Contributed by Susan S. Yamada
Current Protocols in Cell Biology (2003) 2.6.1-2.6.5

When the epidermis is ready to be removed (as described in step 4), there is a change from the shiny and transparent appearance of the fresh epidermis to a more dull and white/opaque surface. Alternatively, the trypsin incubation can be performed at 37°C for several hours. Exposure times must be carefully controlled to curtail trypsin damage to the cells (see Critical Parameters and Troubleshooting).

Remove epidermis

4. Using two pairs of forceps, peel the epidermis from the pieces of tissue and place the detached epidermis in a dish containing 5 ml keratinocyte primary culture medium.

   If the epidermis does not detach from the tissue, incubate as in step 3 for another 8 to 24 hr in fresh trypsin at 4°C. The dermis can be used to isolate fibroblasts (see UNIT 2.1).

5. Release cells from the epidermis by pipetting up and down ten times with a 5-ml disposable plastic pipet.

   The pieces of epidermis may have a tendency to stick to the inside of the pipet. Coating the inside of the pipet with serum proteins by rinsing it with keratinocyte primary culture medium may help prevent this problem.

6. Filter out the epidermal sheets and other debris by passing the cell suspension through a coarse filter such as a tea strainer or several layers of cheesecloth and collecting the filtrate in a 50-ml disposable centrifuge tube.

7. Centrifuge the filtered suspension 5 min at 200 × *g*, room temperature. Aspirate the supernatant and resuspend the cells in 10 ml keratinocyte primary culture medium. Plate the cells in a 75-cm^2 flask (see *UNIT 1.1* for basic cell culture techniques).

8. Place the culture in a humidified 37°C, 5% CO_2 incubator. The next day, inspect for cells attached to the flask.

   The cells may not spread overnight, but there should be some cells attached to the flask. If there are few or no cells attached after an overnight incubation, leave the culture in keratinocyte primary culture medium for up to 1 additional day before changing the medium. Many or most of the cells will never attach.

9. Wash the flask gently three times with Hanks' balanced salt solution and add 15 ml keratinocyte growth medium.

10. Incubate the flask, changing the medium every other day, until the keratinocytes have reached no more than 70% to 80% confluency.

    IMPORTANT NOTE: *Do not permit the cells to become confluent. Confluent cells are very difficult to remove and can begin to differentiate and stop dividing (see Critical Parameters and Troubleshooting).*

Subculture cells

11. To subculture the cells, wash the flask twice with Hanks' balanced salt solution. Add 5 ml trypsin/EDTA warmed to 37°C and incubate at room temperature for about 5 min or until most of the cells are rounded up. Rap the side of the flask against the palm of the hand to release the cells from the flask. If most of the cells do not come off, wait 1 to 2 min and rap again.

    Not all of the cells will detach easily, and it is better to leave a few behind than to overtrypsinize the rest. In general, keratinocytes are more difficult to remove than fibroblasts, and they are also more sensitive to being overtrypsinized (see Critical Parameters and Troubleshooting). It is therefore important that the trypsin/EDTA solution be very fresh. One may wish to freeze aliquots and defrost only as much as needed.

12. After the cells are released from the flask surface, add an equal volume of soybean trypsin inhibitor solution to the trypsinized cell suspension and transfer to a 15-ml disposable centrifuge tube. Centrifuge 5 min at 200 × *g*, at room temperature. Aspirate the supernatant and resuspend the pellet of cells in keratinocyte growth medium. Count cells (*UNIT 1.1*).

13. Plate at ~6×10^3 cells per cm^2 in 75-cm^2 tissue culture flasks.

    This represents about a 1:5 split ratio, and the cells will usually be ready to trypsinize again in about 4 or 5 days. At this split ratio, keratinocyte cultures will usually stop growing at around passage 10 or 11. However, other characteristics of the cultures may begin to change well before this time, and investigators should determine the maximum useful lifespan of these cells for their individual purposes.

14. Change the medium every other day.

Freeze cells and defrost at time of use

15. Resuspend the pellet of trypsinized cells in freezing medium. Dispense into 1-ml sterile cryotubes at 5×10^5 cells/ml, and place in a styrofoam box in a −80°C freezer overnight. Transfer the cryotubes to liquid nitrogen the next day.

16. To defrost cells, thaw a tube of cells in a 37°C water bath. As soon as the cell suspension is thawed, transfer to a 75-cm^2 tissue culture flask containing 15 ml keratinocyte growth medium and incubate at 37°C. As soon as most of the cells have attached, which should take several hours, wash gently several times with Hanks' balanced salt solution and add fresh keratinocyte growth medium.

    The addition of serum to the freezing medium ensures better survival of the frozen cells, but results in high calcium levels once they have been defrosted. Therefore, it is important to wash the cells and change into fresh keratinocyte growth medium as soon as they have attached.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

HEPES-buffered saline

480 ml water
0.5 ml 1 M HEPES buffer solution (1 mM final)
0.9 g glucose (10 mM final)
1.5 ml 1 M KCl (3 mM final)
1 ml 0.5 M $Na_2HPO_4 \cdot 7H_2O$ (1 mM final)
3.8 g NaCl (130 mM final)
0.1 ml 0.5% (w/v) phenol red solution (final 1 µg/ml)
Adjust the volume to 500 ml
Filter sterilize through a 0.22-µm filter
Store up to 1 year at 4°C

Keratinocyte primary culture medium

500 ml DMEM (*APPENDIX 2B*)
12 ml 1 M HEPES (20 mM final)
100 ml fetal bovine serum (FBS; heat-inactivated 30 min at 55°C)
0.6 ml 100 µg/ml EGF (Sigma-Aldrich; 10 ng/ml final)
0.6 ml 10^{-7} M cholera toxin (*Vibrio cholerae*, lyophilized powder; Sigma-Aldrich; 10^{-9} M final)

continued

2.4 ml 100 μg/ml hydrocortisone (Sigma-Aldrich; 0.4 μg/ml final)
6 ml 100× penicillin/streptomycin (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate final)
0.6 ml 250 μg/ml amphotericin B (0.25 μg/ml final)
0.6 ml 10 mg/ml gentamicin (10 μg/ml final)
Store up to 1 month at 4°C in the dark

As an alternative to the 100 ml of FBS, it is possible to use 50 ml calf serum and 50 ml FBS, both heat-inactivated.

CAUTION: *Cholera toxin is very toxic. Use appropriate precautions when handling.*

COMMENTARY

Background Information

Early attempts to grow keratinocytes used medium supplemented with serum and frequently required fibroblast feeder layers (Rheinwald and Green, 1975). The development of serum-free MCDB 153 (Boyce and Ham, 1983) permitted keratinocyte culture without feeder layers. Both of the commercially available serum-free media recommended in this unit (Keratinocyte-SFM from Invitrogen and KGM from Cambrex) are modifications of MCDB 153. They feature low Ca^{2+} concentrations to inhibit keratinocyte differentiation and suppress fibroblast growth and are supplemented with bovine pituitary extract as the primary mitogen. If the undefined composition of bovine pituitary extract is a problem in the experimental system, a medium that replaces it with several defined mitogens is available (Defined Keratinocyte-SFM from Invitrogen).

This protocol can also be adapted to use biopsies or samples of skin from surgical operations or cadavers. A similar protocol has been used to obtain murine keratinocytes (Dlugosz et al., 1995). However, murine keratinocytes are difficult to passage successfully, which can limit their usefulness.

Neonatal human foreskins from circumcisions may be available locally, or they can be obtained through the Cooperative Human Tissue Network (*http://www-chtn.ims.nci.nih.gov/*). Foreskins can be stored immersed in HEPES-buffered saline or serum-free medium such as DMEM (*APPENDIX 2B*) with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, at 4°C for up to 3 days before use.

Critical Parameters and Troubleshooting

This protocol specifies two media: keratinocyte primary culture medium and keratinocyte growth medium. Keratinocyte primary culture medium contains DMEM with 20% serum and is used initially to encourage the keratinocytes from the epidermis to attach. As soon as they attach, the medium is changed to keratinocyte growth medium, a commercially available low-calcium, serum-free medium, which is used in all subsequent cultures.

The concentration of calcium in the medium can regulate whether keratinocytes differentiate or continue to proliferate. The calcium levels in the recommended growth media are low: 0.09 mM in Keratinocyte-SFM from Invitrogen, and 0.15 mM in KGM from Cambrex. Raising the calcium concentration, either intentionally, e.g., by adding $CaCl_2$ to 1 mM, or unintentionally, e.g., by adding factors dissolved in solutions containing calcium, will cause keratinocytes to terminally differentiate. Their growth rate will greatly decrease and their morphology will change markedly (Fig. 2.6.1) from flattened cells with ruffled edges that associate only loosely with neighboring cells to more tightly packed and well-circumscribed colonies of less-spread cells. Differentiated cells are often more phase-dense by phase-contrast microscopy, with phase-bright borders where they contact neighboring cells because the cells are more closely packed and thicker. Ruffled cell margins, if present, are seen only in cells with free edges at the perimeter of colonies. Whereas cells in low calcium can be quite motile, particularly those with semicircular ruffles, cells in high calcium move much less and tend to associate strongly with other cells. Permitting the cells to become confluent will also signal them to slow their growth and differentiate, and cultures that have been allowed to grow to confluence should be replaced with new cells from a frozen stock.

Keratinocytes are relatively sensitive to overtrypsinization, so care should be exercised when passaging the cultures. Use fresh trypsin and expose the cultures for a minimal period.

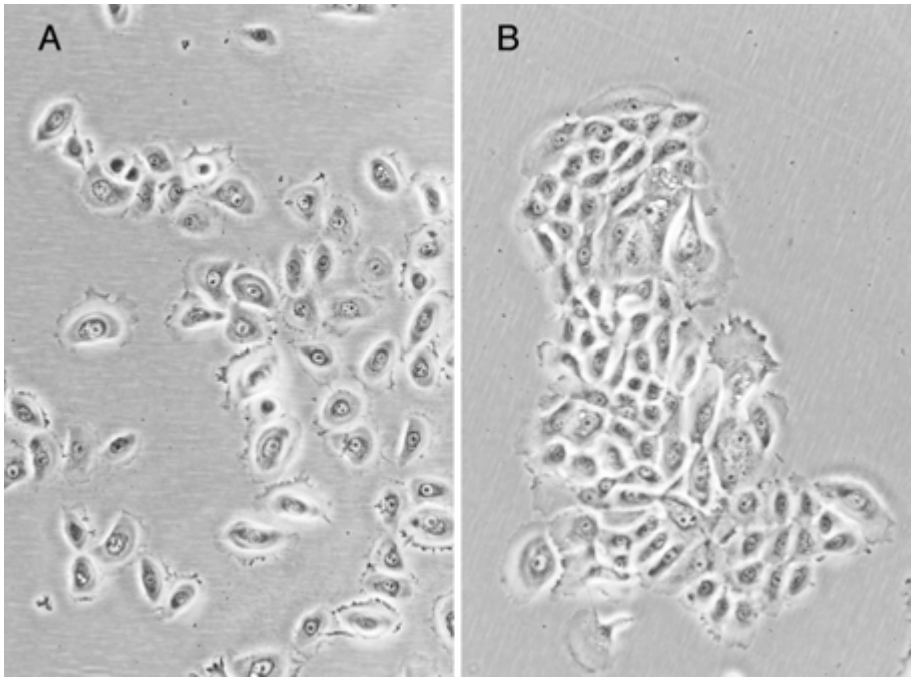


Figure 2.6.1 Human keratinocytes in culture (passage ~6) in: (**A**) low (0.09 mM) or (**B**) high (1 mM) calcium. Magnification: 130×.

Do not trypsinize until all the cells have been removed from the flask; there should always be some cells left attached. Immediately add soybean trypsin inhibitor solution to neutralize the trypsin.

Anticipated Results

After 1 to 2 days in keratinocyte primary culture medium, one should be able to see cells attached to the flask, although they may not be spread. After changing the medium to one of the low-calcium keratinocyte growth media, spread keratinocytes should be visible within 1 to 2 days. Cultures should be ready for passage within 1 or 2 weeks, depending on the initial yield. Early-passage cells should be frozen promptly. From a 75-cm^2 flask at about 80% confluence, it should be possible to harvest ~2 × 10^6 cells.

Time Considerations

Trimming of the foreskin and placing it into trypsin may take 1 to 2 hr, with another hour or less the next day to peel off the epidermis and place the cells into culture. If the epidermis cannot be separated from the dermis after overnight trypsinization, another 8- to 24-hr incubation may be necessary.

Literature Cited

Boyce, S.T. and Ham, R.G. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81:33s-40s.

Dlugosz, A.A., Glick, A.B., Tennenbaum, T., Weinberg, W.C., and Yuspa, S.H. 1995. Isolation and utilization of epidermal keratinocytes for oncogene research. *Methods Enzymol.* 254:3-20.

Rheinwald, J.G. and Green, H. 1975. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell* 6:331-344.

Internet Resources

http://www-chtn.ims.nci.nih.gov/

Web site of the Cooperative Human Tissue Network, which is a program supported by the National Cancer Institute that can provide human tissue at nominal cost.

Contributed by Susan S. Yamada
National Institute of Dental and Craniofacial Research, National Institutes of Health
Bethesda, Maryland

Figure 2.6.1 Human keratinocytes in culture (passage ~6) in: (**A**) low (0.09 mM) or (**B**) high (1 mM) calcium. Magnification: 130×.

Preparation and Coculture of Neurons and Glial Cells

In the development and maintenance of the nervous system there is a complex interdependency between neurons and glial cells. This relationship is vital for their individual differentiation, development, and functionality but also seems to play an important role in progressive neurodegeneration and in the modulation of neurotoxic effects.

Glial cells maintain normal functioning of the nervous system both by controlling the extracellular environment and by supplying metabolites and growth factors. Neurons may interfere with the proliferation and maturation of glial elements (Steward et al., 1991; Gegelashvili et al., 1997) and dynamically regulate the glial signaling pathway through the release of substances such as glutamate (Bezzi et al., 1998). There is also evidence that glia can be neurotoxic both in vivo and in vitro and can exacerbate neuronal damage caused by a variety of agents (Brown et al., 1996; Meucci and Miller, 1996; Rogove and Tsirka, 1998; Viviani et al., 1998). This effect may be attributable not only to an altered supply of trophic factors to neurons, establishment of contacts, and an altered buffering of the extracellular microenvironment by glia, but also to direct release of substances toxic to neurons such as reactive oxygen species (ROS), glutamate, and some cytokines—e.g., interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).

Physiologically, the bidirectional communication existing between glia and other cells of the nervous system (i.e., neurons, other glial cells, and blood vessel cells) allows glia to link cells and structures that are not functionally connected and to continuously monitor and modulate their activity as a function of local needs (for a review, see Volterra and Meldolesi, 2005). The consequences of such interactions are the modulation of synaptic transmission, neuronal synchronization, and the regulation of cerebral blood flow. The recognition of this connection led to the definition of a new function—gliotransmission. The term gliotransmission describes the release of factors from physiologically stimulated glia; these factors are able to activate a rapid response in neighboring cells (Volterra and Meldolesi, 2005).

Cocultures of different cells of the nervous system (i.e., neurons, astrocytes, and microglia) represent the easiest approach to: (1) study intercommunication between the different cell populations of the nervous system, (2) evaluate its relevance in several physiological responses and/or propagation of the damage, and (3) study the molecular mechanisms involved. Other possible approaches are to use aggregate cultures of neural and glial cells and the more complex organotypic slices of hippocampus (Harry et al., 1998).

This unit describes procedures to set up a sandwich coculture system with a combination of neurons and glial cells (see Basic Protocol 1) or astrocytes and microglia cells (see Basic Protocol 2), as well as methods for the preparation of hippocampal neurons (see Support Protocol 1), glial cells (see Support Protocol 2), coated glass coverslips (see Support Protocol 4), and the separation of astrocytes and microglia from glial cells (see Support Protocol 3). A sandwich coculture is an in vitro cell system formed by two different cell populations growing on different surfaces, usually a coverslip and a petri dish. These surfaces are separated by small paraffin dots at the edges of the coverslip, on which one of the cell populations is seeded. In this way the two cell populations face each other without touching (Fig. 2.7.1), and soluble substances can diffuse between them. This cell system is therefore suitable for the study of biological responses that are due to the release of soluble mediators but not of those dependent on the contact of the two cell types.

Contributed by Barbara Viviani

Current Protocols in Cell Biology (2006) 2.7.1-2.7.21

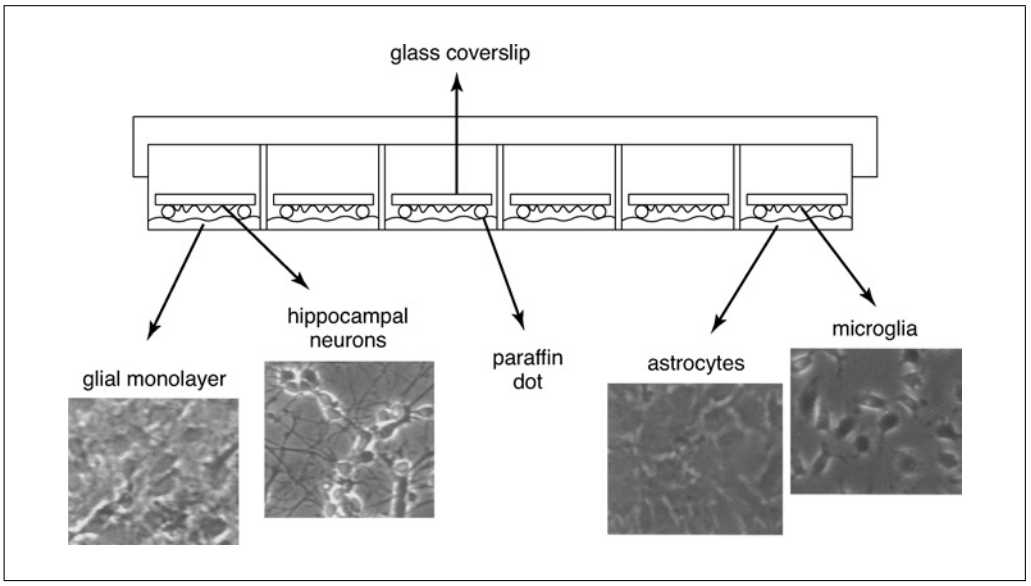


Figure 2.7.1 Scheme of a typical coculture (i.e., hippocampal neurons–glia or microglia-astrocytes) in a 24-well plate. Bright-field photographs are at 10× magnification. Note that cell populations are separated by paraffin dots on the edges of 12-mm round glass coverslips.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: Working conditions must ensure the highest degree of sterility. Thus, brain tissues should be dissected and cells plated and grown in a laminar flow hood. Equipment must be sterilized before use and stored in 95% ethanol when not in use. All solutions and reagents that come into contact with tissues or cells must be sterile.

NOTE: All culture incubations should be performed in a 37°C, 5% CO_2 incubator with 95% relative humidity unless otherwise specified. As soon as prepared, cultures must be maintained in this type of incubator.

BASIC PROTOCOL 1

PREPARATION OF HIPPOCAMPAL NEURON-GLIA SANDWICH COCULTURES

In the author's laboratory, the neuron-glia sandwich coculture is used not only to study the interaction between glia and neural cells, but also to obtain a highly differentiated neural culture. Since glial cells are a valuable source of neurotrophic substances, cultures of cortical glial cells (see Support Protocol 2), consisting mainly of astrocytes and a small number of microglial cells, are prepared and allowed to reach confluence (∼10 days). These monolayers can be used for up to 1 month from their preparation. Next, cultures of hippocampal neurons are prepared by plating onto glass coverslips. These coverslips are then placed above the glial monolayer as shown in Figure 2.7.1. In the author's laboratory, the coculture is set up in 24-well plates, with one coverslip for each well. This avoids overlapping of different coverslips, which can occur if petri dishes are used. The main steps in preparing a neuron-glia sandwich coculture are summarized in Table 2.7.1. The dissection and culture techniques described here are largely based on the method of Goslin and Banker (1991) but have undergone minor changes in the author's laboratory.

Table 2.7.1 Preparation of Neuron-Glia Sandwich Coculture

Day	Action
1[a] (Thursday)	Prepare primary glial cultures (see Support Protocol 2)
8 (Friday)	Add paraffin dots and coat glass coverslips (see Support Protocol 4)
10 (Monday morning)	Wash and condition glass coverslips
	Substitute DMEM with SFM in glial cells
10 (Monday afternoon)	Prepare and seed hippocampal neurons (see Support Protocol 1)
11 (Tuesday)	Transfer coverslips with neurons over glial cells and add cytosine arabinoside to the coculture
18 (Tuesday)	Perform experiments

[a]Note this is 10 days before hippocampal dissection.

Materials

Confluent cortical glial monolayer in 24-well plates (see Support Protocol 2)
Serum-free medium (SFM; see recipe)
Coated glass coverslips seeded with hippocampal neurons (see Support Protocol 1)
2 mM cytosine arabinoside (see recipe)
Sharpened forceps

1. On the day hippocampal neurons are to be prepared (see Table 2.7.1), replace the medium in confluent cortical glial monolayers with 1 ml SFM and return them to the incubator overnight.

   The addition of SFM to glia prior to adding glass coverslips with hippocampal neurons allows conditioning to favor early phases of neural maturation. SFM is chosen because the ingredients are totally defined, and the absence of serum limits the growth of glial cells together with neurons on the glass coverslip.

   Since the author's laboratory usually prepares a number of 24-well glial plates, an amount sufficient for cocultivation with neurons produced over 1 month (i.e., more than will probably be used in one experiment), the medium is replaced only in wells that will receive a coverslip plated with neural cells the following day.

2. The next morning, use one tip of a pair of sharpened forceps to lift the edge of a coated glass coverslip plated with hippocampal neurons. Seize the coverslip with the forceps, and transfer and turn it over the glial monolayer. Repeat for each well to be used.

3. To each well containing neurons and glia, add 2 mM cytosine arabinoside to a final concentration of 5 μM to reduce the proliferation of glial cells.

   Addition of cytosine arabinoside, toxic to dividing cells, is important to block glial proliferation in the neuronal culture.

   In other workers' hands, cytosine arabinoside is usually added on the second to fourth day of culture. Under the experimental conditions used in the author's laboratory, such delayed addition of arabinoside did not satisfactorily reduce glial contamination. This is probably because of the large number of neural cells seeded by the author (160,000/coverslip), which enriches the cell suspension with rapidly dividing astrocytes. Addition of cytosine arabinoside immediately after neuronal cells are attached allows this laboratory to obtain a 98% pure neuronal culture on the glass coverslip, as assessed by immunocytochemistry of microtubule-associated protein 2 (a marker for neurons) and glial fibrillary acidic protein (a marker for astrocytes).

   Under these experimental conditions cytosine arabinoside is not toxic to neurons.

4. Maintain the cocultures, routinely feeding once every 7 to 10 days with SFM.

Neurons are very sensitive to temperature and environmental conditions. Thus, if the incubator is opened often during the day it is better to keep the plates towards the bottom where the temperature, percentage CO_2, and humidity are better preserved.

During feeding, it is important not to change the culture medium completely, since neurons depend upon glial cells to condition the medium for long-term survival. Thus, replace $\sim\frac{1}{3}$ of the medium each time. Under such conditions neurons survive for several weeks and become richly innervated.

These cells reach a high degree of maturation (i.e., developed neuronal network, functional glutamatergic system, and almost complete development of the post-synaptic density) after ~7 to 9 days of culture.

SUPPORT PROTOCOL 1

ISOLATION AND SEEDING OF HIPPOCAMPAL NEURONS

The author prepares hippocampal cultures from 18-day-old fetal rats (Table 2.7.1, day 10). At this stage, the generation of pyramidal neurons is complete while the generation of dentate granule cells has only just begun. Thus, the culture obtained will consist mainly of pyramidal neurons, which constitute the principal cell type in the hippocampus (85% to 90% of total neurons). From one litter of 18-day-old embryos (i.e., nine to twelve embryos), $\sim 7 \times 10^6$ cells for 30 to 40 coverslips 12 mm in diameter (160,000 cells/coverslip) are obtained. The number of coverslips is of course related to the plating density, which has to be chosen according to the experiment to be performed and the techniques available in the laboratory.

Low-density cultures suitable for the study of the development of individual cells and their synaptic interactions or the distribution of antigens within single cells require the application of microscopic techniques because of their sparse distribution. The author's group found that a plating density of 160,000 cells/coverslip yielded a highly differentiated hippocampal culture with a sufficient number of cells for the independent measurement of several parameters of neurotoxicity by nonmicroscopic and quantifiable techniques (Table 2.7.2).

Table 2.7.2 Parameters Measured in Hippocampal Cultures at Low and High Density

Parameter	Low-density cultures	High-density cultures
Neural cell death	Vital dye exclusion and count of living cells	MTT test (Denizot and Lang, 1986)
Apoptosis	Conformation of nuclei using Hoechst 33258 or propidium iodide dyes (Zhivotovsky et al., 1999)	Quantification of oligonucleosomal fragments
Intracellular Ca^{2+} homeostasis	Confocal microscopy (*UNIT 4.5*), video imaging	Spectrofluorimetric measurement in cell population stained with Fura-2 AM (Gunter and Gunter, 1999)
Reactive oxygen species production		Spectrofluorimetric measurement in cell population stained with 6-carboxy-2′,7′-dichlorohydro-fluorescein diacetate
Production of cytokines	Immunohistochemistry (Watkins et al., 2000)	Quantification by ELISA and/or RT-PCR (Bookout et al., 2006)

The procedures performed for isolating hippocampal neurons from rat embryos in the author's laboratory are similar to those described by Goslin and Banker (1991). This section provides an alternative for the dissociation of the hippocampi obtained and for growth in order to set up a sandwich coculture. The highest number of healthy neurons will be obtained by minimizing the total time between removal of uterine horns and dissociation of neurons, which must not exceed ~2 hr.

Materials

Pregnant female rats (Sprague-Dawley) at gestational day 18 (E18)
95% ethanol
HBSS (see recipe)
1× trypsin/EDTA solution (Sigma)
10 mg/ml DNase I stock solution (see recipe)
High-glucose MEM/10% (v/v) FBS (see recipe)
0.04% (w/v) trypan blue

Dissecting tools, sterile:
- Stainless steel scissors with ~4- and ~2-cm blades
- Curved forceps (2 pairs)
- Dumont forceps, no. 3c and no. 5

100- (two), 60- (one) and 35-mm (six) petri dishes
Dissecting microscope, e.g., Zeiss Stemi DV4
Anesthetizing chamber connected to a CO_2 tank
1.5-ml microcentrifuge tubes, sterile
1-ml and 200-µl pipet tips, sterile
24-well tissue culture plate containing coated coverslips (see Support Protocol 4)
75-cm^2 canted-neck flasks with screw caps

Additional reagents and equipment for determination of cell number and viability with a hemacytometer (*UNIT 1.1*)

Prepare for dissections

1. Arrange all the sterile dissecting tools on a sterile surface (e.g., sterile lid of a petri dish).
2. Fill all the petri dishes required for the preparation with the appropriate amount of HBSS (10 ml for 100-mm dishes, 5 ml for 60-mm dishes, and 2 ml for 35-mm dishes).
3. Set up the dissecting microscope.

Remove uterine horns and isolate embryo head

4. Sacrifice the day 18 pregnant animal by an officially approved procedure.

   Anesthetization is often required prior to decapitation. Anesthetize the pregnant rat in an anesthetizing chamber filled with CO_2. Wait until the rat has stopped breathing for a few seconds.

   Longer anesthesia is unnecessary and may result in damage to fetal neurons.

5. Remove the rat from the anesthetizing chamber and transfer it to a dissecting table, ventral side up, and sterilize the abdomen by pouring 95% ethanol over it.
6. Rinse forceps and scissor in ethanol, grasp the abdominal skin with forceps, and cut the abdomen completely open from the vagina to the thoracic cavity. Cut the diaphragm.

7. Gently grasp uterine horns at one of the constrictions and lift up. Remove the horns by cutting the attachments to the abdominal cavity and place them in a 100-mm petri dish filled with 10 ml cold HBSS. Keep on ice.

   Keeping embryos, brains, and later the hippocampi cold reduces metabolic activity and associated cellular damage.

   Typically there are ten to twelve fetuses in a Sprague-Dawley litter.

8. Remove the fetuses from the uterine horns and place them in a 100-mm petri dish with cold HBSS. Keep on ice.

   To remove fetuses grasp at the upper constriction of each yolk sac and cut along one side, the fetus will slip out of it.

9. Decapitate the fetuses with scissors and place the heads in a 60-mm petri dish with cold HBSS. Keep on ice.

Dissect out embryo brain

10. Place one head in a 35-mm petri dish (2 ml HBSS), with the neck down and the skullcap towards the operator. Under the dissection microscope, gently grasp the cut edges of skin and skull with both the Dumont no. 3c and no. 5 forceps and pull in opposite direction to expose the brain.

    Place anterior end of the head towards the top side of the petri dish and posterior end towards the bottom. This orientation will greatly facilitate the dissection.

11. Sever the cerebral hemispheres from the cerebellum and the spinal cord with Dumont no. 5 forceps.

12. Remove the brain by lifting upward out of the skull with curved-tip forceps and place it in a 60-mm petri dish with 5 ml HBSS. Keep on ice.

    Place the curved tips of the forceps under the brain and gently lift it upward.

13. Repeat steps 10 to 12 for each head.

Isolate hippocampi

14. Place the brain in a 35-mm petri dish (2 ml HBSS), with the dorsal side up and the posterior end toward the operator. Working under the dissecting microscope, place the two tips of Dumont no. 5 forceps along the brain midline under the cortices, one at the anterior and the other at the posterior end. Use a single cut to isolate the cortices from the brain stem. Repeat for the other hemisphere.

15. Discard the brainstem and orient one of the hemispheres with the medial surface upwards.

    The cerebral hemisphere is defined by a dorsal side that is much more rounded than the ventral side and usually by the olfactory bulb that projects anteriorly. The hippocampus is located in the medial-posterior part of the cerebral cortex on the back of the olfactory bulb. When the medial cortex is perfectly clean, the hippocampus is clearly visible as a C-shaped structure, with its dorsal margin separated from the adjoining cortex by a fissure while the ventral margin is free.

16. Gently remove meninges by pulling them off with Dumont no. 3c and no. 5 forceps.

17. To obtain the hippocampus, make a curving incision with Dumont no. 5 forceps along the dorsal hippocampal fissure. Release the hippocampus by cutting at the end of the curved incision.

18. Transfer the hippocampus to a 35-mm petri dish with 1 ml HBSS and keep on ice.

19. Repeat steps 14 to 18 for each brain.

Prepare hippocampi

20. Collect hippocampi isolated from rat embryos in a sterile 1.5-ml microcentrifuge tube.

21. Centrifuge 4 min at 100 to 150 × *g*, room temperature. Carefully remove the supernatant.

    Aspiration with a pipet is recommended over a vacuum system to avoid aspirating the hippocampi along with the medium.

Trypsinize hippocampi

22. Add 400 µl of 1× trypsin/EDTA solution and 80 µl of 10 mg/ml DNase I solution. Shake gently (do not vortex) and incubate 5 min at 37°C.

    DNase is added to prevent the DNA released by damaged cells from making the dissociation medium too viscous during digestion.

23. While hippocampi are incubating, prepare four 1.5-ml microcentrifuge tubes with 400 µl high-glucose MEM/10% FBS each.

24. Gently aspirate trypsin/DNase solution with a sterile 1-ml pipet (do not use vacuum). Add 400 µl MEM/10% FBS and shake until hippocampi are floating in the medium.

    The serum in the MEM inhibits residual trypsin and prevents overdigestion of the cells.

***Wash out trypsin and DN*ase**

25. Allow the hippocampi to pellet at the bottom of the tube.

26. Gently collect the pelleted hippocampi with a 1-ml pipet in the smallest possible volume of medium and transfer to the first of the four tubes containing MEM/10% FBS (step 23).

    Take care not to destroy the hippocampi during this and the immediately following steps.

27. Gently shake and allow the hippocampi to pellet at the bottom of the tube.

28. Repeat steps 26 and 27 another three times using the remaining tubes in succession.

    Repetitive passage through microcentrifuge tubes with fresh medium washes the hippocampi free from trypsin and DNase I.

Disaggregate and assess cell number and viability

29. In the last microcentrifuge tube, disaggregate the hippocampi by gently drawing up and down in a 1-ml pipet first and then a 200-µl pipet tip. Adjust the volume of the cell suspension to 1 ml using MEM/10% FBS.

    It is important to be very gentle in pipetting: too vigorous or extensive pipetting causes significant cell death. Pipetting in sequence through a 1-ml pipet and then a 200-µl pipet tip gradually yields a single-cell suspension. Note that pipet tips must be sterile.

30. Gently mix 10 µl cell suspension with 10 µl of 0.04% (w/v) trypan blue. Determine the total cell number and viability with a hemacytometer and an inverted phase-contrast microscope (see *UNIT 1.1*).

    Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained (dark blue).

Plate and prepare hippocampal neurons for the sandwich coculture

31. Pipet 160,000 cells into each well of a 24-well plate containing a coated coverslip.

32. Incubate plates overnight and place the coverslip over the confluent glial monolayer the next morning.

Remember to keep the plates towards the bottom of the incubator where temperature, percentage CO_2, and humidity are better preserved. Neural cells will attach to the coverslip surface during this incubation.

Since seeding medium is not suitable for hippocampal neuron survival and differentiation, remember to transfer the coverslips the day after plating.

BASIC PROTOCOL 2

PREPARATION OF ASTROCYTE-MICROGLIA SANDWICH COCULTURES

Glia include three cell types: astrocytes, microglia, and oligodendrocytes. All glial subtypes can greatly influence not only neuronal, but also neighboring glial cell activity. Astrocytes, long considered to be primarily supportive of neurons, are now thought to be active participants in neural circuit functions. Recently it has been observed that astrocytes are organized in distinct territories and may influence the surrounding cells (including other glial cells) due to the release of diffusible extracellular signals (Volterra and Meldolesi, 2005). Thus, astrocytes influence spontaneous astrocytic excitation by releasing glutamate (Nett et al., 2002), which in turn may result in the excitation of neighboring neurons. The astrocytes may control the release of several modulators (i.e., TNF-α, SDF-1α, and glutamate) through the production of eicosanoids (Bezzi et al., 1998).

Under physiological conditions microglia have a "resting" phenotype adapted to the microenvironment of the central nervous system. However, microglia are able to respond quickly to a variety of signaling molecules. Activation at a very early stage in response to damage or alterations to the microenvironment that precede pathological changes is characteristic of microglia, and its activation often precedes reaction of any other cell type in the brain. The author has recently observed that microglial cells can influence the response of astrocytes in producing neurotoxic substances. For instance, the production of tumor necrosis factor-α (TNF-α) after a neurotoxic insult is much higher in a culture of astrocytes and microglia than with astrocytes or microglia alone (Viviani et al., 1998; Bezzi et al., 2001). Thus, the progression of neurodegenerative events is also modulated by glia-glia interactions.

On the basis of the experience gained by the author's group in coculturing neurons and glial cells (Basic Protocol 1), a similar coculture system of astrocytes and microglial cells has been set up in order to study the molecular mechanisms involved in communication between these two cell population. First, the MEM/FBS medium present in a 24-well plate seeded with astrocytes (see Support Protocol 3) is replaced with SFM (see recipe) 1 to 2 days after the preparation of the culture. Next, the tip of a pair of sharpened forceps is used to lift the corner of a coated glass coverslip seeded with microglia (see Support Protocol 3), seize it, and transfer it over the astrocyte monolayer. In this fashion, microglial cells are juxtaposed, without direct contact, with a monolayer of astrocytes. Note that in the author's laboratory, the astrocyte-microglia coculture is usually used within 48 hr of its preparation.

SUPPORT PROTOCOL 2

ISOLATING AND CULTURING CORTICAL GLIAL CELLS

Glial cells (astrocytes, microglia, and oligodendrocytes) are obtained by mechanical and enzymatic dissociation (e.g., trypsin) of cerebral tissue from 1- to 2-day-old old rat pups (Sprague Dawley; day 1, Table 2.7.1). The use of such young rats ensures the absence of viable neurons in the cell suspension. From one 2-day-old pup, the author usually obtains $\sim 5 \times 10^6$ cells.

The author uses confluent cultures of glial cells both to obtain astrocytes and microglia, and to cocultivate hippocampal neurons. Shaking a confluent glial culture makes it possible to separate astrocytes from microglial cells and obtain two purified cultures (see Support Protocol 3). For this purpose the author's laboratory seeds 4×10^6 glial cells in 75-cm^2 culture flasks. To obtain a glial monolayer for neuron-glia coculture, cells are seeded in 24-well plates at a density of 50,000 cells/ml per well. Both the cells seeded in the wells and those seeded in the flasks reach confluence in ~10 days.

Materials

1- to 2-day-old Sprague-Dawley rat pups.
95% (v/v) ethanol
HBSS (see recipe)
10× trypsin/EDTA (Sigma)
10 mg/ml DNase I (see recipe)
High-glucose MEM/10% and 20% (v/v) FBS (see recipe)
0.04% (w/v) trypan blue

Dissecting tools, sterile:
- Stainless steel scissors with ~4- and ~2-cm blades
- 2 pair curved forceps
- Dumont forceps, no. 3c and no. 5
- Bistoury (Aesculap)

100- (one), 60- (two) and 35-mm (four) petri dishes
Dissecting microscope, e.g., Zeiss Stemi DV4
100-μm nylon cell strainer (Falcon)
50-ml conical polystyrene centrifuge tubes
24-well tissue culture plates
75-cm^2 canted-neck tissue culture flasks with screw caps

Additional reagents and equipment for determining total cell number and viability with a hemacytometer (*UNIT 1.1*)

Prepare for dissections

1. Arrange all the sterile dissecting tools on a sterile surface (e.g., sterile lid of a petri dish).
2. Fill all the petri dishes required for the preparation with the appropriate amount of HBSS (10 ml for 100-mm dishes, 5 ml for 60-mm dishes, 2 ml for 35-mm dishes).
3. Set up the dissecting microscope.

Isolate heads from neonatal pups

4. Transport 1- to 2-day-old Sprague-Dawley pups from the cage to the laboratory.

   The authors order a number of pups, usually three, adequate to provide the amount of glial cells needed for 1 month of experiments.

   Neonatal rats can become hypothermic very quickly. To avoid this, place the pups in a nest of cotton in a small open box and cover them as much as possible to help maintain their body temperature.

5. Sacrifice the animal by an officially approved procedure.

   Anesthetization is often required prior to decapitation. To anesthetize pups, place a small piece of cotton wool soaked in halothane or other approved anesthetic in a tube. Place the pup into the tube until it is anesthetized. Replenish the anesthetic between uses if necessary.

6. Gently hold the pup with thumb and forefinger and rinse it with 95% ethanol.

7. Decapitate the pup with scissors over a 100-mm petri dish with 10 ml HBSS. Keep head on ice.

   Keeping head, brains, and later the cortices cold reduces metabolic activity and the associated cellular damage.

8. Repeat steps 5 to 7 for each pup.

   Sterile scissors should be placed on a sterile petri dish lid or in a beaker with 95% ethanol.

Dissect out brains

9. Place one head in a 35-mm petri dish with 2 ml HBSS. Secure the head by holding down the snout with a curved-tip forceps and cut skin and skull along the midline from the base of the skull to the snout with sharpened scissors. To ease removal of the brain, cut left and right side of the skull in the center perpendicularly to the midline.

   Place the head in the petri dish with neck down and skullcap towards the operator. Place anterior end of the head towards the top side of a petri dish and posterior end towards the bottom. This orientation will greatly facilitate the dissection.

   To avoid damaging the underlying brain tissue, place one scissors tip just under the skull bone and lift upward with the scissor blade while cutting.

10. Still keeping the head secured, remove the skull with a curved-tip forceps and expose the brain. Sever the olfactory bulbs at the anterior end of the brain and the spinal cord at the posterior end with Dumont no. 5 forceps.

11. Remove the brain with a curved-tip forceps and collect it in a 60-mm petri dish with 5 ml HBSS. Keep on ice.

    Place the curved tips of the forceps under the brain and gently lift it upward.

12. Repeat steps 9 to 11 for each head.

Isolate cortices

13. To separate the cortices from the rest of the brain, move one brain to a 35-mm petri dish with 2 ml HBSS. Working under the dissecting microscope, place the two tips of Dumont no. 5 forceps along the brain midline under the cortices, one at the anterior and the other at the posterior end. Use a single cut to isolate the cortices from the brain stem.

14. Repeat this step for each brain and store all the cortices on ice in a 60-mm petri dish with 5 ml HBSS.

15. Place one cortex in a 35-mm petri dish (2 ml HBSS) and, still working under the dissecting microscope, gently remove any extraneous tissue as well as the meningeal coverings on the cortical surface by pulling them off gently with Dumont no. 3c and no. 5 forceps. Take care not to destroy the tissue by pressing too hard with the forceps.

    A semitransparent tissue, the meninges, surrounds the surface of the cortex. They are recognizable for their slightly pink color due to the presence of blood vessels. When the meninges are completely removed, the surface of the cortex appears completely white.

16. Repeat step 15 for each cortex.

Disassociate cells

17. Place cortices from 1- to 2-day-old rat pups in a 35-mm petri dish on ice (no medium). To obtain a homogenate of finely minced cortices, accumulate them together in the center of the dish and cut several times in different directions with a bistoury.

18. Add 2 ml HBSS and resuspend the minced cortices by drawing the mixture in and out of a disposable, sterile, 2-ml plastic pipet.

19. Remove the suspension and transfer to a 50-ml centrifuge tube.

20. Repeat steps 2 and 3 twice more to collect all of the minced cortices from the dish. Resuspend minced cortices in 6 ml HBSS.

Trypsinize cells

21. Add 750 µl of 10× trypsin/EDTA and 750 µl of 10 mg/ml DNase I to the suspension.

    DNase is added to prevent the DNA released by damaged cells from making the dissociation medium too viscous during digestion.

22. Seal the capped tube with Parafilm. Vigorously agitate 15 min in a 37°C water bath to favor enzymatic digestion of the tissue. While the tube is being agitated, prepare a 50-ml centrifuge tube containing 12 ml high-glucose MEM/10% FBS.

23. After agitation, remove the tube from the shaking water bath and allow the undissociated tissue to collect at the bottom.

    Avoid centrifugation; dissociated cells must remain suspended so they can be collected.

24. Collect 5 ml dissociated cells with a disposable, sterile, plastic 12-ml pipet and transfer to the 50-ml centrifuge tube containing MEM/10% FBS (step 22).

    Take care not to include undissociated tissue pieces.

Repeat trypsinization and consolidate products

25. Add 6 ml HBSS, 750 µl of 10× trypsin, and 750 µl DNase I to the 50-ml tube containing the remaining undissociated tissue.

    The FBS in MEM (step 22) inhibits trypsin and thus prevents overdigestion of the dissociated cells.

26. Repeat steps 22 and 23, and add these dissociated cells to the 50-ml tube containing the cells from the first digestion as described in step 24.

***Remove trypsin and DN*ase**

27. Filter dissociated cells through a 100-µm nylon cell strainer and collect in a fresh 50-ml centrifuge tube.

    Filtration eliminates any pieces of tissue accidentally collected with dissociated cells.

28. Pellet pooled cells by centrifuging in a swinging-bucket rotor 5 min at 200 to 300 × *g*, room temperature.

29. Carefully remove the medium from the pelleted cells.

Disaggregate and assess cell number and viability

30. Disaggregate the pellet by first adding 2 ml MEM/10% FBS and then gently drawing in and out of a 2-ml disposable plastic pipet until the solution becomes homogeneous. Add an additional 3 ml MEM/10% FBS for a total resuspension volume of ~5 ml.

31. Gently add 100 µl cell suspension to 200 µl of 0.04% (w/v) trypan blue. Determine total cell number and viability with a hemacytometer and an inverted phase-contrast microscope (*UNIT 1.1*).

    Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained (dark blue).

Plate and prepare glia for the sandwich coculture

32. From the 5-ml cell suspension (step 30) aspirate a volume containing a sufficient number of viable cells to prepare the desired number of 24-well plates at a ratio of 50,000 cells/well. Dilute the cell suspension to 50,000 cells/ml with high-glucose MEM/20% FBS.

    The author usually plates a sufficient number of wells for the cocultivation of neurons prepared over 1 month.

    Glial cells are plated and kept in MEM/20% FBS for 5 days, then the medium is changed to MEM/10% FBS. The cells are maintained in this medium up to 1 month.

33. Pipet 1 ml cell suspension into each well of a 24-well plate.

Plate and prepare glia to obtain astrocytes and microglia

34. From the 5-ml cell suspension (step 30), aspirate a volume containing a number of cells sufficient to prepare the desired number of 75-cm^2 canted-neck flasks with screw caps at a ratio of $\sim 4 \times 10^6$ cells/flask. Dilute 4×10^5 cells/ml in MEM/20% FBS.

    Astrocyte and microglial cells are obtained by shaking the glial culture to divide the two cell populations (see Support Protocol 3). Thus, it is important to use culture flasks having screw caps that can be completely tightened to prevent medium spillage during the shaking period.

35. Pipet 10 ml cell suspension into each flask.

Grow cultures to confluence

36. Incubate plates and flasks 24 hr.

37. To eliminate undetached cells, remove the medium and add 1 or 10 ml fresh MEM/20% FBS for each well or flask, respectively. Repeat 5 days later.

38. Five days later, remove MEM/20% FBS, replace with MEM/10% FBS, and continue to grow to confluence, changing the medium twice a week (e.g., Monday and Thursday or Tuesday and Friday).

    Confluence will be reached in ~10 days.

SUPPORT PROTOCOL 3

ISOLATION AND SEEDING OF CORTICAL ASTROCYTES AND MICROGLIA CELLS

The techniques described here to prepare and separate astrocytes and microglial cells are based on the methods of McCarthy and DeVellis (1980) and Giulian and Baker (1986), which have undergone some changes in this laboratory. The method is based on the selective detachment of microglial cells from astrocytes by shaking mixed glial cultures on an orbital shaker; taking advantage of the difference in degree of attachment of the two cell types to tissue-culture plastic. Astrocytes are then further depleted of microglial cells by treatment with L-leucine methyl ester (L-LME), which selectively kills microglia.

Both astrocytes and microglial cultures appear >97% pure, as assessed by immunocytochemistry of glial fibrillary acidic protein (GFAP), a cytoskeletal protein found only in astrocytes, and with *Griffonia simplicifolia* isolectin B4, a selective marker of both resting and activated microglia (Cheepsunthorn et al., 2001). From each flask of confluent glial cells the author's group usually obtains $\sim 5 \times 10^6$ astrocytes. The number of microglial cells separated from each flask is extremely variable and changes with different preparations (i.e., from $\sim 5 \times 10^5$ to $\sim 3 \times 10^6$ cells).

Materials

High-glucose MEM/10% and 15% FBS (see recipe)
0.04% (w/v) trypan blue solution
1× PBS (Sigma; *APPENDIX 2A*)
High-glucose MEM/10% FBS containing 5 mM L-LME (Sigma)
1× trypsin/EDTA solution (Sigma)
24-well plate containing coated glass coverslips (see Support Protocol 4)
Additional reagents and solutions for preparing confluent glial cultures in 75-cm^2 cantered-neck flasks (see Support Protocol 2) and determining cell number and viability with a hemacytometer (*UNIT 1.1*)

Detach microglia

1. Prepare confluent glial cultures in 75-cm^2 canted-neck flasks (see Support Protocol 2).

   Use culture flasks since the caps can be completely tightened to prevent medium spill during the shaking period.

   In the author's laboratory, usually ~4 × 10^6 cells are seeded per flask. Under these conditions glial cells reach confluence after 10 days (i.e., to obtain a confluent monolayer on Monday prepare glial cells on the Thursday two weeks before).

2. Use Parafilm to seal the flask caps and vigorously agitate on an orbital shaker 1.5 hr at 260 rpm, 37°C.

   Within 2 hr of shaking, most of the microglia can be detached from the glial monolayer and subcultured. Astrocytes remain adherent to the flask.

3. Collect the medium from the flasks in one or more 50-ml centrifuge tubes.

   Collect up to 50 ml in each tube; one 50-ml tube is enough for six flasks.

Equilibrate astrocytes and pellet microglia

4. Add 10 ml fresh high-glucose MEM/10% FBS to each flask with a sterile pipet.

5. To pellet the suspended cells, centrifuge the 50-ml tubes 5 min in a swinging-bucket rotor at 200 to 300 × *g*, room temperature. Meanwhile, transfer the flasks to an incubator for at least 1 hr.

   To obtain purified astrocytes, the flasks have to be shaken further overnight. Since it is usually impossible to shake the flasks in an incubator, it is better to allow the fresh medium to equilibrate before shaking again. This period usually suffices for the preparation of microglial cultures.

Disaggregate and assess cell number and viability

6. At the end of the centrifugation discard the supernatant, add 2 ml MEM/10% FBS to microglial-cell pellet using a sterile pipet and disaggregate by drawing up and down. Add an additional 3 ml MEM/10% FBS for a total of 5 ml and resuspend to obtain a homogeneous suspension.

7. Add 100 µl cell suspension to 200 µl of 0.04% trypan blue solution and mix gently. Determine total cell number and viability with a hemacytometer and inverted phase-contrast microscope (*UNIT 1.1*).

   Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained (dark blue).

Prepare and plate microglia for the sandwich coculture

8. Gently resuspend sedimented cells, then dilute to 200,000 cells/ml with MEM/10% FBS.

9. Add 1 ml suspension to each well of a 24-well plate containing coated glass coverslips.
10. Incubate 30 min.

    This short incubation period allows the adhesion of chiefly microglial cells to the substrate.

11. Gently shake the plate, remove the medium, and wash once with 1× PBS.

    By shaking and washing, loosely adhering cells like oligodendroglia are removed.

12. Add 1 ml high-glucose MEM/15% FBS to each well and transfer plates to an incubator.

Purify astrocytes

13. Transfer the flasks containing partially purified astrocytes (step 5) to an orbital shaker and agitate overnight at 260 rpm, 37°C.
14. The next morning, remove the medium and wash the monolayer three times with 1× PBS.
15. Add 10 ml high-glucose MEM/10%FBS/5 mM L-LME to each flask.

    L-LME selectively kills microglial cells and is used to reduce any microglial contamination of astrocytes.

16. Incubate 2 hr.
17. Wash twice with PBS.

Trypsinize astrocytes

18. Add 2 ml of 1× trypsin/EDTA to each flask to detach astrocytes and gently rotate so that the trypsin can spread over the surface where astrocytes are attached. Incubate 5 min.

    Do not exceed this time, otherwise there is danger of overdigestion. If it is necessary to detach astrocytes from several flasks, do not add trypsin to all of them at the same time.

    To detach cells without using trypsin, incubate the monolayer 5 min in 5 ml PBS without calcium and magnesium, supplemented with 5 mM EDTA; however, this procedure gives lower cell recovery than trypsinization.

19. Detach astrocytes by gently shaking and washing the surface of the flask.
20. Transfer the trypsin solution containing astrocytes to a tube containing 2 ml MEM/10% FBS.

    The serum contained in the medium inactivates trypsin. Thus, to avoid overdigestion the cells resuspended in trypsin are immediately added to an equal volume of cell culture medium. If more than one flask is to be trypsinized, the cells can be pooled in a single tube containing cell culture medium. Add 1 ml of cell culture medium for each milliliter trypsin.

Prepare and plate astrocytes for the sandwich coculture

21. To pellet cells, centrifuge 10 min in a swinging bucket rotor at 100 to 200 × *g*, room temperature. Aspirate the medium and resuspend the pellet in 2 ml of MEM/10% FBS.
22. Repeat steps 7 and 8 with the astrocyte suspension.
23. Add 1 ml cell suspension to each well of a 24-well plate.
24. Transfer to an incubator.

    Astrocytes can be maintained up to 1 month.

PREPARATION OF GLASS COVERSLIPS FOR SANDWICH COCULTURES

SUPPORT PROTOCOL 4

Hippocampal neurons and microglial cells do not readily adhere to glass. Thus, to promote their attachment, maturation, and survival, coverslips are coated using poly-L-ornithine, or poly-L-lysine. In addition, three dots of paraffin are placed on each coverslip to create a narrow gap between the two cultivated cell populations.

The use of 24-well plates and 12-mm coverslips to obtain a coculture does not allow an open-face sandwich coculture. This is basically because the cell monolayer below the coverslip degenerates. In 24-well plates, where the well is slightly bigger than the coverslip, this means the destruction of the glial or astrocyte monolayer.

The coverslips are prepared at least one day before the hippocampal neurons (see Support Protocol 1) or microglial cells (see Support Protocol 2) are dissociated. In the author's laboratory, if hippocampi are dissected on Monday, coverslips are prepared the Friday before and kept in polyornithine at room temperature until Monday morning. Next they are washed and incubated in growth medium before being seeded with cells.

Materials

Paraffin wax
1× poly-L-ornithine solution (see recipe)
1× PBS (Sigma)
High-glucose MEM/10% FBS (see recipe)

12-mm glass coverslips
24-well tissue-culture plates
Microwave oven
5- to 10-ml sterile syringe with 0.95 × 40–mm needle
Germicide lamp (e.g., as equipped on a flow hood)

1. Place one 12-mm glass coverslip in each well of a 24-well tissue-culture plate.

   Each time, prepare the exact number of coverslips to be used. This will reduce the possibility of contamination.

2. Sterilize the coverslips by microwaving 10 min at the highest power setting.

3. Place the plates next to a beaker half full of water in order to avoid melting the plastic plates.

4. Heat paraffin wax to ∼100°C. Take an ∼2-ml aliquot in a 5- or 10-ml syringe and apply three small drops near the outer edge of each coverslip at roughly equal distances from each other.

   To melt paraffin, place four pieces in a beaker and place the beaker on a hot plate.

   By working rapidly, it is possible to place several dots over two to three coverslips. The temperature of the paraffin is very important. If it is too hot, it spreads too thin and wide, while if it is too cool, the dots do not adhere to the coverslip and will subsequently detach.

5. Resterilize the coverslips by UV irradiation for 30 min with a germicide lamp.

   The germicide lamp of a flow hood is sufficient. Avoid exposing coverslips to UV after they have been coated with polyornithine.

6. In a laminar flow hood, add 1 ml of 1× poly-L-ornithine solution in each well containing the coverslips.

   Check that the coverslips do not float in the well but are completely covered by polyornithine. To prevent floating, be sure to eliminate any air bubble under the coverslip by gently pressing over it.

7. Incubate the plates up to 2 days at room temperature or 2 hr at 37°C.

   When plates are out of the laminar flow hood, store them sealed with Parafilm in the dark.

8. Immediately before isolating cells (see Support Protocol 2), remove polyornithine and rinse twice with 1× PBS.

   To obtain the best result in the attachment of cells and their development do not let the coverslips dry at any stage.

9. After the final rinsing add 1 ml high-glucose MEM/10% FBS and incubate until the end of cell preparation.

   When isolated and dissociated, cells will be seeded in this same medium.

   The coverslips are now ready to be seeded. Add the desired number of cells over the coverslips without changing the incubation medium.

   In the author's laboratory unused coverslips are discarded.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent for all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cytosine arabinoside, 2 mM

Dissolve 2 mg cytosine-1-β-D-arabino-furanoside (cytosine arabinoside) in 5 ml water. Store up to 6 months at −20°C.

DNase I, 10 mg/ml

Dissolve 100 mg of 536 Kunitz units/mg DNase I (Sigma) in 10 ml HBSS (see recipe). Store up to 6 months in 1.5-ml aliquots at −20°C.

HBSS

To 850 ml H_2O add:
Hanks Balanced Salts powder (Sigma), enough for 1 liter
10 ml 10 mM HEPES (Sigma)
10 ml penicillin/streptomycin stock solution (Sigma)
Adjust volume to 1 liter with H_2O
Sterilize using a 0.22-μm cellulose-acetate disposable vacuum-filtration system (Millipore)
Store up to 1 month at 4°C

The stock solution of penicillin/streptomycin contains 10,000 U/ml penicillin and 10 mg/ml streptomycin. Store this stock solution up to reported expiration date in 5-ml aliquots at −20°C.

High-glucose MEM/10%, 15%, or 20% FBS

500 ml minimal essential medium with Earle's salts (MEM; Sigma)
0.6% (w/v) D(+)-glucose (Sigma)
5 ml penicillin/streptomycin stock solution (Sigma)
5 ml of 200 mM L-glutamine
Shake vigorously to dissolve glucose
10%, 15%, or 20% (v/v) FBS (Sigma)
Sterilize using a 0.22-μm cellulose-acetate disposable vacuum-filtration system (Millipore)
Store up to 1 month at 4°C

Do not add FBS before shaking, otherwise there is excessive foam formation.

continued

The stock solution of penicillin/streptomycin contains 10,000 U/ml penicillin and 10 mg/ml streptomycin (100 U and 100 μg/ml final, respectively). Store this stock solution up to indicated expiration date in 5-ml aliquots at −20°C.

See UNIT 1.1 for more information regarding preparation of medium.

Insulin, 5 mg/ml

100 mg insulin from bovine pancreas (Sigma)
20 ml sterile H_2O
100 μl glacial acetic acid
Store up to 6 months at 4°C

Poly-L-ornithine solution, 1×

Prepare a 100× stock solution by dissolving 10 mg poly-L-ornithine (Sigma) in 6.67 ml water. Store up to 6 months in 1-ml aliquots at −20°C. Dilute to 1× with water.

Progesterone, 0.1 mM

Dissolve 0.314 mg progesterone in 10 ml of ethanol. Store up to 6 months at −20°C.

Putrescine, 1 M

Dissolve 1.6 g putrescine (Sigma) in 10 ml water. Store up to 6 months at −20°C.

Serum free medium (SFM)

To 850 ml H_2O add:
Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham powder (Sigma), enough for 1 liter
3.7 g $NaHCO_3$
0.11 g sodium pyruvate (1 mM final; Sigma)
1 ml 5 mg/ml insulin (see recipe)
100 mg >97% human apotransferrin (Sigma)
100 μl 1 M putrescine (1 μM final; see recipe)
120 μl 0.25 M sodium selenite (30 nM final; see recipe)
200 μl 0.1 mM progesterone (20 nM final; see recipe)
10 ml penicillin/streptomycin (100 U/ml and 100 μg/ml final, respectively; Sigma)
Adjust pH to 7.2
Adjust volume to 1 liter with H_2O
Sterilize using a 0.22-μm cellulose-acetate disposable vacuum-filtration system (Millipore)
Store for up to 1 month at 4°C.

See UNIT 1.1 for more information regarding preparation of culture medium.

Sodium selenite, 0.25 mM

Dissolve 1 mg sodium selenite (Sigma) in 10 ml water. Store up to 1 month at room temperature.

COMMENTARY

Background Information

Coculture systems provide an easy controlled way to study how a cell population can influence the function, viability, and response of another cell population. Other in vitro systems suitable for the study of the interactions between different cell populations are brain reaggregate cultures or organotypic explants. Brain reaggregate cultures are rotation-mediated aggregating cultures constructed from single-cell suspensions of fetal brain characterized by an organotypic cell association.

A more complex situation is represented by the organotypic cultures. These are derived from explants of undifferentiated embryonic brain and retain some of the structural and functional characteristics of the area of origin.

Both organotypic and reaggregate cultures differ from cocultures since they retain a three-dimensional organization. While a flat coculture system is particularly well suited to detect changes in cell function or differentiation and the interference of a compound on these parameters, organotypic and reaggregate cultures are designed to detect changes in cellular organization by morphological and electrophysiological features. Thus, the choice of the cell system largely depends on the question to be answered.

The great advantage of a sandwich coculture system in studies over the other in vitro systems is the possibility of separating the two cell populations at any time (e.g., prior to or after a treatment) while retaining their integrity. This allows the investigator to (1) manipulate the cell types differently before they are treated together, thus providing information on the involvement of specific mediators or biochemical pathways, (2) perform different biochemical measurements on the two cell populations separately at the end of the treatment, and (3) evaluate the activity on highly differentiated neurons in the presence or absence of the glial feeder layer. For example, the author used this system to study the molecular mechanisms involved in the glia-mediated neuronal death triggered by the human immunodeficiency virus glycoprotein 120 (gp-120). The question of whether the production of reactive oxygen species (ROS) induced by gp-120 in glial cells could be responsible for an increased production of IL-1β from glia and subsequent neural death was assessed (Viviani et al., 2001). For this purpose, glial cells were loaded with an antioxidant, washed, and exposed together with hippocampal neurons to gp-120. In this way, the ability of glial cells to produce ROS as a consequence of gp-120 exposure was blocked without altering the neural cells. As a control, hippocampal neurons and unloaded glia were exposed to gp-120. At the end of the treatment, glia and neurons were separated, neural cell death was assessed by the MTT test, and the results compared with the death rate of neurons exposed to gp-120 in the presence of an unloaded glial monolayer. The synthesis of IL-1β in glial cells was also measured by RT-PCR. It was observed that antioxidant pretreatment of glial cells reduced gp-120-induced production of IL-1β and neural cell death.

This model has also been used to monitor the influence of neural degeneration on glial response in modulating neural cell death (Viviani et al., 2000; Villa et al., 2003).

Because there is no contact between the cell populations, sandwich coculture allows one to study how different cell populations can reciprocally influence their functions/viability through the release of soluble mediators. The lack of contact between neurons and glia, or astrocytes and microglia may, however, represent a disadvantage since it does not mirror physiological conditions. Hippocampal neurons can be plated directly onto glial cells, as can microglia onto astrocytes, but the advantage of manipulating one of the two cell populations forming the coculture would be lost.

Critical Parameters and Troubleshooting

Three very important parameters to obtain successful primary cultures are sterility, rapidity in dissecting brain tissues and plating the obtained cells, and satisfactory reagents. Problems encountered during cell isolation and growth, their possible causes, and possible remedies are reported in Table 2.7.3.

Sterility

Working conditions must ensure the highest degree of sterility. This means that, wherever possible, cell isolation, culturing, and preparation of all solutions should be performed in a laminar flow hood. If nonsterile ingredients are added to a solution, the product has to be filtered through a 0.22-μm filter. To maintain sterility, solutions must never be opened outside the laminar flow hood and must be stored in small aliquots. The addition of antibiotics such as penicillin and streptomycin to the culture medium helps to avoid bacterial contamination.

The use of disposable materials for culturing cells reduces the possibility of contamination and is strongly recommended. All disposable materials (even plastic pipets) should be used only once.

Autoclave surgical instruments (i.e., 21 min at 121°C) to sterilize and store in 95% ethanol when not in use. If, during dissection, tools touch nonsterile surfaces or materials, spray them with 70% ethanol and let the ethanol evaporate before using again.

If any contamination occurs, it is wise to eliminate the contaminated cultures, prepare fresh reagents, and clean the laminar flow hood

Table 2.7.3 Troubleshooting Guide for Preparation and Coculture of Neurons and Glial Cells

Problem	Possible cause	Solution
Low cell viability	Prolonged dissection time	Never exceed 2 hr to obtain brain and dissect hippocampi or cortices
Poor neuronal differentiation	SFM incomplete (e.g., without apotransferrin)	Prepare fresh SFM and check that all ingredients are added
	L-Polyornithine lot unsatisfactory	Test different lots and choose the best
	Glial feeder layer too old	To cocultivate hippocampal neurons use a glial feeder layer within 3 to 4 weeks
	Damaged glial feeder layer	Check the morphology of glial cultures every time before adding neurons
Floating cells in cultures	Contamination	See Critical Parameters and Troubleshooting, Sterility

prior to new culture preparation. To clean the laminar flow hood, wash with a detergent first and then with 70% ethanol. Contaminated solutions can be filtered, but sterility has to be checked prior to use by incubating in culture dishes for a few days at 37°C.

Tissue dissection

Rapid dissection of both hippocampi and cortices is essential to obtain a successful neural or glial culture. In the author's experience the shorter the time of dissection the better the culture obtained. The author's group never exceeds 2 hr to obtain brains and dissect hippocampi and cortices. Preparation time for cultures can be considerably shortened by using a team of two people, one removing brains from the skull and the other cleaning, removing, and dissociating hippocampi or cortices.

Reagent lots

Lot-to-lot variability of reagents such as trypsin, poly-L-ornithine, and all the ingredients of SFM as well as FBS, influences the viability, development, and functionality of glia and particularly of hippocampal neurons. Wherever possible, it is better to test different lots of the same reagent before using it for the routine cultivation of glia and neurons. Measurements to identify the best lot are: the number of cells obtained after tissue dissociation (which can depend on the lot of trypsin used), growth rate of glial cells (dependent on serum), cell viability, and differentiation of neural cells (dependent on the poly-L-ornithine or the ingredients of SFM). If poor neural differentiation is observed, also check that all the requisite ingredients have been added to the culture medium.

Anticipated Results

Healthy hippocampal neurons readily attach to coated glass coverslips and are initially characterized as small round shapes. Within a day of coculture they extend short processes (Fig. 2.7.1). A well-developed network is clearly evident at the third or fourth day of coculture. In the following weeks, even if great morphological changes are not evident any more, hippocampal neurons develop a functional glutamatergic system and a complete post-synaptic density. Hippocampal neurons become sensitive to glutamate, showing an increase in cell death and intracellular calcium, starting from the eighth day of culture.

Glial cultures at confluence are characterized by the presence of an overwhelming percentage of astrocytes and ~5% of microglia. Astrocytes have a flat polygonal appearance and form a uniform layer over which small, dark microglial cells can be observed. Microglial cell numbers can increase considerably with aging of the culture. Once detached from astrocytes and plated on a glass coverslip, microglial cells acquire a round shape that tends to ramify as the number of days in culture increases. A "ramified" morphology is typical of resting microglia. The author's group observed no morphological difference in microglia or astrocytes cultivated alone or in coculture <48 hr from their preparation.

Time Considerations

Preparation of hippocampal neuron-glia sandwich cocultures (Basic Protocol 1) or of astrocyte-microglia sandwich cocultures (Basic Protocol 2) requires 35 to 45 min. Hippocampal neuron-glia cocultures can be maintained up to 3 to 4 weeks. In the author's laboratory, the astrocyte-microglia coculture is used within 48 hr from its preparation.

Isolation and seeding of hippocampal neurons (Support Protocol 1) require 1.5 hr to 2 hr. Hippocampal neurons seeded by following the described protocol can be maintained without glial cells up to a couple of days.

Isolating and culturing cortical glial cells (Support Protocol 2) require 2 hr. Cortical glial cells can be maintained up to 1 month.

Isolation and seeding of cortical astrocytes (Support Protocol 3) require 19 hr. Astrocytes can be maintained up to 1 month. Isolation and seeding of microglial cells (Support Protocol 3) require 2 hr. In the author's laboratory, microglia are maintained up to 48 hr from preparation.

Preparation of glass coverslips for sandwich cocultures (Support Protocol 4) requires 2 days when glass coverslips are incubated with poly-L-ornithine at room temperature or 3 hr when glass coverslips are incubated with poly-L-ornithine at 37°C.

Literature Cited

Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Rizzini, B.L., Pozzan, T., and Volterra, A. 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 391:281-285.

Bezzi, P., Domercq, M., Brambilla, L., Galli, R., Schols, D., De Clercq, E., Vescovi, A., Bagetta, G., Kollias, G., Meldolesi, J., and Volterra, A. 2001. CXCR4-activated astrocyte glutamate release via TNF-α: Amplification by microglia triggers neurotoxicity. *Nature Neurosci*. 4:702-710.

Bookout, A.L., Cummins, C.L., Mangelsdorf, D.J. Pesola, J.M., and Kramer, M.F. 2006. High-throughput real-time quantitative reverse transcription PCR. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 15.8.1-15.8.28. John Wiley & Sons, Hoboken, N.J.

Brown, D.R., Schmidt, B., and Kretzschmar, H.A. 1996. Role of microglia and host prion protein in neurotoxicity of prion protein fragment. *Nature* 380:345-347.

Cheepsunthorn, P., Rador, L., Menzies, S., Reid, J., and Connor, S.R. 2001. Characterization of a novel brain-derived microglial cell line isolated from neonatal rat brain. *Glia* 35:53-62.

Denizot, F. and Lang, R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89:271-277.

Gegelashvili, G., Danbolt, N.C., and Schousboe, A. 1997. Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. *J. Neurochem.* 69:2612-2615.

Giulian, D. and Baker, T.J. 1986. Characterization of ameboid microglia isolated from developing mammalian brain. *J. Neurosci.* 6:2163-2178.

Goslin, K. and Banker, G. 1991. Rat hippocampal neurons in low-density culture. *In* Culturing Nerve Cells (G. Banker and K. Goslin, eds.) pp. 251-281. MIT Press, Cambridge, Mass.

Gunter, K.K., and Gunter, T.E. 1999. Measurements of intracellular free calcium concentration in biological systems. *In* Current Protocols in Toxicology (M.D. Maines, L.G. Costa, E. Hodgson, D.J. Reed, and I.G. Sipes, eds.) pp. 2.5.1-2.5.52. John Wiley & Sons, Hoboken, N.J.

Harry, G.J., Billingsley, M., Bruink, A., Campbell, I.L., Classen, W., Dorman, D., Galli, C.L., Ray, D., Smith, R.A., and Tilson, H.A. 1998. In vitro techniques for the assessment of neurotoxicity. *Environ. Health Persp.* 106:131-158.

McCarthy, K.D. and de Vellis, J. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* 85:890-902.

Meucci, O. and Miller, R.J. 1996. gp-120-induced neurotoxicity in hippocampal pyramidal neuron cultures: Protective action of TGF-α1. *J. Neurosci.* 16:4080-4088.

Nett, W.J., Oloff, S.H., and McCarthy, K.D. 2002. Hippocampal astrocytes in situ exhibit calcium oscillations that occur independent of neuronal activity. *J. Neurophysiol.* 87:528–537.

Rogove, A.D. and Tsirka, S.E. 1998. Neurotoxic responses by microglia elicited by excitotoxic injury in the mouse hippocampus. *Curr. Biol.* 8:19-25.

Steward, O., Torre, E.R., Tomasulo, R., and Lothman, E. 1991. Neuronal activity upregulates astroglial gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 88:6819-6823.

Villa, P., Bigini, P., Mennini, T., Agnello, D., Laragione, T., Cagnotto, A., Viviani, B., Marinovich, M., Cerami, A., Coleman, T.R., Brines, M., and Ghezzi, P. 2003. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *J. Exp. Med.* 198:971-975.

Viviani, B. and Corsini, E., Galli, C.L., and Marinovich, M. 1998. Glia increase degeneration of hippocampal neurons through release of tumor necrosis factor-α. *Toxicol. Appl. Pharmacol.* 150:271-276.

Viviani, B., Corsini, E., Galli, C.L., Padovani, A., Ciusani, E., and Marinovich, M. 2000. Dying

neural cells activate glia through the release of a protease product. *Glia* 32:84-90.

Viviani, B., Corsini, E., Binaglia, M., Galli, C.L., and Marinovich, M. 2001. Reactive oxygen species generated by glia are responsible for neuron death induced human immunodeficiency virus–glycoprotein 120 in vitro. *Neurosci*. 107:51-58.

Volterra, A. and Meldolesi, J. 2005. Astrocytes, from brain glue to communication elements: The revolution continues. *Nat. Rev. Neurosci*. 6:626–640.

Watkins, S. Immunohistochemistry. 2000. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 14.6.1-14.6.13. John Wiley & Sons, Hoboken, N.J.

Zhivotovsky, B., Samali, A., and Orrenius, S. 1999. Determination of apoptosis and necrosis. *In* Current Protocols in Toxicology (M.D. Maines, L.G. Costa, E. Hodgson, D.J. Reed, and I.G. Sipes, eds.) pp. 2.2.1-2.2.34. John Wiley & Sons, Hoboken, N.J.

Key References

Goslin and Banker, 1991. See above.

An excellent text and manual describing point-to-point hippocampal cell preparation and astrocyte-neuron coculture.

Harry et al., 1998. See above.

An extensive overview on several in vitro methods to study neurotoxicity, their advantages and disadvantages. A description of organ, slice, and aggregate cultures is also provided.

Contributed by Barbara Viviani
University of Milan
Milan, Italy

CHAPTER 3
Subcellular Fractionation and Isolation of Organelles

INTRODUCTION

In the early 1700s, Leeuwenhoek noted that avian and amphibian blood cells contained a "clear area," almost certainly corresponding to the structure we now know as the nucleus. This represents probably the earliest recognition that eukaryotic cells are not simply sacs of protoplasm, but instead contain subcellular structures. Although the concept of the nucleated cell became firmly established in the 18th and 19th centuries, the real beginning of subcellular fractionation had to await the emergence in the mid-1940s of the art form known as electron microscopy. The electron microscope revealed much more complexity than Leeuwenhoek could have imagined. Concurrent with improvements in the techniques of electron microscopy was the development of methodologies for subcellular fractionation. Through the 1950s, these parallel approaches resulted in the discovery and isolation of the major organelles that comprise the eukaryotic cell. Finally, as biochemical functions were associated with specific subcellular compartments, a much clearer picture of the eukaryotic cell began to emerge, and with it the field of modern cell biology.

Chapter 3 is dedicated to methods for subcellular fractionation. The chapter begins with an overview of cell fractionation (*UNIT 3.1*) which covers some basic principles as well as the instrumentation of centrifugation. The various centrifugation media, ranging from sucrose to the newer media (Ficoll, Percoll, metrizamide, Nycodenz, and Iodixanol), are described.

Much of the earliest work in subcellular fractionation utilized rat liver as starting material. The second unit of Chapter 3 provides protocols for the isolation of plasma membrane sheets and domains from rat liver (*UNIT 3.2*). This method is used to isolate plasma membrane sheets in sufficient yield and purity to be suitable for a variety of analytical and preparative purposes. For example, these preparations can be used as starting material for the isolation of hepatocyte plasma membrane proteins. The sheets also serve as the starting material for the protocols within *UNIT 3.2* that yield the apical and basolateral domains comprising the polarized hepatocyte plasma membrane.

The densities of mitochondria, peroxisomes, and lysosomes are very similar, and as a result, these organelles cosediment in density gradients as the so-called "light mitochondrial fraction." The development of separation media has significantly improved the ability to separate these organelles from each other. *UNITS 3.3–3.6* deal with separation of these organelles.

UNITS 3.3 & 3.4 describe methods for isolating mitochondria from rat liver as well as other sources including other tissues, cultured mammalian cells, and yeast. *UNIT 3.3* focuses on methods involving differential centrifugation for rapid isolation of metabolically active mitochondria, whereas methods involving density-gradient centrifugation for further purification of mitochondria are described in *UNIT 3.4*.

UNIT 3.5 provides methods for the isolation of peroxisomes from mammalian tissues and cells in tissue culture. Also included in this unit are a method for preparation of

Current Protocols in Cell Biology 3.0.1-3.0.8, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb0300s48

peroxisomes from yeast and descriptions of assays for assessing the success of subcellular fractionation of the various starting materials.

The yeast *Saccharomyces cerevisiae* contains all the membrane-bound subcellular organelles that are characteristic of higher eukaryotes, yet this organism has a haploid genome that is less than four times the size of that of *E. coli*. The availability of the entire *S. cerevisiae* genome sequence, together with the ease with which yeast are cultured (*UNIT 1.6*) and the simplicity with which genetic manipulations are performed have made this organism a popular model in eukaryotic cell biology. Indeed, it is yeast cell biology that may provide insights into the function of many yeast genes and their mammalian counterparts. While some attention to certain subcellular organelles from yeast is given in earlier units of this chapter, *UNIT 3.7* provides a comprehensive overview of yeast subcellular fractionation.

UNIT 3.8 describes centrifugation-based methods for isolating most of the major organelles of the yeast *Saccharomyces cerevisiae*. Most of these procedures begin with the preparation of spheroplasts as described in the Support Protocol near the beginning of the unit. Lysates for subcellular fractionation can also be generated by glass-bead lysis to disrupt intact yeast cells (also described). Because of the fragility of most yeast organelles, lysates of spheroplasts are best used for most protocols; however, glass-bead lysates can be used for preparation of cytosol and plasma membranes. Several of the protocols are analytical for assessing the distribution of proteins along exocytic, endocytic, and biosynthetic pathways in yeast cells. The unit also includes preparative methods for isolating yeast nuclei, vacuoles, mitochondria, peroxisomes, endoplasmic reticulum, plasma membranes, and cytosol.

The Golgi, an organelle named for the pioneering cell biologist, Camillo Golgi, was identified on the basis of a then current protocol developed by Golgi for fixing and staining tissues. Although observations of what he then referred to as the "internal reticular apparatus" probably date even earlier in the 19th century, the Golgi remains an intracellular site of intense investigation by today's cell biologists. *UNIT 3.9* describes the isolation of Golgi membranes on a preparative basis. Individual protocols describe isolation of dextrose-treated Golgi stacks from rat liver using a sucrose density barrier and by flotation from a light mitochondrial fraction. Isolation from cultured cells is also described, as is a method to assay for the Golgi marker enzyme, UDP-galactose galactosyl transferase.

UNIT 3.10 focuses on the "clear area" of Leeuwenhoek—the nucleus. As noted above, rat liver has been the most frequently used source of material for subcellular fractionation. This unit contains two protocols for isolation of nuclei from rat liver. The first utilizes a sucrose density barrier, whereas the second employs Nycodenz as a density medium. Although the vast majority of methods for preparing nuclei use soft tissue (e.g., liver), the protocols described in this unit can be applied to other source materials (e.g., cultured cells, plants), provided appropriate homogenization can be achieved. *UNIT 3.10* also contains two protocols for purification of nuclear membranes from isolated nuclei and Support Protocols describing methods for assaying DNA and RNA.

Free-flow electrophoresis (*UNIT 3.11*) provides opportunities for both analytical and preparative applications for isolating subcellular organelles such as rat endosomes. Endosome-enriched fractions are prepared from rat liver using discontinuous sucrose gradients, and these are used to prepare endosomal subpopulations by free-flow electrophoresis. The analytical approach can be used to study the kinetics and cellular subpopulations involved in endocytic traffic. Three distinct subpopulations of endosomes are revealed in such studies. Support Protocols include methods for analyzing markers for endosomal subpopulations and for demonstrating retention of endosomal function (acidification)

in vitro. Methods are also included for preparing and labeling ligands that traffic through endosomes.

UNIT 3.12 describes protocols for the isolation of synaptic vesicles—small, electron-lucent organelles contained within presynaptic nerve terminals. Synaptic vesicles function to store neurotransmitters and to release them into the synaptic cleft upon stimulation. Methods for the purification of synaptic vesicles take advantage of the vesicle's small size and low density. The first two protocols describe the use of differential centrifugation to enrich synaptic vesicles 8- to 10-fold relative to brain homogenate. The next three protocols allow further purification to ~20-fold using sedimentation velocity or chromatography on controlled-pore glass beads or Sephacryl S-1000. This unit ends with protocols for the sedimentation of synaptic vesicles at high speed, immunoisolation of synaptic vesicle subpopulations, and siliconization of tubes.

Transport of proteins, lipids, and other "cargo" molecules between different compartments of the secretory, endocytic and lysosomal targeting pathways is mediated by membrane-enclosed carriers. The best characterized of these carriers are clathrin-coated vesicles (CCVs), which play critical roles in endocytosis from the plasma membrane and sorting from the trans-Golgi network and endosomes. CCVs are small (50 to 100 nm in diameter) and are encased within a dense proteinaceous coat—properties that are the bases for their isolation by ultracentrifugation procedures. *UNIT 3.13* presents a series of protocols for the isolation of coated vesicles from different sources, including adult and developing rat brain, adult rat liver, and cultured cell lines. In addition, this unit contains a procedure for analyzing the purity of isolated CCVs by electron microscopy. The Commentary section shows representative electron micrographs of these preparations, as well as SDS-PAGE analyses of the protein composition of the purified CCVs.

UNIT 3.14 deals with melanosomes, which are membrane-bound organelles filled with the pigment melanin. These organelles are only found in specialized cell types such as melanocytes and retinal epithelial cells, and they function to protect cells and tissues from the harmful effects of ultraviolet radiation. Oncogenic transformation of melanocyte precursors results in melanomas, which are particularly aggressive cancers. This unit presents protocols for the isolation of melanosomes from melanoma cells and tissues. The biogenesis of melanosomes involves maturation of precursors through a series of well-defined stages; the protocols in this unit allow for the separation of early and late stage melanosomes by ultracentrifugation and free-flow electrophoresis. This unit also describes a method for monitoring the purification of melanosomes by measuring the activity of the melanosomal enzyme, tyrosinase. Finally, the unit lists antibody reagents to melanosomal proteins that can be used to monitor purification and assess the purity of the isolated melanosomes.

Most cells in culture contain discernable lipid droplets, although the size and number of these droplets vary depending on cell type and culture conditions. Lipid droplets can provide a source of metabolic energy and also play a role in cholesterol homeostasis required for membrane biogenesis. In steroidogenic cells, the lipid droplets provide substrates for hormone synthesis. *UNIT 3.15* describes the isolation of lipid droplets based on their high buoyancy, which is reflective of their low mass of protein and high relative mass of neutral lipids. At present, no specific enzyme markers have been localized exclusively to lipid droplets. The droplets do, however, contain certain characteristic structural proteins, and protocols for immunoblotting of these proteins are included for assessment of the isolated lipid droplets.

UNIT 3.16 describes the isolation of rat serosal mast cells by peritoneal lavage and the use of these cells as a source of mast cell granules. These granules are modified lysosomes that release their contents upon degranulation of the activated mast cells. Protocols

are provided for the isolation of mast cell granules enveloped in their perigranular membrane and of granule remnants released upon degranulation. Several protocols aimed at assessing mast cell activation are included, as is a protocol for verification of granule remnant morphology and purity via electron microscopy. A future unit in Chapter 3 will deal with the isolation of the analogous organelle from chromaffin cells (i.e., chromaffin granules).

All previous units in this chapter that deal with organelle isolation have described isolation of organelles that exist in multiple copies per cell. In contrast, *UNIT 3.17* provides protocols related to the isolation of centrosomes, of which there is only one per cell. As starting material for this challenging task, the protocols utilize early (0- to 3.5-hr) *Drosophila* embryos. Centrosomes are isolated by a protocol involving gradient centrifugation and a protocol for further enrichment via immunomagnetic purification. Protocols for assessing the centrosome preparation by immunofluorescence microscopy are included.

In contrast to the situation with the centrosome (one copy per cell), pancreatic zymogen granules have been estimated to constitute as much as 25% of the total cellular volume based on electron microscopy. The relative abundance of these organelles resulted in their being among the first to be the subject of cellular subfractionation protocols, dating to the mid-1950's. *UNIT 3.18* provides more modern protocols for the purification of these important cellular bodies from both rat and dog pancreas, using both sucrose and Percoll gradients. The pancreas contains other secretory granules in lower abundance, most notably the endocrine granules responsible for insulin release. A future unit of this chapter will provide protocols related to isolation of the insulin granules of the pancreas.

An early unit in this chapter (*UNIT 3.5*) deals with the organelles known as peroxisomes. Glyoxysomes represent a specialized form of the peroxisome found in the cotyledons of seedling plants. In this context, these organelles sequester the enzymes of the glyoxylate cycle that provides energy to the rapidly growing seedling. Later in plant development, the peroxisomes of the green leaves have a distinct complement of enzymes reflecting their role in photorespiration. *UNIT 3.19* provides protocols for growing pumpkin seedlings and for isolation of glyoxysomes from their cotyledons via density gradient centrifugation.

The glucose transporter isoform known as GLUT4 functions to facilitate uptake of glucose by both fat and muscle tisssues. GLUT4 is translocated in response to insulin from an intracellular compartment to the plasma membrane as the mechanism for insulin-mediated enhancement of glucose transport. The investigation of trafficking of GLUT4 has gained considerable attention since the discovery that GLUT4 translocation is compromised in type 2 diabetes. *UNIT 3.20* provides protocols for isolation of GLUT4 storage vesicles from primary rat adipocytes, cultured adipocytes, and skeletal muscle.

Another type of membrane vesicle is the subject of *UNIT 3.21*, which describes isolation of vesicles from intestinal brush border from three species (rat, pig, and cow). These vesicles are derived from the apical surface of the polarized epithelial cells of the small intestine, which also contain integral membrane proteins involved in glucose uptake. Whereas the GLUT4 vesicles of *UNIT 3.20* are part of a regulated trafficking of cellular membranes, those of *UNIT 3.21* represent spontaneous sealing of membrane fragments derived from the intestinal microvilli upon disruption of the tissue architecture. These vesicles can nonetheless be utilized for membrane transport studies.

The theme of isolation of membrane-derived vesicles continues in *UNIT 3.22*, which provides several protocols related to exosomes. These vesicles are derived from the plasma membrane via an endocytosis-exocytosis mechanism, but they are isolated from extracellular fluids (either tissue culture media or bodily fluids). Methods are provided whereby exosomes are isolated by either ultracentrifugation, ultrafiltration, or immunoisolation.

The protocols of *UNIT 3.22* should prove useful in studies aimed at understanding exosome function and the possible use of exosomes in therapeutic applications e.g., as immunogens in vaccines.

UNIT 3.23 describes the isolation of intermediate filaments from bovine spinal cord. Although intermediate filaments from different sources contain a diverse complement of intermediate filament proteins, all intermediate filaments share the property of being insoluble in conventional extraction buffers. It is this property that serves as the basis of the isolation protocol. Intermediate filaments can also be produced after assembly of a recombinant intermediate filament protein, and an Alternate Protocol describes this method. With intermediate filaments isolated from either tissue or assembled recombinant protein, it is possible to study the assembly process in vitro and to investigate, in vitro, possible interaction partners that participate in intermediate filament–mediated processes in vivo.

Skeletal and cardiac muscle cells contain an unusual system of tubular invaginations of their sarcolemma known as the transverse tubule (T-tubule), which are critical participants in the process of muscle contraction. *UNIT 3.24* provides a Basic Protocol that enables the isolation of highly purified T-tubules from muscle tissue using density gradient ultracentrifugation. A second, related Basic Protocol takes advantage of the glycoproteins in the membranes to separate sarcolemma membrane vesicles from T-tubule vesicles via aggregation of the former by wheat germ agglutinin. An Alternate Protocol involves immunoisolation T-tubule vesicles utilizing antibodies that recognize the intracellular domain of T-tubule integral membrane proteins (e.g., GLUT4).

Another very specialized membrane is the myelin sheath that surrounds vertebrate axons in both the central and peripheral nervous system. CNS myelin is elaborated as a plasma membrane extension by oligodendroglial cells of the brain and spinal cord, whereas PNS myelin is generated by Schwann cells. Methods for isolating myelin go back over three decades, and the methods of *UNIT 3.25* are in fact based on the classical isolation method of Norton and Podusulo published in 1973. The methods here are modified to minimize proteolysis and contamination with the axon plasma membrane. Although the protocols are related, separate protocols are provided for isolation of myelin from the CNS and from the PNS, reflecting the morphological and biochemical distinction in the two forms of myelin. In both cases, the relatively low density of myelin-derived membrane vesicles allows for a high degree of purification. A support protocol for assay of a marker enzyme is also included.

Brush borders also represent a specialization of the cell surface. In this case, rather than wrapping around axons in a spiral fashion as does myelin, the brush border is characterized by characteristic folds of the plasma membrane to form finger-like projections termed microvilli. These structures markedly increase the surface area of the membrane, which enables enhancement of absorption of substances. *UNIT 3.26* provides protocols for the isolation of brush borders from the kidney where the brush border structure participates in glomular filtration. The microvilli that make up the renal brush border are closely associated with actin filaments, explaining why brush borders have been utilized as a source of actin-binding proteins. It also accounts for the fact that brush borders are relatively resistant to homogenization, and this feature is exploited in the isolation protocols. Isolated brush borders can be further studied and purity/integrity assessed by flow cytometry, marker enzymes, and/or electron microscopy.

The boundary between the fields of virology and cell biology has always been blurry due to the fact that viruses infect cells. The cellular pathways of protein trafficking are among those that are "hijacked" by many viruses. Indeed, the trafficking of viral proteins has been instrumental in understanding these pathways. To localize and examine the

trafficking of the proteins of human cytomegalovirus (HCMV), the ability to isolate subcellular fractions from virally infected cells has proven to be highly informative. *UNIT 3.27* provides procedures for isolating mitochondria, endoplasmic reticulum, and mitochondria-associated membranes from primary fibroblasts and HeLa cells infected with HCMV. The unit also includes a protocol for visualization of the disruption of the mitochondrial network based on subcellular fractionation in Percoll gradients.

Amyloplasts are organelles wherein starch is synthesized and stored in plants. The starch in amyloplasts can subsequently be converted to sugar to meet the energy needs of the plant. Specialized amyloplasts are also thought to participate in gravitropism (sensing of gravity) that determines the growth of root tips. Much less is known about the biochemistry and function of these organelles than other, more studied plastids (e.g., chloroplasts). In *UNIT 3.28*, protocols are provided for the isolation of amyloplasts from maize endosperm and oilseed rape embryos as starting material. These methods could be adapted for other sources.

Microtubules are one of the components of the cytoskeleton. They are dynamic structures undergoing an assembly-disassembly process that contribute to cell motility, movement of organelles within the cell, and segregation of chromosomes into the daughter cells during mitosis. Microtubules are polymers of α-tubulin and β-tubulin with associated proteins (the microtubule-associated proteins or MAPs). The α-β heterodimer is the basic building block of microtubules, and a classical method for the isolation of the soluble heterodimer is provided in *UNIT 3.29*. Also described in this unit are protocols aimed at analysis of tubulin and MAPs. These protocols include the chromatographic and electrophoretic methods that have been traditionally employed, but they also include a new protocol for visualizing microtubules by atomic force microscopy.

Perhaps the best known organelle in plants is the chloroplast, which is responsible not only for energy production via photosynthesis but also for other critical metabolic functions, including synthesis of amino acids, vitamins, and lipids. *UNIT 3.30* provides protocols for the purification of chloroplasts from both *Arabidopsis* and spinach leaves. The purification is accomplished using differential centrifugation followed by Percoll gradient centrifugation and yields intact organelles suitable for additional characterization and experimentation. Whereas chloroplasts have been classically isolated from spinach leaves, the inclusion of an isolation protocol tailored for using *Arabidopsis* leaves as starting material is of significance, owing to the widespread use of this organism in plant genetics and molecular biology.

Melanins are pigmented polymers that occur widely in both plants and animals. Cutaneous melanin is responsible for skin color. Melanin in the brain has been termed neuromelanin and is located in the human midbrain region known as the substantia nigra pars compacta. Indeed the name of this portion of the brain is related to its pigmented nature (from the Latin meaning “black body”). This region of the brain is related to control of movement, and loss of the pigmentation in this region is a feature of Parkinson’s disease. Neuronal neuromelanin granules are distinct from melanosomes (see *UNIT 3.14*), which are located in melanocytes and are thought to be related to lysosomes. The neuromelanin granules exhibit a wider range of size than do melanosomes and have a distinct lipid composition. *UNIT 3.31* describes the isolation of neuromelanin granules with human midbrain as the starting material.

Dense core secretory vesicles (also termed secretory granules) are present in endocrine and neuroendocrine cells where they serve as storage sites for hormones (e.g., insulin), proteases, and signaling molecules. It appears that the intracellular sorting of peptide hormone precursors to the dense core secretory vesicles is an essential step in the bioactivation of these precursors. *UNIT 3.32* details the isolation of dense core vesicles from

pancreatic exocrine cells utilizing differential and density-gradient centrifugation. The unit describes isolation of the vesicles from pancreatic islets, as well as from tissue culture lines and transplantable tumors.

Alzheimer's Disease is characterized by lesions in the brain that contain deposits of amyloid-β protein (plaques) as well as neurofibrillary tangles consisting of abnormally phosphorylated microtubule-associated tau protein. A fuller understanding of these structures will help elucidate the pathology of the disease. *UNIT 3.33* provides protocols for the isolation, biochemical analysis, and characterization of both amyloid plaques and neurofibrilar tangles.

Legionella pneumophila is the bacterial pathogen responsible for the disease known as Legionnaire's disease. The name is based on an outbreak of deadly pneumonia at a convention of the American Legion in Philadelphia in 1976. The previously unknown bacterium was isolated the following year. This organism replicates intracellularly within phagocytes in a unique compartment that is called the *Legionella*-containing vacuole (LCV). It is now appreciated that the LCV is formed by hijacking the host signaling and vesicle trafficking pathways and involves a large number of bacterial effector proteins. The method of isolation of LCVs described in *UNIT 3.34* utilizes immunomagnetic separation using an antibody to one of the bacterial proteins on the surface of the vacuoles, followed by density gradient centrifugation. The unit also provides a means of monitoring the enrichment of the LCVs via fluorescence microscopy.

Immunomagnetic methods are also employed in one of the protocols of *UNIT 3.35* for the isolation of a distinct intracellular structure, the platelet granule. Platelets possess alpha granules that contain several growth factors, as well as dense (delta) granules. An alternative protocol utilizes more traditional differential centrifugation/sedimentation methodology. The degree of purification is monitored via immunoblotting using antibodies that recognize specific granule proteins.

Earlier in this chapter, there is a unit which is focused on isolation of nuclei from rat liver (see *UNIT 3.10*). Within nuclei, the nucleoli are non-membrane-bound structures composed of RNA and protein. These domains within the nucleus are involved in a number of important cellular functions, notably ribosome biogenesis and tRNA maturation. The purification of these relatively large and dense structures from the nuclei of cultured cells is described in *UNIT 3.36*.

Killer lymphocytes have been known for about 50 years, following their discovery in the context of transplant rejection in vivo. Two of the varieties of killer lymphocytes are cytotoxic T cells and natural killer cells. Each of these cell types contains cytotoxic granules, the contents of which are responsible for elimination of target cells. The protocols of *UNIT 3.37* describe purification of such granules utilizing Percoll density gradients following disruption of the killer lymphocytes. The unit also contains protocols for purification via liquid chromatography of key proteins involved in the cytolytic process.

Proteinaceous inclusion bodies formed when the cellular proteosome degradation mechanism is impaired are known as aggresomes. These structures form in the vicinity of the microtubule organizing center and centrosome and appear to be associated with intermediate filaments. The aggresomal response is thought to be a protective mechanism for dealing with abnormally high cytosolic loads of abnormal (e.g., misfolded) or damaged protein. Neuronal cytosolic inclusion bodies seen in Parkinson's disease are thought to be aggresomes. *UNIT 3.38* provides a protocol for isolating aggresomes from a yeast model.

Chromaffin cells are neuroendocrine cells found in the medulla of the adrenal gland. These cells contain secretory vesicles (granules) that contain a complex mixture of catecholamine neurotransmitters, peptide hormones, and a number of enzymes. Understanding of the molecular composition of chromaffin granules continues to evolve. The two protocols of *UNIT 3.39* should further this understanding as relatively large amounts of the granules can be obtained in a relatively short time. One protocol utilizes an iso-osmotic medium and differential centrifugation and yields intact granules. The second is used for isolation of chromaffin granule membranes or contents for further characterization.

Joe B. Harford and
Juan S. Bonifacino

CHAPTER 3
Subcellular Fractionation and Isolation of Organelles

INTRODUCTION

In the early 1700s, Leeuwenhoek noted that avian and amphibian blood cells contained a "clear area," almost certainly corresponding to the structure we now know as the nucleus. This represents probably the earliest recognition that eukaryotic cells are not simply sacs of protoplasm, but instead contain subcellular structures. Although the concept of the nucleated cell became firmly established in the 18th and 19th centuries, the real beginning of subcellular fractionation had to await the emergence in the mid-1940s of the art form known as electron microscopy. The electron microscope revealed much more complexity than Leeuwenhoek could have imagined. Concurrent with improvements in the techniques of electron microscopy was the development of methodologies for subcellular fractionation. Through the 1950s, these parallel approaches resulted in the discovery and isolation of the major organelles that comprise the eukaryotic cell. Finally, as biochemical functions were associated with specific subcellular compartments, a much clearer picture of the eukaryotic cell began to emerge, and with it the field of modern cell biology.

Chapter 3 is dedicated to methods for subcellular fractionation. The chapter begins with an overview of cell fractionation (*UNIT 3.1*) which covers some basic principles as well as the instrumentation of centrifugation. The various centrifugation media, ranging from sucrose to the newer media (Ficoll, Percoll, metrizamide, Nycodenz, and Iodixanol), are described.

Much of the earliest work in subcellular fractionation utilized rat liver as starting material. The second unit of Chapter 3 provides protocols for the isolation of plasma membrane sheets and domains from rat liver (*UNIT 3.2*). This method is used to isolate plasma membrane sheets in sufficient yield and purity to be suitable for a variety of analytical and preparative purposes. For example, these preparations can be used as starting material for the isolation of hepatocyte plasma membrane proteins. The sheets also serve as the starting material for the protocols within *UNIT 3.2* that yield the apical and basolateral domains comprising the polarized hepatocyte plasma membrane.

The densities of mitochondria, peroxisomes, and lysosomes are very similar, and as a result, these organelles cosediment in density gradients as the so-called "light mitochondrial fraction." The development of separation media has significantly improved the ability to separate these organelles from each other. *UNITS 3.3-3.6*, deal with separation of these organelles.

UNITS 3.3 & 3.4 describe methods for isolating mitochondria from rat liver as well as other sources including other tissues, cultured mammalian cells, and yeast. *UNIT 3.3* focuses on methods involving differential centrifugation for rapid isolation of metabolically active mitochondria, whereas methods involving density-gradient centrifugation for further purification of mitochondria are described in *UNIT 3.4*.

UNIT 3.5 provides methods for the isolation of peroxisomes from mammalian tissues and cells in tissue culture. Also included in this unit are a method for preparation of

Current Protocols in Cell Biology 3.0.1-3.0.7, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0300s47

peroxisomes from yeast and descriptions of assays for assessing the success of subcellular fractionation of the various starting materials.

The yeast *Saccharomyces cerevisiae* contains all the membrane-bound subcellular organelles that are characteristic of higher eukaryotes, yet this organism has a haploid genome that is less than four times the size of that of *E. coli*. The availability of the entire *S. cerevisiae* genome sequence, together with the ease with which yeast are cultured (*UNIT 1.6*) and the simplicity with which genetic manipulations are performed have made this organism a popular model in eukaryotic cell biology. Indeed, it is yeast cell biology that may provide insights into the function of many yeast genes and their mammalian counterparts. While some attention to certain subcellular organelles from yeast is given in earlier units of this chapter, *UNIT 3.7* provides a comprehensive overview of yeast subcellular fractionation.

UNIT 3.8 describes centrifugation-based methods for isolating most of the major organelles of the yeast *Saccharomyces cerevisiae*. Most of these procedures begin with the preparation of spheroplasts as described in the Support Protocol near the beginning of the unit. Lysates for subcellular fractionation can also be generated by glass-bead lysis to disrupt intact yeast cells (also described). Because of the fragility of most yeast organelles, lysates of spheroplasts are best used for most protocols; however, glass-bead lysates can be used for preparation of cytosol and plasma membranes. Several of the protocols are analytical for assessing the distribution of proteins along exocytic, endocytic, and biosynthetic pathways in yeast cells. The unit also includes preparative methods for isolating yeast nuclei, vacuoles, mitochondria, peroxisomes, endoplasmic reticulum, plasma membranes, and cytosol.

The Golgi, an organelle named for the pioneering cell biologist, Camillo Golgi, was identified on the basis of a then current protocol developed by Golgi for fixing and staining tissues. Although observations of what he then referred to as the "internal reticular apparatus" probably date even earlier in the 19th century, the Golgi remains an intracellular site of intense investigation by today's cell biologists. *UNIT 3.9* describes the isolation of Golgi membranes on a preparative basis. Individual protocols describe isolation of dextrose-treated Golgi stacks from rat liver using a sucrose density barrier and by flotation from a light mitochondrial fraction. Isolation from cultured cells is also described, as is a method to assay for the Golgi marker enzyme, UDP-galactose galactosyl transferase.

UNIT 3.10 focuses on the "clear area" of Leeuwenhoek—the nucleus. As noted above, rat liver has been the most frequently used source of material for subcellular fractionation. This unit contains two protocols for isolation of nuclei from rat liver. The first utilizes a sucrose density barrier, whereas the second employs Nycodenz as a density medium. Although the vast majority of methods for preparing nuclei use soft tissue (e.g., liver), the protocols described in this unit can be applied to other source materials (e.g., cultured cells, plants), provided appropriate homogenization can be achieved. *UNIT 3.10* also contains two protocols for purification of nuclear membranes from isolated nuclei and Support Protocols describing methods for assaying DNA and RNA.

Free-flow electrophoresis (*UNIT 3.11*) provides opportunities for both analytical and preparative applications for isolating subcellular organelles such as rat endosomes. Endosome-enriched fractions are prepared from rat liver using discontinuous sucrose gradients, and these are used to prepare endosomal subpopulations by free-flow electrophoresis. The analytical approach can be used to study the kinetics and cellular subpopulations involved in endocytic traffic. Three distinct subpopulations of endosomes are revealed in such studies. Support Protocols include methods for analyzing markers for endosomal subpopulations and for demonstrating retention of endosomal function (acidification)

in vitro. Methods are also included for preparing and labeling ligands that traffic through endosomes.

UNIT 3.12 describes protocols for the isolation of synaptic vesicles—small, electron-lucent organelles contained within presynaptic nerve terminals. Synaptic vesicles function to store neurotransmitters and to release them into the synaptic cleft upon stimulation. Methods for the purification of synaptic vesicles take advantage of the vesicle's small size and low density. The first two protocols describe the use of differential centrifugation to enrich synaptic vesicles 8- to 10-fold relative to brain homogenate. The next three protocols allow further purification to ~20-fold using sedimentation velocity or chromatography on controlled-pore glass beads or Sephacryl S-1000. This unit ends with protocols for the sedimentation of synaptic vesicles at high speed, immunoisolation of synaptic vesicle subpopulations, and siliconization of tubes.

Transport of proteins, lipids, and other "cargo" molecules between different compartments of the secretory, endocytic and lysosomal targeting pathways is mediated by membrane-enclosed carriers. The best characterized of these carriers are clathrin-coated vesicles (CCVs), which play critical roles in endocytosis from the plasma membrane and sorting from the trans-Golgi network and endosomes. CCVs are small (50 to 100 nm in diameter) and are encased within a dense proteinaceous coat—properties that are the bases for their isolation by ultracentrifugation procedures. *UNIT 3.13* presents a series of protocols for the isolation of coated vesicles from different sources, including adult and developing rat brain, adult rat liver, and cultured cell lines. In addition, this unit contains a procedure for analyzing the purity of isolated CCVs by electron microscopy. The Commentary section shows representative electron micrographs of these preparations, as well as SDS-PAGE analyses of the protein composition of the purified CCVs.

UNIT 3.14 deals with melanosomes, which are membrane-bound organelles filled with the pigment melanin. These organelles are only found in specialized cell types such as melanocytes and retinal epithelial cells, and they function to protect cells and tissues from the harmful effects of ultraviolet radiation. Oncogenic transformation of melanocyte precursors results in melanomas, which are particularly aggressive cancers. This unit presents protocols for the isolation of melanosomes from melanoma cells and tissues. The biogenesis of melanosomes involves maturation of precursors through a series of well-defined stages; the protocols in this unit allow for the separation of early and late stage melanosomes by ultracentrifugation and free-flow electrophoresis. This unit also describes a method for monitoring the purification of melanosomes by measuring the activity of the melanosomal enzyme, tyrosinase. Finally, the unit lists antibody reagents to melanosomal proteins that can be used to monitor purification and assess the purity of the isolated melanosomes.

Most cells in culture contain discernable lipid droplets, although the size and number of these droplets vary depending on cell type and culture conditions. Lipid droplets can provide a source of metabolic energy and also play a role in cholesterol homeostasis required for membrane biogenesis. In steroidogenic cells, the lipid droplets provide substrates for hormone synthesis. *UNIT 3.15* describes the isolation of lipid droplets based on their high buoyancy, which is reflective of their low mass of protein and high relative mass of neutral lipids. At present, no specific enzyme markers have been localized exclusively to lipid droplets. The droplets do, however, contain certain characteristic structural proteins, and protocols for immunoblotting of these proteins are included for assessment of the isolated lipid droplets.

UNIT 3.16 describes the isolation of rat serosal mast cells by peritoneal lavage and the use of these cells as a source of mast cell granules. These granules are modified lysosomes that release their contents upon degranulation of the activated mast cells. Protocols

are provided for the isolation of mast cell granules enveloped in their perigranular membrane and of granule remnants released upon degranulation. Several protocols aimed at assessing mast cell activation are included, as is a protocol for verification of granule remnant morphology and purity via electron microscopy. A future unit in Chapter 3 will deal with the isolation of the analogous organelle from chromaffin cells (i.e., chromaffin granules).

All previous units in this chapter that deal with organelle isolation have described isolation of organelles that exist in multiple copies per cell. In contrast, *UNIT 3.17* provides protocols related to the isolation of centrosomes, of which there is only one per cell. As starting material for this challenging task, the protocols utilize early (0- to 3.5-hr) *Drosophila* embryos. Centrosomes are isolated by a protocol involving gradient centrifugation and a protocol for further enrichment via immunomagnetic purification. Protocols for assessing the centrosome preparation by immunofluorescence microscopy are included.

In contrast to the situation with the centrosome (one copy per cell), pancreatic zymogen granules have been estimated to constitute as much as 25% of the total cellular volume based on electron microscopy. The relative abundance of these organelles resulted in their being among the first to be the subject of cellular subfractionation protocols, dating to the mid-1950's. *UNIT 3.18* provides more modern protocols for the purification of these important cellular bodies from both rat and dog pancreas, using both sucrose and Percoll gradients. The pancreas contains other secretory granules in lower abundance, most notably the endocrine granules responsible for insulin release. A future unit of this chapter will provide protocols related to isolation of the insulin granules of the pancreas.

An early unit in this chapter (*UNIT 3.5*) deals with the organelles known as peroxisomes. Glyoxysomes represent a specialized form of the peroxisome found in the cotyledons of seedling plants. In this context, these organelles sequester the enzymes of the glyoxylate cycle that provides energy to the rapidly growing seedling. Later in plant development, the peroxisomes of the green leaves have a distinct complement of enzymes reflecting their role in photorespiration. *UNIT 3.19* provides protocols for growing pumpkin seedlings and for isolation of glyoxysomes from their cotyledons via density gradient centrifugation.

The glucose transporter isoform known as GLUT4 functions to facilitate uptake of glucose by both fat and muscle tisssues. GLUT4 is translocated in response to insulin from an intracellular compartment to the plasma membrane as the mechanism for insulin-mediated enhancement of glucose transport. The investigation of trafficking of GLUT4 has gained considerable attention since the discovery that GLUT4 translocation is compromised in type 2 diabetes. *UNIT 3.20* provides protocols for isolation of GLUT4 storage vesicles from primary rat adipocytes, cultured adipocytes, and skeletal muscle.

Another type of membrane vesicle is the subject of *UNIT 3.21*, which describes isolation of vesicles from intestinal brush border from three species (rat, pig, and cow). These vesicles are derived from the apical surface of the polarized epithelial cells of the small intestine, which also contain integral membrane proteins involved in glucose uptake. Whereas the GLUT4 vesicles of *UNIT 3.20* are part of a regulated trafficking of cellular membranes, those of *UNIT 3.21* represent spontaneous sealing of membrane fragments derived from the intestinal microvilli upon disruption of the tissue architecture. These vesicles can nonetheless be utilized for membrane transport studies.

The theme of isolation of membrane-derived vesicles continues in *UNIT 3.22*, which provides several protocols related to exosomes. These vesicles are derived from the plasma membrane via an endocytosis-exocytosis mechanism, but they are isolated from extracellular fluids (either tissue culture media or bodily fluids). Methods are provided whereby exosomes are isolated by either ultracentrifugation, ultrafiltration, or immunoisolation.

The protocols of *UNIT 3.22* should prove useful in studies aimed at understanding exosome function and the possible use of exosomes in therapeutic applications e.g., as immunogens in vaccines.

UNIT 3.23 describes the isolation of intermediate filaments from bovine spinal cord. Although intermediate filaments from different sources contain a diverse complement of intermediate filament proteins, all intermediate filaments share the property of being insoluble in conventional extraction buffers. It is this property that serves as the basis of the isolation protocol. Intermediate filaments can also be produced after assembly of a recombinant intermediate filament protein, and an Alternate Protocol describes this method. With intermediate filaments isolated from either tissue or assembled recombinant protein, it is possible to study the assembly process in vitro and to investigate, in vitro, possible interaction partners that participate in intermediate filament–mediated processes in vivo.

Skeletal and cardiac muscle cells contain an unusual system of tubular invaginations of their sarcolemma known as the transverse tubule (T-tubule), which are critical participants in the process of muscle contraction. *UNIT 3.24* provides a Basic Protocol that enables the isolation of highly purified T-tubules from muscle tissue using density gradient ultracentrifugation. A second, related Basic Protocol takes advantage of the glycoproteins in the membranes to separate sarcolemma membrane vesicles from T-tubule vesicles via aggregation of the former by wheat germ agglutinin. An Alternate Protocol involves immunoisolation T-tubule vesicles utilizing antibodies that recognize the intracellular domain of T-tubule integral membrane proteins (e.g., GLUT4).

Another very specialized membrane is the myelin sheath that surrounds vertebrate axons in both the central and peripheral nervous system. CNS myelin is elaborated as a plasma membrane extension by oligodendroglial cells of the brain and spinal cord, whereas PNS myelin is generated by Schwann cells. Methods for isolating myelin go back over three decades, and the methods of *UNIT 3.25* are in fact based on the classical isolation method of Norton and Podusulo published in 1973. The methods here are modified to minimize proteolysis and contamination with the axon plasma membrane. Although the protocols are related, separate protocols are provided for isolation of myelin from the CNS and from the PNS, reflecting the morphological and biochemical distinction in the two forms of myelin. In both cases, the relatively low density of myelin-derived membrane vesicles allows for a high degree of purification. A support protocol for assay of a marker enzyme is also included.

Brush borders also represent a specialization of the cell surface. In this case, rather than wrapping around axons in a spiral fashion as does myelin, the brush border is characterized by characteristic folds of the plasma membrane to form finger-like projections termed microvilli. These structures markedly increase the surface area of the membrane, which enables enhancement of absorption of substances. *UNIT 3.26* provides protocols for the isolation of brush borders from the kidney where the brush border structure participates in glomular filtration. The microvilli that make up the renal brush border are closely associated with actin filaments, explaining why brush borders have been utilized as a source of actin-binding proteins. It also accounts for the fact that brush borders are relatively resistant to homogenization, and this feature is exploited in the isolation protocols. Isolated brush borders can be further studied and purity/integrity assessed by flow cytometry, marker enzymes, and/or electron microscopy.

The boundary between the fields of virology and cell biology has always been blurry due to the fact that viruses infect cells. The cellular pathways of protein trafficking are among those that are "hijacked" by many viruses. Indeed, the trafficking of viral proteins has been instrumental in understanding these pathways. To localize and examine the

trafficking of the proteins of human cytomegalovirus (HCMV), the ability to isolate subcellular fractions from virally infected cells has proven to be highly informative. *UNIT 3.27* provides procedures for isolating mitochondria, endoplasmic reticulum, and mitochondria-associated membranes from primary fibroblasts and HeLa cells infected with HCMV. The unit also includes a protocol for visualization of the disruption of the mitochondrial network based on subcellular fractionation in Percoll gradients.

Amyloplasts are organelles wherein starch is synthesized and stored in plants. The starch in amyloplasts can subsequently be converted to sugar to meet the energy needs of the plant. Specialized amyloplasts are also thought to participate in gravitropism (sensing of gravity) that determines the growth of root tips. Much less is known about the biochemistry and function of these organelles than other, more studied plastids (e.g., chloroplasts). In *UNIT 3.28*, protocols are provided for the isolation of amyloplasts from maize endosperm and oilseed rape embryos as starting material. These methods could be adapted for other sources.

Microtubules are one of the components of the cytoskeleton. They are dynamic structures undergoing an assembly-disassembly process that contribute to cell motility, movement of organelles within the cell, and segregation of chromosomes into the daughter cells during mitosis. Microtubules are polymers of α-tubulin and β-tubulin with associated proteins (the microtubule-associated proteins or MAPs). The α-β heterodimer is the basic building block of microtubules, and a classical method for the isolation of the soluble heterodimer is provided in *UNIT 3.29*. Also described in this unit are protocols aimed at analysis of tubulin and MAPs. These protocols include the chromatographic and electrophoretic methods that have been traditionally employed, but they also include a new protocol for visualizing microtubules by atomic force microscopy.

Perhaps the best known organelle in plants is the chloroplast, which is responsible not only for energy production via photosynthesis but also for other critical metabolic functions, including synthesis of amino acids, vitamins, and lipids. *UNIT 3.30* provides protocols for the purification of chloroplasts from both *Arabidopsis* and spinach leaves. The purification is accomplished using differential centrifugation followed by Percoll gradient centrifugation and yields intact organelles suitable for additional characterization and experimentation. Whereas chloroplasts have been classically isolated from spinach leaves, the inclusion of an isolation protocol tailored for using *Arabidopsis* leaves as starting material is of significance, owing to the widespread use of this organism in plant genetics and molecular biology.

Melanins are pigmented polymers that occur widely in both plants and animals. Cutaneous melanin is responsible for skin color. Melanin in the brain has been termed neuromelanin and is located in the human midbrain region known as the substantia nigra pars compacta. Indeed the name of this portion of the brain is related to its pigmented nature (from the Latin meaning “black body”). This region of the brain is related to control of movement, and loss of the pigmentation in this region is a feature of Parkinson’s disease. Neuronal neuromelanin granules are distinct from melanosomes (see *UNIT 3.14*), which are located in melanocytes and are thought to be related to lysosomes. The neuromelanin granules exhibit a wider range of size than do melanosomes and have a distinct lipid composition. *UNIT 3.31* describes the isolation of neuromelanin granules with human midbrain as the starting material.

Dense core secretory vesicles (also termed secretory granules) are present in endocrine and neuroendocrine cells where they serve as storage sites for hormones (e.g., insulin), proteases, and signaling molecules. It appears that the intracellular sorting of peptide hormone precursors to the dense core secretory vesicles is an essential step in the bioactivation of these precursors. *UNIT 3.32* details the isolation of dense core vesicles from

pancreatic exocrine cells utilizing differential and density-gradient centrifugation. The unit describes isolation of the vesicles from pancreatic islets, as well as from tissue culture lines and transplantable tumors.

Alzheimer's Disease is characterized by lesions in the brain that contain deposits of amyloid-β protein (plaques) as well as neurofibrillary tangles consisting of abnormally phosphorylated microtubule-associated tau protein. A fuller understanding of these structures will help elucidate the pathology of the disease. *UNIT 3.33* provides protocols for the isolation, biochemical analysis, and characterization of both amyloid plaques and neurofibrilar tangles.

Legionella pneumophila is the bacterial pathogen responsible for the disease known as Legionnaire's disease. The name is based on an outbreak of deadly pneumonia at a convention of the American Legion in Philadelphia in 1976. The previously unknown bacterium was isolated the following year. This organism replicates intracellularly within phagocytes in a unique compartment that is called the *Legionella*-containing vacuole (LCV). It is now appreciated that the LCV is formed by hijacking the host signaling and vesicle trafficking pathways and involves a large number of bacterial effector proteins. The method of isolation of LCVs described in *UNIT 3.34* utilizes immunomagnetic separation using an antibody to one of the bacterial proteins on the surface of the vacuoles, followed by density gradient centrifugation. The unit also provides a means of monitoring the enrichment of the LCVs via fluorescence microscopy.

Immunomagnetic methods are also employed in one of the protocols of *UNIT 3.35* for the isolation of a distinct intracellular structure, the platelet granule. Platelets possess alpha granules that contain several growth factors, as well as dense (delta) granules. An alternative protocol utilizes more traditional differential centrifugation/sedimentation methodology. The degree of purification is monitored via immunoblotting using antibodies that recognize specific granule proteins.

Earlier in this chapter, there is a unit which is focused on isolation of nuclei from rat liver (see *UNIT 3.10*). Within nuclei, the nucleoli are non-membrane-bound structures composed of RNA and protein. These domains within the nucleus are involved in a number of important cellular functions, notably ribosome biogenesis and tRNA maturation. The purification of these relatively large and dense structures from the nuclei of cultured cells is described in *UNIT 3.36*.

Killer lymphocytes have been known for about 50 years, following their discovery in the context of transplant rejection in vivo. Two of the varieties of killer lymphocytes are cytotoxic T cells and natural killer cells. Each of these cell types contains cytotoxic granules, the contents of which are responsible for elimination of target cells. The protocols of *UNIT 3.37* describe purification of such granules utilizing Percoll density gradients following disruption of the killer lymphocytes. The unit also contains protocols for purification via liquid chromatography of key proteins involved in the cytolytic process.

Joe B. Harford and
Juan S. Bonifacino

Overview of Cell Fractionation

Cell fractionation has enjoyed widespread use among cell biologists for half a century. It continues to be a fruitful, if not essential, approach in the reductionistic efforts to define the composition and functions of the multiple compartments in eukaryotic cells. It also provides the essential ingredients for the increasing number of cell-free assays now being used in test-tube reconstructions of complex cellular events involving intercompartmental interactions. Much of the knowledge regarding the composition and function of cell organelles has resulted from fractionation of mammalian tissues, where cells are both abundant and highly differentiated (and thus organelle-rich). However, with new techniques in molecular biology and widened interest in combining studies of intact cells and functional reconstitution, interest in fractionation has spread to cultured cell lines and genetically tractable lower eukaryotic cells.

The goals of cell fractionation often differ depending on the nature of the experiments being conducted. In preparative procedures, in which the intent is to isolate quantities of a particular cell organelle for further study or for subfractionation, the emphasis is on purity and (secondarily) on yield. In analytical experiments, in which the intent is not isolation of organelles but evaluation of associations of selected macromolecules with particular organelles, the emphasis is on using one-step procedures that result in different distributions of various organelles (as defined by marker activities) rather than on separating organelles outright. Finally, in preparing organelles for cell-free reconstitution, the goal is to maintain them in a functional state. The investigator generally has developed a specific assay for intercompartmental interaction that does not rely on organelle purity, so the extent of contamination by irrelevant organelles is less important.

The separation of distinct organelles during cell fractionation results from their differing physical properties—size (and shape), buoyant density, and surface charge density—which reflect their differing compositions. Particular fractionation techniques capitalize on one or more of these properties. For example, gel filtration separates on the basis of size, centrifugation separates on the basis of size and density, and electrophoresis separates on the basis of surface charge density. As knowledge of the specific composition of particular organelles has developed, it has become possible to apply newer techniques such as affinity chromatography and selective density-shift perturbation. Whichever fractionation method is used, the procedures may have to be modified to adapt them to individual needs. Even isolation of a particular kind of organelle from different tissue or cell sources may necessitate adjustment of fractionation conditions. This also means that in addition to isolating the organelle of interest, one should also plan on confirming the identity of what has been isolated.

Centrifugation is the most widely used procedure in cell fractionation. It is the only approach commonly used to separate crude tissue homogenates (often having quite large volumes) into subfractions as starting material for more refined purification procedures. Further, the technology available, using rotors with a variety of geometries and diverse media that enable separation according to size, density, or both, now routinely permits refined separations on volumes ranging from submilliliter to several liters. No other technology is this versatile. Gel filtration is limited by the pore sizes of available resins, such that only regularly shaped vesicles with diameters <100 to 200 nm can be purified away from larger or irregularly shaped organelles. Use of electrophoresis for organelle purification, especially at the preparative level, is relatively recent, and, as with gel filtration, its success relies on generating starting material by centrifugation. Although it shows promise for purifying selected organelles (e.g., endosomes) that have been difficult to obtain otherwise, surface charge densities on most organelles may not be different enough (or able to be manipulated sufficiently) to make this a versatile procedure. For these reasons, the remaining comments in this overview focus primarily on fractionation of organelles by centrifugation. For more detailed coverage of each of the topics, the reader is referred to the excellent book edited by Rickwood (1984).

BASIC PRINCIPLES OF CENTRIFUGATION

One of the best ways to understand the rationale for various cell fractionation proce-

Contributed by J. David Castle

Current Protocols in Cell Biology (1998) 3.1.1-3.1.9

dures is to examine the Svedberg equation, which describes mathematically the sedimentation of a spherical particle in a viscous fluid:

$$\frac{dx/dt}{\omega^2 x} = \frac{2r^2 (\rho_p - \rho_m)}{9\eta}$$

where x is the distance from axis of rotation, t is time, ω is the angular velocity, r is the radius of the particle, ρ_p and ρ_m are the densities of the particle and medium, and η is the viscosity of the medium.

This equation states that the sedimentation velocity, dx/dt, per unit centrifugal field $\omega^2 x$ (which is set by the instrumentation) increases with the square of the particle radius and the difference in density between the particle and the medium, and decreases with increasing viscosity. In practical terms, when sedimentation is performed by differential velocity in a medium such as 0.25 M sucrose, which is less dense than all particles and has a low viscosity, the radius of the particle is the dominant factor that determines the sedimentation rate. In contrast, when isopycnic density gradient centrifugation is used, the density of the medium changes from top to bottom in the centrifugation tube, and the range of densities typically includes the buoyant densities of most particles (vesicles). Thus individual types of vesicles will sediment until $\rho_p = \rho_m$, and the vesicles will then stop at their isopycnic densities. Alternatively, in flotation procedures $\rho_p < \rho_m$ initially and dx/dt is negative, meaning that particles will move toward the axis of rotation (i.e., toward the top of the tube). Clearly, the equation also indicates that sedimentation or flotation, as the case may be, is slowed in media of higher viscosity. As discussed below, various media used in centrifugation are distinguished by their viscosities (and osmolarities) at high densities.

The Svedberg equation can also be used to discuss three other basic relationships encountered in using centrifugation. First, the velocity of a particle per unit centrifugal field (left side of the equation) is the sedimentation coefficient. Its dimensions are in seconds, and it is generally reported in units known as Svedbergs (S). 1 S = 10^{-13} sec, the same order of magnitude as the sedimentation coefficient of several biological macromolecules (e.g., 5S RNA). Second, the centrifugal field, $\omega^2 x$ is usually normalized to gravity and is expressed in the literature as a relative centrifugal force (RCF = $\omega^2 x/g$). Centrifugation conditions are best reported as $\times g$. RCF is usually expressed at the midpoint of the tube (g_{av}). The centrifugal field term includes the angular velocity in units of radians per second, which is easily converted to revolutions per minute (rpm), the unit most often given in the literature, as follows: RCF = 11.18 $r(N/1000)^2$, where N is rpm. Third, integration of the Svedberg equation over time yields the following relationship:

$$t = \frac{1}{s\omega^2} \ln\left(\frac{X_{max}}{X_{min}}\right) = \frac{k}{s}$$

$$\text{where} \quad k = \frac{1}{\omega^2} \ln\left(\frac{X_{max}}{X_{min}}\right)$$

This is a measure of the clearance time, the time required for a particle of given sedimentation coefficient to travel from the top to the bottom of the tube. Included in k are the geometry of the rotor (X_{max} and X_{min}) and the angular velocity. k-factors are reported in the printed information that accompanies most rotors. They are useful for estimating the length of centrifugation required to pellet a particular particle and for translating centrifugation conditions between two rotors. For a given particle or vesicle, k/t (rotor 1) = k/t(rotor 2).

INSTRUMENTATION

Several pieces of equipment must be available to carry out cell fractionation by centrifugation. These include homogenizers, centrifuges (generally one low-speed and one ultracentrifuge) with compatible rotors, a refractometer for measuring the refractive index of centrifugation media (before centrifugation) and gradient fractions (after centrifugation), a gradient-forming device for generating preformed density gradients, and a gradient-collecting device for unloading the gradients after the run. The refractometer, gradient-forming device, and gradient-collection device are very useful, if not essential, for density gradient and rate zonal sedimentation.

Centrifuges. Most organelle purification procedures require that samples be maintained at ≤4°C, so the centrifuges used need to be refrigerated. The low-speed centrifuge is used in the early steps of the typical purification procedure for sedimenting large particulates such as nuclei and unbroken cells, frequently from large volumes. The ultracentrifuge is used for subsequent spins at higher speeds and often with smaller volumes. These instruments have a vacuum system as the rotors are spun in an evacuated chamber to reduce friction (and thus heating).

Rotors. Commonly used rotors come in three configurations: fixed-angle, vertical (or near-vertical), and swinging-bucket (Fig. 3.1.1). Continuous-flow and zonal rotors, which are loaded and unloaded during the run, are less widely used. Fixed-angle rotors emphasize speed and capacity. Many of these rotors generate RCFs in excess of $600{,}000 \times g$, meaning low *k*-factors, and can handle a few hundred milliliters of solution at one time. As a result, they are used frequently for pelleting fractions, particularly from large volumes. Most fixed-angle rotors hold tubes in a fixed incline substantially away from the vertical. When the

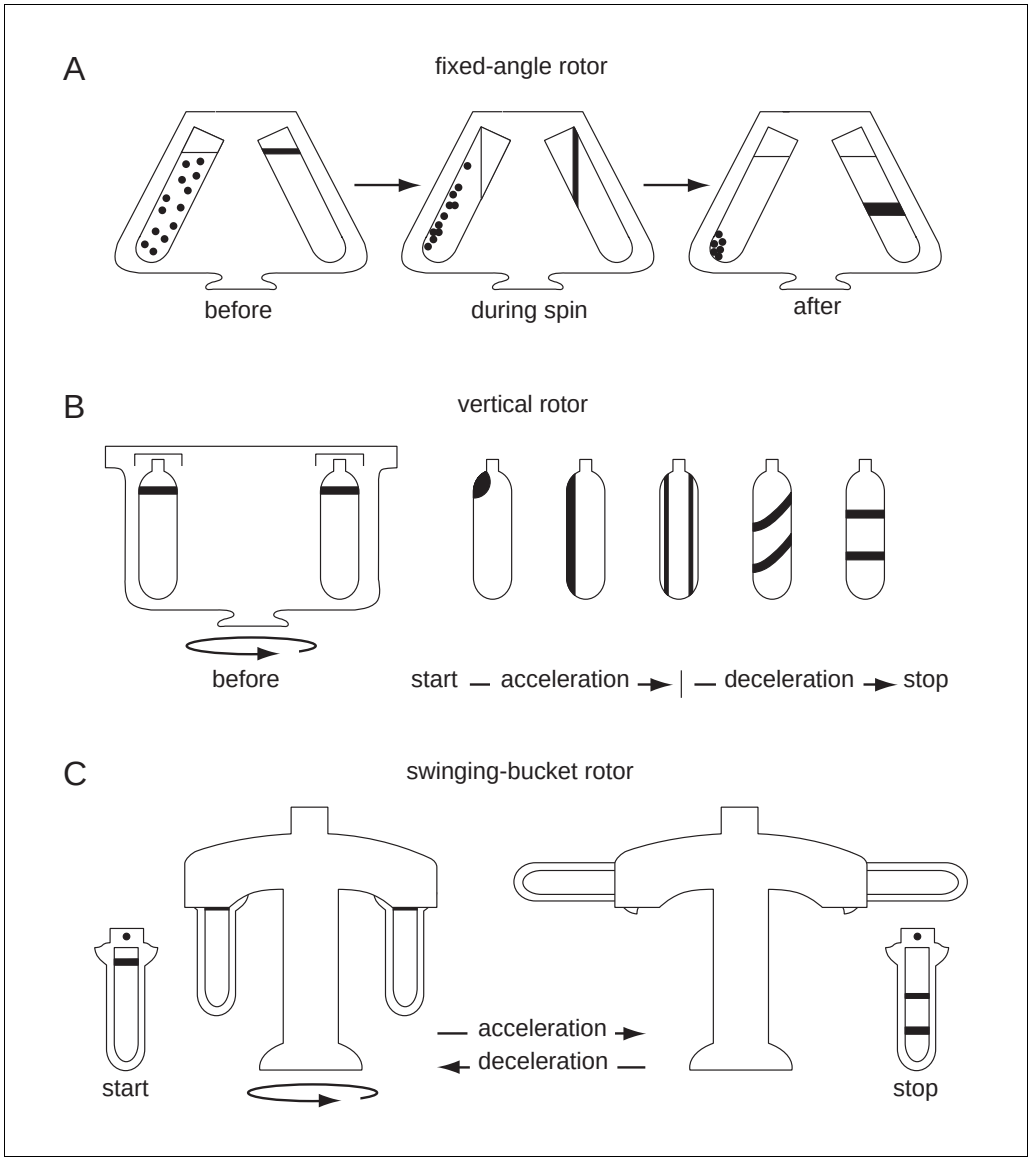


Figure 3.1.1 Rotors used in ultracentrifuges. (**A**) Fixed-angle rotor. In each profile, the tube on the left shows pelleting from a uniform suspension and the tube on the right shows sedimentation of a band layered at the top above a higher-density fluid. Sedimentation is radial (particles move toward the outer wall of the tube and then down this wall to the pellet). Note how the band in the right tube reorients during the spin and then again at the end of the run. (**B**) Vertical rotor. The rotor profile shows tubes with sample layered above a higher-density solution before a run. Note that during the run (successive tube profiles shown at right), the layer reorients along the inner wall of the tube and then the bands resolve vertically. During deceleration the bands reorient to a horizontal position. (**C**) Swinging-bucket rotor. Note the tube on the left in the upper profile has a sample layered at the top. The buckets are mounted vertically on the rotor. During the run, the buckets reorient to the horizontal position, and the bands separate along the length of the tube. Because sedimentation is radial, the particulates in the bands are more concentrated at the walls facing and away from the viewer than in the center of the tube. Toward the end of deceleration, the buckets reorient to the vertical position.

Table 3.1.1 Density and Refractive Index of Aqueous Sucrose Solutions at 20°C[a]

Percent (w/w)	Molarity	Density	Refractive index	Percent (w/w)	Molarity	Density	Refractive index
2	0.06	1.006	1.3359	38	1.30	1.166	1.3958
4	0.12	1.014	1.3388	40	1.38	1.176	1.3997
6	0.18	1.022	1.3418	42	1.46	1.187	1.4036
8	0.24	1.030	1.3448	44	1.54	1.197	1.4076
10	0.30	1.038	1.3479	46	1.62	1.208	1.4117
12	0.37	1.046	1.3510	48	1.71	1.219	1.4158
14	0.43	1.055	1.3541	50	1.80	1.230	1.4200
16	0.50	1.064	1.3573	52	1.88	1.241	1.4242
18	0.56	1.072	1.3606	54	1.98	1.252	1.4285
20	0.63	1.081	1.3639	56	2.07	1.263	1.4329
22	0.70	1.090	1.3672	58	2.16	1.275	1.4373
24	0.77	1.099	1.3706	60	2.26	1.286	1.4418
26	0.84	1.108	1.3740	62	2.35	1.298	1.4464
28	0.91	1.118	1.3775	64	2.45	1.310	1.4509
30	0.99	1.127	1.3811	66	2.55	1.322	1.4558
32	1.06	1.137	1.3847	68	2.65	1.335	1.4605
34	1.14	1.146	1.3883	70	2.76	1.347	1.4651
36	1.22	1.156	1.3920				

[a]Modified from Hofmann (1977) by permission of ISCO, Inc.

centrifugal force is applied, it is radial rather than along the length of the tube. Thus the path length is across the tilted tube and is reasonably short; particles travel outward until they hit the outer wall and then "slide" down the wall to form a pellet (see Fig. 3.1.1). In vertical rotors, the path is only the width of the tube. As is evident from the figure, in both fixed-angle and vertical rotors the solution in the tubes reorients from the horizontal to the vertical during acceleration and reverses during deceleration. These rotors can be advantageous for density gradients because equilibrium is reached quite rapidly with the short path, and the separation of bands of different density increases as the contents of the tube reorient during deceleration. With swinging-bucket rotors, the tube and its contents reorient from vertical to horizontal during acceleration and reverse during the end of deceleration. The centrifugal force in this case is along the length of the tube. Note, however, that because the force is directed radially, sedimenting particles tend to concentrate peripherally near the tube walls. Also, the long path length and the generally lower centrifugal forces required for these rotors mean that *k*-factors are usually much higher than with fixed-angle rotors. Thus these rotors have been used more for density gradient and rate zonal centrifugations and less for pelleting fractions.

Refractometers. A refractometer measures refractive indices of solutions used for organelle separations. Refractive index is linearly related to density, and tables relating these two parameters over a large range of concentrations have been prepared for sucrose (Table 3.1.1) and can readily be constructed for other gradient media by weighing aliquots of accurately prepared solutions of different concentrations. The measurement of refractive index/density is particularly useful for characterizing the position of organelles in gradients after centrifugation and for ensuring reproducibility of gradients from one experiment to another.

Gradient-forming devices. These devices consist of two chambers with an interconnecting channel along the bottom and an outlet from one chamber that connects to a peristaltic pump on its way to the centrifuge tube (see Fig. 3.1.2). The channel is gated by a stopcock. For linear gradients (the type most frequently used), the chambers have the same cross-sectional area. Solutions representing the extremes in density of the desired gradient are each placed in one chamber, and a stirring device is placed in the chamber with the outlet going to the tube. As

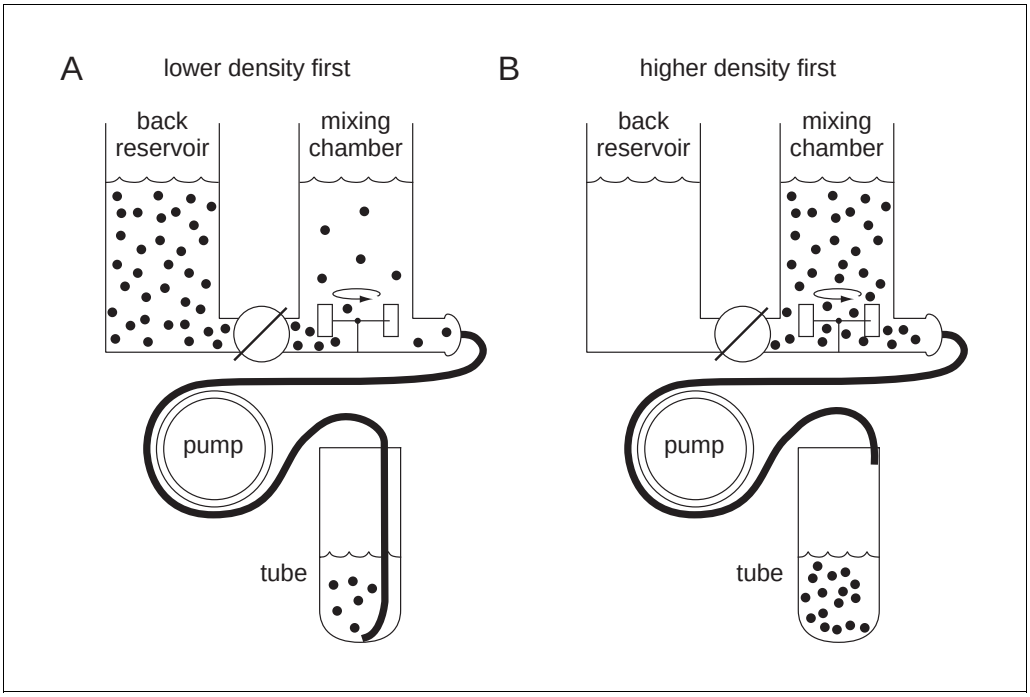


Figure 3.1.2 Linear gradient-forming device showing the back reservoir connected to the mixing chamber via a channel with a stopcock. A delivery tube leads from the mixing chamber to a peristaltic pump and then to the centrifuge tube. (**A**) Configuration used when the lower-density solution is delivered first; the lower-density solution is in the mixing chamber and the outlet is at the bottom of the centrifuge tube. (**B**) Configuration used when the higher-density solution is delivered first; the higher-density solution is in the mixing chamber and the outlet tube is always above the surface of the liquid entering the tube.

fluid is pumped out of the mixing chamber, fluid also flows through the connecting channel from the reservoir to maintain the same pressure head in the two chambers. Consequently, the density of the solution in the mixing chamber changes progressively. Gradients can be generated either highest density first, if the higher-density solution is placed in the mixing chamber, or lowest density first, if the lower-density solution is placed in the mixing chamber. Note that for highest density first, the delivery point must be located above the level of the solution filling the centrifuge tube, whereas for lowest density first, the delivery point must be at the bottom of the tube.

Gradient-collection devices. Gradient collection at the end of centrifugation requires a systematic and reproducible means of emptying the tube. Gradients can be collected from the top or from the bottom. With care, a pipettor (e.g., Pipetman, Rainin) of suitable volume can be used for manual collection from the top, taking care to withdraw equal-volume fractions from just below the meniscus. Alternatively, Buchler markets a pumping device (Autodensiflow) that continually adjusts to the height of the meniscus and withdraws gradients from the top, and both MSE Scientific and Nyegaard market devices that pump high-density solution beneath the gradient and progressively displace the gradient, which is again collected at the top. For collection from the bottom, the centrifuge tube is punctured with a needle and the gradient is collected dropwise.

FRACTIONATION MEDIA

Early in the development of centrifugation as a tool for purification of cellular organelles, it was established that nonelectrolyte-based media are preferable to electrolyte-based media for reducing organelle aggregation (Hogeboom et al., 1948). Most media used today are nonelectrolyte-based, although some do have a substantial electrolyte content (e.g., STKM medium, which contains 50 mM Tris·Cl, 25 mM KCl, and 5 mM $MgCl_2$ in addition to 0.25 M sucrose; Adelman et al., 1973). Moreover, media are generally isoosmotic or hyperosmotic as compared to 0.15 M NaCl. Organelles are osmometers, with water moving across their limiting membranes to maintain osmotic equilibrium; thus lysis can occur in hypoosmotic media.

The nonelectrolyte solute most commonly used in subcellular fractionation is sucrose. High-quality sucrose (free of RNase) is reasonably inexpensive, very soluble, and can be used readily to prepare solutions that span the range of densities of most biological organelles (Table 3.1.1). In addition, procedures have been developed for purifying most biological organelles in sucrose. Sucrose, however, has limitations; at higher concentrations solutions are quite viscous and hyperosmotic. Isoosmotic sucrose (0.25 M; ~9% w/v) has a density of only 1.03 g/ml, which is at the lower-density end for biological organelles. Thus all isopycnic density gradient centrifugation performed in sucrose constitutes hyperosmotic conditions; intraorganellar water redistributes to the surrounding medium, and the organelles shrink and increase in density. These changes are reversible for some, but not all, organelles.

Various sugar alcohols have been used in place of sucrose in some procedures. Glycerol is one, but although its viscosity is lower than that of sucrose, so is its density at a given concentration, and it also permeates some organelles. Mannitol has been used in a few procedures where the use of sucrose is not feasible—e.g., for isolation of epithelial brush border membranes that contain sucrase (Malathi et al., 1979)—and sorbitol has been a favorite for fractionating yeast, which secrete invertase (a form of sucrase; e.g., Walworth and Novick, 1987).

Polysaccharides have also been employed as substitutes for sucrose. Ficoll 400 (Pharmacia Biotech), a chemical polymer of sucrose with epichlorohydrin that has an average molecular weight of 400,000, has been a popular choice (a 70,000-MW form is also available, as Ficoll 70). At low concentrations, it has very low osmolarity, but as can be seen in Figure 3.1.3, the osmolarity rises sharply above 30% (w/v; density ~1.09 g/ml), and it is quite viscous at all concentrations above 10% (w/v). Ficoll has been used widely for separating different cell populations and is frequently most useful as an additive to media containing other density-modifying agents.

Iodinated nonelectrolytes increase the density of fractionation media through most of the range of biological organelles while maintaining reasonably low viscosity and osmolarity as compared to sucrose. Three popular and closely related compounds are metrizamide, Nycodenz, and iodixanol (OptiPrep; marketed by Nyegaard; Accurate Chemical—listed in the *SUPPLIERS APPENDIX*—is the U.S. outlet). As seen from Figure 3.1.3, 40% (w/v) solutions of both metrizamide and Nycodenz have a density >1.2 g/ml (above that of most organelles) and an osmolarity ≤400 mOsm with reasonable viscosity. 60% iodixanol has a density of 1.32 g/ml and an osmolarity of 260 mOsm with only moderate viscosity, making this medium especially useful for density gradient fractionation under isoosmotic conditions. With all these advantageous features, why aren't they used more? They are rather expensive, which certainly limits their use for large-scale work. In addition, they absorb in the UV and have been reported to inhibit selected enzyme activities.

A different alternative for achieving high densities at low viscosity and osmolarity involves the use of Percoll, colloidal silica coated with polyvinylpyrrolidone to reduce its adsorption to biological organelles. For fractionation of cellular organelles, Percoll is almost always used in combination with isoosmotic sucrose, because pure Percoll is only 10 mOsm. As can be seen from Figure 3.1.3, Percoll-containing media can achieve very high densities with reasonably low viscosities. Because Percoll particles have a significant size (range 30 to 150 Å) and density, they sediment during centrifugation. Thus, concentration gradients self-form fairly rapidly during a spin and the profile of density along the length of the tube continuously changes with time. The shallow gradients achieved with Percoll can be very advantageous in separating organelles whose densities differ only slightly; however, for reproducibility, the centrifugation conditions must be quite carefully set. Percoll also has its limitations: it absorbs in the UV, interferes with protein assays, precipitates in acid and organic solvents—do *not* attempt to precipitate Percoll-containing fractions with trichloroacetic acid (TCA) or acetone—is difficult to get rid of entirely.

EVALUATION OF FRACTIONATION

In setting up a procedure for separating or purifying cellular organelles, it is advisable to evaluate the steps of the protocol to confirm that the outcome is as desired and as stated. This means setting up assays for marker activities and protein and accounting for the totality of both throughout the procedure. This meticulousness enables the investigator to evaluate the homogenization, yield, fold purification, and extent of contamination by other undesirable organelles, and to account for 100% of the activity at each step. The extent of cell breakage

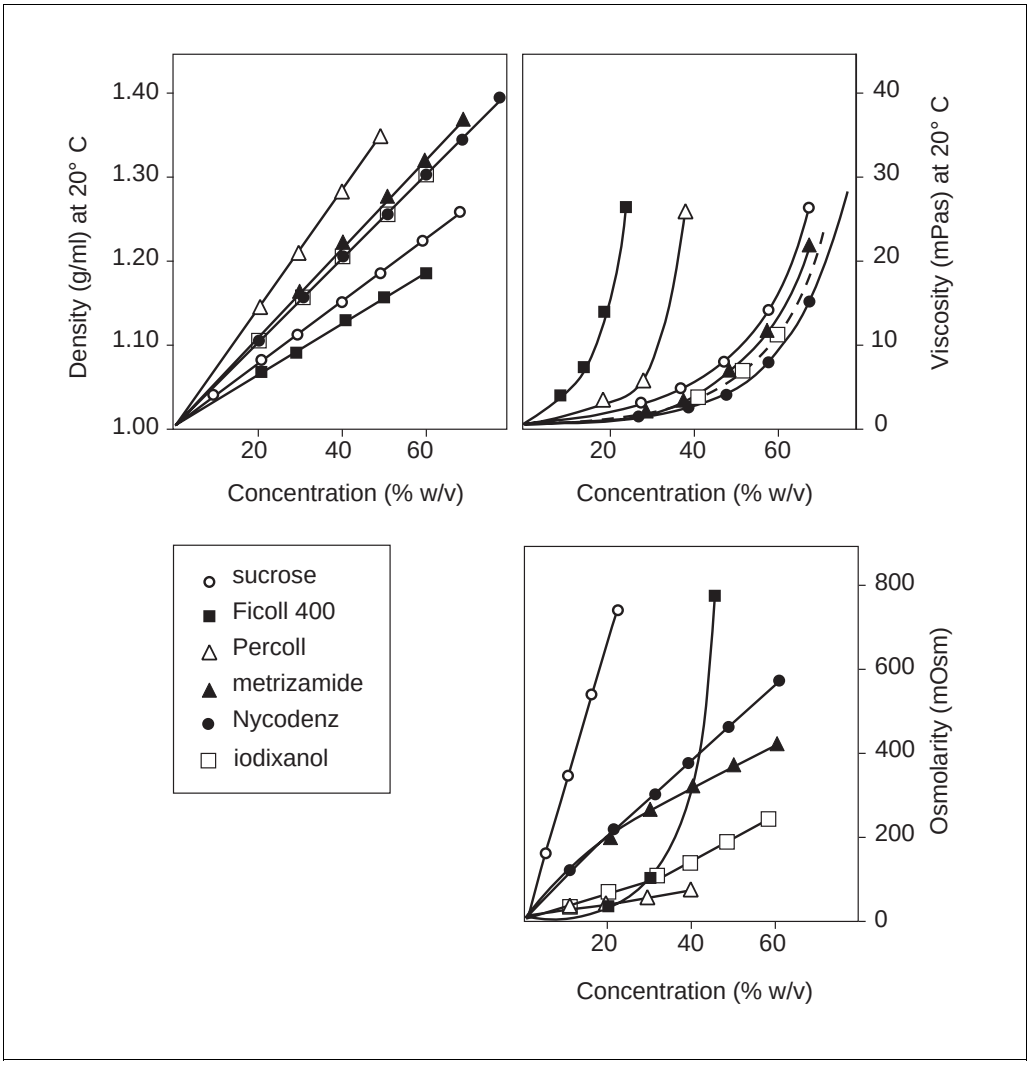


Figure 3.1.3 Plots of density, viscosity, and osmolarity of gradient media used to fractionate cellular organelles as a function of concentration. Data are shown for sucrose, Ficoll 400, Percoll, metrizamide, Nycodenz, and iodixanol. Modified from Rickwood (1984) by permission of IRL Press. Data for iodixanol by permission of Nycomed Pharma AS Diagnostics (Nycomed Pharma, 1996).

during homogenization can be estimated by determining the fraction of a marker activity of a particular organelle that pellets in the first low-speed centrifugation step (and is likely still to be associated with unbroken cells), while the extent of organelle breakage can be estimated by determining the fraction of the total amount of a soluble intraorganellar protein that is not sedimented by a high-speed spin that pellets all of the organelle. Following marker activity throughout purification enables the investigator to determine the yield of the desired organelle, and, if markers of other organelles are followed, the extent of residual contamination. Finally, if protein concentration is determined in parallel, calculation of the ratio of activity to protein (known as specific activity) at each step provides an estimate of fold purification or enrichment. In the event that analytical rather than preparative procedures are used, determination of marker activity in every fraction is essential for specifying the distribution of a particular organelle. Finally, it is very important to note that subcellular fractionation is never perfect! The entirety of a particular organelle is never obtained in completely pure form. In fact, the term "fraction" signifies both incomplete yield and incomplete purity. The careful investigator will always be cognizant of the limitations of the procedures and the consequent limitations of the results.

DEFINITIVE PROCEDURES

A useful starting point in isolating a particular cellular organelle may be to examine a procedure that has been designed for the spe-

Table 3.1.2 Preparing Organelle Fractions from Mammalian Tissues and Cells

Organelle	Procedure	Reference
Nucleus	Centrifugation through high-density sucrose	Blobel and Potter (1966)
Endoplasmic reticulum	Discontinuous sucrose gradient	Adelman et al. (1973)
Golgi complex	Sucrose gradient, continuous and discontinuous	Bergeron et al. (1982)
Secretion granules		
Endocrine	Metrizamide gradient	Loh et al. (1984)
Exocrine	Discontinuous sucrose gradient	Cameron and Castle (1984)
Synaptic vesicles	Chromatography on controlled-pore glass	Carlson et al. (1978) Huttner et al. (1983)
Plasma membrane	Discontinuous sucrose gradient	Hubbard et al. (1983); *UNIT 3.2*
Endosomes	Free flow electrophoresis Density shift with sucrose gradient	Marsh et al. (1987) Beaumelle et al. (1990)
Lysosomes	Metrizamide gradient	Wattiaux et al. (1978)
Mitochondria	Velocity sedimentation in sucrose	Schnaitman and Greenawalt (1968)
Peroxisomes	Sucrose gradient, discontinuous and continuous	Leighton et al. (1968)

cific organelle of interest. Table 3.1.2 lists references to procedures for purifying most of the membranous organelles found in eukaryotic cells. Unfortunately, it is not possible to make this listing comprehensive, but the procedures selected have achieved unusually good purity and in many cases have documented their achievement by bookkeeping of marker activities. Although several of the procedures are reasonably old, they have been used either as starting points for subfractionation (e.g., nucleus, endoplasmic reticulum, secretion granules, plasma membranes, lysosomes, mitochondria, and peroxisomes) or for functional studies in cell-free assays (e.g., Golgi, endoplasmic reticulum, and mitochondria). The adventurous investigator may want to consider the newer-generation reagent iodixanol (OptiPrep). The ability to fractionate under isoosmotic conditions over a large density range seems quite appealing. Sample procedures for various fractionations in iodixanol can be obtained from Nycomed/Accurate Chemical Co.

LITERATURE CITED

Adelman, M.R., Blobel, G., and Sabatini, D.D. 1973. An improved cell fractionation procedure for the preparation of rat liver membrane-bound ribosomes. *J. Cell Biol.* 56:191-205.

Beaumelle, B.D., Gibson, A., and Hopkins, C.R. 1990. Isolation and preliminary characterization of the major membrane boundaries of the endocytic pathway in lymphocytes. *J. Cell Biol.* 111:1811-1823.

Bergeron, J.J.M., Rachubinski, R.A., Sikstrom, R.A., Posner, B.I., and Paiement, J. 1982. Galactose transfer to endogenous acceptors within Golgi fractions of rat liver. *J. Cell Biol.* 92:139-146.

Blobel, G. and Potter, V.R. 1966. Nuclei from rat liver: Isolation method that combines purity with high yield. *Science* 154:1662-1665.

Cameron, R.S. and Castle, J.D. 1984. Isolation and compositional analysis of secretion granules and their membrane subfraction from the rat parotid gland. *J. Memb. Biol.* 79:127-144.

Carlson, S.S., Wagner, J.A., and Kelly, R.B. 1978. Purification of synaptic vesicles from elasmobranch electric organ and the use of biophysical criteria to demonstrate their purity. *Biochemistry* 17:1188-1199.

Hofmann, G. (ed.) 1977. ISCO Tables, 7th ed. ISCO, Lincoln, Neb.

Hogeboom, G.H., Schneider, W.C., and Palade, G.E. 1948. Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particular material. *J. Biol. Chem.* 172:619-635.

Hubbard, A.L., Wall, D.A., and Ma, A.K. 1983. Isolation of rat hepatocyte plasma membranes: I. Presence of the three major domains. *J. Cell Biol.* 96:217-229.

Huttner, W.B., Schiebler, W., Greengard, P., and DeCamilli, P. 1983. Synapsin I (Protein I), a nerve terminal–specific phosphoprotein: III. Its association with synaptic vesicles studied in a

highly purified synaptic vesicle preparation. *J. Cell Biol.* 96:1374-1388.

Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S., and DeDuve, C. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. *J. Cell Biol.* 37:482-513.

Loh, Y.P., Tam, W.W.H., and Russell, J.T. 1984. Measurement of pH and membrane potential in secretory vesicles isolated from bovine pituitary intermediate lobe. *J. Biol. Chem.* 259:8238-8245.

Malathi, P., Preiser, H., Fairclough, P., Mallett, P., and Crane, R.K. 1979. A rapid method for the isolation of kidney brush border membranes. *Biochim. Biophys. Acta* 554:259-263.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* 104:875-886.

Nycomed Pharma. 1996. Centrifugation techniques II. *In* Excellence in Separations—Density Gradient Media (brochure), 4th ed. Nycomed Pharma AS, Diagnostics, Oslo.

Rickwood, D. 1984. Centrifugation, A Practical Approach, 2nd ed. IRL Press, Oxford.

Schnaitman, C. and Greenawalt, J.W. 1968. Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *J. Cell Biol.* 38:158-175.

Walworth, N.C. and Novick, P.J. 1987. Purification and characterization of constitutive secretory vesicles from yeast. *J. Cell Biol.* 195:163-174.

Wattiaux, R., Wattiaux-DeConnick, S., Ronveaux-Dupal, M.-F., and Dubois, F. 1978. Isolation of rat liver lysosomes by isopycnic centrifugation in a metrizamide gradient. *J. Cell Biol.* 78:349-368.

Contributed by J. David Castle
University of Virginia
Charlottesville, Virginia

Isolation of Rat Hepatocyte Plasma Membrane Sheets and Plasma Membrane Domains

The plasma membrane of polarized epithelial cells is separated into discrete domains (apical and basolateral) that are functionally distinct. In polarized hepatocytes, the basolateral surface includes the sinusoidal front, which is specialized for the exchange of metabolites with the blood, and the lateral surface, which is adjacent to neighboring hepatocytes. The apical or bile canalicular plasma membrane domain is separated from the basolateral surfaces by tight junctions and is specialized for bile secretion. The functional and morphological differences between the two domains are matched by differing biochemical compositions. In order to examine the constituents of the hepatocyte plasma membrane, isolation of preparative amounts of this organelle is required.

This unit describes isolation of purified plasma membrane sheets from rat hepatocytes (see Basic Protocol 1). Since relatively high yields of plasma membrane sheets are obtained from this procedure, these preparations are suitable for a variety of analytical or preparative uses. For example, integral plasma membrane proteins can be extracted from preparative amounts of the sheets and further purified. The sheets also serve as the starting material for the separation of the two membrane domains (see Basic Protocol 2). The sheets are vesiculated by sonication, applied to continuous sucrose gradients, and centrifuged to equilibrium. The fractionation is generally an analytical procedure, and is useful for determining the distributions of plasma membrane–associated molecules between the two domains. The recovery and purity of plasma membrane sheets can be determined by measuring alkaline phosphodiesterase I activity (see Support Protocol 1); basolateral membrane recovery and purity can be assessed by measuring K^+-stimulated *p*-nitrophenylphosphatase activity (see Support Protocol 2); and apical membrane recovery and purity can be assessed by measuring 5′-nucleotidase activity (see Support Protocol 3). Distributions of proteins at the hepatocyte plasma membrane can also be determined morphologically (see Support Protocol 4). The large plasma membrane sheets readily adhere to glass coverslips and are easily processed for indirect immunofluorescent detection of specifically labeled antigens. The biochemical and morphological procedures described in this unit provide a relatively simple, yet powerful approach to examining molecules associated with the plasma membrane in rat hepatocytes.

ISOLATION OF PLASMA MEMBRANE SHEETS

BASIC PROTOCOL 1

This protocol describes a rapid and effective method for the purification of plasma membrane sheets from rat hepatocytes. Rat livers are first gently homogenized in buffered sucrose, and the plasma membrane sheets are separated from other intracellular compartments by a series of four differential centrifugations followed by a single flotation through a one-step sucrose gradient.

NOTE: All solutions and glassware should be prechilled to 4°C before the procedure, and kept on ice throughout.

Materials

125- to 150-g male Sprague-Dawley rats
Ether
0.9% (w/v) NaCl, ice cold (store 1 to 2 weeks at 4°C)
0.25 and 2.0 M STM solutions (see recipes), ice cold

continued

Contributed by Pamela L. Tuma and Ann L. Hubbard
Current Protocols in Cell Biology (1999) 3.2.1-3.2.16

Protease inhibitor solutions (see recipe)
0.25 M sucrose solution (see recipe), ice cold

40-ml Dounce homogenizer (Wheaton) with tight- and loose-fitting pestles (size A and B, respectively, although certain manufacturers reverse these designations)
Cheesecloth, grade 60
Abbe refractometer (Bausch and Lomb)
7-ml Dounce homogenizer (Wheaton) with loose-fitting pestle (size B)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), quantitative immunoblotting (*UNIT 6.2*), and densitometry (*UNIT 6.3*)

Subfractionate liver homogenate

1. Starve a 125- to 150-g male rat for 18 to 24 hr.

   For analytical work, one 6- to 8-g rat liver provides sufficient material. For preparative work, use up to 40 g of liver at a time (five to six rats and scale up solution volumes proportionally to number of livers used). The protocol described here and the volumes of sucrose solutions given (see Reagents and Solutions) are for the isolation of plasma membrane sheets from one rat liver.

2. Anesthetize the rat with ether and sacrifice by decapitation. Drain the blood from the animal under cold running water and carefully excise the liver.

3. Rinse the liver with ice-cold 0.9% NaCl to remove excess blood and hair. Using a squirt bottle, perfuse the liver with 0.9% NaCl via the portal vein until the liver is blanched. Quickly weigh the perfused liver and place it in a prechilled beaker on ice.

   This perfusion will remove any contaminating blood trapped within the liver. All subsequent steps are performed at 4°C.

4. Use scissors to mince the liver into ~0.5-cm^3 pieces and add 4.5 vol (4.5 ml/g tissue) of 0.25 M STM solution with protease inhibitors. Pour the mixture into a 40-ml Dounce homogenizer.

   If the liver weighs more than 8 g, divide it in half and homogenize separately.

5. Homogenize with 10 up-and-down strokes with the loose-fitting pestle (B).

   Avoid producing bubbles during homogenization by moving the pestle slowly and steadily. Wipe off any connective tissue that sticks to the pestle.

6. Filter homogenate into a graduated cylinder through a funnel lined with four layers of grade-60 cheesecloth premoistened with 0.25 M STM solution. Add 0.25 M STM solution to adjust the filtered volume to five times the original wet weight (5 ml/g) of the liver, producing a 20% (w/v) homogenate. Mix by gently inverting 3 to 5 times.

7. Pour 25- to 30-ml aliquots of homogenate into 50-ml conical tubes. Centrifuge 5 min at 260 × *g* (e.g., 1100 rpm in Beckman GS-6R), 4°C.

8. Carefully decant supernatant (S1) into a fresh tube, avoiding contamination from the soft pellet, and place on ice.

9. Resuspend pellet in one-half the original homogenate volume (step 6) of 0.25 M STM solution with protease inhibitors, using three strokes of the loose-fitting pestle (B) in a 40-ml Dounce homogenizer. Centrifuge as in step 7.

10. Decant supernatant and pool with supernatant from step 8 (S1). Centrifuge 25- to 30-ml aliquots of pooled supernatant per 50-ml conical tube, 10 min at 1500 × *g* (e.g., 2600 rpm in Beckman GS-6R), 4°C.

11. Pour off supernatant (S2).

    If assaying all fractions for recoveries, save S2 on ice and use on the same day.

Float plasma membrane sheets

12. Add two-thirds the original homogenate volume (step 6) of 0.25 M STM solution with protease inhibitors to the pellet (P2). Resuspend in a 40-ml Dounce homogenizer with three strokes of the loose-fitting pestle (B) followed by one stroke of the tight-fitting pestle (A).
13. Pour the suspension into a graduated cylinder and adjust to 2 times the original homogenate volume (step 6) with 2.0 M STM solution. Mix by gently inverting 3 to 5 times.
14. Check the density of the mixture with an Abbe refractometer, and adjust with either 0.25 M or 2.0 M STM solution until a density of 1.18 g/cm^3 (refractive index = 1.4016) is achieved.
15. Fill ultracentrifuge tubes equally with the resuspended P2 to ~90% total volume. Carefully overlay each tube to within a few millimeters from the top with ice-cold 0.25 M sucrose solution.

    For the Beckman SW28 rotor, 90% is 32 ml.

16. Centrifuge 60 min at 113,000 × *g* (e.g., 25,000 rpm in Beckman SW28 rotor), 4°C, in a swinging-bucket rotor with no brake.
17. Collect and pool the pellicules at the interface of each tube with a blunt-ended plastic transfer pipet.

    If assaying all fractions for recoveries, pour off and save the load fraction and resuspend the pellet (P3) to the desired volume (e.g., 5 to 10 ml).

18. Resuspend pellicules in 0.25 M sucrose solution at 0.8 to 1.0 times the original homogenate volume (step 6) and gently homogenize with three strokes of the loose-fitting pestle in the 40-ml Dounce homogenizer.
19. Check density of the suspension and adjust to ≤1.05 g/cm^3 (refractive index ≤1.3500) by diluting with 0.25 M sucrose solution, if necessary.

Recover and characterize plasma membrane sheets

20. Centrifuge 25- to 30-ml aliquots of resuspended pellicules per 50-ml conical tube, 10 min at 1500 × *g* (e.g., 2600 rpm in Beckman GS-6R), 4°C.
21. Carefully remove supernatant with a pipet and resuspend the pellet (plasma membrane sheets) in 1 to 2 ml of 0.25 M sucrose solution per liver in a 7.0-ml Dounce homogenizer with a loose-fitting pestle. Divide the purified plasma membrane sheets into 0.5- to 1.0-ml aliquots and save indefinitely at −80°C.

    Use caution when removing the supernatant, as this pellet is loose.

22. Apply ~20 µl of resuspended membrane sheets to a glass slide and examine at 25× magnification by phase-contrast microscopy.

    There should be relatively few small (≤1-µm) vesicles and membrane fragments, and an abundance of relatively large (20- to 40-µm), Y-shaped structures. If large (10-µm) spheres are also observed, these are nuclei. To avoid this form of contamination, pour less supernatant off of the pellet in step 8.

23. Check recovery and purity of an aliquot of the plasma membrane sheets by SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*), assaying immunoreactivity with antibodies specific for various intracellular membrane marker proteins (see Table 3.2.1). Determine distributions by densitometric analysis (*UNIT 6.3*) of immunoreactive bands.

    Alternatively, recovery and purity can be checked by using the relatively simple colorimetric assay for the plasma membrane marker alkaline phosphodiesterase I (see Support Protocol 1) and by assaying protein concentration (APPENDIX 3).

Table 3.2.1 Recovery and Enrichment of Various Organellar Markers During the Isolation of Plasma Membrane Sheets[a]

Marker[b]	% Recovery	Fold-enrichment
Protein	0.4 ± 0.13	—
5′-nucleotidase (PM)	17.4 ± 6	39 ± 10
Alkaline phosphodiesterase I (PM)	17.0 ± 5.6	40 ± 9
Asialoglycoprotein binding activity (PM)	16.3 ± 6	47.8 ± 18
NADH-cytochrome *c* reductase (ER)	0.3 ± 0.2	1 ± 0.5
Glucose-6-phosphatase (ER)	0.5	0.8
β-*N*-acetylglucosaminidase (lysosomes)	0.22 ± 0.13	0.66 ± 0.3
Cytochrome oxidase (mitochondria)	0.12 ± 0.07	0.22 ± 0.1
DNA (nuclei)	0.26	0.3
Galactosyltransferase (Golgi)	1.0	1.9

[a]Values are reported as the mean ± standard deviation ($n \geq 3$), when available. Reproduced from Hubbard et al. (1983) by copyright permission of the Rockefeller University Press.
[b]PM, plasma membrane; ER, endoplasmic reticulum.

SUPPORT PROTOCOL 1

ASSAY FOR ALKALINE PHOSPHODIESTERASE I ACTIVITY

Alkaline phosphodiesterase I is a plasma membrane–associated enzyme that distributes equally between the two surface domains. Since its enzymatic activity is easily assayed (Touster et al., 1970), alkaline phosphodiesterase is an excellent marker for total plasma membranes in biochemical subcellular fractions. In general, the assay described below is used to determine the recovery and purity of plasma membrane sheets in the preparative fractions obtained during the isolation procedure (Basic Protocol 1).

Materials

Preparative fractions and isolated plasma membrane sheets (see Basic Protocol 1)
Alkaline phosphodiesterase I reaction buffer (see recipe)
5% and 10% (w/v) trichloroacetic acid (TCA), ice cold
2 N NaOH
0.25 mM *p*-nitrophenol in 5% (w/v) TCA
1-cm-pathlength cuvettes

Analyze samples

1. Determine the necessary volume of alkaline phosphodiesterase I reaction buffer by multiplying the number of assay points plus one substrate blank by 0.8 ml (0.2 ml/assay tube × 4 tubes/fraction) and prepare it fresh.

   In general, all preparative fractions from the isolation of plasma membrane sheets, as well as the substrate blank, are assayed in duplicate for alkaline phosphodiesterase activity. Also, two incubation times are usually used.

   Substrate blanks are processed along with the other fractions and account for the amount of substrate hydrolyzed without added enzyme.

2. Set up four disposable glass tubes per fraction and substrate blank, and distribute 0.2 ml reaction buffer to each tube at room temperature.

3. Add 50 µl of each fraction (kept on ice until ready to assay) to the appropriate sample tubes and 50 µl water to the substrate blank tubes. Vortex gently.

4. Incubate fractions and blanks at 37°C in a shaking water bath, half for 30 min and half for 60 min.

5. Stop the reaction by adding 0.25 ml ice-cold 10% TCA. Gently vortex and let stand on ice.
6. Add 1.5 ml of 2 N NaOH to each tube and gently vortex.
7. Read A_{400} using a 1-cm-pathlength cuvette.

Analyze standards

8. Distribute 0, 0.1, 0.25, 0.3, 0.4, and 0.5 ml of 0.25 mM *p*-nitrophenol in 5% TCA to duplicate tubes.

   The corresponding amounts of p-nitrophenol are 0.0, 25.0, 62.5, 75, 100, and 125 nmoles/tube, respectively.

9. Adjust the volume of each tube to 0.5 ml with 5% TCA.
10. Add 1.5 ml of 2 N NaOH to each tube and read A_{400} as in step 7.

Perform calculations

11. Generate a standard curve by plotting A_{400} versus the amount of *p*-nitrophenol.
12. Subtract the substrate blank from each sample absorbance reading.
13. Determine the *p*-nitrophenol released in each sample tube from the standard curve.

    If the unknown alkaline phosphodiesterase activity is too high, dilute the fraction, assay a smaller aliquot, or generate another standard curve in a higher concentration range. Do the opposite if the activity is too low.

14. Calculate enzyme activity (μmoles substrate hydrolyzed/hr/ml) by multiplying the number of micromoles of *p*-nitrophenol in the samples by 20 (to correct the sample volume to 1.0 ml), by 2 (for the 30-min sample only, to correct the time to 1 hr), and by 1/dilution (if sample was diluted before addition to tube in the 50-μl aliquot).

ISOLATION OF PLASMA MEMBRANE DOMAINS

BASIC PROTOCOL 2

The isolated sheets prepared above (see Basic Protocol 1) are used as the starting material for the separation of the apical and basolateral domains, which constitute the intact rat hepatocyte plasma membrane. Gentle sonication of the plasma membrane sheets results in the formation of vesicles derived from either the apical or the basolateral domains. The two resultant vesicle populations are separated by equilibrium centrifugation on linear sucrose gradients and are identified by immunodetection of domain-specific antigens.

Materials

Plasma membrane sheets (see Basic Protocol 1)
0.25, 0.46, and 1.42 M sucrose solutions (see recipes)
Protease inhibitor solutions (see recipe)
Antibodies specific for apical and basolateral domains (e.g., anti–dipeptidyl peptidase IV for apical domain and CE9 antibody for basolateral domain; Hubbard et al., 1985)

Sonicating water bath (e.g., Laboratory Supplies)
Peristaltic pump
Gradient maker
Abbe refractometer (Bausch and Lomb)

Additional reagents and equipment for preparing and collecting sucrose gradients (*UNIT 5.3*), SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and densitometry (*UNIT 6.3*)

1. Dilute purified plasma membrane sheets to 1.0 mg protein/ml with 0.25 M sucrose solution containing protease inhibitors.

   If using a Beckman SW28 rotor, use 4 mg plasma membrane protein/32-ml gradient.

2. Sonicate 2.0-ml aliquots in 15-ml conical tubes using a sonicating water bath containing an ice/water slurry. Sonicate for 10-sec bursts with 1-min pauses on ice between bursts. Monitor vesiculation by phase-contrast microscopy (25× magnification; see Basic Protocol 1, step 22) every 1 to 5 bursts.

   Vesiculation is complete when no, or very few, large Y-shaped structures are observed. In general, this takes 3 to 20 bursts, depending on the strength of sonication. If a sonicating water bath is not available, vesiculation can also be achieved using a Polytron homogenizer (Brinkmann) fitted with a 12-mm tip at a setting of 8.

3. Pour linear gradients from 0.46 to 1.42 M sucrose (*UNIT 5.3*).

   If using a Beckman SW28 rotor, each gradient is 32.0 ml.

4. Layer the vesiculated plasma membrane sheets onto the gradients to within 2 to 5 mm from the top.

   If using a Beckman SW28 rotor, this volume is ~3.9 to 4.0 ml. Scale sucrose sample volumes proportionally for other types of tubes.

5. Centrifuge 16 to 20 hr at 72,000 × *g* (e.g., 38,000 rpm in Beckman SW28 rotor) with no brake at 4°C.

6. Collect fractions of desired volume (*UNIT 5.3*). Resuspend the pellet in 0.25 M sucrose to the same volume as the fractions. Invert each fraction to mix the sucrose solution and measure the refractive index of each with an Abbe refractometer. Store fractions indefinitely at −80°C.

   For 36-ml gradients, 3.0-ml fractions are usually collected.

7. Determine the distributions of apical and basolateral plasma membrane proteins by SDS-PAGE (*UNIT 6.1*) and quantitative immunoblotting (*UNIT 6.2*) with specific antibodies (e.g., dipeptidyl peptidase IV antibodies for the apical plasma membrane marker and CE9 antibodies for the basolateral), followed by densitometric analysis (*UNIT 6.3*) of immunoreactive bands.

   Alternatively, domain distributions can be determined by assaying for marker enzyme activities. For the basolateral vesicles, assay K^+-stimulated p-nitrophenyl phosphatase activity (see Support Protocol 2), and for the apical vesicles, assay 5′-nucleotidase activity (see Support Protocol 3).

   A typical plot of domain distribution is shown in Figure 3.2.1. For discussion, see Commentary.

SUPPORT PROTOCOL 2

ASSAY FOR K^+-STIMULATED *p*-NITROPHENYLPHOSPHATASE ACTIVITY

K^+-stimulated *p*-nitrophenylphosphatase activity is primarily associated with the basolateral plasma membrane in polarized epithelial cells. The assay outlined below (Stieger et al., 1986) is a straightforward method to determine the distribution of basolateral plasma membranes in sucrose gradient fractions containing vesiculated plasma membrane sheets (Basic Protocol 2). When used in combination with the assay for 5′-nucleotidase activity (Support Protocol 3), the distributions of both domains are easily determined for a single gradient.

Materials

Domain gradient fractions and resuspended pellet fraction (see Basic Protocol 2)
p-Nitrophenylphosphatase reaction buffer, with and without K^+ (see recipe)
Substrate mix (see recipe)

continued

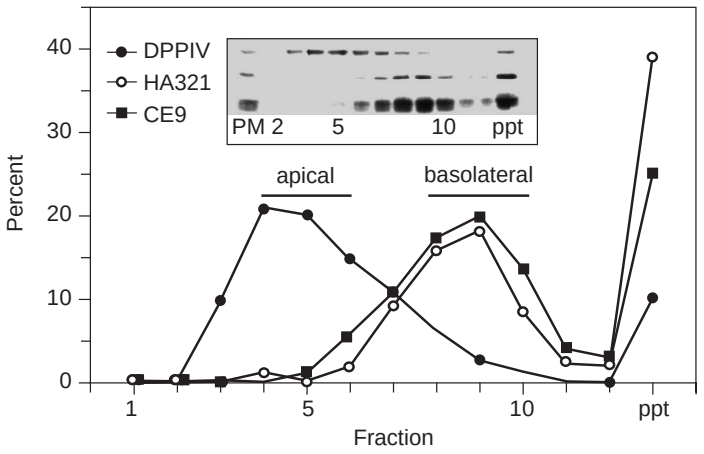


Figure 3.2.1 Distribution of one apical and two basolateral markers in the plasma membrane domain gradient. Isolated hepatocyte plasma membrane sheets were sonicated and the resulting vesicles separated on linear sucrose gradients. Collected fractions were analyzed by SDS-PAGE and quantitative immunoblotting with antibodies against known plasma membrane marker proteins. The data obtained from densitometry of the immunoreactive species detected in each fraction relative to the total recovered were plotted and indicated as percent distribution. The blotting data for each of the proteins are shown in the inset. The majority of the apical plasma membrane marker DPPIV was distributed to fractions 4 to 6, while the basolateral markers HA321 and CE9 were detected in fractions 8 to 10. The data shown are representative of three to ten experiments. Abbreviations: DPPIV, dipeptidyl peptidase IV; PM, plasma membrane; ppt, pellet. Reproduced from Fujita et al. (1998) by copyright permission of Portland Press.

1 and 10 N NaOH
0.25 mM *p*-nitrophenol in 1 N NaOH
1-cm-pathlength cuvette

Analyze samples

1. Determine the necessary volumes of K^+ and K^+-free *p*-nitrophenylphosphatase reaction buffers by multiplying the number of assay points plus one substrate blank by 3.28 ml (0.82 ml/assay tube × 4 tubes/fraction). Freshly prepare this volume of each reaction buffer.

   In general, all gradient fractions (including the resuspended pellet fraction) and a substrate blank are assayed in duplicate for both K^+-stimulated and K^+-independent activity. Also, two incubation times are usually used.

   Substrate blanks are processed along with the other fractions and account for the amount of substrate hydrolyzed without added enzyme.

2. Set up eight disposable glass tubes per fraction and substrate blank. Distribute 0.82 ml of each reaction buffer to four tubes/fraction. Distribute 0.87 ml of each reaction buffer to four substrate blank tubes.
3. Add 50 µl of each gradient fraction and the pelleted fraction (kept on ice until ready to assay) to the appropriate sample tubes and vortex gently.
4. Add 30 µl of substrate mix to all sample and blank tubes to begin the reaction. Incubate at 37°C in a shaking water bath, half for 30 min and half for 60 min.
5. Stop reaction by adding 100 µl of 10 N NaOH and vortex.
6. Read A_{405} using a 1-cm-pathlength cuvette.

Analyze standards

7. Distribute 0, 0.1, 0.25, 0.3, 0.4, and 0.5 ml of 0.25 mM *p*-nitrophenol in 1 N NaOH to duplicate tubes.

   The corresponding amounts of p-nitrophenol are 0, 25.0, 62.5, 75, 100, and 125 nmoles/tube, respectively.

8. Adjust the volume of each tube to 1.0 ml with 1 N NaOH and read A_{405} as in step 5.

Perform calculations

9. Generate a standard curve by plotting A_{405} versus the amount of *p*-nitrophenol.
10. Subtract the substrate blank from each sample absorbance reading.
11. Determine the *p*-nitrophenol released in each sample tube from the standard curve.

    If the unknown phosphatase activities are too high, dilute the fraction, assay a smaller aliquot, or generate another standard curve in a higher concentration range. Do the opposite if the activity is too low.

12. Calculate enzyme activity (μmoles substrate hydrolyzed/hr/ml) by multiplying the number of micromoles of *p*-nitrophenol in the samples by 20 (to correct the sample volume to 1.0 ml), by 2 (for the 30 min sample only, to correct the time to 1 hr), and by 1/dilution (if sample was diluted before addition to tube in the 50-μl aliquot).
13. To determine the K^+-stimulated *p*-nitrophenylphosphatase activity (the basolateral domain marker enzyme), subtract the values obtained using the buffer containing K^+ from those obtained using the K^+-free buffer.

SUPPORT PROTOCOL 3

ASSAY FOR 5′-NUCLEOTIDASE ACTIVITY

5′-nucleotidase is a plasma membrane–associated enzyme that distributes exclusively to the apical domain in many polarized epithelial cells. The assay described below (Widnell and Unkeless, 1968) serves as a straightforward means of determining the distributions of the apical plasma membranes in sucrose gradients containing vesiculated plasma membrane sheets (Basic Protocol 2).

Materials

Domain gradient fractions and resuspended pellet fraction (see Basic Protocol 2)
5′-Nucleotidase reaction buffer (see recipe)
5% and 30% (w/v) trichloroacetic acid (TCA), ice cold
10% (w/v) ascorbic acid, prepared fresh
0.42% (w/v) ammonium molybdate (see recipe)
0.1 mM KH_2PO_4 in 10% (w/v) TCA
1-cm-pathlength cuvette

Analyze samples

1. Determine the necessary volume of 5′-nucleotidase reaction buffer by multiplying the number of assay points plus one substrate blank by 1.8 ml (0.45 ml/assay tube × 4 tubes/fraction) and prepare it fresh.

   In general, all gradient fractions (including the resuspended pellet fraction) and the substrate blank are assayed in duplicate for 5′-nucleotidase activity. Also, two incubation times are usually used.

   A substrate blank consists of a mixture of 0.45 ml reaction buffer and 50 μl of H_2O also done in duplicate. These samples are processed along with the other fractions and account for the amount of substrate hydrolyzed without added enzyme. Two such blanks are required: one for the 30-min reaction and one for the 60-min reaction.

2. Set up four disposable glass tubes per fraction and substrate blank, and distribute 0.45 ml reaction buffer to each tube.
3. Add 50 µl of each gradient fraction (kept on ice until ready to assay) to the appropriate sample tubes and 50 µl water to the substrate blank tubes. Vortex gently.
4. Incubate at 37°C in a shaking water bath, half for 30 min and half for 60 min.
5. Stop reaction by adding 0.1 ml ice-cold 30% TCA and vortexing. Keep samples on ice.
6. Mix one part 10% ascorbic acid with six parts 0.42% ammonium molybdate (AA/AM).
7. To the tubes in step 4, add 1.5 ml AA/AM, cover with aluminum foil, and incubate an additional 20 min at 45°C.
8. Read A_{750} using a 1-cm-pathlength cuvette.

Analyze standards

9. Distribute 0, 0.05, 0.1, 0.2, 0.3, and 0.4 ml of 0.1 mM KH_2PO_4 in 10% TCA to duplicate tubes.

   The corresponding amounts of PO_4 are 0, 5, 10, 20, 30, and 40 nmoles/tube, respectively.

10. Adjust the volume of each tube to 0.6 ml with 5% TCA.
11. Add 1.5 ml AA/AM to each tube and incubate at 45°C as in step 6.
12. Read A_{750} as in step 8.

Perform calculations

13. Generate a standard curve by plotting A_{750} versus the amount of PO_4.
14. Subtract the substrate blank from each sample absorbance reading.
15. Determine the PO_4 released in each sample tube from the standard curve.

    If the unknown 5′-nucleotidase activity is too high, dilute the fraction, assay a smaller aliquot, or generate another standard curve in a higher concentration range. Do the opposite if the activity is too low.

16. Calculate enzyme activity (µmoles substrate hydrolyzed/hr/ml) by multiplying the number of micromoles of PO_4 in the samples by 20 (to correct the sample volume to 1.0 ml), by 2 (for the 30 min sample only, to correct the time to 1 hr), and by 1/dilution (if sample was diluted before addition to tube in the 50-µl aliquot).

INDIRECT IMMUNOFLUORESCENT DETECTION OF PROTEINS ASSOCIATED WITH PLASMA MEMBRANE SHEETS

SUPPORT PROTOCOL 4

The distribution of plasma membrane–associated molecules between the two domains can be easily determined by processing the purified sheets for indirect immunofluorescence. The sheets are settled onto glass coverslips by gravity and fixed with methanol at –20°C. The sheets are labeled with specific primary antibodies and visualized by indirect immunofluorescent detection of fluorophore-conjugated secondary antibodies. For additional discussion of immunofluorescent detection, see *UNIT 4.3*.

Materials

Plasma membrane sheets (see Basic Protocol 1)
0.25 M sucrose solution (see recipe)
PBS (*APPENDIX 2A*)

continued

Methanol, prechilled to −20°C
PBS/1% (w/v) BSA, prepared fresh
Primary antibody for marker protein
PBS/0.2% (w/v) BSA, prepared fresh
Fluorochrome-conjugated secondary antibody specific for Ig of the species of the primary antibody
Phenylenediamine mounting medium (see recipe)

Nail polish
22 × 22–mm glass coverslips

Adhere plasma membrane sheets to glass coverslips

1. Dilute plasma membrane sheets to 1.0 mg protein/ml with 0.25 M sucrose solution. Apply 100 to 200 µl to a 22 × 22–mm glass coverslip and distribute evenly across the surface with the side of a pipet tip.
2. Allow the plasma membrane sheets to settle onto the coverslip by gravity for 20 to 30 min at room temperature.

   The solution should not dry out during this time.

Prepare fixed samples

3. Wash off unattached plasma membrane sheets by rapidly dunking the coverslip 10 times in a small beaker of PBS. Remove excess liquid by touching an edge of the coverslip to a paper towel.
4. Quickly place the coverslip in prechilled (−20°C) methanol and incubate 5 min at −20°C.
5. Remove the coverslip from the methanol and rehydrate by washing three times in PBS for 5 min each.

   This and all subsequent steps are carried out at room temperature.

Stain samples for immunofluorescence

6. Block the plasma membrane sheets by incubating 15 min with PBS/1% BSA.
7. Remove excess blocking reagent as in step 3 and replace with 100 µl fresh PBS/1% BSA containing the desired primary antibody. Incubate 30 min.
8. Wash the coverslip three times with PBS/0.2% BSA, 5 min each, and remove excess wash buffer as in step 3.
9. Incubate 15 min in 100 µl PBS/1% BSA containing the desired concentration of fluorochrome-conjugated secondary antibody.
10. Wash coverslip as in step 8.
11. Place a drop of the phenylenediamine mounting medium in the middle of a glass slide. Place the coverslip with plasma membrane sheets face down on top of the drop.
12. Wick excess liquid with filter paper placed adjacent to the coverslip. Seal the coverslip into place with nail polish.
13. Allow specimen to dry at least 15 min and view with a fluorescent microscope equipped with a 63× or 100× objective.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Alkaline phosphodiesterase I reaction buffer

5.0 ml 100 mM Na_2CO_3/$NaHCO_3$, pH 10.5 (40 mM final)
1.25 ml 1% (v/v) Triton X-100 (0.1% final)
1.0 ml 20 mM TMP solution (see recipe; 2 mM final)
H_2O to 10.0 ml
Prepare fresh and use at room temperature

This volume is sufficient for assaying all the preparative fractions and substrate blanks in duplicate for two incubation times (enough for 50 assay tubes).

Ammonium molybdate, 0.42% (w/v)

27.8 ml concentrated H_2SO_4 (1 N final)
4.2 g ammonium molybdate
H_2O to 1 liter
Store indefinitely at room temperature

p-Nitrophenylphosphatase reaction buffer, with and without K^+

5 ml 100 mM KCl (10 mM final)
1.5 ml 100 mM $MgCl_2$ (3 mM final)
25 ml 100 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*; 50 mM final)
45 mg theophylline (5 mM final)
H_2O to 50 ml
Adjust pH to 7.4
Prepare fresh and use at room temperature

For buffer without K^+: Use 5 ml of 100 mM NaCl (10 mM final) in place of KCl.

This volume is sufficient for assaying all the gradient fractions and substrate blanks in duplicate for two incubation times (enough for 60 assay tubes).

5′-Nucleotidase reaction buffer

27.0 ml 100 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*; 90 mM final)
3.0 ml 100 mM $MgCl_2$ (10 mM final)
15 mg adenosine-5′-monophosphate
Prepare fresh and use at room temperature

This volume is sufficient for assaying all the gradient fractions and substrate blanks in duplicate for two incubation times (enough for 66 assay tubes).

Phenylenediamine mounting medium

2.5 ml 2× TBS (see recipe)
2.5 ml glycerol
10 mg phenylenediamine (2 mg/ml final)

Adjust pH to between 9.5 and 10.5. Place medium in a 5-ml syringe, attach a 0.22-μm syringe-tip filter, and wrap in aluminum foil. Prepare fresh and keep on ice. Pass solution directly onto slide through the filter when needed.

This medium is very light sensitive.

Protease inhibitor solutions

Antipain: Prepare a 5-ml aqueous solution containing 5 mg antipain (1 mg/ml final) and 10% (v/v) dimethyl sulfoxide (DMSO). Divide into aliquots and store up to 1 year at −20°C. Add immediately before use at a 1:1000 dilution (1 μg/ml final).

continued

Aprotinin: Prepare a 10-ml aqueous solution containing 13.6 mg (1.36 mg/ml final) aprotinin. Store up to 1 year at 4°C. Add immediately before use at a 1:200 dilution (6.8 mg/ml final).

Benzamidine: Prepare a 10-ml aqueous solution containing 0.313 g benzamidine (200 mM final). Divide into aliquots and store up to 1 year at −20°C. Add immediately before use at a 1:200 dilution (1 mM final).

Leupeptin: Prepare a 5-ml aqueous solution containing 5 mg leupeptin (1 mg/ml final) and 10% (v/v) DMSO. Divide into aliquots and store up to 1 year at −20°C. Add immediately before use at a 1:1000 dilution (1 µg/ml final).

Phenylmethylsulfonyl fluoride (PMSF): Prepare a 10-ml solution of 100% ethanol containing 0.348 g PMSF (200 mM final). Store indefinitely at 4°C. Add immediately before use at a 1:200 dilution (1 mM final).

STM (sucrose/Tris/$MgCl_2$) solution, 0.25 M

17.12 g sucrose (0.25 M final)
2.0 ml 1.0 M Tris·Cl (*APPENDIX 2A*), pH 7.4 (10 mM final)
0.2 ml 1.0 M $MgCl_2$ (*APPENDIX 2A*; 1.0 mM final)
H_2O to 200 ml

Adjust to pH 7.4. Determine density (refractive index) at room temperature. Filter through a 0.2-µm nitrocellulose filter and store up to 48 hr at 4°C.

Refractive index = 1.3453 ± 0.0005. Solution can also be stored up to 1 year at −20°C, but density and pH should be checked before use.

STM solution, 2.0 M

Prepare as for 0.25 M STM solution (see recipe), but use 68.4 g sucrose per 100 ml (2.0 M final), and filter with a 1.2-µm nitrocellulose filter.

Refractive index = 1.4295 ± 0.0005.

Substrate mix

1.0 ml 10% (w/v) saponin (0.5% final)
0.92 g *p*-nitrophenylphosphate ditris salt (100 mM final)
H_2O to 20 ml
Store for up to 1 year at −20°C

Sucrose solution, 0.25 M

12.84 g sucrose
H_2O to 150 ml

Adjust to pH 7.4. Determine density (refractive index) at room temperature. Filter through a 0.2-µm nitrocellulose filter and store up to 48 hr at 4°C.

Refractive index = 1.3453 ± 0.0005. Solution can also be stored up to 1 year at −20°C, but density and pH should be checked before use.

Sucrose solution, 0.46 M

7.87 g sucrose
0.5 ml 1.0 M Tris·Cl (*APPENDIX 2A*), pH 7.5 (10 mM final)
H_2O to 50 ml

Adjust to pH 7.5. Determine density (refractive index) at room temperature. Filter through a 0.2-µm nitrocellulose filter and store up to 48 hr at 4°C.

Refractive index = 1.3557 ± 0.0005. Solution can also be stored up to 1 year at −20°C, but density and pH should be checked before use.

Sucrose solution, 1.42 M

Prepare as for 0.46 M sucrose solution (see recipe) but use 24.3 g sucrose.

Refractive index = 1.4016 ± 0.0005.

Thymidine-5′-monophospho-p-nitrophenyl ester (TMP), 20 mM

92 mg TMP
H_2O to 10.0 ml
Store up to 1 year at −20°C

Tris-buffered saline (TBS), 2×

2.42 g Tris base (100 mM final)
3.51 g NaCl (300 mM final)
H_2O to 200 ml
Adjust pH to 10.5 with concentrated HCl
Store indefinitely at 4°C

COMMENTARY

Background Information

The plasma membrane of polarized epithelial cells is divided into two functionally and compositionally distinct plasma membrane domains: the apical domain and the basolateral domain. In order to examine the biochemical constituents of each domain, isolation of highly purified plasma membranes is required. Not only must the preparations be pure, they must also contain each of the domains in similar amounts to that found in intact hepatocytes.

Early stereological studies revealed that the basolateral surface accounts for ~87% of the total plasma membrane surface area in hepatocytes; the apical domain for only ~13% (Weibel, 1976; Weibel et al., 1969; Blouin et al., 1977). However, early purification methods were deficient in substantial amounts of the basolateral surface, as discussed in Hubbard et al. (1983). This is largely due to the fact that the plasma membranes were vesiculated in the initial homogenization step, rendering the basolateral-derived vesicles both physically and morphologically indistinguishable from other intracellular vesicular structures. To circumvent this problem, the method presented here (first presented in Hubbard et al., 1983) avoids vesiculation of plasma membranes in the initial purification. Rather, using gentle homogenization, entire plasma membrane sheets are prepared and purified. These purified sheets retain both the apical and basolateral domains in near-normal surface area ratios (Hubbard et al., 1983), as well as a full complement of intercellular junctions and the subplasmalemmal cytoskeletal network (Hubbard and Ma, 1983).

This isolation procedure offers several other advantages over previously published methods. For one, this procedure is fast, taking a total of only 4 to 5 hr. It is also simple, requiring only four low-speed centrifugation steps and a single flotation through a one-step sucrose gradient. Perhaps most importantly, this method is effective, with routinely high yields and substantial enrichment of plasma membrane marker proteins (see Anticipated Results). This preparation also has substantially lower recoveries of membranes derived from other organelles, including the plasma membranes of other liver cell types (e.g., Kupffer and endothelial cells; see Table 3.2.1).

Once isolated, the plasma membrane sheets are useful for a variety of preparative and analytical procedures ranging from the identification of plasma membrane–associated molecules to their purification. Because domain-specific integral membrane proteins of the rat hepatocyte maintain their membrane localizations during isolation (Hubbard et al., 1983), the sheets are also useful as the starting material for purification of plasma membrane domains and determination of the distribution of molecules between domains. For this procedure, the plasma membrane sheets are vesiculated by sonication, applied to linear sucrose gradients, and centrifuged to equilibrium (Bartles et al., 1985). The basolateral-derived vesicles are more dense, probably due to associated cytoskeletal elements, and are readily separated from apical membrane vesicles. However, since the apical and basolateral vesicles partially overlap in the gradients (see Fig. 3.2.1), this method is only appropriate for analytical purposes.

The distributions of plasma membrane–associated molecules between domains can also be determined by processing isolated sheets for indirect immunofluorescence (Fujita et al., 1998). The large sheets readily adhere to uncoated glass coverslips and can be fixed in place with methanol. Conventional methods are then used to specifically label antigens with primary antibodies and visualize them with fluorophore-conjugated secondary antibodies. This method provides a much less time-consuming and labor-intensive alternative to density centrifugation followed

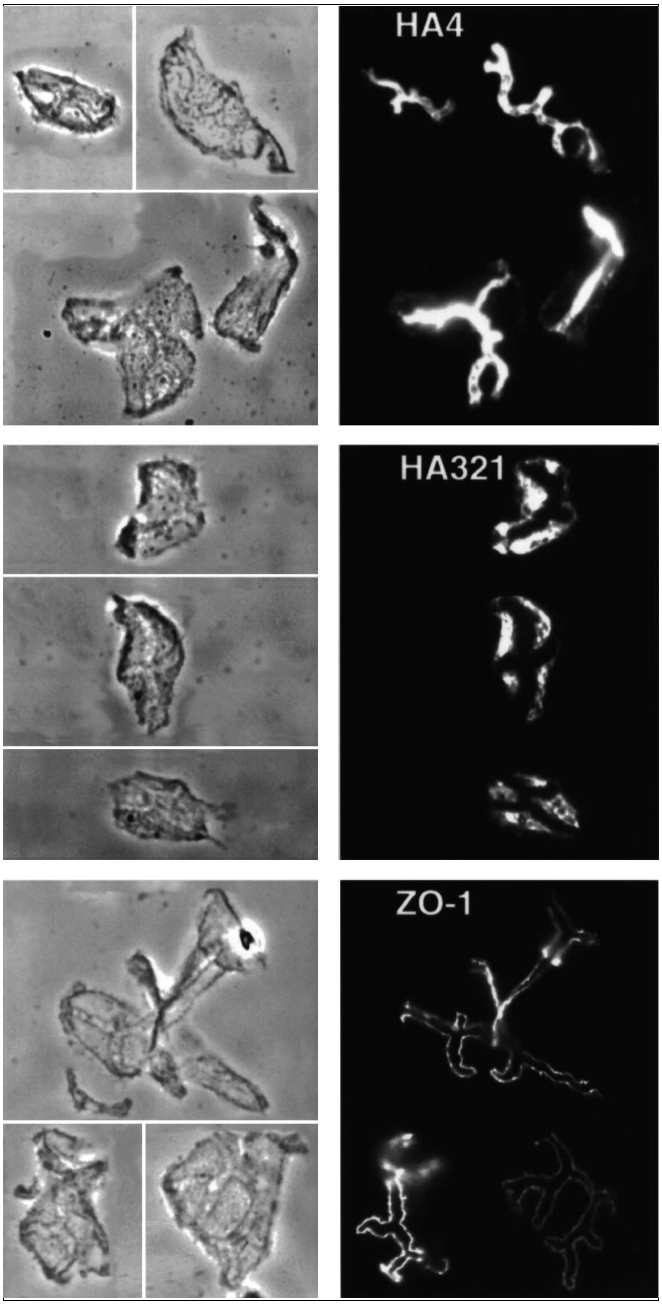


Figure 3.2.2 Immunofluorescent detection of domain-specific proteins and a tight junction–associated molecule in isolated plasma membrane sheets. Isolated plasma membrane sheets (~0.1 mg) were absorbed to glass coverslips, fixed with methanol for 5 min at –20°C, and processed for indirect immunofluorescence. (**A**), (**B**), and (**C**) are phase images of the plasma membrane sheets labeled for HA4 (**D**), HA321 (**E**), and ZO-1 (**F**), respectively. HA4 (an apical plasma membrane protein) was localized strictly to the bile canalicular area, whereas HA321 (a basolateral plasma membrane marker) was excluded from these membranes. The tight junctions, as indicated by ZO-1 staining, were detected in narrow regions immediately adjacent to the canalicular membranes. Reproduced from Fujita et al. (1998) by copyright permission of Portland Press.

by immunoblotting. In addition, the fluorescent staining patterns for domain-specific markers and tight junction components are dramatic and distinct (see Anticipated Results and Fig. 3.2.2). The authors have also found that plasma membrane proteins that were not observed in intact cells can often be detected in isolated sheets (Fujita et al., 1998). The reasons for this are not clear, but may be linked to increased accessibility of antigens for antibody binding. The sheets can also be processed for immunostaining by electron microscopy (Hubbard et al., 1983). However, the time and expertise required for ultrastructural analysis far exceed that required for light-level evaluation, making the latter approach much more accessible to the average experimenter.

Critical Parameters and Troubleshooting

Several features of the isolation procedure are essential to the successful preparation of purified plasma membrane sheets. To reduce aggregation of subcellular organelles by glycogen, rats must be starved at least 18 hr prior to sacrifice. Rat livers from younger rats (125 to 150 g) are preferable for isolation and should be used to avoid the increased amounts of connective tissue and plasma membrane–associated filaments found in preparations from larger rats. Homogenization of the perfused livers should be performed within 5 min of excision to avoid autolysis. To reduce both the vesiculation of the plasma membrane sheets and/or the production of membrane fragments, homogenization should be gentle, with minimal generation of bubbles or vacuum during the up and down strokes with the pestle. To reduce contamination by endoplasmic reticulum, it is recommended to resuspend the second pellet (P2, after centrifugation at 1500 × *g*) in twice the initial homogenate volume in preparation for flotation (see Basic Protocol 1, steps 12 and 13).

The most commonly encountered problem in the domain separation procedure is incomplete vesiculation of the membrane sheets resulting from undersonication. Incomplete vesiculation is characterized by >25% of the plasma membrane markers being found in the pelleted fraction and by the apical plasma membrane markers having more basolateral-like distributions. This problem can be corrected by increasing the number of sonication bursts until no Y-shaped membrane structures are visible by phase-contrast microscopy. Oversonication is a less prevalent problem, but results in basolateral markers having a more apical-like distribution in the gradient. This problem can be avoided by more closely monitoring vesiculation (e.g., after every sonication burst) by phase-contrast microscopy.

The assays to determine the recovery and purity of the plasma membrane sheets and of the two domains are relatively straightforward and easily interpreted. The most common problem is determining the appropriate dilution of the preparative fractions such that their activity levels fall within the values of the standard curve. In Table 3.2.2, suggested dilutions for preparative fractions are listed for both the enzyme assays and protein concentration determination using BCA reagent (Pierce). Alternatively, different standard curves can be prepared shifting the concentrations either higher or lower, or different incubation times can be used. In general, dilution of the domain gradient fractions is not suggested when assaying enzyme activities. Thus, altering the standard curve concentrations and incubation times are advised if problems are encountered.

Anticipated Results

The plasma membrane sheets purified according to Basic Protocol 1 are enriched 20- to 40-fold in plasma membrane markers. This preparation contains substantial amounts of both domains in continuity with each other in ratios approaching those of intact hepatocytes. The yield is 10% to 20% of total plasma membranes. The protein concentration of the purified sheets generally ranges from 1 to 2 mg/ml, corresponding to ~1 mg of plasma membrane protein/g of starting liver wet weight. The major contaminant of the isolated sheets is endoplasmic reticulum, which is enriched in these fractions 1-fold (see Table 3.2.1).

The vesicles derived from the apical and basolateral domains are partially resolved on the basis of differences in equilibrium density (see Fig. 3.2.1). The density profile for apical vesicles is characterized by a single peak with its center at a density of 1.10 g/cm^3 (refractive index = 1.3713). Basolateral vesicles have a bimodal distribution, with a peak centered at 1.14 g/cm^3 (refractive index = 1.3859) and a smaller (and variable) amount found in the pelleted fraction.

The apical and basolateral plasma membrane antigens, as well as tight junction components, exhibit distinct staining patterns in plasma membrane sheets. As shown in Fig. 3.2.2, the relatively intense staining for HA4 (an apical protein) is restricted to the bile canalicular membranes in an evenly distributed pattern. In contrast, staining for HA321 (a basolateral protein) is excluded from the canalicular structures and is detected in the surrounding membranes as a more diffuse and less intense signal. The tight junction protein ZO-1 is detected in regions immediately adjacent

Table 3.2.2 Suggested Dilutions for Analysis of Plasma Membrane Preparative Fractions

Fraction	Dilutions for enzyme assays[a]	Dilutions for BCA protein assay
H	1:50; 1:100	1:50; 1:100
S1	1:25; 1:50	1:25; 1:50
P1	1:50; 1:100	1:20; 1:40
S2	1:20, 1:40	1:25; 1:50
P2	1:10; 1:20	1:5; 1:10
I	1:10; 1:20	No dilution; 1:2
1.18 g/m^3	1:2; 1:4	1:2; 1:4
P3	1:5; 1:10	1:50; 1:100
S3	1:2; 1:4	No dilution
PM	1:50; 1:100	1:5; 1:10

[a]In these listings, "1:50" indicates 1 part enzyme in a total of 50 parts (i.e., 1 part enzyme plus 49 parts diluent).

to (outside) the apical plasma membrane in a ring-like pattern, indicating the location of the junctional complexes that form the barrier between plasma membrane domains.

Time Considerations

The isolation of plasma membrane sheets starting from the excision of the rat liver to the final plasma membrane pellet takes only 4 to 5 hr. Preparation of the sheets for density centrifugation by sonication generally takes 15 to 30 min. Each of the enzyme assays takes approximately 3 to 4 hr to perform, including setting up the assay, incubating the samples, reading the absorbances, and performing the calculations. Processing the sheets for indirect immunofluorescence is accomplished within 2 to 3 hr. SDS-PAGE also takes 2 to 3 hr and immunoblotting takes 18 to 24 hr.

Literature Cited

Bartles, J.R., Braiterman, L.T., and Hubbard, A.L. 1985. Endogenous and exogenous domain markers of the rat hepatocyte plasma membrane. *J. Cell Biol.* 100:1126-1138.

Blouin, A., Bolender, R.P., and Weibel, E.R. 1977. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. *J. Cell Biol.* 72:441-455.

Fujita, H., Tuma, P.L., Finnegan, C.M., Locco, L., and Hubbard, A.L. 1998. Endogenous syntaxins 2, 3 and 4 exhibit distinct but overlapping patterns of expression at the hepatocyte plasma membrane. *Biochem. J.* 329:527-538.

Hubbard, A.L. and Ma., A. 1983. Isolation of rat hepatocyte plasma membranes. II. Identification of membrane-associated cytoskeletal proteins. *J.Cell Biol.* 96:230-239.

Hubbard, A.L., Wall, D.A., and Ma, A. 1983. Isolation of rat hepatocyte plasma membranes. I. Presence of the three major domains. *J. Cell Biol.* 96:217-229.

Hubbard, A.L., Bartles, J.R., and Braiterman, L.T. 1985. Identification of rat hepatocyte plasma membrane proteins using monoclonal antibodies. *J. Cell Biol.* 100:1115-1125.

Stieger, B., Marxer, A., and Hauri, H.-P. 1986. Isolation of brushborder membranes from rat and rabbit colonocytes: Is alkaline phosphatase a marker enzyme? *J. Membr. Biol.* 2:19-31.

Touster, O., Aronson, N.N., Dulaney, J.T., and Hendrickson, H. 1970. Isolation of rat liver plasma membranes: Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* 47:604-618.

Weibel, E.R. 1976. Stereological approach to the study of cell surface morphometry. Sixth European Congress on Electron Microscopy, Jerusalem, pp. 6-9.

Weibel, E.R., Satubli, W., Gnagi, H.R., and Hess, F.A. 1969. Correlated morphometric and biochemical studies of the liver cell. *J. Cell Biol.* 42:68-91.

Widnell, C.C. and Unkeless, J.C. 1968. Partial purification of a lipoprotein with 5′ nucleotidase activity from membranes of rat liver cells. *Proc. Natl. Acad. Sci. U.S.A.* 61:1050-1057.

Contributed by Pamela L. Tuma and Ann L. Hubbard
Johns Hopkins University School of Medicine
Baltimore, Maryland

Isolation of Mitochondria from Tissues and Cells by Differential Centrifugation

The protocols in this unit are simple and rapid methods for the isolation of a mitochondrial fraction from three different mammalian tissues (liver, heart, and skeletal muscle), from cultured cells, and from yeast. Unlike the protocols in *UNIT 3.4*, they only require routine differential centrifugation in low- and high-speed centrifuges and should be accessible to any laboratory. These mitochondrial fractions will be contaminated to varying degrees by smaller particles (lysosomes and peroxisomes), although the heavy mitochondrial fraction from rat liver is relatively pure (~90%). These preparations can be used as starting material for the density-gradient separations described in *UNIT 3.4*.

Basic Protocol 1 describes the isolation of the heavy mitochondrial fraction from rat liver; this fraction has high respiratory control and can be used in oxygen electrode studies for ≥4 hr after preparation. Centrifuging the postnuclear supernatant at only 3000 × *g* avoids significant contamination of the pellet by other more slowly sedimenting organelles (e.g., lysosomes, peroxisomes, endoplasmic reticulum). Contamination is reduced further by gently washing the 3000 × *g* pellet. A mannitol-containing buffer is the medium of choice for this preparation.

Other protocols describe methods for obtaining crude total mitochondrial fractions from bovine heart (see Basic Protocol 2), rat skeletal muscle (see Basic Protocol 3), cultured cells (see Basic Protocol 4), and yeast (see Basic Protocol 5). The major differences between these protocols is their mode of homogenization. Although the easy availability of rat liver in most laboratories makes it a popular choice as a source of mitochondria, those from bovine heart are also often used for respiratory studies. Indeed, they may even be more tightly coupled than liver mitochondria, and they can generally be stored for longer periods while maintaining good functional integrity. This may be allied to the lower levels of proteases and slower release of fatty acids in this tissue. Heart mitochondria also tend to provide better yields of the various structural components of electron transport and ATP synthesis. Basic Protocol 2 also provides a strategy for large-scale preparation. The increasing use of yeast as a model for mammalian membrane and organelle synthesis points to the importance of Basic Protocol 5. Although rat brain is another widely used source of mitochondria, they are rarely purified by differential centrifugation alone (see *UNIT 3.4*).

Methods for measuring succinate dehydrogenase, catalase, and β-galactosidase (as mitochondrial, peroxisomal, and lysosomal markers, respectively) in density-gradient fractions are given in *UNIT 3.4*, but they can also be applied to assessing the purity of mitochondria prepared by differential centrifugation.

Protease inhibitors (see *UNIT 3.4*, Reagents and Solutions) can be included in any or all of the media at the discretion of the investigator, except in the protease-containing solutions used in Basic Protocols 3 and 5.

NOTE: For all protocols, all *g* values are given as *g*.

PREPARATION OF THE HEAVY MITOCHONDRIAL FRACTION FROM RAT LIVER

BASIC PROTOCOL 1

Young adult male animals (150 to 200 g) are routinely used, providing livers of ~10 g wet weight. This protocol is designed for one such liver, but can be scaled up or down proportionally for different amounts of liver. The animals are normally deprived of food overnight to reduce the glycogen content of the liver; this facilitates the separation process.

Contributed by John M. Graham
Current Protocols in Cell Biology (1999) 3.3.1-3.3.15

A homogenization medium containing mannitol and sucrose, a chelating agent (either EGTA or EDTA), and a buffer (normally HEPES or MOPS) is best suited to respiratory studies.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin, but also to protect the hand in the unlikely event that the vessel breaks.

Materials

150- to 200-g male Sprague-Dawley rat
Liver homogenization medium (LHM; see recipe), ice cold

Dissecting tools
Potter-Elvehjem homogenizer (~0.09-mm clearance; 25-ml working volume)
Overhead high-torque electric motor (thyristor-controlled)
Low-speed centrifuge with swinging bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor and 40- to 50-ml polycarbonate tubes
Vacuum pump
Dounce homogenizer (30- to 40-ml volume) with loose-fitting pestle (Wheaton type B)

Isolate liver

1. Deprive a 150- to 200-g male Sprague-Dawley rat of food overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.

   This must be supervised or carried out by an experienced animal technician.

3. Open the abdominal cavity and transfer the liver to a chilled beaker containing ~20 ml LHM.
4. Decant the medium and finely mince the liver using scissors.

   The pieces of liver should be no more than ~25 mm^3.

5. Agitate the minced tissue in ~30 ml ice-cold LHM and allow the pieces to settle out.
6. Decant the medium and replace with ~40 ml fresh medium.

Homogenize liver

7. Transfer half the suspension to the chilled glass vessel of a Potter-Elvehjem homogenizer.
8. Attach the cold pestle to an overhead high-torque electric motor and homogenize the minced liver using five to six up-and-down strokes of the pestle, rotating at ~500 rpm. Decant the homogenate into a beaker on ice.

   The motor should be securely mounted either to a wall, to a bench via a G clamp, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

9. Rinse the homogenizer with medium and wipe the pestle to remove any adhering connective tissue. Repeat the procedure with the other half of the suspension.

Isolate mitochondria

10. Centrifuge the homogenate 10 min at 1000 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.

11. Aspirate the supernatant and transfer to 40- to 50-ml polycarbonate tubes.

    It is convenient to use a 20- to 30-ml syringe attached to a metal filling cannula (i.d. 0.8 to 1.0 mm) to aspirate supernatants that are to be recentrifuged.

12. Centrifuge the supernatant 10 min at 3000 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.

13. Using a glass Pasteur pipet attached to some form of vacuum pump, aspirate the supernatant from each tube, keeping the tip of the pipet at the meniscus to remove as much of the floating lipid layer as possible. Also remove as much as possible of the loose-packed pinkish layer that overlies the brown mitochondria.

14. Wipe away any remaining lipid adhering to the wall of the tube with a paper tissue.

    Removal of this lipid is essential, as free fatty acids are potent uncouplers of phosphorylation from electron transport.

15. Add a small amount of LHM (~8 ml) to each pellet and crudely resuspend the pellet with a glass rod. Then resuspend fully using 3 to 4 very gentle strokes in a Dounce homogenizer.

16. Make up to the original volume with LHM, transfer to new tubes, and recentrifuge 10 min at 3000 × *g* in the high-speed centrifuge.

17. Repeat steps 13 to 16 twice more.

    For large-scale preparations, the total volume of LHM used to resuspend the pellet can be reduced by ~50% for the second and third washes.

18. Resuspend the purified mitochondria in a buffer whose composition is compatible with any subsequent analysis or processing; in many instances, LHM will be satisfactory.

    See Time Considerations for information about storage of mitochondria prior to further processing.

LARGE-SCALE PREPARATION OF MITOCHONDRIA FROM BOVINE HEART

BASIC PROTOCOL 2

It is important that fresh slaughterhouse material be used for this preparation, and that any adhering connective and adipose tissue be carefully removed. Because of the scale of the preparation and the size of the homogenization equipment, the procedure must be carried out in a cold room. The procedure is suitable for 500 to 600 g of material, and is adapted from Smith (1967) and Rice and Lindsay (1997).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice or in a cold room throughout.

Materials

Bovine heart, freshly isolated
Heart wash buffer (see recipe)
2.0 M Tris base
Sucrose/succinate solution (SS; see recipe)

Commercial mincer with a total capacity of 2 to 3 liters
Waring blender or other large-capacity rotating blades homogenizer
Cotton muslin
Low-speed centrifuge with swinging-bucket rotor and 250- to 750-ml bottles
High-speed centrifuge with fixed-angle rotor
Glass rod
Dounce homogenizer (50-ml volume) with loose-fitting pestle (Wheaton type B)

Prepare the heart

1. Cut freshly isolated bovine heart tissue into small cubes (~4 cm^3) and pass once through a commercial mincer.
2. Suspend in 800 ml ice-cold heart wash buffer. While stirring, adjust the pH to 7.8 by adding 2.0 M Tris base.
3. Pour through two layers of cotton muslin and then squeeze to remove as much of the liquid as possible.
4. Transfer minced tissue to a clean beaker and suspend in 800 ml ice-cold SS.

Homogenize the heart

5. Transfer half the suspension to a Waring blender and homogenize at high speed for 20 sec.
6. Readjust the pH to 7.8 using 2.0 M Tris base and then reblend for 60 sec.
7. Repeat the procedure with the other half of the suspension. Combine the two homogenates and dilute with ice-cold SS to ~2.2 liters.

Isolate mitochondria

8. Transfer homogenate to 250- to 750-ml centrifuge bottles and centrifuge 20 min at 800 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.
9. Carefully decant the supernatants and recentrifuge 20 min at 26,000 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.

   A rotor such as the Sorvall SLA-1500 will allow this to be carried out in two centrifugations.

10. Decant and discard the supernatant.

    The pellet is clearly tripartite.

11. Tilting the bottles, gently pour ~10 ml SS on top of each pellet and gently swirl the contents to resuspend the top light-brown layer of partially disrupted mitochondria. Discard this material.
12. Crudely resuspend the remaining dark-brown mitochondria in ~20 ml SS using a glass rod, avoiding the almost-black hard-packed button at the bottom.
13. Completely resuspend mitochondria using 2 to 3 gentle strokes in a Dounce homogenizer.
14. Dilute the suspension to ~300 ml with SS and recentrifuge 20 min at 26,000 × *g*, 4°C.
15. Collect and resuspend the middle layer of the pellet as in steps 10 to 13.

    The upper and lower layers of this second pellet should be relatively minor components.

16. If the composition of the SS medium is incompatible with any subsequent analysis, centrifuge the suspension 20 min at 26,000 × *g* and resuspend the pellet in a suitable medium.

    See Time Considerations for information about storage of mitochondria prior to further processing.

PREPARATION OF MITOCHONDRIA FROM SKELETAL MUSCLE

This protocol uses a commercially available protease to facilitate the homogenization of the muscle tissue. Using this approach, the severity of the shear forces used to disrupt the tissue can be reduced, thus minimizing any damage to the mitochondria. The protocol is adapted from Bhattacharya et al. (1991). The protocol is designed for 4 to 5 g muscle tissue, but can be scaled up or down proportionally.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice or in a cold room throughout. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin, but also to protect the hand in the unlikely event that the vessel breaks.

Materials

150- to 200-g male Sprague-Dawley rat
Muscle wash buffer (see recipe)
Muscle homogenization medium I (see recipe)
Muscle homogenization medium II (see recipe)

Dissecting tools
Potter-Elvehjem homogenizer (~0.3-mm clearance, 40-ml working volume)
Overhead high-torque electric motor (thyristor-controlled)
Fine nylon mesh (200-μm pore size)
Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor
Dounce homogenizer (30-ml volume) with loose-fitting pestle (Wheaton type B)

Isolate muscle tissue

1. Sacrifice a 150- to 200-g male Sprague-Dawley rat by cervical dislocation or decapitation.

   This must be supervised or carried out by an experienced animal technician.

2. Rapidly dissect out 4 to 5 g of striated leg muscle and wash it twice in ~50 ml muscle wash buffer.

3. Mince the muscle finely on a cold surface (e.g., a glass plate on crushed ice) using two scalpels.

   The muscle pieces should be <30 mm^3. The smaller the muscle pieces, the more efficient is the enzyme softening.

Homogenize tissue

4. Add 40 ml muscle homogenization medium I to the tissue and incubate for 5 min at 0° to 4°C.

5. Transfer to an ice-cold glass vessel of a Potter-Elvehjem homogenizer.

6. Attach the cold pestle to an overhead high-torque electric motor and homogenize using eight up-and-down strokes of the pestle, rotating at ~700 rpm.

   The motor should be securely mounted either to a wall, to a bench via a G clamp, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

7. Incubate the homogenate with stirring for 5 min at 0° to 4°C.

8. Dilute with an equal volume of muscle homogenization medium II and rehomogenize as in step 6.

Isolate mitochondria

9. Filter the homogenate through fine nylon mesh and then centrifuge the filtrate 10 min at 2000 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.
10. Decant the supernatant and keep on ice.
11. Rehomogenize the pellet in 20 ml muscle homogenization medium II using three up-and-down strokes of the pestle, recentrifuge, and combine the supernatant with the first supernatant.
12. Centrifuge mitochondria 10 min at 10,000 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.
13. Wash the pellet by resuspending in ~20 ml muscle homogenization medium II using 2 to 3 gentle strokes of the pestle in a Dounce homogenizer.
14. Repeat centrifugation as in step 12 and resuspend the pellet in a medium whose composition is compatible with any subsequent analysis or processing; in many instances muscle homogenization medium II or LHM (see recipe) will be quite satisfactory.

See Time Considerations for information about storage of mitochondria prior to further processing.

BASIC PROTOCOL 4

PREPARATION OF MITOCHONDRIA FROM CULTURED CELLS

This protocol, which is adapted from Attardi and Ching (1979) and Rice and Lindsay (1997), is designed for cultured cells (~2×10^8) grown as a monolayer. The method may also be satisfactory for suspension culture cells, as the homogenization medium is hypoosmotic. Inclusion of Mg^{2+} and K^+ in this medium is crucial; not only do these cations protect the nuclei from breakage, the KCl helps prevent some of the cytoplasmic proteins from forming a gel. To protect the released organelles from the hypoosmotic environment, the volume ratio of cells to medium should be as high as possible.

NOTE: All solutions (except PBS), glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice or in a cold room throughout. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin, but also to protect the hand in the unlikely event that the vessel breaks.

Materials

Confluent monolayer cultured cells (total 2×10^8)
PBS (*APPENDIX 2A*)
Cell homogenization medium (CHM; see recipe)
CHM containing 1 M sucrose
Sucrose/Mg^{2+} medium (see recipe)
Mitochondrial suspension medium I (see recipe)

Rubber policeman
Potter-Elvehjem homogenizer (~0.09-mm clearance, 10- to 15-ml working volume)
Overhead high-torque electric motor (thyristor-controlled)
Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor and appropriate tubes
Dounce homogenizer (5- to 10-ml volume) with loose-fitting pestle (Wheaton type B)

Prepare cells

1. Wash the monolayer cultures twice with ~60 ml PBS per wash at room temperature.
2. Using a rubber policeman, scrape the cells into 60 to 80 ml PBS and pellet the cells at 1000 × *g*, room temperature for 15 min.

   It is important to form a compact pellet so that all of the PBS can be removed in step 3.

3. Aspirate or decant all of the supernatant and resuspend the cells in ice-cold cell homogenization medium. Use a volume of medium equal to six times the volume of the pellet.
4. Leave on ice for 2 min.

Homogenize cells

5. Attach the cold pestle of a Potter-Elvehjem homogenizer to an overhead high-torque electric motor and homogenize the cells using five up-and-down strokes at 500 rpm. Confirm that ≥90% cell breakage has occurred by examining the homogenate under a phase-contrast microscope.

   The motor should be securely mounted either to a wall, to a bench via a G clamp, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

   Some cells may require up to ten strokes of the pestle. If adequate cell breakage has not occurred at this stage, a tight-fitting Dounce homogenizer (Wheaton type A) should be used.

6. Add ⅓ vol ice-cold CHM containing 1 M sucrose (final 0.25 M) and mix gently by repeated inversion.

   Do not create foaming by rapid agitation.

Isolate mitochondria

7. Pellet nuclei by centrifuging 5 min at 1000 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.

   Washing the nuclear pellet to recover more mitochondria cannot be uniformly recommended for all cultured cells, as the nuclei of many cell lines tend to be rather fragile and may release DNA upon being resuspended.

8. Decant or aspirate the supernatant and centrifuge 10 min at 5000 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.
9. Resuspend the pellet in ~10 ml ice-cold sucrose/Mg^{2+} medium using two to three gentle strokes of the pestle in a Dounce homogenizer.
10. Recentrifuge at 5000 × *g*, 10 min, 4°C. Resuspend the pellet in 2 to 3 ml ice-cold mitochondrial suspension medium I or other suitable medium for further processing.

    See Time Considerations for information about storage of mitochondria prior to further processing.

PREPARATION OF MITOCHONDRIA FROM YEAST (*SACCHAROMYCES CEREVISIAE*)

BASIC PROTOCOL 5

This protocol uses Zymolase to digest the tough outer coat of yeast to produce spheroplasts. It is probably best suited to the relatively small-scale cultures used in cell and molecular biology research. The rather harsh mechanical shear forces imposed by commercial homogenizers and ball mills that are often employed for large-scale cultures are not very well suited to the preparation of mitochondria. Zymolase works well with most wild-type yeast strains (e.g., D273-10B or KL14-4A). The yeast should be harvested in early- or mid-log growth phase, as the tougher coat that develops in late-log or stationary phase renders the enzyme digestion less satisfactory.

The yeast should be grown in YPD medium (*UNIT 1.6*) and washed in distilled water and a dithiothreitol-containing buffer. This common strategy seems to facilitate subsequent spheroplast formation (Goud et al., 1988). The protocol is adapted from Rice and Lindsay (1997).

Materials

Yeast cultures in early- to mid-log phase, grown in YPD medium (1% yeast extract/2% Bacto Peptone/2% glucose; see *UNIT 1.6*)
DTT buffer (see recipe)
Yeast sorbitol buffer (see recipe)
Zymolase-100T (from *Arthrobacter luteus*, 100,000 U/g; e.g., ICN Biomedicals, Sigma)
Spheroplast homogenization medium (see recipe), ice cold
Mitochondrial suspension medium II (see recipe), ice cold

Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor and appropriate tubes
30°C incubator
Dounce homogenizer (5- to 10-ml volume) with loose-fitting pestle (Wheaton type B)

Harvest yeast

1. Harvest yeast by centrifuging the culture (~1 liter) 10 min at 1000 × *g*, room temperature, in a swinging-bucket rotor using a low-speed centrifuge. Use a preweighed centrifuge tube, and calculate the wet weight of yeast by reweighing the tube after removing the supernatant.

   An early- to mid-log phase yeast culture is equivalent to an OD_{600} of ~0.6. Such a culture contains ~10^7 cells/ml.

2. Wash cells once in 3 to 4 vol distilled water, centrifuge again, and resuspend in 2 vol DTT buffer.

3. Incubate 30 min at 30°C and then centrifuge again.

4. Wash the cells once in 3 to 4 vol yeast sorbitol buffer and then resuspend in this medium at 0.15 g wet weight/ml.

Make spheroplasts

5. Stir in Zymolase-100T at 0.25 mg/100 g wet weight and incubate for ~1 hr at 30°C.

   Spheroplast formation can be checked by adjusting a small sample of the suspension to 0.1% (w/v) sodium dodecyl sulfate (SDS) to solubilize the spheroplasts and observing the remaining intact yeast cells under a microscope.

   Zymolase is sold by Sigma under the trade name Lyticase.

6. Harvest spheroplasts by centrifuging 10 min at 1000 × *g*, 4°C, and then wash the pellet once in 3 to 4 vol yeast sorbitol buffer.

Homogenize spheroplasts

7. Resuspend spheroplasts in 2 vol ice-cold spheroplast homogenization medium and carry out all subsequent operations at 0° to 4°C.

8. Homogenize in a Dounce homogenizer using no more than ten up-and-down strokes of the pestle.

Isolate mitochondria

9. Dilute homogenate with an equal volume of spheroplast homogenization medium and remove the nuclei and unbroken spheroplasts by centrifuging 10 min at 1000 × *g*, 4°C.

10. Decant or aspirate the supernatant and centrifuge 10 min at 6500 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.

11. Wash the pellet once in ~40 ml ice-cold mitochondrial suspension medium II, recentrifuge, and resuspend in ~10 ml of the same buffer.

    See Time Considerations for information about storage of mitochondria prior to further processing.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocols (except where indicated). For common stock solutions see ***APPENDIX 2A****; for suppliers see* ***SUPPLIERS APPENDIX****.*

Cell homogenization medium (CHM)

To 100 ml H_2O add:
30 µl 1 M $MgCl_2$ (150 mM final)
0.15 g KCl (10 mM final)
2.0 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to 6.7
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

For CHM containing 1 M sucrose, add 68.4 g sucrose to 200 ml CHM.

Dithiothreitol (DTT) buffer

To 100 ml H_2O add:
0.31 g dithiothreitol (10 mM final)
20 ml 1 M Tris base (0.1 M final)
Adjust pH to 9.3 with HCl
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Heart wash buffer

To 500 ml H_2O add:
85.5 g sucrose (0.25 M final)
10 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to pH 7.8
Add H_2O to 1 liter
Store up to 1 to 2 days at 4°C

Liver homogenization medium (LHM)

To 250 ml H_2O add:
18.2 g mannitol (0.2 M final)
8.55.g sucrose (50 mM final)
0.37 g KCl (10 mM final)
5.0 ml 100 mM Na_2EDTA (1 mM final)
50 ml 100 mM HEPES (10 mM final)
Adjust pH to 7.4 with KOH
Add H_2O to 500 ml
Store up to 1 to 2 days at 4°C

Mitochondrial suspension medium I

To 50 ml H_2O add:
8.5 g sucrose (0.25 M final)
1.0 ml 1 M Tris base (10 mM final)
Adjust pH to 7.0 with acetic acid
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Mitochondrial suspension medium II

To 100 ml H_2O add:
21.9 g mannitol (0.6 M final)
40 ml 100 mM HEPES (20 mM final)
Adjust pH to 7.4 with KOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Muscle homogenization medium I

To 100 to <200 ml H_2O add:
6.84 g sucrose (0.1 M final)
0.686 g KCl (46 mM final)
20 ml 100 mM Na_2EDTA (10 mM final)
2.0 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C
Immediately before use, add 1.0 g bovine serum albumin (BSA; 0.5% w/v final) and 40 mg Nagarse (0.2 mg/ml final)

Sigma now markets Nagarse under the name Protease Type VII.

Muscle homogenization medium II

To 100 ml H_2O add:
6.84 g sucrose (0.1 M final)
0.686 g KCl (46 mM final)
20 ml 100 mM Na_2EDTA (10 mM final)
2.0 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Muscle wash buffer

To 100 ml H_2O add:
7.64 g mannitol (0.2 M final)
4.78 g sucrose (70 mM final)
0.2 ml 100 mM Na_2EDTA (0.1 mM final)
2.0 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Spheroplast homogenization medium

To 100 ml H_2O add:
21.9 g mannitol (0.6 M final)
40 ml 100 mM HEPES (20 mM final)
Adjust pH to 7.4 with KOH

continued

Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C
Immediately before use add 1 ml 200 mM phenylmethylsulfonyl fluoride (PMSF; 1 mM final)

Sucrose/Mg^{2+} medium

To 100 ml H_2O add:
30 µl 1 M $MgCl_2$ (0.15 M final)
17.1 g sucrose (0.25 M final)
2 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to 6.7
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Sucrose/succinate solution (SS)

To 1.5 liter H_2O add:
256.5 g sucrose (0.25 M final)
0.81 g disodium succinic acid hexahydrate (1 mM final)
6.0 ml 100 mM Na_2EDTA (0.2 mM final)
30 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust to pH 7.8
Add H_2O to 3 liters
Store up to 1 to 2 days at 4°C

Yeast sorbitol buffer

To 100 ml H_2O add:
43.7 g sorbitol (1.2 M final)
40 ml 100 mM KH_2PO_4 (20 mM final)
Adjust pH to 7.4 with KOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

COMMENTARY

Background Information

Differential centrifugation is often used merely as a preliminary procedure to prepare a crude fraction of membranes or subcellular organelles prior to further purification in a density gradient. This technique separates particles according to their velocity of sedimentation (v), which depends principally on the diameter of the particle (d), although particle density (ρ_p) can also be important in some cases (e.g., nuclei). For the other parameters, ρ_l (the density of the liquid) and μ (its viscosity), are constant, and g is the applied centrifugal force.

$$v = \frac{d^2\left(\rho_p - \rho_l\right)}{18\mu} g$$

Table 3.3.1 gives the dimensions of some of the major particles in mammalian liver. Since v is proportional to d^2, it is clear from these values that the sedimentation rate of the majority of nuclei is much greater than that of the other particles, while only the largest mitochondria are likely to be separable from organelles such as lysosomes and peroxisomes. This is enhanced by the tendency of the larger mitochondria to be slightly more dense than lysosomes and peroxisomes. Thus centrifugation of a homogenate at 500 to 1000 × g for 5 to 10 min will pellet essentially all of the nuclei, a slightly higher relative centrifugal force (RCF) of 3000 × g for 10 min will pellet the "heavy" fraction of the mitochondria, and the considerably higher RCFs (e.g., 10,000 to 15,000 × g) required to pellet all of the smallest mitochondria will also pellet all lysosomes and peroxisomes.

The heterogeneity in the size of different organelles is bound to compromise separations of particles on the basis of sedimentation rate, but differential centrifugation (as opposed to rate-zonal centrifugation) imposes a further problem that arises from the sedimentation path length of the rotor and the radial increase in g

Table 3.3.1 Size (d) and d^2 of Major Organelles from Rat Liver

Particle	Size (μm)	d^2
Nucleus	4-12	16-144
Plasma membrane sheet[a]	3-20	9-400
Golgi membranes	1-2	1-4
Mitochondria	0.4-2.5	0.16-6.25
Lysosomes	0.4-0.8	0.16-0.64
Peroxisomes	0.4-0.8	0.16-0.64
Most vesicles	0.05-0.3	0.0025-0.09

[a]Only organized tissues such as liver produce plasma membrane sheets under certain homogenization conditions.

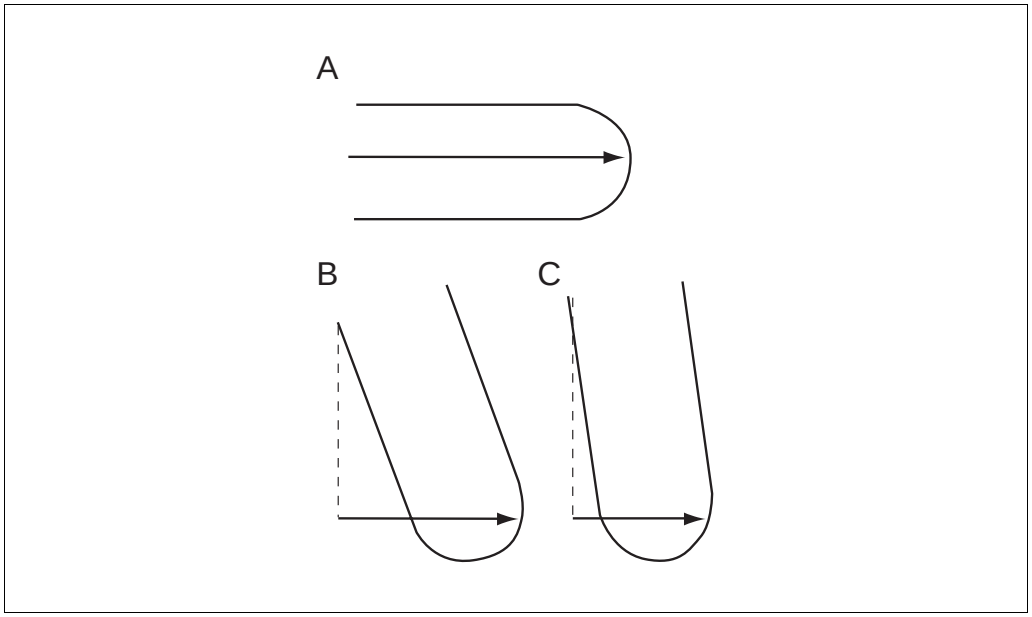


Figure 3.3.1 Sedimentation path length of rotors: (**A**) swinging-bucket; (**B**) high-angle fixed-angle; (**C**) low-angle fixed-angle. The sedimentation path length is indicated by the arrowed line.

force in the spinning rotor. If the homogenate fills the centrifuge tube, then the RCF at the top of the sample is considerably less than that at the bottom of the sample. On the other hand, particles at the top of the sample have the furthest distance to travel to form a stable pellet. The above equation shows that a particle with a diameter of 5 μm will sediment four times faster than one with a diameter of 2.5 μm and equal density. In such cases, all the smaller particles in the lower quarter of the centrifuge tube will sediment in the time taken for the larger particles, initially at the top of the tube, to pellet. Thus, in differential centrifugation, if a rotational speed and centrifugation time are chosen to allow all of the nuclei to pellet, then some the mitochondria towards the bottom of the tube—which are exposed to the highest RCF and have the shortest distance to travel—will also pellet. It is for this reason that "washing" of a differential centrifugation pellet is a common practice; resuspending any pellet in medium and repeating the centrifugation will reduce contamination by the smaller particles. However, it will also reduce the yield of the particle of interest.

The sedimentation path length and the difference in RCF between the top and bottom of the sample are both less in a fixed-angle rotor than in a swinging-bucket rotor of the same tube volume (Fig. 3.3.1). Thus, differential centrifugation should always be carried out in a fixed-angle rotor for maximum resolution, and the lower the tube angle the better. However, as the tube angle decreases, the pellet tends to spread up the wall of the tube and become less compact and sometimes less stable, making removal of the supernatant more difficult. In practice,

therefore, a tube angle of ~30° is probably a good compromise. It might, therefore, be surprising that in all of the protocols presented here, the first low-speed centrifugation to remove the nuclei is often executed in a swinging-bucket rotor. In this case, the difference in the rate of sedimentation between the bulk of the nuclei and the mitochondria is so great that a fixed-angle rotor offers no significant advantage. More information on the use of differential centrifugation for the isolation of subcellular organelles can be obtained from Evans (1992), Graham (1997), and Hinton and Mullock (1997).

For studies on oxidative phosphorylation, mitochondria prepared by simple differential centrifugation are often preferred to those purified by gradient centrifugation. Here the aim is not to achieve high recoveries, but to prepare the organelles as rapidly and as gently as possible in a medium known to promote the retention of a highly coupled state. Hence, it is best to avoid the use of time-consuming density gradients, which may also expose the mitochondria to potentially damaging high *g* forces and unsuitable media. Bovine heart (see Basic Protocol 2) and the heavy fraction from rat liver (see Basic Protocol 1) are widely used for this purpose. See Rice and Lindsay (1997) for a review of methods for the measurement of functions associated with electron transport and oxidative phosphorylation and the preparation of submitochondrial particles.

Purification of subcellular organelles such as mitochondria has been given fresh impetus recently from the increasing number of projects aimed at mapping all proteins within a tissue (proteomics).

Critical Parameters and Troubleshooting

The composition of solutions used for isolating mitochondria is very much dependent on the source material. While a mannitol/sucrose–containing medium (see Basic Protocol 1) appears to offer good retention of functional integrity for liver mitochondria, it is not apparently a universal requirement. Although the inclusion of divalent cations (e.g., Mg^{2+}) is usually avoided (indeed, addition of EDTA or EGTA is often beneficial), the selection of a suitable homogenization medium may be dictated by the choice of material. This is most clearly the case with cultured cells, which may require a hypoosmotic medium to effect cell breakage; in these instances, Mg^{2+} and K^+ are present to protect the nuclei from disruption. The medium used in Basic Protocol 4 is a good general-purpose medium for adherent cultured cells (Attardi and Ching, 1979) that may also be suitable for suspension cultured cells, its composition being not unlike that used by Goldberg and Kornfeld (1983) for isolating membrane compartments of the secretory system. The presence of KCl is quite compatible with the isolation of functionally intact mitochondria, and it is often included to solubilize proteins that tend to form gels in homogenates of cultured cells and skeletal muscle (Rice and Lindsay, 1997). However, some monolayer cells can be homogenized in an isoosmotic sucrose medium containing 0.25 M sucrose, 1 mM EDTA, 10 mM triethanolamine/acetic acid, pH 7.4 (Marsh et al., 1987; Graham, 1997). The efficacy of the medium is related to the particular buffer, for which there is a strict requirement. If a hypoosmotic Mg^{2+}-containing medium is used, the suspending medium must be made isoosmotic as soon as possible and the mitochondria suspended in a Mg^{2+}-free medium. Once the nuclei have been removed, it is acceptable to add EDTA to the postnuclear supernatant to chelate the Mg^{2+}.

Scaling up of procedures should always be accompanied by a proportional scaling up of the homogenization volume, but the volumes of buffer used for washing centrifugation pellets may be reduced. For example, in Basic Protocol 1, the ratio of liver to homogenization medium must be maintained at ~40 ml per 10 g of liver; for washing the $3000 \times g$ pellets, the volume of LHM can be reduced by about one third.

The centrifugation conditions used to isolate mitochondria from the postnuclear supernatant are tailored to individual tissues or cells; however, any RCF between 5,000 and $10,000 \times g$ is generally adequate, and the reason for using $26,000 \times g$ for bovine heart mitochondria (Smith, 1967) is not entirely clear. Lysosome and peroxisome contamination will tend to increase as the RCF increases, although the yields of mitochondria will also increase. The RCFs required for the pelleting of mitochondria from cultured cells may well depend on the type of cell and the homogenization schedule used. With this type of material, therefore, it may be prudent to determine the RCFs empirically by investigating the composition of pellets produced at a series of RCFs such as 500, 1000, 3000, 6000, 10,000, and $15,000 \times g$ (each for 10 min).

Respiratory studies on mitochondria are particularly demanding on the integrity of the product. Thus, at all stages in their preparation, great attention must be paid to the use of the mildest and gentlest of manipulations. It cannot be stressed too strongly that excessive shear forces during homogenization can be highly deleterious to these organelles, and the resuspension of mitochondrial pellets must be carried out with the very minimum of liquid shear forces.

Anticipated Results

The yield of succinate dehydrogenase in the heavy mitochondrial pellet from rat liver may be as much as 80% of the total, with relatively little contamination from lysosomes and peroxisomes. The contamination levels vary with the efficacy of the washing procedure, but 5% to 10% of the total is not uncommon. Levels are much higher in the unwashed material (20% to 25% of the total). Oxygen electrode studies on this fraction reveal that oxygen uptake in the presence of either succinate or glutamate plus malate is very low upon depletion of endogenous ADP, and that addition of exogenous ADP increases the rate of oxygen consumption by as much as ten to fifteen fold, indicating that they are highly coupled. Recoveries of mitochondria in the other protocols are routinely 90% to 95%. However, because the RCFs used to produce the mitochondrial pellet are higher, the contamination by peroxisomes and lysosomes is correspondingly higher (25% to 30%), and these levels are less easily reduced by washing.

Time Considerations

There are no points at which these relatively short protocols may be suspended. Indeed, rapid preparation is the key to the recovery of functionally intact mitochondria. All solutions can be made ahead of time and stored at 4°C for 1 to 2 days or frozen at −20°C for longer period (e.g., 2 to 3 months). Note that any enzymes, BSA, PMSF, or other protease inhibitors should be added immediately prior to use.

Basic Protocols 1, 3, and 4 should be accomplished in 2 to 3 hr, while Basic Protocol 5 will require ≥4 hr because of the two incubation steps. The time required for Basic Protocol 2 will depend on the availability of suitable large-capacity rotors; it may be necessary to split the material between two centrifugations, if the 2.2 liters cannot be accommodated in the rotor. Large-capacity rotors also decelerate slowly (up to 15 min) so the whole procedure may take ~3.5 hr.

Functional assays should be carried out on the mitochondria as soon as possible after their preparation: this applies particularly to studies on oxidative phosphorylation and other measurements which rely on the permeability properties of the mitochondrial membranes. Storage for up to 4 hr at 0° to 4°C (<8 hr for heart mitochondria) is permissible but not recommended. Other functional assays (e.g., the succinate dehydrogenase assay described in *UNIT 3.4*) which are less demanding on structural integrity may be performed on material stored at 0° to 4°C for <16 h. Such assays and compositional measurements may also be carried out on material frozen to −20°C or −80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those made on the fresh organelles.

NOTE: If the mitochondria are to be further purified using one of the Basic Protocols described in *UNIT 3.4*, this must be carried out without delay.

Literature Cited

Attardi, G. and Ching, E. 1979. Biogenesis of mitochondrial protein in HeLa cells. *Methods Enzymol.* 56:66-79.

Bhattacharya, S.K., Thakar, J.H., Johnson, P.L., and Shanklin, D.R. 1991. Isolation of skeletal muscle mitochondria from hamsters using an ionic medium containing EDTA and Nagarse. *Anal. Biochem.* 192:344-349.

Evans, W.H. 1992. Isolation and characterization of membranes and cell organelles. *In* Preparative Centrifugation: A Practical Approach (D. Rickwood, ed) pp. 233-270. IRL Press, Oxford.

Goldberg, D.E. and Kornfeld, S. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159-3165.

Goud, B., Salminen, A., Walworth, N.C., and Novick, P.J. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell* 53:753-768.

Graham, J.M. 1997. Homogenization of tissues and cells. *In* Subcellular Fractionation: A Practical Approach (J.M. Graham and D. Rickwood, eds.) pp. 1-29. IRL Press, Oxford.

Hinton, R.H. and Mullock, B.M. 1997. Isolation of subcellular fractions. *In* Subcellular Fractionation: A Practical Approach. (J.M. Graham and D. Rickwood, eds.) pp. 31-69. IRL Press, Oxford.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* 104:875-886.

Rice, J.E. and Lindsay, J.G. 1997. Subcellular fractionation of mitochondria. *In* Subcellular Fractionation: A Practical Approach (J.M. Graham and D. Rickwood, eds.) pp. 107-142. IRL Press, Oxford.

Smith, A.L. 1967. Preparation and properties and conditions for assay of mitochondria: Slaughterhouse material small scale. *Methods Enzymol*. 10:81-86.

Contributed by John M. Graham
Liverpool John Moores University
Liverpool, United Kingdom

Purification of a Crude Mitochondrial Fraction by Density-Gradient Centrifugation

Metabolic studies of mitochondria are often carried out on organelles prepared by differential centrifugation (*UNIT 3.3*), avoiding the use of gradients, which extend the preparation time. However, to obtain high yields of mitochondria in a relatively pure form, or to determine the specificity of the localization of a particular component or function to mitochondria (as compared to other organelles), density-gradient centrifugation is the most obvious strategy. Most mitochondrial fractions produced by differential centrifugation, with the exception of the heavy fraction from some mammalian tissues (*UNIT 3.3*), are contaminated to a significant degree by other organelles—lysosomes, peroxisomes, tubular Golgi membranes, and small amounts of endoplasmic reticulum. The Basic Protocols in this unit are therefore aimed at the optimal resolution of mitochondria from these other membrane particles in density gradients that can be used either preparatively or analytically.

A continuous sucrose gradient system for mammalian liver mitochondria is described in Basic Protocol 1. Although discontinuous sucrose gradients seem to lack the resolution necessary for mammalian systems, they can be used for yeast (also see Basic Protocol 1). Because of the hyperosmotic nature of all sucrose gradient systems, they lack the resolution possible with media such as Nycodenz, iodixanol, and Percoll, which can form isoosmotic (or nearly isoosmotic) gradients. Basic Protocol 2 describes a discontinuous Percoll gradient as applied to the isolation of rat brain mitochondria. Although the Percoll gradient in this protocol is probably satisfactory for mitochondria from sources other than rat brain, an alternative discontinuous gradient designed for rat liver is also included (see Alternate Protocol). Basic Protocol 3 describes a self-generated Percoll gradient for rat liver mitochondria. Some of the variations of these Percoll-based methods are discussed later (see Commentary). Although iodixanol can also be used in self-generated mode, the *g* forces required are rather high ($\geq$180,000 $\times$ *g*); therefore, Basic Protocol 4 describes the use of a preformed continuous iodixanol gradient. All of the gradients covered in these protocols should be broadly applicable to most material sources; however, because the sedimenting properties and density of organelles from different tissues and cells can vary, it may be necessary to modulate either the density profile of the gradient or the centrifugation conditions.

With the exception of Basic Protocol 2 (for rat brain), the methods do not include homogenization schedules, and any differential centrifugation is designed for mammalian liver. Thus, the homogenization medium is also used for all stages of the differential centrifugation and for suspending the final crude mitochondrial fraction. This is often not the case with other material such as cultured cells, skeletal muscle, or yeast (*UNIT 3.3*). Therefore, the strategy and media recommended in *UNIT 3.3* should be used to prepare crude mitochondria from sources other than mammalian liver.

It should be pointed out that the precise composition of the homogenization medium, the medium used to suspend the crude mitochondrial pellet, and the medium used to prepare the gradients from Percoll and OptiPrep (Basic Protocols 2-4 and Alternate Protocol) can be adapted to subsequent operational requirements. For example, because Percoll itself has no significant osmotic activity, Reinhart et al. (1982) were able to use medium containing 0.21 M mannitol, 60 mM sucrose, 10 mM succinate, 10 mM KCl, 1 mM ADP, 0.25 mM DTT, 10 mM HEPES-KOH, pH 7.4, throughout the entire procedure. As OptiPrep has an osmolarity of approx 170 mOsm, it could also be diluted with a similar medium. Such media doubtless have many beneficial effects on the retention of respiratory competence by the organelles. Because of the high concentrations of sucrose used in the gradients of Basic Protocol 1, however, the use of mannitol in the homogenization and differential centrifugation stages is

Contributed by John M. Graham
Current Protocols in Cell Biology (1999) 3.4.1-3.4.22

probably of little benefit, although the inclusion of KCl, ADP, DTT in all the solutions might be worthwhile. If the aim of the gradient purification is to identify the localization of some functional or compositional parameter or it is a preliminary process to some subsequent extraction, then the use of a simple general-purpose medium throughout the preparation is probably satisfactory. In the following protocols therefore basic general-purpose media containing either sucrose or mannitol, a chelating agent, and an organic buffer have generally been recommended, but these may be adjusted by the investigator to include low concentrations ≤10 mM) of substrates, co-factors, and sulphydryl reagents. Note, also, that the relative centrifugal force (RCF) used to pellet this mitochondrial fraction can be between 6,000 and 20,000 × *g*, as the purpose of the gradient is to resolve mitochondria from contaminating lysosomes, peroxisomes, and Golgi, if present.

Support Protocols describe simple assays for succinate dehydrogenase (see Support Protocol 1), β-galactosidase (see Support Protocol 2), and catalase (see Support Protocol 3), which have been adapted specifically for the detection of mitochondria, lysosomes, and peroxisomes, respectively, in gradient fractions, but they can be used for any mitochondrial fraction.

NOTE: For all protocols, all *g* values are given as g_{au}.

BASIC PROTOCOL 1

RESOLUTION OF A RAT LIVER MITOCHONDRIAL FRACTION IN A CONTINUOUS SUCROSE GRADIENT

This protocol, adapted from Diczfalusy and Alexson (1988) and Vamecq and Van Hoof (1984), was designed for mitochondria from mammalian liver. A crude light mitochondrial fraction should be suspended in a medium that is suitable to mitochondria, but there is little merit in using a mannitol-containing medium, as the mitochondria will be exposed to high hyperosmotic concentrations of sucrose in the gradient. The protocol is best carried out on Triton WR1339–treated rats (see Background Information) in order to reduce the density of the lysosomes. Optimal resolution in these sucrose gradients is carried out in a large-volume vertical rotor. Although this protocol is designed for mitochondria from rat liver, it can be modified for other mammalian tissues and for yeast. In the latter case, discontinuous sucrose gradients are used in a swinging-bucket rotor according to the method of Beauvoit et al. (1989).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all of the solutions.

Materials

General-purpose homogenization medium (GHM; see recipe), ice cold
Sucrose gradient solutions (see recipe)
Sucrose cushion (see recipe)
Protease inhibitors (optional; see recipe)

Dounce homogenizer (5- to 10-ml with loose-fitting, Wheaton type B pestle)
Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor and appropriate tubes
Gradient maker (two-chamber or Gradient Master)
Ultracentrifuge (e.g., Beckman VTi 50 with vertical rotor) and 39-ml tubes
Gradient unloader (optional)

Additional reagents and equipment for preparing homogenates (*UNIT 3.3*)

1. Prepare rat liver homogenate as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold GHM instead of LHM.

   For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case GHM should be used only for resuspending the final crude mitochondrial pellet (step 4 below).

2. Pellet nuclei by centrifuging 5 min at 1000 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge. If desired, wash the nuclei once to maximize the recovery of mitochondria.

3. Pellet mitochondria from the postnuclear supernatant by centrifuging 10 min at 15,000 × *g*, 4°C in a fixed-angle rotor using a high-speed centrifuge.

4. Resuspend the mitochondrial pellet in ~8 ml ice-cold GHM using 3 to 4 gentle strokes of the Dounce homogenizer.

5. Using a two-chamber gradient maker, prepare linear 28-ml sucrose gradients from 34% and 64% (w/v) sucrose gradient solutions in 39-ml tubes for a Beckman VTi 50 ultracentrifuge. Underlay 4 ml sucrose cushion in each tube.

   Alternatively, a Gradient Master can be used to prepare the gradients.

6. Layer 7 to 8 ml sample on top of the gradients and centrifuge 65 min at 170,000 × *g*, 4°C.

   For a discontinuous gradient (yeast), layer 11 ml each of 30%, 40%, and 55% (w/v) sucrose gradient solutions (see recipe) in 38-ml centrifuge tubes for an ultracentrifuge swinging-bucket rotor (e.g., Beckman SW28 or 28.1, or Sorvall AH 629). Then layer 5 ml sample on top of the gradients and centrifuge 20 to 30 min at 150,000 × g, 4°C. For 17-ml tubes, use 5 ml of each sucrose solution and 2 ml mitochondrial fraction.

7. Collect the gradient in 1- to 2-ml fractions using a gradient unloader or harvest the brownish mitochondrial band just above the middle of the tube (continuous gradient).

   For discontinuous gradients, harvest mitochondria from the 40%/55% interface.

8. Dilute sample(s) with ≥2 vol ice-cold GHM.

9. Centrifuge 20 min at 20,000 × *g*, 4°C.

10. Resuspend pellet(s) in ice-cold GHM or another suitable medium at 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to –20°C or –80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

ISOLATION OF MITOCHONDRIA FROM RAT BRAIN USING A DISCONTINUOUS PERCOLL GRADIENT

BASIC PROTOCOL 2

Methods for isolating rat brain mitochondria rarely rely solely on differential centrifugation as a purification process, probably because of contamination by synaptosomes and myelin. This particular purification is thus aimed at the isolation of nonsynaptosomal mitochondria. The method, derived from Sims (1990), appears to work optimally when ≥500 mg of tissue is processed. A discontinuous Percoll gradient for liver is described in the Alternate Protocol.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all solutions.

Materials

150- to 200-g Sprague-Dawley rats
Brain homogenization medium (BHM; see recipe), ice cold
Percoll solutions in BHM: 15% (v/v), 23% (v/v), and 40% (v/v) (see recipe)
Protease inhibitors (optional; see recipe)

10-ml Dounce homogenizer with tight-fitting and loose-fitting pestles (Wheaton type A and B, respectively)
Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor and appropriate tubes
Ultracentrifuge with swinging-bucket rotor for 12-ml tubes

Prepare homogenate

1. Starve 150- to 200-g Sprague-Dawley rats overnight and sacrifice by decapitation.

   This must be supervised or carried out by an experienced animal technician.

2. Dissect out brain tissue, place ~500 mg in ice-cold BHM, and wash in three changes of this medium.
3. After mincing the tissue finely with scissors, suspend it in ~5 ml BHM and homogenize using five up-and-down strokes with the loose-fitting pestle of a 10-ml Dounce homogenizer, followed by ten more up-and-down strokes with the tight-fitting pestle.
4. Centrifuge 3 min at 1300 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge. Decant and retain the supernatant.
5. Resuspend the pellet up to the original volume with BHM and rehomogenize using 10 strokes with the tight-fitting pestle.
6. Repeat centrifugation, decant the supernatant, and combine it with the first (step 4).

Isolate mitochondria

7. Centrifuge 10 min at 21,000 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.
8. Discard the supernatant and resuspend the pellet in 5 ml of 15% Percoll solution.
9. In a suitable tube for a swinging-bucket rotor, layer 4.0 ml each of 23% and 40% Percoll solutions in BHM. Layer 3 ml mitochondrial suspension on top and centrifuge 5 min at 31,000 × *g*, 4°C.

   Add additional BHM on top if necessary to fill the tube.

10. Harvest the mitochondria that band at the lowest interface, and dilute with 4 vol ice-cold BHM.
11. Centrifuge 10 min at 17,000 × *g*, 4°C in a fixed-angle rotor.

12. Remove the supernatant and resuspend the mitochondrial pellet as appropriate at 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to −20°C or −80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

ISOLATION OF MITOCHONDRIA FROM RAT LIVER USING A DISCONTINUOUS PERCOLL GRADIENT

ALTERNATE PROTOCOL

A similar discontinuous Percoll gradient, covering a slightly higher density range, has been used by Reinhart et al. (1982) to isolate a mitochondrial fraction from a total rat liver homogenate; this approach eliminates the resuspension of a crude mitochondrial pellet which may damage the organelles. Presumably to avoid contamination of the gradient by erythrocytes, the authors perfused the liver with a standard balanced salt medium containing 1.3 mM $CaCl_2$. Alternatively a post-nuclear supernatant could be used from an unperfused liver (as described in this protocol). The method is restrictive inasmuch as only 2 ml of homogenate can be processed on a single gradient, and the authors used only the median lobe of the liver in a total homogenate volume of ~10 ml. The method is adapted from Reinhart et al. (1982).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all solutions.

Materials

150- to 200-g Sprague-Dawley rats
Mannitol-sucrose homogenization medium (MSHM; see recipe), ice-cold
Percoll solutions in MSHM: 19%, 31%, 42%, and 52% (v/v) (see recipe)
Protease inhibitors (optional; see recipe) can be added to any or all of the solutions

Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor (e.g., Sorvall SS34) with ~14-ml tubes

Additional reagents and equipment for preparing liver homogenate (*UNIT 3.3*)

1. Prepare rat liver homogenate from the median liver lobe as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold MSHM (10 ml) instead of LHM.

   For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case only use MSHM to prepare the Percoll solutions.

2. Pellet the nuclei by centrifuging 5 min at 1000 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.
3. Remove the postnuclear supernatant into a beaker and keep on ice
4. In a tubes for the ultracentrifuge swinging-bucket rotor layer 3 ml of each of the 31%, 42% and 52% Percoll solutions and 2 ml each of the 19% Percoll and post nuclear supernatant.

5. Centrifuge at 36,000 × *g* for 30 sec at speed.

   The acceleration time should be ~1.5 min and the deceleration time ~3 min.

6. Collect the mitochondria that band at the lowest interface and dilute with 4 vol of MSHM. Centrifuge 10 min at 17,000 × *g*, 4°C, in a fixed-angle rotor.

7. Remove the supernatant and resuspend the mitochondria as appropriate at 1 to 5 mg protein/ml.

   Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to −20°C or −80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

BASIC PROTOCOL 3

RESOLUTION OF A MITOCHONDRIAL FRACTION IN A SELF-GENERATED PERCOLL GRADIENT

A mitochondrial fraction prepared by differential centrifugation is loaded on to a 30% (v/v) solution of Percoll containing 225 mM mannitol and any other additives that may be required for the retention of respiratory activity. Gradient formation is normally carried out in a fixed-angle rotor at 95,000 × *g*. The protocol for rat liver mitochondria is adapted from Hovius et al. (1990).

Mitochondria band at ~1.09 g/ml in Percoll. It is important that the differential centrifugation fraction that is used contain as little endoplasmic reticulum (ER) as possible, as the density of mitochondria and ER tend to overlap quite significantly in these gradients. It is therefore recommended that the centrifugation speed be no more than 10,000 × *g* to produce the crude mitochondrial fraction.

Compared to the discontinuous Percoll gradients described in Basic Protocol 2 and Alternate Protocol, the centrifugation time for self-generated gradients is considerably longer, but the time taken in the preparation of the gradients is much reduced and considerably more simple to execute. Self-generated gradients may therefore be the method of choice for large numbers of samples. The gradients that are formed by self-generation are also highly reproducible. Alternate Protocol allows the use of a whole homogenate but this necessarily restricts the amount of mitochondria which can be purified on one gradient.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all solutions.

Materials

Mannitol buffer A (see recipe), ice cold
30% (v/v) Percoll solution (see recipe), ice cold
Protease inhibitors (optional; see recipe)

Low-speed centrifuge with swinging-bucket rotor and appropriate tubes (~30 ml)
5- and 30-ml Dounce homogenizers with loose-fitting pestles (Wheaton type B)
High-speed centrifuge with fixed-angle rotor and appropriate tubes (~30 ml)
Ultracentrifuge with fixed-angle rotor (e.g., Beckman 60 Ti or Sorvall T-1250) and appropriate tubes

Additional reagents and equipment for preparing homogenates (*UNIT 3.3*)

Isolate light mitochondrial fraction

1. Prepare rat liver homogenate as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold mannitol buffer A instead of LHM.

   For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case mannitol buffer A should be used only for resuspending the final crude mitochondrial pellet (step 9 below).

2. Centrifuge homogenate 10 min at 1000 × *g*, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.

3. Aspirate supernatant into new centrifuge tubes and set aside.

4. Resuspend pellets in ~20 ml ice-cold mannitol buffer A using two to three gentle strokes in a 30-ml Dounce homogenizer.

5. Centrifuge 10 min at 1000 × *g*, aspirate supernatant, and combine with the supernatant in step 3.

6. Centrifuge supernatants 15 min at 10,000 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.

7. Discard supernatant and resuspend pellet in ~20 ml ice-cold mannitol buffer A as in step 4.

8. Centrifuge 15 min at 10,000 × *g*, 4°C, and discard the supernatant.

9. Resuspend the light mitochondrial pellet in 5 ml mannitol buffer A using 2 to 3 gentle strokes in a 5-ml Dounce homogenizer.

Purify mitochondria

10. Transfer 20 ml of 30% Percoll solution into each of four tubes for a suitable fixed-angle rotor (e.g., a Beckman 60Ti or Sorvall T-1250) and layer the resuspended mitochondria on top.

11. Centrifuge 30 min at 95,000 × *g*, 4°C. During deceleration below 1000 rpm, either switch off the brake or use a controlled deceleration program (if available on the ultracentrifuge).

12. Collect the gradient in 1- to 2-ml fractions.

    The lower part of the dense brown/yellow band contains the bulk of the purified mitochondria; once their position has been confirmed, they can, in future separations, be recovered simply by aspiration. A syringe and long metal cannula is the best tool for this task.

13. Dilute the gradient fraction(s) with ≥2 vol ice-cold mannitol buffer A.

14. Pellet organelles by centrifuging 10 min at 6300 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.

15. Wash the mitochondrial pellet at least twice with ~10 ml of this buffer and resuspend in a suitable medium at 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to −20°C or −80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

RESOLUTION OF A RAT LIVER MITOCHONDRIAL FRACTION IN AN IODIXANOL GRADIENT

Although this protocol is carried out with a light mitochondrial fraction, any appropriate differential centrifugation fraction can be used. The fraction is adjusted to $\rho = 1.177$ g/ml, layered beneath a shallow linear gradient of iodixanol $\rho = 1.124$ to 1.162 g/ml, and centrifuged 2 hr at $110{,}000 \times g$ in a swinging-bucket rotor. As an alternative (if preferred), the mitochondrial fraction can be layered on top in the homogenization medium.

This protocol has been devised for mammalian liver mitochondria in a general-purpose medium. Any of the mitochondrial fractions from other sources covered in *UNIT 3.3* should behave in a similar manner, although the detail of the organelle banding may vary slightly and require modulation for optimal results. See Commentary for variations of this separation strategy.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to media used in this protocol are optional and can be added to any or all solutions.

Materials

Iodixanol buffer A (see recipe), ice cold
50% (w/v) iodixanol solution: 1 vol iodixanol buffer B (see recipe) in 5 vol OptiPrep (60% iodixanol; Nycomed Pharm, Life Technologies, Accurate Chemicals, Mediatech)
19% and 27% iodixanol solutions: dilute 50% iodixanol with iodixanol buffer A
Protease inhibitors (optional; see recipe)

Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
5- and 20-ml syringes and metal cannulae (i.d. ~0.7 mm)
5- and 30-ml Dounce homogenizers with loose-fitting pestles (Wheaton type B)
High-speed centrifuge with fixed-angle rotor and appropriate tubes
Ultracentrifuge with swinging-bucket rotor (e.g., Beckman SW 28.1 or Sorvall AH-629) and appropriate tubes
Refractometer (optional)
Gradient maker: two-chamber or Gradient Master

Additional reagents and equipment for preparing homogenates (*UNIT 3.3*)

Isolate mitochondrial fraction

1. Prepare rat liver homogenate as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold iodixanol buffer A instead of LHM.

   For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case iodixanol buffer A should be used only for resuspending the final crude mitochondrial pellet (step 10 below).

2. Centrifuge homogenate 10 min at $1000 \times g$, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.

3. Transfer the supernatant to new centrifuge tubes using a 20-ml syringe and metal cannula, and set aside.

4. Resuspend pellets in ~20 ml ice-cold iodixanol buffer A using two to three gentle strokes in a 30-ml Dounce homogenizer.

5. Centrifuge 10 min at 1000 × *g*, remove supernatant, and combine with the supernatant in step 3.
6. Centrifuge supernatants 10 min at 3000 × *g*, 4°C, in a fixed-angle rotor using a high-speed centrifuge.

   This step can be omitted if the heavy mitochondrial fraction is not required as a separate fraction or if the source material does not exhibit such a fraction.

7. Remove supernatants and centrifuge 15 min at 15,000 × *g*, 4°C.
8. Discard supernatant and resuspend pellet in ~20 ml ice-cold iodixanol buffer A using three to four gentle strokes in the 30-ml Dounce homogenizer.
9. Centrifuge 15 min at 15,000 × *g*, 4°C, and discard the supernatant.
10. Resuspend the light mitochondrial pellet in 3 ml iodixanol buffer A using 2 to 3 gentle strokes in a 5-ml Dounce homogenizer.

Purify by Iodixanol gradient

11. Mix the suspension with 4.5 ml of 50% iodixanol solution by repeated inversion.

    Do not shake the tube vigorously to create foaming. If the sample is going to be loaded on top of the gradient rather than beneath it, then omit this step and the next.

12. Check that the refractive index is 1.3864 (± 0.0004) and adjust with 50% iodixanol solution or iodixanol buffer A, if necessary.
13. Prepare a linear iodixanol gradient in a ~17-ml centrifuge tube for a swinging-bucket rotor (e.g., a Beckman SW28.1 or a Sorvall AH-629) using 6 ml each of 19% and 27% iodixanol solution.

    If the gradient is prepared with a Gradient Master, use an 80° angle at 20 rpm and a time of 1 min 50 sec.

14. Underlayer the gradient with 3 ml sample (step 12), using a 5-ml syringe and a metal cannula.
15. Overlayer the gradient with 1 ml iodixanol buffer A.

    Alternatively, underlayer the gradient with 1 ml of 30% iodixanol (3 vol iodixanol stock solution plus 2 vol iodixanol buffer A), and then layer 3 ml sample on top of the gradient.

16. Centrifuge 1.5 hr at 110,000 × *g*, 4°C.
17. Collect the gradient in 1-ml fractions starting at the low-density end, using upward displacement with a dense liquid. Alternatively, collect the mitochondria that band just above the middle of the gradient using a syringe and cannula.
18. Dilute the sample with ≥2 vol iodixanol buffer A.
19. Centrifuge 20 min at 20,000 × *g*, 4°C, and resuspend the pellet in this buffer or another suitable medium at 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to –20°C or –80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

SUPPORT PROTOCOL 1

SUCCINATE DEHYDROGENASE ASSAY FOR MITOCHONDRIA

Although any component of the tricarboxylic acid cycle can be used as a functional marker for mitochondria, the most frequently used marker is succinate dehydrogenase with either the native electron acceptor (cytochrome *c*) or the artificial acceptor *p*-iodonitrotetrazolium violet (INT). Measuring the reduction of cytochrome *c* ideally requires a recording spectrophotometer, while the INT variant can be performed with a nonrecording instrument. INT is also far more stable than cytochrome *c* and avoids the use of cyanide, which is used to prevent reoxidation of cytochrome *c*. The INT method presented here is from Graham (1993).

Sample handling depends on the type of gradient and the concentration of mitochondria (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed by centrifugating as described in the final steps of each Basic Protocol. The gradient fractions should be resuspended in 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4, or an equivalent buffer.

Materials

Mitochondrial gradient fraction (see Basic Protocols 1 to 4)
Succinate solution (see recipe)
INT solution (see recipe)
Stop solution I (see recipe)
Spectrophotometer and glass cuvettes

1. Place 0.3 ml succinate solution in 1.5- to 2.0-ml microcentrifuge tubes, including a set of tubes to provide blanks for each fraction. Perform the assay in duplicate.

   As long as the protein content of each assay is approximately the same, a blank for each fraction may not be necessary. A single blank containing buffer instead of sample may suffice for many gradients. Alternatively, blanks may be prepared by adding stop solution to the gradient fraction before adding INT solution in step 3.

2. Add 10 to 20 μl of each gradient fraction to separate sample tubes. Incubate samples and blanks 10 min at 37°C.

   The sample should contain ~20 μg protein.

3. Add 0.1 ml INT solution and incubate for another 10 to 20 min at 37°C.
4. Terminate the reaction by adding 1 ml stop solution I.
5. Remove any precipitate by microcentrifuging for 2 min at maximum speed.
6. Using glass cuvettes, measure the absorbance at 490 nm against a suitable blank.
7. Calculate the enzyme activity as μmoles INT reduced per milligram protein.

   The molar extinction coefficient of reduced INT is 19,300 $cm^{-1}M^{-1}$.

SUPPORT PROTOCOL 2

β-GALACTOSIDASE ASSAY FOR LYSOSOMES

β-Galactosidase is one of several glycosidases that can be used to detect lysosomes. The assay is facilitated by the availability of *p*-nitrophenol derivatives of a number of monosaccharides, which mimic the native oligosaccharide substrates. It is linked to a simple spectrophotometric measurement of the released nitrophenol. The method is as described by Graham (1993).

Sample handling depends on the type of gradient and the concentration of lysosomes (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed by

centrifugation as described in the final steps of each Basic Protocol. The gradient fractions should be resuspended in 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4, or an equivalent buffer.

Materials

Mitochondrial gradient fraction (see Basic Protocols 1 to 4)
Substrate solution (see recipe)
Stop solution II (see recipe)
Spectrophotometer and plastic cuvettes

1. Add 20 to 40 µl of each gradient fraction (containing ~30 µg protein) to separate microcentrifuge tubes containing 0.5 ml substrate solution. Perform the assay in duplicate.
2. Prepare a blank for each fraction by adding 1 ml stop solution II before adding the gradient fraction.

   As long as the protein content of each assay is approximately the same, a blank for each fraction may not be necessary. A single blank containing buffer instead of sample may suffice for many gradients.
3. Incubate test samples 30 min at 37°C.
4. Add 1 ml stop solution II to test samples.
5. Remove any precipitate by microcentrifuging 1 to 2 min at maximum speed.
6. Using plastic cuvettes, measure the absorbance of tests at 410 nm against the chosen blank(s).

   The molar extinction coefficient of nitrophenol is 9620 $cm^{-1}M^{-1}$.

CATALASE ASSAY FOR PEROXISOMES

SUPPORT PROTOCOL 3

The titanium oxysulfate method, adapted from Baudhuin (1974), is the method of choice for peroxisomes and provides very reliable data. It is more sensitive than the permanganate-based method and is certainly easier to execute.

In peroxisomes from rat liver, catalase is an extremely active enzyme, and purified fractions from gradients may require dilution of up to 100 fold. Assays are usually carried out at 0°C. Sample handling depends on the type of gradient and the concentration of peroxisomes (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed by centrifuging as described in the final steps of each Basic Protocol. The gradient fractions should be resuspended in 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4, or an equivalent buffer.

CAUTION: Exercise care when handling titanium oxysulfate, as this reagent is extremely corrosive.

Materials

Mitochondrial gradient fraction (see Basic Protocols 1 to 4)
Stock peroxide solution (see recipe)
Tris/BSA solution (see recipe)
Titanium oxysulfate reagent (see recipe)
Sample buffer (see recipe)
Spectrophotometer

NOTE: Carry out all operations on ice in 2-ml microcentrifuge tubes.

1. Prepare substrate mixture by diluting 8.5 ml stock peroxide solution to 100 ml with Tris/BSA solution and keep at 0°C.
2. Add 1.0 ml titanium oxysulfate reagent to 0.5 ml substrate mixture and measure the absorbance at 405 nm.

   A_{405} should be ~1.5; if it is not, adjust the concentration of hydrogen peroxide accordingly.
3. Mix 10 µl gradient fraction with 30 µl sample buffer. Also set up a reagent control using 40 µl sample buffer. Perform assay in duplicate.

   With mammalian liver, because it is necessary to dilute the gradient fractions 10 to 100 fold, it is never necessary to set up blanks for each fraction. A single reagent blank is adequate. If a sample blank is necessary, replace 0.5 ml substrate mixture with buffer.

   Catalase activities in fractions from other sources may be much lower; test the pregradient mitochondrial fraction first.
4. Add 0.5 ml substrate mixture to all tubes. Do this in batches of 6 tubes, adding the assay mixture to successive tubes at timed 10-sec intervals.
5. After exactly 1 min, add 1.0 ml titanium oxysulfate reagent (also at 10-sec intervals).
6. Remove any precipitate by microcentrifuging 2 min at maximum speed, 4°C.
7. Transfer the tubes to room temperature and measure the absorbance at 405 nm against a blank containing 0.5 ml Tris/BSA solution and 1.0 ml titanium oxysulfate reagent.
8. Calculate the activity of the enzyme by deducting the test absorbance of each gradient fraction from that of the reagent control (see Commentary).

   Note that catalase is estimated by "back-titration"; i.e., the residual H_2O_2 is measured in the assay mixture after being acted on by the enzyme.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocols (except where indicated). For common stock solutions see **APPENDIX 2A**; for suppliers see **SUPPLIERS APPENDIX**.*

Brain homogenization medium (BHM)

To 100 ml H_2O add:
21.9 g sucrose (0.32 M final)
2.0 ml 100 mM K_2EDTA (1 mM final)
2.0 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

BHM, 2×

To 50 ml H_2O add:
21.9 g sucrose (0.64 M final)
2.0 ml 100 mM K_2EDTA (2 mM final)
2.0 ml 1 M Tris·Cl (*APPENDIX 2A*; 20 mM final)
Adjust to pH 7.4
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Citrate/phosphate buffer

Dissolve 0.71 g Na_2HPO_4 in 100 ml H_2O (50 mM final). Dissolve 0.96 g citric acid in another 100 ml H_2O (50 mM final). Add Na_2HPO_4 to citric acid to reach pH 4.3.
Store up to 1 to 2 days at 4°C

General-purpose homogenization medium (GHM)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
2.0 ml 100 mM Na_2EDTA (1 mM final)
2.0 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Iodixanol buffer A

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
2.0 ml 100 mM Na_2EDTA (1 mM final)
20 ml 100 mM HEPES (10 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Iodixanol buffer B

To 50 ml H_2O add:
8.55 g sucrose (0.25 M final)
6.0 ml 100 mM Na_2EDTA (6 mM final)
60 ml 100 mM HEPES (60 mM final)
Adjust pH to 7.4
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

p-Iodonitrotetrazolium violet (INT) solution

25 mg INT (2.5 mg/ml final)
Phosphate buffer, pH 7.4 (see recipe), to 10 ml
Store up to 2 to 3 months at −20°C

Mannitol buffer A

To 100 ml H_2O add:
9.13 g mannitol (0.25 M final)
38 mg EGTA (0.5 mM final)
0.2 g bovine serum albumin (BSA; 0.1% w/v final)
10 ml 100 mM HEPES (5 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store 1 to 2 days at 4°C

Mannitol buffer B

To 100 ml H_2O add:
16.4 g mannitol (0.45 M final)
152 mg EGTA (2.0 mM final)
0.4 g BSA (0.2% w/v final)
100 ml 100 mM HEPES (50 mM final)
Adjust pH to 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Mannitol-sucrose homogenization medium (MSHM)

To 100 ml H_2O add:
7.64 g mannitol (0.21 M final)
4.10 g sucrose (60 mM final)
0.15 g KCl (10 mM final)
2.0 ml 1 M HEPES-KOH (10 mM final)
Adjust to pH 7.4
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

MSHM, 4×

To 50 ml H_2O add:
15.3 g mannitol (0.21 M final)
8.2 g sucrose (60 mM final)
0.3 g KCl (10 mM final)
4.0 ml 1 M HEPES-KOH (10 mM final)
Adjust to pH 7.4
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Percoll solution, 30% (v/v)

3 vol Percoll
2 vol water
5 vol mannitol buffer B (see recipe)
Store up to 1 to 2 days at 4°C

Percoll solutions in BHM

Prepare a 50% (v/v) Percoll stock solution by adding 20 ml Percoll to 20 ml 2× BHM (see recipe). Store up to 1 to 2 days at 4°C

To prepare gradient solutions, dilute 50% Percoll stock with BHM to provide 15%, 23%, and 40% (v/v) Percoll. Store up to 1 to 2 days at 4°C

Percoll solution in MSHM

Prepare a 75% (v/v) Percoll solution by adding 30 ml Percoll to 10 ml of 4× MSHM (see recipe). Store up to 1 to 2 days at 4°C

To prepare gradient solutions, dilute Percoll (75% v/v) stock with MSHM to provide 19%, 31%, 42%, and 52% (v/v) Percoll. Store up to 1 to 2 days at 4°C

Phosphate buffer, pH 7.4

Dissolve 0.71 g Na_2HPO_4 in 100 ml water (50 mM final). Dissolve 0.68 g KH_2PO_4 in another 100 ml water (50 mM final). Add KH_2PO_4 to Na_2HPO_4 to reach pH 7.4. Store up to 1 to 2 days at 4°C

Protease inhibitors

Add the following to solutions as required so that the final concentrations are 1 mM for PMSF and 2.0 μg/ml for each of the other inhibitors.

PMSF:
0.348 g phenylmethylsulfonyl fluoride (200 mM final)
10 ml dried propan-2-ol or ethanol
Store up to 2 to 3 months at 4°C

Antipain:
10 mg antipain (1 mg/ml final)
10 ml 10% (v/v) dimethyl sulfoxide (DMSO)
Store in aliquots up to 2 to 3 months at −20°C

continued

Aprotinin:
10 mg aprotinin (1 mg/ml final)
10 ml H_2O
Store in aliquots up to 2 to 3 months at −20°C
Leupeptin:
10 mg leupeptin (1 mg/ml final)
10 ml of 10% (v/v) dimethyl sulfoxide (DMSO)
Store in aliquots up to 2 to 3 months at −20°C

Sample buffer

2 g Triton X-100 (2% w/v final)
H_2O to 100 ml
Mix 1 vol of this solution with 2 vol Tris/BSA solution (see recipe)
Store up to 1 to 2 days at 4°C

Stock peroxide solution

0.67 g 30% (w/w) H_2O_2 (30 mM final)
Tris/BSA solution (see recipe) to 200 ml
Prepare fresh

Stop solution I

10 g trichloroacetic acid
50 ml ethyl acetate
50 ml ethanol
Store up to 1 month at room temperature

Stop solution II

To 50 ml H_2O add:
1.87 g glycine (0.25 M final)
25 ml 1.0 M NaOH (0.25 M final)
Adjust pH to 10 (if necessary)
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Substrate solution

90 mg *o*-nitrophenyl β-D-galactopyranoside (6 mM final)
0.25 g Triton X-100 (0.5% w/v final)
Citrate/phosphate buffer (see recipe) to 50 ml
Store up to 1 to 2 days at 4°C

Succinate solution

0.27 g sodium succinic acid hexahydrate (10 mM final)
Phosphate buffer, pH 7.4 (see recipe), to 100 ml
Adjust to pH 7.4 if necessary
Store up to 1 to 2 days at 4°C

Sucrose cushion (continuous gradient only)

67 g sucrose (67% w/w final)
Dissolve the sucrose in 33 ml of 10 mM Tris·Cl, pH 7.4
Store up to 1 to 2 days at 4°C

Sucrose gradient solutions

Dilute 70% (w/v) sucrose stock (see recipe) with sucrose stock diluent (see recipe) to provide 34% and 64% (w/v) sucrose (continuous gradient); or 30%, 40%, and 54% (w/v) sucrose (discontinuous gradient)

Sucrose stock diluent

1.0 ml 100 mM Na_2EDTA (1 mM final)
1.0 ml 1 M Tris·Cl stock (10 mM final)
H_2O to 100 ml
Adjust pH to 7.4 after adding half the water
Store 1 to 2 days at 4°C

Sucrose stock solution, 70% (w/v)

70 g sucrose (70% w/v final)
1.0 ml 100 mM Na_2EDTA (1 mM final)
1.0 ml 1 M Tris·Cl stock (10 mM final)
H_2O to 100 ml
Adjust pH to 7.4 after adding most of the water
Store 1 to 2 days at 4°C

Supplements

0.27 g sodium succinic acid hexahydrate (10 mM final)
50 mg ADP (potassium salt) (1 mM final)
3.8 mg DTT (0.25 mM final)

Any or all of the above may be added to 100 ml of the homogenization, suspension, and gradient media, before adjusting the pH.

Titanium oxysulfate reagent

Dissolve 5.56 ml of 18 M H_2SO_4 in ~90 ml H_2O (1 M final). Add 0.225 g titanium oxysulfate (2.25 g/liter final). Bring to 100 ml with H_2O. Prepare fresh.

CAUTION: *Both the acid and the titanium oxysulfate are highly corrosive. Exercise great care when adding pure H_2SO_4 to water. Use a face mask and protective gloves (not disposable plastic gloves, as any splashes will cause them to "melt" and adhere to the skin).*

Tris/BSA solution

To 100 ml H_2O add:
0.2 g bovine serum albumin (1 g/liter final)
4.0 ml 1.0 M Tris·Cl (*APPENDIX 2A*; 20 mM final)
Adjust pH to 7.0
Add H_2O to 200 ml
Prepare fresh

COMMENTARY

Background Information

As a general rule, metabolic studies on liver, heart, or muscle mitochondria are carried out on organelles prepared by differential centrifugation (see *UNIT 3.3*). Density-gradient separations, although undoubtedly able to provide a more pure preparation, are frequently avoided simply because the speed of preparation is the prime consideration. With the exception of the heavy mitochondrial fraction (*UNIT 3.3*), however, these preparations contain significant contamination by other organelles (lysosomes, peroxisomes), sometimes by tubular Golgi elements (if present in the homogenate), and also by some of the more rapidly sedimenting endoplasmic reticulum vesicles. Such contamination can only be reduced by gradient centrifugation. The use of higher relative centrifugal forces (RCFs) to sediment the mitochondria from the postnuclear supernatant results in higher contamination. Gradients are also very useful if analytical work is to be carried out to determine the localization of a particular component or function. In this case, the distribution

Table 3.4.1 Density (g/ml) of Major Organelles from Mammalian Liver in Iodixanol, Sucrose, and Percoll

Particle	Density in iodixanol	Density in sucrose	Density in Percoll
Nuclei	1.23-1.25	>1.32	NA[a]
Peroxisomes	1.18-1.21	1.19-1.23	1.075-1.085
Mitochondria	1.14-1.16	1.17-1.21	1.085-1.100
Lysosomes	1.11-1.13	1.19-1.21	1.105-1.111
Golgi	1.03-1.08	1.05-1.12	1.03-1.105

[a]Not available.

of the component or function in the gradient is compared with known markers for the different organelles.

Table 3.4.1 compares the density of mammalian liver mitochondria and other organelles in sucrose, iodixanol, and Percoll. In sucrose, the density of mitochondria overlaps that of lysosomes and peroxisomes. As mitochondria (and lysosomes) sediment through a sucrose gradient, their densities rise to a limiting value as water is removed from their enclosed spaces by the high and rising osmolarity of the gradient. In other media that are true solutes (e.g., Nycodenz and iodixanol) and that can provide gradients of much lower osmolarity, the density of these organelles are lower and more distinctive (Ford et al., 1983; Graham et al., 1990, 1994). Peroxisomes present an exception to this rule; because they do not have an osmotic space in these media, their density is almost identical in sucrose, Nycodenz, and iodixanol (Graham et al., 1994). Although both Percoll and iodixanol can provide isoosmotic gradients over the entire density range of these organelles, the densities of organelles in Percoll are lower. In iodinated density-gradient media, the density of the major organelles always increases in the order lysosomes, mitochondria, peroxisomes. In Percoll the order is sometimes completely reversed (Symons and Jonas, 1987), and in other cases the mitochondria are denser than the lysosomes (Singh et al., 1987). The reason for these differences is not clear. Hence, the popularity of Percoll methods whose efficiency relies as much on the higher sedimentation rate of the mitochondria as on their banding density.

The continuous sucrose gradient system in a vertical rotor used by Vamecq and Van Hoof (1984) can resolve mitochondria and peroxisomes reasonably well. Lysosomes, however, band broadly between these two organelles unless their density can be reduced by prior injection of the animals with Triton WR1339 (85 mg/100 g body weight) 3 to 5 days before sacrifice (Leighton et al., 1968). Diczfalusy and Alexson (1988) used this approach, which makes the lysosomes significantly less dense than the mitochondria. A discontinuous sucrose gradient system was developed by Beauvoit et al. (1989) for yeast and might be applicable to other source materials (see Basic Protocol 1).

A major advantage of using Percoll for the density purification of mitochondria is that the medium forms self-generated gradients at relatively low *g* forces. Thus, the centrifugation conditions that are required are not unlike those used for purification through preformed gradients of other gradient media. One of the problems of using Percoll, however, is the significant overlap of mitochondria and endoplasmic reticulum (ER; Patel et al., 1991). The method of Hovius et al. (1990) uses a relatively low RCF (10,000 × *g* for 10 min) to produce the crude mitochondrial fraction in order to reduce the ER content (see Basic Protocol 3). In an alternative approach, Reinhart et al. (1982) separated the mitochondria principally on the basis of sedimentation rate in a preformed four-step discontinuous gradient of 19%, 31%, 42%, and 52% (v/v) Percoll (Alternate Protocol). Centrifugation was carried out at 37,000 × *g* for just 30 sec, so that the ER vesicles did not have time to reach their banding density. Although this method permits the use of a whole homogenate, only 2 ml can be loaded onto a single 11-ml gradient. A similar sedimentation rate approach was used by Sims (1990) for rat brain mitochondria (see Basic Protocol 2), and the slightly longer centrifugation time of 5 min is probably easier to standardize. Because of the speed of these Percoll systems, the mitochondria tend to display good respiratory control.

Generally speaking, although Percoll gradients can be tailored to the isolation of a particular organelle (e.g., mitochondria), they are less well suited to producing relatively pure fractions of other organelles simultaneously. Both Nycodenz and iodixanol are better suited to this

purpose. A linear gradient of iodixanol from 10% to 30% (w/v) was first developed by Graham et al. (1994), in which the crude mitochondrial fraction was loaded in 35% iodixanol beneath the gradient and centrifuged at ~50,000 × *g* for 1.5 hr. Although this may be a good starting point, it is now recognized that a more shallow gradient (19% to 27%) is probably better for resolving the mitochondria and lysosomes, and indeed this range of gradient concentration is not unlike the discontinuous metrizamide system used by Wattiaux et al. (1983). Note that the density of liver mitochondria in iodixanol is slightly lower than in Nycodenz (Graham et al., 1994).

A significant advantage of iodixanol (and Nycodenz) over Percoll is that, as long as the organelles are sufficiently concentrated in the gradient fractions, the gradient solute does not have to be removed prior to enzyme analysis using visible wavelength spectrophotometry. Because of the light-scattering properties of Percoll, this medium must be removed prior to such analysis, and since the RCFs required to pellet both Percoll particles and organelles are similar, significant loss of organelles can occur (Osmundsen, 1982).

Critical Parameters and Troubleshooting

Density-gradient centrifugation

See *UNIT 3.3* for information regarding the preparation of crude mitochondrial fractions by differential centrifugation.

The preparation of a preformed continuous gradient (see Basic Protocols 1 and 4) using a two-chamber gradient maker requires considerable practice to achieve highly reproducible gradients; some of the important points in operating such devices are covered by Dobrota and Hinton (1992). A Gradient Master, which involves simply layering the most dense and lightest solutions in the centrifuge tube, provides a more expensive but reproducible alternative. If neither of these are available, a discontinuous gradient constructed from three or four solutions spanning the required density range can be prepared and allowed to diffuse. Note that the time required for complete linearity to be achieved will depend on the tube diameter and the volume of each layer. Often this carried out at 4°C overnight without any consideration as to whether the timing is optimal. Always collect a blank gradient and check (by refractive index) that the gradient is indeed linear and covers the appropriate density range.

Although the sample is layered under the iodixanol gradient (see Basic Protocol 4), overlayering can be used as an alternative and may even be beneficial if the separation of the organelles needs to be carried out partly on the basis of sedimentation rate (as with Basic Protocol 2). However, with overlaying, care may be required to avoid overloading the gradient, especially if the top of the gradient is adjusted to a lower density in order to customize the method to a particular source material. Overloading occurs when the total density of the sample layer (liquid plus particles) is greater than that of the top of the gradient; as a consequence, the sample falls through the gradient as a droplet. This is very unlikely to occur in Basic Protocol 1, as the top of the gradient is quite dense.

Self-generated gradients (see Basic Protocol 3) form optimally in tubes of relatively short sedimentation path length. Use of a rotor and gradient volume other than those recommended will mean that the density profile of the gradient, and consequently the separation of the organelles, will be altered. For self-generated Percoll gradients, fixed-angle rotors with an angle of ~24° appear to be the best suited.

It is possible that the density of organelles from other sources (e.g., cultured cells) may be sufficiently different for sucrose gradients to provide some degree of resolution. Poor resolution of organelles may occur as a result of an inappropriate density gradient, but it is just as likely that the basic problem lies with the earlier homogenization and differential centrifugation. A homogenate containing 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4/1 mM EDTA is suitable; the presence of KCl is permissible, but buffers containing Mg^{2+} should be avoided. If Mg^{2+} is essential for the homogenization of a cultured cell, then the mitochondria should be treated as described in *UNIT 3.3*. It is particularly difficult to predict the precise density of organelles separated from cells that have been homogenized in hypoosmotic conditions. In such circumstances, the Basic Protocols should only be regarded as a suitable starting point.

Enzyme assays

Prior to the execution of an enzyme assay (or other measurement), it is common practice

to remove the gradient medium from each gradient fraction by dilution with 2 to 4 volumes of a low-density medium, centrifugation to pellet the organelles, and resuspension in the same low-density medium. The latter is often the homogenization medium or, if it is isoosmotic, a medium used for the subsequent assay. This practice eliminates any possible interference of the gradient medium on the subsequent procedure(s), and it is described in the final steps of each Basic Protocol. In many instances, however, the sample volumes used in the assay are sufficiently small (as in the Support Protocols), that the gradient solute is sufficiently diluted not to cause any interference in the assay, even at the highest density. Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without washing. If, however, either of these requirements are not met, then dilution, pelleting, and resuspension of the pellet in a smaller volume are unavoidable. In the case of Percoll gradients, it is essential to remove the gradient medium prior to any spectrophotometric analysis to avoid variable interference from light scattering. It should also be pointed out that during these postgradient manipulations losses of functional activity can occur. If the assays are carried out in microcentrifuge tubes (as in the Support Protocols) a convenient strategy, which minimizes these losses, is to dilute and centrifuge (maximum speed) the organelles from an appropriate volume of gradient fraction and to use the resuspended pellet directly in the assay.

The only notable source of error in the enzyme assays themselves concerns the β-galactosidase assay. The stop solution for this assay should not have a pH >10; higher pH causes hydrolysis of the substrate.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Most gradient media interfere to varying extents with methods using the Folin-Ciocalteu reagent, although as with the enzyme assays, if by using small volumes (≤100 µl) of fraction in a 1 to 2 ml assay volume the concentration of gradient solute in the assay solution is <10% (w/v), the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of gradient solute, although because of light-scattering problems, it is always advantageous to remove any Percoll.

With the sample volumes used in Support Protocols 1 to 3, the gradient solute is sufficiently diluted to not cause any inhibition of enzyme activity, even at the highest density. If the gradient fraction must be concentrated, the organelles must be pelleted after dilution of the fraction with 2 volumes of an isoosmotic buffer (buffered 0.25 M sucrose is normally adequate). As the assays are carried out in microcentrifuge tubes, a good and convenient strategy is to pellet the particles in a microcentrifuge for 15 to 20 min at 4°C, then suspend the pellet directly in the enzyme assay buffer.

Anticipated Results

Basic Protocol 1

With rat liver, Triton WR1339 shifts the density of lysosomes in sucrose to ~1.15 to 1.17 g/ml (~0.4 g/ml lower than in the normal animal; Table 3.4.1), while mitochondria and peroxisomes are unchanged at 1.17 to 1.21 g/ml and 1.19 to 1.23 g/ml, respectively. Typical separations that might be achieved from normal and Triton-treated animals are shown in Figure 3.4.1 and Figure 3.4.2, respectively. The lysosomes in the Triton WR1339–treated animals are clearly and significantly shifted to lower densities (Diczfalusy and Alexson, 1988) compared to those from the untreated animal (Vamecq and Van Hoof, 1984).

Basic Protocol 2 and Alternate Protocol

In the case of liver mitochondria (Reinhart et al., 1982), ~60% of total mitochondria band at the lowest interface. The major contamination is from peroxisomes and lysosomes (~10% of total; see Table 3.4.2), whereas there is essentially no contamination from either endoplasmic reticulum or plasma membrane. All the enzyme markers for organelles other than mitochondria are broadly distributed across the other interfaces. Although the corresponding band from rat brain (Sims, 1990) only contains ~12% of the total mitochondria, many of these are present in synaptosomes, which band at a much lower density. The author found only 0.3% of the total lactate dehydrogenase and 0.1% of the total 2′,3′-cyclic nucleotide-3′-phosphodiesterase in the bottom interfacial material, attesting to very low contamination by synaptosomes or myelin, respectively.

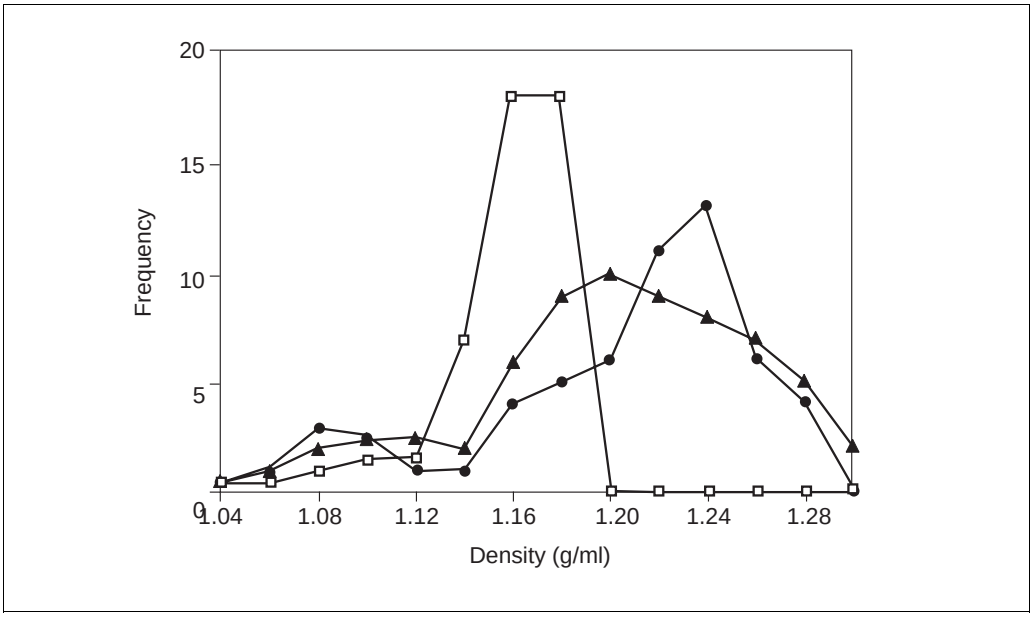


Figure 3.4.1 Frequency distribution of enzyme markers after separation of a crude mitochondrial fraction in a sucrose gradient. Cytochrome *c* oxidase (mitochondria; open squares); *N*-acetyl-β-glucosaminidase (lysosomes; filled triangles); catalase (peroxisomes; filled circles). Data from Vamecq and Van Hoof (1984). Frequency is the amount of enzyme in a fraction divided by the density interval covered by the fraction.

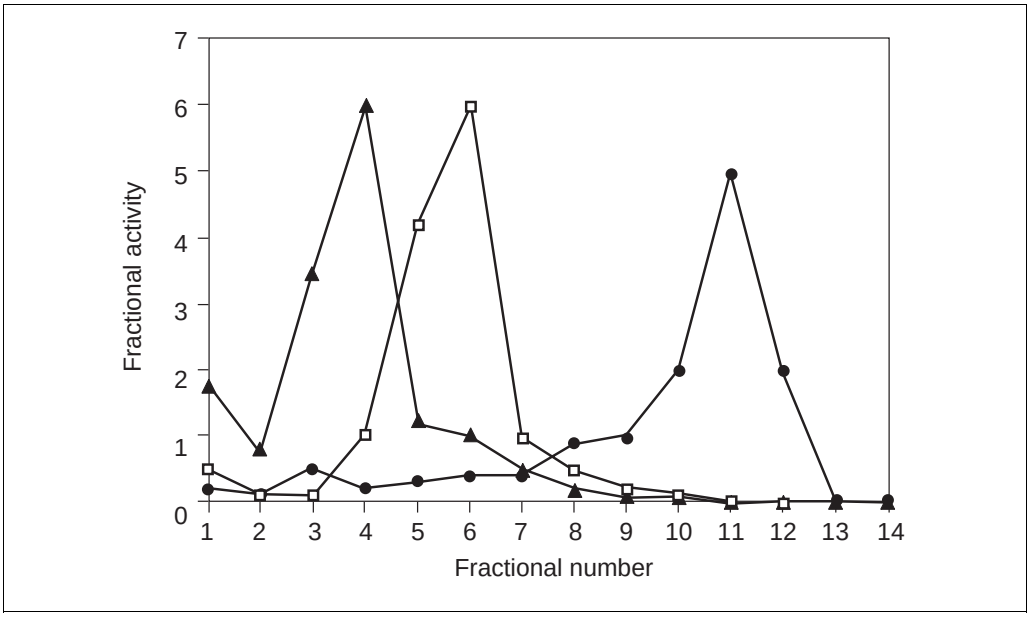


Figure 3.4.2 Fractional activity of enzyme markers after separation of a crude mitochondrial fraction from Triton WR1339–treated rats. Cytochrome *c* oxidase (mitochondria; open squares); *N*-acetyl-β-glucosaminidase (lysosomes; filled triangles); catalase (peroxisomes; filled circles). Fraction 1 is the top of the gradient; data from Diczfalusy and Alexson (1988).

Basic Protocol 3

An ~5-fold enrichment of succinate dehydrogenase is expected in the mitochondrial band over the homogenate, while marker enzymes characteristic of contaminating organelles are significantly depleted (Hovius et al., 1990).

Basic Protocol 4

Figure 3.4.3 describes a typical separation with rat liver on a preformed iodixanol gradient. Fractions 6 to 8 in the middle of the gradient contain >50% of the total mitochondria recovered from the gradient. This gradient also provides an excellent enrichment of lysosomes in fraction 2, and although the peroxisomes are rather broadly banded in the bottom of the gradient, the bottom five fractions contain very little contaminating enzyme markers. This gradient would therefore be very useful analytically as well as preparatively.

Time Considerations

There are no obvious points at which any of the Basic Protocols can be discontinued. Preformed gradients should be made up during the initial differential centrifugation of the homogenate and kept on ice until required. If they are prepared at room temperature, they must be given time to cool down to 4°C. The enzyme marker assays need not be carried out immediately; overnight storage of mitochondria at 4°C is permissible unless metabolic studies are also to be carried out. Enzyme markers can be measured on previously frozen material, but any concentration of the organelle fractions and/or removal of gradient medium must be carried out prior to freezing.

Table 3.4.2 Marker Enzyme Content of a Rat Liver Mitochondrial Fraction after Discontinuous Percoll Gradient Separation[a]

Enzyme marker	Percent of total recovered
Cytochrome *c* oxidase	55
Glucose-6-phosphatase	2
5′-Nucleotidase	4
Acid phosphatase	8
Catalase	11

[a]Data from Reinhart et al. (1982).

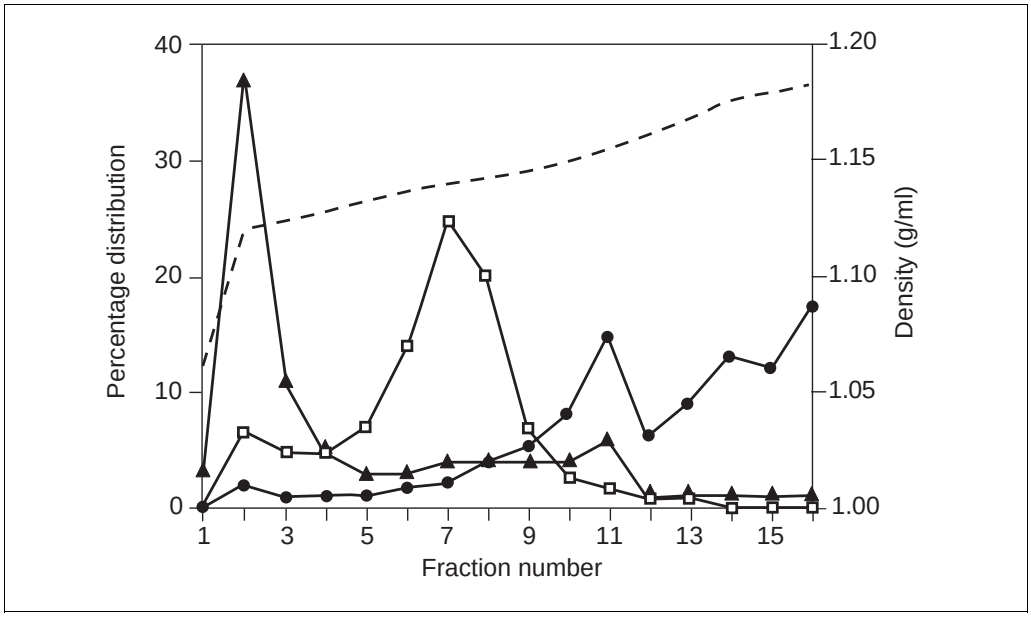


Figure 3.4.3 Percent distribution of enzyme markers after separation of a crude mitochondrial fraction in a preformed continuous iodixanol gradient. Succinate dehydrogenase (mitochondria; open squares); β-galactosidase (lysosomes; filled triangles); catalase (peroxisomes; filled circles).

The figures for the total time (given below) required to complete the gradient separation of mitochondria, include preparation of the homogenate (but omit the optional step of removing the gradient medium) and are calculated for a single rat liver. It is assumed that any preparation of either preformed or continuous gradients is carried out during the periods of differential centrifugation. With the exception of Basic Protocol 2 and Alternate Protocol, total preparation time is probably 3.5 to 4.5 hr. The very short gradient centrifugation times in Basic Protocol 2 and Alternate Protocol mean that overall times are reduced to 2 to 3 hr and 1 to 2 hr respectively. Although the number of pre-gradient operations in Alternate Protocol are also reduced, time has to be taken for preparing a larger number of multi-step discontinuous gradients.

Harvesting the banded organelles from the gradients with a syringe and metal cannula only takes a few minutes, while complete unloading of a single tube into a series of equivolume fractions will take 15 min per tube. Removal of the medium, by washing the pellet once, will add a further 0.5 to 1.0 hr depending on the number of fractions being handled.

All solutions can be made up ahead of time and stored at 4°C for 1 to 2 days or frozen to –20°C for longer periods (2 to 3 months). However, note that any enzymes, BSA, PMSF, or other protease inhibitors should be added immediately prior to use.

Literature Cited

Baudhuin, P. 1974. Isolation of rat liver peroxisomes. *Methods Enzymol.* 31:356-368.

Beauvoit, B., Rigoulet, M., Guerin, B., and Canioni, P. 1989. Polyphosphates, a source of high energy phosphate in yeast mitochondria: A ^{31}P NMR study. *FEBS Lett.* 252:17-21.

Diczfalusy, U. and Alexson, S.E.H. 1988. Peroxisomal chain shortening of prostaglandin $F_{2\alpha}$. *J. Lipid Res.* 29:1629-1636.

Dobrota, M. and Hinton, R. 1992. Conditions for density gradient separations. *In* Preparative Centrifugation: A Practical Approach (D. Rickwood, ed.) pp. 77-142. IRL Press, Oxford.

Ford, T., Rickwood, D., and Graham, J. 1983. Buoyant densities of macromolecules, macromolecular complexes and cell organelles in Nycodenz gradients. *Anal. Biochem.* 128:232-239.

Graham, J.M. 1993. The identification of subcellular fractions from mammalian cells. *In* Methods in Molecular Biology, Vol. 19 (J.M. Graham and J.A. Higgins, eds.) pp. 1-18. Humana Press, Totowa, N.J.

Graham, J., Ford, T., and Rickwood, D. 1990. Isolation of the major subcellular organelles from mouse liver using Nycodenz gradients without the use of an ultracentrifuge. *Anal. Biochem.* 187:318-323.

Graham, J., Ford, T., and Rickwood, D. 1994. The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol. *Anal. Biochem.* 220:267-373.

Hovius, R., Lambrechts, H., Nicolay, K., and de Kruijff, B. 1990. Improved methods to isolate and subfractionate rat liver mitochondria. Lipid composition of the inner and outer membrane. *Biochim. Biophys. Acta* 1021:217-226

Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S., and de Duve, C. 1968. The large-scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR 1339. *J. Cell Biol.* 37:482-513.

Osmundsen, H. 1982. Factors which can influence β-oxidation by peroxisomes isolated from livers of clofibrate-treated rats. Some properties of peroxisomal fractions isolated in a self-generated Percoll gradient by vertical rotor centrifugation. *Int. J. Biochem.* 14:905-914.

Patel, B., Costi, A., Hardy, L., and Mowbray, J. 1991. The characterization of a new enzyme from rat liver mitochondria, oligophosphoglyceroyl-ATP 3′-phosphodiesterase. *Biochem. J.* 274:275-279.

Reinhart, P.H., Taylor, W.M., and Bygrave, F.L. 1982. A procedure for the rapid preparation of mitochondria from rat liver. *Biochem. J.* 204:731-735.

Sims, N.R. 1990. Rapid isolation of metabolically active mitochondria from rat forebrain and subregions using Percoll density gradient centrifugation. *J. Neurochem.* 55:698-705.

Singh, H., Derwas, N., and Poulos, A. 1987. Very long chain fatty acid β-oxidation by rat liver mitochondria and peroxisomes. *Arch. Biochem. Biophys.* 259:382-290.

Symons, L.J. and Jonas, A.J. 1987. Isolation of highly purified rat liver lysosomal membranes using two Percoll gradients. *Anal. Biochem.* 164:382-390.

Vamecq, J. and Van Hoof, F. 1984. Implication of a peroxisomal enzyme in the catabolism of glutaryl-CoA. *Biochem. J.* 221:203-211.

Wattiaux, R. and Wattiaux-De Coninck, S. 1983. Separation of cell organelles. *In* Iodinated Density Gradient Media: A Practical Approach (D. Rickwood, ed.) pp. 119-137. IRL Press, Oxford.

Contributed by John M. Graham
Liverpool John Moores University
Liverpool, United Kingdom

Isolation of Peroxisomes from Tissues and Cells by Differential and Density Gradient Centrifugation

Almost without exception, the purification of peroxisomes from a mammalian tissue homogenate is carried out in two stages: (1) differential centrifugation to produce a light mitochondrial fraction and (2) resolution of the light mitochondrial fraction in some form of density gradient. Iodinated density gradient media (e.g., iodixanol) are preferred for the second phase of the purification (see Commentary). Moreover, because the difference in density between mitochondria and peroxisomes is greater in iodixanol than in Nycodenz, and because the osmolarity of the gradients can be more easily controlled with iodixanol, it is the medium of choice. The much wider use of Nycodenz merely reflects the fact that it has been commercially available for more than 15 years, while iodixanol (OptiPrep) has only been available since 1994.

The unit first describes the homogenization and differential centrifugation process for the most commonly used source material—rat liver (see Basic Protocol 1). This protocol serves as a preliminary methodology for all of the density gradient separations subsequently outlined. Next described is a preformed, continuous iodixanol gradient system for isolation of peroxisomes (see Basic Protocol 2). Iodixanol can also be used as a self-generated gradient (see Alternate Protocol 1). Methods are also presented for the use of Nycodenz: a protocol for a continuous Nycodenz gradient (see Basic Protocol 3) and for a simple Nycodenz barrier (see Alternate Protocol 2) are included. The relative merits of these protocols are discussed in the Commentary. There are a large number of published methods based on Nycodenz and there is variation in both the precise density range covered by the gradients and the centrifugation conditions; some of these are described in the italicized annotations to the appropriate steps of the Nycodenz protocols.

Lastly, the unit describes the isolation of peroxisomes from yeast (see Basic Protocol 4) and cultured monolayer cells (see Basic Protocol 5). In the case of mammalian cultured cells, the differential centrifugation system is truncated and a post-nuclear supernatant is used as the gradient input.

After collecting the banded material in the gradients, the success of the fractionation is routinely measured by assaying for various marker enzymes: catalase (peroxisomes), succinate dehydrogenase (mitochondria), and acid phosphatase or a glycosidase such as β-galactosidase (lysosomes). These assays are provided in *UNIT 3.4*. Although the endoplasmic reticulum (ER) is not a major contaminant of the light mitochondrial fraction, some ER is always present, and therefore an assay for a suitable marker such as NADPH–cytochrome *c* reductase or glucose-6-phosphatase is recommended. This problem becomes more significant if a post-nuclear fraction is loaded on to the gradient.

NADPH–cytochrome *c* reductase is ubiquitously found in the ER, while glucose-6-phosphatase is only present in significant amounts in the ER from liver and kidney. NADPH–cytochrome *c* reductase is also considerably easier to measure than is glucose-6-phosphatase; a method is included for assay of this oxidoreductase (see Support Protocol).

Contributed by John M. Graham
Current Protocols in Cell Biology (2000) 3.5.1-3.5.22

BASIC PROTOCOL 1

ISOLATION OF A LIGHT MITOCHONDRIAL FRACTION FROM RAT LIVER

A standard homogenization medium such as 0.25 M sucrose/1 mM EDTA/10 mM HEPES-NaOH, pH 7.4, is satisfactory for a tissue such as liver, and may be chosen particularly if other organelles are to be isolated from the same homogenate. However if the aim is solely to isolate peroxisomes, the medium is supplemented with 0.1% ethanol to aid the recovery of functionally intact organelles, and the preferred buffer is often MOPS rather than HEPES (or Tris). Sometimes 1 to 5 mM KCl is also included, and the supplementation of any medium with protease inhibitors is at the discretion of the investigator. A standard three-step differential centrifugation system is described, although some workers use a two-step system (see Commentary for more information).

The following protocol is suitable for a single 10- to 12-g liver.

NOTE: All protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to government regulations regarding the care and use of laboratory animals.

Materials

150- to 200-g Sprague-Dawley rats
Homogenization medium (HM; see recipe)
Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe

Dissecting equipment
Overhead high-torque electric motor (thyristor-controlled) for homogenizers
Potter-Elvehjem homogenizer (clearance ~0.09 mm), 25-ml working volume, prechilled
Dounce homogenizer (loose-fitting Wheaton type B pestle, ~30 ml), prechilled
Dounce homogenizer (loose-fitting Wheaton type B pestle, ~5 ml), prechilled
Low-speed refrigerated centrifuge with swinging-bucket rotor accommodating 50-ml tubes
40- to 50-ml polycarbonate centrifuge tubes
High-speed refrigerated centrifuge with fixed-angle rotors accommodating 50-ml tubes (e.g., Sorvall SS-34)

NOTE: All solutions, glassware, centrifuge tubes and equipment should be pre-cooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin but also to protect the hand in the unlikely event of breakage of the vessel.

Isolate liver

1. Starve the animal overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.

   This must be supervised or carried out by an experienced animal technician.

3. Open the abdominal cavity and remove the liver to a chilled beaker containing ~20 ml of ice-cold HM.
4. Decant the medium and finely mince the liver using scissors.

   The pieces of liver should be no more than ~25 mm^3 each.

Homogenize liver

5. Suspend the mince in ~40 ml of HM and transfer half of this suspension to the glass vessel of the Potter-Elvehjem homogenizer.

   The motor should be mounted either to a wall, via a G-clamp to the bench, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

6. Attach the cold pestle to the electric motor and homogenize the liver mince using 4 to 5 up-and-down strokes of the pestle, rotating at ~500 rpm.

7. Decant the homogenate into a beaker on ice, and after rinsing the homogenizer with medium and wiping the pestle to remove any connective tissue that may be adhering, repeat steps 5 and 6 with the other half of the mince and combine the homogenates.

Isolate light mitochondrial fraction

8. Centrifuge the homogenate 10 min at 750 × *g*, 0° to 4°C, in the low-speed centrifuge.

   Steps 8 to 12 are omitted in the two-step protocol (see Commentary). Proceed directly to step 13, centrifuging the homogenate from step 7 in the high-speed centrifuge at 3500 × g.

9. Decant and retain the supernatant.

10. Resuspend the pellet in 20 ml of HM, first using a glass rod and then more completely using 3 to 4 gentle strokes of the pestle of the 30-ml Dounce homogenizer.

11. Centrifuge, decant, and retain the supernatant (steps 8 and 9).

12. Combine the supernatants from steps 9 and 11 and transfer to 40- to 50-ml polycarbonate tubes.

13. Centrifuge 10 min at 3500 × g, 0° to 4°C, in the high-speed centrifuge.

14. Decant and retain the supernatant.

    In the two-step method the pellet from this step should be resuspended in HM as in step 10, centrifuged at 3500 × g as in step 13, the supernatant decanted as in step 14, and the two supernatants combined.

Fractionate liver homogenate

15. Centrifuge the 3500 × *g* supernatant(s) from step 14 in the high-speed centrifuge, 20 min at 23,000 × *g*, 0° to 4°C.

16. Decant and discard the supernatant.

    If a microsomal fraction is required for complete bookkeeping, centrifuge this supernatant plus the supernatant from step 18 for 45 min at 100,000 × g.

17. Resuspend the light mitochondrial pellet (LMP) in HM (~20 ml) using a glass rod followed by 3 to 4 gentle strokes of the pestle of the 30-ml Dounce homogenizer.

18. Recentrifuge 20 min at 23,000 × *g*, 0° to 4°C, and discard the supernatant.

19. Resuspend the LMP in HM (~6 ml) using a glass rod and then 3 to 4 gentle strokes of the pestle of the 5-ml Dounce homogenizer (do this in two batches).

    This is equivalent to ~0.5 ml per gram of liver tissue. Use this for all subsequent density-gradient separations of rat liver peroxisomes.

BASIC PROTOCOL 2

ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION USING A PREFORMED CONTINUOUS IODIXANOL GRADIENT

In the past, both metrizamide (e.g., Völkl and Fahimi, 1985; Wanders et al., 1986) and Nycodenz gradients (see Basic Protocol 3) have been used to purify peroxisomes from mammalian liver, but improved resolution can be achieved with iodixanol gradients (Graham et al., 1994; Van Veldhoven et al., 1996). Peroxisomes can be purified from a light mitochondrial fraction in preformed continuous 20% to 40% (w/v) iodixanol gradients at high yield (80% to 90%), with no detectable contamination from any other organelle. In this protocol, which is adapted from the method of Van Veldhoven et al. (1996), the gradient has been designed to be accurately isoosmotic over its whole range, by inclusion of an inverse gradient of sucrose. Because of the large difference in density between the peroxisomes and the other organelles, the separation can be carried out easily in open-topped tubes for a fixed-angle rotor. This considerably simplifies the loading procedures and also increases the capacity of the system. Since the peroxisomes are the densest organelle in the light mitochondrial fraction, it is normal practice to unload the gradient dense-end-first.

Materials

Iodixanol gradient solutions A, B, and C (see recipe)
Light mitochondrial pellet (LMP) suspension (see Basic Protocol 1)
Homogenization medium (HM; see recipe)
Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe

Gradient maker: two-chamber or Gradient Master (Accurate Chemical)
5-ml syringe with metal cannula (1-mm i.d.).
Ultracentrifuge and thick-walled polycarbonate tubes (30-ml) for a fixed-angle rotor (e.g., Beckman 60 Ti or Sorvall T-865)
Gradient unloader for dense-end-first collection (thin metal cannula connected to a peristaltic pump)

Additional reagents and equipment for protein assay (*APPENDIX 3B*)

NOTE: All solutions, glassware, centrifuge tubes and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Using the two-chamber gradient maker (or Gradient Master) prepare two linear gradients from 9 ml each of iodixanol gradient solutions B and C in the thick-walled polycarbonate tubes for the ultracentrifuge rotor.

   Alternatively, a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 20%, 25%, 30%, 35%, and 40% (w/v) iodixanol and let stand 16 hr at 4°C.

2. Using a syringe and metal cannula, underlayer each gradient with 2 ml of gradient solution A.

   If thin-walled tubes, which require complete filling, are used the volume of solution A may need to be increased.

3. Layer 3 ml of the LMP suspension over each gradient and ultracentrifuge 1 hr at 105,000 × *g*, 0° to 4°C.

   If the centrifuge has a slow acceleration facility use a program that allows the rotor speed to increase from 0 to 2000 rpm over a period of ~4 min.

4. Allow the rotor to decelerate from 2000 rpm without the brake (or on a controlled deceleration program) and collect the gradient in 1-ml fractions, dense-end-first, by aspiration using the gradient unloader.

   Thin-walled tubes may be unloaded by tube puncture.

   Peroxisomes have a median density of ρ *= 1.17 g/ml in iodixanol; the peak fraction is 7 to 8 ml from the bottom.*

5. Dilute the fraction(s) with ≥2 vol of HM and centrifuge 20 min at 30,000 × *g*, 0° to 4°C.

6. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to –20° or –80°C; always check that any measurements are unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A SELF-GENERATED IODIXANOL GRADIENT

ALTERNATE PROTOCOL 1

The LMP suspension is mixed with OptiPrep to a starting iodixanol concentration of 25% (w/v) and centrifuged in a vertical, near-vertical, or low-angle fixed-angle rotor in tubes of ~12 ml volume at ~180,000 × *g* for 2 to 3 hr (depending on rotor type). The protocol is adapted from Graham et al. (1994).

Additional Materials *(also see Basic Protocol 2)*

OptiPrep (60%, w/v iodixanol; Life Technologies, Accurate Chemical, Sigma, or Aldrich)
Diluent medium (DM; see recipe)
Vertical (e.g., Beckman VTi 65.1 or Sorvall 65V13), near-vertical (e.g., Beckman NVT 65), or low-angle (20° to 26°) fixed-angle rotor for an ultracentrifuge with 11- to 12-ml sealed tubes

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Make a 50% (w/v) iodixanol working solution by diluting 5 vol of OptiPrep with 1 vol of DM.

2. Mix equal volumes of 50% (w/v) iodixanol stock and LMP suspension by repeated gentle inversion.

3. Transfer to a suitable sealed tube.

4. Centrifuge 1.5 to 3 hr at 180,000 × *g*, 0° to 4°C, in vertical, near-vertical, or low-angle fixed angle rotor.

   The time will depend on the sedimentation path length of the rotor. For a vertical or near-vertical rotor with a path length of 17 to 25 mm, 1.5 hr will be sufficient; for longer path-length fixed-angle rotors the time will be up to 3 hr. The aim is to generate a gradient that is shallow in the middle to allow satisfactory separation of peroxisomes and mitochondria (see Commentary).

5. Decelerate the rotor from 2000 rpm to rest either using a controlled deceleration program or without the brake to allow a smooth reorientation of the gradient.
6. Collect the gradient, dense-end-first, in ~1-ml fractions by tube puncture or aspiration.

   The peroxisomes band in the bottom third of the gradient.
7. Dilute the fraction(s) with ≥2 vol of HM and centrifuge 20 min at 30,000 × *g*, 0° to 4°C.
8. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20° or −80°C; always check that any measurements are unaffected by the storage conditions, by comparing with those made on fresh material.

BASIC PROTOCOL 3

ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION USING A PREFORMED CONTINUOUS NYCODENZ GRADIENT

Because the difference in density between peroxisomes and mitochondria in Nycodenz is less than that in iodixanol, it is common practice to use the high resolving power of a vertical rotor. The short sedimentation path length of such rotors permits the use of relatively short centrifugation times at low relative centrifugal forces of 35,000 to 75,000 × *g*. This approach also reduces potential contamination of the peroxisomes by vesicles of the endoplasmic reticulum. Published methods use a variety of gradients and centrifugation conditions, and the following protocol is adapted from a number of these (Appelqvist et al., 1990; Kase and Björkhem, 1989; Prydz et al., 1988; Wilcke and Alexson, 1994).

Materials

20% (w/v) Nycodenz in homogenization medium (HM; see recipe)
50% and 60% (w/v) Nycodenz in high-density diluent (HD; see recipe)
Light mitochondrial pellet (LMP) suspension (see Basic Protocol 1)
Homogenization medium (HM; see recipe)
Protease inhibitors (optional; see recipe) added to any or all of the solutions at concentrations indicated in recipe

Gradient maker: two-chamber or Gradient Master (Accurate Chemical)
5-ml syringe with metal cannula (1-mm i.d.)
Ultracentrifuge with vertical rotor (e.g., Beckman VTi50 or Sorvall 50V39) with appropriate sealed tubes (~39 ml)
Gradient unloader for dense-end-first collection (tube-puncturing device or thin metal cannula connected to a peristaltic pump)

Additional reagents and equipment for protein assay (*APPENDIX 3B*)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Using the two-chamber gradient maker (or Gradient Master) prepare linear gradients from 17 ml each of the 20% and 50% Nycodenz solutions in tubes appropriate for the vertical rotor of the ultracentrifuge.

Alternatively, a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 20%, 30%, 40% and 50% (w/v) Nycodenz and let stand for 16 hr at 4°C.

Variants of this gradient include 18% to 50% (Hartl et al., 1985); 13% to 48% (Kase and Björkhem, 1989); 15% to 48% (Prydz et al., 1988); and 25% to 50% (Wilcke and Alexson, 1994).

2. Using the syringe and metal cannula, underlayer the gradient with 2 ml of the 60% Nycodenz as a density cushion.

   A number of published methods used the nonaqueous, low-viscosity Maxidens as a density cushion, but this is no longer readily available commercially.

3. Layer 2 to 3 ml of the LMP suspension on top of the gradient.

4. Centrifuge 75 min at 60,000 × *g*, 0° to 4°C, using programmed acceleration and deceleration to ensure a smooth reorientation of the gradient in the tubes.

   If programmed deceleration is not available then turn off the brake below 2000 rpm.

   Variants of the centrifugation parameters include 20 min at 74,000 × g (Prydz et al., 1988) and 75 min at 35,000 × g (Appelqvist et al., 1990).

   If a vertical rotor is not available, use a swinging-bucket rotor for 2 hr at 70,000 × g (Hartl et al., 1985).

5. Collect the gradient, dense-end-first, in 1- to 2-ml fractions, using a gradient unloader.

   The peroxisomes band close to the bottom of the gradient.

6. Dilute the fraction(s) with ≥2 vol of HM and centrifuge 20 min at 30,000 × *g*, 0° to 4°C.

7. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20° or −80°C; always check that any measurements are unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION USING A NYCODENZ BARRIER

ALTERNATE PROTOCOL 2

This is the simplest of all the systems. There is a tendency for the contamination from mitochondria to be slightly higher than with other systems, and the formation of a pellet rather than a band in a gradient may be detrimental to the integrity of the rather fragile organelles. The method is adapted from Ghosh and Hajra (1986).

Additional Materials *(also see Basic Protocol 3)*

30% (w/v) Nycodenz in high-density diluent (HD; see recipe)
Fixed-angle rotor with ~25-ml thick-walled polycarbonate tubes (e.g., Beckman 55.2 Ti or Sorvall T-865)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Transfer 15 ml of 30% Nycodenz to a 25-ml polycarbonate centrifuge tube.

2. Layer 2 ml of the LMP suspension over the Nycodenz barrier.

3. Centrifuge 1 hr at 130,000 × *g*, 0° to 4°C.

4. Remove all of the supernatant carefully by aspiration and resuspend the pellet in 1 to 2 ml of HM.

   Do not decant the supernatant, since the pellet is not firmly packed.

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20° or −80°C; always check that any measurements made are unaffected by the storage conditions by comparing with those made on fresh material.

BASIC PROTOCOL 4

ISOLATION OF PEROXISOMES FROM YEAST SPHEROPLASTS USING A PREFORMED CONTINUOUS NYCODENZ GRADIENT

Although wild-type yeast appears to contain rather few peroxisomes, they can be induced to proliferate, and there are a number of peroxisome gene–deletion strains of the organism (Thieringer et al., 1991; Watkins et al., 1998). Yeast has therefore become an increasingly popular means of investigating peroxisome development. In the following protocol, spheroplasts are homogenized in a sorbitol-containing medium using a Dounce homogenizer. Some variation exists in the sorbitol concentration in this medium; Watkins et al. (1998) used 0.6 M, while Crane et al. (1994) used 1.0 M. As with mammalian liver, a continuous Nycodenz gradient is used to fractionate a light mitochondrial fraction prepared from the homogenate. This method is adapted from Crane et al. (1994) and Watkins et al. (1998).

Materials

Yeast homogenization medium (YHM; see recipe)
15% (w/v) Nycodenz in yeast low-density diluent (YLD; see recipe)
42.5% and 50% (w/v) Nycodenz in yeast high-density diluent (YHD; see recipe)
Yeast low-density diluent (YLD; see recipe)
Yeast high-density diluent (YHD; see recipe)
Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe

Dounce homogenizer (Wheaton Type B, 40-ml)
Dounce homogenizer (Wheaton Type B, 5- to 10-ml)
Gradient maker: two-chamber or Gradient Master (Accurate Chemical)
5-ml syringe with metal cannula (1-mm i.d.)
Ultracentrifuge with vertical rotor (e.g., Beckman VTi65.1 or Sorvall 65V13) with appropriate sealable tubes (~13 ml)
Gradient unloader for dense-end-first collection (tube-puncturing device or thin metal cannula connected to a peristaltic pump)
High-speed refrigerated centrifuge with fixed-angle rotor to accommodate 50-ml tubes (e.g., Sorvall SS34)

Additional reagents and equipment for preparation of yeast spheroplasts (*UNIT 3.3*) and protein assay (*APPENDIX 3B*)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Prepare light mitochondrial fraction from spheroplasts

1. Prepare spheroplasts from 1 liter of yeast culture, grown in YPD medium to an OD_{600} of 0.5 to 1.0 (*UNIT 3.3*).
2. Suspend the spheroplasts in 35 ml of yeast homogenization medium (YHM) and homogenize in the 40-ml Dounce homogenizer, using 10 up-and-down strokes of the pestle.

3. Centrifuge the homogenate 10 min at 1500 × *g*, 0° to 4°C.
4. Aspirate and retain the supernatant on ice.
5. Resuspend the pellet in 35 ml of YHM and repeat steps 2 to 4.
6. Combine the two supernatants and centrifuge 30 min at 25,000 × *g*, 0° to 4°C.
7. Resuspend the light mitochondrial pellet in 6 ml of YHM using 10 gentle strokes of the pestle of the small volume Dounce homogenizer.

Separate peroxisomes in Nycodenz gradient

8. Using a two-chamber gradient maker (or Gradient Master) prepare 10.5-ml linear gradients from equal volumes of the 15% and 42.5 % Nycodenz in tubes for the vertical rotor.

   Alternatively, a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 15%, 25%, 35%, and 45% (w/v) Nycodenz and let stand for 16 hr at 4°C.

9. Using a syringe and metal cannula, underlayer the gradient with a cushion of 0.5 ml of 50% Nycodenz.
10. Layer the yeast light mitochondrial pellet (step 7) on top to fill the tube.
11. Ultracentrifuge 75 min at 174,000 × *g*, 0° to 4°C. Use controlled acceleration and deceleration programs to ensure a smooth reorientation of the gradient. If these are not available turn off the brake below 2000 rpm.

    Alternative systems include 15% to 37% Nycodenz for 90 min at 118,000 × g in Beckman 50.2Ti rotor (McAlister-Henn et al., 1995) and 15% to 36% Nycodenz and 42% cushion, 1 hr at 100,000 × g in a Sorvall 865B vertical rotor (Thieringer et al., 1991).

12. Collect the gradient in 0.5-ml fractions, dense-end-first, using a gradient unloader.
13. Dilute the fraction(s) with ≥2 vol of YHM and centrifuge 20 min at 30,000 × *g*, 0° to 4°C.
14. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of YHM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20° or −80°C; always check that any measurements are unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF PEROXISOMES FROM CULTURED CELLS (HepG2) USING A PREFORMED CONTINUOUS NYCODENZ GRADIENT

BASIC PROTOCOL 5

The cultured cells most widely used in peroxisome studies are the human hepatoblastoma line HepG2, and various skin fibroblast lines that are used in investigations into Zellweger syndrome. Both of these lines are grown routinely as a monolayer culture. In view of the fragility of peroxisomes from cultured cells, homogenization techniques such as nitrogen cavitation (Schrader et al., 1994) or the ball-bearing homogenizer (Watkins et al., 1991) devised by Balch and Rothman (1985) are to be preferred. If these are not available, a standard Potter-Elvehjem homogenizer (Cohen et al., 1992) may be used (see Commentary for more information). The following protocol is adapted from Cohen et al. (1992) and Watkins et al. (1991). The buffer used for all the solutions below is the same as that described for rat liver (i.e., MOPS) but Tris and HEPES are also commonly used with cultured cells. Some workers (Aikawa et al., 1991; Wanders et al., 1991; Cohen et al.,

1992) use a post-nuclear supernatant rather than a light mitochondrial fraction—in these cases a larger volume gradient may be necessary (see annotations to the corresponding steps below).

Materials

Suspension of cells (~10^8 cells) in 3 to 5 ml of homogenization medium (HM; see recipe)
Homogenization medium (HM; see recipe)
10% (w/v) Nycodenz in homogenization medium (HM; see recipe)
40% and 50% (w/v) Nycodenz in high-density diluent (HD; see recipe)
Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe

Ball-bearing homogenizer (see Balch and Rothman, 1985, for details of construction)
Low-speed centrifuge with swinging-bucket rotor to accommodate 10- to 20-ml tubes
Dounce homogenizer (5- to 10-ml, Wheaton type B)
High-speed centrifuge with fixed-angle rotor to accommodate 10- to 14-ml tubes (e.g., Sorvall SE12)
Gradient maker: two-chamber or Gradient Master (Accurate Chemical)
5-ml syringe with metal cannula (1-mm i.d.)
Ultracentrifuge with vertical rotor (e.g., Beckman VTi65.1or Sorvall 65V13) with appropriate sealable tubes (~13-ml)
Gradient unloader for dense-end-first collection (tube-puncturing device or thin metal cannula connected to a peristaltic pump)

Additional reagents and equipment for protein assay (*APPENDIX 3B*)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Isolate the light mitochondrial fraction

1. Homogenize the cells using five passes from syringe to syringe in the ball-bearing homogenizer.

   Alternatives include a nitrogen cavitation vessel (e.g Artisan Industries) or a Potter-Elvehjem homogenizer (clearance ~0.09 mm), 10-ml working volume.With nitrogen cavitation, use 400 psi for 10 min; with the Potter-Elvehjem homogenizer use 50 up-and-down strokes of the pestle at 1000 rpm.

2. Check by phase-contrast microscopy that cell breakage is ≥90%.

3. Centrifuge the homogenate 5 min at 500 × *g*, 0° to 4°C, in the low-speed centrifuge.

4. Aspirate the supernatant and retain on ice.

5. Resuspend the pellet in HM using 4 to 5 strokes of the pestle of the Dounce homogenizer and repeat steps 3, 4, and 5.

6. Centrifuge the combined supernatants 10 min at 6000 × *g*, 0° to 4°C, in the high-speed centrifuge.

7. Aspirate and recentrifuge the supernatant 15 min at 20,000 × *g*, 0° to 4°C.

8. Aspirate and discard the supernatant and resuspend the light mitochondrial pellet in 3 to 4 ml of HM using 3 to 4 gentle strokes of the pestle of the Dounce homogenizer.

Separate the peroxisomes in Nycodenz gradient

9. Using the two-chamber gradient maker (or Gradient Master) prepare 10-ml linear gradients from equal volumes of the 10% and 40% Nycodenz in tubes for the vertical rotor.

   Alternatively a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 10%, 20%, 30% and 40% (w/v) Nycodenz and let stand for 16 hr at 4°C.

   If a post-nuclear supernatant is used rather than a light mitochondrial pellet, then prepare 34-ml gradients.

10. Using the syringe and metal cannula, underlayer the gradient with 0.5 ml of 50% Nycodenz.

11. Layer ~2 ml of the pellet suspension from step 8 on top of the gradient, to fill the tube.

    Alternatively use 4 to 5 ml of a post-nuclear supernatant on the larger gradients.

12. Centrifuge 25 to 35 min at 75,000 × *g*, 0° to 4°C. Use a controlled acceleration and deceleration programs to ensure a smooth reorientation of the gradient. If these are not available turn off the brake below 2000 rpm.

    For fibroblasts, Aikawa et al. (1991) used a 15% to 30% Nycodenz gradient and 1 hr at 305,000 × g in the same rotor.

13. Collect the gradient in 0.75-ml fractions, dense-end-first, using a gradient unloader.

    The peroxisomes band close to the bottom of the tube.

14. Dilute the fraction(s) with ≥2 vol of HM and centrifuge 20 min at 30,000 × *g*, 0° to 4°C.

15. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20°C or −80°C; always check that any measurements made are unaffected by the storage conditions by comparing with those made on fresh material.

ASSAY FOR ENDOPLASMIC RETICULUM MARKER ENZYME NADPH-CYTOCHROME *c* REDUCTASE

SUPPORT PROTOCOL

NADPH cytochrome *c* reductase is an important oxidoreductase linked to the oxidative status of the biosynthetic pathways present in the endoplasmic reticulum. Most methods measure the production of reduced cytochrome *c* by monitoring the absorbance at 550 nm. Sometimes NADH is used as an alternative substrate to NADPH, in which case it is normal to include an inhibitor of the mitochondrial oxidation pathway—normally rotenone—to which the endoplasmic reticulum enzyme is insensitive. Indeed, the inclusion of rotenone in the assay is not uncommon when using NADPH as the substrate, to avoid any interference from mitochondrial electron transport. The method is taken from Graham (1993) and adapted from Williams and Kamin (1962).

Materials

Assay buffer (see recipe)
25 mg/ml cytochrome *c* in assay buffer (prepare fresh; keep on ice)
10 mM EDTA (dilute 1 ml 100 mM EDTA stock to 10 ml with H_2O)
1 mg/ml rotenone in ethanol (store up to 1 month at 4°C)
2 mg/ml NADPH in assay buffer (prepare fresh; keep on ice away from light)
Recording spectrophotometer (visible wavelength) with 1-ml cuvettes

1. Bring the assay buffer to room temperature and carry out all operations at this temperature.
2. Adjust the chart recorder to give a 0.2 absorbance unit full-scale deflection.
3. In a 1-ml cuvette, add 50 µl of cytochrome *c*, 10 µl 10 mM EDTA, and 10 µl of 1 mg/ml rotenone to 1 ml of assay buffer.
4. Add up to 50 µl of sample and mix well.

   Neither Nycodenz nor iodixanol interfere with this enzyme at the concentrations that are likely to occur in the assay mixture. If the gradient fractions need to be concentrated, they should be diluted with 2 vol of HM, sedimented by centrifuging 20 min at 35,000 × g, and resuspended in a smaller volume of HM.

5. Record the absorbance at 550 nm until the baseline is steady, then add 0.1 ml of NADPH.

   A double-beam recording spectrophotometer is ideal, but, as single-beam instruments are more commonly available, these steps are designed for such an instrument.

6. Mix well and continue to record the absorbance until a linear increase in value can be measured over a period of 1 to 2 min.
7. Calculate the activity of the enzyme (µmol cytochrome *c* reduced/min) by measuring the slope of the trace.

   The molar extinction coefficient of reduced cytochrome c is 27,000.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Assay buffer

Dissolve 0.71 g Na_2HPO_4 in 100 ml water (50 mM final). Dissolve 0.68 g KH_2PO_4 in another 100 ml water (50 mM final). Add KH_2PO_4 to Na_2HPO_4 to reach pH 7.7. Store up to 1 to 2 days at 4°C.

Diluent medium (DM)

To 100 ml H_2O add:
12 ml 100 mM disodium EDTA (6 mM final)
60 ml 100 mM MOPS (see recipe; 30 mM final)
1.2 ml ethanol (0.6% v/v final)
Adjust to pH 7.2 with 1 M NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

High-density diluent (HD)

To 100 ml H_2O add:
2 ml 100 mM disodium EDTA (1 mM final)
10 ml 100 mM MOPS (see recipe; 5 mM final)
0.2 ml ethanol (0.1% v/v final)
Adjust to pH 7.4 with 1 M NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Homogenization medium (HM)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
2 ml 100 mM disodium EDTA (1 mM final)
10 ml 100 mM MOPS (see recipe; 5 mM final)
0.2 ml ethanol (0.1% v/v final)
Adjust to pH 7.2 with 1 M NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Iodoxinol gradient solutions A, B, and C

These gradient solutions are prepared by mixing the following stock solutions at the volume ratios listed below:

OptiPrep (60% w/v iodixanol; available from Life Technologies, Accurate Chemical and Scientific, Sigma, and Aldrich)
Diluent medium (DM; see recipe)
1 M sucrose (see recipe)

Gradient solution A (50% w/v iodixinol):
5 vol OptiPrep
0.6 vol DM
0.4 vol 1 M sucrose

Gradient solution B (40% w/v iodixanol):
4 vol OptiPrep
0.6 vol DM
0.7 vol 1 M sucrose
0.7 vol H_2O

Gradient solution C (20% w/v iodixanol):
2 vol OptiPrep
0.6 vol DM
1.1 vol 1 M sucrose
2.3 vol H_2O

MES, 100 mM

4.16 g 2-(*N*-morpholino)ethanesulfonic acid (MES)
H_2O to 200 ml
Store up to 2 to 3 months at −20°C

MOPS, 100 mM

4.18 g 3-(*N*-morpholino)propanesulfonic acid (MOPS)
H_2O to 200 ml
Store 2 to 3 months at −20°C

Protease inhibitors

Add the following to solutions as required so that the final concentrations are: 1 mM PMSF and 2.0 µg/ml for each of the other inhibitors. Also see *APPENDIX 1B* for general information on these compounds. All of these stock solutions may be stored in aliquots up to 2 to 3 months at −20°C, except PMSF which should be stored at 4°C.

PMSF: Dissolve 0.348 g phenylmethylsulfonyl fluoride (PMSF; 200 mM final) in 10 ml of dried propan-2-ol or ethanol.

Antipain: Dissolve 10 mg antipain (1 mg/ml final) in 10 ml of 10% (v/v) dimethyl sulfoxide (DMSO).

Aprotinin: Dissolve 10 mg antipain (1 mg/ml final) in 10 ml water.

Leupeptin: Dissolve 10 mg leupeptin (1 mg/ml final) in 10 ml of 10% (v/v) dimethylsulfoxide (DMSO).

Sucrose, 1 M

34.2 g sucrose (1 M final)
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Yeast high-density diluent (YHD)

To 100 ml H_2O add:
8.55 g sucrose (0.125 M final)
15 mg KCl (1 mM final)
2 ml 100 mM disodium EDTA (1 mM final)
10 ml 100 mM MES (see recipe; 5 mM final)
0.2 ml ethanol (0.1% v/v final)
Adjust to pH 6.0 with 1 M NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Yeast homogenization medium (YHM)

To 100 ml H_2O add:
21.9 g sorbitol (0.6 M final)
15 mg KCl (1 mM final)
2 ml 100 mM disodium EDTA (1 mM final)
10 ml 100 mM MES (see recipe; 5 mM final)
0.2 ml ethanol (0.1% v/v final)
Adjust to pH 6.0 with 1 M NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Yeast low-density diluent (YLD)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
15 mg KCl (1 mM final)
2 ml 100 mM disodium EDTA (1 mM final)
10 ml 100 mM MES (see recipe; 5 mM final)
0.2 ml ethanol (0.1% v/v final)
Adjust to pH 6.0 with 1 M NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

COMMENTARY

Background information

Differential centrifugation

As with most subcellular organelles, methods for the isolation of peroxisomes have come largely from work with rat liver. With the exception of nuclei and rough endoplasmic reticulum (RER), peroxisomes are the densest of the major subcellular organelles. Peroxisomes are also one of the major components of the light mitochondrial fraction, which is obtained from a homogenate by differential centrifugation. The "classical" differential centrifugation scheme involves four steps: 600 to 1000 × *g* for 10 min (nuclear pellet); 3000 to 4000 × *g* for 10 min (heavy mitochondrial pellet); 15,000 to 25,000 × *g* for 20 min (light mitochondrial fraction); and 50,000 to 100,000 × *g* for 45 min (microsomal pellet). There is considerable variation in the actual RCFs used for each step, and sometimes the isolation of the heavy mitochondrial fraction is omitted. Moreover since the size of peroxisomes is quite diverse (0.2 to 1.0 μm), and as there is evidence for subpopulations of these organelles (Schrader et al., 1994; van Roermund et al., 1995), it is not unusual for the homogenate to be centrifuged at 2000 × *g* for 10 min, followed by 22,000 to 25,000 × *g* for 20 min, in order to pellet as many of the peroxisomes as possible. Unavoidably, some of the more rapidly sedimenting microsomes will also be pelleted. This situation is exacerbated if the RCF is increased; Lyons et al. (1991) used 41,000 × *g* for 30 min to pellet the peroxisomes from a hepatoma homogenate. Although in this case most of the microsomes formed a layer on top of the main organelle pellet, which could be recovered separately, increased microsomal contamination of the light mitochondrial pellet is inevitable with this technique. Some protocols, notably those for cultured cells (e.g., Aikawa et al., 1991; Wanders et al., 1991; Cohen et al., 1992) often use a post-nuclear supernatant rather than a light mitochondrial fraction; in these cases all of the endoplasmic reticulum (ER) will also be present. This unavoidable contamination of the peroxisome-containing fraction by vesicles from the ER is a significant factor, since this influences the choice of density gradient medium.

Density gradient centrifugation

The densities of the major components of the light mitochondrial fraction in a variety of media are given in Table 3.5.1. In iodinated density gradient media such as Nycodenz or iodixanol, the density of peroxisomes is only slightly lower than that in sucrose; on the other hand the densities of the other organelles—mitochondria, lysosomes and Golgi membranes—are significantly higher in sucrose (Graham et al., 1994). The limiting membrane of peroxisomes is freely permeable to small solute molecules; these organelles therefore do not behave as osmotically sensitive particles in low-molecular mass solutes. Hence their observed density is relatively little affected by the osmolarity of the suspending solution and reflects only the macromolecular composition of the particle. The observed density of the mitochondria, lysosomes, and ER vesicles, on the other hand, includes the enclosed water component and is consequently dependent on the osmolarity of the gradient.

Although sucrose gradients are still occasionally used to purify peroxisomes from yeast (e.g., Crane et al., 1994), in the case of mammalian tissues and cells they have been totally superseded by iodinated density gradient media. In sucrose gradients, peroxisomes are significantly contaminated by lysosomes, and this can only be effectively reduced, in the case of rat liver, if the animals are injected with Triton

Table 3.5.1 Density of Organelles of the Light Mitochondrial Fraction in Different Gradient Media

	Density (g/ml)			
Organelle	Sucrose	Nycodenz	Iodixanol	Percoll
Endoplasmic reticulum	1.06-1.23	1.05-1.16	1.03-1.13	1.03-1.06
Golgi membranes	1.05-1.12	1.03-1.08	1.03-1.06	1.03-1.05
Lysosomes	1.19-1.21	1.12-1.15	1.11-1.14	1.06-1.12
Mitochondria	1.17-1.21	1.13-1.16	1.13-1.15	1.05-1.08
Peroxisomes	1.18-1.23	1.17-1.20	1.16-1.19	1.04-1.06

WR1339 to lower the density of the lysosomes (Leighton et al., 1968). Although contamination by mitochondria is less marked, the densest of these organelles consistently overlap the lighter peroxisomes. Consequently, the lower densities of both mitochondria and lysosomes in iodinated density gradient media make these media ideally suited to the purification of peroxisomes. Since about 1985, Nycodenz has been the gradient medium of choice, and the use of preformed gradients in vertical rotors has become an almost standard technique. Vertical rotors, with their short sedimentation path lengths, allow efficient and rapid separations of organelles. Moreover, the hydrostatic pressures in vertical rotors (and low-angle fixed-angle rotors) are much lower than those in swinging-bucket rotors, thus minimizing any potential deleterious effects on the organelles. Although the various published methods using Nycodenz show some minor differences in the density range of the gradient, generally they run from ~1.10 to 1.19 g/ml and include a cushion of density 1.22 to 1.26 g/ml to prevent any peroxisomes reaching the wall of the tube. This is essential for vertical rotors. The centrifugation conditions show the largest discrepancy, varying from 30,000 × *g* for 75 min (Kase and Björkhem, 1989; Appelqvist et al., 1990) to 309,000 × *g* for 60 min (Aikawa et al., 1991). Without exception, the gradients are top-loaded with the sample, and many gradients are centrifuged below 60,000 × *g* for <1 hr. At these relatively low RCFs and times, any endoplasmic reticulum vesicles will probably not reach their equilibrium density banding position, and this may virtually eliminate any potential contamination of the peroxisomes.

Although inverse gradients of sucrose are often used to balance the osmolarity of Nycodenz gradients, at concentrations above 30% w/v (~1.16 g/ml) any Nycodenz solution is hyperosmotic. Iodixanol solutions on the other hand can be made isoosmotic at all densities (Graham et al., 1994); consequently mitochondria and lysosomes have significantly lower densities (Table 3.5.1) in iodixanol than in Nycodenz, and the mitochondria in particular are easier to resolve from peroxisomes. Moreover, the latter are also isolated under isoosmotic conditions. Most organelle separations with iodixanol are carried out in preformed gradients. Although the formation of self-generated gradients of iodixanol is normally carried out at RCFs above 300,000 × *g*, gradients with a relatively shallow median section, which are ideal for peroxisome purification (see Anticipated Results), can be formed at RCFs of 180,000 × *g*, and although the centrifugation time maybe 1.5 to 3 hr (depending on rotor type), the method has the considerable merits of ease of sample preparation and high reproducibility (Graham et al., 1994).

Peroxisomes can also be isolated under isoosmotic conditions in Percoll gradients. Probably because Percoll is a colloidal suspension of silica, rather than a true solute, mammalian peroxisomes behave rather differently: they exhibit a much lower density than in sucrose, Nycodenz, or iodixanol, and they are generally well resolved from both mitochondria or lysosomes (see Table 3.5.1). However, why the density of the major organelles in iodinated density gradient media (lysosomes<mitochondria<peroxisomes) should be essentially reversed in Percoll is not clear. In some nonmammalian systems, however, for example the filamentous hemiascomycete *Ashbya gossypii* (Maeting, et al., 1999), peroxisomes are denser than mitochondria in Percoll.

Although the formation of Percoll gradients has the great merit of self-generation at low RCFs and centrifugation times, a major problem with these gradients is that endoplasmic reticulum vesicles in the light mitochondrial fraction from mammalian tissues always copurify with the peroxisomes (see Table 3.5.1), as evinced by the coincidence of catalase and glucose-6-phosphatase profiles (see for example Schepers et al., 1989). One other problem that is rarely considered is the potential contamination from Golgi membranes in Percoll gradients. In the case of liver in particular, a large proportion of the Golgi will sediment in the LMP. According to Schweizer et al. (1991) the Golgi membranes also band at a low density in Percoll, as they do in iodixanol and Nycodenz (see Table 3.5.1). In Percoll, therefore, they are likely to contaminate the peroxisomes, while in iodixanol (and Nycodenz), Golgi membranes band at the opposite end of the gradient from the peroxisomes.

Moreover, before spectrophotometric assays for peroxisomes (and other organelles) can be carried out, it is usually necessary to remove the Percoll by centrifugation, because of the light-scattering properties of this medium. During this procedure the peroxisomes will also sediment and they are recovered as a loose layer above the Percoll pellet. However Osmundsen (1982) established that this procedure accounted for an approximately 50% loss of the organelles. As long as the organelles are at a

sufficiently high concentration in the gradient fractions, removal of the medium is generally not a strict requirement for either Nycodenz or iodixanol gradients. If the peroxisomes do need to be concentrated or if the medium does interfere with some subsequent procedure, then a simple dilution of the fraction(s) with buffer to reduce the density, followed by pelleting of the organelles, will give high recoveries.

Critical Parameters and Troubleshooting

Homogenization

Only cultured cells may provide a significant homogenization problem. Many monolayer cells can be disrupted in an isoosmotic sucrose medium using relatively mild liquid shear conditions; this minimizes any damage to peroxisomes and loss of intra-organelle components. Watkins et al. (1991) observed that the standard liquid shear techniques of Dounce or Potter-Elvehjem homogenization led to the release of soluble peroxisomal macromolecules from HepG2 cells, and stressed the need to use the more gentle disruptive forces provided by the ball-bearing device of Balch and Rothman (1985). Nevertheless, other workers using the same cells have used up to 50 strokes of the pestle of a Potter-Elvehjem homogenizer (Wanders et al., 1991; Cohen et al., 1992). Nitrogen cavitation is another alternative and was used by Völkl and Fahimi (1985). If only Dounce or Potter-Elvehjem homogenizers are available, a homogenization medium of 0.25 M sucrose/1 mM EDTA/10 mM triethanolamine-acetic acid, pH 7.4 (Marsh et al., 1987), combined with no more than 10 strokes of the pestle of a tight-fitting Dounce homogenizer, has been found to be widely applicable to monolayer cells (and fibroblasts in particular). For a more complete discussion of these problems see Evans (1992) and Graham (1997).

Differential centrifugation

The aim of the homogenization should be to devise conditions such that ≥90% of cells are disrupted with the minimum release of peroxisomal markers into the medium. To check this out, all of the differential centrifugation fractions, including the microsomes and the cytosol (100,000 × *g*/45 min pellet and supernatant), should be assayed for organelle markers and proteins (see Anticipated Results). For tissues or cells other than those described in this unit, always carry out a more thorough differential centrifugation (e.g., 600 × *g*/10 min, 3000 × *g*/10 min, 15,000 × *g*/10 min; 25,000 × *g*/10 min, 40,000 × *g*/10 min) in order to devise the optimal protocol.

Density gradient centrifugation

For a tissue or cell other than one of those described in this unit, it may also be necessary to modulate some of the gradient and/or centrifugation conditions in the light of experience. For example, try making the gradient more or less dense at the bottom to take account of organelles that may be correspondingly more or less dense. Modifications will also need to be made if the type of rotor described in the protocol is not available. If a swinging-bucket or fixed-angle rotor is substituted for a recommended vertical rotor, then the centrifugation times will have to be increased to take account of the longer sedimentation path lengths.

The preparation of preformed continuous gradients using a two-chamber gradient maker requires considerable practice to achieve highly reproducible gradients, and some of the important points in operating such devices are covered by Dobrota and Hinton (1992). A Gradient Master, which involves simply layering the densest and lightest solutions in the centrifuge tube, provides a more expensive but reproducible alternative. If neither of these are available, then a discontinuous gradient constructed from three or four solutions, spanning the required density range, can be prepared and allowed to diffuse, usually overnight at 4ÉC. Note that the time required for complete linearity to be achieved will depend on the tube diameter and the volume of each layer. Always collect a blank gradient and check (using refractive index) that the gradient is indeed linear and covers the appropriate density range.

In self-generated gradients of iodixanol (Alternate Protocol 1), the light mitochondrial fraction is mixed with OptiPrep to a uniform starting concentration of 25% iodixanol; the gradient forms and the particles move to their banding density during the centrifugation. It is important that the gradient generated be relatively shallow in the middle of the gradient (ρ = 1.14 to 1.16 g/ml) to achieve a satisfactory linear separation between the mitochondria and peroxisomes (see Anticipated Results).

Enzyme and other assays

Prior to the execution of an enzyme assay (or other measurement), it is common practice to remove the gradient medium from each gradient fraction by dilution with 2 to 4 volumes of a low-density medium, followed by centrifugation to pellet the organelles and resuspension in

the same low-density medium. The latter is often the homogenization medium, or, if it is isoosmotic, a medium used for the subsequent assay. This practice eliminates any possible interference of the gradient medium on the subsequent procedure(s), and it is described in the final steps of each Basic Protocol. In many instances, however, the sample volumes used in the assay are sufficiently small (as in the Support Protocol) that the gradient solute is adequately diluted so as not to cause any interference in the assay, even at the highest density. Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without washing. If, however, either of these requirements are not met, then dilution, pelleting and resuspension of the pellet in a smaller volume are unavoidable.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Nycodenz and iodixanol interfere with methods using the Folin-Ciocalteu reagent; however, if small volumes (≤100 µl) of fraction are used in a 1- to 2-ml assay volume, the concentration of gradient solute in the assay solution is <10% (w/v) and the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of gradient solute.

The only significant interference of common peroxisome functional assays by Nycodenz (and by implication by iodixanol too) is phytanic acid α-oxidation (Singh et al., 1993).

Anticipated Results

Basic Protocol 1

The expected distribution of the major organelle enzyme markers in the nuclear, heavy mitochondrial, light mitochondrial, microsomal, and cytosolic fractions from a rat liver homogenate is given in Table 3.5.2. If the first 750 × *g* is omitted, the figures for the percent protein and percent enzyme content of the 3500 × *g* pellet will be the sum of the nuclear and light mitochondrial fractions. If the actual RCFs and times used are different from those in Basic Protocol 1, then the data will also be similarly different. Nevertheless, the overall pattern of enzyme content should remain the same. In the case of HepG2 cells, the percent of the total catalase in the light mitochondrial fraction is reported (Watkins et al., 1991) at 32%, and the mitochondria tend to be more prevalent in the heavy mitochondrial fraction (48%) compared to the nuclear fraction (16%). Organelles from a yeast homogenate also conform to this overall pattern.

Basic Protocol 2

Fractions 5 to 14 of the gradient (~10 ml) contain ~85% of the total catalase activity recovered from the gradient (Fig. 3.5.1). There is no detectable contamination from mitochondria, lysosomes, or endoplasmic reticulum in these fractions whatsoever.

Alternate Protocol 1

The bottom seven fractions of the gradient (~5 ml) contain nearly 90% of the total catalase activity recovered from the gradient (Fig. 3.5.2). There is no detectable contamination from mitochondria, lysosomes, or endoplasmic reticulum (not shown), which co-bands with the lysosomes in this gradient.

Basic Protocol 3

There will be some small differences in the precise banding position of the peroxisomes (in terms of the distance from the bottom of the tube) which reflect the density profile of the

Table 3.5.2 Protein and Enzyme Distribution in Rat Liver Differential Centrifugation Fractions[a,b]

	Percent (relative sp. act.)				
Fraction	Protein	Catalase	SDH	NADPH cyt *c* red	AP
Nuc	30-35	20-25 (<1.0)	50-60 (<2.0)	20-25 (<1.5)	20-25 (<2.0)
H Mit	15-25	7.5-12.5(<0.5)	20-25 (>3.0)	5-7.5 (<0.2)	10-12.5 (<1.0)
L Mit	7.5-12.5	20-25 (>3.0)	5-10 (<2.0)	5-10 (<1.0)	20-25 (>3.0)
Mic	12.5-17.5	1-2 (<0.2)	10-15 (<0.5)	50-55 (>3.0)	2-5 (<0.2)
Cytosol	25-30	25-30 (<1.0)	0	5-10 (<0.5)	20-25 (<1.5)

[a]Figures are percent of protein and enzyme in each fraction and figures in parenthesis are relative specific activity of enzymes (percent enzyme/percent protein).

[b]Abbreviations: AP, acid phosphatase; H Mit, heavy mitochondrial; L Mit, light mitochondrial; Mic, microsomal; NADPH cyt *c* red, NADPH cytochrome *c* reductase; Nuc, nuclear; SDH, succinate dehydrogenase.

gradient, the concentration of Nycodenz cushion (if present), and the centrifugation conditions. Nevertheless there will be an overall similarity of organelle banding, with the peroxisome peak being very close to the bottom of the gradient. The example given in Figure 3.5.3 is from Wilcke and Alexson (1994) who used a 25% to 50% Nycodenz gradient, a 60% cushion, and 60,000 × *g* for 35 min in a vertical rotor. Approximately 70% of the total catalase

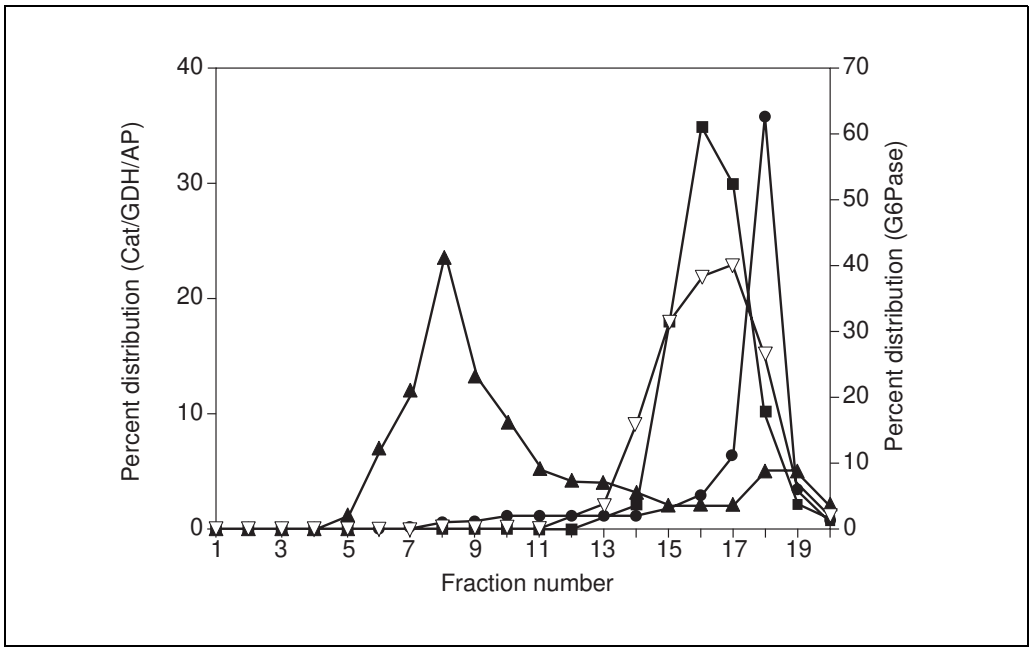


Figure 3.5.1 Isolation of peroxisomes in a preformed continuous gradient of iodixanol in a fixed-angle rotor. Fraction 1 is the dense end of the gradient. Distribution of enzymes: GDH, glutamate dehydrogenase (filled squares); Cat, catalase (filled triangles); AP, acid phosphatase (open inverted triangles); G6Pase, glucose-6-phosphatase (filled circles). Data is adapted from Van Veldhoven et al. (1996) with permission of Academic Press.

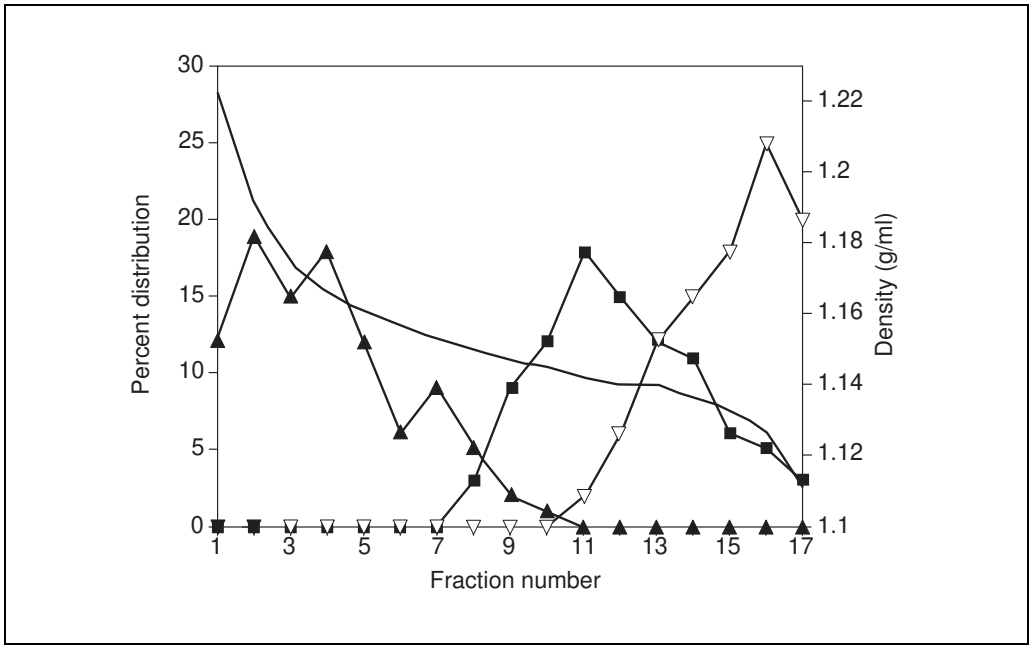


Figure 3.5.2 Isolation of peroxisomes in a self-generated gradient of iodixanol in a 12-ml fixed-angle rotor. Fraction 1 is the dense end of gradient. Distribution of enzymes: Cat, catalase (filled triangles); SDH, succinate dehydrogenase (filled squares); βGal, β-galactosidase (open inverted triangles). Data is adapted from Graham et al. (1994) with permission of Academic Press.

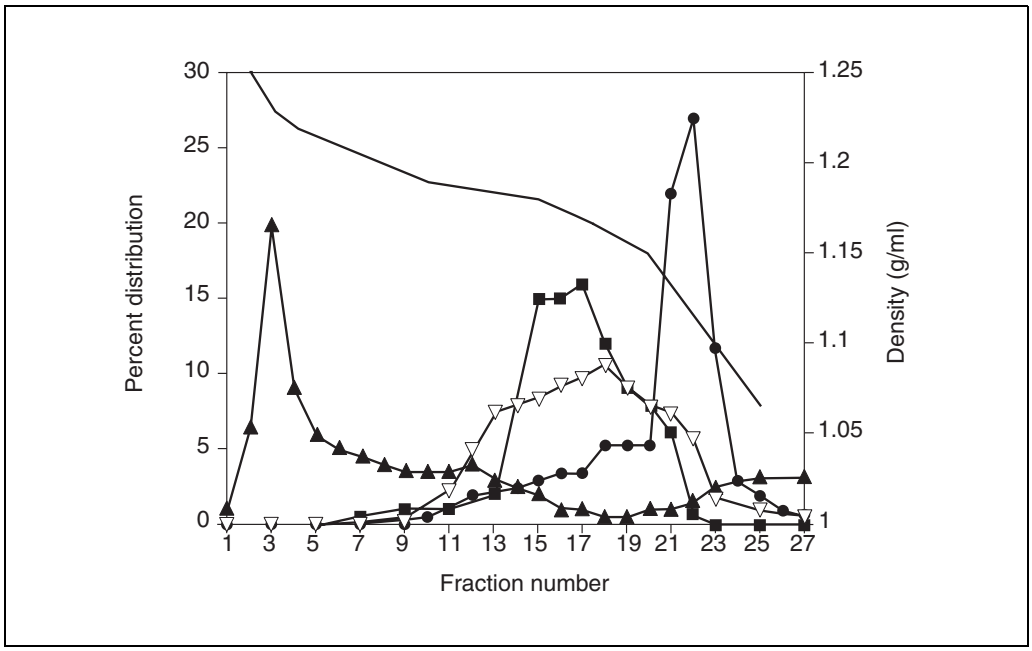


Figure 3.5.3 Isolation of peroxisomes in a 25% to 50% (w/v) Nycodenz gradient (60,000 × *g* for 35 min) in a vertical rotor. Fraction 1 is the dense end of the gradient. Distribution of enzymes: Cat, catalase (filled triangles); Cytox, cytochrome oxidase (filled squares); AP, acid phosphatase (open inverted triangles); Est, esterase (filled circles; endoplasmic reticulum marker). Data is adapted from Wilcke and Alexson (1994) with permission of European Journal of Biochemistry.

in the gradient is recovered from the first eleven fractions, with essentially no contamination from any other organelle. Compared to iodixanol, there is a greater tendency for some of the lighter peroxisomes to overlap the denser lysosomes and mitochondria.

Alternate Protocol 2

Because density barriers are less discriminating than continuous gradients, it is difficult to choose a single density that will give both good yields and lack of contamination by other organelles. Nevertheless the 30% Nycodenz barrier devised by Ghosh and Hajra (1986) should give a yield of ~50% of the catalase from the light mitochondrial fraction, with <2% of the lysosomes, mitochondria, and endoplasmic reticulum.

Basic Protocol 4

In this yeast protocol, ~55% of the total catalase is located in the bottom half of the gradient with no detectable contamination from mitochondria (Watkins et al., 1998). Unlike the mammalian systems, however, the rest of the lighter catalase-containing particles form a second well defined peak, which co-bands with the mitochondria. Moreover the median banding densities of all of the particles in Nycodenz gradients appear significantly lighter than their mammalian counterparts. Peroxisomes have a median density of ~1.16 g/ml and that of the mitochondria is as low as 1.12 g/ml (Thieringer et al., 1991).

Basic Protocol 5

The density of the organelles from HepG2 cells is almost identical to that of those from rat liver. Approximately 55% of the total catalase bands in the lowest third of the gradient; this peroxisome band is devoid of succinate dehydrogenase, but contains ~8% of the total ER in the gradient (Watkins et al., 1991). The overall distribution of enzyme markers is, however, essentially identical to that of Basic Protocol 3.

Time Considerations

There are no points at which any of the basic protocols can be discontinued. The light mitochondrial fraction produced in Basic Protocol 1 must be used immediately for any subsequent gradient separation. Preformed gradients should be made up during Basic Protocol 1 and kept on ice until required. If they are prepared at room temperature, they must be given time to cool down to 4°C.

Basic Protocol 1 should be accomplished in 2 to 2.5 hr. If the preformed iodixanol or Nycodenz gradients have been made up either the previous night (diffusion of discontinuous gra-

dients) or during Basic Protocol 1, Basic Protocols 2 and 3 should take ~3 hr. Alternate Protocol 1 will also take ~3 hr (depending on the rotor), but the preparations prior to centrifugation are considerably simpler and shorter. Basic Protocol 3 should take no more than 2 hr. Because of the ~4 hr spheroplast preparation time, Basic Protocol 4 will require in total ~7 hr. Basic Protocol 5 should be completed within 3.5 hr.

With experience and practice it may be unnecessary to unload the gradients in a series of equal fractions; instead the peroxisome band might be removed using a Pasteur pipet or syringe—this will save at least 15 min per tube.

Functional assays should be carried out as soon as possible after the preparation, but simple marker enzyme assays (such as described in this unit and in *UNIT 3.4*) can be carried out on material stored at 0° to 4°C for up to 16 hr without much loss of activity. These assays and compositional measurements may also be carried out on material frozen to −20° or −80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those obtained with the fresh organelles. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All the solutions can be made up ahead of time and stored at 4°C for 1 to 2 days or frozen to −20°C for longer periods. But note that any enzymes, as well as PMSF or other protease inhibitors, should be added immediately prior to use.

Literature Cited

Aikawa, J., Chen, W.W., Kelley, R.I., Tada, K., Moser, H.W., and Chen, G.L. 1991. Low-density particles (W-particles) containing catalase in Zellweger syndrome and normal fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 88:10084-10088.

Appelqvist, E-L., Reinhart, M., Fischer, R., Billheimer, J., and Dallner, G. 1990. Presence of individual enzymes of cholesterol biosynthesis in rat liver peroxisomes. *Arch. Biochem. Biophys.* 282:318-325.

Balch, W.E. and Rothman, J.E. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: Asymmetric properties of donor and acceptor activities in cell-free systems. *Arch. Biochem. Biophys.* 240:413-425.

Cohen, L.H., Griffioen, M., van Roermund, C.W.T., and Wanders, R.J.A. 1992. Subcellular localization of squalene synthase in human hepatoma cell line HepG2. *Biochim. Biophys. Acta* 1126:114-118.

Crane, D.I., Kalish, J.E., and Gould, S.J. 1994. The *Pichia pastoris PAS4* gene encodes a ubiquitin-conjugating enzyme required for peroxisome assembly. *J. Biol. Chem.* 269:21835-21844.

Dobrota, M. and Hinton, R. 1992. Conditions for density gradient separations. *In* Preparative Centrifugation—A Practical Approach (D. Rickwood, ed.) pp. 77-142. Oxford University Press, Oxford.

Evans, W.H. 1992. Isolation and characterization of membranes and cell organelles. *In* Preparative Centrifugation—A Practical Approach. (D. Rickwood, ed.) pp. 233-270. Oxford University Press, Oxford.

Ghosh, M.K. and Hajra, A.K. 1986. A rapid method for the isolation of peroxisomes from rat liver. *Anal. Biochem.* 159:169-174.

Graham, J.M. 1993. The identification of subcellular fractions from mammalian cells. *In* Methods in Molecular Biology 19 (J.M. Graham and J.A. Higgins, eds.) pp. 1-18. Humana Press, Totowa, N.J.

Graham, J.M. 1997. Homogenization of cells and tissues. *In* Subcellular Fractionation—A Practical Approach. (J.M. Graham and D. Rickwood, eds.) pp. 1-29. Oxford University Press, Oxford.

Graham, J., Ford, T., and Rickwood, D. 1994. The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol. *Anal. Biochem*. 220:267-373.

Hartl, F.-U., Just, W.W., Köster, A., and Schimassek, H. 1985. Improved isolation and purification of rat liver peroxisomes by combined rate zonal and equilibrium density centrifugation. *Arch. Biochem. Biophys.* 237:124-134.

Kase, B.F. and Björkhem, I. 1989. Peroxisomal bile acid-CoA: Amino-acid *N*-acyltransferase in rat liver. *J. Biol. Chem.*. 264:9220-9223.

Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S., and de Duve, C. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR 1339. *J. Cell Biol.* 37:482-513.

Lyons, H.T., Kharroubi, A., Wolins, N., Tenner, S., Chanderbhan, R.F., Fiskum, G., and Donaldson, R.P. 1991. Elevated cholesterol and decreased carrier protein-2 in peroxisomes from AS-30D hepatoma compared to normal rat liver. *Arch. Biochem. Biophys.* 285:238-245.

Maeting, I., Schmidt, G., Sahm, H., Revuelta, J.L., Stierhof, Y-D., and Stahmann, K-P. 1999. Isocitrate lyase of *Ashbya gossypii*—transcriptional regulation and peroxisomal localization. *FEBS Lett*. 444:15-21.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol*. 104:875-886.

McAlister-Henn, L., Steffan, J.S., Minard, K.I., and Anderson, S.L. 1995. Expression and function of a mislocalized form of peroxisomal malate dehydrogenase (MDH3) in yeast. *J. Biol. Chem.* 270:21220-21225.

Osmundsen, H. 1982. Factors which can influence β-oxidation by peroxisomes isolated from livers of clofibrate-treated rats. Some properties of peroxisomal fractions isolated in a self-generated Percoll gradient by vertical rotor centrifugation. *Int. J. Biochem.*. 14:905-914.

Prydz, K., Kase, B.F., Björkhem, I., and Pedersen, J.I. 1988. Subcellular localization of 3α,7α-dihydroxy- and 3α,7α,12α-trihydroxy-5β-cholestanoyl-coenzyme A ligase(s) in rat liver. *J. Lipid Res*. 29:997-1004.

Schepers, L., Casteels, M., Verheyden, K., Parmentier, G., Asselberghs, S., Eyssen, H.J., and Mannaerts, G.P. 1989. Subcellular distribution and characteristics of trihydroxycoprostanoyl-CoA synthetase in rat liver. *Biochem. J.* 257:221-229.

Schrader, M., Baumgart, E., Volkl, A., and Fahimi, H.D. 1994. Heterogeneity of peroxisomes in human hepatoblastoma cell line HepG2. Evidence of distinct subpopulations. *Eur. J. Biochem.* 64:281-294.

Schweizer, A., Matter, K., Ketcham, C.M., and Hauri, H.-P. 1991. The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and *cis*-Golgi. *J. Cell Biol.* 113:45-54.

Singh, I., Pahan, K., Dhaunsi, G.S., Lazo, O., and Ozand, P. 1993. Phytanic acid α-oxidation. Differential subcellular localization in rat and human tissues and its inhibition by Nycodenz. *J. Biol. Chem.* 268:9972-9979.

Thieringer, R., Shio, H., Han, Y., Cohen, G., and Lazarow, P.B. 1991. Peroxisomes in *Saccharomyces cerevisiae*: Immunofluorescence analysis and import of catalase A into isolated peroxisomes. *Mol. Cell. Biol.* 11:510-522.

van Roermund, C.W.T., van den Berg, M., and Wanders, R.J.A. 1995. Localization of peroxisomal 3-oxoacyl-CoA thiolase in particles of varied density in rat liver: Implications for peroxisome biogenesis. *Biochim. Biophys. Acta* 1245:348-358.

Van Veldhoven, P.P., Baumgart, E., and Mannaerts, G.P. 1996. Iodixanol (OptiPrep), an improved density gradient medium for the iso-osmotic isolation of rat liver peroxisomes. *Anal. Biochem.*. 237:17-23.

Völkl, A. and Fahimi, H.D. 1985. Isolation and characterization of peroxisomes from the liver of normal untreated rats. *Eur. J. Biochem.*. 149:257-265.

Wanders, R.J.A., van Roermund, C.W.T., de Vries, C.T., van den Bosch, H., Schrakamp, G., Tager, J.M., Schram, A.W., and Schutgens, R.B.H. 1986. Peroxisomal β-oxidation of palmitoyl-CoA in human liver homogenates and its deficiency in the cerebro-hepato-renal (Zellweger) syndrome. *Clin. Chim. Acta* 159:1-10.

Wanders, R.J.A., van Roermund, C.W.T., Griffioen, M., and Cohen, L. 1991. Peroxisomal enzyme activities in the human hepatoblastoma cell line HepG2 as compared to human liver. *Biochim. Biophys. Acta* 1115:54-62.

Watkins, P.A., Ferrell, E.V., Pedersen, J.I., and Hoefler, G. 1991. Peroxisomal fatty acid β-oxidation in HepG2 cells. *Arch. Biochem. Biophys.* 289:329-336.

Watkins, P.A., Lu, J.-F., Steinberg, S.J., Gould, S.J., Smith, K.D. and Braiterman, L.T. 1998. Disruption of the *Saccharomyces cerevisiae FAT1* gene decreases very long chain fatty acyl-CoA synthetase activity and elevates intracellular very long chain fatty acid concentrations. *J. Biol. Chem.* 273:18210-18219.

Wilcke, M. and Alexson, S.E.H. 1994. Characterization of acyl-CoA thioesterase activity in isolated rat liver peroxisomes. *Eur. J. Biochem.* 222:803-811.

Williams, C.H. and Kamin, H. 1962. Microsomal triphosphopyridinenucleotide-cytochrome *c* reductase of liver. *J. Biol. Chem.* 237:587-595.

Contributed by John M. Graham
Liverpool John Moores University
Liverpool, United Kingdom

Isolation of Lysosomes from Tissues and Cells by Differential and Density Gradient Centrifugation

Lysosomes are widely studied organelles; they are an integral part of the complex traffic of membrane vesicles between intracellular and surface membranes. Most notably, they are the site of degradation of macromolecules which have been brought into the cell by endocytosis. Most of the biochemical and structural analysis of lysosomes has been carried out on organelles from rat liver. A Percoll gradient method for this still widely-used tissue is described in this unit (see Basic Protocol 1). In the italicized annotations to this protocol, there are some comments regarding the application to other mammalian tissues (i.e., brain and kidney). More recently, the study of lysosomes as part of the endocytic and synthetic processes has been performed on organelles from a wide range of cultured cells. Because of the diversity of homogenization procedures and minor variations in the Percoll centrifugation regime used for cultured cells, it is not practical to present each cell type in a detailed protocol. A second procedure (see Basic Protocol 2) describes the isolation of lysosomes from HL-60 cells and the italicized annotations, a few of the many variants. The Commentary also presents some of the protocol variations used for cultured cells.

Since lysosomes, mitochondria, and peroxisomes have similar and overlapping densities in sucrose gradients, in the past, density perturbation of the lysosomes by Triton WR1339 loading was a prerequisite to achieving satisfactory purification in these gradients. With the advent of low-osmolarity Percoll and Nycodenz gradient media, the use of sucrose gradients declined and they are little used today, being generally regarded as unsatisfactory for lysosome purification. Nevertheless, to obtain the highest yields and purities of lysosomes, density perturbation can improve the resolution of Percoll gradients. This approach is presented in Alternate Protocol 1. Alternate Protocol 2 describes the use of a discontinuous gradient of Nycodenz for rat liver lysosomes. None of these systems are recommended for concomitant purification of the other major organelles in the light mitochondrial fraction (mitochondria and peroxisomes); this is best carried out by flotation of the light mitochondrial fraction through a continuous iodixanol gradient, described in Alternate Protocol 3.

After collecting the banded material from the gradients, the success of the fractionation is routinely measured by assaying for various marker enzymes; acid phosphatase (see Support Protocol 1) or a glycosidase such as β-galactosidase (*UNIT 3.4*) are commonly used for lysosomes. For peroxisomes, mitochondria, and endoplasmic reticulum, respectively, catalase, succinate dehydrogenase (*UNIT 3.4*), and NADPH-cytochrome *c* reductase (*UNIT 3.5*) may be used as markers.

IMPORTANT NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

ISOLATION OF LYSOSOMES FROM RAT LIVER USING A SELF-GENERATED PERCOLL GRADIENT

BASIC PROTOCOL 1

From a mammalian tissue homogenate, the lysosomes are always purified from a light mitochondrial fraction (LMF), this is also occasionally true of cultured cell homogenates. Routinely, lower relative centrifugal forces (RCFs) are required to produce the LMF for lysosome purification than for peroxisome purification (*UNIT 3.5*). A medium containing

Contributed by John M. Graham
Current Protocols in Cell Biology (2000) 3.6.1-3.6.21

buffered 0.25 M sucrose and 1 mM EDTA is routinely used both for the homogenization and as the osmotic balancer for the Percoll gradient.

The LMF is either mixed with a Percoll stock solution or layered on top of a uniform concentration of Percoll, normally in the range 25% to 40% (v/v), and most commonly centrifuged in a fixed-angle rotor for 30 to 90 min at 20,000 to 35,000 × *g*. A gradient is generated by the centrifugal field and the lysosomes are the densest organelle in this system. The normal mode of harvesting the gradient is either tube puncture (for thin-walled centrifuge tubes only) or aspiration from the bottom of the gradient. In a technique requiring practice and experience, the principal lysosome band can be removed using a syringe attached to a long metal cannula. See Commentary for more information on the manner in which the lysosomes are banded in these gradients. The protocol below is adapted from Jonas (1986) and Symons and Jonas (1987).

Percoll must normally be removed from gradient fractions before any spectrophotometric assay for marker enzymes can be carried out, because of the light-scattering properties of the medium. It is also advisable to remove the medium before carrying out any electrophoresis.

While isolation from liver is the focus of this protocol, the use of both brain (Caimi et al., 1989) and kidney (Ohshita et al., 1992) tissues are described in the annotations where appropriate.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: The following protocol is suitable for a single rat liver of wet weight 10 to 12 g.

Materials

150- to 200-g Sprague-Dawley rats
Homogenization medium (HM; see recipe)
Percoll stock solution (see recipe)

Dissecting equipment
~40- and ~5 ml Dounce homogenizers with loose-fitting Wheaton type B pestles (e.g., Kontes)
Low-speed centrifuge with swinging-bucket rotor to take 50-ml tubes
Sorvall RC series centrifuge and SE-12 and SS-34 rotors or equivalents
Gradient unloader: thin metal cannula connected to a peristaltic pump
Ultracentrifuge with fixed-angle rotor (e.g., Beckman 50 Ti or 70.1 Ti or Sorvall T-865.1)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all of the solutions.

Isolate and homogenize liver

1. Starve the animal overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.

   This must be supervised or carried out by an experienced animal technician.

3. Open the abdominal cavity and remove the liver to a chilled beaker containing ~20 ml of homogenization medium (HM).
4. Decant the medium and finely mince the liver using scissors.

   The pieces of liver should be no more than ~25 mm^3.

5. Suspend the mince in ~40 ml of HM and homogenize the liver using 15 to 20 strokes of the type B pestle of the 40-ml Dounce homogenizer.

   If only a smaller-volume Dounce homogenizer is available, carry out the homogenization in two batches. Brain tissue should be homogenized in 3 vol of HM containing a higher sucrose concentration (0.32 M) using a 50-ml Potter-Elvehjem homogenizer (0.2 mm clearance) with five gentle strokes at 500 rpm . For kidney tissue use 9 vol of HM (0.25 M sucrose) in a Potter-Elvehjem homogenizer.

Isolate light mitochondrial fraction

6. Centrifuge the homogenate 10 min at 750 × *g*, 0° to 4°C, in a low-speed centrifuge with a swinging-bucket rotor.
7. Decant and retain the supernatant.
8. Crudely resuspend the pellet in 20 ml of HM using a glass rod. Complete the resuspension using 3 to 4 gentle strokes of the type B pestle of the Dounce homogenizer
9. Repeat steps 6 and 7.
10. Combine the supernatants and transfer to ~50-ml high-speed centrifuge tubes.

    This is the post-nuclear supernatant (PNS).

11. Centrifuge 10 min at 20,000 × g in Sorvall RC series centrifuge and SS-34 or equivalent fixed-angle rotor, 0° to 4°C.
12. Decant and discard the supernatant
13. Suspend the light mitochondrial pellet (LMP) in ~40 ml HM using a glass rod followed by 3 to 4 gentle strokes of the pestle of the Dounce homogenizer.
14. Centrifuge 10 min at 20,000 × *g*, 4°C, and discard the supernatant.
15. Resuspend the LMP in ~6 ml HM using a glass rod. Complete resuspension with 3 to 4 gentle strokes of the pestle of the 5 ml Dounce homogenizer.

    The homogenate (light mitochondrial fraction; LMF) should contain ~25 mg/ml protein.

Purify lysosomes

16. Mix 4.5 ml of Percoll stock solution with 5.5 ml LMF from step 15.

    This is equivalent to ~40% (v/v) Percoll.

17. Transfer 10 ml of the Percoll/LMF mixture to each high-speed centrifuge tube and centrifuge 90 min at 35,000 × *g*, 0° to 4°C, in a Sorvall SE-12 or equivalent fixed-angle rotor.

    The gradient can also be generated in an ultracentrifuge fixed-angle rotor. Such a rotor is required to remove the Percoll particles from the gradient fractions (see step 19).

    Because the gradient reorients in the tube during deceleration from 2,000 rpm to rest, use a slow deceleration during the braking phase so deceleration occurs smoothly and disturbance of the gradient and the banded material is avoided.

    For brain lysosomes, layer 4 ml of LMF over 36 ml of 27% (v/v) Percoll and centrifuge 90 min at 20,000 × g. Kidney lysosomes: mix LMF with Percoll stock to 40% (v/v) Percoll and centrifuge 30 min at 47,000 × g.

18. Harvest the gradient (preferably dense-end-first) in ~1 ml fractions from the top using a gradient unloader; the lysosomes band in the bottom 1 to 2 ml.

    Alternatively, a syringe (1 to 2 ml) with metal cannula (i.d. 1 mm) can be used to collect equal-volume fractions from the top or bottom of the gradient. Thin-walled tubes can be unloaded by tube puncture.

19. Ultracentrifuge the fractions 1 hr at 100,000 × *g*, 0° to 4°C, and collect the turbid layer of lysosomes just above the Percoll pellet.

20. Perform metabolic assays for lysosomes (see Support Protocols 1 and 2), peroxisomes (*UNIT 3.4*), mitochondria (*UNIT 3.4*), or endoplasmic reticulum (*UNIT 3.5*) as soon as possible upon preparation.

    If necessary dilute the lysosomes to 1 to 5 mg protein/ml with HM.

    Some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20°C or −80°C; however, always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained from fresh material.

ALTERNATE PROTOCOL 1

ISOLATION OF LYSOSOMES FROM RAT LIVER USING DENSITY PERTURBATION AND A SELF-GENERATED PERCOLL GRADIENT

After intraperitoneal injection of the carbohydrate polymer dextran, the macromolecule is endocytosed by the liver and delivered to the lysosomes, thereby increasing their density. On the other hand, if the post-nuclear supernatant of a liver homogenate is made 1 mM with respect to Ca^{2+}, the mitochondria take up water and become considerably less dense. This bimodal density perturbation approach was devised by Arai et al. (1991) to improve the yield and resolution of lysosomes from mitochondria in Percoll gradients. These dextran-perturbed lysosomes are sometimes called dextranosomes. The following method is adapted from Arai et al. (1991).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Additional Materials *(also see Basic Protocol 1)*

100 mg/ml dextran solution (see recipe)
Homogenization medium (HM; see recipe) without EDTA
100 mM $CaCl_2$ (see recipe)
Percoll stock solution (see recipe) without EDTA

2-ml syringe and 23-G needle
Beckman 70.1 Ti rotor

1. Inject rats intraperitoneally with 1 mg dextran per gram of body weight using a 2-ml syringe and 23-G needle.

   This must be carried out by a properly trained and licensed operative.

2. Sacrifice the animal by decapitation or cervical dislocation.
3. Blanch the liver by perfusion from the portal vein with 20 to 30 ml ice-cold HM.
4. Prepare the PNS (see Basic Protocol 1, steps 3 to 10).
5. Measure the volume of PNS; add 0.01 vol of 100 mM $CaCl_2$, and incubate 5 min at 37°C.
6. Cool the suspension back to 0° to 4°C and prepare the LMF (see Basic Protocol 1, steps 11 to 15).

7. Mix 5.5 ml of Percoll stock solution with 4.5 ml of the LMF and ultracentrifuge 15 min at 60,000 × g in a 70.1 Ti or equivalent fixed-angle rotor, 0° to 4°C.

   This is equivalent to ~50% (v/v) Percoll.

8. Collect the gradient and process the fractions as described (see Basic Protocol 1, steps 18 to 20).

   The dextranosomes band close to the bottom of the gradient.

ISOLATION OF LYSOSOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A DISCONTINUOUS GRADIENT OF NYCODENZ

ALTERNATE PROTOCOL 2

The discontinuous metrizamide gradient devised by Wattiaux and Wattiaux-De Coninck (1983) has been superseded by a Nycodenz gradient covering the same density range (Olsson et al., 1989). The light mitochondrial fraction is adjusted to a high density and layered under the gradient; bottom-loading has been shown (Wattiaux and Wattiaux-De Coninck, 1983) to be preferable for isolation of lysosomes in this system.

As Nycodenz is a true solute, the gradient fractions do not require centrifugation at 100,000 × *g* (as for Percoll) to remove the gradient medium; if necessary the organelles can be pelleted 15 min at 20,000 × *g* after dilution of the fractions with 2 vol of HM.

Additional Materials *(also see Basic Protocol 1)*

45% Nycodenz stock solution (see recipe)
Ultracentrifuge with swinging-bucket rotor to take ~38-ml tubes (e.g., Beckman SW28, Sorvall AH629, or equivalent)
Gradient unloader: 1- to 2-ml syringe attached to metal cannula (i.d. 1 mm)

1. Prepare LMF as described (see Basic Protocol 1, steps 1 to 15). To keep the sample volume to a minimum, resuspend the LMP in 3 to 4 ml of HM.
2. To 1 vol of LMF add 2 vol 45% Nycodenz stock solution.
3. Prepare 19%, 24%, 26%, and 30% Nycodenz by dilution of 45% Nycodenz stock solution in HM.
4. Transfer 8 ml of 19% Nycodenz to a tube for the SW-28 rotor (or equivalent), and, using a syringe and metal cannula, underlayer 7 ml each of the other three gradient solutions in order of increasing density. Underlayer 9 ml of LMF in the bottom of the tube.
5. Ultracentrifuge 2 hr at 95,000 × *g*, 0° to 4°C.
6. Harvest the lysosomes from the 19%/24% interface using a syringe and metal cannula.
7. If required, dilute the fraction with ≥2 vol of HM and centrifuge 20 min at 30,000 × g, 0° to 4°C.
8. Resuspend the pellet in HM to 1 to 5 mg protein/ml.
9. Process the fractions as described (see Basic Protocol 1, step 20).

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20°C or −80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained with fresh material.

ALTERNATE PROTOCOL 3

FRACTIONATION OF A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A CONTINUOUS IODIXANOL GRADIENT

Occasionally it is necessary to at least partially purify the other organelles in the light mitochondrial fraction (as well as the lysosomes), as, for example, when the localization of a particular component or function is being determined (i.e., a gradient is being used analytically rather than preparatively). In this case, none of the gradients described in Basic Protocol 1 or Alternate Protocols 1 and 2 are really suited to this task, because mitochondria, peroxisomes, and endoplasmic reticulum tend to copurify (particularly in Percoll gradients). The continuous iodixanol gradient described in this protocol overcomes this problem to a large extent. Bottom-loading of the sample in a dense medium is described, but top loading can be used.

Additional Materials *(also see Basic Protocol 1)*

OptiPrep diluent (see recipe)
OptiPrep (Accurate Chemical)
Dense gradient unloading liquid (e.g., Maxidens; Lipotek, U.K.)
Two-chamber gradient maker or Gradient Master

Gradient unloader
Ultracentrifuge with Beckman SW28.1 or equivalent swinging bucket rotor

1. Prepare the LMF (see Basic Protocol 1, steps 1 to 15), but suspend the final pellet in 3.5 ml HM.
2. Prepare 50% (w/v) iodixanol stock by combining 5 vol of OptiPrep (60% iodixanol) and 1 vol OptiPrep diluent. Mix 3.0 ml of LMF with 4.5 ml of 50% iodixanol stock by repeated inversion.

   NOTE: *Do not shake the tube so vigorously as to create foaming.*

   If the sample is going to be loaded on top of the gradient rather than beneath it, then omit this step.
3. Prepare a linear iodixanol gradient in the 17-ml centrifuge tubes from 6 ml each of 19% and 27% (w/v) iodixanol.

   If the gradient is prepared with a Gradient Master use an angle of 80°, 20 rpm, and a time of 1 min 50 sec.
4. Underlayer the gradient with 3 ml of the LMF/iodixanol from step 2, using a syringe and metal cannula.
5. Overlayer the gradient with 1 ml of HM.

   Alternatively underlayer the gradient with 1 ml of 30% iodixanol (3 vol iodixanol stock and 2 vol HM) and then layer 3 ml of LMF on top of the gradient.
6. Ultracentrifuge 2 hr at 110,000 × *g*, 0° to 4°C. Collect the gradient in 1-ml fractions, low-density end first by upward displacement with a dense liquid (e.g., Maxidens).

   The lysosomes band close to the top of the gradient.
7. If required, dilute the fraction with 2 vol of HM and centrifuge 20 min at 30,000 × *g*, 0° to 4°C.
8. Resuspend the pellet in HM to 1 to 5 mg protein/ml.
9. Process the fractions as described (see Basic Protocol 1, step 20).

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to −20° or −80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained with fresh material.

ISOLATION OF LYSOSOMES FROM HUMAN HL-60 CULTURED CELLS USING A SELF-GENERATED PERCOLL GRADIENT

This is an example of the use of a post-nuclear supernatant from a cultured cell homogenate. While similar homogenization media and centrifugation and analysis protocols, with minor modifications, have been applied to the isolation of lysosomes from a variety of cultured cells, the form of homogenization that has been used is extremely variable. The protocol below has been adapted from the method of Carlsson and Fukuda (1992), as these authors used the ball-bearing homogenizer of Balch and Rothman (1985), which causes very little damage to subcellular organelles. The homogenization procedures used for cultured cells are too diverse to be covered comprehensively in this text (see Graham, 1997 for more information), but a few alternatives are suggested in the italicized annotations and discussed more fully in the Commentary. Whatever homogenization method is used, the aim should be to produce ~90% cell breakage using the minimum shearing forces.

Materials

HL-60 cells (ATCC#CCL-240) in homogenization medium
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Homogenization medium (HM; see recipe)
10% (w/v) bovine serum albumin (BSA)
Percoll stock solution
10% (w/v) Nonidet-40 (NP-40) in water

Ball-bearing cell homogenizer (see Balch and Rothman, 1985, for more details)
Low-speed centrifuge with swinging-bucket rotor to take 10-ml tubes
Sorvall high-speed centrifuge with SE-12 and SS-34 or equivalent fixed-angle rotors
Beckman ultracentrifuge with 50Ti, 70Ti, T865 (Sorvall), or equivalent fixed-angle rotor
Gradient unloader: thin metal cannula connected to a peristaltic pump

NOTE: An ultracentrifuge rotor is required to pellet Percoll (step 12), but gradient formation (step 10) can be carried out in a high-speed centrifuge or ultracentrifuge.

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all of the solutions.

1. Wash 1-3 × 10^8 HL-60 cells once with 5 ml phosphate buffered saline (PBS), and again with 5 ml of HM, pelleting cells by centrifugation at 500 × *g* for 10 min.

   In some methods the EDTA is omitted from the homogenization medium (and the Percoll diluent). The inclusion of EDTA is dependent on the subsequent procedures to be done on purified lysosomes, and how well cells homogenize (some cells will not homogenize without EDTA). An alternative medium in which 10 mM triethanolamine-acetic acid, pH 7.4 replaces the HEPES buffer has been found to be suitable to many monolayer cultures (Marsh et al., 1987).

2. Suspend the cells in 3 ml of HM.

3. Homogenize in the ball-bearing homogenizer using 5 passes.

   See Balch and Rothman (1985), for more details on the use of ball-bearing homogenizers. A Dounce (Finley and Kornfeld, 1994; Lipman et al., 1990; Muno et al., 1990) or Potter-Elvehjem homogenizer (Akasaki et al., 1993), or nitrogen cavitation (Kelly et al., 1989) may be used if the ball-bearing homogenizer is not available.

4. Rinse the homogenizer with 0.5 ml of HM and add to the homogenate.

5. Pellet the nuclei, cell debris and any unbroken cells by centrifuging at 800 × g for 10 min in a low-speed centrifuge.
6. Decant or aspirate the supernatant and keep on ice.
7. Suspend the nuclear pellet in 1 ml of HM (use gentle vortexing). Repeat steps 5 and 6.
8. Combine the supernatants, adding adding HM to a final total volume of 3.5 ml if necessary.
9. Mix 3 ml of supernatant with 0.18 ml 10% bovine serum albumin (BSA), 1.0 ml of Percoll stock solution, and HM to a final volume of 4.5 ml.

   The final Percoll concentration is 20%.

   In some methods the post-nuclear fraction is layered over the Percoll solution and other Percoll concentrations that have been used are ~27% and 35% (v/v).
10. Ultracentrifuge 30 min at 36,000 × *g* in a Beckman 50 Ti rotor or equivalent fixed-angle rotor, 0° to 4°C.

    Centrifugation conditions vary from 60 min at 15,000 × g to 40 min at 62,500 × g and the gradient volume may be as high as 35 ml.

    The density profile required to achieve high resolution will depend on cell type and HM composition. Centrifugation conditions required to produce this profile will depend on rotor type.
11. Collect the gradient dense-end-first (0.4-ml factions) using a gradient unloader.

    The lysosomes band close to the bottom of the gradient.

    A thin metal cannula connected to a peristaltic pump can be used as a gradient unloader.
12. Add 10% Nonidet-40 (NP-40) to each gradient fraction (final concentration 0.5%) before pelleting the Percoll by ultracentrifuging 1 to 2 hr at 100,000 × *g*, 0° to 4°C.

    By adding a nonionic detergent to solubilize the organelles before the Percoll is pelleted, very high recoveries of lysosomal proteins may be obtained. If the aim, however, is to recover intact organelles, the detergent must be omitted.
13. Perform metabolic assays for lysosomes (see Support Protocols 1 and 2), peroxisomes (*UNIT 3.4*), mitochondria (*UNIT 3.4*), or endoplasmic reticulum (*UNIT 3.5*) as soon as possible upon preparation.

    Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to –20° or –80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained with fresh material.

SUPPORT PROTOCOL 1

ASSAY FOR ACID PHOSPHATASE

Acid phosphatase is an established marker for lysosomes. The assay is simple to execute and uses *p*-nitrophenyl phosphate as a substrate. The released nitrophenol is measured spectrophotometrically in an alkaline solution. *p*-Nitrophenyl phosphate is quite stable under alkaline conditions, thus if the reaction is terminated by addition of NaOH, background hydrolysis is negligible. Because of the ubiquity of nonspecific phosphatases, glycosidases such as β-galactosidase (e.g., Graham, 1997; *UNIT 3.4*) or β-*N*-acetylglucosaminidase (e.g., Berg et al., 1985; Support Protocol 2) are increasingly used today as markers.

Sample handling depends on the type of gradient and the concentration of lysosomes (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed and the gradient fractions suspended in 0.25 M sucrose, 10 mM HEPES/NaOH, pH 7.4 or equivalent.

Materials

Assay buffer I (see recipe)
Substrate solution I (see recipe)
Gradient fractions (see Basic Protocol 1 or 2 or Alternate Protocol 1 or 2)
0.25 M NaOH

1. Prepare assay solution by mixing equal volumes of assay buffer I and substrate solution I. Add 50 µl of each gradient fraction (containing ~20 µg protein) to separate microcentrifuge tubes containing 0.2 ml of assay solution. Perform the assay in duplicate.
2. Prepare a blank for each fraction by adding 0.2 ml of assay solution to 0.6 ml of 0.25 M NaOH before adding 50 µl gradient fraction.

   As long as the protein content of each assay is approximately the same, a blank for each fraction may not be necessary; a single blank containing buffer instead of sample may suffice for many gradients. This simple approach cannot be adopted for Percoll-containing fractions.
3. Incubate assay tubes at 37°C for 30 min.
4. Add 0.6 ml of 0.25 M NaOH to each test sample to stop the reaction.
5. Remove any precipitate by microcentrifuging 1 to 2 min at maximum speed.
6. Using plastic cuvettes, measure the absorbance of the assay reactions at 410 nm against the appropriate blank.

   The molar extinction coefficient of nitrophenol is 9620 $cm^{-1}M^{-1}$.

ASSAY FOR β-*N*-ACETYLGLUCOSAMINIDASE

SUPPORT PROTOCOL 2

This sensitive fluorometric method is based on the release of 4-methylumbelliferone from 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide at an acid pH followed by determination of the fluorescence of the aglycone at pH 10 or 11. It may be necessary to remove protein by precipitation with trichloroacetic acid before measuring the fluorescence. A less sensitive spectrophotometric method, which uses the *p*-nitrophenyl derivative, is also available (see β-galactosidase assay in *UNIT 3.4*).

Sample handling depends on the type of gradient and the concentration of lysosomes (and other organelles) in the fraction (see Commentary). Ideally, the protocols should be carried out on fractions from which the gradient solute or Percoll has been removed; the gradient fractions are resuspended in 0.25 M sucrose, 10 mM HEPES/NaOH, pH 7.4, or equivalent.

This method is adapted from Barrett and Heath (1977).

Materials

Assay buffer II (see recipe)
Substrate solution II (see recipe)
Bicarbonate buffer (see recipe)
1.2 mM (21 mg/100 ml) 4-methylumbelliferone, prepare fresh
Fluorimeter and appropriate cuvettes

1. Mix 0.5 ml each of assay buffer II and substrate solution II with 0.45 ml water.
2. Add 50 µl sample.

   It is strongly advised that some preliminary experimentation be carried out to determine the optimum range of protein concentrations that will provide a satisfactory fluorescent emission, detectable by the available instrument and falling on the standard curve (see step 5).

3. Prepare a blank for each fraction by repeating step 1, adding 3 ml bicarbonate buffer, and then adding 50 µl of sample.

   Unless the samples are clarified by the addition of TCA and sedimentation prior to fluorescent measurement (step 6), light scattering in the sample may require a separate blank for each sample. However, if the protein content of each clarified assay reaction is approximately the same, a blank for each fraction may be avoided, in which case a single blank containing 0.5 ml assay buffer, substrate solution, and water (and no sample) may suffice.

4. Incubate 30 to 60 min at 37°C.
5. Prepare a standard curve by diluting 1.2 mM 4-methylumbelliferone with water to make 1 ml standards from 1.2 to 12 nmol and adding 0.5 ml assay buffer II to each.
6. Add 3 ml bicarbonate buffer to all sample and standard reactions (not blanks). Measure the fluorescence emission at 448 nm using an excitation wavelength of 360 nm.

   If it is necessary to clarify the samples, add 1.5 ml of 3.3% (w/v) TCA to each tube. Let stand 5 min, then centrifuge 2 min in a microcentrifuge, to remove precipitate. To 2 ml of supernatant add 2 ml bicarbonate buffer and measure fluorescence.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Assay buffer I

To 100 ml H_2O add:
2.94 g sodium acetate (180 mM final)
Adjust to pH 5.0 with glacial acetic acid
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Assay buffer II

To 100 ml H_2O add:
11.52 g citric acid (0.3 M final)
17.64 g trisodium citrate dihydrate (0.3 M final)
3.5 g NaCl (0.3 M final)
H_2O to 200 ml
Store 1 to 2 days at 4°C

Bicarbonate buffer

To 250 ml H_2O add:
26.5 sodium carbonate (0.5 M final)
21 g sodium bicarbonate (0.5 M final)
H_2O to 500 ml
The pH should be ~9.9
Store 1 to 2 weeks at 4°C

$CaCl_2$, 100 mM

To 50 ml H_2O add:
1.47 g $CaCl_2 \cdot 2H_2O$
Add H_2O to 100 ml
Store up to 3 months at 4°C

Dextran solution, 100 mg/ml

To 50 ml H_2O add:
0.85 g NaCl
10 g dextran (M_r = 60,000 to 90,000)
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

HEPES, 1 M and 100 mM

23.83 g N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)
Adjust volume to 100 ml with H_2O for 1 M and to 1000 ml for 100 mM HEPES.

Homogenization medium (HM)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
2 ml 100 mM Na_2EDTA (1 mM final)
20 ml 100 mM HEPES (10 mM final; see recipe)
Adjust to pH 7.0 with NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Nycodenz stock solution, 45% (w/v)

To 100 ml H_2O add:
90 g Nycodenz (Accurate Chemicals)
2 ml 100 mM Na_2EDTA (1 mM final)
20 ml 100 mM HEPES (10 mM final; see recipe)
Adjust to pH 7.4 with NaOH
Add H_2O to 200 ml
Store up to 3 months at −20°C

OptiPrep diluent

To 50 ml H_2O add:
8.55 g sucrose (0.25 M final)
6 ml 100 mM Na_2EDTA (6 mM final)
12 ml 1 M HEPES (120 mM final; see recipe)
Adjust to pH 7.4 with NaOH
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Percoll diluent

To 75 ml H_2O add:
171 g sucrose (2.5 M final)
20 ml 100 mM Na_2EDTA (10 mM final)
20 ml 1 M HEPES (100 mM final)
Adjust to pH 7.0 with NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

For protocols where EDTA is omitted, replace with 20 ml water.

Percoll stock solution, 90% (v/v)

Combine 9 vol Percoll and 1 vol Percoll diluent (with or without EDTA depending on protocol). Store up to 1 to 2 days at 4°C.

Substrate solution I

To 100 ml H_2O add:
0.59 g *p*-nitrophenyl phosphate hexahydrate
Store up to 3 months at −20°C

Substrate solution II

To 100 ml H_2O add:
56.8 mg 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (1.5 mM final)
Prepare fresh

COMMENTARY

Background Information

Homogenization and differential centrifugation

For all mammalian tissues, a routine homogenization medium containing 0.25 M sucrose and 1 mM EDTA, usually buffered with HEPES, is used with no special supplements, although for brain tissue the sucrose concentration is invariably increased to 0.32 M (Caimi et al., 1989). This medium has also commonly been used for cultured cells, although sometimes the EDTA is omitted. Dounce homogenization of the cells is widely used, although nitrogen cavitation of human skin fibroblasts was used by Kelly et al. (1989) and a ball-bearing homogenizer (Balch and Rothman, 1985) by Carlsson and Fukuda (1992) for HL-60 cells. As with peroxisomes (*UNIT 3.5*), the gentlest possible homogenization protocol should be adopted, to avoid any potential damage to the organelles.

With tissues such as rat liver, the first stage of the lysosome purification is always the isolation of a light mitochondrial fraction (LMF), which is then further fractionated on some form of density gradient. This is also true of other tissues such as brain (Caimi et al., 1989; Ohshita and Kido, 1995) and kidney (Ohshita et al., 1992). In the case of cultured cells, a postnuclear supernatant (PNS) is often used as the source of crude lysosomes for gradient centrifugation. This was also used by Oliver et al. (1989) for rat parotid acinar cells. If a LMF is used, then it is normally sedimented from the PNS by centrifuging 10 to 15 min at 18,000 to 20,000 × *g*; slightly lower than that used for the purification of peroxisomes (*UNIT 3.5*) which is routinely 25,000 × *g* for 10 to 15 min. The reason for this may be that the smallest peroxisomes are ~0.2 μm, while the smallest lysosomes are ~0.4 μm. There are also a few examples of the use of much lower RCFs: 20 min at 12,500 × g for chicken liver (Nakabayashi and Ikezawa, 1988) and 20 min at 11,000 × g for macrophages (Muno et al., 1990). Since lysosomes contain many degradative enzymes, resuspension of any differential centrifugation pellets must be carried out as gently as possible; indeed the lack of any pellet resuspension is a clear advantage of using a PNS.

Density gradients

Sucrose gradients. Because the density of lysosomes is very similar to that of mitochondria and peroxisomes in sucrose gradients (Table 3.5.1), that method is now regarded as unsatisfactory for lysosome purification. Sucrose gradients can only achieve a partial resolution of lysosomes from other organelles, unless their density is reduced by loading them with Triton WR1339 (Leighton et al., 1968). This involves injection of the experimental animal with the detergent. It is now known that the lysosomes (or tritosomes) that are produced behave abnormally, particularly in regard to their enzyme composition (Wattiaux and Wattiaux-De Coninck, 1983). Therefore, it is now considered more acceptable to use one of the low-osmolarity media to isolate these organelles, because in such media the density of lysosomes is sufficiently different from that of the other organelles (see Table 3.5.1) to permit easy isolation. In iodinated density gradient media such as Nycodenz or iodixanol, lysosomes are less dense than either mitochondria or peroxisomes, while in Percoll they tend to be more dense; however, the reason for this is not clear. Because Percoll can be used as a self-generated gradient at relatively low RCFs (20,000 to 60,000 × *g* is common) and because

a band of lysosomes is invariably formed towards the bottom of the gradient with relatively little contamination from other organelles, the use of this medium is very popular.

Percoll gradients. In the handling of Percoll gradients, the osmolarity of the commercial medium (which is close to zero) has to be adjusted to ~290 mOsm by the addition of 0.1 vol of a solution containing 2.5 M sucrose. Normally this osmotic balancer is simply a 10× solution of the homogenization medium. In this way, the organelles are always exposed to the same isoosmotic medium. Usually, the final 90% (v/v) Percoll stock solution contains 0.25 M sucrose, 10 to 20 mM HEPES/NaOH, pH 7.0 to 7.4, with or without 1 mM EDTA (see recipe in Reagents and Solutions); however, as an alternative, 0.24 M mannitol, 10 mM MOPS/NaOH, pH 7.0 (Pisoni et al., 1987), and 1 mM EDTA, can be used instead.

As with all self-generated gradients, the centrifugation conditions which are used to produce the Percoll gradient control its profile. That is, lower RCFs and shorter centrifugation times will tend to produce sigmoidal gradients which are very shallow in the middle, while higher RCFs and times cause the gradient to become more linear (although a truly linear gradient is difficult to achieve with Percoll). Usually a fixed-angle rotor is used to create the gradient, but there are examples which use a vertical rotor (Kelly et al., 1989; Oliver et al., 1989). Oliver et al. (1989) included a cushion of 2.5 M sucrose, presumably to prevent any silica particles pelleting on to the vertical wall of the tube. RCFs normally vary from 20,000 to 65,000 × *g* and times from 30 to 90 min, although some methods fall outside these ranges. For lower RCFs, it is possible to use a high-speed centrifuge. The starting concentration of the Percoll, normally 20 to 40% (v/v), affects the density range of the gradient. The lower the starting concentration, the nearer the bottom of the tube the lysosomes will band. Some of the conditions for lysosome separation from cultured cells are summarized in Table 3.6.1.

The PNS or LMF is either simply mixed with the Percoll stock, or layered over a Percoll solution prepared from the Percoll stock and (usually) HM. Overlayering will minimize any contamination of the lysosomes by soluble proteins, which under the given centrifugation conditions will remain at the top of the gradient.

There are relatively few instances of Percoll being used in a non-self-generated gradient mode. Examples include PNS from guinea-pig alveolar cells fractionated on a preformed 0% to 50% Percoll gradient, 1 hr at 19,000 × *g* (12,000 rpm) in a Beckman SW-28 swinging-bucket rotor (Yamaguchi and Kaneda, 1988), and an 8% Percoll barrier used for pelleting the lysosomes from a K562 erythroleukemia cell PNS, 20 min at 19,000 × g (Bridges, 1987).

Nycodenz and iodixanol gradients. The discontinuous Nycodenz gradient (Olsson et al., 1989), based on the metrizamide system developed by Wattiaux and Wattiaux-De Coninck (1983), provides a useful alternative to Percoll. Nycodenz was also used by Graham et al. (1990) for isolation of organelles. Lack of a simple self-generated gradient protocol, however, is a disadvantage. Although iodixanol, which is a dimer of Nycodenz, forms self-generated gradients rather more quickly than Nycodenz and can be used to fractionate the organelles from a LMF (Graham et al., 1994), the RCFs required are in excess of 150,000 × *g*, with centrifugation times of 1 to 3 hr (depending on the RCF and the rotor). However, if it is a requirement that the separation system should adequately resolve both the mitochondria and peroxisomes, as well as the lysosomes, then a continuous iodixanol gradient can achieve this aim more effectively than Percoll.

Table 3.6.1 Percoll Centrifugation Conditions for Selected Cultured Cells

Cell type	% Percoll (v/v)	Volume (ml)	RCF/time	Reference
Human fibroblasts	27	35	35,000 × *g*/60 min	Kelly et al., 1989
	34	10	17,500 × *g*/35 min	Pisoni et al., 1987
Macrophages	35	7.5	62,500 × *g*/40 min	Muno et al., 1990
	27	30	15,000 × *g*/60 min	Lipman et al., 1990
Monkey COS	36	12	35,000 × *g*/90 min	Finley and Kornfeld, 1994
Rat hepatocytes	27	10	25,000 × *g*/40 min	Akasaki et al., 1993

Critical Parameters and Troubleshooting

Homogenization

As with all membrane fractionations, the use of cultured cells, rather than intact tissues, may provide problems in devising a suitable homogenization method. Although most monolayer cultured cells seem to homogenize reasonably effectively in the widely-used sucrose/EDTA/HEPES medium, the homogenization medium developed by Marsh et al. (1987) of 0.25 M sucrose, 1 mM EDTA, and 10 mM triethanolamine/10 mM acetic acid, pH 7.4, is more widely applicable (the triethanolamine-acetic acid buffer being the critically important component). This was used for the preparation of lysosomes from human fibroblasts (Pisoni et al., 1987), while for the J774 mouse macrophage-like cells, Lipman et al. (1990) used 0.25 M sucrose, 1 mM EGTA, 1 mM Mg^{2+}, 1 mM dithiothreitol, and 20 mM HEPES/NaOH, pH 7.4. If, upon homogenization, the cell homogenate acquires a gelatinous quality, this may be due either to nuclear breakage or to the release of proteins from cytoskeletal elements. Inclusion of 1 mM Mg^{2+} with or without DNase I (μg/ml) and/or 10 mM KCl may ameliorate the problems, although these measures should be regarded as a last resort.

Although a Dounce homogenizer is often used to break cultured cells, it is strongly recommended that the optimal conditions for disrupting the cells be thoroughly investigated. The aim must always be to use the minimum force to achieve ~90% cell breakage; therefore, protocols which use more than 20 strokes of the pestle should be avoided.

For cells that resist the liquid shear forces either of the Balch and Rothman (1985) ball-bearing homogenizer or of Dounce or Potter-Elvehjem homogenizers, nitrogen cavitation is probably the best option, as an isoosmotic sucrose medium can always be used. Standard conditions are 300 to 600 psi for 10 to 15 min, although Kelly et al. (1989) reported the use of much lower pressures. For a more complete discussion of these problems see Evans (1992) and Graham (1997).

Differential centrifugation

The aim of the homogenization should be to devise conditions such that 90% of cells are disrupted with the minimum possible release of organelle markers into the medium. To determine organelle integrity, all of the differential centrifugation fractions, including the microsomes and the cytosol (45 min, 100,000 × *g* pellet and supernatant) should be assayed for organelle markers and proteins (see Anticipated Results). For a tissue or cell other than those described in this unit, always carry out a more thorough differential centrifugation (e.g., 10 min at 600 × *g*, 10 min at 3000 × *g*, 10 min at 15,000 × *g*, 10 min at 25,000 × g, and 10 min at 40,000 × g) in order to devise the optimal protocol.

Density gradient centrifugation

For a tissue or cell other than those described in this unit, it may also be necessary to modulate some of the gradient and/or centrifugation conditions in the light of experience, for example, making the gradient more or less dense at the bottom to take account of organelles which may be correspondingly more or less dense. Modifications will also need to be made if the type of rotor described in this unit (see Basic Protocol 1) is not available. The sedimentation path length of the rotor will influence the shape of the density gradient profile—the longer the path length, the more shallow the gradient will be in the middle, and the steeper it will be at both ends (under the same centrifugation conditions). With Percoll, the density profile of the gradient can be checked by using colored density marker beads, which are available from Amersham-Pharmacia Biotech.

The preparation of preformed continuous gradients of iodixanol using a two-chamber gradient maker requires considerable practice to achieve highly reproducible gradients. Some of the important points in operating such devices are covered by Dobrota and Hinton (1992). A Gradient Master, which involves simply layering the densest and lightest solutions in the centrifuge tube, provides a more expensive but reproducible alternative. If neither of these is available, then a discontinuous gradient constructed from three or four solutions spanning the required density range can be prepared and allowed to diffuse, usually overnight at 4°C. Note that the time required to achieve complete linearity will depend on the tube diameter and the volume of each layer. Always collect a blank gradient and check (using refractive index) that the gradient is indeed linear and covers the appropriate density range.

Enzyme and other assays

Prior to the execution of an enzyme assay (or other measurement), it is common practice to remove Percoll, Nycodenz, or iodixanol from each gradient fraction. In the case of

Percoll, the silica colloid is normally pelleted from the gradient fraction at 100,000 × *g*, while the other two media are removed in the supernatant after dilution of the fraction with 2 to 4 vol of a low-density medium and centrifugation of the organelles 15 to 20 min at ~20,000 × *g*. These practices eliminate any possible interference of the gradient medium with the subsequent procedure(s) and are described in the final steps of the appropriate protocols. Osmundsen (1982) warned, however, that at least with peroxisomes, organelles may be lost into the Percoll pellet and that this loss may be as much as 50% of the total.

If the sample volumes used in an enzyme assay are sufficiently small (see Supports Protocol 1 and 2), the gradient solute may be adequately diluted so as not to cause any interference in the assay, even at the highest density. Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without removal. If, however, either of these requirements are not met, then dilution, pelleting, and resuspension of the pellet in a smaller volume are unavoidable. If Percoll is not removed from the gradient fraction, a blank containing the sample is essential.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Percoll, Nycodenz, and iodixanol interfere with methods using the Folin-Ciocalteu reagent, although, if by using small volumes of Nycodenz or iodixanol-containing fractions (<100 µl) in a 1 to 2 ml assay volume, the concentration of gradient solute in the assay solution is ≤10%, the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of these gradient media, while Percoll-containing fractions may be used so long an appropriate Percoll blank is used.

Anticipated Results

Basic Protocol 1

Differential centrifugation. Table 3.6.2 provides an analysis of the enzyme content of three principal differential centrifugation fractions. In particular it should be noted that values for acid phosphatase in the 20,000 × *g* supernatant, in excess of those in Table 3.6.2 might indicate that there is considerable lysosomal breakage during the homogenization. There will be some variation depending on the type of tissue or cell, and it is interesting that for brain tissue Caimi et al. (1989) reported only 4% of the acid phosphatase in a 17,500 × *g* supernatant. The authors used a very mild homogenization method using only 5 very slow strokes of the pestle of a Potter-Elvehjem homogenizer.

Density gradient. In Percoll in particular, the density of the lysosomes (Table 3.5.1) reveals a considerable heterogeneity, with the result that those lysosomes banding at a lower density in Percoll gradients overlap other organelles and are not recoverable in anything approaching a pure form. The profile of the lysosomal enzyme markers in the gradient, while always demonstrating this broad range of density banding, varies with the gradient-forming conditions and the enzyme. In the gradient system described in this protocol (Symons and Jonas, 1987) the β-hexosaminidase of rat liver lysosomes shows only one clear band in the bottom 2 ml of the gradient, while the enzyme is broadly distributed at a low level in the top 8 ml of the gradient; nevertheless, this represents ~40% of the total enzyme in the gradient (Fig. 3.6.1).

In the system of Nakabayashi and Ikezawa (1988), on the other hand, the lysosomal enzymes from chicken liver showed a very pronounced biphasic distribution (Fig. 3.6.2); moreover *N*-acetyl-β-D-glucosaminidase was concentrated in the dense band, while β-D-

Table 3.6.2 Enzyme Content in Differential Centrifugation Fractions

Enzyme/protein	Enzyme content (% of total in homogenate)		
	750 × *g* pellet	20,000 × *g* pellet	20,000 × *g* supernatant
Acid phosphatase	20-25	30-35	35-40
Catalase	20-25	35-40	25-30
NADPH-cyt *c* reductase	15-20	5-10	70-80
Protein	25-30	15-20	55-60
Succinate dehydrogenase	40-50	40-50	5-10

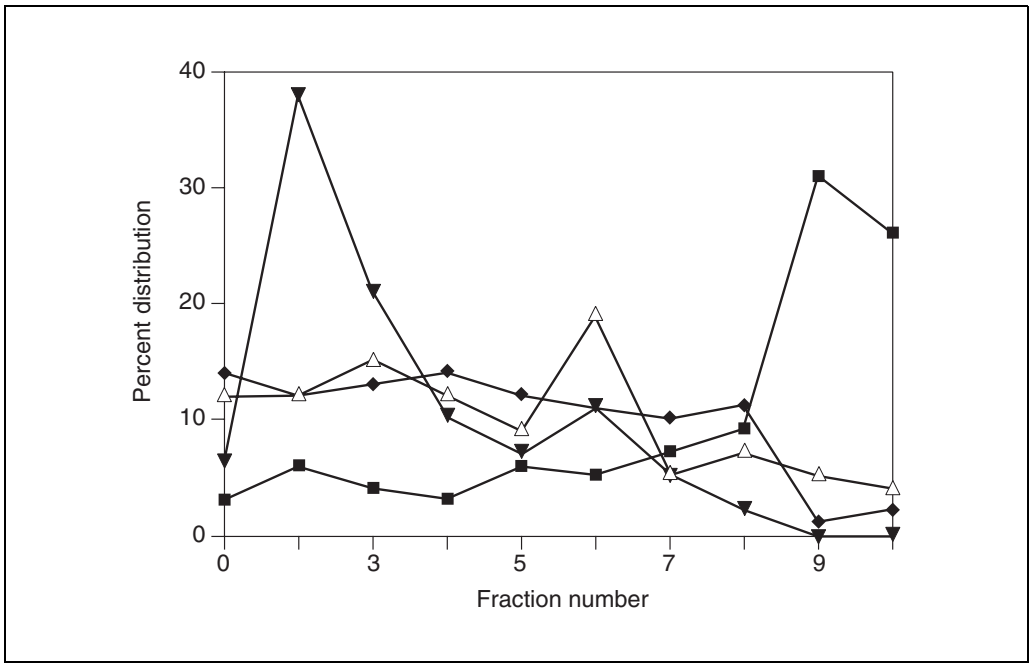


Figure 3.6.1 Isolation of rat liver lysosomes in a Percoll gradient: distribution of enzyme markers. Rat liver LMF in 40% Percoll was centrifuged for 90 min at 35,000 × g, 4°C. The gradient was collected low-density end first (1-ml fractions). β-hexosaminidase (filled squares); cytochrome oxidase (filled triangles); catalase (filled-diamonds); and glucose-6-phosphatase (filled inverted triangles). Data adapted from Symons and Jonas (1987) with kind permission.

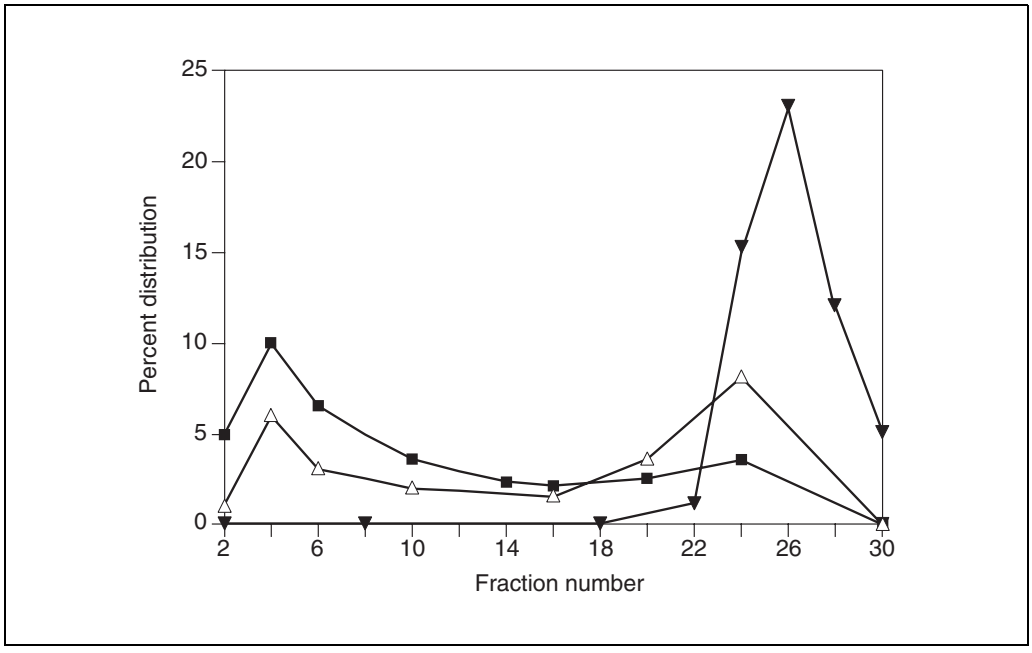


Figure 3.6.2 Isolation of chicken liver lysosomes in a Percoll gradient: distribution of enzyme markers. Chicken liver LMF in 36% Percoll was centrifuged 30 min for 25,000 × *g*, 4°C. The gradient was collected dense-end first. *N*-acetyl-β-glucosaminidase (filled squares); β-galactosidase (open triangles) and succinate dehydrogenase (filled inverted triangles). Data adapted from Nakabayashi and Ikezawa (1988) with kind permission.

galactosidase predominated in the light band. The actual enzyme profiles may be dictated by the gradient density profile, which in turn is controlled by the centrifugation conditions. At a low RCF and short centrifugation time (Nakabayashi and Ikezawa, 1988) the density profile is more sigmoidal (shallow in the middle), while at higher RCFs and longer times the profile will be more linear (Symons and Jonas, 1987).

Although there is clear heterogeneity of the lysosome population in Percoll gradients, it is most likely that the production of two distinct bands is an artifact of the gradient density profile. This was elegantly demonstrated by Draye et al. (1987), who banded rat liver lysosomes in a Percoll gradient and discovered that when the enzyme marker (*N*-acetyl-β-glucosaminidase) was plotted as a relative concentration (concentration in gradient fraction/theoretical concentration if homogenously distributed through the gradient) versus gradient volume, the typical biphasic profile was obtained (Fig. 3.6.3). When the data is replotted as frequency (enzyme activity/density interval of fraction) versus density, a single asymmetrical peak is obtained (Fig. 3.6.4). The authors confirmed this by banding the lysosomes in a linear gradient and obtained the same single asymmetrical peak.

The overall yield of lysosomes (as a percentage of the homogenate) in the Symons and Jonas (1987) gradient is ~20%, although for most Percoll gradients, the figure is closer to 15%. The relative specific activity (RSA) of lysosomal enzyme markers (specific activity in the fraction/specific activity in the homogenate) is ~60.

Alternate Protocol 1

When the lysosomes are density-perturbed with dextran, 70% to 80% of the total *N*-acetyl-β-glucosaminidase in the gradient is recovered in a single very sharp band in the bottom two fractions, and all of the $CaCl_2$-swollen mitochondria are at the top of the gradient (Arai et al., 1991). The RSA of lysosomal markers (~100) is accordingly higher than in Basic Protocol 1.

Alternate Protocol 2

The relative specific activity of lysosomal enzyme markers is ~100 (Olsson et al., 1989) in the 19%/24% Nycodenz interfacial material, while that for enzyme markers of mitochondria, peroxisomes, and endoplasmic reticulum is <0.5, showing a high degree of purification of the lysosomes. Typical yields of lysosomes (as a percent of the total homogenate) are ~10%. Thus, while the yield is lower than with the

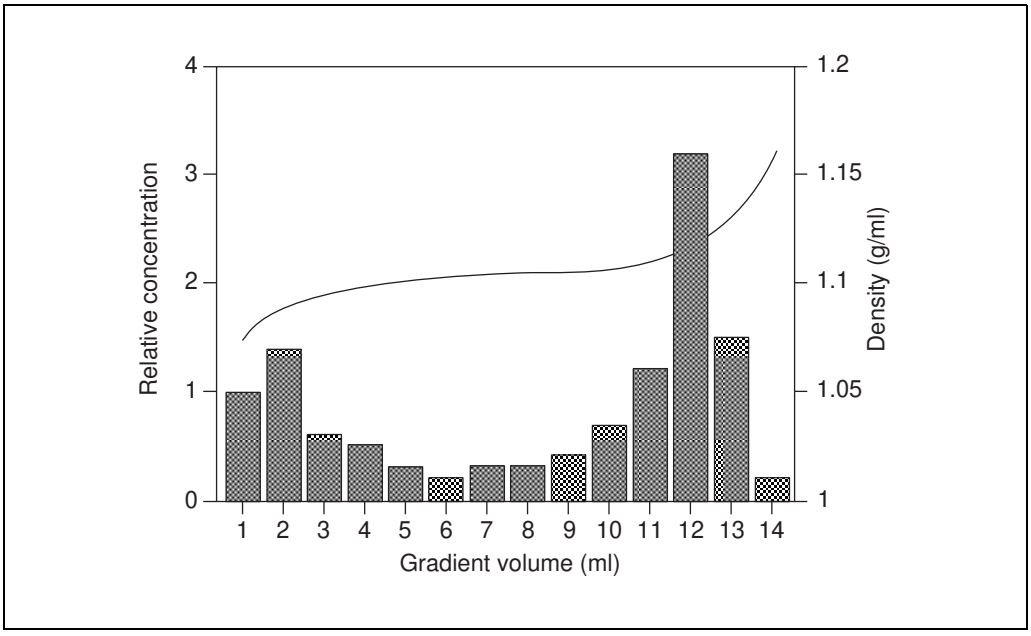


Figure 3.6.3 Biphasic banding of lysosomes in Percoll gradient. The bars show the relative concentration (concentration in gradient fraction/theoretical concentration if homogenously distributed through the gradient) of *N*-acetyl-β-glucosaminidase plotted against gradient volume. The continuous line is the density profile. Data from Draye et al. (1987) with kind permission.

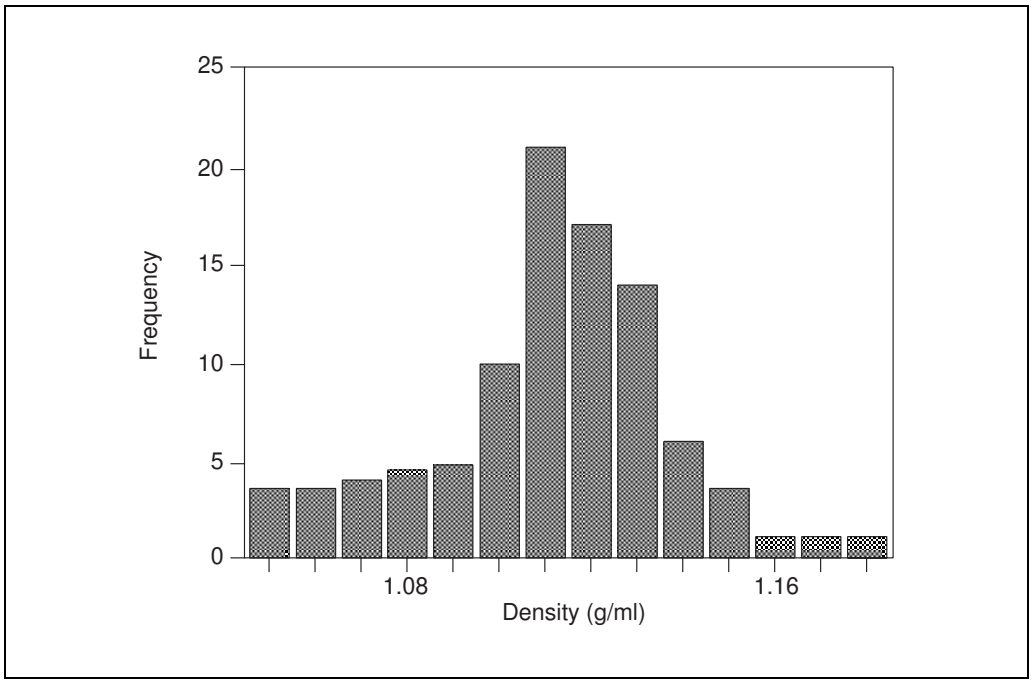


Figure 3.6.4 Real density heterogeneity of lysosomes in Percoll gradient. The data from Figure 3.6.3 were replotted as frequency (enzyme activity divided by density interval of fraction) versus density. Data from Draye et al (1987) with kind permission.

native lysosomes on a Percoll gradient, the purity is as high as that of dextranosomes.

Alternate Protocol 3

A typical separation of the organelles from the LMF on a continuous iodixanol gradient is shown in Figure 3.6.5. The lysosomes band at the top of the gradient and there is a concomitant separation of the mitochondria and peroxisomes in the lower density region of the gradient. Although the iodixanol gradient was designed primarily as an analytical gradient, it could be used preparatively for lysosomes.

Basic Protocol 2

Figure 3.6.6 shows the very satisfactory separation of lysosomes from plasma membrane (leukosialin) and Golgi membranes (galactosyl transferase) in the Percoll gradient (Carlsson and Fukuda, 1992). Approximately 50% of the total *N*-acetyl-β-hexosaminidase is recoverable in the bottom four fractions of the gradient. The mitochondria (not shown) band with the plasma and Golgi membranes.

Time Considerations

There are no points at which either of the basic protocols can be discontinued and then resumed. The light mitochondrial fraction produced in Basic Protocol 1 must be used immediately for any subsequent gradient separation. The preformed gradients in Alternate Protocols 2 and 3 should be made up during the preparation of the LMF in Basic Protocol 1, and kept on ice until required. If they are prepared at room temperature they must be given time to cool down to 4°C. The continuous iodixanol gradient of Alternate Protocol 3 can also be made from a discontinuous gradient the previous day and stored overnight at 4°C.

Basic Protocol 1 and Alternate Protocol 1 should be accomplished in ~4 hr. Because of the shorter density gradient centrifugation time, Basic Protocol 2 will take ~3.5 hr. As long as the gradients for Alternate Protocols 2 and 3 have been prepared ahead of time, these protocols will take ~4.5 hr. These values assume the use of a Percoll gradient. Protocols will take more or less time if one of the alternative gradients is chosen.

With experience and practice it may be unnecessary to unload the gradients in a series of equal fractions. Instead, the lysosome band might be removed using a Pasteur pipet or syringe and this will save ≥15 min per tube.

Functional assays should be carried out as soon as possible after the preparation, but for simple marker enzyme assay (such as described in this unit, and *UNIT 3.4*) material can be stored at 0° to 4°C for up to 16 hr without much loss of activity. These assays and compositional measurements may also be carried out on ma-

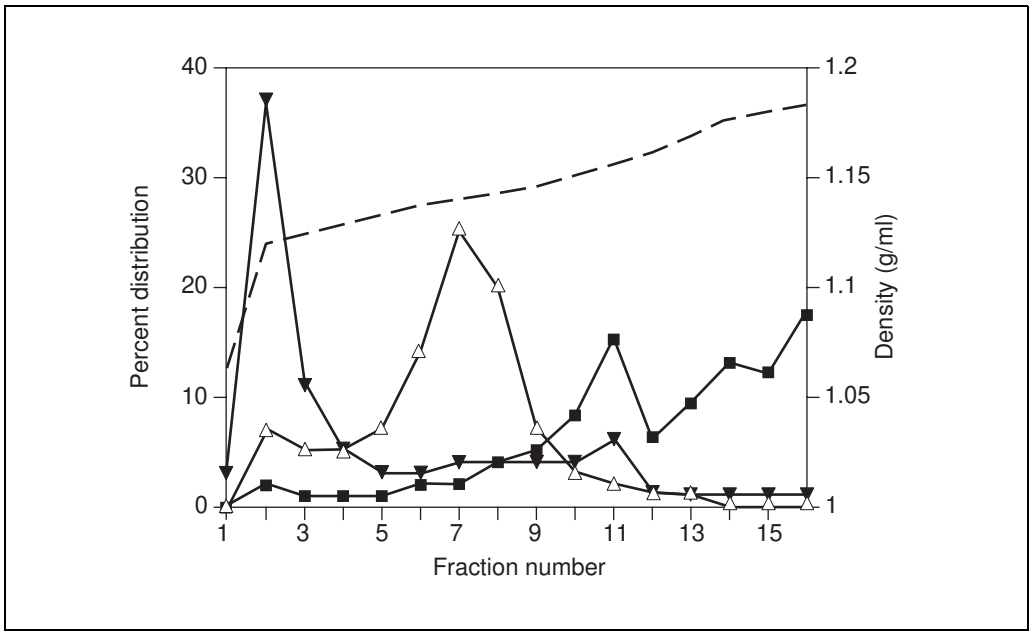


Figure 3.6.5 Fractionation of a rat liver LMF in a continuous iodixanol gradient. Rat liver LMF was centrifuged through a 19% to 27% iodixanol gradient for 2 hr at 110,000 × *g*, 4°C. Gradient collected in 1 ml fractions. β-galactosidase (filled inverted triangles); succinate dehydrogenase (open triangles), and catalase (filled squares). The discontinuous line is the density profile.

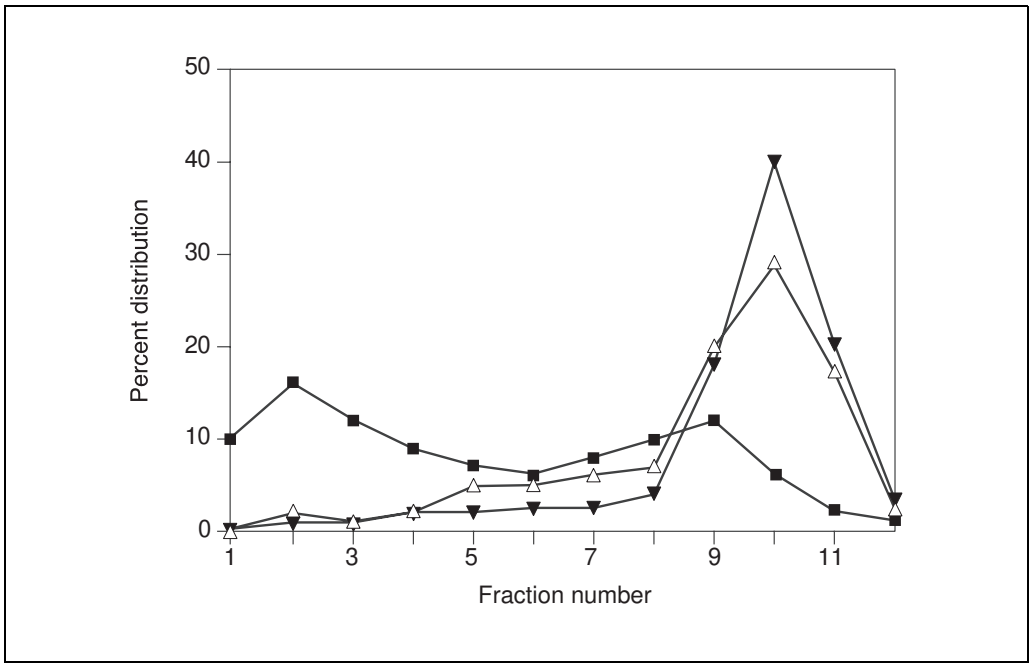


Figure 3.6.6 Isolation of HL-60 cell lysosomes in a Percoll gradient. PNS was layered over 20% Percoll and centrifuged 30 min at 36,000 × *g*, 4°C. The gradient was collected dense-end first in 0.38 ml fractions. β-*N*-acetylhexosaminidase (filled squares); galactosyl transferase (open triangles) and leukosialin (filled inverted triangles). Data from Carlsson and Fukuda (1992) with kind permission.

terial frozen to −20°C or −80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with results obtained with the fresh organelles. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All of the solutions can be made up ahead of time and stored at 4°C for 1 to 2 days, or frozen to −20°C for longer periods, but note that any enzymes and PMSF (or other protease inhibitors) should be added immediately prior to use.

Literature Cited

Akasaki, K., Fukuzawa, M., Kinoshita, H., Furuno, K., and Tsuji, H. 1993. Cycling of two endogenous lysosomal membrane proteins, lamp-2 and acid phosphatase, between the cell surface and lysosomes in cultured rat hepatocytes. *J. Biochem*. 114:598-604.

Arai, K., Kanaseki, T., and Ohkuma, S. 1991. Isolation of highly purified lysosomes from rat liver: Identification of electron carrier components on lysosomal membranes. *J. Biochem.* 110:541-547.

Balch, W.E. and Rothman, J.E. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: Asymmetric properties of donor and acceptor activities in cell-free systems. *Arch. Biochem. Biophys*. 240:413-425.

Barrett, A.J. and Heath, M.F. 1977. Lysosomal enzymes. *In* Lysosomes—A Laboratory Manual. 2nd. ed. (J.T. Dingle, ed.) pp. 19-145. North Holland Publishing Co., Amsterdam.

Berg, T., Kindberg, G.M., Ford, T., and Blomhoff, R. 1985. Intracellular transport of asialoglycoproteins in rat hepatocytes. Evidence for two subpopulations of lysosomes. *Exp. Cell Res*. 161:285-296

Bridges, K.R. 1987. Ascorbic acid inhibits lysosomal autophagy of ferritin. *J. Biol. Chem.* 262:14773-14778.

Caimi, L., Marchesini, S., Aleo, M.F., Bresciani, R., Monti, E., Casella, A., Giudici, M.L., and Preti, A. 1989. Rapid preparation of a distinct lysosomal population from myelinating mouse brain using Percoll gradients. *J. Neurobiol*. 52:1722-1728.

Carlsson, S.R. and Fukuda, M. 1992. The lysosomal membrane glycoprotein Lamp-1 is transported to lysosomes by two alternative pathways. *Arch. Biochem. Biophys.* 296:630-639.

Dobrota, M. and Hinton, R. 1992. Conditions for density gradient separations. *In* Preparative Centrifugation: A Practical Approach (ed D. Rickwood) pp. 77-142. Oxford University Press, Oxford

Draye, J.-P., Courtoy, P.J., Quintart, J. and Baudhuin, P. 1987. Relations between plasma membrane and lysosomal membrane. 2. Quantitative evaluation of plasma membrane marker enzymes in the lysosomes. *J. Biochem*. 170:405-411.

Evans, W.H. 1992. Isolation and characterization of membranes and cell organelles. *In* Preparative Centrifugation: A Practical Approach. (D. Rickwood, ed.) pp. 233-270. Oxford University Press, Oxford.

Finley, E.M. and Kornfeld, S. 1994. Subcellular localization and targeting of cathepsine. *Eur. J. Biol. Chem*. 269:31259-31266.

Graham, J.M. 1997. Homogenization of cells and tissues. *In* Subcellular Fractionation: A Practical Approach. (J.M. Graham and D. Rickwood, eds.) pp. 1-29. Oxford University Press, Oxford

Graham, J., Ford, T., and Rickwood, D. 1990. Isolation of the major subcellular organelles from mouse liver using Nycodenz gradients without the use of an ultracentrifuge. *Anal. Biochem*. 187:318-323.

Graham, J., Ford, T. and Rickwood, D. 1994. The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol. *Anal. Biochem*. 220:367-373.

Jonas, A.J. 1986. Cystine transport in purified rat liver lysosomes. *Biochem. J*. 236:671-677.

Kelly, B.M., Yu, C.Z., and Chang, P.L. 1989. Presence of a lysosomal enzyme, arylsulfatase-A, in the prelysosome-endosome compartments of human cultured fibroblasts. *Eur. J. Cell Biol*. 48:71-78.

Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S., and de Duve, C. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR 1339. *J. Cell Biol.* 37:482-513.

Lipman, B.J., Silverstein, S.C. and Steinberg, T.H. 1990. Organic anion transport in macrophage membrane vesicles. *J. Biol. Chem*. 265: 2142-2147.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. 1987. Rapid analytical and preparative isolation of functional endosomes by free-flow electrophoresis. *J. Cell Biol.* 104:875-886.

Muno, D., Sutoh, N., Watanabe, T., Uchiyama, Y., and Kominami, F. 1990. Effect of metabolic alterations on the density and the contents of cathepsins B, H, and L of lysosomes in rat macrophages. *Eur. J. Biochem*. 191:91-98.

Nakabayashi, T. and Ikezawa, H. 1988. Isolation and characterization of chicken liver lysosomes. *Biochem. Int*. 16:1119-1125

Ohshita, T and Kido, H. 1995. Simple preparation of rat brain lysosomes and their proteolytic properties. *Anal. Biochem*. 230:41-47.

Ohshita, T., Ike, Y., and Katunuma. N. 1992. Incorporation and degradation by kidney lysosomes of cystatin α injected intravenously. *Eur. J. Biochem.* 205:347-353.

Oliver, C., Dromy, R., and Hart, T.K. 1989. Density gradient separation of two populations of lysosomes from rat parotid acinar cells. J. *Histochem. Cytochem*. 37:1645-1652.

Olsson, G.M., Svensson, I, Zdoisek, J.M., and Brunk, U.T. 1989. Lysosomal enzyme leakage during hypoxanthine/xanthine oxidase reaction. *Virchows Arch. B Cell Pathol*. 56:385-391.

Osmundsen, H. 1982. Factors which can influence β-oxidation by peroxisomes isolated from livers of clofibrate-treated rats. Some properties of peroxisomal fractions isolated in a self-generated Percoll gradient by vertical rotor centrifugation. *Int. J. Biochem*. 14:905-914.

Pisoni, R.L., Flickinger, K.S., Thoene, J.G., and Christensen, H.N. 1987. Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes. *J. Biol. Chem.* 262:610-617.

Symons, L.J. and Jonas, A.J. 1987. Isolation of highly purified rat liver lysosomal membranes using two Percoll gradients. *Anal. Biochem.* 164:382-390.

Wattiaux, R. and Wattiaux-De Coninck S. 1983. Separation of cell organelles. *In* Iodinated Density Gradient Media: A Practical Approach (D. Rickwood, ed.) pp. 119-137, Oxford University Press, Oxford.

Yamaguchi, T. and Kaneda, M. 1988. Presence of cytochrome b-558 in guinea pig alveolar macrophages-subcellular localization and relationship with NADPH oxidase. *Biochim. Biophys. Acta* 933:450-459.

Contributed by John M. Graham
Liverpool John Moores University
Liverpool, United Kingdom

Overview of Subcellular Fractionation Procedures for the Yeast *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* is an excellent model organism for the study of eukaryotic cell biology. This unicellular organism shares many features with higher eukaryotic cells including all of the major membrane-bounded subcellular organelles. Accumulating evidence indicates that many cellular processes are mechanistically conserved between yeast and other eukaryotes. Despite the cellular complexity of *S. cerevisiae*, its haploid genome is only 3.5 times larger than that of *E. coli* and has been completely sequenced (Cherry et al., 1997; Clayton et al., 1997; Mewes et al., 1997). This incredible wealth of genetic information, together with powerful genetic and molecular biology techniques, has made *S. cerevisiae* an extremely valuable organism in eukaryotic cell biology (Botstein et al., 1997; Winzeler et al., 1999). The subcellular fractionation techniques for *S. cerevisiae* discussed in this unit are critical to the investigation of the molecular mechanisms of complex cellular functions.

The basic methods used to isolate and characterize organelles and organelle-derived membranes from *S. cerevisiae* are very similar to those used for animal cells and cells from other higher eukaryotes (see *UNITS 3.1-3.6*). This unit provides an overview of centrifugation-based fractionation procedures used to separate the subcellular components of *S. cerevisiae* according to their size and/or buoyant densities. The critical steps include: (1) cell growth under appropriate conditions, (2) preparation of the cell lysate with minimal damage to organelle integrity, (3) selection of a fractionation protocol that separates the subcellular components of interest into distinct sets of fractions, and (4) identification and characterization of the membranes/organelles in the resulting fractions.

Yeast cells are cultured using relatively simple, rapid, and inexpensive techniques (see *UNIT 1.6*). Vigorous strains grown in rich medium at 30°C have a doubling time of ~90 min. Yeast are very versatile. They can be maintained in either their haploid or diploid state and grown under either aerobic and anaerobic conditions. The abundance of certain enzymes and organelles that are subject to metabolic regulation can be manipulated by altering the nutrient composition of the medium. In contrast to animal cells, yeast cells have a thick cell wall that must be disrupted or removed to generate cell lysates. The released subcellular components are then subjected to one or more centrifugation steps, and their distribution among the resulting fractions is determined through their biochemical and morphological characteristics.

Disruption of the cell wall by shearing with glass beads is a rapid and effective method to prepare yeast cell homogenates for the isolation of cytosol or plasma membranes. However, glass-bead lysis is not suitable for most subcellular fractionation experiments, because strong mechanical forces damage the intracellular organelles and can disrupt critical protein and membrane interactions. To minimize the disruption of subcellular integrity, yeast cells are usually enzymatically converted to spheroplasts (yeast cells lacking cell walls) prior to cell lysis. For example, yeast cells can be incubated with enzyme preparations such as Zymolyase 100T (Seikagaku America, ICN), which destabilizes cell walls by cleaving the β-glucan linkages. Subsequently, the spheroplasts are lysed under iso- or hypoosmotic conditions, usually in combination with moderate mechanical force (e.g., several strokes in a Dounce homogenizer).

The most appropriate subcellular fractionation procedure depends on the goals of the particular experiment and the desired levels of organelle yield, purity, and function. Analytical protocols are primarily used to evaluate the subcellular location of one or more proteins. These techniques are designed to obtain discernible differences in the distribution of two or more organelles, while organelle purity, yield, and function are of secondary importance. Ideally, such procedures provide a relatively rapid and simple means to fractionate subcellular components and to subsequently compare the distribution of a protein of interest with the distribution of various organelle membranes. Preparative procedures are generally used to isolate specific organelles, mem-

Contributed by Stephanie E. Rieder and Scott D. Emr
Current Protocols in Cell Biology (2000) 3.7.1-3.7.25

branes, and/or cytosol. These protocols have a greater emphasis on purity, yield, and/or function than do most analytical protocols. For example, when preparing organelles for reconstitution of a cellular process in a cell-free system, the primary requirement is that the organelle(s) of interest remain functional, while organelle yield and purity are important secondary goals. In contrast, the analysis of the protein or lipid composition of a particular organelle requires that the preparation be of very high purity. If the isolated subcellular components will be fractionated further (e.g., for the separation of mitochondrial inner and outer membranes), and/or used in multiple subsequent experiments, then high yields become especially important.

The following sections provide an overview of several key techniques used during yeast subcellular fractionation experiments. The critical parameters, merits, and limitations of various procedures are discussed to facilitate the development and optimization of centrifugation-based fractionation strategies for yeast. Topics include yeast cell growth, cell lysate preparation, differential velocity centrifugation, density gradient centrifugation, and the analysis of subcellular fractions.

YEAST STRAIN SELECTION AND GROWTH CONDITIONS

Yeast Strains

All references to "yeast" within this unit refer to the budding yeast *S. cerevisiae*, unless otherwise indicated. Many of the protocols developed for *S. cerevisiae* are not directly applicable to other yeast (e.g., *Schizosaccharomyces pombe*) without significant modification. *S. cerevisiae* protocols can be applied to many different strains; however, variations among strains and growth conditions may require adjustments to a given protocol.

The desired *S. cerevisiae* strains can be generated using a variety of genetic and molecular biology techniques (Sherman, 1991). In addition, published strains can be obtained from centralized sources such as the Yeast Genetic Stock Center (*http://www.atcc.org/searchengine/ygsc.html*) or from the investigators that first generated the strain and described it in the literature. In many cases, a limited set of strains is used most frequently by a given laboratory or a group of investigators in a common field. If the strain used in a published protocol is available and compatible with the goals of the experiment, using it can minimize the need for protocol modifications. All other things being equal, it is usually best to use the healthiest strain and the most optimal growth conditions that meet the goals of the experiment.

For many subcellular fractionation procedures, it is best to use a strain that is deficient in vacuolar protease activity in order to minimize problems with proteolysis. The three major vacuolar proteases are proteinase A (PrA), proteinase B (PrB), and carboxypeptidase Y (CPY), which are encoded by the *PEP4, PRB1,* and *PRC1* genes, respectively (Jones, 1991a,b; Van Den Hazel et al., 1996). These proteases, as well as most other vacuolar hydrolases, are first synthesized as inactive precursors and are activated by the cleavage of their pro domain upon arrival in the acidic, protease-rich environment of the vacuole (Klionsky and Emr, 1989; Horazdovsky et al., 1995). The minimal requirement for low vacuolar protease activity is the deletion or inactivation of PrA, because this proteinase self-activates under acidic conditions and subsequently initiates the activation cascade for PrB, CPY, and multiple other vacuolar hydrolases (Zubenko et al., 1983; Jones, 1990, 1991a,b). Protease activity levels can be further reduced by eliminating PrB and CPY expression, as these proteases can become activated in the absence of PrA activity under certain conditions (Jones, 1991a,b; Romanos et al., 1992; Westphal et al., 1996).

Cell Culture Conditions

Most *S. cerevisiae* strains can be cultured with relatively little difficulty and expense (see UNIT 1.6; Sherman, 1991). Unless there are special nutritional indications, the medium of choice is a rich broth, such as yeast extract/peptone/dextrose (YPD; UNIT 1.6), that promotes rapid growth. Minimal or synthetic medium supplemented with dextrose (SD) and the appropriate amino acids is frequently used if plasmid maintenance is required. Alternate carbon sources can be used to enhance organelle function and proliferation (e.g., peroxisomes, mitochondria) or to regulate the expression of proteins under the control of certain inducible or repressible promoters. The growth of strains carrying mutations in the adenine deficiency genes *ADE1* or *ADE2* can be improved by adding adenine hemisulfate at 100 mg/liter, which minimizes the accumulation of the red metabolic intermediates. (The pH of the medium should be adjusted to 6.0 with NaOH after the addition of adenine hemisulfate.)

The optimal temperature for culturing yeast is 30°C, unless strains sensitive to high or low temperature are being used. Yeast growth is most vigorous under aerobic conditions. Adequate aeration is achieved by culturing yeast in an Erlenmeyer flask (filled to no more than one-fourth capacity) on a rotary shaker. If extensive foaming occurs, the cultures can be safely treated with a standard antifoam solution. Smaller cultures (2 to 10 ml) can be grown in tubes, which should be secured to a rotary shaker at an angle to allow greater movement of the culture. Fermenters can be used to culture the many cells required for large-scale preparations.

To ensure that a culture consists of a homogeneous population of vigorously growing cells, yeast should be cultured for ≥16 to 24 hr prior to the start of an experiment. This is often best achieved by inoculating a small volume (~5 to 10 ml) of rich medium with a single yeast colony from a fresh plate. Incubation of this initial culture, sometimes referred to as a preculture, allows viable cells to emerge from the stationary phase and enter the growth phase. Once the cell number has doubled at least twice (two generations), the preculture is used to inoculate a larger volume of medium with cells that will be cultured for an extended period (usually overnight) prior to the start of the experiment. If the culture surpasses its target cell density during the incubation period and its growth rate begins to slow, it is best to dilute the culture and allow the cells to grow for several hours (at least two generations) prior to harvesting.

Monitoring Growth Phase and Cell Titer

For most applications, yeast cells are harvested by centrifugation during their mid- to late-logarithmic growth phase. During this time, the cells are growing and dividing vigorously, and have reached a concentration that permits a reasonable yield. In addition, rapidly dividing cells have lower protease levels and thinner cell walls that are more susceptible to disruption than cells exiting or approaching stationary phase.

The cell concentration that correlates with the mid-logarithmic growth phase can vary considerably for different strains and culture conditions. For example, mutant strains with slower growth kinetics or strains grown in minimal medium will reach the logarithmic growth and stationary phases at significantly lower cell concentrations than heartier wild-type strains or strains grown in rich medium. Thus, growth curves should be generated for each strain and each set of culture conditions.

The approximate cell concentration of a culture can be determined using a microscope and hemacytometer (*UNIT 1.1*) or by measuring the optical density of the suspension at 600 nm (OD_{600}; *UNIT 1.6*). Although the latter technique provides only an estimate of the cell concentration, it is rapid and simple. The OD_{600} measurement of a culture, also referred to as the absorbance (A_{600}), is proportional to the number of cells and their size.

The relationship between the OD_{600} value, viable cell number, and growth phase is dependent on the strain and culture conditions. To generate a standard curve for each strain and culture condition to be used, remove samples of the culture at regular intervals throughout its growth and measure the OD_{600} value. The most linear range of the relationship between OD_{600} values and cell number is at OD_{600} values between 0.1 and 0.3; thus, it is usually recommended to measure a diluted culture sample (e.g., 1:5 to 1:10 dilution). Next, determine the corresponding viable cell number by plating appropriate dilutions onto YPAD plates (*UNIT 1.6*).

In general, each 0.1 OD_{600} unit corresponds to ~2–4 × 10^6 cells/ml for haploid cells and 0.5–2 × 10^6 cells/ml for diploid cells. An OD_{600} unit of haploid cells, defined as 1 ml of culture with an OD_{600} value of 1, typically represents 3 × 10^7 cells. In many cases, a haploid yeast culture (grown with appropriate preculturing as described above) will reach its mid-logarithmic growth phase when its OD_{600} value is between 0.4 and 0.9. However, these values are estimates, and it is strongly recommended that the correlation between the OD_{600} value, cell density, and growth phase be determined empirically for each strain and growth condition to be used.

An unusually rapid increase in the OD_{600} of a yeast culture, as well as an unpleasant odor, may indicate that the culture is contaminated with bacteria. Contamination can be confirmed by inspection with a phase-contrast microscope.

PREPARATION OF YEAST CELL LYSATES

The preparation of the cell homogenate is a crucial step in any subcellular fractionation procedure. The major aim of a homogenization

technique is to reproducibly achieve a high degree of cell breakage with a minimum of damage to the organelles or organelle membranes of interest. To analyze yeast organelle membranes, the cell must be stressed sufficiently to cause the disruption of the cell wall and plasma membrane to release the cytosol or the other subcellular components in an intact, dispersed state. Ideally, the cells are disrupted in such a way that the subcellular compartments and membranes: (1) retain their normal functional and structural integrity, (2) are freely suspended in the homogenate, and (3) are in a state that is compatible with the goals and techniques of the subsequent experiments.

The extent of cell lysis and the quality of a yeast cell homogenate depend largely on the culture conditions, cell lysis and homogenization techniques, and lysis buffer. Culture conditions have been discussed above (see Yeast Strain Selection and Growth Conditions), and should be standardized to ensure optimal and reproducible results. In addition, the experimental conditions to which cells are subjected (e.g., extended incubations on ice, treatment with energy poisons) should be noted and standardized.

All lysis and homogenization techniques cause some degree of membrane fragmentation, which reduces organelle integrity and exacerbates the preexisting problem of organelle heterogeneity. Some organelle membranes will rupture and release their lumenal contents; subsequently, the damaged membranes can also reseal and trap inappropriate constituents. The release of hydrolytic enzymes from the vacuole/lysosome can result in extensive protein degradation, while the release of DNA from nuclei increases the viscosity of the suspension and promotes aggregation. Moreover, the normal interactions between organelles and cytoskeletal elements, as well as the formation of aggregates during the homogenization process, can cause difficulties in the preparation of an optimal homogenate. Realistically, homogenization procedures are an imperfect compromise between complete homogenization and minimal damage to intracellular components.

Cell Lysis with Glass Beads

Mechanical disruption of the cell wall by shearing with glass beads is a simple and rapid method to prepare homogenates for the isolation of cytosol or plasma membranes (see Table 3.7.1). This technique is very flexible, as it can be applied to multiple small cultures using a vortex mixer, as well as to very large cultures using a Bead Beater (BioSpec). However, glass-bead lysis is not suitable for most subcellular fractionation experiments, as the strong mechanical forces damage the intracellular organelles and can disrupt critical protein and membrane interactions.

Spheroplast Preparation and Lysis

The optimal lysates for most subcellular fractionation experiments are prepared by enzymatically removing the yeast cell wall prior to lysis (see Table 3.7.1). Yeast cells lacking cell walls, referred to as spheroplasts, are fragile and can be lysed with far more gentle methods than can whole yeast cells. Thus, the preparation of lysates from spheroplasts causes much less damage to organelles, organelle membranes, protein complexes, and other intracellular components than does glass-bead lysis.

A number of hydrolytic enzyme preparations that rapidly and efficiently destabilize yeast cell walls are available. The key enzyme in these preparations is a glucanase that hydrolyzes the bonds between β1-3-linked glucans, the primary structural components of yeast cell walls. In addition, the enzymatic preparations have an alkaline protease activity that plays a more minor role in cell wall digestion. Hydrolysis of the cell walls is enhanced by treatment with dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). These sulfhydral reagents break the disulfide bonds within cell wall proteins, thereby giving the β1-3-glucanase greater access to the glucan linkages. It is often beneficial to determine the optimal digestion conditions empirically in a small-scale experiment, as they can vary considerably with different yeast strains, culture conditions, and enzyme preparations.

The most widely used enzyme preparation is Zymolyase 100T (Seikagaku America, ICN), a highly purified β1-3-glucanase preparation derived from cultured filtrates of *Arthrobacter luteus*. Zymolyase 20T is also used, but its specific activity is one-fifth that of Zymolyase 100T. Several investigators use lyticase preparations to minimize costs, because it can be isolated from cultures of *Oerskovia xanthineolytica* using a straightforward protocol (Scott and Schekman, 1980) or purchased (Sigma). The use of crude enzyme preparations such as β-glucuronidase is usually not recommended for subcellular fractionation ex-

Table 3.7.1 Yeast Cell Lysis Techniques: Advantages and Disadvantages

Method	Advantages	Disadvantages
Glass-bead lysis	Rapid, efficient homogenization Can be applied to many small cultures (using a vortex mixer) or large cultures (using a Bead Beater) Glass beads are inexpensive and easy to prepare Does not require an incubation step prior to cell lysis Plasma membrane is not exposed to hydrolytic enzymes for extended times	Extensive damage to organelles and organelle membranes Agitation generates heat (increased proteolysis) and foam (due to protein denaturation) Can disrupt membrane and protein interactions Lysis efficiency is less reproducible than spheroplast lysis
Spheroplast preparation and lysis	Minimal damage to organelles and protein complexes, as spheroplasts readily lyse when exposed to osmotic and/or mechanical stress Spheroplast lysis is highly efficient and reproducible Multiple methods for lysing spheroplasts: DEAE-facilitated isoosmotic lysis (the most gentle method), exposure to hypoosmotic conditions (osmotic shock), Dounce homogenization, and/or multiple passes through a syringe needle or pipet tip The degree of lysis and homogenization can be easily adjusted (e.g., by changing number of strokes or pestle clearance when using a Dounce homogenizer) High-quality glucanase preparations are readily available (e.g., Zymolyase 100T) Lyticase is very economical if prepared in the laboratory	Takes longer than glass-bead lysis Extended incubation time (30-60 min) before cell lysis can conflict with the goals and steps of some experiments Spheroplasts are fragile; osmotic support and gentle handling are required to prevent premature lysis Enzyme preparations used to digest cell wall can damage some plasma membrane proteins Highly purified glucanase preparations (e.g., Zymolyase 100T) are relatively expensive Lyticase is more economical than Zymolyase, but time and effort are required to prepare it

periments, since they have much higher protease levels than do Zymolyase or lyticase preparations. For example, β-glucuronidase preparations ("snail-gut juice"), which are isolated from the alimentary canal of the Roman snail *Helix pomatia*, can have at least thirty different enzyme activities. Although Zymolyase and lyticase are highly purified preparations, even they can contain residual protease, phosphatase, and nuclease activities; thus, it is always important to wash the spheroplasts well prior to cell lysis.

Spheroplasts are very sensitive to mechanical and osmotic stress. They must be suspended in an isotonic solution during and after the digestion procedure to prevent lysis. The nonmetabolizable sugar sorbitol is frequently used at concentrations of 1.0 to 1.2 M (up to 1.5 M for particularly unstable strains) to provide osmotic support. In addition, spheroplasts must be handled with care to prevent premature cell lysis.

Spheroplasts can be lysed using several techniques. In many procedures, the spheroplasts are resuspended in a hypoosmotic solution, which causes them to swell and potentially lyse. A dramatic and rapid decrease in osmotic support is sufficient to promote cell lysis; however, it also promotes the lysis of intracellular organelles. To disrupt the plasma membrane with a minimum of damage to subcellular components, mild hypoosmotic conditions are used in combination with moderate homogenization techniques—e.g., glass/glass (Dounce) or glass/Teflon (Potter-Elvehjem) homogenization, or passage through a small-gauge syringe needle or pipet tip. Even milder conditions with very little osmotic and mechanical stress are required to maintain the integrity of a high percentage of very fragile

organelles (such as vacuoles). Cell lysis under isoosmotic conditions can be facilitated by incubating the spheroplasts with a cationic polymer such as DEAE-dextran, which interacts with the negatively charged phospholipids in the plasma membrane. Treatment with triethanolamine/acetic acid, pH 7, also improves cell lysis efficiency under mild hypotonic conditions.

Lysis Buffers

The optimal lysis buffer facilitates cell lysis and simultaneously protects the structural and functional integrity of the organelles, organelle membranes, and protein complexes of interest. Ideally, a lysis buffer should be isoosmotic to the organelles within the homogenate; however, most lysis buffers are hypoosmotic to some degree to facilitate efficient cell lysis. In some cases, the osmolarity of the buffer can be adjusted after cell lysis to protect organelles from extended osmotic stress. In addition to minimizing damage to subcellular components, the lysis buffer should interfere as little as possible with subsequent fractionation steps and biochemical assays.

The most appropriate buffer composition depends on the cell lysis method and the purpose for which the lysate is being prepared. Standard buffers consist of several components, including: (1) a suitable organic buffer to maintain a physiological pH, (2) sorbitol or another osmotic balancer to stabilize the organelle membranes, (3) salts to provide the appropriate ionic strength, and (4) a cocktail of protease inhibitors to minimize the damage caused by hydrolytic enzymes. Most lysis buffers include the cation chelator EDTA, as Mg^{2+} serves as a cofactor for several proteases and can promote membrane aggregation at high concentrations. However, in a few cases, Mg^{2+} is added to the lysis buffers to stabilize the composition, membrane association, and function of certain protein complexes and enzymes.

Protein degradation can be a severe problem during subcellular fractionation experiments. Subcellular components are inevitably exposed to degradative enzymes upon cell lysis and homogenization, as these manipulations disrupt the in vivo regulation and compartmentalization of hydrolase activities. The primary source of active degradative enzymes in cell lysates is usually the vacuole, which is an acidic lysosome-like compartment. A cocktail of protease inhibitors with a broad specificity range should be included in the lysis buffer and all subsequent solutions (even when using a vacuolar protease–deficient strain). Because protease inhibitors such as phenylmethylsulfonyl flouride (PMSF) are unstable in aqueous solutions, they should be added just before the solutions are to be used and should be replenished at multiple steps during the fractionation process. In addition, the lysis buffer and all other solutions and equipment required to process the lysate should be prechilled to 0° to 4°C before and during their use (unless otherwise indicated). Damage caused by hydrolytic enzymes can be minimized further by (1) working quickly to limit the potential exposure time to active hydrolases, (2) separating material of interest from the remaining cell lysate as soon as possible, (3) maintaining the integrity of the vacuole, and/or (4) using mutant yeast strains that lack most vacuolar hydrolase activities (e.g., *pep4*Δ*prb1*Δ yeast strains).

It is also critical to be aware that different lysis buffers can destabilize protein-protein interactions to varying degrees and promote the release of sensitive peripheral membrane proteins. The destabilization of protein-protein interactions can disrupt the function, composition, and other biochemical characteristics of subcellular components. In addition, the release of peripheral membrane proteins can change both the absolute and the relative densities of organelles and organelle membranes. It can be particularly difficult to determine the subcellular localization of a peripheral membrane protein, since these proteins can dissociate from their resident membranes during the fractionation procedure and thus cofractionate with soluble cytoplasmic proteins.

The buffer composition often needs to be optimized for a given purpose, since even a slight change can have dramatic effects. Certain buffer characteristics can be either advantages or disadvantages depending on the goals of the experiment. For example, the use of buffers with relatively high ionic strengths minimizes protein and membrane aggregation during cell lysis and homogenization; however, these buffers also tend to destabilize certain protein-protein interactions and promote the release of peripheral membrane proteins. Similarly, inclusion of triethanolamine in the lysis buffer facilitates spheroplast lysis under very mild hypoosmotic conditions, but it can also destabilize some protein-protein interactions.

ISOLATION OF SUBCELLULAR FRACTIONS: PROCEDURAL OPTIONS

Subcellular fractionation procedures capitalize on the differing physical properties of individual organelle and membrane types. The most versatile fractionation procedures are based on centrifugation techniques, which effectively exploit the differences in size, shape, and/or density of distinct organelles and membranes (see Table 3.7.2 and the text below). A number of more-specialized fractionation protocols use gel filtration, immunoisolation, or electrophoresis to separate certain subcellular components according to their size, surface antigen composition, or surface charge, respectively (see Table 3.7.3). However, even these alternate procedures usually begin with centrifugation-based steps to enrich the starting material with the subcellular components of interest and to remove some of the undesirable components.

The most appropriate fractionation procedure will depend on the primary goals of the particular experiment. Parameters to consider include: (1) the properties of the organelles/membranes of interest (e.g., their abundance, fragility, size, density), (2) the properties of the other subcellular components that need to be separated from the organelles/membranes of interest, (3) the required yield, (4) the required purity, (5) the degree to which certain contaminants are harmful, (6) the need for organelle integrity and function, (7) the need to maintain the association of peripheral membrane proteins with their resident compartments, and (8) the need to maintain enzymatic activity levels. The importance of each of these factors should be prioritized for a given experiment, since some desirable qualities will need to be sacrificed to meet the most important goals. For example, the yield of a given organelle within a fraction will generally decrease as its purity increases. Thus, fractionation procedures often need to be evaluated and modified to meet particular requirements.

Centrifugation-Based Subcellular Fractionation Techniques

There are three primary centrifugation procedures used for subcellular fractionation experiments: (1) differential centrifugation by velocity, (2) equilibrium density gradient centrifugation, and (3) rate-zonal gradient centrifugation. Centrifugation-based fractionation procedures separate organelles and membranes according to their sedimentation velocities and/or buoyant densities. These techniques are very versatile, since the sedimentation velocities and densities of subcellular components are determined both by their intrinsic properties and the characteristics of the surrounding medium. Some of the merits and limitations of these procedures are summarized in Table 3.7.2. Several excellent reviews on the theory and practice of centrifugation-based fractionation techniques are also available (Dobrota and Hinton, 1992; Evans, 1992; Hinton and Mullock, 1997).

The absolute densities of subcellular components, as well as their densities relative to one another, can vary with the composition of the surrounding solution. One cause of this variability is the semipermeable nature of membranes. While the mass of the membrane (i.e., the lipids and integral membrane proteins) and the nondiffusible components within the lumen remain constant, the mass of the water and diffusible solutes within the organelle can change as the diffusible molecules move into or out of the organelle to reach equilibrium. Also, the volume of the organelle will change as it swells with water in a hypoosmotic solution, shrinks in a hyperosmotic solution, or bursts due to osmotic or mechanical stress. The mass of an organelle is also dependent on the amounts of associated peripheral membrane proteins and macromolecular complexes (e.g., ribosomes), which can vary in different buffers and density media. For example, if a density medium destabilizes protein-protein interactions, peripheral membrane proteins may be released from their resident membranes, and the mass and density of the organelle will decrease.

The relative sedimentation velocities of subcellular components are primarily determined by the mass and shape of the components and by their density relative to that of the surrounding medium. If the organelles have significantly higher densities than the surrounding medium, their relative sedimentation velocities will be principally determined by their mass, so that larger organelles will have a faster sedimentation velocity than will smaller ones. Shape usually plays a more minor role. However, if the organelles are subjected to centrifugation in a density gradient, their sedimentation velocities will decrease as the density of the surrounding medium approaches their own density. The movement of

Table 3.7.2 Centrifugation-Based Subcellular Fractionation Techniques: Advantages and Disadvantages

Method	Advantages	Disadvantages
Differential centrifugation (basis: sedimentation velocity[a], primarily size)	Simple and rapid method to enrich membranes in fractions corresponding to broad size classes (Fig. 3.7.1) Effective separation of membranes with large size differences (see Table 3.7.4) Relatively high yield Initial low-speed centrifugation permits removal of unbroken cells and large aggregates Excellent preparation step for further fractionation: can enrich components of interest, remove unwanted material, and concentrate membranes from large lysate volumes Provides a rapid means to determine the potential subcellular location of a protein of interest (Table 3.7.4)	Low resolution and purity[a] Poor separation of membranes of similar sizes Pellets containing larger subcellular components will be contaminated with smaller ones[a] Pelleting of organelles and membranes can cause aggregation and structural damage (minimized by pelleting on a cushion) Isolation of particular membrane types requires further fractionation steps
Equilibrium density gradient (basis: density)	Extremely versatile: well suited for both preparative and analytical fractionation experiments Relative densities of organelles/membranes can vary in different gradient media (high flexibility and versatility) Gradients can be either continuous or discontinuous Not sensitive to changes in centrifugation times as long as equilibrium status is reached (good for overnight centrifugations)	Extended centrifugation times may be detrimental to labile organelles and enzyme activities (centrifugation times can be minimized by using a vertical tube rotor) Sample size should be relatively low to maximize resolution and minimize aggregation Very dense organelles/membranes may be exposed to hyperosmotic conditions (especially in sucrose gradients)
Rate-zonal (velocity) gradient (basis: sedimentation velocity and density[b])	Multiparameter separation based on size, shape, and density Well suited for the separation of multiple subcellular components of different sizes Superior to differential centrifugation for separation of components based on size and sedimentation velocities, since sample is layered on top of the gradient Results vary with centrifugation time and gradient composition and shape (high flexibility) Shorter centrifugation runs than for equilibrium gradients (lower risk of damage to subcellular components)	Results depend on centrifugation times and gradient shape and composition (can be more difficult to design and optimize) Less well suited for organelle/membrane purification unless procedure also includes equilibrium gradient fractionation step Sample size should be relatively low to maximize resolution and minimize aggregation Very large or dense components may be exposed to hyperosmotic conditions

[a]Relative sedimentation velocity in a buffer of low density and viscosity is primarily determined by size. Shape and density play a more minor role. The resolution is quite low because components are suspended throughout the medium prior to centrifugation, and thus have a range of migration distances to the bottom of the tube.

[b]Relative sedimentation velocity and final banding position in a nonequilibrium density gradient is determined by both particle size and density relative to the surrounding medium. Size is the primary determinant of sedimentation velocity when the density of the component is significantly greater than the surrounding medium. As the component approaches medium equal to its own density, the particle velocity will decrease. If the particle reaches medium with a density equal to its own, the particle will stop, and its final position within the gradient will be based on its buoyant density alone, irrespective of size or shape.

Table 3.7.3 Several Noncentrifugal Fractionation Techniques: Advantages and Disadvantages

Method	Advantages	Disadvantages
Gel filtration (basis: size and shape)	Excellent for purification of small spherical vesicles (<200 nm) from larger or irregularly shaped organelles High resolution	Not suited for fractionation of larger or irregularly shaped organelles/vesicles Poor separation of different vesicle types with similar sizes
Affinity isolation (basis: surface epitope)	Highly selective for compartment or membrane that has appropriate surface epitope (high purity) Relatively gentle, facilitates isolation of intact organelles	Highly specialized; may need extensive optimization Not well suited for large-scale preparations Limited by antibody specificity and avidity (controls for nonspecific interactions are critical) Limited by epitope accessibility
Electrophoresis (basis: surface charge and size)	Can be used after gel filtration to separate different vesicle types that are similar in size, shape, and density	Requires specialized, expensive equipment Not suitable for larger organelles Rarely used for yeast organelles (purification of mammalian endosomes has been the most common application)

a subcellular component will stop once the density of the surrounding medium is equal to its own density; in this case, the position of the component within the gradient is based on its density, irrespective of size.

The fractionation of subcellular components is never perfect or complete. Centrifugation-based fractionation procedures are limited by the fact that different organelle or membrane populations can have similar sizes and/or densities. In addition, members of a given organelle population vary to some degree both in mass and density. Both of these complications (i.e., the differences within an individual population and the similarities between different populations) are exacerbated by the organelle/membrane fragmentation caused by lysate preparation and fractionation procedures.

Differential centrifugation

Differential centrifugation protocols rapidly fractionate cell lysates into broad size classes through sequential centrifugation steps of increasing force and duration (Fig. 3.7.1). This technique is also referred to as differential pelleting. Because the subcellular components have a substantially higher density than the surrounding buffer, their relative sedimentation velocities are primarily determined by their mass; larger organelles and membranes will pellet faster and at lower centrifugal forces than will smaller organelles. Some of the merits and limitations of differential centrifugation are summarized in Table 3.7.2.

Differential centrifugation is particularly useful to generate fractions that are significantly enriched for the component(s) of interest. The use of enriched fractions (instead of crude cell lysates) facilitates subsequent purification or analysis by density gradient centrifugation and other fractionation techniques. Differential centrifugation is also a convenient way to assess the potential subcellular locations of a protein of interest.

The resolution of size separation by differential centrifugation is quite low, because subcellular components are randomly dispersed throughout the medium prior to centrifugation. Thus, the resulting separation of subcellular components is based both on their sedimentation velocity (which is proportional to their mass) and on their migration distance to the bottom of the centrifugation tube (a random characteristic). Small organelles with short migration distances (and relatively slow sedimentation velocities) will pellet with larger organelles that have faster sedimentation velocities. In other words, a pellet intended to consist of relatively large organelles will also contain smaller organelles that happened to be close to the bottom of the centrifugation tube. Some of the smaller organelles found in low-speed pellets can be removed by resuspending

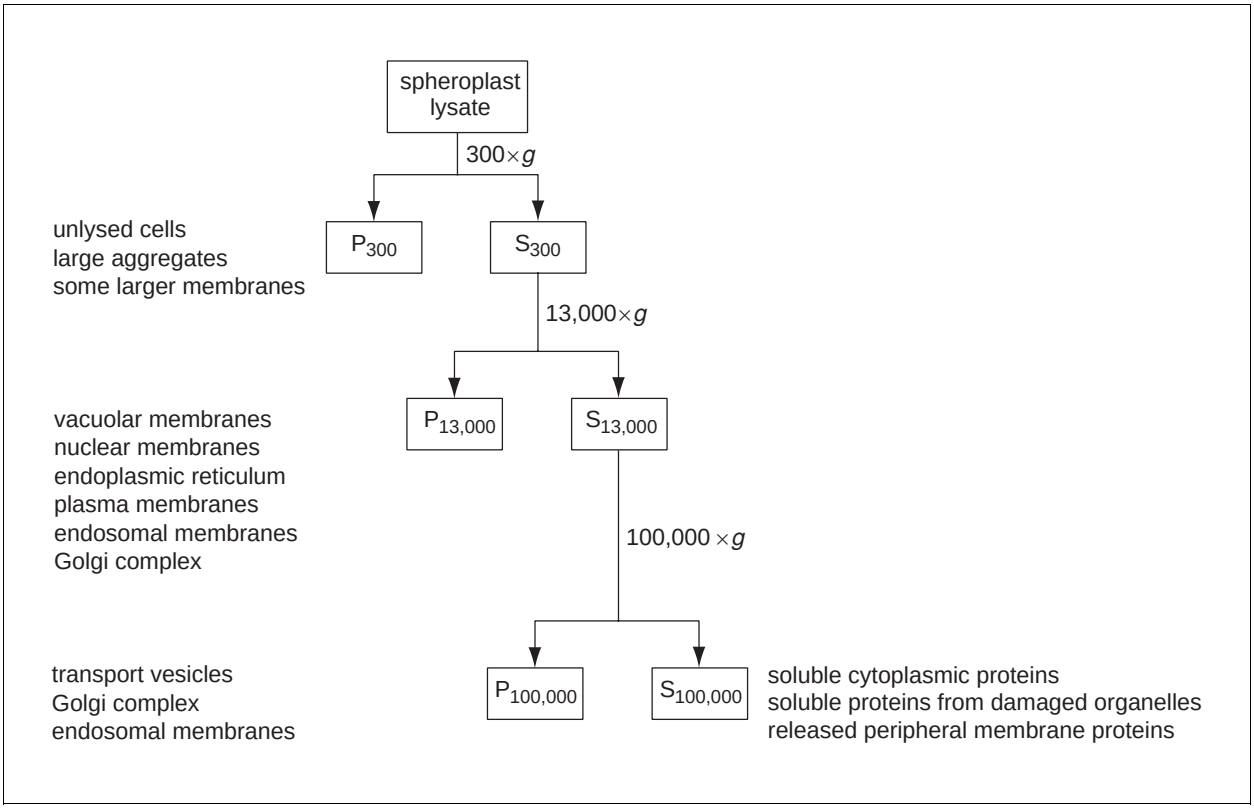


Figure 3.7.1 A procedure for differential centrifugation of yeast cells (see Table 3.7.4 for further details).

the pellet in fresh medium and repeating the centrifugation step, a technique often referred to as "washing" the pellet; however, this step also increases the likelihood of organelle damage.

A typical differential centrifugation scheme is a presented in Figure 3.7.1. First, the yeast cell lysates are subjected to a low centrifugal force (e.g., 300 × *g*) to pellet any unlysed cells and large aggregates. This centrifugation step is often referred to as a "clearing spin." The cleared lysate (S_{300}) is then subjected to sequential centrifugation steps to generate a 13,000 × *g* pellet ($P_{13,000}$), a 100,000 × *g* pellet ($P_{100,000}$), and a 100,000 × *g* supernatant ($S_{100,000}$). The resulting distribution of subcellular components is summarized in Table 3.7.4. The $P_{13,000}$ fraction primarily contains larger organelles and membranes (e.g., plasma membrane, vacuole, endoplasmic reticulum, mitochondria, nuclei, peroxisomes), while the $P_{100,000}$ fraction contains smaller compartments (e.g., Golgi, transport vesicles). Heterogeneous or moderate-sized organelles can distribute between the two fractions (e.g., endosomal membranes). The $S_{100,000}$ fraction contains soluble cytoplasmic proteins, soluble proteins released from the lumen of damaged organelles, and released peripheral membrane proteins.

Equilibrium density gradient centrifugation

Subcellular components can be separated according to their buoyant densities by centrifugation to equilibrium in a density gradient. This technique is referred to as equilibrium density gradient centrifugation or isopycnic gradient centrifugation. In response to centrifugation, a given organelle or membrane will migrate within the gradient until it reaches the region of the gradient that matches its own buoyant density. The subcellular component will then remain at this position for the duration of the centrifugation irrespective of its size, as there is no net force exerted on a particle that is suspended in a liquid of its own density (Archimedes' principle). Some of the merits and limitations of equilibrium density gradient centrifugation are summarized in Table 3.7.2.

Equilibrium density gradient centrifugation techniques are very versatile and are well suited for both analytical and preparative procedures. Often the material to be separated by equilibrium density gradient centrifugation is first subjected to differential centrifugation

Table 3.7.4 Distribution of Organelle Membranes after Cleared Yeast Spheroplast Lysate (S_{300}) is Subjected to Sequential Centrifugation at 13,000 × *g* and 100,000 × *g* [a]

Subcellular component	$P_{13,000}$	$P_{100,000}$
Endosomal membranes[b]	~30%-70%	~30%-70%
Endoplasmic reticulum[c]	>95%	
Golgi (*cis* cisternae)	~30%-60%	~40%-70%
Golgi (medial and *trans* cisternae)	~10%-30%	~70%-90%
Mitochondria	>95%	
Nuclei[c]	>95%	
Peroxisomes (oleate induced)	>95%	
Plasma membrane[c]	>95%	
Transport vesicles		>95%
Vacuolar membranes	>95%	

[a]Strain: SEY6210. Selected references: Gaynor et al. (1994); Marcusson et al. (1994); Cereghino et al. (1995); Zinser and Daum (1995); Rieder and Emr (1997); Babst et al. (1998).

[b]The endosomal membrane system is thought to consist of at least two distinct compartment types. The later prevacuolar endosomes appear to pellet at lower speeds than the earlier post-Golgi endosomal compartments.

[c]Up to 50% of these membranes may pellet during the clearing centrifugation step at 300 × *g*. Yields can be improved by resuspending and recentrifuging the 300 × *g* pellet material, but some loss is unavoidable.

and is thus enriched for components of the appropriate size. The combination of differential centrifugation and equilibrium density gradient centrifugation allows subcellular components to be fractionated according to both mass and buoyant density. The material to be fractionated can be loaded on the top, bottom, or middle of the density gradient, since the components will sediment or float until they reach the appropriate region of the gradient. (Loading a gradient at the bottom, however, may damage sensitive organelles if the gradient is hyperosmotic at the dense regions of the gradient.)

Rate-zonal gradient centrifugation

Rate-zonal gradient centrifugation is a nonequilibrium method in which subcellular components loaded on top of a density gradient move into enriched regions within the gradient. This technique is also referred to as velocity gradient centrifugation. The absolute and relative banding positions of subcellular components are primarily determined by each component's mass, the density of the component relative to the surrounding medium, the centrifugation time (duration), and the composition of the gradient. The mass is the primary determinant of the sedimentation velocity (and thus of position) when the density of the component is significantly greater than that of the surrounding medium. As the component approaches medium equal to its own density, its velocity will decrease. If the particle reaches medium with a density equal to its own, it will stop, and its final position within the gradient will be based on its buoyant density alone, irrespective of its mass or shape.

Like equilibrium density gradient centrifugation techniques, rate-zonal density gradient centrifugation techniques are very versatile and well suited for both analytical and preparative procedures. The separation between different subcellular components can be manipulated empirically to some extent by changing the length of centrifugation, the density limits of the gradient, or the gradient medium. Often the material to be separated by rate-zonal density gradient centrifugation is first subjected to differential centrifugation and is thus enriched for components of the appropriate size. Rate-zonal centrifugation procedures are particularly useful to fractionate subcellular components within a relatively short time, since the rate-zonal centrifugation times are typically much shorter than density equilibrium centrifugation times.

Continuous Versus Discontinuous Density Gradients

The density gradients used for density gradient centrifugation procedures can be either continuous or discontinuous. Some of the mer-

Table 3.7.5 Advantages and Disadvantages of Continuous and Discontinuous Density Gradients

Density gradients	Advantages	Disadvantages
Continuous gradient	Especially well suited for the separation of multiple organelle/membrane types Very versatile: useful for both analytical and preparative fractionation procedures Appropriate for both equilibrium and velocity density gradients Higher resolution than step gradients Less sensitive to overloading than step gradients	Fractions containing organelles of interest are usually not identifiable without monitoring distribution over several fractions Material of interest may be spread over several fractions and may require concentration Preparation is more time consuming
Discontinuous gradient (step gradient)	Gradient preparation is simple and rapid Especially well suited for preparative fractionation procedures to isolate a subcellular component of interest Concentrated organelle/membrane layers are often visible and may have distinguishing characteristics (e.g., color, "fluffiness") Subcellular components of interest can be rapidly harvested by collecting fractions at the appropriate region of the gradient. (All gradient fractions should be collected and examined to assess the success of the experiment.)	Lower resolution than continuous gradients Preliminary experiments using continuous density gradients are usually required to determine optimal densities Accumulation of membranes/organelles at an interface can block movement of other membranes/organelles Sample quantities loaded on gradients must be relatively low to minimize aggregation at interfaces Generally less well suited for analytical purposes (e.g., separation of multiple organelle types on a single gradient)

its and limitations of continuous and discontinuous density gradients are summarized in Table 3.7.5.

In continuous density gradients, the density of the medium changes gradually along the length of the gradient tube. Such gradients are particularly useful for equilibrium or rate-zonal centrifugation to analyze a complex mixture of subcellular components, some of which may have unique but overlapping distributions along the density gradient. The relationship between the density of the gradient medium and the positions of given densities along the gradient, sometimes referred to as the shape of the gradient, can be linear or nonlinear. The optimal shape depends on the densities of the key components that need to be separated from each other.

Discontinuous density gradients, also referred to as step gradients, consist of two or more layers of gradient media with significantly different densities. The interfaces between the layers should be very sharp; therefore, discontinuous gradients must be prepared just before use. Discontinuous density gradients are most frequently used for preparative procedures (e.g., the purification of a subcellular component of interest) but have also been used for analytical experiments. In most cases, the appropriate density and volume of the gradient layers (or steps) are determined by first examining the distribution of the subcellular components within a continuous density gradient. For preparative procedures, discontinuous density gradients are often designed in such a way that the components of interest accumulate in a small and easily identifiable region of the gradient (e.g., an interface between two layers), while other components either do not reach this region or pass through it.

Gradient Preparation

The accurate and reproducible preparation of density gradients is critical to the success of density gradient fractionation procedures. Continuous density gradients are often generated using a gradient maker that mixes two solutions of different densities in the appropriate proportions. Alternatively, continuous density gradients can be formed by preparing a multilayer step gradient and allowing the density medium to diffuse for several hours to eliminate the interfaces. Regardless of whether a continuous gradient is prepared with a gradient maker or by diffusion, it is best to analyze the density distributions of at least two test

gradients to ensure that the densities are as planned and are reproducible. A refractometer is the best way to accurately measure the densities of media that are true solutions (e.g., sucrose, Nycodenz, iodixinol).

Discontinuous gradients are prepared just before use by sequentially adding layers of gradient media with significantly different densities to the centrifugation tube. Some investigators prefer to start with the most dense layer, then gently add the subsequent layer of lower density on top (overlaying). Other investigators find it easier to add the least-dense layer first and then introduce layers of increasing density underneath it, using a very long, slim cannula or needle (underlaying). With either technique, it is critical that the interface between the layers remains sharp. If mixing between layers becomes apparent during gradient preparation, the gradient should be discarded and a new one should be made.

Gradient Media

The optimal density gradient medium should facilitate the separation of the subcellular components of interest into distinct sets of fractions. It should also protect the functional and structural integrity of the organelles and membranes, as well as the appropriate protein-protein and protein-membrane interactions, to the greatest extent possible.

The characteristics of several different density gradient media are summarized in Table 3.7.6. To generate the required density range of the gradient medium, the primary constituent is dissolved or suspended in a buffered solution at varying concentrations. Some density gradient media also contain sucrose to provide osmotic support for organelle membranes, a cocktail of protease inhibitors to minimize damage due to hydrolytic enzymes, and occasionally salts to provide the appropriate ionic strength.

The density gradient media listed in Table 3.7.6 are all very soluble in aqueous buffers and are nontoxic, chemically inert, transparent to visible light, and available at high purity. In addition, each of the media has one or more (but not all) of the following desirable characteristics: (1) has a low viscosity-to-density ratio to minimize the required centrifugation time and/or force, (2) has a low osmolarity-to-density ratio to minimize exposure of dense organelles to hyperosmotic conditions, (3) is transparent to UV light, (4) is inexpensive, (5) is not metabolized by yeast cells, (6) can be used to form gradients with osmolarity levels that support organelle membranes and vary by <10% across the gradient, (7) does not interfere with subsequent analyses of the resulting fractions, and (8) can be easily removed from isolated subcellular components if required.

For several decades, sucrose density gradients have been the most common method for separating organelles on the basis of both size and density. More recently, density gradient solutions prepared with nonionic iodinated compounds (e.g., metramizide, Nycodenz, iodixinol) have increased in popularity, since these solutions have lower viscosity and osmolarity levels than do sucrose solutions of equal density. Density gradients prepared from sucrose or nonionic iodinated compounds are well suited for analytical experiments designed to examine the distribution of multiple organelles and membranes along the same gradient, as well as for preparative experiments used to isolate a particular organelle or membrane type at higher yield and purity levels. In contrast, density gradients prepared from Percoll or Ficoll suspensions are primarily used for preparative procedures. Ficoll consists of highly branched polymers of sucrose and epichlorhydrin, while Percoll consists of colloidal silica beads coated with polyvinylpyrrolidone. Percoll and Ficoll suspensions both have low osmolarity and viscosity levels; in fact, sucrose is usually added to the suspensions to provide the required osmotic support. Density gradients prepared with sorbitol or glycerol, or with D_2O instead of H_2O as the solvent, are used less frequently.

The most appropriate density gradient composition depends on the purpose of the experiment. For example, certain protein-protein interactions will be more stable in one medium than in another. In addition, the absolute densities of organelles and membranes, as well as their densities relative to each other, depend on both the intrinsic characteristics of the organelles and the properties of the surrounding gradient medium. Important factors include differential levels of membrane permeability to gradient solutes, osmolarity of the density medium relative to the organelles, and degree of peripheral protein loss. Thus, subcellular components that are unresolvable in one gradient medium may be resolvable in another.

Ideally, the entire density gradient should be isoosmotic to the organelles within the homogenate. Hyperosmotic solutions tend to draw water out of organelles, and thus can

Table 3.7.6 Characteristics of Several Density Gradient Media: Advantages and Disadvantages

Medium	Advantages	Disadvantages
Sucrose	Most common medium, used for many years Inexpensive and available at high purity Many applications (preparative and analytical) Does not absorb UV light Differential membrane permeability to sucrose[a] Removed by centrifugation, dialysis, or ultrafiltration	High osmotic-pressure-to-density ratio Moderate to high viscosity Can disrupt some protein and membrane interactions Can pass through organelle membranes to some extent[a] Interferes with some enzyme assays
Nycodenz, iodixinol[b]	Exert moderate osmotic pressure (less than sucrose) Isoosmotic density gradients can be generated with osmotic balancers (e.g., sucrose, sorbitol, NaCl) Low to moderate viscosity Many applications (preparative and analytical) Iodixinol gradients can be formed through centrifugation Do not penetrate organelle membranes Removed by centrifugation, dialysis, or ultrafiltration	Moderate osmotic-pressure-to-density ratio Relatively expensive Can disrupt some protein and membrane interactions Moderate viscosity at high concentrations Absorb UV light Interfere with some enzyme assays
Ficoll 400[c]	Very low osmotic pressure at concentrations <25% Does not penetrate organelle membranes Generally used for preparative procedures Usually does not interfere with enzyme assays	Osmotic pressure increases rapidly at concentrations >25% High osmotic pressure at concentrations >30% High viscosity (longer centrifugation times) Cannot be removed by dialysis or ultrafiltration
Percoll[d]	Exerts essentially no osmotic pressure (required osmotic support is usually provided by adding sucrose) Very low viscosity (shorter centrifugation times) Continuous density gradients form during centrifugation Gradient shape is sensitive to changes in rotor geometry, centrifugation speed, and duration (offers flexibility) Permits simple, rapid isolation of larger organelles Does not penetrate organelle membranes	Adheres to membranes and is difficult to remove Cannot be removed by dialysis or ultrafiltration Absorbs UV light and interferes with some assays Gradient shape usually changes during centrifugation Gradient shape is sensitive to changes in centrifugation speed, time, and rotor (more difficult to design and reproduce) Generally not useful for evaluating multiple organelles Not suitable for small organelles or membrane vesicles

continued

change their size, shape, and density. Exposure to hyperosmotic conditions can also promote the loss of protein coats and other peripheral membrane proteins, thereby altering the composition, function, and density of the affected organelle membranes. In addition, organelles suspended in hyperosmotic solutions are more susceptible to lysis if subsequently transferred to a buffer of lower osmolarity. Organelles can also be damaged through exposure to hypoosmotic conditions, which can induce organelle swelling, membrane discontinuities, and organelle lysis. However, hypoosmotic conditions are rarely a practical problem during gradient fractionation, since osmotic balancers such as sorbitol or sucrose can be added to gradient solutions to increase their osmolarity.

Information booklets provided by the manufacturers of gradient media can serve as an excellent resource for details on gradient media

Table 3.7.6 Characteristics of Several Density Gradient Media: Advantages and Disadvantages, continued

Medium	Advantages	Disadvantages
Sorbitol	Advantages are similar to those of sucrose Unlike sucrose, sorbitol is not metabolizable Slightly less membrane permeable than sucrose[a]	Not used as frequently as sucrose More expensive than sucrose Other disadvantages are similar to those of sucrose
Glycerol	Used for separating large protein complexes Freely penetrates membranes (organelle/vesicle density primarily determined by membrane composition)[a] Removable by centrifugation, dialysis, or ultrafiltration	Rarely the best medium for membrane separations Very high viscosity (longer centrifugation times) Freely penetrates membranes (high banding densities) Interferes with some enzyme assays
D_2O solvent[e]	Lower osmolarity and viscosity levels than with H_2O-based media of equal density Extent of exchange between D^+/OD^- and H^+/OH^- molecules varies with membrane composition, changing their relative densities[a]	Expensive and benefits can be insignificant D^+/OD^- molecules can exchange with membrane- and protein-associated H^+/OH^- molecules, increasing the density of components

[a]The absolute densities of subcellular organelles/membranes, as well as their densities relative to each other, can change in different gradient media (e.g., because of differential levels of membrane permeability to the solute, osmolarity of the medium, or peripheral protein loss). Thus, components of interest that have overlapping densities in one medium can have differentiable densities in another gradient medium.

[b]Nycodenz (Nycomed Pharma) and iodixinol (Optiprep, Nycomed Pharma) are nonionic iodinated density compounds with similar characteristics. Nycodenz is a nonionic derivative of triiodobenzoic acid with three aliphatic side chains. Iodixinol is essentially a dimeric form of Nycodenz, and thus has a higher density-to-osmolarity ratio. These nonionic compounds have largely replaced the nonionic medium metramizide, as well as the ionic iodinated density compounds.

[c]Ficoll 400 is a neutral, highly branched polymer of sucrose and epichlorhydrin (average mol. wt. 400,000).

[d]Percoll consists of colloidal silica beads coated with polyvinylpyrrolidone (PVP).

[e]Preparation of gradient density media in heavy water (D_2O; 1.1 g/ml) instead of regular water (1.0 g/ml).

characteristics, gradient preparation techniques, and subcellular fractionation procedures. For example, Nycomed Pharma has several booklets that discuss Nycodenz and Optiprep (iodixinol) gradient media, centrifugation-based fractionation procedures, and several cellular and subcellular fractionation protocols. While the focus of these texts is on mammalian systems, many of the principles are also applicable to yeast fractionation experiments.

ANALYSIS OF SUBCELLULAR FRACTIONS

The specialized functions of subcellular organelles and their membranes are reflected by their unique protein compositions, enzymatic activities, and morphologies. These characteristics can be used to follow the distribution of particular organelles and membranes during the subcellular fractionation procedure, to characterize the resulting fractions, and to analyze the success of an experiment. The most common method used to monitor the distribution and integrity of particular organelles and membranes after subcellular fractionation is to analyze the distribution of their characteristic marker proteins and enzyme activities (Tables 3.7.7 and 3.7.8). To establish the purity and yield of an organelle preparation or the success of an analytical fractionation experiment, it is of central importance to determine the levels of the components of interest, as well as the levels of the potential contaminants. The distribution and integrity of fractionated organelles and membranes can also be characterized by examining their morphologies, functions, and overall protein compositions.

Marker Proteins and Enzymatic Activities

An optimal marker is a well-characterized protein and/or enzyme activity that (1) is associated with a single subcellular compartment or membrane type, (2) remains tightly associated with its resident compartment/membrane

Table 3.7.7 Marker Proteins Used to Identify Fractionated Subcellular Components

Subcellular fraction	Markers (and corresponding genes)[a,b]	Selected references[c]
Plasma membrane:		
	Gas1p (*GAS1*)	Nuoffer et al., 1991. *MCB* 11:27
	H^+-ATPase (*PMA1*)	Goffeau and Dufour, 1988, *Methods Enzymol*. 157:528; Serrano, 1988. *Methods Enzymol.* 157:533
	Syntaxin complex (*SSO1*, *SSO2*)	Aalto et al., 1993. *EMBO J.* 12:4095
	Chitin synthetases (*CHS1*, *CHS2*, *CHS3*); also associated with chitosomes/endosomes	Chuang and Schekman, 1996. *JCB* 135:597; Ziman et al., 1996. *MBC* 7:1909; Ziman et al., 1998. *MBC* 9:1565
	α1-3-Glucan synthase (*GLS1*, *GLS2*)	Mazur et al., 1995. *MCB* 15:5671
	PM receptors and transport proteins	Andre, 1995. *Yeast* 11:1575; van der Rest et al., 1995. *Microbiol. Rev*. 59:304
	a-factor receptor (*STE3*, α strains); transient	Davis et al., 1993. *JCB* 127:53
	α-factor receptor (*STE2*, **a** strains); transient	Blumer et al., 1988. *JBC* 263:10836
Endosomes:		
Membrane	Pep12p (*PEP12*)	Becherer et al., 1996. *MBC* 7:579
	a-factor receptor (*STE3*, α strains); transient	Davis et al., 1993. *JCB* 127:53; Wendland and Emr, 1998, *JCB* 141:71
	α-factor receptor (*STE2*, **a** strains); transient	Blumer et al., 1988. *JBC* 263:10836; Wendland and Emr, 1998. *JCB* 141:71
Lumen	Exogenous [^{35}S]α-factor (MFα1); transient	Singer and Riezman, 1990. *JCB* 110:1911; Dulic et al., 1991. *Metholds Enzymol.* 194:697; Wendland and Emr, 1998. *JCB* 141:71
Golgi complex:		
Membrane, *cis*	α1-6-Mannosyltransferase (*OCH1*); also in ER	Nakayama et al., 1992. *EMBO J.* 11:2511; Gaynor et al., 1994. *JCB* 127:653
Membrane, medial	Guanosine diphosphatase (*GDA1*)	Vowels and Payne, 1998. *MBC* 9:1351
	α1-3-Mannosyltransferase (*MNN1*)	Cunningham and Wickner, 1989. *Yeast* 5:25
	α1-2-Mannosyltransferase (*KRE1*)	Lussier et al., 1995. *JCB* 131:913
Membrane, *trans*[d]	Endoprotease yscF (*KEX2*)	Redding et al., 1991. *JCB* 113:527; Cooper and Bussey, 1992. *JCB* 119:1459
	Carboxypeptidase ysc-α (*KEX1*)	Bryant and Boyd, 1993. *J Cell Sci.* 106:815
	Dipeptidyl aminopeptidase A (*STE13*)	Bryant and Boyd, 1993. *J Cell Sci.* 106:815; Nothwehr et al., 1993. *JCB* 121:1197
	Vacuolar protein receptor (*VPS10*)	Marcusson et al., 1994. *Cell* 77:579; Cereghino et al., 1995. *MBC* 6:1089
Peroxisomes:		
Membrane	ABC transporters (e.g., *PXA1, PXA2*)	Hettema et al., 1996. *EMBO J.* 15:3813; Shani and Valle, 1998. *Methods Enzymol.* 292:753
	Peroxins (e.g., *PEX3, PEX13)*	Hohfeld et al., 1991. *JCB* 114:1167; Gould et al., 1996. *JCB* 135:86; Waterham and Cregg, 1997. *Bioassays* 19:57
Matrix	Peroxisomal enzymes: e.g., acyl-CoA oxidase (*POX1*), malate dehydrogenase (*MDH3*), catalase A (*CTA1*), 3-ketoacyl-CoA thiolase (*POT1*), enoyl-CoA hydratase (*EDH1*, *EDH2*), 3-hydroxy-acyl-CoA epimerase (*FOX2*), citrate synthase (*CIT2*)	Dmochowska et al., 1990. *Gene* 88:247; Kispal and Srere, 1991. *Arch. Biochem. Biophys.* 286:132; Kunau and Hartig, 1992. *Antonie van Leuwenhook* 62:63; Singh et al., 1992. *MCB* 12:5593; Kunau et al., 1993. *Biochimie* 75:209; Subramani, 1993. *Annu. Rev. Cell Biol.* 9:445; Erdmann and Kunau, 1994. *Yeast* 10:1173; McAlister-Henn et al., 1995. *JBC* 270:21220; Geisbrecht et al., 1998. *JBC* 273:33184; Gurvitz et al., 1998. *JBC* 273:31366

continued

Table 3.7.7 Marker Proteins Used to Identify Fractionated Subcellular Components, continued

Subcellular fraction	Markers (and corresponding genes)[a,b]	Selected references[c]
Mitochondria:		
Outer membrane	Porin (*POR1, POR2*)[e]	De Pinto et al., 1987. *BBA* 894:109
	Outer membrane translocase (e.g., *TOM72, TOM20, TOM40, TOM7*)[f]	Lithgow et al., 1995. *Trends Biochem. Sci.* 20:98; Pfanner et al., 1997. *Annu. Rev. Cell Devel. Biol.* 13:25; Pfanner and Meijer, 1997. *Curr. Biol.* 7:R100; Kunkele et al., 1998. *Cell* 93:1009
	OM45p (*OM45*)	Yaffe et al., 1989. *JBC* 264:21091
Inner membrane	Cytochrome oxidase (e.g., *COX1, COX2, COX3, COX4*)[f]	Taanman and Capaldi, 1995. *Eur. J. Biochem.* 227:22481; Geier et al., 1997. *FEBS Lett.* 412:296
	Succinate dehydrogenase (e.g., *SDH3, SDH4*)[f]	Daignan-Fornier et al., 1994. *JBC* 269:15469; Oyedotun and Lemire, 1996. *MCB* 16:31382; Bullis and Lemire, 1997. *JBC* 272:6543
	Mitochondrial H^+-ATPase (e.g., *ATP5, ATP9*)[f]	Jean-Francois et al., 1988. *BBA* 933:223; Uh et al., 1990. *JBC* 265:19047
	Inner membrane translocase (e.g., *TOM17, TOM23, TOM54*)[e,f]	Lithgow et al., 1995. *TBS* 20:98; Pfanner et al., 1997. *Annu. Rev. Cell Devel. Biol.* 13:25; Pfanner and Meijer, 1997. *Curr. Biol.* 7:R100; Blom et al., 1998. *MCB* 18:309
	Inner membrane protease (*IMP1, IMP2*)	Nunnari et al., 1991. *EMBO J.* 10:1997; Schneider, 1991. *MCB* 34:401
Intermembrane space	Cytochrome *b2* (*CYB2*) and cytochrome *c* peroxidase (*CCP1*)	Mathews and Wittenberg, 1979. *JBC* 254:5991; Daum et al., 1982. *JBC* 257:13028; Daum et al., 1982. *JBC* 257:13075; Brown and Trumpower, 1995. *J. Bacteriol.* 177:1380
Matrix	Fumarase (*FUM1*) and aconitase (*ACO1*); both also in cytoplasm	Daum et al., 1982. *JBC* 257:13075; Gangloff et al., 1990. *MCB* 10:3551; Stein et al., 1994. *MCB* 14:4770
Nucleus:		
Matrix	DNA, histones, other DNA-binding proteins	Fukuma et al., 1994. *Yeast* 10:319; Patterton et al., 1998. *JBC* 273:7268
Nucleolus	38-kDa yeast fibrillarin (*NOP1*)	Aris and Blobel, 1988. *JCB* 107:17; Henriquez et al., 1990. *JBC* 265:2209
Membrane	Nuclear pore components (e.g., *POM152*)[f]	Wozniak et al., 1994. *JCB* 125:31
	Membrane proteins (e.g., *SPO7, NEM1*); also in ER	Siniossoglou et al., 1998. *EMBO J.* 17:6449
Vacuoles:		
Membrane	Alkaline phosphatase (*PHO8*)[e]	Klionsky and Emr, 1989. *EMBO J.* 8:2241; Horazdovsky et al., 1995. *Curr. Opin. Cell Biol.* 7:544
	Dipeptidyl aminopeptidase B (*DAP2*)	Roberts et al., 1989. *JCB* 108:1363; Klionsky et al., 1990. *Microbiol. Rev.* 54:266
	Vacuolar SNARE (*VAM3*)	Darsow et al., 1997. *JCB* 138:517
	V-ATPase[f]: e.g., 100-kDa subunit (*VPH1*)[e], 60- and 69-kDa subunits (*VMA1*, *VMA2*)[e,g]	Kane et al., 1992. *JBC* 267:442; Manolson et al., 1992. *JBC* 267:14294; Nelson and Klionsky, 1996. *Experentia* 52:1101
Lumen	Vacuolar enzymes: e.g., carboxypeptidase Y (*PRC1*)[e], proteinase A (*PEP4*), proteinase B (*PRB1*), aminopeptidase I (*LAP4*), carboxypeptidase S (*CPS1*)[h]	Jones, 1990. *Methods Enzymol.* 185:372; Klionsky et al., 1990. *Microbiol Rev.* 54:266; Klionsky et al., 1992. *JCB* 119:287; Spormann et al., 1992. *JBC* 267:8021; Horazdovsky et al., 1995. *Curr. Opin. Cell Biol.* 7:544; Van Den Hazel et al., 1996. *Yeast* 12:1; Odorizzi et al., 1998. *Cell* 95:847

continued

Table 3.7.7 Marker Proteins Used to Identify Fractionated Subcellular Components, continued

Subcellular fraction	Markers (and corresponding genes)[a,b]	Selected references[c]
Endoplasmic reticulum:		
Membrane	Dolichol P-mannosyltransferase (*DPM1*)[e]	Orlean et al., 1988. *JBC* 263:17499; Forsee et al., 1997. *Eur. J. Biochem.* 244:953
	Dolichol P-glucose synthetase (*ALG5*)	Heesen et al., 1994. *Eur. J. Biochem.* 224:71
	NAPDH-cytochrome P-450 reductase (*NCP1*)	Venkateswarlu et al., 1998. *JBC* 273:4492
	HMG-CoA reductase (*HMG1*, *HMG2*)	Hampton and Rine, 1994. *JCB* 125:299
	Protein translation complex (e.g., *SEC61*, *SEC62*, *SSS1*, *SSH1*)[f]	Corsi and Schekman, 1996. *JBC* 271:30299; Lyman and Schekman, 1996. *Experentia* 52:1042
	Oligosaccharyl transferase (e.g., *WBP1, OST1-5, STT3, SWP1*)	te Heesen et al., 1993. *EMBO J.* 12:279; Silberstein and Gilmore, 1996. *FASEB J.* 10:849; Karaoglu et al., 1997. *JBC* 272:32513
	Ribosome and translocation complex (RER)[f]	Sanderson and Meyer, 1991. *JBC* 266:13423; Planta and Mager, 1998. *Yeast* 14:471
	Mannosyltransferase (e.g., *PMT1-4*)	Gentzsch and Tanner, 1996. *EMBO J.* 15:5752
Lumen	BiP (*KAR2*)	Normington et al., 1989. *Cell* 57:1223; Rose et al., 1989. *Cell* 57:1211
	Protein disulfide isomerase (*PDI1*)	LaMantia and Lennarz, 1993. *Cell* 74:899
Cytoplasm	3-Phosphoglycerate kinase (*PGK1*)[i]	Perkins et al., 1983. *Biochem. J.* 1211:199
	Glycerol-3-*P*-dehydrogenase (*GPD1*)	Albertyn et al., 1994. *MCB* 14:4135
	Glucose-6-*P*-dehydrogenase (*ZWF1*)[i]	Nogae and Johnston, 1990. *Gene* 96:161
Transport vesicles:		
ER-Golgi	COPII proteins (e.g., *EMP24*, *SEC23*[g], *SEC24*[g]); Emp24p is also in ER	Schimmoller et al., 1995. *EMBO J.* 14:1329; Schekman and Orci, 1996. *Science* 271:1526; Matsuoka et al., 1998. *Cell* 93:263
Golgi-ER	COPI proteins (e.g., *COP1*, *RET2*, *RET3*)[g]	Letourneur et al., 1994. *Cell* 79:1199; Gaynor et al., 1998. *BBA* 1404:33
Intra-Golgi	Sec7p (*SEC7*)[g]	Franzusoff et al., 1991. *Methods Enzymol.* 194:662
Post-Golgi	Clathrin (*CHC1*, *CLC1*)[g]	Lemmon et al., 1988. *J. Cell Biochem.* 36:329; Chu et al., 1996. *JBC* 271:33123
	SNARE proteins (*SNC1, SNC2*)	Protopopov et al., 1993. *Cell* 74:855
Secretory (lumen)	Invertase (*SUC2*); induced in low glucose	Klionsky et al., 1988. *MCB* 8:2105
	Acid phosphatase (*PHO5*); induced in low phosphate	Vogel and Hinnen, 1990. *Mol. Microbiol*. 4:2013

[a]The examples of membrane-associated proteins are integral membrane proteins unless otherwise indicated.
[b]Abbreviations: ER, endoplasmic reticulum; PM, plasma membrane; RER, rough endoplasmic reticulum.
[c]Journal abbreviations: *BBA*, *Biochim. Biophys. Acta*; *JBC*, *J. Biol. Chem.*; *JCB*, *J. Cell Biol.*; *MBC*, *Mol. Biol. Cell*; *MCB*, *Mol. Cell Biol.*
[d]Although these proteins are primarily located in the late Golgi, they also traffic to and from the endosome.
[e]Specific monoclonal antibodies can be purchased from Molecular Probes.
[f]Complex of peripheral and integral membrane proteins.
[g]Peripheral membrane protein(s).
[h]The processed vacuolar form of carboxypeptidase S is a soluble protein, whereas the precursor form is an integral membrane protein. Note that in protease-deficient mutant yeast, carboxypeptidase S is not processed into its soluble form.
[i]Polyclonal antiserum can be purchased from Sigma.

throughout the fractionation experiment, and (3) can be detected by immunological or enzymatic techniques. Proteins that associate with more than one organelle can also serve as markers if they are subjected to compartment-specific posttranslational modifications (e.g., glycosylation, propeptide cleavage). An overview of compartment-specific posttranslational modifications is presented in Table 3.7.9.

Examples of marker proteins for various organelles are supplied in Table 3.7.7. The distribution of marker proteins within a series of fractions can be followed using immunoprecipitation (*UNIT 7.2*) or immunoblotting (*UNIT 6.2*) techniques. To ensure the highest possible accuracy, it is important to determine the relative protein levels among the fractions within the linear ranges of the detection methods.

Several enzyme activities that are characteristic of specific organelles are listed in Table 3.7.8. In most cases, the use of marker proteins to analyze the distribution of organelles/membranes is now favored over the use of enzyme activities if the appropriate antibodies are available. Enzyme activity levels are more sensitive than are protein levels to differing experimental conditions. For example, enzyme activities can be significantly impaired by the lysis buffer or gradient medium, the loss of a needed cofactor, or partial proteolysis.

Protein Profiles

The proteins found in several different highly purified yeast organelle preparations have been analyzed by SDS-PAGE (reviewed in Zinser and Daum, 1995; *UNIT 6.1*). The typical protein pattern (or protein profile) can be used to analyze the purity of an organelle preparation. In addition, the apparent molecular weights and isoelectric points of characterized resident proteins have been used to generate theoretical two-dimensional protein distributions for particular organelles (Yeast Protein Database; Hodges et al., 1998). Several two-dimensional profiles of *S. cerevisiae* proteins have been published (Singer-Kruger et al., 1993; Shevchenko et al., 1996; Garrels et al., 1997; Perrot et al., 1999), and it is likely that experimentally derived two-dimensional protein profiles for various organelle preparations will become increasingly available and informative in the near future.

Analysis of Protein Localization

To determine the subcellular localization of a novel protein (or a mutant form of a characterized protein), the distribution of the protein of interest is compared with the distribution of markers (proteins or enzyme activities) that have a well-characterized subcellular localization. The cofractionation of the protein of interest with a marker suggests, but does not prove, that the two components may be associated with the same organelle or membrane in vivo. A second method (e.g., immunofluorescence) should be used to confirm the steady-state localization of the protein of interest (Pringle et al., 1991; Hasek and Streiblova, 1996). In contrast, if the protein of interest fractionates away from a given marker protein, one can usually conclude that the two components have different subcellular distributions in vivo. However, this generalization does not apply when the protein of interest primarily cofractionates with soluble cytoplasmic marker proteins, because these data alone do not rule out the possibility that the protein is a released peripheral membrane protein or a soluble protein that normally resides within the lumen of an organelle instead of a native cytoplasmic protein.

Analysis of Organelles Along Protein Transport Pathways

The dynamic compartments along the secretory, endocytic, and vacuolar protein transport pathways can be particularly difficult to characterize (especially endosomes and transport vesicles), as they tend to have few proteins or enzyme activities that can serve as reliable steady-state markers. Many of the proteins associated with these organelles are either: (1) cargo molecules that transit along the transport pathways (e.g., newly synthesized membrane proteins, internalized components from the cell surface), (2) proteins that cycle between two or more of the compartments (e.g., cargo receptors, vesicle targeting molecules), or (3) molecules that only transiently associate with the compartment membranes (e.g., small GTPases, coat proteins).

Despite these complications, several techniques can be used to follow the organelles along the secretory, endocytic, and vacuolar protein transport pathways. For example, proteins that are actively retained within a compartment (and retrieved if they escape) have been used as markers for the endoplasmic reticulum (ER), Golgi complex, and plasma membrane, since their steady-state distribution strongly favors a single organelle type (see Table 3.7.7 for examples). In addition, proteins

Table 3.7.8 Enzyme Activities Associated with Subcellular Organelles

Primary location	Marker enzyme activities	Selected references[a]
Endoplasmic reticulum:		
Membrane	NAPDH-cytochrome *c* reductase	Kubota et al., 1977. *J. Biochem.* 81:197; Roberts et al., 1991. *Methods Enzymol.* 194:644
	Dolichyl phosphate mannose synthetase	Marriott and Tanner, 1979. *J. Bacteriol.* 139:566; Heesen et al., 1994. *Eur. J. Biochem.* 224:71
	Translocation activity (rough ER)[b]	Sanderson and Meyer, 1991. *JBC* 266:13423
Golgi:		
Membrane: *cis*	α1-6-Mannosyltransferase (Och1p)	Nakayama et al., 1992. *EMBO J.* 11:2511
	GDPase	Abeijon et al., 1989. *PNAS* 86:6935
Membrane: medial	α1-3-Mannosyltransferase (Mnn1p)	Nakajima and Ballou, 1975. *PNAS* 72:3912
Membrane: *trans*	Dipeptidyl aminopeptidase A (heat stable)	Julius et al., 1984. *Cell* 36:309; Roberts et al., 1991. *Methods Enzymol.* 194:644
	Kex2p	Brenner et al., 1994. *Methods Enzymol.* 244:152
Post-Golgi secretory vesicles:		
Lumen	Invertase; induced in low glucose	Goldstein and Lampen, 1975. *Methods Enzymol.* 42:504; Holcomb et al., 1987. *Anal. Biochem.* 166:328; Walworth and Novick, 1987. *JCB* 105:163
	Acid phosphatase; induced in low phosphate	Van Rijn et al., 1972. *BBA* 268:431; Holcomb et al., 1988. *JCB* 106:641
Plasma membrane:		
Membrane	H^+-ATPase (vanadate sensitive)[c]	Willsky, 1979. *JBC* 254:3326; Serrano, 1988. *Methods Enzymol.* 157:533; Serrano, 1988. *BBA* 947:1
	Chitin synthetase; also in chitosomes	Bowman and Slayman, 1979. *JBC* 254:2928
Vacuoles:		
Membrane	Alkaline phosphatase (ALP)	Roberts et al., 1991. *Methods Enzymol.* 194:644
	α-Mannosidase	Opheim, 1978. *BBA* 524:121; Roberts et al., 1991. *Methods Enzymol.* 194:644
	Dipeptidyl aminopeptidase B (heat sensitive)	Roberts et al., 1991. *Methods Enzymol.* 194:644
	H^+-ATPase (bafilomycin sensitive)[c]	Roberts et al., 1991. *Methods Enzymol.* 194:644

continued

that transit through multiple compartments can serve as transient markers for the transport intermediate of interest. Several techniques used to generate and monitor transient markers are summarized in Table 3.7.10. In some cases, the transit of a limited pool of proteins is followed until it reaches the compartment of interest. In addition, multiple investigators have successfully used temperature-sensitive mutants that block protein transport at specific steps and accumulate cargo molecules within a particular compartment. Multiple temperature-sensitive mutants that inhibit specific transport steps are available for the secretory pathway (e.g., *sec* mutants), the endocytic pathway (e.g., *end* mutants), and vacuolar protein transport pathway (e.g., *vps* mutants; see Table 3.7.10 for selected references). However, the results derived from mutant cells should only be extrapolated to normal cells after careful evaluation of the mutant cell properties and potential artifacts.

Analysis of Organelle Morphology, Integrity, and Function

The organelles and membranes within subcellular fractions can also be analyzed by electron microscopy. Some organelles, such as

Table 3.7.8 Enzyme Activities Associated with Subcellular Organelles, continued

Primary location	Marker enzyme activities	Selected references[a]
Matrix	Carboxypeptidase Y (CPY)	Jones, 1991. *JBC* 266:7963
	Carboxypeptidase S (CPS)	Distel et al., 1983. *BBA* 741:128
	Proteinase A (PrA), proteinase B (PrB)	Jones, 1991. *JBC* 266:7963
	Aminopeptidase I (API)	Distel et al., 1983. *BBA* 741:128
Peroxisomes:		
Peripheral membrane	Acyl-CoA oxidase[b]	Dommes et al., 1981. *JBC* 256:8259; Kionka and Kunau, 1983. *J. Bacteriol.* 161:153
Matrix	Catalase A	Luck, 1963 (see Literature Cited); Ueda et al., 1991. *Methods Enzymol.* 188:463
	3-Oxoacyl-CoA thiolase	Kionka and Kunau, 1983. *J. Bacteriol.* 161:153
	Citrate synthase	Lewin et al., 1990. *MCB* 10:1399
Mitochondria:		
Inner membrane	Cytochrome *c* oxidase	Mason et al., 1973. *JCB* 248:1346; Poyton et al., 1995. *Methods Enzymol.* 260:97
	Succinate dehydrogenase	Ackrell et al., 1978. *Methods Enzymol.* 53:466
	H^+-ATPase (oligomycin sensitive)[c]	Roberts et al., 1991. *Methods Enzymol.* 194:644
Intermembrane space	Cytochrome *b2*	Daum et al., 1982. *JBC* 257:13075
	Cytochrome *c* peroxidase	Daum et al., 1982. *JBC* 257:13028
Matrix	Fumarase	Daum et al., 1982. *JBC* 257:13028
Cytoplasm:		
	Glucose-6-phosphate dehydrogenase	Roberts et al., 1991. *Methods Enzymol.* 194:644

[a]Journal abbreviations: *BBA*, *Biochim. Biophys. Acta*; *JBC*, *J. Biol. Chem.*; *JCB*, *J. Cell Biol.*; *MCB*, *Mol. Cell Biol.*; *PNAS*, *Proc. Natl. Acad. Sci. U.S.A.*

[b]Peripheral membrane protein(s).

[c]Complex of peripheral and integral membrane proteins.

intact mitochondria, retain recognizable features after their isolation (e.g., Aris and Blobel, 1991; Glick and Pon, 1995). However, most organelles and membranes have similar tubular and/or vesicular appearances after homogenization and fractionation, and thus cannot be identified by their morphology alone. In these cases, analysis by immunocytochemistry, immunoelectron microscopy (*UNITS 4.3, 4.4 & 4.6*), or other marker-based techniques is advisable (e.g., Byers and Goetsch, 1991; Clark, 1991; Hasek and Streiblova, 1996; Rieder et al., 1996; Hicke et al., 1997; Prescianotto-Baschong and Riezman, 1998).

The structural integrity of an organelle can be monitored by measuring the latency of marker proteins or enzymes that normally reside within the lumen of an organelle. Morphological analysis can also reveal the integrity of certain organelles, especially mitochondria and nuclei. In addition, the integrity and orientation of organelle and vesicle membranes can be examined using protease- and antibody-accessibility experiments (see *UNIT 5.4*).

The intracellular functions of several organelles have been successfully reconstituted in a test tube (e.g., Daum et al., 1982a,b; Baker et al., 1988; Vida et al., 1990; Lazarow et al., 1991; Brodsky et al., 1993). These in vitro assays are essential tools for studying complex cellular processes, as the conditions and corresponding consequences can be manipulated and analyzed. In addition, in vitro assays can be used to monitor the functional integrity of the organelle of interest and thus the quality of an organelle preparation.

Table 3.7.9 Compartment-Specific Posttranslational Protein Modifications

Compartment	Posttranslational modification
Mitochondria:	
Intermembrane space	Heme attachment
	Membrane anchor cleavage
Matrix	Leader peptide cleavage
Endoplasmic reticulum:	
Lumen	Signal peptide cleavage
	Core N-linked[a] oligosaccharide addition
	Core O-linked[a] oligosaccharide addition
	Cleavage of glucose from N-linked oligosaccharides
Golgi:	
cis cisternae	Initial α1-6-linked oligosaccharide addition
	α1-6-linked oligosaccharide elongation
Medial cisternae	α1-3-linked oligosaccharide addition
	α1-2-linked oligosaccharide addition
	Elongation of O-linked oligosaccharides
trans cisternae	Lysine-arginine dibasic endoproteolysis
	Cleavage by dipeptidyl aminopeptidase A (DPAP-A)
Vacuoles:	
Lumen	Propeptide cleavage and activation of hydrolases

[a]N-linked, asparagine-linked; O-linked, serine- or threonine-linked.

Table 3.7.10 Application of Transient Markers to Identify Compartments Along the Secretory, Endocytic, and Vacuolar Protein Transport Pathways[a]

General principle	Specific techniques
Follow transit of limited protein pool until it reaches compartment of interest	Briefly label newly synthesized proteins with [^{35}S]cysteine/methionine and analyze compartments after appropriate chase times Briefly label cell surface proteins with ^{125}I and analyze endocytic compartments after appropriate chase times Follow radiolabeled ligand (e.g., [^{35}S]α-factor) to analyze endocytic compartments after appropriate chase times Transiently induce/derepress translation of protein known to transit through compartment(s) of interest Localize cargo proteins by analyzing their compartment-specific posttranslational modifications (Table 3.7.9)
Slow or block protein transport at specific step to accumulate cargo proteins in compartment of interest	Block protein transport at specific step along transport pathway using temperature-conditional mutants (e.g., *sec*, *end*, *vps* mutants) Slow protein transport through endosomal system by cooling cells to 15°C

[a]Selected references: Schekman (1985); Rothblatt and Schekman (1989); Walworth et al. (1989); Singer and Riezman (1990); Vida et al. (1990); Dulic et al. (1991); Franzusoff et al. (1991); Raths et al. (1993); Horazdovsky et al. (1995); Wuestehube et al. (1996); Hicke et al. (1997); Rieder and Emr (1997).

LITERATURE CITED

Aris, J.P. and Blobel, G. 1991. Isolation of yeast nuclei. *Methods Enzymol.* 194:735-749.

Babst, M., Wendland, B., Estepa, E.J., and Emr, S.D. 1998. The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. *EMBO J.* 17:2982-2993.

Baker, D., Hicke, L., Rexach, M., Schleyer, M., and Schekman, R. 1988. Reconstitution of *SEC* gene product-dependent intercompartmental protein transport. *Cell* 54:335-344.

Botstein, D., Chervitz, S.A., and Cherry, J.M. 1997. Yeast as a model organism. *Science* 277:1259-1260.

Brodsky, J.L., Hamamoto, S., Feldheim, D., and Schekman, R. 1993. Reconstitution of protein translocation from solubilized yeast membranes reveals topologically distinct roles for BiP and cytosolic Hsc70. *J Cell Biol.* 120:95-102.

Byers, B. and Goetsch, L. 1991. Preparation of yeast cells for thin-section electron microscopy. *Methods Enzymol.* 194:602-608.

Cereghino, J.L., Marcusson, E.G., and Emr, S.D. 1995. The cytoplasmic tail domain of the vacuolar protein sorting receptor Vps10p and a subset of *VPS* gene products regulate receptor stability, function, and localization. *Mol. Biol. Cell* 6:1089-1102.

Cherry, J.M., Ball, C., Weng, S., Juvik, G., Schmidt, R., Adler, C., Dunn, B., Dwight, S., Riles, L., Mortimer, R.K., and Botstein, D. 1997. Genetic and physical maps of *Saccharomyces cerevisiae*. *Nature* 387:67-73.

Clark, M.W. 1991. Immunogold labeling of yeast ultrathin sections. *Methods Enzymol.* 194:608-626.

Clayton, R.A., White, O., Ketchum, K.A., and Venter, J.C. 1997. The yeast genome directory: The first genome from the third domain of life. *Nature* 387:459-462.

Daum, G., Bohni, P.C., and Schatz, G. 1982a. Import of proteins into mitochondria. Cytochrome *b2* and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* 257:13028-13033.

Daum, G., Gasser, S.M., and Schatz, G. 1982b. Import of proteins into mitochondria. Energy-dependent, two-step processing of the intermembrane space enzyme cytochrome *b2* by isolated yeast mitochondria. *J. Biol. Chem.* 257:13075-13080.

Dobrota, M. and Hinton, R. 1992. Conditions for density gradient separations. *In* Preparative Centrifugation: A Practical Approach (D. Rickwood, ed.) pp. 77-137. IRL Press, New York.

Dulic, V., Egerton, M., Elguindi, I., Raths, S., Singer, B., and Riezman, H. 1991. Yeast endocytosis assays. *Methods Enzymol.* 194:697-710.

Evans, W.H. 1992. Isolation and characterization of membranes and cell organelles. *In* Preparative Centrifugation: A Practical Approach (D. Rickwood, ed.) pp. 233-270. IRL Press, New York.

Franzusoff, A., Rothblatt, J., and Schekman, R. 1991. Analysis of polypeptide transit through yeast secretory pathway. *Methods Enzymol.* 194:662-674.

Garrels, J.I., McLaughlin, C.S., Warner, J.R., Futcher, B., Latter, G.I., Kobayashi, R., Schwender, B., Volpe, T., Anderson, D.S., Mesquita-Fuentes, R., and Payne, W.E. 1997. Proteome studies of *Saccharomyces cerevisiae*: Identification and characterization of abundant proteins. *Electrophoresis* 18:1347-1360.

Gaynor, E.C., te Heesen, S., Graham, T.R., Aebi, M., and Emr, S.D. 1994. Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* 127:653-665.

Glick, B.S. and Pon, L.A. 1995. Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol.* 260:213-23.

Hasek, J., and Streiblova, E. 1996. Fluorescence microscopy methods. *Methods Mol. Biol.* 53:391-405.

Hicke, L., Zanolari, B., Pypaert, M., Rohrer, J., and Riezman, H. 1997. Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires an active secretory pathway and Sec18p/N-ethylmaleimide-sensitive fusion protein. *Mol. Biol. Cell* 8:13-31.

Hinton, R.H. and Mullock, B.M. 1997. Isolation of subcellular fractions. *In* Subcellular Fractionation: A Practical Approach (J. M. Graham and D. Rickwood, eds.) pp. 31-69. IRL Press, New York.

Hodges, P.E., Payne, W.E., and Garrels, J.I. 1998. The Yeast Protein Database (YPD): A curated proteome database for *Saccharomyces cerevisiae*. *Nucl. Acids Res.* 26:68-72.

Horazdovsky, B.F., DeWald, D.B., and Emr, S.D. 1995. Protein transport to the yeast vacuole. *Curr. Opin. Cell Biol.* 7:544-551.

Jones, E.W. 1990. Vacuolar proteases in yeast *Saccharomyces cerevisiae*. *Methods Enzymol.* 185:372-386.

Jones, E.W. 1991a. Tackling the protease problem in *Saccharomyces cerevisiae*. *Methods Enzymol.* 194:428-453.

Jones, E.W. 1991b. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* 266:7963-7966.

Klionsky, D.J. and Emr, S.D. 1989. Membrane protein sorting: Biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J.* 8:2241-2250.

Lazarow, P.B., Thieringer, R., Cohen, G., Imanaka, T., and Small, G. 1991. Protein import into peroxisomes in vitro. *Methods Cell Biol.* 34:303-326.

Luck, H. 1963. Catalase. *In* Methods of Enzymatic Analysis (H.U. Bergmeyer, ed.) pp. 885-894. Academic Press, New York.

Marcusson, E.G., Horazdovsky, B.F., Cereghino, J.L., Gharakhanian, E., and Emr, S.D. 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the *VPS10* gene. *Cell* 77:579-586.

Mewes, H.W., Albermann, K., Bahr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S.G., Pfeiffer, F., and Zollner, A. 1997. Overview of the yeast genome. *Nature* 387:7-65.

Perrot, M., Sagliocco, F., Mini, T., Monribot, C., Schneider, U., Shevchenko, A., Mann, M., Jeno, P., and Boucherie, H. 1999. Two-dimensional gel protein database of *Saccharomyces cerevisiae* (update 1999). *Electrophoresis* 20:2280-2298.

Prescianotto-Baschong, C. and Riezman, H. 1998. Morphology of the yeast endocytic pathway. *Mol. Biol. Cell* 9:173-189.

Pringle, J.R., Adams, A.E., Drubin, D.G., and Haarer, B.K. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* 194:565-602.

Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. 1993. *end3* and *end4*: Two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:55-65.

Rieder, S.E. and Emr, S.D. 1997. A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. *Mol. Biol. Cell* 8:2307-2327.

Rieder, S.E., Banta, L.M., Kohrer, K., McCaffery, J.M., and Emr, S.D. 1996. Multilamellar endosome-like compartment accumulates in the yeast *vps28* vacuolar protein sorting mutant. *Mol. Biol. Cell* 7:985-999.

Romanos, M.A., Scorer, C.A., and Clare, J.J. 1992. Foreign gene expression in yeast: A review. *Yeast* 8:423-488.

Rothblatt, J. and Schekman, R. 1989. A hitchhiker's guide to analysis of the secretory pathway in yeast. *Methods Cell Biol.* 32:3-36.

Schekman, R. 1985. Protein localization and membrane traffic in yeast. *Annu. Rev. Cell Biol.* 1:115-143.

Scott, J.H. and Schekman, R. 1980. Lyticase: Endoglucanase and protease activities that act together in yeast cell lysis. *J. Bacteriol.* 142: 414-423.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol.* 194:3-21.

Shevchenko, A., Jensen, O.N., Podtelejnikov, A.V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H., and Mann, M. 1996. Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci. U.S.A.* 93:14440-14445.

Singer, B. and Riezman, H. 1990. Detection of an intermediate compartment involved in transport of alpha-factor from the plasma membrane to the vacuole in yeast. *J. Cell Biol.* 110:1911-1922.

Singer-Kruger, B., Frank, R., Crausaz, F., and Riezman, H. 1993. Partial purification and characterization of early and late endosomes from yeast. Identification of four novel proteins. *J. Biol. Chem.* 268:14376-14386.

Van Den Hazel, H.B., Kielland-Brandt, M.C., and Winther, J.R. 1996. Review: Biosynthesis and function of yeast vacuolar proteases. *Yeast* 12:1-16.

Vida, T.A., Graham, T.R., and Emr, S.D. 1990. In vitro reconstitution of intercompartmental protein transport to the yeast vacuole. *J. Cell Biol.* 111:2871-2884.

Walworth, N.C., Goud, B., Ruohola, H., and Novick, P.J. 1989. Fractionation of yeast organelles. *Methods Cell Biol.* 31:335-356.

Westphal, V., Marcusson, E.G., Winther, J.R., Emr, S.D., and van den Hazel, H.B. 1996. Multiple pathways for vacuolar sorting of yeast proteinase A. *J. Biol. Chem.* 271:11865-11870.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H., Chu, A.M., Connelly, C., Davis, K., Dietrich, F., Dow, S.W., El Bakkoury, M., Foury, F., Friend, S.H., Gentalen, E., Giaever, G., Hegemann, J.H., Jones, T., Laub, M., Liao, H., Davis, R.W., et al. 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 285:901-906.

Wuestehube, L.J., Duden, R., Eun, A., Hamamoto, S., Korn, P., Ram, R., and Schekman, R. 1996. New mutants of *Saccharomyces cerevisiae* affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. *Genetics* 142:393-406.

Zinser, E. and Daum, G. 1995. Isolation and biochemical characterization of organelles from the yeast, *Saccharomyes cerevisiae. Yeast* 11:493-536.

Zubenko, G.S., Park, F.J., and Jones, E.W. 1983. Mutations in *PEP4* locus of *Saccharomyces cerevisiae* block final step in maturation of two vacuolar hydrolases. *Proc. Natl. Acad. Sci. U.S.A.* 80:510-514.

KEY REFERENCES

Dobrota and Hinton, 1992. See above.

This review provides an excellent overview of the theory and design of subcellular fractionation experiments. Other helpful reviews include Evans (1992) and Hinton and Mullock (1997; see above).

Sikorski, R. and Peters, R. 1998. Yeast sites on the net. *Science* 279:1968.

This review provides information on multiple Internet resources available for S. cerevisiae.

Walworth et al., 1989. See above.

This older review provides an excellent summary of the subcellular fractionation techniques applied to S. cerevisiae, with a focus on organelles along the secretory pathway.

Zinser and Daum, 1995. See above.

This review provides an excellent summary of the subcellular fractionation techniques applied to S. cerevisiae and the corresponding references.

INTERNET RESOURCES

http://genome-www.stanford.edu/Saccharomyces/

Saccharomyces Genome Database (SGD) provides access to the complete sequence of the S. cerevisiae genome, confirmed and predicted open reading frames (ORFs), protein information, and a number of useful Internet links.

http://www.atcc.org/searchengine/ygsc.html

Yeast Genetic Stock Center at the ATCC, which can provide many published yeast strains.

http://www.proteome.com/

The Yeast Protein Database (Proteome, Inc.) is an excellent source of comprehensive and up-to-date information on S. cerevisiae proteins. The information is derived from the published literature and DNA sequence databases. The protein information provided, including summaries of the protein and gene characteristics and the corresponding literature references, can be searched and categorized in several convenient ways (e.g., according to subcellular location, gene name, or size).

Contributed by Stephanie E. Rieder and Scott D. Emr
University of California, San Diego
La Jolla, California

Isolation of Subcellular Fractions from the Yeast *Saccharomyces cerevisiae*

The refinement of subcellular fractionation techniques for the budding yeast *Saccharomyces cerevisiae* has provided a valuable approach for investigating the molecular mechanisms of complex cellular functions. The protocols presented in this unit cover a range of centrifugation-based procedures used to fractionate subcellular components from *S. cerevisiae* according to their sizes and/or buoyant densities. The key goals and techniques of each protocol are summarized in Table 3.8.1. In addition, many other *S. cerevisiae* subcellular fractionation procedures and the corresponding references are listed in Table 3.8.2. An overview of centrifugation-based fractionation procedures for *S. cerevisiae* is presented in *UNIT 3.7*.

The basic methods used to isolate and characterize organelles from *S. cerevisiae* are very similar to those used for mammalian cells and other higher eukaryotes. The most appropriate subcellular fractionation procedure will vary with the goals of the particular experiment and the desired levels of organelle yield, purity, and function. The merits, limitations, and critical parameters of various *S. cerevisiae* techniques are discussed in *UNIT 3.7* to facilitate the development of successful subcellular fractionation experiments.

Yeast cells can be cultured using relatively simple techniques (*UNIT 1.6*) and are extremely versatile. For example, the abundance of certain enzymes and organelles that are subject to metabolic regulation can be manipulated by varying the nutrient composition of the medium. The optimal development of mitochondria and peroxisomes induced by growth on lactate or oleate, respectively, is critical to the effective isolation of these organelles (see Basic Protocols 6 and 7). In addition, the availability of mutant strains is also beneficial. For example, the use of a strain that is deficient in vacuolar protease activity can minimize problems with proteolysis.

In contrast to animal cells, yeast cells have a thick cell wall that must be disrupted or removed in order to generate cell lysates. The disruption of the cell wall by shearing with glass beads is a rapid and effective method to prepare yeast cell homogenates for the isolation of plasma membranes (see Basic Protocol 9) and cytosol (see Basic Protocol 10). However, glass bead lysis is not suitable for most subcellular fractionation experiments, since the strong shear forces required to disrupt the cell wall can also damage intracellular organelles and disrupt protein and membrane interactions. To minimize the disruption of subcellular integrity, yeast cells are usually first enzymatically converted to spheroplasts (yeast cells lacking cell walls) prior to cell lysis (see Support Protocol). Unlike intact yeast cells, spheroplasts are fragile and osmotically sensitive; thus they can be lysed under isoosmotic or hypoosmotic conditions, usually in combination with moderate shear force (e.g., several strokes in a Dounce glass homogenizer). Multiple enzyme preparations that digest the cell wall by cleaving the critical β-glucan linkages are available, such as Zymolyase 100T (see Support Protocol) and oxalyticase (see Basic Protocol 4).

NOTE: All references to "yeast" within this unit refer to the budding yeast *S. cerevisiae*. Many protocols developed for *S. cerevisiae* are not directly applicable to other yeasts (e.g., *Schizosaccharomyces pombe*) without significant modification. *S. cerevisiae* protocols can be applied to many different *S. cerevisiae* strains; however, variations among strains and growth conditions may require adjustments to a given protocol.

Contributed by Stephanie E. Rieder and Scott D. Emr
Current Protocols in Cell Biology (2000) 3.8.1-3.8.68

Table 3.8.1 Summary of the Primary Goals and Techniques of the Protocols[a]

Protocol	Primary goals	Key techniques
Basic Protocol 1	Separate lysate into a cytosol fraction and several membrane fractions (roughly according to size; see Table 3.7.2, Table 3.7.4, and Figure 3.7.1) Prepare enriched membrane fractions for subsequent procedures	Spheroplast preparation (Support Protocol) Dounce homogenization Sequential centrifugation steps
Support Protocol	Generate spheroplasts (for subsequent lysate preparation)	Cell culture Cell wall digestion (Zymolyase 100T)
Basic Protocol 2	Separate endosomal and Golgi membranes from vacuolar membranes	Spheroplast preparation (Support Protocol) Homogenization and centrifugation (Basic Protocol 1) Equilibrium Nycodenz gradients
Alternate Protocol	Separate endosomal and vacuolar membranes from Golgi membranes	Spheroplast preparation (Support Protocol) Homogenization and centrifugation (Basic Protocol 1) Equilibrium sucrose gradients
Basic Protocol 3	Subfractionate 13,000 × *g* pellet: separate PM and ER membranes from vacuolar and Golgi membranes	Spheroplast preparation (Support Protocol) Homogenization and centrifugation (Basic Protocol 1) Sucrose step gradients
Basic Protocol 4	Isolate intact vacuoles	Spheroplast preparation (oxylyticase) DEAE-isoosmotic spheroplast lysis Ficoll 400 step gradients
Basic Protocol 5	Isolate intact nuclei	Spheroplast preparation (Zymolyase 100T and Glusulase) Homogenization and differential centrifugation Ficoll 400 step gradients
Basic Protocol 6	Isolate lactate-induced mitochondria	Induction of mitochondria (lactate as carbon source) Spheroplast preparation (Zymolyase 20T) Homogenization and differential centrifugation Nycodenz step gradients
Basic Protocol 7	Isolate oleate-induced peroxisomes	Induction of peroxisomes (oleate as carbon source) Spheroplast preparation (Zymolyase 100T) Homogenization and differential centrifugation Sucrose step gradient
Basic Protocol 8	Isolate ER membranes	Spheroplast preparation (Support Protocol) Homogenization and differential centrifugation Sucrose step gradient
Basic Protocol 9	Isolate plasma membranes	Glass bead lysis Differential centrifugation Sucrose step gradient
Basic Protocol 10	Prepare “active” cytosol (e.g., for functional in vitro assays)	Glass bead lysis Centrifugation

[a]Abbreviations: ER, endoplasmic reticulum; PM, plasma membrane.

NOTE: Operations during and after cell lysis should be conducted at 0° to 4°C (unless otherwise indicated). In addition, solutions and equipment to be used during and after cell lysis should be chilled to 0° to 4°C prior to their use (unless otherwise indicated).

NOTE: Phenylmethylsulfonyl fluoride (PMSF) and other protease inhibitors are toxic and should be handled with care. They should be added to solutions just before use.

FRACTIONATION OF SPHEROPLASTS BY DIFFERENTIAL CENTRIFUGATION

BASIC PROTOCOL 1

The protocol presented below is a differential centrifugation protocol that separates subcellular components from yeast spheroplasts into several fractions, roughly according to their size. Differential centrifugation is a component of most subcellular fractionation experiments, since it provides a way to enrich for the organelle or membrane of interest, to deplete undesired components, and/or reduce the sample volume. Differential centrifugation is also used to analyze the potential subcellular location(s) of a protein of interest (see Table 3.7.4).

Yeast cells in their exponential growth phase are first converted to spheroplasts (see Support Protocol, steps 4 to 15). Alternate spheroplast preparation procedures can also be used; e.g., Basic Protocol 4, Basic Protocol 5, or numerous other protocols. The spheroplasts are lysed using a combination of osmotic shock and Dounce homogenization. Subsequently, the lysates are subjected to sequential centrifugation runs at increasing speeds (see Figure 3.7.1). The resulting fractions include the 300 × *g* pellet (P_{300}) and supernatant (S_{300}), the 13,000 × *g* pellet ($P_{13,000}$) and supernatant ($S_{13,000}$), and the 100,000 × *g* pellet ($P_{100,000}$) and supernatant ($S_{100,000}$) fractions.

If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., BJ3505, a *pep4 prb1* mutant yeast strain). The cell number required will depend on the goals of the experiment. This protocol is written for relatively small-scale experiments (10 to 40 OD_{600} units of cells) but can be adapted to experiments requiring more cells. When investigating the localization of a protein of interest, it is helpful to use enough cells so that one can set aside an aliquot of each fraction and monitor the yield of each centrifugation step.

Materials

Desired yeast cultures in their exponential growth
Stop solution or spheroplast medium B (see recipe; optional), ice cold
HEPES/potassium acetate (HEPES/KAc) lysis buffer (see recipe), ice cold
500× protease inhibitor cocktails A and B (500× PIC-A and 500× PIC-B; see recipes)
Protease inhibitor stock solutions A (see recipe)

Dounce glass homogenizer, tight-fitting pestle, prechilled to 4°C
1.6-ml microcentrifuge tubes, prechilled to 4°C
Microcentrifuge prechilled to 4°C
1.6-ml polycarbonate tubes for ultracentrifugation at 100,000 × *g*, prechilled on ice
Beckman refrigerated tabletop ultracentrifuge (or equivalent) and rotor (e.g., Beckman TLA100.3 or equivalent)

Additional reagents and equipment for growing yeast cells (see *UNIT 1.6*) and preparation of spheroplasts (see Support Protocol)

Prepare and harvest cells

1. Culture desired yeast strains and prepare spheroplasts (Support Protocol, steps 1 to 15).

   From this point on, keep samples on ice or at 4°C.

   Spheroplasts are fragile and must be handled very gently.

2. Resuspend the spheroplasts at a concentration of 5 to 10 OD_{600} units/ml in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

   The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

   Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

   Stop solution contains two energy poisons (i.e., NaN_3 and NaF) that are toxic and should be handled with care. The inclusion of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

3. To harvest spheroplasts centrifuge 5 min at 1500 × *g*, 4°C. Aspirate or decant the supernatants.

   Use care while removing supernatants, because spheroplast pellets will be relatively loose and fluffy. In addition, the emergence of CO_2 bubbles from the spheroplasts can significantly disrupt the pellets.

Lyse spheroplasts

4. Gently resuspend spheroplasts at a concentration of 5 OD_{600} units/ml in ice-cold HEPES/KAc lysis buffer.

   The spheroplasts can also be lysed at higher concentrations (i.e., 5 to 15 OD_{600} units/ml) for large-scale preparations or if it is crucial to minimize the lysate volume.

5. Transfer spheroplast suspension to a Dounce homogenizer that has been prechilled in an ice bucket. Disrupt spheroplasts with 10 up-and-down strokes of a tight-fitting pestle.

   Keep the Dounce homogenizer on ice throughout this process. Move the pestle slowly and maintain contact between pestle and suspension to minimize foaming.

6. Transfer lysate to multiple prechilled 1.6-ml microcentrifuge tubes.

   It is often convenient to make 1-ml aliquots of the lysate (e.g., each containing 5 OD_{600} cell equivalents). Larger microcentrifuge tubes can also be used; however, the use of 1.6-ml microcentrifuge tubes and a refrigerated microcentrifuge is often faster and more convenient.

   In order to monitor recovery levels in the fractions generated by the subsequent microcentrifugation steps, it is often beneficial to set aside an aliquot of the lysate that will not be fractionated (e.g., 1 ml or 5 OD_{600} cell equivalents).

Clear lysates

7. Clear the lysates of unbroken cells, partially disrupted cells, and aggregates by centrifuging 5 min at 300 × *g*, 4°C.

8. Carefully withdraw the supernatant (S_{300}) without disturbing the very loose, fluffy pellet (P_{300}), and transfer to new prechilled 1.6-ml tubes. Record the volumes of the transferred S_{300}.

   Usually only ~80% of S_{300} can be harvested from each tube without disturbing the pellets.

   In order to monitor the recovery levels in the fractions generated by the subsequent centrifugation steps at 13,000 × g and 100,000 × g, it is often beneficial to set aside an aliquot of the S_{300} that will not be fractionated further.

   Also, it is critical to record the volume of harvested supernatant at each step (as it is never 100%) in order to follow the yield of each centrifugation step.

9. *Optional:* Resuspend the P_{300} by pipetting up and down ten times using a micropipettor with a 200-µl pipet tip. Add HEPES/KAc lysis buffer to a total volume of ~500 µl and mix with a cut pipet tip or by vortexing. Repeat steps 7 and 8. Pool the first and second S_{300} supernatants.

   Alternatively, the sample can be transferred to a clean, chilled Dounce homogenizer and dispersed with several gentle strokes.

   This washing step can improve the yield of intracellular membranes and purity of P_{300} fractions by removing some of the smaller organelles trapped within P_{300} pellets. This step is critical if the P_{300} will be retained for further analyses (e.g., to examine ER and plasma membrane distributions, since up to 50% of these membranes can pellet at 300 × g; see Table 3.7.4). However, it is not always recommended, since it increases the sample volume, processing time, and likelihood of organelle damage.

   The P_{300} pellets not required for subsequent analyses are often discarded since they contain a crude mixture of unlysed and partially disrupted cells, as well as a very heterogeneous mixture of intracellular membranes.

Prepare 13,000 × g fractions

10. Microcentrifuge S_{300} supernatants 10 min at 13,000 × *g*, 4°C.

11. Carefully transfer the supernatants ($S_{13,000}$) to prechilled 1.6-ml polycarbonate ultracentrifuge tubes and keep on ice.

    In order to monitor the recovery in the fractions generated by the subsequent centrifugation at 100,000 × g, it is often beneficial to set aside an aliquot of the $S_{13,000}$ that will not be fractionated further.

12. To remove any residual supernatant from the $P_{13,000}$ pellets and tube walls, microcentrifuge the tubes containing the $P_{13,000}$ samples ~30 sec at 13,000 × *g,* 4°C. Carefully remove any remaining $S_{13,000}$ above pellet.

    If the residual $S_{13,000}$ has a small volume (e.g., ≤10 µl), discard it; otherwise, add it to the appropriate $S_{13,000}$ fraction.

13. Add ice-cold HEPES/KAc lysis buffer (or another appropriate buffer) with freshly added protease inhibitors (PIC-A, PIC-B, protease inhibitor stock solution A) to the $P_{13,000}$ pellets. Briefly mix using a micropipettor or by vortexing. Cap tubes and store on ice. Quickly move on to step 14.

    The appropriate buffer and volume will depend on the goals and procedures of subsequent experiments. It is critical, however, that the buffer contain fresh protease inhibitors.

Prepare 100,000 × g fractions

14. Balance the $S_{13,000}$ samples by adding ice-cold HEPES/KAc lysis buffer as needed. Centrifuge $S_{13,000}$ samples 60 min at 100,000 × *g,* 4°C, in a fixed-angle rotor (e.g., 55,000 rpm for the Beckman TLA100.3 rotor).

15. *Optional:* During the 60-min centrifugation, process the $P_{13,000}$ pellets further as needed.

    If it is critical to disperse the membranes for subsequent fractionation steps, transfer the suspension to a small chilled Dounce homogenizer and apply several up-and-down strokes until the pellet is disrupted.

16. After ultracentrifugation, carefully transfer the supernatants ($S_{100,000}$) to clean, pre-chilled microcentrifuge tubes and store on ice.

17. To remove residual supernatant from pellets ($P_{100,000}$) and tube walls, centrifuge tubes with $P_{100,000}$ pellets ~30 sec at 13,000 × *g*, 4°C. Carefully remove and discard any remaining supernatant above the $P_{100,000}$ pellet.

18. Add ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors (or another appropriate buffer) to $P_{100,000}$ pellets. Quickly mix with a pipettor or vortexer. Keep on ice until needed.

    The appropriate buffer and volume will depend on the goals and procedures of subsequent experiments. It is critical, however, that the buffer be ice-cold and contain fresh protease inhibitors.

    The $P_{100,000}$ pellets are usually very difficult to resuspend. If it is critical to disperse the membranes for subsequent experiments, transfer the suspension to a small chilled Dounce homogenizer and apply several up-and-down strokes until the pellet is disrupted. It also can be helpful to allow the pellets to "soften" by incubating the pellets with buffer on ice. If the membranes do not need to be intact for further fractionation experiments, the pellet can be quickly resuspended in a small volume of buffer (~100 µl) using a water sonication bath.

SUPPORT PROTOCOL

PREPARATION OF YEAST SPHEROPLASTS USING ZYMOLYASE

The preparation of yeast spheroplasts through enzymatic digestion of the cell wall is the first step for most subcellular fractionation experiments. This protocol describes a method to prepare exponentially growing yeast cultures and convert yeast cells into spheroplasts using Zymolyase 100T, a β-glucanase preparation. Digestion of the cell wall is monitored by spectrophotometry and microscopy. This protocol can be adapted to any cell number or strain.

Additional Materials *(also see Basic Protocol 1)*

Exponentially growing yeast cell cultures in yeast extract/peptone/dextrose (YPD) medium (see *UNIT 1.6*) or appropriate medium
TSD reduction buffer (see recipe)
Spheroplast medium A (see recipe)
5 mg/ml Zymolyase 100T (ICN Immunobiochemicals) in spheroplast medium A, (store in aliquots at −20°C)
Spheroplast medium B (see recipe), ice cold

50-ml glass culture tubes *or* 100-ml Erlenmeyer flasks
Shaker platform at 30°C (or appropriate growth temperature of the strains being used)
250-ml centrifuge bottles
50-ml centrifuge tubes
Centrifuge and rotor (e.g., Sorvall GS-3 rotor)
30°C water bath

Additional reagents and equipment for culturing yeast cells (*UNIT 1.6*)

Preculture cells

1. In the morning, a day before the experiment, start precultures of the desired yeast strains by inoculating 10 ml YPD (or appropriate medium) with a single yeast colony from a fresh plate.

   The cultures can be grown in 50-ml glass culture tubes or 100-ml Erlenmeyer flasks.

   If using strains that grow very slowly or that have been stored on a plate at 4°C for more than a few days, it is advised that the preculture be started with a single colony the night before.

2. Culture cells throughout the day while shaking at 225 to 325 rpm at 30°C (or appropriate permissive temperature).

3. In the evening, a day before the experiment, determine cell density of the precultures by measuring the OD_{600}.

   For the most accurate measurement, dilute the culture at 1:5 or 1:10 in water, measure OD_{600}, and then multiply the reading by 5 or 10, respectively. The correlation between the cell number and the OD_{600} is usually most linear between an OD_{600} of 0.1 to 0.3 (see UNIT 3.7).

4. After completing the calculations below, use the appropriate amount of the preculture to inoculate fresh cultures for overnight growth.

   The suitable yeast cell concentration is determined by the growth rate of each strain (i.e., the doubling time), the desired incubation time (usually overnight), and the target cell density. For most applications, the target OD_{600} corresponds to the cell density when the cultures are in their mid-logarithmic growth phase. The culture OD_{600} during this rapid growth phase varies with the strain and growth conditions, but is often between 0.6 and 1.0. The required culture volumes will depend on the number of cells needed. It is best to start slightly larger cultures than required in case the cell density does not quite reach the expected level (see calculations below and UNITS 1.6 & 3.7).

   a. Calculate desired culture volume as follows:

   minimum culture volume (ml) = (required OD_{600} equivalents of cells/target OD_{600}) $\times$ 1.25

   Example: If 20 OD_{600} equivalents of a strain are required and the target cell density is at an OD_{600} of 0.8, then a 30-ml culture should be started, since (20/0.8) $\times$ 1.25 = 31.25 ml.

   b. Calculate the inoculation cell density (OD_{600}):

   inoculation OD_{600} = target $OD_{600} \times 2^{(\text{no. of doublings})}$

   Examples: A strain with a doubling time of 2.5 hr should be inoculated at an OD_{600} of ~0.008 in order to reach an OD_{600} of ~1 after 17.5 hr of growth (7 generations), since $1 \times 2^{-4} = 0.008$. A strain with a doubling time of 1.75 hr should be inoculated at an OD_{600} of ~0.001 in order to reach an OD_{600} of ~1 after 17.5 hr of growth (10 generations), since $1 \times 2^{-10} = 0.001$.

5. In the morning of the day that the spheroplasts are needed, measure the OD_{600} of cultures. Continue to culture the cells until they are within their target density range (i.e., mid-logarithmic growth phase).

   If the cultures have significantly exceeded the maximum target density (i.e., by >20%), dilute the cultures (e.g., 1:4 to 1:6 dilution in medium) and allow them to grow for at least two more generations before harvesting.

6. Harvest cells by centrifuging 5 min at 4000 $\times$ *g* (e.g., 5000 rpm in a Sorvall GS-3 rotor), room temperature, and carefully remove supernatant.

Prepare spheroplasts

7. Resuspend cells at 5 to 10 OD_{600} units/ml in TSD reduction buffer and incubate 10 min at room temperature.

   The DTT treatment facilitates cell wall digestion by breaking disulfide bonds and making the β-glucan linkages more accessible to the β-glucanase activity present in the Zymolyase preparation.

8. Harvest cells by centrifuging 5 min at ~4000 × *g*, room temperature. Aspirate supernatant.

9. Resuspend cells at ~20 to 30 OD_{600} units/ml in spheroplast medium A.

10. Remove 10 µl of each cell suspension and dilute in 990 µl water. Mix by briefly vortexing. Measure and record the OD_{600} value.

    The decrease in the OD_{600} value of the diluted cell suspension will be used to determine the efficiency of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol in the spheroplast medium), yeast cells will lyse and thus will no longer contribute to the optical density.

11. Add Zymolyase 100T (as 5 mg/ml stock) to the cell suspension at a concentration of 1 to 5 µg per OD_{600} unit of cells. Incubate at 30°C (or appropriate permissive temperature) for ~20 min.

    The optimal Zymolyase concentration and digestion time depend on the strains and growth conditions. Thus, these parameters should ideally be determined empirically for the specific strains and culture conditions to be used.

12. To monitor the efficiency of cell wall removal, dilute 10 µl cell suspension into 990 µl water. Mix by vortexing. After 1 or 2 min, mix again and then measure the OD_{600} value. Continue cell wall digestion until the OD_{600} measurement of the diluted cell suspension is <5% of the original value (step 9), indicating that over 95% of the cells have been converted to spheroplasts.

    The generation of spheroplasts can also be monitored by light microscopy. Spheroplasts will appear as spherical cells with bright halos, while yeast with intact cell walls will have a darker appearance and a slightly more oval shape. Spheroplast lysis can also be observed under the microscope by adding a drop of water to the sample.

Harvest and wash spheroplasts

13. Harvest spheroplasts by centrifuging 5 min at 1500 × *g*, room temperature.

    Spheroplasts are fragile and must be handled very gently.

    If working with a very osmotically sensitive strain, the sorbitol concentration can be increased to 1.2 M or even 1.5 M. In addition, the spheroplasts can be pelleted through a cushion of 1.1 M sorbitol/7.5% (v/v) Ficoll 400 to minimize damage during the following wash steps.

14. Aspirate or decant supernatants.

    Use care while removing the supernatants, since the spheroplast pellets will be relatively loose and fluffy. In addition, the emergence of CO_2 bubbles from the spheroplasts can significantly disrupt the pellets.

15. Resuspend spheroplasts at 1 to 5 OD_{600} units/ml in spheroplast medium B by gently swirling tube by hand or gently stirring with a glass rod.

    Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding remaining solution.

    This washing step is critical to remove contaminating proteases and any other enzymes present in the Zymolyase preparation.

16. Harvest the spheroplasts by centrifuging 5 min at 1500 × *g,* 4°C. Decant or aspirate the supernatant.

17. *Optional:* Repeat steps 15 and 16 to ensure that the Zymolyase has been completely removed from the spheroplast preparation.

18. Resuspend spheroplasts in ice-cold spheroplast medium B, stop solution, or another solution with the appropriate osmotic support. Keep the suspension on ice.

    The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

    Stop solution contains two energy poisons (NaN_3 and NaF) that are toxic and should be handled with care. The inclusion of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

EQUILIBRIUM DENSITY GRADIENT FRACTIONATION USING NYCODENZ

BASIC PROTOCOL 2

In this protocol, equilibrium Nycodenz density gradient centrifugation is used to separate vacuolar membranes from endosomal and late Golgi compartments (Rieder and Emr, 1997). The distribution of subcellular components among the resulting fractions can be analyzed using marker proteins and activities (see *UNIT 3.7*). The fractions can also be used to analyze the subcellular localization of proteins of interest.

Yeast cells are first converted to spheroplasts (see Support Protocol). The spheroplasts are lysed using a combination of osmotic shock and Dounce homogenization. Subsequently, the lysates are subjected to centrifugation at 300 × *g* to remove unlysed cells and aggregates. These cleared cell lysates are loaded onto Nycodenz density gradients for overnight centrifugation.

About 15 to 20 OD_{600} units of cells in their mid-logarithmic growth phase are recommended for each gradient. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain). Since the membranes are not separated from the cytosol in this protocol, use of a protease-deficient strain is particularly advantageous to minimize damage by vacuolar hydrolases released from ruptured vacuoles.

Materials

20 OD_{600} units of cells in mid-logarithmic growth phase per gradient
HEPES/potassium acetate (HEPES/KAc) lysis buffer (see recipe), ice cold
50% (w/v) Nycodenz/sorbitol stock solution (see recipe)
37%, 31%, 27%, 23%, 20%, 17%, 13%, and 9% (w/v) Nycodenz in HEPES/KAc lysis buffer (see recipe), 4°C
500× protease inhibitor cocktails A and B (500× PIC-A and 500× PIC-B; see recipes)
Protease inhibitor stock solution A (see recipe)
100 mg/ml bovine serum albumin (BSA; optional)
Stop solution (see recipe) or another appropriate buffer, ice cold

Trimmed 1-ml pipet tips (cut ~5 to 10 mm from the tips to increase the size of the opening)
Clear 14 × 89–mm ultracentrifuge tubes (e.g., Ultraclear 14 × 89–mm tubes or equivalent) and rack
Centrifuge and microcentrifuge at 4°C
Dounce homogenizer with a tight-fitting pestle, prechilled

1.6-ml microcentrifuge tubes, prechilled

Refrigerated ultracentrifuge with swinging bucket rotor (e.g., Beckman SW41 Ti or equivalent), 4°C

Refractometer

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and preparation of spheroplasts (Support Protocol)

Culture cells

1. On the day before the experiment, culture desired yeast strains (Support Protocol, steps 1 to 5).

   About 20 OD_{600} units of cells are usually needed for each gradient.

2. In the morning on the day of the experiment, dilute the cultures so that the cells will be ready to harvest in the early afternoon (see Support Protocol, step 4 for sample calculations).

Prepare gradients

3. In the morning on the day of the experiment, prepare gradients with a micropipettor and trimmed 1-ml pipet tips. Add protease inhibitors to each Nycodenz gradient solution. In a 14 × 89–mm ultracentrifuge tube carefully layer the following volumes of Nycodenz gradient media on top of one another in the following order:

   1 ml 37%
   1.5 ml 31%
   1.5 ml 27%
   1.5 ml 23%
   1.5 ml 20%
   1 ml 17%
   1 ml 13%
   1 ml 9%.

4. Store the gradients undisturbed for 2 to 3 hr at room temperature. After several hours, transfer gradients to 4°C so that they are chilled prior to use.

   During the storage time, the concentration differences at the interfaces decrease through diffusion, and the gradient becomes essentially continuous.

Prepare cleared spheroplast lysates (S_{300})

5. Once the cells have reached their target density, prepare spheroplasts (Support Protocol, steps 6 to 15).

   Spheroplasts are fragile and must be handled very gently.

6. Resuspend spheroplasts at a concentration of 1 to 5 OD_{600} units/ml in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

   The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

   Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

   Stop solution contains two energy poisons (NaN_3 and NaF) that are toxic and should be handled with care. The use of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

7. Harvest spheroplasts by centrifuging 5 min at 2000 × *g*, 4°C. Aspirate the supernatant.

   From this point on, keep the samples on ice or at 4°C. Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

8. Resuspend spheroplasts at a concentration of ~15 OD_{600} units/ml in ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors (PIC-A, PIC-B, and protease inhibitor stock solutions A).

   The spheroplasts are lysed at a higher concentration than in Basic Protocol 1 in order to minimize the load volume for step 13.

9. Transfer the spheroplast suspension to a Dounce homogenizer that has been pre-chilled in an ice bucket. Disrupt the spheroplasts with 10 up-and-down strokes of a tight-fitting pestle.

   Keep Dounce homogenizer on ice throughout this process. Move the pestle slowly and maintain contact between pestle and suspension to minimize foaming.

10. Transfer lysate to multiple chilled 1.6-ml microcentrifuge tubes. Dispense convenient (~1.35-ml) aliquots of lysate (~20 OD_{600} cell equivalents) into each tube.

    Larger centrifugation tubes can also be used; however, use of 1.6-ml microcentrifuge tubes and a refrigerated microcentrifuge is often faster and more convenient.

    In order to monitor recovery levels in fractions generated by the subsequent centrifugation steps, it is often beneficial to set aside an aliquot of the lysate that will not be fractionated.

11. Clear the lysates of unbroken cells, partially disrupted cells, and aggregates by centrifuging 5 min at 300 × *g*, 4°C, in a microcentrifuge.

12. Carefully withdraw supernatants (S_{300}), without disturbing the very loose, fluffy pellet (P_{300}), and transfer them to new prechilled 1.6-ml tubes.

    Usually only ~80% of the S_{300} can be harvested from each tube without disturbing the pellets.

    In order to monitor the recovery levels in the gradient fractions, it is often beneficial to set aside a small aliquot of the S_{300} that will not be fractionated further.

    The yield of intracellular membranes can be improved by "washing" the P_{300} pellet; however, this practice increases the sample volume and handling times, and is not recommended for this protocol.

Fractionate lysate (S_{300}) on Nycodenz density gradients

13. Carefully layer supernatant (S_{300}) on the previously prepared gradient (step 4) using trimmed 1-ml pipet tip. Record volume of transferred S_{300}.

14. Balance appropriate pairs of buckets, gradients, and lids by adding a small amount of ice-cold HEPES/KAc lysis buffer to lighter tubes. Place the loaded gradient into prechilled buckets of the swinging bucket rotor.

    Slowly add buffer using a Pasteur pipet. Keep the tip near the surface of the gradient, when adding buffer, to minimize disruption of the gradient.

15. Centrifuge the gradients in a Beckman SW41 Ti rotor (or equivalent) 15 to 18 hr at 170,000 × *g*, 4°C, using the slow acceleration and deceleration settings to minimize disruption of the gradients.

Collect and process fractions

16. While the rotor is slowly decelerating, label 14 microcentrifuge tubes for the fractions to be collected from each gradient and place the tubes on ice.

    To prepare for manual fraction collection, cut ~5 to 10 mm off the tips of 1-ml pipet tips (~15 per gradient). Alternatively, an automated fraction collector system can be assembled.

17. After centrifugation is completed, carefully remove gradients from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregation or other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C.
18. Collect ~14 fractions starting at the top of the gradient, using either a micropipettor and the trimmed 1-ml pipet tips or an automated fraction collector.

    If desired, 200 μg of BSA or another well-characterized protein source can be added to increase the protein concentration of the collected fractions.

19. Mix each sample well by vortexing. Centrifuge 15 sec at 13,000 × *g*, 4°C, to clear the sample droplets from lids. Transfer 30 to 40 μl of each fraction to new microcentrifuge tubes and set aside at room temperature (for step 21).
20. Process fractions as desired.

    For example, membranes can be collected by diluting the fractions 2- to 3-fold with ice-cold HEPES/KAc lysis buffer and then subjecting the samples to centrifugation ≥60 min at 100,000 × g, 4°C. The proteins in each fraction can be precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%, and subsequently analyzed using immunoprecipitation (UNIT 7.2) or immunoblotting (UNIT 6.2) techniques.

21. Determine Nycodenz density of each fraction by measuring refractive index of the 30- to 40-μl samples set aside in step 19.

ALTERNATE PROTOCOL

EQUILIBRIUM DENSITY GRADIENT FRACTIONATION USING SUCROSE

In this protocol, equilibrium sucrose density gradient centrifugation is used to separate vacuolar and endosomal membranes from late Golgi compartments (Becherer et al., 1996). The resulting fractions can be used to analyze the subcellular localization of proteins of interest.

Yeast cells are first converted to spheroplasts (see Support Protocol). The spheroplasts are lysed under hypoosmotic conditions with a glass Dounce homogenizer. Differential centrifugation is used to generate 13,000 × *g* pellet and supernatant fractions ($P_{13,000}$ and $S_{13,000}$, respectively). The $P_{13,000}$ and $S_{13,000}$ fractions are subsequently loaded onto sucrose density gradients for overnight centrifugation.

About 15 to 25 OD_{600} units of cells in their mid-logarithmic growth phase are recommended for each gradient set ($P_{13,000}$ and $S_{13,000}$). If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain). Since the membranes in the $S_{13,000}$ fraction are not separated from the cytosol in this protocol, the use of a protease-deficient strain is particularly advantageous to minimize damage by vacuolar hydrolases released from ruptured vacuoles.

Additional Materials *(also see Basic Protocol 2 and Support Protocol)*

60% (w/v) sucrose stock solution, pH 6.8 (see recipe)
Sucrose solutions for equilibrium gradients (see recipe)
Refractometer

Prepare spheroplasts and sucrose gradients

1. Culture desired yeast strains (Support Protocol, steps 1 to 5).
2. Aliquot 37%, 34%, 32%, 29%, 27%, 22%, 10% (w/v) sucrose solutions for equilibrium gradients to separate 5-ml centrifuge tubes and add protease inhibitors just before preparing gradients.

    The volumes for these aliquots will depend on the number of gradients to be prepared. Always aliquot a bit more than the amount required.

3. In the morning on the day of the fractionation experiment, prepare the sucrose gradients in Ultra-Clear 14 × 89–mm tubes. Carefully layer the following sucrose solutions on top of each other with a trimmed 1-ml pipet tip:

   1 ml 60%
   1 ml 37%
   1.5 ml 34%
   2 ml 32%
   2 ml 29%
   1 ml 27%
   1.5 ml 22%
   0.5 ml 10% sucrose.

   Store, undisturbed at room temperature for 2 to 3 hr and then carefully transfer to 4°C.

4. Harvest ~20 OD_{600} units of cells in their mid-logarithmic growth phase for each gradient by centrifuging 5 min at ~5000 × *g*, room temperature.

5. Prepare spheroplasts (Support Protocol, steps 6 to 15).

   Spheroplasts are fragile and must be handled very gently.

6. Resuspend the spheroplasts at a concentration of 1 to 5 OD_{600} units/ml in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

   The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

   Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

   Stop solution contains two energy poisons (NaN_3 and NaF) that are toxic and should be handled with care. The use of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for some functional studies).

Prepare spheroplast lysates and generate $P_{13,000}$ and $S_{13,000}$ fractions

7. Harvest spheroplasts by centrifuging 5 min at 2000 × *g*, 4°C. Aspirate the supernatant.

   From this point on, keep the samples on ice or at 4°C. Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

8. Gently resuspend spheroplasts at a concentration of ~15 OD_{600} units/ml in ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors.

9. Transfer spheroplast suspension to a Dounce homogenizer that has been prechilled in an ice bucket. Disrupt spheroplasts with 10 up-and-down strokes of a tight-fitting pestle.

   Keep the Dounce homogenizer on ice throughout this process. Move the pestle slowly and maintain contact between pestle and suspension to minimize foaming.

10. Transfer lysate to multiple chilled 1.6-ml microcentrifuge tubes.

    In order to monitor recovery levels in the fractions generated by the subsequent centrifugation steps, it is often beneficial to set aside an aliquot of the lysate that will not be fractionated.

11. Clear lysate of unbroken cells, partially disrupted cells, and aggregates by microcentrifuging 5 min at 300 × *g*, 4°C.

12. Carefully withdraw supernatant (S_{300}), without disturbing the very loose, fluffy pellets (P_{300}), and transfer to new chilled 1.6-ml microcentrifuge tube.

    Usually only ~80% of S_{300} can be harvested from each tube.

13. Microcentrifuge S_{300} supernatant 10 min at 13,000 × *g*, 4°C.

14. Transfer supernatant ($S_{13,000}$) to prechilled 1.6-ml microcentrifuge tube and store on ice.

    If desired, $S_{13,000}$ can be subjected to centrifugation 60 min at 100,000 × g, 4°C, to generate $P_{100,000}$ and cytosol $S_{100,000}$ fractions. $P_{100,000}$ membranes can then be fractionated on the density gradients. The $S_{13,000}$ fraction is used instead of the $P_{100,000}$ in this protocol, since membranes can be very difficult to resuspend after centrifugation at 100,000 × g.

15. To remove any residual supernatant from $P_{13,000}$ pellets and tube, centrifuge the tube containing $P_{13,000}$ sample ~45 sec at 13,000 × *g*, 4°C. Carefully remove any remaining liquid above pellet and discard.

16. Resuspend ~20 OD_{600} unit equivalents of $P_{13,000}$ membranes in ~800 µl ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors (PIC-A, PIC-B, and protease inhibitor stock solutions A). Pipet the suspension up-and-down at least 20 times to ensure complete dispersion of the $P_{13,000}$ pellet.

Fractionate $P_{13,000}$ and $S_{13,000}$ samples on sucrose density gradients

17. Carefully layer the $P_{13,000}$ and $S_{13,000}$ samples (i.e., products from steps 13 and 15) on separate previously prepared, separate sucrose gradients using trimmed 1-ml pipet tips.

18. Balance the appropriate pairs of gradients by adding a small amount of ice-cold HEPES/KAc lysis buffer to the lighter tubes. Place loaded gradient into the prechilled bucket of the swinging-bucket rotor.

19. Centrifuge the gradient in a Beckman SW41 Ti rotor (or equivalent) 15 to 18 hr at 170,000 × *g*, 4°C, using the slow acceleration and deceleration settings to minimize disruption of the gradients.

Collect and process fractions

20. While the rotor is slowly decelerating, label fourteen 1.6-ml microcentrifuge tubes for the fractions to be collected from each gradient and place tubes on ice.

    To prepare for manual fraction collection, cut ~5 to 10 mm from the pointed ends of 1-ml pipet tips (~15 per gradient). Alternatively, an automated fraction collector system can be assembled.

21. After centrifugation is completed, carefully remove gradients from the rotor buckets and place in a stable rack. Note any apparent membrane aggregation or other characteristics. While processing each gradient, store the remaining gradients, undisturbed, at 4°C.

22. Collect ~14 fractions from top of gradient, using either a micropipettor and trimmed 1-ml pipet tips or an automated fraction collector.

    If desired, 200 µg of BSA or another well-characterized protein source can be added to increase protein concentration of the collected fractions.

23. Mix each sample well with a vortexer. Centrifuge 15 sec at 13,000 × *g* to clear the sample droplets from the lids. Transfer 30 to 40 µl of each fraction to new 1.6-ml microcentrifuge tube and set aside at room temperature for step 25.

24. Process remaining fractions as desired.

    For example, membranes can be collected by diluting the fractions 3- to 10-fold with ice-cold HEPES/KAc lysis buffer and then subjecting samples to centrifugation ≥60 min at 100,000 × g, 4°C. The proteins in each fraction can be precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% (v/v) and subsequently analyzed using immunoprecipitation (UNIT 7.2) or immunoblotting (UNIT 6.2) techniques.

25. Determine sucrose density of each fraction by measuring refractive index of the 30- to 40-µl samples from step 22.

FRACTIONATION OF $P_{13,000}$ MEMBRANES ON SUCROSE STEP GRADIENTS

BASIC PROTOCOL 3

This protocol was designed to analyze the distribution of one or more proteins among vacuoles, plasma membrane (PM), early Golgi, and endoplasmic reticulum (ER) membranes (Gaynor et al., 1994). Yeast cells are converted to spheroplasts with the β-glucanase Zymolyase 100T (see Support Protocol). The spheroplasts are lysed under hypoosmotic conditions with a glass Dounce homogenizer. Differential centrifugation is used to generate a 13,000 × *g* pellet that is enriched for vacuoles, PM, early Golgi, and ER membranes ($P_{13,000}$). This membrane pellet is subsequently fractionated on a sucrose density step gradient.

About 30 OD_{600} units of cells in their mid-logarithmic growth phase are recommended for each gradient. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain).

Materials

Desired yeast strains (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain)
Stop solution or spheroplast medium B, ice cold (see recipe; or another buffer with the appropriate osmotic support)
HEPES/potassium acetate (HEPES/KAc) lysis buffer, ice cold (see recipe)
500× protease inhibitor cocktails A and B (500× PIC-A and 500× PIC-B, see recipes)
Protease inhibitor stock solution A (see recipe)
1.2 and 1.5 M sucrose gradient solutions in sucrose gradient buffer (see recipe)

Ultra-clear 11 × 34–mm centrifuge tubes (e.g., Beckman or equivalent)
Benchtop refrigerated centrifuge and swinging bucket rotor, 4°C (e.g., Beckman TLS55 or equivalent)
Dounce homogenizer with a tight-fitting pestle, prechilled
1.6-ml microcentrifuge tubes
Microcentrifuge, 4°C
Trimmed 1-ml and 200-µl pipet tips (~5 to 10 mm cut from the tips)

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and preparation of spheroplasts (Support Protocol)

Culture cells and prepare spheroplasts

1. Culture desired yeast strains (Support Protocol, steps 1 to 5).

   About 30 OD_{600} units of cells in their mid-logarithmic growth phase are recommended for each gradient.

2. Prepare spheroplasts (Support Protocol, steps 6 to 15).

   Spheroplasts are fragile and must be handled very gently.

3. Resuspend spheroplasts in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

   The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

   Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

   Stop solution contains two energy poisons (NaN_3 and NaF) that are toxic and should be handled with care. The use of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for some functional studies).

Prepare lysates and generate $P_{13,000}$ membrane pellets

4. Harvest spheroplasts by centrifuging 5 min at 2000 × *g*, 4°C. Aspirate supernatant.

   From this point on, keep samples on ice or at 4°C. Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

5. Gently resuspend spheroplasts at a concentration of 5 OD_{600} units/ml in ice-cold HEPES/KAc lysis buffer.

6. Transfer spheroplast suspension to a Dounce homogenizer that has been prechilled in an ice bucket. Disrupt spheroplasts with 10 up-and-down strokes with a tight-fitting pestle.

   Keep Dounce homogenizer on ice throughout this process. Move pestle slowly and maintain contact between pestle and suspension to minimize foaming.

7. Transfer lysate to multiple chilled 1.6-ml microcentrifuge tubes.

   To monitor recovery levels in the fractions generated by subsequent centrifugation steps, it is often beneficial to set aside an aliquot of lysate that will not be fractionated (e.g., 1 ml or 5 OD_{600} cell equivalents).

8. Clear lysates of unbroken cells, partially disrupted cells, and aggregates by centrifuging 5 min at 300 × *g*, 4°C.

9. Carefully withdraw supernatant (S_{300}), without disturbing the very loose, fluffy pellets (P_{300}), and transfer to new chilled 1.6-ml tube.

   Usually only ~80% of the S_{300} can be harvested from each tube.

10. Resuspend P_{300} by pipetting up-and-down 10 times using a micropipettor and a 200-µl trimmed pipet tip. Add ice-cold HEPES/KAc lysis buffer to a total volume of 1 ml. Mix by pipetting up and down a few more times.

    Alternatively, the sample can be transferred to a clean, chilled Dounce homogenizer and dispersed with several gentle strokes.

    This washing step can improve the yield of intracellular membranes and the purity of the P_{300} fractions by removing some of the smaller organelles trapped within P_{300} pellets.

11. Repeat steps 8 and 9.

    The P_{300} pellets not required for subsequent analyses are often discarded since they contain a crude mixture of unlysed and partially disrupted cells, as well as a very heterogeneous mixture of intracellular membranes.

12. Pool the first and second S_{300} supernatants (from step 9 and 11) and mix. Transfer the pooled supernatant (S_{300}) to prechilled 1.6-ml microcentrifuge tube and place on ice.

13. Microcentrifuge the S_{300} supernatant 10 min at 13,000 × *g*, 4°C.

14. Remove the supernatant ($S_{13,000}$) to new 1.6-ml microcentrifuge tube. Store $S_{13,000}$ on ice until the gradient fractionation of $P_{13,000}$ membranes is in progress (step 20).

    Alternatively, the supernatants can be discarded.

    If desired, an aliquot (e.g., 5 OD_{600} units equivalents) of the $S_{13,000}$ can then be subjected to centrifugation 60 min at 100,000 × g, 4°C, to generate $P_{100,000}$ and cytosol $S_{100,000}$ fractions.

15. To remove any residual supernatant from $P_{13,000}$ pellet, centrifuge tubes containing $P_{13,000}$ samples ~45 sec at 13,000 × *g*, 4°C. Carefully remove any remaining liquid above pellet and discard.

16. Resuspend ~20 OD_{600} units equivalents of $P_{13,000}$ membranes in 200 µl ice-cold HEPES/KAc buffer with freshly added protease inhibitors (PIC-A, PIC-B, and protease inhibitor stock solutions A). Pipet each individual $P_{13,000}$ suspension up-and-down at least 20 times with a micropipettor and 200-µl pipet tip to ensure complete dispersion of $P_{13,000}$ pellet. Then combine pellets and mix by pipetting up-and-down a few more times.

Prepare and centrifuge sucrose step gradients

17. Carefully pipet 800 µl of 1.5 M sucrose step gradient solution into each ultra-clear 11 × 34–mm centrifuge tube. Using a trimmed 1-ml pipet tip, carefully layer 800 µl of 1.2 M sucrose step gradient solution on top of the 1.5 M sucrose layer.

    Prepare the sucrose gradients just prior to their use so that the interface remains crisp.

    If the interface is disturbed during or after preparation of the gradient, a new step gradient will need to be prepared.

18. Carefully layer 180 µl $P_{13,000}$ suspension (from step 16) over the 1.2 M sucrose solution with a trimmed 200-µl pipet tip.

19. Balance appropriate pairs of gradients by adding more of the $P_{13,000}$ suspension to the lighter tube if necessary. Put gradient tubes into prechilled buckets of the swinging bucket rotor.

20. Centrifuge gradients for 1 hr at 85,000 × *g*, 4°C (35,000 rpm for TLS55 rotor), using the slow acceleration and deceleration settings to minimize disruption of the gradients.

Collect and process fractions

21. While the rotor is slowly decelerating, prepare six labeled 1.6-ml microcentrifuge tubes for each gradient and keep on ice. Cut ~5 to 10 mm off the tips of 1-ml pipet tips (i.e., ~6 tips per gradient).

22. Carefully remove tubes from rotor buckets and place in a rack. Place gradients, undisturbed, at 4°C and process one gradient at a time. Make a note of position and appearance of bands and any other visible characteristics.

    Two discrete membrane bands should be visible, one in the 1.2 M sucrose layer and one at the interface between the 1.2 M and 1.5 M sucrose layers.

23. Collect the six fractions listed below with a micropipettor and cut 1-ml pipet tips. Store fractions in labeled microcentrifuge tubes on ice.

    Fraction 1: the top of gradient to just above the first membrane layer
    Fraction 2: first membrane band within the 1.2 M sucrose layer
    Fraction 3: sucrose solution between first membrane layer (Fraction 2) and second membrane layer (Fraction 4)
    Fraction 4: membrane band at the 1.2 M/1.5 M sucrose interface
    Fraction 5: 1.5 M sucrose solution below the interface
    Fraction 6: membrane pellet.

    The fractions will not have identical volumes.

    The vacuolar membranes are found in fractions 1 and 2; Golgi membranes are primarily in fraction 2; and ER membranes and the plasma membrane are split between fractions 2 (~35%) and 4 (~65%).

    If ER membranes and/or plasma membranes are the only features of interest, one can simply harvest the two membrane bands and discard the remaining solutions.

24. Increase the volume of each fraction to ~1.4 ml with ice-cold HEPES/KAc lysis buffer and mix.

25. Process fractions as desired.

    For example, the membranes can be collected by diluting the fractions 3- to 10-fold with ice-cold HEPES/KAc lysis buffer and then subjecting samples to centrifugation ≥50 min at 100,000 × g, 4°C. The proteins in each fraction can be precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% (v/v) and subsequently analyzed using immunoprecipitation (UNIT 7.2) or immunoblotting (UNIT 6.2) techniques.

BASIC PROTOCOL 4

ISOLATION OF INTACT VACUOLES USING FICOLL STEP GRADIENTS

This protocol was designed to purify intact, functional vacuoles from yeast spheroplasts (Haas, 1995). Yeast cells are converted to spheroplasts by incubation with oxalyticase, a β-glucanase preparation. The spheroplasts are lysed under isoosmotic conditions after treatment with the polybase DEAE-dextran. This gentle lysis procedure facilitates the isolation of intact vacuoles. Subsequently, the vacuoles are isolated through flotation on a Ficoll density step gradient.

The use of ~550 to 800 OD_{600} units of cells is recommended for each gradient. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY1, a *pep4* mutant yeast strain). The protocol is optimized for the yeast strains BJ3505 (a *pep4 prb1* mutant strain, deficient for most vacuolar hydrolase activities) and DKY6281 (a *pho8* mutant strain that does not express vacuolar alkaline phosphatase but has normal vacuolar protease activity levels).

Materials

Desired yeast strain
0.2× YPD (*UNIT 1.6*)
PIPES/DTT buffer (see recipe)
Oxalyticase buffer (see recipe)
Oxalyticase (45,000 U/mg, Enzogenetics; 0.5 to 1.0 mg/gradient) or an alternate β-glucanase enzyme (e.g., Zymolyase 100T; Support Protocol)
15%, 8%, 4%, and 0% (w/v) Ficoll solutions, 4°C (see recipe)
50× protease inhibitor cocktail (PIC, see recipe)
0.4 mg/ml dextran solution, freshly made (see recipe)
Protein assay reagent kit (e.g., BioRad; or an alternate technique, see *APPENDIX 3B*)

250-ml and 2-liter Erlenmeyer flasks
Shaker platform at 30°C (or appropriate growth temperature)
Centrifuge and rotor, room temperature and 4°C (e.g., Beckman JA-20 and JA-10 rotors or equivalents)
500-ml centrifuge bottles (e.g., for Beckman JA-10 rotor or equivalent)
10-ml glass pipets
30-ml glass Corex tubes (1 tube per gradient) or equivalent glass centrifugation tubes
Water bath at 30°C
Adapters for 30-ml glass Corex tubes (e.g., Beckman JA-20 rotor or equivalent)
Polyallomer centrifuge tubes, prechilled (e.g., 14 × 89–mm for SW41 Ti rotor)
Ultracentrifuge and rotor, 4°C (e.g., Beckman SW41 Ti rotor or equivalent)
200-µl pipet tips with ~5 to 8 mm trimmed off each tip
1.6-ml microcentrifuge tubes

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow cells

1. In the morning, the day before the experiment, start precultures of desired yeast strains by inoculating 20-ml of YPD (or appropriate medium) in 250-ml Erlenmeyer flasks. Culture cells throughout the day at 30°C (or appropriate permissive temperature) while shaking at 225 to 350 rpm.

   It is best to start precultures with a single yeast colony from a fresh plate.

   If using a strain that grows very slowly or if using a strain that has been stored on a plate at 4°C for more than a few days, it is wise to start the preculture with a single colony the night before.

2. In the evening, the day before the experiment, measure and record the OD_{600} of precultures.

3. Inoculate 1 liter of fresh medium with an appropriate volume of each preculture.

   A 1-liter culture is required for each gradient, since 550 to 800 OD_{600} units of cells from a rapidly growing culture (e.g., OD_{600} of 0.6 to 0.8) are needed per gradient. The suitable yeast cell concentration at this inoculation step depends on the growth rate of each strain and length of the incubation time. See Support Protocol, step 3 for sample calculations.

   Example: Haas and coworkers recommend inoculating 1 liter of YPD with 0.25 OD_{600} equivalents of DKY6281 cells or 0.9 OD_{600} equivalents of BJ3505 cells when incubating for 16.5 hr. (Protease-deficient pep4 mutant strains grow more slowly than the corresponding wild-type strains.)

4. Culture cells overnight at 30°C (or appropriate permissive temperature) while shaking at 225 to 325 rpm.

Generate spheroplasts with oxalyticase treatment

5. In the morning, the day of the experiment, analyze cell density.

   If the OD_{600} is >1.2, dilute cultures to an OD_{600} of 0.2 and allow growth to an OD_{600} of 0.6 to 0.8.

   Ideally, cultures should have an OD_{600} of 0.6 to 0.8 to begin the experiment.

6. Harvest cells at an OD_{600} of 0.6 to 0.8 by centrifuging 5 min at 4000 × *g* (5000 rpm in JA-10 rotor), room temperature.

   As noted earlier, ~550 to 800 OD_{600} units are needed per vacuole isolation gradient.

7. Decant the supernatant. Resuspend pellet in freshly prepared PIPES/DTT buffer at a concentration of ~15 OD_{600} units/ml (i.e., ~50 ml of buffer per liter of original culture).

8. Pool or divide cell suspension for each gradient into an individual 500-ml centrifuge bottle (i.e., one bottle of cells per gradient).

9. Incubate the cells for 10 min in a 30°C water bath.

10. Harvest cells by centrifuging 5 min at 4000 × *g* (5000 rpm in a JA-10 rotor), room temperature.

11. Decant supernatants and gently resuspend each pellet in oxalyticase buffer at a concentration of ~40 OD_{600} units/ml (e.g., ~15 ml/liter of original culture) using a 10-ml glass pipet.

12. Transfer suspension from each centrifuge bottle into separate, labeled 30-ml glass Corex tubes (i.e., one glass tube per gradient).

    It is important to use glass tubes, since they allow for more rapid temperature changes than plastic tubes.

13. Transfer 10 µl from each cell suspension to a labeled microcentrifuge tube and set aside to use as controls in step 16.

14. Add oxalyticase (or an alternate β-glucanase enzyme) to a concentration of 1800 to 3600 U/ml.

    Oxalyticase is often supplied at 45,000 U/mg. The amount of oxalyticase required to efficiently digest the cell wall needs to be determined empirically for each strain and growth condition.

    For DKY6281 cells, 1800 U/ml of oxalyticase is usually sufficient to generate spheroplasts efficiently (i.e., ~0.04 mg/ml or 0.8 mg for each 1000 OD_{600} units of cells). However, a higher concentration of ~2700 U/ml of oxalyticase is recommended for BJ3505 cells (i.e., ~0.06 mg/ml or 1.2 mg for each 1000 OD_{600} units of cells).

15. Incubate 25 min in 30°C water bath (or appropriate permissive temperature). Prepare the fresh 0.4 mg/ml dextran solution during the oxalyticase digestion and keep on ice until ready to use.

16. To monitor the efficiency of cell wall removal, dilute 10 µl of each cell suspension with oxalyticase into 990 µl water and mix by briefly vortexing. Also, dilute the 10-µl control samples set aside at step 13. After 1 or 2 min, briefly vortex again and measure OD_{600} values of all samples.

    The decrease in OD_{600} values of diluted oxalyticase cell suspension indicates the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to optical density.

    The extent of cell-wall digestion can also be examined by phase-contrast microscopy (Support Protocol, step 11).

17. Continue to incubate at 30°C (or appropriate permissive temperature) until ≥95% of cells have been converted to spheroplasts.

    The total incubation time for preparation of spheroplasts should be ≤60 min.

18. Place 30-ml glass Corex tubes in a JA-20 rotor with appropriate adapters. Harvest spheroplasts by centrifuging 1 min at 800 × *g* (2500 rpm in a JA-20 rotor), 4°C, followed by centrifugation for 1 min at 1500 × *g* (3500 rpm in a JA-20 rotor), 4°C.

    Spheroplasts are fragile and must be handled very gently.

19. *Optional:* Wash spheroplasts by gently resuspending each pellet in ~15 ml oxalyticase buffer. Resuspend by gently swirling tube by hand or by gently stirring with a glass rod. Do not vortex. Harvest spheroplasts as in step 18.

Perform dextran-mediated spheroplast lysis

20. Carefully decant supernatant and place tube on ice. Add 2.5 ml 15% Ficoll solution, 4°C, to each glass tube. Resuspend spheroplasts by gently swirling tube by hand or by gently stirring with a glass rod. Do not vortex.
21. Add 100 to 200 µl freshly made 0.4 mg/ml dextran solution to each tube. Immediately mix by gently swirling tubes by hand or by gently stirring with a glass rod. Do not vortex.

    The optimal amount of dextran for each strain needs to be determined empirically. (For DKY6281 strains, 100 µl per tube is recommended, while 180 to 200 µl per tube is recommended for BJ3505.)

22. Place tube on ice for 2 min to allow dextran to bind to the plasma membrane.
23. Disrupt the cell membrane by placing tube in a 30°C water bath for 60 to 75 sec, then immediately place tube on ice.
24. Examine cell lysates by phase contrast microscopy. Do not repeat the heat-shock step.

    Haas (1995) has found that lysates with one free-floating vacuole per 5 to 10 unlysed cells seem to yield the most vacuoles.

Prepare Ficoll step gradient

25. Transfer cell lysate from the glass tube to prechilled 14 × 89–mm polyallomer centrifuge tube and keep on ice.
26. Carefully layer 3 ml 8% Ficoll solution and 3.5 ml 4% Ficoll solution over cell lysate. Fill tube with 0% Ficoll solution (i.e., up to ~5 mm from top of tube).
27. Balance tubes using 0% Ficoll solution and place in prechilled ultracentrifuge rotor buckets (e.g., Beckman SW41 Ti).
28. Centrifuge 90 min at 110,000 × *g* (30,000 rpm for SW41 Ti rotor), 4°C. Use the slow acceleration and deceleration rate settings.

    To minimize mixing of the layers, allow the rotor to coast to a stop after slowing to 1000 rpm.

Collect and assess fractions

29. First, carefully remove top layer consisting of lipid with trimmed 200-µl tip and place in a microcentrifuge tube on ice (or discard).

    Remember to change the tip after each pipetting step.

    The vacuoles primarily accumulate in the interface between the 4% Ficoll and 0% Ficoll layers.

30. Carefully remove the vacuolar fraction from the 0% Ficoll/4% Ficoll interface using trimmed 200-µl tip and place in a microcentrifuge tube on ice.

    Avoid harvesting any of the 4% Ficoll layer, since Ficoll can interfere with subsequent steps. Collect a maximum of ~600 µl to avoid overdiluting the sample and to minimize Ficoll contamination.

31. Check for organelle integrity and purity by phase-contrast microscopy. Carefully mix 2 µl vacuole preparation with 10 µl 15% Ficoll solution for viewing.

    The vacuoles will appear as bright disks on a darker background, while lipid bodies that often associate with vacuolar membranes will appear as smaller dark spheres.

32. Determine protein concentration using 10 µl of each vacuole preparation and the BioRad protein assay reagent kit (or an alternate technique, see *APPENDIX 3B*).

Each sample of ~600 µl should have a protein concentration of ~0.25 to 0.9 mg/ml.

Vacuoles that will be used for functional assays should not be stored on ice for more than a few hours. Vacuoles remain functional in in vitro vacuole-vacuole fusion assays for up to several weeks if stored at –80°C as described in Haas (1995).

BASIC PROTOCOL 5

ISOLATION OF INTACT NUCLEI WITH FICOLL STEP GRADIENTS

This protocol was designed to isolate intact nuclei from yeast spheroplasts (Dove et al., 1998). Yeast cells are converted to spheroplasts by incubation with two β-glucanase preparations, Glusulase and Zymolyase 100T. The spheroplasts are lysed under hypoosmotic conditions. The lysate is cleared of unbroken cells and aggregates by centrifugation. Subsequently, the nuclei are isolated by fractionation on two sequential Ficoll step gradients.

The protocol is written for the preparation of nuclei from 6 liters of yeast culture in the exponential growth phase (i.e., ~3×10^{11} cells), but can be scaled up or down as needed. It has been optimized for the haploid strain BJ2168, which is deficient for vacuolar protease activity. BJ5465, another protease-deficient strain, also yields good results. Other strains can also be used; however, the yields will vary from strain to strain, and the protocol may need to be optimized (Aris and Blobel, 1991; Dove et al., 1998). If the nuclei will be used for functional studies, a recovery incubation after spheroplast preparation is needed for optimal activity.

Materials

Desired yeast strain
YPD medium (*UNIT 1.6*; or appropriate growth medium)
Sterile H_2O, ice cold
Pretreatment buffer (see recipe)
1.1 M sorbitol solution (see recipe), ice cold
Glusulase (NEN Life Science Products)
10 mg/ml Zymolyase 100 T (or 100,000 U/g yeast lytic enzyme can also be used; ICN)
Spheroplast recovery medium (optional; see recipe)
1000× protease inhibitor cocktail-D (1000× PIC-D; see recipe)
1000× protease inhibitor cocktail-W (1000× PIC-W; see recipe)
Ficoll cushion solution (see recipe), 4°C
20%, 30%, 40%, and 50% (w/v) Ficoll lysis solutions with 1× PIC (see recipe)
1× and 2× PM buffer (see recipe), 4°C
PSM 1 solution (see recipe)
TE/SDS solution (see recipe; optional)

Shaker platform in incubator at 30°C (or appropriate permissive temperature)
250-ml and 2-liter Erlenmeyer flasks
250-ml centrifuge bottles
Hemacytometer
Sorvall GSA rotor or equivalent; prechill to 4°C
1.6-ml microcentrifuge tubes
30°C water bath
50-ml polycarbonate centrifuge tubes (e.g., Oakridge or equivalent)
Swinging bucket rotor (e.g., Sorvall HB-4 rotor or equivalent), prechill to 4°C

Homogenizer (e.g., Potter-Elvehjem or a 40-ml glass Dounce homogenizer with a loose-fitting pestle), prechilled

Beckman Ultra-Clear 25 × 89–mm centrifuge tubes (or equivalent), prechill to 4°C

Beckman SW28 rotor (or equivalent), 2°C

20-ml syringes with 16-G needles

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow cells

1. In the morning, 2 days before the experiment, start precultures of desired yeast strains by inoculating 5 ml YPD (or appropriate medium) with a single yeast colony from a fresh plate.

2. Culture cells throughout the day at 30°C (or appropriate permissive temperature) while shaking at 225 to 350 rpm.

3. In the evening, measure and record the OD_{600} of precultures. Use 5-ml preculture to inoculate 20 ml of growth medium in 250-ml Erlenmeyer flasks to yield a culture with an ~1 OD_{600} unit by the following morning.

   The suitable yeast cell concentration at this inoculation step depends on the growth rate of each strain and length of incubation time. See Support Protocol, step 3 for sample calculations.

   The doubling time of BJ2168 and BJ5465 in YPD at 30°C is ~110 min.

4. Culture cells overnight at 30°C (or appropriate permissive temperature) while shaking at 225 to 350 rpm.

5. In the morning, the day before the experiment, measure and record the OD_{600} of precultures. Use the 20-ml precultures to inoculate 50 ml of growth medium in 250-ml Erlenmeyer flasks so that the culture will reach an OD_{600} of 0.5 to 1.0 by the late afternoon.

6. Measure OD_{600} of the culture in the evening. Prewarm six 1-liter volumes of YPD to 30°C. Add appropriate volume of 50-ml preculture to each of six 1-liter volumes of YPD so that the cultures will reach an OD_{600} of 0.6 to 0.7 by morning. Culture cells overnight at 30°C while shaking at 225 to 350 rpm.

Harvest cells

7. Measure OD_{600} of 1-liter cultures in the morning of the experiment. Harvest cells when cultures reach an OD_{600} of 0.6 to 0.7.

   If cultures exceed an OD_{600} of 0.8, dilute cultures to ~0.15 and continue growth until cultures reach an OD_{600} of 0.6 to 0.7.

8. Chill cells by placing flasks in an ice-water bath with occasional swirling.

9. Transfer cells to 250-ml centrifuge bottles and harvest by centrifuging 5 min at ~4000 × *g* (Sorvall GSA rotor at 5000 rpm), 4°C.

   If a Pellicon (Millipore) or similar cell concentrator is available, it is convenient to concentrate cells before centrifugation.

10. Decant supernatant and place tubes on ice. Resuspend cell pellets in ice-cold water to a total of 200 ml.

11. Transfer cell suspension to a single, preweighed 250-ml centrifuge bottle. Centrifuge for 5 min at ~4000 × *g* (Sorvall GSA rotor at 5000 rpm), 4°C.

12. Decant supernatant and aspirate remaining supernatant. Determine wet weight of cell pellet.

    The cell pellet usually weighs ~9 g.

Pretreat cells

13. Resuspend pellet in freshly-prepared pretreatment buffer, using 4 ml for every gram of wet cell pellet (e.g., add 36 ml pretreatment buffer to a 9-g cell pellet). Incubate 10 min at room temperature with intermittent swirling.

14. Add ice-cold water to a final volume of 250 ml and mix well. Transfer 10 µl to a microcentrifuge tube and store on ice for step 16.

15. Centrifuge 5 min at ~4000 × *g* (Sorvall GSA rotor at 5000 rpm), 4°C.

16. During centrifugation, determine the cell yield (i.e., cell number) using a hemacytometer. Dilute the 10-µl sample in 990 µl water to facilitate counting.

    BJ2168 typically yields 1.6×10^{10} cells/g. The number of cells per gram varies with different strains, but is usually between 1.2 and 1.7×10^{10} cells/g.

17. Resuspend cells in ~200 ml ice-cold 1.1 M sorbitol solution. Centrifuge 5 min at ~4000 × *g* (Sorvall GSA rotor at 5000 rpm), 4°C.

18. Decant supernatant and aspirate remaining supernatant. For every 1×10^{11} cells, add 25 ml 1.1 M sorbitol solution (e.g., if there are 3×10^{11} cells, add 75 ml sorbitol solution). Disperse well by pipetting.

19. Remove 100 µl of cell suspension and transfer to a microcentrifuge tube. Store on ice until step 22.

    This sample will be used as a control when monitoring cell wall integrity.

Prepare spheroplasts

20. To digest cell wall, add 1.5 ml Glusulase (at concentration supplied by manufacturer) and 0.3 ml of 10 mg/ml Zymolyase 100T for every 1×10^{11} cells (e.g., if there are 3×10^{11} cells in 75 ml of sorbitol solution, add 4.5 ml Glusulase and 0.3 ml 10 mg/ml Zymolyase 100T). Mix cell suspension with a glass rod and loosely cap the bottle.

21. Incubate 1.5 to 2.5 hr in a 30°C water bath with gentle swirling. Monitor the digestion microscopically (under 400× magnification) every 15 to 20 min. Continue digesting until cells are round and clumped cells separate.

    After ~1 hr of digestion, cells should be spherical and show extensive clumping. After further digestion, the spheroplasts will separate.

22. To monitor the efficiency of cell wall removal, dilute 10 µl of each cell suspension into 990 µl water and mix by briefly vortexing. Also dilute the 9.4-µl samples stored at step 19 to serve as controls. After 1 or 2 min, briefly vortex again and measure the OD_{600} values.

    The decrease in the OD_{600} values of the digested cell suspensions indicate the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to the optical density.

Wash spheroplasts with sorbitol

23. During cell-wall digestion and subsequent steps, prepare first set of Ficoll step gradients as time permits (see step 39).

24. *Optional:* If the nuclei will be used for functional studies, a recovery incubation is needed for optimal activity. Incubate spheroplasts in prewarmed spheroplast recovery medium for 30 min at 30°C with gentle swirling.

    Other growth media supplemented with sorbitol at a final concentration of 1 to 1.5 M can be used as well.

    Optimum recovery conditions should be determined empirically for each application.

Chill and wash spheroplasts

25. Divide spheroplast suspension into 50-ml polycarbonate centrifuge tubes adding 1×10^{11} cells per tube. Store on ice for 10 min to chill the cells.

26. Centrifuge in a swinging-bucket rotor for 5 min at ~4000 × *g* (e.g., Sorvall HB-4 rotor at 5000 rpm), 4°C. Aspirate supernatants.

27. Gently resuspend each pellet with ~25 ml ice-cold 1.1 M sorbitol solution. Use a glass rod to carefully resuspend pellet. Do not vortex.

28. Centrifuge in a swinging-bucket rotor 5 min at ~4000 × *g* (e.g., Sorvall HB-4 rotor at 5000 rpm), 4°C. Aspirate supernatant.

29. Resuspend each pellet with ~20 ml ice-cold 1.1 M sorbitol solution. Use a glass rod to carefully resuspend pellet. Do not vortex.

Prepare cushion solution and filter cells

30. Add 20 µl of each 1000× PIC-W and PIC-D to a 6-ml aliquot of Ficoll cushion solution and vortex. Carefully load 6 ml Ficoll cushion solution under spheroplast suspension using a Pasteur pipet or a syringe with a long needle or canula. Repeat for each tube.

    To load the Ficoll cushion solution, slowly move the pipet, needle, or canula through the lysate until the tip is at the bottom of the tube. Slowly expel 6 ml of the Ficoll cushion solution. Carefully remove the pipet, needle, or canula by lifting straight up.

31. Centrifuge spheroplasts through cushion solution 10 min at ~4000 × *g* (e.g., Sorvall HB-4 rotor at 5000 rpm), 4°C. Aspirate supernatants and store wet pellets on ice.

Isolate enriched nuclear fraction

32. To lyse spheroplasts, add 25 ml of 20% Ficoll solution (room temperature) to each pellet. Quickly disperse pellet with a 25-ml pipet.

    For optimal lysis, it is important that the Ficoll solution is at room temperature, not 4°C.

33. Immediately transfer suspension to a prechilled motorized Potter-Elvehjem homogenizer. Lyse cells with 5 strokes and a fast-spinning pestle using a motorized drive.

    Alternatively, cells can be lysed in a prechilled 40-ml Dounce homogenizer. Use 20 slow strokes with a loose-fitting pestle (3 to 4 min total).

34. Immediately transfer lysate to a 50-ml polycarbonate centrifuge tube that is submerged to the neck in ice.

    Work quickly so that each pellet is processed within 3 to 5 min.

35. Chill lysates on ice for 10 to 15 min. Examine lysates by microscopy (400× magnification).

    The spheroplasts should be completely disrupted. The nuclei will be relatively small, round, and dark. The edges may look slightly ruffled and the nucleolus may appear as a dark crescent. Released vacuoles will look larger and refractile.

36. Centrifuge lysate in a precooled swinging-bucket rotor for 5 min at 13,000 × *g* (e.g., Sorvall HB-4 rotor at 9000 rpm), 2°C.

37. Carefully transfer supernatant into new, prechilled 50-ml polycarbonate centrifuge tubes without transferring any of the loose pellet. Centrifuge supernatant 10 min at 13,000 × *g* (e.g., 9000 rpm in a Sorvall HB-4 rotor), 2°C.

    After centrifugation, a tight pellet should be visible.

38. Do not transfer supernatant; place tube on ice until step 40.

Prepare Ficoll step gradients

39. Prepare the first set of Ficoll step gradients as time permits during previous steps. Warm the 50%, 40%, and 30% Ficoll solutions to ~25°C. Transfer ~20 ml of each solution into a separate 50-ml tube. Add PIC-W and PIC-D solutions and vortex.

40. Place a 25 × 89–mm ultracentrifuge tube and support base on a balance. Tare. Slowly pipet 6.5 g of 50% Ficoll solution to the bottom of the tube. Place the first tube on ice and repeat for the remaining tubes.

    It is important to chill each Ficoll layer for a few minutes before adding another layer. At lower temperatures Ficoll increases in viscosity and it becomes less likely that addition of the next layer will disrupt the layer below.

41. Add 6.5 g of 40% Ficoll solution to each tube as in step 40. Chill tubes on ice.

42. Add 6.5 g of 30% Ficoll solution to each tube as in step 40. Chill gradients >10 min on ice before use.

    One gradient is needed for every 1×10^{11} cells. Typically, three gradients are needed for a 6-liter preparation.

    Ficoll solutions are very viscous so it is more accurate to aliquot by weight.

Purify and collect nuclei

43. Carefully layer ~25 ml supernatant from each tube (from step 38) onto each of the chilled Ficoll gradients. Do not transfer any of the pellet.

44. Fill and balance tubes with cold 1× PM buffer as necessary. Centrifuge the gradients 60 min at 58,400 × *g* (18,000 rpm in a Beckman SW28 rotor), 2°C.

45. During the last 30 min of centrifugation, prepare second set of Ficoll step gradients as in steps 39 to 42, except use 5.5 g of Ficoll solution. Chill on ice for ≥10 min before using gradients.

    Typically, two gradients are needed for a 6-liter preparation.

46. After centrifugation (step 44), inspect gradients and note any visible characteristics.

    Bands containing nuclei are usually visible at the 30%/40% and 40%/50% Ficoll interfaces. (Nuclei are also found throughout the 40% Ficoll layer.) A layer of white film should be visible at the top of the gradient. A thick layer should be visible at the 20%/30% interface. A small, halo-like pellet should occur at the bottom.

47. Collect nuclei with a 20-ml syringe and a 16-G needle. Insert needle through tube wall just below the 40%/50% Ficoll interface. With needle tip in the center of tube and the beveled edge facing up, carefully remove ~8 ml while slowly moving needle back and forth.

    Cold Ficoll solutions are quite viscous, so collection should be done smoothly and slowly. The 8-ml volume will include the 40% Ficoll layer and some of the 50% Ficoll layer. Avoid the 30% Ficoll layer.

    If an enriched (but less pure) preparation of nuclei is sufficient for the purposes of the experiment, one can collect ~6 ml from each gradient and omit the second Ficoll gradient. However, this is not recommended when purity is of importance.

48. Remove needle and slowly expel harvested nuclear suspension into a 150-ml glass beaker chilled on ice.

49. Repeat steps 46 to 48 for each gradient and pool harvested nuclei in the 150-ml beaker.

50. Dilute suspension with 1 volume of cold 1× PM buffer by gently stirring with a glass rod.

51. Carefully layer harvested nuclear suspension over two prechilled second set Ficoll step gradients (prepared in step 45). Fill and balance tubes with cold 1× PM buffer plus PIC as necessary.

52. Centrifuge gradients in a 60 min at 58,400 × *g* (18,000 rpm in a Beckman SW28 rotor), 2°C.

53. After centrifugation, collect ~10 ml from each gradient as described in step 47.

54. Pool harvested nuclei in a 50-ml tube on ice. Mix by stirring gently with a glass rod.

55. Transfer 50 µl nuclear preparation to a chilled microcentrifuge tube for protein determination. Store on ice until step 57.

    If the nuclei are not needed right away, aliquot harvested nuclei while still in Ficoll solution, freeze in liquid nitrogen, and store at −70°C.

56. To remove Ficoll solution, dilute nuclei in 10 vol cold 1× PM buffer plus PIC. Mix by gently stirring with a glass rod. Pellet nuclei by centrifuging 10 min at 10,000 × *g*, 4°C. Process nuclei as desired.

    For most purposes (e.g., TCA protein precipitation, SDS-PAGE analysis, functional assays), the Ficoll will need to be removed from harvested nuclei.

Determine protein concentration

57. To determine protein concentration, dilute 50 µl of harvested nuclei in 10 volumes PSM 1 solution and mix well. Centrifuge 30 min at 12,000 × *g*, 4°C. Resuspend pellet in 50 µl of water or TE/SDS solution. Determine protein concentration using a modified Lowry procedure or Bradford assay (e.g., *APPENDIX 3B*).

ISOLATION OF LACTATE-INDUCED MITOCHONDRIA USING NYCODENZ STEP GRADIENTS

BASIC PROTOCOL 6

This protocol is designed to isolate intact, functional mitochondria from lactate-grown D273-10B spheroplasts (Glick and Pon, 1995). Yeast cells are precultured in a glucose medium for a day and then transferred to a lactate medium to encourage optimal development of mitochondria. After ~15 hr in lactate medium, the cells are converted to spheroplasts using the β-glucanase preparation Zymolyase 20T and lysed by osmotic shock. Differential centrifugation is used to generate a mitochondria-enriched pellet. This

pellet is subsequently fractionated on a Nycodenz density step gradient to purify the mitochondria.

This protocol calls for four 1-liter cultures of lactate-grown D273-10B cells, but can be scaled-up or scaled-down as needed. Cells other than lactate-grown D273-10B cells can be used; however, the mitochondrial density and abundance will vary with strain and growth conditions, and thus the protocol may need to be optimized. If the mitochondria will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Materials

D273-10B cells (e.g., or desired yeast strain)
Semi-synthetic lactate medium (see recipe)
TSD reduction buffer (see recipe)
Zymolyase 20T (e.g., ~75 mg)
Buffer A: 1.2 M sorbitol/20 mM potassium phosphate, pH 7.4 (see *APPENDIX 2A* for phosphate buffer), room temperature
Semi-synthetic lactate medium (see recipe) supplemented with 1.2 M sorbitol (optional)
200 mM phenylmethylsulfonyl fluoride (PMSF; 34.5 mg/ml) in absolute ethanol; prepare fresh
Buffer B: 0.6 M sorbitol/20 mM potassium MES (pH 6.0), ice cold
0.6% (w/v) SDS solution
2× Buffer B (see recipe)
Buffer C: 0.6 M sorbitol/20 mM potassium HEPES (pH 7.4), ice cold
18% and 14.5 % (w/v) Nycodenz solution (see recipe), prechilled
100 mg/ml fatty-acid free bovine serum albumin (BSA)

200-ml Erlenmeyer flask
Platform shaker, 30°C (or permissive growth temperature)
Sorvall GS-3 rotor (or equivalent)
250-ml centrifuge bottles (e.g., Sorvall GS-3 rotor or equivalent)
30°C water bath
1.6-ml microcentrifuge tubes
40- and 1-ml glass Dounce homogenizers with a tight-fitting pestles (or Teflon homogenizer), 4°C
40-ml centrifuge tubes (e.g., for Sorvall SS-34 rotor or equivalent)
Sorvall SS-34 rotor (or equivalent)
Clear ultracentrifuge tubes (e.g., Beckman SW-41 14 × 89–mm Ultra-Clear centrifuge tubes)
Beckman ultracentrifuge with a SW-41 rotor (or equivalent)
Cut 1-ml pipet tips

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow D273-10B cells and induce mitochondrial development

1. In the evening, 2 days before fractionation experiment, start a preculture of D273-10B cells (or desired yeast strain) by inoculating 40 ml semi-synthetic lactate medium in a 200-ml Erlenmeyer flask.

   It is best to use a single colony from a fresh plate.

2. Culture cells for ~24 hr at 30°C while shaking at 225 to 325 rpm.

   Good aeration is critical for optimal cell growth and proliferation of mitochondria.

3. In the evening of the day before fractionation experiment, use 40-ml stationary-phase preculture to inoculate 4 liters semi-synthetic lactate medium (e.g., use 10 ml preculture per 1-liter culture).
4. Culture cells at 30°C overnight with good aeration until OD_{600} value is ~3.

   For strain D273-10B, cells should be grown for ~15 hr.

Harvest cells

5. In the morning, on the day of the experiment, check OD_{600} value of cultures. Allow cells to grow until OD_{600} reaches ~3.
6. Harvest cells by centrifuging 5 min at 4000 × *g* (5000 rpm in a Sorvall GS-3 rotor), room temperature, and removing the supernatant.
7. Resuspend cells in a total volume of 120 ml sterile water. Transfer suspension to a preweighed 250-ml centrifuge bottle.
8. Pellet cells by centrifuging 5 min at 2000 × *g* (3500 rpm in a Sorvall GS-3 rotor), room temperature.
9. Decant supernatant and weigh the wet pellet. Determine the wet weight of pellet by subtracting bottle weight.

   Four liters of a D273-10B culture at an OD_{600} of 3.0 usually yields ~30 g of packed cells.

Prepare spheroplasts

10. Resuspend cells in 40 ml freshly prepared reduction buffer.
11. Incubate cell suspension for 15 min in a 30°C water bath with gentle shaking. During this incubation, weigh out the required amount of Zymolyase 20T to add 2.5 mg Zymolyase 20 T per gram of packed cells.

    Alternatively, 0.5 mg Zymolyase 100T per gram of packed cells can be used.

12. Pellet cells by centrifuging 5 min at 2000 × *g* (3500 rpm in a Sorvall GS-3 rotor), room temperature. Remove supernatant.
13. Gently resuspend pellet in buffer A, using 2 ml buffer A per gram of packed cells (e.g., 60 ml buffer A for a 30-g cell pellet).
14. Remove 10 µl suspension and store in a 1.6-ml microcentrifuge tube on ice until step 18.
15. Pellet cells by centrifuging 5 min at 2000 × *g*, room temperature. During centrifugation, dissolve weighed Zymolyase 20T in buffer A at a concentration of 1.25 mg/ml.

    If using Zymolyase 100T, dissolve it in buffer A at a concentration of 0.25 mg/ml.

16. Decant supernatant and resuspend pellet in Zymolyase solution.
17. Incubate suspension for 30 min in a 30°C water bath with gentle shaking.
18. After 30 min, monitor the efficiency of cell wall removal. Dilute 10 µl of each cell suspension into 990 µl water and mix by briefly vortexing. Also dilute the 10-µl samples stored after step 14 to serve as controls. After 1 or 2 min, briefly vortex again and measure OD_{600} values.

    The decrease in OD_{600} values of diluted cell suspension indicates the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to the optical density.

    The extent of cell-wall digestion can also be examined by phase-contrast microscopy as described in Support Protocol, step 11.

19. Continue incubation until >95% of cells have been converted to spheroplasts.

Wash spheroplasts

20. Centrifuge spheroplasts 5 min at 2000 × *g* (3500 rpm in a Sorvall GS-3), 4°C. Remove supernatant.

21. Gently resuspend spheroplast pellet in 30 ml buffer A. Pellet spheroplasts by centrifuging 5 min at 2000 × *g* (Sorvall GS-3 rotor, 3500 rpm), 4°C.

22. Wash the spheroplasts again by repeating step 21.

23. *Optional:* Incubate washed spheroplasts for 30 to 60 min at 30°C in semi-synthetic lactate medium supplemented with 1.2 M sorbitol (final concentration). Shake gently. Pellet spheroplasts (step 21).

    Optimal recovery conditions should be determined empirically for each application.

    Other growth media supplemented with sorbitol to a final concentration of 1 to 1.5 M can be used.

    A recovery period after cell wall removal is essential for optimal translation/transcription activity.

24. Prepare buffer B by adding 0.5 ml of 200 mM PMSF drop-wise to 200 ml of ice-cold buffer B while stirring. Filter through Whatman paper into a flask on ice.

    It is important to keep the suspensions, solutions, and equipment at 0° to 4°C from this step on.

Prepare spheroplast lysates

25. Resuspend spheroplasts in 40 ml ice-cold buffer B with PMSF. Divide suspension into two equal portions and keep on ice.

26. Homogenize each portion in an ice-cold 40-ml glass Dounce homogenizer, using 15 strokes with a tight-fitting pestle.

    Keep Dounce homogenizer on ice throughout this process. Move pestle slowly and maintain contact between pestle and suspension to minimize foaming.

27. Combine lysates in a prechilled beaker on ice. Add buffer B with PMSF to a total volume of 100 ml.

Collect mitochondria

28. Transfer lysate to four 40-ml centrifuge tubes. Centrifuge 5 min at 1500 × *g* (3500 rpm in a Sorvall SS-34 rotor), 4°C.

29. Carefully transfer supernatant to a prechilled beaker on ice, being careful to avoid the loose pellet. Store supernatants on ice until step 33.

30. Resuspend each pellet in 10 ml buffer B with PMSF. Combine suspensions in a single tube, swirl to mix, and then divide into two 20-ml portions.

31. Homogenize each 20-ml suspension in the 40-ml glass Dounce homogenizer, using 15 strokes with a tight-fitting pestle, without introducing air.

32. Combine lysates in a prechilled beaker on ice. Add buffer B with PMSF to a total volume of 100 ml.

33. Transfer lysate to four new 40-ml centrifuge tubes. Centrifuge 5 min at 1500 × *g*, 4°C.

34. Transfer supernatant to the beaker containing the stored supernatants (step 28), being careful to avoid the loose pellets. Discard pellets.

Isolate enriched mitochondrial fraction

35. Divide supernatants between six 40-ml centrifuge tubes. Centrifuge 10 min at 12,000 × *g* (10,000 rpm in Sorvall SS-34 rotor), 4°C.

36. Decant supernatants and resuspend each pellet in 7 ml ice-cold buffer B (without PMSF) and combine into two tubes.

37. Homogenize each suspension (gently, but thoroughly) in a clean, prechilled 40-ml glass Dounce homogenizer, using 5 to 10 slow strokes with a tight-fitting pestle.

38. Transfer suspensions to two clean 40-ml tubes. Centrifuge 5 min at 1500 × *g*, 4°C.

39. Transfer supernatants into two fresh 40-ml centrifuge tubes, avoiding the loose pellets. Centrifuge 10 min at 12,000 × *g* (10,000 rpm in a Sorvall SS-34 rotor), 4°C.

40. Decant supernatant and gently resuspend pellets in 0.5 ml ice-cold buffer B (without PMSF) using a prechilled 1-ml glass Dounce homogenizer or Teflon homogenizer. Transfer suspensions to 1.6-ml microcentrifuge tubes and store on ice.

    These suspensions are crude preparations that are enriched for mitochondria.

    The mitochondria prepared from lactate-grown D273-10B cells usually have a dark brown color but may also look orange or pink.

41. Estimate protein concentration by measuring A_{280} of 10 µl of crude mitochondrial suspension diluted in 990 µl of 0.6% SDS. Mix 10 µl buffer B with 990 µl of 0.6% SDS to use as a reference standard.

    An A_{280} value of 0.21 corresponds to ~10 mg/ml protein in the undiluted suspension. A 4-liter culture typically yields 120 mg of crude mitochondrial protein.

    If no further purification is desired, dilute the crude mitochondrial suspension to 15 ml with ice-cold buffer C. Centrifuge 10 min at 12,000 × g, 4°C. Gently resuspend crude mitochondrial pellet to the desired concentration in ice-cold buffer C.

Purify mitochondria

42. Centrifuge crude mitochondrial suspension generated in step 40 for 5 min at 3000 × *g*, 4°C, in a microcentrifuge, to remove aggregated material. Transfer supernatant to new microcentrifuge tube and store on ice.

43. Prepare two Nycodenz step gradients just before use. Transfer 5 ml prechilled 18% Nycodenz solution to each clear ultracentrifuge tube. Carefully add 5 ml prechilled 14.5% Nycodenz solution, taking care to obtain a sharp interface. Keep gradients on ice.

    Two Nycodenz gradients are usually sufficient for purifying mitochondria obtained from 4 liters of cells. For optimal separation, the amount of material loaded on each 10-ml gradient should not exceed 50 to 75 mg protein (as measured in step 41).

44. Gently layer ~1 ml crude mitochondrial preparation from step 42 onto each gradient.

45. Ultracentrifuge 30 min at 284,000 × *g* (40,000 rpm in Beckman SW-41 rotor), 2°C, using the slow acceleration and deceleration settings.

46. After centrifugation is completed, carefully remove gradient from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregations and other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C.

The purified mitochondria should collect at the 18%/14.5% Nycodenz interface and form a brown band. The mitochondrial layer may also appear to be orange or pink.

Collect mitochondria

47. First remove buffer layer and most of the 14.5% Nycodenz layer using cut 1-ml pipet tips. Harvest mitochondrial layer with a cut 1-ml pipet tip and transfer to a 50-ml tube on ice.

    Change pipet tips with each pipetting step to minimize contamination.

    Alternatively, the mitochondrial layer can be recovered using a syringe with a 19-G needle. Place a piece of clear tape on each tube at the 18%/14.5% Nycodenz interface and puncture the side of each tube to collect mitochondria.

48. Pool harvested mitochondria and dilute to 15 ml with buffer C. Centrifuge 10 min at 12,000 × *g*, 4°C.

49. Discard supernatant and gently resuspend pellet in 0.5 ml ice-cold buffer C (0.25 ml buffer C per Nycodenz gradient), using a glass Dounce or Teflon homogenizer to resuspend mitochondria completely. Store suspension in a microcentrifuge tube on ice.

Determine protein concentration

50. To determine approximate protein concentration, dilute 10 µl purified mitochondrial suspension from step 49 in 0.5 ml buffer C. Centrifuge 3 min at 12,000 × *g*, 4°C.

    This centrifugation step is required to remove any residual Nycodenz from the mitochondrial suspension, since Nycodenz absorbs ultraviolet light and will distort the A_{280} measurements.

51. Discard supernatant, resuspend pellet in 1.0 ml of 0.6% SDS, and measure the A_{280}.

    For gradient-purified mitochondria, an A_{280} value of 0.12 corresponds to a protein concentration of ~10 mg/ml in the undiluted suspension.

52. To store mitochondria, dilute mitochondria with a combination of cold buffer C and cold buffer C plus BSA to yield final concentrations of 25 mg/ml mitochondrial protein and 10 mg/ml BSA. Aliquot, freeze in liquid nitrogen, and store at −70°C or −80°C. Just before use, rapidly thaw the mitochondrial suspension in a 30°C water bath and immediately place on ice.

    Alternatively, mitochondria can be frozen in a DMSO-mannitol solution as described by Yaffe (1991).

BASIC PROTOCOL 7

ISOLATION OF OLEATE-INDUCED PEROXISOMES USING SUCROSE STEP GRADIENTS

This protocol was designed to purify intact, functional peroxisomes from the wild-type yeast strain D273-10B after growth in oleate medium (Distel et al., 1996). Yeast cells are precultured in glucose medium for two days and then transferred to oleate medium to induce the proliferation and growth of peroxisomes. After 12 to 18 hr in oleate medium, they are converted to spheroplasts using the β-glucanase preparation Zymolyase 100T and lysed by osmotic shock. Differential centrifugation is used to generate a peroxisome-enriched pellet. This pellet is subsequently fractionated on a sucrose density step gradient to purify the peroxisomes.

The protocol presented here calls for a 1-liter culture of D273-10B cells in oleate medium, but can be scaled up or down as needed. The extent of peroxisome proliferation in response

to growth on oleate medium can vary significantly among different *S. cerevisiae* strains. If using a strain other than D273-10B, it is wise to conduct a pilot experiment and monitor peroxisome induction using peroxisomal enzyme activity assays and electron microscopy. If the peroxisomes will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Materials

Rich growth medium (see recipe)
Yeast strain D273-10B (or another strain of interest)
Peroxisome induction medium (see recipe)
TSD reduction buffer (see recipe)
1.2 M sorbitol/phosphate solution (see recipe)
100,000 U/g Zymolyase 100T (ICN Biochemicals)
1.2 M sorbitol in MES buffer (see recipe for buffer), 4°C
MES buffer (see recipe), 4°C
0.65 M sorbitol/MES solution (see recipe), ice cold
20%, 30%, 40%, 44%, 46%, and 60% (w/w) sucrose/MES solution: ultrapure sucrose in MES buffer (see recipe), 4°C

100-ml and 2-liter flasks
50-ml centrifuge tubes
250-ml centrifuge bottles
1.6-ml microcentrifuge tubes
Sorvall GS-3 rotor
Centrifuge and Sorvall SS-34 rotor (or equivalent)
Dounce homogenizer with a loose-fitting pestle (optional), chilled
Beckman polyallomer Quick-seal tubes (25 × 89–mm) or equivalent
Vertical rotor (Beckman VTi 50 or equivalent), 4°C
30°C water bath
Syringe and wide-gauge needle

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Preculture cells

1. Three to four days before fractionation, inoculate 10 ml of rich growth medium in a 100-ml flask with a single colony of desired yeast strain from a fresh plate. Culture cells overnight at 28°C with vigorous shaking.

2. Measure OD_{600} the following morning. Use preculture to inoculate 10 ml of rich growth medium (in a new 100-ml flask) to an OD_{600} of 0.15. Culture cells during the day at 28°C with vigorous shaking.

3. Once culture reaches an OD_{600} of 1.0 to 1.5, inoculate 10 ml of rich growth medium (in a new 100-ml flask) with cells to an OD_{600} of ~0.010 to 0.015 (i.e., a 1:100 dilution). Incubate at 28°C overnight with vigorous shaking.

4. *Optional:* Repeat steps 2 and 3.

   Extended preculturing will generate a relatively homogeneous population of healthy, rapidly dividing cells that will respond well when transferred to the peroxisome proliferation medium; however, the third day of preculturing is usually not necessary.

Culture and harvest cells

5. In the morning, the day before the fractionation experiment, measure the OD_{600} of the 100-ml preculture from step 3. Inoculate 300 ml of rich growth medium (in a

2-liter flask) to an OD_{600} of 0.15. Culture cells at 28°C with vigorous shaking until culture reaches an OD_{600} of 1.0.

6. Once the culture reaches an OD_{600} of 1.0, harvest cells by centrifuging 5 min at ~4000 × *g* (4900 rpm in Sorvall GS-3 rotor), room temperature.
7. Decant supernatant and resuspend cells in 10 ml induction medium. Transfer 5 ml suspension to a flask containing 1 liter induction medium. Incubate overnight at 28°C with vigorous shaking.

Prepare cells to generate spheroplasts

8. In the morning of the day of fractionation experiment, harvest cells by centrifuging 5 min at 4000 × *g*, room temperature.

   For best results, harvest cells 12 to 18 hr after the shift to induction medium.

9. Decant supernatant and resuspend cells in a total of ~100 ml sterile water. Pellet cells by centrifuging 5 min at ~4000 × *g*, room temperature.
10. Decant supernatant and resuspend cells in a total of 40 ml sterile water. Transfer suspension to a single preweighed 50-ml centrifuge tube. Pellet cells by centrifuging 5 min at ~4000 × *g*, room temperature.
11. Decant supernatant and aspirate any remaining liquid. Weigh centrifuge tubes containing pellets to determine the wet weight of cell pellet.
12. Resuspend cells in freshly prepared TSD reduction buffer at 0.125 g/ml (i.e., add 8 ml TSD reduction buffer per gram wet cell pellet). Incubate 15 min at 30°C with gentle shaking.
13. Harvest cells by centrifuging 5 min at ~3000 × *g* (e.g., 5000 rpm in Sorvall SS-34 rotor), room temperature.

Generate spheroplasts

14. Decant supernatant and resuspend cells with ~30 ml 1.2 M sorbitol/phosphate buffer. Harvest cells by centrifuging 5 min at ~3000 × *g*, room temperature.
15. Decant supernatant and resuspend cells to 0.125 g/ml in 1.2 M sorbitol/phosphate buffer. Transfer 10 µl cell suspension to a 1.6-ml microcentrifuge tube and store on ice until step 17.
16. Add Zymolyase 100T to a final concentration of 1 mg enzyme per gram of wet cell pellet. Incubate 20 min at 30°C with occasional gentle agitation.
17. After 20 min, monitor cell wall digestion. Dilute 10 µl of each cell suspension into 990 µl water and mix by briefly vortexing. Also dilute the 10-µl samples stored after step 15 to serve as controls. After 1 or 2 min, briefly vortex again and measure the OD_{600} values.

    The decrease in OD_{600} values of diluted cell suspension indicates the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to the optical density.

    The extent of cell-wall digestion can also be examined by phase-contrast microscopy as described in Support Protocol, step 11.

18. Continue incubation until >95% of cells have been converted to spheroplasts, which usually takes 30 to 60 min. Harvest spheroplasts by centrifuging 5 min at ~1000 × *g* (e.g., 3000 rpm in Sorvall SS-34 rotor), room temperature.

19. Decant supernatant and carefully resuspend pellet in ice-cold 1.2 M sorbitol/MES buffer by swirling and gently mixing with a glass rod. Do not vortex, as spheroplasts are fragile.

    All subsequent steps are performed at 4°C with prechilled solutions and equipment.

20. Pellet spheroplasts by centrifuging 5 min at 1000 × *g*, 4°C. Transfer cell suspension to a preweighed centrifuge tube. Repeat steps 19 and 20.

21. *Optional*: Incubate spheroplasts 30 to 60 min at 28° to 30°C in rich growth medium supplemented with sorbitol to a final concentration of 1.2 M. Shake gently. Pellet spheroplasts by centrifuging 5 min at ~1000 × *g*, 4°C.

    Optimal recovery conditions should be determined empirically for each application.

    Other growth media supplemented with sorbitol to a final concentration of 1 to 1.5 M can be used.

22. Repeat steps 19 and 20.

Isolate enriched peroxisome fraction

23. Decant supernatant and weigh wet pellet. Gently resuspend spheroplasts in ice-cold 1.2 M sorbitol/MES buffer to a concentration of 0.125 g/ml (i.e., add 8 ml of solution per g wet cell pellet determined in step 11). Resuspend with a glass rod. Do not vortex.

24. To generate hypoosmotic conditions, slowly add ice-cold MES buffer with freshly added PMSF dropwise to spheroplast suspension while gently stirring until the final sorbitol concentration is 0.65 M.

    The required volume ice-cold MES buffer will need to be calculated based on the volume of 1.2 M sorbitol/MES added in step 23.

25. Monitor the degree of cell lysis microscopically.

    If >20% of the spheroplasts remain intact after several minutes, induce further lysis with a few gentle strokes in a chilled Dounce homogenizer with a loose-fitting pestle.

26. Centrifuge homogenate 10 min at 2000 × *g* (e.g., 4100 rpm in Sorvall SS-34), 4°C, to remove unlysed cells and large aggregates. Transfer supernatant to a new tube and store on ice until step 28.

27. Using a pipet, resuspend pellet in ice-cold 0.65 M sorbitol/MES solution with PMSF to a concentration of 0.125 g/ml. Centrifuge 10 min at 2000 × *g*, 4°C.

28. Pool supernatants from steps 26 and 27 in a 50-ml centrifuge tube prechilled on ice.

29. Pellet peroxisomes (as well as mitochondria and other organelles) from pooled supernatants by centrifuging 30 min at 20,000 × *g* (e.g., 13,000 rpm in Sorvall SS-34 rotor), 4°C.

30. After 30-min centrifugation, aspirate supernatant. Carefully resuspend the organelle pellet in 2 ml cold 30% sucrose/MES solution and store organelle suspension on ice.

    In order to minimize damage to the fragile peroxisomes, resuspend pellet using a glass rod or a pipet with a wide tip opening.

Determine protein concentration

31. Determine approximate protein concentration of organelle suspension by any standard method (e.g., *APPENDIX 3B*).

32. Add cold 30% sucrose/MES solution to organelle suspension to reach a final protein concentration of ~5 mg/ml. Carefully mix with a glass rod or a pipet with a wide tip opening.

Purify peroxisomes

33. Carefully prepare sucrose step gradients in Quick-seal centrifuge tubes by layering 5 ml of 60% sucrose/MES solution, 12 ml of 46% sucrose/MES solution, 12 ml of 44% sucrose/MES solution, and 5 ml of 40% sucrose/MES solution. Cool on ice for ≥10 min.

    It is important that gradients be prepared <30 min before use and interfaces between sucrose layers are sharp.

    One gradient will be needed for every 2 ml of organelle suspension.

34. Apply 2 ml suspension from step 30 to each sucrose step gradient. Fill and balance tubes with cold 20% sucrose/MES solution and carefully seal tubes.

35. Centrifuge 2.5 hr at 34,500 × *g*, 4°C, in a vertical rotor (Beckman VTi 50 or equivalent), using the slow acceleration and deceleration modes.

36. After centrifugation is completed, carefully remove gradient from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregations and other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C.

    Two bands will be visible in the gradient: (1) a narrow band at the 60%/46% sucrose interface consisting primarily of peroxisomes, and (2) a broader band at the 46%/44% sucrose interface consisting primarily of mitochondria.

37. Collect peroxisomes at the 60%/46% sucrose interface using a syringe with a wide-gauge needle.

    Alternatively, collect 2-ml fractions with an automated collector or a cut 1-ml pipet tip. Ideally the membrane layers at the 60%/46% and 46%/44% sucrose interfaces should each be in a single fraction.

    If the peroxisomes are not immediately needed, the sucrose fraction(s) can be rapidly frozen in liquid nitrogen and stored at −80°C.

BASIC PROTOCOL 8

ISOLATION OF ENDOPLASMIC RETICULUM USING SUCROSE STEP GRADIENTS

This protocol was designed to isolate functional ER membranes from yeast spheroplasts (Wuestehube and Schekman, 1992). Yeast cells are converted to spheroplasts with the β-glucanase Zymolyase 100T as in Support Protocol. The cells are lysed under hypoosmotic conditions with a motor-driven Potter-Elvehjem homogenizer (or a glass Dounce homogenizer). Differential centrifugation is used to generate a pellet that is enriched for ER membranes. This pellet is subsequently fractionated on a sucrose density step gradient to isolate ER membranes.

The protocol presented here calls for 5000 OD_{600} units of cells in mid-logarithmic growth phase, but it can be scaled-up or scaled-down as needed. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain).

Materials

Desired yeast strain (~5000 OD_{600} units of cells in exponential growth phase)
HEPES lysis buffer (see recipe), 4°C
1 M DTT
Protease inhibitor stock solutions B (see recipe)
1.5 M and 1.2 M sucrose/HEPES solution (see recipe)

250-ml centrifugation bottles
Motor-driven Potter-Elvehjem homogenizer (or a glass Dounce homogenizer)
1.6-ml centrifuge tubes
1.6-ml polycarbonate ultracentrifuge microcentrifuge tubes, prechilled
Sorvall GS-3 rotor
Trimmed 1-ml and 200-µl pipet tips (i.e., ~5 to 10 mm cut from the tips)
4-ml Dounce homogenizer, prechilled
Beckman Ultraclear 11 × 34–mm centrifuge tubes (or equivalent), prechilled
Swinging bucket rotor (e.g., Beckman SW 50.1 or equivalent), 4°C

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*), spheroplast preparation (see Support Protocol), and Lowry assay for proteins (*APPENDIX 3B*)

Culture cells and prepare spheroplasts

1. Culture desired yeast strains as described in Support Protocol, steps 1 to 4 (also see *UNIT 1.6*).

2. Harvest ~5000 OD_{600} units of cells in mid-logarithmic growth phase by centrifuging 5 min at ~4000 × *g* (5000 rpm in Sorvall GS-3 rotor), room temperature.

3. Prepare spheroplasts as described in Support Protocol, steps 5 to 15.

   Alternatively, spheroplasts can be prepared as in Basic Protocol 4 or by other methods.

   Spheroplasts are fragile and must be handled very gently.

4. Resuspend spheroplasts in ~100 ml ice-cold cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

   The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

   Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

   Spheroplasts can be stored at −80°C. Resuspend at 1000 OD_{600} units/ml in 0.7 mM sorbitol/20 mM HEPES (pH 7.4). Slowly freeze to −80°C in an insulated container and then store at −80°C.

Prepare spheroplast lysates

5. Harvest spheroplasts by centrifuging 5 min at 1500 × *g*, 4°C, and aspirate supernatant.

   From this point on, keep samples on ice or at 4°C. Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

6. Resuspend spheroplasts at a concentration of 1000 OD_{600} units/ml in ice-cold HEPES lysis buffer with freshly-added DTT and protease inhibitors.

7. Prepare lysate with a motor-driven Potter-Elvehjem homogenizer (i.e., 4 to 10 strokes). Keep lysate at 4°C.

   Alternatively, spheroplasts can be disrupted with 10 up-and-down strokes of a tight-fitting pestle in an ice-cold Dounce homogenizer. Keep Dounce homogenizer on ice throughout this process. Move pestle slowly and maintain contact between pestle and suspension to minimize foaming.

8. Inspect 10 µl of the lysate by phase microscopy.

   Intact spheroplasts have characteristic bright halos that are easily distinguished from lysed spheroplast and spheroplast fragments.

   Usually, >95% lysis is achieved.

9. Transfer lysate to multiple chilled 1.6-ml centrifuge tubes.

10. Clear lysate of unbroken cells, partially disrupted cells, and aggregates by microcentrifuging 10 min at 1000 × *g*, 4°C.

11. Carefully withdraw supernatants (S_{1000}) and transfer to new chilled tubes, taking care not disrupt loose pellets (P_{1000}).

    Usually only 80% to 90% of S_{1000} can be harvested from each tube.

12. Resuspend P_{1000} in ~2.5 ml ice-cold HEPES lysis buffer. Repeat homogenization and low-speed centrifugation (steps 7 to 10).

    This second homogenization step can improve the yield of intracellular membranes by ~30%.

Prepare $P_{27,000}$ membrane pellets

13. Pool the first and second S_{1000} supernatants (steps 11 and 12). Mix and transfer the pooled supernatants (S_{1000}) to polycarbonate ultracentrifuge tubes, chilled on ice.

    When not required for subsequent analyses, the P_{1000} pellets are usually discarded since they contain a crude mixture of unlysed and partially disrupted cells, as well as a very heterogeneous mixture of intracellular membranes.

14. Centrifuge the S_{1000} supernatants 10 min at 27,000 × *g*, 4°C. Remove the supernatants ($S_{27,000}$).

    The $S_{27,000}$ can either be saved for analysis or discarded.

15. To remove any residual supernatant from $P_{27,000}$ pellets, centrifuge tubes containing $P_{27,000}$ samples ~45 sec at 13,000 × *g*, 4°C. Carefully remove any remaining liquid above pellet and discard.

16. Resuspend $P_{27,000}$ membranes in 1.0 ml HEPES lysis buffer (~5000 OD_{600} equivalents per ml). Use five strokes in a Dounce homogenizer to ensure complete dispersion of $P_{27,000}$ pellet.

Prepare and run sucrose step gradients

17. Prepare four sucrose step gradients just prior to use so that the interface remains crisp. Carefully pipet 1.0 ml of 1.5 M sucrose/HEPES solution into each 11 × 34–mm ultracentrifuge tube. Using a trimmed 1-ml pipet tip, carefully layer 1.0 ml of 1.2 M sucrose/HEPES solution on top of the 1.5 M sucrose layer.

    If the interface is disturbed during or after preparation of gradient, a new step gradient will need to be prepared.

18. Carefully layer $P_{27,000}$ suspension (step 16) over the four sucrose gradients using a trimmed 200-µl pipet tip.

    Distribute the $P_{27,000}$ suspension among the four gradients so that they are loaded with similar volumes. Up to 0.5 ml of suspension can be loaded on each gradient.

19. Balance the appropriate pairs of gradients by adding ice-cold lysis buffer as necessary. Put gradient tubes into the prechilled buckets of the swinging bucket rotor (e.g., SW 50.1 rotor or equivalent).

20. Centrifuge gradient 1 hr at 100,000 × *g*, 4°C, using the slow acceleration and deceleration settings to minimize disruption of gradients.

Collect and process fractions

21. Carefully remove tubes from the rotor buckets and place in a rack. Make a note of the position and appearance of bands and any other visible characteristics.

    Two discrete membrane bands should be visible, one in the 1.2 M sucrose layer and one at the interface between the 1.2 M and 1.5 M sucrose layers.

22. Collect ER membranes at the 1.2 M/1.5 M sucrose interface. First remove the load layer and most of the 1.2 M sucrose layer by aspiration. Then collect the band at the interface.

    Alternatively, all of the gradient can be harvested as described in Basic Protocol 3, step 23.

23. Dilute ER membrane suspension 10-fold in ice-cold HEPES lysis buffer.

24. Harvest membranes by centrifuging 10 min at 27,000 × *g*, 4°C. Remove supernatant and process pellet fractions as desired.

25. Determine the protein concentration with the Lowry assay (*APPENDIX 3B*) after resuspending a small amount of the preparation in 1% SDS.

    Typically, this protocol yields ~7 mg of protein per 5000 OD_{600} units of cells.

ISOLATION OF PLASMA MEMBRANES FROM WHOLE YEAST CELLS USING SUCROSE STEP GRADIENTS

BASIC PROTOCOL 9

This protocol is an efficient method to isolate plasma membranes from intact yeast cells (Panaretou and Piper, 1996). Yeast cells are rapidly disrupted by vortexing with glass beads. The resulting lysate is fractionated by differential centrifugation to generate a pellet enriched for plasma membrane. The plasma membrane–enriched pellet is then fractionated on a sucrose density step gradient.

The protocol presented here calls for a 1-liter culture of yeast cells in exponential growth phase, but it can be scaled-up or -down as needed. If compatible with the goals of the experiment, it is often beneficial to use a strain that is deficient in vacuolar protease activity to minimize protein degradation (e.g., TVY1, a *pep4* mutant yeast strain), especially when working with heat-shocked cells.

Materials

1-liter culture of desired yeast cells in exponential growth phase
0.4 M, 1.1 M, 1.65 M, 2.25 M sucrose/imidazole solutions (see recipe), 4°C
Protease inhibitor stock solutions C (see recipe)
Breakage buffer (see recipe), 4°C
Plasma membrane storage buffer (optional; see recipe)

250-ml centrifuge bottles
50-ml polycarbonate centrifuge tubes
Acid-washed glass beads (0.45-mm diameter; see recipe), prechilled to 4°C
Sorvall GS-3 rotor
Sorvall SS-34 rotor (or equivalent) and appropriate centrifugation tubes, 4°C
14 × 89–mm Beckman Ultra-clear centrifugation tubes
Beckman SW41 or SW40Ti rotor (or equivalent), 4°C
Beckman 50 Ti rotor (or equivalent)

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and culture preparation (Support Protocol)

Grow cells

1. Prepare a 1-liter yeast culture in exponential growth phase (see Support Protocol, steps 1 to 4).
2. Harvest cells by centrifuging 5 min at 5000 × *g* (5000 rpm in Sorvall GS-3 rotor), 4°C.

   All subsequent steps should be performed at 4°C with prechilled solutions and equipment.

3. Decant supernatant and resuspend cells in 80 ml cold 0.4 M sucrose/imidazole solution with freshly added protease inhibitors.
4. Transfer cell suspension to two 50-ml polycarbonate centrifuge tubes. Centrifuge 10 min at 5000 × *g* (e.g., 6500 rpm in Sorvall SS-34 rotor), 4°C.

Perform glass-bead lysis

5. Decant supernatant and add two times the pellet volume of prechilled glass beads to pellets. Add sufficient cold 0.4 M sucrose/imidazole solution to just cover pellet and glass beads.
6. Vortex 5 min at maximum speed. Keep lysate cold by alternating between 30 sec of vortexing followed by 30 to 60 sec in an ice-water bath.

   Ice can be used instead of an ice-water bath; however, an ice-water bath will cool the lysates more efficiently.

7. Monitor degree of cell breakage under a microscope.

   The percentage of lysed cells should be between 50% and 80%. If the lysis efficiency is significantly below 50%, repeat step 6.

8. Place tubes on ice ~2 min to allow glass beads to settle and cool the lysate. Transfer supernatant to a new tube and store on ice.
9. Add ~2 times volume of cold 0.4 M sucrose/imidazole solution to glass beads. Vortex for 30 sec. Allow glass beads to settle while the tube is on ice.
10. Remove supernatant and pool with the supernatant collected in step 8. Repeat steps 9 and 10.

Isolate enriched plasma membrane fraction

11. Clear the pooled lysate of unbroken cells, cell wall debris, and aggregates by centrifuging 20 min at 530 × *g* (2150 rpm in Sorvall SS-34 rotor), 4°C.
12. During the centrifugation step, prepare two sucrose step gradients in 14 × 89–mm Beckman Ultra-clear centrifugation tubes. Add 4 ml of 2.25 M sucrose/imidazole solution to each tube. Then carefully overlay 4 ml of 1.65 M sucrose/imidazole solution, followed by 4 ml of 1.1 M sucrose solution. Chill gradients on ice until ready for use.

    Crisp interfaces are essential to the success of the experiment.

13. Transfer supernatant from step 11 to fresh centrifuge tubes. Centrifuge 30 min at 22,000 × *g* (13,500 rpm in Sorvall SS-34 rotor), 4°C, to obtain a pellet that includes plasma membranes (as well as nuclei, mitochondria, and vacuoles).
14. Carefully remove supernatant and resuspend pellet in 2 ml cold breakage buffer by vortexing for 30 sec at slow-medium speed.

    The use of <2 ml breakage buffer is not recommended, as membranes will tend to clump.

Purify plasma membranes

15. Carefully layer 1 ml resuspended membranes onto each of the two sucrose step gradients (prepared in step 12). Balance tubes with breakage buffer.

16. Centrifuge 14 hr at 80,000 × *g* (~22,000 rpm in Beckman SW-41 rotor), 4°C.

    Alternatively, centrifuge for 6 hr at 284,000 × g (~40,000 rpm in Beckman SW-41 rotor).

17. After centrifugation is completed, carefully remove gradient from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregations and other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C.

    A layer primarily consisting of plasma membrane should form at the 2.25 M/1.65 M sucrose solution interface; this band represents up to two-thirds of the plasma membranes loaded onto the gradient. The remaining plasma membranes will colocalize with mitochondria at the 1.65 M/1.10 M sucrose solution interface.

18. Collect membranes at the 2.25 M/1.65 M sucrose interface using a Pasteur pipet. Dilute membranes with 4 vol cold breakage buffer and store on ice.

    If desired, the other fractions can also be harvested and analyzed.

19. Transfer suspensions to centrifuge tubes. Pellet membranes by centrifuging 40 min at 30,000 × *g* (~17,000 rpm in Beckman 50 Ti rotor), 4°C.

20. Decant supernatant and resuspend pellet in desired buffer for immediate use.

    For storage, resuspend the pellet in plasma membrane storage buffer and store at −80°C.

21. *Optional*: Determine protein concentration by indirect means (*APPENDIX 3B*).

PREPARATION OF CYTOSOL FROM WHOLE YEAST CELLS

BASIC PROTOCOL 10

This protocol is designed to prepare cytosol from intact yeast cells (Haas, 1995). Yeast cells are rapidly disrupted by vortexing with glass beads. The resulting lysate is subjected to high-speed centrifugation to separate the cytosol from membrane components.

The protocol presented here calls for 2000 OD_{600} units of yeast cells grown to a concentration of 1 to 2 OD_{600} units/ml, but it can be scaled up or down as needed. If compatible with the goals of the experiment, it is often beneficial to use a strain that is deficient in vacuolar protease activity to minimize protein degradation (e.g., TVY1, a *pep4* mutant yeast strain).

Materials

Desired yeast strains
YPD (*UNIT 1.6* or appropriate growth medium)
PIPES lysis buffer (see recipe), prechilled
50× protease inhibitor cocktail (50× PIC; see recipe) or equivalent
Acid-washed glass beads (~0.45-mm diameter; BDH or Sigma; see recipe), prechilled to 4°C
Methylene blue
BioRad protein assay reagent kit (or equivalent)

250-ml and 2-liter Erlenmeyer flasks
Platform shaker, 30°C (or appropriate permissive temperature)
Centrifuge rotors (e.g., Beckman JA-10 and JA-20 rotors or equivalents)
500-ml centrifuge bottles
30-ml glass Corex tubes (1 tube per cytosol preparation), prechilled

Ice-water bath
Adapters for 30-ml glass Corex tubes (for Beckman JA-20 rotor or equivalent)
Ultracentrifuge tubes, 4°C
Ultracentrifuge rotor (Beckman TLA 100.2 rotor or equivalent)
Tabletop ultracentrifuge, 4°C
Microcentrifuge tubes, prechilled

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow cells

1. In the morning, the day before the experiment, start precultures of desired yeast strains by inoculating 20-ml of YPD (or appropriate medium) in 250-ml Erlenmeyer flasks. Grow throughout the day at 30°C (or appropriate permissive temperature) shaking at 225 to 335 rpm.

   It is best to start with a single yeast colony from a fresh plate.

   If using a strain that grows very slowly or if using a strain that has been stored on a plate at 4°C for more than a few days, it is wise to start the preculture with a single colony the night before.

2. In the evening, the day before the experiment, measure and record the OD_{600} values of precultures.

3. Inoculate two 1-liter cultures (in 2-liter Erlenmeyer flasks) with the appropriate volume of each preculture.

   Cultures of 1 to 2 liters are recommended for each strain, since 2000 OD_{600} units of cells at 1 to 2 OD_{600} units/ml are needed for each batch of cytosol. The suitable yeast cell concentration at this step depends on the growth rate of each strain and the length of incubation time. See Support Protocol step 3 for sample calculations.

   For some purposes, cytosol can be prepared from yeast cell cultures with a higher cell density (OD_{600} of 2 to 6); however, cytosol preparations from lower-density cultures (OD_{600} 1 to 2) generally seem to have higher activities as determined by vacuolar fusion assays (Haas, 1995).

4. Culture cells overnight at 30°C (or appropriate permissive temperature) while shaking at 225 to 325 rpm. In the morning, the day of experiment, analyze cell density.

   Ideally the cultures should have an OD_{600} of 1 to 2 to begin the experiment.

5. Harvest 2000 OD_{600} units of cells in 500-ml centrifugation bottles by centrifuging 5 min at 4400 × *g* (5000 rpm in JA-10 rotor), room temperature. Decant and discard supernatants.

Prepare cell lysates using glass beads

6. Resuspend pellets in a total of 50 ml prechilled PIPES lysis buffer (50 ml per 2000 OD_{600} units of cells). Transfer cells to smaller centrifuge tubes and vortex at maximum speed for 15 sec. Transfer cell suspensions to a single 500-ml centrifugation bottle.

7. Harvest the washed cells by centrifuging 5 min at 4400 × *g* (e.g., 5000 rpm in JA-10 rotor), room temperature. Decant and discard supernatants.

8. Resuspend cells in 2 ml prechilled PIPES lysis buffer (2 ml per 2000 OD_{600} units of cells). Add 80 µl 50× PIC to each 2-ml suspension and mix by vortexing.

9. Transfer the cell suspension into a prechilled, labeled 30-ml glass Corex tube (one tube/2000 OD_{600} units suspension). Add 3 g prechilled acid-washed glass beads. Seal tubes tightly with Parafilm and keep tubes 2 min in an ice-water bath.

10. Vortex at maximum speed for 30 sec. Then immediately return to ice-water bath for 1 min. Repeat 15 times for each tube.

    Ice can be used instead of an ice-water bath; however, an ice-water bath will cool the lysate more efficiently.

11. Monitor the degree of cell lysis by phase-contrast microscopy. Dilute 10 µl cell lysate in 90 µl PIPES lysis buffer. Add ~5 µl methylene blue and mix. View lysate to determine the percentage of cells that stain with the reagent.

    The stained cells have been lysed. Ideally, 80% of the cells should be lysed. If <50% of cells are lysed, repeat step 10.

Prepare cytosol

12. Clear lysate of unlysed cells, larger aggregates, and glass beads by centrifuging 10 min at 3000 × g (5000 rpm in JA-20 rotor with adapters for the Corex tubes), 4°C.

13. Carefully transfer supernatant to prechilled ultracentrifuge tubes (~1.2 ml per tube).

    If required, balance tubes using cold PIPES lysis buffer.

14. Centrifuge samples 1 hr at 150,000 × *g* (65,000 rpm in Beckman TLA 100.2 rotor), 4°C.

15. Carefully remove and discard the lipid layer visible on top of each sample. Then transfer supernatant to prechilled microcentrifuge tubes, being careful not to include any of the pellet.

16. Determine protein concentration of each cytosol sample using the BioRad protein assay reagent kit (or equivalent; see *APPENDIX 3B*). Prepare several dilutions of cytosol in water (e.g., 1:10 to 1:100; 10 µl total).

    The cytosol preparations usually have protein concentrations of 10 to 30 mg/ml.

    If the cytosol is not needed immediately, aliquot cytosol in prechilled tubes (e.g., 100 µl per 0.6-ml microcentrifuge tube). Quickly freeze the samples in liquid nitrogen and store at −80°C. Haas and coworkers (1995) have found that cytosol can be stored for months at −80°C and still support an in vitro vacuole-vacuole fusion assay.

REAGENTS AND SOLUTIONS

Use double-distilled water and Ultrapure reagents in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acid-washed glass beads

For yeast glass bead lysis, it is best to use glass beads between 0.4 and 0.5 mm (BDH or Sigma). Soak the glass beads in concentrated nitric acid or 1 M hydrochloric acid for 2 hr in a fume hood (use caution; wear safety goggles, nitrile gloves, and a long lab coat). During incubation, carefully mix beads a few times using a long glass stirring rod. Wash beads extensively with deionized water. Continue washing until the water has the same pH before and after addition to the beads (it is helpful to use a filter apparatus for the washing). Transfer beads to a glass dish or metal tray. Place them in a vacuum oven at ~200°C. Stir a few times to ensure that all of the beads will dry completely. Once the beads are completely dry and have cooled to room temperature, store them in a glass bottle at 4°C.

Breakage buffer

2 mM EDTA
25 mM imidazole-HCl, pH 7.0
Store up to a few weeks at 4°C

Just before use, add PMSF and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) to a final concentration of 1 mM each. Also add pepstatin A to a final concentration of 2.5 µg/ml (see recipe for protease inhibitor stock solutions C).

Keep buffer at 4°C, not 0°C, to prevent precipitation of protease inhibitors.

Buffer B, 2×

1.2 M sorbitol
40 mM potassium MES, pH 6.0
Filter sterilize
Store up to several weeks at 4°C

Dextran solution, 0.4 mg/ml (Basic Protocol 4)

Dissolve 2 mg dextran in 5 ml of 15% Ficoll solution (see recipe). Mix by vortexing. Prepare fresh and keep on ice until ready to use.

Less than 0.3 ml is required for each gradient.

Ficoll cushion solution (Basic Protocol 5)

5.5 g sorbitol
1.25 g Ficoll 400
H_2O to 25 ml
Prepare fresh and store at 0° and 4°C until used

Ficoll gradient buffer (Basic Protocol 4)

10 mM PIPES/KOH, pH 6.8
200 mM sorbitol
Store a few weeks at 4°C
Add 50× PIC and PMSF (1 mM; see recipes) just before use

Ficoll solutions

0% Ficoll solution: Ficoll gradient buffer (see recipe) without Ficoll. Add 50× protease inhibitor cocktail (50× PIC; see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

4% (w/v) Ficoll solution: Dissolve 4 g Ficoll 400 in 50 ml Ficoll gradient buffer (see recipe); adjust final volume to 100 ml with Ficoll gradient buffer. Add 50× PIC (see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

8% (w/v) Ficoll solution: Dissolve 8 g Ficoll 400 in 50 ml Ficoll gradient buffer (see recipe); adjust final volume to 100 ml with Ficoll gradient buffer. Add 50× PIC (see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

15% (w/v) Ficoll solution: Dissolve 15 g Ficoll 400 in 50 ml Ficoll gradient buffer (see recipe); adjust final volume to 100 ml with Ficoll gradient buffer. Add 50× PIC (see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

Prepare solutions on the day before the experiment (or earlier) since Ficoll takes a long time to dissolve. To minimize clumping, slowly add the indicated amount of Ficoll 400 to the Ficoll gradient buffer while stirring. Continue to stir the Ficoll solutions without heat. Once Ficoll has dissolved, adjust final volume of each solution to 100 ml with Ficoll gradient buffer. Sterilize by filtration. Store up to a few weeks at 4°C.

Ficoll lysis solutions (Basic Protocol 5)

50% (w/v) Ficoll lysis solution. Heat 100 ml 2× PM buffer (see recipe) and 30 ml water in a 400-ml beaker to ~60°C (not boiling). Slowly add 100.0 g Ficoll 400 with continuous, rapid stirring over a period of 5 to 10 min. Keep solution hot (but avoid boiling) and cover with a glass petri dish. Continue moderate stirring and gentle heating until Ficoll has completely dissolved (often takes ~1 hr). Allow the solution to cool to room temperature. Transfer Ficoll solution to a clean glass 250-ml cylinder. Use multiple washes with water to transfer all of Ficoll from beaker to cylinder. Once all of the bubbles have reached the surface, add water to cylinder to a final volume of 200 ml. Carefully seal cylinder with Parafilm and tape. Mix on a rotator until Ficoll stock is completely homogeneous (30 to 60 min). Aliquot into five sterile, plastic 50-ml tubes and store at −20°C. Remember to mix well after thawing. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

It is critical to prepare the 50% Ficoll lysis stock solution accurately.

20% (w/v) Ficoll lysis solution. Mix 40.0 ml of 50% Ficoll lysis solution (see recipe) and 30 ml of 2× PM buffer (see recipe), and add water to 100 ml. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

30% (w/v) Ficoll lysis solution. Mix 27.0 ml of 50% Ficoll lysis solution (see above) and 9.0 ml of 2× PM buffer (see recipe), and add water to 45 ml. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

40% (w/v) Ficoll lysis solution. Mix 36.0 ml of 50% Ficoll lysis solution (see above) and 4.5 ml of 2× PM buffer (see recipe), and add water to 45 ml. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

HEPES/KAc lysis buffer (Basic Protocols 1, 2, 3, and Alternate Protocol)

20 mM HEPES/KOH, pH 6.8
50 mM potassium acetate
200 mM sorbitol
1 mM EDTA
Autoclave and store up to 1 month at 4°C

Just before use, aliquot the desired amount of HEPES/KAc lysis buffer to a tube. Add 500× protease inhibitor cocktails A and B (PIC-A and PIC-B, see recipes) to a final concentration of 1×. Add 1 mM PMSF and 5 μg/ml α-macroglobulin (final concentrations; protease inhibitor cocktail stock solutions A, see recipe). If desired, add DTT to 1 mM but omit α-macroglobulin.

HEPES lysis buffer (Basic Protocol 8)

20 mM HEPES/KOH, pH 6.8
50 mM potassium acetate
100 mM sorbitol
2 mM EDTA

Autoclave and store up to 1 month at 4°C

Add DTT and protease inhibitors just before use at final concentrations of 1 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin, and 1 μg/ml leupeptin (see recipe for protease inhibitor stock solutions B).

MES buffer (Basic Protocol 7)

5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5
1 mM EDTA
1 mM KCl
Store up to 1 month at 4°C

Nycodenz solutions for equilibrium gradients (Basic Protocol 2)

Prepare the following solutions by diluting the 50% (w/v) Nycodenz/sorbitol stock solution (see recipe) with the appropriate volume of HEPES/KAc lysis buffer (see recipe): 37%, 31%, 27%, 23%, 20%, 17%, 13%, and 9% (w/v) Nycodenz solutions. Just before use, aliquot the desired volume of each Nycodenz solution to individual 15-ml tubes. Add 500× protease inhibitor cocktails A and B (PIC-A and PIC-B, see recipes) to a final concentration of 1×. Add 1 mM PMSF and 5 µg/ml α-macroglobulin (final concentrations; protease inhibitor cocktail stock solutions A, see recipe). If desired, add DTT to 1 mM but omit α-macroglobulin.

Alternatively, each solution can be prepared in a manner similar to 60% sucrose stock solution (see recipe).

Nycodenz stock solution, 50% (w/v) (Basic Protocol 6)

Dissolve 50% (w/v) Nycodenz (Life Technologies) in sterile water by slowly adding powder while stirring. Store several months at −20°C.

It may take several hours for the Nycodenz to dissolve completely.

Nycodenz/sorbitol stock solution, 50% (w/v)

50% (w/v) Nycodenz powder
50 mM HEPES/KOH, pH 6.8
1 mM EDTA
200 mM sorbitol
Aliquot and store several weeks at −20°C

Prepare well in advance because Nycodenz powder can take several hours to dissolve. To minimize formation of clumps, stir while adding Nycodenz powder.

Nycodenz step gradient solutions

18% (w/v) Nycodenz step gradient solution: Mix 10 ml of 2× buffer B (see recipe) with 7.2 ml of 50% Nycodenz stock solution (see recipe). Adjust final volume to 20 ml with water. Prepare fresh. Add PMSF to 1 mM final concentration just before use.

14.5% Nycodenz step gradient solution: Mix 10 ml of 2× buffer B (see recipe) with 5.8 ml of 50% Nycodenz stock solution (see recipe). Adjust final volume to 20 ml with water. Prepare fresh. Add PMSF to 1 mM final concentration just before use.

Oxalyticase buffer (Basic Protocol 4)

7.5 ml 1 M potassium phosphate buffer, pH 7.5 (*APPENDIX 2A*)
22.5 ml 4 M sorbitol
120 ml 0.2× YPD medium (*UNIT 1.6*)
Store up to a few weeks at 4°C

Final concentrations are 50 mM potassium phosphate, pH 7.5, 0.6 M sorbitol, and 0.16× YPD. About 15 ml is required for each gradient.

Peroxisome induction medium (Basic Protocol 7)

0.5% (w/v) Bacto peptone
0.3% (w/v) yeast extract
0.12% (w/v) oleic acid
0.2% (v/v) Tween 40

continued

0.5% (w/v) KH_2PO_4
Adjust to pH 6.0 with NaOH
Autoclave
Store up to several weeks at room temperature

At least 1.1 liter of induction medium will be needed per strain.

This medium is best prepared within a few days of use.

PIPES/DTT buffer (Basic Protocol 4)

Prepare 100 mM PIPES/KOH (pH 9.4) and 1 M DTT stock solutions in sterile water at room temperature. Mix 10 ml of 1 M PIPES/KOH (pH 9.4), 1 ml of 1 M DTT, and water for a final volume of 100 ml. Prepare fresh before use.

About 50 ml is required for each gradient.

PIPES lysis buffer (Basic Protocol 10)

20 mM PIPES/KOH (pH 6.8)
250 mM sorbitol
100 mM potassium acetate
50 mM KCl
5 mM $MgCl_2$
Store several weeks at 4°C
Just before use, add DTT, PMSF, and 50× PIC to final concentrations of 2 mM, 1 mM, and 2× respectively

Prepare for sterile components or filter sterilize.

PM buffer, 2× and 1× (Basic Protocol 5)

For 2×:
2.5 ml 1 M K_2HPO_4
5.5 ml 1 M KH_2PO_4
0.4 ml 1 M $MgCl_2$
H_2O to 200 ml
Filter sterilize
Store up to several weeks at 4°C

For 1×: Dilute 2× PM buffer 1:1 with water. Chill to 4°C. Add 1000× protease inhibitor cocktails PIC-W and PIC-D (see recipe) at 1:1000 just before use.

PMSF, 200 mM

Dissolve 34.5 mg phenylmethylsulfonyl fluoride (PMSF) in 9 ml absolute ethanol. Prepare fresh or store aliquots at −20°C for a few weeks.

Pretreatment buffer (Basic Protocol 5)

100 mM Tris·Cl, pH 9.4
50 mM DTT
5 mM EDTA, pH 9.0
Prepare fresh at room temperature from sterile stock solutions

Protease inhibitor cocktail (PIC), 50× (Basic Protocols 4 and 10)

10 µl leupeptin (0.5 mg/ml in water)
50 µl 1,10-phenanthroline (500 mM in ethanol)
25 µl pepstatin A (1 mg/ml in methanol)
50 µl Pefabloc (100 mM in water)
865 µl sterile water
Mix thoroughly, dispense into 1-ml aliquots, and store several months at −80°C
Dilute 1:50 or 1:25 in the indicated buffers just before use

Protease inhibitor cocktails (PICs), 500× and 1000× (Basic Protocols 1, 2, 3, and Alternate Protocol)

500× PIC-A: Dissolve 5 mg/ml antipain, 1 mg/ml leupeptin, and 1 mg/ml aprotinin in water. Prepare 1-ml aliquots and store several months at −20°C.

500× PIC-B: Dissolve 5 mg/ml pepstatin A and 5 mg/ml chymostatin in methanol. Prepare 1-ml aliquots and store several months at −20°C.

1000× PIC-D: Dissolve 880 mg phenylmethylsufonyl fluoride (PMSF; final concentration 0.5 M), 10 mg pepstatin A, and 10 mg chymostatin in 10 ml dimethylsulfoxide (DMSO). Prepare ten 1-ml aliquots and store up to several months at −20°C.

1000× PIC-W: Dissolve 1.5 g benzamidine (final concentration 1 M), 131 mg ε-aminocaproic acid (final concentration 1 M), 5 mg bestatin, and 5 mg leupeptin in 10 ml water. Prepare ten 1-ml aliquots and store up to several months at −20°C.

Dilute PIC solutions at 1:1000 in the indicated buffers solutions just before use. Add PIC-D below the surface of the solution while rapidly stirring to minimize precipitation.

Protease inhibitor cocktail solutions A

5 mg/ml α-macroglobulin in H_2O (optional; omit if using DTT)
200 mM PMSF in absolute ethanol (200×)
Store a few weeks at −20°C

Protease inhibitor cocktail solutions B

100 mM PMSF in absolute ethanol (100×)
1 mg/ml pepstatin in methanol (1000×)
1 mg/ml leupeptin in water (1000×)
Store a few weeks at −20°C

Protease inhibitor stock solutions C (Basic Protocol 9)

Prepare 100 mM PMSF in absolute ethanol

Prepare 100 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in absolute ethanol

Prepare 2.5 mg/ml pepstatin A in methanol (1000×)
Store protease inhibitor stocks in aliquots up to several months at –20°C

PSM 1 solution (Basic Protocol 5)

1.6 ml 1 M K_2HPO_4
2.4 ml 1 M KH_2PO_4
0.2 ml 1 M $MgCl_2$
17.12 g ultrapure sucrose (final concentration 250 mM)
H_2O to 200 ml
Filter sterilize
Store up to several weeks at 4°C

Rich growth medium (Basic Protocol 7)

0.67% (w/v) yeast nitrogen base without amino acids
0.1% (w/v) yeast extract
0.3% (w/v) glucose

Autoclave. Add required amino acids (usually to a final concentration of 20 µg/ml) once medium has cooled. Store up to a few weeks at room temperature.

At least 350 ml of growth medium will be needed per strain.

Semi-synthetic lactate medium (Basic Protocol 6)

In a total volume of 2.5 liters, mix:
15 g yeast extract
2.5 g glucose
2.5 g $CaCl_2 \cdot 2H_2O$
2.5 g NaCl
3 g $MgCl_2 \cdot 2H_2O$
5 g KH_2PO_4
5 g NH_4Cl
220 ml 90% (w/v) DL-lactic acid
40 g NaOH
Adjust volume to 5 liters with water
Autoclave
Store up to several weeks at room temperature

This medium is best prepared within a few days of use.

1.2 M Sorbitol/phosphate solution (Basic Protocol 7)

50 mM potassium phosphate, pH 7.5 (*APPENDIX 3B*)
1 mM EDTA, pH 7.5
Prepare with sterile stock solutions, autoclave, or filter sterilize
Store several months at 4°C

Sorbitol solution, 1.1 M (Basic Protocol 5)

Dissolve 50 g ultrapure sorbitol in water and dilute to 250 ml. Autoclave. Store up to a few months at 4°C.

Spheroplast medium A, pH 7.5 (Support Protocol)

1× yeast nitrogen base (YNB; Difco)
2% (w/v) glucose
1× amino acids
1 M sorbitol
20 mM Tris·Cl, pH 7.5
Store up to a few weeks at room temperature

Prepare from sterile stock solutions or filter sterilize.

Spheroplast medium B (Support Protocol, optional for other protocols)

1× yeast nitrogen base (YNB; Difco)
2% (w/v) glucose
1× amino acids
1 M sorbitol
Store up to a few weeks at room temperature

Spheroplast recovery medium (Basic Protocol 5, optional)

1 M sorbitol
1% (w/v) Bacto peptone (Difco)
1% (w/v) glucose
0.5% (w/v) Bacto yeast extract (Difco)
20 mM potassium phosphate, pH 6.5 (*APPENDIX 2A*)
Store up to a few weeks at room temperature

Stop solution (Support Protocol, optional for other protocols)

1 M sorbitol
20 mM NaN_3
20 mM NaF
Prepare shortly before use and chill to 4°C

Stop solution contains two energy poisons (NaN_3 and NaF) that are toxic and should be handled with care. The use of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

Sucrose gradient buffer (Basic Protocol 5)

50 mM potassium acetate
20 mM HEPES, pH 6.8
2 mM EDTA
Autoclave
Store up to several weeks at room temperature

Sucrose gradient solutions, 1.2 M and 1.5 M in sucrose gradient buffer (Basic Protocol 3)

Stock solutions: Prepare 1.516 M and 1.213 M sucrose solutions in sucrose gradient buffer (see recipe). Filter sterilize. Store up to several weeks at room temperature. Chill prior to use.

Working solutions: Prepare required amounts of 1.5 M and 1.2 M sucrose solutions on day of the experiment by adding 1 M DTT and 100 mM PMSF to a final concentration of 1 mM each (i.e., for 3 ml of 1.5 M sucrose, add 3 µl 1 M DTT and 30 µl 100 mM PMSF).

Sucrose/HEPES gradient solutions, 1.5 M and 1.2 M (Basic Protocol 8)

Prepare 1.516 M and 1.213 M stock sucrose solutions in HEPES lysis buffer (see recipe). Filter sterilize. Store at room temperature. Chill prior to use. Prepare required amounts of 1.5 M and 1.2 M sucrose solutions just before use by adding 1 M DTT and 100 mM PMSF to a final concentration of 1 mM each (i.e., for 3 ml of 1.5 M sucrose, add 3 µl 1 M DTT and 30 µl 100 mM PMSF).

Sucrose solutions for equilibrium gradients (Alternate Protocol)

Prepare the following sucrose solutions by diluting 60% sucrose stock solution (see recipe) with the appropriate volume of HEPES/KAc lysis solution (see recipe): 37%, 34%, 32%, 29%, 27%, 22%, and 10% (w/v) sucrose solutions. Just before use, aliquot the desired volumes of solutions to individual 15-ml tubes. Add 500× protease inhibitor cocktails A and B (PIC-A and PIC-B, see recipes) to a final concentration of 1×. Add 1 mM PMSF and 5 µg/ml α-macroglobulin (final concentrations; protease inhibitor cocktail stock solutions A, see recipe). If desired, add DTT to 1 mM but omit α-macroglobulin.

Alternatively, each solution can be prepared in a manner similar to 60% sucrose stock solution (see recipe).

Sucrose stock solution, 60% (w/v; Alternate Protocol)

60% (w/v) ultrapure sucrose
20 mM HEPES/KOH, pH 6.8
50 mM potassium acetate
1 mM EDTA
1 mM DTT (optional)
Store up to several months at −20°C

Sucrose/imidazole solutions (Basic Protocol 9)

0.4 M sucrose/imidazole solution: 0.4 M sucrose/2 mM EDTA/25 mM imidazole·HCl (pH 7.0)
1.1 M sucrose/imidazole solution: 1.1 M sucrose/2 mM EDTA/25 mM imidazole·HCl (pH 7.0)
1.65 M sucrose/imidazole solution: 1.65 M sucrose/2 mM EDTA/25 mM imidazole·HCl (pH 7.0)
2.25 M sucrose/imidazole solution: 2.25 M sucrose/2 mM EDTA/25 mM imidazole·HCl (pH 7.0)
Store up to several weeks at 4°C
Prechill to 4°C
Add protease inhibitor stock solutions C (see recipe) just before use

TE/SDS solution (Basic Protocol 5, optional)

10 mM Tris·Cl, pH 8 (*APPENDIX 2A*)
1 mM EDTA
0.1% (w/v) SDS
Store at up to several months at room temperature

100 mM Tris·SO_4, pH 9.4

Solubilize 12.11 g Tris in water. Adjust pH to 9.4 with sulfuric acid (H_2SO_4). Adjust final volume to 1 liter with water. Autoclave or filter sterilize. Store up to several months at room temperature.

TSD reduction buffer

0.1 M Tris sulfate, pH 9.4 (see recipe)
10 mM DTT added just before use

COMMENTARY

Background Information

A range of centrifugation-based subcellular fractionation procedures have been successfully applied to the yeast *Saccharomyces cerevisiae*. This unit includes several analytical procedures that are primarily designed to analyze the distribution of proteins along the exocytic, endocytic, and biosynthetic protein transport pathways (see Basic Protocols 1 to 3; Alternate Protocol). Preparative protocols for the isolation of yeast nuclei, vacuoles, mitochondria, peroxisomes, endoplasmic reticulum, plasma membrane, and cytosol are also presented (see Basic Protocols 4 to 10). In addition, numerous alternative *S. cerevisiae* subcellular fractionation procedures and the corresponding references are listed in Table 3.8.2.

These centrifugation-based fractionation procedures separate organelles and membranes according to their sedimentation velocities and/or buoyant densities. An overview of yeast subcellular fractionation procedures and the key considerations are presented in *UNIT 3.7*. In addition, several excellent reviews on yeast subcellular fractionation techniques (Walworth et al., 1989; Zinser and Daum, 1995) and on the theory and practice of centrifugation-based fractionation techniques (Dobrota and Hinton, 1992; Evans, 1992; Hinton and Mullock, 1997) are available.

Most yeast subcellular fractionation procedures begin with spheroplast preparation (i.e., the enzymatic removal of the yeast cell wall) in order to facilitate cell lysis and minimize damage to cellular components (e.g., see Support Protocol). Lysates can also be prepared by agitating intact yeast cells with glass beads. While glass bead lysis works well for the isolation of plasma membrane and cytosol (see Basic Protocols 9 and 10), this technique is not recommended for other fractionation procedures because the strong shear forces required to break the cell wall also damage subcellular components. Once cell lysates are prepared, most protocols then utilize differential centrifugation (e.g., see Basic Protocol 1) in order to enrich the starting material with the components of interest and to remove some of the undesirable components. Further fractionation is achieved by density gradient centrifugation (see Tables 3.8.1 and 3.8.2). Density gradient separation techniques are especially versatile,

since the sedimentation velocities and densities of subcellular components are determined both by their intrinsic properties and the characteristics of the surrounding medium. Thus, the optimal density gradient medium, density range, and shape will vary depending on which components one is trying to separate.

Preparation of yeast spheroplasts using Zymolyase

Most subcellular fractionation procedures begin with preparation of spheroplasts. Standard spheroplast preparation protocols include a reduction step (i.e., with DTT or 2-mercaptoethanol) to break disulfide bonds, followed by incubation with a glucanase enzyme preparation that disrupts the critical β-glucan linkages of the cell wall. Multiple glucanase preparations are commercially available (e.g., Zymolyase, lyticase). In addition, lyticase can be prepared in the laboratory (Scott and Schekman, 1980).

The conditions for optimal cell wall digestion will vary with the strain and growth conditions and thus often need to be determined empirically. The choice of a spheroplast preparation is primarily a matter of preference and expertise of the lab. Zymolyase and oxalyticase are generally preferred since they are highly purified. In contrast, Glusulase is very effective but is much less pure and consequently, contaminating proteases are more of a concern. In Support Protocol, DTT and Zymolyase treatment are used to prepare spheroplasts. Similar methods are used in Basic Protocols 6 and 7. Oxalyticase is used to digest the cell wall in Basic Protocol 4, while a combination of Zymolyase and Glusulase is used in Basic Protocol 5. In all cases, it is wise to monitor the cell wall digestion process by microscopy and/or optical density (OD_{600}) (lysis in water due to osmotic shock) as described in the Support Protocol.

Fractionation of spheroplasts by differential centrifugation

Differential centrifugation protocols rapidly fractionate cell lysates into broad size classes through sequential centrifugation steps with each step increasing in force and duration. The relative sedimentation velocities of the subcellular components are primarily determined by their mass; thus larger organelles and membranes will pellet faster and at lower centrifugal forces than smaller organelles. Differential centrifugation is particularly useful to generate fractions that are significantly enriched for the component(s) of interest and thus facilitates the subsequent fractionation steps. Differential centrifugation is also a convenient way to assess the potential subcellular locations of a protein of interest.

The resolution of the size separation by differential centrifugation is quite low, since the subcellular components are randomly dispersed throughout the medium prior to centrifugation. While the low-speed and medium-speed pellets will primarily consist of relatively large components, they will contain smaller components that were near the bottom of the tube before the centrifugation began.

Equilibrium density gradient fractionation

Basic Protocol 2, Basic Protocol 3, and Alternate Protocol are used to analyze the distribution of one or more proteins among the dynamic compartments of the exocytic, endocytic, and biosynthetic protein transport pathways. The protocols separate these subcellular components into sets of distinct, yet often overlapping fractions.

Basic Protocol 2 was designed to separate vacuolar membranes from endosomal and late Golgi compartments using equilibrium Nycodenz density gradient centrifugation (Rieder and Emr, 1997). The cleared cell lysate is loaded onto gradients, this minimizes the manipulation of lysate and the potential for membrane aggregation. However, abundant cytosolic proteins and unneeded membrane fractions can increase the potential for contamination and can limit the amount of the organelle of interest that can be loaded on a gradient. Thus it is often beneficial to use differential centrifugation (see Basic Protocol 1) to prepare a sample for density gradient fractionation. Pelleting of the membranes allows the removal of cytosolic proteins and can achieve some separation of membrane-bound organelles. A disadvantage to this approach is that it can be difficult to resuspend pelleted membranes well.

The samples can also be loaded onto the bottom of the density gradient if a concentrated Nycodenz stock is added to the sample to increase its density. Singer and co-workers have been able to differentiate between two different endocytic transport intermediates using Nycodenz floatation gradient (Singer and Riezman, 1990). The purification of endosomes, however, has been difficult. Endosome-enriched fractions contain significant levels of other membranes (e.g., ER and Golgi; Singer-Kruger et al., 1993), and the identification of new endosomal proteins using endosome-enriched fractions has remained elusive.

The equilibrium sucrose density gradient centrifugation protocol (Alternate Protocol) is particularly useful to separate vacuolar and endosomal membranes from late Golgi membranes (monitored using mALP, Pep12p, and Vps10p, respectively; see Table 3.7.9); however, the endosomal and vacuolar membranes overlap significantly (Becherer et al., 1996). In contrast, the Nycodenz gradient presented in Basic Protocol 2 can separate vacuolar membranes from endosomal and late Golgi membranes, but in this case the vacuolar and endosomal membranes overlap (Rieder and Emr, 1997). A similar sucrose equilibrium density gradient has been used to separate plasma membranes, ER membranes, and Golgi membranes into distinct sets of fractions (Bowser and Novick, 1991).

Fractionation of $P_{13,000}$ membranes on sucrose step gradients

Basic Protocol 3 was designed to analyze the distribution of one or more proteins among vacuoles, plasma membrane, early Golgi, and ER membranes (Gaynor et al., 1994). The sucrose step gradient is especially useful to determine whether a protein is associated with ER or Golgi compartments. The protocol is very similar to Basic Protocol 8, but on a smaller scale. A continuous sucrose velocity gradient has also been used to obtain discernible differences in the distribution of Golgi, ER, and vacuolar membranes (Antebi and Fink, 1992).

Isolation of intact vacuoles using Ficoll step gradients

Yeast vacuoles are acidic, lysosome-like compartments that occupy ~25% of the cell volume. These single-membrane-bound organelles harbor numerous hydrolases (e.g., proteases, phosphatases, lipases), as well as metabolites (e.g., basic amino acids, polyphosphates, and *S*-adenosylmethionine). Yeast vacuoles are very fragile and are easily damaged during subcellular fractionation experiments. This has two key consequences: (1) it is difficult to isolate intact vacuoles unless very gentle lysis and fractionation techniques are used (see Basic Protocol 4); and (2) the active hydrolases released from ruptured vacuoles can cause significant problems with protein degradation and other damage to subcellular components. Difficulties with protein degradation by vacuolar enzymes can be minimized by using yeast strains with deficient protease activity (e.g., *pep4 prb1* strains; see UNIT 3.7).

Basic Protocol 4 was designed to isolate intact, functional vacuoles. The spheroplasts are lysed with a dextran-mediated isoosmotic lysis procedure, a more gentle lysis technique that allows the isolation of intact vacuoles. However, even with this technique, some of the vacuoles inevitably rupture. The vacuoles and vacuolar membranes are isolated by floatation on a Ficoll gradient. The buoyant density of vacuoles is due to their relatively low protein density and the association of lipid particles or lipid storage granules with the vacuolar membrane (Zinser and Daum, 1995).

An alternate protocol for the isolation of vacuoles uses an hypoosmotic spheroplast lysis and two sequential Ficoll gradients (Roberts et al., 1991; Uchida et al., 1988). The hypoosmotic lysis procedure disrupts the integrity of the majority of vacuoles, as indicated by the loss of lumenal contents and the relatively small size of the isolated vacuoles (Uchida et al., 1988). However, the vacuoles are primarily right-side-out and still functional in several enzyme and transport assays.

Isolation of intact nuclei with Ficoll step gradients

The yeast nucleus is a relatively large (~1 μm), dense organelle that consists of a crescent-shaped nucleolus, chromatin, RNA, and numerous nuclear proteins surrounded by the nuclear envelope. The nuclear envelope is composed of two membrane bilayers that are interconnected at the nuclear pores. The outer membrane is continuous with portions of the rough ER (RER); indeed the nuclear envelope represents ~30% of the functional RER. Thus it is not possible to completely separate ER membranes and nuclei.

Basic Protocol 5 was designed to isolate intact nuclei from yeast spheroplasts using Ficoll step gradients (Dove et al., 1998). Nuclei have also been isolated using Ficoll-glycerol gradients and sucrose gradients (see Table 3.8.2). For example, Kalinich and co-workers developed a protocol that includes cytochalasin B treatment to minimize the interactions between nucleus and the cytoskeleton, followed by glycerol/Ficoll-glycerol step gradients to isolate the nuclei (Kalinich and Douglas, 1989).

Isolation of lactate-induced mitochondria using Nycodenz step gradients

Mitochondria are double-membrane-bound organelles that play a key role in eukaryotic cell metabolism. The enzymes of the electron transport and oxidative phosphorylation pathways are localized to mitochondria, which also contain mitochondrial DNA and the enzymes re-

quired for its replication, transcription, and translation. Growth of yeast on non-fermentable carbon sources such as lactate allows for optimal development of mitochondria. In contrast, the proliferation of mitochondria is repressed under anaerobic conditions, and the formation of pro-mitochondria is observed instead (Pon et al., 1989).

Basic Protocol 6 was designed to isolate intact mitochondria from lactate-grown D273-10B cells on Nycodenz step gradients. (Glick and Pon, 1995). If using cells other than lactate-grown D273-10B cells, it may be necessary to modify the Nycodenz density gradient procedure, as mitochondrial characteristics can vary between yeast strains. In addition, density of the mitochondria can vary significantly depending on growth conditions of the cells, such as the carbon source, temperature, and growth phase. A continuous Nycodenz gradient fractionation procedure to empirically determine the optimal Nycodenz concentrations for a step density gradient is presented by Glick and Pon (1995). Alternatively, mitochondria can be purified using Percoll gradients (Yaffe, 1991), as well as other gradients (see Table 3.8.2). Isolated mitochondria can be fractionated further into outer membrane, inner membrane, intermembrane space and matrix fractions (see Table 3.8.2).

Isolation of oleate-induced peroxisomes using sucrose step gradients

Peroxisomes are single-membrane-bound organelles that range from 0.1 to 1 μm in diameter. The enzymes of the β oxidation and glyoxylate pathways are localized to peroxisomes, as well as enzymes that generate and degrade hydrogen peroxide. The abundance, size, and protein composition of peroxisomes can vary dramatically in response to the cells' environment. When *S. cerevisiae* are grown under standard conditions on a fermentable carbon source (e.g., glucose), peroxisomes and their proteins are barely detectable. In contrast, when *S. cerevisiae* are cultured on fatty acids (e.g., oleate), the expression of peroxisomal proteins is induced and the peroxisomes proliferate and increase in size (Veenhuis et al., 1987).

Basic Protocol 7 was designed to purify intact peroxisomes from D273-10B cells (Distel et al., 1996). The peroxisomes are induced by growth on oleate medium and purified on sucrose step gradients. Alternatively, peroxisomes can be purified on continuous Nycodenz gradients, Nycodenz step gradients, or continuous sucrose gradients (see Table 3.8.2). Some researchers have found that peroxisomes purified on continuous Nycodenz gradients have slightly higher activities and lower levels of contamination with mitochondria than peroxisomes purified on sucrose gradients (Kunau et al., 1993). If an especially pure peroxisome preparation is required, peroxisomes can be purified on two sequential gradients, a sucrose gradient followed by a Nycodenz/sucrose gradient (Erdmann and Blobel, 1995).

Isolation of endoplasmic reticulum using sucrose step gradients

The endoplasmic reticulum (ER) is a complex network of compartments that plays a key role in the transport, folding, and post-translational modification of proteins, as well as in lipid metabolism and several other processes. ER compartments cannot be isolated in a completely intact state, and thus ER preparations primarily consist of ER membrane vesicles, often referred to as microsomes. Basic Protocol 8 was developed to isolate ER membranes for an in vitro protein transport assay (Wuestehube and Schekman, 1992). The protocol is very similar to Basic Protocol 3, but on a larger scale. Rough ER membrane vesicles can be separated from smooth ER vesicles using density gradients, since the ribosomes associated with the rough ER membranes increase their density relative to smooth ER membranes (Sanderson and Meyer, 1991).

Isolation of plasma membranes from whole yeast cells using sucrose step gradients

The plasma membrane forms a selective barrier between the cell's surroundings and its interior. Plasma membrane contains many signaling molecules, as well as transport proteins and channels that move molecules in and out of cells. The plasma membrane is very dynamic, due to the fast growth and division rates of yeast and the continuous exocytic and endocytic transport processes. Like ER membranes, plasma membrane preparations consist of vesicles and sheets.

Basic Protocol 9 provides a method to isolate plasma membranes from intact yeast cells after glass bead lysis (Panaretou and Piper, 1996). The use of intact yeast cells instead of spheroplasts has several benefits beyond ease and speed of the technique. Plasma membranes isolated from glass-bead cell lysates tend to have lower levels of ER contamination. In addition, Zymolyase and other β-glucanase preparations are contaminated with low levels

of proteases that could damage cell surface proteins. Indeed, plasma membrane preparations from spheroplasts have a slightly lower density than those prepared from whole cells.

Plasma membranes can also be successfully prepared from yeast spheroplasts, which are osmotically lysed and subjected to density gradient fractionation (see Table 3.8.2). Several of these procedures specifically alter the sedimentation properties of the plasma membrane. For example, a procedure using cationic silica beads that specifically bind to the plasma membrane is well suited for large-scale isolation of plasma membranes (Panaretou and Piper, 1996). In addition, researchers have used the lectin concanavalin A to facilitate the separation of plasma membrane from other cellular components (e.g., Ziman et al., 1996). Concanavalin A interacts with the glycoproteins found on the cell surface, thereby increasing the plasma membrane density and also minimizing its vesiculation.

Preparation of cytosol from whole yeast cells

The preparation of yeast cytosol is critical to the success of in vitro functional assays. Basic Protocol 10 is a simple and efficient method to prepare cytosol from yeast cells lysed by agitation with glass beads (Haas, 1995). The resulting lysate is subjected to high-speed centrifugation to separate the cytosol from cell wall fragments and membrane components. This cytosol preparation supports an in vitro vacuole-vacuole fusion assay and is likely to support other functional assays as well (Haas, 1995). Cytosol is also frequently prepared from spheroplast lysates (see Table 3.8.2). In addition, a large number of intact yeast cells can be processed by freezing them in liquid nitrogen and then disrupting the cells in a blender or with a mortar and pestle (see Table 3.8.2). Small molecules, such as residual ATP or ions, can be removed from cytosol by passing it over a Sephadex G-25 column (e.g., Wuestehube and Schekman, 1992).

Critical Parameters and Troubleshooting

Subcellular fractionation experiments share several key steps, including, (1) cell growth under appropriate conditions; (2) preparation of cell lysate with minimal damage to subcellular components; (3) selection of a fractionation protocol that separates subcellular components of interest into distinct sets of fractions; and (4) identification and characterization of the membranes/organelles in the resulting fractions. Table 3.8.3 summarizes some of the important factors that are relevant to most subcellular fractionation experiments. Many of these points are discussed in more detail in *UNIT 3.7*. Table 3.8.4 provides a summary of problems that can arise during subcellular fractionation experiments and suggestions for potential solutions. Some key steps that are especially important for the preparative protocols presented in this unit are summarized below.

Isolation of intact vacuoles

In Basic Protocol 4, vacuoles are isolated from spheroplast lysates prepared using a dextran-mediated isoosmotic lysis technique. This lysis procedure is more gentle than hypoosmotic lysis, leaving more of the vacuoles intact. The timing and temperature shifts are critical to the success of this lysis technique. It is important to use glass tubes, since they allow for more rapid temperature changes than plastic tubes. After adding the freshly prepared dextran and swirling to mix, the tubes are placed on ice for 2 min to allow the dextran to bind to the plasma membrane. The optimal amount of dextran for each strain needs to be determined empirically. The cell membrane is disrupted by placing tubes in a 30°C water bath for 60 to 75 sec and the tubes are then immediately placed on ice again. This heat-shock step should not be repeated.

After Ficoll gradient centrifugation, it is important to collect the vacuoles without harvesting the Ficoll solution. Ficoll can be difficult to remove and interferes with many subsequent analyses. If necessary, the Ficoll can be removed by dilution and centrifugation as described in Basic Protocol 5.

Isolation of intact nuclei

For optimal results, the cell cultures need to be in their early log growth phase. The extensive incubation time with glucanase preparations is important to maximize yields. The digestion should be continued until the cells are round and the clumped cells have separated. The lysis buffer should be at room temperature, not 4°C as in most other protocols. Lysis at low temperature will reduce the yield by 50%. However, after the lysis step, the preparation should be kept at 4°C. If the nuclei will be used for functional experiments, it is critical to allow the spheroplasts to recover from the glucanase treatment by incubating in spheroplast recovery medium (or another rich medium supplemented with 1.2 M sorbitol). Optimal conditions for the recovery often need to be determined empirically. For most purposes (e.g.,

Table 3.8.2 Subcellular Fractionation Protocols for *Saccharomyces cerevisiae*

Focus	Primary techniques	Selected references[a]
Endocytic and secretory pathways		
	Sucrose velocity gradient	Antebi and Fink (1992) *MBC* 3:633; Schimmoller et al. (1995) *EMBO J.* 14:1329; Schroder et al. (1995) *JCB* 131:895
	Sucrose equilibrium gradient[b]	Bowser and Novick (1991) *JCB* 112:1117; Becherer et al. (1996) *MBC* 7:579
	Sucrose equilibrium gradient, ConA treatment	Chuang and Schekman (1996) *JCB* 135:597; Ziman et al. (1996) *MBC* 7:1909; Ziman et al. (1998) *MBC* 9:1565
	Nycodenz equilibrium gradient[b]	Rieder and Emr (1997) *MBC* 8:2307
	Nycodenz equilibrium floatation gradient	Singer and Riezman (1990) *JCB* 110:1911
	Sucrose/D_2O step gradient	Singer-Kruger et al. (1993) *JBC* 268:14376
	Sucrose 2-step gradient[b]	Dean and Pelham (1990) *JCB* 111:369; Gaynor et al. (1994) *JCB* 127:653
Organelle isolation		
Nucleus	Sucrose step gradient	Strambio-de-Castillia et al. (1995) *JCB* 131:19
	Sucrose step gradient[b]	Aris and Blobel (1991) *Methods Enzymol.* 194:735; Dove et al. (1998) *Methods Cell Biol.* 53:33; Mann and Mecke (1980) *BBA* 687:57
	Ficoll/glycerol gradient	Kalinich and Douglas (1989) *JBC* 264:17979; Chang et al. (1999) *Methods Enzymol.* 304:76
Nuclear envelope	PVP-sucrose step gradient	Strambio-de-Castillia et al. (1995) *JCB* 131:19
	Ficoll-sucrose gradient	Mann and Mecke (1982) *BBA* 687:57; Hurt et al. (1988) *Eur. JCB* 46:554
Endoplasmic reticulum	Sucrose 2-step gradient[b]	Wuestehube and Schekman (1992) *Methods Enzymol.* 219:124
	Sucrose multistep gradient	Zinser et al. (1991) *J. Bacteriol.* 173:2026
	Continuous sucrose gradient (RER and SER)	Sanderson and Meyer (1991) *JBC* 266:13423
Golgi	Sorbitol gradient	Cleves et al. (1991) *Cell* 64:789; McGee et al. (1994) *J. Bacteriol*. 176:6861
	Sucrose/D_2O step gradient	Lupashin et al. (1996) *JCB* 132:277
Vacuoles	Hypoosmotic lysis, two Ficoll step gradients	Uchida et al. (1988) *Methods Enzymol.* 157:544; Roberts et al. (1991) *Methods Enzymol.* 194:644
	Isoosmotic lysis, Ficoll step gradient[b]	Haas (1995) *Methods Cell Sci.* 17:283; Mayer et al. (1996) *Cell* 85:83
Intravacuolar vesicles	Iodixinol gradient (Optiprep)	Harding et al. (1995) *JCB* 131:591; Oda et al. (1996) *JCB* 132:999

continued

Table 3.8.2 Subcellular Fractionation Protocols for *Saccharomyces cerevisiae,* continued

Focus	Primary techniques	Selected references[a]
Mitochondria	Self-forming Percoll gradient	Yaffe (1991) *Methods Enzymol.* 194:627
	Nycodenz step gradients[b]	Glick and Pon (1995) *Methods Enzymol.* 260:213
	Sucrose gradient	Daum et al. (1982) *JBC* 257:13028; Zinser et al. (1991) *J. Bacteriol.* 173:2026; Poyton et al. (1995) *Methods Enzymol.* 260:97; Martin et al. (1998) *Anal. Biochem.* 265:123
Mitochondrial membranes	Sucrose step gradients (inner membrane)	Jascur (1991) *Methods Cell Biol.* 34:359
	Continuous sucrose gradients (outer membrane and contact sites)	Pon et al. (1989) *JCB* 109:2604; Simbeni et al. (1991) *JBC* 266:10047
	Sucrose step gradients (outer membrane)	Alconada et al. (1995) *Methods Enzymol.* 260:263
Peroxisomes	Nycodenz/sucrose gradient	Thieringer and Kunau (1991) *JBC* 266:13110; Kunau et al. (1993) *Biochimie* 75:209; Erdmann and Blobel (1995) *JCB* 128:509
	Sucrose step gradient[b]	McCammon et al. (1990) *J. Bacteriol.* 172:5816; Distel et al. (1996) *Methods Mol. Biol.* 53:133
	Nycodenz gradient	Lewin et al. (1990) *MCB* 10:1399
Plasma membrane	Glass bead lysis, sucrose step gradient[b]	Serrano (1988) *Methods Enzymol.* 157:533; Panaretou and Piper (1996) *Methods Mol. Biol.* 53:117.
	Cationic bead isolation from spheroplasts	Schmidt et al. (1983) *BBA* 732:421; Panaretou and Piper (1996) *Methods Mol. Biol.* 53:117
Post-Golgi secretory vesicles[c]	Sephacryl S-1000	Walworth and Novick (1987) *JCB* 105:163
	Sephacryl S-1000, electrophoresis	Holcomb et al. (1987) *Anal. Biochem.* 166:328
	Sephacryl S-1000, Nycodenz gradients	Harsay and Bretscher (1995) *JCB* 131:297
ER-to-Golgi vesicles	Permeabilized cells, affinity-isolation	Groesch et al. (1990) *JCB* 111:45; Groesch et al. (1992) *Methods Enzymol.* 219:137
	Purified membrane and COPII components	Barlowe et al. (1994) *Cell* 77:895; Yeung et al. (1995) *JBC* 270:30567
	Permeabilized *ypt1*^{ts} cells[c], affinity-isolation	Rexach et al. (1994) *JCB* 126:1133
Clathrin-coated vesicles	Sephacryl S-1000 column	Mueller and Branton (1984) *JCB* 98:341
Cell lysates/cytosol	Glass bead lysis of whole cells[b]	Wuestehube and Schekman (1992) *Methods Enzymol.* 219:124; Haas (1995) *Methods Cell Sci.* 17:283
	Osmotic lysis of spheroplasts	Cheng et al. (1990) *Methods Enzymol.* 181:89; Hartley et al. (1996) *Methods Mol. Biol.* 53:249; Schultz (1999) *Methods* 17:161
	Disruption of whole cells frozen in liquid nitrogen	Dunn and Wobbe (1993) *CPMB UNIT 13.13*

[a]Abbreviations: *BBA*, Biochimica Biophysica Acta; *CPMB*, Current Protocols in Molecular Biology; *Eur JCB*, European Journal of Cell Biology; *JBC*, Journal of Biological Chemistry; *JCB*, Journal of Cell Biology; *MBC*, Molecular Biology of the Cell; *MCB*, Molecular Cell Biology.

[b]These types of protocols are included in this unit.

[c]Vesicles are isolated from certain temperature-sensitive mutant yeast strains that accumulate vesicles under nonpermissive conditions.

Table 3.8.3 Critical Parameters Common to Most Subcellular Fractionation Experiments

Procedure	Critical parameters
Strain selection and growth	The use of a vacuolar protease deficient strain can minimize proteolysis
	If available and compatible with goals of the experiment, use the strain(s) that the protocol was optimized for, as results can vary with strain and growth conditions
	Inoculate the culture with a single colony from a fresh plate (ideally prepared from frozen stock within the past week)
	Determine the correlation between the growth phase, OD_{600} value, and viable cell number for the strains, growth conditions, and spectrophotometer in use
	To generate a homogeneous, active cell population, culture the cells so that they are in their exponential growth phase at least 16 to 24 hr
	For the purification of peroxisomes and mitochondria, preculture cells in the appropriate induction medium prior to harvesting (i.e., oleate or lactate medium)
	Harvest cells in their mid-logarithmic growth phase (unless otherwise indicated)
Reagents and equipment	Use Ultrapure (or equivalent) reagents and double-distilled water for all solutions
	Be sure that glassware is clean and completely free of detergents
	Prepare reagents and solutions ahead of time (unless otherwise indicated)
	Bring reagents to the appropriate temperature in advance
	Label tubes ahead of time to save time and minimize mistakes
	Reserve critical equipment and prechill as needed
Spheroplast preparation	Start with a homogeneous population of dividing cells (unless otherwise indicated)
	Optimal conditions may need to be determined for each strain and growth condition in a small-scale pilot experiment
	Pretreat cells with DTT or 2-ME to break disulfide bonds within the cell wall
	Provide sufficient osmotic support (usually with 1 to 1.5 M sorbitol)
	Digest cell wall with Zymolyase 100T or another glucanase preparation
	Handle the spheroplasts gently (e.g., do not vortex)
	Monitor cell wall digestion by microscopy or lability in water (Support Protocol)
	Wash spheroplasts to remove residual glucanase and contaminating enzymes before continuing to the cell lysis step
Glass bead cell lysis	Use acid-washed beads of ~0.45 mm diameter
	Prechill beads to 4°C
	Resuspend the cell pellet in buffer before adding glass beads
	Use ~1:1 ratio of cell suspension to bead volume
	Cool the lysate every 30 to 45 sec in an ice-water bath
	Minimize foaming. If agitating the sample with a vortexer, be aware of how speed, pressure, and angle affect foaming
	Monitor lysis by microscopy (and methylene blue treatment)
	A Bead Beater is recommended for larger volumes (2 to 200 ml) as there is better heat dissipation and less foaming

continued

Table 3.8.3 Critical Parameters Common to Most Subcellular Fractionation Experiments, continued

Procedure	Critical parameters
Lysate preparation	Keep samples at 0° to 4°C (unless otherwise indicated)
	Add fresh protease inhibitors to solutions just before use
	Use a buffer with the appropriate composition and osmotic support (may need to be determined empirically)
	Use a homogenization technique that balances the need for cell lysis and the need for intact subcellular components
	Minimize foaming
	Handle the lysate gently as many subcellular components are fragile (e.g., avoid narrow pipet tips, vigorous pipetting, mixing with a vortexer)
Fractionation	Set aside samples at each step in order to monitor the recovery levels and marker distribution at each step
	Work as quickly, carefully, and consistently as possible
	Keep samples at 0° to 4°C (unless otherwise indicated)
Gradient preparation	Test whether gradient medium will disrupt protein interactions of interest
	Prepare mock gradients and measure their densities to ensure accuracy and reproducibility of gradient preparation
	Chill the gradients to 4°C before using
	Prepare step gradients just before use so that interface is sharp
	Use the slow acceleration and deceleration rate settings. To minimize mixing of layers, allow the rotor to coast to a stop after slowing to 1000 rpm
Gradient harvesting	Note any bands, pellets, and other characteristics before unloading the gradient
	Save all fractions until confident that they are not needed
	Save a small amount of each fraction to test its density
	When unloading one gradient, store the others at 4°C in a safe place
	If harvesting with a pipettor, change the pipet tip with every fraction
	If the organelles need to be intact, handle the fractions gently (e.g., avoid narrow pipet tips, vigorous pipetting, mixing with a vortexer)
	Work as quickly, carefully, and consistently as possible
Analysis of fractions	Determine the distribution and enrichment factors of multiple markers (see Tables 3.7.8 and 3.7.9)
	Remove gradient medium if it will interfere with subsequent analyses (e.g., by centrifugation)
	If the organelles need to be intact, handle the fractions gently (e.g., avoid narrow pipet tips, vigorous pipetting, mixing with vortexing)
	Remember that colocalization of subcellular components in a given fraction does not necessarily mean they colocalize in vivo
	Optional: examine the fractions for organelle integrity and/or function
	Ideally, confirm results and interpretations using an alternate technique

Table 3.8.4 Troubleshooting Yeast Fractionation

Problem	Potential causes	Potential solutions[a]
Inefficient cell wall digestion	Presence of older and/or stationary-phase cells (which have thicker cell walls)	Generate growth curves for strains and conditions being used
		Dilute or restart cultures and allow cells to grow for several generations until the desired growth phase is reached
	Bacterial contamination	Check for contamination by light microscopy
		If necessary, start a new yeast culture with sterile reagents
	Insufficient enzyme activity levels or incubation times	Purchase and/or prepare a fresh enzyme stock if required
		Determine the optimal conditions in small-scale experiments (e.g., enzyme concentration, duration of treatment with reducing agent, duration of digestion)
	The use of strains with especially thick, strong cell walls (e.g., mutant cells, cells approaching stationary growth phase)	Determine the optimal conditions in small-scale experiments
Inefficient cell lysis (spheroplasts)	Inefficient cell wall digestion	See above
	Insufficient mechanical force to disrupt spheroplasts and/or too much osmotic support	Increase the homogenization intensity and/or duration (e.g., tighter-fitting pestle; more strokes; different lysis technique; buffer with less osmotic support). However, these steps will tend to increase damage to intracellular components
Inefficient cell lysis (whole cells)	Glass beads not of proper size	Glass beads for cell lysis should be 0.45 to 0.55 mm in diameter
	Cells not resuspended before the addition of the glass beads	Resuspend the cell pellet in the lysis buffer before adding glass beads
	Excessive foaming	Determine the optimal conditions with test samples. Changing the angle of the tube or the vortex used can make a difference
	Insufficient intensity or duration of agitation	Increase the duration of agitation (with intermittent chilling on ice). Determine the optimal conditions with test samples
	Glass bead to cell ratio not optimal	Usually a 1:1 ratio of cell suspension to glass beads works well. The cell suspension is ~1:1 cells to buffer
	Presence of older and/or stationary-phase cells (which have thicker cell walls)	Generate growth curves for strains and conditions being used
		Dilute or restart cultures and allow cells to grow for several generations until the desired growth phase is reached
	The use of strains with especially thick, strong cell walls (e.g., mutant cells, cells in stationary growth phase)	Determine the optimal conditions in small-scale experiments

continued

Table 3.8.4 Troubleshooting Yeast Fractionation, continued

Problem	Potential causes	Potential solutions[a]
Proteolysis	Exposure to temperatures >4°C after cell lysis	Keep samples at 0° to 4°C as much as possible. Prechill all solutions and equipment to 0° to 4°C. Use ice and ice-water baths as possible
	Inadequate levels or specificity range of protease inhibitors	Increase levels or change composition of protease inhibitor cocktail
		Add inhibitor cocktail to all solutions during and after cell lysis
		Add PMSF more than once (as it has a short half-life in aqueous solutions)
		Prepare fresh protease inhibitor stocks (if older or improperly stored)
	Extended exposure to hydrolytic enzymes	Work as quickly as possible and keep samples ice cold
		Use a vacuolar protease–deficient strain (e.g., *pep4 prb1*)
		Minimize vacuole lysis (especially important with *PEP4* strains) by using a more gentle homogenization technique, increasing osmotic support in the lysis buffer, and/or treating samples gently throughout the experiment
		Separate components of interest from proteases as soon as possible (e.g., limit exposure to cytosol containing released vacuolar enzymes)
Poor separation on density gradients	Gradients overloaded	Decrease sample amount loaded onto gradients
	Membrane aggregation	See below
	Inadequate cell lysis	See above
	Gradient density medium, density range and/or shape are not ideal for the application	Determine more optimal conditions in small-scale experiments
Membrane aggregation	Insufficient resuspension of pelleted membranes	Pellet membranes onto a cushion (instead of against tube wall)
		Minimize the number and/or intensity of pelleting steps
		Increase intensity and/or duration of resuspension step (e.g., resuspend membranes in a small Dounce homogenizer or by passage through a small pipet tip or needle)
		Allow resuspended membranes to sit on ice in buffer
	Too much material	Use less material or more buffer
	Omission of a clearing step	The low-speed spin clears the lysate of partially-lysed cells (e.g., 500 × *g*)

continued

Table 3.8.4 Troubleshooting Yeast Fractionation, continued

Problem	Potential causes	Potential solutions[a]
	Buffer composition not optimal	Buffer composition can contribute to aggregation (e.g., high Mg^{2+} levels). Aggregation can be minimized to some extent with the addition of 1-2 mM DTT or 2-ME. EDTA can also be helpful
	Insufficient homogenization	Increase the homogenization intensity and/or duration (e.g., tighter-fitting pestle; more strokes; different lysis technique; buffer with less osmotic support). However, these steps will tend to increase damage to intracellular components
	Release of DNA from nuclei	Minimize damage to nuclei by handling samples gently (see below)
		If required, add DNase to 10 µg/ml
Damage to subcellular components	Insufficient osmotic support or rapid change in osmotic support	Increase osmotic support. Avoid rapid buffer changes
	High levels of proteolysis	See above
	Loss of peripheral membrane proteins and/or disruption of protein complexes	Use an alternate buffer or gradient medium. Some buffers and gradient media can destabilize protein interactions. Determine the optimal conditions in small-scale experiments
	Repeated pipetting, pelleting, and/or resuspension	Pellet membranes onto a cushion (instead of the tube wall)
		Handle suspension gently (e.g., cut pipet tip to enlarge the opening, initially resuspend by gently tapping the tube, pipet slowly, etc.)
		Minimize pelleting steps required
	Homogenization technique not gentle enough	Avoid cell lysis with agitated glass beads
		Use a more gentle method to lyse and homogenize spheroplasts (e.g., DEAE-facilitated isoosmotic lysis, a Dounce homogenizer with greater clearance)
		Monitor the appearance of the lysate with a light microscope
Low recovery of proteins and/or enzyme activities	Exposure to active hydrolases	See above
	Damage to membrane integrity and loss of lumenal contents	See above
	Loss of peripheral membrane proteins and/or disruption of protein complexes	Use an alternate buffer or gradient medium. Some buffers and gradient media can destabilize protein interactions
	Gradient media may interfere with enzymatic assays and/or liquid scintillation assays	Remove gradient media from fractions with an appropriate technique (e.g., centrifugation, dialysis). If necessary, choose an alternative gradient medium
	Low recovery of membranes	See below

continued

Table 3.8.4 Troubleshooting Yeast Fractionation, continued

Problem	Potential causes	Potential solutions[a]
Low recovery of membranes	Inefficient cell lysis	See above
	Inadequate homogenization	Increase the homogenization intensity and/or duration (e.g., tighter-fitting pestle; more strokes; different lysis technique; buffer with less osmotic support). However, these steps will tend to increase damage to intracellular components
		Repeat homogenization of low-speed pellet
	Membrane aggregation	See above
	Poor recovery of spheroplasts due to premature spheroplast lysis	Increase osmotic support (up to 1.5 M sorbitol)
		Handle spheroplasts gently (e.g., pellet spheroplasts onto a cushion or through a cushion, cut pipet tips to increase bore size, resuspend cells by swirling, pipet very slowly and gently, do not vortex)

[a]These potential solutions are not compatible with the goals of all experiments; thus they are only recommended if there are no conflicts with subsequent steps and the desired final result. In most cases, the problems cannot be entirely eliminated. Furthermore, pilot experiments may be needed to determine to optimum conditions for a particular purpose.

TCA protein precipitation, SDS-PAGE analysis, functional assays), Ficoll will need to be removed from the harvested nuclei.

Isolation of lactate-induced mitochondria

Basic Protocol 6 was optimized for lactate-grown D273-10B cells. If using cells other than lactate-grown D273-10B cells, it may be necessary to modify the Nycodenz density gradient procedure (Glick and Pon, 1995). The density of the mitochondria can vary significantly with the strain, growth conditions of the cells—e.g., carbon source, temperature—and growth phase.

Good aeration is critical for optimal cell growth and proliferation of mitochondria. A recovery period after cell wall removal is essential for optimal mitochondrial transcription/translation activity. It is critical that high-quality deionized sorbitol be used in the reagents, since lower quality grades contain trace metal contaminants that can damage mitochondria.

Isolation of oleate-induced peroxisomes

The isolation of intact peroxisomes from *S. cerevisiae* is more difficult than from other yeast strains (e.g., *Pichia*, *Candida*). In *S. cerevisiae*, the peroxisomes do not proliferate as readily and are especially fragile. For optimal peroxisome induction, cells must be dividing rapidly prior to the shift to oleate medium. Thus, extended preculturing (i.e., 2 to 3 days) is recommended in order to generate a relatively homogeneous population of healthy, rapidly-dividing cells that will respond well when transferred to the oleate medium. The cells should then be harvested 12 to 18 hr after the shift to induction medium for optimal results. If working with a strain other than D273-10B, it is wise to do a pilot experiment and monitor peroxisome induction using peroxisomal enzyme activity assays and electron microscopy.

In order to isolate intact peroxisomes from *S. cerevisiae*, it is critical that all buffers used during the isolation procedure be adjusted to a low pH (5.5 to 6.0; Thieringer and Kunau, 1991). The sucrose step gradients need to be prepared shortly before use and must have a sharp interface between the sucrose layers. Since peroxisomes are especially fragile, the density gradients used for the purification of peroxisomes are often centrifuged in a vertical rotor to decrease the required centrifugation time.

Isolation of endoplasmic reticulum

In order to maximize yield, the pellet from the first 1000 × *g* clearing spin should be homogenized a second time. This second homogenization step can improve the yield of intracellular membranes by ~30%. The sucrose step

gradients need to be prepared shortly before use and must have a sharp interface between the sucrose layers.

Isolation of plasma membranes from whole yeast cells

The plasma membranes are isolated from yeast cells after glass bead lysis. For efficient cell lysis, the ratio of glass beads to cell suspension is important. It is also critical to keep the lysate cold by alternating between vortexing and incubation in an ice-water bath. Ice can be used instead of an ice-water bath; however, an ice-water bath will cool the lysate more efficiently. The step gradients need to be prepared shortly before use and must have a sharp interface between the sucrose layers.

Preparation of cytosol from whole yeast cells

In Basic Protocol 10, cytosol is isolated from yeast cells cultured to an OD_{600} of 1 to 2. Cytosol can also be prepared from yeast cell cultures with a higher cell density (OD_{600} of 2 to 6); however, these cytosol preparations tend to have lower activity levels as determined by in vitro vacuolar fusion assays (Haas, 1995). For efficient cell lysis, the ratio of glass beads to cell suspension is important. It is also critical to keep the lysate cold by alternating between vortexing and incubation in an ice-water bath. The tubes need to be sealed tightly with Parafilm. The use of glass tubes allows the lysate to be cooled more rapidly.

A high-speed centrifugation step is used to separate cytosol from cell wall fragments, cellular membranes, and larger protein complexes and assemblies. The lipid layer that forms on top of each sample needs to be carefully removed and discarded. The supernatant should then be transferred to a prechilled microcentrifuge tube with a clean pipet, being careful not to include any of the pelleted material.

Use of protease inhibitors

It is critical to include protease inhibitors in all solutions for every step during and after cell lysis. In most cases a number of different inhibitors are included to ensure that a broad range of protease types are inactivated. The protocols described in this unit require a number of different protease combinations that have been recommended by the authors of published methods. Other combinations may be equally or even more appropriate for a particular application. Also convenient premixed protease inhibitor cocktails are commercially available (e.g., Boehringer-Mannheim, Sigma).

Anticipated Results

The unique protein compositions, enzymatic activities, and morphologies of subcellular organelles can be used to follow their distribution among the resulting fractions and analyze the success of an experiment. The most common method used to monitor the distribution and integrity of particular subcellular components is to analyze the distribution of characteristic marker proteins and enzyme activities (see *UNIT 3.7*, see Tables 3.7.7 and 3.7.8). The fractionated subcellular components can also be characterized by examining their morphologies, functions, and overall protein compositions. In order to establish the purity and yield of an organelle preparation or the success of an analytical fractionation experiment, it is important to determine the levels of the components of interest, as well as the levels of the potential contaminants.

The fractionation of subcellular components is never perfect or complete. Centrifugation-based fractionation procedures are limited by the fact that different organelle or membrane populations can have similar sizes and/or densities. In addition, members of a given organelle population vary both in mass and density to some degree. Both of these complications are exacerbated by the additional fragmentation caused by the lysate preparation and fractionation procedures.

Fractionation of spheroplasts by differential centrifugation

Basic Protocol 1 is a typical differential centrifugation method used to separate subcellular components into three membrane pellet fractions and a cytosol fraction. A schematic is presented in Figure 3.7.1. First, yeast cell lysates are subjected to a low centrifugal force (e.g., $300 \times g$) to pellet any unlysed cells and large aggregates. The lysate (S_{300}) is then subjected to sequential centrifugation at $13,000 \times g$ and $100,000 \times g$ to generate $P_{13,000}$ and $P_{100,000}$ pellets, respectively. The distribution of subcellular components in the resulting fractions is summarized in Table 3.7.4. The $P_{13,000}$ primarily contains large membrane structures, such as vacuolar membranes, plasma membrane, endoplasmic reticulum, mitochondria, and nuclei, while the $P_{100,000}$ fraction contains Golgi membranes and transport vesicles (Marcusson et al., 1994). Endosomal membranes containing the syntaxin-homolog Pep12p is distributed between the $P_{13,000}$ and $P_{100,000}$ pellets (Becherer et al., 1996). Soluble cytoplasmic proteins, soluble proteins released from the lumen of dam-

aged organelles, and released peripheral membrane proteins are found in the $S_{100,000}$ fraction. The P_{300} pellet will contain significant amounts of the larger organelles (e.g., plasma membrane, endoplasmic reticulum). The P_{300} and $P_{13,000}$ pellets will contain some smaller organelles that happen to be close to the bottom of the centrifuge tube or that are trapped within partially lysed cells and larger membrane structures. Some of these smaller organelles found in low-speed pellets can be removed by resuspending the pellet in fresh medium and repeating the centrifugation step.

Preparation of yeast spheroplasts

The optimal conditions for digesting the cell wall will vary with the strains, growth conditions, and glucanase enzyme preparation. It is wise to monitor the digestion process by microscopy or lability in water (Support Protocol). For most applications, at least 95% of the cells should be converted to spheroplasts within 20 to 40 min.

Equilibrium density gradient fractionation using Nycodenz

Basic Protocol 2 is particularly useful to separate vacuolar and endosomal membranes from late Golgi membranes (monitored using mALP, Pep12p, and Vps10p, respectively; see Table 3.7.9); however, the endosomal and late Golgi membranes overlap significantly (Rieder and Emr, 1997). The vacuolar membranes migrate to the top region of the gradient, while the endosomal and Golgi membranes localize to the more central fractions of the Nycodenz gradient.

Equilibrium density gradient fractionation using sucrose

The Alternate Protocol is particularly useful to separate vacuolar and endosomal membranes from late Golgi membranes (monitored using mALP, Pep12p, and Vps10p, respectively; see Table 3.7.9); however, the endosomal and vacuolar membranes overlap significantly (Becherer et al., 1996). The vacuolar and endosomal membranes migrate to the top fractions of the gradient, while the Golgi membranes localize to the more central fractions of the sucrose gradient.

Fractionation of $P_{13,000}$ membranes

Basic Protocol 3 was designed to analyze the distribution of one or more proteins among vacuoles, plasma membranes, early Golgi, and ER membranes (Gaynor et al., 1994). Six fractions of unequal volume are collected (summarized below). Vacuolar membranes are found in fractions 1 and 2. Golgi membranes are primarily in fraction 2. ER membranes and the plasma membrane vesicles are split between fractions 2 (~35%) and 4 (~65%; Gaynor et al., 1994).

Isolation of intact vacuoles

Basic Protocol 4 was designed to purify intact vacuoles that are functional in an in vitro vacuole-vacuole fusion assay (Haas, 1995). The resulting vacuole preparation is ~50-fold enriched in vacuolar proteins with respect to the total cell protein of the spheroplast lysate. Cytosolic and ER markers are found in the vacuole preparation in only trace amounts. The level of endosome contamination has not been investigated and may be significant.

The yield is <5% of the total vacuoles. Each gradient with material from ~500 OD_{600} cell equivalents yields a ~600-µl vacuole preparation with a protein concentration of ~0.25 to 0.9 mg/ml. One of the most prominent proteins in the vacuole preparation is Vph1p, a 95-kDa integral membrane protein that is part of the vacuolar ATPase. It is sensitive to proteolysis, so it also can appear as a 75-kDa fragment on SDS-PAGE gels. Isolated vacuoles can be viewed by staining with the vital dyes carboxydichlorofluorescein diacetate (CDC-FDA; lumen) or *N*-(3-triethylammoniumpropyl)-4-6-(diethylammino)phenyl) hexatrienyl)pyridinium dibromide (FM 4-64; membrane) in the presence of ATP (Roberts et al., 1991; Vida and Emr, 1995).

Isolation of intact nuclei

Basic Protocol 5 was designed to isolate intact nuclei from yeast spheroplasts (Dove et al., 1998). The preparation also contains ER membranes, as well as very low levels of plasma membrane, vacuoles, and sometimes mitochondria. The most prominent proteins include the histones and fibrillarin, the 38-kDa nuclear envelope protein. Six liters of yeast culture grown as described usually yield ~75 mg total nuclear protein. Only 2% to 5% of all nuclei are recovered after Ficoll gradients (Aris and Blobel, 1991). Yield and purity of nuclei can vary depending on strain, carbon source, and life stage. Quality of the preparation can be followed by microscopy due to characteristic size and shape of nuclei. Under a light microscope, the nuclei are uniformly gray with slightly rough edges. A small aliquot of the preparation can also be stained with DAPI (4′,6-diaminidino-2-phenylindone) to monitor the purification. This

protocol yields primarily intact nuclei. The nuclei are functional in several in vitro assays. The nuclei can be fractionated further to isolate nucleoli or nuclear envelope preparations (see Table 3.8.2; Dove et al., 1998).

Isolation of lactate-induced mitochondria

Basic Protocol 6 is designed to isolate intact, functional mitochondria from lactate-grown D273-10B spheroplasts (Glick and Pon, 1995). Cells other than lactate-grown D273-10B cells can be used; however, the mitochondrial density and abundance will vary with the strain and growth conditions, and thus the protocol may need to be optimized. The mitochondria will usually have dark brown color but may also look orange or pink. The gradient-purified mitochondrial preparation usually contains only trace amounts of the marker proteins for vacuoles, nuclei, endoplasmic reticulum, plasma membrane, cytosol, or cytosolic ribosomes. Some of the residual microsomal membranes (that associate with the mitochondrial surface) can be removed with an acidic wash as described in Zinser and Daum (1995). When examined by electron microscopy, the gradient-purified mitochondria preparation primarily consists of mitochondria; however, a minor population of unidentified membranes is present. Common mitochondrial marker proteins include porin (outer membrane), cytochrome *c* (inner membrane), cytochrome b_2 (intermembrane space), and citrate synthase (matrix). A typical yield from four 1-liter cultures of lactate-grown D273-10B cells is 20 to 25 mg of mitochondrial protein. These mitochondria are suitable for organelle translation studies, in vitro protein import studies, and other in vitro assays (Daum et al., 1982). If the mitochondria will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Isolation of oleate-induced peroxisomes

Basic Protocol 7 was designed to purify intact, functional peroxisomes from the wild-type yeast strain D273-10B after growth in oleate medium (Distel et al., 1996). Strains other than D273-10B can be used; however, it is wise to conduct a pilot experiment and monitor peroxisome induction and isolation steps. Peroxisomal fractions are highly enriched with peroxisomal proteins, such as catalase A, thiolase, and acyl-CoA oxidase. Contamination of the peroxisomal fraction with proteins from mitochondria, vacuoles, ER, and plasma membrane is low. Only a fraction of the initial peroxisome population is recovered after the sucrose density gradient steps. A substantial proportion of the peroxisomes are unavoidably lost in the low-speed pellet. A 1-liter culture of oleate-grown D273-10B cells typically yields 5 to 15 mg of peroxisomal protein. By electron microscopy, a homogenous population of single-membrane bound organelles should be visible. The isolated peroxisomes are functional for in vitro protein-import studies. If the peroxisomes will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Isolation of endoplasmic reticulum

Basic Protocol 8 was designed to isolate endoplasmic reticulum (ER) membranes from yeast spheroplasts (Wuestehube and Schekman, 1992). These membranes are functional in in vitro protein import and transport studies. The gradients are similar to those used in Basic Protocol 3. The ER membranes distribute in a membrane layer within the 1.2 M sucrose solution and at the 1.2 M/1.5 M sucrose interface. Both of these fractions have significant amounts of plasma membrane vesicles. Since the membrane band in the 1.2 M sucrose solution also contains vacuolar membranes and Golgi membranes, only the ER vesicles at the 1.2 M/1.5 M sucrose interface are harvested. Typically, this protocol yields ~7 mg of protein per 5000 OD_{600} units of cells.

Isolation of plasma membranes

Basic Protocol 9 isolates plasma membranes from yeast cells that are rapidly disrupted by vortexing with glass beads (Panaretou and Piper, 1996). Glass bead lysis protocols typically lyse 50% to 80% of the cells. The isolated plasma membrane preparation consists of vesicles in both the right-side-out and inside-out orientations. Vacuolar markers are usually not detected, while low levels of mitochondrial, ER, and Golgi markers are found. The plasma membrane vesicles isolated from disrupted whole cells tend to have lower levels of ER contamination than plasma membrane vesicles prepared from spheroplasts. Plasma membranes obtained from ruptured spheroplasts tend to have a lower density, presumably due to degradation of surface glycoproteins. The most prominent plasma membrane protein is the 100-kDa PM-ATPase. This Mg^{2+}-dependent ATPase activity is inhibited by orthovanadate, while the ATPase activities of mitochondria and vacuoles are inhibited by KNO_3 and bafilomycin A1, respectively. The activity of the plasma membrane ATPase can be sensitive to gradient medium, so protein analy-

sis is recommended in addition to enzymatic analysis.

Preparation of cytosol

Basic Protocol 10 is a simple and efficient method to prepare cytosol from yeast cell lysates prepared by glass bead lysis (Haas, 1995). Glass bead lysis protocols typically lyse 50% to 80% of the cells. The resulting cytosol is enriched 10- to 15-fold over the crude lysate in cytosolic proteins. ~2000 OD_{600} units of cells yield 25 to 60 mg of cytosolic protein at a concentration of 10 to 30 mg/ml.

Cytosol prepared by this technique supports in vitro vacuole-vacuole fusion and is likely to be functional in other in vitro assays. The cytosol should be tested and titrated for functional activity every time a fresh batch is prepared. Typically, cytosol prepared from cultures in their early- to mid-logarithmic growth phase has a higher specific activity than that prepared from cultures at later growth stages. Small molecules (e.g., ions, ATP) can be removed by passing the cytosol over a G25-Sephadex column.

Time Considerations

For best results, desired yeast strains should be obtained from glycerol stocks (stored at −80°C) and streaked onto fresh plates 2 to 3 days prior to starting the first cultures. Precultures should be started with a single colony from fresh plates 1 to 3 days before the fractionation experiment. Extensive preculturing (i.e., 2 to 3 days) is especially important prior to the induction of peroxisomes (see Basic Protocol 7). A full day should be allowed for each fractionation experiment, especially when doing the experiment for the first time. Some of the experiments will require several more hours on the following day to harvest fractions from gradients subjected to centrifugation overnight (see Basic Protocol 2, Alternate Protocol, and Basic Protocol 9).

It is wise to prepare well for fractionation experiments, since it is critical to work as quickly as possible once the cells have been lysed. Prepare all the solutions ahead of time (unless otherwise indicated) and store at the appropriate temperature. Reserve the necessary equipment and prechill if required. Prelabel tubes to the extent that it is possible. Minimize the number of strains used, especially when doing the experiment for the first time. It is often helpful to do small pilot experiments to determine the optimal experimental conditions for a given strain and growth regimen.

Literature Cited

Antebi, A. and Fink, G.R. 1992. The yeast Ca(2+)-ATPase homologue, *PMR1*, is required for normal Golgi function and localizes in a novel Golgi-like distribution. *Mol. Biol. Cell* 3:633-654.

Aris, J.P. and Blobel, G. 1991. Isolation of yeast nuclei. *Methods Enzymol.* 194:735-749.

Becherer, K.A., Rieder, S.E., Emr, S.D., and Jones, E.W. 1996. Novel syntaxin homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. *Mol. Biol. Cell* 7:579-594.

Bowser, R. and Novick, P. 1991. Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. *J. Cell Biol.* 112:1117-1131.

Daum, G., Bohni, P.C., and Schatz, G. 1982. Import of proteins into mitochondria. Cytochrome *b2* and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* 257:13028-13033.

Distel, B., van der Leij, I., and Kos, W. 1996. Peroxisome isolation. *Methods Mol. Biol.* 53:133-138.

Dobrota, M. and Hinton, R. 1992. Conditions for density gradient separations. *In* Preparative Centrifugation: A Practical Approach (D. Rickwood, ed.) pp. 77-137. Oxford-IRL Press, New York.

Dove, J.E., Brockenbrough, J.S., and Aris, J.P. 1998. Isolation of nuclei and nucleoli from the yeast *Saccharomyces cerevisiae*. *Methods Cell Biol.* 53:33-46.

Erdmann, R. and Blobel, G. 1995. Giant peroxisomes in oleic acid-induced *Saccharomyces cerevisiae* lacking the peroxisomal membrane protein Pmp27p. *J. Cell Biol.* 128:509-523.

Evans, W.H. 1992. Isolation and characterization of membranes and cell organelles. *In* Preparative Centrifugation: A Practical Approach (D. Rickwood, ed.) pp. 233-270. Oxford-IRL Press, New York.

Gaynor, E.C., te Heesen, S., Graham, T.R., Aebi, M., and Emr, S.D. 1994. Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* 127:653-665.

Glick, B.S. and Pon, L.A. 1995. Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol.* 260:213-223.

Haas, A. 1995. A quantitative assay to measure homotypic vacuole fusion in vitro. *Methods Cell Sci.* 17:283-294.

Hinton, R.H. and Mullock, B.M. 1997. Isolation of subcellular fractions. *In* Subcellular Fractionation: A Practical Approach (J.M. Graham and D. Rickwood, eds.) pp. 31-69. Oxford: IRL Press, New York.

Kalinich, J.F. and Douglas, M.G. 1989. In vitro translocation through the yeast nuclear envelope. Signal-dependent transport requires ATP and calcium. *J. Biol. Chem.* 264:17979-17989.

Kunau, W.H., Beyer, A., Franken, T., Gotte, K., Marzioch, M., Saidowsky, J., Skaletz-Rorowski, A., and Wiebel, F.F. 1993. Two complementary approaches to study peroxisome biogenesis in *Saccharomyces cerevisiae*: Forward and reversed genetics. *Biochimie* 75:209-224.

Marcusson, E.G., Horazdovsky, B.F., Cereghino, J.L., Gharakhanian, E., and Emr, S.D. 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the *VPS10* gene. *Cell* 77:579-586.

Panaretou, B. and Piper, P. 1996. Isolation of yeast plasma membranes. *Methods Mol. Biol.* 53:117-121.

Pon, L., Moll, T., Vestweber, D., Marshallsay, B., and Schatz, G. 1989. Protein import into mitochondria: ATP-dependent protein translocation activity in a submitochondrial fraction enriched in membrane contact sites and specific proteins. *J. Cell Biol.* 109:2603-2016.

Rieder, S.E. and Emr, S.D. 1997. A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. *Mol. Biol. Cell* 8:2307-2327.

Roberts, C.J., Raymond, C.K., Yamashiro, C.T., and Stevens, T.H. 1991. Methods for studying the yeast vacuole. *Methods Enzymol.* 194:644-661.

Sanderson, C.M. and Meyer, D.I. 1991. Purification and functional characterization of membranes derived from the rough endoplasmic reticulum of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 266:13423-13430.

Scott, J.H. and Schekman, R. 1980. Lyticase: Endoglucanase and protease activities that act together in yeast cell lysis. *J. Bacteriol.* 142:414-423.

Singer, B. and Riezman, H. 1990. Detection of an intermediate compartment involved in transport of alpha-factor from the plasma membrane to the vacuole in yeast. *J. Cell Biol.* 110:1911-1922.

Singer-Kruger, B., Frank, R., Crausaz, F., and Riezman, H. 1993. Partial purification and characterization of early and late endosomes from yeast. Identification of four novel proteins. *J. Biol. Chem.* 268:14376-14386.

Thieringer, R. and Kunau, W.H. 1991. The beta-oxidation system in catalase-free microbodies of the filamentous fungus *Neurospora crassa*. Purification of a multifunctional protein possessing 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase activities. *J. Biol. Chem.* 266:13110-13117.

Uchida, E., Ohsumi, Y., and Anraku, Y. 1988. Purification of yeast vacuolar membrane H^+-ATPase and enzymological discrimination of three ATP-driven proton pumps in *Saccharomyces cerevisiae*. *Methods Enzymol.* 157:544-562.

Veenhuis, M., Mateblowski, M., Kunau, W.H., and Harder, W. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast* 3:77-84.

Vida, T.A. and Emr, S.D. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* 128:779-792.

Walworth, N.C., Goud, B., Ruohola, H., and Novick, P.J. 1989. Fractionation of yeast organelles. *Methods Cell Biol.* 31:335-356.

Wuestehube, L.J. and Schekman, R.W. 1992. Reconstitution of transport from endoplasmic reticulum to Golgi complex using endoplasmic reticulum-enriched membrane fraction from yeast. *Methods Enzymol.* 219:124-136.

Yaffe, M.P. 1991. Analysis of mitochondrial function and assembly. *Methods Enzymol.* 194:627-643.

Ziman, M., Chuang, J.S., and Schekman, R.W. 1996. Chs1p and Chs3p, two proteins involved in chitin synthesis, populate a compartment of the *Saccharomyces cerevisiae* endocytic pathway. *Mol. Biol. Cell* 7:1909-1919.

Zinser, E. and Daum, G. 1995. Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. *Yeast* 11:493-536.

Key References

Walworth et al., 1989. See above.

This older review provides an excellent summary of the subcellular fractionation techniques applied to S. cerevisiae, with a focus on organelles along the secretory pathway.

Zinser and Daum, 1995. See above.

This review provides an excellent summary of the subcellular fractionation techniques applied to S. cerevisiae and the corresponding references.

Internet Resources

http://www.proteome.com/

The Yeast Protein Database (Proteome) is an excellent source of comprehensive and up-to-date information on S. cerevisiae proteins. The information is derived from the published literature and DNA sequence databases. The protein information provided, including summaries of the protein and gene characteristics and the corresponding literature references, can be searched and categorized in several convenient ways (e.g., according to subcellular location, gene name, size).

http://genome-www.stanford.edu/Saccharomyces/

Saccharomyces Genome Database (SGD) provides access to the complete sequence of the S. cerevisiae genome, the confirmed and predicted open reading frames (ORFs), protein information, and a number of useful internet links.

Contributed by Stephanie E. Rieder
The Scripps Research Institute
La Jolla, California

Scott D. Emr
University of California San Diego
School of Medicine
La Jolla, California

Isolation of Golgi Membranes from Tissues and Cells by Differential and Density Gradient Centrifugation

Golgi membranes from actively secreting tissues form a major defined organelle and are relatively easy to isolate so long as the method of tissue disruption is as mild as possible, such that fragmentation of the Golgi stacks is minimized. As a general rule, if the Golgi membranes retain their in vivo tubular structure, they will sediment when centrifuged at 10,000 to 15,000 × *g* for 10 to 20 min. They can thus be purified from a light mitochondrial fraction by flotation through a discontinuous sucrose gradient (see Basic Protocol 2). Alternate Protocol 1 adapts this method to a self-generated iodixanol gradient. This may be of particular use for multiple samples because of the simplicity of gradient preparation. In mammalian liver, not only can the homogenization conditions be tailored to the retention of Golgi tubules, but, by inclusion of dextran in the homogenization medium, the entire Golgi stack may be retained so that it sediments at a relative centrifugal force (RCF) as low as 5000 × *g* (see Basic Protocol 1). This not only promotes a high recovery of Golgi membranes but also reduces possible contamination by other membrane particles. Isolation of Golgi membranes is facilitated by their having a distinctively low density, lower than any other membrane except possibly the *trans*-Golgi network and some plasma membranes. Thus, even if the Golgi membranes are present in a homogenate in a vesicular form (e.g., those from cultured cells), they can be isolated either in a discontinuous sucrose density gradient (see Basic Protocol 3), a continuous iodixanol gradient (see Alternate Protocol 2), or a self-generated iodixanol gradient (see Basic Protocol 4). Again the latter may be of particular use for multiple samples.

The aim of the protocols described in this unit is to isolate the Golgi in a preparative manner; the unit does not specifically address the requirements for an analytical investigation of the secretory process, which requires the simultaneous fractionation of a series of membrane compartments through a continuous gradient. Strategies for these studies (including the partial resolution of the *cis*-, medial, and *trans*-Golgi domains; *trans*-Golgi network; and various vesicular intermediates) will be given in a future unit. It is worth noting, however, that the protocols for the isolation of Golgi vesicles (particularly Basic Protocol 4 and Alternate Protocol 2) also permit the simultaneous isolation of other vesicular fractions and can therefore also be used in an analytical mode.

This unit also includes a routine method (see Support Protocol) for the assay of the UDP-galactose galactosyltransferase, which, although strictly a *trans*-Golgi enzyme, is the most commonly used marker in any preparative Golgi method.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

RAPID ISOLATION OF GOLGI MEMBRANES FROM RAT LIVER USING A SUCROSE DENSITY BARRIER

BASIC PROTOCOL 1

The use of dextran in the homogenization medium and a mechanical shear means of homogenization, as opposed to liquid shear, favors the retention of intact Golgi stacks. This permits the recovery of the Golgi membranes in a relatively low-speed pellet (5000 × *g* for 15 min). In the bipartite pellet that is formed, the Golgi forms a quite distinct upper layer, which can be resuspended independently of the much more well-packed material

Contributed by John M. Graham
Current Protocols in Cell Biology (2001) 3.9.1-3.9.24

(containing nuclei, some plasma membrane sheets, and mitochondria) in the lower layer (Morré et al., 1972). Purification of the Golgi is then achieved using a simple sedimentation on a 1.2 M sucrose density barrier. Because dextran effectively cross-links the Golgi tubules, these membranes cannot be further subfractionated into the *cis*-, medial, and *trans*-Golgi domains unless they are unstacked by hydrolyzing the dextran with a mixture of amylases. The unstacking procedure of Hartel-Schenk et al. (1991) is presented below as an option.

If the presence of dextran is undesirable, then one of the alternative methods described in Basic Protocol 2 and Alternate Protocol 1 should be used. The following protocol is adapted from Morré et al. (1972).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

150- to 200-g Sprague-Dawley rat
DHM (see recipe)
1.2 M sucrose density barrier (see recipe)
HM (see recipe), optional
Crude α-amylase Type X-A from *Aspergillus oryzae* (Sigma Aldrich Co.), optional
α-Amylase Type VIII-A from barley (Sigma Aldrich Co.), optional

Dissecting tools, including scissors, razor blade, and forceps
Polytron homogenizer (Brinkmann Instruments)
Phase-contrast microscope
High-speed centrifuge with swinging-bucket rotor and 30- to 50-ml clear plastic tubes
10-ml syringes with long metal cannulas (i.d. ~1 mm) or Pasteur pipet attached to an aspirator
Glass rod
Ultracentrifuge with swinging-bucket rotor (Beckman SW 28.1, Sorvall AH629, or equivalent) and 17-ml tubes

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

Isolate and mince liver

1. Starve a 150- to 200-g Sprague-Dawley rat overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.

   This must be supervised or carried out by an experienced animal technician.

3. Open the abdominal cavity and remove liver to a chilled beaker containing ~20 ml DHM. Record weight of the liver.

   This protocol is suitable for a 10- to 12-g liver.

4. Decant medium and finely mince liver using scissors or a razor blade.

   The pieces of liver should be no more than ~25 mm^3.

Isolate intact stacked Golgi

5. Suspend liver mince in ~20 ml DHM.

6. Set suspension on ice and homogenize with a Polytron homogenizer set at 10,000 rpm (setting 1) for 40 sec, using 10-sec pulses and 10-sec rests. Check for completeness of tissue disruption by phase-contrast microscopy.
7. Centrifuge 15 min in a high-speed centrifuge at 5000 × *g*, 4°C.

   The brake should not be used to decelerate the rotor; rapid changes in rpm may disturb the layering of the pellet.
8. Very carefully remove most of the supernatant using a 10-ml syringe with a long metal cannula or a Pasteur pipet attached to an aspirator. Leave 0.5 to 1.0 ml supernatant.

   The supernatant should not be decanted.

   The Golgi membranes are contained in the upper (yellow-brown) portion of the bipartite pellet.
9. Resuspend upper portion of pellet in the residual supernatant by very gentle stirring with a glass rod.

   Resuspension of the lower part of the pellet, which contains nuclei and whole cells, must be avoided.
10. Transfer resuspended Golgi material to a 10-ml measuring cylinder using the syringe and cannula.
11. Adjust concentration of the suspension with DHM to 6 ml per 10 g liver and layer over 2 vol of 1.2 M sucrose density barrier in a 17-ml tube.
12. Centrifuge 30 min in an ultracentrifuge at 120,000 × *g*, 4°C.
13. Remove Golgi membranes, which collect at the interface, with a fresh syringe and cannula.
14. If required, dilute fraction with ≥2 vol DHM (or HM) and centrifuge 20 min at 10,000 × *g*, 4°C.
15. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet in DHM (or HM) to 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤16 hr. Many assays can be carried out on material frozen to –20° or –80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

Unstack Golgi (optional)

If a subsequent aim is to attempt to resolve various domains of the Golgi membranes, it is necessary to hydrolyze the dextran in order to unstack the Golgi. The following is adapted from Hartel-Schenk et al. (1991).

16. Incubate 4 to 5 ml Golgi membrane suspension with 3 mg each of crude α-amylase Type X-A from *Aspergillus oryzae* and α-amylase Type III-A from barley at 4°C for 45 min.
17. Use gentle liquid shear to complete the destacking process by repeatedly (5 to 6 times) drawing suspension into and expelling it from a Pasteur pipet (1-mm tip i.d.) or a 10-ml syringe with a long metal cannula.

    The success of the destacking process can only be monitored by electron microscopy or by comparing the banding pattern of cis-, medial, and trans-Golgi markers in a suitable density gradient or in some other separative technique before and after treatment.

BASIC PROTOCOL 2

ISOLATION OF GOLGI MEMBRANES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION BY FLOTATION THROUGH A DISCONTINUOUS SUCROSE GRADIENT

If the use of dextran to maintain the stacked format of the Golgi apparatus, as described in Basic Protocol 1, is unacceptable, then a light mitochondrial fraction from a liver homogenate can be used to overcome several of the problems associated with the fragmentation of the Golgi. The relatively gentle homogenization technique used in this protocol is aimed at retaining the tubular structure of the Golgi membranes and avoiding excessive fragmentation. In this manner, at least some of the Golgi membranes will sediment at ~15,000 × *g*, thus facilitating the separation procedure. Purification of Golgi vesicles from vesicles produced from other smooth membrane compartments of the cell (plasma membrane, *trans*-Golgi network, smooth endoplasmic reticulum, and some endocytic structures) may be more problematic. In a microsomal fraction the Golgi vesicles will be both denser and lighter than some of the other vesicle populations. By processing the light mitochondrial fraction rather than a microsomal fraction (or some other microsome-containing fraction) these problems are minimized, as the Golgi is the least dense component of this fraction. This method works very satisfactorily for rat liver but its applicability to other tissues (and to cultured cells) will depend on the behavior of the Golgi membranes during homogenization and differential centrifugation. For the method to be suitable for a particular tissue or cell type, a significant amount of a Golgi marker (e.g., galactosyltransferase) should be recovered in the light mitochondrial fraction rather than the microsomes. The method is adapted from Fleischer and Fleischer (1970) and Graham and Winterbourne (1988).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

150- to 200-g Sprague-Dawley rat
HM (see recipe)
2.0, 1.33, 1.2, 1.1, 0.77, and 0.25 M sucrose gradient solutions (see recipe)

Dissecting tools, including scissors or razor blade
Potter-Elvehjem homogenizer, ~0.09-mm clearance, 40-ml working volume (Fisher Scientific), with chilled pestle
Overhead high-torque electric motor, thyristor controlled (Fisher Scientific)
Low-speed centrifuge with swinging-bucket rotor and 50-ml tubes
~5- and ~30-ml Dounce homogenizers (Wheaton) with loose-fitting type-B conical pestles
High-speed centrifuge with 8 × 50–ml fixed-angle rotor (e.g., Sorvall SS34)
10-ml syringe and metal cannula
Refractometer
Ultracentrifuge with swinging-bucket rotor (Beckman SW 28.1, Sorvall AH629, or equivalent) and 17-ml tubes

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

Isolate and mince liver

1. Starve a 150- to 200-g Sprague-Dawley rat overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.

   This must be supervised or carried out by an experienced animal technician.

3. Open the abdominal cavity and remove liver to a chilled beaker containing ~20 ml HM.

   This protocol is suitable for a 10- to 12-g liver.

4. Decant medium and finely mince liver using scissors or a razor blade.

   The pieces of liver should be no more than ~25 mm^3.

Homogenize liver

5. Suspend mince in ~40 ml HM and transfer half of this suspension to the glass vessel of a Potter-Elvehjem homogenizer.
6. Attach the cold homogenizer pestle to an overhead high-torque electric motor and homogenize liver mince using four to five up-and-down strokes of the pestle, rotating at ~500 rpm.

   The motor should be mounted either to a wall via a G clamp, to the bench, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

7. Decant homogenate into a beaker on ice and rinse homogenizer with HM, wiping pestle to remove any connective tissue that may be adhering.
8. Repeat the procedure with the other half of the mince.

Prepare light mitochondrial fraction

9. Transfer homogenate to a 50-ml conical tube and centrifuge 10 min at $1000 \times g$, 4°C, in a low-speed centrifuge to pellet nuclei.
10. Decant supernatant and place on ice.
11. Transfer nuclear pellet to an ~30-ml Dounce homogenizer in ~20 ml HM. Use four gentle strokes of the loose-fitting pestle to resuspend pellet.
12. Transfer suspension to a clean 50-ml tube and recentrifuge 10 min at $1000 \times g$, 4°C.
13. Combine supernatants from steps 10 and 12, and centrifuge 10 min at $3000 \times g$, 4°C, to pellet the heavy mitochondria.
14. Transfer supernatant to a clean 50 ml tube and centrifuge 20 min at $17{,}000 \times g$, 4°C, in a high-speed centrifuge.
15. Aspirate supernatant using a 10-ml syringe and metal cannula, and discard.

Isolate Golgi membranes

16. Resuspend light mitochondrial pellet in 1 to 2 ml HM by vortex mixing, then add 8 ml of 2.0 M sucrose gradient solution (final concentration ~1.55 M). Transfer to an ~5-ml Dounce homogenizer and homogenize using gentle strokes of the loose-fitting pestle.
17. Use a refractometer to check that the refractive index is 1.4080 and adjust, if necessary, with either HM or 2.0 M sucrose.

    If a refractometer is not available, check that the suspension will layer beneath the 1.33 M sucrose solution.

18. Transfer 5 ml light mitochondrial suspension to a 17-ml tube and overlay it with the following sucrose gradient solutions:

    4.0 ml 1.33 M sucrose
    2 ml 1.2 M sucrose
    2 ml 1.1 M sucrose
    2 ml 0.77 M sucrose
    0.25 M sucrose to fill the tube

19. Centrifuge 1 hr in an ultracentrifuge at 100,000 × *g*, 4°C.

Harvest light Golgi membranes

20. Harvest material that bands at the 0.77 M/1.1 M and 1.1 M/1.2 M sucrose interfaces and any intervening material.

21. If required, dilute fraction with ≥2 vol HM and centrifuge 20 min at 10,000 × *g*, 4°C.

22. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet in HM to 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤16 hr. Many assays can be carried out on material frozen to −20°C or −80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

ALTERNATE PROTOCOL 1

ISOLATION OF GOLGI MEMBRANES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A SELF-GENERATED GRADIENT OF IODIXANOL

In this variation, the light mitochondrial fraction from mammalian liver is suspended in a medium containing 15% iodixanol and centrifuged in a vertical, near-vertical, or low-angle fixed-angle rotor at >180,000 × *g* for 1 to 3 hr. The centrifugation conditions are chosen to produce a slightly sigmoidal density profile; the relatively shallow gradient in the middle of the tube is used to provide a large linear separation between the Golgi membranes (low density) and the other organelles (high density). The precise gravitational forces and times will depend on the rotor type. In high-performance vertical rotors, which can produce ~350,000 × *g*, the separation will take only 1 hr. The method is adapted from Graham et al. (1994). It should be applicable to other tissues and cells, although the iodixanol concentration (step 3) may need to be modulated.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Additional Materials *(also see Basic Protocol 2)*

HME (see recipe), optional
OptiPrep
ODB solution (see recipe)
IWS solution (see recipe)
High-density cushion (20%, w/v, iodixanol): 2:3 (v/v) IWS/HME (or HM)
Ultracentrifuge with vertical (e.g., Beckman VTi 65.1, Sorvall 65V13), near-vertical (e.g., Beckman NVT 65) rotor and ~11-ml sealable ultracentrifuge tubes or low-angle fixed-angle rotor (~20°; e.g., Beckman 50.3Ti) and ~5-ml sealable ultracentrifuge tubes.

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

1. Isolate a rat liver, homogenize, and prepare a light mitochondrial fraction as described (see Basic Protocol 2, steps 1 to 15) in either HM or HME.

   The sample can be prepared in the presence (HME) or absence (HM) of EDTA (see Background Information, discussion of homogenization).

2. Resuspend light mitochondrial pellet in HME or HM to a total volume of 14 ml.

3. Add 6 ml IWS to the suspension (15% w/v iodixanol final).

   EDTA may be omitted from the OptiPrep dilution buffer (ODB) used to make IWS.

4. Transfer 9.5 ml to an ~11-ml sealable ultracentrifuge tube or a 4.5- to 5.0-ml sealable tube.

5. Underlayer suspension with 0.5 ml high-density cushion; omit cushion with fixed-angle rotor.

   The cushion prevents the densest organelles (peroxisomes) from pelleting against the wall of the tube and making unloading difficult. This is particularly important if a vertical rotor is used.

6. Fill the tube by layering HME or HM on top, seal the tube, and centrifuge 1 to 3 hr in an ultracentrifuge at 180,000 to 350,000 × *g*, 4°C.

   The precise centrifugation conditions depend on the rotor type and tube sedimentation path length. In a vertical rotor such as the VTi 65.1, 1 hr at 350,000 × g will be sufficient, while at 180,000 × g, 3 hr will be needed. Longer path-length rotors may need 3 hr at 350,000 × g. Fixed-angle rotors with these tube volumes are not ideal for rapid formation of self-generated gradients because of their relatively long sedimentation path lengths. Many Beckman rotors that are capable of high speeds (e.g., 80 Ti and 90 Ti) can, however, be adapted to accommodate smaller-volume g-Max tubes that have suitable path lengths. Ford et al. (1994) and the Axis-Shield Density Gradient Media Catalog (Axis-Shield, 2000) provide more information on the preparation of self-generated iodixanol gradients.

7. During deceleration below 2000 rpm, either use a programmed deceleration or turn off the brake.

8. Harvest the Golgi membranes, which band near the top of the gradient.

ISOLATION OF GOLGI MEMBRANES FROM CULTURED CELLS BY FLOTATION THROUGH A DISCONTINUOUS SUCROSE GRADIENT

BASIC PROTOCOL 3

This protocol, devised by Balch et al. (1984), is applicable to any cell homogenate (primarily from cultured cells) in an isoosmotic sucrose medium. The whole homogenate is adjusted to 1.4 M sucrose and layered under a discontinuous sucrose density gradient of 1.2 M and 0.8 M sucrose. During centrifugation, the Golgi membranes, which have a much lower density than those of the other membrane particles (with the exception of the *trans*-Golgi network and possibly the plasma membrane), float upwards to band at the 1.2 M/0.8 M sucrose interface. By including a layer of 1.6 M sucrose beneath the homogenate it is also possible to resolve (at least partially) some of the endoplasmic reticulum (ER) from the other denser organelles (mitochondria, lysosomes, peroxisomes, and nuclei), which will sediment into or through this layer (the smooth ER in particular will remain in the load zone). The advantage of this procedure is not only the speed of preparation but the elimination of differential centrifugation; resuspending the pellets also minimizes any comminution of the subcellular organelles. A similar discontinuous sucrose gradient consisting of 8.4% (0.25 M), 32.5% (0.95 M), and 45.2% (1.33 M) sucrose, with the sample (an 11,000 × *g* supernatant) loaded beneath the gradient in ~51% (1.5 M) sucrose, was devised by Macintyre (1992). In Alternate Protocol 2, the use of a continuous iodixanol gradient is described.

The homogenization procedure should be as gentle as possible (in a medium containing 0.25 M sucrose) to avoid excessive fragmentation of internal organelles. A ball-bearing device ("cell cracker") of the type designed by Balch and Rothman (1985) is recommended; it certainly causes the least amount of damage to organelles. A tight-fitting Dounce homogenizer is a possible alternative. The method may also be used for a tissue such as rat liver, but volumes will need to be scaled up (see annotations). The following protocol is adapted from Balch et al. (1984).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

Cultured cells
HM (see recipe)
0.8, 1.2, 1.6, and 2.0 M sucrose gradient solutions (see recipe)

Ball-bearing homogenizer
5-ml syringe and metal cannula
Ultracentrifuge with swinging-bucket rotor (Beckman SW 28.1, Sorvall AH629, or equivalent) and ~17-ml tubes, 4°C

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

1. Wash 1–3 × 10^8 cultured cells twice in 5 ml HM, resuspend in 3 ml HM, and homogenize in a ball-bearing homogenizer using five passes.

   Alternatively use ten to fifteen strokes of a tight-fitting pestle (0.025- to 0.075-mm clearance) in a Dounce homogenizer.

   For a tissue such as rat liver, the liver mince (see Basic Protocol 1, steps 1 to 4) should be suspended in 20 ml HM and homogenized using ten strokes of a loose-fitting pestle in a Dounce homogenizer.

2. Make the homogenate 1.4 M with respect to sucrose by adding 2 vol of 2.0 M sucrose gradient solution.

3. Transfer 6 ml to an ~17-ml tube.

   Use ~2.2× this volume (here and in step 4) for rat liver. A larger-volume rotor will also be required (~38-ml tubes).

4. Overlay sample with 6 ml of 1.2 M sucrose gradient solution and 3 ml of 0.8 M sucrose gradient solution.

5. Use a 5-ml syringe and metal cannula to underlay sample with 2 ml of 1.6 M sucrose gradient solution.

6. Centrifuge 2 hr in an ultracentrifuge at 110,000 × *g*, 4°C.

7. Harvest the Golgi band from the 0.8 M/1.2 M sucrose interface.

   If required, harvest the ER, which bands within the 1.4 M sucrose layer.

8. If required, dilute fraction(s) with ≥2 vol HM and centrifuge 30 min at 100,000 × *g*, 4°C.

9. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet in HM to 1 to 5 mg protein/ml.

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤16 hr. Many assays can be carried out on material frozen to −20°C or −80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

ISOLATION OF GOLGI MEMBRANES FROM CULTURED CELLS IN A CONTINUOUS GRADIENT OF IODIXANOL

ALTERNATE PROTOCOL 2

Yang et al. (1997) devised a continuous iodixanol (0% to 26%, w/v) gradient to study the localization of UBC6 ubiquitin-containing protein in COS-7 cells, and Zhang et al. (1998) used a similar gradient (1% to 20%, w/v) to study the localization of presenilins to the ER or Golgi membranes of CHO cells. Sufficient resolution of the Golgi was obtained that these methods could be used to prepare Golgi on a preparative basis. The following protocol is based on these two published methods and may be used in one of three sample-handling modes: (1) layering a postnuclear fraction (or postmitochondrial supernatant) on top, (2) layering a microsomal fraction on top, or (3) layering any fraction (including a homogenate) at the bottom (see annotations to this protocol). The method also allows simultaneous purification of the ER and plasma membrane. See Commentary for more information on the relative advantages and disadvantages of the three types of sample handling.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Additional Materials *(also see Basic Protocol 3)*

Cellular homogenate (see Basic Protocol 3, step 1)
25% (w/v) iodixanol: 1:1 (v/v) IWS solution (see recipe) and HM (see recipe)

Low-speed centrifuge with swinging-bucket rotor and 10- to 15-ml tubes, all at 4°C
Ultracentrifuge with fixed-angle rotor and 5- to 10-ml tubes (optional), all at 4°C
5- to 10-ml Dounce homogenizer (Wheaton) with loose-fitting type-B pestle (optional)
Two-chamber gradient maker or Gradient Master (e.g., BioComp Instruments)
Gradient unloader (upward displacement; Axis-Shield)
Refractometer

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

1. Transfer a cellular homogenate to a 10- to 15-ml centrifuge tube and centrifuge 10 min at 2000 × *g*, 4°C, in a low-speed centrifuge. Save supernatant.

   This step should be omitted if the whole homogenate is to be layered under the gradient.

2. *Optional:* Transfer supernatant to a 5- to 10-ml tube and centrifuge 40 min at 100,000 × *g*, 4°C, in the fixed-angle rotor of an ultracentrifuge. Resuspend microsomal pellet in 4 to 8 ml HM using a 5- to 10-ml Dounce homogenizer with a loose-fitting pestle.

   This step should be omitted if either the homogenate or the 2000 × g supernatant is used as gradient input.

3. Prepare a 14-ml continuous gradient from 7 ml each HM and 25% iodixanol solution in a 17-ml tube for a swinging-bucket rotor using a two-chamber gradient maker or Gradient Master.

4. Layer 3 ml of 2000 × *g* supernatant (step 1) or resuspended microsomes (step 2) on top of the gradient and centrifuge 2 to 3 hr at 200,000 × *g*, 4°C.

   Alternatively, the homogenate (or any of the other fractions) can be adjusted to 30% (w/v) iodixanol with IWS solution and layered beneath the gradient.

5. Collect gradient in 0.75-ml fractions using a gradient unloader. Check the density of the fractions with a refractometer.

   The Golgi bands between 1.040 and 1.065 g/ml. With practice it is possible to use a syringe or Pasteur pipet to remove the Golgi-containing gradient zone (see Commentary for more details).

6. If required, dilute Golgi fractions with ≥2 vol HM and centrifuge 30 min at 100,000 × *g*, 4°C.

7. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet in HM to 1 to 5 mg protein/ml.

   Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤16 hr. Many assays can be carried out on material frozen to −20°C or −80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

BASIC PROTOCOL 4

ISOLATION OF GOLGI MEMBRANES FROM A MICROSOMAL FRACTION OF HEPATOCYTES IN A SELF-GENERATED GRADIENT OF IODIXANOL

Cartwright et al. (1997) developed a rapid method for the fractionation and subfractionation of the smooth and rough endoplasmic reticulum (SER and RER) from a microsomal fraction of rabbit hepatocytes. The method used a self-generated gradient of iodixanol (starting concentration 20%, w/v) at ~350,000 × *g* for 2 hr in a vertical rotor and was used to study the synthesis of very low density lipoproteins (VLDL) from radiolabeled precursors. Later, Plonné et al. (1999) adapted this method to the simultaneous isolation of Golgi, SER, and RER membranes from both isolated hepatocytes and intact liver. Although it is possible to make the gradient more shallow in the low-density region by increasing the centrifugation time to 3 hr and thus improve the resolution of the Golgi membranes from other intracellular particles, Plonné et al. (1999) preferred an alternative method of generating the gradient from equal volumes of 15% and 20% (w/v) iodixanol without increasing the centrifugation time. Although both layers can contain the microsomal fraction, if it is restricted to the denser 20% iodixanol layer, any residual contaminating soluble proteins remain in this layer and the Golgi membranes float into the soluble-protein-free lower-density region. The following protocol is adapted from Plonné et al. (1999) and uses collagenase-prepared rat hepatocytes as the source material. Some comments regarding the use of rat liver are given in the annotations.

NOTE: With the exception of the Dulbecco's modified Eagle medium with BSA (DMEM/BSA), all solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

95% O_2/5% CO_2 gas mixture
DMEM/BSA: Dulbecco's modified Eagle medium (*APPENDIX 2A*) with 1.0% (w/v) BSA
Collagenase-prepared rat hepatocytes (Plonné et al., 1999)
PBS (*APPENDIX 2A*), 4°C
10 mM HEPES-NaOH buffer, pH 7.8
HB (see recipe)
SB (see recipe)
OptiPrep (Axis-Shield, Life Technologies, Accurate Chemicals)
30% (w/v) iodixanol: 1:1 (v/v) OptiPrep/SB
15% (w/v) iodixanol: 1:3 (v/v) OptiPrep/SB

Low-speed centrifuge with swinging-bucket rotor and 50-ml conical tubes, all at 4°C
~10-ml Dounce homogenizer (Wheaton) with tight-fitting type-A pestle
High-speed centrifuge with fixed-angle rotor and ~15-ml tubes (e.g., Sorvall SE12), 4°C
Ultracentrifuge with the following, all at 4°C:
Fixed-angle rotor (e.g., Beckman 50Ti, SorvallT865.1) and ~10-ml tubes
Vertical rotor (e.g., Beckman VTi 65.1, Sorvall 65V13), near-vertical rotor (e.g., Beckman NVT 65), with ~11-ml sealable tubes or a low-angle fixed-angle rotor (~20°; e.g., Beckman 50.3Ti) and ~5-ml sealable ultrcentrifuge tubes.
~10-ml Dounce homogenizer (Wheaton) with loose-fitting type-B pestle
Gradient unloader (upward displacement; e.g., Axis-Shield)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

Homogenize hepatocytes

1. Bubble a 95% O_2/5% CO_2 gas mixture through DMEM/BSA for 20 min, and then suspend collagenase-prepared rat hepatocytes in this medium at $0.5–1 \times 10^7$ cells/ml.
2. Incubate ≥30 min at 37°C in a shaking incubator with continuous gassing.
3. Transfer cells to a preweighed 50-ml conical tube and centrifuge 2 min in a low-speed centrifuge at 800 × *g*, 4°C, to pellet cells. Discard supernatant and record weight of cell pellet.
4. Wash cell pellet twice in ~20 ml PBS at 4°C.
5. Remove all PBS after last wash and resuspend cells in 10 mM HEPES buffer (5 ml/g cells).
6. Allow cells to swell at 4°C for ~10 min and then centrifuge 2 min at 800 × *g*, 4°C, to pellet cells.
7. Remove and discard 70% of buffer and then add a volume of HB equal to residual supernatant.
8. Resuspend cells and homogenize using 30 strokes of a tight-fitting pestle in an ~10-ml Dounce homogenizer.

For intact liver, isolate and homogenize as described (see Basic Protocol 2, steps 1 to 8) using SB as the homogenization medium.

Isolate microsomal fraction

9. Transfer homogenate to a 15-ml centrifuge tube and add protease inhibitors, if required.
10. Centrifuge homogenate 20 min at 8000 × *g*, 4°C, in a high-speed centrifuge.
11. Transfer supernatant to an ~10-ml tube and centrifuge in the fixed-angle rotor of an ultracentrifuge 40 min at 150,000 × *g*, 4°C, to pellet microsomes.
12. Resuspend microsomes in SB (~2.5 ml/g cells) using the loose-fitting pestle of an ~10-ml Dounce homogenizer.

Purify Golgi

13. Mix 4 vol microsome suspension with 2 vol OptiPrep (20% w/v iodixanol final).
14. Transfer 4.5 ml to an ~11-ml sealable tube for a vertical, near-vertical, or low-angle fixed-angle rotor. Underlayer with 1.8 ml of 30% iodixanol and overlayer with 4.5 ml of 15% iodixanol. Seal tube.

    In fixed-angle rotors the 30% iodixanol may be omitted; fill the tube with equal volumes of the two iodixanol solutions.

    Fixed-angle rotors with these tube volumes are not ideal for rapid formation of self-generated gradients because of their relatively long sedimentation path lengths. Many Beckman rotors that are capable of high speeds (e.g., 80 Ti and 90 Ti) can, however, be adapted to accommodate smaller-volume g-Max tubes that have suitable path lengths. Ford et al. (1994) and Axis-Shield Density Gradient Media Catalog (Axis-Shield, 2000) provide more information on the preparation of self-generated iodixanol gradients.

15. Centrifuge 2 hr at 350,000 × *g*, 4°C.
16. Use a gradient unloader to unload into 1-ml sample tubes by upward displacement in ~0.5-ml fractions.

    The Golgi bands in the top 7 ml of the gradient, smooth ER in the median 8 to 14 ml, and rough ER in the bottom third.

17. If required, dilute Golgi fractions with ≥2 vol SB and centrifuge 30 min at 100,000 × *g*, 4°C.
18. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet SB to 1 to 5 mg protein/ml.

    Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤16 hr. Many assays can be carried out on material frozen to −20°C or −80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

SUPPORT PROTOCOL

ASSAY FOR UDP-GALACTOSE GALACTOSYLTRANSFERASE

The domains of the Golgi (*cis*, medial, and *trans*) are involved in the post-translational modification of oligosaccharide residues of glycoproteins and proteoglycans. As such, any of the enzymes that characterize these reactions can be used as markers. They all have their active sites directed towards the cisternal space of the Golgi, and if a glycoprotein is used as an acceptor molecule in the assay, it is normal practice to include a mild detergent in the medium to allow access of the acceptor to the enzyme. The most commonly used marker is UDP-galactose galactosyltransferase, which is really a marker for the *trans*-Golgi, but in total Golgi membrane preparations it is used as a general marker for these membranes.

UDP-galactose galactosyltransferase can be measured using as an acceptor a suitable glycoprotein (normally) that has had its terminal *N*-acetylneuraminic acid and galactose residues removed chemically (Kim et al., 1971). To avoid this rather lengthy procedure, two alternative acceptors are more commonly used today: *N*-acetylglucosamine (the terminal sugar residue, which is the acceptor in vivo) or ovalbumin (a commercially available glycoprotein, which can be used as an artificial acceptor without modification). With *N*-acetylglucosamine as acceptor, the radiolabeled product, *N*-acetyllactosamine, is separated from UDP-galactose by passage through a small Dowex ion-exchange resin (Fleischer et al., 1969); this is a simple but very tedious technique with large numbers of samples. The ovalbumin method is easier to carry out and is described below (Beaufay et al., 1974).

CAUTION: When working with radioactivity (UDP-gal), take appropriate precautions to avoid contamination of the experimenter and the surroundings (*APPENDIX 1D*). Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer.

Materials

10% (w/v) trichloroacetic acid (TCA)
Acceptor solution (see recipe)
UDP-gal solution (see recipe)

2.4-cm filter paper discs
Polystyrene board
Pins

1. Prepare two 2.4-cm filter paper discs per sample (numbered lightly with a pencil).
2. Use pins to attach discs to a polystyrene board, making sure that the discs do not touch the board.
3. Place 250 ml of 10% TCA in a beaker on ice.
4. In a 0.5-ml tube in an ice/water bath, mix 50 µl acceptor solution with 50 µl sample and add 10 µl UDP-gal solution.
5. At time zero and after a 20-min incubation at 37°C, transfer 50 µl incubation mixture to a filter disc.
6. Plunge disc into the ice-cold 10% TCA.

   If the disc is dropped onto the surface of the TCA, the pencilled number is liable to lift off.
7. Leave for 2 to 3 hr, swirling discs occasionally.
8. Wash discs with several changes of distilled water and leave to dry overnight at room temperature.
9. Count radioactivity in any commercial scintillant for non-aqueous samples.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acceptor solution

To 5 ml assay buffer (see recipe) add:
55 mg Na_2ATP (10 mM final)
0.5 g ovalbumin (5% final)
Add assay buffer to 10 ml
Prepare fresh

Assay buffer

To 50 ml H_2O add:
0.19 g $MnCl_2 \cdot 4H_2O$ (10 mM final)
0.23 g 2-mercaptoethanol (30 mM final)
0.2 g Triton X-100 (0.2% v/v final)
1.6 g sodium cacodylate (0.1 M final)
Adjust to pH 6.2 with 1 M HCl
Add H_2O to 100 ml
Store up to 1 month at 4°C

Dextran-containing homogenization medium (DHM)

To 100 ml H_2O add:
34.2 g sucrose (0.5 M final)
2 g dextran (mol. wt. 225,000; 1% final)
1.21 g Tris base (50 mM final)
1.16 g maleic acid (50 mM final)
Adjust pH to 6.4 with 50 mM Tris base or 50 mM maleic acid as required
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Homogenization medium (HM)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
2 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*; 10 mM final)
Adjust to pH 7.4 with 1 M Tris or 1 M HCl if required
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Homogenization medium with EDTA (HME)

Prepare as for HM (see recipe), but add 2 ml 100 mM Na_2EDTA (*APPENDIX 2A*; 1 mM final) before adjusting pH. Store 1 to 2 days at 4°C.

Hyperosmotic buffer (HB)

To 100 ml H_2O add:
41 g sucrose (0.6 M final)
20 ml 100 mM HEPES (10 mM final)
Adjust to pH 7.8 with NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Iodixanol working stock (IWS) solution

Dilute 5 vol OptiPrep (Axis-Shield, Life Technologies, Accurate Chemicals) with 1 vol ODB (see recipe) to give a final iodixanol concentration of 50% (w/v). Store up to 1-2 days at 4°C.

OptiPrep dilution buffer (ODB)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
12 ml 100 mM Na_2EDTA (6 mM final)
12 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A;* 60 mM final)
Adjust to pH 7.4 with 1 M Tris or 1 M HCl if required
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Sucrose density barrier, 1.2 M

To 50 ml H_2O add:
41.0 g sucrose (1.2 M final)
0.61 g Tris base (50 mM final)
0.58 g maleic acid (50 mM final)
Adjust pH to 6.4 with 50 mM Tris base or 50 mM maleic acid
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Sucrose gradient solutions

2.0 M solution:
To 45 ml H_2O add:
69 g sucrose (2.0 M final)
1 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A;* 10 mM final)
Adjust to pH 7.4 with 1 M Tris or 1 M HCl if required
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

For other concentrations: Dilute as needed with 10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*). Store up to 1 to 2 days at 4°C.

For 1.33 M sucrose, dilute 1.33:0.67, v/v; for 1.2 M, dilute 1.2:0.8, v/v; for 1.1, dilute 1.1:0.9, v/v; and for 0.77 M, dilute 0.77:1.23, v/v.

Suspension buffer (SB)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
20 ml 100 mM HEPES (10 mM final)
Adjust to pH 7.8 with NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Uridine-5′-diphospho[6-^{3}H]galactose (UDP-gal) solution

To 5 ml H_2O add:
76 mg uridine 5′-diphosphogalactose, disodium salt (12.5 mM final)
10 µl uridine 5′-diphospho[6-^{3}H]galactose (70 kBq/ml)
Add H_2O to 10 ml
Store up to 1 month at −80°C

COMMENTARY

Background Information

Homogenization

Most published methods describing the isolation of Golgi membranes from mammalian tissues and cells employ a standard buffered sucrose solution as homogenization medium, although there is apparently no rigorous requirement for the presence of some chelating agent such as EDTA. In this respect, the isolation of the Golgi membranes from a light mitochondrial fraction is different from the isolation of other organelles from this fraction (e.g., mitochondria, lysosomes, peroxisomes), where inclusion of EDTA facilitates the recovery of highly purified and functionally competent organelles. Indeed, the inclusion of EDTA in the homogenization medium is actually detrimental to the isolation of Golgi membranes from a microsomal fraction using the self-generated iodixanol gradient described in Basic Protocol 4 (J.A. Higgins, pers. comm.). Moreover, in those methods that use gradient purification, any fraction containing microsomal material (an actual microsomal fraction, a postnuclear or postmitochondrial supernatant, or a homogenate), the presence of EDTA may also destabilize the ribosomes on the rough endoplasmic reticulum (RER), thus causing these membranes to band anomalously. Such an effect would be particularly undesirable if the density gradient was being used for the simul-

taneous purification of one or more components of the ER system. This is much less of a problem if a light mitochondrial fraction is used as a Golgi source for a density gradient; indeed, inclusion of EDTA will reduce contamination of this fraction by the RER.

The only major exceptions to the use of a standard isoosomotic sucrose homogenization medium are: (1) the method of Morré et al. (1972), where dextran is included in a slightly hyperosmotic (0.5 M) sucrose medium to promote the maintenance of Golgi stacks (from rat liver) during homogenization; and (2) for the isolation of Golgi from yeast, where it is common to use higher concentrations of sorbitol (~0.8 M) for lysis of spheroplasts (Bowser and Novick, 1991).

Rat liver may be homogenized in either a Potter-Elvehjem (four to five strokes of the pestle rotating at ~500 rpm) or in a loose-fitting Dounce homogenizer (ten to fifteen strokes), although in the method of Morré et al. (1972) the use of a Polytron homogenizer (or some similar means of providing controlled mechanical shear) is an absolute requirement, as otherwise the stacks of Golgi tend to fragment unpredictably. Hepatocytes are best homogenized in a tight-fitting Dounce homogenizer. For the often smaller volumes of cultured-cell suspensions, a ball-bearing homogenizer ("cell cracker"), repeated passages through a fine needle of a 1- to 2-ml syringe, or a tight-fitting Dounce homogenizer are commonly used techniques. It is strongly recommended that for any cell suspension (either from cultured cells or from a disaggregated tissue), the homogenization procedure be thoroughly investigated so that ≥90% of the cells can be disrupted reproducibly in the shortest time.

Differential centrifugation

Generally, it is only the Golgi membranes from an intact tissue such as mammalian liver that form highly organized and extensive cytoplasmic structures. When dextran is included in the homogenization medium (Morré et al., 1972) stacked tubules will sediment along with nuclei and heavy mitochondria at 5000 × *g*; otherwise, so long as the homogenization procedure is gentle, tubular elements will sediment in the light mitochondrial fraction (10,000 to 15,000 × *g*). On the other hand, the Golgi from most cultured cells will form fragments and vesicles of a variety of sizes. Hence, in the interest of maximum recovery, it is common to use either a total homogenate or, more commonly, a postnuclear or post–heavy mitochondrial supernatant for gradient fractionation of these cells. Although use of a microsomal fraction (centrifugation of a light mitochondrial supernatant at 100,000 to 150,000 × *g* for 30 to 40 min) may result in the loss of some of the larger fragments in earlier fractions of a differential centrifugation scheme, the elimination of both the majority of large organelles and the cytosolic proteins may be an advantage. The volume of medium required to suspend the microsomes will also be considerably less than that of the homogenate or postnuclear or postmitochondrial supernatant, thus facilitating sample and gradient handling. This may be of particular importance with intact tissues or hepatocytes where volumes tend to be large.

Density gradients

In sucrose gradients (Table 3.9.1), the density of Golgi membranes is predominantly in the range of 1.07 to 1.12 g/ml (although densities as low as 1.05 g/ml are possible). Because Golgi membranes are considerably lighter than other organelles in the light mitochondrial fraction (mitochondria, peroxisomes, and lysosomes), which have densities in sucrose >1.16 g/ml, discontinuous gradients for purifying Golgi membranes were successfully developed by a number of workers between 1970 and

Table 3.9.1 Density (g/ml) of Membrane Fractions from Rat Liver in Sucrose and Iodixanol

Particle	Density in sucrose	Density in iodixanol
Nuclei	>1.32	1.23-1.25
Peroxisomes	1.19-1.23	1.18-1.21
Rough endoplasmic reticulum	1.18-1.26	1.14-1.20
Mitochondria	1.17-1.21	1.14-1.16
Lysosomes	1.19-1.21	1.11-1.13
Smooth endoplasmic reticulum	1.10-1.17	1.08-1.12
Golgi	1.07-1.12	1.04-1.07
Plasma membrane	1.07-1.14	1.02-1.03

1980 (e.g., Fleischer and Fleischer, 1970; Ehrenreich et al., 1973). These have formed the basis for most of the technology based on sucrose (e.g., Basic Protocol 2). Later these methods were adapted to the use of the iodinated density gradient medium metrizamide (Graham and Winterbourne, 1988) and more recently to iodixanol (Graham et al., 1994; Yang et al., 1997; Zhang et al., 1998). Because solutions of iodinated density gradient media have a significantly lower osmolarity than those of sucrose, osmotically sensitive subcellular particles exhibit a much lower density in gradients of these media compared to those of sucrose (Table 3.9.1). The density of Golgi fragments in iodixanol, for example, is principally in the range 1.04 to 1.07 g/ml. As in sucrose, there are some variations: Plonné et al. (1999), for example, reported the same density range for most of the Golgi membranes in rat hepatocytes, but a small fraction of galactosyltransferase activity was observed at ~1.10 g/ml.

The variation in density may at least be partly explained by the fact that the Golgi membranes, and indeed the other membranes of the synthetic and secretory systems, are involved in post-translational modification of proteins and lipids that are destined either for incorporation into other membranes or for secretion. The density of these membranes (or of certain domains of these membranes) will be influenced by the density of the macromolecules (and macromolecular complexes) within them. Thus, the presence of particles such as VLDL, which are synthesized by hepatocytes, will tend to make membrane vesicles containing them have a relatively low density, while the presence of heavily glycosylated molecules (carbohydrate is normally very dense) will have the reverse effect. This may not be true of all glycosylated molecules: many proteoglycans, for example, are highly hydrated, giving them a relatively low density in isoosmotic gradients (Ford et al., 1983), but possibly not in a hyperosmotic medium such as sucrose.

The choice of medium may be influenced less by the attainable resolution, which is very satisfactory in both sucrose and iodixanol, than by the ease of handling, notably the commercial availability of iodixanol as a 60% (w/v) solution (OptiPrep) and the avoidance of the need to prepare highly viscous solutions of sucrose. Iodixanol may also be used as a self-generated gradient (Alternate Protocol 1 and Basic Protocol 4). In a self-generated gradient system, the sample is loaded into the centrifuge tube as a dilute suspension; this practice, together with the absence of any density interfaces, reduces aggregation of particles and promotes high resolution. It is also highly reproducible and facilitates the handling of large numbers of samples. Generation of a suitable density gradient profile depends on the use of an appropriate type of rotor. Vertical, near-vertical, or low-angle fixed-angle rotors can be used. Because the separation of the Golgi from a light mitochondrial fraction (Alternate Protocol 1) requires a gradient containing a relatively shallow median region, g forces as low as $180{,}000 \times g$ are permissible. For more information on the production and use of self-generated gradients, see Ford et al. (1994) and the Axis-Shield Density Gradient Media Catalog (Axis-Shield, 2000). In any system it may be necessary to modulate the density range of the gradients according to the median density of the Golgi membranes.

By using homogenization conditions that allow the Golgi from rat liver to retain its in vivo stacked form, Morré et al. (1972) were able to use a simple density barrier to purify the intact Golgi from the upper layer of a $5000 \times g$ pellet. Yields also tend to be higher than in other methods that use a light mitochondrial fraction (Basic Protocol 2 and Alternate Protocol 1) and in which the size of the Golgi fragments is variable. The only disadvantage of the method is that because the stacking is maintained by the inclusion of dextran in the homogenization medium, this fraction has to be hydrolyzed enzymatically if further subfractionation into domains is to be attempted (see Basic Protocol 1).

As to the separation of Golgi membrane vesicles from other vesicles of the secretory system and from plasma membrane, the situation is rather less clear with sucrose. Although the density in sucrose gradients of the RER is clearly considerably greater than that of the Golgi (Table 3.9.1), the lighter smooth-ER (SER) vesicles clearly overlap the denser Golgi membranes. The overlap of the Golgi with the ER is less significant in iodixanol gradients (Table 3.9.1), making iodixanol the gradient medium of choice for the isolation of Golgi membranes from a microsomal fraction, postnuclear (or postmitochondrial) supernatant, or homogenate (see Anticipated Results).

The advantage of using either a whole homogenate or a postnuclear supernatant for the isolation of Golgi membranes is that the response of the Golgi tubules to the homogenization procedure is irrelevant as all of the membranes will be applied to the gradient. If a homogenate is used, then it is certainly advis-

able to adjust its density such that it can be layered under the gradient; if it were layered on top, the presence of rapidly sedimenting nuclei might disturb the gradient. The situation with a postnuclear supernatant or with a microsomal fraction is less critical, and top-loading is permissible. Nevertheless, it is often considered an advantage, especially when isolating the least-dense membrane from much larger populations of denser particles, that bottom loading provides better resolution (Hinton and Mullock, 1997). In this mode the densest particles either remain in or float very slowly into the gradient from the load zone. The disadvantage to this approach is that there is an increase in sample volume when the sample density is adjusted by adding a high-density solution, and this may require that more gradients be set up to accommodate the volume.

In top loading a fraction that also contains the cytosolic fraction (i.e., any fraction except a resuspended microsomal pellet), it is inevitable that soluble proteins will also move into the gradient due to sedimentation and diffusion. If discrimination of cytosolic proteins from any membrane fraction is important, then bottom loading should alleviate this problem as the proteins will tend to remain in the load zone. Although cytoplasmic proteins may also be subsequently removed by pelleting the membranes from the gradient fractions, this additional step may be undesirable.

Use of a pelleted microsomal fraction will not only remove most of the cytosolic proteins (although, unless the pellet is washed, there will always be some residual contamination), it will also permit reduction of the fluid volume. On the other hand, when the pellet is resuspended, liquid shear forces may cause some transient damage to the membrane vesicles and consequent leakage of their contents.

Critical Parameters and Troubleshooting

Homogenization

Homogenization of the commonly used rat liver is covered in these protocols, and the methodology should be applicable to other soft tissue such as kidney. The homogenization procedures are unsatisfactory for hard tissues such as skeletal or cardiac muscle. These tissues require special treatment, and it is strongly recommended that methods that employ severe mechanical shear be avoided, as they tend to cause organelles such as lysosomes to be disrupted. Use of a protease (Nagarse, marketed by Sigma-Aldrich as Proteuse Type VII) to soften the tissue allows its subsequent homogenization by more gentle means in a Dounce or Potter-Elvehjem homogenizer. For more information see Bhattacharya et al. (1991) and Graham (1997).

Hepatocytes, CHO cells, and COS-7 cells can be homogenized either in a tight-fitting Dounce homogenizer or a ball-bearing homogenizer using a routine homogenization medium (i.e., 0.25 M sucrose with or without 1 mM EDTA, buffered with either Tris or HEPES). Although most monolayer cultured cells seem to homogenize reasonably effectively in this medium, the medium developed by Marsh et al. (1987), in which the buffer was changed to 10 mM triethanolamine/10 mM acetic acid, pH 7.4, is more widely applicable. The triethanolamine/acetic acid buffer is the critically important component. If, upon homogenization, the cell homogenate acquires a gelatinous quality, this may be due either to nuclear breakage or to the release of proteins from cytoskeletal elements. Inclusion of 1 mM Mg^{2+} with or without DNase I (10 μg/ml) and/or 10 mM KCl may alleviate the problems.

For cultured cells, the device of choice that seems to provide the least damage to intracellular organelles is the ball-bearing homogenizer, first described by Balch and Rothman (1985). A Dounce homogenizer (or passage through a fine syringe needle) is often used as an acceptable alternative, but for cells other than those described in this unit, it is strongly recommended that the optimal conditions for disrupting the cells be thoroughly investigated. The aim must always be to use the minimum force to achieve ~90% cell breakage, and protocols, for example, that use more than 20 pestle strokes of a Dounce homogenizer should be avoided, if at all possible.

For cells that resist disruption by liquid shear forces, nitrogen cavitation is probably the best option, as an isoosmotic sucrose medium can always be used. Standard conditions are 300 to 600 psi for 10 to 15 min, although Kelly et al. (1989) reported the use of much lower pressures. For a more complete discussion of these problems, see Evans (1992) and Graham (1997).

Ideally the Polytron homogenizer used in Basic Protocol 1 should not be substituted by any other homogenization device. If another device is used, the homogenate should be centrifuged at 5000 × *g* for 15 min and the supernatant recentrifuged at 100,000 × *g* for 30 min. The recovery of galactosyltransferase in the

5000 × *g* pellet should be ≥50% of the total (Morré et al., 1972).

Density gradient centrifugation

Use of tissues or cells other than those for which the protocols were designed may require some modification to the density range of gradients; this can only be determined by experimentation. In those protocols in which preformed continuous or discontinuous gradients are used, the use of swinging-bucket rotors with tube volumes other than those recommended in the protocols is permissible so long as the volume ratios of sample and gradient are maintained and the rotors are capable of similar relative centrifugal forces (RCFs). However, use of alternative rotors for self-generated gradients is not recommended unless it is first established that the rotor can be used to form a gradient of the correct density profile (see Anticipated Results). The sedimentation path length and maximum RCF of the rotor are the critical factors; generally speaking, path lengths of >30 mm and RCFs <180,000 g_{av} are unacceptable. The gradient-forming capacity of any rotor should be checked by running a blank gradient, fractionating the gradient either by upward displacement or tube puncture, and determining the density of the gradient fractions by refractometry. In the case of iodixanol, density (ρ) and refractive index (η) of solutions produced by diluting OptiPrep with a medium containing 0.25 M sucrose are related by the following equation: $\rho = 3.4713\eta - 3.6393$.

Enzyme and other assays

If the sample volumes from sucrose or iodixanol gradient fractions used in an enzyme assay are sufficiently small (compared to the assay volume), the gradient solute may be adequately diluted so as not to cause any interference in the assay, even at the highest density. Indeed, it is well established that neither iodixanol nor sucrose exhibits any significant inhibition of the standard enzyme markers (Ford et al., 1994). Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without washing. If, however, either of these requirements is not met, or if it is important to suspend the membrane fractions in a medium of constant composition, then dilution, sedimentation, and resuspension of the pellet in a smaller volume of an appropriate medium is the recommended method. In the case of sucrose, dialysis is an acceptable alternative, although any residual dextran in a gradient fraction from Basic Protocol 1 is not dialyzable. For iodixanol, ultrafiltration using a microcentrifuge cone filter can be used.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Both iodixanol and, to a lesser extent, sucrose interfere with methods using the Folin Ciocalteu reagent. However, if the concentration of gradient solute in the assay solution can be reduced to <10%, i.e., by using small volumes of gradient fractions (≤100 µl) in a 1- to 2-ml assay volume, the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of these gradient media.

Any electrophoretic and electroblotting technique can also be carried out on sucrose- or iodixanol-containing fractions without prior removal of the medium, again, as long as the concentration of membrane in the sample is sufficiently high for the analytical procedure.

Anticipated Results

Basic Protocol 1

According to the data of Morré et al. (1972), 40% to 70% of the total *N*-acetylglucosamine galactosyltransferase activity originally in the rat liver homogenate should be recoverable in the material harvested from the top of the 1.2 M sucrose density barrier. The recovery from other tissues or cells will depend very much on the existence of a well-organized Golgi apparatus in the cell and the behavior of the Golgi membranes during homogenization. The fraction purity should be ≥80% with a relative specific activity (ratio of specific activity of N-acetylglucosamine galactosyltransferase in the Golgi fraction to that in the homogenate) of ~90.

Basic Protocol 2

The following information is taken from Fleischer and Fleischer (1970). The Golgi membranes at the 0.77 M/1.1 M sucrose interface should contain the highest activity of galactosyltransferase activity (a specific activity of ~120 relative to that of the homogenate), while the slightly denser membranes should have lower activity (~70). Total yield of galactosyltransferase in the two bands is 30% to 35% of that in the homogenate, while only ~0.3% of the total protein is recovered in these two bands.

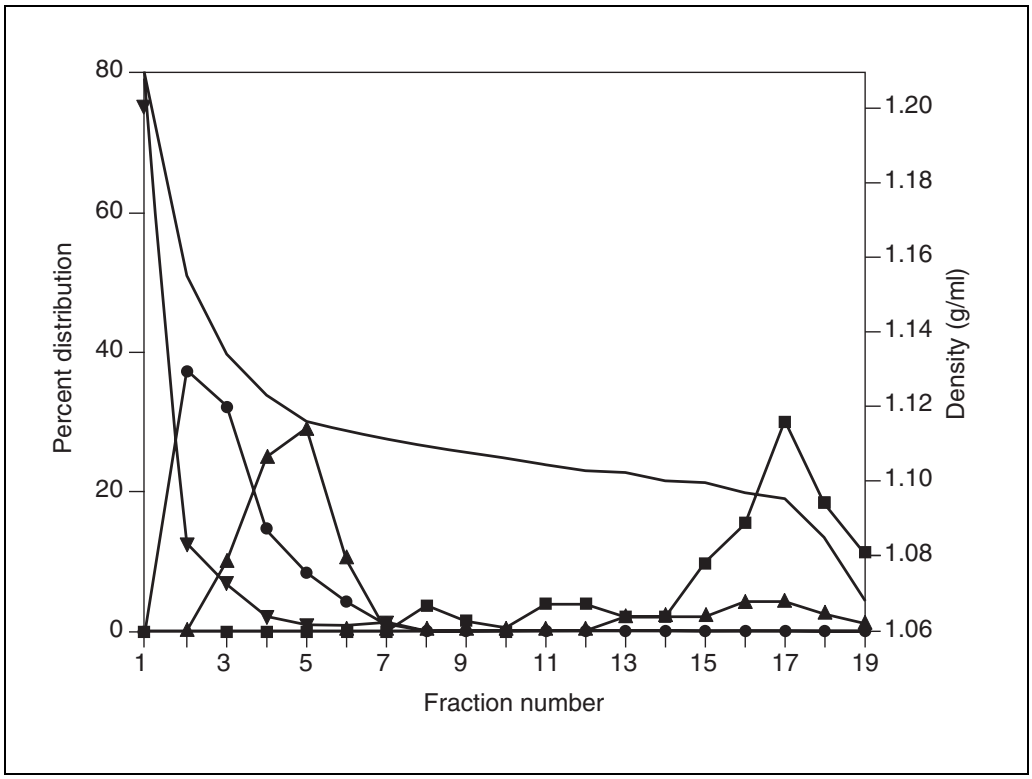


Figure 3.9.1 Fractionation of a light mitochondrial fraction (mouse liver) in a self-generated gradient of iodixanol: percent distribution of enzyme markers (symbols) and density profile (—). Light mitochondrial fraction adjusted to 15% (w/v) iodixanol and centrifuged at 180,000 × *g* for 3 hr in a 20° fixed-angle rotor. Organelle markers and symbols: catalase for peroxisomes (filled inverted triangle), succinate dehydrogenase for mitochondria (filled circle), β-galactosidase for lysosomes (filled triangle), and galactosyltransferase for Golgi (filled square). Adapted from Graham et al. (1994) with permission from Academic Press.

Alternate Protocol 1

Figure 3.9.1 shows a typical distribution of marker enzymes in the iodixanol gradient. At least 80% of the total galactosyltransferase present in the light mitochondrial fraction from mouse liver should be recovered from the top five fractions. The same fractions should contain <10% of any lysosomal marker (β-galactosidase) and negligible amounts of enzyme markers for other organelles (Graham et al., 1994). The relative specific activity of galactosyltransferase (specific activity in gradient fraction relative to that in the light mitochondrial fraction) in the peak Golgi fraction should be ~20.

Basic Protocol 3

Some typical recoveries of Golgi (*N*-acetylglucosamine galactosyltransferase) and ER (glucosidase I) markers are given in Table 3.9.2

Table 3.9.2 Marker Enzyme Activity in Isolated Fractions from CHO Cells Processed by Flotation Through a Discontinuous Sucrose Gradient[a]

Fraction	Protein recovery (%)	*N*-Acetylglucosamine galactosyltransferase		Glucosidase I	
		Total activity	Specific activity	Total activity	Specific activity
Postnuclear supernatant	NA[b]	164	2.3	0.6	0.008
Golgi	3.6	70	26.8	0.026	0.01

[a]Data from Balch et al. (1984).

[b]Abbreviation: NA, not applicable.

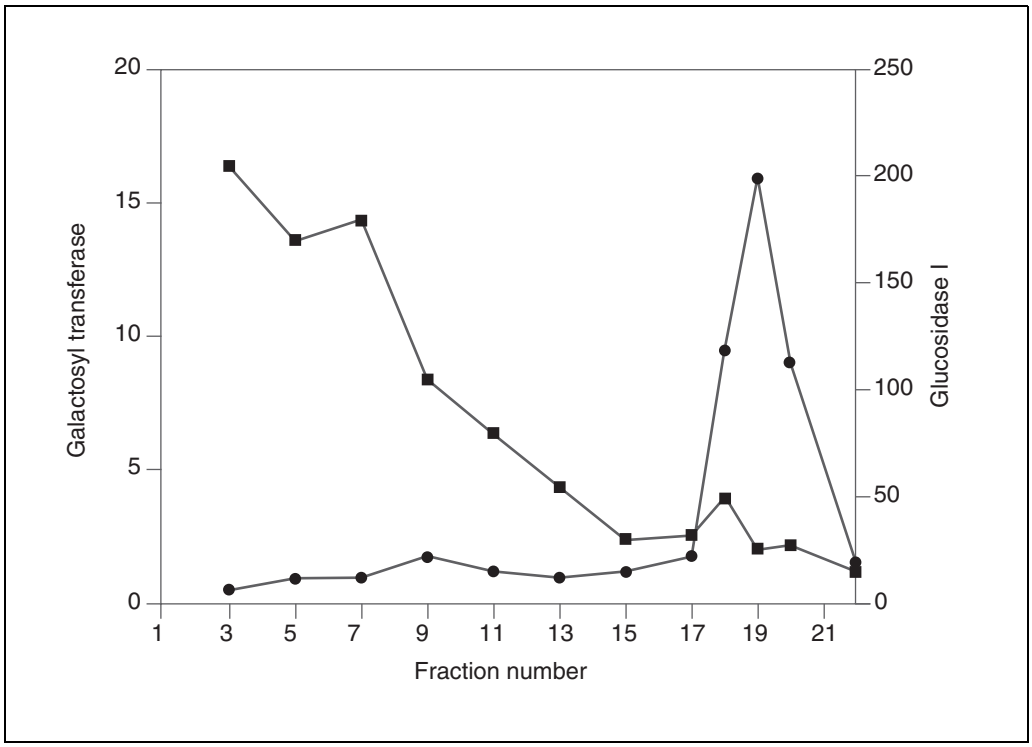


Figure 3.9.2 Fractionation of a homogenate of CHO cells in a 0.8 to 2.0 M discontinuous sucrose gradient: distribution of Golgi (galactosyltransferase) and endoplasmic reticulum (ER; glucosidase I) markers. Gradients were unloaded dense-end first. Activity of galactosyltransferase (filled circle) expressed as cpm $\times 10^{-2}$; activity of glucosidase I (filled square) expressed as cpm. Adapted from Balch et al. (1984) with permission from Elsevier Science.

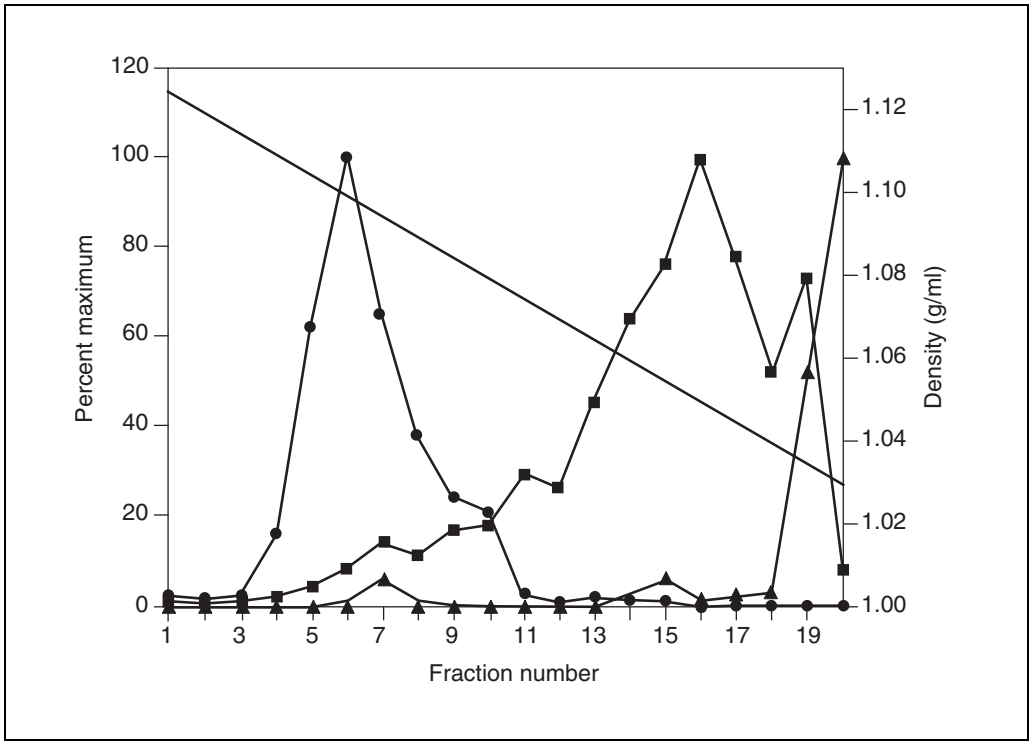


Figure 3.9.3 Fractionation of a postnuclear supernatant from CHO cells on a preformed continuous 0% to 26% (w/v) iodixanol gradient: distribution of markers (symbols) and density profile (—). Activities in each fraction expressed as a percentage of the maximum fraction. Organelle markers and symbols: ribophorin for endoplasmic reticulum (ER; filled circle), galactosyltransferase for Golgi (filled square), and biotinylated surface proteins for plasma membrane (filled triangle). Adapted from Yang et al. (1997) with permission from the American Society for Biochemistry and Molecular Biology.

(Balch et al., 1984). The Golgi fraction contains ~40% of the total *N*-acetylglucosamine galactosyltransferase of the postnuclear supernatant but only ~4% of the glucosidase I. Figure 3.9.2 shows that essentially all of the ER is distributed quite broadly in the bottom half of the gradient, while the Golgi bands sharply at the 0.8 M/1.2 M sucrose interface.

Alternate Protocol 2

Distribution of the Golgi and ER markers (Yang et al., 1997) in the continuous iodixanol gradient (Fig. 3.9.3) is not unlike that observed in the discontinuous sucrose gradient (Basic Protocol 3), but the rather broader distribution of the Golgi may provide some potential for subfractionation of the Golgi into light and dense populations. Moreover, some small contamination of the Golgi by the ER is evident in the sucrose gradient (the specific activity of glucosidase I is indeed slightly raised in the Golgi fraction over the postnuclear supernatant; Table 3.9.2). In the iodixanol gradient, on the other hand, no contamination whatsoever is apparent. If the two lightest fractions containing plasma membrane are discarded, then ≥80% of the galactosyltransferase in the gradient is recovered in a highly purified manner.

Basic Protocol 4

Figure 3.9.4 shows a typical result of the self-generated iodixanol gradient (Plonné et al., 1999): ~80% of the galactosyltransferase is contained in the top seven fractions of the gradient. The system can also be used for the simultaneous isolation of smooth ER (fractions 9 to 13) and rough ER (fractions 15 to 20).

Time Considerations

There are no points at which any of the basic or alternate protocols can be discontinued. The light mitochondrial fraction produced in Basic Protocol 2 must be used immediately for any subsequent gradient separation. Discontinuous gradients may be made up immediately prior to use and underlayered with the dense light mitochondrial fraction (Basic Protocol 2) or homogenate (Basic Protocol 3). Preformed continuous gradients (Alternate Protocol 2) may be made up during the preparation of the postnuclear supernatant and kept on ice until required. If these continuous gradients are prepared at room temperature they must be given time to cool down to 4°C. Alternatively, they can be made from a discontinuous gradient the previous day and stored overnight at 4°C to allow diffusion to occur. Always check that a

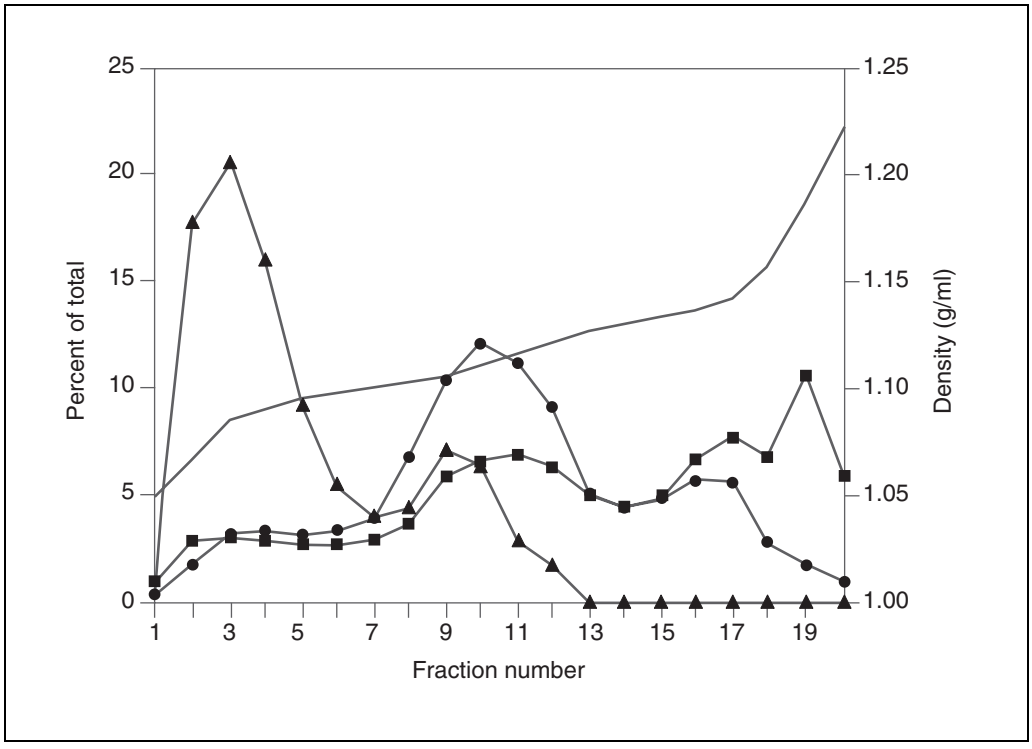


Figure 3.9.4 Fractionation of a rat hepatocyte microsomal fraction in a 15%/20% self-generated iodixanol gradient: percent distribution (symbols) and density profile (—). Centrifugation was carried out in a Beckman VTi 65.1 vertical rotor at 350,000 × *g* for 2 hr. Organelle markers and symbols: NADPH-cytochrome c reductase for ER (filled circle), galactosyltransferase for Golgi (filled triangle), and protein (filled square). Adapted from Plonné et al. (1999) with permission from Academic Press.

continuous gradient has been formed by measuring the refractive index of fractions from a blank gradient.

Basic Protocol 1 is a rapid procedure and Morré et al. (1972) considered that the livers from as many as ten to twelve rats could be processed in 2 hr. Preparation of the light mitochondrial fraction (Basic Protocol 2), which forms the input for gradients described in the same protocol and in Alternate Protocol 1, should take no more than 1 hr for one to two rat livers. Forming, centrifuging, and harvesting the discontinuous gradients (Basic Protocol 2) will take ~2 hr for two tubes. Tube preparation for self-generated gradients (Alternate Protocol 1) is much faster than is the construction of preformed gradients, but because of the longer acceleration and deceleration times, the overall time will be similar if the centrifugation is carried out at 350,000 × *g*. Basic Protocol 3 will take ~3 hr, as will Alternate Protocol 2 if the shorter centrifugation time is used. If hepatocytes are used, the overall time for Basic Protocol 4 will be ~5 hr, but for liver this will be nearer to 4 hr.

For the first couple of preparations it is probably advisable to unload continuous gradients in a number of equivolume fractions (fraction volume should be ~5% of gradient volume). With experience and practice, however, it will be possible to remove the Golgi-containing fractions with a syringe or Pasteur pipet.

Functional assays should be carried out as soon as possible after the preparation. The Support Protocol should take ~4 hr for 20 to 30 samples. For simple marker enzyme assays (such as described in this unit), material can be stored at 0° to 4°C for ≤16 hr without much loss of activity. These assays and compositional measurements may also be carried out on material frozen to −20° or −80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those made on the fresh organelles. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All solutions, except the acceptor solution (Support Protocol), can be made up ahead of time and stored at 4°C for 1 to 2 days or frozen to −20°C for longer periods (e.g., 5 months). Any enzymes or protease inhibitors should be added immediately prior to use.

Literature Cited

Axis-Shield. 2000. Types of centrifugal separations. *In* Axis-Shield Density Gradient Media Catalog, pp. 13-29. Axis-Shield, Oslo, Norway; Life Technologies, Grand Island, N.Y.; Accurate Chemicals, Westbury, N.Y.

Balch, W.E. and Rothman, J.E. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: Asymmetric properties of donor and acceptor activities in cell-free systems. *Arch. Biochem. Biophys*. 240:413-425.

Balch, W.E., Dunphy, W.G., Braell, W.A., and Rothman, J.E. 1984. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of *N*-acetylglucosamine. *Cell* 39:405-416.

Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M., and Berthet, J. 1974. Analytical study of microsomes and isolated subcellular fractions from rat liver. *J. Cell Biol*. 61:188-200.

Bhattacharya, S.K., Thakar, J.H., Johnson, P.L., and Shanklin, D.R. 1991. Isolation of skeletal muscle mitochondria from hamsters using an ionic medium containing EDTA and Nagarse. *Anal. Biochem.* 192:344-349.

Bowser, R. and Novick, P. 1991. Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. *J. Cell Biol.* 112:1117-1131.

Cartwright, I.J., Higgins, J.A., Wilkinson, J., Bellavia, S., Kendrick, J.S., and Graham, J.M. 1997. Investigation of the role of lipids in the assembly of very low density lipoproteins in rabbit hepatocytes. *J. Lipid Res*. 38:531-545.

Ehrenreich, J.H., Bergeron, J.J.M., Siekevitz, P., and Palade, G.E. 1973. Golgi fractions prepared from rat liver homogenates. *J. Cell Biol.* 59:45-72.

Evans, W.H. 1992. Isolation and characterization of membranes and cell organelles. *In* Preparative Centrifugation—A Practical Approach (D. Rickwood, ed.) pp. 233-270. Oxford University Press, Oxford.

Fleischer, B. and Fleischer, S. 1970. Preparation and characterization of Golgi membranes from rat liver. *Biochim. Biophys. Acta* 219:301-319.

Fleischer, B., Fleischer, S., and Ozawa, H. 1969. Isolation and characterization of Golgi membranes from bovine liver. *J. Cell Biol*. 43:59-79.

Ford, T., Rickwood, D., and Graham, J. 1983. Buoyant densities of macromolecules, macromolecular complexes and cell organelles in Nycodenz gradients. *Anal. Biochem.* 128:232-239.

Ford, T., Graham, J., and Rickwood, D. 1994. Iodixanol: A nonionic iso-osmotic centrifugation medium for the formation of self-generated gradients. *Anal. Biochem*. 220:360-366.

Graham, J.M. 1997. Homogenization of cells and tissues. *In* Subcellular Fractionation—A Practical Approach (J.M. Graham and D. Rickwood, eds.) pp. 1-29. Oxford University Press, Oxford.

Graham, J.M. and Winterbourne, D.J. 1988. Subcellular localization of the sulphation reaction of heparan sulphate synthesis and transport of the proteoglycan to the cell surface of rat liver. *Biochem. J*. 252:437-445.

Graham, J., Ford, T., and Rickwood, D. 1994. The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol. *Anal. Biochem*. 220:367-373.

Hartel-Schenk, S., Minnifield, N., Reutter, W., Hanski, C., Bauer, C., and Morré, D.J. 1991. Distribution of glycosyltransferases among Golgi apparatus subfractions from liver and hepatomas of the rat. *Biochim. Biophys. Acta* 1115:108-122.

Hinton, R. and Mullock, B.M. 1997. Isolation of subcellular fractions. *In* Subcellular Fractionation—A Practical Approach (J.M. Graham and D. Rickwood, eds.) pp. 31-69. Oxford University Press, Oxford.

Kelly, B.M., Yu, C.-Z., and Chang, P.L. 1989. Presence of a lysosomal enzyme, arylsulfatase-A, in the prelysosome-endosome compartments of human cultured fibroblasts. *Eur. J. Cell Biol*. 48:71-78.

Kim, Y.S., Perdomo, J., and Nordberg, J. 1971. Glycoprotein biosynthesis in small intestinal mucosa. I. A study of glycosyl transferases in microsomal subfractions. *J. Biol. Chem.* 246:5466-5476.

Macintyre, S.S. 1992. Regulated export of a secretory protein from the ER of the hepatocyte: A specific binding site retaining C-reactive protein within the ER is downregulated during the acute phase response. *J. Cell Biol*. 118:253-265.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol*. 104:875-886.

Morré, D.J., Cheetham, R.D., and Nyquist, S.E. 1972. A simplified procedure for isolation of Golgi apparatus from rat liver. *Prep. Biochem*. 2:61-66.

Plonné, D., Cartwright, I., Linss, W., Dargel, R., Graham, J.M., and Higgins, J.A. 1999. Separation of the intracellular secretory compartment of rat liver and isolated rat hepatocytes in a single step using self-generating gradients of iodixanol. *Anal. Biochem.* 276:88-96.

Yang, M., Ellenberg, J., Bonifacino, J.S., and Weissman, A.M. 1997. The transmembrane domain of a carboxyl-terminal anchored protein determines localization to the endoplasmic reticulum. *J. Biol. Chem.* 272:1970-1975.

Zhang, J., Kang, D.E., Xia, W., Okochi, M., Mori, H., Selkoe, D.J., and Koo, E.J. 1998. Subcellular distribution and turnover of presenilins in transfected cells. *J. Biol. Chem.* 273:12436-12442.

Contributed by John M. Graham
Liverpool John Moores University
Liverpool, United Kingdom

Isolation of Nuclei and Nuclear Membranes From Animal Tissues

The vast majority of methods for the isolation of nuclei and nuclear membranes use soft mammalian tissues (particularly rat liver) as the biological source; however, the protocols described in this unit should be applicable generally to any tissue type, cultured cells, or cells of lower eukaryotes or plants, so long as a suitable homogenization method is available. Generally, a buffered isoosmotic medium containing KCl and $MgCl_2$ is used to stabilize the nuclei of mammalian tissues. Some of the media used for cultured animal cells and nonmammalian sources are described later in the unit (see Commentary). Purification using a sucrose barrier of 2.3 M sucrose is described (see Basic Protocol 1). The use of such dense sucrose solutions has several disadvantages, notably the high viscosity, meaning that particles sediment very slowly through the medium; therefore, the procedure requires an ultracentrifuge. The high osmolarity may also be detrimental to the intranuclear structure, and certainly the organelles loose water and shrink considerably in size. The use of OptiPrep (iodixanol; Axis-Shield) overcomes both the viscosity and osmolarity problems (see Alternate Protocol 1) and permits the use of a high-speed centrifuge and much shorter centrifugation times. A series of alternate steps are given for plant cell (i.e., wheat germ) nuclei, since the dense layer of iodixanol used in this method is able to resolve nuclei from the denser starch granules (see Commentary for more details).

The centrifugation of any subcellular particle through an aqueous medium tends to lead to loss of some proteins that may be important for proper functioning of the organelle. Although nonaqueous media can overcome this problem, they are generally avoided, since they often pose more problems than they solve. Nuclei are probably the only organelle that might benefit from such an approach, so a method employing a gradient of Nycodenz (Axis-Shield) in ethylene glycol is described (see Alternate Protocol 2).

The isolation of nuclear membranes from purified nuclei has been approached via diametrically opposite strategies. High-ionic-strength media generally give high yields of nuclear membranes, and the hollow membranous spheres that are formed seem well suited to permeability studies (see Basic Protocol 2). Low-ionic-strength media produce highly purified nuclear envelopes, which are particularly good for compositional studies (see Basic Protocol 3).

Two simple chemical methods for estimating DNA and RNA (see Support Protocols 1 and 2) and a fluorometric method for measuring both nucleic acids (see Support Protocol 3) are also provided.

ISOLATION OF NUCLEI FROM RAT-LIVER HOMOGENATE USING A SUCROSE DENSITY BARRIER

BASIC PROTOCOL 1

In this procedure the tissue is homogenized in an isoosmotic medium containing 0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$, and 10 mM Tris·Cl, pH 7.4 (Blobel and Potter, 1966). Variations for other tissues, cultured cells, or nonmammalian material are described elsewhere (see Commentary). Filtration of the homogenate is often included to avoid contamination of the subsequent crude nuclear pellet, produced by centrifugation of the homogenate at 800 × *g* for 10 min, by connective tissue and unbroken cells; the connective tissue may also make resuspension of the pellet difficult. The pellet is then resuspended in ~1.6 M sucrose and layered over 2.3 M sucrose. After centrifugation at 100,000 × *g* for 1 hr, the nuclei form a pellet at the bottom of the tube, while any

Contributed by John M. Graham
Current Protocols in Cell Biology (2001) 3.10.1-3.10.19

contaminating material remains in the load zone. In some methods (Widnell and Tata, 1964), the lower barrier is eliminated and the nuclei are simply pelleted from the dense crude nuclear suspension. This alternative is given in the annotations to the respective steps.

If the exposure of the nuclei to such a vastly hyperosmotic medium is undesirable, then a method using an isoosmotic discontinuous gradient of iodixanol can be used (see Alternate Protocol 1). These gradients are also much less viscous and the centrifugation can be carried out at much lower RCFs in a high-speed centrifuge. A gradient that is nonaqueous can also be used (see Alternate Protocol 2). The following protocol is adapted from Blobel and Potter (1966).

Materials

150- to 200-g Sprague-Dawley rats
Nuclear isolation medium (NIM; see recipe)
Sucrose density barrier (SDB; see recipe)

Scissors or razor blade
~30-ml Potter-Elvehjem homogenizer with 0.1-mm clearance and loose-fitting pestle
75-µm pore size nylon gauze or cheesecloth (muslin)
Glass rod
Refrigerated low-speed centrifuge with swinging-bucket rotor and appropriate 50-ml polypropylene or polystyrene tubes
~30-ml Dounce homogenizer with loose-fitting Wheaton Type B pestle
Refractometer
Ultracentrifuge with swinging-bucket rotor (e.g., Beckman SW40Ti, Sorvall TH641) and appropriate 14-ml tubes

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C, and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

NOTE: Protease inhibitors (*UNIT 3.4*) may be added to any or all solutions.

Isolate and homogenize the liver

1. Starve a 150- to 200-g Sprague-Dawley rat overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.

   This must be supervised or carried out by an experienced animal technician.

3. Open the abdominal cavity and remove the liver to a chilled beaker containing ~20 ml nuclear isolation medium (NIM).
4. Decant the medium and finely mince the liver using scissors or a razor blade.

   The pieces of liver should be no more than ~25 mm^3.

5. Suspend the mince in ~40 ml NIM and transfer half of this suspension to the glass vessel of the ~30-ml Potter-Elvehjem homogenizer.

   The motor should be mounted either to a wall, via a G-clamp to the bench, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

6. Attach the precooled pestle to the electric motor and homogenize the liver mince using 4 to 5 up-and-down strokes of the pestle, rotating at ~500 rpm.
7. Decant the homogenate into a beaker on ice. After rinsing the homogenizer with NIM, and wiping the pestle to remove any connective tissue that may be adhering, perform the procedure (i.e., steps 5 to 7) with the other half of the mince.

Prepare crude nuclear pellet

8. Filter the homogenate through a single layer of nylon gauze (pore size of 75 μm), or three layers of cheesecloth (muslin) to remove unbroken cells and any connective tissue.

   Do not force the homogenate through the filter; assist filtration only by stirring with a glass rod.

9. Dilute the homogenate with at least an equal volume of NIM.

   For cultured cells that have been homogenized in a hypoosmotic medium, add an equal volume of buffer containing 0.5 M sucrose. If there is neither KCl nor $MgCl_2$ in the homogenate, double the concentration of these salts in the added buffer.

10. Distribute the suspension equally between two 50-ml centrifuge tubes and centrifuge in a low-speed centrifuge with a swinging-bucket rotor 10 min at 800 × *g*, 4°C.
11. Decant the supernatant, discarding as much of the upper, loosely packed brown layer as possible. Resuspend the pellet in 40 ml NIM using the ~30-ml Dounce homogenizer.
12. Repeat the centrifugation as described in step 10.
13. Decant the supernatant and make a crude resuspension of the combined pellets in NIM, using a glass rod, so that the final volume is ~8 ml.

    If the crude nuclear suspension itself is to be adjusted to ~2.2 M sucrose and centrifuged without layering over a barrier, do not resuspend the pellets in NIM.

Purify nuclei

14. Add 2 vol sucrose density barrier (SDB) to the crudely resuspended nuclear pellet and mix very thoroughly using the Dounce homogenizer.

    Alternatively, resuspend the pellets from step 13 directly in ~9 vol SDB. This critical step is quite difficult because of the viscosity of the SDB. It may be easier to use the Potter-Elvehjem homogenizer rotating at 1000 rpm.

15. Check that the refractive index of the suspension is 1.4117 ± 0.0004, using a refractometer. Adjust concentration with SDB, if necessary, to attain this refractive index.
16. Transfer 9 ml suspension to a 14-ml ultracentrifuge tube. Underlayer with 2.3 M sucrose to fill the tube. Ultracentrifuge in a swinging-bucket rotor 1 hr at 100,000 × *g*, 4°C.

    In the alternative method, simply fill the tubes with the resuspended nuclei and centrifuge in a swinging-bucket or fixed-angle rotor.

17. Remove any material that has floated to the meniscus with a spatula, then decant all the liquid from above the pellet. Leave the tubes inverted for a few minutes to drain as much of the viscous sucrose layer as possible.
18. While the tube is inverted, wipe off as much of the liquid from the walls of the tube as possible, using a tissue wrapped around forceps.

19. Resuspend the pellet in NIM, or other suitable medium compatible with the next process, to ~5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Assays can often be carried out on material frozen to –20° or –80°C. Always check that the measurements made are relatively unaffected by the storage conditions by comparing with those made on fresh material. Note however that thawing a frozen suspension of nuclei may cause release of DNA, and consequently almost irreversible aggregation of the particles.

ALTERNATE PROTOCOL 1

ISOLATION OF NUCLEI FROM ANIMAL OR PLANT (WHEAT GERM) CELLS USING AN IODIXANOL GRADIENT

If elimination of any potential damage to the nucleoprotein complexes is important, then the alternative iodixanol gradient described in this protocol might be relevant. Using these gradients it is possible to isolate nuclei by isopycnic banding in an isoosmotic nonionic environment (Graham et al., 1994; Provost et al., 1996). Because the nuclei retain their normal hydration, their density (ρ = 1.20 to 1.22 g/ml) is much lower than in sucrose (>1.32 g/ml). Buoyant density banding thus requires much lower concentrations of iodixanol; consequently the viscosity of the medium is much lower, as are the centrifugation forces and times needed to purify the nuclei. Also, unlike using a sucrose gradient (see Basic Protocol 1), it is not necessary to produce a crude nuclear fraction first. After the whole homogenate is adjusted to 25% (w/v) iodixanol, this is loaded directly on to a discontinuous iodixanol gradient; therefore, the method is quicker and simpler to execute. The protocol below is adapted from Graham et al. (1994). Ford et al. (1998) reported that a comparable iodixanol gradient is also able to separate the nuclei and starch granules from wheat germ (see alternative steps). This is not possible with sucrose gradients, since both pellet through the 2.3 M sucrose.

Additional Materials *(also see Basic Protocol 1)*

Filtered homogenate (animal cells or tissue; see Basic Protocol 1) or wheat germ
50% (w/v) iodixanol (see recipe; animal cells or tissue)
30% and 35% (w/v) iodixanol gradient solutions (see recipe; animal cells or tissue)
25% and 40% (w/v) iodixanol gradient solutions (see recipe; wheat germ)
Wheat germ medium (WGM; see recipe)
High-speed centrifuge with a swinging-bucket rotor and appropriate 50-ml tubes

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C, and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

NOTE: Protease inhibitors (*UNIT 3.4*) may be added to any or all solutions.

For animal cells or tissue

1a. Prepare the filtered homogenate as described (see Basic Protocol 1, steps 1 to 8).

2a. Mix the homogenate with an equal volume of 50% iodixanol.

3a. In a 50-ml centrifuge tube, layer 15 ml each of 35% and 30% iodixanol solutions, and sample.

If it is necessary to reduce the number of tubes used for this centrifugation, the volume of 35% iodixanol per tube may be reduced to 7.5 ml while increasing the volume of homogenate by a corresponding amount.

4a. Centrifuge in a high-speed centrifuge with a swinging-bucket rotor 20 min at 10,000 × *g*, 4°C.

The nuclei from some tissues or cells may be smaller and/or less dense than those from mammalian liver; therefore, it may be necessary to increase the RCF to 20,000 × g and/or modulate the concentration (w/v) of iodixanol in the three layers (e.g., 20%, 25%, and 30%).

5a. Harvest the nuclei from above the 35% iodixanol and dilute with 2 vol of NIM.

For wheat germ

1b. Make a crude suspension of nuclei by vigorously shaking ~5 g of wheat germ in ~50 ml WGM.

2b. Allow the debris to settle for 10 min. Carefully decant the supernatant.

3b. Layer 20 ml crude nuclear suspension over 5 ml each 25% and 40% iodixanol gradient solutions.

4b. Centrifuge in a high-speed centrifuge with a swinging-bucket rotor 30 min at 5600 × *g*, 4°C.

5b. Harvest the nuclei, which band above the 40% iodixanol.

The starch granules form a pellet.

Concentrate nuclei

6. Centrifuge 10 min at 2000 × *g*, 4°C in a low-speed centrifuge with a swinging-bucket rotor. Resuspend the pellet in NIM (animal cells or tissue) or WGM (wheat germ), or other suitable medium compatible with the next process, to ~5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to –20° or –80°C. Always check that the measurements made are relatively unaffected by the storage conditions by comparing with those made on fresh material. Thawing a frozen suspension of nuclei may cause release of DNA, and consequently almost irreversible aggregation of the particles.

ISOLATION OF NUCLEI FROM RAT-LIVER HOMOGENATE IN A NONAQUEOUS MEDIUM

ALTERNATE PROTOCOL 2

It may be beneficial to use homogenization and gradient media that are nonaqueous in order to eliminate, as far as possible, any loss of proteins from the external surface of the nuclei, or the leaching out of proteins from the matrix. This approach, in particular, eliminates loss of DNA polymerase from the nuclei. The method, developed by Rickwood et al. (1997), in which an aqueous sucrose medium is replaced by ethylene glycol, permits the use of standard Potter-Elvehjem homogenization techniques. Moreover, Nycodenz (Axis-Shield) is freely soluble in the same solvent so purification in a density gradient is also possible.

Additional Materials *(also see Basic Protocol 1)*

Ethylene glycol homogenization medium (EGHM; see recipe)
10% and 40% (w/v) Nycodenz (Axis-Shield; available from Accurate Chemicals) in EGHM
Two-chamber gradient maker or Gradient Master (BioComp Instruments)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C, and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

NOTE: Protease inhibitors (*UNIT 3.4*) may be used in any or all solutions.

1. Prepare a filtered homogenate as described (see Basic Protocol 1, steps 1 to 8), except omit the use of NIM for washing the liver (step 3), replacing the ~40 ml NIM (step 5) with ~50 ml EGHM, and increasing the number of strokes of the pestle from 4 to 5 to 6 to 9 (step 6).
2. Centrifuge in a low-speed centrifuge with a swinging-bucket rotor 10 min at 1000 × *g*, 4°C.
3. Discard the supernatant and resuspend the pellet in 10% (w/v) Nycodenz in EGHM (~2 ml/g liver), using a Dounce homogenizer.
4. Prepare 12-ml continuous gradients from equal volumes 10% and 40% Nycodenz in EGHM in a 14-ml ultracentrifuge tube using a two-chamber gradient maker or Gradient Master, and layer 2 ml resuspended nuclear pellet on top.
5. Ultracentrifuge in a swinging-bucket rotor 90 min at 80,000 × *g*, 4°C.
6. Remove the white band of nuclei from the middle of the gradient (i.e., ~22% Nycodenz) using a syringe or pipet.
7. Dilute the suspension with 2 vol EGHM. Centrifuge in a low-speed centrifuge with a swinging-bucket rotor 10 min at 1000 × *g*, 4°C.
8. Resuspend in EGHM or some other medium appropriate to further analysis.

Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to –20°C or –80°C. Always check that the measurements made are relatively unaffected by the storage conditions, by comparing with those made on fresh material. Note that thawing a frozen suspension of nuclei may cause release of DNA, and consequently almost irreversible aggregation of the particles.

BASIC PROTOCOL 2

ISOLATION OF NUCLEAR MEMBRANES: HIGH-IONIC-STRENGTH METHOD

High-ionic-strength methods for removing chromatin from the nucleus were first developed by Monneron et al. (1972), and more recently, high concentrations (i.e., 1.6 M) of NaCl were used by Kaufmann et al. (1983). In these methods, nucleus-sized hollow spheres are produced that are good for permeability studies. DNase I and RNase are also commonly included to digest the nucleic acids, and phenylmethylsulfonyl fluoride (PMSF) is included as a protease inhibitor.

As with the preparation of nuclei, most nuclear membrane methods are also based on rat liver and application to nuclei from other sources will need to be approached empirically to determine which, if any, of the current methods are suitable. This protocol is adapted from Kaufmann et al. (1983).

Materials

200 mM PMSF (*APPENDIX 2A*)
Nuclei suspension medium (NSM; see recipe)
DNase I
RNase
Incubation buffer (IB; see recipe)
High-NaCl buffer (HNB; see recipe)
2-mercaptoethanol
Nuclear membrane storage medium (NMSM; see recipe)

~20-ml Dounce homogenizer with loose-fitting Wheaton Type B pestle
Refrigerated low-speed centrifuge with swinging-bucket rotor

1. Prepare the nuclei (see Basic Protocol 1 or Alternate Protocol 1), using 1 mM PMSF in all media, diluted from a 200 mM stock.
2. Suspend the nuclei in nuclear suspension medium (NSM) at a concentration of 5 to 8 mg DNA/ml (see Support Protocols 1 and 3), using 3 to 4 gentle strokes of the ~20-ml Dounce homogenizer.
3. Add DNase I and RNase to a final concentration of 250 μg/ml and stir gently for 1 hr at 4°C.
4. Sediment the nuclei in a low-speed centrifuge with a swinging-bucket rotor for 10 min at 1000 × *g*, 4°C. Resuspend the pellet in the same volume incubation buffer (IB) to a concentration of 5 to 8 mg DNA/ml.
5. While stirring at 4°C, add 4 vol high-NaCl buffer (HNB) dropwise. Add 2-mercaptoethanol to a final concentration of 1% (v/v).

   Make sure each drop of HNB is well mixed before adding the next.
6. Stir for a further 15 min, then pellet the nuclear envelopes in a low-speed centrifuge with swinging-bucket rotor for 30 min at 1600 × *g*, 4°C.
7. Resuspend the pellet in IB and repeat steps 5 and 6, but omit the 2-mercaptoethanol.
8. Harvest the membranes by centrifugation in a low-speed centrifuge with swinging-bucket rotor for 30 min at 1600 × *g*, 4°C.
9. If the membranes are not to be analyzed immediately, suspend in nuclear membrane storage medium (NMSM) and maintain at –20°C.

   Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to –20°C or –80°C. Always check that the measurements made are relatively unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF NUCLEAR MEMBRANES: LOW-IONIC-STRENGTH METHOD

BASIC PROTOCOL 3

The low-ionic-strength method described in this protocol uses 0.1 mM $MgCl_2$ and is relatively rapid. This method produces fragments that range from incomplete "ghosts" to much smaller pore-sized fragments that retain the lamina and pores and are good for compositional studies (Kay et al., 1972; Harris and Milne, 1974; Rickwood et al., 1997). The protocol is adapted from Kay et al. (1972).

The application of this and other methods to nuclei from sources other than mammalian liver will need to be approached empirically to determine which of these protocols are suitable.

Materials

200 mM PMSF (*APPENDIX 2A*)
Low-ionic-strength suspension medium (LISM; see recipe)
1.0 mg/ml DNase I
Sucrose buffer, pH 7.4 and 8.5 (SB7.4 and SB8.5; see recipe)
NMSM (see recipe)
~10-ml Dounce homogenizer with loose-fitting Wheaton Type B pestle
High-speed centrifuge with fixed-angle rotor for 15-ml tubes

NOTE: All centrifugations are carried out at 4°C and all incubations are at 22°C.

1. Prepare the nuclei (see Basic Protocol 1, Alternate Protocol 1, or Alternate Protocol 2), using 1 mM PMSF in all media, diluted from a 200 mM stock.
2. Suspend the nuclei in low-ionic-strength suspension medium (LISM) to a concentration of 3 to 4 mg DNA/ml (see Support Protocols 1 and 3), using 4 to 5 gentle strokes of the pestle of the ~10-ml Dounce homogenizer.
3. Add DNase I to a final concentration of 5 µg/ml from the 1 mg/ml stock and add 4 vol sucrose buffer, pH 8.5 (SB8.5).
4. Incubate with gentle stirring for 15 min at 22°C and then add an equal volume ice-cold distilled water.
5. Transfer to an appropriate centrifuge tube and centrifuge in a high-speed centrifuge with fixed-angle rotor 15 min at 38,000 × *g*, 4°C.
6. Resuspend the pellet in the same volume of sucrose buffer, pH 7.4 (SB7.4), and add DNase I to a final concentration of 1 µg/ml.
7. Incubate 15 min with gentle stirring at 22°C and then add an equal volume ice-cold distilled water.
8. Centrifuge 15 min at 38,000 × *g*, 4°C. If the membranes are not to be analyzed immediately, resuspend in NMSM and store at –20°C.

   Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to –20°C or –80°C. Always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with those made on fresh material.

SUPPORT PROTOCOL 1

DIPHENYLAMINE ASSAY FOR DNA

This well-established chemical method for quantifying DNA, devised by Schneider (1957), is still probably one of the best, simplest, and most reliable assays. It measures the deoxyribose released by acid hydrolysis; however, because of interference by other carbohydrates, the DNA of samples in sucrose or any other gradient medium containing a sugar residue (e.g., metrizamide), must be first precipitated with trichloroacetic acid (TCA). Although a later method based on the use of the dye methyl green (Peters and Dahmus, 1979) does not require hydrolysis of the nucleic acid and is compatible with most gradient media, it is necessary to extract the methyl green with chloroform to remove contaminating crystal violet, and some sources of methyl green do not give consistent results (Graham and Ford, 1983).

Materials

500 µg/ml DNA standard solution
5%, 10%, and 20% (w/v) trichloracetic acid (TCA), ice-cold
Samples
Diphenylamine reagent (see recipe)

1. Dilute the 500 µg/ml DNA standard to give 0 to 200 µg DNA in 1.0 ml of 5% (w/v) TCA, diluted from a 20% (w/v) stock.
2. *Optional:* For samples in sucrose, precipitate the DNA by adding an equal volume ice-cold 20% (w/v) TCA.

   Although this is not strictly necessary for iodixanol-containing fractions, it is a useful way of concentrating dilute fractions. Omit steps 2 to 4 if precipitating DNA is not necessary.
3. After 10 min on ice, sediment the DNA by centrifuging in a low-speed centrifuge with a swinging-bucket rotor 20 min at 1000 × *g*, 4°C.
4. Wash the pellet twice in ice-cold 10% TCA.
5. Hydrolyze the DNA in the standards, membrane suspensions, or pellets in 5% (w/v) TCA (1 ml final volume), 20 min at 90°C.
6. Centrifuge 2 min at full speed in a microcentrifuge.
7. To 0.5 ml of each supernatant (and a blank containing 0.5 ml 5% TCA), add 1 ml diphenylamine reagent and heat 10 min at 100°C.
8. Cool to room temperature and measure the absorbance at 595 nm.

ORCINOL ASSAY FOR RNA

SUPPORT PROTOCOL 2

Like the diphenylamine assay for DNA, this simple method was devised by Schneider (1957) and measures the ribose released from RNA by acid hydrolysis. Gradient media (e.g., sucrose, metrizamide) containing carbohydrate residues must be removed by precipitation of the nucleic acid with trichloroacetic acid (TCA) prior to hydrolysis. As the deoxyribose from DNA also interferes, standard curves for both DNA and RNA should be constructed.

Materials

500 µg/ml DNA and RNA standard solutions
5%, 10%, and 20% (w/v) TCA
Samples
Orcinol reagent (see recipe)

1. Dilute the 500 µg/ml RNA and DNA standards to give 0 to 200 µg nucleic acid in 0.5 ml water.
2. *Optional:* For samples in sucrose, precipitate the nucleic acids by adding an equal volume ice-cold 20% TCA.

   Although this is not strictly necessary for iodixanol-containing fractions, it is a useful way of concentrating dilute fractions. Omit steps 2 to 5 if precipitating the DNA is not necessary.
3. After 10 min on ice, sediment the DNA by centrifuging in a low-speed centrifuge with a swinging-bucket rotor 20 min at 1000 × *g*, 4°C.
4. Wash the pellet twice in ice-cold 10% TCA.
5. Resuspend the pellet in 0.5 ml water.
6. To 0.5 ml standards, membrane suspensions, or resuspended pellets, add 0.1 ml water and 0.2 ml 20% (w/v) TCA.
7. Hydrolyze the nucleic acids 20 min at 90°C.

8. Microcentrifuge for 2 min at full speed.
9. To 0.6 ml of all supernatants and a blank containing 0.6 ml of 5% TCA, add 0.6 ml orcinol reagent and heat 20 min at 100°C.
10. Cool to room temperature and measure the absorbance at 660 nm.

To obtain an accurate value of the RNA content of a gradient fraction the contribution of the deoxyribose to the total absorbance must be subtracted. Determine the amount of DNA in the fraction using the diphenylamine method (see Support Protocol 1) and using the orcinol DNA standard curve convert this to an A_{660} value. Deduct this value from the A_{660} of the sample.

SUPPORT PROTOCOL 3

ETHIDIUM BROMIDE ASSAY FOR DNA AND RNA

This very sensitive fluorometric method devised by Karsten and Wollenberger (1972, 1977), although very simple to execute, does require a large number of controls. Although the method avoids the use of the strong acids needed for the chemical method (see Support Protocols 1 and 2), ethidium bromide is extremely toxic. The characteristic fluorescence is produced by the ethidium bromide intercalating the DNA, which therefore indicates that ethidium bromide is a strong mutagen. The alternative fluorescent method (Brunk et al., 1979) using diamidinophenylindole (DAPI) is less potentially harmful, but the binding of the DAPI is highly specific for A-T base pairs, and it is therefore essential that the standard and sample have the same base composition (Graham and Ford, 1983). Both methods are compatible with any gradient medium.

NOTE: Use phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBSCM) to make all solutions and dilutions in this protocol.

Materials

Phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBSCM; see recipe)
25 µg/ml DNA standard solution: store at –20°C for 2 to 3 months
25 µg/ml heparin
Sample
50 µg/ml RNase: heat at 100°C for 10 min to denature DNase
25 µg/ml ethidium bromide

3-ml cuvettes
Spectrophotofluorometer with an emission wavelength of 580 nm and an excitation wavelength of 360 nm

CAUTION: Ethidium bromide is a mutagen and should be handled, stored, and disposed of with appropriate care.

1. Just before use, dilute 25 µg/ml DNA standard with an additional 4 vol PBSCM.
2. In 3-ml cuvettes, labeled a to f, make up the following mixtures:
   a. standard: 0.5 ml of 5 µg/ml DNA standard, 0.5 ml heparin solution and 1.0 ml PBSCM
   b. blank I: 0.5 ml heparin solution and 1.5 ml PBSCM
   c. blank II: 2.5 ml PBSCM
   d. sample (DNA + RNA): 0.5 ml sample, 0.5 ml heparin solution and 1.0 ml PBSCM
   e. sample (DNA): 0.5 ml sample, 0.5 ml heparin solution, 0.5 ml 50 µg/ml RNase, 0.5 ml PBSCM
   f. sample blank: 0.5 ml sample, 2.0 ml PBSCM

   Dilute samples as required with PBSCM, in order to dilute the DNA to a level compatible with the assay.

3. Incubate all cuvettes at 37°C for 20 min.
4. Add 0.5 ml 25 µg/ml ethidium bromide to all cuvettes except c and f.
5. Stir the contents before measuring the fluorescence at an emission wavelength of 580 nm and an excitation wavelength of 360 nm.

   The fluorescence of the standard (a) should be set at 100 and the temperature should remain constant (±0.5°C) during the measurements. Measurements should be made at least 1 min after adding the ethidium bromide, but within an hour. Check the RNase solution for fluorescence and subtract if necessary.
6. Calculate the mass of DNA in the sample using the following equation:

$$A_{DNA} = \frac{A_{std}\left(F_e - F_b - F_f + F_c\right)}{F_a - F_b}$$

Calculate the mass of RNA according to the following equation:

$$A_{RNA} = \frac{A_{std}\left(F_d - F_e\right)}{0.46 \times \left(F_a - F_b\right)}$$

where A_{DNA} is the mass of DNA in sample mixture, A_{RNA} is the mass of RNA in sample mixture, A_{std} is the mass of DNA in standard mixture, and F is the fluorescence intensity.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Diphenylamine reagent

To 100 ml glacial acetic acid add 1 g diphenylamine (1% w/v final) and 2.75 ml concentrated sulfuric acid (H_2SO_4). Store up to 1 month at room temperature.

CAUTION: *Take care when adding the H_2SO_4 and use safety goggles.*

Ethylene glycol homogenization medium (EGHM)

To 100 ml ethylene glycol add 20 mg $MgCl_2 \cdot 6H_2O$ (1 mM final). Store up to 1 month at 4°C.

High-NaCl buffer (HNB)

To 100 ml H_2O add:
23.4 g NaCl (2.0 M final)
40 µl 1 M $MgCl_2$ (*APPENDIX 2A;* 0.2 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A;* 10 mM final)
Adjust to pH 7.4 if necessary
Add H_2O to 200 ml
Store 2 to 3 days at 4°C
Immediately before use, add 1 ml of 200 mM PMSF (*APPENDIX 2A*)

continued

Incubation buffer (IB)

To 100 ml H_2O add:
40 µl 1 M $MgCl_2$ (*APPENDIX 2A;* 0.2 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A;* 10 mM final)
Adjust to pH 7.4 if necessary
Add H_2O to 200 ml
Store 2 to 3 days at 4°C
Immediately before use, add 1 ml of 200 mM PMSF (*APPENDIX 2A*)

Iodixanol, 50% (w/v)

Dilute 5 vol OptiPrep (60% iodixanol; Axis-Shield; available from Accurate Chemical) with 1 vol OptiPrep diluent for nuclei (ODN; see recipe). Store 2 to 3 days at 4°C.

Iodixanol gradient solution, 30% and 35% (animal cells or tissues)

For 30% and 35% iodixanol, dilute 50% (w/v) iodixanol (see recipe) with NIM (see recipe) at a ratio of 6:4 and 7:3 by volume, respectively. Store 2 to 3 days at 4°C.

Iodixanol gradient solutions, 25% and 40% (wheat germ)

For 25% and 40% iodixanol, dilute OptiPrep (60% iodixanol; Axis-Shield; available from Accurate Chemical) with WGM (see recipe) at a ratio of 2.5:3.5 and 4:2 by volume, respectively. Store 2 to 3 days at 4°C.

Low-ionic-strength suspension medium (LISM)

To 200 ml H_2O, add 20 µl 1 M $MgCl_2$ (*APPENDIX 2A;* 0.1 mM final). Store up to 2 weeks at 4°C.

Nuclear isolation medium (NIM)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
0.37 g KCl (25 mM final)
1 ml 1 M $MgCl_2$ (*APPENDIX 2A;* 5 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A;* 10 mM final)
Adjust to pH 7.4 if necessary
Add H_2O to 200 ml
Store 2 to 3 days at 4°C

Nuclear membrane storage medium (NMSM)

To 100 ml H_2O add:
40 ml glycerol (20% v/v final)
2 ml 100 mM disodium EDTA (*APPENDIX 2A;* 1 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A;* 10 mM final)
Adjust to pH 7.5 if necessary
Add H_2O to 200 ml
Store 2 to 3 days at 4°C

Nuclei suspension medium (NSM)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
1 ml 1 M $MgCl_2$ (*APPENDIX 2A;* 5 mM final)
10 ml 1 M Tris·Cl (*APPENDIX 2A;* 50 mM final)
Adjust to pH 7.4 if necessary
Add H_2O to 200 ml
Store 2 to 3 days at 4°C
Immediately before use add 1 ml of 200 mM PMSF (*APPENDIX 2A*)

OptiPrep diluent for nuclei (ODN)

To 100 ml H_2O add:
17.1 g sucrose (0.25 M final)
2.22 g KCl (150 mM final)
6 ml 1 M $MgCl_2$ (*APPENDIX 2A;* 30 mM final)
12 ml 1 M Tris·Cl (*APPENDIX 2A;* 60 mM final)
Adjust to pH 7.4 if necessary
Add H_2O to 200 ml
Store 2 to 3 days at 4°C

Orcinol reagent

To 50 ml concentrated HCl, add 0.5 g orcinol (1% w/v final) and 0.25 g $FeCl_3 \cdot 6H_2O$ (0.5% w/v final). Make up fresh and keep on ice until required.

Phosphate-buffered saline with Ca/Mg (PBSCM)

To 100 ml H_2O add:
0.026 g $CaCl_2 \cdot 2H_2O$ (0.9 mM final)
0.04 g KCl (2.68 mM final)
0.04 g KH_2PO_4 (1.47 mM final)
100 µl 1 M $MgCl_2$ (*APPENDIX 2A*; 0.5 mM final)
0.16 g NaCl (0.137 M final)
0.23 g Na_2HPO_4 (8.1 mM final)
Adjust to pH 7.5 with NaOH or HCl
H_2O to 200 ml
Store up to 1 week at 4°C

Sucrose buffer, pH 7.4 (SB7.4), pH 8.5 (SB8.5)

Sucrose buffer, pH 8.5 (SB8.5)
To 100 ml H_2O add:
20.5 g sucrose (0.3 M final)
20 µl 1 M $MgCl_2$ (*APPENDIX 2A;* 0.1 mM final)
78 mg 2-mercaptoethanol (5 mM final)
0.37 g triethanolamine (10 mM final)
Adjust to pH 8.5 with HCl
Add H_2O to 200 ml
Store 2 to 3 days at 4°C

Sucrose buffer, pH 7.4 (SB7.4)
Prepare as SB8.5, but adjust to pH 7.4

Sucrose density barrier (SDB)

To 95 ml H_2O add:
157 g sucrose (2.3 M final)
0.37 g KCl (25 mM final)
1 ml 1 M $MgCl_2$ (*APPENDIX 2A;* 5 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A;* 10 mM final)
Adjust to pH 7.4 if necessary
Add H_2O to 200 ml
Store 2 to 3 days at 4°C

(continued)

Wheat germ medium (WGM)

To 100 ml H_2O add:
27.4 g sucrose (0.4 M final)
0.37 g KCl (25 mM final)
1 ml 1 M $MgCl_2$ (*APPENDIX 2A*; 5 mM final)
0.39 g 2-(*N*-morpholino)sulfonic acid (MES; 10 mM final)
Adjust pH to 6.2 with NaOH
Add H_2O to 200 ml
Store 2 to 3 days at 4°C

COMMENTARY

Background Information

Isolation of nuclei from a mammalian tissue homogenate has proved to be one of the easier tasks of sucrose density gradient separation technology. In a homogenate the nuclei are generally regarded as the largest organelle (4 to 12 μm in diameter), although some plasma membrane sheets, often present in a tissue homogenate but not in homogenate from cultured cells, may be up to 20 μm in length. Consequently a "low-speed" or "nuclear" pellet, produced by centrifugation of a homogenate at 500 to 1000 × *g* for 10 min, will contain these two components, plus some of the largest mitochondria. Unbroken tissue, cells, and connective tissue also sediment under these conditions, and this is the reason why nuclear isolation methods invariably preface the low-speed centrifugation with a filtration step to remove these elements which would otherwise contaminate the nuclear pellet and compromise the subsequent gradient fractionation. Smaller subcellular particles will also contaminate the pellet because of entrapment and the inefficiency of differential centrifugation (*UNIT 3.3*). These can be partially removed by washing the pellet, but this might be regarded as superfluous, as any subsequent gradient will resolve these contaminants, since the density of nuclei (>1.32 g/ml in sucrose) is also greater than that of any other major subcellular component.

It is also possible to purify the nuclei from a low-speed pellet by washing the crude nuclei in a medium containing 0.5% Triton X-100 in order to solubilize contaminating membranes (Hoffmann and Chalkley, 1978); however, the method tends to make the nuclear membrane rather more fragile, the nuclei prone to aggregation, and endogenous nuclease activity can be activated (Rickwood et al., 1997). Thus, density barrier centrifugation is the most widely used technique. As the nuclei are the largest and densest of all the subcellular organelles, simple density barriers or discontinuous gradients are usually effective for their purification.

Sucrose solutions are not dense enough to band nuclei (ρ > 1.32 g/ml in sucrose) within a gradient; thus, pelleting through a 2.2 to 2.3 M sucrose density barrier is the only option. Because of the high viscosity of the sucrose density barrier, the sedimentation of the particles is very slow and the nuclei lose much of the water from their internal space due to the osmotic gradient across the membrane. This process may disrupt the macromolecular structures, which are normally highly hydrated. Nevertheless, the nuclei do rehydrate when they are transferred back to an isoosmotic medium, and the method is still widely used. After centrifugation of sucrose barriers at 100,000 to 200,000 × *g* (for up to 1 hr), the nuclei are recovered as a pellet. Because resuspension of the pellet is sometimes difficult (aggregation of the nuclei is often a problem), banding the organelles at an interface offers some significant benefits.

Although both CsCl and sodium diatrizoate can provide solutions of sufficient density to band nuclei at an interface, their ionic strength can cause disruption of the nucleoprotein structure, unless the chromatin is first fixed using a cross-linking agent such as formaldehyde to prevent dissociation of the protein from DNA (Gollin and Wray, 1984). However, the nonionic iodinated density gradient media can be used in this mode. With iodixanol, the gradient is isoosmotic, so the nuclei do not become condensed. Consequently, they have a much lower density (i.e., 1.20 to 1.22 g/ml), and the lower viscosity of the density gradient allows the separation to be carried out at much lower RCFs (i.e., 10,000 × *g* for 20 min) than with sucrose. It is also considered unnecessary to produce a crude nuclear fraction first. The recommended strategy is to adjust the homogenate with OptiPrep, or a high-density working solution produced from it, to a density that is higher than most organelles (ρ = ~1.16 g/ml), and layer

it over a double iodixanol barrier (ρ = 1.175 and 1.20 g/ml, respectively). In mammalian cells, the only other organelles that have a density approaching that of nuclei are peroxisomes (Graham et al., 1994), but, as they are much smaller, only the nuclei have the size and density to band above the densest layer at the relatively-low *g* forces (i.e., 10,000 × *g*) and short times (i.e., 20 min) used in this method (Graham et al., 1994; Provost et al., 1996).

Early methods that attempted to use nonaqueous media for the isolation of organelles relied on organic solvents such as benzene and carbon tetrachloride and the preparation of lyophilized cells and tissues, so it is hardly surprising that they never attained any degree of popularity whatsoever (see Rickwood et al., 1997 for more information). Nevertheless, a method such as homogenization of lyophilized cells in pure glycerol (Gurney and Foster, 1977) may be useful for analysis of inorganic ions and soluble proteins which are normally leached out by the normal aqueous media. However, the method developed by Rickwood et al. (1997), in which the liver is homogenized in ethylene glycol and the nuclei are purified in gradients of Nycodenz in the same solvent, probably provides a much more acceptable manner for avoiding the use of aqueous media (see Alternate Protocol 2).

Critical Parameters and Troubleshooting

Homogenization

For mammalian tissues, inclusion of divalent cations in the homogenization medium, usually in the form of $MgCl_2$ ($CaCl_2$ is generally avoided because of the problem of Ca^{2+}-stimulated phospholipases), as well as KCl in buffered 0.25 M sucrose, helps to stabilize and preserve nuclear structure (e.g., the 0.25 M sucrose/25 mM KCl/5 mM $MgCl_2$/10 mM Tris·Cl, pH 7.4 medium; see Basic Protocol 1). This buffer was devised by Blobel and Potter (1966). While KCl is sometimes omitted, inclusion of $MgCl_2$ is invariant; Widnell and Tata (1964), for example, used 0.25 M sucrose/5 mM $MgCl_2$/10 mM Tris·Cl, pH 7.4. For nuclei from nonmammalian sources, inclusion of Mg^{2+} is also common, although the details of the homogenization medium are generally specific for the organism. Yeast spheroplasts have been homogenized in 8% polyvinylpyrrolidone (PVP; M_r 40,000)/1 mM $MgCl_2$/0.02 M KH_2PO_4/0.02% Triton X-100, while for intact yeast cells an Mg^{2+}-free medium has been used (i.e., 1 M sorbitol/20% glycerol/5% PVP), and for plant cells the media are even more diverse. For a review of these media see Rickwood et al. (1997).

With mammalian liver, KCl and $MgCl_2$ can be present at all stages of the procedure, including homogenization. On the other hand, homogenization media for hard tissues (e.g., skeletal muscle) may contain KCl, but they may also contain EDTA rather than Mg^{2+}. In such circumstances, isolation procedures need to be developed empirically. If adequate homogenization does not occur in the absence of EDTA, or in the presence of Mg^{2+}, then a compromise must be reached in which the tissue or cells are homogenized in a suitable medium and then an isoosmotic medium containing KCl and/or $MgCl_2$ might be added to the homogenate. Alternatively the nuclei can be pelleted and then resuspended in a KCl/$MgCl_2$ medium.

The same problem applies to cultured cells in which it is common to use media that are not ideally suited to nuclei (e.g., 0.25 M sucrose/1 mM EDTA/10 mM triethanolamine-acetic acid, pH 7.4 medium; Marsh et al., 1987). If the cells do not disrupt readily in an isoosmotic Mg^{2+}-containing medium, then it may be better to use a hypoosmotic medium such as 15 mM KCl/1.5 mM magnesium acetate/1 mM dithiothreitol/10 mM HEPES-KOH, pH 7.5 (Goldberg and Kornfeld, 1983; Dunphy and Rothman, 1985). Birnie (1978) recommended a swelling medium of 10 mM NaCl/1.5 mM $MgCl_2$/10 mM Tris·Cl, pH 7.4. After homogenization, the medium is returned to isoosmoticity by adjusting to 0.25 M sucrose. With cultured-cell nuclei, it is also common to include a number of washing steps prior to centrifugation through 2.2 to 2.3 M sucrose. Moreover, Birnie (1978) used a Ca^{2+}-containing buffer (0.25 M sucrose/3 mM $CaCl_2$/10 mM Tris·Cl, pH 7.4) as a wash medium; apparently Ca^{2+}-stimulated phospholipases are less of a problem with cultured cells (i.e., as compared to liver).

For cells and tissues that do not homogenize satisfactorily in any of these media, the method adopted by Wray et al. (1977) may be useful. Homogenization of liver in 0.5 M hexylene glycol (2 methyl-2,4-pentanediol)/1.0 mM $CaCl_2$/50 mM PIPES, pH 7.5, allowed the isolation of nuclei with good functional integrity. Although this method only produced a yield of 40% to 50%, it was applicable to a very wide range of tissues, including those which do not respond well to the normal sucrose-based meth-

Table 3.10.1 Enzyme Content of Nuclear Membranes[a]

	Glucose-6-phosphatase activity (μmol phosphate/hr/mg/protein)	NADHCR[b] activity (μmol cytochrome *c* reduced/hr/mg/protein)	DNA polymerase activity (pmol TMP incorporated/min/mg protein)
Nuclei	1.32	6.48	68.3
Nuclear membranes	9.6	16.0	9.0
Microsomes	10.56	45.5	Not applicable

[a]Data adapted from Kay et al. (1972) with kind permission.
[b]NADHCR = NADH cytochrome *c* reductase.

ods (e.g., Novikoff hepatoma cells and mouse brain).

Selection of a suitable nonaqueous system is a difficult problem, and the approach adopted by Rickwood et al. (1997) is something of a compromise in that, although the homogenization medium is nonaqueous, water will be present in the tissue since lyophilization is not used. Although such an approach cannot remove cytosolic water, the water in the liver vasculature can be eliminated by perfusion with ethylene glycol prior to homogenization, if this is regarded as important. Use of a nonaqueous medium generally makes homogenization more difficult, and the liver should be minced very finely before using the Potter-Elvehjem homogenizer; indeed it may be advantageous to use a small mincer rather than scissors.

Isolation of mammalian nuclei by sucrose density gradient centrifugation

At the high concentrations of sucrose used in the first protocol of this unit (see Basic Protocol 1), the extreme viscosity is a major problem, not only in preparing the solutions, but also in accurate dispensing of aliquots into centrifuge tubes. It may be easier to make up the solutions on a weight/weight basis, rather than to try and dissolve the sucrose in a smaller volume than the final, and then make up to the volume (e.g., 2.2 and 2.3 M sucrose are equivalent to 59% and 61% w/w, respectively). However, it should be noted that the liquid used to dissolve the sucrose should contain approximately twice the required concentrations of buffer and salts to allow for "dilution of the water by the sucrose." If the crude nuclear pellet is resuspended directly in 2.3 M sucrose rather than in 0.25 M sucrose first (see Basic Protocol 1, step 14), great care has to be taken to ensure that the pellet is properly resuspended; using such a viscous medium in an homogenizer is technically difficult.

Isolation of mammalian nuclei by iodixanol density gradient centrifugation

For material other than mammalian liver it may be necessary to modulate the density of the three layers of iodixanol and/or increase the time of centrifugation (see Alternate Protocol 1). This separation is based to some extent on the sedimentation rate of the nuclei as well as their density. In dense hyperosmotic sucrose, all nuclei shrink and attain the same limiting density irrespective of their real size and density. In isoosmotic iodixanol gradients, the organelles retain their native size and density; therefore, the rate at which they sediment and their banding density may vary with the material source.

Gradient purification of nonmammalian nuclei

Nuclei from plant cells pose additional problems in that other subcellular particles such as chloroplasts and starch granules sediment at low RCFs and are as dense or denser than nuclei in sucrose gradients. Consequently, sucrose gradients are ineffective for resolving and purifying plant cell nuclei; however, since nuclei are much less dense in iodixanol than in sucrose, the opportunity exists for resolution in these gradients. Ford et al. (1998) reported that starch granules are able to pellet through an iodixanol solution of density 1.234 g/ml, and that centrifugation of a crude nuclear preparation over a discontinuous gradient of iodixanol (i.e., 1.167 and 1.234 g/ml) produces nuclei free of starch granules at the lower interface. It is not known at what density chloroplasts band in this system. Rickwood et al. (1997) also noted that the melanin granules of *Xenopus* liver also pellet with the nuclei in sucrose and that only a 58% (w/v) metrizamide barrier (i.e., 10,000 × *g* for 20 min) was effective in separating these particles (the melanin granules pellet). At this concentration, metrizamide is hyperosmotic

and it may be possible to use a lower concentration of iodixanol.

Nuclear membranes

In the 1970s, two quite different strategies were evolved for the isolation of nuclear envelopes from mammalian liver nuclei. One used high concentrations of $MgCl_2$ (i.e., 0.5 to 0.7 M) to disrupt the nuclear chromatin (Monneron et al., 1972) while the other used low-ionic-strength media (Kay et al., 1972; Harris and Milne, 1974). Since that time, these two basic strategies have been developed almost in parallel, with some workers favoring high ionic strength and others low ionic strength.

There are no particular technical problems associated with either method of nuclear membrane preparation (see Basic Protocols 2 and 3). Either method works well for rat liver; only experimentation will reveal whether this is true for other tissues and cells. Choice of methodology depends to some extent on the subsequent studies to be carried out on the envelopes. The high-ionic-strength method produces nucleus-sized hollow spheres, which are good for permeability studies. The yield of envelopes is very high and they are intact, retaining the lamina and pores. Low-ionic-strength procedures tend to produce high-purity fragments of the nuclear membrane, with intact pores and lamina, and are particularly good for compositional studies. The fragments of membrane have a variety of sizes ranging from incomplete "ghosts" to much smaller pore-sized fragments; therefore, a high-speed centrifuge is required to harvest the membranes in high yield. The high-ionic-strength procedure, on the other hand, in which the whole envelope is retained, requires only a low-speed centrifuge. All the modern methods incorporate the use of enzymes to digest the released nucleic acids.

Assays

Determination of the purity of a nuclear preparation can be assessed by phase-contrast microscopy (i.e., absence of any particles other than nuclei) and measurement of DNA and RNA. The chemical methods that measure the content of either deoxyribose or ribose (Schneider, 1957) are probably the most widely used. Neither Nycodenz nor iodixanol interferes significantly, but sucrose, metrizamide, and other sugar-containing molecules do. The newer fluorometric methods (Karsten and Wollenberger, 1972, 1977) are certainly more sensitive and are unaffected by most gradient media. There are no easily measurable enzyme markers for the nuclear membrane, but enzymes that are characteristic of the endoplasmic reticulum (ER) are found in nuclear and nuclear-membrane preparations. Since there is evidence for some continuity between the outer nuclear membrane and the ER, this is probably not surprising, but the levels of enzymes such as glucose-6-phosphatase, NADH cytochrome *c* reductase, and NADPH cytochrome *c* reductase (*UNIT 3.5*) observed in nuclear membrane preparations suggest that they are true components, rather than merely a reflection of the presence of some "attached" ER. It is more common to express the purity of nuclei in terms of the DNA and RNA content and in addition, protein and phospholipid, for nuclear membrane fractions (see Anticipated Results).

Anticipated Results

Nuclei

The yield of nuclei is measured in terms of percentage recovery of DNA from the homogenate, and for both the sucrose and iodixanol methods a figure of at least 90% is expected. Approximately 4% of the total RNA is recovered in the nuclear pellet, and the DNA/RNA ratio should be ~0.11. By phase-contrast microscopy, the nuclei should appear as characteristic dark-gray structures, 4 to 12 μm in diameter, and although smaller organelles cannot be discerned at the levels of magnification achievable by this technique, there should be a complete absence of any "stippled" background. In neither method can any marker enzymes of any other membrane be detected, except those of the ER (e.g., glucose-6-phosphatase and NADH cytochrome *c* reductase), which may be present at specific activities that are characteristically 10% to 15% of the levels in microsomes.

Nuclear membranes

Because the preparation of nuclear membranes is always made from previously purified nuclei, it is common to express efficacy of the membrane separation procedure in terms of the percentage recovery of protein, DNA, RNA, and phospholipid from the nuclei. Typical values are 8% to 12%, 0.6% to 2%, 6% to 8%, and 55% to 60%, respectively (Kay et al., 1972; Monneron et al., 1972). Phospholipid is only present in the membrane, so 55% to 60% is also a measure of the overall recovery of nuclear membranes. The relative amounts of each of these four components in the membrane is

typically 73%, 0.6%, 3%, and 20%, respectively, by weight.

Table 3.10.1 gives the specific activity of some typical enzyme markers in the nuclear membranes, and, for comparison, levels in the nuclei and in the microsomes (Kay et al., 1972). There is a clear enrichment in the nuclear membrane (i.e., compared to the nuclei) of the two enzyme markers, which are generally regarded as characteristic of the endoplasmic reticulum (glucose-6-phosphatase and NADH cytochrome *c* reductase). The specific activity of the glucose-6-phosphatase, in particular, is close to that of the microsomes, and thus this enzyme would appear to be a true component of the nuclear membrane rather than a result of contamination by the ER. It should be noted that in some preparations the levels of glucose-6-phosphatase are much lower (for a review of this data, see Harris and Agutter, 1976). The situation with the NADH cytochrome *c* reductase is rather less clear, in as much as the membranes contained <10% of the protein present in the nuclei, but the specific activity of this enzyme only increased approximately twofold. Again the reported levels for this enzyme vary considerably (Harris and Agutter, 1976).

Time Considerations

There are no points at which any of the basic or alternate protocols can be interrupted. The crude nuclear fraction (see Basic Protocol 1) must be used immediately for any subsequent density barrier or gradient separation. Discontinuous (see Alternate Protocol 1) and continuous gradients (see Alternate Protocol 2) should be made up immediately prior to use, although time can be saved by preparing these gradients during the immediately previous step or steps. Basic Protocol 1 and Alternate Protocol 2 will take about 2.5 hr, while the shorter centrifugation time and avoidance of use of an ultracentrifuge means that Alternate Protocol 1 can be accomplished in less time (i.e., ~1.75 hr).

Nuclear membrane preparations should be executed as soon as possible upon purification of the nuclei. Basic Protocol 2 will take about 3.15 hr, while Basic Protocol 3 is more rapid (i.e., ~1.75 hr).

Functional assays should be carried out as soon as possible after the preparation, but for simple marker-enzyme assay, material can be stored at 0° to 4°C for up to 16 hr without much loss of activity. Enzyme assays may be carried out on nuclear membrane preparations frozen to –20° or –80°C, but when frozen nuclei are thawed, the tendency to leak DNA may cause serious aggregation. There are no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those made on fresh organelles. Chemical assays for DNA and RNA, which may take up to 3 hr if the DNA is precipitated first, can be carried out on frozen material, although again the aggregation that can occur when frozen suspensions of nuclei are thawed may cause sampling errors. The ethidium bromide assay for DNA and RNA is very rapid and will take no more than 1 hr, and this is also best carried out on unfrozen material. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All the solutions can be made ahead of time and stored at 4°C for 1 to 2 days or frozen to –20°C for longer periods; however, note that any enzymes, PMSF, or other protease inhibitors, should be added immediately prior to use.

Literature Cited

Birnie, G.D. 1978. Isolation of nuclei from animal cells in culture. *Methods Cell Biol.* 17:13-26.

Blobel, G. and Potter, V.R. 1966. Nuclei from rat liver: An isolation method that combines purity with high yield. *Science* 154:1662-1665.

Brunk, C.F., Jones, K.C., and James, T.W. 1979. Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.* 92:497-500.

Dunphy, W. and Rothman, J.E. 1985. Compartmental organization of the Golgi stack. *Cell* 40:463-472.

Ford, T.C., Baldwin, J.P., and Lambert, S.J. 1998. Rapid enzyme-free preparation of starch-free nuclei from plants facilitates studies of chromatin. *Plant Protein Club Annual Symposium, p. 24.* York, U.K.

Goldberg, D.E. and Kornfeld, S. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159-3165.

Gollin, S.M. and Wray, W. 1984. Isopycnic centrifugation of metaphase chromosomes in Nycodenz. *Exp. Cell. Res.* 152:204-211.

Graham, J.M. and Ford, T.C. 1983. Enzymic and chemical assays compatible with iodinated density gradient media. *In* Iodinated Density Gradient Media: A Practical Approach (D. Rickwood, ed.), pp. 195-216. Oxford University Press, Oxford.

Graham, J., Ford, T., and Rickwood, D. 1994. The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol. *Anal. Biochem.* 220:367-373.

Gurney, T. and Foster, D.N. 1977. Nonaqueous isolation of nuclei from cultured cells. *Methods Cell Biol.* 16:45-68.

Harris, J.R. and Agutter, P.S. 1976. The isolation and characterization of the nuclear envelope. *In* Biochemical Analysis of Membranes (A.H. Maddy, ed.), pp. 132-173. Chapman and Hall, London.

Harris, J.R. and Milne, J.F. 1974. A rapid procedure for the isolation and purification of rat liver nuclear envelope. *Biochem. Soc. Trans.* 2:1251-1253.

Hoffmann, P. and Chalkley, R. 1978. Procedures for minimizing protease activity during isolation of nuclei, chromatin, and the histones. *Methods Cell Biol.*17:1-12.

Karsten, U. and Wollenberger, A. 1972. Determination of DNA and RNA in homogenized cells and tissue by surface fluorometry. *Anal. Biochem.* 46:135-148.

Karsten, U. and Wollenberger, A. 1977. Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates. *Anal. Biochem.* 77:464-469.

Kaufmann, S.H., Gibson, W., and Shaper, J.H. 1983. Characterization of the major polypeptides of the rat liver nuclear envelope. *J. Biol. Chem.* 258:2710-2719.

Kay, R.R., Fraser, D., and Johnston, I.R. 1972. A method for the rapid isolation of nuclear membranes from rat liver. *Eur. J. Biochem.* 30:145-154.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* 104:875-886.

Monneron, A., Blobel, G., and Palade, G.E. 1972. Fractionation of nuclei by divalent cations. Isolation of nuclear membranes. *J. Cell Biol.* 55:104-125.

Peters, D.L. and Dahmus, M.E. 1979. A method of DNA quantitation for localization of DNA in metrizamide gradients. *Anal. Biochem.* 93:306-311.

Provost, J.J., Fudge, J., Israelit, S., Siddiqi, A.R., and Exton, J.H. 1996. Tissue-specific distribution and subcellular distribution of phospholipase D in rat: Evidence for distinct RhoA- and ADP-ribosylation factor (ARF)-regulated isoenzymes. *Biochem. J.* 319:285-291.

Rickwood, D., Messent, A., and Patel, D. 1997. Isolation and characterization of nuclei and nuclear subfractions. *In* Subcellular Fractionation: A Practical Approach (J.M. Graham and D. Rickwood, eds.), pp 71-105. IRL Press at Oxford University Press, Oxford.

Schneider, W.C. 1957. Determination of nucleic acids in tissues by pentose analysis. *Methods Enzymol.* 3:680-684.

Widnell, C.C. and Tata, J.R. 1964. A procedure for the isolation of enzymically active rat liver nuclei. *Biochem. J.* 92:313-317.

Wray, W., Conn, P.M., and Wray, V.P. 1977. Isolation of nuclei using hexylene glycol. *Methods Cell Biol.* 16:69-86.

Contributed by John M. Graham
Liverpool John Moores University
Liverpool, United Kingdom

Free-Flow Electrophoretic Analysis of Endosome Subpopulations of Rat Hepatocytes

Endosomes constitute a functionally, morphologically, and biochemically heterogeneous population of intracellular organelles that play a major role in sorting of incoming ligands, receptors, membrane, and content. To characterize their composition and properties, highly purified endosomes are required. Given the heterogeneity of endosomes and the lack of a general and suitable marker, endosome subpopulations have to be specifically labeled by pulse-chase internalization of ligands routed to the pathway of interest. However, due to their heterogeneity and similarity in buoyant density to other intracellular organelles, isolation procedures using standard cell fractionation techniques usually do not result in sufficient purification and adequate resolution of the endosome population of interest. Thus, alternative protocols have to be used for endosome purification. These involve the selective modification of the density of endosomes, immunoisolation, and free-flow electrophoresis (FFE). The latter technique has been applied to isolate highly purified and, most importantly, functional endosomes from cultured cells and tissues (Marsh et al., 1987). Furthermore, endosome subpopulations can be resolved by FFE, allowing for the kinetic analysis of ligand transport through distinct endosome compartments (Schmid et al., 1988).

This unit describes the application of FFE for analysis of endosome subpopulations of polarized rat hepatocytes (see Basic Protocol). As a prerequisite, endosomes involved in transport to lysosomes and transcytosis have to be labeled. In rat liver, asialoorosomucoid (ASOR) and polymeric immunoglobulin A (pIgA) can be used as ligands to selectively label the lysosomal and transcytotic route in hepatocytes. The respective receptors—i.e., the asialoglycoprotein receptor (ASGPR) and the pIgA receptor (pIgR)—are expressed in parenchymal but not in other cells in the liver. Procedures for preparation of ASOR, fluorescein isothiocyanate (FITC)-ASOR, and [^{125}I]pIgA are detailed in Support Protocols 4, 5, and 6, respectively. In vivo labeling of rat liver endosomes is achieved by intravenous injection of the ligands. As starting material for the FFE separation, endosome-enriched fractions have to be prepared from rat liver homogenates. The FFE protocol (see Basic Protocol) can be extended for the preparative isolation of endosomes from rat liver (see Alternate Protocol). The characterization of the endosome-containing FFE-fractions with respect to contamination by other intracellular organelles and the analysis of the protein composition are outlined in Support Protocols 1 and 2, respectively. Highly purified endosomes obtained by FFE are fully functional with respect to in vitro endosome acidification as described in Support Protocol 3.

FREE-FLOW ELECTROPHORESIS OF HEPATOCYTE ENDOSOMES

BASIC PROTOCOL

Endosome subpopulations of rat hepatocytes can be most accurately labeled in the isolated perfused liver using single-pass perfusion and a pulse-chase protocol (Mueller and Hubbard, 1986; Perez et al., 1988). This method, however, requires a skillful person trained in this specific operating technique and the instruments (e.g., pumps, drop counter, bile flow recorder) for the isolated perfusion of the liver. For a detailed description of the method see Exton (1975). Alternatively, markers can be injected into the rat for in vivo endosome labeling (Hoppe et al., 1985). However, using in vivo marker injection, a detailed pulse-chase protocol for two or more endocytic markers is not applicable and consequently the labeling of distinct endosome subpopulations is less precise in comparison to the isolated perfused liver. Nevertheless, the endosomes are well resolved from the

Contributed by Renate Fuchs and Isabella Ellinger
Current Protocols in Cell Biology (2002) 3.11.1-3.11.31

main protein peak and the kinetics of marker transport through distinct subcompartments can also be analyzed.

For FFE separation of rat liver endosomes, the sample injected into the FFE apparatus must already be considerably enriched in endosomes with respect to the original homogenate. This can be achieved by differential centrifugation of the initial homogenate, preparation of microsomes, and flotation of microsomes in discontinuous sucrose density gradients. The protocol for preparation of endosome-enriched fractions described below (also see Fig. 3.11.1) is a modification of the method used by Khan et al. (1986) and has been successfully used as starting material for FFE isolation of rat liver endosomes (Fuchs et al., 1989; Stefaner et al., 1997).

Endosome-enriched fractions prepared from rat liver are used as the starting material for the separation of endosome subpopulations from each other—and from contaminating organelles such as plasma membrane, endoplasmatic reticulum, and lysosomes—by free-flow zone electrophoresis, also known as continuous flow electrophoresis (the

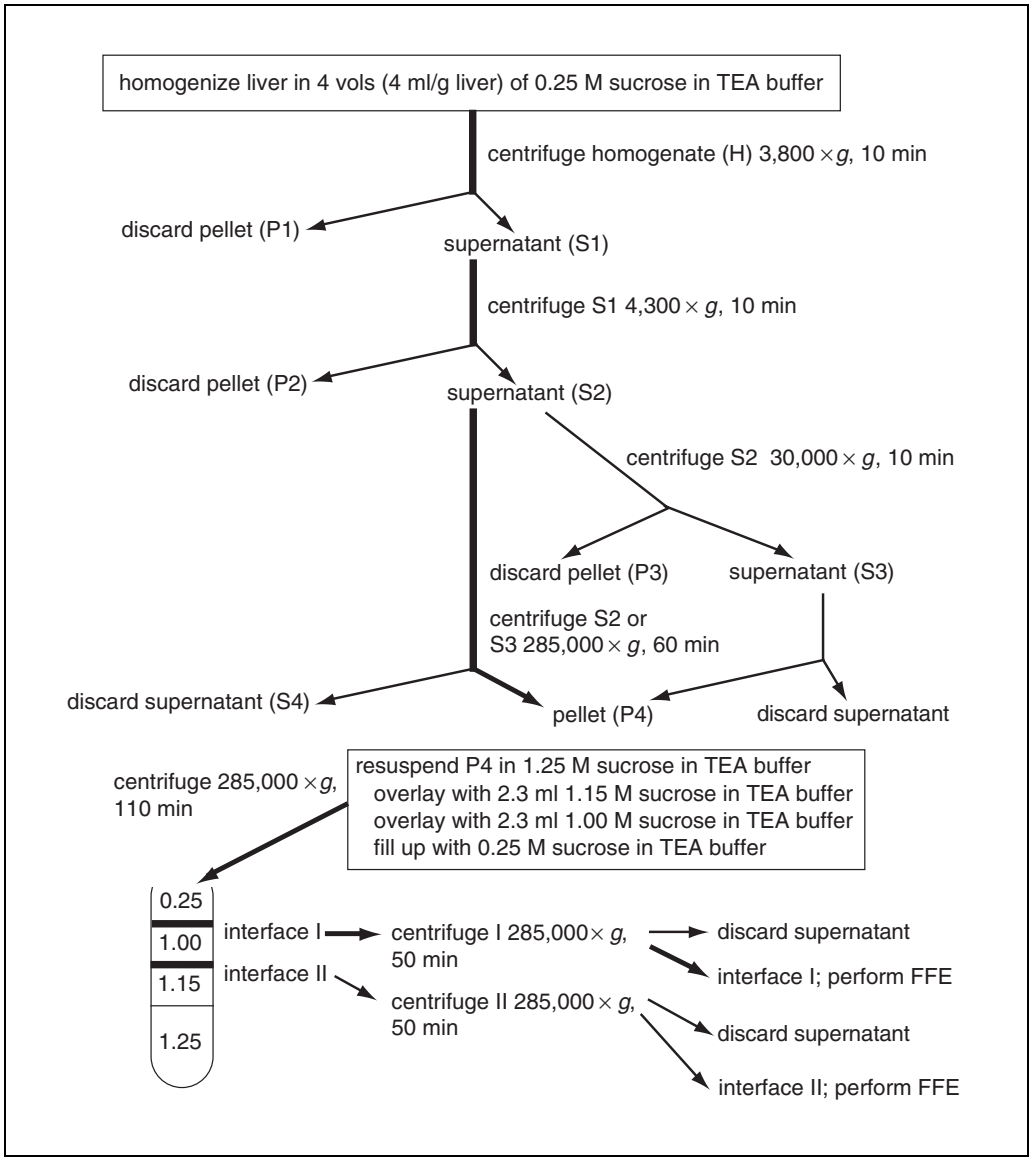


Figure 3.11.1 Flow chart of the fractionation protocol applied to prepare endosome-enriched fractions (interface I and interface II) from rat liver. Interface I and II are subsequently subjected to separation by free-flow electrophoresis (FFE).

abbreviation FFE is used in this protocol for this specific type of free-flow electrophoresis). The starting membrane material is treated with trypsin, a procedure which alters the electrophoretic mobility of endosomes and lysosomes (Marsh et al., 1987). Samples are then injected as a fine jet into the chamber of the FFE apparatus, where they are subjected to separation by the laminar flow of the chamber buffer and an high-voltage electrical field. Endosomes are deflected more towards the anode from the flow direction of the medium and contaminating organelles such as plasma membranes and endoplasmic reticulum. Among the endosome subpopulations, late endosomes are more deflected towards the anode than transcytotic or early endosomes, respectively (Schmid et al., 1988; Stefaner et al., 1997). Once the samples have reached the end of the separation chamber at the top, they can be fractionated continuously and collected by means of a 96-fold splitting device. The presence of endosomes and other organelles in these fractions is then identified by determination of endosomal markers and organelle-specific marker enzyme activities.

Carrier-free high voltage electrophoresis involves high-tech instruments. The user must have well-founded knowledge with regard to the theory and practice of the method and operation of the instrument. The protocol described below has been established for the Elphor VaP 22, Bender and Hobein, FFE apparatus. Application of the protocol to other free-flow electrophoresis instruments (e.g., Octopus) might require modifications with respect to buffer volumes, chamber buffer flow, sample flow rate, and voltage/current.

CAUTION: Wear gloves and carry out experiments in radioactivity-certified areas. This procedure must be performed in a laboratory certified for work with ^{125}I-labeled material. All personnel using radioactive material must be properly trained. Make sure that all radioactive waste (including the rat corpse and perfusion buffer) is disposed appropriately (also see *APPENDIX 1D*).

NOTE: All solutions, rotors, centrifuge tubes and glassware should be prechilled to 4°C and kept on ice throughout the isolation procedure. All manipulations and centrifugations following step 17 are carried out at 4°C.

NOTE: All protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to government regulations regarding the care and use of laboratory animals. Anesthesia, surgery, and killing of the rats should be supervised or carried out by an experienced animal technician.

Materials

150- to 200-g male Sprague-Dawley rats (e.g., Taconic Farms, The Jackson Laboratory)

Urethane anesthetic solution: 10 g urethane ethyl carbamate/10 ml 0.9% (w/v) NaCl (prepare immediately before use)

0.25 M, 1 M, 1.15 M, and 2.5 M sucrose in TEA buffer, ice cold (see recipes)

Endocytic tracers: 220 µg FITC-ASOR (see Support Protocols 4 and 5) and 1.4×10^6 cpm [^{125}I]pIgA (see Support Protocol 6) per 500 µl phosphate-buffered saline (PBS; *APPENDIX 2A*)

Protease inhibitor solutions (*UNIT 3.4*)

1× TEA buffer (see recipe)

FFE chamber buffer (see recipe)

FFE electrode buffer (see recipe)

1 mg/ml trypsin solution (trypsin-TPCK treated; Worthington), freshly prepared

2 mg/ml trypsin inhibitor solution (trypsin inhibitor, Type I-S, from soybean; Sigma-Aldrich), freshly prepared

2-ml syringes and 25-G needles

Plastic dissecting trays (~15 cm width × 30 cm length) with legs (~8 cm height)
Plastic basins (~35 cm width × 35 cm height × 15 cm depth)
500-ml buffer reservoir with outlet at bottom and connecting tube to fit the 18-G cannula (e.g., 500 ml squibb, separatory pear-shaped funnel with stopcock; Cole Parmer No. P-34506-04) or 500 ml aspirator bottle)
Cannula (e.g., 18-G plastic cannula)
Dissecting instruments including: microscissors, scissors, and curved forceps
4-0 surgical silk
15-ml and 40-ml Dounce homogenizers (Kontes Glass) with loose- and tight-fitting pestles (size A and B, respectively)
50-ml thick-walled polycarbonate centrifuge tubes for Beckman J6-B centrifuge
Low-speed centrifuge with swinging bucket-rotor for 50-ml tubes (e.g., Beckman J6-B)
30-ml thick-walled polycarbonate centrifuge tubes for Sorvall SS-34 rotor
High-speed centrifuge (e.g., Sorvall RC-5B) with fixed-angle rotor (e.g., Sorvall SS-34 rotor) for 30-ml tubes
14-ml Ultraclear ultracentrifuge tubes
Ultracentrifuge (e.g., Beckman L8-55) with swinging-bucket rotor (e.g SW 40 or equivalent)
1-ml and 2-ml syringes with blunt-ended 1.8 × 18–mm needles (for underlaying gradients)
Zeiss Refractometer
5-ml syringe with blunt-ended 1.8 × 10–mm needle (for collecting fractions from gradients)
2-ml syringe with 0.6 × 30–mm blunt-ended needle (for resuspending pellets)
Conductivity meter
FFE apparatus (e.g., Elphor VaP 22, Bender and Hobein)

Additional reagents and equipment for determining the protein concentration according to Bradford (*APPENDIX 3B*)

Label hepatocellular endosomes in situ

1. Starve a 150-to 200-g male Sprague-Dawley rat overnight.

   For analytical separation, one 7- to 10-g liver provides sufficient material. For preparative work (see Alternate Protocol), use up to 30 g of liver at a time (three to four rats and scale up volumes proportionally to number of livers used).

2. Anesthetize rats by an intraperitoneal injection of urethane anesthetic solution (1 ml per 100 g body-weight), using an 25-G needle.

3. Shave abdomen of rat.

4. Fasten animal on a plastic tray (~15 cm × 30 cm with legs ~8 cm in height) using adhesive tape and place the tray in a plastic basin (~35 cm × 35 cm × 15 cm) to collect blood and perfusion buffer contaminated with ^{125}I.

   Make sure that the dimensions of the plastic basin are large enough to collect the entire fluid dropping from the plastic tray.

Perfuse the liver

5. Place a buffer reservoir with an outlet at the bottom ~50 cm above the animal. Fix a tube to the outlet that is long enough to allow for connection to a cannula (e.g., an 18-G plastic cannula) to be placed later into the portal vein of the rat (step 9). Fill the reservoir and the tube with ~500 ml ice-cold 0.25 M sucrose in TEA buffer.

6. Open the abdomen widely by a midline incision without injuring liver or diaphragm. Expose the liver by gently moving intestines to the left side of the rat to gain easy access to the liver, the portal vein, and the inferior vena cava.

7. Prepare two ligatures of 4-0 silk in loose knots around the portal vein 1 to 2 cm and 4 to 5 cm, respectively, before the vessel enters the liver. Leave bile duct exterior to the ligatures.

8. Inject endocytic tracers (e.g., 220 µg FITC-ASOR and 1.4×10^6 cpm ^{125}I-pIgA in 500 µl PBS) into the saphenous vein using a 2-ml disposable syringe with a 25-G needle.

   The endocytic tracers FITC-ASOR and [^{125}I]-pIgA can be injected simultaneously in 500 µl PBS or successively (e.g., each tracer in 500 µl PBS) to label the endosome subpopulation of interest. Due to variability of the animals, the injection procedure and the resulting labeling of the individual endosome subpopulation, injection of two or more tracers in the same animal is required.

   If labeling is carried out for more than 5 min, the body temperature of the animal should be stabilized by e.g., covering the body with paper towels and placing the animal under a 60 W light bulb.

9. At the end of the labeling time (1 to 20 min), hold the portal vein with forceps and incise on the ventral surface with a fine pair of scissors between the two prepared ligations. Lift and steady the vein and insert the cannula into the hepatic portal vein. Immediately remove the needle and place the tip of the cannula ~3 mm before the first bifurcation of the portal vein to ensure perfusion of all lobes. Fix the cannula with the ligature proximal to the liver and then also tie the distal ligature.

10. Remove any air bubble at the end of the cannula by adding ice-cold 0.25 M sucrose in TEA buffer from a syringe with a 25-G needle immediately before it is connected to the tube. Then rapidly connect the cannula air bubble-free to the tube and start to perfuse the liver with ice-cold 0.25 M sucrose in TEA buffer by gravity.

    When labeling endosomes, a rapid subsequent perfusion with ice-cold 0.25 M sucrose in TEA buffer is important to halt endocytic processes. In addition, this perfusion will remove any contaminating blood and extracellular marker trapped within the liver. Since binding of ASOR to the ASGPR is calcium dependent, plasma membrane bound FITC-ASOR is also removed using this calcium-free and EDTA-containing TEA buffer.

11. Cut the inferior vena cava soon after connecting the cannula to the tube.

    If this is not done, back-pressure will damage the liver. The perfusion fluid is allowed to drain from the vena caba into the body and then drain into the basin. The fluid remaining in the corpse after removing the liver should be drainedi nto the basin before disposal of the corpse.

12. After perfusion, cut the diaphragm and immediately proceed to prepare endosome-enriched fractions, beginning with step 13.

    All subsequent steps are performed at 4°C.

Isolate and mince liver

13. After in vivo labeling of hepatocyte endosomes and perfusion of the liver with ~500 ml ice-cold 0.25 M sucrose in TEA buffer, carefully excise the liver and cut away connective tissue.

14. Determine the liver weight and place the liver in a prechilled beaker containing 4 vol (4.0 ml/g liver) of 0.25 M sucrose in TEA buffer (i.e., 28 ml) with protease inhibitors on ice.

For the case of 150- to 200-g male Sprague-Dawley rats, the range of liver weight is 7 to 10 g. The conditions described below are based on a liver weight of 7 g.

15. Use scissors to mince the liver into ~0.5-cm^3 pieces. Pour the mixture into a 40-ml Dounce homogenizer.

    If the liver weighs more than 10 g, divide it into two equal portions and homogenize separately.

Homogenize the liver

16. Homogenize the liver with eight up-and-down strokes with loose-fitting pestle (A), followed by three up-and-down strokes with a tight-fitting pestle (B). Measure total volume of the homogenate (H) and remove an aliquot (~100 µl) for determination of radioactivity, fluorescence, protein, and enzymes.

    To avoid producing bubbles during homogenization, move the pestle slowly and steadily. Wipe off any connective tissue that sticks to the pestle.

Prepare microsomes

17. Pour homogenate into 50-ml thick-walled polycarbonate tubes (1 to 2 tubes/liver). Centrifuge 10 min at 3800 × *g*, 4°C, in a low-speed centrifuge to pellet nuclei and unbroken cells. Remove supernatant (S1) and discard pellet (P1).

18. Transfer supernatant (S1) into a another 50-ml thick-walled polycarbonate tube, and centrifuge for 10 min at 4300 × *g*, 4°C, in a low-speed centrifuge. Remove supernatant (S2) and discard pellet (P2).

    These two centrifugation steps remove nuclei and unbroken cells. Furthermore, the resulting supernatant (S2) is depleted in marker for mitochondria and plasma membranes.

19. Measure volume of supernatant S2 and remove an aliquot (~100 µl) for analysis. Continue endosome preparation either at step 20a or step 20b.

20a. *To prepare "microsomes":* Transfer S2 to a 30-ml thick walled polycarbonate tube (one tube per 10 g liver, maximum) and centrifuge 10 min at 30,000 × *g*, 4°C, in a high-speed centrifuge to pellet lysosomes and mitochondria. Discard the pellet and transfer the supernatant (S3) to 14-ml ultraclear ultracentrifuge tubes. Proceed directly to step 21.

    This additional centrifugation step results in a higher enrichment of endosomal markers in the microsomal pellet (P4; see step 24) and consequently in endosome-enriched fractions due to removal of the majority of lysosomes and mitochondria. As a further consequence, the amount of trypsin required to achieve separation of endosomes by free-flow electrophoresis can be reduced from 3% to 0.3% (see steps 39b and 40b). This is of major importance when the acidification properties of the isolated endosomes are analyzed (see Support Protocol 3).

20b. *To prepare "total microsomes":* Transfer S2 to 14-ml Ultraclear ultracentrifuge tubes and proceed directly to step 21.

    If this alternative is chosen, centrifugation of S2 at 30,000 × g is omitted. For quantitative analysis of endosome subpopulations total microsomes are used.

21. Place a 1.3-ml cushion of 2.5 M sucrose in TEA buffer at the bottom of each of the tubes (containing either S2 or S3) using a 2-ml syringe equipped with a 1.8 × 18–mm needle with a blunted end.

    The high-density cushion prevents the breakage of organelles, and therefore the loss of endocytic markers or vesicular content, by preventing the organelles from being pelleted against the walls of the tube. In addition, gentle resuspension of the pellet is facilitated.

22. To prepare total microsomes (i.e., from S2, where step 20a has not been performed) or microsomes (i.e., from S3, where step 20a has been performed), respectively, centrifuge either supernatant S2 or S3 for 50 min at 285,000 × *g* (40,000 rpm in an SW 40 rotor), 4°C.

23. Discard supernatant (S4).

24. Transfer the pellet (P4) and the cushion to a 15-ml Dounce homogenizer. Rinse tube with 2 to 3 ml of 0.25 M sucrose in TEA buffer.

25. Resuspend the membranous material by using 10 up-and-down strokes with a tight-fitting pestle (B). Monitoring with a Zeiss refractometer, adjust the sucrose concentration with 2.5 M sucrose (in TEA buffer) to 1.25 M (refractive index = 1.3934) and to a final volume of 1.3 to 1.5 ml per gram liver.

If assaying all fractions for yield and enrichment, save them on ice and use on the same day.

Prepare endosome-enriched fractions

26. Transfer 5 ml of resuspended total microsomes (or microsomes) to a 14-ml ultraclear ultracentrifuge tube (for 10 g liver, three tubes are required). Overlay with 2.3 ml of 1.15 M sucrose in TEA buffer, then overlay that with 2.3 ml of 1.0 M sucrose in TEA buffer. Finally, fill up tube to 3 mm below the top with 0.25 M sucrose in TEA buffer.

Figure 3.11.1 contains a diagram of the preparation of the sucrose gradient.

27. Centrifuge gradients for 110 min at 285,000 × *g*, 4°C.

28. Using a 5-ml syringe with a blunt-ended 1.8 × 10–mm needle, collect and pool the endosome-enriched fractions at the 0.25/1.0 M sucrose interface (interface I) and the fractions at the 1.0/1.15 M interface (interface II), from each tube. Transfer the pooled interface I fractions and the pooled interface II fractions into separate 14-ml Ultraclear centrifuge tubes.

For a 7-g liver, one tube for the pooled interface I fraction and another tube for the pooled interface II fraction are sufficient.

29. Determine refractive index of the pooled interface I fraction and of the pooled interface II fraction and add TEA buffer to bring the sucrose concentration to 0.25 M in each fraction.

30. Fill up tubes to ~14 ml with 0.25 M sucrose in TEA buffer and underlay each tube with 100 µl 2.5 M sucrose in TEA buffer using a 1-ml syringe with a 1.8 × 18–mm needle with a blunted end.

Concentrate endosome-enriched fractions by centrifugation

31. Ultracentrifuge tubes for 50 min at 285,000 × *g*, 4°C, to concentrate material in the sucrose cushion.

32. Carefully remove the supernatants, add 500 µl of 0.25 M sucrose in TEA buffer, and resupend the pellets and the sucrose cushion using a 2-ml syringe with a 0.6 × 30–mm needle.

33. Determine the sucrose concentration of the resuspended material using a refractometer and adjust the sucrose concentration with TEA buffer to 0.25 M (refractive index = 1.3453).

Adjust protein concentrations of enriched fractions

34. Determine the protein concentration of each fraction using the Bradford method (*APPENDIX 3B*).

    From a 7 g liver, one may expect to obtain ~2.5 mg protein in the final interface I fraction and 7 mg in the final interface II fraction. To quantitate yield and enrichment, remove aliquots of all supernatants and pellets (~100 µl to 500 µl) and also of the fractions applied to the free-flow electrophoresis apparatus (designated interface 1 and interface II). Furthermore, determine and record the volumes of the respective fractions. Either keep fractions at 4°C and assay immediately or store at −20°C.

35. Adjust the protein concentration to 1 mg/ml by adding 0.25 M sucrose in TEA buffer.

    High protein concentrations (>1 mg/ml) result in reduced separation of endosomes from other subcellular organelles by FFE. Low protein concentrations (<1 mg/ml) complicate detection of markers in the FFE fractions.

Set up instrument

36. Make sure that the FFE instrument is assembled correctly, and the conductivities of the chamber and electrode buffers are correct. Fill chamber with FFE chamber buffer without inclusion of air bubbles, and add FFE electrode buffer to the electrodes.

    Degass chamber buffer using a vacuum pump immediately before use. Fill the chamber slowly to prevent generation of air bubbles during filling. Carefully read the manufacturer's manual on how to avoid air bubbles during filling of the chamber.

37. Before turning on the high voltage (and injecting sample), make sure that all 96 collecting tubes are not clogged.

    For the separation profile, a continuous dropping of buffer from all collecting tubes is of great importance. Due to the presence of sucrose in the chamber buffer, the tubes tend to clog as a result of limited washing after the run.

38. Follow the manufacturer's instructions for starting the instrument. Set the following parameters and maintain constantly:

    Chamber buffer flow: 2.0 to 2.5 ml/hr/fraction
    Chamber temperature: 6°C
    Voltage: ~1400 to 1550 V
    Current: ~120 to 135 mA.

    Values indicated have been established for the Elphor VaP 22 (Bender and Hobein) FFE apparatus, and might need to be retested in other systems.

    Stabilization of voltage and current requires that the FFE instrument be run with the settings listed above for 1 to 2 hr before sample injection. This should be considered in the time schedule. For the stability of the field, the conductivities of electrode buffer (~7.2 mS) and chamber buffer (~720 µS) are of great importance and need to be verified.

Treat endosome-enriched fractions with trypsin

For analysis of "total microsomes" (i.e., where step 20a has not been performed)

39a. Immediately before injection of samples into the FFE apparatus, treat interfaces I and II obtained from total microsomes with 3% trypsin (0.03 mg trypsin/mg protein; added from 1 mg/ml trypsin solution) for 5 min at 37°C.

    The amount of trypsin is "standardized as percent trypsin/mg protein; 0.03 mg trypsin/mg protein is therefore equal to 3% trypsin.

40a. Stop the reaction by adding 6% soybean trypsin inhibitor (0.06 mg soybean trypsin inhibitor/mg protein; added from 2 mg/ml soybean trypsin inhibitor solution), and cooling immediately to 4°C.

For analysis of "microsomes" (i.e where step 20a has been performed)

39b. Immediately before injection of samples into the FFE apparatus, treat interfaces I and II derived from microsomes with 0.3% trypsin (0.003 mg trypsin/mg protein) for 5 min at 37°C.

40b. Stop the reaction by adding 0.6% soybean trypsin inhibitor (0.006 mg soybean trypsin inhibitor/mg protein; added from 2 mg/ml soybean trypsin inhibitor solution), and cooling immediately to 4°C.

Subject samples to separation by FFE

41. Inject trypsin-treated sample into the FFE apparatus at a rate of 1 to 1.5 ml/hr and 4°C.

Avoid introducing an air bubble into the chamber. Wash sample inlet with FFE chamber buffer after injection of sample.

Turn off the room light and try to visualize the sample jet in the FFE- chamber using a flashlight. Splitting of the jet into two unequal beams will give a first indication for separation of endosomes (thin beam, deflected more towards the anode) from the majority of other organelles (thick beam, majority of protein). By watching the sample in the chamber one knows when the fraction collection has to be started and stopped, to avoid unnecessary dilution of the fractions.

42. Begin collecting the fractions when the sample reaches the top of the separation chamber. Collect FFE fractions on ice during the entire run.

Keep an eye on the volume in the tubes collecting the 96 fractions. Try to avoid clogged tubes. Usually, ~2 to 3 ml/fraction are obtained from the FFE separation of interface I or II from one rat liver.

Analyze samples

43. At the end of the run, immediately analyze the collected fractions for protein concentration (e.g., Bradford assay; *APPENDIX 3B*), endosomal marker, and marker enzymes (see Support Protocol 1).

Turn off the FFE instrument by exactly following the manufacturer's instructions. Wash extensively with water to prevent deposition of sucrose or salt in the apparatus.

PREPARATIVE FFE

ALTERNATE PROTOCOL

Usually, one rat liver yields enough material to analyze FFE fractions for marker enzymes, for kinetic analysis of transport through endosomal compartments, or for functional studies such as in vitro acidification of endosomes. However, to characterize the composition of endosomes and identify endosomal proteins or lipids—by, e.g., SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*) or by immunoprecipitation (*UNIT 7.2*), larger quantities of purified fractions are required than what are obtained from one rat liver. To avoid the labeling of endosomes in all livers subjected to fractionation, endosomes from one liver are labeled with the appropriate marker (see the Basic Protocol), and two to four livers that have been flushed with buffer by in situ perfusion without prior endosome labeling are processed in parallel.

For materials, see Basic Protocol.

Table 3.11.1 Suggested Dilutions and Volumes Used for Protein, Endocytic Marker, and Marker Enzyme Determination

Fraction[a]	Dilution/volume used			
	Protein (Bradford)	[^{125}I]IgA	FITC-ASOR	Enzymes
H	1:20/5 µl	No dilution/50 µl	-	1:20/10 to 100 µl
S2	1:10/5 µl	No dilution/50 µl	-	1:10/10 to 100 µl
P4	1:10/5 µl	No dilution/5 µl	No dilution/5 µl	1:10/10 to 100 µl
Interface I	No dilution/5 µl	No dilution/10 µl	No dilution/5 µl	No dilution - 1:5/10 to 60 µl
Interface II	No dilution/5 µl	No dilution/10 µl	No dilution/5 µl	No dilution - 1:5/10 to 60 µl
FFE fractions	No dilution/300 µl	No dilution/500 to 1000 µl	No dilution/500 µl	No dilution/60 to 200 µl

[a]See Basic Protocol and Figure 3.11.1 for explanations of the terms used for the various fractions. Abbreviations: H, homogenate; P4, pellet 4; S2, supernatant 2.

1. Label endosomes of one rat liver and halt endocytosis by ice-cold perfusion with 0.25 M sucrose in TEA buffer (see Basic Protocol, steps 1 to 12).
2. Flush one to four livers by in situ perfusion with ice-cold 0.25 M sucrose in TEA buffer (see the Basic Protocol, steps 1 to 12, but omit pulsing with endocytic tracers at step 8)
3. Prepare endosome-enriched fractions from labeled and unlabeled livers, respectively (see Basic Protocol, steps 13 to 35). Prepare the FFE apparatus (see Basic Protocol, steps 36 to 38).
4. Pool interfaces derived from unlabeled livers.
5. Trypsinize interface I or II that contain labeled endosomes (see Basic Protocol, steps 39 to 40).
6. Subject the interfaces containing labeled endosomes (e.g., FITC-ASOR and [^{125}I]pIgA) to FFE separation, and collect the 96 fractions (see Basic Protocol, steps 41 to 42).
7. Trypsinize pooled interface I or II from unlabeled livers (see Basic Protocol, steps 39 to 40).
8. Immediately after the FFE separation of the labeled samples (step 6 of this Alternate Protocol), inject the pooled fractions of the unlabeled samples (step 7 of this Alternate Protocol) and subject to FFE. Collect the 96 fractions.

   According to the amount of livers used, the duration of separation as well as the fraction volumes will be increased.
9. During FFE of unlabeled samples, analyze labeled fractions for protein (*APPENDIX 3B*) and endosome marker distribution (see Support Protocol 1).
10. Analyze FFE fractions of unlabeled samples for the distribution of proteins (see *APPENDIX 3B* and Support Protocol 1).

    The localization of the major protein peak and the endosome marker proteins should be identical in FFE-separations carried out under the same condition.

    Fractions containing endosomes derived from the unlabeled livers can be identified by comparison to the separation profile of labeled fractions.
11. Pool fractions of interest and continue with analysis (see, e.g., Support Protocol 2).

DETERMINATION OF PROTEIN, ENDOSOMAL MARKER, AND MARKER ENZYMES

SUPPORT PROTOCOL 1

The amount and dilution used for analysis of individual fractions is listed in Table 3.11.1. Protein concentration is determined using the Bradford assay (*APPENDIX 3B*). The enrichment of and purity of [^{125}I]pIgA-labeled endosomes is determined by γ counting of individual fractions. Since the liver homogenate and most fractions obtained from it are colored and have a high level of autofluorescence, an accurate determination of the FITC-ASOR concentration and the enrichment of FITC-ASOR labeled endosomes is difficult and requires the parallel analysis of rat liver fractionation where endosome labeling was omitted. Nevertheless, the distribution of FITC-ASOR in interface I and II and in free-flow fractions can be carried out following this protocol. Furthermore, ATP-dependent acidification can be used to determine the enrichment of FITC-ASOR labeled compartments in free-flow fractions in comparison to P3 or P4 (see Support Protocol 3). In order to determine the enrichment of ASOR-labeled endosomes with respect to the initial homogenate it is preferable to use ^{125}I-labeled ASOR.

The following organelle-specific markers can be used to determine the degree of contamination of the endosomal fraction after free-flow electrophoresis. For the apical and basolateral plasma membrane, alkaline phosphodiesterase I (APDE I), which is localized at both plasma membrane domains, is determined (see Support Protocol 1, *UNIT 3.2*). NADPH cytochrome *c* reductase is an oxidoreductase present in the endoplasmic reticulum and is determined according to the Support Protocol in *UNIT 3.5*. As a marker enzyme for lysosomes, β-*N*-acetylglucosaminidase can be assayed using the less sensitive *p*-nitrophenyl derivative as substrate instead of 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (see Support Protocol 2 in *UNIT 3.6*; also see *UNIT 3.4*). It has to be pointed out that lysosomal enzymes are also found in endosomes en route to lysosomes. Furthermore, lysosomal enzymes are also found in the biosynthetic pathway (e.g., Golgi) where they can be partially active. As the *cis, medial,* and *trans* Golgi are involved in glycoprotein and proteoglycan modification carrying out distinct modifications at distinct sites in the Golgi stack, the respective enzymes involved in these modifications exhibit a site-specific localization. Most commonly, UDP-galactose galactosyltransferase, a marker of the *trans*-Golgi, is used to determine contamination by Golgi membranes (see Support Protocol in *UNIT 3.9*). Since most of the enzymes are facing the interior of the organelles and many substrates for the enzymes are membrane impermeable, it is important to include detergents in the reaction mixture. For pratical considerations of the assay procedure see *UNIT 3.4-3.9*.

Materials

FFE fractions enriched in endosomes or other organelles (see Basic Protocol or Alternate Protocol)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4
PBS, pH 7.4 (*APPENDIX 2A*) containing 0.2% (v/v) Triton X-100
0.25 M sucrose in TEA buffer (see recipe)

Disposable plastic cuvettes for spectrofluorometer
Spectrofluorometer (e.g., Jasco FP 777)
2.0 ml scintillation vials
γ-counter

Determine FITC-ASOR fluorescence

1. When required (Table 3.11.1), dilute fractions with PBS, pH 7.4.
2. Use 300 µl to 500 µl of each free-flow fraction and add PBS containing 0.2% (v/v) Triton X-100 up to 2 ml.

3. Use 300 µl to 500 µl of 0.25 M sucrose in TEA buffer as blank.
4. Determine fluorescence of the fractions using a spectrofluorimeter at 485 nm excitation (excitation slit, 10 nm) and at 515 nm emission wavelength (emission slit, 10 nm).
5. Subtract blank.

Calculate and present data

6. To determine the distribution of marker enzymes and endosome subpopulations (radioactivity of [^{125}I]pIgA and FITC-ASOR fluorescence) in free-flow fractions, set the total amount of the respective marker recovered in all free-flow fractions (in arbitary units) to 100% and express the activity in individual fractions, counted in 2-ml scintillation vials in a γ-counter in percent of the total amount.
7. Plot the percent of marker in individual fractions versus fraction number.

SUPPORT PROTOCOL 2

CHARACTERIZATION OF PROTEINS IN ENDOSOME SUBPOPULATIONS

The distribution of proteins of interest associated with endosome subpopulations (or other organelles) separated by FFE can be determined by processing the FFE fractions obtained for SDS-PAGE (*UNIT 6.1*) and immunoblotting with specific antibodies (*UNIT 6.2*). FFE fractions are pooled and concentrated, and proteins are dissolved in SDS sample buffer. In order to detect a protein of interest, the effect of trypsin on its antigenicity needs to be tested, as the cytoplasmic portions of proteins can be cleaved by trypsin during preparation for FFE.

Materials

1 mg/ml trypsin
2 mg/ml soybean trypsin inhibitor
FFE fractions enriched in endosomes or other organelles (Interfaces I and II; see Basic Protocol or Alternate Protocol)
0.25 M sucrose in TEA buffer, ice cold (see recipe)
1× SDS sample buffer (*UNIT 6.1*)
Ultracentrifuge with swinging-bucket rotor (e.g., Beckman SW 40 or equivalent) and 14-ml Ultraclear tubes
Additional reagents and equipment for protein determination (*APPENDIX 3B*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

Determine effect of trypsin on the integrity of proteins by immunoblotting

The incubation of endosome-enriched fractions with trypsin is an absolute requirement for separation of endosomes from other organelles; however, when dealing with a membrane-associated or membrane-spanning protein, the trypsin incubation and the resulting degradation of the protein of interest might result in a loss of antigenicity. Test endosome-enriched fractions (i.e., interfaces I, II) for the effect of trypsin on the protein of interest before subjecting them to FFE separation. When starting the protocol, the conditions for SDS-PAGE and immunoblotting required to detect the protein of interest with the antibody should be known.

1. Prepare endosome-enriched fractions (interfaces I, II; see Basic Protocol, step 29, including step 20a).
2. Determine protein concentration in the interfaces (e.g., Bradford assay, *APPENDIX 3B*).

3. If necessary, adjust protein concentrations of interfaces as described (see Basic Protocol, steps 34 to 35).

4. Digest, e.g., 100 µg protein from interfaces with increasing concentrations of trypsin (e.g., 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%; see Basic Protocol, step 39) from 1 mg/ml trypsin stock.

   The amount of trypsin is "standardized as % trypsin/mg protein; 0.003 mg trypsin/mg protein is therefore equal to 0.1% trypsin.

5. Stop incubation as described in Basic Protocol, step 40 by addition of 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% soybean trypsin inhibitor from 2 mg/ml.

   The results obtained from immunoblotting the samples treated with various concentrations of trypsin should indicate whether trypsin treatment does or does not interfere with detection of the protein of interest by immunoblotting.

Process FFE fractions for SDS-PAGE

6. Determine the protein concentration of pooled FFE fractions of interest (e.g., Bradford assay, *APPENDIX 3B*) and measure the total volume. Calculate total amount of protein in the pooled fractions.

7. Load 14-ml Ultraclear ultracentrifuge tubes with the pooled fractions. Fill up tubes with 0.25 M sucrose in TEA buffer.

8. Ultracentrifuge 50 min at 285,000 × *g* (e.g., 40,000 rpm in Beckman SW 40 rotor), 4°C, to concentrate material.

9. Carefully remove the supernatants and add a small volume (e.g., 50 µl) of 1× SDS sample buffer to the pellet.

10. Perform SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*).

IN VITRO ACIDIFICATION OF ISOLATED ENDOSOMES

SUPPORT PROTOCOL 3

Endosomes purified by free-flow electrophoresis maintain their functional integrity. In vivo, endosomes are acidified by a proton ATPase (so called vacuolar or V-ATPase) resulting in the generation of a pH gradient across the endosomal membrane (Mellman et al., 1986). Due to passive proton efflux, this pH gradient can only be sustained by constant proton influx energized by the proton ATPase. This functional property of endosomes is also found in vitro. Thus, a cell-free acidification assay can be applied to characterize proton transport and ion permeabilities of the isolated endosomes, taking advantage of the pH-dependent fluorescence of FITC-labeled ligands (such as FITC-ASOR), whereby lowering the pH from 7.4 to 5.0 results in a pH-dependent quenching of the fluorescence intensity of FITC. In the absence of permeable ions (such as in 0.25 M sucrose in TEA buffer) the in vivo acidic pH of the endosomes is not dissipated during the isolation procedure and purified endosomes are therefore still acidic. Consequently, this pH gradient has to be dissipated by incubation with permeable cations and anions (e.g., in KCl-containing buffers). Subsequently, ATP is added, and a rapid decrease in the initial fluorescence is indicative of ATP-dependent intravesicular acidification.

Due to trypsinization of the interface I fraction (with 0.03 mg trypsin/mg interface I protein) prior to free-flow electrophoresis, the proton permeability of the endosomes is increased, and, as a consequence, ATP-dependent acidification is decreased. Thus, for acidification experiments, endosome-enriched fractions are prepared that have been depleted in mitochondria and lysosomes (by centrifugation of S2 at 30,000 × *g*; see Fig. 3.11.1). For such endosome fractions (interface I) the amount of trypsin required can be

reduced to 0.003 mg trypsin/mg protein to achieve separation by free-flow electrophoresis. 0.003 mg trypsin/mg protein does not affect ion permeabilities and acidification properties of free-flow purified endosomes (Fuchs et al., 1989).

Materials

FFE fractions from microsomes (Interface I, see Basic Protocol, step 20a) containing FITC-ASOR labeled endosomes (see Basic Protocol), treated with reduced quantity of trypsin (see Basic Protocol, steps 39b and 40b)
Acidification buffer (see recipe)
pH calibration buffers (see recipe)
ATP stock (see recipe)
500 µM nigericin stock (see recipe)
1 mg/ml rabbit anti-fluorescein antibody: IgG(H+L)-fraction (Molecular Probes)

Spectrofluorometer (e.g., Jasco FP 777)
Disposable plastic cuvettes for spectrofluorometer (e.g., Sarstedt)

Generate pH calibration curve of internalized FITC-ASOR

1. Add 0.2 to 10 µl interface I endosome fraction (0.2 to 10 µg protein) to 2 ml of pH calibration buffer (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.4) in disposable plastic cuvettes.

   FITC-ASOR labeled endosomes are mainly enriched at interface I, regardless of whether endosomes were labeled by injection of markers for 1 min or 10 min.

2. Add 1 µM nigericin (from 500 µM stock) and 1 µl anti-fluorescein antibody.

   The K^+/H^+ ionophore nigericin results in pH equilibration between the pH calibration buffer and the interior of the vesicle. Any FITC-ASOR that leaks out during incubation is quenched by the anti-fluorescein antibody and therefore does not contribute to the fluorescence measurements.

3. Incubate for 2 to 3 hr at room temperature in the dark to equilibrate extra- and intravesicular pH.

4. Transfer cuvettes into spectrofluorometer and determine the fluorescence intensities at 485 nm excitation (excitation slit, 10 nm) and at 515 nm emission wavelength (emission slit, 10 nm).

5. Generate a pH calibration curve by setting fluorescence intensitiy at pH 7.4 to 100% and plotting pH versus fluorescence intensities (in percent), to obtain a calibration curve of internalized FITC-ASOR.

Dissipate initial acidic endosomal pH

6. Add endosome fraction (0.1 to 10 µl of interface I; 100 to 500 µl of free-flow fraction) to 2 ml acidification buffer in a plastic cuvette.

7. Add 1 µl anti-fluorescein antibody.

8. Place cuvette into spectrofluorometer and continuously record the fluorescence until it reaches a steady-state level.

   Dissipation of the initial pH to neutrality requires 30 min to 1 hr.

Acidify endosomes

9. Add 10 µl ATP stock (final concentration 2.5 mM) to the cuvette and mix rapidly by carefully pipetting up and down.

10. Record fluorescence decrease until steady-state level is obtained.

Usually, ATP-dependent acidification results in a fluorescence decrease at least to the initial fluorescence prior to pH dissipation. Steady-state low pH is obtained after ~15 min. If a fluorescence increase is then observed, the amount of ATP added was insufficient and must be increased. Due to other ATPases present in the fraction, the ATP concentration decreases, and this may result in insufficient levels to provide the driving force for the V-ATPase.

11. Add 4 µl of 500 µl nigericin stock (final concentration, 1 µM), mix rapidly by carefully pipetting up and down, and record the rapid fluorescence increase.

Nigericin leads to an immediate dissipation of the intravesicular pH. The fluorescence value upon nigericin addition should be identical to the value prior to ATP addition (i.e., neutral pH inside the endosomes).

Caclulate initial endosomal pH and the pH obtained after ATP addition

12. Express initial fluorescence in percent of the value obtained after dissipation of the pH gradient (i.e., after the intravesicular pH has reached 7.4).

13. Express ATP-dependent fluorescence-decrease in percent of the value prior to ATP addition (i.e., at intravesicular pH of 7.4).

14. Use pH calibration curve to calculate the initial (acidic) endosomal pH and the pH obtained after ATP-dependent acidification.

The pH obtained after in vitro acidification amounts to 6.5 to 6.0 for early endosomes and to 5.0 to 5.5 for late FITC-ASOR labeled endosomes, and is comparable to the initial pH. Peak fractions from free-flow fractions have the highest capacity for ATP-dependent acidification/µg protein, demonstrating a high level of enrichment of these fractions.

PREPARATION OF ASIALOOROSOMUCOID (ASOR)

SUPPORT PROTOCOL 4

The unique and high expression of a galactose-specific receptor, i.e., asialoglycoprotein receptor (ASGPR) at the sinusoidal (basolateral) plasma membrane of rat hepatocytes leads to uptake and removal of desialylated glycoproteins from the circulation (Wall et al., 1980). Many desialylated proteins with terminal galactose residues bind to this receptor. High binding affinities are exhibited by proteins such as ASOR that expose at least three terminal galactose residues. ASOR can be obtained by enzymatic desialylation of α1-acid glycoprotein (orosomucoid; Wall et al., 1980), as described in this protocol. Subsequent conjugation of ASOR to fluorescein isothiocyanate (FITC) is described in Support Protocol 5.

Materials

Insoluble neuraminidase (type X-A, attached to beaded agarose; Sigma-Aldrich)
0.1 M sodium acetate buffer, pH 5.5 (*APPENDIX 2A*)
2% (w/v) sodium azide in H_2O, prepare before use
Human α1-acid glycoprotein (Sigma-Aldrich)
0.1 M sodium borate buffer, pH 9.3

Sephadex G-25 M column (prepacked; Pharmacia Biotech PD-10; bed volume 9.1 ml)
Low-speed centrifuge with swinging bucket rotor for 50-ml tubes (e.g., Beckman JA-20 rotor or equivalent)
50-ml conical centrifuge tubes

Prepare neuraminidase

1. Resuspend 0.25 U insoluble neuraminidase in 1.5 ml of 0.1 M sodium acetate buffer, pH 5.5, in a 2.0-ml microcentrifuge tube at room temperature.

2. Microcentrifuge for 10 min at maximum speed, (16,000 × *g*) room temperature.
3. Remove and discard supernatant.
4. Resuspend the pellet of insoluble neuraminidase in 1.5 ml of 0.1 M sodium acetate buffer, pH 5.5.
5. Pellet insoluble neuraminidase by centrifugation as in step 2.
6. Repeat steps 4 and 5 once more.

Desialylate α1-acid glycoprotein with insoluble neuraminidase

7. Dissolve 100 mg α1-acid glycoprotein in 2 ml of 0.1 M sodium acetate buffer, pH 5.5, containing 0.02% (w/v) sodium azide (added from a 2% w/v stock solution).
8. Add this α1-acid glycoprotein solution to the pellet of neuraminidase and incubate 48 hr under rotation at 37°C.
9. Pellet neuraminidase by microcentrifuging 10 min at maximum speed, at room temperature.
10. Remove supernatant containing ASOR and free sialic acid in 0.1 M sodium acetate buffer, pH 5.5 for subsequent spin desalting. Use this solution in 1-ml aliquots for spin desalting (steps 11 – 15).

Spin desalt ASOR

11. Equilibrate prepacked Sephadex G-25 M column with two bed volumes (20 ml) of 0.1 M sodium borate buffer, pH 9.3.

    One column is needed to exchange the buffer and remove free sialic acid from 1 ml of ASOR solution.

12. Centrifuge column for 5 min at 2500 × *g* (in JA-20 rotor) at room temperature to remove excess liquid.

    Centrifugation can be done by cutting a hole in the screw cap of a 50-ml conical plastic tube to fix the column at the top of the tube. Excess liquid is then collected at the bottom of the tube and discarded. It is important to allow liquid to be removed by centrifugation without back-flow into the column, otherwise the protein solution subsequently applied cannot be quantitatively removed by the centrifugation step below.

13. Insert the column into a new 50 ml conical plastic tube and place a 2.0-ml microcentrifuge tube under the outlet of the Sephadex G 25 column at the bottom of the 50-ml centrifuge tube.
14. Apply 1 ml of ASOR solution (in sodium acetate buffer, pH 5.5; see step 10) to the column.
15. Centrifuge column for 5 min at 2500 × *g*, at room temperature, to collect the ASOR in 1 ml 0.1 M sodium borate buffer, 9.3 in the 2.0-ml microcentrifuge tube. Cool ASOR solution to 4°C.

    Since excess liquid has been removed from the column by centrifugation (step 12) the 1 ml ASOR in 0.1 M sodium acetate buffer, pH 5.5, applied should be recovered as ASOR in 1 ml of 0.1 M sodium borate buffer, pH 9.3.

16. Use an aliquot (20 mg) of this solution for FITC-labeling (see Support Protocol 5) and store remaining aliquots (20 mg) at −20°C.

COUPLING OF FLUORESCEIN ISOTHIOCYANATE (FITC) TO ASOR

To follow endocytosis and to determine the endosomal pH, FITC conjugates of various ligands or fluid-phase markers have been used (van Renswoude et al., 1982). The fluorescence intensity of FITC is pH dependent and the fluorophore can be easily conjugated to the protein of interest. Specific receptor binding of FITC-ligands has to be verified by competing internalization using a 10- to 100-fold excess of unconjugated ligand.

Materials

20 mg ASOR (see Support Protocol 4) in 1 ml of 0.1 M sodium borate buffer, pH 9.3
40 mg FITC on celite (Isomer I on celite; ~10% FITC; Sigma-Aldrich): use 2 mg FITC on celite/mg protein for coupling
TBS, pH 7.38 (see recipe)
PBS, pH 7.38 (*APPENDIX 2A*)

Thermomixer for microcentrifuge tubes (e.g., Eppendorf thermomixer)
Dialysis tubing (Spectra/Por 2; 0.32 ml/cm; MWCO, 12,000 to 14,000 Da)
Sephadex G-25 M column (prepacked; Pharmacia Biotech PD-10; bed volume 9.1 ml)
Spectrofluorometer (e.g., Jasco FP 777)
Microcuvettes for spectrofluorometer

Additional reagents and equipment for dialysis (*APPENDIX 3C*)

Label ASOR with FITC

1. Add 40 mg FITC on celite to 20 mg ASOR in 1 ml 0.1 M sodium borate buffer, pH 9.3, at 4°C, in a 1.5-ml microcentrifuge tube.
2. Incubate under constant mixing at maximum mixing frequency in an Eppendorf Thermomixer in the dark for 40 min at 4°C.
3. Pellet celite by microcentrifuging 10 min at maximum speed, (16,000 × *g*), 4°C.
4. Remove supernatant containing FITC-ASOR and free unconjugated FITC with a plastic transfer pipet and transfer this solution into a prewetted (*APPENDIX 3C*) dialysis tubing.

   Avoid aspiration of any celite, which is not removed during dialysis and which would otherwise interfere with the endocytosis of FITC-ASOR.

5. Dialyze supernatant (*APPENDIX 3C*) against eight 2-hr changes of 1 liter TBS at 4°C in the dark.
6. Finally, dialyze against 1 liter PBS at 4°C in the dark.
7. Remove FITC-ASOR (~1 ml of 40 mg/ml in PBS) from the dialysis tubing, determine the coupling ratio (steps 8 to 10), and store aliquots (e.g., 100 µg) at −20°C.

Determine the FITC/ASOR ratio (coupling ratio)

8. Determine the protein concentration of the conjugate using the Bradford method (*APPENDIX 3B*).
9. Dilute the FITC-ASOR solution (~1:40) with PBS, pH 7.38, and measure the absorbance of the conjugate at 496 nm (maximal FITC absorbance, A_{496}).

   It is important to adjust the pH of the PBS to exactly 7.38, as the fluorescence of FITC is strongly pH dependent.

10. Calculate the amount (μg) FITC bound to ASOR (mg) using the following equations (coupling ratio; Jobbágy and Király, 1966):

    μg FITC/mg ASOR = cFITC/cASOR

    cFITC (FITC content of the conjugate; μg/ml) = $6.09 \times A_{496} \times$ dilution

    cASOR = concentration of ASOR in the conjugate (mg/ml)

    Following this protocol, the authors found that ~18 μg FITC bound to 1 mg ASOR; this corresponds to a molar ratio of FITC:ASOR of 2:1. The FITC-to-ASOR ratio should not exceed 8 mol FITC per mol ASOR to ensure specific binding to the ASGP receptor.

Analyze the purity of the FITC-ASOR conjugate by chromatography on Sephadex G-25

11. Equilibrate Sephadex G-25 column with two bed volumes (20 ml) of PBS.
12. Apply 100 μl of diluted (1:100 in PBS) FITC-ASOR solution to the column.
13. Elute column with PBS and collect 2-ml fractions of the eluate.
14. Determine fluorescence of individual fractions in a spectrofluorometer at excitation and emission wavelengths of 485 nm and 515 nm, respectively (slit, 10 nm).

    The FITC-ASOR conjugate elutes at fraction 2, whereas the peak of free FITC, if present, is found in fractions 10 to 11. In the case of free FITC, the conjugate can be further purified by chromatography on Sephadex G-25 (follow steps 11 to 14 of this protocol using undiluted FITC-ASOR as starting material).

    Specific binding of FITC-ASOR can be demonstrated by competing binding and internalization by a 10-fold excess of unlabeled ASOR; under this condition no fluorescence signal in isolated endosomes is obtained (Fuchs et al., 1989).

SUPPORT PROTOCOL 6

PREPARATION OF ^{125}I-LABELED HUMAN POLYMERIC IMMUNOGLOBULIN A ([^{125}I]pIgA)

The pIgA-receptor (pIgR) on rat hepatocytes efficiently transports pIgA from the circulation into bile via vesicular transcellular transport. To label transcytotic endosomes in rat liver, rat, human or mouse pIgA can be used as unmodified or radiolabeled ligand (Hoppe et al., 1985; Giffroy et al., 1998).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

CAUTION: This procedure must be performed in a hood certified for radioiodination. All personnel performing iodinations or using radioactive materials must be properly trained.

Materials

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 0.1% (w/v) bovine serum albumin (BSA)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Iodo-Beads (iodination reagent; Pierce)
Na[^{125}I] (17 mCi/ml; NEN Life Sciences)
Human polymeric immunoglobulin A (pIgA), purified from human myeloma serum (Vaerman and Lemaitre-Coelho, 1979) and kindly provided by Jean-Pierre Vaerman and Pierre J. Courtoy (Catholic University of Louvain, Brussels, Belgium).

TBS (*APPENDIX 2A*)
10% (w/v) trichloroacetic acid (TCA)

Pre-packed anion-exchange cartridges (e.g., Bio-Rad Econo-Pac Q; bed volume 5 ml)
5-ml conical-bottom screw-cap glass reaction vials
2.0-ml scintillation vials
γ counter (e.g., Beckman Instruments)

Prepare the anion exchange column

1. Equilibrate the prepacked anion-exchange column with three bed volumes (15 ml) of PBS/0.1% (w/v) BSA to reduce nonspecific protein absorption.
2. Remove excess BSA with 1 bed volume (5 ml) PBS.

Radiolabel pIgA

3. Wash one Iodo-Bead with 500 µl PBS and dry bead on filter paper.
4. Put 100 µl PBS containing 1 mCi (2.2×10^9 cpm) Na[^{125}I] in a 5-ml glass reaction vial.
5. Add Iodo-Bead to the vial, cap vial, and incubate in the hood for 10 min at room temperature.
6. Add 50 µg pIgA (in 5 µl PBS) to the vial and incubate for 30 min at room temperature in the hood.

   Alternatively, mouse pIgA can be used. The polymer forms of mouse IgA can be purified from clarified ascites (Sigma-Aldrich) or from the purified myeloma immunoglobulin (Sigma-Aldrich) of a plasmacytoma cell line (TEPC) as described by Hoppe et al., 1985.

7. Stop the iodination reaction by applying the mixture to the anion-exchange column.
8. Rinse the vial with 100 µl PBS and transfer the rinse to the anion-exchange column.
9. Elute iodinated pIgA with 100 µl portions of PBS at room temperature and collect ~100-µl fractions in pre-chilled microcentrifuge tubes on ice.

Analyze labeling

10. Quantify radioactivity by counting 3-µl aliquots of each fraction in a γ-counter.
11. To determine protein-bound radioactivity, place a 5-µl sample of each fraction in a microcentrifuge tube and add 1 ml ice cold 10% (w/v) TCA, mix, and incubate on ice for 30 min.
12. Pellet precipitated proteins by microcentrifuging 10 min at maximum speed, 4°C.
13. Remove and count 500 µl of the supernatant (i.e., TCA-soluble radioactivity) as well as the remaining 500 µl containing soluble and precipitable (^{125}I-labeled pIgA) radioactivity.
14. Determine cpm in a γ counter and calculate free (soluble) and precipitable cpm.

    This procedure should yield pIgA with a specific activity of ~1.5 µCi/µg pIgA (3.3×10^6 cpm/µg pIgA).

15. Use the fractions with at least 95% to 98% TCA-precipitable counts for in vivo endosome labeling. Store ^{125}I-labeled pIgA in 50-µl aliquots for up to 1 month at 4°C in a lead container.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acidification buffer

5.6 g KCl (150 mM final)
3.4 g HEPES (*N*-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; 20 mM final)
616.2 mg $MgSO_4.7H_2O$ (5 mM final)
H_2O to 500 ml (final volume)
Adjust pH with tetramethylammonium hydroxide to pH 7.4
Filter through a qualitative filter paper (e.g., Whatman no. 4)
Store up to 1 month at 4°C

ATP stock

2917 mg adenosine 5′-triphosphate (dipotassium salt; Sigma-Aldrich; 500 mM final)
H_2O to 10 ml (final volume)
Adjust pH with 1 M KOH (prepared freshly) to 7.4
Store aliquots (100 µl) indefinitely at −20°C

FFE chamber buffer

Prepare 5 liters of 1× TEA buffer from 10× stock solution (see recipe). Adjust pH to 7.4. Add 427.9 g sucrose (250 mM final) to a final volume of 5 liters of 1× TEA buffer, stir overnight, filter through a qualitative filter paper (e.g., Whatman no. 4) before use, and determine conductivity (should be 720 µS). Prepare fresh and use at 4°C

With a chamber buffer flow rate of 2.0 to 2.5 ml/h/fraction, this volume is sufficient to run the FFE for at least 20 hr nonstop. The FFE separation of one interface by the Elphor Vap 22 takes ~45 to 60 min.

FFE electrode buffer

10× TEA buffer (see recipe), room temperature
Determine conductivity before use (conductivity: 7.2 mS)
Prepare 5 liters for one FFE run

Nigericin stock, 500 µM

5 mg nigericin, sodium salt (Sigma-Aldrich; 500 µM final)
Ethanol to 13.4 ml
Store 1-ml aliquots up to 1 year at −20°C

pH calibration buffers

Mix solution A (see recipe) with solution B (see recipe) until the desired pH (5.0, 5.5, 6.0, 6.5, 7.0, and 7.4) is obtained. Prepare 100 to 200 ml of each buffer. Store up to 1 month at 4°C

Solution A for pH calibration buffer

11.2 g KCl (150 mM final)
9.8 g MES (2-[*N*-Morpholino]ethanesulfonic acid; 50 mM final)
H_2O to 1000 ml (final volume)
Adjust pH with KOH to 5.0
Filter through a qualitative filter paper (e.g., Whatman no. 4)
Store up to 1 month at 4°C

Solution B for pH calibration buffer

11.2 g KCl (150 mM final)
16.9 g HEPES (*N*-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]); 50 mM final)
H_2O to 1000 ml (final volume)
Adjust pH with KOH to 7.4
Filter through a qualitative filter paper (e.g., Whatman no. 4)
Store up to 1 month at 4°C

Sucrose in TEA buffer, 0.25 M

171.2 g sucrose (250 mM final)
1× TEA buffer (prepare from 10×; see recipe) to 2000 ml (final volume)
Adjust to pH 7.4

To control the molarity of the solution, check the refractive index (refractive index = 1.3453 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 48 hr at 4°C.

Sucrose in TEA buffer, 1.00 M

85.6 g sucrose (1.0 M final)
1× TEA buffer (prepare from 10×; see recipe) to 250 ml (final volume)
Adjust to pH 7.4

To control the molarity of the solution, check the refractive index (refractive index = 1.3816 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 48 hr at 4°C.

Sucrose in TEA buffer, 1.15 M

98.4 g sucrose (1.15 M final)
1× TEA buffer (prepare from 10×; see recipe) to 250 ml (final volume)
Adjust to pH 7.4

To control the molarity of the solution, check the refractive index (refractive index = 1.3888 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 48 hr at 4°C.

Sucrose in TEA buffer, 2.5 M

214.0 g sucrose (2.5 M final)
1× TEA buffer (prepare from 10×; see recipe) to 250 ml (final volume)
Adjust to pH 7.4

To control the molarity of the solution, check the refractive index (refractive index = 1.4528 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 1 month at 4°C.

TEA buffer, 10×

66.6 ml triethanolamine, free base (100 mM final)
28.6 ml acetic acid (glacial; 100 mM final)
100 ml 500 mM EDTA (*APPENDIX 2A*; 10 mM final)
H_2O to 5000 ml (final volume)
Adjust pH to 7.4 with NaOH
Store at room temperature

Using the FFE instruments available from Elphor VaP 22, Bender and Hobein, this volume is sufficient for the FFE electrode buffer volume required for one FFE run. An additional 500 ml to 1000 ml will be needed to prepare the FFE chamber buffer and the solutions containing sucrose in TEA buffer used.

continued

Tris-buffered saline (TBS)

87.7 g NaCl (150 mM final)
24.2 g Tris base (20 mM final)
H_2O to 10,000 ml (final volume)
Adjust pH with HCl to 7.38
Store indefinitely at 4°C

This volume is sufficient to dialyze 20 mg FITC-ASOR.

COMMENTARY

Background Information

Free-flow electrophoresis (FFE) is an efficient method providing both analytical capabilities and the opportunity for continuous operation on a preparative scale. The range of substances to be separated is wide, including small ions, proteins, membrane particles, and even viable cells, and the recoveries of membrane and proteins are high (~90%). FFE separates membranous cell components and organelles (e.g., endosomes) based on surface charge (Morré et al., 1994). As the procedure is non-

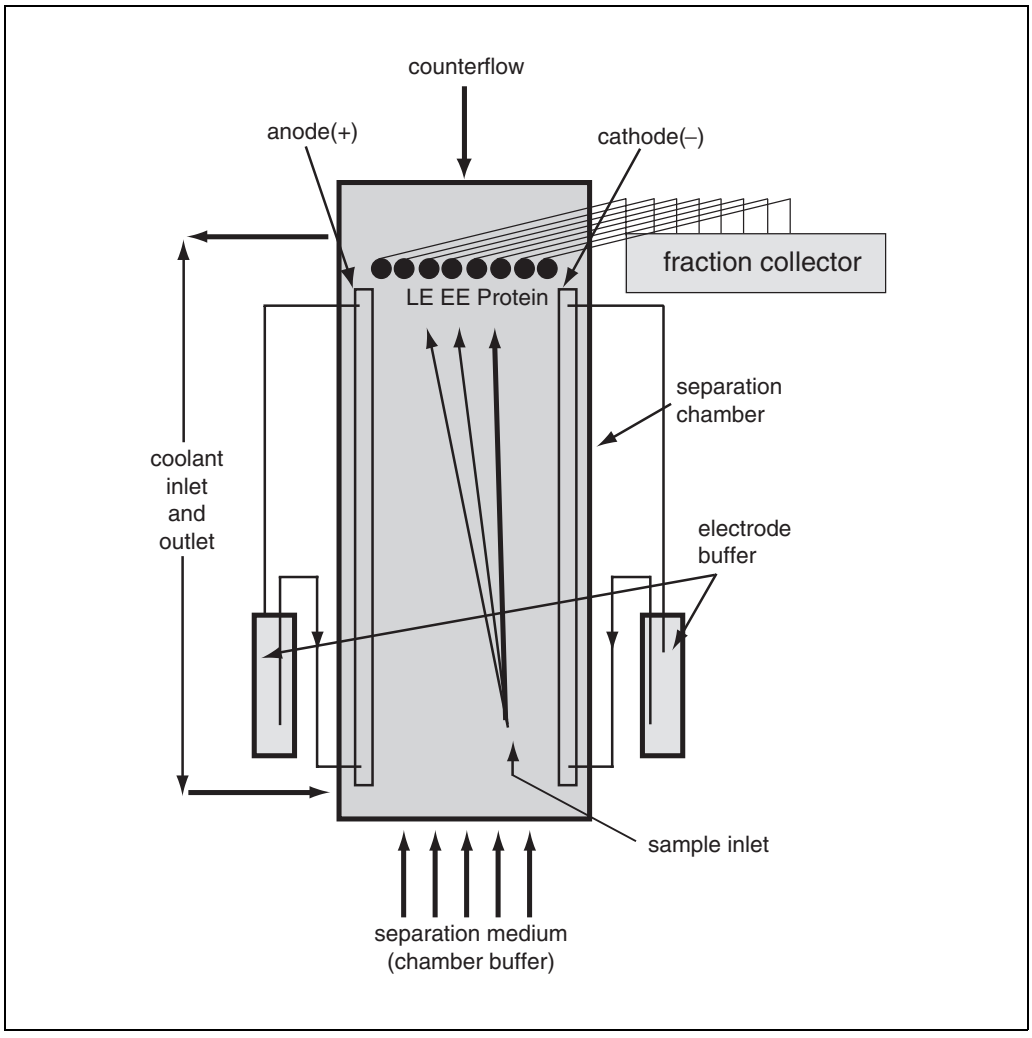


Figure 3.11.2 Basic principle of endosome separation by free-flow zone electrophoresis, referred to as free-flow electrophoresis (FFE), represented in a schematic view. A basic electrolyte with defined pH (chamber buffer) is continuously pumped through the chamber. A fine stream of sample is injected into the laminar flow of the chamber buffer. Due to their different electrophoretic mobilities the different organelles exhibit different angles of deflection and can be collected in different fractions. Early endosomes (EE) are less deflected towards the anode than late endosomes (LE) and the main protein peak elutes nearly undeflected.

destructive and fractions obtained retain full in vitro function, organelles such as endosomes isolated by FFE can be subsequently analyzed for their characteristic functions (e.g., acidification).

A mixture of components to be separated is injected as a fine jet into a separation buffer moving perpendicular to the field lines of an electric field (see Fig. 3.11.2). The electric field leads to deflection of membranes bearing different electrical charge densities according to their mobility or isoelectric point. The sample and the separation buffer enter the separation chamber at one end. On the opposite side, the fractionated sample and the separation buffer are collected. Though FFE can be performed in various modes depending on the electrolyte system applied (e.g., isotachophoretic mode, isoelectric focusing mode), the simplest and most widely used free-flow electrophoresis technique is free-flow zone eletrophoresis, also known as continuous flow electrophoresis. In this mode, the chamber buffer has a constant composition, pH, and conductivity, and the electrically charged components of the samples (e.g., organelles) are deflected from the flow direction of the medium to a defined angle, which is determined by flow velocity and by electrophoretic migration speed (separation according to the size-to-charge ratio).

With regard to the isolation of intracellular organelles from rat liver, FFE has been applied (Krivankova and Bocek, 1998) for the isolation of lysosomes (Stahn et al., 1970), peroxisomes

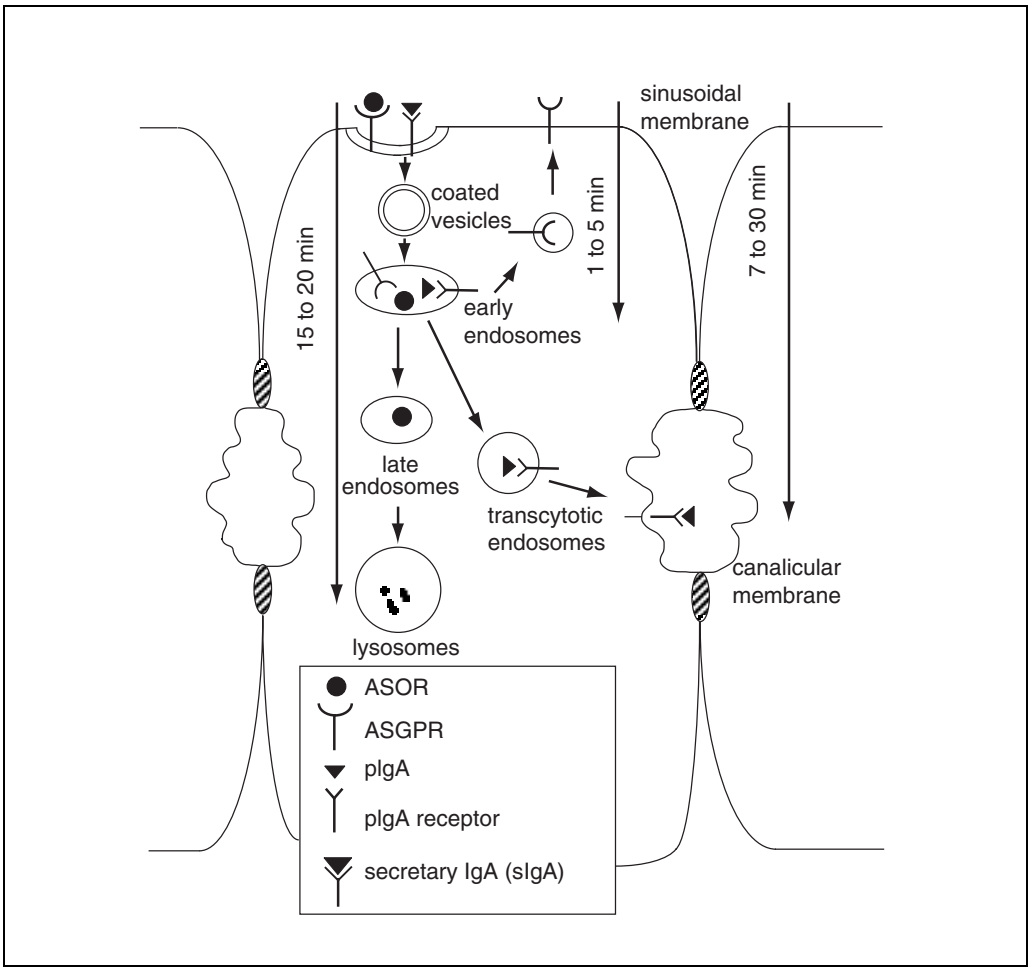


Figure 3.11.3 Kinetics of ASOR and pIgA transport through endocytic compartments in rat hepatocytes. Endocytic subcompartments of rat hepatocytes can be selectively labeled by cointernalization of FITC-ASOR and [^{125}I]pIgA. Within 1 to 5 min after in vivo injection, both ligands can be localized in a common early endosomal compartment. Thereafter, pIgA is sorted into the transcytotic pathway and is secreted into bile (as secretory IgA) 7 to 30 min after injection. In contrast, ASOR is dissociated from its receptor in acidic early endosomes and transported via late endosomes to lysosomes. Lyosomal degradation of ASOR is observed 15 to 20 min after uptake. The authors therefore labeled (common) early endosomes by injection of both markers for 1 min. Late endosomes and transcytotic endosomes are preferentially labeled when the markers are injected for 10 min.

(Volkl et al., 1997) and for subfractionation of the Golgi apparatus (Morré et al., 1983). The buffer system and FFE conditions described in this protocol can also be used for the purification of lysosomes, and with some modifications for peroxisome isolation, and for subfractionation of the Golgi apparatus. In any case, an organelle-enriched fraction is subjected to separation by FFE. Although trypsination is omitted, the starting material is, for example, treated with amylase (Morré, 1998) or reacted with antibodies (Volkl et al., 1997). As marker enzymes β-*N*-acetylglucosaminidase (lysosomes), urate oxidase (peroxisomen), thiamine pyrophosphatase and UDP-galactose galactosyltransferase (trans-Golgi) can be used to follow the organelle purification and enrichment.

An important application of FFE is the isolation and analysis of endosomes and endosome subpopulations. Although endosomes per se are not separated from contaminating material (e.g., endoplasmic reticulum, plasma membranes, mitochondria) when subjected to FFE, separation can be achieved by mild trypsin treatment of the sample, which results in a dose-dependent increase in separation (Marsh et al., 1987). Although the effect of trypsin is still obscure, the electrophoretic mobility of endosomes and lysosomes is enhanced after trypsinization. Applying this technique, it was demonstrated that late endocytic compartments are more shifted to the anode than early compartments (Schmid et al., 1988). Migration of late endosomes to the anode appear to correlate with the internal lower (more acidic) pH of late compartments as compared to the higher (less acidic) pH of early compartments. This lead Morré et al. (1994) to suggest that ATP-dependent vesicle (endosome) acidification generates an outside negative membrane potential and as a consequence results in a more anodal migration of vesicles with a lower internal pH, as compared to those maintaining a higher pH. ATP-dependent acidification was then applied to achieve endosome separation by FFE without the need for trypsinization (Morré et al., 1994). In the authors' experience, rat liver endosomes maintain their initial low internal pH during the isolation procedure; nevertheless no separation is achieved without trypsinization (Fuchs et al., 1989).

FFE has been widely used to characterize endocytic pathways and compartments in non-polarized cells, primarily in tissue culture cells. The isolation of endosomes from polarized tissue culture cells requires the culture of the cells on permeable filters in sufficient quantities to permit subcellular fractionation. This is time-consuming and costly. Alternatively, the rat liver provides a tissue where trafficking in polarized hepatocytes can be studied and where sufficient material is provided from one liver to isolate intracellular organelles. Therefore, the authors describe a protocol for the labeling of endosomal subcompartments of rat hepatocytes and the subsequent FFE isolation and analysis of endosomal fractions.

Polarized hepatocytes express many receptors also commonly found in other cell types (e.g., transferrin receptor, LDL receptor, insulin receptor) and in addition exhibit unique receptor expression (e.g., ASGPR) at their basolateral surface (Courtoy, 1991). Furthermore, hepatocytes are involved in selective transcytosis and secretion of serum proteins (e.g., pIgA) into bile. After internalization via clathrin-coated vesicles, internalized ASGPs are first delivered to kinetically early endosomes that maintain a mildly acidic intravesicular pH. In these compartments, ligands and receptors are sorted into distinct pathways: ASGPs dissociate from their receptors in the acidic endosomal milieu and are transported via kinetically late endosomal compartments to lysosomes, where they are rapidly degraded. Similar to the transferrin receptor, the ASGPR is recycled to the sinusoidal membrane. Although the pIgA-receptor complex is initially delivered via clathrin-coated vesicles to the same early endosomal compartment as ASGPs, the complex is subsequently sorted into transcytotic endosomes and pIgA/pIgR is transported to the canalicular (apical) plasma membrane. Due to proteolytic cleavage near or at the canalicular membrane, the extracellular (ligand-binding) domain of the pIgR (now called secretory component) still bound to pIgA is secreted into bile (hence the term secretory IgA, or sIgA). Although hepatocytes internalize membrane and extracellular material at the canalicular surface (Tuma et al., 1999), this pathway is less characterized due to experimental inaccessibility of the canalicular domain.

Based on the kinetics of ASOR transport through early (receptor-positive) and late (receptor-negative) compartments and its arrival and immediate degradation in lysosomes, early or late endosomes en route to lysosomes can be labeled in a time-dependent manner (see Fig. 3.11.3). Similarly, early and late compartments involved in transcytosis of pIgA from the sinusoidal to the canalicular domain can be marked by short or prolonged internalization

of [^{125}I]pIgA. The labeling procedure applied in the Basic Protocol is based on in vivo studies and cell fractionation experiments reported by various laboratories (Courtoy, 1991; Hoppe et al., 1985; Mueller and Hubbard, 1986; Perez et al., 1988; Sztul et al., 1991; Stefaner et al., 1997). Early endosomes were labeled by coinjection of FITC-ASOR and [^{125}I]pIgA for 1 min, whereas late compartments of the transcytotic and lysosomal pathway, respectively, were labeled by coinjection of the markers for 10 min.

Critical Parameters and Troubleshooting

Due to variability of the animals, the injection procedure and the resulting labeling of the individual endosome subpopulation, injection of two or more tracers in the same animal is required. To obtain reproducible endosome labeling by in vivo marker injection, rapid cooling of the liver by in situ perfusion is very important, especially when early compartments have to be labeled, since markers are rapidly sorted (within 1 to 5 min) from these compartments. Furthermore, the livers have to be processed (minced, homogenized) immediately after cooling. The entire isolation procedure (including homogenization) is carried out in isotonic buffers to maintain the integrity of the organelles and to prevent loss of endocytic markers. Thus, care has to be taken during homogenization, and protease inhibitors are included in the buffer to inhibit damage due to release of proteases. It is also critical for subsequent FFE separation to use the 0.25 M sucrose in TEA buffer, which is also used as FFE chamber buffer, throughout the entire isolation procedure. Most tissue culture cells, and also liver cells, are effectively broken up in this buffer.

To obtain good separation of samples by free-flow zone electrophoresis in general, an accurate preparation of FFE electrode and chamber buffer is of major importance. The composition, the pH, and the conductivity of these buffers are critical parameters; therefore, pH and conductivity should be tested prior to every run. In general, all parameters (buffer composition, coolant volume, filter membranes) directly or indirectly influencing the high-voltage field must be regularly checked. To avoid loss of essential samples, a constant electrical field should be maintained for at least 1 hr before the sample is injected. This might allow one to observe and find a reason for any

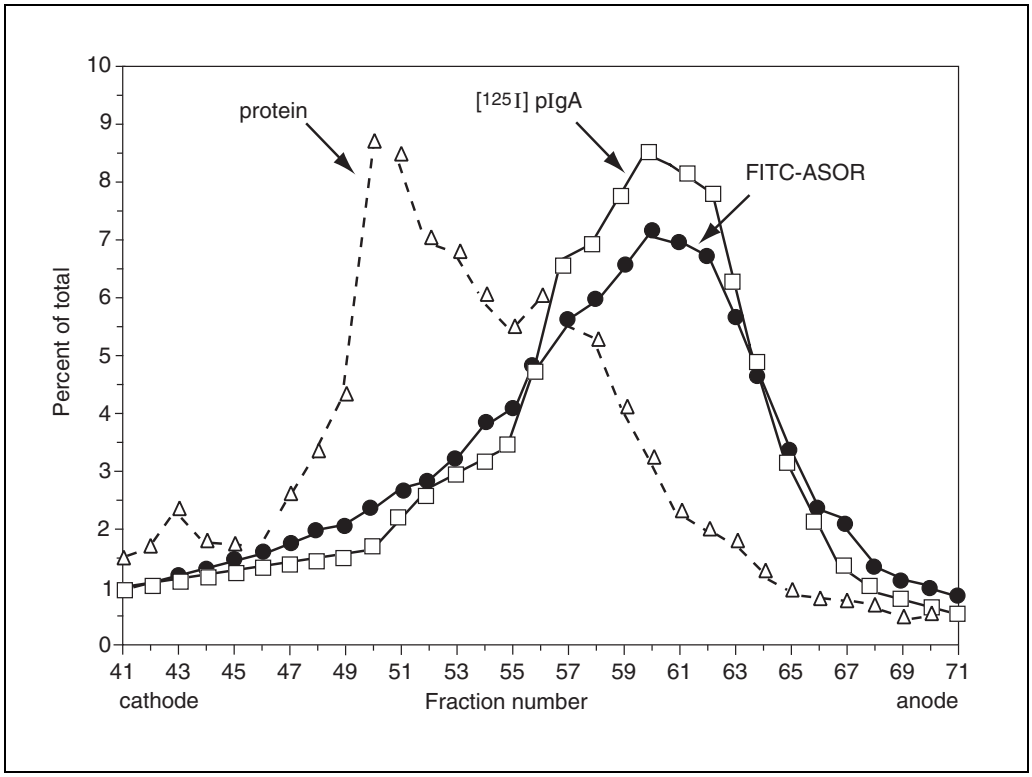


Figure 3.11.4 Distribution of protein and endocytic marker after free-flow electrophoresis of interface I after in vivo injection of FITC-ASOR and [^{125}I]pIgA for 1 min. Endosome-enriched fractions were prepared. Material at the 0.25/1.0 M sucrose interface was collected, trypsin-treated, and subjected to FFE.

problem before the sample is lost. Filtering of chamber buffer is essential, too, as small particles introduced into the thin chamber can disturb the separation by, e.g., deflecting the jets of separated substances. For the same reason, it is essential to accurately clean the chamber before it is filled and to avoid the introduction of small air bubbles either during filling of the chamber with chamber buffer or during injection of the sample. Separated samples are collected via 96 tubes. Clogged tubes (especially several clogged tubes in series) severely alter the separation profile. It is therefore critical to extensively wash the chamber and all connecting tubes with water after every run to remove the sucrose. Tubes blocked at the beginning of a run might be forced to flow again by applying a vacuum (e.g., sucking with a large syringe). Also wash the sample inlet immediately after sample injection with TEA/0.25 M sucrose buffer to prevent clogging. Separation of endosomes from other organelles requires brief trypsinization. As demonstrated by Marsh et al. (1987), even a small increase or decrease in the trypsin concentration can alter the separation profile. Therefore, the amount of trypsin added, the incubation time of samples with trypsin at 37°C, and an exact determination of the protein concentration in the interfaces are critical parameters. The amount of trypsin is "standardized" as percent trypsin per milligram protein. Since the amount of trypsin required to separate endosomes depends on the enrichment of endosomes in the sample, the optimal trypsin concentration has to be experimentally determined, whenever the fractionation protocol is modified. The adjustment of a certain protein concentration (1 mg/ml) in the loading sample has important implications: lower concentrations make it difficult to detect marker proteins in fractions; high concentrations cause broadening of jets and reduced separation of organelles. When the expected separation (~10 fractions) of endosomal markers from the major protein peak is not obtained (e.g., with a new instrument), alter the relevant conditions (sample flow rate, chamber buffer flow rate, trypsin concentration, voltage/current) one by one.

When two different endosome-labeling conditions (e.g., 1 min versus 10 min labeling) are analyzed, these two livers should be processed in parallel and subjected to FFE on the same day, one after another, to have comparable isolation and FFE conditions. When individual FFE runs at different days and liver preparations are compared, the actual fraction num-

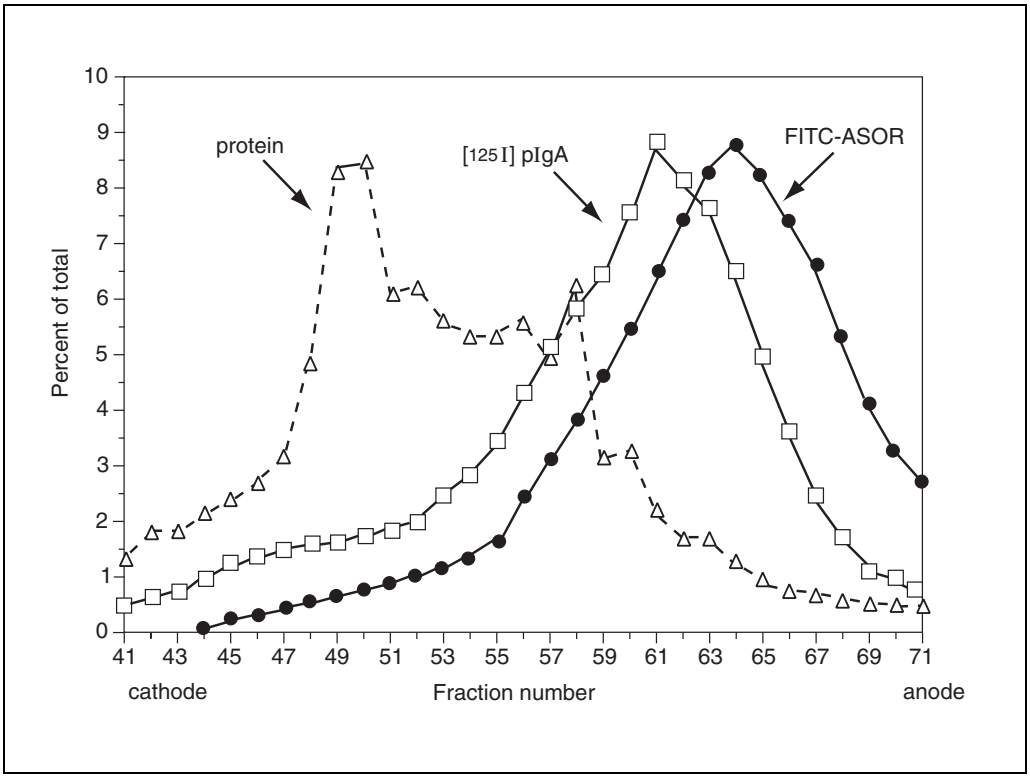


Figure 3.11.5 Distribution of protein and endocytic marker after free-flow electrophoresis of interface I. FITC-ASOR and [^{125}I]pIgA were injected for 10 min. Endosome-enriched fractions were prepared. Material at the 0.25/1.0 M sucrose interface was collected, trypsin-treated, and subjected to FFE.

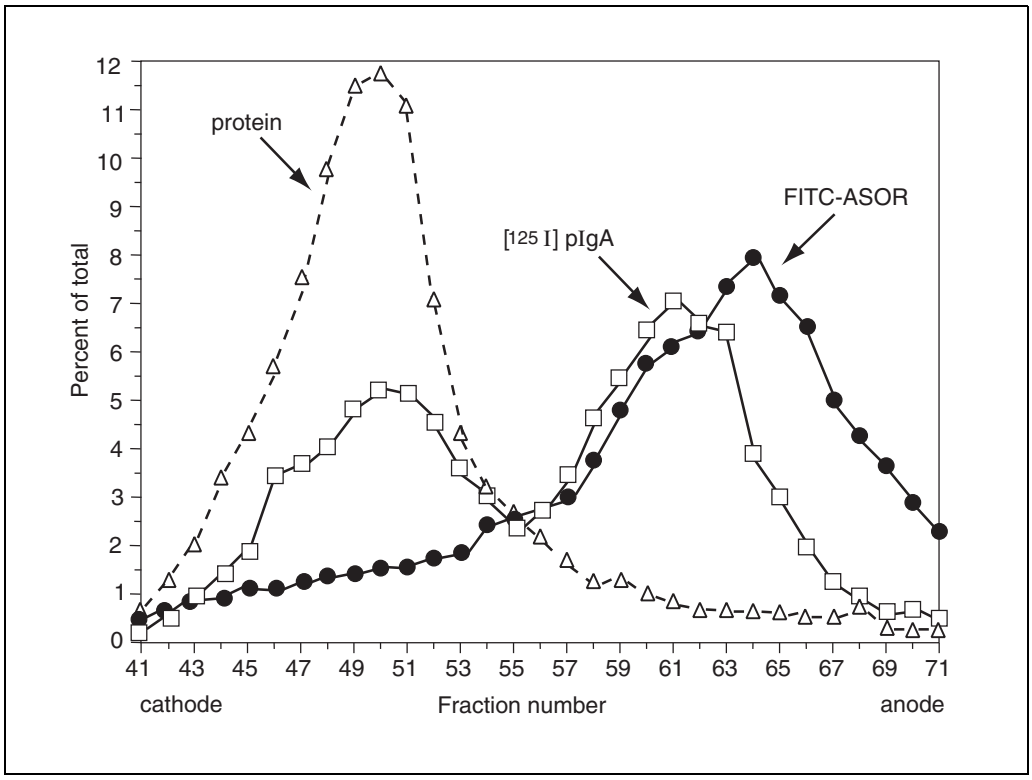


Figure 3.11.6 Distribution of protein and endocytic marker after free flow electrophoresis of interface II. FITC-ASOR and [^{125}I]pIgA were injected for 10 min. Endosome-enriched fractions were prepared. Material at the 1.0/1.15 M sucrose interface was collected, trypsin-treated, and subjected to FFE.

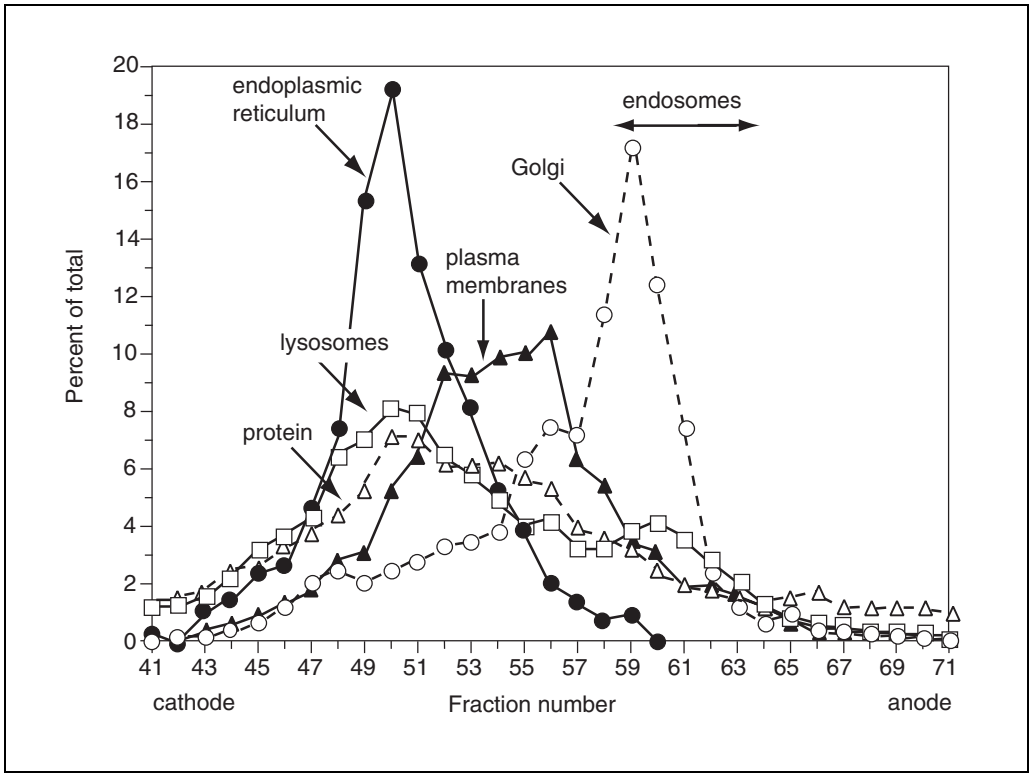


Figure 3.11.7 Distribution of protein and marker enzymes after free-flow electrophoresis of interface I. The following marker enzymes were determined: NADPH cytochrome *c* reductase (endoplasmic reticulum); alkaline phosphodiesterase I (plasma membranes); β-*N*-acetylglucosaminidase (lysosomes); UDP-galactose galactosyltransferase (*trans*-Golgi).

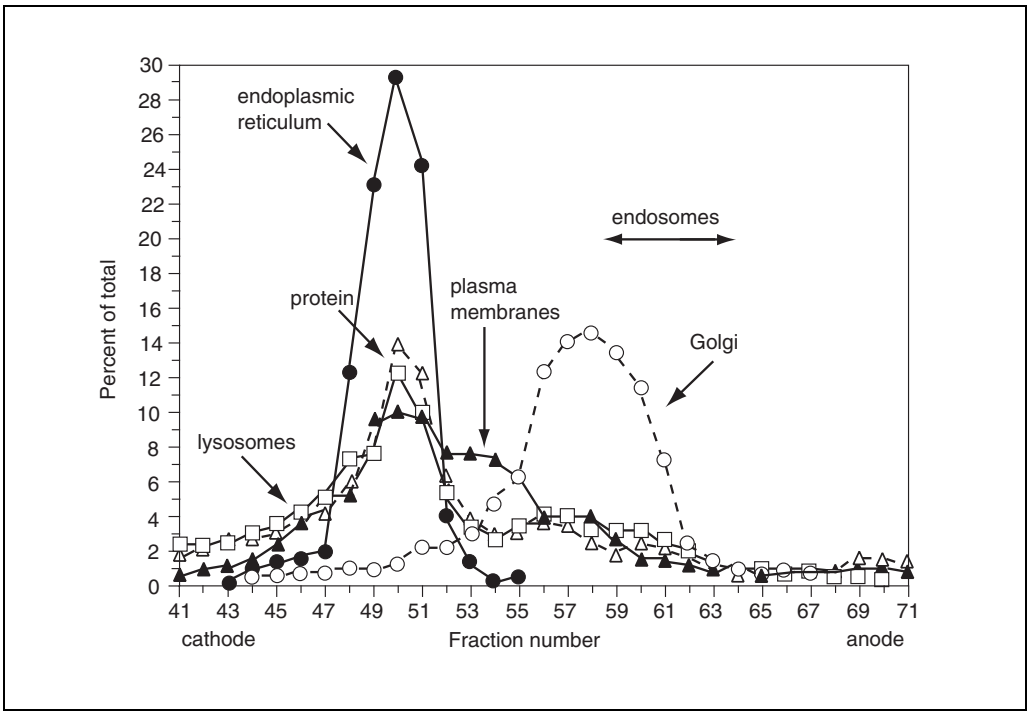


Figure 3.11.8 Distribution of protein and marker enzymes after free flow electrophoresis of interface II. The following marker enzymes were determined: NADPH cytochrome *c* reductase (endoplasmic reticulum); alkaline phosphodiesterase I (plasma membranes); β-*N*-acetylglucosaminidase (lysosomes); UDP-galactose galactosyltransferase (*trans*-Golgi).

ber—i.e., the position at which protein and endosomal peaks elute—varies, but separation (number of fractions between endosomal and protein peaks, and between endosome subcompartments) is fairly constant.

Anticipated Results

Basic Protocol

Preparation of endosome-enriched fractions. Rat liver endosomes are mainly enriched in so-called Golgi fractions that can be obtained by flotation of microsomes or total microsomes (see Fig. 3.11.1) in discontinuous sucrose density gradients. According to the Basic Protocol, a "Golgi fraction" is prepared that corresponds to a combined Golgi light, intermediate, and heavy fraction as described by Khan et al. (1986). The combined Golgi fraction, here called interface I (interface 0.25/1.0 M sucrose), is primarily enriched in endosomes labeled with ASOR. In contrast, pIgA is equally distributed between interfaces I and II (1.0/1.15 M interface) and also within the 1.15 M sucrose layer. Nevertheless, due to the higher amount of protein-contaminating material (primarily endoplasmic reticulum and plasma membranes) present in interface II, pIgA is less enriched in interface II as compared to interface I. Both interface I and interface II have been analyzed by FFE to demonstrate that the distribution and separation of endosome subcompartments is very similar in these interfaces and therefore independent of their density properties. For further functional studies, endosomes derived from interface I are preferentially used. These endosome-enriched fractions are mainly contaminated by the Golgi apparatus, which is highly enriched at interface I. Although the majority of plasma membranes are removed by the initial low-speed centrifugation steps, interfaces I and II are slightly enriched in APDE I with a higher enrichment found in interface II as compared to interface I. This becomes apparent when FFE fractions of interface I and II are compared (see below) and plasma membrane localization of pIgA is evident in interface II but not in interface I. For a detailed characterization (yield, enrichment, purity) of endosome–enriched gradient fractions and FFE fractions see previous reports (Fuchs et al., 1989; Khan et al., 1986; Marsh et al., 1987; Schmid et al., 1988; Stzul et al., 1991; Stefaner et al., 1997).

FFE separation of endosome-enriched fractions: To analyze the kinetics and potential subcompartments involved in endocytic transport, analytical FFE can be applied. For these separations, the distribution of marker enzymes

is first determined to ensure the resolution of the technique. For subsequent analysis, the distribution of protein and endosomal markers is determined. The quality of the separation is usually estimated from the number of fractions between the main protein peak and the endosome peak fractions. Since early endosomes are less deflected to the anode than transcytotic and late endosomes, a separation is considered as "good" if the protein and early endosome peaks are 9 to 10 fractions apart. Late endosomes are separated by 13 to 15 fractions from the protein peak, at best. In general, the separation of rat liver endosomes is usually less when compared to the separation of endosomes from tissue culture cells.

Early endosomes were labeled by coinjection of FITC-ASOR and [^{125}I]pIgA for 1 min. Since ~1 to 2 min are required to cool the liver and halt endocytosis, both ligands are internalized for ~2 to 3 min. The FFE separation of the endosome-enriched fraction at interface I is shown in Figure 3.11.4. The major protein peak elutes at fraction 50, whereas endosomes are deflected towards the anode and are well resolved from the protein peak. As expected, the major peak of ASOR and pIgA-labeled endosomes are localized in the same fraction (60).

Internalization of FITC-ASOR and [^{125}I]pIgA for 10 min and FFE separation of interface I and interface II is shown in Figures 3.11.5 and 3.11.6. In comparison to early endosomes labeled with ASOR, late ASOR-labeled compartments are more deflected to the anode, and the peak fraction of the late ASOR-labeled endosomes now elutes at fraction 64. In contrast, labeling of late transcytotic compartments with pIgA leads to a small but reproducible shift of the peak fraction from 60 (see Fig. 3.11.4) to 61 (see Fig. 3.11.5 and 3.11.6). Similar results were obtained by labeling early, late, and transcytotic endosomes in the isolated perfused liver (Stefaner et al., 1997). The second peak of [^{125}I]pIgA at fraction 50 after FFE separation of interface II (Fig. 3.11.6) is due to pIgA still present in the plasma membrane. This also demonstrates the higher contamination of interface II fractions by plasma membranes, since this peak is largely absent in interface I fractions (see Fig. 3.11.5). In contrast to ASOR, binding of pIgA to its receptor is neither calcium- nor pH-dependent and plasma membrane–associated pIgA is not displaced from the plasma membrane during isolation.

The distribution of marker enzymes after FFE separation of interface I and II (see Fig. 3.11.7 and 3.11.8) is very similar with respect to elution of peak fractions. The major protein peak consists of endoplasmic reticulum and plasma membranes. Lysosomes are a minor contaminant and the lysosomal enzyme activity found in the FFE fractions from rat liver does not colocalize with endosomal fractions, in contrast to FFE of endosomes from tissue culture cells (Marsh et al., 1987). Similar to endosomes, marker for the *trans*-Golgi is also shifted to the anode, eluting at fraction 59. Thus, it is primarily early endosomes that are contaminated by Golgi membranes. Due to removal of the majority of the protein (i.e., endoplasmic reticulum, plasma membranes), upon FFE the enrichment of the endosomal peak fractions is further increased 2- to 4-fold as compared to the starting material (interface I and II).

In conclusion, FFE separation of endosome-enriched fractions from rat liver results in the identification of three distinct endosomal subcompartments: (common) early, late, and transcytotic endosomes. When the acidification properties of these three peak fractions is analyzed using Support Protocol 3, these populations are functional with respect to ATP-dependent proton transport and differ in their internal pH.

Time Considerations

In contrast to the FFE purification of endosomes from tissue culture cells, the isolation procedure for rat liver endosomes (see Basic Protocol) is time-consuming and connot be interrupted at any time. The following time requirements have to be calculated for individual steps (preparation of buffers, ligands, and solutions is not included in this timetable):

In vivo endosome labeling, and liver perfusion: 1 hr;

Preparation of endosome-enriched fractions ready for FFE separation: ~8 to 9 hr;

FFE-set up before separation: 2 to 3 hr (this can be done in parallel during the centrifugation procedure);

Endosome separation: calculate ~1 to 1.5 hr/sample to obtain material for analytical purpose; at least two samples are analyzed, i.e., 2 to 3 hr; For preparative endosome isolation: 3 to 5 hr;

Analysis of protein and endosomal marker in FFE fractions: ~1 to 2 hr; For analysis of marker enzymes the fractions can be frozen and assayed when appropriate;

Enzyme assays: 1 to 2 days.

It has to be pointed out that the result of the separation and the reproducibility largely depend on how fast endosome-enriched fractions ready to be used for the FFE are prepared. Since the entire protocol, starting at marker injection into the rat in the morning and ending after FFE fractions are obtained, has to be carried without interruption, at least 13 to 14 hr must be allotted. When cell-free acidification assays are carried out for functional analysis, these assays have to be performed immediately after analysis of the FFE fractions for endosomal markers and for protein. This functional assay is similarly time-consuming. To dissipate the initial pH gradient and to subsequently acidify endosomes, ~1 hr is required. In most cases, dissipation of the initial gradient does not have to be determined in each fraction, and fractions can be pre-equilibrated consecutively while determining ATP-dependent acidification of the preceding samples. Nevertheless, ~5 hr are required to carry out these assays.

For preparative isolations, at least 15 hr must be allotted. In the case of analysis of endosomes by SDS-PAGE and immunoblotting, protein and endosomal markers have to be analyzed immediately after FFE separation in order to pool the fractions of interest and to concentrate the membranes by centrifugation (2 to 3 hr). Thereafter, the endosomal (membrane) pellets can be stored frozen for further analysis (e.g., SDS-PAGE and immunoblotting: 1 to 2 days).

Disclaimer

This article was written by Renate Fuchs and Isabella Ellinger in their private capacity and without any official support.

Literature Cited

Courtoy, P.J. 1991. Dissection of endosomes. *In* Intracellular Trafficking of Proteins (C. Steer and J. Hanover, eds.) pp. 103-156. Cambridge University Press, Cambridge, U.K.

Exton, J.H. 1975. The perfused rat liver. *Methods Enzymol.* 39:25-36.

Fuchs, R., Male, P., and Mellman, I. 1989. Acidification and ion permeabilities of highly purified rat liver endosomes. *J. Biol. Chem*. 264:2212-2220.

Giffroy, D., Langendries, A., Maurice, M., Daniel, F., Lardeux, B., Courtoy, P.J., and Vaerman, J.-P. 1998. In vivo stimulation of polymeric Ig receptor transcytosis by circulating polymeric IgA in rat liver. *Int. Immunol*. 10:347-354.

Hoppe, C.A., Connolly, T.P., and Hubbard, A.L. 1985. Transcellular transport of polymeric IgA in the rat hepatocyte: Biochemical and morphological characterization of the transport pathway. *J. Cell Biol.* 101:2113-2123.

Jobbágy, A. and Király, K. 1966. Chemical characterization of fluorescein isothiocyanate-protein conjugates. *Biochim. Biophys. Acta* 124:166-175.

Khan, M.N., Savoie, S., Bergeron, J.J.M., and Posner, B.I. 1986. Characterization of rat liver endosomal fractions. In vivo activation of insulin-stimulable receptor kinase in these structures. *J. Biol. Chem.* 261:8462-8472.

Krivankova, L., and Bocek, P.. 1998. Continuous free-flow electrophoresis. *Electrophoresis*. 19:1064-74.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* 104:875-886.

Mellman, I., Fuchs, R., and Helenius, A. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem*. 55:663-700.

Morré, D.J. 1998. Cell-free analysis of Golgi apparatus membrane traffic in rat liver. *Histochem Cell Biol*. 109:487-504.

Morré, D.J., Lawrence, J., Safranski, K., Hammond, T., and Morré, D.M. 1994. Experimental basis for separation of membrane vesicles by preparative free-flow electrophoresis. *J. Chromatogr. A*668:201-213.

Morré, D.J., D.M. Morre, and Heidrich, H.G. 1983. Subfractionation of rat liver Golgi apparatus by free-flow electrophoresis. *Eur J Cell Biol*. 31:263-74.

Mueller, S.C. and Hubbard, A.L. 1986. Receptor-mediated endocytosis of asialoglycoproteins by rat hepatocytes: Receptor-positive and receptor-negative endosomes. *J. Cell Biol.* 102:932-942.

Perez, J.H., Branch, W.J., Smith, L., Mullock, B.M., and Lucio, J.P. 1988. Investigation of endosomal compartments involved in endocytosis and transcytosis of polymeric immunoglobulin A by subcellular fractionation of perfused isolated rat liver. *Biochem. J.* 251:763-770.

Schmid, S.L., Fuchs, R., Male, P., and Mellman, I. 1988. Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. *Cell* 52:73-83.

Stahn, R., K.P. Maier, and Hannig, K. 1970. A new method for the preparation of rat liver lysosomes. Separation of cell organelles of rat liver by carrier-free continuous electrophoresis. *J Cell Biol*. 46:576-91.

Stefaner, I., Klapper, H., Stzul, E., and Fuchs, R. 1997. Free-flow electrophoretic analysis of endosome subpopulations of rat hepatocytes. *Electrophoresis* 18:2516-2522.

Sztul, E., Kaplin, A., Saucan, L., and Palade, G. 1991. Protein traffic between distinct plasma membrane domains-isolation and characterization of vesicular carriers involved in transcytosis. *Cell* 64:81-89.

Tuma, P.L., Finnegan, C.M., Yi, J.-H., and Hubbard, A.L. 1999. Evidence for apical endocytosis in polarized hepatic cells: Phosphoinositide 3-kinase inhibitors lead to the lysosomal accumula-

tion of resident apical plasma membrane proteins. *J. Cell Biol.* 145:1089-1102.

Vaerman, J.-P. and Lemaitre-Coelho, I. 1979. *In* Protein Transmission Through Living Membranes (W.A. Hemmings, ed.) pp. 383-393. Elsevier/North-Holland, New York.

van Renswoude, J., Bridges, K.R., Harford, J.B., and Klausner, R.D. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: Identification of a nonlysosomal acidic compartment. *Proc. Natl. Acad. Sci. U.S.A.* 79:6186-6190.

Volkl, A., H. Mohr, G. Weber, and Fahimi, H.D. 1997. Isolation of rat hepatic peroxisomes by means of immune free flow electrophoresis. *Electrophoresis*. 18:774-80.

Wall, D.A., Wilson, G., and Hubbard, A.L. 1980. The galactose-specific recognition system of mammalian liver: The route of ligand internalization in rat hepatocytes. *Cell* 21:79-93.

Contributed by Renate Fuchs and
Isabella Ellinger
University of Vienna
Vienna, Austria

Isolation of Synaptic Vesicles

Neurons communicate through synaptic contacts organized in presynaptic and postsynaptic domains (see Fig. 3.12.1). A central aspect of synapse organization is that the presynaptic and postsynaptic domains are separated by a cleft. The presence of this synaptic cleft between neurons restricts the nature of their communication to diffusible chemicals called neurotransmitters. Presynaptic terminals are characterized by their capacity to store and secrete neurotransmitters in small packets or quanta. These packets contain a discrete and relatively homogenous number of neurotransmitter molecules. Ultrastructural studies of the presynaptic terminal suggested that the quantal nature of synaptic transmission is due to the presence of numerous synaptic vesicles that are highly regular in size.

Synaptic vesicles are probably the most abundant vesicular organelle. The function and molecular composition of these vesicles is understood in detail largely because they can be purified to a great degree. At the electron microscopic level, synaptic vesicles are spherical, with a characteristic cross-sectional diameter of ~40 nm ± 4% to 9%

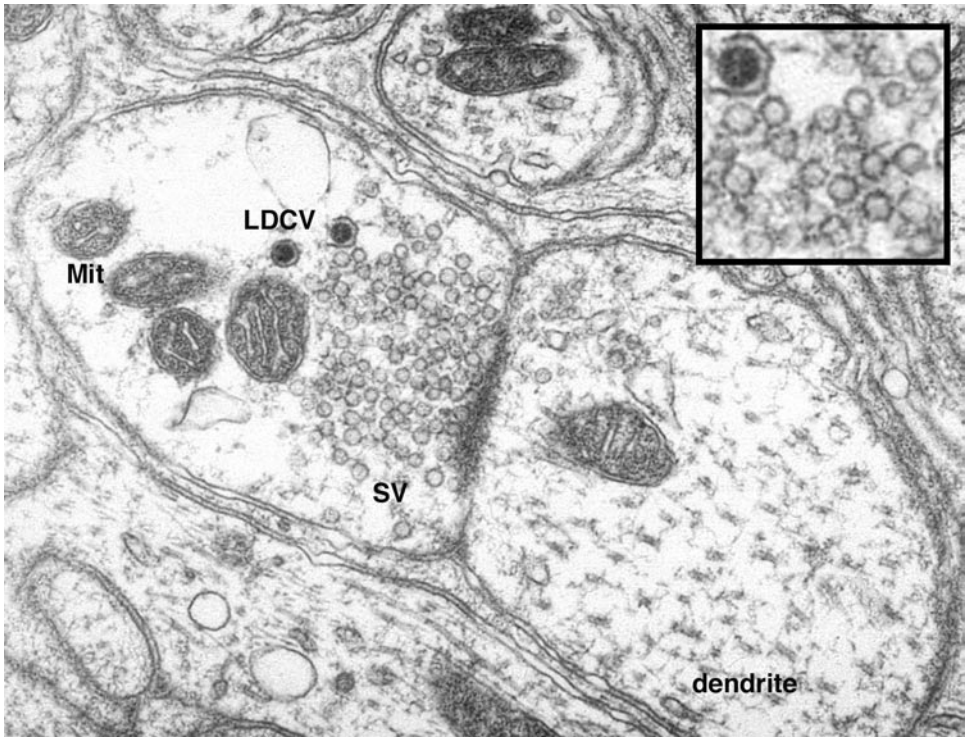


Figure 3.12.1 Ultrastructure of a rat brain synapse. The figure depicts a transmission electron micrograph of the presynaptic (left side of the panel) and the post-synaptic (dendrite, right side of the panel) elements that constitute a typical synapse. Two classes of secretory organelles are present in the presynaptic element, synaptic vesicles (SV) and large dense core vesicles (LDCV). Note the difference in the size and the luminal content between these organelles as well as their abundance. Synaptic vesicles are very homogenous in size, with a clear center (see text). Mitochodria (Mit) are present in both pre and post-synaptic terminals. Insert shows a magnification of vesicles. An LDCV is present on the upper left-hand corner of the insert. Image provided as courtesy of Dr. Jorge Garrido (Facultad de Ciencias Biologicas, Pontificia Universidad Catolica de Chile, Santiago, Chile).

Contributed by Branch Craige, Gloria Salazar, and Victor Faundez
Current Protocols in Cell Biology (2004) 3.12.1-3.12.17

(Kim et al., 2000). Synaptic vesicles can be visually identified in electron micrographs because they have an electron-lucent appearance, which is due to the absence of protein or peptides in their lumen. These characteristics (size and density) are used in almost all the protocols designed to isolate synaptic vesicles. The synaptic vesicle purification methods that are described here take advantage of the characteristic density and size of these organelles. Combinations of differential centrifugation, density-based sedimentation, and size fractionation have been used in several synaptic vesicle isolation procedures, including those presented here. In the past, the efficacy of these synaptic vesicle purification procedures was confirmed by electron microscopy. Today, the degree of synaptic vesicle purification can be easily determined by immunoblot using various antibodies that recognize synaptic-vesicle proteins.

Two synaptic vesicle isolation procedures, developed, respectively, by Clift-O'Grady et al. (1990) and Huttner et al. (1983), are described. Both of these utilize rat brain as the source of tissue. Although both protocols rely on similar biophysical principles to isolate vesicles, they differ in (1) the way the tissue is homogenized; (2) the buffer in which homogenization is performed; and (3) the method of fractionation. Clift-O'Grady's method (see Basic Protocol 1) is performed in buffered physiological saline. In contrast, Huttner's protocol (see Alternate Protocol 1) is performed in the absence of salt, resulting in a lower vesicle yield. However, synaptic vesicles purified by the Huttner protocol retain loosely associated peripheral proteins such as synapsins (Huttner et al., 1983). Thus, the reader is referred to Basic Protocol 1 or Alternate Protocol 1, depending on purification needs. The biochemical and biophysical foundations of these protocols can be found in the classical work of Whittaker and Kelly (Whittaker et al., 1972; Nagy et al., 1976; Carlson et al., 1978; Wagner et al., 1978). Once the synaptic vesicles have been isolated, they can be enriched (Basic Protocol 2 or Alternate Protocol 2 or 3). They can be sedimented at high speed (Support Protocol 1) or immunoprecipitated (Support Protocol 2). Silanized glassware and plasticware (Support Protocol 3) are used for synaptic-vesicle purifications.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: The authors recommend that all surfaces coming in contact with purified vesicles be siliconized (see Support Protocol 3). As one proceeds further into the purification protocols, the vesicles will have a tendency to adsorb to plastic and glass.

BASIC PROTOCOL 1

CLIFT-O'GRADY METHOD OF SYNAPTIC VESICLE ISOLATION

This is a 2-day protocol. Brain tissue is disrupted in a Waring Blendor in a physiological salt buffer; thus, synaptic vesicles are released from whole brain and do not from a crude synaptosomal preparation as in the Huttner method (Alternate Protocol 1). All procedures, buffers, tubes, and equipment must be maintained at 4°C to perform the purification. Microcentrifuge tubes and glass tubes as well as pipets used to collect and handle synaptic vesicles should be siliconized with Sigmacote (see Support Protocol 3). This is particularly critical at the later stages of purification because synaptic vesicles adsorb strongly to untreated glass and plastic surfaces. Addition of carrier protein to the purified vesicles is not recommended. At each fractionation step, keep a record of the approximate volume of the fractions and collect an aliquot of material to determine protein concentration and content of synaptic vesicle antigens by immunoblot (refer to Table 3.12.1 for a list of commonly used antibodies; also see *UNIT 6.2*). With these figures, it will be possible to estimate the enrichment and recovery of synaptic vesicle markers.

Membranes and vesicles fractions should be stored at −80°C after flash freezing in liquid nitrogen.

Table 3.12.1 Antibodies Used to Immunoisolate Synaptic Vesicles

Antigen	Name and isotype	Reactivity	Performance with[a]		Supplier
			Immunomagnetic isolation	Immunoblotting	
VAMP II	69.1/IgG1	Human, mouse, rat	++++	+++	Synaptic Systems
Synaptophysin	SY38/IgG1	Human, mouse, rat	++++	++++	Chemicon
SV2	10H4/IgG1	Human, mouse, rat, frog, fish	+++	++++	Iowa Hybridoma Bank
Rab3a	42.1/IgG1	Mouse, rat	+++	+++	Synaptic Systems
Vacuolar ATPase	P116 rabbit	Mouse, rat	NT	+++	Synaptic Systems
Vglut1/BNP1	Rabbit	Mouse, rat	++	+++	Synaptic Systems
VGAT	Rabbit	Mouse, rat	++	+++	Synaptic Systems

[a]Symbols: ++++, best performance; ++ good performance; NT, not tested.

Materials

200- to 250-g female rats (22 animals needed)
Buffer A (see recipe) prepared in H_2O (light water), with and without 1× protease inhibitors
1000 × protease inhibitors (see recipe) *or* Boehringer Complete protease inhibitor tablets (Roche Applied Science)
Buffer A (see recipe) prepared in 80% (v/v) H_2O/20% D_2O (deuterium oxide, 99% isotopic purity), with and without 1× protease inhibitors
320 mM sucrose/4 mM HEPES, pH 7.4, prepared with 100% D_2O
320 mM sucrose/4 mM HEPES, pH 7.4, prepared with 85% (v/v) D_2O/15% H_2O
320 mM sucrose/4 mM HEPES, pH 7.4, prepared with 70% (v/v) D_2O/30% H_2O

Dissecting instruments
Waring Blendor with small glass cup (kept in cold room)
Refrigerated centrifuge with Sorvall SS-34 rotor (or equivalent), and corresponding polycarbonate centrifuge tubes
Beckman ultracentrifuge with 45 Ti rotor (or equivalent), and corresponding Oak Ridge ultracentrifuge tubes
Small Teflon-coated spatula
Tight-clearance Teflon/glass Potter-Elvehjem homogenizer, ~50 ml
Beckman ultracentrifuge with SW 41 Ti rotor (or equivalent) and corresponding polyallomer ultracentrifuge tubes
Loose-clearance Teflon/glass Potter-Elvehjem homogenizer, ~2 ml
SG series gradient maker (Hoefer Scientific)
Loose-clearance Teflon/glass Potter-Elvehjem homogenizer, ~1 ml

NOTE: Carry out all procedures at 4°C using solutions prechilled to that temperature.

Homogenize rat brains

1. Euthanize 22 rats by CO_2 asphyxiation (Donovan and Brown, 1995). Decapitate rats, dissect out brains, and quickly place them in ice-cold buffer A without protease inhibitors. Wash brains in ice-cold buffer A without protease inhibitors extensively to remove blood.

   Rats should be euthanized two to three at a time and the brains removed so as to minimize the time at room temperature.Wash brains by swirling the cold buffer with a metal spatula, avoiding mechanical damage to the brains.

2. Drain off the buffer used for washing and bring the 22 brains to a final volume of 110 ml with ice-cold buffer A containing 1× protease inhibitors.

3. In the cold room, blend for 2 min in a Waring Blendor with a small glass cup at maximum speed.

Prepare cytosolic suspension

4. Pour the homogenate into six to eight 50-ml polycarbonate SS-34 Sorvall tubes and centrifuge 30 min at 20,250 × *g*, 4°C.

5. Pool all supernatants in a separate tube and bring the volume up to 94 ml with ice-cold buffer A containing 1× protease inhibitors.

Isolate membranes

6. Layer a pad of 20 ml of buffer A prepared in 80% H_2O/20% D_2O, containing 1× protease inhibitors, in each of four to six Oak Ridge ultracentrifuge tubes appropriate for a 45 Ti rotor. Carefully overlay the supernatant from step 5 on top of the pads, distributing it evenly among the tubes. Balance the tubes with chilled buffer A.

7. Ultracentrifuge 4 hr at 225,000 × *g*, 4°C, with centrifuge acceleration and brake set to the maximum setting.

8. Aspirate and discard the supernatants and collect the pellets.

   Pellets are sticky, so the best method of collecting them is to use a Teflon-coated small spatula to slice each pellet in three pieces and scoop them out of the tubes.

Fractionate membranes

9. Transfer pellet pieces to a tight-clearance 50-ml Potter-Elvehjem (Teflon/glass) homogenizer and bring the volume up to 30 ml with buffer A (prepared with light water) containing 1× protease inhibitors. Resuspend pellets with 12 strokes at 600 to 800 rpm. Try to avoid bubble formation.

10. Layer the following consecutively from bottom to top in four SW 41 Ti polyallomer tubes to prepare step gradients:

    Layer 1: 1.5 ml 320 mM sucrose/4 mM HEPES, pH 7.4, prepared with 100% D_2O
    Layer 2: 1.5 ml 320 mM sucrose/4 mM HEPES, pH 7.4, prepared with 85% D_2O/15% H_2O
    Layer 3: 1.5 ml of 320 mM sucrose/4 mM HEPES, pH 7.4, 70% D_2O/30% H_2O.

    Layer 8 to 9 ml of the membrane suspension obtained in step 9 on top of each step gradient.

    Fill tubes completely; otherwise they will collapse during the run.

11. Ultracentrifuge 1.5 hr at 225,000 × *g*, 4°C.

12. Collect the sedimented material in layer 3 and at the interface between layer 3 and layer 2.

13. Transfer the material collected to a 45 Ti Oak Ridge centrifuge tube. Bring the total volume up to 68 ml with buffer A (prepared with light water) containing 1× protease inhibitors. Centrifuge overnight at 225,000 × *g*, 4°C.

Isolate synaptic vesicles

14. Discard the supernatant and collect the pellet as in step 8. Resuspend pellets in 1.6 ml of buffer A containing 1× protease inhibitors and transfer to a 2-ml Potter-Elvehjem homogenizer using the technique described in step 9 and homogenize.

15. Using an SG series gradient maker, prepare four 10-ml continuous gradients in SW 41 Ti polyallomer ultracentrifuge tubes, each containing 5.8 ml of buffer A prepared with 80% H_2O/20% D_2O (without protease inhibitors) and 6.2 ml of 320 mM sucrose/4 mM HEPES, pH 7.4, prepared with 100% D_2O.

    See Basic Protocol 2, step 1, for preparation of continuous gradients using the SG series gradient maker.

16. Layer the membrane suspension obtained in step 14 over the gradients at 1 ml per gradient. Centrifuge 3 hr at 274,000 × *g*, 4°C.

    After this centrifugation step, membranes appear as a white fluffy material distributed all along the gradient.

17. Using a 1-ml automatic pipettor, take the uppermost 2 ml of each gradient and discard. Collect the next 8 ml from each tube and pool. Discard the pellet at the bottom of the tube.

18. Dilute the pooled membranes to 136 ml with buffer A (prepared with light water) containing 1× protease inhibitors. Divide between two 45 Ti Oak Ridge tubes and ultracentrifuge at least 3 hr at 225,000 × *g*, 4°C.

19. Collect each pellet as described in step 8. Transfer to a 1-ml Potter-Elvehjem homogenizer. Resuspend in 0.6 ml of buffer A containing 1× protease inhibitors as described in step 9.

20. Immediately clarify the resuspended membranes by microcentrifuging 4 min at maximum speed, 4°C.

21. Proceed to enrichment of synaptic vesicles (see Basic Protocol 2 or Alternate Protocol 2 or 3).

HUTTNER METHOD OF SYNAPTIC VESICLE ISOLATION

ALTERNATE PROTOCOL 1

This is a 2-day protocol. Brain tissue is disrupted gently with a loose-clearance Potter-Elvehjem (Teflon/glass) homogenizer. The initial differential fractionation steps (steps 1 to 6 below) generate a crude synaptosomal fraction that contains intact presynaptic terminals. Vesicles are released by hyposmotic shock. As mentioned in Basic Protocol 1, all procedures, buffers, tubes, and equipment must be kept at 4°C during the course of the purification. Microcentrifuge and glass tubes, as well as pipets used to collect and handle synaptic vesicles should be siliconized with Sigmacote (see Support Protocol 3). At each fractionation step, keep record of the approximate volume of the fractions and collect an aliquot of material to determine protein concentration and synaptic vesicle content by immunoblot, using the antibodies described in Table 3.12.1. With these figures it will be possible to estimate the enrichment and recovery of synaptic vesicle markers.

Membranes and vesicle fractions should be stored at −80°C after flash freezing in liquid nitrogen.

Additional Materials *(also see Basic Protocol 1)*

200- to 250-g female rats (14 animals needed)
SB buffers (see recipe), with and without 1× protease inhibitors
1 M HEPES buffer, pH 7.4
Loose clearance Potter-Elvehjem (Teflon/glass) homogenizer, ~50 ml
10-ml syringe with 25-G needle
Beckman ultracentrifuge with SW 28 rotor (or equivalent), and corresponding Ultraclear centrifuge tubes

NOTE: Carry out all procedures at 4°C using solutions prechilled to that temperature.

Homogenize rat brains

1. Euthanize 14 rats by CO_2 axphyxiation (Donovan and Brown, 1995). Decapitate rats, dissect out brains, and quickly place them in ice-cold buffer A without protease inhibitors. Wash brains in ice cold SB320 without protease inhibitors extensively to remove blood.
2. Drain off the buffer used for washing and bring the 14 brains to a final volume of 100 ml in ice-cold SB320 containing 1× protease inhibitors.
3. Transfer brains to a 50-ml loose-clearance Potter-Elvehjem (Teflon/glass) homogenizer and homogenize with 12 strokes at 600 to 800 rpm.

   Since the brains are in a 100-ml volume, the homogenization will be done in two installments.

Perform initial fractionation

4. To the (pooled) homogenate generated in step 3, add 40 ml SB320 containing 1× protease inhibitors. Split volume evenly into four SS-34 tubes, then centrifuge 10 min at 1000 × *g*, 4°C.
5. With a pipet, collect the supernatants and pool them in a separate tube. Bring the volume to 100 ml with SB320 containing 1× protease inhibitors. Split volume evenly into four new SS-34 tubes, then centrifuge 15 min at 13,200 × *g*, 4°C.
6. With a pipet, remove and discard the supernatant. Resuspend the pellet, bringing the volume to 13 ml with SB320 containing 1× protease inhibitors.

   The pellet at this stage is almost 13 ml in volume, so be careful not to add too much buffer.

Fractionate synaptosome preparation

7. Add 117 ml of ice-cold double-distilled water containing 1× protease inhibitors and homogenize in a 50-ml tight-clearance Potter-Elvehjem (Teflon/glass) homogenizer with three strokes at 600 to 800 rpm.
8. Add 1 ml of 1 M HEPES buffer pH 7.4, and incubate on ice for 30 min.
9. Transfer to four SS-34 tubes and centrifuge 20 min at 33,000 × *g*, 4°C.
10. Collect the supernatants with a pipet and transfer to two 45 Ti Oak Ridge tubes. Ultracentrifuge at least 5.5 hr at 213,000 × *g*, 4°C.

    This step can be prolonged to overnight.

11. Collect the pellets in a total volume of 6 ml of SB40 containing 1× protease inhibitors.
12. Pass the material in and out through a 25-G needle five times using a 10-ml syringe.
13. In two Ultraclear SW 28 tubes pour a discontinuous gradient by sequentially layering 16 ml of SB800, then 19 ml of SB50.
14. Load the resuspended homogenate from step 12 and layer carefully on top of the gradients, distributing it evenly among the tubes. Centrifuge 5 hr at 95,000 × *g* in SW 28 rotor, 4°C.

    IMPORTANT NOTE*: This is not an equilibrium gradient; it is a velocity sedimentation. Therefore changes in the time of the run or the angular velocity will affect the vesicle sedimentation.*

15. Collect the faint hazy band that extends from approximately half-way to two-thirds of the way down the tube. Discard the top portion of the gradient.

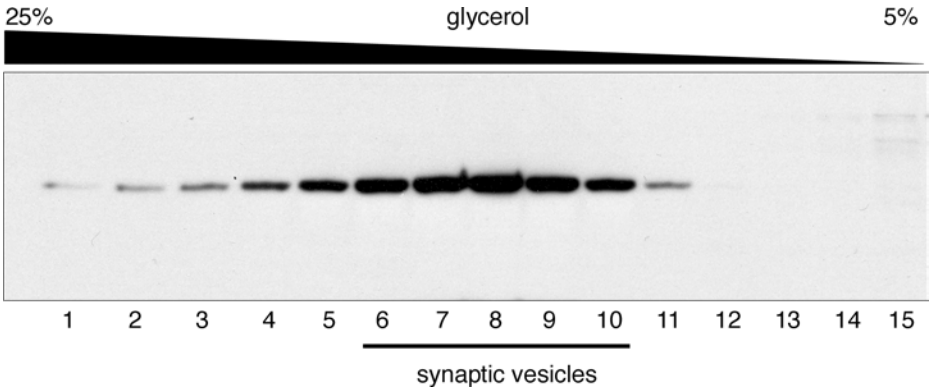


Figure 3.12.2 Size fractionation of brain synaptic vesicles. Synaptic vesicles were purified according to the protocol of Clift-O'Grady and size fractionated in glycerol velocity gradients as described in Basic Protocol 2. Vesicles were resolved in 10% polyacryloamide (PAA)–SDS gel and the presence of synaptic vesicles was determined by immunoblot using the antibody SY38 directed against synaptophysin, also known as p38. Synaptophysin (p38) is one of the most abundant proteins in synaptic vesicles. A vesicle peak is evident between fractions 6 and 10. Electron microscopic examination confirmed that these vesicles possess the features of synaptic vesicles found in nerve tissue.

16. Proceed to enrichment of synaptic vesicles (see Basic Protocol 2 or Alternate Protocol 2 or 3).

If vesicles will be purified by size fractionation they can be loaded directly onto the columns, but if glycerol sedimentation is going to be used, vesicles should be sedimented to wash away the sucrose (see Support Protocol 1).

SYNAPTIC VESICLE ENRICHMENT

Basic Protocol 1 and Alternate Protocol 1 yield membrane preparations in which SV are enriched ~8 to 10 times over brain homogenate. The protocols below take advantage of the uniformity in SV size and their small diameter to further purify these vesicles. These steps increase the yield to ~20 fold over the starting homogenate. Basic Protocol 2 utilizes velocity sedimentation (see Fig. 3.12.2) and Alternate Protocols 2 and 3 each employ a chromatography approach. The chromatography protocols differ in the protein capacity of the column as well as the ease of preparation and maintenance of the different chromatographic media.

NOTE: Carry out all procedures at 4°C using solutions prechilled to that temperature.

SYNAPTIC VESICLE ENRICHMENT BY GLYCEROL VELOCITY SEDIMENTATION

BASIC PROTOCOL 2

Materials

5% and 25% (v/v) glycerol in buffer A (see recipe for buffer)
Synaptic vesicle preparation (see Basic Protocol 1 or Alternate Protocol 1)

SG series gradient maker (Hoefer Scientific)
Transi-stir motor (Talboys Engineering Corp.)
Beckman ultracentrifuge with SW 55 Ti rotor (or equivalent) and corresponding polyallomer centrifuge tubes
Tube-piercing apparatus (Brandel)

1. Layer a pad of 0.5 ml of 50% sucrose in buffer A in an SW 55 Ti polyallomer centrifuge tube. On top of the pad, layer a 5% to 25% continuous glycerol gradient in buffer A as follows. On a SG series gradient maker, first close the communication vessel between chambers. Place 2.2 ml of 25% glycerol solution in the heavy solution chamber. Place 2.2 ml of 5% glycerol solution in the other chamber. Place a stirring rod connected to a Transi-stir motor in the heavy solution chamber. Slightly tilt the gradient maker so the heavy solution is below the level of the light solution. Stir the solution and open the connection between chambers.

   As the gradient is flowing dropwise into the SW 55 Ti tube, the volume of both chambers should come down evenly. The linearity of the gradient can be tested by refractometry. This procedure is highly consistent and reproducible and the linearity of the gradient is optimal.

2. Place the gradients on ice for 20 to 30 min to chill. Gradients can be prepared the night before the experiment and kept at 4°C. Once prepared cover them with Parafilm.
3. Load 200 to 250 μl of the synaptic vesicle preparation (100 to 500 μg of total protein) on top of the gradient.
4. Ultracentrifuge 75 min at 218,309 × *g*, 4°C.
5. Collect gradients from the bottom into sixteen 6-drop fractions using a tube-piercing apparatus.

   Fractions can also be collected from the top using a 1-ml automatic pipettor; collect ~250- tp 270-μl fractions. Synaptic vesicles migrate in fractions 7 to 12 in the middle of the gradient.

6. Flash freeze the fractions in liquid nitrogen and store at −80°C.

ALTERNATE PROTOCOL 2

SYNAPTIC VESICLE ENRICHMENT BY CONTROLLED PORE GLASS (CPG) CHROMATOGRAPHY

Materials

Glycerol-coated controlled pore glass (glyceryl-CPG) beads, 3000 Å particle size (CPG, Inc.)
300 mM glycine/5 mM HEPES, pH 7.4, containing 1× protease inhibitors (see recipe for protease inhibitors)
Synaptic vesicle preparation (see Basic Protocol 1 or Alternate Protocol 1)
Buffer A (see recipe) containing 1× protease inhibitors (see recipe)

85 × 1.6–cm glass chromatography column, siliconized (see Support Protocol 3)
Wall-mounted column rack
Peristaltic pump and fraction collector, with appropriate tubing and connections
Additional reagents and equipment for protein assay (*APPENDIX 3B* or *APPENDIX 3H*) and concentration of protein solutions (*APPENDIX 3C*)

1. Resuspend CPG beads in distilled water and degas.
2. Place an 85 × 1.6–cm i.d. glass chromatography column in a wall-mounted column rack with the bottom part of the column in contact with a Vortex mixer.

   The glass column should be sturdy enough to withstand extensive vortexing.

3. With the vortexer at maximum speed, apply the CPG slurry very slowly, to avoid the formation of settled zones. Once finished pouring the slurry, keep vortexing the column for at least 30 to 60 min to settle all the beads.
4. Attach column to peristaltic pump. Equilibrate column with 10 volumes of 300 mM glycine/5 mM HEPES, pH 7.4.

5. Load the synaptic vesicle preparation (do not exceed 15 mg protein).
6. Run the column at 50 to 80 ml/hr and collect 4-ml fractions.
7. Check protein elution by monitoring the column eluate by spectrophotometry at 280 nm (*APPENDIX 3B*) or by a protein assay (*APPENDIX 3H*).

   There should be two overlapping peaks. The second peak corresponds to a preparation of highly purified synaptic vesicles.

8. Depending on the application, flash freeze vesicles in liquid nitrogen or concentrate using a centrifugal concentrator (*APPENDIX 3C*).

SYNAPTIC VESICLE ENRICHMENT BY GEL-FILTRATION CHROMATOGRAPHY IN SEPHACRYL S-1000

ALTERNATE PROTOCOL 3

Materials

Sephacryl S-1000 resin (Amersham Biosciences)
Buffer A (see recipe) containing 25% (v/v) glycerol and 1× protease inhibitors *or* 300 mM glycine/5 mM HEPES, pH 7.4, containing 1× protease inhibitors (see recipe for protease in hibitors)
Synaptic vesicle preparation (see Basic Protocol 1 or Alternate Protocol 1)
15 × 1–cm plastic chromatography column, siliconized (see Support Protocol 3)

Additional reagents and equipment for concentrating protein solutions (*APPENDIX 3C*)

1. Pour Sephacryl S-1000 into a 15 × 1–cm plastic chromatography column to make an 11-ml packed bed column.
2. Equilibrate column with 5 volumes of 25% glycerol in buffer A or 300 mM glycine/5 mM HEPES, pH 7.4, containing 1× protease inhibitors.
3. Equilibrate with ten volumes of either buffer A or 300 mM glycine 5 mM HEPES, pH 7.4.
4. Apply the synaptic vesicle preparation (1.5 mg protein) and run column by gravity.
5. Collect 0.5-ml fractions in siliconized microcentrifuge tubes.
6. Microcentrifuge the fraction-containing tubes at maximum speed, 4°C, and retain the supernatants.
7. Depending on the application, flash freeze vesicles in liquid nitrogen or concentrate by ultracentrifugation (*APPENDIX 3C*).

SYNAPTIC VESICLE SEDIMENTATION AT HIGH SPEED

SUPPORT PROTOCOL 1

After chromatography, vesicles are diluted in a large volume. Although their concentration may be good for immunoblotting or SDS-PAGE, if larger amounts of concentrated material are needed, vesicles can be concentrated by sedimentation. Tabletop ultracentrifuges or airfuges are appropriate for this purpose. Remember that if vesicles are obtained from a gradient they may have already reached their equilibrium buoyancy. Therefore, the solution in which the vesicles are present ought to be diluted at least 1:1. Failure to do this will result in the vesicles staying in suspension despite the time and g-force applied to sediment them. If the vesicles are in a solution that contains soluble proteins, it should be taken into account that, at the g-force applied here, many protein complexes will also sediment with the vesicles, irrespective of whether or not these proteins interact with synaptic vesicles.

Materials

Synaptic vesicle preparation (see previous protocols)
Buffer A (see recipe)
1000 × protease inhibitors (see recipe) *or* Boehringer Complete protease inhibitor tablets (Roche Applied Science)
Beckman ultracentrifuge with TLA 120.2 rotor (or equivalent), and corresponding ultracentrifuge tubes

NOTE: Carry out all procedures at 4°C using solutions prechilled to that temperature.

1. If required, dilute vesicles in buffer A. Keep all the solutions at 4°C and add protease inhibitors to concentration of 1×.
2. Place the diluted vesicles in siliconized tubes for the TLA 120.2 rotor. Mark the site in which the membrane pellet should be found (i.e., the outermost side of the tube bottom in a fixed-angle rotor).

   This is an important consideration, as the pellet is crystalline and hard to visualize.
3. Centrifuge 1 hr at 200,000 × *g*, 4°C.
4. Carefully remove supernatant with a pipet.
5. Add a small volume of buffer A (~0.2 ml) to wash the pellet. Carefully aspirate.
6. Depending on the use of the vesicles store them at −80°C or resuspend in the appropriate buffer (e.g., buffer A containing 1× protease inhibitors).

SUPPORT PROTOCOL 2

IMMUNOPURIFICATION OF SV SUBPOPULATIONS

Synaptic vesicles or their subpopulations can be isolated by immunoadsortion. Beads coated with antibodies specific to the cytosolic tails of universal synaptic vesicle markers like VAMP II (Baumert et al., 1989) or SV2 (Floor and Feist, 1989) can retrieve synaptic vesicles selectively and with high efficiency. This procedure is helpful to determine if a new antigen suspected to be in synaptic vesicles is indeed present, and not just a protein present in an unidentified membrane population comigrating with synaptic vesicles. Furthermore, the use of transporter-specific synaptic vesicles allows the purification of vesicle subpopulations that contain specific neurotransmitters like glutamate (VGlut1-containing vesicles; Takamori et al., 2000a), GABA (VGAT-containing vesicles; Takamori et al., 2000b; see Table 3.12.1 for antibodies), or zinc (Salazar et al., 2004). A critical element in this protocol is the purity of the vesicles. Crude membrane fractions are not recommended, since some synaptic vesicle antigens might be present in nonsynaptic vesicle membranes as they traffic through different compartments. Also, nonspecific membrane background is substantially higher in cruder membrane preparations.

Materials

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 5% (w/v) BSA (fraction V, Boehringer Mannheim)
1000 × protease inhibitors (see recipe) *or* Boehringer Complete protease inhibitor tablets (Roche Applied Science)
Size-purified synaptic vesicles (Basic Protocol 2 or Alternate Protocol 2 or 3)
Primary antibody to protein of interest (Table 3.12.1)
Magnetic bead slurry (Dyanal): e.g., Dynabeads M450 sheep anti-mouse IgG–coated *or* Dynabeads M2800 sheep anti-rabbit IgG–coated beads, depending on species from which primary antibody was derived
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Laemmli sample buffer (*UNIT 17.3*)

Rotating platform
Dynal MPC-S magnetic stand
Beckman tabletop ultracentrifuge with TLA 120.2 rotor (or equivalent), and corresponding polycarbonate ultracentrifuge tubes

Prepare beads

1. Add 0.5 ml of PBS/5% BSA to a 1-ml screw-cap microcentrifuge tube.

   Snap-cap tubes should be avoided because they have the tendency to trap beads in between the cap and the tube.

2. Add 40 µl of the magnetic bead slurry plus the desired amount of primary antibody.

   Note that the great majority of the bead slurry is buffer. Check that the beads are coupled to the right antibody to recognize the species from which the immunoglobulin of the primary antibody was derived. The anti–synaptic vesicle antibody should be tested at different concentrations. Use a preimmune antibody or an unrelated IgG at the same concentration to determine nonspecific membrane binding to beads. If planning to perform immunoblot on the isolated membranes, set up one tube without any antibody to determine cross-reactivity between the immunoglobulins bound to the beads and the secondary antibodies used in the immunoblot procedure.

3. Incubate at room temperature for 2 hr with slow agitation on a rotating platform.

   Alternatively, incubate overnight at 4°C.

   Prebinding is performed to avoid having free antibody compete with the bead-bound antibody.

4. Microcentrifuge for 2 to 5 sec at maximum speed to remove the beads from the cap.

   This step is not intended to sediment all the beads but rather to avoid losing them on the cap.

5. Put the tubes in the magnetic stand at 4°C. Aspirate and add 1 ml of PBS/5% BSA and wash with slow agitation on a rotating platform for 10 min. Repeat wash.

   IMPORTANT NOTE*: After aspiration, new buffer should be added immediately to avoid drying the beads.*

Bind vesicles

6. Prepare tubes containing 0.5 ml of PBS/5% BSA containing 1× protease inhibitors. Add size-purified synaptic vesicles and the prebound beads from step 5.

   Do not use membrane preparations that have not been size-purified, since the nonspecific background will greatly increase. Between 1 and 5 µg of vesicles is appropriate to start with, but the optimal concentration of vesicles should be determined for each antibody.

7. Incubate with slow rotary agitation for 3 hr at 4°C. Repeat steps 4 and 5 but perform at 4°C.

8. Recover supernatant and save it. Keep supernatant on ice.

9. Add 1 ml of ice cold PBS/5% BSA and rotate beads for 10 min at 4°C. Centrifuge and put in the magnetic stand as described in steps 4 and 5.

10. Repeat washes for a total number of three.

11. Perform a last wash of 10 min in ice-cold PBS to remove the excess BSA.

Prepare samples for storage

12. Add 10 to 40 μl Laemmli sample buffer to the beads and store at −80°C.

13. Transfer supernatants from step 8 to a TLA tabletop ultracentrifuge polycarbonate tube. Dilute the supernatant with 1 volume of PBS and centrifuge 1 hr at 200,000 × *g*, 4°C, to sediment the unbound synaptic vesicles. Add 10 to 40 μl Laemmli sample buffer to pellet and store at −80°C.

SUPPORT PROTOCOL 3

TUBE SILICONIZATION

The authors recommend siliconizing all the surfaces that will be in contact with the purified vesicles. Further into the purification protocol, vesicles will have a tendency to adsorb to plastic and glass. Sigmacote is a special silicone solution in heptane that readily forms a covalent, microscopically thin film that is water resistant.

Materials

Sigmacote (Sigma)
Glass- or plasticware to be siliconized

1. In a fume hood, evenly cover the plastic or glass surface and recover the solution to apply to the next piece of glassware or plasticware.

2. Let stand overnight in a chemical fume hood to dry.

For ultracentrifugation tubes, the authors recommend siliconizing polycarbonate tubes. Microcentrifuge tubes and glass pipets can be autoclaved before use.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

Prepare 10× stock:
100 mM HEPES, pH 7.4
1.5 M NaCl
10 mM EGTA
1 mM $MgCl_2$
Autoclave
Store up to several months at room temperature
1 × working solution: Dilute to 1× with water just before use.

Protease inhibitors, 1000×

Prepare the following together in H_2O:
5 mg/ml leupeptin
10 mg/ml aprotinin
1 mg/ml chymostatin
Store up to 2 to 3 months at −20°C
Separately prepare the following stocks:
10 mM benzamidine in ethanol (store up to 2 to 3 months at −20°C)
2 mg/ml antipain in H_2O (store up to 2 to 3 months at −20°C)
5 mg/ml pepstatin in methanol (store up to 2 to 3 months at −20°C)
1 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol (prepare fresh)
Add to solutions at 1× concentration as recommended in the protocols. PMSF is very sensitive to water, so it should be added fresh to the solutions just before contact with biological material.

SB buffers

SB40:
5 mM HEPES, pH 7.4
40 mM sucrose
SB50:
5 mM HEPES, pH 7.4
50 mM sucrose
SB320:
5 mM HEPES, pH 7.4
320 mM sucrose
SB800:
5 mM HEPES pH 7.4
800 mM sucrose
Prepare fresh

COMMENTARY

Background Information

Synaptic vesicles are the most abundant membrane-enclosed organelle in vertebrate cells. It has been estimated that each neuron possesses, on average, 10^6 synaptic vesicles, and that the total number in the mammalian central nervous system is $\sim 10^{17}$ (Jahn and Sudhof, 1993). About 5% of total nerve tissue protein is associated with synaptic vesicles, implying that these organelles can be enriched ~20 fold over the homogenate. In fact, the enrichment estimated through measurements of the peripherally associated synaptic vesicle protein synapsin I (15.5 fold; Huttner et al., 1983), the integral membrane protein SV2 (18 fold; Floor et al., 1988), or vesicle acetylcholine content (28 fold; Nagy et al., 1976) are in agreement with theoretical calculations.

The above protocols are based on the pioneering work of Whittaker and Kelly (Whittaker et al., 1972; Nagy et al., 1976; Carlson et al., 1978; Wagner et al., 1978). The authors of this unit recommend exploring those papers because of the detailed description of the procedural aspects of vesicle purification. Two chracteristic features of synaptic vesicles are central to all purification protocols. Synaptic vesicles do not contain secretory proteins because they are constantly fusing and rapidly reforming at the presynaptic plasma membrane (Murthy and De Camilli, 2003). The lack of protein in the lumen of a synaptic vesicle results in a density that is lower compared to other organelles (see *UNIT 3.5*, Background Information). Synaptic vesicle density depends on the source of tissue analyzed and the centrifugation medium. However, the density of synaptic vesicles ranges from 1.045 ± 0.002 g/cm^3 by isoosmotic sucrose sedimentation (Carlson et al., 1978) to 1.11 ± 0.003 g/cm^3 by hyperosmotic sucrose sedimentation (Clift-O'Grady et al., 1990). This low density results from the fact that 74% of the synaptic vesicle mass is water, 14% is phospholipids, and only 2.6% is protein (Wagner et al., 1978).

The second characteristic utilized in purification procedures is synaptic vesicle size. Synaptic vesicles are small and highly uniform in size. Morphometric analysis of electron micrographs of fixed synaptic vesicles revealed diameters of ~30 to 50 nm (Kim et al., 2000). The values determined by electron microscopy of fixed tissue have been elegantly confirmed by biophysical analysis of freshly purified synaptic vesicles (Wagner et al., 1978). Vesicle size varies depending on the species in which synaptic vesicles are analyzed and the type of nerve terminal the vesicles are derived from. For example, synaptic vesicles from the *Drosophila* neuromuscular junction differ in size depending on the muscle that they innervate (Karunanithi et al., 2002). The synaptic vesicle's small and uniform size has facilitated size-purification approaches that greatly enhance the purity of these vesicles. Basic Protocol 2 and Alternate Protocols 2 and 3 take advantage of the size of synaptic vesicles.

Homogenization of the tissue is a variable that was extensively analyzed in earlier work on the *Torpedo* fish electric organ (Whittaker et al., 1972; Carlson et al., 1978). Measurements of membrane-enclosed ATP or acetylcholine indicated that blending in the Waring Blendor is more efficient in releasing vesicles than Potter-Elvehjem homogenization. Yield can be increased further by freeze-thawing followed by use of the Waring Blendor (Carlson et al., 1978). Blending of tissue in the Waring Blendor is the basis of Basic Protocol 1, which

was purposely designed to avoid hypotonic lysis of synaptosomes. Tissue is homogenized in a buffer of physiological ionic strength to minimize the nonspecific adsorption of soluble proteins to synaptic vesicles. When vesicles isolated by this procedure are size fractionated by Sephacryl-1000 chromatography, vesicles elute as a peak whose polypeptide composition (by protein gel staining) appears to be similar to that obtained via the Huttner procedure (see Alternate Protocol 1 and Clift-O'Grady et al., 1990). However, Basic Protocol 1 removes loosely associated proteins such as synapsin I from the vesicle's cytoplasmic surface, a process that occurs at ionic strengths as low as 10 mM salt (Huttner et al., 1983).

Basic Protocol 1 can be used with mammalian brain tissue and fish electric organ. The size-purification procedures are universal because the morphological and biophysical features of synaptic vesicles are conserved from invertebrates to humans. In fact glycerol sedimentation has been used in *Drosophila* (van de Goor et al., 1995) as well as neuroendocrine cell lines to identify this type of vesicle (Clift-O'Grady et al., 1990).

Critical Parameters

The two synaptic vesicle purification protocols described here differ in vesicle yield, due to differences in the technique of tissue homogenization and the buffer in which purification is performed. The Huttner method (low salt and gentle homogenization) results in lower yield compared to the Grady protocol, but has an advantage in that it preserves low-affinity protein interactions with synaptic vesicle membranes. In contrast, the Clift-O'Grady protocol utilizes a physiological salt buffer and homogenization is performed in a Waring Blendor. Thus, the Clift-O'Grady protocol results in a higher synaptic-vesicle yield, but proteins that are loosely associated with vesicle membranes are lost. Both protocols are capable of purifying integral membrane proteins residing in synaptic vesicles. However, if there is no available information as to how a protein of interest associates with the membrane, the authors of this unit recommend performing pilot experiments in which the tissue is homogenized in either buffer A or SB40 buffer. Follow steps 1 to 4 of Basic Protocol 1, then sediment the supernatant from step 4 in a TLA centrifuge (see Support Protocol 1). The resulting pellet contains a crude small vesicle membrane fraction that can be tested for the presence of the protein of interest. Proteolytic sensitivity and biological activity of the protein should also be tested to determine if the protein of interest can withstand a 2-day purification procedure.

An equally important consideration is the tendency for synaptic vesicles to adsorb to glass and plastic surfaces. During the initial steps of purification, the high concentration of cytosolic proteins and cellular membranes reduces synaptic vesicle adsorption. However, as synaptic vesicles become increasingly purified, adsorption increases and can compromise the final yield. To avoid this problem the authors recommend siliconizing all glass and plastic materials that will come in contact with synaptic vesicles. To minimize adsorption to the CPG column, it is recommended that glycerol-coated beads, which are available pre-coated from the supplier, be used.

If greater yield is desired, the authors definitely do not recommend increasing the amount of protein loaded into the gradients or changing tissue/buffer ratios. When additional yield is desired, the user is encouraged to perform additional purifications and pool the vesicles.

Assessing vesicle purity

The synaptic vesicle purification protocols presented in this unit have been extensively characterized both for the enrichment of synaptic vesicle markers as well as for the uniformity of the isolated vesicles by electron microscopy (see Fig. 3.12.3). Early work by Whittaker explored whether classical enzymatic markers of mitochondria, ER, and lysosomes were present in the synaptic-vesicle fraction. Although these markers are excluded from purified synaptic vesicles, fractions containing vesicles should not be considered absolutely pure, but rather highly enriched. Other vesicle intermediaries present in brain tissue cells may have identical biophysical properties to synaptic vesicles and could possibly copurify with synaptic vesicles. Users should test whether their preparations contain other vesicles or organelles in which the protein of interest is suspected to be present. Alternatively, immunoisolation of vesicles using synaptic vesicle–specific antibodies can provide a solid indication as to the presence of a certain protein in synaptic vesicles.

The authors strongly encourage the user to characterize the final vesicle preparation not only for the enrichment of synaptic vesicles using the antibodies described in Table 3.12.1, but also to ensure that other organelle markers are absent. A good collection of compartment-specific antibodies available commercially can be found at *http://www.bdbiosciences.com*.

Table 3.12.2 Proteins Identified in Size-Purified Synaptic Vesicles

Protein	Reference (PMID no.)
Casein kinase I	7622570
Chloride channel ClC3	11182090, 15073168
Cysteine string protein	12956868
High-affinity choline transporter (CHT1)	14585997
Munc 18	8247129, 15217342
Myosin V	9199173
Phosphatidylinositol 4-kinase type II α	12646710
rab3	9530492
rab11	14627637
SCAMPs	11050114
SNAP-25	15217342
SV2A	1519064
SV2B	7681585
SV2C	10625067
SVOP	9801366
Synapsin I	8430330, 10212475
Synapsin II	8430330, 10212475
Synapsin III	8430330, 10212475
Synaptogyrin 1	8557746
Synaptogyrin 3	8557746
Synaptophysin	15057942
Synaptoporin	2203533
Synaptotagmin I	9530492
Syntaxin 1	15217342
Synuclein α	12461550
Vacuolar ATPase subunits of sector V0	11836511
Vacuolar ATPase subunits of sector V1	11836511
VAMP I	12154365
VAMP II	12154365
VAT1	2483112
Vesicular acetylcholine transporter (VAchT)	7938002
Vesicular GABA transporter (VGAT)	9349821
Vesicular glutamate transporter I (VGlut1)	15102489
Vesicular glutamate transporter II (VGlut2)	15102489
Vesicular glutamate transporter III (VGlut3)	15102489
Vti1a-β	10908612
Zinc transporter ZnT3	8962159, 14657250

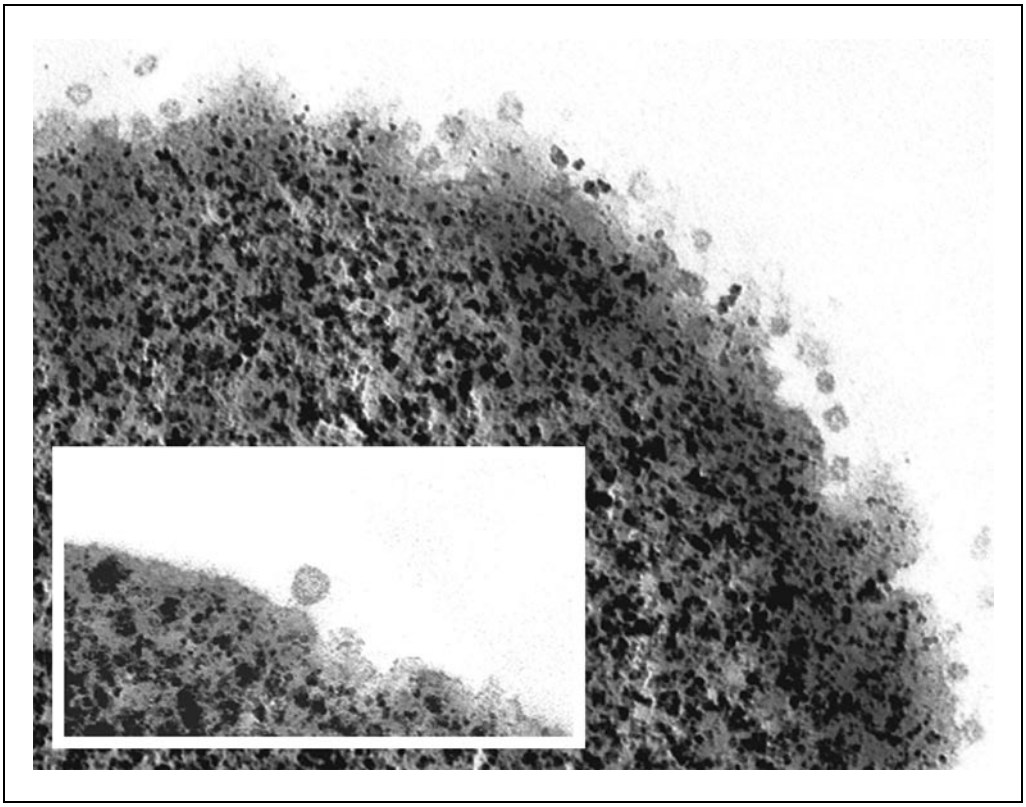


Figure 3.12.3 Electron microscopy of purified synaptic vesicles. Synaptic vesicles isolated by glycerol velocity sedimentation (fractions 6 to 10) were incubated with magnetic beads coated with antibodies against the cytosolic tail of synaptophysin (SY38) as described in Support Protocol 2. Beads were extensively washed and processed for transmission electron microscopy. The dark and granular circular profile that covers two thirds of the image and extends from the lower left-hand corresponds to a sectioned magnetic bead. Note the presence of multiple vesicle profiles of ~40 nm in diameter on the bead horizon. Control beads with mouse IgG do not display synaptic vesicles on their surface. Insert shows a magnification of an archetypical synaptic vesicle.

Anticipated Results

The outcome of the purification should be an enrichment of synaptic vesicle antigens to about 20- to 30-fold over the starting homogenate. A good record of the volumes and protein concentrations of all the fractions should be maintained. The preparations yielded by the protocols described here have been analyzed for the presence of contaminant membranes using electron microscopy and biophysical criteria, as well as for the presence of classical enzymatic markers of plasma membrane, mitochondria, and endoplasmic reticulum. Although these protocols are well established and characterized, users should determine whether the synaptic vesicles obtained contain contaminant membranes. The use of antibodies against compartment-specific antigens is strongly encouraged. It should be noted that for accurate immunoblot analysis it is important to maintain sample integrity throughout the procedure. Always maintain protease inhibitors in all buffers and store fractions at −80°C. After adding sample buffer, samples must under no circumstances be boiled, because many synaptic vesicle proteins form aggregates that do not efficiently enter the gel or that run as smears. In the authors' laboratory, samples are routinely heated to 70°C for 5 min. In addition, some antigens (SV2, for example) electrophorese differently due to species, post-translational modifications (Buckley et al., 1983; Buckley and Kelly, 1985), or differential isoforms (Bajjalieh et al., 1994; Janz and Sudhof, 1999).

Table 3.12.2 tabulates proteins identified in size-purified synaptic vesicles.

Literature Cited

Bajjalieh, S.M., Frantz, G.D., Weimann, J.M., McConnell, S.K., and Scheller, R.H. 1994. Differential expression of synaptic vesicle protein 2 (SV2) isoforms. *J. Neurosci.* 14:5223-5235.

Baumert, M., Maycox, P.R., Navone, F., De Camilli, P., and Jahn, R. 1989. Synaptobrevin: An integral membrane protein of 18,000 daltons present in

small synaptic vesicles of rat brain. *EMBO J.* 8:379-384.

Buckley, K. and Kelly, R.B. 1985. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J. Cell Biol.* 100:1284-1294.

Buckley, K.M., Schweitzer, E.S., Miljanich, G.P., Clift-O'Grady, L., Kushner, P.D., Reichardt, L.F., and Kelly, R.B. 1983. A synaptic vesicle antigen is restricted to the junctional region of the presynaptic plasma membrane. *Proc. Natl. Acad. Sci. U.S.A.* 80:7342-7346.

Carlson, S.S., Wagner, J.A., and Kelly, R.B. 1978. Purification of synaptic vesicles from elasmobranch electric organ and the use of biophysical criteria to demonstrate purity. *Biochemistry* 17:1188-1199.

Clift-O'Grady, L., Linstedt, A.D., Lowe, A.W., Grote, E., and Kelly, R.B. 1990. Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12. *J. Cell Biol.* 110:1693-1703.

Donovan, J. and Brown, P. 1995. Euthanasia. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.8.1-1.8.4. John Wiley & Sons, Hoboken, N.J.

Floor, E. and Feist, B.E. 1989. Most synaptic vesicles isolated from rat brain carry three membrane proteins, SV2, synaptophysin, and p65. *J. Neurochem.* 52:1433-1437.

Floor, E., Schaeffer, S.F., Feist, B.E., and Leeman, S.E. 1988. Synaptic vesicles from mammalian brain: Large-scale purification and physical and immunochemical characterization. *J. Neurochem.* 50:1588-1596.

Huttner, W.B., Schiebler, W., Greengard, P., and De Camilli, P. 1983. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III: Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J. Cell Biol.* 96:1374-1388.

Jahn, R. and Sudhof, T.C. 1993. Synaptic vesicle traffic: Rush hour in the nerve terminal. *J. Neurochem.* 61:12-21.

Janz, R. and Sudhof, T.C. 1999. SV2C is a synaptic vesicle protein with an unusually restricted localization: Anatomy of a synaptic vesicle protein family. *Neuroscience* 94:1279-1290.

Karunanithi, S., Marin, L., Wong, K., and Atwood, H.L. 2002. Quantal size and variation determined by vesicle size in normal and mutant *Drosophila* glutamatergic synapses. *J. Neurosci.* 22:10267-10276.

Kim, S., Atwood, H.L., and Cooper, R.L. 2000. Assessing accurate sizes of synaptic vesicles in nerve terminals. *Brain Res.* 877:209-217.

Murthy, V.N. and De Camilli, P. 2003. Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci.* 26:701-28.

Nagy, A., Baker, R.R., Morris, S.J., and Whittaker, V.P. 1976. The preparation and characterization of synaptic vesicles of high purity. *Brain Res.* 109:285-309.

Salazar, G., Love, R., Werner, E., Doucette, M.M., Cheng, S., Levey, A., and Faundez, V. 2004. The zinc transporter ZnT3 interacts with AP-3 and it is preferentially targeted to a distinct synaptic vesicle subpopulation. *Mol. Biol. Cell.* 15:575-587.

Takamori, S., Rhee, J.S., Rosenmund, C., and Jahn, R. 2000a. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 407:189-194.

Takamori, S., Riedel, D., and Jahn, R. 2000b. Immunoisolation of GABA-specific synaptic vesicles defines a functionally distinct subset of synaptic vesicles. *J. Neurosci.* 20:4904-4911.

van de Goor, J., Ramaswami, M., and Kelly, R. 1995. Redistribution of synaptic vesicles and their proteins in temperature-sensitive shibire(ts1) mutant *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 92:5739-5743.

Wagner, J.A., Carlson, S.S., and Kelly, R.B. 1978. Chemical and physical characterization of cholinergic synaptic vesicles. *Biochemistry* 17:1199-1206.

Whittaker, V.P., Essman, W.B., and Dowe, G.H. 1972. The isolation of pure cholinergic synaptic vesicles from the electric organs of elasmobranch fish of the family Torpedinidae. *Biochem J.* 128:833-45.

Contributed by Branch Craige,
Gloria Salazar, and Victor Faundez
Emory University
Atlanta, Georgia

Isolation of Clathrin-Coated Vesicles by Differential and Density Gradient Centrifugation

Endocytosis of nutrients, signaling receptors, and other cell surface regulatory proteins via clathrin-coated vesicles (CCVs) is essential for normal cellular function. CCVs are also involved in the transport of proteins, such as lysosomal hydrolases, from the *trans*-Golgi network to the endosomal/lysosomal system. CCVs are relatively uniform in size and, at 50 to 100 nm in diameter, they are among the smallest membranous organelles. Moreover, they are encased in a dense, proteinaceous coat, which contributes to the formation of the vesicular structure. Protocols for isolating CCVs take advantage of the small size and high density of these organelles by using differential centrifugation coupled with velocity and equilibrium gradients to separate CCVs from contaminating membranes.

The first reliable protocol for isolating CCVs was described by Pearse (1975), working with pig brain. CCVs were enriched in a microsomal fraction prepared by differential centrifugation. They were subsequently separated from larger and less dense membranes using velocity and equilibrium sedimentation, respectively, on linear sucrose gradients. Pearse (1982) later introduced a protocol (for purification of CCVs from human placenta) in which Ficoll and D_2O were substituted for sucrose. Most protocols currently in use are derived from these original protocols.

In Basic Protocol 1, the authors describe a procedure for isolating CCVs from adult rat brain. This procedure, which is based on the protocol of Maycox et al. (1992), uses differential centrifugation coupled with Ficoll and D_2O-sucrose density gradient centrifugation. The application of an additional step involving velocity sedimentation in linear sucrose gradients, as originally described by Wasiak et al. (2002), is also outlined. In Alternate Protocols 1 and 2, the authors describe how the same basic approach can be applied to the isolation of CCVs from developing rat brain and cell lines, respectively.

When applied to other tissues, such as rat liver, the steps outlined in Basic Protocol 1 yield CCV preparations that exhibit only modest enrichment and purity. In Basic Protocol 2, therefore, the authors describe a fractionation procedure that has been used to purify CCVs from rat liver. This protocol, based on that of Pilch et al. (1983), involves differential centrifugation coupled with velocity and equilibrium centrifugation using discontinuous sucrose gradients.

Following fractionation, it is necessary to characterize CCV enrichment and purity. One important method for doing so is the analysis of equal protein aliquots of the various subcellular fractions by SDS-PAGE (*UNIT 6.1*) using Coomassie blue staining and/or immunoblotting (*UNIT 6.2*) with antibodies against specific CCV proteins (e.g., clathrin). Thus, for each protocol, we indicate fractions from which aliquots should be retained for analysis. The purity of CCVs is best assessed by electron microscopy (EM). In the Support Protocol, we describe an EM procedure, based on that of Baudhuin et al. (1967), that involves filtration of the isolated vesicle fractions onto nitrocellulose filters. This procedure ensures a random sampling of membranes in the pellet fraction and is thus appropriate for quantitative evaluation of CCV purity.

NOTE: All protocols using live animals must first be approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for laboratory animal care and use.

Contributed by Martine Girard, Patrick D. Allaire, Francois Blondeau, and Peter S. McPherson
Current Protocols in Cell Biology (2005) 3.13.1-3.13.31

NOTE: All prepared solutions, glassware, centrifuge tubes, and equipment should be precooled, and samples should be kept on ice throughout unless otherwise noted.

NOTE: It is recommended that the purification of CCVs be monitored using clathrin as a marker. Each fraction that should be included in subsequent analyses is named, and the need to reserve aliquots of various fractions in screw-cap tubes is indicated throughout the protocols that follow. All reserved aliquots should be snap-frozen in liquid nitrogen and stored at −70°C.

BASIC PROTOCOL 1

ISOLATION OF CCVs FROM ADULT RAT BRAIN USING DIFFERENTIAL AND DENSITY GRADIENT CENTRIFUGATION

In this protocol, adult rat brains are homogenized in MES buffer at pH 6.5 (buffer A) using a glass-Teflon homogenizer. Figure 3.13.1 provides a flow chart summarizing the subsequent steps used to isolate CCVs. A microsomal fraction is first generated from the homogenate by performing two differential centrifugation steps. Next, the CCVs are separated from larger microsomal contaminants by velocity sedimentation through a Ficoll-sucrose solution. The CCVs are subsequently separated from less dense membranes by pelleting through a sucrose cushion prepared in D_2O. These steps represent the basic procedure as described by Maycox et al. (1992). The D_2O pellets are further fractionated on linear sucrose gradients (20% to 50%) as originally described by Wasiak et al. (2002).

Materials

10 Sprague-Dawley rats (150 to 200 g each)
1× buffer A (see recipe) containing protease inhibitors (see recipes)
Ficoll-sucrose solution (see recipe)
Deuterium oxide (D_2O; heavy water)–sucrose solution (see recipe)
20% and 50% sucrose solutions in 1× buffer A (see recipe)

Glass-Teflon homogenizers (assorted sizes; Wheaton) fitted to a power head
Sorvall high-speed centrifuge equipped with SS-34 fixed-angle rotor (or equivalent)
Battery-operated pipet filler
Ultracentrifuge with fixed-angle (Sorvall T-865, Beckman 45Ti, or equivalent) and swinging-bucket (Sorvall AH-629, Beckman SW-28, or equivalent) rotors
25-G, $\frac{5}{8}$-in. needle
13-ml thin-walled centrifuge tubes
2-mm-diameter glass capillary tubes
Two-chamber gradient maker
Peristaltic pump

Additional reagents and equipment for standard protein assays (*APPENDIX 3H*)

NOTE: Protease inhibitors are added (to a concentration of 1×) to 1× buffer A within 30 min of buffer use, except for PMSF, which should be added immediately after the buffer comes into contact with a protein sample.

Prepare a crude homogenate

1. Sacrifice 10 Sprague-Dawley rats (150 to 200 g), by decapitation or, alternatively, by CO_2 asphyxiation followed by decapitation.

   The sacrificing of rats must be supervised or carried out by an experienced animal technician according to specific animal care protocols at the investigator's institution.

2. Open the rats' skulls with scissors, and remove and weigh the brains. After weighing, transfer the brains to a beaker and add enough ice-cold 1× buffer A so that they are completely covered.

   Ten rat brains typically weigh 15 to 20 g.

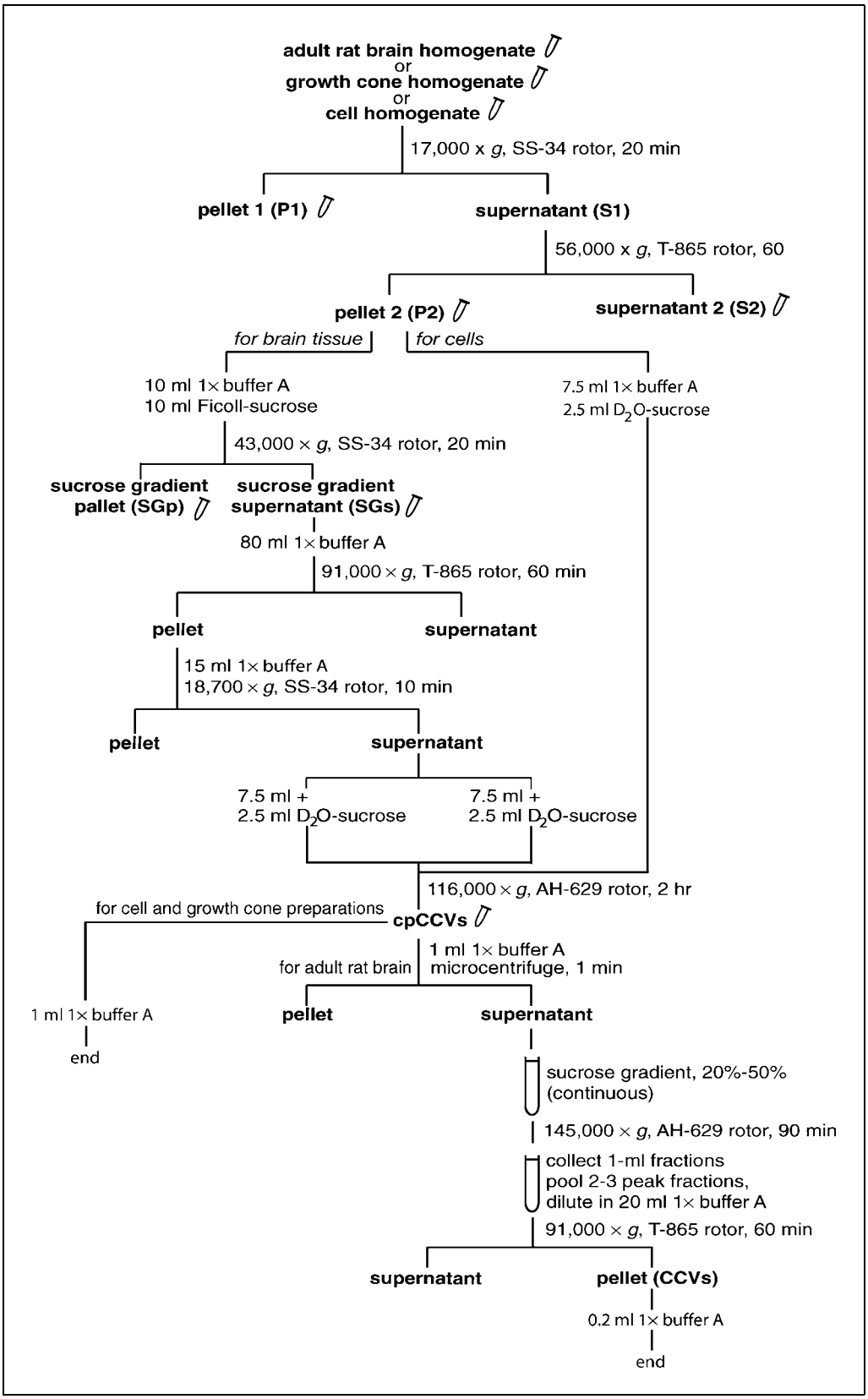


Figure 3.13.1 Flow chart summarizing Basic Protocol 1 and Alternate Protocol 2. The flow chart also covers Alternate Protocol 1 after the lysis of growth cones. The stylized microcentrifuge tube denotes fractions from which an aliquot should be retained for analysis by SDS-PAGE. CCVs, clathrin-coated vesicles; cpCCVs, cusion-pellet clathrin-coated vesicles.

3. Once all of the brains have been removed and weighed, decant and discard the 1× buffer A and transfer the brains to the mortar of a 55-ml glass-Teflon homogenizer.

   A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process all of the rat brains that have been collected.

4. Add 5 ml of 1× buffer A for every gram of rat brain in the homogenizer. Homogenize using 10 strokes of the Teflon pestle with the power head set at 1500 rpm.

5. Pool homogenates in a 250-ml graduated cylinder and add ice-cold 1× buffer A to a final volume of 10 ml for every gram of rat brain present (e.g., if 20 g of rat brain is present, add ice-cold 1× buffer A to a final volume of 200 ml). Reserve a 0.5-ml aliquot of the pooled homogenate, but do not discard the rest.

Prepare a microsomal (P2) fraction by differential centrifugation

6. Transfer pooled homogenate to six 40-ml centrifuge tubes and centrifuge 20 min at 17,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

7. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a 250-ml glass beaker on ice.

8. Resuspend one of the pellets (P1; step 6) in 10 ml of 1× buffer A by pipetting up and down using a pipet filler and pipet. Transfer the suspension to the mortar of a 35-ml glass-Teflon homogenizer. Prepare an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

9. Transfer S1 (step 7) to eight 25-ml centrifuge tubes and centrifuge 60 min at 56,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

10. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot of S2 from one of the tubes and discard the rest.

11. Combine all pellets (P2; step 9) and use a pipet filler and pipet to resuspend in a total of 10 ml of 1× buffer A.

    The head of a small Teflon homogenizer can be used to scrape the pellets out of their individual centrifuge tubes so that they can be combined.

12. Transfer the resuspended P2 pellets to the mortar of a 35-ml glass-Teflon homogenizer and prepare an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

13. Transfer the homogenized material to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the material once through the needle by slowly and evenly applying pressure to the plunger. Reserve a 0.25-ml aliquot of the P2 suspension, but do not discard the rest.

Centrifuge microsomal fraction on a Ficoll-sucrose density gradient

14. Add 10 ml ice-cold Ficoll-sucrose solution to the P2 suspension (∼10 ml) and gently mix by pipetting up and down. Transfer to a 40-ml centrifuge tubes.

15. Centrifuge 20 min at 43,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

16. Using a pipet filler and pipet, remove the supernatant (sucrose gradient supernatant, or SGs) and transfer to a 100-ml graduated cylinder.

17. Resuspend the pellet (sucrose gradient pellet, or SGp) in 5 ml of 1× buffer A by pipetting up and down. Transfer the suspension to the mortar of a 10-ml glass-Teflon

homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

18. Dilute the SGs from step 16 (20 ml) by adding 80 ml ice-cold 1× buffer A. Transfer to six 25-ml centrifuge tubes.

19. Centrifuge diluted SGs 60 min at 91,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

20. Using a pipet filler and pipet, remove and discard all supernatants.

21. Combine all pellets and use a pipet filler and pipet to resuspend in a total of 15 ml of 1× buffer A.

    The head of a small Teflon homogenizer can be used to scrape the pellets out of their individual centrifuge tubes so that they can be combined.

22. Transfer the resuspended material to the mortar of a 30-ml glass-Teflon homogenizer and homogenize using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

23. Transfer the homogenized material to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the material once through the needle by slowly and evenly applying pressure to the plunger, and collect in a 40-ml centrifuge tube.

24. Centrifuge 10 min at 18,700 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

25. Using a pipet filler and pipet, remove the supernatant (still referred to as the SGs) and transfer to a 50-ml graduated cylinder. Reserve a 0.5-ml aliquot and discard pellet.

Pellet CCVs through sucrose cushion

26. Split SGs into 2 aliquots of 7.5 ml and transfer each aliquot to a 13-ml thin-walled centrifuge tube designed for a Sorvall AH-629 swinging-bucket rotor.

27. Underlay each SGs sample with 2.5 ml D_2O-sucrose solution in the following way.

    a. Use a piece of Tygon tubing to attach a 5-ml syringe barrel to a 2-mm-diameter glass capillary tube.

    b. Load the 5-ml syringe barrel with D_2O-sucrose solution.

    c. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the centrifuge tube containing the 7.5-ml sample.

    d. Slowly eject 2.5 ml D_2O-sucrose solution into the centrifuge tube from the syringe.

28. Centrifuge samples 2 hr at 116,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

29. Using a pipet filler and pipet, remove and discard the supernatants.

30. Combine pellets (cushion-pellet CCVs, or cpCCVs) and resuspend in a total of 1 ml of 1× buffer A.

    The head of a small Teflon homogenizer can be used to scrape the pellets out of their individual centrifuge tubes so that they can be combined.

31. Transfer suspension to the mortar of a 3-ml glass-Teflon homogenizer.

32. Generate even cpCCV suspensions using 3 strokes of a Teflon pestle (from the glass-Teflon homogenizer) with the power head set at 1500 rpm. After homogenization, transfer the suspended material to a single microcentrifuge tube. Reserve a 100-µl aliquot of the homogenized suspension, but do not discard the rest.

The protocol can be stopped at this point, with the cpCCVs having been collected; if so, the cpCCV suspension can be divided into aliquots (to eliminate the need for repeated freezing/thawing in the future), transferred to screw-cap tubes, snap-frozen in liquid nitrogen, and stored at −70°C for up to 1 year. Otherwise, proceed to linear sucrose gradient fractionation.

Perform linear sucrose gradient fractionation

33. Transfer the cpCCV suspension to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the suspension once through the needle by slowly and evenly applying pressure to the plunger, and collect suspension in a microcentrifuge tube.

34. Microcentrifuge the cpCCV suspension 1 min at maximum speed, 0° to 4°C.

35. In a 13-ml thin-walled centrifuge tube designed for a Sorvall AH-629 swinging-bucket rotor, prepare a 12-ml linear gradient from equal volumes of 20% and 50% sucrose in 1× buffer A using a two-chamber gradient maker. Use a peristaltic pump to ensure an even flow rate (from the gradient maker to the centrifuge tube) of ∼2 to 3 ml/min.

36. Using a Pasteur pipet, remove the supernatant and gently layer it onto the top of the linear gradient. Discard pellet.

37. Centrifuge 90 min at 145,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

38. Collect approximately twelve fractions of 1 ml from the centrifuged gradient in the following way.

    a. Use a piece of Tygon tubing to attach a 2-mm-diameter glass capillary tube to a peristaltic pump.
    b. Gently position the exposed end of the capillary tube so that it rests on the bottom of the tube containing the centrifuged sucrose gradient.
    c. Activate the peristaltic pump and collect each fraction at a flow rate of ∼2 to 3 ml/min.

39. Use a standard protein assay (*APPENDIX 3H*) to determine which of the isolated fractions yield the highest protein signals.

    The highest protein signals should be found near the middle of the gradient (around fractions 6 to 8). The peak protein signal is directly correlated with the peak concentration of CCVs.

40. Pool the 2 to 3 fractions that yield the highest protein signals and dilute to a total volume of 20 ml in 1× buffer A. Transfer to a 25-ml centrifuge tube.

41. Centrifuge the pooled and diluted sample 60 min at 91,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

42. Decant and discard supernatant. Resuspend pellet (which contains CCVs) in 0.2 ml of 1× buffer A.

43. Transfer the resuspended CCVs to the mortar of a 1-ml glass-Teflon homogenizer and generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

44. Transfer the suspension to a screw-cap tube, snap-freeze in liquid nitrogen, and store at −70°C for up to 1 year.

    This CCV suspension can be divided into aliquots before freezing to eliminate the need for repeated freezing/thawing in the future.

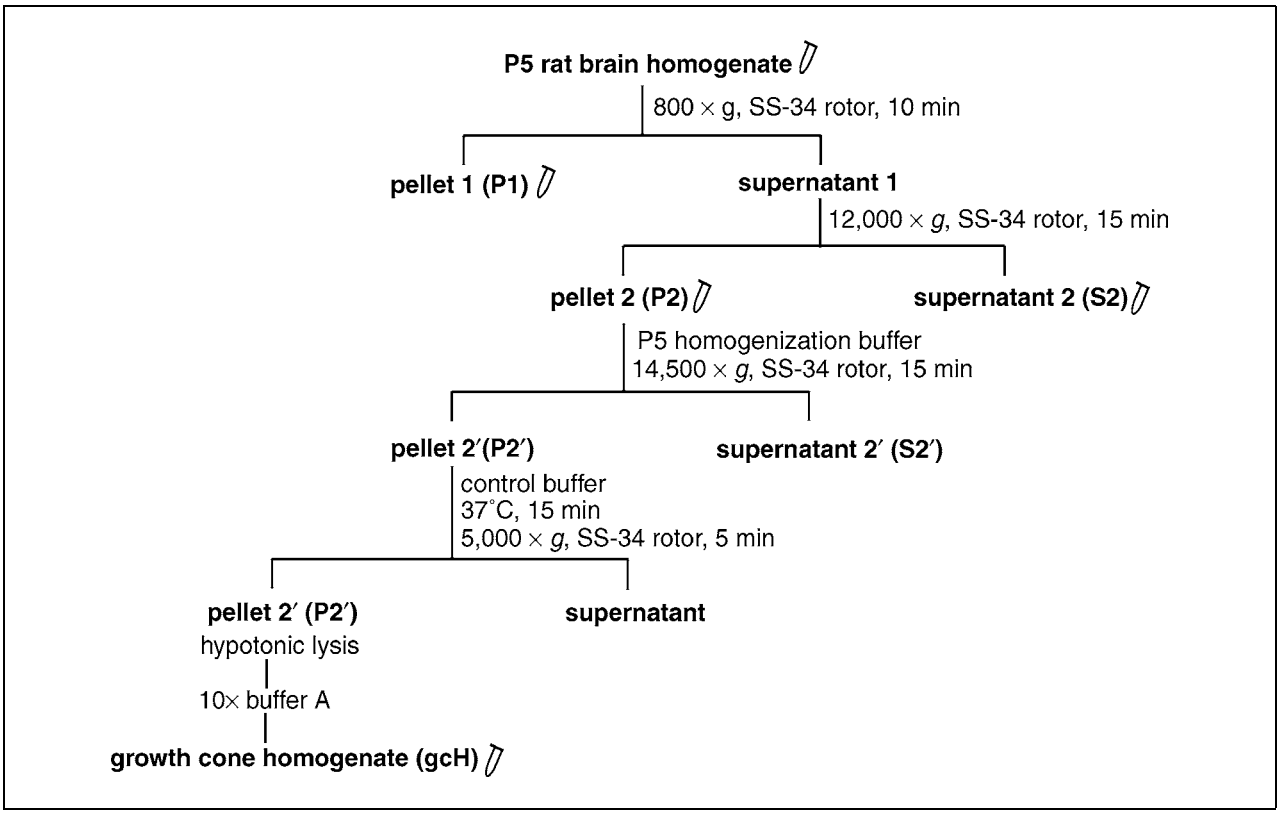


Figure 3.13.2 Flow chart summarizing the generation of crude growth cones in Alternate Protocol 1. The stylized microcentrifuge tube denotes fractions from which an aliquot should be retained for analysis by SDS-PAGE.

ISOLATION OF CCVs FROM DEVELOPING RAT BRAIN USING DIFFERENTIAL AND DENSITY GRADIENT CENTRIFUGATION

ALTERNATE PROTOCOL 1

This protocol is similar to Basic Protocol 1. It is derived from a procedure developed by Maycox et al. (1992) to isolate CCVs from synaptosomes, nerve terminals that are pinched off during homogenization. However, because Alternate Protocol 1 starts with P5 (postnatal day 5) rat brains, CCVs are being isolated primarily from growth cones, nerve terminal structures that guide growing neurites prior to synapse formation (Saito et al., 1992). Figure 3.13.2 presents a flow chart for the initial steps of the protocol. P5 rat brains are homogenized in HEPES buffer containing 0.3 M sucrose (P5 homogenization buffer). With gentle homogenization and isotonic conditions, the growth cones remain intact and are pelleted out at moderately low *g* forces. Next, the crude growth cones are lysed by hypotonic shock to release CCVs, and buffer A is added to stabilize the clathrin coats. The lysate is then subjected to a series of differential and density gradient centrifugation steps as described in Basic Protocol 1 and outlined in Figure 3.13.1.

Additional Materials *(also see Basic Protocol 1)*

15 litters of P5 rat pups
P5 homogenization buffer (see recipe) containing protease inhibitors (see recipes)
Control buffer (see recipe) containing protease inhibitors (see recipes)
10× buffer A (see recipe)

NOTE: Protease inhibitors are added (to a concentration of 1×) to P5 homogenization buffer, control buffer, and 1× buffer A within 30 min of buffer use, except for PMSF, which should be added to a given solution immediately after the solution comes into contact with protein samples.

Prepare crude growth cones

1. Sacrifice 15 litters of P5 rat pups by decapitation or, alternatively, by CO_2 asphyxiation followed by decapitation.

   The sacrificing of rats must be supervised or carried out by an experienced animal technician according to specific animal care protocols at the investigator's institution.

2. Open the rats' skulls with scissors, and remove and weigh the brains. After weighing, transfer the brains to a beaker and add enough ice-cold P5 homogenization buffer so that they are completely covered.

   In 15 litters, there will be ~200 pups total. The total wet weight of the brains collected will be ~110 g.

3. Once all brains have been removed and weighed, decant and discard the P5 homogenization buffer and transfer the brains to the mortar of a 55-ml glass-Teflon homogenizer.

   A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process all of the rat brains that have been collected.

4. Add 5 ml P5 homogenization buffer for every gram of rat brain in the homogenizer. Homogenize using 10 strokes of the Teflon pestle with the power head set at 900 rpm.

5. Pool homogenates in a 1000-ml graduated cylinder. Reserve a 0.5-ml aliquot of the pooled homogenate.

6. Transfer pooled homogenate to sixteen 40-ml centrifuge tubes and centrifuge 10 min at 800 × *g*, 0° to 4°C, in a Sorvall SS-34 (or equivalent) fixed-angle rotor.

   The SS-34 rotor fits eight tubes, so two rounds of centrifugation will be necessary.

7. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a glass beaker on ice.

   It is recommended that the supernatants be removed at the centrifuge, as transport of the tube can disturb the pellet, which is very soft.

8. Resuspend one of the pellets (P1; step 6) in 5 ml P5 homogenization buffer. Transfer the suspension to the mortar of a 10-ml glass-Teflon homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

9. Transfer S1 to sixteen 40-ml centrifuge tubes and centrifuge 15 min at 12,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

   The SS-34 rotor fits eight tubes, so two rounds of centrifugation will be necessary.

10. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot from one of the tubes and discard the rest.

11. Combine all pellets (P2; step 9) and use a pipet filler and pipet to resuspend in a total of 400 ml P5 homogenization buffer. Reserve a 0.5-ml aliquot of the P2 suspension.

12. Transfer the P2 suspension to sixteen 40-ml centrifuge tubes and centrifuge 15 min at 14,500 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

13. Using a pipet filler and pipet, remove and discard all supernatants.

14. Gently pipet 10 ml control buffer up and down over each pellet until only the soft, beige outer component of the pellet (P2′) is resuspended, leaving behind a dense, red core. Pool all of these suspensions in a 500-ml beaker and add control buffer up to a total volume of 200 ml.

The soft, beige outer component, which is weakly adherent to the walls of the centrifuge tube, contains the growth cones. The dense, red core that is left behind is mainly mitochondrial and is strongly adherent to the walls of the tube.

15. Incubate the pooled P2′ suspension 15 min in a 37°C water bath.

Lyse growth cones

16. Transfer the P2′ suspension to sixteen 40-ml centrifuge tubes and centrifuge 5 min at 5000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

17. Using a pipet filler and pipet, remove and discard all supernatants.

18. Combine all pellets and resuspend in a total of 20 ml of 1× buffer A.

19. Transfer suspension to a 500-ml graduated cylinder and add 160 ml ice-cold water. Immediately thereafter, homogenize the diluted suspension (in aliquots not exceeding the capacity of the homogenizer mortar) in a 55-ml glass-Teflon homogenizer using 10 strokes of the Teflon pestle with the power head set at 1500 rpm. Pool homogenized aliquots in a 500-ml graduated cylinder.

    All 160 ml of ice-cold water should be added at once in order to rapidly dilute the sample and lyse the growth cones.

    A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process the entire suspension.

20. Immediately after homogenization, add 20 ml of 10× buffer A to the suspension. Reserve a 0.5-ml aliquot of this suspension (referred to as the growth cone homogenate, gcH), but do not discard the rest.

    The addition of 10× buffer A stabilizes the clathrin coats that are present in the sample.

21. Proceed from Basic Protocol 1, step 6 through step 32, treating the gcH as if it were the homogenate suspension obtained in Basic Protocol 1, step 5. Reserve aliquots as directed throughout this portion of the Basic Protocol.

ISOLATION OF CCVs FROM CELL LINES

ALTERNATE PROTOCOL 2

In this protocol, as in Basic Protocol 1, cells are homogenized in 1× buffer A. As outlined in the flow chart in Figure 3.13.1, differential centrifugation is used to generate a microsomal fraction, and enrichment for CCVs is performed by centrifuging this microsomal fraction through a sucrose cushion prepared in D_2O.

Additional Materials *(also see Basic Protocol 1)*

HEK-293, COS-7, or HeLa cells
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Rubber stopper cut in half lengthwise (to yield straight edges along top and bottom)
Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

NOTE: Protease inhibitors are added (to a concentration of 1×) to 1× buffer A within 30 min of buffer use, except for PMSF, which is added immediately after the buffer comes into contact with a protein sample.

Prepare a cell extract

1. Grow 5 dishes (diameter, 10 cm) of HEK-293, COS-7, or HeLa cells to confluence in a 37°C incubator (*UNIT 1.1*).

2. Remove confluent cells from incubator. Using a vacuum source, aspirate culture medium from cells.

3. Wash cells twice, each time by gently adding 10 ml room temperature PBS to each dish of cells and then using a vacuum source to aspirate the PBS.

4. Add 4 ml of 1× buffer A to each plate. Detach cells from plate surface by scraping the bottom of the plate with the straight edge of the cut rubber stopper (i.e., the edge where the flat face that results from cutting and the bottom face of the stopper meet).

5. Transfer cell suspensions to the mortar of a 55-ml glass-Teflon homogenizer and homogenize using 10 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the homogenate.

Prepare a microsomal (P2) fraction

6. Transfer the homogenate to one to two 40-ml centrifuge tubes and centrifuge 20 min at 17,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

7. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a glass beaker on ice.

8. Resuspend one of the pellets (P1; step 6) in 5 ml of 1× buffer A. Transfer the suspension to the glass mortar of a 10-ml glass-Teflon homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

9. Transfer S1 to two 25-ml centrifuge tubes and centrifuge 60 min at 56,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

10. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot from one of the tubes and discard the rest.

11. Combine all pellets (P2; step 9) and use a pipet filler and pipet to resuspend in a total of 7.5 ml of 1× buffer A.

12. Transfer the resuspended P2 pellets to the mortar of a 10-ml glass-Teflon homogenizer and generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

13. Transfer the homogenized material to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the suspension through the needle once by slowly and evenly applying pressure to the plunger, and collect in a 13-ml thin-walled centrifuge tube designed for a Sorvall AH-629 swinging-bucket rotor. Reserve a 0.25-ml aliquot.

Pellet CCVs through sucrose cushion

14. Underlay the sample with 2.5 ml D_2O-sucrose solution in the following way.

    a. Use a piece of rubber tubing to attach a 5-ml syringe barrel to a 2-mm-diameter glass capillary tube.

    b. Load the 5-ml syringe barrel with D_2O-sucrose solution.

    c. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the centrifuge tube containing the 7.5-ml sample.

    d. Slowly eject 2.5 ml D_2O-sucrose solution into the centrifuge tube from the syringe.

15. Centrifuge sample 2 hr at 116,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

16. Using a pipet filler and pipet, remove and discard the supernatant. Resuspend the pellet (CCVs) in 0.25 ml of 1× buffer A.

17. Transfer the resuspended CCV pellet to the mortar of a 1-ml glass-Teflon homogenizer and generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

18. Transfer the suspension to a screw-cap tube, snap-freeze in liquid nitrogen, and store at −70°C for up to 1 year.

    This CCV suspension can be divided into aliquots before freezing to eliminate the need for repeated freezing/thawing in the future.

ISOLATION OF CCVs FROM ADULT RAT LIVER USING DIFFERENTIAL AND DENSITY GRADIENT CENTRIFUGATION

BASIC PROTOCOL 2

In this protocol, adult rat livers are homogenized in HEPES buffer, pH 7.4, containing 0.25 M sucrose (liver homogenization buffer) using a glass-Teflon homogenizer. Figure 3.13.3 provides a flow chart summarizing the subsequent steps used to isolate CCVs. First, a microsomal fraction is obtained from the homogenate by two differential centrifugation steps. (MES buffer, pH 6.5, is added after the first centrifugation step in order to stabilize the clathrin coat.) Next, CCVs are separated from larger microsomal contaminants by velocity sedimentation through discontinuous sucrose gradients. The CCVs are subsequently separated from less dense membranes by equilibrium sedimentation on discontinuous sucrose gradients, and they can be further purified by performing an additional velocity sedimentation step.

Basic Protocol 2 is based on the procedure described by Pilch et al. (1983), as modified from Blitz et al. (1977).

Materials

Ten Sprague-Dawley rats (150 to 200 g)
Liver homogenization buffer (see recipe) containing protease inhibitors (see recipes)
10× and 1× MES buffers (see recipes) containing protease inhibitors (see recipes)
5%, 10%, 20%, 30%, 40%, 45%, 50%, and 55%, and 60% (w/v) sucrose solutions in 1× MES buffer (see recipe)
Glass-Teflon homogenizers (assorted sizes; Wheaton) fitted with power heads
Sorvall high-speed centrifuge equipped with SS-34 fixed-angle rotor (or equivalent)
Battery-operated pipet filler
Ultracentrifuge with fixed-angle (Sorvall T-865, Beckman 45Ti, or equivalent) and swinging-bucket (Sorvall AH-629, Beckman SW-28, or equivalent) rotors
16- and 36-ml polypropylene tubes for Sorvall AH-629 (or equivalent) swinging-bucket rotor
2-mm-diameter glass capillary tubes

NOTE: Protease inhibitors are added to liver homogenization buffer and 1× MES buffer within 30 min of buffer use, except for PMSF, which is added to a given solution immediately after the solution comes into contact with protein samples.

Prepare crude homogenate

1. Starve 10 Sprague-Dawley rats overnight. After overnight starvation, sacrifice rats by decapitation or, alternatively, by CO_2 asphyxiation followed by decapitation.

   The starvation and sacrificing of rats must be supervised or carried out by an experienced animal technician in accordance with specific animal care protocols at the investigator's institution.

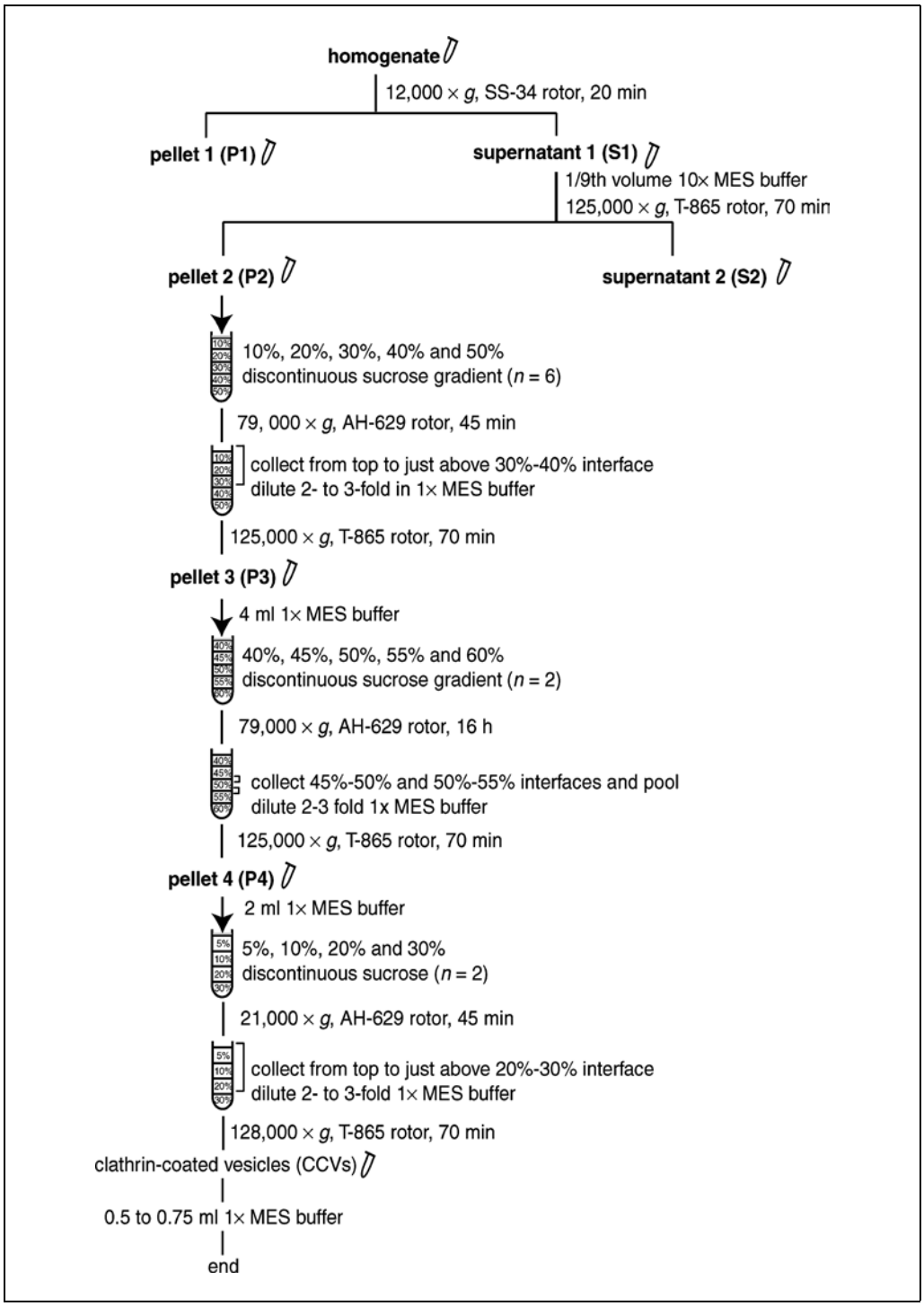


Figure 3.13.3 Flow chart summarizing Basic Protocol 2. The stylized microcentrifuge tube denotes fractions from which an aliquot should be retained for analysis by SDS-PAGE.

2. Open the rats' abdominal cavities and remove their livers. Transfer the livers to a chilled beaker containing ~100 ml liver homogenization buffer.
3. Once all brains have been removed and weighed, decant and discard the liver homogenization buffer. Cut the livers into small pieces with scissors and weigh.

   Ten rat livers typically weigh 60 to 100 g.

4. Transfer the liver tissue to the mortar of a 55-ml glass-Teflon homogenizer.

   A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process all of the rat liver tissue that has been collected.

5. Add 2 ml liver homogenization buffer for every gram of rat liver in the homogenizer. Homogenize using 10 to 15 strokes of the Teflon pestle with the power head set at 1500 rpm.

6. Pool homogenates in a 500-ml graduated cylinder and add ice-cold liver homogenization buffer to a final volume of 4 ml for every gram of rat liver present (e.g., if 100 g of rat liver is present, add ice-cold liver homogenization buffer to a final volume of 400 ml). Reserve a 0.5-ml aliquot of the pooled homogenate, but do not discard the rest.

Prepare a microsomal (P2) fraction by differential centrifugation

7. Transfer the pooled homogenates to eight to ten 25-ml centrifuge tubes and centrifuge 20 min at 12,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

8. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a 500-ml graduated cylinder on ice.

9. Resuspend one of the pellets (P1; step 7) in 25 ml of 1× MES buffer. Transfer the suspension to the mortar of a glasss-Teflon homogenizer. Generate an even suspension using 5 to 10 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

10. Add 1/9th volume of 10× MES buffer to the pooled S1 from step 8. Reserve a 0.5-ml aliquot.

11. Transfer the pooled S1 (with MES buffer added) to eight to ten 25-ml centrifuge tubes and centrifuge 70 min at 125,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

    The SS-34 rotor fits eight tubes, so two rounds of centrifugation may be necessary.

12. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot from one of the tubes and discard the rest.

13. In the following way, combine the pellets (P2; step 11) and resuspend them in a maximum of 30 ml of 1× MES buffer.

    a. Scrape the pellets out of their centrifuge tubes using the head of a small Teflon homogenizer (adding a combined total of 15 to 20 ml of 1× MES buffer to the tubes to facilitate pellet removal), and transfer the pellets (along with the added 1× MES buffer) to a chilled beaker. Resuspend the transferred pellets using a 1-ml pipet tip.

    b. Add a combined total of 10 to 15 ml of 1× MES buffer to the tubes from which the P2 pellets were removed, use the head of a small Teflon homogenizer to scrape the remnants of the pellets from the walls of these tubes, and then transfer the contents of the tubes to the chilled beaker from substep a.

       This procedure ensures maximum recovery of the P2 pellet, which is very thick.

14. Transfer the P2 suspension to the mortar of a 30-ml glass-Teflon homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.25-ml aliquot of the homogenized P2 suspension.

Isolate CCVs from P2 microsomes

15. Prepare six discontinuous sucrose gradients (sucrose concentrations: 10%, 20%, 30%, 40%, and 50%; 5 ml per layer), with each gradient being constructed in the following way.

    a. To a 36-ml polypropylene tube designed for a Sorvall AH-629 swinging-bucket rotor, add 5 ml of a 10% sucrose solution.

    b. Use a piece of Tygon tubing to attach a 10-ml syringe barrel to a 2-mm-diameter glass capillary tube.

       Alternatively, a syringe equipped with a blunt-end pipetting needle can be used to dispense the gradient solutions.

    c. Load the syringe barrel with the 20% sucrose solution.

    d. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the 36-ml polypropylene tube in which the sucrose gradient is being constructed.

    e. Slowly eject 5 ml of the 20% sucrose solution. Discard the sucrose solution remaining in the syringe barrel.

    f. Repeat substeps c to e three times, first using a 30% sucrose solution, then using a 40% sucrose solution, and finally using a 50% sucrose solution.

    g. Mark all interfaces with an indelible marker.

       It is important to mark all interfaces with an indelible marker, as this will facilitate sample recovery.

16. Using a Pasteur pipet, gently layer 5 ml of the P2 suspension on top of each of the six discontinuous sucrose gradients prepared in step 15.

17. Centrifuge gradients 45 min at 79,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

18. For each gradient, using a Pasteur pipet and starting from the top of the tube, collect all of the liquid down to (but not including) the interface between the 30% and 40% sucrose solutions. Transfer collected liquid to a 250-ml graduated cylinder.

19. Add sufficient 1× MES buffer to dilute the collected sample 2- to 3-fold.

20. Transfer diluted sample to eight to twelve 25-ml centrifuge tubes and centrifuge 70 min at 125,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

21. Decant and discard all supernatants. Resuspend pellets (P3) in a total of 4 ml of 1× MES buffer and combine.

    The head of a small Teflon homogenizer can be used to scrape the pellets from their individual centrifuge tubes so that they can be combined.

22. Transfer P3 suspension to the mortar of a 10-ml glass-Teflon homogenizer and generate an even suspension using 3 to 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.25-ml aliquot of the P3 suspension.

23. Prepare two discontinuous sucrose gradients (sucrose concentrations: 40%, 45%, 50%, 55%, and 60%; 5.5 ml per layer) using the procedure outlined in step 15.

    Note that the gradients constructed in this step differ from those constructed in step 15 only in terms of their layer volumes and sucrose concentrations.

24. Using a Pasteur pipet, layer 2 ml of the P3 suspension on top of each of the two discontinuous sucrose gradients.

25. Centrifuge gradients 16 hr at 79,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

26. From each gradient, collect the interface between the 45% and 50% sucrose solutions and the interface between the 50% and 55% sucrose solutions in the following way.

    a. Using a Pasteur pipet and starting from the top of the tube, remove and discard all of the contents of the tube down to a point just above the interface between the 45% and 50% sucrose solutions.

    b. Gently remove the material at the interface between the 45% and 50% sucrose solutions and transfer it to a glass beaker on ice.

    c. Continue by removing and discarding the contents of the tube down to a point just above the interface between the 50% and 55% sucrose solutions.

    d. Gently remove the material at the interface between the 50% and 55% sucrose solutions and add it to the material from the interface between the 45% and 50% sucrose solutions.

    e. Determine the volume of the collected material using a pipet filler and pipet, and then return the material to the glass beaker.

27. Dilute the sample 2- to 3-fold with 1× MES buffer.

28. Transfer diluted sample to four to eight 25-ml centrifuge tubes and centrifuge 70 min at 125,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

29. Decant and discard supernatants. Resuspend pellets (P4) in a total of 2 ml of 1× MES buffer and combine.

    The head of a small Teflon homogenizer can be used to scrape the pellets from their individual centrifuge tubes so that they can be combined.

30. Transfer P4 suspension to the mortar of a 10-ml glass-Teflon homogenizer and generate an even suspension using 3 to 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.25-ml aliquot of the P4 suspension.

31. Prepare two discontinuous sucrose gradients (sucrose concentrations: 5%, 10%, 20%, and 30%; 3.0 ml per layer), with each gradient being constructed in the following way.

    a. To a 16-ml polypropylene tube designed for a Sorvall AH-629 swinging-bucket rotor, add 3.0 ml of a 5% sucrose solution.

    b. Use a piece of Tygon tubing to attach a 10-ml syringe barrel to a 2-mm-diameter glass capillary tube.

        Alternatively, a syringe equipped with a blunt-end pipetting needle can be used to dispense the gradient solutions.

    c. Load the syringe barrel with the 10% sucrose solution.

    d. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the 16-ml polypropylene tube in which the sucrose gradient is being constructed.

    e. Slowly eject 3.0 ml of the 10% sucrose solution. Discard the remaining sucrose solution in the syringe barrel.

f. Repeat substeps c to e twice, first using a 20% sucrose solution and then using a 30% sucrose solution.

g. Mark all interfaces with an indelible marker.

It is important to mark all interfaces with an indelible marker, as this will facilitate sample recovery.

32. Using a Pasteur pipet, layer 1 ml of the P4 suspension on top of each of the two discontinuous sucrose gradients.

33. Centrifuge gradients 45 min at 21,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

34. For each gradient, using a Pasteur pipet and starting from the top of the tube, collect the contents of the tube down to a point just above the interface between the 20% and 30% sucrose solutions. Transfer collected material to a 100-ml graduated cylinder.

35. Dilute the sample 2- to 3-fold with 1× MES buffer.

36. Transfer diluted sample to two to four 25-ml centrifuge tubes and centrifuge 70 min at 128,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

37. Decant and discard supernatants. Using a pipettor, resuspend pellets (CCVs) in a total of 0.5 to 0.75 ml of 1× MES buffer and combine.

38. Transfer the suspension to a screw-cap tube, snap-freeze in liquid nitrogen, and store at −70°C for up to 1 year.

This CCV suspension can be divided into aliquots before freezing to eliminate the need for repeated freezing/thawing in the future.

SUPPORT PROTOCOL

ANALYSIS OF CCV PURITY BY ELECTRON MICROSCOPY

This protocol is derived from the method of Baudhuin et al. (1967). It is specifically designed to analyze subcellular particles generated by centrifugation and to ensure random sampling throughout a membrane pellet. Isolated membrane fractions that have been enriched for CCVs are fixed using glutaraldehyde and then applied to Millipore filters, generating very thin pellicles of packed particles. The CCVs and contaminating membranes in these samples are further fixed with osmium tetroxide and stained with tannic acid and uranyl acetate (Simionescu and Simionescu, 1976), after which the filters are embedded in Epon for EM analysis.

Materials

Fresh CCVs in 1× buffer A or 1× MES buffer (see Basic Protocol 1, Basic Protocol 2, Alternate Protocol 1, or Alternate Protocol 2)
1× buffer A (see recipe), filtered
1× MES buffer (see recipe), filtered (use only if CCVs were prepared using Basic Protocol 2)
25% (w/v) glutaraldehyde solution
Nitrogen gas
Sodium cacodylate buffer (see recipe), 4°C
Osmium tetroxide solution (see recipe), 4°C
Tannic acid solution (see recipe), 4°C
Sodium sulfate solution (see recipe), 4°C
Uranyl acetate solution (see recipe), 4°C, filtered
Maleate buffer (see recipe), 4°C

50%, 70%, 90%, 95%, and 100% (all v/v) ethanol
Propylene oxide
Epon solution (see recipe)
3:1, 1:1, and 1:3 (all w/w) Epon solution (see recipe)/propylene oxide
Platform rocker
Filter unit (Baudhuin et al., 1967; also see annotation to step 3), including 13-mm-diameter Millipore filter discs (pore size, 0.22 μm)
Glass vial
Small paraffin-coated polypropylene containers (Peel-A-Way disposable embedding molds; Polysciences) with (see recipe) and without a coating of hardened Epon on the inside bottom surface

NOTE: Osmium tetroxide, glutaraldehyde, and propylene oxide must be handled and disposed of in accordance with protocols approved by Institutional Chemical Safety and Chemical Waste Committees. Uranyl acetate is radioactive and therefore must be handled and disposed of in accordance with protocols approved by an Institutional Radiation Safety Committee, as well as in accordance with protocols approved by Institutional Chemical Safety and Chemical Waste Committees.

NOTE: All steps involving propylene oxide should be performed in a fume hood, as this compound is highly volatile.

Fix sample in glutaraldehyde

1. Add 0.2 mg of CCV fractions (as measured using a standard protein assay; *APPENDIX 3H*) to a microcentrifuge tube. Dilute to a final volume of 0.9 ml in ice-cold, filtered 1× buffer A or ice-cold, filtered 1× MES buffer, depending on which buffer the CCV fractions are suspended in.

2. Add 100 μl of ice-cold 25% glutaraldehyde solution. Incubate sample overnight at 4°C with gentle rocking.

   Once the glutaraldehyde solution has been added, the sample can be kept at 4°C for up to 72 hr.

   The authors have only processed fresh CCV fractions using this protocol; thus, they perform steps 1 and 2 immediately following the preparation of CCVs.

Filter sample

3. Wash the filter unit with double-distilled water.

   The filter unit is a custom-built apparatus modeled on the one described by Baudhuin et al. (1967). In brief, a Millipore filter is placed between two Teflon O-rings and then seated on top of a support screen at mid-height in a stainless steel centrifuge tube, and a Plexiglas chamber with a cylindrical opening of matching diameter is fitted to the top of this tube. After the sample of interest has been applied to the filter, the Plexiglas chamber can be closed with a plug (held securely to the Plexiglas chamber by a screw) that allows the filter unit to be connected to a tank of nitrogen gas.

4. Install a 13-mm-diameter Millipore filter (pore size, 0.22 μm) in the filter unit.

5. Apply 1× buffer A to the filter, and adjust the nitrogen flow rate such that 2 ml of the buffer passes through the filter in 30 sec.

6. Apply the fixed sample from step 2 to the filter.

7. Using a Pasteur pipet, gently layer 4 ml of 1× buffer A on top of the fixed sample.

   The 1× buffer A must be layered on top of the fixed sample very gently. The difference in density between the 1× buffer A and the fixed sample prevents mixing of the two layers.

8. Close the filter unit and apply constant nitrogen pressure (using the flow rate determined in step 5). When 2 ml of liquid has passed through the filter (as estimated by eye), cut off the nitrogen flow and open the filter unit.

   Allowing 2 ml of liquid to pass through the filter will ensure that the fixed membranes are collected on the filter while also ensuring that the filter does not dry out. As membranes collect on the filter, the rate at which liquid passes through will decrease to as low as 2 ml every 20 to 30 min.

9. Add 2 to 4 ml ice-cold sodium cacodylate buffer to a glass vial kept at 4°C. Using forceps, transfer the filter to this glass vial such that the sample side faces up.

   In this step and all subsequent steps, take care to ensure that the filter does not dry out.

10. Wash the filter three times, each time by adding 2 to 4 ml ice-cold cacodylate buffer, incubating 15 min at 4°C, and then removing the buffer.

    To avoid damaging the filter, add and remove the cacodylate buffer gently.

    All wash and incubation times presented here are intermediate recommended times and can be changed by ~30% without affecting sample preparation.

Fix sample in osmium tetroxide

11. After removing the last cacodylate buffer wash, add 2 ml ice-cold osmium tetroxide solution. Incubate 1 hr at 4°C, and then remove the solution.

12. Wash the filter three times, each time by adding 2 ml ice-cold cacodylate buffer, incubating 10 min at 4°C, and then removing the buffer.

Treat with tannic acid

13. After removing the last cacodylate buffer wash, add 2 ml ice-cold tannic acid solution. Incubate 1 hr at 4°C, replacing the old 2-ml aliquot of tannic acid solution with a new 2-ml aliquot every 20 min. At the end of the incubation period, remove all of the tannic acid solution.

14. Wash the filter twice, each time by adding 2 ml ice-cold sodium sulfate solution, incubating 10 min at 4°C, and then removing the solution.

Treat sample with uranyl acetate

15. After removing the last sodium sulfate wash, add 2 ml ice-cold, filtered uranyl acetate solution. Incubate in the dark for 2 hr at 4°C, and then remove the solution.

    Precipitate particles will appear as dark spots when the sample is viewed under EM. Thus, it is critical that the uranyl acetate solution be passed through a 0.22-μm filter prior to use. It is also critical that the sample be kept in the dark throughout the course of the incubation, as uranyl acetate will precipitate if it is exposed to light.

16. Wash the filter twice, each time by adding 2 to 4 ml ice-cold maleate buffer, incubating 10 min at 4°C, and then removing the buffer.

    During these two washes, handle the sample under the assumption that it is contaminated with uranyl acetate.

17. Add 2 to 4 ml ice-cold maleate buffer. Incubate overnight at 4°C, and then remove the buffer.

Dehydrate sample

18. Add 2 to 4 ml of ice-cold 50% ethanol to the vial containing the filter. Incubate 10 min at 4°C.
19. Replace the 50% ethanol with 2 to 4 ml of ice-cold 70% ethanol. Incubate 10 min at 4°C.
20. Replace the 70% ethanol with 2 to 4 ml of ice-cold 90% ethanol. Incubate 10 min at 4°C.
21. Replace the 90% ethanol with 2 to 4 ml of ice-cold 95% ethanol. Incubate 10 min at 4°C, and then remove the ethanol solution.
22. Wash the filter twice more, each time by adding 2 to 4 ml of ice-cold 95% ethanol to the vial, incubating 10 min at 4°C, and then removing the ethanol solution.
23. Add 2 to 4 ml of ice-cold 100% ethanol to the vial. Incubate 10 min at 4°C, and then remove the ethanol.
24. Wash the filter once by adding 2 to 4 ml of ice-cold 100% ethanol to the vial, incubating 10 min at 4°C, and then removing the ethanol.

Treat sample with propylene oxide

25. Wash the filter once more by adding 2 to 4 ml of ice-cold 100% ethanol to the vial and incubating 10 min at 4°C. At the end of the 10-min incubation period, fill a small, paraffin-coated polypropylene (Peel-A-Way) disposable embedding mold with propylene oxide and transfer the filter to this mold.

    Do not transfer the filter to an empty mold and then add propylene oxide, as this will cause the filter to fragment.

26. Incubate the filter in propylene oxide for 1 to 2 hr at room temperature.

    Propylene oxide is highly volatile. Therefore, the propylene oxide in the mold will need to be replenished periodically to replace any liquid that has evaporated over the course of the incubation.

    Incubation in propylene oxide makes the filter extremely fragile, and in many cases, the filter will break into pieces as a result. In the remaining steps, the filter should be treated very gently, so as to minimize fragmentation.

Embed samples

27. Gently pour the filter and propylene oxide into a Peel-A-Way embedding mold whose inside bottom surface has been coated with a layer of hardened Epon.
28. Replace the propylene oxide solution in the Epon-coated mold with 2 to 4 ml of 1:3 (w/w) Epon solution/propylene oxide. Incubate 1 hr at room temperature.
29. Replace the 1:3 Epon solution/propylene oxide with 2 to 4 ml of 1:1 (w/w) Epon solution/propylene oxide. Incubate 1 hr at room temperature.
30. Replace the 1:1 Epon solution/propylene oxide with 2 to 4 ml of 3:1 (w/w) Epon solution/propylene oxide. Incubate 1 hr at room temperature.
31. Replace the 3:1 Epon solution/propylene oxide with 2 to 4 ml Epon solution. Incubate 76 hr at 60°C to generate a hard block surrounding the sample.
32. Trim the block, and then section and analyze using standard EM protocols (Baudhuin et al., 1967).

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

For 10× stock

To 80 ml H_2O, add:
19.52 g 2-(*N*-morpholino)ethanesulfonic acid (MES; 1 M final)
5 ml of 200 mM ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA; 10 mM final)
0.25 ml of 2 M $MgCl_2$ (5 mM final)
Adjust to pH 6.5 with 10 N NaOH
Add H_2O to 100 ml
Store up to 4 weeks at 4°C

For 1× working solution

To 400 ml H_2O, add 50 ml of 10× buffer A
Adjust to pH 6.5 with 1 N NaOH if necessary
Add H_2O to 500 ml
Store up to 2 days at 4°C

To prevent the appearance of dust or other particles in the sample to be analyzed, pass 1× buffer A through a 0.22-μm filter before use.

Control buffer

To 400 ml H_2O, add:

10 ml of 1 M HEPES, pH 7.4 (20 mM final)
16 ml of 4 M NaCl (128 mM final)
0.6 ml of 2.5 M KCl (3 mM final)
0.3 ml of 2 M $MgCl_2$ (1.2 mM final)
0.05 ml of 1 M $CaCl_2$ (0.1 mM final)
18.8 g glucose (11 mM final)
Add H_2O to 500 ml
Store up to 24 hr at 4°C

D_2O-sucrose solution

To 5 ml deuterium oxide (D_2O; heavy water), add:

0.8 g sucrose (8% w/v final)
1 ml of 10× buffer A (see recipe)
Add D_2O to 10 ml
Prepare fresh and use immediately

Epon solution

12.6 g Epon 812 resin (specific density, 1.256 g/cm^3; MECA Lab)
6.7 g dodecenylsuccinic anhydride (specific density, 1.024g/cm^3; MECA Lab)
6.6 g nadic methyl anhydride (specific density, 1.237 g/cm^3; MECA Lab)
0.3 g 2,4,6-tris[(dimethylamino)methyl]phenol (DMP30; specific density, 0.977 g/cm^3; MECA Lab)
Make solution fresh and use immediately

With the exception of DMP30, the components used to make Epon are very viscous and thus hard to measure by volume. Therefore, in the preparation of Epon, measurement of these components by weight is recommended.

The recipe provided here yields 26.2 g Epon, which is sufficient for the preparation of four samples in the Support Protocol.

Ficoll-sucrose solution

To 20 ml of 1× buffer A (see recipe), add:
3.75 g sucrose (12.5% w/v final)
3.75 g Ficoll 400 (12.5% w/v final)
Add 1× buffer A to 30 ml
Stir or gently rock until sucrose and Ficoll 400 are completely dissolved
Prepare fresh and use immediately

Liver homogenization buffer

To 800 ml H_2O, add:
10 ml of 1 M HEPES, pH 7.4 (10 mM final)
85.6 g sucrose (0.25 M final)
Adjust to pH 7.4 if necessary
Add H_2O to 1000 ml
Store up to 24 hr at 4°C

Maleate buffer

To 400 ml H_2O, add:
4 g NaOH
11.6 g maleic acid (50 mM final)
Adjust pH to 6 with 1 N HCl
Add H_2O to 500 ml
Store up to 3 months at 4°C

MES buffer

For 10× stock
To 150 ml H_2O, add:
39.04 g MES (1 M final)
20 ml of 200 mM EGTA (20 mM final)
1.5 ml of 2 M $MgCl_2$ (15 mM final)
Adjust to pH 6.5 with 10 N NaOH
Add H_2O to 200 ml
Store up to 4 weeks at 4°C

For 1× working solution
To 400 ml H_2O, add 50 ml of 10× MES buffer
Adjust to pH 6.5 with 1 N NaOH
Add H_2O to 500 ml
Store up to 2 days at 4°C

Osmium tetroxide solution

To 1 ml of 4% (w/v) osmium tetroxide, add (in the order presented):
2 ml of 3% (w/v) potassium ferrocyanide
1 ml of 0.2 M sodium cacodylate buffer (see recipe)
Prepare fresh and use immediately

P5 homogenization buffer

To 800 ml H_2O, add:
10 ml of 1 M HEPES, pH 7.4 (10 mM final)
102.9 g sucrose (0.3 M final)
Add H_2O to 1000 ml
Store up to 24 hr at 4°C

Peel-A-Way disposable embedding mold with Epon-coated inside bottom surface

Add just enough Epon solution (see recipe) to cover the bottom of a Peel-A-Way disposable embedding mold (Polysciences). Incubate 76 hr at 60°C.

Protease inhibitors

Aprotinin, 10,000× stock:
To 1 ml H_2O, add 5 mg aprotinin powder (5 mg/ml final)
Store up to 6 months at −20°C
Benzamidine, 100× stock:
To 100 ml H_2O, add 1 g benzamidine powder (64 mM final)
Pass solution through a 0.22-μm filter
Store up to 6 months at 4°C
Leupeptin, 10,000× stock:
To 1 ml H_2O, add 5 mg leupeptin powder (5 mg/ml final)
Store up to 6 months at −20°C
Phenylmethylsulfonyl fluoride (PMSF), 500× stock:
To 100 ml of 100% ethanol, add 1 g PMSF powder (10 mg/ml final)
Store up to 3 months at room temperature

Sodium cacodylate buffer

To 400 ml H_2O, add:
21.4 g sodium cacodylate (0.2 M)
Adjust pH to 7.4 with 1 N HCl
Add H_2O to 500 ml
Store up to 3 months at 4°C
Dilute 1:1 with H_2O immediately prior to use in the Support Protocol (final sodium cacodylate concentration, 0.1 M)

Sodium sulfate solution

To 80 ml of 0.1 M sodium cacodylate buffer (see recipe), add 1 g sodium sulfate (1% w/v final). Add 0.1 M sodium cacodylate buffer to 100 ml. Prepare fresh and use immediately.

Sucrose solution, 20% or 50% (w/v), in 1× buffer A

To a 50-ml conical tube containing 25 ml of 1× buffer A (see recipe), add 10 g (20% final) or 25 g (50% final) sucrose. Add 1× buffer A to 50 ml. Stir or gently rock until sucrose is completely dissolved. Prepare fresh and use immediately.

Sucrose solutions in 1× MES buffer

Add the following amounts of sucrose to individual 50-ml conical tubes, each containing 25 ml of 1× MES buffer (see recipe).
2.5 g (5% final)
5 g (10% final)
10 g (20% final)
15 g (30% final)
20 g (40% final)
22.5 g (45% final)
25 g (50% final)
27.5 g (55% final)
Add 1× MES buffer to 50 ml
Prepare fresh and use immediately

Tannic acid solution

To 80 ml of 0.1 M sodium cacodylate buffer (see recipe), add:
1 g tannic acid (1% w/v final)
0.1 M sodium cacodylate buffer to 100 ml
Prepare fresh and use immediately

Uranyl acetate solution

To 100 ml H_2O, add:
25 ml maleate buffer (see recipe)
2 g uranyl acetate (2% w/v final)
Store up to 3 months at 4°C in the dark

To prevent the appearance of dust or other particles in the sample to be analyzed, pass uranyl acetate solution through a 0.22-μm filter prior to use.

COMMENTARY

Background Information

Homogenization and differential centrifugation

CCVs are generally purified in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffers at mildly acidic pH, as originally described by Pearse (1975). These buffers usually contain $MgCl_2$ and EDTA, although the inclusion of these chemicals appears to be primarily a matter of convention (Daiss and Roth, 1983). MES buffers are used at mildly acidic pH, as the stability of the clathrin coat is sensitive to pH. Clathrin coats are also sensitive to protonated amines, and Tris-containing buffers are therefore avoided (Keen et al., 1979). In fact, Tris-containing buffers are routinely used to strip clathrin coats following the isolation of CCVs; this technique is useful in determining whether a protein of interest is associated with the coat or with the vesicle fraction. Moreover, Tris-mediated stripping of clathrin from isolated CCVs is often used in the purification of clathrin. Keen et al. (1979) describe protocols for stripping clathrin coats from CCVs using Tris and other buffers.

Due to their small size, CCVs are resistant to shear forces, and high–shear force methods of homogenization are therefore acceptable (Daiss and Roth, 1983). Protocols originally described by Pearse (1975) used a Waring blender for the homogenization of pig brain tissue. Subsequent protocols for isolating CCVs from placental and lymphoma cells (Pearse, 1976; Pearse, 1982), calf brain (Blitz et al., 1977), rat liver (Pilch et al., 1983), and bovine adrenal cortex (Mello et al., 1980) used similar homogenization techniques. The protocols presented in this unit call for a Wheaton glass-Teflon homogenizer, as previously described by Maycox et al. (1992).

For adult brain and cultured cells, homogenization is performed directly in 100 mM MES, pH 6.5, containing 1 mM $MgCl_2$ and 0.5 mM EDTA (buffer A). The homogenizer power head is set at 1500 rpm, and the pestle is pushed through the sample rapidly and forcefully. These conditions are sufficient to lyse cultured cells and, in the case of brain tissue, to lyse nerve terminals as well as cell somata. When P5 rat brain samples are used, CCVs are isolated from crude growth cones using a protocol derived from the one described by Maycox et al. (1992) for purifying CCVs from synaptosomes. Thus, the homogenization step in that protocol is designed to lyse the somata of neurons but to leave growth cones (or synaptosomes) intact. To this end, homogenization is performed in isotonic sucrose using a glass-Teflon homogenizer. The power head is set at 900 rpm, and the pestle is passed through the sample slowly, using little force. Thus, populations of CCVs and other membrane components, as well as soluble proteins, are trapped inside the growth cones or synaptosomes. Spins of moderate force (12,000 to 14,500 × *g* in sucrose, 5000 × *g* in isotonic saline) are sufficient to pellet these membranes, whereas CCVs and other microsomal components from cell bodies are not pelleted at such *g* forces. After separation from membranes, the growth cones/synaptosomes are washed of peripherally associated vesicles and lysed using hypotonic shock. In Basic Protocol 2, homogenization is performed as in Basic Protocol 1, except that a neutral-pH homogenization buffer containing 0.25 M sucrose is initially employed, with MES buffer (100 mM

MES, pH 6.5; 2 mM $MgCl_2$; 1.5 mM EGTA) being added to the supernatant from the first centrifugation step.

In each protocol, the first step of purification involves differential centrifugation to generate crude microsomes, which are enriched for CCVs. Thus, the homogenates (or lysed growth cones, when P5 brain samples are used) are centrifuged at moderate *g* forces (12,000 to 17,000 × *g*), and the supernatants are collected and centrifuged at 56,000 to 125,000 × *g* (Figs. 3.13.1 to 3.13.3). These steps reduce contamination by nuclei, mitochondria, and other large organelles, such that the majority of CCV contamination comes from vesiculated endoplasmic reticulum, Golgi bodies, membranes of the endosomal/lysosomal system, and other transport vesicles, including synaptic vesicles when brain tissue samples are used.

Density gradients

The various forms of density gradient centrifugation employed in these protocols all take advantage of the small size and high density of CCVs to separate these vesicles from contaminating membranes present in the crude microsomal fractions. Pearse (1975) developed the first protocols of this type. In those protocols, microsomal membranes were applied to 5%-to-60% linear sucrose gradients and centrifuged at 50,000 × *g* for 2 hr (Pearse, 1975). Under velocity sedimentation conditions such as these, organelles will separate based on their size, and CCVs, because of their small size, have a low sedimentation rate. Pearse (1975) found that CCVs banded near the middle of the gradient, with larger contaminating membranes appearing at the bottom of the gradient. Once the CCVs were recovered by pelleting, they were centrifuged for 16 hr at 50,000 × *g* through a linear 20%-to-60% sucrose gradient (Pearse, 1975). Under equilibrium sedimentation conditions such as these, organelles have time to reach their isopycnic points and are thus separated based primarily on their densities. Because of their protein coat, CCVs have a high density (1.20 to 1.25 mg/ml; Daiss and Roth, 1983). Thus, as observed by Pearse (1975), they equilibrate at ~50% to 55% sucrose. Many of the small microsomal vesicles not removed in the velocity gradient step will equilibrate at lower sucrose concentrations and remain above the CCVs in the equilibrium gradient. Basic Protocol 2 takes advantage of these principles using an equilibrium sedimentation step sandwiched between two velocity sedimentation steps on discontinuous sucrose gradients.

One potential problem with sucrose density gradients is that osmotic stress encountered by an organelle at high sucrose concentrations can cause partial disruption of membranes. In fact, it was reported that the sucrose density gradient procedures originally described by Pearse (1975) caused partial disruption of CCVs, with some loss of contents (Pearse, 1982). Pearse (1982) thus introduced techniques for purifying CCVs using Ficoll and D_2O gradients. Maycox et al. (1992) adapted these procedures for the purification of CCVs from rat brain. As described in Basic Protocol 1 and Alternate Protocol 1, microsomal membranes are first fractionated by centrifugation in a Ficoll-sucrose solution in which the sucrose concentration is 12.5%. Centrifugation at 43,000 × *g* for 20 min leads to pelleting of larger microsomal membranes, while CCVs remain in the supernatant. The CCVs are then collected by centrifugation and pelleted through a cushion of 8% sucrose in D_2O. Only dense CCVs are pelleted under these conditions. This step is particularly important when working with brain tissue, since synaptic vesicles, which are abundant in brain, are major contaminating organelles in microsomal fractions. Maycox et al. (1992) were able to determine that these Ficoll-sucrose and D_2O-sucrose centrifugation steps were effective in separating CCVs from synaptic vesicles.

EM analysis of CCVs

Negative-stain electron microscopy (EM) is used routinely to examine CCV purity following subcellular fractionation (Pearse, 1975). Typically, a drop of a resuspended pellet is placed on a carbon-coated copper grid and negatively stained with 1% uranyl acetate, as originally described by Huxley (1963). However, the purity of CCVs may be overestimated using this procedure, as protein-coated vesicles are selectively retained on the grid (Daiss and Roth, 1983). Specimens prepared by fixing pellets prior to embedding and sectioning give a more faithful representation of the purity of CCVs (Daiss and Roth, 1983). However, as noted by Baudhuin et al. (1967), when particles in a given preparation have differing sedimentation coefficients or densities, the distribution of those particles within a pellet will be far from homogeneous. Thus, in the Support Protocol, we describe a procedure for analyzing CCV purity that is based on protocols developed by Baudhuin et al. (1967) and Simionescu and Simionescu (1976). Resuspended CCV fractions are fixed in glutaraldehyde and then passed through Millipore

nitrocellulose filters using nitrogen pressure (Baudhuin et al., 1967). This generates very thin pellicles of packed particles, with fraction heterogeneity solely in the direction perpendicular to the filter surface. The vesicles on the filters are then processed using osmium tetroxide and tannic acid treatments prior to staining with uranyl acetate (Simionescu and Simionescu, 1976). This procedure better preserves membranes as the samples go through the dehydration and embedding steps and results in increased contrast, especially for membranes, on EM (Simionescu and Simionescu, 1976). Taking pictures of large numbers of random EM fields and determining the percentages of CCVs in these fields should yield an unbiased estimate of CCV purity (Blondeau et al., 2004). However, it is possible that the Support Protocol leads to underestimation of CCV purity, since CCVs are small, and larger membranes may be preferentially retained on the filters.

Critical Parameters and Troubleshooting

Tissue source

The species of animal used for CCV preparations is an important consideration. The authors have isolated CCVs exclusively from rat. Rat is a convenient laboratory source of tissue, and the animals are easy to manipulate experimentally; for example, CCVs can be prepared from tissues following the injection of drugs or receptor ligands of interest. Moreover, rat proteins are more likely to be reactive with a broad range of commercial or investigator-generated antibodies. The authors recently performed a proteomic analysis of CCVs isolated from rat brain, with that study being aided by the relative completeness of rat databases (Blondeau et al., 2004). However, CCVs have been successfully isolated from a variety of species. For example, CCVs have also been isolated from the brains of rabbits, cows, and pigs (Pearse, 1975; Blitz et al., 1977; Woods et al., 1978); one advantage of using these species is that large amounts of tissue can be obtained. The use of human tissues is limited, given the relatively large amount of starting material required, although CCVs have been prepared from human placenta (Pearse, 1982).

Another advantage to using rat is that an extensive array of frozen tissues are available. The protocols described here use fresh tissue. However, the authors have also used commercially available frozen tissue from adult rat brain and liver. Based on Coomassie blue staining and immunoblot analysis of subcellular fractions, no obvious differences between fresh and frozen tissue in terms of the protein profiles of the CCVs isolated have been observed. However, Daiss and Roth (1983) have noted that fresh tissue yields a better product for studies of the assembly and disassembly of clathrin coats.

Vesicle isolation

Overall, the protocols presented here are reliable and reproducible. However, there are several points to note. Given the importance of mildly acidic pH to the stability of clathrin coats, the pH of each buffer should be checked immediately prior to starting the preparations. Tissues other than brain should be minced before homogenizing with a glass-Teflon homogenizer. During homogenization, the glass mortar should be held in such a way that the investigator does not warm the tissue with his or her hands. Also, when starting with adult brain synaptosomes (Maycox et al., 1992) or P5 growth cones (Alternate Protocol 1), the pestle should be moved through the sample with limited force in order to minimize shear forces. For the lysis of these structures, water should be added quickly to ensure hypotonic shock, and the pestle should then be passed through the samples forcefully.

Ensure that pellets are completely scraped from the walls of centrifuge tubes. The head of a Teflon pestle is useful in this regard, and the authors generally rinse the walls of the tube with the resuspension buffer as well. Certain pellets, as noted in the protocols, are very soft, and it is important to remove the supernatant from these pellets at the centrifuge in order to avoid disturbing the pellet. Step gradients are easy to prepare, and with some practice, linear gradients can also be generated reproducibly using a conventional two-chamber gradient maker. When using high concentrations of sucrose, ensure that the solution in the front chamber of the gradient maker (i.e., the chamber with the exit port) is adequately stirred. An automatic gradient maker (for example, the BioComp system) that generates linear gradients following the layering of the densest and lightest solutions in a centrifuge tube can also produce reliable gradients.

EM analysis

When following the Support Protocol for the analysis of CCVs by EM, it is important to remember that several of the reagents are somewhat dangerous and must be handled with care. In addition, as noted in the annotation

to step 26, the filter becomes translucent and very fragile once treated with propylene oxide. Thus, it must be handled carefully to avoid losing sample. Even if the filter does fragment, keep all of the pieces that show a spot of brown color (the sample). Finally, do not exceed the recommended nitrogen flow rate, as doing so can cause the filter to collapse.

Anticipated Results

Basic Protocol 1

Using 10 rat brains with a combined wet weight of 15 to 20 g, Basic Protocol 1 yields approximately 0.5 mg of CCVs. The protocol can be stopped following the D_2O-sucrose cushion step (step 32), yielding 1 mg of cushion-pellet CCVs (cpCCVs). Figure 3.13.4A demonstrates the enrichment of clathrin heavy chain (CHC; as seen on Coomassie blue staining and immunoblotting) going from the crude homogenate to the cpCCV fraction. Other components of the clathrin coat, including the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor complexes, as well as the clathrin light chain (CLC), are readily detectable in the cpCCV fractions. Cosedimentation of these components is seen when the cpCCVs are fractionated by velocity sedimentation on linear 20%-to-50% sucrose gradients (Fig. 3.13.4B). These patterns are highly reproducible and can be used to easily and rapidly assess the quality of the preparation.

Figure 3.13.4C shows an EM image (Support Protocol) of CCVs following linear sucrose gradient centrifugation. When randomly chosen EM images from multiple preparations were counted, cpCCVs and CCVs were found to represent 64.9% ± 1.2% and 72.7% ± 1.7% (mean ± SD; $n = 3$ CCV preparations), respectively, of all membrane profiles (Blondeau et al., 2004). On sensitive tandem mass spectrometric analysis, CCVs prepared using this protocol were found to be essentially free of contamination by endoplasmic reticula, nuclei, mitochondria, Golgi bodies, endosomes, peroxisomes, and lysosomes (Blondeau et al., 2004).

Alternate Protocols 1 and 2

The Coomassie blue staining pattern of retained fractions from P5 rat growth cone CCV (gcCCV) preparations (Alternate Protocol 1) is shown in Figure 3.13.5A. As with adult CCV preparations, clathrin coat components, including α-, β-, γ-, and μ-adaptin and CLCs, are readily detectable in the gcCCVs. Immunoblotting for CHC in the same fractions shows clathrin enrichment (Fig. 3.13.5A). An EM image of gcCCVs is presented in Figure 3.13.5B. Counting of randomly sampled fields revealed that these vesicle fractions were 79% ± 2.3% (mean ± SD; $n = 4$ CCV preparations) pure. The exact same protocol applied to adult rat brain synaptosomes yields similar clathrin enrichment (data not shown). The authors routinely process the brains of P5 pups from ~15 litters (combined weight of brain tissue, ~110 g); doing so yields ~0.3 mg of gcCCVs.

Rothman and Fine (1980) have developed a protocol for the purification of CCVs from cultured CHO cells using the procedure of Pearse (1975). This procedure requires approximately 40 g of packed cells, corresponding to 24 liters of CHO cells in culture (Rothman and Fine, 1980). The procedure for the isolation of CCVs described in Alternate Protocol 2 is valuable, because it can be performed using as few as five plates of cells. The protocol can thus easily be applied to transfected cells to obtain valuable information regarding the mechanisms by which a given protein associates with CCVs (see Metzler et al., 2001, for example). This protocol, which leads to membranes that are significantly enriched for clathrin (Metzler

Figure 3.13.4 *(at right)* Summary of results from Basic Protocol 1. (**A**) Analysis of adult rat brain fractions on a Coomassie-stained gel (top) and on immunoblot with a clathrin heavy chain (CHC) antibody (bottom). The migratory positions of CHC, clathrin light chains (CLCs), and the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor protein complexes are indicated. P1, P2, and S2 are fractions obtained before isolation of the final clathrin-coated vesicle (CCV) fraction; see Basic Protocol 1 for additional details. (**B**) Cushion-pellet CCVs (cpCCVs) were prepared and fractionated on linear 20%-to-50% sucrose gradients, and 1-ml fractions were collected from the bottom of each gradient as described in Basic Protocol 1. Proteins from aliquots of fractions 1 through 11 were separated by SDS-PAGE and analyzed by Coomassie blue staining. The migratory positions of CHC, CLCs, and the α-, β-, γ-, and μ-adaptin subunits of AP-1 and AP-2 are indicated. (**C**) Fractions that yielded peak protein signals (i.e., CCV fractions; see Basic Protocol 1) were analyzed by EM as described in the Support Protocol. A representative image is shown. H, crude homogenate; SGp, suspension of pellet from Ficoll-sucrose density gradient centrifugation; SGs, supernatant from Ficoll-sucrose density gradient centrifugation.

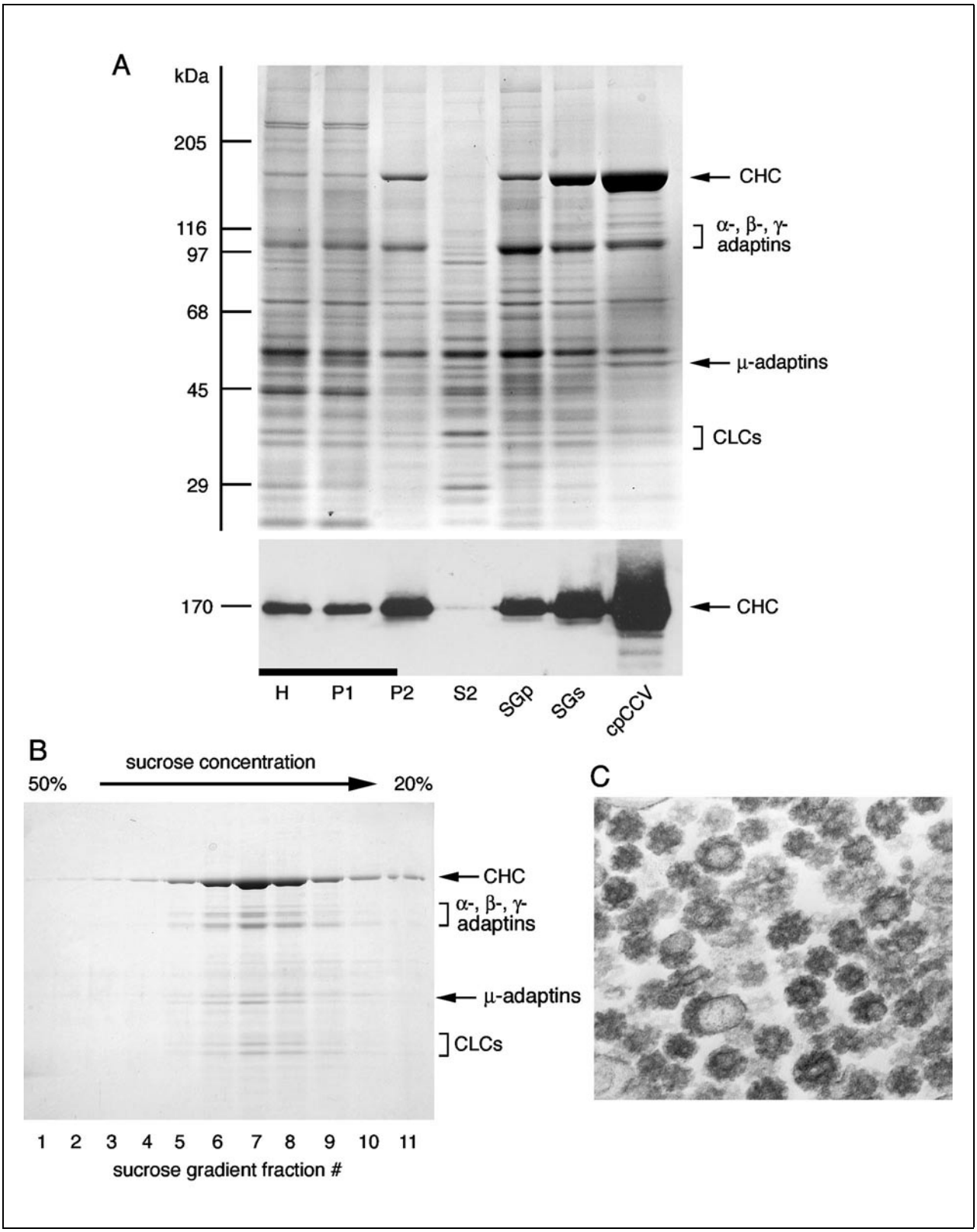


Figure 3.13.4 Legend at left.

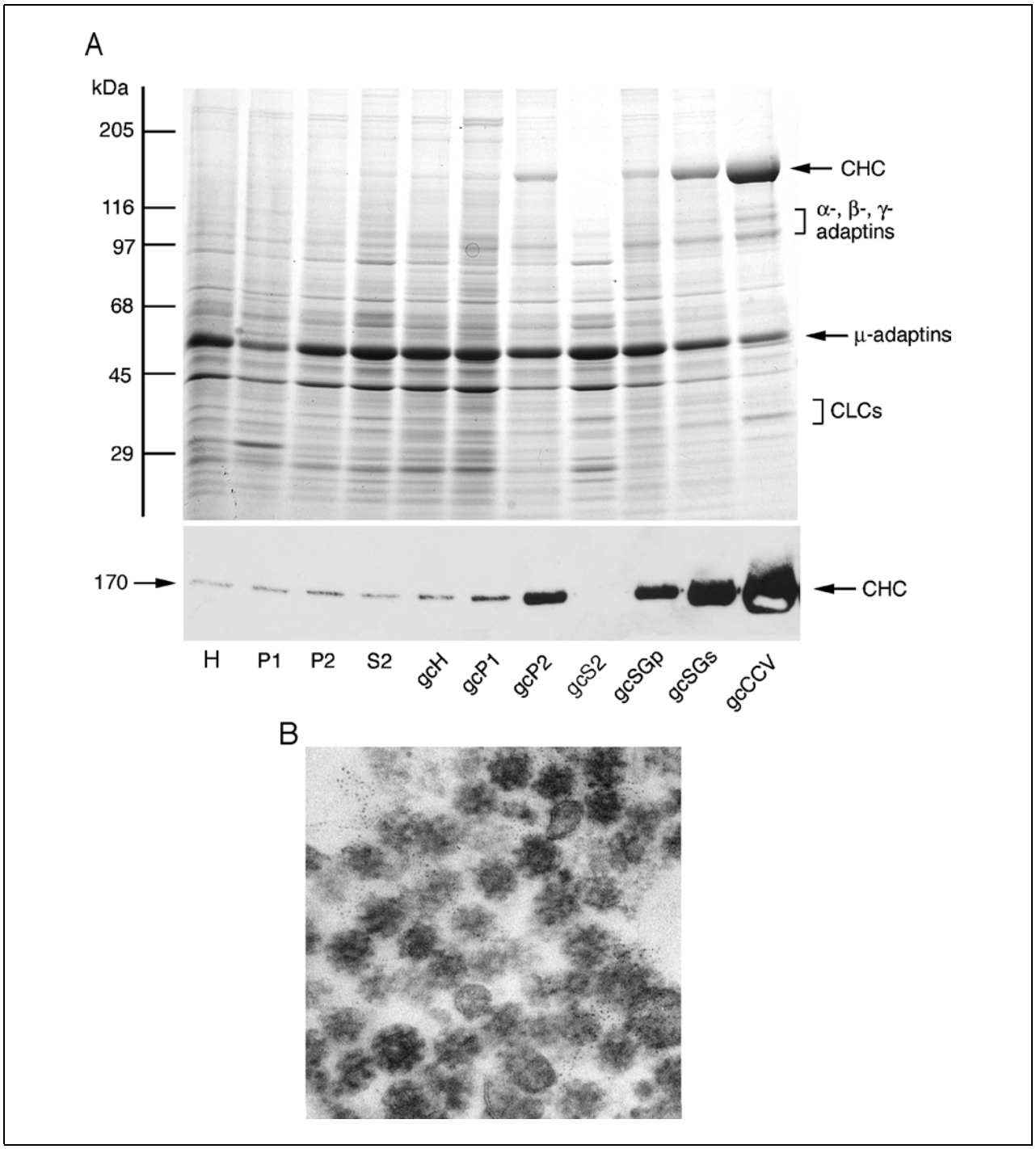


Figure 3.13.5 Summary of results from Alternate Protocol 1. (**A**) Analysis of P5 rat brain fractions on a Coomassie-stained gel (top) and on immunoblot with a clathrin heavy chain (CHC) antibody (bottom). The migratory positions of CHC, clathrin light chains (CLCs), and the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor protein complexes are indicated. P1, P2, S2, gcP1, gcP2, and gcS2 are fractions obtained before isolation of the final clathrin-coated vesicle (CCV) fraction; see Alternate Protocol 1 for additional details. (**B**) Growth cone clathrin-coated vesicles (gcCCVs) were analyzed by EM as described in the Support Protocol. A representative image is shown. H, crude homogenate; gcH, growth cone homogenate; gcSGp, suspension of pellet from Ficoll-sucrose density gradient centrifugation; gcSGs, supernatant from Ficoll-sucrose density gradient centrifugation.

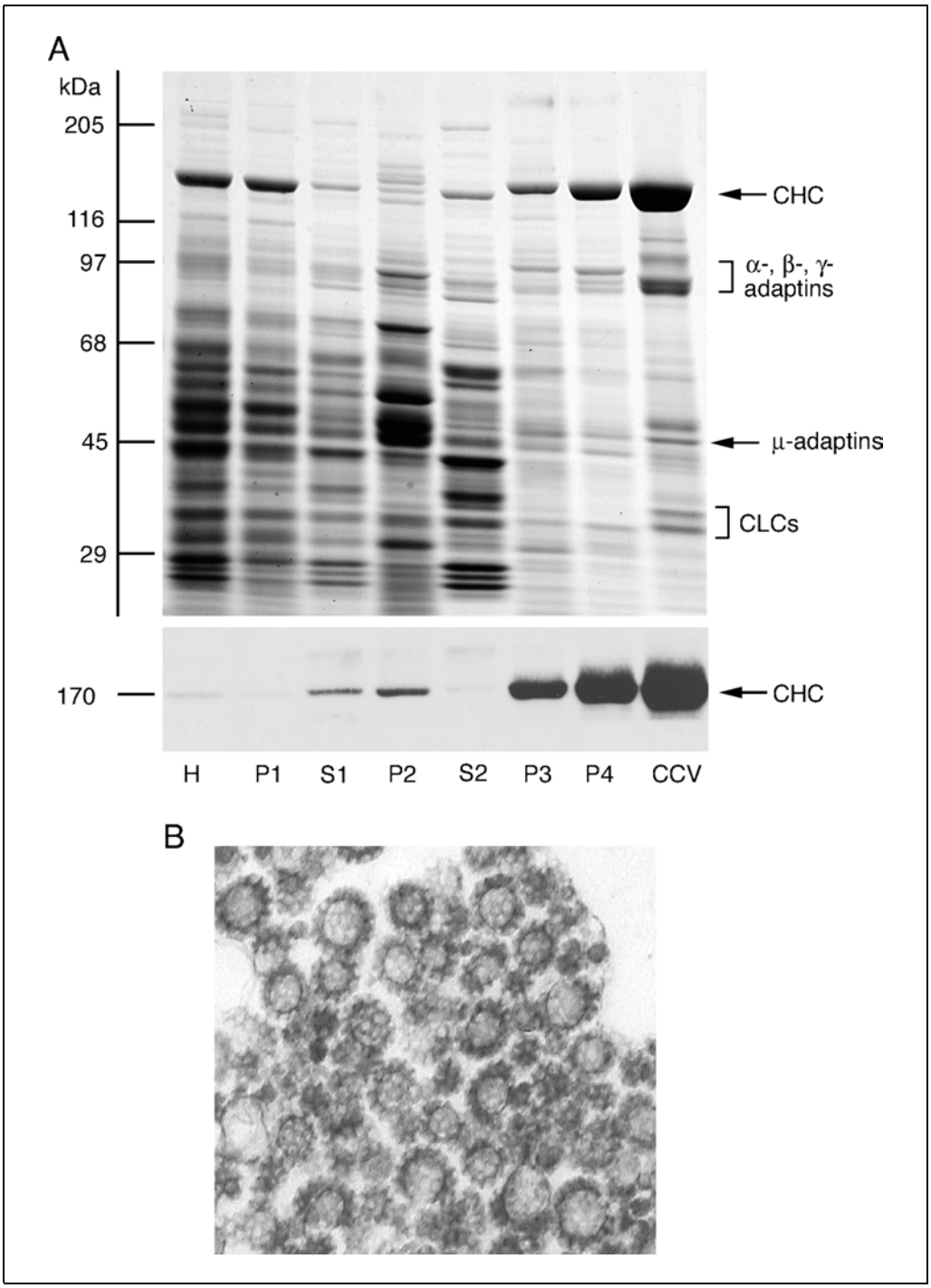


Figure 3.13.6 Summary of results from Basic Protocol 2. (**A**) Analysis of adult rat liver fractions on a Coomassie-stained gel (top) and on immunoblot with a clathrin heavy chain (CHC) antibody (bottom). The migratory positions of CHC, clathrin light chains (CLCs), and the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor protein complexes are indicated. P1, S1, P2, S2, P3, and P4 are fractions obtained before isolation of the final clathrin-coated vesicle (CCV) fraction; see Basic Protocol 2 for additional details. (**B**) Liver CCVs were analyzed by EM as described in the Support Protocol. A representative image is shown. H, crude homogenate.

et al., 2001), has been applied to COS-7 and HeLa cells, as well as HEK-293 cells. Five plates of confluent cells should yield ~0.5 mg of relatively crude CCVs.

Basic Protocol 2

Basic Protocol 2 starts with 10 rat livers (combined wet weight, 60 to 100 g) and yields 2 to 3 mg of CCVs. Figure 3.13.6A shows a Coomassie blue–stained gel of the fractions from a typical preparation. Major components of CCV coats, including CHCs, CLCs, and the various subunits of the AP-1 and AP-2 protein complexes, are easily detected in the CCV fraction. Enrichment for CHC throughout the preparation is monitored by Coomassie staining and immunoblotting (Fig. 3.13.6A). EM analysis of the CCV fraction revealed a nearly homogeneous CCV population (Fig. 3.13.6B), and random sampling of EM images showed that the fraction had a CCV purity of 89.2% ± 3.2% (mean ± SD; $n = 6$ CCV preparations).

Basic Protocol 2, which closely resembles the protocol originally described by Pearse (1975), should be applicable to the isolation of CCVs from a wide variety of tissues. The authors have used it to purify CCVs from rat kidney and brain, and they have observed similar enrichment for CHC in both cases. Similar or identical protocols have been used successfully to isolate CCVs from rabbit and calf brain (Blitz et al., 1977), human placenta (Pearse, 1982), lymphoma and CHO cells (Pearse, 1976; Rothman and Fine, 1980), and adrenal medulla and cortex (Pearse, 1976; Mello et al., 1980).

Time Considerations

There is no point at which any of the CCV purification protocols can be stopped and then resumed. Basic Protocol 1 will take ~14 hr, although this is shortened to ~10 hr if the last linear sucrose gradient step is omitted. Basic Protocol 2 takes ~35 hr. However, this includes a 16-hr (overnight) equilibrium sucrose gradient centrifugation. Basic Protocol 2 is thus very manageable over a 2-day period. Alternate Protocol 2 is the most straightforward and can be completed in ~6 hr, including the 2-hr centrifugation step. The most problematic protocol in terms of time management is Alternate Protocol 1. This protocol takes ~16 hr but contains no extended centrifugation steps. An important consideration with regard to this protocol is the time required to sacrifice and dissect brains from nearly 200 P5 rat pups.

The Support Protocol requires 6 days, not including the time needed for sectioning and EM observation. However, this time estimate includes 2 overnight incubations and 1 incubation of 76 hr. Days 2 and 3 both require hands-on time, with day 2 requiring ~8 hr for filtration and multiple wash steps.

Literature Cited

Baudhuin, P., Evrard, P., and Berthet, J. 1967. Electron microscopic examination of subcellular fractions: I: The preparation of representative samples from suspensions of particles. *J. Cell Biol.* 32:181-191.

Blitz, A.L., Fine, R.E., and Toselli, P.A. 1977. Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. *J. Cell Biol.* 75:135-147.

Blondeau, F., Ritter, B., Allaire, P.D., Wasiak, S., Girard, M., Hussain, N.K., Angers, A., Legendre-Guillemin, V., Roy, L., Boismenu, D., Kearney, R.E., Bell, A.W., Bergeron, J.J., and McPherson, P.S. 2004. Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. *Proc. Natl. Acad. Sci. U.S.A.* 101:3833-3838.

Daiss, J.L. and Roth, T.F. 1983. Isolation of coated vesicles: Comparative studies. *Methods Enzymol.* 98:337-349.

Huxley, H.E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. *J. Mol. Biol.* 77:281-308.

Keen, J.H., Willingham, M.C., and Pastan, I.H. 1979. Clathrin-coated vesicles: Isolation, dissociation and factor-dependent reassociation of clathrin baskets. *Cell* 16:303-312.

Maycox, P.R., Link, E., Reetz, A., Morris, S.A., and Jahn, R. 1992. Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. *J. Cell Biol.* 118:1379-1388.

Mello, R.J., Brown, M.S., Goldstein, J.L., and Anderson, R.G. 1980. LDL receptors in coated vesicles isolated from bovine adrenal cortex: Binding sites unmasked by detergent treatment. *Cell* 20:829-837.

Metzler, M., Legendre-Guillemin, V., Gan, L., Chopra, V., Kwok, A., McPherson, P.S., and Hayden, M.R. 2001. HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. *J. Biol. Chem.* 276:39271-39276.

Pearse, B.M. 1975. Coated vesicles from pig brain: Purification and biochemical characterization. *J. Mol. Biol.* 97:93-98.

Pearse, B.M. 1976. Clathrin: A unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 73:1255-1259.

Pearse, B.M. 1982. Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G. *Proc. Natl. Acad. Sci. U.S.A.* 79:451-455.

Pilch, P.F., Shia, M.A., Benson, R.J., and Fine, R.E. 1983. Coated vesicles participate in the receptor-mediated endocytosis of insulin. *J. Cell Biol.* 96:133-138.

Rothman, J.E. and Fine, R.E. 1980. Coated vesicles transport newly synthesized membrane glycoproteins from endoplasmic reticulum to plasma membrane in two successive stages. *Proc. Natl. Acad. Sci. U.S.A.* 77:780-784.

Saito, S., Fujita, T., Komiya, Y., and Igarashi, M. 1992. Biochemical characterization of nerve growth cones isolated from both fetal and neonatal rat forebrains: The growth cone particle fraction mainly consists of axonal growth cones in both stages. *Brain Res. Dev. Brain Res.* 65:179-184.

Simionescu, N. and Simionescu, M. 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy: I: Procedure, and evidence for mordanting effect. *J. Cell Biol.* 70:608-621.

Wasiak, S., Legendre-Guillemin, V., Puertollano, R., Blondeau, F., Girard, M., de Heuvel, E., Boismenu, D., Bell, A.W., Bonifacino, J.S., and McPherson, P.S. 2002. Enthoprotin: A novel clathrin-associated protein identified through subcellular proteomics. *J. Cell Biol.* 158:855-862.

Woods, J.W., Woodward, M.P., and Roth, T.F. 1978. Common features of coated vesicles from dissimilar tissues: Composition and structure. *J. Cell Sci.* 30:87-97.

Contributed by Martine Girard,
Patrick D. Allaire, Francois Blondeau,
and Peter S. McPherson
Montreal Neurological Institute
McGill University
Montreal, Quebec, Canada

Isolation of Melanosomes

Melanosomes are specific membrane-bound organelles that are produced only by melanocytes and retinal pigment epithelial cells, both types of cells being derived from the embryonic neural tube. Melanosomes are specialized intracellular organelles in which the biopolymer pigment melanin is synthesized and stored. Melanosomes have been shown to be closely related to lysosomes and platelet dense bodies, primarily because of the effects of certain diseases, such as Hermansky-Pudlak syndrome (HSP) and Chediak-Higashi syndrome (CHS), on the function of all three of these types of organelles (Dell'Angelica et al., 2000; Raposo and Marks, 2002). Pigmentation of the hair, skin, and eyes results from the concerted action of a number of melanocyte-specific enzymes required for the biosynthesis of melanin, most of which are localized in the melanosomes. Melanin protects underlying cells and tissues from the harmful effects of ultraviolet radiation.

Mammalian pigmentation is regulated directly or indirectly by more than 120 distinct genes, and many of the corresponding gene products modulate the quality or quantity of melanin produced, the structure of the melanosomes, and/or the processing and distribution of melanosomes. Melanosomal proteins can be structural proteins, such as Pmel17/gp100, or enzymatic proteins, such as tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and tyrosinase-related protein 2 (DCT/TYRP2). TYR, which is absolutely required for melanogenesis, catalyzes the rate-limiting reactions in melanin synthesis, converting tyrosine to dopaquinone and subsequently oxidizing 5,6-dihydroxyindole to indole-5,6-quinone (Wakamatsu and Ito, 2002). Pmel17/gp100 has been identified as a specific marker for melanoma and is processed by proteolysis. It is localized within fibrils in the melanosomal lumen (Kwon et al., 1991; Kobayashi et al., 1994; Kushimoto et al., 2001).

The biogenesis of melanosomes and the regulation of melanin synthesis have been studied extensively by means of electron microscopy, as well as by biochemical and immunohistochemical methods. On the basis of their morphology and degree of pigmentation, melanosomes have been classified into four developmental stages. Stage I melanosomes, originally termed premelanosomes, are characterized by a poorly organized internal structure and lack melanin. Stage II melanosomes display a well organized structure and exhibit lumenal fibrillar striations. In stage III melanosomes, some deposition of electron-opaque melanin on the fibrillar striations becomes evident, while stage IV melanosomes are fully melanized and have all of their internal structure obscured by this pigment (Fig. 3.14.1; Seiji et al., 1963a; Marks and Seabram, 2001; Dell'Angelica, 2003; Kushimoto et al., 2003). During their maturation, melanosomes are transported toward the tips of the dendrites and, in the skin, are then transferred to keratinocytes by an as yet poorly understood mechanism.

The protocols in this unit are simple and relatively rapid methods for isolating melanosomes of different stages from melanoma cells in culture and from melanoma tissues. These protocols describe the isolation of early (stage I and II) and late (stage III and IV) melanosomes from pigmented melanoma cells in culture (see Basic Protocol 1; Kushimoto et al., 2001; Basrur et al., 2003) and from pigmented melanoma tissues (see Basic Protocol 2; Seiji et al., 1963b; Araki et al., 2000). Alternate Protocol 1 describes a method for obtaining early-stage melanosomes from pigmented or nonpigmented melanoma cells in culture (Basrur et al., 2003; Watabe et al., 2004). Early melanosomes can be contaminated to varying degrees by lysosomes and by other

Contributed by Hidenori Watabe, Tsuneto Kushimoto, Julio C. Valencia, and Vincent J. Hearing
Current Protocols in Cell Biology (2005) 3.14.1-3.14.16

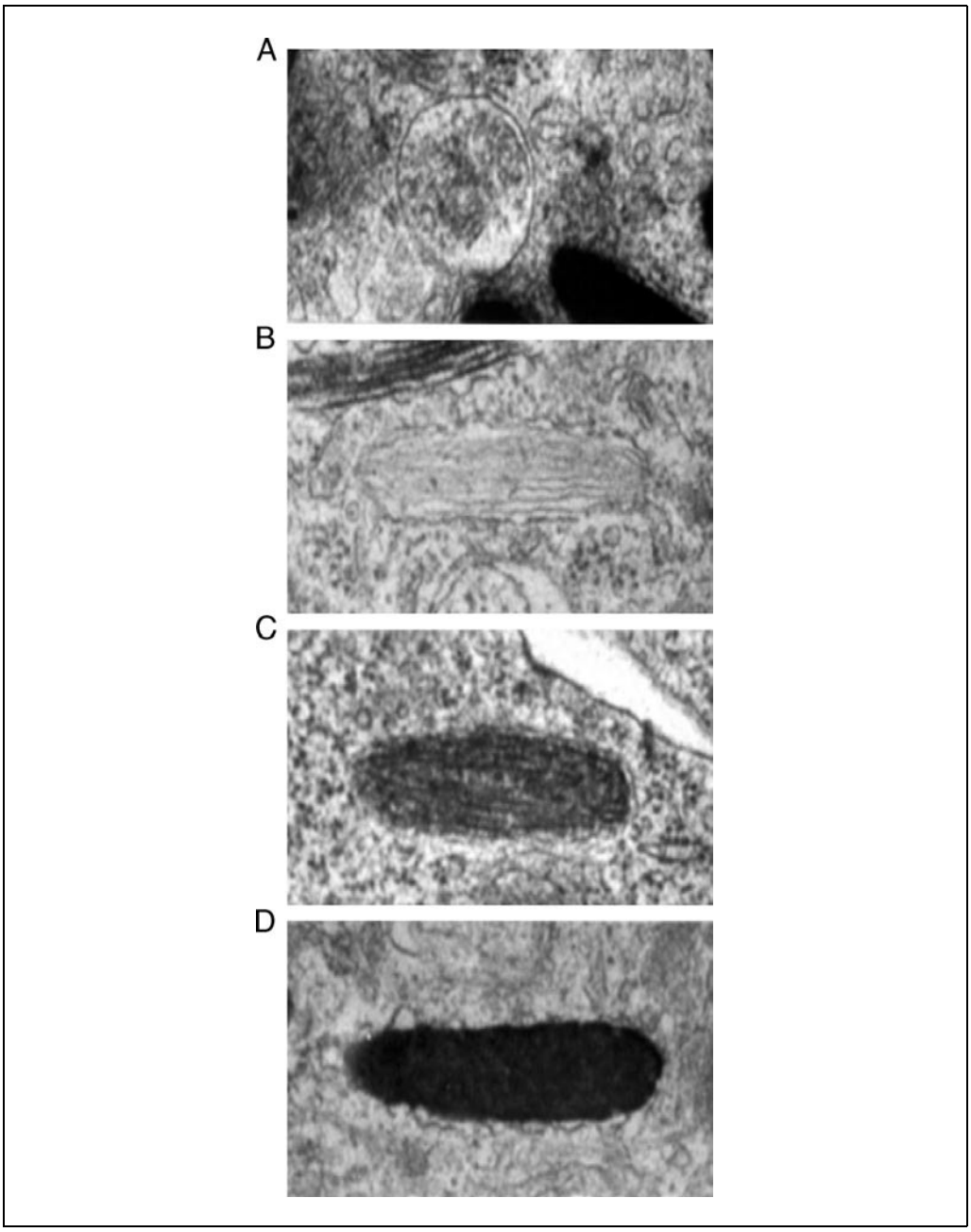


Figure 3.14.1 Ultrastructural appearance of (**A**) stage I, (**B**) stage II, (**C**) stage III, and (**D**) stage IV melanosomes. Adapted from: Watabe et al. (2003).

subcellular organelles, whereas late melanosomes can be completely separated from other membrane-bound organelles by virtue of their high density, which is caused by melanin deposition. To isolate early melanosomes from other smaller vesicular components (lysosomes and endosomes) and other organelles (late melanosomes and mitochondria), the authors describe the use of free-flow electrophoresis (FFE; see Alternate Protocol 2; Kushimoto et al., 2001; Basrur et al., 2003). The fractions obtained can be analyzed for various types of enzymatic activity, with tyrosinase activity being the most specific (see Support Protocol); immunohistochemistry using various melanosome-specific antibodies is also useful (see Table 3.14.1). These approaches provide essentially pure melanosome fractions, the likes of which have yielded important clues to melanosome biogenesis and function.

Table 3.14.1 Melanosome-Specific Antibodies[a]

Antigen	Antibody name[b]	Source[c]
Tyrosinase	αPEP7, αPEP7h	—[d]
	T311	Novocastra Labs
	C-19	Santa Cruz Biotechnology
Tyrp1	αPEP1, aPEP1h	—[d]
	TA99	Neomarkers[e]
	A-20	Santa Cruz Biotechnology
Dct	αPEP8, αPEP8h	—[d]
	G-15	Santa Cruz Biotechnology
gp100/Pmel17	αPEP13, αPEP13h	—[d]
	HMB45	Dako
	HMB50	Neomarkers[e]
	K-18	Santa Cruz Biotechnology
MART-1	M2-9E3	Neomarkers[e]
	Ab-3	Neomarkers[e]

[a]The authors do not attest to the specificity of the commercially available antibodies listed herein.
[b]The appearance of the letter 'h' at the end of the antibody name indicates that the antibody was made against the sequence of the human protein. All other antibodies were made against the mouse sequence.
[c]See ***SUPPLIERS APPENDIX***.
[d]hearingv@nih.gov
[e]http://www.labvision.com

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

NOTE: All solutions, glassware, centrifuge tubes, centrifuge rotors, and other equipment should be precooled to 0 to 4°C, and samples should be kept on ice throughout processing.

NOTE: Some marker enzyme assays (e.g., TYR; see Support Protocol) can be carried out using material that has been stored at 4°C for up to 1 to 2 days. Many assays can be performed on material frozen to −20°C or −80°C for storage; however, care should be taken to ensure that the measurements made using such materials are relatively unaffected by the storage conditions. This can be confirmed by comparing results with those obtained using fresh material.

BASIC PROTOCOL 1

PREPARATION OF EARLY AND/OR LATE MELANOSOMES FROM CULTURED MELANOMA CELLS BY DENSITY GRADIENT CENTRIFUGATION

Cultured melanoma cells are collected, homogenized, and then fractionated on a sucrose step gradient to allow isolation of early and/or late melanosomes.

Materials

MNT-1 human melanoma cells
Dulbecco's modified Eagle medium (DMEM), supplemented (see recipe)
PBS (*APPENDIX 2A*)
Cell homogenization medium–1 (CHM-1; see recipe)
Sucrose step gradient, 1.0 to 2.0 M (see recipe)
Melanosome wash buffer (MWB; see recipe)
150-cm^2 culture dishes (Becton Dickinson Labware)
Tabletop centrifuge, 4°C
Dounce glass/glass homogenizer
15-ml centrifuge tubes
36.5-ml polyallomer centrifuge tubes (Seton)
Beckman SW-28 swinging-bucket rotor
3-in., 20-G needle attached to a 12-ml syringe
50-ml Oak Ridge centrifuge tubes (Nalge Nunc)
Sorvall SS-34 centrifuge rotor
Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

Culture MNT-1 cells

1. Using 150-cm^2 culture dishes and supplemented DMEM, grow MNT-1 cells to confluent monolayers (*UNIT 1.1*).

   Inoculating with 1 × 10^6 cells will result in a confluent monolayer in 7 to 10 days.

   MNT-1 cells are highly pigmented human melanoma cells (Kushimoto et al., 2001). Other pigmented cultured melanocytes or melanoma cells can also be used, but MNT-1 cells have been shown to consistently give high yields

   This protocol typically calls for a minimum of 30 culture dishes of MNT-1 cells and will yield ~1 × 10^7 cells/dish.

Homogenize cultured cells

2. Wash confluent monolayers of MNT-1 cells once with PBS (10 ml/dish). Harvest cells by incubating with trypsin/EDTA (6 ml/dish; see *UNIT 1.1*) for 2 to 3 min, and then adding serum-containing medium (6 ml/dish) to stop the reaction. Pool cells in suitable tubes or flasks. Centrifuge the resulting cell suspensions 5 min at 700 × *g*, 4°C. Remove supernatant.

3. Wash each pellet once with 10 ml CHM-1 and centrifuge for 10 min at 1000 × *g*, 4°C. Remove supernatant. Resuspend each pellet in 10 ml CHM-1 and homogenize on ice using 20 strokes in 2 min with a Dounce glass/glass homogenizer.

Isolate postnuclear supernatant

4. Using a pipettor, transfer homogenates (~10 ml each) from step 3 to 15-ml centrifuge tubes and centrifuge for 10 min at 1000 × *g*, 4°C.

Isolate melanosomes

5. Recover supernatants (~8 ml per tube) from step 4. Using a pipettor, gently layer each supernatant on top of a 1.0 to 2.0 M sucrose step gradient (sucrose concentrations, from top to bottom: 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, and 2.0 M; 4 ml each) in a 36.5-ml polyallomer centrifuge tube.

   A permanent marker can be used to mark the outside of the tube to make subsequent identification of gradient fractions easier.

6. Centrifuge gradients 1 hr at 100,000 × *g*, 4°C, in a Beckman SW-28 swinging-bucket rotor.

7. After centrifugation, carefully recover each layer of the sucrose gradient from the top of the tube (e.g., using a 3-in., 20-G needle attached to a 12-ml syringe). Transfer each recovered layer to a 36.5-ml polyallomer centrifuge tube.

8. Resuspend each recovered layer in 30 ml melanosome wash buffer (MWB), transfer to a 50-ml Oak Ridge centrifuge tube, and centrifuge 30 min at 12,000 × *g*, 4°C, in a Sorvall SS 34 rotor. Remove supernatant.

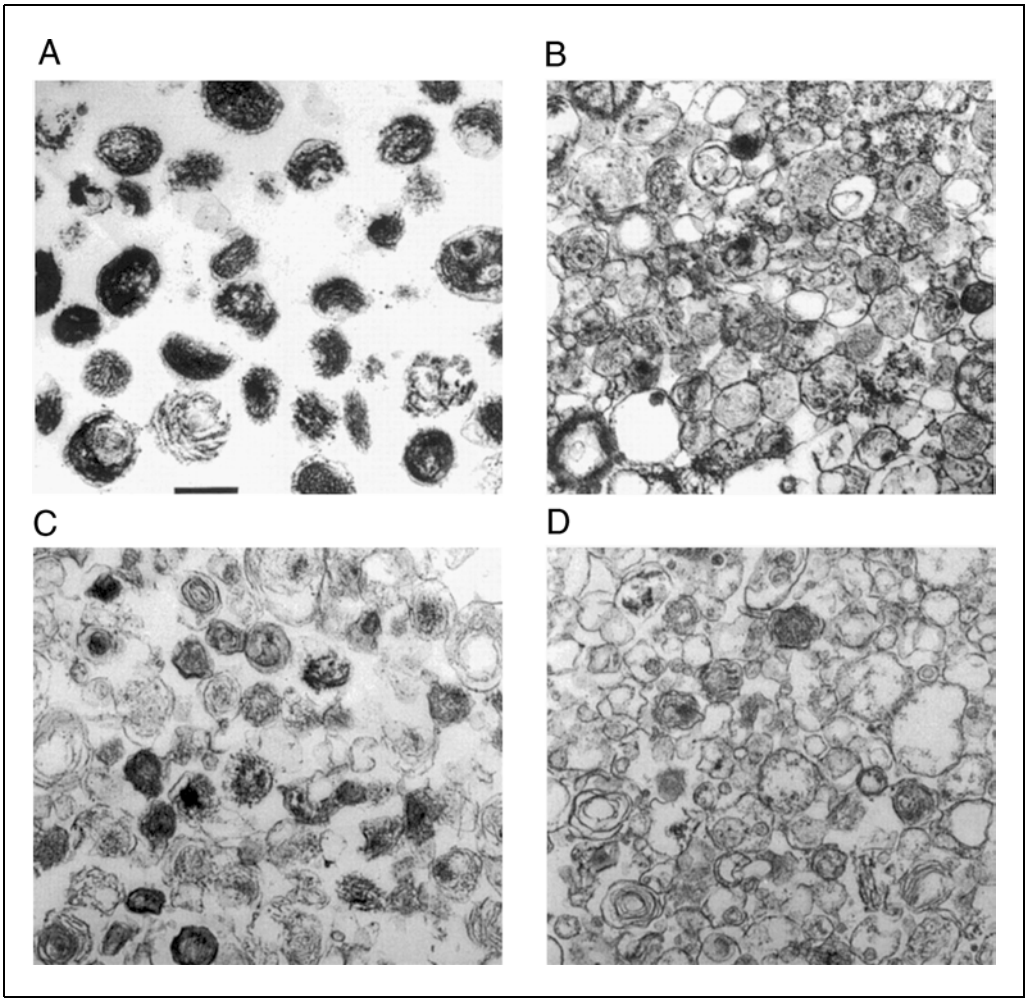


Figure 3.14.2 Ultrastructural appearance of melanosomes in the sucrose density gradient and in free-flow electrophoresis (FFE) fractions purified from the 1.0 M layer of the sucrose density gradient. (**A**) 1.8 M sucrose fraction. (**B**) 1.0 M sucrose fraction. (**C**) FFE fraction II. (**D**) FFE fraction I. Bar = 0.5 μm. Adapted from: Kushimoto et al. (2001).

9. Resuspend each pellet in 1 ml PBS. Wash once with 5 ml PBS, centrifuge 20 min at 5000 × *g*, 4°C, and then remove supernatant. Analyze resulting pellets using enzyme assays, electron microscopy (Fig. 3.14.2A,B), and/or immunoblotting (*UNIT 6.2*) as necessary.

ALTERNATE PROTOCOL 1

PREPARATION OF EARLY MELANOSOMES FROM CULTURED MELANOMA CELLS BY INVERSE DENSITY GRADIENT CENTRIFUGATION

In this protocol, early melanosomes are isolated from cultured cells using an inverse sucrose step gradient.

Additional Materials *(also see Basic Protocol 1)*

MNT-1 human melanoma cells or SK-MEL-28 amelanotic human melanoma cells (American Type Culture Collection)
DMEM, supplemented (for MNT-1 cells; see recipe), or minimal essential medium, supplemented (for SK-MEL-28 cells; see recipe)
2.0 M sucrose (see recipe)
Sucrose step gradient, 0.25 to 1.8 M (see recipe)
Sucrose step gradient, 0.25 to 1.4 M (see recipe)

Culture MNT-1 or SK-MEL-28 cells

1. Using 150-cm^2 culture dishes and supplemented DMEM, grow MNT-1 cells to confluent monolayers. Alternatively, using 150-cm^2 culture dishes and supplemented minimal essential medium, grow SK-MEL-28 cells to confluent monolayers (*UNIT 1.1*).

   This protocol typically calls for a minimum of 30 culture dishes of MNT-1 cells.

Homogenize cultured cells

2. Wash confluent monolayers of cells once with PBS (10 ml/dish). Harvest cells by incubating with trypsin/EDTA (6 ml/dish; see *UNIT 1.1*) for 2 to 3 min, and then adding serum-containing medium (6 ml/dish) to stop the reaction. Pool cells in suitable tubes or flasks. Centrifuge the resulting cell suspensions 5 min at 700 × *g*, 4°C. Remove supernatant.

3. Wash each pellet once with 10 ml CHM-1 and centrifuge 10 min at 1000 × *g*, 4°C. Remove supernatant. Resuspend each pellet in 10 ml CHM-1 and homogenize on ice using 20 strokes in 2 min with a Dounce glass/glass homogenizer.

Isolate postnuclear supernatant

4. Using a pipettor, transfer homogenates (~10 ml each) from step 3 to 15-ml centrifuge tubes and centrifuge 10 min at 1000 × *g*, 4°C.

Isolate early melanosomes

5. Recover supernatants (~8 ml each) from step 4, transfer to 50-ml Oak Ridge centrifuge tubes, and centrifuge 30 min at 19,000 × *g*, 4°C, in a Sorvall SS-34 rotor. Remove supernatant.

6. Resuspend each pellet in 8 ml of 2.0 M sucrose. Using a pipettor, gently layer each of the resulting suspensions onto the bottom of a 0.25 to 1.8 M sucrose step gradient (sucrose concentrations, from top to bottom: 0.25, 1.0, 1.2, 1.4, 1.5, 1.6, and 1.8 M sucrose; 4 ml each) in a polyallomer centrifuge tube.

   A permanent marker can be used to mark the outside of the tube to make subsequent identification of gradient fractions easier.

7. Centrifuge gradients 1 hr at 100,000 × *g*, 4°C, in a Beckman SW-28 swinging-bucket rotor.
8. After centrifugation, carefully recover each sucrose layer (~4 ml each) of each gradient from the top of the tube (e.g., using a 3-in., 20-G needle attached to a 12-ml syringe). Transfer each recovered layer to a suitable tube or flask.
9. Layer each 1.0 M fraction (volume, ~4 ml) into the middle (i.e., between the 0.8 and 1.0 M layers) of a 0.25 to 1.4 M sucrose step gradient (sucrose concentrations, from top to bottom: 0.25, 0.8, 1.2, and 1.4 M; 8 ml each) in a 36.5-ml polyallomer centrifuge tube.
10. Centrifuge gradients 1 hr at 100,000 × *g*, 4°C, in a Beckman SW-28 swinging-bucket rotor.
11. After centrifugation, carefully recover the 1.0 M sucrose fraction from the top of the tube as in step 8. Transfer each recovered layer to a suitable tube or flask.
12. Resuspend each recovered layer in 30 ml MWB, transfer to a 50-ml Oak Ridge centrifuge tube, and centrifuge 30 min at 12,000 × *g*, 4°C, in a Sorvall SS-34 rotor. Remove supernatant.
13. Resuspend each pellet in 1 ml PBS. Wash once with 5 ml PBS, centrifuge 20 min at 5000 × *g*, 4°C, and then remove supernatant. Analyze resulting pellets using enzyme assays, electron microscopy (Fig. 3.14.2A,B), and/or immunoblotting (*UNIT 6.2*) as necessary.

PREPARATION OF EARLY MELANOSOMES FROM CULTURED PIGMENTED MELANOMA CELLS BY DENSITY GRADIENT CENTRIFUGATION AND FREE-FLOW ELECTROPHORESIS (FFE)

ALTERNATE PROTOCOL 2

In this protocol, cultured melanoma cells are homogenized, and the melanosomes within are isolated on a sucrose step gradient. Early melanosomes are further purified by free-flow electrophoresis.

Additional Materials *(also see Basic Protocol 1)*

0.3% (w/v) trypsin
0.6% (w/v) soybean trypsin inhibitor
FFE chamber buffer: 0.25 M sucrose in 1× TEA, pH 7.4 (see recipe for 100×)
Octopus-PZE FFE apparatus (FFE Weber)

Culture MNT-1 cells

1. Using 150-cm^2 culture dishes and supplemented DMEM, grow MNT-1 cells in confluent monolayers (*UNIT 1.1*).

This protocol typically calls for a minimum of 30 culture dishes of MNT-1 cells.

Homogenize cultured cells

2. Wash confluent monolayers of MNT-1 cells once with PBS (10 ml/dish). Harvest cells by incubating with trypsin/EDTA (6 ml/dish; see *UNIT 1.1*) for 2 to 3 min, and then adding serum-containing medium (6 ml/dish) to stop the reaction. Pool cells in suitable tubes or flasks. Centrifuge the resulting cell suspensions 5 min at 700 × *g*, 4°C. Remove supernatant.
3. Wash each pellet once with 10 ml CHM-1 and centrifuge for 10 min at 1000 × *g*, 4°C. Remove supernatant. Resuspend each pellet in 10 ml CHM-1 and homogenize on ice using 20 strokes in 2 min with a Dounce glass/glass homogenizer.

Isolate postnuclear supernatant

4. Using a pipettor, transfer homogenates (~10 ml each) from step 3 to 15-ml centrifuge tubes and centrifuge 10 min at 1000 × *g*, 4°C.

Isolate melanosomes

5. Recover supernatants (~8 ml per tube) from step 4. Using a pipettor, gently layer each supernatant on top of a 1.0 to 2.0 M sucrose step gradient (sucrose concentrations, from top to bottom: 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, and 2.0 M; 4 ml each) in a 36.5-ml polyallomer centrifuge tube.

   A permanent marker can be used to mark the outside of the tube to make subsequent identification of gradient fractions easier.

6. Centrifuge gradients 1 hr at 100,000 × *g*, 4°C, in a Beckman SW-28 swinging-bucket rotor.

7. After centrifugation, carefully recover each 1.0 M sucrose layer from the top of the tube (e.g., using a 3-in., 20-G needle attached to a 12-ml syringe). Transfer each recovered layer to a suitable tube or flask.

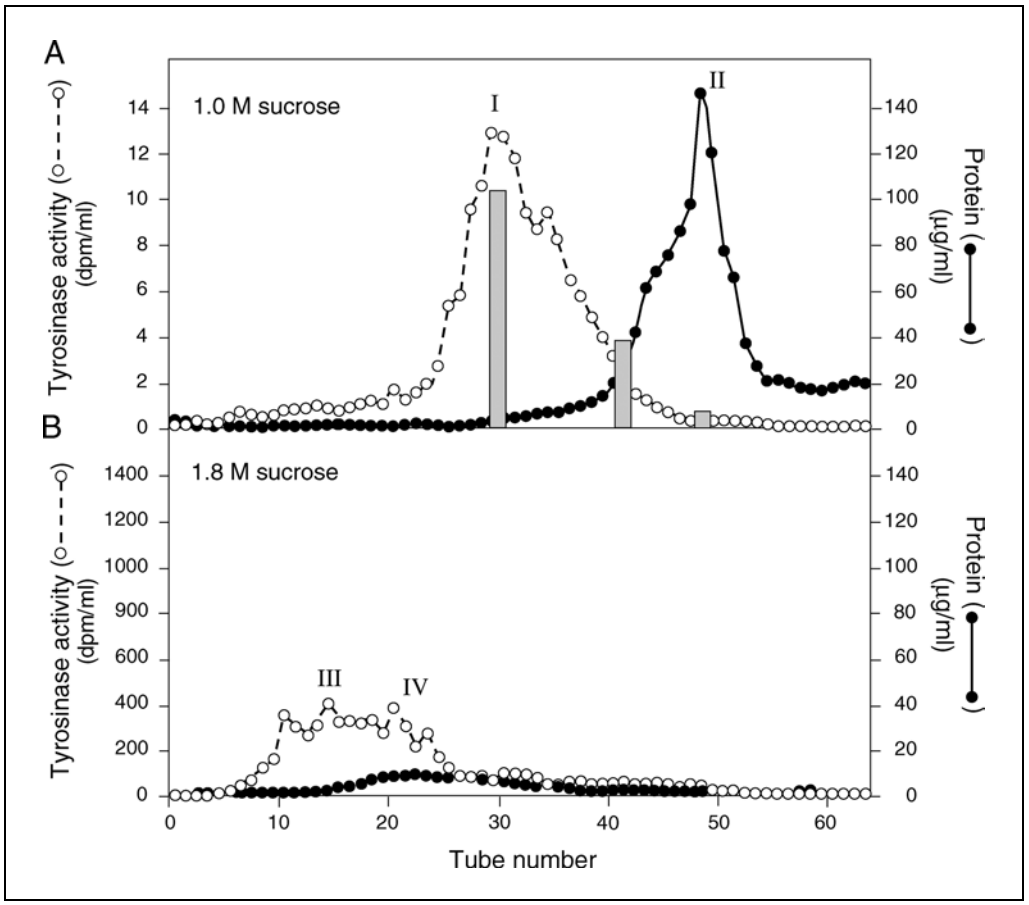


Figure 3.14.3 Distribution curves for tyrosinase (TYR), tyrosinase-related protein 2 (DCT), and other proteins after purification by free-flow electrophoresis (FFE). Elution of the (**A**) 1.0 M and (**B**) 1.8 M sucrose fractions after separation by FFE is shown. Broken lines with open symbols represent TYR enzyme activity, bars represent DCT enzyme activity, and solid lines with closed symbols represent protein content. Enzyme activity data in dpm/ml; protein concentration data in µg/ml. Adapted from: Kushimoto et al. (2001).

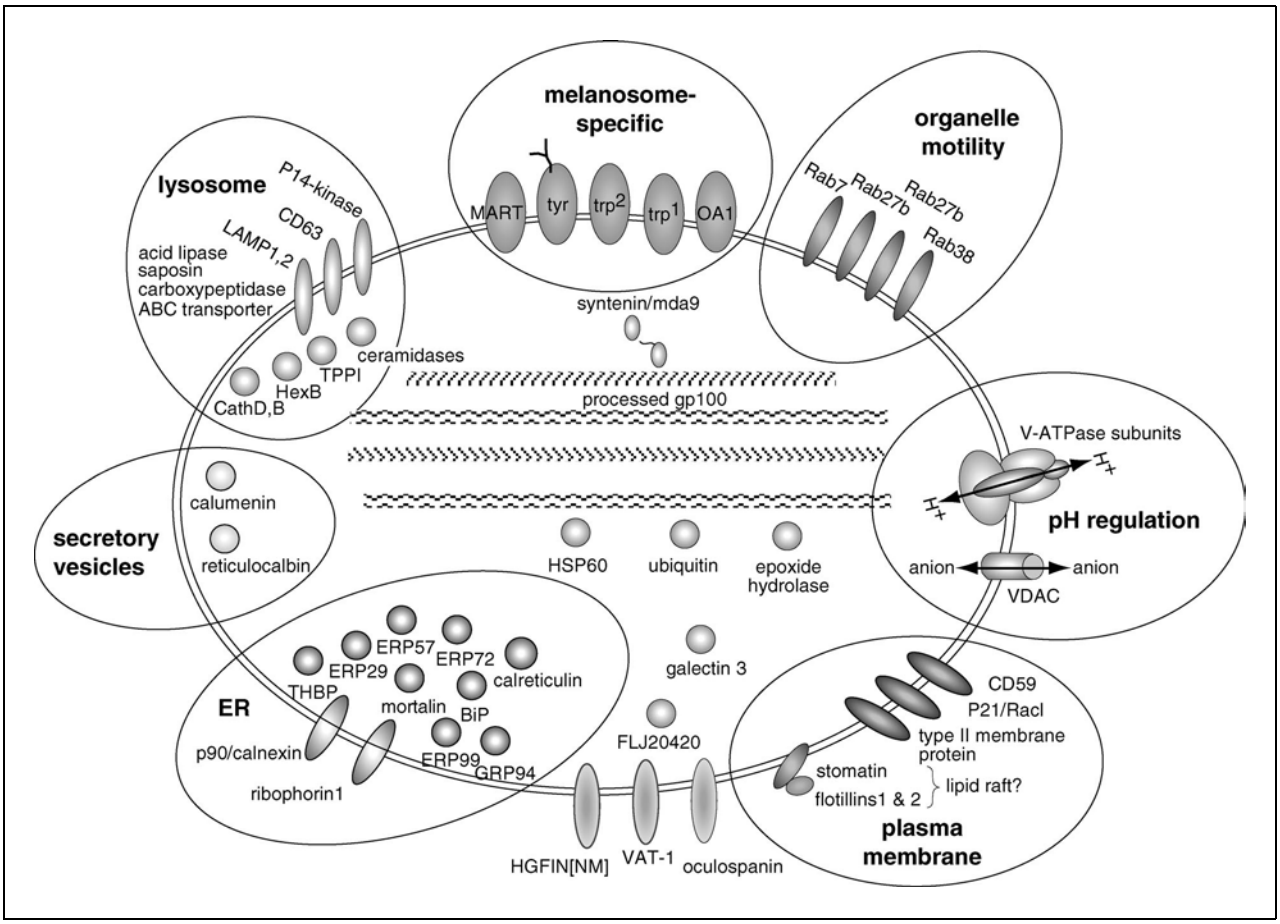


Figure 3.14.4 Proteomic analysis in early melanosomes. Proteins detected in the 1.0 M sucrose fraction after separation by free-flow electrophoresis (FFE) and in fraction I as analyzed by mass spectrometry are shown. Adapted from: Basrur et al. (2003).

8. Resuspend each sample in 30 ml MWB, transfer to a 50-ml Oak Ridge centrifuge tube, and centrifuge 30 min at 12,000 × *g*, 4°C, in a Sorvall SS-34 rotor. Remove supernatant.

9. Resuspend each pellet in 1 ml PBS. Wash once with 5 ml PBS, centrifuge 20 min at 5000 × *g*, 4°C, and then remove supernatant.

Isolate early melanosomes using free-flow electrophoresis (FFE)

10. Immediately before injection into the FFE apparatus, treat each sample from step 9 with 0.5 ml of 0.3% trypsin and incubate at 37°C. After 5 min, stop the reaction by adding 0.5 ml of 0.6% soybean trypsin inhibitor, and immediately cool each sample to 4°C.

11. Taking care not to introduce air bubbles into the FFE chamber, inject the early melanosome fractions into the right inlet of an Octopus-PZE FFE apparatus at a rate of 2.0 ml/hr. After injection, wash the sample inlet by injecting FFE chamber buffer. Perform FFE at 1000 to 1100 V and ~110 to 125 mA, with an elution flow rate of 3 to 4 ml/hr and a chamber temperature of 10°C.

12. Once the sample has reached the top of the separation chamber, begin collecting fractions in 5-ml plastic tubes on ice. Analyze fractions using enzyme assays (Fig. 3.14.3), electron microscopy (Fig. 3.14.2C, D), immunoblotting (*UNIT 6.2*), and/or mass spectrometry (Fig. 3.14.4) as necessary.

PREPARATION OF EARLY AND/OR LATE MELANOSOMES FROM MELANOMA TISSUES BY DENSITY GRADIENT CENTRIFUGATION

In this protocol, melanoma cells are injected into a mouse host. When the resulting tumor is of sufficient size, the tumor tissue is harvested. Tissue fragments are subsequently homogenized, and melanosomes are isolated using a sucrose step gradient.

Materials

B16 melanoma cells
Eagle's minimal essential medium (Sigma) containing 10% (v/v) heat-inactivated FBS (*UNIT 1.2*; HyClone)
C57BL/6 mice
PBS (*APPENDIX 2A*)
Cell homogenization medium–2 (CHM-2; see recipe)
Sucrose step gradient, 1.0 to 2.0 M (see recipe)
MWB (see recipe)
150-cm^2 culture dishes (Bacton Dickinson Labware)
Potter-Elvehjem glass homogenizer
15-ml centrifuge tubes
Tabletop centrifuge, 4°C
36.5-ml polyallomer centrifuge tubes (Seton)
Beckman SW-28 swinging-bucket rotor
3-in., 20-G needle attached to a 12-ml syringe
50-ml Oak Ridge centrifuge tubes (Nalge Nunc)
Sorvall SS-34 centrifuge rotor
Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

NOTE: All protocols using live animals must first be reviewed and approved by an institutional animal care and use committee (IACUC) and must conform to governmental regulations regarding the care and use of laboratory animals.

Culture melanoma cells

1. Using 150-cm^2 culture dishes, culture B16 melanoma cells in Eagle's minimal essential medium containing 10% (v/v) heat-inactivated FBS (*UNIT 1.1*).

   B16 melanoma cells are pigmented mouse melanoma cells. Other pigmented melanoma cells can be used in a similar manner.

Homogenize cultured cells

2. Harvest B16 melanoma cells (Basic Protocol 1, step 2). Implant cells (10^6 per mouse) by injecting subcutaneously (Donovan and Brown, 1995) into the flanks of C57BL/6 mice.

3. When a tumor reaches 1 cm in size, sacrifice the mouse using an approved protocol (preferably CO_2 asphyxiation) and excise the tumor with scissors and forceps. Wash the excised tumor twice, using at least 50 ml PBS for each wash.

4. For each excised tumor, use small scissors to carefully trim away excess connective tissue, and then cut the tumor into small pieces (~1 mm^3) using a sterile surgical blade.

5. For each tumor, resuspend the small pieces in a total of 10 ml CHM-2 and homogenize on ice using 20 strokes of a Potter-Elvehjem glass homogenizer.

Isolate postnuclear supernatant

6. Using a pipettor, transfer homogenates from step 5 (~10 ml per mouse) to 15-ml centrifuge tubes and centrifuge 10 min at 700 × *g*, 4°C.

Isolate melanosomes

7. Recover supernatants from step 6 (~8 ml each). Using a pipettor, gently layer each supernatant on top of a 1.0 to 2.0 M sucrose step gradient (sucrose concentrations, from top to bottom: 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, and 2.0 M; 4 ml each) in a 36.5-ml polyallomer centrifuge tube.

   A permanent marker can be used to mark the outside of the tube to make subsequent identification of gradient fractions easier.

8. Centrifuge sucrose gradients 1 hr at 100,000 × *g*, 4°C, in a Beckman SW-28 swinging-bucket rotor.

9. After centrifugation, carefully recover each layer of each gradient from the top of the tube (e.g., using a 3-in., 20-G needle attached to a 12-ml syringe). Transfer each recovered layer to a 36.5-ml polyallomer centrifuge tube.

10. Resuspend each sample in 30 ml MWB, transfer to a 50-ml Oak Ridge centrifuge tube, and centrifuge 30 min at 12,000 × *g*, 4°C, in a Sorvall SS-34 rotor. Remove supernatant.

11. Resuspend each pellet in 1 ml PBS. Wash once with 5 ml PBS, centrifuge 20 min at 5000 × *g*, 4°C, and then remove supernatant. Analyze resulting pellets using enzyme assays, electron microscopy (Fig. 3.14.2A,B), and/or immunoblotting (*UNIT 6.2*) as necessary.

TYROSINASE ASSAY

SUPPORT PROTOCOL

In this support protocol, the melanosome fractions isolated in the protocols described above are exposed to radiolabeled tyrosine. Because tyrosinase catalyzes the rate-limiting steps in the eventual conversion of tyrosine to melanin, tyrosinase activity can be evaluated by monitoring the appearance of radiolabeled melanin in the assay mixture.

Materials

Melanosome fractions to be assayed (see Basic Protocols 1 and 2 and Alternate Protocols 1 and 2)
Extraction buffer (see recipe)
Tyrosinase assay buffer (see recipe)
25 μCi/ml L-[U-^{14}C]tyrosine solution (100 mCi/mmol)
0.1 N HCl
1 g/liter unlabeled tyrosine in 0.1 N HCl
95% ethanol
Acetone
Scintillation fluid

Vortex mixer
96-well microtiter plates
2.5-cm Whatman 3MM filter paper discs
1-liter Erlenmeyer flasks
Liquid scintillation counter
Scintillation vials

1. Resuspend melanosome fractions in extraction buffer. Mix thoroughly using a vortex mixer, and then store at 4°C for at least 1 hr.

   The volume of extraction buffer added depends on the number of cells used to prepare the sample. Typically, a ratio of ~1 ml/10^6 cells or 1 ml/1 mg protein is used.

2. Transfer samples to microcentrifuge tubes. Centrifuge 2 min at 10,000 × *g*, 4°C. Recover supernatants for tyrosinase assays.

3. For each melanosome fraction, add 30 µl supernatant from step 2, 10 µl tyrosinase assay buffer, and 10 µl of 2.5 µCi L-[U-^{14}C]tyrosine solution to a well in a 96-well microtiter plate. Mix thoroughly by pipetting the resulting solution up and down in the microtiter well. Incubate for 1 hr at 37°C.

   Samples are routinely run at least in duplicate or triplicate.

   Longer incubation times are possible for samples with low tyrosinase activity.

4. Using a pipettor, remove 40 µl from each microtiter well. Apply each 40-µl aliquot to a separate 2.5-cm disc of Whatman 3MM filter paper, and allow filter paper to air dry.

5. Remove unincorporated background label by washing filters (with gentle agitation) in 1-liter beakers (50 filters/liter), using the following washing fluids in sequence:

   200 ml of 0.1 N HCl containing 1 g/liter unlabeled tyrosine (once, for 15 min)
   200 ml of 0.1 N HCl (twice; 15 min per wash)
   100 ml of 95% ethanol (twice; 5 min per wash)
   100 ml acetone (once, for 5 min).

6. Allow filters to dry in air, transfer each filter to a scintillation vial containing 10 ml scintillation fluid, and then assay sample radioactivity using a liquid scintillation counter.

7. Calculate tyrosinase activity using the following standard parameters: time, protein amount, and pmol of product ([^{14}C]melanin) generated (Hearing and Ekel, 1976).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cell homogenization medium–1 (CHM-1)

42.79 g sucrose (0.25 M final)
5 ml 1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Invitrogen; 10 mM final)
Add H_2O to 500 ml
Adjust pH to 7.5 with 5 N NaOH
Store up to 1 week at 4°C
Immediately before use, add 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml of solution
Filter using a 0.45-µm filter unit

Cell homogenization medium–2 (CHM-2)

42.79 g sucrose (0.25 M final)
5 ml 1 M HEPES (Invitrogen; 10 mM final)
1 ml 0.5 M EDTA (1 mM final)
Add H_2O to 500 ml
Adjust pH to 7.5 with 5 N NaOH
Store up to 1 week at 4°C
Immediately before use, add 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml of solution
Filter using a 0.45-µm filter unit

Dulbecco's modified Eagle medium (DMEM), supplemented

Dulbecco's modified Eagle medium (DMEM; Invitrogen) containing:
20% (v/v) heat-inactivated FBS (Atlanta Biologicals)
10% (v/v) AIM V medium (Life Technologies)
20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Invitrogen)
0.1 mM nonessential amino acids (Invitrogen)
1 mM sodium pyruvate (Invitrogen)
2 mM L-glutamine (Invitrogen)
3.7 μg/ml sodium bicarbonate (Invitrogen)
Store up to 1 month at 4°C

The addition of these supplemental ingredients to DMEM helps to maximize the growth and pigmentation of the cells to be cultured.

Extraction buffer

1% (v/v) Nonidet P-40 (NP-40)
0.01% (w/v) SDS
0.1 M Tris·Cl, pH 7.2 (*APPENDIX 2A*)
1 μg/ml aprotinin
100 μM phenylmethylsulfonyl fluoride (PMSF)
Store up to 1 month at 4°C

Melanosome wash buffer (MWB)

42.79 g sucrose (0.25 M final)
5 ml 1 M Tris·Cl, pH 7.5 (10 mM final; *APPENDIX 2A*)
Add filtered H_2O to 500 ml
Store up to 1 week at 4°C
Immediately before use, add 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml of solution
Filter using a 0.45-μm filter unit

Minimal essential medium, supplemented

Minimal essential medium (Invitrogen) containing:
10% (v/v) heat-inactivated FBS (Atlanta Biologicals)
0.1 mM nonessential amino acids (Invitrogen)
1 mM sodium pyruvate (Invitrogen)
2 mM L-glutamine (Invitrogen)
2.8 μg/ml sodium bicarbonate (Invitrogen)
Store up to 1 month at 4°C

See Watabe et al. (2004).

The addition of these supplemental ingredients to minimal essential medium helps to maximize the growth and pigmentation of the cells to be cultured.

Sucrose stock solution, 2 M

To 200 ml H_2O, add:
136.92 g sucrose (2 M final)
Adjust pH to 7.5 with 5 N NaOH
Filter using a 500-ml filter system (pore size, 0.45 μM)
Store up to 1 week at 4°C

Table 3.14.2 Composition of Sucrose Step Gradient Solutions

Step concentration (M)	Composition of step solution		
	2 M sucrose stock solution (ml)	H_2O (ml)	1 M HEPES[a] (μl)
2.0	20	—	—
1.8	18	1.8	200
1.6	16	3.8	200
1.5	15	4.8	200
1.4	14	5.8	200
1.2	12	7.8	200
1.0	10	9.8	200
0.8	8	11.8	200
0.25	2.5	17.3	200

[a]HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Sucrose step gradient

Make appropriate sucrose step gradient solutions (see Table 3.14.2)
Immediately before use, add complete mini protease inhibitor tablets (1 tablet/50 ml)
Store individual solutions up to 1 week at 4°C

To construct the gradient, layer the appropriate volume of each sucrose step gradient solution into a 36.5-ml polyallomer tube, beginning with the most concentrated solution in the gradient and proceeding in order of decreasing concentration. During layering, take care not to allow adjacent layers to mix with one another.

TEA stock solution, 100×

66.6 ml triethanolamine (1 M final)
28.75 ml acetic acid (1 M final)
100 ml 0.5 M EDTA (100 mM final)
Add H_2O to 500 ml
Adjust pH to 7.4 with 5 N NaOH
Filter using a 500-ml filter system (pore size 0.46 μm)
Store up to 3 months at 4°C

Tyrosinase assay buffer

0.1 M sodium phosphate buffer (APPENDIX 2A), pH 7.2, containing:
0.25 mM L-3,4-dihydroxyphenylalanine
0.1 mg/ml cycloheximide
0.1 mg/ml chloramphenicol
0.1 mg/ml BSA
100 units/ml penicillin
Store up to 1 month at −20°C

COMMENTARY

Background Information

Homogenization

Most published methods describing the isolation of melanosomes from melanoma tissues and cells employ standard buffered sucrose solution as the homogenization medium, although there is apparently no rigorous requirement for the presence of a chelating agent such as EDTA. For mammalian tissues, a routine homogenizing buffer containing 0.25 M sucrose/1 mM EDTA, usually buffered with HEPES, is used without additional supplementation. This buffer has also commonly been used for cultured melanoma cells or melanocytes, although EDTA is sometimes omitted. Melanoma cells and melanocytes are best homogenized in a tight-fitting Dounce homogenizer. Melanoma tissues may be homogenized in a Potter-Elvehjem homogenizer.

Density gradient centrifugation

Melanosomes are one of the major components of pigmented melanoma tissues and cultured melanoma cells, and they are obtained from cell homogenates by sucrose density gradient centrifugation. The classical density gradient centrifugation scheme involves two steps: 1000 × *g* for 10 min (nuclear pellet), and 100,000 × *g* for 60 min (pellet containing melanosomes and other membrane-bound organelles). The density of late melanosomes (stages III and IV) is very similar to that of mitochondria in a sucrose gradient, and as a result, the classical method is now regarded as unsatisfactory for melanosome purification. On the other hand, the inverse density gradient centrifugation scheme involves four steps—1000 × *g* for 10 min (nuclear pellet), 19,000 × *g* for 30 min (pellet containing late-stage melanosomes and mitochondria), 100,000 × *g* for 60 min, and 100,000 × *g* for 60 min (pellet containing early melanosome fraction)—because the density of early melanosomes is significantly lower than that of late melanosomes and mitochondria in the sucrose gradient. However, the density of early melanosomes is very similar to that of lysosomes in the sucrose gradient, because the structures of both of these organelles are closely related, and because both initially evolve via the same biogenesis pathway.

Free-flow electrophoresis (FFE)

FFE is an efficient method that provides both analytical capabilities and the opportunity for continuous operation on a preparative scale. The range of substances that can be separated is wide, including small ions, proteins, membrane particles, and even viable cells, and yields of recovered membrane and protein are high. FFE separates membranous cell components and organelles, such as melanosomes, lysosomes, and endosomes, based on their surface charge. This method allows the recovery of organelles and their constituent proteins in a form suitable for studying enzyme activity, morphology, and cytochemistry, among other features.

Critical Parameters and Troubleshooting

Care must be taken to include protease inhibitors in all solutions and to keep these solutions at 4°C; melanosomes contain most, if not all, proteases normally found in lysosomes, and degradation of the sample can occur quickly. The procedures outlined work equally well for purifying melanosomes from normal skin or other pigmented tissue or for purifying these organelles from normal melanocytes in tissue culture, although the amounts recovered from cultured cells will be dramatically reduced compared with the amount recovered from melanoma cells.

Anticipated Results

The purified fraction of melanosomes can be used for enzymatic and/or proteomic analyses or for structural characterization. Despite the fact that the pigmentation of cells and fractions can look very dark, small amounts of melanin can cause very dense pigmentation. Recoveries of early and late melanosomes are usually very low, and significant protein levels in the purified fractions usually indicate poor purity. As a guideline, 30 confluent 150-cm^2 dishes of heavily pigmented MNT-1 melanoma cells (usually 1–2 × 10^8 cells) will usually provide only 30 μg of protein from late (stage III or IV) melanosomes and only 2 μg from early (stages I or II) melanosomes.

Time Considerations

There is no point at which any of these relatively short protocols may be suspended. Indeed, rapid preparation is the key to the recovery of functionally intact melanosomes. All solutions can be made ahead of time and stored at 4°C for 1 week or frozen at −20°C for longer periods (up to 1 month).

Basic Protocols 1 and 2 can be carried out in 2 to 3 hr each, while Alternate Protocol 1 will require ~4 hr because of the additional centrifugation step. FFE assays should be performed as soon as possible after melanosome purification and will require a total of ~7 hr. The tyrosinase asasay (Support Protocol) requires ~4 hr.

Literature Cited

Araki, K., Horikawa, T., Chakraborty, A.K., Nakagawa, K., Itoh, H., Oka, M., Funasaka, Y., Pawelek, J.M., and Ichihashi, M. 2000. Small GTPase Rab3A is associated with melanosomes in melanoma cells. *Pigment Cell Res.* 13:332-336.

Basrur, V., Yang, F., Kushimoto, T., Higashimoto, Y., Yasumoto, K., Valencia, J., Muller, J., Vieira, W.D., Watabe, H., Shabanowitz, J., Hearing, V.J., Hunt, D.F., and Appella, E. 2003. Proteomic analysis of early melanosomes: Identification of novel melanosomal proteins. *J. Protein Res.* 2:69-79.

Dell'Angelica, E.C. 2003. Melanosome biogenesis: Shedding light on the origin of an obscure organelle. *Trends Cell Biol.* 13:503-506.

Dell'Angelica, E.C., Mullins, C., Caplan, S., and Bonifacino, J.S. 2000. Lysosome-related organelles. *FASEB J.* 14:1265-1278.

Donovan, J. and Brown, P. 1995. Parenteral injections. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.6.1-1.6.10. John Wiley & Sons, Hoboken, N.J.

Hearing, V.J. and Ekel, T.M. 1976. Mammalian tyrosinase: A comparison of tyrosine hydroxylation and melanin formation. *Biochem. J.* 157:549-557.

Kobayashi, T., Urabe, K., Orlow, S.J., Higashi, K., Imokawa, G., Kwon, B.S., Potterf, S.B., and Hearing, V.J. 1994. The pmel17/silver locus protein: Characterization and investigation of its role in mammalian melanogenesis. *J. Biol. Chem.* 269:29198-29205.

Kushimoto, T., Basrur, V., Valencia, J., Matsunaga, J., Vieira, W.D., Ferrans, V.J., Muller, J., Appella, E., and Hearing, V.J. 2001. A new model for melanosome biogenesis based on the purification and mapping of early melanosomes. *Proc. Natl. Acad. Sci. U.S.A.* 98:10698-10703.

Kushimoto, T., Valencia, J.C., Costin, G.E., Toyofuku, K., Watabe, H., Yasumoto, K., Rouzaud, F., Vieira, W.D., and Hearing, V.J. 2003. The Seiji Memorial Lecture—The melanosome: An ideal model to study cellular differentiation. *Pigment Cell Res.* 16:237-244.

Kwon, B.S., Chintamaneni, C.D., Kozak, C.A., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Barton, D.E., Francke, U., Kobayashi, Y., and Kim, K.K. 1991. A melanocyte-specific gene, Pmel 17, maps near the silver coat color locus on mouse chromosome 10 and is a syntenic region on human chromosome 12. *Proc. Natl. Acad. Sci. U.S.A.* 88:9228-9232.

Marks, M.S. and Seabra, M.C. 2001. The melanosome: Membrane dynamics in black and white. *Nat. Rev. Mol. Cell Biol.* 2:738-748.

Raposo, G. and Marks, M.S. 2002. The dark side of lysosome-related organelles: Specialization of the endocytic pathway for melanosome biogenesis. *Traffic* 3:237-248.

Seiji, M., Fitzpatrick, T.B., Simpson, R.T., and Birbeck, M.S. 1963a. Chemical composition and terminology of specialized organelles (melanosomes and melanin granules) in mammalian melanocytes. *Nature* 197:1082-1084.

Seiji, M., Shimao, K., Birbeck, M.S., and Fitzpatrick, T.B. 1963b. Subcellular localization of melanin biosynthesis. *Ann. N.Y. Acad. Sci.* 100:497-533.

Wakamatsu, K. and Ito, S. 2002. Advanced chemical methods in melanin determination. *Pigment Cell Res.* 15:174-183.

Watabe, H., Valencia, J.C., Yamaguchi, Y., and Hearing, V.J. 2003. Physiological regulation of skin and hair pigmentation. *J. Clin. Dermatol.* 32:S2-S8.

Watabe, H., Valencia, J.C., Yasumoto, K., Kushimoto, T., Ando, H., Muller, J., Vieira, W.D., Mizoguchi, M., Appella, E., and Hearing, V.J. 2004. Regulation of tyrosinase processing and trafficking by organellar pH and by proteasome activity. *J. Biol. Chem.* 279:7971-7981.

Contributed by Hidenori Watabe,
Tsuneto Kushimoto, Julio C. Valencia,
and Vincent J. Hearing
National Institutes of Health
Bethesda, Maryland

Isolation of Lipid Droplets from Cells by Density Gradient Centrifugation

Lipid droplets are organelles found in most cultured cells, particularly when the cells have been cultured in medium containing serum or exogenous fatty acids (see Fig. 3.15.1). Due to a relatively low protein content and the presence of a neutral lipid core composed of primarily triacylglycerol or cholesterol esters, lipid droplets are more buoyant than other cellular compartments and can be isolated easily by centrifugation. Brief low-speed centrifugation can rapidly float lipid droplets, but other cellular membranes and particularly mitochondria may adhere to the lipid droplets. This unit describes a method to isolate a highly purified lipid droplet fraction with minimal contaminating membranes (see Basic Protocol). The unit also describes an alternate method to disrupt cells while maintaining the integrity of lipid droplets (see Alternate Protocol), a method for loading cells with lipid (see Support Protocol 1), and two methods for solubilizing lipid droplet membrane proteins (see Support Protocols 2 and 3).

ISOLATION OF LIPID DROPLETS FROM CULTURED CELLS BY DENSITY GRADIENT CENTRIFUGATION

BASIC PROTOCOL

Nuclei are removed by low-speed centrifugation and the density of the post-nuclear supernatant is adjusted with sucrose prior to flotation of the lipid droplets through a single discontinuous sucrose gradient. The lipid droplet fraction is characterized by immunoblotting of component proteins.

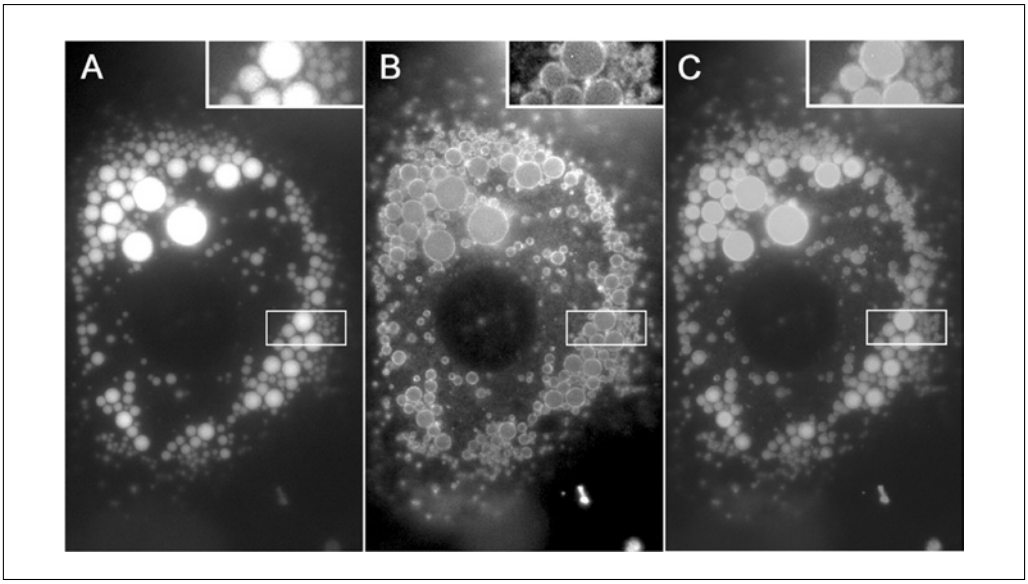


Figure 3.15.1 Cultured 3T3-L1 adipocyte labeled with fluorescent fatty acids (**A** and **C**: green) and stained for perilipin (**B** and **C**: red). Cultured 3T3-L1 adipocytes were differentiated for 6 days with the addition of a BODIPY-labeled 12-carbon fatty acid (Molecular Probes, Invitrogen detection technologies, D3822, BODIPYFL C_{12}) for the final 18 hr. Cells were fixed with 2% paraformaldehyde in PBS prior to staining with guinea pig polyclonal antibody to perilipin (Research Diagnostics, Inc., RDI-PROGP29) followed by AlexaFluor 594 goat anti-guinea pig IgG (Molecular Probes, Invitrogen detection technologies, A11076). Images were captured with a Zeiss Axioplan-2 microscope equipped with a Hamamatsu Orca CCD camera. For the color version of this figure go to *http://www.currentprotocols.com.*

Contributed by Dawn L. Brasaemle and Nathan E. Wolins
Current Protocols in Cell Biology (2005) 3.15.1-3.15.12

NOTE: Protease inhibitors (*UNIT 3.4*) may be included in any or all of the media at the discretion of the investigator.

NOTE: All solutions and glassware should be prechilled to 4°C before the procedure, and kept on ice throughout. Centrifuges, centrifuge rotors, and buckets should be precooled to the same temperature.

Materials

4 to 10 100-mm dishes containing confluent monolayer cells (1–2.5 × 10^7 cells)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice cold
Hypotonic lysis medium (HLM; see recipe), ice cold
HLM containing 60% and 5% (w/w) sucrose (see recipes), ice cold
Rubber policeman or cell-scraper
15-ml plastic tubes with caps
Potter-Elvehjem tissue homogenizer with loose-fitting Teflon pestle (Wheaton), 4-ml capacity, 0.01 to 0.02 cm clearance
Low-speed refrigerated centrifuge with swinging-bucket rotor and appropriate centrifuge tubes
Beckman or Sorvall ultracentrifuge with SW41Ti or Th-641 swinging-bucket rotor
13.2-ml thin-walled polyallomer or polycarbonate ultracentrifuge tubes
Beckman tube slicer with two metal shim rings and two rubber rings to fit ultracentrifuge tubes
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

Prepare cells

1. Wash cells two times with 10 ml ice-cold PBS per dish.
2. Using a rubber policeman or cell scraper, scrape cells from all dishes into 10 to 15 ml ice-cold PBS in a single 15-ml plastic tube with cap and pellet cells by centrifuging 10 min at 1000 × *g*, 4°C.
3. Remove the supernatant with a pipet to a new 15-ml plastic tube with cap and gently and thoroughly resuspend the cells in ice-cold HLM by pipetting the cells up-and-down using a pipet tip with a wide opening. Use a volume of about five times the cell pellet volume.

   Total volume will be 1.5 to 2.5 ml.

4. Incubate the suspended cells on ice for 10 min.

Homogenize cells

5. Transfer the resuspended cells to a Potter-Elvehjem tissue homogenizer on ice. Keeping the homogenizer on ice, insert the Teflon pestle and slowly homogenize the cells by six to eight gentle strokes with the hand-driven pestle. Transfer the homogenate to a 15-ml tube.
6. Centrifuge the cell lysate 10 min at 1000 × *g*, 4°C.
7. Collect the supernatant and the floating fat layer into a separate 15-ml tube.
8. Add 1/3 volume of ice-cold HLM containing 60% sucrose (final 20% sucrose), and mix by gentle pipetting using a pipet tip with a wide opening until aggregates of lipid droplets are finely and thoroughly dispersed.

Isolate lipid droplets

9. Layer density adjusted cell lysate into the bottom of a 13.2-ml ultracentrifuge tube for an SW41Ti rotor, or equivalent.

Table 3.15.1 Adipocyte Lipid Droplet–Associated Proteins and Corresponding Commercially Available Antibodies

Protein	Gel:band no.[a]	Antibody[b]	Product no.	Source[c]
Perilipin A	A:17-19; B:16-17	Guinea pig polyclonal	RDI-PROGP29	Research Diagnostics
		Rabbit polyclonal	RDI-PERLIPABabr	Research Diagnostics
		Rabbit polyclonal	PA1-1052	Affinity BioReagents
		Rabbit polyclonal	ab3527	Novus Biologicals
Adipophilin	B:23	Mouse monoclonal	RDI-PRO651102	Research Diagnostics
		Guinea pig polyclonal	RDI-PROGP40	Research Diagnostics
TIP47	A:21; B:20	Guinea pig polyclonal	RDI-PROGP30	Research Diagnostics
Hormone-sensitive lipase	A:11; B:9, 10	Chicken polyclonal	ab17652	Novus Biologicals
Vimentin	A:20; B:18	Mouse monoclonal	RDI-PRO61013	Research Diagnostics
		Mouse monoclonal	MA3-745	Affinity BioReagents
		Mouse monoclonal	V2258	Sigma-Aldrich
		Guinea pig polyclonal	RDI-PROGP53	Research Diagnostics
		Goat polyclonal	V4630	Sigma-Aldrich
Calnexin	A:9; B:8	Mouse monoclonal	RDI-CALNEXabm	Research Diagnostics
		Mouse monoclonal	MA3-027	Affinity BioReagents
		Rabbit polyclonal	SPA-860/865	Stressgen Bioreagents
		Rabbit polyclonal	C4731	Sigma Aldrich
Caveolin-1	B:36	Mouse monoclonal	RDI-CAVEOL1abm	Research Diagnostics
		Mouse monoclonal	610406	BD Transduction Laboratories
		Rabbit polyclonal	RDI-CAVEOL1abrX	Research Diagnostics
		Rabbit polyclonal	PA1-064	Affinity BioReagents
		Rabbit polyclonal	C3237	Sigma-Aldrich
		Rabbit polyclonal	610059	BD Transduction Laboratories

[a] Band numbers refer to gels A and B depicted in Figure 3.15.2.
[b] Listed antibodies may not work well for all applications.
[c] Additional antibodies are available from sources that are not listed.

10. Gently layer 5 ml ice-cold HLM containing 5% sucrose over sample.
11. Gently add 5 to 6.5 ml ice-cold HLM over the sucrose layers to fill the tube.
12. Centrifuge tube 30 min at 28,000 × *g*, 4°C. Allow rotor to coast to a stop.

    A balance tube containing equivalent volumes of the solutions and equal mass will be required if a single sample is prepared.

Collect lipid droplets

13. Remove ultracentrifuge tubes from ultracentrifuge rotor and place into Beckman tube slicer with blade placement 5 to 8 mm below the floating opaque lipid droplet layer. Slice tube firmly and steadily.
14. Collect the lipid droplet fraction from the top chamber of the tube slicer using ice-cold HLM to rinse residual lipid droplets from the sides of the tube and surface of the cutting blade.

Characterize lipid droplets

15. Check recovery and purity of an aliquot of the lipid droplet fraction by SDS-PAGE (*UNIT 6.1*) of solubilized proteins (see Support Protocol 2) and immunoblotting (*UNIT 6.2*), assaying for immunoreactivity against lipid droplet–associated proteins including adipophilin (~47 kDa for mouse) for all types of cells except differentiated adipocytes, and perilipin (~57 kDa for mouse) for differentiated adipocytes.

    The sizes of the two proteins adipophilin and perilipin A provided here, ~47 kDa and ~57 kDa, respectively, pertain to mouse proteins. These protein sizes vary between species.

16. Check for contamination of lipid droplet fractions with other membranes by immunoreactivity with antibodies specific for various intracellular membrane marker proteins.

ALTERNATE PROTOCOL

ISOLATION OF LIPID DROPLETS WITH LYSIS OF CELLS USING A CELL DISRUPTION BOMB

Nitrogen cavitation is particularly effective in lysing cells while keeping lipid droplets intact.

Additional Materials *(also see Basic Protocol)*

Suspended cells in HLM (see Basic Protocol, step 4)
45-ml cell disruption bomb (Parr)
15- and 50-ml plastic tubes

1. Transfer suspended cells in HLM into the chamber of a cell disruption bomb. Pressurize the chamber with nitrogen at 450 psi for 15 min.
2. Release the sample dropwise and collect in a 50-ml plastic tube.
3. Centrifuge tube 5 min at 100 × *g*, 4°C, to collect the sample at the bottom of the tube; transfer to a 15-ml plastic tube with cap.
4. Continue with Basic Protocol, step 6.

SUPPORT PROTOCOL 1

LIPID LOADING OF CULTURED CELLS

Many cultured cells store very low mass of neutral lipids in lipid droplets. To promote the synthesis of triacylglycerols and the formation of lipid droplets, the culture medium can be supplemented with 100 μM to 1 mM fatty acids complexed to albumin. Oleic acid is commonly used for lipid loading of cells because it is a good substrate for triacylglycerol biosynthesis (Coleman and Lee, 2004). The most critical step of the procedure is to assure complete complexation of fatty acids to the albumin to prevent detergent effects of unbound fatty acids. One mole of albumin may bind up to 7 mol of fatty acids, depending upon the acyl chain length of the fatty acid (Petitpas et al., 2001); the accompanying procedure guides complex formation of 6 mol of fatty acids to 1 mol of bovine serum albumin. The starting albumin must be fatty acid–free to avoid the addition of an excess of fatty acids. The formation of complexes is accompanied by a visible clearing of the solution; cloudy solutions contain aggregates of uncomplexed fatty acids.

Materials

Fatty acid–free bovine serum albumin
0.1 M Tris·Cl, pH 8.0
Oleic acid
50-ml screw-capped polypropylene tubes
Rotisserie shaker
0.2- or 0.45-μm filter unit

1. Dissolve 3.36 g fatty acid–free bovine serum albumin in 24 ml of 0.1 M Tris·Cl, pH 8.0.
2. Transfer 84 mg oleic acid into a clean 50-ml screw-capped tube using a pipet tip with a wide opening.

   A wide-opening tip should be used because the oleic acid is viscous and difficult to pipet.
3. Add 24 ml of albumin solution to the oleic acid, and mix using a rotisserie shaker, or by gentle inversion and swirling to avoid excessive foaming.

   Complex formation is complete when cloudiness in the solution disappears and there is no visible uncomplexed oleic acid on the side or bottom of the tube.
4. Sterile filter the solution through a 0.2- or 0.45-μm filter unit.
5. Add solution to culture medium to a final concentration of 100 μM to 1 mM oleic acid.

SOLUBILIZATION OF LIPID DROPLET–ASSOCIATED PROTEINS FOR IMMUNOBLOTTING

SUPPORT PROTOCOL 2

Lipid droplet fractions contain a low mass of protein, but high relative mass of neutral lipids, including either triacylglycerols or cholesterol esters, in addition to phospholipids. The high lipid content of the samples interferes with the resolution of proteins by SDS-PAGE. When the lipid droplet fractions are fresh and have not been frozen, the component proteins can be solubilized using detergent solutions with warming and sonication of the sample.

Materials

10% (w/v) sodium sodecyl sulfate (SDS; see recipe)
Fresh lipid droplet fraction (see Basic Protocol)
2× SDS sample buffer (for discontinuous systems; see *UNIT 6.1*)
Sonicating water bath with adjustable temperature
Vortex mixer
1.5-ml microcentrifuge tubes
Gel-loading pipet tip or wide-guage needle attached to a small syringe
Additional reagents and equipment for a discontinuous SDS-PAGE gel (see *UNIT 6.1*)

1. Add 1 vol of 10% SDS to the fresh (not frozen) lipid droplet fraction in HLM. Incubate for at least 1 hr at 37°C in a sonicating water bath. Remove the sample every 5 to 10 min and agitate on a vortex mixer before returning to the bath.
2. Transfer the sample to a 1.5-ml microcentrifuge tube and microcentrifuge 10 min at maximum speed, room temperature.
3. Use a gel-loading pipet tip or a wide-gauge needle attached to a small syringe to collect the infranatant containing the solubilized proteins from beneath the floating lipid layer without removing any of the opaque floating lipid or any precipitated insoluble material from a visible pellet. Transfer to a new 1.5-ml microcentrifuge.

   If the floating lipid layer becomes disrupted, repeat microcentrifugation to consolidate the lipid layer.
4. Add an equivalent volume of 2× SDS sample buffer, and boil the sample for 10 min prior to loading onto a discontinuous SDS-PAGE gel (see *UNIT 6.1*).

DELIPIDATION AND SOLUBILIZATION OF LIPID DROPLET–ASSOCIATED PROTEINS

Alternatively, frozen lipid droplet fractions, or fractions containing excessive lipid can be delipidated using solvents, and the precipitated proteins solubilized in concentrated detergent solutions with warming and sonication. The extent of solvent delipidation required is dependent upon the lipid content of the sample; whereas most samples require only a single delipidation step, lipid droplets isolated from adipocytes and highly lipid-enriched cells require multiple solvent delipidation steps.

Materials

Acetone, −80°C and room temperature
Frozen lipid droplet fraction, thawed
1:1 (v/v) acetone/ether
Ether
2× SDS sample buffer (for discontinuous systems; see *UNIT 6.1*)
Extra reducing reagent (e.g., β-mercaptoethanol or dithiothreitol)

Polypropylene screw-capped centrifuge tubes (Sarstedt)
High-speed refrigerated centrifuge with Sorvall SS34 rotor and tube adapter sleeves, or equivalent
Sonicating water bath with adjustable temperature
1.5-ml microcentrifuge tubes

NOTE: All procedures using organic solvents should be carried out in a fume hood. Tubes should be tightly capped before removing samples from the fume hood for incubation or centrifugation steps. Glass pipets and storage containers should be used to transfer solvents, because disposable polystyrene laboratory pipets will dissolve in many organic solvents. Solvent waste should be disposed of in accordance with institutional policy.

Delipidate sample with acetone

1. Add at least 10 vol of cold acetone that has been stored at −80°C to the thawed or fresh lipid droplet fraction in a screw-capped polypropylene tube. Cap the tube and mix the sample thoroughly by inversion.
2. Incubate the sample overnight at −20°C, or for 4 hr on dry ice.
3. Mix the sample by inversion and centrifuge 1 hr at 4300 × *g*, 4°C.
4. Carefully remove acetone from the loose pellet by pouring off the solvent, or removing with a glass pipet. Discard the acetone in a suitable container.

If samples do not require additional delipidation

5a. Remove residual solvent residue from the pellet using a gentle stream of nitrogen in a fume hood. Continue to step 12.

If additional delipidation is required (adipocyte lipid droplet fractions and samples yielding large fluffy appearing pellets)

5b. Check the tube carefully for stress fractures. Add 10 vol of room temperature acetone to the protein pellet and vortex to mix. Centrifuge 30 min at 4300 × *g*, 4°C.

6b. Carefully remove acetone from the loose pellet by pouring off the solvent, or removing with a glass pipet. Discard the solvent in an appropriate container.

7b. Add 10 vol of 1:1 acetone/ether to the protein pellet at room temperature and vortex to mix. Centrifuge 30 min at 4300 × *g*, 4°C.

8b. Carefully remove the solvent from the loose pellet. Discard the solvent in an appropriate container.

9b. Add 10 vol ether to the pellet at room temperature and vortex to mix. Centrifuge 30 min at 4300 × g, 4°C.

10b. Carefully remove the solvent from the loose pellet. Discard the solvent in an appropriate container.

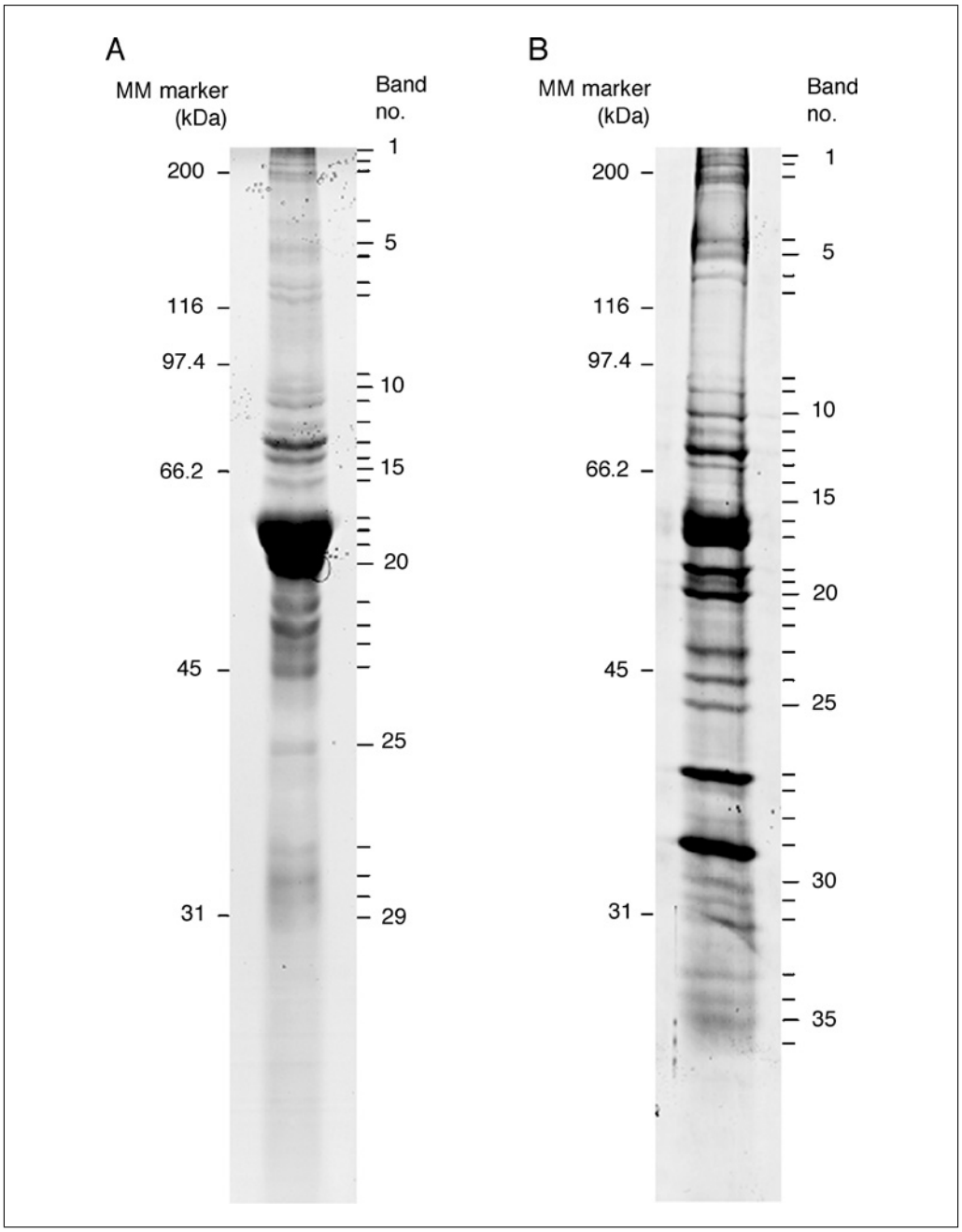


Figure 3.15.2 Coomassie Blue-stained proteins of lipid droplets isolated from 3T3-L1 adipocytes incubated under basal, lipid-storing conditions (**A**) and lipolytically stimulated conditions (**B**). Positions of molecular mass markers and stained bands are depicted on the left and right sides of the panels, respectively. Figure reprinted with permission of *The Journal of Biological Chemistry*, from "Proteomic Analysis of Proteins Associated with Lipid Droplets of Basal and Lipolytically Stimulated 3T3-L1 Adipocytes," Dawn L. Brasaemle, Georgia Dolios, Lawrence Shapiro, and Rong Wang, Vol. 279 (2004) 46835-46842 (original manuscript); Correction Vol. 280 (2005) 4004; permission conveyed through Copyright Clearance Center, Inc.

11b. Remove residual solvent residue using a gentle stream of nitrogen in a fume hood.

Prepare samples for SDS-PAGE gel

12. Add 2× SDS sample buffer to the dry pellet using the largest volume that will fit into sample wells of gel; do not further dilute 2× SDS sample buffer to a 1× concentration.

13. Incubate sample 4 to 6 hr at 60°C in a sonicating water bath. Remove the sample every 5 to 15 min and agitate vigorously using a vortex mixer before returning to the water bath.

14. Transfer the sample to a 1.5-ml microcentrifuge tube and microcentrifuge 10 min at 14,000 × *g*, room temperature.

    The presence of a pellet indicates incomplete solubilization of proteins. Repeat sonication at 60°C, if necessary.

15. Add sufficient additional reducing reagent (e.g., β-mercaptoethanol or dithiothreitol) to replace the majority of reducing reagent in the initial volume of 2× SDS sample buffer (much of the reagent will have been lost during solubilization of the proteins). Load samples onto an SDS-PAGE gel in 2× SDS sample buffer without dilution. Run the gel.

    All samples and molecular weight standards must contain the same volume and 2× concentration of SDS sample buffer.

    Samples may be boiled prior to loading, or loaded onto gels without further processing.

    A full-size SDS-PAGE gel is recommended rather than a mini-gel due to the larger size of the sample wells; it is advantageous to use as large a volume of 2× SDS sample buffer as possible to fully solubilize delipidated lipid droplet proteins. Failure to adequately delipidate the samples will result in one of two possible outcomes: (1) proteins within the samples will be retarded in the wells or stacking gel, and will not migrate sufficiently into the gel, or (2) vertical streaking or smearing of the samples will occur, yielding poor resolution of protein bands. Insufficient solubilization of the samples will yield a low signal of lipid droplet–associated proteins.

    See Figure 3.15.2 for a gel showing proteins associated with lipid droplets and Table 3.15.1 for antibodies to those proteins and their suppliers.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

HLM containing 5% (w/w) sucrose

2.54 g sucrose
48.34 g HLM (see recipe)
Store 1 to 2 days at 4°C

HLM containing 60% (w/w) sucrose

38.6 g sucrose
25.73 g HLM (see recipe)
Store 1 to 2 days at 4°C

Hypotonic lysis medium (HLM)

1 ml 1 M Tris·Cl, pH 7.4 (20 mM final)
100 µl 0.5 M EDTA (1 mM final)
1 ml 0.5 M sodium fluoride (10 mM final; *optional*)

continued

Protease inhibitors (see *UNIT 3.4*)
H_2O to 50 ml
Prepare fresh and keep on ice

Sodium fluoride is a general phosphatase inhibitor.

Sodium dodecyl sulfate (SDS) 10% (w/v)

10 g SDS
H_2O to 100 ml
Store 1 month at room temperature

COMMENTARY

Background Information

Most cultured cells and many types of cells within tissues have lipid droplets composed of a core of neutral lipids surrounded by a membrane monolayer of phospholipids and cholesterol, into which proteins are embedded. The majority of cells have a few tiny lipid droplets of 0.5 to 5 μm in diameter and containing cores of primarily cholesterol esters, whereas adipocytes store triacylglycerol in large lipid droplets that can reach diameters of 100 μm. In adipocytes and muscle cells, triacylglycerol stored in lipid droplets serves as an important source of energy, whereas in the majority of cells, stored cholesterol esters serve a role in the maintenance of cellular cholesterol homeostasis and are used for membrane synthesis and repair. In steroidogenic cells found in the adrenal cortex, testes, and ovaries, stored cholesterol esters are a source of substrate for steroid hormone synthesis. In stellate cells of the liver and in the retina of the eye, lipid droplets are enriched with retinyl esters.

Identification of the PAT family of lipid droplet–associated proteins (perilipin, adipophilin, also called ADRP, and TIP47; also including S3-12) that contain highly conserved sequences of amino acids has created interest in the proteomics of lipid droplets and the study of lipid droplets as a dynamic metabolic compartment.

Additionally, several enzymes required for the biosynthesis of sterols and for acylation of neutral lipids and phospholipids have been localized to lipid droplets (Caldas and Herman, 2003; Ohashi et al., 2003; Brasaemle et al., 2004; Fujimoto et al., 2004; Liu et al., 2004; Umlauf et al., 2004).

In general, the culture medium used to promote the growth of most cell lines contains a relatively low concentration of fatty acid and cholesterol substrates for neutral lipid synthesis. Under these conditions, most cell lines display few or no lipid droplets per cell, when visualized by staining of cells with the lipophilic dyes Nile Red (Greenspan et al., 1985), or Bodipy 493/503 (Gocze and Freeman, 1994). The addition of either decimillimolar concentrations of fatty acids complexed to albumin, or a combination of fatty acids bound to albumin and cholesterol delivered via liposomes or cyclodextrins promotes increased synthesis of neutral lipids and subsequent storage of these lipids in lipid droplets.

The isolation of lipid droplets relies upon the buoyant density of lipid droplets, which is <1 g/cm^3. Two low-speed centrifugation steps and a single ultracentrifugation step using a discontinuous density gradient will collect >95% of the lipid droplets from a cell lysate. The isolated lipid droplets may be used as a source of lipid substrates for studying the activities of lipid metabolic enzymes. To date, no specific enzyme activities have been localized exclusively to lipid droplets. The PAT family proteins adipophilin and perilipin are structural proteins that are used as specific markers of the lipid droplet compartment in immunoblotting and indirect immunofluorescence applications. The use of isolated lipid droplet fractions for proteomics studies necessitates the washing of the initial lipid droplet fraction through several additional centrifugation steps to remove traces of contaminating membranes including microsomes, mitochondria, and fragments of plasma membranes. The described methods may also be scaled up and used to isolate lipid droplets from tissue samples, following the digestion of the tissue to yield a uniform suspension of intact, disaggregated cells.

Critical Parameters and Troubleshooting

The isolation of lipid droplets by centrifugation is a relatively simple procedure. A very low protein-to-lipid ratio renders lipid droplets more buoyant than all other subcellular structures; lipid droplets can be separated from

more dense subcellular compartments using discontinuous density gradients. The most critical step in the isolation of lipid droplets is making an appropriate choice for the method of cell disruption to keep the droplets intact, particularly when working with cells containing very large lipid droplets. Gentle disruption of cells is required to preserve lipid droplet structure. The use of rotor-stator homogenizers or sonication to lyse cells will disrupt and emulsify lipid droplets. Likewise, the extrusion of cells or isolated lipid droplets through small openings exerts sufficient shear force to disrupt lipid droplets. Gentle homogenization using a hand-operated homogenizer with a loose-fitting Teflon pestle, or the use of a nitrogen cavitation bomb will yield better results.

The buoyant density of lipid droplets facilitates flotation during centrifugation of any aqueous medium. The use of solutions lacking electrolytes may reduce the aggregation of lipid droplets with other subcellular organelles; the use of hypotonic solutions does not compromise the integrity of lipid droplets because they have no aqueous compartment. Since segments of endoplasmic reticulum and numerous mitochondria are often closely apposed to the droplets in intact cells, isolated lipid droplet fractions contain low levels of these membranes. Contamination of lipid droplets with these membranes can be minimized by layering density-adjusted cell lysates beneath one or two layers of decreasing density in a discontinuous gradient prior to centrifugation. The movement of the lipid droplets upwards through the layers of the gradient reduces the adherence of contaminant membranes to the droplets and resolves the droplets from soluble proteins. Further removal of contaminating membranes may be enhanced by flotation of the isolated lipid droplets through additional discontinuous density gradients, which may include solutions containing a low concentration of glycerol, 100 mM to 1 M sodium chloride, or 100 mM sodium carbonate, pH 11.5 (Fujiki et al., 1982). The use of sodium carbonate wash solutions, however, may remove loosely adherent proteins that normally localize to the outer membrane monolayer of lipid droplets, and may denature the proteins. While lipid droplets can be isolated using tubes with volumes smaller than those described, and with correspondingly reduced volumes of the gradient solutions, higher levels of contaminating membranes may be floated with the lipid droplet fraction. Wide-diameter tubes are not recommended for the isolation of lipid droplets from relatively small numbers of cells, because the lipid droplet layer will be diffuse and more easily disrupted during sample collection. Additionally, swinging-bucket rotors are recommended to band the floating lipid droplets compactly at the top of the tube; rotors should be allowed to coast to a stop through the final deceleration to minimize disruption of the lipid droplet layer.

Following centrifugation, lipid droplets will appear as a milky layer at the top of the tube. If there is a clear oily layer at the top of the tube, the lipid droplets have broken during sample preparation; this is indicative of a poor isolation. Gentle homogenization with fewer strokes and slower insertion of the pestle will reduce disruption of the lipid droplets. There will be a pellet containing dense membranes at the bottom of the tube and one or two translucent bands of membranes at the interfaces of the density phases. If these bands appear very cloudy and white, this indicates breakage and emulsification of the lipid droplets, and a poor preparation of lipid droplets. A portion of the floating droplet fraction will adhere to the sides of the tube, and to the surfaces of any pipetting device used to collect the fraction. Additionally, if the layer is disrupted during handling of the tube or collection of the samples, some of the droplets may swirl down into the top layer of the gradient, making sample collection more difficult.

The best way to collect the majority of the lipid droplet fraction in a small volume is to use a Beckman tube slicer in combination with thin-walled polyallomer or polycarbonate tubes. The tube is inserted into the tube slicer to position the cutting blade several millimeters below the lipid droplet layer. The steady and firm insertion of the blade through the tube isolates the lipid droplet layer in a small volume of the top gradient solution in the sliced top portion of the tube. Rubber rings within the tube slicer form a seal with the blade, keeping the top solution from leaking out. Use of a tube slicer facilitates the most efficient collection of the lipid droplets in a minimal volume, and it permits rinsing of the surfaces of the blade and tube with additional solution to more completely collect the fraction. When a tube slicer is unavailable, the lipid droplet fraction can be collected using either a pipetting device or a syringe with a wide-bore needle; however, it is more difficult to collect the entire lipid droplet layer in a minimal volume. Additionally, there

will be significant loss of lipid droplets that are adherent to the sides of the tube. Gradient collectors that puncture the bottoms of tubes and collect the most dense fractions first are unsuitable for the collection of lipid droplets, because the majority of the lipid droplets will adhere to the sides of the tube as the volume of liquid drops.

Lipid droplets from chordates are characterized by their content of neutral lipids and by the presence of one or more members of the PAT family of proteins. The neutral lipid content may be assessed by quantitative thin layer chromatography (Brasaemle et al., 1997a) of solvent extracts of lipids (Bligh and Dyer, 1959), or by the adaptation of commercially available clinical assays for the quantitation of triacylglycerol or cholesterol for use with extracted lipids. The content of PAT family proteins is best assessed by immunoblotting of proteins extracted from lipid droplet fractions. Since lipid droplets have an extremely low content of protein relative to lipid, it is imperative to remove the excess lipid before applying samples to SDS-PAGE gels (see Support Protocols 2 and 3). Detergent solubilization of proteins in preparation for SDS-PAGE must be conducted using freshly isolated lipid droplet fractions; the recovery of PAT family proteins from frozen and thawed samples of lipid droplets is significantly reduced. A buffered solution containing 1% Triton X-100 and 0.5% sodium deoxycholate can substituted for the SDS-containing sample buffer. Lipid droplet fractions that have been frozen and thawed can be delipidated with solvents and the proteins solubilized in 2× concentrated sample buffer; the higher content of SDS and reducing reagent in undiluted 2× sample buffer helps to solubilize the relatively insoluble component proteins. Effective solubilization of precipitated proteins requires extensive warming and sonication of the samples; however, the use of a probe sonicator will cause foaming of the sample and should be avoided. Solubilization of samples for several hours at 60°C with sonication in a bath sonicator is more effective than brief boiling of the samples. Samples should be loaded on gels immediately after the solubilization steps to avoid precipitation of the component proteins. For most types of cells, immunoblotting for adipophilin will identify lipid droplets; however, immunoblotting for perilipin is required for lipid droplets isolated from mature adipocytes, since adipophilin is excluded from the droplets when the expression of perilipin is induced during adipose differentiation (Brasaemle et al., 1997b).

Anticipated Results

This procedure will yield a lipid droplet fraction that contains >95% of the total neutral lipid content of the cells, and >95% of adipophilin or perilipin. The lipid droplet fraction will contain an extremely low protein mass relative to that of lipid; the relative protein mass will vary with the size of the lipid droplet and type of cell used. The lipid droplet fraction will also contain low levels of contamination with mitochondria and membrane fragments from endoplasmic reticulum and plasma membrane. Typical preparations of adipocyte lipid droplets using this procedure contain ≤5% of the total content of calnexin, a marker for endoplasmic reticulum, when assessed by immunoblotting. The relative purity of the lipid droplet fraction will be influenced by several factors including the size of the lipid droplets and the number of lipid droplets in the cell lysate. Lower levels of contaminants may be anticipated when isolating lipid droplets from cells that have very small lipid droplets, and when centrifugation of the cell lysates yields a distinct, but relatively thin lipid layer.

If the isolated lipid droplets are being used to establish the relative content in lipid droplets of a protein that is found in other subcellular compartments, then it is essential to characterize the levels of contaminant membranes using either immunoblotting methods, or marker enzyme assays. When analyzing the distribution of a protein that localizes to both lipid droplets and another subcellular compartment, care must be taken to make appropriate comparisons. Lipid droplets contain an extremely low protein content relative to lipid, whereas other membrane compartments are relatively protein dense. Thus, comparing lipid droplets to other membrane fractions on the basis of total protein content of the compartments is inappropriate, as it will require the use of lipid droplets from 20- to 100-fold greater cell mass than for the other subcellular compartments. It is more appropriate to compare a volume of the lipid droplet fraction that represents an equivalent fraction of the whole cell lysate.

Time Considerations

The isolation of intact lipid droplets requires fresh and not frozen cell or tissue samples. It is necessary to proceed from harvest of

cells through the collection of lipid droplets without storage of samples at intermediate steps of the procedure. Since the centrifugation steps are relatively brief, all steps from the harvest of cells through the initial collection of the lipid droplet fraction can be completed within 4 hr. Additional washes of the lipid droplet fraction should be done on freshly isolated material, immediately following the initial isolation. The lipid droplet fraction can then be stored for several hours at 4°C, or several weeks at −80°C.

Assessment of lipid droplet components can be performed upon frozen and thawed samples of isolated lipid droplets, although recovery of proteins using the detergent solubilization procedure will be reduced for samples that have been stored at −80°C. Protein precipitation by solvent delipidation, and the subsequent solubilization of the proteins will require at least 8 hr, if a one-step delipidation is completed. SDS-PAGE may be completed in 2 to 4 hr and transfer of proteins to membranes and subsequent immunoblotting takes 10 to 24 hr.

Literature Cited

Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Med. Sci.* 37:911-917.

Brasaemle, D.L., Barber, T., Kimmel, A.R., and Londos, C. 1997a. Post-translational regulation of perilipin expression. Stabilization by stored intracellular neutral lipids. *J. Biol. Chem.* 272:9378-9387.

Brasaemle, D.L., Barber, T., Wolins, N.E., Serrero, G., Blanchette-Mackie, E.J., and Londos, C. 1997b. Adipose differentiation-related protein is a ubiquitously expressed lipid storage droplet-associated protein. *J. Lipid Res.* 38:2249-2263.

Brasaemle, D.L., Dolios, G., Shapiro, L., and Wang, R. 2004. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J. Biol. Chem.* 279:46835-46842.

Caldas, H. and Herman, G.E. 2003. NSDHL, an enzyme involved in cholesterol biosynthesis, traffics through the Golgi and accumulates on ER membranes and on the surface of lipid droplets. *Hum. Mol. Genet.* 12:2981-2991.

Coleman, R.A. and Lee, D.P. 2004. Enzymes of triacylglycerol synthesis and their regulation. *Prog. Lipid Res.* 43:134-176.

Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. *J. Cell Biol.* 93:97-102.

Fujimoto, Y., Itabe, H., Sakai, J., Makita, M., Noda, J., Mori, M., Higashi, Y., Kojima, S., and Takano, T. 2004. Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7. *Biochim. Biophys. Acta* 1644:47-59.

Gocze, P.M. and Freeman, D.A. 1994. Factors underlying the variability of lipid droplet fluorescence in MA-10 Leydig tumor cells. *Cytometry* 17:151-158.

Greenspan, P., Mayer, E.P., and Fowler, S.D. 1985. Nile red: A selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* 100:965-973.

Liu, P., Ying, Y., Zhao, Y., Mundy, D.I., Zhu, M., and Anderson, R.G. 2004. Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *J. Biol. Chem.* 279:3787-3792.

Ohashi, M., Mizushima, N., Kabeya, Y., and Yoshimori, T. 2003. Localization of mammalian NAD(P)H steroid dehydrogenase-like protein on lipid droplets. *J. Biol. Chem.* 278:36819-36829.

Petitpas, I., Grune, T., Bhattacharya, A.A., and Curry, S. 2001. Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. *J. Mol. Biol.* 314:955-960.

Umlauf, E., Csaszar, E., Moertelmaier, M., Schuetz, G.J., Parton, R.G., and Prohaska, R. 2004. Association of stomatin with lipid bodies. *J. Biol. Chem.* 279:23699-23709.

Contributed by Dawn L. Brasaemle
Rutgers, The State University of New Jersey
New Brunswick, New Jersey

Nathan E. Wolins
Washington University of St. Louis School of Medicine
St. Louis, Missouri

Isolation of Mast Cell Granules

This unit describes the means to isolate and use rat serosal mast cell granules in experiments aimed at determining the role of mast cell activation and degranulation in disease. Upon activation, mast cells exocytose, through an energy-requiring process, cytoplasmic secretory granules into their extracellular microenvironment. These granules are modified lysosomes containing preformed mast cell mediators, such as histamine, neutral proteases, cytokines, and growth factors embedded in a heparin proteoglycan matrix (Fig. 3.16.1). Isolated rat serosal mast cells are activated by compound 48/80 (Thon and Uvnäs, 1967), a commercially available basic polyamine (a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde) that has been shown to activate mast cells through activation of G protein–coupled receptors (Aridor et al., 1990). Upon activation, a part of the secretory granules become swollen and their individual perigranular membranes fuse to form a tubular channel in which the granules to be exocytosed lie in chains. The outer end of a degranulation channel fuses with the plasma membrane, and a pore connecting the tubule with the extracellular fluid is formed (Fig. 3.16.2B). The outermost granules are expelled, whereas the innermost granules remain in the degranulation channel. The soluble components of the granules exposed to the extracellular fluid, such as histamine, diffuse away, whereas the heparin proteoglycans and the mast cell–specific neutral proteases (e.g., chymase) remain tightly bound to each other, forming proteolytically active intra- and extracellular granule remnants (Fig. 3.16.3; Kovanen, 1993; Kokkonen et al., 1995). The whole process of degranulation, from stimulation to exocytosis, occurs within a few minutes. This unit focuses on the preparation of serosal mast cells from the peritoneal and pleural cavities of rats for the isolation of mast cell granules (Kokkonen and Kovanen, 1985). It also describes a procedure to obtain intact cytoplasmic granules, which are enveloped by their individual perigranular membranes (Krüger et al., 1980; Lindstedt et al., 1992). These protocols concern rat serosal mast cells only.

The first step in the procedure is isolation of rat serosal mast cells (see Basic Protocol 1). Rat serosal mast cell granules can be isolated by different procedures. One involves mast cell activation and degranulation, i.e., mimicking the in vivo situation of granule secretion (see Basic Protocol 2). Upon exposure to the extracellular fluid containing physiological levels of ions, the soluble components loosely bound to the negatively charged proteoglycan component of the granules (also those remaining within the degranulation channels) diffuse away due to lost ionic interactions (Bergendorff and Uvnäs, 1972). The heparin proteoglycan protease–containing residues are called granule remnants. Furthermore, intact mast cell granules enveloped by their individual perigranular membranes, i.e., containing both heparin proteoglycan–bound and soluble mediators, can be isolated using a mild sonication procedure (see Alternate Protocol 1). The isolation of intact membrane-covered granules allows one to study all preformed granule mediators, i.e., both bound and soluble, present within the granule organelle. In addition, mast cell granules can be isolated simply by disruption of mast cells using freezing and thawing, and isolation of the granule remnants by a combination of low- and high-speed centrifugations (see Alternate Protocol 2). Since activation of mast cells with compound 48/80 only leads to release of a fraction of the cytoplasmic granules, the advantage of using the freezing and thawing method is a higher yield of isolated granule remnants. The composition of the granule remnants is, however, identical whether the remnants have been isolated after mast cell stimulation or by the freezing and thawing method. Assays for histamine (see Support Protocol 1), lactate dehydrogenase (LDH, see Support

Contributed by Ken A. Lindstedt and Petri T. Kovanen
Current Protocols in Cell Biology (2005) 3.16.1-3.16.13

Protocol 2), granule morphology and purity (see Support Protocol 3), heparin proteoglycans (see Support Protocol 4), and chymase activity (see Support Protocol 5) are included to assess degranulation of mast cells.

BASIC PROTOCOL 1

ISOLATION OF RAT SEROSAL MAST CELLS

Loosely associated serosal mast cells are isolated from the peritoneal and pleural cavities of male rats by gentle lavage with albumin-containing phosphate buffer. Typically, ~15% of the cells are mast cells, and ~85% are macrophages. The obtained cell suspension is then plated on plastic petri dishes in the presence of rat serum to allow the contaminating macrophages to adhere to the plastic. The purity of the nonadherent cells, i.e., mast cells, is then checked by staining with Moore & James stain, and normally exceeds 90% to 95%.

Materials

Male Wistar rats, 300 to 500 g
CO_2 gas
Ethanol
Buffer A (see recipe)
Heparin (Sigma)
Medium A (see recipe)
PBS with magnesium and calcium (GIBCO)
Moore & James staining solution (see recipe)

Glass tank with cover
Sterilized scissors and forceps (surgical and anatomical)
20- and 10-ml syringes
10-ml disposable pipets
18-G needles
15- and 50-ml centrifuge tubes
56°C water bath
Centrifuge (e.g., Eppendorf 5810R)
150 × 15–mm petri dishes
37°C, 5% CO_2 humidified incubator
Hemacytometer

NOTE: Treat the mast cells gently throughout the protocol to avoid spontaneous degranulation and loss of granules.

Isolate rat serosal cells

1. Sacrifice male Wistar rats weighing 300 to 500 g by CO_2-asphyxiation in a covered glass tank followed by cervical dislocation.
2. Spray the abdomen and breast area with ethanol and remove the fur from the sprayed area by lifting the dermis with surgical forceps and cutting with scissors in a longitudinal direction.
3. Lift the abdominal muscles with surgical forceps and make a small incision through the abdominal wall with the scissors. Carefully inject 20 ml of buffer A containing 0.05 mg/ml heparin into the peritoneal cavity using a 20-ml syringe (without a needle). Keep the injection site lifted with forceps and gently massage the abdomen by hand for 30 sec.
4. Open the abdomen by cutting with surgical scissors in a longitudinal direction and carefully aspirate the buffer containing the peritoneal cells using a disposable 10-ml pipet. To recover most of the injected buffer A from the peritoneal cavity, carefully lift the intestines out of the peritoneal cavity with anatomical forceps.

Avoid touching the internal organs with the pipet because it may cause damage to the liver or intestines and contaminate the mast cell suspension.

5. Make a small incision in the upper part of the diaphragm avoiding visible blood vessels and inject 10 ml of buffer A containing 0.05 mg/ml heparin into the pleural cavity. Again, gently massage the torso for 30 sec by hand, and aspirate the mast cell–containing buffer with a disposable 10-ml pipet.

Prepare rat serum

6. Puncture the heart with a 10-ml syringe equipped with an 18-G needle and draw blood into a 15-ml centrifuge tube. Allow the blood to coagulate at room temperature for 10 min and centrifuge 10 min at 700 × *g*, room temperature.

7. Recover the serum, inactivate it 30 min at 56°C, and add it (final concentration 5% v/v) to medium A when plating the mast cells (step 9).

Remove macrophages

8. Pool the cells from the peritoneal and pleural cavities in 50-ml centrifuge tubes. Sediment the cells by centrifuging 5 min at 150 × *g*, room temperature. Gently resuspend the cells in 15 ml of medium A.

9. Seed aliquots of the cells (1×10^6 mast cells) onto plastic petri dishes in medium A with 5% rat serum (step 7) and incubate 1 hr in a 37°C, 5% CO_2 humidified incubator to allow contaminating macrophages to adhere to the plastic.

10. Recover the non-adherent cells (mast cells) by shaking the petri dishes for 30 sec and transfering the mast cells into a new 50-ml centrifuge tube.

Wash the mast cells

11. Carefully wash the recovered mast cells two times by adding 10 ml PBS and centrifuging 5 min at 150 × *g*, 22°C, avoiding spontaneous activation.

12. Resuspend 1×10^6 mast cells in 200 µl PBS by gently flicking the tube with a finger.

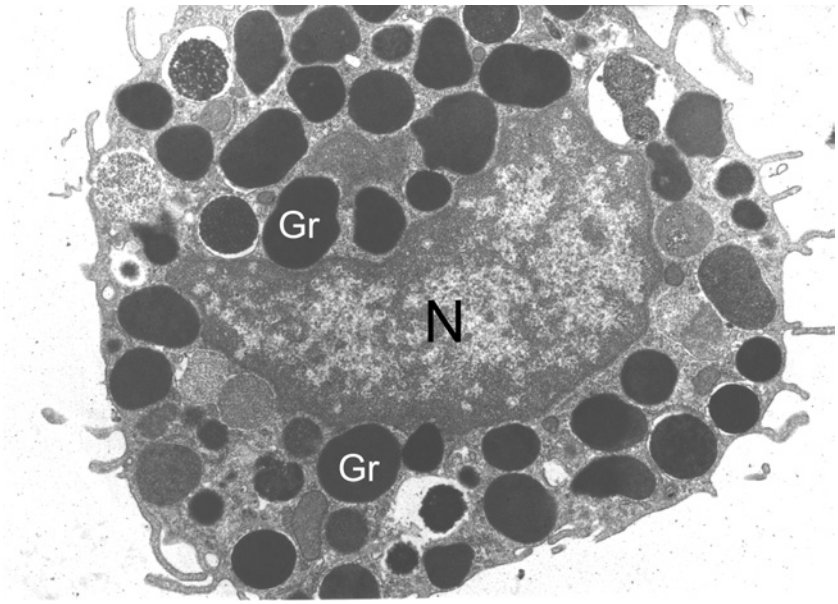


Figure 3.16.1 A transmission electron micrograph showing an isolated rat serosal mast cell filled with cytoplasmic electron dense granules (black spheres, Gr). One rat serosal mast cell typically contains ~1000 secretory granules. N = nucleus; Gr = granules

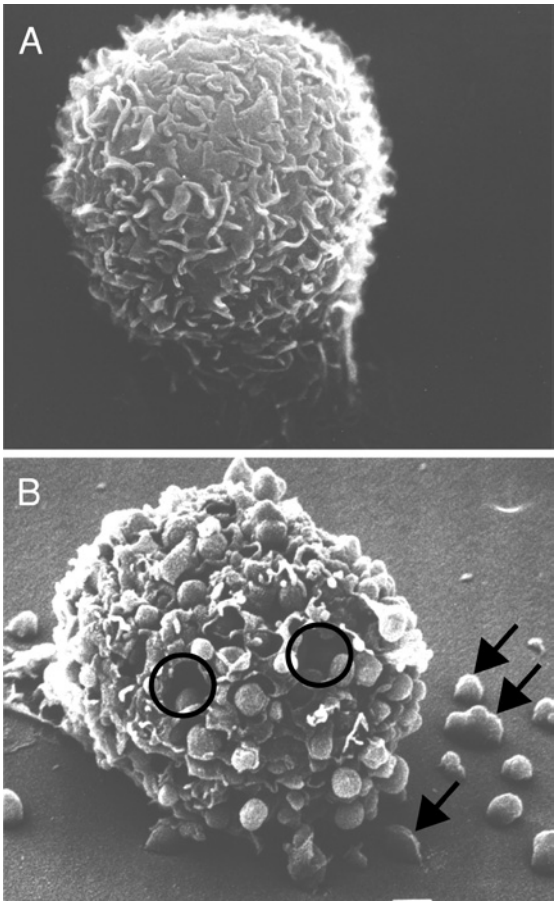


Figure 3.16.2 Scanning electron micrographs of isolated rat serosal mast cells. (**A**) Resting mast cell. (**B**) Activated and degranulated mast cell. Arrows point to expelled granule remnants. Two open degranulation channels showing intracellular granule remnants are encircled.

13. Judge the purity of the mast cell suspension by adding 5 µl of the mast cell suspension to 5 µl Moore & James staining solution and immediately count an aliquot on a hemacytometer.

    One should find 90% to 95% of the cells to be mast cells (see Fig. 3.16.1), the remaining cells are lymphocytes, eosinophils, and occasional macrophages.

BASIC PROTOCOL 2

ISOLATION OF MAST CELL GRANULES

Mast cell granules are isolated from activated or disrupted mast cells by low- and high-speed centrifugation steps (Thon and Uvnäs, 1966; Kokkonen and Kovanen, 1985). All centrifugation steps are performed using 1.5-ml microcentrifuge tubes and a microcentrifuge.

Mast cells can be activated to release a fraction of their granules by several distinct mechanisms. IgE-mediated activation is the classical pathway known to activate mast cells in vivo in, for example, allergic reactions. It involves the crosslinking of cell surface–bound antigen-specific IgE by the respective antigens or by anti-IgE antibodies. Mast cell activation can also be accomplished using a commercially available activator, compound 48/80, which causes extensive mast cell degranulation by activating G protein–coupled receptors (Aridor et al., 1990). Because compound 48/80 mimicks the physiological pathway of activation, it has been used in this unit for isolation of mast cell granules.

Materials

Mast cells (see Basic Protocol 1)
PBS with magnesium and calcium (GIBCO), cold and room temperature
Compound 48/80 (Sigma)

1.5-ml microcentrifuge tubes
Microcentrifuge
37°C, 5% CO_2 humidified incubator

Activate mast cells

1. For the standard assay, preincubate 1×10^6 mast cells in 200 μl of PBS in a 1.5-ml microcentrifuge tube 15 min in a 37°C, 5% CO_2 humidified incubator.
2. Stimulate the mast cells to degranulate by adding compound 48/80 to a final concentration of 1 μg/ml.
3. Continue incubation for 5 min in a 37°C, 5% CO_2 humidified incubator to allow the mast cells to completely activate and degranulate.
4. Sediment the cells by centrifuging 5 min at 150 × *g*, 4°C. Save the supernatant.
5. Determine the histamine contents of both supernatant and sediment as described in Support Protocol 1.

Recover granules and remnants

6. Recover any extracellular granules loosely associated with the stimulated mast cells by gently resuspending the sediment ten times in 200 μl of PBS using a micropipet.
7. Sediment the cells by centrifuging 5 min at 150 × *g*, 4°C. Collect the supernatant.
8. Determine intactness of the mast cells by measuring the presence of lactate dehydrogenase in the supernatants from steps 4 and 7 (see Support Protocol 2).
9. Pool the supernatants from steps 4 and 7. Recover the granule remnants by centrifuging the supernatants 5 min at 15,000 × *g*, 4°C. Wash one time with 200 μl deionized water and repeat centrifugation.
10. Resuspend the granule remnants in 200 μ1 of deionized water and determine the amounts of protein, heparin proteoglycans, and chymase (see Support Protocol 5).

ISOLATION OF MEMBRANE-COVERED GRANULES

ALTERNATE PROTOCOL 1

Intact mast cell granules enveloped by their individual perigranular membranes, i.e., containing both heparin proteoglycan bound and soluble mediators can be isolated using a mild sonication procedure. The isolation of intact membrane-covered granules allows for the study of all preformed granule mediators, i.e., both insoluble and soluble mediators, present within the granule organelle.

Materials

Purified mast cells (see Basic Protocol 1)
Buffer B (see recipe)
0.1% (v/v) Triton X-100 in buffer B (see recipe for buffer B)
PBS with magnesium and calcium (GIBCO)

1.5-ml microcentrifuge tubes
Sonicator (e.g., Model Pul 125, Kerry Ultrasonics)

1. Wash purified mast cells (5–10 × 10^6) three times with 4 ml buffer B, centrifuging 5 min at 150 × *g*, 22°C. Resuspend the cells in 500 µl buffer B in a 1.5-ml microcentrifuge tube.
2. Subject the mast cell suspension to mild sonication in a Kerry water bath sonicator for 30 sec at 4°C. Determine the duration of sonication necessary to rupture the plasma membranes but leave the membrane-covered granules intact by checking an aliquot of the cells with a light microscope at intervals.
3. Sediment cellular debris by centrifuging 10 min at 150 × *g*, 4°C.
4. Transfer the supernatant containing the membrane-covered granules to a new 1.5-ml microcentrifuge tube.
5. Treat 250 µl of membrane-covered granules with 1 ml of 0.1% Triton X-100 in buffer B for 10 min at room temperature to remove the granule membranes.
6. Obtain membrane-free granule remnants by centrifuging 5 min at 15,000 × *g*, 4°C.
7. Analyze the insoluble granule remnants and the soluble granule remnant–free supernatant for their individual components using various techniques, e.g., SDS-PAGE, western blotting, and others.

ALTERNATE PROTOCOL 2

BULK PREPARATION OF MAST CELL GRANULES

If mast cell granules are used for experiments involving the effects of chymase and heparin proteoglycans, they can also be prepared in advance (in bulk) and stored for up to 6 months at –20°C.

Materials

Purified mast cells (see Basic Protocol 1)
0.3 M sucrose
Liquid nitrogen
Buffer A (see recipe)
1.5-ml microcentrifuge tubes
37°C water bath
Additional reagents and equipment for determination of protein concentration by the method of Lowry (*APPENDIX 3H*)

1. Resuspend purified mast cells isolated from ten rats in 1 ml of 0.3 M sucrose in a 1.5-ml microcentrifuge tube.
2. Lyse the cells by six freeze/thaw cycles using liquid nitrogen and a 37°C water bath.

   The freeze/thaw cycles should include freezing samples thoroughly (~5 sec) in liquid nitrogen and thawing (~10 sec) in the 37°C water bath.
3. Sediment the cellular debris from the lysate by centrifuging 5 min at 450 × *g*, 22°C.
4. To obtain a practically pure granule fraction, transfer the supernatant to a new 1.5-ml microcentrifuge tube and centrifuge 20 min at 3000 × *g*, 22°C.
5. Wash the granule pellet two times in 1 ml buffer A by centrifuging 5 min at 15,000 × *g*, 22°C. Resuspend the granule pellet in 100 µl of deionized water.
6. Measure the protein concentration by the method of Lowry (*APPENDIX 3H*) and express the quantity of granules in terms of micrograms granule proteins per milliliter.

HISTAMINE ASSAY

SUPPORT PROTOCOL 1

The yield of granules obtained during the process of mast cell degranulation (see Basic Protocol 2) is dependent on the degree of mast cell activation. This can easily be measured by analyzing the amount of soluble histamine both in the supernatant and in the remaining cell pellet.

Materials

Activated mast cells (see Basic Protocol 2)
Trichloroacetic acid (TCA)
Orthopthalaldehyde (OPTA, Fluka)
Methanol
Histamine (Sigma)
0.01, 0.05, and 2 N H_2SO_4
1 N NaOH

1.5-ml microcentrifuge tubes
Fluorometer (340-nm excitation and 443-nm emission filters)

1. After mast cell activation, transfer 200 µl (1×10^6 mast cells) to a 1.5-ml microcentrifuge tube. Centrifuge 5 min at 150 × *g*, 4°C.
2. Remove the supernatant by pipetting it into a new 1.5-ml microcentrifuge tube. Incubate the cell pellet with 200 µl deionized water 30 min at room temperature to lyse the cells.
3. Treat 50 µl of the supernatant and 50 µl of the resuspended pellet with 11 µl of ice-cold 50% TCA (10% final concentration), shake the tubes vigorously and incubate 20 min at 4°C.
4. Sediment the precipitated material by centrifuging 10 min at 12,000 × *g*, 4°C.
5. Determine the histamine content by fluorometry according to the method of Bergendorff and Uvnäs (1972). Briefly, prepare a fresh solution of 10 mg/ml fluorescent OPTA by dissolving it in methanol.
6. Dissolve the histamine standard in 0.05 N H_2SO_4 at 100 ng/µl and prepare a 1 ng/µl histamine working solution by diluting the stock solution in water.
7. Incubate samples and histamine standards (0 to 300 ng histamine) in a mixture of 2 ml of 0.1 N H_2SO_4, 400 µl of 1 N NaOH, and 100 µl of 10 mg/ml OPTA 4 min at room temperature.
8. Add 200 µl of 2 N H_2SO_4 and incubate 5 min at room temperature. Measure the fluorescence using a 340-nm excitation filter and a 443-nm emission filter.
9. Calculate the percentage of histamine released from the mast cells into the incubation medium by using the following formula: histamine release (%) = $S/(S + P) \times 100$, where *S* is the histamine content of the supernatant and *P* is the histamine content of the mast cell pellet.

A concentration of 1×10^6 mast cells contains ~14 µg of histamine.

QUANTIFICATION OF LACTATE DEHYDROGENASE USING THE CYTOTOXICITY DETECTION KIT (ROCHE)

SUPPORT PROTOCOL 2

To verify that the mast cells have been properly activated, resulting in the release of physiological mast cell mediators and not components released due to cell rupture or damage, the level of lactate dehydrogenase (LDH) in the cell-free supernatant is measured. In this protocol, a commercially available Roche kit is used for LDH-measurement, but the assay

may also be performed using a standard method for LDH and reagents prepared in the laboratory. The assay is a colorimetric assay for the quantification of cell death and cell lysis based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The percentage of cytotoxicity is determined by calculating the average absorbance values of triplicates and subtracting from each of these the value obtained in the background control (LDH activity present in the assay medium). Low control absorbance provides the spontaneous LDH released from untreated cells and high control absorbance provides the maximum LDH released from lysed cells. The following equation is used for calculating cytotoxicity: cytotoxicity (%) = [experimental value – low control/(high control – low control)] × 100.

Materials

Cell-free supernatant from mast cells (see Basic Protocol 2; 1×10^6 activated mast cells)
PBS
Cytotoxicity detection kit (Roche) containing:
Catalyst: diaphorase/NAD^+ mixture and lyophilizate, stabilized
Dye solution: iodotetrazolium chloride (INT) and sodium lactate

96-well microtiter plates with flat-bottomed wells
Microtiter plate reader (490 nm)

1. Transfer 10 µl of cell-free supernatant per well of a 96-well clear flat-bottomed microtiter plate. Add PBS up to 100 µl. Prepare samples in triplicate.
2. Prepare the reaction mixture from the cytotoxicity detection kit as follows: add 1 part of catalyst to 45 parts of dye solution. Add 100 µl of the reaction mixture to each well.
3. Incubate ~10 to 30 min at room temperature until color is visible. Protect the plate from light during the incubation.
4. Measure absorbance at 490 nm using a microtiter plate reader and calculate cytotoxicity (see protocol introduction).

SUPPORT PROTOCOL 3

VERIFICATION OF GRANULE MORPHOLOGY AND PURITY

The morphology and purity of the isolated mast cell granules can be verified using transmission electron microscopy (TEM).

Materials

Mast cell granules (see Basic Protocol 2)
2.5% (v/v) glutaraldehyde/100 mM sodium phosphate buffer, pH 7.2
1% (w/v) osmium tetroxide/100 mM sodium phosphate buffer, pH 7.2
50%, 70%, 94%, and 99% ethanol
LX-112 embedding medium (Ladd Research)
Uranyl acetate (Ultrostain I, Leica)
Lead citrate (Ultrostain II, Leica)

Ultramicrotome
Automated Leica EMStain apparatus (Leica)
Electron microscope

1. Fix 10 µg mast cell granules 60 min at 22°C in 200 µl of 2.5% glutaraldehyde/100 mM sodium phosphate buffer, pH 7.2
2. Postfix the sample 30 min at 22°C in 200 µl of 1% osmium tetroxide/100 mM sodium phosphate buffer, pH 7.2.

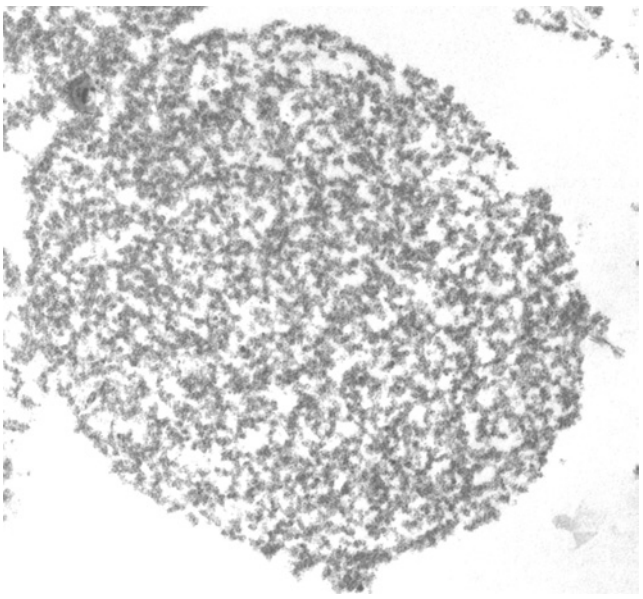


Figure 3.16.3 A transmission electron micrograph showing an isolated rat serosal mast cell granule remnant.

3. Dehydrate samples in an increasing ethanol series: 5 min in 50% ethanol, 10 min in 70% ethanol, 20 min in 94% ethanol, and 45 min in 99% ethanol. Embed in LX-112 embedding medium.
4. Cut 70- to 90-nm sections for analysis with an ultramicrotome.
5. Using an automated Leica EMStain staining apparatus, post stain sections with Ultrostain I 30 min at 20°C and Ultrostain II 1.2 min at 20°C with water wash in between the stainings.
6. Visualize the preparation using a transmission electron microscope.

Figure 3.16.3 shows a typical granule remnant isolated from rat serosal mast cells visualized by the transmission electron microscopic technique.

DETERMINATION OF HEPARIN PROTEOGLYCANS

SUPPORT PROTOCOL 4

This method of determining the amount of proteoglycans in mast cell granules and fragments is adapted from Bartold and Page (1985).

Materials

Sepraphore III cellulose polyacetate electrophoresis strips (2.5 × 15.2–cm, Pall Corporation)
Heparin standards (Sigma)
Mast cell granule remnants (see Basic Protocol 2)
0.2% (w/v) Alcian blue staining solution (see recipe)
Destaining solution (see recipe)
DMSO

10-ml disposable plastic tubes (DMSO-resistant)
Platform shaker
Whatman no. 3 filter paper
Spectrophotometer

1. Mark a Sepraphore III cellulose polyacetate electrophoresis strip in 2-cm sections with a soft pencil.
2. Apply heparin standards (1.25 and 2.5 μg) and granule remnants (10 to 30 μl), in duplicate, onto the middle of the marked strip and let them air dry. Include two empty sections for blanks.
3. Stain the strip in 10 to 100 ml of 0.2% Alcian blue staining solution 30 min at room temperature on a platform shaker.
4. Destain strip through three changes, 15 min each, in 10 to 100 (depending on the container used) ml destaining solution on a platform shaker.
5. Dry the strip using Whatman no. 3 filter papers.
6. Cut the marked strips into 2-cm pieces. Place into 10-ml disposable plastic tubes and dissolve the pieces in 2 ml of DMSO by vigorous vortexing. Repeat the vortexing until the pieces have dissolved completely.
7. Measure the absorbance at 678 nm in a spectrophotometer.
8. Calculate the amount of heparin proteoglycan in the granule remnants using the known concentrations of the heparin standards.

SUPPORT PROTOCOL 5

MEASUREMENT OF CHYMASE ACTIVITY

A major protein component in the granule remnants is the neutral serine protease, chymase, which constitutes ~30% of the total granule remnant protein. The proteolytic activity of chymase in the isolated granule remnants can be determined spectrophotometrically using *n*-benzoyl-L-tyrosine ethyl ester (BTEE; Sigma) as substrate (Woodbury et al., 1981).

Materials

Isolated mast cell granules (see Basic Protocol 2)
0.5 mM *n*-benzoyl-L-tyrosine ethyl ester (BTEE; Sigma) in 100 mM NaCl/50 mM Tris·Cl, pH 7.4
Spectrophotometer (256 nm)

1. Resuspend 1 to 5 μg of isolated mast cell granule protein in 2.9 ml of 100 mM NaCl/50 mM Tris·Cl (pH 7.4) and add 100 μl of BTEE substrate immediately before starting the measurement.
2. Measure the change in absorbance at 256 nm for 5 min at room temperature using 30-sec measuring intervals.
3. Calculate the activity (arbitrary U/min) as the change in absorbance measured as the Δ-slope value × 1000, and using purified or recombinant chymase as a standard.

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common reagents, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alcian blue staining solution, 0.2% (w/v)

200 mg Alcian blue
100 ml destaining solution (see recipe)
Filter using a piece of Whatman no. 3 filter paper
Store up to 2 weeks at 4°C

Buffer A

PBS (GIBCO) supplemented with:
0.5 mg/ml bovine serum albumin
5.6 mM glucose, pH 7.3
Prepare fresh and keep at 4°C

Buffer B

PBS (GIBCO) supplemented with:
1.75 mg/ml bovine serum albumin
5.6 mM glucose
Prepare fresh and keep at 4°C

Destaining solution

0.05 M $MgCl_2$
0.025 M sodium acetate
50% (v/v) ethanol
Store up to 2 weeks at 4°C

Medium A

RPMI 1640 (with 25 mM HEPES) supplemented with:
10 mg/ml bovine serum albumin
5% (v/v) fresh rat serum
25 mM NaCl
100 IU/ml penicillin
2 mM L-glutamine
Store up to 2 weeks at 4°C

Moore & James staining solution

4 ml 0.05% (w/v) toluidine blue in 0.9% (w/v) NaCl
100 μl saturated saponin (Merck) in 5% methanol
1.1 ml 95% ethanol
Filter through Whatman no. 3 filter paper
Store up to 2 months at 22°C
Use the stain by mixing 1:1 (v/v) with cell suspension.

COMMENTARY

Background Information

The mast cell is a multipotent inflammatory cell, which has been shown to participate in the pathogenesis of a variety of diseases, such as immediate hypersensitivity reactions, arthritis, atherosclerosis, and heart failure. Mast cells are long-lived cells (weeks to months) that under normal conditions are present in tissues in a resting, i.e., non-degranulating, state. Their localization at cutaneous and mucosal surfaces where exogenous antigens penetrate the organism has suggested a role as sentinels at the interface of the host and the environment for mast cells. The sentinel function of mast cells is essentially accomplished through an energy requiring process of activation, in which they release preformed cytoplasmic granules into their microenvironment. These secretory granules consist of a heparin proteoglycan matrix embedded with preformed mediators, such as histamine, neutral proteases, growth factors, and cytokines, which together trigger acute allergic and ensuing sustained inflammatory reactions. Mast cell activation in vivo or in vitro occurs by exogenous triggers, including IgE-mediated crosslinking of FcεRI (high affinity IgE receptors), histamine-releasing factors secreted by neighboring T-lymphocytes or macrophages, and anaphylatoxin components of the complement system (C3a, C5a). Upon activation, a part of the secretory cytoplasmic granules become swollen, and their individual membranes fuse to form tubular channels in which the granules to be secreted lie in chains.

The degranulation channels then rapidly open to the extracellular fluid and some of the peripheral granules are expelled. The soluble components of the granules, such as histamine, diffuse away, whereas the heparin proteoglycans and the mast cell–specific neutral proteases (e.g., chymase and carboxypeptidase A) remain tightly bound to each other through ionic interactions, forming proteolytically active extracellular granule remnants. The extracellular remnants are eventually phagocytosed by macrophages, smooth muscle cells, fibroblasts, or even endothelial cells in the close vicinity of the activated mast cells. Finally, the granule remnants remaining in the degranulation channels are converted into individual membrane-covered mature granules, when the lost preformed mediators are replenished through a rapid onset of de novo synthesis. Depending on the tissue and type of stimulation, the degree of mast cell degranulation may greatly vary from being almost complete, i.e., involving most of the granules as in anaphylactic shock, to being only minimal and directed only towards the site of activation.

Critical Parameters

The procedure to isolate rat mast cell granules is straightforward and only few critical parameters exist. To obtain uncontaminated mast cells, all media injected into the cavities and all instruments need to be sterile, and the mast cells need to be recovered without perforating the bowel, and thus, avoiding

Table 3.16.1 Amounts of Granule Components in 1×10^6 Mast Cells

		Experimental yield	
Granule component	100% recovery	Compound 48/80	Freezing and thawing
Protein	50 μg	~20 μg (~40%)	~45 μg (~90%)
Heparin proteoglycan	18 μg	~7 μg (~40%)	~16 μg (~89%)
Chymase	15 μg	~6 μg (~ 40%)	~13 μg (~87%)

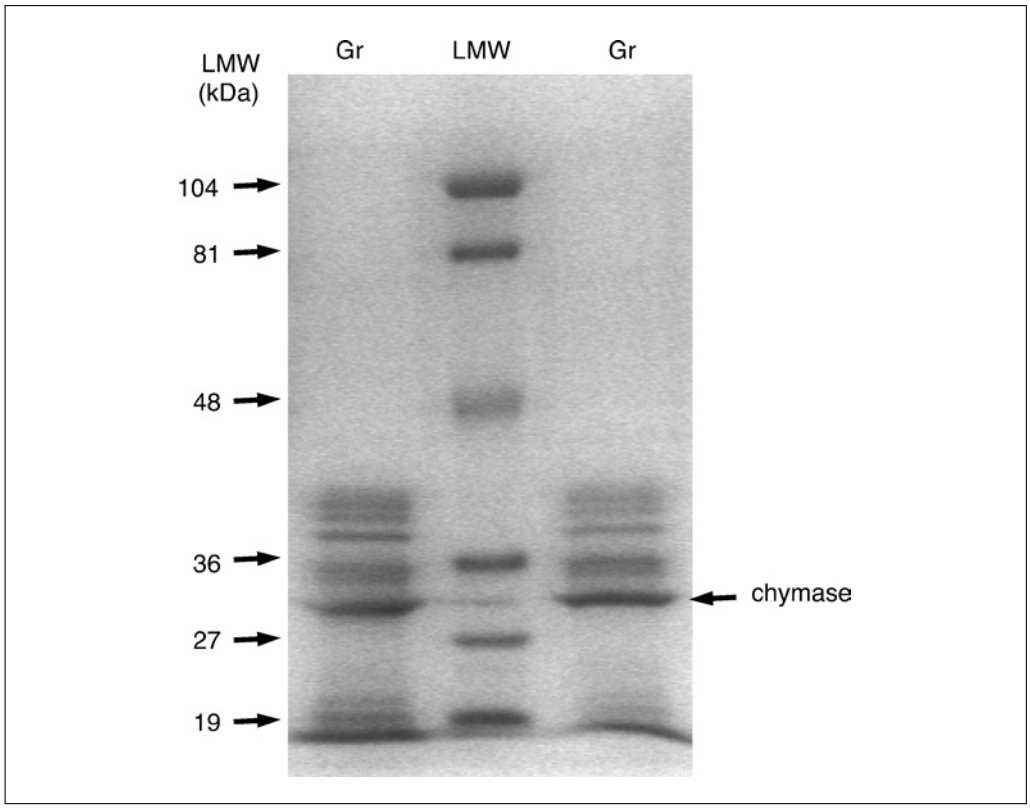


Figure 3.16.4 A Coomassie blue–stained SDS-PAGE (4% to 20%) gel describing the protein composition of isolated mast cell granule remnants (Gr, 5 μg/lane). The size of the low-molecular weight standard (LMW, Bio-Red) is indicated on the left side of the gel and the major granule protein, chymase, is indicated on the right side of the gel.

contamination with enteric bacteria. Throughout the isolation procedure, the mast cells have to be treated gently not to induce spontaneous degranulation. When isolating granules using the physiological method of mast cell activation, a confirmation of mast cell activation can easily be performed with a microscope. The process of degranulation is clearly visible in a microscope at low magnification immediately after the addition of compound 48/80. To isolate intact granules embedded within perigranular membranes, the sonication time is critical, since it depends on the efficiency of the sonication apparatus used. The optimal time interval needs to be determined empirically in each laboratory.

Troubleshooting

Low yield of mast cells: Use older and heavier animals and improve the lavage of the cavities. Use prewarmed buffer A and aim at maximal recovery of the buffer.

Low purity of mast cells: Do not use the cell-suspension if it is yellow or reddish, which is a sign of contamination. Increase the plating time for more efficient removal of macrophages.

Low yield of granules: Check the level of histamine release. It should be high (up to 80%). Repeat the stimulation and check the process of degranulation visually by microscopy.

Anticipated Results

A successful isolation of mast cells will give $\sim 1 \times 10^6$ mast cells/rat.

After plating, the purity of mast cells in the suspension will be ~90% to 95%. Table 3.16.1 describes the amount of granule components in 1×10^6 mast cells. See Figure 3.16.4 for a Coomassie blue–stained SDS-PAGE gel with low-molecular-weight standards and 20 μg of granule protein.

Time Considerations

The whole procedure can be performed within 1 working day. The isolated granules can be rapidly frozen in liquid nitrogen and stored up to 6 months at –20°C to be analyzed later for their content of protein, heparin proteoglycans, and protease activities.

Literature Cited

Aridor, M., Traub, L.M., and Sagi-Eisenberg, R. 1990. Exocytosis in mast cells by basic secretagogues: Evidence for direct activation of GTP-binding proteins. *J. Cell Biol.* 111:909-917.

Bartold, P.M. and Page, R.C. 1985. A microdetermination method for assaying glycosaminoglycans and proteoglycans. *Anal. Biochem.* 150:320-324.

Bergendorff, A. and Uvnäs, B. 1972. Storage of 5-hydroxytryptamine in rat mast cells. Evidence for an ionic binding to carboxyl groups in a granule heparin-protein complex. *Acta Physiol. Scand.* 84:320-331.

Kokkonen, J.O. and Kovanen, P.T. 1985. Low density lipoprotein degradation by rat mast cells: Demonstration of extracellular proteolysis caused by mast cell granules. *J. Biol. Chem.* 260:14756-14763.

Kokkonen, J.O., Lindstedt, K.A., and Kovanen, P.T. 1995. Role of mast cell proteases and proteoglycans in lipoprotein metabolism. *In* Mast Cell Proteases in Immunology and Biology. Clinical Allergy and Immunology. (G.H. Caughey, ed.) pp. 257-287. Marcel Dekker, Inc. New York.

Kovanen, P.T. 1993. The mast cell—A potential link between inflammation and cellular cholesterol deposition in atherogenesis. *Eur. Heart J.* 14:105-117.

Krüger, P.G., Lagunoff, D., and Wan, H. 1980. Isolation of rat mast cell granules with intact membranes. *Exp. Cell. Res.* 129:83-93.

Lindstedt, K.A., Kokkonen, J.O., and Kovanen, P.T. 1992. Soluble heparin proteoglycans release from stimulated mast cells induce uptake of low density lipoproteins by macrophages via scavenger receptor-mediated phagocytosis. *J. Lipid Res.* 33:65-75.

Thon, I.-L. and Uvnäs, B. 1966. Mode of storage of histamine in mast cells. *Acta Physiol. Scand.* 67:455-470.

Thon, I.-L. and Uvnäs, B. 1967. Degranulation and histamine release, two consecutive steps in the response of rat mast cells to compound 48/80. *Acta Physiol. Scand.* 71:303-315.

Woodbury, R.G., Everitt, M.T., and Neurath, H. 1981. Mast cell proteases. *Methods Enzymol.* 80:588-609.

Contributed by Ken A. Lindstedt and Petri T. Kovanen
Wihuri Research Institute
Helsinki, Finland

Immunoisolation of Centrosomes from *Drosophila melanogaster*

The isolation of centrosomes from cells has been, for a long time, a challenging task. The main reason is that this small organelle (~0.3 μm in diameter in mammalian cells) is present only in a single copy in the cell and therefore represents only a minuscule part of the whole cell proteome. Another technical difficulty is that the density of this organelle ($\rho > 1.32$ g/ml) is too high for it to be purified in a continuous gradient using isopycnic density centrifugation. Hence, step-gradient centrifugation has been the method of choice for most of the isolation approaches used to date. Both Ficoll and sucrose density gradients, as well as a combination of the two, have been employed. Most recent methods use sucrose gradient centrifugation only. Density gradient centrifugation is followed by immunofluorescence assays, microtubule nucleation assays, or electron microscopy to evaluate enrichment of cell organelles in particular fractions of the gradient.

Currently, isolation of centrosomes from *Drosophila* embryos, although relatively inefficient (i.e., only ~10% of total centrosomes present in starting material are isolated), results in some of the best total yields compared to isolation of centrosomes from other organisms. In short, this method permits production of excellent quantities for many biochemical, functional, and structural studies.

The method described here focuses on the isolation of centrosomes from the syncytial stages of the early *Drosophila* embryo (0 to 3.5 hr). This has the particular advantage that cell organelles are not bounded by cellular membranes and that lysis can be performed on a relatively small scale, keeping the resulting volume low. This is an essential factor for subsequent gradient centrifugation steps, because ultracentrifugation of large buffer volumes is impractical. The abundance of pericentriolar material of these centrosomes isolated from the highly mitotic embryos produces excellent total protein yields. Moreover, maintenance of large fly populations that produce grams of starting material within a few hours is relatively cost efficient.

Basic Protocol 1 describes isolation of centrosomes from early-syncytial-stage *Drosophila* embryos by sucrose step gradient centrifugation. Basic Protocol 2 details an immunomagnetic isolation procedure for further purification of the centrosomes isolated in Basic Protocol 1. Support Protocol 1 details an immunofluorescence microscopy procedure for quantitatively tracking the gradient purification of centrosomes, and, finally, Support Protocol 2 describes fluorescence microscopy to assess the purification of the immunomagnetically isolated centrosome preparations.

ISOLATION OF CENTROSOMES FROM THE EARLY SYNCYTIAL STAGES OF THE *DROSOPHILA* EMBRYO USING SUCROSE STEP GRADIENT CENTRIFUGATION

BASIC PROTOCOL 1

This protocol outlines the production of *Drosophila* embryo extract as starting material for the isolation of centrosomes. Embryos are homogenized in homogenization buffer (HB), centrifuged at low speed, and filtered twice to remove large aggregates of membranes, debris, and other cell organelles. The two consecutive low-speed centrifugations also eliminate the large quantities of lipids that float on top of the embryo extract; these lipids need to be carefully removed to avoid contamination in subsequent isolation steps. The first two steps of density centrifugation serve to pool centrosomes for the subsequent immunoisolation, rather than to enrich cell organelles in a particular fraction. The

Contributed by Verena Lehmann, Hannah Müller, and Bodo M.H. Lange
Current Protocols in Cell Biology (2005) 3.17.1-3.17.13

affinity-isolation step then removes contaminants that copurify in the sucrose gradient to obtain a high degree of purity.

This protocol was adapted from Moritz et al. (1995) and Moritz and Alberts (1999) for use with the immunoisolation protocols developed by the authors of this unit.

Materials

Large population of *Drosophila* (e.g., ~250,000 strain W118 flies) maintained in large environmental incubator or temperature- and humidity-controlled room with fixed day (12 hr) and night (12 hr) cycles (Greenspan, 1997; Bonte and Becker, 1999; Sullivan et al., 2000)
Apple juice/molasses agar plates (see recipe)
Yeast paste: ordinary baker's yeast dissolved in warm water to form a paste
Embryo wash: 0.7% (w/v) NaCl/0.04% (v/v) Triton X-100
3% (v/v) sodium hypochlorite in embryo wash (see above)
Homogenization buffer (HB, see recipe)
Liquid N_2
5× BRB80 with 100 mM KCl (see recipe), diluted to 1×
25% (v/v) Triton X-100
100× protease inhibitor mix (PIM, see recipe)
55% and 70% (w/v) sucrose solutions (see recipes)
Fine strainer
Filter unit fitted onto vacuum flask
Motor-driven Wheaton homogenizer with tight-fitting Teflon pestle (60-ml volume)
50-ml conical polypropylene centrifuge tubes
Refrigerated low-speed centrifuge
Miracloth (Calbiochem)
15-ml snap-cap polypropylene tubes for freezing and storage of supernatants
39-ml thin-walled polyallomer ultracentrifuge tubes (e.g., Beckman)
Large-volume ultracentrifuge (e.g., Beckman) with swinging-bucket rotor (e.g., Beckman SW 32)
Additional reagents and equipment for assessing purify of centrosome fractions from gradient (see Support Protocol 1)

Homogenize embryo material

1. Collect 30 to 50 g of 0- to 3.5-hr-old *Drosophila* embryos by placing a fresh apple juice/molasses agar plate with a lump of yeast paste on the surface into the population cage every 3 hr.

   Depending on the size of the fly population, three collections of 3 hr each will be needed in order to obtain a total of ~30 to 50 g of embryo material.

2. After the 3-hr collection period for each plate, wash embryos off the plate with embryo wash using a paint brush to aid in removing the embryos, collecting them in a fine strainer, and store the embryos in ice-cold embryo wash until all embryos have been collected.

3. Remove the chorion layer by stirring embryos for 3 min in 250 ml of 3% sodium hypochlorite solution.

4. Intensively wash the embryos with a forceful stream of cold tap water, then three times with a total volume of 1 liter of ice-cold distilled, deionized water, on a filter unit fitted onto a vacuum flask. Determine the dry weight after removing all excess water.

5. Resuspend dechorionated embryos in HB at a ratio of 1:5 (w/v).

6. Homogenize material on ice using five strokes of the motor-driven Wheaton homogenizer at a speed of 1500 rpm in a cold room.

CAUTION: *Wear protective gloves and goggles when using the homogenizer.*

Remove lipids and prepare/store HSS

7. Transfer homogenate to 50-ml conical polypropylene centrifuge tubes. Centrifuge 10 min at 1800 × *g*, 4°C. Remove floating lipids by aspirating with a suction pump and filter supernatant through Miracloth. Repeat the centrifugation, lipid removal, and filtration a second time.

8. Divide the resulting supernatant (referred to as HSS: i.e., "homogenate, supernatant, supernatant") into 10-ml aliquots in 15-ml conical polypropylene tubes, snap-freeze in liquid nitrogen, and store at −80°C until further use.

   The extract can be conveniently frozen at this stage if the isolation procedure must be interrupted. Freezing also gives the opportunity to produce stockpiles of embryo extracts without affecting the functional and structural integrity of the centrosome.

Perform sucrose-gradient purifications

9. Prepare six 50-ml conical polypropylene centrifuge tubes, each containing:

   20 ml 70% (w/v) sucrose
   1 ml 1× BRB80 with 100 mM KCl
   640 µl 25% Triton X-100
   400 µl 100× PIM.

10. Drop the contents of each 15-ml tube containing a frozen 10-ml HSS aliquot into one of the 50-ml tubes containing the mixture prepared in the previous step, and thaw on ice. Collect a 30-µl aliquot for immunofluorescence microscopy (see below).

11. For each of the extract mixtures prepared in the previous step, pour a sucrose gradient in a 39-ml thin-walled polyallomer ultracentrifuge tube by carefully overlaying 3 ml of 70% sucrose solution with 4 ml of 55% sucrose, and, finally, at the top, the HSS mixture from the preceding step. Mark the upper margin of the 55% sucrose layer of the sucrose gradient on the plastic wall of the tube.

12. Centrifuge samples for 1.5 hr at 100,000 × *g* in a SW 32 rotor with slow acceleration and slow braking.

    Slow acceleration and braking correspond to "setting 4" on the Beckman instrument.

13. Aspirate the supernatant above the marked line with a vacuum aspirator. Pool the gradients (all of the sucrose solutions below the mark; Pool 1) and discard the pellets. Take a 30-µl sample for immunofluorescence microscopy.

14. Dilute the ~35 to 40 ml Pool 1 to 70 ml with 1× BRB80 containing 100 mM KCl and add 100× PIM to a final concentration of 1×.

15. Prepare two sucrose gradients in 39-ml thin-walled polyallomer tubes ultracentrifuge tubes by carefully overlaying 3 ml of 70% sucrose solution with 1 ml of 55% sucrose solution. Split the diluted Pool 1 (step 14) into two equal portions, and carefully overlay each of these portions over the 55% sucrose layer in each of the two respective tubes. Mark the upper margin of the 55% sucrose layer of the sucrose gradient on the plastic wall of the tube.

16. Ultracentrifuge the gradients 1 hr at 100,000 × *g*, 4°C. Remove the supernatant from each tube with a vacuum aspirator and discard the pellets. Pool the sucrose gradients (resulting in Pool 2). Take a 30-µl sample for immunofluorescence microscopy.

17. Assess the sample aliquots from each purification stage by immunofluorescence microscopy (see Support Protocol 1). Use the remainder of Pool 2 for further purification by immunoisolation (Basic Protocol 2).

In principle, these enriched preparations can also be used for functional in vitro microtubule nucleation assays and for standard immunofluorescence colocalization studies. However, the preparations are too crude to be used for biochemical analysis of centrosome composition. For convenience, samples can also be snap-frozen in liquid nitrogen until used in Basic Protocol 2.

SUPPORT PROTOCOL 1

IMMUNOFLUORESCENCE MICROSCOPY OF ISOLATED CENTROSOMES

This protocol is used to assess the enrichment and yield of aliquots from the intermediate steps in preparation of centrosomes.

Materials

30-µl aliquots from Pool 1 and Pool 2 (see Basic Protocol 1)
PBS-T: phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4, containing 0.003% (v/v) Triton X-100
Methanol, −20°C
Primary antibody: anti-γ-tubulin (Sigma)
Secondary antibody: fluorochrome-conjugated antibody specific for IgG of the species from which the primary anti-γ-tubulin antibody was obtained
Mounting medium (e.g., Mowiol from Calbiochem, or 50% v/v glycerol)
20 mg/ml (10×) *p*-phenylendiamine in H_2O
Nail polish

Plastic inserts to support a 11-mm round glass coverslip (Evans et al., 1985)
15-ml Corex tubes
11-mm round glass coverslips (grade 1)
Refrigerated centrifuge with swinging-bucket rotor (e.g., Sorvall HB-6 or Beckman JS 13.1, or equivalent) and adapters
24-well tissue culture plates
Spatula bent at the tip for removing the plastic insert
Needle with bent tip
Forceps
Microscope slides
Epifluorescence microscope equipped with immunofluorescence filters and appropriate optics

NOTE: To avoid staining artifacts, never allow the coverslips to dry out at any point during the following protocol.

Centrifuge centrosome sample onto coverslip

1. Resuspend the 30-µl aliquot of *Drosophila* centrosomes from HSS mix Pool 1 or Pool 2 in 11 ml PBS-T.

   Keep samples on ice until centrifugation.

2. Place a pair of plastic inserts into a 15 ml-Corex glass tube to support a 11-mm round glass coverslip.

3. Transfer the 11-ml diluted centrosome sample into a 15-ml Corex tube and centrifuge onto the coverslips for 15 min at 15,000 × *g*, 4°C.

4. Remove the coverslip by lifting the top insert out of the Corex tube with a spatula bent at the tip. Transfer the coverslip with a pair of forceps immediately into −20°C methanol in a well of a 24-well tissue culture plate and incubate at least 5 min at −20°C to fix the sample.

5. Aspirate methanol and rehydrate sample in the well by covering with 1.5 ml PBS-T and incubating 15 min at room temperature. Aspirate PBS-T.

Stain with antibodies

6. Add ~80 μl of the anti-γ-tubulin primary antibody, appropriately diluted in PBS-T (e.g., 1:400 for polyclonal antisera, depending on antibody used), to each coverslip-containing well. Incubate 1 hr at room temperature in a moist environment.

7. Wash four times, each time by filling the sample-containing well(s) with an excess (e.g., 1.5 ml) of PBS-T, incubating 2 min at room temperature, and aspirating the solution.

   Apply the PBS along the wall of each well and not directly onto the sample, to avoid rinsing the centrosomes away.

8. Add ~80 μl of the fluorophore-labeled secondary antibody diluted in PBS-T according to the manufacturer's recommendations to each coverslip-containing well. Incubate 45 min at room temperature in the dark in a moist environment.

   The antibody dilution will vary; e.g., anti-mouse IgG whole-molecule dilutions will range between 1:400 and 1:2000.

Mount coverslip on slide and examine centrosome preparation

9. Remove coverslip(s) from well(s) with a bent needle and a pair of forceps and dip each coverslip briefly in water to rinse off excess PBS.

   Coverslips can more easily be removed from the plastic wells using a needle with a slightly bent tip.

10. Add 20-mg/ml *p*-phenylenediamine to the mounting medium (e.g., Mowiol or 50% glycerol) for a final concentration of 1 mg/ml. Mount each coverslip on a microscope slide with 6 μl of the phenylenediamine-containing mounting medium. Carefully remove excess mounting medium with tissue, and allow to set for 20 min at room temperature in the dark. Seal with nail polish.

11. Check preparations under a fluorescence microscope or store at 4°C in the dark until samples are viewed.

    The centrosomes should be homogenous in size and shape, and should be enriched through each step of the isolation procedure.

IMMUNOPURIFICATION OF CENTROSOMES WITH MAGNETIC BEADS

BASIC PROTOCOL 2

Prepare magnetic beads cross-linked with affinity-purified anti-γ-tubulin antibody. For negative control, magnetic beads are cross-linked with the relevant preimmune antibody. Centrosomes should be well enriched and quite similar in size. A ratio of 1:6 (v/v) between beads and centrosomes is recommended.

Materials

Protein G magnetic bead suspension (Dynal)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
0.2 M triethanolamine, pH 8.2, with and without 20 mM dimethyl pimelidate dihydrochloride (DMP); prepare fresh
Anti-γ-tubulin antibody (anti-peptide antibody; not commercially available; affinity-purified; Tavosanis et al., 1997)
Tris·Cl, pH 7.5 (*APPENDIX 2A*)
PBS (*APPENDIX 2A*) containing 0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*) containing 0.1% (v/v) Tween 20 and 0.02% (w/v) thimerosal
Pool 2 of centrosomes (see Basic Protocol 1)
Dilution buffer (see recipe), 4°C

PBS-T: phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4, containing 0.003% (v/v) Triton X-100, 4°C

Methanol, −20°C

SDS sample buffer (*UNIT 6.1*)

Magnetic particle collector (magnet and magnetic stand) suitable for 1.5-ml microcentrifuge tubes (e.g., Dynal)

End-over-end rotator

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and determining purity of centrosome preparations from immunomagnetic purification (see Support Protocols 1 and 2)

Prepare the antibody-coated magnetic beads

1. Place 120 μl of protein G magnetic bead suspension in a 1.5-ml microcentrifuge tube. Place the tube on the magnetic stand to immobilize the beads with the magnet according to the manufacturer's documentation, and remove the supernatant. Add 100 μl of PBS, resuspend the beads, and immobilize the beads on the magnet again. Repeat this wash four times.
2. Remove the supernatant and add 100 μg of anti-γ-tubulin antibody to the washed protein G beads. Incubate 45 min on an end-over-end rotator at room temperature. Immobilize beads and remove supernatant.
3. Wash the beads three times, each time with 1 ml of 0.2 M triethanolamine, pH 8.2, by immobilizing the beads on the magnet.
4. Cross-link by incubating the antibody-coupled beads with 1 ml of freshly prepared 0.2 M triethanolamine, pH 8.2, containing 20 mM DMP, on an end-over-end rotator at room temperature for 30 min.
5. Place tube on the magnetic stand and remove supernatant. Add 1 ml of 50 mM Tris·Cl, pH 7.5, and incubate on an end-over-end rotator at room temperature for 15 min.
6. Remove supernatant and wash beads three times, each time with 1 ml PBS containing 0.1% Tween 20, by immobilizing the beads on the magnet. Store antibody-coupled beads at 4°C in the original bead-suspension volume of PBS containing 0.1% Tween 20 and 0.02% thimerosal.

   Antibody binding and cross-linking efficiency can be checked by SDS-PAGE (UNIT 6.1) of 20-μl samples of beads before and after cross-linking.

Perform immunomagnetic separation

7. Take 120 μl antibody-coupled beads suspension and wash twice, each time with 1 ml wash buffer using the technique described above. Take a sample of 20 μl for later analysis by SDS-PAGE (*UNIT 6.1*).
8. Thaw 0.5 ml of centrosome Pool 2 on ice, then mix with 0.5 ml dilution buffer. Take a 20-μl sample for further analysis (see Support Protocol 1).
9. Add the 1 ml of diluted centrosomes to 100 μl washed, cross-linked beads. Mix gently by inverting the tube. Incubate 1 hr at 4°C on an end-over-end rotator. Immobilize the beads on the magnetic collector and carefully remove the supernatant. Keep the supernatant on ice for later analysis.
10. Wash beads three times, each time with 1 ml cold wash buffer, using the technique described above.
11. Wash beads twice, each time with 1 ml cold PBS-T, using the technique described above. Resuspend in 80 μl (original bead volume) of PBS-T. Remove an aliquot of 10 μl for immunofluorescence microscopy and an aliquot of 20 μl for SDS-PAGE.

12. Remove the PBS-T from the bead suspension with bound centrosomes (50 µl), resuspend in 55% sucrose solution, snap-freeze in liquid nitrogen, and store at −80°C.

    The purified centrosome preparation is used subsequently in various assays. These include, for example, functional tests such as microtubule nucleation assay, electron microscopic study of centrosome morphology, antigen localization, or antigen for the generation of monoclonal or polyclonal antibodies, and biochemical assays.

13. Fix the 10-µl aliquot of beads reserved for immunofluorescence microscopy for at least 5 min in 1 ml of −20°C methanol and store at −20°C until analyzed according to Support Protocol 2.

Assess purity

14. Immobilize beads and remove supernatant as described above. Add SDS sample buffer, and boil 5 min. Perform magnetic collection to remove beads and run the supernatant on a 10% SDS-PAGE gel (*UNIT 6.1*).

15. Assay diluted centrosome fractions before (see Support Protocol 1) and after immunomagnetic separation (see Support Protocol 2) by fluorescence microscopy.

IMMUNOFLUORESCENCE MICROSCOPY OF IMMUNOPURIFIED CENTROSOMES ON MAGNETIC BEADS

SUPPORT PROTOCOL 2

Materials

Centrosomes immunopurified on magnetic beads (see Basic Protocol 2)
Methanol, −20°C
PBS-T: phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4, containing 0.003% (v/v) Triton X-100
Primary antibody: anti-γ-tubulin (Sigma)
PBS-T containing 0.1% (w/v) bovine serum albumin (BSA)
Secondary antibody: fluorochrome-conjugated antibody specific for IgG of the species from which the primary anti-γ-tubulin antibody was obtained
Mounting medium: Mowiol (Calbiochem)
20 mg/ml (10×) *p*-phenylendiamine in H_2O
Clear nail polish

Magnetic particle collector (magnet and magnet stand) suitable for 1.5-ml microcentrifuge tubes (e.g., Dynal)
End-over-end rotator
Microscope slides
Round coverslips (grade 1, 11 mm diameter)
Epifluorescence microscope equipped with immunofluorescence filters and appropriate optics

NOTE: Handle samples with care and avoid vigorous mixing or vortexing.

1. Fix a 10-µl magnetic bead–centrosome sample in 1 ml −20°C methanol by incubating for at least 5 min at −20°C. Place the test tube containing the beads on the magnetic stand, immobilize the beads with the magnet as described in the manufacturer's documentation, and remove the supernatant.

2. Add 1 ml PBS-T to the beads in the tube. Mix gently by inverting the tube, then incubate 15 min on an end-over-end rotator. Collect beads and remove supernatant as described above.

3. Dilute the anti-γ-tubulin primary antibody 1:500 in PBS-T containing 0.1% BSA. Add 300 µl of the diluted primary antibody to the beads in the tube and mix gently

by inversion. Incubate 1 hr at room temperature with gentle rotation. Collect beads and remove supernatant as described above.

4. Wash beads four times, each time by immobilizing the beads on the magnet, removing the supernatant, resuspending the collected beads in 1 ml PBS-T, then removing the supernatant.
5. Dilute the fluorochrome-conjugated secondary antibody appropriately in PBS-T containing 0.1% BSA. Add 300 µl of the diluted secondary antibody to the beads in the tube and mix gently by inverting the tube. Incubate 45 min at room temperature on an end-over-end rotator in the dark (cover tubes with aluminum foil). Collect beads and remove supernatant as described above.
6. Wash beads four times, each time with 1 ml PBS-T using the technique described in step 4.
7. Add 20 mg/ml *p*-phenylenediamine to Mowiol mounting medium for a final concentration of 2 mg/ml. Add ~30 µl of this medium to the tube and mix gently by inverting the tube (solution is viscous).
8. Place 6 µl of bead/Mowiol-mix on a microscope slide and carefully mount a coverslip on top. Let set for ~20 min at room temperature in the dark. Seal the edges of the coverslip with nail polish.
9. Examine preparation under the epifluorescence microscope or store at 4°C in the dark until samples are viewed.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Apple juice/molasses agar plates

For 200 plates:
11.5 liters H_2O
5 liters apple juice
750 ml molasses
500 g agar
420 ml 10% (w/v) Nipagin (Fluka) in absolute ethanol

BRB80, 5×

To 100 ml H_2O add:
24.2 g PIPES (400 mM final)
2 ml 0.5 M EGTA (5 mM final)
1 ml 1 M $MgCl_2$ (5 mM final)
Adjust pH to 6.8 with KOH pellets.
Add H_2O to 200 ml
Store up to 2 months at 4°C

Dilution buffer

To 200 ml phosphate-buffered saline (PBS; APPENDIX 2A) add:
0.4 g bovine serum albumin (BSA, fraction V; 0.2% w/v final)
100× protease inhibitor mix (see recipe) to 2× final
20 mg/ml stock of DNase I, RNase free to final concentration of 20 µg/ml (stock stored at −20°C)
Prepare fresh just prior to use

Homogenization buffer

To 140 ml H_2O add:
40 ml 5× BRB80 (1× final)
20 ml 1 M KCl (100 mM final)
28 g sucrose (14% w/v final)
100× protease inhibitor mix (see recipe) to 2× final
Prepare fresh

Protease inhibitor mix (PIM), 100×

To 3 ml of methanol add:
4 ml of 1 mg/ml aprotinin (dissolved in H_2O)
4 ml of 1 mg/ml leupeptin (dissolved in H_2O)
4 ml of 1 mg/ml pepstatin A (dissolved in methanol)
5 ml of 100 mg/ml Pefabloc SC (Roche; dissolved in H_2O)
Store up to 2 weeks at −20°C

Sucrose solution, 55% (w/v)

To 140 ml H_2O add:
40 ml 5× BRB80 (1× final)
20 ml 1 M KCl (100 mM final)
110 g sucrose (55% w/v final)
Store up to 2 months at 4°C

Sucrose solution, 70% (w/v)

40 ml 5× BRB80 buffer (1× final)
20 ml 1 M KCl (100 mM final)
140 g sucrose (70% w/v final)
H_2O to 200 ml
Heat without boiling to dissolve
Store up to 2 months at 4°C

Wash buffer

To 200 ml phosphate-buffered saline (PBS; APPENDIX 2A), add:
0.2 g bovine serum albumin (BSA, fraction V; 0.1% w/v final)
100× protease inhibitor mix (see recipe) to 1× final
20 mg/ml stock of DNase I, RNase free to final concentration of 20 μg/ml (stock stored at −20°C)
Prepare fresh just prior to use

Yeast Paste

For 90 plates:
220 ml H_2O
150 g baker's yeast
1.4 ml propionic acid (Merck)

COMMENTARY

Background Information

Protocols for the isolation of centrosomes from higher eukaryotic cells are classically based on cell organelle enrichment via gradient centrifugation. Various successful protocols have been described that isolate centrosomes from mammalian tissue culture cells (Mitchison and Kirschner, 1986; Bornens et al., 1987; Blomberg-Wirschell and Doxsey, 1998; Bornens and Moudjou, 1999), tissue (Komesli et al., 1989; Lange and Gull, 1995), clam oocytes (Palazzo and Vogel, 1999), *Drosophila* (Moritz and Alberts, 1999; Lange et al., 2000), and yeast

(Wigge et al., 1998), to mention only some of the more frequently used sources. Most of the employed methods involve a lysis step in a low-ionic-strength buffer followed by density gradient centrifugation. The material thus produced is used subsequently in various assays. These include functional tests such as microtubule nucleation assay, electron microscopic study of centrosome morphology, antigen localization, or antigen production for the generation of monoclonal or polyclonal antibodies. Furthermore, centrosomal preparations have been used for the characterization of protein content of the organelle (protein complex); this application requires preparations in which the centrosomes are both highly enriched and present in biochemically meaningful quantities—i.e., micrograms of centrosomes for subsequent gel electrophoresis and mass spectrometry analysis (Wigge et al., 1998; Lange et al., 2000; Andersen et al., 2003).

Critical Parameters and Troubleshooting

Lysis and homogenization

Correct lysis and homogenization are critical for yield as well as for purity of centrosome preparations. Alternative methods using either tissue or mammalian tissue culture cells as starting material carry out the lysis in a low-ionic-strength buffer. Pretreatment of cells with anti-actin (e.g., cytochalasin B) and anti-microtubule drugs (e.g., nocodozole) has been employed in order to release the nuclear-centrosome interaction; this technique was initially proposed to efficiently dissociate the centrosome from the cytoskeleton and nuclei. However, cytochalasin treatment might not be an essential prerequisite for cytoskeletal dissociation (Bornens and Moudjou, 1999). Here, for *Drosophila* lysis and homogenization, buffer conditions are derived from the classical PIPES-based microtubule reassembly buffer (BRB80) but are modified (Moritz and Alberts, 1999) to include additional salt and sucrose, probably bringing them closer to isotonic conditions. An additional condition determining the outcome of the preparations is the efficient removal of the outer protective chorion layer of *Drosophila* embryos using washes with sodium hypochlorite solution. Incomplete removal can cause blockage or overheating of the tight-fitting Teflon pestle and render the material produced nearly useless.

Isolation of centrosomes by density gradient centrifugation

In the protocol developed by Moritz and Alberts (1999) for the isolation of *Drosophila* centrosomes, cell organelles are enriched in a single sucrose step gradient. The isolated centrosomes have been used in structural assays (electron microscopy) and functional assays (in vitro microtubule nucleation). Here centrifugation is carried out in two consecutive rounds, which represent concentration steps rather than purifications. These steps are then followed by affinity immunoisolation of centrosomes to remove co-migrating contaminants. Other groups employ, albeit for mammalian centrosome isolation, Ficoll gradients in a concentration step (Michison and Kirschner, 1986) or a rapid isolation step (Blomberg-Wirschell and Doxsey, 1998). This step can be followed by a purifying sucrose gradient centrifugation. The gradient is then fractionated and assayed by immunofluorescence microscopy using an anti-γ-tubulin antibody (Support Protocol 1) to select for the most enriched fractions for subsequent experiments. In the protocol in this unit, this assay is used to calculate the yield and determine the quality of isolated organelles. To perform this calculation, the number of centrosomes per relative area of the coverslip are counted, i.e., a given volume of the preparation is centrifuged down onto the coverslip, the count is performed, and the results extrapolated to the total volume of the preparation. A similar assay, though based on electron microscopy negative staining, can also be employed for rapidly checking the quality and structural integrity of centrosome preparations (Lange and Gull, 1996).

Purification of centrosomes by immunoaffinity purification

Because the avenue of density banding of centrosomes is blocked due to the high density of the organelle, the purity of most of the current centrosome isolation protocols is limited. Therefore, the authors of this unit developed a protocol for the affinity purification of centrosomes. The critical parameter here is the quality of the antibody that is used for the immunoprecipitation. Each antibody will need to be tested to determine if the antibody will or will not work under certain buffer or detergent concentrations and antibody concentrations, and protocols will have to be adapted accordingly. The initial protocol for affinity isolation

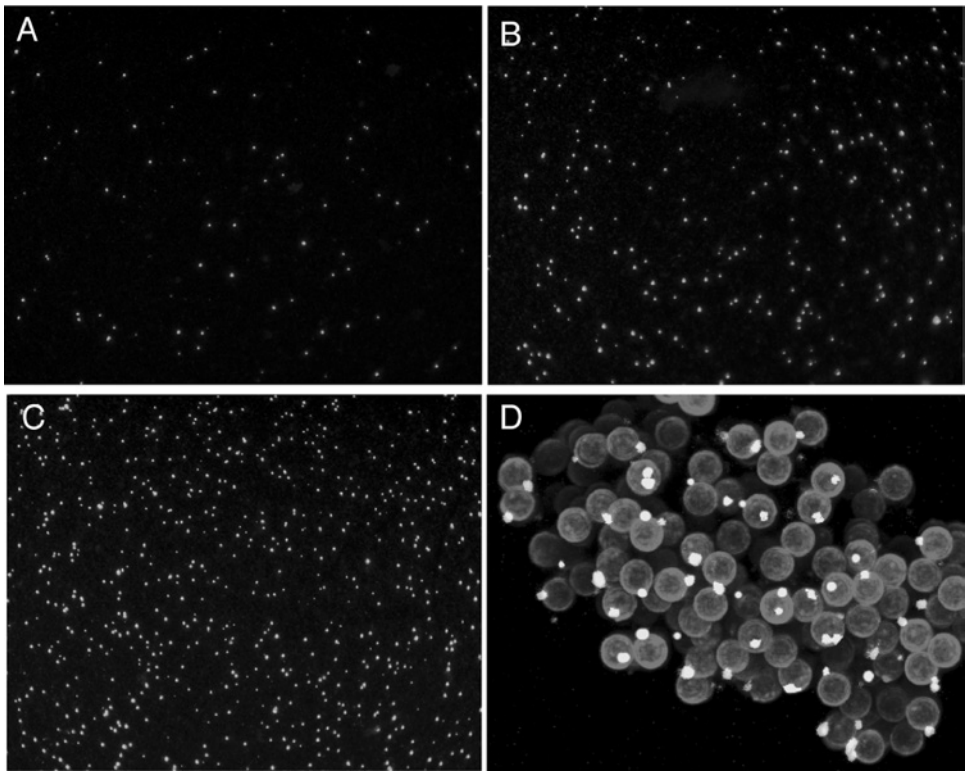


Figure 3.17.1 These images show the consecutive isolation steps assayed by immunofluorescence microscopy (Support Protocols 1 and 2) using an anti-γ-tubulin antibody to evaluate number and integrity of isolated centrosomes. (**A**) Centrosomes in embryo homogenate; (**B**) Pool 1; (**C**) Pool 2; (**D**) immunopurified centrosomes. Centrosomes are shown in yellow, beads are shown in red. For the color version of this figure go to *http://www.currentprotocols.com*.

of centrosomes was based on an indirect affinity method (Lange et al., 2000). In this technique, centrosomes were first coated with antibody, then anti-immunoglobulin beads were employed to isolate the centrosomes in a second step. In the protocol described in this unit, the authors use direct coupling of antibodies to magnetic beads to affinity purify centrosomes from enriched sucrose gradient fractions. This reduces the number of steps involved and consequently improves the yield.

Anticipated Results

With the described method microgram quantities of centrosomal protein can easily be obtained from ~10 g of embryo extract. Centrosomes should be intact as assayed by immunofluorescence microscopy (Support Protocols 1 and 2) and electron microscopy (Lange and Gull, 1996). Immunofluorescence images of centrosomes from consecutive isolations steps labeled with an anti-γ-tubulin antibody (see Fig. 3.17.1, panels A to D) are quantified to control enrichment and yield. In the Coomassie-stained SDS-PAGE analysis (see Fig. 3.17.2) of the immunopurified preparations, a major band should be CNN, a centrosomal protein of about 130 kD (see Fig. 3.17.2, asterisk) indicating the level of enrichment of the preparations.

Time Considerations

In well maintained fly populations, large quantities of embryo material can be obtained almost on a daily basis. Collection of material and production of embryo extract can be streamlined so that extracts are stockpiled for future use. The actual preparation of centrosomes by density gradient centrifugation can be easily achieved within 4 hr, and samples can be safely frozen without losing microtubule nucleation activity. Affinity purification, including all of the washing and coupling steps, will consume ~4 hr, not taking into account buffer and antibody preparation time. Assays monitoring the quality of the

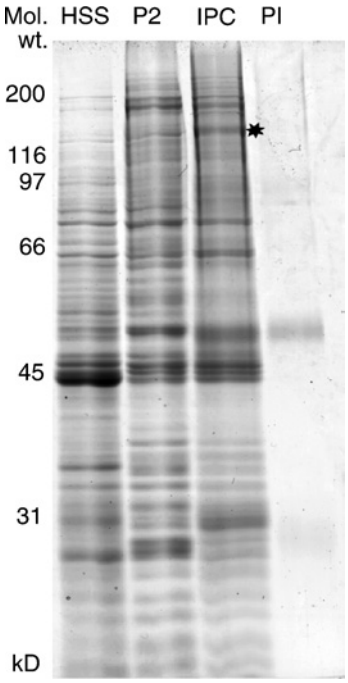


Figure 3.17.2 Isolation profile showing the protein composition of samples from the major consecutive steps in a Coomassie-stained gel. Abbreviations: HSS, homogenate supernatant supernatant; P2, Pool 2; IPC, immunopurified centrosomes; PI, preimmune isolation negative control. The asterisk at ~130 kDa labels the position of one of the major centrosome components (CNN) that is enriched throughout the isolation process.

preparation using immunofluorescence microscopy and SDS-PAGE are performed within 3 to 4 hr, not including microscope viewing time. Most solutions can be made ahead of time and stored either at 4°C or −20°C as indicated. Some solutions, such as proteinase inhibitors and DNase, must be added fresh from stock solutions to the solutions prior to use.

Literature Cited

Andersen, J.S., Wilkinson, C.J., Mayor, T., Mortensen, P., Nigg, E.A., and Mann, M. 2003. Characterization of the human centrosome by protein correlation profiling. *Nature* 426:570-574.

Blomberg-Wirschell, M. and Doxsey, S.J. 1998. Rapid isolation of centrosomes. *Methods Enzymol*. 298:228-238.

Bonte, E. and Becker, P.B. 1999. Preparation of chromatin assembly extracts from preblastoderm *Drosophila* embryos. *Methods Mol. Biol*. 119:187-194.

Bornens, M., Paintrand, M., Berges, J., Marty, M.C., and Karsenti, E. 1987. Structural and chemical characterization of isolated centrosomes. *Cell Motil. Cytoskel*. 8:238-249.

Bornens, M. and Moudjou, M. 1999. Studying the composition and function of centrosomes in vertebrates. *Methods Cell Biol*. 61:13-34.

Evans, L., Mitchison, T., and Kirschner, M. 1985. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol*. 100:1185-1191.

Greenspan, R.J. 1997. Fly Pushing: The Theory and Practice of Drosophila Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Komesli, S., Tournier, F., Paintrand, M., Margolis, R.L., Job, D., and Bornens, M. 1989. Mass isolation of calf thymus centrosomes: Identification of a specific configuration. *J. Cell Biol*. 109:2869-2878.

Lange, B.M.H. and Gull, K. 1995. A molecular marker for centriole maturation in the mammalian cell cycle. *J. Cell Biol*. 130:919-927.

Lange, B.M.H. and Gull, K. 1996. A structural study of isolated mammalian centrioles using negative staining electron microscopy. *J. Struct. Biol*. 117:222-226.

Lange, B.M.H., Bachi, A., Wilm, M., and Gonzalez, C. 2000. Hsp90 is a core centrosomal component and is required at different stages of the centrosome cycle in *Drosophila* and vertebrates. *EMBO J*. 19:1252-1262.

Mitchison, T.J. and Kirschner, M.W. 1986. Isolation of mammalian centrosomes. *Methods Enzymol*. 134:261-268.

Moritz, M. and Alberts, B.M. 1999. Isolation of centrosomes from *Drosophila* embryos. *Methods Cell Biol*. 61:1-12.

Moritz, M., Braunfeld, M.B., Fung, J.C., Sedat, J.W., Alberts, B.M., and Agard, D.A. 1995. Three-dimensional structural characterization of centrosomes from early *Drosophila* embryos. *J. Cell Biol.* 130:1149-1159.

Palazzo, R.E. and Vogel, J.M. 1999. Isolation of centrosomes from *Spisula solidissima* oocytes. *Methods Cell Biol.* 61:35-56.

Sullivan, W., Ashburner, M., and Hawley, R.S. (eds.) 2000. Drosophila Protocols. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Tavosanis, G., Llamazares, S., Goulielmos, G., and Gonzalez, C. 1997. Essential role for gamma-tubulin in the acentriolar female meiotic spindle of *Drosophila*. *EMBO J.* 16:1809-1819.

Wigge, P.A., Jensen, O.N., Holmes, S., Soues, S., Mann, M., and Kilmartin, J.V. 1998. Analysis of the *Saccharomyces* spindle pole by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. *J. Cell Biol.* 141:967-977.

Contributed by Verena Lehmann, Hannah Müller, and Bodo M.H. Lange
Max Planck Institute for Molecular Genetics
Berlin, Germany

Isolation of Zymogen Granules from Rat Pancreas

Zymogen granules are storage organelles in pancreatic acinar cells that contain digestive enzymes to be released into the pancreatic duct for eventual transport, along with bile salts from the liver, to the small intestine where they aid in digestion (see Fig. 3.18.1). The content proteins include amylase, the most abundant protein in the granules, as well as trypsinogen, chymotrypsinogen, carboxypeptidases, esterases, and lipases. The membranes of the granules contain proteins involved in granule exocytosis as well as enzymes that are released into the pancreatic juice. Many of the enzymes are stored in the granules as inactive proenzymes. Conversion of trypsinogen to trypsin occurs in the small intestine via a proteolytic cleavage by enterokinase, an enzyme of the duodenal mucosa. Activated trypsin, in turn, proteolytically cleaves and activates the other zymogen enzymes. The major stimuli for release of the contents of the granules from the cells, which occurs soon after ingestion of food, are cholecystokinin and acetylcholine.

This unit includes protocols for the isolation of crude zymogen granules from rat pancreas, further purification on sucrose gradient (see Basic Protocol) and/or Percoll gradient (see

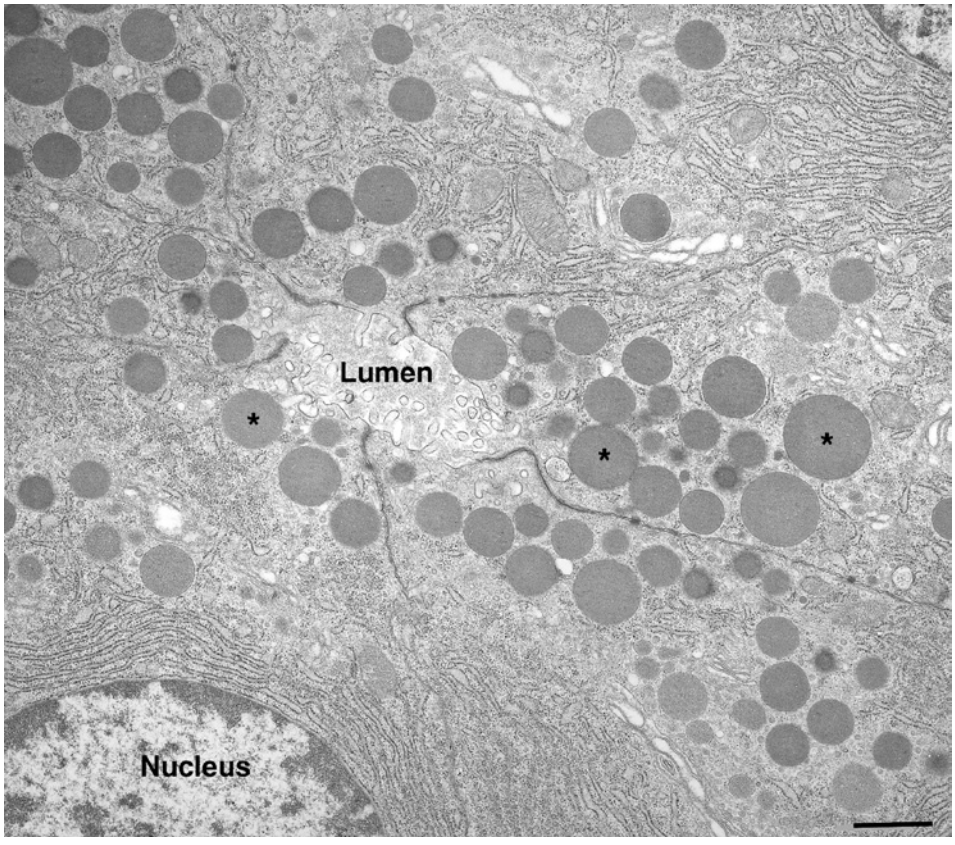


Figure 3.18.1 Electron micrograph of exocrine pancreas. Mouse pancreas was fixed and processed for conventional electron microscopy. Shown are exocrine cells containing abundant large zymogen granules near their apical plasma membrane (asterisks). Secretory contents are released into the acinar lumen and transported to the duodenum via the pancreatic ducts. Courtesy of H. Plesken and Dr. David Sabatini, NYU School of Medicine. Bar = 1 μm.

Contributed by Michael J. Rindler
Current Protocols in Cell Biology (2005) 3.18.1-3.18.16

Alternate Protocol 1), and purification of granule contents and membranes (see Alternate Protocol 2). There is also a protocol for purification of zymogen granules from dog pancreas (see Alternate Protocol 3). Additional protocols are provided for preparing a continuous sucrose gradient (see Support Protocol 1) and using a refractometer to assess the density of gradient fractions (see Support Protocol 2).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL

ISOLATION OF ZYMOGEN GRANULES FROM RAT PANCREAS

The early steps of this protocol can be used to prepare crude zymogen granules from rat pancreas. These granules are useful for structural and functional studies. If a higher degree of purity is required, the sucrose gradient fractionation steps described further along in the protocol can be used (see Fig. 3.18.2).

Materials

Rats (>200 g; Sprague-Dawley)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Homogenization buffer (see recipe)
30-ml continuous sucrose gradients in 25 × 89–mm Beckman Ultraclear centrifuge tubes (0.8 to 2 M sucrose; see Support Protocol 1)
Homogenization buffer (see recipe) containing 1 mM EDTA
CO_2 tank and vented chamber for inducing narcosis
Rat guillotine
Dissecting instruments including:
  Dissecting scissors and small scissors
  Scalpels or single-edge razor blades
150- and 250-ml beakers
Glass plates, clean
Balance accurate to 0.1 g
30-ml piston-type glass homogenizer-tissue grinder with Teflon pestle (Thomas) powered by variable-speed rotary drill (Wheaton Instruments)
15- and 50-ml conical polypropylene screw-cap centrifuge tubes
Eppendorf 5810C centrifuge (or equivalent low-speed refrigerated centrifuge) with swinging-bucket rotor
150-ml Erlenmeyer flask
Nitex 20-μm nylon mesh (Fisher)
30-ml glass Sorvall centrifuge tubes
Sorvall RC-5B centrifuge with SS-34 rotor (or equivalent)
7-ml Dounce homogenizer with B pestle (Kontes Glass)
Optima L-90K ultracentrifuge with SW-28 rotor (Beckman Coulter) or equivalent ultracentrifuge with swinging-bucket rotor
Additional reagents on equipment for preparing a sucrose gradient (Support Protocol 1) and using a refractometer (Support Protocol 2)

NOTE: Wear a laboratory coat and gloves throughout the procedure. All steps should be performed at 4°C, in a cold room if possible.

Collect pancreas

1. Feed 6 to 12 rats ad libitum (or starve overnight if required).

   The procedure may be scaled up or down to accommodate from 1 to 36 rats.

2. Anesthetize the first rat by CO_2 narcosis, then decapitate using a guillotine.

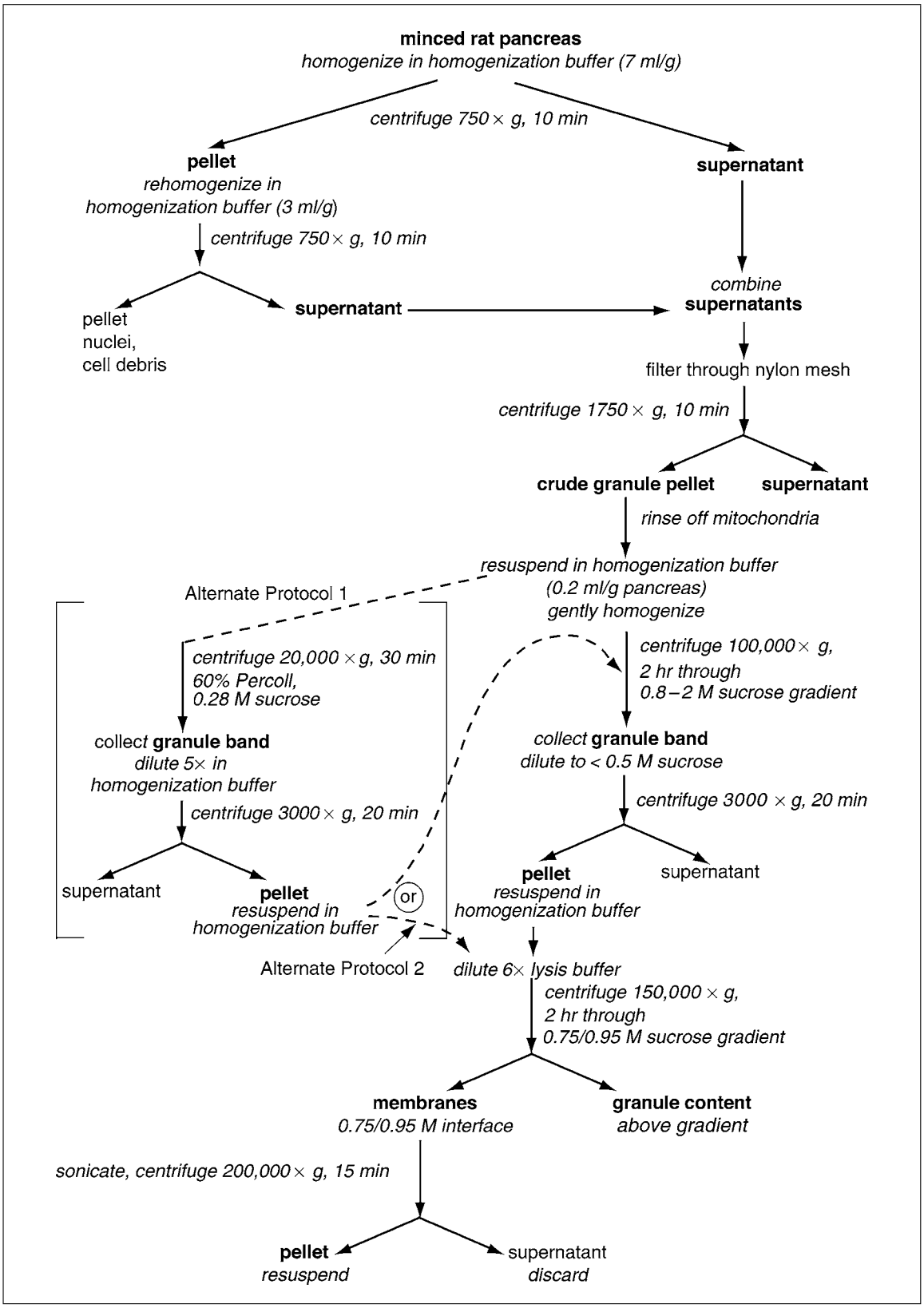


Figure 3.18.2 Flowchart of Basic and Alternate Protocols 1 and 2, providing a schematic summary of the major steps (described in detail in the text) of the protocols for purification of zymogen granules from rat pancreas and for separation of content and membrane proteins.

3. Excise pancreas (~1 g wet weight per animal) within 10 min. Using dissecting scissors make a vertical incision in the midline through the skin and underlying abdominal wall up to the thorax, then make lateral incisions just beneath the rib cage.

   This will allow access to the abdominal cavity. The pancreas in rats and mice is located in the mesogastrium just inferior to the stomach, also attached to the spleen and the duodenum.

4. Using small scissors, cut along the edge of the spleen and continue cutting along the inferior edge of the stomach and duodenum. Remove the entire mesentery, which contains the pancreas.

5. Place the pancreas in ice-cold PBS (10 ml per pancreas) in a 250-ml beaker.

6. Repeat steps 1 to 5 for each rat. When finished with all of the animals, transfer the pancreata to the same amount of fresh ice-cold PBS in another beaker.

Weigh pancreas

7. Prepare a 250-ml beaker containing 10 ml homogenization buffer per pancreas and obtain the tare weight to an accuracy of 0.1 g. Transfer each pancreas to a clean glass plate and trim away the fat and mesentery using a scalpel, then place the trimmed pancreas in the tared beaker. When finished, weigh the beaker to determine the amount of pancreas present.

   Protease inhibitors (present in the homogenization buffer; see Reagents and Solutions) are critical in the isolation procedure to prevent spontaneous activation of the inactive protease precursors, e.g., trypsinogen and chymotrypsinogen, to their active forms. It is possible to substitute a commercial protease inhibitor cocktail (Roche Biomedical).

Homogenize pancreas

8. Remove one or two pancreata at a time and place on another glass plate in the cold room or on ice. Mince finely, first with two scalpels or razor blades and subsequently with fine scissors.

9. Gather the minced material in a 150-ml beaker containing 7 ml homogenization buffer for each 1 g of pancreas

10. Homogenize with three strokes of a 30-ml Teflon/glass homogenizer powered by a variable-speed rotary drill at 3000 rpm. Repeat until all of the material has been homogenized.

    CAUTION: *Wear safety glasses or goggles during this procedure.*

    Smaller homogenizers can be used if only one or two animals are sacrificed.

Collect PNS

11. Transfer homogenate to disposable 15-ml (10 ml homogenate per tube) or 50-ml (40 ml homogenate per tube) conical polypropylene screw-cap centrifuge tubes. Centrifuge 10 min at 750 × *g*, 4°C, in an Eppendorf 5810C centrifuge with a swinging-bucket rotor (or equivalent) to sediment unbroken cells, nuclei, and large particles.

12. Remove as much of the floating fat and debris as possible from each tube with a pipet. Collect the remaining post-nuclear supernatant (PNS) carefully from each tube and pool all of the supernatants in a 150-ml Erlenmeyer flask.

13. Resuspend and pool pellets in 3 vol of homogenization buffer by pipetting up and down. Rehomogenize the pellets using three strokes of the Teflon/glass homogenizer at 3000 rpm. Centrifuge homogenate 10 min at 750 × g, 4°C.

14. Collect the PNS as above and combine with the first. Filter the PNS through a layer of 20-μm nylon mesh by covering the top of the Erlenmeyer flask and inverting over a 250-ml beaker.

For comparison or quantification purposes, it is useful to take an aliquot (e.g., 50 to 100 μl) of the PNS. To determine if a protein is present in zymogen granules, its enrichment in the purified granule fraction can be compared to that of amylase and other granule proteins.

Isolate zymogen granules

15. Transfer the filtrate to 15- or 30-ml glass Sorvall tubes. Centrifuge 20 min at 1750 × *g*, 4°C, in a Dupont Sorvall RC-5B with an SS-34 rotor, to sediment the zymogen granules.

16. Remove supernatants. Add 2 ml homogenization buffer to each pellet and swirl briefly to mobilize and dislodge the upper tan layer of mitochondria. Remove the supernatants by aspiration. Repeat the procedures in this step for a second rinse.

A small amount of the white granule pellet will be lost and not all the mitochondria will be removed.

17. Resuspend the pellets in a small volume of homogenization buffer (0.2 ml per g of pancreas) by manually shaking the tubes. Do not vortex.

This can take 15 to 30 min and is best done in a cold room. If necessary, pellets can be dislodged and crushed gently with a Teflon policeman during resuspension.

18. Gently homogenize the resuspended pellet (crude granule fraction) with five strokes of a 7-ml Dounce homogenizer with a loosely fitting (B) pestle.

This crude granule fraction is >90% pure. For many purposes, this may be sufficient. If not proceeding further, store granules at −80°C. after flash freezing in liquid nitrogen.

Further purify zymogen granules

19. Load 7 ml of resuspended granules onto the top of a continuous 30-ml sucrose gradient (0.8 to 2 M sucrose).

Alternatively, a step gradient using 0.2 M sucrose increments can be used (4.3 ml per step).

20. Ultracentrifuge gradient(s) 2 hr at 100,000 × *g*, 4°C, in an SW-28 rotor (or equivalent).

21. Collect the white granule layer about three-quarters of the way down the tube.

It is helpful to remove most of the gradient first and collect the band with a Pasteur pipet. It is also possible to collect the fraction by carefully puncturing the tube from the side with a syringe needle just below the granule layer and quickly collecting the granules in the syringe.

22. After collecting the granules, dilute them slowly in 3 vol of homogenization buffer containing 1mM EDTA.

It is best to put a small magnetic stirring bar in the tube and stir slowly while adding the homogenization buffer. If necessary, check sucrose concentration on a refractometer (see Support Protocol 2).

23. Transfer the diluted granules to 30-ml Sorvall tubes. Centrifuge the diluted granules 20 min at 3000 × *g*, 4°C, in an RC-5B centrifuge with SS-34 rotor.

24. Discard the supernatants. Resuspend the pellets in 2 ml homogenization buffer. Further purify zymogen granules (see Alternate Protocol 1) and/or separate content

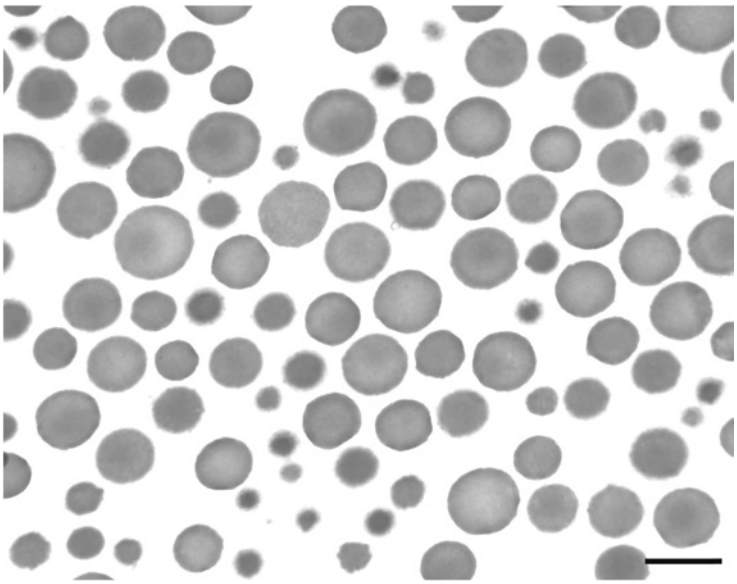


Figure 3.18.3 Electron micrograph of zymogen granule preparation. Granules prepared by the Basic Protocol, including the sucrose gradient fractionation, were fixed in a mixture of paraformaldehyde and glutaraldehyde and processed for electron microscopy. Courtesy of H. Plesken and Dr. David Sabatini, NYU School of Medicine. Bar = 1 μm.

and membrane fractions (see Alternate Protocol 2), or, if not proceeding further, freeze granules in liquid nitrogen and store at −80°C.

After this step, the granules appear to be pure by electron microscopy (Fig. 3.18.3).

SUPPORT PROTOCOL 1

POURING A CONTINUOUS SUCROSE GRADIENT

Continuous sucrose gradients are used to purify zymogen granules. They are poured using an Auto Densi-Flow device from Buchler Instruments. This device has an electric eye probe controller that senses the air/water interface and provides for precise gradient formation.

Materials

0.8 and 2 M sucrose gradient solutions (see recipe)
39-ml, 25 × 89–mm Beckman Ultraclear centrifuge tube
Auto Densi-Flow device (Buchler Instruments)
Peristaltic pump (Buchler Instruments)
Gradient maker (Hoefer Instruments SG30)
Magnetic stirrer and stir bars
Parafilm

1. Clamp a 39-ml, 25 × 89–mm Beckman Ultraclear centrifuge tube into the Auto Densi-Flow device.
2. Set the device on "Up" and manually lower the sensor so that it is almost touching the bottom of the tube.
3. Attach the device to the tubing of a peristaltic pump and connect the pump tubing to the outflow spout of a gradient maker on top of a magnetic stirrer.
4. With the stopcocks closed, add 15 ml of 0.8 M sucrose solution to the far chamber and 15 ml of 2 M sucrose solution, along with a small stirring bar to the outflow chamber of the gradient maker.

5. Set the stirrer to spin at a medium speed.
6. Open the outflow stopcock with the pump set ~5 ml/min flow. After a few seconds, open the stopcock between the chambers and check for free flow between the chambers.

   If the flow is not present, apply slight pressure to the outer chamber by covering it Parafilm and pressing down with a finger or thumb.
7. Allow the gradient to pour slowly, and keep at 4°C (up to 24 hr) until use.

USING A REFRACTOMETER

SUPPORT PROTOCOL 2

A refractometer is used to check the density of gradient fractions.

Materials

Sucrose gradient fractions (Basic Protocol)
Refractometer (Bausch & Lomb)

1. First calibrate the refractometer to zero by pipetting 50 μl water onto the quartz surface.
2. Close the chamber and read the refractive index.
3. Wipe the quartz surface clean with tissue paper (e.g., Kimwipes).
4. Read 50 μl of each sample from the sucrose gradient.
5. Convert the refractive index reading to density or molarity using a standard table.

   Among other sources, Beckman Coulter publishes a chart in the back of their ultracentrifuge brochure.

FURTHER PURIFICATION OF ZYMOGEN GRANULES ON A PERCOLL GRADIENT

ALTERNATE PROTOCOL 1

This protocol adds a Percoll gradient step to the purification procedures described in the Basic Protocol, instead of the sucrose gradient, in order to allow the isolation of granules of a slightly higher purity. Either the Percoll or the sucrose gradient, or both, can be used, in any order. The granules obtained from the Percoll gradient can also be used directly in assays, such as measurements of ion transport, where the high concentrations of sucrose are undesirable. Other investigators have reported methods to separate zymogen granules effectively from mitochondria using 40% Percoll gradients (De Lisle et al., 1984). The gain in purity is minor but there is a decrease in contaminant levels.

Additional Materials (see Basic Protocol)

Crude zymogen granule fraction (Basic Protocol, step 18)
Percoll gradient solution (see recipe)

50 ml, 29 × 102–mm flanged polycarbonate tubes centrifuge tubes (Fisher)
Sorvall RC-5B with SS-34 rotor or equivalent centrifuge with fixed-angle rotor

1. Prepare and resuspend the crude zymogen granule fraction (see Basic Protocol, steps 1 to 18).
2. Load 1 to 5 ml of the resuspended granules on top of 20 ml of Percoll gradient solution in a 29 × 102–mm thick-walled polycarbonate tube.

3. Centrifuge 30 min at 20,000 × *g*, 4°C, in an RC-5B centrifuge with an SS-34 rotor.

   Percoll forms a gradient when centrifuged. This procedure allows for only a shallow gradient to form, so that the lighter organelles remain in the top half of the tube.

4. Collect the white granule band near the bottom of the gradient and dilute slowly five-fold with homogenization buffer in a 30-ml glass Sorvall centrifuge tube.

   The lighter-density mitochondrial layer can be collected and sedimented to produce a mitochondria-enriched fraction.

5. Centrifuge the diluted granules 20 min at 3000 × *g*, 4°C, using an RC-5B centrifuge with SS-34 rotor.

   If it is necessary to remove Percoll entirely, this centrifugation can be repeated after resuspension of the white granule pellet in step 6.

6. Remove the supernatant and gently resuspend the pellet in 2 ml homogenization buffer without dissolving the Percoll below the pellet.

7. *Optional.* If greater purity is desired, continue with steps 19 to 24 of the Basic Protocol.

8. *Optional.* If desired, proceed with Alternate Protocol 2 for separating content and membrane fractions.

9. Freeze the granule fraction in liquid nitrogen and store at −80°C.

ALTERNATE PROTOCOL 2

ISOLATION OF ZYMOGEN GRANULE CONTENT AND MEMBRANE FRACTIONS

Content and membrane fractions can be separated on sucrose step gradients after granule lysis. Zymogen granules spontaneously lyse when the pH is raised to pH 8. This gentle procedure has the advantage of not disturbing other organelles that might be very minor contaminants, such as rough endoplasmic reticulum and mitochondria, which will sediment during centrifugation of the lysed granules.

Additional Materials *(also see Basic Protocol)*

Percoll-purified zymogen granules (Alternate Protocol 1)
Lysis buffer (see recipe)
0.75 M and 0.95 M sucrose gradient solutions (see recipe)
25 mM Tris·Cl, pH 7.5 containing 1 mM EDTA

13.2 ml, 14 × 89–mm Ultraclear centrifuge tubes
Beckman ultracentrifuge with SW-41 rotor (or equivalent)
15-ml conical polypropylene centrifuge tubes
Probe sonicator (Heat Systems Ultrasonics Model 185 or equivalent) with pencil probe
Beckman TL-100 centrifuge and TLA 100.3 rotor (or equivalent)
13 × 31–mm thick-walled polycarbonate centrifuge tubes or polyallomer microcentrifuge tubes

1. In a beaker, dilute resuspended gradient-purified granules slowly with 6 vol of lysis buffer while stirring slowly with a magnetic stirrer and a small stir bar. Incubate at least 45 min at 4°C.

   The solution should clarify with time. 0.2 M $NaHCO_3$, pH 8.3 (with protease inhibitors), can be substituted for the lysis buffer.

2. Prepare a step gradient by layering 3 ml of 0.75 M sucrose gradient on top of 3 ml of 0.95 M sucrose gradient solution in a 14 × 89–mm Ultraclear centrifuge tube. Mark

the interface to facilitate collection. Layer 6.5 ml of the diluted, lysed granules on top of the gradient and centrifuge 2 hr at 150,000 × *g*, 4°C, using an SW-41 rotor.

3. Collect the granule lysate (soluble content) above the gradient. Store at −80°C.
4. Collect the membranes at the 0.75/0.95 M sucrose interface and transfer to a 15-ml centrifuge tube. Dilute the membranes 1:1 in lysis buffer and sonicate using a probe sonicator with a pencil tip for 5 sec at maximum power.
5. Recover membranes by centrifuging 15 min at 200,000 × *g*, 4°C, in 13 × 31–mm thick-walled polycarbonate tubes (or microcentrifuge polyallomer tubes) in a Beckman TL-100 centrifuge with a TLA 100.3 rotor.
6. Discard the supernatants and resuspend the pellets by brief sonication (see step 4) in 0.1 ml 25 mM Tris·Cl, pH 7.5/1 mM EDTA. Freeze at −80°C for storage.

Expect a yield of <1% of the total granule protein.

ISOLATION OF ZYMOGEN GRANULES FROM DOG PANCREAS

ALTERNATE PROTOCOL 3

Zymogen granules can be isolated from dog pancreas, which is a more abundant source of material, using procedures similar to those described in this unit for rat pancreas. With canine pancreas, the connective tissue in the organ makes the procedure more cumbersome. It is necessary to use a tissue press and scale up the procedure, because the pancreas can be 100 g or more depending on the size of the animal. Otherwise, the procedure here makes use of the sucrose and Percoll gradient procedures described in the protocols above.

Additional Materials *(also see Basic Protocol)*

Dog, newly sacrificed
1-liter beakers
Tissue press consisting of a piston that drives the tissue through a 1-mm stainless steel mesh (a good-quality garlic press may be substituted)
250-ml disposable plastic conical screw-cap centrifuge tubes (Corning)
Sorvall RC-5B centrifuge with GSA rotor (or equivalent refrigerated centrifuge with fixed-angle rotor)
40-ml Dounce homogenizer with B pestle

Obtain and mince pancreas

1. From a newly sacrificed dog, dissect out the pancreas. Place in 500 ml ice-cold PBS in a 1-liter beaker to wash away blood and other debris.

Generally, animals used for experiments in open-heart surgery or other medical procedures are sacrificed at the end of the procedure. Control animals not treated with drugs or subjected to invasive procedures are preferred. The pancreas should be excised within 20 min of sacrifice. If the animal must be sacrificed in the laboratory, check with the institutional animal facility for the best procedures.

2. Remove the pancreas from PBS and cut away fat and large blood vessels with scissors and scalpels. Cut tissue into 2 to 3-cm pieces and put into a 1-liter beaker with 500 ml homogenization buffer.
3. Remove pancreas pieces from beaker one piece at time, cut the tissue into small fragments, and mince with scalpels or razor blades.

Press tissue and perform first homogenization

4. Prepare a 1-liter beaker containing 500 ml homogenization buffer and obtain the tare weight to an accuracy of 1 g. Put minced pieces in tissue press and force through the

press over the tared beaker. Repeat until all pieces have been pressed, cleaning out the screen mesh in between uses.

It can take a great deal of pressure to force the tissue through the press. Connective tissue will clog the mesh. Clean the press frequently.

5. Reweigh the beaker to determine the amount of tissue. Dilute to a volume of 10 ml per g of tissue using homogenization buffer.

6. Homogenize using three to five strokes of a 30-ml piston-type Teflon/glass homogenizer powered by a variable-speed rotary drill at 2500 rpm. Repeat until all the tissue is homogenized. Combine homogenates.

Even after the tissue press step, there will be considerable amounts of connective tissue present which will make homogenization difficult. Be very careful in the homogenization not to force the pestle down to the base of the glass vessel, as this may cause the homogenizer to shatter. Wear safety goggles.

An alternative is to use a Polytron homogenizer (Brinkmann Instruments) at a medium speed setting. Insert the head of the Polytron directly into the beaker and homogenize until the solution is uniformly pink. Connective tissue can also clog the Polytron, so it is important to check and clean the probe if it does not function effectively.

Obtain and PNS

7. Transfer homogenate to disposable plastic 50-ml (40 ml homogenate per tube) conical polypropylene screw-cap tubes or to 250-ml conical centrifuge tubes. Centrifuge 10 min at 750 × *g*, 4°C, in an Eppendorf 5810C centrifuge with a swinging-bucket rotor (or equivalent).

8. Remove as much of the floating fat and debris as possible with a pipet. Collect the remaining post-nuclear supernatant (PNS) carefully from each tube and pool all of the supernatants in a 500-ml Erlenmeyer flask.

Because of the large amount of PNS obtained and the fact that there will be connective tissue in the pellet, it is neither necessary nor desirable to rehomogenize the pellet.

Collect zymogen granules

9. Filter the PNS through a layer of 20-µm nylon mesh by covering the top of the Erlenmeyer flask and inverting it over a 500-ml beaker.

10. Transfer the filtrate to 250-ml plastic centrifuge tubes (<200 ml filtrate/tube) and centrifuge 30 min at 1750 × *g*, 4°C, in a fixed-angle (e.g., GSA) rotor, to bring down the zymogen granules.

11. Discard supernatant. Add 10 ml homogenization buffer and swirl briefly to remove the upper tan layer of mitochondria from the pellet. Remove the solution. Repeat.

A small amount of the white granule pellet will be washed away.

Further homogenize and purify zymogen granules

12. Resuspend the pellet in a small volume (0.2 ml per g of pancreas) of homogenization buffer by manually shaking the tubes. Dislodge the pellets and mash them gently with a Teflon policeman during resuspension. Do not vortex.

13. Gently homogenize the resuspended pellet (crude granule fraction) with five strokes of a loosely fitting Dounce homogenizer with a loosely fitting (B) pestle.

14. Further purify the granules according to Alternate Protocol 1, steps 1 to 8.

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Homogenization buffer

0.3 M sucrose
2 mM MOPS, pH 6.8
1× protease inhibitors (see recipe for 1000×)
1 mM EDTA
Prepare fresh

Lysis buffer

150 mM KCl
25 mM HEPES, pH 8
1 mM EDTA
1× protease inhibitors (see recipe for 1000×)
Prepare fresh

Percoll gradient solution

60% (v/v) Percoll (Amersham Biosciences)
0.28 M sucrose
20 mM MOPS, pH 6.8
1 mM EDTA
1× protease inhibitors (see recipe for 1000×)
Store up to 2 hr at 4°C

Percoll consists of colloidal silica coated with polyvinylpyrrolidone.

Protease inhibitors, 1000×

Prepare the following 1000× solutions separately:
10 mg/ml aprotinin in H_2O (store up to 3 months at −20°C)
5 mg/ml pepstatin in methanol (store up to 3 months at −20°C)
0.5 M phenylmethylsulfonyl fluoride (PMSF) in DMSO (prepare fresh)
Add inhibitors to solutions at 1× concentration as recommended

PMSF is unstable in water and should be added to the solutions just prior contact with biological material.

Sucrose gradient solutions, 0.75 M, 0.8 M, 0.95 M, and 2 M

Solutions for purification of zymogen granules (see Basic Protocol; see Support Protocol 1 for continuous gradient preparation):
0.8 M sucrose
10 mM HEPES, pH 6.8
1 mM EDTA
1× protease inhibitors (see recipe for 1000×)
Store up to 24 hr at 4°C

2 M sucrose
10 mM HEPES, pH 6.8
1 mM EDTA
1× protease inhibitors (see recipe for 1000×)
Store up to 24 hr at 4°C

Solutions for separation of zymogen granule content and membranes (see Alternate Protocol 2):
0.75 M sucrose
25 mM HEPES, pH 8

(continued)

1 mM EDTA
1× protease inhibitors (see recipe for 1000×)
Store up to 24 hr at 4°C

0.95 M sucrose
25 mM HEPES, pH 8
1 mM EDTA
1× protease inhibitors (see recipe for 1000×)
Store up 24 hr at 4°C

COMMENTARY

Background Information

Pancreatic zymogen granule isolation procedures were first reported by Hokin (1955) for dog pancreas and later extended to bovine and guinea pig pancreas by Palade and collaborators (Greene et al., 1963; Tartakoff et al., 1974). These procedures were similar to the crude granule preparation outlined in the early steps of the Basic Protocol of this unit. Sucrose gradient fractionation was later introduced (Paquet et al., 1982). All of the procedures to purify zymogen granules take advantage of their large size and density as compared to other cellular organelles. Variations on the theme include procedures that use sucrose/Ficoll gradients for granule isolation (Cameron et al., 1986). These procedures are similar to those used to purify secretory granules from salivary glands, whose granules resemble those of the pancreas and have exocrine secretions that include amylase and several other proteins also found in the pancreatic zymogen granules. Percoll gradient fractionation has been useful for preparing zymogen granules competent for ion transport because Percoll, unlike sucrose, has very low osmolality (De Lisle et al., 1984). Zymogen granules are more susceptible to lysis after being exposed to high concentrations of sucrose.

Zymogen granules lyse as the pH is raised above 7 and the content proteins become soluble (Hokin, 1955). The pH-dependent solubilization of the granule content correlates with the physiological function of the pancreatic duct as a secretor of bicarbonate to neutralize stomach acid in the duodenum. Thus, the granule content, which is in an acidic environment as long as the granules remain within the cells in order to ensure that the zymogens remain inactive and in a condensed state, is exposed to a pH of ∼8 in the lumen of the duct after release from the acinar cells.

Acinar cells, whose exocrine products are secreted into the pancreatic juice, are the predominant cells in the pancreas. However, there are also pancreatic duct cells, connective tissue cells, vascular endothelial cells, and islets of Langerhans, whose cells secrete endocrine hormones such as insulin and glucagon. The islet cells contain secretory granules, but constitute a minor percentage of the total pancreatic volume (∼1% in rats; Bonner-Weir et al., 1989). Hence zymogen granules will be far more abundant than any other type of granule, including the Weibel-Palade bodies of the endothelial cells. The question arises as to whether endocrine granules are present in the zymogen granule preparations. Because the endocrine granules are smaller than the zymogen granules, they do not sediment efficiently at 1750 × g. Indeed, procedures for preparing insulin granules take advantage of the fact that zymogen granules sediment at 3000 × g while the insulin granules do not (Rhodes et al., 1987). Although the endocrine contamination is minimal, in order to determine if a protein is a bona fide zymogen granule protein, it is essential to compare its enrichment in the granule fractions with known zymogen granule content proteins.

Table 3.18.1 lists the major proteins of the zymogen granule. Amylase is the most abundant. Figure 3.18.4 shows the pattern of Coomassie blue staining of a SDS/PAGE gel with some of the major proteins indicated. Two-dimensional SDS/PAGE has been used to identify many of the proteins listed in Table 3.18.1 (Scheele, 1981; Kleene et al., 1999).

Critical Parameters

The pancreatic zymogen granules include proteases that may become activated at a low rate during granule isolation. Thus, it is essential to keep the samples at 4°C and to use protease inhibitors.

Contaminants are the major concern. Nuclei are quantitatively eliminated in the initial low-speed centrifugation. However, the crude granule fraction will be contaminated with mitochondria, as previously mentioned, and by some large fragments of the rough endoplasmic reticulum (ER), which is abundant

Table 3.18.1 Major Proteins Identified in Pancreatic Zymogen Granules

Protein	Mol. wt. (kDa)
Secreted proteins (content and peripheral membrane)	
Amylase isoforms	56
Carboxyl ester hydrolase/lysophospholipase/bile salt–dependent lipase	70–78
Chymotrypsinogen isoforms	26
Colipase	10
DNase I	35
Kallikrein I (glandular)	30
Lipase Related Protein 1 (PLRP1)	45–53
GP3/GPIII/PLRP2	54
Pancreatic lipase (triacylglycerol lipase)	53
Pancreatic thread protein (stone protein, reg, lithostathine)	14–19
Phospholipase A2	14
Procarboxypeptidase (PCP) A1, A2, B/ZAP47 isoforms	44–47
Proelastase (proteinase E) isoforms	32
RNase	50
Serglycin proteoglycan	>300
Serpin (ZG46)	46
Serpin (ZP21p)	21
Syncollin	18
Trypsinogen I (cationic), II (anionic), III (cationic), VB	25
ZG16p	16
ZG29	29
Integral membrane proteins	
CLCA-1 (calcium-activated HCO_3^- channel)	90
GP2 (luminal domain released into content)	78–90
ITMAP (luminal domain released into content)	110
Multi-drug resistance transporter MDR-related protein	65
Muclin/CRP-ductin/DBMT1/hensin/gp-340 (luminal domain released)	300
Rab 3D	27
Syntaxin 3	35

in pancreas. These organelles are not as dense as zymogen granules and therefore can be separated from granules during sucrose and/or Percoll gradient fractionation. Mitochondria will appear as a tan layer above the white granules. The rough ER is more variable in density but will also be in lighter fractions. It is important to gently resuspend the crude granule pellet and homogenize with a Dounce homogenizer so as to minimize nonspecific absorption of these organelles to the outside of the granules. Mitochondrial contamination can be monitored using mitochondrial enzyme assays, such as succinate dehydrogenase (Pennington, 1961; *UNIT 3.4*). Mitochondrial enzyme–specific activity is usually below the threshold of detectability in granules purified through sequential Percoll and sucrose gradient steps. ER contamination can be measured by immunoblotting with antibodies to rough ER marker proteins, such as BiP/GRP78, available commercially through a number of sources.

The ER is difficult to eliminate entirely, especially from granule membrane fractions. Granule content is generally highly purified,

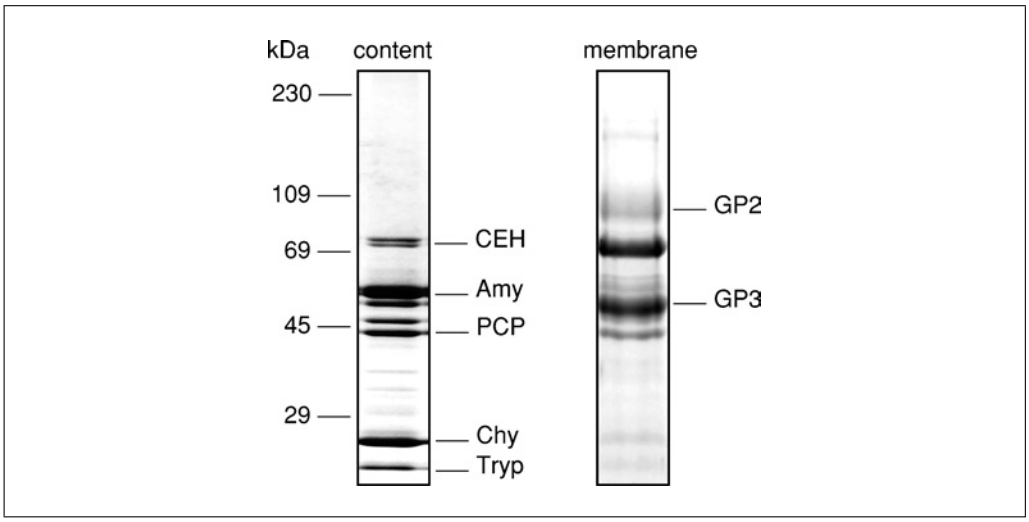


Figure 3.18.4 Profile of content and membrane proteins. Zymogen granules from rat pancreas were prepared according to the Basic Protocol, including sucrose gradient steps, and Alternate Protocol 1 (the Percoll gradient procedure). Membranes and content fractions (30 μg) were subjected to SDS/PAGE on 10% gels and stained with Coomassie blue. The migration positions of some of the major content proteins, including amylase (Amy), procarboxypeptidase (PCP), chymotrypsinogen (Chy), trypsinogen (Try), and carboxyl ester hydrolase (CEH, also known as lysophospholipase and bile salt–dependent lipase) are indicated. The migration positions of two of the major membrane proteins, GP2 and GP3, are indicated in the right panel.

but membranes are much less so. This is because the contaminants, such as ER and even mitochondrial remnants, often adhere nonspecifically to the granule membranes, which represent <1% of the total zymogen granule protein. Even when the membranes are isolated on a step gradient, contaminants can remain with them. However, all of the major proteins visible upon staining of polyacrylamide gels with Coomassie blue or silver after SDS-PAGE are granule membrane proteins or content proteins; hence the contamination is usually very minor and will not be detected unless very sensitive methods are used.

In addition, lysosomes are large and dense organelles. Some of them are large enough to cosediment in the crude granule pellet and to remain in the high-density granule fraction of the sucrose and/or Percoll gradients. Lysosomal enzyme assays such as that of aryl sulfatase, β-hexosaminidase (*UNIT 15.8*), or β-glucuronidase can be monitored to assess lysosomal contamination (a protocol for the assay of β-glucuronidase is available from Worthington Biochemical at *http://www.worthington-biochem.com/GL/default.html*). In general, lysosomes are represented in the isolated gradient-purified granule fraction to about the same extent as they are in the initial homogenate, whereas zymogen granules will be enriched ~4 fold, as noted above.

Granule content proteins are present in the granule membrane fraction and vice versa. In the case of the content, a number of major membrane proteins, including the major membrane protein GP2, are known to be enzymatically cleaved from the membrane and released in a soluble form in the pancreatic juice (Rindler and Hoops, 1990). In addition, several granule peripheral membrane proteins are also known to be present in the granule content, including GP-III/PLRP2 (Wagner et al., 1994). Therefore, their presence in the granule content is expected. However, the presence of major content proteins in the membrane fraction is more complex. In part, their presence could represent trapping of soluble proteins as granules lyse and their membranes subsequently reseal after incubation at pH 8. After isolation of these membranes on the sucrose step gradient, the protocol calls for the membranes to be sonicated and sedimented. This eliminates much of the nonspecific trapping as the large granule membrane ghosts are broken up into smaller vesicles. However, even if these sonication and washing steps are repeated, amylase and other content proteins can be observed in the membrane fraction. Their presence may be related to the mechanisms by which granules form in the *trans*-Golgi network (TGN), and similar findings have been reported for endocrine granules. During granule formation in the TGN, aggregating content proteins need to

interact with membrane proteins prior to immature granule detachment (Rindler, 1992). Zymogen granule content proteins as well peripheral membrane proteins can be extracted by sonication in 0.1 M $NaCO_3$, pH 11 (Rindler and Hoops, 1990).

Anticipated Results

Assessment of the efficacy of purification is essential in any cell fractionation scheme. Aliquots of the homogenate, PNS, crude granule pellet, and purified granules are saved for this purpose. Because amylase and other major granule content proteins are easily identified after staining polyacrylamide gels with Coomassie blue, it is relatively easy to assess the extent of purification using SDS-PAGE. After determining the protein content of each fraction by standard methods (e.g., Bradford; *APPENDIX 3H*), equal amounts of protein are run in parallel lanes on the gel. The intensity of the content protein bands can be quantitated using the NIH Image computer program (*http://rsb.info.nih.gov/nih-image/*) and digitally scanned images of the gels. Generally, a ~4 fold enrichment in the specific activity of the content proteins is expected in the granule preparations during purification because the granules constitute ~25% of the total cellular volume in electron micrographs. However, the procedures are designed for high purity rather than maximum yields, which can be low (<20% for the entire procedure including the Percoll gradient step) because many granules lyse during the homogenization procedure and subsequent steps.

Classically, the specific activity of amylase has been used to monitor the purification. Enzymatic activity is based on the ability of α-amylase to hydrolyze starch and release maltose. There have been a number of methods used to quantify this. A spectrophotometric assay based on detection of reducing groups liberated from starch using 3,5-dinitrosalicylic acid has been commonly used. It is inexpensive, but cumbersome and linear only in a relatively narrow range (see the Worthington Enzyme Manual, *http://www.worthington-biochem.com/AA/default.html*, and references therein). Alternatively, a diagnostic kit using a *p*-nitrophenyl maltoheptaside derivative as a substrate is available from Sigma (cat no. 568-20) and can be scaled down to use on 96-well plates and read on a standard plate reader at 405 nm. Recently, a BODIPY-derivatized starch has been used as an enzyme substrate (Molecular Probes). This assay is sensitive and easy to use but is costly and requires a fluorescent plate reader.

Time Considerations

The time that the procedure requires depends critically on the number of animals used. For a standard preparation using 12 rats, it should take ~2 hr to obtain a PNS and an additional 1 hr to sediment and resuspend the crude granule pellet. Further purification on sucrose gradients will add an additional 3 hr, and a Percoll gradient step will add 1 hr. If membranes and content proteins are separated, then allow 3 additional hr. Hence, a high-purity preparation of content and membrane fractions would take ~10 hr to prepare.

It is possible to centrifuge sucrose gradients longer than the recommended times, even overnight, without adverse effects. This applies both to the initial continuous gradients and the step gradients used to separate membrane from content proteins. If pressed for time, this course would be appropriate. Granules cannot be fractionated after being frozen. While membrane and content proteins can be separated readily from frozen and thawed granules by Alternate Protocol 3, this is not recommended because other organelles that may be minor contaminants of the preparation, like lysosomes and mitochondria, will lyse, and their proteins will further contaminate the preparations.

Literature Cited

Bonner-Weir, S., Deery, D., Leahy, J.L., and Weir, G.C. 1989. Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion. *Diabetes* 38:49-53.

Cameron, R.S., Cameron, P.L., and Castle, J.D. 1986. A common spectrum of polypeptides occurs in secretion granule membranes of different exocrine glands. *J. Cell Biol.* 103:1299-1313.

De Lisle, R.C., Schulz, I., Tyrakowski, T., Haase, W., and Hopfer, U. 1984. Isolation of stable pancreatic zymogen granules. *Am. J. Physiol.* 246:G411-G418.

Greene, L.J., Hirs, C.H., and Palade, G.E. 1963. On the protein composition of bovine pancreatic zymogen granules. *J. Biol. Chem.* 238:2054-2070.

Hokin, L.E. 1955. Isolation of the zymogen granules of dog pancreas and a study of their properties. *Biochim. Biophys. Acta* 18:379-388.

Kleene, R., Dartsch, H., and Kern, H.F. 1999. The secretory lectin ZG16p mediates sorting of enzyme proteins to the zymogen granule membrane in pancreatic acinar cells. *Eur. J. Cell Biol.* 78:79-90.

Paquet, M.R., St-Jean, P., Roberge, M., and Beaudoin, A.R. 1982. Isolation of zymogen granules from rat pancreas and characterization of their membrane proteins. *Eur. J. Cell Biol.* 28:20-26.

Pennington, R.J. 1961. Biochemistry of dystrophic muscle. Mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem. J.* 80:649-654.

Rhodes, C.J., Lucas, C.A., Mutkoski, R.L., Orci, L., and Halban, P.A. 1987. Stimulation by ATP of proinsulin to insulin conversion in isolated rat pancreatic islet secretory granules. Association with the ATP-dependent proton pump. *J. Biol. Chem.* 262:10712-10717.

Rindler, M.J. 1992. Biogenesis of storage granules and vesicles. *Curr. Opin. Cell Biol.* 4:616-622.

Rindler, M.J. and Hoops, T.C. 1990. The pancreatic membrane protein GP-2 localizes specifically to secretory granules and is shed into the pancreatic juice as a protein aggregate. *Eur. J. Cell Biol.* 53:154-163.

Scheele, G.A. 1981. Analysis of the secretory process in the exocrine pancreas by two-dimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis. *Methods Cell Biol.* 23:345-358.

Tartakoff, A., Greene, L.J., and Palade, G.E. 1974. Studies on the guinea pig pancreas. Fractionation and partial characterization of exocrine proteins. *J. Biol. Chem.* 249:7420-7431.

Wagner, A.C., Wishart, M.J., Mulders, S.M., Blevins, P.M., Andrews, P.C., Lowe, A.W., and Williams, J.A. 1994. GP-3, a newly characterized glycoprotein on the inner surface of the zymogen granule membrane, undergoes regulated secretion. *J. Biol. Chem.* 269:9099-9104.

Contributed by Michael J. Rindler
New York University School of Medicine
New York, New York

Isolation of Glyoxysomes from Pumpkin Cotyledons

The isolation of organelles is essential for a variety of biochemical procedures. The Basic Protocol in this unit delineates the efficient isolation and purification of glyoxysomes from pumpkin cotyledons. It has been used, with minor modifications, to isolate peroxisomes from multiple plant tissues and species. In addition, effective means to quantify and determine the integrity of the isolated organelles are discussed. A Support Protocol for growing pumpkin seedlings for use in the Basic Protocol is also provided.

ISOLATION OF GLYOXYSOMES

BASIC PROTOCOL

Glyoxysomes are found in germinating seedlings and contain the enzymes of the glyoxylate cycle. They are isolated by differential and density gradient centrifugation.

Materials

5- to 7-day, dark-grown pumpkin seedlings (Support Protocol)
1× grinding buffer working solution (see recipe)
2 M sucrose
28% (v/v) Percoll/resuspension buffer solution (see recipe)
1× resuspension buffer working solution (see recipe)

250-ml beakers
Balance accurate to 0.1 g
Waring blender (2-speed) and 500-ml blender cup, precooled to 4°C
Miracloth (Calbiochem)
Glass funnel, precooled to 4°C
50-ml round-bottom polypropylene centrifuge tubes
Sorvall RC5C centrifuge with HB-6 swinging-bucket rotor (or equivalent refrigerated centrifuge and rotor)
15-ml Corex tubes, cooled to 4°C
Small, soft paintbrush (natural hair)
UV spectrophotometer

NOTE: Keep all equipment and all solutions cold (4°C) for the duration of the procedure.

Prepare cotyledons

1. Record the age and growth conditions of the pumpkin seedlings.
2. Harvest the pumpkin cotyledons (Fig. 3.19.1) in dim room light by manually separating the cotyledons from the hypocotyls. For very small seedlings, remove the seed coat and radicle as well.
3. Preweigh a 250-ml beaker to the nearest 0.1 g, cool the beaker on ice, then place the cotyledons inside the beaker. Reweigh the beaker, and record the tissue weight to the nearest 0.1 g.

   Be sure to keep the tissue cold. Avoid exposure to bright light, as this will cause the cotyledons to become green. Normal room lighting can be resumed during the first centrifugation step.

Contributed by Nicola Harrison-Lowe and Laura J. Olsen
Current Protocols in Cell Biology (2005) 3.19.1-3.19.8

Figure 3.19.1 Five-day-old dark-grown pumpkin seedlings**.** 1, seed coat; 2, cotyledons; 3, hypocotyl; 4, radicle.

Homogenize the tissues

4. Put the tissue and enough 1× grinding buffer working solution (with BSA) to cover the tissue (usually about 150 to 170 ml) in the blender cup. Homogenize the tissue with the Waring Blender, using three short bursts of ∼3 sec each, on low speed. Do not overgrind.

5. Filter the homogenate through a piece of Miracloth that has been folded in half and used to line the inside of a precooled funnel placed over a 250-ml beaker on ice. Collect the filtered homogenate in the beaker below the funnel. Squeeze the macerated tissue inside the Miracloth bag gently, to extract all the liquid. Discard the remaining tissue and used Miracloth.

6. Distribute the filtrate evenly between four 50-ml round-bottom polypropylene centrifuge tubes and centrifuge in a swinging-bucket rotor 10 min at 3000 × *g*, 4°C.

7. Remove the thick lipid layer floating on top of each supernatant using a Kimwipe wrapped around the wide end of a 1000-µl plastic pipet tip.

8. Decant the supernatant into a second set of four clean 50-ml round-bottom polypropylene centrifuge tubes.

9. Centrifuge this supernatant in a swinging-bucket rotor 20 min at 10,500 × *g*, 4°C. Carefully decant and discard the supernatant from each tube, retaining the pellets (keep on ice).

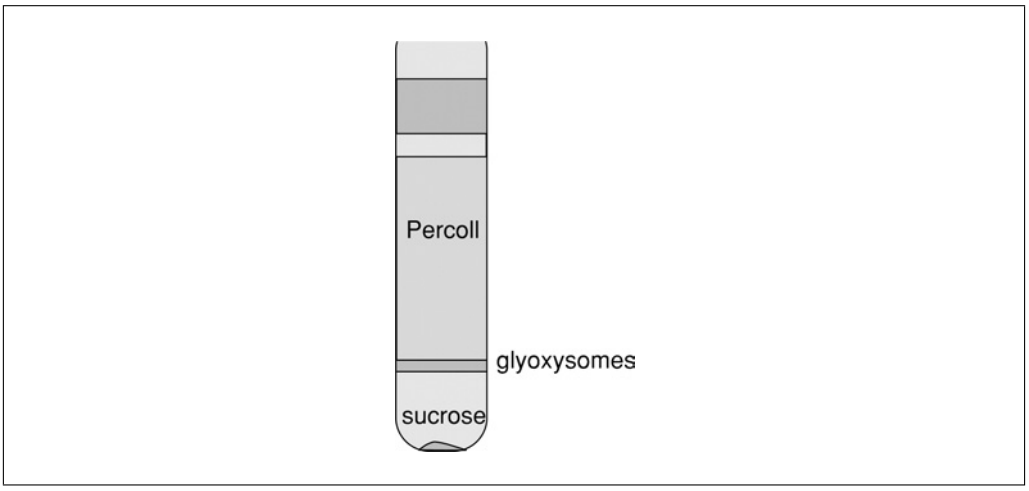


Figure 3.19.2 Percoll gradient separation of glyoxysomes.

Centrifuge the homogenate

10. Prepare a Percoll gradient by placing 1.5 ml of 2 M sucrose solution in a chilled 15-ml Corex tube. Carefully overlay with 10 ml 28% Percoll/1× resuspension buffer solution, being very careful not to mix the sucrose and Percoll layers.

11. Using a small paintbrush, gently resuspend each pellet (from step 9) in 250 µl of 1× resuspension buffer working solution. Pool the resulting suspension in one tube and swirl gently to mix.

12. Carefully overlay the pooled suspension on to the top of the prepared Percoll gradient.

13. Centrifuge the supernatant in a swinging-bucket rotor 30 min at 18,000 × *g*, 4°C, without the brake.

    Using the brake on this step will disturb the gradient and greatly diminish the final glyoxysome yield.

Purify the glyoxysomes

14. Carefully remove the upper layers of lipid and broken organelles in the gradient with a Pasteur pipet (Fig. 3.19.2). Finally, carefully collect and transfer the glyoxysomes (the visible yellowish band at the Percoll/sucrose interface) with a clean pipet to a 15-ml Corex tube.

    Removing too much Percoll or sucrose cushion will reduce the purity of the glyoxysomes, while removing too little will decrease the overall yield.

15. Dilute the glyoxysomes 3- to 5-fold with 1× resuspension buffer (usually ~8 ml is required).

16. Centrifuge glyoxysomes in a swinging-bucket rotor 16 to 20 min at 7000 × *g*, 4°C, with the brake on.

    A shorter (16-min) centrifugation will result in a soft organelle pellet, making it harder to remove all of the supernatant without losing part of the pellet. A longer (20-min) centrifugation will yield a tighter pellet.

17. Carefully remove and discard the supernatant. Gently resuspend the pellet of purified glyoxysomes in 100 µl of 1× resuspension buffer.

18. To approximate the yield, take an aliquot of the glyoxysome suspension, dilute it 1:500 with 1× resuspension buffer, and measure the absorbance at A_{280} and A_{235}. Use these values in the equations below to obtain the approximate concentration and yield.

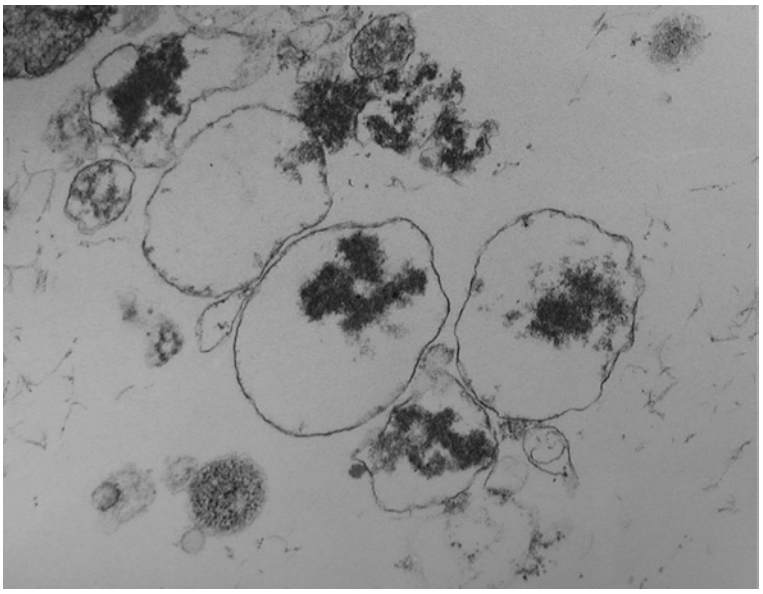


Figure 3.19.3 Electron micrograph of purified pumpkin glyoxysomes. Magnification, 10,000× (micrograph by Mary Alice Webb, Purdue University).

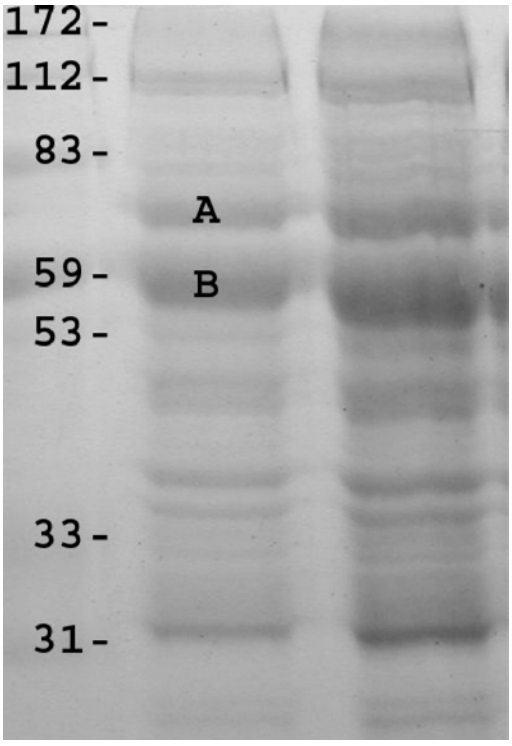


Figure 3.19.4 Coomassie blue–stained gel of proteins from purified pumpkin glyoxysomes. Proteins from freeze-thawed, lysed glyoxysomes were separated by 10% SDS-PAGE: left lane, 25 μg; right lane 50 μg. The major glyoxysomal protein bands indicated in the figure are: (**A**) isocitrate lyase (64 kDa) and (**B**) catalase (55 kDa).

Approx. conc. (mg protein/ml) $= [(A_{235} - A_{280})/2.51] \times 500$

Approx. yield (mg total protein) = (mg protein/ml)
× (final resuspension volume in ml).

These numbers are only approximate, but they are quick and easy to obtain and are consistent between organelle preparations. The authors use this calculation to standardize the amount of glyoxysomes used in each experiment. If more accurate protein concentrations are needed, one can do a biochemical protein assay, such as Pierce's BCA assay or any other protein assay of choice (APPENDIX 3H).

19. Determine integrity of the organelle preparation.

In addition to morphological analysis by electron microscopy (Fig. 3.19.3), there are many enzymatic assays that can be used to verify the presence of intact glyoxysomes, including catalase assays (Cooper and Beevers, 1969; Aebi, 1984; also see UNIT 3.4) and isocitrate lyase assays (Cooper and Beevers, 1969). Mitochondrial contamination can be assessed by performing fumarase assays (Cooper and Beevers, 1969; Hatch, 1978). The intactness of the organelles can be determined by performing the enzyme assays in the presence (to disrupt the membrane) and absence of 0.5% (v/v) Triton X-100. Though substantially enriched for glyoxysomes, the final organelle pellet usually contains some mitochondrial contamination. The proteins can be analyzed by SDS-PAGE and Coomassie blue staining (Fig. 3.19.4).

GROWING SEEDLINGS FOR GLYOXYSOME ISOLATION

SUPPORT PROTOCOL

Seedlings are required for isolation of the glyoxysomes.

Materials

Pumpkin seeds (or other plant seeds depending on desired organelle source)
Medium-coarse vermiculite (available from gardening supply stores)
Flat for germination (available from gardening supply stores)
Plastic wrap

1. Place ~75 ml pumpkin seeds on about 2 cm of medium-coarse vermiculite in a germination flat and sprinkle with a thin layer of vermiculite just sufficient to cover the seeds. Water the seeds liberally and cover the flat loosely with plastic wrap to maintain high humidity for germination.

The amount of seeds may need to be adjusted for other plant types to generate 25 to 35 g of tissue.

2. Germinate pumpkin seeds (or desired organelle source) in the dark at 22° to 25°C.

Optimize the temperature for the particular tissue source.

Germination and growth routinely takes 5 to 7 days, depending on the time of year (shorter during the summer months).

3. Water the seedlings 3 to 4 days after planting to promote germination.

4. Harvest the seedlings when they are ~5 to 8 cm tall.

Seeds that have just begun to germinate can also be harvested, as long as the seed coat is removed prior to grinding.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Grinding buffer

2× stock solution:
17.844 g tetrasodium pyrophosphate (40 mM final)
0.744 g EDTA (2 mM final)
109.32 g D-mannitol (0.6 M final)
Add 950 ml H_2O
Adjust pH to 7.5 with glacial acetic acid
Add H_2O to 1 liter final volume
Sterilize through 0.45-μm filter
Store up to 6 months at 4°C
1× working solution (170 ml): Add 85 ml of 2× grinding buffer stock solution to 85 ml water. Add 170 mg BSA (1 mg/ml) to the buffer and mix well. Make fresh working solution for each glyoxysome preparation.

The final concentrations in the working solution are 20 mM tetrasodium pyrophosphate, 1 mM EDTA, and 0.3 M D-mannitol.

Percoll, 28% (v/v)/resuspension buffer, 1×

28 ml Percoll
50 ml 2× resuspension buffer stock solution (see recipe)
22 ml H_2O
Store up to 6 months at 4°C

Resuspension buffer

2× stock solution:
4.776 g HEPES (20 mM final)
109.32 g D-mannitol (0.6 M final)
Add 950 ml H_2O
Adjust pH to 7.2 with KOH
Adjust volume to 1 liter with H_2O
Sterilize through 45-μm filter
Store up to 6 months at 4°C
1× working solution (20 ml): Add 10 ml of 2× resuspension buffer stock solution to 10 ml distilled water. Make fresh working solution for each glyoxysome preparation.

The final concentrations in the working solution are 10 mM HEPES and 0.3 M D-mannitol.

Sucrose, 2 M

Dissolve 85.575 g sucrose in water. Bring final volume to 125 ml. Filter the solution through a 45-μm filter. Store up to 6 months at 4°C.

Filtration can be tricky because of the high viscosity of the solution. A vacuum or syringe filter may be used. Filtering may be easier if the solution is warm.

COMMENTARY

Background Information

In the late 1960s, cell biologists determined that the spherical particles observed in plant cell electron micrographs were in fact classes of a distinct and vital organelle, the peroxisome (Tolbert, 1971). Peroxisomes derive their name from their critical function in the metabolism of hydrogen peroxide. For example, the degradation of hydrogen peroxide is catalyzed by the matrix protein catalase, arguably the most abundant peroxisomal enzyme. Catalase is also a major constituent of the distinctive peroxisomal core or crystalline inclusion that is sometimes observed in

plant peroxisomes (Heinze et al., 2000). Peroxisomes are small organelles that are bound by a single phospholipid membrane. They can range in size from 0.5 to 1.5 μm. Although they have been observed in a variety of shapes, they are most commonly spherical. While all plant peroxisomes have some enzymes in common (e.g., catalase, thiolase), they are typically divided into several classes based upon their physiological roles, as defined by spatial and temporal parameters (Olsen and Harada, 1995; Beevers, 2002). For example, cotyledons of oilseeds, such as pumpkins, contain a special class of peroxisomes, called glyoxysomes. These sequester all of the enzymes required for the glyoxylate cycle, to supply the growing seedling with energy. In contrast, peroxisomes found later in leaf development have a partially different enzyme complement because of their function in other pathways, including photorespiration (Olsen, 1998).

An interesting feature of peroxisomes is their lack of an organellar genome. This means that nuclear genes encode all constituent proteins. Peroxisomal proteins are synthesized in the cytoplasm and are post-translationally translocated into peroxisomes (Brickner et al., 1997). The biogenesis of peroxisomes most likely involves the growth and division of preexisting peroxisomes (Olsen, 1998; Purdue and Lazarow, 2001). Intense study has elucidated the molecular machinery (PEX proteins) required for the interrelated processes of peroxisome biogenesis and protein import. Import of peroxisomal matrix proteins occurs through two receptor-mediated import pathways. Each pathway is defined by one of two peroxisome targeting signals (PTS1, PTS2) on the cargo proteins that the receptors recognize and transport. Thus, there are two cytosolic receptors (Pex5p, Pex7p) that recognize the targeting signals and bind to the cargo proteins (Subramani, 1996; Johnson and Olsen, 2001; Brown and Baker, 2003). In vitro protein import assays have been invaluable in studies of the dynamics of peroxisomal protein import, addressing questions about the interactions between the receptors and cargo proteins, the energy requirements, the roles of protein chaperones, and the molecular mechanisms of translocation (Mori and Nishimura, 1989; Behari and Baker, 1993; Brickner et al., 1997; Brickner and Olsen, 1998; Crookes and Olsen, 1998; Johnson and Olsen, 2003). The success of these assays relies on the reproducible purification and manipulation of intact peroxisomes and the presence of the relevant protein cargo, receptors, and other biochemical constituents.

In addition to questions of organelle biogenesis, peroxisome biochemistry and function has received recent attention. Many previously uncharacterized enzymes, including alanine aminotransferase, alanine:glyoxylate aminotransferase, and sarcosine oxidase, have been localized to plant peroxisomes (Liepman and Olsen, 2001, 2003; Goyer et al., 2004). A role for peroxisomal β-oxidation in auxin metabolism has been extensively studied (e.g., Zolman et al., 2000, 2001; Zolman and Bartel, 2004). An isozyme of 12-oxophytodienoate reductase, OPR3, which catalyzes the final step of jasmonate biosynthesis, also possesses a peroxisomal targeting signal, suggesting a role for peroxisomes in jasmonic acid signaling (Sanders et al., 2000; Stintzi and Browse, 2000). Thus, peroxisomes have many critical physiological functions throughout the life cycle of plants.

Critical Parameters and Troubleshooting

There are several critical points for consideration during the glyoxysome purification procedure. Care must be taken to ensure that plants are grown in the dark, that the seedlings are ~5 to 8 cm tall, and that exposure to light during harvesting is minimized to get a good yield of glyoxysomes. This is because, when seedlings begin greening and producing the first true leaves, some glyoxysomes transition into leaf peroxisomes while others are probably degraded (there are fewer peroxisomes present in leaves than there are glyoxysomes present in cotyledons). The tissue must remain cold throughout the protocol to help reduce endogenous protease activities that can cause the degradation of glyoxysomes and glyoxysomal peripheral membrane proteins. The tissue should not be overground during the homogenization step. Instead subject the tissue to just enough homogenization needed to break up the majority of the tissue. When homogenization is done correctly, there should be no large chunks of intact tissue evident after homogenization, though smaller pieces of ground tissue should be present.

Anticipated Results

A preparation of 25 g of pumpkin cotyledons should yield 100 μl of glyoxysomes with an approximate protein concentration of 100 mg/ml. The yield largely depends on the successful removal of the glyoxysome layer from the Percoll gradient.

Time Considerations

An efficient glyoxysome preparation should be completed within 2 to 2.5 hr. Purified glyoxysomes can be stored on ice, but will lose structural and metabolic integrity within hours. Intact glyoxysomes do not survive freezing, though many glyoxysomal enzymes will continue to have measurable activity.

Literature Cited

Aebi, H. 1984. Catalase in vitro. *Methods Enzymol.* 105:121-126.

Beevers, H. 2002. Early research on peroxisomes in plants. *In* Plant Peroxisomes. Biochemistry, Cell Biology and Biotechnological Applications. (A. Baker and I.A. Graham, eds.) pp. 1-17. Kluwer Academic Publishers. New York.

Behari, R. and Baker, A. 1993. The carboxyl terminus of isocitrate lyase is not essential for import into glyoxysomes in an in vitro system. *J. Biol. Chem.* 268:7315-7322.

Brickner, D.G. and Olsen, L.J. 1998. Nucleotide triphosphates are required for the transport of glycolate oxidase into peroxisomes. *Plant Physiol.* 116:309-317.

Brickner, D.G., Harada, J.J., and Olsen, L.J. 1997. Protein transport into higher plant peroxisomes. In vitro import assay provides evidence for receptor involvement. *Plant Physiol.* 113:1213-1221.

Brown, L.A. and Baker, A. 2003. Peroxisome biogenesis and the role of protein import. *J. Cell. Molec. Med.* 7:388-400.

Cooper, G. and Beevers, H. 1969. Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. *J. Biol. Chem.* 244:3507-3513.

Crookes, W.J. and Olsen, L.J. 1998. The effects of chaperones and the influence of protein assembly on peroxisomal protein import. *J. Biol. Chem.* 273:17236-17242.

Goyer, A., Johnson, T.L., Olsen, L.J., Collakova, E., Shachar-Hill, Y., Rhodes, D., and Hanson, A.D. 2004. Characterization and metabolic function of a peroxisomal sarcosine and pipecolate oxidase from *Arabidopsis*. *J. Biol. Chem.* 279:16947-16953.

Hatch, M.D. 1978. A simple spectrophotometric assay for fumarate hydratase in crude tissue extracts. *Anal. Biochem.* 85:271-275.

Heinze, M., Reichelt, R., Kleff, S., and Eising, K. 2000. High resolution scanning electron microscopy of protein inclusions (cores) purified from peroxisomes of sunflower (*Helianthus annuus* L.) cotyledons. *Cryst. Res. Technol.* 35:877-886.

Johnson, T.L. and Olsen, L.J. 2001. Building new models for peroxisome biogenesis. *Plant Physiol.* 127:731-739.

Johnson, T.L. and Olsen, L.J. 2003. Import of the peroxisomal targeting signal type 2 protein 3-ketoacyl-coenzyme A thiolase into glyoxysomes. *Plant Physiol.* 133:1991-1999.

Liepman, A.H. and Olsen, L.J. 2001. Peroxisomal alanine:glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*. *Plant J.* 25:487-498.

Liepman, A.H. and Olsen, L.J. 2003. Alanine aminotransferase homologs catalyze the glutamate:glyoxylate aminotransferase reaction in peroxisomes of Arabidopsis. *Plant Physiol.* 131:215-227.

Mori, H. and Nishimura, M. 1989. Glyoxysomal malate synthase is specifically degraded in microbodies during greening of pumpkin cotyledons. *FEBS Lett.* 244:163-166.

Olsen, L.J. 1998. The surprising complexity of peroxisome biogenesis. *Plant Mol. Biol.* 38:163-189.

Olsen, L.J. and Harada, J.J. 1995. Peroxisomes and their assembly in higher plants. *Annu Rev. Plant Physiol.* 46:123-146.

Purdue, P.E. and Lazarow, P.B. 2001. Peroxisome biogenesis. *Annu. Rev. Cell Devel. Biol.* 17:701-752.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., and Goldberg, R.B. 2000. The Arabidopsis *DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. *Plant Cell* 12:1041-1061.

Stintzi, A. and Browse, J. 2000. The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 97:10625-10630.

Subramani, S. 1996. Protein translocation into peroxisomes. *J. Biol. Chem.* 271:32483-32486.

Tolbert, N.E. 1971. Microbodies: peroxisomes and glyoxysomes. *Annu. Rev. Plant Physiol.* 21:45-74.

Zolman, B.K. and Bartel, B. 2004. An Arabidopsis indole-3-butyric acid-response mutant defective in PEROXIN 6, an apparent ATPase implicated in peroxisomal function. *Proc. Natl. Acad. Sci. U.S.A.* 101:1786-1791.

Zolman, B.K., Yoder, A., and Bartel, B. 2000. Genetic analysis of indole-3-butyric acid responses in *Arabidopsis thaliana* reveals four mutant classes. *Genetics* 156:1323-1337.

Zolman, B.K., Monroe-Augustus, M., Thompson, B., Hawes, J.W., Krukenberg, K.A., Matsuda, S.P., and Bartel, B. 2001. *chy1* an Arabidopsis mutant with impaired beta-oxidation, is defective in a peroxisomal beta-hydroxyisobutyryl-CoA hydrolase. *J. Biol. Chem.* 276:31037-31046.

Contributed by Nicola Harrison-Lowe and Laura J. Olsen
University of Michigan
Ann Arbor, Michigan

Isolation of GLUT4 Storage Vesicles

The regulation of blood glucose following a meal is achieved by the insulin-dependent mobilization of the glucose transporter isoform GLUT4, which resides primarily (>95%) in intracellular membranes in the basal state. In response to insulin it moves to the cell surface where it can then function to transport glucose into fat and muscle, the tissues that express GLUT4. In type 2 diabetes this process of GLUT4 translocation is compromised. For this and other fundamental reasons, there is considerable interest in GLUT4 trafficking.

The process is similar to two other well-studied vesicular trafficking paradigms: (1) the cellular itinerary of nutritional receptors such as those for transferrin and LDL and (2) synaptic vesicle movement. However, unlike the former, GLUT4 movement is highly insulin dependent, and unlike the latter, it has a relatively slow time course of ~20 min to complete a journey from its intracellular storage site to the cell surface and back again. It is thought that ~50% of GLUT4 represents the immediately mobilizable pool, called GLUT4 storage vesicles (GSVs), although these have not yet been isolated on a preparative scale sufficient for proteomic analysis.

Nevertheless, the isolation procedures described in this unit for all GLUT4 pools and their subsequent biochemical analysis has been instrumental to our understanding of GSV trafficking (see Fig. 3.20.1). The Basic Protocol describes immunoisolation of GLUT4 pools using primary rat adipocytes where the majority of this work has been performed. Alternate Protocols 1 and 2 describe appropriate adjustments to the Basic Protocol for cultured adipocytes and primary skeletal muscle.

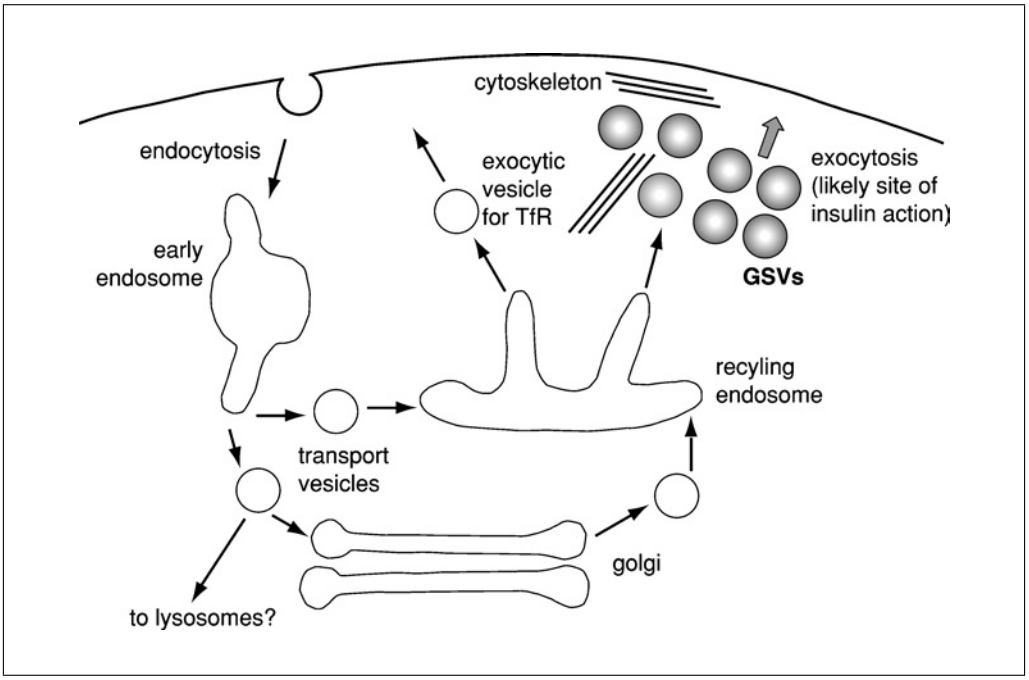


Figure 3.20.1 Known and speculative features of GLUT4 trafficking. Some steps are depicted as being unidirectional although they may operate bidirectionally (Karylowski et al., 2004). Abbreviations: GSVs, GLUT4 storage vesicles; TfR, transferrin receptor.

Contributed by Konstantin V. Kandror and Paul F. Pilch

Current Protocols in Cell Biology (2006) 3.20.1-3.20.13

IMMUNOISOLATION OF GLUT4 VESICLES FROM RAT ADIPOSE CELLS

Primary rat adipocytes are highly insulin responsive cells that can be isolated in exceptional purity (<98%) and sufficient quantity (4 to 25 ml packed cells, density <0.9g/ml) for extensive biochemical characterization. They are easily fractionated into relatively pure membrane compartments enriched, respectively, in plasma membrane, light membranes containing Golgi markers and GSVs, and heavier membranes containing ER markers. Thus, they are a particularly good tissue source for the isolation of GSVs and analysis of their component proteins and lipids.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

Materials

1 mg/ml protein A–purified mouse monoclonal anti-GLUT4 antibody (e.g., R & D Systems 1F8) in 0.1 M sodium borate, pH 8.6 (see James et al., 1988)
1 mg/ml protein A–purified nonspecific mouse IgG in 0.1 M sodium borate, pH 8.6
Reacti-Gel (GF-2000) beads (Pierce)
PBS (*APPENDIX 2A*)
PBS (*APPENDIX 2A*)/0.02% (w/v) NaN_3
Male Sprague-Dawley rats, 150 to 175 g
KRH buffer (see recipe), 37°C
Type I collagenase specified for adipocyte isolation (Worthington) or equivalent
PBS with inhibitors (see recipe), 37°C, 18°C to 20°C, and 4°C
PBS (*APPENDIX 2A*)/1% (w/v) BSA
PBS (*APPENDIX 2A*)/1% (v/v) Triton X-100
Laemmli SDS-PAGE sample buffer without reducing agents (*UNIT 6.1*)
CO_2 chamber (or other equipment for IACUC-approved protocols for sacrificing rats)
Dialysis tubing
1.5-ml microcentrifuge tubes or cryovials
−80°C freezer
Sharp scissors
50-ml plastic vials with tight caps
37°C water bath with orbital and reciprocal shaker
Low-speed medical centrifuge
300-μm nylon mesh
Long-tip, 9-in. Pasteur pipets
Vacuum pump with trap
50-ml centrifuge tube
Motor-driven tissue grinder with a Teflon pestle (Potter-Elvehjem type, Thomas model #3431, size B or C)
High-speed centrifuge (e.g., Sorvall RC-5C with SA-600 rotor)
Gel-loading tip (optional)
Spectrophotometer
1.5-ml microcentrifuge tubes
Tube rotator in a 4°C incubator
Additional reagents and equipment for protein quantification (*APPENDIX 3B & 3H*) and SDS-PAGE (*UNIT 6.1*) or immunoprecipitation (*UNIT 7.2*) and immunoblotting (*UNIT 6.2*)

Prepare antibody-coupled beads

1. Couple protein A–purified 1F8 antibody and non-specific mouse IgG, in separate reactions, to Reacti-Gel beads according to manufacturer's instructions.

   The resulting beads should contain ~0.8 to 1.0 mg of either 1F8 or IgG per ml.

2. Remove the beads by centrifuging 10 sec at 300 × *g*, collect unbound 1F8, dialyze the antibody against three changes of PBS, measure the protein concentration (*APPENDIX 3B & 3H*), separate the stock into 200-µl aliquots, and store at −80° for unlimited periods of time (>10 years).

   This residual antibody can be used for immunoblotting (UNIT 6.2) at 2 µg/ml.

3. Prepare 50% suspension of immunobeads in PBS/0.02% (w/v) NaN_3 and store them at 4°C for up to 1 year.

Prepare tissue

4. Sacrifice male rats using a chamber with CO_2 or another IACUC-approved protocol.

5. Dissect epididymal fat pads from 10 to 12 rats (5 to 10 g of tissue) and place them pooled together into a plastic vial with ~20 ml warm KRH.

Isolate cells

6. Use scissors to mince fat pads into small (<1-mm^3) pieces. Add dry collagenase to 1.75 mg/ml, place vials into a 37°C shaking water bath, adjust speed to achieve intense but gentle shaking, and incubate vials for 45 min.

   The time and concentration of collagenase digestion may vary with the lot and should be adjusted to yield a smooth suspension of adipocytes; also see next step.

7. Filter cells through 300-µm nylon mesh. Gently mix cells with an equal volume of warm KRH in a 50-ml centrifuge tube and centrifuge 10 sec at 100 to 300 × *g*, room temperature.

   Lipid-filled fat cells will float because their buoyant density is less than water. A Pasteur pipet should be inserted through the cell layer to the bottom of the tube and the buffer should be carefully aspirated from underneath the cells.

   Handle primary cells with extreme care. A lipid layer on top of adipocytes after the centrifugation step indicates that a significant number of cells have been broken due to either harsh handling or low temperature that causes solidification of intracellular lipids and cell lysis. Certain batches of collagenase can also cause this problem.

8. Resuspend the cell layer (not the pellet) in warm KRH and aspirate KRH with a long-tip Pasteur pipet attached to a vacuum pump with a trap. Repeat wash three times, then incubate cells 45 min at 37°C.

9. Aspirate KRH and wash cells by adding three volumes of PBS with inhibitors warmed to room temperature (18°C to 20°C).

10. Add 2 vol warm (18°C to 20°C) PBS with inhibitors (twice the volume of lightly packed adipocytes), place cells in a 50-ml centrifuge tube, and spin 20 min at 16,000 × *g*, 4°C.

    Primary adipocytes will break open without homogenization. Alternatively, homogenize cells with seven strokes in a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle (Simpson et al., 1983).

Collect the homogenate

11. Place cell homogenate on ice immediately.

    Another protocol for subcellular fractionation of adipocytes without homogenization is described in Lee et al. (1999).

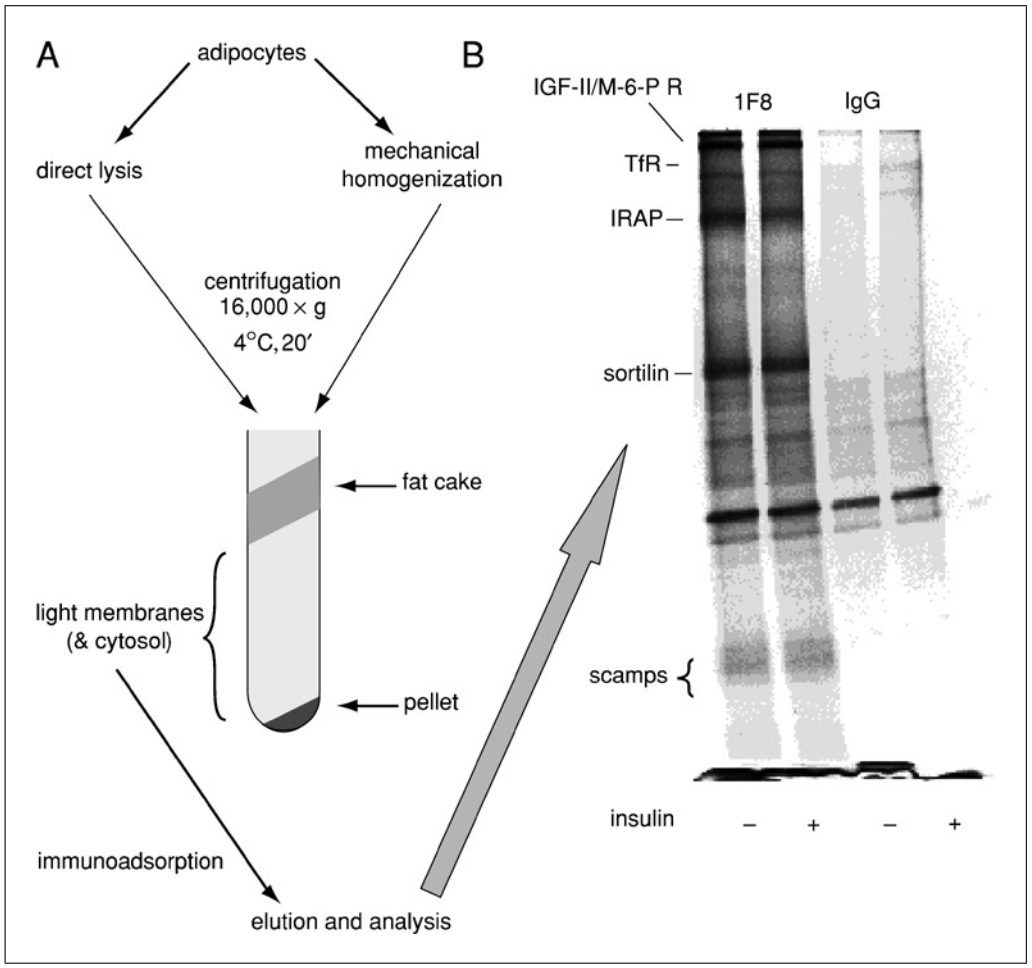


Figure 3.20.2 Vesicle isolation scheme (**A**) and the results of silver staining of proteins eluted with Triton X-100 (**B**). The identity of the bands in this experiment was determined by immunoblotting with corresponding antibodies and has been confirmed by protein sequencing in other experiments (Laurie et al., 1993; Thoidis et al. 1993; Kandror et al., 1994, 1995; Keller et al., 1995; Kandror and Pilch, 1996, 1998; Lin et al., 1997; Morris et al., 1998; Larance et al., 2005). The proteins listed on the left of panel B are discussed in Background Information, along with additional proteins implicated in vesicle association.

12. Centrifuge homogenized cells 20 min at 16,000 × *g*, 4°C.

    The triglycerides will form a congealed fat cake on top, and the heavy membrane will pellet (Fig. 3.20.2A). Centrifugation for 20 min at 16,000 × g results in pellets that contain the heavier GLUT4-containing membranes such as the plasma membrane and endosomes. The substantial majority of GLUT4 in the 16,000 × g supernatant is localized in homogenous 60 to 80 S vesicles. In addition to these vesicles, the 16,000 × g supernatant contains light membranes (LM) that do not contain GLUT4 and all soluble proteins from the cytosol.

13. Using a gel-loading tip or a Pasteur pipet and with minimum disturbance of the fat cake and pellet, collect as much as possible of the light membranes plus cytosol from below the fat cake and above the pellet.

14. Measure the protein concentration (*APPENDIX 3B & 3H*) in the light membranes plus cytosol fraction (LM + cytosol) which will be applied to the beads for immunoadsorption.

Block antibody-bound beads

15. Calculate the amount of immunobeads required for the experiment based on a ratio of ~1 mg of the starting material (LM + cytosol) to 15 μg of immobilized antibody. Resuspend immunobeads in 1 ml PBS/1% BSA in 1.5-ml microcentrifuge tubes and rotate for 30 min at 4°C.

    Blocking beads with BSA is required in order to decrease nonspecific sorption of protein and membrane material on the beads.

16. Rinse blocked beads with PBS three times, centrifuging 10 sec at 300 × *g*, room temperature, between rinses.

Isolate GLUT4 storage vesicles

17. Add LM + cytosol to the beads in the amounts calculated in step 15. Gently rotate immunobeads with the LM overnight at 4°C.

    Use same amounts of the starting material for specific and nonspecific immunobeads. Save an aliquot (25 to 50 μg) of the starting material for analysis of the efficiency of immunoadsorption (see step 18).

18. At the end of the overnight incubation, place microcentrifuge tubes in a rack and wait till the beads sediment to the bottom of the tubes.

    This process should take about a minute and may be expedited by a 4–5 sec centrifugation at ~1000 × g in a microcentrifuge. Avoid high speed centrifugation, which can lead to contamination of the settled beads with membranes and protein aggregates.

19. Collect postadsorptive supernatant, which contains unbound material.

    The volume and the total protein concentration of the postadsorptive supernatant should be equal to the volume and the protein concentration of the starting material because the GLUT4 protein represents such a small portion of the total material that the difference is undetectable.

20. Wash immunobeads four times with 1 ml cold PBS by gentle mixing and microcentrifuging 10 sec at 300 × *g*, room temperature.

    Avoid vortexing and high-speed centrifugation of the beads. Wash the beads at room temperature, but use cold PBS.

21. Add at least 3 bead vol of PBS/1% Triton X-100 to washed beads and elute bound material by rotating the tubes 1 hr at 4°C.

22. Collect the Triton eluate for SDS-PAGE (*UNIT 6.1*) or immunopreciptation (*UNIT 7.2*).

    1% Triton X-100 will solubilize the vesicular membrane and elute vesicular proteins with the exception of GLUT4 itself, which will remain bound to immobilized antibody. Alternatively, all protein can be eluted with SDS (Laemmli) sample buffer.

23. Rinse the beads with 1 ml PBS/1% Triton X-100, then with 1 ml PBS alone with centrifugation 10 sec at 300 × *g*, room temperature, after each rinse.

24. Add Laemmli SDS-PAGE sample buffer without reducing agents to the beads. Vortex the beads five times and collect SDS eluate that contains GLUT4.

    For convenience sake, use the same volume of Laemmli sample buffer as the volume of the starting material and postadsorptive supernatant. Do not add reductants such as β-mercaptoethanol or DTT to Laemmli sample buffer in order to avoid dissociation of the immobilized antibody and elution of the heavy and light antibody chains from the beads. The IgG heavy chain migrates in SDS-PAGE immediately above GLUT4, and if immunoblotting is performed with other mouse antibodies, they will interfere with it.

Analyze the fractions

25. Analyze the starting material, postadsorptive supernatant, Triton eluate, and SDS eluate by SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*) with 1F8 antibody.

    If volumes of all these fractions are similar, the distribution of GLUT4 between the fractions will immediately show the efficiency of immunoadsorption (see Fig. 3.20.2B).

ALTERNATE PROTOCOL 1

IMMUNOISOLATION OF GLUT4 VESICLES FROM CULTURED ADIPOSE CELLS

Cultured adipocytes are similar to primary cells in that they are highly insulin responsive and can be relatively easily fractionated for biochemical analysis of GSVs. Moreover, they can be more easily manipulated genetically (e.g. by transfection) than primary cells, and this potentially allows new avenues for understanding GSV composition and behavior. One disadvantage is the large number of cell culture dishes needed to obtain the cultured cells compared to the few animals needed to obtain primary cells. In addition, the GLUT4 content of cultured adipocytes is significantly less than in primary cells.

Additional Materials *(also see Basic Protocol)*

3T3-L1 preadipocytes (ATTC CL-173; Green and Kehinde, 1976) or other adipocyte cell lines (Gross et al., 2004)
DMEM supplemented with 10% (v/v) bovine calf serum (DMEM-10)
Differentiation medium (see recipe)
Maintenance medium (see recipe)
1 mg/ml protein A–purified mouse monoclonal anti-GLUT4 antibody (1F8) in 0.1 M sodium borate, pH 8.6
1 mg/ml protein A–purified nonspecific mouse IgG in 0.1 M sodium borate, pH 8.6
Reacti-Gel (GF-2000) beads (Pierce)
PBS with inhibitors (see recipe)
Cell scraper
Ball-bearing homogenizer (Isobiotec; Heidelberg, Germany) with a 12-μm clearance.
Tube rotator
Additional reagents and equipment for tissue culture (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media, e.g., DMEM, require higher levels of CO_2 to maintain pH 7.4.

1. Grow 3T3-L1 pre-adipocytes (Green and Kehinde, 1976) in DMEM-10 until confluent.
2. Wait 48 hr after confluence and replace the medium with the differentiation medium. Continue incubation.
3. After 48 hr, replace the differentiation medium with the maintenance medium and change it every 48 hr. Use differentiated cells after 5 to 8 days of differentiation.
4. Using a cell scraper, harvest differentiated cells in PBS with inhibitors (0.8 to 1 ml per 10-cm dish).

    Up to four 10-cm plates may be harvested and combined in 1 ml.

5. Homogenize the cells with 10 strokes using a ball-bearing homogenizer with a 12-μm clearance. Rinse the homogenizer with 0.2 to 0.4 ml PBS with inhibitors and combine this fraction with the homogenate.

   The Potter-Elvehjam tissue grinder described in the Basic Protocol is also suitable for this purpose.

6. Isolate GLUT4 as in Basic Protocol beginning with step 6.

IMMUNOISOLATION OF GLUT4 VESICLES FROM RAT SKELETAL MUSCLE

ALTERNATE PROTOCOL 2

The major source of GLUT4 in animals is skeletal muscle where >80% of glucose is transported after a meal. Therefore, it is quantitatively the most important target tissue for insulin action, but it has been relatively less studied with regard to its GSV content. This is largely due to the lack of well defined and accepted muscle-fractionation protocols similar to those extant for adipocytes for over 20 years. The procedures described in this protocol (from Kandror et al. 1995) result in the isolation of highly enriched GSVs for comparison to those from adipocytes and possible identification of muscle-specific GSV components.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male Sprague-Dawley rats, 150 to 175 g
PBS with inhibitors (see recipe)
1 mg/ml protein A–purified anti-GLUT4 antibody (e.g., R & D Systems 1F8) in 0.1 M sodium borate, pH 8.6
1 mg/ml protein A–purified non-specific mouse IgG in 0.1 M sodium borate, pH 8.6
Reacti-Gel (GF-2000) beads (Pierce)
10% to 30% (w/w) linear sucrose gradients
Chamber with CO_2 or other equipment for IACUC-approved protocol for sacrificing rats
Sharp scissors
Polytron homogenizer, 0.5 in. generator
50-ml centrifuge tubes
High-speed centrifuge (e.g., Sorvall RC-5C)
Ultracentrifuge with SW-55Ti rotor and 5-ml tubes, or comparable
Peristaltic pump
1.5-ml collection tubes

Dissect and process muscles

1. Sacrifice two rats using a chamber with CO_2 or another IACUC-approved protocol.
2. Remove gastrocnemius and/or soleus muscles from rat hindlimb.
3. Trim muscles of connective tissue, fat, and nerves, pool them in a 50-ml centrifuge tube, mince with scissors into small pieces, and rinse three times with PBS with inhibitors.

Homogenize muscle tissue

4. Homogenize in 10 ml PBS with inhibitors on ice three times (10-sec each) using a Polytron homogenizer set at 13,500 rpm.
5. Remove nonhomogenized tissue by centrifuging 10 min at 2000 × *g*, 4°C, and collect the supernatant.

Fractionate the homogenate

6. Centrifuge supernatant 20 min at 9000 × *g*, 4°C. Collect supernatant in a fresh 50-ml tube.

   The pellet of this second centrifugation step contains muscle fibers, cell nuclei, mitochondria, and large fragments of sarcolemma.

7. Resuspend the pellet in 1 ml PBS with inhibitors and store up to 7 days at 4°C for further analysis.

8. Centrifuge the second supernatant 90 min at 180,000 × *g*, 4°C. Solubilize the pellet in 0.25 ml PBS with inhibitors using a flattened yellow pipet tip.

   This is an important step. Try to resuspend the pellet as thoroughly as possible with at least 60 strokes of the pipet tip. The resulting solution should be completely homogeneous.

9. Load solubilized pellet (3 to 4 mg of protein in a total volume of 0.2 ml) on a 5-ml 10% to 30% (w/w) linear sucrose gradient and centrifuge 55 min at 48,000 rpm (250,000 × *g*) in a Beckman SW-55Ti rotor.

   Because skeletal muscle is not fractionated as readily as adipocytes by differential centrifugation, sucrose gradient centrifugation is required in order to separate GSVs from other GLUT4-containing intracellular membranes.

Identify GLUT4-containing fractions

10. Separate each gradient into ~25 fractions using a peristaltic pump. Collect the pellet formed during gradient centrifugation.

    This pellet contains most of the plasmalemma and T-tubules.

11. Analyze the 9000 × *g* pellet from step 6 and gradient fractions and pellets by SDS-PAGE (10% gel; *UNIT 6.1*) and immunoblotting (*UNIT 6.2*) with 1F8 antibody.

    GLUT4 is a 55 kDa protein.

12. Combine GLUT4-containing fractions and use this material for immunoadsorption beginning with step 7 of the Basic Protocol.

ALTERNATE PROTOCOL 3

IMMUNOISOLATION OF GLUT4-CONTAINING VESICLES USING MAGNETIC BEADS

The use of antibody-coupled magnetic beads rather than acrylic beads may offer some advantages in obtaining vesicles of higher purity and/or ease of manipulation.

Additional Materials *(also see Basic Protocol)*

Dynabead M-280-sheep anti-mouse IgG (Dynal Biotech)

Magnetic bead separator (Dynal Biotech)
Tube rotator

1. Incubate protein A–purified 1F8 antibody and nonspecific mouse IgG (2 μg each), in separate reactions, with 30 μl Dynabeads M-280 sheep anti-Mouse IgG according to manufacturer's instructions.

2. Prepare the cell extract for immunoadsorption (Basic Protocol, steps 4 to 14).

3. Block the beads by incubating with PBS/1% BSA for 30 min with rotation at 4°C as in the Basic Protocol, steps 15 and 16.

4. Wash the beads three times with PBS, centrifuging 10 sec at 300 × *g*, room temperature.

5. Use 800 μg of the cell extract per 30 μl of either specific or nonspecific beads. Rotate immunobeads with the starting material overnight at 4°C.

6. Isolate GLUT4-containing vesicles as in Basic Protocol, steps 14 to 24 *except* use a magnetic holder to separate the beads form the liquid phase during the wash steps.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Differentiation medium

DMEM containing:
10% (v/v) fetal bovine serum
0.5 mM 3-isobutyl-1-methylxanthine
1 μM dexamethasone
1.7 μM insulin
Prepare on day of use

KRH buffer

12.5 mM HEPES
120 mM NaCl
6 mM KCl
1.2 mM $MgSO_4$
1 mM $CaCl_2$
0.6 mM Na_2HPO_4
0.4 mM NaH_2PO_4
2.5 mM D-glucose
2% (w/v) bovine serum albumin

Add the glucose and bovine serum albumin on the day of use, and adjust to pH 7.4 with HCl

Maintenance medium

DMEM containing:
10% (v/v) fetal bovine serum
Prepare on day of use

PBS with inhibitors

PBS (phosphate-buffered saline; APPENDIX 2A) containing:
1 μM aprotinin (American Bioanalytics)
2 μM leupeptin (American Bioanalytics)
1 μM pepstatin (American Bioanalytics)
5 mM benzamidine (Sigma)
1 mM PMSF (phenylmethylsulfonyl fluoride; Sigma; 100× in ethanol stock or see *APPENDIX 2A*)
10 mM Na_3VO_4 (Sigma)
50 mM NaF
1 mM β-glycerophosphate
1 mM sodium pyrophosphate
Add inhibitors on day of use

COMMENTARY

Background Information

In adipose and skeletal muscle cells, insulin, within minutes, dramatically increases glucose uptake. This effect of insulin is caused by translocation of the glucose transporter isoform 4 (GLUT4) from the intracellular storage pool to the plasma membrane. In basal adipocytes ~75% of the total GLUT4 pool is accumulated in small (<100 nm in diameter) 60S to 80S membrane vesicles (GLUT4 storage vesicles, or GSVs) with the rest of the transporter being present in ubiquitous intracellular transport vesicles and large (120 to 500 nm) rapidly sedimenting intracellular membranes that are likely to represent endosomes and, possibly, TGN (*trans*-Golgi network) structures (Slot et al., 1991; Ploug et al., 1998; Shewan et al., 2003). Upon insulin administration, GSVs rapidly fuse with the plasma membrane thus increasing the amount of GLUT4 molecule at the cell surface (Bryant et al., 2002) and allowing enhanced glucose uptake. The precise mechanism of insulin-dependent translocation of GSVs is currently under intense investigation in numerous laboratories around the world.

In addition to GLUT4 itself, several integral membrane proteins have been found in the GSVs in adipose cells. The most abundant of these is the insulin-regulated aminopeptidase (IRAP; Kandror et al., 1994; Keller et al., 1995), which is actually more abundant than GLUT4 (Kupriyanova et al., 2002) and serves as a surrogate marker for GSVs (Gross et al., 2004). Why this protein is such a major component of GSVs remains unclear. Other cargo proteins found in GSVs include receptors for transferrin (Kandror and Pilch, 1998) and IGFII (insulin-like growth factor)/ mannose 6-phosphate (Kandror and Pilch, 1996), sortilin (a sorting receptor; Lin et al., 1997; Morris et al., 1998), the tetraspanners called secretory compartment-associated membrane proteins (SCAMPs; Laurie et al., 1993; Thoidis et al., 1993), vesicle-associated membrane proteins VAMP2 (Cain et al., 1992), and VAMP3 (Volchuk et al., 1995). VAMP2 appears to be the v-snare (vesicular soluble-NSF-attaching protein receptor) required for fusion of the GSVs with the plasma membrane (Cheatham et al., 1996), and sortilin functions in GSV formation (Shi and Kandror, 2005). The exact biochemical/physiological role of the rest of the GSV proteins in the context of GSVs is not so clear, although a teleological argument can be made that iron uptake via the transferrin receptor makes sense as a postprandial, insulin-dependent event.

Peripheral membrane proteins must also associate with the GSVs and play a role in vesicle formation and targeting. These may include vesicle tethers such as p115 (Hosaka et al., 2005), TUG (tether, containing the UBX domain, for GLUT4; Bogan et al., 2003) and others (Kao et al., 1999; Chi and Lodish, 2000), rab proteins (Cormont et al., 1991), adaptors (Gillingham et al., 1999; Li and Kandror, 2005), motors (Bose et al., 2002; Semiz et al., 2003) and probably others. The biological significance of these associations is also a topic of much current investigation.

Along with synaptic vesicles, GSVs from adipose cells represent a well-characterized and widely used cell biological model for the study of membrane compartmentalization, sorting, and trafficking.

Critical Parameters

Basic Protocol

Preparation of immunobeads is extremely important for the success of vesicle immunoisolation. For experiments on a small scale (0.5 to 2 mg of light microsomes plus cytosol), use Alternate Protocol 3 with magnetic beads. For the preparative isolation of GLUT4-vesicles, use covalently immobilized antibody (Basic Protocol). An important difference between the two protocols is that SDS will elute the primary antibody from magnetic beads. This result should be minimal when the antibody is covalently coupled to the beads, but some antibody leakage commonly occurs. When choosing the beads for coupling with the antibody, remember that acrylamide beads, such as Reacti-Gel GF-2000, tend to have less nonspecific adsorption, than agarose (Sepharose) beads. Do not use immunobeads that have less than 0.5 mg of antibody per ml, which may lead to unacceptable levels of nonspecific adsorption.

Thorough mincing of fat pads with sharp scissors prior to addition of collagenase (as well as the good quality of the enzyme itself) is absolutely required for the efficient collagenase digestion. In general, it is better to spend more time on the preparation of the tissue, than to prolong incubation with collagenase which can be damaging to cells. Too vigorous shaking and/or prolonged collagenase treatment may trigger translocation of the intracellular GLUT4-vesicles to the plasma

membrane, thus complicating data analysis. Be sure to reequilibrate the cells for 45 min after collagenase digestion to avoid this artifactual translocation.

Alternate Protocol 1

When working with cultured adipocytes, make sure that the cells are well differentiated. Keep in mind that different lines of 3T3-L1 cells may express different amounts of GLUT4 and differentiate at various rates. A good cell line is a key to success, along with using cells passaged <12 times. In preliminary experiments, make sure that cells express sufficient amounts of GLUT4 and increase their glucose uptake at least 5- to 6-fold in response to insulin stimulation (day 6 to 10 after initiation of differentiation).

Alternate Protocol 2

As is the case with adipocytes, tissue preparation and homogenization are very important steps for isolation of GSVs from skeletal muscle. Usually, the results of sucrose-gradient centrifugation show whether or not homogenization has gone well and the experiment may be continued. GLUT4-vesicles should form a distinct peak in the middle of the gradient and be well separated from the bulk of protein and heavy GLUT4-containing membranes in the gradient pellet (Kandror et al., 1995).

Troubleshooting

Insufficient yield of GLUT4

The most common reason for the problem of insufficient yield of GLUT4 is a low ratio between 1F8 antibody and the amount of GLUT4 in the starting material. Try to use more immunobeads, keeping in mind, however, that an increase in the bead volume is likely to increase nonspecific adsorption as well.

High level of nonspecific adsorption

To avoid a high level of nonspecific adsorption, increase stringency of the washing step by elevating the ionic strength of washing buffer to 200 to 250 mM NaCl and/or by performing two to three additional washes.

Most likely, not all of the proteins isolated by immunoadsorption are components of GSVs. Immunoadsorption will isolate all GLUT4-containing membranes regardless of their biological origin. Thus, if intracellular membranes, such as endosomes, are fragmented during homogenization, these membrane fragments will also be isolated. Although artificial fragmentation of membranes does not appear to be a significant factor upon proper cell homogenization (Kupriyanova et al., 2002), this possibility should not be overlooked. In addition, a significant fraction of GLUT4 is localized in ubiquitous intracellular transport vesicles that do not demonstrate insulin responsiveness (Lee et al., 1999; Kupriyanova et al., 2002).

Thus, additional approaches, such as sucrose gradient centrifugation, are required in order to determine whether or not the protein of interest is indeed localized in the GSVs. Also, if a protein is localized in the GSVs, it should be translocated to the cell surface in response to insulin stimulation. This is routinely assessed by immunofluorescence staining (*UNIT 4.3*) and/or cell surface labeling (*UNITS 7.10 & 15.4*).

Anticipated Results

Immunoisolation procedures allow for isolation of 80% to 90% of GLUT4-vesicles, ~0.1% to 0.2% of the total protein content of adipocytes. One can then calculate what quantity of adipocytes will provide sufficient material for various studies, e.g., proteomic analysis, although small-scale analytical experiments are always a good idea to start with, and scale-up seems to be a linear function of starting material and beads. For analytical-scale experiments, the protein yield is too low to bother measuring, as it will use up too much material. For example, for a scaled-up protocol for proteomic analysis, adipocytes from ten or more animals or more than ten 100-cm dishes of cultured cells will yield 50 to 100 μg of immunoadsorbed membrane protein. The amount of GLUT4 in the eluate from nonspecific beads (Triton eluate + SDS eluate) should be <5% of its amount in the starting material. Because some of the membrane material may be lost during washing steps, the protein balance between the starting material, postadsorptive supernatant, and eluates may not always be complete. Usually, recovery of 80% to 90% of any individual protein is within acceptable range.

IRAP (see Background Information) represents a good alternative marker for GSVs, and the yield of IRAP is usually equal to the yield of GLUT4. Note that IRAP and other vesicular proteins are present in the Triton eluate from immunobeads (Fig. 3.20.2). Thus, one can test for the presence of candidate proteins in GSVs by immunoblotting, using either GLUT4 or IRAP as monitors for the specificity and yield of the protocol.

When analyzing the purity of the immunoisolated vesicles, keep in mind that immunoblotting is more specific than silver staining. In other words, it is quite common to have a good and specific yield of GLUT4, IRAP, and other vesicular proteins by immunoblotting and to see relatively small differences in the total protein composition between 1F8- and IgG-bound material when analyzed by protein staining (see Troubleshooting section). The use of sufficient starting material for immunoadsorption, e.g., as in Figure 3.20.2, will minimize this problem.

Time Considerations

The whole procedure from obtaining the rats to the isolation of the light microsomes plus cytosol fraction will take one person ~4 to 6 hr, depending on the nature of the experiment. The addition of the albumin and glucose to the KRH should be done on the day of the experiment and the pH adjusted. Then, ~3 hr are required for preparation, digestion, and resting of the adipocytes, 1 hr or more for experimental manipulation, if needed (addition of insulin, inhibitors, etc.), and 45 to 60 min. for centrifugation and fractionation. It is convenient, therefore, to carry out immunoadsorption overnight at the end of the day and to run the gel next morning; however, the time for immunoadsorption can be reduced to 5 to 6 hr without noticeable changes in yield, and therefore, the whole procedure of vesicle immunoisolation can be achieved in one day.

For preparations from skeletal muscle, homogenize the tissue and perform gradient centrifugation on the first day and run the gel with gradient fractions overnight. Carry out immunoblotting during the second day and perform immunoadsorption overnight. The results of immunoadsorption should be available by the end of day 3. Alternatively, gradient fractions may be kept at 4°C (do not freeze them!) for 2 to 3 days.

Literature Cited

Bogan, J.S., Hendon, N., McKee, A.E., Tsao, T.S., and Lodish, H.F. 2003. Functional cloning of TUG as a regulator of GLUT4 glucose transporter trafficking. *Nature* 425:727-733.

Bose, A., Guilherme, A., Robida, S.I., Nicoloro, S.M., Zhou, Q.L., Jiang, Z.Y., Pomerleau, D.P., and Czech, M.P. 2002. Glucose transporter recycling in response to insulin is facilitated by myosin Myo1c. *Nature* 420:821-824.

Bryant, N.J., Govers, R., and James, D.E. 2002. Regulated transport of the glucose transporter glut4. *Nat. Rev. Mol. Cell Biol.* 3:267-277.

Cain, C.C., Trimble, W.S., and Lienhard, G.E. 1992. Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes. *J. Biol. Chem.* 267:11681-11684.

Cheatham, B., Volchuk, A., Kahn, C.R., Wang, L., Rhodes, C.J., and Klip, A. 1996. Insulin-stimulated translocation of Glut4 glucose transporters requires SNARE-complex proteins. *Proc. Natl. Acad. Sci. U.S.A.* 93:15169-15173.

Chi, N.W. and Lodish, H.F. 2000. Tankyrase is a golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles. *J. Biol. Chem.* 275:38437-38444.

Cormont, M., Tanti, J.F., Gremeaux, T., Obberghen, E.V., and Marchand-Brustel, Y.L. 1991. Subcellular distribution of low molecular weight guanosine triphosphate-binding proteins in adipocytes: colocalization with the glucose transporter Glut4. *Endocrinology* 129:3343-3350.

Gillingham, A.K., Koumanov, K., Pryor, P.R., Reaves, B.J., and Holman, G.D. 1999. Association of AP1 adaptor complexes with GLUT4 vesicles. *J. Cell Sci.* 112:4793-4800.

Green, H. and Kehinde, O. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* 7:105-113.

Gross, D.N., Farmer, S.R., and Pilch, P.F. 2004. Glut4 storage vesicles without Glut4: Transcriptional regulation of insulin-dependent vesicular traffic. *Mol. Cell Biol.* 24:7151-7162.

Hosaka, T., Brooks, C.C., Presman, E., Kim, S.K., Zhang, Z., Breen, M., Sztul, E., and Pilch, P.F. 2005. p115 interacts with the GLUT4 vesicle protein, IRAP, and plays a critical role in insulin-stimulated GLUT4 translocation. *Mol. Biol. Cell* 16:2882-2890.

James, D.E., Brown, R., Navarro, J., and Pilch, P.F. 1988. Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. *Nature* 333:183-185.

Kandror, K.V. and Pilch, P.F. 1996. The insulin-like growth factor II/mannose 6-phosphate receptor utilizes the same membrane compartments as Glut4 for insulin-dependent trafficking to and from the rat adipocyte cell surface. *J. Biol. Chem.* 271:21703-21708.

Kandror, K.V. and Pilch, P.F. 1998. Multiple endosomal recycling pathways in rat adipose cells. *Biochem. J.* 331:829-835.

Kandror, K.V., Yu, L., and Pilch, P.F. 1994. The major protein of GLUT4-containing vesicles, gp160, has aminopeptidase activity. *J. Biol. Chem.* 269:30777-30780.

Kandror, K.V., Coderre, L., Pushkin, A.S., and Pilch, P.F. 1995. Comparison of glucose transporter-containing vesicles from rat fat and muscle tissues: Evidence for a unique endosomal compartment. *Biochem. J.* 307:383-390.

Kao, A.W., Noda, Y., Johnson, J.H., Pessin, J.E., and Saltiel, A.R. 1999. Aldolase mediates the association of F-actin with the

insulin-responsive glucose transporter Glut4. *J. Biol. Chem.* 274:17742-17747.

Karylowski, O., Zeigerer, A., Cohen, A., and McGraw, T.E. 2004. GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. *Mol. Biol. Cell* 15:870-882.

Keller, S.R., Scott, H.M., Mastick, C.C., Aebersold, R., and Lienhard, G.E. 1995. Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from GLUT4 vesicles. *J. Biol. Chem.* 270:23612-23618.

Kupriyanova, T.A., Kandror, V., and Kandror, K.V. 2002. Isolation and characterization of the two major intracellular Glut4 storage compartments. *J. Biol. Chem.* 277:9133-9138.

Larance, M., Ramm, G., Stockli, J., van Dam, E.M., Winata, S., Wasinger, V., Simpson, F., Graham, M., Junutula, J.R., Guilhaus, M., and James, D.E. 2005. Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. *J. Biol. Chem.* 280:37803-37813.

Laurie, S.M., Cain, C.C., Lienhard, G.E., and Castle, J.D. 1993. The glucose transporter GLUT4 and secretory carrier membrane proteins (SCAMPs) colocolize in rat adipocytes and partially segregate during insulin stimulation. *J. Biol. Chem.* 268:19110-19117.

Lee, W., Ryu, J., Souto, R.P., Pilch, P.F., and Jung, C.Y. 1999. Separation and partial characterization of three distinct intracellular Glut4 compartments in rat adipocytes. Subcellular fractionation without homogenization. *J. Biol. Chem.* 274:37755-37762.

Li, L.V. and Kandror, K.V. 2005. Golgi-localized, gamma-ear-containing, Arf-binding protein adaptors mediate insulin-responsive trafficking of glucose transporter 4 in 3T3-L1 adipocytes. *Mol. Endocrinol.* 19:2145-2153.

Lin, B.Z., Pilch, P.F., and Kandror, K.V. 1997. Sortilin is a major protein component of Glut4-containing vesicles. *J. Biol. Chem.* 272:24145-24147.

Morris, N.J., Ross, S.A., Lane, W.S., Moestrup, S.K., Petersen, C.M., Keller, S.R., and Lienhard, G.E. 1998. Sortilin is the major 110-kDa protein in GLUT4 vesicles from adipocytes. *J. Biol. Chem.* 273:3582-3587.

Ploug, T., vanDeurs, B., Ai, H., Cushman, S.W., and Ralston, E. 1998. Analysis of Glut4 distribution in whole skeletal muscle fibers: Identification of distinct storage compartments that are recruited by insulin and muscle contractions. *J. Cell Biol.* 142:1429-1446.

Semiz, S., Park, J.G., Nicoloro, S.M., Furcinitti, P., Zhang, C., Chawla, A., Leszyk, J., and Czech, M.P. 2003. Conventional kinesin KIF5B mediates insulin-stimulated GLUT4 movements on microtubules. *EMBO J.* 22:2387-2399.

Shewan, A.M., van Dam, E.M., Martin, S., Luen, T.B., Hong, W., Bryant, N.J., and James, D.E. 2003. GLUT4 recycles via a trans-Golgi network (TGN) subdomain enriched in syntaxins 6 and 16 but not TGN38: Involvement of an acidic targeting motif. *Mol. Biol. Cell* 14:973-986.

Shi, J. and Kandror, K.V. 2005. Sortilin is essential and sufficient for the formation of Glut4-storage vesicles in 3T3-L1 adipocytes. *Dev. Cell* 9:99-108.

Simpson, I.A., Yver, D.R., Hissin, P.J., Wardzala, L.J., Karnieli, E., Salans, L.B., and Cushman, S.W. 1983. Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: Characterization of subcellular fractions. *Biochim. Biophys. Acta.* 763:393-407.

Slot, S.W., Geuze, H.J., Gigengack, S., Lienhard, G.E., and James, D.E. 1991. Immunolocalization of the insulin-regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* 113:123-135.

Thoidis, G., Kotliar, N., and Pilch, P.F. 1993. Immunological analysis of GLUT4-enriched vesicles. Identification of novel proteins regulated by insulin and diabetes. *J. Biol. Chem.* 268:11691-11696.

Volchuk, A., Sargeant, R., Sumitani, S., Liu, Z. He, L., and A. Klip, A. 1995. Cellubrevin is a resident protein of insulin-sensitive GLUT4 glucose transporter vesicles in 3T3-L1 adipocytes. *J. Biol. Chem.* 270:8233-8240.

Contributed by Konstantin V. Kandror and Paul F. Pilch
Boston University School of Medicine
Boston, Massachusetts

Isolation of Intestinal Brush-Border Membranes

This unit describes how to prepare isolated brush-border membrane vesicles from the small intestine of rat, pig, and cow. The procedure is based upon cation precipitation which causes intracellular organelles to coalesce and takes ~3 hr to complete (Hopfer et al., 1973). Membranes isolated in this way can be used for the identification of integral membrane proteins found at the apical pole of the intestinal epithelium or for transport assays because the microvilli spontaneously form sealed membrane vesicles. The technique does not require much specialized equipment apart from a high-speed centrifuge and a Polytron homogenizer. The enrichment of apical membrane proteins is about twenty-fold and will be a product primarily of the enterocyte (absorptive cell) population which makes up ~80% of the cells. There will also be a small contribution from endocrine and mucus-secreting cells.

The Basic Protocol describes isolation of brush-border membranes from rat, while Alternate Protocols 1 and 2 describe isolation of the same material from pig and cow intestines, respectively. A Support Protocol describes methods for assessing the enrichment of the membrane fraction.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Maintain all solutions at 4°C during preparations. Increased stability of rat membranes has been reported by including phospholipase inhibitors in the initial homogenization medium (Maenz et al., 1991), and the inhibitors should also be used in the initial homogenization of pig and cow membranes.

ISOLATION OF INTESTINAL BRUSH-BORDER MEMBRANES FROM RAT

BASIC PROTOCOL

This protocol provides the procedure for taking mucosal scrapings from the small intestine and isolating the apical (brush-border) membranes (see Fig. 3.21.1) from the epithelial cells with minimal contamination of intracellular or basolateral membranes.

Materials

Anesthetic (e.g., sodium pentobarbital; MTC Pharmaceuticals, Ontario, Canada)
Rats (e.g., male Sprague-Dawley rats, ~300 g)
Isotonic saline (0.9% w/v NaCl) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), ice-cold
Water, ice cold
Rat collection solution A (see recipe)
Formaldehyde
100 mM $MgCl_2$
Rat homogenization solution B (see recipe)
Final suspension medium (see recipe)

1-ml syringe with 26-G needle
Surgical instruments for laproscopic surgery: scissors and forceps
30 × 20–cm Plexiglas or other flat plate, chilled on ice
Glass microscope slides, chilled on ice

Contributed by Chris I Cheeseman and Debbie O'Neill
Current Protocols in Cell Biology (2006) 3.21.1-3.21.10

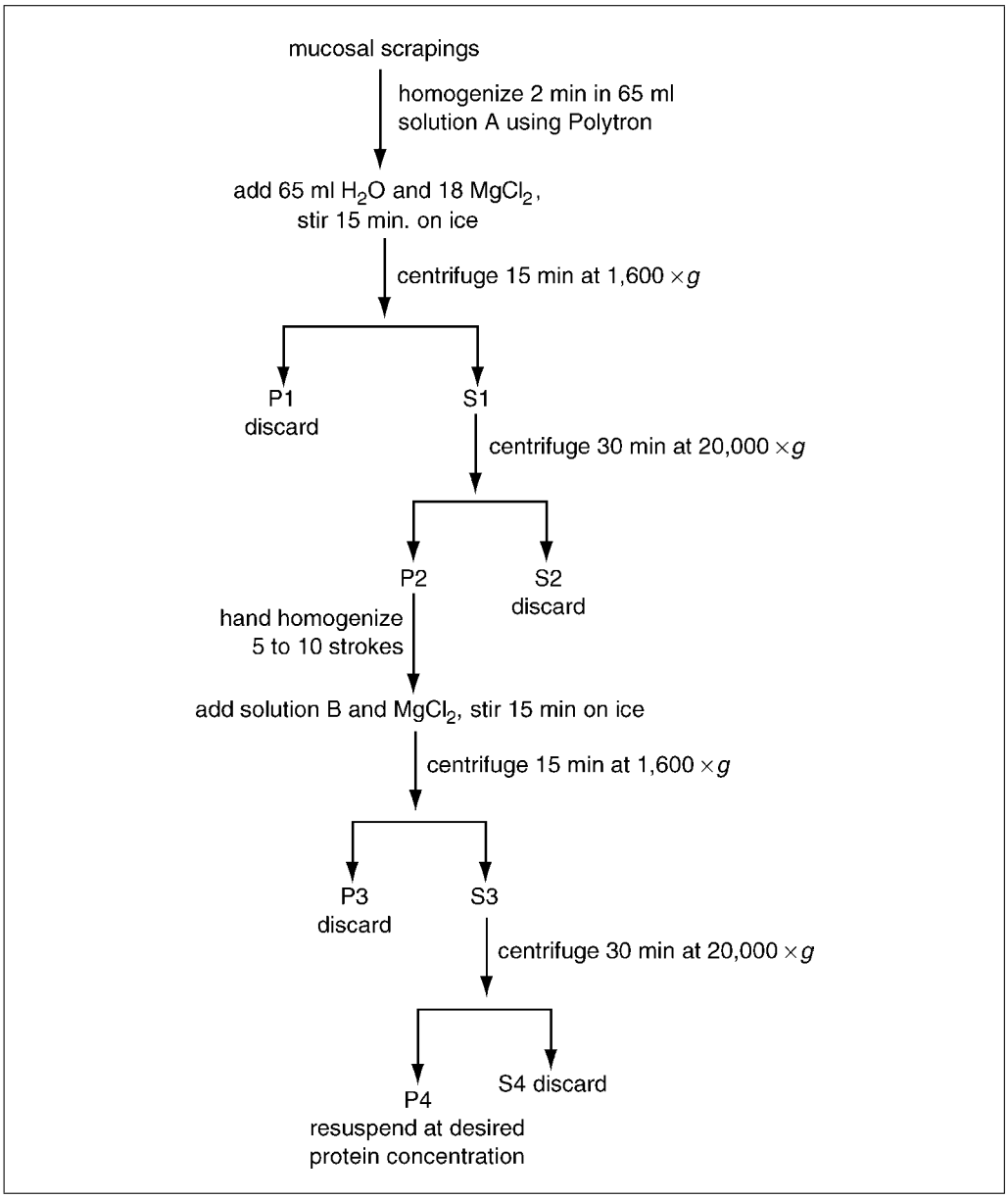


Figure 3.21.1 Flow chart for procedures to isolate rat intestinal brush-border membranes. Pig and bovine preparations differ in the details (see text).

100- and 250- ml beakers
Polytron homogenizer (Brinkmann) or equivalent
Magnetic stirrer and stir bar
50-ml polycarbonate centrifuge tube
High-speed centrifuge capable of generating 20,000 × *g* and handling samples up to 50 ml
15-ml hand held glass homogenizer
Additional materials for determination of protein concentration (*APPENDIX 3H*) and fixing, sectioning, and staining tissue for light microscopy (optional)

Prepare rat intestinal mucosa

1. Anesthetize each rat using sodium pentobarbital, 45 mg/kg body weight, for intraperitoneal injection with a 1-ml syringe with 26-G needle.

   The strain, weight, and sex of the rats will vary, depending on the objectives of a particular study.

The authors use Somnotol (sodium pentobarbital), but the choice of anesthesia will depend upon the jurisdiction and local animal regulations.

If the animals are fasted overnight there will be less lumenal content and the remaining material can be removed more easily. However, it must be remembered that fasting may change the expression of surface enzymes and integral membrane proteins.

2. Expose the small intestine by laproscopic surgery, allowing for blood flow to be maintained.

   Rat intestine is very sensitive to anoxia, and membranes are best prepared from tissue that has the blood flow interrupted as late as possible.

3. Identify the region of interest (small intestine) and make small incisions at both ends of the required section (normally no longer than 30 cm).

   The best way to remove the epithelium from the underlying tissues (muscle, neuronal, and connective tissue) is to take mucosal scrapings (see below).

4. Flush the lumen gently, in situ, with ~15 to 30 ml ice-cold isotonic saline containing 0.1 mM PMSF.

   It is necessary to cool the tissue in this way while maintaining the blood flow in order to reduce stress and avoid possible reinternalization of membrane proteins. It is essential to have the protease inhibitor present at this initial step or the resulting isolated membranes will degrade very rapidly. It is also essential that cooling proceed rapidly and that no delays occur once this procedure has started.

5. Remove the section of intestine from the animal and place it on an ice-cold plate.

   A piece of Plexiglas, sitting on ice, will serve this purpose very well.

   The rat is killed, after the dissection with an anesthetic overdose.

6. Open up the intestine along its antimesenteric border, i.e., along the surface opposite to the blood and nerve supply.

7. Lay this sheet of tissue flat, but avoid touching the exposed mucosal surface.

Collect and homogenize mucosal cells

8. Scrape off the mucosa using an ice-cold glass slide held at a 45° angle.

   This is best done using short strokes.

9. Shake the collected cells into a 100-ml beaker containing 65 ml of collection solution A. Proceed along the length of the sheet of intestine.

   This procedure takes practice to achieve the degree of harvesting required. A light pressure and a few strokes will result in harvesting only the villus tips. More pressure and a greater number of strokes will remove cells from further down the villi towards the crypts.

10. Determine the extent of harvesting the epithelium by fixing the remaining tissue overnight with formaldehyde, sectioning (with basic paraffin sectioning technique), staining with hematoxylin and eosin, and examining by conventional light microscopy.

    Microscopic examination need not be performed for every preparation but is useful when establishing the technique. Only the villi should be harvested without cells being taken from the crypts.

11. Homogenize the cell suspension from step 9 using a Polytron homogenizer 2 min at setting 5, on ice.

    For enrichment assays (see Support Protocol) hold back a 1-ml sample of this initial homogenate.

Precipitate intracellular and basolateral membranes

12. Add 65 ml ice-cold double distilled water and 18 ml of 100 mM $MgCl_2$ to the homogenate. Keep this solution on ice for 15 min and gently stir continuously using a stir bar and a magnetic stirrer.

    The magnesium will cause a large proportion of the intracellular and basolateral membranes to aggregate.

13. Pellet these membranes by centrifuging the solution for 15 min at 1600 × *g*, 4°C.

14. Collect the supernatant (S1 in Fig. 3.21.1) in a 50-ml centrifuge tube. Discard the pellet (P1). Centrifuge S1 30 min at 20,000 × *g*, 4°C. Discard the resulting supernatant (S2).

15. Homogenize the pellet (P2) with a hand-held 15-ml glass homogenizer in 2 to 3 ml homogenization solution B, using 10 strokes.

16. Add up to 30 ml homogenization solution B and repeat the magnesium precipitation step by adding 4 ml of 100 mM $MgCl_2$ to the suspension and stirring on ice for another 15 min.

17. Pellet the aggregated membranes by centrifuging 15 min at 1,600 × *g*, 4°C. Collect the supernatant (S3) and discard the pellet (P3).

Isolate brush-border membranes

18. Pellet the brush-border membranes in S3 by centrifuging this supernatant 30 min at 20,000 × *g*, 4°C.

19. Finally, resuspend the resulting pellet, P4, in the required final resuspension medium and determine the protein concentration using standard protocols (see *APPENDIX 3H*).

    These isolated brush-border membranes spontaneously form vesicles with a trapped volume normally of about 1.5 to 2.0 μl per mg protein.

    If these vesicles are to be used for rapid filtration flux studies a maximum protein concentration of 8 mg/ml is recommended or the filters clog, lengthening filtration time and making flux assays inaccurate.

20. Proceed to the Support Protocol to analyze membrane enrichment.

    Membranes can now used for enzyme assays, flux studies with rapid filtration, or for protein identification with (western) immunoblotting.

SUPPORT PROTOCOL

ASSESSMENT OF MEMBRANE ENRICHMENT

There are several methods which can be used to determine the degree of enrichment of the resulting membrane preparation, most of which rely on enzyme assays. Sucrase-isomaltase and alkaline phosphatase are both enzymes associated with the intestinal brush-border membrane. Assays for these enzymes can be used to determine the level of enrichment of the isolated membranes (Fredricksen and Wirsen, 1956; Tsuboi et al., 1985). Normally, the degree of enrichment is about fifteen- to twenty-fold compared to the original homogenate.

ALTERNATE PROTOCOL 1

ISOLATION OF INTESTINAL BRUSH-BORDER MEMBRANES FROM PIG

This is a modification of the procedure employed for rat tissue. It requires slightly different solutions and takes account of the greater yield. Pig BBM vesicles are ideal for use in rapid filtration uptake assays because they are very robust and give very reproducible data.

Additional Materials *(also see Basic Protocol)*

Pig intestine (freshly isolated from slaughter or animal house)
Pig and cow collection solution A, ice cold
Pig and cow homogenization solution B

25-G needle
10-ml syringe
−70°C freezer

Isolate membranes

1. Flush the intestinal lumen of freshly isolated pig intestine pieces (30 cm long) with 60 to 70 ml ice-cold isotonic saline containing 0.1 mM PMSF.

   Intestine can be collected at the slaughter or animal house and must be chilled immediately. Ideally it should be sectioned and flushed, with mucosal scrapings taken on site and kept in ice-cold saline until they can be used. If mucosal scrapings cannot be prepared immediately, flush out the lumenal contents of the intestinal segments with ice-cold saline and keep the tissue on ice in the saline for no more than 2 hours.

   Note that the flow chart in Figure 3.21.1 does not fully match the isolation steps for pig or cow.

2. Open up the intestine along its antimesenteric border, i.e., along the surface opposite to the blood and nerve supply and lay it flat on an ice-cold plate or piece of Plexiglas.

Collect and homogenize mucosal cells

3. Take mucosal scrapings, as for rat tissue (Basic Protocol, step 8), using a microscope slide held at 45° angle.

4. Collect the scrapings in ice-cold pig and cow collection solution A, at a ratio of ∼20 ml solution for each gram of tissue.

5. Homogenize the suspension from step 4 with a Polytron homogenizer 90 sec at setting 5, on ice.

Remove intracellular and basolateral membranes

6. Centrifuge this suspension (in 50-ml tubes) 15 min at 1,600 × *g*, 4°C. Collect the supernatant (S1) and discard the pellet (P1).

7. Add the 100 mM $MgCl_2$ to S1 bringing the magnesium concentration to a final concentration of 10 mM and stir 15 to 20 min on ice.

8. Centrifuge the suspension 15 min at 1,600 × *g*, 4°C.

9. Collect the supernatant (S2) discarding the pellet (P2). Centrifuge S2 30 min at 20,000 × *g*, 4°C. Retain pellet (P3). Discard supernatant (S3).

10. To resuspend P3 effectively, add 1 ml of ice-cold pig and cow homogenization solution B and draw the solution back and forth 5 to 10 times through a 25-G needle on a 10-ml syringe.

    At this stage aliquots of this samples can be frozen up to 4 months at −70°C with little deterioration.

Isolate brush-border membranes

11. Dilute the 1 ml of P3 suspension to 35 ml with pig and cow homogenization solution B and centrifuge 30 min at 20,000 × *g*, 4°C. Retain P4 and discard S4.

12. Resuspend P4 in a small volume of homogenization solution B, measure the protein concentration (see *APPENDIX 3H*) and then dilute to the required protein concentration in final suspension medium.

    The desired concentration for rapid filtration assays is 6 to 8 mg/ml.

ISOLATION OF INTESTINAL BRUSH-BORDER MEMBRANES FROM COW

Clearly, the tissue cannot be flushed *in situ* as with smaller species but, tissue samples store well for subsequent membrane isolation.

Additional Materials *(also see Basic Protocol)*

Cow intestine
Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 0.1 mM PMSF and 0.1 mM aprotonin
Pig and cow collection solution A (see recipe)
Pig and cow homogenization solution B (see recipe)
Aluminum foil or plastic vials suitable for freezing
Liquid nitrogen or −70°C freezer
25-G needle
10-ml syringe

Collect intestinal tissue

1. Cut the intestine into 10-cm lengths. Flush pieces of intestine with 30 ml ice-cold PBS containing 0.1 mM PMSF and 0.1 mM aprotonin. Place in flask containing ice-cold flushing solution and transport rapidly to preparation site.

   Tissue will keep for a couple of hours on ice.

2. Blot the pieces and wrap in aluminum foil before dropping into liquid nitrogen for storage.

   Alternatively mucosal scrapings can be prepared as below and then placed in plastic vials for rapid freezing under liquid nitrogen. These will store well under liquid nitrogen for several weeks. If they are subsequently kept at −70°C they will only keep for 1 week.

Isolate membranes

3. Thaw samples on ice and collect mucosal scrapings as in Basic Protocol, step 8. Add 1 g of mucosal scrapings to 20 ml of pig and cow collection solution A.
4. Homogenize the material with a Polytron homogenizer 2 min at setting 5, on ice.
5. Then centrifuge the suspension for 15 min at 1,600 × *g*, 4°C. Retain supernatant (S1) and discard the pellet (P1).

Precipitate intracellular and basolateral membranes

6. Add stock 100 mM $MgCl_2$ to a final concentration of 10 mM $MgCl_2$. Stir the solution slowly on ice for 15 to 20 min.
7. Centrifuge 15 min at 1,600 × g, 4°C. Collect S2 and discard P2.
8. Centrifuge S2 30 min at 20,000 × *g*, 4°C, and retain P3 while discarding S3.

Homogenize brush-border membranes

9. Wash P3 with 1 ml ice-cold pig and cow homogenization solution B and mix by drawing back and forth through a 25-G needle on a 10-ml syringe five to ten times.

   At this stage aliquots of this sample can be frozen up to 4 months at −70°C with little deterioration.

Isolate brush-border vesicles

10. Dilute the 1 ml of P3 suspension to 35 ml in pig and cow homogenization solution B and centrifuge 30 min at 20,000 × g, 4°C. Retain P4 and discard S4.

11. Resuspend P4 in a small volume of pig and cow homogenization solution B, measure the protein concentration (see *APPENDIX 3H*) and then dilute to the required protein concentration using final suspension medium.

For rapid filtration assays, 6 to 8 mg protein/ml is the desired concentration for rapid filtration assays.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Final suspension medium

Using solution C (see recipe), add components, including protease inhibitors, buffers at the appropriate pH, and chemicals to provide the ionic composition that will be required for the experiment to be performed. Store up to 1 week at 4°C.

The composition of this solution will determine what the vesicles contain because this is the point at which they seal and equilibrate. Mannitol is often used as the osmolyte so that the effects of different extravesicular cations can be tested on transport processes; however, the disadvantage of using the sugar is that the ionic strength is not maintained across the membrane.

Pig and cow collection solution A

For 500 ml: 50 mM mannitol
2 mM Tris base, pH 7.4
Store up to 1 month at 4°C
Add 200 µl of 250 mM PMSF just before use (0.1 mM. final)

Pig and cow homogenization solution B

30 ml 500 mM mannitol (300 mM final)
5 ml of 500 mM HEPES (50 mM final), adjusted to pH 7.4 with Tris base
H_2O to 50 ml
Store up to 1 month at 4°C

Rat collection solution A

300 mM mannitol
5 mM EGTA
12 mM Tris·Cl
Adjust to pH 7.4 with 5 M NaOH
0.1 mM PMSF (add just before use; see *APPENDIX 2A* for stock solution recipe)
Store up to 1 month at 4°C

Rat homogenization solution B

150 mM mannitol
2.5 mM EGTA
6 mM Tris·Cl
Adjust to pH 7.4 with 5 M NaOH
0.05 mM PMSF (added just before use)
Store up to 1 month at 4°C

Solution C

300 mM mannitol
5 mM Tris·Cl, pH 7.4
Store up to 1 month at 4°C

COMMENTARY

Background Information

Polarized epithelial cells, such as those of the small intestine, have two distinct membrane domains. The basolateral membrane (BLM) bounds the sides and basal surface of the epithelial cells while the apical surface is often referred to as the brush-border membrane (BBM). This name comes from the appearance of this surface as bristles of a brush under a low-power light microscope. These bristles are formed by the plasma membrane having numerous uniform projections called microvilli, which have a very complex cytoskeletal structure in their core (Fig. 3.21.2A and 3.21.2B). A key feature of polarized cells is their asymmetrical expression of membrane proteins which allows them to mediate vectorial transport of numerous solutes and ions. In the case of the small intestine, amino acid and hexose absorption is achieved by the expression in the BBM of proteins like SGLT1 (sodium-coupled glucose transporter) and GLUT5 (a facilitated fructose transporter). These proteins are not expressed in the BLM; instead we find GLUT2, a low-affinity glucose and fructose facilitated transporter, and sodium/potassium ATPase, which pumps sodium out of the cell into the blood stream. This differential expression then promotes the uptake of fructose and the coupled entry of glucose and sodium, followed by the exit of these hexoses via GLUT2 and the pumping out of sodium by the ATPase. The end result is the absorption of hexoses and sodium from the lumen into the blood stream.

The isolation of BBMs then allows one, using a variety of biochemical and molecular techniques, to determine the expression of proteins at the apical surface of the epithelial cells. The intestine is a very plastic tissue, varying the expression of numerous proteins in response to environmental conditions, e.g., starvation, alterations in diet, and some disease states like diabetes. In addition, there is evidence for regulatory processes in which transport proteins are rapidly inserted or removed from the BBM. Isolated BBMs permit quantification of the degree of changes in protein expression and also allow for measurement of the time course of expression or turnover. The major technique for determining transporter protein expression would be immunoblotting (*UNIT 6.2*) with densitometric scanning (*UNIT 6.3*) which allows for at least semiquantitative analysis. Confirmation of the identity of proteins expressed in the BBM can also be achieved by using mass spectrometry. In addition to the embedded membrane proteins, the intestine also expresses a number of surface enzymes which are anchored by a single membrane-spanning domain (Hauser and Semenza, 1983). Their activity can be assayed using isolated BBMs and a variety of substrates depending upon the enzyme to be assayed, e.g., sucrase-isomaltase (Tsuboi et al., 1985).

The second major use of these isolated BBMs is for transport studies. During the homogenization processes the individual microvilli are sheared off from the cells producing tubes of plasma membrane containing microtubules. The majority of these individual microvilli then seal at their base by the fusion of the lipid bilayer around the cytoskeleton. During the final centrifugation the suspension medium equilibrates with the intramicrovillus space giving vesicles containing a solution of known composition. The trapped volume of these isolated BBM vesicles is usually in the range of 1 to 2 μl per mg membrane protein. If these vesicles are then suspended in a medium containing a radiolabeled substrate of known concentration, incubated for a few seconds and then separated from the medium by filtration, it is possible to measure the functional activity of transport proteins expressed in the BBM.

Critical Parameters and Troubleshooting

This protocol is based upon the principle of divalent cation precipitation (calcium or magnesium) and can be used to isolate BBMs from a variety of different species (Hopfer et al., 1973). The initial description is for rat small intestine with later protocols indicating modifications for pig and cow. It must be recognized that the intestinal epithelium is heterogeneous, with ~20% of the cells being non-epithelial, including endocrine cells, mucus secreting goblet cells, and immunoreactive Paneth cells. Thus, any preparation will at best have a contribution of 80% from intestinal epithelial cells (enterocytes). Potential contamination from basolateral and intracellular membranes must also be allowed for.

Rat BBM vesicles are quite labile and are best used fresh, although they can be frozen under liquid nitrogen for up to a week or so. Pig BBMs are far more robust and will store well under liquid nitrogen for extended periods. Bovine material can be stored frozen as tissue, scrapings, or fully isolated

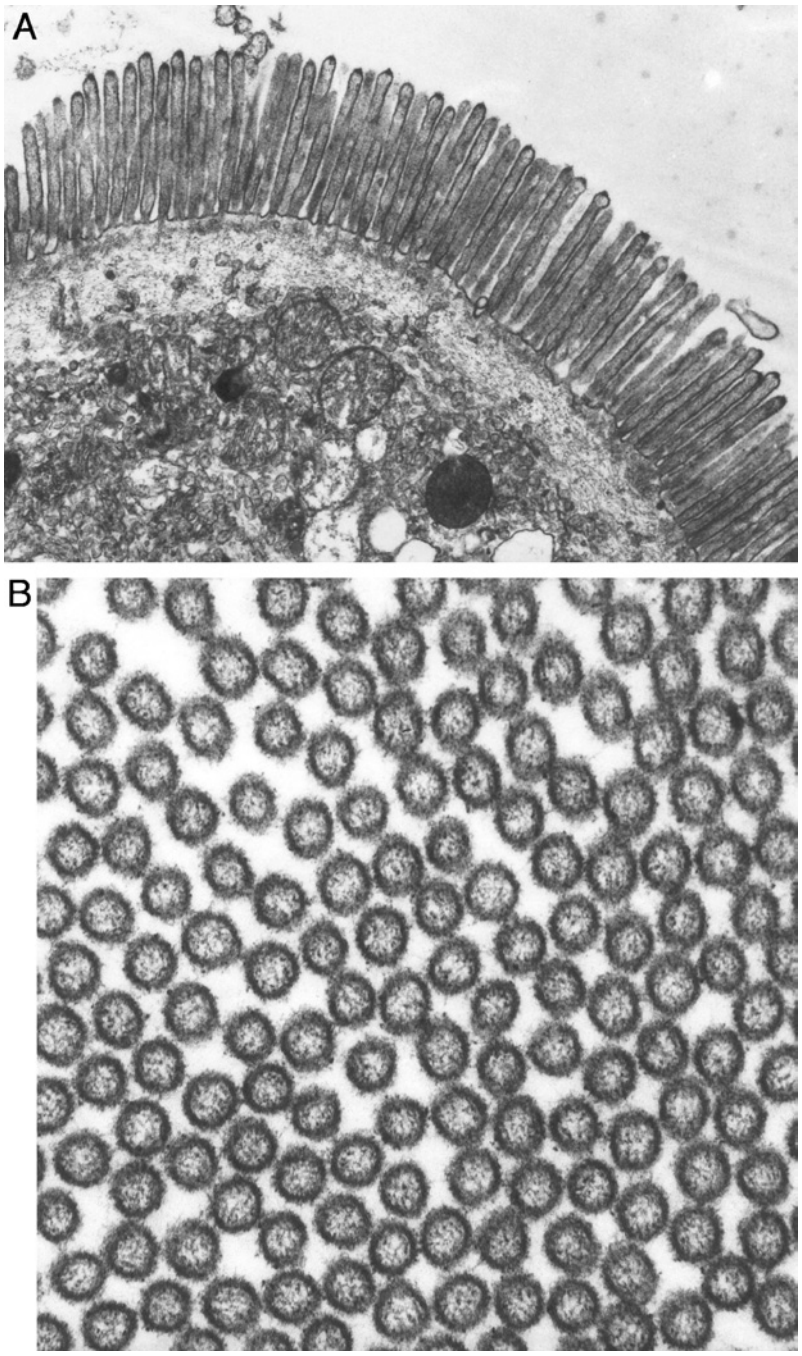


Figure 3.21.2 (**A**) Low-power electron micrograph of isolated rat enterocytes (intestinal epithelial cells) showing highly specialized apical membrane organized into microvilli. (**B**) Cross-section electron micrograph of intestinal microvilli showing plasma membrane surrounding a core of structural filaments.

membranes. It should be noted that a new protocol has been reported using polyethylene glycol (PEG) which may produce vesicles with a better phospholipid profile than is obtained with the divalent cation method (Prabhu and Balasubramanian, 2001). This may be a consequence of the possible stimulation of cation-dependent phospholipases when using the divalent cation precipitation method, and it may be avoided using PEG precipitation.

The isolated membranes are sheared-off microvilli, which will seal spontaneously at their bases forming vesicles that are almost invariably oriented outside out (Hearn et al., 1981); thus, they can be used for rapid uptake experiments. They usually trap ~1 to 2 μl per

mg protein, and so even with a suspension of 8 to 10 mg protein/ml the total trapped volume is negligible compared to the extravesicular suspension volume. The sealing process and equilibration of intravesicular medium with the suspension medium takes place during the final centrifugation of S3. Thus, in these protocols the vesicles will contain an isotonic mannitol medium. If a different composition is required, an additional step is required in which P4 is resuspended in 10 ml of the medium of choice, mixed by repeated flushing through a plastic pipet tip, and then given a final 15-min centrifugation at 20,000 × *g*, 4°C. Discard the supernatant and resuspend in the same solution using a minimal volume (~ 500 µl) to allow for final dilution to the required protein concentration. If the yield cannot give 8 to 10 mg protein/ml for flux assays it is possible to go as low as 5 mg/ml. However, the transport assays will not be as reliable.

When designing an uptake experiment where the composition of the intravesicular medium may be different from that of the uptake medium, it is necessary to take into account the ratio of intravesicle medium versus uptake medium to determine the actual final concentration of substrates and electrolytes. Normally, the ratio of vesicle suspension to uptake medium is in the range of two to five parts uptake medium to one part vesicles.

Anticipated Results

The yield of isolated BBMs varies significantly among species. Three 30-cm pieces of rat jejunum will yield ~8 to 10 mg membrane protein. The ileum will give a slightly lower yield, but there is ~70 cm available in the adult animal. The BBMs can be suspended to the desired protein concentration depending upon whether they are to be used for vesicle transport assays, enzyme assays, or immunoblotting. Normally, for transport assays 8 to 10 mg protein would be resuspended in 1 ml incubation medium. A higher concentration will clog the filters used in the assay.

One 30-cm segment of pig small intestine will generate up to 5 to 6 mg isolated BBM membrane protein.

One 30-cm segment of cow small intestine will generate up to 5 to 6 mg isolated BBM protein.

Time Considerations

Total preparation time from harvesting of the tissue to the final suspension of purified material takes 4 to 5 hr. Ideally, preparation should be commenced at the start of the day and the experiments with the isolated membranes can then be performed in the afternoon.

Literature Cited

Fredricksen, B. and Wirsen, C. 1956. In-vivo effect of colchicine on alkaline phosphatase of rat intestinal epithelium. *Exp. Cell Res.* 10:749-751.

Hauser, H. and Semenza, G. 1983. Sucrase-isomaltase: A stalked intrinsic protein of the brush border membrane. *CRC Crit. Rev. Biochem.* 14:319-345.

Hearn, P.R., Russell, R.G., and Farmer, J. 1981. The formation and orientation of brush-border vesicles from rat duodenal mucosa. *J. Cell Sci.* 47:227-236.

Hopfer, U., Nelson, K., Perrotto, J., and Isselbacher, K.J. 1973. Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* 248:25-32.

Maenz, D.D., Chenu, C., Bellemare, F., and Berteloot, A. 1991. Improved stability of rabbit and rat intestinal brush border membrane vesicles using phospholipase inhibitors. *Biochim. Biophys. Acta* 1069:250-258.

Prabhu, R. and Balasubramanian, K.A. 2001. A novel method of preparation of small intestinal brush-border membrane vesicles by polyethylene glycol precipitation. *Anal. Biochem.* 289:157-161.

Tsuboi, K.K., Kwong, L.K., Yamada, K., Sunshine, P., and Koldovsky, O. 1985. Nature of elevated rat intestinal carbohydrase activities after high-carbohydrate diet feeding. *Am. J. Physiol.* 249:G510-G518.

Contributed by Chris I Cheeseman and Debbie O'Neill
University of Alberta
Edmonton, Alberta Canada

Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids

Exosomes are small vesicles secreted by most cell types in culture. Exosomes form intracellularly by inward budding of the limiting membrane of endocytic compartments, leading to vesicle-containing endosomes, called multivesicular bodies (MVBs). MVBs eventually fuse with the plasma membrane, thus releasing their internal vesicles (i.e., exosomes) into the extracellular medium. What, then, may the physiological function of exosomes be? On one hand, exosome secretion could be a function per se, e.g., exosome secretion by reticulocytes allows the elimination of proteins such as transferrin receptor or integrins, which are useless in differentiated red blood cells (Pan et al., 1985; Vidal et al., 1997). On the other hand, exosomes could be involved in intercellular communication, allowing exchange of proteins and lipids between the exosome-producing cells and target cells. Such a function has been exemplified in the immune system where exosomes allow exchange of antigen or major histocompatibility complex (MHC)-peptide complexes between antigen-bearing cells and antigen-presenting cells (e.g., dendritic cells; Wolfers et al., 2001; Andre et al., 2002, 2004; Théry et al., 2002). Nevertheless, the physiological functions of exosomes remain a matter of debate.

The purpose of this unit is to give simple and reliable methods for purifying and characterizing exosomes. Cell culture supernatants (conditioned media; CM) contain several types of shed membrane fragments and vesicles; therefore, before performing any functional analysis, it is critical to ensure that the purified vesicles are exosomes and not other contaminating material. The first part of this unit describes the most common protocols used to purify exosomes from cell culture conditioned media or from physiological fluids, and the second part describes different methods for characterizing and assessing the purity of the isolated exosomes.

Exosomes have been successfully purified from cell culture conditioned medium or bodily fluids. Support Protocols 1 and 2 provide all details and precautions to take in collecting materials from which exosomes will be purified. Starting from this material, the original and most commonly used protocol for exosome purification (Raposo et al., 1996) is described in Basic Protocol 1. It involves several centrifugation and ultracentrifugation steps. In some cases, the first centrifugation steps can be replaced by a single filtration step: this option is described in an Alternate Protocol. A slightly modified version of Basic Protocol 1, designed for purifying exosomes from viscous fluids (e.g., plasma) is described in Basic Protocol 2. An extra purification step that provides extremely pure exosomes can be added to these protocols, and is described in Support Protocol 3. A different purification procedure, involving trapping exosomes on beads bearing an antibody specific for exosomal surface molecules, has more recently been described (Clayton et al., 2001) and is provided in Basic Protocol 3. It is easy to use and useful for rough characterization of exosomes, but it is not intended for purification of large amounts of exosomes. An additional new method for purifying exosomes by ultrafiltration instead of ultracentrifugation is not described in this unit. This method employs ultrafiltration cartridges and pumps and is especially useful for purifying exosomes from large volumes (>1 liter) of conditioned medium. It is suitable for clinical applications of purified exosomes, but it is not the easiest option for laboratory applications. Interested readers should see Lamparski et al. (2002) for details.

Contributed by Clotilde Théry, Aled Clayton, Sebastian Amigorena, and Graça Raposo
Current Protocols in Cell Biology (2006) 3.22.1-3.22.29

Identification of membrane vesicles as exosomes requires morphological analysis. Given their small size (50 to 90 nm), exosomes can be visualized only with an electron microscope; thus, evaluation of the purity of exosome preparations as well as their characterization should be determined by electron microscopy (see Support Protocols 4, 5, and 6). Separation of exosomes by SDS-PAGE, followed by Coomassie blue staining of all exosomal proteins also gives an idea of the quality of the exosome preparation: exosomes should contain a large number of proteins, i.e., at least a few dozen, many of which are distinct from the proteins detected in whole cell lysates. Further characterization involves analysis of the physical properties of exosomes on a continuous sucrose gradient (Support Protocol 7) and of protein quantity (Support Protocol 9) and composition by immunoblotting (Support Protocol 8) and FACS analysis (Support Protocol 10).

BASIC PROTOCOL 1

PURIFICATION OF EXOSOMES BY DIFFERENTIAL ULTRACENTRIFUGATION

The general idea of exosome purification by ultracentrifugation is depicted in Figure 3.22.1. The first steps are designed to eliminate large dead cells and large cell debris by successive centrifugations at increasing speeds (steps 1 to 5 below). At each of these steps, the pellet is thrown away, and the supernatant is used for the following step (Fig. 3.22.1). The final supernatant is then ultracentrifuged at 100,000 × *g* to pellet the small vesicles that correspond to exosomes. The pellet is washed in a large volume of PBS, to eliminate contaminating proteins, and centrifuged one last time at the same high speed.

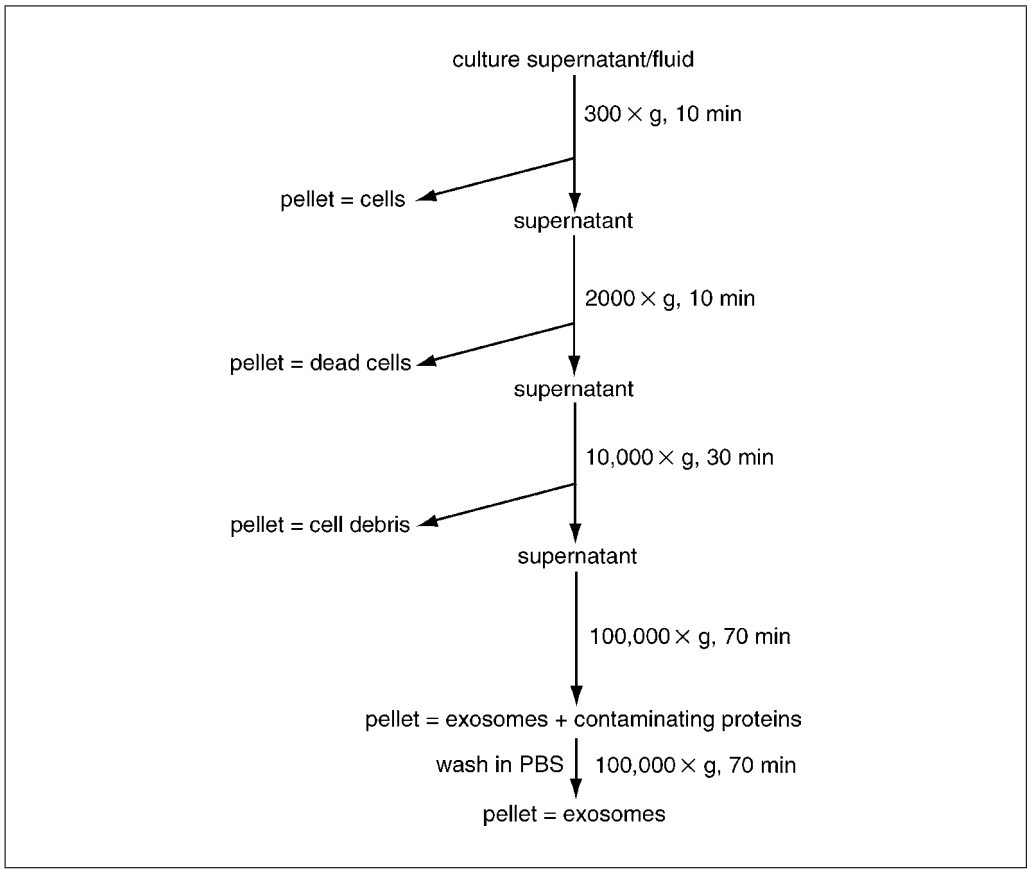


Figure 3.22.1 Flow chart for the exosome purification procedure based on differential ultracentrifugation. The speed and length of each centrifugation are indicated to the right of the arrows. After each of the first three centrifugations, pellets (cells, dead cells, cell debris) are discarded, and the supernatant is kept for the next step. In contrast, after the two 100,000 × *g* centrifugations, pellets (exosomes + contaminant proteins, exosomes) are kept, and supernatants are discarded.

Table 3.22.1 Ultracentrifuge and Rotor Information for Exosome Purification

Rotor (Beckman)	Tubes (Beckman)	Max vol/tube (ml)	Max vol/rotor (ml)	rpm for 10,000 × *g*	rpm for 12,000 × *g*	rpm for 100,000 × *g*	rpm for 110,000 × *g*
SW 41 or 40 (swinging bucket)	Polyallomer	12	72	7,500	8,200	24,000	25,000
SW 28 or 32 (swinging bucket)	Polyallomer	30	180	7,500	8,200	24,000	25,000
70 Ti	Polycarbonate bottle	22	180	10,000	11,000	31,000	32,000
45 Ti	Polycarbonate bottle	68	400	9,000	10,000	30,000	31,000
TLA-100.3	Thick-walled	3	18	13,000	15,000	43,000	45,000
TLA-110	polycarbonate	5	40				

Materials

Conditioned medium (Support Protocol 1, step 5), cleared
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Tris-buffered saline (TBS; *APPENDIX 2A*), optional

Refrigerated centrifuge
50-ml polypropylene centrifuge tubes
Ultracentrifuge and fixed-angle or swinging-bucket rotor (see Table 3.22.1)
Polyallomer tubes or polycarbonate bottles, appropriate for the ultracentrifuge rotor (see Table 3.22.1)
Micropipettor (e.g., Pipetman)
Tabletop ultracentrifuge (e.g., Beckman TL-100)
–80°C freezer

NOTE: All centrifugations should be performed at 4°C.

NOTE: It is only necessary to use sterile equipment if the final use of exosomes is going to require sterility (e.g., functional in vivo or in vitro assay). If only biochemical analyses will be performed (e.g., immunoblot, proteomics, FACS analysis), very clean, but not necessarily sterile, tubes are required.

NOTE: If sterility is required, sterile centrifuge and ultracentrifuge tubes must be used, and all steps up to the time when CM-containing tubes or tube holders are closed must be performed in a tissue culture hood. To sterilize ultracentrifuge tubes, wash the clean tubes and their lids briefly in 70% ethanol, rinse twice in sterile PBS, pour PBS off, and drain the remaining drops of PBS with a pipet before use. The rotor lid (45Ti rotor) or the lid for each tube holder (SW41, SW28 rotors) must also be cleaned with 70% ethanol before closing the rotors.

Remove cells, dead cells, and cellular debris

1. Transfer the cleared, conditioned medium to 50-ml centrifuge tubes.
2. Centrifuge 20 min at 2,000 × *g*, 4°C.
3. Pipet off the supernatant, and transfer it to polyallomer tubes or polycarbonate bottles appropriate for the ultracentrifugation rotor to be used.

Ensure that none of the pellet is collected and contaminates the supernatant. Use a pipet, rather than pouring off the supernatant, and leave behind half a centimeter of liquid above the pellet.

4. Mark one side of each ultracentrifuge tube with a waterproof marker, orient the tube in the rotor with the mark facing up, and centrifuge 30 min at 10,000 × *g*, 4°C (see Table 3.22.1).

   The mark is a reference for the location of a pellet at the end of the centrifugation.

Collect exosome fraction

5. Transfer the supernatant to a fresh tube or bottle the same size as in step 3.

   Ensure that none of the pellet is collected and contaminates the supernatant.

   There will probably not be a visible pellet at this step. For swinging-bucket rotors, the pellet is at the bottom of the tube. For fixed-angle rotors, the pellet is on the side of the tube facing up (marked by the marker pen) near the bottom of the tube.

   When removing the supernatant with the pipet, hold the tube at an angle, so that the pellet is always covered with supernatant, and stop removing supernatant when half a centimeter of liquid is still covering the pellet.

6. Centrifuge at least 70 min at 100,000 × *g*, 4°C.

   For this high-speed centrifugation, all tubes should be at least three-quarters full. If one of the tubes is not three-quarters full, add PBS.

   Centrifugation time is calculated to allow a full hour at 100,000 × g, i.e., ~10 min for the centrifuge to reach 100,000 × g plus 1 hr at the final speed. A longer time (up to 3 hr) will not damage the exosomes.

7. Remove the supernatant completely.

   For fixed-angle rotors, at this step, pour off the supernatant rather than use a pipet.

   For swinging-bucket rotors, remove the supernatant with a pipet and leave 2 mm of supernatant above the pellet.

Wash exosomes

8. Resuspend the pellet in each tube in 1 ml PBS, using a micropipettor. Pool the resuspended pellets from all the tubes containing materials from the same cells in a single centrifuge tube. Then add PBS to fill the tube completely.

   There will probably not be a visible pellet at this step. For fixed-angle rotors, resuspend by flushing up and down where the pellet should be (upper side of the tube, towards the bottom). For swinging-bucket rotors, flush the bottom of the tube.

9. Centrifuge 1 hr at 100,000 × *g*, 4°C.

10. Remove the supernatant as completely as possible. For fixed-angle rotors, pour off the supernatant, keep the tube upside down, and aspirate the remaining liquid on the sides and the mouth of the tube with a micropipettor. Proceed to step 11a or step 11b

11a. *To resuspend the pellet* (*i.e., exosomes)*: Add a small volume (50 to 100 µl) of PBS or TBS and resuspend.

   If the final volume of exosomes is too large (>1/2000 of the initial volume of conditioned medium) or if there was no visible pellet at step 10, use step 11b instead.

11b. *To concentrate the exosome preparation*: Centrifuge the supernatant (step 10) 1 hr at 100,000 × *g*, 4°C in a tabletop ultracentrifuge, using a TLA-100.3 rotor and the corresponding thick-walled polycarbonate tubes. Remove most of the PBS above the visible pellet and resuspend exosomes in 20 to 50 µl of fresh PBS.

12. Store exosomes up to 1 year at –80°C in 100-µl aliquots. Avoid repeated freezing and thawing.

ELIMINATION OF LARGE CELL DEBRIS AND MEMBRANES BY FILTRATION

ALTERNATE PROTOCOL

For some cells (e.g., mouse dendritic cells) it is possible to replace steps 1 to 6 of Basic Protocol 1 with a single filtration step using a 0.22-µm filter; this will eliminate dead cells and large debris while keeping small membranes for further purification by ultracentrifugation. To determine whether this Alternate Protocol can be used, perform Basic Protocol 1 once using the same volume of conditioned medium obtained from the same cells at the same time in parallel with this protocol, to compare the yields and the quality of exosomes by electron microscopy and immunoblotting (Support Protocols 4, 5, 6, 7, 8, 9, and 10), to ensure that the exosome preparations are identical.

Materials *(also see Basic Protocol 1)*

0.22-µm filter sterilization device (e.g., Steritop; Millipore)
100-ml to 1-liter glass bottle, sterile

1. Filter sterilize the conditioned medium by passing through a vacuum-connected 0.22-µm filter on top of a sterile bottle, using a vacuum.
2. Store the filtered supernatant up to 1 week at 4°C before proceeding to exosome purification (Basic Protocol 1, steps 8 to 12).

COLLECTING CULTURE SUPERNATANTS FOR EXOSOME PURIFICATION

SUPPORT PROTOCOL 1

The exosome-containing conditioned medium from cultured cells is used as the starting material for exosome purification. Total cell lysate of the cells that produce the conditioned medium is prepared at the same time and is used later in parallel with exosomes for immunoblot analysis (Support Protocol 8).

Materials

Cells in culture (*UNIT 1.1*)
Exosome-production medium (Support Protocol 2)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Cell lysis buffer (see recipe)

Refrigerated centrifuge
50-ml polypropylene centrifuge tubes
500-ml or 1-liter glass bottle, sterile
1.5-ml microcentrifuge tubes

Additional materials for growing and counting cells in culture (*UNIT 1.1*) and clearing conditioned medium by filtration (Alternate Protocol; optional)

Collect and clear conditioned medium

1. Grow cells of interest under the usual conditions (see *UNIT 1.1*) until they reach 70% to 80% confluency for adherent cells, or 60% to 70% of their maximum concentration for cells grown in suspension.

   Use as many cells as necessary to produce at least 70-ml of conditioned medium (i.e., a minimum of seven 10-cm dishes and up to twenty 15-cm dishes); it is always better to purify exosomes from large volumes of conditioned medium because the yield of the purification procedure increases with the starting volume.

2a. *For adherent cells:* Remove the culture medium, and replace it with a similar volume of exosome-production medium.

2b. *For cells in suspension:* centrifuge cells 10 min at 300 × *g*, 4°C, pour off the supernatant, and resuspend cells in the same volume of exosome-production medium.

3. Return the cells to the incubator for 24 to 48 hr.

Cells that grow fast may be overconfluent and start dying after 48 hr. In this case, take the conditioned medium after 24 hr.

Cells that are very sensitive may not like growing in exosome production medium or in FBS-free medium. In this case, take the supernatant after 24 hr. For instance, dendritic cells tend to mature spontaneously if kept for 48 hr in exosome-production medium.

4a. *To clear conditioned medium from adherent cells:* Collect the conditioned medium with a pipet and transfer to 50-ml polypropylene centrifuge tubes. Centrifuge cells 10 min at 300 × *g*, 4°C, and collect the conditioned medium with a pipet.

4b. *To clear conditioned medium from cells in suspension:* Centrifuge cells 10 min at 300 × *g*, 4°C, and collect the conditioned medium with a pipet and transfer to 50-ml polypropylene centrifuge tubes.

The centrifugations will pellet the cells from suspension cultures or remove the large dead cells from adherent cultures.

5. Transfer the conditioned medium to a sterile glass bottle. Pool supernatants from all dishes or flasks containing the same cell type into a single bottle and store up to 1 week at 4°C.

Once supernatant is collected, the authors strongly recommend proceeding with exosome purification (Basic Protocol 1, Basic Protocol 3, or Alternate Protocol) as soon as possible. Keeping the supernatant at 4°C for up to a week may lead to loss of some exosomes (C. Théry and S. Amigorena, unpub. observ.). Alternatively, some investigators have successfully isolated exosomes from supernatants kept at −80°C for several months, but this also probably leads to exosome loss and should be avoided.

Prepare total cell lysate

6a. *To collect adherent cells:* Trypsinize adherent cells from one to two dishes and count cells with a hemacytometer (see *UNIT 1.1*).

6b. *To collect cells in suspension:* Use cells from the pellet remaining in the tube after step 4b. Resuspend and count cells with a hemacytometer (see *UNIT 1.1*).

7. Put 1–5 × 10^7 cells in a microcentrifuge tube. Centrifuge 5 min at 300 × *g*, 4°C, discard supernatant, and resuspend the cell pellet in 1-ml of PBS. Centrifuge and repeat the PBS wash and centrifugation.

8. Add 200-µl (for 1 × 10^7 cells) or 1-ml (for 5 × 10^7 cells) of cell lysis buffer to the cell pellet and incubate 20 min on ice, vortexing at the beginning and end of the incubation.

9. Centrifuge 20 min at 20,000 × *g*, 4°C. Collect the supernatant in a fresh microcentrifuge tube. Store up to 6 months at −20°C.

The total cell lysate is used in parallel with exosomes for immunoblot analysis (Support Protocol 8).

PREPARING EXOSOME-PRODUCTION MEDIUM

Exosomes are present in the serum generally used for tissue culture. To avoid contamination by these exosomes, culture medium used to grow cells for conditioned media from which the cell-derived exosomes will be purified must be depleted of the contaminating exosomes. There are two options for depleting medium of serum-derived exosomes: (1) if the cells grow in the absence of serum, exosome production-medium can be the basal culture medium, supplemented with all the nutrients and antibiotics, but no fetal bovine serum (FBS). If the cells need some proteins to survive, 1% (w/v) bovine serum albumin (BSA) can be added instead of FBS. (2) If the cells do not survive in serum-free conditions, predeplete the FBS-derived exosomes from FBS-containing medium following this protocol to obtain FBS-exosome-free, exosome-production medium.

Materials

Culture medium complete with required nutrients (e.g., antibiotics, L-glutamine, HEPES, 2-mercaptoethanol, FBS
Ultracentrifuge and fixed-angle or swinging-bucket rotor (see Table 3.22.1)
Polyallomer tubes or polycarbonate bottles, appropriate for the ultracentrifuge rotor (see Table 3.22.1)
0.22-μm filter-sterilization device (e.g., Steritop; Millipore)
100-ml to 1-liter glass bottle, sterile

1. Prepare medium supplemented with all nutrients, plus 20% (v/v) FBS.
2. Centrifuge the medium overnight at 100,000 × *g*, 4°C.

   Follow the same general instructions for centrifugation as explained in the exosome purification section;. i.e., mark a side of each tube with a waterproof marker, and orient the tubes in the centrifuge with mark facing up, indicating where to look for a pellet at the end of the centrifugation.

   If using reusable centrifuge tubes, keep a specific batch of centrifuge tubes for medium depletion, and another different batch for exosome purification to avoid potential contamination with serum endosomes.

3. Filter sterilize the supernatant (i.e., depleted medium) by pouring the contents of each tube into a vacuum-connected 0.22-μm filter on top of a sterile bottle.

   Keep an eye on the pellet (on the marked side of the tube), to make sure that it does not become loose. If it does, stop pouring and throw away the medium remaining in the tube.

4. Dilute the depleted medium with medium supplemented with all the nutrients and antibiotics, but no FBS, in order to reach the final FBS concentration required to make the exosome production medium.

   For instance, if the cells are normally cultured in 10% FBS, dilute 1 vol depleted medium with 1 vol FBS-free medium.

   Depleting medium with 20% FBS allows depletion of a large volume of culture medium in a single round of centrifugation.

   Do not deplete pure FBS because the viscosity of pure FBS will cause elimination of many proteins and factors, which will pellet as aggregates. The cells may not like it!

5. Store exosome-depleted medium after step 4 (sterilized, but not diluted) or step 5 (sterilized and diluted) up to 4 weeks at 4°C.

BASIC PROTOCOL 2

PURIFYING EXOSOMES FROM VISCOUS FLUIDS

To purify exosomes from bodily fluids (e.g., urine, broncho-alveolar lavage, serum, plasma, tumor ascites), simply collect the fluid by the usual means. Store up to 5 days at 4°C in a glass bottle until proceeding with exosome purification.

The principle for exosome purification is the same as when starting from tissue culture conditioned media, but because of the viscosity of some fluids it is necessary to dilute them, and to increase the speed and lengths of centrifugations. This protocol has been used in the authors' laboratories for purification of exosomes from human plasma (Caby et al., 2005).

All precautions indicated in Basic Protocol 1 apply to this protocol as well.

Materials

Fluid (e.g., plasma: separate from blood cells by Ficoll centrifugation; lymph, serum, urine, bronchiolar lavage, or tumor ascites)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Refrigerated centrifuge
50-ml polypropylene centrifuge tubes
0.22-μm filter device (e.g., Steritop, Millipore)
Ultracentrifuge and fixed-angle or swinging-bucket rotor (see Table 3.22.1)
Polyallomer tubes or polycarbonate bottles, appropriate for the ultracentrifuge rotor (see Table 3.22.1)

NOTE: All centrifugations should be performed at 4°C.

1. Dilute fluid with an equal volume of PBS. Transfer diluted fluid in 50-ml tubes. Centrifuge 30 min at 2,000 × *g*, 4°C.
2. Carefully transfer supernatant to ultracentrifuge tubes or bottles without pellet contamination (see Basic Protocol 1, step 3 annotation). Centrifuge 45 min at 12,000 × *g*, 4°C.
3. Carefully transfer supernatant to ultracentrifuge tubes or bottles (See Table 3.22.1 for tubes to choose according to the volume of fluid handled.). Centrifuge 2 hr at 110,000 × *g*, 4°C.
4. Resuspend pellets in 1 ml PBS and pool them in one of the tubes. Fill the tube with PBS to dilute the resuspension in a large volume.
5. Filter the suspension through a 0.22-μm filter, and collect in a fresh ultracentrifuge tube or bottle.
6. Centrifuge 70 min at 110,000 × *g*, 4°C. Pour off the supernatant.
7. Resuspend the pellet in 1 ml PBS, and then fill the tube with PBS. Centrifuge 70 min at 110,000 × *g*, 4°C. Pour off the supernatant.
8. Resuspend pellet in 50 to 200 μl PBS. Store up to 1 year at −80°C.

SUPPORT PROTOCOL 3

PREPARATION OF EXOSOMES ON A 30% SUCROSE CUSHION

Although Basic Protocols 1 and 2 provide reasonably pure exosomes, for some applications it may be advisable to include an extra purification step using a sucrose cushion. This step eliminates more contaminants, such as proteins nonspecifically associated with exosomes, or large protein aggregates, which are sedimented by centrifugation but do not float on a sucrose gradient.

Materials

Partially purified exosome pellet (Basic Protocol 1, step 10 or Basic Protocol 2, step 7)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Tris/sucrose/D_2O solution (see recipe)

Ultracentrifuge with SW 28 and 45 Ti rotors
Polyallomer tubes appropriate for the SW 28 rotor (Table 3.22.1)
Thick-walled polycarbonate tubes appropriate for the 45 Ti rotor (Table 3.22.1)
5-ml syringe
18-G needle

NOTE: All centrifugations should be performed at 4°C.

1. Resuspend partially purified exosome pellet in 25 ml PBS total.
2. Load 4 ml of Tris/sucrose/D_2O solution at the bottom of a SW 28 tube, to make a cushion.
3. Add the diluted exosomes gently above the sucrose cushion without disturbing the interface. Centrifuge 75 min at 100,000 × *g*, 4°C.
4. With a 5-ml syringe fitted with an 18-G needle, collect ~3.5 ml of the Tris/sucrose/D_2O cushion, which now contains exosomes, from the side of the tube.
5. Transfer the exosomes to a fresh ultracentrifuge tube. Dilute exosomes to 60 ml with PBS. Centrifuge 70 min at 100,000 × *g*, 4°C, in a 45 Ti rotor.
6. Resuspend the pellet in 50 to 100 µl PBS.

PURIFICATION OF EXOSOMES BY IMMUNOISOLATION

BASIC PROTOCOL 3

A simple and rapid method, suitable for the routine isolation and analysis of exosomes, is based upon immuno-magnetic extraction of exosomes bearing human MHC Class II molecules as described by Clayton et al. (2001). (See Table 3.22.2 for exosome markers that are not immune or MHC molecules.). This method has been used by the authors and others (Rabesandratana et al., 1998; Wubbolts et al., 2003; Caby et al., 2005) using magnetic beads coated with antibodies directed against proteins exposed on exosomal membranes. It is, therefore, a mode of isolating exosomes without the need for ultracentrifugation. Furthermore, this method demonstrates colocalization of the protein recognized by the antibody-coated beads with other identified proteins, strengthening the evidence that the analysis is that of exosomes and not, for example, soluble contaminating proteins present in the exosome preparation.

There are several advantages and some disadvantages to this approach. The bead-exosome complexes can be analyzed by flow cytometry, using fluorophore-conjugated antibodies; this facilitates a rapid semiquantitative characterization of the exosome surface phenotype. Alternatively, bead-exosome complexes can be incubated in SDS sample buffer and analyzed by immunoblot (Support Protocol 8). In addition, the loaded beads can be processed for electron microscopy, which allows investigation of the morphology of the adsorbed membranes. Given their size, beads can only be observed after sectioning, which can be accomplished with standard electron microscopy methods such as resin embedding and ultrathin sectioning or, preferably, ultracryomicrotomy to avoid dehydration steps (Raposo et al., 1997). Do not use Support Protocols 4 and 5 for electron microscopy of exosome-bead complexes.

The choice of exosome marker for immobilization is a key issue. For MHC Class II–positive cells, the use of beads precoated with a pan class II–specific antibody is ideal. However, this marker is unsuitable for exosome isolation from non antigen-presenting cells which are MHC class II–negative. In theory, a good candidate marker would be MHC Class I molecules expressed on most if not all exosomes. Unfortunately, MHC class I molecules are also shed from the plasma membrane as a soluble molecule, especially in certain cancerous cells, so that the MHC class I–capturing antibody on the bead surface captures soluble class I as well as exosomally expressed class I molecules, significantly reducing the efficiency of exosome capture. Some suggested alternative exosome-capture molecules include members of the tetraspanin family such as CD81 or CD63, for MHC class II–negative cells (Table 3.22.2). Dynal products (available now from Invitrogen) provide naked beads for coating with antibodies of choice. There are at least 2 types: M-450 Epoxy and M-450 Tosylactivated. Other manufacturers make beads of a similar size, which may also be suitable.

Although selecting exosomes from a conditioned medium based on chosen exosome-expressed proteins is an advantage in several ways, it is important to be aware that this approach may isolate only a subpopulation of marker-positive exosomes, so that subsequent analysis does not reflect the exosome population as a whole. Immunoisolation is not appropriate for purification of large amounts of exosomes. Finally, once captured on beads, exosomes retaining full functionality may not be successfully eluted from the bead surface.

Materials

Conditioned medium from cultured cells (Support Protocol 1)
Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 3 mg/ml bovine serum albumin (BSA), filter sterilized and stored up to 1 month at 4°C
50-ml centrifuge tubes, sterile
Refrigerated centrifuge
4.5 μM paramagnetic Dynabeads M-450 (Dynal), ready-coated with antibodies (e.g., anti-human MHC II)
Magnet (Dynal)
Test tube rolling machine holding 50-ml tubes

1. Transfer the conditioned medium (Support Protocol 1, step 5) to 50-ml centrifuge tubes. Centrifuge 10 min at 2,000 × *g*, 4°C.
2. Transfer supernatant to fresh 50-ml tubes, and centrifuge again.
3. Wash the antibody-coated Dynabeads as recommended by the manufacturer (i.e., five times in 5 ml of PBS containing 3 mg/ml BSA) using magnetic separation.
4. Pellet the beads using the magnet, and remove all buffer. To this pellet add the supernatant from step 2.

   The easiest method for defining conditions for bead saturation is to perform a dilution series (of cell numbers or of exosome-rich supernatant), and to capture exosomes using a fixed number of beads, in a fixed volume of medium. After washing, beads are stained (preferably with direct-conjugated antibody against a strongly expressed exosome protein e.g., MHC Class I), and the fluorescence intensity measured by flow cytometry. A plot of bead:cell ratio versus mean fluorescence intensity should be drawn, and from this graph, it should be clear at around which point a maximum (plateau) is reached—i.e., adding more exosomes to the beads results in no further increase in signal because the bead surface is already completely saturated. Examples are shown in Clayton et al. (2001).

   For cells such as B cells, which produce abundant exosomes, cells are typically seeded at 1–5 × 10^6 cells/ml and allowed to condition the medium for 24 to 48 hr. About 10 to

50 ml of precleared supernatant is added to 0.5–5 × 10^6 beads. (A half million beads is usually sufficient for the analysis of 10 to 20 different surface markers i.e., 10 to 20 FACS tubes, each representing staining with a particular antibody). For nonimmune cells, start with the same ratio as for B cells and adjust as necessary.

Dynal provides the concentration of the bead stock (usually 400,000 beads/ml). The beads can also be counted using a hemacytometer.

5. Incubate 24 hr at room temperature or at 4°C (to limit proteolysis) on a test tube rolling machine to allow complete bead saturation.

   Do not use a test tube rotating shaker because it may be very harsh and encourage formation of bubbles in protein rich supernatants. The bubbles will trap the magnetic beads and make it difficult to pull them into a pellet using a magnet.

   In almost all situations, it is desirable to obtain beads that are completely saturated with exosomes (for examples of the possible use of partially coated beads see Clayton et al., 2001). Subsequent analysis of exosome-bead complexes, will therefore give the maximum possible signal. It is especially important to be sure that the saturating condition has been achieved if comparing exosome phenotypes from different cell lines, for example. Two key factors dictate the bead coupling efficiency: (1) the coupling time and (2) the ratio of beads to exosomes.

   For an exosome in solution to bind to the surface of a bead it is essential that the exosome makes contact with the bead. If the coupling time is long, the likelihood that such an interaction occurs is increased; hence, long coupling times result in better bead coating.

   However, coupling is entirely dependent on the concentration of exosomes in the supernatant, and beads reach saturation more quickly in exosome-rich medium. In practical terms, the authors have found that an overnight coupling gives the best bead-coating results and extending the time beyond 27 hr has no beneficial effect on the extent of bead coating. If time is short, however, incubations of ~1 to 3 hr give ~50% to 70% bead saturation. If the exosomes are particularly dilute (i.e., cell density is low, or cells produce few exosomes) extending the coupling time will be of little benefit. Optimal bead saturation occurs if there is a vast excess of exosomes present in the supernatant relative to beads.

   In practice, use only as much of the bead preparation as is required (~20,000 to 50,000 beads per flow cytometry antibody, or 5–10 × 10^6 beads per well for immunoblotting), and condition the medium with as many cells as possible. As a guide, the authors suggest using at least 100 cells for every bead, but this ratio will vary considerably among different cell types and with duration of cell culture.

6. Collect the beads with the magnet, and wash at least three times with 2 to 10 ml PBS containing 3 mg/ml BSA to remove material that is nonspecifically bound.
7. Finally, wash once in ~10 ml PBS as in step 6 to reduce BSA content.
8. Resuspend beads in 100 µl of PBS for FACS (Support Protocol 10) or continuous sucrose gradient (Support Protocol 7) analysis, SDS sample buffer for immunoblotting (Support Protocol 8), or fixative for electron microscopy (Clayton et al. 2001).

ELECTRON MICROSCOPE ANALYSIS OF WHOLE-MOUNTED EXOSOMES

SUPPORT PROTOCOL 4

Standard electron microscopic (EM) methods requiring fixation, dehydration, resin embedding, and ultrathin sectioning that are currently used for tissues, cells, and subcellular fractions are, in the authors' opinions, difficult to apply to exosomes and do not give a representative view of the preparation because membranes may be lost during dehydration and repeated centrifugation steps related to dehydration and embedding. This protocol describes a quick and reliable contrasting and embedding method that permits EM analysis of whole-mount exosome preparations.

Materials

100,000 × *g* exosome pellet or frozen concentrated exosome preparations (Basic Protocol 1 or Support Protocol 3)
2% or 4% (w/v) paraformaldehyde (PFA; see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1% glutaraldehyde (see recipe)
Uranyl-oxalate, pH 7 (see recipe)
Methyl cellulose-UA, pH 4: 9 parts 2% methyl cellulose (see recipe) and 1 part 4% uranyl acetate (see recipe) mixed just before use
Formvar-carbon coated EM grids (Support Protocol 6; also see Video 11 for *UNIT 4.7* at *http://www.currentprotocols.com*)
Parafilm
Forceps (Dumont no. 5), clean
Glass dish
Stainless steel loops (homemade; see Video 1 for *UNIT 4.7* at *http://www.currentprotocols.com*), slightly larger than grids
Whatman no. 1 filter paper
Grid storage boxes (e.g., PELCO; *http://tedpella.com*)
Transmission electron microscope (TEM)

Fix exosomes on electron-microscope grids

1. Resuspend the 100,000 × *g* exosome-containing pellet in 50 to 100 µl of 2% PFA.

   Purified concentrated exosomes frozen at –20°C or –80°C are thawed and mixed with an equal volume of 4% PFA.

   Exosomes in 2% PFA can be stored up to 1 week at 4°C before proceeding further.

2. Deposit 5 µl resuspended pellets on Formvar-carbon coated EM grids. Prepare two or three grids for each exosome preparation. Cover and let the membranes adsorb for 20 min in a dry environment.

   Alternatively, put a drop of 5 to 10 µl of exosome suspension on clean Parafilm. In this case float the grids on the drop with their coated side facing the suspension.

3. Put 100-µl drops of PBS on a sheet of Parafilm. Transfer the grids (membrane side down) to drops of PBS with clean forceps to wash.

   IMPORTANT NOTE: *It is important to keep the grids wet on the side of membrane adsorption during all steps, but dry on the opposite side.*

   Subsequent procedures are performed in the same manner: placing drops of reagents on a sheet of Parafilm and transferring the grids from drop to drop with forceps.

   Grids with the same sample can be handled in the same drop.

4. Transfer the grids to a 50-µl drop of 1% glutaraldehyde for 5 min.

5. Transfer to a 100-µl drop of distilled water and let grids stand for 2 min. Repeat seven times for a total of eight water washes.

Contrast and embed samples

6. Transfer grids to a 50-µl drop of uranyl-oxalate solution, pH 7, for 5 min.

   Samples are contrasted first in a solution of uranyl oxalate, pH 7 and then contrasted and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose in a ratio of 100µl/900µl, respectively.

7. Transfer grids to a 50-µl drop of methyl cellulose-UA for 10 min on ice.

   To keep the grids cold; use a glass dish covered with Parafilm on ice.

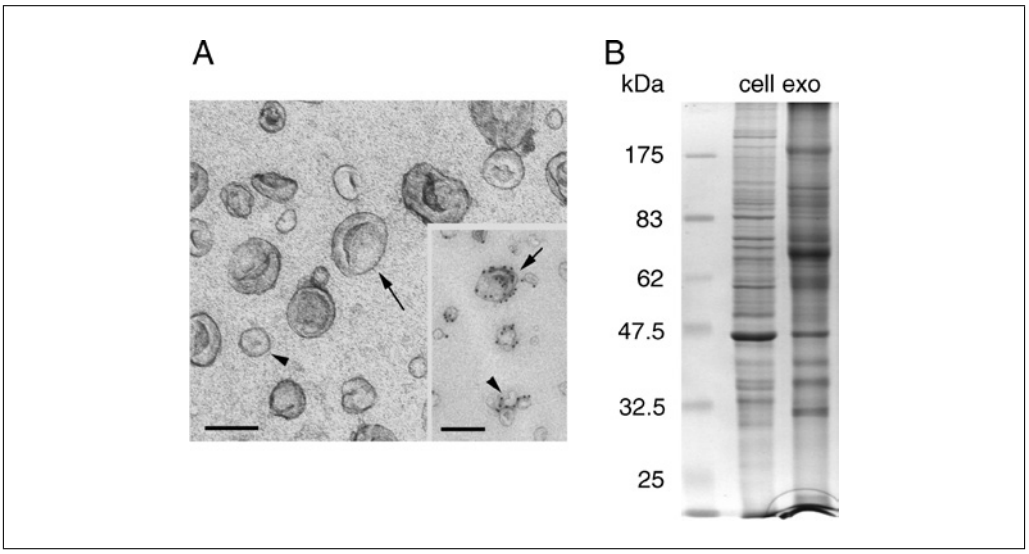


Figure 3.22.2 Typical characteristics of exosomes. (**A**) Electron-microscopic observation of whole-mounted exosomes purified from mouse dendritic cells. Arrows indicate exosomes, arrowheads point to smaller nonexosomal vesicles. Insert: Immunogold labeling of MHC class II molecules (10-nm gold particles). Scale bar = 100 nm. (**B**) Coomassie blue–stained SDS polyacrylamide gel after separation of 30 μg of total cell lysates (Cell) or exosomes (Exo) from mouse dendritic cells. Molecular weight markers were loaded in the first lane (kDa).

This contrasting and embedding procedure ("positive-negative" contrast) was originally developed for ultrathin cryosections. This method is reproducible and combines both increase in contrast and stabilization of the membranes.

8. Remove the grids with stainless steel loops and blot excess fluid by gently pushing the loop sideways on Whatman no. 1 filter paper so that a thin film is left behind over the exosome side of the grid. (See Video 3 of *UNIT 4.7* at *http://www.currentprotocols.com.*)

   It is essential to control the thickness of the methyl-cellulose film. The final thickness of the film is critical in obtaining optimal contrast and fine structure preservation. This can be controlled by the speed of adsorption onto the filter paper (the faster this goes the thicker the film). In the authors' laboratory a sheet of Whatman no. 1 filter paper is placed on top of Parafilm to avoid contamination of the bench with uranyl acetate.

   Loops are slightly larger than the grids and are generally mounted on 1-ml plastic micropipettor tips.

9. Air dry the grid 5 to 10 min while still on the loop.

   After drying, a blue-gold interference color is indicative of a homogeneous and appropriate thickness of the methyl-cellulose film.

10. Store the grids in appropriate grid storage boxes for many years.

11. Observe under the electron microscope at 80 kV.

    Electron microscopy of negatively stained exosomes reveals cup-shaped membrane vesicles of 50 to 90 nm (Fig. 3.22.2). Often, smaller 10- to 20-nm lipid particles are also observed within the same preparations. It is possible that the morphological appearance of exosomes may be influenced by chemical fixation and/or embedding and contrasting with methyl cellulose and uranyl acetate. Unpublished observations of cryo-fixed no-contrasted exosomes visualized with cryo-electron microscopy reveal rather round membrane vesicles.

    Analysis of the 100,000 × g pellets at the electron microscopic level is also essential in evaluating contamination of exosome preparations by other membranes that may be present in culture supernatants as a consequence of cell lysis and may be pelleted during centrifugation.

SUPPORT PROTOCOL 5

IMMUNOGOLD LABELING OF WHOLE-MOUNT EXOSOMES

For further characterization of exosomes, immunolabeling with antibodies against proteins known to be exposed on exosomal membranes is recommended before electron microscopy. A standard immunolabeling procedure is difficult to establish because each antibody/antigen combination may require a slightly different approach.

The most commonly used single antigen-specific labeling procedures are specific antibody + Protein A–gold (PAG) or specific antibody + bridging antibody + PAG. IgG conjugated to gold probes can also be used. The specific antibody can be either a monoclonal or a polyclonal antibody, the bridging antibody or IgG conjugated to the gold probe must originate from a different species. A bridging antibody can be used either to amplify the signal or in situations where the specific antibody does not react with PAG or the IgG-gold. The labeling efficiency, specificity, and background of bridging antibodies depend on the source and should be tested when handling antibodies from different manufacturers.

Multiple antigen-specific immunolabeling procedures can be accomplished using two or more consecutive single-labeling procedures. Up to three different antigens can be visualized (double- and triple-immunolabeling). When performing double or triple immunolabeling, one should consider the best choice of the size of gold particles to be used in the first, second, or third step and for the sequence of gold probes and antibodies. This should be tested first in a single-labeling procedure. Also take into account that, given the small size of exosomes, steric hindrance may hide epitopes and give a negative result.

The procedure described in this protocol was used by Escola et al. (1998), Raposo et al. (1996), Théry et al. (1999), Van Niel et al. (2001), and Zitvogel et al. (1998), to localize proteins exposed at the surface of exosomes. To visualize proteins on the cytosolic side of exosomes (e.g., Tsg101) one may introduce a permeabilization step with saponin after the fixation procedure and during incubation with antibodies and PAG (Fevrier et al., 2004). However, permeabilization of exosomal membranes is difficult to control and may affect exosome morphology.

Materials

100,000 × *g* exosome pellet (Basic Protocol 1)
2% or 4% (w/v) paraformaldehyde (PFA; see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS/50 mM glycine *or* PBS/50 mM NH_4Cl
Blocking buffer: PBS/5% (w/v) BSA, PBS/10% (v/v) fetal calf serum (FCS), *or* PBS/1% (w/v) cold-water fish skin gelatin (CFG; Sigma Aldrich)
Primary antibody
Antibody diluent: PBS/1% (w/v) BSA, PBS/5% (v/v) FCS, *or* PBS/1% (w/v) cold-water fish skin gelatin
Washing buffer: PBS/0.1% (w/v) BSA, PBS/0.1% (v/v) FCS, *or* PBS/0.1% (w/v) CFG
Secondary (bridging) antibody (optional; e.g., Dakopatt)
PBS/0.5% (w/v) BSA
Protein A–gold conjugates (Cell Microscopy Center, Utrecht, The Netherlands)
1% glutaraldehyde (see recipe)
Formvar-carbon coated EM grids (Support Protocol 6 or Pelco International, *http://www.pelcoint.com*)
Parafilm
Clean forceps (Dumont no. 5)
Additional reagents and equipment for contrasting, embedding, and electron microscopy (Support Protocol 4)

Adsorb exosomes on electron-microscope grids

1. Resuspend the 100,000 × *g* exosome-containing pellet in 50 to 100 µl of 2% PFA.

   Purified concentrated exosomes frozen at –20°C or –80°C are thawed and mixed with an equal volume of 4% PFA.

   Exosomes in 2% PFA can be stored up to 1 week at 4°C before proceeding further.

2. Deposit 5 µl of the resuspended pellets on Formvar-carbon coated electron-microscopy grids. Prepare two or three grids for each exosome preparation. Cover and let the membranes adsorb for 20 min in a dry environment.

   Alternatively, put a drop of 5 to 10 µl of exosome suspension on clean Parafilm. In this case float the grids on the drop with their coated side facing the suspension.

3. Put 100-µl drops of PBS on a sheet of Parafilm. Transfer the grids to drops of PBS with clean forceps.

   IMPORTANT NOTE: *It is important to keep the grids wet on the side of membrane adsorption during all steps, but dry on the opposite side.*

   Grids with the same sample can be handled in the same drop.

   The immunolabeling procedure is performed by floating the exosome-coated electron-microscope grids on successive drops of washing, blocking, and labeling solutions disposed on a Parafilm sheet. Each rinsing step is performed on drops of at least 100 µl, while 5-µl drops are sufficient for immunoreagents.

   The following steps proceed by floating grids on 100- to 200-µl droplets of solutions.

Wash and block exosomes on grids

4. Transfer grid to PBS and wash twice for 3 min.

5. Transfer grid to PBS/50 mM glycine for 3 min. Repeat transfers to fresh drops of PBS/50 mM glycine three times for a total of four washes.

   The glycine quenches free aldehyde groups. As an alternative use a 50 mM solution of ammonium chloride (NH_4Cl).

6. Transfer grids to a drop of blocking buffer for 10 min

   Bovine serum albumin (BSA) and other proteins saturate nonspecific binding sites. Alternative blocking solutions are 10% fetal calf serum (FCS) or 1% cold-water fish skin gelatin (CFG) in PBS.

   Diluents and washing buffers in following steps should contain the same proteins as the blocking buffer.

Incubate exosomes with antibodies

7. Transfer the grid to a 5-µl drop with the first antibody diluted in the appropriate antibody diluent for the blocking solution and incubate 30 min.

   Test a range of antibody dilutions from 2 to 20 µg/ml.

   As in any labeling procedure it is critical to avoid contamination of solutions. Buffers should be freshly prepared, and antibody dilutions can be kept from 1 week to 1 month, depending on the antibody.

8. Transfer grids to the appropriate washing buffer and wash 3 min. Repeat transfers to fresh drops of washing buffer five times for a total of six washes.

   It is important to keep the grids wet on the specimen side during all steps, but dry at the opposite side. When the samples are allowed to dry during the immunoincubation, high background levels or other contamination can result.

If the first antibody is not directly reactive with protein A (for example mouse monoclonal IgG1, rat monoclonals, goat polyclonals), use a bridging antibody: for example, rabbit anti-mouse IgM, rabbit anti-goat IgG, rabbit anti-rat (commercially available from Dakopatt).

9. (*Optional*): If it is necessary to perform bridging, incubate 30 min with a secondary antibody diluted in the appropriate blocking solution.

10. Transfer grids to 100-µl drops of PBS/0.5% BSA (blocking buffer) and wash 3 min. Repeat transfers to fresh drops of PBS/0.5% BSA five times for a total of six washes.

Label exosomes with protein A-gold conjugates

11. Incubate in 5-µl drops of protein A-gold conjugates diluted in the appropriate blocking buffer 20 min.

    For a single labeling the authors generally use 10-nm gold particles.

    Protein A-gold of excellent quality with appropriate dilutions indicated by the supplier can be purchased from Cell Microscopy Center, Department of Cell Biology, Utrecht University, The Netherlands. These reagents are extremely pure, the dilutions have been perfectly controlled, and the same batches (made four times a year) are used by different labs.

    The authors recommend a 1:200 dilution when using Dako bridging antibodies.

12. Transfer grids to 100-µl drops of PBS and wash 2 min. Repeat transfers to fresh drops of PBS seven times for a total of eight washes.

13. Transfer grids to 50-µl drops of 1% glutaraldehyde for 5 min.

    This step is necessary to stabilize the immunoreaction.

14. Transfer grids to 100-µl drops of water and wash 2 min. Repeat transfers to fresh drops of water seven times for a total of eight washes.

    This step is necessary to remove ions responsible for precipitates during contrast enhancement.

    When performing a double labeling, proceed with steps 3 to 14 with the second label.

Prepare labeled exosomes for electron microscopy

15. Contrast and embed the grids as in Support Protocol 4, steps 6 to 10.

16. Observe under the electron microscope at 80 kV. See Figure 3.22.2.

SUPPORT PROTOCOL 6

PREPARATION OF FORMVAR-CARBON COATED GRIDS

The successful adsorption of exosomal membranes onto grids depends on the quality of the support film. Commercially available Formvar-carbon coated grids can be purchased from Leica or Pelco International (*http://www.pelcoint.com*). This protocol describes a method that is currently used in several laboratories to prepare clean and strong Formvar films. (Also see Video 11 for UNIT 4.7 at *http://www.currentprotocols.com*.)

Materials

Ethanol
Acetone
Formvar powder (Agar Scientific, *http://www.agarscientific.com*)
Chloroform
EM grids, 200 mesh/copper-palladium, hexagonal specimen (e.g., PELCO; *http://tedpella.com*)
Warming plate or 37°C drying oven

100-ml volumetric flask: rinse with chloroform before use
Funnel-shaped glass column with a stopcock (e.g., PELCO; *http://tedpella.com*; Electron Microscopy Sciences): rinse with chloroform before use
Glass microscope slides
Lens tissue
Deep glass dish: rinse with chloroform before use
Clean forceps
Razor blades
Adhesive label (e.g., address label) or Parafilm
Petri dishes with tops
Filter paper
Carbon vacuum evaporator (Bal-tec, *http://www.bal-tec.com*)

Create Formvar film on microscope slide

1. Clean EM grids in a 200 ml glass flask, by rinsing them ~5 min with ethanol and then three times, ~3 min each, in acetone. Remove acetone.
2. Dry over a 37°C warming plate or in a drying oven.
3. Put 1.2 g Formvar powder into a 100-ml volumetric flask and add 100 ml chloroform with stirring. Let stand overnight wrapped in aluminum foil to protect from light.

   In order to prepare a clean solution all materials should be previously rinsed with chloroform.

4. Clean a glass slide with ethanol and dry it immediately with lens tissue.
5. Fill the glass column with Formvar solution. Using clean forceps, introduce the clean slide upright in the solution.

   The glass column is funnel-shaped and specially designed to accommodate the slide. The column should be previously rinsed with chloroform.

6. Open the stopcock to allow the Formvar to drain over the slide.

   This leaves a thin film of Formvar on the slide. The draining time determines the thickness of the film, and is usually 12 to 15 sec.

7. Remove the slide from the funnel and let the slide dry vertically.

Transfer Formvar film to EM grid

8. Fill a deep glass dish with water and place a light so that it reflects on the water surface. Clean the water surface by passing a piece of lens tissue over the surface.
9. Cut the edges of the dry Formvar film around the slide with a razor blade.
10. Put the slide on a water surface in an upright position. Push the slide gently into the water to allow the film to be released from the slide and to float on the water surface.

    The thickness of the film can be judged by its color, which ideally should be grey/white. If the films are yellow-purple, they are too thick. Either dilute the solution with chloroform or decrease the speed of draining with the stopcock.

11. Place the grids on the film floating on the water surface.
12. Remove the grids/film from the water by putting a microscope slide covered with an adhesive label in an upright position on the edge of the film, and pushing it downwards into the water.

    The film with the grids will stick to the label. Clean Parafilm can be used instead of the label.

13. Place the slides filled with grids in plastic dishes over a filter paper and let dry overnight at room temperature.

14. Evaporate a thin layer of carbon on the grids using a carbon-vacuum evaporator according to the manufacturer's instructions. Store in a covered petri dish.

SUPPORT PROTOCOL 7

DETERMINATION OF THE DENSITY OF AN EXOSOME PREPARATION ON A CONTINUOUS SUCROSE GRADIENT

This method measures the density of exosomes in a sucrose gradient. Exosomes have been found to float at densities ranging from 1.15 to 1.19 g/ml on continuous sucrose gradients. By comparison, vesicles purified from the endoplasmic reticulum float at 1.18 to 1.25 g/ml, and vesicles from the Golgi at 1.05 to 1.12 g/ml.

Since exosomes are usually spread among three to five fractions of the sucrose gradient, it is best to perform this separation on at least five times the amount of exosomal proteins needed to detect exosomes after this procedure. For example, to perform an immunoblot on the sucrose-gradient fractions using an antibody that detects its target among 3 μg of exosomal proteins, perform the sucrose gradient on 15 to 20 μg of exosomal proteins. To visualize exosomal proteins in the sucrose-gradient fractions by Coomassie blue staining on gels, use 100 μg of exosomal proteins, to obtain about 20 μg in each exosomal sucrose gradient fraction.

Materials

Exosome preparation (Basic Protocol 1, step 12 or Basic Protocol 2, step 8)
HEPES/sucrose stock solution (see recipe)
HEPES stock solution (see recipe)
4× SDS sample buffer (*APPENDIX 2A*)

30-ml gradient maker
Ultracentrifuge with SW 41 rotor
3-ml ultraclear tubes
Tabletop ultracentrifuge with TLA-100.3 rotor
3-ml thick-walled polycarbonate ultracentrifuge tubes
Refractometer

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), protein staining (*UNIT 6.6*), and immunoblotting (*UNIT 6.2*)

Prepare and pour exosome-containing sucrose gradient

1. For each gradient (i.e., each exosome preparation to be analyzed) prepare 5 ml of 2 M sucrose solution by mixing 4 ml HEPES/sucrose stock solution and 1 ml HEPES stock solution.

2. For each gradient, prepare 5 ml of 0.25 M sucrose solution by mixing 0.5 ml HEPES/sucrose stock solution and 4.5 ml HEPES stock solution.

3. Resuspend exosomes in 2 ml of HEPES/sucrose stock solution.

4. Pour exosomes at the bottom of an SW 41 centrifuge tube.

5. In the gradient maker with all shutters closed, load 5 ml of 2 M sucrose solution in the proximal compartment with a magnetic stir bar, and 5 ml of 0.25 M sucrose solution in the distal compartment.

6. Put the gradient maker on a high shelf, on a magnetic stir plate. Open shutter between proximal and distal compartments and turn on the magnetic stir plate.

7. Place the outer tubing in the SW 41 tube, just above the 2 ml of exosomes.
8. Open the outer shutter, and slowly pour a continuous 2 M (bottom) to 0.25 M (top) sucrose gradient on top of the exosomes.

   Lower the SW 41 tube as the gradient is poured, so that the tubing is always slightly above the top of the liquid.

   It is often worth pouring a test gradient on top of non-exosome-containing sucrose first, to get used to the procedure.
9. Balance all tubes containing gradient with each other, or with other tubes containing the same weight of sucrose solutions.

Centrifuge gradients and collect fractions

10. Centrifuge the gradients overnight (≥14 hr) at 210,000 × *g*, 4°C, in the SW 41 swinging-bucket rotor with the brake set on low.

    Total centrifugation time must be at least 14 hr. Set the centrifuge on hold in the evening, and turn it off on arrival in the morning
11. With a micropipettor, collect eleven 1-ml fractions, from top to bottom. Put each fraction in a 3-ml tube for the TLA-100.3 rotor

Measure refractive index

12. Set aside, in separate wells of a 96-well plate, 50 µl of each fraction to use to measure the refractive index. Cover the plate with adhesive foil to prevent evaporation. Store up to 1 hr at room temperature.
13. Use a refractometer to measure the refractive index (hence the sucrose concentration, and the density) of 10 to 20 µl of each fraction from the material saved in the 96-well plate.

    A table for converting the refractive index into g/ml is available in the ultracentrifugation catalog downloadable from the Beckman website.

Prepare fractions for analysis by SDS-PAGE

14. Add 2 ml of 20 mM HEPES, pH 7.4, to each 1-ml gradient fraction, and mix by pipetting up and down two to three times.
15. Mark one side of each tube with a permanent marker, and place the tubes marked side up in a TLA-100.3 rotor.
16. Centrifuge the 3 ml-tubes containing diluted fractions 1 hr at 110,000 × *g*, 4°C.

    The TLA-100.3 rotor holds six tubes, so it is necessary to perform two centrifugations for each gradient. Keep the other tubes at 4°C until they can be centrifuged.
17. Aspirate the supernatant from each of the 3-ml tubes, leaving a drop on top of the pellet.

    The pellet most probably is not visible, but its location can be inferred from the mark on the tube. See Basic Protocol 1.
18. Resuspend the invisible pellet and transfer to microcentrifuge tube.
19. Store half of each resuspended fraction up to 1 year at −80°C. To the other half of each resuspended fraction add one third of that volume of 4× SDS sample buffer.

    This will result in a 1 × final concentration of SDS sample buffer.

    The stored fractions can be used for other purposes (e.g. functional analysis, or further purification by immunoisolation, step 22) once gel analysis (step 21) has revealed which fractions contain exosomes.

20. Heat samples in SDS sample buffer from step 19a to 70°C for 10 min or 95°C for 5 min, and load half onto a 10% or 12% SDS-PAGE gel for further analysis (*UNIT 6.1*).

21. Analyze the gel by immunoblotting (*UNIT 6.2*) to visualize different exosome markers (Table 3.22.2), or Coomassie blue staining (*UNIT 6.6*) to visualize all proteins.

    Most proteins should be recovered in fractions with densities of 1.15 to 1.20 g/ml. Contaminants that are not specifically associated with exosomes will be recovered in the densest fractions collected from the bottom of the tube (>1.25 g/ml)

Purify fractions by immunoisolation

22. Thaw frozen fractions from step 19 on ice, and pool exosome-containing gradient fractions (as determined in step 21). Dilute to 1.5 ml final with PBS.

23. In one microcentrifuge tube, combine 750 µl of the pooled fractions with 200 µl of antibody-coated Dynabeads (8×10^7 beads). In another microcentrifuge tube, combine 750 µl with 200 µl of goat anti mouse IgG-coated Dynabeads as a negative control.

24. Proceed further as in Basic Protocol 3.

    See Wubbolts et al., 2003 for more details.

SUPPORT PROTOCOL 8

IMMUNOBLOT ANALYSIS OF EXOSOMES

The overall protein composition of exosomes from various cellular sources has been investigated by several groups, using extensive proteomic analysis, immunoblotting (western blots), or FACS. Exosome sources include various immortalized cell lines or tumor cells (Clayton et al., 2001; Skokos et al., 2001; Blanchard et al., 2002; Van Niel et al., 2003; Wubboltz et al., 2003; Amzallag et al., 2004; Fevrier et al., 2004; Hegmans et al., 2004; Mears et al., 2004; Segura et al., 2005), primary cells (Heijnen et al., 1999; de Gassart et al., 2003), or bodily fluids (Bard et al., 2004; Pisitkun et al., 2004; Caby et al., 2005). Several proteins were consistently found in exosomes from many of these different sources.

To characterize the purified vesicles as exosomes, it is important to show that most of the common exosomal proteins are present. Immunoblotting is a convenient way to accomplish this. Table 3.22.2 gives an overview of the exosomal proteins that can be identified by immunoblotting. To show that a given protein is specifically enriched in exosomes, it is important to compare on the same gel identical amounts of protein from exosomes and from total lysates prepared from the producing cells. To give a more accurate quantification and to allow detection of proteins which are not very abundant in either exosomes or cells, two different amounts of exosomal or total cell proteins are run side by side on the SDS-gel for subsequent immunoblot analysis.

Materials

Exosomes (pellet from Basic Protocol 1, step 10; Basic Protocol 2, step 8; or Basic Protocol 3, step 8)
Whole cell lysates: prepared from same cell source as exosomes (Support Protocol 1, step 8)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
4× SDS sample buffer, reducing or nonreducing (i.e., with or without DTT or 2-mercaptoethanol; *APPENDIX 2A*)

Additional reagents and equipment for quantifying protein (Support Protocol 9), SDS-PAGE (*UNIT 6.1*); and western blots (immunoblotting; *UNIT 6.2*)

Table 3.22.2 Characteristic Protein Markers of Exosomes, as Assessed by Immunoblot

Protein	MW (kDa)	Cells types with exosomes containing the protein[a]	Enrichment in exosomes	Remarks
Alix	96	DC, M, U	Medium	MVB marker
Annexin II	38	DC, M, IEC, MC, Mov, U	Low	
B7-2	80	DC, B, Mast	High	Not all cells express B7-2
Calnexin	96		Absent in exosomes	Endoplasmic reticulum marker
CD9	25	DC, IEC, U, P	High	Do not use 2-mercaptoethanol or DTT in samples
CD63, CD81	50-60	Human: DC, B, IEC	High	Do not use 2-mercaptoethanol or DTT in samples
Clathrin	192	DC, IEC, B, HEK	Medium	
Flotillin-1	48	DC, Mov, U, Ret	Medium	Associated with lipid rafts
Gi2α	40	DC, Mov, U, B, Ret	Medium	
Grp94 (or gp96)	96		Absent in exosomes	Endoplasmic reticulum marker
Hsc70	70	DC, M, MC, Mov, U, B, Ret, Mast	Low	
ICAM-1	90	Mature DC, B	Variable, depending on the cell type	Do not use 2-mercaptoethanol or DTT in samples
Lamp-1 or 2	90	DC, B	Low	Enrichment of lamp-1 or lamp-2 on exosomes depends on the cell type
MFG-E8	70 + 60	DC, IEC, Mov	Very high (not detectable in cell lysates)	Not all cells express MFG-E8
MHC I	47	DC, IEC, MC, U, B, T, HEK	Medium	
MHC II	30 (α chain)	DC, IEC, B, T, Mast	High	Not all cells express MHC II
Transferrin receptor	90	DC, Mov, Ret	Variable, depending on the cell type	
Tsg101	44	DC, Mov, U	Medium	MVB marker

[a]Abbreviations for cell types: B, B cells; DC, dendritic cells; HEK, human embryonic kidney cells; IEC, intestinal epithelial cells; Mast, mast cells; M, melanoma cells; MC, mesothelioma cells; Mov, immortalized Schwann cells; MVB, multivesicular bodies; P, platelets; Ret, reticulocytes; T, T cells; U, urine.

1. Quantify the protein content of all the samples (exosomes and cell lysates) in the same assay (Support Protocol 9).
2. For each sample, prepare a tube with 10 μg and a tube with 3 μg of cell lysate, a tube of 3 μg and a tube of 10 μg (if possible) of exosomes.
3. Add PBS to a final volume of 20 μl in each tube.
4. Add 6 μl of 4× SDS sample buffer to each tube.
5. Heat to 70°C for 10 min or 95°C for 5 min.
6. Load samples on a 10% or 12% gel, putting the two exosome samples and the two lysate samples next to each other.

   See UNIT 6.1 for more information about SDS-PAGE.

7. Run the gel, transfer to a nylon membrane, and analyze by immunoblots (*UNIT 6.2*) with antibodies specific for exosomes markers, as listed in Table 3.22.2.

SUPPORT PROTOCOL 9

MEASURING THE PROTEIN CONTENT OF EXOSOMES USING THE BRADFORD ASSAY

Measuring the amount of total proteins present in the exosome preparations gives a rough idea of the amount of exosomes secreted by the cells. When performing immunoblots with exosomes and total cell lysates, or when comparing different exosome preparations on the same immunoblot, it is important to perform the protein quantification by the Bradford assay on all the samples at the same time. The protocol given below is a classical version of the assay adapted to a large number (≤80) of precious samples (it uses only 10 μl of each sample). It allows accurate quantification of many samples at once, by running them side by side with a standard BSA dilution curve. (Also see *APPENDIX 3H.*)

Materials

BSA standards (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Cell lysates (Support Protocol 1, step 8)
Exosome preparations (Basic Protocol 1, step 12 or Basic Protocol 2, step 8)
Bradford concentrate solution (Bio-Rad)

0.5-ml microcentrifuge tubes
Flat-bottom 96-well plates
Microplate reader with 590 nm filter

1. Thaw a set of BSA standards, and store up to 2 weeks at 4°C.

2a. *For Bradford assay of total cell lysates*: Thaw the cell lysate in ice and dilute 10 μl in 90 μl PBS (i.e., 1/10).

   This step will reduce the detergent used to lyse cells to a level that will not interfere with the color reaction of the Bradford assay.

2b. *For Bradford assay of exosome preparations*: Thaw exosomes on ice.

3. In a flat-bottom 96-well plate, load 10 μl of PBS in the first well (blank) and 10 μl of each standard BSA dilution in the eight following wells.

   Perform all protein determinations for standards and samples in duplicate.

4. Load 8 μl of PBS to each well for cell lysate samples, and 6 μl of PBS to each well for exosome samples.

5. Add 2 μl of cell lysates to the wells containing 8 μl PBS.
6. Homogenize each exosome sample by pipetting up and down several times, then load 5 μl in the wells containing 5 μl PBS.
7. Dilute Bradford concentrate solution 1/5 in distilled water. Prepare enough for 200 μl per well.
8. Add 200 μl of diluted Bradford solution to each blank, standard, and sample well.

   The color changes immediately. If the color of some samples is obviously out of line with the BSA standard curve, prepare new wells immediately with more or less sample, PBS to 10 μl, and 200 μl of diluted Bradford solution.

9. Read OD at 590 nm within 10 min.
10. Estimate the amount of exosomes purified per cell number based on the standard curve.

    The authors usually obtain 0.5 μg exosomes/10^6 immature dendritic cells in 24 hr. The amounts are quite variable with other cell lines.

ANALYSIS OF EXOSOMES BY FACS OF LABELED EXOSOMES BOUND TO BEADS

SUPPORT PROTOCOL 10

Exosomes are too small to be reliably analyzed by direct cell sorting. To overcome this problem, exosomes are fixed to beads of a size that is in the detection range of a flow cytometer. The exosomes are then labeled with fluorophore-conjugated antibodies and analyzed by FACS.

Materials

Purified exosomes (Basic Protocol 1, step 12; or Basic Protocol 2, step 8; or Basic Protocol 3)
3.9-μm latex beads, surfactant-free aldehyde/sulfate, 4% solids (Interfacial Dynamics 12-4000; *http://www.idclatex.com*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS/1 M glycine
PBS/0.5% (w/v) BSA
Fluorochrome-conjugated primary or secondary antibodies

Test tube rotator wheel for 1.5-ml microcentrifuge tubes
Microcentrifuge
Flow cytometer (e.g., FACScan; BD)

1a. *To analyze exosomes purified by antibody-coated bead*: Proceed directly to step 8.

1b. *To analyze exosomes purified by ultracentrifugation*: Incubate 5 μg purified exosomes as measured by Bradford assay (Support Protocol 9) with 10 μl latex beads 15 min at room temperature, in a 1.5-ml microcentrifuge tube.

   A nonspecific adsorption of all exosomal proteins to the latex beads occurs in this step.

2. Add PBS to a final volume of 1 ml, and incubate on a test tube rotator wheel 2 hr at room temperature.

   Overnight incubation at 4°C is also possible.

3. Add 110 μl of 1 M glycine (i.e., 100 mM final), mix gently and let stand on the bench at room temperature for 30 min.

   The purpose of this step is to saturate any remaining free binding sites on beads.

4. Microcentrifuge 3 min at 4000 rpm, room temperature. Remove the supernatant and discard.

5. Resuspend the bead pellet in 1 ml PBS/0.5% BSA and microcentrifuge 3 min at 4000 rpm, room temperature. Remove the supernatant and discard.

   If not enough proteins are used to coat beads, the bead pellet may be spread along the side of the tube. In this case, resuspend the beads by flushing the side of the tube with PBS/0.5% (w/v) BSA; the pellet will become visible after the next centrifugation

6. Resuspend the bead pellet and microcentrifuge as in step 5 to wash two more times.

7. Resuspend beads in 0.5 ml PBS/0.5% BSA.

8. Incubate 10 µl coated beads with 50 µl anti-exosomal protein antibody diluted in PBS/0.5% BSA 30 min at 4°C. Wash twice with 150 µl PBS/0.5% BSA. If necessary, incubate with 50 µl secondary antibody diluted in PBS/0.5% BSA 30 min at 4°C. Wash twice in PBS/BSA, resuspend in 200 µl of PBS/BSA.

   Use fluorophore-coupled primary antibodies, or noncoupled primary antibodies followed by fluorophore-coupled secondary antibodies. Always perform negative-control staining on another 10 µl of beads, using an irrelevant isotype-matched primary antibody.

   When analyzing exosomes purified with antibody-coated beads (Basic Protocol 3), use directly conjugated fluorescent antibodies for FACS, rather than antibodies requiring a secondary antibody step.

   If the fluorescence signal is weak with antibodies specific for abundant exosomal proteins (e.g., MHC II on dendritic cell exosomes), try to increase the amount of exosomes and/or decrease the amount of beads in step 1.

9. Analyze antibody-stained exosome-coated beads on a flow cytometer. Adjust the forward scatter (FSC) and side scatter (SSC) to see both single beads and bead doublets. Gate on both single and doublet beads to analyze fluorescence. Compare fluorescence obtained with specific antibody and with irrelevant isotype control.

   When performing flow cytometric analysis of exosome-coated Dynal beads it is important to be aware that these paramagnetic beads may cause blockages in certain flow cytometric instruments. This is due to the use of electro-magnetic valves controlling the fluidics of certain instruments. This may present a problem for some instruments including BD FACScalibur range and BD FACScanto. The authors have never experienced any such problems when using BD FACScan.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BSA standards

Prepare a set of standard dilutions with BSA, starting with 500 µg/ml, performing two-fold dilutions in PBS to 4 µg/ml (seven dilutions). Dispense 100-µl aliquots of the diluted BSA standard curve solutions into microcentrifuge tubes and store up to 6 months at –20°C.

Cell lysis buffer

300 mM NaCl
50 mM Tris, pH 7.4 (adjust pH of Tris base with drops of 6 N HCl)
0.5% Triton X-100 or NP-40
Store up to 1 year at room temperature
Add antiproteases cocktail (Roche) just before use

Glutaraldehyde, 1% (v/v)

Dilute EM-grade glutaraldehyde fixatives (commercially available as 8%, 25% or 70% aqueous solutions; Polysciences or Sigma) in 0.1 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*), to the appropriate dilution. Store up to 6 months at −20°C or up to 1 week at 4°C after thawing.

0.1 M PIPES or HEPES can also be used. The buffers used to prepare fixatives need to have a good buffering capacity to maintain a pH of about 7.4 during fixation.

HEPES stock solution

2.4 g *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 20 mM final)
300 H_2O
Adjust pH to 7.4 with 10 N NaOH, added slowly
Adjust volume to 500 ml with H_2O
Sterilize by passing through a 0.22-μm filter
Store up to 2 years at 4°C

HEPES/sucrose stock solution

2.4 g hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 20 mM final)
428 g protease-free sucrose (ICN; 2.5 M final)
150 ml H_2O
Adjust pH to 7.4 with 10 N NaOH
Adjust volume to 500 ml with H_2O
Store up to 2 years at 4°C

Methyl cellulose, 2% (w/v)

Heat 196 ml of distilled water to 90°C and add 4 g methyl cellulose (Sigma, 25 centipoise, M-6385) with stirring. Rapidly cool on ice with stirring, until the solution has reached 10°C. Continue slow stirring overnight at 4°C. Stop stirring and allow the solution "ripen" for 3 days at 4°C. Bring to a final volume of 200 ml with water. Centrifuge using polycarbonate centrifuge bottles with cap assemblies 95 min at 100,000 × *g*, 4°C. Collect the supernatant and store up to 3 months at 4°C.

Paraformaldehyde (PFA), 2% and 4% (w/v)

Dissolve 4 g of PFA powder (Polysciences) in 90 ml of 0.1 M sodium phosphate buffer (see *APPENDIX 2A*) and heat to 65°C while stirring, If needed, add drops of 1 N NaOH until the solution becomes clear. Bring to 100 ml with 0.1 M sodium phosphate buffer. Cool and filter. Store at −20°C in 4- to 10-ml aliquots. Use thawed aliquots immediately and do not refreeze. To make 2% glutaraldehyde, dilute 4% glutaraldehyde with 0.1 M sodium phosphate buffer.

Tris/sucrose/D_2O solution

30 g protease-free sucrose
2.4 g Tris base
50 ml D_2O
Adjust pH to 7.4 with 10 N HCL drops
Adjust volume to 100 ml with D_2O
Sterilize by passing through a 0.22-μm filter
Store up to 2 months at 4°C

Uranyl acetate (4% w/v), pH 4

Weigh 2 g of uranyl acetate (Polysciences) and dissolve in 50 ml distilled water. Store up to 4 months at 4°C, in a 20-ml plastic syringe protected from light. Just before use, filter the amount needed of uranyl solution with a 0.22-μm filter.

CAUTION: *Radioactive materials require special handling. See the institutional Radiation Safety Office for guidelines concerning proper handling and disposal.*

The uranyl acetate crystals are difficult to dissolve and it may be necessary to use a rotating wheel for mixing.

Uranyl-oxalate, pH 7

Mix 4% uranyl acetate, pH 4 (see recipe) with 0.15 M solution of oxalic acid (0.945 g in 50 ml distilled water), in a 1:1 ratio. Adjust the pH to 7 by adding 25% (w/v) NH_4OH in drops to prevent formation of insoluble precipitates. Store in the dark up to 1 month at 4°C.

COMMENTARY

Background Information

The purification of exosomes from cell culture supernatants or other biological fluids is not trivial. The most characteristic feature of exosomes that should be used both for their purification and characterization is their density. The most usual way of determining exosomes' density is on continuous sucrose gradients. A buoyant density of 1.23 to 1.16 g/liter, together with the presence of endocytic markers (e.g., tsg101 or alix), and images in whole-mount electron microscopy showing the characteristic cup shaped morphology are good evidence that one is dealing with exosomes. Some groups have argued that the ultrafiltration method (Lamparski et al., 2002) is less harmful and more reliable than the ultracentrifugation method described in this unit. The authors have compared the two preparation methods for the same dendritic cell supernatants side by side, using biochemical, morphological, and functional assays for exosomes. They found no significant differences. The ultrafiltration method is advisable mainly for clinical-grade applications of exosomes, because it allows rigorous control of the environment during the purification procedure.

The issue of exosome quantification also deserves some attention. Two approaches to quantifying the amount of exosomes in a given preparation have been used. One is to quantify the amount of a particulate exosomal protein using ELISA or immunoblotting. The main limitation of this approach is that it is very hard to distinguish between a modification of the number of molecules per exosome (e.g., the proportion of exosomes expressing any given marker) and a real modification in the amount of exosomes. The other approach for quantifying exosomes is to measure the protein concentration in the exosome preparations. In the authors' experience, if the FBS used for the production of the conditioned media is properly cleared and if one carries out the experiment carefully, the protein concentration has proven to be a reliable and reproducible approach to quantification.

Recent work on exosomes from antigen-presenting cells has caused renewed interest in this controversial field. It is the authors' feeling that the study of exosomes is now in a critical phase of scientific development. Their identity as a bone fide secreted subcellular compartment is established. The challenge for the next few years is to understand their physiological mode of action and to learn how to use them in therapeutic strategies (e.g., vaccination).

Critical Parameters and Troubleshooting

When preparing exosomes from cell culture supernatants, it is important to make sure that the cells are healthy (including no contamination with mycoplasma) and viable at the end of the exosome production period. If not, the product will be heavily contaminated with membrane fragments that are not exosomes. Fragments of apoptotic cells are bigger than exosomes when observed by whole-mount EM, do not migrate at the proper density on sucrose gradients, and contain high amounts of nuclear proteins, such as histones (Théry et al., 2001).

When designing experiments involving exosome purification, it is important to make sure that there is an assay to characterize the preparations and show that they contain exosomes and no other contaminants. Whole-mount

EM combined with immunoblotting is the best choice.

Aggregates of exosomes can often be observed in preparations obtained by ultracentrifugation. Resuspending the pellet in PBS containing EDTA may help, but it can also be detrimental if exosomes are subsequently used in functional assays.

Particular attention should be given to the centrifugation tubes used to prepare exosomes. Exosomal membranes may stick to some ultracentrifugation tubes, such as ultraclear tubes from Beckman (W. Stoorvogel, Faculty of Veterinary Medicine, Utrecht University, pers. comm.).

Anticipated Results

The amount of exosomes produced varies with the cell type. The authors usually obtain 0.3 to 0.5 μg of exosomes per 10^6 dendritic cells in 24 hr (Segura et al., 2005). Amounts of exosomes obtained from most murine tumor cell lines (e.g., fibrosarcomas, melanomas, carcinomas) are in the same range. In contrast, very small amounts of exosomes seem to be produced by a mouse mastocytoma (P815) or thymoma (EL4; C. Théry, A. Clayton, S. Amigorena, and G. Raposo, unpub. observ.) Intestinal epithelial adenocarcinoma cell lines seem to produce slightly fewer exosomes (~0.05 μg/10^6 cells; Van Niel et al., 2001). Given the lack of details in the literature, it is difficult to say today whether all cell types produce exosomes and at what level, but exosome production seems to be a rather general mechanism, and an average level of production of ~0.1 μg/10^6 cells may be taken as a rough general estimate.

Purified exosomes should appear by EM as 50- to 100-nm vesicles surrounded by a lipid layer with a cup-shaped morphology (Fig. 3.22.2A) and exosomal proteins on their surfaces. Smaller vesicles (<50 nm), not displaying the cup-shaped morphology, are sometimes observed in preparations of exosomes, especially if the centrifugation is performed at >100,000 × *g*. Such vesicles cannot be considered exosomes.

When separating 30 to 50 μg of exosomal proteins on 10% or 12% SDS-gels, there should be a pattern of bands ranging from the largest to the smallest sizes, different from the pattern obtained with whole cell lysates (Fig. 3.22.2B). Be aware that exosome preparations showing mainly one band at ~60 kDa cannot be considered as good preparations because they probably contain mostly BSA from the culture medium.

Time Considerations

Exosome purification

The time required to produce cell culture conditioned medium from which exosomes will be purified is 24 to 48 hr after the cells have reached about 70% confluency (which varies greatly with the cell type). The time required to prepare the exosome collection medium is 14 hr (overnight). It takes ~30 min to 1 hr to collect the conditioned medium, depending on the number of different cells and the volume of conditioned medium. The conditioned medium can then be stored for up to a week at 4°C. From this conditioned medium, the whole procedure to purify exosomes by ultracentrifugation takes ~4 hr (Basic Protocol 1). If it has been demonstrated that Alternate Protocol can be used, the whole exosome purification procedure will take 3 hr instead of 4 hr. For the extra sucrose-cushion step (Support Protocol 3), allow an extra 2.5 to 3 hr. To purify exosomes from viscous fluids (Basic Protocol 2) it takes 6 hr in total. Purification of exosomes by immunoisolation (Basic Protocol 3) requires 1 hr the first day and 45 min the next day (with a 24-hr incubation in between).

Exosome analysis

Preparation of whole-mounted exosomes for EM analysis takes a maximum of 1.5 hr. Immunohistochemical staining of exosomes for EM requires 2.5 hrs for single labeling. If the primary antibody is not directly recognized by protein A, an extra 1 hr is required for the use of a bridging antibody. For double labeling the time required is doubled. Analysis of exosomes by immunoblotting takes ~1 hr to prepare the samples, plus the time required to perform SDS-PAGE followed by electrotransfer and antibody incubations; the total time varies depending on the systems used and the laboratory's preferred protocols. For exosomes analysis by FACS, it takes 2.5 hr to prepare exosome-coated beads, followed by the time required for incubation in antibodies (30 to 60 min) and flow cytometric analysis (30 min to 1 hr).

Literature Cited

Amzallag, N., Passer, B.J., Allanic, D., Segura, E., Théry, C., Goud, B., Amson, R., and Telerman, A. 2004. TSAP6 facilitates the secretion of translationally controlled tumor protein/histamine-releasing factor via a nonclassical pathway. *J. Biol. Chem.* 279:46104-46112.

Andre, F., Schartz, N.E., Movassagh, M., Flament, C., Pautier, P., Morice, P., Pomel, C., Lhomme, C., Escudier, B., Le Chevalier, T., Tursz, T.,

Amigorena, S., Raposo, G., Angevin, E, and Zitvogel, L. 2002. Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* 360:295-305.

Andre, F., Chaput, N., Schartz, N.E., Flament, C., Aubert, N., Bernard, J., Lemonnier, F., Raposo, G., Escudier, B., Hsu, D.H., Tursz, T., Amigorena, S., Angevin, E., and Zitvogel. L. 2004. Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J. Immunol.* 172:2126-2136.

Bard, M.P., Hegmans, J.P., Hemmes, A., Luider, T.M., Willemsen, R., Severijnen, L.A., van Meerbeeck, J.P., Burgers, S.A., Hoogsteden, H.C., and Lambrecht, B.N. 2004. Proteomic analysis of exosomes isolated from human malignant pleural effusions. *Am. J. Respir. Cell Mol. Biol.* 31:114-121.

Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G., and Hivroz, C. 2002. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J. Immunol.* 168:3235-3241.

Caby, M.P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G., and Bonnerot, C. 2005. Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.* 17:879-887.

Clayton, A., Court, J., Navabi, H., Adams, M., Mason, M.D., Hobot, J.A., Newman, G.R., and Jasani, B. 2001. Analysis of antigen presenting cell derived exosomes, based on immunomagnetic isolation and flow cytometry. *J. Immunol. Methods* 247:163-174.

de Gassart, A., Geminard, C., Fevrier, B., Raposo, G., and Vidal, M. 2003. Lipid raft-associated protein sorting in exosomes. *Blood* 102:4336-4344.

Escola, J.M., Kleijmeer, M.J., Stoorvogel, W., Griffith, J.M., Yoshie, O., and Geuze, H.J. 1998. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* 273:20121-20127.

Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., Laude, H., and Raposo, G. 2004. Cells release prions in association with exosomes. *Proc. Natl. Acad. Sci. U.S.A.* 101:9683-9688.

Hegmans, J.P., Bard, M.P., Hemmes, A., Luider, T.M., Kleijmeer, M.J., Prins, J.B., Zitvogel, L., Burgers, S.A., Hoogsteden, H.C., and Lambrecht, B.N. 2004. Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am. J. Pathol.* 164:1807-1815.

Heijnen, H.F., Schiel, A.E., Fijnheer, R., Geuze, H.J., and Sixma, J.J. 1999. Activated platelets release two types of membrane vesicles: Microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 94:3791-3799.

Lamparski, H.G., Metha-Damani, A., Yao, J.Y., Patel, S., Hsu, D.H., Ruegg, C., and Le Pecq, J.B. 2002. Production and characterization of clinical grade exosomes derived from dendritic cells. *J. Immunol. Methods* 270:211-226.

Mears, R., Craven, R.A., Hanrahan, S., Totty, N., Upton, C., Young, S.L., Patel, P., Selby, P.J., and Banks, R.E. 2004. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics* 4:4019-4031.

Pan, B.T., Teng, K., Wu, C., Adam, M., and Johnstone, R.M. 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* 101:942-948.

Pisitkun, T., Shen, R.F., and Knepper, M.A. 2004. Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. U.S.A.* 101:13368-13373.

Rabesandratana, H., Toutant, J.P., Reggio, H., and Vidal, M. 1998. Decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) are released within exosomes during in vitro maturation of reticulocytes. *Blood* 91:2573-2580.

Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., Harding, C.V., Melief, C.J., and Geuze, H.J. 1996. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* 183:1161-1172.

Raposo, G., Kleijmeer, M.J., Posthuma, G., Slot, J.W., and Geuze, H.J. 1997. Immunogold labeling of ultrathin cryosections: Application in immunology. *In* Handbook of Experimental Immunology, 5th ed. (M.A. Cambridge, L.A. Herzenberg, D. Weir, L.A. Herzenberg, C. Blackwell, eds.), pp. 1-11. Blackwell Scientific, Oxford.

Segura, E., Nicco, C., Lombard, B., Véron, P., Raposo, G., Batteux, F., Amigorena, S., and Théry, C. 2005. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T cell priming. *Blood* 106:216-223.

Skokos, D., Le Panse, S., Villa, I., Rousselle, J.C., Peronet, R., David, B., Namane, A., and Mecheri, S. 2001. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J. Immunol.* 166:868-876.

Théry, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. 1999. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J. Cell Biol.* 147:599-610.

Théry, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J., and Amigorena, S. 2001. Proteomic analysis of dendritic cell-derived exosomes: A secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* 166:7309-7318.

Théry, C., Duban, L., Segura, E., Veron, P., Lantz, O., and Amigorena, S. 2002. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat. Immunol.* 3:1156-1162.

Van Niel, G., Mallegol, J., Bevilacqua, C., Candalh, C., Brugiere, S., Tomaskovic-Crook, E., Heath, J.K., Cerf-Bensussan, N., and Heyman, M. 2003. Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut* 52:1690-1697.

Van Niel, G., Raposo, G., Candalh, C., Boussac, M., Hershberg, R., Cerf-Bensussan, N., and Heyman, M. 2001. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* 121:337-349.

Vidal, M., Mangeat, P., and Hoekstra, D. 1997. Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation. *J. Cell Sci.* 110:1867-1877.

Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Théry, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., Tursz, T., Angevin, E., Amigorena, S., and Zitvogel, L. 2001. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat. Med.* 7:297-303.

Wubbolts, R., Leckie, R.S., Veenhuizen, P.T., Schwarzmann, G., Mobius, W., Hoernschemeyer, J., Slot, J.W., Geuze, H.J., and Stoorvogel, W. 2003. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J. Biol. Chem.* 278:10963-10972.

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. 1998. Eradication of established murine tumors using a novel cell-free vaccine: Dendritic cell-derived exosomes. *Nat. Med.* 4:594-600.

Contributed by Clotilde Théry, Sebastian Amigorena, and Graça Raposo
Institut Curie
Paris, France

Aled Clayton
Cardiff University
Whitchurch, Cardiff United Kingdom

Isolation of Intermediate Filaments

Intermediate filaments (IFs) are 8- to 12-nm-diameter filaments that are made up of intermediate filament proteins (IFPs). IFPs constitute a family of proteins that have similarities in structure and are defined by their ability to assemble into IFs (Parry and Steinert, 1995). There are at least 65 distinct human IFPs, and each exhibits a distinct tissue distribution. Therefore, this unit covers the purification of a large and diverse group of related proteins. The conditions used to purify each of these proteins have to be optimized depending on the particular IFP. Nonetheless, all IFs share a basic property of being highly insoluble in conventional extraction buffers. As a result, most of the unwanted soluble proteins can easily be removed by extracting with nondenaturing buffers. IFPs are commonly purified under denaturing conditions using chaotropic reagents such as urea or guanidinium hydrochloride. Purifications are performed by ion-exchange chromatography, and the purified proteins can be reassembled into filaments by dialysis. This reassembly procedure can also be used as an additional purification step. The procedures for purification of IFs are illustrated by the example of purification of neurofilament proteins from bovine spinal cord (see Basic Protocol) as well as the purification of recombinant neurofilament protein from transformed bacteria with a prokaryotic expression system (see Alternate Protocol). Neurofilaments are the predominant IF network in mature neurons and are composed of the light (NFL), medium (NFM), and heavy (NFH) subunits of neurofilament triplet proteins (NFTPs). A discussion of various purification methods for other IF proteins is included in the Commentary.

ISOLATION OF NEUROFILAMENTS FROM BOVINE SPINAL CORD

BASIC PROTOCOL

This protocol describes the purification of neurofilament proteins (NFL, NFM, and NFH subunits) from bovine spinal cord and the procedure for reassembling the purified proteins into neurofilaments in vitro (Liem, 1986).

Materials

Fresh bovine spinal cord (can be obtained from a local slaughterhouse)
Solution A (see recipe), ice cold
Solution A-TSP (see recipe), ice cold
Buffers 1, 2, 3, and 4 (see recipes)
Bio-Gel HTP resin (Bio-Rad) or equivalent hydroxylapatite resin
10 mM sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*), degassed under vacuum
Assembly buffer (see recipe)
7.5% SDS-PAGE gel (*UNIT 6.1*)
DEAE-cellulose resin (Sigma or Whatman)
Buffer 4 (see recipe) containing 1% (v/v) 2-mercaptoethanol
Buffer 4 (see recipe), pH 6.5, containing 55 mM NaCl
Buffer 4 (see recipe), pH 7.0, containing 66 mM NaCl

Dounce homogenizer
Refrigerated centrifuge and centrifuge bottles
1.5 × 10–cm glass barrel chromatography column (Bio-Rad)
Peristaltic pump and fraction collector
0.45-μm syringe filters
Amicon Ultra-15 centrifugal filter units, 30-kDa MWCO (Millipore), or equivalent
Dialysis cassette, 10-kDa MWCO
Ultracentrifuge

Contributed by Conrad L. Leung and Ronald K.H. Liem
Current Protocols in Cell Biology (2006) 3.23.1-3.23.11

Additional reagents and equipment for spectrophotometric determination of protein (*APPENDIX 3B*) and SDS-PAGE (*UNIT 6.1*)

Prepare crude neurofilament proteins

1. Clean up a bovine spinal cord by removing the meningeal sheath and rinsing it with 4 liters of ice-cold solution A. Mince the spinal cord into small (~0.5-cm^3) pieces.

   CAUTION: *In order to minimize the danger of bovine spongiform encephalopathy (BSE), make sure to wear gloves when handling the animal tissues and always purchase the spinal cord from a slaughterhouse accredited for scientific research.*

   One spinal cord ~20 cm in length is sufficient for one neurofilament preparation. The bovine spinal cord can be replaced by bovine brain, rat or mouse brains, or mouse spinal cords. About 50 rat brains will provide the equivalent of one bovine spinal cord.

   The spinal cord should be transported on ice. Some vendors will deliver the spinal cord to the laboratory. If it must be picked up directly from the slaughterhouse, transport it in a styrofoam box with "blue ice."

2. Homogenize the spinal cord (~300 g) in 400 ml solution A in a Dounce homogenizer and centrifuge 30 min at 10,000 × g, 4°C.

   The volume of solution A should be adjusted based on the initial amount of starting material. However, the exact volume is not critical because the neurofilament proteins are insoluble under these conditions.

3. Remove and discard the supernatant and resuspend the pellet in 250 ml ice-cold solution A-TSP.

4. Centrifuge the lysate 1 hr at 10,000 × *g*, 4°C.

5. Discard the supernatant and resuspend the pellet again in 250 ml ice-cold solution A-TSP.

   The supernatant contains myelin and undesirable soluble proteins. Myelin and other membranous material will float to the top of the sucrose.

6. Repeat step 4 and 5 until no more floating material (myelin) is extracted in the supernatant.

7. Dissolve the pellet, which contains the crude neurofilament proteins, in 50 ml room temperature Buffer 1. Centrifuge 30 min at 10,000 × *g*, 4°C, to remove insoluble material, and retain supernatant for purification of neurofilament proteins as described in steps 8 to 15, reserving an aliquot of supernatant for analysis by SDS-PAGE (see step 14).

   The preparation will contain considerable amounts of neurofilament proteins, as well as other cytoskeletal proteins such as glial fibrillary acidic protein (GFAP), vimentin, tubulins, fodrin, and microtubule-associated proteins. It can be stored at −80°C for future use.

Purify neurofilament proteins

8. Rehydrate Bio-Gel HTP with 10 mM NaH_2PO_4, pH 7.4, according to the manufacturer's protocol. Remove the fines by letting the resin settle and decanting several times.

9. Pour the hydrated Bio-Gel HTP into a 1.5 × 10–cm column and allow the resin to pack under gravity. When the bed is stable, attach the column to the peristaltic pump and fraction collector and equilibrate the column with two bed volumes of Buffer 1 at a flow rate of ~1 ml/min.

10. To prevent clogging of the column, filter the solution containing the crude neurofilament preparation (supernatant from step 7) with a 0.45-μm syringe filters.

11. Load a quantity of the filtered preparation containing up to 50 mg of protein onto the HTP column and wash the column with room temperature Buffer 1 at a flow rate of ~1 ml/min until all unbound proteins have been removed, as determined by monitoring the OD_{280} of the eluate (*APPENDIX 3B*).

    Chromatography should be done at room temperature, as the urea in Buffer 1 will precipitate at 4°C.

    The flowthrough and the eluate from the washing step should be saved for later SDS-PAGE analysis (see step 14). The flowthrough will contain GFAP, as well as other contaminants. GFAP can be further purified by a reassembly step, described below.

12. Elute the proteins by washing with Buffer 2 at a flow rate of ~1 ml/min, collecting the eluate in 2-ml fractions and monitoring the OD_{280} (*APPENDIX 3B*) until all protein has been eluted.

13. Elute remaining proteins with Buffer 3 at a flow rate of ~1 ml/min, collecting the eluate in 2-ml fractions and monitoring the OD_{280} (*APPENDIX 3B*) until all the protein has been eluted.

    Chromatography should be performed with a peristaltic pump and a fraction collector.

14. Analyze the fractions with the highest OD_{280} by electrophoresis on a 7.5% SDS-PAGE gel (*UNIT 6.1*).

15. Pool the fractions that contain the neurofilament proteins. If necessary, concentrate the protein to 1mg/ml using an Amicon Ultra-15 centrifugal filter unit (MWCO 30-kDa) according to the manufacturer's instructions.

    Figure 3.23.1A shows a gel of purified neurofilament proteins. The neurofilament proteins are usually eluted in Buffer 3, which contains 300 mM sodium phosphate. The preparation contains all three neurofilament proteins, NFL, NFM, and NFH. Their apparent molecular weights are about 67 kDa, 150 kDa, and 200 kDa, respectively. The three subunits can be purified by additional ion-exchange chromatography, but the bacterial expression systems described below (see Alternate Protocol), have simplified these more difficult steps.

Perform in vitro assembly of neurofilaments

16. Dialyze 10 ml of the purified neurofilament proteins in a 10-kDa MWCO dialysis cassette against 2 liters assembly buffer for 4 hr at 4°C, then change the buffer and continue dialyzing overnight at 4°C to assemble proteins into filaments.

17. Collect the assembled filaments by ultracentrifuging 1 hr at 100,000 × *g*, 4°C, and removing the supernatant.

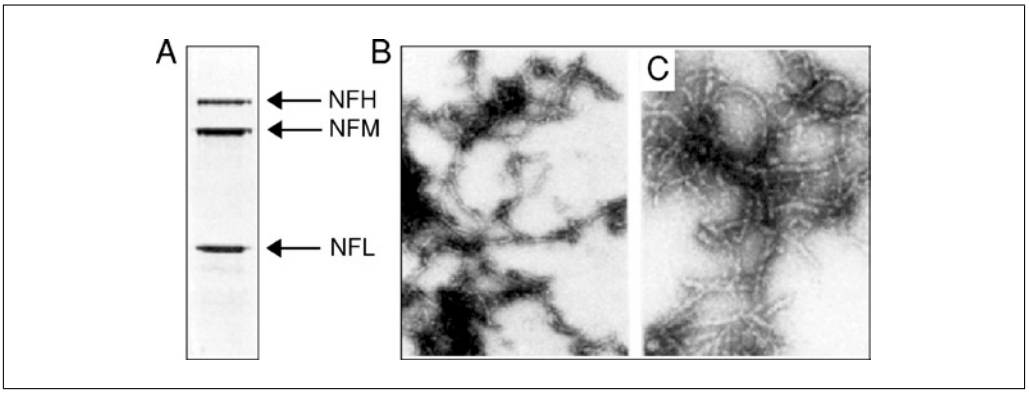


Figure 3.23.1 (**A**) Gel electrophoretic profile of purified neurofilament triplet proteins. (**B** and **C**) Electron micrographs of negatively stained reassembled 10-nm neurofilaments, (B) 37,500× and (C) 60,000×. Reproduced and modified with permission from *Biochemistry* 1982, 21:3221-3226. Copyright 1982 Am. Chem. Soc.

18. *Optional*: Visualize assembled filaments by negative staining under the electron microscope (see Fig. 3.23.1B and C; also see Liem and Hutchison, 1982).

Purify individual neurofilament subunits

After the initial purification and reassembly, the three neurofilament subunits can be further separated from each other by additional ion-exchange chromatography.

19. Prepare DEAE-cellulose resin according to the manufacturer's instructions. Pack a 1.5 × 10–cm column with ~40 ml of resin under gravity.

    The ion-exchange chromatography is performed at room temperature using a peristaltic pump set at a flow rate of 1 ml/min.

20. Connect the column to the peristaltic pump and equilibrate the column with 100 ml of Buffer 4.

21. Dissolve the assembled neurofilaments (pellet from step 17) at a concentration of ~1 mg/ml in Buffer 4 containing 1% (v/v) 2-mercaptoethanol. Apply 20 ml of the neurofilament solution to the column.

22. Wash the column with 80 ml of Buffer 4. Collect the void volume in 2-ml fractions, which will contain NFH.

23. Add 80 ml of 55 mM NaCl/Buffer 4, pH 6.5, and collect the eluate in 2-ml fractions, which will contain NFM.

24. Add 80 ml of 66 mM NaCl/Buffer 4, pH 7, and continue to collect the eluate in 2-ml fractions, which will contain NFH.

25. Analyze the collected fractions by SDS-PAGE (*UNIT 6.1*) and select those that contain the highest amount of the purified protein of interest.

    The purified proteins can be stored up to several months at –80°C.

    NFL obtained from this procedure usually still contains some NFM that has remained on the column. The two can be further separated by additional HTP chromatography. However, as described below (see Alternate Protocol), the use of recombinant proteins obtained from bacterial expression systems has made these further chromatography steps unnecessary. The main advantage of using the material from brain or spinal cord is that the proteins will be post-translationally modified, whereas the recombinant bacterial proteins will not. NFM and NFH are very heavily phosphorylated and NFL and NFM are known to be modified by O-linked N-acetylglucosamine (O-GlcNAc). Purified bovine neurofilament proteins are shown in Figure 3.23.2.

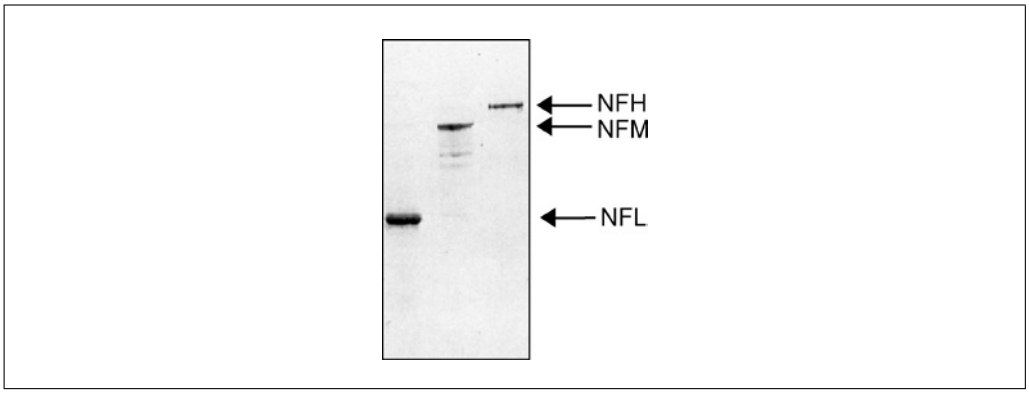


Figure 3.23.2 Gel electrophoretic profile of the separated neurofilament subunits. Reproduced with permission from *Biochemistry* 1982, 21:3221-3226. Copyright 1982 Am. Chem. Soc.

ISOLATION OF RECOMBINANT NEUROFILAMENT LIGHT SUBUNIT (NFL)

ALTERNATE PROTOCOL

This protocol describes the partial purification of NFL protein using a prokaryotic expression system. Crude NFL preparation can be further purified by HTP column chromatography and assembled into homopolymeric filaments by dialysis as described in the Basic Protocol.

Materials

NFL cDNA: generated by reverse-transcription PCR using RNA from brain of mouse (or other species) *or* obtained as EST clone from I.M.A.G.E. Consortium (*http://image.llnl.gov/*)

pET16d vector (Novagen) or equivalent

BL21(DE3) *E. coli* competent cells or equivalent

LB-ampicillin plates and LB-ampicillin liquid medium (*APPENDIX 2A*; see *APPENDIX 3A* for cross-references to selection methods)

100 mM isopropyl-β-D-thiogalactopyranoside (IPTG; see *APPENDIX 3A* for cross-references to protein expression methods)

2× SDS sample buffer (*APPENDIX 2A*)

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

Buffer, 1, 2, and 3 (see recipes)

Refrigerated centrifuge

Probe sonicator

Additional reagents and equipment for molecular biology techniques (cloning, restriction digestion, transformation of *E. coli*, selection of transformants and growth in LB-ampicillin media, induction of expression with IPTG; see *APPENDIX 3A*), and SDS-PAGE (*UNIT 6.1*)

Establish a bacterial culture that expresses NFL

APPENDIX 3A provides cross-references for the molecular biology techniques used in the following steps.

1. Clone NFL cDNA into the pET16d vector. Utilize the *Nco*I site of the vector for cloning so that the N-terminal His_6-Tag will be removed.

   One should avoid introducing a tag onto the protein because it may affect the ability of the IFPs to assemble into filaments.

2. Transform the pET-NFL construct into BL21(DE3) *E. coli* competent cells by heat shock or electroporation (*UNIT 20.5*) and incubate the transformants on LB-ampicillin plates overnight (*APPENDIX 3A*).

3. The following morning, pick 12 colonies with a loop and grow each of them in a 2-ml LB-ampicillin liquid culture to an OD_{600} of 0.4 (which usually takes ∼6 hr). When culture has reached that density, add 8 µl of 100 mM IPTG to induce expression.

4. Incubate cultures an additional 4 hr, then harvest 1.5 ml of each of the 12 cultures by centrifuging 3 min at 5000 × *g*, 4°C. Save the remaining 0.5 ml of each culture at 4°C.

5. Resuspend each cell pellet in 50 µl of 20 mM Tris·Cl, pH 7.5, then add 50 µl of 2× SDS sample buffer to lyse the cells. Heat lysates 5 min in a boiling water bath, then chill on ice and analyze 10 µl of each lysate by SDS-PAGE (*UNIT 6.1*) to evaluate expression levels of NFL.

6. Identify the bacterial colony that expresses the highest amount of NFL, streak out the remaining culture from that colony (see step 4) on an LB-ampicillin plate, and incubate overnight at 37°C to obtain new colonies.

7. Select a colony from the plate and grow overnight in a 2-ml LB-ampicillin liquid culture. The following day, mix 0.2 ml glycerol with 1 ml of that culture and freeze at –80°C to provide a frozen stock for future use.

A detailed protocol to evaluate protein expression in bacteria can be found in the pET system manual (Novagen, http://www.emdbiosciences.com/docs/docs/PROT/TB055.pdf). The authors have used this system to express a number of IFPs, including NFM, NFH, α-internexin, peripherin, and vimentin, and found that all of them are expressed very well in bacteria.

Express proteins

8. Inoculate a 2-ml LB-ampicillin liquid culture with the bacterial colony that has the highest level of NFL expression. Incubate overnight at 37°C.
9. The following day, collect the bacteria by centrifuging 2 min at 5000 × *g*, 4°C. Discard supernatant.
10. Resuspend the bacterial pellet in a small volume of LB-ampicillin medium and transfer the bacteria into 500 ml of LB-ampicillin medium. Grow the cultures at 37°C.
11. After the culture reaches OD_{600} ~0.6 (usually after ~4 hr), add 2 ml of 100 mM IPTG to induce NFL expression. Incubate 4 hr at 37°C.

Harvest and purify proteins

12. After 4 hr, harvest the cells by centrifuging 5 min at 5000 × *g*, 4°C. Proceed to the purification steps or store the bacterial pellet at −80°C for future use.
13. Resuspend the bacterial pellet in 20 ml of 20 mM Tris·Cl, pH 7.5. Lyse the cells by sonication on ice using a probe sonicator with the power level set between 4 and 5, at 40% to 50% duty cycle for 15 to 20 bursts.
14. Centrifuge the lysate 10 min at 14,000 × *g*, 4°C, and discard the supernatant.

The bulk of the NFL, as well as other IFPs are always found in the insoluble fraction.

15. Resuspend the insoluble protein pellet in 20 ml Buffer 1. Perform hydroxylapatite (HTP) column chromatography and SDS-PAGE analysis as described in the Basic Protocol, steps 8 to 14. Store purified NFL up to 3 months at –80°C.

NFL and other neurofilament proteins (NFM, NFH and α-internexin) will elute with Buffer 3. GFAP and vimentin usually elute in the flowthrough with Buffer 1.

NFL can be reassembled by dialysis as described in the Basic Protocol; the dialysis functions as an additional purification step. This reassembly step is not possible for the purification of NFM and NFH or other IFPs, such as the keratins, that are obligate heteropolymers, because they require a partner for coassembly. Many other IFPs (such as GFAP and vimentin) can also be reassembled.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Assembly buffer

0.1 M KCl
10 mM sodium phosphate buffer, pH 6.8 (*APPENDIX 2A*)
1 mM EGTA
1 mM $MgCl_2$
Store with above components up to 6 months at room temperature
Immediately before use, add:

continued

1 mM ATP
1× protease inhibitors (see recipe for protease inhibitor stock solutions)
Adjust pH to 7.4

Buffer 1

8 M urea, deionized and filtered (see recipe)
10 mM sodium phosphate buffer, pH 7.4
0.1% (v/v) 2-mercaptoethanol
1× protease inhibitors (see recipe for protease inhibitor stock solutions)
Prepare fresh

Buffer 2

8 M urea, deionized and filtered (see recipe)
100 mM sodium phosphate buffer, pH 7.0
0.1% (v/v) 2-mercaptoethanol
1× protease inhibitors (see recipe for protease inhibitor stock solutions)
Prepare fresh

Buffer 3

8 M urea, deionized and filtered (see recipe)
300 mM sodium phosphate buffer, pH 7.0
0.1% (v/v) 2-mercaptoethanol
1× protease inhibitors (see recipe for protease inhibitor stock solutions)
Prepare fresh

Buffer 4

8 M urea, deionized and filtered (see recipe)
300 mM sodium phosphate buffer, pH 6.5 or 7.0 (depending on use in protocol; see Basic Protocol materials list)
1× protease inhibitors (see recipe for protease inhibitor stock solutions)
Prepare fresh

Protease inhibitor stock solutions

500× phenylmethylsulfonyl fluoride (PMSF) stock: Prepare 250 mM PMSF in ethanol; store in aliquots at room temperature.

5000× leupeptin stock: Prepare 5 mg/ml leupeptin in water; store in aliquots at –20°C.

5000× aprotinin stock: Prepare 5 mg/ml aprotinin in water; store in aliquots at –20°C.

Alternatively, complete Protease Inhibitor Cocktail Tablets (Roche) may be used.

Solution A

0.1 M NaCl
1 mM EDTA
10 mM NaH_2PO_4
Adjust pH to 6.8 using NaOH
Store up to 6 months at room temperature

Solution A-TSP

Solution A (see recipe) containing:
1% (v/v) Triton X-100
0.85 M sucrose
1× protease inhibitors (see recipe for protease inhibitor stock solutions)
Prepare fresh

Urea, 8 M, deionized and filtered

Prepare an 8 M solution of urea in water. Deionize by stirring with a mixed-bed ion exchanger resin (TMD 8, Sigma or equivalent) for at least 3 hr at room temperature (until the color of the resin changes from greenish blue to yellowish brown). Remove the resin by filtering through Whatman filter paper in a funnel. Store up to 3 months at room temperature. Filter through a 0.45-μm filter immediately before use in the preparation of other solutions.

COMMENTARY

Background Information

IFs are composed of a diverse family of proteins that are expressed in a highly cell-type-specific manner, e.g., keratins in epithelial cells, neurofilaments in neurons, and vimentin in mesenchymal cells. Hence, different types of IFs can be isolated from different kinds of tissues or cultured cells. As illustrated in the Basic Protocol, neurofilament proteins can be purified from bovine spinal cord, although they can also be readily purified from spinal cord or brain from other species (Liem and Hutchison, 1982; Liem, 1986). Spinal cords are a more abundant source for the neurofilament proteins. Microtubule proteins and other cytoskeletal components are for the most part removed by the early cold extraction steps, as are most other soluble proteins. Myelin and other membrane proteins are removed by the sucrose-flotation step. After the repeated extractions and flotations, the major proteins left will be IFPs, i.e., GFAP and neurofilament proteins. NFTPs can be readily separated from GFAP by hydroxylapatite (HTP) chromatography. Advances in molecular biology have made the use of transformed bacteria that produce the IFP of interest the preferred method for isolating IFs (see Alternate Protocol). Subsequently, the purified recombinant proteins are assembled back into filaments in vitro. The advantage of this method is that it is relatively simple to perform and easy to scale up, and the assembled IFs are entirely composed of the desired IFPs. However, the method also has inherent disadvantages. First, it may be difficult to express some of the mammalian proteins in bacteria. Although many IFPs have been successfully purified from transformed bacteria, some others may be toxic to the bacteria and make it difficult for them to be expressed. For example, Heitlinger et al., (1991) experienced difficulty in expressing chicken lamin B_2 in the bacteria strain BL21 (DE3). Second, purified IFPs are not post-translationally modified in bacteria. Lack of post-translational modification does not seem to affect the polymerization process in vitro; however, in vivo, modifications such as phosphorylation have long been known to play regulatory roles in IF assembly. For example, phosphorylation of vimentin has been shown to inhibit filament formation (Eriksson et al., 2004). Furthermore, if the objective is to study IF-associated proteins, the target protein might only bind to post-translationally modified native IFs. Therefore, in some situations, it is important to execute the downstream experiments using both native and recombinant IFs.

IFPs have been divided into six major groups on the basis of their gene structure. Keratins belong to the type I and type II groups. There are at least 50 keratin genes, including the epithelial keratins (also designated "cytokeratin" or "soft" keratins) and the hair keratins (also known as the "hard keratins"). The keratin filaments in epithelial cells are obligate heteropolymers composed of at least one type I and one type II keratin protein. Isolation of epithelial keratins is quite similar to the isolation of neurofilament proteins, except epithelial cells or tissues are used as the starting materials. For crude keratin preparations, one can use the high-salt extraction protocol described by Chou et al. (1993); a step-by-step protocol can be found in Omary and Coulombe (2004). To further purify keratins, ion-exchange chromatography with Q-Sepharose columns has been commonly used; the bound proteins are eluted by a linear salt gradient (Coulombe and Fuchs, 1990; Fradette et al., 1998; Herrmann et al., 1999). To assemble keratin proteins into filaments in vitro, one needs to combine the matching keratin pair and dialyze them together in a Tris-based buffer (10 mM Tris·Cl, pH 7.4/0.2% 2-mercaptoethanol, with protease inhibitors).

Type III IFPs include vimentin, desmin, glial fibrillary acidic protein (GFAP), and peripherin; Type IV includes α-internexin and the neurofilament proteins. These IFPs were originally isolated from different sources: vimentin from BHK21 cells (Steinert et al., 1981), lens tissue (Geisler and Weber, 1981), and mastocytoma cells (Inagaki et al., 1987); desmin from chicken gizzard, porcine stomach, and skeletal muscle (Geisler and Weber,

1980; O'Shea et al., 1981); peripherin from PC12 cells (Parysek and Goldman, 1987); GFAP from bovine brain (Rueger et al., 1979); α-internexin from rat spinal cord (Pachter and Liem, 1985); and neurofilament proteins from bovine spinal cords (Liem and Hutchison, 1982). All of these IFPs have also been successfully purified as recombinant proteins from transformed bacteria. The purification procedures are similar to the protocols described above. With the exception of the neurofilament proteins, all of the type III and IV IFPs are able to self-polymerize in vitro into homopolymeric filaments. NFL can self-assemble into homopolymeric filaments in vitro, but NFM and NFH require NFL to form filaments.

Type V IFPs are nuclear lamins. Unlike other IFPs that form filamentous networks in the cytoplasm, lamins are the major structural constituents of the nuclear lamina. Hence, purification of nuclei should precede the isolation of lamins. Detailed protocols for isolating nuclear lamins from liver, transformed bacteria, and baculovirus expression systems can be found in Omary and Coulombe (2004).

Type VI IFPs include nestin, synemin, and paranemin. Paranemin is probably the avian ortholog of mammalian nestin. Nestin has been successfully purified from BHK-21 cells (Steinert et al., 1999), while synemin and paranemin were originally isolated from avian muscles (Sandoval et al., 1983; Bilak et al., 1998; Hemken et al., 1997). Detailed protocols for isolating these IFPs are described in Omary and Coulombe (2004). Recently, a muscle protein that shows sequence homology to IFPs has been isolated in a two-hybrid screen (Newey et al., 2001). The protein was named syncoilin and was found to be able to bind to desmin. However, desmin and syncoilin do not coassemble into heterofilaments, and syncoilin does not appear to participate in filament formation with any IFPs (Poon et al., 2002). Hence, syncoilin should not be considered as an IFP, and its isolation may be very different from isolating IFPs as described above.

In addition to these six general types of IFPs, lens fiber cells express two specific IFPs, filensin (also called CP115) and phakinin (also called CP49). Filensin and phakinin form a distinct filament network called beaded filaments in lens fiber cells and they can coassemble into 10-nm filaments in vitro. As expected, filensin and phakinin are purified from lens. Hydroxylapatite columns have been used successfully for purifying filensin (Quinlan et al., 1992), while DEAE-cellulose columns have been used for isolating phakinin (Goulielmos et al., 1996).

There are two common reasons for isolating IFs: (1) to study their in vitro assembly properties; and (2) to study their interaction partners (Omary and Coulombe, 2004). The assembly properties can be studied by electron microscopy, cross-linking analysis, and ultracentrifugation. To investigate potential interactions of associated proteins with IFs, one can perform overlay binding assays or centrifugation-based pull-down experiments.

Critical Parameters

The insolubility of IFs simplifies the purification procedures. In general, soluble proteins can be extracted with nondenaturing buffers before starting the purification of the IFPs. The IFPs are generally dissolved in chaotropic reagents such as urea or guanidium hydrochloride. Ionic detergents such as SDS are not recommended, because this will make it more difficult to reassemble the proteins. Reassembly of IFPs into filaments is accomplished rather easily by dialyzing against assembly buffer. The dialysis can also serve as an important purification step and can also remove some degraded proteins. However, this step is not possible for those proteins that cannot self-assemble, such as NFM and NFH, or for the keratins, which are obligate heteropolymers.

Troubleshooting

If protein degradation is observed: (1) make sure the bacterial lysate is not overheated during sonication, and/or (2) increase the concentration of protease inhibitors. Phenylmethylsulfonyl fluoride (PMSF; see *APPENDIX 1B*) is conventionally used at 0.1 mM; it may be necessary to increase its concentration up to 3 mM (Pytela and Wiche, 1980). Be cautious when handling PMSF; it is very toxic.

If impurities are detected: (1) perform a couple more rounds of extractions with solution A-TSP before loading onto the HTP columns; (2) further purify the IFPs by performing another round of ion-exchange chromatography with a different type of column such as the DEAE-cellulose column; or (3) conduct a couple of cycles of assembly-disassembly.

If there is no recombinant protein expression in the transformed BL21(DE3) bacteria: (1) try using other bacterial derivatives such as the Rosetta (Novagen) or BL-21-Codon Plus (Stratagene) *E. coli* strains, which are designed to alleviate codon bias when expressing heterologous proteins; (2) try using another

prokaryotic expression system, such as the pMAL system (New England Biolabs); or (3) consider using eukaryotic expression systems such as baculovirus or yeast.

If the IFP does not pellet well after centrifugation, this is probably due to contamination with genomic DNA. Add DNase I to the extraction buffer at 5 μg/ml or sonicate the crude cell lysate.

If the columns get clogged: (1) be sure to filter the crude IFP lysate before loading onto the column, and (2) degas all the chromatography solutions.

Anticipated Results

The yield of crude neurofilament proteins is about 14 mg per g bovine spinal cord. The yield from HTP chromatography is about 90 mg purified proteins per gram of crude neurofilament proteins. Hence, for a 300-g spinal cord, the total yield of neurofilament proteins is about 375 mg.

Time Considerations

Using a bovine spinal cord as the starting material, the entire purification procedure should take ~5 working days. The first day involves isolation of the crude neurofilament proteins. At the end of the day, crude neurofilament extracts can be stored in Buffer 1 at −80°C up to 3 months. The second day should be used for preparation of the HTP column, and chromatography should be done on day 3. The eluted material can also be stored in Buffer 2 or 3 at −80°C up to 3 months. Analysis of the eluted fractions by SDS-PAGE should be done on day 4. To prevent degradation, one should not assemble the filaments until one is ready to conduct the downstream experiments. It takes ~1 day to dialyze and assemble the filaments. To prepare recombinant proteins from bacteria, it will take ~1 week for cloning the cDNA into the bacterial expression vector and another week to test the optimal conditions for expressing the proteins. After identifying the bacterial colony that expresses the highest amounts of the desired IFP, one should prepare bacterial stocks with glycerol (15% final concentration) for long-term storage at −80°C.

Literature Cited

Bilak, S.R., Sernett, S.W., Bilak, M.M., Bellin, R.M., Stromer, M.H., Huiatt, T.W., and Robson, R.M. 1998. Properties of the novel intermediate filament protein synemin and its identification in mammalian muscle. *Arch. Biochem. Biophys.* 355:63-76.

Chou, C.F., Riopel, C.L., Rott, L.S., and Omary, M.B. 1993. A significant soluble keratin fraction in 'simple' epithelial cells. Lack of an apparent phosphorylation and glycosylation role in keratin solubility. *J. Cell Sci.* 105:433-444.

Coulombe, P.A. and Fuchs, E. 1990. Elucidating the early stages of keratin filament assembly. *J. Cell Biol.* 111:153-169.

Eriksson, J.E., He, T., Trejo-Skalli, A.V., Harmala-Brasken, A.S., Hellman, J., Chou, Y.H., and Goldman, R.D. 2004. Specific in vivo phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments. *J. Cell Sci.* 117:919-932.

Fradette, J., Germain, L., Seshaiah, P., and Coulombe, P.A. 1998. The type I keratin 19 possesses distinct and context-dependent assembly properties. *J. Biol. Chem.* 273:35176-35184.

Geisler, N. and Weber, K. 1980. Purification of smooth-muscle desmin and a protein-chemical comparison of desmins from chicken gizzard and hog stomach. *Eur. J. Biochem.* 111:425-433.

Geisler, N. and Weber, K. 1981. Isolation of polymerization-competent vimentin from porcine eye lens tissue. *FEBS Lett.* 125:253-256.

Goulielmos, G., Gounari, F., Remington, S., Muller, S., Haner, M., Aebi, U., and Georgatos, S.D. 1996. Filensin and phakinin form a novel type of beaded intermediate filaments and coassemble de novo in cultured cells. *J. Cell Biol.* 132:643-655.

Heitlinger, E., Peter, M., Haner, M., Lustig, A., Aebi, U., and Nigg, E.A. 1991. Expression of chicken lamin B2 in *Escherichia coli*: Characterization of its structure, assembly, and molecular interactions. *J. Cell Biol.* 113:485-495.

Hemken, P.M., Bellin, R.M., Sernett, S.W., Becker, B., Huiatt, T.W., and Robson, R.M. 1997. Molecular characteristics of the novel intermediate filament protein paranemin: Sequence reveals EAP-300 and IFAPa-400 are highly homologous to paranemin. *J. Biol. Chem.* 272:32489-32499.

Herrmann, H., Haner, M., Brettel, M., Ku, N.O., and Aebi, U. 1999. Characterization of distinct early assembly units of different intermediate filament proteins. *J. Mol. Biol.* 286:1403-1420.

Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M., and Sato, C. 1987. Site-specific phosphorylation induces disassembly of vimentin filaments in vitro. *Nature* 328:649-652.

Liem, R.K. 1986. Purification of neurofilament and their constituent polypeptides. *In* The Contractile Apparatus and the Cytoskeleton, Vol. 134 (R.B. Vallee ed.) pp. 380-387. Elsevier Academic Press, Orlando, Fla.

Liem, R.K. and Hutchison, S.B. 1982. Purification of individual components of the neurofilament triplet: Filament assembly from the 70,000-dalton subunit. *Biochemistry* 21:3221-3226.

Newey, S.E., Howman, E.V., Ponting, C.P., Benson, M.A., Nawrotzki, R., Loh, N.Y., Davies, K.E., and Blake, D.J. 2001. Syncoilin, a novel member

of the intermediate filament superfamily that interacts with alpha-dystrobrevin in skeletal muscle. *J. Biol. Chem.* 276:6645-6655.

O'Shea, J.M., Robson, R.M., Hartzer, M.K., Huiatt, T.W., Rathbun, W.E., and Stromer, M.H. 1981. Purification of desmin from adult mammalian skeletal muscle. *Biochem. J.* 195:345-356.

Omary, B. and Coulombe, P.A. 2004. Intermediate Filament Cytoskeleton. Methods in Cell Biology, Vol. 78 (L. Wilson and P. Matsundaira, eds.). Elsevier Academic Press, San Diego, Calif.

Pachter, J.S. and Liem, R.K. 1985. Alpha-Internexin, a 66-kD intermediate filament-binding protein from mammalian central nervous tissues. *J. Cell Biol.* 101:1316-1322.

Parry, D.A. and Steinert, P.M. 1995. Intermediate Filament Structure. R.G. Landes, Austin, Tx.

Parysek, L.M. and Goldman, R.D. 1987. Characterization of intermediate filaments in PC12 cells. *J. Neurosci.* 7:781-791.

Poon, E., Howman, E.V., Newey, S.E., and Davies, K.E. 2002. Association of syncoilin and desmin: Linking intermediate filament proteins to the dystrophin-associated protein complex. *J. Biol. Chem.* 277:3433-3439.

Pytela, R. and Wiche, G. 1980. High molecular weight polypeptides (270,000-340,000) from cultured cells are related to hog brain microtubule-associated proteins but copurify with intermediate filaments. *Proc. Natl. Acad. Sci. U.S.A.* 77:4808-4812.

Quinlan, R.A., Carter, J.M., Hutcheson, A.M., and Campbell, D.G. 1992. The 53 kDa polypeptide component of the bovine fibre cell cytoskeleton is derived from the 115 kDa beaded filament protein: Evidence for a fibre cell specific intermediate filament protein. *Curr. Eye Res.* 11:909-921.

Rueger, D.C., Huston, J.S., Dahl, D., and Bignami, A. 1979. Formation of 100 ~filaments from purified glial fibrillary acidic protein in vitro. *J. Mol. Biol.* 135:53-68.

Sandoval, I.V., Colaco, C.A., and Lazarides, E. 1983. Purification of the intermediate filament-associated protein, synemin, from chicken smooth muscle: Studies on its physicochemical properties, interaction with desmin, and phosphorylation. *J. Biol. Chem.* 258:2568-2576.

Steinert, P.M., Idler, W.W., Cabral, F., Gottesman, M.M., and Goldman, R.D. 1981. In vitro assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblastic cells. *Proc. Natl. Acad. Sci. U.S.A.* 78: 3692-3696.

Steinert, P.M., Chou, Y.H., Prahlad, V., Parry, D.A., Marekov, L.N., Wu, K.C., Jang, S.I., and Goldman, R.D. 1999. A high molecular weight intermediate filament-associated protein in BHK-21 cells is nestin, a type VI intermediate filament protein: Limited co-assembly in vitro to form heteropolymers with type III vimentin and type IV alpha-internexin. *J. Biol. Chem.* 274:9881-9890.

Contributed by Conrad L. Leung and Ronald K.H. Liem
Columbia University
New York, New York

Isolation of T-Tubules from Skeletal Muscle

The transverse tubule (T-tubule) system of a skeletal and cardiac muscle cell is a network of tubular invaginations of the sarcolemma that forms specifically functional associations with the sarcoplasmic reticulum, and they are responsible for conducting the action potential to the interior of the muscle fiber (Fig. 3.24.1). Thus, the signal for excitation-contraction coupling is transferred across the triad junction from T-tubules to the terminal cisternae of the sarcoplasmic reticulum to trigger Ca^{2+} release and muscle contraction.

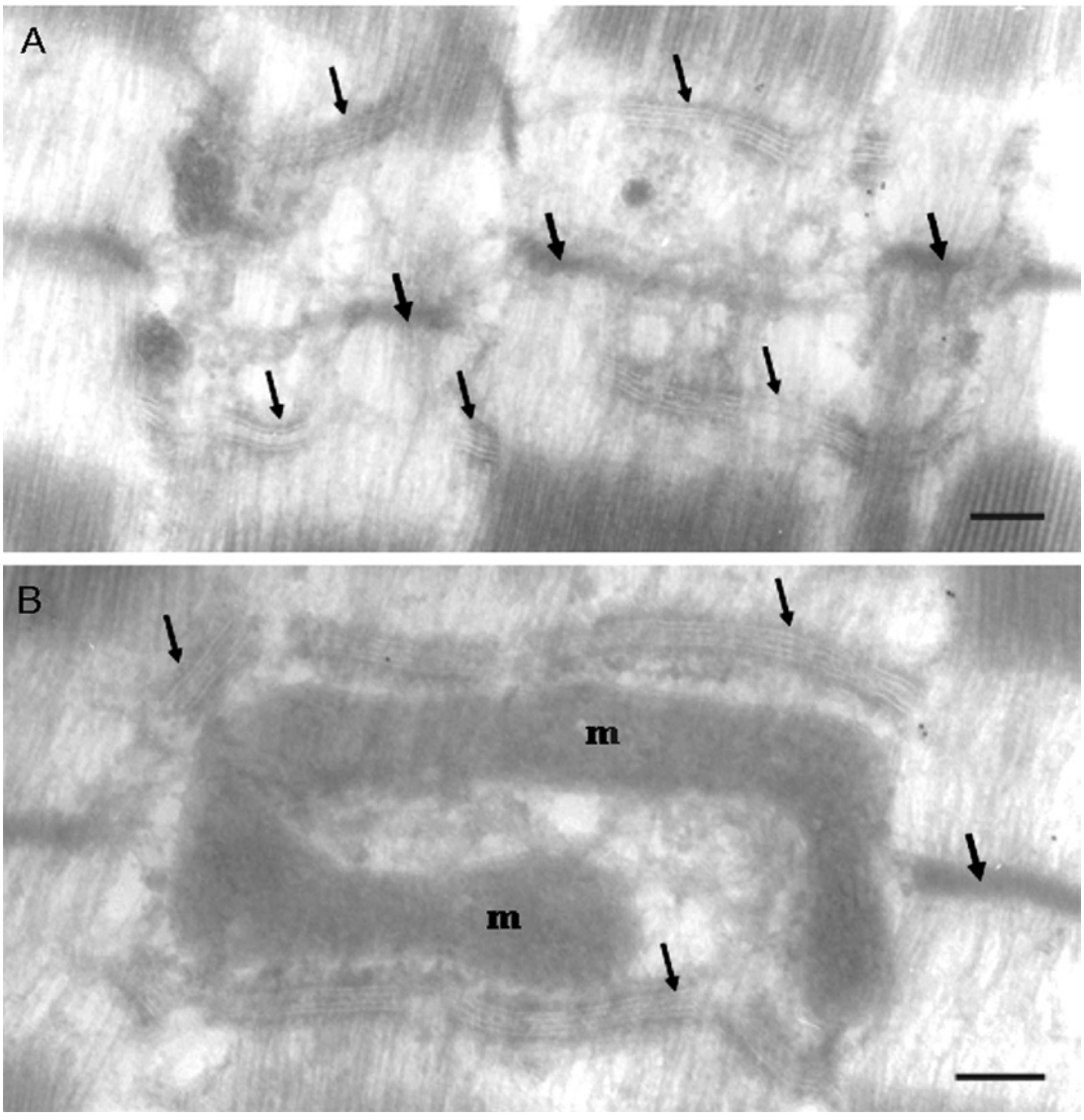


Figure 3.24.1 Electron micrograph of transverse tubules in rat skeletal muscle. Extensor digitorum longus muscle cryo-ultrasections were obtained from overnight-fasted rats and processed for electron microscopy. (**A**) Long sections of transverse tubules (T-tubules), indicated by arrows, run parallel to the Z-line. (**B**) Long stretches of T-tubules are close to mitochondria (m). Scale bars, 250 nm.

Contributed by Antonio Zorzano and Marta Camps
Current Protocols in Cell Biology (2006) 3.24.1-3.24.14

Two types of surfaces are distinguished in T-tubule membranes. A portion of T-tubule membranes faces into the junctional area with the sarcoplasmic reticulum, and that portion is called the junctional T membrane. The rest of the T-tubule membrane is in fragments of the network that do not participate in junction formation or that, if they are a junctional portion, face away from the junction itself.

T-tubules contain proteins relevant in the handling of Ca^{2+}, such as L-type Ca^{2+} channels (Carl et al., 1995; Brette and Orchard, 2003) and in the handling of Na^{+}, such as Na^{+}-K^{+}-ATPase or Na^{+} channels (Petrecca et al., 1999). In addition, T-tubules are sites for the initiation of certain signaling pathways that might be connected with the role of T-tubules in the transmission of membrane depolarization. T-tubules contain enzymes and other components important for the phosphoinositide pathway, which includes phospholipase C activity, G proteins, protein kinase C, phosphatidylinositol 4,5 bisphosphate, and inositol 1,4,5-trisphosphate (Di Virgilio et al., 1986; Toutant et al., 1990; Angelica et al., 1993; Hidalgo et al., 1993; Luise et al., 1993; Salvatori et al., 1993; Milting et al., 1994; Petrecca et al., 1999). T-tubules contain β-adrenergic receptors (Caswell et al., 1978). Colocalization of protein kinase A and calcineurin has also been detected in this compartment (Gao et al., 1997; Laflamme and Becker, 1999). T-tubules also contain abundant levels of insulin receptors and GLUT4 glucose transporters; therefore, insulin increases the abundance of GLUT4 in T-tubules (Marette et al., 1992; Munoz et al., 1995a; Wang et al., 1996). These data indicate that T-tubules participate in a large number of biological processes.

This unit focuses on the biochemical methods that permit one to obtain highly purified T-tubule membranes. Basic Protocol 1 focuses on the subcellular fractionation of rat skeletal muscle membranes to obtain membranes enriched in T-tubule markers. Two different methods for further purification of T-tubules are also described: wheat germ agglutination (Basic Protocol 2) and vesicle immunoisolation (Alternate Protocol).

BASIC PROTOCOL 1

SUBCELLULAR FRACTIONATION OF RAT SKELETAL MUSCLE MEMBRANES

This protocol is based in the sequential preparation of two crude membrane fractions, accomplished by a two-step homogenization from a single sample of rat skeletal muscle. These two different crude membrane fractions (F1 and F2) are washed with KCl, loaded with Ca^{2+}, and centrifuged on a sucrose gradient to obtain a variety of cell surface and intracellular membranes and, specifically, a fraction highly enriched in T-tubules (Figure 3.24.2). Of note, Ca^{2+} loading of sarcoplasmic reticulum membranes by incubation of fractions with ATP and $CaCl_2$ (Rosemblatt et al., 1981; Hidalgo et al., 1983) results in a T-tubule-enriched fraction that is free from sarcoplasmic reticulum membranes.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Rats (Wistar, male, ~250 g)
Homogenization buffer (see recipe), ice cold
4 M KCl
Ca^{2+}-loading buffer 1 (see recipe)
1 M ATP
150 mM $CaCl_2$
Ca^{2+}-loading buffer 2 (see recipe).
50 mM Tris·Cl, pH 7.2 (*APPENDIX 2A*)

Solution C (see recipe)
35%, 29%, 26%, and 23% (w/v) sucrose in sucrose gradient buffer (see recipe for buffer)
Tris-sucrose buffer (see recipe)
HEPES-sucrose buffer (see recipe)
Liquid nitrogen

Petri dish
Dissecting instruments
Polytron homogenizer with 1-cm-diameter probe
Sorvall refrigerated centrifuge with SA-600 rotor (or equivalent fixed-angle rotor)
Sorvall refrigerated high-speed centrifuge with T-647.5 rotor (or equivalent fixed-angle rotor)
Orbital shaker
Sorvall refrigerated high-speed centrifuge with TA-641 rotor (or equivalent swinging-bucket rotor) and 13-ml ultracentrifuge tubes

Additional reagents and equipment for Bradford protein assay (*APPENDIX 3H*)

Homogenize skeletal muscle

1. Excise 12 g of skeletal muscle tissue from the gastrocnemius and quadriceps muscles of three rats and place in a petri dish on ice. Trim muscles of fat and tendons, and mince into 2- to 4-mm^2 pieces.

2. Prepare six separate samples, each by homogenizing 2 g of minced skeletal muscle in 8 ml of ice-cold homogenization buffer using two 5-sec bursts of a Polytron homogenizer at low speed (setting 4; 6100 rpm) on ice.

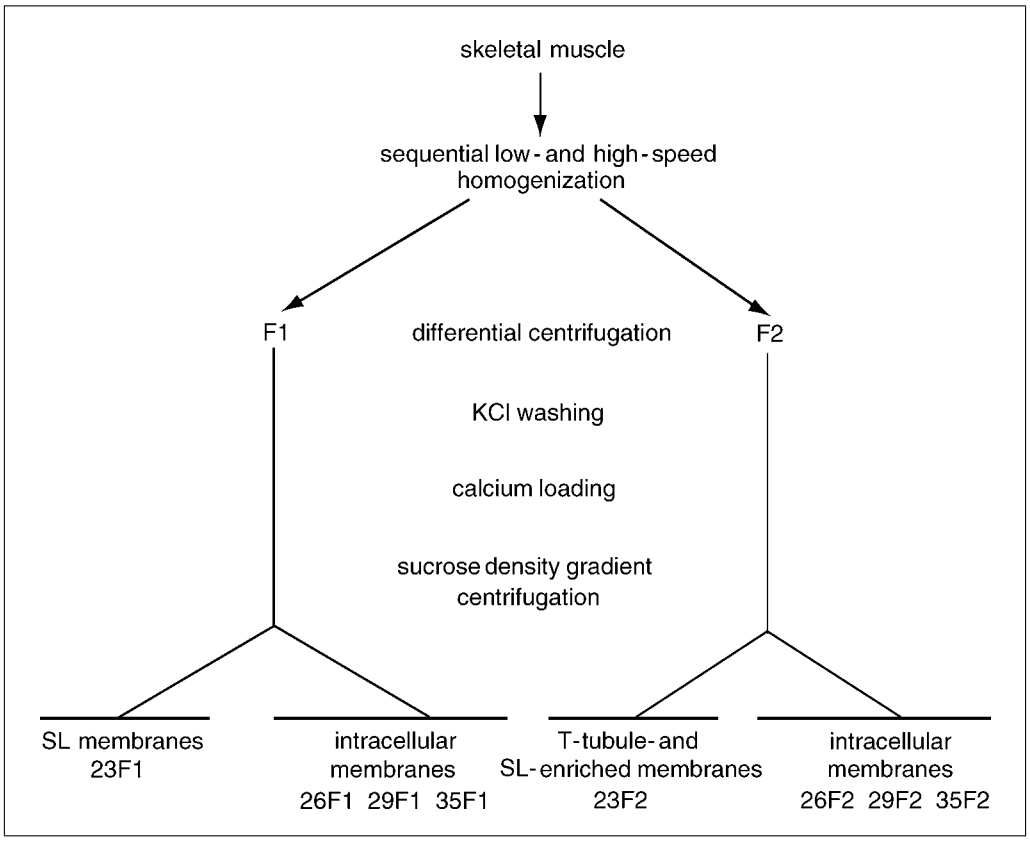


Figure 3.24.2 Flow chart of procedure used to isolate different membrane fractions from rat skeletal muscle. SL, sarcolemma.

Better control over homogenization can be achieved by splitting the sample into 2-g portions and homogenizing separately.

The speed of the homogenizer can be measured by a speed regulator (RECO-60) from Kinematica (http://www.kinematica.ch).

3. Centrifuge the homogenates separately for 20 min at 12,000 × *g* in a fixed-angle rotor, 4°C.

4. Collect the supernatants and store on ice.

5. Resuspend each pellet in 8 ml of homogenization buffer and centrifuge again for 20 min at 12,000 × *g*, 4°C.

6. Collect the supernatants of the second centrifugation and pool them with the supernatants from step 4.

   This pool is referred to as the F1 fraction (~100 ml).

7. Resuspend each of the six pellets in 8 ml ice-cold homogenization buffer and homogenize using two 30-sec bursts of the Polytron homogenizer at high speed (setting 6; 15,100 rpm) on ice.

   This second homogenization further disrupts muscle structures that may have resisted the initial homogenization step.

8. Centrifuge the homogenates 20 min at 12,000 × *g*, 4°C. Collect the supernatants and pool them together. Discard the pellets.

   This pool is referred to as the F2 fraction (~50 ml).

   The process described in steps 7 and 8 eliminates most of nuclei and mitochondria in the discarded pellet.

Solubilize myofibrils

9. Add 4 M KCl to F1 and F2 fractions for a final concentration of 0.6 M KCl. Incubate 1 hr at 4°C on an orbital shaker.

   This process solubilizes the myofibrils present in the membrane fractions.

10. Centrifuge each fraction 1 hr at 150,000 × *g* in fixed-angle rotor, 4°C.

11. Discard supernatants, which contain soluble myofibrillar proteins. Separately resuspend the pellets of F1 and F2 membrane fractions in ~2 ml Ca^{2+}-loading buffer 1.

12. Determine the protein concentration of each of the KCl-washed fractions using the Bradford method (*APPENDIX 3H*).

    Usually 25 mg and 35 mg of protein are recovered in F1 and F2 fractions, respectively.

13. Dilute the F1 and F2 fractions each to a concentration of 2 mg protein/ml with Ca^{2+}-loading buffer 1.

Load sarcoplasmic reticulum vesicles

14. To the KCl-washed F1 and F2 fractions, add 1 M ATP for a final concentration of 2 mM and 150 mM $CaCl_2$ for a final concentration of 0.3 mM.

15. Incubate membrane fractions for 20 min at room temperature on an orbital shaker. Every 5 min (i.e., at time points of 5, 10, and 15 min), add additional 1 M ATP and 150 mM $CaCl_2$ at volumes equivalent to 2 mM ATP and 0.3 mM $CaCl_2$.

    The process described in steps 14 and 15 loads the sarcoplasmic reticulum vesicles with calcium, thereby increasing their density.

Obtain membrane fractions

16. After the 20-min incubation, place the F1 and F2 fractions on ice and bring each solution to 60 ml with Ca^{2+}-loading buffer 2.
17. Centrifuge fractions 60 min at 150,000 × *g* in a fixed-angle rotor, 4°C.
18. Discard supernatants and resuspend each pellet in 1.5 ml of 50 mM Tris·Cl, pH, 7.2.
19. Determine the protein concentration of the resuspended membrane fraction by the Bradford method (*APPENDIX 3H*). Keep one 50-µl aliquot frozen for future analysis.

    Usually 15 and 25 mg of protein are recovered in F1 and F2 fractions, respectively.

20. Add 1.8 ml of solution C to each of the resuspended membrane fractions (F1 and F2).

Separate membranes

21. Prepare two discontinuous density gradients (designated for F1 and F2, respectively) in 13-ml ultracentrifuge tubes by carefully layering 3 ml of 35% sucrose at the bottom of the tube, then layering 2 ml of 29% sucrose over that, followed by 2 ml of 26% sucrose, and finally 2 ml of 23% sucrose as the upper layer. Keep gradients on ice.
22. Carefully layer the resuspended membrane fraction (F1 or F2) on top of its designated gradient. Centrifuge the gradients 12 hr at 77,000 × *g* in a swinging-bucket rotor, 4°C.
23. After centrifugation collect four protein fractions from each gradient:

    Fraction 23 on top of the 23% layer
    Fraction 26 from the 23%/26% interface
    Fraction 29 from the 26%/29% interface
    Fraction 35 from the 29%/35% interface.

    Also collect the pellets (pellet F1 and pellet F2).

24. Dilute all the collected fractions up to 60 ml with Tris-sucrose buffer. Centrifuge the diluted fractions 60 min at 150,000 × *g* in a fixed-angle rotor, 4°C, and remove the supernatants.
25. Resuspend pellets in HEPES-sucrose buffer at the following (typical) volumes:
    a. Add 0.3 ml to pellets obtained from the F1 and F2 fractions 23 and 35.
    b. Add 0.2 ml to pellets obtained from the F1 and F2 fractions 26 and 29.
    c. Add 0.6 ml to pellet F1 and pellet F2 (collected at the bottom of the original gradient; see step 23).
26. Determine the protein concentrations of each of the fractions by the Bradford method (*APPENDIX 3H*).

    F2 fraction 23 is highly enriched in T-tubule membranes and its protein yield is ~0.5 mg.

27. Divide fractions into 30- to 50-µl aliquots in microcentrifuge tubes and freeze in liquid nitrogen. Store at −80°C.

BASIC PROTOCOL 2

SEPARATION OF T-TUBULES FROM SARCOLEMMAL VESICLES BY WHEAT GERM AGGLUTINATION

This protocol is based on previously reported methods used in T-tubule preparations from heart and skeletal muscle (Charuk et al., 1989; Ohlendieck et al., 1991). The method consists of the separation of cell surface membranes that are susceptible to agglutination by wheat germ agglutinin (WGA), a lectin with high affinity for N-acetyl-D-glucosamine and sialic acid, which are components of membrane glycoproteins.

WGA-induced agglutination requires that the cell surface membrane vesicles be right-side-out so the carbohydrates are exposed to the incubation medium. Whereas most sarcolemmal vesicles are right-side-out, vesicles originating from T-tubule membranes show an inverse orientation such that the cytosolic side of the membranes are localized in the lumen of the vesicle (inside-out vesicles). As a consequence, T-tubule vesicles cannot be agglutinated by WGA, because the carbohydrates are located in the vesicular lumen. This protocol leads to efficient separation of T-tubule membranes from sarcolemmal membranes which, otherwise, share similar biophysical properties.

Materials

F2 fraction 23 (Basic Protocol 1)
50 mM potassium phosphate buffer, pH 7.4 (*APPENDIX 2A*), containing 160 mM NaCl
1 mg/ml wheat germ agglutinin (WGA; lectin from *Triticum vulgaris*, lyophilized powder, Sigma) in 50 mM phosphate buffer (pH 7.4)/160 mM NaCl
Tris-sucrose buffer (see recipe)
Deagglutination buffer (see recipe)
Liquid nitrogen

Beckman ultracentrifuge with TLS-55 rotor (or equivalent)
10-ml ultracentrifuge tubes for TLS-55 rotor

Expose T-tubule-enriched fractions to WGA

1. Thaw 300 μg of protein from F2 fraction 23 (enriched in T-tubules) by warming the tube at 37°C.
2. Dilute samples to 1 mg protein/ml with 50 mM phosphate buffer (pH 7.4)/160 mM NaCl.
3. Add 0.3 ml of 1 mg/ml WGA. Shake gently and incubate 10 min on ice.
4. Microcentrifuge 1.5 min at 15,000 × *g*, room temperature. Transfer the supernatant (WGA^- fraction, enriched in T-tubules) to a separate microcentrifuge tube on ice.
5. Resuspend the pellet containing the lectin-agglutinated vesicles (WGA^+ fractions) in 1 ml of 20 mM Tris-sucrose buffer and microcentrifuge 1.5 min at 15,000 × *g*, room temperature, and discard the supernatant. Repeat twice more for a total of three washes.

Harvest WGA^+ fraction (sarcolemmal vesicles)

6. Resuspend the WGA^+ pellet in 0.5 ml of deagglutination buffer. Incubate 20 min on ice.
7. Microcentrifuge the deagglutinated suspension 1.5 min at 15,000 × *g*, 4°C. Collect the supernatant, transfer to a 10-ml ultracentrifuge tube, and ultracentrifuge 60 min at 150,000 × *g*, 4°C.
8. *Optional:* Resuspend the WGA^+ pellet (sarcolemmal vesicles) in 50 μl Tris-sucrose buffer and freeze in liquid nitrogen. Store at −80°C.

   This fraction is harvested if a comparative analysis of sarcolemmal and T-tubule proteins is to be done.

Harvest WGA⁻ fraction (T-tubule vesicles)

9. Ultracentrifuge the nonagglutinated vesicles (supernatant from step 4) 60 min at 150,000 × *g*, 4°C. Remove the supernatant.

10. Resuspend the WGA⁻ pellet in 50 µl Tris-sucrose buffer and freeze in liquid nitrogen. Store at −80°C.

PURIFICATION OF T-TUBULES BY VESICLE IMMUNOISOLATION

ALTERNATE PROTOCOL

An alternative to the purification of T-tubules by WGA agglutination is the use of vesicle immunoisolation techniques. Central to these techniques is the use of antibodies that specifically recognize intracellular domains of membrane proteins selectively localized in T-tubules. This protocol makes use of an antibody raised against protein tt28, a major component of the transverse tubular membrane vesicles (Rosemblatt et al., 1981; Horgan and Kuypers, 1987). Antibodies can be coupled to different type of beads such as agarose or acrylamine (Zorzano et al., 1989, 1996; Munoz et al., 1995a,b; Munoz et al., 1996; Fischer et al., 1997). This protocol leads to efficient separation of T-tubule membranes from sarcolemmal membranes and, in addition, permits the separation of T-tubule membranes from intracellular membranes that also contaminate those membrane fractions.

Materials

Goat anti–mouse IgG coupled to agarose beads, saline suspension (5 to 10 mg antibody/ml bead suspension; Sigma)

Phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4

Anti-tt28 antibody, monoclonal (Rosemblatt and Scales, 1989), or other antibody directed against tt-28 or against other proteins such as DHPR (Morton and Froehner, 1987, 1989; Flucher et al., 1991)

Blocking solution: 1% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4 (prepare fresh)

T-tubule-enriched fraction (typically fraction 23 from F2; see Basic Protocol 1)

2× immunoadsorption solution (see recipe)

3× Laemmli sample buffer (see recipe)

Orbital shaker

95°C water bath or heat block

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

Couple anti-tt28 antibody to anti-IgG-coupled agarose beads

1. Pipet 75 µl of the suspension of goat anti-mouse IgG coupled to agarose beads into a microcentrifuge tube. Wash the beads by adding 1 ml PBS, shaking gently, and microcentrifuging 6 sec at maximum speed, room temperature. Discard supernatant and repeat washing step a second time.

2. Add 5 µg anti-tt28 antibody to the bead pellet, then bring to a final volume of 50 µl with PBS. Incubate overnight at 4°C with constant shaking on an orbital shaker.

3. Microcentrifuge 6 sec at maximum speed. Discard the supernatant (containing free anti-tt28 antibody) and wash the pellet twice with 1 ml of PBS using the technique described in step 1.

4. Wash the coupled beads by adding 1 ml blocking solution to the pellet, incubating 15 min at room temperature with sporadic shaking, then microcentrifuging 6 sec at maximum speed, room temperature, and discarding the supernatant. Repeat this wash procedure twice with blocking solution, then once with PBS using the same technique.

Immunoadsorb and collect vesicles

5. Add to 25 μg of protein from T-tubule-enriched fraction in 200 μl of 1× immunoadsorption solution to the coupled beads. Incubate beads overnight at 4°C with shaking on an orbital shaker.

6. Collect the agarose beads and the vesicles bound to them by microcentrifuging 6 sec at maximum speed, room temperature. Transfer the supernatant, which contains the unadsorbed membrane vesicles, to a new microcentrifuge tube. Also save the pellet, which contains the T-tubule vesicles.

7. Add 3× Laemmli sample buffer to the supernatant for a final concentration of 1×, incubate 5 min at 95°C, then microcentrifuge 10 sec at maximum speed. Collect the supernatant and analyze by SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*).

   Analysis of the unadsorbed membranes in the supernatant by SDS-PAGE and subsequent immunoblotting will allow the efficacy of immunoadsorption to be determined.

8. Wash the pellet from step 6 (which contains the vesicles that are bound to the immobilized antibody) twice, each time by adding 1 ml PBS, microcentrifuging 6 sec at maximum speed, room temperature, and discarding the supernatant.

9. Add 0.5 ml PBS to the pellet and incubate for 30 min at room temperature. Microcentrifuge 6 sec at maximum speed, room temperature, and discard the supernatant.

10. Elute the adsorbed material from the beads by adding 0.1 ml of 1× Laemmli sample buffer. Incubate 5 min at 95°C.

11. Microcentrifuge 6 sec at maximum speed, room temperature. Collect the supernatant (eluted material from the beads, containing the proteins that were bound as well as the eluted anti-tt20 antibody) and analyze by SDS-PAGE (*UNIT 6.1*).

    This fraction contains protein tt28 (28 kDa), the α_2 subunit of DHPR (170 kDa), GLUT4 (45 kDa), or the β subunit of insulin receptors (95 kDa).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ca^{2+}-loading buffer 1

50 mM potassium phosphate buffer, pH 7.5 (APPENDIX 2A), containing:
5 mM $MgCl_2$
150 mM KCl
Store up to 1 month at 4°C

Ca^{2+}-loading buffer 2

50 mM potassium phosphate buffer, pH 7.5 (APPENDIX 2A), containing:
5 mM $MgCl_2$
150 mM KCl
0.3 mM $CaCl_2$
Prepare fresh just before use

This buffer can be prepared immediately before use from Ca^{2+}-loading buffer 1 (see recipe) by adding 150 mM $CaCl_2$ to a final concentration of 0.3 mM $CaCl_2$.

Deagglutination buffer

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
250 mM sucrose
300 mM N-acetyl-D-glucosamine
Prepare fresh just before use

HEPES-sucrose buffer

30 mM HEPES, pH 7.4
250 mM sucrose
Prepare just before use and store on ice

Homogenization buffer

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
250 mM sucrose
1 mM EDTA
1 μM pepstatin
1 μM leupeptin
Prepare fresh just before use and store on ice

Immunoadsorption solution, 2×

Phosphate-buffered saline (PBS), pH 7.4 (APPENDIX 2A) containing:
0.2% (w/v) BSA
2 mM EDTA
Prepare fresh just before use

Laemmli sample buffer, 3×

0.3 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
60% (v/v) glycerol
6% (w/v) SDS
Pinch of bromphenol blue
Store in aliquots at –20°C

Solution C

90 mM $Na_4P_2O_7$
540 mM KCl
Store with above ingredients at 4°C
Just before use add 450 mM sucrose
pH should be 7.2 (determined by the 90 mM pyrophosphate)

Sucrose gradient buffer

20 mM Tris·Cl, pH 7.2 (*APPENDIX 2A*)
50 mM $Na_4P_2O_7$
300 mM KCl
Store at 4°C

Tris-sucrose buffer

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
250 mM sucrose
Prepare just before use and store on ice

COMMENTARY

Background Information

It has been reported that gentle homogenization of skeletal muscle favors the isolation of sarcolemmal membranes, which do not contain T-tubules (Burdett et al., 1987), whereas a more energetic homogenization of skeletal muscle favors the isolation of T-tubule membranes (Lau et al., 1977; Dunn, 1989). Based on these observations, a protocol involving two sequential homogenization steps differing in the speed at which the Polytron operates seems best suited for obtaining T-tubules that are less contaminated with sarcolemmal membranes. However, this procedure is not sufficient to bypass all of the problems associated with the purification of membranes from skeletal muscle. One difficulty is related to the high abundance of myofibrillar proteins in this tissue. In fact, myofibrillar proteins interact with many other cellular proteins and membranes, and

this is particularly the case after tissue homogenization. In order to prevent this nonspecific sequestering of membranes by myofibrillar proteins, homogenates are treated with high concentrations of KCl at 4°C, which destabilizes myofibrillar proteins and reduces their binding to membranes (Barchi et al., 1979). Another technical problem is the extremely high abundance of sarcoplasmic reticulum membranes in skeletal muscle; these contaminate most membrane fractions obtained from this tissue. To minimize this problem, the density of the sarcoplasmic reticulum vesicles is increased in vitro by loading them with Ca^{2+} in a process catalyzed by the high Ca^{2+}-ATPase activity.

The procedure explained in Basic Protocol 1 permits the generation of a membrane fraction highly enriched in T-tubules (named 23F2, referring to fraction 23 of F2; see Basic Protocol 1); this membrane fraction is characterized by the high abundance of the α_2

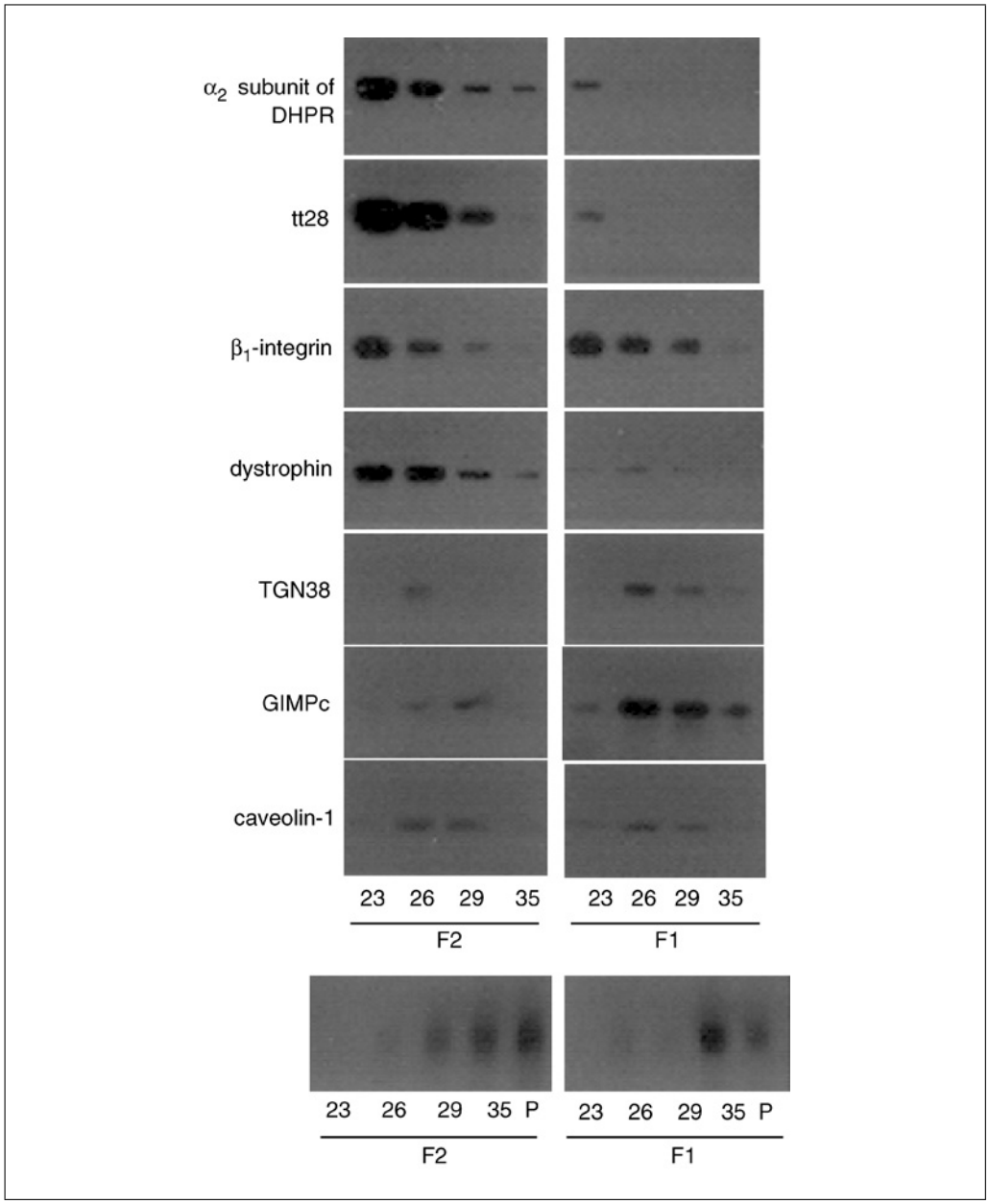


Figure 3.24.3 Demonstration that the fraction 23F2 is highly enriched in T-tubule markers and is essentially free from sarcoplasmic reticulum. The abundance of T-tubule markers (α_2 subunit of dihydropyridine receptors, DHPR, and protein tt28), sarcolemmal markers (β_1-integrin and dystrophin), trans-Golgi network (TGN38), cis-Golgi (GIMPc), sarcoplasmic reticulum (Ca^{2+}-ATPase), and endothelial cells (caveolin-1) is assayed in different membrane fractions obtained from rat skeletal muscle. The distribution of the different proteins is studied by immunoblot assays.

subunit of the dihydropyridine receptor and of the protein tt28, both of which are known markers of transverse tubules (Fig. 3.24.3). This membrane fraction is free from intracellular membrane components or sarcoplasmic reticulum, as indicated (see Fig. 3.24.3) by the absence of Ca^{2+}-ATPase or TGN38 (marker of the *trans*-Golgi network) or GIMPc (marker of the *cis*-Golgi). However, this fraction also contains proteins that are markers of sarcolemmal membranes, such as β_1-integrin or dystrophin (Fig. 3.24.3). In addition, a membrane protein that is only detected in endothelial cells in skeletal muscle, caveolin-1, is detected in very low levels in the membrane fraction 23F2 (Fig. 3.24.3). These data indicate that the membrane fraction highly enriched in T-tubules is also contaminated with sarcolemmal vesicles, and that it contains very low levels of endothelial cell–derived membranes. These characteristics are not specific to preparations obtained via the procedure explained in Basic Protocol 1, but are general properties of methods that permit the isolation of T-tubule membranes.

Purification of T-tubules from sarcolemmal membranes can be achieved through two different methods: separation of T-tubules in a nonagglutinable fraction (WGA^-) after treatment with wheat germ agglutinin (Basic Protocol 2) or immunoisolation of T-tubule vesicles using immobilized antibodies directed against proteins specific of the T-tubules (Alternate Protocol).

Treatment of fraction 23F2 (Basic Protocol 1) with WGA renders two fractions, an agglutinable fraction (WGA^+) and a nonagglutinable fraction (WGA^-). Usually these two fractions account for 74% and 26%, respectively, of the total protein recovered after WGA treatment. Most importantly, the WGA^- fraction is markedly enriched in the T-tubule protein markers tt28 and dihydropyridine receptors and shows no sarcolemmal protein markers such as β_1-integrin, dystrophin, or GLUT1 glucose transporters (Fig. 3.24.4). Thus, the WGA^- fraction constitutes a highly purified T-tubule fraction that is essentially devoid of sarcolemmal markers. It is also likely that this fraction shows low contamination with membranes from endothelial origin, as assessed by the presence of caveolin-1 (Fig. 3.24.4).

Immunoisolation of T-tubule vesicles has been accomplished by using monoclonal antibodies directed against protein tt28 or a cytosolic domain of GLUT4 glucose transporters (Munoz et al., 1995a,b). Immobilized anti-tt28 antibody immunoprecipitates vesicles that are characterized by high tt28 protein, dihydropyridine receptors, and GLUT4 content

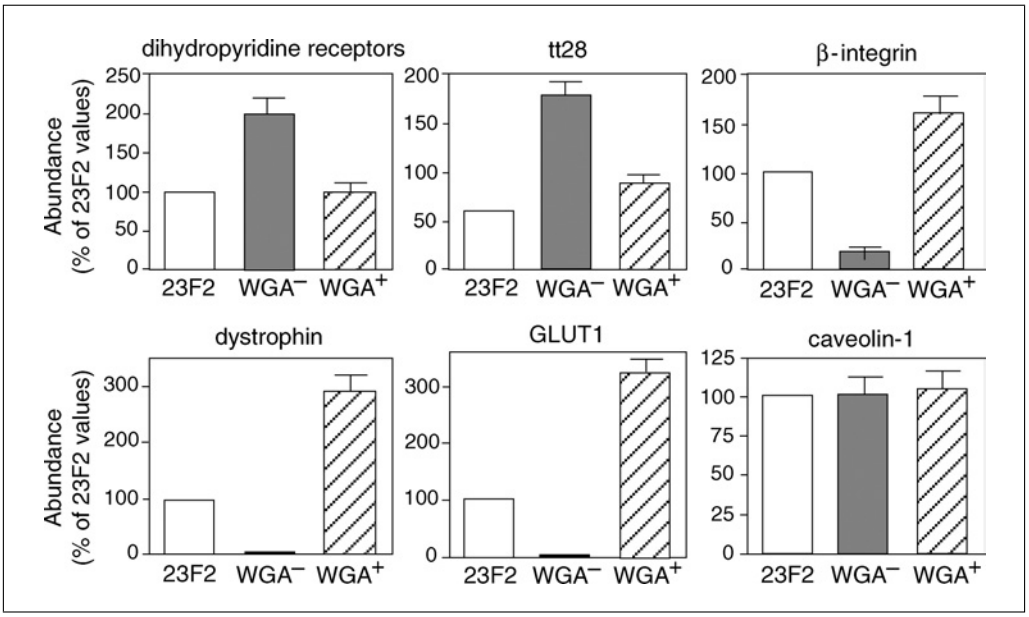


Figure 3.24.4 Purification of T-tubule membrane vesicles by WGA agglutination. Dihydropyridine receptors, protein tt28, β_1-integrin, dystrophin, GLUT1, and caveolin-1 were assayed before and after agglutination with WGA lectin. 23F2 fractions were incubated with WGA, and subsequently the nonagglutinated fraction (WGA^-) was separated from the agglutinated vesicles (WGA^+). The distribution of surface markers was studied by immunoblot assays. Equal amounts of membrane protein from the different fractions were loaded on the gels. The results are mean ±SEM of six experiments and are expressed as percentage of levels detected in fraction 23F2.

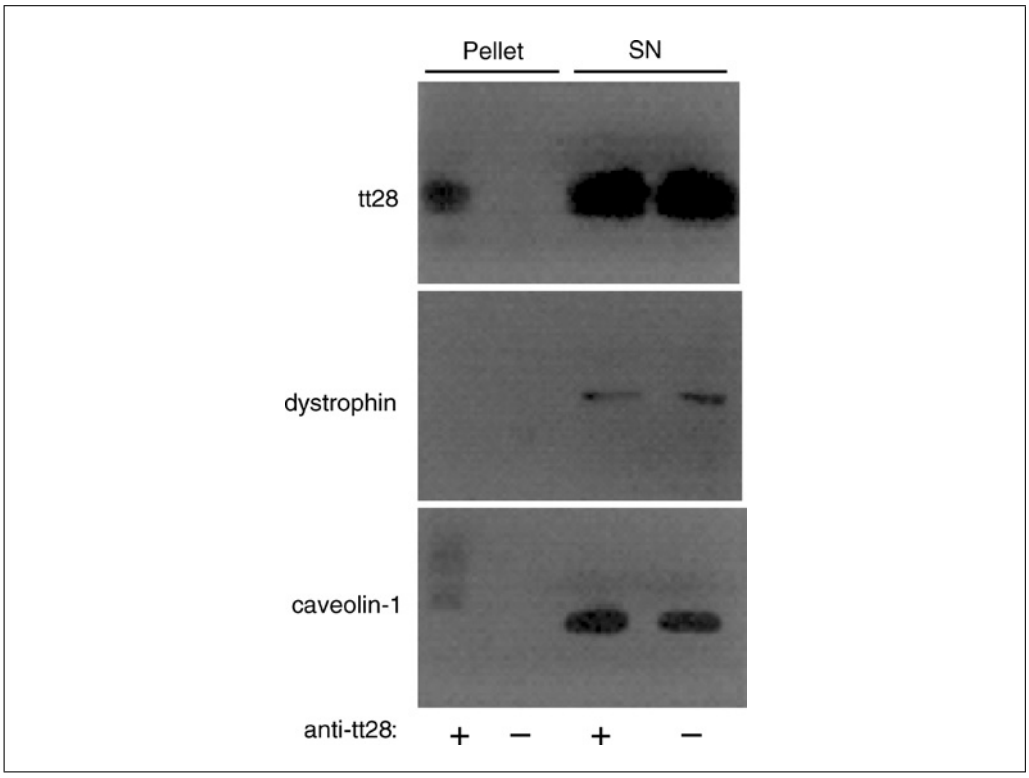


Figure 3.24.5 Purification of T-tubule membranes by vesicle immunoisolation. Membrane vesicles 23F2 highly enriched in T-tubules were incubated with or without immobilized antibody against protein tt28. After incubation, the immunoadsorbed fractions (pellet) and the nonadsorbed fraction (SN) were electrophoresed and immunoblotted to determine the abundance of proteins tt28, dystrophin, and caveolin-1.

(Fig. 3.24.5 and data not shown) and that lack caveolin-1 and dystrophin (Fig. 3.24.5). These data indicate that the purification of T-tubule vesicles by vesicle immunoisolation renders a fraction that is free from contamination by sarcolemmal or endothelial membranes vesicles.

Critical Parameters and Troubleshooting

The methods explained above require substantial training on the part of the researcher, and there are a number of critical parameters that should be kept in mind. The method explained in Basic Protocol 1 has been set up using rat skeletal muscle; therefore, changes in the species being used may lead to different results. More specific concerns relate to three key parameters: (1) the intensity of the homogenization, (2) the effectiveness of the myofibril solubilization, and (3) the increase in the density of the sarcoplasmic reticulum achieved by calcium loading.

In Basic Protocol 1, the yield of membrane protein and the quality of the membrane protein extract critically depend on the Polytron speed and homogenization time. In this regard, an increase in the Polytron speed or duration in the first homogenization may release T-tubules and lower the yield of T-tubule membrane proteins in fraction 23F2. Special attention should be paid to calibration of the speed of the Polytron. Similarly, poor myofibril solubilization or destabilization will reduce the yield of all membrane proteins, including T-tubule membranes. Finally, poor calcium loading of sarcoplasmic reticulum vesicles will markedly reduce the purity of T-tubule membranes.

Based on these considerations, changes in protein yield and in the purity of the different membrane fractions may indicate the existence of a problem at any of these steps. In this regard, membrane yields using Basic Protocol 1 have been reported in the literature (Munoz et al., 1995b).

Anticipated Results

Starting with 12 g of rat muscle, a crude preparation of T-tubules (23F2) equivalent to 0.5 mg protein will be obtained. Wheat germ agglutination starting with 0.3 mg of 23F2 will yield ~0.08 mg of highly purified T-tubules. Immunoadsorption permits a high level of purification of membrane T-tubules, but the preparation will also contain the antibody against the T-tubule protein tt28.

Time Considerations

The amount of time required to perform Basic Protocol 1 is rather long. Performance of the whole procedure takes 1.5 days of continuous work. In contrast, Basic Protocol 2 takes ~3 hr. The Alternate Protocol takes 3 days if both steps, coupling of antibodies to agarose beads and immunoadsorption, are done overnight.

Literature Cited

Angelica, C.M., Sierralta, J., and Hidalgo, C. 1993. Phospholipase C activity in membranes and a soluble fraction isolated from frog skeletal muscle. *Biochim. Biophys. Acta* 1152**:**44-48.

Barchi, R.L., Weigele, J.B., Chalikian, D.M., and Murphy, L.E. 1979. Muscle surface membranes: Preparative methods affect apparent chemical properties and neurotoxin binding. *Biochim. Biophys. Acta* 550**:**59-76.

Brette, F. and Orchard, C. 2003. T-tubule function in mammalian cardiac myocytes. *Circ. Res.* 92**:**1182-1192.

Burdett, E., Beeler, T., and Klip, A. 1987. Distribution of glucose transporters and insulin receptors in the plasma membrane and transverse tubules of skeletal muscle. *Arch. Biochem. Biophys.* 253**:**279-286.

Carl, S.L., Felix, K., Caswell, A.H., Brandt, N.R., Ball, W.J. Jr., Vaghy, P.L., Meissner, G., and Ferguson, D.G. 1995. Immunolocalization of sarcolemmal dihydropyridine receptor and sarcoplasmic reticular triadin and ryanodine receptor in rabbit ventricle and atrium. *J. Cell Biol.* 129**:**672-682.

Caswell, A.H., Baker, S.P., Boyd, H., Potter, L.T., and Garcia, M. 1978. Beta-adrenergic receptor and adenylate cyclase in transverse tubules of skeletal muscle. *J. Biol. Chem.* 253**:**3049-3054.

Charuk, J.H., Howlett, S., and Michalak, M. 1989. Subfractionation of cardiac sarcolemma with wheat-germ agglutinin. *Biochem. J.* 264**:**885-892.

Di Virgilio, F., Salviati, G., Pozzan, T., and Volpe, P. 1986. Is a guanine nucleotide-binding protein involved in excitation-contraction coupling in skeletal muscle? *EMBO J.* 5**:**259-262.

Dunn, S.M. 1989. Voltage-dependent calcium channels in skeletal muscle transverse tubules: Measurements of calcium efflux in membrane vesicles. *J. Biol. Chem.* 264**:**11053-11060.

Fischer, Y., Thomas, J., Sevilla, L., Munoz, P., Becker, C., Holman, G., Kozka, I.J., Palacin, M., Testar, X., Kammermeier, H., and Zorzano, A. 1997. Insulin-induced recruitment of glucose transporter 4 (GLUT4) and GLUT1 in isolated rat cardiac myocytes: Evidence of the existence of different intracellular GLUT4 vesicle populations. *J. Biol. Chem.* 272**:**7085-7092.

Flucher, B.E., Terasaki, M., Chin, H., Beeler, T., and Daniels, M.P. 1991. Biogenesis of transverse tubules in skeletal muscle in vitro. *Dev. Biol.* 145:77-90.

Gao, T., Puri, T.S., Gerhardstein, B.L., Chien, A.J., Green, R.D., and Hosey, M.M. 1997. Identification and subcellular localization of the subunits of L-type calcium channels and adenylyl cyclase in cardiac myocytes. *J. Biol. Chem.* 272**:**19401-19407.

Hidalgo, C., Gonzalez, M.E., and Lagos, R. 1983. Characterization of the Ca^{2+}- or Mg^{2+}-ATPase of transverse tubule membranes isolated from rabbit skeletal muscle. *J. Biol. Chem.* 258**:**13937-13945.

Hidalgo, C., Jorquera, J., Tapia, V., and Donoso, P. 1993. Triads and transverse tubules isolated from skeletal muscle contain high levels of inositol 1,4,5-trisphosphate. *J. Biol. Chem.* 268**:**15111-15117.

Horgan, D.J. and Kuypers, R. 1987. Isolation of transverse tubules by fractionation of sarcoplasmic reticulum preparations in ion-free sucrose density gradients. *Arch. Biochem. Biophys.* 253**:**377-387.

Laflamme, M.A. and Becker, P.L. 1999. G(s) and adenylyl cyclase in transverse tubules of heart: Implications for cAMP-dependent signaling. *Am. J. Physiol.* 277**:**H1841-H1848.

Lau, Y.H., Caswell, A.H., and Brunschwig, J.P. 1977. Isolation of transverse tubules by fractionation of triad junctions of skeletal muscle. *J. Biol. Chem.* 252**:**5565-5574.

Luise, M., Presotto, C., Senter, L., Betto, R., Ceoldo, S., Furlan, S., Salvatori, S., Sabbadini, R.A., and Salviati, G. 1993. Dystrophin is phosphorylated by endogenous protein kinases. *Biochem. J.* 293:243-247.

Marette, A., Burdett, E., Douen, A., Vranic, M., and Klip, A. 1992. Insulin induces the translocation of GLUT4 from a unique intracellular organelle to transverse tubules in rat skeletal muscle. *Diabetes* 41**:**1562-1569.

Milting, H., Heilmeyer, L.M. Jr., and Thieleczek, R. 1994. Phosphoinositides in membranes that build up the triads of rabbit skeletal muscle. *FEBS Lett.* 345**:**211-218.

Morton, M.E. and Froehner, S.C. 1987. Monoclonal antibody identifies a 200-kDa subunit of the dihydropyridine-sensitive calcium channel. *J Biol Chem.* 262:11904-11907.

Morton, M.E. and Froehner, S.C. 1989. The α_1 and α_2 polypeptides of the dihydropyridine-sensitive calcium channel differ in developmental expression and tissue distribution. *Neuron* 2:1499-1506.

Munoz, P., Rosemblatt, M., Testar, X., Palacin, M., Thoidis, G., Pilch, P.F., and Zorzano, A. 1995a. The T-tubule is a cell-surface target for insulin-regulated recycling of membrane proteins in skeletal muscle. *Biochem. J.* 312:393-400.

Munoz, P., Rosemblatt, M., Testar, X., Palacin, M., and Zorzano, A. 1995b. Isolation and characterization of distinct domains of sarcolemma and T-tubules from rat skeletal muscle. *Biochem. J.* 307**:**273-280.

Munoz, P., Mora, S., Sevilla, L., Kaliman, P., Tomas, E., Guma, A., Testar, X., Palacin, M.,

and Zorzano, A. 1996. Expression and insulin-regulated distribution of caveolin in skeletal muscle: Caveolin does not colocalize with GLUT4 in intracellular membranes. *J. Biol. Chem.* 271**:**8133-8139.

Ohlendieck, K., Ervasti, J.M., Snook, J.B., and Campbell, K.P. 1991. Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. *J. Cell Biol.* 112**:**135-148.

Petrecca, K., Atanasiu, R., Grinstein, S., Orlowski, J., and Shrier, A. 1999. Subcellular localization of the Na^+/H^+ exchanger NHE1 in rat myocardium. *Am. J. Physiol.* 276**:**H709-H717.

Rosemblatt, M.S. and Scales, D.J. 1989. Morphological, immunological and biochemical characterization of purified transverse tubule membranes isolated from rabbit skeletal muscle. *Mol. Cell Biochem.* 87:57-69.

Rosemblatt, M., Hidalgo, C., Vergara, C., and Ikemoto, N. 1981. Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle. *J. Biol. Chem.* 256**:**8140-8148.

Salvatori, S., Furlan, S., Millikin, B., Sabbadini, R., Betto, R., Margreth, A., and Salviati, G. 1993. Localization of protein kinase C in skeletal muscle T-tubule membranes. *Biochem. Biophys. Res. Commun.* 196**:**1073-1080.

Toutant, M., Gabrion, J., Vandaele, S., Peraldi-Roux, S., Barhanin, J., Bockaert, J., and Rouot, B. 1990. Cellular distribution and biochemical characterization of G proteins in skeletal muscle: Comparative location with voltage-dependent calcium channels. *EMBO J.* 9**:**363-369.

Wang, W., Hansen, P.A., Marshall, B.A., Holloszy, J.O., and Mueckler, M. 1996. Insulin unmasks a COOH-terminal Glut4 epitope and increases glucose transport across T-tubules in skeletal muscle. *J. Cell Biol.* 135**:**415-430.

Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wadzinkski, B.E., Ruoho, A.E., and Pilch, P.F. 1989. Insulin-regulated glucose uptake in rat adipocytes is mediated by two transporter isoforms present in at least two vesicle populations. *J. Biol. Chem.* 264**:**12358-12363.

Zorzano, A., Munoz, P., Camps, M., Mora, C., Testar, X., and Palacin, M. 1996. Insulin-induced redistribution of GLUT4 glucose carriers in the muscle fiber: In search of GLUT4 trafficking pathways. *Diabetes* 45**:**S70-S81.

Contributed by Antonio Zorzano and Marta Camps
Departament de Bioquimica i Biologia Molecular, Universitat de Barcelona, and Institute for Research in Biomedicine, Barcelona
Science Park
Barcelona, Spain

Isolation of Myelin

The methods used to prepare myelin involve homogenization of the tissue in isotonic sucrose solution, followed by the isolation of myelin membranes by a series of steps that include density gradient centrifugation and differential centrifugation. Homogenization of nervous tissue in isotonic sucrose causes the myelin sheath to peel from the axon and form myelin vesicles relatively large in size. The large size of the myelin vesicles, together with the fact that myelin has a lower density than other biological membranes, make differential centrifugation and density gradient centrifugation the main tools for the isolation of this membrane. Three protocols are outlined in this unit. The Basic Protocol describes the isolation of a highly-purified myelin fraction from the central nervous system (CNS). Alternate Protocol 1 outlines the separation of a highly-purified CNS myelin fraction into subfractions of different densities. Alternate Protocol 2 describes the isolation of myelin from the peripheral nervous system (PNS).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

ISOLATION OF CENTRAL NERVOUS SYSTEM MYELIN

BASIC PROTOCOL

This protocol is based on the Norton and Poduslo method (Norton and Poduslo, 1973). Two modifications to the original Norton and Poduslo protocol are incorporated. One is the inclusion of 20 mM Tris·Cl buffer (pH 7.45 at 25°C), 1 mM dithiothreitol (DTT), and a mixture of protease inhibitors (Larocca et al., 1987, 1991; *UNIT 3.4*) in all solutions. The other is the inclusion of an extra density gradient centrifugation (Haley et al., 1981). These two modifications result in better conservation of the enzymatic activities present in myelin and increased purity of the myelin fraction. In particular, the degree of axolemma contamination is reduced (Haley et al., 1981).

This protocol can be used for the isolation of myelin from different sources including developing and adult whole brain, spinal cord, and white matter from animals of different species including rodent, bovine, and human. This protocol can be used to isolate myelin from fresh or frozen tissue. The following describes the isolation of myelin from fresh adult rat whole brain. The brain is homogenized in 0.3 M sucrose solution containing 20 mM Tris·Cl buffer (pH 7.45), 1 mM EDTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 10 μg/ml antipain (these compounds are present in all steps of the procedure). The homogenate is layered over 0.83 M sucrose and centrifuged 35 min at high speed (75,000 × *g*). The band of crude myelin membranes formed at the 0.3 M /0.83 M sucrose interface is collected, and after washing out the sucrose with hypotonic buffer, this myelin fraction is subjected two times to a cycle of hypoosmotic shock and low-speed centrifugation (15 min at 12,000 × *g*) to remove cytoplasmic and microsomal contaminants. Then myelin is further purified by subjecting the crude myelin fraction to a repetition of the first density gradient centrifugation, and the cycle of hypoosmotic shock and low-speed centrifugation. Finally, a highly-purified myelin fraction is prepared by a third density gradient centrifugation, but in this step, myelin is resuspended in 0.83 M sucrose, and the 0.83 M sucrose solution overlaid with 0.30 M sucrose. During centrifugation, myelin floats upward to band at the 0.30/0.83 M sucrose interface.

Contributed by Jorge N. Larocca and Williams T. Norton
Current Protocols in Cell Biology (2006) 3.25.1-3.25.19

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

200- to 300-g Sprague-Dawley rats (30 to 60 days old)
Anesthesia
0.30 M and 0.83 M sucrose solutions (see recipe)
Tris·Cl buffer solution (see recipe)

Glass plate
Dissecting tools including: scissors, razor blades, spatulas, and forceps
50- and 100-ml beakers
Razor blades
40-ml Dounce homogenizer (Kontes) with both loose (clearance 0.0030 to 0.0060 in.) and tight (clearance 0.0010 to 0.0030 in.) pestles
100- and 250-ml graduated cylinder
38.5-ml thick-walled ultracentrifuge tubes
Ultracentrifuge with swinging-bucket rotor (Beckman SW 28, or equivalent)
Pasteur pipets
Glass rod

Additional reagents and equipment for determining protein concentration (*APPENDIX 3H*)

Dissect brains

1. Place a glass plate on a bed of ice in an ice bucket. Ensure that the glass plate will fit flat inside the ice bucket.
2. Anesthetize three animals and then euthanize by decapitation.

   This must be supervised or carried out by an experienced animal technician according to an approved animal protocol.

3. Open the cranial cavity using scissors and forceps, and remove the brain (with the use of a spatula) to a chilled beaker containing 20 ml of 0.30 M sucrose solution, which has been weighed.
4. Reweigh the beaker. Record weight of the brains.
5. Transfer the brains to the glass plate, use a razor blade to quickly remove the meninges and then mince the brains into cubes of ~0.25 × 0.25 × 0.25–cm.

   Mincing the brains is not strictly necessary, but facilitates homogenization.

Homogenize the tissue

6. Place the minced brains in a cold beaker containing 60 ml of 0.30 M sucrose solution.
7. Pour ~30 ml of the minced brain suspenson into the cold vessel of the Dounce homogenizer.
8. Homogenize the tissue suspension by using five strokes of the loose pestle and five to seven strokes of the tight pestle, on ice.
9. Transfer the tissue homogenate to a 100-ml graduated cylinder and proceed to homogenize the rest of the tissue suspension.
10. Combine both homogenates and bring the suspension to a final volume of 72 ml with 0.30 M sucrose solution.

    The suspension should not contain more than 5 g of wet tissue per 100 ml.

Carry out density gradient centrifugation

11. Layer 18 ml of homogenate over 18 ml of 0.83 M sucrose solution in each of four 38.5-ml ultracentrifuge tubes, and ultracentrifuge 30 min at 75,000 × *g*, 4°C.

    When thin-walled tubes are used, be sure that the tubes are filled to within 2 to 3 mm from the top (~2 ml more of 0.30 M sucrose solution should be added).

12. Collect the layers of crude myelin, which form at the interface of the two sucrose solutions, with a Pasteur pipet and transfer to the cold homogenizer vessel. Discard the rest of the gradient.

    To facilitate the harvesting of the crude myelin fraction, first discard the 0.30 M sucrose solution and then collect the crude myelin preparation.

Subject the myelin fraction to osmotic shock

13. Resuspend the combined myelin layers in 20 ml of Tris·Cl buffer by homogenization using five to seven strokes of the tight pestle.

    Osmotic shock and differential centrifugation remove small microsomal membrane fragments and cytoplasmic contaminants.

14. Transfer myelin membranes to a 250-ml graduated cylinder, and bring the suspension to a final volume of 228 ml with Tris·Cl buffer solution.

15. Transfer the suspension to six 38.5-ml ultracentrifuge tubes and centrifuge the suspension 15 min at 75,000 × *g*, 4°C.

16. Discard the supernatant and resuspend the pellet in Tris·Cl buffer solution. To facilitate pellet resuspension, add 2 ml of Tris·Cl buffer solution to the ultracentrifuge tubes and use a glass rod to gently stir the pellet. Using a Pasteur pipet, transfer the resuspended membrane to a 40-ml homogenizer.

    Alternatively, the pellet can be resuspended by pipeting up and down using a Pasteur pipet.

17. Disperse the myelin membranes in 20 ml Tris·Cl buffer solution by homogenization using five to seven strokes of the tight pestle.

18. Transfer myelin membranes to a 250-ml graduated cylinder, and bring the suspension to a final volume of 228 ml with Tris·Cl buffer solution.

19. Transfer the suspension to six 38.5-ml ultracentrifuge tubes and ultracentrifuge the suspension 15 min at 12,000 × *g*, 4°C.

20. Discard the cloudy supernatant, then resuspend and disperse the loosely packed pellets in 228 ml Tris·Cl buffer solution as described in steps 16 to 18.

21. Centrifuge the crude myelin suspension 10 min at 12,000 × *g*, 4°C.

Purify myelin from a crude myelin preparation

22. Combine the crude myelin pellets and suspend them in 72 ml of 0.30 M sucrose solution. Layer 18 ml of this suspension over 18 ml of 0.83 M sucrose solution in four 38.5-ml ultracentrifuge tubes and ultracentrifuge 30 min at 75,000 × *g*, 4°C.

23. Harvest the purified myelin from the interface with a Pasteur pipet and suspend the combined myelin layers in 228 ml Tris·Cl buffer solution.

24. Wash out the sucrose solution and subject the myelin preparation to further purification by hypoosmotic shock and differential centrifugation as described in steps 13 to 21.

A third discontinuous gradient followed by a cycle of hypoosmotic shock and differential centrifugation results in a slight increase in myelin purity. Therefore, consider the possibility of using the myelin fraction obtained in step 24 as purified myelin.

Perform further myelin purification

25. Resuspend the myelin pellet in 72 ml of 0.83 M sucrose solution.
26. Transfer 18 ml of the myelin suspension to 38.5-ml centrifuge tubes, overlay the myelin suspension with 18 ml 0.30 M sucrose and centrifuge 30 min at 75,000 × *g*, 4°C.
27. Harvest the myelin fraction at the interface and subject the myelin fraction to hypoosmotic shock as described in steps 13 to 21.
28. Determine protein concentration (*APPENDIX 3H*) and resuspend pellet in Tris·Cl buffer solution to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage for 1 to 16 hr at 4°C. Many assays can be carried out on material frozen at −20°C or −80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions. For determination of myelin dry weight, carry out steps 27 and 28 using water instead of buffer. Then lyophilize and weigh the myelin preparation and store up to 1 year at −80°C.

ALTERNATE PROTOCOL 1

SEPARATION OF HIGHLY PURIFIED CNS MYELIN FRACTION INTO SUBFRACTIONS OF DIFFERENT DENSITIES

Highly-purified myelin (see Basic Protocol, step 28) contains a heterogeneous population of membranes that have different morphologies and chemical compositions (Norton and Cammer, 1984). Purified myelin can be subfractionated either by continuous or discontinuous densitygradient centrifugation. This protocol outlines the myelin subfractionation by discontinuous density gradient centrifugation and is based in the method described by Cammer et al. (1977). Isolated CNS myelin is suspended in 0.30 M sucrose solution and layered on a discontinuous gradient. After centrifugation, the myelin subfractions contained at the interfaces and in the pellet are collected, resuspended in Tris·Cl buffer solution, and pelleted. The light myelin subfractions contain mainly multilamellar fragments; the dense subfractions are enriched in single membrane vesicles and fragments with only two or three membrane layers (Norton and Cammer, 1984).

NOTE: All solutions contain 20 mM Tris·Cl buffer (pH 7.45), 1 mM EDTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml antipain.

Additional Materials *(also see Basic Protocol)*

Highly-purified myelin fraction (see Basic Protocol, step 28)
0.30, 0.62, and 0.70 M sucrose solutions (see recipe)

1. Prepare a highly-purified myelin fraction as described in Basic Protocol, step 28.
2. Resuspend the highly-purified myelin in 25 ml of 0.30 M sucrose solution.
3. Prepare two discontinuous gradients using two 38.5-ml ultracentrifuge tubes. In each tube, layer 12.5 ml each of 0.62 M sucrose solution, 0.70 M sucrose solution, and sample.
4. Ultracentrifuge the gradients 35 min at 75,000 × *g*, 4°C.

5. Collect the myelin subfractions, which form at the interfaces of the sucrose solutions (i.e., between 0.30 M/0.62 M and 0.62 M/0.70 M) and in the pellet, with a Pasteur pipet.

   This step will generate three different subfractions: (1) low-density myelin (L), myelin collected from the 0.30 M/0.62 M sucrose interface, (2) medium-density myelin (M), myelin collected from the 0.62 M/0.70 M sucrose interface, and (3) high-density myelin (H), myelin collected from the pellet.

6. Resuspend each myelin fraction in 38 ml Tris·Cl buffer solution.

7. Transfer each myelin suspension to two 38.5-ml ultracentrifuge tubes and ultracentrifuge 35 min at 75,000 × *g*, 4°C.

8. Resuspend pellets, separately, in 2 ml Tris·Cl buffer solution, stir with a glass rod, and store the membrane suspensions as in Basic Protocol, step 28.

ISOLATION OF PNS MYELIN

ALTERNATE PROTOCOL 2

The methods used to isolate PNS myelin are based on protocols designed to isolate CNS myelin. However, isolation of myelin from PNS is more difficult than isolation of myelin from CNS. The presence of a large amount of collagen and connective tissue in PNS makes it necessary to use a more drastic method of tissue homogenization using a Polytron homogenizer, which leads to the formation of smaller myelin vesicles. Because PNS myelin has a lower density than CNS myelin, the concentration of sucrose in the density gradient solutions is modified to avoid the loss of myelin during the isolation. Substantial amounts of small myelin vesicles are lost through the isolation process, in particular during the hypoosmotic shock.

This protocol is based on the methods of Cammer et al. (1981), Wiggins and Fuller, (1981), and Wiggins et al. (1975). The sciatic nerve is cut into pieces with a razor blade on a cold glass plate, the small nerve segments are suspended in 0.27 M sucrose solution containing 20 mM Tris·Cl buffer (pH 7.45), and they are homogenized with a Polytron homogenizer. Then connective tissue is eliminated by passing the homogenate through layers of cheese cloth. The tissue suspension is layered over 0.83 M sucrose solution and centrifuged. Myelin forms a layer at the interface. To prevent loss of myelin in the upper phase of the gradient, the concentration of sucrose used in this protocol is lower than that used in the Basic Protocol. The crude myelin fraction is subjected to hypoosmotic shock and centrifuged to remove microsomal contamination. The crude preparation of myelin obtained after the first cycle of hypoosmotic shock and differential centrifugation is a reasonably pure myelin fraction. Thus, depending on the degree of purity required, it may be possible to consider this myelin fraction as purified myelin. A second discontinuous gradient followed by a second cycle hypoosmotic shock and differential centrifugation results in a slightly increase in the myelin purity; however, a large amount of myelin is lost during this procedure.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0°C to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature. All solutions contain 20 mM Tris·Cl buffer (pH 7.45), 1 mM EDTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml antipain.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

25- and 50-ml beaker
50-ml Pyrex tube
Polytron homogenizer (Brinkmann Instruments)
Cheesecloth
Funnel

Dissect sciatic nerve

1. Place a glass plate on a bed of ice in an ice bucket. Ensure that the glass plate will fit flat inside the ice bucket.
2. Anesthetize the rat and euthanize by decapitation. Repeat for a total of four rats.

   This must be supervised or carried out by an experienced animal technician according to an approved animal protocol.

3. Dissect the sciatic nerves using scalpel and forceps, and remove sciatic nerves to a chilled 25-ml beaker containing 10 ml of 0.27 M sucrose solution.
4. Record weight of the sciatic nerves (~28 to 35 mg each).
5. Transfer the four pairs of sciatic nerves to the glass plate, and cut the nerves in segments of 2 to 3 mm using a razor blade or scissors.

Homogenize the neural tissue

6. Place the minced sciatic nerves in a cold 50-ml beaker containing 18 ml of 0.27 M sucrose solution.
7. Pour the minced sciatic nerve suspension into the cold 50-ml Pyrex tube and homogenize the tissue with a Polytron homogenizer, using four 15-sec pulses.
8. To eliminate connective tissue, pour the sciatic nerve homogenate through four layers of cheese cloth placed inside a funnel resting in a 100-ml graduated cylinder surrounded by ice.
9. Pour 18 ml of 0.27 M sucrose solution through the four layers of cheese cloth to recover any tissue suspension that may have been trapped in the cheese cloth, and add the filtrate to the tissue homogenate (total volume 36 ml)
10. Overlay 18 ml of the homogenate over 18 ml of 0.83 M sucrose solution in each of two 38.5-ml ultracentrifuge tubes and ultracentrifuge 45 min at 82,000 $\times$ g, 4°C.
11. With a Pasteur pipet, harvest the crude myelin fraction, which forms at the 0.27M/083M sucrose interface.

Carry out osmotic shock

12. Resuspend the combined myelin layers in 20 ml Tris·Cl buffer solution using a Dounce homogenizer using five to seven strokes with the tight pestle.
13. Transfer myelin membranes to a 100-ml graduated cylinder, and bring the suspension to a final volume of 76 ml with Tris·Cl buffer solution.
14. Transfer the suspension to two 38.5-ml ultracentrifuge tubes and ultracentrifuge the suspension 15 min at 82,000 $\times$ g, 4°C.
15. Discard the supernatant and resuspend the pellet in Tris·Cl buffer solution. To facilitate pellet resuspension, add 2 ml Tris·Cl buffer solution and gently stir the pellet with a glass rod.

    Alternatively, the pellet can be resuspended by pipeting up and down repeatedly using a Pasteur pipet.

16. Transfer the resuspended membrane using a Pasteur pipet to a 100-ml graduated cylinder and bring the suspension to a final volume of 76 ml with Tris·Cl buffer solution.
17. Transfer the suspension to 38.5-ml centrifuge tubes and centrifuge the suspension 10 min at 17,000 $\times$ g, 4°C.

18. Discard the cloudy supernatant and disperse the two crude myelin pellets in a total volume of 76 ml Tris·Cl buffer solution.

19. Transfer the suspension to 38.5-ml centrifuge tubes and centrifuge the suspension 10 min at 17,000 × *g*, 4°C.

    A second discontinuous gradient followed by a cycle of hypoosmotic shock and differential centrifugation results in a slight increase in myelin purity. However, a large amount of myelin is lost during this procedure. Therefore, consider the possibility of using the myelin fraction obtained in step 18 as purified myelin.

    To avoid loss of myelin membranes, it is recommended that the two myelin pellets be combined and purified using only one discontinuous sucrose gradient.

Isolate myelin from a crude preparation

20. Resuspend the combined myelin pellet in 18 ml of 0.27 M sucrose solution by homogenization using five to seven strokes of the tight pestle

21. Layer 18 ml of this suspension over 18 ml of 0.83 M sucrose in one 38.5-ml ultracentrifuge tube and ultracentrifuge exactly as described in step 10.

    The tube containing the myelin fraction has to be balanced with a tube containing just the sucrose gradient.

22. Harvest the purified myelin from the interface with a Pasteur pipet and wash out the sucrose as described in steps 12 to 14.

23. Subject the myelin fraction to further purification by hypoosmotic shock and differential centrifugation as described in steps 15 to 18.

24. Determine protein concentration (*APPENDIX 3H*).

    Metabolic assays should be carried out as soon as possible after preparation; some marker enzyme assays can be carried out after storage for ~16 hr at 4°C. Many assays can be carried out on material frozen at –20°C or –80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

ASSAY FOR 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHODIESTERASE ACTIVITY

SUPPORT PROTOCOL

Two enzymes, and a cholesterol ester hydrolase (Eto and Suzuki, 1973), have been shown to be enriched in CNS myelin. The neutral cholesterol ester hydrolase specific activity in myelin is approximately ten times higher than in total homogenate.

The enzyme 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; Kurihara and Tsukada, 1967) has been extensively used as a marker during isolation of CNS myelin, or to estimate myelin contamination of other subcellular fractions (Norton and Cammer, 1984). CNPase catalyzes the hydrolysis of several 2′,3′-cyclic nucleoside monophosphates to their respective 2′-nucleoside monophosphates. These include the 2′,3′-cyclic monophosphates of adenosine, cytidine, and uridine as well as certain oligonucleotides containing a 2′,3′-cyclic nucleotide terminus. Although studies are made with 2′,3′-cyclic AMP, the physiological substrate(s) remains unknown, and the physiological role of CNP has not been established. Several methods have been used to determine CNPase activity (Prohaska et al., 1973), which the authors recommend. In this method the samples are first treated with deoxycholate, and CNPase activity is measured as an inorganic phosphate released from the product, 2′-AMP, by *E. coli* alkaline phosphatase. Measurements are carried out under optimal conditions of temperature (30°C) and pH (6.2). CNPase specific activity in isolated myelin is between three to six times higher than the CNPase specific activity present in the total brain homogenate.

Materials

Myelin sample with 0.2 to 1 mg/ml protein
0.2 M Tris·Cl buffer, pH 7.5 (see recipe)
1% (w/v) sodium deoxycholate solution (see recipe)
CNPase reaction solution (see recipe)
0.3 M Tris·Cl, pH 9.00, containing 21 mM $MgCl_2$
E. coli alkaline phosphatase solution (see recipe)
1:1 (v/v) isobutanol/benzene
1.5% (w/v) ammonium molybdate solution (see recipe)

5-ml Dounce homogenizer (Kontes) with tight (clearance 0.0010 to 0.0030 in.) pestle
Pyrex test tubes (10 × 75–mm)
30° and 100°C water baths

Prepare the samples

1. Dilute 0.2 ml of the myelin sample (containing 0.2 mg to 1 mg of myelin protein) with 0.1 ml of 0.2 M Tris·Cl, pH 7.5.
2. Add 0.2 ml of 1% sodium deoxycholate solution to the mix, and incubate the tube 10 min at 4°C.
3. Add 2 ml of water to the sample treated with deoxycholate, and transfer the mix to the vessel of a 5-ml Dounce homogenizer.
4. Disperse the membranes by homogenization using five to seven strokes of the tight pestle.

Perform the assay

5. Add 20 µl of the dispersed sample membranes (1.5 to 8 µg of myelin protein) to 10 × 75–mm Pyrex test tubes containing 180 µl CNPase reaction solution.
6. Incubate the reaction mix 10 min at 30°C.
7. Terminate the reaction by placing the Pyrex tubes 30 sec in a boiling water bath.
8. Return the tubes to the 30°C water bath, and add 0.1 ml of 0.3 M Tris·Cl (pH 9.00) containing 21 mM $MgCl_2$ and 0.72 U *E. coli* alkaline phosphatase (30 to 60 U/mg), and maintain the tubes 20 to 30 min in the 30°C water bath.
9. Add 1.2 ml of 1:1 isobutanol/benzene and 1.2 ml of 1.5% ammonium molybdate solution.

   This part of the protocol should be carried out under a chemical hood. Because the reagent solution contains O.5 N H_2SO_4 take extra precaution in the handling of the tubes.

10. Vigorously vortex the tubes for 20 sec.
11. Centrifuge tubes 5 min at 800 × *g*, 4°C.

Measure absorbance

12. Collect the upper phase with a syringe, and measure the absorbance at 410 nm against an isobutanol/benzene blank.
13. Determine the amount of phosphate liberated by comparing the value of absorbance obtained with values of absorbance obtained using phosphate standards.

    To construct a phosphate standard curve, add 0.3-ml aliquots of: 5, 3.4, 2.5, 1.5, 1, 0.5, 0.25, and 0.125 mM Na_2PO_4H solutions to 10 × 75–mm Pyrex test tubes. This results in a standard curve of eight points containing 1.5, 1, 0.75, 0.45, 0.3, 0.150, 0.075, and 0.0375 µmol of phosphate, respectively. To prepare the Na_2PO_4H solutions, dilute 10 mM Na_2PO_4H solution with water, as needed.

REAGENTS AND SOLUTIONS

Use distilled and deionized water in all recipes and protocols steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alkaline phosphatase buffer

To 20 ml of H_2O, add:
30 ml 1 M Tris·Cl, pH 9.00 (0.3 M final)
0.427 g $MgCl·6H_2O$ (21 mM final)
Adjust to pH 9.00 with 1 M Tris·Cl or 1 M HCl if required
Add H_2O to 100 ml

Ammonium molybdate solution

To 100 ml of H_2O add:
3 g $(NH_4)6Mo_7O_{24}·4H_2O$ (1.5%, w/v final)
2.81 ml concentrated (35.64 N) H_2SO_4 (0.5 N final)
Add H_2O to 200 ml

CAUTION: *Take extra precaution when preparing this reagent, H_2SO_4 is highly corrosive. Concentrated acid must be slowly added to water. Never add water to concentrated acid because the heat generates a violent reaction that leads to spattering.*

CNPase reaction solution

To 5 ml of Tris-maleate buffer (see recipe) add:
0.035 g of adenosine 2′,3′-cyclic monophosphate sodium salt (10 mM final)
Add Tris-maleate buffer (see recipe) to 10 ml
Prepare fresh

E. coli alkaline phosphatase solution

For ten reactions:
To 0.5 ml of alkaline phosphatase buffer (see recipe), add:
7.2 U *E. coli* alkaline phosphatase (~220 μg)
Add alkaline phosphatase buffer (see recipe) to 1 ml
Prepare fresh

Sodium deoxycholate solution, 1% (w/v)

To 50 ml of H_2O, add:
1 g deoxycholate
Add H_2O to 100 ml

Sucrose solution, 1 M

To 100 ml of H_2O, add:
68.46 g sucrose (1 M final)
4 ml 1 M Tris·Cl, pH 7.45, at 25°C (20 mM final)
4 ml 100 mM Na_2EDTA (2 mM final)
0.031 g dithiothreitol (1 mM final)
Adjust to pH 7.45 with 1 M Tris·Cl or 1 M HCl if required
Add H_2O to 200 ml
Add protease inhibitors according to *UNIT 3.4*
Store up to 1 to 2 days at 4°C

Sucrose solutions for CNS myelin isolation:
To prepare 0.30 M and 0.83 M sucrose solutions, dilute 1 M sucrose solution with Tris·Cl buffer solution (see recipe), as needed. For 0.83 M sucrose, dilute 0.83:0.27 (v/v) 1 M sucrose solution/Tris·Cl buffer solution; for 0.30 M sucrose, dilute 0.30:0.70 (v/v) 1 M sucrose solution/Tris·Cl buffer solution.

Sucrose solutions for PNS myelin isolation:
To prepare 0.27 M and 0.83 M sucrose solutions, dilute 1 M sucrose solution with Tris·Cl buffer solution (see recipe), as needed. For 0.83 M sucrose, dilute 0.83:0.27 (v/v) 1 M sucrose solution/Tris·Cl buffer solution (see recipe); for 0.27 M sucrose, dilute 0.27:0.73 (v/v) 1 M sucrose solution/Tris·Cl buffer solution (see recipe).

Sucrose solutions for subfractionation of CNS myelin:
To prepare 0.30 M, 0.62 M, and 0.70 M sucrose solutions, dilute 1 M sucrose solution with Tris·Cl buffer solution, as needed. For 0.70 M sucrose, dilute 0.70:0.30 (v/v) 1 M sucrose solution/Tris·Cl buffer solution (see recipe); for 0.62 M sucrose, dilute 0.62:0.38 (v/v) 1 M sucrose solution/Tris·Cl buffer solution (see recipe), and for 0.30 M, dilute 0.30:0.70 (v/v) 1 M sucrose solution/Tris·Cl buffer solution (see recipe).

Tris·Cl buffer, 0.2 M (pH 7.5)

To 50 ml of H_2O, add:
20 ml 1 M Tris·Cl, pH 7.50 (0.2 M final)
Add H_2O to 100 ml

Tris·Cl buffer solution

To 100 ml of H_2O, add:
4 ml 1 M Tris·Cl, pH 7.45, at 25°C (20 mM final)
4 ml 100 mM Na_2EDTA (2 mM final)
0.031 g dithiothreitol (1 mM final)
Adjust to pH 7.45 with 1 M Tris·Cl or 1 M HCl if required
Add H_2O to 200 ml
Add protease inhibitors according to *UNIT 3.4*
Store up to 1 to 2 days at 4°C

Tris-maleate buffer

To 100 ml of H_2O, add:
1.16 g maleic acid (50 mM final)
Adjust to pH 6.20 with Tris base solution
Add H_2O to 200 ml
Store up to 30 days at 4°C

COMMENTARY

Background Information

Methods of myelin isolation

Myelin is a highly specialized multilamellar structure that surrounds, in a spiral fashion, segments of axons of the central and peripheral nervous systems of vertebrates (Fig. 3.25.1). In the CNS, myelin is produced by oligodendrocytes, while in the PNS, myelin is generated by Schwann cells (Norton and Cammer, 1984; Morell and Quarles, 1999). Although myelin is present all through the CNS, this membrane is particularly enriched in the brain white matter and spinal cord. PNS contains a high amount of myelin as well. Myelin may account for 75% of the dry weight of some peripheral nerves.

CNS and PNS myelin are morphologically and biochemically different (for detailed analyses of myelin composition, see Norton and Cammer, 1984 and Morell and Quarles, 1999). The protein composition of CNS myelin is more simple than other brain membranes, with the proteolipid protein (PLP) and myelin basic proteins (MBP) making up 60% to 80%. Other proteins include myelin-associated glycoprotein (MAGs) and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase). PNS myelin does not contain PLP and the amount of MBP present is low. A single protein, Po, accounts for >50% of the PNS myelin proteins. PNS myelin contains a 170-kDa glycoprotein that constitutes 5% of the total protein. MAG is also present in PNS myelin but to a lesser extent than in CNS myelin. Additionally, both CNS and PNS

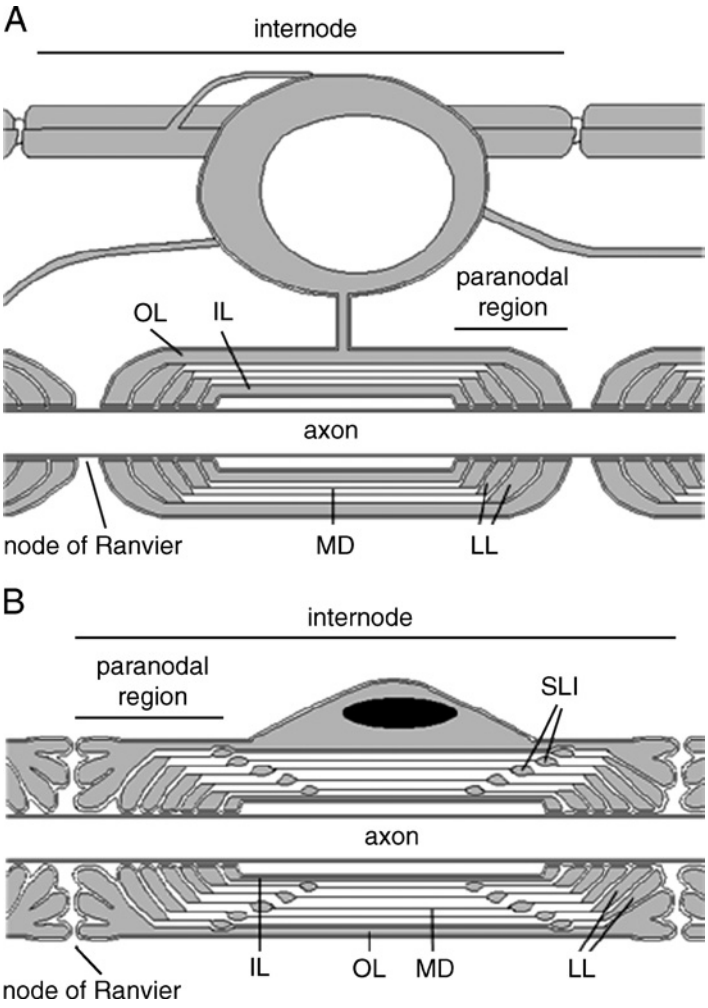


Figure 3.25.1 Composite diagrams showing the morphological characteristics of CNS and PNS myelins. (**A**) CNS. As shown, the myelin sheath is connected to an oligodendrocyte by a process. The longitudinal cross section shows a myelin sheath covering an internode. The paranodal region of the myelin sheath is in close contact with the axon. Apposition of the inner faces of the myelin membranes (cytoplasmatic faces) form the major dense line (MD). Apposition of the outer faces (extracellular faces) form the intraperiod line (not drawn for clarity). Cytoplasm is present in the lateral (LL), inner (IL), and outer (OL) loops. An oligodendrocyte can produce several myelin sheaths and wrap several axon segments. (**B**) PNS. Diagram showing a longitudinal cross section through a Schwann cell and its myelin wrapping around an axon segment. PNS and CNS myelins have similar morphologic characteristics, mainly a multilayer of compact membranes and cytoplasm in the lateral (LL), inner (IL), and outer (OL) loops. Cytoplasm is also present in the Schmidt-Laterman incisures (SLI). The Schmidt-Laterman cleft has islands of cytoplasm appearing between openings in the major dense line (MD). Note that in the CNS, Schmidt-Laterman incisures are absent from brain myelin, and in spinal cord myelin are present only in very small numbers. A Schwann cell only produces one myelin sheath.

myelin contain numerous quantitatively minor proteins (Fig. 3.25.2). Both CNS and PNS myelin have a large ratio of lipids to proteins (lipids amount to 70% to 85% of the myelin weight, while proteins represent 15% to 30%), and both PNS and CNS myelin have the lowest density of any nervous systems membrane.

The abundance of myelin in the mammalian nervous systems, together with its particular physical properties, makes it feasible to purify large quantities of myelin in high yield and purity by standard techniques of subcellular fractionation.

All the methods for isolating myelin require homogenization of the tissue in sucrose solution of low ionic strength. Homogenization of nervous tissue in sucrose solution leads to the swelling of the myelin sheath, disruption of the

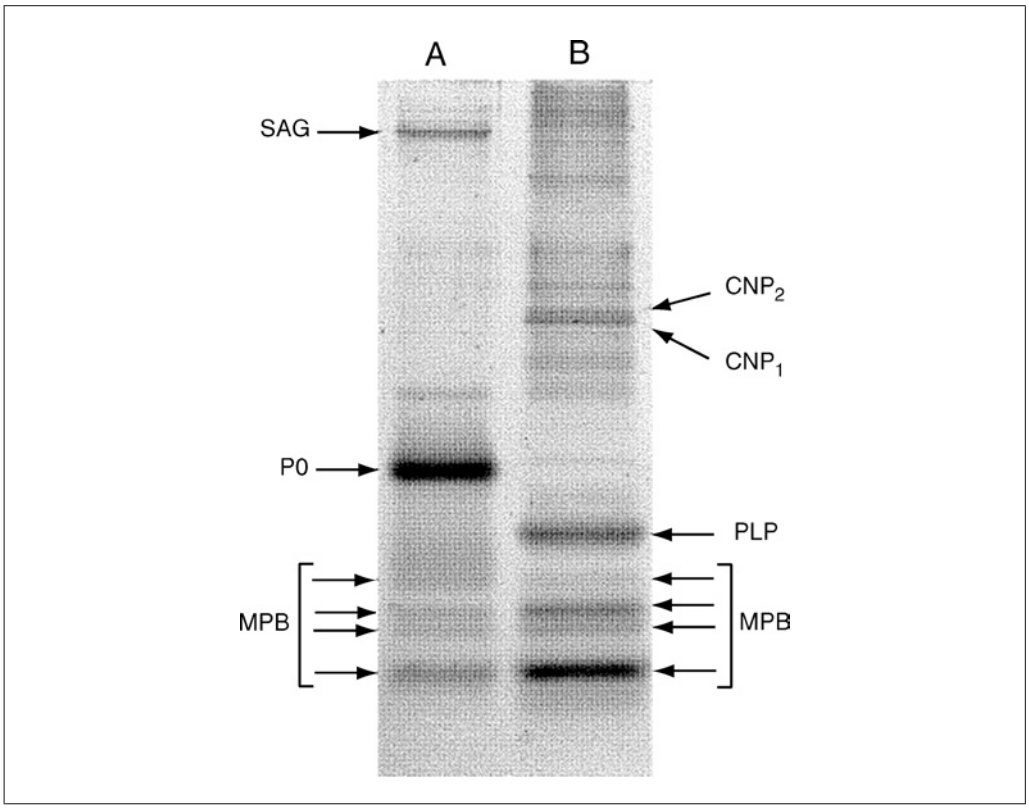


Figure 3.25.2 Protein patterns of rat PNS myelin (**A**) and rat CNS myelin (**B**). Samples of isolated myelin containing 20 μg of proteins were solubilized with SDS, electrophoresed on a 12.5% polyacrylamide gel, and stained with Coomassie brilliant blue. Myelin basic protein (MBP), proteolipid protein (PLP), 2:3-cyclic nucleotide-3′-phosphodiesterase (CNPase), protein zero (Po), and Schwann cell membrane glycoprotein (SAG) are shown. Four MBP isoforms (14.0-, 17.0-, 18.5-, and 21.5-kDa) are present in both CNS and PNS myelins. The PLP band is seen only in CNS myelin. PLP of ~30-kDa migrates anomalously fast on SDS gels. Po (30-KDa) is present only in PNS. Two isoforms of CNPase, CNP1 (~46 kDa) and CNP2 (~48 kDa), are present in CNS myelin, and to a lesser extent in PNS myelin. SAG (170-kDa) is present only in PNS. Several minor bands on the gel are probably myelin components, but are not identifiable solely from their position. Myelin protein patterns may differ depending on species and age of the animal. The four MBP isoforms are generated by alternative splicing of mRNA. The 18.5-kDa MBP in CNS is called P_1 in PNS. Small amounts of PLP are synthesized in PNS but not incorporated into myelin in appreciable amounts. CNP1 and CNP2 are generated by alternative splicing of mRNA. Both CNS and PNS myelin contain numerous minor proteins that are not seen on gels stained with Coomassie blue.

axon myelin interaction, and the formation of relatively large myelin vesicles (up to 20 μm in diameter).

The methods to isolate myelin rely on two properties of the myelin vesicles, their large size and their low density (Norton and Cammer, 1984; Morell and Quarles, 1999). There are mainly two groups of methods of CNS myelin isolation. One group takes advantage of vesicle size to obtain a crude myelin preparation by differential centrifugation of the tissue homogenate (De Robertis et al., 1962; Eichberg et al., 1964). The other group takes advantage of myelin density to obtain an initial enrichment by density gradient centrifugation of the tissue homogenate (Norton and Poduslo, 1973).

In the first group of protocols, a fraction enriched in myelin membranes is obtained by carrying out two differential centrifugations. First, total brain homogenate is centrifuged for 10 min at low speed ($900 \times g$), which results in a supernatant that contains the majority of the myelin membranes, microsomes, and mitochondria, and a pellet containing nuclei. However, a fraction of myelin is also recovered in the nuclear fraction. The amount of myelin recovered in the supernatant or in the nuclear fraction depends on the size of the myelin vesicles, a physical property that depends on the strength of the homogenization and the age of the animal. Second, a fraction enriched in myelin and mitochondria is obtained by centrifugation of the low-speed supernatant for

15 min at 13,500 × *g*. Myelin and mitochondria co-purify in the pellet, while microsomes remain in the supernatant.

Finally, myelin is isolated by centrifugation of the crude mitochondrial fraction for 2 hr at 50,000 × *g* on a sucrose gradient, usually having concentrations of 0.32, 0.8 M, and 1.2 M sucrose. Myelin will layer out at the 0.32 M sucrose/0.8 M sucrose interface.

These protocols permit the simultaneous separation of all the brain fractions. Additionally, the possibility of contamination by microsomes is reduced by the early elimination of the microsomal membranes in the second differential centrifugation. However, they present the disadvantage that to recover all the myelin, the mitochondria and nuclear fractions have to both be processed, and if only myelin is wanted, some of the centrifugation steps are unnecessary.

The protocols outlined in this unit belong to the second group of myelin-isolation methods, in which a crude myelin fraction is isolated in the first step by density gradient centrifugation. These methods take advantage of the low density of myelin. In a sucrose gradient, myelin has a density of ∼1.08 g/ml, and the minimal sucrose concentration required to float all the myelin is 0.8 M (density 1.103 g/ml). A crude myelin fraction is prepared from brain homogenate in 0.32 M sucrose by density gradient centrifugation on a step of 0.85 M sucrose. Myelin will layer out at the 0.32 M/0.85 M sucrose interface, while nuclei, mitochondria and synaptosomes will move through the 0.85 M sucrose phase and form a pellet. Similarly, microsomal membranes will form a pellet or remain resuspended on 0.85 M sucrose. However, after centrifugation, particulate material remains in the 0.32 M sucrose phase and contaminants are trapped in the myelin layer. The amount of contaminants trapped in the myelin layer depends on the tissue from which the myelin is isolated. Tissues such as white matter that are enriched in myelin yield a relatively pure crude myelin fraction. In contrast, tissues containing low amounts of myelin, such as whole brain from animals in the early period of myelination, yield an impure crude myelin fraction. The major contaminant might be glial membranes, endoplasmic reticulum, and axoplasm. Because of their large size, myelin vesicles move through the 0.32 M sucrose ahead of other membrane fractions, and form a layer on the 0.32M/0.85 M sucrose interface. Therefore, it is possible that part of the microsomal fraction has to pass through a layer of myelin membranes, which may result in the retention of some microsomal membranes in the myelin layer. Myelin vesicles formed during the tissue homogenization might also trap microsomal membranes. Moreover, the paranodal areas of the myelin sheath have pockets of cytoplasm containing intracellular organelles such as polyribosomes, mitochondria, and endoplasmic reticulum. Reduction of such contaminants is carried out by osmotically shocking the myelin in hypotonic buffer. The trapped contaminants are released, and the larger myelin particles are separated from the smaller membranous material by low-speed centrifugation. In most methods, the final purification of myelin is carried out by repetitions of both the first density gradient centrifugation on a discontinuous gradient of 0.32 M to 0.85 M sucrose, and of the cycle of hypoosmotic shock and low-speed centrifugation. These protocols yield a reasonably pure myelin fraction that contains no more than 5% contamination. The fact that there are axon-glial intercellular junctions connecting the myelin lateral loops to the axolemma leads to the possibility that fragments of axolemma could also be present in the fraction of purified myelin (Haley et al., 1981). The level of axolemmal contamination can be reduced significantly (0.6% to 1.2 % of the myelin protein) by the addition of a third centrifugation on a discontinuous gradient, in which myelin is resuspended in 0.85 M sucrose. In this case, myelin rises and layers out in the interface (Haley et al., 1981). The level of axolemmal contamination can also be reduced by treatment of the myelin fraction with EGTA (Haley et al., 1981; Norton and Cammer, 1984).

Most of the published protocols for myelin isolation do not include protease inhibitors. Analyses of the myelin proteins by SDS-PAGE gel electrophoresis indicate that significant protein degradation does not occur during myelin isolation. Also, a large number of enzymatic activities have been found in myelin preparations isolated using sucrose solutions (Norton and Cammer, 1984; Morell and Quarles, 1999). However, the inclusion of dithiothreitol and of a mixture of protease inhibitors in all the solutions used during the isolation of myelin is essential for the recovery of some intrinsic proteins, including muscarinic receptors and GTP-binding proteins (Larocca et al., 1987; 1991).

Most of the methods used to isolate CNS myelin can be used to isolate PNS myelin once the problem of homogenization of the nerve tissue is solved. The presence of connective

tissue and collagen in PNS makes the homogenization of the PNS tissue difficult. Two different approaches have been used to overcome this difficulty. One approach is that the tissue is pulverized in liquid nitrogen before homogenization (Greenfield et al., 1973). In another approach, the tissue is homogenized using a high-speed motor-driven homogenizer (Polytron; Wiggins and Fuller, 1981). After homogenization, connective tissue can be removed by low-speed centrifugation or by filtration of the homogenate through gauze.

The protocols used to isolate PNS myelin derive from Norton and Poduslo's method (Norton and Poduslo, 1973). However, because PNS myelin has lower density than CNS myelin, the concentration of sucrose used to homogenize PNS tissue is lower than that used by Norton and Poduslo. Wiggins and Fuller homogenized sciatic nerve in 0.29 M sucrose and overlaid the tissue suspension on 0.80 M sucrose (Wiggins and Fuller, 1981). Roomi et al. (1978) homogenized sciatic nerve in 0.17 M sucrose, and the tissue homogenate was overlaid on 0.65 M sucrose.

The PNS myelin preparation obtained by a first discontinuous-density gradient followed by several washes has been found sufficient to yield myelin with relatively little contamination. Although a second density gradient seems to increase the purity of the myelin fraction (Wiggins et al., 1975), it is important to note that a significant amount of myelin membranes are recovered with the membrane contaminant (Wiggins and Fuller, 1981). Similarly, during hypoosmotic shock and differential centrifugation, it seems that the material discarded contains as much myelin as contaminant (Wiggins and Fuller, 1981).

PNS myelin has also been isolated using a continuous gradient of sucrose (Matthieu et al., 1979). In this protocol, the sciatic nerve is homogenized in water and the tissue suspension is centrifuged at high speed. The supernatant containing the cytoplasmic material is discarded, and the pellet containing a total membrane fraction is resuspended in water and layered on a continuous gradient of sucrose (0.1 M to 1.2 M sucrose, using 1.4 M sucrose as a cushion). Centrifugation is carried out for 4 hr at 31,000 $\times$ *g*. Most of the PNS tissue membranes localizes between 0.32 M and 0.85 M sucrose. Three peaks are obtained: peak A (located at 0.1 M sucrose) contained mostly contaminating adipose material, peak B (located at 0.30 M sucrose) containing mainly membrane fragments with occasional large multilamellar structures, and peak C (located at 0.57 M sucrose) containing large multilamellar myelin fragments and smaller myelin fragments. Biochemical analysis of the gradient fractions confirms that the majority of the myelin is present in peak C. It also indicates that some of the fragments of membranes present in the peak B originate from myelin.

Heterogeneity of the myelin fraction

Highly purified CNS myelin fractions can be separated into subfractions by continuous or discontinuous density gradient centrifugation. The less-dense fractions are enriched in multilamellar fragments, whereas the heavy fractions have a large proportion of single-membrane vesicles and fragments with two or three membrane layers (Norton and Cammer, 1984; Morell and Quarles, 1999).

These fractions have different lipid-to-protein ratios but the lipid compositions are similar. Their protein compositions are different; myelin basic proteins are present in higher amounts in the lighter fractions than in the heavy fractions. The heavier fractions contain more CNPase and carbonic anhydrase enzymatic activities than lighter fractions, while the amount of PLP remains relatively constant among the different fractions. The fact that the different membrane fractions contain myelin components, but in different proportions, points to true heterogeneity, and that heterogeneity in the population of isolated myelin membranes is not just a reflection of contaminant membranes (Norton and Cammer, 1984; Morell and Quarles, 1999).

It is not possible to precisely define the origin of the heterogeneity of the myelin preparation. However, various data indicate that heterogeneity of the myelin fractions can be caused by diverse factors.

(1) A number of regional specializations are present in the myelin sheath. The internodal region consists mainly of a multilayer of condensed membranes. However, internodal clefts are present in which the membranes are not compacted and contain pockets of cytoplasm. Cytoplasm is present also in the lateral loops, regions close to the nodes of Ranvier, and in the outer and inner loops of the myelin sheath (Fig. 3.25.1). Intracellular organelles and elements of the cytoskeleton are present in these uncompacted areas of myelin. Similarly, immunocytochemistry shows that the proteins that form myelin have distinct localizations in the myelin sheath. For example, PLP/DM20 and MBP are localized in the compacted internodal region, CNPase is localized periaxonally and in the outer loops. MAG is

in the periaxonal loop, and MOG (myelin oligodendrocytes glycoprotein) in the outer loop (Norton and Cammer, 1984; Morell and Quarles, 1999).

(2) Different oligodendrocytes might produce myelin sheath of different composition, or depending on the diameter of the axon, an oligodendrocyte might produce myelin of different composition. In particular areas of the brain, some very large fibers contain more MBP that PLP, while small fibers contain more PLP than MBP (Hartman et al., 1982). Moreover, oligodendrocytes of different areas of the CNS produce myelin of different composition. Spinal cord myelin has different composition than cerebral myelin (Lees and Paxman, 1974).

(3) In developing animals, the myelin heterogeneity can be caused by differences in the stage of myelin maturation. The degree of myelin compaction and the myelin composition change during maturation (Danks and Matthieu, 1979).

Critical Parameters and Troubleshooting

Homogenization

The use of a homogenization medium containing low concentration of salt is essential for disruption of the structure of the myelin sheath and the subsequent formation of myelin vesicles. Inclusion of 0.1 M NaCl and 0.05 M potassium phosphate in isotonic sucrose solution preserved the axon-myelin relation, leading to a significant contamination of the myelin preparation with axolemma.

Homogenization of central nervous tissues does not present any difficulty. However, it may be beneficial to chop the tissue into smaller pieces on an ice-cold plate, and the tissue should be kept wet by adding homogenization medium. The ratio of tissue to homogenization buffer should be ~5% (1 g of wet tissue/20 ml of homogenization medium). An increase of this ratio may cause an increase in the levels of contaminant of the isolated myelin, especially when myelin is isolated from brains of animals in development.

On the contrary, homogenization of the PNS tissue is difficult and requires more extreme methods. In this case, homogenization can be performed using a Polytron homogenizer. However, excessive mechanical stress of the tissue leads to the rupture of intracellular organelles and generates small myelin vesicles, which leads to a higher level of contamination and the loss of significant amounts of myelin, in particular, during the hypoosmotic shock and low-speed centrifugation.

Homogenization can be facilitated by grinding the tissue in liquid nitrogen before homogenization (Greenfield et al., 1973). It is important to note that fresh tissue should be used when it is possible. Po, the major protein of PNS myelin, is rapidly degraded by endogenous proteases during dissection and thawing of frozen tissue (Cammer et al., 1981). Therefore, it is important to carry out the tissue dissection quickly and submerge the tissue sample in medium containing protease inhibitors. Similarly, if frozen tissue is used, it is recommended that the frozen tissue be allowed to thaw in sucrose solution containing a mixture of protease inhibitors.

Density gradient centrifugation

The use of swinging-bucket rotors with tube volumes other than those recommended in the protocols is permissible, as long as the volume ratios of sample and gradient are maintained and the rotors are capable of similar relative centrifugal forces.

Discontinuous sucrose density gradient methods, similar to those described here, have been used to isolate myelin from fish nervous systems. In such protocols, changes in the sucrose concentration and centrifugation time were performed to improve myelin yield (Morris et al., 2004).

The large majority of laboratories use discontinuous sucrose gradients as the method of choice for the isolation of myelin. However, special precaution should be taken when myelin is isolated from brains of animals with conditions that affect myelin. Alteration in the myelin composition might lead to both changes in the density of the myelin membranes and changes in the size of the myelin vesicles formed during the homogenization of the tissue (Bourre et al., 1980; Jurevics et al., 2003). Modification of the density and size of myelin vesicles can lead to a reduction of the amount of myelin recovered. Therefore, it may be necessary to modify the concentrations of the sucrose solutions used to make the discontinuous gradient, and the time and speed of centrifugation. In this case, it might be convenient to use a continuous sucrose density gradient to determine changes in the density of the myelin fraction and avoid losing part of the myelin. Myelin membranes can be separated from other CNS membranes using continuous sucrose density gradient zonal centrifugation (Bourre et al., 1980). Total CNS membranes can be separated by zonal centrifugation on

a continuous gradient of 0.1 M to 1.2 M of sucrose. Four peaks of membranes are obtained, two minor (at 0.12 M and at 0.35 M sucrose), and two major (at 0.68 M and at 1.1 M sucrose). The myelin fraction is present mainly in the peak of the 0.68 M region. The degree of contamination of the myelin fractions seems to be higher than the myelin isolated by the methods of Norton and Poduslo. The level of contamination can be reduced significantly by preparing the total membrane fraction by homogenizing the CNS tissue in water instead of isotonic sucrose solutions. In particular, the level of axolemmal contamination is greatly reduced by this hypoosmotic treatment (Bourre et al., 1980).

CsCl continuous density gradients have been used to isolate myelin (Greenfield et al., 1971). In this procedure, myelin forms a band at 0.85 M CsCl, equal to a density of 1.11 g/ml. The fact that myelin has a different density in sucrose than in CsCl might be the result of several factors, including packing of the membranes, degree of hydration, the amount of bound ions, changes in the myelin composition, or extraction of proteins at high ionic strength. Therefore, the authors do not recommend the use of CsCl gradient.

Enzyme and other assays

CNS myelin contains high activity of both CNPase and cholesterol ester hydrolase (Kurihara and Tsukada, 1967; Eto and Suzuki, 1973). In particular, CNPase has been used as a myelin marker and to follow myelin purification (Prohaska et al., 1973).

Nevertheless, the assessment of the degree of purity of isolated myelin is a conceptually difficult task. In part, this problem is generated by the ambiguity of the criteria used to define myelin. The best criterion to define myelin is morphological, not chemical or operational. As shown by electron microscopy, the myelin sheath is a morphologically heterogeneous structure derived from the plasma membrane of the oligodendrocytes that cover discontinuous segments of the axons.

However, the morphologic criteria cannot be applied to assess the purity of the myelin fraction isolated. The methods to isolate myelin involve procedures that disrupt the structures of the myelin sheath and eliminate the cytoplasm and organelles present in the non-compact areas of the myelin sheath. Electron microscopic analysis of the myelin fraction shows the presence of multilamellar membrane fragments, fragments with only

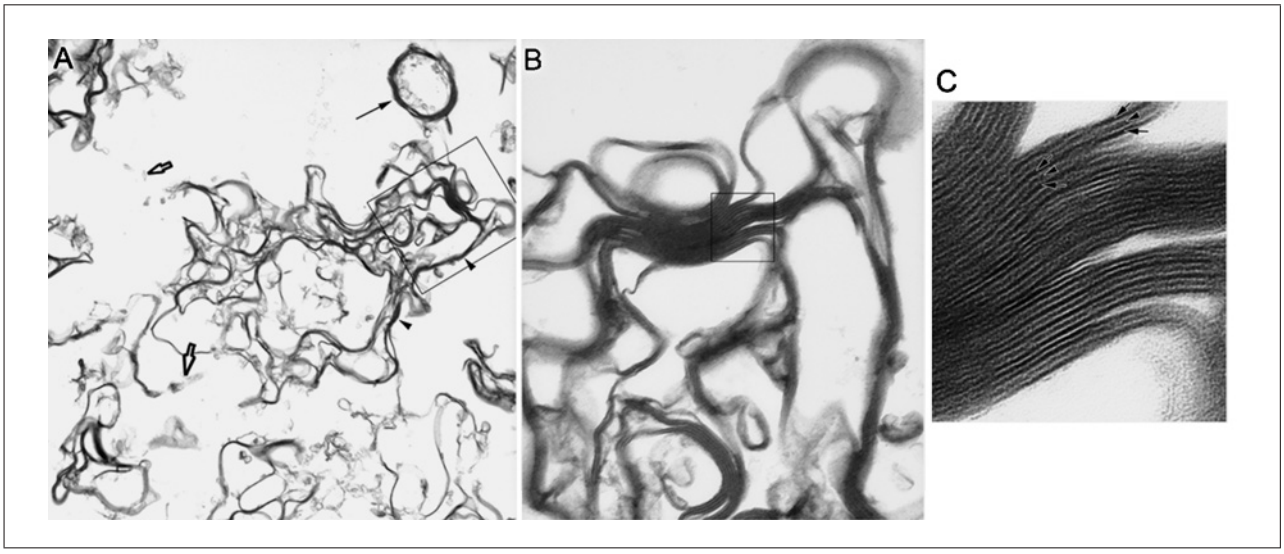


Figure 3.25.3 Electron microscopic analysis of isolated CNS myelin. A pellet of myelin membranes isolated from adult rat brain according to the Basic Protocol was fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4), post-fixed with 1% osmic acid, dehydrated through a graded series of ethyl alcohol, cleared in propylene oxide, and embedded in epoxy resin. (**A**) Electron micrograph showing myelin membranes broken up into rings (black arrow) and bands (black arrowheads), which split and rejoin irregularly to form a random net. Small units of membrane fragments (white arrows), appear to originate from split myelin layers (box marks the area shown in **B**). Magnification at 13,000×. (**B**) High-magnification electron micrograph showing the multilamellar nature of myelin fragments (box marks the area shown in **C**). Magnification at 50,000×. (**C**) Electron micrograph showing structural details of an isolated multilamellar myelin membrane. Major dense lines (arrows), intraperiod lines (arrowheads). Magnification at 227,000×. Courtesy of Dr. Cedric Raine.

two or three membrane layers, and single membrane vesicles (Fig. 3.25.3). Morphologic analysis could not determine whether all of these organelles are part of the myelin sheath. However, biochemical analysis supports the fact that these small vesicles are derived from the myelin sheath. Lastly, it is an insoluble problem to define where the oligodendrocyte plasma membrane finishes and the myelin sheath begins.

The best approach to determine myelin purity is to assess the presence in the isolated fraction of myelin of potential contaminant, including membrane fragments from cellular organelles as well as absorbed cytosolic proteins. Several markers can be used to determine contaminants including, nuclei (DNA), mitochondria (succinate dehydrogenase, *UNIT 3.4*), lysosomes (beta glucosidase), endoplasmic reticulum (NADH cytochrome *c* reductase, *UNIT 3.5*), neuronal membranes (acetylcholinesterase), and cytosolic proteins (lactate dehydrogenase, *UNIT 11.18*). Enzyme markers should be used with caution, because markers of plasma membranes, such as Na-K ATPase and 5′-nucleotidase enzymes, have been reported to be present in myelin (Norton and Cammer, 1984).

To determine the yield of the myelin isolation and specific activities of measured enzymes, it is necessary to estimate the protein content of the myelin fractions. The Markwell's method is widely used to determine the amount of proteins present in myelin preparations (Markwell et al., 1978). This method is a modification of the Lowry method; an increase in the cooper tartrate reagent allows assay of samples containing a substantial amount of sucrose or EDTA. Sodium dodecyl sulfate is added to the reagent to alleviate possible lipid interference and to promote rapid solubilization of the myelin proteins. However, because this method does not eliminate the interference of DTT, it is recommended to first precipitate the samples using the deoxycholate-trichloroacetic acid (DOC-TCA) protein precipitation technique (Peterson, 1979), and then solubilize the pellet using Markwell's reagent (2%, w/v, CO_3Na_2, 0.4%, w/v, NaOH, 0.16%, w/v, sodium tartrate, 1%, w/v, SDS, and 0.04% $CuSO_4 \cdot 5\ H_2O$).

Myelin samples can be directly subjected to SDS polyacrylamide gel electrophoresis (*UNIT 6.1*). However, myelin samples containing >25 μg of protein should be delipidated to avoid artifacts produced by the presence of high concentrations of lipids during electrophoresis. In this case, samples are treated with 10 vol of cold acetone and centrifuged; the pellet is washed once with an equal volume of cold acetone, and then resuspended in 2 to 3 ml of ethyl ether/ethanol (2:3 v/v). The insoluble material is recovered by centrifugation and the pellet is washed with 2 ml of ethyl ether and then dried under nitrogen. The delipidated sample can be directly dissolved with SDS in sample buffer.

Anticipated Results

Basic Protocol

Approximately 50% to 55% of the myelin present in the CNS tissue homogenate is recovered when isolation of myelin is carried out according to the Basic Protocol. The amount of myelin obtained depends on both the area of the nervous systems from which the myelin is isolated and the age of the animals from which the tissues are obtained.

Myelin is present throughout the nervous system, but is largely concentrated in areas containing mostly fiber tracts including the white matter of the brain and spinal cord. Myelination is a developmentally regulated process; it begins in the PNS, then the spinal cord, and lastly the brain. Myelination in different areas of the brain does not start and finish at the same time. The myelination pattern is similar for all mammals, but the time of birth in relation to the myelination progression is different in different species. Some animals, such as horses, cows, and sheep have a high degree of myelination at birth. Myelination in animals such as mice and rats starts at ∼9 to 10 days after birth.

Having delineated all the factors that influence the amount of myelin recovered, the Basic Protocol yields ∼3.75 mg of myelin (0.55 to 1.13 mg of myelin proteins per brain) from a brain of a 15-day-old rat, and 60 mg of myelin (9 to 18 mg of myelin protein per brain) from the brain of a 6-month-old rat. From bovine white matter, it is possible to obtain 100 mg of total myelin (15 to 30 mg of myelin protein) per gram of fresh tissue.

Isolated CNS myelin should contain high CNPase specific activity, relative specific activity (RSA) = 2 to 3 (enzymatic specific activity in myelin divided by the enzymatic specific activity in the unfractionated homogenate). In contrast, none or very low activity (RSA <0.04 to 0.06) of other membrane enzyme markers should be detected in the isolated myelin fraction.

Alternate Protocol 1

A very small amount of myelin membranes is lost during this procedure, probably <5%.

Alternate Protocol 2

The percentage of PNS myelin recovered with this protocol is low. Approximately 16% of the myelin present in the PNS homogenate is recovered when the entire protocol is carried out. However, when the isolation is carried out using only one discontinuous gradient, rather than two, 32% of the sciatic nerve myelin is recovered.

The amount of myelin obtained depends on the age of the animal. Myelination in rat sciatic nerve begins early in the first postnatal week, the amount of myelin formed increases 100 fold between birth and day 16, and continuously increases beyond the third month after birth.

One can expect to recover 0.8 to 1.8 mg of myelin protein per pair of rat (30 to 60 days old) sciatic nerves, or ~4 to 9 mg of total myelin.

PNS myelin contains some CNPase enzymatic activity. However, CNPase RSA in rat PNS myelin is ~0.5 to 0.8; a large amount of CNPase in sciatic nerve is present in the plasma membrane of the Schwann cell. CNPase activity in PNS myelin is approximately ten-fold lower than in CNS myelin. No or very low activity (<0.04 to 0.06 RSA) of other membrane enzyme marker should be detected in the isolated myelin fraction.

Time Considerations

The Basic Protocol involves twelve centrifugation steps with a combined time of 3.25 hr; allowing more than two times that time for acceleration, deceleration, and other manipulations, it is possible to obtain purified myelin in ~6.50 hr. It is not recommended, but if a preparation must be interrupted, it should be done after the second sucrose density gradient (after Basic Protocol, step 25). Preparations that have been left overnight at 0°C after this step and then continued on the next day, show no obvious differences in chemical composition, but no studies have been made of the enzymes activities of such preparations. Therefore, it is strongly recommended that the validity of results on myelin obtained by interrupted isolation be checked by comparison with those made on myelin obtained without interrupted isolation. Alternate Protocol 1 should take ~2 hr. Discontinuous sucrose gradients should be prepared immediately before use. Alternate Protocol 2 can be performed in ~5 hr, and if it is decided to purify PNS myelin using only one sucrose density gradient, it should take ~2.5 hr.

Assay of CNPase activity should be completed in <1 hr. Enzyme assays can be carried out in freshly obtained myelin. Alternatively isolated myelin fractions can be stored at 0°C to 4°C for 16 hr without significant loss of activity. Enzymatic assays and compositional measurements may also be carried out on material frozen at −20°C or −80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with measurements made on a fresh membrane preparation.

All the solutions can be kept for 1 to 2 days at 4°C or frozen for longer periods at –20°C. However, note that any enzymes, PMSF, or other protease inhibitors, should be added immediately prior to use.

Literature Cited

Bourre, J.M., Jacque, C., Delassalle, A., Nguyen-Legros, J., Dumont, O., Lachapelle, F., Raoul, M., Alvarez, C., and Baumann, N. 1980. Density profile and basic protein measurements in the myelin range of particulate material from normal developing mouse brain and from neurological mutants (Jimpy; quaking; Trembler; shiverer and its mld allele) obtained by zonal centrifugation. *J. Neurochem.* 35:458-464.

Cammer, W., Bieler, L., Fredman, T., and Norton, W.T. 1977. Quantitation of myelin carbonic anhydrase-development and subfractionation of rat brain myelin and comparison with myelin from other species. *Brain Res.* 138:17-28.

Cammer, W., Brosnan, C.F., Bloom, B.R., and Norton, W.T. 1981. Degradation of the P0, P1, and Pr proteins in peripheral nervous system myelin by plasmin: Implications regarding the role of macrophages in demyelinating diseases. *J. Neurochem.* 36:1506-1514.

Danks, D.M. and Matthieu, J.M. 1979. Hypotheses regarding myelination derived from comparisons of myelin subfractions. *Life Sci.* 24:1425-1440.

DeRobertis, E., Pellegrino De Iraldi, A., I, Rodriguez de Lores Garnaiz, G., and Salganicoff, L. 1963 Cholinergic and non-cholinergic nerve endings in rat brain. I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase. *J. Neurochem.* 9:23-35.

Eichberg, J., Whittaker, V.P., and Dawson, R.M. 1964. Distribution of lipids in subcellular particles of guinea-pig brain. *Biochem. J.* 92:91-100.

Eto, Y. and Suzuki, K. 1973. Cholesterol ester metabolism in rat brain. A cholesterol ester hydrolase specifically localized in the myelin sheath. *J. Biol. Chem.* 248:1986-1991.

Greenfield, S., Brostoff, S., Eylar, E.H., and Morell, P. 1973. Protein composition of myelin of the peripheral nervous system. *J. Neurochem.* 20:1207-1216.

Greenfield, S., Norton, W.T., and Morell, P. 1971. Quaking mouse: Isolation and characterization of myelin protein. *J. Neurochem.* 18:2119-2128.

Haley, J.E., Samuels, F.G., and Ledeen, R.W. 1981. Study of myelin purity in relation to axonal contaminants. *Cell Mol. Neurobiol.* 1:175-187.

Hartman, B.K., Agrawal, H.C., Agrawal, D., and Kalmbach, S. 1982. Development and maturation of central nervous system myelin: Comparison of immunohistochemical localization of proteolipid protein and basic protein in myelin and oligodendrocytes. *Proc. Natl. Acad. Sci. U. S. A.* 79:4217-4220.

Jurevics, H., Hostettler, J., Sammond, D.W., Nave, K.A., Toews, A.D., and Morell, P. 2003. Normal metabolism but different physical properties of myelin from mice deficient in proteolipid protein. *J. Neurosci. Res.* 71:826-834.

Kurihara, T. and Tsukada, Y. 1967 The regional and subcellular distribution of 2′,3′-cyclic nucleotide 3′-phosphohydrolase in the central nervous system. *J. Neurochem.* 14:1167-1174.

Larocca, J.N., Golly, F., and Ledeen, R.W. 1991. Detection of G proteins in purified bovine brain myelin. *J. Neurochem.* 57:30-38.

Larocca, J.N., Ledeen, R.W., Dvorkin, B., and Makman, M.H. 1987. Muscarinic receptor binding and muscarinic receptor-mediated inhibition of adenylate cyclase in rat brain myelin. *J. Neurosci.* 7:3869-3876.

Lees, M.B. and Paxman, S.A. 1974. Myelin proteins from different regions of the central nervous system. *J. Neurochem.* 23:825-831.

Markwell, M.A., Haas, S.M., Bieber, L.L., and Tolbert, N.E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87:206-210.

Matthieu, J.M., Waehneldt, T.V., Webster, H.D., Beny, M., and Fagg, G.E. 1979. Distribution of PNS myelin proteins and membrane enzymes in fractions isolated by continuous gradient zonal centrifugation. *Brain Res.* 170:123-133.

Morell, P. and Quarles, R.H. 1999. Myelin formation, structure and biochemistry. *In* Basic Neurochemistry: Molecular, Cellular and Medical Aspects, 6th ed. (G.J. Siegel, B.W. Agranoff, R.W. Albers, S.K. Fisher, and M.D. Uhler, eds.) Lippincott-Raven, New York.

Morris, J.K., Willard, B.B., Yin, X., Jeserich, G., Kinter, M., and Trapp, B.D. 2004. The 36K protein of zebrafish CNS myelin is a short-chain dehydrogenase. *Glia* 45:378-391.

Norton, W.T. and Cammer, W. 1984. Isolation and characterization of myelin. *In* Myelin (P. Morell ed.) pp. 147-195. Plenum Press, New York.

Norton, W.T. and Poduslo, S.E. 1973. Myelination in rat brain: Method of myelin isolation. *J. Neurochem.* 21:749-757.

Peterson, G.L. 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr, and Randall. *Anal. Biochem.* 100:201-220.

Prohaska, J.R., Clark, D.A., and Wells, W.W. 1973. Improved rapidity and precision in the determination of brain 2′,3′-cyclic nucleotide 3′-phosphohydrolase. *Anal. Biochem.* 56:275-282.

Roomi, M.W., Ishaque, A., Khan, N.R., and Eylar, E.H. 1978. The PO protein. The major glycoprotein of peripheral nerve myelin. *Biochim. Biophys. Acta* 536:112-121.

Wiggins, R.C., Benjamins, J.A., and Morell, P. 1975. Appearance of myelin proteins in rat sciatic nerve during development. *Brain Res.* 89:99-106.

Wiggins, R.C. and Fuller, G.N. 1981. Analysis of distribution of rat sciatic nerve protein among soluble, insoluble, and myelin subfraction. *Neurochem. Res.* 6:719-727.

Contributed by Jorge N. Larocca and Williams T. Norton
Albert Einstein College of Medicine, Yeshiva University
Bronx, New York

Isolation of Renal Brush Borders

The renal brush border has a prominent role in determining the specificity and rate of reabsorption from the glomular filtrate. Thus, the availability of isolated fractions of renal brush border has been instrumental in elucidating the molecular mechanisms that underlie tubular reabsorption (Murer and Kinne, 1980; Murer and Gmaj, 1986; Cutillas, 2005).

Historically, the first methods described for the isolation of brush border fragments were based mainly on the relative rigidity of the brush border and its resistance to homogenization as contrasted to the lability of the basal-lateral membranes (Murer and Kinne, 1980). With a higher sedimentation rate and greater density, differential centrifugation and simple density gradients were sufficient to obtain preparations enriched in large brush border fragments separated from the bulk of the remainder of the epithelial cell homogenates.

The early methods have gradually been supplanted by methods based on the use of divalent ions, chiefly magnesium or calcium to precipitate extra brush border membranes that are then discarded in an initial centrifugation. The brush border membranes, which do not precipitate due to high negative charges, are then collected by a second centrifugation. Homogenization on ice in 20 volumes of 150 mM KCI and 25 mM MES, pH 7.4, containing 0.01 M $MgCl_2$ is accomplished by four passes through a tight-fitting Teflon pestle in ground glass, followed by three 30-sec bursts with a Polytron homogenizer. The pellet obtained by centrifugation for 10 min at 2000 $\times$ *g* is discarded, and the brush borders are collected by centrifugation of the 2000 $\times$ *g* supernatant for 20 min at 35,000 $\times$ *g*. Then, 1 ml of 150 mM KCl and 25 mM MES is added to the resultant pellet and the membranes are resuspended using a Dounce hand homogenizer.

The selection of the method of membrane isolation is usually dictated by the aim of the study. For reconstitution of transport systems, a fast isolation method providing high yield of sealed vesicles containing the transport system as intact as possible is of primary importance. Often, small amounts of cross contaminating membranes are of little consequence so that purity is sacrificed for the sake of speed and yield. For analytical studies, where integrity of transport systems may be of secondary importance, additional purification steps are included based on different physical criteria.

To isolate brush borders from renal absorptive cells, a number of approaches are available: zonal sucrose gradients (see Basic Protocol), bivalent metal ion precipitation (see Alternate Protocol 1), isolation for dye/buffer uptake (see Alternate Protocol 2), aqueous two-phase preparations (see Alternate Protocol 3), and incremental two-phase isolation for separation of brush border and endosomes (see Alternate Protocol 4). These can be started from fresh or frozen materials. Protocols for assessment of brush border purity include flow cytometry (see Support Protocol 1), enzyme analysis (see Support Protocol 2), and electron microscopy (see Support Protocol 3).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

Contributed by D. James Morré and Timothy Hammond
Current Protocols in Cell Biology (2007) 3.26.1-3.26.14

BASIC PROTOCOL

ISOLATION OF RENAL BRUSH BORDER BY RATE ZONAL SUCROSE GRADIENTS

Of the many methods to isolate renal brush borders, the simplest are the traditional methods employing rate-zonal sucrose gradients (Berger and Sacktor, 1970). This protocol is popular because it is simple and highly reproducible. A major advantage of brush border membrane vesicles is that if they are not kept strictly ice cold during preparation, they entrap in their lumen the buffer in which they are homogenized, making it easy to deliver dyes and experimentally desirable solutions. These advantages are offset by the disadvantage that there is a tradeoff between yield and purity during membrane isolation.

Materials

New Zealand white male rabbits (2 to 3 kg)
0.5, 1.4, and 1.7 M sucrose solutions in distilled or deionized water

Forceps
Scalpel blade or scissors
Ground glass homogenizers (hand-held and motor-driven e.g., 15-ml Dounce homogenizer and Potter-Elvehjem with Teflon pestle, respectively)
Polytron homogenizer
Phase-contrast microscope
35-ml polyallomer ultracentrifuge tubes
Refrigerated centrifuge (90,000 × *g*) with a swinging-bucket rotor

Dissect the kidney cortices

1. Quickly remove kidneys from an anesthetized (IACUC-approved method) New Zealand white male rabbits weighing 2 to 3 kg, and place in sufficient cold 0.5 M sucrose to cover the kidneys.

2. Decapsulate and defat the kidneys by simple blunt dissection tearing the fascia between forceps.

   The vessels can be removed by cutting a wedge out of the hilum of the kidney. Bisection of the kidney along the long axis then allows clean removal of the papilla and much of the medulla, leaving behind highly enriched renal cortex.

3. Keeping the materials cold throughout this and the following steps, dissect out cortices. Weigh the cortices.

4. Finely slice the cortices with scalpel blade or scissors until the consistency of minced meat is reached.

Homogenize the tissue

5. Homogenize the tissue by hand at a ratio of 1 g tissue to 6 ml cold 0.5 M sucrose with a 15-ml Dounce homogenizer.

   Homogenization by hand is done by stroking up and down until the pestle reaches the bottom of the homogenizer. An extremely gentle homogenization is necessary to obtain large, intact brush borders. Over homogenization results in breaking the brush borders into fragments, which then enter the microsomal fraction.

6. Homogenize the suspension further with three complete strokes of a motor-driven 15-ml Potter-Elvehjem homogenizer with a tight-fitting Teflon pestle at 1000 rpm. Monitor the extent of the homogenization by examining the homogenate by phase-contrast microscopy.

   The objective is to preserve intact, generally rounded brush borders in good yield and fraction purity.

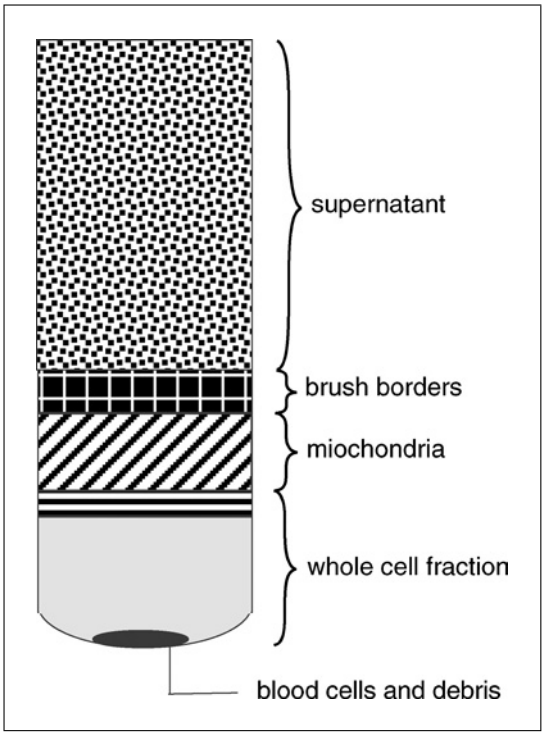


Figure 3.26.1 Schematic of sucrose gradient for separation of the brush border fraction from rabbit renal cortex homogenates. From Berger and Saktor (1970).

7. Layer the homogenate (~6 to 12 ml in 0.5 M sucrose) on a discontinuous gradient comprising 8 ml each of 1.7 and 1.4 M sucrose in 35-ml polyallomer ultracentrifuge tubes.

8. Centrifuge the tubes 60 min at 90,000 × *g*, 4°C.

   The bands resolved are shown in Figure 3.26.1. A thin layer of dense cells is sedimented at the bottom of the tube, and a thicker band of predominantly whole cells is distributed in the 1.7 M sucrose zone. The brownish, mitochondrial-enriched fraction is localized in the 1.4 M sucrose band, with large cell fragments plus many nuclei at the interface of the 1.7 and 1.4 M sucrose zones. The pinkish fluffy layer containing the brush borders is distributed at the interface of the 1.4 M and 0.5 M sucrose zones. A supernatant with increasing turbidity toward the brush border layer remains in the 0.5 M sucrose zone.

Remove the mitochondria

9. Carefully aspirate the brush border–enriched fraction (the entire 0.5 M layer and 0.5/1.4 M interface) from the top.

10. Centrifuge the brush border–enriched fraction from the sucrose gradient 30 min at 4000 × *g*, 4°C.

    This separates the brush borders from the microsomal fraction, yet effectively prevents the loss of small brush borders to the supernatant.

11. To separate brush borders from mitochondrial contamination, centrifuge 5 min at 32,000 × *g*, 4°C, to yield a small, tightly packed pellet containing contaminating mitochondria.

12. Remove the light pink layer containing the brush borders by gentle agitation and careful aspiration without disturbing the mitochondrial pellet.

Prepare brush borders

13. Further separate the intact brush borders from membranous material and disrupted brush borders by approximately six low-speed centrifugations for 10 min at 15,000 to 30,000 × *g*, 4°C.

At each step in the procedure, trace contamination by mitochondrial fragments collecting at the bottom of the centrifuge tube may be discarded as described above.

The final washed fraction is essentially a pure preparation of intact brush borders. Approximately 11 mg of protein (range of 10 to 13 mg) may be obtained from ~1 g renal cortex protein.

Preparations should be held at ice-bath temperatures or, if appropriate, frozen prior to use in an experiment (i.e., an experiment run at room temperature or 37°C, as required).

ALTERNATE PROTOCOL 1

ISOLATION OF KIDNEY BRUSH BORDERS BY BIVALENT METAL PRECIPITATION

Most methods to isolate brush border membrane vesicles from kidney cortex are modifications of the method originally introduced by Booth and Kenny (1974), reproduced in Figure 3.26.2, which is based on differential centrifugation following the addition of 10 to 20 mM $CaCl_2$ or $MgCl_2$ to the initial homogenate.

***Additional Materials** (also see Basic Protocol)*

1 M $MgCl_2$
Homogenate of rabbit kidney cortex (prepared from frozen rabbit kidney, see Basic Protocol, steps 1 to 6)
300 mM mannitol/12 mM HEPES, pH 7.4 (adjusted with Tris)

1. Add solid or 1 M $MgCl_2$ to a homogenate of cortical tissue prepared from frozen rabbit kidneys to a final concentration of 10 mM. Incubate 15 min with stirring in an ice bath.

   The method depends on the preferential aggregation of other subcellular structures by bivalent metal ions.

2. Remove the aggregated material by a low-speed centrifugation of the supernatant 20 min at 1500 × *g*, 4°C, and discard pellet.
3. Centrifuge the supernatant 12 min at 15,000 × *g*, 4°C.
4. Remove supernatant and resuspend pellet in 100 µl of 300 mM mannitol/12 mM Tris·Cl, pH 7.4, with $MgCl_2$ (10 mM final concentration).
5. Centrifuge 12 min at 2200 × *g*, 4°C, and discard pellet.
6. Centrifuge the supernatant 12 min at 15,000 × *g*, 4°C.
7. Resuspend pellet in 10 ml of 300 mM mannitol/12 mM HEPES, pH 7.4.

   Two treatments with Mg^{2+} and four short centrifugations give a product by sucrose gradient centrifugation comparable with brush-border preparations in enrichment and yield of marker enzymes (Hammond and Verroust, 1994; Fig. 3.26.4).

ALTERNATE PROTOCOL 2

ISOLATION OF RENAL BRUSH BORDER FOR DYE/BUFFER UPTAKE

Many investigators have modified the brush border membrane vesicle preparation by using much more vigorous homogenizations (Hammond, 1990; Hammond et al., 1993; Karniski, 2002), which facilitate the uptake of dyes, buffers, or other substances. Follow the Basic Protocol with the following modifications. This is the most commonly utilized brush border membrane vesicles methodology for renal membranes and is described stepwise in Figure 3.26.2. The disadvantage of this more vigorous protocol is that it compromises purity to some extent, which has led to attempts to further purify brush border membrane vesicles by an aqueous two-phase partition or flow cytometry (see Alternate Protocol 3).

For materials, see Basic Protocol.

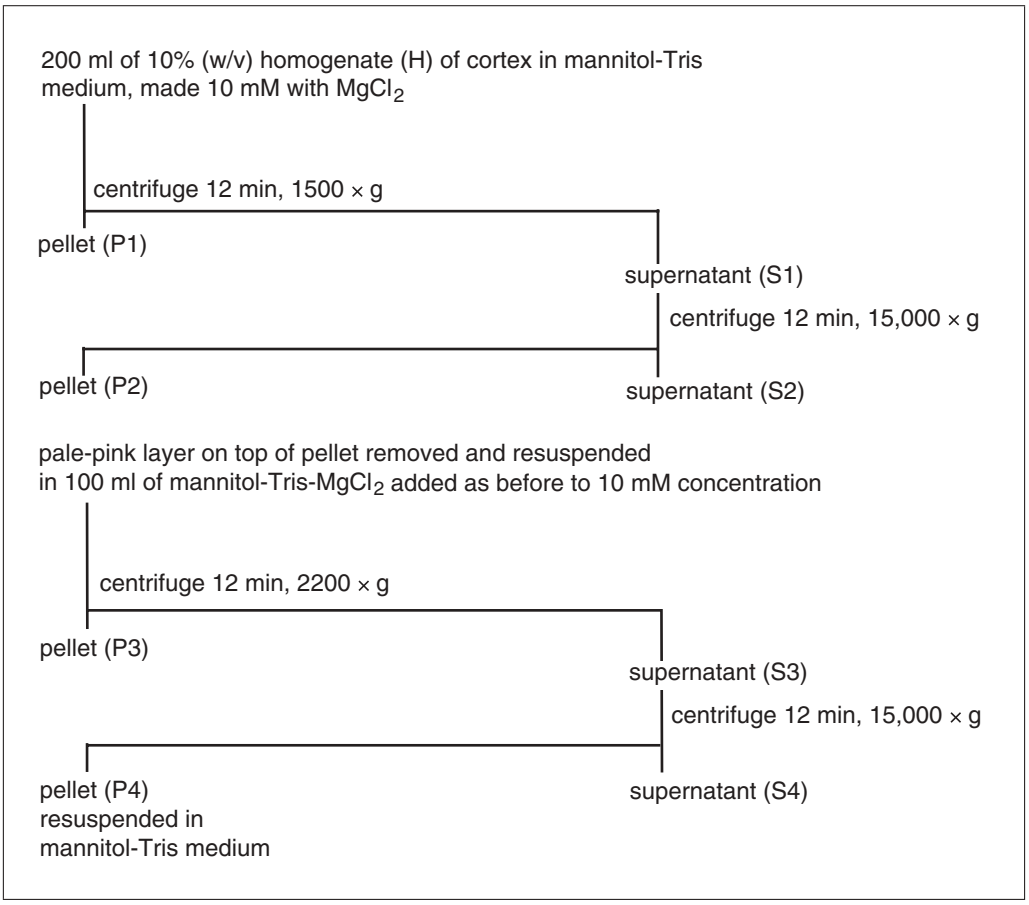


Figure 3.26.2 Preparation of brush border membranes by the divalent ion precipitation method with Mg^{2+} according to the original protocol of Booth and Kenny (1974).

1. Mince the initial kidney cortex with scissors to give the consistency of paté. Add the minced renal cortex to 6 vol of buffer that contains the dye or electrolyte solution intended to be encapsulated.

   Unlike cell isolation, mechanical grinding techniques such as Waring blenders can be substituted for hand mincing, but care must be taken to control the volume of buffer to about six times the volume of the packed tissue to facilitate the subsequent isolation steps.

2. Following motorized glass-Teflon homogenization (see Basic Protocol, step 6), homogenize each ~10-ml aliquot on ice using a Polytron for three bursts of 30 sec at top speed with 30 sec between bursts.

   This creates smaller brush border fragments, but facilitates uptake of the homogenization buffer into the core to the brush borders, which spontaneously vesiculate below a certain membrane size. This allows for controlled delivery of buffers and dyes to the core of the final product, which is critical for diverse studies such as transport properties, and use of indirect dye reporter molecules (Hammond et al., 1993; Karniski, 2002).

ISOLATION OF RENAL CORTICAL MEMBRANES USING AN AQUEOUS TWO-PHASE PARTITION TECHNIQUE

ALTERNATE PROTOCOL 3

Aqueous two-phase partition techniques have been applied to isolation of renal brush border membrane vesicles in an attempt to correct for the increased mitochondrial contamination inherent in protocols using vigorous homogenization, as these protocols are valued for the facile delivery of homogenization buffer to the core of the isolated membrane vesicles. The disadvantage of aqueous two-phase partition is the necessity to validate that the exposure to polyethylene glycols and dextran inherent to the isolation method does not affect the properties of the membranes to be studied.

***Additional Materials** (also see Basic Protocol)*

Kidneys harvested from one or more (depending on requirements) pentobarbital-anaesthetized male Sprague-Dawley rats, 200 to 250 g
20% (w/v) dextran T-500 (Pharmacia)
40% (w/v) polyethylene glycol 3350 (PEG; Union Carbide or Fisher)
0.2 M potassium phosphate, pH 7.2

30-ml Pyrex tube
Parafilm
Pasteur pipets
50-ml polyallomer tubes

Homogenize kidney cortices

1. Place harvested rat kidneys into ice-cold 300 mM mannitol/12 mM HEPES, pH 7.4.
2. Decapsulate each kidney and dissect the cortexes free from the medulla and hilum as described above.
3. Mince the cortex with scissors to give the consistency of paté.

Homogenize tissue

4. Homogenize the tissue as in Basic Protocol, step 6.
5. Continue homogenization using three 30-sec bursts of a Polytron homogenizer at top speed separated by 30 sec. Keep the homogenate on ice throughout.
6. Add 100 µl of 1 M $MgCl_2$/10 ml of homogenate.
7. Centrifuge the resulting cortical homogenate 10 min at 1700 × *g*, 4°C.

Isolate brush border vesicles

8. Remove the supernatant to a 50-ml polyallomer tube and centrifuge 20 min at 14,500 × *g*, 4°C. Discard the supernatant.
9. Resuspend the pellet of brush border membrane vesicles in <2 ml of buffer.
10. Repeat steps 8 and 9.

Perform aqueous two-phase partition fractionation

11. Prepare stock solutions of 20% (w/w) dextran T-500 and 40% (w/w) PEG in double-distilled water.
12. Prepare a 6.4% (w/w) two-phase systems by adding 5.1 g of 20% dextran to 2.56 g of 40% PEG in a tared 30-ml Pyrex tube on ice. Then, add 0.32 ml of 0.2 M potassium phosphate at pH 7.1.
13. Make the total weight of the contents up to 14 g with distilled water or the medium used for homogenate preparation (Table 3.26.1).

    Other weight ratios are prepared by varying the amounts of dextran and PEG added to the system as described (Morré and Morré, 1989).

14. Resuspend the membranes to be separated in a minimum volume (e.g., <2 ml) of the medium used for homogenate preparation, e.g., 300 mM mannitol/12 mM HEPES, pH 7.4.
15. Adjust the final weight of the two-phase system to 16 g with medium after adding the membranes (total volume <2 ml).
16. Cover the Pyrex tube with Parafilm and invert vigorously 40 times in the cold (9°C).

Table 3.26.1 Composition of a 16 g (6.0%) Two-phase System

Reagent	Amount
20% (w/v) dextran T-500 (Pharmacia)[a]	4.80 g
40% (w/v) polyethylene glycol 3350 (PEG; Union Carbide)	2.40 g
0.2 M potassium phosphate buffer, pH 7.2	0.32 ml
Sufficient distilled water or isolation buffer to bring the weight of the mixture to 14 g	
Brush border membranes (~250 mg) resuspended in isolation buffer	2 g

[a]It is practical to prepare stock solutions of the phase system and store them frozen as aliquots. The stock solutions of dextran and polyethylene glycol are prepared on a weight basis, while sucrose, buffer and salt stock solutions are prepared on a molar basis.

17. Centrifuge the resultant mixture 5 min at 750 × *g*, 4°C, to separate the phases.
18. Remove the upper phase using a Pasteur pipet to a 50-ml tube.
19. Dilute the upper phase ten-fold with 300 mM mannitol/12 mM HEPES, pH 7.4.
20. Collect the membranes from the upper phase by centrifuging 30 min at 20,000 × *g*, 4°C.

The yield of brush border protein is ~10 mg per gram of kidney. The brush borders can be used in many ways according to the investigator's interests. Popular examples include immediate use for function studies such as electrolyte uptake studies using fluorophores or radioactive electrolytes, labeling with antibodies, or SDS-PAGE analysis. The brush borders can be frozen at –80°C for later use, but this lyses the vesicles, which encapsulate the surrounding buffer when thawed.

INCREMENTAL AQUEOUS TWO-PHASE PARTITION FRACTIONATION OF RAT KIDNEY TO YIELD BOTH BRUSH BORDER AND ENDOSOMES

ALTERNATE PROTOCOL 4

In studies where endosome contamination is to be minimized or where brush border microvilli-endosome comparisons are a study objective, the brush border microvilli may be preferentially collected from the upper dextran-rich phase by aqueous two-phase partition. Other membranes, including basolateral plasma membranes and endosomes remain in the polyethylene glycol–rich lower phase and are subsequently extracted from the lower phase by employing less concentrated dextran-PEG phase systems.

For materials, see Alternate Protocol 3.

1. For incremental aqueous two-phase partitioning, prepare a 6.0% (w/w) two-phase system containing 4.80 g of 20% dextran and 2.40 g of 40% PEG in a tared 30-ml Pyrex tube on ice.
2. Add 0.32 ml of 0.2 M potassium phosphate at pH 7.2.
3. Adjust the total mass of the contents to 14 g with distilled water.

Other mass ratios are prepared by varying the amounts of dextran and PEG added to the system as described (Morré and Morré, 1989).

4. Resuspend membranes to be separated in 300 mM mannitol/12 mM HEPES, pH 7.4, buffer (total volume <2 ml).
5. Add the membranes to the two-phase system, and adjust the final mass of the contents to 16 g with 12 mM HEPES, pH 7.4, buffer.
6. Cover the Pyrex tube with Parafilm, and invert vigorously 40 times in the cold (9°C).

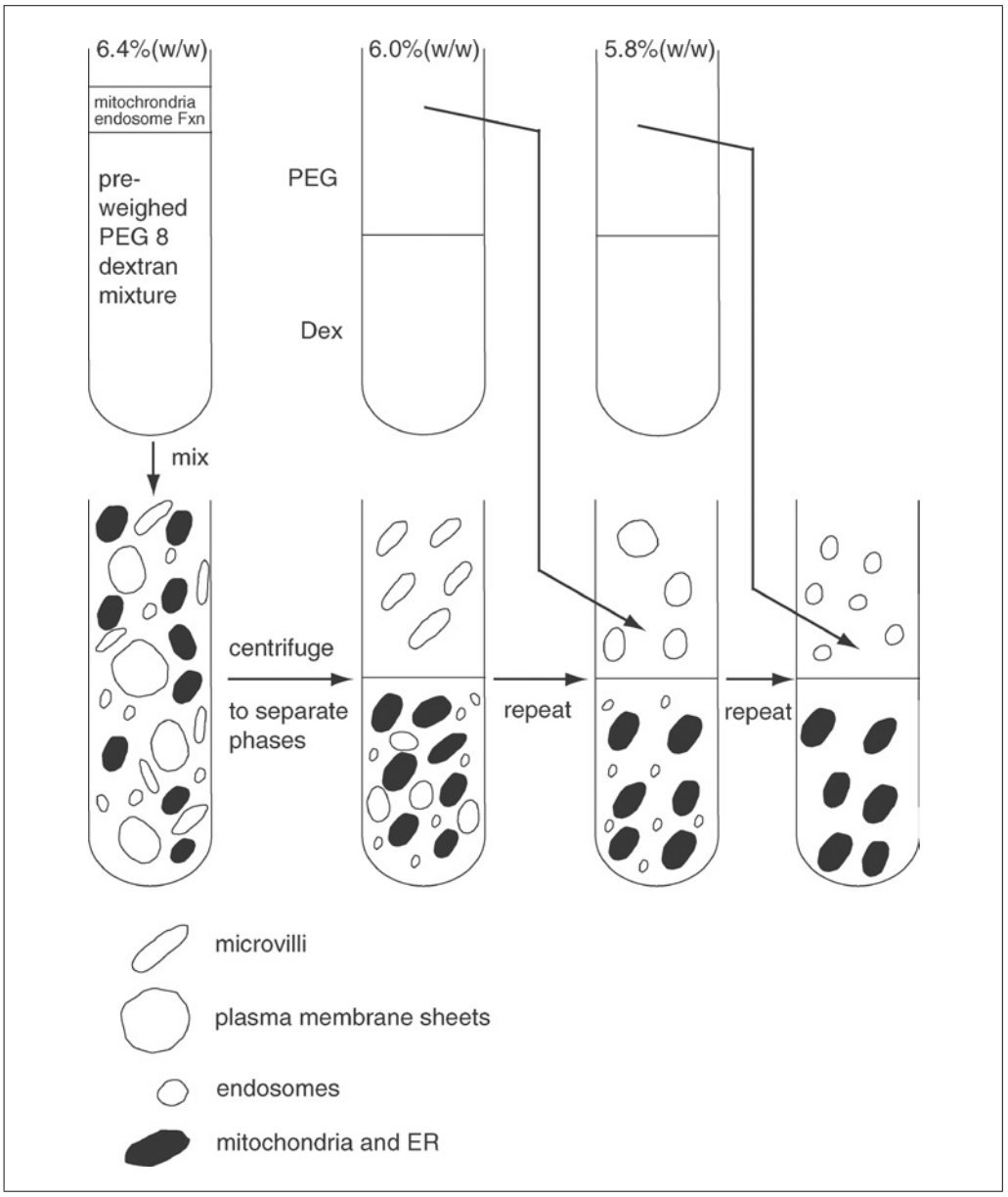


Figure 3.26.3 The incremental two-phase partition for the resolution of different plasma membrane domains and a partially purified endosome fraction from a rat kidney particulate fraction (Morré et al., 1994). The initial partition used a 6.4% PEG-dextran phase system. Brush border membranes partitioned into the upper phase. A 6.0% phase system was equilibrated in parallel and the upper phase of this phase system replaced the brush border-containing upper phase of the 6.4% system. After mixing and resolution of the two new phases, the upper phase now contained the remainder of the plasma membrane including basolateral-derived vesicles. A 5.8% phase system also was equilibrated in parallel and the upper phase replaced the plasma membrane–containing upper phase of the second partition. After mixing and resolution into two phases, a third upper phase enriched in endosomes and depleted in plasma membrane was obtained. The fraction, however, was still contaminated by mitochondrial fragments. The bulk of the mitochondria, endoplasmic reticulum, and nuclei remained in the lower phase after the last partitioning step. Fxn, fraction.

7. Centrifuge the resultant mixture 5 min at 1000 × *g*, 4°C, to separate the phases.
8. Collect the upper phase using a Pasteur pipet and dilute it ten-fold with 300 mM mannitol/12 mM HEPES, pH 7.4.
9. Collect membranes from the upper phase by centrifuging 30 min at 20,000 × *g*, 4°C.

Brush border membranes are concentrated in the upper phase of the initial 6.4% dextran-PEG phase separation (Fig. 3.26.3A). The lower phase still contains basolateral plasma membranes, endosomes, mitochondria, and endoplasmic reticulum (ER). An upper phase equilibrated from a 6.0% dextran-PEG phase system extracts the basolateral plasma membranes (Fig. 3.26.3B). An upper phase equilibrated from a 5.8% dextran-PEG phase system extracts the endosomes for further purification in a third partitioning step (Fig. 3.26.3C). The bulk of the mitochondria, endoplasmic reticulum, nuclei, and other internal membranes are retained in the lower phase (Fig. 3.26.3D).

Density gradient centrifugation (Boffeli et al., 1997; Yoshioka et al., 1997), density gradient centrifugation phase partitioning (Hammond et al., 1993; Gingras et al., 1994), differential centrifugation (Biber et al., 1981; Yusufi et al., 1994), flow cytometry (Hammond, 1990), and free-flow electrophoresis (Mircheff et al., 1984) all have been employed to separate brush border membranes and basolateral membranes, which exhibit subtle differences in density and net surface charge.

STUDY OF BRUSH BORDER MEMBRANE VESICLES BY FLOW CYTOMETRY

SUPPORT PROTOCOL 1

Membranes isolated by the vigorous homogenization techniques and divalent ion precipitation have been further imaged and isolated by flow cytometry (Hammond, 1990). Using the technique of fluorescence-activated cell sorting, direct comparison can be made of enzymatic marker purity of rat renal cortical brush border membrane vesicles prepared by divalent ion precipitation, with and without flow sorting based on enzymatic or antibody probes to detect the brush border membrane enzyme marker gamma-glutamyl-transferase. Flow-sorted membrane vesicles are characterized by larger amounts of brush border membrane markers, no detectable mitochondrial or basolateral markers, and greatly reduced Golgi and lysosomal markers. The flow-sorted membrane vesicles are functional for transport studies as they take up at least as much ^{3}H-proline and ^{3}H-glucose per milligram protein as divalent ion–precipitated membrane vesicles. Preparation of membrane vesicles from superficial and deep cortex allows imaging of the different distributions of gamma-glutamyl transferase in membrane vesicles from these areas. Hence, membrane vesicle populations of exceptional purity can be separated according to fluorescent markers using flow cytometry. High-speed observations on large numbers of individual vesicles allow identification of subpopulations, and statistical comparison within a single heterogeneous sample. Unlike today when flow cytometry has become a standard, a near-fully automated analytical tool in biological laboratories, at the time these techniques were reported, there were only a few flow cytometers in use, and they were complex and expensive to run. The isolation and study of membrane vesicles by flow cytometry has had only a few dedicated followers, mostly for highly specific renal or intestinal biological applications (Gorvel et al., 1984; Hammond et al., 1993; Hammond and Verroust, 1994).

ANALYSIS OF MARKER ENZYMES FOR RENAL BRUSH BORDER

SUPPORT PROTOCOL 2

1. Measure protein by using the biocinchoninic acid method (*APPENDIX 3H;* Smith et al., 1985).
2. Determine the activity of K^+-stimulated, ouabain-inhibited, nitrophenyl phosphatase, a basolateral marker enzyme that co-localizes with Na^+/K^+-ATPase, by measuring cleavage of *p*-nitrophenylphosphate (Kashiwamata et al., 1979; Colas and Marous, 1980).
3. Measure the activity of γ-glutamyltranspeptidase by the cleavage of glutamyl *p*-nitroanilide (Tsao and Curthoys, 1980) or L-leucyl-nitroanilide (Ferracci and Maroux, 1980).

4. Measure alkaline phosphatase by cleavage of *p*-nitrophenyl phosphate at pH 9.0 (Shirazi et al., 1980).
5. Assay succinyl dehydrogenase, a mitochondrial marker, based on succinate 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolum reductase (Pennington, 1961).
6. Measure mannosidase activity according to the method of Tulsiani et al. (1977).
7. Assay glucose-6-phosphatase in fresh membrane preparations (Nordlie and Arion, 1966) using Leon's reagent for secondary phosphate analysis.

SUPPORT PROTOCOL 3

ANALYSIS OF RENAL BRUSH BORDER PREPARATIONS BY ELECTRON MICROSCOPY

In addition to evaluation by phase-contrast microscopy, the purity and integrity of the brush borders may be evaluated by electron microscopy (Fig. 3.26.4). Samples of membrane vesicles are fixed for electron microscopy with 2% (v/v) glutaraldehyde in

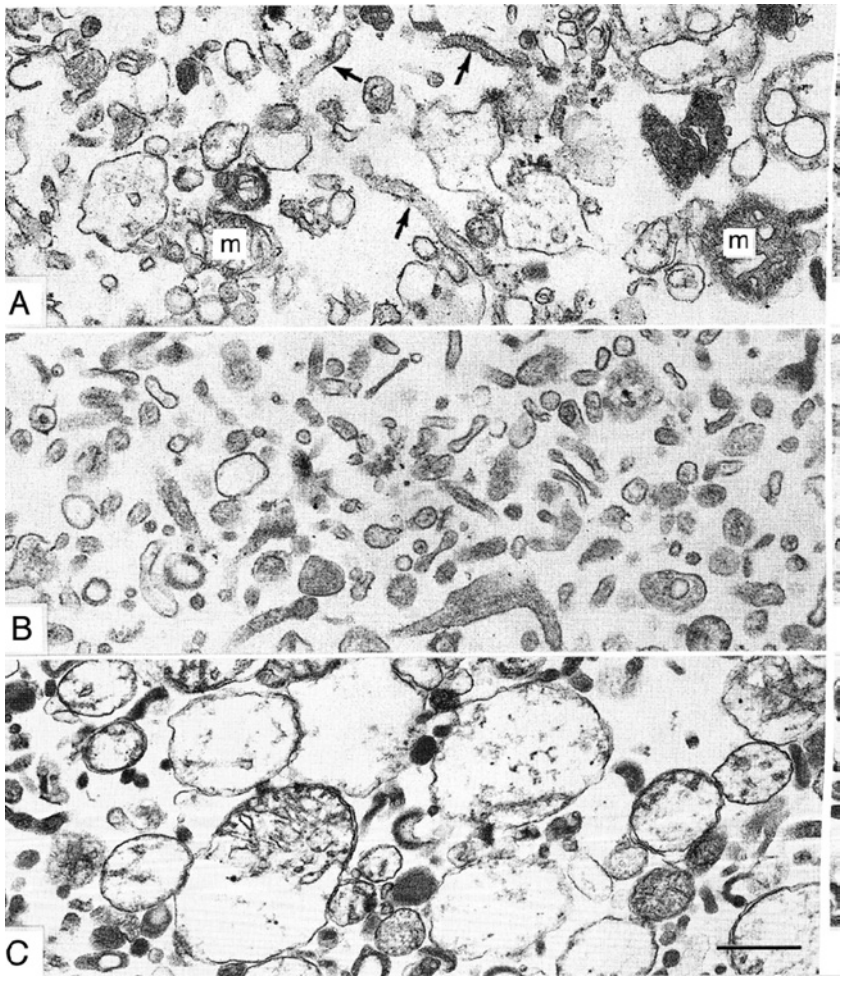


Figure 3.26.4 Electron micrographs of brush-border membrane vesicles following aqueous two-phase partition fractionation. (**A**) Total starting brush-border membrane vesicles fraction prior to aqueous two-phase partition. M = mitochondria. Arrows denote microvilli. (**B**) A 6.8% upper phase enriched in microvilli. (**C**) 5.8% lower phase consisting of mitochondria and membrane contaminants. Scale bar = 0.5 μm. Modified from Hammond et al. (1993).

phosphate-buffered saline. The samples are transferred to 1% (w/v) osmium tetroxide in 0.05 M sodium phosphate, pH 7.2, for 1 hr to overnight at 4°C. Dehydrate the samples in an acetone series and embed in Epon. Lead-stained thin sections are examined and photographed.

COMMENTARY

Background Information

Brush borders are cell-surface structures facing the lumens of the small intestine and kidney cortex specialized for the absorption of nutrients. The name derives from the characteristic folds in the plasma membranes that delineate a large number of fingerlike projections called microvilli. These microvilli greatly increase the surface area of absorptive cells to enhance their absorption rates. In addition to carrying out transport functions, the plasma membrane of the brush border contains many enzymes that assist in the degradation of sucrose into glucose and fructose and of proteins into amino acids. As part of their transport functions, the brush border membrane also contains specific permeases that allow the epithelial cells to absorb these nutrients from the kidney or intestinal lumen.

Absorptive microvilli from epithelial cells represent one of the few nonmuscle systems in which actin microfilaments occur in well-ordered patterns. Each microvillus contains a bundle of 20 to 30 actin microfilaments anchored in the plasma membrane at the tip of the microvillus.

Just under the microvilli are two additional networks also rich in actin and actin-binding proteins. The terminal web is a layer of filaments that crisscrosses the cytosol just under the microvilli. Another is the belt desmosome, which contains a set of actin filaments that encircles the plasma membrane and is linked to the plasma membrane at the level of the terminal web. Study of these networks in brush borders has led to an understanding of how actin filaments cause rigid fingerlike extensions of the plasma membrane and has provided valuable information on the roles of various actin-binding proteins in non-muscle cells.

The bundle of specialized actin filaments at the core of each microvillus lacks myosin, tropomyosin, and α-actinin. The actin bundles presumably play a structural role, one in which the shapes of the microvilli are maintained. The (+) ends of these microfilaments point toward the site of membrane insertion at the tip of the microvillus. In addition to these actin microfilaments, microvilli contain several major proteins that contribute to generating their rigid structure. For the most part, the presence and location of these proteins is similar for brush borders of kidney and intestine (Coudrier et al., 1988). Thus, brush borders are complex cellular structures consisting not only of a plasma membrane but underlying cytoplasmic and cytoskeletal elements. Normally, the objective is to isolate brush borders with microvilli intact for use primarily in structural and in transport studies.

The initial impression that the isolation and application of renal brush border membrane vesicles is straightforward stands in marked contrast to the frustrations of many investigators utilizing the techniques. A lot of the confusion can be explained in two false assumptions. First, the kidney has a highly specialized endosomal pathway characterized by intramicrovillar clefts arising at the base of the brush borders, and delivering membrane elements into small endosomal vesicles and larger endosmal vacuoles, before selectively delivering membrane elements back to the apical membrane surface or to lysosomes (Maunsback, 1976; Christensen and Nielsen, 1990; Verroust et al., 1996). This system of endosomal vesicles and vacuoles is a highly specialized reabsorptive system shared only with cells in the inner ear, and the placenta (Maunsback, 1976; Christensen and Nileson, 1990; Verroust et al., 1996). This phenomenon leads to confusion because when renal brush borders are directly compared with brush border membrane elements from the gastrointestinal tract, or cultured cell systems, the renal membranes have unexpected properties. Second, there is an assumption that compartmental markers such as enzymes and transporters heavily concentrated in the brush border should not traffic into other membrane compartments. In reality, many proteins that are concentrated in the brush border are transported, albeit at lower levels, through the specialized renal endosomal pathway (Maunsbach, 1976; Christensen and Nielsen, 1990; Verroust et al., 1996). Hence, using enzyme enrichment as the sole source of indices of membrane purity can lead to disappointment and uncertainty (Hammond and Verroust, 1994). Several lines of evidence

suggest that fractions very heavily enriched in renal brush border membrane vesicles (Hammond, 1990), intermicrovilar clefts (Hammond et al., 1994), clathrin-coated vesicles (Hammond and Verroust, 1994), endosomal vesicles and vacuoles (Hammond et al., 1994), and lysosomes (Piqueras et al., 1994) can be isolated from the kidney, but their properties are uniquely organ specific for the kidney cortex, and the distribution of compartmental markers is far from absolute (Verroust et al., 1996).

Critical Parameters and Troubleshooting

Assuming all solutions are correctly prepared, low yield, poor morphology, and/or reduced function is most often traced to loss of function in the kidney cortex prior to homogenization, usually attributed to untimely delays between animal sacrifice and transfer of the chopped cortex into the cold homogenization buffer. Further delays in homogenization or over homogenization may also be a factor. Surprisingly, once the homogenate is prepared, further changes are minimal as the initial centrifugation steps are completed. However, even when working in the cold, speed is an essential condition of successful membrane isolation.

Incomplete homogenization as determined visually or by microscopic examination of debris fraction is usually a result of not dicing the tissue finely enough prior to homogenization or the use of an overly loose-fitting homogenizer.

The brush border region of epithelial cells is relatively resistant to homogenization. This permits initial isolation of large fragments of the apical cell structures. These may be disrupted further to form smaller brush border membrane vesicles. However, even under aggressive Polytron homogenization, structures filled with electron-dense material resembling intact microvilli are still present.

For analytical separations based on two-phase partition, it is important to completely resuspend the initial pellet to be applied to the two-phase system. A poorly fitting homogenizer or insufficient homogenization will result in large pieces of unsuspended pellet. Drive the pestle completely to the bottom of the homogenizer and observe the suspension of large particles between glass and pestle. Confirm homogeneity by light microscopy.

Two-phase systems are extremely sensitive to conditions of pH, temperature, polymer-concentrations, and ionic composition. Directions for preparation must be carefully followed with addition by weight using an accurate top-loading balance with sufficient sensitivity to detect milligram differences.

Care should be taken to not allow the temperature of the two-phase system to change during mixing because two-phase partition is temperature dependent. It is best to work consistently either from an ice bucket or in a cold room. It is essential that the contents of the tubes do mix and that an unmixed cushion of dextran does not remain along the wall of the tube. Subsequent separation into two phases occurs too slowly at unit gravity so that the separation is usually accelerated by centrifugation in a swinging-bucket rotor at 750 $\times$ g, 4°C. The speed can range from 250 to 2500 $\times$ g and the duration can range from 3 to 10 min.

A common initial difficulty in phase separation may involve inaccurate preparation of the polymer stocks. If the polymers have picked up excess water in storage, they will be underrepresented and the compositions will not be those required. This can be compensated for by increasing the phase compositions until separation into two equal phases is achieved.

To accurately prepare a stock solution, access to a polarimeter to determine optical rotation is necessary. A detailed description of the procedure for preparation of the dextran stock solution is given by Larsson (1985).

The bulk, $>$90%, of the membranes should remain in the upper phase. If there is nothing or $>$10% of the membranes in the lower phase, the phase composition should be lowered to increase or increased to lower the proportion of membranes entering the lower phase.

Normally, the phases are diluted 1:1 with buffer and the membranes are collected by centrifugation. If the membranes fail to sediment, especially from the lower phase, it is usually the result of incomplete mixing after dilution and prior to centrifugation.

The vesicles are 15- to 20-fold enriched in marker enzymes for the brush border such as alkaline phosphatase with a yield of $\sim$50% (Biber et al., 1981).

Anticipated Results

The anticipated outcome is a preparation of functional isolated kidney brush border membranes suitable for use in transport studies. The yield of purified intact brush borders from rat or rabbit renal cortex is in the range of 10 to 20 mg protein per gram renal cortex protein. Brush border membranes are isolated largely oriented right-side out, and they are extremely resistant to inversion. Protein can be assayed

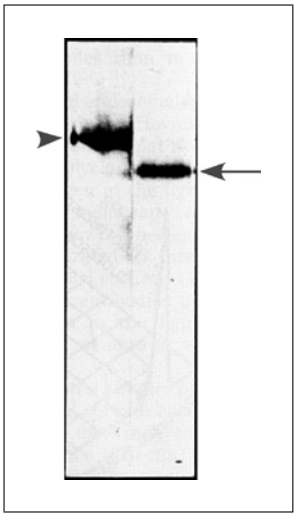


Figure 3.26.5 Immunoblot analysis of glycoprotein content of kidney brush border membrane vesicles and kidney endosomes. (**A**) 330-kDa Heymann antigen (arrowhead) and a 280-kDa protein derived from intermicrovillar clefts (arrow) are present. Modified from Hammond and Verroust (1994).

following isolation of the final brush border fraction using standard biochemical protein assays (Smith et al., 1985).

If specific antibodies are available, analysis of specific proteins can also be undertaken, such as a western blot of renal brush border shown in Figure 3.26.5, which identifies megalin and cubilin that are highly concentrated on the apical brush border of renal cortical cells.

For analytical preparations with reduced contents of mitochondrial and internal and basal-lateral membranes, where more extensive disruption may be required, yield, intactness, and suitability for transport studies will be correspondingly less.

Time Considerations

The key to functional brush borders in high yield is to work fast and not interrupt the procedure. For intact brush borders, the complete protocol from animal sacrifice to resuspension of the final pellet should be completed in 2 to 3 hr. It is essential that all materials, solutions, supplies, and equipment be prepared and at the appropriate temperatures before sacrificing the animal.

Literature Cited

Berger, S.J. and Sacktor, B. 1970. Isolation and biochemical characterization of brush borders from rabbit kidney. *J. Cell Biol.* 47:637-645.

Biber, J., Steiger, B., Haase, W., and Murer, H. 1981. A high yield preparation for rat kidney brush border membranes: Different behaviour of lysosomal markers. *Biochim. Biophys. Acta.* 647:169-176.

Boffelli, D., Weber, F.E., Compassi, S., Werder, M., Schultess, G., and Hauser, H. 1997. Reconstitution and further characterization of the cholesterol transport activity of the small-intestinal brush border membrane. *Biochemistry* 36:10784-10792.

Booth, A.G. and Kenny, A.J. 1974. A rapid method for the preparation of microvilli from rabbit kidney. *Biochem. J.* 142:575-581.

Christensen, E.I. and Nielsen, S. 1991. Structural and functional features of protein handling in the kidney proximal tubule. *Semin. Nephrol.* 11:414-439.

Colas, B. and Marous, S. 1980. Simultaneous isolation of brush border and basolateral membrane from rabbit enterocytes. *Biochim. Biophys. Acta* 600:406-420.

Coudrier, E., Kerjaschki, D., and Louvard, D. 1988. Cytoskeleton organization and submenbranous interactions in intestinal and renal brush borders. *Kidney Int.* 34:309-320.

Cutillas, P.R., Biber, J., Marks, J., Jacob, R., Steiger, B., Cramer, R., Waterfield, M., Burlingame, A.L., and Unwin, R.J. 2005. Proteomic analysis of plasma membrane vesicles isolated from the rate renal cortex. *Proteomics* 5:101-112.

Ferracci, H. and Maroux, S. 1980. Rabbit intestinal amino peptidase N. Purification and molecular properties. *Biochim. Biophys. Acta* 599:448-463.

Gingras, D., Boivin, D., and Beliveau, R. 1994. Asymmetrical distribution of L-isoaspartyl protein carboxyl methyltransferases in the plasma membranes of rat kidney cortex. *Biochem. J.* 297:145-150.

Gorvel, J.P., Mawas, C., Maroux, S., and Mishal, Z. 1984. Flow cytometry is a new method for the characterization of intestinal plasma membrane. *Biochem. J.* 221:453-457.

Hammond, T.G. 1990. Study of rat renal brush border membrane vesicles by flow cytometry. *Kidney Int.* 38:5-11.

Hammond, T.G. and Veroust, P.J. 1994. Heterogeneity of endosomal populations in the rat renal cortex: Light endosomes. *Am. J. Physiol.* 266:C1783-C1794.

Hammond, T.G., Majewski, R.R., Onorato, J.J., Brazy, P.C., and Morré, D.J. 1993. Isolation and

characterization of renal cortical membranes using aqueous two-phase partition technique. *Biochem. J.* 292:743-748.

Hammond, T.G., Verroust, P.J., Majewski, R.R., Muse, K.E., and Oberley, T.D. 1994. Heavy endosomes isolated from the rat renal cortex show attributes of intermicrovillar clefts. *Am. J. Physiol.* 267:F516-F527.

Karniski, L.P. 2001. Mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene: Correlation between sulfate transport activity and chondrodysplasia phenotype. *Hum. Mol. Genet.* 10:1485-1490.

Kashiwamata, S., Goto, S., Semba, R.K., and Suzuki, F.N. 1979. Inhibition by bilirubin of $(Na^+ + K^+)$-activated adenosine triphosphatase and K^+-activated *p*-nitrophenylphosphatase activities of NaI-treated microsomes from young rat cerebrum. *J. Biol Chem.* 254:4577-4584.

Larsson, C. 1985. Plasma membranes. *In* Modern Methods of Plant Analysis. New Series, Vol. 1. (H.F. Linskins and J.F. Jackson, eds.) pp. 85-104. Springer. Berlin, Heidelberg.

Maunsbach, A.B. 1976. Cellular mechanisms of tubular protein transport. *Int. Rev. Physiol.* 11:145-167.

Mircheff, A.K., Ives, H.E., Yee, V.I., and Warnoch, D.G. 1984. Na^+/H^+ antiporter in membrane populations resolved from a renal brush border vesicle preparation. *Am. J. Physiol* 246:F853-F858.

Morré, D.J. and Morré, D.M. 1989. Preparation of mammalian plasma membranes by aqueous two-phase partition. *BioTechniques* 7:946-958.

Morré, D.J., Lawrence, J., Safranski, K., Hammond, T., and Morré, D.M. 1994. Experimental basis for separation of membrane vesicles by preparative free-flow electrophoresis. *J. Chromatog.* 668:201-214.

Murer, H. and Gmaj, P. 1986. Transport studies in plasma membrane vesicles isolated from renal cortex. *Kidney Int.* 30:171-186.

Murer, H. and Kinne, R. 1980. The use of isolated membrane vesicles to study epithelial transport processes. *J. Memb. Biol.* 55:81-95.

Nordlie, R.C. and Arion, W.J. 1966. Glucose-6-phosphatase. *Methods Enzymol.* 9:619-625.

Pennington, R.J. 1961. Biochemistry of dystrophic muscle. Mitochondrial succinic tetrazolium reductase and adenosine triphosphatase. *Biochem.* 80:649-654.

Piqueras, A.I., Somers, M., Hammond, T.G., Strange, K., Harris, H.W., Jr, Gawryl, M., and Zeidel, M.L. 1994. Permeability properties of rat renal lysosomes. *Am. J. Physiol.* 266:C121-C133.

Shirazi, S.P., Bechey, R.B., and Butterworth, P.J. 1980. The use of potent inhibitors of alkaline phosphatase to investigate the role of the enzyme in intestinal transport or inorganic phosphate. *Biochem. J.* 194:803-809.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Garnter, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.

Tsao, B. and Curthoys, N.P. 1980. The absolute asymmetry of orientation of gamma-glutamyltranspeptidase and aminopeptidase on the external surface of the rat renal brush border membrane. *J. Biol. Chem.* 255:7708-7711.

Tulsiani, D.R.P., Opheim, D.J., and Touster, O. 1977. Purification and characterization of α-D-mannosidase from rat liver Golgi membranes. *J. Biol. Chem.* 252:3227-3233.

Verroust, P.J., Christense, E.I., and Hammond, T.G. 1996. Endocytosis and receptors:molecular and physiological aspects. *Nephrologie* 17:359-364.

Yoshioka, S., Suzuki, T., and Kawakita, M. 1997. Analysis of the distribution of Na+/H+ exchanger isoforms among the plasma membrane subfractions of bovine kidney cortex: Reevaluation of methods for fractionating the brush-border and the basolateral membranes. *J. Biochem.* 122:641-646.

Yusufi, A.N., Murayama, N., Gapstur, S.M., Szczepanska-Konkel, M., and Dousa, T.P. 1994. Differential properties of brush-border membrane vesicles from early and late proximal tubules of rat kidney. *Biochim. Biophys. Acta* 1191:117-132.

Contributed by D. James Morré
Purdue University
West Lafayette, Indiana

Timothy Hammond
Tulane University
School of Medicine and Southeast Louisianna Veterans Health Care System
New Orleans, Louisianna

Isolation of Endoplasmic Reticulum, Mitochondria, and Mitochondria-Associated Membrane Fractions from Transfected Cells and from Human Cytomegalovirus-Infected Primary Fibroblasts

Petros Bozidis,[1] Chad D. Williamson,[1, 2] and Anamaris M. Colberg-Poley[1, 2, 3]

[1]Center for Cancer and Immunology Research, Children's Research Institute, Washington, DC
[2]Department of Biochemistry and Molecular Biology, George Washington University School of Medicine and Health Sciences, Washington, DC
[3]Department of Pediatrics, George Washington University School of Medicine and Health Sciences, Washington, DC

ABSTRACT

Increasingly mechanistic virology studies require dependable and sensitive methods for isolating purified organelles containing functional cellular sub-domains. The mitochondrial network is, in part, closely apposed to the endoplasmic reticulum (ER). The mitochondria-associated membrane (MAM) fraction provides direct physical contact between the ER and mitochondria. Characterization of the dual localization and trafficking of human cytomegalovirus (HCMV) UL37 proteins required establishing protocols in which the ER and mitochondria could be reliably separated. Because of its documented role in lipid and ceramide transfer from the ER to mitochondria, a method to purify MAM from infected cells was also developed. Two robust procedures were developed to efficiently isolate mitochondria, ER, and MAM fractions while providing the substantial protein yields from HCMV-infected primary fibroblasts and from transfected HeLa cells. Moreover, this unit includes a protocol that allows visualization of the mitochondria network disruption that occurs in permissively infected cells by their optimal resolution in Percoll gradients. *Curr. Protoc. Cell Biol.* 37:3.27.1-3.27.23. © 2007 by John Wiley & Sons, Inc.

Keywords: subcellular fractionation • human fibroblasts • ER • mitochondria • MAM • HCMV • protein localization • sucrose gradient • Percoll gradient • differential centrifugation

INTRODUCTION

Fractionation, the mechanical separation and purification of subcellular compartments, is an invaluable tool for studying protein localization, trafficking, processing, and functions. Subcellular fractionation is also useful for the concentration of relatively low-abundance proteins, isolation of enzymatic complexes, or proteomic identification of organelle components. Increasingly mechanistic analyses of cellular biology and virology require dependable and sensitive methods for isolating purified organelles from which functional subcellular domains, such as translocation complexes, lipid rafts, and viral envelopment sites, can be studied. Moreover, functional contacts between distinct organelles have been identified and fractionated. For example, the contact sites between endoplasmic reticulum (ER) and mitochondria are being characterized as sites for exchange of calcium (Rizzuto et al., 1998) and lipids (Stone and Vance, 2000) between these organelles. In all, ~5% to 20% of the mitochondrial network surface within a cell is in close apposition to the

Current Protocols in Cell Biology 3.27.1-3.27.23, December 2007
Published online December 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0327s37

ER (Rizzuto et al., 1998). The mitochondria-associated membrane (MAM) fraction, a subdomain of the ER, which consists of membrane tubules that provide direct physical contact between the ER and mitochondria, has been fractionated to high purity (Vance, 1990). Importantly, the purified MAM fraction is enriched in lipid synthetic enzymes, which produce phosphatidylserine and ceramide, and transport these products from the ER into mitochondria (Stone and Vance, 2000; Ardail et al., 2003; Bionda et al., 2004).

The authors' laboratory investigates human cytomegalovirus (HCMV) UL37 proteins, which dually target the ER and mitochondria of transfected and of HCMV-infected human foreskin fibroblasts (HFFs; Al-Barazi and Colberg-Poley, 1996; Goldmacher et al., 1999; Colberg-Poley et al., 2000; Mavinakere and Colberg-Poley, 2004a,b). The authors recently found that HCMV UL37 proteins traffic sequentially from the ER into the mitochondrial outer membrane (Mavinakere et al., 2006). Characterization of the subcellular localization and trafficking of HCMV UL37 proteins required establishing a protocol in which ER and mitochondria could be reliably separated from one another, despite their prevalent interconnection. Because of the documented role of MAM in lipid and ceramide transfer from the ER to mitochondria, the authors also sought to highly purify the MAM from transfected and from infected cells, adapting an established protocol from Vance (1990). The Vance protocol was originally used for the isolation of microsomes, mitochondria, and MAM from rat liver tissue (Vance, 1990). The following protocols were developed (see Basic Protocol 1) to maximally isolate mitochondria, ER, and MAM fractions while providing the best protein recoveries from HCMV-infected HFFs and from transfected, non-permissive HeLa cells. Basic Protocol 2 has proved especially valuable because it allows visualization of the mitochondria network disruption that occurs in permissively infected cells (McCormick et al., 2003) by their resolution in Percoll gradients.

BASIC PROTOCOL 1

DIFFERENTIAL SUCROSE GRADIENT ISOLATION OF ER AND MITOCHONDRIA

This protocol utilizes discontinuous sucrose gradients to band purified ER and mitochondrial organelles. Initially, cells are lysed mechanically with sonication and, then, a low-speed centrifugation (700 × *g*) is used to remove large cellular debris. Supernatant from this step is collected as a total lysed protein fraction. A subsequent 15,000 × *g* centrifugation crudely pellets mitochondria and separates it from ER and other organelles. The supernatant is loaded onto a three-layered sucrose gradient and purified ER is banded by centrifugation at 152,000 × *g*. Separately, the pellet is washed and loaded onto a two-layer sucrose gradient and purified mitochondria are banded using a 40,000 × *g* centrifugation. The high protein yields and considerable purity of banded organelles makes this fractionation of great utility for studies involving ER- or mitochondrial-resident proteins. The critical steps are shown in Figure 3.27.1.

Materials

Human foreskin fibroblasts (HFFs; Viromed SF cells)
HeLa cells (ATCC CCL-2)
HCMV (strain AD169 or desired strain) or DNA for transfection
2% and 10% (w/v) FBS
Lipofectamine 2000 (Invitrogen; *UNIT 20.6*)
Opti-MEM I (Invitrogen)
1.0, 1.3, 1.5, 1.7, and 2.0 M sucrose solutions (see recipes), sterile
1× PBS, pH 7.4 (*APPENDIX 2A*)
0.25% trypsin/EDTA
1× mannitol/Tris/EDTA (1× MTE) buffer (see recipe)
100 mM PMSF stock (see recipe)
Ultrapure water
70% ethanol

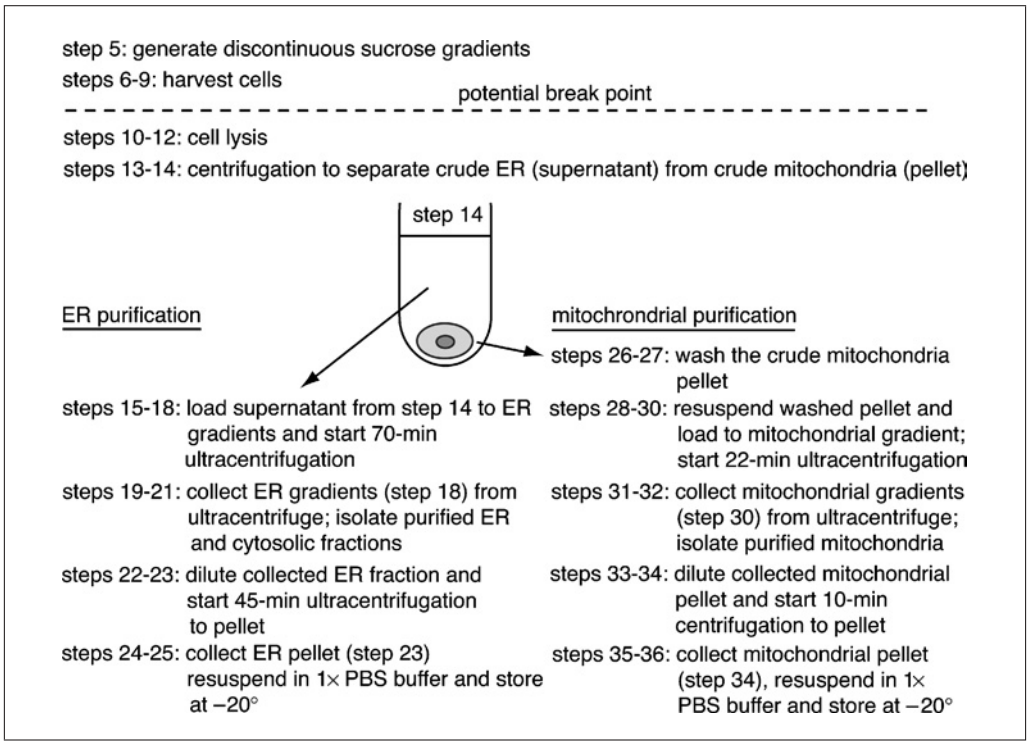


Figure 3.27.1 A flow chart for Basic Protocol 1 is shown. Basic Protocol 1, step 14, separates crude ER (supernatant) from crude mitochondria (pellet). Subsequent steps are grouped by the organelle which is to be purified for clarity and to provide a sense of continuity. To streamline the timing of the procedure and to reduce protein degradation, however, ER and mitochondrial purification steps should be carried out simultaneously.

175-cm^2 flasks (~0.8–2 × 10^7 cells/flask)
37°C incubator
Pre-sterilized (autoclaved) Beckman polyallomer centrifuge tubes: 14 × 89–mm (cat. no. 331372) or 11 × 60–mm (cat. no. 328874)
5-ml serological pipets
Aspirator
Sterile 15-ml conical tubes
Beckman GS-6R centrifuge with GH-3.8 swinging-bucket rotor
Analog sonicator with 1/8-in. microtip (Branson Ultrasonics model 250)
250-ml glass beakers
1.5-ml microcentrifuge tubes
14-ml polypropylene, round-bottom, snap-cap tubes (17 × 100–mm; Falcon cat. no. 2059)
Beckman J2-MI centrifuge with JA20.1 rotor
Beckman XL-90 ultracentrifuge with SW60 Ti and SW41 Ti rotors
1-ml syringes and 20-G needles
Parafilm
Beckman GS-15R centrifuge with F2402 rotor

Culture cells

1. For HCMV infection, seed two 175-cm^2 flasks of 60% to 70% density, actively growing primary HFFs. For transfection, seed two 175-cm^2 flasks of HeLa cells, 25% to 30% density and actively growing cells.

   For best results, use HFFs at or less than passage 18 as primary HFFs undergo senescence following 50 cell doublings or ~25 passages.

Transfer vital DNA to cells

To infect HFF cells with HCMV

2a. Twenty-four hr after seeding, infect permissive HFFs with HCMV (strain AD169 or desired strain), for 1 hr at 37°C in low-serum (2% FBS) medium, at a multiplicity of three plaque forming units (pfu) per cell (Mavinakere and Colberg-Poley, 2004a).

A cell density of ~80% the day of infection is best for maximal virus infectivity, as HCMV grows better in actively growing cells.

3a. After 1 hr, remove virus inocula by suction and overlay cells with complete medium (10% FBS). Proceed to step 4.

To transfect HeLa cells

2b. Transfect HeLa cells with Lipofectamine 2000 (*UNIT 20.6*) according to manufacturer's protocol. For each 175-cm^2 flask, use 20 μg DNA, 35 μl Lipofectamine 2000, and a total of 8.8 ml Opti-MEM I.

3b. Proceed to step 4.

A cell density of 80% to 90% is critical for maximal transfection efficiency using Lipofectamine 2000. If it is necessary to attain the correct cell density, incubate cultures for an extra day after seeding before transfecting.

Lipofectamine 2000 was found to give reliably higher transfection efficiencies in HeLa cells and HFFs than either Oligofectamine or Lipofectamine.

4. Incubate HCMV-infected cells according to the temporal class of examined viral protein. Allow transfected cells to incubate 24 hr at 37°C.

Depending on the abundance of the protein of interest or the kinetics of its movement through the cell, the incubation time may be adjusted to anywhere between 16 to 72 hr.

Prepare discontinuous sucrose gradients

5a. *For mitochondria gradients*. Dispense 1 ml of 1.7 M sucrose into a sterile 11 × 60–mm Beckman polyallomer ultracentrifuge tube. Mark the top of sucrose layer on the outside of the tube with an indelible felt-tip marker. Using a 5-ml serological pipet, carefully overlay with 1.6 ml of 1.0 M sucrose.

Hold the tube at a slight angle and slowly add top layer, to prevent sucrose layers from mixing.

5b. *For ER gradients*. Dispense 2 ml of 2.0 M sucrose to the bottom of a sterile 14 × 89–mm Beckman polyallomer ultracentrifuge tube. Using a 5-ml serological pipet, slowly layer 3 ml of 1.5 M sucrose onto the 2.0 M sucrose. Overlay with 3 ml of 1.3 M sucrose on top of the gradient.

The 14 × 89–mm polyallomer tubes used for ER gradients fit snugly into a polypropylene holder/dryer rack with 102 drying pins (made for 10- to 13-mm tubes).

Use the discontinuous sucrose gradients within 10 hr of preparation, for best results. The fine demarcation between sucrose layers may fade rapidly, but gradients remain stable at room temperature for several hours.

Harvest cells

6. Remove medium from cells by aspiration and wash the monolayers with 10 ml of 1× PBS, pH 7.4. Add 2 ml of 0.25% trypsin/EDTA and incubate 5 min at 37°C.

7. Inactivate the trypsin by adding 8 ml complete medium (10% FBS) to each flask and resuspend cells by vigorous pipetting. Transfer the resuspended cells from each flask into separate, sterile 15-ml conical tubes.

8. Pellet cells by centrifuging 5 min at 200 × *g* (1000 rpm in tabletop Beckman GS-6R centrifuge), 4°C. Aspirate the supernatant and resuspend the cell pellet in 10 ml of 1× PBS, pH 7.4.

9. Centrifuge cell suspension 5 min at 1400 × *g* (2500 rpm in tabletop Beckman GS-6R centrifuge), 4°C. Remove the supernatant by aspiration and store the cell pellet on ice (average pellet size is ~0.145 g).

   Cell pellets are stable on ice for up to 2 hr. Alternatively, the cell pellets can be frozen up to 1 month at –80°C at this stage and saved for later processing.

Lyse cells

10. Dispense 15 ml of 1× MTE and add fresh PMSF to a final concentration of 1 mM. Keep solution on ice. Add 2 ml of this solution to each 15-ml conical tube (step 9), and resuspend cell pellets.

11. Wash the sonicator microtip with ultrapure water and then with 70% ethanol. Blot dry with a Kimwipe before use. Set the sonicator for continuous pulse on a power setting of 3.5.

12. Place the 15-ml conical tube with resuspended cells into a 250-ml glass beaker partially filled with a slurry of ice and cold water. Submerge the cleaned, dry sonicator tip into the cell suspension to just above the bottom of the tube, avoiding touching the bottom. Sonicate the cell suspension on ice three times for 10 sec each, separated by 10-sec rest intervals. Keep the lysed cells on ice.

    Cell pellets from each flask should be kept separately up to this point to maximize the lysis efficiency. In step 14, however, combine the lysed cells from two 175-cm^2 flasks to maximize protein yields during fractionation.

    The 10-sec lysis condition had the best combination of high protein yields and fraction purity. If one wishes to sacrifice protein yield to obtain maximal purity of subcellular fractions, Basic Protocol 2 is recommended using homogenization lysis.

13. Centrifuge the lysed cells in 15-ml conical tubes 10 min at 1400 × *g* (2500 rpm in a tabletop Beckman GS-6R centrifuge), 4°C. Collect 100 µl of supernatant from each 15-ml conical tube, pool together duplicate samples into a single 1.5-ml microcentrifuge tube and label as "total protein." Store immediately at −20°C.

    Depending upon the cell lysis procedure, a pellet may not be seen at this step (corresponding to large cellular debris). A large pellet can be seen after gentle Dounce homogenization, while no pellet is usually visualized after long sonication times (see Table 3.27.1).

14. Decant remaining supernatant into a sterile, pre-chilled 14-ml polypropylene round-bottom snap-cap tube. Combine material from both 175-cm^2 flasks into a common chilled 14-ml tube. Centrifuge tubes without a snap-cap in the inner row of a JA20.1 rotor 10 min at 15,000 × *g*, 4°C.

    Use only polypropylene tubes for this procedure, as polystyrene tubes will crack and leak because of centrifugal stress.

    This centrifugation separates crude ER (supernatant) from crude mitochondria (pellet). Subsequent steps are grouped by the organelle which is to be purified for clarity and to provide a sense of continuity. To streamline the timing of the procedure and to reduce protein degradation, however, ER and mitochondrial purification steps should be carried out simultaneously.

Load ER gradient for ER purification

15. After centrifugation, transfer tubes to ice.

    There should be a large yellowish-brown pellet containing mitochondrial proteins.

Table 3.27.1 Cellular Markers for Verification and Assessment of ER, Mitochondria, and MAM Subcellular Fractions

Subcellular compartment	Marker	Antibody	Vendor	Reference
ER (microsomes)	DPM1	I-20	Santa Cruz Biotechnology cat. no. sc-15836	Maeda et al., 1998
	Calreticulin		Affinity Bioreagents cat. no. PA3-900	Johnson et al., 2001; Gelebart et al., 2005
Mitochondria	Grp75		Stressgen cat. no. SPS-825	Manning-Krieg et al., 1991
	COXII	K-20	Santa Cruz Biotechnology cat. no. sc-23984	Scheffler, 2001
MAM	mEFGP-huPPS-1	B-2 (anti-GFP)	Santa Cruz Biotechnology cat. no. sc-9996	
	FACL4		Abgent cat. no. AP2536b	Simmen et al., 2005

16. Using a micropipet, carefully withdraw 1.7 ml of the supernatant (containing crude ER) and layer it onto the top of the ER sucrose gradient, creating a new layer. Pipet slowly to keep from mixing the top layer of sucrose with the sample. Decant and discard any excess supernatant remaining in the 14-ml tubes containing the protein pellet, and then return the pellets to ice.
17. Transfer an additional 1.3 ml of ice-cold 1× MTE plus PMSF onto the top of the ER gradient.
18. Ultracentrifuge ER gradients 70 min at 152,000 × *g* (35,000 rpm in an SW41 rotor), 4°C. Set acceleration and deceleration profiles to 1 (transition speed of 170 rpm for 2 min).

    It is important to add the extra 1× MTE plus PMSF buffer on top of the samples to keep the polyallomer tubes from collapsing during ultracentrifugation.

Isolate ER fractions

19. Collect the ER gradient tubes from the ultracentrifuge. Withdraw the upper 1 ml of solution from the tube, using a micropipet, and transfer into a sterile 1.5-ml microcentrifuge tube. Label as "cytosol" and store immediately at −20°C.
20. Extract 0.4 to 0.6 ml volume of the large band at the interface of the 1.3 M sucrose gradient layer, using a 20-G needle and 1-ml syringe (Fig. 3.27.2).

    The samples can be stored for 1 to 2 months at –20°C prior to their use for western analysis.

21. Remove the needle from the syringe, and transfer the extracted band to a sterile 11 × 60–mm Beckman polyallomer tube. Add an additional 3.6 to 3.8 ml of ice-cold 1× MTE plus PMSF buffer to dilute out the sucrose.

    It is important to remove the needle from the syringe before dispensing liquid into the new ultracentrifuge tube, to avoid shear forces that would damage the sample.

22. Cover the top of the tube with Parafilm and mix by inversion until the suspension looks homogeneous (until the highly viscous sucrose swirling within the tube is no longer distinguishable).
23. Ultracentrifuge 45 min at 126,000 × *g* (35,000 rpm in an SW60 Ti rotor), 4°C.

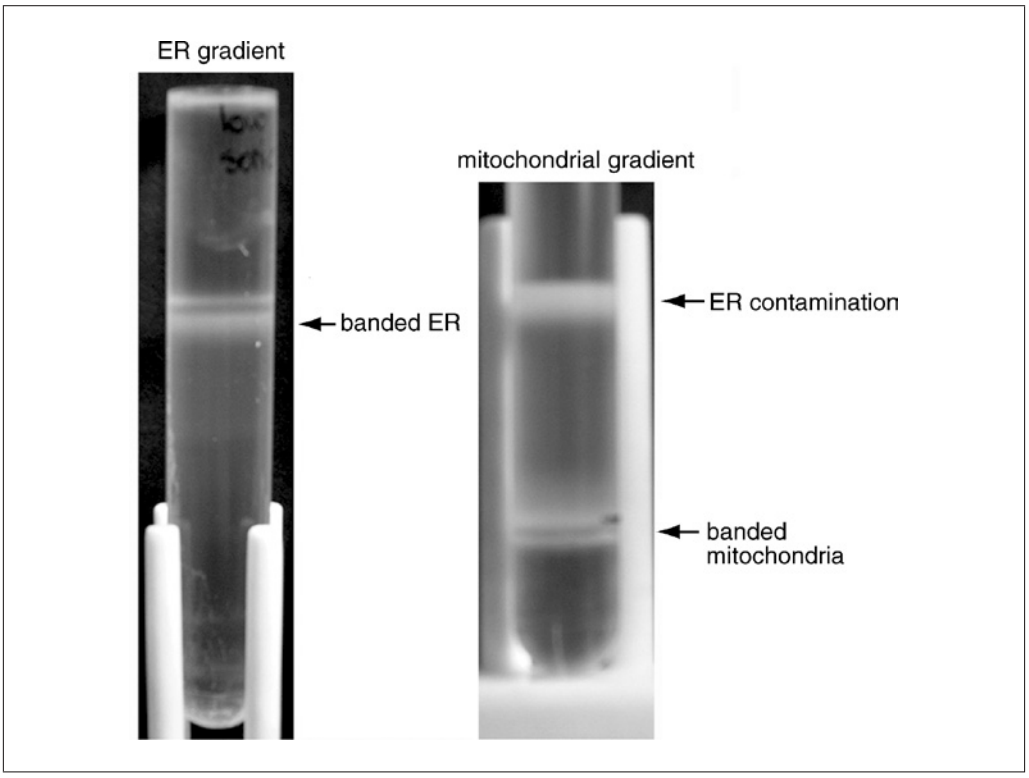


Figure 3.27.2 Representative pictures of visible bands seen upon ultracentrifugation as described in Basic Protocol 1. The ER and mitochondria bands are indicated on their respective gradients following ultracentrifugation.

24. Collect tube from ultracentrifuge. Decant and discard the supernatant.

    There will be a large, translucent pellet at the bottom of the tube.

25. Allow tubes to dry, inverted, for a few minutes. Resuspend the pellet in 100 µl of 1× PBS, pH 7.4, and label as "ER." Store immediately at −20°C.

    The samples can be stored for 1 to 2 months at −20°C prior to their use for western analysis.

Load mitochondria gradient for mitochondrial purification

26. For the mitochondrial pellet wash 1, return to the 14-ml tube on ice containing the protein pellets (step 16). Gently wash the inside sides of the tube with 0.5 ml of ice-cold 1× MTE plus PMSF, being careful not to disrupt the protein pellet. Wash the sides three to five times using the same aliquot of buffer. Decant and discard wash solution.

27. For mitochondrial pellet wash 2, use a fresh aliquot of 0.5 ml of ice-cold 1× MTE plus PMSF buffer to carefully wash the periphery of the protein pellet, which contains ER contaminant proteins. Wash three to five times using the same aliquot of buffer. Decant and discard the wash.

    The ER contaminant proteins make a large, loose ring around the more stable mitochondrial pellet. The mitochondrial pellet will appear more yellow in color, while the surrounding contamination appears whiter in color. A large amount of contaminant protein towards the bottom of the pellet will be seen.

28. Resuspend the washed mitochondrial pellet in 0.8 ml of ice-cold 1× MTE plus PMSF. Load slowly on top of the mitochondrial sucrose gradient so that it forms a new layer.

29. Top off gradient with an additional 0.8 ml of ice-cold 1× MTE plus PMSF buffer.

30. Ultracentrifuge the mitochondrial gradient 22 min at 40,000 × *g* (19,500 rpm in an SW60 Ti rotor), 4°C. Set acceleration and deceleration profiles to 1 (transition speed of 170 rpm for 2 min).

Isolate mitochondrial fractions

31. Collect mitochondrial gradients from the ultracentrifuge (step 30). Using a 1-ml syringe with a 20-G needle, extract a volume of 0.4 ml from the band at the interface of the 1.7 M and 1.0 M sucrose layers (Fig. 3.27.2).

    The mitochondrial band can vary in visibility and is often very thin and hard to see, which is why marking the location of the interface with an indelible felt tip marker before centrifugation is important.

32. Remove the needle and dispense the extracted volume into a sterile 1.5-ml microcentrifuge tube.

33. Add 1.1 ml of ice-cold 1× MTE plus PMSF, cap microcentrifuge tube, and mix well by inversion until suspension is homogeneous.

34. Centrifuge 10 min at 15,000 × *g*, 4°C (in tabletop Beckman GS-15R centrifuge).

35. Decant and discard supernatant (a small, stable yellowish-brown pellet should be visible).

36. Allow tube to dry, inverted, for a few minutes. Resuspend pellet in 30 µl of 1× PBS, pH 7.4, and label as "mitochondria." Store immediately at −20°C.

    The samples can be stored for 1 to 2 months at −20°C prior to their use for western analysis.

BASIC PROTOCOL 2

SEPARATION OF MITOCHONDRIA AND MITOCHONDRIA-ASSOCIATED MEMBRANE FRACTION

This procedure combines differential and Percoll gradient centrifugations. Its critical steps are underscored in Figure 3.27.3. During the first steps, the post-nuclear supernatant (PNS) is separated from nuclei and cellular debris by differential centrifugation at low *g* forces. The post-nuclear supernatant is then subjected to centrifugation at 10,300 × *g* during which the crude mitochondrial fraction is separated from the total microsomal fraction. The total microsomal fraction consists mainly of vesicles derived from rough and smooth ER and membranes from the Golgi apparatus and plasma membrane. The microsomal fraction is then recovered as a pellet after centrifugation of the total microsomal fraction at 100,000 × *g*. The post-microsomal (high-speed) supernatant is generally used as "cytosol" in downstream applications. The crude mitochondrial fraction is subjected to a density gradient fractionation through a self-generating Percoll gradient (Pertoft et al., 1978). Because both mitochondria and mitochondria-associated membrane (MAM) have similar densities, a second step of purification that includes a centrifugation at 6300× *g* follows their collection from the gradient. Finally, the supernatant containing MAM is subjected to centrifugation at 100,000 × *g* and the MAM is isolated as a pellet above the tight Percoll pellet.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be pre-cooled to 0°C to 4°C and kept on ice throughout the procedure.

Materials

Untransfected or transfected HeLa cells (3×10^7 cells, ten 100 × 20–mm tissue culture dishes, 100% confluent) or uninfected or HCMV-infected HFF cells (5×10^7 cells, four 850-cm^2 roller bottles, 90% confluent)

Phosphate-buffered saline (PBS; see recipe)

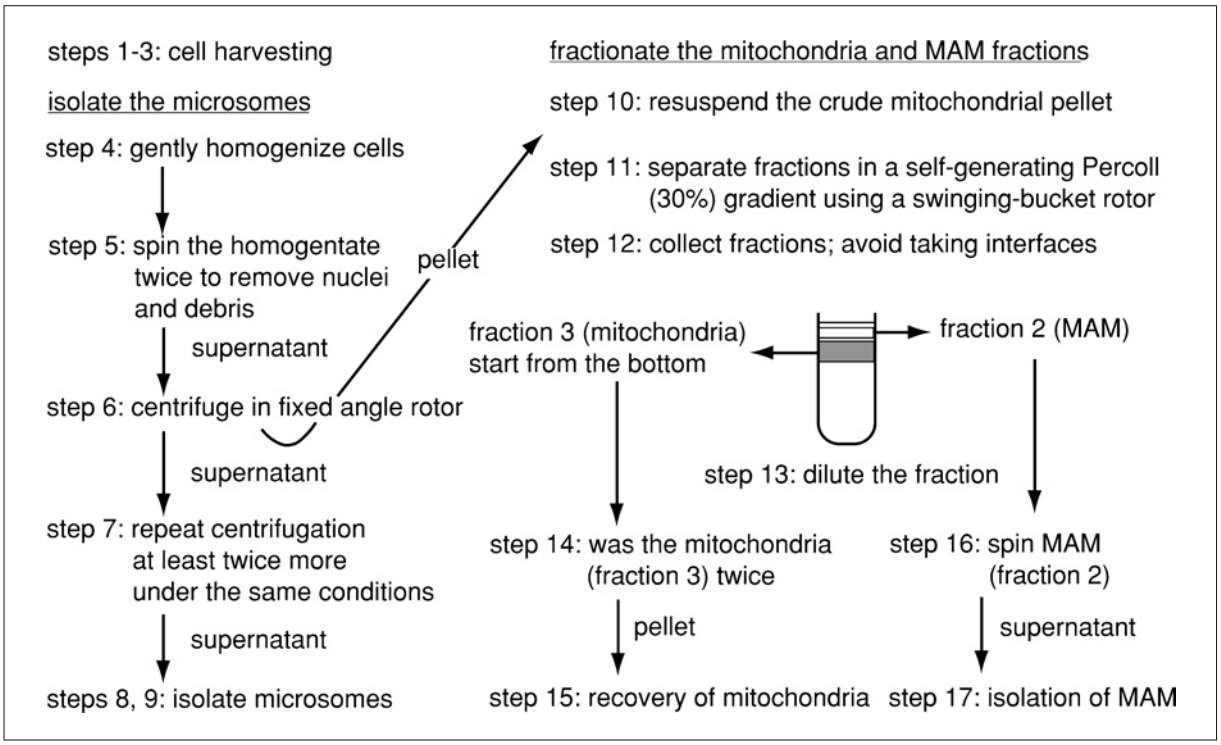


Figure 3.27.3 Schematic representation of Basic Protocol 2. Critical steps in the procedure, which improve purity of the microsomes, mitochondria, and MAM, are emphasized by bold font.

Sucrose homogenization medium (SHM; see recipe), ice cold
Protease inhibitors (optional but recommended, see recipe)
Mannitol buffer A (see recipe), ice cold
30% (v/v) Percoll suspension in mannitol buffer B (see recipe), ice cold
Mannitol buffer B (see recipe), ice cold

Beckman GS-15R tabletop centrifuge with swinging-bucket rotor (e.g., S4180)
14-ml polypropylene round-bottom centrifuge tubes (e.g., Falcon)
Potter-Elvehjem plastic-coated tissue grinder with ball-shaped Teflon pestle (e.g., Wheaton safe-grind type, cat. no. 358003)
Overhead stirrer for tissue grinder (Wheaton, cat no. 903475)
Phase-contrast microscope
1.5-ml microcentrifuge tubes
Beckman GS-15R centrifuge with fixed-angle rotor (e.g., F2402)
Centrifuge tubes, 1.5-ml microcentrifuge tubes
Beckman XL-90 ultracentrifuge with swinging-bucket rotor (e.g., SW 41)
Ultracentrifuge SW41 tubes (ultraclear tubes are highly recommended, e.g., 14 × 89–mm, cat. no. 344059)
1-ml syringes and 20-G needles

Harvest cells

1. Wash the cell monolayers with ~50 ml PBS at room temperature. Remove PBS by suction.
2. Harvest cells (90% to 100% confluent), using a sterile scraper, into 50 ml PBS and pellet cells by centrifuging 10 min at 1000 × *g*, room temperature, using a tabletop centrifuge and four 14-ml centrifuge tubes.
3. Decant the supernatant and resuspend cell pellets in 8 ml ice-cold sucrose homogenization medium containing freshly added protease inhibitors.

Isolate microsomes

4. Attach the pre-cooled pestle of a Potter-Elvehjem homogenizer to an overhead stirrer and gently homogenize the cells by ten up-and-down strokes at 500 rpm. Confirm that ≥90% cell breakage has occurred by examining the homogenate under a phase-contrast microscope.

   It is best to keep the cells on ice throughout the homogenization.

   To check the efficiency of the homogenization, pipet 2 to 3 μl of the homogenized suspension onto a glass slide, overlay with coverslip, and observe using a microscope. A shiny ring around the nuclei indicates that cells are still intact. If >90% of the nuclei do not have the shiny ring, proceed to the next step. Otherwise, repeat the homogenization at 1000 rpm.

5. Transfer homogenates to 1.5-ml microcentrifuge tubes. Pellet the nuclei, cell debris and any unbroken cells by low-speed centrifugation. Centrifuge the homogenates two times in a fixed-angle rotor (e.g., F2402) 5 min at 600 × *g*, 4°C.

   This step is more convenient using a 10-ml centrifuge tube. The centrifugation steps will then be under the same conditions using a different rotor (S4180) but the same centrifuge (Beckman GS-15R). However, it is still recommended that the homogenate should be transferred in 1.5-ml microcentrifuge tubes for the centrifugation that is described in step 6 because of the better separation of the pellet from the supernatant.

6. Transfer the supernatant into new 1.5-ml microcentrifuge tubes and centrifuge 10 min at 10,300 × *g*, 4°C, using the tabletop centrifuge (e.g., Beckman GS-15R) with a fixed-angle rotor (e.g., F2402) and retain the pellet for subsequent steps.

7. Centrifuge supernatant at least two additional times under the same conditions (10 min at 10,300 × *g*, 4°C) until a pellet is no longer visible. Save supernatant on ice for subsequent steps.

   These additional centrifugation steps under the same conditions are needed to ensure that the microsomal fraction will be efficiently separated from any residual mitochondrial membranes and MAM.

8. Pellet the microsomes by ultracentrifuging resultant supernatant 60 min at 100,000 × *g*, 4°C, using a swinging-bucket rotor (e.g., SW41).

9. Resuspend the pellet in 0.5 ml of sucrose homogenization medium.

Fractionate mitochondria and MAM fractions

10. Resuspend the pellet from step 6 in 0.3 ml of mannitol buffer A using two or three strokes in a Potter-Elvehjem homogenizer at 500 rpm, as above in step 4, and carefully layer on top of 10 ml of 30% Percoll suspension in a 14 × 89–mm SW41 ultraclear tube.

11. Centrifuge 65 min at 95,000 × *g*, 4°C, in an ultracentrifuge with a swinging-bucket rotor (e.g., a Beckman SW41).

    The generation of Percoll gradients is an automatic process occurring during centrifugation of the colloid. Therefore, the use of an acceleration program (e.g., 500 rpm for 3 min) in the beginning of the ultracentrifugation is recommended. Although Percoll gradients are considered to be very stable, a similar deceleration program would be the best choice, if such a program is available on the ultracentrifuge.

    A total of 1 to 5 mg of protein (~0.5 ml) on 10 ml of gradient material results in satisfactory separation of banded material without overloading the gradient.

12. Collect the banded organelles in 0.5- to 1-ml fractions (Fig. 3.27.4). To collect the fractions, attach a sterile metallic needle of appropriate diameter (e.g., 20-G) to a sterile 1-ml syringe and carefully puncture the wall of the tube just above fractions

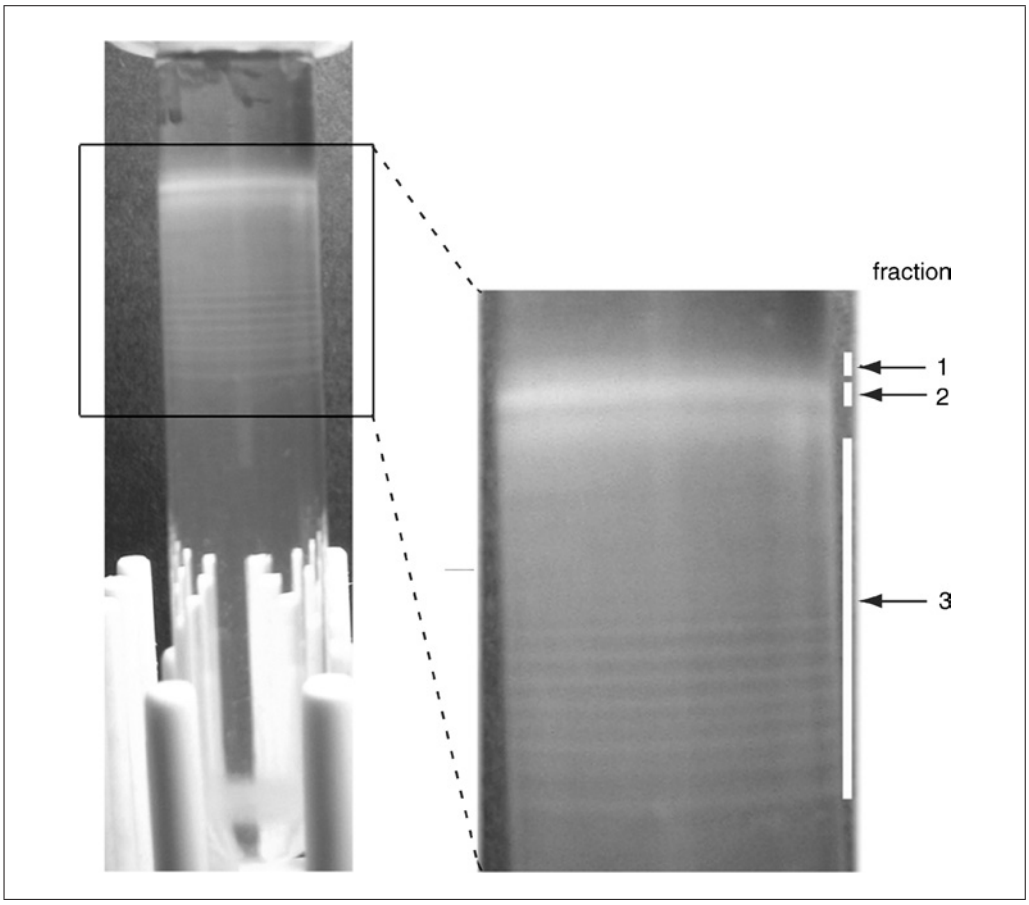


Figure 3.27.4 Separation of MAM and mitochondrial fractions using a self-generating Percoll (30%) gradient. Crude mitochondrial extract from HeLa cells was subjected to ultracentrifugation in a Percoll gradient (see Basic Protocol 2). Three fractions were isolated as indicated on the enlarged section of the gradient. Fraction 2 was identified as the MAM fraction, which is immediately above the top of the mitochondrial fraction (fraction 3). The heterogeneous mitochondrial fraction was conservatively taken.

1 and 2 and below fraction 3 (Fig. 3.27.4). Slowly and conservatively suction the fractions with a fan-like movement, avoiding other bands.

The MAM fraction (fraction 2) is the compact, white band, which is located immediately above the multi-band mitochondrial fraction (fraction 3), ~$^{1}/_{3}$ down the tube.

13. Transfer each sample to a 14 × 89–mm ultraclear SW41 tube. Dilute the fractions with ≥5 vol of ice-cold mannitol buffer B (~10 ml total) to dilute Percoll.

14. Wash the diluted mitochondrial band (fraction 3), at least two times, with 5 ml of ice-cold mannitol buffer B. Pellet mitochondria after each wash by centrifuging 10 min at 6300 × *g* (~6000 rpm using an SW41 rotor), 4°C.

    These washing steps are necessary to remove the Percoll from the fraction, which otherwise will interfere with subsequent analysis.

15. Resuspend the mitochondrial pellet after the last washing step in ice-cold mannitol buffer B at a concentration of 0.5 to 2 mg protein/ml (usually 0.05 to 0.2 ml).

16. Centrifuge the diluted MAM fraction (fraction 2) in SW41 ultraclear tubes 10 min at 6300 × *g* (~6000 rpm), 4°C.

    This step is needed to ensure that the mitochondria-associated membrane fraction will be detectably free from any residual mitochondria.

17. Ultracentrifuge the resulting supernatant 60 min at 100,000 × *g*, 4°C, to pellet the MAM.

    After the ultracentrifugation, the mitochondria-associated membranes form a loose pellet, which is located above the hard pellet of Percoll particles.

18. Resuspend the MAM in ice-cold homogenization buffer at a concentration of 0.5 to 2 mg protein/ml (usually 0.05 to 0.2 ml). Freeze the isolated fractions at −80°C or submit immediately to SDS-PAGE (*UNIT 6.1*) or proteomics analyses.

    The loose MAM pellet is carefully resuspended using a micropipette and transferred to a new 1.5-ml microcentrifuge tube. These fractions may be stored up to 6 months at –80°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Mannitol buffer A

To 100 ml ultrapure H_2O, add:
9.13 g mannitol (0.25 M final)
38 mg EGTA (0.5 mM final)
10 ml 100 mM HEPES (5 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add ultrapure H_2O to 200 ml
Sterilize by autoclaving for 20 min using a liquid cycle
Store up to 2 or 3 months at 4°C

Mannitol buffer B

To 100 ml ultrapure H_2O, add:
8.22 g mannitol (0.225 M final)
76 mg EGTA (1 mM final)
50 ml 100 mM HEPES (25 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add ultrapure H_2O to 200 ml
Sterilize by autoclaving for 20 min using a liquid cycle
Store up to 2 or 3 months at 4°C

MTE solution, 1×

In 60 ml ultrapure H_2O, dissolve:
4.914 g D-mannitol (270 mM final)
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.4 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize through a 0.22-μm vacuum filter
Store 3 to 6 months at room temperature

Percoll solution, 30% (v/v)

1 vol 90% (v/v) stock isotonic Percoll (see recipe)
2 vol mannitol buffer B (see recipe)
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

Phosphate-buffered saline, pH 7.4

3 liters distilled water
32 g NaCl (140 mM final)
0.8 g KH_2PO_4 (1.5 mM final)
8.7 g $Na_2HPO_4 \cdot 7H_2O$ (8.1 mM final)
0.8 g KCl (2.7 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add H_2O to 4 liters
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

PMSF, 100 mM

In 500 μl absolute ethanol, dissolve:
8.71 mg PMSF
Store up to 1 year at −80°C

Protease inhibitors

PMSF (200 mM final):
0.348 g phenylmethylsulfonyl fluoride
10 ml 2-propanol or ethanol
Store up to 1 year at −20°C

Add to solutions as required so that the final concentrations are 1 mM.

Stock isotonic Percoll, 90% (v/v)

9 vol Percoll (Pharmacia cat. no. P1644)
1 vol 2.5 M sucrose (0.25 M final)
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

Sucrose, 1.0 M

In 60 ml ultrapure H_2O, dissolve:
34.23 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature
Open only in a laminar flow hood to avoid contamination

Sucrose, 1.3 M

In 60 ml ultrapure H_2O, dissolve:
44.50 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose, 1.5 M

In 60 ml ultrapure H_2O, dissolve:
51.35 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose, 1.7 M

In 60 ml ultrapure H_2O, dissolve:
58.19 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose, 2.0 M

In 60 ml ultrapure H_2O, dissolve:
68.46 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose homogenization medium (SHM)

To 100 ml ultrapure H_2O, add:
17.1 g sucrose (0.25 M final)
20 ml 100 mM HEPES (10 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add ultrapure H_2O to 200 ml
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

COMMENTARY

Background Information

Highly purified mitochondria are very difficult to obtain in appreciable amounts. The discontinuous sucrose gradient protocol (see Basic Protocol 1) is valuable for experiments that require higher yields of mitochondrial samples. It is often imperative to use the same pool of purified mitochondria to observe fraction purity, probe for the presence of mitochondrially localized viral proteins, and examine the post-translational modifications of those proteins. Obtaining enough purified mitochondria for all of these experiments by the Percoll protocol (see Basic Protocol 2) presented here would not be feasible. The discontinuous sucrose gradient protocol takes slightly less time, is more user-friendly, requires less starting material (number of cells), and produces higher yields of mitochondrial fractions. These mitochondrial fractions are well-purified and practical for almost any application. Also, the utility of starting with fewer cells is that one can compare more experimental conditions and controls within a single fractionation experiment. However, when one is willing to sacrifice protein yield for even higher purity mitochondrial fractions, or when one needs to isolate MAM fractions to compare to either

ER or mitochondria, then the Percoll protocol (see Basic Protocol 2) is the preferred method.

Points of contact between the ER and mitochondria make up 5% to 20% of the total mitochondrial network (Rizzuto et al., 1998). These connections, as well as the ER and mitochondria organelles themselves, are quite variable and can undergo rapid changes in overall morphology, size, and composition (Bereiter-Hahn and Vöth, 1994; Collins et al., 2002). Factors such as calcium homeostasis, cell metabolism, and perceived stress can have dramatic impacts on both the form and function of these organelles. Thus, it is imperative to monitor fraction purity for each experiment by analyzing total, ER, mitochondria, and MAM pools by utilizing suitable organelle markers (Table 3.27.1). Not only will this ensure that the identity and purity of the fractions are sufficient for unequivocal conclusions, but appropriate controls can provide additional information about cell status at the time of fractionation.

Following lipofection, it is important to check transfected cells often. Lipofectamine 2000 was the least toxic, in the authors' hands, to both HeLa and HFF cells, but cell viability can still change significantly in response to overexpression of a transfected plasmid. When using control plasmids encoding innocuous or beneficial (anti-apoptotic) proteins, Lipofectamine 2000 can be left in the culture medium for up to 24 hr. However, for transfection of most plasmids, it is prudent to change Lipofectamine 2000 transfection medium after 4 hr, replacing it with complete cell medium so as to minimize the stress or apoptotic signals on the cells. Furthermore, overexpression of some plasmids may be more toxic to cells, in which case one must wash out the lipofection medium after 4 hr (as before) and, moreover, may have to decrease the total incubation time before cell harvesting. Prior to harvesting, monitor the cells for viability as cells that are heavily stressed or apoptotic are not reliable for fractionation experiments.

The need to obtain intact and highly purified subcellular fractions, including MAM, imposes the additional use of density gradient centrifugation to remove contamination by broken membranes or organelles of similar size as in Basic Protocol 2. Percoll is considered to be one of the best density gradient medium available for the separation of cells, organelles, viruses, and subcellular particles. Because of its low osmolarity (<25 mOs/kg water), Percoll forms a density gradient without generating an osmolarity gradient (Pertoft et al., 1978). Osmolarity of the gradient medium must be taken into consideration since cellular membrane-bound organelles such as mitochondria and MAM act as osmometers. A high external osmolarity will result in shrinkage of membrane-bound organelles while low osmolarities will result in swelling of organelles, hence altering their buoyant densities. Differences in the osmolarity of the gradient medium can explain differences in the size and apparent buoyant densities of subcellular compartments when different gradient media are used. Buoyant densities of organelles in sucrose rise as water is removed from their enclosed spaces. In contrast, organelles in Percoll gradients in physiological range (280 to 320 mOs/kg) have lower apparent buoyant densities than in sucrose (Pertoft et al., 1978).

Another unique feature of Percoll is that it can form self-generated gradients by centrifugation at moderate *g* forces. Fixed-angle rotors are most commonly used for Percoll gradients. The advantage of using fixed-angle or vertical rotors is that the path-length for formation of the gradient is shorter and the gradient forms more rapidly. Both Vance (1990) and Hovius et al. (1990) used a fixed-angle rotor to isolate purified mitochondria from a self-generated Percoll (30% v/v) gradient. Conversely, the authors used a swinging-bucket rotor for the formation of the Percoll gradient. There are two main reasons for this choice: first, because both fractions (mitochondria and MAM) have similar densities and they are banded within the density range of ~1.039 to 1.051 g/ml (Fig. 3.27.4), a better separation of this density range can be achieved by using a rotor with a longer path-length. Second, a good spatial separation between resolved mitochondria subpopulations, which differ slightly in density and size from one another, can be achieved (Collins et al., 2002). The latter feature is especially desirable in the case of HCMV-infected cells in which disruption of mitochondria networks (McCormick et al., 2003) can be visualized by density banding of mitochondrial species in these Percoll gradients.

Based upon the authors' experience, the transfection protocol does not affect the pertinent physical properties of the organelles and their constituents during fractionation. Thus, no difference should be expected between fractions that are isolated from either untransfected or transfected cells. However, HCMV infection markedly alters the fractionation protocol.

A change in the density of mitochondrial bands after HCMV infection has been observed. This density change in fraction 3 is detected as a significant increase of the mitochondrial markers in the pellet that is formed after the first centrifugation of the diluted MAM fraction at 6300 × *g* (see Basic Protocol 2, step 16). Usually, only background levels of the mitochondrial markers can be detected in this pellet. It appears that HCMV infection causes a shift of mitochondrial densities such that some mitochondria band in the density range of MAM. This is probably related to the documented disruption of mitochondria networks by HCMV. Therefore, it is highly recommended that the study of the MAM should always be based on the more rigorous fraction that is isolated after the final ultracentrifugation step at 100,000 × *g*, as it is described in Basic Protocol 2, step 17. This fraction is consistently devoid of mitochondrial contamination in both uninfected and HCMV-infected cells.

Critical Parameters and Troubleshooting

Sucrose gradient fractionation

Sometimes a mitochondrial band will not be seen after centrifugation, so make sure to mark the gradient interface between the 1.7 M and 1.6 M sucrose layers when preparing the gradients. If a band is not seen after centrifugation, insert the collecting needle into the side of the 11 × 60–mm Beckman tube at the marked gradient interface and carefully withdraw ~0.4 ml of liquid.

When collecting protein fractions with needles, the use of small-bore needles should be avoided as they increase the shear force on the extracted samples. Extract the protein band slowly into the syringe, then remove the needle before dispensing the protein fraction into a microcentrifuge tube for storage. This saves the sample from the shear force of flowing a second time through the needle.

Proteins can be degraded extremely rapidly. Once the cells have been lysed, work as quickly as possible. Also make sure to keep samples on ice at all times. Pre-cooling buffers to 4°C can also help. Furthermore, PMSF is a serine protease inhibitor. It does not inhibit other proteases, and does not even inhibit all serine proteases. If protein degradation appears to be a problem, it may be necessary to add a commercially available protease inhibitor cocktail to the 1× MTE buffer in addition to PMSF. Freeze fractionated samples immediately upon collection to avoid unwarranted degradation.

Differential pelleting by centrifugation

In the initial steps of Basic Protocol 2, we use differential centrifugation to separate the mitochondrial and MAM fractions from the microsomal fractions using a centrifugation at 10,300 × *g*. This centrifugation step proved to be critical. The method of Hovius et al. (1990) uses pelleting at a lower RCF (10,000 × *g*) to reduce the ER content in the resulting crude mitochondrial fraction. This lower centrifugal force will be insufficient to efficiently pellet the MAM with mitochondria and will decrease the subsequent yield of MAM on Percoll gradients. The proposed RCF (10,300 × *g*), however, greatly improves the yields of MAM in subsequent Percoll gradient.

Another critical step of the method involves the centrifugal separation of the mitochondrial-MAM fraction from the microsomal fraction. Care must be taken in this step to remove all traces of unpelleted mitochondrial-MAM. It is therefore recommended to use a fixed-angle rotor and at least two centrifugations under the same conditions (Fig. 3.27.3). In the authors' experience, if the above conditions are not followed, the supernatant microsomal fraction is still contaminated with detectable mitochondrial-MAM fraction.

Percoll density gradients

To generate Percoll gradients, stock isotonic Percoll (SIP) solution is prepared at the desired density in 0.25 M sucrose. To accurately measure buoyant density, samples are premixed with the gradient material. However, if samples are layered onto the top of the Percoll gradient, better resolution of subcellular particles from soluble proteins is attained. Soluble proteins tend to remain above the Percoll gradient and subcellular particles will sediment into the gradient, thus achieving better separation.

Percoll gradients continuously change during high-speed centrifugation. Therefore, depending on the centrifugation conditions (type of rotor, RCF, time of centrifugation), the density profile of the gradient and, consequently, the separation or the organelles will be altered. Thus, using density marker beads (Amersham Biosciences cat. no. 17–0459-01) to monitor densities in a duplicate gradient is recommended. Their use verifies the consistency of density gradient formation and greatly facilitates identification of the desired fractions.

In spite of their many advantages, Percoll particles are difficult to remove from purified fractions. A simple approach is to separate subcellular particles from Percoll-coated silica particles by high-speed centrifugation in a swinging-bucket rotor or fixed-angle rotor (Calaminus et al., 1979). The organelles can be separated from Percoll by centrifugation at 100,000 × *g* for 2 hr (in a swinging-bucket rotor) or 90 min (in a fixed-angle rotor). Percoll is in the tight pellet; whereas, the biological material remains loosely packed and can be gently resuspended.

Anticipated Results

Different lysis methods

The effects of various lysis conditions were evaluated using untransfected HeLa cells fractionated by discontinuous sucrose gradients (Table 3.27.2). Five conditions were surveyed: low sonication (three, 5-sec pulses), medium sonication (three, 10-sec pulses), high sonication (three, 15-sec pulses), homogenization (10 strokes with Dounce homogenizer), or freeze/thaw cycles (three cycles of 1-hr incubation at −80°C followed by a rapid thaw in a 37°C water bath).

Homogenization, the gentlest lysis procedure, had the lowest ER and mitochondrial yields of the lysis protocols tested. The low-speed spin after lysis, to remove large cellular debris, produced large pellets of intact cells after homogenization, which decreased the material available for subsequent banding on ER and mitochondrial gradients.

To determine the purity of the fractionated mitochondria and ER from the various lysis procedures, an examination of the presence of ER (DPM1), MAM (FACL4), and mitochondrial (Grp75, COXII) markers in the banded ER and mitochondria fractions (Fig. 3.27.5) was done. Sonication resulted in good separation of ER and mitochondria although the anti-DPM1 antibody detected mitochondrial DPM (Gasnier et al., 1992) in this experiment. However, the other markers tested (FACL4, Grp75, and COXII) showed good separation of ER and mitochondria. Medium or high sonication disrupted the association between the MAM and mitochondrial compartments, as the MAM marker (FACL4) was detected in the ER fraction and was barely detected in the mitochondrial fraction. Conversely, the freeze/thaw lysis showed an equal distribution of MAM in both ER and mitochondrial fractions. Of the lysis procedures tested, homogenization was the most effective at preserving the association of the MAM with mitochondria. Nonetheless, the highest yields of ER and mitochondrial fractions were obtained from low and medium sonication procedures. Medium sonication was therefore chosen as the preferred lysis condition for discontinuous sucrose gradient fractionation (see Basic Protocol 1). This procedure produces superior yields of well-purified ER and mitochondrial fractions, with MAM membranes predominantly appearing in the ER fraction.

Purification of mitochondria and mitochondria-associated membranes

The ultracentrifugation of the crude mitochondrial extract in a self-generating Percoll (30%) gradient using a swinging-bucket rotor produces three distinct fractions (Fig. 3.27.4). Using density marker beads (Pharmacia), the authors found that MAM and mitochondria from cultured human cells span a range of densities between 1.039 and 1.051 g/ml (data not shown). The higher density beads ($\geq$1.069 g/ml) sediment near the bottom of the Percoll gradient. Because of this broad span of 1.039 to 1.051 g/ml densities, the self-generating 30% Percoll gradient is excellent for effective resolution of MAM from mitochondria.

Fraction 1 appears as a faint, diffuse layer just above fraction 2; whereas, fraction 2 is the more compact white in color band, less dense than mitochondria. Fraction 3 consists of multiple denser mitochondrial bands. Because the borders of the fractions usually are partially overlapping, care must be taken during the collection of the bands to minimize mixing of the samples. If a needle and syringe is used for this purpose, it is recommended that the collection should start from the top in the case of fraction 1 and from the bottom for fraction 3. In every case, the interface between the fractions should be carefully avoided.

The isolated fractions were verified by western blot analysis using known cellular protein markers for cytosol (Hsp70), microsomes (DPM1, calreticulin), MAM (mEGFP-huPSS-1), and mitochondria (COXII, Grp75) (Fig. 3.27.6 and Table 3.27.1). The MAM is physically associated and pelleted with mitochondria. Upon resolution in Percoll gradients, its position is consistent with previously reported relative densities of MAM (Vance, 1990). Most compellingly, the high relative abundance of mEGFP-huPSS-1 fusion protein in fraction 2 as well as the presence of the other ER markers (DPM1, calreticulin) indicate that it is an ER subcompartment, enriched for phosphatidylserine synthetic enzymes as

Table 3.27.2 Mitochondrial and ER Yields and Purity from Differentially Lysed HeLa Cells

		Average						
Lysis method	Yield samples	Initial cell pellet (g)	Total protein fraction (mg)	Mitochondria yield (μg)	ER yield (μg)	Yield	MAM detected in	Purity
Sonication								
Low (3 × 5 sec)	1, 2	0.153	9.87	415.86	842.6	High	ER + mitochondria	Good
Medium (3 × 10 sec)	1, 2	0.148	9.584	350.34	824.4	High	ER	Good
High (3 × 15 sec)	1, 2	0.146	8.778	163.14	820.5	Medium	ER	Good
Freeze/thaw	1, 2		7.634	ND[a]	195.2	Medium	ER + mitochondria	Good
Homogenization	1, 2	0.146	1.484	ND[a]	ND[a]	Low	MAM/ mitochondria	Excellent
	3, 4	0.147	1.392	ND[a]	ND[a]	Requires large scale-up	Mitochondria	

[a]ND, not determined as extract protein concentrations were below detection level of the BCA assay.

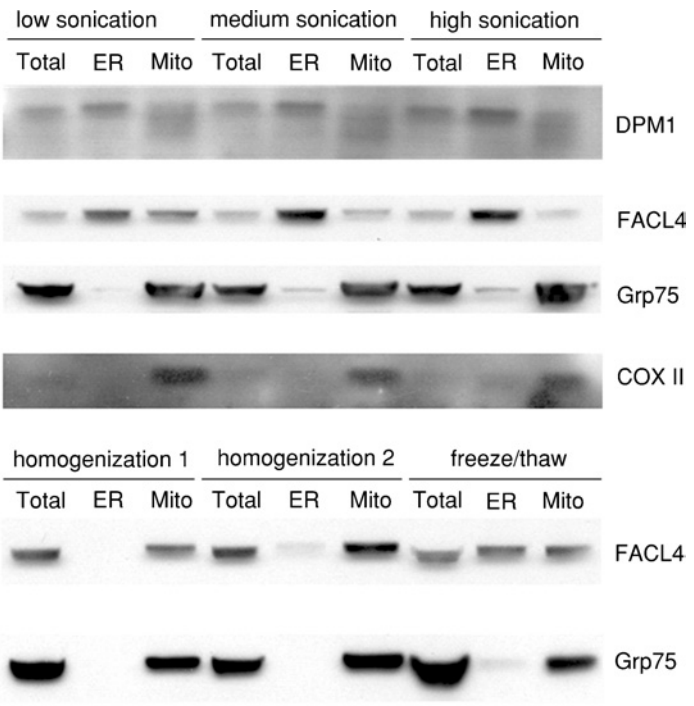


Figure 3.27.5 Western analyses of ER (DPM1), mitochondrial (Grp75, COXII), and MAM (FACL4) markers in HeLa cells lysed by different methods. HeLa cells were fractionated using discontinuous sucrose gradients (see Basic Protocol 1) as detailed in Table 3.27.2. Briefly, cells were lysed using sonication (three times, 5, 10, or 15 sec each), Dounce homogenization (ten strokes), or freeze/thaw cycles (three times 1 hr at –80°C, followed by rapid thawing). Twenty micrograms of total, ER, and mitochondrial protein fractions from each sonication condition were subjected to SDS-PAGE in 10% Bis-Tris NuPage gels (Invitrogen). For homogenization and freeze/thaw lysis conditions, 40 μl of each fraction were used (due to low protein concentrations). Proteins were then transferred to nitrocellulose membranes and probed for DPM1 (1:100 goat anti-DPM1, 1:2000 donkey anti-goat HRP). Membranes were stripped and reprobed for Grp75 (1:2000 mouse anti-Grp75, 1:2500 goat anti-mouse). Similarly, membranes were then stripped and reprobed sequentially for FACL4 (1:250 rabbit anti-FACL4, 1:3000 goat anti-rabbit HRP) then COX II (1:200 goat anti-COXII, 1:2500 donkey anti-goat HRP).

previously documented in rat liver tissue (Stone and Vance, 2000). Therefore, it has been concluded that fraction 2 contains the purified mitochondria-associated membranes from cultured human cells.

Western analyses identified fraction 3 as the purified mitochondria (Fig. 3.27.6). The parallel bands that can be observed within this fraction reflect mitochondria of slightly different sizes and densities. Mitochondria in the cell differ in size and morphology (Bereiter and Vöth, 1994; Collins et al., 2002). It should be noted that the position of the mitochondria in the gradient, exactly below fraction 2, is another indication that fraction 2 represents the mitochondria-associated membranes.

The identity of fraction 1 is less clear. Western analyses clearly indicate that it is ER-related as it shares markers (Fig. 3.27.6, calreticulin) with the ER and MAM. Nonetheless, fraction 1 notably differs in the presence of an alternative DPM species detected by the anti-DPM1 antibody. Moreover, fraction 1 has markedly less mEGFP-huPSS-1 than the MAM fraction. Because of its association with the MAM and mitochondria and the retention of some ER markers, fraction 1 could represent a transition between the rough ER and the MAM or from the ER to another secretory compartment.

Cellular markers for verification of subcellular compartment identity and purity

To verify the purity of the isolated microsomal and mitochondria fractions, established organelle-specific markers that were previously used (Colberg-Poley et al., 2000; Mavinakere and Colberg-Poley, 2004a,b; Mavinakere et al., 2006), and others, were used for the same purpose. To unequivocally

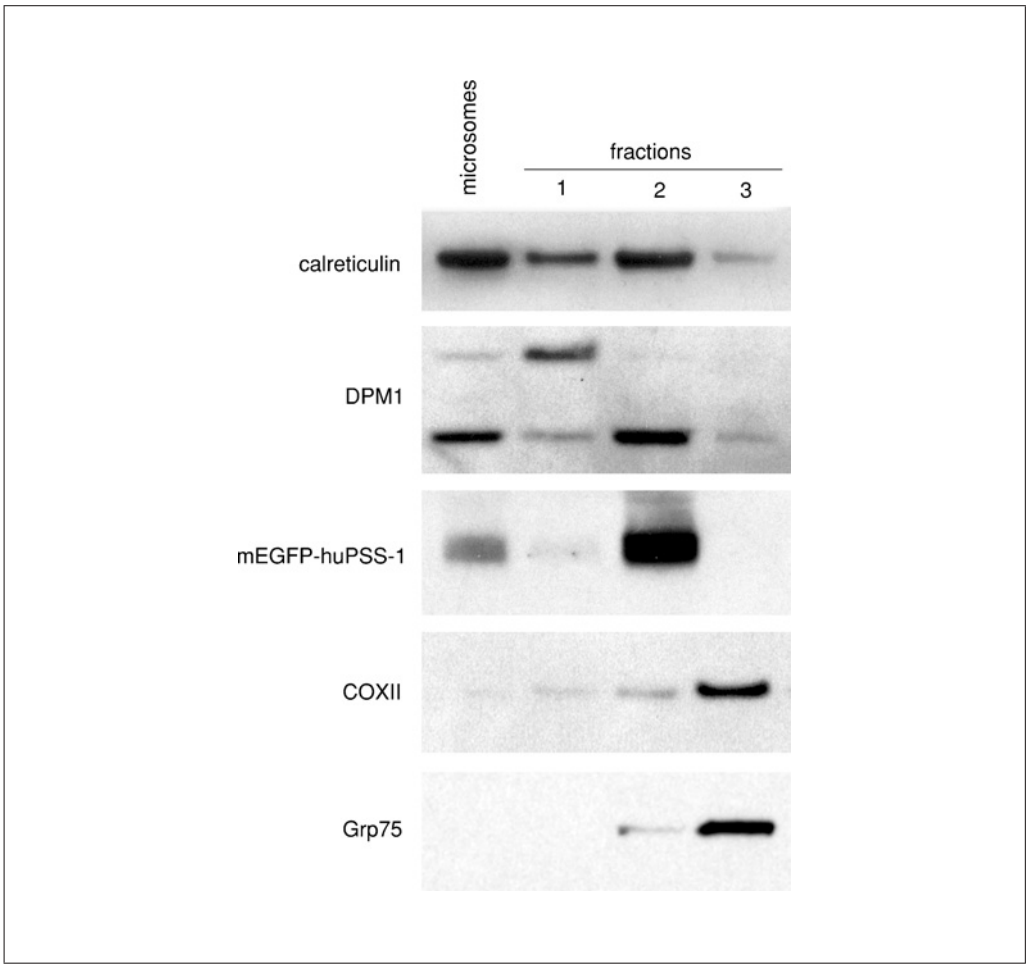


Figure 3.27.6 Western analyses of fractionated HeLa cells that stably express mEGFP-huPSS-1 fusion protein. Stably transfected cells were fractionated according to the procedure described in Basic Protocol 2 and subcellular fractions were isolated. Fractionated proteins (10 μg) were separated by 10% SDS PAGE and transferred onto nitrocellulose membranes using a semi-dry protein transfer apparatus (BioRad). Blotted proteins were probed against markers for microsomes (anti-DPM1, 1:100 or anti-calreticulin, 1:1000), MAM (mEGFP-huPSS-1 using anti-GFP, 1:100), and mitochondria (anti-COX, 1:100 or anti-Grp75, 1:2500) and with the corresponding horseradish peroxidase-conjugated secondary Ab (1:2000). Reactivity was detected using the chemiluminescent method (Amersham, GE Healthcare).

identify the MAM fraction (fraction 2) from Percoll gradients, phosphatidyl-serine synthase 1 (PSS-1), which has been shown to be enriched in the rat liver MAM (Stone and Vance, 2000), was chosen. However, neither commercial antibodies nor human PSS-1 cDNA clones were available. Therefore, the complete human PSS-1 cDNA was cloned from a HeLa cDNA library and its open reading frame was tagged with EGFP (data not shown). The cellular markers, antibodies, commercial sources and original references for ER, mitochondria and MAM are listed in Table 3.27.1. A HeLa cell transfectant stably expresses the fusion protein mEGFP-huPSS-1 fusion protein. The fractionated subcellular compartments from HeLa-PSS-1_{20} cells verified the identity of fraction 2 as the MAM by virtue of the selective presence of PSS-1 in fraction 2 but not in fraction 3 (Fig. 3.27.6).

Microsomes

Dolichol phosphate-mannose synthase 1 (DPM1). DPM synthase 1, a transmembrane protein, which catalyzes mannosyl transfer from GDP-mannose hydrophobic long-chain acceptor dolichol-phosphate, is present in the rough ER (Maeda et al., 1998). In human cells, DPM1, which is located at the cytosolic side of the ER, associates with DPM2 and DPM3 to form the complete multicomponent enzyme of human DPM synthase (Maeda et al., 2000). In addition, a mitochondrial DPM synthase is located on the cytosolic face of

the outer membrane of mitochondria (Gasnier et al., 1992). On occasion, the anti-DPM1 (I-20, Santa Cruz Biotechnology) antibody used detected these different DPM species in the ER or in mitochondrial fractions.

Calreticulin. Calreticulin is a Ca^{2+}-binding chaperone that contains an N-terminal amino acid signal sequence and a C-terminal KDEL ER retrieval sequence, which are responsible for its localization in the ER lumen (Gelebart et al., 2005). However, calreticulin has also been detected in cellular compartments, other than ER and including the cell surface (Johnson et al., 2001). Anti-calreticulin (Affinity Bioreagents) antibody detected calreticulin in the cytosol; whereas, other markers for the ER (anti-DPM1), MAM (anti-mEGFP-PSS-1), or mitochondria (anti-GRP75 and anti-COXII) did not.

Mitochondria

Grp75 (glucose regulated protein 75 kDa). Grp75 is a molecular chaperone that is localized in the mitochondrial matrix, where, in concert with Hsp60, it is thought to participate in the refolding of proteins translocated into this organelle (Manning-Krieg et al., 1991). Very recently, it was shown that Grp75 is also present in a macromolecular complex with VDAC and IP_3Rs at the ER-mitochondria interface (Szabadkai et al., 2006).

COXII (cytochrome c oxidase subunit II). COXII is one of three mitochondrial DNA–encoded subunits (MTCO1–3) of respiratory complex IV. Complex IV localizes to the mitochondrial inner membrane and is the terminal enzyme of the electron transport chain (Scheffler, 2001). Although COXII is mainly localized in the mitochondria, recently, it was shown that the protein is present at extra-mitochondrial sites (Sadacharan et al., 2005).

Mitochondria-associated membranes

mEGFP-huPSS-1 (monomeric-enhanced green fluorescent protein- human phosphatidylserine synthase). Both the PSS-1 and PSS-2 enzymes are involved in phosphatidylserine biosynthesis in mammalian cells. It has been shown that PSS-1 is highly enriched in MAM of rat liver and is largely excluded from the bulk of the ER (Stone and Vance, 2000). The fusion protein mEGFP-huPSS-1 has the same localization like the wild-type protein in human cells.

FACL4 (fatty acid CoA ligase, long chain 4). FACL4 converts long-chain fatty acids into fatty acyl-CoA esters for use in synthesizing complex lipids (Piccini et al., 1998). Similar to PSS-1, it is enriched in MAM compartments and is a marker for MAM fractions in western blot analyses (Simmen et al., 2005).

Time Considerations

Basic Protocol 1 can easily be accomplished within 1 full working day. The time required heavily depends on the number of transfection or infection conditions being tested. It is best to limit the scale of the experiment to ultracentrifuge availability (i.e., each ultracentrifuge rotor can only hold six tubes). For a point of reference, twelve 175-cm^2 flasks (six duplicate sets of experimental conditions) can be processed at once using a single rotor for each ultracentrifugation set, taking a total of ~6 to 7 hr. Preparation and sterilization of buffers is done prior to the day of fractionation. PMSF is added fresh to 1× MTE buffer just before use. Creation of sucrose gradients and harvesting of cell pellets are done first. Cell pellets are stable on ice for up to 2 hr, providing a convenient resting point. Alternatively, cell pellets can be frozen, providing a stopping point in the protocol. After sonication of cells, there are no more stopping points in the protocol, and one should work as quickly as possible. The ultracentrifugation for the ER fraction is relatively long (70 min), and this should be started first. After beginning this ultracentrifugation, go back and perform the pellet washes and load the gradients for the mitochondrial fractions. Both centrifugations will probably finish around the same time. Again, work with the ER fractions first, as they require an additional 45 min ultracentrifugation. Then, go back and collect banded mitochondria from the other tubes.

There are no points at which Basic Protocol 2 can be stopped. All of the fractions that are isolated through this procedure must be stored at −80°C upon recovery. All the solutions can be made ahead of time and stored for 1 to 2 days at 4°C or for longer period (e.g., 2 to 3 months) frozen at −20°C. Note that PMSF or other protease inhibitors should be added immediately prior to use.

Cell preparation and homogenization can be accomplished in 30 min. The separation of crude mitochondrial fraction and total microsomal fraction should take an additional ~30 min. The estimated time of both the isolation of the fractions from the Percoll gradient and the recovery of the pure microsomal pellet is ~2 hr. Another 90 min are needed for the isolation of pure mitochondria and MAM fractions. The total time of this protocol is

estimated to be ~4 hr. Functional assays should be carried out as soon as possible after the preparation, but for simple marker enzyme assay material can be stored up to 16 hr at 0° to 4°C without much loss of activity.

Literature Cited

Al-Barazi, H.O. and Colberg-Poley, A.M. 1996. The human cytomegalovirus UL37 immediate-early regulatory protein is an integral membrane N-glycoprotein which traffics through the endoplasmic reticulum and Golgi apparatus. *J. Virol.* 70:7198-7208.

Ardail, D., Popa, J., Bodennec, J., Louisot, P., Schmitt, D., and Portoukalian, J. 2003. The mitochondria-associated endoplasmic-reticulum subcompartment (MAM fraction) of rat liver contains highly active sphingolipid-specific glycotransferases. *Biochem. J.* 371:1013-1019.

Bereiter-Hahn, J. and Vöth, M. 1994. Dynamics of mitochondria in living cells: Shape changes, dislocations, fusion, and fission of mitochondria. *Microsc. Res. Tech.* 27:198-219.

Bionda, C., Portoukalian, J., Schmitt, D., Rodriguez-Lafrasse, C., and Ardail, D. 2004. Subcellular compartmentalization of ceramide metabolism: MAM (mitochondria-associated membrane) and/or mitochondria? *Biochem. J.* 382:527-533.

Calaminus, J.M., Brüggen, J., and Sorg, C. 1979. Isolation, characterization and cultivation of human trophoblastic cells. *Immunobiology* 156-287.

Colberg-Poley, A.M., Patel, M.B., Erezo, M.P.P., and Slater, J.E. 2000. Human cytomegalovirus UL37 immediate-early regulatory proteins traffic through the secretory apparatus and to mitochondria. *J. Gen. Virol.* 81:1779-1789.

Collins, T.J., Berridge, M.J., Lipp, P., and Bootman, M.D. 2002. Mitochondria are morphologically and functionally heterogeneous within cells. *EMBO J.* 21:1616-1627.

Gasnier, F., Rousson, R., Lerme, F., Vaganay, E., Louisot, P., and Gateau-Roesch, O. 1992. Mitochondrial dolichyl-phosphate mannose synthase. Purification and immunogold localization by electron microscopy. *Eur. J. Biochem.* 206:853-858.

Gelebart, P., Opas, M., and Michalak, M. 2005. Calreticulin, a Ca^{2+}-binding chaperone of the endoplasmic reticulum. *Int. J. Biochem. Cell Biol.* 37:260-266.

Goldmacher, V.S., Bartle, L.M., Skaletskaya, A., Dionne, C.A., Kedersha, N.L., Vater, C.A., Han, J.-W., Lutz, R.J., Watanabe, S., Cahir McFarland, E.D., Kieff, E.D., Mocarski, E.S., and Chittenden, T. 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. *Proc. Natl. Acad. Sci. U.S.A.* 96:12536-12541.

Hovius, R., Lambrechts, H., Nicolay, K., and de Kruijff, B. 1990. Improved methods to isolate and subfractionate rat liver mitochondria. Lipid composition of the inner and outer membrane. *Biochiml. Biophys. Acta* 1021:217-226.

Johnson, S., Michalak, M., Opas, M., and Eggleton, P. 2001. The ins and outs of calreticulin: From the ER lumen to the extracellular space. *Trends Cell Biol.* 11:122-129.

Maeda, Y., Tomita, S., Watanabe, R., Ohishi, K., and Kinoshita, T. 1998. DPM2 regulates biosynthesis of dolichol phosphate-mannose in mammalian cells: Correct subcellular localization and stabilization of DPM1, and binding of dolichol phosphate. *EMBO J.* 17:4920-4929.

Maeda, Y., Tanaka, S., Hino, J., Kangawa, K., and Kinoshita, T. 2000. Human dolichol-phosphate-mannose synthase consists of three subunits, DPM1, DPM2 and DPM3. *EMBO J.* 19:2475-2482.

Manning-Krieg, U.C., Scherer, P.E., and Schatz, G. 1991. Sequential action of mitochondrial chaperones in protein import into the matrix. *EMBO J.* 10:3273-3280.

Mavinakere, M.S. and Colberg-Poley, A.M. 2004a. Dual targeting of the human cytomegalovirus UL37 exon 1 protein during permissive infection. *J. Gen. Virol.* 85:323-329.

Mavinakere, M.S. and Colberg-Poley, A.M. 2004b. Internal cleavage of the human cytomegalovirus UL37 immediate-early glycoprotein and divergent trafficking of its proteolytic fragments. *J. Gen. Virol.* 85:1989-1994.

Mavinakere, M.S., Williamson, C.D., Goldmacher, V.S., and Colberg-Poley, A.M. 2006. Processing of human cytomegalovirus UL37 mutant glycoproteins in the endoplasmic reticulum lumen prior to mitochondrial importation. *J. Virol.* 80:6771-6783.

McCormick, A.L., Smith, V.L., Chow, D., and Mocarski, E.S. 2003. Disruption of mitochondrial networks by the human cytomegalovirus UL37 gene product viral mitochondrion-localized inhibitor of apoptosis. *J. Virol.* 77:631-641.

Pertoft, H., Laurent, T.C., Laas, T., and Kagedal, L. 1978. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). *Anal. Biochem.* 88:271-282.

Piccini, M., Vitelli, F., Bruttini, M., Pober, B.R., Jonsson, J.J., Villanova, M., Zollo, M., Borsani, G., Ballabio, A., and Renieri, A. 1998. FACL4, a new gene encoding long-chain acyl-CoA synthetase 4, is deleted in a family with Alport syndrome, elliptocytosis, and mental retardation. *Genomics* 47:350-358.

Rizzuto, R., Pinton, P., Carrington, W., Fay, F.S., Fogarty, K.E., Lifshitz, L.M. Tuft, R.A., and Pozzan, T. 1998. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* 280:1763-1766.

Sadacharan, S.K., Singh, B., Bowes, T., and Gupta, R.S. 2005. Localization of mitochondrial DNA encoded cytochrome c oxidase subunits I and II in rat pancreatic zymogen granules and pituitary growth hormone granules. *Histochem. Cell Biol.* 124:409-421.

Scheffler, I.E. 2001. A century of mitochondrial research: Achievements and perspectives. *Mitochondrion* 1:3-31.

Simmen, T., Aslan, J.E., Blagoveshchenskaya, A.D., Thomas, L., Wan, L., Xiang, Y., Feliciangeli, S.F., Hung, C.-H., Crump, C.M., and Thomas, G. 2005. PACS-2 controls endoplasmic reticulum–Mitochondria communication and Bid-mediated apoptosis. *EMBO J.* 24:717-729.

Stone, S.J. and Vance J.E. 2000. Phosphatidylserine synthase-1 and -2 are localized to mitochondria-associated membranes. *J. Biol. Chem.* 275:34534-34540.

Szabadkai, G., Bianchi, K., Várnai, P., De Stefani, D., Wieckowski, M.R., Cavagna, D., Nagy, A.I., Balla, T., and Rizzuto, R. 2006. Chaperone-mediated coupling of endoplasmic reticulum and mitochondria Ca^{2+} channels. *J. Cell. Biol.* 175:901-911.

Vance, J.E. 1990. Phospholipid synthesis in a membrane fraction associated with mitochondria. *J. Biol. Chem.* 265:7248-7256.

Isolation of Amyloplasts

Kay Denyer[1] and Marilyn Pike[1]

[1]John Innes Centre, Norwich Research Park, Norwich, Norfolk

ABSTRACT

Two different methods for the preparation of starch-rich plastids are described together with protocols for the determination of plastid yield, purity, and intactness. The preparation of amyloplasts from maize endosperm and oilseed rape embryos are given as examples, but the protocols could be adapted for the isolation of starch-rich plastids from other plant organs. A method for the determination of the quantitative distribution of an enzyme between the plastids and cytosol is given. Typical results and references for marker enzymes for a range of subcellular compartments are listed. *Curr. Protoc. Cell Biol.* 38:3.28.1-3.28.15. © 2008 by John Wiley & Sons, Inc.

Keywords: amyloplast • plastid • starch

INTRODUCTION

Amyloplasts are nonphotosynthetic starch-containing plastids. They accumulate starch in the form of insoluble granules. Some amyloplasts, such as those in pea embryos, contain a single large starch granule (see Figure 3.28.1). Other amyloplasts, such as those in rice endosperm, contain numerous separate granules. The starch granule(s) in amyloplasts usually occupy a large proportion of the volume of the plastid. Some plastids, such as those in oilseed rape embryos, contain large amounts of starch only at some developmental stages. When considering plastid isolation protocols, these starch-rich plastids are equivalent to amyloplasts and will be referred to as such in this unit.

All plastids are fragile and therefore difficult to isolate from the plant without breakage. Intact amyloplasts are more difficult to isolate than chloroplasts because of their high

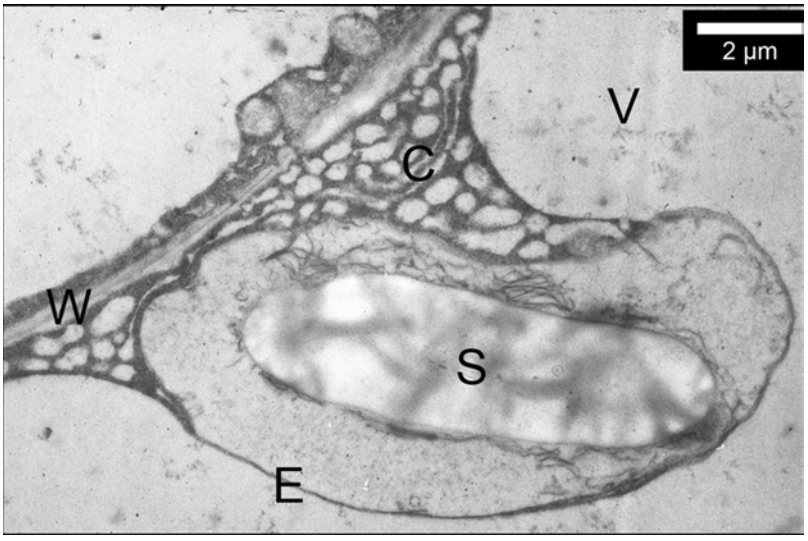


Figure 3.28.1 Ultrastructure of an amyloplast in a developing pea embryo. The figure is an electron micrograph of an amyloplast containing a single starch granule (S). The plastid envelope (E), cytosol (C), cell wall (W), and cell vacuole (V) are indicated.

Current Protocols in Cell Biology 3.28.1-3.28.15, March 2008
Published online March 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0328s38

starch content. Starch granules tend to rupture through the plastid envelope if they are subjected to abrupt changes in gravity or to agitation. Even for chloroplast isolation, leaves with low starch content are favored (Walker, 1971). However, the presence of starch granules can also aid plastid isolation: amyloplasts sediment more readily than other less dense organelles such as mitochondria. Thus, although starch granules make amyloplasts fragile, they also ease their purification from other organelles.

Chloroplast isolation protocols were developed before those for amyloplasts. The standard chloroplast isolation protocols had to be modified to enable the less robust amyloplasts to be isolated without damage (see Background Information). Whereas intact chloroplasts can be isolated from tissue that has been pulverized in a mortar or macerated in an electric blender, many amyloplasts will not survive such violent extraction methods. The first requirement for successful amyloplast isolation, therefore, is to use a method that will release the amyloplasts from the cells without breaking them. Slicing the tissue with a sharp razor blade is the least damaging extraction method. More robust amyloplasts with relatively low starch content, such as those from rape embryos, will survive extraction with a Polytron tissue homogenizer.

To separate the amyloplasts from pieces of tissue, whole cells, and cell debris, the preferred method is filtration through cloth of a mesh size just large enough to allow the passage of the plastids. Cloth is preferred to nylon membrane because it absorbs some of the cell debris. After filtration, the amyloplasts are separated from soluble cellular components, such as cytosolic enzymes, by low-speed centrifugation. The exact centrifugation conditions will be determined by the sedimentation properties of the amyloplasts. Those containing large amounts of starch will require lower centrifugation speeds and times than amyloplasts with relatively less starch. Refrigerated centrifuges with swing-out rotors that are capable of slow acceleration and deceleration are preferred. The purpose for which the amyloplasts are to be used will determine how the plastids are resuspended following centrifugation. If the amyloplasts are to be assayed for enzyme activity, then it is no longer important to maintain plastid integrity after the supernatant has been removed from the plastid pellet. The plastid pellet can be resuspended in a suitable medium, and the plastids deliberately broken by vigorous mixing, passage through a small-bore syringe needle, or addition of detergent. If the isolation of intact amyloplasts is necessary, the plastids must be resuspended very gently to avoid rupture. It will be necessary to resuspend the plastids without breaking them, before they can be purified further. The shape of the centrifuge tube can influence the ease of resuspension. Wide, round-bottom centrifuge tubes will aid resuspension and maximize the yield of intact plastids.

Below, we describe two slightly different amyloplast isolation protocols that we have used for the isolation of amyloplasts from different tissues: maize endosperm (Basic Protocol) and rape embryos (Alternate Protocol). These methods are likely to be applicable to other types of plant organ. Support protocols describe methods for determining the yield, purity (Support Protocol 1), and intactness by latency (Support Protocol 2) and protection (Support Protocol 3) of the amyloplasts. Both require the assay of enzymes known to be located exclusively in particular subcellular compartments (marker enzymes). By quantifying the distribution of the marker enzymes, the contributions of their subcellular compartments to each fraction can be deduced. A selection of suitable marker enzymes and references to protocols for their assay are listed in Table 3.28.1. In Support Protocol 4, we give an example of the methods and calculations required to quantify the subcellular distribution of an enzyme that is present in both plastids and cytosol.

NOTE: The authors recommend that all equipment that will come into contact with the plastids or with solutions used on the plastids be rinsed several times with distilled water to remove all traces of detergent.

Table 3.28.1 Assay Procedures for Marker Enzymes

Enzyme	Compartment	Reference
Phosphoenolpyruvate carboxylase (EC 4.1.1.31)	Cytosol	Wedding and Kline (1994)
Alcohol dehydrogenase (EC 1.1.1.1)	Cytosol	Macdonald and ap Rees (1983); El-Shora and ap Rees (1991)
UDP-glucose pyrophosphorylase (EC 2.7.7.9)	Cytosol	Hansen et al. (1965)
Pyrophosphate:fructose 6-P phosphotransferase (EC 2.7.1.90)	Cytosol	Journet and Douce (1985)
NADP-glyceraldehyde-3-P dehydrogenase (EC 1.2.1.13)	Plastid	Holtum and Winter (1982)
Alkaline pyrophosphatase (EC 3.6.1.1)	Plastid	Gross and ap Rees (1986)
ADP-glucose pyrophosphorylase (EC 2.7.7.27)	Plastid	Entwistle and ap Rees (1988)
Fumarase (EC 4.2.1.2)	Mitochondria	Hatch (1978); El-Shora and ap Rees (1991)
Citrate synthase (EC 4.1.3.7)	Mitochondria	El-Shora and ap Rees (1991)
Succinate dehydrogenase[a] (EC 1.3.99.1)	Mitochondria	Bonner (1953)
Cytochrome *c* oxidase (EC 1.9.3.1)	Mitochondria	Bowles and Kauss (1976); Appelmans et al. (1954)
Cytochrome *c* reductase[b] (EC 1.6.2.1)	ER	Bowles and Kauss (1976)
Hydroxypyruvate reductase (EC 1.1.1.81)	Peroxisomes	El-Shora and ap Rees (1991)

[a]See *UNIT 3.4* for assay protocol.
[b]See *UNIT 3.5* for assay protocol.

ISOLATION OF AMYLOPLASTS FROM MAIZE ENDOSPERM

BASIC PROTOCOL

This protocol results in a crude preparation of amyloplasts suitable for studies of the subcellular distributions of enzyme activities. This is a gentle and quick procedure designed to maximize yield and minimize loss of enzyme activity. The starting material for this protocol, endosperm dissected from developing maize kernels, is shown in Figure 3.28.2. We have used very similar protocols to that described here to isolate plastids from pea embryos and from wheat and barley endosperm.

Materials

Amyloplast isolation medium 1 (AIM 1; see recipe), ice cold
~10 g of developing endosperm harvested 11 to 22 days after pollination

Pasteur pipets and rubber pipet bulbs
Single-edged razor blades
90-mm petri dish
Filtration cloth (e.g., Miracloth, pore size 22 to 25 μm; Calbiochem, or equivalent)
Small funnel
30-ml glass centrifuge tube
Refrigerated benchtop centrifuge (e.g., Beckman RT 6000D, or equivalent)
Measuring cylinder
Small (1-ml) syringes and fine (23-G) needles

NOTE: Carry out all procedures at 0° to 4°C using solutions and equipment prechilled to that temperature.

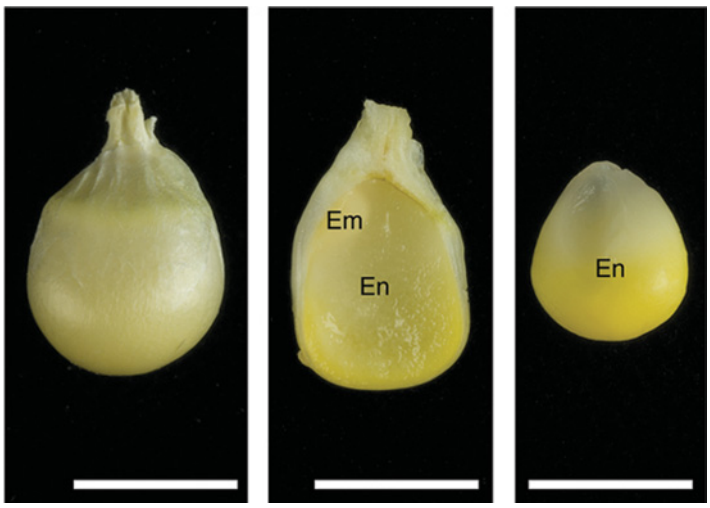


Figure 3.28.2 Developing maize kernels. The panel on the left shows a single developing maize kernel. The panel in the center shows a kernel cut in half to reveal the embryo (Em) and the endosperm (En). The panel on the right shows endosperm dissected from one kernel. The scale bars are 0.5 cm. For the color version of this figure go to *http://www.currentprotocols.com*.

Prepare equipment and plant materials

1. Make a wide-ended glass Pasteur pipet as follows: break off the narrow end of a standard Pasteur pipet by wrapping the end in tissue and applying pressure with a gloved hand. Invert the broken pipet and place the fractured end into a rubber pipet bulb.

   Alternatively, use an automatic pipettor with a wide-ended disposable tip. Widen the tip by cutting off the end with scissors.

2. Place a razor blade and a 90-mm petri dish on ice to cool.

3. Place a small piece of Miracloth in a small funnel and suspend over a 30-ml glass centrifuge tube in ice. Prewet the Miracloth and the tube with ~1 ml AIM 1.

4. Immediately before the experiment, harvest the maize kernels and place on ice. Remove the pericarp (the seed coat surrounding the endosperm and embryo) and embryo from each kernel and discard.

5. Place the endosperm in the 90-mm petri dish on ice. Add sufficient AIM 1 to cover the endosperm and incubate for 20 to 60 min.

Chop endosperm to release intact plastids

6. Replace the AIM 1 with 4 to 5 ml fresh AIM 1 and chop the endosperms repeatedly with a very sharp single-edged razor blade until the endosperms are coarsely diced.

7. Tilt the petri dish slightly and carefully remove the liquid (homogenate) surrounding the pieces of endosperm with the wide-ended Pasteur pipet.

   The homogenate, containing intact plastids, will look pale yellow. Try not to suck up bits of the endosperm. Pipet the homogenate slowly and avoid vortex mixing, which promotes the introduction of air bubbles.

8. To remove pieces of tissue and intact cells, filter the homogenate through the prepared Miracloth and into the 30-ml glass centrifuge tube. Add the homogenate to the funnel slowly, with the pipet in contact with the Miracloth, to avoid the formation of drips. Position the funnel and the tube in the ice so that the plastids glide down the side of the tube (rather than dropping from the funnel and splashing to the bottom of the tube).

9. Add 4 to 5 ml more AIM 1 to the petri dish, chop the endosperm more finely, and transfer the homogenate to the filter/tube as before.

10. Repeat chopping and filtering once or twice more until the endosperm is finely divided and all the homogenate has been transferred to the tube. Remove the funnel and discard the chopped endosperm.

11. Cover the top of the tube with Parafilm, then mix the filtered homogenate in the tube by very slow and gentle inversion. Measure the volume and take a 0.5-ml sample of the homogenate for enzyme analysis.

Prepare the plastid-enriched pellet and the supernatant fractions

12. Centrifuge the homogenate at 100 × *g* for 10 min, 4°C.

    The intact plastids will collect in the pellet. The pellet will also contain other subcellular fractions such as cell walls and perhaps even intact cells.

    The pellet should be pale yellow throughout. If the bottom of the pellet is white, this indicates that some of the plastids have ruptured, releasing their starch granules.

13. Remove the supernatant to a measuring cylinder and note the volume. Take a 0.5-ml sample of the supernatant for enzyme analysis.

    The volume of the supernatant should be only slightly less than the volume of the homogenate. The supernatant will contain most of the enzymes from the cytosol, intact organelles that are less dense than amyloplasts (such as mitochondria), and enzymes from broken plastids.

Resuspend the plastids

14. Carefully layer 1 ml AIM 1 above the plastid-enriched pellet. Gently and slowly resuspend the pellet by rocking and revolving the tube repeatedly. Take a 0.5-ml sample of the resuspended pellet for enzyme analysis and retain the remaining resuspended pellet for use in downstream experiments.

    The resuspended pellet should contain intact plastids. To determine plastid intactness, see Support Protocol 2 or 3.

    It is important to use the intact plastids quickly once they are prepared, so do not try to do too many assays with any one plastid preparation. Also, randomize the order of your replicate assays in case there are changes, e.g., in the integrity of the plastids with time.

Prepare samples for enzyme assays

15. Rupture the plastids in the 0.5-ml samples of homogenate, supernatant, and pellet by squirting 10 times up and down through a fine (23-G) syringe needle attached to a small (1-ml) syringe.

    Alternatively, you could add Triton X-100 to 0.1% (v/v), but you must check that this does not interfere with your enzyme assays.

16. To remove insoluble material such as starch granules, centrifuge the samples 5 min at 14,000 × *g*, 4°C.

17. Assay the resulting supernatant for marker enzymes and other enzymes of interest (Support Protocols 1 and 4).

ISOLATION OF AMYLOPLASTS FROM OILSEED RAPE EMBRYOS

ALTERNATE PROTOCOL

This protocol is similar to Basic Protocol, but it includes, in addition, two alternative methods for the purification of the plastids after the initial low-speed centrifugation. This method is suitable for amyloplasts that are reasonably robust and where purity as well as yield is important. We have used this method to isolate plastids from oilseed

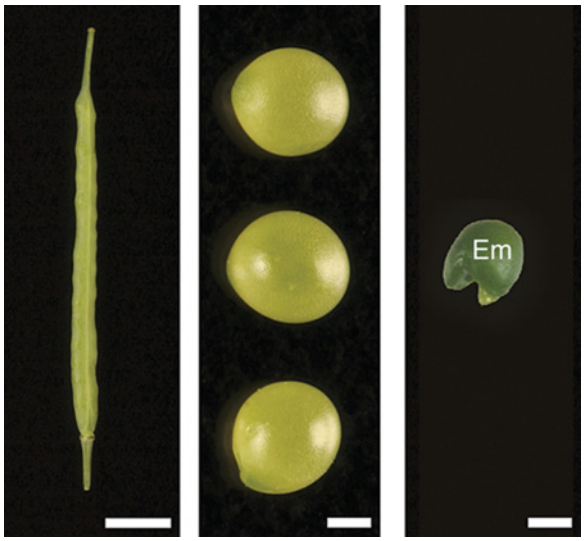


Figure 3.28.3 Developing oilseed rape (mid-developmental stage). The panel on the left shows a single developing silique (scale bar = 1 cm). The panel in the center shows three individual seeds removed from a silique (scale bar = 1 mm). The panel on the right shows an embryo (Em) dissected from a seed (scale bar = 1 mm). For the color version of this figure go to *http://www.currentprotocols.com*.

rape embryos at a number of stages during seed development (Fig. 3.28.3). The plastids contain starch only during mid embryo development. At later stages of development, the starch is degraded and replaced by oil.

Materials

1× and 2× amyloplast isolation medium 2 (AIM 2; see recipe), ice cold
Developing embryos of oil seed rape (*Brassica oleracea* variety Topas)
Percoll

Polytron homogenizer (e.g., model PT 3000, Kinematica, *http://www.kinematica-inc.com/*; or equivalent)
90-mm petri dish
Filtration cloth (e.g., Miracloth, pore size 22 to 25 μm; Calbiochem, or equivalent)
15- and 30-ml glass centrifuge tubes
50-ml plastic graduated cylinder, prechilled
Small funnel
Wide-ended pipet (see Basic Protocol, step 1) or small paintbrush
Refrigerated centrifuge (e.g., Sorvall RC5C with an HB4 rotor, or equivalent)
Small (1-ml) syringes and fine (23-G) needles

NOTE: Carry out all procedures at 0° to 4°C using solutions and equipment prechilled to that temperature.

Prepare equipment and plant materials

1. Place the Polytron probe and a 90-mm petri dish containing 5 to 10 ml 1× AIM 2 on ice to cool.

2. Place a small piece of Miracloth in a small funnel and suspend over a 30-ml glass centrifuge tube in ice. Prewet the Miracloth and tube with approximately 1 ml 1× AIM 2.

3. Immediately before the experiment, harvest developing siliques (Fig. 3.28.3) and place on ice. Choose siliques containing seeds at the mid-stage of development (siliques at this stage have attained their maximum length). Remove 500 to 1000 seeds from the siliques and place in the medium-containing petri dish prepared in step 1, on ice.

4. Using a wide-bore (23-G) syringe needle to hold each seed, remove the testa (seed coat surrounding the embryo) and place the embryo into 5 to 10 ml ice cold 1× AIM 2 in a petri dish on ice.

5. After preparing all the embryos, remove the 1× AIM 2 with a pipet and replace with 5 to 10 ml of fresh 1× AIM 2.

Homogenize embryos to release intact plastids

6. Pour the embryos in 1× AIM 2 into a cold 50-ml plastic measuring cylinder.

7. Using a Polytron, homogenize the embryos for 5 sec at 9,500 to 10,000 rpm on ice.

8. To remove large pieces of tissue and intact cells, filter the homogenate through the prepared Miracloth and into the 30-ml glass centrifuge tube.

9. Remove the material from the filter and suspend in 5 to 10 ml fresh 1× AIM 2. Homogenize and filter as before (steps 7 and 8), combining the first and second filtrates.

   The homogenate, containing intact plastids, will look milky green.

10. Adjust the volume of the filtered homogenate to 50 to 100 ml with 1× AIM 2. Mix gently and take a 0.5-ml sample of homogenate for enzyme analysis.

Prepare the plastid-enriched pellet and the supernatant fractions

11. Divide the homogenate between two to four 30-ml glass centrifuge tubes and centrifuge 5 min at 750 × *g*, 4°C.

    The intact plastids will collect in the pellets.

12. Remove the supernatants and combine in a measuring cylinder. Note the volume and take a 0.5-ml sample for enzyme analysis.

    During this procedure, some plastids will rupture and release starch granules. The starch granules will collect in the pellet and can be seen as a small white pellet below the green plastid pellet.

13. Resuspend the plastid-enriched pellets, carefully layer 1 ml of 1× AIM 2 above each pellet, and gently resuspend them using a wide-ended pipet (see Basic Protocol, step 1) or a small paintbrush.

Purify the plastids

Two alternative plastid purification methods are given below. The second method (steps 14b to 19b) generally gives cleaner plastids, but a lower yield compared to the first method (steps 14a to 19a).

Higher-yield method

14a. Add 20 ml of 1× AIM 2 to each of the resuspended pellets, mix gently, and centrifuge 5 min at 750 × *g*, 4°C.

15a. Discard the supernatants.

16a. Repeat steps 14a and 15a once more.

17a. Carefully layer 0.5 ml of 1× AIM 2 above each of the plastid pellets and gently resuspend the plastids as before.

18a. Combine the purified plastids and take a 0.5-ml sample for enzyme analysis.

19a. Determine plastid intactness (Support Protocol 2 or 3 or use the plastids in downstream experiments).

Cleaner-plastid method

14b. Prepare 5 ml of 35% Percoll in a cold 15-ml centrifuge tube by mixing 1.75 ml Percoll, 2.5 ml of 2× AIM 2, and 0.75 ml H_2O.

15b. Combine the resuspended plastid-enriched pellets and dilute to 5 ml with 1× AIM 2. Mix gently and layer the plastids over the 35% Percoll.

16b. Centrifuge 8 min at 1000 × *g*, 4°C, in a centrifuge with the brakes off to allow slow deceleration. Discard the supernatant (including the interface and the Percoll) and carefully resuspend the plastid pellet in 1 ml AIM 2 as before.

Thylakoid membranes will collect at the interface between the AIM 2 and the Percoll. Intact plastids will collect in the pellet. Both the pellet and the interface should be green.

17b. Add 4 ml 1× AIM 2 to the resuspended pellet, mix gently, and centrifuge 5 min at 750 × *g*, 4°C. Discard the supernatant.

The pellet should contain purified plastids.

18b. Carefully layer 1 ml 1× AIM 2 above the plastid pellet and gently resuspend the plastids as before. Take a 0.5-ml sample for enzyme analysis.

19b. Determine plastid intactness (Support Protocol 2 or 3 or use the plastids in downstream experiments).

Prepare samples for enzyme assays

20. Rupture plastids (and other organelles) in the 0.5-ml samples of homogenate, supernatant, pellet, and purified plastids by squirting these liquids 10 times up and down through a fine (23-G) syringe needle attached to small (1-ml) syringe.

21. To remove insoluble material such as starch granules, centrifuge the samples 5 min at 14,000 × *g*, 4°C.

22. Assay the resulting supernatants for marker enzymes and other enzymes of interest (Support Protocols 1 and 4).

SUPPORT PROTOCOL 1

DETERMINING THE YIELD AND PURITY OF AMYLOPLASTS

The pellets obtained in the amyloplast isolation procedures should be enriched in amyloplasts and depleted in cytosolic enzymes and non-plastid organelles (such as mitochondria) relative to the initial homogenate. The proportion of plastids in the homogenate that are recovered in the plastid-enriched pellet (the yield of plastids) can be determined by measuring the distribution of enzymes known to be confined to the plastids (plastid marker enzymes). Similarly, the purity of the plastids can be assessed by measurement of the activities of other marker enzymes in these fractions. Aliquots of each fraction are reserved during the amyloplast preparation procedure for this purpose (see Basic Protocol and Alternate Protocol). For suitable plastid marker enzymes see Table 3.28.1.

Materials

Aliquots of homogenate, supernatant, and pellet fractions (see Basic Protocol and Alternate Protocol)

Additional reagents and equipment for assays of amyloplast marker enzymes (see references in Table 3.28.1)

NOTE: Carry out all procedures at 0° to 4°C using solutions and equipment prechilled to that temperature unless otherwise stated.

1. Measure the activity of each marker enzyme in the 0.5-ml aliquots of homogenate, supernatant, and pellet.

   The literature references in Table 3.28.1 describe assay procedures for amyloplast marker enzymes.

2. Calculate the activity of each enzyme in the total volumes of these fractions.

3. Check that there have been no significant losses of enzyme activity during plastid isolation by calculating, for each enzyme, the recovery of activity in the supernatant plus pellet as a proportion of that in the homogenate.

   This recovery value should be close to 100%.

4. For each enzyme, calculate the activity in the pellet as a proportion of that in the supernatant plus pellet.

   The proportion of the plastid marker enzyme in the pellet shows the yield. The proportion of the marker enzymes for other subcellular compartments that are in the pellet shows the degree to which the plastids are contaminated with those compartments.

DETERMINATION OF PLASTID INTACTNESS BY LATENCY

SUPPORT PROTOCOL 2

This approach relies on the inability of the substrates of a plastidial marker enzyme to cross the plastid envelope and enter the plastid. This means that assays of intact plastids will give low values compared to assays of ruptured plastids. Only enzymes with substrates that cannot cross the envelope in vitro (e.g., NADP-glyceraldehyde 3-phosphate dehydrogenase) can be used in these latency experiments. The difference between the values found for intact and deliberately ruptured plastid preparations, expressed as a percentage of the activity in the ruptured plastids, is called the latency (Stitt and ap Rees, 1979), and is a measure of the proportion of intact plastids in the preparation. To prevent plastid rupturing during the assay, osmoticum (e.g., sorbitol) is added to the assay mix to the same final concentration as in the plastid preparation, which has been resuspended in sorbitol-containing AIM 1 or AIM 2. The assays must be mixed very carefully to avoid plastid rupture. To deliberately rupture the plastids, the osmoticum is omitted from the assay and the assay and plastids are thoroughly mixed. A high concentration of osmoticum is then added so that the final concentrations of osmoticum in both the intact and ruptured assay mixes are the same.

As an example, the following method is given for a marker enzyme that is normally assayed spectrophotometrically in a 1-ml assay volume (e.g., NADP-glyceraldehyde 3-phosphate dehydrogenase). It is assumed that the plastids used have been resuspended in medium (i.e., AIM 1 or AIM 2; see Reagents and Solutions) containing 0.5 M sorbitol.

Materials

2 M sorbitol
Resuspended, intact plastids (Basic Protocol and Alternate Protocol)
Cuvettes suitable for spectrophotometry

Additional reagents and equipment for assays of amyloplast marker enzymes (see references in Table 3.28.1)

1. Set up two cuvettes by adding all of the components of the assay (except the substrate used to start the reaction and the plastids; see references in Table 3.28.1) in a final volume of 0.70 ml.
2. To one of the cuvettes (intact plastid assay), add 0.25 ml of 2 M sorbitol and mix well.
3. Using a wide-ended pipet, carefully add 0.05 ml plastids, cover the opening of the cuvette with Parafilm, and mix by gentle inversion two to three times.
4. To the second cuvette (ruptured plastid assay), before adding sorbitol, add 0.05 ml plastids, mix vigorously by vortexing, and then, after mixing, add 0.25 ml 2 M sorbitol.
5. Measure the activities in the two cuvettes using a spectrophotometer, ensuring that after adding the starting reagent, the samples are mixed by gentle inversion two to three times.
6. Determine the percentage of intact plastids in the preparation (% latency) using the following equation:

   $[(\text{activity of ruptured} - \text{activity of intact})/(\text{activity of ruptured})] \times 100 = \%\text{latency}$
7. Repeat the estimate of latency for several independently-prepared batches of plastids.

SUPPORT PROTOCOL 3

DETERMINATION OF PLASMID INTACTNESS BY PROTECTION

This method relies on the assumption that enzymes within intact amyloplasts are protected from proteolysis because the added trypsin cannot cross the amyloplast envelope (Stitt et al., 1978). Treatment of intact plastids with trypsin will therefore result in the removal of proteins released from ruptured plastids, but it will not alter the activity of enzymes that are contained within intact plastids. After trypsin treatment, an inhibitor of trypsin is added, plastids are deliberately ruptured, and the activity of a plastidial marker enzyme is measured. To show that the trypsin and inhibitor themselves have no effect on activity of the marker enzyme, they are added together to a parallel sample. To demonstrate that the marker enzyme is sensitive to trypsin when not protected by an intact plastid envelope, deliberately ruptured plastids are also treated with trypsin. Provided these two controls give the expected results, the proportion of the plastid marker enzyme that is protected from trypsin indicates the proportion of intact plastids in the amyloplast preparation.

As an example, the following method is given for a marker enzyme that is normally assayed spectrophotometrically in a 1-ml assay volume (e.g., NADP-glyceraldehyde 3-phosphate dehydrogenase).

Materials

Resuspended, intact plastids (Basic Protocol and Alternate Protocol)
400 mg/ml trypsin (Type IX, 6000 U/mg, from bovine pancreas) stock solution in H_2O (prepare immediately before use)
600 mg/ml trypsin inhibitor (Glycine max, 12,000 U/mg) stock solution in H_2O (prepare immediately before use)

Small (1-ml) syringes and fine (23-G) needles
25°C water bath
Cuvettes suitable for spectrophotometry

Additional reagents and equipment for assays of amyloplast marker enzymes (see references in Table 3.28.1)

1. Deliberately rupture a sample of the plastids by squirting 10 times up and down through a fine (23-G) syringe needle attached to small (1-ml) syringe.
2. Measure the enzyme activity (see references in Table 3.28.1) of the both intact and ruptured plastids (initial activity).
3. To 0.2-ml samples of the intact and ruptured plastids, add trypsin alone (to a final concentration of 4 mg/ml) or trypsin (final concentration, 4 mg/ml) together with trypsin inhibitor (final concentration, 6 mg/ml). Incubate at 25°C for 30 min.
4. Add trypsin inhibitor (6 mg/ml) to the samples that contain trypsin alone.
5. Measure the enzyme activity of each sample (final activity).
6. Provided that the final activity of the samples incubated with trypsin and inhibitor was the same as the initial activity, calculate the percentage of intact plastids in the preparation (% protection) using the following equation:

   $$[(\text{activity after trypsin alone})/(\text{activity after trypsin+inhibitor})] \times 100 = \%\ \text{protection}$$

7. Repeat the estimate of the extent of protection for several independently prepared batches of plastids.

DETERMINATION OF THE DISTRIBUTION OF AN ENZYME BETWEEN THE AMYLOPLASTS AND CYTOSOL

SUPPORT PROTOCOL 4

The amyloplast isolation protocols described in this unit can be used to investigate the subcellular location of an enzyme. This is because a plastidial enzyme will sediment with the plastids and be enriched in the pellet fraction. In contrast, a cytosolic enzyme will not sediment with plastids and will be found mainly in the supernatant. Some enzymes are located in both the cytosolic and plastidial compartments. For example, ADP-glucose pyrophosphorylase in cereal endosperms is both plastidial and cytosolic (Denyer et al., 1996). In such cases, the amyloplast isolation procedures can be used to quantify the distribution of the enzyme between these two compartments. To illustrate the methodology, in this section, we will consider an enzyme X that is located in both the plastids and the cytosol. We will describe how to use an amyloplast isolation protocol to determine the how much of the activity of X is plastidial. In principle, the same method could be used to determine the distribution of an enzyme between the plastids and any other compartment for which marker enzymes distributions can be determined (see Denyer and Smith, 1988).

Using the data for the average distributions of plastidial and cytosolic marker enzymes (Support Protocol 1), the percentage of the total activity of X that is plastidial can be calculated. This calculation relies upon the fact that the distribution of plastidial X between the supernatant and pellet fractions will be the same as that of the plastid markers and the distribution of cytosolic X will be the same as that of the cytosolic markers. Additionally, the total activity of X in the pellet will be the sum of plastidial X in the pellet plus cytosolic X in the pellet. In theory, the calculation is not influenced by the yield of plastids, provided that the distributions of cytosolic and plastidial marker enzymes are significantly different statistically. The accuracy of the calculation depends on there being no loss of enzyme activity during the amyloplast isolation procedure. It is important therefore to establish that the sum of the activity of each enzyme in the pellet plus supernatant fraction equals that in the initial homogenate.

Materials

Aliquots of homogenate, supernatant, and pellet fractions (see Basic and Alternate Protocols)

Assay mix for enzyme X (the enzyme of interest; see Table 3.28.1)

Additional reagents and equipment for determining yield and purity of amyloplasts (Support Protocol 1) and assays of amyloplast marker enzymes (see references in Table 3.28.1)

1. Measure the distributions of plastid and cytosolic marker enzymes and enzyme X in the homogenate, supernatant, and pellet as in Support Protocol 1.
2. Calculate the average distributions of the plastid marker enzymes and the cytosolic marker enzymes.
3. Calculate the activity of enzyme X in the pellet as a percentage of that in the supernatant plus pellet.
4. Solve the following equation:

$$\%X \text{ in plastids} =$$

$$[(\%X \text{ in pellet} - \%CM \text{ in pellet})/(\%PM \text{ in pellet} - \%CM \text{ in pellet})] \times 100$$

where PM = plastidial marker and CM = cytosolic marker.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Amyloplast isolation medium 1 (AIM 1)

50 mM HEPES
0.8 M sorbitol
1 mM disodium EDTA
1 mM KCl
2 mM $MgCl_2$
Adjust to pH 7.5 with NaOH
Store at 4°C for up to 1 week
On the day of use add:
1 g/liter bovine serum albumin (BSA)
2 mM dithiothreitol (DTT)

Amyloplast isolation medium 2 (AIM 2), 2× and 1×

40 mM HEPES
1 M sorbitol
20 mM KCl
2 mM $MgCl_2$
2 mM disodium EDTA
20% (v/v) ethanediol
Adjust to pH 7.4 with NaOH
Store at 4°C for up to 1 week
On the day of use add:
10 g/liter bovine serum albumin (BSA)
5 mM dithiothreitol (DTT)
1× AIM 2:
Dilute 2× AIM 2 1:1 with water before use.

COMMENTARY

Background Information

The first plastids to be isolated successfully were chloroplasts. Some of the techniques developed for chloroplasts were later also used for amyloplast isolation. For example, the use of sorbitol as an osmoticum, which is widespread in amyloplast isolation protocols today, was first used in chloroplast isolation protocols (Walker, 1964). Sorbitol was considered superior to sugars as an osmoticum since there is less chance of it being metabolized (Walker, 1971).

Initial attempts to separate chloroplasts from other cellular organelles used sucrose density gradients. Density gradients were also used subsequently for amyloplast isolation. In the earliest protocols, the extracts containing plastids were loaded onto the gradient and centrifuged until the organelles reached a position where the density of the gradient was equal to their own. For chloroplasts, however, this method was not very successful, since the density of chloroplasts is little different from that of many other organelles. Thus, the chloroplasts prepared by such methods were highly contaminated. An improvement on this technique was made by Miflin and Beevers (1974), and was based on the observation that chloroplasts sedimented in sucrose gradients more rapidly than other organelles (probably because of their starch content). They used much shorter centrifugation times, such that few organelles other than the chloroplasts attained their equilibrium positions in the gradient. Early attempts to isolate amyloplasts involving density gradient centrifugation used high-density reagents such as Urografin (Duffus and Rosie, 1975; Gaynor and Galston, 1983), Nycodenz (Entwistle and ap Rees, 1988), or Ficoll (Echeverria et al., 1985), rather than sucrose.

Few of these early amyloplast isolation experiments used marker enzymes to measure the yield, purity, and intactness of the amyloplasts. Sometimes only qualitative assessment of the plastids by microscopy was used, and plastid-free starch granules, rather than intact amyloplasts, may have been the main product. An exception was the work of MacDonald and ap Rees (1983), who isolated amyloplasts from soybean cultures. These amyloplasts were shown to be substantially free from contamination (using marker enzymes) and intact (as determined by latency and protection experiments). This work set the standard for later attempts to isolate amyloplasts from other tissues.

The isolation of starch-rich amyloplasts from cereal endosperm was achieved using methods based on those developed for soybean plastids (MacDonald and ap Rees, 1983). As with the soybean amyloplasts, the cereal endosperm amyloplast isolations used protoplasts as the starting material (Echeverria et al. 1985, 1988; Entwistle and ap Rees, 1988; Tyson and ap Rees, 1988). Interestingly, ADP-glucose pyrophosphorylase was used as one of the marker enzymes for the cereal endosperm plastids, having been shown earlier to be confined to plastids in soybean (MacDonald and ap Rees, 1983). Later it was discovered that, in cereal endosperm, most of the activity of this enzyme is cytosolic (Denyer et al., 1996), and so now we know that, for cereal endosperm, this is not an appropriate enzyme to use as a plastid marker.

A significant simplification to the plastid isolation procedures was made by Emes and England (1986) for the purification of pea root plastids. Instead of using density gradient centrifugation, they used a brief, low-speed centrifugation step to remove cell debris, followed by centrifugation of the resulting supernatant containing plastids through a layer of Percoll. Unfortunately, this method is not suitable for the isolation of starch-rich amyloplasts, since they would sediment in the first centrifugation step. The pea embryo amyloplast protocol developed by Denyer and Smith (1988) represented a further simplification of the method of Emes and England (1986), and it is suitable for starch-rich amyloplasts. A single centrifugation step is used to sediment the amyloplasts directly from the homogenization medium. This method was later used to isolate amyloplasts from maize endosperm (Denyer et al., 1996), and similar methods, involving the isolation of plastids by sedimentation from the homogenization medium, were used to isolate amyloplasts from potato tuber (Mohabir and John, 1988), wheat endosperm (Tetlow et al., 1993), and oilseed rape embryos (Alternate Protocol; Kang and Rawsthorne, 1994). These one-step methods of amyloplast isolation have the advantage that, as well as being quick and simple, they give high yields of amyloplasts. However, their disadvantage is that the amyloplasts produced are not completely free of contamination from other cellular compartments. They should be regarded as fractions enriched in amyloplasts rather than as purified amyloplasts. Enriched fractions are suitable for many experiments, including

enzyme localization (see Support Protocol 4). If required, preparations of greater purity can be generated by washing the plastids by resuspension and re-centrifugation as in the Alternate Protocol, or by subjecting the plastids to density gradient centrifugation (e.g., Neuhaus et al., 1993). However, the price for greater purity is a decrease in yield, and it is necessary to question whether or not the properties of the few purified plastids obtained are similar to those of the bulk of the plastids in the original tissue.

Critical Parameters

The starch content of plastids has a critical influence on their yield, low starch content favoring higher yields. Thus, the choice of starting material is an important consideration. We have found that the highest yields of amyloplasts from cereal endosperm are obtained from endosperms harvested at an early stage in seed development, before they attain maximum starch content. The accurate analysis of the yield and purity of the amyloplast preparation is also critical. This depends upon the appropriate use of marker enzymes. We recommend using two marker enzymes for each subcellular compartment of interest. The distributions of the two markers should be in close agreement. A list of marker enzymes likely to be suitable for amyloplast isolation from a range of different plant organs is given in Table 3.28.1. The original references describing the methods are given. To adapt these assay methods for your plant extracts, we recommend that you check that the pH and the concentrations of all substrates and effectors are optimal for activity with extracts of your plant material and that the activity is linear with respect to both assay time and the amount of extracted added.

Other important parameters include the following:

1. Minimizing the time taken from harvest of the plant material to completion of the last assay will improve the yield of plastids and minimize loss of enzyme activity.
2. All transfers of intact plastids should be done gently and smoothly to avoid plastid rupture.
3. The osmotic strength of the medium in which the plastids are suspended should be optimized, and abrupt changes in osmotic strength, pH, and temperature should be avoided.
4. Detergent should be avoided—even traces on glassware can lead to plastid rupture.

Anticipated Results

Values for yield, intactness, and contamination of maize endosperm and oilseed rape amyloplast preparations obtained previously using the methods described here are shown in Table 3.28.2. Reported values for plastids made from a range of other plant organs are also shown for comparison.

Time Considerations

The isolation and purification of amyloplasts from maize endosperm requires 1 to 2 hr and from oilseed rape embryos 3 to 4 hr. Analysis of yield, purity, and intactness takes 1 to 2 hr.

Table 3.28.2 Published Values for Yield, Intactness, and Contamination of Amyloplast Preparations[a]

Reference	Plant organ	Yield of plastids	Intactness	Contamination with cytosol
MacDonald and ap Rees (1983)	Suspensions cultures of soybean	23%	44%-74%	0.2%
Denyer and Smith (1988)	Pea cotyledons	43%	65%-67%	3.6%
Neuhaus et al. (1993)	Various heterotrophic plant tissues	ND	89%-96%	0.6%-1.5%
Tetlow et al. (1993)	Wheat endosperm	23%	50%	<0.7%
Kang and Rawsthorne (1994)	Oilseed rape	10%	63%-65%	3.9%
Denyer et al. (1996)	Maize endosperm	24%-47%	ND	0.7%-1.4%

[a] Abbreviations: ND, not determined.

Literature Cited

Appelmans, R., Wattiaux, R., and de Duve, C. 1954. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem. J.* 59:438-445.

Bonner, W.D. 1953. Succinate dehydrogenase. *Methods Enzymol.* 1:722-727.

Bowles, D.J. and Kauss, H. 1976. Characterization, enzymatic and lectin properties of isolated membranes from *Phaseolus aureus*. *Biochim. Biophys. Acta* 443:360-374.

Denyer, K. and Smith, A.M. 1988. The capacity of plastids from developing pea cotyledons to synthesize acetyl CoA. *Planta* 173:172-182.

Denyer, K., Dunlap, F., Thorbjornsen, T., Keeling, P., and Smith, A.M. 1996. The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra- plastidial. *Plant Physiol.* 112:779-785.

Duffus, C.M. and Rosie, R. 1975. Purification and fractionation of potato amyloplasts. *Anal. Biochem.* 65:11-18.

Echeverria, E., Boyer, C., Kang-Chien Lie., and Shannon, J. 1985. Isolation of amyloplasts from developing maize endosperm. *Plant Physiol.* 77:513-519.

Echeverria, E., Boyer, C., Thomas, P.A., Kang-Chien Lie., and Shannon, J. 1988. Enzyme activities associated with maize kernel amyloplasts. *Plant Physiol.* 86:786-792.

El-Shora, H.M. and ap Rees, T. 1991. Intracellular location of $NADP^+$-linked malic enzyme in C_3 plants. *Planta* 185:362-367.

Emes, M.J. and England, S. 1986. Purification of plastids from higher plant roots. *Planta* 168:161-166.

Entwistle, G. and ap Rees, T. 1988. Enzymatic capacities of amyloplasts from wheat (*Triticum aestivum*) endosperm. *Biochem. J.* 255:391-396.

Gaynor, J.J. and Galston, A.W. 1983. Purification and characterization of amyloplasts from etiolated epicotyls of *Pisum sativum*. *Plant Cell Physiol.* 24:411-421.

Gross, P. and ap Rees, T. 1986. Alkaline inorganic pyrophosphatase and starch synthesis in amyloplasts. *Planta* 167:140-145.

Hansen, R.G., Albrecht, G.J., Bass, S.T., and Seifert, L.L. 1965. UDP-Glucose pyrophosphorylase (crystalline) from liver. *Methods Enzymol.* 8:248-253.

Hatch, M.D. 1978. A simple spectrophotometric assay for fumerate hydratase in crude tissue extracts. *Anal. Biochem.* 85: 271-275.

Holtum, J.A.M. and Winter, K. 1982. Activity of enzymes of carbon metabolism during the induction of crassulacean acid metabolism in *Mesembrynthemum crystallinum* L. *Planta* 155:8-16.

Journet, E.-P. and Douce, R. 1985. Enzymatic capacities of purified cauliflower bud plastids for lipid synthesis and carbohydrate metabolism. *Plant Physiol.* 79:458-467.

Kang, F. and Rawsthorne, S. 1994. Starch and fatty acid synthesis in plastids from developing embryos of oilseed rape (*Brassica napus* L.). *Plant J.* 6:795-805.

Macdonald, F.D. and ap Rees, T. 1983. Enzymatic properties of amyloplasts from suspension cultures of soybean. *Biochem. Biophys. Acta* 755:81-89.

Miflin, B.J. and Beevers, H. 1974. Isolation of intact plastids from a range of plant tissues. *Plant Physiol.* 53:870-874.

Mohabir, G. and John, P. 1988. Effect of temperature on starch synthesis in potato tuber tissue and in amyloplasts. *Plant Physiol.* 88:1222-1228.

Neuhaus, H.E., Batz, O., Thom, E., and Schiebe, R. 1993. Purification of highly intact plastids from various heterotrophic plant tissues: Analysis of enzymatic and precursor dependency for starch biosynthesis. *Biochem. J.* 296:395-401.

Stitt, M. and ap Rees, T. 1979. Capacities of pea chloroplasts to catalyse the oxidative pentose phosphate pathway and glycolysis. *Phytochemistry* 18:1905-1911.

Stitt, M., Bulpin, P.V., and ap Rees, T. 1978. Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. *Biochem. Biophys. Acta* 544:200-214.

Tetlow, I.J., Blissett, K.J., and Emes, M.J. 1993. A rapid method for the isolation of purified amyloplasts from wheat endosperm. *Planta* 189:597-600.

Tyson, R.H. and ap Rees, T. 1988. Starch synthesis by isolated amyloplasts from wheat endosperm. *Planta* 175:33-38.

Walker, D.A. 1971. Chloroplasts (and grana): Aqueous (including high carbon fixation ability). *Methods Enzymol.* 23:211-220.

Walker, D.A. 1964. Improved rates of carbon dioxide fixation by illuminated chloroplasts. *Biochem. J.* 92:22-23

Wedding, R.T. and Kline, K. 1994. Comparative studies of coupled assays for phosphoenolpyruvate carboxylase. *Physiol. Plant.* 92:197-200.

Isolation of Microtubules and Microtubule Proteins

J. Avila,[1] H. Soares,[2] M.L. Fanarraga,[3] and J.C. Zabala[3]

[1]Centro de Biología Molecular (CSIC). Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain
[2]Instituto Gulbenkian de Ciência, 2781-901, Oeiras, and Escola Superior de Tecnologia da Saúde de Lisboa, Lisboa, Portugal
[3]Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria-IFIMAV, Santander, Spain

ABSTRACT

This unit describes various protocols for the isolation and purification of the main constituents of microtubules, chiefly α- and β-tubulin, and the most significant microtubule associated proteins (MAPs), specifically MAP1A, MAP1B, MAP2, and tau. We include a classical isolation method for soluble tubulin heterodimer as the first basic purification protocol. In addition, we show how to analyze the tubulin and MAPs obtained after a phosphocellulose chromatography purification procedure. This unit also details a powerful and simple method to determine the native state of the purified tubulin based on one-dimensional electrophoresis under nondenaturing conditions (*UNIT 6.5*). The last protocol describes the application of a new technique that allows visualizing the quality of polymerized microtubules based on atomic force microscopy (AFM). *Curr. Protoc. Cell Biol.* 39:3.29.1-3.29.28. © 2008 by John Wiley & Sons, Inc.

Keywords: tubulin • microtubule • MAP1A • MAP1B • MAP2 • tau • native gel electrophoresis • atomic force microscopy

INTRODUCTION

The different morphologies displayed by cells from different tissues—for example the great diversity of neurons—is the result of the assembly of different cell scaffolds.

There are three different components in the cell scaffold known as the cytoskeleton. These are microtubules (Fig. 3.29.1), microfilaments, and intermediate filaments. Microtubules are particularly abundant in brain tissue and their components represent ∼20% of the total protein content from soluble brain extract, with α- and β-tubulin heterodimers the major components.

Microtubules are tubular cytoplasmic fibers with a diameter of 24 nm (Fig. 3.29.2). In addition to their role in the determination of cell shape they also play fundamental roles in other common cellular functions, such as intracellular transport, chromosome segregation (a specific form of transport), and flagella/cilia-mediated cell motility. In neurons, besides their function in determining neuron shape, microtubules seem to play essential roles in the formation and maturation of the axon and dendrites. The majority, if not all, of the microtubule functions are based on the capacity of these polymers to polymerize/depolymerize. The capacity of microtubules for assembly-disassembly was mimicked in vitro in the pioneer work of Weisenberg (1972), who reproduced conditions for the assembly of these polymers using a brain cell extract. In this work it was established that microtubule polymerization depends on the amount of tubulin and that, at least in vitro, a critical concentration of this protein is needed for polymerization into microtubules. Since there is a large amount of tubulin in a brain cell extract, it is possible

Current Protocols in Cell Biology 3.29.1-3.29.28, June 2008
Published online June 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0329s39

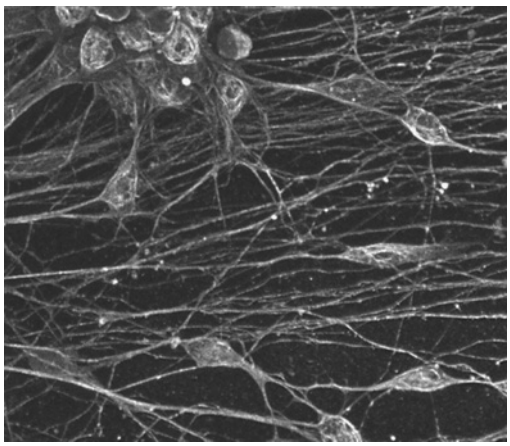


Figure 3.29.1 Microtubule networks of cerebellar cultured granule cells, as visualized by immunofluorescence microscopy using a specific antibody against the α-tubulin subunit (DM1A).

Figure 3.29.2 Electron micrograph of brain microtubules assembled after a single polymerization cycle. Scale bar represents 1 μm. For color version of this figure see *http://www.currentprotocols.com.*

to induce microtubule assembly in vitro. In addition, Weisenberg also found that the presence of GTP and the absence of calcium were both needed to properly carry out microtubule assembly. These observations served as the basis of various methods for microtubule protein purification (Borisy and Olmsted, 1972; Shelanski et al., 1973). At that time it was also found that microtubules are induced to assemble at warm temperatures. In contrast to tubulins from Antarctic fishes that polymerize at low temperatures, mammalian microtubules are not cold-stable and depolymerize very fast at 4°C. The observed temperature dependence seems to reside in differences in the polymerization energetics and kinetics of specific tubulin polypeptides. Thus, warming a cytosolic brain extract promotes microtubule polymerization, and the microtubules can be easily collected by centrifugation. But, because in vitro–polymerized mammalian microtubules depolymerize in the cold, after centrifugation, collected microtubules can be depolymerized using a cold buffer. After depolymerization, the solution can again be centrifuged to remove contaminants and those microtubule fragments that are not competent for depolymerization.

This sequence of warm-polymerization and cold-depolymerization can be repeated two or more times until the desired degree of purity of the microtubules is achieved.

The quality and quantity of tubulins and proteins associated with microtubules (MAPs) obtained can be followed by SDS-PAGE analysis (*UNIT 6.1*). From this analysis it is possible to observe the main microtubule components—two polypeptides or subunits, α- and β-tubulin (for reviews see: Vallee et al., 1986; Matus, 1988; Avila, 1990). MAPs are classified in three major families. The first protein family comprises the MAP1 polypeptides: MAP1A, MAP1B, and MAP1C (or dynein, a motor protein). The second family is composed of four structurally related proteins derived from a single gene: MAP2A, MAP2B, MAP2C, and MAP2D. The third family, present in a lower amount, is composed of several related polypeptides (tau proteins), or isoforms expressed from a unique gene. All these MAPs preferentially bind to the C-terminus of tubulins (Serrano et al., 1984a,b, 1986).

In this unit, we describe protocols for the purification of tubulins (Basic Protocols 1 and 3 and Alternate Protocol 2), MAP1 (Basic Protocol 4), MAP2 (Basic Protocol 5), and tau proteins (Basic Protocol 6), but not for other MAPs or motor proteins (such as dynein). We also describe how to assemble microtubules in vitro in the presence of paclitaxel (Taxol; Basic Protocol 2 and Alternate Protocol 1) and protocols for native gel electrophoresis (Support Protocol 1) and atomic force microscopy (AFM; Support Protocol 2).

ISOLATION OF MICROTUBULE PROTEIN BY CYCLES OF POLYMERIZATION AND DEPOLYMERIZATION

BASIC PROTOCOL 1

Many similar microtubule protein purification methods have been developed, published, and used for the purification of microtubule proteins (Shelanski et al., 1973; Williams and Lee, 1982; Vallee, 1986a). Most of these are based on the reversible assembly capacity of microtubule proteins in the presence or in the absence of assembly-promoting agents, such as glycerol or dimethyl sulfoxide (DMSO). Here we describe the isolation of brain microtubule proteins by temperature-dependent cycles of polymerization-depolymerization using glycerol and DMSO in the first and second polymerization cycles, respectively (Fig. 3.29.3). Figure 3.29.3 illustrates the results of the step-by-step protocol that we follow in our laboratories. This method can be applied to brain tissues from different sources, i.e., porcine, bovine, rat, etc. The protocol is appropriate for a medium-scale preparation (1 to 3 cow or pig brains).

Materials

One to three brains (100 to 500 g total start-up tissue; the weight of a pig brain is ~100 g)
Phosphate-buffered saline, pH 6.7 (PBS; *APPENDIX 2A*), 4°C
Isotonic buffer (see recipe), 4°C
Protease inhibitors (see recipe)
Buffer A (see recipe), 4°C
Glycerol
PMSF
GTP (see recipe)
Dimethyl sulfoxide (DMSO)

Plastic wrap
Ice-water bath
No.10 scalpel blade
500-ml beaker
Potter homogenizer (Teflon-in-glass homogenizer), as large as possible
Centrifuge (if possible two centrifuges, one at 4° to 6°C and the other at 25° to 30°C): Sorvall RC-5B or equivalent Beckman J2, J21 series, or Avanti J-25

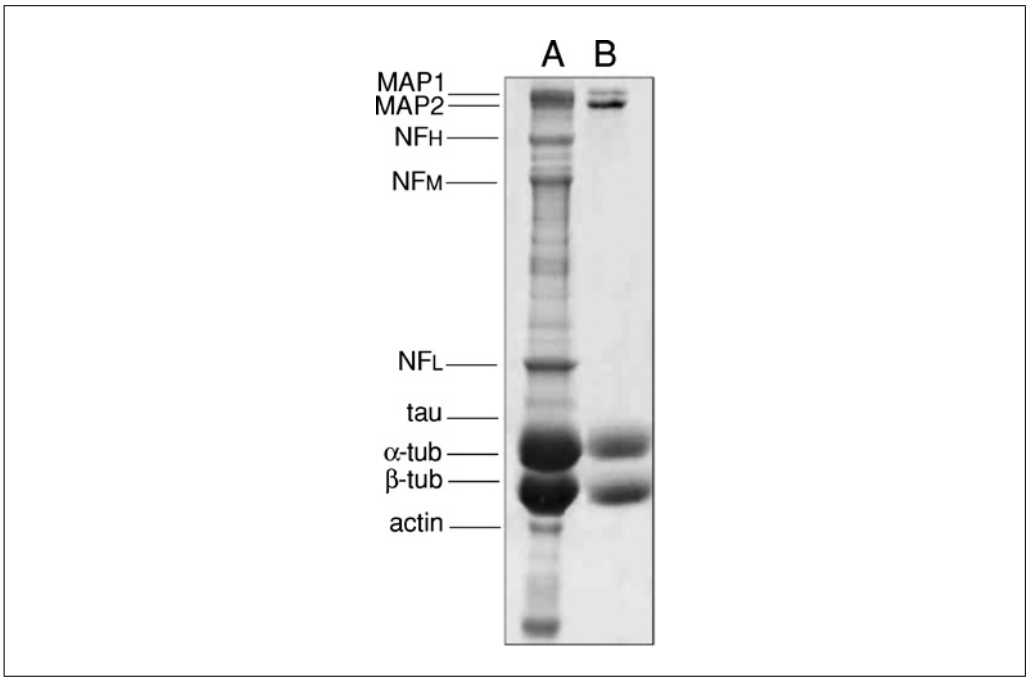


Figure 3.29.3 Characterization of pelleted protein after one (**A**), and three polymerization cycles. (**B**) In (A), the presence of MAP1, MAP2, neurofilament proteins (NF_H, NF_M and NF_L), tau protein, tubulin subunits, and actin are indicated. The gel was stained with Coomassie blue as explained in *UNIT 6.6.*

Centrifuge rotors: GSA or JA-10; type 50.2Ti and 70.1 Ti for the Beckman
Graduated flask
26.3-ml ultracentrifuge tubes
35°C rocking incubator
Ultracentrifuge Beckman L8-70, Optima XL-100 or 120

Additional reagents and equipment for measuring protein concentration (*APPENDIX 3B*)

Obtain the brains

1. Make an appointment with veterinarians at the slaughterhouse and request fresh brains to be picked up immediately after animal slaughter.
2. Wrap brains carefully with plastic before introducing in the ice-water bath. Place brains on ice during transport to the laboratory (<1 hr in our case). After arriving at the laboratory start the preparation of the cytosolic extracts (immediately).

Prepare brains

3. Rinse brains well, three times, each time with 100 ml cold PBS buffer and keep them cold throughout the following steps until the first polymerization cycle.
4. Remove the meninges, the blood clots, and most of the peripheral blood vessels from the brain.
5. Chop the brains into smaller pieces (1- to 2-cm) using a no. 10 scalpel blade.
6. Weigh the tissue in a tared 500-ml cold beaker.

Homogenize the brain tissue

7. Add 0.75 to 1 ml of isotonic buffer per gram of tissue.

   The use of the isotonic buffer for tissue homogenization facilitates the removal of neurofilaments in the following microtubule isolation process.

8. Add PMSF to a final concentration of 1 mM. Add aprotinin, leupeptin, and pepstatin to a final concentration of 1 μM each (see recipe for protease inhibitors in Reagents and Solutions).

9. Disrupt brain tissues at 4°C by using a Potter homogenizer, which allows a fast and reproducible homogenization.

   Use the largest Potter you have and homogenize as many times as required depending on the total volume (see Critical Parameters).

10. Clear the crude brain homogenate by centrifuging 30 min at 25,000 × *g*, 4°C.

    If necessary, since the volume is too high, a rotor type GSA (Dupont-Sorvall) or equivalent JA-10 in a Beckman J2-21, or Avanti J-25 centrifuge can be used.

11. Decant the supernatant in a cold 500-ml beaker and, if necessary for further clarification, centrifuge again for 30 to 45 min at 25,000 × *g*, 4°C.

12. Discard the pellet and take the supernatant. Take a small aliquot for analysis in SDS-PAGE (*UNIT 6.1*), native electrophoresis (Support Protocol 1), and for protein determination (*APPENDIX 3B*).

    From an initial 100 g we usually get 150 ml at 40 mg/ml at this point, and, of this amount, ~20% represents microtubule proteins.

13. Measure the total volume of the cytosolic extract in a graduated flask and make it 1× buffer A by adding for each 90 ml of extract, 10 ml of 10× buffer A. Add glycerol to a final concentration of 25%(v/v) to 30%(v/v) (Krauhs et al., 1981).

    Be aware that some commercial glycerol preparations have a concentration of 87%.

14. Add PMSF to a final concentration of 1 mM to prevent protein degradation by nonspecific proteolysis. Finally, add 1 M GTP (1000×) to a final concentration of 1 mM.

Perform first polymerization

15. Transfer the solution to 26.3-ml ultracentrifuge tubes and balance them to ±0.01 g. Incubate solution for 30 min at 35°C with gentle agitation.

16. Ultracentrifuge the mixture 45 min at 100,000 × *g*, 25°C, using a prewarmed (30°C) rotor.

    We use the 50.2 Ti rotor in a Beckman centrifuge.

17. Discard the supernatant and take the pellet, which should be translucent.

    If necessary, pellets can be frozen at −80°C at this point without much loss of function of the polymerized tubulin. We usually get 300 to 400 mg protein at this point, determined by Lowry's method.

Perform first depolymerization

18. Resuspend the pellet in cold buffer A at 10 to 20 mg/ml. Let the mixture to stand in an ice-water bath for 30 min.

    Intermittent homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate microtubule depolymerization.

19. Centrifuge 15 to 20 min at 25,000 × *g*, 4°C using a precooled rotor Beckman 50.2 Ti. Discard the pellet and keep the supernatant.

    The pellet at this point is enriched in the so-called cold-resistant microtubule fraction and denatured protein. It is not advisable to centrifuge at very high speed, to avoid loss of "competent" microtubule proteins.

20. Add PMSF and GTP to the supernatant to a final concentration of 1 mM (add 1/1000 volume of 1 M GTP). Add DMSO to a final 10% concentration.

CAUTION: DMSO is hazardous: see APPENDIX 2A for guidelines on handling, storage, and disposal.

Perform second polymerization

21. Transfer the solution to 26.3-ml ultracentrifuge tubes and balance them to within ±0.01 g. Incubate the tubes 30 min at 35°C with gentle agitation.

22. Collect the microtubules polymerized in the second cycle by ultracentrifuging 40 min at 180,000 × *g* (Beckman 70.1 Ti at 50,000 rpm), 25° to 30°C.

If at this point there is only one rotor available, wash the rotor with hot water to rapidly reach the appropriate temperature.

23. Discard the supernatant. To finish the second cycle resuspend the pellet in 6 ml of cold 0.5× buffer A. Let the mixture stand in an ice-water bath for 30 min.

The pelleted protein can be frozen in liquid nitrogen or at −80°C for further use. We usually get 60 to 80 mg protein at this point.

Occasional homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate depolymerization of microtubules.

24. Centrifuge 20 min at 25,000 × *g*, 4°C, using a precooled rotor. Discard the pellet. Measure protein concentration in the supernatant by using a spectrophotometer absorbance at 280 nm and 260 nm. (*APPENDIX 3B*)

The extinction coefficient of tubulin at 280 nm is 115,000 $M^{-1}cm^{-1}$ [1.15 $(mg/ml)^{-1}$ cm^{-1}]. This extinction coefficient is calculated from tubulin sequences and includes the contribution of the two bound guanine nucleotides to the absorbance at 280 nm. For this reason, we use the formula $A_{280}/1.15$ = mg/ml of the sample. Also, this formula might be improved if we take into account the contribution of nucleotides to the absorbance at 260 nm: $1.45 \times A_{280} - 0.74 \times A_{260}$ = mg/ml of tubulin. The isolated tubulin competent to polymerize can be stored at −80°C for several months or until further steps.

BASIC PROTOCOL 2

ISOLATION OF MICROTUBULE PROTEIN USING PACLITAXEL (TAXOL)

An alternative single-step method to isolate polymerized microtubule proteins is based on the fact that microtubules assemble and remain stable in the presence of paclitaxel (Shiff et al., 1979). This allows one to sediment them and to resuspend them in a cold buffer without depolymerization (Borisy and Olmsted, 1972). Moreover, an advantage of this procedure is the fact that MAPs can be easily dissociated from these stable microtubules by exposing them to an elevated ionic strength. Additionally, the use of paclitaxel permits the purification of tubulin from sources where the amount of this protein is low.

Materials

Bovine or porcine brain tissue (10 g)
Phosphate-buffered saline (*APPENDIX 2A*)
Buffer A (see recipe)
Protease inhibitors (see recipe)
Paclitaxel (Taxol; see recipe)
GTP (see recipe)
PMSF
Sucrose underlayer solution (1 ml per centrifuge tube containing 10% sucrose, 10 μM paclitaxel, and 0.5 mM GTP)
Scalpel blade, No.10
500-ml beaker

Potter homogenizer (Teflon-in-glass homogenizer)
Centrifuge rotors: GSA or JA-10. Type 50.2Ti and 70.1 Ti for the Beckman Ultracentrifuge Beckman L8-70, Optima XL-100 or 120
13.5-ml ultracentrifuge tubes
30°C rocking incubator
Pasteur pipet

Prepare cytosolic extract

1. Obtain fresh brains from the slaughterhouse as described in Basic Protocol 1.
2. Place and keep brains on ice until arrival at the laboratory, as described in Basic Protocol 1.

   Although we use fragments of any region of porcine brain, whole brains from other sources can also be used.

3. Rinse brains well, three times, each time with 100 ml cold PBS buffer and keep them cold throughout the following steps until the first polymerization cycle.
4. Remove the meninges, the blood clots, and most of the peripheral blood vessels from the brain.
5. Chop the brains into smaller pieces (1 to 2 cm) using a scalpel blade, no. 10. Weigh the tissue in a tared 500-ml cold beaker.
6. Add 15 ml buffer A (1.5 ml per g of tissue).
7. Disrupt brain tissues at 4°C by using a Potter homogenizer.

   Follow recommendations in Basic Protocol 1.

8. Add freshly prepared PMSF to a final concentration of 1 mM in DMSO. Also add the following protease inhibitors to a final concentration of 1 μM: aprotinin, leupeptin and pepstatin (see recipe for protease inhibitors in Reagents and Solutions).
9. Clear the crude brain homogenate by ultracentrifuging 40 min at 100,000 × *g*, 4°C. Use a precooled rotor (Beckman 50.2 Ti or similar).

Polymerize microtubules

10. Discard the pellet and add paclitaxel to the supernatant to a final concentration of 20 μM, GTP to a final concentration of 1 mM, and PMSF to a final concentration of 1 mM.
11. Transfer the solution to 13.5-ml ultracentrifuge tubes and balance them to within ±0.01 g. Incubate at 30 min at 30°C with gentle agitation.
12. With a Pasteur pipet introduce 1 ml of a 30°C prewarmed sucrose solution very gently at the bottom of the tube.
13. Collect the assembled microtubules in the polymerization cycle by ultracentrifuging 30 min at 180,000 × *g* (prewarmed rotor Beckman 70.1 Ti), 25° to 30°C.
14. Discard the supernatant and keep the translucent pellet. Resuspend pellet in 3 ml buffer A containing 20 μM paclitaxel and 1 mM GTP.

    These microtubule preparations, containing assembled microtubules, can be used as a source for isolating MAPs, such as MAP1B (see below) or they can be stored in the refrigerator (4°C). You must be aware that storage for >1 week will enrich the preparation in unpolymerized tubulin. To store samples for longer periods keep the preparation at −80°C.

ALTERNATE PROTOCOL 1

MICROTUBULE POLYMERIZATION IN THE PRESENCE OF PACLITAXEL

??

Microtubule proteins (tubulin and MAPs) are prepared by repeated cycles of assembly/disassembly using a temperature-dependent procedure as described in Basic Protocol 1. Purified microtubule heterodimers are incubated 30 to 40 min with 20 to 25 μM paclitaxel (taxol) at 35°C in BRB80 buffer (see recipe) containing 1 mM GTP and 30% glycerol. The pellet corresponding to the polymerized microtubules is obtained by centrifuging 30 min at 45,000 × *g*, 30°C through a 50% sucrose cushion containing 1 mM GTP.

BASIC PROTOCOL 3

ISOLATION OF TUBULIN DIMERS FROM MICROTUBULE PROTEINS

Tubulin contains very acidic regions, mainly in its C-terminal domain (Krauhs et al., 1981), which allows its fractionation from many other proteins by ion-exchange chromatography (anionic or cationic). Two types of resins have been successfully used to purify tubulin: cationic resins such as DEAE-cellulose (or DEAE-Sephadex) and anionic resins like phosphocellulose. However, it is possible to use any other type of ion-exchange resins such as Mono-Q or Mono-S from Pharmacia, or HiTrap columns. Nevertheless, all these chromatographic columns show advantages and disadvantages. For example, while the use of DEAE-cellulose is a slow procedure, the use of phosphocellulose columns might result in a partial inactivation of tubulin due to magnesium ion chelation (Williams and Detrich, 1979). Likewise, highly purified tubulin can be obtained from cationic columns, but the fact that tubulin binds to these resins requires the use of a salt gradient to elute the protein, which might result in tubulin denaturation. Therefore, tubulin elution from these columns requires a further step of salt removal by dialysis or ultrafiltration. In this protocol we describe the classical procedure to purify tubulin using phosphocellulose chromatography. This method has been chosen because it is the fastest and does not need the additional dialysis step that could also result in a partial denaturation of tubulin.

Materials

Resin: Whatman P11 Cellulose Phosphate
0.5 M NaOH
0.5 M HCl
Buffer A (see recipe)
50 mg of porcine brain microtubules stored at −80°C or in liquid nitrogen (Basic Protocol 1)

Buchner funnel
XK50/20 (Length 20 cm, i.d. 50 mm; Pharmacia) column including:
  Thermostat jacket
  Flow adaptor
  Flanged tubing at both ends for direct connection to valves
  Pumps
  UV monitors if using an FPLC or an AKTA system
Teflon-in-glass homogenizer
Ice-water bath
Ultracentrifuge

Additional reagents and equipment for isolating microtubule proteins (Basic Protocol 1), determining protein concentration (*APPENDIX 3B*), SDS-PAGE (*UNIT 6.1*), and nondenaturing gel electrophoresis (Support Protocol 1)

Precycle the ion exchanger

1. Take 100 g of resin and stir into 1.5 liter of 0.5 M NaOH for 30 min at room temperature. Filter the resin in a Buchner funnel and wash with water several times.

   Alternatively, stir the resin in a beaker and let it settle by gravity for a few minutes and decant the supernatant.

2. After the wash, treat the resin with 1.5 liter of 0.5 M HCl as described above. Filter or decant and wash several times with water.
3. Repeat this procedure until a neutral pH is attained.
4. Next, filter and stir the resin in 0.5× buffer A.
5. Add the resin to an XK 50 column (~150 ml) and equilibrate with 0.5× buffer A until the absorbance at 280 nm returns to baseline.

Prepare sample

6. Collect microtubule proteins (10 mg/ml), isolated following Basic Protocol 1, by depolymerization of microtubules preparing a homogenate in cold 0.5× buffer A with the help of a Teflon-in-glass homogenizer.
7. Leave homogenized sample in ice-water for 30 min.

   Intermittent homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate microtubule depolymerization.

8. Ultracentrifuge the homogenate 15 min at 25,000 × *g*, 4°C, using a precooled rotor. Discard the pellet and keep the supernatant.

   We currently use 50.2 Ti or 70.1 Ti rotors in a Beckman centrifuge in our laboratory.

9. Adjust the final protein concentration of the supernatant to ~10 mg/ml and load it onto a prepacked XK 50 phosphocellulose column (1 ml column/mg protein) equilibrated in 0.5× buffer A.

   The first time you use the resin you must take into account that you will probably lose some tubulin. This is due to the fact that some will irreversibly bind to the phosphocellulose. This could be overcome or minimized by previously loading in the column with 1 g of BSA diluted in the same buffer, followed by several washes with the salt-containing buffer before re-equilibration.

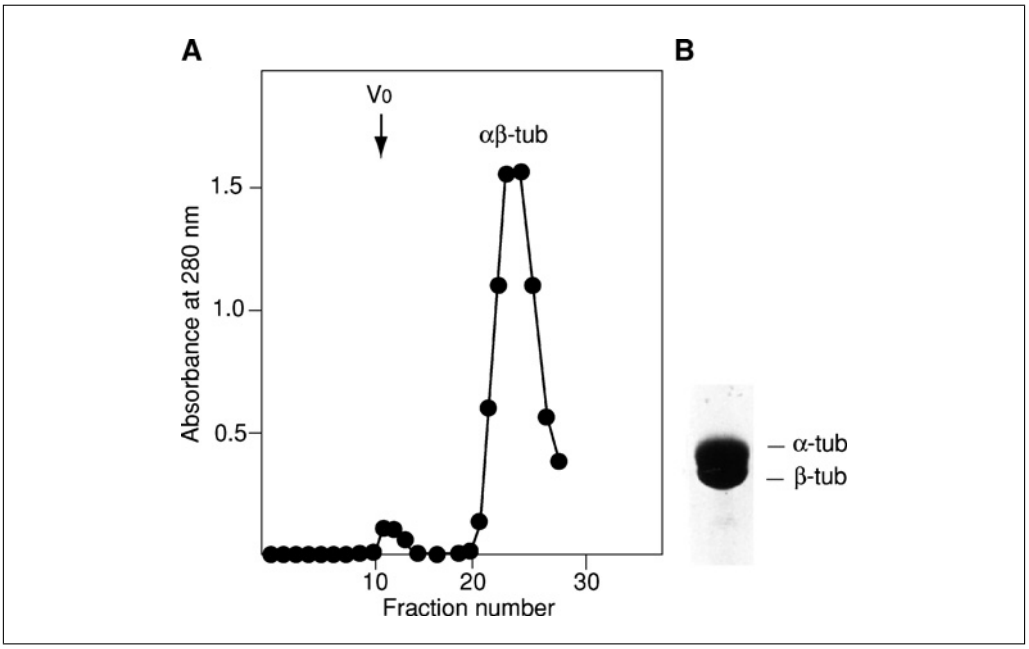


Figure 3.29.4 (**A**) Chromatographic elution profile obtained at 280 nm of absorbance from a gel filtration analysis of phosphocellulose-purified tubulin chromatographed through a Sepharose 4B column. (**B**) Denaturing SDS gel (8.5% polyacrylamide) analysis of the peak corresponding to a molecular mass of 110 kDa eluted from the column. The gel was stained with Coomassie blue as explained in *UNIT 6.6.* Abbreviations: V_o, void volume.

10. Collect 0.5-ml fractions from the column.

    Tubulin, which will be present in the first fractions after the void volume of the column, can be detected by measuring absorbance at 280 nm (Fig. 3.29.4).

11. Determine protein concentration by Bradford, Lowry, or BCA, and measure the absorbance at 280/260 as described in *APPENDIX 3B*.

12. To finish, analyze the quality of purified tubulin by SDS-PAGE (*UNIT 6.1*) and by nondenaturing gel electrophoresis (Support Protocol 1).

13. Add to the tubulin-containing fractions the amount of 10× buffer A to obtain 1× buffer A, and GTP to a final concentration of 1 mM. Freeze fractions immediately in liquid nitrogen or at −80°C.

    Other microtubule associated proteins (MAPs) can be eluted from the column using buffer A containing 2 M NaCl (Basic Protocol 2 and Alternate Protocols 4 and 5).

BASIC PROTOCOL 4

ISOLATION OF MAP1A/1B

Microtubule-associated proteins comprise a heterogeneous group of polypeptides which display different structural characteristics, are differentially expressed during development, and are localized at different sites in neurons. A good example of these differences can be found between proteins termed MAP1A and MAP1B (Bloom et al., 1984; 1985). MAP1B is localized in the developing rat brain (Tucker et al., 1989), decreasing its expression dramatically at the end of the neuronal differentiation. On the other hand, MAP1A is expressed at later developmental stages and its level of expression remains constant through adulthood. MAP1B and the protein tau are mostly axonal proteins, whereas the high-molecular-weight forms of MAP2 are mostly found in dendrites (Matus, 1988).

Different MAPs display different characteristics which have served in developing diverse strategies and schemes to purify them. For instance, MAP2 and tau proteins are heat-resistant proteins, whereas MAP1A/1B denature at high temperature. Yet, since MAP1A/1B preferentially bind to a cluster of acidic amino acids present at the carboxy-terminus of tubulin (Tucker et al., 1989; Cross et al., 1991), molecules like poly-L-aspartic acid can be employed to compete with the interaction of these MAPs with tubulin (Fujii et al., 1990). Moreover, MAP1B can be isolated from the brain of newborn rats, whereas the most appropriate source for other MAPs is the adult brain. Similarly, the differential proportion of axons/somatodendritic tissues between white and gray matters can also be used to differentially purify MAP1 (white matter) and MAP2 (gray matter), minimizing contaminations (Vallee, 1986b), while either of the two matters can be used to purify MAP1A. In the case of tau, the expression pattern of the different isoforms is clearly different between white and gray matter, and there is a dramatic decrease in the amount of two tau isoforms in gray matter (de Ancos and Avila, 1993).

MAP1B/1A are both thermolabile proteins and cannot be isolated through a heat-treatment step. Instead, the addition of poly-L-aspartic to taxol-stabilized microtubules detaches MAP1A/1B proteins from these polymers. This step is performed in a PIPES buffer where the interaction of MAP1 proteins with tubulin is weakened (Pedrotti et al., 1993). MAP1 proteins are next fractionated from taxol-polymerized microtubules by centrifugation, being further purified using an FPLC (fast protein liquid chromatography) or AKTA system (Pazzagli and Avila, 1994). This particular protocol uses adult rat or porcine brains as the source of MAP1A. MAP1B is isolated from whole newborn-rat brains. These sources were chosen taking into consideration the information described above.

Materials

White matter from a cow or ten brains from adults rats (MAP1A)
Twenty brains from newborns rats (MAP1B)

Buffer B (see recipe)
GTP
Paclitaxel (Taxol; see recipe)
Poly-L-aspartic
Buffer C (see recipe)
Buffer D (see recipe)
Buffer A (see recipe)

10.4-ml centrifuge tubes
Ice-water bath
Centrifuge
Centrifuge rotors: GSA or JA-10. Type 50.2Ti and 70.1 Ti for the Beckman
100-ml Erlenmeyer flask
37°C incubator
Ultracentrifuge Beckman L8-70, Optima XL-100 or 120
1-ml Mono-Q column (or another with similar characteristics) connected to a FPLC or AKTA system

Additional reagents and equipment for preparing and homogenizing the brains (Basic Protocol 1), FPLC (Pazzagli and Avila, 1994), and SDS-PAGE (*UNIT 6.1*)

1. Follow steps 1 to 17 of Basic Protocol 1.
2. Resuspend the pelleted protein in buffer B to a final concentration of 4 mg/ml. Transfer to 10.4-ml centrifuge tubes. Let stand 30 min in an ice-water bath.
3. Centrifuge 15 min at 20,000 × *g*, 4°C.
4. Collect the supernatant in a 100-ml Erlenmeyer flask and add GTP, paclitaxel, and poly-L-aspartic acid to final concentrations of 1 mM, 10 μM, and 50 μM, respectively. Incubate for 30 min at 37°C.
5. Ultracentrifuge at 30 min at 100,000 × *g*, 25° to 30°C.

   Prewarm the rotor.

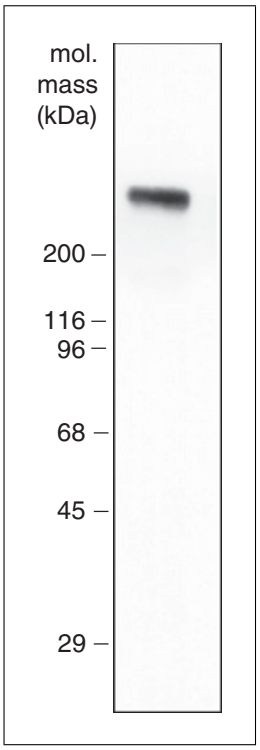


Figure 3.29.5 Characterization of the isolated MAP1A protein (Basic Protocol 4) by denaturing SDS-PAGE (7% polyacrylamide). The gel was stained with Coomassie blue as explained in *UNIT 6.6*.

6. Load the MAP1-containing supernatant (at a concentration of 0.5 mg/ml) onto a 1-ml mono-Q column and fractionate by fast protein liquid chromatography (FPLC; Pazzagli and Avila, 1994). Wash and equilibrate the column with buffer C until the A_{280} of the eluent returns to the baseline.
7. Next, elute using a linear gradient from buffer C to buffer D.
8. Characterize the protein content of the different collected fractions by SDS-PAGE (*UNIT 6.1*).
9. Select only MAP1-containing fractions (Fig. 3.29.5) and dialyze against buffer A. Store samples at −20°C or −70°C.

Be aware that usually MAP1 proteins are eluted in more than one peak.

BASIC PROTOCOL 5

ISOLATION OF HIGH-MOLECULAR-WEIGHT MAP2

The MAP2 gene gives rise to different isoforms that are developmentally regulated. For instance, those showing a higher molecular weight are the major isoforms in adult brain (Matus, 1988). As already mentioned, MAP2 is heat resistant and remains stable and fully functional after a high temperature treatment. Consequently, the inclusion of a heat-treatment step in the purification protocol facilitates the separation of MAP2 from other microtubule-associated proteins that should denature and precipitate (Herzog and Weber, 1978; Sloboda and Rosembaum, 1982). An alternative protocol for MAP2 purification has also been published (Williams and Lee, 1982).

Materials

Porcine or bovine brains or alternatively microtubule protein stored at −80°C or liquid nitrogen (Basic Protocol 1)
Buffer A (see recipe)
NaCl
2-mercaptoethanol
Ammonium sulfate
Sepharose 4B resin (GE Healthcare-Pharmacia)

Teflon-glass homogenizer
Ice-water bath
Centrifuge
Centrifuge rotors: GSA or JA-10, Type 50.2 Ti and 70.1 Ti for the Beckman
Boiling water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

Isolate MAP2

1. Follow steps 1 to 17 of Basic Protocol 1.
2. Depolymerize microtubules in cold buffer A using a Teflon-glass homogenizer to obtain microtubule proteins (10 to 20 mg/ml).

Intermittent homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate microtubule depolymerization.

3. Incubate in an ice-water bath for 30 min.
4. Centrifuge the mixture 15 min at 25,000 × *g*, 4°C.
5. Collect the supernatant and dilute to a protein concentration of 1 mg/ml with buffer A. Then, add NaCl and 2-mercaptoethanol to a final concentration of 1 M and 10 mM, respectively.
6. Heat the resulting protein solution for 5 min at 100°C in a boiling water bath.

7. Centrifuge 10 min at 10,000 × *g*, 25°C.

   A white precipitate containing denatured protein should be obtained.

8. Then, collect the supernatant in an Erlenmeyer flask and add ammonium sulfate salt to 35% (w/v) saturation. Incubate 10 min at room temperature.

   A total of 20.9 g of ammonium sulfate should be added to 100 ml of solution to obtain a 35% saturated solution.

9. Centrifuge 10 min at 10,000 × *g*, 4°C, then discard the supernatant and resuspend the pellet in 2 to 5 ml of buffer A containing 1 M NaCl.

10. Load resuspended protein onto a Sepharose 4B column equilibrated with buffer A containing 1 M NaCl.

11. Characterize the proteins present in the different collected fractions by SDS-PAGE (*UNIT 6.1;* Fig. 3.29.6).

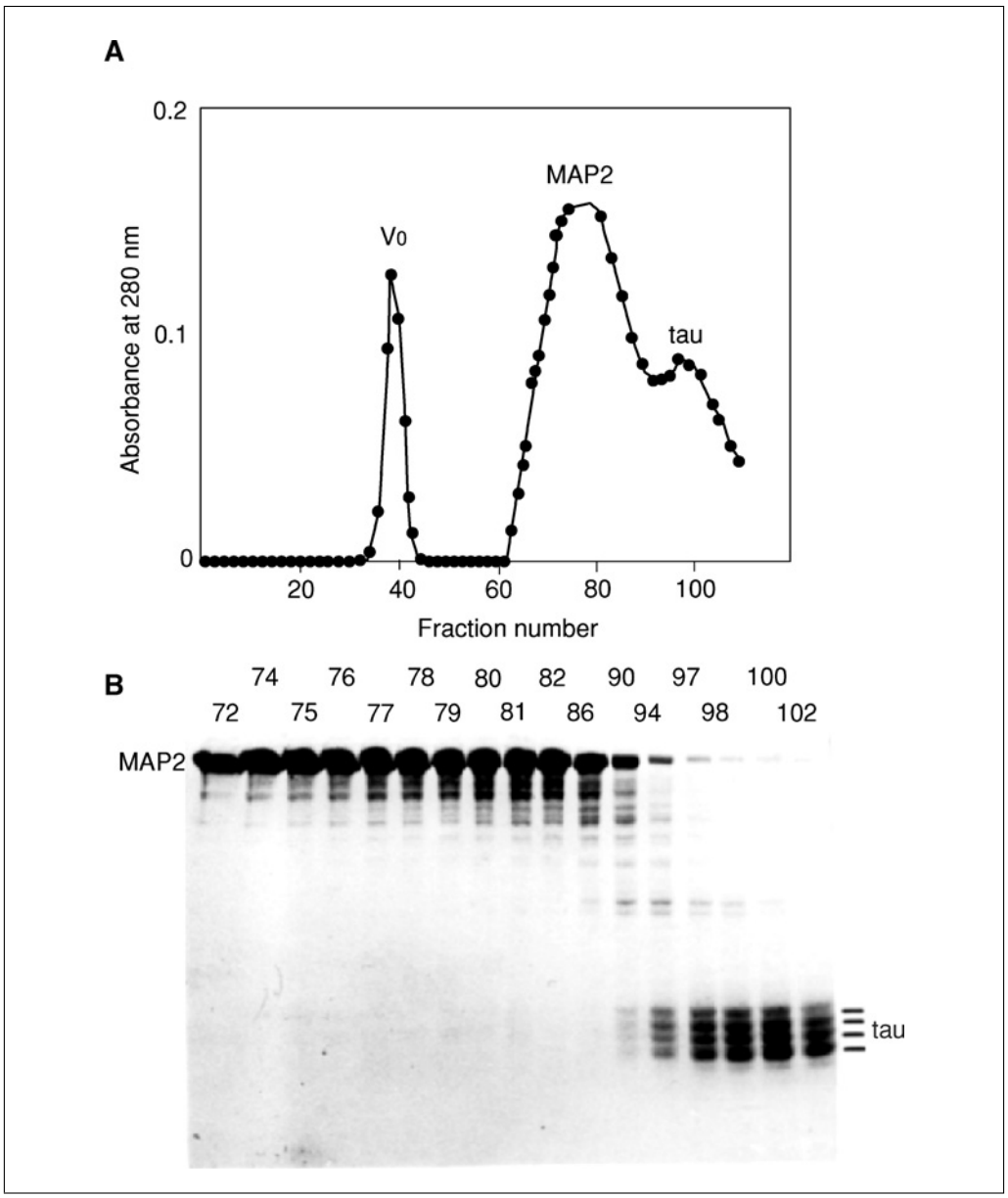


Figure 3.29.6 (**A**) Fractionation of heat-resistant MAPs by chromatography on Sepharose 4B (Basic Protocol 5). (**B**) SDS-PAGE analysis of the corresponding eluted fractions. The gel was stained with Coomassie blue as explained in *UNIT 6.6.* Abbreviations: V_o, void volume.

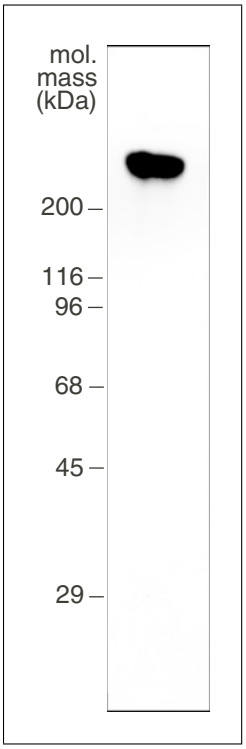


Figure 3.29.7 Characterization of the isolated high-molecular-weight MAP2 protein (Basic Protocol 5) by denaturing SDS-PAGE (7% acrylamide). The gel was stained with Coomassie blue as explained in *UNIT 6.6.*

12. Pool fractions containing MAP2 and precipitate protein by the addition of ammonium sulfate to 50% saturation. Collect the precipitated protein by centrifuging 10 min at 15,000 × *g*, room temperature.

    A total of 31.3 g of ammonium sulfate should be added to 100 ml to obtain a 50% saturated solution.

13. Add 1 ml of buffer A to resuspend the pellet and dialyze against 1 liter of the same buffer.

14. Store protein samples in a freezer at −20°C (Fig. 3.29.7).

BASIC PROTOCOL 6

ISOLATION OF TAU PROTEIN

The primary gene transcript of tau is a well-known example of alternative splicing in the mammalian brain (Himmler et al., 1989). Furthermore, tau possesses multiple phosphorylation sites that are selectively used to create polypeptides with different functions. The post-translational modifications of the various tau isoforms result in multiple protein behaviors (Lindway and Cole, 1984). For example, not all the phosphorylated tau isoforms bind to microtubules. For this reason there are two alternate procedures depending on whether we want to isolate all the isoforms, or just those that bind to microtubules (Garcia de Ancos et al., 1993).

To isolate total tau isoforms (Garcia de Ancos et al., 1993), adjust the concentration of NaCl and DTT to 1 M and 1 mM, respectively in the homogenate obtained in step 12 of Basic Protocol 1, and boil for 5 min and chill on ice. Further steps of the purification are indicated below. In this protocol we describe the isolation of microtubule binding tau isoforms. In addition to this protocol, there are other tau purification procedures that have also been described (Herzog and Weber, 1978; Sobue et al., 1981; Drubin and Kirschner, 1986; Garcia de Ancos et al., 1993).

Materials

Porcine brain
Buffer A (see recipe)
Perchloric acid
Ammonium sulfate
Glycerol

Ice-water bath
Boling water bath
Centrifuge
Centrifuge rotors: GSA or JA-10. Type 50.2Ti and 70.1 Ti for the Beckman

Isolate tau

1. Follow steps 1 to 17 of Basic Protocol 1.
2. Resuspend microtubule proteins in 25 ml of buffer A. Leave 30 min in an ice-water bath.
3. Centrifuge 15 min at 20,000 × *g*, 4°C.
4. Collect the supernatant in a 100-ml Erlenmeyer flask and correct the concentrations of NaCl and DTT to 1 M and 1 mM, respectively. Then boil samples for 5 min and immediately chill on ice for 5 min.
5. Centrifuge samples 30 min at 35,000 × *g*, 4°C.
6. Add perchloric acid to the supernatant to a 2.5% (v/v) final concentration. Keep on ice for 10 min.
7. Centrifuge 15 min at 35,000 × *g*, 4°C.
8. Recover the supernatant, collecting it in a 100-ml Erlenmeyer flask, and add ammonium sulfate up to 50% saturation. Incubate 30 min at 4°C.

   A total of 313 g of ammonium sulfate should be added to 1 liter to obtain a 50% saturated solution.

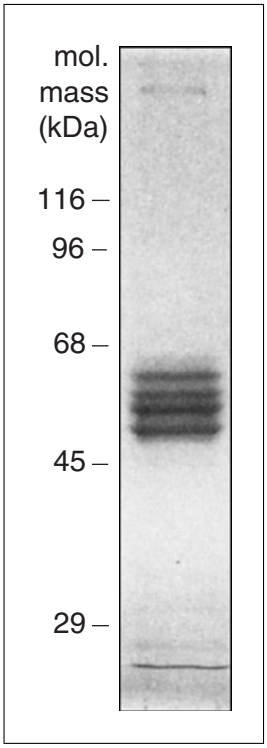


Figure 3.29.8 Characterization of different isoforms of the isolated tau protein by denaturing SDS-PAGE (10% acrylamide). The gel has been stained with Coomassie blue as explained in *UNIT 6.6.*

9. Centrifuge 15 min at 35,000 × *g*, 4°C.

10. Dissolve the pellet in buffer A (to a final concentration of 1 to 5 mg/ml), bring up to 2.5% perchloric acid, and centrifuge 15 min at 35,000 × *g*, 4°C.

11. Decant supernatant and add glycerol to a final concentration of 25% (v/v). Centrifuge 15 min at 35,000 × *g*, 4°C.

12. Resuspend the pellet—which should appear as a translucent film stuck to the centrifuge tube wall—in buffer A to a final concentration of 0.5 to 2 mg/ml. Store samples in a freezer at −20°C (Fig. 3.29.8).

SUPPORT PROTOCOL 1

NONDENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS AS A SIMPLE METHOD TO QUANTIFY/CHECK POLYMERIZATION-COMPETENT TUBULIN

The determination of the purity of the different protein fractions from the various purification steps can be monitorized using SDS-denaturing gels (*UNIT 6.1*). Purified tubulin is usually analyzed by SDS-PAGE. Under denaturing conditions, α- and β-tubulin polypeptides have different mobilities, both polypeptides in the molecular mass range of 55 kDa. However, the analysis and quantification of the amount of tubulin by most of the known biochemical methods does not provide information about the native state of the tubulin in the preparation. To distinguish between folded and unfolded tubulin (noncompetent for microtubule assembly) we can take advantage of an old, but still very important, nondenaturing gel electrophoresis technique first applied by Ornstein (1964) for the analysis of water-soluble serum proteins (see *UNIT 6.5*). This method can be used only under conditions that preserve the native state of soluble individual proteins or complexes. In contrast to classic SDS-denaturing electrophoresis, the migration of proteins in native gels is essentially dependent on their isoelectric point and the pH of the buffer (Safer, 1994). Consequently, in native electrophoresis it is crucial to select the appropriate buffer for each protein under analysis. The behavior of native tubulin under nondenaturing conditions was established by Zabala and Cowan (1992). Although many times it is advantageous to use a discontinuous system to maximize the resolution of the protein bands, we have found that this is not required for tubulin, and it is easier and faster to prepare a continuous system. To prepare and to run a native electrophoresis, carefully follow the safety indications explained in Chapter 6.

Materials

Gel solutions (Table 3.29.1)
4× loading buffer [50 mM MES, pH 6.7, containing 30%(v/v) glycerol or 25%(w/v) sucrose
Electrophoresis (running) buffer (see recipe)
GTP
Protein sample to be analyzed
10× transfer buffer (see recipe), optional
Electrophoresis equipment (Chapter 6; e.g., Miniprotean from Bio-Rad and 0.75-mm spacers)

Additional reagents and equipment for gel staining (*UNIT 6.2*)

1. Assemble the gel glass-plate sandwich and fit it to the gel electrophoresis unit casting stand according to manufacturer's instructions.

2. Prepare the gel solution as indicated in Table 3.29.1.

3. Pour the gel mix to the top of the gel mold and insert the comb (0.75-mm spacers and 10 wells per comb). Avoid trapping air bubbles under the comb teeth. Allow gel to polymerize for 30 to 60 min.

Table 3.29.1 Recipe for Gel Preparation Using a Native Continuous Buffer System[a]

Stock solution (ml)[b]	Final acrylamide concentration in gel (%)[c]			
	4	5	6	7
Distilled water	6.48	6.15	5.81	5.48
30% acrylamide/0.8% bisacrylamide[d]	1.33	1.66	2	2.33
0.5 M MES, pH 6.7	2	2	2	2
1 M $MgCl_2$	0.01	0.01	0.01	0.01
0.5 M EGTA	0.02	0.02	0.02	0.02
1 M GTP	0.01	0.01	0.01	0.01
10% (w/v) APS (ammonium persulfate)[e]	0.14	0.14	0.14	0.14
TEMED[f]	0.01	0.01	0.01	0.01

[a]Preparation of the gel: Use a 75 or 100-ml Erlenmeyer flask or in order to speed polymerization, degas the mix under vacuum several minutes in a side-arm flask. Mix the ProtoGel[c] solution with 0.5M MES pH 6.7, 1M $MgCl_2$, 1M GTP and 0.5M EGTA. Add 10% (w/v) ammonium persulfate and TEMED.
[b]All reagents and solutions used must be prepared with Milli-Q purified water or equivalent.
[c]The recipe is for 10 ml solution, which is adequate for 2 minigels (7 cm × 8 cm) 0.75 mm thickness.
[d]The polyacrylamide can be prepared as described in Chapter 6, but it is more convenient to use a ready-to-use commercial solution, we usually use ProtoGel 30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide from National Diagnostics, England.
[e]It can be prepared and stored frozen at −20°C in aliquots but we prefer to use freshly made.
[f]Add just before polymerization.

4. Mix the protein sample to be analyzed with 4× native loading buffer [50 mM MES, pH 6.7, containing 30% (v/v) glycerol or 25% (w/v) sucrose] for a final concentration of 1×.

   For better sample visualization small amounts [0.025% (w/v)] of bromphenol blue can be added in the loading buffer.

5. Assemble the gel unit and fill the upper tank with enough electrophoresis buffer with GTP added to 0.01 M. Use the rest of the 800 ml of electrophoresis buffer, without GTP, to fill the rest of the cell. Remove the comb and rinse wells with buffer.

6. Carefully load a maximum of 25 μl of the samples starting up from the bottom of the wells.

   Use a micropipet or alternatively a Hamilton syringe to load the gel.

7. Connect the unit to the power supply. Set at constant voltage of 80 V for 2 hr.

   We usually run one gel per cell unit, for no longer than 2 hr at room temperature.

   Gels can run longer than 2 hr if the electrophoresis buffer is replaced with freshly prepared electrophoresis buffer in order to maintain the pH. The apparatus needs to be turned off before exchanging the buffer.

8. Turn off the power supply. Disassemble the unit and remove gel from glass sandwich. Stain the gel according to *UNIT 6.6*.

   Coomassie blue or silver staining is the usual method to detect tubulin depending on the amount of tubulin loaded into the gel (Fig. 3.29.9). Alternatively, gels can be transferred onto nitrocellulose or PVDF membranes prior to antibody detection in immunoblots (UNIT 6.2) by using a typical transfer buffer where methanol is omitted.

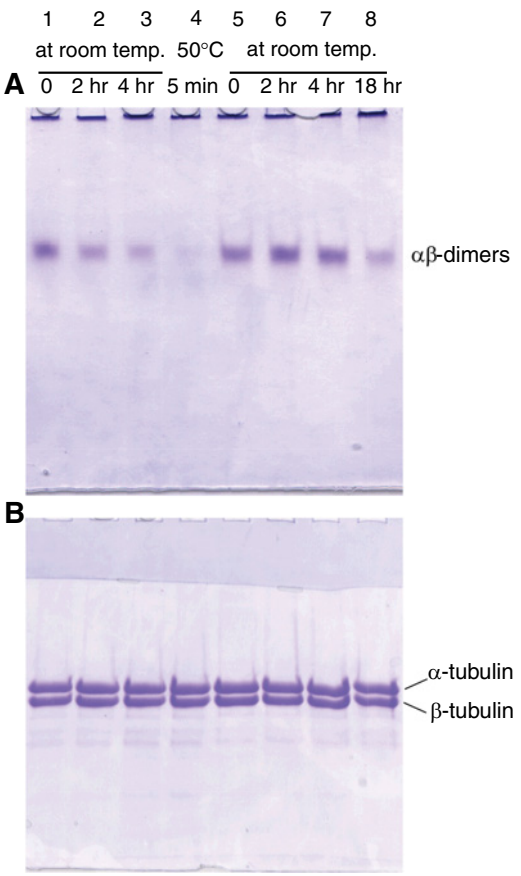


Figure 3.29.9 Assessment of the quality of native tubulin heterodimers by non-denaturing and denaturing gel electrophoresis. (**A**) Nondenaturing PAGE (8.5% acrylamide) characterization of 1-μl aliquots (Support Protocol 1) of purified tubulin (at 6 μg/μl) diluted to 10 μl with water Milli-Q (lanes 1 to 4) or 50 mM MES, pH 6.7 (lanes 5 to 8), and incubated for the indicated times at room temperature with the exception of the aliquot analyzed in lane 5, which was incubated 5 min at 50°C. (**B**) The same tubulin samples were loaded on a gel prepared and run as described in Support Protocol 1. Both gels were stained with Coomassie blue and then scanned before drying. For color version of this figure see *http://www.currentprotocols.com*.

SUPPORT PROTOCOL 2

VISUALIZATION OF POLYMERIZED MICROTUBULES BY ATOMIC FORCE MICROSCOPY (AFM)

Atomic Force Microscopy (AFM) is a powerful technique with a wide range of applications in biology, allowing not only imaging, but also different types of probing and manipulation at nanometric scale. AFM systems are becoming widely available, and new developments are reported everyday, many of them in biological research. In AFM a sharp tip (local probe) is moved over a surface at constant (van der Waals) interaction force between the tip and the surface, allowing the reconstruction of the surface topography. This measurement process can be performed in different ambient conditions, e.g., in vacuum, air, or liquid, over a broad temperature range. These characteristics and the possibility to observe features at nanometric scale in a nondestructive manner are a strong motivation for the use of AFM on biological systems. As compared to existing microscopy techniques in the same range (as electron microscopy), sample preparation is also much easier and less time-consuming, allowing one to perform measurements even on live systems (Fotiadis et al., 2002). The list of examples is now almost endless. For example, AFM

techniques have been extensively used to image the surface of cells (Dufrêne et al., 1999; Charras and Horton, 2002), nucleic acids (Pande et al., 1998; Scheuring et al., 1999), proteins (Round et al., 2002), chromosomes (Radmacher et al., 1994; Fritzsche et al., 1997), viruses (Dubrovin et al., 2007), and biomaterials (Wallwork et al., 2001). Besides being used as a powerful imaging technique, AFM shows a strong potential as a unique tool for nanometric probing of physical (e.g., elasticity or viscosity of surfaces, binding forces, strength of protein folding) and chemical properties (by using a chemically activated tip) and for precise local manipulation (Rief, et al., 1997; Sritharan et al., 1998; Raab et al., 1999; Dufrêne, 2000). It is nowadays a fundamental tool for nanomedicine (medicine approached at a molecular level) and its relevance can no longer be overlooked

Microtubules may be characterized by AFM (Ramalho et al., 2007), which permits nanometer-scale resolutions and topographic characterization of the sample, allowing one to visualize the assembled microtubules.

Here we introduce a protocol adapted from Martins et al. (2005) and Moreno-Herrero et al., (2002, 2005) describing how to adsorb microtubules on a substrate to visualize them by AFM. In this protocol, microtubules are adsorbed to mica functionalized with APTES, a silane with an amine group that is positively charged at around pH 7. Since mica is composed of layers, which can be cleaved ("peeled" off) to expose a new clean surface and, unlike more traditional glass, no preliminary washing procedures are necessary.

Materials

Mica (muscovite; SPI Supplies)
Nail polish or water-resistant glue (e.g., epoxy resin)
Silanization solution: 0.1% (v/v) 3-aminopropyl-triethoxysilane (APTES; Fluka, 96%) freshly prepared with Milli-Q water
Milli-Q water; if unavailable, bidistilled water filtered through a 0.22-μm pore filter
GTP
Paclitaxel (taxol)
Sample of purified tubulin (Basic Protocol 3)
0.1 M PIPES, pH 6.9
1 mM EGTA
1 mM $MgCl_2$
1 mM PMSF

Sharp scissors
Glass coverslips or slides
Duct tape
Micropipet
30°C thermostatic bath
AFM Si tapping mode tips (Olympus, OTESP)

Functionalize the mica

1. Use sharp scissors to cut mica squares of appropriate dimensions (2 to 5 mm) and mount them on glass coverslips or slides (for easier handling) with nail polish or water-resistant glue (e.g., epoxy resin). Remove the upper layers of the mica squares by pulling off the top layer with a piece of duct tape.

2. Immediately apply 10 μl silanization solution on the exposed surface, taking care not to spill on the sides. To complete silanization let it rest for 10 min.

   While the nail polish will secure the mica even if wet, silanization solution may still get between the mica layers and distort them.

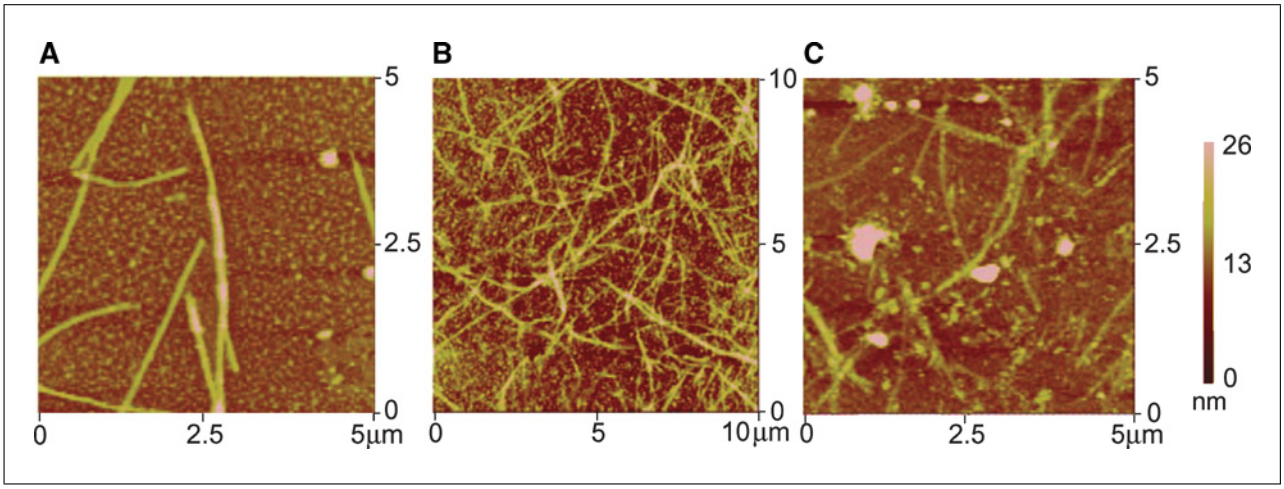


Figure 3.29.10 (**A**) AFM topography images of polymerized microtubules adsorbed to silanized mica where a few individual microtubules with a well preserved structure are easily observed. (**B**) A microtubule-rich region in the sample showing contaminant material. (**C**) Distortion of measurements due to the contaminants in a dirty sample. For color version of this figure see *http://www.currentprotocols.com*.

3. Remove the silanization solution as much as possible with a micropipet and avoid scratching the mica surface. Wash off the mica three times, each time with 10 µl of Milli-Q water and allow the mica to air dry.

   It is quite important that mica surface is maintained clean during this process in order to guarantee that environmental dust does not stick to it during the process of silanization.

Polymerize and adsorb microtubules

4. Add 2 µl of 100 mM GTP (to a final concentration of 1 mM) to 200 µl of a purified tubulin solution in 0.1 M PIPES pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, at 1 mg/ml on ice.

5. Transfer to a 30°C thermostatic bath and incubate for 45 min.

6. Add 4 µl of 1 mM paclitaxel (taxol) to give a final concentration of 20 µM. Incubate for another 15 min at the same temperature.

   Optionally, microtubules may be concentrated by sedimenting them at 15,000 × g for 120 min. This will usually require more than one aliquot to give a visible pellet.

   If you are not going to immediately use the assembled microtubule suspension then aliquot it in suitable volumes, promptly freeze in liquid nitrogen, and store at −80°C.

7. When appropriate, thaw the microtubule suspension by placing on ice. Apply 5 µl of the microtubule suspension on the surface of the functionalized mica. Incubate for 5 to 10 sec. Immediately carefully remove the liquid with a micropipettor.

8. Gently wash the preparation twice with 10 µl Milli-Q water. Allow remaining water to air dry and observe with AFM as soon as possible.

   Avoid touching the mica surface with the tip of the micropipet in steps 1 and 2.

   For AFM measurements we use a Dimension 31 00 (Veeco/Digital Instruments) microscope with nanoscope III a controller and silicon nitride tips (SiN_2). All samples are observed in tapping mode at atmosphere in order to minimize de-adsorption (Fig. 3.29.10).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BRB80 buffer

80 mM PIPES, pH 6.8
1 mM EGTA
1 mM $MgCl_2$
Store up to 2 months at 4°C

Buffer A, 10×

1 M MES, pH 6.7
20 mM EGTA
10 mM $MgCl_2$
Store up to 2 months at 4°C

Buffer B

0.15 M PIPES, pH 7.0 (see recipe)
2 mM EGTA
0.1 mM $MgCl_2$
Store up to 2 months at 4°C

Buffer C

0.1 M PIPES, pH 7.0 (see recipe)
2 mM EGTA
Store up to 2 months at 4°C

Buffer D

0.1 M PIPES, pH 7.0 (see recipe)
2 mM EGTA
1 M NaCl
Store up to 2 months at 4°C

EGTA, 0.5 M

Dissolve 19 g EGTA [ethylene glycol-bis-(2-aminoethyl)-N,N,N′,N′-tetraacetic acid] in 80 ml water. Titrate to pH 8 with KOH or NaOH. Adjust to a final volume of 100 ml. Store up to 2 years at room temperature.

Electrophoresis (running) buffer

0.1 M MES, pH 6.7
1 mM $MgCl_2$
1 mM EGTA

Dilute and adjust with distilled water 160 ml 0.5 M MES, pH 6.7 (see recipe), 800 µl 1 M $MgCl_2$, and 1.6 ml 0.5 M EGTA (see recipe) to 800 ml. Prepare fresh just prior to use.

GTP, 1 M

1.5 ml distilled H_2O
1 g Na_2GTP
Bring to final volume of 1.91 ml with H_2O.

Do not let stand at room temperature or at 4°C. Aliquot in small volumes and store at −80°C.

Isotonic buffer

0.32 M sucrose
1 mM EGTA
1 mM $MgCl_2$
10 mM phosphate buffer, pH 7 (*APPENDIX 2A*)
Store up to 2 months at 4°C

MES, 0.5 M, pH 6.7

Dissolve 53.3 g MES monohydrate in 400 ml water. Titrate to pH 6.7 with KOH or NaOH. Adjust to a final volume of 500 ml with water. Store up to 6 months at 4°C.

Paclitaxel, 10 mM

10 mM paclitaxel (taxol; Sigma). Prepare in DMSO. Store 50-µl aliquots at −80°C and use at final concentration of 20 µM.

PIPES, 1 M (pH 7.0)

151.2 g PIPES (free acid)
H_2O to 400 ml
Add concentrated KOH dropwise to achieve pH 7.0.
Bring to final volume of 500 ml with H_2O
Store up to 2 months at 4°C

Protease inhibitors

100 mM phenylmethylsulfonyl fluoride, (PMSF; *APPENDIX 2A*) prepare fresh, use at 1 mM, dissolve in DMSO
1000× leupeptin, prepare 1 µg/µl in Milli-Q water
1000× pepstatin A, prepare 1 µg/µl in DMSO
1000× aprotinin, 10 µg/ml in Milli-Q water (sodium azide or 0.2% Triton X-100 can be added to avoid contamination)

CAUTION: *Phenylmethylsulfonyl fluoride is toxic.*

Transfer buffer, 10×

Dissolve the following in water:
15.13 g Tris base (250 mM)
72.067 g glycine (1.92 M)
Adjust to a final volume of 500 ml with H_2O

COMMENTARY

Background Information

The generation of complex neuronal morphologies during vertebrate neurodevelopment is characterized by the production of long cytoplasmic processes known as neurites. These processes, referred to as axons and dendrites, will eventually generate a complex network of neuronal synaptic contacts. Although microtubules are universal polymers in all eukaryotic cell types, in neurons they are highly specialized and more abundant, and play crucial roles in the determination of the cell shape, being organized in long bundles within the axons and dendrites of mature neurons. Microtubules are dynamic structures in vitro, where possible regulatory factors of the process have been thoroughly studied. These investigations led to the discovery of a group of proteins that bind to tubulin "in vitro" during microtubule polymerization assays, promoting the polymerization and further stabilizing the polymer structure. These proteins are referred to as microtubule-associated proteins or MAPs. Three major families of MAPs have been identified and characterized: MAP1 (Vallee, 1990), MAP2 (Murphy et al., 1977), and tau proteins (Cleveland et al., 1977).

MAP1 proteins

The MAP1 protein family consists of two distinct, but related, polypeptides, MAP1A and MAP1B (Schoenfeld et al., 1989). MAP1A has a molecular mass of 299 kDa, whereas MAP1B has a molecular mass of 255 kDa as predicted from their respective amino acid sequences (Noble et al., 1989; Langkopf et al., 1992).

There are also three low-molecular-weight proteins associated with the microtubule-binding domains of both MAP1A and MAP1B; these are referred to as light chains: LC1 (34 kDa), LC2 (30 kDa), and LC3 (19 kDa; Schoenfeld et al., 1989).

In the mammalian brain there is a strong developmental control of the expression patterns of these MAPs. The first MAP expressed in neurons is MAP1B (Tucker et al., 1988). Immunohistochemical analyses in developing brain tissue have shown that this protein is highly concentrated in the growing axons of the developing neurons, exhibiting a more moderate expression in both axons and dendrites of mature neurons (Schoenfeld et al., 1989). Indeed, MAP1B expression is down-regulated during brain development (Binder et al., 1984; Bloom et al., 1985; Riederer et al., 1986; Tucker et al., 1989; Garner et al., 1990). On the contrary, MAP1A is more abundant in mature neurons (Bloom et al., 1984).

MAP2

There are several developmentally regulated isoforms of MAP2 arising from alternative mRNA splicing. MAP2B is a high-molecular-weight isoform which contains 1828 amino acids and has an apparent molecular mass of 270 kDa as determined by SDS-PAGE (Lewis et al., 1988). Also, MAP2A shows a slower electrophoretic mobility (Matus, 1988). There is a smaller form, referred to as MAP2C that contains 467 amino acids and has an apparent molecular mass of 70 kDa as determined by SDS-PAGE. MAP2B is a neuron-specific phosphoprotein which is selectively localized in the cell bodies and dendrites (Caceres et al., 1984; Huber and Matus, 1984). MAP2C is expressed perinatally in rats, coincident with the period of maximal dendritic outgrowth and synaptogenesis (Riederer and Matus, 1985). There is an additional MAP2 isoform which is more abundant in glial cells named MAP2D (Doll et al., 1993).

Tau

There are several tau proteins with apparent molecular masses ranging from 55 kDa to 68 kDa (as determined by SDS-PAGE); they are found in the central nervous system (Weingarten et al., 1975). This large number of tau isoforms is generated by alternative splicing from a primary transcript (Himmler et al., 1989; Goedert et al., 1992). Based on their domain characteristics, two classes of tau isoforms have been described. One class contains three tubulin-binding motifs that are predominantly expressed in the developing brain; a second class contains four motifs and is mostly expressed in the adult brain (Kosik et al., 1989; Goedert et al., 1991).

An additional tau isoform, with an apparent molecular mass of 110 kDa, has also been identified in the peripheral nervous system (Georgieff et al., 1991). This high-molecular-weight tau contains four repeated motifs in its microtubule-binding domain; it is similar to the adult brain tau isoform but has an additional insertion of 254 amino acids in its amino-terminal region (Goedert et al., 1991; Couchie et al., 1992).

Critical Parameters and Troubleshooting

Our experience suggests that, although a higher yield of microtubule proteins is usually obtained from porcine brain tissue, cow brains do also give good tubulin yields. Tubulin has traditionally been considered an extremely labile protein that becomes noncompetent for polymerization if left on ice for long periods of time. However, as Figure 3.29.9 shows, tubulin is a stable dimer for >4 hr at room temperature in MES buffer at pH 6.7 (at a concentration as low as 0.6 μg/μl, with no magnesium ions or GTP added). In fact, the tubulin dimer becomes unstable only after 18 hr or longer incubation periods. Our experience also shows that tubulin can be stored at temperatures between −70 and −80°C. Regarding the size of the preparation, we usually prepare medium-scale tubulin preparations. This guarantees a faster protein extract preparation, which is key to preserving a good-quality tubulin, as explained in the Time Considerations section. The intermediate-step tubulin aliquots obtained can be fast-frozen and stored at −80°C instead of in liquid nitrogen. For each experiment, appropriate-size aliquots can be thawed, mixed well by flicking, and centrifuged 20 min at 20,000 × *g*, 4°C, before use, to discard insoluble material. Although repeated freezing and thawing of aliquots is not recommended, we find that tubulin will still work fine in freeze-thawed aliquots. Another

crucial point to obtaining a good homogenate when isolating tubulin from brain extracts is not to destroy the tissue excessively. The use of a classical blender, even at low speed, is not recommended since vigorous homogenization leads to the formation of aggregates that persist during the purification. Moreover, this procedure also provokes the formation of tubulin aggregates. We recommend the use of a Polytron set at low speed, or just a Teflon-in-glass homogenizer at 2,000 rpm for 5 to 10 sec, with the pestle moving up and down the homogenizer. A commercial drill adapted as a homogenizer is a cheaper alternative.

Preparation of microtubule samples for AFM entails adsorption onto an adequate substrate (positively charged) and washing off the buffer as much as possible in order to avoid artifacts resulting from salt crystallization. Otherwise, salt crystals will rapidly grow over the mica surface after the microtubule suspension has dried, completely impairing the AFM measurements. Washes must be carefully performed to avoid microtubule damage or removal from the surface. So, drop and remove water from the surfaces containing microtubules during washes.

Since microtubules are adsorbed to mica functionalized with APTES, the time of incubation of microtubules on the functionalized mica is one of the most important parameters in this method. Longer incubation times tend to yield "dirty" samples due to the adsorption onto the surface of nonpolymerized tubulin and other contaminants (even if they are scarce in the sample). On the other hand, shorter incubation times will result in sparser distributions of microtubules on the surface, allowing a better visualization. The more microtubules are adsorbed onto the surface, the more difficult it will be to observe detailed features. Incubation times of 5 to 10 sec will usually give good results with microtubules polymerized from ~1 mg/ml brain tubulin solution. However, longer times may be necessary for lower concentrations. Attention should also be paid to the concentration of microtubules in suspension, since an increase in polymer concentration would lead to aggregation and microtubule bundle formation.

Anticipated Results

The cytosolic extract obtained after the first centrifugation of the isolation procedure of microtubule proteins usually contains a protein concentration in the range of 20 to 40 mg/ml of protein and ~4 to 8 mg/ml of tubulin. Phosphocellulose-FPLC chromatography purified bovine brain tubulin should give a single band upon electrophoresis under native conditions—in the presence of GTP—which serves as a reference marker for native tubulin heterodimers. The amount of tubulin in this unique band is extremely useful to quantify the amount of tubulin dimers capable of polymerization. Proteins that have denatured or aggregated during these purification steps will remain at, or close to, the origin of the native gel (Fig 3.29.9). Therefore, this type of electrophoresis will serve to estimate the amount of tubulin that should be used in any other experiment requiring native tubulin. The use of GTP during native gel electrophoresis is critical for this type of analysis. In the absence of GTP we have found that the intensity of the native tubulin heterodimer band decreases, indicating that tubulin dimers have became unstable in the absence of GTP. This could be due to denaturation of the β-tubulin that loses GTP during the running of native gel electrophoresis in the absence of this nucleotide (Zabala and Cowan, 1992). Be aware that ATP (used instead of GTP for actin) cannot replace GTP in this type of electrophoresis.

There are a number of different gel staining protocols that can be used (*UNIT 6.6*). Coomassie blue or silver staining are the usual ways to detect tubulin depending on the amount of sample loaded on the gel. Protein in these gels can also be transferred to nitrocellulose or PVDF membranes prior to antibody detection by immunoblots (*UNIT 6.2*). In this case, a typical transfer buffer without methanol should be used.

In vitro polymerized microtubule samples observed by AFM are typically heterogeneous. These samples will characteristically present areas with fewer microtubules, but also fewer interfering contaminants (Fig. 3.29.10A), along with areas denser in microtubules but presenting more contaminants (Fig. 3.29.10B) or areas with many amorphous structures (precipitates from the microtubule solution) that will make the analysis more difficult (Fig.3.29.10C). Denser areas, as well as bundles of microtubules, and generally more contaminants, are often observed in samples prepared from more concentrated microtubule solutions. On the other hand, concentrations lower than those recommended will result in very scattered samples where microtubules may be difficult to find.

Time Considerations

The most critical step in the tubulin protein purification protocol is the time spent from the

time of slaughter to the end of the first microtubule polymerization. It is crucial to obtain brains of freshly sacrificed animals and to remove the skulls as soon as possible. These must be immediately stored at 4°C wrapped in plastic bags or similar wrapping material, to avoid direct contact with ice. The brain cytosolic protein extract should be prepared as soon as possible. Everything needed for the isolation/purification procedure should be already prepared in the laboratory. In the same way, extreme care should be taken to avoid any delay in washing and cleaning the brains.

For AFM, waiting periods for sample drying should typically be of 0.5 to 1 hr, depending on room temperature and humidity. For reproducibility, microtubule incubation times must be carefully observed, even if chosen times differ from those detailed in this protocol. Nonetheless, this procedure should take a maximum of 4 hr. Similarly, samples must be observed with AFM as soon as microtubules are adsorbed to functionalized mica, thus avoiding artifacts caused by dust or degradation. Samples can be kept for at least a week at 4°C (in a sealed container) although some degradation and contamination can occur.

Literature Cited

Avila, J. 1990. Microtubule dynamics. *FASEB J.* 4:3284-3290.

Binder, L.I., Frankfurter, A., Kim, H., Caceres, A., Payne, M.R., and Rebhun, L.I. 1984. Heterogeneity of microtubule-associated protein 2 during rat brain development. *Proc. Natl. Acad. Sci. U.S.A.* 81:5613-5617.

Bloom, G.S., Schoenfeld, T.A., and Vallee, R.B. 1984. Widespread distribution of the major polypeptide component of MAP 1 microtubule-associated protein 1 in the nervous system. *J. Cell Biol.* 98:320-330.

Bloom, G.S., Luca, F.C., and Vallee, R.B. 1985. Microtubule-associated protein 1B: identification of a major component of the neuronal cytoskeleton. *Proc. Natl. Acad. Sci. U.S.A.* 82:5404-5408.

Borisy, G.G. and Olmsted, J.B. 1972. Nucleated assembly of microtubules in porcine brain extracts. *Science* 177:1196-1197.

Caceres, A., Banker, G., Steward, O., Binder, L., and Payne, M. 1984. MAP2 is localized to the dendrites of hippocampal neurons which develop in culture. *Brain Res.* 315:314-318.

Charras, G.T. and Horton, M.A. 2002. Single cell mechanotransduction and its modulation analyzed by atomic force microscope indentation. *Biophys. J.* 82:2970-2981.

Cleveland, D.W., Hwo, S.Y., and Kirschner, M.W. 1977. Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. *J. Mol. Biol.* 116:207-225.

Couchie, D., Mavilia, C., Georgieff, I.S., Liem, R.K., Shelanski, M.L., and Nuñez, J. 1992. Primary structure of high molecular weight tau present in the peripheral nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 89:4378-4381.

Cross, D., Dominguez, J., Maccioni, R.B., and Avila, J. 1991. MAP-1 and MAP-2 binding sites at the C-terminus of beta-tubulin. Studies with synthetic tubulin peptides. *Biochemistry* 30:4362-4366.

de Ancos, J.G. and Avila, J. 1993. Differential distribution in white and grey matter of tau phosphoisoforms containing four tubulin-binding motifs. *Biochem J.* 296:351-354.

Doll, T., Meichsner, M., Riederer, B.M., Honegger, P., and Matus, A. 1993. An isoform of microtubule-associated protein 2 MAP2. containing four repeats of the tubulin-binding motif. *J. Cell Sci.* 106:633-639.

Drubin, D. and Kirschner, M. 1986. Purification of tau protein from brain. *Methods Enzymol.* 134:156-160.

Dubrovin, E.V., Drygin, Y.F., Novikov, V.K., and Yaminsky, I.V. 2007. Atomic force microscopy as a tool of inspection of viral infection. *Nanomedicine* 3:128-131.

Dufrêne, Y.F. 2000. Direct characterization of the physicochemical properties of fungal spores using functionalized AFM probes. *Biophys. J.* 78:3286-3291.

Dufrêne, Y.F., Boonaert, C.J.P., Gerin, P.A., Asther, M., and Rouxhet, P.G. 1999. Direct probing of the surface ultrastructure and molecular interactions of dormant and germinating spores of *Phanerochaete chrysosporium*, *J. Bacteriol.* 181:5350-53544.

Fotiadis, D., Scheuring, S., Müller, S. A., Engel, A., and Müller, D. J. 2002. Imaging and manipulation of biological structures with the AFM. *Micron* 33:385-97.

Fritzsche, W., Takac, L., and Henderson, E. 1997. Application of atomic force microscopy to visualization of DNA, chromatin, and chromosomes. *Crit. Rev. Eukaryot. Gene Expr.* 7:231-240.

Fujii, T., Nakamura, A., Ogoma, Y., Kondo, Y., and Arai, T. 1990. Selective purification of microtubule-associated proteins 1 and 2 from rat brain using poly L-aspartic acid. *Anal. Biochem.* 184:268-273.

Garcia de Ancos, J., Correas, I., and Avila, J. 1993. Differences in microtubule binding and self-association abilities of bovine brain tau isoforms. *J. Biol. Chem.* 268:7976-7982.

Garner, C.C., Garner, A., Huber, G., Kozak, C., and Matus, A. 1990. Molecular cloning of microtubule-associated protein 1 (MAP1A) and microtubule-associated protein 5 (MAP1B): Identification of distinct genes and their differential expression in developing brain. *J. Neurochem.* 55:146-154.

Georgieff, I.S., Liem, R.K., Mellado, W., Nunez, J., and Shelanski, M.L. 1991. High molecular weight tau: Preferential localization in the peripheral nervous system. *J. Cell Sci.* 100:55-60.

Goedert, M., Crowther, R.A., and Garner, C.C. 1991. Molecular characterization of microtubule-associated proteins tau and MAP2. *Trends Neurosci.* 14:193-199.

Goedert, M., Spillantini, M.G., and Crowther, R.A. 1992. Cloning of a big tau microtubule-associated protein characteristic of the peripheral nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 89:1983-1987.

Herzog, W. and Weber, K. 1978. Fractionation of brain microtubule-associated proteins. Isolation of two different proteins which stimulate tubulin polymerization in vitro. *Eur. J. Biochem.* 92:1-8.

Himmler, A., Drechsel, D., Kirschner, M.W., and Martin, D.W., Jr. 1989. Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. *Mol. Cell. Biol.* 9:1381-1388.

Huber, G. and Matus, A. 1984. Differences in the cellular distributions of two microtubule-associated proteins, MAP1 and MAP2, in rat brain. *J. Neurosci.* 4:151-160.

Kosik, K.S., Orecchio, L.D., Bakalis, S., and Neve, R.L. 1989. Developmentally regulated expression of specific tau sequences. *Neuron* 2:1389-1397.

Krauhs, E., Little, M., Kempf, T., Hofer-Warbinek, R., Ade, W., and Ponstingl, H. 1981. Complete amino acid sequence of beta-tubulin from porcine brain. *Proc. Natl. Acad. Sci. U.S.A.* 78:4156-4160.

Langkopf, A., Hammarback, J.A., Muller, R., Vallee, R.B., and Garner, C.C. 1992. Microtubule-associated proteins 1A and LC2. Two proteins encoded in one messenger RNA. *J. Biol. Chem.* 267:16561-16566.

Lewis, S.A., Wang, D.H., and Cowan, N.J. 1988. Microtubule-associated protein MAP2 shares a microtubule binding motif with tau protein. *Science* 242:936-939.

Lindwall, G. and Cole, R.D. 1984. Phosphorylation affects the ability of tau protein to promote microtubule assembly. *J. Biol. Chem.* 259:5301-5305.

Martins, V., Fonseca, L.P., Ferreira, H.A., Graham, D.L., Freitas, P.P., and Cabral, J.S. 2005. Use of magnetoresistive biochips for monitoring of pathogenic microorganisms in water through bioprobes: Oligonucleotides and antibodies. *Technical Proceedings of the 2005 NSTI Nanotechnology Conference and Trade Show* Volume 1. pp. 493-496.

Matus, A. 1988. Microtubule-associated proteins: Their potential role in determining neuronal morphology. *Annu. Rev. Neurosci.* 11:29-44.

Moreno-Herrero, F., de Jager, M., Dekker, N.H., Kanaar, R., Wyman, C., and Dekker, C. 2005. Mesoscale conformational changes in the DNA-repair complex Rad50/Mre11/Nbs1 upon binding DNA. *Nature* 437:440-443.

Moreno-Herrero, F., de Pablo, P.J., Fernández-Sánchez, R., Colchero, J., Gómez-Herrero, J., and, Baró, A.M. 2002. Scanning force microscopy jumping and tapping modes in liquids. *Appl. Phys. Let.* 81:2620-2622.

Murphy, D.B., Vallee, R.B., and Borisy, G.G. 1977. Identity and polymerization-stimulatory activity of the nontubulin proteins associated with microtubules. *Biochemistry* 16:2598-2605.

Noble, M., Lewis, S.A., and Cowan, N.J. 1989. The microtubule binding domain of microtubule-associated protein MAP1B contains a repeated sequence motif unrelated to that of MAP2 and tau. *J. Cell Biol.* 109:3367-3376.

Ornstein, L. 1964. Disc Electrophoresis. I. Background and theory. *Ann N Y Acad. Sci.* 121:321-349.

Pande, R., Ruben, G.C., Um, J.O., Tripathy, S., and Marx, K.A. 1998. DNA bound to polypyrrole films: High-resolution imaging, DNA binding kinetics and internal migration. *Biomaterials* 19:1657-1667.

Pazzagli, C. and Avila, J. 1994. Purification of brain microtubule-associated protein MAP1A. *Neurochem. Res.* 19:1195-1198.

Pedrotti, B., Soffientini, A., and Islam, K. 1993. Sulphonate buffers affect the recovery of microtubule-associated proteins MAP1 and MAP2: Evidence that MAP1A promotes microtubule assembly. *Cell Motil. Cytoskeleton* 25:234-242.

Raab, A., Han, H, Badt, D., Smith-Gill, S.J., Lindsay, S.M., Schindler, H., and Hinterdorfer, P. 1999. Antibody recognition imaging by force microscopy. *Nature Biotechnology* 17:901-905.

Radmacher, M., Fritz, M., Hansma, H.G., and Hansma, P.K. 1994. Direct observation of enzyme activity with the atomic force microscope. *Science* 265:1577-1579.

Ramalho, R.R., Soares, H., and Melo, L.V. 2007. Microtubule behavior under strong electromagnetic fields. *Mat. Sci. Eng.* 27:1207-1210.

Riederer, B. and Matus, A. 1985. Differential expression of distinct microtubule-associated proteins during brain development. *Proc Natl. Acad. Sci. U.S.A.* 82:6006-6009.

Riederer, B., Cohen, R., and Matus, A. 1986. MAP5: A novel brain microtubule-associated protein under strong developmental regulation. *J. Neurocytol.* 15:763-775.

Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J.M., and Gaub, H.E. 1997. Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science* 276:1109-1112.

Round, A.N., Berry, M., McMaster, T.J., Stoll, S., Gowers, D., Corfield, A.P., and Miles, M.J. 2002. Heterogeneity and Persistence Length in Human Ocular Mucins. *Biophys. J.* 83:1661-1670.

Safer, D. 1994. Nondenaturing Polyacrylamide Gel Electrophoresis (NPAGE) as a method for studying protein interactions. *In* Cell Biology, A Laboratory Handbook, Vol. 3 (J.E. Celis, ed.) pp. 218-221. Academic Press, Inc., San Diego, Calif..

Scheuring, S., Ringler, P., Borgnia, M., Stahlberg, H., Müller, D.J., Agre, P., and Engel, A.

1999. High resolution AFM topographs of the *Escherichia coli* water channel aquaporin Z. *EMBO J.* 18:4981-4987.

Schiff, P.B., Fant, J., and Horwitz, S.B. 1979. Promotion of microtubule assembly in vitro by taxol. *Nature* 277:665-667.

Schoenfeld, T.A., McKerracher, L., Obar, R., and Vallee, R.B. 1989. MAP 1A and MAP 1B are structurally related microtubule associated proteins with distinct developmental patterns in the CNS. *J. Neurosci.* 9:1712-1730.

Serrano, L., and Avila, J. 1990. Structure and function of tubulin regions. *In* Microtubule Proteins (J. Avila, ed.) pp. 67-88. CRC Press, Boca Raton, Fla.

Serrano, L., de la Torre, J., Maccioni, R.B., and Avila, J. 1984a. Involvement of the carboxyl-terminal domain of tubulin in the regulation of its assembly. *Proc. Natl. Acad. Sci. U.S.A.* 81:5989-5993.

Serrano, L., Avila, J., and Maccioni, R.B. 1984b. Controlled proteolysis of tubulin by subtilisin: Localization of the site for MAP2 interaction. *Biochem.* 23:4675-4681.

Serrano, L., Valencia, A., Caballero, R., and Avila, J. 1986. Localization of the high affinity calcium-binding site on tubulin molecule. *J. Biol. Chem.* 261:7076-7081.

Shelanski, M.L., Gaskin, F., and Cantor, C.R. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 70:765-768.

Sloboda, R.D. and Rosenbaum, J.L. 1982. Purification and assay of microtubule-associated proteins MAPs. *Methods Enzymol.* 85:409-416.

Sobue, K., Fujita, M., Muramoto, Y., and Kakiuchi, S. 1981. The calmodulin-binding protein in microtubules is tau factor. *FEBS Lett.* 132:137-140.

Sritharan, K.C., Quinn, A.S., Taatjes, D.J., and Jena, B.P. 1998. Binding contribution between synaptic vesicle membrane and plasma membrane proteins in neurons: An AFM Study. *Cell Biology Intern*. 22:650-656.

Tucker, R.P., Binder, L.I., and Matus, A.I. 1988. Neuronal microtubule-associated proteins in the embryonic avian spinal cord. *J. Comp. Neurol.* 271:44-55.

Tucker, R.P., Garner, C.C., and Matus, A.I. 1989. In situ localization of microtubule-associated protein mRNA in the developing and adult rat brain. *Neuron* 2:1245-1256.

Vallee, R.B. 1982. A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins MAPs. *J. Cell. Biol.* 92:435-442.

Vallee, R.B. 1986a. Reversible assembly purification of microtubules without assembly-promoting agents and further purification of tubulin, microtubule-associated proteins, and MAP fragments. *Methods Enzymol.* 134:89-104.

Vallee, R.B. 1986b. Purification of brain microtubules and microtubule-associated protein 1 using taxol. *Methods Enzymol.* 134:104-115.

Vallee, R.B. 1990. Molecular characterization of high molecular weight microtubule-associated proteins: some answers, many questions. *Cell Motil. Cytoskeleton* 15:204-209.

Vallee, R.B., Bloom, G.S., and Luca, F.C. 1986. Differential structure and distribution of the high molecular weight brain microtubule-associated proteins, MAP-1 and MAP-2. *Ann. N.Y. Acad. Sci.* 466:134-144.

Wallwork, M., Kirkham, J., Zhang, J., Smith, D.A., Brookes, S.J., Shore, R.C., Wood, S.R., Ryu, O., and Robinson, C. 2001. Protein binding to biological hydroxyapatite crystals characterised by atomic force microscopy. *Langmuir* 17:2508-2513

Weingarten, M.D., Lockwood, A.H., Hwo, S.Y., and Kirschner, M.W.,1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U.S.A.* 72:1858-1862.

Weisenberg, R.C. 1972. Microtubule formation in vitro in solutions containing low calcium concentrations. *Science* 177:1104-1105.

Williams, R.C., Jr. and Detrich, H.W., 3rd 1979. Separation of tubulin from microtubule-associated proteins on phosphocellulose. Accompanying alterations in concentrations of buffer components. *Biochemistry* 18:2499-2503.

Williams, R.C., Jr. and Lee, J.C. 1982. Preparation of tubulin from brain. *Methods Enzymol.* 85:376-385.

Zabala, J.C. and Cowan, N.J. 1992. Tubulin dimer formation via the release of alpha- and beta-tubulin monomers from multimolecular complexes. *Cell Motil. Cytoskeleton.* 3:222-230.

Internet Resources

Web sites for protocols and advice on microtubule preparation, tubulin purification, and handling:

http://mitchison.med.harvard.edu/protocols/tubprep.html

Web site for large scale tubulin preparation protocol.

http://mitchison.med.harvard.edu/protocols/recycle.html

http://www.bio.com/protocolstools/protocol.jhtml?id = p1426

http://www.ciwemb.edu/labs/koshland/Protocols/MICROTUBULE/cyclingmicrotub.html

Web sites for handling recycled tubulin.

http://www.bio.com/protocolstools/protocol.jhtml?id = p1434

Protocol for tubulin/MAP co-sedimentation under high ATP conditions and MAP/motor/tubulin co-sedimentation.

http://www.bio.com/protocolstools/protocol.jhtml?id = p1600

In this protocol purified tubulin is stabilized with Taxol.

http://www.bio.unc.edu/faculty/salmon/lab/protocolsporcinetubulin.html

Web site for tubulin purification from 3 pig brains similar to Basic Protocol 1.

http://www.borisylab.northwestern.edu/pages/protocols.html

Web page for microtubule protein preparation.

Purification of Intact Chloroplasts from *Arabidopsis* and Spinach Leaves by Isopycnic Centrifugation

Daphné Seigneurin-Berny,[1] Daniel Salvi,[1] Jacques Joyard,[1] and Norbert Rolland[1]

[1]Laboratoire de Physiologie Cellulaire Végétale, CNRS and Université Joseph Fourier, Grenoble, France

ABSTRACT

Chloroplasts are plant-specific organelles. They are the site of photosynthesis but also of many other essential metabolic pathways, such as syntheses of amino acids, vitamins, lipids, and pigments. This unit describes the isolation and purification of chloroplasts from *Arabidopsis* and spinach leaves. Differential centrifugation is first used to obtain a suspension enriched in chloroplasts (crude chloroplasts extract). In a second step, Percoll density gradient centrifugation is used to recover pure and intact chloroplasts. The Basic Protocol describes the purification of chloroplasts from *Arabidopsis* leaves. This small flowering plant is now widely used as a model organism in plant biology as it offers important advantages for basic research in genetics and molecular biology. The Alternate Protocol describes the purification of chloroplasts from spinach leaves. Spinach, easily available all through the year, remains a model of choice for the large-scale preparation of pure chloroplasts with a high degree of intactness. *Curr. Protoc. Cell Biol.* 40:3.30.1-3.30.14. © 2008 by John Wiley & Sons, Inc.

Keywords: chloroplast • *Arabidopsis* • spinach • purification • leaves

INTRODUCTION

Plastids are semiautonomous organelles found in plants and some protists. In plant leaves, plastids are photosynthetically active and named chloroplasts. They are also the site of essential metabolic pathways, such as syntheses of amino acids, vitamins, lipids, and pigments (Wise and Hoober, 2006). Biochemical, physiological, and proteomic analyses of the chloroplast can only be achieved using pure and intact chloroplasts that have conserved their metabolic activities and functional surrounding envelope membranes (Block et al., 2007). Localized at the interface between the plastid stroma and the cytosol, the envelope membranes have to remain intact to allow, for instance, physiological characterization of the various transport systems regulating plastid metabolism. Therefore, the isolation of pure and intact organelles becomes essential when characterization of plastid functions is expected. The procedure described here couples differential centrifugation to obtain a crude extract of chloroplasts with isopycnic centrifugation (Percoll-based density gradients) to recover pure and intact chloroplasts.

The Basic Protocol describes the step-by-step purification procedure to isolate chloroplasts from *Arabidopsis* leaves. Since the complete sequencing of its genome (The AGI, 2000), *Arabidopsis thaliana*, a small flowering plant, has become a widely used model organism in plant biology. The generation of large collections of insertion mutants, together with growing numbers of gene expression databases, offer important advantages for basic research in genetics and molecular biology. This Basic Protocol, to isolate chloroplasts from *Arabidopsis thaliana*, is provided with some tricks to optimize yield and purity. Conditions to grow *Arabidopsis* plants suitable for chloroplast isolation are also described in the Support Protocol.

Current Protocols in Cell Biology 3.30.1-3.30.14, September 2008
Published online September 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0330s40

The Alternate Protocol describes the purification of chloroplasts from spinach (*Spinacia oleracea* L.) leaves. Due to its almost permanent availability in markets, this plant remains a traditional model plant for physiologists and biochemists. Spinach also contains low levels of phenolics and other inhibitory compounds (prevalent in numerous cultivated species), and thus is a good choice to easily obtain fully competent CO_2-fixing chloroplasts. Finally, the Alternate Protocol allows isolating large amounts of pure and intact chloroplasts.

BASIC PROTOCOL

ISOLATION OF PURE AND INTACT CHLOROPLASTS FROM *ARABIDOPSIS* LEAVES

The chloroplast is, in general, an extremely fragile structure. Thus, skill and expertise are required in order to avoid large-scale rupture of the two envelope membranes during chloroplast isolation. The media used throughout the procedure must contain an osmoticum (0.4 M sorbitol for *Arabidopsis* chloroplast) to avoid osmotic shock and thus, disruption of the organelles. The homogenization of the tissue is another critical step. The grinding process must be as short as possible. Longer blending, while apparently strongly improving the yield of recovered chlorophyll, rapidly increases the proportion of broken chloroplasts in the crude cell extract and strongly affects further purification steps. Filtration of the homogenate then allows limiting contamination with cellular debris and unbroken cells. A first centrifugation allows obtaining a crude chloroplast suspension. Intact chloroplasts are then purified by isopycnic centrifugation on preformed continuous Percoll gradients. The outline of the strategy is described in Figure 3.30.1.

Materials

Percoll gradients solution (see recipe)
Four to six boxes containing 3- to 4-week-old *Arabidopsis* plantlets (400 to 500 g of rosette material, see Support Protocol)
Grinding medium (see recipe)
Washing medium (see recipe)
80% (v/v) acetone

50-ml polypropylene tubes
Superspeed refrigerated centrifuge (e.g., Sorvall RC5), with the following rotors and corresponding tubes: fixed angle rotors GS-3 with six 500-ml plastic bottles and SS34 with eight 50-ml polypropylene tubes; swinging bucket rotor HB-6 with six 50-ml polycarbonate tubes or equivalent
1000-ml and 5-liter beakers
Ice and ice buckets
Motor-driven blender, 3 speeds, 1 gallon (3.785 liter; e.g., Waring blender)
Muslin or cheesecloth, 80-cm
Nylon blutex (50-μm aperture; Tripette et Renaud)
Pasteur pipet
Vacuum aspirator with a flask as a liquid trap
Curved plastic spatula
1- and 10-ml pipets
1-ml microcentrifuge tube
Vortex
1-ml spectrophotometer glass cuvette
UV spectrophotometer

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: All operations are carried out at 0° to 5°C either by keeping samples on ice or by working in a cold room.

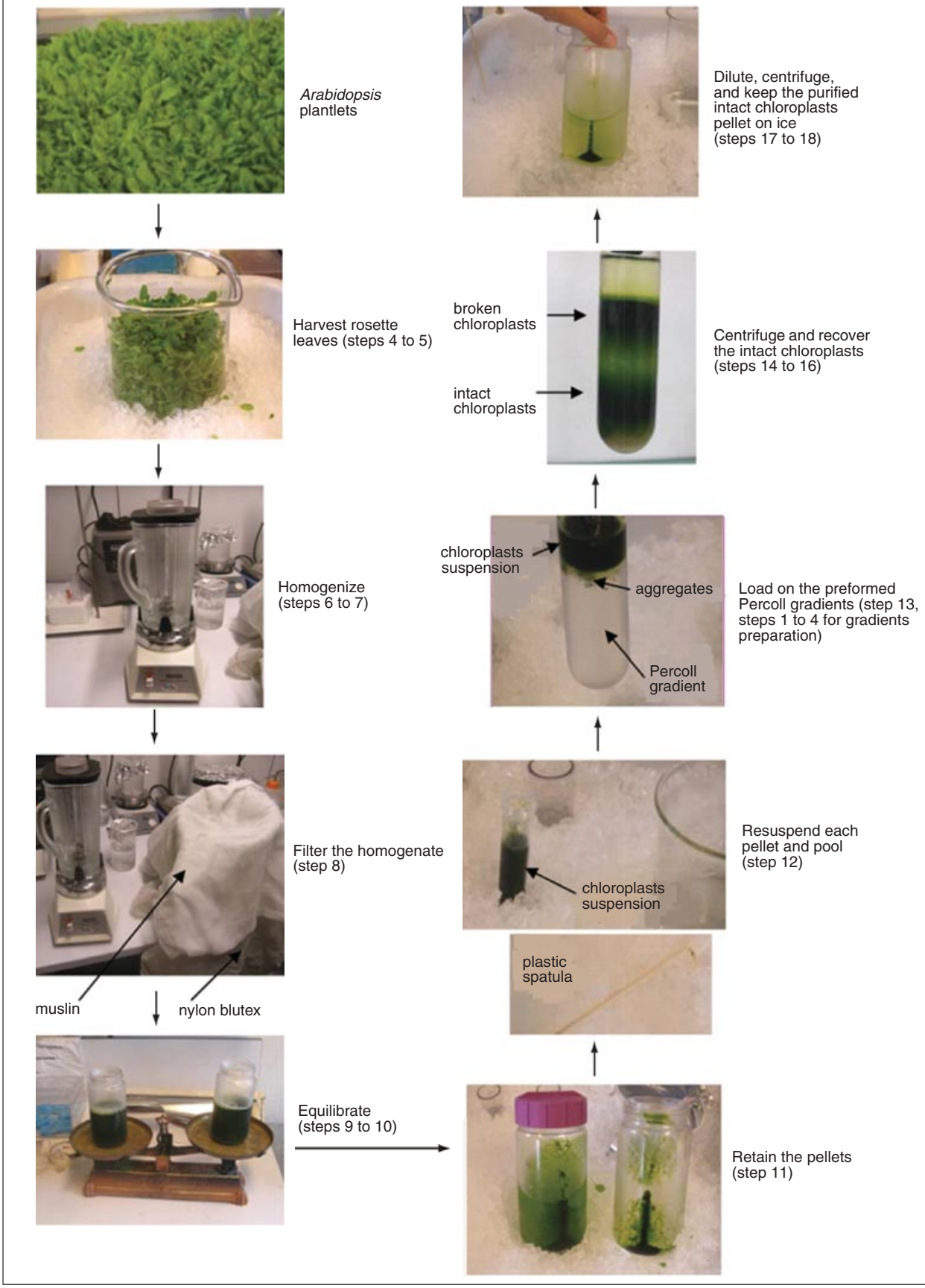


Figure 3.30.1 Outline of the strategy used to purify intact chloroplasts from *Arabidopsis* leaves. For color version of this figure see *http://www.currentprotocols.com*.

Prepare preformed continuous Percoll gradient

1. Prior to the experiment, prepare six 50-ml polypropylene tubes containing 30 ml Percoll gradients solution.

2. Preform Percoll gradients for chloroplast purification by centrifuging 55 min at 38,700 × *g* (Sorvall SS-34 rotor), 4°C.

   Vertical rotors can easily be used to obtain preformed Percoll gradients and subsequently purify chloroplasts (Douce and Joyard, 1982). It is recommended that the brake be disconnected or that the automatic rate controller (if available) of the centrifuge be used to prevent mixing of the gradients at the critical stage of deceleration.

3. Store the tubes containing preformed continuous Percoll gradients in the cold room until use.

   Percoll gradient can be prepared 1 or 2 days before the experiment.

Prepare Arabidopsis leaves and homogenize the tissue

4. Harvest 400 to 500 g of rosette leaves from boxes containing 3- to 4-week-old *Arabidopsis* plantlets.

   It is tempting to start from large amounts of material (huge rosettes with large leaves). It however appears that staring from younger leaves (3- to 4-week-old) improves yield, purity, and integrity of the purified organelles.

   The number of starch granules present in chloroplasts is critical for the preparation of intact chloroplasts: chloroplasts containing large starch grains will usually be broken during centrifugation (Douce and Joyard, 1982). Therefore, prior to the experiment, the plants should be kept for several hours in a dark and cold room (4°C) to reduce the amount of starch. A good way to proceed is to place the plants under such conditions the day before the extraction (we usually perform this at the beginning of the afternoon prior to the day of the experiment).

5. Preweigh a 1000-ml beaker, cool the beaker on ice, then place the rosette leaves inside the beaker. Reweigh the beaker and record the tissue weight. Transfer the beaker to a cold room for the next steps.

6. Put the leaf material and enough leaf grinding buffer (with BSA) to cover the leaves (usually ~2 liters of grinding medium for 400 to 500 g leaves) in the blender cup.

   The buffer to tissue ratio is 4 ml/g.

   A minimum of 60 g of leaves is required to obtain intact and purified chloroplasts.

7. Homogenize the leaf material three times, each time 2 sec in a Waring blender at high speed (see Video 1 at *http://www.currentprotocols.com*).

   The grinding process should be as short as possible. Longer blending improves the yield of recovered chlorophyll, but increases the proportion of broken chloroplasts in the homogenate, and then affects further purification steps.

8. Rapidly filter the homogenate through 4 to 5 layers of muslin and one layer of nylon blutex that line the inside of a precooled funnel placed over a 5-liter beaker. Collect the filtered homogenate in the beaker below the funnel. Gently squeeze the homogenate leaves inside the muslin/nylon blutex bag (see Video 1 at *http://www.currentprotocols.com*), to extract all the liquid.

   The filtration process should also be rapidly performed. After the grinding process, organelles are released in a crude extract consisting of broken cells, having released some very aggressive compounds (e.g., proteases, lipases, and phenolic compounds). Storing the organelles for too long in such a homogenate strongly affects their integrity and functioning. A short delay between grinding (step 7) and concentration (step 10) is thus expected to improve yield of intact chloroplasts.

9. Put the remaining tissue in the blender cup with enough grinding buffer to cover the tissue, and repeat steps 7 and 8. Collect the filtered homogenate in the same beaker.

Centrifuge the homogenate

10. Equally distribute the filtered suspension into six 500-ml bottles for centrifugation and centrifuge them 2 min at 2070 × *g* (Sorvall GS-3 rotor), 4°C.

    Do not forget to equilibrate the pairs of bottles on a balance prior to centrifugation.

11. Carefully discard the supernatant from each tube, retaining the pellets (pour out the liquid holding the tube with the pellet on the top of the tube; see Video 1 at *http://www.currentprotocols.com*). Remove the remaining supernatant with a Pasteur pipet connected to a vacuum aspirator with a flask as a liquid trap. Keep the pellets on ice.

    These pellets contain crude chloroplast fractions.

Purify the chloroplasts

12. Carefully resuspend each pellet, representing crude chloroplast fraction, by adding a minimal volume of washing medium using a curved plastic spatula (see Video 1 at *http://www.currentprotocols.com*).

    Use a 10-ml pipet to add 2 to 3 ml of washing medium in each tube. Carefully resuspend each pellet and gently aspirate the resuspended chloroplasts with a 10-ml pipet (do not use pipets with very fine tips, to limit mechanical breaking of the organelles).

    In order to homogenize chloroplast aggregates found in the bottom of the tube, carefully mix the suspension.

13. Pool the resuspended chloroplasts in one tube. Rinse each tube with additional washing medium.

    The final volume should not exceed 36 ml.

14. Load the crude chloroplast suspension on the top of the six preformed Percoll gradients (6 ml per tube).

    For this step, carefully tilt the Percoll gradient tube and load the chloroplast suspension very slowly, trying not to mix the chloroplast suspension with the upper Percoll layer. Again, do not use pipets with too fine tips.

15. Centrifuge the gradients 10 min at 13,300 × *g* (Sorvall swinging HB-6 rotor), 4°C. Carefully remove the upper part of the gradient containing the broken chloroplasts (Fig. 3.30.1) by aspirating with a Pasteur pipet connected to a vacuum aspirator with a flask as a water trap (see Video 1 at *http://www.currentprotocols.com*).

    It is recommended the brake be disconnected or that the automatic rate controller (if available) be used to prevent mixing of the gradients at the critical stage of deceleration.

16. Recover intact chloroplasts (a broad dark green band in the lower part of the gradient) with a 10-ml pipet. Gently pool the intact chloroplasts in a 500-ml plastic bottle.

    Broken chloroplasts are present in the upper part as a broad band. A small pellet containing cell pieces and large debris is found at the bottom of the tube. To limit cross-contamination with other cell fractions, do not aspirate this debris with the intact chloroplasts.

17. Dilute the chloroplast suspension 3- to 4-fold (up to a final volume of 500 ml) with 200 to 300 ml washing medium. Centrifuge the suspension 2 min at 2070 × *g* (Sorvall GS-3 rotor), 4°C.

18. Discard the supernatant and keep the purified intact chloroplasts pellet on ice.

    At this stage, the yield of intact chloroplasts is 50 to 60 mg protein.

19. Before use, add a minimal volume (~1 ml) of washing medium, or any other buffer containing an osmoticum, to resuspend the purified chloroplasts. Keep the purified chloroplast as a concentrated suspension on ice.

The excellent purity of the Arabidopsis chloroplasts prepared by the Percoll purification step was confirmed using immunological detection of marker enzymes from other subcellular compartments (Seigneurin-Berny et al., 2006). It is worth mentioning that mitochondrial and plasma membrane markers were not detected in the Percoll-purified Arabidopsis chloroplasts. This is also in good agreement with proteomic analyses, which demonstrated that only 5% (6 out of 112) of the Arabidopsis proteins identified in purified chloroplast envelope membranes (Ferro et al., 2003) may correspond to nonplastid proteins. Among them, only one protein appeared to correspond to a previously characterized major plasma membrane component; four proteins may indicate contamination by major tonoplast proteins, and one from glyoxysomes. Considering the high sensitivity of present mass spectrometers it is not surprising to detect minute amounts of these few extra-plastidial contaminants, which are major proteins in their respective subcellular compartments.

Quantify chlorophyll content

20. Quantify the chlorophyll content according to Bruinsma (1961). Add 10 µl of the purified chloroplasts to 1 ml 80% (v/v) acetone in a 1-ml microcentrifuge tube.

21. Vortex the mixture, incubate on ice and in the dark for 15 min, and centrifuge 15 sec at 16000 × *g*, 4°C.

22. Pour in a 1-ml spectrophotometer glass cuvette. Measure the absorbance at 652 nm against a tube containing 80% (v/v) acetone for the zero.

A ratio of $OD_{652}/36 = 1$ corresponds to 1 mg chlorophyll ml^{-1}.

23. Determine integrity of the organelle preparation.

In addition to morphological analysis by microscopy, the integrity of the purified chloroplasts can be confirmed using an oxygen electrode and performing the ferricyanide test for intactness (Walker, 1990). When lacking microscopy or oxygen electrode facilities, a simple way to estimate the integrity of the purified chloroplasts relies on the use of an SDS-PAGE analysis. This method relies on the demonstration that soluble proteins from the stroma are not lost during the purification procedure (Fig. 3.30.2). A protein profile obtained from pure and intact chloroplast preparations reveals that the amount of major soluble protein (RbcL or RubisCO) from the stroma is higher than the amount of the major thylakoid membrane proteins (LHCPs or Light Harvesting Complex Proteins). When broken chloroplasts are analyzed, and due to the loss of soluble proteins resulting from the breakage of the limiting envelope membranes, the ratio of RbcL/LHCPs is strongly reduced. This is illustrated in the Fig. 3.30.2, using broken or intact chloroplasts as obtained, respectively, from upper and lower layers of the Percoll-density gradient.

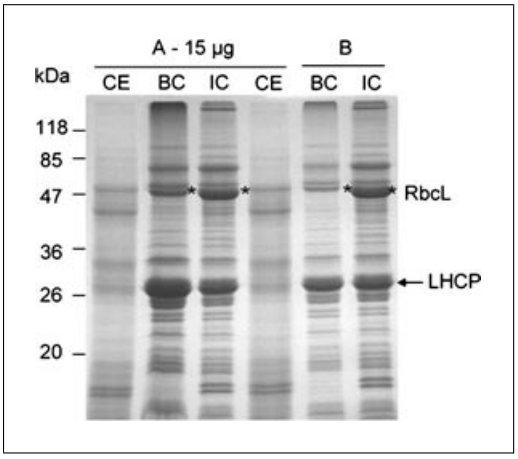


Figure 3.30.2 SDS-PAGE analysis of Percoll-purified chloroplasts from *Arabidopsis*. Fractions (15 µg protein) were analyzed on a 12% SDS-PAGE followed by Coomassie-blue staining. In lanes B, the amount of protein was calculated in order to load the same amount of LHCP in the two lanes of the gel. The star and the arrow respectively indicate the major soluble protein from the stroma, RbcL, and the major membrane proteins from the thylakoids, the LHCPs. Note that the ratio of RbcL/LHCPs is strongly reduced in broken chloroplasts, as a consequence of breakage of the limiting envelope membranes and thus of loss of soluble proteins from the stroma. Abbreviations: CE: crude extract, BC: broken chloroplasts, IC: intact chloroplasts.

ISOLATION OF PURE AND INTACT CHLOROPLASTS FROM SPINACH LEAVES

ALTERNATE PROTOCOL

Spinach leaves remain a tissue of choice for the preparation of chloroplasts with a high degree of intactness. They can be obtained in large amount from markets all through the year, and thus do not require plant growth facilities. Furthermore, chloroplasts isolated from spinach leaves are less fragile than chloroplasts purified from *Arabidopsis* leaves, and due to the unlimited availability of starting material, large amounts of chloroplasts can be obtained daily. Several important differences exist between the protocols used to purify spinach or *Arabidopsis* (Basic Protocol) chloroplasts. First, as starting material can be obtained in large amount, volumes of buffers or numbers of bottles and tubes need to be adapted. Second, the composition of buffers varies (the osmoticum used is sucrose instead of sorbitol). Finally, and as another difference between the two protocols described here, spinach chloroplasts are purified on a discontinuous Percoll gradient (Douce and Joyard, 1982) instead of preformed continuous Percoll gradient.

Materials

3 kg of spinach leaves from the market
Spinach leaf grinding medium (see recipe)
Ice
Spinach washing medium (see recipe)
40% (v/v) Percoll and 80% (v/v) Percoll solutions (see recipes)

Motor-driven blender, 3 speeds, 1 gallon (3.785 liter; Waring blender)
Muslin or cheesecloth, 80-cm
Nylon blutex (50-μm aperture; Tripette et Renaud)
5-liter beakers
Superspeed refrigerated centrifuge (Sorvall RC5), with the following rotors and corresponding tubes: fixed angle rotors GS-3 with six 500-ml plastic bottles and SS34 with eight 50-ml polypropylene tubes; swinging bucket rotor HS-4 with four 150-ml polycarbonate tubes or equivalent
Pasteur pipet
Vacuum aspirator with a flask as a liquid trap
Ice buckets
Curved plastic spatula
1- and 10-ml pipets
250-ml cylinder

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: All operations are carried out at 0° to 5°C either by keeping samples on ice or by working in a cold room.

Prepare spinach leaves and homogenize the tissue

1. Wash the spinach leaves and then remove large veins from the leaves. Strain the leaves and store them in the dark and in a cold room (4°C) overnight.

   The overnight storage in the dark reduces the amount of starch granules in the spinach leaves (as for Arabidopsis leaves).

2. Put the leaf material and enough spinach leaf grinding buffer (with BSA) to cover the leaves (usually ~3 liters of spinach leaf grinding medium for 3 kg leaves) in the blender cup.

3. Homogenize the leaf material two times, each time 2 to 3 sec in a Waring blender at low speed and a third time (2 to 3 sec) at high speed.

   The grinding process should be as short as possible (see Basic Protocol, step 7).

4. Rapidly filter the homogenate through 4 to 5 layers of muslin and one layer of nylon blutex as described in Basic Protocol, step 8. Collect the filtered homogenate in a 5-liter beaker.

   Proceed rapidly.

Centrifuge the homogenate

5. Equally distribute the filtered suspension into 500-ml bottles for centrifugation and centrifuge them 10 min at 1200 × *g* (Sorvall GS-3 rotor), 4°C.

   Do not forget to equilibrate the pairs of bottles on a balance.

6. Carefully discard the supernatant from each bottle, retaining the pellets. Remove the remaining supernatant with a Pasteur pipet connected to a vacuum aspirator with a flask as a liquid trap. Keep the pellets on ice.

   These pellets contain crude chloroplast fraction.

Purify the chloroplasts by isopycnic centrifugation

7. Carefully resuspend each pellet, containing a crude chloroplast fraction, by adding 10 ml spinach washing medium using a curved plastic spatula.

   Carefully resuspend each pellet and gently aspirate the resuspended chloroplasts with a 10-ml pipet (do not use pipets with very fine tips).

8. Pool the chloroplast suspension in one tube. Rinse all tubes with additional spinach washing medium.

   The final volume should not exceed 200 ml.

9. Filter the crude chloroplast suspension through one layer of nylon blutex in order to separate the aggregates, and adjust the final volume to 200 ml with spinach washing buffer using a 250-ml cylinder.

10. Prepare the discontinuous Percoll gradients by successively layering 20 ml of 80% and 50 ml of 40% Percoll solutions in four separate 150-ml polycarbonate tubes.

11. Load the crude chloroplast suspension on the top of the discontinuous Percoll gradients (50 ml per tube).

    Carefully layer the chloroplast suspension on the top of the gradients trying not to mix the chloroplast suspension with the upper Percoll layer. Again, do not use pipets with too fine tips.

12. Centrifuge the gradients 20 min at 3000 × *g* (Sorvall swinging-bucket HS-4 rotor), 4°C.

    Intact chloroplasts are recovered at the bottom interface (80%/40% Percoll) and broken chloroplasts + extrachloroplastic membrane systems form a band at the upper interface (sample/40% Percoll; Fig. 3.30.3).

    It is recommended that the brake be disconnected or that the automatic rate controller (if available) be used to prevent mixing of the gradients at the critical stage of deceleration.

13. Carefully remove the upper part of the gradient containing the broken chloroplasts by aspirating with a Pasteur pipet connected to a vacuum aspirator with a flask as a liquid trap.

14. Recover intact chloroplasts (at the interface 80%/40% Percoll) with a 10-ml pipet. Gently pool the intact chloroplasts in a 500-ml plastic bottle.

15. Dilute the chloroplast suspension 3- to 4-fold (up to a final volume of 500 ml) with the spinach washing medium (500 ml final). Centrifuge the suspension 5 min at 4000 × *g* (Sorvall GS-3 rotor), 4°C.

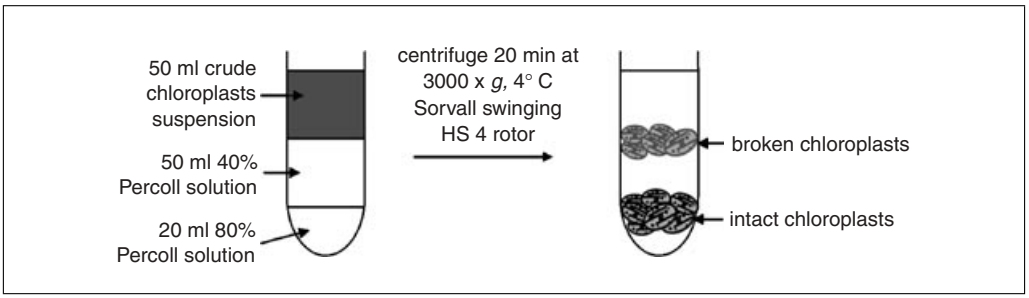


Figure 3.30.3 Schematic representation of the discontinuous Percoll-gradient fractionation of the crude spinach chloroplasts preparation to yield intact and broken chloroplasts.

16. Carefully discard the supernatant and repeat the wash step. Gently resuspend the purified pellet with 150 ml of spinach washing medium and equally distribute the suspension in 50-ml polypropylene tubes.
17. Centrifuge 5 min at 3000 × *g* (Sorvall SS34 rotor), 4°C.
18. Discard the supernatant and keep the pellet (purified intact chloroplasts) on ice.

At this stage, the yield of intact spinach chloroplasts is 150 to 200 mg chlorophyll or 2.5 to 4 g proteins.

After this purification step, the chloroplast preparation is devoid of enzymatic activities such as NADH:cytochrome c oxidoreductase, fumarase, catalase, glycolate oxidase, or nitrate reductase, indicating that it is essentially free of contamination by endoplasmic reticulum, mitochondria, peroxisomes, or cytoplasmic proteins.

Integrity of the chloroplast obtained is determined by phase contrast microscopy (Douce and Joyard, 1982), using an oxygen electrode and the ferricyanide test for intactness (Walker, 1990), or by SDS-PAGE analysis (Fig. 3.30.2).

GROWING OF *ARABIDOPSIS* PLANTLETS

SUPPORT PROTOCOL

Rosette leaves from 3- to 4-week-old *Arabidopsis thaliana* plantlets are required for isolation of the chloroplasts.

Materials

Arabidopsis thaliana seeds
Compost

Large plastic cases (30-cm × 45-cm)
Growth rooms

1. Fill large (30-cm × 45-cm) plastic cases with compost and water.
2. Sow seeds onto the surface of the compost by scattering them carefully at a high density (~30 mg of seeds for a whole box).
3. Grow *A. thaliana* plantlets in growth rooms with a 12-hr light cycle at 23°C (day)/18°C (night) with a light intensity of 150 μmol.m^{-2}sec^{-1}.

Four to six boxes containing 3- to 4-week-old Arabidopsis plantlets are expected to provide 400 to 500 g of rosette material.

4. Harvest the rosette leaves when they are ~1 to 2 cm (~3 to 4-week-old).

The chloroplasts purification procedure described in this unit was applied efficiently to both Columbia and Wassilewskija ecotypes of Arabidopsis.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

EDTA, pH 8

104.05 g EDTA (0.5 M final)
Adjust pH to 8 using NaOH
Add H_2O to 500 ml
Store up to 6 months at 4°C

Grinding medium

291.5 g sorbitol (0.4 M final)
80 ml 1 M Tricine buffer, pH 8.4 (20 mM final; see recipe)
80 ml 0.5 M EDTA, pH 8 (10 mM final; see recipe)
3.36 g $NaHCO_3$ (10 mM final)
Adjust to pH 8.4 using NaOH
Add H_2O to 4 liters
Store up to 1 to 2 days at 4°C
Add 4 g BSA (0.1% w/v) just before use

MOPS buffer, pH 7.8

41.85 g MOPS (1 M final)
Adjust to pH 7.8 using NaOH
Add H_2O to 200 ml
Store up to 6 months at 4°C

Percoll gradients solution

Mix 1 vol of Percoll with 1 vol of 2× washing medium stock solution (see recipe) to obtain a 50% (v/v) Percoll/0.4 M sorbitol solution.

Percoll solution, 40% (v/v)

20.6 g sucrose (0.33 M final)
80 ml Percoll (Pharmacia; 40% v/v final)
2 ml 1 M MOPS (10 mM final; see recipe)
Adjust to pH 7.8 using NaOH
Add H_2O to 200 ml
Store up to 1 to 2 days at 4°C

Percoll solution, 80% (v/v)

10.3 g sucrose (0.33 M final)
80 ml Percoll (80% v/v final)
1 ml 1 M MOPS (10 mM final; see recipe)
Adjust to pH 7.8 using NaOH
Add H_2O to 100 ml
Store up to 1 to 2 days at 4°C

Spinach leaf grinding medium

451 g sucrose (0.33 M final)
30 g pyrophosphate (30 mM final)
Adjust to pH 7.8 using NaOH
Add H_2O to 4 liters
Store up to 1 to 2 days at 4°C
Add 4 g BSA (0.1% w/v final) just before use

Spinach washing medium

112 g sucrose (0.33 M final)
20 ml 1 M MOPS (20 mM final; see recipe)
Adjust to pH 7.8 using NaOH
Add H_2O to 1 liter
Store up to 1 to 2 days at 4°C

Tricine buffer, pH 7.6

89.6 g Tricine (1 M final)
Adjust to pH 7.6 with KOH
Add H_2O to 500 ml
Store up to 6 months at 4°C

Tricine buffer pH 8.4

89.6 g Tricine (1 M final)
Adjust to pH 8.4 with KOH
Add H_2O to 500 ml
Store up to 6 months at 4°C

Washing medium

2× stock solution:
73 g sorbitol (0.8 M final)
20 ml 1 M Tricine buffer, pH 7.6 (40 mM final; see recipe)
5 ml 1 M $MgCl_2$ (5 mM final)
5 ml 0.5 M EDTA, pH 8 (2.5 mM final; see recipe)
Adjust to pH 7.6 using NaOH
Add H_2O to 500 ml
Store up to 1 to 2 days at 4°C

1× working solution:
Add 100 ml of 2× washing medium stock (see recipe) solution to 100 ml distilled water.

The final concentrations in the working solution are 0.4 M sorbitol, 20 mM Tricine, 2.5 mM $MgCl_2$, and 1.25 mM EDTA.

COMMENTARY

Background Information

The development of methods for the isolation of functional chloroplasts from plant tissue has been a long process, starting from the late 1930s when Robert Hill prepared chloroplast fractions shown to evolve oxygen in presence of an artificial oxidant, but not in presence of CO_2 (Hill, 1937, 1939). For a long time, chloroplast preparation was restricted to the isolation of green particles capable of supporting the Hill reaction. Arnon and coworkers (for instance Arnon et al., 1954; Allen et al., 1955) demonstrated CO_2-dependent O_2 evolution by chloroplast suspension, but the rates were considerably lower than those of intact leaves. It is only when the importance of maintaining chloroplast integrity throughout the preparation process was fully recognized that preparations showing high rates of CO_2-dependent O_2 evolution were obtained (Walker, 1964; Kalberer et al., 1967; Walker and Hill, 1967; Walker, 1971). Indeed, the use of sugar as an osmoticum (as suggested initially by Hill) together with a very brief homogenization and rapid separation allowed the isolation of chloroplasts with an intact envelope (Walker, 1964). Furthermore, Jensen and Bassham (1966) significantly improved the medium, especially the buffer, used for chloroplast isolation. Thus, in the late 1960s, methods to prepare physiologically active chloroplasts by differential centrifugation were widely available. Indeed, such chloroplast preparations paved the way for an extensive characterization of metabolite transport and mechanisms across the envelope membranes, studies of protein,

and lipid synthesis. For instance, Heber and Santarius (1970) demonstrated the exchange of ATP and ADP across the chloroplast envelope, whereas Heldt and Sauer (1971) identified the inner chloroplast envelope membrane as the site of specific metabolite transport. One should also mention the parallel development of non-aqueous methods to prepare chloroplast (for example, see Stocking, 1959), with the aim of analyzing the intracellular distribution of water-soluble molecules between plastids and the cytosol. Despite their interest, the development of such procedures remained limited.

Although physiologically and structurally intact, chloroplast fractions prepared by differential centrifugation were actually rather crude since they contained pieces derived from other cell compartments (e.g., nuclei and mitochondria) and even a few intact cells. The next step was therefore to remove these contaminants and prepare chloroplasts as pure as possible. Sucrose was first used for preparing gradients to separate chloroplasts (Leech, 1964), and then various media like colloidal silica-derived compounds (such as Ludox) were developed (for example, see Morgenthaler and Price, 1974). Unfortunately, chloroplasts purified on sucrose gradients were unable to perform CO_2-dependent oxygen evolution, mostly because the envelope became leaky during the course of the centrifugation at a high sucrose concentration. Actually, the development of Percoll (review by Pertoft, 2000) was the major breakthrough that allowed (around 1980) the development of procedures to prepare intact and almost pure chloroplasts from a wide variety of tissues (for example, see Takabe et al., 1979; Grant and Wright, 1980; Ortiz et al., 1980; Mourioux and Douce, 1981). Percoll-purified chloroplasts were able to achieve CO_2-dependent oxygen evolution almost identical (on a chlorophyll basis) to that of the leaves (Mourioux and Douce, 1981). Percoll has several key characteristics for the purification of physiologically active organelles: (1) Percoll is made of silica beads of various size and is highly suitable to the formation of density gradients that can be self generated just by high-speed centrifugation; (2) Percoll is an inert compound, its low osmolarity does not change that of the assay medium, even at a high concentration, thus maintaining the integrity of the organelle structure during density gradient centrifugation; (3) in Percoll, silica beads are coated with polyvinylpyrrolidone, which helps remove harmful phenolic compounds from the medium. In the early 1990s, a large number of publications were produced with the term "Percoll" as a keyword (i.e., 50 papers in 1990, 241 in 1991, and 224 in 1992). Screening the literature with the term "Percoll" now identifies >5000 published papers, thus demonstrating the importance of a powerful method—density gradient centrifugation—applicable to any cells or organelles in suspension for which differences in size or buoyant density exist (Pertoft, 2000).

Chloroplast purification was an essential step to prepare and characterize envelope membranes (Douce and Joyard, 1979). Since in chloroplasts, envelope membranes only represents a minor fraction (1% to 2% of the total chloroplast proteins), any contamination of the initial chloroplast fraction is expected to represent, at the end, a major proportion of the fraction enriched in envelope membranes. Indeed, Douce et al. (1973) used sucrose-purified chloroplasts to prepare and characterize envelope membranes from spinach chloroplasts, whereas Percoll-purified chloroplasts were used by Cline et al. (1981) and Block et al. (1983a,b) to prepare and characterize inner and outer envelope membranes from pea and spinach, respectively.

The Alternate Protocol described here to perform Percoll purification of pure and intact chloroplasts from spinach leaves essentially relies on a method published by Douce and Joyard (1982). More recently, and since the complete sequencing of the *Arabidopsis* genome (The AGI, 2000), *Arabidopsis thaliana* has become a widely used model organism, supplanting spinach and pea as model plants. Somerville et al. (1981) had already described the isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*. However, the method did not rely on the use of Percoll-gradients to isolate the organelles. More recently, Kuntz (1998) and Ferro et al. (2003) published alternative methods for the Percoll-based purification of chloroplasts from *Arabidopsis*. The Basic Protocol presented here, to isolate chloroplasts from *Arabidopsis thaliana*, is adapted from these previously published protocols, and provided with some tricks to optimize yield and purity.

Critical Parameters and Troubleshooting

There are several essential points to take into consideration for successful purification of chloroplasts, e.g., the leaf material, medium composition, and critical steps of the protocol.

The best results are obtained when starting from 3- to 4-week-old *Arabidopsis* rosette leaves. Leaves that are too old are enriched in phenolic compounds that are known to affect integrity of the chloroplasts (Walker, 1990). The quantity of leaves is also critical. No intact chloroplasts can be recovered when the leaf starting material is <50 g. The day before the extraction, put the plants (*Arabidopsis* or spinach) overnight at 4°C in the dark to reduce the amount of starch in leaves. Chloroplasts containing large starch grains will generally be broken during centrifugation (Douce and Joyard, 1982).

For *Arabidopsis* chloroplast purification, we found that the optimal osmoticum concentration was 0.4 M sorbitol instead of 0.33 M (Kuntz, 1998). Addition of EDTA in the grinding and washing media is necessary to recover intact *Arabidopsis* chloroplasts as EDTA has a protective effect (Sommerville et al., 1981).

When purification is performed with less material than the amount described in the protocols described here, the number of Percoll gradient tubes should also be reduced (e.g., use only one preformed Percoll gradient when starting from 60 g of *Arabidopsis* leaves). The tissue must remain cold throughout the protocol to help reduce endogenous protease activities. The grinding process must be as short as possible. Longer blending improves the yield of recovered chlorophyll, but increases the proportion of broken chloroplasts.

The crude chloroplast suspension obtained after homogenization of leaves that are too young is mainly composed of broken chloroplasts. Once the leaf material has been homogenized, rapidly filter and centrifuge the homogenate to protect chloroplasts from activities of proteases released after disruption of the cells. Addition of BSA immediately before use of the grinding medium helps to limit the activity of proteases.

Anticipated Results

Using the Basic Protocol for *Arabidopsis* chloroplast purification, an average of 54 mg chloroplast proteins can be purified from 500 g of 3- to 4-week-old *Arabidopsis* leaves. Excellent purity of these Percoll-purified *Arabidopsis* chloroplasts was confirmed through immunological detection of markers enzymes (Seigneurin-Berny et al., 2006) and proteomic analysis (Ferro et al., 2003). Only 5% (6 out of 112) of the identified *Arabidopsis* proteins may correspond to non-plastid proteins. On a chlorophyll basis, we determined that the yield (~2% to 3%) of Percoll-purified and intact chloroplasts is equivalent in *Arabidopsis* and spinach (Douce and Joyard, 1982), the major limit for *Arabidopsis* being the availability of large amounts of starting material. The yield of intact spinach chloroplasts obtained from 3 kg of leaves is ~150 to 200 mg chlorophyll or 2.5 to 4 g proteins. A classical preparation of *Arabidopsis* chloroplasts starts from 500 g of leaves. On average, the yield of intact *Arabidopsis* chloroplasts obtained from 3 kg of leaves would be ~45 to 55 mg chlorophyll or 0.18 to 0.32 g proteins.

Time Considerations

An efficient chloroplast preparation should be completed within 1 to 1.5 hr. Preformed continuous Percoll gradients can be prepared 1 or 2 days before. Concentrated purified chloroplasts can be stored on ice for a few hours before loss of their structural and metabolic integrity. *Arabidopsis* chloroplasts are more fragile than spinach chloroplasts and can be stored intact for 3 to 4 hr only. Intact chloroplasts do not survive freezing. Buffers with osmoticum must be used to keep the chloroplasts intact.

Literature Cited

Allen, M.B., Arnon, D.I., Capindale, J.B., Whatley, F.R., and Durham, L.J. 1955. Photosynthesis by isolated chloroplasts. III. Evidence for complete photosynthesis. *J. Am. Chem. Soc.* 77:4149-4155.

Arnon, D.I., Allen, M.B., and Whatley, F.R. 1954. Photosynthesis by isolated chloroplasts. *Nature* 174:394-396.

Block, M.A., Dorne, A.J., Joyard, J., and Douce, R. 1983a. Preparation and characterization of membrane-fractions enriched in outer and inner envelope membranes from spinach-chloroplasts. 1. Electrophoretic and immunological analyses. *J. Biol. Chem.* 258:3273-3280.

Block, M.A., Dorne, A.J., Joyard, J., and Douce, R. 1983b. Preparation and characterization of membrane-fractions enriched in outer and inner envelope membranes from spinach-chloroplasts. 2. Biochemical-characterization. *J. Biol. Chem.* 258:3281-3286.

Block, M.A., Douce, R., Joyard, J., and Rolland, N. 2007. Chloroplast envelope membranes: A dynamic interface between plastids and the cytosol. *Photosynth. Res.* 92:225-244.

Bruinsma, J. 1961. A comment on the spectrophotometric determination of chlorophyll. *Biochim. Biophys. Acta* 52:576-578.

Cline, K., Andrews, J., Mersey, B., Newcomb, E.H., and Keegstra, K. 1981. Separation and characterization of inner and outer envelope membranes of pea-chloroplasts. *Proc. Nat. Acad. Sci. U.S.A.* 78:3595-3599.

Douce, R., Holtz, R.B., and Benson, A.A. 1973. Isolation and properties of envelope of spinach-chloroplasts. *J. Biol. Chem.* 248:7215-7222.

Douce R. and Joyard J. 1979. Structure and function of the plastid envelope. *Adv. Bot. Res.* 7:1-116.

Douce, R. and Joyard, J. 1982. Purification of the chloroplast envelope. *In* Methods in Chloroplast Molecular Biology (M. Edelman, R. Hallick, and N.H. Chua, eds.) pp. 139-256. Elsevier/North-Holland, Amsterdam.

Ferro, M., Salvi, D., Brugière, S., Miras, S., Kowalski, S., Louwagie, M., Garin, J., Joyard, J., and Rolland, N. 2003. Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. *Mol. Cell Proteomics* 2:325-345.

Grant, B.R. and Wright, S.W. 1980. Purity of chloroplasts prepared from the siphonous green alga, *Caulerpa simpliciuscula*, as determined by their ultrastructure and their enzymic content. *Plant Physiol*. 66:130-138.

Heber, U. and Santarius, K.A. 1970. Direct and indirect transfer of ATP and ADP across chloroplast envelope. *Z. Naturforsch*. Part B 25:718-728.

Heldt, H.W. and Sauer, F. 1971. Inner membrane of chloroplast envelope as site of specific metabolite transport. *Biochim. Biophys. Acta.* 234:83-91.

Hill, R. 1937. Oxygen evolution by isolated chloroplasts. *Nature* 139 S:881-882.

Hill, R. 1939. Oxygen produced by isolated chloroplasts. *Proc. R. Soc. London Ser. B* 127:192-210.

Jensen, R.G. and Bassham, J.A. 1966. Photosynthesis by isolated chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 56:1095-1101.

Kalberer, P.P., Buchanan, B.B., and Arnon, D.I. 1967. Rates of photosynthesis by isolated chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 57:1542-1549.

Kunst, L. 1998. Preparation of physiologically active chloroplasts from Arabidopsis. *In* Methods in Molecular Biology, Vol. 82: *Arabidopsis* Protocols (J. M. Martinez-Zapater, and J. Salinas, eds.) pp. 43-48. Humana Press Inc, Totowa, N.J.

Leech, R.M. 1964. The isolation of structurally intact chloroplasts. *Biochim. Biophys. Acta*. 79:637-639.

Morgenthaler, J.J. and Price, C.A. 1974. Photosynthetic activity of spinach chloroplasts after isopycnic centrifugation in gradients of silica. *Plant Physiol*. 54:532-534.

Mourioux, G. and Douce, R. 1981. Slow passive diffusion of orthophosphate between intact isolated chloroplasts and suspending medium. *Plant Physiol*. 67:470-473.

Ortiz, W., Reardon, E.M., and Price, C.A. 1980. Preparation of chloroplasts from Euglena highly active in protein synthesis. *Plant Physiol*. 66:291-294.

Pertoft, H. 2000. Fractionation of cells and subcellular particles with Percoll. *J. Biochem. Biophys. Methods* 44:1-30.

Seigneurin-Berny, D., Gravot, A., Auroy, P., Mazard, C., Kraut, A., Finazzi, G., Grunwald, D., Rappaport, F., Vavasseur, A., Joyard, J., Richaud, P., and Rolland, N. 2006. HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. *J. Biol. Chem.* 281:2882-2892.

Somerville, C.R., Somerville, S.C., and Ogren, W.L. 1981. Isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*. *Plant Sci. Lett.* 21:89-96.

Stocking, CR. 1959. Chloroplast isolation in non-aqueous media. *Plant Physiol.* 34:56-61.

Takabe, T., Nishimura, M., and Akazawa, T. 1979. Structure and function of chloroplast proteins. 50. Isolation of intact chloroplasts from spinach leaf by centrifugation in gradients of the modified silica Percoll. *Agricul. Biol. Chem.* 43:2137-2142.

The *Arabidopsis* Genome Initiative (AGI) 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana. Nature* 408:796-815.

Walker, D.A. 1964. Improved rates of carbon dioxide fixation by illuminated chloroplasts. *Biochem. J.* 92:22C-23C.

Walker, D.A. 1971. Chloroplast (and grana): Aqueous (including high carbon fixation ability). *Methods Enzymol.* 23:211-220.

Walker, D. 1990. The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. Oxygraphics Ltd, University of Sheffield, South Yorkshire, UK.

Walker, D.A. and Hill, R. 1967. The relation of oxygen evolution to carbon assimilation with isolated chloroplasts. *Biochim. Biophys. Acta.* 131:330-338.

Wise, R.R. and Hoober, J.K. 2006. The Structure and Function of Plastids. Advances in Photosynthesis and Respiration, vol 23. Springer, Dordrecht, The Netherlands.

Isolation of Neuromelanin Granules

Florian Tribl[1]

[1]Medizinisches Proteom-Center, Ruhr-Universitaet Bochum, Bochum, Germany

ABSTRACT

Neuromelanin granules are pigmented organelles in the human midbrain that give name to a brain area, substantia nigra pars compacta, which macroscopically appears as a dark brown region in the midbrain due to the insoluble pigment neuromelanin. The substantia nigra pars compacta massively degenerates in Parkinson's disease and gives rise to severely disabling movement symptoms. It has been suggested that neuromelanin granules play an important role in the neurodegenerative events in Parkinson's disease: redox-active iron is bound to neuromelanin and thereby retained within this compartment, but in Parkinson's disease it is thought to be increasingly released into the cytosol, promoting oxidative stress. This unit includes a methodological workflow for the isolation of neuromelanin granules from the human midbrain. This top-down approach (describes an approach that reduces the complexity of the sample stepwise from the level of tissue to cell, and from cell to organelle) encompasses the organelle isolation by sequential density gradient centrifugation and the assessment of the isolation efficacy by western blotting. *Curr. Protoc. Cell Biol.* 41:3.31.1-3.31.15. © 2008 by John Wiley & Sons, Inc.

Keywords: human brain • neuromelanin • lysosome-related organelle • organelle isolation • density gradient

INTRODUCTION

In the human brainstem, a region termed "substantia nigra pars compacta" is detectable by the naked eye due to its brown to black color (see Fig 3.31.3A, B). The characteristic pigmentation of the human substantia nigra pars compacta arises from specific organelles termed neuromelanin granules.

These organelles contain the insoluble pigment neuromelanin, which is biosynthesized from dopamine or noradrenalin. Interestingly, neuromelanin granules are absent in laboratory animals, such as mice and rats. In contrast, neuromelanin granules are found in most catecholaminergic neurons of primates and reach their maximal appearance in humans. A contribution of neuromelanin granules to the pathomechanisms of Parkinson's disease has been suggested. In Parkinson's disease, neuromelanin granules are engaged in iron-related oxidative stress and in the selective deposition of α-synuclein on the organellar lipid components. The lack of accessible model systems, however, has so far impeded the functional analysis of neuromelanin granules by molecular approaches.

Recently, the successful isolation of neuromelanin granules from human brain tissue led to their fundamental characterization as a lysosome-related organelle. The scope of this unit is to provide a detailed protocol for the isolation of neuromelanin granules from the human brain. Briefly, neuromelanin granules are isolated via a top-down approach by reducing the complexity of the sample stepwise. First, human substantia nigra pars compacta tissue is mechanically dissociated into a suspension of cells. Then, the pigmented neurons are enriched by centrifugation through a discontinuous sucrose gradient. While the glial cells and membranous matter accumulates at the sucrose layer interfaces, the dark brown neuronal cell bodies can be collected as a pellet, isolated, and homogenized. The homogenate containing a variety of cellular organelles is then centrifuged through

Current Protocols in Cell Biology 3.31.1-3.31.15, December 2008
Published online December 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0331s41

a Percoll cushion. This step separates the very dense neuromelanin granules from the remaining cellular components.

Neuromelanin granules isolated by this manner were initially used for proteomic investigations (Tribl et al., 2005). Nevertheless, isolated neuromelanin granules could be applied in cellular assays, e.g., to compare the function of neuromelanin in conditions of health and disease. Furthermore, detailed biochemical analyses of the lipidic components of neuromelanin granules, or the metal ions present in the organelle, should be feasible.

Although the purpose of the Basic Protocol is to isolate neuromelanin granules from the human brain, an application of an adapted protocol on, e.g., brains of human and nonhuman primates, is thinkable.

NOTE: All protocols using human tissue must be reviewed and approved by an ethics committee, and must conform to governmental regulations regarding the acquisition and distribution of human post-mortem tissue.

BASIC PROTOCOL

ISOLATION OF NEUROMELANIN GRANULES FROM HUMAN SUBSTANTIA NIGRA PARS COMPACTA TISSUE

The protocol described here is optimized for the isolation of neuromelanin granules from human brain and yields this organelle in high purity. The purity of the isolated organelles is sufficiently high for proteomic analyses (Tribl et al., 2005). Additionally, this procedure is very mild and preserves the major morphological features of neuromelanin granules; in particular the lipidic components remain attached to the granules after isolation, as demonstrated by transmission electron microscopy (see Fig. 3.31.1).

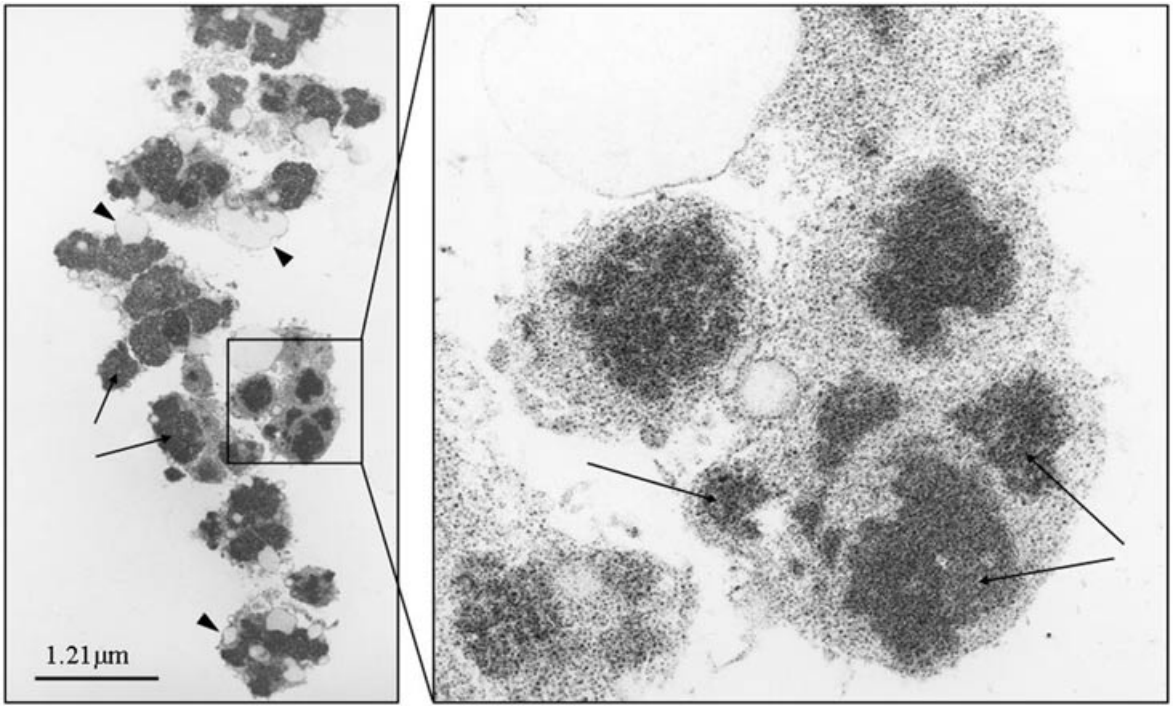


Figure 3.31.1 Quality assessment of the organelle preparation on a morphological level by transmission electron microscopy. Isolated neuromelanin granules are virtually free of contaminating organelles and retained their morphological appearance after isolation. Dark areas represent the electron-dense neuromelanin (highlighted by arrows), which is embedded in a protein matrix. Lipid component (arrowheads) that are characteristic for neuromelanin granules are still attached to the organelles. Reproduced from Tribl et al., 2005.

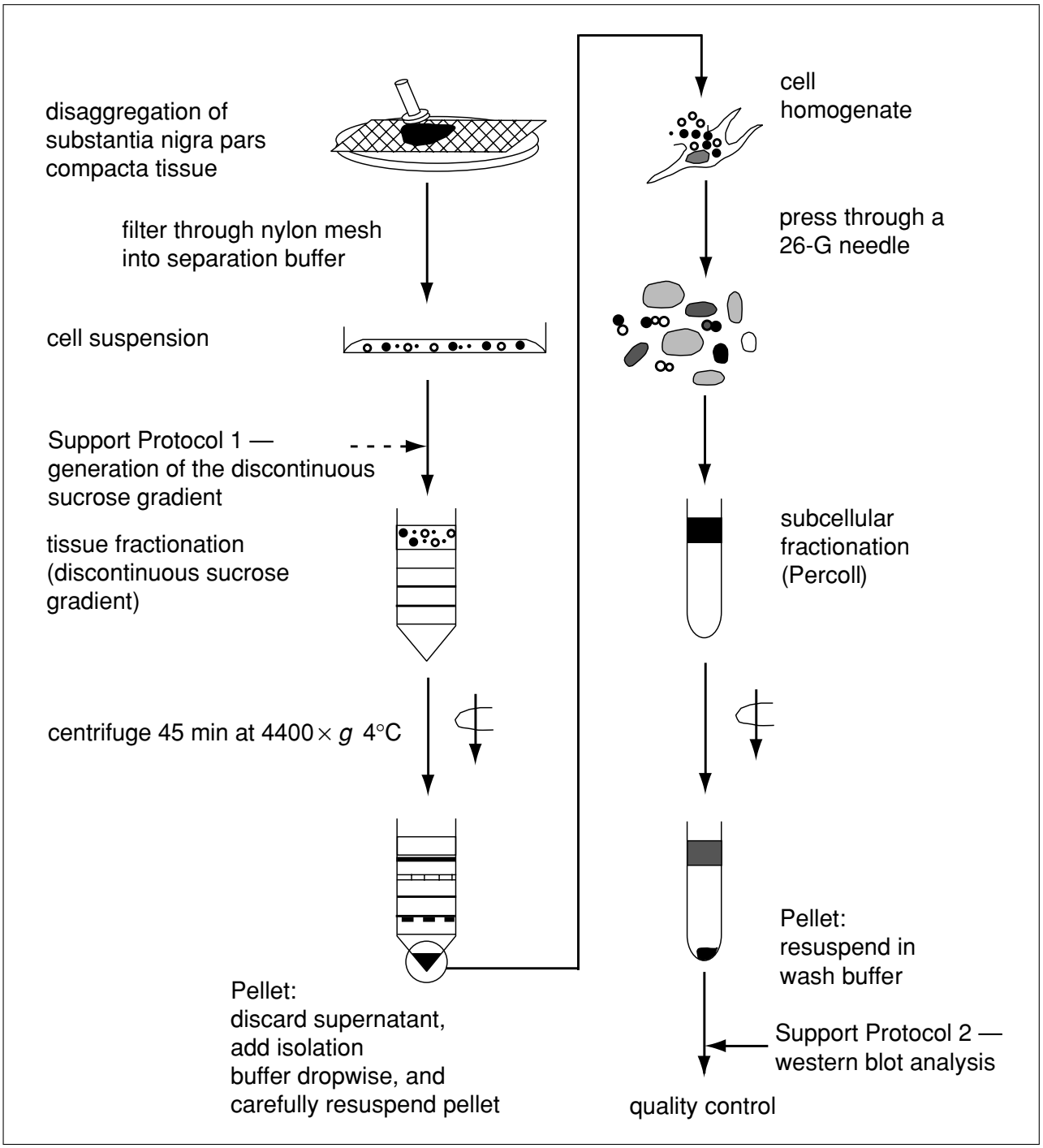


Figure 3.31.2 Flowchart of the Basic Protocol and Support Protocols 1 and 2, providing a schematic overview of the major steps to purify neuromelanin granules from human post-mortem substantia nigra pars compacta. Reproduced from Tribl et al., 2005.

The schematic flowchart provides a visual summary of the major purification steps (see Fig. 3.31.2).

Materials

Disinfecting agents for dissecting instruments and surfaces (e.g., any liquid formulation used to clean metal surgical instruments is appropriate)

Dissected, unfixed human substantia nigra pars compacta, prepared at the Brain Bank (stored at −80°C)

Separation buffer (see recipe), cooled to 4°C

Ice
20-ml discontinuous sucrose gradients in 50-ml conical polyethylene screw-cap centrifuge tubes (see Support Protocol 1)
Isolation buffer (see recipe)
80% (v/v) Percoll solution (see recipe)
Wash buffer (see recipe)
Liquid nitrogen

Cooling plate
Plastic sack (e.g., an autoclave bag)
250- and 500-ml beakers
Petri dishes, 100-mm in diameter
Scissors
150-μm nylon mesh
Balance accurate to ±0.1 g
Dissecting instruments including:
Scalpels or single-edge razor blades
Forceps
10-ml syringes (B. Braun Melsungen AG), single-use
15- and 50-ml conical polyethylene screw-cap centrifuge tubes
Styropor boxes
Refrigerated Rotanta 96 RS centrifuge (Hettich GmbH) with swing-out rotor inserts for 15-ml and 50-ml conical polyethylene screw-cap centrifuge tubes
1.5-ml microcentrifuge tubes (Eppendorf)
1-ml syringe equipped with a 26-G needle
−80°C freezer

Additional reagents and equipment for preparing a discontinuous sucrose gradient (Support Protocol 1)

NOTE: It is highly recommended to use human substantia nigra pars compacta that is prepared by experienced pathologists. Substantia nigra pars compacta should be dissected from transverse brain tissue plates of the brain stem (see Fig. 3.31.3A, B) and should be kept intact.

NOTE: For biosafety reasons, during the tissue preparation steps use personal protective equipment including a laboratory coat, a surgical mask, safety glasses, and gloves to avoid dermal and mucous membrane contact with blood and brain tissue. Wear a laboratory coat, safety glasses, and gloves throughout the other procedures. All steps should be performed at 4°C.

Disaggregate human substantia nigra pars compacta

1. Set the cooling plate to −10°C.
2. Cover the metal surface of the cooling plate with a plastic sack.

   This will allow the most secure disposal of residual tissue from the cooling plate.
3. Prepare a 500-ml beaker with disinfecting agent for the scalpels and forceps.
4. Place the 100-mm petri dish on the cooled metal plate of the cooler and cover the dish with a nylon mesh.

   Using scissors, cut the nylon mesh into 12 × 12–cm squares. The nylon mesh should be large enough to cover the entire petri dish properly. It is not necessary to additionally fix the mesh on the petri dish.

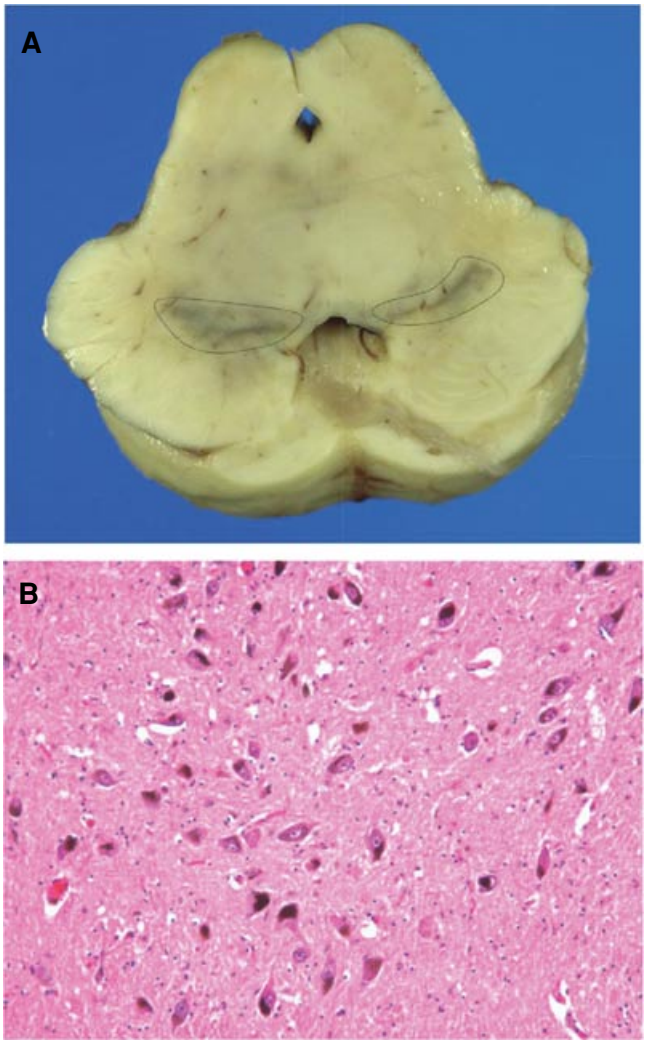


Figure 3.31.3 (**A**) A macroscopic aspect of a tissue slice of a human brain stem. The dark areas represent the substantia nigra pars compacta that is additionally highlighted by dotted lines. (**B**) Human substantia nigra pars compacta tissue under the light microscope (hematoxylin-eosin stain, 10×). The brownish neuromelanin granules are clearly visible within the cytosol of the dopaminergic neurons.

5. Place the pieces of frozen human substantia nigra pars compacta into separation buffer (cooled to 4°C). Add 5 g of intact tissue and bring to 7.5 ml with separation buffer. Let the tissue thaw in the buffer.

   All buffers should be supplemented with protease inhibitors (see Reagents and Solutions) to prevent proteolytic activity of endogenous proteases in the tissue. It is also possible to use a commercial protease inhibitor cocktail.

6. Using the forceps, place one total piece of brain tissue on top of the nylon mesh (~500 to 1000 mg per substantia nigra pars compacta). If necessary, remove excessive white matter and trim the substantia nigra pars compacta using a scalpel. Add 500 to 1000 µl of separation buffer to the tissue.

7. Using the plunger of the 10-ml single-use syringe, press the brain tissue through the nylon mesh. Make sure that the tissue stays in contact with the ice-cold liquid.
8. As soon as the piece of tissue has been disaggregated completely, remove the nylon mesh from the petri dish and transfer the suspension into a 50-ml conical polyethylene screw-cap centrifuge tube. Place the centrifuge tube into a styropor box filled with ice.
9. Repeat this procedure with the remaining pieces of brain tissue and combine the tissue suspensions.

Fractionate tissue

10. Overlay 7.5 ml of the suspension dropwise onto one ice-cold discontinuous sucrose gradient (see Support Protocol 1).
11. Transfer the centrifuge tubes into the refrigerated centrifuge (e.g., Rotanta 96 RS) equipped with swing-out rotor inserts for 50-ml conical polyethylene screw-cap centrifuge tubes (or equivalent). Centrifuge 45 min at 4400 × *g*, 4°C, to sediment the cells.

    After centrifugation, a dark brown (neuromelanin-containing) pellet is visible, while fine layers of pale particles (debris, myelin sheaths, etc.) are visible at the sucrose solution interfaces.

Prepare cell homogenate

12. Carefully aspirate the supernatant with a pipet and discard into a 250-ml beaker containing a disinfecting agent.
13. Add 500 µl of isolation buffer dropwise to the dark brown pellet and gently resuspend by pipetting up and down.
14. Centrifuge in the Rotanta 96 RS centrifuge 15 min at 4400 × *g*, 4°C, and discard the supernatant.
15. Again, add 500 µl of isolation buffer dropwise to the dark brown pellet and gently resuspend by pipetting up and down. Transfer the suspension into a 1.5-ml micro-centrifuge tube.
16. Coat a 1-ml syringe equipped with a 26-G needle with the isolation buffer. Pass the buffer several times through the needle.
17. Homogenize the dark brown pellet by passing it through the needle ten times to release cellular organelles.

    Avoid the generation of air bubbles. Slowly aspirate the suspension into the syringe and slowly press back the solution into the 1.5-ml reaction tube.

Perform subcellular fractionation

18. Add 5 ml ice-cold 80% (v/v) Percoll solution into a 15-ml conical polyethylene screw-cap centrifuge tube.
19. Layer the homogenate dropwise on top of the 80% Percoll solution.
20. Transfer the centrifuge tube into the refrigerated centrifuge (Rotanta 96 RS) equipped with swing-out rotor inserts for 15-ml conical polyethylene screw-cap centrifuge tubes (or equivalent). Centrifuge 15 min at 4400 × *g*, 4°C, to sediment the neuromelanin granules.

    After centrifugation, a dark brown pellet is visible, while a fine layer of pale particles is visible at the Percoll solution interface.

21. Aspirate the supernatant with a pipet and discard into a 250-ml beaker containing a disinfecting agent.
22. Add 500 µl of wash buffer dropwise to the dark brown pellet and gently resuspend by pipetting up and down.
23. Centrifuge in the Rotanta 96 RS centrifuge 15 min at 4400 × *g*, 4°C, and discard the supernatant.
24. If not proceeding further, freeze the isolated neuromelanin granules in liquid nitrogen and store at −80°C.

Based on a transmission electron microscopic inspection, neuromelanin granules appear to be pure (Fig. 3.31.1).

Neuromelanin granules isolated following this protocol were used for proteomic analyses (Tribl et al., 2005). Nevertheless, neuromelanin granules isolated in this manner could be applied in cellular assays to compare the function of neuromelanin in health and disease. Furthermore, biochemical investigations of the lipidic components of neuromelanin granules, or the metal ions present in the organelle, should be feasible.

PREPARATION OF A DISCONTINUOUS SUCROSE GRADIENT

SUPPORT PROTOCOL 1

The discontinuous sucrose gradient is used to prefractionate the tissue suspension.

Materials

1.0 M sucrose gradient solution, ice cold (see recipe)
1.2 M sucrose gradient solution, ice cold (see recipe)
1.4 M sucrose gradient solution, ice cold (see recipe)
1.6 M sucrose gradient solution, ice cold (see recipe)
Ice

50-ml conical polyethylene screw-cap centrifuge tubes
Test tube rack
150-mm glass Pasteur pipets with a straight tip, constricted, but unplugged
Styropor box

1. Place a 50-ml conical polyethylene screw-cap centrifuge tube into a test tube rack.
2. Place the Pasteur pipet into a 50-ml conical polyethylene screw-cap centrifuge tube. Using an e.g., automatic pipettor with a 1-ml tip, add five 1-ml aliquots stepwise of ice-cold 1.0 M sucrose gradient solution into the Pasteur pipet and let the solution flow into the 50-ml centrifuge tube (see Fig. 3.31.4).

NOTE: *After the last aliquot is added, quickly close the Pasteur pipet with a thumb before the meniscus of the sucrose solution would enter the constriction of the tip. This largely prevents the formation of air bubbles after addition of the second layer. Air bubbles may disturb the formation of sharp interfaces between two sucrose layers.*

3. Keep the Pasteur pipet from step 2 in the 50-ml centrifuge tube and underlay the 1.0 M sucrose layer by adding 5 ml of ice-cold 1.2 M sucrose gradient solution.
4. Underlay the 1.2 M sucrose layer by adding 5 ml of ice-cold 1.4 M sucrose gradient solution.
5. Underlay the 1.4 M sucrose layer by adding 5 ml of ice-cold 1.6 M sucrose gradient solution.
6. Place the discontinuous sucrose gradients on ice into the styropor box until the application of the sample.

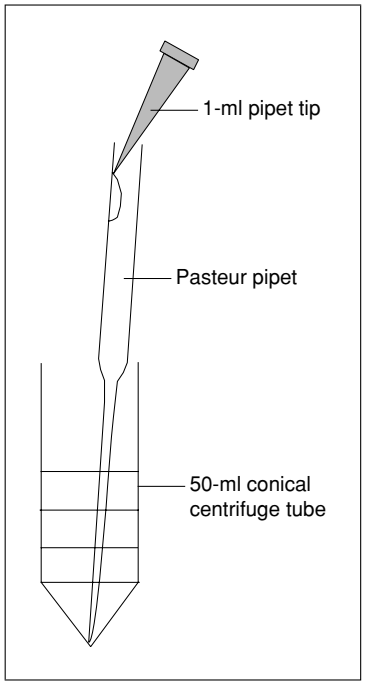


Figure 3.31.4 Figure shows the preparation of the discontinuous sucrose gradient. The sucrose solutions are pipetted into a Pasteur pipet and allowed to flow into the 50-ml conical polyethylene screw-cap centrifuge tube.

SUPPORT PROTOCOL 2

QUALITY CONTROL BY IMMUNOBLOT ANALYSIS

Quality control of organelle preparations is indispensable to monitor the success of the isolation and to exclude the presence of contaminating organelles as opposed to detecting neuromelanin markers.

An extensive quality control for organelle preparations on a molecular level, e.g., by immunoblot analyses, is crucial to evaluate the degree of enrichment. Antibodies against several organelle marker proteins, which are specific for a given cellular compartment, are commercially available and allow for visualizing the purity of the sample (see Fig. 3.31.5).

Materials

2× tricine/SDS sample buffer (Novex)
Polyacrylamide gels, e.g., precast 10% to 20% gradient tricine gels (Novex), or equivalent gels (*UNIT 6.1*)
10 × tricine/SDS running buffer (Novex)
10× reducing agent (Novex)
Isolated neuromelanin granules sample (see Basic Protocol)
Molecular weight markers
Ice
Tris-glycine transfer buffer, 20× (Novex)
Ponceau red solution (Pierce)
Tris-buffered saline (TBS; *APPENDIX 2A*)
Blocking solution (see recipe)
Primary antibodies
Secondary antibodies (HRP-conjugated)
Tris-buffered saline-Triton X-100 (TBS-T; see recipe)
ECL solutions (Pierce)

Heater
Power supply
XCell II Mini Gel Electrophoresis System (Novex)
XCell II blot module, blotting cassette, and sponges (Novex)

Benchtop centrifuge
Gel knife
Precut nitrocellulose membranes (Invitrogen GmbH)
Sponges
Whatman 3MM Chr paper (Schleicher & Schuell)
Rocking platform
Gel documentation system

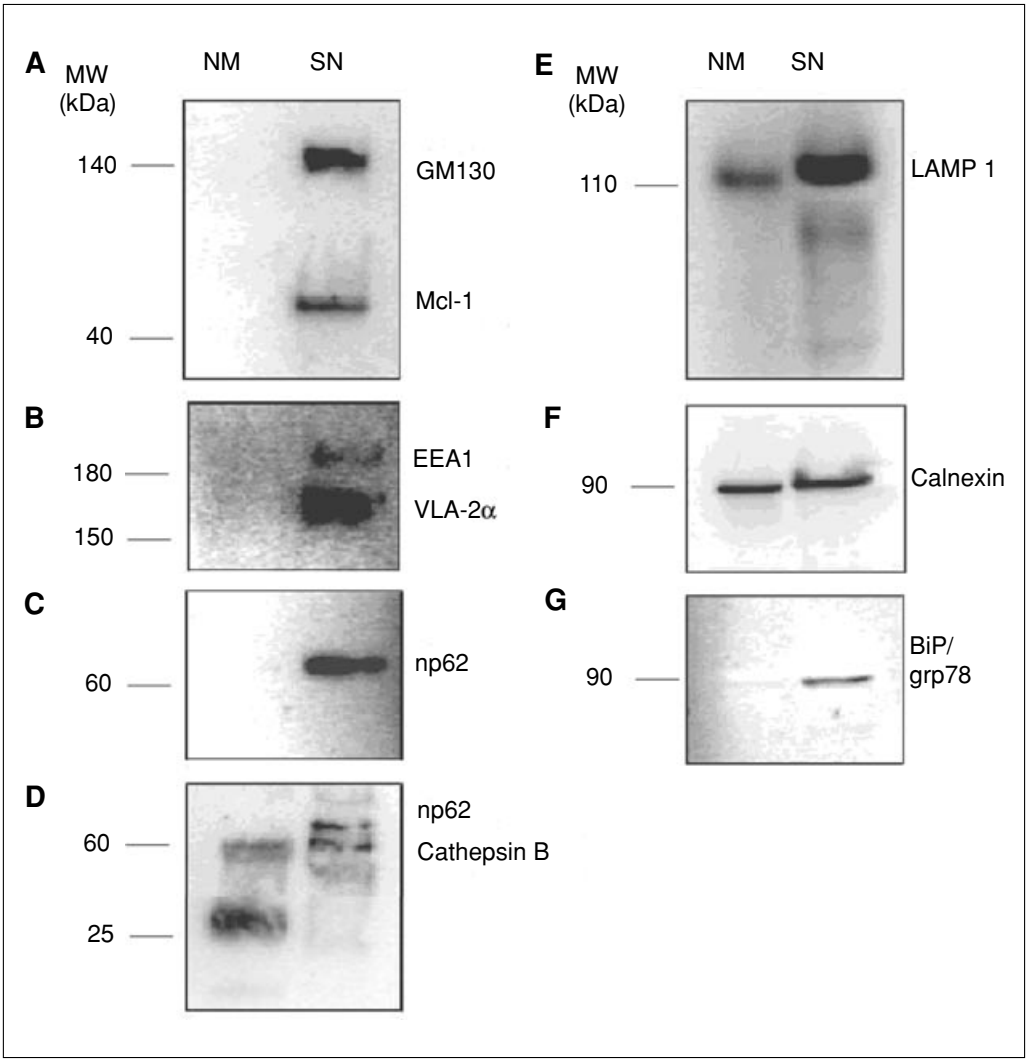


Figure 3.31.5 Assessment of the neuromelanin granule purification on a molecular level by western blot analyses. Probing for organelle marker proteins enables visualization of the purity of isolated neuromelanin granules (NM) compared to total substantia nigra pars compacta tissue homogenate (SN). Marker proteins for (**A**) the Golgi network (GM130), mitochondria (Mcl-1), (**B**) early endosomes (EEA-1), the plasma membrane (VLA-2a), and (**C**) the nucleus (np62) are not detected in the neuromelanin granule preparation, while (**D, E**) late endosomes and lysosomal marker proteins (cathepsin B, LAMP1) are present. (**F**) Calnexin is predominantly detected in the endoplasmic reticulum, but also plays a role in pigmented organelles as a melanogenic chaperone and is detected in considerable amounts in neuromelanin granules. (**G**) BiP/grp78, however, a classical marker protein for the endoplasmic reticulum, is absent. Reproduced from Tribl et al., 2005.

Perform SDS-polyacrylamide gel electrophoresis

These instructions assume the use of precast gels, e.g., 10% to 20% gradient tricine gels and the XCell II Mini Gel Electrophoresis System (Novex), but are easily adaptable to other formats, including self-cast minigels.

1. Set a heater at 90°C and bring the tricine/SDS sample buffer (2×) to room temperature.

   NOTE: *SDS precipitates at 4° C. Incomplete dissolution may hamper proper solubilization of the proteins.*

2. Remove the gel from the bag, briefly rinse with water, peel the tape from back of gel, and remove the comb.

3. Pour ~100 ml of the 1× tricine/SDS running buffer into the electrophoresis chamber; place the gel into the XCell II module, fill the upper chamber with the 1× tricine/SDS running buffer, and check for leaks. Remove air bubbles, if necessary.

4. Prepare the isolated neuromelanin granules sample for electrophoresis by adding 2× tricine/SDS sample buffer (1× final concentration) and an aliquot of the 10× reducing agent (1× final concentration), and then incubate the sample 5 to 10 min at 90°C.

5. Incubate the sample on ice 10 sec to briefly cool the heated sample and spin in a benchtop centrifuge 10 min at 16,000 × *g*, room temperature.

6. Load 20 µl of the sample onto the gel and include one well for molecular weight markers.

7. Run the gel at 200 V constant voltage for ~50 min.

   Expect a current of 100 to 120 mA at the beginning and 60 to 70 mA at the end of the electrophoresis.

Immunoblot the gel

8. Remove the gel from the plastic cassette and cut off the wells with a gel knife. Then cover the gel with 1× Tris-glycine transfer buffer.

9. Wet the precut nitrocellulose membranes in 1× Tris-glycine transfer buffer.

   The membrane should be completely covered with buffer.

10. Presoak 6 to 7 sponges in 1× Tris-glycine transfer buffer. Gently press to remove air from the sponges, which accomplishes the complete uptake of buffer.

11. Prepare the XCell II blot module and transfer three sponges into the blotting cassette.

12. Prepare the gel sandwich as follows: wet a sheet of Whatman paper by briefly dipping into 1× Tris-glycine transfer buffer and place the gel onto it. Cover the gel with the nitrocellulose membrane and put a second sheet of wet Whatman paper onto the nitrocellulose membrane. Remove air bubbles by applying gentle pressure to allow uniform protein transfer onto the membrane. Then, to transfer the gel sandwich onto the sponges, cover it with the remaining sponges and close the cassette.

13. Place the cassette into the XCell II blot module and perform the protein transfer onto the membrane at 70 V for 90 min.

14. Monitor the transfer efficacy briefly by dipping the membrane into the Ponceau Red solution, which reversibly visualizes red protein bands. Then, destain the membrane either briefly in TBS or directly transfer it into 30 ml of blocking solution.

15. Block nonspecific antibody binding sites 1 hr at room temperature on a rocking platform (10 to 15 cycles/min).
16. Discard the blocking solution. Dilute the primary antibody appropriately in 10 ml blocking solution for probing. Gently pour the antibody solution onto the membrane and incubate for 1 hr at room temperature on a rocking platform.

    Antibodies are diluted in a range of 1:1000 (v/v) to 1:2000 (v/v).
17. Remove the primary antibody and wash the membrane three times, each time for 15 min with 30 ml TBS-T.
18. Dilute the secondary antibody in TBS-T. Discard the blot washing buffer and incubate the membrane with the secondary antibody for 1 hr at room temperature on a rocking platform.
19. Finally, remove the secondary antibody and wash the membrane three times, each time for 15 min with 30 ml TBS-T.
20. During the last washing step, warm 1 to 2 ml of the ECL solutions to room temperature and prepare a 1:1 (v/v) mixture.
21. Briefly rinse the membrane in TBS, and then add the mixed ECL solution onto the membrane and disperse evenly.

    Exposure time in ECL solution ranges between 30 sec to 1 min.
22. Finally, monitor chemiluminescence in a gel documentation system.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking solution

TBS-T (see recipe)
5% (w/v) non-fat dry milk
Store up to 2 days at 4°C

Isolation buffer

100 mM KCl
10 mM HEPES, pH 7.5
1 mM disodium EDTA
10% (m/v) sucrose
0.01% (v/v) protease inhibitor cocktail (see recipe)
Filter through a 0.2-μm filter device (Millipore)
Store up to several days at 4°C

Percoll solution

80% (v/v) Percoll (Fluka)
0.25 M sucrose
10 mM HEPES, pH 7.5
0.01% (v/v) protease inhibitor cocktail (see recipe)
Store up to 6 months at room temperature

Percoll consists of colloidal silica beads coated with polyvinlpyrrolidone.

Protease inhibitor cocktail

1 mg/ml aprotinin in H_2O
0.5 mg/ml pepstatin in methanol
0.05 M phenylmethylsulfonyl fluoride (PMSF) in DMSO
Store up to 2 years at −20°C
Add the protease inhibitor cocktail to the buffer solutions at 0.01% (v/v)

Separation buffer

100 mM KCl
10 mM HEPES, pH 6
10% (w/v) glucose
0.01% (v/v) protease inhibitor cocktail (see recipe)
Filter through a 0.2-μm filter device (Millipore)
Store up to several days at 4°C

Sucrose gradient solutions, 1.0 M, 1.2 M, 1.4 M, and 1.6 M

1 M sucrose
10 mM KCl
10 mM HEPES, pH 7.5
0.01% (v/v) protease inhibitor cocktail (see recipe)

1.2 M sucrose
10 mM KCl
10 mM HEPES, pH 7.5
0.01% (v/v) protease inhibitor cocktail (see recipe)

1.4 M sucrose
10 mM KCl
10 mM HEPES, pH 7.5
0.01% (v/v) protease inhibitor cocktail (see recipe)

1.6 M sucrose
10 mM KCl
10 mM HEPES, pH 7.5
0.01% (v/v) protease inhibitor cocktail (see recipe)
Store sucrose gradient solutions up to 2 months at 4°C

Tris-buffered saline, supplied with Tween (TBS-T)

Tris-buffered saline (TBS; see *APPENDIX 2A*)
0.1% (v/v) Tween-20
Store up to 3 months at 4°C

Wash buffer

250 mM NaCl
10 mM HEPES, pH 7.5
0.01% (v/v) Triton X-100
0.01% (v/v) protease inhibitor cocktail (see recipe)
Prepare fresh

COMMENTARY

Background Information

Neuromelanin granules are absent in the brains of most laboratory animals currently used, e.g., fish, rodents, or rabbits, but these pigmented organelles are found in most primates, and in highest quantities in humans (Marsden, 1961). In the human brain, neuromelanin granules are especially found in the dopaminergic substantia nigra pars compacta, and also in the catecholaminergic locus

ceruleus. Neuromelanin granules appear at the age of 3 to 5 years and are formed until ~20 years of age (Halliday et al., 2006). Different from melanosomes that form the basis of oculo-cutaneous pigmentation and develop by a maturation process from unpigmented stage I melanosomes via striated stage II melanosomes to pigmented stage III and IV melanosomes, no precursor compartment of neuromelanin granules is currently known. After the age of 20, no further generation of neuromelanin granules takes place, while the pigment continues to be formed.

Neuromelanin is an amorphous, insoluble pigment of unknown structure that is suggested to be a polymer formed from dopamine (substantia nigra pars compacta) or from noradrenalin (locus ceruleus; Fedorow et al., 2005). Comparable to the melanin in highly pigmented stage IV melanosomes, the pigment neuromelanin is responsible for the high density of neuromelanin granules. For isolation, density gradient centrifugation has been widely applied to isolate a great variety of cellular organelles (Whittaker, 1965; Graham and Rickford, 1997). Due to the exceptional high density of pigmented organelles, this approach has been applied very successfully to yield oculo-cutaneous melanosomes in high purity in sucrose gradients (Seiji et al., 1963; Gahl et al., 1995), and in an isotonic solution of 80% (v/v) Percoll (Rogers et al., 1998). In addition, Percoll is applicable to isolate neuromelanin granules. Affinity-based purification, in contrast, is not recommendable for neuromelanin granules, since no membrane protein unique for these organelles has been found so far.

Critical Parameters

Neuromelanin granules are absent in most laboratory animals. Moreover, there is no cell culture system that reliably forms neuromelanin granules. Unlike other organelles or subcellular compartments, highest quantities of neuromelanin granules are present in human brain substantia nigra pars compacta. Although high-quality human brain tissue is collected and preserved in brain bank centers, the availability of human substantia nigra pars compacta tissue with a post-mortem delay of up to 12 hr, however, may represent the bottleneck of the entire isolation process. Additionally, it is currently not known to what extent factors such as the post-mortem delay, the agonal status, gender, age, and storage time may affect the isolation of neuromelanin granules from human autopsy tissue. Fortunately, most nucleic acids and proteins are reasonably stable in the brains, allowing reliable analyses (Hynd et al., 2003).

Neuromelanin granules belong to the group of lysosome-related organelles, which are not primarily engaged in the digestion of macromolecular polymers such as proteins, lipids, or glycosides, as this is accomplished by conventional lysosomes (Tribl et al., 2006). Nevertheless, hydrolytic enzymes, most importantly proteases, are still present and eventually active in these lysosome-related organelles. It is thus critical to keep the sample at 4°C and to perform all experimental steps on ice. Moreover, it is recommended to use a cocktail of protease inhibitors to keep the protease activity at a minimum.

Contamination of neuromelanin granules by other organelles is not a major concern, since the extraordinary density of neuromelanin granules is far beyond that of organelles such as mitochondria or lysosomes. Nevertheless, quality control is crucial to monitor the success and the efficacy of the isolation procedure. Since there is currently no unique protein available to allow for an unambiguous identification of neuromelanin granules, several methodologies should be applied, most importantly immunoblot analyses to exclude contaminations at the molecular level (see Support Protocol 2), and transmission electron microscopy to identify neuromelanin granules by their characteristic morphology (see Fig. 3.31.1).

Anticipated Results

One gram of substantia nigra pars compacta yields ~1 to 2 mg neuromelanin granules. Both substantia nigra pars compacta tissue and isolated neuromelanin granules should be saved for quality control analyses. These include immunoblot analysis of organellar marker proteins of the endoplasmic reticulum (BiP/grp78), the Golgi complex (GM180), early endosomes (EEA1), lysosomes (Lamp-1, cathepsin B), mitochondria (mcl-1), the nucleus (np62), and the plasma membrane (VLA-2α; Fig. 3.31.5). Although lysosomal marker proteins are present in the sample, all other organellar marker proteins are absent.

When monitoring the isolation efficacy by transmission electron microscopy, the neuromelanin sample should be virtually free of contaminating organelles or subcellular structures.

Table 3.31.1 Troubleshooting Guide for Neuromelanin Granule Isolation

Problem	Cause	Solution
No pellet after sucrose gradient centrifugation	Wrong sucrose density	Check that the sucrose densities are correct
	Inefficient homogenization process	Extend the homogenization and thoroughly sieve the tissue through the mesh
Neuromelanin granules enter inefficiently into the Percoll layer	Granules are bound to less-dense cellular components	This is regularly observed and may be improved by repeated passage of the interface fraction through the 26-G syringe
Protein degradation visualized by western blot	Protein degradation	Use protease inhibitors, extend the already applied protease mix; use tissue with a smaller post-mortem delay

Troubleshooting

Table 3.31.1 highlights the most commonly encountered problems and provides suggestions for their solutions.

Time Considerations

The time required to isolate neuromelanin granules depends on the brain tissue used. Homogenization of substantia nigra pars compacta tissue requires 10 to 15 min per gram. The preparation of the gradients takes ~30 to 45 min, if the underlaying procedure (see Support Protocol 1) is used. Since the weight of the sucrose layers is increased step by step, underlaying additional sucrose layers will take more time. Centrifugation of the tissue suspension will require 45 min, but may be extended to 90 min to eventually obtain higher yields. The isolation of the pellet after this first centrifugation step will require an additional 30 to 45 min. If several gradients are to be handled, additional time is required.

The homogenization of this pellet by passage through a 26-G needle may require 30 min, since the aspiration of the suspension into the narrow needle is rather slow. Overlaying the sample onto the Percoll layer and the sedimentation of the neuromelanin granules are estimated to require 30 min. Additional time has to be considered, if the yield in this step is not satisfactory (see Table 3.31.1). In general, a high-purity preparation of neuromelanin granules requires 6 to 8 hr including the preparation of buffers and solutions.

Acknowledgments

The author is grateful to his mentors Prof. Peter Riederer, Prof. Manfred Gerlach, and Prof. Gerhard Bringmann for constant encouragement and support, and to Prof. Katrin Marcus and Prof. Helmut E. Meyer for a successful cooperation and an excellent scientific environment. The author is grateful to Dr. Esther Asan and Mrs. Sigi Schenk for their expert assistance with transmission electron microscopy, and to Dr. Thomas Arzberger and Dr. Thomas Tatschner for their expertise in human brain tissue preservation.

This work was supported by the Austrian Academy of Sciences, by the *BrainNet Europe*, by the BMBF Grant 031U102F, the Deutsche Parkinson Vereinigung, and the Fond der Chemischen Industrie.

Literature Cited

Fedorow, H., Tribl, F., Halliday, G., Gerlach, M., Riederer, P., and Double, K.L. 2005. Neuromelanin in human dopamine neurons: Comparison with peripheral melanins and relevance to Parkinson's disease. *Prog. Neurobiol.* 75:109-124.

Gahl, W.A., Potterf, B., Durham-Pierre, D., Brilliant, M.H., and Hearing, V.J. 1995. Melanosomal tyrosine transport in normal and pink-eyed dilution murine melanocytes. *Pigment Cell Res.* 8:229-233.

Graham, J.M. and Rickwood, D. 1997. Subcellular Fractionation: A Practical Approach. pp. 339. Oxford University Press, New York, N.Y.

Halliday, G.M., Fedorow, H., Rickert, C.H., Gerlach, M., Riederer, P., and Double, K.L. 2006. Evidence for specific phases in the

development of human neuromelanin. *Neurobiol. Aging.* 27:506-512.

Hynd, M.R., Lewohl, J.M., Scott, H.L., and Dodd, P.R. 2003. Biochemical and molecular studies using human autopsy brain tissue. *J. Neurochem.* 85:543-562.

Marsden, C.D. 1961. Pigmentation in the nucleus substantiae nigrae of mammals. *J. Anat.* 95:256-261.

Rogers, S.L., Tint, I.S., and Gelfand, V.I. 1998. In vitro motility assay for melanophore pigment organelles. *Methods Enzymol.* 289:361-372.

Seiji, M., Shimao, K., Birbeck, M.S.C., and Fitzpatrick, T.B. 1963. Subcellular localization of melanin biosynthesis. *Ann. NY Acad. Sci.* 100:497-533.

Tribl, F., Gerlach, M., Marcus, K., Asan, E., Tatschner, T., Arzberger, T., Meyer, H. E., Bringmann, G., and Riederer, P. 2005. Subcellular proteomics of neuromelanin granules isolated from the human brain. *Mol. Cell. Proteomics* 4:945-957.

Tribl, F., Marcus, K., Meyer, H.E., Bringmann, G., Gerlach, M., and Riederer, P. 2006. Subcellular proteomics reveals neuromelanin granules to be a lysosome-related organelle. *J. Neural Transm.* 113:741-749.

Whittaker, V.P. 1965. The application of subcellular fractionation techniques to the study of brain function. *Prog. Biophys. Mol. Biol.* 15:39-96.

Isolation of Dense Core Secretory Vesicles from Pancreatic Endocrine Cells by Differential and Density Gradient Centrifugation

John C. Hutton,[1] Randall Wong,[1] and Howard W. Davidson[1]

[1]Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado

ABSTRACT

Methods are presented for the separation of dense core secretory vesicles from insulin-secreting tissues (insulin granules) based on a combination of differential and density gradient centrifugation on various media. Emphasis is given to the use of transplantable tumors, tissue culture cell lines, and pancreatic islets as a tissue source. *Curr. Protoc. Cell Biol.* 42:3.32.1-3.32.20. © 2009 by John Wiley & Sons, Inc.

Keywords: insulin granules • DCV • insulinoma • Min6 • INS1 • homogenization • centrifugation

INTRODUCTION

The techniques in this unit have been tailored for the isolation of insulin secretory granules under conditions that retain their integrity through the use of isoosmolar conditions. The same procedures can, in principle, be adapted to isolate dense core vesicles (DCVs) from other tissue types but require careful empirical analyses of the behavior of the organelles relative to other major cellular components. Different density media, even under isoosmotic conditions, have dramatically different capacities to separate DCVs from lysosomes and mitochondria that presumably relate to the interaction of the density media components with the granule membrane. Protocols need to be developed for the specific organelle and tissue by application of quantitative analyses of organelle markers so that the purity and relative specific activity of markers can be calculated. The literature abounds with examples of claims of co-localization of specific proteins to DCVs based on co-sedimentation on a density gradient though often this has been based on fractions in which the organelle contributes <5% of the total protein. In such cases other supporting data such as electron microscopy, co-immunoprecipitation, or the use of multiple analytical gradients should be used.

Procedures for the isolation of insulin granules from pancreatic β-cells are detailed below. Basic Protocol 1 describes isolation of purified granules from a transplantable rat insulinoma (Hutton et al., 1982; Roep et al., 1991) that can be propagated in gram quantities (2 to 20 g wet wt.) in an inbred Wistar rat strain from the New England Deaconess Hospital (NEDH rats; Chick et al., 1977). The tumors have a high hormone content (3% to 10% of total cellular protein) and give preparations of high purity and yield. There are several variants on the gradients that are used for granule purification (see Alternate Protocols 1 through 4) depending on the experimental objective. Subsequent protocols outline the adaptation of the procedures to INS-1E and Min6 cell lines (see Basic Protocol 2) that can be maintained in tissue culture (Konrad et al., 1995; Mohlig et al., 1997). The latter have the advantage of starting with a more homogeneous tissue source containing fewer dead cells, and the avoidance of animal husbandry. The cell lines

Current Protocols in Cell Biology 3.32.1-3.32.20, March 2009
Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0332s42

are practical for routine isolation of sub-milligram quantities of granules starting from 0.2 to 0.5 g wet weight. The lines are less granulated than either solid tumors or pancreatic islets, and the purity and yield of material is correspondingly lower. An adaptation for pancreatic islets is also given. Key considerations are the homogenization procedure and the choice of density gradient medium. Emphasis is given to the use of isoosmotic media so that the organelles remain intact and can be recovered for functional studies such as ion uptake measurements (Hutton, 1982). Vesicles derived from disrupted intracellular membranes distribute over a broad range of densities under isoosmotic conditions and the distribution is directly proportional to the volume/surface area ratio of the vesicle. Fractionation under hyperosmotic conditions (e.g., sucrose density gradients) reduces the complexity of density gradient profiles because such vesicles shrink and their densities approximate their protein/lipid ratio. Granules remain intact under such conditions and thus sucrose has its place as a gradient medium. Insulin granules, however, do not separate well from lysosomes or mitochondria on sucrose, and they lyse if diluted in isoosmolar solutions. Finally, Support Protocols 1 through 5 describe enzyme assays, which are used to characterize the gradient fractions.

BASIC PROTOCOL 1

PREPARATION OF DCVs FROM INSULINOMAS

The transplantable rat insulinoma was used to develop the insulin granule isolation protocol described here (Hutton et al., 1982; Roep et al., 1991). The tumor was generated by whole body irradiation of an animal that was linked to a parabiont and was derived from a number of different spontaneous tumors that subsequently arose (Chick et al., 1977). It was subsequently maintained and propagated by serial transplantation in the same strain of inbred rats (New England Deaconess Hospital—NEDH—rats) for >30 years (see Support Protocol 1). Tumor pieces can be cryopreserved in 10% (v/v) DMSO in RPMI in liquid nitrogen storage to preserve early passage number. One variant of the tumor that was propagated from a liver metastasis, MSL-G2 (Madsen et al., 1986), has been shown to have the interesting property of expressing insulin in tissue culture but glucagon as a tumor in vivo. The tumor was first successfully adapted to monolayer tissue culture as the RINm5F cell line, which secreted insulin in response to cAMP and alanine but not glucose (Praz et al., 1983) and later to a glucose-sensitive line INS1-E, which remains popular as an in vitro model of insulin gene transcription and cell biology (Asfari et al., 1992). Such cells, while slow growing, can be expanded to large numbers and have been used for subcellular fractionation (Bhathena et al., 1982). INS1-E cells can be passaged in the NEDH rat in vivo. The principal advantage of using the original tumor lies with an insulin content that is up to 30% of pancreatic islet tissue and the fact that large quantities of tissue can be harvested on a single day (10 to 20 g), which makes it ideal for homogenization and subcellular fractionation.

Percoll (a colloidal suspension of polyvinylpyrrolidone-coated silica particles) has negligible osmolarity (<20 mosmol/liter), produces a self-forming gradient up to $\rho = 1.16$, and is used to separate insulin granules from plasma membrane, ER, Golgi, endosomes, peroxisomes and synaptic-like vesicles, and mitochondria. Partial overlap of granules with lysosomes occurs at the higher density end of gradients. Percoll's major drawback is the additional steps needed to separate granules from the medium. It is suited for experiments where time is of the essence and subsequent experimentation requires freshly prepared organelles. Differential centrifugation is combined with the density gradient step. Equipment is pre-cooled to 4°C and all steps are performed at 4°C or on ice.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

Rats (NEDH strain, either sex, 150 to 200 g)
70% (v/v) ethanol or iodine
Sterile phosphate-buffered saline: 10 mM Na phosphate in 150 mM NaCl, pH 7.4, on ice
Ice-cold β-cell homogenization medium (β-HM; see recipe)
27% Percoll medium (see recipe)

Sterile dissection instruments (large and small scissors, large and small forceps, scalpels)
Sterile Petri dishes
50-ml sterile plastic centrifuge tubes
Refrigerated low-speed centrifuge accepting 50-ml capacity tubes (2.5-cm diameter)
Dounce homogenizer (40-ml capacity with loose (B) and tight (A) glass pestles; Wheaton)
Cheesecloth
Potter-Elvehjem homogenizer (25-ml capacity, Teflon pestle, 0.1-mm nominal clearance—a bench or wall-mounted variable speed drive capable of 2500 rpm for homogenization or a domestic drill mounted in a drill press)
9 × 2.5–cm polycarbonate centrifuge tubes
30-ml syringe fitted with lumbar puncture needle or peristaltic pump
High-speed refrigerated centrifuge with Beckman Type 30 and SW28 rotors

Remove tumor

1. Identify tumor-bearing rats by inspection of the inoculation site or from symptoms of hypoglycemia (ataxia or hind limb paralysis).

2. Euthanize tumor-bearing rat in accordance with an IACUC-approved method.

   Carbon dioxide asphyxiation, if permitted, is convenient.

3. Sterilize skin with 70% ethanol or iodine. Identify tumors that have developed as non-invasive subcutaneous adenomas at the inoculation site. Remove tumor after exposing the tumor on a flap of skin and dissect from surrounding connective tissue. Reject any obviously necrotic or cystic tumors.

4. Transfer tumor to a Petri dish. Trim off and remove fat and connective tissue, cut tumor open with scalpel, and scrape tissue into cold PBS. Cut tissue into pieces of ~1 to 5 mm using small scissors, transfer to a 50-ml plastic centrifuge tube, and fill with cold PBS.

5. Remove debris and blood by letting tissue sediment under gravity or centrifuge for 10 sec at 1000 × *g*, 4°C. Wash two additional times adding 40 ml PBS per wash.

Homogenize tissue

6. Suspend the insulinoma pieces harvested from 5 to 20 animals (~2.5 to 10 g wet weight) in 40 ml ice-cold β-HM and centrifuge 5 min at 800 × *g*, 4°C.

7. Resuspend pellet in 5 vol of 1× β-HM.

8. Homogenize in Dounce homogenizer with four passes (four times down and up) with loose pestle and four passes with tight pestle.

9. Centrifuge 10 min at 1700 × *g*, 4°C. Retain supernatant.

10. Resuspend pellet in 5 vol of ice-cold β-HM, filter through two layers of cheesecloth.

11. Homogenize filtrate with Potter-Elvehjem homogenizer 10 to 20 passes at 2500 rpm.

12. Centrifuge 10 min at 1700 × *g*, 4°C. Combine supernatant with that from step 9 above–post-nuclear supernatant (PNS).

Prepare granules on self-forming Percoll density gradients

13. Pipet 10-ml aliquots of PNS into a series of 9 × 2.5–cm polycarbonate centrifuge tubes.

14. Underlay the sample with 25 ml of 27% Percoll medium using a 30-ml syringe fitted with lumbar puncture needle or pumped in by peristaltic pump.

15. Centrifuge in Beckman Type 30 rotor for 40 min at 35,000 × *g*, 4°C, with slow deceleration below 775 × *g*.

16. Fractionate the gradient by pumping out or displacement into 25 tubes (1-ml each).

17. Discard the first dense fractions (clear but containing lysosomes) and pool the following white turbid fractions (two to seven fractions) that contain the granules (ρ = 1.09 to 1.14).

18. Dilute the pooled fraction to 35 ml with β-HM and centrifuge 15 min at 20,000 × *g* in a Beckman SW28 rotor, 4°C.

19. Recover the granules from the floating pellicle in the bottom of the tube.

20. Repeat steps 17 and 18 above until the granules form a pellet in the base of the tube.

    At the indicated centrifugal forces, Percoll forms a gradient with a steep profile at the top and bottom of the tube with a near-linear intervening segment. A glassy pellet of Percoll forms in the bottom of the tube and should be avoided when removing fractions with a pump or syringe as it will occlude the tubing.

    Other angle-type rotors may be used such as Sorvall SS34 and Beckman 50.2 rotors. In each case the best centrifugation conditions need to be established empirically by varying the initial Percoll concentration (25% to 30% range), centrifugation speed, and time. Optimization of the shape of the gradient and the resolution in the ρ = 1.09 to 1.14 range can be performed on dummy gradients using the refractive index to monitor the sedimentation of the Percoll particles. Colored Percoll density marker beads are useful for this exercise.

    Percoll does not generally affect marker enzyme assays or SDS-PAGE of gradient fractions. Concentration of proteins from fractions by ultrafiltration, or trichloroacetic acid or organic solvent precipitation, is impractical as the medium also precipitates.

21. Resuspend final pellet in 1 ml β-HM for further analysis or store at −80°C.

PREPARATION OF GRANULES ON PREFORMED NYCODENZ GRADIENTS

Nycodenz (5-(*N*-2,3-dihydroxypropoylacetamido)-2,4,6-tri-iodo-*N*,*N*′-bis(2,3-dihydroxypropoyl)-isophthalamide) is used to prepare isoosmotic gradients up to ρ = 1.15 (27.6% w/v solution). It is superior to Percoll in separating insulin granules from lysosomes but contamination with intracellular membranes is greater. ER and mitochondria are also well separated but, unlike Percoll, sediment at a higher density than granules. Nycodenz can be used for preformed, self-formed continuous gradients, and step gradients. It is easily removed by dilution and centrifugation, gel filtration, and dialysis. Gradient profiles can be easily determined from the refractive index of the fractions and using the refractive index of the homogenization medium and the isotonic 27.6% Nycodenz solutions as reference values.

Preparation of Granules Using Nycodenz Continuous Gradients

There are many commercially available devices for pouring continuous gradients; however, they can be generated simply and reproducibly with common laboratory equipment. Two approaches for preparing isotonic 10% to 27.6% Nycodenz gradients are described (also see Alternate Protocol 2). The benefit of the continuous gradient over the step gradient is the case of preparation. The use of the continuous gradient over the step gradient may be due to lack of equipment or time.

Materials

β-HM medium (see recipe)
Nycodenz
Post-nuclear supernatant (PNS; see Basic Protocol 1)

9 × 2.5–cm polycarbonate centrifuge tubes
Parafilm
Multichannel peristaltic pump (Fig. 3.32.1)
Beakers
Stir plate and magnetic stir bar
Velcro
Pasteur pipets with 0.5-cm wide bores
Centrifuge with a Beckman SW28
5-ml syringe with long (8-cm) needle, 16-G lumbar puncture set

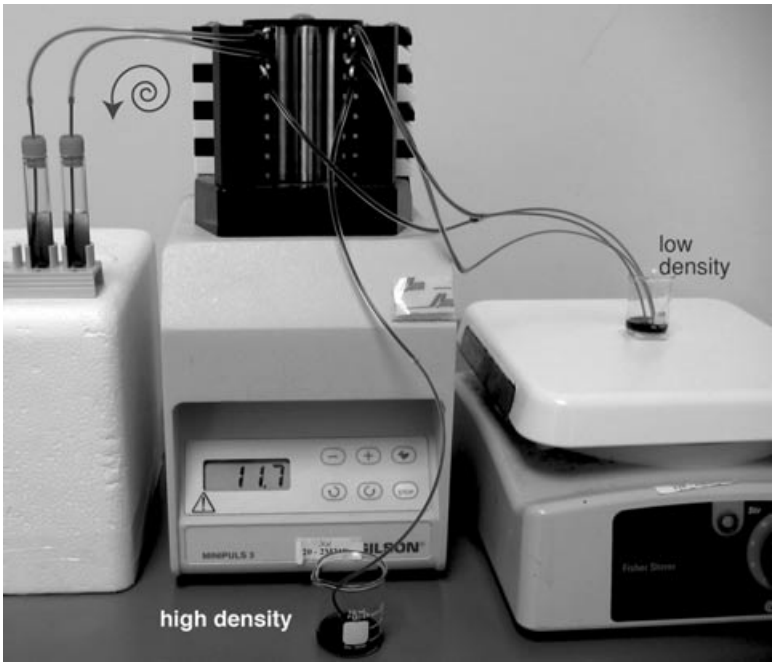


Figure 3.32.1 Gradient former. The setup shown can be used to generate pairs of matched gradients with a density range that spans the two starting solutions. The pump tubing must be of the same diameter as the tubes positioned in the two beakers, and the stirrer speed controlled so as to avoid the introduction of bubbles. The initial volume in the mixing beaker is equivalent to the final volume of each.

Prepare gradients

1. Prepare a series of mixtures of β-HM and buffered isotonic Nycodenz in the ratios of 9:1, 7:3, 5:5, 3:7, and 1:9 by transfering 5 ml of the first solution into the bottom of a 9 × 2.5–cm polycarbonate centrifuge tube, then underlay successively with 5 ml of the solutions of increasing density, ending with buffered isotonic Nycodenz.
2. Cap or seal with Parafilm, lay horizontally, and leave at room temperature or 4°C to allow diffusion of the layers (>2 hr).

   The process can be accelerated by inverting the tube once and returning it to the upright position.
3. Assemble a multichannel peristaltic pump with three tubes of equal diameter, a beaker with a stir bar fastened by a Velcro strip to a stir plate, and a reservoir of isotonic Nycodenz in a second beaker as shown in Figure 3.32.1. Ensure that the tubing from the reservoir to the mixing beaker is primed with liquid and the two tubes from the mixer are primed with air.
4. Place 36 ml low-density solution into the beaker and direct the two exit tubes to the bottom of two 9 × 2.5–cm polycarbonate centrifuge tubes. Start the stirrer and pump, and stop just before air is about to enter the centrifuge tube.

   Two identical linear gradients of ~25 ml each will be generated. The procedure can be repeated for additional gradients and can be used to prepare gradients of any volume; each gradient will approximate 0.7 of the volume initially placed in the stir plate beaker.
5. Leave the gradients at 4°C until use.

   Gradients are stable for up to 24 hr. Figure 3.32.1 shows a typical setup.

Centrifuge

6. Transfer 10-ml aliquots of PNS onto the top of each gradient. Use a Pasteur pipet with a wide bore (0.5-cm) immersed just under the top of the gradient to minimize mixing.
7. Centrifuge in a Beckman SW28 rotor for 1 hr at 100,000 × *g*, 4°C, with slow deceleration below 12 × *g*.
8. Fractionate the gradient by pumping out or by displacement into 25 tubes (1 ml each fraction).

   Granules appear as a diffuse cloudy band in the middle of the tube ($\rho = 1.10$ to 1.13).
9. Recover granules with a 5-ml syringe fitted with a long 8-cm needle (16-G lumbar puncture set). Avoid picking up the flocculent band of ER/lysosome/mitochondria that sediments as a tight layer below the granules.
10. Dilute the pooled fraction to 35 ml with β-HM and centrifuge 15 min at 20,000 × *g*, 4°C, in a Beckman SW28 rotor to recover the granules as a pellet. Resuspend the pellet for further analysis or store at −80°C.

ALTERNATE PROTOCOL 2

Using Nycodenz Step Gradients for Crude Granule Preparations

Alternatively, a Nycodenz step gradient can be used to prepare a crude granule fraction.

Materials

Post-nuclear supernatant (PNS; see Basic Protocol 1)
4.4%, 8.8%, and 17.2% (v/v) Nycodenz solutions (see recipes)
β-HM medium (see recipe)

9 × 2.5–cm polypropylene centrifuge tubes
Centrifuge with a Beckman SW28 rotor
5-ml syringe with 16-G needle

1. Transfer 20 ml of post-nuclear supernatant into two 9 × 2.5–cm polypropylene centrifuge tubes (40 ml total PNS).
2. Carefully underlay a step gradient comprised of 5 ml each of 4.4%, 8.8%, and 17.2% isotonic Nycodenz solution into each tube.
3. Centrifuge in a Beckman SW28 rotor 1 hr at 100,000 × *g*, 4°C, with slow deceleration below 12 × *g*.

   Mitochondria, lysosomes, and ER form a pellet in the tube. The interface between the original sample and the 4.4% Nycodenz layer is enriched in plasma membrane markers.
4. Recover the granules from the interface between the 8.8% and 17.2% Nycodenz layers using a 5-ml syringe fitted to a 16-G needle.
5. Resuspend granules in 35 ml β-HM medium and recover by centrifugation for 15 min at 20,000 × *g*, 4°C, in a Beckman SW28 rotor to recover as a pellet. Resuspend cell pellet for further analysis or store at −80°C.

   Step gradients are best designed after an initial analysis of the PNS on a continuous Nycodenz gradient and measurement of marker proteins (Fig. 3.32.2). There is variation between batches of Nycodenz probably related to the water content, and it is better to prepare the steps based on the refractive index and density. The steps can also be tailored to the experimental requirements as there is an inevitable trade off between recovery and purity of the fractions.

Granule Purification by Combined Nycodenz-Percoll Gradients

ALTERNATE PROTOCOL 3

Combined gradients of Nycodenz and Percoll are used for the additional isolation of granules as a more purified fraction at the expense of less material.

Materials

Nycodenz step gradient (see Alternate Protocol 2)
27% (v/v) Percoll (see recipe)
β-HM medium (see recipe)

9 × 2.5–cm polycarbonate centrifuge tube
Refrigerated centrifuge with Beckman Type 30 and SW28 rotors

1. Perform the Nycodenz step gradient procedure as in Alternate Protocol 2 omitting the final washing step 5.
2. Recover material from the 8.8%/17.2% interface in a total volume of 4 ml.
3. Transfer recovered material to a 9 × 2.5–cm polycarbonate centrifuge tube and suspend uniformly with 32 ml of 27% (v/v) Percoll.
4. Centrifuge in a Beckman Type 30 rotor for 40 min at 35,000 × *g*, 4°C, with slow deceleration below 11 × *g*.
5. Remove the clear layer below the granule band (1 to 2 ml) and discard. Pool the following white turbid fraction (∼5 to 7 ml) that contains the granules (ρ = 1.09 to 1.14).
6. Dilute the pooled fraction to 35 ml with β-HM and centrifuge 15 min at 20,000 × *g*, 4°C, in a Beckman SW28 rotor.

7. Recover the granules from the floating pellicle in the bottom of the tube.
8. Repeat steps 6 and 7 above until the granules form a pellet in the base of the tube. Resuspend pellet for further analysis or store at −80°C.

ALTERNATE PROTOCOL 4

Separations Using Self-Forming Nycodenz Gradients

Nycodenz gradients can be self-formed by centrifugation at high speeds; however, they will inevitably be somewhat hypotonic at the low-density end and hypertonic at the high-density end. It is nevertheless a convenient approach for preparing gradients in small volumes (2 to 4 ml) with benchtop ultracentrifuges. A protocol for an 8-place Beckman TLA 110 rotor that generates an approximation of a 10% to 30% Nycodenz gradient is described here.

Materials

27.6% isotonic Nycodenz
β-HM (see recipe)
Post-nuclear supernatant (PNS; see Basic Protocol 1)

13 × 50–mm centrifuge tubes
Refrigerated ultracentrifuge with a Beckman SW50.1 swing-out rotor

1. Mix 16 ml of 27.6% isotonic Nycodenz and 16 ml β-HM.
2. Transfer 3.3 ml to 13 × 50–mm centrifuge tubes.
3. Centrifuge 5 hr at 440,000 × *g*, 4°C.
4. Carefully remove the top 0.8 ml from each tube and replace with 0.8 ml PNS or other tissue fraction suspended in β-HM.
5. Centrifuge 60 min at 100,000 × *g*, 4°C, in a Beckman SW50.1 swing-out rotor.

The tube used is shorter than the conventional 13 × 56–mm TLA 110 rotor tube. Gradients can be prepared in advance and can be stored at 4°C for at least 24 hr. The gradient profile can be manipulated by changing the starting concentration of Nycodenz and the cumulative g × min for which the centrifuge is run. The Nycodenz will eventually clear from the top down. Other angle-head rotors can be used but in each case pilot studies are required to optimize the gradient shape based on measurement of the refractive index or density of gradient fractions.

INSULIN GRANULE PREPARATIONS FROM OTHER TISSUE SOURCES

Tumors

The INS1 cell line can usually be propagated in the NEDH rat as a solid tumor if required; however, results are variable in terms of the insulin content of the solid tumors that arise.

The procedures used to isolated rat insulinoma granules have been applied to mouse insulinomas (Bergman et al., 2000) using primary insulinomas from mice carrying a T antigen oncogene driven by the rat insulin II promoter (Efrat et al., 1988). This study used the non-obese diabetic (NOD) mouse on an immunodeficient *scid* background, a strain combination that poses logistical problems in terms of the longevity and care of the mice, but alternative insulinoma-bearing mice can be developed on other backgrounds by intercrossing with RIP T antigen transgenic mice. Once established, tumors and the derived cell lines can be passaged in mice of the same background, or more conveniently on a *scid* background, and harvested from animals after 3 to 6 weeks.

Cell Lines

A number of insulin-secreting cell lines derived from tumors can be grown in sufficient bulk to generate on the order of 0.1 to 1 g wet weight of tissue. These include the hamster insulinoma, HIT cell (Wang, 1989), rat INS-1 (Asfari et al., 1992), RINm5F (Bhathena et al., 1982) lines, and mouse β-TC (Efrat et al., 1988) and Min6 lines (Miyazaki et al., 1990), and derivatives thereof. Granules have been prepared from each of these sources using essentially the same procedures as described in Basic Protocol 1 for the solid tumors. Such procedures are best performed in smaller volumes using smaller rotors with modified homogenization procedures. A major determinant of the purity of the isolated granule fraction is the extent of granulation of the starting material, which for many lines may be <1% of pancreatic islets or solid tumors. It is thus essential to establish the suitability of the cell source based on insulin content before embarking on the isolation procedures. The buoyant density of granules can be expected to vary with the cellular source and species and, probably, clonal variation and passage number of the cell line in question. Pilot studies using continuous density gradient centrifugation are essential before proceeding to procedures based on step gradients. A procedure for isolation of granules from Min6 cells is detailed in Basic Protocol 2.

Pancreatic Islets

Pancreatic islets, in principle, should be an ideal source of starting material. The β-cell approximates 60% to 70% of the islet cell population, the rest being comprised of cells that store glucagon, somatostatin, pancreatic polypeptide, and ghrelin in dense core vesicles. Islets comprise only 2% to 5% of the pancreas volume and need to be isolated first by collagenase digestion and gradient purification. Inevitably there is some contamination with pancreatic acinar tissue, a cell type that is also extensively granulated. It is feasible to generate ∼1 mg wet weight of islets from a single mouse, and 5 to 10 mg from a rat. In both cases up to ten animals can be processed on a single day. The islet yield from a single human cadaveric pancreas can exceed 1 g wet weight, although the contamination with ductal and acinar tissue is usually high (10% to 50%).

PREPARATION OF GRANULES FROM CULTURED Min6 INSULINOMA CELLS

BASIC PROTOCOL 2

The following protocol uses the same gradient materials as those above for solid tumors but are adapted to the smaller amounts of tissue that are available from cell lines and isolated islets. The other major modification is the homogenization technique. Cultured cells appear more difficult to break than solid tissue and once dissociated into single cells appear resistant to homogenization by methods that rely on shearing such as the Potter-Elvejhem homogenizer. INS-1E monolayer cultures are used as an example. This harvesting, homogenization, and centrifugation protocol has been applied successfully to mouse β-TC3 and Min6 cells.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

50× media supplement stock (see recipe)
Basal RPMI 1640 medium (see recipe)
Mm 6 cells in culture (75% to 80% confluent in 75-cm^2 tissue culture flask; Miyazaki)
0.25% (w/v) trypsin-EDTA (GIBCO cat. no. 25200)

Calcium- and magnesium- free PBS (GIBCO cat. no. 10010)
Cell dissociation medium (GIBCO cat. no. 13150-016)
β-HM (see recipe)
DNase I
30% Percoll (see recipe)

150-cm^2 tissue culture flasks
150-mm Petri dishes treated for tissue culture (Nunc cat. no. 168381)
Cell lifter (Costar cat. no. 3008)
2-ml Dounce homogenizer with loose-fitting (A) and tight-fitting (B) glass pestles (Wheaton), pre-chilled
1.5-ml microcentrifuge tubes
70-μm mesh filter *or* insulin syringe and 28-G needle (Becton Dickenson cat. no. 309309)
3-ml syringe
Ball-bearing cell cracker (0.1576-in. ball; 0.1586-in. bore; Industrial Tectonics)
13 × 56–mm polycarbonate centrifuge tubes
Centrifuge with Beckman TLA110 rotor
Screw-capped microcentrifuge tubes

Split cells

1. Add 10 ml media supplement stock solution to a 500-ml bottle of basal RPMI 1640 medium.
2. Split Mm 6 cells in a 1:4 ratio for culture in 150-cm^2 flasks under 5%CO_2/95% air atmosphere.
3. The day preceding the granule isolation recover the cells from each flask by trypsinization with 0.25% trypsin-EDTA (*UNIT 1.1*) and re-seed on 150-mm diameter Petri dishes treated for tissue culture at 5×10^6 cells per flask.

Harvest cells in GIBCO cell dissociation medium

4a. Prepare 5 to 20 flasks/dishes for each isolation ($0.2–1 \times 10^9$ cells in total).

5a. Rinse monolayers with Ca^{2+} and Mg^{2+}-free PBS to remove serum proteins and non-adherent cells.

6a. Dissociate cells from plastic with 2 to 5 ml cell dissociation medium.

7a. Pool tissue from all flasks and collect by centrifuging 5 min at 800 × *g*, 4°C.

8a. Rinse pellet two times by resuspending in 20 to 30 ml PBS and centrifuging 5 min at 800 × *g*, 4°C.

9a. Rinse once in 20 to 30 ml β-HM. Proceed to step 10.

Harvest cells in hypotonic medium

4b. Prepare 5 to 20 dishes for each isolation ($0.2–1 \times 10^9$ cells).

5b. Rinse monolayers with ≥4 ml Ca^{2+}- and Mg^{2+}-free PBS to remove serum proteins and non-adherent cells.

6b. Place each dish on ice and add 2.5 ml β-HM diluted 1:5 with water for 10 min.

7b. Harvest cells by scraping dish with cell lifter. Rinse with an additional 0.5 ml of 0.2× β-HM.

8b. Recover cells by centrifuging for 5 min at 800 × *g*, 4°C.

9b. Resuspend cell pellet in 5 vol β-HM just before proceeding to the homogenization step 10.

Homogenize cells

10. Estimate the volume of the cell pellet, resuspend in 5 vol β-HM containing 10 μg/ml DNase I and transfer 2-ml aliquots to the chilled 2-ml Dounce homogenizer.
11. Homogenize with 20 up and down strokes with the tight-fitting pestle (B).
12. Transfer homogenate to a series of 1.5-ml microcentrifuge tubes and microcentrifuge 5 min at 800 × *g*, 4°C.
13. Retain supernatant.
14. Resuspend pellet in original volume, pass through 70-μm mesh filter to remove any large aggregates of debris.

    Alternatively, aspirate and express through a 28-G needle attached to an insulin syringe.

15. Recover the filtrate in a 3-ml capacity syringe and pass five to ten times (back and forth) through a ball-bearing cell cracker (0.1576-in. ball; 0.1586-in. bore).
16. Transfer homogenate to a series of 1.5-ml microcentrifuge tubes and centrifuge 5 min at 800 × *g*, 4°C.
17. Recover supernatant and pool with supernatant obtained from step 13.

Perform differential and density gradient centrifugation

18. Transfer supernatants to a 13 × 56–mm polycarbonate centrifuge tube and centrifuge 10 min at 20,000 × *g*, 4°C, in a Beckman TLA110 rotor.
19. Gently resuspend the pellet in 1.0 ml β-HM using the loose-fitting pestle of the Dounce homogenizer. Transfer to a chilled 2-ml Dounce homogenizer and homogenize with several passes with a loose-fitting pestle (A) to ensure that pelleted material is well dispersed.

Perform Percoll gradient

20. Load 13 × 56–mm polycarbonate centrifuge tubes each with 0.5 ml of the resuspended 20,000 × g pellet from step 19.
21. Underlay with 3 ml isotonic 30% (v/v) Percoll. Mix and centrifuge 60 min at 25,000 × *g*, 4°C, in a TLA 110 rotor.
22. Fractionate the gradient into eighteen 1.5-ml screw-capped microcentifuge tubes either by pumping out the gradient from the bottom of the tube or by carefully removing 200-μl samples from the top of the gradient.

    The two-step homogenization procedure should release up to 60% to 80% of the secretory granules from cells as measured by the granule marker enzyme carboxypeptidase E (CPE) activity in the 10-min 800 × g supernatant. A CPE activity of 50% to 75% should be recoverable in a pellet produced by centrifugation of the supernatant 10 min at 20,000 × g.

    The harvest of tissue using the hypotonic medium does not cause cell lysis and the cells remain in clumps but are obviously swollen. A greater proportion of the cells can be broken with the Dounce homogenizer by this method, and the granules band at the same density on Percoll gradients without loss of yield.

PROPAGATION OF SOLID TUMORS

SUPPORT PROTOCOL 1

This is best performed at the time of harvesting but will require additional assistance. Select freshly harvested solid tumors that developed to a size of ∼0.3 g or less after 5 to 6 weeks from hypoglycemic animals as the source. Alternatively, use material from archived sources that were frozen at −80°C in 10% (v/v) DMSO in PBS and stored in liquid nitrogen. They can be thawed by dilution in PBS immediately prior to use.

Materials

Ethanol
Tumor pieces in PBS (see Basic Protocol 1) in 1-ml syringe and 16- or 18-G needle

1. Sterilize the injection site (scruff of neck or groin) with ethanol.
2. Inject ~0.1 ml of a suspension of tumor pieces prepared as in Basic Protocol 1 in PBS aiming at transferring 1 to 3 mg wet weight tissue per animal.

   Recipients were typically 8 weeks old of mixed sex.

   It is essential to monitor the stability of the tissue line. Tumor lines that develop quickly (<3 weeks) or grow to a large size (>2 g) without inducing hypoglycemia indicate a reduction of insulin biosynthetic capacity and should be rejected. Select against tumors that appear to be infiltrated by lymphocytic tissue (cheese like nodules) or small tumors that produce severe hypoglycemic symptoms. The phenotype can change at any time and it is advisable to periodically monitor the insulin content of the tumor fragment line (usually 10 to 40 μg/mg protein). Blood glucose levels are conveniently measured using any commercially available glucose monitor as used by diabetes patients by nicking the tip of the tail of the animal with a razor blade or the point of a 18- to 29-G hypodermic needle and expressing ~50 μl of blood. A normal random blood glucose in the rat is 5 to 10 mM (80 to 200 mg/dl).

ENZYME MARKER ASSAYS

Density gradient fractions should be analyzed for a series of marker proteins to evaluate the success of the procedure and the overlap between fractions. Quantitative analyses allow the determination of specific activity and enrichment. Immunoblotting is inferior in this regard but can provide information regarding compartmentalization as revealed by different molecular sizes of precursor and mature forms. A simple Coomassie or silver stained gel of fractions is useful in revealing major contamination arising from subcellular membranes that do not have specific markers. For pancreatic endocrine cells, immunoblots for Na/K ATPase (plasma membrane), Golgin 48 (Golgi), carboxypeptidase E (granules), and Cathepsin B (lysosomes) provide good signals. Enzyme assays for 5′ nucleotidase (plasma membrane), NADPH cytochrome *c* reductase (ER, see Support Protocol 3), cytochrome oxidase (mitochondria, see Support Protocol 4), CPE (granules, see Support Protocol 5), and *N*-acetylglucosaminidase (lysosomes; see Support Protocol 2) are sensitive and simple to perform. Examples of four such assays are given.

SUPPORT PROTOCOL 2

Enzyme Assay for β,*N*-Acetyl Glucosaminidase

β,*N*-acetyl glucosaminidase is a lysosomal marker.

Materials

Sample material: gradient fractions
β,*N*-acetyl glucosaminidase reaction mixture (see recipe)
1 M Na_2CO_3
4-Methylumbelliferone (standard)

96-well plate suitable for fluorimeter
37°C heating block
Fluorimeter

1. Transfer samples (1 to 10 μl) to a 96-well plate suitable for fluorimeter in use (black for top reading instrument; clear for bottom-reading instrument).
2. Add 50 μl β,*N*-acetyl glucosaminidase reaction mixture.

3. Incubate 10 to 30 min at 37°C.
4. Add 200 µl of 1 M Na_2CO_3 per well to stop the reaction.
5. Read fluorescence at excitation 360 nm, emission 488 nm against the 4-methylumbelliferone standard.

The fluorescence is greatly enhanced by the addition of Na_2CO_3 at the end of the incubation. Nevertheless the time course of substrate hydrolysis can be monitored at excitation 360 nm, emission 500 nm to establish the optimal sample volumes and incubation times for the assay.

Enzyme Assay for NADPH–Cytochrome *c* Reductase

SUPPORT PROTOCOL 3

NADPH-cytochrome *c* reductase is a marker for endoplasmic reticulum and Golgi.

Materials

Samples
NADPH-cytochrome *c* reductase reaction mixture (see recipe)

96-well plate
Spectrophotometer

1. Transfer samples (1 to 10 µl) to a 96-well plate.
2. Add 200 µl NADPH-cytochrome *c* reductase reaction mixture.
3. Monitor the increase in OD_{550nm} for 10 to 30 min at 37°C.

It is important to set the spectrophotometer at 550 nm as absorption peak is very narrow.

Reduction is monitored at 550 nm using molar absorbance indices of 27.7 × 10^3 $cm^2m^{-1\text{-}1iter}$. NADPH-linked assays should not be confused with the rotenone insensitive NADH-cytochrome c reductase that is mainly located in the outer mitochondrial membrane (Sottocasa et al., 1967).

Enzyme Assay for Cytochrome Oxidase

SUPPORT PROTOCOL 4

Cytochrome oxidase is a marker for mitochondria.

Materials

Samples
Cytochrome oxidase reaction mixture (see recipe)
100 mM potassium ferricyanide

96-well plate
Spectrophotometer

1. Transfer samples (5 to 25 µl) to a 96-well plate.
2. Add 200 µl of cytochrome oxidase reaction mix.
3. Monitor the OD_{550nm} for 2 to 10 min and calculate the enzyme rate from the linear segment of the time course.
4. Determine the absorbance of the oxidized form by the subsequent addition of 5 µl of 100 mM potassium ferricyanide and the difference in the OD_{550nm} used to quantitate the rate of reaction.

If no activity is detected in the assay, try shaking the reaction mixture again to remove residual dithionite.

SUPPORT PROTOCOL 5

Enzyme Assay for Carboxypeptidase E

Carboxypeptidase E is a marker for secretory granules.

Materials

Samples: granules isolated from insulinomas or cell lines
Carboxypeptidase E reaction mix A (see recipe)
Carboxypeptidase E reaction mix B (see recipe)
Acidified chloroform (see recipe)

1.5-ml microcentrifuge tubes
37°C heating block
96-well silica glass plate
Fluorimeter (360-nm excitation; 500-nm emission)

1. Transfer samples (1 to 10 μl) to each of two series of 1.5-ml microcentrifuge tubes: (A) total activity; (B) inhibited activity.
2. Add 200 μl carboxypeptidase E reaction mix A or B to the appropriate tubes and incubate 30 min to 1 hr at 37°C.
3. Add 300 μl acidified chloroform, vortex vigorously for 1 min.
4. Centrifuge 1 min at 2000 × *g*, room temperature and aspirate majority of upper phase.
5. Transfer 200 μl of the lower phase to 96-well silica glass plate.
6. Read the fluorescence at excitation 360 nm/emission 500 nm.

Although the fluorescence signal is not high, the signal-to-noise ratio is ~100:1 for a 100 μM solution of product. The CPE activity is the difference between the total (A) and GEMSA-inhibited (B) activity.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acidified chloroform

Add 10 μl of 10 M HCl to 50 ml chloroform in glass bottle. Shake to dissolve. Prepare fresh.

Basal RPMI 1640 medium

Basal RPMI 1640 medium (Sigma cat. no. R 8758) containing:
11.1 mM D-glucose
10% (v/v) fetal bovine serum
500 U penicillin
0.5 mg streptomycin
Store up to 2 months at 4°C

β-HM, 2×

14 ml 60% (w/w) sucrose (see recipe)
1 ml 100 mM EGTA-K^+ (see recipe)
1 ml 1 M $MgSO_4$
2 ml 100 mM MES-K^+, pH 6.5 (see recipe)
Add H_2O to 50 ml
Store for 1 to 2 days at 4°C

β-HM

Add equal volumes of 2× β-HM (see recipe) and water. Store for 1 to 2 days at 4°C.

β, N-acetyl glucosaminidase reaction mixture

0.1 M citric acid NaOH, pH 5.6
0.1 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside
0.1% (v/v) Triton X-100
Prepare fresh

β, N-acetyl glucosaminidase or 4-methyl umbelliferone standard

Prepare a 10 mM stock solution of 4-methyl umbelliferone or β, *N*-acetyl glucosaminidase in water. Store frozen and protected from light. Dilute 1:100 in the reaction mix and prepare a standard curve with 1, 2, 5, 10, 20, and 50 μl in the reaction mix and make to a final volume of 50 μl.

Carboxypeptidase E reaction mix A

100 mM acetate buffer, pH 5.5
0.2 mM $CoCl_2$
0.2 mM dansyl phenylalanyl, alanyl, arginine (from 10 mM aqueous stock)
Store up to 3 months at −20°C

Carboxypeptidase E reaction mix B

Carboxypeptidase E reaction mix A (see recipe) with 10 μM GEMSA (guanidinyl ethane mercaptosulfonic acid; Calbiochem)
Store up to 3 months at −20°C

Carboxypeptidase E standard

Prepare 5 mM dansyl chloride solution in acidified chloroform (Sigma; see recipe). Prepare standard curve by addition of 1, 2, 5, and 10 μl of 5 mM dansyl solution to 200 μl final volume of acidified chloroform. Prepare fresh.

Cytochrome oxidase reaction mix

Add 10 μl of freshly prepared 1.2 M sodium dithionite solution (0.238 mg/ml in 50 mM sodium phosphate, pH 7.5) to 5 ml of 17 μM cytochrome *c*.

The solution will change color from orange to pink reflecting the reduction of the cytochrome.

Inactivate any excess dithionite by shaking the solution in the flask vigorously for 10 to 30 sec to allow atmospheric oxygen to oxidize the dithionite.

If the enzyme reaction appears to fail with a sample that is known to contain cytochrome oxidase activity, it is usually because there is residual dithionite, in which case repeat the shaking procedure.

EGTA-K^+, 100 mM

Add 3.8 g EGTA free acid in 80 ml water
Adjust to pH 7 with 1 M KOH
Adjust to 100 ml
Store 2 weeks at 4°C

The solid dissolves as neutrality is reached.

Isotonic Nycodenz, 4.4% w/v (pH 6.5)

3.2 ml 27.6% isotonic Nycodenz (see recipe)
8.4 ml 2× β-HM (see recipe)
8.4 ml H_2O
Store up to 1 to 2 days at 4°C

Isotonic Nycodenz, 8.8% w/v (pH 6.5)

6.4 ml 27.6% isotonic Nycodenz (see recipe)
6.8 ml 2× β-HM (see recipe)
6.8 ml H_2O
Store up to 1 to 2 days at 4°C

Isotonic Nycodenz, 17.6% w/v (pH 6.5)

12.8 ml 27.6% isotonic Nycodenz (see recipe)
3.6 ml 2× β-HM (see recipe)
3.6 ml H_2O
Store up to 1 to 2 days at 4°C

Isotonic Nycodenz, 27.6% w/v (pH 6.5)

Nycoprep Universal (500 g Nycodenz; Axis-shield cat. no. 1113058)

Media supplement stock, 50×

0.5 M Na^+-HEPES
100 mM L-glutamine
50 mM Na-pyruvate
2.5 mM β-mercaptoethanol
Filter sterilize through 0.2-μm filter and store up to 3 months at −20°C in 10-ml aliquots

MES-K^+, 100 mM (pH 6.5)

1.95 g mercaptoethanesulfonic acid hydrate
Adjust with 1 M KOH to pH 6.5
Add H_2O to 100 ml
Store 2 weeks at 4°C

$MgSO_4$, 1 M

12 g anhydrous $MgSO_4$ salt to 100 ml water. Store indefinitely at 4°C.

NADPH cytochrome c reductase reaction mixture

0.1 mM NADPH
0.3 mM KCN
0.1 mM horse heart cytochrome
50 mM sodium phosphate buffer, pH 7.5
Prepare fresh

Percoll, 27% (v/v)

13.5 ml Percoll suspension (Sigma)
Mix well by repeated inversion
25 ml 2× β-HM (see recipe)
11.5 ml H_2O
Stable for 48 hr at 4°C

Percoll, 30% (v/v)

15 ml Percoll suspension
Mix well by repeated inversion
25 ml 2× β-HM (see recipe)
10 ml H_2O
Stable for 48 hr at 4°C

Sucrose, 60% (w/w)

Slowly add 300 g sucrose to 500 ml water while stirring
Stable indefinitely at 4°C

COMMENTARY

Background Information

Dense core vesicles (DCVs) are the primary storage organelle for the secretion products of endocrine and exocrine tissues involved in regulated release of proteins such as polypeptide hormones and digestive hormones. They share common features: a simple phospholipid bilayer envelops a core comprised of condensed, often crystalline, protein that is usually specific for the cell type in question and often heterogeneous in composition. The dimensions of these vesicles are usually characteristic of the cell type in question, presumably related to the physico-chemical properties of the major protein components. They range from spheres of constant diameter (50-nm to 1-μm diameter) filled with cargo of uniform composition to cigar-shaped structures with longitudinally arranged fibrils. The high protein-to-water content and high protein-to-lipid ratio place these organelles among those with the highest buoyant densities ($\rho > 1.10$) in the cell, and thus they are generally readily separable from other organelles in high yield. That said, the pool of granules is usually comprised of immature, mature, and senescent populations and in some cell types mixed populations of granules occur that contain different proportions of major secretory products. The granule membranes are sites of active transport of protons and ions and susceptible to osmotic changes; the membranes are reactive to the ionic composition of the isolation medium. The spherical shape and close approximation of the membrane to the core make these organelles particularly susceptible to osmotic distension and breakage; thus they are best isolated under isoosmotic or hyperosmotic conditions.

Critical Parameters and Troubleshooting

The single most important variable that affects the success of the organelle isolation is the tissue homogenization process. The objective is to disrupt the majority of cells and release the organelles without breaking them. Homogenization procedures that work well with millimeter size pieces of soft tissues that depend on shearing in a motor-driven pestle and glass tube are inefficient with tissue culture cells. Conversely, ball-bearing homogenizers that work well with culture cells have limited application for tissue pieces. The authors' experience with blade homogenizers and nitrogen decompression methods with tissues has been that they disrupt cells efficiently but also cause excessive disruption of organelles, particularly nuclei and dense core vesicles. An approach that uses two or three cycles of tissue disruption, each with increasing severity and followed by centrifugation to separate released organelles from debris and unbroken cells, is preferable. This typically involves one round with a Dounce homogenizer, aspiration of the cells through a hypodermic syringe needle of 25- to 28-G, and finally, several passages through a ball-bearing homogenizer. It is important to determine the effectiveness of each step, and it usually suffices to determine the activity of a marker such as CPE (see Support Protocol 5) in the pellet generated by the 5-min, 800 $\times g$ centrifugation pellet, the corresponding supernatant, and the pellet and supernatant obtained by further centrifugation of the PNS for 10 min at 20,000 $\times$ g. Excessive homogenization not only reduces the yield of organelles but also leads to the generation of artifacts, which manifest as multiple sharply defined layers on density gradient centrifugation or as aggregated material of high density. With cultured cells it is usually better to err on the side of less efficient cell disruption and sacrifice yield.

The other important parameter to follow on gradient centrifugation is the density of the fractions. This is best achieved indirectly by measuring the refractive indices of the component solution of the gradient and the gradient

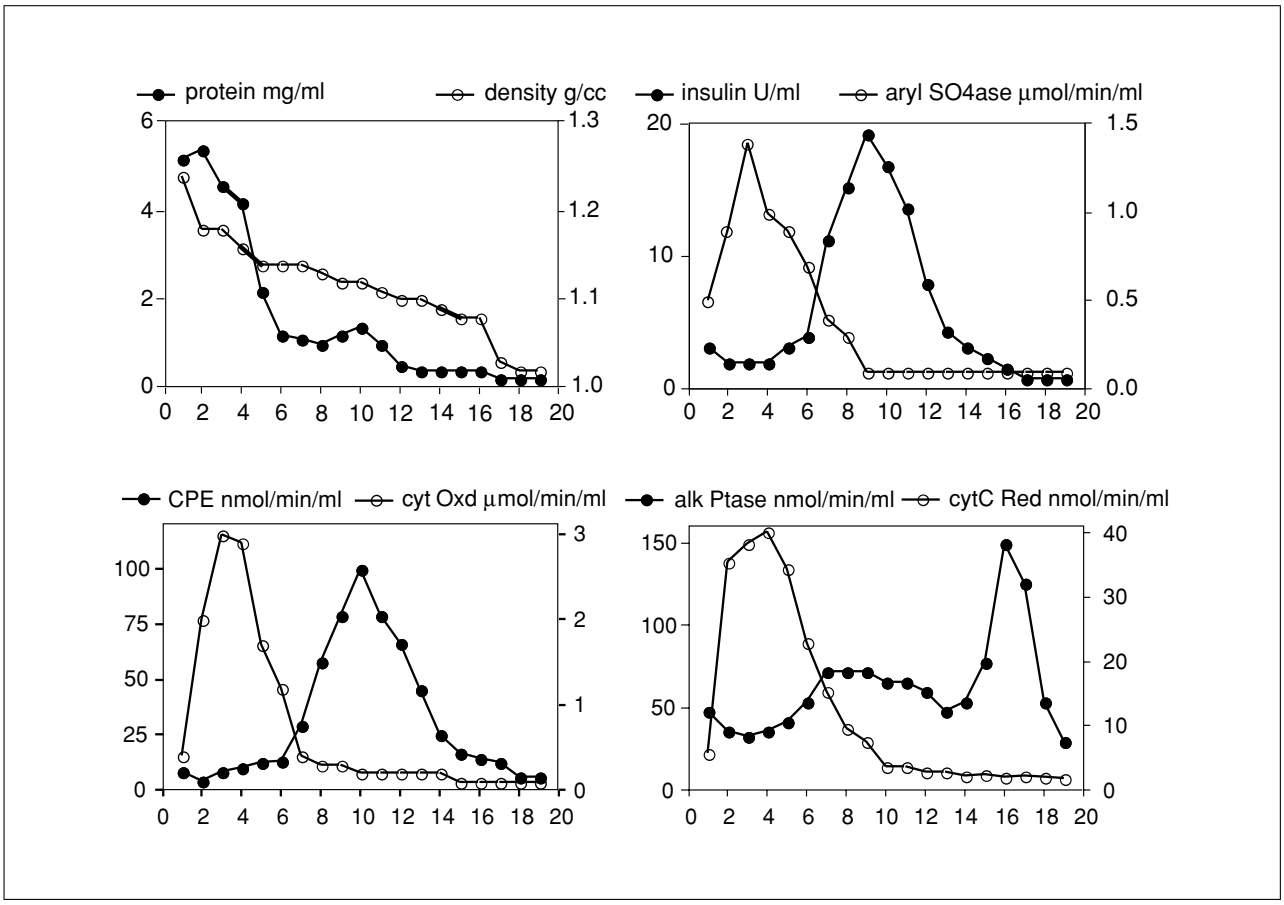


Figure 3.32.2 Ultrastructure and electrophoretic profile of granules isolated from a transplantable rat insulinoma by Percoll gradient centrifugation. An electron micrograph of a typical rat insulinoma cell is shown alongside the final preparation of granules equivalent to fraction B in Fig. 3.32.4 and the SDS PAGE separation of the major fractions A through G that result from the procedure (see Fig. 3.32.4 and Anticipated Results).

fraction. A useful device for these measurements are hand-held refractometers calibrated to measure sucrose concentrations in the 0% to 28% and 28% to 62% range (e.g., SHILAC models SH/MNL 1125 and SH/MNL2862; Camlab).

Ball-bearing homogenizer are not generally available commercially but can be manufactured in a well-equipped machine shop. The original design was prepared by Hans Issel (1667 Anamor Street, Redwood City, CA 94061, tel. 650-368-5417).

Anticipated Results

Insulin granule preparations from solid tumors typically yield on the order of 10 mg insulin granule protein (fraction B) from 10 to 20 g wet weight of washed tumor pieces. Typical marker protein profiles can be found in the literature (Hutton et al., 1982, 1983; Hutton and Peshavaria, 1983). The ultrastructural appearance of the final preparation is illustrated in Figure 3.32.2 along with the starting material and an SDS-PAGE analysis of the major fractions that are recovered from the gradient.

Preparations on Nycodenz gradients generally yield more material as there is less loss of granules at the final steps that involve repeated washes to remove gradient medium. An example of marker profiles is shown in Figure 3.32.3 and reported in Roep et al. (1991).

Granule preparation from cell lines is dependent both on the amount of starting material and the extent of granulation. Figure 3.32.4 illustrates results that were obtained from a Percoll gradient–based preparation from Min6 cells that contained ~10% of the insulin content (10 µg/mg protein) of rodent islet. The banding pattern observed on the gradient (Fig. 3.32.4B) is very similar to that obtained with solid tumors prepared on a larger scale, and the marker enzymes distributed in essentially the same way. The side fractions generated by the Percoll fraction while containing a mixture of organelles are often useful for other studies and can be recovered by the same procedure of repeated washing. They can be broadly described as follows:

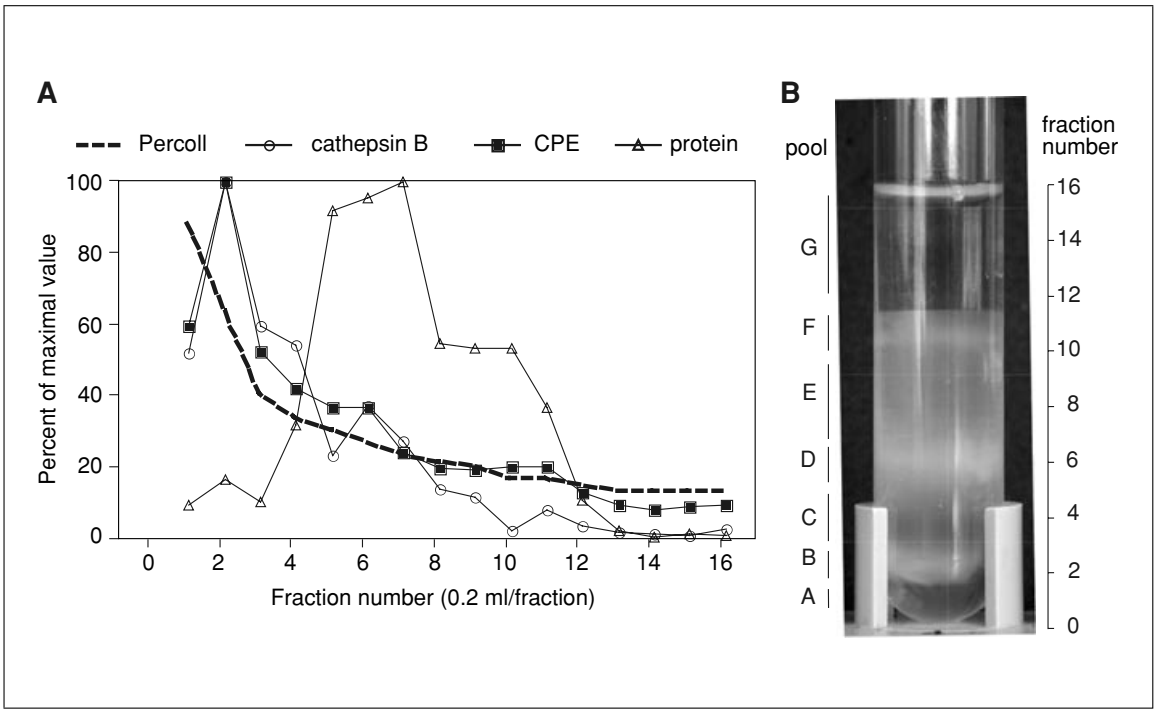


Figure 3.32.3 Percoll density gradient subfractionation of mouse Min6 cell homogenate A panel of lysosomal (cathepsin B) and granule (CPE) markers are shown alongside the gradient that was analyzed. The equivalent positions of fractions A through G that are shown in Figure 3.32.4 are indicated alongside the centrifuge tube.

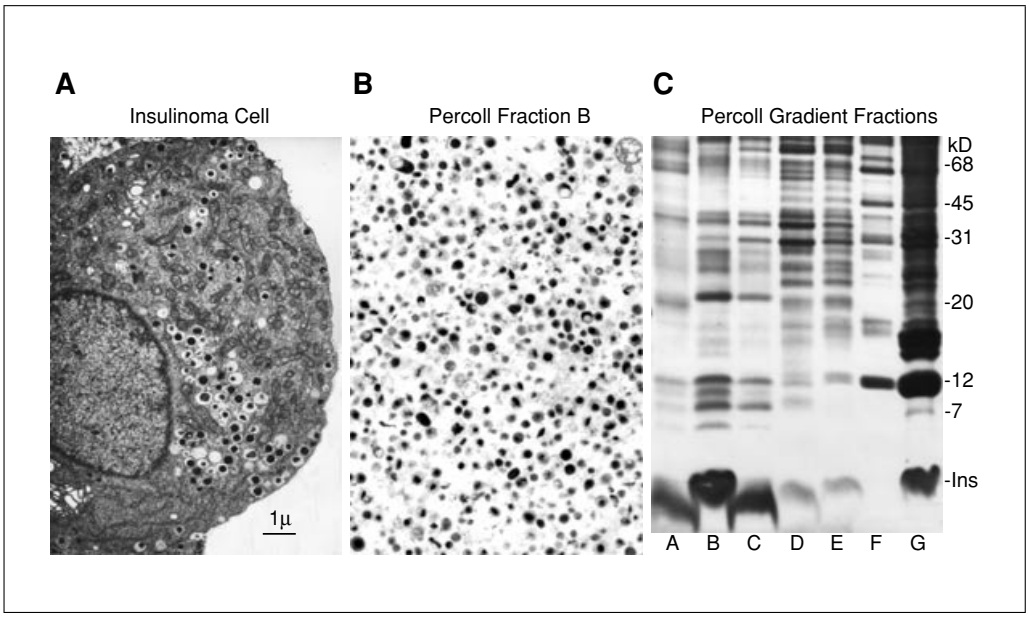


Figure 3.32.4 Nycodenz density gradient subfractionation of rat insulinoma tissue. Tissue was fractionated according to the protocol above and analyzed for markers of secretory granules (insulin and CPE), lysozomes (aryl sulfatase), mitochondria (cytochrome oxidase), ER (NADPH cytochrome *c* reductase), and plasma membrane (alkaline phosphatase). Profiles such as these are used in the design of step gradients. Steps corresponding to the Nycodenz concentration in fractions 7 and 15 would be a reasonable compromise between purity and yield. The purest fractions appear to correspond to the interval between fractions 12 and 15.

1. A secretory granule fraction containing the majority of cellular lysosomes.

2. The highest purity secretory granule fraction.

3. Granules with the lowest lysosomal contamination but containing significant mitochondrial and endoplasmic reticulum.

4. A mixed mitochondrial, ER, Golgi, and endosomal fraction with the highest specific activity of mitochondrial markers.

5. A mixed mitochondrial, ER, Golgi, and endosomal fraction with the highest specific activity of ER markers.

6. A mixed ER, Golgi, and endosomal fraction with the highest specific activity of plasma membrane markers.

7. A fraction containing all the cytosolic proteins together with proteins released from rupture of intracellular organelles.

Time Considerations

The time taken to complete the entire fractionation procedure from transplantable tumors is ~7 hr. Solution preparations, tissue harvesting, and initial preparation of tissue require ~120 min. Homogenization and preparation of the nuclear-free supernatant takes 60 min, and a single gradient centrifugation procedure another 60 min. Fractionation of the gradient is achieved in <60 min but the granule washing steps and clean up requires an additional 120 min. The procedure based on Nycodenz gradients can save 60 min principally as the wash steps are shorter.

Procedures based on the use of cell lines require ~6 hr, the time-saving part being on the initial tissue propagation.

Literature Cited

Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P.A., and Wollheim, C.B. 1992. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167-178.

Bergman, B., McManaman, J.L., and Haskins, K. 2000. Biochemical characterization of a beta cell membrane fraction antigenic for autoreactive T cell clones. *J. Autoimmun.* 14:343-351.

Bhathena, S.J., Oie, H.K., Gazdar, A.F., Voyles, N.R., Wilkins, S.D., and Recant, L. 1982. Insulin, glucagon, and somatostatin receptors on cultured cells and clones from rat islet cell tumor. *Diabetes* 31:521-531.

Chick, W.L., Warren, S., Chute, R.N., Like, A.A., Lauris, V., and Kitchen, K.C. 1977. A transplantable insulinoma in the rat. *Proc. Natl. Acad. Sci. U.S.A.* 74:628-632.

Efrat, S., Linde, S., Kofot, H., Spector, D., Delannoy, M., Grant, S., Hanahan, B., and Baekeskov, S. 1988. Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc. Natl. Acad. Sci. U.S.A.* 85:9037-9041.

Hutton, J.C. 1982. The internal pH and membrane potential of the insulin-secretory granule. *Biochem. J.* 204:171-178.

Hutton, J.C. and Peshavaria, M. 1983. Nucleotide and bivalent cation specificity of the insulin-granule proton translocase. *Biochem. J.* 210:235-242.

Hutton, J.C., Penn, E.J., and Peshavaria, M. 1982. Isolation and characterisation of insulin secretory granules from a rat islet cell tumour. *Diabetologia* 23:365-373.

Hutton, J.C., Peshavaria, M., and Tooke, N.E. 1983. 5-Hydroxytryptamine transport in cells and secretory granules from a transplantable rat insulinoma. *Biochem. J.* 210:803-810.

Konrad, R.J., Young, R.A., Record, R.D., Smith, R.M., Butkerait, P., Manning, D., Jarett, L., and Wolf, B.A. 1995. The heterotrimeric G-protein Gi is localized to the insulin secretory granules of beta-cells and is involved in insulin exocytosis. *J. Biol. Chem.* 270:12869-12876.

Madsen, O.D., Larsson, L.I., Rehfeld, J.F., Schwartz, T.W., Lernmark, A., Labrecque, A.D., and Steiner, D.F. 1986. Cloned cell lines from a transplantable islet cell tumor are heterogeneous and express cholecystokinin in addition to islet hormones. *J. Cell Biol.* 103:2025-2034.

Miyazaki, J., Araki, K., Yamato, A., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. 1990. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: Special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126-132.

Mohlig, M., Wolter, S., Mayer, P., Lang, J., Osterhoff, M., Horn, P.A., Schatz, H., and Pfeiffer, A. 1997. Insulinoma cells contain an isoform of Ca2+/calmodulin-dependent protein kinase II delta associated with insulin secretion vesicles. *Endocrinology* 138:2577-2584.

Praz, G.A., Halban, P.A., Wollheim, C.B., Blondel, B., Straus, A.J., and Renold, A.E. 1983. Regulation of immunoreactive-insulin release from a rat cell line (RINm5F). *Biochem. J.* 210:345-352.

Roep, B.O., Kallan, A.A., Hazenbos, W.L., Bruining, G.J., Baileys, E.M., Arden, S.D., Hutton, J.C., and de Vries, R.R. 1991. T-cell reactivity to 38-kD insulin-secretory-granule protein in patients with recent-onset type 1 diabetes. *Lancet.* 337:1439-1441.

Sottocasa, G.L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J. Cell Biol.* 32:415-438.

Wang, S.Y. 1989. The acute effects of glucose on the insulin biosynthetic-secretory pathway in a simian virus 40-transformed hamster pancreatic islet beta-cell line. *Endocrinology* 124:1980-1987.

Isolation and Biochemical Characterization of Amyloid Plaques and Paired Helical Filaments

Agueda Rostagno[1] and Jorge Ghiso[1, 2]

[1]Department of Pathology, New York University, New York, New York
[2]Department of Psychiatry, New York University, New York, New York

ABSTRACT

Extracellular deposits of amyloid fibrils in the form of parenchymal plaques and cerebrovascular lesions, as well as intracellular accumulation of paired-helical filaments in the form of neurofibrillary tangles (NFT) in selected neuronal populations are the main neuropathologic hallmarks of Alzheimer's disease. Amyloid fibrils composed of polymeric structures of the amyloid-β (Aβ) concentrate at the center of senile plaques and accumulate in the walls of cerebral blood vessels, exhibiting extensive Congo red/thioflavin S staining. Intraneuronal NFT are composed of building blocks of aberrantly hyperphosphorylated species of the microtubule-associated protein tau, which accumulate in the perinuclear cytoplasm of vulnerable neurons in the form of paired helical filaments (PHF). This unit presents a variety of protocols for the isolation, biochemical analysis, and characterization of amyloid fibrils and neurofibrillary tangles. *Curr. Protoc. Cell Biol.* 44:3.33.1-3.33.33. © 2009 by John Wiley & Sons, Inc.

Keywords: Alzheimer's disease • amyloid • neurofibrillar tangles • paired-helical filaments • laser capture microdissection • immunoblotting • immunoprecipitation • mass spectrometry • amino acid sequence

INTRODUCTION

The profuse deposition of amyloid fibrils in the parenchymal and vascular extracellular spaces of the cerebral cortex and leptomeningeal vessels is one of the hallmark lesions of Alzheimer's disease (AD). Fibrillar, compact deposits composed of amyloid-β (Aβ) and exhibiting extensive Congo red/thioflavin S staining concentrate at the center of the senile plaques or accumulate around cerebral blood vessels. In addition to compact plaques, typically surrounded by dystrophic neurites, activated microglia, and reactive astrocytes, most Alzheimer brains contain amorphous and less dense deposits, which are commonly referred to as diffuse or pre-amyloid plaques (reviewed in Selkoe, 1994; Ghiso and Frangione, 2002; Fig. 3.33.1). Under electron microscopy, these diffuse lesions—negative under Congo red or thioflavin S staining—display very few, if any, structurally altered neurites, astrocytes, or microglial cells, and in spite of their immunoreactivity with anti-amyloid antibodies, fibrillar components are very sparse or absent.

The presence of neurofibrillary tangles (NFTs), another histopathological trademark of AD, was described by Alzheimer himself in affected limbic and cerebral cortices via light microscopic evaluation following silver tissue impregnation by the Bielschowsky method (Fig. 3.33.1). Tangles are composed of building blocks of aberrantly phosphorylated species of the microtubule associated protein tau, which accumulates in the perinuclear cytoplasm of selected neurons in the form of paired, helically wound filaments (PHF; Grundke-Ikbal et al., 1986a,b; Ballatore et al., 2007).

Current Protocols in Cell Biology 3.33.1-3.33.33, September 2009
Published online September 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0333s44

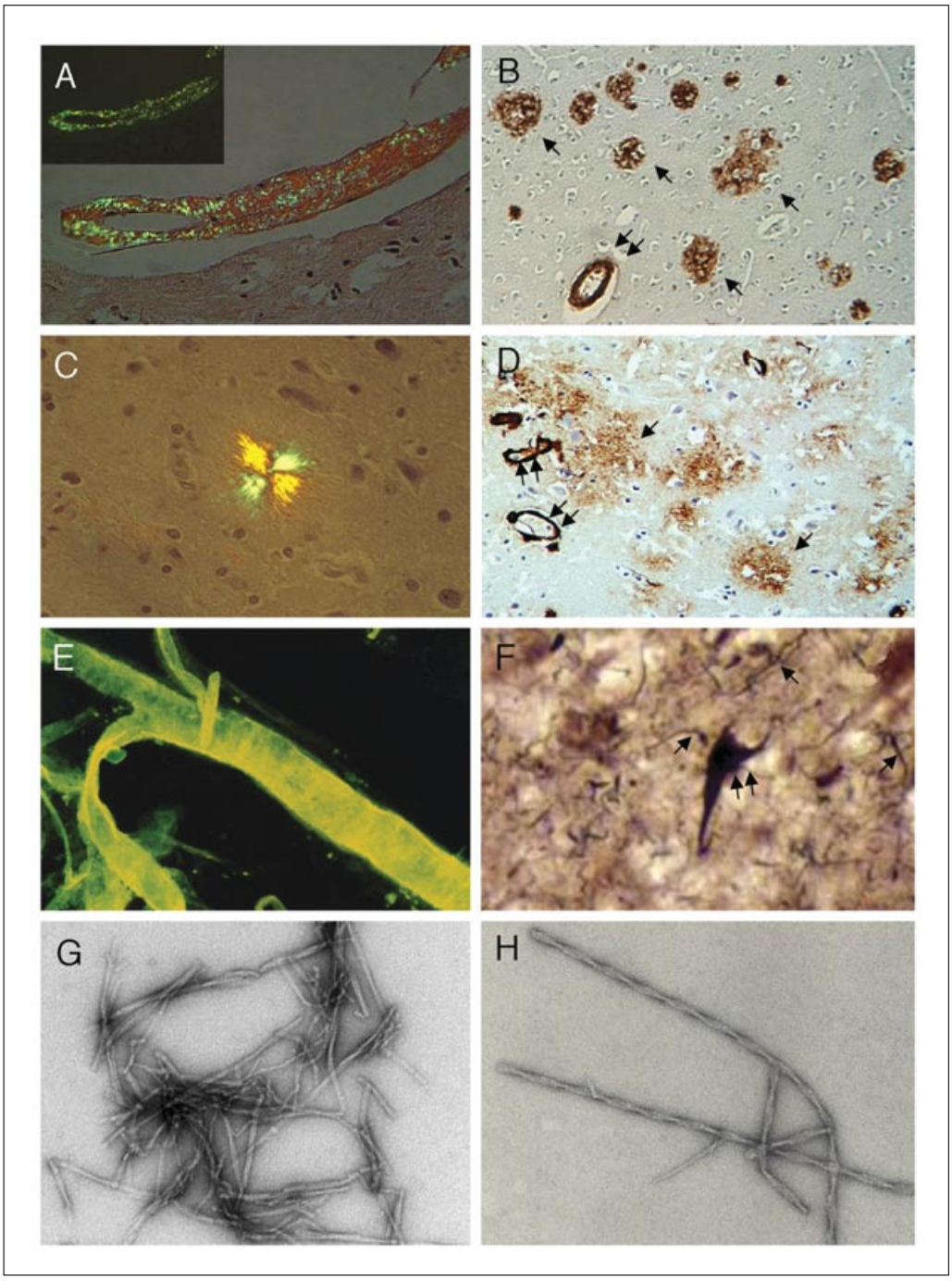


Figure 3.33.1 Microscopic examination of fibrillar deposits in Alzheimer's disease. (**A**) Vascular amyloid deposits in a medium-size leptomeningeal artery visualized by polarized microscopy after Congo red staining. Note the change in color from red to apple green (inset) when the light is polarized (100×). (**B**) Immunohistochemical detection of parenchymal amyloid plaques (arrow) and cerebrovascular Aβ lesions (double arrow) using specific anti-Aβ antibodies (100×). (**C**) Typical maltese cross aspect of a parenchymal plaque stained with Congo red and visualized by polarized microscopy (400×). (**D**) Parenchymal pre-amyloid lesions (arrows) and vascular deposits (double arrow) stained with anti-Aβ antibodies as in B (100×). (**E**) Leptomeningeal amyloid deposits in an isolated vessel observed under fluorescence microscopy after thioflavin S staining (100×). (**F**) Neurofibrillary tangles (double arrow) and dystrophic neurites (arrows) are visualized by Bielschowsky silver impregnation (1000×). (**G, H**) Electron microscopy images of amyloid fibrils (G) and PHF (H) obtained after negative staining with uranyl acetate (110,000×). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0333*.

The biochemical characterization of both amyloid deposits and NFTs, initially hampered by their poor solubility properties, revealed a high degree of molecular heterogeneity and enrichment in post-translationally modified species. Due to their tissue localization and fibrillar configuration, protocols developed to isolate and purify amyloid fibrils and PHF primarily relied on physical methodologies (e.g., homogenization, filtration, standard and gradient centrifugation, FACS analysis), whereas improvements in solubilization were based on the use of detergents (SDS, sarkosyl), chaotropes (guanidine-HCl, guanidine-SCN, urea), or concentrated formic acid. Below, we describe different combinations of these approaches that have been successfully implemented in numerous laboratories (Iqbal et al., 1984; Gorevic et al., 1986; Grundke-Ikbal et al., 1986a,b; Roher et al., 1986; Selkoe et al., 1986; Tomidokoro et al., 2005).

Tissue Source For the Isolation of Amyloid and PHF

The isolation of amyloid and PHF requires the use of frozen brain tissue obtained at autopsy from AD patients with short post-mortem delay, preferable 4 to 8 hr (ideally $<$2 to 4 hr), to minimize the mostly enzymatically driven protein modifications occurring after death. The magnitude of the neuropathological lesions is highly variable not only among different AD cases but also among different regions of the same brain. This makes it necessary to select specimens with abundant AD-related pathology, as assessed by histopathological standard protocols (Hyman and Trojanowski, 1997), to ensure a high yield of purified amyloid and/or PHF. In general, most published protocols perform extractions from cortical regions after gross dissection of the gray matter, since plaques and NFTs are typically absent from the white matter (Braak and Braak, 1991; Lee et al., 1999).

AMYLOID/ PRE-AMYLOID PURIFICATION

Aβ deposits in brain parenchyma and cerebral blood vessels are challenging lesions to study. Their heterogeneous composition and rather poor solubility properties are perhaps the most important reasons to rely in a combination of procedures rather than in a single approach at the time of performing the studies. Whereas new technical approaches have been incorporated—particularly at the detection level—many of the current purification procedures were adapted from existing protocols to study systemic amyloid lesions. Classical protocols to study amyloid deposits generally include physical purification steps (e.g., centrifugation, filtration, and microdissection) combined with differential solubilization approaches (e.g. solubility in water, use of various chaotropes, organic solvents, and strong acids). The following protocols describe step-by-step procedures to isolate Aβ from different lesions and use an array of analytical detections.

Extraction and Solubilization of Amyloid and Pre-Amyloid Deposits for Subsequent Biochemical and Mass-Spectrometry Studies

BASIC PROTOCOL 1

The following extraction strategy takes advantage of the differential solubility properties of pre-amyloid deposits, which are usually poorly soluble in water-based solutions but extractable with SDS-containing buffers, in comparison with fibrillar amyloid structures, which are highly insoluble under both prior conditions but able to be solubilized by treatment with 70% to 99% formic acid. This differential-solubility based protocol for the extraction of amyloid and pre-amyloid materials has been widely used for the subsequent biochemical analysis of the Aβ species composing the lesions, typically by amino acid sequence analysis and more recently by a combination of immunoprecipitation and mass spectrometry. The latter is an extremely sensitive methodology, and has allowed the further identification of numerous truncated and post-translationally modified Aβ species as components of the deposits (Golde et al., 2000; Tomidokoro et al., 2005).

Materials

Frozen brain tissue
Tissue homogenization buffer 1 (THB-1; see recipe)
2% (w/v) SDS in 20 mM Tris·Cl, pH 7.4: prepared by diluting 1:5 a commercially available 10% SDS solution (BioRad) in 20 mM Tris·Cl, pH 7.4
70% (v/v) formic acid: prepared by diluting 99% formic acid (Sigma) with de-ionized water

Sterile dissecting instruments including:
Scalpel
Tweezers
Small spatula
Razor blades
Dounce glass homogenizer
300- and 70-μm Spectra/mesh nylon filters (Spectrum Laboratories)
10.4-ml polycarbonate bottles (Beckman) for ultracentrifugation (step 4)
XL100K ultracentrifuge (Beckman Coulter) equipped with a Beckman 70.1 Ti rotor, or equivalent
Ice bath
Vortex
5417 refrigerated microcentrifuge (Eppendorf), or equivalent

NOTE: All solutions should be ice-cold and procedures should be carried out on ice or inside a 4°C cold room.

Dissect brain (Fig. 3.33.2)

1. From frozen brain tissue, remove leptomeninges and large blood vessels. Separate gray from white matter with the aid of sterile dissecting tools (scalpel, tweezers, small spatula).

   To obtain a high yield in the extraction of amyloid, it is indispensable to verify the magnitude of the deposits using immunohistochemistry (Support Protocol 1) prior to the start of the isolation procedure.

2. Finely mince the gray matter (∼2.5 g) with a razor blade, add 12.5 ml of ice-cold THB-1, and homogenize in a Dounce glass homogenizer immersed in ice until no large pieces are visible.

   A useful alternative to the Dounce glass homogenizer, particularly when many samples are processed at once, is the use of a mechanical homogenizer equipped with disposable probes for soft tissues (e.g., Omni International).

Fractionate brain tissue

3. After homogenization, eliminate tissue debris via filtration through a 300-μm mesh nylon filter followed by subsequent removal of small vessels by filtration through a 70-μm nylon mesh.

   The vessel fraction may be reserved for further analysis of vascular amyloid (see Basic Protocol 3).

4. Subject the filtrate of the 70-μm filter to ultracentrifugation in a XL100K ultracentrifuge (Beckman Coulter) with a Beckman 70.1 Ti rotor 1 hr at 112,000 × *g*, 4°C, and save the THB-1-extracted supernatant enriched in soluble Aβ species for further analysis.

   The PBS supernatant typically contains soluble brain proteins and a minor proportion of soluble Aβ species (primarily monomers/dimers).

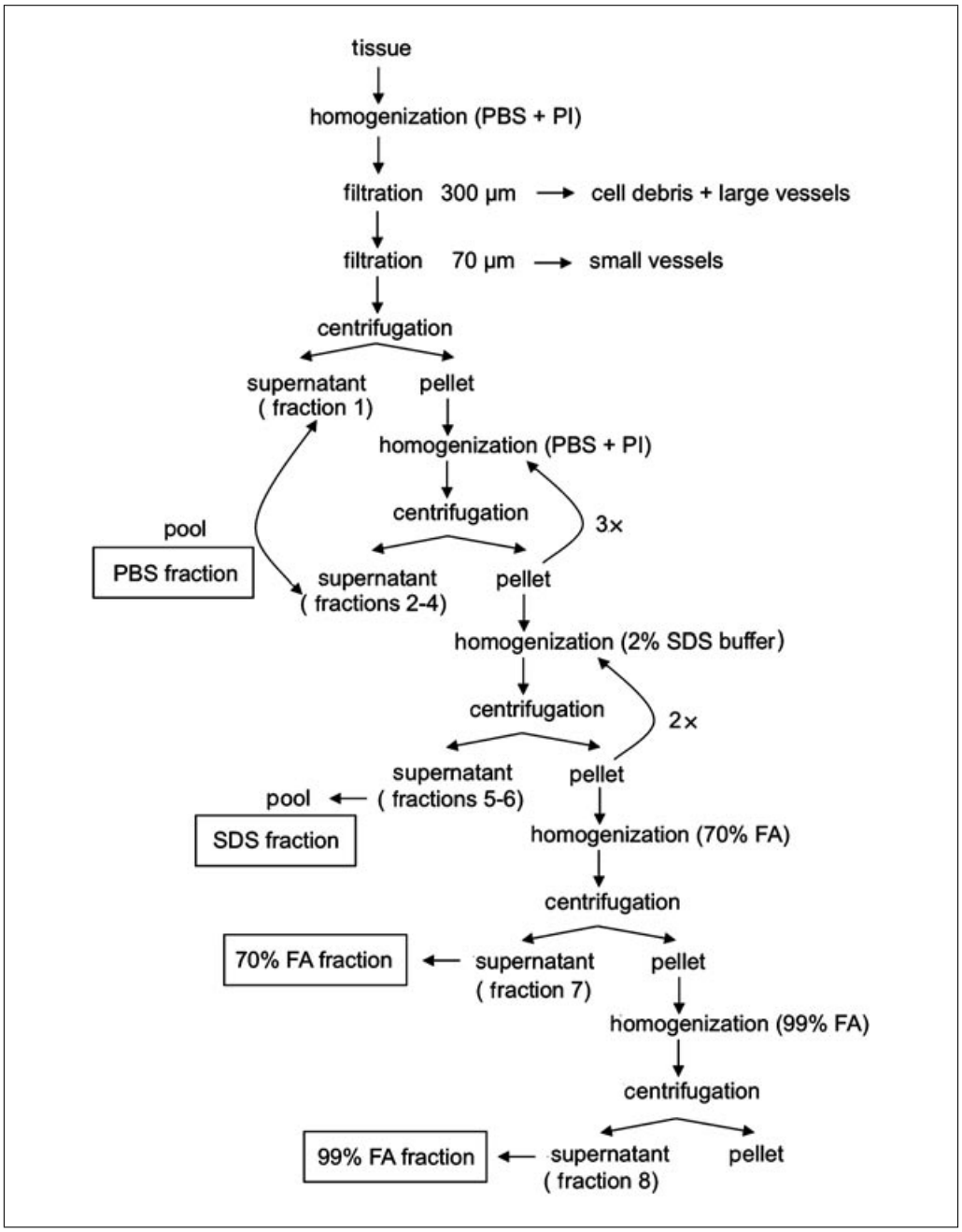


Figure 3.33.2 Schematic representation of the sequential extraction of pre-amyloid and amyloid species for subsequent biochemical and mass spectrometry studies, as described in Basic Protocol 1.

5. To ensure removal of undesirable water-soluble proteins, and extraction of most of the soluble Aβ, repeat the homogenization/centrifugation procedure at least 3 times. Combine and analyze the resulting supernatants as the PBS fraction.

6. Resuspend the PBS-insoluble pellet containing amyloid and pre-amyloid materials in 12.5 ml of 2% SDS in 20 mM Tris·Cl, pH 7.4, at room temperature.

   IMPORTANT NOTE: *The use of this solution at 4°C will result in crystallization of SDS.*

7. Ultracentrifuge samples 1 hr at 112,000 × *g*, 10°C, as above, and save the SDS-extracted supernatant rich in pre-amyloid species for further analysis.

Depending on the pre-amyloid load of the specimen, steps 6 to 7 should be repeated 2 to 3 times not only to increase pre-amyloid yield in the SDS-extracts but to minimize contamination of the fibrillar amyloid to be extracted in step 8.

8. Resuspend the pellet in 1 ml 70% formic acid, vortex 2 min, and centrifuge in a 5417 microcentrifuge 15 min at 18,000 × *g*, 4°C.

   The resulting supernatant (70% FA fraction) contains solubilized amyloid-rich fraction for further analysis.

   Depending on the load of dense plaques in the specimen, it might be necessary to add an additional extraction (step 9) using 99% FA to maximize the yield of solubilized amyloid. Use the initial immunohistochemical analysis to assess compact plaque density.

9. Resuspend the remaining pellet in 1 ml 99% formic acid, vortex 2 min, and centrifuge in a 5417 microcentrifuge 15 min at 18,000 × *g*, 4°C, as above.

   The resulting supernatant (99% FA fraction) also contains solubilized amyloid-rich fraction for further analysis.

   Regardless of the compact plaque density, proceed with step 9 if the pellet after step 8 is still significant. Typically, after 99% FA the remaining pellet is minimal or not existent.

ALTERNATE PROTOCOL 1

Extraction of Amyloid Fibrils and Plaque Cores

The isolation of amyloid plaque cores from homogenized gray matter tissue from AD cases (see Basic Protocol 1, steps 1 and 2) primarily rely on gradient density centrifugation. The most commonly used are discontinuous sucrose gradients (Selkoe et al., 1982; Gorevic et al., 1986; Selkoe et al., 1986; Roher and Kuo, 1999), although Ficoll density gradients have also been successfully used (Roher et al., 1986).

Materials

Frozen brain tissue
Tissue homogenization buffer 2 (THB-2 see recipe)
1.0, 1.2, 1.4, 2.0 M sucrose gradient solutions (see recipe)

Dounce glass homogenizer
100°C water bath
300-μm, 70-μm, and 35-μm Spectra/mesh nylon filters (Spectrum Laboratories)
Ultracentrifuge XL100K (Beckman Coulter) equipped with 70.1 and SW40 Ti rotors, or equivalent
10.4-ml polycarbonate bottles (Beckman) for ultracentrifugation (step 5)
Ultra clear tubes (14 × 95–mm; Beckman) for ultracentrifugation (step 7)

Additional reagents and equipment for dissecting gray matter (Basic Protocol 1) and microscopic detection of amyloids using Congo red staining (Support Protocol 2), Thioflavin S staining (Support Protocol 3), and electron microscopy (Support Protocol 4)

Prepare tissue sample (Fig. 3.33.3)

1. Select cerebral cortex rich in dense plaque cores (assessed by standard immunohistochemical analysis of the AD brain specimen), remove leptomeninges and large blood vessels, and dissect gray matter as described in Basic Protocol 1.

Homogenize tissue

2. Add THB-2 to the dissected tissue at a 1:1 (w/v) ratio and homogenize at 4°C in a Dounce glass homogenizer until a homogeneous suspension is obtained.

   A mechanical homogenizer equipped with disposable probes for soft tissues (e.g., Omni International) provides a useful alternative to the Dounce glass homogenizer.

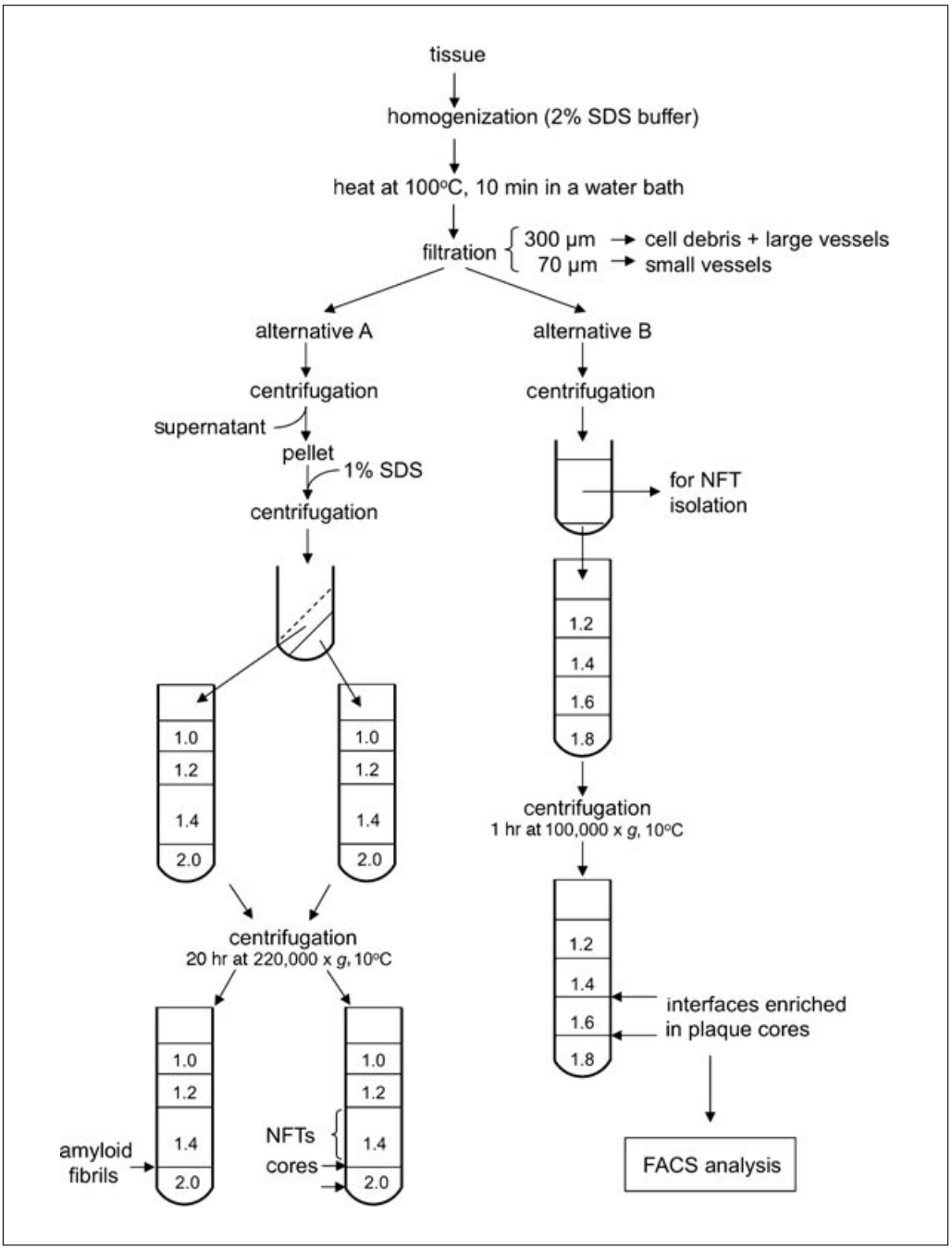


Figure 3.33.3 Schematic representation of the extraction procedure of amyloid fibrils and plaque cores, as described in Alternate Protocol 1 (alternative A) and Alternate Protocol 2 (alternative B).

3. Heat the mixture 10 min at 100°C in a water bath.
4. Sieve the homogenate through sequential nylon-meshes (300-µm and then 70-µm) to remove debris and blood vessels.

Fractionate the tissue

5. Centrifuge 1 hr at 100,000 × *g*, 10°C using a Beckman XL100K ultracentrifuge and a 70.1 Ti rotor.

   Depending on the number of samples and the starting material a 50.2 Ti rotor may be used to handle larger volumes or number of fractions.

6. Discard the supernatant and resuspend the pellet in THB-2, this time containing 1% SDS (ratio buffer to pellet 2:1) and repeat the centrifugation of step 5.

   The resulting pellet will be composed of two well-defined layers: an upper beige fluffy layer and a lower dark-brown hard layer. Both layers can be easily separated by carefully resuspending the top layer in THB-2 containing 1% SDS with the aid of a Pasteur pipet. Each layer will be used separately for further purification.

7. Load each layer separately onto a discontinuous sucrose gradient (1.0/1.2/1.4/2.0 M sucrose in 50 mM Tris·Cl, pH 7.6, containing 1% SDS).

   Layering of the sucrose solutions should be carefully performed with the aid of a Pasteur pipet and without disturbing the bottom layer. The tube should be kept at eye-level and observed against a light-source to allow visualization of the interfaces created by the different density levels of the various sucrose solutions. Start with 2 ml of the 2.0 M solution at the bottom of the tube. Sequentially, layer carefully 3 ml of the 1.4 M solution followed by 2 ml of the 1.2 M and 2 ml of the 1.0 M sucrose solution. Finally, load 2.3 ml of the resuspended layer on the top of the sucrose gradient.

8. Centrifuge 20 hr at 220,000 × *g*, 10°C in a Beckman XL100K ultracentrifuge using a SW 40 Ti rotor.

   The resulting interfaces and layers are easily visualized with indirect light against a dark background. Removal of each fraction can be achieved with the aid of a Pasteur pipet.

   The 1.4/2.0 interface obtained from the upper beige fluffy layer in step 6 is highly enriched in amyloid fibrils, whereas the same 1.4/2.0 interface from the dark-brown layer in step 6 contains mainly amyloid cores and some loose fibrils (Fig. 3.33.2). The 1.4 sucrose layer from the latter is enriched in PHF and could be saved for further purification.

9. Resuspend the 1.4/2.0 interface fractions containing either fibrils or a mixture of fibrils and cores in 8 ml distilled water and centrifuge 1 hr at 100,000 × *g*, 4°C, using a 70.1 Ti rotor.

10. Confirm the presence of fibrillar-amyloid-rich structures by light microscopy after Congo Red staining (Support Protocol 2), fluorescence microscopy following Thioflavin S staining (Support Protocol 3), or by electron microscopy after negative staining with uranyl acetate (Support Protocol 4) (Gorevic et al., 1986; Westermark et al., 1999).

11. Store purified amyloid fractions at −80°C until use.

ALTERNATE PROTOCOL 2

Extraction of Plaque Cores

The following protocol is based on the classical approach first described by Selkoe et al. (1986). As in the case of Alternate Protocol 1, it consists of a series of density centrifugation steps following tissue homogenization in the presence of SDS and with heating at high temperature to disrupt the tissue architecture and allow the release of the amyloid material.

Additional Materials *(see Alternate Protocol 1)*

Wash buffer (see recipe)
1.2, 1.4, 1.6, 1.8 M sucrose gradient solutions (see recipe)

100-μm and 35-μm Spectra/mesh nylon filters (Spectrum Laboratories)
J-6B refrigerated centrifuge (Beckman Coulter)
XL100K Ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with SW40 Ti rotors, or equivalent
Ultra-clear tubes (14 × 95–mm; Beckman) for gradient centrifugation (step 6)

Additional reagents and equipment for preparing brain homogenates (Alternate Protocol 1)

Prepare brain homogenates (Fig. 3.33.3, Alternative B)

1. Dissect gray matter, homogenize tissue, heat at 100°C, and sieve to remove debris and blood vessels as described in Alternate Protocol 1 (steps 1 to 4).

Fractionate the tissue

2. Centrifuge the filtrate 30 min at 300 × *g*, 100°C in a J-6B centrifuge. The supernatant, which contains abundant NFTs, may be saved for further purification of PHF (steps 5 to 16 of Basic Protocol 3). For amyloid core isolation, continue with steps 4 to 10.
3. Wash the pellet by resuspending in wash buffer and pelleting as described in step 2.
4. Repeat washes (steps 2 and 3) two times.
5. Resuspend the pellet thoroughly, sieve through a 35-μm nylon filter, and load on a gradient composed of 1.2 M, 1.4 M, 1.6 M, and 1.8 M sucrose.
6. Centrifuge 60 min at 72,000 × g, 10°C using an XL100K ultracentrifuge and SW40 Ti rotors.
7. Collect the 1.6/1.8 and 1.4/1.6 M sucrose interfaces, which typically show the greatest enrichment in plaque cores, separately.
8. Dilute each interface five-fold with wash buffer and pellet as described in step 2.
9. Store plaque cores at −80°C until further use.

Further purification of the amyloid core preparation may be achieved by fluorescence-activated cell sorting (FACS), if desired.

This procedure is highly specialized and it requires sophisticated and expensive equipment. It is therefore performed primarily at dedicated Flow Cytometry Facilities. For processing of the amyloid samples (step 9) for subsequent FACS analysis, please see (Roher et al., 1986; Selkoe et al., 1986). For detailed protocols on flow cytometry, please refer to Robinson et al. (2009).

Extraction of Amyloid from Leptomeningeal Vessels and Microvessels

ALTERNATE PROTOCOL 3

The presence of cerebrovascular amyloid is a common finding in AD and other vascular dementias (reviewed in Ghiso and Frangione, 2002). Interestingly, and driven by mechanisms still to be elucidated, there is not a completely homogeneous distribution of Aβ species between parenchymal and vascular AD deposits. While Aβ peptides ending at position 42 are found predominantly associated with parenchymal plaques, vessel-associated components are highly enriched in Aβ species ending at residue 40. Therefore, when a precise biochemical analysis of the vascular amyloid components is required, vessels need to be isolated prior to amyloid extraction. Sometimes the degree of amyloid load in leptomeningeal vessels is so overwhelming that just the dissection of a few large vessels provides enough material for biochemical studies. When leptomeninges are either not available or the leptomeningeal involvement is minimal, a thorough isolation of brain microvessels is mandatory.

Materials

Cortical brain tissue (e.g., frontal lobe)
Tissue homogenization buffer 3 (THB-3; see recipe)
Protease inhibitor cocktail (Complete; Roche Applied Science)
26% (w/v) dextran (MW 65,000-85,000) in THB-2
Percoll (Sigma)

Dounce glass homogenizer
J-6B refrigerated centrifuge (Beckman Coulter), or equivalent

350-, 150-, and 70-μm nylon mesh filters
50-ml polypropylene tubes (Fisher Scientific)
XL100K ultracentrifuge (Beckman Coulter) equipped with a Beckman SW40 Ti rotor, or equivalent (for Percoll gradient)
15 × 95–mm ultraclear tubes (Beckman)

Prepare tissue (Pardridge et al., 1987; Morelli et al., 2004)

1. Dissect cortical tissue (typically from frontal lobe of affected individuals) from leptomeningeal vessels.

   Not all sporadic AD cases present with the same level of vascular involvement and, in some cases, amyloid is mainly restricted to parenchymal distribution. Therefore, to obtain a high yield in the extraction of vascular amyloid, it is indispensable to verify the magnitude of the vascular deposits by immunohistochemistry, prior to the start of the isolation procedure (Support Protocol 1).

   For the isolation of amyloid from leptomeningeal vessels, skip steps 2 to 5. The leptomeningeal fractions obtained in step 1 of Basic Protocol 1, and Alternate Protocols 1 and 2 can be used for the isolation.

Homogenize tissue

2. Homogenize cortical tissue devoid of leptomeninges in 5 vol of ice-cold THB-3 containing protease inhibitor cocktail (see Basic Protocol 1) using ∼15 up and down strokes of a Dounce glass homogenizer immersed in ice.

3. Add an equal volume of a 26% dextran solution prepared dissolving dextran of 65,000 to 85,000 molecular weight in THB-3, and homogenize.

4. Centrifuge 15 min at 5,800 × *g*, 4°C in a J-6B Beckman centrifuge (or equivalent).

5. Resuspend the pellet in 10 vol THB-3 and sieve sequentially through 350-, 150-, and 70-μm nylon meshes.

   From this point onwards, microvessel fractions obtained in either step 3 from Basic Protocol 1 or step 4 in Alternate Protocol 1 can be also used in the purification procedure.

Isolate the vessel fractions

6. Collect the material that remained on top of the 150- and 70-μm meshes by inverting the filters on top of a 50-ml polypropylene tube and washing them with a stream of ice-cold THB-3 (∼20 to 30 ml).

7. Pellet these fractions 10 min at 1,000 × *g* in the J-6B refrigerated centrifuge.

8. Resuspend in 1 to 2 ml THB-3 and load on top of a preformed Percoll gradient (50% in THB-3, pre-generated by centrifuging 1 hr at 27,000 × *g*, 4°C, in the ultracentrifuge using 14 × 95–mm ultraclear tubes.

9. Centrifuge 10 min at 1,000 × *g*, 4°C, collect the middle white layer, and repeat the centrifugation on the Percoll gradient one more time.

Extract the vessel fractions

10. Combine the vessel fractions, homogenize, and proceed to sequential extraction with SDS and formic acid, as described in Basic Protocol 1 (steps 6 to 9).

    At this point many published protocols insert an extra step in which collagenase (1:20 to 1:50 enzyme/extract ratio) is added to the vessel preparation in order to degrade the collagen matrix and release the fibrils from the deposits, therefore increasing the yield of amyloid in the extracts (Roher et al., 1986; Tennent, 1999). However, keep in mind that collagenase also degrades Aβ generating proteolytic fragments that are not usually present in the deposits. If the operator is interested in the assessment of all Aβ species present in the various lesions, the use of collagenase should be avoided.

Plaque Isolation by Laser Capture Microdissection

Traditional biochemical methods like the ones described above have certain limitations since they fail to achieve the isolation of plaques to homogeneity. These restrictions were recently overcome with the advent of the laser capture microdissection (LCM) methodology allowing the collection of microscopic regions as small as 3- to 5-μm in diameter (Ahram et al., 2003; *UNIT 2.5*). It is possible to use LCM to capture neuropathological structures (e.g., plaques) with high purity, but the amount of sample material collected by this methodology is often limited, complicating the subsequent analysis. The recent development of highly sensitive proteomic technologies like liquid chromatography combined with tandem mass spectrometry (LC-MS/MS), which can directly analyze thousands of proteins from complex protein samples with sensitivity levels in the low femtomolar range, have made possible the successful use of LCM–obtained samples (Liao et al., 2004; Gozal et al., 2006).

Materials

Post-mortem blocks of frontal or temporal cortex
Cryostat mounting medium (Tissue-Tek O.C.T.; Jed Pella)
2-methylbutane (Fisher Scientific)
75% ethanol
Thioflavin S (Sigma)
Graded ethanol solutions: 50%, 95%, and 100% (v/v) ethanol
Xylene

HM500OM cryotome (MICROM International), or equivalent
Uncoated and uncharged glass slides (Fisher Scientific)
Fluorescence microscope equipped with a Pixcell II laser capture device (Arcturus), or similar
CapSure Macro LCM cap (Arcturus)

Additional reagents and equipment for laser capture microdissection (Liao et al., 2004)

Prepare tissue

1. Embed fresh post-mortem blocks of frontal and/or temporal cortex obtained at autopsy from neuropathologically diagnosed AD cases in cryostat mounting medium to allow for the cryopreservation of the tissue architecture.
2. Freeze tissue on dry, ice-cold 2-methylbutane, and store at −80°C until sectioning.
3. Prepare 10-μm tissue sections in a HM500OM cryotome (or equivalent).

   Laser capture microscopy may be conducted on tissue sections ranging from 5- to 10-μm thickness, too thin a section may prevent complete removal of Aβ plaques with concomitant loss of material, whereas too thick a section may result in the acquisition of contaminating excess material.

4. Mount the cryosections on uncoated and uncharged glass slides, and thaw at room temperature.
5. Briefly fix sections for 1 min at room temperature with 75% ethanol to histologically preserve the section while maintaining the quality of macromolecules for subsequent proteomic profiling.

   Despite being considered the "gold standard" for histopathological preservation, the use of aldehyde-based fixatives (e.g., formalin) should be avoided because the high level of covalently cross-linked proteins typically observed with such fixation is incompatible with current proteomic analysis protocols (Ahram et al., 2003; Gozal et al., 2006).

6. Visualize Aβ plaques by staining cryosections 1 min at room temperature with 1% thioflavin S in deionized water.
7. Differentiate by removing excess thioflavin S by incubating in 75% ethanol for 1 min at room temperature.
8. Dehydrate by sequential incubations of 2 min each in graded ethanol solutions (50%, 95%, and 100%,), clear for 5 min in fresh xylenes, air-dry for 5 min, and desiccate sections before LCM.

Perform laser capture microdissection (Liao et al., 2004)

9. Perform LCM the same day the cryosections are stained with thioflavin S and desiccated (as described in steps 6 to 8). Carry out the procedure in a fluorescence microscope attached to a Pixcell II laser capture device with the following settings: excitation wavelength, 495 nm; laser power, 60 to 80 mW; duration, 1 msec; laser spot size, 7.5 μm.
10. Collect ~500 to 2000 amyloid plaques from the tissue sections.

    The number of plaques dissected depends on the sensitivity of the analytical procedure to follow. A total of 2000 plaques allowed the complete proteomic analysis of plaque components with the individualization of >400 constituents of the amyloid deposits (Liao et al., 2004).

11. Capture a similar number of spots in nonplaque areas as control tissue.

    Each CapSure Macro LCM cap is used for the capture of ~500 plaques or control tissue spots from one slide.

12. *Optional:* Employ microdissected samples for mass spectrometry analysis.

    Typically, quadrupole time-of-flight mass spectrometry and liquid chromatography coupled with ion-trap tandem mass spectrometry (LC-MS/MS) are the most sensitive and widely used methodologies. Due to the sophisticated equipment and specific methodology involved in these analyses, readers should refer to specially dedicated reviews in the field (Gozal et al., 2006; Nesvizhskii, 2007).

MICROSCOPIC DETECTION OF AMYLOID

Amyloid and pre-amyloid deposits are easily visualized by immunohistochemical analysis with specific anti-amyloid antibodies and light microscopy evaluation. All amyloid deposits, irrespective of the amyloid subunit that compose the fibrils, share common physical, tinctoreal, and structural properties that include: (1) high degree of insolubility, (2) β-pleated sheet rich structures, (3) apple-green birefringence under polarized light after Congo red staining, (4) yellow fluorescence after Thioflavin S staining, and (5) fibrillar appearance after negative staining. Thus, microscopes with different levels of detection and resolution in conjunction with staining techniques with a diverse degree of specificity are very useful tools in amyloid research. Light and/or fluorescence microscopy are the methods of election for the general visualization of amyloid (e.g. using general amyloid dyes like Congo red or Thioflavin S), as well as for the identification of their individual components (e.g. through the use of specific antibodies). Electron microscopy provides the necessary resolution to visualize the actual fibrils and assess morphological parameters. The following are protocols to perform immunohistochemical, histological, and electron microscopic analysis of amyloid lesions and/or tissue extracts.

Aβ Immunohistochemistry

Immunohistochemical analysis of the brain tissues to be employed for amyloid isolation is indispensable for the evaluation of amyloid load and the adequate selection of the brain areas to be used. It is beyond the scope of this unit to describe the protocols for fixation and paraffin embedding of the brain specimens, as well as the preparation of adequate sections (see Hofman, 2002). This protocol describes a procedure for the immunohistochemical staining of amyloid and pre-amyloid Aβ deposits for subsequent light microscopy visualization.

Materials

8-μm thick Paraffin-embedded brain tissue sections on glass microscopy slides
Xylene
100%, 95%, and 75% ethanol
De-ionized water
98% (w/v) formic acid
Ready-to-use peroxidase blocking reagent (DakoCytomation)
Vecstatin ABC kit (Vector Laboratories) including:
  Blocking reagent
  Secondary antibody
  Vectastatin ABC
Phosphate-buffered saline (PBS; see recipe)
4G8 and 6E10 mouse monoclonal anti-Aβ antibodies (Covance)
Ready-to-use antibody diluent (DakoCytomation)
Tween 20
DAB Chromogen (DakoCytomation)
3% hydrogen peroxide
Hematoxylin (DakoCytomation)
Cytoseal-60 low-viscosity mounting medium (Richard-Allan Scientific)

60°C oven
Paper towels
Olympus BX60 microscope, or equivalent

Deparaffinize the sections

1. Dry slides 1 hr in a 60°C oven.

   This drying procedure removes water from underneath the tissue sections and makes them adhere better to the slide.

2. Deparaffinize the sections by placing the slides for three 5-min changes each in xylene and 100% ethanol followed by one 5-min change in 95% ethanol. Between changes, quickly blot the edge of the slide on a paper towel to remove excess liquid, but do not allow the slide to dry.

   Immersion steps are performed using Coplin jars or staining dishes with slide racks, depending on the number of slides that are being processed. The containers are filled so that the slides are completely immersed when they are inside the container. The solutions are reusable and should usually be changed after every 50 slides.

3. Rinse with de-ionized water.

Perform tissue pretreatment

4. Incubate with 98% (w/v) formic acid in de-ionized water for 10 min at room temperature.

   This step is necessary to increase access of the antibodies to the Aβ epitopes, which may be masked not only due to the protein cross-linking caused by paraformaldehyde but also due to the highly insoluble and fibrillar conformation of the protein within

the deposits. This pretreatment is commonly performed not only for Aβ but also for the immunohistochemical analysis of other amyloids. The incubation time is critical as prolonged incubations may result in the loss of the protein immunoreactivity.

5. Rinse thoroughly with de-ionized water.
6. Incubate 10 min at room temperature with ready-to-use peroxidase blocking reagent.

Immunohistochemical procedure

7. Block for 20 min with the blocking reagent of the Vecstatin kit diluted by adding 3 drops of the stock solution to 10 ml PBS.
8. Incubate 1 hr at room temperature with a combination of 4G8 and 6E10 antibodies diluted in ready-to-use antibody diluent at 1/200 each.
9. Wash the slides for three 5-min changes of PBS containing 0.1% Tween 20.
10. Incubate 30 min at room temperature with the secondary antibody (anti-mouse IgG) included in the Vecstatin kit diluted as indicated by the manufacturer.

    During this incubation prepare the ABC reagent, which needs to stand for 30 min before it can be used, following the manufacturer's instructions.

11. Wash the slides for three 5-min changes of PBS containing 0.1% Tween 20.
12. Incubate with Vecstatin ABC for 30 min at room temperature.

    At the time of setting this incubation, start dissolving the DAB. For this, add 1 tablet DAB to 10 ml PBS, and vortex. Protect from light until use.

13. Wash the slides with three 5-min changes of PBS containing 0.1% Tween 20.
14. Prepare the DAB chromogen solution by combining 2 ml DAB (prepared as in step 12) with 15 µl of 3% hydrogen peroxide.

    The DAB chromogen should be protected from light by covering the tube with aluminum foil. It is only stable for 2 hr at room temperature, therefore it is better to prepare it immediately before use and discard any remnant solution.

15. Coat sections with DAB chromogen solution and incubate 3 to 5 min at room temperature.
16. Rinse immediately with abundant de-ionized water.
17. Counterstain with hematoxylin for 6 min at room temperature.
18. Rinse with de-ionized water until wash solution is uncolored.

Mounting slides

19. Dehydrate sections by placing the slides for one 5-min change in 95% ethanol followed by three 5-min changes each in 100% ethanol and xylene.
20. Mount with low-viscosity mounting medium, coverslip, and visualize the staining in an Olympus BX60 microscope, or equivalent.

SUPPORT PROTOCOL 2

Congo Red Staining

Staining with Congo red in association with polarization microscopy is the most universally used methodology for the demonstration of amyloid in tissue specimens. The procedure is sensitive and simple to perform, two factors that have contributed to its incorporation to routine staining panels in pathological examinations. In order to be reliable, not only must the staining be performed under well-controlled conditions, but also tissue examination and staining evaluation require experienced operators using appropriate microscopy equipment and filters for polarization.

Materials

Congo red (Sigma)
80% (v/v) ethanol
Tissue samples
1 M NaOH
4% (v/v) paraformaldehyde in PBS
Deionized water

Whatman no. 1 filter paper
Positively charged microscope slides (Fisher Scientific)
Super Pap Pen (Invitrogen)
Olympus BX60 microscope equipped with crossed polarizers, or equivalent

Prepare the Congo red solution

1. Prepare 100 ml of a 1% (w/v) Congo red solution in 80% ethanol.
2. Stir 1 hr at room temperature.
3. Add 1 ml of 1 M NaOH.
4. Filter the solution through Whatman no.1 filter paper.

Fix and stain the isolated amyloid-fractions

5. Spot 5 to 10 µl of the resuspended fractions to be checked for the presence of fibrillar amyloid materials on a positively charged microscope slide.
6. Air dry for 30 min.
7. Circle the area of the dry drop with a Super Pap Pen.

   Circling the pertinent areas with a Pap Pen prior to histological or immunohistochemical procedures creates a water-repellent circle around the section preventing the waste of valuable reagents and at the same time ensuring that the staining solution remains in contact with the section during the staining procedure. The chemistry involved is formulated to withstand alcohol treatment.

8. Fix the material by incubating with 4% (v/v) paraformaldehyde (in PBS) 10 min at room temperature.
9. Rinse the slides with deionized water and air dry.
10. Stain the slides for 30 min at room temperature with the Congo red solution prepared in step 4.
11. Rinse the slides briefly (~10 sec) in 80% ethanol, and air dry.
12. Observe by polarization microscopy in an Olympus BX60 microscope equipped with crossed polarizers, or equivalent.

    Polarized light is a contrast-enhancing technique that improves the quality of the image obtained with birefringent materials. Under these conditions, amyloid shows a bright green birefringence, often referred to as apple-green birefringence (see Fig. 3.33.1).

    For polarization analysis, the microscope must be equipped with both a polarizer, positioned in the light path before the specimen, and an analyzer (a second polarizer) placed in the optical pathway between the objective rear aperture and the observation tubes. Image contrast arises from the interaction of plane-polarized light with a birefringent (or doubly refracting) specimen to produce two individual wave components that are each polarized in mutually perpendicular planes (Howie et al., 2008). After exiting the specimen, the light components become out of phase, but they are recombined with constructive and destructive interference when they pass through the analyzer.

SUPPORT PROTOCOL 3

Thioflavin S Staining

Thioflavin S and thioflavin T are sulfur-containing compounds that bind to amyloid fibrils; the latter is mostly used in spectrofluorometric studies while the former is preferably employed in histological protocols. Thioflavin staining is currently very popular for amyloid studies, not only for the detection of amyloid in tissue sections, but also for the study of fibril formation in vitro. The staining is easy to perform, but the requirement of fluorescence microscopy creates limitations for some facilities.

Materials

Amyloid-containing tissue sections
4% (v/v) paraformaldehyde in PBS
Thioflavin S (Sigma)
80% (v/v) ethanol
Mounting medium for fluorescence microscopy (Vector Laboratories)
Olympus BX60 epi-fluorescence microscope or equivalent

1. Fix the isolated amyloid-containing materials to be analyzed with 4% (v/v) paraformaldehyde 10 min at room temperature (as described in steps 5 to 9 of Support Protocol 2).

   The type of fixation procedure is not critical; different available methodologies are compatible with the immunofluorescence staining.

2. Incubate sections in 1% (w/v) thioflavin S in distilled water for 5 to 10 min at room temperature protected from light.

   IMPORTANT NOTE: *It is recommended that a new solution is freshly prepared the day of use and maintained protected from light (e.g., in amber-color or aluminum-foil-covered containers).*

3. Differentiate the sections in 80% (v/v) ethanol to remove excess fluorochrome and facilitate visualization.

4. Mount with fluorescence microscopy mounting medium (or equivalent).

5. Visualize in an Olympus BX60 or comparable fluorescence microscope, equipped for evaluation of green fluorescence (excitation 495 nm/emission 519 nm; see Fig. 3.33.1).

SUPPORT PROTOCOL 4

Electron Microscopy

Electron microscopy (EM), with a resolution of ~2 nm, offers a useful technique for the ultrastructural characterization of proto-filaments and mature fibrils formed during in vitro fibrillogenesis. The method also constitutes an invaluable tool to follow-up the isolation of amyloid plaque material in the various fractions as described above. For contrast enhancement of specimens, the samples to be evaluated are typically subjected to negative-staining techniques (Gorevic et al., 1986; Ward et al., 2000).

It is beyond the scope of this unit to describe the preparation of EM-grids and the use of the EM. Below is a description of the procedure to load the samples on the grid and perform the negative staining.

Materials

Sample to be analyzed, e.g., resuspended fraction
Milli-Q water (purified water with resistivity values at 25°C <18.2 Mho/cm), sterile
1% uranyl acetate, pH 6.0 (Electron microscopy grade, Spi-Chem)
300 mesh, electron microscope cooper or nickel grids (Canemco & Marivac, Quebec)

Filter paper
CM12 Philips Transmission Electron Microscope or equivalent

1. Apply 5 μl of the sample to be analyzed (e.g., a suspension of the washed pellet containing the amyloid fraction isolated from the sucrose gradient) on Formvar-carbon-coated, copper or nickel, 300 mesh, electron microscope grids and let stand for ~2 min.
2. Gently rinse with sterile milli-Q filtered water.
3. Add 5 μl of freshly prepared aqueous (milli-Q filtered water) 1% (w/v) uranyl acetate, pH 6.0, and incubate 15 to 20 sec for negative-contrast stain

   CAUTION: *Uranyl salts are both toxic and radioactive (low-intensity emitter of α-particles). Follow general safety precautions for handling radioactive compounds and dispose of contaminated waste in accordance with Institutional Policies.*
4. Blot dry with a strip of filter paper until the last drop is drained off, leaving only a thin film on the grid, and air dry.

   The stained grids can be stored at room temperature without adverse effects.
5. Evaluate amyloid fibrillar structures by transmission electron microscopy on a CM12 Philips Transmission Electron Microscope, or equivalent.

   Electron microscopy requires specialized equipment, handled by experienced operators and it is typically performed at dedicated facilities. For detailed description of microscope operation, image capture, and evaluation consult dedicated reviews (Holm et al., 1999; Westermark et al., 1999).

BIOCHEMICAL ANALYSIS OF AMYLOID IN THE BRAIN EXTRACTS

Biochemical analysis of the brain extracts are usually aimed to identify the main constituent of the amyloid deposits. Therefore, it combines the use of specific antibodies to either retrieve the molecule of interest from the extracts (e.g. using immunoprecipitation procedures) or identify its presence (e.g. by immunoblot analysis). This is done using methodologies that provide additional information about the analytes under study (e.g. a more precise molecular mass and/or amino acid sequence data using mass spectrometry techniques and automated amino acid sequence analysis). The following protocols provide a detailed description of the methodologies that can be carried out in a non-specialized research laboratory and the preparation of samples to be further analyzed at dedicated facilities.

Immunoprecipitation of Amyloid and Pre-Amyloid Extracted Fractions

BASIC PROTOCOL 2

Immunoprecipitation is a widely used methodology to enrich a preparation in a given protein, allowing subsequent elution of the precipitated protein and further analysis by gel electrophoresis (*UNIT 6.1*), mass spectrometry, immunoblotting (*UNIT 6.2*), amino acid sequence analysis, or any other method for identifying protein constituents in a sample. Different methods are available for the immobilization of the formed antigen-antibody complex, among them those based on its capture by immobilized Protein A or Protein G. More recently, new methods have been developed based on the use of paramagnetic beads precoated with secondary antibodies, which can be easily immunoreacted with the desired primary antibodies. After immunoprecipitation and following respective washes, paramagnetic beads are rapidly and efficiently collected with the aid of a magnet yielding high recoveries.

Materials

Paramagnetic beads coated with goat anti-mouse IgG (Dynabeads M-280, Dynal/Invitrogen)
Phosphate-buffered saline (PBS; see recipe)
4G8 and 6E10 monoclonal anti Aβ antibodies (Covance)
Blocking buffer: PBS containing 0.1% (w/v) bovine serum albumin (BSA; Sigma)
SDS-OUT (Pierce Biotechnology)
0.5 M Tris·Cl, pH 11.0
10% 2-mercaptoethanol (Sigma)
Milli-Q water (Purified water with resistivity values at 25°C <18.2 Mho/cm)
4:4:1 mixture of isopropyl alcohol/water/formic acid

1.5-ml microcentrifuge tubes
Eppendorf tube-rack equipped with a removable slide-out magnet (Dynal MPC-S)
Mini labRoller bi-directional rotator (Labnet International)
Savant SpeedVac concentrator (Global Medical Instrumentation)

Additional reagents and equipment for immunoblot assay (Support Protocol 5)

Coat paramagnetic beads with anti-Aβ antibodies

1. Resuspend the mouse-IgG-coated paramagnetic beads by inverting the vial several times. Transfer 50 µl of the suspension to a 1.5-ml microcentrifuge tube and position the tube in the rack equipped with a removable slide-out magnet.

   It is very important to use specially designed racks, which are equipped with potent magnets allowing the compacting of the beads at the bottom of the tube for volumes of 0.5 µl to 2 ml. The strong magnetic field permits total recovery of the beads without losses of material in the subsequent steps of the immunoprecipitation.

2. Wash the beads three times, each time with 1 ml PBS.

   For washing, remove the tube from the magnetic rack, add the PBS, resuspend by inverting the tube, position it back in the magnet-equipped rack, and remove the supernatant after the beads have compacted down.

3. Incubate pelleted beads with 3 µg each of anti-Aβ 4G8 and 6E10 overnight, at 4°C with bi-directional rotation in a Mini labRoller rotator or equivalent in a final volume of 100 µl.

   Coating with primary antibodies is also successfully performed by incubating 2 to 3 hr at room temperature. The proportions shown above are enough for one immunoprecipitation; if more samples are to be processed, the protocol should be scaled up accordingly.

4. Collect the beads by placing the tube on the magnet rack for 2 min. Remove and discard the supernatant.

5. Block the beads by resuspension in 1 ml blocking buffer and subsequently wash three times, each time with 1 ml of the same solution.

   Blocked beads can now be safely stored at 4°C; otherwise, proceed with the immunoprecipitation steps below.

Immunoprecipitate

6. Pretreat the brain extracts to allow for a successful immunoprecipitation. Treat SDS-extracts with SDS-OUT reagent, following the manufacturer's instructions to eliminate SDS. Neutralize formic acid extracts using 0.5 M Tris·Cl, pH 11 (Tomidokoro et al., 2005).

   Incomplete neutralization or lack of SDS removal will result in partial or total failure to immunoprecipitate Aβ from the brain extracts. Immunoprecipitation is based on the specific antigen-antibody reaction, in this case the interaction of Aβ species with the

immobilized anti-Aβ 4G8 and 6E10 antibodies. Extreme pH values and presence of detergents are among the known elements that disrupt antigen-antibody interactions.

7. Incubate anti-Aβ coated beads with neutralized amyloid- or SDS-free pre-amyloid-rich extracts overnight in a 4°C cold room and with bi-directional rotation, as above.

   The volumes of the tissue-extracted fractions to be incubated with the beads depend on the Aβ load of the different cases, as well as to the detection method that follows. Typically, immunoprecipitation of ~4% of the formic-acid- and ~20% of the SDS-extracts prepared in accordance to Basic Protocol 1 yield enough material for subsequent immunoblot and mass spectrometry analyses.

8. Wash the magnetic beads three times, each time with 1 ml PBS.

9. Elute the material bound to the paramagnetic beads in accordance to the assays that will be performed subsequently on the eluted material.

10a. *For immunoblot analysis:* Resuspend the beads in 10 µl of Tris-tricine SDS sample buffer containing 10% 2-mercaptoethanol and directly apply onto 16% Tris-Tricine gels for SDS-PAGE and immunoblot analysis, as described in Support Protocol 5.

   2-mercaptoethanol may be substituted by 5 µl of 1 M dithiothreitol (DTT; Sigma).

10b. *For MALDI-TOF mass spectrometry:* Wash the beads three times, each time with Milli-Q water and dry them in a SpeedVac concentrator system. Elute bound Aβ peptides with 5 µl of a 4:4:1 mixture of isopropyl alcohol/water/formic acid (Ghiso et al., 2001). Store the eluate at −80°C until MALDI-TOF mass spectrometry analysis.

   This analysis requires specialized equipment and it is typically performed at dedicated facilities; therefore, it will not be described herein.

Immunoblot Analysis

SUPPORT PROTOCOL 5

Immunoblot analysis is used to assess the presence of amyloid in purification fractions. Chemiluminescent substrates have been steadily gaining in popularity throughout the past decade to become the detection method of choice in most protein laboratories due to several advantages they have over other detection methods. ECL not only allows multiple exposures to obtain the best image, but also, the detection reagents can be stripped away and the entire blot reprobed to optimize detection conditions, test different antibodies, or visualize other proteins, including control proteins for normalization purposes. A large linear response range allows detection and quantitation over a large range of protein concentrations. Most importantly, chemiluminescence yields the greatest sensitivity of any available detection method with detection limits in the low picogram range.

Materials

Acrylamide stock: 40% acrylamide/Bis solution, 29:1 (3.3% C; BioRad)
Gel buffer: 3 M Tris·Cl, 0.3% (w/v) SDS, pH 8.45
Glycerol
Deionized water
10% (w/v) ammonium persulfate (APS)
TEMED (N,N,N,N′-tetra-methyl-ethylendiamine; BioRad)
Isopropanol
Samples to be analyzed
Tris-tricine sample buffer (BioRad)
Cathode buffer: Tris-Tricine-SDS buffer 10× concentrate (Sigma)
Anode buffer (10× concentrate): 2 M Tris·Cl, pH 8.9

Transfer buffer: 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS, Sigma), pH 11.0, containing 10% (v/v) methanol
Blocking solution: 5% non-fat dry milk in PBS containing 0.1% Tween 20 (Sigma)
Monoclonal anti-Aβ antibodies, 4G8 and 6E10 (Covance)
Tris-buffered saline (Fisher Scientific) containing 0.1% Tween 20 (TBS-T)
HRP-labeled F(ab′)2 anti–mouse IgG (GE Lifesciences)
Western Blotting ECL Detection Substrate (SuperSignal West Pico, Pierce Biotechnology)

Hoeffer MiniVE Mini Vertical Electrophoresis Unit (Fisher Scientific)
Nitrocellulose membranes for chemiluminescence (Hybond ECL; GE Lifesciences), prewet
Hoeffer TE22 Mini Tank Blotting Unit (Fisher Scientific)
Orbital Shaker (Bellco Biotechnology) or equivalent
Film for chemiluminescence (Hyperfilm ECL, GE Lifesciences)
M35A X-OMAT Film Processor (Eastman Kodak Company) or equivalent
Power supply Power Station 300 plus (Labnet International, Inc.) or equivalent

Prepare slab gels for Tris-Tricine SDS-PAGE (Schagger and Von Jagow, 1987)

1. For two 16% running gels (12 × 10–cm × 1.5–mm) combine:

   12.4 ml acrylamide stock solution
   10 ml gel buffer
   3.2 ml glycerol
   4.5 ml deionized water.

2. Add 150 µl 10% APS and 15 µl TEMED and pour immediately between the two previously set up glass plates. Overlay with isopropanol and leave at room temperature until polymerized.

3. Once polymerized, remove the isopropanol layer, and pour the stacking gel prepared as follows:

   1.6 ml acrylamide stock solution
   3 ml gel buffer
   7.4 ml deionized water.

4. Add 80 µl 10% APS and 14 µl TEMED, pour on top of the running gel, and carefully insert the comb avoiding the formation of bubbles.

Perform electrophoresis

5. Resuspend the samples to be analyzed—either purified and lyophylized amyloid and pre-amyloid extracts or paramagnetic beads containing the immunoprecipitated material (see Basic Protocol 2, step 10a)—in ~20 µl Tris-tricine sample buffer containing 10% 2-mercaptoethanol.

6. Boil for 5 min and load onto the gel previously assembled into the electrophoresis unit with the cathode buffer in the upper chamber and the anode buffer in the lower chamber.

7. Run electrophoresis at ~45 V (20 mA) for 15 to 30 min until samples have entered the running gel; at this point, increase voltage to 120 to 150 V until completion (~2 to 3 hr).

   For organization purposes, sometimes it is convenient to run the gels overnight. In this case, after the sample is inside the running gel, lower the voltage to ~20 V (<10 mA) for the overnight run.

Transfer the proteins

8. Remove the gel, equilibrate in transfer buffer for a few minutes, and assemble the transfer cassette positioning the prewetted nitrocellulose membrane carefully on top of the gel.

   Avoid touching the gel, nitrocellulose, transfer sponges, and cassettes without gloves. Make sure there are no air bubbles trapped between the gel and the nitrocellulose, which will prevent successful transfer.

9. Electrotransfer 45 min at 400 mA in a Hoeffer TE22 Mini Tank Blotting Unit, or equivalent

   To analyze the degree of Aβ oligomerization in the extracts increase the transfer time up to 2 hr to allow proper electrotransfer of higher-molecular-mass assemblies.

Expose the blot to antibodies

10. Carefully disassemble the transfer cassette and remove the nitrocellulose sheet. Rinse the membrane with 20 to 30 ml PBS, and block with 15 ml blocking solution for 3 hr at 4°C while rocking in a rotating platform.

    Blocking time is not critical and it can be extended overnight or even performed during the weekend depending on individual schedules.

11. Remove the blocking solution and incubate 3 hr at room temperature with a 10 ml combination of 4G8 and 6E10 at a final concentration of 1 μg/ml each in TBS-T, while on a shaker.

12. Wash the membrane three times, each time for 10-min with 20 ml TBS-T while rocking vigorously.

13. Incubate with 10 ml of the secondary antibody at a 1:10,000 dilution in TBS-T for 1 hr at room temperature.

14. Wash the membranes three times, each time for 10 min with 20 ml TBS-T while rocking vigorously.

Visualize the signal

15. Prepare the two-component ECL substrate, consisting of a stable luminol solution with an enhancer and a stable peroxide solution, by mixing the two components together at a 1:1 ratio.

    Once prepared, the ECL substrate working solution is stable for a minimum of 24 hr at room temperature.

16. Immerse the nitrocellulose sheet in the ECL substrate working solution and incubate for 2 min at room temperature. Assemble the autoradiography cassette positioning the drained nitrocellulose membrane between regular plastic sheet protectors making sure that no bubbles remain on top of the nitrocellulose membrane.

17. In a darkroom, assemble the autoradiography cassette by putting a sheet of film on top of the sheet protector sandwich containing the transferred membrane covered by the ECL substrate. Close the cassette and expose the membrane for ~30 sec to 1 min.

    Exposure times should be determined empirically and range from 3 to 5 sec to 10 to 20 min depending on the intensity of the signals, which in turn are related to the protein load on the gel, the concentration of Aβ of the samples analyzed, as well as the strength of the primary antibody. It is recommended to start with a medium time, ~30 sec to 1 min, each time developing the film as described below. Depending on the quality of the image obtained, either decrease or increase the exposure time, accordingly.

18. Develop the film in a M35A X-OMAT Film Processor, or equivalent.

SUPPORT PROTOCOL 6

Amino Acid Sequence Analysis

Determination of the primary amino acid sequence of a protein is the gold standard for protein identification delivering picomole-order sensitivity. Most sequence analyzers determine the N-terminal amino acid sequence through the Edman degradation reaction, labeling the amino terminal residue, which is then cleaved from the rest of the peptide, derivatized, and identified through the elution time in high-pressure liquid chromatography in comparison with standards (Edman, 1950; Niall, 1973). In this unit, we will restrict our discussion to the preparation of the samples for amino acid sequencing since the procedure requires specialized equipment and it is performed at dedicated facilities.

Materials

100% methanol
Milli-Q water (Purified water with resistivity values at 25°C <18.2 Mho/cm)
Transfer buffer: 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS, Sigma), pH 11.0, containing 10% (v/v) methanol
Sample to be sequenced
Protein stain solution: 0.125% (w/v) Coomassie Blue R-250 in 50% methanol
Destaining solution: 50% methanol

Polyvinylidene difluoride membranes (PVDF; Immobilon-P; Millipore)
Scalpel
Microcentrifuge tubes
494 Procise Protein Sequencer (Applied Biosystems) or equivalent

Additional reagents and equipment for immunblot assay (Support Protocol 5)

Prepare samples

1. PVDF membranes are hydrophobic and need to be pre-wetted in 100% methanol for 15 seconds before being transferred to a container with Milli-Q water for 2 min, and equilibrated in CAPS transfer buffer prior to the assembly of the transfer cassette. Do not leave any dry spots that can result in an unsuccessful transfer.
2. Separate the sample of interest in SDS-PAGE and electrotransfer to PVDF membranes following the procedure for immunoblot analysis (see Support Protocol 5).
3. After transfer, rinse the membrane with Milli-Q water, stain for 1 min with protein stain, and differentiate with several washes (15-sec each) of 10 ml destaining solution.

   The destaining procedure should be performed under continuous, vigorous shaking and with several changes of the destaining solution for more efficient removal of excess dye.
4. Rinse the transferred membrane with 30 ml Milli-Q water, excise the bands of interest with a scalpel, and keep them in a microcentrifuge tube under Milli-Q water at 4°C until sequencing at a dedicated facility.

   It is recommended that PVDF membranes do not dry at any time, since due to their hydrophobicity they will require re-wetting in 100% methanol to re-establish their hydrophilicity.

PURIFICATION AND CHARACTERIZATION OF PAIRED HELICAL FILAMENTS

Neurofibrillary tangles found in Alzheimer's disease brain are composed of twisted fibers known as paired helical filaments (PHFs). The biochemical characterization of the components of PHF eluded investigators for many years principally due to the difficulties in disrupting their structure and achieving solubilization. Eventually, it was demonstrated that PHF was constituted by self-assembled tangles of hyperphosphorylated tau proteins, a family of various isoforms of microtubule-associated proteins abundant in neurons. Through their interaction with tubulin, tau proteins promote tubulin assembly

into microtubules and stabilize these microtubules mostly by phosphorylation (reviewed in Ballatore et al., 2007).

Protocols employed for the purification of PHF are, like in the case of amyloid, mostly based on a combination of different solubilization strategies (e.g., use of various detergents, sonication and boiling, and addition of chaotropes) together with enrichment by sedimentation centrifugation and in some cases column chromatography. Below we describe two basic procedures successfully employed for the isolation of PHF from AD tissues (Iqbal et al., 1984; Lee et al., 1999).

Isolation of PHF by Sedimentation Centrifugation

BASIC PROTOCOL 3

This method for isolating PHFs is adapted from Lee et al. (1999) and summarized in Figure 3.33.4.

Materials

Frozen brain tissue enriched in NFT, as determined by standard immunohistochemistry
Tissue homogenization 4 (THB-4; see recipe)
PHF extraction buffer (see recipe)
Sarkosyl (Sodium Lauroyl Sarcosinate)
RAB buffer (see recipe)
Sucrose gradient solutions (see recipe)
Protease Inhibitors Cocktail (Roche)
2 M guanidine isothiocyanate

Dounce glass homogenizer
Ultracentrifuge XL100K (Beckman Coulter, Fullerton, CA) equipped with 70.1 and SW40 Ti rotors, or equivalent
Sonicator

Additional reagents and equipment for preparing a discontinuous sucrose gradient (Alternate Protocol 1)

Prepare tissue (Figure 3.33.4)

1. Dissect the gray matter clean of leptomeninges and large blood vessels.
2. Homogenize in tissue homogenization buffer 4 at a ratio of 1.5 ml buffer per gram of tissue using a Dounce homogenizer (20 strokes).
3. Incubate 20 min at 4°C to depolymerize any residual microtubules.
4. Centrifuge 20 min at 11,000 × *g*, 4°C, using the 70.1 rotor.

Separate PHF

5. Collect the supernatant and recentrifuge 60 min at 100,000 × *g* for 60 min, 4°C using the same rotor.

   The resulting supernatant contains primarily soluble normal tau while PHF remains in the pellet.

6. Combine the pellets from the first and the second cold centrifugations and resuspend in PHF extraction buffer at 1:10 (w/v) ratio (e.g., for 1 g starting material use 10 ml extraction buffer).
7. Centrifuge 20 min at 15,000 × *g*, 4°C.

   In the presence of 10% sucrose and at low-speed centrifugation, isolated PHF, as well as small PHF aggregates remain in the supernatant whereas intact or fragmented NFTs and larger PHF aggregates are pelleted.

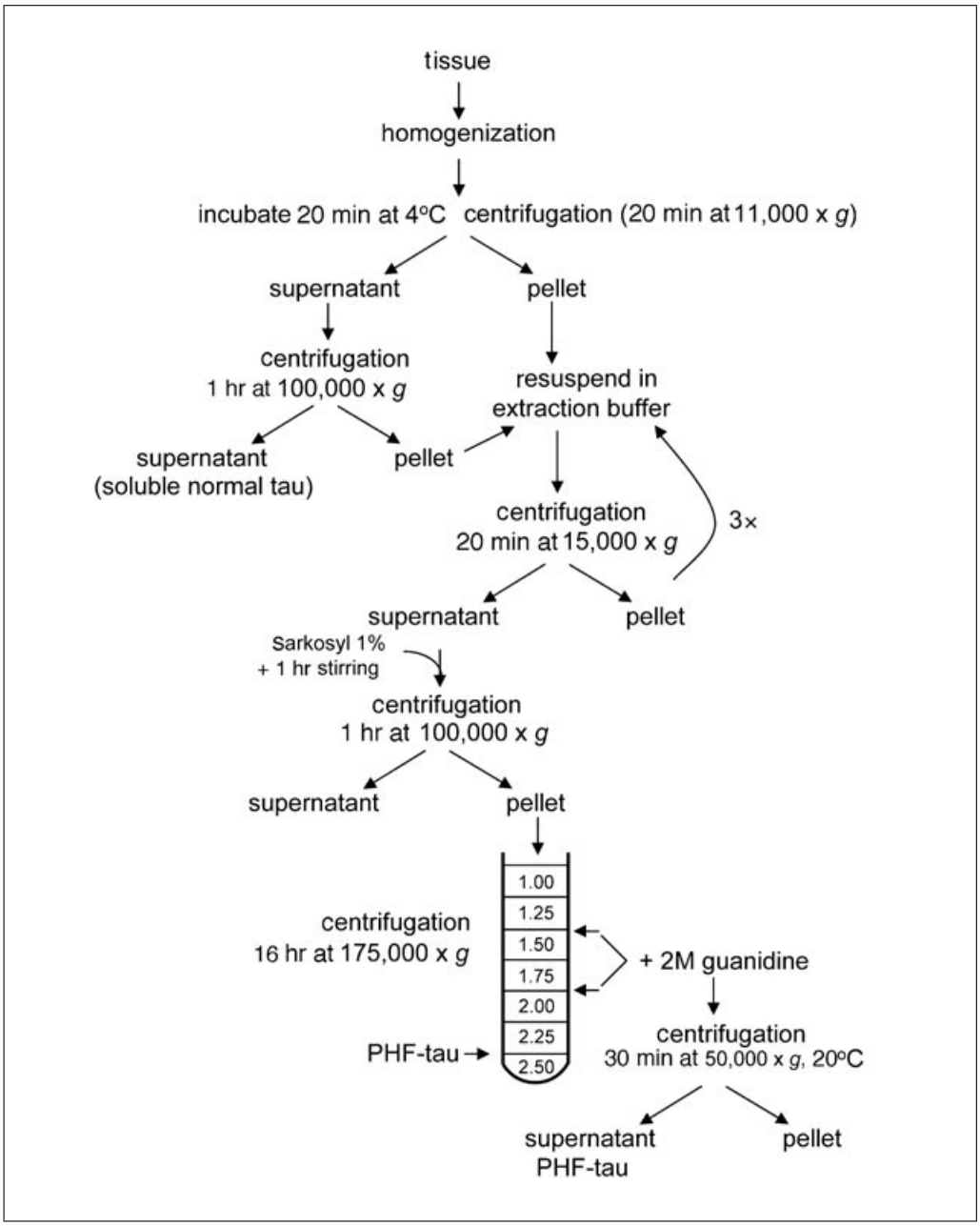


Figure 3.33.4 Schematic representation of the isolation of PHF by the sedimentation centrifugation procedure described in Basic Protocol 3.

8. Re-extract the pellet repeating steps 6 and 7 and pool the supernatants.

Remove membranes

9. Add Sarkosyl to a final concentration of 1% and stir 1 hr at room temperature.

   Sarkosyl treatment removes membranous material from the crude preparation thereby enriching the solution in PHF-tau.

10. Centrifuge for 30 min at 100,000 × *g*, 4°C, in the 70.1 Ti rotor.

11. Resuspend the pellet containing PHF in 1 to 5 ml THB-4 depending on the amount of starting material (1 ml buffer for 25 g of starting gray matter), sonicate until smooth, and boil for 5 min.

Purify PHF fraction

12. Load 0.5-ml aliquots onto a discontinuous sucrose gradient (1.0, 1.25, 1.5, 1.75, 2.0, 2.25, and 2.0 M sucrose in THB-3).

13. Centrifuge 16 hr at 175,000 × *g*, 10°C, using the SW40 Ti swinging bucket rotor.

14. Discard the white flocculent layer containing mostly membranes on the top of the gradient. Collect the 1.25/1.5 M interface, the 1.75/2.0 M interface, and the 2.25/2.5 M interface.

    A thick brown band is recovered between the 1.25 and 1.5 M sucrose interface, thick brownish material is found on the 1.75 and 2.0 M interface, and a small light-brown material between the 2.25 and 2.5 M sucrose layers.

    Typically, only the heaviest fraction contains PHF uncontaminated with amorphous material. However, this fraction represents <20% of the total PHF-tau recovered from all three fractions of the sucrose gradient—from 10 g of enriched gray matter only ∼1 μg of nearly pure intact PHFs is generated from this heaviest fraction.

15. If fractions from the two lightest sucrose interfaces are to be further purified for PHF-tau, after collecting the material, add 2 M guanidine isothiocyanate (∼1 ml per gram wet tissue) and stir for 1 hr at 37°C.

16. Centrifuge 30 min at 50,000 × *g*, 25°C, discard the pellet, dialyze the supernatant against several changes of distilled water to remove the guanidine isothiocyanate, and lyophilize.

    PHF-tau proteins are ready for further use; the fibrillar structure may be confirmed by Thioflavin S fluorescence assay (Support Protocol 3) or EM (Support Protocol 4) as described for amyloid preparations.

    The measurement of Thioflavin S fluorescence is a good indicator of PHF formation and it is generally employed in in vitro assays of tau assembly. Nevertheless, it is not an absolute proof of PHF existence since not only is it positive for tau aggregation into amorphous structures but also a shift in the thioflavin fluorescence is seen in the presence of amyloid fibrils and protofibrils, as described above.

    The ultimate demonstration of PHF purification is achieved by EM, revealing the typical 10 to 20 nm-wide filaments and showing the characteristic twist with a cross-over repeat of ∼80 nm (Wisniewski and Wen, 1985; Barghorn et al., 2005; von Bergen et al., 2005).

An Alternative for Isolation of PHF by Sedimentation Centrifugation

ALTERNATE PROTOCOL 5

This alternative protocol is also based on density centrifugation enrichment of NFTs, but it incorporates an additional glass beads chromatography step to eliminate contamination with capillary vessels (Iqbal et al., 1984).

Materials

Tissue
0.32, 1, 1.05, 1.1, 1.15, 1.2, 1.8, 2, 2.2 M sucrose (see recipe)
0.32 M sucrose-equilibrating buffer (see recipe)
Sodium dodecyl sulfate (SDS)

Dounce homogenizer
J-6B refrigerated centrifuge (Beckman Coulter), or equivalent
150-, 70-, 35-μm nylon mesh
150- and 30-ml tubes
Glass bead column (bead sizes 0.25 to 0.30-mm and 0.45 to 0.5-mm)

Additional reagents and equipment for transmission electron microscopy (Support Protocol 4)

Prepare tissue

1. Separate ~60 g of gray matter from meninges and white matter, homogenize in a 5-fold volume of 0.32 M sucrose in a Dounce homogenizer, and sieve through 150- and 70-μm nylon meshes (as in Basic Protocol 1).
2. Bring up the sucrose concentration of the filtrate to 1 M by adding 3.4 ml of 2 M sucrose for every 10 ml of filtrate.
3. Centrifuge 40 min at 4,800 × *g*, 10°C.
4. Remove the floating material, as well as the supernatant by aspiration and discard.

Fractionate the pellet

5. Resuspend the pellet in 1 M sucrose, rehomogenize, and load over a discontinuous density gradient of 20 ml each of 1.8, 1.2, 1.15, 1.1, and 1.05 M sucrose in 150-ml capacity tubes.
6. Centrifuge 50 min at 4800 × *g*, 10°C.
7. Remove the floating pad and all material above the 1.2/1.8 M sucrose interface and discard it. Pool together the layer banding at the 1.2/1.8 M sucrose interface and the pellet, which are enriched in NFTs, for further purification.
8. Dilute the pooled NFTs fractions five- to six-fold with deionized water, centrifuge as above (step 3), and discard the supernatant.
9. Resuspend the pellet in 0.32 M sucrose (as described in step 1) using a Dounce homogenizer, and further sieve the suspension through a 35-μm nylon mesh.

Run a glass bead column

10. Pass the filtrate through a glass bead column to remove the bulk of the capillaries present as a contaminant. Elute the column with the 0.32 M sucrose-equilibrating buffer.
11. Centrifuge the eluate as described in step 3, discard the supernatant, and re-homogenize the pellet in 0.32 M sucrose.
12. Add SDS to a 2% (w/v) final concentration and incubate at room temperature 3 to 5 min.

    Non-ionic detergents like Triton X-100 are considerably milder in their solubilization capacity and consequently leave a number of subcellular structures intact (e.g., neurofilaments). Therefore, their use for PHF isolation is not recommended.

13. Bring the sucrose concentration to 1 M by addition of 2 M sucrose (as in step 2). Layer on a discontinuous gradient of 5 ml of 2.2 M and 10 ml of 1.1 M sucrose in a 30-ml tube. Centrifuge as above (step 6), but increase the temperature to 20°C.

    SDS-containing samples cannot be maintained at low temperatures (see comments in Basic Protocol 1 for amyloid isolation).

14. Harvest NFTs, typically they are in the 1.1/2.2 M sucrose interface; a minor fraction may be found at the 1.0/1.1 M interface.

Assess the purity

15. Assess the purity of NFTs by EM using the same procedure as described above for amyloid and visualization by transmission electron microscopy (Support Protocol 4).

    PHFs extracted from Alzheimer's brain exhibit a characteristic twist with a cross-over repeat of ~80 nm (Fig. 3.33.1). The filaments have a typical width of 10 to 20 nm and a minimum width at crossing points of 9 to 9.5 nm. Single filaments twist over themselves to give the appearance of flat ribbons.

NFT Isolation by Laser Capture Microdissection

LCM provides advantages for the purification of NFTs, as in the case of amyloid plaques, being the most significant method with the capability of obtaining samples without cross-contamination with other tissue structures. This protocol is essentially the same as that described for the LCM of amyloid plaques (Alternate Protocol 4), also using 10-μm-thick cryostat sections of AD brain tissue. The individualization of NFT prior to the dissection is performed by routine immunohistochemistry using, as the primary antibody, mouse monoclonal anti-tau antibodies (tau-2, Sigma; Wang et al., 2005) or equivalent. This may be followed by either immunofluorescence detection via secondary antibodies conjugated to fluorescent dyes or by traditional HRP-labeled secondary antibodies followed by ABC complex and diaminobenzidine detection.

Materials

Tissue sections
Acetone, cold
0.1% Triton X-100
70% formic acid

Sonicator
Vacuum centrifugation system

1. Follow steps 1 to 4 of Alternate Protocol 4 for tissue preparation for LCM.
2. Fix sections in cold acetone for 10 min and permeabilize with 0.1% Triton X-100 for 10 min, and follow routine immunohistochemical procedures for NFT detection (Wang et al., 2005).
3. Follow step 9 of the microdissection procedure in Alternate Protocol 4. Collect ~2000 NFT-containing cells for a successful proteomic analysis, as well as a similar number of cells free of NFT to be used as controls.
4. Solubilize microdissected samples in 70% formic acid with 1 min sonication. Dry samples through vacuum centrifugation and proceed with mass spectrometry analysis.

   Typically, liquid chromatography coupled with ion-trap tandem mass spectrometry (LC-MS/MS) is one of the most sensitive and widely used methodologies for minute samples of complex protein mixtures. Due to the sophisticated equipment and elaborate methodology involved in these analyses, readers are referred to specially dedicated reviews in the field (Gozal et al., 2006; Nesvizhskii, 2007).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Phosphate-buffered saline

10 mM phosphate buffer, pH 7.4, containing 2.7 mM KCl and 137 mM NaCl. Dissolve one tablet of phosphate-buffered saline (PBS; Sigma) in 200 ml of de-ionized water. Scale up as needed. Store up to 6 months at 4°C.

PHF extraction buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
10% sucrose
0.85 M NaCl
1 mM EGTA

continued

Adjust pH to 7.4 using 0.5 M NaOH
Store up to 3 months at 4°C

Sucrose-equilibrating buffer, 0.32 M

0.32 M sucrose
50 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.1 M 2-mercaptoethanol
2% (w/v) SDS
Protease inhibitors cocktail (Complete; Roche Applied Sciences; prepared in accordance to the manufacturer's specifications)

Sucrose gradient solutions

Sucrose to appropriate molarity
50 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
Prepare fresh

Tissue homogenization buffer 1

10 mM phosphate buffer, pH 7.4, containing 2.7 mM KCl and 137 mM NaCl. Dissolve one tablet of phosphate-buffered saline (PBS; Sigma) in 200 ml of deionized water. Scale up as needed. Add:

Protease inhibitors cocktail (Complete; Roche)
Dissolve one tablet of protease inhibitors cocktail per 50 ml of PBS
Store up to 1 week at 4°C

Tissue homogenization buffer 2

50 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
2% (w/v) SDS
Protease inhibitor cocktail (Complete; Roche Applied Sciences; prepared in accordance to the manufacturer's specifications)
0.1 M 2-mercaptoethanol (add immediately prior to use)
Store up to 3 months at room temperature (without the addition of 2-mercaptoethanol)

Tissue homogenization buffer 3

15 mM HEPES, pH 7.4
103 mM NaCl
4.7 mM KCl
2.5 mM $CaCl_2$
1.2 mM $KHPO_4$
25 mM $NaHCO_3$
10 mM glucose
10 IU/ml heparin
Store up to 3 weeks at 4°C

Tissue homogenization buffer 4

100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)
0.75 M NaCl
1 mM EGTA
0.5 mM $MgSO_4$
2 mM DTT
Adjust pH 6.8 using 0.5 M NaOH
Store up to 3 months at 4°C

Wash buffer

150 mM NaCl
0.1% SDS
0.02% NaN_3
Store up to 3 months at room temperature

COMMENTARY

Background Information

Cerebral amyloid diseases are considered part of an emerging complex group of chronic and progressive entities collectively known as "Disorders of Protein Folding" that include sporadic and familial Alzheimer's disease, cerebellar ataxias, Parkinson's and prion diseases, tauopathies, type-II diabetes, systemic amyloidosis, Huntington's disease, and cataracts, among others (Dobson, 2001; Taylor et al., 2002; Lovestone and McLoughlin, 2002; Temussi et al., 2003). In these diseases, through mechanistic pathways poorly understood, soluble proteins normally found in biological fluids change their conformation and form insoluble structures that accumulate in the form of either intra- and extra-cellular aggregates or fibrillar lesions usually resulting in cell damage, organ dysfunction, and eventually death. Only about one-third of amyloid proteins known to be linked with human disease are associated with neurodegenerative disorders (Taylor et al., 2002; Rostagno and Ghiso, 2003). In AD two proteins aggregate in an abnormal fashion, the Aβ peptide, which builds the plaques in the parenchymal extracellular space and deposit around blood vessels, and the microtubule-associated protein tau, which polymerizes in the form of intraneuronal paired helical filaments, the building blocks of NFTs (Johnson and Bailey, 2002; Selkoe, 2003).

Regardless of the nature of the misfolded proteins involved, all extracellular fibrillar deposits, generically referred to as amyloid, share common physical, structural, and tinctorial properties (see Fig. 3.33.1). In general, they are (1) highly polymerized and poorly soluble assemblies, features that require the use of strong detergents, harsh acid conditions, or concentrated chaotropes to partially extract them from the tissue deposits; (2) structurally rich in β-pleated sheet conformations, a property responsible for the apple-green birefringence of the deposits when observed under polarized light after Congo red staining, as well as for their yellow-green fluorescence after thioflavin S staining; and (3) fibrillar in shape when negatively stained and observed under the electron microscope (LeVine, 1995; Klunk et al., 1999; Ghiso and Frangione, 2002). These properties are also shared by PHF, which show a high degree of insolubility, an enrichment in β-sheet configuration when compared with the unfolded structure of soluble tau (Vallet et al., 1992), and a typical fibrillar structure under the electron microscope (Gorevic, 1986; Barghorn et al., 2004; Fig. 3.33.1).

The high insolubility of the amyloid deposits and NFTs challenged investigators for many years and prevented the individualization of the respective proteins constituting the lesions. The methods employed to isolate plaques and tangles were mostly based on their physical properties, and consisted in various combinations of sieving through different sized meshes to eliminate microvascular components, density centrifugation to enrich preparations in heavier components (plaques and tangles), addition of different detergents and chaotropic agents, and heating and sonication in attempts to solubilize the materials (Selkoe et al., 1982, 1986; Masters et al., 1985; Gorevic et al., 1986; Roher et al., 1986; Greenberg and Davies, 1990; Iqbal and Grundke-Iqbal, 2006). Above we have presented a group of protocols exemplifying the use of these different methodological approaches. Other protocols, not described here, represent variations of the main procedures and are based on the same principles (Ihara et al., 1983; Masters et al., 1985; Rubenstein et al., 1986; Greenberg and Davies, 1990; Roher and Kuo, 1999). An exception is constituted by a few methods that in addition to the traditional tissue homogenization and gradient centrifugation steps employ fluorescence-activated cell sorting (FACS) techniques to separate plaques and tangles after their immunoreaction with either anti-Aβ or anti-tau antibodies, respectively, followed by secondary antibodies conjugated to fluorescent dyes (Roher et al., 1986; Selkoe et al., 1986). We did not describe the FACS-based methods in these protocols due to their requirement for cell sorting equipment, which is not always widely available.

The advent of mass spectrometry methodologies provided invaluable tools for the analysis of the components of plaques and tangles and were instrumental in the assessment of post-translational modifications associated with both deposits. In the case of Aβ, mass spectrometry corroborated the presence of cyclic pyroglutamate-modified fragments, exclusively associated with deposited N-terminally truncated Aβ species not detected in biological fluids, and demonstrated an abundance of soluble N-, and C-terminal degraded species likely representing the action of an array of diverse proteases and clearance mechanisms (Vigo-Pelfrey et al., 1993; Kuo et al., 1997; Tomidokoro et al., 2005). Mass spectrometry was also instrumental in the identification of many of the abnormal hyperphosphorylated sites of PHF-tau protein extracted from AD cases (Hasegawa et al., 1992; Hanger et al., 1998; Iqbal and Grundke-Iqbal, 2006). The recent development of proteomic technologies based on the use of more sophisticated mass spectrometry methods, capable of identifying minute components in complex protein mixtures, became an invaluable tool for the analysis of AD-related lesions. Their extraordinary sensitivity, in combination with the high selectivity of laser-capture microdissection techniques, allows the isolation of plaques and tangles without cross-contamination with other tissue structures. Using this approach, >400 proteins have been demonstrated in AD senile plaques (Liao et al., 2004; Gozal et al., 2006) while 155 different components have been shown to form part of the protein deposits in NFTs (Wang et al., 2005).

We have limited the scope of the protocols presented above to the isolation of amyloid and NFTs from AD brains. Nevertheless, the same methodological approaches may be adapted to the isolation of plaques and tangles found in other cerebral amyloidosis. It should be emphasized that although AD is by far the most common brain amyloidosis, other disorders share the presence of parenchymal amyloid and pre-amyloid deposits and, in some cases, even NFTs with identical pattern of PHF-tau (reviewed in Ghiso and Frangione, 2002). Among the non-Aβ amyloidosis, two diseases, familial British and Danish dementias, show striking clinical and neuropathological similarities to AD (reviewed in Ghiso et al., 2006). We have adapted the protocols described above for the extraction and biochemical identification of the vascular and parenchymal amyloid and pre-amyloid deposits in familial British and Danish dementia cases, and successfully achieved the identification of the deposited species via mass spectrometry (Tomidokoro et al., 2005). Through slight modifications, the methodology described above may also be employed for other amyloid disorders either cerebral or systemic, with differences in solubility being the major consideration.

Critical Parameters and Troubleshooting

The accurate biochemical evaluation of amyloid and PHF extracted from brain specimens of affected individuals requires minimization of the mostly enzymatic-driven protein modifications occurring after death. It is therefore imperative to use tissue that was collected with the shortest post-mortem delay—ideally no more than 2 to 4 hr—to ensure high recovery and minimal changes. In addition, the appropriate selection of tissues is perhaps one of the most critical factors in determining a good recovery. The extension of the neuropathological lesions is highly variable not only among different AD cases but also among various regions of the same brain. It is therefore advisable to select specimens with abundant AD-related pathology to guarantee high yields in the preparations. The use of widely available standardized protocols and abundant commercial sources of antibodies for both Aβ and PHF-tau allows the immunohistochemical evaluation of the neuropathological compromise, which should be performed prior to starting the isolation procedure and should also serve as a guidance as to the starting amount of tissue.

All the procedures described in this unit involve tissue homogenization prior to the different extraction protocols. The use of protease inhibitors and the maintenance of the specimens at 4°C are imperative to prevent undesired enzymatic degradation after the release of intracellular proteases occurring during cell lysis. We have described the use of a cocktail of inhibitors, which is routinely employed in our laboratory. Nevertheless, there are multiple options available on the market (including individual inhibitors) that could replace the one we suggest. After homogenization, many of the protocols require density gradient centrifugation. It should be emphasized that it is essential to observe the recommended temperatures during the full procedure, since the density of the different solutions composing the various gradients, either sucrose, Percoll, or Ficoll depends on the temperature. The

localization of amyloid and PHF in the different gradient interfaces may vary slightly with the preparation of the different solutions. It is advised that, at least the first time the protocols are run in the laboratory, the presence of fibrillar materials in the fractions—either amyloid or PHF—be confirmed by electron microscopy.

Anticipated Results

A typical purification of amyloid plaques results in their enrichment in the 1.4 M/2.0 M sucrose interfaces (Alternate Protocol 1). In the case of PHF isolation (Basic Protocol 2), the most pure fraction is typically located between the 2.25 M and 2.5 M sucrose layers, although representing only a minor part (~20%) of the total PHF-tau; the rest of PHF is recovered from the 1.25/1.5 and 1.75/2.0 M interfaces. The ultimate confirmation of the presence of amyloid and PHF requires electron microscopy evaluation after negative staining with uranyl acetate as described above (Support Protocol 4). Figure 3.33.1 shows the expected morphological features of the purified elements. Amyloid fibrils, irrespective of their origin, are arranged in random arrays and are nonbranching with a typical 8- to 10-nm width. PHF exhibits a characteristic twist with a crossover repeat of ~80 nm; the filaments have a typical width of 10 to 20 nm and a minimum width at crossing points of 9 to 9.5 nm. Single PHF filaments twist over themselves to give the appearance of flat ribbons.

The biochemical evaluation of amyloid Aβ deposits by mass spectrometry typically demonstrates high heterogeneity. The major species expected are Aβ1-40 and Aβ1-42, for vascular and parenchymal deposits, respectively. Nevertheless, N-, and C-terminal truncated species representing proteolytic degradation of the deposited species, as well as a variety of different post-translationally modified fragments including oxidation-derived and pyroglutamate derivatives, are common findings.

Time Considerations

The protocols for the isolation of plaques and PHF involve several rounds of sedimentation centrifugation and require ~1 week for completion, although much of this time is spent waiting for centrifugations and does not involve continual lab-bench work. On the contrary, the sequential extraction of amyloid and pre-amyloid deposits described in Basic Protocol 1 utilizing short-duration centrifugations, requires a more constant dedication to the procedure, which will take ~3 days for completion.

Extra time should be allotted to the characterization of the purified material by immunoblot, mass spectrometry, amino acid sequence analysis, and electron microscopy studies, which will easily use up 7 to 10 days, depending on the experience of the operator, his/her work-day schedule, and the availability of the equipment. In many circumstances, the evaluation of the isolated material by mass spectrometry, sequence analysis, and electron microscopy requires the use of specially dedicated facilities. Although the analyses themselves do not require more than a few hours, the workload of the dedicated facilities in some cases may add extra time to the overall procedure.

Acknowledgements

This work was supported in part by NIH grants NS051715, AG010491, AG008051 ADC Pilot and by the Alzheimer's Association.

Literature Cited

Ahram, M., Flaig, M.J., Gillespie, J.W., Duray, P.H., Linehan, W.M., Ornstein, D.K., Niu, S., Zhao, Y., Petricoin, E.F., 3rd, and Emmert-Buck, M.R. 2003. Evaluation of ethanol-fixed, paraffin-embedded tissues for proteomic applications. *Proteomics* 3:413-421.

Ballatore, C., Lee, V., and Trojanowski, J.Q. 2007. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat. Rev. Neurosci.* 8:663-672.

Barghorn, S., Davies, P., and Mandelkow, E. 2004. Tau paired helical filaments from Alzheimer's disease brain and assembled in vitro are based on β-structure in the core domain. *Biochemistry* 43:1694-1703.

Barghorn, S., Biernat, J., and Mandelkow, E. 2005. Purification of recombinant tau protein and preparation of Alzheimer-paired helical filaments in vitro. *Methods Mol. Biol.* 299:35-51.

Braak, H. and Braak, E. 1991. Neuropathological stageing of Alzheimer-related changes. *Acta neuropathol.* 82:239-259.

Dobson, C.M. 2001. Protein folding and its links with human disease. *Biochem. Soc. Symp.* 68:1-26.

Edman, P. 1950. Method for the determination of amino acid sequences in peptides. *Acta Chem. Scand.* 4:283-293.

Ghiso, J. and Frangione, B. 2002. Amyloidosis and Alzheimer's disease. *Adv. Drug Delivery Rev.* 54:1539-1551.

Ghiso, J., Holton, J., Miravalle, L., Calero, M., Lashley, T., Vidal, R., Houlden, H., Wood, N., Neubert, T.A., Rostagno, A., Plant, G., Revesz,

T., and Frangione, B. 2001. Systemic amyloid deposits in Familial British Dementia. *J. Biol. Chem.* 276:43909-43914.

Ghiso, J., Rostagno, A., Tomidokoro, Y., Lashley, T., Bojsen-Moller, M., Braendgaard, H., Plant, G., Holton, J., Lal, R., Revesz, T., and Frangione, B. 2006. Genetic alterations of the BRI2 gene: Familial British and Danish dementias. *Brain Pathol.* 16:71-79.

Golde, T.E., Eckman, C.B., and Younkin, S.G. 2000. Biochemical detection of Abeta isoforms: Implications for pathogenesis, diagnosis, and treatment of Alzheimer's disease. *Biochim. Biophys. Acta* 1502:172-187.

Gorevic, P., Goni, F., Pons-Estel, B., Alvarez, F., Peress, N., and Frangione, B. 1986. Isolation and partial characterization of neurofibrillary tangles and amyloid plaque core in Alzheimer's disease: Immunohistological studies. *J. Neuropathol. Exp. Neurol.* 45:647-664.

Gozal, Y.M., Cheng, D., Duong, D.M., Lah, J.J., Levey, A.I., and Peng, J. 2006. Merger of laser capture microdissection and mass spectrometry: A window into the amyloid plaque proteome. *Methods Enzymol.* 412:77-93.

Greenberg, S.G. and Davies, P. 1990. A preparation of Alzheimer paired helical filaments that displays distinct tau proteins by polyacrylamide gel electrophoresis. *Proc. Natl. Acad. Sci. U.S.A.* 87:5827-5831.

Grundke-Ikbal, I., Iqbal, K., Quinlan, M., Tung, Y.C., Zaidi, M.S., and Wisniewski, H.M. 1986a. Microtubule-associated protein tau. *J. Biol. Chem.* 261:6084-6089.

Grundke-Ikbal, I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M., and Binder, L.I. 1986b. Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. U.S.A.* 83:4913-4917.

Hanger, D.P., Betts, J.C., Loviny, T.L.F., Blackstock, W.P., and Anderton, B. 1998. New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nano-electrospray mass spectrometry. *J. Neurochem.* 71:2465-2476.

Hasegawa, M., Morishima-Kawashima, M., Takio, K., Suzuki, M., Titani, K., and Ihara, Y. 1992. Protein sequence and mass spectrometric analyses of tau in the Alzheimer's disease brain. *J. Biol. Chem.* 267:17047-11754.

Hofman, F. 2002. Immunohistochemistry. *Curr. Protoc. Immunol.* 49:21.4.1-21.4.23.

Holm Nielsen, E., Nybo, M., and Svehag, S.-E. 1999. Electron microscopy of prefibrillar structures and amyloid fibrils. *Methods Enzymol.* 309:491-496.

Howie, A.J., Brewer, D.B., Howell, D., and Jones, A. 2008. Physical basis of colors seen in Congo red-stained amyloid in polarized light. *Lab. Invest.* 88:232-242.

Hyman, B.T. and Trojanowski, J.Q. 1997. Consensus recommendations for the postmortem diagnosis of Alzheimer disease from the National Institute on Aging and the Reagan Institute Working Group on diagnostic criteria for the neuropathological assessment of Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 56:1095-1097.

Ihara, Y., Abraham, C.R., and Selkoe, D.J. 1983. Antibodies to paired helical filaments in Alzheimer's disease do not recognize normal brain proteins. *Nature* 3034:727-730.

Iqbal, K. and Grundke-Ikbal, I. 2006. Discoveries of tau, abnormally hyperphosphorylated tau and others of neurofibrillary degeneration: A personal historical perspective. *J. Alz. Dis.* 9:219-242.

Iqbal, K., Zaidi, T., Thompson, C.H., Merz, P.A., and Wisniewski, H.M. 1984. Alzheimer pair helical filaments: Bulk isolation, solubility, and protein composition. *Acta Neuropathol.* 62:167-177.

Johnson, G.V. and Bailey, C.D. 2002. Tau, where are we now? *J. Alzheimers Dis.* 4:375-398.

Klunk, W.E., Jacob, R.F., and Mason, R.P. 1999. Quantifying amyloid by Congo red spectral shift assay. *Methods Enzymol.* 309:285-305.

Kuo, Y.M., Emmerling, M.R., Woods, A.S., Cotter, R.J., and Roher, A.E. 1997. Isolation, chemical characterization, and quantitation of A beta 3-pyroglutamyl peptide from neuritic plaques and vascular amyloid deposits. *Biochem. Biophys. Res. Comm.* 237:188-191.

Lee, V., Wang, J., and Trojanowski, J.Q. 1999. Purification of paired helical filament tau and normal tau from human brain tissue. *Methods Enzymol.* Wetzel, R., editor. New York: Elsevier Inc. 81-89.

LeVine, H. 1995. Thioflavine T interaction with amyloid-sheet structures. *Amyloid: Intl. J. Exp. Clin. Invest.* 2:1-6.

Liao, L., Cheng, D., Wang, J., Duong, D.M., Losik, T.G., Gearing, M., Rees, H.D., Lah, J.J., Levey, A.I., and Peng, J. 2004. Proteomic characterization of postmortem amyloid plaques isolated by laser capture microdissection. *J. Biol. Chem.* 279:37061-37068.

Lovestone, S. and McLoughlin, D.M. 2002. Protein aggregates and dementia: Is there a common toxicity? *J. Neurol. Neurosurg. Psychiatry* 2002:152-161.

Masters, C.L., Multhaup, G., Sims, G., Pottigiesser, J., Martins, R.N., and Beyreuther, K. 1985. Neuronal origin of cerebral amyloid: Neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. *EMBO J.* 4:2757-2763.

Morelli, L., Llovera, R., Mathov, I., Lue, L.-F., Frangione, B., Ghiso, J., and Castaño, E.M. 2004. Insulin-degrading enzyme in brain microvessels: Proteolysis of amyloid β vasculotropic variants and reduced activity in cerebral amyloid angiopathy. *J. Biol. Chem.* 279:56004-56013.

Nesvizhskii, A.I. 2007. Protein identification by tandem mass spectrometry and sequence

database searching. *Methods Mol. Biol.* 367:87-119.

Niall, H.D. 1973. Automated Edman degradation: The protein sequenator. *Methods Enzymol.* 27:942-1010.

Pardridge, W.M., Vinters, H.V., Yang, J., Eisenberg, J., Choi, T.B., Tourtellotte, W.W., Huebner, V., and Shively, J.E. 1987. Amyloid angiopathy of Alzheimer's disease: Amino acid composition and partial sequence of a 4,200-dalton peptide isolated from cortical microvessels. *J. Neurochem.* 49:1394-1401.

Robinson, J.P., Darzynkiewicz, Z., Hoffman, R., Nolan, J.P., Orfao, A., Rabinovitch, P.S., and Watkins, S. (eds.). 2009. Current Protocols in Cytometry. John Wiley & Sons, Hoboken, N.J.

Roher, A. and Kuo, Y.-M. 1999. Isolation of amyloid deposits from brain. *Methods Enzymol.* 309:58-67.

Roher, A., Wolfe, D., Palutke, M., and Kukuruga, D. 1986. Purification, ultrastructure, and chemical analysis of Alzheimer disease amyloid plaque core protein. *Proc. Natl. Acad. Sci. U.S.A.* 83:2662-2666.

Rostagno, A. and Ghiso, J. 2003. Amyloidosis. *In* Encyclopedia of Neurological Sciences (M. Aminoff and R. Daroff, eds.) pp. 129-135. Academic Press, San Diego.

Rubinstein, R., Kascsak, R.J., Merz, P.A., and Wisniewski, H.M. 1986. Paired helical filaments associated with Alzheimer disease are readily soluble structures. *Brain Res.* 373:80-88.

Selkoe, D.J. 1994. Normal and abnormal biology of the beta-amyloid precursor protein. *Annu. Rev. Neurosci.* 17:489-517.

Selkoe, D.J. 2003. Folding proteins in fatal ways. *Nature* 426:900-904.

Selkoe, D.J., Ihara, Y., and Salazar, F.J. 1982. Alzheimer's disease insolubility of partially purified helical filaments in sodium dodecyl sulfate and urea. *Science* 215:1243-1245.

Selkoe, D.J., Abraham, C.R., Podlisny, M.B., and Duffy, L.K. 1986. Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease. *J. Neurochem.* 46:1820-1834.

Schagger, H. and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379.

Taylor, J.P., Hardy, J., and Fischbeck, K.H. 2002. Toxic proteins in neurodegenerative disease. *Science* 296:1991-1995.

Temussi, P.A., Masino, L., and Pastore, A. 2003. From Alzheimer to Huntington: Why is a structural understanding so difficult? *EMBO J.* 22:355-361.

Tennent, G.A. 1999. Isolation and characterization of amyloid fibrils from tissue. *Methods Enzymol.* 309:26-47.

Tomidokoro, Y., Lashley, T., Rostagno, A., Neubert, T.A., Bojsen-Moller, M., Braendgaard, H., Plant, G., Holton, J., Frangione, B., Revesz, T., and Ghiso, J. 2005. Familial Danish dementia: Co-existence of ADan and Aβ amyloid subunits in the absence of compact plaques. *J. Biol. Chem.* 280:36883-36894.

Vallet, P.G., Guntern, R., Hof, P.R., Golaz, J., Delacourte, A., Robakis, N.K., and Bouras, C. 1992. A comparative study of histological and immunohistochemical methods for neurofibrillary tangles and senile plaques in Alzheimer's disease. *Acta Neuropathol.* 83:170-178.

Vigo-Pelfrey, C., Lee, D., Keim, P., Lieberburg, I., and Schenk, D.B. 1993. Characterization of β-amyloid peptide from human cerebrospinal fluid. *J. Neurochem.* 61:1965-1968.

von Bergen, M., Li, L., and Mandelkow, E. 2005. Intrinsic fluorescent detection of tau conformation and aggregation. *Methods Mol. Biol.* 299.

Wang, Q., Woltjer, R.L., Cimino, P.J., Pan, C., Montine, K.S., Zhang, J., and Montine, T.J. 2005. Proteomic analysis of neurofibrillary tangles in Alzheimer disease identifies GAPDH as a detergenti-insoluble paired helical filament tau binding protein. *FASEB J.* 19:869-871.

Ward, R.V., Jennings, K.H., Jepras, R., Neville, W., Owen, D.E., Haukins, J., Christie, G., Davis, J.B., George, A., Karran, E.H., and Howlet, D.R. 2000. Fractionation and characterization of oligomeric, protofibrillar and fibrillar forms of β-amyloid peptide. *Biochem. J.* 348:137-144.

Westermark, G.T., Johnson, K.H., and Westermark, P. 1999. Staining methods for identification of amyloid in tissue. *Methods Enzymol.* 309:3-25.

Wisniewski, H.M. and Wen, G.Y. 1985. Substructures of paired helical filaments from Alzheimer's disease neurofibrillary tangles. *Acta Neuropathol.* 66:173-176.

Isolation of *Legionella*-Containing Vacuoles by Immuno-Magnetic Separation

Simon Urwyler,[1] Ivo Finsel,[1] Curdin Ragaz,[1] and Hubert Hilbi[1]

[1]Institute of Molecular Life Sciences, University of Zürich, Zürich, Switzerland

ABSTRACT

The environmental bacterium *Legionella pneumophila* naturally parasitizes free-living amoebae. *L. pneumophila* is an opportunistic human pathogen that grows in macrophages, thus causing a life-threatening pneumonia termed Legionnaires' disease. The bacteria replicate intracellularly in environmental and immune phagocytes within a unique compartment, the *Legionella*-containing vacuole (LCV). Formation of LCVs is a complex and robust process involving >150 secreted bacterial effector proteins, which are believed to subvert host cell signaling and vesicle trafficking pathways. This unit describes a simple approach to purify intact LCVs from *Dictyostelium discoideum* amoebae. The method comprises a two-step purification protocol that includes immuno-magnetic separation by means of an antibody against an effector protein specifically binding to LCVs, followed by density gradient centrifugation. The use of *D. discoideum* producing a fluorescent LCV marker and fluorescently labeled *L. pneumophila* allow tracking the enrichment of LCVs by light microscopy. *Curr. Protoc. Cell Biol.* 46:3.34.1-3.34.14. © 2010 by John Wiley & Sons, Inc.

Keywords: bacterial pathogenesis • *Dictyostelium* • GTPase • macrophage • pathogen vacuole • phagosome • phosphoinositide • vesicle trafficking

INTRODUCTION

Many intracellular pathogens reside and replicate in vacuolar compartments, which are custom-tailored for each invader. A model used for vacuolar pathogens is *Legionella pneumophila*, the causative agent of the severe pneumonia Legionnaires' disease (Fields et al., 2002). *L. pneumophila* is a Gram-negative environmental bacterium that parasitizes free-living amoebae, including the social soil amoeba *Dictyostelium discoideum*. The bacteria grow mechanistically similarly within amoebae and macrophages in a membrane-bound compartment termed the *Legionella*-containing vacuole (LCV; Hilbi et al., 2007). The formation of LCVs is a complex and robust process involving the bacterial Icm/Dot type IV secretion system and >150 secreted effector proteins, which subvert multiple host-cell signaling and vesicle trafficking pathways (Segal et al., 2005; Isberg et al., 2009).

In previous attempts to investigate the process of phagocytosis and the formation of pathogen-containing vacuoles, these compartments were enriched, and their proteome was determined by 2-D gel electrophoresis or liquid chromatography coupled to mass spectrometry. Subcellular vacuoles isolated from mammalian macrophages include latex bead phagosomes (Garin et al., 2001) and vacuoles containing intracellular (pathogenic) microorganisms, such as *Mycobacterium avium* (Sturgill-Koszycki et al., 1994, 1997), *Salmonella enterica* serovar Typhimurium (Mills and Finlay, 1998), *Listeria innocua* (Lührmann and Haas, 2000), *Rhodococcus equi* (Fernandez-Mora et al., 2005), or *Leishmania* parasites (Kima and Dunn, 2005). Furthermore, *D. discoideum* amoebae have been used to enrich phagosomes containing latex beads (Gotthardt et al., 2002, 2006) or *Legionella pneumophila* (Shevchuk et al., 2009).

Published online March 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0334s46

The enrichment protocols employed in these studies were based on density gradient centrifugation using sucrose or iodinated non-ionic materials such as Optiprep (iodixanol) or Histodenz/Nycodenz (iohexol). In these preparations, no specific molecular features of the pathogen vacuoles were exploited, and thus, the discrimination between functionally relevant vacuole components and co-purified irrelevant proteins was difficult. Moreover, due to the lack of fluorescent or other labels, the intact vacuoles had to be visualized by tedious and time-consuming electron microscopy.

To overcome these intrinsic limitations, a purification scheme was developed for pathogen vacuoles, which exploits the presence of a bacterial effector protein that specifically localizes to the compartment to be enriched (Urwyler et al., 2009b). The microbial vacuole marker is detected by a polyclonal antibody, followed by a secondary antibody coupled to magnetic beads. Thus, the pathogen vacuole can be enriched by immuno-magnetic separation using a MACS (magnetic cell sorting) column in the presence of a strong magnet. Furthermore, by using fluorescently labeled bacteria and an ectopically produced fluorescent host cell marker of the pathogen vacuole, the purification can be easily followed by light microscopy rather than by electron microscopy.

D. discoideum producing green fluorescent calnexin-GFP (see Support Protocol 1), a marker of the endoplasmic reticulum (ER) and LCVs, was used, and the amoebae were infected with *L. pneumophila* labeled with the red fluorescent protein DsRed-Express (see Support Protocol 2). After homogenization, green fluorescent LCVs harboring red fluorescent *L. pneumophila* were enriched by immuno-magnetic separation, followed by Histodenz density gradient centrifugation. For characterizing pathogen vacuoles, this approach has a number of advantages: (1) *D. discoideum* grows clonally in tissue culture flasks, and thus, large numbers of identical host cells are available; (2) *L. pneumophila* growing in liquid culture yields a morphologically and metabolically homogenous population of infectious bacteria, which forms relatively homogenous LCVs of ~2 μm in diameter in the amoeba host; (3) the infection can be synchronized and controlled kinetically; (4) intact LCVs can be isolated and purified by a simple and fast two-step protocol, exploiting the presence of markers specific for LCVs; and (5) fluorescently labeled bacteria and host cells allow tracking the LCV purification by light rather than electron microscopy (see Support Protocol 3). Purified LCVs can be readily used for further analysis, including SDS-PAGE/protein staining (*UNITS 6.1 & 6.6*), western blotting (*UNIT 6.2*), immuno-fluorescence microscopy (*UNIT 4.2*), and mass spectrometry.

BASIC PROTOCOL

ISOLATION OF *LEGIONELLA*-CONTAINING VACUOLES

D. discoideum amoebae producing calnexin-GFP (see Support Protocol 1) are infected with DsRed-Express–labeled *L. pneumophila* grown to early stationary phase (see Support Protocol 2) and incubated at 25°C. All subsequent steps are performed at 4°C using buffers, tubes, and equipment pre-cooled to this temperature. The infected *D. discoideum* amoebae are washed and subsequently lysed with a ball homogenizer. For the immuno-magnetic separation step, the homogenate is blocked and incubated with affinity purified primary antibody against a bacterial LCV marker, followed by a secondary antibody coupled to magnetic beads. Antibody decorated LCVs are retained on a magnetic cell sorting (MACS) column by a magnet, eluted upon removal of the magnet, and subjected to density centrifugation through a Histodenz gradient. Purification of LCVs is followed by fluorescence microscopy (see Support Protocol 3).

CAUTION: L. pneumophila is a Biosafety Level 2 microorganism, and thus, the corresponding biosafety regulations need to be observed.

Materials

Dictyostelium discoideum (see Support Protocol 1)
HL5 medium (see recipe)
AYE medium (see recipe)
Legionella pneumophila (see Support Protocol 2)
SorC buffer (see recipe), ice cold
HS buffer (see recipe), cold
Blocking reagent (e.g., NHS: normal human serum; Blutspendezentrum Zürich)
Primary antibody against a bacterial LCV marker (e.g., affinity-purified polyclonal rabbit anti-SidC serum; NeoMPS SA)
Secondary antibody coupled to MACS micro-beads (e.g., MACS goat anti-rabbit IgG micro-beads; Miltenyi Biotec)
Histodenz (Sigma-Aldrich) solution in PBS (see recipe)

75-cm^2 tissue culture flasks
Incubators (23°C, 25°C, 37°C) with and without wheel or shakers
15-ml test tubes
Spectrophotometer
Tissue culture centrifuge with swing-out rotors for plates, flasks, and 15-ml tubes
Plastic cell scraper
3-ml plastic Luer-lok syringes
Stainless-steel ball homogenizer (8-μm clearance, 0.5-ml chamber; Isobiotec), cold
15-ml centrifuge tubes with screw caps
Overhead spinning wheel at 4°C
MACS-MS separation columns (Miltenyi Biotec, cat. no. 130-042-201)
MACS separator (e.g., MACS Multistand; Miltenyi Biotec)
1.5-ml microcentrifuge tubes
Glass Pasteur pipets (230-mm, plugged)

Infect Dictyostelium discoideum with Legionella pneumophila

1. Seed 1×10^7 *D. discoideum* in a 75-cm^2 tissue culture flask and grow overnight in 10 ml HL5 medium at 23°C.

   The yield is ~2×10^7 cells per flask (70% to 80% confluency). Up to three flasks per infection, and a total of 6×10^7 D. discoideum per sample (bacterial strain) are used.

   Importantly, any antibiotic used to grow D. discoideum (e.g., G418; see Support Protocol 1) must be removed prior to infection with L. pneumophila.

2. Inoculate 3 ml AYE/Cm medium in a 15-ml test tube with *L. pneumophila* ($OD_{600} = 0.1$) and grow on a wheel or shaker for 21 to 22 hr at 37°C (final $OD_{600} > 3.0$).

3. Add 100 μl of *L. pneumophila* culture ($OD_{600} = 3.0$) per 75-cm^2 tissue culture flask (from step 1) and gently swirl.

   The multiplicity of infection (MOI) is 100 bacteria per D. discoideum cell.

   An MOI of 100 is appropriate for infections lasting up to 6 hr. Due to intracellular bacterial replication, lower MOIs should be used for longer infections (e.g., an MOI 20 for a 14-hr infection). L. pneumophila does not grow in amoeba medium.

4. Centrifuge the 75-cm^2 tissue culture flask containing *L. pneumophila* and *D. discoideum* 10 min at $500 \times g$, 25°C, to synchronize the infection.

5. Incubate 1 hr at 25°C.

   An incubation period of 1 hr is the standard infection time for the isolation of LCVs (Fig. 3.34.1). However, LCVs have been successfully purified after infections ranging from 15 min up to 14 hr.

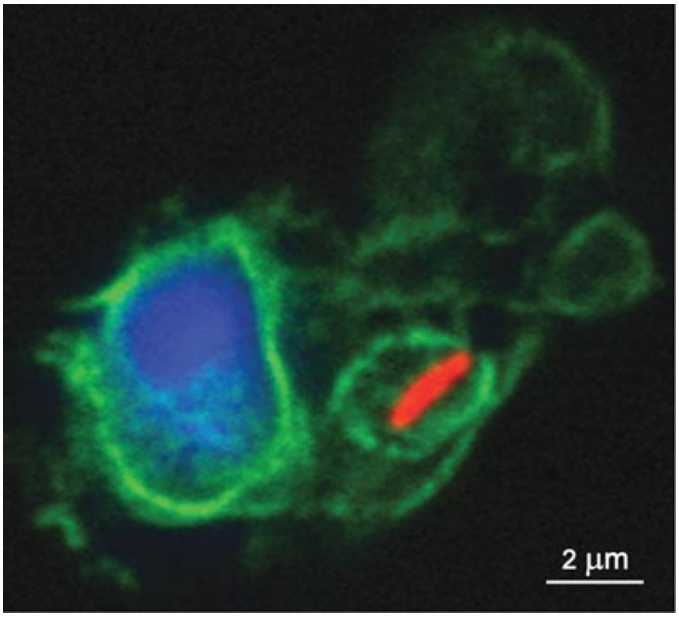


Figure 3.34.1 *D. discoideum* amoebae infected with *L. pneumophila*. *D. discoideum* producing the ER/LCV marker calnexin-GFP (green) was infected with DsRed-Express labeled *L. pneumophila* Philadelphia-1 wild-type strain JR32 (red) at an MOI of 100 and incubated 1 hr at 25°C. The nucleus of *D. discoideum* is stained with DAPI. Image kindly provided by Stefan S. Weber. For color figure go to *http://www.currentprotocols.com/protocol/cb0334*.

Homogenize infected D. discoideum

6. Place infected *D. discoideum* on ice, and wash cells two to three times with 10 ml ice-cold SorC buffer to remove medium and non-phagocytosed bacteria. To wash, replace HL5 medium with SorC buffer and gently agitate without dislocating the cells.

   If the infected D. discoideum cells lift off the tissue culture flask, carefully wash only once.

7. Resuspend up to 2×10^7 infected *D. discoideum* cells in 3 ml ice-cold HS buffer using a plastic cell scraper. Transfer the suspension of infected amoeba in HS buffer into a 3-ml disposable Luer-lok syringe. Set aside 180 µl suspension of infected *D. discoideum* for fluorescence microscopy.

   For 6×10^7 infected D. discoideum, a total of 9 ml suspension is obtained. The protease inhibitor in the HS buffer may be omitted, without significantly changing the results.

8. Rinse ball homogenizer extensively with distilled water before use to remove any contaminating detergent. Flush the chilled ball homogenizer (8-µm clearance) with cold HS buffer and remove any air bubbles with buffer.

9. Mount the syringe containing the infected *D. discoideum* cells, and press the suspension of infected amoebae through the homogenizer into a second syringe. Passage the suspension back and forth seven to eleven times, until the homogenate becomes clear. Keep homogenate on ice. Set aside 180 µl homogenate of infected *D. discoideum* for fluorescence microscopy.

   Use an odd number of strokes to homogenize the suspension; thereby, allowing unbroken cells to remain in the first syringe and not end up in the second syringe (homogenate).

   Before processing a different sample (bacterial strain), dismantle and thoroughly clean the ball homogenizer.

Purify Legionella-containing vacuoles by immuno-magnetic separation

10. Pool homogenates of infected *D. discoideum* (~9 ml per 6×10^7 infected *D. discoideum*) into a 15-ml plastic tube.

11. Add NHS as blocking reagent to a final concentration of 2% (w/v), invert tube, and incubate 30 min on an overhead spinning wheel (10 to 20 rpm) at 4°C.

    The blocking reagent and concentration should be optimized for each primary antibody. Other reagents such as BSA may be more appropriate for other antibodies.

12. Vortex the primary antibody solution prior to use. Add primary antibody directed against a bacterial marker specifically binding to LCVs, invert tube, and incubate 1 hr on an overhead spinning wheel at 10 to 20 rpm, 4°C.

    Affinity-purified polyclonal rabbit antiserum directed against the L. pneumophila effector protein SidC (Weber et al., 2006; Ragaz et al., 2008) was used at a dilution of 1:3000. However, antibodies against other markers exclusively present on LCVs should also be suitable.

13. Centrifuge homogenate 15 min at $600 \times g$, 4°C. Remove supernatant completely, resuspend pellet in 1.5 ml HS buffer per homogenate of 6×10^7 *D. discoideum*, and transfer to a new 15-ml centrifuge tube.

    After the centrifugation step, resuspend only the pellet and not the debris on the wall of the tube.

14. Vortex the micro-beads suspension prior to use. Add secondary antibody coupled to MACS magnetic micro-beads, invert tube, and incubate 30 min on an overhead spinning wheel at 10 to 20 rpm, 4°C.

    MACS goat anti-rabbit IgG micro-beads were used for the polyclonal rabbit antiserum directed against SidC at a concentration of 20 µl magnetic bead slurry per 0.5 ml concentrated homogenate.

15. Place a magnetic MACS-MS separation column (60 µl void volume) into a MACS separator and equilibrate with 0.5 ml ice-cold HS buffer. Allow the buffer to pass through the column by gravity flow.

16. Load the suspension of antibody-treated homogenate (1.5 ml, corresponding to 6×10^7 *D. discoideum*) onto an equilibrated MACS-MS column by gravity flow. Set aside 30 µl of the antibody-treated homogenate for fluorescence microscopy.

17. Wash loaded MACS columns three times with 0.5 ml ice-cold HS buffer by gravity flow. Set aside 30 µl of the flow-through for fluorescence microscopy (Fig. 3.34.2A).

18. Remove loaded MACS columns from the MACS separator (and thus, the magnetic field), and firmly press 0.5 ml ice-cold HS buffer through the column to elute the bound beads linked to LCVs into a 1.5-ml microcentrifuge tube. Set aside 10 µl of the eluate for fluorescence microscopy (Fig. 3.34.2B).

    IMPORTANT NOTE: Apply firm pressure to elute the column; this will substantially increase the yield of LCVs.

Purify Legionella-containing vacuoles by Histodenz gradient centrifugation

19. Prepare a linear gradient of 10% to 35% Histodenz in a total of 11.5 ml PBS. To this end, fill 5.75 ml of 35% Histodenz/PBS into a 15-ml capped centrifuge tube and carefully top with 5.75 ml of 10% Histodenz/PBS without mixing the two solutions. Cap the tube.

20. Gently lay the tube down horizontally for 1 hr and then slowly return it back into a vertical position.

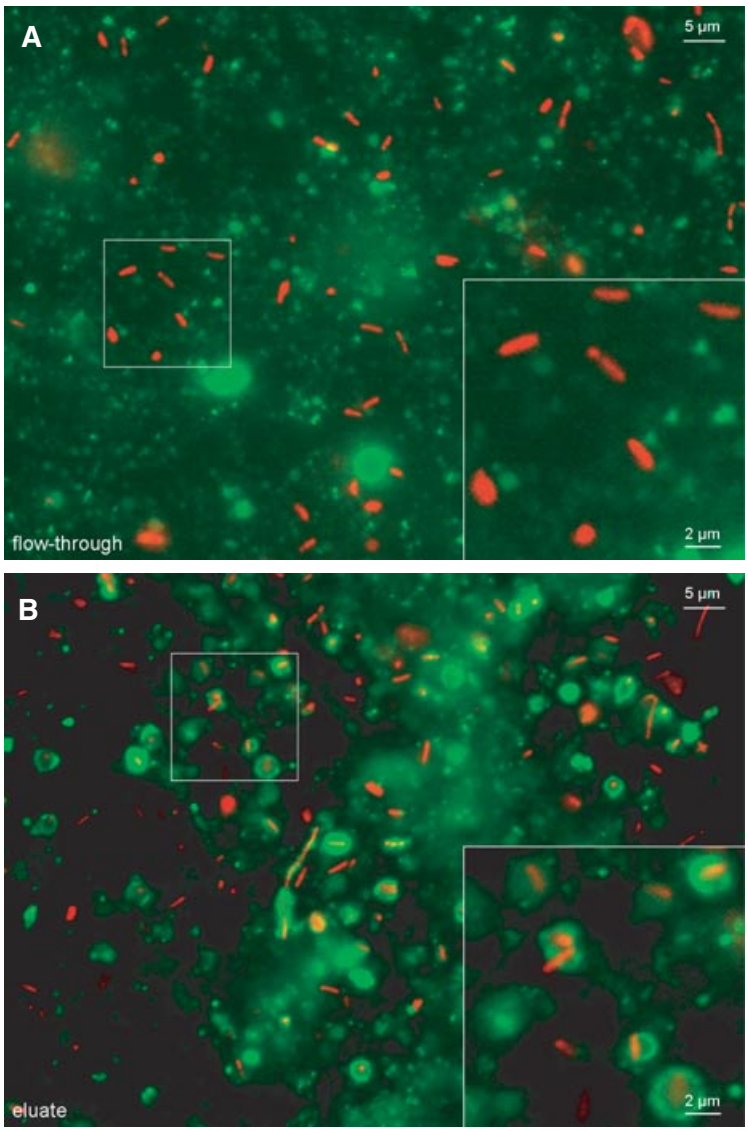


Figure 3.34.2 Immuno-magnetic separation of *Legionella*-containing vacuoles. (**A**) Flow-through and (**B**) eluate of a MACS (magnetic cell separation) column loaded with antibody-treated homogenate of *L. pneumophila*-infected *D. discoideum*. Homogenates of *D. discoideum* producing calnexin-GFP (green) infected with DsRed-labeled *L. pneumophila* (red) were treated with a primary antibody (polyclonal serum) against the bacterial LCV marker SidC and a secondary antibody coupled to magnetic micro beads. Intact LCVs on a MACS column were selectively retained by a magnet and eluted upon removal of the magnet. The insets show magnifications of the marked areas. For color figure go to *http://www.currentprotocols.com/protocol/cb0334*.

21. Load 0.5 ml of LCVs enriched by immuno-magnetic separation (step 18) on top of the 11.5 ml gradient of 10% to 35% Histodenz and centrifuge 1 hr at 3350 × *g*, 4°C.

    If the yield of LCVs after the immuno-magnetic separation step is high, multiple aliquots of 0.5-ml LCV preparation can be loaded onto separate Histodenz gradients.

22. From the bottom of the 15-ml tube holding the 12-ml Histodenz gradient, collect eight 1.5-ml fractions using a Pasteur pipet, and place on ice. Set aside 150 µl of each of the eight Histodenz fractions for fluorescence microscopy.

    Always collect the bottom fraction first. In cases where several tubes are used to separate aliquots of the same sample, the identical fractions can be pooled.

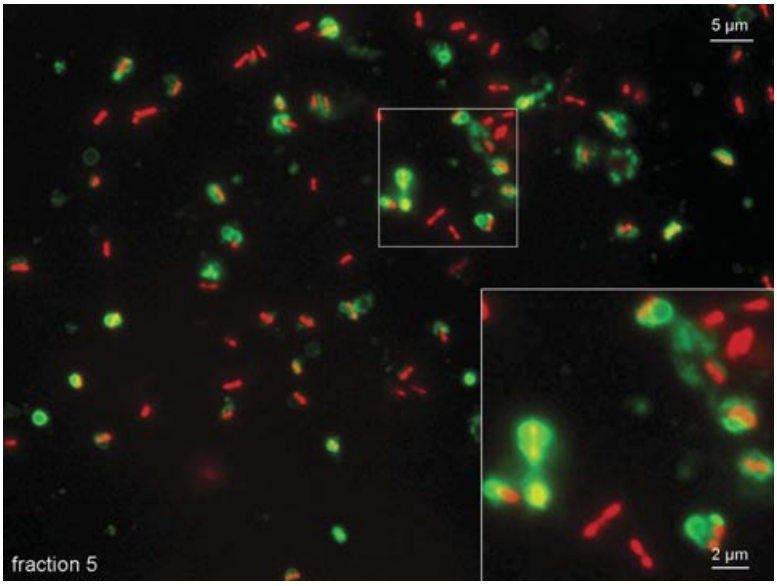


Figure 3.34.3 Density centrifugation of *Legionella*-containing vacuoles. The eluate from a MACS (magnetic cell separation) column was centrifuged through a linear (10% to 35%) Histodenz density gradient and collected in 8 fractions of 1.5 ml. Intact LCVs accumulate in the fractions 4 and 5 (image shown). The inset shows a magnification of the marked area. For color figure go to *http://www.currentprotocols.com/protocol/cb0334*.

To assess the quality of the LCV preparation, smaller volumes (e.g., 30 µl) of the fractions of interest (fractions 4 and 5) are sufficient (Fig. 3.34.3).

23. Follow the enrichment of LCVs by fluorescence microscopy (see Support Protocol 3), examining the reserved fractions.

24. Analyze the purified LCVs (fractions 4 and 5 after Histodenz density centrifugation, step 22) by SDS-PAGE (*UNIT 6.1*)/protein staining (*UNIT 6.6*), western blotting (*UNIT 6.2*), immuno-fluorescence microscopy (*UNIT 4.2*), and mass spectrometry.

GROWTH OF *DICTYOSTELIUM DISCOIDEUM*

SUPPORT PROTOCOL 1

The amoebae used for this protocol are *D. discoideum* wild-type strain Ax3 producing calnexin-GFP, an ER-resident protein that also localizes to LCVs (Müller-Taubenberger et al., 2001; Lu and Clarke, 2005; Weber et al., 2006).

Materials

HL5 medium (see recipe)
G418 (geneticin; Invitrogen)
D. discoideum wild-type strain Ax3 bearing a plasmid for expression of calnexin-GFP (Müller-Taubenberger et al., 2001)
SM/5 agar plates (see recipe)
Klebsiella pneumoniae (grown in LB medium; *APPENDIX 2A*)

75-cm^2 tissue culture flasks
23°C incubator

1. Supplement HL5 medium (see recipe) with 20 µg/ml G418 to maintain the plasmid encoding calnexin-GFP.

The stock concentration of G418 (geneticin) is 20 mg/ml in water. Filter-sterilize 500-µl aliquots using 0.22-µm filters and store upto 2 years at −20°C.

2. Grow *D. discoideum* in 10 ml HL5/G418 medium in 75-cm^2 tissue culture flasks at 23°C, and split the culture two times a week.

3. Prior to infections, split the culture, seed 1×10^7 *D. discoideum* cells in a 75-cm^2 tissue culture flask and allow growth overnight in 10 ml HL5 medium without antibiotic at 23°C, to yield $\sim 2 \times 10^7$ cells per flask.

   Prior to infection, the D. discoideum cells should have reached a confluency of 80%; a higher cell density will negatively affect the uptake efficiency. To this end, the inoculum and the growth time of the amoebae may need to be adjusted. Any antibiotic used to grow D. discoideum (e.g., G418) must be removed prior to infection with L. pneumophila.

4. For optional viability assays, plate *D. discoideum* cells on SM/5 agar plates together with *Klebsiella pneumoniae* (grown in LB medium; *APPENDIX 2A*), and count plaque-forming units (pfu) after 3 to 4 days incubation at 23°C (Sussman, 1987).

   D. discoideum grows axenically (in absence of bacteria) in a complex medium (e.g., HL5) at 21° to 23°C. Above 25°/26°C, the amoebae will respond with a heat shock reaction and eventually die. D. discoideum is light sensitive, and therefore, frequent alternation between light and dark should be avoided (turn on light in incubator, if possible).

SUPPORT PROTOCOL 2

GROWTH OF *LEGIONELLA PNEUMOPHILA*

The bacteria used for this method are *L. pneumophila* wild-type strain JR32 Philadelphia-1, producing the red fluorescent protein DsRed-Express encoded on plasmid pSW001 (Mampel et al., 2006). An OD_{600} of 0.3 corresponds to 2×10^9 bacteria/ml.

CAUTION: L. pneumophila is a Biosafety Level 2 microorganism, and thus, the corresponding biosafety regulations must be observed.

Materials

Frozen glycerol stocks of *Legionella pneumophila* bearing Ds-Red Express on plasmid pSW001
Charcoal yeast extract (CYE) agar plates (see recipe)
Chloramphenicol (Cm)
ACES yeast extract (AYE) broth (see recipe)

37°C incubator with and without shaker
15-ml test tubes
Spectrophotometer

1. Streak out *L. pneumophila* from frozen glycerol stocks onto a CYE agar plate containing 5 μg/ml chloramphenicol to maintain plasmid pSW001. Incubate 3 days at 37°C (*L. pneumophila* grown to stationary growth phase).

   The stock concentration of Cm is 30 mg/ml ethanol. Filter-sterilize aliquots and store at −20°C.

2. Prepare AYE broth and freshly add 5 μg/ml Cm.

3. Inoculate 3 ml AYE/Cm medium in a 15-ml test tube with *L. pneumophila* scraped from CYE agar plates ($OD_{600} = 0.1$), and allow to grow on a wheel (or shaker) for 21 to 22 hr (final $OD_{600} > 3.0$) at 37°C.

   L. pneumophila bacteria grown to post-exponential/early stationary growth phase in liquid AYE medium are morphologically uniform (~2 × 0.5 μm), i.e., the proportion of long, filamentous L. pneumophila (>20 μm) is much smaller than in bacterial cultures grown on CYE agar plates. The morphology of the bacteria can be easily checked by light microscopy using a small volume (10 μl) of the bacterial culture. The final OD_{600} should not be <3.0, otherwise the infection efficiency is severely compromised.

   The glassware used should not be contaminated with residual detergents, as L. pneumophila is very sensitive.

4. As a control for *L. pneumophila* viability, plate 20 µl of a 10^5/ml bacterial solution on CYE agar plates and count colony forming units (cfu) after incubation for 3 days at 37°C.

ANALYSIS OF LCV PURIFICATION BY FLUORESCENCE MICROSCOPY

SUPPORT PROTOCOL 3

The purification of LCVs formed by DsRed-Express-labeled *L. pneumophila* upon infection of *D. discoideum* producing calnexin-GFP can be followed by epifluorescence microscopy. The enrichment is quantified by counting (100×) intact LCVs per field of view.

Materials

0.1% (w/v) poly-L-lysine sterile solution (Sigma)
D. discoideum Infected with *L. pneumophila* (see Basic Protocol)
HS medium (see recipe), ice cold
4% (w/v) paraformaldehyde in PBS (see recipe)
SorC buffer (see recipe)
Mounting medium (e.g., Vectashield; Vector Laboratories)

Round microscope coverslips, sterile
24-well flat-bottomed tissue culture plate
Microscope slides
Epifluorescence microscope equipped with filters for GFP and Ds-Red

1. Coat a sterile, round microscopy coverslip with poly-L-lysine (sterile 0.01% solution), and place it into the well of a 24-well flat-bottomed tissue culture plate.
2. Prepare samples of adherent *D. discoideum* infected with *L. pneumophila* (see Basic Protocol, steps 1 to 5; Fig. 3.34.1) by seeding cells directly onto the coated coverslips.

   The infection efficiency should be checked by fluorescence microscopy prior to proceeding with the purification protocol.

3. Add samples containing suspensions of infected *D. discoideum* or cell-free LCVs [e.g., from Basic Protocol, steps 7, 9, 16 to 18 (Fig. 3.34.2), and 22 (Fig. 3.34.3)] to the poly-L-lysine-coated coverslips in the wells. Fill wells containing samples from step 22 with 1 ml of ice-cold HS buffer to dilute high Histodenz concentrations and centrifuge 15 min at 600 × *g*, 4°C.
4. Carefully remove supernatant, add 0.5 ml/well 4% paraformaldehyde in PBS and incubate 30 min at 4°C.
5. Wash coverslip two times with 0.5 ml/well SorC buffer each time, removing the supernatant.
6. Mount coverslip on microscope slide using mounting medium (e.g., Vectashield).
7. Analyze the morphology, integrity, and quantity of LCVs per field of view with an epifluorescence microscope equipped with filters for GFP and Ds-Red.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AYE medium (Feeley et al., 1979)

Prepare in 1 liter water the following:
55 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)

continued

10 g Bacto yeast extract (Difco)
3.3 mM L-cysteine
0.6 mM $Fe(NO_3)_3 \cdot 9H_2O$

Dissolve ACES and yeast extract in 900 ml H_2O. While stirring, slowly add 10 ml cysteine, followed by 10 ml iron nitrate. Adjust pH to 6.9 with 10 M KOH, and add H_2O to 1 liter. Filter six to eight times through a glass fiber filter and filter-sterilize afterwards (500-ml Stericup; Millipore). Store up to 1 year at 4°C in the dark. If required, add filter-sterilized chloramphenicol (final concentration: 5 μg/ml; 30 mg/ml stock concentration in ethanol) immediately before use.

CYE agar plates

Prepare in 1 liter water the following:
55 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)
10 g Bacto yeast extract (Difco)
2 g activated charcoal powder (puriss. p.a.)
3.3 mM L-cysteine
0.6 mM $Fe(NO_3)_3 \cdot 9H_2O$
15 g agar (Serva)

Suspend ACES, yeast extract, charcoal, and agar in 1 liter water. Adjust pH to 6.9 with 10 M KOH and autoclave. Allow to cool to ~50°C, and add filter-sterilized cysteine as well as iron nitrate in 10 ml water each. If required, add filter-sterilized chloramphenicol (final concentration: 5 μg/ml; 30 mg/ml stock concentration in ethanol). Pour plates, allow them to dry for 1 day, and store up to 6 months inverted at 4°C.

Histodenz (10% or 35%) solution

Dissolve 10 g or 35 g Histodenz (Sigma-Aldrich) in PBS (*APPENDIX 2A*) to an end volume of 100 ml. Store up to 2 years at 4°C in the dark.

HL5 medium (Watts and Ashworth, 1970)

Prepare in 1 liter water the following:
2.5 mM KH_2PO_4
2.5 mM Na_2HPO_4
11 g glucose
5 g BBL yeast extract (Becton Dickinson)
5 g Bacto proteose peptone (Becton Dickinson)
5 g BBL thiotone E peptone (Becton Dickinson)
Adjust pH to 6.5 ± 0.1 with 1 M NaOH or 1 M HCl
Autoclave and store 1 year at 4°C

HS buffer

20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)
250 mM sucrose
0.5 mM ethylene glycol tetraacetic acid (EGTA)
Complete, EDTA-free protease inhibitor cocktail tablet (Roche)
Adjust to pH 7.2 with 1 M KOH
Filter sterilize using a 0.22-μm filter (do not autoclave)
store 6 months at 4°C

(Optional) Add protease inhibitor cocktail tablet freshly according to the manufacturer's instructions.

Paraformaldehyde, 4% (w/v)

Wear gloves and mask and weigh 40 g paraformaldehyde (Sigma-Aldrich) in a chemical fume hood. Dissolve in 1 liter PBS (*APPENDIX 2A*), while constantly stirring and heating the solution to 50°C. Store up to 2 years at −20°C.

SM/5 agar plates

Prepare in 1 liter water the following:
14 mM KH_2PO_4
5.7 mM K_2HPO_4
0.4 mM $MgSO_4 \cdot 7H_2O$
0.2 g Bacto yeast extract (Difco)
2 g Bacto peptone (Oxoid)
2.2 g glucose
Adjust pH with 1 M KOH or 1 M HCl to 6.9, and then add 15 g agar (Serva)
Autoclave and pour plates after cooling to ∼50°C
Allow plates to dry for 1 day and store up to 6 months inverted at 4°C

SorC buffer (Sörensen phosphate buffer; Malchow et al., 1972)

15 mM KH_2PO_4
2 mM Na_2HPO4
50 μM $CaCl_2$
Adjust to pH 6.0 with 1 M KOH or 1 M HCl
Autoclave and store up to 2 years at room temperature

COMMENTARY

Background Information

The social soil amoeba *D. discoideum* is haploid and genetically tractable. Many genetic and cell biological tools are available, including defined mutants and strains stably producing GFP fusion proteins. *D. discoideum* is a phagocytic predator that naturally feeds on bacteria. However, a number of environmental bacteria avoid being digested and instead grow within the amoebae. *L. pneumophila* is such an amoebae-resistant bacterium, and *D. discoideum* has been established as a valuable model system to analyze the virulence of this pathogen on a cellular and molecular level (Hägele et al., 2000; Solomon et al., 2000). As an example, *D. discoideum* has been used to investigate the role of phosphoinositide (PI)-metabolizing enzymes during uptake and intracellular replication of *L. pneumophila* (Weber et al., 2006, 2009), as well as to study the recruitment kinetics to LCVs of several small GTPases fused to GFP (Urwyler et al., 2009b).

The mechanism of intracellular replication of *L. pneumophila* within free-living protozoa (*D. discoideum* and other amoeba) and metazoan macrophages is very similar. Indeed, an analysis of *D. discoideum* phosphoinositide (PI) 5-phosphatases implicated in intracellular replication and LCV formation revealed that the *D. discoideum* PI 5-phosphatase Dd5P4 inhibits bacterial growth, and Dd5P4 as well as its mammalian ortholog OCRL1 are recruited to LCVs via an N-terminal domain (Weber et al., 2009). In agreement with a similar function of these PI 5-phosphatases during LCV formation in protozoan and mammalian phagocytes, OCRL1 is also recruited to the LCV membrane in macrophages. These findings illustrate the usefulness of *D. discoideum* as a model to analyze the virulence of *L. pneumophila* and other human pathogenic bacteria (Hilbi et al., 2007).

Further underscoring the similarity of LCV formation within amoeba and macrophages, the protocol outlined here was successfully employed to enrich intact LCVs from murine RAW264.7 macrophage-like cells (data not shown). The protocol used was identical to the Basic Protocol, except that the addition of the protease inhibitor cocktail prior to homogenization and a constant temperature of 4°C during the immuno-magnetic separation were essential for a successful enrichment. Based on these results, the protocol described here is likely useful (with some appropriate modifications) for the isolation of vacuoles established by other pathogens in *D. discoideum* or

macrophages. The immuno-magnetic separation method should, in principle, be applicable for any marker exclusively present on a distinct pathogen vacuole. To this end, the optimal concentration and conditions will have to be determined for each antibody.

To analyze the proteome of purified pathogen vacuoles, powerful liquid chromatography-tandem mass spectrometry techniques are available (Urwyler et al., 2009b). Given the ease and speed of vacuole purification and further proteome analysis, the immuno-magnetic separation method is likely useful for comparative proteomics. It would be of interest to analyze and compare proteomes of vacuoles harboring distinct bacterial mutant strains, or proteomes of vacuoles formed by the same bacterial strain in different isogenic *D. discoideum* mutant strains.

Critical Parameters

To isolate intact LCVs from *D. discoideum* infected with *L. pneumophila*, a number of parameters are critical for a high yield and purity. Prior to an infection, the *D. discoideum* cells should have grown to a confluency of $\leq$80%, since a higher cell density will decrease the uptake efficiency of bacteria. *L. pneumophila* liquid cultures should consist of a mostly homogenous population of small rod-shaped cells ($\sim 2 \times 0.5$ μm). A uniform bacterial culture is a prerequisite for the formation of a homogenous population of LCVs. Furthermore, antibiotics used to grow *D. discoideum* interfere with a productive infection by *L. pneumophila* and must be removed.

For the infection, some points must also be considered. To synchronize the infection, the centrifugation force should not exceed 500 × *g*. Since the plate holders mounted onto the swing-out rotor are not in a fully horizontal position, an MOI gradient will be formed. At high MOIs, the infected *D. discoideum* cells tend to lift off the tissue culture flasks, and this process is promoted upon increasing the *g*-force during centrifugation. Along the same line, the infected amoebae must be washed very gently to prevent the loss of too many cells.

Critical for the homogenization and immuno-magnetic separation steps is that even traces of detergent will lyse the LCV membrane. Therefore, prior to use, care should be taken to rinse all equipment and glassware well with distilled water or buffer. Moreover, the application of pressure while eluting the MACS column will increase the yield of purified LCVs substantially. If the problem of a low yield persists, perform Basic Protocol, steps 6 through 21, at 4°C and in the presence of a protease inhibitor to minimize the proteolytic degradation of the surface marker targeted by the primary antibody for immuno-magnetic separation.

Anticipated Results

Approximately 6×10^7 *D. discoideum* amoebae grown in three tissue culture flasks are infected with *L. pneumophila* wild-type JR32 at an MOI of 100. After an incubation period, the infected amoebae are lysed, and intact LCVs are purified in a two-step process, involving immuno-magnetic separation and density centrifugation. The purification by magnetic immuno-separation yields $\sim 1 \times 10^6$ intact LCVs per 1×10^7 infected amoebae. LCVs harboring wild-type *L. pneumophila* are about seven-fold enriched in the eluate compared to the flow-through (Urwyler et al., 2009b). In contrast, LCVs harboring an *L. pneumophila sidC-sdcA* mutant strain do not accumulate in the eluate, indicating that the enrichment using an anti-SidC antibody is strictly dependent on the bacterial LCV marker SidC. LCVs eluting from the MACS column are further purified by centrifugation through a cushion of a linear 10% to 35% gradient of Histodenz. The majority of the LCVs accumulated in fractions 4 and 5 of a total of 8 fractions at a ratio of $\sim 2 \times 10^5$ intact LCVs per 1×10^7 infected amoebae (Urwyler et al., 2009b). Thus, using the protocol described here, the overall yield of purified LCVs is $\sim 1 \times 10^6$ intact LCVs per 6×10^7 infected *D. discoideum* cells.

Purified LCVs tested positive for markers of different subcellular compartments, such as the ER (calnexin-GFP), mitochondria (porin), and *L. pneumophila* SidC, while some markers of the Golgi apparatus and late endosomes were not detected. The proteome of purified LCVs is determined by reversed-phase micro-capillary liquid chromatography (RP-μLC) electrospray ionization tandem mass spectrometry (ESI MS/MS). This approach reveals the presence of >560 host proteins (Urwyler et al., 2009b). In the LCV proteome, many mitochondrial proteins, as well as proteins localizing to phagocytic vesicles or the ER-Golgi compartment and close to 70 ribosomal components, were identified. These findings are in agreement with the notion that LCVs sequentially interact with mitochondria, smooth vesicles, and rough vesicles/ER. A number of proteins previously not recognized to be associated with LCVs are

also identified. These include the coat protein clathrin and several small GTPases or GTPase-associated proteins (Arf1, Rab1a, -1d, -7a, -8a, -14, RabC, RabGDI, RasG, RanA, RanGAP, RhoGDI). Only Arf1 and Rab1, which promote the formation of LCVs, were previously identified on this compartment. To confirm the results obtained upon analyzing the LCV proteome by LC-MS/MS, GFP fusion proteins of Rab1a, Rab7a, Rab8a, and Rab14 in *D. discoideum* were produced and it was found that the proteins selectively localize to LCVs harboring wild-type but not *icmT* mutant *L. pneumophila*. In summary, these findings indicate that LCVs not only communicate with the early secretory pathway, but also with the late, endosome-dependent secretory pathway, as well as with the early and late endocytic pathway (Urwyler et al., 2009a).

Time Considerations

The two-step vacuole isolation protocol outlined here takes ~5 to 6 hr (post-infection) to be completed, and thus is straight-forward and rapid.

Literature Cited

Feeley, J.C., Gibson, R.J., Gorman, G.W., Langford, N.C., Rasheed, J.K., Mackel, D.C., and Baine, W.B. 1979. Charcoal-yeast extract agar: Primary isolation medium for *Legionella pneumophila*. *J. Clin. Microbiol.* 10:437-441.

Fernandez-Mora, E., Polidori, M., Lührmann, A., Schaible, U.E., and Haas, A. 2005. Maturation of *Rhodococcus equi*-containing vacuoles is arrested after completion of the early endosome stage. *Traffic* 6:635-653.

Fields, B.S., Benson, R.E., and Besser, R.E. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin. Microbiol. Rev.* 15:506-526.

Garin, J., Diez, R., Kieffer, S., Dermine, J.F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., and Desjardins, M. 2001. The phagosome proteome: Insight into phagosome functions. *J. Cell Biol.* 152:165-180.

Gotthardt, D., Warnatz, H.J., Henschel, O., Bruckert, F., Schleicher, M., and Soldati, T. 2002. High-resolution dissection of phagosome maturation reveals distinct membrane trafficking phases. *Mol. Biol. Cell* 13:3508-3520.

Gotthardt, D., Blancheteau, V., Bosserhoff, A., Ruppert, T., Delorenzi, M., and Soldati, T. 2006. Proteomics fingerprinting of phagosome maturation and evidence for the role of a G_{α} during uptake. *Mol. Cell Proteomics* 5:2228-2243.

Hägele, S., Kohler, R., Merkert, H., Schleicher, M., Hacker, J., and Steinert, M. 2000. *Dictyostelium discoideum*: A new host model system for intracellular pathogens of the genus *Legionella*. *Cell Microbiol.* 2:165-171.

Hilbi, H., Weber, S.S., Ragaz, C., Nyfeler, Y., and Urwyler, S. 2007. Environmental predators as models for bacterial pathogenesis. *Environ. Microbiol.* 9:563-575.

Isberg, R.R., O'Connor, T.J., and Heidtman, M. 2009. The *Legionella pneumophila* replication vacuole: Making a cosy niche inside host cells. *Nat. Rev. Microbiol.* 7:13-24.

Kima, P.E. and Dunn, W. 2005. Exploiting calnexin expression on phagosomes to isolate Leishmania parasitophorous vacuoles. *Microb. Pathog.* 38:139-145.

Lu, H. and Clarke, M. 2005. Dynamic properties of *Legionella*-containing phagosomes in *Dictyostelium* amoebae. *Cell Microbiol.* 7:995-1007.

Lührmann, A. and Haas, A. 2000. A method to purify bacteria-containing phagosomes from infected macrophages. *Methods Cell Sci.* 22:329-341.

Malchow, D., Nagele, B., Schwarz, H., and Gerisch, G. 1972. Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. *Eur. J. Biochem.* 28:136-142.

Mampel, J., Spirig, T., Weber, S.S., Haagensen, J.A.J., Molin, S., and Hilbi, H. 2006. Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. *Appl. Environ. Microbiol.* 72:2885-2895.

Mills, S.D. and Finlay, B.B. 1998. Isolation and characterization of *Salmonella typhimurium* and *Yersinia pseudotuberculosis*-containing phagosomes from infected mouse macrophages: *Y. pseudotuberculosis* traffics to terminal lysosomes where they are degraded. *Eur. J. Cell Biol.* 77:35-47.

Müller-Taubenberger, A., Lupas, A.N., Li, H., Ecke, M., Simmeth, E., and Gerisch, G. 2001. Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. *EMBO J.* 20:6772-6782.

Ragaz, C., Pietsch, H., Urwyler, S., Tiaden, A., Weber, S.S., and Hilbi, H. 2008. The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cell Microbiol.* 10:2416-2433.

Segal, G., Feldman, M., and Zusman, T. 2005. The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. *FEMS Microbiol. Rev.* 29:65-81.

Shevchuk, O., Batzilla, C., Hägele, S., Kusch, H., Engelmann, S., Hecker, M., Haas, A., Heuner, K., Glockner, G., and Steinert, M. 2009. Proteomic analysis of *Legionella*-containing phagosomes isolated from *Dictyostelium*. *Int. J. Med. Microbiol.* 299:489-508.

Solomon, J.M., Rupper, A., Cardelli, J.A., and Isberg, R.R. 2000. Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of

host-pathogen interactions. *Infect. Immun.* 68:2939-2947.

Sturgill-Koszycki, S., Schlesinger, P., Chakraborty, P., Haddix, P., Collins, H., Fok, A., Allen, R., Gluck, S., Heuser, J., and Russell, D. 1994. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* 263:637-639.

Sturgill-Koszycki, S., Haddix, P.L., and Russell, D.G. 1997. The interaction between *Mycobacterium* and the macrophage analyzed by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* 18:2558-2565.

Sussman, M., 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* 28:9-29.

Urwyler, S., Brombacher, E., and Hilbi, H. 2009a. Endosomal and secretory marker of the *Legionella*-containing vacuole. *Commun. Integr. Biol.* 2:107-109.

Urwyler, S., Nyfeler, Y., Ragaz, C., Lee, H., Mueller, L.N., Aebersold, R., and Hilbi, H. 2009b. Proteome analysis of *Legionella* vacuoles purified by magnetic immunoseparation reveals secretory and endosomal GTPases. *Traffic* 10:76-87.

Watts, D.J. and Ashworth, J.M. 1970. Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* 119:171-174.

Weber, S.S., Ragaz, C., Reus, K., Nyfeler, Y., and Hilbi, H. 2006. *Legionella pneumophila* exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. *PLoS Pathog.* 2:E46.

Weber, S.S., Ragaz, C., and Hilbi, H. 2009. The inositol polyphosphate 5-phosphatase OCRL1 restricts intracellular growth of *Legionella*, localizes to the replicative vacuole and binds to the bacterial effector LpnE. *Cell Microbiol.* 11:442-460.

Isolation of Platelet Granules

Juliane Nießen,[1] Gabriele Jedlitschky,[1] Andreas Greinacher,[2] and Heyo K. Kroemer[1]

[1]Department of Pharmacology, Research Center of Pharmacology and Experimental Therapeutics, Ernst-Moritz-Arndt-University, Greifswald, Germany
[2]Department of Immunology and Transfusion Medicine, Ernst-Moritz-Arndt-University, Greifswald, Germany

ABSTRACT

Functional analysis of platelet intracellular structures requires isolation and purification of these cellular compartments. With regard to the function of platelets, both, dense (delta) and alpha granules are relevant target structures. However, the availability of sufficient purification protocols for these structures is rather limited. This unit describes two protocols for isolation and purification of platelet granule structures. The Basic Protocol describes a new technique based on immunolabeling with target-specific antibodies followed by magnetic sorting, whereas the Alternate Protocol describes the more traditional procedure based on differential centrifugation and density-based sedimentation. For both methods, the degree of granule purification can be most easily determined by immunoblotting using various antibodies that recognize structure-specific proteins. The immunomagnetic sorting method is especially good for studies requiring highly purified material (e.g., for the identification of specific transporters and receptors). *Curr. Protoc. Cell Biol.* 46:3.35.1-3.35.14. © 2010 by John Wiley & Sons, Inc.

Keywords: human platelets • subcellular fractionation • magnetic sorting • sucrose density gradient • granule-structures • immunopurification

INTRODUCTION

Besides red blood cells, platelets are the second anuclear blood compartment. They have a crucial role in primary hemostasis, as well as in a variety of other physiological processes. To fulfill these functions, platelets contain a variety of mediators, which are stored in intracellular vesicles or granules. Using morphologic criteria, three secretory granule classes are distinguished: alpha granules, delta or dense granules, and lysosome-like granules (Fig. 3.35.1; Bentfeld and Bainton, 1975; Fukami and Salganicoff, 1977; White, 1994). Both types of granules and the lysosomes contain specific mediators and molecules. While small molecules as ADP, ATP, GTP, GDP, calcium, and serotonin are stored in the dense granules, polypeptides like von Willebrand Factor (vWF), fibrinogen, thrombospondin, and platelet factor 4 are found in α-granules. Lysosomes contain a number of specific hydrolases. The localization of secretory substances within the platelets is of paramount importance for understanding platelet function. To study the underlying processes, methods for subfractionation of human platelets resulting in highly purified fractions are required. The membrane of the granules and the plasma membrane also contain specific proteins as receptors and transport proteins. For example, the lysosome-associated membrane glycoprotein 2 (LAMP2) is mainly located on dense granules and lysosomes, whereas P-selectin is specific for the membrane of α-granules, and the surface glycoproteins GPIb and GPIIb/IIIa are marker proteins for the plasma membrane (Fig. 3.35.1). The organelle-specific membrane proteins are the basis for the subfractionation by immunomagnetic sorting (Basic Protocol), as depicted in Figure 3.35.2A. Alternatively, the organelles can be separated by centrifugation in a density gradient (Alternate Protocol). Here, the subfractions form zones according to their density (Fig. 3.35.2B).

Current Protocols in Cell Biology 3.35.1-3.35.14, March 2010
Published online March 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0335s46

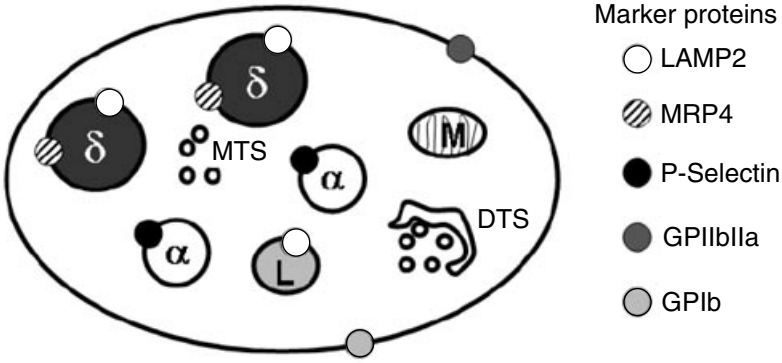


Figure 3.35.1 Scheme of the cellular compartments in a platelet and the marker proteins in the respective membrane. α, δ, L: alpha, dense, and lysosomal granules; MTS: microtubular system; DTS: dense tubular system.

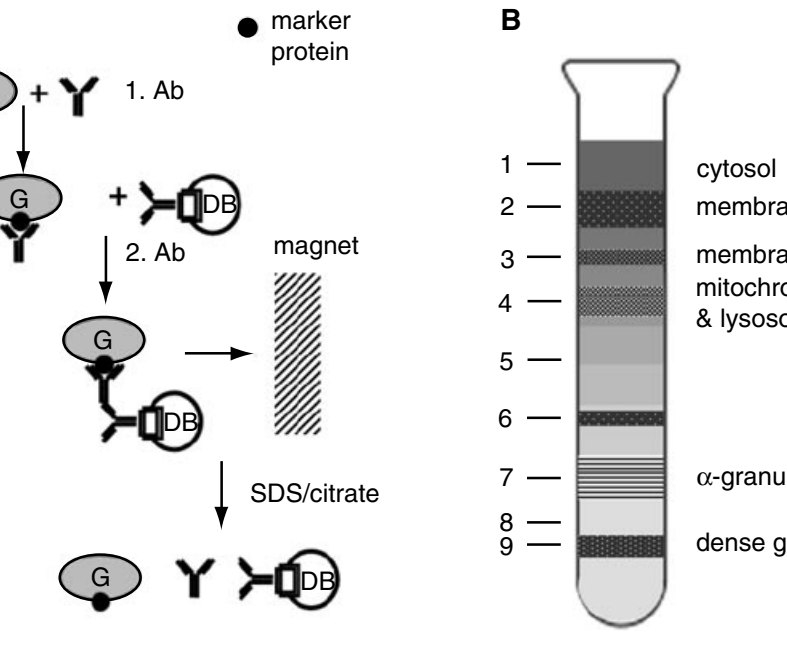


Figure 3.35.2 Biophysical principles of the two separation methods. (**A**) Immunomagnetic sorting: Platelet lysate including the granules (G) is incubated with the primary antibody (1. Ab). This binds to a marker protein on the target granule. Dynabeads (DB) coated with the secondary antibody (2.Ab) are added to form the Dynabead-antibody-organelle complex. This complex is extracted by a magnet. Separation of the Dynabead-antibody-organelle-complex is achieved by SDS (Support Protocol 1) or mild citrate elution (Support Protocol 2). (**B**) Sucrose density gradient separation (modified scheme according to Broekman, 1992). Nine zones are indicated. Visible bands/zones can be isolated and are enriched in cytosol (1), plasma membrane (2,3), mitochondria and lysosomes (4), alpha granules (7) and dense granules (9).

STRATEGIC PLANNING

General

Two major platelet granule isolation procedures, based on the methods published by Broekman (1992) and Niessen et al. (2007) are described in this unit. Both of these utilize platelet homogenates made from platelet concentrates. The protocols differ mainly in the biophysical principles. In the protocol according to Broekman (1992; see Alternate

Protocol), granules are separated by centrifugation of the cell homogenate in a sucrose density gradient. This procedure is based on previous work published by Marcus et al. (1966), Siegel et al. (1971), Klempner et al. (1980), and Kjeldsen et al. (1999). In contrast, Basic Protocol (Niessen et al., 2007) is based on immunomagnetic sorting. This technique separates the subcellular fractions based on granule-specific marker proteins, thereby avoiding problems associated with separating structures of similar sizes or densities compared with the density gradient technique. Platelet granules purified by the density gradient method retain fraction losses and mixed fractions (Zhang et al., 2003; Lawson et al., 2006). However, depending on the further application as well as the yield and the purity required, the reader may select the Basic Protocol or the Alternate Protocol. The degree of the granule purification can be easily determined today by immunoblot using various antibodies that recognize granule-specific proteins (Support Protocols 1 and 2). The assessment of platelet granule purity based on marker enzyme activities or on the evaluation of the fractions by electron microscopy represent older alternative methods (Broekman et al., 1992).

The very high sensitivity of platelets to various physical and chemical factors is linked to the specific functions of these cells (Warren et al., 1975). The presence of a contractile apparatus, similar to the actomyosin complex in muscles, in platelets enables them to change the shape of their surface actively from a smooth disc to a contoured sphere, with the formation of pseudopodia (Bottechia et al., 1977). This property makes it difficult to isolate platelets in an inactivated form, i.e., in the morphological and functional state in which they circulate in the blood stream. Successful preparation of platelet subcellular structures requires knowledge and experience with platelet isolation and platelet handling.

A prerequisite for both fractionation protocols is the preparation of platelet-rich plasma (PRP) from blood samples according to an established standard protocol (e.g., Greinacher et al., 1991). Briefly, platelets are obtained by double-arm cytapheresis, in which adenine-citrate dextrose is used as an anticoagulant. The resulting platelet concentrate is filtered via a leukocyte depletion filter (99.999% reduction of leukocytes). By cell counting using a Nagotte chamber or flow cytometry, leukocyte contaminations should be $<1/\mu l$. If even higher purity of platelets is desired, (e.g., for concomitant RNA studies), the leukocyte-depleted platelet concentrate can again be filtered over a second leukocyte filter.

Homogenization of the platelets is achieved in both protocols given below by repeated cycles of freezing and rapid thawing shock. Alternatively, nitrogen cavitation ("French press") may be applied as described by Broekman (1992).

ISOLATION OF PLATELET GRANULES BY IMMUNOMAGNETIC SORTING

BASIC PROTOCOL

In this protocol, platelet granules are isolated using compartment-specific antibodies.

This protocol will take 1 to 2 days. All samples, buffers, and equipment must be maintained at 4°C. Because of the risk of platelet activation, the initial centrifugation steps must be performed without brake.

Materials

Adenine-citrate-dextrose-anticoagulant (ACD-A; code RDB8652, Baxter Healthcare)
Platelet-rich plasma (PRP)
Washing buffer A (see recipe)
Apyrase (Sigma)
Hirudin (Pharmion)

Tyrode buffer (see recipe)
Washing buffer B (see recipe)
Homogenization buffer (see recipe)
1000× protease inhibitors (stock solution, see recipe) or Boehringer Complete protease inhibitor (Roche Applied Science)
Liquid nitrogen
Tris/sucrose buffer (see recipe)
Target-specific primary antibody (see Table 3.35.1)
Dynabeads M-280 Sheep anti-rabbit IgG (Invitrogen)
Dynabeads Pan anti-mouse IgG (Invitrogen)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Centrifuge
10-ml polystyrol centrifuge tubes
37°C incubator
Refrigerated Hettich Rotixa 50 RS Centrifuge (Hettich) with 4256 swing-out rotor (or equivalent)
Loose-fitting Dounce homogenizer, 15-ml capacity (Wheaton)
Beckman ultracentrifuge with 70.1Ti rotor and corresponding polycarbonate ultracentrifuge tubes (12-ml or equivalent)
Tight-fitting Dounce homogenizer, 15-ml capacity (Wheaton)
Teflon pestle glass homogenizer (Glas-Col)
DynaMag-2 magnet (Invitrogen)

NOTE: Carry out all procedures at 4°C using solutions prechilled to that temperature.

Wash the platelets

1. Add 111 μl/ml ACD-A and 5 μl/ml of 1000 U/ml apyrase to PRP in a 12-ml polystyrol tube. Centrifuge 7 min at 650 × *g*, room temperature, without brake.
2. Discard the supernatant and wash platelets three times, each time with 10 ml washing buffer A containing 5 U/ml apyrase and 2 U/ml hirudin.
3. Incubate platelets for 15 min at 37°C.
4. Centrifuge platelets 7 min at 650 × *g*, room temperature, without brake.
5. Discard the supernatant and resuspend the platelets in ∼2 ml Tyrode buffer to obtain 300,000 platelets/μl.

Prepare the platelet homogenate

6. Add 4 ml of washing buffer B to the platelet suspension and centrifuge 10 min at 2800 × *g*, 4°C.
7. Resuspend the pellet in 1 to 2 ml of homogenization buffer supplemented with 1× protease inhibitors (stock solution diluted 1:1000).
8. Lyse the platelet suspension by repeated cycles of rapid freezing (in liquid nitrogen) and thawing (5 times) and bring it to a final volume of 10 ml with ice-cold Tris-sucrose buffer supplemented with 1× protease inhibitors.
9. Homogenize the lysate with 30 strokes of a loose-fitting Dounce homogenizer.
10. Transfer the homogenate to a 15-ml tube. Centrifuge the platelet homogenate 5 min at 90 × *g*, 4°C, to remove cell debris. Following the centrifugation, transfer the supernatant into a 12-ml ultracentrifuge tube.
11. Ultracentrifuge the supernatant 30 min at 100,000 × g, 4°C, with centrifuge acceleration and brake set to the maximum.

12. Resuspend the pellet in 10 to 15 ml Tris-sucrose buffer and homogenize with 30 strokes of a tight-fitting Dounce homogenizer. Transfer the homogenate into a 12-ml ultracentrifuge tube.
13. Ultracentrifuge the homogenate 30 min at 100,000 × g, 4°C, with centrifuge acceleration and brake set to the maximum.
14. Resuspend the pellet in 1.5 to 2 ml Tris-sucrose buffer. Transfer to the homogenizer and homogenize with 20 strokes of a Teflon pestle glass homogenizer.

The obtained crude preparation of granules and plasma membrane can be shock-frozen in liquid N_2 and stored in aliquots at –80°C.

Perform immunomagnetic separation

15. Add the target-specific (alpha- and dense-granule, plasma membrane) primary antibody (see Table 3.35.1 for antibodies with best performance for immunomagnetic isolation) to the platelet pellet homogenates (100 µg per reaction batch) in 1/100 dilution (for all used primary antibodies) and bring it to a final volume of 1 ml with ice-cold PBS supplemented with 1× protease inhibitors.
16. Incubate the platelet-primary antibody homogenate for 1.5 hr at 4°C, with gentle tilting and rotation of the tube.
17. Ultracentrifuge 30 min at 100,000 × g, 4°C.
18. Discard the supernatant. Resuspend the pellet by gentle repeated pipetting and bring it to a final volume of 1 ml with ice-cold PBS buffer supplemented with 1× protease inhibitors.
19. Ultracentrifuge 30 min at 100,000 × g, 4°C.
20. Discard the supernatant. Resuspend the pellet by repeated pipetting and bring it to a final volume of 1 ml with ice-cold PBS supplemented with 1× protease inhibitors.
21. Add 50 µl of coated Dynabeads and incubate for 1.5 hr at 4°C with gentle tilting and rotation.

Table 3.35.1 Antibodies Used for Immunomagnetic Isolation and Immunoblotting of Platelet Granules[a]

Antigen	Name and isotype	Reactivity	Performance with		Supplier
			Immunomagnetic isolation	Immunoblotting	
MRP4	SNG rabbit	Human	(X)	X	NC
	M_4I-10 rat	Human	NT	X	Axxora
GPIb	Gi27 mouse	Human	X	(X)	NC
GPIIb/IIIa	Gi5 mouse	Human	X	(X)	NC
P-selectin	CD62P mouse	Human	X	(X)	Beckman Coulter
LAMP2	Mouse	Human	(X)	X	Santa Cruz
GPIb	CD42Iβ goat	Human	NT	X	Santa Cruz
vWF	Goat	Human	NT	X	Santa Cruz
PDI	Mouse	Human	(X)	X	ABCAM
P-selectin	Goat	Human	(X)	X	Santa Cruz

[a]Abbreviations: X, best performance; (X), satisfactory performance; NT, not tested; NC, not for sale; PDI, protein disulfide isomerase.

22. Place the sample in the magnetic field for 2 min, thereafter discard the liquid phase and remove the tube from the magnet.
23. Resuspend the Dynabead fraction in ice-cold PBS with protease inhibitors and bring it to a final volume of 1 ml.
24. Repeat steps 22 and 23 two times.
25. Elute the target from the magnetic bead complex (see Support Protocol 1 or 2).

ELUTE TARGET PROTEIN FROM THE MAGNETIC BEAD-COMPLEX

Conventional elution methods can be applied for the elution of target structure from the Dynabeads. One of the major advantages using Dynabeads in subcellular isolation is the ability to elute in small volumes. Boiling the bead-target complex in SDS-PAGE application buffer for direct characterization of proteins using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is the most efficient elution method. Also low pH (2.8 to 3.5; Support Protocol 2), change in ionic strength, affinity elution, electrophoresis, polarity reducing agents, or deforming eluants can be applied. The method of choice depends on the affinity of the immunoglobulins for the specific target protein, stability of the target structures, and the downstream applications and detection methods. Most complexes will be eluted at pH 3.1. Some protein and organelle functionality might be lost under these conditions. If maintaining functionality is important, milder elution conditions such as high salt (e.g., 2 M NaCl) or stepwise elution, reducing pH from 6 down to 3 can be used. This is also recommended if the bead-bound antibody must remain functional to allow reuse of the Dynabeads. Here, the denaturing method with SDS and the milder citrate elution are described as possible techniques for the elution of the target subcellular fraction from the magnetic bead–complex.

If the target protein to be investigated is in the range of a molecular weight of 50 or 100 kDa, covalent binding of the Fc-part of the IgG by a hydrazide reaction to the bead reduces the load of IgG (heavy chain) on the gel.

SUPPORT PROTOCOL 1

Elute Target Protein from the Magnetic Bead Complex by Denaturing Elution

This method of eluting the target protein from the complex is used for subsequent direct characterization of proteins by SDS-PAGE.

Materials

Platelet fraction–Dynabead complex (see the Basic Protocol)
4% (w/v) SDS (*APPENDIX 2A*)

95°C heating apparatus
DynaMag-2 magnet
−80°C freezer

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

1. Resuspend the pelleted Dynabead complex (Basic Protocol) in 0.5 ml 4 % (w/v) SDS.
2. Mix well by tilting and rotation for 2 min.
3. Boil 5 min at 95°C for elution of the bead-target complex.
4. Place the tube in the magnetic field for 2 min.

5. Recover the liquid phase and remove the tube from the magnet.

   The supernatant now contains the respective subcellular structure and the denatured antibodies.

6. Again, add 0.5 ml 4% (w/v) SDS to the beads to elute any remaining bead-target complexes.
7. Mix well by tilting and rotation for 2 min.
8. Place the tube on a magnet and pool the supernatants containing the purified target structure.
9. Store fractions in 50-µl aliquots at −80°C.
10. For further characterization, perform SDS-PAGE (*UNIT 6.1*).

    NOTE: *Dynabeads cannot be reused after elution with denaturing agents.*

Elute Target Protein from the Magnetic Bead Complex by Mild Elution with Citric Acid

SUPPORT PROTOCOL 2

This protocol is used for milder elution conditions that allow reuse of the Dynabeads.

Materials

Target protein–Dynabead complex (see the Basic Protocol)
0.1 M citric acid
1 M NaOH
DynaMag-2 magnet
−80°C freezer

1. Add 0.5 ml 0.1 M citric acid to the pelleted Dynabead complex (Basic Protocol).
2. Mix well by tilting and rotating for 2 min.
3. Place the test tube on a magnet and transfer the supernatant, containing the purified subcellular structure and immunoglobulins to a clean tube.
4. Again, add 0.5 ml 0.1 M citric acid to the beads to elute any remaining ligands.
5. Mix well by tilting and rotating for 2 min.
6. Place the test tube on a magnet, recover the eluate, and pool the supernatants.
7. Using 1 M NaOH, adjust the pH to 7.0-7.4 and store in 50-µl aliquots at −80°C.

   Dynabeads can be reused at least five times after this elution. For re-use, the beads should immediately be brought to neutral pH after elution using PBS/BSA (5%) buffer and should be stored in this buffer.

ISOLATION OF PLATELET GRANULES USING A DENSITY GRADIENT

ALTERNATE PROTOCOL

This protocol will take 1 to 2 days to complete. All procedures, buffers, and equipment must be maintained at 4°C to perform the purification. Due to the risk of platelet activation, the initial centrifugation steps must be performed without brake.

Materials

Platelet-rich plasma (PRP)
Washing buffer B (see recipe)
Homogenization buffer (see recipe)
1000× protease inhibitors (stock solution; see recipe) or Boehringer Complete protease inhibitor (Roche Applied Science)

Tris/sucrose buffer (see recipe)
60% (w/v) sucrose solution in 5 mM EDTA, pH 7.4

Refrigerated Hettich Rotixa 50 RS centrifuge (Hettich) with 4256 swing out rotor (or equivalent)
Ultrasonic processor UW60 (Bandelin Electronic)
Loose-fitting Dounce homogenizer, 15-ml capacity (Wheaton)
15-ml centrifuge tubes
Ultracentrifuge (Beckmann) with 70.1Ti fixed-angle rotor and a swing-out rotor (Beckmann SW40 or Sorvall TH-641) and corresponding polycarbonate ultracentrifuge tubes (or equivalent)
Tight-fitting homogenizer Dounce, 15-ml capacity (Wheaton)
Teflon pestle glass homogenizer (Glas-Col)

NOTE: Carry out all procedures at 4°C using solutions prechilled to that temperature, except when noted otherwise.

Wash the platelets

1. Wash the platelet-rich plasma as described in Basic Protocol (steps 1 to 5).

Prepare platelet homogenate

2. Suspend 2 ml washed platelets in 4 ml of washing buffer B in a 15-ml tube and centrifuge 10 min at 2800 × *g*, 4°C.

3. Resuspend the pellet in 1 to 2 ml of homogenization buffer supplemented with 1× protease inhibitors.

4. Lyse the platelet suspension by repeated cycles of rapid freezing and thawing (5 times).

5. *Optional:* To achieve complete homogenization, sonicate platelet suspension using an ultrasonic processor two times (4°C, 3 sec of sonication followed by a 15-sec period on ice that allows the sample to chill down).

6. Bring the homogenate to a final volume of 10 ml with ice-cold Tris/sucrose buffer supplemented with 1× protease inhibitors.

7. Transfer the homogenate to a 15-ml Dounce homogenizer with loose-fitting pestle. Homogenize using 15 strokes.

8. Transfer the homogenate to a 15-ml tube and centrifuge the platelet homogenate 5 min at 90 × g, 4°C.

9. Collect the supernatant in a new tube.

Perform sucrose density gradient separation

10. Prepare sucrose solutions with concentrations stepwise decreasing from 60% to 30% sucrose (60%, 55%, 50%, 45%, 40%, 35%, and 30%) by diluting the 60% sucrose stock solution with 5 mM EDTA.

    IMPORTANT NOTE: *The 60% sucrose solution should be used at room temperature, to decrease its viscosity!*

11. Prepare a stepwise sucrose gradient in an ultracentrifuge tube for the swing-out rotor by layering 1 ml of each sucrose concentration in decreasing order (from the bottom up) over a cushion of an extra 0.5 ml of 60% (w/w) sucrose/EDTA.

    The cushion prevents pelleting of the heaviest fraction (enriched in dense granules) to the bottom of the gradient tube.

12. Leave this stepwise gradient at 4°C overnight to allow diffusion to result in an approximately linear gradient by the following day.

    Alternatively, a commercial time-saving gradient-forming apparatus (e.g., cat. no. 350052, Spinco Division, Beckman Instrument) may be used for formation of continuous gradients.

13. Carefully layer 3 to 4 ml of platelet homogenate onto the linear gradient.

14. Place the gradient tubes in the swing-out rotor and ultracentrifuge for 90 min at 200,000 × *g*, 4°C.

15. Following ultracentrifugation, identify the nine bands as indicated in Figure 3.35.2B. Collect the bands by carefully pipetting from the top.

    Commercial fraction collection equipment can also be used.

16. Dilute fractions to a final volume of 8 ml each with ice-cold Tris/sucrose buffer and homogenize with 30 strokes of a tight-fitting homogenizer.

17. Centrifuge 30 min at 100,000 × *g*, 4°C, using the 70.1Ti (fixed-angle) rotor.

18. Resuspend the pellet in 1.5 to 2 ml of Tris/sucrose buffer and homogenize with 20 strokes of Teflon pestle-glass homogenizer.

    The fractions can be stored in 50-µl aliquots at −80°C.

CHARACTERIZATION OF PLATELET SUBCELLULAR FRACTIONS BY IMMUNOBLOT ANALYSIS

SUPPORT PROTOCOL 3

The Basic Protocol and Alternate Protocol yield subcellular fractions in which the respective organelles are highly enriched over the platelet homogenate. To determine the degree of purification, fraction-specific assays and markers should be utilized to determine the contamination by other organelles. Today the purity of the fractions can be easily determined by immunoblot using various organelle-specific antibodies. Formerly, the efficacy of the purification procedures was confirmed by electron microscopy or by assaying specific marker enzymes, as described by Broekman (1992).

Immunoblot analysis (*UNIT 6.2*) is a standard procedure and therefore is not described in detail in this unit. An example is given in Niessen et al. (2007). Briefly, 50 µg protein of each fraction—protein concentrations of subcellular fractions can be analyzed e.g., by the BCA method (Smith et al., 1985)—is resuspended in SDS-PAGE sample buffer (see recipe) and loaded on a 7.5% sodium dodecyl sulfate–polyacrylamide gel after denaturation at 95°C for 5 min. Immunoblotting to PVDF or nitrocellulose membranes should be performed using a tank blotting system (Bio-Rad or equivalent). The membrane can be blocked with 5% (w/v) dry milk and 5% (v/v) fetal bovine serum (FBS) in Tris-buffered saline containing 0.05% (v/v) Tween 20 and 1% (w/v) BSA (TBST). Primary antibodies (see Table 3.35.1, antibodies with best performance for immunoblotting) are diluted in TBST to the following final concentrations: MRP4 serum 1:1000 (alternatively commercially available mouse monoclonal antibody 1:500), anti-PDI 1:1000; anti-LAMP2, polyclonal P-selectin, and anti-vWF 1:500; and anti-GPIbβ 1:50. Secondary horseradish peroxidase–conjugated goat anti-rabbit and goat anti-mouse IgG antibodies (e.g., Bio-Rad) and horse anti-goat IgG antibody (e.g., Vector Laboratories) are used at 1:2000 dilutions. Signals are detected on X-ray film using an enhanced chemiluminescence detection system (G.E. Healthcare). Results can be quantified by densitometric analysis (e.g., software Image Quant 5.0, Molecular Dynamics), as shown in Figure 3.35.3.

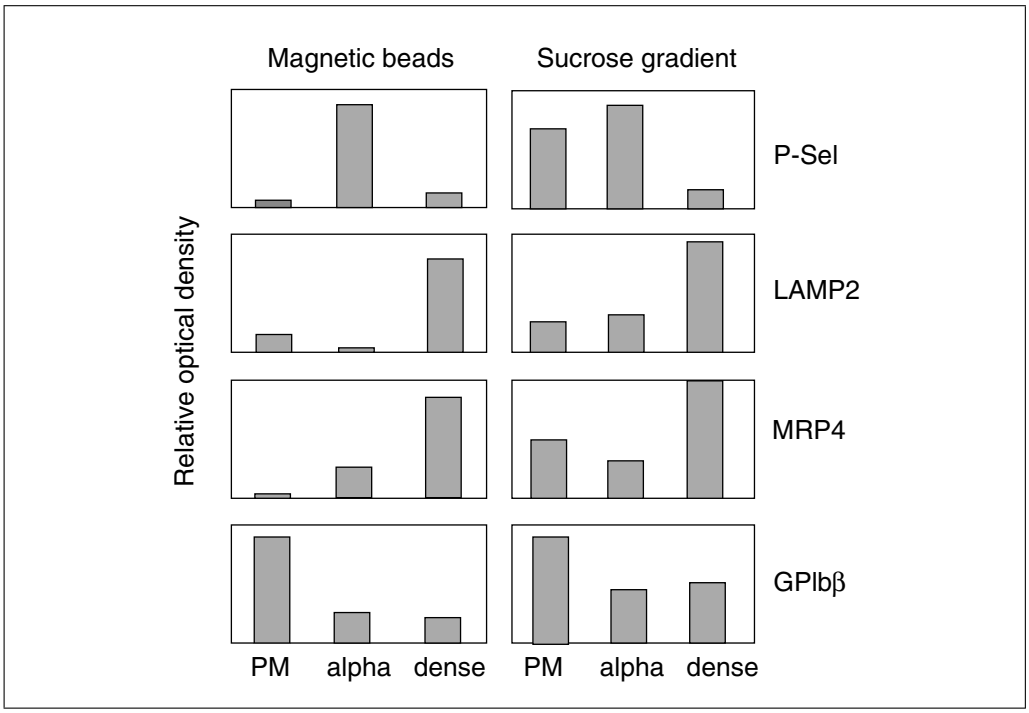


Figure 3.35.3 An example of analysis of platelet subcellular fractions. Platelet subcellular fractions are separated by sucrose density gradient or by immunomagnetic sorting. Equal protein amounts of each fraction are separated by SDS-PAGE and transferred to a PVDF membrane. The blots are probed with antibodies against fraction-specific markers: anti-P-selectin antibody for the alpha granules, anti-LAMP2 and anti-MRP4 for the dense granules, and an antibody against the GPIbβ receptor for the outer plasma membrane (PM). The results are quantified by densitometric analysis and the immunodetection in each fraction is here expressed as percentage of the sum of the optical densities of the respective proteins in all fractions (relative optical density). For original data and blots, see Jedlitschky et al. (2004) and Niessen et al. (2007).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Homogenization buffer

100 mM KCl
25 mM NaCl
2 mM $MgSO_4$
12 mM sodium-citrate
10 mM glucose
25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]
5 mM ATP
0.35% (w/v) bovine serum albumin (BSA)
Adjust pH to 7.0 with 1 M HCl or 1 M NaOH
Filter sterilize
Store for only a few days at 4°C

Protease inhibitors, 1000×

Prepare the following together in H_2O:
5 mg/ml leupeptin
10 mg/ml aprotinin
Store up to 3 months at −20°C

continued

Separately prepare the following stocks fresh:
1 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol
Add to solutions at 1× concentration as recommended in the protocols

PMSF is very sensitive to water, so it should be added fresh to the solutions just before contact with biological material.

SDS-PAGE sample buffer (4× Laemmli buffer)

0.25 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
1.92 M glycine
8% (w/v) SDS
5% (v/v) 2-mercaptoethanol
0.005% (w/v) bromphenol blue
Store up to 1 month at room temperature

Tris/sucrose buffer

10 mM Tris ultra [tris(hydroxymethyl)aminomethane]
250 mM sucrose
Adjust pH to 7.4 using 1 M HCl or 1 M NaOH
Filter sterilize
Store for a few days at 4°C

Tyrode buffer

150 mM NaCl
12 mM $NaHCO_3$
2.5 mM KCl
1 mM $MgCl_2$
2 mM $CaCl_2$
5.5 mM D-glucose
1 mg/ml BSA
Adjust pH to 7.4 with 1 M HCl or 1 M NaOH
Filter sterilize
Store for only a few days at 4°C

Washing buffer A

150 mM NaCl
12 mM $NaHCO_3$
2.5 mM KCl
1.5 mM NaH_2PO_4
5.5 mM D-glucose
1 mg/ml bovine serum albumin (BSA)
Adjust pH to 6.3 with 1 M HCl or 1 M NaOH
Filter sterilize
Store for only a few days at 4°C

Washing buffer B

0.9% (w/v) NaCl
10 mM tetrasodium EDTA
0.2% (w/v) BSA added just before use
Prepare fresh

COMMENTARY

Background Information

Platelets circulate in the blood of mammals and are involved in hemostasis, leading to the formation of blood clots. These anuclear blood cells are also surprisingly multifunctional and play a fundamental role in many physiological and pathophysiological processes including hemostasis and thrombosis, clot retraction, vessel constriction and repair, inflammation including promotion of atherosclerosis, host defense, even tumor growth/metastasis. They are also a natural source of growth factors (Bottechia et al., 1977; Rodgers, 1999; Michelson, 2002; Sunitha and Munirathnam, 2008). One of the central platelet functions is the so-called release reaction where material stored in granules is secreted into the extracellular medium on exposure of the platelets to stimuli like thrombin, collagen, or high levels of ADP (Davey and Luscher, 1968; Keenan and Solum, 1972; Koutts et al., 1978; Kaplan et al., 1979; Zucker et al., 1979).

The high mediator concentrations inside the platelet granules, as ADP in dense granules (0.6 M), suggest the localization of active transport proteins in the granular membrane. The multidrug-resistance protein MRP4 (ABCC4) represents such an active pump localized in the granule membrane (Jedlitschky et al., 2004). Furthermore, transport proteins (Niessen et al., 2009) and membrane receptors like GPIIb/IIIa or GPIb in the platelet plasma membrane play a crucial role in platelet function.

While the molecular consistence of subcellular platelet structures are of high physiological, therapeutic, and pharmacological interest, detailed analyses of these structures are difficult as no easily applicable method has been developed to obtain highly purified fractions of the respective structures. Traditional fractionation protocols make use of physical properties of intracellular organelles and membranes such as their density and a density gradient ultracentrifugation of platelet cell homogenates can be performed. Gogstad characterized a procedure with respect to recovery of intact granules using a French pressure cell (nitrogen cavitation) to prepare a granule-rich homogenate followed by further purification of the granules by centrifugation in metrizamide gradients (Gogstad, 1980). For preparation of dense granules, a method using mepacrine-labeled granules isolated by a short metrizamide gradient was described (Rendu et al., 1982). Fukami and Salganicoff (1977) presented a modified procedure with a pretreatment of human platelets with metabolic inhibitors to prevent activation before French press homogenization, which led to the isolation of dense granules in an overall yield of ~20%. Furthermore, a method for isolating platelet plasma membranes using polylysine-coated beads has been described (Jacobson and Branton, 1977; Kinoshita et al., 1979). With this method, surface membranes of cells attach to the polylysine beads and, after cell disruption, relatively pure preparations of plasma membranes remain attached to the beads.

The most common methods for subcellular fractionation consist mainly of a homogenization step e.g., by nitrogen cavitation and, after elimination of remaining cell debris, ultracentrifugation in a sucrose gradient (Marcus et al., 1966; Klempner et al., 1980, Broekman, 1992; Kjeldsen et al., 1999). Siegel et al. (1971) compared the ultracentrifugation in Ficoll and sucrose density gradients and found the sucrose gradient as the preferable one, because of its better ability to form complexes with cellular components. Given the overlap in size and density of the different granules, fraction losses and mixed fractions are common problems with techniques based on these attributes (Zhang et al., 2003; Lawson et al., 2006). Therefore, this unit describes a new method based on immunomagnetic sorting (Niessen et al., 2007) and, in addition, a protocol based on a common sucrose density gradient. The new purification method is based on immunolabeling with target-specific antibodies and magnetic sorting (Niessen et al., 2007) and has already been used to separate and isolate different cells and other cellular compartments like mitochondria (Safarik and Safarikova, 2004; Lawson et al., 2006). In contrast to the methods mentioned above, which discriminate between the different fractions based on their density, this technique separates the fractions based on specific marker proteins.

Critical Parameters

The two platelet granule isolation protocols described here differ in complexity and granule yield and quality due to differences in the technique. The density gradient method is relatively time- and labor-intensive, in which one of the important problems is the small difference in density between the respective structures. Therefore, the resulting fractions are often of lesser purity because of

cross-contamination by other compartments (Zhang et al., 2003; Lawson et al., 2006). In fact, even within well-defined and adequately separated fractions, the admixture of organelles from adjacent zones is frequently observed. Furthermore, high-density sucrose solutions put an osmotic stress on organelles, and repeated and prolonged centrifugations must be avoided. Many, if not most, organelles are rather labile structures which are particularly easily affected by a disturbed osmotic equilibrium.

Concerning the immunomagnetic sorting, the selection of the primary antibodies is the most important factor for successful subcellular fractionation. Some antibodies showed reduced antigen-binding efficacy when coated onto beads, even though they show clear signals when used in other immunological assays like immunofluorescence. So, a prerequisite of this technique is applicable and target-specific antibodies. Not insignificant disadvantages that have been identified so far are the expensive price of magnetic beads, which can be a limiting factor for isolating large quantities and problems with the disintegration of the Dynabead-protein complex, depending on the further applications.

Anticipated Results

The outcome of both purification methods of platelet granules should be an enrichment of platelet granule markers to some magnitudes over the starting homogenate. Although these protocols are well established, users should check the purity of platelet granules after the purification procedure. A good record of volumes and protein concentrations of all different fractions should be maintained. The preparations yielded by the protocols described here have been analyzed for the presence of contaminant membranes using immunoblot analysis (Fig. 3.35.3; original data are taken from Jedlitschky et al., 2004 and Niessen et al., 2007). In general, a higher fraction purity was observed by immunomagnetic sorting (as indicated from the immunoblot data). As indicated by immunoblotting for the lysosomal marker beta-glucuronidase (not shown), only a minor contamination of the dense fraction with lysosomes in the immunomagnetic sorting using LAMP2 as separating antibody was observed. It should be noted that for accurate immunoblot analysis it is important to maintain sample integrity throughout the procedure. Always maintain protease inhibitors in all buffers and store fractions at −80°C.

Time Considerations

Platelet granule preparation by sucrose density gradient centrifugation is relatively time-consuming (~8 hr). In comparison to this technique, the Dynabead method requires less time (~4 hr). The preparation of washed platelets, as well as platelet homogenates is a prerequisite for both methods and requires ~2 to 3 hr. The preparation of a stepwise sucrose density gradient and leaving this at 4°C to get an approximately linear gradient requires an overnight step. With a commercial gradient-forming apparatus for continuous gradient formation, time can be saved. The main separation steps in both methods are manageable in 3 to 4 hr. Finally, the characterization of the obtained subcellular fractions by immunoblot analysis with fraction-specific antibodies needs another day. Platelet subcellular granule fractions can be stored at −80°C for a reasonably long time (>1 year).

Literature Cited

Bentfeld, M.E. and Bainton, D.F. 1975. Cytochemical localization of lysosomal enzymes in rat megakaryocytes and platelets. *J. Clin. Invest.* 56:1635-1649.

Bottechia, D., Fantin, G., and Nassuato, G. 1977. Platelets: A Multidisciplinary Approach (G. de Gaetano and S. Garratini, eds.). p. 111. Raven Press, New York.

Broekman, M.J. 1992. Homogenization by nitrogen cavitation technique applied to platelet subcellular fractionation. *Methods Enzymol.* 215:21-32.

Davey, M.G. and Luscher, E.F. 1968. Release reaction of human platelets induced by thrombin and other agents. *Biochim. Biophys. Acta* 165:490-506.

Fukami, M.H. and Salganicoff, L. 1977. Human platelet storage organelles. *Thromb. Haemostasis.* 38:963-970.

Gogstad, G.O. 1980. A method for the isolation of alpha-granules from human platelets. *Thromb. Res.* 20:669-681.

Greinacher, A., Michels, I., Kiefel, V., and Mueller-Eckhardt, C. 1991. A rapid and sensitive test for diagnosing heparin-associated thrombocytopenia. *Thromb. Haemost.* 66:734-736.

Jacobson, B.S. and Branton, D. 1977. Plasma membrane: Rapid isolation and exposure of the cytoplasmic surface by use of positively charged beads. *Science* 195:302-304.

Jedlitschky, G., Tirschmann, K., Lubenow. L.E., Nieuwenhuis. H.K, Akkerman. J.W, Greinacher. A., and Kroemer, H.K. 2004. The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage. *Blood* 104:3603-3610.

Kaplan, K.L., Broekman, M.J., Chernoff, A., Lesznik, G.R., and Drillings, M. 1979. Platelet α-granule proteins: Studies on release and subcellular localization. *Blood* 53:604-608.

Keenan, J.P. and Solum, N.O. 1972. Quantitative studies on the release of platelet fibrinogen by thrombin. *Brit. J. Haematol.* 23:461-466.

Kinoshita, T., Nachman, R.L., and Minick, R. 1979. Isolation of human platelet plasma membranes with polylysine beads. *J. Cell. Biol.* 82:688-696.

Kjeldsen, L., Sengelov, H., and Borregaard, N. 1999. Subcellular fractionation of human neutrophils on Percoll density gradients. *J. Immunol. Methods* 232:131-143.

Klempner, M.S., Mikkelsen, R.B., Corfman, D.H., and André-Schwartz, J. 1980. Neutrophil plasma membranes. I. High-yield purification of human neutrophil plasma membrane vesicles by nitrogen cavitation and differential centrifugation. *J. Cell. Biol.* 86:21-28.

Koutts, J., Walsh, P.N., Plow, E.F., Fenton, J.W., Bouma, B.N., and Zimmermann, T.S. 1978. Active release of human platelet factor VIII-related antigen by adenosin diphosphate, collagen and thrombin. *J. Clin. Invest.* 62:1255-1263.

Lawson, E.L., Clifton, J.G., Huang, F., Li, X., Hixson, D.C., and Josic, D. 2006. Use of magnetic beads with immobilized monoclonal antibodies for isolation of highly pure plasma membranes. *Electrophoresis* 27:2747-2758.

Marcus, A.J., Zucker-Franklin, D., Safier, L.B., and Ullman, H.L. 1966. Studies on human platelet granules and membranes. *J. Clin. Invest.* 45:14-28.

Michelson, A.D. 2002. Platelets. Academic Press, San Diego.

Niessen, J., Jedlitschky, G., Grube, M., Bien, S., Strobel, U., Ritter, C.A., Greinacher, A., and Kroemer, H.K. 2007. Subfractionation and purification of intracellular granule-structures of human platelets: An improved method based on magnetic sorting. *J. Immunol. Methods* 328:89-96.

Niessen, J., Jedlitschky, G., Grube, M., Bien, S., Schwertz, H., Ohtsuki, S., Kawakami, H., Kamiie, J., Oswald, S., Starke, K., Strobel, U., Siegmund, W., Rosskopf, D., Greinacher, A., Terasaki, T., and Kroemer, H.K. 2009. Human platelets express organic anion-transporting peptide 2B1, an uptake transporter for atorvastatin. *Drug Metab. Dispos.* 37:1129-1137.

Rendu, F., Lebret, M., Nurden, A.T., and Caen, J.P. 1982. Initial characterization of human platelet mepacrine-labelled granules isolated using a short metrizamide gradient. *Br. J. Haematol.* 52:241-251.

Rodgers, G.M. 1999. Overview of platelet physiology and laboratory evaluation of platelet function. *Clin. Obstet. Gynecol.* 42:349-359.

Safarik I. and Safarikova M. 2004. Magnetic techniques for the isolation and purification of proteins and peptides. *Biomagn. Res. Technol.* 2:7.

Siegel, A., Burri, P.H., Weibel E.R., Bettex-Galland, M., and Lüscher, E.F. 1971. Density gradient centrifugation and electron microscopic characterization of subcellular fractions from human blood platelets. *Thromb. Diath. Haemorrh.* 25:252-267.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.

Sunitha, R. and Munirathnam, N. 2008. Platelet-rich fibrin: Evolution of a second-generation platelet concentrate. *Indian J. Dent. Res.* 19:42-46.

Warren, B.A., Valcs, O., and Khan, S. 1975. Platelets. *In* Recent Advances in Basic Research and Clinical Aspects (P. Elutin, ed.) pp. 43. Amsterdam.

White, J.G. 1994. Anatomy and structural organization of the platelet. *In* Hemostasis and Thrombosis Basic Principles and Clinical Practice, 3rd ed. (R.W. Colman, J. Hirsch, V.J. Marder, E.W. Salzmann, H.R. Roberts, and J.B. Lefkowitz, eds.) pp. 397-413. Lipincott, Philadelphia.

Zhang, W., Zhou, G., Zhao, Y., White, M., and Zhao, Y., 2003. Affinity enrichment of plasma membrane for proteomic analysis. *Electrophoresis* 24:2855-2863.

Zucker, M.B., Broekman, M.J., and Kaplan, K.L. 1979. Factor VIII related antigen in human blood platelets. Localization and release by thrombin and collagen. *J. Lab. Clin. Med.* 94:675-682.

Isolation of Nucleoli

Sabine Hacot,[1] Yohann Coute,[2] Stéphane Belin,[1] Marie Alexandra Albaret,[1] Hichem C. Mertani,[1] Jean-Charles Sanchez,[3] Manuel Rosa-Calatrava,[1] and Jean-Jacques Diaz[1]

[1]Université de Lyon, Lyon, France
[2]CEA, DSV, iRTSV, Laboratoire d'Etude de la Dynamique des Protéomes, Grenoble, France and Université Joseph Fourier, Grenoble, France
[3]Biomedical Proteomics Research Group, Département de Biologie Structurale et Bioinformatique, Centre Médical Universitaire, Geneva, Switzerland

ABSTRACT

Nucleoli are now recognized as multi-functional nuclear domains involved in several fundamental cell processes such as ribosome biogenesis, regulation of the assembly of non-ribosomal ribonucleoprotein complexes, tRNA maturation, sequestration of protein, viral infection, and cellular ageing. Extensive proteomic analyses of these nucleolar domains after their purification have contributed to the description of their multiple biological functions. Because nucleoli are the largest and densest nuclear structures, they are easily amenable to purification from nuclei of cultured animal cells using the protocol described in this unit. *Curr. Protoc. Cell Biol.* 47:3.36.1-3.36.10. © 2010 by John Wiley & Sons, Inc.

Keywords: nuclear domain • nucleoli • cell fractionation

INTRODUCTION

Nucleoli are the largest and densest nuclear structures. They were the first nuclear domains to be observed by contrast microscopy (Fontana, 1781). The number and size of nucleoli vary depending on cell type, metabolic activity, and physiopathological conditions (Carter et al., 1993; Lewis and Tollervey, 2000; Dundr and Misteli, 2001). Observations by electron microscopy show that mammalian nucleoli are organized in three distinct domains: fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC; Granboulan and Granboulan, 1965; Stoykova et al., 1985; Melese and Xue, 1995; Olson et al., 2000; Fig. 3.36.1). Nucleoli are dynamic nuclear domains that constantly communicate with the cytoplasm and other nuclear domains (e.g., Cajal body, perinucleolar compartment; Sleeman and Lamond, 1999; Phair and Misteli, 2000). It was demonstrated in the 1960s that nucleoli are the sites of ribosome biogenesis. Moreover, nucleoli are also implicated in the regulation of several other major cellular processes such as the biogenesis of non-ribosomal ribonucleoprotein complexes, tRNA maturation, sequestration of protein, viral infection, and cellular ageing (Coute et al., 2006). The activity, number, and morphology of nucleoli vary greatly during cell cycle progression. In fact, nucleoli are assembled at the end of mitosis, upon entry into telophase when ribosomal DNA (rDNA) transcription is reinitiated around the nucleolar organizing regions (NOR). They remain active and assembled during the interphase and completely disassemble at pro-metaphase when ribosomal gene expression ceases. At this stage they lose their structural integrity and their components are dispersed throughout the mitotic cells (Comai, 1999; Olson, 2004).

The development of large-scale purifications of nucleoli from cells of animal and plant origins permitted the establishment of their protein content using proteomic approaches (Andersen et al., 2002, 2005; Scherl et al., 2002; Gonzalez-Camacho and Medina, 2004; Brown et al., 2005; Pendle et al., 2005). To date, >700 different proteins have been

Current Protocols in Cell Biology 3.36.1-3.36.10, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0336s47

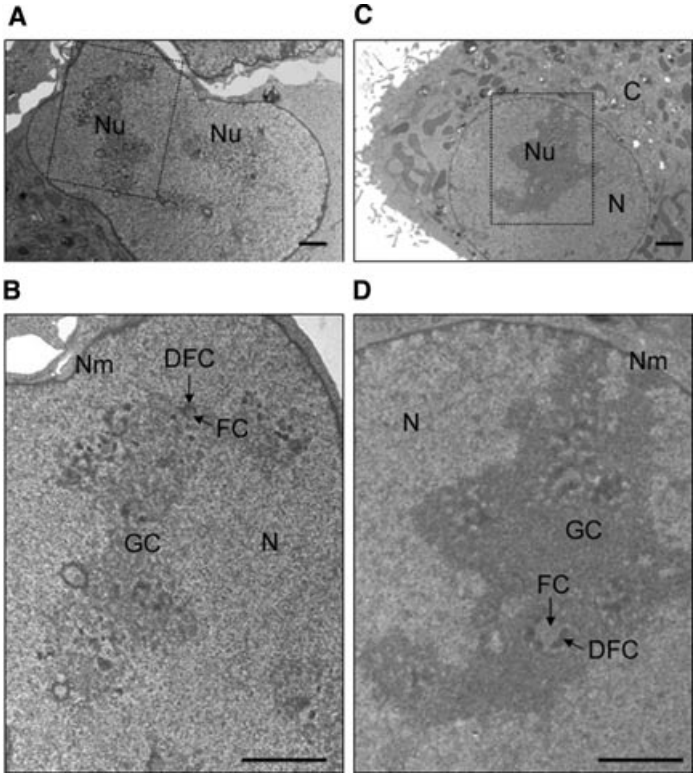


Figure 3.36.1 Electron micrograph of human epithelial polarized cells (**A,B**) and enlargement of respective nucleoli (**C,D**). Three constitutive nucleolar components are observed: the central fibillar center (FC) is surrounded by the dense fibrillar component (DFC) and both are embedded within the granular component (GC). Abbreviations: C, cytoplasm; ch, perinuclear layer of condensed chromatin; GC, granular components; N, nucleus; Nm, nuclear membrane; Nu, nucleolus. Scale bars = 2000 nm.

identified in human cells. A function for ~85% of these proteins has been proposed either after extensive biological studies or after bioinformatics analyses, whereas the roles of the remaining 15% remain elusive.

Figure 3.36.1, which presents an electron micrographic view of a human epithelial cell line, illustrates the ultrastructural organization of nucleoli and the variations of their morphological aspects depending on the physiological state of the cell. The nucleolus of actively growing cells is large and occupies a significant part of the nucleus (Fig. 3.36.1A-D). The number of FC also correlates with the cellular activity, as observed in Figure 3.36.1C. The DFC forms irregular strands, which are intermingled with GC and the border between nucleolus and nucleoplasm is irregular. A compacted large nucleolus (Fig. 3.36.1D, framed in 3.36.1B) reflects enhanced cellular activity and usually contains multiple FC that are surrounded by thick DFC and large areas of GC.

BASIC PROTOCOL

ISOLATION OF NUCLEOLI FROM ADHERENT CELL CULTURES

This protocol is adapted from previously published protocols (Muramatsu et al., 1963; Ochs, 1998; Scherl et al., 2002). The main steps for purification performed with HeLa cells are illustrated in Figure 3.36.2. After harvesting, HeLa cells are subjected to osmotic shock by incubation in an appropriate hypotonic buffer, resulting in swelling of cytoplasm and detachment from the nuclei. The efficiency of this treatment can be monitored by visual inspection of the cells by phase contrast microscopy. The swelling of the cytoplasm is clearly distinguishable. Cells are then lysed by addition of NP-40 and homogenized using a Dounce homogenizer. Nuclei are purified through a sucrose cushion

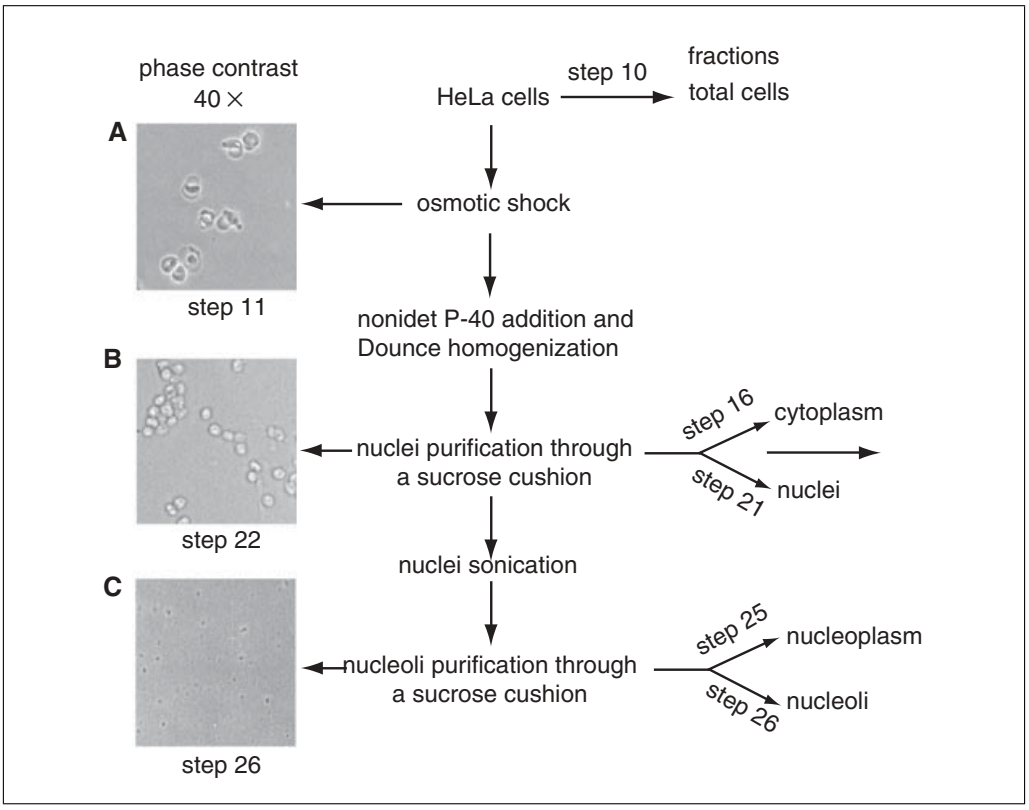


Figure 3.36.2 Main steps of the Basic Protocol. Left section of figure is the verification under phase contrast microscopy of the appearance of different fractions using a 40× objective lens. (**A**) Appearance of the sample after treatment with hypotonic buffer (step 9). (**B**) Appearance of the nuclei after addition of the sucrose cushion (step 18). (**C**) Appearance of the nucleoli after addition of the sucrose cushion (step 24). Middle section of the figure is a succinct description of the main steps of the protocol. The right section of the figure shows steps at which samples should be collected.

and the efficiency of nuclei purification can be monitored by visual inspection using phase contrast microscopy. Nuclei appear as rounded particles devoid of filamenteous structures. Finally, nucleoli are purified through a sucrose cushion after sonication of the purified nuclei and the efficiency of nucleoli purification can be monitored by visual inspection using phase contrast microscopy. Nucleoli appear as very small birefringent dots. No intact nuclei should be visible.

NOTE: Carry out all procedures at 4°C using pre-chilled solutions and equipment.

NOTE: This protocol was optimized for HeLa cells.

Materials

15×10^6 cultured adherent cells plated on 100- or 150-mm Petri dishes
Dulbecco's phosphate buffered saline for cell culture (DPBS; *APPENDIX 2A*), ice cold
Nucleoli standard buffer (NSB; see recipe)
10% (v/v) Nonidet P-40 (NP-40)
250 mM sucrose/10 mM $MgCl_2$
880 mM sucrose/5 mM $MgCl_2$
340 mM sucrose/5 mM $MgCl_2$
0.34 M sucrose buffer

Cell scrapers (Biologix Research Company)
15-ml polypropylene centrifuge tubes
Refrigerated centrifuge (Jouan, CR422)

Micropipets
Phase contrast microscope
0.4-mm clearance Dounce homogenizer (Wheaton type; Kimble/Kontes)
Sonicator (Vibracell 72434; Bioblock Scientific)

Culture and harvest cells

1. Plate cells on 100-mm diameter Petri dishes in the usual medium 48 hr before purification of the nucleoli.

   For HeLa cells, plate 1.5 × 10^6 cells per dish 48 hr before purification of nucleoli to obtain ∼6 × 10^6 cells per 100-mm Petri dish the day of nucleoli isolation. See Critical Parameters for comments about timing.

   Good nucleolar preparations require a minimum of 15 × 10^6 cells; 100 × 10^6 cells yield 150 μg of nucleolar protein.

2. At 80% confluence, wash cells two times with 10 ml of ice-cold DPBS, pH 7.4, per dish.

3. Scratch the Petri dishes with a cell scraper in a minimal volume (1 ml) of ice-cold DPBS.

4. Pool cells three plates (to obtain 30 μg nuclear protein) in a pre-cooled 15-ml centrifuge tube. Collect cells by centrifuging 5 min at 500 × *g*, 4°C.

Determine reference volume

5. Discard supernatant.

6. To determine the reference volume (RV), first visually determine the volume of the pellet that contains the cells. Add ∼2 vol ice-cold NSB and resuspend the cells by gentle pipetting up and down.

7. At this stage, measure exactly, using a micropipettor, the new volume obtained that represents the initial volume of the cells and the volume of added NSB.

   See Critical Parameters for comments on RV.

8. Calculate the volume of cells (RV) as the difference between this measured total volume and the added volume of NSB.

   The volume of the cells, which has been precisely determined, becomes the RV for the following steps. Each volume of buffer, which will be added, will be calculated based on this RV.

9. Adjust the volume of the cellular suspension with ice-cold NSB to 15 times the RV. Incubate for 30 min on ice.

   In this step, cells are in hypotonic buffer and their cytoplasm swells and the cytoplasm starts to detach from the nuclei.

10. Save ∼1/20 of the total cell suspension and store up to 6 months at −80°C for future SDS-PAGE analysis (see Support Protocol; also see *UNIT 6.1*).

    See Critical Parameters for comments on samples to save for evaluating purification.

11. Collect a drop of the homogenate with a micropipet and check the purification by visualizing under a phase contrast microscope (see Fig. 3.36.2).

Prepare cytoplasmic fraction

12. To perform cell lysis, add the appropriate volume of 10% NP-40 solution in the homogenate to obtain a final concentration of 0.3%.

    NP-40 is a nonionic detergent that facilitates cell lysis by inducing weakness of the plasma membrane.

13. Invert the suspension in the tube four to six times to homogenize.

14. Transfer the homogenate (~15 ml) immediately to a 0.4-mm clearance 15-ml Dounce homogenizer for gentle separation of nuclei and cytoplasm.

15. Carry out ten strokes with the Dounce pestle and verify under a phase contrast microscope that nuclei appear devoid of plasma membrane remnants.

    See Critical Parameters for comments on homogenization.

16. Transfer homogenate into a 15-ml centrifuge tube and centrifuge 5 min at 1200 × *g*, 4°C. Collect the supernatant to a new 15-ml tube.

    The supernatant contains the cytoplasmic fraction that is harvested for further analyses.

Fractionate nuclear fraction

17. Resuspend the pellet containing the nuclear fraction in 10 RV of the 250 mM sucrose solution containing 10mM $MgCl_2$.

18. Add 10 RV of 880 mM sucrose containing 5 mM $MgCl_2$ under the nuclear fraction.

19. Purify nuclei by centrifuging 10 min at 1200 × *g*, 4°C, through this sucrose cushion.

    See Critical Parameters for comments on nuclear purification.

20. Discard the supernatant. Resuspend the nuclear pellet in 10 RV of 340 mM sucrose solution containing 5mM $MgCl_2$.

21. Collect 1/20 of the nuclear fraction and store up to 6 months it at −80°C for future SDS-PAGE analysis (see Support Protocol; *UNIT 6.1*).

    See Critical Parameters for comments on sample to save for purification.

22. Collect a drop of nuclear fraction with a micropipet and verify purification by visualizing under a phase contrast microscope (see Fig. 3.36.2).

Prepare nucleoplasmic and nucleolar fractions

23. Break the nuclei by sonicating the nuclear fraction on ice using several bursts of 30 sec at 40 V at 5-min intervals. After each burst, verify the appearance of the suspension by examining a drop of the suspension by phase contrast microscopy. Stop the sonication when intact nuclei cannot be detected in the suspension.

    Nucleoli should be visible as dark dense structures under phase contrast microscopy (see Fig. 3.36.2).

24. Very gently add 10 RV of 880 mM sucrose under the sonicated nuclei with a micropipettor and then centrifuge 20 min at 2000 × *g*, 4°C.

25. After centrifugation, collect the supernatant, which contains the nucleoplasmic fraction, for further analysis. Save a sample of the nucleoplasm for further purification analysis and store up to 6 months at −80°C.

26. Resuspend the purified nucleolar fraction in a minimal volume of 0.34 M sucrose buffer and check the quality of purification by visualizing under a phase contrast microscope. Save a sample for purification analysis and store up to 6 months at −80°C.

    Nucleoli appear as very small birefringent structures (see Fig. 3.36.2).

    For an RV of 0.5 ml (three plates), the minimum volume is 20 μl and the maximum volume would be 200 μl for 100 × 10^6 cells.

Validate purification by one-dimensional gel electrophoresis

27. Thaw the samples collected in steps 10, 16, 21, 25, and 26 corresponding to each fraction obtained during the purification and stored at −80°C.

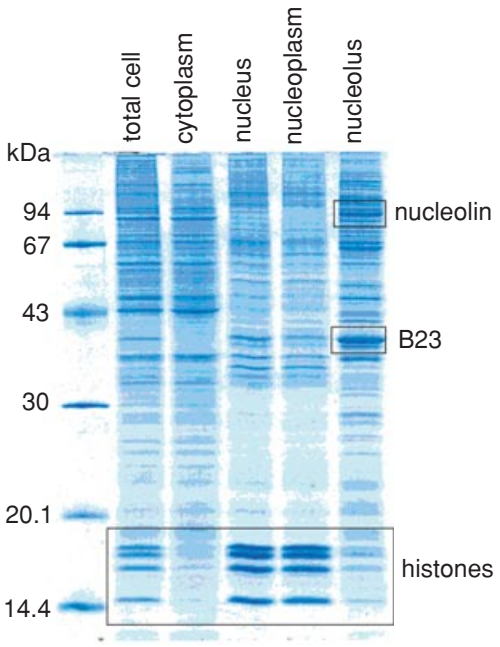


Figure 3.36.3 Analysis of the cellular fractions obtained during nucleoli purification. One-dimensional electrophoretic separation of 10 μg of proteins extracted from total cells, cytoplasmic fraction, nuclear fraction, nucleoplasmic fraction, and nucleolar fraction on a 12.5% polyacrylamide gel. Proteins are stained with Coomassie blue R250. Nucleolin, B23, and histones are indicated by insert and stars for histones. Size of molecular weight markers are indicated on the left.

28. Quantify proteins using the Bradford method (Simonian and Smith, 2006; *APPENDIX 3H*).

29. Separate 10 μg of each fraction by 12.5% SDS-PAGE and stain with Coomassie blue (see Support Protocol).

    An image of a typical gel is presented in Figure 3.36.3.

    A visual inspection of the gel makes it easy to monitor the efficiency of the purification.

    In general, the SDS-PAGE protein profiles of total and cytoplasmic fractions are very similar, except for histones (indicated by stars in Fig. 3.36.3), which are more enriched in the total fraction. The low abundance of these proteins in the cytoplasmic fraction is the basis for verification of the efficiency of the separation of nuclei and cytoplasm.

    In contrast, the SDS-PAGE profile of the cytoplasmic fraction is very different from the nuclear SDS-PAGE protein profiles (nucleus and nucleoplasm). In particular, there is an important enrichment in the four bands of low-molecular mass (indicated by stars in Fig. 3.36.3) that correspond to histones 1 to 4.

    Finally, the nucleolus fraction exhibits a very specific SDS-PAGE profile with a very clear enrichment of B23 (35 to 40 kDa) and of nucleolin (90 to 100 kDa).

    Obtaining an SDS-PAGE profile similar to that presented in Figure 3.36.3 is classically sufficient to assess the purity of the nucleolar fraction.

SUPPORT PROTOCOL

ONE-DIMENSIONAL SDS GEL ELECTROPHORESIS OF PROTEINS

To prepare the samples for SDS-PAGE, thaw each aliquot of fractions obtained in Basic Protocol steps 10, 16, 21, 25, and 26 during the purification slowly on ice. Quantify the protein in each samples using the Bradford protocol (Simonian and Smith, 2006; *APPENDIX 3H*) using a blank for each aliquot that contains the correct buffer: for total fraction, use NSB blank; for cytoplasm, use NSB plus 10% NP-40 blank; for nuclear

fraction, nucleoplasm fraction, and nucleolar fraction, use 340 mM sucrose solution for blank. After the quantification of the proteins, add 2 vol of 2× Laemmli electrophoretic sample buffer (see recipe) to ~10 μg of each fraction. Separate the proteins on a 12.5% one-dimensional SDS-PAGE gel (*UNIT 6.1*) and stain by Coomassie blue (*UNIT 6.6*). An image of a typical gel is presented in Figure 3.36.3.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Laemmli electrophoretic sample buffer, 2×

62.5 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
1% (w/v) SDS
10% (v/v) glycerol
0.1 M DTE
Traces of bromophenol blue
Dispense into 500-μl aliquots and store up to 6 months at −20°C

Nucleoli standard buffer (NSB), pH 7.4

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
10 mM NaCl
0.5 to 2 mM $MgCl_2$
Add water to 50 ml
1 tablet of Boehringer complete protease inhibitor (Roche Applied System)
Store up to 2 to 3 weeks at 4°C

COMMENTARY

Background Information

Nuclei of eukaryotic cells are highly organized organelles in which tens of different transient non-membrane bound structures, called nuclear domains or nuclear bodies, support most of the nuclear functions (Dundr and Misteli, 2001). Nucleoli are the most prominent of these nuclear domains with diameters ranging from 0.5 to 5 μm. The history, from their first description by Fontana in the second half of the 18th century to the modern research on the functions of nucleoli, has been properly summarized by Raska et al. (2006).

Nucleoli are present in every eukaryotic cell. They are formed around specific genetic loci, the nucleolar organizing regions, each of them containing tens of repeated ribosomal DNA genes (Henderson et al., 1972). The main biological role of nucleoli as ribosome subunit factories was proposed 50 years ago. Ribosome biogenesis is a very complex process requiring the contribution of numerous accessory factors, probably >100 factors that are not part of the ribosome itself (Kressler et al., 1999; Venema and Tollervey, 1999; Coute et al., 2006). This function gives rise to the characteristic ultrastructural organization of nucleoli consisting of three main compartments—the fibrillar center, the dense fibrillar component, and the granular component (Melese and Xue, 1995). The fibrillar center contains hundreds of rRNA genes not being transcribed. The dense fibrillar component contains rRNA genes that are actively transcribed by RNA polymerase I. The granular component contains rRNA being processed, and proteins added to it, to form ribosomal subunits that will be exported into the cytoplasm to initiate protein translation. More recently, the discovery of several molecules, with no obvious relation to ribosome biogenesis, were accumulating within nucleoli, which has promoted the emergence of a modern concept of nucleoli, where they are now considered as plurifunctional nuclear domains (Pederson, 1998; Olson et al., 2000; Raska et al., 2006). Nucleoli play crucial roles in several cellular processes such as the biogenesis of nonribosomal ribonucleoprotein complexes, cell cycle regulation, cellular ageing, and stress sensing. At the biomedical level, more and more evidence suggests their involvement in cancer (Montanaro et al., 2008), as well as viral infections (Hiscox, 2007).

The extreme concentrations of proteins and RNAs within nucleoli explain their very

high density, rendering them highly robust and quite easy to purify. The first efficient preparations of nucleoli from cells of various origins were obtained by pioneer works by Busch, Muramatsu, and colleagues (Busch et al., 1963, 1965, 1966; Muramatsu et al., 1963). Most of the isolation methods described later were based on the principles they proposed. First, highly purified nuclei have to be prepared. Then, nuclear organization has to be disrupted in order to free nucleoli; for this, controlled sonication in a solution containing a well-defined concentration of divalent cations is used. Finally, controlled centrifugation makes it possible to separate nucleoli from the rest of the nuclear components.

The efficiency of nucleoli purification can then be evaluated using various techniques: electron microscopy to ensure the conservation of classical nucleolar ultrastructure, immunoblotting to evaluate contamination from other cell compartments, or incorporation of labeled nucleotides to prove the maintenance of transcriptional activity.

After obtaining the nucleolar fraction, proteins can be identified by mass spectrometry as well. The first nucleolar proteomes were obtained in 2002. Two separate analyses identified 271 proteins (Andersen et al., 2002) and 213 nucleolar proteins (Scherl et al., 2002) for a total of 350 proteins after merging the two studies. Complementary studies were performed in 2005 that allowed the identification of 489 proteins (Andersen et al., 2005). Partial lists of nucleolar proteins as well as pictures of nucleolar proteins separated by one- or two-dimensional gel electrophoresis are available in SWISS-2D PAGE: *http://www.expasy.ch/swiss-2dpage/map=nucleoli_hela_1d_human* and *http://www.expasy.ch/swiss-2dpage/map=nucleoli_hela_2d_human*.

A nucleolar proteome database is also available (*http://www.lamondlab.com/NOPdb3.0/*) containing all the proteins identified in the nucleolar fraction by mass spectrometry (Ahmad et al., 2009).

Critical Parameters

In Basic Protocol steps 11, 15, 16, 22, 23, and 26, verify the appearance of the fractions by phase contrast microscopy (40× objective) using a drop of the fraction deposited between the microscope slide and a coverslip. The appearance of each fraction under phase contrast microscopy is illustrated in Figure 3.36.2.

In Basic Protocol step 1, the time at which the cells are plated before beginning the purification of nucleoli is critical. This can vary according to the cell line used. This time should be adapted to obtain 80% cell confluence at the beginning of the purification.

In Basic Protocol step 9, an aliquot of cell fraction should be analyzed by SDS–PAGE for the verification of the purification. This aliquot should be taken before adding 15 vol of NSB buffer to the remaining cell fraction for an optimal protein concentration.

In Basic Protocol step 15, the number of strokes should be optimized for each experiment. After four strokes, observe the cells under the phase contrast microscope. If the majority of the cells appear without their cytoplasm, proceed to the next step. If numerous nuclei are still encircled with remnants of cytoplasm (that appears under these conditions as more or less homogeneous filamentous structures attached to nuclei), subject the cells to another four strokes, then observe under the microscope. If necessary, repeat until nuclei are free of cytoplasm. For some cell lines (C2C12), >40 strokes are necessary. The concentration of $MgCl_2$ at this step is crucial (see below).

In Basic Protocol step 18, avoid mixing the suspension of nuclei with the sucrose cushion, usually the sucrose cushion is underlaid below nuclei suspension using a Pasteur pipet.

In Basic Protocol step 23, the number of sonicator bursts and the concentration of $MgCl_2$ (divalent cation) in NSB buffer must be adapted to each cell line and each physiological condition.

The concentration of divalent cation in hypotonic buffer is a very important parameter that allows lysis while preserving the nuclear membranes. A too high divalent cation concentration does not allow effective lysis; although too low a concentration leads to very effective cell lysis, it also induces a high destabilization of the nuclear membranes, which results in the disruption of nuclei at the centrifugation step.

Sonication of the nuclear suspension allows disruption of the nuclear membrane and the chromatin matrix and should be adapted for each cell line in terms of power and timing period (control by observing the suspension with a microscope).

Anticipated Results

A concentration of 1×10^8 adherent cells are expected to yield ~150 μg of nucleolar proteins. It is recommended to initiate the purification using 1 mM $MgCl_2$ and then optimize the concentration if the quality of the

fractionation is not satisfactory. It is not possible to obtain an efficient purification with a number of cells that was $<15 \times 10^6$.

Nucleolar fractions of good quality with good yields can be obtained using the following cell lines: HeLa (ATCC #CCL-2), IMR32 (ATCC #CCL-127), HEK-293 (ATCC #CRL-1573), MCF7 (ATCC #HTB-22), HEp-2 (ATCC #CCL-23), C2C12 (ATCC #CRL-1772), and RKO (ATCC #CRL-2577).

For nucleoli purification from these cell lines, the $MgCl_2$ concentration was experimentally determined to be between 0.5 mM and 2 mM.

The number of homogenizer strokes varied from 4 to 45 for these cells lines (see Critical Parameters).

The number of sonication bursts varied from one time for 30 sec at 40 V to five times for 30 sec at 40 V.

Time Considerations

Generally, 48 hr are sufficient to obtain each plate of $\sim 6 \times 10^6$ adherent cells at 80% confluency. However, this time could vary depending on the cell type used.

Nucleoli purification and storage of the purified fractions requires 1 day.

Quality control of the purification using one-dimensional electrophoresis and immunoblot analyses takes 1 to 2 days. The immunoblotting is not systematically done; however, it might insure that all steps were correctly performed. For immunoblotting, antibodies for beta actine (Sigma, cat. no. A 5228, diluted 1/1000), Fibrillarin (Abcam, cat. no. 4566, diluted 1/1000), and Histone H3 (Abcam, cat. no. 1791, diluted 1/1000) are regularly used. A detailed protocol for immunobloting can be found in Gallagher et al. (2008).

Note that it is difficult to perform nucleoli purifications from different cell lines in parallel due to the different steps of optimization that can vary a lot from one cell line to another.

Acknowledgements

This work was supported by INCa projet libre RIBOCAN, Région Rhône-Alpes, La Ligue National Contre le Cancer, and Association pour la Recherche contre le Cancer.

Literature Cited

Ahmad, Y., Boisvert, F.M., Gregor, P., Cobley, A., and Lamond, A.I. 2009. NOPdb: Nucleolar Proteome Database—2008 update. *Nucleic Acids Res.* 37:D181-D184.

Andersen, J.S., Lyon, C.E., Fox, A.H., Leung, A.K., Lam, Y.W., Steen, H., Mann, M., and Lamond, A.I. 2002. Directed proteomic analysis of the human nucleolus. *Curr. Biol.* 12:1-11.

Andersen, J.S., Lam, Y.W., Leung, A.K., Ong, S.E., Lyon, C.E., Lamond, A.I., and Mann, M. 2005. Nucleolar proteome dynamics. *Nature* 433:77-83.

Brown, J.W., Shaw, P.J., Shaw, P., and Marshall, D.F. 2005. Arabidopsis nucleolar protein database (AtNoPDB). *Nucleic Acids Res.* 33:D633-D636.

Busch, H., Muramatsu, M., Adams, H., Steele, W.J., Liau, M.C., and Smetana, K. 1963. Isolation of nucleoli. *Exp. Cell Res.* 24:S9:150-163.

Busch, H., Lane, M., Adams, H.R., Debakey, M.E., and Muramatsu, M. 1965. Isolation of nucleoli from human tumors. *Cancer Res.* 25:225-233.

Busch, H., Desjardins, R., Grogan, D., Higashi, K., Jacob, S.T., Muramatsu, M., Ro, T.S., and Steele, W.J. 1966. Composition of nucleoli isolated from mammalian cells. *J. Natl. Cancer Inst. Monogr.* 23:193-212.

Carter, K.C., Bowman, D., Carrington, W., Fogarty, K., McNeil, J.A., Fay, F.S., and Lawrence, J.B. 1993. A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. *Science* 259:1330-1335.

Comai, L. 1999. The nucleolus: A paradigm for cell proliferation and aging. *Brazilian J. Med. Biolog. Res.* 32:1473-1478.

Coute, Y., Burgess, J.A., Diaz, J.J., Chichester, C., Lisacek, F., Greco, A., and Sanchez, J.C. 2006. Deciphering the human nucleolar proteome. *Mass Spectrom. Rev.* 25:215-234.

Dundr, M. and Misteli, T. 2001. Functional architecture in the cell nucleus. *Biochem. J.* 356:297-310.

Fontana, F. 1781. Traité sur le venin de la vipère. Florence.

Gallagher, S., Winston, S.E., Fuller, S.A., and Hurrell, J.G.R. 2008. Immunoblotting and immunodetection. *Curr. Protoc. Mol. Biol.* 83:10.8.1-10.8.28.

Gonzalez-Camacho, F. and Medina, F.J. 2004. Identification of specific plant nucleolar phosphoproteins in a functional proteomic analysis. *Proteomics* 4:407-417.

Granboulan, N. and Granboulan, P. 1965. Ultrastructure cytochemistry of the nucleolus. II. Study of the sites of RNA synthesis in the nucleolus and the nucleus. *Exp. Cell Res.* 38:604-619.

Henderson, A.S., Warburton, D., and Atwood, K.C. 1972. Location of ribosomal DNA in the human chromosome complement. *Proc. Natl. Acad. Sci. U.S.A.* 69:3394-3398.

Hiscox, J.A. 2007. RNA viruses: Hijacking the dynamic nucleolus. *Nat. Rev. Microbiol.* 5:119-127.

Kressler, D., Linder, P., and de La Cruz, J. 1999. Protein trans-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 19:7897-7912.

Lewis, J.D. and Tollervey, D. 2000. Like attracts like: Getting RNA processing together in the nucleus. *Science* 288:1385-1389.

Melese, T. and Xue, Z. 1995. The nucleolus: An organelle formed by the act of building a ribosome. *Curr. Opin. Cell Biol.* 7:319-324.

Montanaro, L., Trere, D., and Derenzini, M. 2008. Nucleolus, ribosomes, and cancer. *Am. J. Pathol.* 173:301-310.

Muramatsu, M., Smetana, K., and Busch, H. 1963. Quantitative aspects of isolation of nucleoli of the Walker carcinosarcoma and liver of the rat. *Cancer Res.* 23:510-518.

Ochs, R.L. 1998. Methods used to study structure and function of the nucleolus. *Methods Cell Biol.* 53:303-321.

Olson, M.O. 2004. The Nucleolus. In Molecular Biology Intelligence Unit (R.G. Landes). Springer-verlag, Heidelberg, Germany.

Olson, M.O., Dundr, M., and Szebeni, A. 2000. The nucleolus: An old factory with unexpected capabilities. *Trends Cell Biol.* 10:189-196.

Pederson, T. 1998. The plurifunctional nucleolus. *Nucleic Acids Res.* 26:3871-3876.

Pendle, A.F., Clark, G.P., Boon, R., Lewandowska, D., Lam, Y.W., Andersen, J., Mann, M., Lamond, A.I., Brown, J.W., and Shaw, P.J. 2005. Proteomic analysis of the *Arabidopsis* nucleolus suggests novel nucleolar functions. *Mol. Biol. Cell* 16:260-269.

Phair, R.D. and Misteli, T. 2000. High mobility of proteins in the mammalian cell nucleus. *Nature* 404:604-609.

Raska, I., Shaw, P.J., and Cmarko, D. 2006. New insights into nucleolar architecture and activity. *Int. Rev. Cytol.* 255:177-235.

Scherl, A., Coute, Y., Deon, C., Calle, A., Kindbeiter, K., Sanchez, J.C., Greco, A., Hochstrasser, D., and Diaz, J.J. 2002. Functional proteomic analysis of human nucleolus. *Mol. Biol. Cell* 13:4100-4109.

Simonian, M.H. and Smith, J.A. 2006. Spectrophotometric and colorimetric determination of protein concentration. *Curr. Protoc. Mol. Biol.* 76:10.1A.1-10.1A.9.

Sleeman, J.E. and Lamond, A.I. 1999. Nuclear organization of pre-mRNA splicing factors. *Curr. Opin. Cell Biol.* 11:372-377.

Stoykova, A.S., Dabeva, M.D., Dimova, R.N., and Hadjiolov, A.A. 1985. Ribosome biogenesis and nucleolar ultrastructure in neuronal and oligodendroglial rat brain cells. *J. Neurochem.* 45:1667-1676.

Venema, J. and Tollervey, D. 1999. Ribosome synthesis in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* 33:261-311.

Isolation of Cytotoxic T Cell and NK Granules and Purification of Their Effector Proteins

Jerome Thiery,[1,2] Michael Walch,[1,2] Danielle K. Jensen,[1,2] Denis Martinvalet,[1,2] and Judy Lieberman[1,2]

[1]Program in Cellular and Molecular Medicine, Children's Hospital and Immune Disease Institute, Boston, Massachusetts
[2]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Killer lymphocytes induce apoptosis by the release of cytotoxic mediators from specialized secretory lysosomes, called cytotoxic granules, into the immunological synapse formed with a cell targeted for elimination. Methods are presented here for isolating CTL and NK cell cytotoxic granules using cell disruption by nitrogen cavitation followed by continuous Percoll density gradient fractionation. Protocols are also given for purifying the key cytolytic molecules (perforin, granzyme A, granzyme B, and granulysin) from isolated cytotoxic granules by fast protein liquid chromatography. *Curr. Protoc. Cell Biol.* 47:3.37.1-3.37.29. © 2010 by John Wiley & Sons, Inc.

Keywords: cytotoxic granules • CTL • NK cells • perforin • granzyme • granulysin

INTRODUCTION

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells play key roles in the immune response against virus-infected cells and tumors. These killer cells eliminate infected or transformed cells principally by releasing the contents of cytotoxic granules that contain programmed cell death–inducing serine proteases, called granzymes (for granule enzyme), and membranolytic pore-forming molecules, perforin (PFN) and granulysin (GNLY; Russell and Ley, 2002; Voskoboinik et al., 2006; Pipkin and Lieberman, 2007; Chowdhury and Lieberman, 2008). Recognition and binding of target cells by CTL and NK cells leads to highly polarized exocytosis of cytotoxic granules into the immunological synapse formed with the target cell. Granzyme entry into the target cell requires perforin.

Cytotoxic granules are complex secretory lysosomes that contain both effector molecules responsible for target cell elimination and resident lysosomal proteins (Table 3.37.1). These include PFN, calreticulin, and granzymes, which are complexed with a chondroitin sulfate–rich proteoglycan termed serglycin (for its alternating serine-glycine backbone; Lieberman, 2003). The dense core of activated human CTL and NK cells cytotoxic granules also contain GNLY, a member of the saposin-like protein (SAPLIP) family that exhibits a broad spectrum of antimicrobial activity, killing bacteria, fungi, and parasites (Stenger et al., 1998). Other than calreticulin, the expression of these proteins is largely restricted to activated CTL and NK cells. However, some of the granzymes, especially granzyme B, are also expressed together with mast cell proteases, but without perforin, in activated myeloid cells and some other cell types (Rissoan et al., 2002; Strik et al., 2007).

Protocols have been developed for cytotoxic granule isolation in order to study the composition of these organelles, as well as to purify native PFN and granzymes for in vitro studies of their respective roles in inducing target cell apoptosis. There is also increasing evidence for noncytotoxic functions of the granzymes in inflammation. Methods to

Current Protocols in Cell Biology 3.37.1-3.37.29, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0337s47

Table 3.37.1 CTL and NK Cell Cytotoxic Granule Contents

Protein	Function
Dense core	
Perforin	Pore formation; internalization of granzymes
Granzymes	Serine proteases
Granulysin	Microbicidal activity
Calreticulin	Calcium storage and perforin inhibitor
Cathepsin C	Granzyme processing
Serglycin	Proteoglycan matrix
Periphery (resident lysosomal proteins)	
Lamp-1	Lysosomal membrane proteins
Lamp-2	Lysosomal membrane proteins
CD63	Lysosomal membrane proteins
H^+ ATPase	Lysosomal acidification
Cathepsin B	Protection of killer cell from perforin
Cathepsin D	Lysosomal hydrolases
A-glucosidase	Lysosomal hydrolases
B-glucuronidase	Lysosomal hydrolases

express and purify recombinant versions of these proteins are also available (Sun et al., 1999; Martinvalet et al., 2008b; Sutton et al., 2008), but the procedure for purifying recombinant perforin is very challenging and not reproducible in our hands.

Basic Protocol 1 describes the isolation of cytotoxic granules from human lymphokine-activated killer (LAK) cells obtained from interleukin-2 and phytohemagglutinin (IL2/PHA)-activated peripheral blood mononuclear cells (PBMC) or human NK cell lines (i.e., YT-Indy) using a modification of the Borregaard method (Borregaard et al., 1983). This method (and all of the procedures described here) can also be used to isolate rodent granules and purify their cytolytic molecules. The Borregaard method is based on nitrogen cavitation of activated effector cells designed to disrupt the plasma membrane without lysing cytotoxic granules. The cytotoxic granules are then separated from other cell components by Percoll density gradient fractionation. Cytotoxic granules may also be isolated by the same method from RNK-16 cells, a rat NK-like leukemia cell line, grown as ascites for higher yields (see Alternate Protocol 1). Once the cytotoxic granules have been isolated, they can be lysed and either used directly to treat target cells to induce apoptosis (Support Protocols 9 and 10), or their cytolytic effector molecules can be purified by fast protein liquid chromatography (FPLC; Basic Protocol 2 and Alternate Protocol 2). We describe the purification of PFN (Basic Protocol 2), and the major granzymes—GzmA, GzmB, and GNLY (Alternate Protocol 2). PFN and granzyme expression can be detected immunologically (Support Protocols 1 and 2). After purification, PFN activity may be measured by different methods, including hemoglobin release assay (Support Protocol 3) or propidium iodide uptake (Support Protocol 4). GzmA and GzmB proteolytic activity is tested by peptide substrate (BLT and AAD) hydrolysis (Support Protocols 5 and 6), and GNLY activity by a colony-forming unit assay (Support Protocol 7) and turbidimetry (Support Protocol 8). We also describe two common assays (chromium release assay and annexin-V assay) to assess PFN and granzyme-induced cell death and apoptosis obtained by these methods (Support Protocols 9 and 10).

BORREGAARD METHOD OF CTL AND NK CELL CYTOTOXIC GRANULE ISOLATION

BASIC PROTOCOL 1

This is a 1-day protocol. CTL or NK cells are disrupted by nitrogen cavitation in a physiological buffer to release cytotoxic granules from whole cells. Isolated granules obtained from human LAK cells contain PFN and both major granzymes, GzmA and GzmB, as well as GNLY. YT-Indy NK cells contain PFN, GzmB, and GNLY, but not GzmA or K, and they are especially recommended for high-yield hGzmB purification. Alternatively, cytotoxic granules can be isolated from rat RNK-16 NK-like leukemia cells, serially passaged in vivo as ascites in Fischer F344 rats for higher yield purification (Alternate Protocol 1).

Granules may be purified from various sources, three of which are described in this protocol. The advantage of using human cells is that all effector molecules will be of the same species and from the same source. Additionally, primary human cells are rapidly dividing, and the transformed human NK lines highly over-express granzymes. The disadvantages of these techniques are the cost of IL-2 when expanding primary human cells and that the transformed human NK lines expand slowly and require a large amount of culture medium.

Materials

Human blood *or* buffy coats for LAK cell production *or* YT-Indy NK cells (ATCC)
Ficoll-Paque PLUS (GE Healthcare)
K10 medium (see recipe)
K10 + IL-2: K10 medium plus 1000 IU/ml human IL-2 (Chiron)
PHA-P (Sigma)
Hanks' balanced salt solution (HBSS) without calcium, magnesium, and phenol red
Relaxation buffer (see recipe)
Continuous 40% (w/v) adjusted Percoll gradient (see recipe)
Solubilization buffer (see recipe)

50-ml centrifuge tubes
Centrifuge
1000-µl pipettor *or* serological pipet
Cavitation/disruption bomb (Parr Instrument Company)
Magnetic stirrer
Refrigerated centrifuge with Sorvall SS-34 rotor (or equivalent), and corresponding 26-ml rigid polycarbonate tube (Beckman Instruments)
20-G spinal needle (Popper & Sons)
Beckman ultracentrifuge with Sw28Ti rotor (or equivalent) and thick-wall polycarbonate tubes (cat. no. 355631)

Additional reagents and equipment for counting the cells (*UNIT 1.1*), flow cytometry (Support Protocol 2), and fluorescence microscopy (Support Protocol 1)

NOTE: All procedures, buffers, tubes, and equipment must be maintained at 4°C during the purification unless otherwise noted.

NOTE: Make sure to handle human blood products with universal precautions following the rules of your institution.

Isolate and culture cells

1. For purification from NK cell lines, skip to step 8. For purification from PBMC or human buffy coats, layer 30 ml of whole blood or buffy coat onto 20 ml prewarmed Ficoll in a 50-ml centrifuge tube. Layer the blood on top of the Ficoll by slowly

running the blood down the side of the tube, making sure not to disrupt the phase layers during layering.

2. Centrifuge the tubes 30 min at 950 *g*, room temperature, and turn off the brake of the centrifuge.

   Active braking will disturb the lymphocyte layer. Do not centrifuge at 4°C, as this will alter cell/Ficoll density.

3. Remove tubes carefully from the centrifuge.

   The top layer will be plasma, the middle white layer (interface) will be PBMC, the clear layer will be Ficoll, and the bottom layer will be red blood cells.

4. Carefully remove the lymphocyte layer from the tubes with a 1000-µl pipettor or serological pipet, making sure to remove the least amount of Ficoll possible.

5. Wash the PBMC three times, each time for 5 min in 50-ml 37°C K10 medium to remove Ficoll and resuspend in 10 to 50 ml K10. Count an aliquot of the cells (*UNIT 1.1*).

6. Culture cells at 10^6 cells/ml for 3 days in K10+IL-2 supplemented with 4 µg/ml PHA-P in 75-cm^2 cell culture flasks at 37°C, 5% CO_2.

   Alternatively, one may select for $CD3^+CD8^+$ cells to enrich for cytotoxic T cells or for $CD3^-CD56^+$ cells to enrich for NK cells. Reduced doses of IL-2 may be used, but high doses of IL-2 improve yields by up-regulating expression of the effector proteins.

7. On the fourth day, resuspend the cells in fresh K10+IL-2 (without PHA-P) and reseed at 10^6 cells/ml and expand culture, splitting every several days to maintain a density of 1×10^6 cells/ml, until the desired cell number is achieved.

   At least 5×10^9 total cells are suggested for granule purification.

   These LAK cells should grow very rapidly; expanding from $0.1–1 \times 10^8$ cells (the expected yield of PBMCs is at least 10^6 cells/ml of blood) should take less than 2 weeks.

   Cells should be harvested 1 to 3 weeks after PHA stimulation for optimal expression of cytotoxic molecules.

8. For cultures beginning with NK cell lines, maintain YT-Indy cells in K10 medium at densities of $0.1–1 \times 10^6$ cells/ml. Split the cells by diluting 1:2 to 1:3, adding new medium to older medium.

   Do not completely change medium as the cells rely on secreted IL-2 to proliferate.

9. Expand the cells to $3–5 \times 10^9$ total cells, which is adequate for granule purification.

   The cells do not divide very rapidly, so expect the expansion to take ~3 weeks.

10. After cell expansion, test expression of PFN and/or granzymes contained in cytotoxic granules by flow cytometry (Support Protocol 2) or fluorescence microscopy (Support Protocol 1) before isolation of cytotoxic granules or effector molecule purification (Fig. 3.37.1).

Disrupt cells by nitrogen cavitation

11. Prechill the cavitation/disruption bomb at 4°C overnight before procedure.

12. Collect at least $3–5 \times 10^9$ cells, wash the cells twice with cold HBSS (keep at 4°C at all times), and then resuspend at 10^8 cells/ml in relaxation buffer.

13. Place cells in the cavitation bomb on top of a magnetic stirrer.

    If the cell suspension is ≤50 ml, place the cells in a 50-ml centrifuge tube and then place the tube in the bomb with ice surrounding the tube of cells.

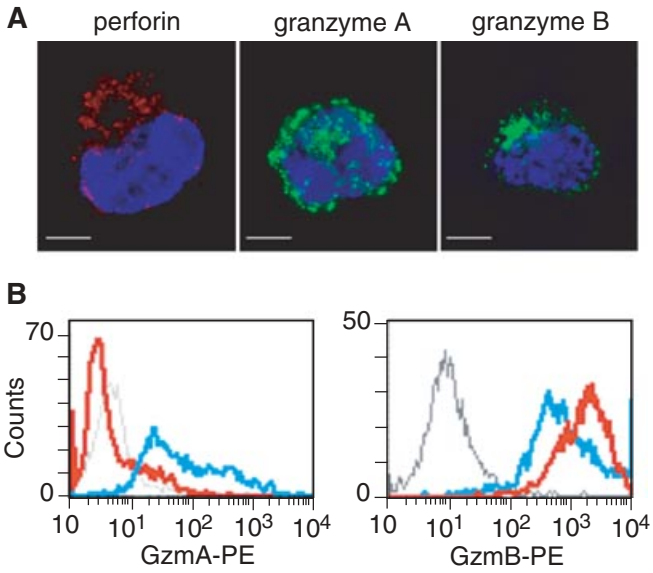


Figure 3.37.1 Analysis of cytotoxic granule content. (**A**) Cytotoxic granules of day 10 LAK cells were stained with anti-PFN, anti-GzmA, or anti-GzmB monoclonal antibodies before fluorescence microscopy analysis. Scale bars: 5 μm. (**B**) Jurkat (gray), YT-Indy (red), or day 10 LAK cells (blue) cells were stained with GzmA (left panel) or GzmB (right panel) monoclonal antibodies and expression was determined by FACS analysis. Jurkat cells do not express GzmA or GzmB while YT-Indy cells only express GzmB. LAK cells express both GzmA and GzmB. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0337*.

14. Slowly pressurize to 450 psi with N_2 and stir at 4°C for 20 min. Then, gradually release pressure and harvest the contents dropwise into a new tube on ice. If pressure drops below 250 psi, release remaining pressure quickly through the intake valve and collect remaining sample.

    The yield of granules is markedly influenced by the cavitation process. Excessive cavitation will rupture the cytotoxic granules. The stir plate must be adjusted to a speed that minimizes foaming.

Concentrate granules and remove nuclear and cytoplasmic contamination

15. To remove nuclei by differential centrifugation, centrifuge the sample 10 min at 400 × *g*, 4°C, and retain the supernatant.

16. Wash the pellet twice in equal volumes of relaxation buffer, centrifuge 5 min at 400 × *g*, 4°C, and add supernatants to the original supernatant.

17. Centrifuge the pooled supernatants 5 min at 400 × *g*, 4°C.

    The final post-nuclear supernatant (PNS) should be free of nuclei and whole cells.

18. Centrifuge the PNS 10 min at 15,000 × *g*, 4°C, to pellet granules and heavy organelles and resuspend the pellet in 20 ml relaxation buffer with a 20-G spinal needle.

Fractionate and collect cytotoxic granules

19. Preform a 20-ml continuous gradient of 40% adjusted Percoll (4°C) in four rigid polycarbonate tubes by centrifuging 10 min at 48,000 × *g*, 4°C, in an SS-34 rotor.

    "Adjusted" means that the Percoll concentration is adjusted with relaxation buffer to a final concentration of 40%.

20. Carefully, layer 5 ml of PNS/tube onto the 20 ml of preformed gradient of 40% adjusted Percoll. Centrifuge for 35 min at 48,000 × *g*, 4°C.

21. Extract cytotoxic granules with a 20-G spinal needle from the bottom of the gradient by harvesting the bottom 5 to 7 ml from each tube.

    Granules should form a visible refractile layer.

22. Pool the granule fractions and then remove Percoll by pelleting it by centrifugation overnight at 64,000 × *g* in 28-ml thick-wall polycarbonate tubes, 4°C, in a Sw28Ti rotor.

    Granules form a thin layer above the Percoll pellet.

Pellet granules

23. Remove the supernatant and resuspend the granules in solubilization buffer with a 20-G spinal needle (2 ml per 10^9 original cells) and store at −80°C.

    After resuspension, the granules can be stored indefinitely at −80°C before further protein purification.

    Disruption of the granule membranes and solubilization of granule proteins is optimal after at least one freeze/thaw cycle after storage overnight at −80°C followed by two freeze/thaw cycles in liquid N_2.

    The solubilized granules can also be used directly to treat cells for apoptosis assays provided the total salt is diluted to 150 mM NaCl with HE buffer (Support Protocol 9).

ALTERNATE PROTOCOL 1

PURIFICATION OF CYTOTOXIC GRANULES FROM RAT RNK-16 CELLS

The advantage of this method is that RNK-16 granule isolation described below is best suited for high-yield purification. The disadvantage of this technique is the need to use rats to serially passage RNK-16 NK-like leukemia cells as ascites in vivo since these cells do not grow in tissue culture. Typically, ascites from each rat yields 1–5 × 10^9 cells.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

F344 rats (200 to 225 g; Harlan Laboratories)
Pristane (2,5,10,14-tetramethyl-pentadecane; Sigma)
RNK-16 cells
Hanks' balanced salt solution (HBSS) without calcium, magnesium, and phenol red
HHH buffer (see recipe)
Ice
Red blood cell lysis buffer (Sigma)

3-ml syringe and 23-G needle
Dissecting pad
Scissors
10-ml pipet

Additional reagents and equipment for euthanizing the rat (Donovan and Brown, 2006)

Prepare the peritoneal cavity

1. Inject F344 rats intraperitoneally with 1.5 ml of sterile Pristane using a 3-ml syringe with a 23-G needle 4 to 7 days before RNK-16 cell injection to prepare the peritoneal cavity to host the cells.

Grow RNK-16 cells in rats

2. Wash 5–10 $\times$ 10^7 RNK-16 cells/rat twice, each time in 50 ml HBSS and resuspend in 2 ml of HBSS per rat for intraperitoneal (i.p.) injection.
3. Inject about 5–10 $\times$ 10^7 RNK-16 cells into each rat i.p. using a 3-ml syringe and 23-G needle.
4. Beginning 11 days post i.p. injection of RNK-16 cells, monitor rats for ascites production. Monitor rats every day for ascites production by abdominal palpation and progressive ascites fluid distention of the abdomen.

   Most of the time, rats are sacrificed 12 to 15 days post i.p. injection and RNK-16 cells are harvested immediately.

Harvest RNK-16 cells

5. Sacrifice the rat using CO_2 asphyxiation (Donovan and Brown, 2006) and perform bilateral thoracotomy (a small incision into the pleural space of the chest) 5 min after CO_2 exposure for euthanasia before harvesting the cells.
6. To harvest cells, pin the rat on its back to a dissecting pad and using scissors make a midline vertical slit in the abdominal skin to expose the peritoneal membrane without damaging it.
7. Then make a small incision with scissors into the left peritoneal membrane and slowly inject 10 ml of cold HHH buffer with a 10-ml pipet into the peritoneal cavity.
8. Mix carefully with the pipet to mobilize the ascitic fluid from the cavity, withdraw the peritoneal wash fluid containing RNK16 cells, and place on ice until the end of the procedure.
9. Repeat washing and harvesting cells until washes are clear.

   Expect to use 100 to 150 ml HHH buffer to extract all RNK-16 cells in one rat.

10. Centrifuge 5 min at 200 $\times$ *g*, 4°C, and remove the supernatant (carefully take the top layer first, which is pristane, and then remove the HHH buffer).

    The pristane is a distinguishable white layer above the HHH buffer.

    If RNK-16 cells are contaminated with blood, incubate the pellet with red blood cell lysis buffer for 10 min at 4°C.

11. Wash the cells three times, each time with at least 100 ml cold HBSS (keep at 4°C at all times) and then resuspend the cells at 10^8/ml in relaxation buffer.

    Aliquots of RNK-16 cells that appear viable by trypan blue staining from rats that produce a high yield of cells without blood contamination are used for re-injection or immediately frozen for future use.

Isolate granules

12. Proceed immediately with cell disruption by nitrogen cavitation and Percoll gradient fractionation of cytotoxic granules (Basic Protocol 1, steps 11 through 23).

PURIFICATION OF PERFORIN FROM CYTOTOXIC GRANULES

BASIC PROTOCOL 2

Rodent and human granzymes and PFN are cross-reactive at inducing cell death across species, although with some differences in substrate activity (Casciola-Rosen et al., 2007; Cullen et al., 2007). PFN can be purified from cytotoxic granules isolated from human LAK cells, YT-Indy NK cells, or rat RNK-16 cells. Because YT-Indy NK cells lack GzmA and GzmK expression and have high GzmB yields, those cells are preferred for GzmB purification, but GzmB can also be purified from rat RNK-16 cells. Because

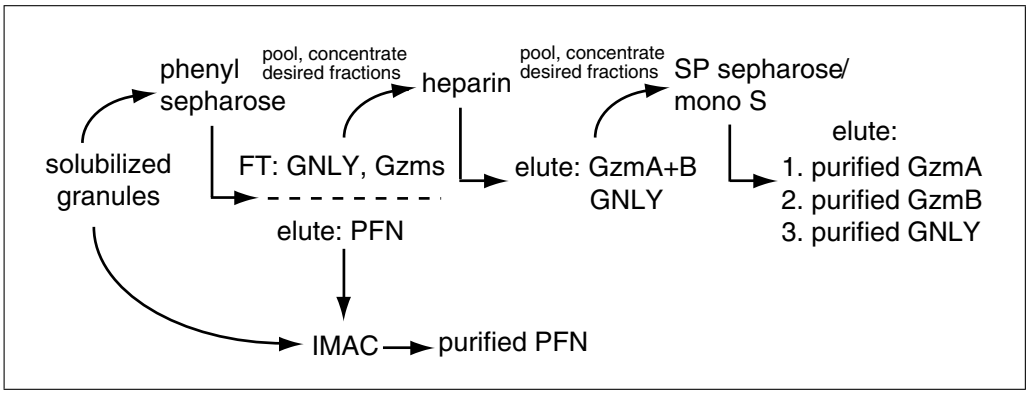


Figure 3.37.2 Procedure for purification of cytotoxic granule effector proteins by fast protein liquid chromatography (see Basic Protocol 2 and Alternate Protocol 2 for details). Abbreviations: FT, flow through; IMAC, immobilized metal affinity chromatography.

GNLY is not expressed in rodents, YT-Indy or human LAK cells preferentially are used for GNLY purification. A schematic of the different purification procedures is described in Figure 3.37.2.

For perforin isolation, an IMAC (immobilized metal affinity chromatography) purification method has been optimized by Winkler et al. (1996) to separate PFN from other granule components.

Materials

Cytotoxic granules (Basic Protocol 1), frozen
Ice
Equilibration buffer A1 (see recipe)
IMAC resin charged with Cobalt (TALON Superflow Metal Affinity Resin; Clontech)
0.5 M EGTA in H_2O
Elution buffer B1 (see recipe)
BCA assay (Pierce)

Rabbit anti-PFN (Cell Signaling, cat. no. 3693)
0.45-μm low-protein-binding syringe filter (Millipore)
10-ml desalting columns (Econo-Pac 10DG disposable chromatography columns; Biorad)
50-ml Superloop (Amersham)
Fast Protein Liquid Chromatography (FPLC) system (Biorad) and columns (Pharmacia Biotechnology)
Ultrafiltration concentrator (Amicon Ultra centrifugal filter device with low binding; Millipore)
Nitrocellulose membrane

Additional reagents and equipment for hemoglobin release assay (Support Protocol 2), BCA assay (*APPENDIX 3H*), SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and silver staining (*UNIT 6.6*)

NOTE: All buffers and suspensions are kept on ice.

Prepare granules

1. Place the frozen tube of solubilized granules (Basic Protocol 1, step 23) on ice for 2 hr, mixing the suspension by inverting the tube every 15 to 20 min.
2. Centrifuge the suspension 10 min at 15,000 × *g*, 4°C.

3. Carefully decant the supernatant and filter through a 0.45-μm low-protein-binding syringe filter to remove membrane fragments

   If the filter is blocked, carefully change the filter.

Desalt the suspension

4. During step 1, equilibrate desalting columns with 25 ml of cold equilibration buffer A1. Prepare one column for every 2.5 ml of supernatant.

5. Run 2.5 ml of supernatant through each desalting column, discard the flow-through, and then add 3.5 ml of equilibration buffer A1. Retain the flow-through and combine all samples for FPLC purification.

6. Filter the sample again through a 0.45-μm low-protein binding syringe filter.

   It is very important to note that at this point perforin is no longer in a chelated buffer and can be inactivated by multimerizing in the presence of trace amounts of Ca^{2+}. Therefore, the FPLC step must be performed as quickly as possible.

Perform FPLC

7. During step 5, prepare a 5-ml column of IMAC TALON Superflow resin and equilibrate it with 10 column volumes (CV) of equilibration buffer A1.

8. Load filtered sample into a superloop and attach to an FPLC workstation.

   Add 50 or 20 μl of 0.1 M EGTA into each fraction collector tube before FPLC run (if collecting 5- or 2-ml fractions to yield 1 mM final concentration of EGTA) to prevent perforin inactivation.

9. Run the following FPLC program with equilibration buffer A1 and elution buffer B1:

   a. Wash the column with 100% buffer A1 at 1.5 ml/min for 1 CV.
   b. Inject sample at 1 ml/min.
   c. Wash the column with a linear gradient of 1% to 2% buffer B1 at 1 ml/min for 5 CVs.
   d. Then elute with a linear gradient of 2% to 40% buffer B1 at 1.5 ml/min for 7 CVs and 40% to 100% buffer B1 at 1.5 ml/min for 5 CVs.

   Elution fractions are continuously monitored for absorbance at 280 nm. With this method one peak, absorbing at 280 nm, containing perforin is observed (Fig. 3.37.3A).

Identify PFN-containing fractions

10. Assay 10 to 20 μl of each fraction for hemolytic activity by a hemoglobin release assay (see Support Protocol 2; Fig. 3.37.3B).

11. Then, collect and pool all hemolytic positive fractions (containing perforin).

12. Concentrate with an ultracentrifugation concentrator to a final volume of 200 to 500 μl (10 mg/ml) to prevent lost of PFN due to binding to concentrator tube membrane.

13. Determine protein concentration by BCA assay (*APPENDIX 3H*) using BSA for calibration.

14. Divide purified active perforin and store indefinitely at −80°C.

    Since PFN is not stable with freeze thawing and loses activity at 4°C within a week, it is important to store it in small aliquots in an amount that will be used for just a few experiments. We generally divide our samples into 5-μl aliquots.

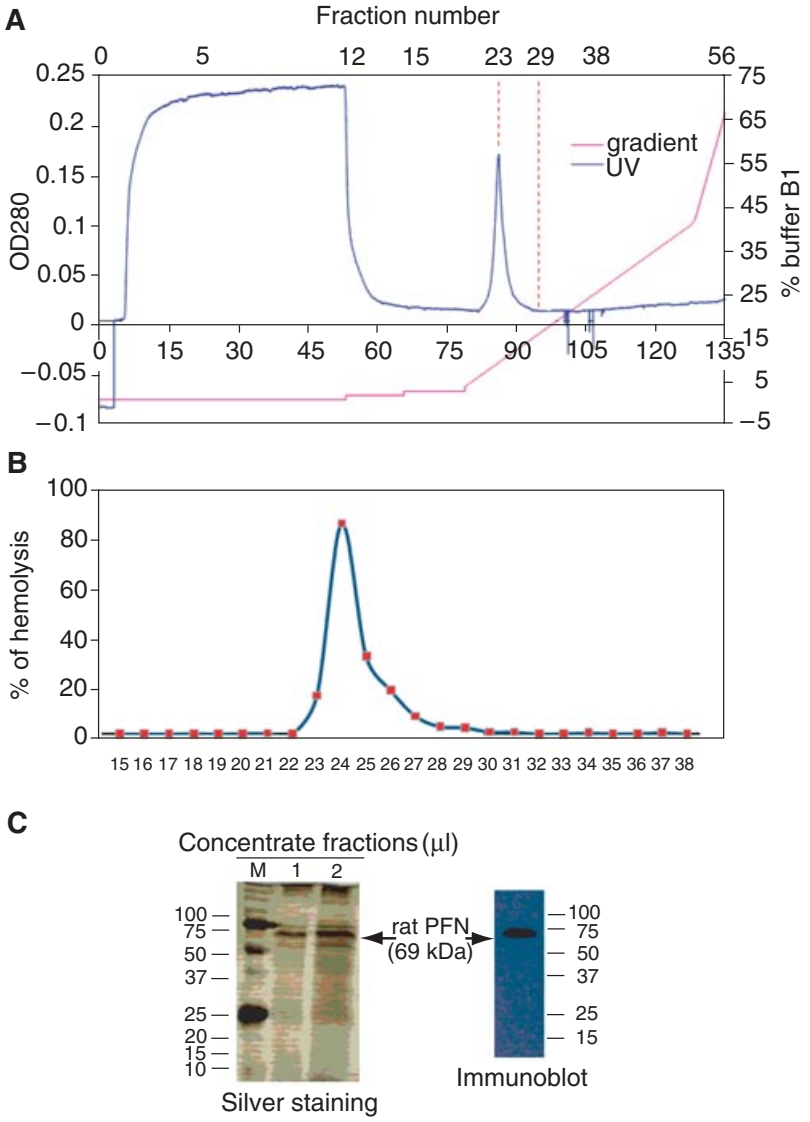


Figure 3.37.3 Purification of PFN from cytotoxic granules by fast protein liquid chromatography. (**A**) The profile of eluted protein from the FPLC is shown. Perforin activity is eluted over an imidazole gradient (pink line) and coincides with a single absorption peak at 280 nm (blue line) as shown by (**B**) testing the hemolytic activity of collected fractions by hemoglobin release assay. (**C**) After concentration of hemolytic positive fractions, 1 and 2 µl were used to assess the purity of the purified PFN by electrophoresis on a 12% polyacrylamide gel. Rat PFN (69 kDa) is visualized by silver staining (left) and an immunoblot (right). Abbreviations: M, protein markers. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0337*.

Analyze purity

15. Analyze purified perforin by SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*) to assess purity. Stain the protein gel by silver staining (*UNIT 6.6*). Transfer to nitrocellulose membrane and detect with rabbit anti-PFN (Fig. 3.37.3C).

    The staining is performed according to the manufacturer's protocol (dilution 1:1000, incubation overnight at 4°C).

    Immunoblots can also be probed for granzymes and GNLY to verify that there is no cross-contamination.

    It is important to note that cell sensitivity to PFN follows a very steep dose response curve that differs from cell to cell and varies even for the same cell under different culture

conditions (such as variations in cell density; Keefe et al., 2005). At high concentrations (termed lytic) perforin on its own triggers necrosis by causing irreparable membrane damage. At lower concentrations (termed sublytic) perforin on its own triggers necrosis in only a minority of cells (generally 5% to 20%, assessed by propidium iodide incorporation; Support Protocol 4), but is able to deliver granzymes into cells to induce apoptotic death. At still lower concentrations (when PI incorporation is <5%), perforin is not active at delivering granzymes. Sublytic concentrations are used to load granzymes into target cells to mimic CTL or NK cell-mediated cell death (which is apoptotic rather than necrotic). Because of this steep dose dependence (often a factor of 2 is all that distinguishes an inactive concentration from a lytic concentration), our practice is to titrate the perforin concentration to be used for each experiment on the day of the experiment or the day before (see Support Protocol 4).

PURIFICATION OF GRANZYME AND GRANULYSIN FROM CYTOTOXIC GRANULES

ALTERNATE PROTOCOL 2

A method adapted from Shi et al. (2000) is detailed here for purifying human GzmB and GNLY from the cytotoxic granules of YT-Indy cells, an autonomously growing human NK cell line expressing high levels of GzmB, perforin, and GNLY. YT-Indy cells do not express GzmA or GzmK (Froelich et al., 1996), which facilitates the purification of GzmB. However, RNK cell granules can also be used as starting material. RNK cells do not express GNLY, but express GzmA, GzmK, and GzmB. The granzymes are all highly basic (they are among the most basic proteins in mammalian cells with pI >9), making them easy to separate from other granule components. However, because of the similar chemical properties of these homologous enzymes, the purified granzymes need to be carefully screened by immunoblot and activity assay for cross-contamination. Since GzmA and GzmB have distinct substrate activity (GzmA is a tryptase, while GzmB cuts after aspartic acid residues), either antibodies or protease assays can be used to detect contamination. It is possible to separate GzmA and GzmB from LAK or RNK cell granules using a Mono-S column for the final step purification as previously described (Hanna et al., 1993). However, this must be done with care. An alternative is expression and purification of recombinant GzmA or GzmB as described elsewhere (Martinvalet et al., 2008b).

Materials

Granules from YT-Indy cells (Basic Protocol 1)
Phenyl Sepharose chromatography elution buffers A2 and B2 (see recipes)
Liquid nitrogen
Heparin chromatography buffers A3 and B3 (see recipes)
SP Sepharose (or MonoS) chromatography elution buffers A4 and B4 (see recipes)
BCA protein assay kit (Pierce)

Fast Protein Liquid Chromatography (FPLC) and columns (GE Healthcare)
Centrifuge
0.45-μm low-protein-binding syringe filter (Millipore)
Phenyl Sepharose High Performance, SP Sepharose High Performance, 5 ml of each densely packed in a column (GE Healthcare)
Ultracentrifugation concentrator
HiTrap heparin column, 5 ml (GE Healthcare)
MonoS column (GE Healthcare)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), staining with Coomassie brilliant blue (*UNIT 6.6*), and immunoblotting (*UNIT 6.2*)

NOTE: For the purification of rat granzymes the use of a MonoS column (GE Healthcare) instead of SP Sepharose is highly recommended.

Prepare for purification

1. Attach all columns to the FPLC system in a cold room.
2. Solubilize cytotoxic granules from YT-Indy cells in 10 ml buffer A2.
3. Subject the samples to three freeze/thaw cycles in liquid N_2.
4. Centrifuge 15 min at 15,000 × *g*, 4°C.
5. Collect the supernatant, filter it through a 0.45-μm low-protein-binding filter, and store on ice until use.

Remove PFN

6. Equilibrate the Phenyl Sepharose column with 10 CV of buffer A2.

   A Phenyl Sepharose column is used to separate PFN, which binds to the column, from granzymes and GNLY, which do not bind.

7. Load the filtered supernatant manually at ∼1 ml/min.
8. After the sample loading, wash the column with 10 ml buffer A2 until the UV detector baseline is reached (usually 2 CVs). Collect flowthrough and initial wash fractions, which contain granzymes and GNLY.

   Collect 2-ml fractions for a total of 20 ml (10 fractions).

9. Pool and concentrate these fractions to a volume <1 ml using an ultracentrifugation concentrator with a molecular cutoff <10 kDa before loading on the heparin column.
10. Elute perforin.

    PFN can be eluted from the Phenyl Sepharose column using the following program (flow: 1 ml/min): wash 10 min with buffer A2, elute with a 0–2 M NaCl (0% to 100% buffer B2) linear gradient for 20 min. Peak fractions contain perforin and are pooled, concentrated to a final volume of ∼2.5 ml, and stored at −80°C until IMAC purification (Basic Protocol 2).

Pass the sample through the heparin column

11. Equilibrate the HiTrap heparin column with 10 CVs of buffer A3.
12. Dilute the concentrated flowthrough and wash fractions from the Phenyl Sepharose column 20-fold in buffer A3 prior to loading manually on the heparin column at 1 ml/min.
13. Wash the column until the UV absorbance baseline is reached.
14. Elute granzymes and GNLY from the heparin column using the following program at a flow rate of 1 ml/min:

    a. Wash with 2 CVs of buffer A3.
    b. Elute with a 0−2 M NaCl (0%–100% buffer B3) steep linear gradient for 10 min.
    c. Finally, elute with 100% buffer B3 for 10 min.

    A typical chromatogram for YT-Indy cell granules is shown in Figure 3.37.4A.

    AAD esterolytic activity (GzmB, see Support Protocol 4) is found in elution peak 3 and 4 while antibacterial activity is mainly found in the peak 4. Since measuring GNLY antibacterial activity takes at least 12 hr, we recommend pooling the GzmB-containing fractions with the last elution peak without testing for GNLY before further purification on the S-column.

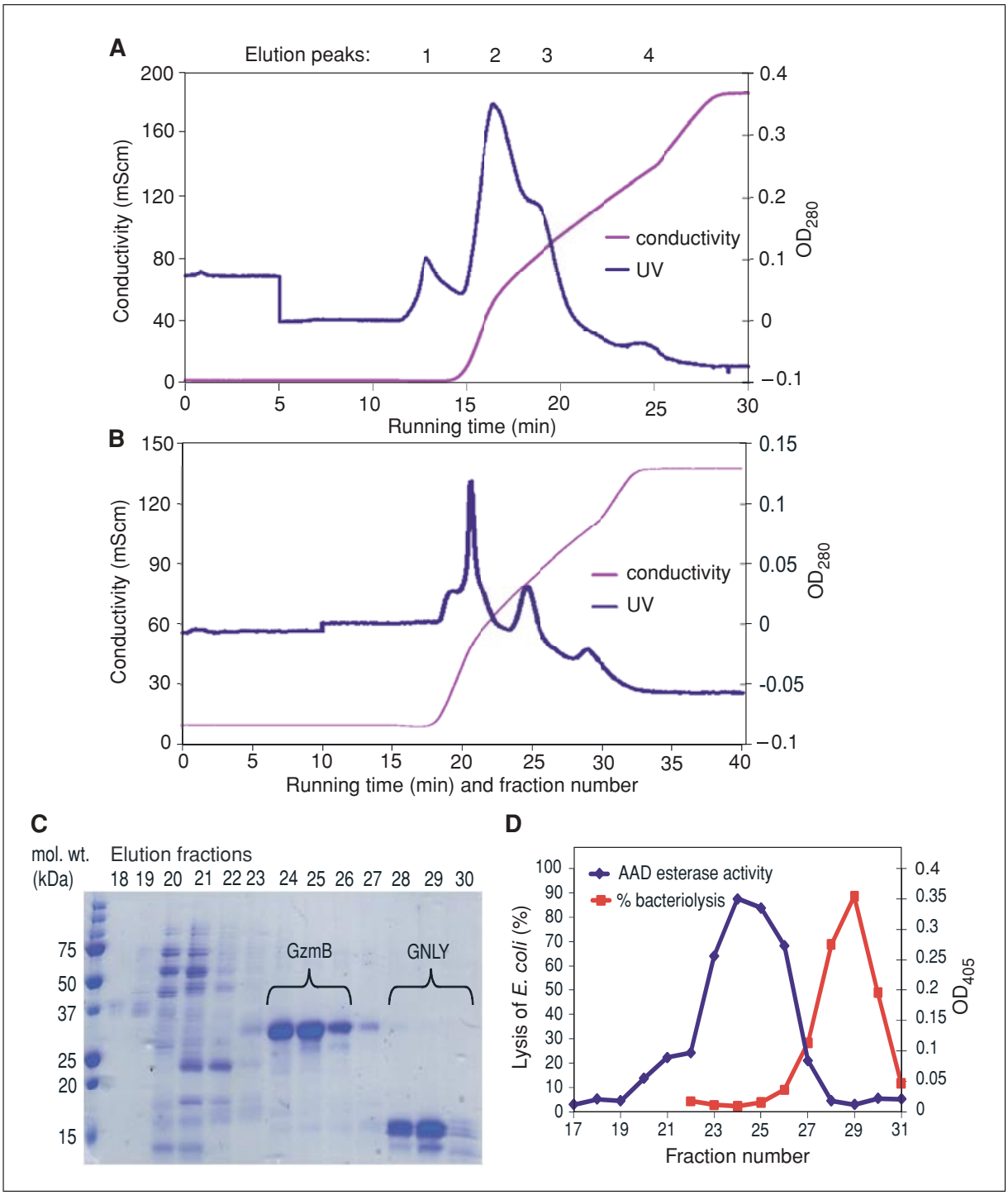


Figure 3.37.4 GzmB and GNLY purification from YT Indy cells. A Chromatogram (OD_{280} and conductivity) of the protein elution from the heparin column is shown in (**A**), and from the SP Sepharose column in (**B**). The eluted fractions from the SP Sepharose column were further analyzed by electrophoresis on a 12.5% polyacrylamide gel and by Coomassie blue staining (**C**) by AAD esterase assay and bacteriolytic activity (CFU assay) (**D**). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0337*.

15. Concentrate the pooled fractions to a final volume ≤1 ml using an ultracentrifugation concentrator with a molecular cutoff ≤10 kDa.

    If granules containing GzmA and GmzB are used as starting material, the eluate fractions should also be screened for BLT activity (see Support Protocol 5).

16. Equilibrate the SP Sepharose column with 10 CV of buffer A4.

17. Dilute the pooled and concentrated fractions of the heparin column 20-fold in buffer A4.

18. Manually load the sample on the S-column at 1 ml/min.

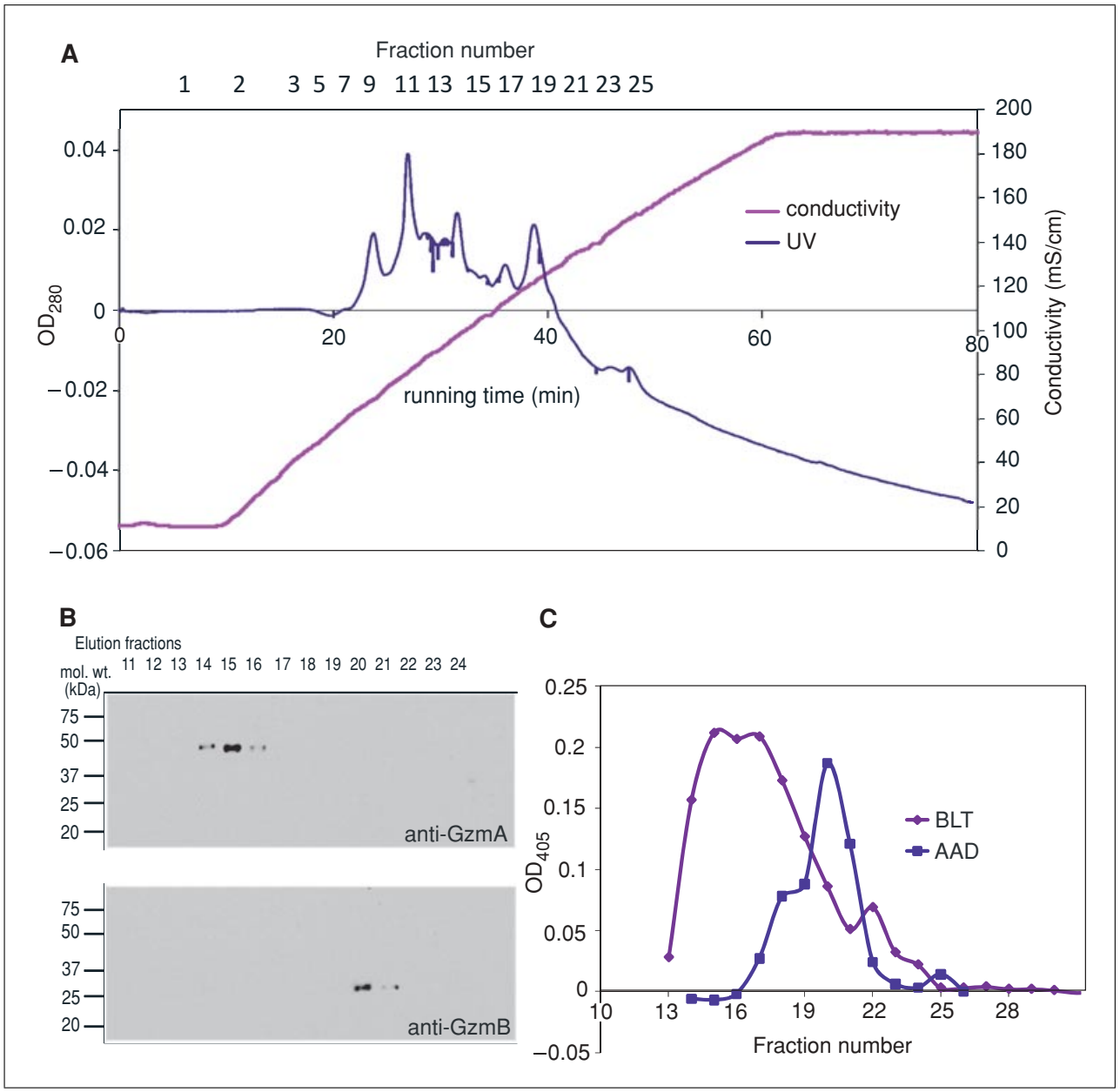


Figure 3.37.5 GzmA and GzmB purification from human LAK cells. The chromatogram (OD_{280} and conductivity) of the protein elution from the Mono-S column is shown in (**A**). The fractions were analyzed by electrophoresis on a 12.5% polyacrylamide gel and immunoblotting using anti-GzmA and anti-GzmB antibodies (**C**). The elution fractions were further screened for esterase activity (BLT and AAD) (**D**). Note there is BLT activity in fractions that stained negative for GzmA (fractions 17 and 18). The source of the esterase activity is most likely GzmK (not specifically tested in this purification). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0337*.

19. Wash the column with buffer A4 until the UV baseline is reached (2 to 3 CV).

Substitute a MonoS column if granules contain GzmA and GzmB to separate these.

Separate granzymes and GNLY on SP Sepharose column

20a. Use the following program at a flow rate of 1 ml/min to elute granzymes and GNLY from the SP Sepharose column using the following program:

i. Wash with an additional 2 CVs of buffer A4.
ii. Elute with a 0–1 M NaCl (0%–100% buffer B4) linear gradient for 20 min.
iii. Finally. elute with 100% buffer B4 for 10 min. GzmB elutes at ~60%–70% of buffer B4, GNLY at ~90%–100% buffer B4 (Fig. 3.37.4B).

21a. Analyze elution fractions using SDS-PAGE (*UNIT 6.1*) and stain with Coomassie brilliant blue (*UNIT 6.6*; Fig. 3.37.4C). Test purity by immunoblotting (*UNIT 6.2*). Perform activity testing (Support Protocols 3 to 9; Fig. 3.37.4D).

22a. Separately pool and concentrate fractions containing GzmB (28 kDa) or GNLY (15 and 10 kDa) to a final volume of ~250 µl before dividing into 5-µl aliquots and storing at −80°C.

Separate GymA and GzmB on Mono-S column

20b. If GzmA and GzmB–containing granules are used as starting material, perform the purification on a Mono-S column to allow better resolution of the cationic granule proteins.

21b. Apply the following program at a flow rate of 0.5 ml/min for elution:

i. Wash 20 min with buffer A4.
ii. Elute with a 0.05–1 M NaCl (0%–100% buffer B4) linear gradient for 60 min.
iii. Finally, elute with 100% buffer B4 for 20 min.

GzmA elutes at ~50% to 60% and GzmB at 60% to 70% of buffer B4 (Fig. 3.37.5A).

Be aware that GzmK (found in LAK cells as well as RNK granules) elutes also in this salt concentration range and exhibits BLT activity (see Support Protocol 4; Fig. 3.37.5B). The eluting fractions should therefore be carefully screened using SDS-PAGE under reducing and non-reducing conditions to distinguish between monomer- and dimer-containing fractions (GzmA is a dimer and GzmK a monomer under non-reducing conditions), as well as immunoblotting using GzmA, B, and K antibodies (Fig. 3.37.5C).

22b. Proceed to step 23.

23. Determine the protein concentration with the BCA protein assay kit.

24. Test protein activity as described in Support Protocols 5 and 6.

IMMUNOSTAINING ASSAY FOR PERFORIN AND/OR GRANZYME EXPRESSION

SUPPORT PROTOCOL 1

Immunostaining of cells can be used to assess expression of perforin and granzymes.

Materials

Poly-L-lysine (Sigma)
Cultures of CTL or NK cells
Phosphate-buffered saline (PBS; Cellgro, cat. no. 21-031-CV)
4% (w/v) PFA in PBS, pH 7.4
Permeabilization buffer: 0.2% (v/v) Triton X-100 in PBS
Blocking buffer: 10% (v/v) FBS in PBS
Mouse anti-human PFN, clone Pf80/164 (Mabtech); 1:600 dilution for microscopy

Mouse anti-human GzmB, clone GB11 (Caltag Laboratories); 1:300 dilution for microscopy
Mouse anti-human GzmA, clone CB9 (BD Pharmingen); 1:300 dilution for microscopy
Mouse anti-granulysin (BD Pharmingen)
Incubation buffer: PBS/0.05% (v/v) Triton X-100
Fluorochrome-conjugated secondary antibody (Molecular Probe)
Vectashield mounting medium containing DAPI (Vector Laboratories)

18-mm circle coverslips
96-well plates
Centrifuge, adaptors for plates
Fluorescence microscope

1. One day before staining, prepare Poly-L-lysine–coated 18-mm circle coverslips in 12-well plates.
2. Wash CTL or NK cells in 10 ml PBS and resuspend in 0.5 ml PBS.
3. Add 50,000 cells to the coated coverslips or into a 96-well plate for flow cytometry analysis, centrifuge 3 min at 500× *g*, room temperature, and remove supernatants.
4. Fix the cells 20 min in PBS containing 2% PFA at room temperature.
5. Wash the cells three times, each time with 1 ml PBS and permeabilize for 5 min in 1 ml permeabilization buffer.
6. Wash twice, each time in 1 ml PBS and add 1 ml blocking buffer for 30 min.
7. Wash one time in 1 ml PBS.
8. Add the required dilution of primary antibody (see Materials list) for PFN, GzmB, GzmA, or GNLY to the cells and incubate 1 hr at room temperature in 0.2 ml incubation buffer.

   Use incubation buffer to achieve the appropriate antibody dilutions.
9. Wash the cells three times, each time with 1 ml incubation buffer and incubate 1 hr at room temperature with fluorochrome-conjugated secondary antibodies (diluted 1:500 in incubation buffer or according to the manufacturer's protocol) in 0.2 ml incubation buffer.
10. Wash cells three times, each time in 1 ml PBS and mount in a small drop (~10 to 20 μl) Vectashield mounting medium before fluorescence microscopy analysis (see Fig. 3.37.1A).

SUPPORT PROTOCOL 2

FLOW CYTOMETRY ASSAY FOR PERFORIN AND/OR GRANZYME EXPRESSION

As an alternative to microscopy, flow cytometry can be used to screen for PFN, granzyme, and GNLY expression.

Materials

Jurkat (ATCC), LAK cells, *or* YT-Indy cells from Basic Protocol 1 (step 10)
4% (w/v) PFA in PBS, pH 7.4
FACS buffer (see recipe)
Phosphate-buffered saline (PBS; Cellgro, cat. no. 21-031-CV)
Saponin (Sigma)
Mouse anti-human PFN, clone δG9 (BD Pharmingen) for flow cytometry

Mouse anti-granulysin (BD Pharmingen) for flow cytometry
Mouse anti-GzmA (clone CB9, BD Pharmingen)
Mouse anti-GzmB (clone GB11, Catlag)
Secondary antibody

Microcentrifuge tubes *or* 96-well V-bottom plates
Centrifuge
Flow cytometer

1. Dispense 1–5 × 10^5 cells in microcentrifuge tubes or in 96-well V-bottom plates. Centrifuge cells 3 min at 400 × *g*, room temperature, to pellet cells.
2. Resuspend the cells in 50 µl of PBS containing 2% paraformaldehyde. Incubate 20 min at room temperature.
3. Wash the cells with 1 ml FACS buffer (microcentrifuge tubes) or twice with 200 µl FACS buffer (96-well plate), centrifuging the cells 5 min at 400 × *g*, room temperature, each time, and aspirating the supernatants.
4. Resuspend the cells in 50 µl PBS containing 0.1% (w/v) saponin to permeabilize the cells for intracellular staining.
5. Add 1 µl anti-GzmA, 5 µl anti-GzmB, 2.5 µl anti-perforin, or 2.5 µl anti-GNLY antibody. Incubate 30 min at room temperature in the dark.
6. If a secondary antibody is required, wash the cells as in step 3, and resuspend in 50 µl PBS containing 0.1% (w/v) saponin and the appropriate dilution of fluorochrome-conjugated secondary antibody.
7. Wash the cells as in step 3, and resuspend the cells in 100 µl FACS buffer.

   The cells are now ready for flow cytometry analysis (Fig. 3.37.1B).
8. Using a nonstained control sample, determine the optimal Forward Scatter and Side Scatter settings and adjust the fluorophore channels to baseline levels. Using stained samples, measure the fluorescence intensity of effector molecule-stained cells compared to the appropriate isotype control-stained samples.

MEASURING PERFORIN ACTIVITY BY HEMOGLOBIN RELEASE ASSAY

SUPPORT PROTOCOL 3

Perforin induces membrane damage by calcium-dependent multimerization to form pores in target cell membranes. Hemoglobin release from sheep red blood cells is commonly used to detect perforin activity during purification.

Materials

Sheep red blood cells (Rockland Immunochemicals)
Hanks' balanced salt solution (HBSS), without calcium, magnesium, and phenol red, ice cold
HBSS + 4 mM $CaCl_2$, ice cold
FPLC fractions of perforin purification (Basic Protocol 2)
Saponin (Sigma)

96-well round-bottom microplate
96-well flat-bottom microplates
Microplate reader

1. Wash sheep red blood cells three times, each time with 50 ml cold HBSS and resuspend at 1% (v/v) in HBSS containing 4 mM $CaCl_2$.

2. Transfer 100 μl of red blood cell solution to a well of a 96-well round-bottom microplate.
3. Add 10 to 20 μl of each FPLC fraction to the well.
4. Incubate the plate 20 min at room temperature.
5. Centrifuge the plate 5 min at 400 × *g*, 4°C.
6. Transfer 50 μl of cell-free supernatant from each well to a 96-well flat-bottom microplate.
7. Detect the hemoglobin release into supernatant by reading absorbance at 412 nm (Fig. 3.37.3B).
8. Calculate the percent release relative to the maximal hemolysis determined by adding 0.01% (v/v) saponin to the red blood cells as (experimental hemolysis − spontaneous hemolysis)/(maximal hemolysis − spontaneous hemolysis) × 100.

SUPPORT PROTOCOL 4

MEASURING PERFORIN ACTIVITY BY PROPIDIUM IODIDE UPTAKE

Propidium iodide uptake is usually used to determine the sublytic level of PFN for a given cell type to establish the conditions for efficient delivery of granzymes for apoptosis assays.

Materials

Target cells
Buffer C (see recipe)
FPLC fractions from perforin purification (Basic Protocol 2)
Buffer P (see recipe)
An5 buffer
Propidium iodide (PI; Sigma)

37°C incubator
Flow cytometer

1. Wash 5 × 10^4 target cells of interest in 0.5 ml buffer C and resuspend in 30 μl of the same buffer.
2. Make dilutions of purified perforin in 30 μl of buffer P.

   The diluted form at this stage is at a concentration of 2×.
3. Add 30 μl of the diluted perforin to 30 μl of cells from step 1.

   Because different PFN dilutions/concentrations need to be tested, it is convenient to use a 96-well V-bottom microplate for this assay.
4. Incubate 15 min at 37°C.
5. Wash cells once with 100 μl An5 buffer.
6. Resuspend cells in 50 μl An5 buffer with 2 μg/ml propidium iodide.
7. Measure PFN-induced necrosis immediately by flow cytometry by measuring PI uptake (Fig. 3.37.6).

   A PFN concentration that causes ∼5% to 20% cell death is considered a sublytic dose that is ideal for granzyme delivery and induction of apoptosis (Keefe et al., 2005). It is also important to note that the sublytic dose of PFN varies from cell to cell and must be determined for each experiment.

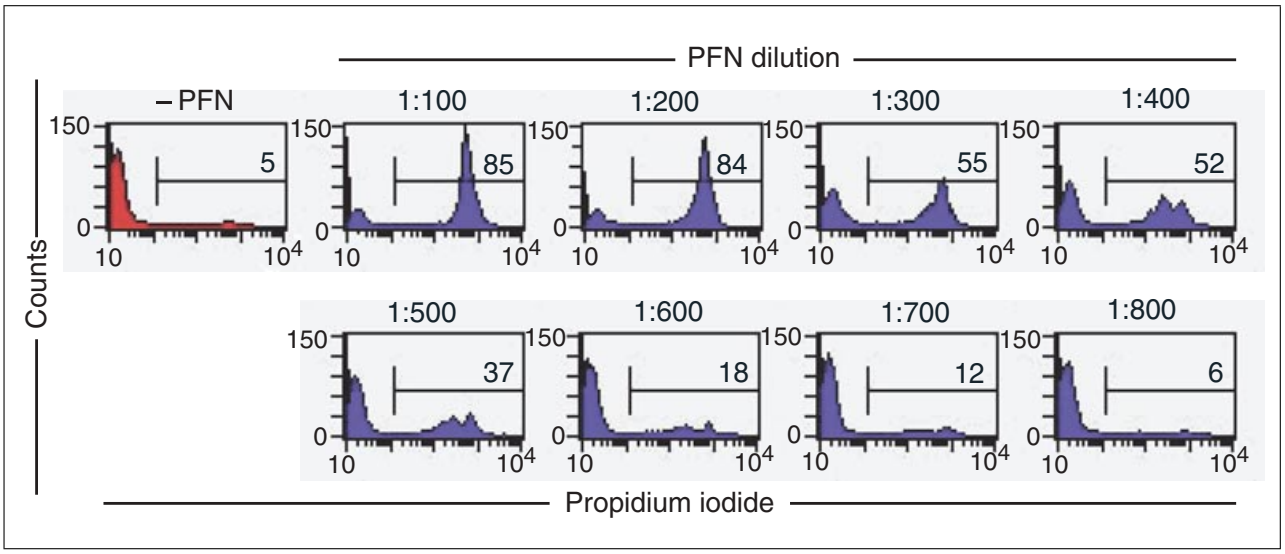


Figure 3.37.6 Measuring PFN activity by flow cytometry by propidium iodide uptake. HeLa cells were treated with different dilutions of purified native rat PFN for 15 min at 37°C before staining with propidium iodide (PI) and analysis by flow cytometry. Percentage of PI positive cells is indicated. In this experiment, the sublytic PFN concentration is between 1:600 and 1:700.

MEASURING GzmB ACTIVITY

SUPPORT PROTOCOL 5

The GzmB activity is measured by assessing its proteolytic activity. The measurement of peptide substrate (AAD) hydrolysis is commonly used during the purification of GzmB. GzmB-induced apoptosis can also be detected by various methods (see Support Protocols 9 and 10).

Materials

AAD assay buffer (see recipe)
FPLC fractions of GzmB purification (Alternate Protocol 2)

96-well flat-bottom plates
Microplate reader

1. Make fresh AAD assay buffer. Make sure to mix thoroughly.
2. Combine 5 µl of FPLC fractions or control buffer (for blank control) with 200 µl of AAD assay buffer in a 96-well flat-bottom plate.
3. Incubate the plate for 5 min at 37°C.
4. Measure $OD_{405\ nm}$ using a microplate reader.

Fractions with OD >0.1 are considered positive for GzmB activity (see Fig. 3.37.4D).

MEASURING GzmA ACTIVITY

SUPPORT PROTOCOL 6

GzmA activity is measured by assessing its proteolytic activity. The measurement of peptide substrate (BLT) hydrolysis is commonly used during the purification of GzmA. GzmA-induced apoptosis can also be detected by various methods (see Support Protocols 9 and 10).

Materials

BLT assay buffer (see recipe)
FPLC fractions for GzmA purification (Alternate Protocol 2)

96-well flat-bottom plate
37°C incubator
Microplate reader

1. Freshly add BLT and DTNB to make BLT assay buffer. Make sure to mix thoroughly.
2. Combine 5 µl of FPLC fractions or control buffer with 200 µl of BLT assay buffer in a 96-well flat bottom plate.
3. Incubate the plate for 5 min at 37°C.
4. Measure $OD_{405\ nm}$ using a microplate reader.

   Fractions with $OD_{405\ nm}$ >0.2 are considered positive for GzmA activity. These fractions may be pooled and concentrated using the Centricon MWCO 10 kDA concentrating units (Fig. 3.37.5C).

SUPPORT PROTOCOL 7

MEASURING GNLY-MEDIATED ANTIBACTERIAL ACTIVITY BY COLONY-FORMING UNIT ASSAY

The effect of GNLY on bacteria can be measured by quantifying bacterial numbers by colony formation on agar plates.

Materials

Bacteria: *E. coli, Listeria, Salmonella*, or whatever lab-strain is available
LB medium and LB agar plates (*APPENDIX 2A*)
Colony forming and turbidimetry unit assay buffer (see recipe)
Fractions for GNLY purification (Alternate Protocol 2)
37°C incubator

NOTE: NaCl concentrations higher than 50 mM interfere with GNLY activity.

1. Grow bacteria to mid-log phase ($OD_{600\ nm} = 0.5$ equaling $\sim 10^8$/ml viable bacteria) in LB medium in a bacterial culture shaker at 37°C.
2. Wash bacteria three times, each time in 1 ml colony forming and turbidimetry unit assay buffer and dilute to 5×10^5 bacteria/ml.
3. Prepare serial dilutions (between 2 µM and 0.06 µM) of GNLY in 25 µl of colony forming and turbidimetry unit assay buffer. Use buffer only as the negative control.

   Serial dilutions of antibiotics or of human beta-defensin 1 (Innavogen) can serve as positive control.

4. Add 25 µl of bacterial dilutions (equaling $\sim 1.25 \times 10^4$ viable bacteria) to each reaction, mix gently, and incubate 1 hr at 37°C. Include a no-bacteria control (assay buffer only).
5. Add 250 µl of LB medium to each reaction and prepare 10-fold serial dilutions from each reaction.
6. Plate 50 µl of the dilutions on LB agar plates and incubate at 37°C overnight prior to colony counting.
7. Calculate the percent lysis using the following equation: % specific lysis = $CFU_{GNLY\text{-}treated}/CFU_{buffer\text{-}treated} \times 100$ (Fig. 3.37.4D).

MEASURING GNLY-MEDIATED ANTIBACTERIAL ACTIVITY BY TURBIDIMETRY

The effect of GNLY on bacteria can also be measured by quantifying bacterial numbers by the change in turbidity of liquid cultures.

Materials

Bacteria: *E. coli, Listeria, Salmonella*, any non-virulent bacterial lab-strain
LB medium (*APPENDIX 2A*)
Colony forming and turbidimetry unit assay buffer (see recipe)
Fractions for GNLY purification (Alternate Protocol 2)

96-well transparent flat-bottomed plates
37°C incubator
Microplate reader (with ability to monitor kinetics at 37°C and discontinuous shaking; Spectra Max 340PC, Molecular Devices)

1. Grow bacteria to mid-log phase ($OD_{600} = 0.5$ equaling $\sim 10^8$/ml viable bacteria) in LB medium
2. Wash bacteria three times in 1 ml colony forming and turbidimetry unit assay buffer and dilute to 10^5 bacteria/ml
3. Prepare serial dilutions of GNLY in 25 µl of colony forming and turbidimetry unit assay buffer in a flat-bottomed, transparent 96-well plate. Use buffer only as a negative control.

   Serial dilutions of antibiotics or human beta-defensin 1 (Innavogen) can serve as positive control.

4. Add 25 µl of bacterial dilutions (equaling $\sim 2.5 \times 10^3$ viable bacteria) to each well, mix gently, and incubate 1 hr at 37°C.
5. Add 150 µl of LB medium to each well. Monitor $OD_{600\ nm}$ in the plate reader while shaking at intervals for 12 hr or until the steady-state of the bacterial growth curve is reached.

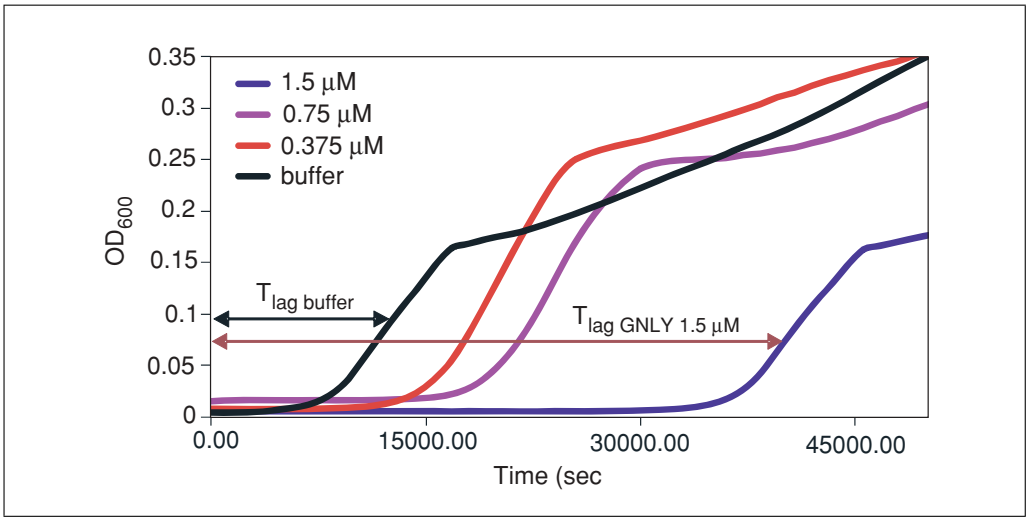


Figure 3.37.7 Measuring the antibacterial activity mediated by GNLY using turbidimetry. Typical bacterial growth curves from buffer- as well as GNLY-treated *E. coli* are shown. A possible read-out from these curves is to measure the time until a certain threshold OD is reached (T_{Lag}) and compare control versus test curves. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0337*.

6. Analyze the bacterial growth curves.

   The length of the lag-phase (T_{Lag}) of the bacterial growth curves (Fig. 3.37.7) reflects the initial bacteria count and can therefore be used to quantify the antibacterial activity of GNLY.

SUPPORT PROTOCOL 9

GRANZYME-MEDIATED CHROMIUM RELEASE APOPTOSIS ASSAY

To measure the activity of granzymes in cells, purified granzymes and PFN are added and cell death can then be monitored by a number of assays. Here, we provide the most sensitive measure of total cell death (apoptosis + necrosis), which is a chromium release assay.

Materials

Target cells
K10 medium: RPMI 1640 + 10% (v/v) FBS
^{51}Cr-labeled Na_2CrO_4 (NEN BioLabs), 5 μCi/μl
Hanks' balanced salt solution (HBSS)
Buffer C (see recipe)
Purified granzymes and perforin
Buffer P (see recipe)

96-well V-bottom plate
37°C incubator
TopCount scintillation counter and LumaPlate 96 (PerkinElmer)

1. Harvest target cells and resuspend the cells in 1 ml K10 medium and label with 100 μCi of ^{51}Cr for 1 hour at 37°C.
2. Wash the labeled target cells three times, each time in 10 ml HBSS.
3. Resuspend cells in buffer C at the same density used to determine the sublytic perforin concentration (see Support Protocol 4).

   Generally, 10^4–10^5 cells per well in 30 μl are dispensed in triplicate in a 96-well V-bottom plate.

4. Dilute perforin in 30 μl buffer P to 2× the sublytic dose previously determined.
5. Dilute the purified granzyme to 2× the final concentration in the same tube of buffer P and add 30 μl to cells.

   Include perforin only and granzyme only controls. Additionally, include buffer only controls (spontaneous release) and cells in 1% NP-40 (maximal release).

6. Incubate the plate for 1 to 4 hr at 37°C.
7. Pellet cells and cell debris by centrifuging the plate for 5 min at 400 × *g*, room temperature.
8. Remove 30 μl of supernatant, taking care not to disturb the cell pellet, and add to a LumaPlate 96.
9. Allow the supernatant to dry several hours or overnight before measuring ^{51}Cr release into the supernatant using a TopCount counter.

   Calculate the percent release relative to the maximal release determined by adding NP40 to the target cells as (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100.

GRANZYME-MEDIATED ANNEXIN-V/PROPIDIUM IODIDE (PI) ASSAY

To measure the activity of granzymes in cells, purified granzymes and PFN are added and cell death can then be monitored by a number of assays. Here we provide a flow cytometry–based assay that distinguishes apoptotic cells (generally Annexin V^+) from necrotic cells (generally Annexin V^-).

Materials

Target cells
Buffer C (see recipe)
Buffer P (see recipe)
Purified granzymes and perforin
An5 buffer (see recipe)
Annexin-V-APC (Caltag)
Propidium iodide (PI)

96-well V-bottom plates
37°C incubator
Centrifuge with plate-adaptor

1. Wash cells once with 5 ml buffer C.
2. Resuspend cells and dispense 10^5 cells/well of 96-well V-bottom plates in 30 µl buffer C.
3. Dilute perforin in 30 µl buffer P to 2× the sublytic dose previously determined.
4. Dilute the purified granzyme to 2× the final concentration in the same tube of buffer P and add 30 to cells.

   Make sure to include PFN only, granzyme only, and buffer-only treated cells.

5. Incubate the treated cells for 30 to 60 min at 37°C.
6. Add 100 µl An5 buffer to each well.
7. Centrifuge the plate 3 min at 500 × *g*, room temperature, in a centrifuge containing a plate-adaptor.
8. Resuspend the cells in 100 µl An5 buffer containing APC-conjugated Annexin V (1:33 dilution) and incubate 10 min at room temperature in the dark.
9. Wash the cells twice, each time in 100 µl An5 buffer.
10. Resuspend the pellet in An5 buffer containing 2 µg/ml propidium iodide and analyze cells by flow cytometry (Fig. 3.37.8).

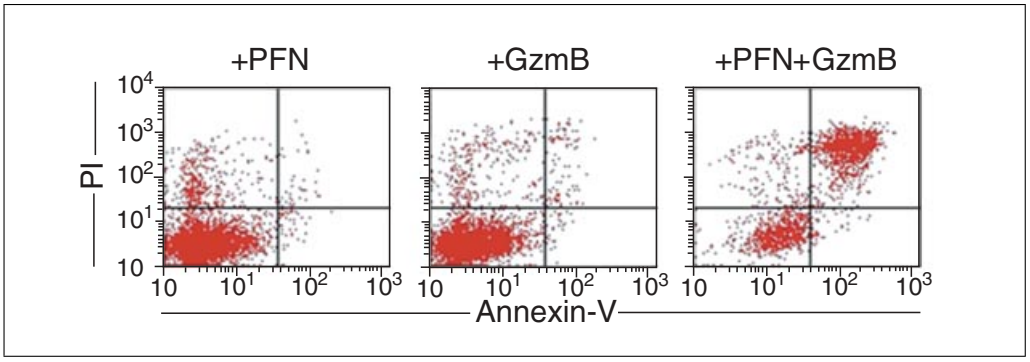


Figure 3.37.8 Analysis of PFN and GzmB-mediated apoptosis by Annexin-V/PI assay. HeLa cells were treated for 1 hr at 37°C with sublytic rat PFN and purified human GzmB before staining with Annexin-V and propidium iodide. Cells were analyzed by flow cytometry. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0337*.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AAD assay buffer

H_2O containing:
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.2 mM Boc-Ala-Ala-Asp-Thiobenzyl ester (AAD; MP Biomedicals), 20 mM stock solution in DMSO (Sigma)
0.22 mM of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma), 0.55 M stock solution in DMSO
Prepare fresh

An5 buffer

H_2O containing:
10 mM HEPES, pH 7.5
140 mM NaCl
2.5 mM $CaCl_2$
Store up to 6 months at room temperature

BLT assay buffer

H_2O containing:
50 mM Tris·Cl, pH 7.5
0.2 mM Nα-benzylozycarbonyl-L-lysine thiobenzyl ester (BLT; Sigma), 0.5 M stock solution in DMSO
0.22 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma), 0.55 M stock solution in DMSO
Prepare fresh

Buffer C

Hanks' balanced salt solution (HBSS)
10 mM HEPES, pH 7.5
4 mM $CaCl_2$
0.4% (w/v) bovine serum albumin (BSA)
Store up to 1 month at 4°C

Buffer P

Hanks' balanced salt solution (HBSS)
10 mM HEPES, pH 7.5
Store up to 1 month at 4°C

Colony forming and turbidimetry unit assay buffer

H_2O containing:
20 mM HEPES, pH 7.4
10 mM NaCl
Store up to 1 month at 4°C

Continuous 40% adjusted Percoll gradient

Relaxation buffer (see recipe) containing:
40% (w/v) Percoll (Sigma)
250 mM sterile sucrose
10 mM HEPES pH 7.2
7 mM HCl
Prepare fresh

Elution buffer B1

Equilibration buffer A1 (see recipe) containing 1 M imidazole, pH 7.5. Store up to 1 month at 4°C.

Elution buffer B2

H_2O containing:
20 mM Tris·Cl, pH 7.2 (*APPENDIX 2A*)
1 mM EGTA
Store up to 1 month at 4°C

Elution buffer B3

H_2O containing:
20 mM Tris·Cl, pH 7.2 (*APPENDIX 2A*)
0.1 mM EGTA
2 M NaCl
Store up to 1 month at 4°C

Elution buffer B4

H_2O containing:
20 mM bis-Tris, pH 5.8
1 M NaCl
Store up to 1 month at 4°C

Equilibration buffer A1

Distilled H_2O containing:
1 M NaCl
20 mM HEPES, pH 7.2
10% (w/v) Betaine (Sigma)
Store up to 1 month at 4°C

Equilibration buffer A2

H_2O containing:
20 mM Tris·Cl, pH 7.2 (*APPENDIX 2A*)
1 mM EGTA
2 M NaCl
Store up to 1 month at 4°C

Equilibration buffer A3

H_2O containing:
20 mM Tris·Cl, pH 7.2 (*APPENDIX 2A*)
0.1 mM EGTA
Store up to 1 month at 4°C

Equilibration buffer A4

H_2O containing:
20 mM bis-Tris, pH 5.8
50 mM NaCl
Store up to 1 month at 4°C

FACS buffer

Phosphate-buffered saline (PBS; Cellgro, cat. no. 21-031-CV)
2% (v/v) fetal bovine serum (FBS; heat-inactivated)
Store up to 6 months at 4°C

HE buffer

H_2O containing:
150 mM NaCl
10 mM HEPES, pH 7.5
2 mM EDTA, pH 8.0 (*APPENDIX 2A*)
Store up to 1 month at 4°C

HHH buffer

Hanks' balanced salt solution (HBSS) containing:
10 mM HEPES, pH 7.2
100 U/ml heparin (Sigma)
Prepare fresh

K10 medium

RPMI-1640 medium supplemented with:
10% (v/v) fetal bovine serum (FBS; heat-inactivated)
100 U/ml penicillin (from a 20× stock)
100 μg/ml streptomycin (from a 20× stock)
6 mM HEPES
1.6 mM L-glutamine
50 μM 2-mercaptoethanol
Store up to 1 to 2 months at 4°C

Relaxation buffer

H_2O containing:
10 mM KCl
3.5 mM $MgCl_2$
10 mM PIPES pH 6.8
1.25 mM EGTA pH 8.0
1 mM ATP (Sigma)

Relaxation buffer can be prepared in advance without ATP. This buffer can be stored 1 to 2 days at 4°C prior to addition of the ATP. Once ATP is added the buffer must be discarded after each use.

Solubilization buffer

H_2O containing:
1 M NaCl
20 mM sodium acetate, pH 4.5
2 mM EDTA, pH 8.0 (*APPENDIX 2A*)
Store up to 6 months at 4°C

COMMENTARY

Background Information

Cytotoxic T lymphocytes and NK cells deliver their effector molecules via specialized secretory lysosomes. These lysosomes, or cytotoxic granules, are dual-function organelles, containing both lysosomal proteases, which function at acidic pH, and secretory products such as perforin, which function at neutral pH when they are released from the killer cells to induce target cell death. Indeed, when cytotoxic T lymphocytes and natural killer cells form an immune synapse with a specifically recognized target cell destined for elimination, cytotoxic granules move to the immune synapse where the cytotoxic granule membrane fuses with the killer cell membrane, releasing the granule contents into the synaptic cleft (Voskoboinik and Trapani, 2006; Chowdhury and Lieberman, 2008). These cytotoxic granules contain perforin (PFN), needed to deliver the granzymes into the target cell (Keefe et al., 2005), and a family of highly homologous serine proteases named granzymes (Gzms), whose major job is to

induce programmed cell death to eliminate virus-infected and tumor cells (Stinchcombe et al., 2001). By activating apoptosis instead of necrosis, inflammation is minimized to focus on the intended target and minimize effects on bystander cells. Cytotoxic granules of human NK cells and cytotoxic T cells also contain granulysin (GNLY), a protein with antimicrobial activity against a broad spectrum of microbial pathogens. Isolation of cytotoxic granules and further purification of native PFN, granzymes, and/or GNLY allows researchers to study how the granzymes initiate programmed target cell death pathways, how PFN induces internalization of granzymes into the target cell cytosol, and how GNLY induces the death of microbial pathogens.

The protocols described in this unit are based on isolation of cytotoxic granules from CTL and NK cells by nitrogen cavitation as originally developed by Borregaard (Borregaard et al., 1983). CTL and NK cells are resuspended in relaxation buffer designed to mimic the conditions found in the cytosol of neutrophils, facilitating the separation of cytoplasmic organelles from nuclei. Cells are lysed by nitrogen cavitation and cytotoxic granules are purified over a Percoll density gradient. Although purified granules can be used for some studies, further FPLC purification of effector molecules is often necessary for other purposes. Active recombinant mouse GzmB and human GzmA can also be expressed and purified (Martinvalet et al., 2008b). A method was also developed to express and purify active recombinant GNLY (Ernst et al., 2000). However, active recombinant PFN is difficult to purify and even purified native PFN is tricky to work with (as discussed above). For that reason, many researchers substitute a bacterial pore-forming protein such as streptolysin O for PFN as the delivery agent to introduce granzymes into target cells. However, we feel that using native PFN is a more physiologically relevant system. Thus, isolation of cytotoxic granules is a reliable source of active native PFN, granzymes, as well as GNLY. For perforin isolation, IMAC (immobilized metal affinity chromatography) procedure is preferred. The advantage of cobalt IMAC for PFN purification is that there are few other co-purifying proteins, and this method allows better separation between granzymes and PFN (Winkler et al., 1996). Both GzmB and GNLY are purified by a 3-step method adapted from Shi et al. (2000), taking advantage of hydrophobic interactions of PFN with phenyl Sepharose to separate PFN from the other granule components, followed by two consecutive cation-exchange columns (heparin and SP Sepharose or MonoS) to separate the more basic granule components.

Critical Parameters

The two-step cytotoxic granule isolation protocol described here is particularly suitable for use of granules and/or purified effector proteins in studies that aim to define the different mechanisms of target cell death by the CTL/NK cells granule pathway. Cytotoxic granules can also be isolated using a protocol described by Millard et al. (1984). This method includes heparin in the cell lysis buffer to optimize granule yield, and DNase I to digest DNA released from nuclei damaged during the cell lysis step. However, the use of these granules for further studies of granzyme-mediated cell death is not recommended because DNase I added during purification can contribute to nonspecific DNA fragmentation. By comparison, cytotoxic granule extracts by the Borregaard method are free of non-specific nuclease activity (Davis et al., 2003). Before any cytotoxic granule purification, LAK, CTL, or NK cells activation state should be tested by a PFN and/or granzyme expression assay.

One of the most important variables that affect the success of granule isolation is the cell lysis by nitrogen cavitation. Indeed, as mentioned before, the yield of granules is markedly influenced by the cavitation process and excessive cavitation ruptures the cytotoxic granules.

The other important step is the continuous Percoll density gradient. The number of cell equivalents loaded into a preformed 20-ml Percoll continuous gradient should not exceed 10^{10} in 5 ml. When more cells are used, the users are encouraged to prepare additional tubes of Percoll gradient. Moreover, users are strongly encouraged to test harvested granule fractions for activity. Usually granules are harvested by withdrawing the bottom 5 ml from each tube. The highest granule titer is in the first 4 ml, but it is sometimes necessary to harvest 7 ml in order to fully recover granules. Isolated cytotoxic granules must be properly stored in solubilization buffer at -80°C.

At present, rat RNK-16 cells are the best source of high-quality and high-yield native PFN. The use of YT-Indy NK cells also offers a reliable source of human PFN that avoids in vivo propagation, but with a much lower yield. Because NK cell lines expand slowly, it can be difficult to reach the number of cells necessary for purification of high-yield protein. One of the critical steps before

chromatography purification of PFN from cytotoxic granules is granule solubilization. At least two freeze/thaw cycles are necessary to solubilize granules fully. Best results are obtained with a freeze cycle at −80°C overnight, followed by thawing for 2 hr on ice, and a freeze/thaw cycle with liquid nitrogen. After buffer exchange with a desalting column it is also crucial to note that PFN is no longer in a Ca^{2+}-chelated buffer and can be inactivated by traces of Ca^{2+}. Hence, the FPLC step must be performed as quickly as possible. Moreover, EGTA must be added into each fraction collector tube before the FPLC run to prevent PFN inactivation by traces of Ca^{2+}. Concentrated purified PFN must be stored at −80°C. PFN must be handled on dry ice, and PFN remaining after an assay can be saved at −80°C, pooled, and activity retitered before using.

The best results for isolating GzmB and GNLY using YT-Indy cells as the source of granules are usually obtained if the protocol is completed within 1 day. Furthermore, to optimize the yield, it is critical that the NaCl concentration of the loading samples for the cation exchange columns not exceed 100 mM; the granzymes and GNLY will otherwise not bind to the heparin or S-column. To improve activity in subsequent experiments using purified GzmB and GNLY, it is necessary to concentrate the proteins to >20 μM. High NaCl concentration (almost 1 M) in the eluate fractions will interfere with protein activity. Concentrated, purified granzymes must be stored at −80°C. It is also important to note that measuring purified GzmA activity with BLT is highly sensitive but might not be as specific as substrate cleavage. We highly recommend to test purified GzmA for cleavage of its substrate SET (Beresford et al., 2001) either using recombinant SET (Martinvalet et al., 2008a) or whole-cell lysate (Bovenschen et al., 2009).

Anticipated Results

Cytotoxic granule preparation depends on the amount of starting material. Typically, preparation of cytotoxic granules from RNK-16 harvested from ten F344 rats yields 50 to 100 μg of purified native PFN. We also usually purify about 500 μg GzmB and about 300 μg GNLY from 3×10^9 YT Indy cells and about 100 to 150 μg GzmA from 7.5×10^9 LAK cells. Although these protocols are well established and characterized, users should take care to assess their purified PFN, granzymes, or GNLY for contaminants by SDS-PAGE and silver or Coomassie staining and/or by probing with antibodies specific for PFN, GzmB, GzmA, or GNLY.

Time Considerations

The protocols for isolation of cytotoxic granules from human LAK cells or YT-Indy cells (Basic Protocol 1) or from rat RNK-16 cells (Alternate Protocol 1) involve several steps and require about 1 day. The in vitro growth of LAK/YT-Indy or in vivo amplification of RNK-16 cells require about 3 weeks to reach the desired number of cells. Extra time should also be allotted to characterize PFN and Gzms expression in the cells used for granule extraction, either by microscopy (6 hr; Support Protocol 1) or by flow cytometry (2 hr; Support Protocol 2).

Purification of the effector proteins (PFN, GzmA, GzmB, GNLY) by FPLC requires an additional day to complete (Basic Protocol 2 and Alternate Protocol 2). Although analysis of the activity of purified proteins does not require more than a few hours, this analysis must be performed before pooling positive fractions and adds extra time to the overall procedure. During purification of effector proteins, measuring PFN activity in isolated fractions by hemoglobin release assay (Support Protocol 3) requires ∼1 hr. Then, measurement of PFN activity in concentrated, pooled positive fractions and determination of the sublytic concentration can be easily performed by propidium iodide uptake (Support Protocol 4) in ∼30 min. Similarly, measuring GzmB (Support Protocol 5) or GzmA (Support Protocol 6) activity by AAD or BLT assays, respectively, can be performed in 30 min. Finally, measuring GNLY activity by colony-forming assay (Support Protocol 7) or turbidimetry (Support Protocol 8) requires ∼16 hr (which includes an overnight incubation).

Purified effector proteins (PFN/GzmA/GzmB) can then be used to induce apoptosis of target cells. The chromium release assay described in Support Protocol 9 necessitates ∼6 hr for completion, whereas measuring Gzm-induced cell death by Annexin-V/PI staining (Support Protocol 10) can be performed in 2 hr.

Acknowledgments

This work was supported by NIH grants AI 045587 and AI 063430 (JL).

Literature Cited

Beresford, P.J., Zhang, D., Oh, D.Y., Fan, Z., Greer, E.L., Russo, M.L., Jaju, M., and Lieberman, J. 2001. Granzyme A activates an endoplasmic reticulum-associated caspase-independent

nuclease to induce single-stranded DNA nicks. *J. Biol. Chem.* 276:43285-43293.

Borregaard, N., Heiple, J.M., Simons, E.R., and Clark, R.A. 1983. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. *J. Cell Biol.* 97:52-61.

Bovenschen, N., Quadir, R., van den Berg, A.L., Brenkman, A.B., Vandenberghe, I., Devreese, B., Joore, J., and Kummer, J.A. 2009. Granzyme K displays highly restricted substrate specificity that only partially overlaps with granzyme A. *J. Biol. Chem.* 284:3504-3512.

Casciola-Rosen, L., Garcia-Calvo, M., Bull, H.G., Becker, J.W., Hines, T., Thornberry, N.A., and Rosen, A. 2007. Mouse and human granzyme B have distinct tetrapeptide specificities and abilities to recruit the bid pathway. *J. Biol. Chem.* 282:4545-4552.

Chowdhury, D. and Lieberman, J. 2008. Death by a thousand cuts: Granzyme pathways of programmed cell death. *Annu. Rev. Immunol.* 26:389-420.

Cullen, S.P., Adrain, C., Luthi, A.U., Duriez, P.J., and Martin, S.J. 2007. Human and murine granzyme B exhibit divergent substrate preferences. *J. Cell Biol.* 176:435-444.

Davis, J.E., Sutton, V.R., Browne, K.A., and Trapani, J.A. 2003. Purification of natural killer cell cytotoxic granules for assaying target cell apoptosis. *J. Immunol. Methods* 276:59-68.

Donovan, J. and Brown, P. 2006. Euthanasia. *Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Ernst, W.A., Thoma-Uszynski, S., Teitelbaum, R., Ko, C., Hanson, D.A., Clayberger, C., Krensky, A.M., Leippe, M., Bloom, B.R., Ganz, T., and Modlin, R.L. 2000. Granulysin, a T cell product, kills bacteria by altering membrane permeability. *J. Immunol.* 165:7102-7108.

Froelich, C.J., Turbov, J., and Hanna, W. 1996. Human perforin: Rapid enrichment by immobilized metal affinity chromatography (IMAC) for whole cell cytotoxicity assays. *Biochem. Biophys. Res. Commun.* 229:44-49.

Hanna, W.L., Zhang, X., Turbov, J., Winkler, U., Hudig, D., and Froelich, C.J. 1993. Rapid purification of cationic granule proteases: application to human granzymes. *Protein Expr. Purif.* 4:398-404.

Keefe, D., Shi, L., Feske, S., Massol, R., Navarro, F., Kirchhausen, T., and Lieberman, J. 2005. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity* 23:249-262.

Lieberman, J. 2003. The ABCs of granule-mediated cytotoxicity: New weapons in the arsenal. *Nat. Rev. Immunol.* 3:361-370.

Martinvalet, D., Dykxhoorn, D.M., Ferrini, R., and Lieberman, J. 2008a. Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death. *Cell* 133:681-692.

Martinvalet, D., Thiery, J., and Chowdhury, D. 2008b. Granzymes and cell death. *Methods Enzymol.* 442:213-230.

Millard, P.J., Henkart, M.P., Reynolds, C.W., and Henkart, P.A. 1984. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. *J. Immunol.* 132:3197-3204.

Pipkin, M.E. and Lieberman, J. 2007. Delivering the kiss of death: Progress on understanding how perforin works. *Curr. Opin. Immunol.* 19:301-308.

Rissoan, M.C., Duhen, T., Bridon, J.M., Bendriss-Vermare, N., Peronne, C., de Saint Vis, B., Briere, F., and Bates, E.E. 2002. Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. *Blood* 100:3295-3303.

Russell, J.H. and Ley, T.J. 2002. Lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 20:323-370.

Shi, L., Yang, X., Froelich, C.J., and Greenberg, A.H. 2000. Purification and use of granzyme B. *Methods Enzymol.* 322:125-143.

Stenger, S., Hanson, D.A., Teitelbaum, R., Dewan, P., Niazi, K.R., Froelich, C.J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S.A., Bloom, B.R., Krensky, A.M., and Modlin, R.L. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 282:121-125.

Stinchcombe, J.C., Bossi, G., Booth, S., and Griffiths, G.M. 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* 15:751-761.

Strik, M.C., de Koning, P.J., Kleijmeer, M.J., Bladergroen, B.A., Wolbink, A.M., Griffith, J.M., Wouters, D., Fukuoka, Y., Schwartz, L.B., Hack, C.E., van Ham, S.M., and Kummer, J.A. 2007. Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation. *Mol. Immunol.* 44:3462-3472.

Sun, J., Bird, C.H., Buzza, M.S., McKee, K.E., Whisstock, J.C., and Bird, P.I. 1999. Expression and purification of recombinant human granzyme B from Pichia pastoris. *Biochem. Biophys. Res. Commun.* 261:251-255.

Sutton, V.R., Waterhouse, N.J., Baran, K., Browne, K., Voskoboinik, I., and Trapani, J.A. 2008. Measuring cell death mediated by cytotoxic lymphocytes or their granule effector molecules. *Methods* 44:241-249.

Voskoboinik, I. and Trapani, J.A. 2006. Addressing the mysteries of perforin function. *Immunol. Cell Biol.* 84:66-71.

Voskoboinik, I., Smyth, M.J., and Trapani, J.A. 2006. Perforin-mediated target-cell death and immune homeostasis. *Nat. Rev. Immunol.* 6:940-952.

Winkler, U., Pickett, T.M., and Hudig, D. 1996. Fractionation of perforin and granzymes by immobilized metal affinity chromatography (IMAC). *J. Immunol. Methods* 191:11-20.

Isolation of Aggresomes and Other Large Aggregates

Anatoli B. Meriin,[1] Yan Wang,[1] and Michael Y. Sherman[1]

[1]Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts

ABSTRACT

Upon permanent stresses and in various diseases, small protein aggregates may accumulate in cells and cause toxicity. A recently discovered protective system transports these aggregates to the centrosome location via microtubules, to form a large agglomerate of aggregates called the aggresome. Here, we describe a newly developed method for isolating aggresomes. This principle can also be used for purification of other large structures and even organelles. *Curr. Protoc. Cell Biol.* 48:3.38.1-3.38.9. © 2010 by John Wiley & Sons, Inc.

Keywords: aggresome • aggregates • subcellular fractionation • yeast

INTRODUCTION

Molecular chaperones and the ubiquitin-proteasome system (UPS; *UNIT 15.9*) play an important role in handling soluble abnormal polypeptides that arise as a result of misfolding, damage, or mutations. However, under certain conditions these systems fail to repair or destroy abnormal species, leading to formation of small cytoplasmic aggregates. Mechanisms of intracellular protein aggregation attract growing attention because of their relevance to a number of neuropathological conditions. In many major neurodegenerative diseases, such as amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and Huntington's disease, the pathology and the eventual death of specific neuronal populations occur due to accumulation of certain abnormal polypeptides, which can form insoluble aggregates (for review see Sherman and Goldberg, 2001).

Recently, it was discovered that special machinery has evolved that transports small protein aggregates in a microtubules-dependent manner to the centrosome, forming an organelle called the aggresome (Chung et al., 2001; Corboy et al., 2005). The aggresome serves as a storage compartment for protein aggregates, and it could be actively involved in their refolding and degradation. In fact, major chaperones, like Hsp70 or Hsp27, and components of the UPS are recruited to the aggresome (Garcia-Mata et al., 1999; McNaught et al., 2002; Ahn and Jeon, 2006; Kovacs et al., 2006). Furthermore, recently it was demonstrated that autophagic clearance of protein aggregates also occurs in association with the aggresome (Garcia-Mata et al., 2002; Pankiv et al., 2007; Olzmann and Chin, 2008).

There is a notion in the field that aggresome formation represents a protective cellular response to a buildup of aggregating abnormal polypeptides under the conditions when chaperones and UPS machineries fail to handle abnormal species (Tanaka et al., 2004; Olzmann and Chin, 2008). Indeed, it was reported that there is a close correlation between aggresome formation and cell survival (Taylor et al., 2003). Furthermore, toxicity of abnormal proteins is strongly enhanced by inhibition of the microtubule-dependent transport, which is required for aggresome formation. In line with this concept, inhibition of aggresome formation was recently suggested as an approach to enhance the cytotoxicity of proteasome inhibitors to facilitate their anti-cancer activity (Nawrocki et al., 2006; Piazza, et al., 2007).

Current Protocols in Cell Biology 3.38.1-3.38.9, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb0338s48

Beside aggresome formation, other protein aggregation pathways also appear to exist in mammalian cells. For example, mutant glial fibrillary acidic protein (GFAP) expressed in cells seems to be unable to form aggresomes, and usually forms small multiple aggregates all around the cytoplasm (Quinlan et al., 2007). In another example, we have recently demonstrated that while synphilin 1, a protein associated with Parkinson's disease, forms aggresomes, its mutant form lacking an ankyrin repeat domain can only form multiple cytoplasmic aggregates (Zaarur et al., 2008). To understand the aggresome response it is critical to use clear mechanistic criteria of aggresomes. The characteristic features of aggresomes that distinguish them from other types of protein aggregates include the microtubules-dependence of aggresome formation and its co-localization with the centrosome.

A number of factors have been implicated in aggresome formation. For example, a microtubule-associated histone deacetylase, HDAC6, was shown to interact with cytoplasmic aggregates of ubiquitinated proteins via its ubiquitin-binding BUZ domain, and facilitate their association with the dynein motor protein that drives this cargo to the aggresome (Kawaguchi et al., 2003). Other proteins also play a role in aggresome formation, e.g., PLIC, ataxin 3 (Burnett and Pittman, 2005; Heir et al., 2006), p62/sequestosome (Donaldson et al., 2003; Seibenhener et al., 2004; Lim et al., 2005), and Parkin (Lim et al., 2006). Nevertheless, our current knowledge about the mechanisms of aggresome formation is very limited. Among the major questions in the field are: (1) how the aggresome machinery recognizes protein aggregates and distinguishes them from monomeric abnormal proteins; (2) how these aggregates are recruited to microtubules; (3) how small aggregates in the aggresome are kept together; and (4) how aggresome formation suppresses the toxicity of protein aggregates. One of the approaches to address these questions would be to identify factors associated with the aggresome-forming abnormal polypeptide.

We have developed a novel method for isolation of aggresomes and other types of aggregates, utilizing a recently established yeast model of aggresome formation (Wang et al., 2007). In this model, we used as a substrate a fragment of huntingtin, a pathological protein that causes Huntington's disease. Upon expansion of its polyglutamine (polyQ) domain, huntingtin becomes toxic and aggregation-prone. An important mechanistic insight into the aggresome machinery was the finding that aggresome formation by the huntingtin fragment requires a distinct proline-rich region of this protein, which serves as a special aggresome-targeting signal (Wang et al., 2009). Accordingly, the polyQ polypeptide without the proline-rich region (103Q) formed multiple toxic aggregates, while the polyQ polypeptide with this domain (103QP) formed typical nontoxic aggresomes. This model allows separately isolating aggresomes and multiple aggregates and analyzing differences in their composition.

BASIC PROTOCOL

ISOLATION OF AGGRESOMES FROM YEAST

Yeast are grown and induced to express the GFP-containing plasmid. The cells are lysed and fractionated. Fractions expressing GFP are collected.

Materials

Wild-type yeast strain W303 (*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1-100 ssd1-d*)

pYES2 vector (used as a negative control) *or* pYES2-based plasmids for expression of 103QP or 103Q constructs under control of the *Gal*1 promoter (Meriin et al., 2001); 103QP encodes the full exon 1 of mutant (103 glutamines extension) htt tagged with FLAG at the N-terminus and with EGFP at the C-terminus; 103Q is the same construct missing the proline-rich domain of the exon 1

Selective medium with 2% (w/v) glucose (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Selective medium with 2% (w/v) galactose (see recipe)
Lysis buffer (see recipe)
Glass beads (Sigma, cat. no. G8772)
Mouse anti-FLAG monoclonal antibody (Sigma)
AffiniPure rabbit anti–mouse and goat anti–rabbit IgGs (H+L; Jackson ImmunoResearch Laboratories)
50% (w/v) sucrose in lysis buffer

30°C incubator
Bullet blender (NextAdvance)
50-ml Sephacryl S-400 HR 2.5 (dia.) × 10–cm gel filtration column
Fluorescent microscope
15-ml test tubes

Grow, induce, and collect yeast

1. Grow 250 ml of the yeast W303 cells, transfected with the appropriate plasmid, on a shaker (250 rpm) overnight at 30°C on the selective minimal medium with 2% glucose.
2. In the morning, wash the cells as follows:
   a. Collect the cells by centrifuging 10 min at 3000 × *g*, room temperature.
   b. Bring the cells into suspension with 50 ml PBS (without calcium and magnesium).
   c. Collect the cells again.
   d. Repeat the procedure once more.
3. Transfer the collected cells into 2 liters of selective medium with 2% galactose and grow 6 hr at 30°C to logarithmic phase. Collect the cells by centrifuging (see above).

   At this point, the cell pellet can be frozen.

Lyse the cells

4. To 1 g of wet cells, add 1 ml of the lysis buffer.

   This step and ALL of the following processes are conducted at 4°C.
5. Disrupt with glass beads using the Bullet blender according to the manufacturer's instruction.
6. Collect the cell lysates into an appropriate centrifugation tube and centrifuge 2.5 min at 1000 × *g*, to remove the debris.
7. Collect the supernatant, measure the total protein concentration in the clarified lysate, and adjust with the lysis buffer to equalize protein concentrations in all samples.

Fractionate the sample

8. Load the sample onto a the 2.5 (dia.) × 10–cm gel filtration column (50-ml Sephacryl S-400 HR) with a cut-off range of ~8 MDa.
9. Collect 1.7-ml fractions at 0.85 ml/min flow rate.
10. Analyze the fractions using a fluorescent microscope for the presence and abundance of the GFP-labeled aggregates.
11. Combine four fractions with the highest content of the aggresomes.

    As a control, use the corresponding fractions from the lysates of cells with vector only.

Treat the fractions with the antibody

12. Transfer the pooled fractions into a 15-ml test tube. Add the primary antibody (mouse anti-FLAG IgG) to the pooled fractions to achieve a final concentration of 70 μg/ml and incubate the solution for 1.5 hr with rotation.

    Of note, if aggresomes of a distinct protein are isolated, corresponding primary antibodies should be used for decoration of aggregates.

13. Add the secondary antibody (rabbit anti–mouse IgG) to a final concentration of 140 μg/ml and incubate the solution for 1 hr.

14. Add the tertiary antibody (goat anti–rabbit IgG) at 300 μg/ml and incubate the solution for 1.5 hr.

Isolate the aggregates

15. Overlay the samples on the 2 ml of the 50% sucrose dissolved in the same lysis buffer in 15-ml test tubes.

16. Centrifuge 2.5 min at 600 × *g*, 4°C.

17. Aspirate the supernatant and store the purified aggregate pellet at −20°C.

    We have never studied the stability of the aggregates. Obviously, it is determined by the stability of the most unstable protein(s) in the aggregates. If there are some doubts, the aggregates can be kept at −80°C. On the other hand, if the aggregates are to be assayed for enzymatic activities, freezing should be avoided altogether and isolated samples should be assayed instantly.

    The most obvious reason for isolation of aggregates is to determine their protein composition. It can be examined by one- or two-dimensional protein gel and/or by mass-spectrometry (Wang et al., 2007; Wang et al., 2009). Additionally, enzymatic activities of the particular components of the aggregates may be assayed in vitro. The aggregates are not homogeneous in regard of both their size and composition; such heterogeneity, as we explained in the Background Information, necessitated the development of the new approach to aggregates isolation.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Lysis buffer

50 mM HEPES, pH 7.5
150 mM NaCl
1% Triton X-100
1 mM PMSF
1 mM benzamidine
5 μg/ml of each: leupeptin, pepstatin A and aprotinin
Prepare fresh

Selection medium

For 1 liter of medium combine the following:
1.7 g yeast nitrogen base (w/o amino acids and ammonium sulfate)
5.0 g ammonium sulfate
20 mg adenine sulfate
Add the following amino acids (final concentrations given):
40 mg/liter L-tryptophan-40
20 mg/liter L-histidine-HCl
20 mg/liter L-arginine-HCl

continued

20 mg/liter L-methionine
30 mg/liter L-tyrosine
60 mg/liter L-leucine
30 mg/liter L-isoleucine
30 mg/liter L-lycine-HCl
50 mg/liter L-phenylalanine
100 mg/liter L-aspartic acid
100 mg/liter L-glutamic acid
150 mg/liter L-valine
200 mg/liter L-threonine
400 mg/liter L-serine
Store the medium at room temperature prior to the addition of the amino acids; with amino acids, store at 4°C
Bring to 900 ml with water
Filter-sterilize
Store up to 1 week at room temperature
To make selection medium with 2% glucose: Add 100 ml of autoclaved 20% glucose (keep at room temperature)
To make selection medium with 2% galactose: Add 100 ml of autoclaved 20% galactose (keep at room temperature)

COMMENTARY

Background Information

Multiple attempts have been undertaken to identify proteins associated with various types of protein aggregates, including aggresomes; most have employed two-hybrid screens (Faber et al. 1998, Kaltenbach et al. 2007; *UNIT 17.3*). The two-hybrid approach, however, does not allow a comprehensive search for proteins sequestered in various types of aggregates, since some of these proteins may interact only with the soluble forms of abnormal polypeptides, or be recruited via indirect interactions.

Another approach would be to isolate the aggresome and determine its composition, since factors involved in various stages of aggresome formation, as well as factors involved in the aggresome-mediated cell protection, may physically associate with the aggresome. Broad analysis of the components associated with the aggresome and other types of aggregates is hampered by the difficulties in isolation of aggregates. These difficulties, which include extreme heterogeneity of size and charge of the aggregates, preclude application of conventional biochemical methods to the purification of aggregates, e.g., gel filtration or ion-exchange chromatography. In addition, enormous sizes of aggregates practically preclude using various types of affinity chromatography for their isolation. In fact, aggregates are washed-out from affinity chromatography columns due to a shear force upon wash, even if they are covalently cross-linked to the beads. Previously, isolation of aggregates was performed by utilizing the ionic detergent insolubility of amyloids (Doi et al., 2004) or density gradient fractionation (Suhr et al., 2001). While these methods may be useful to address certain questions about the structure of protein aggregates, they are inadequate for identification of the aggregate-associated proteins. In fact, SDS treatment causes dissociation of most of the associated proteins, while density gradient isolation yields a high fraction of non-specifically associated polypeptides. Another method using a fluorescence-activated cell sorter to separate GFP-tagged aggregates overcame some of these problems. However, the cell sorter approach has several obvious limitations: it requires very large and highly homogeneous aggregates, and provides low yields (Mitsui et al., 2006). Furthermore, this method is not applicable to the isolation of aggregates from clinical samples.

Our method of aggresome isolation is based on affinity purification without involvement of a solid phase. It allows isolation of aggresomes under mild conditions that preserve associated proteins. The yields are sufficient for a broad proteomics study.

Following yeast cell lysis, the unbroken cells and cell debris are sedimented by centrifugation, which leaves aggresomes and large aggregates in the supernatant. The first step

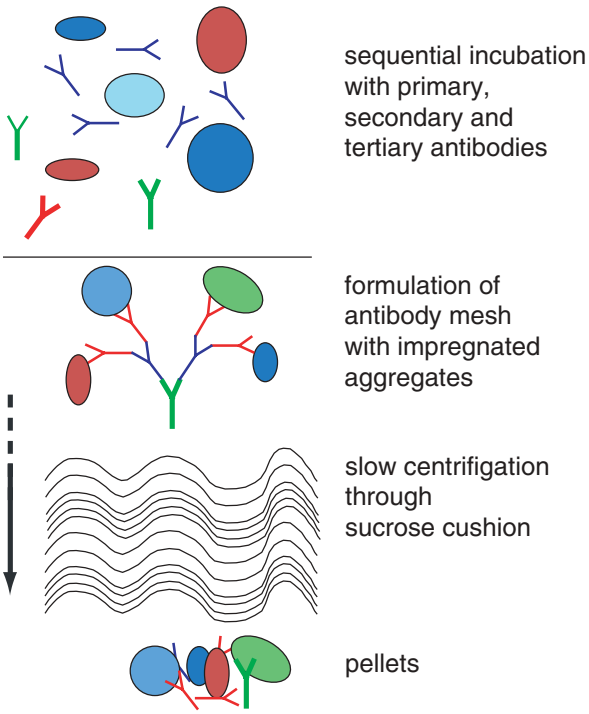


Figure 3.38.1 Scheme of Basic Protocol steps 12 through 16 of aggresome isolation.

is separation of the aggresomes from soluble mono- and oligomeric forms of the same polypeptide, as well as from soluble cellular proteins using gel filtration and collecting void volume fractions highly enriched with aggresomes. The cut-off range of the column is chosen to yield in void volume only very large structures, including aggresomes and other types of large aggregates.

The second and the main isolation step employs an approach eliminating the need for a solid support in affinity purification (Fig. 3.38.1). We first decorated aggresomes with a primary antibody (anti-FLAG, since our aggresome-forming polypeptides were FLAG-tagged). Then, we built a three-dimensional antibody mesh that incorporated aggresomes by consequent additions of an excess of secondary antibody, and finally of an excess of tertiary antibody. As a result of the consecutive incubations, the aggregates became impregnated into an IgG mesh (Fig. 3.38.2). The size of this mesh is much greater than the sizes of free large intracellular particles, like components of cytoskeleton that remain in the supernatant after the first centrifugation. Therefore, the mesh could be effectively separated from other large particles by a very low-speed centrifugation through a dense sucrose cushion. In fact, since the centrifugation through the cushion was run at a speed lower than the speed used to clarify yeast lysates before gel filtration, even the largest particles un-associated with the IgG mesh did not sediment under these conditions, and remained in supernatant.

The method allows isolation of aggresomes and other types of protein aggregates together with associated proteins. Indeed, we identified more than thirty associated proteins, including a set of molecular chaperones, several glycolytic enzymes, components of the ubiquitin-proteasome machinery, and other polypeptides (Wang et al., 2007). Beside aggresomes, this approach can be applied to isolation of any types of large structures, like other types of aggregates, components of cytoskeleton, and even organelles.

Critical Parameters

Upon adaptation of this methodology to isolation of various types of structures, one should consider that it involves two centrifugation steps. At the first centrifugation, extremely large complexes and unbroken cells are removed, and structures of interest should remain in supernatant. Then these structures are incorporated into the IgG mesh, become much larger, and could sediment at lower speeds through the dense sucrose cushion. The critical consideration is that in order to avoid contamination with unrelated complexes,

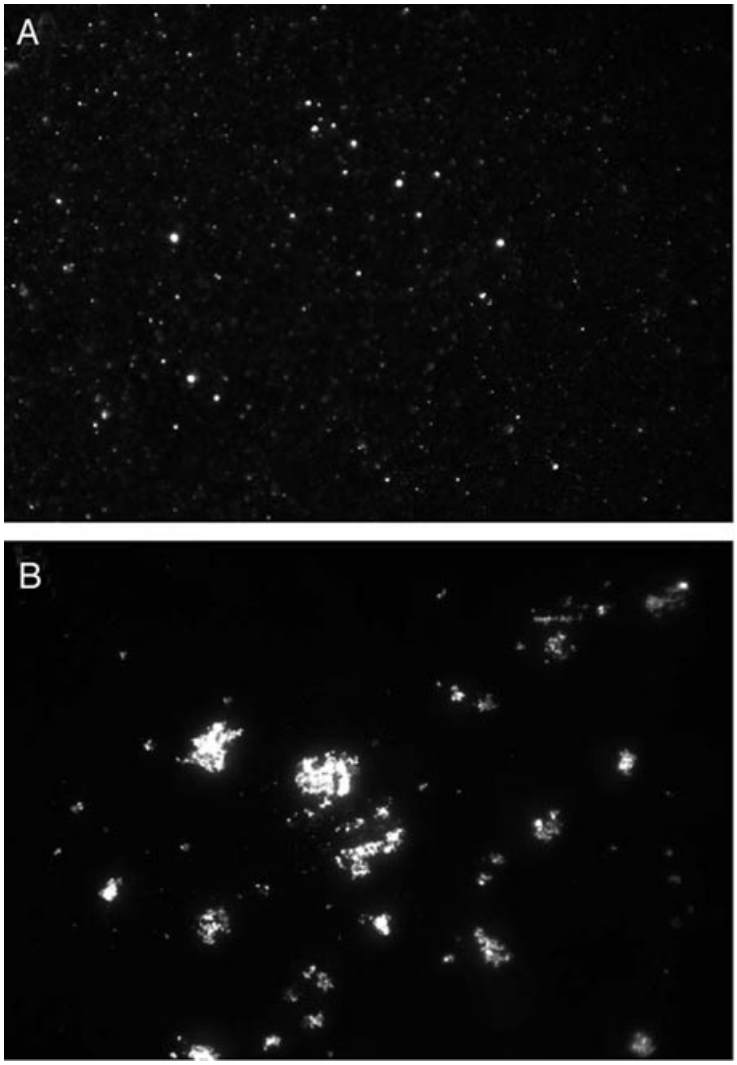


Figure 3.38.2 Appearance of aggregates before (**A**) and after (**B**) formation of the IgG mesh.

the speed of the first centrifugation must be higher than the speed of the second centrifugation. This speed difference ensures that the unrelated complexes remain in supernatant while the IgG-impregnated structures sediment.

To separate the aggresome and the associated proteins from the IgG molecules, biotinylated IgG could be used in the experiment, allowing their efficient removal after solubilization of pellets using streptavidin beads.

Anticipated Results

The described method provides yield of ~2 mg of aggresome preparation from 1 g of yeast protein. The first step of size-exclusion chromatography purifies aggresomes about 7-fold, and the second step of immunoisolation purifies them about 20-fold.

Time Considerations

Yeast growth and collection of cells takes 24 hr. The entire purification procedure should take one working day. At the end of the day, purified aggresome fractions can be frozen and stored. Analysis of the isolated fractions by two-dimensional SDS-PAGE should take another day. *Optional:* Before the gel analysis, one extra day could be spent for the removal of the antibodies using streptavidin column.

Literature Cited

Ahn, T.B. and Jeon, B.S. 2006. Protective role of heat shock and heat shock protein 70 in lactacystin-induced cell death both in the rat substantia nigra and PC12 cells. *Brain Res.* 1087:159-167.

Burnett, B.G. and Pittman, R.N. 2005. The polyglutamine neurodegenerative protein ataxin 3

regulates aggresome formation. *Proc. Natl. Acad. Sci. U.S.A.* 102:4330-4335.

Chung, K.K., Dawson, V.L., and Dawson, T.M. 2001. The role of the ubiquitin-proteasomal pathway in Parkinson's disease and other neurodegenerative disorders. *Trends Neurosci.* 24:S7-S14.

Corboy, M.J., Thomas, P.J., and Wigley, W.C. 2005. Aggresome formation. *Methods Mol. Biol.* 301:305-327.

Doi, H., Mitsui, K., Kurosawa, M., Machida, Y., Kuroiwa, Y., and Nukina, N. 2004. Identification of ubiquitin-interacting proteins in purified polyglutamine aggregates. *FEBS Lett.* 571:171-176.

Donaldson, K.M., Li, W., Ching, K.A., Batalov, S., Tsai, C.C., and Joazeiro, C.A. 2003. Ubiquitin-mediated sequestration of normal cellular proteins into polyglutamine aggregates. *Proc. Natl. Acad. Sci. U.S.A.* 100:8892-8897.

Faber, P.W., Barnes, G.T., Srinidhi, J., Chen, J., Gusella, J.F., and MacDonald, M.E. 1998. Huntingtin interacts with a family of WW domain proteins. *Hum. Mol. Genet.* 7:1463-1474.

Garcia-Mata, R., Bebok, Z., Sorscher, E.J., and Sztul, E.S. 1999. Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J. Cell Biol.* 146:1239-1254.

Garcia-Mata, R., Gao, Y.S., and Sztul, E. 2002. Hassles with taking out the garbage: Aggravating aggresomes. *Traffic* 3:388-396.

Heir, R., Ablasou, C., Dumontier, E., Elliott, M., Fagotto-Kaufmann, C., and Bedford, F.K. 2006. The UBL domain of PLIC-1 regulates aggresome formation. *EMBO Rep.* 7:1252-1258.

Kaltenbach, L.S., Romero, E., Becklin, R.R., Chettier, R., Bell, R., Phansalkar, A., Strand, A., Torcassi, C., Savage, J., Hurlburt, A., Cha, G.H., Ukani, L., Chepanoske, C.L., Zhen, Y., Sahasrabudhe, S., Olson, J., Kurschner, C., Ellerby, L.M., Peltier, J.M., Botas, J., and Hughes, R.E. 2007. Huntingtin interacting proteins are genetic modifiers of neurodegeneration. *PLoS Genet.* 3:e82.

Kawaguchi, Y., Kovacs, J.J., McLaurin, A., Vance, J.M., Ito, A., and Yao, T.P. 2003. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* 115:727-738.

Kovacs, I., Lentini, K.M., Ingano, L.M., and Kovacs, D.M. 2006. Presenilin 1 forms aggresomal deposits in response to heat shock. *J. Mol. Neurosci.* 29:9-19.

Lim, K.L., Chew, K.C., Tan, J.M., Wang, C., Chung, K.K., Zhang, Y., Tanaka, Y., Smith, W., Engelender, S., Ross, C.A., Dawson, V.L., and Dawson, T.M. 2005. Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: Implications for Lewy body formation. *J. Neurosci.* 25:2002-2009.

Lim, K.L., Dawson, V.L., and Dawson, T.M. 2006. Parkin-mediated lysine 63-linked polyubiquitination: A link to protein inclusions formation in Parkinson's and other conformational diseases? *Neurobiol. Aging* 27:524-529.

McNaught, K.S., Shashidharan, P., Perl, D.P., Jenner, P., and Olanow, C.W. 2002. Aggresome-related biogenesis of Lewy bodies. *Eur. J. Neurosci.* 16:2136-2148.

Meriin, A.B., Mabuchi, K., Gabai, V.L., Yaglom, J.A., Kazantsev, A., and Sherman, M.Y. 2001. Intracellular aggregation of polypeptides with expanded polyglutamine domain is stimulated by stress-activated kinase mekk1. *J. Cell Biol.* 153:851-864.

Mitsui, K., Doi, H., and Nukina, N. 2006. Proteomics of polyglutamine aggregates. *Methods Enzymol.* 412:63-76.

Nawrocki, S.T., Carew, J.S., Pino, M.S., Highshaw, R.A., Andtbacka, R.H., Dunner, K. Jr., Pal, A., Bornmann, W.G., Chiao, P.J., Huang, P., Xiong, H., Abbruzzese, J.L., and McConkey, D.J. 2006. Aggresome disruption: A novel strategy to enhance bortezomib-induced apoptosis in pancreatic cancer cells. *Cancer Res.* 66:3773-3781.

Olzmann, J.A. and Chin, L.S. 2008. Parkin-mediated K63-linked polyubiquitination: A signal for targeting misfolded proteins to the aggresome-autophagy pathway. *Autophagy* 4:85-87.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* 282:24131-24145.

Piazza, F.A., Gurrieri, C., Trentin, L., and Semenzato, G. 2007. Towards a new age in the treatment of multiple myeloma. *Ann. Hematol.* 86:159-172.

Quinlan, R.A., Brenner, M., Goldman, J.E., and Messing, A. 2007. GFAP and its role in Alexander disease. *Exp. Cell Res.* 313:2077-2087.

Seibenhener, M.L., Babu, J.R., Geetha, T., Wong, H.C., Krishna, N.R., and Wooten, M.W. 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. *Mol. Cell Biol.* 24:8055-8068.

Sherman, M.Y. and Goldberg, A.L. 2001. Cellular defenses against unfolded proteins: A cell biologist thinks about neurodegenerative diseases. *Neuron* 29:15-32.

Suhr, S.T., Senut, M.C., Whitelegge, J.P., Faull, K.F., Cuizon, D.B., and Gage, F.H. 2001. Identities of sequestered proteins in aggregates from cells with induced polyglutamine expression. *J. Cell Biol.* 153:283-294.

Tanaka, M., Kim, Y.M., Lee, G., Junn, E., Iwatsubo, T., and Mouradian, M.M. 2004. Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. *J. Biol. Chem.* 279:4625-4631.

Taylor, J.P., Tanaka, F., Robitschek, J., Sandoval, C.M., Taye, A., Markovic-Plese, S., and Fischbeck, K.H. 2003. Aggresomes protect

cells by enhancing the degradation of toxic polyglutamine-containing protein. *Hum. Mol. Genet.* 12:749-757.

Wang, Y., Meriin, A.B., Costello, C. and Sherman, M.Y. 2007. Characterization of proteins associated with polyglutamine aggregates: A novel approach towards isolation of aggregates from protein conformation disorders. *Prion* 1:128-135.

Wang, Y., Meriin, A.B., Zaarur, N., Romanova, N.V., Chernoff, Y.O., Costello, C.E., and Sherman, M.Y. 2009. Abnormal proteins can form aggresome in yeast: aggresome-targeting signals and components of the machinery. *FASEB J.* 23:451-463.

Zaarur, N., Meriin, A.B., Gabai, V.L., and Sherman, M.Y. 2008. Triggering aggresome formation. Dissecting aggresome-targeting and aggregation signals in synphilin 1. *J. Biol. Chem.* 283:27575-27584.

Isolation of Chromaffin Granules

Carl E. Creutz[1]

[1]Department of Pharmacology, University of Virginia, Charlottesville, Virginia

ABSTRACT

Adrenal medullary chromaffin granules (dense core secretory vesicles) have been a valuable model system for the study of the proteins and membrane components involved in the process of exocytosis. Because of the abundance of chromaffin granules in a readily available tissue source, bovine adrenal medullae, and their unique sedimentation properties, it is possible to obtain large quantities of highly purified granules and granule membranes in a short period of time. Two protocols are presented here for the isolation of chromaffin granules: a basic protocol based on differential centrifugation in an iso-osmotic medium that yields intact chromaffin granules, and an alternate protocol based on sedimentation through a density step gradient that provides a greater yield of more highly purified chromaffin granules. Since in the latter case the granules cannot be returned to a medium of physiological osmolarity without lysis after purification on the step gradient, the alternate protocol is more useful to obtain the granule membranes or contents for further study. *Curr. Protoc. Cell Biol.* 48:3.39.1-3.39.10. © 2010 by John Wiley & Sons, Inc.

Keywords: chromaffin granule • chromaffin granule membrane • secretory vesicle • dense core vesicle • adrenal medulla • catecholamine • chromogranin • exocytosis • cytochrome b562 • dopamine-β-hydroxylase • bovine

INTRODUCTION

Two protocols are presented here for the isolation of chromaffin granules, the dense core secretory vesicles of the adrenal medulla. Although the protocols can be applied to other species, the granules are usually isolated from bovine adrenals. Using 25 to 50 glands, about 50 mg of chromaffin granule protein can readily be obtained in 4 hr after adrenal glands arrive in the laboratory. The first procedure is based on differential centrifugation in an iso-osmotic medium and yields intact chromaffin granules (Hillarp, 1958; Taugner and Hasselbach, 1966; Pollard et al., 1976; see Basic Protocol). The second procedure is based on sedimentation through a density step gradient and provides a greater yield of more highly purified chromaffin granules (Smith and Winkler, 1967; Bartlett and Smith, 1974; see Alternate Protocol). Since in this case the granules cannot be returned to a medium of physiological osmolarity without lysis after purification on the step gradient, the second protocol is more useful to obtain the granule membranes or contents for further study.

ISOLATION OF CHROMAFFIN GRANULES BY DIFFERENTIAL CENTRIFUGATION IN ISO-OSMOTIC MEDIUM

BASIC PROTOCOL

This protocol yields intact chromaffin granules useful for studies of the properties and interactions of the whole organelle in vitro. The alternate protocol described below provides a greater yield of granules for biochemical studies of the membrane components or granule contents.

Materials

5 to 30 bovine adrenal glands, freshly collected and transported to the laboratory
Cold 0.3 M sucrose solution (store at 4°C to inhibit bacterial growth), 1 liter

Surgical scissors, 2-in. blades
500-ml beakers

Current Protocols in Cell Biology 3.39.1-3.39.10, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb0339s48

Kitchen cutting board
Surgical scissors, 1-in. blades
Mouse-tooth forceps, 5-in.
Waring blender
Potter-Elvehjem glass homogenizer with a loose-fitting Teflon pestle, 15 to 20 ml volume (a loose fitting pestle is one that shows little or no resistance or suction when moved up and down in the glass tube in air)
1/4-in. electric hand drill for Potter-Elvehjem pestle
Gauze sponges
Rubber band
8-in. stainless steel spatula with a 1/4-in. wide, rounded blade
Clear, 40-ml polycarbonate centrifuge tubes
Refrigerated high speed centrifuge with Sorvall SS-34 rotor or equivalent
40-ml Dounce homogenizer with loose-fitting (type B) pestle
15-ml Dounce homogenizer with loose fitting (type B) pestle

NOTE: All glassware and equipment must be completely free of detergents in order to preserve the integrity of the chromaffin granule membranes.

NOTE: After initial dissection of the adrenal glands, all materials are kept on ice.

Obtain and prepare the bovine adrenal glands

1. Obtain bovine adrenal glands at a slaughterhouse not more than 90 min away from the laboratory. Call the slaughterhouse just before going in order to ensure that they are slaughtering that day.

   Small, local slaughterhouses often operate erratically.

2. To obtain the most rapid post-mortem chilling stand on the killing room floor and receive the adrenals as they are removed.

   This requires dressing with rubber boots, laboratory coat, hairnet, hard hat, safety glasses, and surgical gloves.

   The glands are removed by slaughterhouse workers along with the kidneys right after the carcass is sawn in half. The glands are often embedded in fat and not visible. An experienced floor worker will be able to slice the gland from the kidney. A monetary gratuity may help provide the most intact glands.

3. Trim additional fat from the gland with surgical scissors (2-in. blade) to expose the gland and promote rapid cooling before plunging it into a plastic bag in an ice bucket.

   Some companies offer to remove adrenals at a slaughterhouse and ship them on ice overnight (not frozen which would destroy the chromaffin granules). However, there appears to be little or no published experience with trying to isolate chromaffin granules from such a source. In general, it is felt that the best practice is to minimize the length of time from killing to chilling, and subsequently to dissecting the medullae from the glands.

Dissect the adrenal medullae from the glands

4. Record the weight of one 500-ml beaker and place it on ice.

5. Remove the glands from the ice bucket one at a time and dissect them on the cutting board at room temperature.

   The bovine adrenal gland is about the size of a human thumb. It is also similar in shape to a thumb, but the actual shape varies considerably. Adrenal medullary tissue is pink-orange due to the presence of cytochrome b561 in the chromaffin granule membrane (responsible for electron transport supporting the activity of dopamine-β-hydroxylase in the granule interior; Fleming and Kent, 1991), and so can easily be distinguished from the liver-colored adrenal cortical tissue that forms a 3- to 5-mm layer surrounding the medulla. Outside the cortex, the gland is encased in a fairly tough, membranous capsule.

6. Remove any significant remaining chunks of fat with the 2-in. surgical scissors.
7. With the 1-in scissors, cut directly into the gland through the capsule 3- to 5-mm deep along the longest circumference of the gland.

   That is, if the gland lies flat on the cutting board, the incision would be all the way around the edge, parallel to the cutting board surface (although it is easier to pick up the gland when making this cut). The object is to cut through the cortex, all the way around the gland, but not deeply into the medulla.

8. Now, lay the gland flat on the cutting board. With the mouse-tooth forceps, grasp the top edge of the incision at one end of the gland. Lift the cortical tissue to reveal the medullary tissue.
9. Carefully cut between the pink-orange medulla and the brown cortex with the 1-in. surgical scissors all the way across the top of the gland. Discard the cortical tissue.
10. Then grasp the pink-orange medullary tissue with the forceps and lift it up as you cut with the scissors between the medulla and the cortex on the bottom half of the gland, all the way across the gland. Discard the remaining cortical tissue, and immediately drop the medullary tissue into the 500-ml weighed beaker chilled on ice.

    With practice, the dissection takes about 5 min per gland. When dissecting multiple glands it is helpful to recruit others in the laboratory to assist with the dissection.

    Each gland will yield 1 to 2 g of medullary tissue (beef steer have smaller glands; culled bulls and dairy cows have larger glands). The dissection does not have to be perfect. It is more important to minimize the loss of medullary tissue than to remove every trace of cortical tissue.

Homogenize the adrenal medullary tissue

11. Determine the mass of adrenal medullary tissue by weighing the previously tared and chilled 500-ml beaker, and add 5 vol of ice-cold 0.3 M sucrose.
12. Homogenize the tissue in an ice-cold Waring blender on high setting for two 3-sec bursts.
13. Pour the homogenate, 15 to 20 ml at a time, into the Potter-Elvehjem homogenizer.
14. Homogenize with the pestle driven by the electric drill at high speed, one pass, down and up.

    Because of the high speed and force of the motor-driven pestle in the glass homogenizer, this step is potentially dangerous as the glass homogenizer may shatter if the pestle is inadvertently pushed into the glass tube at an angle. If this step is done with a hand drill, wear a heavy glove, like an autoclave glove, on the hand holding the glass tube. An alternative that reduces the chance of shattering the glass homogenizer is to drive the pestle with a small drill press.

15. Take four gauze sponges, unfold them, and stack them to form a layer of gauze four-ply thick. Attach the gauze layer to the top of a 500-ml beaker on ice with a rubber band.
16. Pour the homogenate fractions from the Potter-Elvehjem homogenizer onto the gauze. Promote the drainage of the homogenate through the gauze by using the stainless steel spatula to spread the homogenate across the surface of the gauze.
17. After all of the homogenate has been poured onto the gauze, extract the rest of the well-dispersed material by removing the rubber band, picking up the gauze and squeezing out the remaining liquid to add to the filtered homogenate. Transfer to 40-ml centrifuge tubes.

Isolate the chromaffin granules by differential centrifugation.

18. Centrifuge the homogenate 10 min at 500 × *g* (e.g., 2000 rpm in the SS-34 rotor), 4°C, to sediment nuclei and partially broken cells.

    If, for example, 25 ml of medullary tissue were homogenized initially, about 120 ml of homogenate will be obtained and that will fit conveniently into four SS-34 tubes.

19. Decant the supernatant into a new set of 40-ml tubes and centrifuge 30 min at 20,000 × *g* (13,000 rpm in the SS-34 rotor), 4°C.

20. Discard the supernatant and add 5 ml of cold 0.3 M sucrose to wash the upper part of the pellet in each tube. Use a gentle swirling motion that removes the looser, brownish part of the pellet. Discard the wash.

21. Resuspend the pellet (known as the "large granule fraction") from each tube with 30 ml of 0.3 M sucrose per tube. Add the sucrose solution, and then use the spatula to slice up and free the pellet from the tube wall. Do not disturb, and leave behind, any dark brown material or red blood cells that may appear at the very bottom of the pellet.

22. With a swirling motion, decant the resuspended pellet into a cold 40-ml Dounce homogenizer with a loose-fitting pestle. Homogenize the pellet with 3 to 4 gentle strokes of the pestle.

23. Pour the suspensions into a new set of centrifuge tubes and centrifuge 30 min at 10,000 × *g* (9,400 rpm in the SS-34 rotor), 4°C.

    After this centrifugation, the pellets will tend to have two zones, a firm pink zone at the bottom of the pellet enriched in chromaffin granules, and a looser, brownish upper layer enriched in mitochondria.

24. Discard the supernatant and swirl off the upper layer, as before, with 5 ml of cold 0.3 M sucrose solution.

    As much as half of the volume of the pellet may be washed away at this step.

25. Resuspend the remaining, pink part of the pellet, as before, with 30 ml of 0.3 M sucrose solution and repeat the Dounce homogenization.

26. Centrifuge the homogenized pellets 30 min at 7000 × *g* (7700 rpm in the SS-34 rotor), 4°C.

27. Discard the supernatant, wash the pellet as before with 5 ml of 0.3 M sucrose solution, then resuspend all pellets together (e.g., from four tubes) in a total of 10 ml of 0.3 M sucrose using a 15-ml Dounce homogenizer with a loose-fitting pestle. Store the purified granules at 4°C, or on ice, in the 0.3 M sucrose for 2 or 3 days maximum.

    The preparation can be frozen indefinitely at −70°C if intact granules are no longer needed (e.g., to isolate membranes or granule contents at a later time).

ALTERNATE PROTOCOL

ISOLATION OF CHROMAFFIN GRANULES BY SEDIMENTATION THROUGH A SUCROSE DENSITY STEP

Chromaffin granules of higher purity can be obtained by sedimenting the large granule fraction obtained after the 20,000 × *g* centrifugation in the Basic Protocol on a sucrose step gradient.

***Additional Materials** (also see Basic Protocol)*

250 ml cold 1.6 M sucrose solution, store at 4°C to inhibit bacterial growth
Clear, thick-walled, 60-ml polycarbonate ultracentrifuge tubes
10-ml pipet

Refrigerated ultracentrifuge and large-capacity fixed-angle rotor, e.g., Beckman Type 45 Ti

Tight-fitting, type A pestle for the 40-ml Dounce homogenizer

NOTE: All additional steps below are performed at 4°C or on ice. These steps must be undertaken immediately after the preparatory steps in the Basic Protocol above; granules kept for hours or longer gradually lose their contents and will not sediment properly in a density gradient.

Collect the chromaffin granules

1. Carry out steps 1 through 20 of the Basic Protocol.
2. Pour 40 ml of 1.6 M sucrose into each of several 60-ml ultracentrifuge tubes (number equal to the number of chromaffin granule pellets in step 20 of the Basic Protocol).
3. Resuspend each pellet from step 20 of Basic Protocol in 20 ml 0.3 M sucrose, leaving behind brown matter and red cells, and homogenize with a Dounce homogenizer with a loose-fitting pestle.
4. Carefully overlay the 20 ml of granule suspension from each tube on top of the 1.6 M sucrose in a 60-ml ultracentrifuge tube. Use a 10-ml pipet and gently allow the suspension to run down the inner wall of the centrifuge tube without stirring the underlying 1.6 M sucrose.
5. Centrifuge the step gradients for 60 min at 100,000 × *g* (e.g., 35,000 in the Beckman Type 45 Ti rotor), 4°C.
6. After the centrifugation, the pink chromaffin granules will be in the pellet and the other organelles, mainly mitochondria, will be in a band at the interface between the 0.3 M and 1.6 M sucrose fractions. Decant the supernatant, and save the pellet.

   The purified granules from the steps above can be stored intact in 1.6 M sucrose for a few days. However, most applications of these granules involve studies of the membranes from the granules, or the secretory contents of the granules (catecholamines, ATP, chromogranins, bioactive peptides, dopamine-β-hydroxylase, etc.).

Prepare chromaffin granule membranes and lysate

7. To separate the chromaffin granule membranes from secretory contents, resuspend each pellet in 25 ml deionized water with eight vigorous strokes with a 40-ml Dounce homogenizer, using a tight-fitting pestle.
8. Collect the membranes by centrifuging 30 min at 39,000 × *g* (e.g., 18,000 RPM in a Sorvall SS-34 rotor), 4°C.
9. Resuspend the pellets in 5 ml water or appropriate experimental buffer with a 15-ml Dounce homogenizer.
10. Save the supernatant for studies of the granule contents and the resuspended pellets for studies of the membranes.

    Both membranes and supernatant may be stored indefinitely at −70°C.

COMMENTARY

Background Information

"Previous studies have emphasized the necessity to get more exact information on the structure, composition, and enzyme activities of the specific granules storing the catechol amines of the adrenal medulla. Without such information, it does not seem possible to elucidate some essential problems concerning the storage and release of the amines. Several data of interest in this respect are, however, difficult to obtain unless specific granules of high purity are available. The present work is a first report of some experiments to separate the large granules of the adrenal medulla into

fractions and to isolate the specific granules. (Hillarp, 1958)"

This opening paragraph of Nils-Åke Hillarp's landmark paper on the isolation of chromaffin granules reflected the aspirations of many who pioneered the field of molecular cell biology in the 1950s and 60s. Organelle isolation was to become a sine qua non for understanding the molecular basis of intracellular activities. As a model secretory vesicle, the chromaffin granule (so named due to the affinity of the organelle for chromium used in histological studies of the adrenal medulla) has played an important role in the development of our understanding of the secretory pathway and the process of exocytosis. Indeed, the determination that the contents of isolated chromaffin granules are identical to the materials that are secreted by the adrenal medulla is one of the critical experimental underpinnings of the concept that exocytosis is a process in which a secretory vesicle membrane fuses with the plasma membrane and its contents are expelled from the cell (Douglas and Poisner, 1966; Kirshner et al., 1966).

Several characteristics have made chromaffin granules a particularly rewarding target for cell fractionation and organelle isolation. First, they are abundant. The cytoplasm of the highly differentiated chromaffin cell of the adrenal medulla is densely packed with the organelles (Fig. 3.39.1), and bovine adrenal medullary tissue itself is readily available in 100-g quantities. Second, they are dense. This unusual

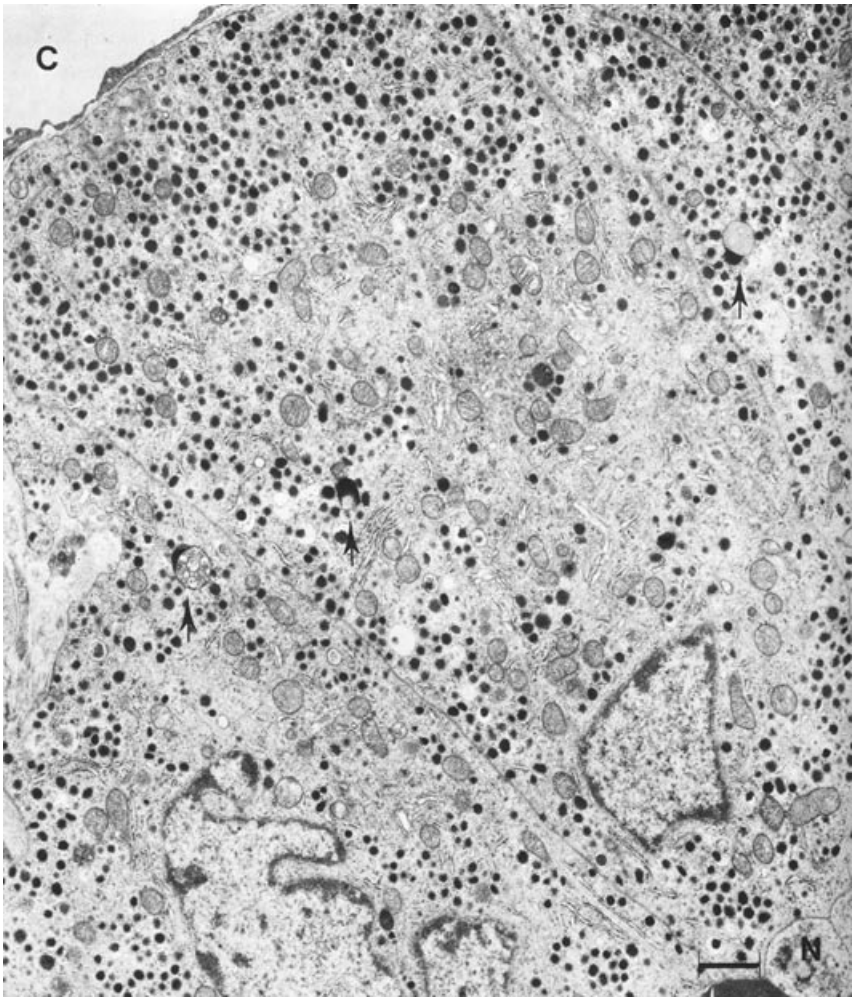


Figure 3.39.1 Electron micrograph of a thin section of several chromaffin cells of the adrenal medulla of the hamster. The cytoplasm of the chromaffin cells is seen to contain numerous, dense-core chromaffin granules. C, lumen of an adrenal medullary capillary, which receives the contents of the chromaffin granules when they are discharged by exocytosis. N, nerve terminal involved in chromaffin cell stimulation. Length of scale bar on the lower right, 1 μm. (Reproduced from Grynszpan-Winograd, 1975 with permission from the American Physiological Society).

density compared to other organelles, which is enhanced by dehydration in hyperosmotic sucrose solutions, greatly facilitates their isolation by differential and/or density gradient centrifugation. Third, they are pink. Due to the presence of cytochrome b561 in their membranes, they carry a readily visible marker that aids in following their purification.

Critical Parameters

Of the many published procedures for the purification of chromaffin granules, almost all are based on differential or gradient purification methods with minor variations. The procedures described here are essentially those that were standardized in the laboratory of Harvey Pollard at the NIH during the 1970s. An essential element is the use of iso-osmotic solutions (0.3 M sucrose) to maintain the integrity of the granules, which are osmotically quite labile. If they lose their contents, their characteristic behavior on centrifugation is lost.

Another critical parameter is the use of low-ionic-strength solutions (again, 0.3 M sucrose). In the presence of physiological ionic strengths (even as little as 30 mM KCl) the organelles in the large granule fraction (pellet from 20,000 × *g* centrifugation in the Basic Protocol) remain bound to one another by residual cytoskeletal components, and the chromaffin granules do not separate well from the other organelles during centrifugation. This is why the granules are mixed throughout the first high-speed pellet with other organelles, and only begin to separate with the repeated washes and the series of centrifugations at descending *g*-forces (Taugner and Hasselbach, 1966; Pollard et al., 1976).

When working with the unbuffered sucrose solutions, the pH of the homogenate is around pH 6.0. This is close to the internal pH of chromaffin granules (Pollard et al., 1979) and is likely due to the natural buffering provided by granule contents leaked from broken granules. This slightly acidic pH tends to stabilize the granules and aids further in separating organelles from cytoskeletal components. However, in some cases buffering of the homogenate may be desirable, for example when isolating calcium-dependent, chromaffin granule–binding proteins (the chromobindins; Creutz, 1981; Creutz et al., 1983) from the soluble fraction of the same homogenate used to isolate granules, or to ensure that the purified organelles do not have residual amounts of these peripheral proteins bound to them. In this case, one can add 2 mM EGTA to the sucrose solution to chelate calcium and 5 to

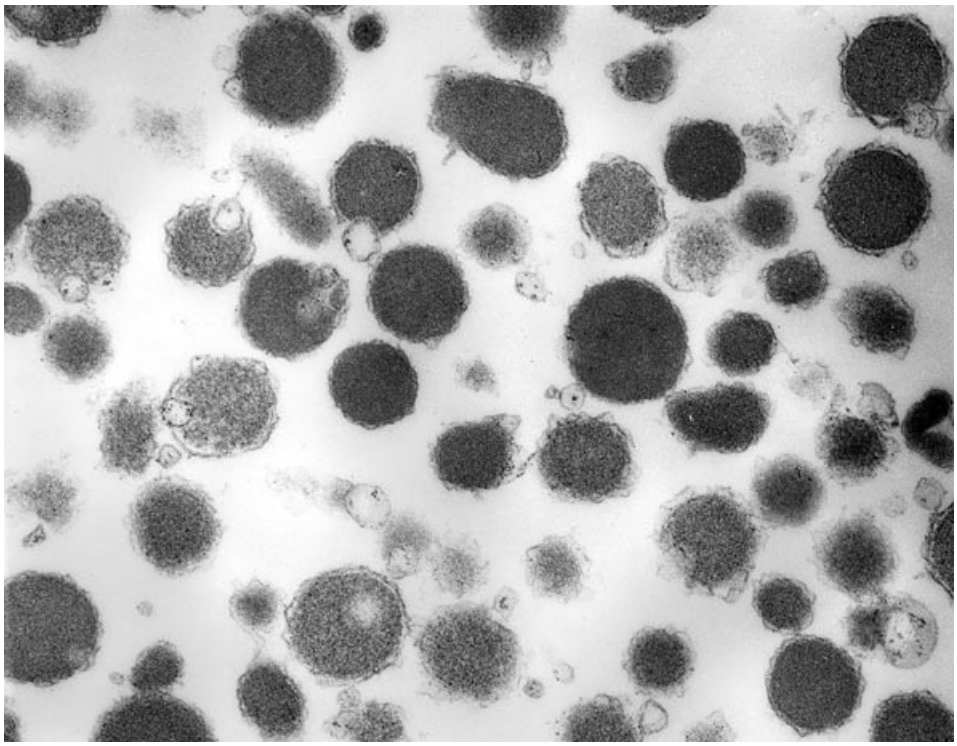


Figure 3.39.2 Electron micrograph of a thin section of a preparation of isolated bovine adrenal medullary chromaffin granules.

10 mM HEPES-NaOH, pH 7.3, to ensure that the affinity of EGTA for calcium, which is reduced at acidic pH, will remain high. These additions may reduce the purity and yield of granules slightly, but this does not seem to be prohibitive for most applications. The addition of protease inhibitors at the low concentrations usually used in biochemical extractions is also not a significant problem.

The two protocols described here provide two types of granule preparations. The Basic Protocol yields intact organelles that can be used in more physiological experimentation, but these granules are of lower purity (around 80% to 90% chromaffin granules), with the major contaminant being mitochondria. Examples of electron micrographs of granules prepared this way can be seen in Creutz et al., 1978, and in Figure 3.39.2. The Alternate Protocol yields more and higher purity granules (more than 95% chromaffin granules) useful primarily for study of the biochemistry of the granule membrane and membrane proteins. Figure 3.39.3 shows a representative two-dimensional gel of the membrane proteins. If higher purity, intact chromaffin granules are needed, one may use alternative procedures based on the use of iso-osmotic density gradients of Metrizamide (Morris and Schovanka, 1977; Pollard et al., 1979), Percoll (Gratzl et al., 1981), or Ficoll (Trifaro and Dworkind, 1970).

The protocols described here are preparative in nature. Some contaminating organelles are still present in the final products. If one wishes to determine, for example, if a given protein or lipid is a specific component of the chromaffin granule, one must resort to traditional, analytical, continuous density gradient centrifugation, using a sucrose gradient (e.g., Parsons and Creutz, 1986) or one of the iso-osmotic gradient methods cited above. The distribution of the marker of interest is then compared with accepted markers for

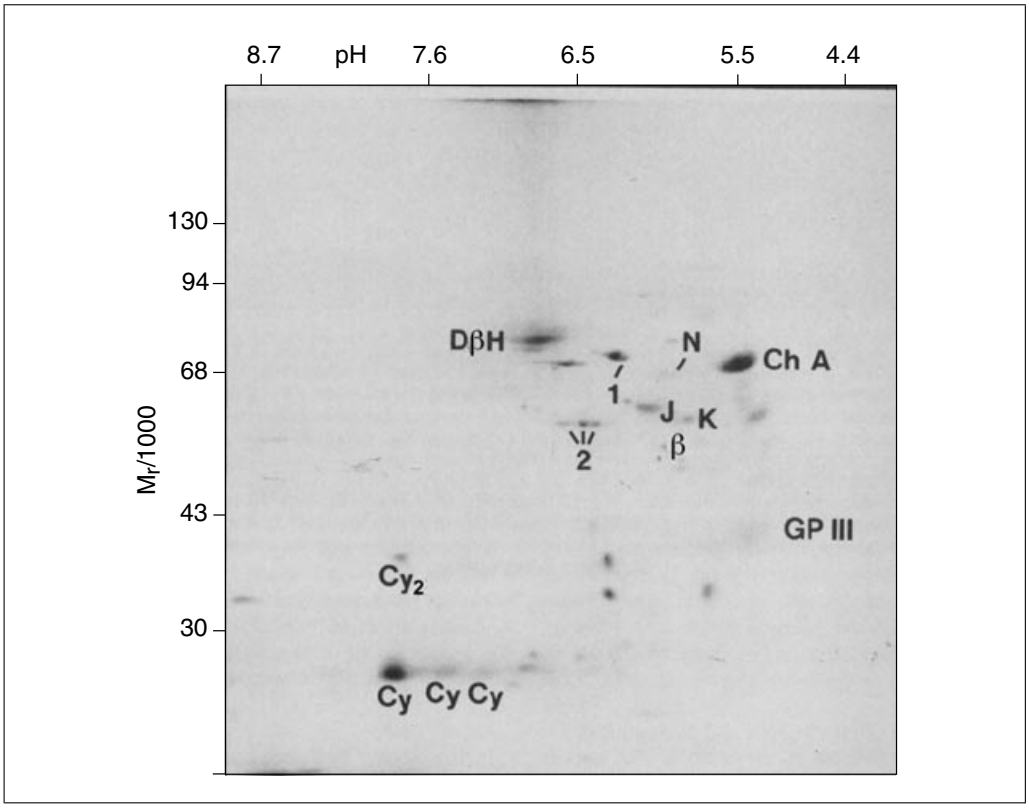


Figure 3.39.3 Two-dimensional electrophoretic gel of bovine chromaffin granule membrane proteins stained with Coomassie blue. Major proteins identified are Ch A, chromogranin A (a residual content protein); DβH, dopamine-β-hydroxylase; Cy and Cy_2, cytochrome b561 (monomeric and dimeric forms); GP III, glycoprotein III (poorly stained with Coomassie blue); J,K, glycoproteins J and K; N, nucleotide carrier; β, subunit of F1-ATPase from contaminating mitochondrial membranes; 1,2, subunits of the chromaffin granule ATPase I. Also known to be present from functional studies but unidentified or unstained: synaptobrevin, a catecholamine transporter, phosphatidyl inositol kinase, and a calcium transporter. (Reproduced from Winkler et al., 1986 with permission from Elsevier).

chromaffin granules, such as the catecholamines, chromogranin A, cytochrome b561, or dopamine-β-hydroxylase.

Procedures similar to those described here have been used to isolate chromaffin granules from a range of animals including cows, sheep, humans, dogs, cats, and rats. For systems where the amount of tissue is quite limited, the procedures must be correspondingly reduced in scale because chromaffin granules are less stable in highly dilute homogenates. With the probable development of genetically engineered mice with interesting mutations in chromaffin granule proteins, it seems likely that isolation of chromaffin granules on a very small scale may become increasingly important in the future. However, the principles and practice of isolation described here should still apply.

Troubleshooting

In actual practice, all of the following problems have caused these protocols to fail:

1. Too long a post-mortem delay before the glands are removed and chilled.

2. Too long a delay before beginning the dissection.

3. Homogenizing medium is not iso-osmotic: Solution incorrectly prepared or some of the sucrose consumed by bacteria.

4. Contamination of solutions with detergent.

5. Failure of the ultracentrifuge to complete the run with the sucrose gradients.

Anticipated Results

Starting with 50 g of adrenal medullary tissue, the Basic Protocol should yield a final 10-ml suspension of 5 mg/ml chromaffin granule protein. Using Alternate Protocol, the yield will be about twice as great. The yield of chromaffin granule membrane protein is about 20% of the total granule protein.

Time Considerations

After the adrenal glands arrive in the laboratory, purified chromaffin granules are obtained in ~4 hr.

Literature Cited

Bartlett, S.F. and Smith, A.D. 1974. Adrenal chromaffin granules: Isolation and disassembly. *Methods Enzymol.* 31:379-389.

Creutz, C.E. 1981. Secretory vesicle-cytosol interactions in exocytosis: Isolation by Ca^{2+}-dependent affinity chromatography of proteins that bind to the chromaffin granule membrane. *Biochem. Biophys. Res. Commun.* 103:1395-1400.

Creutz, C.E., Pazoles, C.J., and Pollard, H.B. 1978. Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. *J. Biol. Chem.* 253:2858-2866.

Creutz, C.E., Dowling, L.G., Sando, J.J., Villar-Palasi, C., Whipple, J.H., and Zaks, W.J. 1983. Characterization of the chromobinins: Soluble proteins that bind to the chromaffin granule membrane in the presence of Ca^{2+}. *J. Biol. Chem.* 258:14664-14674.

Douglas, W.W. and Poisner, A.M. 1966. Evidence that the secreting adrenal chromaffin cell releases catecholamines directly from ATP-rich granules. *J. Physiol.* 183:236-248.

Fleming, P.J. and Kent, U.M. 1991. Cytochrome b561, ascorbic acid, and transmembrane electron transfer. *Am. J. Clin. Nutr.* 54:1174S-1178S.

Gratzl, M., Krieger-Brauer, H., and Ekerdt, R. 1981. Latent acetylcholinesterase in secretory vesicles isolated from adrenal medulla. *Biochim. Biophys. Acta* 649:355-366.

Grynszpan-Winograd, O. 1975. Ultrastructure of the chromaffin cell. *In* Handbook of Physiology, Section 7: Endocrinology, Volume VI: Adrenal Gland (H. Blaschko, G. Sayers, and A.D. Smith, eds.) pp. 295-308. American Physiological Society, Bethesda, Md.

Hillarp, N.-Å. 1958. Isolation and some biochemical properties of the catechol amine granules in the cow adrenal medulla. *Acta Physiol. Scand.* 43:82-96.

Kirshner, N., Sage, H.J., Smith, W.J., and Kirshner, A.G. 1966. Release of catecholamines and specific protein from adrenal glands. *Science* 154:529-531.

Morris, S.J. and Schovanka, I. 1977. Some physical properties of adrenal medulla chromaffin granules isolated by a new continuous iso-osmotic density gradient method. *Biochim. Biophys. Acta* 464:53-64.

Parsons, S.J. and Creutz, C.E. 1986. *p60c-src* activity detected in the chromaffin granule membrane. *Biochem. Biophys. Res. Commun.* 134:736-742.

Pollard, H.B., Zinder, O., Hoffman, P.G., and Nikodejevic, O. 1976. Regulation of the transmembrane potential of isolated chromaffin granules by ATP, ATP analogs, and external pH. *J. Biol. Chem.* 251:4544-4550.

Pollard, H.B., Shindo, H., Creutz, C.E., Pazoles, C.J., and Cohen, J.S. 1979. Internal pH and state of ATP in adrenergic chromaffin granules determined by ^{31}P nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* 254:1170-1177.

Smith, A.D. and Winkler, H. 1967. A simple method for the isolation of adrenal chromaffin granules on a large scale. *Biochem. J.* 103:480-482.

Taugner, G. and Hasselbach, W. 1966. On the mechanism of catecholamine storage in the chromaffin granules of the adrenal medulla. *Naunyn Schmiedebergs Arch. Exp. Pathol. Pharmakol.* 255:266-286.

Trifaro, J.M. and Dworkind, J. 1970. A new and simple method for isolation of adrenal chromaffin granules by means of an isotonic density gradient. *Anal. Biochem.* 34:403-412.

Winkler, H., Apps, D.K., and Fischer-Colbrie, R. 1986. The molecular function of adrenal chromaffin granules: Established facts and unresolved topics. *Neuroscience* 18:261-290.

Key References

Pollard, H.B., Pazoles, C.J., Creutz, C.E., and Zinder, O. 1979. The chromaffin granule and possible mechanisms of exocytosis. *Int. Rev. Cytol.* 58:159-197.

Winkler et al., 1986. See above.

Winkler, H. and Smith, A.D. 1975. The chromaffin granule and the storage of catecholamines. *In* Handbook of Physiology, Section 7: Endocrinology, Volume VI: Adrenal Gland (H. Blaschko, G. Sayers, and A.D. Smith, eds.) pp. 321-339. American Physiological Society, Bethesda, MD.

The above reviews provide a guide to the extensive literature on the chromaffin granule and the role it has played in the development of our understanding of secretory vesicle structure and function.

Purification of Ribosomes from Human Cell Lines

Stéphane Belin,[1,2] Sabine Hacot,[1] Lionel Daudignon,[1] Gabriel Therizols,[1] Stéphane Pourpe,[1] Hichem C. Mertani,[1] Manuel Rosa-Calatrava,[1] and Jean-Jacques Diaz[1]

[1]Université de Lyon, Lyon, France
[2]Boston Children Hospital/Harvard Medical School, Boston, Massachusetts

ABSTRACT

Highly conserved during evolution, the ribosome is the central effector of protein synthesis. In mammalian cells, the ribosome is a macromolecular complex composed of four different ribosomal RNAs (rRNA) and about 80 ribosomal proteins. Requiring more than 200 factors, ribosome biogenesis is a highly complex process that takes place mainly within the nucleoli of eukaryotic cells. Crystallographic data suggest that the ribosome is a ribozyme, in which the rRNA catalyses the peptide bond formation and ensures quality control of the translation. Ribosomal proteins are involved in this molecular mechanism; nonetheless, their role is still not fully characterized. Recent studies suggest that ribosomes themselves and/or the mechanisms underlying their synthesis, processing, and assembly play a key role in the establishment and progression of several human pathologies. The protocol described here is simple, efficient, and robust, and allows one to purify high-quality ribosomes from human cultured cell lines. Ribosomes purified with this protocol are adequate for most of the subsequent analyses of their RNA and protein content. *Curr. Protoc. Cell Biol.* 49:3.40.1-3.40.11. © 2010 by John Wiley & Sons, Inc.

Keywords: ribosome • cell fractionation • translation

INTRODUCTION

The ribosome is a cytoplasmic ribonucleoprotein machine involved in a fundamental cell process: protein synthesis. George E. Palade made the first observation of ribosomes in 1955 (Palade, 1955). Transmission electron microscope analyses resulted in the description of ribosomes as spherical and dense structures of about 10 to 15 nm within the cytoplasm of eukaryotic cells. The number of ribosomes is variable and dependent upon the physiological and pathological status of the cell. There are more ribosomes in highly proliferative cells, whereas the number is less important in quiescent cells. The ribosomal production rate in normal cells (yeast) is about 2000 ribosomes per min, and the energy requirement for this may represent ∼60% of the total cell energy (Warner, 1999; Rudra and Warner, 2004).

The structure of the ribosome has been highly conserved during evolution. The ribosome is composed of two subunits: a small 40S subunit (30S in prokaryotes) and a large 60S subunit (50S in prokaryotes), which form the 80S ribosome (70S in prokaryote) when associated together. Each subunit contains ribosomal RNA (rRNA) and ribosomal proteins (RP). In mammals, the small subunit comprises the 18S rRNA (1869 nucleotides) and 33 proteins (RPS). The large subunit comprises three rRNAs—the 28S (5035 nucleotides), the 5.8S (157 nucleotides), and the 5S (121 nucleotides)—and 47 proteins (RPL).

The three-dimensional structure of the ribosome was initially determined in prokaryotes. The complete crystal structure of the 70S complex from *Thermus thermophilus* was

Current Protocols in Cell Biology 3.40.1-3.40.11, December 2010
Published online December 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb0340s49

obtained in 2001 at 5.5-Å resolution by X-ray crystallography (Yusupov et al., 2001). The eukaryotic ribosome from yeast was obtained the same year by cryo-electron microscopy (cryo-EM) at a 15-Å resolution (Spahn et al., 2001). The structure of the ribosome in mammals was determined recently from the dog by cryo-EM at a resolution of 8.7 Å (Chandramouli et al., 2008).

Decoding the mRNA and synthesizing the polypeptide chain requires, in addition to the ribosome, transfer RNA (tRNA) and multiple regulatory protein factors for initiation, elongation, and termination. Structural data obtained mainly in prokaryotes strongly support the notion that the ribosome is a ribozyme (Cech et al., 2000; Moore and Steitz, 2002). The rRNAs are organized in functional domains which catalyze the peptide bond formation and enable quality control of the translation. Nonetheless, proteins and tRNAs may be involved in these processes, but their role is still unclear (Nissen et al., 2000). Most of these studies have been performed in prokaryotes, and many data are still missing to elucidate the structure-function relationship of eukaryote ribosomes, in particular human ribosomes, and to complete the scheme of their evolutionary adaptation. In addition, numerous recent studies suggest that the ribosome could be involved in different pathological processes, such as cancer (Ruggero and Pandolfi, 2003; Belin et al., 2009), genetic diseases (Yoon et al., 2006; Idol et al., 2007; Narla and Ebert, 2010), and viral infections (Diaz et al., 1989, 1993, 1996, 2002; Simonin et al., 1995a,b, 1997; Greco et al., 2001; Belin et al., 2010). However, in most of these studies, the link between ribosome composition (RNA and proteins) and the pathological process is still not established in great detail.

In this unit, we describe a protocol to purify ribosomes from human cell lines that allow their subsequent biochemical analyses (see Figure 3.40.1). This protocol is easily adaptable to many cultured animal cell lines.

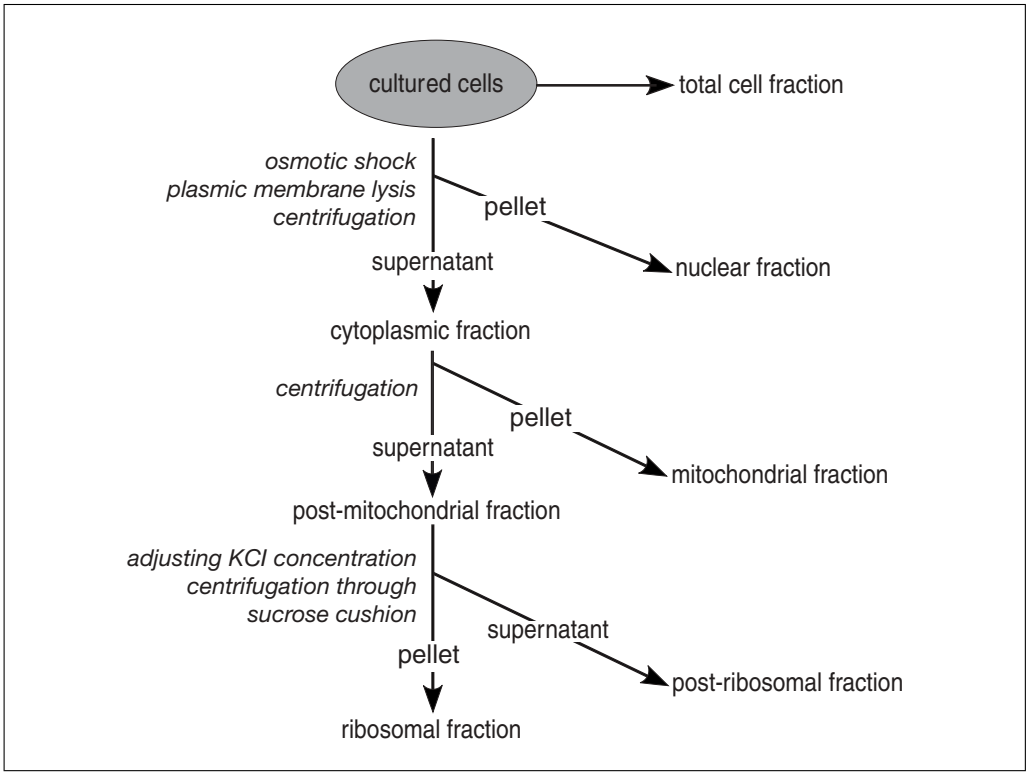


Figure 3.40.1 Synopsis of the Basic Protocol. The technical manipulations underlying this protocol are described in details in the text.

PURIFICATION OF RIBOSOMES

BASIC PROTOCOL

This protocol was adapted from previously published protocols (Madjar et al., 1977; 1979; Madjar and Fournier, 1981; Diaz et al., 1989; Massé et al., 1990a,b; Madjar, 1994; Simonin et al., 1995b, 1997; Chan et al., 1996). The main steps of the basic protocol are illustrated in Figure 3.40.1 for a purification performed from human cultured cell lines. After harvesting, cells are submitted to an osmotic shock by incubation in an appropriate hypotonic buffer containing NP-40. After a first centrifugation at 750 × *g*, the nuclear fraction is removed and stored. The cytoplasmic fraction is submitted to centrifugation at 12,500 × *g* to remove the mitochondrial fraction. The concentration of the post-mitochondrial fraction is then adjusted to 0.5 M KCl to disrupt most interactions between ribosomes and other proteins of the other cell compartments. Finally, ribosomes are purified through a sucrose cushion (containing also 0.5 M KCl) by ultracentrifugation. The ribosomes are in the pellet that appears translucent and very compact.

Materials

Cultured adherent cells
Appropriate medium for cells to be cultured
Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*)
Buffers A, B, and C (see recipes)
10% (v/v) Nonidet P-40
4 M KCl
Sucrose cushion (see recipe)
12.5% SDS-PAGE gel (Gallagher, 2006)

150-mm diameter Petri dishes
15- and 50-ml conical polypropylene centrifuge tubes (e.g., BD Falcon)
Cell lifters (Biologix Research Company; *http://biologixresearch.com/*)
Refrigerated centrifuge (e.g., Jouan CR422) for cell harvesting
3-ml thick-walled polycarbonate tubes (Beckman)
Refrigerated ultracentrifuge with fixed-angle rotor (e.g., Beckman TL-100.3) for ribosome harvesting
Spectrophotometer capable of measuring at 260 nm

Additional reagents and equipment for protein quantification (see Support Protocol and Simonin and Smith, 2008), SDS-PAGE (see Support Protocol and Gallagher, 2006), and staining gels (see Support Protocol and Sasse and Gallagher, 2009)

NOTE: Carry out all procedures at 4°C using prechilled solutions.

NOTE: This protocol is optimized for dissociated cytoplasmic ribosomal subunits. To purify polysomes, 40S and 60S subunits, or monosome 80S, buffers have to be modified. See Critical Parameters for more details.

Culture and harvest cells

1. At a time point 48 hr before purification of ribosomes, plate 8×10^6 cells per 150-mm diameter Petri dish in the appropriate medium.
2. At 80% confluence, wash cells three times with 15 ml per dish of cold DPBS, pH 7.4.
3. Scrape the Petri dish with a cell scraper into 10 ml of DPBS.
4. Pool cells in a cold 50-ml conical polypropylene centrifuge tube.

5. Collect cells by centrifugation 5 min at 500 × *g*, 4°C.

   Cells should not be spin at more than 500 × g.

6. Discard supernatant, saving an aliquot for subsequent SDS-PAGE analysis.

Prepare cytoplasmic and nuclear fractions

7. Resuspend gently by pipetting (do not use vortex) the cellular pellet with cold buffer A added in three sequential steps with gentle pipetting (homogenization) between additions of buffer A.

   The volume of buffer A is three times the volume of the cell pellet. It is very important to obtain a homogeneous cell suspension.

8. Save ∼1/20th of the suspension and store it at −80°C for subsequent SDS-PAGE analyses.

9. To perform cell lysis, add the appropriate volume of NP-40 from a 10% solution in the cell suspension to obtain a final concentration of 0.7% (v/v).

10. Incubate on ice for 10 to 15 min. Homogenize the suspension by gentle pipetting at the beginning of the incubation and 5 min following the incubation.

    For example, add 28 μl of 10% NP-40 solution for 300 μl of homogenate.

    In this step, the cytoplasm detaches from the nuclei. NP-40 is a nonionic detergent that facilitates the cell lysis by inducing weakness of the plasma membrane.

11. Centrifuge the cell lysate 10 min at 750 × *g*, 4°C, to pellet nuclei.

    The supernatant contains the cytoplasmic fraction with cytoplasmic ribosomes. The pellet contains nuclei.

12. Save the nuclear pellet and store it at −80°C for further SDS-PAGE analyses.

Prepare cytoplasmic fraction without mitochondria

13. Centrifuge the cytoplasmic fraction 10 min at 12,500 × *g*, 4°C, to obtain a pellet containing the mitochondria.

14. Carefully decant and retain the post-mitochondrial fraction (PMT), which contains the ribosomes. Save an aliquot for further analysis and store at −80°C.

Determine PMT volume to adjust KCl concentration

15. Accurately measure the volume of the PMT fraction (PMT_{vol}) using a graduated pipet.

16. Calculate the amount of 4 M KCl solution to add to the PMT fraction to give a final concentration of 0.5 M using a 4 M KCl solution as:

    $V_{KCL} = PMT_{vol}/14$

17. Slowly add the 4 M KCl solution to the PMT fraction.

    The volume of the KCl-adjusted PMT is between 100 μl and 600 μl and should not exceed 600 μl.

Harvest the ribosome pellet

18. For a TL100.3 rotor (Beckman) use a 3-ml polycarbonate tube. Add 1 ml of the sucrose cushion.

19. Carefully add the KCl-adjusted PMT fraction above the 1-ml sucrose cushion.

20. Carefully balance the tubes very accurately by weight (within 0.01 g) using buffer B.

    It is not necessary to fill the tubes.

21. Ultracentrifuge these tubes for 2 hr at 250,000 × *g* at 4°C.

22. Discard the supernatant which is the post-ribosomal fraction.

    The pellet appears translucent, and contains ribosome. The pellet is very compact and very dense.

23. Quickly rinse the pellet twice by carefully adding 200 µl cold water to the pellet and removing it immediately without losing the pellet.

24. Resuspend the ribosome pellet with three 100-µl additions of buffer C. After each addition of buffer, gently homogenize the pellet by pipetting, to obtain a ribosome suspension. Save an aliquot for further analysis and store at −80°C.

25. To estimate the amount of ribosomes, measure the optical density of the ribosome suspension obtained with a spectrophotometer at 260 nm.

    OD_{260} = 14 corresponds to 1 mg of ribosomes and about 500 µg of ribosomal proteins.

    Protein concentration of this fraction can also be estimated using the Bradford protocol (Simonin and Smith, 2008).

Validate the purification by one-dimensional gel electrophoresis

26. Thaw samples reserved in steps 8, 12, and 24 corresponding to total cells and to nuclear, post-mitochondrial supernatant, and ribosomal fractions, keeping the samples on ice.

27. Quantify the proteins in each sample using the Bradford assay (see Support Protocol and Simonin and Smith, 2008).

28. Separate 10 µg of each fraction by SDS-PAGE (see Support Protocol and Gallagher, 2006) using a 12.5% gel, then stain with Coomassie blue (see Support Protocol and Sasse and Gallagher, 2009).

    An image of a typical gel stained with Coomassie blue is presented in Figure 3.40.2. Panel A presents the fraction corresponding to total cell fraction (TC), nuclear fraction (N), and post-mitochondrial fraction (PMT). The three first lanes in panel B present the ribosomal fractions purified with 0.5 M KCl. The three next lanes present the same ribosomal fractions but purified with 0.025 M KCl in order to illustrate that the KCl concentration is critical to obtain a ribosome fraction highly enriched with ribosome subunits.

    A visual inspection of the gel makes it easy to monitor the efficiency of the purification. In general, the SDS-PAGE protein profiles of total and nuclear protein profiles are very different. In particular, there is an important enrichment in the four bands of low molecular mass (indicated by asterisks) that correspond to histones in the nuclear fraction. The high abundance of these proteins in the nuclear fraction allows for the verification of the efficiency of the separation of nuclei of total cells.

    The PMT fraction appears very different from those of total cell (TC) and nuclear fraction (N). This fraction is very poor in low-molecular-weight proteins and appears without histones. This is in agreement with an efficient separation of the cytoplasmic fraction.

    The post-nuclear fraction is informative for checking the purity of the cytoplasmic fraction.

    Finally, the ribosomal fraction purified with 0.5 M KCl exhibits a very specific SDS-PAGE profile with a very clear enrichment in low- and medium-kDa proteins, which correspond to ribosomal proteins that have molecular masses between 47.3 kDa for the higher (L4)–mass and 11.5 kDa for the lower (P1)–mass proteins (Wool et al., 1996). This fraction is highly enriched in the 80 ribosomal proteins.

    The SDS-PAGE protein profiles of ribosomal fraction purified with 0.025 M KCl and of ribosomal fraction purified with 0.5 M KCl are very different. In the 0.025 M KCl protein fraction, the ribosomal proteins are visible, as they are in the 0.5 M KCl profile, but there are also a lot of other proteins of high molecular weights. Obtaining an SDS-PAGE profile

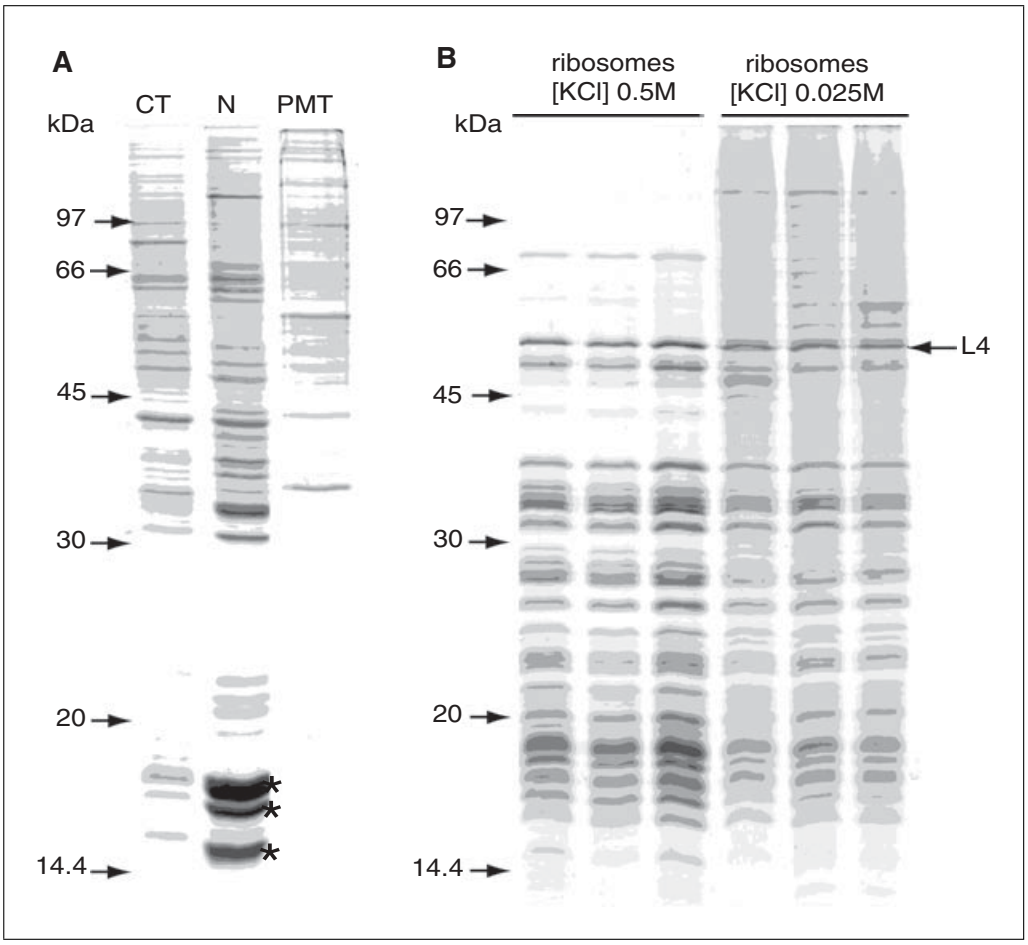


Figure 3.40.2 Analysis of the cellular fractions obtained during ribosomes purification. (**A**) 1-D separation on a 12.5% polyacrylamide gel of 10 μg of proteins extracted from total cells (TC), nuclear fraction (N), and post-mitochondrial fraction (PMT). Histones are indicated by asterisks. (**B**) 1-D separation on a 12.5% polyacrylamide gel of 10 μg of proteins extracted from ribosomal fraction purified at high stringency (0.5 M KCl) and at low stringency (0.025 M KCl). Proteins are stained with Coomassie blue R250. Sizes of molecular weight markers are indicated to the left of the panels.

similar to that presented in Figure 3.40.2 is classically sufficient to assess a satisfactory purity of the ribosomal fraction for most of the further applications, notably analyses of their RNA and protein content. In this protocol it is not necessary to analyze RNA purity, as the protein analysis is necessary and sufficient for estimating the purity of the ribosomal fraction.

SUPPORT PROTOCOL

ONE-DIMENSIONAL SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Thaw aliquots of fractions obtained in steps 8, 12, 14, and 24 and during the purification, slowly on ice. Quantify the proteins by the Bradford method (Simonin and Smith, 2008) using the appropriate blank for each aliquot: for the total cell fraction use buffer A as a blank; for the nuclear fraction use buffer A containing 0.7% NP-40 as a blank; for the PMT fraction use buffer B as a blank; and finally, for the ribosomal protein fraction, use buffer C as a blank. After the quantification of the proteins, add 2 volumes of 2× Laemmli electrophoretic sample buffer (see recipe). Separate about 10 μg of each fraction by SDS-PAGE on a 12.5% gel (Gallagher, 2006) and stain by Coomassie blue (Sasse and Gallagher, 2009). An image of a typical gel stained with Coomassie blue is presented in Figure 3.40.2.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

250 mM sucrose
250 mM KCl
5 mM $MgCl_2$
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Store up to 1 week at 4°C

Buffer B

250 mM sucrose
0.5 M KCl
5 mM $MgCl_2$
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Store up to 1 week at 4°C

Buffer C

25 mM KCl
5 mM $MgCl_2$
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Store up to 1 week at 4°C

Laemmli electrophoretic sample buffer, 2×

62.5 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
1% (w/v) SDS
10% (v/v) glycerol
0.1 M dithioerythritol (DTE)
Traces (e.g., tip of spatula full) of bromphenol blue
Store up to 1 year at −20°C

Sucrose cushion

1 M sucrose
0.5 M KCl
5 mM $MgCl_2$
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Store up to 1 year at 4°C

COMMENTARY

Background Information

Genetic information is contained in genes. The first step of gene expression is the transcription of DNA into RNA. Some of these RNAs are dedicated to producing proteins. As such, they contain a coding sequence flanked by regulatory 5′ and 3′ untranslated region (5′ and 3′ UTR). These RNAs are called messenger RNA (mRNA). The translation apparatus is composed of transfer RNA (tRNA), translation regulatory factors, and ribosomes. tRNAs are small RNAs with a sequence of 3 nucleotides at the 3′ end linked to a defined amino acid (aminoacyl-tRNA) and a sequence of three nucleotides, the anticodon that interacts with the mRNA. The translation-regulatory factors are numerous and act at different stages of translation. Ribosomes are the central effector elements, allowing the synthesis of polypeptide chain under strict quality control.

George E. Palade made the first observation of ribosomes in 1955 by performing transmission electron microscopy experiments using 40 different vertebrate cells (Palade, 1955). The same observation was made later in prokaryotes by Tissieres and Watson (1958). The ribosomal function was clearly established during the late 1950s (Balis et al., 1958).

The fine structure of the ribosome has been determined following the achievement of two main goals. First, during the 1980s, many groups worked to obtain the crystal structure of the two subunits or the entire ribosome, but the resolution was not sufficient to have a complete atomic view of the ribosome (Yonath et al., 1984, Makowski et al., 1987, Trakhanov et al., 1989). Then, better resolution was obtained at the end of the 1990s. The structure of the prokaryotic 50S large subunit was obtained at 2.4 Å (Ban et al., 2000; Nissen et al., 2000), the structure of the 30S small subunit was obtained at 3 Å (Wimberly et al., 2000), and the 70S complete structure was obtained at 3.5 Å (Schuwirth et al., 2005).

The structure of the ribosome is highly conserved during evolution. The ribosome is composed of two subunits: a small subunit 40S (30S in prokaryote) and a large subunit 60S (50S in prokaryote) to form a complete ribosome 80S (70S in prokaryote). rRNAs undergo numerous post-transcriptional modifications of three types: base-methylations, 2′-*O*-methylations of the ribose, and pseudouridylations. The position of these modifications on rRNA is highly conserved during evolution, although their numbers increase between prokaryotes and eukaryotes. In eukaryotes and archaeans, post-transcriptional modifications are carried out by small nucleolar ribonucleo-particles (snoRNP), which consist of an RNA guide (snoRNA) and proteins that catalyze the modification. In prokaryotes, each modification is carried out by a specialized enzyme (Decatur and Fournier, 2002). Proteins can also be modified post-translationally, e.g., by phosphorylation. In mammals, the small subunit is constituted by the 18S rRNA (1869 nucleotides) and 33 proteins (RPS). The large subunit contains three rRNAs—the 28S (5035 nucleotides), the 5.8S (157 nucleotides), and the 5S (121 nucleotides)—and 47 proteins (RPL). Ribosome biogenesis is a complex and multistep process, which takes place mainly in the nucleolus of the eukaryotes, but also in the nucleus and the cytoplasm (Fromont-Racine et al., 2003). In mammals, ribosome biogenesis requires more than 200 protein factors and more than 100 snoRNA (Couté et al., 2006). This process is highly regulated to ensure the production of a sufficient supply of ribosomes for the cell.

Although the function of ribosomes in protein synthesis has been demonstrated for over 50 years, the precise mechanisms of translation and the implication of the ribosome became possible only when the high-resolution three-dimensional structure of ribosomes was performed by various groups; these studies were awarded the Nobel Prize in Chemistry in 2009 (V. Ramakrishnan, T.A. Steitz, and A.E. Yonath). According to these structural studies, it has been definitively demonstrated that ribosomes are ribozymes, i.e., the catalysis of peptide bond formation is performed by the RNA (Cech et al., 2000). Today, more and more studies provide data suggesting that ribosomes, at least in humans, are more or less directly involved in different physiopathological processes (Yoon et al., 2006; Idol et al., 2007; Belin et al., 2009, 2010). Furthermore, a recent study performed in yeast has demonstrated that the composition of ribosomal protein in the ribosome could influence the pool of mRNA that is translating, suggesting the existence of a "ribosomal code" (Komili et al., 2007). However, at present, many studies remain to be performed in order to validate this new provocative concept in other organisms.

Many protocols have been developed to purify ribosomes from different eukaryotic species and from different animal tissues. In general, these protocols allow investigators to obtain high-quality purified ribosomal complexes to study ribosomal protein as well as the ribosomal RNA. Here we present a highly robust protocol that we have optimized and validated with numerous cultured human cell lines. The efficiency of ribosome purification can be evaluated using various techniques: electron microscopy to ensure the conservation of classical ribosome ultrastructure; one- and two-dimensional gel electrophoresis; or even immunoblot analysis to evaluate contamination from other cell structures. After obtaining the ribosomal fraction, proteins can be identified by mass spectrometry as well. Several two-dimensional maps of ribosomal proteins have been published for the rat (Madjar et al., 1979) and for cancer cells (Diaz et al., 1989; Görg et al., 1997; Belin et al., 2009). Here, we describe a simple (one-dimensional SDS-PAGE) standard method to check the quality of the purified ribosomes.

Critical Parameters

The time at which the cells are plated before the beginning of the purification of ribosomes is critical. This can vary according to the cell lines used. The time and plating density should be adjusted to obtain about 80% cell confluence at the beginning of the purification (48 hr).

This protocol is optimized for cells that have been harvested by scrapping with a cell lifter and not by trypsinization. Harvesting cells by trypsinization could induce a partial degradation of ribosomal proteins.

This protocol is optimized for purification of ribosomes from cells immediately after harvesting, not from a cell pellet that has been frozen. Freezing the cell pellets could prevent an efficient cell lysis and a good separation of nuclear and cytoplasmic fractions. However, once the protocol is well optimized with "fresh culture" you can try to perform the purification starting with cells that have been frozen directly at −80°C (dry frozen pellet).

This protocol is intended to obtain ribosomes devoid of interacting proteins. This protocol is not suitable for polysome purification. For purification of polysomes it is necessary to use different buffers; in particular, the KCl concentration needs to be lowered to 0.025 M. For this type of purification and analyses see the numerous references in Warner and Knopf (2002).

The final concentration of NP-40 can range from 0.3% to 2%, depending on the cell line used. Plasma membrane lysis can be evaluated by visualizing the nuclei, devoid of cytoplasmic fractions under phase-contrast microscopy.

To obtain the PMT fraction, a first centrifugation at 750 × *g* is necessary to spin down the nuclei. A direct centrifugation at 12,500 × *g* could induce a disruption of nuclei and therefore cause contamination with nuclear content. This contamination is demonstrated by the presence of histones among ribosomal proteins after separation by SDS-PAGE.

The concentration of KCl at 0.5 M is critical to obtain a ribosome fraction with the lowest level of contamination. The stringency of this solution is one of the more important parameters to obtain ribosomes devoid of contamination from other cell fractions. Be aware that the 4 M KCl solution has a tendency to crystallize.

Any centrifuge and rotor that allow ultracentrifugation with tubes containing ∼3 ml can be used. However it is better to use the smallest possible volume (1 ml) for the sucrose cushion.

The ribosome pellet must be resuspended very carefully, to avoid a loss of ribosomes and to obtain a good yield of protein in this fraction. For this, it is very important to pipet the ribosomes resuspended in buffer C up and down many times, with the tubes on ice. Also be aware that the ribosomes are in suspension, and they have a tendency to sediment at the bottoms of the tubes. Therefore, homogenize very carefully by pipetting (try to avoid vortexing) the ribosome suspension before the following steps (i.e, quantification, SDS-PAGE).

Generally, the protein concentration of the fractions is very high, and it is not necessary to concentrate these fractions for further SDS-PAGE analyses.

Anticipated Results

The expected yield from 15×10^6 adherent HeLa cells is ∼2.8 A_{260} units, or about 100 μg of ribosomal proteins.

Time Considerations

It takes 5 hr to purify ribosomes from 80% confluent dishes of adherent cells. Quantification of protein content requires 1 hr and one-dimensional gel electrophoresis and staining require 6 hr

Literature Cited

Balis, M.E., Samarth, K.D., Hamilton, M.G., and Petermann, M.L. 1958. Role of the ribonucleoprotein particle in protein synthesis and the effects of growth hormone. *J. Biol. Chem.* 233:1152-1155.

Ban, N., Nissen, P., Hansen, J.H., Moore, P.B., and Steitz, T.A. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* 289:905-920.

Belin, S., Beghin, A., Solano-Gonzalez, E., Bezin, L., Brunet-Manquat, S., Textoris, J., Prats, A.C., Mertani, H.C., Dumontet, C., and Diaz, J.J. 2009. Dysregulation of ribosome biogenesis and translational capacity is associated with tumor progression of human breast cancer cells. *PLoS ONE* 4:e7147.

Belin, S., Kindbeiter, K., Hacot, S., Albaret, M.A., Roca-Martinez, J.X., Therizols, G., Grosso, R., and Diaz, J.J. 2010. Uncoupling ribosome biogenesis regulation from RNA polymerase I activity during herpes simplex virus type 1 infection. *RNA* 16:131-140.

Cech, T.R. 2000. Structural biology. The ribosome is a ribozyme. *Science* 289:878-879.

Chan, Y.L., Diaz, J.J., Denoroy, L., Madjar, J.J., and Wool, I.G. 1996. The primary structure of rat ribosomal protein L10: Relationship to a Jun-binding protein and to a putative Wilms' tumor suppressor. *Biochem. Biophys. Res. Commun.* 225:952-956.

Chandramouli, P., Toof, M., Menetret, J.F., Eswar, N., Cannone, J.J., Gutell, R.R., Sali,. A., and Akev, C.W. 2008. Structure of the mammalian 80S ribosome at 8.7 Å resolution. *Structure* 16:535-548.

Couté, Y., Burgess, J.A., Diaz, J.J., Chichester, C., Lisacek, F., Greco, A., and Sanchez, J.C. 2006.

Deciphering the human nucleolar proteome. *Mass Spectrom. Rev.* 25:215-234.

Decatur, W.A. and Fournier, M.J. 2002. rRNA modifications and ribosome function. *Trends Biochem. Sci.* 27:344-351.

Diaz, J.J., Gandrillon, O., Hentzen, D., Leguellic, D., Samarut, J., and Madjar, J.J. 1989. Increase in ribosomal protein S6 phosphorylation is due to v-erbB-transforming activity and not to v-erbA mitogenic activity in avian erythroblastosis virus-infected chicken embryo fibroblasts. *Oncogene Res.* 4:163-175.

Diaz, J.J., Simonin, D., Masse, T., Deviller, P., Kindbeiter, K., Denoroy, L., and Madjar, J.J. 1993. The herpes simplex virus type 1 US11 gene product is a phosphorylated protein found to be non-specifically associated with both ribosomal subunits. *J. Gen. Virol.* 74:397-406.

Diaz, J.J., Dodon, M.D., Schaerer-Ulthurralt, N., Simonin, D., Kindbeiter, K., Gazzolo, L., and Madjar, J.J. 1996. Post-transcriptional transactivation of human retroviral envelope glycoprotein expression by herpes simplex virus Us11 protein. *Nature* 379:273-277.

Diaz J.J., Giraud, S., and Greco, A. 2002. Alteration of ribosomal protein maps in herpes simplex virus type 1 infection. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 771:237-249.

Fromont-Racine, N.M., Sanger, B., Saveanu, C., and Pasiolo, F. 2003. Ribosome assembly in eukaryotes. *Gene* 313:17-42.

Gallagher, S.R. 2006. One-dimensional SDS gel electrophoresis of proteins. *Curr. Protoc. Mol. Biol.* 75:10.2A.1-10.2A.37.

Görg, A., Obermaier, C., Boguth, G., Csordas, A., Diaz, J.J., and Madjar, J.J. 1997. Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. *Electrophoresis* 18:328-337.

Greco, A., Bienvenut, W., Sanchez, J.C., Kindbeiter, K., Hochstrasser, D., Madjar, J.J., and Diaz, J.J. 2001. Identification of ribosome-associated viral and cellular basic proteins during the course of infection with herpes simplex virus type 1. *Proteomics* 1:545-549.

Idol, R.A., Robledo. S., Du, H.Y., Crimmins, D.L., Wilson, D.B., Ladenson, J.H., Besler, M., and Mason, P.J. 2007. Cells depleted for RPS19, a protein associated with Diamond Blackfan Anemia, show defects in 18S ribosomal RNA synthesis and small ribosomal subunit production. *Blood Cells Mol. Dis.* 39:35-43.

Komili, S., Farny, N.G., Roth, F.P., and Silver, P.A. 2007. Functional specificity among ribosomal proteins regulates gene expression. *Cell* 131:557-571.

Madjar, J.J. 1994. Preparation of ribosomes and ribosomal proteins from cultured cells. *In* Cell Biology: A Laboratory Handbook (J.E. Celis, ed.) pp. 657-661. Academic Press, New York.

Madjar, J.J. and Fournier, A. 1981. *Bombyx mori* L. ribosomal proteins: Resolution, nomenclature, molecular weights and in vivo phosphorylation. *Mol. Gen. Gene.* 182:273-278.

Madjar, J.J., Arpin, M., Marion, M.J., and Reboud, J.P. 1977. Comparison of the protein content of free and membrane-bound rat liver polysomes and of the derived subunits. *Mol. Biol. Rep.* 3:289-296.

Madjar, J.J., Arpin, M., Buisson, M., and Reboud, P. 1979. Spot position of rat liver ribosomal proteins by four different two-dimensional electrophoreses in polyacrylamide gel. *Mol. Gen. Genet.* 171:121-134.

Makowski, I., Frolow, F., Saper, M.A., Shoham, M., Whitmann, H.G., and Yonath, A. 1987. Single crystals of large ribosomal particles from *Halobacterium marismortui* diffract to 6 Å. *J. Mol. Biol.* 193:819-822.

Massé, T., Garcin, D., Jacquemont, B., and Madjar, J.J. 1990a. Herpes simplex virus type-1-induced stimulation of ribosomal protein S6 phosphorylation is inhibited in neomycin-treated human epidermoid carcinoma 2 cells and in ras-transformed cells. *Eur. J. Biochem.* 194:287-291.

Massé, T., Garcin, D., Jacquemont, B., and Nadjar, J.J. 1990b. Ribosome and protein synthesis modifications after infection of human epidermoid carcinoma cells with herpes simplex virus type 1. *Mol. Gen. Genet.* 220:377-388.

Moore, P.B. and Steitz, T.A. 2002. The involvement of RNA in ribosome function. *Nature* 418:229-235.

Narla, A. and Ebert, B.L. 2010. Ribosomopathies: Human disorders of ribosome dysfunction. *Blood* 115:3196-3205.

Nissen, P., Hansen, J., Ban, N., Moore, P.B., and Steitz, T.A. 2000. The structural basis of ribosome activity in peptide bond synthesis. *Science* 289:920-930.

Palade, G.E. 1955. A small particulate component of the cytoplasm. *J. Biophys. Biochem. Cytol.* 1:59-68.

Rudra, D. and Warner, J.R.. 2004. What better measure than ribosome synthesis? *Genes Dev*. 18: 2431-2436.

Ruggero, D. and Pandolfi, P.P. 2003. Does the ribosome translate cancer? *Nat. Rev. Cancer* 3:179-192.

Sasse, J. and Gallagher, S.R. 2009. Staining proteins in gels. *Curr. Protoc. Mol. Biol.* 85:10.6.1-10.6.27.

Schuwirth, B.S., Borovinskaya, M.A., Hau, C.W., Zhang, W., Vila-Sanjurjo, A., Holton, J.M., and Cate, J.H. 2005. Structures of the bacterial ribosome at 3.5 Å resolution. *Science* 310:827-834.

Simonin, M.H. and Smith, J.A. 2008. Spectrophotometric and colorimetric determination of protein concentration. *Curr. Protoc. Mol. Biol.* 76:10.1A.1-10.1A.9.

Simonin, D., Diaz, J.J., Kindbeiter, K., Pernas, P., and Madjar, J.J. 1995a. Phosphorylation of herpes simplex virus type 1 Us11 protein is independent of viral genome expression. *Electrophoresis* 16:1317-1322.

Simonin, D., Diaz, J.J., Kindbeiter, K., Denory, L., and Madjar, J.J. 1995b. Phosphorylation of ribosomal protein L30 after herpes simplex virus type 1 infection. *Electrophoresis* 16:854-859.

Simonin, D., Diaz, J.J., Masse, T., and Madjar, J.J. 1997. Persistence of ribosomal protein synthesis after infection of HeLa cells by herpes simplex virus type 1. *J. Gen. Virol.* 78:435-443.

Spahn, C.M., Beckmann, R., Eswar, N., Penczek, P.A., Sali, A., Blobel, G., and Frank, J. 2001. Structure of the 80S ribosome from *Saccharomyces cerevisiae*—tRNA-ribosome and subunit-subunit interactions. *Cell* 107:373-386.

Tissieres, A. and Watson, J.D. 1958. Ribonucleoprotein particles from *Escherichia coli*. *Nature* 182:778-780.

Trakhanov, S., Yusupov, M., Shirokov, V., Garber, M., Mitschlker, A., Ruff, M., Thiery, J.C., and Moras, D. 1989. Preliminary X-ray investigation of 70S ribosome crystals from *Thermus thermophilus*. *J. Mol. Biol.* 209:327-328.

Warner, J.R. 1999 The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* 24:437-440.

Warner, J.R. and Knopf, P.M. 2002. The discovery of polyribosomes. *Trends Biochem. Sci.* 27:376-380.

Wimberly, B.T., Brodersen, D.E., Clemons, W.M. Jr., Morgan-Warren, R.J., Carter, A.P., Vonrhein, C., Hartsch, T., and Ramakrishnan, V. 2000. Structure of the 30S ribosomal subunit. *Nature* 407:327-339.

Wool, I.G., Chan, Y.-L., and Glück, A. 1996. Mammalian ribosomes: the structure and the evolution of the proteins. *In* Translational Control (J.W.B. Hershey and M.B. Mathews, eds.) pp. 685-732. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Yonath, A., Bartunik, H.D., Bartels, K.S., and Witmann, H.G. 1984. Some x-ray diffraction patterns from single crystals of the large ribosomal subunit from *Bacillus stearothermophilus*. *J. Mol. Biol.* 177:201-206.

Yoon, A., Peng, G., Brandenburger, Y., Zoillo, O., Xu, W., Rego, E., and Ruggero, D. 2006. Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita. *Science* 312:902-906.

Yusupov, M.M., Yusupova, G.Z., Baucom, A., Lieberman, K., Earnest, T.N., Cate, J.H., and Noller, H.F. 2001. Crystal structure of the ribosome at 5.5 Å resolution. *Science* 292:883-896.

CHAPTER 4
Microscopy

INTRODUCTION

Light microscopy historically has been an important technique of scientific discovery because it provides enlarged images of small objects. Recent advances in optical design and the development of probes have made light microscopy an essential tool for both research and diagnosis, enabling analysis of structure, physiology, and function. This chapter deals with methods for using the microscope, including the principles underlying different applications of light microscopy—e.g., phase-contrast, differential interference contrast (DIC), and fluorescence microscopy—as well as how to align and adjust the microscope for these different applications, prepare and label specimens, and image them accurately.

Although the structural detail in images resolved by the microscope is continually being improved by video and digital cameras and electronic imaging, the quality and accuracy of the image is primarily dependent on proper microscope alignment and adjustment. *UNIT 4.1* details methods for aligning a typical research light microscope for bright-field, phase-contrast, DIC, and epifluorescence microscopy. The unit emphasizes how proper alignment and adjustment are essential for optimal image quality and accurate quantitative measurements. The principles underlying Köhler illumination are explained, providing the background for discussions of image formation and microscope alignment for bright-field, phase-contrast, and DIC imaging. *UNIT 4.1* also describes methods for camera attachment, calibration of image formation, care and cleaning of the microscope, and assessment of microscope performance.

UNIT 4.2 describes fluorescence microscopy and its use as a tool for imaging biological specimens. Fluorescence microscopy has long been used to characterize cellular organization and is based on the property of fluorescent molecules whereby they absorb light of a particular wavelength and emit light at longer wavelengths. This unit includes methods for aligning the arc lamp and provides information on how to choose wavelength selection devices and objectives (also see *APPENDIX 1E*). *UNIT 4.2* also explains how to image fluorescent specimens and optimize image brightness and resolution. The availability in recent years of economical camera and software systems for capturing, storing, and analyzing digital images obtained from fluorescence microscopes has led to the replacement of photography by digital analysis as the preferred method for documenting images. This is described in the section of *UNIT 4.2* entitled "The Digital Darkroom," in which the advantages of digital images over conventional photography are described. These include convenience of image storage, retrieval, and manipulation. In addition, digital imaging allows quantitative analysis of fluorescence data and greatly facilitates the analysis of large numbers of images.

UNIT 4.3 provides methods for what is perhaps the most widely used application of fluorescence microscopy, immunofluorescent staining. In this technique, fluorophore-conjugated antibodies are used as sensitive and specific probes for staining antigens in fixed and/or permeabilized cells, and the location of specific proteins can be determined. When two or more antibodies labeled with different fluorophores are used to stain cells, multiple protein distributions can be visualized simultaneously. This unit provides the basic methodology for fixation and optimal staining of cells and a variety of tips for troubleshooting potential problems.

Current Protocols in Cell Biology 4.0.1-4.0.6, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0400s47

The advent of fluorescent dyes that selectively label intracellular organelles has resulted in an additional application of light microscopy whereby the spatial distribution of specific cellular components may be examined in vivo. *UNIT 4.4* describes protocols for fluorescently labeling three subcellular organelles—endoplasmic reticulum (ER), Golgi complex, and mitochondria—using selective fluorescent probes, including $DiOC_6(3)$, C_6NBD-ceramide, and BODIPY-ceramide. These lipid-based dyes have been shown to incorporate into the membranes of the ER, Golgi complex, and mitochondria in living cells so they can be used to follow the dynamics of these organelles in real time. This unit includes an extensive troubleshooting guide for staining, as well as detailed descriptions of anticipated results.

Another powerful system for imaging specimens is the confocal microscope, which produces sharp images of structures by selectively collecting light from a thin focal plane within the specimen. *UNIT 4.5* provides important background information for the use of such microscopes in cell biological applications. It details how confocal optical sectioning is accomplished and how the technique can be used to reconstruct a three-dimensional view of a specimen. The unit also describes the different types of confocal microscope systems (including laser-scanning, rapid-scan, and two-photon) and their advantages and disadvantages, and provides practical advice for using these instruments.

UNIT 4.6 describes immunoperoxidase methods for localizing intracellular antigens. With these methods, the distribution of proteins can be characterized at both the light- and electron-microscopic levels, providing insight into cellular organization at different size scales. Included in the description are tips for ensuring that antibodies have access to the intracellular environment where antigen is localized, preserving antigenicity, and maintaining good cellular morphology during fixation and staining. Also described are the advantages and disadvantages of immunoperoxidase staining and practical tips for ensuring its success.

Cryo-immunogold electron microscopy (*UNIT 4.7*) has become the method of choice for high-resolution detection of antigens at the subcellular level. This technique involves preparation of ultrathin cryosections from cells or tissues followed by immunogold labeling to localize antigens. Because harsh organic solvents such as those used for plastic embedding are not required, preservation of protein antigenicity and ultrastructural details is better than with conventional electron microscopic embedding. In cryo-immunogold labeling, gold particles of different sizes conjugated to *Staphylococcus* protein A can be used to label different populations of antibodies that have been bound to antigens. This allows several antigens to be localized simultaneously, which is not possible with other immuno–electron microscopy labeling techniques. *UNIT 4.7* describes how to perform cryo-immunogold electron microscopy labeling of samples, including fixation, embedding, cryosectioning, and labeling. The unit also provides useful discussions on methods for improving the structural integrity of cryosections, the common problems and artifacts that arise during section preparation, and the choice of antibodies and gold particle sizes for optimal immunolabeling results. *UNIT 4.7* has been updated to include silver enhancement of the signal, as well as eleven new videos that show how to perform various steps in this technique, e.g., embedding samples and performing thin sectioning.

UNIT 4.8 describes a method known as correlative video light electron microscopy (CVLEM) that combines fluorescent microscopy and electron microscopy for analyzing dynamic structures with complex morphology within cells. In this technique, cells expressing a fluorescent reporter protein such as a GFP protein chimera are first monitored to identify and record a structure of interest. The cells are fixed, and the same structure of interest is then analyzed by electron microscopy using three-dimensional reconstruction methods. This technique requires considerable skill at the various steps involved

in specimen preparation, but it can yield tremendous insight into the high-resolution organization and morphology of complex structures within the cell, such as the Golgi complex, membrane transport intermediates, and cytoskeletal elements.

UNIT 4.9 describes polarization microscopy, a technique that allows the organization and dynamics of cells and organelles to be studied based on their anisotropic properties, such as birefringence and dichroism. These properties derive from differences in the fine structural detail of an object that make it reflect light in a polarized manner (i.e., differently in different directions). The unit starts out with a brief explanation of the phenomenon of polarized light and birefringence. It then describes how to optimally use a polarizing microscope, which is an ordinary microscope equipped with a polarizer underneath a condenser, an analyzer above the objective, and a compensator in between. Several examples of applications of the polarizing microscope are discussed, including study of the assembly and disassembly dynamics of spindle microtubules and the packing arrangement of DNA in sperm chromosomes.

UNIT 4.10 characterizes a fluorescent imaging technique for visualizing the dynamics, movement, and turnover of intracellular protein assemblies like actin and tubulin—fluorescent speckle microscopy (FSM). The technique is based on the incorporation of low concentrations of microinjected fluorescent subunits into a macromolecular structure through co-assembly with endogenous unlabeled subunits. This results in the structure of interest acquiring a fluorescent speckled pattern that can be observed in resolution-limited fluorescent images. Changes in the fluorescent speckle pattern can then be used to quantitatively analyze the assembly, disassembly, and movement of the structure. The virtue of this technique compared to conventional fluorescent imaging is that it significantly reduces out-of-focus fluorescence so the visibility of the speckles of a fluorescently labeled structure is greatly improved. Moreover, FSM can provide information about protein dynamics throughout the cell in contrast to methods like photoactivation and photobleaching, which follow a small, marked region of a structure. Use of FSM to study microtubules and actin cytoskeleton dynamics in living cells is presented as an example of the usefulness of this technique, so methods for fluorescently labeling tubulin and actin and time-lapse imaging are also detailed.

UNIT 4.11 describes the basic principles and uses of two-photon excitation microscopy. This technique allows three-dimensional imaging of a specimen without absorption above and below the plane of focus. The result is that thick samples, including brain slices, whole organs, and embryos, can be readily imaged, since there is no out-of-focus light. This unit discusses the advantages and limitations of its use relative to confocal and deconvolution microscopy and includes selected applications of two-photon microscopy to illustrate the unique features of this technique, particularly its depth penetration in a specimen, avoidance of phototoxic effects from ultraviolet irradiation, and use in fluorescence lifetime imaging.

UNIT 4.12 discusses total internal reflection microscopy (TIR-FM), also known as evanescent wave microscopy. The fundamental principle of TIR-FM is that light will be totally internally reflected back into a medium with higher refractive index when the incident angle reaches a critical angle. A standing field is then generated into the medium of lower refractive index on the side opposite the incident wave. This field, called the evanescent field, has a short characteristic distance for decay, so it can be used to limit the depth of penetration of excitation light into the specimen. This permits imaging of the specimen within 50 nm of the cell surface. Recent technical advances in fast imaging and image analysis, together with the availability of TIR fluorescence microscopes, has popularized this technique. It is now used in a variety of live-cell applications, including the study of cell-surface contact zones, exocytosis and endocytosis of vesicles, and submembrane

actin dynamics. This unit describes the theory behind TIR-FM; it provides tips for optimizing this method and describes the necessary steps for setting up a TIR-FM system. Finally, the unit discusses how TIR-FM can be used for addressing simple cell biological questions.

UNIT 4.13 describes a range of labeling techniques for fluorescent labeling of yeast cells, which are quite small and difficult to visualize by fluorescence microscopy. Among the techniques described in this unit are fluorescent dye labeling of membranes and visualization of expressed GFP fusion proteins in both fixed and living yeast cells. With these approaches, combined with the use of newer confocal imaging instruments, yeast organelle structure and protein localization can be characterized. Specific applications described include labeling of yeast vacuoles, filamentous actin patterns, and yeast budding morphologies.

UNIT 4.14 describes the technique of fluorescence lifetime imaging microscopy (FLIM), which allows information about the local molecular environment and chromophore photophysics of a fluorescent molecule to be obtained. FLIM is usually used to enhance the image contrast of biological samples, but it can also be used to study molecular interactions of fluorescently labeled biomolecules by fluorescence resonance energy transfer (FRET). This unit focuses on the latter. It describes how to perform FRET imaged by FLIM under physiological conditions using interacting molecules labeled with suitable fluorescent dyes that have the proper optical characteristics. The result is the possibility of mapping interaction events that are on the 10- to 100-Å scale, which significantly increases the diffraction-limited spatial resolution offered by normal light microscope systems. The unit further describes the limitations, pitfalls, and other considerations for FLIM and FRET when used in the study of molecular events in biological samples.

UNIT 4.15 details the technique of second and third harmonic generation microscopy (HGM). This method, which relies on two-photon excitation (*UNIT 4.11*), provides a powerful way to image numerous structural elements of cells and tissues at the resolution of visible light without the use of dyes or other stains. Among the structures that can be observed with this technique are collagen fibers, bone, lipid bodies, blood vessels, and hair. Because the technique involves multiphoton microscopy, it allows deep tissue penetration without phototoxicity, making it suitable for imaging with living organisms. Details of the experimental setup for HGM imaging, as well as examples of its use in three-dimensional tissue reconstruction and dynamic imaging of cell motility in three-dimensional tissues, are presented.

Many protrusive structures play important roles in the interaction between the cell and its environment. These include microvilli, filopodia, lamellipodia, and surface ruffles. *UNIT 4.17* describes an important technique for imaging such cell surface morphologies: scanning electron microscopy (SEM). The high resolution of SEM makes it an ideal technique for imaging the cell surface under different physiological conditions. Depending on the instrument, the scanning electron microscope has a magnification range from 15× to 200,000×, with a resolution between 1 and 20 nm. This unit describes the preparation of cells for SEM examination. In addition, it discusses several critical factors in sample preparation that are necessary to ensure optimal high-resolution imaging of the surface of mammalian cells and tissues with this technique.

UNIT 4.18 describes the fluorescence imaging techniques that are available for studying *Drosophila* embryo development. This unit includes protocols for generating flies expressing fluorescently tagged proteins, as well as for preparing embryos for fluorescent imaging. A detailed description of how to perform time-lapse confocal imaging of live fly embryos is provided. Specific challenges and considerations for fluorescent imaging within a live embryo, including optimizing image acquisition and performing

three-dimensional imaging, are discussed. Techniques that permit optical highlighting of specific subsets of fluorescently tagged proteins and organelles in the embryo, including fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), and photoactivation techniques are presented; these techniques permit analysis of the specific movements of fluorescently tagged proteins within cells. This set of protocols permit researchers to express and image tagged proteins in a specific tissue or at specific developmental times.

Determining the spatial relationship among different molecules within cells is a fundamental aspect of describing biological systems. An important approach for deciphering these relationships is through co-expression of different fluorescently tagged markers within cells and determination by fluorescence microscopy of whether the two molecules are co-localized. *UNIT 4.19* describes protocols for quantifying the extent of co-localization among different fluorescent labels observed in confocal fluorescence microscopy images. The unit discusses a variety of ways to remove background and to adjust brightness and contrast settings to ensure reliable calculation of the extent of co-localization of different fluorescent labels.

UNIT 4.20 describes an in vitro live cell–based method for imaging and quantitatively measuring the degradation of extracellular matrix (ECM) components by live cells. Since proteolytic degradation of ECM components by cells is required for cells to grow, remodel, and migrate through the ECM, this methodology is useful for analyzing ECM degradation in the study of developmental processes as well as pathologies such as cancer. In the protocol, cells are grown in the presence of fluorescent dye-quenched protein substrates that are mixed with protein matrices. Upon proteolytic cleavage, fluorescence is released that directly reflects the level of proteolysis by the cells. Confocal microscopy and advanced imaging software can be used to quantify fluorescence that is released and thereby determine the extent of proteolytic degradation in space and time.

UNIT 4.21 describes the technique of photoactivated localization microscopy (PALM). This super-resolution imaging technique improves the spatial resolution of light microscopy by over an order of magnitude (10 to 20 nm resolution), breaking the diffraction barrier of light. PALM is based on the controlled activation and sampling of sparse subsets of photoconvertible fluorescent molecules whose illumination centroids are fitted and then summed into a final super-resolution image, revealing the complex distribution of dense populations of molecules within subcellular structures with nanometer precision. This unit describes the instrumention for PALM as well as the photoactivatable fluorescent protein (PA-FP) tags that are used as probes in this technique. Both single- and dual-color PALM super-resolution imaging of biological structures are described, with specific focus on adhesion complexes at the leading edge of crawling cells.

UNIT 4.22 describes methods for culturing polarized kidney cells in three dimensions (3-D) and ways to image these cells using new-generation confocal microscopy. Unlike polarized kidney cells that are grown on tissue culture plates, those grown in 3-D cultures of extracellular matrix differentiate into a multicellular structure of polarized cells. This process shares many characteristics with the physiological development of an epithelial tissue and the formation of polarity in epithelial cells. By imaging cells that have polarized in this 3-D context, new insights into the development and maintenance of epithelium becomes possible. Technical aspects for culturing and imaging MDCK 3-D culture for both fixed 3-D cultures and live-cell imaging are discussed.

UNIT 4.23 describes the technique known as Interference Reflection Microscopy (IRM). This technique uses the interference of reflected light waves to generate images with high contrast and definition. Although it is most useful for examining sites of close contact between a cell and substratum, IRM can be used to examine almost any cell attached to

a glass surface. The unit discusses optimal ways of obtaining IRM images using a laser scanning confocal microscope (LSCM). In addition, techniques are presented for imaging fixed and live cells, as well as simultaneous multi-channel capture of fluorescence and reflection images.

UNIT 4.24 focuses on fluorescence correlation spectroscopy (FCS), a technique that uses single-molecule fluorescence to monitor molecular dynamics in living cells. Practical methods are presented for expressing fluorescently labeled proteins and lipids to determine the diffusion timescales and the total number of diffusing fluorescent molecules in the cell. A step-step protocol for performing FCS with a commercial spectroscopy/microscopy system, the Zeiss Confocor 3, is provided, as well as instructions on how to set up live-cell FCS experiments, acquire reliable data, and analyze the data.

UNIT 4.25 describes imaging approaches for analyzing mitochondrial dynamics and function. Mitochondria serve many important cellular functions, including roles in aerobic metabolism, lipid modifications, redox balance, calcium maintenance, and controlled cell death. Mitochondria are motile and highly dynamic organelles due to their ability to undergo fission and fusion among themselves. This unit describes three imaging techniques useful for characterizing mitochondrial dynamism and membrane potential in living cells. The first technique, time-lapse confocal imaging of mitochondria, permits high-resolution, three-dimensional images of mitochondria to be obtained in live cells. The second technique, fluorescence recovery after photobleaching (FRAP), enables the connectivity among mitochondrial elements across the cell to be assessed. The third technique, microirradation of mitochondria loaded with a membrane, potential dye, permits analysis of mitochondrial membrane potential and its loss of potential upon local depolarization of mitochondrial membrane.

Chapter 4 also contains the Organelle Atlas, which is a collection of figures that shows organelles of the cell visualized using a variety of reagents and techniques.

Jennifer Lippincott-Schwartz

Proper Alignment and Adjustment of the Light Microscope

Optical microscopes can be powerful tools in biomedical research and diagnosis if properly aligned and adjusted. This is essential for optimal image quality and accurate quantitative measurements. Video cameras, digital cameras, and electronic image processing can improve visibility of structural detail resolved by the microscope optics in comparison to viewing by eye. However, the quality and accuracy of the image still depends critically on proper microscope alignment and adjustment.

This unit presents protocols for alignment and adjustment of a typical research compound light microscope for transillumination and epi-illumination imaging modes typically used today in biomedical research. The transillumination light modes include bright-field, phase-contrast, and differential interference contrast (DIC). The primary epi-illumination mode is fluorescence microscopy.

The described procedures are for alignment of a research upright microscope (Fig. 4.1.1). The procedures are applicable to inverted microscopes that have similar imaging and illumination light paths to the upright microscope. In either case, the specimen image is produced by an objective lens and the image is projected either to the eye with an eyepiece or to a camera with (and sometimes without) a projection lens. One lamp attached to the back of the microscope provides light for transillumination of the specimen through a condenser lens (Fig. 4.1.2 and Fig. 4.1.3). Another lamp attached to the back of the microscope provides light for epi-illumination of the specimen through the objective using a mirror in a filter cube to bring the illuminating light into the objective light path (Fig. 4.1.4). For both the transillumination and epi-illumination paths, there are field diaphragms for controlling the specimen region illuminated and condenser diaphragms for controlling illumination from the condenser. The lamps, the field diaphragm, and the condenser diaphragm, as well as the condenser and the objective, must be properly focused and centered for the best image formation. In addition, each different mode of image formation has special optical components that also require alignment and adjustment for optimal performance.

The first section in this unit (see discussion of Major Components of the Light Microscope) describes the location and basic functions of the most important features of the upright compound microscope. The second section (see discussion of Basic Imaging and Köhler Illumination Light Paths For Bright-Field and Fluorescence Microscopy) introduces the imaging and illumination light paths of the microscope and describes the principles of Köhler illumination, which is the alignment used typically for both transillumination and epi-illumination in the light microscope. Basic Protocol 1 lists steps in microscope alignment for transmitted-light Köhler illumination. Basic Protocol 2 lists steps in microscope alignment for epifluorescence Köhler illumination. Basic Protocols 3 and 4 provide brief descriptions of the principles of image formation and microscope alignment for phase-contrast and differential interference contrast (DIC) microscopy; these optical modes produce contrast of transparent specimens using transmitted-light illumination. Support Protocol 1 deals with mating cameras to the microscope and Support Protocol 2 deals with calibrating image magnification. Several procedures (see Support Protocols 3, 4, and 5) are given for testing the optical performance of the microscope; these also describe test specimens for microscope performance and their sources. Finally, Support Protocol 6 deals with the care and cleaning of microscope optics. The Commentary refers the reader to references that provide more comprehensive

Contributed by Edward D. Salmon and Julie C. Canman

Current Protocols in Cell Biology (1998) 4.1.1-4.1.26

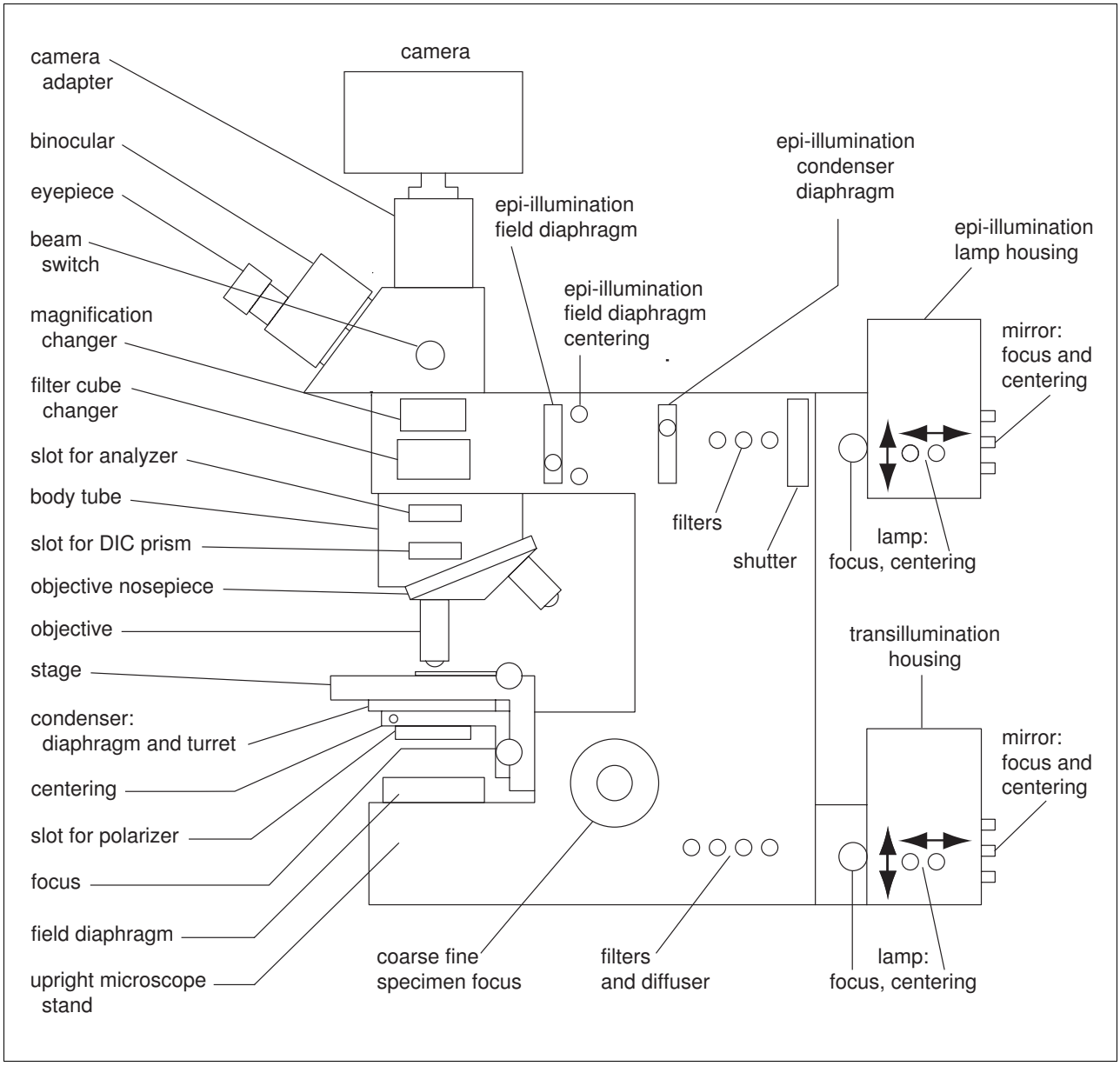


Figure 4.1.1 Diagram of the major component parts and centering screws for a research upright light microscope.

treatments of the basic modes and methods of light microscopy as well as advanced microscope imaging methods like multiwavelength, confocal, or multiphoton imaging, and electronic imaging techniques, including video and digital microscopy.

MAJOR COMPONENTS OF THE LIGHT MICROSCOPE

Familiarizing oneself with the components of the light microscope is best done using the manual for the microscope as reference. Figure 4.1.1 provides a diagram of an upright research compound light microscope equipped for both transmitted light and epifluorescence microscopy. Locate the following components on the microscope, identify their adjustment screws (if applicable) and movements (e.g., condenser focus), and ascertain that they appear correctly mounted. In this and the following sections, z is a direction along the microscope axis; x and y are perpendicular directions.

Image-Forming Components

Specimen stage and focus

A mechanical carrier on the stage holds the specimen slide. Knobs control movement in the *x*–*y* direction. Look for the vernier scales that mark the *x* and *y* positions. Rotatable stages are typically used for DIC microscopy. The objective is usually fixed and the specimen is focused by moving the stage along the *z* axis using coarse- and fine-focus knobs on the microscope body. Check the scale on the fine focus. On research microscopes, this is usually is 1 μm/unit.

Objectives and revolving nosepiece

Examine the different objectives on the nosepiece. Each objective is usually labeled with the following designations: magnification (e.g., 60×); the degree of optical correction (Apochromat is better than Fluor which is better than an Achromat); the label Plan (if both the center and edges of the field are in focus); numerical aperture (NA, which measures the half-angle of the cone of light from the specimen accepted by the objective; Fig. 4.1.3); immersion medium to be used (e.g., air, oil, water, or glycerin between the objective front lens and the specimen); coverslip thickness; optical tube length (previously 160 mm, currently infinity); and other special features like phase contrast, DIC, or long working distance. Working distance refers to the distance from the front element of the objective to the specimen. Working distance usually decreases with increasing objective magnification and NA. Check that the objectives are mounted in series from the objective of the lowest magnification to the objective of the highest magnification. It is usually best to find the specimen or region of a specimen with a low-magnification objective, and then swing in objectives with higher magnifications and shorter working distances. The high-NA, short-working distance oil-immersion objectives often have the ability to lock up the nosepiece when changing objectives. This prevents running the front element of the objective into the mounting medium on the edges of the coverslip. Check that the nosepiece is down for imaging.

Specimen slides and coverslips

Microscope condenser lenses are usually corrected optically for 1-mm-thick glass slides. The objectives are corrected optically for coverslips with a thickness of 0.17 mm; no. 1.5 coverslips are on average this thick. The image quality decreases for thinner (e.g., no. 1) or thicker (e.g., no. 2) coverslips, particularly for non–oil immersion ("dry") objectives with high NA. For oil-immersion objectives, this problem is not critical when the refractive index of the oil and coverslip (~1.515 to 1.52) match. Coverslips thinner than no. 1.5 are often used with oil immersion, to allow greater range of focus.

Body tube

In the modern research light microscope, the imaging light leaving the objective and nosepiece is unfocused and the image is projected to "infinity" (Fig. 4.1.2, left). This infinity space above the objective allows insertion of filters without changing the focal position of the image at the intermediate image plane. A positive focusing lens, the tube lens, above the infinity space, is used to bring the imaging light into focus for the eyepieces or camera.

Check for the following possible inserts in the infinity space of the microscope.

1. DIC prisms for the DIC objectives, which are usually right above the objective.
2. Analyzer; used for DIC microscopy (which should be removed from the light path for bright field, phase-contrast, and fluorescence microscopy).

3. Epi-illuminator filter-cube changer. These devices usually hold 2 to 4 filter cubes for fluorescence microscopy, each of which contains an excitation filter, an emission filter, and a dichroic mirror (Fig. 4.1.4) designed specifically for different fluorophores. Check the numbers on the filter cubes and mark the corresponding positions on the outside of the filter changer to identify the proper cube position for a given fluorophore.
4. Magnification changer and Bertrand lens. Body tube magnifications are selected from a turret, and possible values are 1.0×, 1.25×, 1.5×, and 2.0×. Often one position of the turret contains a Bertrand lens. This lens is used in combination with the eyepieces to produce a telescope view of the objective back focal plane (Fig. 4.1.2, far right). This is an important device for checking that the lamp image is centered and in focus during alignment for Köhler illumination, and for adjustment of the condenser diaphragm (see discussion of Transillumination Components).

Beam switch

This device switches the light between the binocular and the camera port. Check for the percentage of light in each direction. For fluorescence microscopy, it is important to be able to send 100% of the imaging light either to the eye or to the camera.

Eyepieces

Eyepiece magnification to the eye is marked on the barrel (e.g., 10×). Note that there are two and maybe three adjustments. The interpupillary distance for the eyes is adjusted by grasping the bases of the eyepiece tubes and moving them closer or further apart. At least one of the eyepieces is adjustable so that the eyes are parfocal.

Camera adapters

Check the type of camera adapter and whether it matches the detector. There are several different types of camera adapters. One type uses an eyepiece tube (not the binocular tube) and a projection eyepiece combined with a camera lens to project an image onto the camera detector. This method is common in photographic film cameras (which use ≥35-mm film) and for some video cameras with large-size detectors (e.g., 1-in., equivalent to 2.54-cm). The recent video and cooled slow and progressive scan charge-coupled device (CCD) cameras have small detectors (≤⅔ in., equivalent to 1.7 cm). These cameras require smaller projection magnifications or none—in the latter case the detector is mounted at the intermediate image plane, the focal plane of the objective (Fig. 4.1.2), and no eyepiece or projection lens is used.

Transillumination Components

Lamp and housing

The lamp is typically a low-voltage 100-W quartz halogen bulb with a tungsten-filament light source and variable control. Some lamp housings have no adjustments for centering the bulb; the socket is prefixed. Most lamp housings have *x*–*y* adjustments for the bulb socket, while an advanced lamp housing also has a mirror in the back for reflecting an image of the lamp back along the microscope axis. This mirror usually has adjustments for *x*, *y*, and *z* positions of the mirror image of the lamp. There is usually a knob on the side of the lamp housing for moving the collector lens back and forth along the *z* axis to focus the tungsten-filament image onto the condenser diaphragm plane.

Diffuser and filters

A diffuser and other filters are often inserted in slots in the base of the microscope. The diffuser helps spread the image of the source at the condenser diaphragm plane in order to uniformly fill the condenser aperture. This is important for achieving high resolution.

A heat-reflecting filter (e.g., BG 58) blocks infrared light. Video cameras, but not the eye, are often sensitive to this wavelength of light. A green filter is frequently preferred for illuminating living cells. High-efficiency interference filters with a 40-nm bandwidth around 540 nm are often best. Various neutral-density (ND) filters, which are not wavelength selective, are useful for attenuating light to cameras.

Field diaphragm

This is usually located just above or beneath the mirror that deflects the light up to the condenser lens. It controls the specimen area illuminated by the condenser (Fig. 4.1.2, left).

Polarizer

This is inserted below the condenser diaphragm for polarization and DIC microscopy. Otherwise, it is removed from the light path.

Condenser focus knob

This translator moves the condenser along the microscope *z* axis to focus an image of the field diaphragm on the specimen.

Condenser centering screws

There are usually two screws on the condenser carrier which move the condenser in an *x–y* plane. These screws are used to center the image of the field diaphragm on the *z* axis.

Condenser diaphragm

The condenser diaphragm is located on the bottom of the condenser. It controls the angle (NA) of the condenser cone of illumination of the specimen (Fig. 4.1.3).

Condenser turret

Condensers have turrets with inserts for special image-contrast techniques such as phase-contrast and DIC microscopy. Each insert matches certain objectives. In phase-contrast microscopy, the inserts are annuli of different diameters designed to match the phase ring in phase-contrast objectives (Fig. 4.1.5). For DIC, the inserts are typically DIC prisms designed for certain objectives (Fig. 4.1.7). To see these inserts, as well as the opening and closing of the condenser diaphragm, remove the condenser from the condenser carrier by loosening the locking screen and turning it upside down.

Condenser lens

The condenser's main function is to provide bright, uniform illumination of both the specimen field (Fig. 4.1.2, right) and objective aperture (Fig. 4.1.3) for objectives with different NAs. Check the markings on the condenser to see if it is designed for air (dry) or oil immersion with the glass slide. Dry (no oil-immersion) condensers have NA values of ≤0.9 and should not be used with immersion oil. Oil-immersion condensers usually have NA values of 1.0 to 1.4 when using immersion oil.

Epi-illumination Components

Lamp and housing

The light source for the epifluorescence illumination is typically an HBO 50-W or HBO 100-W mercury arc lamp or a xenon lamp of similar wattage. These lamps need to be handled with care because they can blow up if mistreated. Their glass envelopes should be carefully cleaned with 70% ethanol before installation to prevent fingerprints or other materials from inducing hot points that may result in fracturing of the bulbs. Look for the adjustment screws to adjust the *x–y* position of the lamp. Look also for the rear mirror

and its *x*, *y*, and *z* adjustment screws. Identify the focusing knob for the lamp collector lens. Do not turn the lamp on until the bulb has been installed according to the manufacturer's instructions, the collector lens has been inserted properly, and the lamp housing has been installed on the back of the microscope. These lamps produce intense light, and the mercury bulbs have large peaks in the UV range. Therefore, UV-protective glasses should always be worn when handling and installing these lamps. The light intensity decreases and the probability of explosion increases with the number of hours of operation and lamp starts. Check the timer on the lamp power supply to see that the recommended limits are not exceeded.

Shutter
Photobleaching is a big problem in fluorescence microscopy. A shutter is used to block the light from the specimen when not taking exposures on a camera or viewing by eye.

Filters
A heat-reflecting filter is used to prevent infrared illumination of the specimen and to keep infrared scattered light from reaching the camera. Neutral-density (ND) filters are used to reduce the light intensity of fluorescence illumination by the amount indicated on each filter.

Epi-illumination condenser diaphragm
This diaphragm provides variable adjustment of the illumination intensity. Some microscopes lack this diaphragm.

Epi-illumination field diaphragm
This diaphragm controls the area of the specimen illuminated. Look for the centering screws that control the *x*–*y* position of the field diaphragm.

Filter cubes
See discussion of Image-Forming Components.

BASIC IMAGING AND KÖHLER ILLUMINATION LIGHT PATHS FOR BRIGHT-FIELD AND FLUORESCENCE MICROSCOPY

The second major step in learning proper alignment of the light microscope is to understand the basic image-forming and Köhler illumination light paths and the functions of the key optical components and diaphragms. These are outlined in Figure 4.1.2, Figure 4.1.3, and Figure 4.1.4.

Imaging-Ray Paths

The upper left section of Figure 4.1.2 shows ray paths for the image-forming light from the objective. The objective, in combination with the tube lens, produces a real, magnified image of the specimen at the intermediate image plane. The eyepiece provides a second stage of magnification to the eye. The magnification to the eye is the product of objective magnification and eyepiece magnification multiplied by any magnification provided by additional lenses in the body tube of the microscope between the objective and the eyepieces.

When a camera detector—film, video, or charge coupled device (CCD)—is mounted at the intermediate image plane, the magnification to the camera is the magnification produced by the objective and body tube. When a camera is mounted above an eyepiece, then the unfocused light leaving the eyepiece must be focused onto the camera detector by a camera lens, which modifies the total magnification to the detector. Special adapters (see Image-Forming Components, Camera adapters) are also available for projecting the

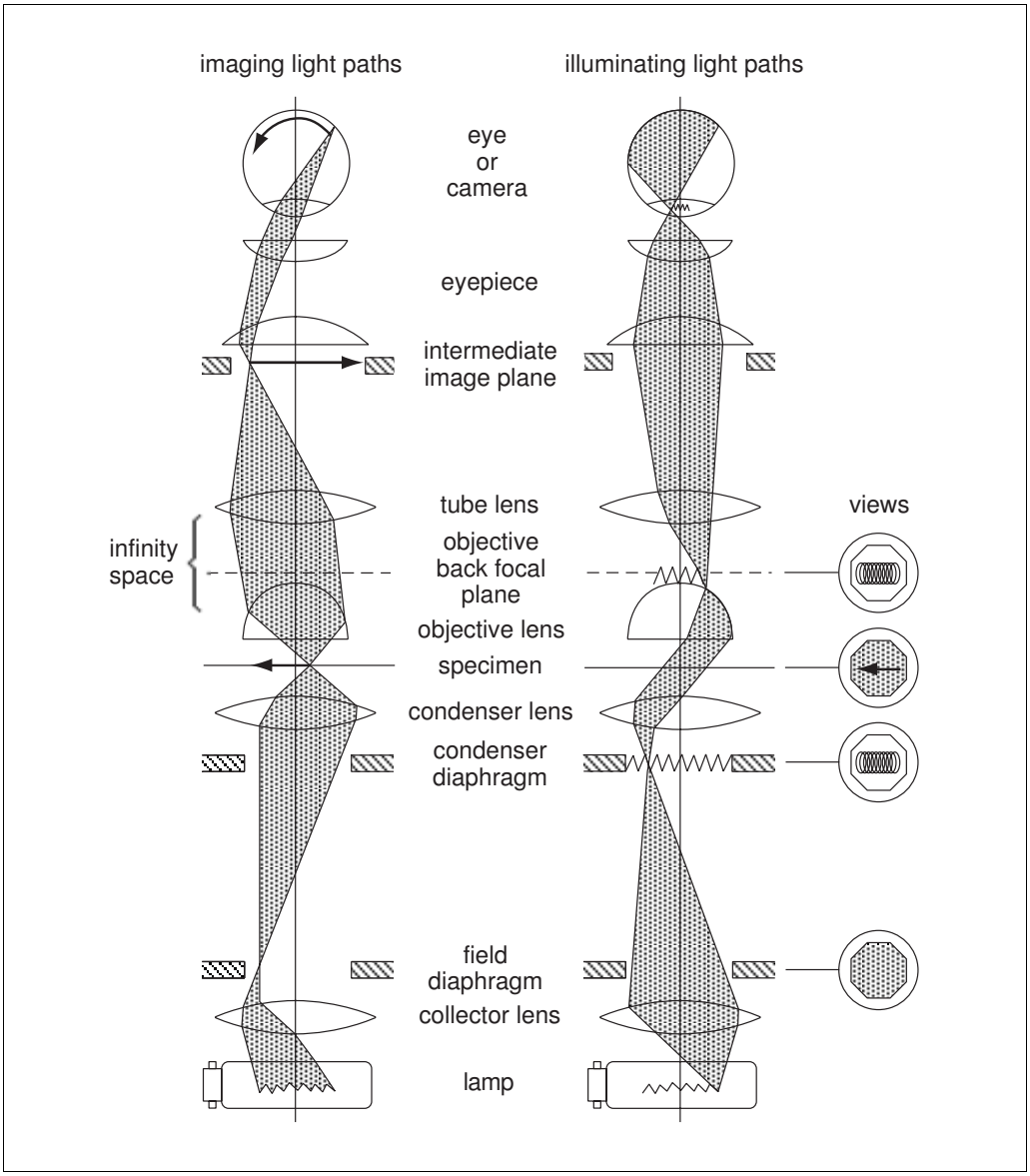


Figure 4.1.2 The imaging and illuminating light paths for a bright-field microscope aligned for transmitted light Köhler illumination. Modified from Keller (1998).

objective image onto a camera detector with magnification, but without the need for an eyepiece.

Transillumination Ray Paths

In the illustration of the standard Köhler method (Fig. 4.1.2), trace the illumination ray paths for a bright-field microscope aligned for Köhler transillumination (right side). A point on the light source is focused by the lamp collector lens onto the front focal plane of the condenser lens, where the condenser diaphragm is located. This light is projected, out of focus, through the specimen by the condenser lens, producing uniform illumination of the specimen. The objective collects the unfocused illumination light and brings an image of the light source into focus at its back focal plane, which is at the back aperture of the objective (Fig. 4.1.3). The light source is again out of focus at the intermediate image plane and at the retina of the eye or the detector of the camera. In between these two points, the light source is in focus at the exit pupil of the eyepiece, at a position ~15

mm above the eyepiece. This position is also called the eyepoint, the position of eye placement above the eyepiece (Fig. 4.1.2, right).

The condenser diaphragm controls the NA (cone angle) of specimen illumination by the condenser lens (Fig. 4.1.3). Opening the diaphragm increases the aperture of illumination, which increases both the light intensity and resolution in bright-field light microscopy (see Support Protocol 3). Note on the right side of Figure 4.1.2 that the image of the condenser diaphragm is in focus where the lamp image is in focus along the microscope axis, at the objective back focal plane and the exit pupil of the eyepiece.

Trace the imaging light rays from the field diaphragm through the microscope (Fig 4.1.2, left). Note that the field diaphragm controls the specimen area illuminated by the condenser. Note also that the field diaphragm is placed between the lamp collector lens and the condenser lens in a plane where the lamp image is out of focus (compare Fig. 4.1.2, left and right). When the condenser lens has focused the image of the field diaphragm onto the specimen, then the field diaphragm will be in focus with the specimen at the intermediate image plane and at the eye or detector.

Epi-illumination Ray Paths

Figure 4.1.4 shows the optical alignment for an epi-illuminator and Köhler illumination through the objective. As in transillumination (Fig. 4.1.2), the light source is focused by a collector lens onto the condenser diaphragm of a condenser lens in the light path. The condenser lens illuminates a field diaphragm. Another lens collects this light and projects it off a reflective mirror into the objective. The image of the light source is focused at the back focal plane of the objective so that it is out of focus at the specimen plane. In epifluorescence microscopy, filter cubes containing dichroic mirrors are used in combination with excitation and emission filters to efficiently reflect the excitation light into the objective and transmit to the eyepiece or camera only the longer-wavelength fluorescent light emitted from the specimen (see *UNIT 4.2* and Taylor and Salmon, 1989).

BASIC PROTOCOL 1

ALIGNMENT FOR KÖHLER ILLUMINATION IN BRIGHT-FIELD, TRANSMITTED LIGHT MICROSCOPY

The following procedures assume that the positions of the quartz-halogen tungsten filament and collector lens are adjustable. Inexpensive, non-research compound microscopes may have the illuminator built into the stand, with no adjustment for the lamp. Such microscopes depend on a ground-glass filter for even illumination. For these microscope stands, skip steps 2, 3, 4, and 8.

Focus the light source

1. If possible, remove the diffusion filter in the transillumination pathway during alignment so that a crisp image of the light source can be viewed at the condenser diaphragm plane and at the objective back focal plane. When alignment is complete, reinsert the diffusion filter.

2. Center and focus the lamp filament near the condenser diaphragm plane. Remove the condenser lens. Place a sheet of lens paper on the microscope stage. Close down the field diaphragm and adjust the intensity of the lamp so that the lens paper is moderately illuminated. Use the lamp-focusing knob to move the condenser lens (or lamp) along the *z* axis until an image of the filament is in focus on the lens paper. Roughly center the lamp on the microscope axis with the adjustment screws on the lamp housing, then adjust the mirror image (if there is a mirror) using its adjustment screws on the lamp housing.

Table 4.1.1 Suppliers for Items Used In Light Microscopy

Test specimen	Supplier
Hematoxylin/eosin-stained skeletal muscle	Carolina Biological Supply
Diatom test plate	Carolina Biological Supply
Squamous cheek cells	Freshly prepared (see Support Protocol 4)
Stage micrometer	Fisher
Red, green, and blue fluorescent tissue culture cells	Molecular Probes

3. Place the lens paper on the condenser carrier and raise the condenser carrier until it is ~20 mm from the top of the stage.

   This is the approximate position of the condenser diaphragm when a condenser is installed and in focus.

4. Refocus the lamp and mirror images at this position of the lens paper.

   If there is no mirror image, center the lamp image. If there is a mirror image, then position the images so that they sit side by side to fill the condenser aperture. Remove the lens paper and replace the condenser.

Focus for low-power viewing

5. Obtain an in-focus image of a specimen with the low-power objective (10× or 16×) by placing a test specimen that absorbs light on the stage (e.g., stained muscle section; see Table 4.1.1).

   The focus position can be estimated from the working distance of the objective lens. For a standard 10× lens, this is ~4 mm. So, use the coarse focus to bring the specimen to ~16 mm from the coverslip by viewing the objective position from the side of the microscope.

6. Move the condenser lens up close to the lower surface of the slide and open the field and condenser diaphragms all the way. Look down the eyepieces, and use the coarse- and fine-focus knobs to bring the specimen into sharp focus.

7. Focus the image of the field diaphragm centered on the specimen by adjusting the condenser *x–y* screws and the condenser focus. Initially close down the field diaphragm until an edge of the image can be focused by the condenser, then close the field diaphragm further as the image is centered, using the *x–y* translation screws.

   Usually, the field diaphragm is opened just enough to match the field of view in the eyepiece or the camera. However, the best image contrast is obtained if the field diaphragm is opened just enough to illuminate the region of interest. This eliminates the presence of scattered light from outside the region of interest.

8. Center an in-focus image of the lamp and mirror images at the objective back focal plane using the focus and adjustment screws on the lamp housing. View the objective back aperture (the position of the back focal plane; Fig. 4.1.3) by using the Bertrand lens in the magnification changer in combination with the eyepieces, by replacing one eyepiece with a telescope, or by simply removing one eyepiece and peering down the body tube. Open the condenser diaphragm all the way. Readjust the collector lens and the mirror so that the lamp images are in focus and sit side by side to fill the objective aperture.

   This is a rough adjustment with the low-power objective and will need to be fine-tuned using the objective with the highest NA.

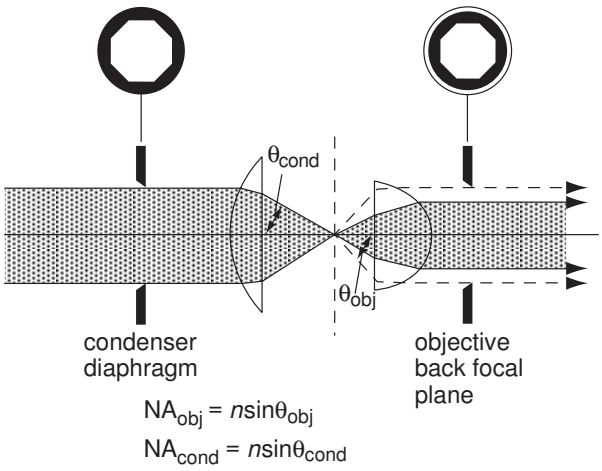


Figure 4.1.3 Numerical aperture (NA) of objective light collection and condenser illumination. The objective numerical aperture (NA_{obj}) depends on the angle of the cone of light from the specimen, which is accepted by the objective aperture while the numerical aperture of condenser illumination (NA_{cond}) is controlled by the condenser diaphragm and limited by the maximum NA of the condenser when the condenser diaphragm is wide open.

9. Adjust the opening of the condenser diaphragm so that the diameter of its image at the objective back focal plane (see far right of Fig. 4.1.2; also see Fig. 4.1.3) is slightly less than the diameter of the objective back aperture.

   Some research condensers also have centering adjustments for the condenser diaphragms. The image of the condenser diaphragm should be centered in the objective back aperture.

10. Adjust the light intensity for comfortable viewing of the specimen by adjustment of the light-source intensity, either using the power-supply rheostat or by inserting neutral-density filters in the illumination light path.

    The microscope is now adjusted properly for the low-power objective.

Adjust for 40× viewing

11. Switch the turret to the 40× high-dry objective.

    Note that because of parfocal objective design the specimen is almost in focus. Focus on the specimen with the fine focus.

12. Focus and center the field diaphragm with the condenser focus and the condenser-carrier *x*–*y* adjustment screws.

13. View the objective back aperture (Fig. 4.1.3).

    Notice that the image of the condenser diaphragm adjusted for the low-power objective is only about half the diameter of the 40× back aperture. The ratio of the diameter of this image to the diameter of the objective back aperture is equal to the ratio of the NA of the condenser illumination to the NA of the objective (Fig. 4.1.3). When the condenser illumination NA equals the objective NA, the aperture of the objective is filled with light and maximum resolution will be achieved for the objective NA (see Commentary).

14. Adjust the condenser diaphragm so that the condenser illumination NA just about matches the objective NA.

    Each time objectives are changed, these procedures should be repeated.

Adjust for high-power viewing

15. Rotate the nosepiece so that the high power (60× to 100×, NA 1.25 to 1.4) oil-immersion lens is coming into place, but stop before it clicks into place. Place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the point upon which the condenser beam is focused.

    This oil drop must be free of air bubbles and dirt. The refractive index of the immersion oil should be close to that of the glass coverslip.

16. Complete the rotation of the nosepiece so that the oil-immersion objective clicks into place. Lower the objective nosepiece if appropriate.

    The space between its front lens and the coverslip is now filled with immersion oil.

    IMPORTANT NOTE: *Use only the fine adjustment knob when working with the oil-immersion lenses.*

17. Remove the eyepiece (or use the telescope or Bertrand lens) and inspect the back aperture of the objective. Open the condenser diaphragm as much as possible to try and match its image with the objective aperture (Fig. 4.1.3).

    This will be impossible if the condenser is dry and not designed for oil immersion, since dry condensers have NA ≤0.9 and the relative size of the image of the condenser aperture or condenser diaphragm seen in the objective back focal plane is given by the ratio of NA_{cond}/NA_{obj} *(Fig. 4.1.3).*

18. View the objective back aperture and tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

19. Replace the eyepiece (or remove the Bertrand lens) and examine the specimen. Adjust the field diaphragm until its margins just match the field of view.

    With inexpensive condensers, the image of the edge of the field diaphragm will not be in good focus, even at the optimum position. If a condenser designed for oil immersion is being used, enormous improvement in the field iris image can be achieved by oil immersion.

20. Oil the condenser (if possible) to achieve the highest resolution and image quality. To oil a condenser, rotate the objective out of the way, remove the slide, oil the condenser (it takes much more air-bubble-free oil than the objective), then replace the slide and refocus the objective.

    Before oiling the condenser, make sure it is not designed to be a "dry" condenser by checking that it is marked with an NA >0.9.

    The image of the field diaphragm should now be much sharper when in focus and centered.

21. Look at the objective back aperture. Again, tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

    After oiling, the aperture should be filled by the condenser illumination when the condenser diaphragm is wide open.

    Notice also that viewing the objective back aperture is the best way to see if air bubbles have become trapped in the oil. If so, carefully wipe off the oil with lens paper and repeat steps 15 through 21.

22. Reinsert the diffusion glass in the illumination light path.

    This will reduce light intensity, but make the illumination of the objective aperture more uniform.

23. When finished for the day, use lens paper to remove excess oil from the objective and condenser lens surfaces to prevent dripping on specimens.

    It is not necessary, however, to completely clean oil from the optics after every use (see Support Protocol 6).

BASIC PROTOCOL 2

ALIGNMENT OF THE EYEPIECES

The binocular usually has adjustments for the inter-eyepiece distance and visual acuity in each eye. The goal is for the image to be in focus for each eye without any eyestrain or discomfort. Only the left eyepiece tube or eyepiece is adjustable on a typical microscope. However, if the microscope has a target reticle in the microscope stand that can be rotated into view, then usually both eyepieces are adjustable.

1. With a low-power objective (10× or 16×), focus on a stained specimen placed on the stage of the microscope (e.g., stained muscle section, see Table 4.1.1) and align the microscope for Köhler illumination.
2. Move the bases of the eyepiece tubes together or apart to set the proper interpupillary distance for your eyes.

3a. *If the microscope has a target reticle:* Rotate the reticle into the field of view. Close the left eye and use the diopter-adjustment ring on the right eyepiece until the target is in sharp focus for the right eye. Then close the right eye and use the diopter-adjustment ring on the left eyepiece until the target is in sharp focus for the left eye. Remove the target to view the specimen.

3b. *If the microscope has no target reticle:* Set the right eyepiece tube to the inter-eyepiece setting (if possible). Close the left eye and bring into sharp focus a structural detail near the center of the field of view with the right eye. Close the right eye and use the diopter-adjustment ring on the left eyepiece tube to bring the specimen detail into sharp focus for the left eye. With both eyes open, fine tune the focus of the left eyepiece tube.

BASIC PROTOCOL 3

ALIGNMENT FOR KÖHLER ILLUMINATION IN EPIFLUORESCENCE MICROSCOPY

The eye is most sensitive to green light. Hence, the following protocol is best done using a filter cube that produces green excitation light (e.g., a filter cube for rhodamine).

Focus the lamp

1. Remove one objective, and rotate the nosepiece so the open position is centered on the microscope axis. Place a white card on the microscope stage, on top of supports that position the card at ~2 to 3 cm from the nosepiece.

   This is approximately the position of the objective back focal plane, where the images of the light source should be in focus and centered after alignment (Fig. 4.1.4).

   Some microscopes have a special device for lamp alignment. It screws into the objective nosepiece and projects an image of the epi-illumination arc and electrodes onto a small diffusion screen within the barrel of the device.

2. Install the mercury bulb in the lamp housing and attach the lamp housing on the back of the microscope according to the manufacturer's instructions.
3. Turn on the power source and ignite the lamp.

   It takes ~10 min for the arc to brighten.

   CAUTION: *It is usually a good idea to make sure computers in the vicinity are turned off before starting the lamp because the high voltage pulse used to ignite the arc plasma may damage the electronics. Laboratory personnel should protect their eyes against UV light from the lamp.*

4. Close down the field diaphragm and open the condenser diaphragm (if there is one) all the way.

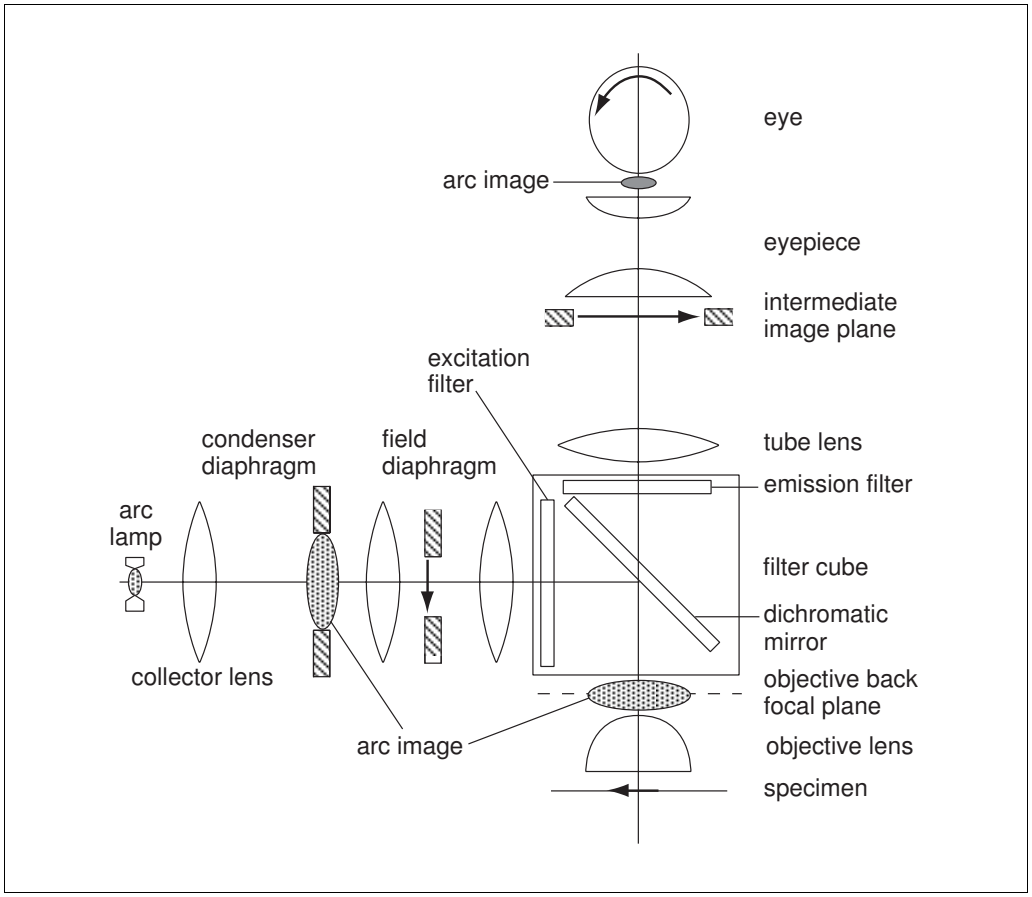


Figure 4.1.4 Microscope alignment for epifluorescence Köhler illumination.

5. On the diffusion glass screen or on the white card on the stage, the image of the arc between the tips of the two electrodes should be visible. Use the lamp focusing knob to obtain an in-focus image of the lamp arc and electrodes. Use the lamp *x–y* adjustment screws to approximately center the image of the arc and electrode tips (see Figure 4.1.2).

6. If there is a lamp mirror, use its adjustment screws to focus the mirror image of the arc and electrode tips, centered on the target.

   Often the primary image and the mirror image of the arc are adjusted side-by-side and slightly overlapping, but centered on the microscope axis.

Focus the field diaphragm

7. Place a test fluorescent specimen (see Table 4.1.1) on the stage and rotate the objective nosepiece to a low-power objective. Select the appropriate filter cube for the fluorescent specimen. Open up the epi-illuminator field diaphragm all the way. Open the shutter and view the specimen.

8. Focus the specimen, then close down the field diaphragm until an edge comes into the field of view. Use the *x–y* adjustment screws for the field diaphragm to center the field diaphragm image within the field of view.

9. Close down the field diaphragm until only the region of interest is illuminated.

   This prevents photobleaching of areas outside the region of interest and reduces the amount of scattered fluorescent light in the image from outside the region of interest.

10. Switch to a high-power objective. For an oil-immersion objective, place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the region of interest. Carefully swing in the objective. Open the shutter and focus on the specimen. Readjust the size and centration of the field diaphragm.

    This oil drop must be free of air bubbles and dirt.

11. To prevent photobleaching, be sure to close the epi-illumination shutter when not viewing or taking camera exposures.

BASIC PROTOCOL 4

ALIGNMENT FOR PHASE-CONTRAST MICROSCOPY

Phase-contrast microscopy is often used to produce contrast for transparent, non–light-absorbing, biological specimens. The technique was discovered by Zernike, in 1942, who received the Nobel prize for his achievement (Zernike, 1942, 1955, 1958). The last of these references describes the principles of phase contrast and provides an excellent introduction to the wave optics of image formation, resolution, and contrast in the microscope.

The phase-contrast microscope is a bright-field light microscope with the addition of special phase-contrast objectives (Fig. 4.1.5) containing a phase plate or ring and a condenser annulus instead of a diaphragm; the annulus is usually located on a condenser turret because it has to be selected for different objectives. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method. However, there is no condenser diaphragm to adjust. Instead, the phase annulus must be selected and adjusted properly. Modern phase-contrast objectives have a phase plate containing a ring in the back focal plane within the barrel of the objective. This ring

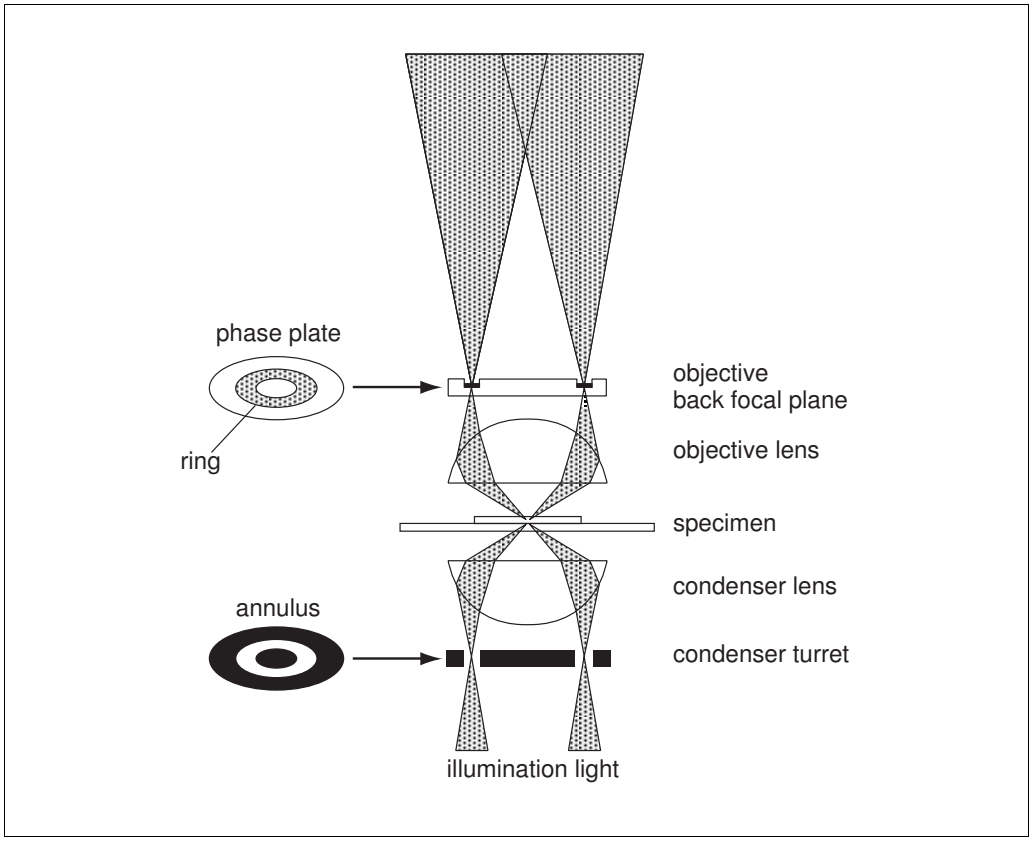


Figure 4.1.5 Illumination light path through the condenser annulus and objective phase ring in a microscope aligned for phase-contrast microscopy.

absorbs and advances the phase of the light passing through it by ¼ wavelength in comparison to light passing through the rest of the objective aperture. For each phase objective, there is a corresponding annulus in the condenser turret that has about the same relative size as the phase ring in the objective. Light passing through this annulus passes through the phase ring in the objective when the annulus is selected and aligned properly. Light scattered by the specimen mainly passes through the objective aperture outside the phase ring. Light scattered from a thin transparent specimen is ~¼ wavelength retarded from the unscattered illumination light. The additional ¼ wavelength retardation between the scattered and illumination light produced by the objective phase ring makes the scattered and illumination light 180° out of phase. They destructively interfere with each other at the image plane to produce the "dark" contrast of structural detail typical of phase contrast images of biological specimens.

Because the phase annulus and the phase ring reduce the intensity of the background light, a bright illuminator—e.g., a 100-W quartz-halogen illuminator—is necessary at high magnifications (because image intensity decreases as 1/magnification^2). For living cells, heat reflection and green illumination filters should be used.

To examine the objective phase ring and matching condenser phase annulus, remove the low- and medium-power phase objectives and the phase condenser from the microscope. View the phase ring (phase plate) within the objective by looking in the back end. The phase ring is located at the position of the objective back focal plane; its diameter is usually ~⅔ that of the objective aperture. Note that it is situated within the objective body and is visible because it absorbs light. Locate the annulus for each objective in the condenser turret. The annulus is located at the condenser diaphragm plane, which is situated at the condenser front focal plane. Notice that as the objective NA increases, the diameter of the corresponding annulus in the condenser turret increases. The NA of condenser illumination from an annulus is designed to match that of the phase ring in the corresponding objective.

Replace the condenser on the microscope and illuminate with white light. Hold a piece of tissue paper near the top of the condenser and examine the illumination cone. Notice that it is an annular cone of illumination. Change the annulus from the setting for the low-power objective to the 40× setting. Note that the NA or angle of illumination increases. Points of illumination further from the central axis in the condenser diaphragm plane produce higher-aperture rays passing through the specimen.

Perform alignment using the following steps.

1. Align the microscope for bright-field Köhler illumination using the low-power phase objective (10× or 16×) and a cheek cell preparation for the specimen (see Support Protocol 4).

   The cheek cell specimen is an excellent transparent test specimen for alignment of the microscope for phase contrast and DIC. The cheek cells are transparent and only barely visible by bright-field illumination. To find the plane of focus in bright field, initially close down the condenser diaphragm and look for the edges of air bubbles in the preparation. These edges scatter much light and appear dark in the image.

2. Align the condenser annulus with the phase ring by rotating the condenser turret to the position where an annulus matches the phase ring in the objective; use the telescope, your eye, or the Bertrand lens to view the objective back aperture.

   As the condenser turret is rotated, notice in the objective back focal plane the images of the different-diameter annuli designed for the different numerical aperture objectives. The correct annulus may not be properly aligned with the objective phase ring.

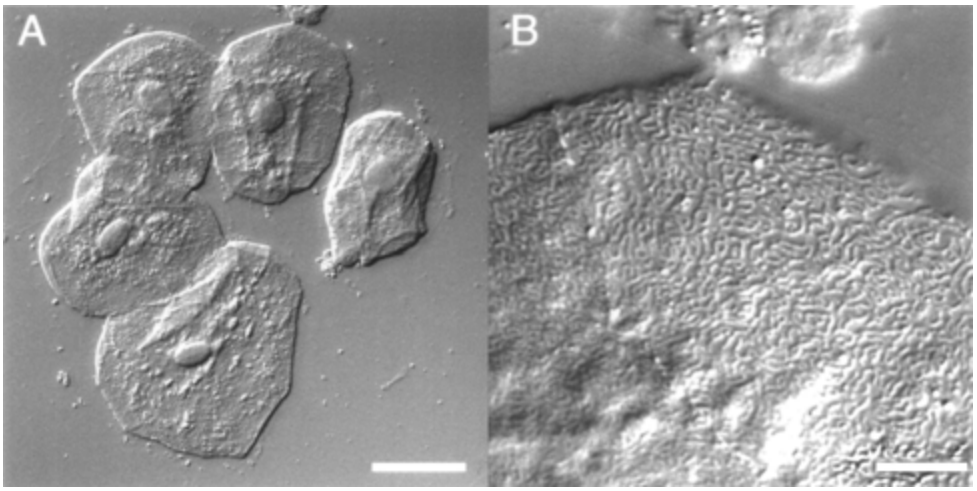


Figure 4.1.6 DIC images of a human cheek cell test specimen. (**A**) Low magnification of cheek cell preparation with a 20× objective. Bar = 20 µm. (**B**) High-resolution image of the surface of the cell at the top of (A) using a 60×/(NA = 1.4) Plan Apochromat objective and matching condenser ilumination. The ridges on the cell surface are often diffraction limited in width. Bar = 5 µm. From Salmon and Tran (1998), reprinted with permission from Academic Press.

3. Use the adjustment screws in the condenser (and the special tools if necessary) to move the annulus in the *x*–*y* plane to achieve alignment with the phase ring in the objective.

   Note that the phase ring is slightly wider than the image of the correct annulus. It is critical that the image of the annulus be within the phase ring, but it is not critical if it is very slightly off center.

4. When the objective phase ring and condenser annulus are aligned, view the specimen and properly adjust the focus and centration of the field diaphragm.

   Notice that if the phase ring and annulus are slightly misaligned (rotate the turret slightly), the background light intensity goes up. This is because the phase ring is designed to absorb much of the illumination light. The unscattered illumination light becomes closer to the intensity of the light scattered by the specimen, which passes through the objective aperture outside of the phase ring. Minimizing the background light intensity while viewing the specimen image can also be used to align the annulus with its phase ring or to touch up the alignment done by viewing down the body tube.

5. Switch to the 40× phase objective and then the high-power 60× or 100× phase objective (NA 1.25 to 1.4) and repeat steps 1 to 4.

   It is necessary to increase light intensity at the higher magnifications. It should be possible to see the ridges on the cell surface (see Fig. 4.1.6 for a DIC image of the ridges). Notice the "phase halos" at discrete edges, a problem that limits conventional phase contrast in high-resolution imaging.

BASIC PROTOCOL 5

ALIGNMENT FOR DIC MICROSCOPY

Since its introduction in the late 1960s (e.g., Allen et al., 1969), DIC microscopy has been popular in biomedical research because it highlights edges of specimen structural detail, provides high-resolution optical sections of thick specimens—including tissue cells, eggs, and embryos—and does not suffer from the "phase halos" typical of phase-contrast images. See Salmon and Tran (1998) for details of image formation and video-contrast enhancement.

Proper Alignment and Adjustment of the Light Microscope

The DIC microscope is a bright-field light microscope with the addition of the following elements (Fig. 4.1.7, middle): a polarizer beneath the condenser; a DIC beam-splitting

prism (Nomarski or Wollaston) in the condenser turret; a DIC beam-combining prism (Nomarski) just above the objective; an analyzer above the objective prism in the infinity body tube space; a compensator after the polarizer or before the analyzer in some microscopes (not shown in Fig. 4.1.7); and a rotatable stage. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method.

The polarizer, which produces plane-polarized light (Fig. 4.1.7, left), is typically oriented with its transmission azimuth in an east-west direction facing the front of the microscope. Polarizers with high transmission efficiency are preferred. Polarizers are usually high-quality polaroid material held between thin optical glass flats. Another polarizer is used as an analyzer. The transmission azimuth of the analyzer is oriented north-south at 90° with respect to the polarizer azimuth to produce extinction of the illumination light in the absence of the DIC prisms.

The specimen is held on a rotating stage. Contrast depends on orientation, and frequently specimens must be reoriented to achieve maximum contrast of the structures of interest. Either the stage or the objective and condenser must be centerable.

For the highest sensitivity, the objective and condenser lenses should be selected free of birefringence (Inoué and Spring, 1997). Rectifiers can be used to correct for the rotation of the plane of polarization of light which occurs at the periphery of lens surfaces (Inoué and Spring, 1997).

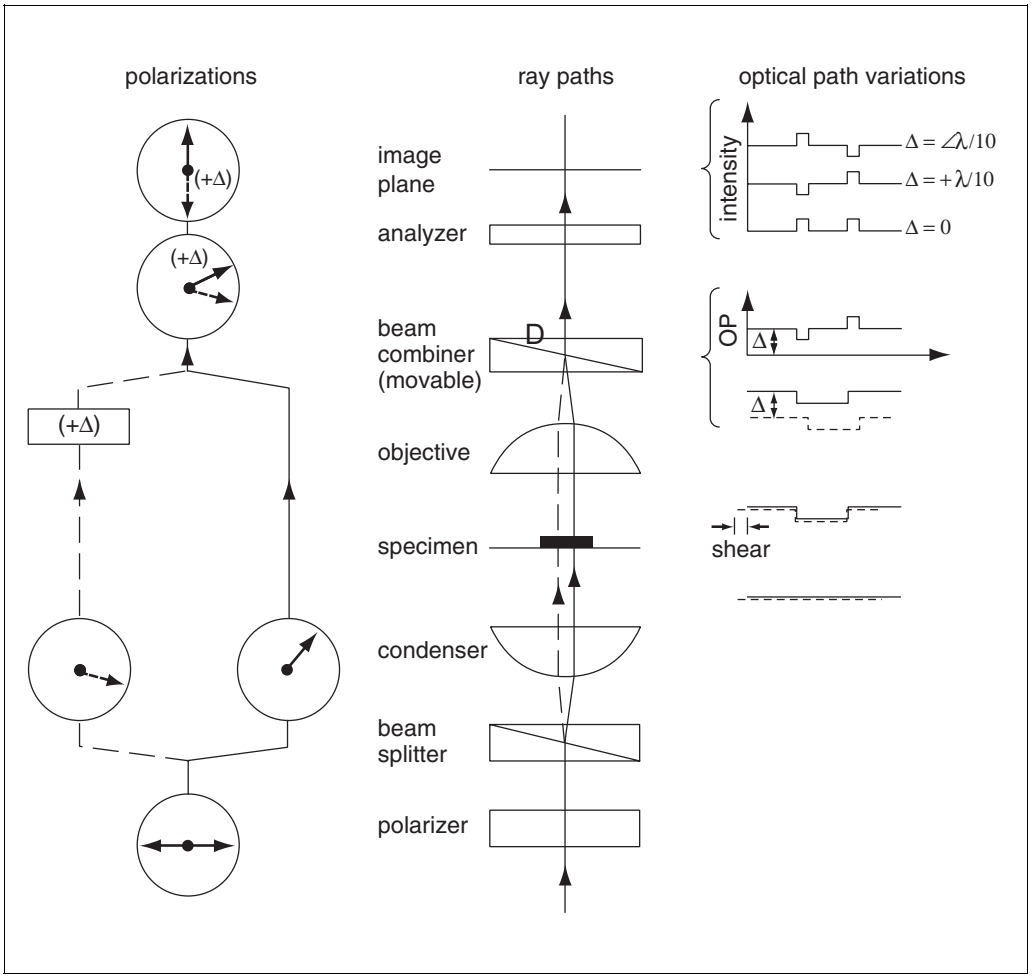


Figure 4.1.7 The optical system for DIC microscopy. From Salmon and Tran (1998), reprinted with permission from Academic Press.

Figure 4.1.7 outlines the principles of DIC image formation and contrast. The DIC beam-splitting prisms are located at the condenser diaphragm plane, just above the polarizer in the condenser turret. Objectives of different magnification and/or NA usually require condenser prisms of different optical characteristics.

The condenser prism splits the light coming from the polarizer into divergent polarized light wavefronts whose planes of polarization are orthogonal to each other and at 45° with respect to the initial plane of polarization (Fig. 4.1.7, left). The divergent beams are converted by the condenser into two wavefronts, which pass through the specimen separated laterally from each other in the direction of the prism wedge (the shear direction, Fig. 4.1.7, middle) by a tiny amount that is usually less than the resolution limit of the condenser-objective lens combination (Fig. 4.1.7, right). These two wavefronts are recombined just above the objective by a beam-combining prism. Often, each objective has its own prism so that it accurately matches the action of the condenser prism. In some microscopes, there is one beam-combining prism for all objectives and a different condenser prism for each objective. Check which is the case for your microscope.

DIC image contrast depends on the "compensation" or "bias retardation" (Δ) between the two wavefronts along the microscope axis (Fig. 4.1.7, right). When the objective beam-combining prism is perfectly aligned with the condenser beam-splitting prism and there is no compensation ($\Delta = 0$), the background light is extinguished and the edges of objects are bright against a black background (Fig. 4.1.7, upper right). When one wavefront is retarded relative to the other by Δ, this increases the optical path (OP) between the wavefronts (Fig. 4.1.7, middle right) and brightens the background light. One edge of an object becomes brighter than the background while the opposite edge becomes darker (Fig. 4.1.7, upper right). This produces the "shadow cast" appearance of DIC images. Reversing the sign of retardation, reverses the contrast of the specimen edges.

In some microscopes, the objective beam-combining prism (Fig. 4.1.7, middle) is used as a compensator by translating the prism in the direction of prism wedge away from the position of background light extinction. One direction produces positive while the other produces negative retardation (Δ). In other microscopes, both the objective and condenser prisms are fixed at positions that produce background light extinction, and typically a deSenarmont compensator (a birefringent quarter-wave retarder in combination with a rotatable polarizer or analyzer; Inoué and Spring, 1997) is inserted just above the polarizer or beneath the analyzer.

Bright illumination sources are required for high magnification because of the crossed polarizer and analyzer; at least the 100-W quartz-halogen illuminator is usually needed.

To examine the DIC prisms, remove the objective prisms and the DIC condenser from the microscope. Examine the objective prism. Notice that it is very thin and wide enough to cover the objective aperture. Also notice if there is a screw that can be used to translate the prism back and forth in one direction across the objective aperture. Examine the prisms in the condenser turret. Notice that there are different prisms for low- and high-NA objectives. The highest-resolution (NA) objectives often have special prisms designed only for the optical properties of that objective. In aligning the microscope for DIC, be careful to use the condenser Wollaston prism that matches the objective in use. Reinstall the condenser.

Steps 1 to 6 in the following procedure are for the initial alignment of the microscope. Once this is done, then begin at step 7 for routine use.

Perform initial alignment

1. Align the microscope initially for Köhler illumination using the low-power objective and the cheek cell preparation. Again look for the bright air-bubbles in the preparation to find the specimen plane.

2. Check that the polarizer is oriented with its transmission azimuth in an east-west direction as determined facing the front of the microscope (look for the line or double-headed arrow on the polarizer mount). Rotate the deSenarmount compensator (if there is one) so that it is at its zero position (no compensation).

3. Remove the objective prism and rotate the condenser turret to an open position. Make sure that the analyzer transmission azimuth is crossed to the polarizer by checking that the background light is at extinction.

   This exercise is best done with the brightest light position of a 100-W quartz-halogen illuminator.

4. Insert the objective DIC prism (observe caution as the image will be very bright). Observe the extinction fringe in the middle of the prism (you must use the telescope, remove an eyepiece, or insert the Bertrand lens to focus on the objective focal plane). Make a drawing of your view of the fringe in the back aperture.

   The fringe should be at 45° with respect to the analyzer-polarizer transmission azimuths.

5. Remove the objective DIC prism and rotate into place the condenser prism that matches the objective. Observe the objective back focal plane.

   The orientation of the fringe should match the orientation of the extinction fringe for the objective prism in your drawing.

6. Insert the objective prism and observe the objective back focal plane. Rotate in the other condenser prisms and notice that the extinction fringe is no longer spread across the objective aperture. Rotate in the correct condenser prism.

   If the condenser and objective prisms are properly matched and oriented in the same direction, the fringe should become spread across the objective aperture (a dark cross will still occur in high-power, unrectified objectives; Inoué and Spring, 1997).

Align for specimen viewing

7. View the specimen with the correct objective and condenser prisms in place. Translate the objective prism (or rotate the deSenarmount compensator if the objective prism is fixed) to induce a retardation (Δ) between the two wavefronts to brighten the background light and make the edges of the cell appear shaded. If possible, rotate the specimen and observe that contrast is directional—45° with respect to the analyzer-polarizer orientations.

   As the upper prism is translated (or the deSenarmount compensator is rotated) to compensation of opposite sign, the initially bright edges become darker and the initially dark edges become brighter than the background.

8. Adjust for proper bright-field, transmitted-light Köhler illumination.

   The quality of the DIC image depends critically on the field diaphragm being sharply focused on the specimen (Fig. 4.1.2, left), since this puts the condenser prism in the proper place. The highest possible resolution is achieved when the condenser diaphragm is adjusted to match the diameter of the objective aperture (view the objective back focal plane during this adjustment).

   Optimum edge contrast is produced when the retardation between the wavefronts is adjusted to extinguish the light coming from one set of edges in the specimen. Further retardation increases light intensity, but not contrast to the eye. When using video cameras,

it is often important to view the specimen by eye and adjust the compensation for best contrast, then change illumination intensity to provide the camera with enough light for good image quality.

9. Repeat steps 2 to 8 for the other objectives.

   Once you are sure of the correct prisms and their orientation, then only steps 7 and 8 are necessary for each objective.

SUPPORT PROTOCOL 1

MATCHING MICROSCOPE MAGNIFICATION TO DETECTOR RESOLUTION

This procedure uses the diatom test slide (see Table 4.1.1 for supplier information). For more details on matching a camera to the microscope see Hinsch (1998) or Inoué and Spring (1997).

1. Look down the eyepieces and obtain an in-focus image of the frustrule pores of the diatom *Pleurosigma angulatum* (Fig. 4.1.8, number 6 in panel A) using 40×/(NA ~0.7) phase-contrast or DIC and proper Köhler illumination.

   The pores should be clearly visible by eye.

2. Project the diatom image onto the faceplate of a video or CCD camera.

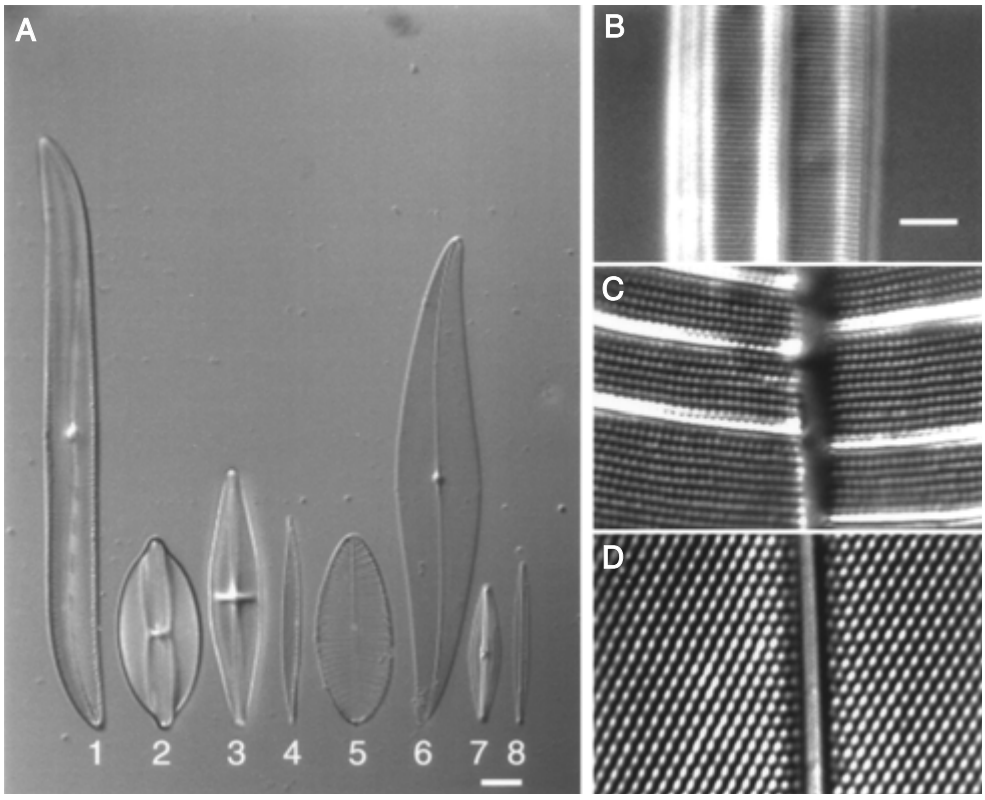


Figure 4.1.8 (**A**) The diatom test plate. The rows of pores are spaced in the silica shell by ~0.25 μm in *Amphipleura pellucida* (panel **B**; also number 8 on panel A), 0.41 μm in *Surrella gemma* (panel **C**; also number 5 on panel A), and 0.62 μm in *Pleurasigma angulatum* (panel **D**; also number 6 on panel A). Bar: panel A, 10 μm; panels B, C, and D, 2.5 μm. From Salmon and Tran (1998), reprinted with permission from Academic Press.

3. Adjust the gain and contrast camera controls for optimum image brightness and contrast.

   As you increase contrast, you will need either more camera gain or brighter illumination. Usually, a better signal-to-noise ratio is achieved by leaving the gain control in the middle of its adjustment and increasing illumination intensity.

   If the projection magnification to the detector is too small, the frustrule pore lattice will be invisible in the video image, although it will be clearly visible when viewing the image by eye.

4. Increase the magnification to the camera as much as possible.

   As magnification increases, resolution in the image becomes less limited by the resolution of the camera. However, the size of the field of view decreases inversely with magnification. In addition, the intensity of light in the image decreases as 1/magnification^2. The image will become noisy at low light intensities. It will be necessary to increase illumination intensity as much as possible at high magnification or increase the integration period for the camera exposure.

CALIBRATING IMAGE MAGNIFICATION WITH A STAGE MICROMETER

SUPPORT PROTOCOL 2

1. Insert the stage micrometer (see Table 4.1.1) on the microscope stage.
2. With the low-power objective, find the region of the micrometer with 10-μm scale intervals.

   The larger intervals are 100 μm.

3. Use the 100- or 10-μm scales to calibrate distance in images taken with your objectives.

   The magnifications on the objective barrel and projection lenses are only approximations; accurate measurements of distances in images require a calibration scale.

4. Acquire images of the scale in both the horizontal and vertical directions to check if the camera has square pixels.

TESTS FOR THE OPTICAL PERFORMANCE OF THE MICROSCOPE

Test slides are used to evaluate the performance of the microscope under different conditions.

Testing Phase-Contrast and DIC Using Diatom Testing Slide

SUPPORT PROTOCOL 3

Diatoms have silica shells shaped like pillboxes. There are pores in the shell arranged in a lattice pattern specific for each diatom species. Figure 4.1.8 shows a low-magnification view of the eight diatoms on the test slide (panel A) plus higher-magnification views of the lattices of three diatoms most useful in testing the resolution performance of microscope optics using phase-contrast or DIC. Number 6 in panel A of Figure 4.1.8, *Pleurosigma angulatum*, has a triangular pore lattice with spacing of ~0.61 μm between rows (illustrated in Fig. 4.1.8, panel D). Number 5, *Surrella gemma*, has rows of pores where the rows are separated by ~0.41 μm (illustrated in Fig. 4.1.8, panel C). Number 8 is *Amphipleura pellucida*, which has horizontal rows of pores separated by ~0.25 μm (illustrated in Fig. 4.1.8, panel B). In transmitted light, the diffraction limit to lateral resolution, *r*, is given by (Inoué, 1989):

$$r = \lambda/(NA_{obj} + NA_{cond})$$

where λ is the wavelength of light, NA_{obj} is the objective NA, and NA_{cond} is the condenser NA (see Fig. 4.1.3). The lateral resolution, *r*, is equal to 0.195 μm for the highest objective

NA, which is equal to 1.4, with $NA_{cond} = NA_{obj}$ and 546 nm green light. As seen in Figure 4.1.8B, this objective is capable of resolving the rows of pores in the shell of *Amphipleura*, but not the individual pores, which are slightly <0.19 µm apart.

Use the above equation to calculate the diffraction limit of resolution for your other objectives and use the diatoms to test whether they achieve their theoretical limits.

SUPPORT PROTOCOL 4

Testing Phase-Contrast and DIC Using Squamous Cheek Cell Test Slide

Cheek cells are a convenient specimen for testing the performance of phase-contrast or DIC microscopes. As seen in the low-magnification view (Fig. 4.1.6A) they are large and flat, ≤3 µm thick except near the cell center which contains the nucleus. The upper and lower surfaces have fine ridges which swirl around much like fingerprints. Many of the ridges are <0.2 µm in width and separated by <0.5 to 1.0 µm.

To prepare the cheek cells, gently scrape the inside of your mouth with the tip of a plastic pipet or similar soft tool and spread the cells and saliva on the center of a clean no. 1.5 coverslip. Invert the coverslip quickly onto a clean slide and press down to spread the cell preparation into a thin layer. Seal the edges with a thin layer of nail polish.

SUPPORT PROTOCOL 5

Testing Fluorescence Using Red, Green, and Blue Fluorescent Tissue Culture Cell Test Slide

The cells in Figure 4.1.9 are triple labeled: DAPI stained nuclei and chromosomes (blue fluorescence; Fig. 4.1.9A); Alexa 488-phalloidin labeled actin filaments (green fluorescence; Fig 4.1.9B); and X-Rhodamine immunofluorescently labeled microtubules (red fluorescence; Fig. 4.1.9C). The microtubules and fine actin filamentous arrays are <100 nm in width, but they should appear sharp and in high contrast in the microscope. There should be no "bleed-through" of fluorescence from one fluorescence channel to another if the filters are chosen properly (Taylor and Salmon, 1989).

In fluorescence microscopy, the diffraction-limited lateral resolution is given by (Inoué 1989; Inoué and Spring, 1997):

$$r = 0.61\lambda/NA_{obj}$$

and the intensity of the image, I_{image}, is given approximately by:

$$I_{image} \approx I_{ex}NA_{obj}^{4}/M_{p}^{2}$$

where I_{ex} is the excitation light intensity entering the objective and M_p is the projection magnification from the objective to the eye or camera. The rate of photobleaching, R_p, of a specimen depends on:

$$R_p \approx I_{ex}NA_{obj}^{2}M_{obj}^{2}$$

where M_{obj}^{2} is the magnification of the objective.

Note from these equations that resolution depends inversely on NA_{obj} (r = 0.24 µm for NA_{obj} = 1.4 and 546 nm green light), image intensity depends on the fourth power of NA_{obj}, while loss of intensity by photobleaching of the fluorophore increases with the square of objective NA (a measure of the excitation light collected by the objective) and magnification (which concentrates the excitation light on the specimen).

Verify these equations by imaging the fluorescent specimen (the microtubules in the spread cells are a good choice) for objectives with different magnifications and numerical apertures. It will quickly be seen why in fluorescence one wants to use the minimum total

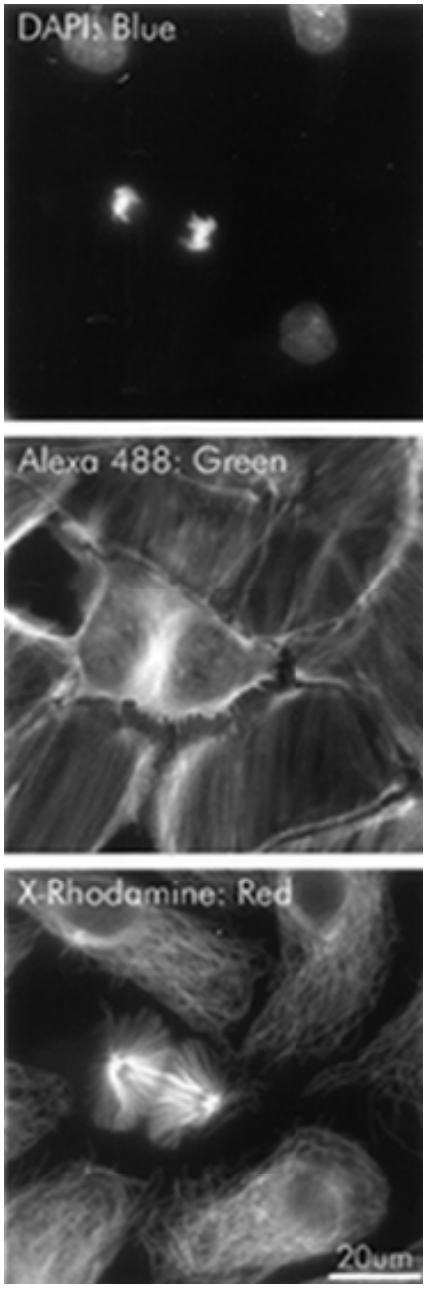


Figure 4.1.9 Epifluorescent images of fixed tissue culture cells stained with (**A**) DAPI, making DNA fluorescent blue; (**B**) Alexa 488 bound to phalloidin to label actin filaments fluorescent green; and (**C**) X-rhodamine labeled antibodies against tubulin to label microtubules fluorescent red. Bar = 20 μm. Images recorded with a 40×/(NA = 1.4) Plan Fluor objective, 1.5 magnification, to a cooled CCD camera and the multi-modem multiwavelength microscope described by Salmon et al. (1998).

magnification necessary to resolve the structures of interest in the detector, as well as use the maximum NA_{obj} in order to maximize light intensity for the least amount of excitation light (and the least amount of photobleaching).

CARE AND CLEANING OF MICROSCOPE OPTICS

SUPPORT PROTOCOL 6

Keeping the microscope optics clean is important for high-quality imaging. Dust, fingerprints, excess immersion oil, or mounting medium on or in a microscope causes reduction in contrast and resolution. DIC is especially sensitive to contamination and scratches on the lens surfaces. Below are steps for keeping the microscope clean.

1. Always keep microscopes covered when not in use.
2. Make sure that all ports, tubes, and unoccupied positions on the lens turrets are plugged.

   Plastic plugs are usually supplied with the microscope.

3. Store objectives in screw-top containers when not on the microscope. Keep accessories—e.g., condensers and compensators—in plastic bags or boxes. Keep slides and coverslips covered.
4. Be careful with salt water, corrosive liquids, and all solvents.
5. When cleaning lens surfaces, avoid touching the lens surface with anything (even lens paper if possible).

   IMPORTANT NOTE: *Never use Kimwipes or commercial facial tissue, because they may contain a filler that is part diatomaceous material (glass). One pass of a Kleenex could ruin an objective.*
6. Remove dust by gently brushing with an oil-free (ether-washed) camel's hair brush or by using a low-velocity stream of purified air.
7. Remove water-soluble contamination using distilled water with a small amount of detergent, such as Kodak Photoflow solution.

   Much (modern) immersion oil can also be removed by washing with detergent.
8. Remove most immersion oil by passing a high-quality lens tissue over the objective or condenser front element.

   IMPORTANT NOTE: *Do not rub. No area on the tissue should come in contact with the lens twice. This prevents dust and dirt removed from the lens from coming back and possibly scratching it. This is easily accomplished by passing the tissue over the lens in a "Z" pattern or by making parallel passes.*
9. Clean objective lenses by holding a piece of doubled lens paper over the objective and placing a few drops of solvent on the paper. Draw the paper across the lens surface so that the solvent flows rapidly in a circular pattern over the recessed lens surface (see Inoué and Spring, 1997). Finish the stroke with a dry portion of the paper. Repeat as necessary.

   In this way, the solvent contacts the lens but the paper does not, because the lens is recessed. For solvent, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface. Avoid soaking a lens with solvent, to prevent damage to lens cements.
10. To clean recessed front elements of dry objective lenses or to remove stubborn dirt, use a cotton-tipped applicator that has been soaked in cleaning solution and then shaken to remove excess fluid. Rotate the cotton tip over the lens surface to clean. Again, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface.
11. Use a detergent solution or ethanol to clean the surfaces of the eyepiece lenses.

    Do not use xylene as it may solubilize enamel surfaces.

COMMENTARY

The development of lasers, electronic cameras, digital image analysis, and specific fluorescent molecular probes have recently made light microscopy an enormously powerful tool in the biomedical sciences. There are a number of excellent books and review articles about these applications, as well as the optical principles of light microscope design and image formation. Listing of all these references is beyond the scope of this unit; only a few of these books and reviews are listed, and these can be used to find other useful references.

Spencer (1982), Zernike (1958), and Keller (1995, 1997, 1998) provide excellent introductions to the principles of image formation and contrast in the light microscope for beginners,

while Pluta (1988, 1989) provides a more comprehensive treatment. Inoué and Oldenbourg (1995) also review the basic concepts and principles of microscope design and different methods of image formation. The abovementioned references describe the many modes of transmitted-light imaging in the microscope, including bright-field, phase-contrast, and DIC, which are described in this unit, as well as dark-field, polarization, interference contrast, Hoffman modulation, and reflection interference contrast methods. Taylor and Salmon (1989) and *UNIT 4.2* give clear introductions to fluorescence microscopy. The recent edition of *Video Microscopy* by Inoué and Spring (1997) is a comprehensive introduction to the principles and practical aspects of light microscopy, video, and digital imaging. The *Video Microscopy* volume of *Methods in Cell Biology* edited by Sluder and Wolf (1998) also has contributions that cover many basic concepts and practical aspects of light microscopy in the biomedical sciences; it also includes chapters on multiwavelength, multimode digital imaging methods, camera selection, ion ratio imaging, and specimen chambers. Salmon and Tran (1998) review the principles of video-enhanced DIC methods used to image macromolecular complexes invisible in the microscope when viewed by eye. Taylor et al. (1992), Salmon et al. (1998), and Rizzuto et al. (1998) review microscope design for multiwavelength, multimode digital imaging of fluorescent specimens, and four-dimensional microscopy. Advanced quantitative fluorescence methods like ratio imaging, photobleaching, photoactivation, resonance energy transfer, chromophore-assisted laser ablation, and fluorescence lifetime imaging are also reviewed in the *Methods in Cell Biology* volumes edited by Taylor and Wang (1989), and Sluder and Wolf (1998) as well as in Herman and Jacobson (1990) and Inoué and Spring (1997). Reviews on imaging cells containing expressed protein coupled to green fluorescent protein (GFP) include Heim and Tsien (1996), and Sullivan and Kay (1998). Agard et al. (1989), Carrington et al. (1995), and Rizzuto et al. (1998) review how deconvolution of three-dimensional image stacks can produce super resolution in the light microscope. The recent edition of *Handbook of Biological Confocal Microscopy* edited by Pawley (1995) is an excellent reference on this important method for imaging fluorescent structures in thick specimens (Smith, 1997). The new technique of multiphoton laser scanning microscopy is described by Denk et al. (1994), while biological applications of optical traps to manipulate organelles and measure molecular forces is reviewed by Svoboda and Block (1994).

LITERATURE CITED

Agard, D.A., Hiraoka Y., Shaw, P., and Sedat, J.W. 1989. Fluorescence microscopy in three dimensions. *Methods Cell Biol.* 30:353-377.

Allen, R.D., David, G., and Nomarski, G. 1969. The Zeiss-Nomarski differential interference equipment for transmitted-light microscopy. *Z. Wiss. Mikf. Microtech.* 69:193-221.

Carrington, W.A., Lynch, R.M., Moore, E.D.W., Isenberg, G., Forarty, K.E., and Fay, F.S. 1995. Superresolution three-dimensional images of fluorescence in cells with minimal light exposure. *Science* 268:1483-1487.

Denk, W., Delatner, K.R., Gelperin, A., Keleinfeld, D., Strowbridge, B.W., Tank, D.W., and Yuste, R. 1994. Anatomical and functional imaging of neurons using 2-photon laser scanning microscopy. *J. Neurosci. Methods* 54:151-162.

Heim, R. and Tsien, R. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* 6:178-182.

Herman, B. and Jacobson, K. 1990. Optical Microscopy for Biology. Wiley-Liss, New York.

Hinsch, J. 1998. Mating camera to microscopes. *Methods Cell Biol.* 56:147-152.

Inoué, S. 1989. Imaging unresolved objects, superresolution and precision in distance measurement with video microscopy. *Methods Cell Biol.* 30:112.

Inoué, S. and Oldenbourg, R. 1995. Chapter 17 *In* Handbook of Optics, vol. 2 (Optical Society of America; M. Bass, ed.). McGraw-Hill, New York.

Inoué, S. and Spring, K. 1997. Video Microscopy. 2nd ed., Plenum Press, New York.

Keller, H.E. 1995. *In* Handbook of Biological Confocal Microscopy (J.B. Pawley, ed) 2nd ed, pp. 111-126. Plenum, New York.

Keller, H.E. 1997. Contrast enhancement in light microscopy. *In* Current Protocolsin Cytometry (J.P. Robinson, Z. Darzynkiewicz, P.N. Dean, A. Orfao, P.S. Rabinovitch, C.C. Stewart, H.J. Tanke, and L.L. Wheeless, eds.) pp. 2.1.1-2.1.11. John Wiley & Sons, New York.

Keller, H.E. 1998. Proper Alignment of the Microscope. *Methods Cell Biol.* 56:135-146.

Pawley, J. (ed). 1995. Handbook of Biological Confocal Microscopy, 2nd ed., Plenum, New York.

Pluta, M. 1988. Advanced Light Microscopy, Vol. I: Principles and Basic Properties. Elsevier/North-Holland, Amsterdam.

Pluta, M. 1989. Advanced Light Microscopy Vol. II: Specialized Methods. Elsevier/North-Holland, Amsterdam.

Rizzuto, R., Carrington, W., and Tuft, R. 1998. Digital imaging microscopy of living cells. *Trends Cell Biol.* 8:288-292.

Salmon, E.D. and Tran, P. 1998. High resolution video-enhance differential interference contrast (VE-DIC) light microscopy. *Methods Cell Biol.* 56:153-183.

Salmon, E.D., Shaw, S.L., Waters, J., Waterman-Storer, C.M., Maddox, P.S., Yeh, E., and Bloom, K. 1998. A high-resolution multimode digital microscope system. *Methods Cell Biol.* 56:185-215.

Sluder, G. and Wolf, D.E. (eds). 1998. Video microscopy. *Methods Cell Biol.* vol. 56.

Smith, C. L. 1997. Basic confocal microscopy. *In* Current Protocols in Neuroscience (J.N. Crawley, C.R. Gerfen, R. McKay, M.A. Rogawski, D.R. Sibley, and P. Skolnick, eds.) pp. 2.2.1-2.2.13. John Wiley & Sons, New York.

Spencer, M. 1982. Fundamentals of Light Microscopy. Cambridge University Press, Cambridge.

Sullivan, K.F. and Kay, S.A. 1998. *Methods Cell Biol.*. In press.

Svoboda, K. and Block, S.M. 1994. Biological applications of optical forces. *Annu. Rev. Biophys. Biomol. Struct.* 23:247-285.

Taylor, D.L. and Salmon, E.D. 1989. Basic fluorescence microscopy. *Methods Cell Biol.* 29:207-237.

Taylor, D.L. and Wang, Y.-L. (eds). 1989. Fluorescence Microscopy of Living Cells in Culture. *Methods Cell Biol.* Vols. 29 and 30.

Taylor, D.L., Nederlof, M., Lanni, F., and Waggoner, A.S. 1992. The new vision of light microscopy. *Am. Sci.* 80:322-335.

Zernike, F. 1942. Phase contrast: a new method for the microscopic observation of transparent objects. *Physica* 9:686-693.

Zernike, F. 1955. How I discovered phase contrast. *Science* 121:345-349.

Zernike, F. 1958. The wave theory of microscope image formation. *In* Concepts in Classical Optics (J. Strong, ed.) pp. 525-536. W.H. Freeman, San Francisco.

Contributed by Edward D. Salmon
and Julie C. Canman
University of North Carolina
Chapel Hill, North Carolina

Fluorescence Microscopy

Fluorescence is a property exhibited by some molecules whereby energy of a particular wavelength (λ) is absorbed, and, after a certain period of time (termed the fluorescence lifetime), a portion of this absorbed energy is emitted. The amount of energy emitted as fluorescence is always less than the amount of energy absorbed. Absorption of energy by molecules in the form of light and emission of energy in the form of fluorescence can occur only at certain wavelengths, which are characteristic for a given molecule. The difference in the maximal wavelengths (corresponding to energy levels) of absorption and emission is known as the Stokes shift. Each fluorescent molecule is characterized by its own distinct absorption and emission energy levels (i.e., excitation and emission spectrum). This fact—combined with the fact that a number of processes can affect the emission of fluorescence from a fluorophore during its excited-state lifetime—allow the specificity, quantitative and environmental sensitivity, and high temporal resolution provided by fluorescence to be brought to bear in the examination of microscopic structures. To accomplish this, the fluorescence microscope is constructed in such a way as to permit the delivery of excitation energy to the specimen in the form of light of specific energy levels corresponding to wavelengths that match the maximal absorbance band of the fluorescing species. The microscope must also have the capacity to separate the weaker emitted fluorescence light from the brighter excitation light for observation.

FLUORESCENCE MICROSCOPE OPTICS

Fluorescence microscopes must accomplish the following four functions: (1) deliver excitation light of the appropriate wavelengths to the specimen; (2) separate the excitation light from the emitted fluorescence; (3) collect as much of the emitted fluorescence given off by the emitted fluorophores as is possible; and (4) allow observation of fine detail in the specimen.

Fluorescence microscopes have been designed with these functions in mind. Excitation light sources and wavelength-selection devices allow selection of appropriate excitation wavelengths. Excitation light is delivered to the specimen through the microscope objective. A special mirror, known as a dichroic beam-splitting mirror, allows separation of the excitation light from the emitted fluorescence. The dichroic beam-splitting mirror has the special property of being able to reflect light below a specific wavelength, yet allow light above this specific wavelength to pass through the mirror unobstructed. Objective lenses have been developed that allow maximal collection of a portion of the emitted fluorescence from the sample, as well as magnification and observation of fine detail (resolution) of the specimen.

The most commonly employed illumination system is that invented by Ploem, termed Ploem illumination, incident-light illumination, or epiillumination. In epiillumination, a dichroic beam-splitting mirror reflects the excitation light into the back aperture of the objective. The objective acts as a condenser and focuses the excitation light onto the specimen. A portion of the emitted fluorescence (the amount being determined by the numerical aperture, or NA, of the objective) is collected by the objective and passes through the dichromatic beam-splitting mirror either to eyepieces or a detector. The existence of Stokes shift and the special property of the dichroic beam-splitting mirror described above allow the separation of the excitation light from the emitted fluorescence.

COMPONENTS OF THE FLUORESCENCE MICROSCOPE

Excitation Light Sources

There is a variety of excitation light sources that can be used for fluorescence microscopy. The choice of which light source to employ will depend on the fluorescent probes being used. Because many of the available fluorophores (see Table A.1E.1) require excitation in the blue/green portion of the visible light spectrum, mercury (Hg), xenon (Xe), or Hg/Xe combination lamps are generally employed. Lasers can also be used. Each light source has benefits, depending on the wavelength and strength of excitation energy required in a given experimental situation. For example, Hg light sources emit energy at discrete wavelengths (e.g., 365, 400, 440, 546, and 580 nm) making this light source useful for caged compound release—i.e., the release of chemically caged (inert) molecules such as Ca^{2+} and inositol 1,4,5-triphosphate (IP_3) inside living cells with UV excitation—as well as for Hoescht-, 4′,6-di-

Contributed by Brian Herman

Current Protocols in Cell Biology (1998) 4.2.1-4.2.10

amidino-2-phenylindole (DAPI)–, fluorescein- and rhodamine-based fluorophores. Conversely, Xe lamps have a spectrally uniform intensity profile from the ultraviolet to the far red; this light source is particularly suited for use of Fura-2, a calcium-sensitive dye. Metal halide lamp spectral output is the same as that of the Hg arc lamp, but the energy output per unit area for metal halide is less than that of the Hg arc lamp. However, the 150-W metal halide bulb has an average lifespan five times that of an Hg lamp (1000 versus 200 hr). A Xe lamp (75-W) lasts ~400 hr. These times are estimates, as the lifetime of a bulb is determined by the total hours that it is on and the number of times it is turned on and off. When first used or when first ignited, the spark of these lamps can flicker or wander. This can be minimized by an initial 2-hr "burn in" or 10-min warm-up period. If the lamp has been turned off and is to be used again, ≥20 min must pass before turning on the lamp again.

Lasers (light amplification by stimulated emission of radiation) offer monochromatic light of very high energies and can be used in continuous-wave (CW) or pulsed modes of operation. Laser light is coherent, monochromatic (although a single laser can output multiple wavelengths of light), and polarized (propagates in one plane). Pulsed lasers are finding more and more applications in fluorescence microscopy, especially in two-photon excitation microscopy (TPEM) and in the two- and three-dimensional measurement of fluorescence lifetimes in intact cells and tissues. An important concern with the use of lasers in microscopy is the potential for scattering and diffraction due to dirt and interference on optical surfaces in the beam path. This can be overcome by using a multimode fiber-optic or liquid light pipe.

Köhler illumination

When using a fluorescence microscope, it is necessary to provide bright and even illumination of the whole field of view, while at the same time not illuminating any areas outside of the field of view. This reduces scattered light and photodamage. The optical design that can accomplish this, named after its inventor, is known as Köhler illumination (Fig. 4.2.1). In Köhler illumination, a collector lens in front of the excitation light source focuses an image of the excitation light onto a condenser field diaphragm. The condenser lens then focuses an image of the field diaphragm into the plane of the specimen (for a step-by-step procedure see *UNIT 4.1*). When this is done, the microscope is aligned in such a way that the planes carrying the illumination information are separate from the set of planes carrying the specimen information. The advantages of Köhler illumination are that the back focal plane of the objective is fully illuminated, providing a microscope field that is homogeneously bright. In addition, this system makes possible maximal lateral and axial resolution, optimal contrast, and reduction of flare from the microscope optics.

Arc lamp alignment

Alignment of the arc lamp is required each time a new lamp is inserted into the lamp

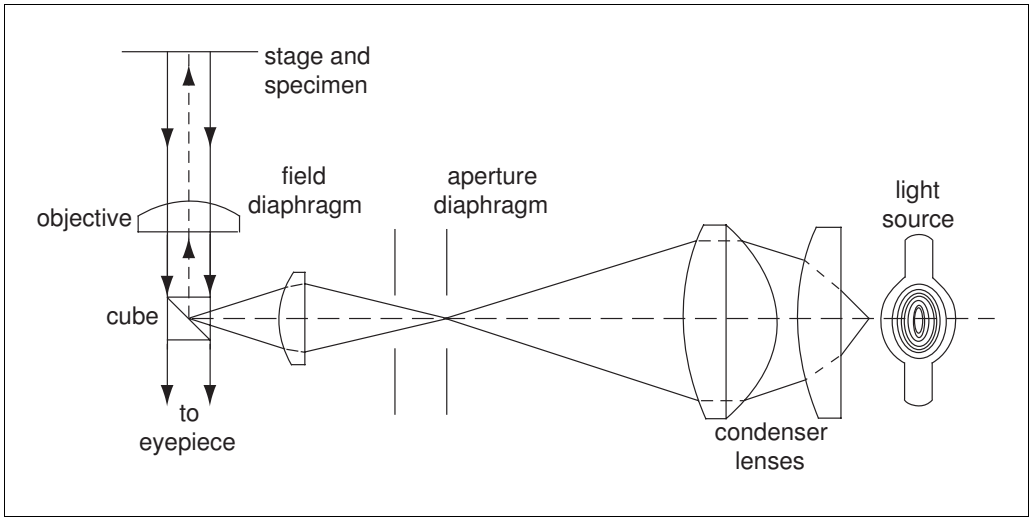


Figure 4.2.1 Köhler illumination. Condenser lenses are placed in front of the light source to make sure that the image of the centerable aperture diaphragm is focused on the back aperture of the objective, which is then focused on the specimen.

housing, and occasionally during routine usage. It is important to remember, when replacing an arc lamp, that the bulb should never be touched with one's hands (lens tissue or lint-free gloves should be used) or when it is hot (because it might explode). Also, whenever an arc lamp is replaced, the lamp power supply should be unplugged, protective eye gear should be worn, the lamp should never be used outside of its housing, and one should never look directly at or expose one's skin to the lamp (because it could cause burns and/or skin cancer). After placing a new lamp bulb in its housing, perform the following steps.

1. Reattach the lamp to the microscope, turn it on, and remove one of the objective lenses. Put a white piece of paper (a business card works well), on the microscope stage to visualize and align the lamp arc.

2. To align the arc (e.g., Hg or Xe) lamp, first focus the real and mirror images of the lamp arc to their smallest size using the collector lens in the lamp housing (Fig. 4.2.2).

3. Next, adjust the real and mirror images so that they are visible in the same field and are at the same focus or size. Adjust the real image until it is centered in the vertical direction in the field and is just to the side of the center. Move the mirror image so that it is also centered vertically in the image, but is on the opposite side of center from the real arc image.

4. Lastly, using the collector lens focusing knob on the lamp housing, defocus the arc image to provide an evenly illuminated field.

Photobleaching

Photobleaching or "fading" is the loss of fluorescence intensity following illumination of the specimen. Photobleaching decreases the fluorescence signal and hence the signal-to-noise (S/N) ratio of the microscope image. Photodynamic photobleaching is the most common type of photobleaching that occurs in fluorescence microscopy; it involves the interaction of the fluorophore with light and oxygen. In their excited state, fluorescent molecules can interact with nearby oxygen (O_2) molecules, resulting in oxidation of the fluorophore and loss of fluorescence. Fluorophores in their excited state can collide with molecular oxygen and "sensitize" singlet oxygen production. Singlet oxygen is highly reactive and can interact with the fluorophore to bleach it. The amount of photobleaching or fading that occurs in a given situation will depend on the concentration of molecular oxygen, the fluorescence life-

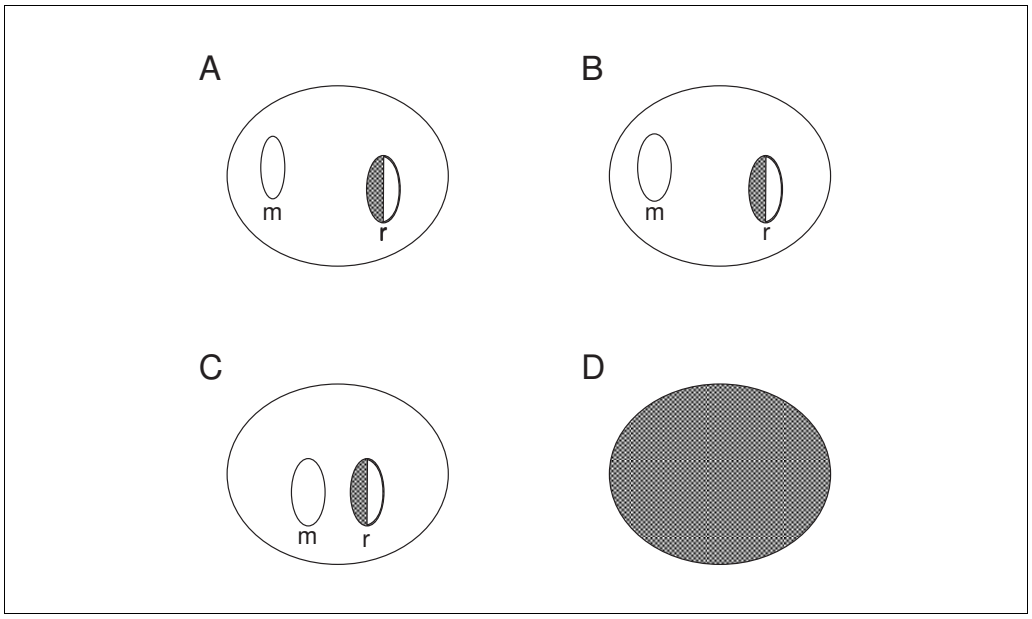


Figure 4.2.2 Alignment of arc lamp. (**A**) After placing a new lamp bulb in the lamp housing, use the collector lens focusing knob on the lamp housing so that the real (r) and mirror (m) images of the lamp bulb are at their smallest size (i.e., most focused). (**B**) Next, using a socket wrench and the ports on the lamp housing, adjust the real and mirror images so that they are visible in the same field and are at the same focus or size. (**C**) Adjust the real image until it is centered in the vertical direction in the field and is just to the side of the center. Move the mirror image so that it is also centered vertically in the field, but is on the opposite side of center from the real arc image. (**D**) Using the collector lens-focusing knob on the lamp housing, defocus the arc image to provide an evenly illuminated field. Once the lamp is focused, replace the objective.

time of the excited state (the longer the lifetime the more chance for interaction with molecular oxygen), and the distance between the fluorophore, molecular oxygen, and any other cellular constituents.

From a theoretical standpoint, whether the excitation energy is delivered in a pulsed versus continuous fashion should not affect the photochemical lifetime (rate of photobleaching). Therefore, at low excitation energy levels, photobleaching is not prevented, but the rate of photobleaching is merely reduced. However, anecdotal reports suggest that the delivery of excitation light in a pulsed versus continuous fashion may have experimental advantages in certain situations. For example, it may be advantageous to get several high signal-to-noise digital video images before photobleaching has occurred, using intense short-duration pulses. Alternatively, for certain cell physiological events, one may need to illuminate for much longer times to study the time evolution of a phenomenon, but at considerably lower S/N ratios in the individual images.

Protection against photobleaching involves reducing exposure time or excitation energy. However, these actions will also reduce the measurable signal. Solutions of molecules or suspensions of cells could also be deoxygenated, although this is not feasible for living cells and tissues. Antifade reagents such as *n*-propyl gallate, or others that are commercially available, can be added to fixed specimens. Singlet oxygen quenchers such as histidine, diphenylisobenzofuran, or crocetin (a water-soluble carotenoid) can also be employed. The use of a computer-controlled electronic shutter that is open only when experimental data is being collected will prolong filter life as well as keep exposure of the sample to harmful radiation at a minimum.

While photobleaching is usually to be avoided at all costs, there is one experimental technique that uses photobleaching to examine the lateral diffusional mobility of fluorescent molecules. This technique, termed fluorescence recovery after photobleaching (FRAP), involves irreversible photobleaching of a small area of fluorescent molecules with a short, intense burst of a laser, and observation of the reappearance of fluorescence in the bleached area (Fig. 4.2.3). The reappearance of fluorescence is due to the diffusion of unbleached fluorescent molecules into the bleached region. The kinetics of this process are related to the lateral diffusional mobility of the fluorescent material.

Wavelength Selection Devices

In the fluorescence microscope, excitation light of a specific energy level (wavelength) from Hg or Xe arc lamps or a laser is isolated using an exciter filter and reflected onto the specimen by a dichroic beam-splitting mirror. The emitted fluorescence is viewed through a cutoff or barrier filter that blocks all wavelengths below a set value from reaching the observer.

Filters can consist of a variety of substances such as absorption glass (including gelatin) or

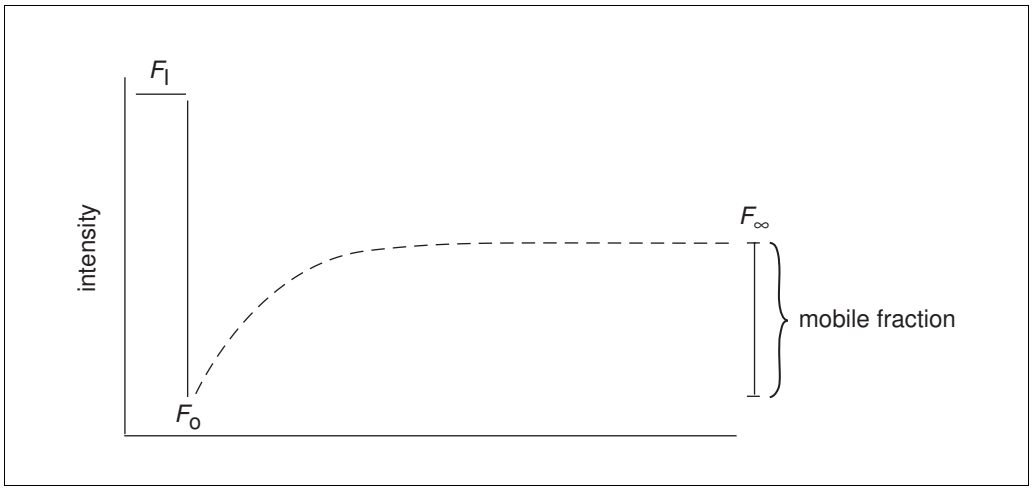


Figure 4.2.3 Fluorescence recovery after photobleaching (FRAP). A small region of the sample containing a fluorophore of some intensity value (F_i) is irreversibly bleached by a strong laser beam resulting in a drop in the observed fluorescence intensity (F_0). A much weaker laser beam then monitors the recovery of fluorescence into the bleached area as a function of time, until there is no further increase in fluorescence. The mobile fraction is defined as the difference between F_∞ and F_0.

thin-film coatings. Thin-film coatings may be either of a metallic nature (for fully reflective mirrors and neutral-density filters) or interference coatings (for interference filters). Several standard exciter filter/dichroic beam-splitting mirror/barrier filter combinations are available from each microscope manufacturer. The choice of which combination to use will depend on the fluorochrome(s) employed.

Filters are described according to their transmission properties. A short-pass filter will pass light up to a certain wavelength, but will not allow light of higher wavelengths to pass through the filter. A long-pass filter will pass only wavelengths above a certain value, and will prevent light of lower wavelengths from passing through the filter. Both short-pass and long-pass filters are named by the wavelength at which the transmission of light is 50% of maximum.

Filters also exist that can transmit only a specific range of wavelengths (interference or band-pass filters). Both wide band-pass and narrow band-pass filters exist. The narrower the range of wavelengths that can pass through the filter, the more specific is its ability to discriminate individual fluorophores in a mixture (i.e., the higher its contrast is). However, higher specificity means less intensity. Broad-band filters provide more signal but less contrast (i.e., where contrast is defined as the ability to distinguish specific versus nonspecific fluorescence or the ability to distinguish individual fluorophores when more than one fluorophore is present). These filters are named by their center wavelength (the arithmetic means of the wavelengths at 50% of peak transmission) and by the range of wavelengths (bandwidth) of light they transmit at 50% peak transmittance (full width half maximum, or FWHM). For example, BP 490/30 is an interference filter with maximal transmission at 490 nm (for fluorescein excitation) and which transmits light from 475 to 505 nm. A short-pass and long-pass filter can be sandwiched together to generate specific band-pass filters.

Dichroic beam-splitting mirrors reflect the shorter wavelengths of excitation light to the specimen and allow the long wavelength emitted fluorescence to pass to the detector, while at the same time preventing the passage of the shorter excitation wavelengths to the detector. Optimally, >90% reflectance of the excitation light and >90% transmission of the emission light is desired. Dichroic beam-splitting mirrors are identified by the highest wavelength of light that will be reflected onto the specimen.

Often more than one fluorophore will be visualized in a single specimen. In order to visualize each fluorophore individually without any contamination from the other fluorophores, microscopes have been designed to accommodate multiple exciter/dichroic/emission filter units, each optimally matched for its respective fluorophore. Of course this type of arrangement does not allow observation of all of the fluorophores simultaneously. To accomplish this, single filters with multiple band passes have been designed that can be used to image more than one fluorophore simultaneously. In addition to filters, monochromators and electro-optic instruments can be employed as wavelength-selection devices.

Fluorescein and rhodamine represent two of the most common fluorophores utilized in fluorescence microscopy. Typical fluorescein and rhodamine filter sets might be constructed according to the following considerations. Fluorescein absorbs maximally at 490 nm and emits maximally at 525 nm. Rhodamine absorbs maximally at 550 nm and emits maximally at 580 nm. Therefore, the two filter sets have to be constructed in such a way that the appropriate wavelengths of excitation (490 nm and 550 nm) can be delivered to the specimen, and the emitted fluorescence (525 nm and 580 nm) can be separated from the excitation light and any other scattered light before reaching the detector. Using an Hg lamp, an excitation interference filter is placed in the light path—comprised of a 450 to 490 nm filter for fluorescein and a 546/12 nm filter for rhodamine—to select the appropriate wavelengths of excitation for the two probes. After passing through the excitation filter, the excitation light encounters a dichroic beam-splitting mirror (DM)—comprised of a DM 510 nm mirror for fluorescein and a DM 580 nm mirror for rhodamine. These dichroic mirrors will reflect light below 510 nm and 580 nm, respectively, into the back focal plane of the objective and then to the sample. The emissions of fluorescein and rhodamine are then isolated using an emission filter—comprised of a long-pass (LP) 520 nm and an LP 590 nm filter. While these filter units work to enable observation of fluorescein and rhodamine independently in the same sample, they do not correct for the following possibility (which actually occurs). Some excitation of the rhodamine can occur at the wavelengths of excitation of fluorescein, and a small portion of the emission spectrum of fluorescein overlaps a portion of the emission spectrum of rhodamine. To overcome this problem, emission

interference filters—515 to 565 nm for fluorescein and LP 610 nm for rhodamine—can be employed to visually isolate, specifically, the emission of fluorescein from rhodamine.

There are other types of filters that are employed in fluorescence microscopy—i.e., heat and neutral-density filters. Heat filters are used to protect the excitation filters and detectors from infrared radiation. They are placed in front of the light source to reduce heat transfer to the excitation interference filter and can also be used to reduce the UV excitation intensity. Neutral-density filters reduce the intensity of all wavelengths of the exciting light equally, and are commonly used for reducing the intensity of the exciting light to prevent photobleaching of fluorophores and photodamage of living cells and tissue. The neutral-density filter should be interposed between the heat filter and excitation filter.

Objectives

In fluorescence microscopy, the microscope objective serves the functions of a condenser, a magnifier, and a collector of the emitted fluorescence. In epifluorescence microscopy, reflected-light objectives have been developed, which feature specially coated glass surfaces to avoid reflection in the optics ("antireflection coating"). Most (if not all) objectives in use today are infinity-focused objectives. In infinity-focused objectives, light rays emanating from any point in the specimen are parallel between the objective and the eyepiece. The advantage of infinity-designed objectives is their insensitivity to other components introduced in the beam path (e.g., filters).

When selecting an objective for use in fluorescence microscopy, it is important to remember that objectives differ in magnification, light-gathering ability (numerical aperture or NA), wavelengths of light that they transmit, immersion medium required, and the specific applications for which they were developed. Table 4.2.1 lists some current types of objectives and their special properties.

Numerical aperture

In most biological applications involving living cells, it is important to collect as much of the emitted fluorescence as possible. Employing the largest NA possible will allow maximal collection of emitted fluorescence. NA (where $NA = \eta \sin\alpha$, η being the refractive index of the medium between the specimen and the objective) is defined as the angle, α, of the emitted fluorescence given off from a fluorophore relative to the original direction of the exciting light (Fig. 4.2.4). Objectives that are capable of collecting light over a large angle α will provide brighter images. Such objectives are said to possess a large NA. However, there is a limit to the size of the NA, resulting from a mismatch between the refractive indices of the medium (air) between the front surface of the objective and the coverslip. To increase the NA of the objective, a better match between the refractive indices of the front surface of the

Table 4.2.1 Properties of Objective Lenses[a]

Lens	Properties	Uses
Plan achromat	Flat field; color-corrected; short working distance	Routine fluorescence
Ultrafluar[b]	Broad spectral transmission (200-700 nm)	Calcium measurements with Fura-2
Quartz	UV transmission	UV fluorescence
Multi-immersion	For use with or without coverslip	Low-magnification, high-NA applications
Water-immersion	High NA	Live cell and tissue physiology
Achromat	Low-cost; partially color-corrected	Phase-contrast microscopy
Plan/Epiplan[b]	Good image contrast; flat field; long working distance	Photomicroscopy
Plan/Epiplan Neofluar[b]	Flat field; corrected for spherical and chromatic errors over broad range of visible spectrum	Calcium measurements with Fura-2

[a]Abbreviation: NA, numerical aperture.

[b]Ultrafluar, Neofluar, and Epiplan are trademarks of Carl Zeiss, Inc.

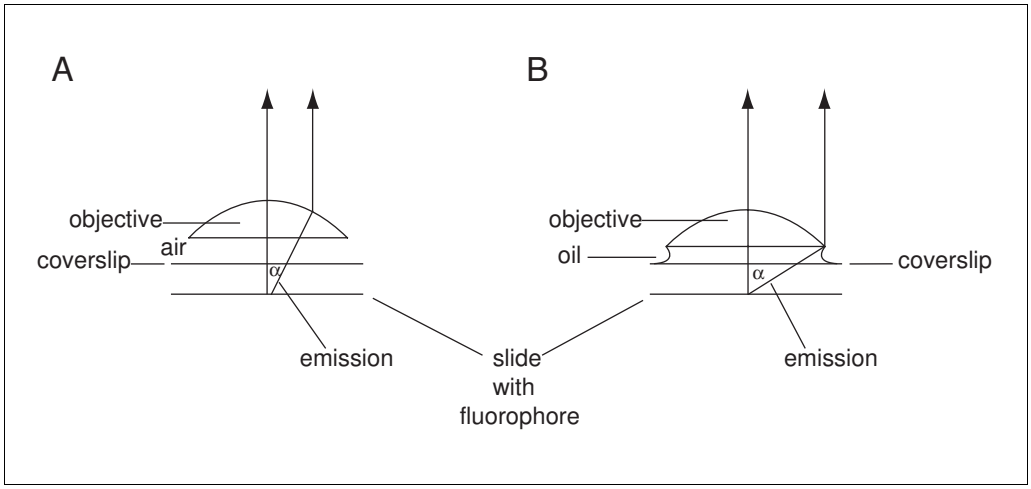


Figure 4.2.4 The numerical aperture (NA) is a measure of the light-collecting ability of an objective lens. The larger the NA, the greater the quantity of light collected by the objective lens. Larger NA is associated with shorter working distance of the objective lens (distance between the front of the objective lens and the sample). Since NA is proportional to the refractive index (η) of the medium between the specimen and the objective, matching the refractive indices of the objective lens and the sample results in a larger NA and collection of more of the emitted fluorescence. Compare (**A**), representing an objective without oil immersion and (**B**), representing an objective with oil immersion to match the refractive indices of lens and sample. Note the shorter working distance and larger angle (α) between the excitation and emission beams in B.

objective and cover glass must be made. This is done by placing immersion oil between the front lens of the objective and the coverslip. The η of both glass and immersion oil is 1.515, thereby eliminating all reflections on the path from the object to the objective and increasing the NA of the objective as well as the amount of light that can be captured by the objective.

Resolution

Resolution (R) is defined as the smallest distance (d) between two small objects that still can be discerned as two separate objects. $R = 0.61(\lambda/\text{NA})$, where λ is the wavelength of light and NA is the numerical aperture of the objective lens. Because R is determined by the wavelength of the light and the objective NA, objectives with higher NA values result in smaller d (i.e., greater resolving power). Shorter wavelengths are also beneficial for increasing objective resolving power.

Magnification

Magnification is defined as the power to cause objects to appear larger than they are. The useful range of overall magnification of a microscope should be >500 times but <1000 times the objective aperture. The magnification and NA of an objective are related to each other in such a way that the brightness of the image decreases as the magnification increases. In the typical fluorescence microscope employing epiillumination, the objective lens serves both as a condenser and an objective. This results in the light intensity (brightness) being proportional to $(\text{NA})^4$. Brightness is also related to the magnification but in the opposite direction—i.e., brightness is proportional to $1/(\text{Mag})^2$. Therefore, the overall brightness is proportional to $(\text{NA})^4/(\text{Mag})^2$. Thus, when performing fluorescence microscopy, it is important to use the highest NA and the lowest overall magnification objective possible.

Optimizing image brightness and resolution

Optimizing image brightness involves the following considerations.

1. The sample should be supplied with sufficient light energy for excitation (but not photobleaching) at the appropriate (fluorophore-dependent) wavelength.
2. The emitted fluorescence should be observed without any contamination from the excitation light.
3. The light source must provide a large amount of excitation energy in very narrow ranges of the spectrum.
4. Appropriate filters that transmit the required wavelengths but block the unrequired ones should be employed.

5. The objectives should have high transmission from the UV through a large part of the visible spectrum.

6. Objectives with the highest NA should be employed. A good rule of thumb is that if the objective aperture is doubled in size, approximately four times more fluorescence light can be gathered.

7. Nonautofluorescent, PCB-free immersion oil with the proper refractive index (η = 1.51), to eliminate light loss caused by light reflection on surfaces, should be used.

8. None of the optical components of the microscope should be autofluorescent. Autofluorescence will increase the brightness of the background and reduce the contrast in the image.

9. The objective lenses must be clean.

10. Coverslips of the appropriate thickness (0.17 mm) should be used. For objectives with NA > 0.7, the thickness of the coverslip can vary ±0.01 mm (from 0.17 mm) and still provide a high-resolution image. When the NA of the objective is between 0.3 and 0.7, coverslip thickness can vary by ±0.03 mm and still provide high-resolution images.

11. Air bubbles must be avoided in the immersion-oil layer.

12. To improve contrast, the field diaphragm should be closed down.

Cleaning the microscope optics

The following are some general rules that should always be followed when working with any optical microscope to maintain optimal performance.

1. Always work in as clean an environment as possible.

2. Avoid dust at all costs.

3. Keep the microscope covered at all times when not in use.

4. Store all microscope components in appropriate containers and avoid salt water, corrosive solvents, and all solvents if possible.

5. Clean the microscope thoroughly after each use.

6. Do not smoke.

7. Before cleaning an objective lens, directly inspect the lens using an inverted ocular from the microscope as a magnifier in room light. It is also possible to inspect the objective lens by placing the lens under a dissecting microscope and focusing on the lens to observe any imperfections.

8. Use oil only on lenses designed to be used with oil. It is a tedious process to clean immersion oil off a dry lens so as not to damage the lens.

9. Properly store objectives that are not in use in the container that they came in.

The first rule of cleaning microscope optics is do not touch the lens surface if at all possible, not even with lens paper. Dust particles in the air and even components of cleaning tissues can contain abrasive materials that will scratch the lens surface. The best way to clean an objective lens is as follows.

1. Soak a previously unused Q-tip in any of the following solvents: a mixture of 55% acetone/30% ether/15% ethanol; pure alcohol; the glass cleaner Sparkle; distilled H_2O; lighter fluid; or a well-shaken 1:1:1 mixture of chloroform/alcohol/water. Avoid benzene, toluene and xylene. The organic solvents are usually more effective, but bear in mind that some of these solvents are carcinogens and flammable and need to be used in a well-ventilated environment with no nearby flames (i.e., no smoking).

2. Very gently roll the solvent-laden Q-tip over the surface of the lens once.

3. Take another fresh Q-tip dipped in solvent and pass it over the lens very gently, barely touching the lens surface. Another option is to place a drop of solvent onto the objective lens (without directly touching the lens), and then gently blot the drop of solvent up using lens paper, but not touching the lens directly.

If you are using oil-immersion objectives and scanning a number of slides, it is not necessary to clean the objective after each slide. Rather, wipe off excess oil with lens paper and place the new slide (with a drop of oil on it) onto the objective so that the oil on the slide and the residual oil on the objective meet without producing air bubbles.

THE DIGITAL DARKROOM

Photographs of images viewed through the microscope have long been the preferred means of disseminating microscopic information. This has historically meant using film as the medium for recording images. However, the use of computers for acquisition and display of images, along with the explosion of the World Wide Web (WWW), has led to the rapid expansion of the use of computer-based image analysis and presentation programs for production of permanent hard-copy microscopic images. Digital imaging provides the user with great latitude with respect to speed of image acquisition and data presentation. While it is a great improvement over film, the use of computer-

based image-analysis and presentation software provides a ready means for altering image information, and care must be taken to maintain the original data in the final image. There are also other problems with electronic image presentation—e.g., there often exists incompatibility between the data formats of the software used for image acquisition and presentation, and the electronic components themselves can induce distortion of the original image information.

The overall design of a typical digital darkroom for fluorescence microscopy is as follows. A one- or two-dimensional detector (e.g., various cameras, photomultiplier tubes, or photodiode arrays) is attached to the microscope port at the in-focus plane of image formation. The image formed by the microscope is recorded by the detector, whose output is sent to a computer. The computer may require specific hardware to receive the image from the camera as well as a substantial amount of fast access RAM and hard disk space to store the image(s). Since images contain a large amount of information (and therefore require huge amounts of storage space), a number of storage media can be employed to store microscopic images for subsequent display and processing in digital format. These include optical memory disk recorder (OMDR), computer floppy disks, Zip drives, Jazz drives, and CD-ROM disks, as well as hard drives, laser disks, and optical disks.

Once in computer memory or on the computer hard disk, a number of software programs (e.g., Image-1, Photoshop, Adobe Illustrator, Designer, and Corel Draw) can be used to create publication-quality images. These programs allow a number of operations, some of which include sharpening or blurring the image, removal of background and/or nonspecific noise, pseudocoloring, creating montages, and labeling of the figure. A word of caution is required regarding these programs. It may be the case that the input/output file formats used by these programs are not compatible with other image presentation-programs. As a general consideration, image-processing software is most efficient and useful if it contains the flexibility of user-defined parameters and multiple data and file formats. It is very advantageous to be able to generate digital data sets for computer-based processing in a uniform format that is easily understood by the variety of image analysis/processing software currently available. The tagged image file format (TIFF) is one such universal file format. Other important needs for software used for presentation of microscopic images include bitmap manipulation capabilities to allow adjustment of the printed color image so that it matches the image seen on the computer monitor. For printing color images, another important feature is the ability to print in both CMYK and RGB format. It is also necessary to make sure that, if the software uses PostScript drivers for color printing, the PostScript color interpreter exploits the full ability of the printing objects.

Once completed, a publication-quality hard copy of the computer image can then be produced using a number of techniques. These include photographic printing and color copiers as well as a variety of types of computer printers—e.g, color or black-and-white laser, ink-jet (which are becoming quite good and cost-effective), and dye-sublimation (which require special paper but have higher resolution and better color rendition). LCD projectors, which allow display of computer acquired/processed images directly from the computer, are also available.

SUGGESTED READING

Abramowitz, M. 1993. Fluorescence Microscopy: The Essentials. Olympus-America, New York.

Adams, S.R. and Tsien, R.Y. 1993. Controlling cell chemistry with caged compounds. *Annu. Rev. Physiol.* 55:755-784.

Becker, E. 1989. Fluorescence Microscopy. Wild Leitz, Wetzlar, Germany.

Heim, R. and Tsien, R.Y. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* 6:178-182.

Herman, B. 1998. Fluorescence Microscopy. BIOS Scientific Publishers, Oxford.

Herman, B. 1989. Resonance energy transfer microscopy. *Methods Cell Biol.* 30:219-243.

Kapitza, H.G. 1996. Microscopy From the Very Beginning. Carl Zeiss, Jena, Germany.

Lakowicz, J.R. 1983. Principles of Fluorescence Spectroscopy. Plenum, New York.

Mason, W.T. 1993. Fluorescent and Luminescent Probes for Biological Activity. Academic Press, London.

Nuccitelli, R. (ed.) 1994. A Practical Guide to the Study of Calcium in Living Cells. *Methods Cell Biol.* vol. 40.

Pawley, J. 1995. Handbook of Biological Confocal Microscopy, 2nd ed. Plenum, New York.

Periasamy, A. and Herman, B. 1994. Computerized microscopic vision in cell biology. *J. Comput. Assisted Microsc*. 6:1-26.

Pesce, A.J., Rosen, C.-G., and Pasby, T.L. 1971. Fluorescence Spectroscopy: An Introduction for Biology and Medicine. Marcel Dekker, New York.

Piston, D.W., Masters, B.R., and Webb, W.W. 1995. Three-dimensionally resolved NAD(P)H cellular metabolic redox imaging of the in situ cornea with two-photon excitation laser scanning microscopy. *J. Microsc*. 178:20-27.

Reichman, J. 1994. Glossary of Related Terminology with Page References to the Handbook of Optical Filters for Fluorescence Microscopy. Chroma Technology Corporation, Brattleboro, Vt.

Reichman, J. 1994. Handbook of Optical Filters for Fluorescence Microscopy. Chroma Technology Corporation, Brattleboro, Vt.

Slavik, J. (ed.) 1996. Fluorescence Microscopy and Fluorescent Probes. Plenum, New York.

Taylor, D.L. and Wang, Y.L. (eds.) 1989. Fluorescence Microscopy of Living Cells in Culture, parts A and B. *Methods Cell Biol.* vols. 29 and 30.

Tsien, R.Y. 1994. Fluorescence imaging creates a window on the cell. *Chem. Eng. News* 72:34-36.

Wu, P. and Brand, L. 1994. Resonance energy transfer: Methods and applications review. *Anal. Biochem.* 218:1-13.

Yguerabide, J. 1972. Nanosecond fluorescence spectroscopy of macromolecules. *Methods Enzymol*. 26:498-578.

Contributed by Brian Herman
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

Immunofluorescence Staining

This unit describes the widely used and powerful technique of localization of proteins in cells by immunofluorescence (see Basic Protocol). The location can be determined by double labeling with an antibody directed against a protein of known location. The technique can be used as a supplement to immunolocalization by electron microscopy and subcellular fractionation (Chapter 3). It allows not only identification of the antigen distribution in the cell, but also a survey of the dynamic aspects of protein movements in the cell—on and off membranes, into and out of the nucleus, and through membrane traffic pathways.

IMMUNOFLUORESCENCE LABELING OF CULTURED CELLS

BASIC PROTOCOL

The following is a basic "generic" method for localizing proteins and other antigens by indirect immunofluorescence. The method relies on proper fixation of cells to retain cellular distribution of antigen and to preserve cellular morphology. After fixation, the cells are exposed to the primary antibody directed against the protein of interest, in the presence of permeabilizing reagents to ensure antibody access to the epitope. Following incubation with the primary antibody, the unbound antibody is removed and the bound primary antibody is then labeled by incubation with a fluorescently tagged secondary antibody directed against the primary antibody host species. For example, incubation with a mouse IgG primary antibody might be followed by incubation with a RITC (rhodamine isothiocyanate)–labeled goat anti–mouse IgG secondary antibody. After removal of the secondary antibody, the specimen is ready for viewing on the fluorescence microscope. Once the conditions for observing specific immunolocalization have been identified for a given antibody and cell type, double labeling with two antibodies can be employed to compare localizations. To do this, primary antibody incubation can contain two antibodies generated in two species (e.g., mouse and rabbit), followed by incubation with two secondary antibodies coupled to different fluorophores. Care should be taken, however, that the two antibody combinations, especially the secondary antibodies, do not cross-react.

Materials

Cells of interest, growing in tissue culture
2% formaldehyde (see recipe)
Phosphate-buffered saline (PBS; see recipe), pH 7.4
PBS/FBS: PBS, pH 7.4, containing 10% fetal bovine serum (FBS)
0.1% (w/v) saponin in PBS/FBS: prepare fresh from 10% (w/v) saponin stock solution (*APPENDIX 2A*; store stock up to 2 months at 4°C or in aliquots up to 1 to 2 years at –20°C)
Primary antibody
Controls: preimmune serum (if using rabbit polyclonal antibody) or antigen added in excess to primary antibody
Secondary antibodies (against Ig of species from which primary antibody was obtained) coupled to fluorophore: e.g., RITC (rhodamine isothiocyanate), FITC (fluorescence isothiocyanate) Cy3, or Texas Red
Mounting medium (see recipe)

10-cm diameter tissue culture dishes
12-mm no. 1 round glass coverslips, sterilized by autoclaving or soaking in 70% ethanol
12-well tissue culture plates
150-mm petri dishes

Contributed by Julie G. Donaldson
Current Protocols in Cell Biology (1998) 4.3.1-4.3.6

Watchmaker's forceps
Microscope slides
Nail polish
Fluorescence microscope with 63× oil-immersion lens

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. For adherent cells, 1 to 2 days prior to experiment trypsinize cells and seed onto 10-cm culture dishes, each containing 15 to 20 sterilized coverslips, so that on day of experiment cells are 20% to 50% confluent.

   Nonadherent cells can be coaxed into adhering to the coverslips by precoating the coverslip with poly-L-lysine (see recipe). Apply 10 to 20 µl of suspended cells to each coverslip, let sit 10 min, then proceed with fixation (step 3).

   Alternatively, cells can be attached to coverslips using a cytocentrifuge by following the manufacturer's instructions.

2. On day of experiment, transfer each coverslip individually to a well of a 12-well tissue culture dish containing 1 ml culture medium. Subject cells to the desired experimental conditions (e.g., treat with various drugs, inhibitors, or temperatures prior to fixation and immunostaining).

3. Aspirate medium and add 1 ml of 2% formaldehyde to each well. Allow cells to fix at room temperature for 10 min.

4. Aspirate the formaldehyde fixative and wash coverslips twice, each time by adding 1 ml PBS, pH 7.4, letting stand 5 min, then aspirating the PBS. Add 1 ml PBS/FBS to the fixed coverslips and let stand 10 to 20 min to block nonspecific sites of antibody adsorption.

   NOTE: *Throughout the procedure, do not let cells dry out.*

5. In 1.5-ml microcentrifuge tubes dilute primary antibodies in 0.1% saponin/PBS/FBS.

   Typically affinity-purified antibodies are diluted in the range of 1 to 10 µg/ml, and rabbit antisera are diluted between 1:100 and 1:1000. If using a commercial antibody, follow suggested dilutions from manufacturer.

   During initial characterization, it is wise to try a range of dilutions of antibody.

6. Prepare controls containing only 0.1% saponin/PBS/FBS or (if available) containing preimmune antiserum (if rabbit polyclonal antibody is being used) or specific (primary) antibody with the antigen added in excess.

   Controls are often the most important part of an immunofluorescence experiment.

7. Microcentrifuge antibody dilutions and control solutions 5 min at maximum speed, room temperature, to bring down aggregates in pellet.

   Pipet the antibody solution from above the aggregate pellet.

8. Place a 10 × 10–cm piece of Parafilm in the bottom of a 150-mm petri dish. In a grid pattern that replicates the 12 wells used to incubate the coverslips, label the appropriate place on the Parafilm for each coverslip with a marker.

9. Apply a 25-µl drop of appropriate primary antibody solution to each numbered section. Carefully remove each coverslip from the 12-well plate with watchmaker's forceps, blot the excess fluid by touching the edge to a Kimwipe, then invert the coverslip over the appropriate 25-µl drop, making sure that the side with the cells is down. Place the top on the petri dish and incubate 1 hr at room temperature.

   Sometimes proper labeling will require a longer incubation time or the petri dish will be incubated >1 hr for convenience. For longer incubations, add some wetted Kimwipes to the dish to maintain a humid atmosphere. The incubation can be extended overnight at 4°C, if necessary.

   NOTE: *It is important to always be aware of which side of the coverslip the cells are on. Cells should be facing up when in the 12-well plate, but facing down when placed on the antibody. Picking up coverslips with the forceps is awkward at first but becomes easier with practice.*

10. Carefully pick up each inverted coverslip and flip it over so that it is cell-side-up, then place in a well of a 12-well plate. Wash each coverslip three times to remove unbound antibody, each time by adding 1 ml PBS/FBS, letting stand 5 min, then aspirating the solution.

11. Dilute fluorophore-conjugated secondary antibodies in 0.1% saponin/PBS/FBS. Mix, then microcentrifuge as in step 7 to remove aggregates.

    Typically, commercial preparations are diluted between 1:100 and 1:500.

12. Prepare an incubation chamber as in step 8. Apply 25 µl of appropriate secondary antibody solution to each numbered section and invert coverslip over drop as in step 8. Cover petri dish and protect from light with aluminum foil or place chamber in drawer. Incubate 1 hr at room temperature.

13. Wash coverslips as in step 10. After removal of last PBS/FBS wash, add 1 ml PBS.

14. Label slides and place 1 drop of mounting medium onto slide. Pick up coverslip from well, gently blot off excess PBS by touching the edge to a Kimwipe, then invert coverslip, cell-side-down, onto drop. Gently blot mounted coverslip with paper towel, then seal edge of coverslip onto slide by painting the edge with a rim of nail polish. Let dry.

    The fixed, mounted, and nail polish–sealed coverslips can be stored in the dark for 6 months to 1 year at 4°C.

15. View specimen on fluorescence microscope using an 63× oil immersion objective.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Formaldehyde, 2%

In a chemical fume hood, dilute 2 ml 37% reagent-grade formaldehyde into 35 ml PBS (see recipe), pH 7.4.

As reagent-grade formaldehyde contains 11% methanol, as an alternative (or if necessary) make up formaldehyde from paraformaldehyde by dissolving 0.4 g paraformaldehyde powder in 10 ml H_2O that has been heated to 60°C, then diluting 1:1 with 2× PBS, pH 7.4. It might be desirable to try both procedures for preparing the formaldehyde solution and see which gives better results.

Mounting medium

Use Fluormount G (Southern Biotechnology) or prepare 50% (w/v) glycerol and 0.1% (w/v) *p*-phenylenediamine in PBS (see recipe), pH 8.0.

Phosphate-buffered saline (PBS)

0.144 g KH_2PO_4
9.0 g NaCl
0.795 g $Na_2HPO_4 \cdot 7H_2O$
H_2O to 1 liter
Adjust to desired pH with 1 M NaOH or 1 M HCl
Store indefinitely at room temperature

Poly-L-lysine-coated coverslips

Apply 25 µl of 1 mg/ml poly-L-lysine to each sterilized no. 1 coverslip in a hood and allow to stand ~10 min. Carefully rinse coverslips three times with water, then allow to air dry.

COMMENTARY

Background Information

The first use of fluorescently labeled antibodies to localize a protein in cells occurred over 50 years ago (see Coons, 1961, for a reminiscence). Since that time, the wide availability of numerous antibodies and improvements in indirect labeling methods and fluorophores has made immunofluorescent localization of proteins in cells both a routine and a vital component in any study. Immunofluorescence labeling is quite effective when combined with biochemical or ultrastructural studies because the technique is rapid and so many parameters can be assessed. Furthermore, in contrast to biochemical studies, which assume uniformity of the sample, immunofluorescence technique allows analysis of individual cell differences.

Immunofluorescence labeling has been employed in a variety of cell-biological studies, including the first description of peptide targeting sequences specifying retention of endoplasmic reticulum (ER) lumenal proteins (Munro and Pelham, 1987) and initial studies describing the dynamic membrane trafficking between the ER and Golgi complex (Lippincott-Schwartz et al., 1990). The first study describing the family of Rab GTPases used immunofluorescence to demonstrate distinct localization of the different Rabs to different organelles in the cell (Chavrier et al., 1990). Finally, immunofluorescent localization of proteins encoded by novel genes, including those associated with human diseases and cancer (Nathke et al., 1996), will provide critical information for determining the cellular function of these proteins.

Critical Parameters

To ensure success with immunofluorescence, three parameters are critical—fixation, permeabilization, and determination of the specificity of labeling. The fixation and permeabilization conditions must be assessed individually for each antibody and each cell type investigated. Refer to Griffiths (1993) for an excellent discussion on fixation and issues related to specificity of antibody labeling.

Different fixatives might be investigated to optimize preservation of the antigen, its distribution, and the morphology of other cellular constituents. For example, some epitopes are lost upon aldehyde fixation but preserved with alcohol fixation, and vice versa. Alternative fixatives to try include methanol at –20°C (5 min exposure) or formaldehyde fixation followed by a brief (1-min) exposure to methanol at 0°C. Methanol fixation is often quite effective for localizing cytoskeletal elements. Alcohols work by extracting lipids and precipitating remaining proteins, whereas aldehydes are cross-linking reagents that generally preserve membranous structures better (McCaffery and Farquhar, 1995).

Sometimes, even when material is appropriately fixed, the epitope is obscured and not accessible to antibody binding. Permeabilizing reagents are typically detergents that partially denature fixed proteins, exposing the epitope. In addition to saponin, which the authors include throughout the staining procedure, treatments after fixation with 0.2% to 0.5% Triton X-100 or SDS can also be tried. Often a short treatment with these reagents prior to antibody incubations is sufficient to expose the epitope. Special attention should be directed toward ensuring that the access to the epitope is the same even if the protein has undergone a translocation; for example proteins that shuttle between the cytoplasm and nucleus are not always equally accessible to antibody labeling (Pines, 1997).

The primary antibody should be purified to the extent that it recognizes only the protein of interest on immunoblots. Although affinity pu-

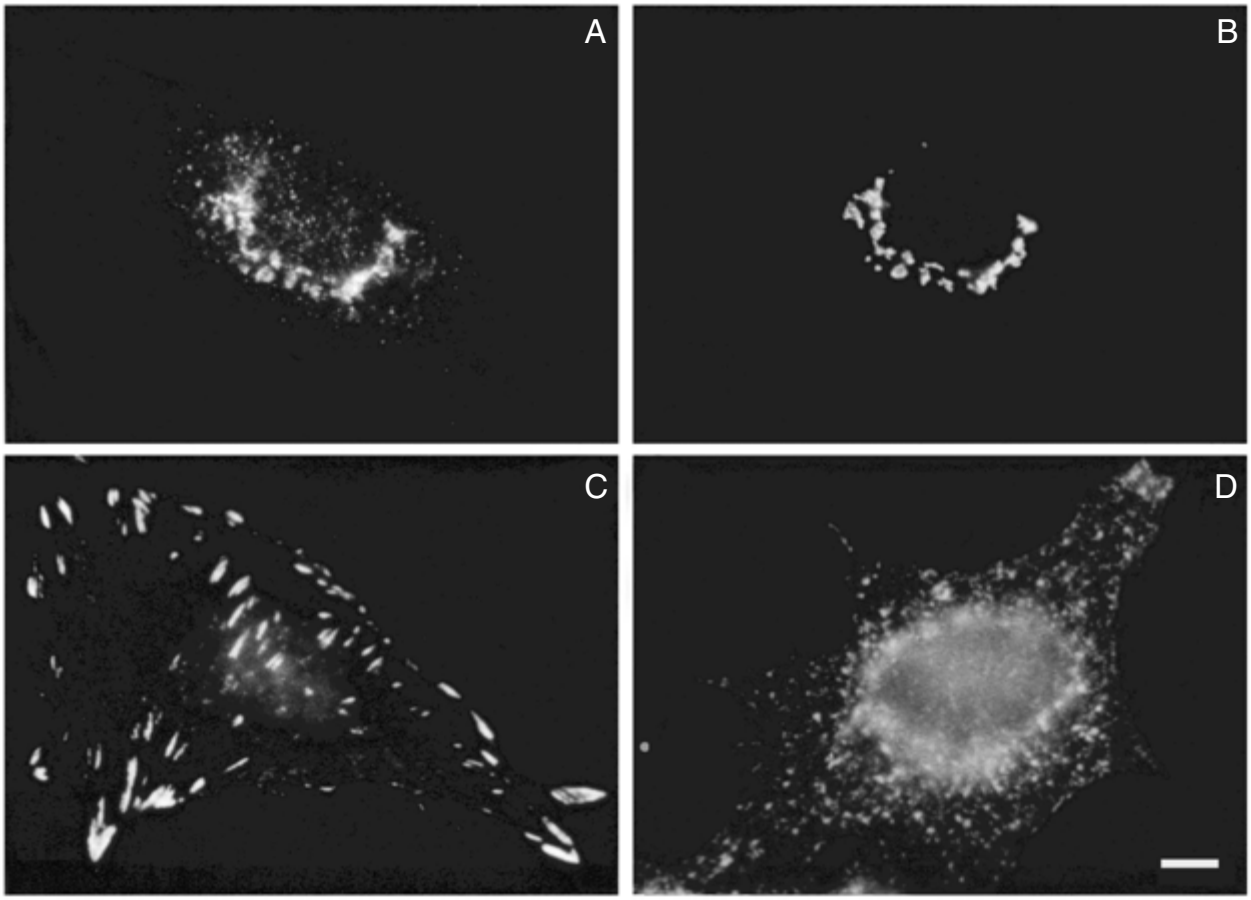


Figure 4.3.1 Examples of immunofluorescence labeling of formaldehyde-fixed cells. (**A**) and (**B**) Double labeling of a normal rat kidney cell with a mouse monoclonal antibody to (**A**) the β-COP component of coatomer and (**B**) a rabbit polyclonal antibody to mannosidase II. (**C**) Distribution of vinculin in a formaldehyde-fixed normal rat kidney cell using a mouse monoclonal antibody. (**D**) Distribution of transferrin receptor in formaldehyde-fixed HeLa cells using a mouse monoclonal antibody. Bar is equal to 10 μm.

rification often resolves this, be aware that "affinity-purified" antibodies do not always result in antibodies that recognize only a single protein. Especially in the case of anti-peptide antibodies, multiple bands are sometimes labeled even after affinity purification. By immunoblotting on a gel (*UNIT 6.2*), it is easy to determine on the basis of molecular weight whether it is the protein of interest that is recognized. However, not all antibodies that can be used to detect a specific protein by immunoblotting work for immunofluorescence detection. Likewise, there are antibodies that recognize the protein by immunofluorescence that do not recognize the separated, denatured proteins transferred to nitrocellulose.

Next, it is important to be able to determine what constitutes "specific labeling" with the antibody. To assess this, control-stained slides (incubated with secondary antibody only or with preimmune serum) should first be viewed to determine what constitutes "nonspecific staining." Ideally this is negligible, and, by contrast, the staining pattern obtained with the specific antibody is much brighter and more distinct. When initially characterizing immunolocalization using a particular antibody, a range of dilutions of the primary and secondary antibodies should be examined to determine optimal concentrations that minimize nonspecific staining and maximize specific staining. Minimal background staining from secondary antibodies can often be achieved by dilution or trying different secondary antibodies made in different host species or obtained from different commercial suppliers. Then, a dilution of the primary antibody is selected to optimally label the specific protein. At this point, further controls should be analyzed to ensure that the staining observed is due to the presence of the protein of

interest. If a rabbit antiserum is used, preimmune serum should yield a pattern of staining similar to that of the secondary antibody alone. If the immunizing antigen is available, it can be added to the antibody dilution to compete for the specific staining; and it should yield "background" levels of fluorescence. Lastly, support for specific labeling can be confirmed by lack of staining in a cell type that does not express the protein of interest and increased labeling in cells overexpressing the protein by transient transfection. Finally, the use of different antibodies to the protein, if available, can independently confirm the localization pattern.

Troubleshooting

If background staining with secondary antibodies is too high, there are several possible remedies. The secondary antibodies may be diluted further, or different secondary antibodies, different hosts, or different fluorophores may be tried. Also, it is possible to try other "sorbing" reagents. PBS/FBS is generally a good sorbing reagent that effectively blocks out nonspecific sites, but other agents can be used—e.g., 1% (w/v) BSA (immunoglobulin-free) or gelatin.

If no specific staining is observed, it is possible to increase the concentration of or the time of exposure to the primary antibody. One can also try alternative fixation and permeabilization regimens to visualize specific staining.

If specific staining is observed, but it is very dim, it is possible to increase the concentration of the primary and/or secondary antibodies. As long as background staining is low, the signal can be enhanced by using a fluorescence double-sandwich technique. To do this, following incubation with primary antibody (e.g., a mouse monoclonal antibody) and washing, incubate first in FITC-conjugated goat anti–mouse IgG and then in FITC-conjugated donkey anti–goat IgG. Caution should be observed that secondary antibodies do not alter the pattern of single antibody labeling.

Anticipated Results

Once conditions for specific localization have been optimized, the distribution of the protein in a variety of cells under a variety of conditions can be observed (see Fig. 4.3.1). Double labeling of two antigens allows the distribution of one protein to be compared with that of another protein in the same sample using two secondary antibodies attached to fluorophores that can be monitored in two different channels by flow cytometry. Thus, the location of a known protein, say an antibody to a Golgi-resident protein, can be used as a marker for the Golgi complex to see whether the antigen under study colocalizes with it (Fig. 4.3.1, panels A and B).

Time Considerations

The entire procedure can be performed in ~3 hr. Fixed, washed coverslips can be stored at 4°C for several days prior to immunolabeling. Incubations with primary antibodies can be extended to overnight at 4°C if necessary (or if it is desirable to increase staining).

Disclaimer

This article was written by Julie Donaldson in her private capacity. No official support or endorsement by the NHLBI or NIH is intended and none should be inferred.

Literature Cited

Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K., and Zerial, M. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* 62:317-329.

Coons, A.H. 1961. The beginnings of immunofluorescence. *J. Immunol.* 87:499-503.

Griffiths, G. 1993. Fine Structure Immunocytochemistry. Springer-Verlag, Heidelberg.

Lippincott-Schwartz, J., Donaldson, J.G., Schweizer, A., Berger, E.G., Hauri, H.P., Yuan, L.C., and Klausner, R.D. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell* 60:821-836.

McCaffery, J.M. and Farquhar, M.G. 1995 Localization of GTPases by indirect immunofluorescence and immunoelectron microscopy. *Methods Enzymol.* 257:259-279.

Munro, S. and Pelham, H.R.B. 1987. A C-terminal signal prevents secretion of lumenal ER proteins. *Cell* 48:899-907.

Nathke, I.S., Adams, C.L., Polakis, P., Sellin, J.H., and Nelson, W.J. 1996. The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. *J. Cell Biol.* 134:165-179.

Pines, J. 1997. Localization of cell cycle regulators by immunofluorescence. *Methods Enzymol.* 283:99-113.

Contributed by Julie G. Donaldson
National Heart, Lung, and Blood Institute/NIH
Bethesda, Maryland

Fluorescent Staining of Subcellular Organelles: ER, Golgi Complex, and Mitochondria

The ability to distinguish and identify different subcellular compartments is crucial for understanding organelle function, biogenesis, and maintenance within cells, as well as for defining protein trafficking pathways. This unit describes protocols for labeling three subcellular organelles—endoplasmic reticulum (ER), Golgi complex, and mitochondria—using fluorescent dyes and fluorescently labeled lipid derivatives. Many fluorescent lipid derivatives have been shown to preferentially partition into membrane compartments of living cells. This finding has been used to generate protocols for fluorescently labeling specific intracellular organelles in vivo. Such protocols have been enormously valuable for enabling researchers to identify different organelles and to study their dynamics and spatial organization within cells (Terasaki et al., 1984; Cooper et al., 1990; Pagano et al., 1991).

The unit first describes how to stain the endoplasmic reticulum (ER) by using the fluorescent dye $DiOC_6(3)$. This dye can be used to stain fixed cells (see Basic Protocol 1) or living cells (see Alternate Protocol), and the protocols are very simple. Since the dye stains many other organelles in addition to the ER, $DiOC_6(3)$ is particularly useful for staining the thin peripheral regions of cultured cells, where it is usually easy to distinguish the ER on the basis of morphology.

Fluorescent analogs of ceramide, including *N*-[7-(4-nitrobenzo-2-oxa-1,3-dizole)]-6-aminocaproyl D-*erythro*sphingosine (C_6-NBD-Cer) and *N*-[5-(5,7-dimethyl BODIPY)-1-pentanoyl]-D-*erythro*sphingosine (BODIPY-Cer), have proved to be particularly valuable for labeling the Golgi complex (reviewed in Pagano, 1989), which receives, processes, and sorts newly synthesized proteins exported from the ER. These dyes readily accumulate in the Golgi complex of most cell types by a preferential membrane partitioning process (Lipsky and Pagano, 1983, 1985a; Pagano et al., 1989). They can be used to image the Golgi complex in fixed or living cells (see Basic Protocol 2).

Mitochondria are unique among intracellular organelles in that they have a large membrane potential, which is negative inside. This characteristic makes it relatively easy to specifically stain mitochondria in living cells. Many fluorescent lipophilic cationic molecules can be used. These molecules pass through the plasma membrane by their lipophilicity and then accumulate in the mitochondria as a result of the attraction of the plus charge on the molecule to the high negative mitochondrial membrane potential. This unit describes the use of TMRE (tetramethylrhodamine, ethyl ester) to stain mitochondria in mammalian cells in culture (see Basic Protocol 3). Accumulation of this dye follows the Nernst equation and is therefore independent of other factors such as binding to mitochondrial components (Ehrenberg et al., 1988; Farkas et al., 1989). This establishes TMRE as a good monitor for the mitochondrial membrane potential (Loew et al., 1993). TMRE has an additional advantage in that live cell staining is accomplished simply by equilibrating cells with the dye, then mounting them for observation in the presence of the dye. Other fluorescent dyes are available for investigating mitochondrial properties in interesting ways and are discussed briefly in the Commentary.

Contributed by Mark Terasaki, Leslie Loew, Jennifer Lippincott-Schwartz, and Kristien Zaal

Current Protocols in Cell Biology (1998) 4.4.1-4.4.18

BASIC PROTOCOL 1

STAINING THE ENDOPLASMIC RETICULUM IN FIXED CELLS

In this protocol, cells are fixed in glutaraldehyde for 3 min, stained with the fluorescent dye $DiOC_6(3)$ for 10 sec, then mounted in dye-free buffer. Live cell staining is accomplished by mounting cells directly in the fluorescent dye (see Alternate Protocol).

Cells growing on coverslips are handled in a similar way in this protocol as for immunofluorescence, with two major differences. One is that the cells must be kept away from detergents, organic solvents, and nail polish, since these will extract $DiOC_6(3)$. The second is that the time scale is much shorter.

Materials

Fixative: prepare 0.25% glutaraldehyde in PBS (*APPENDIX 2A*) from 7% to 70% commercial stock solution (store up to 1 week at 4°C)
Cells of interest, growing on coverslips
2.5 µg/ml $DiOC_6(3)$ working solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Silicone high-vacuum grease

Small petri dish
Watchmaker's forceps
Microscope slides
Silicon rubber chamber (see recipe)
Fluorescence microscope with fluorescein filters and either 63× or 100× oil-immersion objective (*UNIT 4.2*)

Additional reagents and equipment for fluorescence microscopy (*UNIT 4.2*)

1. Put some fixative in a small empty petri dish. Using watchmaker's forceps, remove coverslip containing cells from culture dish and put it into the fixative. Let cells fix 3 to 5 min.

2. Remove coverslip from fixative and place cell-side-up on a piece of Parafilm. Remove excess fixative using a Pasteur pipet, but do not let the coverslip dry out.

3. Apply enough 2.5 µg/ml $DiOC_6(3)$ working solution to cover the coverslip.

   A volume of 100 µl should be sufficient for most coverslip sizes.

4. After ~10 sec, remove the $DiOC_6(3)$ solution with a Pasteur pipet, then apply sufficient PBS to cover the coverslip.

5. Grease both sides of a silicon rubber chamber sparingly with silicone high-vacuum grease. Press the greased chamber firmly on a microscope slide, avoiding air bubbles. Fill the well with $DiOC_6(3)$ working solution, then place the coverslip, cell-side-down, on the chamber. Press down lightly on the coverslip to make contact between the glass and the silicon rubber and make a seal that keeps the coverslip adherent to the chamber (Fig. 4.4.1). Dry off excess fluid with a Kimwipe.

   One way to do this is to hold the Pasteur pipet used for filling the chamber in the left hand and hold the coverslip with watchmaker's forceps in the right hand.

   Use a minimal amount of grease, since this can be very difficult to remove if it gets onto the microscope objective lens. For short-term observations it is possible to mount coverslips without the silicone grease.

   An alternative method (not involving a silicon rubber chamber) is to gently place the coverslip cell-side-down directly on a microscope slide with a minimum of bubbles. Carefully dry off excess fluid at the sides and especially on top of the coverslip. Sealing the sides is not required for short-term observations. Do not let the microscope immersion oil

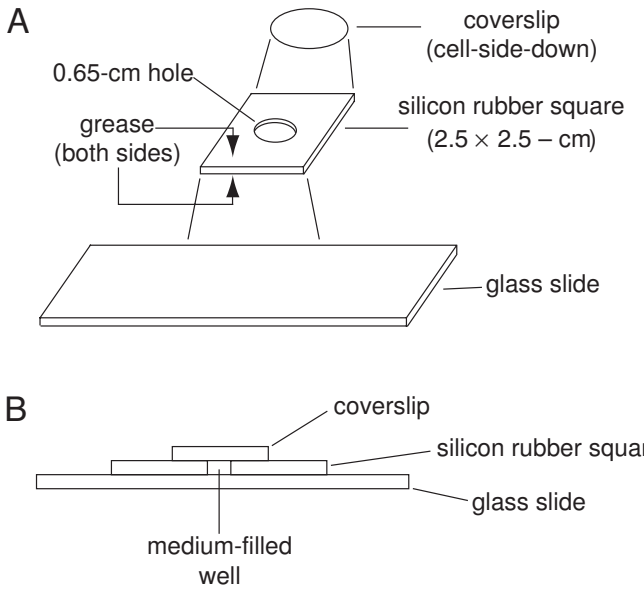


Figure 4.4.1 Microscope slide with silicon rubber chamber. (**A**) Assembly; (**B**) side view in cross-section.

leak underneath the coverslip. Also, be aware that the coverslip will start to dry out from the edges, and that dried cells are not suitable for viewing. If sealing of the coverslip is desired, use paraffin or Valap (UNIT 13.1), not nail polish, because the organic solvents in the nail polish will extract the stain.

6. Observe cells using fluorescence microscope with fluorescein filters and oil-immersion 63× or 100× objective lens.

   The results are best observed during the first 10 to 20 min after staining. Afterwards, the dye starts to accumulate in lysosomes. After longer periods of time, autofluorescence from glutaraldehyde begins to obscure the staining.

STAINING THE ENDOPLASMIC RETICULUM IN LIVING CELLS

ALTERNATE PROTOCOL

The ER of cells that have not been fixed can also be labeled with $DiOC_6(3)$.

Additional Materials *(also see Basic Protocol 1)*

0.5 µg/ml $DiOC_6(3)$ working solution in growth medium appropriate for cells (see recipe)

1. Grease both sides of silicon rubber chamber sparingly with silicone high-vacuum grease and press onto microscope slide.

   The silicon rubber chamber does not need to be sterilized for short-term observations of a few hours.

2. Using watchmaker's forceps, remove coverslip containing cells from culture dish. Fill chamber with 0.5 µg/ml $DiOC_6(6)$ working solution, then put coverslip cell-side-down on chamber. Press down carefully on the coverslip to make a seal that keeps the coverslip adherent to the chamber, and dry off excess fluid with a Kimwipe.

   If a large bubble is seen in the chamber, remount the coverslip. If a chamber with temperature control and ability to perfuse is desired, see Basic Protocol 3, introduction, for information on live cell chamber.

3. Observe cells using fluorescence microscope with fluorescein filters and oil-immersion 63× or 100× objective lens.

It should take 5 to 10 min for ER staining to develop. The mitochondria are usually swollen at dye concentrations that stain the ER. After 10 min, if only the mitochondria are stained, try 1.0 μg/ml $DiOC_6(3)$ staining solution. If the cells look sick and are starting to detach from the coverslip, use a lower concentration of dye. To stain only the mitochondria with $DiOC_6(3)$, use 0.1 μg/ml.

If photodynamic damage or bleaching is hindering the experiments, scavenge the oxygen in the medium with Oxyrase, a bacterial enzyme system. The enzyme is used at 0.3 U/ml (1:100 dilution of enzyme as purchased from Oxyrase, Inc.; Waterman-Storer et al., 1993).

BASIC PROTOCOL 2

STAINING THE GOLGI COMPLEX IN LIVING CELLS

Vital staining of the Golgi complex with C_6-NBD-Cer or BODIPY-Cer involves incubation of cells with either of these fluorescent lipids at low temperature, followed by washing. The ER, nuclear envelope, and mitochondria are initially fluorescent, but over time the Golgi complex begins to fluoresce as a result of preferential insertion of the fluorescent lipids into its membranes. Upon warming the cells to 37°C, intense labeling of the Golgi complex occurs, but only for a finite period of time. This is because the ceramide derivatives within the Golgi complex are metabolized at 37°C to the corresponding fluorescent analogs of sphingomyelin and glucosylceramide. These traffic to the cell surface, causing fluorescent labeling of the plasma membrane over time. Fluorescent labeling of the cell surface can be reduced by incubating cells in medium containing nonfluorescent liposomes or defatted BSA. This results in "back-exchange" of fluorescent sphingomyelin and glucosylceramide into the medium (Lipsky and Pagano, 1985b; van Meer et al., 1987). Labeled cells can be fixed and further processed for double labeling with fluorescently tagged antibodies (see *UNIT 4.3*), to compare the localization of the Golgi complex with other markers within the cell.

Golgi membranes labeled with the fluorescent ceramide derivatives are viewed on a fluorescent microscope equipped with filter combinations appropriate for fluorescein. BODIPY-Cer can also be visualized with filter combinations appropriate for rhodamine optics, since at high concentrations within Golgi membranes BODIPY-Cer forms excimers, which emit >590-nm light when excited with blue light.

Materials

Ethanol
0.05% trypsin in HBSS (*APPENDIX 2A*) without calcium and magnesium
1 mM ceramide fluorescent derivative working solution in ethanol (see recipe)
Serum-free medium appropriate to cells
Cells of interest
Phosphate-buffered saline (PBS; *APPENDIX 2A*), 4°C
10% (w/v) defatted BSA (Sigma) in serum-free medium appropriate to cells
Fluoromount G (Southern Biotechnology Associates or Electron Microscopy Sciences) *or* mounting medium (see recipe)
HEPES-buffered culture medium appropriate to cells, pH 7.0, with 10% serum and without phenol red

12-mm diameter no. 1 round glass coverslips
Watchmaker's forceps
10-cm sterile tissue culture dishes
Hamilton syringe
Silicon rubber chambers (see recipe)
Microscope slides

Conventional fluorescence microscope with standard fluorescein and rhodamine filter cubes *or* confocal microscope with Kr/Ar laser (*UNIT 4.2*)
63× (1.4 NA) or 100× (1.3 NA) oil-immersion objective
Microscope air-stream incubator (Nevtek)

Additional reagents and equipment for growing cells in tissue culture and trypsinization of cells (*UNIT 1.1*) and fluorescence microscopy (*UNIT 4.2*)

Prepare cells on coverslips

1. Sterilize 12-mm diameter no. 1 round glass coverslips by soaking in 95% ethanol and flaming. Handling the coverslips with watchmaker's forceps, place in a 10-cm sterile tissue culture dish (which will accommodate up to eight coverslips).

   Be sure the coverslips are no. 1 thickness for viewing at high magnification on the microscope. Thicker coverslips will not match the numerical aperture of the objective and can give significant spherical aberration, resulting in poor microscopic images.

2. One to two days prior to start of experiment, trypsinize cells (*UNIT 1.1*) in 0.05% trypsin and seed onto the culture dishes containing the coverslips.

   For optimal viewing, the cells should have grown to only ~50% confluency by the day of the experiment.

   If cells fail to grow well, the coverslips can be treated prior to seeding with 1 mg/ml poly-D-lysine or poly-L-lysine for 15 min.

Label cells

3. Take a small aliquot of the 1 mM ceramide derivative in ethanol and disperse it into serum-free medium at room temperature by injecting it into the medium with a Hamilton syringe while rapidly vortexing. Use quantities of Cer-derivative solution and medium such that the final working concentration of Cer-derivative in the medium will be 5 to 10 μM and the final concentration of ethanol will be <0.5% (in order to prevent damage to the cells).

   This step causes the Cer-derivatives to form small vesicles which are more easily delivered to the cell.

4. Place coverslips in a tissue culture dish containing ice-cold PBS for 2 min. Remove PBS and replace with fresh ice-cold PBS, then repeat one more time.

   This step removes serum from outside the cells. Cer-derivatives have a very high affinity for serum, so any serum present on the cells will lower the effective concentration of the Cer-derivative.

5. Add medium containing the Cer-derivative to cells so that the bottom of the dish, along with the surface of the coverslip, is completely covered with medium and incubate 30 min at 4°C.

6. Wash cells three times with cold PBS to remove unbound lipid, then incubate with medium containing 10% defatted BSA for 30 min at 4°C.

   This removes or "back exchanges" the excess NBD- or BODIPY-Cer that is not incorporated into intracellular membranes.

7. Warm cells to 37°C, and continue incubating at that temperature, examining cells under a fluorescence microscope to determine when optimal labeling of the Golgi complex and minimal labeling of other intracellular membranes occurs.

   Golgi labeling should be prominent after 15 to 40 min.

View cells by fluorescence microscopy

8. Place a small drop of Fluoromount G or mounting medium on the cell side of the coverslip. Invert coverslip onto a silicon rubber chamber attached to a microscope slide (see Basic Protocol 1, step 5) that contains HEPES-buffered medium with serum, pH 7.0, without phenol red.

   Phenol red is autofluorescent and therefore increases the background level of fluorescence within cells.

   Alternatively, the cells can be grown in commercially supplied coverslip–bottom open dishes (Lab-Tek chamber coverglass system, item nos. 136307, 136420, Nunc). These dishes allow drugs or other reagents to be easily added while the cells are being imaged. Such chambers, however, can only be used with an inverted microscope.

9. Place a drop of immersion oil on the non-cell side of the coverslip and view cells with a fluorescent microscope using a 63× or 100× oil immersion objective.

   a. View C_6-NBD-Cer–labeled cells using a filter combination on the microscope appropriate for fluorescein optics (i.e., one that excites the fluorophore at 450 to 490 nm and collects its emission at 520 to 560 nm).

   b. View BODIPY-Cer–labeled cells using the above filter combination or a filter combination that excites the fluorophore at 450 to 490 nm and collects its emission at >590 nm. For confocal microscopes, image with the 488-nm line of the Kr/Ar laser.

      The filter set collecting emission at >590 nm allows the distribution of BODIPY-Cer excimers to be visualized. These usually only form in Golgi membranes, where the lipid has become greatly concentrated. Because BODIPY-Cer excimer formation usually only occurs in the Golgi complex, imaging the red-shifted BODIPY-Cer signal rather than the green BODIPY-Cer signal provides a convenient way to avoid imaging the green signal from non-Golgi membranes (including the ER and plasma membrane) containing BODIPY-Cer.

   To keep cells at 37°C while viewing under the microscope, warm microscope stage by blowing warm air upon it with an air-stream incubator.

BASIC PROTOCOL 3

STAINING MITOCHONDRIA

This staining protocol (which is for living cells only, not fixed cells) is simple, since it merely involves incubating the cells in the presence of the dye. An additional consideration, however, is how to maintain normal cell behavior on the microscope stage. For physiological experiments on mammalian cells, where mitochondrial potential is to be measured or where the motility or distribution of mitochondria is being studied, it is necessary to regulate the temperature. The ability to perfuse different solutions or media may also be necessary. Several temperature-regulated chambers are commercially available. For experiments on an inverted microscope, the authors have used a thermoregulated microscope chamber from Bioptechs equipped for perfusion and solution exchange. Coverslips (no. 1, 31-mm diameter) were also obtained from Bioptechs. For experiments in which mitochondria are to be observed at room temperature, simpler mounting strategies can be used (e.g., see Chen, 1989).

Materials

100 nM TMRE working solution (see recipe) in NB
Normal buffer (NB; see recipe)

No. 1, 31-mm diameter coverslips (Bioptechs)
60-mm petri dishes
Forceps

Q tips or Kimwipes
Temperature-regulated microscope chamber (Bioptechs)
Conventional fluorescence microscope with conventional rhodamine filter *or* confocal microscope (*UNIT 4.2*)
40× or greater oil-immersion objective

1. Grow cells on no. 1, 31-mm glass coverslips.

   Culturing procedures will depend on the application. For example, to clearly observe the endoplasmic reticulum network of fibroblasts, trypsinize the cells, plate them on the coverslip at a relatively low density so that most cells are not in contact with another cell, and observe them after allowing them to spread overnight.

2. Fill three 60-mm petri dishes with NB and one tissue culture dish with 100 nM TMRE working solution in NB. Handling the coverslip with forceps, gently wash by serially dipping in the three NB dishes and finally in the TMRE solution. Return the coverslip to a new dish and incubate 10 min in 100 nM TMRE working solution in NB.

3. Remove the coverslip from the culture dish, dry the bottom of the coverslip with a Q-tip or Kimwipe, and mount in a microscope chamber. Fill the chamber with 100 nM TMRE in NB.

4. Observe cells using either a conventional rhodamine filter on a conventional fluorescence microscope or the 540- or 568-nm laser excitation line on a confocal microscope, and a 40× or greater oil-immersion objective lens.

   The staining should be sufficient for observing mitochondrial distribution, motility, or relative levels of mitochondrial membrane potential. Quantitative measurements of the membrane potential requires low-light-level imaging and digitization of images. These topics are covered in Loew et al. (1993) and Loew (1998).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ceramide fluorescent derivatives

Stock solution: For long-term storage, dissolve an appropriate amount of powdered Cer-derivative (C_6NBD-Cer or BODIPY-Cer; Molecular Probes) in a glass scintillation vial with a Teflon-lined screw cap containing 19:1 (v/v) chloroform/methanol. Blow argon gas on top of the liquid before sealing to prevent oxidation. Keep the tubes sealed and store up to 2 years at −20°C.

Working solution (1 mM): When ready to label cells, dry an aliquot of the chloroform stock under nitrogen and redissolve in absolute ethanol to make a solution of 1 mM.

For short-term storage (i.e., 1 to 2 weeks), dissolve the Cer-derivatives in 100% ethanol to a concentration of 1 mM, seal, and keep at −20°C.

$DiOC_6(3)$ stock and working solutions

Stock solution (0.5 mg/ml): Prepare ~10 ml of 0.5 mg/ml $DiOC_6(3)$ (D-273 from Molecular Probes) in ethanol. Store in a scintillation vial wrapped in aluminum foil at room temperature (probably good for several years).

Working solution for Basic Protocol 1 (2.5 μg/ml): Make a 0.5% (v/v) dilution in PBS (*APPENDIX 2A*) of the 0.5 mg/ml $DiOC_6(3)$ stock solution [e.g., 5 μl $DiOC_6(3)$ stock in 1 ml PBS].

Working solution for Alternate Protocol (0.5 μg/ml): Make a 1:1000 dilution of the 0.5 mg/ml stock solution in the growth medium appropriate to the cells of interest.

This working solution can be used for a few hours, but do not store overnight.

Mounting medium

PBS (*APPENDIX 2A*) containing:
50% (w/v) glycerol
0.1% (w/v) *p*-phenylenediamine
Adjust pH to pH 8.0 using NaOH
Store up to 1 to 2 weeks at 4°C

Normal buffer (NB)

7.6 g NaCl (130 mM final)
0.41 g KCl (5.5 mM final)
0.2 g $CaCl_2$ (1.8 mM final)
0.2 g $MgCl_2 \cdot 7H_2O$ (1.0 mM final)
4.5 g glucose (25 mM final)
4.8 g HEPES (20 mM final)
H_2O to 1 liter
Adjust pH to pH 7.4 with 1 N NaOH
Store up to 1 month at 4°C

Silicon rubber chamber

Using a razor blade, cut a 2.5 × 2.5–cm piece of Ronsil calendared sheet, 0.3 in. (0.76 mm) thick (purchase from North American Reiss; 100 feet minimum order). Using a hole puncher, cut a hole ~0.65 cm in diameter in the middle of the square over which the coverslip will be placed.

After the experiment, the silicon rubber chamber can be peeled off the slide, wiped clean with a Kimwipe, and reused.

TMRE

Stock solution (0.5 mM): To make 10 ml, add 2.5 mg TMRE (T-669 from Molecular Probes) to 10 ml of 95% ethanol. Store up to 1 year at −20°C.

Working solution (100 nM): Add 20 µl of 0.5 mM TMRE stock to 100 ml normal buffer (NB; see recipe). Use only on day of preparation.

One may consider using growth medium instead of NB to prepare the working solution, but this often produces higher background fluorescence, probably from phenol red or from serum binding of dyes.

COMMENTARY

Background Information

Endoplasmic reticulum

The endoplasmic reticulum (ER) was discovered by electron microscopy in the peripheral regions of osmium-fixed cells grown on formvar-coated grids (Porter et al., 1945; Porter, 1953). The peripheral regions of cells are thin enough that the organization can be imaged without sectioning. The name ER was given to a network (reticulum) that was more abundant in the central (endoplasmic) portion as opposed to the motile periphery (ectoplasm). Therefore, the ER was first defined purely in terms of morphology. With the advent of thin-section electron microscopy and differential centrifugation, the functions of the membranes became established.

The use of fluorescent dyes to stain the ER grew out of the use of dyes for monitoring membrane potential (Cohen et al., 1974). Dicarbocyanine dyes are of the "slow" class of potential-sensitive dyes (Sims et al., 1974). Rhodamine 123, like many of the dicarbocyanine dyes, was found to label mitochondria in living cells (Johnson et al., 1980). Michael Weiss found that one of the dicarbocyanines labeled a network when viewed at high magnification (Terasaki et al., 1984). Rhodamines and dicarbocyanines were screened, and $DiOC_6(3)$ was chosen as the brightest and most photostable stain for the network. The network was identified as ER, based on its morphology. In addition, conditions for staining fixed cells were described. It was initially stated that the dye did not stain Golgi or endosomes, but it was

later concluded that it did stain these organelles (Terasaki et al., 1986; Terasaki and Reese, 1992). There was some uncertainty among cell biologists whether the network stained was actually the ER, even though this network had been originally defined as the ER. Double imaging of BiP, a protein agreed to be in the ER, proved that the peripheral network stained by $DiOC_6(3)$ was identical to the BiP immunofluorescence pattern (Terasaki and Reese, 1992).

This two-dimensional network of ER in the periphery of many cultured cell types has some noteworthy characteristics. All of the ER network in this region is close to the plasma membrane. This is in contrast to the bulk of the ER of most cells, which is in the interior and not close to the plasma membrane. The ER network is usually composed of tubules that are connected by three-way junctions; sometimes, large sheets that correspond to ER cisternae are also seen (Terasaki et al., 1986). The tubules near the leading edge of the cell are often aligned with microtubules that are responsible for extending the ER tubules outward (Terasaki et al., 1986; Dailey and Bridgman, 1989). It is also noteworthy that the ER network in the periphery is relatively sparse. First of all, due to the limitations in light microscopy, the fluorescence image of the ER tubules is larger than its actual width, which is on the order of 100 nm. When the ER is seen in whole-mount electron micrographs, it looks surprisingly thin (Dailey and Bridgman, 1991). Secondly, it is easy to see that the total amount of ER membrane in the peripheral regions is less than the amount of plasma membrane in those regions. This is in contrast to the situation in most cells, where the area of the ER is many times as large as that of the plasma membrane.

Another dicarbocyanine dye, DiI [$DiIC_{18}(3)$] has been used to label the ER (Terasaki and Jaffe, 1991). This method results in more specific labeling of the ER and is well suited for three-dimensionally arranged ER, but requires microinjection (Terasaki and Dailey, 1995).

Golgi complex

The Golgi complex plays a central role in secretory protein processing and trafficking within cells. It receives membrane-enclosed packets of protein cargo from the ER, modifies and processes cargo proteins, and then repackages them into transport intermediates for delivery to the plasma membrane and other intracellular destinations (Mellman and Simons, 1992). To perform these tasks, the Golgi complex is enriched in a variety of resident enzymes involved in carbohydrate processing. It also has a unique lipid composition that includes high concentrations of cholesterol and other lipids relative to the concentrations found in the ER. This property is the basis for vital staining of the Golgi with fluorescent lipids. The C_6-NBD- and BODIPY-fluorescent analogs of ceramide have proved to be particularly valuable for this purpose. C_6-NBD-Cer is formed by *N*-acylation of the long-chain base, sphingosine, with the short-chain fluorescent fatty acid, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproic acid (C6-NBD-FA). C_6-NBD-Cer can be synthesized in the laboratory (Kishimoto, 1975; Schwarzmann and Sandhoff, 1987) or purchased from Molecular Probes. The newer fluorescent ceramide analog, BODIPY-Cer, is a conjugate of D-*erythro*-sphingosine and a fluorescent fatty acid containing the fluorophore boron dipyrromethene difluoride (BODIPY). It has ~2- to 3-fold higher fluorescent yield and greater photostability than NBD-ceramide (Pagano et al., 1991). An additional advantage of the BODIPY-Cer analog is that its fluorescence emission spectrum is dramatically red shifted as the probe concentrates in membranes. This is due to excimer formation of the BODIPY-Cer molecules. The Golgi complex is usually the only membrane system where BODIPY-Cer accumulates to high enough levels to form excimers and hence is the only organelle where the red shift occurs (Pagano et al., 1991). By using microscope filters appropriate for visualizing the red-shifted BODIPY-Cer that has formed excimers in Golgi membranes, non-Golgi membranes containing low concentrations of the dye will not be visualized.

The mechanism whereby C_6NBD-Cer and BODIPY-Cer initially accumulate in the Golgi complex is not understood. It has been suggested that Golgi labeling by C_6-NBD-Cer and BODIPY-Cer is due to preferential partitioning into Golgi membranes as a result of the specific physical properties of these membranes. This could explain why NBD-Cer labeling of the Golgi complex occurs at low temperature or in the presence of metabolic inhibitors, and can even occur in cells that have been fixed before incubation.

The finding that C_6-NBD-ceramide labels the Golgi complex after cells have been fixed (Pagano et al., 1989) is the basis for a novel methodology for visualizing the Golgi complex at the electron microscope (EM) level (Pagano and Martin, 1998). In this protocol, fixed cells labeled with C_6-NBD-ceramide are photobleached in the presence of diaminoben-

zadine (DAB). The photooxidation products catalyze the polymerization of DAB to yield a high-molecular-weight osmiophilic compound that can be visualized at the EM level. Previous work has shown that under these conditions, the C_6-NBD-ceramide product labels the trans-Golgi elements (Pagano et al., 1989, 1991).

In addition to being useful markers for Golgi membranes in localization studies (Lipsky and Pagano, 1985a; van Meer et al., 1987; Kobayashi and Pagano, 1989; Rosenwald and Pagano, 1993; van't Hof and van Meer, 1990), C_6-NBD-Cer and BODIPY-Cer have been used in fluorescent time-lapse imaging studies to analyze the dynamics of the Golgi complex in cultured cells (Cooper et al., 1990; Sciaky et al., 1997; see Fig. 4.4.2). These studies have shown that while Golgi elements have a large-scale stability in organization, they are remarkably dynamic. Long tubule processes were often seen extending off the rims of Golgi elements during time-lapse imaging. The Golgi tubule processes appeared to serve both to interconnect adjacent Golgi elements and to carry Golgi membrane out to the cell periphery.

Mitochondria

Mitochondria in tissue culture cells can be seen by phase-contrast or bright-field microscopy. Johnson et al. (1980) described the use of rhodamine 123 for viewing mitochondria by fluorescence microscopy. Many fluorescent dyes that had been developed for monitoring plasma-membrane potential have turned out to stain mitochondria similarly. Fluorescence staining of mitochondria produced images of much higher contrast than available with transmitted light microscopy, and it was also realized that they could be used for monitoring mitochondrial membrane potential. Some mitochondrial dyes have been reported to selectively kill cultured cancer cells that retain them longer (Bernal et al., 1982). Molecular Probes has generated the greatest number of new mitochondrial probes in recent years.

Critical Parameters

Endoplasmic reticulum

The best way to fluorescently label the ER is probably to express a GFP chimera targeted to the ER (Hampton et al., 1996; Terasaki et al., 1996). However, it may be difficult to express GFP constructs in a given cell, or the fluorescence may not be bright enough. $DiOC_6(3)$ is an older method for staining the ER, but is still useful because of the ease of use and brightness of staining. Its great weakness is the lack of specificity of staining; $DiOC_6(3)$ will stain all of the ER but will stain other organelles as well.

It must be stressed that $DiOC_6(3)$ does not stain the ER specifically. $DiOC_6(3)$ is a positively charged lipophilic molecule that permeates through the plasma membrane. At low concentrations, it accumulates in mitochondria due to the effect of the large negative mitochondrial membrane potential on the positively charged dye. At higher concentrations, the dye stains other membranes, including the ER. The mitochondria are swollen at these higher concentrations, suggesting that they are no longer concentrating dye, and that the dye is free to associate with other membranes. In fixed cells, the dye stains ER, mitochondria, and large vesicles even at the lowest concentrations. The simplest interpretation is that the dyes are associating with all intracellular membranes. Some of the membranes may be so small that the amount of dye contained in them is not visible. Since $DiOC_6(3)$ probably stains all membranes, it is only useful as an ER stain when the ER pattern can be distinguished from the other membranes. The thin peripheral region of many cultured cells has often been used, because in that region the ER is a single-layered network that is easily identified.

Other dyes with the same staining properties as $DiOC_6(3)$ include rhodamine 6G, $DiOC_6(5)$, $DiIC_6(3)$, and $DiSC_6(3)$ (all screened in Terasaki et al., 1984), as well as hexyl ester rhodamine B (Terasaki and Reese, 1992). All of these are lipophilic cationic dyes that stain only mitochondria at low doses in living cells. If it is desired to observe ER staining with a rhodamine filter set, hexyl ester rhodamine B (Molecular Probes R-648, also known as "R6") may be the best dye to try first.

The quality of the images obtained with $DiOC_6(3)$ is generally much better than that obtained with immunofluorescence staining. One reason is that the glutaraldehyde used for $DiOC_6(3)$ staining is a superior fixative that is usually not used for immunofluorescence because of the autofluorescence that develops with time. $DiOC_6(3)$ methods use lower glutaraldehyde concentrations and images are observed before autofluorescence develops. Another reason is that immunofluorescence requires permeabilization of the cells with detergents or organic solvents. These treatments generally disrupt the delicate membranes of the ER, so that they are no longer continuous, or are so disfigured that the network pattern is no longer discernible. Immuno-

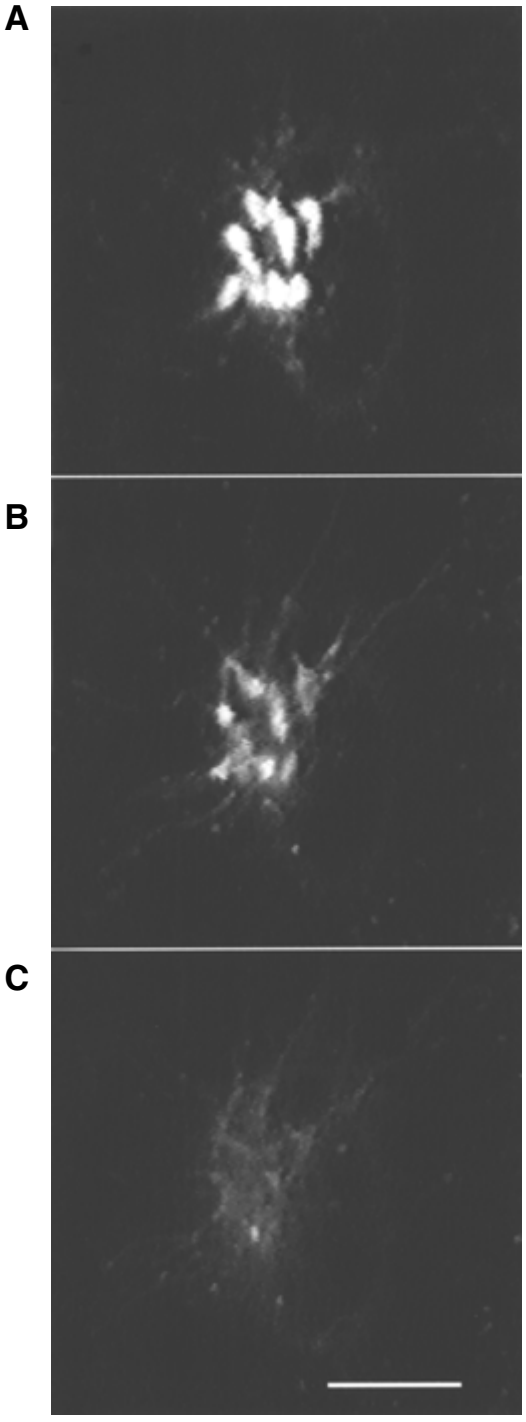


Figure 4.4.2 BODIPY-Cer labeling of Golgi membranes to study the effects of brefeldin A (BFA). Golgi membranes were labeled with BODIPY-Cer and then treated with brefeldin A before imaging at 37°C on a cooled CCD microscope system. Note that Golgi membranes tubulated in response to the drug and then dispersed (Sciaky et al., 1997). (**A**) Image collected immediately after addition of BFA; (**B**) image collected 5 min later; and (**C**) image collected 10 min after addition of drug. Bar is equal to 10 μm.

fluorescence has the advantage, however, of labeling specific molecules in the ER. Immunofluorescence can also show compartmentalization in the ER, and is also much better suited than $DiOC_6(3)$ for screening the results of multiple experimental variations, because of the short period during which the dye staining is optimal.

$DiOC_6(3)$ is well suited for observations in living cells. One drawback is that the mitochondria are swollen at dye concentrations required to stain the ER, so that there is a possibility that the cells are not behaving normally. Nevertheless, $DiOC_6(3)$ has been used successfully for observing ER dynamics at video rates (Lee and Chen, 1988), in time-lapse (Dailey and Bridgman, 1989; Terasaki and Reese, 1994), and during mitosis (Waterman-Storer et al., 1993). It is particularly good for observing ER movements during cytoplasmic streaming in plant cells (Allen and Brown, 1988; Knebel et al., 1990; McCauley and Hepler, 1990). When using the fluorescent dyes on a new organism, it is important to optimize the staining conditions. Koning et al. (1993) give a good description of how the cell chamber and optical conditions were optimized for yeast. For some discussion of other cell preparations and of other aspects of this method, see earlier reviews (Terasaki, 1989, 1993, 1998). For live-cell imaging by confocal microscopy, see Terasaki and Dailey (1995).

Golgi complex

Attention to several critical parameters is important for ensuring the success of C_6-NBD- and BODIPY-Cer labeling of cells. These parameters include the amount of fluorescent lipid added to cells, the length of time for incorporation of the lipid into cells, and the method of imaging cells. Each of these parameters needs to be optimized to ensure that Golgi labeling is bright and specific, and that cells are not damaged during the labeling or imaging process.

Although incubation of cells with medium containing 5 to 10 μM fluorescent Cer-derivative for 30 min at 4°C, washing, and warming to 37°C for 30 min is recommended, the optimal conditions for Golgi labeling vary depending on cell type and therefore need to be tested. The level of brightness of Golgi labeling also depends on the purpose of the experiment. If cells are to be used for time-lapse imaging of the Golgi complex, for example, a brighter signal than normal is recommended since the fluorescent lipid is quickly photobleached during repetitive imaging. To increase levels of Golgi labeling, simply incubate cells with a higher concentration of fluorescent-ceramide solution, and vice-versa for lower levels of Golgi labeling.

Higher levels of Golgi labeling are not always desirable. Labeling of non-Golgi membranes also usually increases under conditions where Golgi labeling increases. In addition, if the Golgi complex is too bright, images will be saturated (with low signal-to-noise ratios) and the risk of photobleaching damage upon imaging is greater.

For time-lapse imaging studies, BODIPY-Cer is preferable to C_6-NBD-Cer because it has a slower rate of photobleaching. Even BODIPY-Cer photobleaches quickly relative to other fluorophores (e.g., rhodamine) so imaging with a low-light detection system is essential. To reduce photobleaching of fluorescent ceramide derivatives in time-lapse studies, minimal exposure of the specimen to bleaching radiation is essential. Control of the exposure time during imaging is critical in these types of experiments. This can be done by using a computer-controlled shutter to control exposure time and by using neutral-density filters during illumination to reduce the light reaching the specimen. Imaging with low light intensity through the use of neutral-density filters will lead to significantly less photobleaching. This is essential for time-lapse studies of C_6NBD- or BODIPY-Cer labeled cells.

Photobleaching results in permanent inactivation of the fluorophore due to its interaction with neighboring molecules. This frequently involves the generation of free radicals that can damage the cell. In time-lapse imaging studies where the specimen becomes photobleached due to repetitive imaging, it is essential to monitor the overall appearance of the cell to assess potential light-induced damage to the cell. A typical response by cells that are being damaged by photobleaching radiation is for them to round up and then eventually die.

The process of finding a labeled cell and focusing it can cause extensive photobleaching if performed without neutral-density filters. A useful approach is to find and focus cells in phase optics before imaging the specimen with fluorescent light. Cells that are fixed after staining for double-label studies should be immediately processed using as little light exposure as possible. The fixed cells should be imaged immediately when they are ready, since they do not store for very long periods. If double labeling with an antibody is being performed after fixa-

Table 4.4.1 Troubleshooting Guide for Fluorescent Staining of Organelles

Problem	Possible causes and/or solution(s)
Endoplasmic reticulum	
Staining pattern is too dense to see a network	This means that the the staining of the ER and/or other organelles is too dense relative to the resolution of light microscopy. If ER membranes are separated from each other or from other organelles by <0.5 μm or so, it will be difficult to resolve them by light microscopy. If the ER has a three-dimensional organization, the overlapping membranes compound the problem. Sometimes, using a 100× lens or a confocal microscope can help. It may be necessary to use another cell type or to resort to other means to localize the ER, such as electron microscopy.
Vesiculation of ER network	One possible cause is poor fixation. Fixation with formaldehyde often causes vesiculation of the ER network. If glutaraldehyde is being used, it may help to try a fresh stock solution. Another cause of vesiculation is the fixation buffer. Make sure that the tonicity and pH are physiological. For fixed cells, it may help to replace PBS with another buffer (100 mM sucrose/100 mM sodium cacodylate, pH 7.4). Another cause of vesiculation is drying of the cells during the processing. It is important to keep the cells hydrated at all times.
Smeared-out network	This can be caused by organic solvents or detergents that extract the membranes and the dye staining. Do not use nail polish to seal coverslips to microscope slides since the organic solvents in the nail polish will have this same effect.
Out-of-focus, distorted network	This is usually a problem of the microscope optics. Make sure that there is no water in the immersion oil on the coverslip. Also check for dried material, broken pieces of coverslip, and other debris on the objective lens.
No network visible	In one cell type, fish chromatophores, $DiOC_6(3)$ did not stain the ER and gave a diffuse background staining (M. McNiven, pers. comm.). This was surprising, since preparations from many higher vertebrates, as well as many invertebrates and unicellular organisms, have been successfully stained with $DiOC_6(3)$. Perhaps the lipid composition of the ER in the fish chromatophores somehow prevents the partitioning of $DiOC_6(3)$ into the membrane bilayer.
Golgi complex	
Background labeling with fluorescent lipid is too high	Use lower concentrations of fluorescent lipid (<5 μM) during initial labeling of cells.
There is no specific labeling	Check stock solutions of fluorescent lipids to see if they were properly prepared and are not too old. Check to see if incubation with fluorescent lipid solution for longer times at 4°C and/or 37°C result in specific labeling.
Nonspecific labeling is too high	Reduce concentration of fluorescent lipid added to cells. Wait for longer times after shift to 37°C before viewing cells. Check to make sure cells are not autofluorescent (i.e., whether unlabeled cells show the same non-specific labeling). Consider labeling with BODIPY-Cer and viewing cells in rhodamine optics so that fluorescence from the BODIPY-Cer excimers is only being imaged.
Specific staining is too dim	Add higher concentrations of fluorescent lipid to cells and incubate at 4°C for longer periods.

continued

Table 4.4.1 Troubleshooting Guide for Fluorescent Staining of Organelles, continued

Problem	Possible causes and/or solution(s)
Cells round up and appear unhealthy during imaging	Use attenuated light to image cells. Image for only short time periods. Label cells with lower concentrations of fluorescent lipid.
Fluorescence is lost too quickly because of photobleaching	Find cells and focus them using phase optics to reduce the extent of photobleaching prior to imaging. Use attenuated light to image cells and image for as short a time as possible. Imaging systems that detect low light levels are useful since photobleaching is reduced when specimens are imaged with less light. Use BODIPY-Cer instead of NBD-Cer since the former photobleaches less rapidly than the latter.
Mitochondria	
Dim labeling	This probably means that the dye staining concentration or incubation period needs to be increased. If this does not help, perhaps this is due to a very low mitochondrial membrane potential. This in turn may be due to poor health of cells.
Swollen mitochondria; labeling of ER (fine reticular network throughout cell) in addition to mitochondria; cell death	These are indications that there is too much dye in the cells. The solution is to decrease dye staining concentration.

tion of the C_6-NBD-Cer or BODIPY-Cer labeled cells, it is crucial to check that there is minimal cross-over of the fluorescent Cer into the channel used for revealing the fluorescent antibody staining. BODIPY-Cer labeled cells usually cannot be be used in double-labeling experiments, since BODIPY-Cer emits light at multiple wavelengths due to excimer formation.

Mitochondria

Since the first report of fluorescent dye staining of mitochondria (Johnson et al., 1980), many dyes have been found that have similar properties. Generally, these dyes are all small, lipophilic cationic molecules. The lipophilicity allows them to cross the plasma membrane, and the positive charge causes them to be accumulated in the mitochondria, which have a high negative membrane potential.

Rhodamine 123, the first fluorescent dye for mitochondria (Johnson et al., 1980), is used to stain cells at 10 μg/ml for 10 min followed by a brief wash and mounting in dye-free growth medium (Chen, 1989). The stock solution used for this dye is 1 mg/ml in water. Because the staining is observed in dye-free medium, mitochondrial staining eventually decreases due to leakage from the cells. The leakage rate is highly dependent on cell type. In some cases, the dye is retained for over an hour, but in others, it leaks out within 10 min.

Several rhodamine and dicarbocyanine dyes have similar staining properties to those of rhodamine 123, although rhodamine 123 may be less toxic (Chen, 1989). Two examples are rhodamine 6G and the dicarbocyanine dye $DiOC_6(3)$. The staining concentrations are lower for several of these dyes, and some of them can be left in the medium because they have a relatively low background. Other reasons for using one of these other dyes are brightness, slower bleaching rate, or convenience of fluorescence spectrum. Rhodamine 123 has an advantage in that it does not stain the ER at high doses, whereas most of the other dyes do. The dye described in Basic Protocol 3, TMRE, is well documented for quantitative measurements of the mitochondrial membrane potential (Loew et al., 1993).

Several other mitochondrial dyes are available for studying mitochondrial properties in interesting ways. The MitoTracker series of dyes can be fixed in place once they have been stained (Poot et al., 1996; Poot, 1998). JC-1 dyes change fluorescence after they form "J aggregates" which is potential dependent (Smiley et al., 1991). The CM-H2TMRos dye is nonfluorescent until it becomes oxidized in

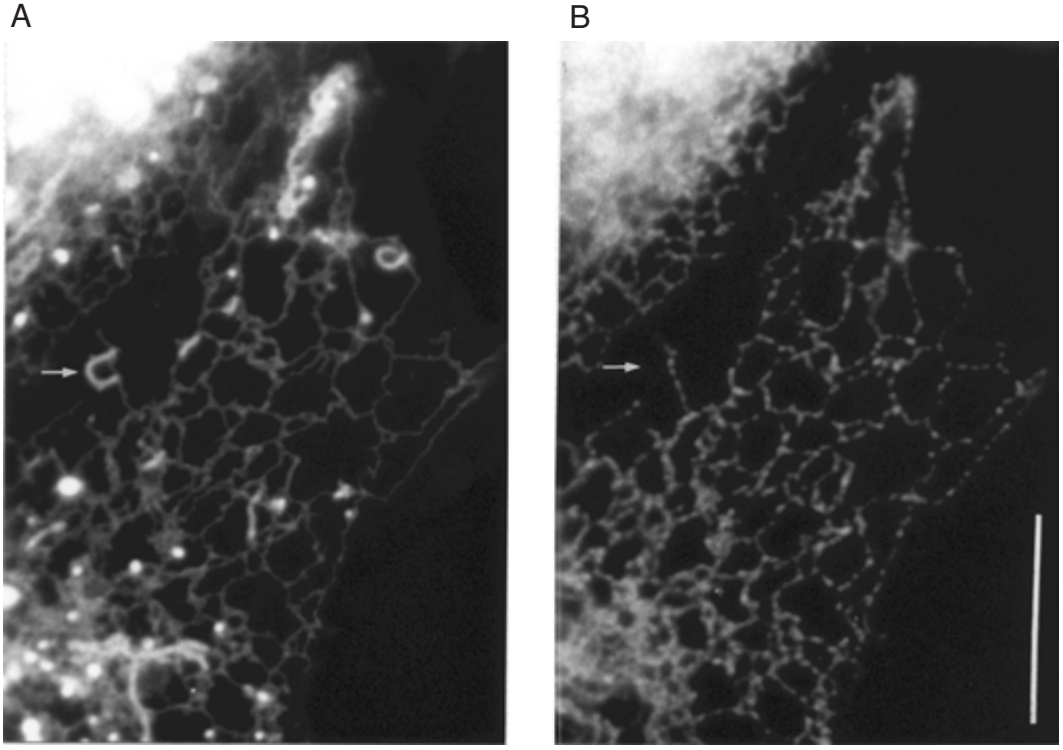


Figure 4.4.3 CV-1 cell double-labeled with (**A**) $DiOC_6(3)$ and (**B**) immunofluorescent staining of BiP, a protein in the ER. Bar is equal to 10 μm.

the mitochondria. Nonyl acridine orange is thought to bind to cardiolipin rather than respond to membrane potential. A good place to get a more detailed overview of the different kinds of mitochondrial stains is the Molecular Probes catalog (Haugland, 1996).

Troubleshooting

Table 4.4.1 lists some problems that may be encountered in fluorescent staining the ER, Golgi complex, and mitochondria along with some possible causes and solutions.

Anticipated Results

Endoplasmic reticulum

The ER is best seen in the thin peripheral region of cultured cells, where it has a two-dimensional distribution. In some cell types, this peripheral region is very narrow or nonexistent, in which case $DiOC_6(3)$ will probably not be of use. The ER pattern differs considerably from cell type to cell type, and also from cell to cell in a population. It is usually a network of tubes, but can also contain sheets or membrane cisternae. The overall pattern often seems to mirror underlying stresses or morphogenetic processes of the cytoplasm, and can be anything from a closely spaced isotropic meshwork to a highly unrestrained pattern (seen more often in primary cultures).

Figure 4.4.3 shows a CV-1 cell (from African green monkey kidney), double-labeled with $DiOC_6(3)$ (panel A) and immunofluorescence of BiP (panel B), a protein in the ER. Note that $DiOC_6(3)$ stains a continuous network as well as mitochondria (arrow). Anti-BiP labels a vesiculated network, but does not label the mitochondria. The cells were first fixed lightly with glutaraldehyde (0.025% for 5 min), stained with $DiOC_6(3)$, photographed, then permeabilized with −20°C methanol and processed for immunofluorescence (Terasaki and Reese, 1992). The vesiculated network seen in immunofluorescence was caused by the methanol permeabilization.

Golgi complex

Once conditions for specific labeling of the Golgi complex have been optimized, the localization and dynamics of the Golgi complex can be studied. Golgi membranes usually appear as a ribbon-like structure near the microtubule organizing center adjacent to the nucleus (Rogalski and Singer, 1984; Cole et al., 1996). Frequently, thin tubule processes interconnect the larger Golgi structures (Cooper et al., 1990). Because the normal distribution of Golgi mem-

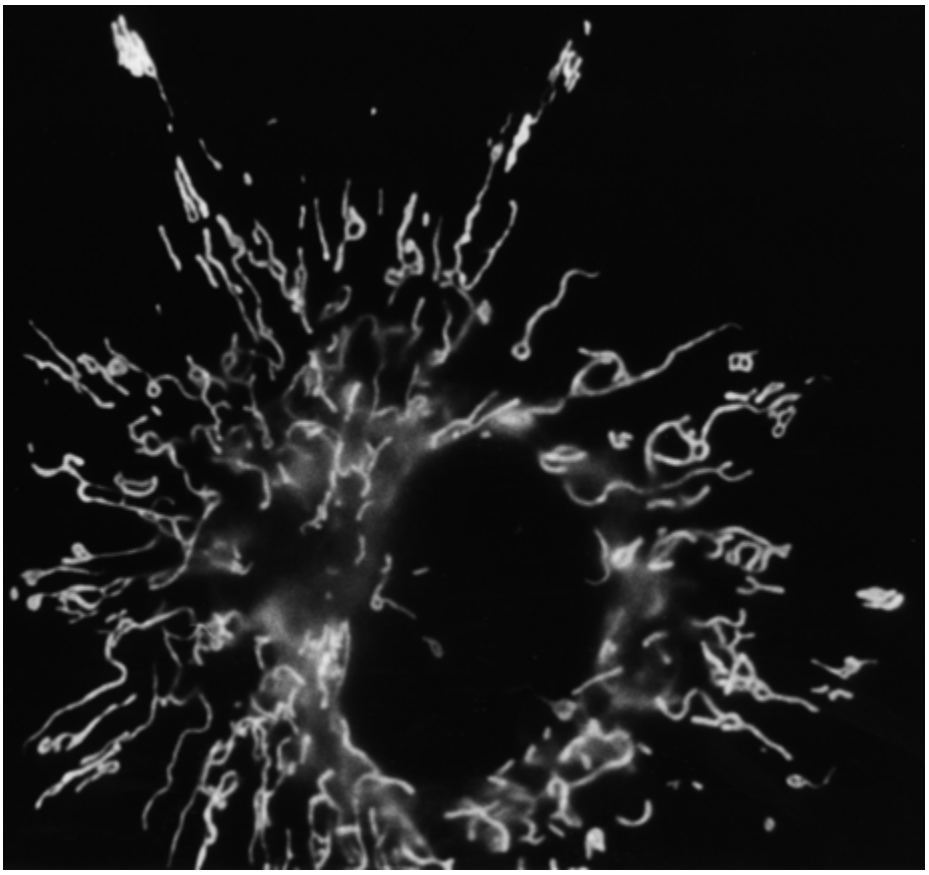


Figure 4.4.4 Labeling of the mitochondria in a CV-1 cell.

branes within cells represents a dynamic steady state of forward and recycling pathways connecting the Golgi and ER, any condition that perturbs forward traffic into the Golgi complex without a corresponding effect on recyling will result in redistribution of Golgi material to the site of perturbation (Cole et al., 1996). One example where the Golgi complex undergoes such reorganization is during microtubule disruption, which causes extensive fragmentation of the Golgi into hundreds of elements scattered throughout the cell (Turner and Tartakoff, 1989). Microtubules are known to serve as tracks for delivery of ER-derived transport intermediates into the centrosomal Golgi region (Presley et al., 1997; Scales et al., 1997) and are not as important for Golgi-to-ER traffic (Cole et al., 1996), therefore when net forward transport into the Golgi is blocked during microtubule depolymerization, Golgi proteins accumulate at ER exit sites and regenerate short Golgi stacks at these sites (Cole et al., 1996).

Mitochondria

Labeling of the mitochondria in a CV-1 cell (African green monkey kidney) is shown in Figure 4.4.4. The appearance of the mitochondria can be very variable. In some cell lines, they are short, in others very long. Mitochondria are often aligned with cytoskeletal elements (Summerhayes et al., 1983).

Time Considerations

This is a rapid staining procedure once all the equipment and materials are in place. Equilibration time for staining is ~10 min.

The time required for making the chamber and getting the microscope set should be 5 to 10 min. Staining the ER with $DiOC_6(3)$ is very rapid as compared to immunofluorescence. A certain amount of preparation is required, but once everything is in place, fixation, staining and microscopy can be done in 20 min or so.

The entire labeling procedure for the Golgi complex can be performed in ~1 hr. Although samples can be subsequently fixed and used for double-labeling studies with antibodies, they usually cannot be stored for very long (due to signal loss from photobleaching), so immediate use in imaging studies is recommended.

Once the equipment and reagents are prepared, mitochondrial staining is rapid.

Literature Cited

Allen, N.S. and Brown, D.T. 1988. Dynamics of the endoplasmic reticulum in living onion epidermal cells in relation to microtubules, microfilaments and intracellular particle movement. *Cell Motil. Cytoskeleton* 10:153-163.

Bernal, S.D., Lampidis, T.J., Summerhayes, I.C., and Chen, L.B. 1982. Rhodamine-123 selectively reduces clonal growth of carcinoma cells in vitro. *Science* 218:1117-1119.

Chen, L.B. 1989. Fluorescent labeling of mitochondria. *Methods Cell Biol.* 29:103-123.

Cohen, L.B., Salzberg, B.M., Davila, H.V., Ross, W.N., Landowne, D., Waggoner, A.S., and Wang, C.H. 1974. Changes in axon fluorescence during activity: Molecular probes of membrane potential. *J. Membr. Biol.* 19:1-36.

Cole, N.B., Sciaky, N., Marotta, A., Song, J., and Lippincott-Schwartz, J. 1996. Golgi dispersal during microtubule disruption: Regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. *Mol. Biol. Cell* 7:631-650.

Cooper, M.S., Cornell-Bell, A.H., Chernjavsky, A., Dani, J.W., and Smith, S.J. 1990. Tubulovesicular processes emerge from the trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans-Golgi elements into a reticulum. *Cell* 61:135-145.

Dailey, M.E. and Bridgman, P.C. 1989. Dynamics of the endoplasmic reticulum and other membranous organelles in growth cones of cultured neurons. *J. Neurosci.* 9:1897-1909.

Dailey, M.E. and Bridgman, P.C. 1991. Structure and organization of membrane organelles along distal microtubule segments in growth cones. *J. Neurosci. Res.* 30:242-258.

Ehrenberg, B., Montana, V., Wei, M.D., Wuskell, J.P., and Loew, L.M. 1988. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys. J.* 53:785-794.

Farkas, D.L., Wei, M.D., Febbroriello, P., Carson, J.H., and Loew, L.M. 1989. Simultaneous imaging of cell and mitochondrial membrane potentials. *Biophys. J.* 56:1053-1069.

Hampton, R.Y., Koning, A., Wright, R., and Rine, J. 1996. In vivo examination of membrane protein localization and degradation with green fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 93:828-833.

Haugland, R.P. 1996. Handbook of Fluorescent Probes and Research Chemicals, 6th ed. pp. 266-274. Molecular Probes, Eugene, Ore.

Johnson, L.V., Walsh, M.L., and Chen, L.B. 1980. Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. U.S.A.* 77:990-994.

Kishimoto, Y. 1975. A facile synthesis of ceramides. *Chem. Phys. Lipids* 15:33-36.

Knebel, W., Quader, H., and Schnepf, E. 1990. Mobile and immobile endoplasmic reticulum in onion bulb epidermis cells: Short- and long-term observations with a laser confocal laser scanning microscope. *Eur. J. Cell Biol.* 52:328-340.

Kobayashi, T. and Pagano, R.E. 1989. Lipid transport during mitosis: Alternative pathways for delivery of newly synthesized lipids to the cell surface. *J. Biol. Chem.* 264:5966-5973.

Koning, A.J., Lum, P.K., Williams, J.M., and Wright, R. 1993. DiOC6 staining reveals organelle structure and dynamics in living yeast cells. *Cell Motil. Cytoskeleton* 25:111-128.

Lee, C. and Chen, L.B. 1988. Dynamic behavior of endoplasmic reticulum in living cells. *Cell* 54:37-46.

Lipsky, N.G. and Pagano, R.E. 1983. Sphingolipid metabolism in cultured fibroblasts: Microscopic and biochemical studies employing a fluorescent analogue of ceramide. *Proc. Natl. Acad. Sci. U.S.A.* 80:2608-2612.

Lipsky, N.G. and Pagano, R.E. 1985a. A vital stain for the Golgi apparatus. *Science* 228:745-747.

Lipsky, N.G. and Pagano, R.E. 1985b. Intracellular translocation of fluorescent sphingolipids in cultured fibroblasts: Endogenously synthesized sphingomyelin and glucocerebroside analogs pass through the Golgi apparatus *en route* to the plasma membrane. *J. Cell Biol.* 100:27-34.

Loew, L.M. 1998. Measuring membrane potential in single cells with confocal microscopy. *In* Cell Biology: A Laboratory Handbook, 2nd ed. (J. Celis, ed.) vol. 3, pp. 375-379. Academic Press, Orlando, Fla.

Loew, L.M., Tuft, R.A., Carrington, W., and Fay, F.S. 1993. Imaging in five dimensions: Time-dependent membrane potentials in individual mitochondria. *Biophys. J.* 65:2396-2407.

McCauley, M.M. and Hepler, P.K. 1990. Visualization of the endoplasmic reticulum in living buds and branches of the moss *Funaria hygrometrica* by confocal laser scanning microscopy. *Development* 109:753-764.

Mellman, I. and Simons, K. 1992. The Golgi complex: In vitro veritas? *Cell* 68:829-840.

Pagano, R.E. 1989. A fluorescent derivative of ceramide: Physical properties and use in studying the Golgi apparatus of animal cells. *Methods Cell Biol.* 29:75-85.

Pagano, R.E. and Martin, O.C. 1998. Use of fluorescent analogs of ceramide to study the Golgi apparatus of animal cells. *In* Cell Biology: A Laboratory Handbook, 2nd ed. (J. Celis, ed.) vol. 2, pp. 507-512. Academic Press, Orlando, Fla.

Pagano, R.D., Sepanski, M.A., and Martin, O.C. 1989. Molecular trapping of a fluorescent ceramide analog at the Golgi apparatus of fixed cells: Interaction with endogenous lipids provides a trans-Golgi marker for both light and electron microscopy. *J. Cell Biol.* 109:2067-2079.

Pagano, R.E., Martin, O.C., Kang, H.C., and Haugland, R.P. 1991. A novel fluorescent ceramide analog for studying membrane traffic in animal cells: Accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J. Cell Biol.* 113:1267-1279.

Poot, M. 1998. Staining of mitochondria. *In* Cell Biology: A Laboratory Handbook, 2nd ed. (J. Celis, ed.) vol. 2, pp. 513-517. Orlando, Fla.

Poot, M., Zhang, Y.-Z., Kramer, J., Wells, K.S., Jones, L.J., Hanzel, D.K., Lugade, A.G., Singer, V.L., and Haugland, R.P. 1996. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J. Histochem. Cytochem.* 44:1363-1372.

Porter, K.R. 1953. Observations on a submicroscopic basophilic component of the cytoplasm. *J. Exp. Med.* 97:727-750.

Porter, K.R., Claude, A., and Fullam, E. 1945. A study of tissue culture cells by electron microscopy. *J. Exp. Med.* 81:233-241.

Presley, J.F., Cole, N.B., Schroer, T.A., and Lippincott-Schwartz, J. 1997. ER-to-Golgi transport visualized in living cells. *Nature* 389:81-85.

Rogalski, A.A. and Singer, S.J. 1984. Associations of elements of the Golgi apparatus with microtubules. *J Cell Biol.* 99:1092-1100.

Rosenwald, A.G. and Pagano, R.E. 1993. Intracellular transport of ceramide and its metabolites at the Golgi complex: Insights from short-chain ceramides. *Adv. Lipid Res.* 26:101-118.

Scales, S.J., Pepperkok, R., and Kreis, T.E. 1997. Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell* 90:1137-1148.

Schwarzmann, G. and Sandhoff, K. 1987. Lysogangliosides: Synthesis and use in preparing labeled gangliosides. *Methods Enzymol.* 138:319-341.

Sciaky, N., Presley, J., Smith, C., Zaal, K.J.M., Cole, N., Moreira, J.E., Terasaki, M., Siggia, E., and Lippincott-Schwartz, J. 1997. Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J. Cell Biol.* 139:1137-1156.

Sims, P.J., Waggoner, A.S., Wang, C.H., and Hoffman, J.F. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry* 13:3315-3330.

Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D. Jr., and Chen, L.B. 1991. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc. Natl. Acad. Sci. U.S.A.* 88:3671-3675.

Summerhayes, I.C., Wong, D., and Chen L.B. 1983. Effect of microtubules and intermediate filaments on mitochondrial distribution. *J. Cell Sci.* 1:87-105.

Terasaki, M. 1989. Fluorescent labeling of endoplasmic reticulum. *Methods Cell Biol.* 29:125-135.

Terasaki, M. 1993. Probes for endoplasmic reticulum. *In* Fluorescent Probes of Living Cells: A Practical Manual. (W.T. Mason, ed.) pp. 120-123. Academic Press, London.

Terasaki, M. 1998. Labeling of endoplasmic reticulum with $DiOC_6(3)$. *In* Cell Biology: A Laboratory Handbook, 2nd ed. (J. Celis, ed.) vol. 2, pp. 501-506. Academic Press, Orlando, Fla.

Terasaki, M. and Dailey, M.E. 1995. Confocal microscopy of living cells. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley ed.) pp. 327-246. Plenum, New York.

Terasaki, M. and Jaffe, L.A. 1993. Imaging endoplasmic reticulum in living sea urchin eggs. *Methods Cell Biol.* 38:211-220.

Terasaki, M. and Reese, T.S. 1992. Characterization of endoplasmic reticulum by co-localization of BiP and dicarbocyanine dyes. *J. Cell Sci.* 101:315-322.

Terasaki, M. and Reese, T.S. 1994. Interactions among endoplasmic reticulum, microtubules and retrograde movement of the cell surface. *Cell Motil. Cytoskeleton* 29:291-300.

Terasaki, M., Song, J., Wong, J.R., Weiss, M.J., and Chen, L.B. 1984. Localization of endoplasmic reticulum in living and glutaraldehyde fixed cells with fluorescent dyes. *Cell* 38:101-108.

Terasaki, M., Chen, L.B., and Fujiwara, K. 1986. Microtubules and the endoplasmic reticulum are highly interdependent structures. *J. Cell Biol.* 103:1557-1568.

Terasaki, M., Henson, J., Begg, D., Kaminer, B., and Sardet, C. 1991. Characterization of sea urchin egg endoplasmic reticulum in cortical preparations. *Dev. Biol.* 148:398-401.

Terasaki, M., Jaffe, L.A., Hunnicutt, G.R., Hammer, J.A. III. 1996. Structural change of the endoplasmic reticulum during fertilization: Evidence for loss of membrane continuity using the green fluorescent protein. *Dev. Biol.* 179:320-328.

Turner, J.R. and Tartakoff, A.M. 1989. The response of the Golgi complex to microtubule alterations: The roles of metabolic energy and membrane traffic in Golgi complex organization. *J. Cell Biol.* 109:2081-2088.

van't Hof, W. and van Meer, G. 1990. Generation of lipid polarity in intestinal epithelial (Caco-2) cells: Sphingolipid synthesis in the Golgi complex and sorting before vesicular traffic to the plasma membrane. *J. Cell Biol.* 111:977-986.

van Meer, G., Stelzer, E.H.K., Wijnaendts-van-Resandt, W., and Simons, K. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* 105:1623-1635.

Waterman-Storer, C.M., Sanger, J.W., and Sanger, J.M. 1993. Dynamics of organelles in the mitotic spindles of living cells: Membrane and microtubule interactions. *Cell Motil. Cytoskeleton* 26:19-39.

Contributed by Mark Terasaki (ER and mitochondria) and Leslie Loew (mitochondria)
University of Connecticut Health Center
Farmington, Connecticut

Jennifer Lippincott-Schwartz and Kristien Zaal (Golgi complex)
National Institute of Child Health & Human Development/NIH
Bethesda, Maryland

Basic Confocal Microscopy

Confocal microscopy produces sharp images of structures within relatively thick specimens (up to several hundred microns). It is particularly useful for examining fluorescent specimens. Thick fluorescent specimens viewed with a conventional widefield fluorescent microscope appear blurry and lack contrast because fluorophores throughout the entire depth of the specimen are illuminated, and fluorescence signals are collected not only from the plane of focus but also from areas above and below. Confocal microscopes selectively collect light from thin (~1-μm) optical sections representing single focal planes within the specimen. Structures within the focal plane appear more sharply defined than they would with a conventional microscope because there is essentially no flare of light from out-of-focus areas. A three-dimensional view of the specimen can be reconstructed from a series of optical sections at different depths.

The confocal microscope is the instrument of choice for examining fluorescence-stained cells in tissue slices or small, intact organisms such as *Drosophila* (Fig. 4.5.1A,B) and zebrafish embryos. It is also useful for localizing fluorescent-tagged molecules in dissociated cells (Fig. 4.5.1C,D). Its sensitivity even allows fluorescence in living specimens to be monitored, making it feasible to follow the movements in living cells of fluorescent probes such as the green fluorescent protein (GFP; Fig. 4.5.1D). In addition, some types of confocal microscopes can be configured to perform photobleach experiments (Fig. 4.5.1D) and to photoactivate "caged" molecules (molecules that are inactive until released with UV illumination).

Biologists use confocal microscopy in a number of creative ways that are beyond the scope of this article. The information presented herein is intended to provide background and practical tips needed to get started with confocal microscopy. An excellent source of theoretical and technical information is the *Handbook of Biological Confocal Microscopy* (1995; edited by J. Pawley). Also recommended are *Cell Biological Applications of Confocal Microscopy* (1993; edited by B. Matsumoto), a good source of practical information; *Confocal Microscopy* (1990; edited by T. Wilson), for theoretical background; and *Video Microscopy* (1997; Inoué and Spring) for fundamentals of microscopy.

BASIS OF OPTICAL SECTIONING

Confocal microscopes accomplish optical sectioning by scanning the specimen with a focused beam of light and collecting the fluorescence signal from each spot via a spatial filter (generally a pinhole aperture) that blocks signals from out-of-focus areas of the specimen. The physical basis of optical sectioning in fluorescence confocal microscopy is illustrated in Figure 4.5.2. A point light source (typically a laser) evenly illuminates the back focal plane of the objective, which focuses the light to a diffraction-limited spot in the specimen. The irradiation is most intense at the focal spot, although areas of the specimen above and below the focal spot are also illuminated. Fluorescent molecules excited by the incident light emit fluorescence in all directions. The fluorescence collected by the objective comes to focus in the image plane, which is conjugate (confocal) with the focal plane in the specimen. A pinhole aperture in the image plane allows fluorescence from the illuminated spot in the specimen to pass to the detector but blocks light from out-of-focus areas.

The diameter of the pinhole determines how much of the fluorescence emitted by the illuminated spot in the specimen is detected, and the thickness of the optical section. From wave optics we know that a point light source in the plane of focus of an objective produces a three-dimensional diffraction pattern in the image plane. The cross section at the image plane is an Airy disk, a circular diffraction pattern with a bright central region. The radius of the bright central region of the Airy disk in the reference frame of the specimen is given by

$$R_{\text{Airy}} = 0.61\lambda/\text{NA}$$

where λ is the emission wavelength and NA is the numerical aperture of the objective (see *UNIT 4.1* for a discussion of NA). At the image plane (the location of the pinhole aperture), the radius of the central region is R_{Airy} multiplied by the magnification at that plane (for a more complete explanation see Wilson, 1995).

Adjustment of the pinhole to a diameter slightly less than the diameter of the central region of the Airy disk allows most of the light from the focal point to reach the detector and reduces the background from out-of-focus areas by ~1000-fold relative to widefield microscopy (Sandison et al., 1995). The separation of the in-focus signal from the out-of-focus back-

Contributed by Carolyn L. Smith

Current Protocols in Cell Biology (1999) 4.5.1-4.5.12

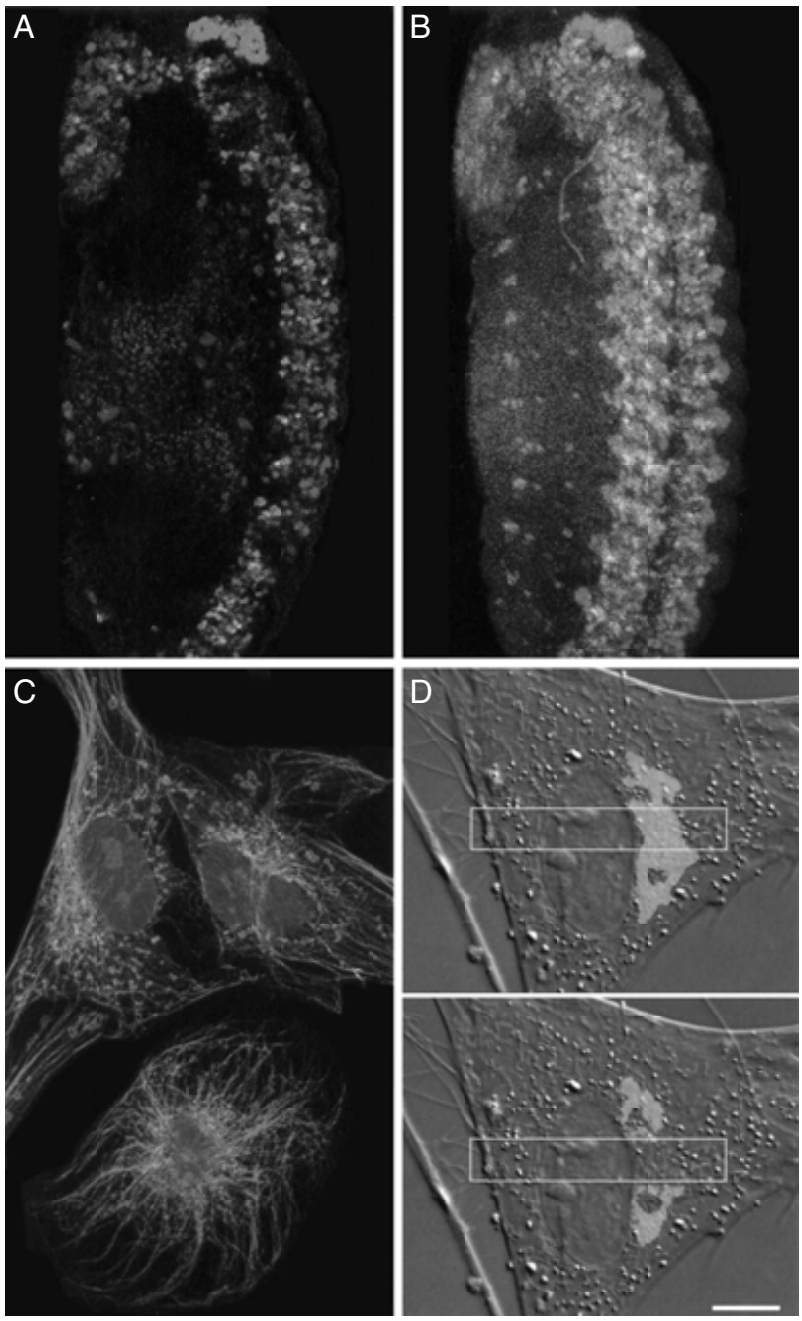


Figure 4.5.1 Applications of laser scanning microscopy. Top images (from W. Odenwald; see Kamabadur et al., 1998) show three-dimensional analysis of thick specimens. Different neuronal populations of an ~250-μm-thick *Drosophila* embryo were immunolabeled with antibodies against three transcription factors. (**A**) Approximately 2.5-μm optical section collected with 25×, 0.8-NA objective using a detector pinhole diameter of ~1.3 Airy units. Labeled neurons in the plane of focus appear sharply defined, while those outside it are not visualized. (**B**) Projection (superimposition) of 65 optical sections collected at 2-μm intervals in the *z* axis. Neurons at different focal planes appear to overlap in this flattened image, but are distinct in a 3-D reconstruction. (**C**) Localization of intracellular structures. Dissociated rat fibroblasts were immunolabeled with anti-tubulin antibodies to visualize microtubules (green) and stained with fluorescent probes for mitochondria (Mitotracker, red) and DNA (DAPI, blue). The image is a projection of 20 optical sections collected at 0.3-μm intervals in the *z* axis with 100×, 1.4-NA objective. (**D**) Measuring molecular motility. In a living fibroblast expressing a Golgi membrane protein (galactosyltransferase) fused to GFP (S65T-GFP), GFP fluorescence (green) localized to the Golgi complex, shown superimposed on a DIC image of the cell. After the first image was collected, the boxed region (yellow) was scanned with full laser power; this photobleached the GFP in the boxed area as shown in the second image collected ~2 sec later. The rate of fluorescence recovery into the photobleached zone (not illustrated) indicated that the GFP-galactosyltransferase fusion is highly mobile in Golgi membranes. **See color figure.**

ground achieved by a properly adjusted pinhole is the principle advantage of confocal microscopy for examination of thick specimens (see Fig. 4.5.1A,B).

Point illumination and the presence of a pinhole in the detection light path also produce improved lateral and axial resolution relative to conventional microscopy (Table 4.5.1). The actual extent of improvement depends on the size of the pinhole. Near-maximal axial resolution is obtained with a pinhole radius ~$0.7 \times R_{Airy}$ whereas optimal lateral resolution is obtained with a pinhole smaller than $0.3 \times R_{Airy}$ (Wilson, 1995). However, a pinhole smaller than ~$0.7 \times R_{Airy}$ significantly reduces the total signal, a sacrifice that may not be worth the gain in resolution, especially when imaging dim samples. In fluorescence imaging, resolution also is influenced by the emission and excitation wavelengths (Table 4.5.1).

TYPES OF CONFOCAL MICROSCOPES

Several types of confocal microscopes are available, each having unique features and advantages. Those most commonly used for examining fluorescence specimens are laser-scanning confocal microscopes. These microscopes, as their name implies, use lasers as light sources and collect images by scanning the laser beam across the specimen.

Lasers provide intense illumination within a narrow range of wavelengths. The emission wavelengths of several types of lasers, together with the excitation spectra of familiar fluorophores, are illustrated in Figure 4.5.3. Mixed krypton-argon gas lasers are popular for multi-wavelength confocal microscopy because they emit at three well-separated wavelengths (488, 568, and 647 nm) that can be used to simultaneously image two or three fluorophores (e.g., FITC, lissamine rhodamine, and Cy5). The disadvantage of krypton-argon lasers is that

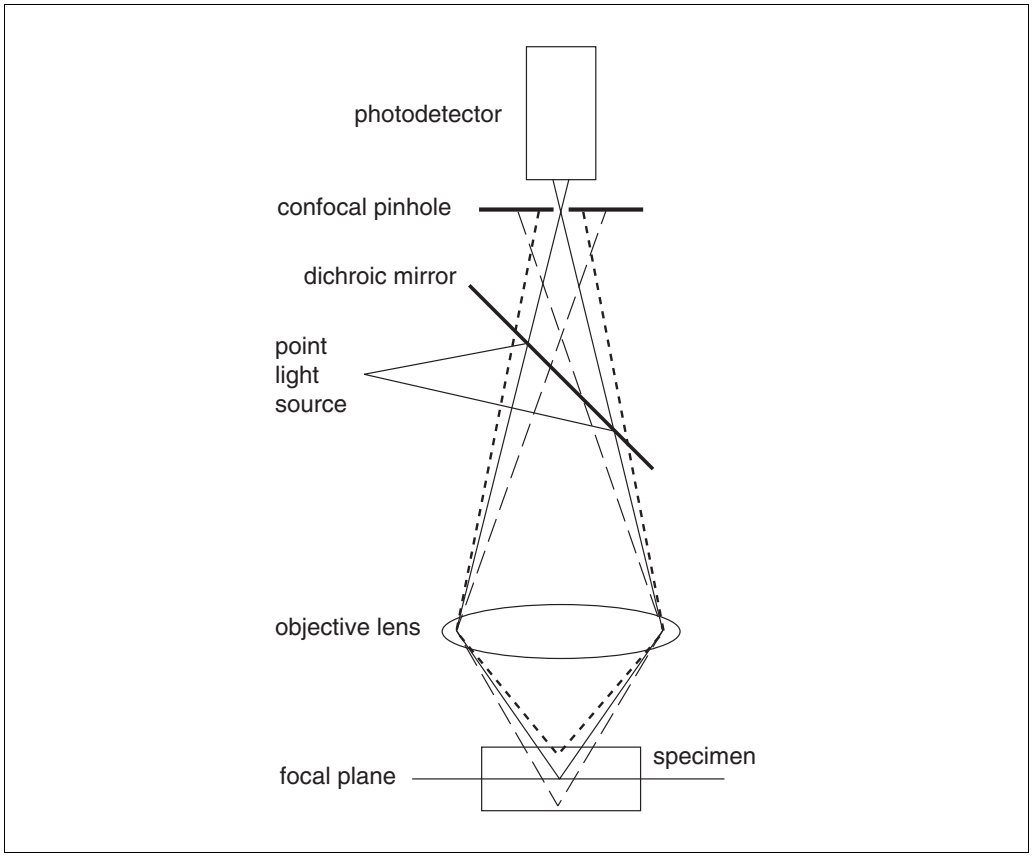


Figure 4.5.2 The basis of optical sectioning in confocal epifluorescence microscopy. Illumination from the point light source is reflected by the dichroic mirror and focused by the objective lens to a diffraction-limited spot within the specimen. Fluorophores within the focal spot as well as in the cone of light above and below it are excited, emitting fluorescence at a longer wavelength than the incident light. The fluorescence captured by the objective passes through the dichroic mirror because of its longer wavelength. The confocal pinhole allows fluorescence from the plane of focus in the specimen to reach the photodetector but blocks fluorescence from areas above and below the plane of focus. Redrawn from Shotton (1993).

Table 4.5.1 Theoretical Resolutions of Confocal and Conventional Microscopes[a]

$\lambda_{ex}/\lambda_{em}$	Objective					
	10×, 0.4 NA, air		40×, 0.85 NA, air		60×, 1.4 NA, oil	
	Lat. res.	Ax. res.	Lat. res.	Ax. res.	Lat. res.	Ax. res.
Confocal fluorescence microscope						
488/518	0.55	4.50	0.26	0.99	0.16	0.56
568/590	0.64	5.17	0.30	1.09	0.18	0.64
647/677	0.72	5.88	0.34	1.28	0.21	0.72
Conventional fluorescence microscope						
518	0.79	6.48	0.37	1.43	0.24	0.93
590	0.90	7.38	0.42	1.63	0.28	1.06
680	1.04	8.50	0.49	1.88	0.32	1.22

[a]Data reprinted from Brelje et al. (1993) by permission of Academic Press. λ_{ex} and λ_{em}, excitation and emission wavelengths; lat. res. and ax. res., lateral and axial resolutions.

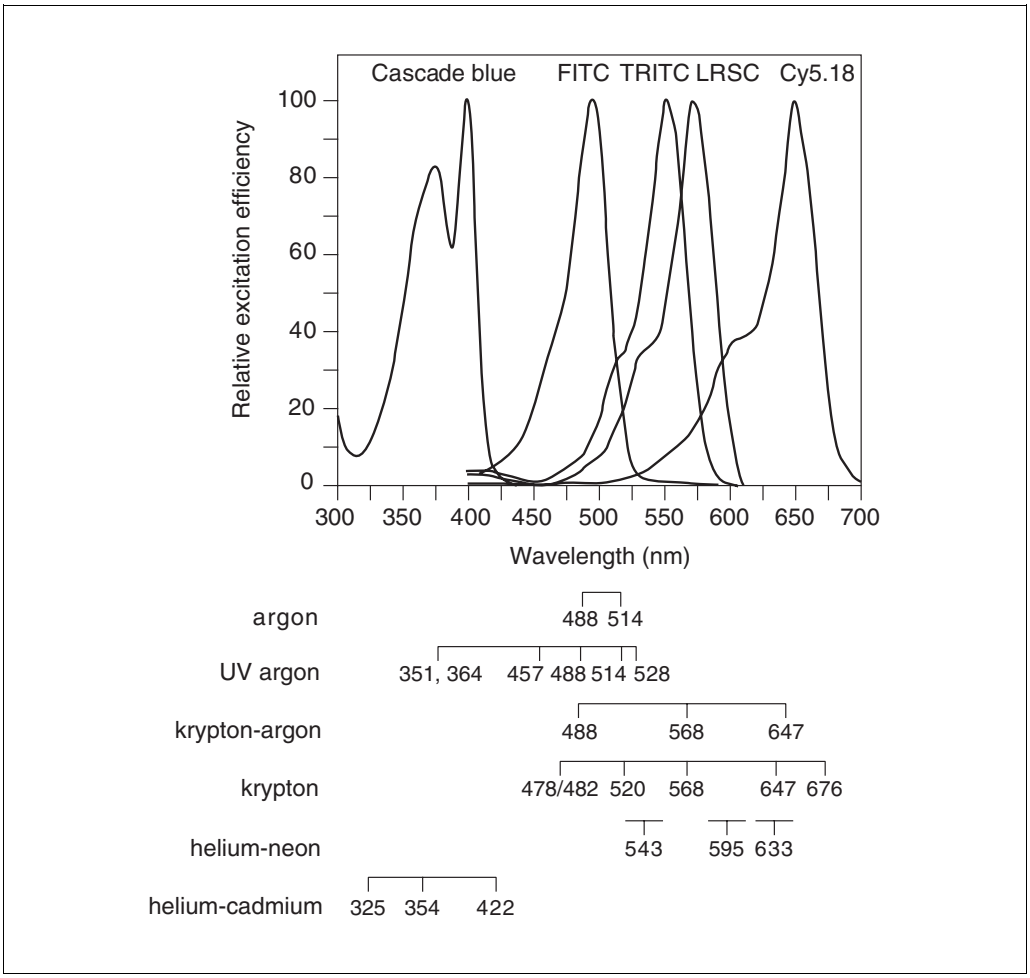


Figure 4.5.3 Comparison of the emission wavelengths of various lasers and the excitation spectra of representative fluorophores. The lasers most commonly used for laser-scanning confocal microscopy are air-cooled argon (488 and 514 nm), krypton-argon, and helium-neon lasers. UV argon lasers generally require water cooling and are more expensive. They may be configured to provide only UV wavelengths (351 nm and 364 nm) or both UV and longer wavelengths. Data for the excitation spectra of Cascade blue, fluorescein (FITC), tetramethylrhodamine (TRITC), lissamine rhodamine (LRSC), and cyanine 5.18 (Cy5.18) are from Wessendorf and Brelje (1993) and were downloaded from the web page of Aryeh Weiss, *http://optics.jct.ac.il/~aryeh/Spectra*. Modified from Brelje et al. (1993).

their life spans are short (~2000 hr). Another way to achieve multiwavelength excitation is to combine the outputs of two or more lasers.

Several methods have been devised for scanning the sample with the laser beam to illuminate different positions in the specimen. The most common method employs a pair of galvanometer mirrors to both scan the laser beam across the specimen and collect the fluorescence emitted from the specimen (Fig. 4.5.4). One galvanometer mirror scans sequential spots along the *x* axis, and the second mirror moves from line to line in the *y* axis. The fluorescence emission is separated from the illuminating beam by a dichroic beam splitter and is directed to a photomultiplier tube which collects the fluorescence produced as each spot in the specimen is illuminated. The photodetector output is converted to a digital image that can be displayed on a monitor and stored as a digital image file for later analysis. Most laser-scanning confocal microscopes have 8-bit digitizers that encode 256 gray levels, although some recent models have 12- or 16-bit digitizers. Collection of a full-size image (typically 1024 × 1024 pixels) takes ~2 sec. Laser-scanning microscopes that employ galvanometer mirror scanners sometimes are called "slow-scan" microscopes because of their relatively slow image acquisition rates. Slow-scan microscopes are available from several sources (Bio-Rad, Zeiss, Leica, Olympus, Nikon, Molecular Dynamics, and Meridian; see *SUPPLIERS APPENDIX*).

The movements of the galvanometer mirrors in laser-scanning microscopes are under the control of a computer, providing flexibility in the scanning pattern. For example, it is possible to "zoom" a region of interest (visualize it at higher magnification) by reducing the scan area and the distances between sample points. In addition, many laser-scanning microscopes have the ability to repetitively scan a single line or to "park" the scanner to monitor fluores-

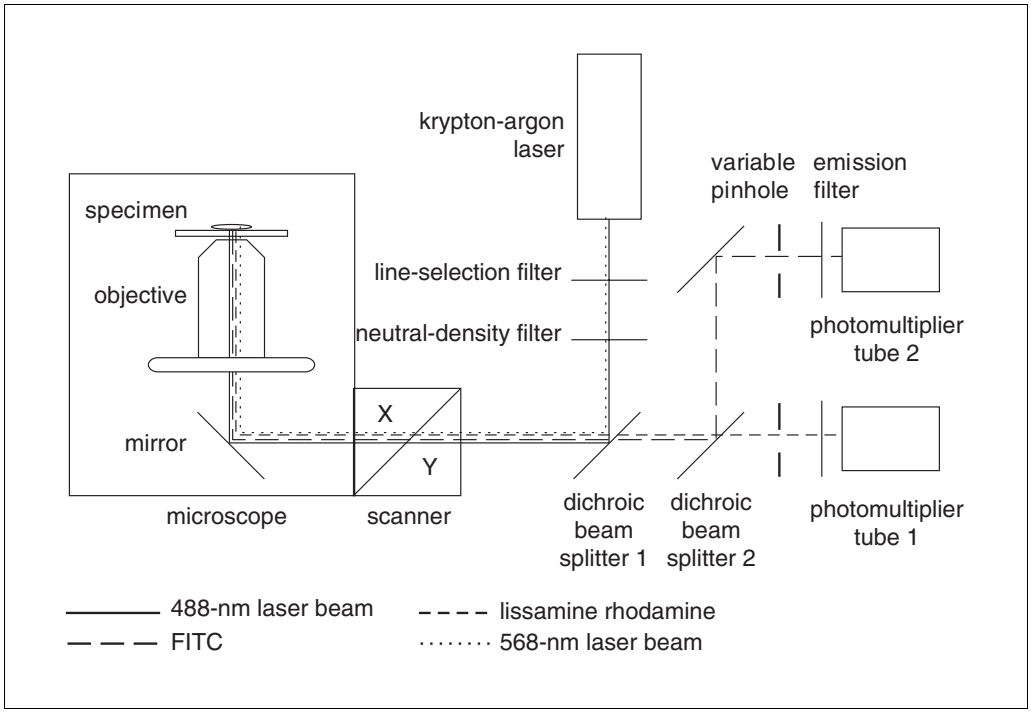


Figure 4.5.4 The light path of a laser-scanning confocal microscope set up for simultaneous imaging of FITC and lissamine rhodamine. The 488-nm and 568-nm lines of a krypton-argon laser are reflected by dichroic beam splitter 1 into the optical axis of the microscope. The scanner contains two galvanometer mirrors, which generate the *x* and *y* axis movements of the beam. The beam is reflected by a mirror into the objective which focuses the beam onto the specimen. The specimen is scanned line by line in a raster pattern. Fluorescence emitted by the specimen as each spot is illuminated travels the reverse path through the scanning system. The FITC fluorescence (peak at 520 nm) and lissamine rhodamine fluorescence (peak at 590 nm) pass through dichroic beam splitter 1 to dichroic beam splitter 2, which transmits the lissamine rhodamine fluorescence to photomultiplier tube 1 and reflects the FITC fluorescence to photomultiplier tube 2. A variable pinhole in front of each photodetector blocks light from out-of-focus areas of the specimen while allowing light from the illuminated spot to reach the detector.

cence at a single spot. The latter technique is particularly useful for studying rapidly changing fluorescence signals, such as those produced by a Ca^{2+} indicator in an active neuron.

Laser-scanning microscopes are available (from Noran, Life Sciences Resources, and Meridian; see *SUPPLIERS APPENDIX*) that can collect images at video rates (30 frames/sec) or faster. Several methods for achieving rapid scanning rates have been employed, such as acousto-optical deflection devices, rotating mirrors, or resonating mirrors (reviewed by Art and Goodman, 1993; Tsien and Bacskai, 1995). The gain in imaging speed always comes at a cost, however. For example, rapid-scan confocal microscopes do not provide the degree of control over the scan pattern offered by top-of-the-line slow-scan microscopes, and some video-rate confocal microscopes are incapable of multiwavelength illumination. Video-rate microscopes that rely on slit apertures rather than pinhole apertures have slightly poorer lateral and axial resolution.

A type of rapid-scan confocal microscope that deserves mention because of its lower cost (among other reasons) uses a spinning disk with multiple pinholes (~200,000) to simultaneously illuminate and detect emission from many spots in the specimen. The light source can be a laser or a broad-spectrum lamp like that used for conventional epifluorescence microscopy. The principle advantage of this type of confocal microscope is that it is capable of collecting images very rapidly (up to 700 frames/sec at 5000 lines resolution; Kino, 1995). The images can be examined directly by eye or captured with a sensitive camera. The main disadvantage is that the disk transmits only ~1% of the available light because the holes in the spinning disk need to be widely spaced. A new type of spinning-disk confocal microscopy has recently become available that uses "microlenses" to improve optical throughput and achieve high-speed confocal imaging with better sensitivity (Ultra View; Life Sciences Resources; see *SUPPLIERS APPENDIX*).

Another form of laser-scanning microscopy that promises to be of great value uses two-photon (and three-photon) excitation to induce fluorescence emission (Denk et al., 1995). Two-photon excitation occurs when a fluorophore absorbs two photons, each having half the energy needed to raise the fluorophore to the excited state. The light intensities required for simultaneous absorption occur only at the focal point, so only fluorophores at the focal point are excited. Therefore, two-photon excitation allows optical sectioning without a spatial filter in front of the detector. Moreover, since fluorophores outside the focal point are not excited, the specimen is less subject to photobleaching than in a conventional laser-scanning microscope. The wavelengths needed to excite standard visible light fluorophores by two-photon absorption are longer and penetrate tissue better than the wavelengths used for one-photon excitation, making it possible to look deeper into a specimen. In addition, UV fluorophores can be imaged without many of the problems that arise when UV wavelengths are used in conventional laser-scanning microscopes. A current drawback of two-photon confocal microscopy is the high cost of an appropriate laser (~$100,000). Two-photon scanning microscopes are now available from commercial sources (Bio-Rad, Leica; see *SUPPLIERS APPENDIX*).

PRACTICAL GUIDELINES

Sample Preparation: Immunofluorescence in Fixed Specimens

Additional guidelines for sample preparation are discussed in *UNIT 4.3*.

Fixation

The best fixative is one that accurately preserves the three-dimensional geometry of the specimen. The standard fixative for fluorescence microscopy (2% to 4% formaldehyde in PBS) is not ideal because it can cause blebbing of the plasma membrane, vesiculation of intracellular membrane compartments, and other alterations in cellular morphology. Moreover, some commercial preparations of formaldehyde contain methanol, which shrinks cells. Techniques for optimizing formaldehyde fixation are described by Bacallao et al. (1995). The buffer should be chosen to match the osmolality and pH of the specimen. Fixatives containing 0.125% to 0.25% glutaraldehyde in addition to formaldehyde preserve cellular morphology better than those containing formaldehyde alone. Some investigators avoid using glutaraldehyde for fluorescence microscopy because it induces autofluorescence. However, autofluorescence can be reduced by treating the sample after fixation with $NaBH_4$ (1 mg/ml in PBS, pH 8.0, using two treatments of 5 min each for dissociated cells, longer for thicker samples). A more serious drawback of glutaraldehyde for immunofluorescence studies is that it destroys the antibody recognition sites of some antigens.

An alternative fixation technique that preserves tissue better than chemical fixation is rapid freezing followed by freeze substitution (Bridgman and Reese, 1984).

Choices of fluorophores

Criteria to consider in selecting fluorophores for fluorescence microscopy are described in *UNIT 4.2 & APPENDIX 1E*. The only additional consideration for confocal microscopy is to choose fluorophores that can be excited by the wavelengths provided by the available lasers. However, it is not essential for the excitation spectrum peak to precisely match the laser wavelength because the lasers on most microscopes are sufficiently powerful to maximally excite fluorophores at off-peak wavelengths. For experiments that depend on imaging two fluorophores, it is best to select fluorophores whose excitation and emission spectra have minimal overlap. Good choices for multiwavelength imaging with a krypton-argon laser are: FITC/Oregon green/Alexa 488 (Molecular Probes) for excitation at 488 nm; lissamine rhodamine/Cy3/Texas red/Alexa 568 (Molecular Probes) for excitation at 568 nm; and Cy5 for excitation at 647 nm. UV fluorophores also are good for multicolor imaging (with absorption at 350 to 390 nm; some of the best dyes for DNA are UV fluorophores).

Control samples

Confocal microscopes rely on electronic image enhancement techniques that can make even a dim autofluorescence signal or nonspecific background staining look bright. In order to be able to distinguish a real signal from background it is essential to prepare appropriate control samples. For immunofluorescence experiments with one primary antibody, the appropriate control samples are unstained specimens and specimens treated with the secondary antibody but no primary antibody. Experiments with two primary and secondary antibodies require additional controls to test whether the secondary antibodies cross-react with the "wrong" primary antibody. Other control experiments may be required to verify the specificity of labeling.

Mounting the specimen

The mounting medium should preserve the three-dimensional structure of the specimen. PBS (*APPENDIX 2A*) or a mounting medium consisting of 50% glycerol/50% PBS preserves the shapes of cells quite well, but Mowiol and gelvatol cause a 10% decrease in height (Bacallao et al., 1995). Adding an antioxidant to the mounting medium helps to alleviate photobleaching. One of the best antioxidants is 100 mg/ml 1,4-diazabicyclo[2,2,2]octane (DABCO; Sigma; Bacallao et al., 1995). *n*-propyl gallate (Giloh and Sedat, 1982) and *p*-phenylenediamine (PPD; Johnson et al., 1982) also are effective antibleaching agents, but the former may cause dimming of the fluorescence while the latter may damage the specimen (Bacallao et al., 1995). ProLong, a new antifade reagent from Molecular Probes, hardens upon drying and is exceptionally effective in preserving the fluorescence of most fluorophores.

The choice of mounting medium should take into account the type of microscope objective that will be used to observe the specimen. In order for an objective to perform optimally, the mounting medium should have the same refractive index as the objective immersion medium. Table 4.5.2 gives the refractive indexes of standard objective immersion media and mounting media. Mismatches in the refractive indexes produce spherical aberration leading to loss of light at the detector, as well as decreased *z* axis resolution and incorrect depth discrimination. Image deterioration caused by spherical aberration increases with depth into the specimen. Significant losses of signal intensity and axial resolution are apparent at distances of just 5 to 10 μm when an oil immersion objective is used to examine a specimen in an aqueous medium (Keller, 1995).

Most microscope objectives are designed for viewing specimens through a glass coverslip of a specific thickness (typically 0.17 μm, a no. 1½ coverslip). Correct coverslip thickness is especially critical for high-NA (>0.5) dry objectives and water immersion objectives (Keller, 1995). Use of a coverslip that differs from the intended thickness by only 5% causes significant spherical aberration. High-NA dry and water immersion objectives typically have an adjustable collar to correct for small variations in coverslip thickness.

The specimen should be mounted as close to the coverslip as possible, especially for observation with immersion objectives, which have short working distances (~100 to 250 μm, depending on the type of objective). This also helps to avoid image deterioration due to spherical aberration. Fragile specimens should be protected by supporting the coverslip; for example, using a thin layer of nail polish, strips of coverslips, or a gasket made from a sheet of

Table 4.5.2 Refractive Indexes of Common Immersion and Mounting Media

Medium	Refractive index (RI)
Immersion media	
Air	1.00
Water	1.338
Glycerol	1.47
Immersion oil	1.518
Mounting media	
50% glycerol/PBS/DABCO	1.416[a]
5% *n*-propyl gallate/0.0025% *p*-phenylene diamine (PPD) in glycerol	1.474[a]
0.25% PPD/0.0025% DABCO/5% *n*-propyl gallate in glycerol	1.473[a]
VectaShield (Vector Labs)	1.458[a]
Slow Fade (Molecular Probes)	1.415[b]
ProLong (Molecular Probes)	1.3865[b,c]

[a]Data from Bacallao et al. (1995).
[b]Data from Molecular Probes.
[c]RI for liquid medium (RI for solidified medium will be higher).

silicon rubber (Reiss; see *SUPPLIERS APPENDIX*). Sealing the edges of the coverslip—with nail polish or silicon vacuum grease (Dow Corning; see *SUPPLIERS APPENDIX*)—helps to prevent specimen desiccation and movement.

Living Specimens

Confocal microscopy of living preparations is challenging for several reasons. The specimen must be mounted in a chamber that keeps it healthy and immobile while at the same time providing access for the objective. For high-resolution transmitted-light imaging (e.g., by laser-scanning differential interference contrast microscopy), the chamber must be thin enough to accommodate a high-NA (oil immersion) condenser. Fluorescence signals in living specimens generally are weak and the illumination levels needed to detect them can be damaging to the specimen. Photobleaching inevitably is a problem for experiments that require collecting many images. Temperature fluctuations in specimens kept at nonambient temperatures make it difficult to maintain accurate focus.

A simple chamber for culture preparations grown on glass coverslips can be made by forming a well on a glass slide with a gasket cut from a sheet of silicon rubber or a plastic ruler. To prevent the well from leaking, it should be sealed with silicon vacuum grease, a mixture of melted paraffin and petroleum jelly, or Sylgard (Dow Corning; see *SUPPLIERS APPENDIX*). The well is filled with medium and then the coverslip with attached cells is placed, cell side down, on top of the well. The preparation can be kept warm during observation on the microscope with a heated air blower—e.g., a hair dryer with variable power source or a commercial air-stream incubator (e.g., Neutek; see *SUPPLIERS APPENDIX*)—or with infrared lamps. More elaborate chambers, some of which have built-in heaters and ports for changing solutions, are available from commercial sources (see Terasaki and Dailey, 1995, for a partial listing of manufacturers). An important factor to consider in choosing a chamber is whether it maintains the desired temperature while in contact with an immersion objective that acts as a heat sink. One solution to this problem is to heat the objective as well as the chamber. A heated chamber and objective warmer designed for microscopy with a high-NA objective and condenser are available from Bioptechs (see *SUPPLIERS APPENDIX*).

Addition of an oxygen quencher to the medium can help to alleviate photobleaching of the fluorophores. Photobleaching not only leads to dimming of the signal but also to generation of oxygen radicals that can damage cells. Several oxygen quenchers have been re-

ported to be effective, including oxyrase (0.3 U/ml; Oxyrase [see *SUPPLIERS APPENDIX*]; Waterman-Storer et al., 1993); ascorbic acid (0.1 to 3.0 mg/ml; Sigma; Terasaki and Dailey, 1995); a mixture of Trolox (10 μM; Aldrich) and *N*-acetylcysteine (50 μM; Sigma; M. Burack and G. Banker, pers. comm.); and crocetin (Tsien and Waggoner, 1995).

Optimizing Imaging Parameters

Choice of objectives

High-NA objectives generally are preferable for fluorescence microscopy because they collect more light than low-NA objectives (brightness is proportional to NA^4). Most high-quality high-NA objectives have >80% transmission at visible wavelengths, but some have low transmission at UV wavelengths (Keller, 1995).

Water immersion objectives are the best choice for visualizing specimens in aqueous solutions (e.g., living specimens). Several microscope manufacturers recently have introduced high-NA water immersion objectives specifically designed for confocal microscopy of biological specimens. These objectives differ from previously available types of water immersion objectives in that they are intended for viewing specimens mounted under a coverslip. They have working distances of ~250 μm.

Oil immersion objectives can have higher NAs than water immersion objectives. Most have fairly short working distances (~100 μm) although some recently introduced oil objectives have working distances of ~200 μm. A long-working-distance oil objective will be useful only if the specimen is mounted in a medium that matches the refractive index of immersion oil (η = 1.518). If an aqueous mounting medium is used, images from depths at more than ~20 μm into the specimen will be noticeably degraded by spherical aberration. Also, distance measurements in the *z* axis will need to be corrected. The actual movement of the focal plane in the specimen (d_s) produced by a movement of the objective (d_{obj}) depends on the ratio of the refractive indexes of the specimen and immersion medium. A reasonable approximation (Majilof and Forsgren, 1993) of the relationship is given by:

$$d_s/d_{obj} = \eta_s/\eta_{obj}$$

Pinhole size

As was explained above (see Basis of Optical Sectioning), the size of the pinhole has a critical influence on image quality. A pinhole with a radius equal to the radius of the first minimum of the Airy disk—which is approximately equivalent to the diameter at half maximal intensity (Amos, 1995)—will let most of the light from the plane of focus reach the detector, while blocking most of the out-of-focus flare. The lateral resolution will be ~20% better than that obtainable by conventional microscopy with the same optics (Centonze and Pawley, 1995), although not as good as can be achieved with a smaller pinhole. Lateral resolution continues to improve as pinhole radius is decreased down to a pinhole size of ~0.2 × Airy disk radius, but a pinhole this small excludes ~95% of the signal (Wilson, 1995). Axial resolution improves as pinhole size decreases, down to ~0.7 × Airy disk radius, then levels off. The best trade-off between signal intensity and resolution will depend on the characteristics of the sample and aims of the experiment.

Zoom factor

The zoom setting on a confocal microscope determines the size of the scan region and the apparent magnification of the image. A zoom factor of 2 will scan an area half as long and wide as a zoom factor of 1. Images are made up of the same number of samples (points along the horizontal axis, lines along the vertical axis) and are displayed on the image monitor by a fixed number of pixels regardless of the zoom factor. Therefore, the pixels in a zoom-2 image will represent areas within the specimen half as large in each dimension as the areas represented by the pixels at zoom 1. If the pixel size for an objective at zoom 1 represents 0.25 μm × 0.25 μm, then the pixel size at zoom 2 will be 0.125 × 0.125 μm. The pixel dimensions (referring to the specimen) are inversely related to the zoom setting.

For each objective, there is an optimal zoom setting which yields pixel dimensions small enough to take advantage of the full resolution of the objective but large enough to avoid oversampling. In order for the minimum resolvable entity to be visible on the display monitor, the pixel dimensions need to be smaller than (less than one-half) the optical resolution. However, if the pixel size is made too small by using a higher-than-optimal zoom factor, the specimen is subjected to more irradiation than necessary with an increased risk of photobleaching. The rate of photobleaching increases proportionally to the square of the zoom factor (Centonze and Pawley, 1995). A guideline for selecting an appropriate zoom factor derived from informa-

tion theory (the Nyquist Sampling Theorem) states that the pixel dimensions should be equal to the optical resolution divided by 2.3 (see Webb and Dorey, 1995). However, pixel dimensions smaller than this may produce more informative images.

Z axis sectioning interval

In order to study the three-dimensional structure of a specimen, images are collected at a series of focal levels at intervals determined by the commands sent to the focus motor. The most straightforward way to ensure that the reconstructed images have correct proportions in the *x*, *y*, and *z* axes is to collect optical sections at *z* axis intervals equal to the *x*,*y* pixel dimension. However, the interfocal plane interval needed to adequately sample the specimen in the *z* axis is not as small as the *x*,*y* pixel dimension because the axial resolution is poorer than the lateral resolution (see Table 4.5.1). The optimal interfocal plane interval (according to the Nyquist Sampling Theorem) is equal to the axial resolution divided by 2.3. Collecting images at shorter intervals results in oversampling with an increased risk of photobleaching.

Illumination intensity

Fluorescence emission increases linearly with illumination intensity up to a level at which emission saturates. Optimal signal-to-background and signal-to-noise ratios are obtained with illumination levels well below saturation (Tsien and Waggoner, 1995). The illumination intensity on a laser-scanning microscope can be adjusted by inserting neutral-density filters into the light path and/or by operating the laser at submaximal power. In general, the best images are obtained with illumination levels that are as high as possible without producing unacceptable rates of photobleaching.

PMT black level and gain

The contrast and information content of confocal images are influenced by the black level and gain of the photomultiplier tube (PMT) amplifiers. To obtain maximal information, the black level and gain should be adjusted to take advantage of the full dynamic range of the PMTs. The appropriate black level setting can be found by scanning while the light path to the PMT is blocked. The image that appears on the display monitor should be just barely brighter than the background, which is black (gray level = 0). To set the gain, scan the specimen and adjust the gain so that the brightest pixel in the image is slightly below white (gray level = 255). Selecting black level and gain settings which ensure that all signals fall within the dynamic range of the PMT is important for quantitative imaging experiments. The software provided with many confocal microscopes includes a pseudocolor image display mode that facilitates selection of appropriate black level and gain settings by highlighting pixels with intensity values near absolute black and absolute white.

Averaging

Confocal images of dimly fluorescent specimens captured at typical scan rates (1 to 2 sec/frame for a slow-scan confocal microscope) appear noisy because of the small numbers of photons collected from each spot. In some instances, it may be possible to improve the signal-to-noise ratio by scanning the specimen at slower rates. Another way to obtain a better image is by summing and averaging the signals obtained in multiple scans (frame averaging). Some confocal microscopes provide a second averaging method (line averaging), in which individual lines are repeatedly scanned and averaged. Line averaging generally produces sharper images than frame averaging (which averages full frames) because there is less risk of blurring due to movements or changes in the specimen.

Image display

Commercial confocal microscope packages provide software for some types of image enhancement and display. The display options for three-dimensional datasets typically include "*z* projections" (see Fig. 4.5.1B), which are two-dimensional displays formed by superimposition of stacks of optical sections, and stereoscopic views, which are made by combining two image stacks, one aligned in the *z* axis and the other with a displacement between successive images. Many systems also have the capability to compute cross-sections and projections of the specimen from varying angles. Computed projections for a sequence of view angles can be played as a movie in which the specimen appears to rotate around an axis. Such movies give the viewer a striking impression of the three-dimensional geometry of the specimen. Additional display options are available in various integrated software/hardware packages specifically designed for visualization and analysis of three-dimensional images.

Anticipated Results

Fluorescence in fixed specimens protected with an antifade agent is often sufficiently bright and resistant to photobleaching to make it possible to reconstruct three-dimensional images using imaging parameters that provide optimal resolution. Superb three-dimensional views may be obtained of structures as small and complex as a cell's cytoskeleton or the terminal arbor of an axon. The maximum depth in the specimen at which adequate images can be obtained depends on a number of factors (e.g., the match in refractive indexes of the immersion and mounting media, the wavelength of light, and the extent of scattering and absorption by the specimen). Under optimal conditions, it may be possible to image structures at depths near the limit allowed by the working distance of the objective; in practice, image quality usually deteriorates at depths in the range of a few hundred micrometers or less.

Although confocal microscopy on living cells is more difficult and damage to the tissue may preclude extensive three-dimensional reconstruction, the added time dimension and confidence in the reality of the images makes it well worth the effort. In addition, it is possible to study dynamic processes lasting for hours by collecting sequences of time-lapse images. Robust fluorophores such as certain variants of GFP (S65T, EGFP) can be imaged repeatedly with minimal loss of fluorescence (see, for example, Ellenberg et al., 1997). In addition, modern laser-scanning confocal microscopes provide a versatile optical bench and sophisticated specimen positioner which permit a wide range of experiments with the controlled application of laser light to living tissues. Current examples of these approaches are photobleaching (Cole et al., 1996; Wedekind et al., 1996) and release of caged compounds (Callaway and Katz, 1993; Svobodoa et al., 1996). These are only the harbingers of many future applications of light probe physiology made possible by the versatility of the confocal microscope.

Resources Available via Internet

NIH Image, a powerful image analysis program for Macintosh computers developed by W. Rasband (Research Services Branch, National Institute of Mental Health, NIH), has many useful tools for analysis of confocal images. It can be downloaded from *http://rsb.info.nih.gov/nih-image/* or obtained via FTP from *zippy.nimh.nih.gov*. A version of NIH Image modified for operation under Windows also is available. Much information about fluorescent probes can be obtained from the Molecular Probes Web Site at *http://www.probes.com/*.

Many topics of interest to confocal microscopists are discussed on the confocal e-mail listserver network. To subscribe to the list, send the message "subscribe confocal<your name>" to *listserv@ubvm.cc.buffalo.edu*.

LITERATURE CITED

Amos, W.B. 1995. Appendix 1: Optical units. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 579-580. Plenum, New York.

Art, J.J. and Goodman, M.B. 1993. Rapid scanning confocal microscopy. *In* Cell Biological Applications of Confocal Microscopy (B. Matsumoto, ed.) pp. 47-78. Academic Press, San Diego.

Bacallao, R., Kiai, K., and Jesaitis, L. 1995. Guiding principles of specimen preservation for confocal fluorescence microscopy. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 311-326. Plenum, New York.

Brelje, T.C., Wessendorf, M.W., and Sorenson, R.L. 1993. Multicolor laser scanning confocal immunofluorescence microscopy: Practical applications and limitations. *In* Cell Biological Applications of Confocal Microscopy (B. Matsumoto, ed.) pp. 98-182. Academic Press, San Diego.

Bridgman, P.C. and Reese, T. 1984. The structure of cytoplasm in directly frozen cultured cells. 1. Filamentous meshworks and the cytoplasmic ground substance. *J. Cell. Biol.* 99:1655-1668.

Callaway, E.M. and Katz, L.C. 1993. Photostimulation using caged glutamate reveals functional circuitry in living brain slices. *Proc. Natl. Acad. Sci. U.S.A.* 90:7661-7665.

Centonze, V. and Pawley, J. 1995. Tutorial on practical confocal microscopy and use of the confocal test specimen. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 549-570. Plenum, New York.

Cole, N., Smith, C., Sciaky, N., Terasaki, M., Edidin, M., and Lippincott-Schwartz, J. 1996. Diffusional mobility of Golgi proteins in membranes of living cells. *Science* 237:797-801.

Denk, W., Piston, D.W., and Webb, W.W. 1995. Two-photon molecular excitation in laser-scanning microscopy. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 445-458. Plenum, New York.

Ellenberg, J., Siggia, E.D., Moreira, J.E., Smith, C.L., Presley, J.F., Worman, J.J., and Lippincott-Schwartz, J. 1997. Nuclear membrane dynamics and reassembly in living cells: Targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell Biol.* 138:1193-1206.

Giloh, H. and Sedat, J.W. 1982. Fluorescence microscopy: Reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. *Science* 217:1252-1255.

Inoué, S. and Spring, K.R. 1997. Video Microscopy: The Fundamentals, 2nd ed. Plenum, New York.

Johnson, G.D., Davidson, R.S., McNamee, K.C., Russell, G., Goodwin, D., and Holborow, E.J. 1982. Fading of immunofluorescence during microscopy: A study of the phenomenon and its remedy. *J. Immunol. Methods.* 55:231-242.

Kamabadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S., and Odenwald, W. 1998. Regulation of *POU* genes by *castor* and *hunchback* establishes layered compartments in the *Drosophila* CNS. *Genes & Devel.* 12:246-260.

Keller, E. 1995. Objective lenses for confocal microscopy. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 111-126. Plenum, New York.

Kino, G.S. 1995. Intermediate optics in Nipkow disk microscopes. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 155-166. Plenum, New York.

Majilof, L. and Forsgren, P. 1993. Confocal microscopy: Important considerations for accurate imaging. *In* Cell Biological Applications of Confocal Microscopy (B. Matsumoto, ed.) pp. 79-97. Academic Press, San Diego.

Matsumoto, B. (ed.) 1993. Cell Biological Applications of Confocal Microscopy. Academic Press, London.

Sandison, D.R., Williams, R.M., Wells, K.S., Strickler, J., and Webb, W.W. 1995. Quantitative fluorescence confocal laser scanning microscopy. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 39-54. Plenum, New York.

Shotton, D.M. 1993. Electronic acquisition of light microscope images. *In* Electronic Light Microscopy (D.M. Shotton, ed.) pp. 1-38. Wiley-Liss, New York.

Svobodoa, K., Tank, D.W., and Denk, W. 1996. Direct measurement of coupling between dendritic spines and shaft. *Science* 272:716-719.

Terasaki, M. and Dailey, M.E. 1995. Confocal microscopy of living cells. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 327-346. Plenum, New York.

Tsien, R.Y. and Bacskai, B.J. 1995. Video-rate confocal microscopy. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 459-478. Plenum, New York.

Tsien, R.Y. and Waggoner, A. 1995. Fluorophores for confocal microscopy. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 267-280. Plenum, New York.

Waterman-Storer, C.M., Sanger, J.W., and Sanger, J.M. 1993. Dynamics of organelles in the mitotic spindles of living cells: Membrane and microtubule interactions. *Cell Motil. Cytoskel.* 26:19-39.

Webb, R.H. and Dorey, C.K. 1995. The pixilated image. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 55-68. Plenum, New York.

Wedekind, P., Kubitscheck, U., Heinrich, O., and Peters, R. 1996. Line-scanning microphotolysis for diffraction-limited measurements of lateral diffusion. *Biophys. J.* 71:1621-1632.

Wessendorf, M.W. and Brelje, T.C. 1993. Multicolor fluorescence microscopy using the laser-scanning confocal microscope. *Neuroprotocols* 2:121-140.

Wilson, T. (ed.) 1990. Confocal Microscopy. Academic Press, London.

Wilson, T. 1995. The role of the pinhole in confocal imaging system. *In* Handbook of Biological Confocal Microscopy, 2nd ed. (J. Pawley, ed.) pp. 167-182. Plenum, New York.

KEY REFERENCES

Inoué and Spring, 1997. See above.

Covers basics of light microscopy, video microscopy, and much more.

Matsumoto, 1993. See above.

Good source of practical information.

Pawley, J. (ed.) 1995. Handbook of Biological Confocal Microscopy, 2nd ed. Plenum, New York.

Excellent source of theoretical and technical information.

Russ, J. 1995. The Image Processing Handbook. CRC Press, Boca Raton, Fla.

Excellent source of information about digital image processing.

Shotton, D.M. (ed.) 1993. Electronic Light Microscopy. John Wiley and Sons, New York.

Covers many aspects of light microscopy, including confocal microscopy.

Wilson, 1990. See above.

Good source of theoretical background information.

INTERNET RESOURCES

zippy.nimh.nih.gov

Use to obtain NIH Image via FTP.

http://optics.jct.ac.il/~aryeh/Spectra

Source of excitation and emission spectra for common fluorophores.

http://rsb.info.nih.gov/nih-image/

Use to obtain NIH Image.

http://www.probes.com

Molecular Probes web site, including product listings and much more.

http://www.mwrn

Microworld Resources, a comprehensive list of microscopy products and vendors.

listserv@ubvm.cc.buffalo.edu

Confocal e-mail listserv network.

Contributed by Carolyn L. Smith
National Institute of Neurological Disorders and Stroke
Bethesda, Maryland

Immunoperoxidase Methods for Localization of Antigens in Cultured Cells and Tissues

This unit describes the localization of intracellular antigens by the immunoperoxidase staining technique at the electron microscopic (EM) level. These methods provide a potentially valuable alternative, or in some cases a complementary approach, to immunogold labeling. Labeling with immunogold provides a higher level of antigen resolution and can be made reasonably quantitative. However, immunogold labeling is usually best conducted on ultrathin, frozen sections, which requires a special microtome and an extremely high degree of specialized technical skill. In contrast, immunoperoxidase labeling methods are relatively simple in that no special equipment or skills are needed beyond those required for standard transmission EM of plastic-embedded samples. Furthermore, they provide the opportunity for enzymatic amplification of what might otherwise be weak immunoreactive signals. The immunoperoxidase method is limited by its inherently qualitative nature and by the potential for diffusion of the immunoreactive signal from the site of antigen location. These advantages and limitations make immunoperoxidase labeling particularly valuable for localizing intracellular antigens that are contained within membrane-bounded organelles. Under certain circumstances, however, the method can also be successfully applied to cytoplasmic and extracellular antigens.

The immunoperoxidase methods described here are typically referred to as preembedding labeling methods, meaning that the antibodies bind to antigens before the cells or tissues are embedded for sectioning. The methods are also of the indirect type, meaning that the antigen is localized by a two-step antibody binding protocol: antigens are first bound by primary antibodies, which are in turn bound by a secondary antibody that is covalently coupled with horseradish peroxidase (HRP). An electron-dense immunoreaction product at the sites of antigen-antibody binding is then generated in two steps: first, the bound HRP-conjugated secondary antibody catalyzes the formation of oxidized and polymerized diaminobenzidine (DAB) using H_2O_2 as an electron donor, and second, the oxidized and polymerized DAB is rendered electron dense by binding to reduced osmium tetroxide (OsO_4). Labeled cells are then embedded in plastic resin and sectioned for standard transmission EM.

This unit describes two protocols for immunoperoxidase staining: one specific for adherent, cultured cells (see Basic Protocol 1) and one specific for cells within tissue sections (see Basic Protocol 2). Three embedding methods are described for adherent cultured cells. (1) For antigens located anywhere in the cell except the endoplasmic reticulum (ER), cells can be easily detached using propylene oxide and embedded with a standard plastic embedding resin in a microcentrifuge tube (see Basic Protocol 1). (2) For antigens located in the ER, cells can be detached by scraping in ethanol before embedding (see Alternate Protocol 1). (3) Alternatively, to prepare sections through the full depth of the cultured monolayer, cells can be embedded while they are still attached to the culture dish (see Alternate Protocol 2).

STRATEGIC PLANNING

Preliminary Light Microscopy Experiments

Immunoelectron microscopy, by either immunogold or immunoperoxidase labeling, is generally a labor-intensive and time-consuming enterprise. The rate-limiting step is thin sectioning, which is required for EM observations. Therefore, it is strongly recommended that several preliminary studies establishing near-optimal staining parameters be carried

Contributed by William J. Brown
Current Protocols in Cell Biology (1999) 4.6.1-4.6.17

at the light microscopic (LM) level, first by immunofluorescence staining (*UNIT 4.3*) and then by LM-level immunoperoxidase localization. Immunofluorescence is relatively rapid and simple, and it can be used to optimize a number of potentially important variables. For example, one can easily screen by immunofluorescence to optimize fixation time, detergent concentration for cell permeabilization, antibody concentrations, and incubation times. Experience has shown that experimental conditions (for these variables) that generate a bright immunofluorescence signal can generally be applied directly to the immunoperoxidase labeling protocol. Once these conditions are established, a few preliminary immunoperoxidase labeling experiments should be conducted at the LM level. This second series of experiments is valuable for establishing the optimal concentration of HRP-conjugated secondary antibody and the optimal DAB reaction time.

A large amount of time can be saved by optimizing conditions through these preliminary immunofluorescence and immunoperoxidase LM experiments rather than at the EM level. Also, the results obtained by the LM methods can serve as an invaluable comparison for those obtained by EM, either providing increased confidence that the antibodies are accurately localizing the antigen of interest or providing cause for concern. In other words, the results should be consistent irrespective of the method used. Finally, preliminary screening by immunofluorescence or LM immunoperoxidase labeling should be used to ensure that the primary antibody is of sufficiently high quality to generate a high signal-to-noise ratio. Meeting this goal may require production of an IgG fraction, affinity purification of the antibodies, or production of a high-titer ascites fluid for monoclonal antibodies. A successful immunoperoxidase localization at the EM level is very unlikely to be realized if the antibodies cannot first generate an easily detectable immunofluorescence and LM immunoperoxidase signal.

Monitoring the DAB Reaction

Monitoring development of the DAB reaction can easily be done with an upright light microscope and a low-power or long-working-distance objective lens (so it won't get wet), or with an inverted microscope. If using a standard upright microscope, place the petri dish on a microscope slide on the microscope stage to move the dish around. The development of the DAB reaction is best viewed by plain brightfield optics, rather than by phase contrast, because the formation of the contrasting brown/black reaction product is more easily detected.

For most antigens, by the time the reaction looks really good by LM, it will probably be overdone for EM, because the DAB reaction product may have diffused away from the immediate area of the antigen-antibody complexes. This is especially true for antigens contained within membrane-bounded organelles (e.g., the Golgi complex and endosomes), which can lyse following formation of extensive amounts of reaction product. However, antigens contained within the endoplasmic reticulum are sometimes more difficult to detect at the EM level and, therefore, require that the reaction product be quite dark and obvious at the LM level. Thus, it is sometimes difficult to know exactly how far to let the reaction go at the LM level in order to generate a well-contained signal by EM. Therefore, for each experimental condition, two to three dishes should be carried through the fixation and antibody steps, so that each can be subjected to the DAB reaction for a different period of time. The following sequence is suggested: first, react one dish until the reaction product is robust and obvious, then react another dish until the brown/black DAB reaction product is just barely visible by LM, and react a third dish to a point somewhere in between the first two. With experience, two dishes will usually suffice for each experimental condition. The optimal length of time for the DAB reaction can vary greatly from 2 to 30 min. Generally, if the reaction has not developed by ~30 min, something may have gone wrong.

Some special considerations apply to DAB reactions in tissue sections. The length of reaction time can be quite variable. It is best to not have an explosive reaction, as this leads to DAB diffusion in the tissue. It is better to have the reaction proceed slowly (typically, 5 to 30 min at room temperature). If reactions proceed very quickly (e.g., within 1 to 2 min), place the tubes on ice to chill the DAB substrate before adding H_2O_2, and perform the reaction on ice. During the reaction time, the tissue will probably turn brown or black, depending upon the antigen and antibody. Affinity-purified or monoclonal antibodies will often not turn sections as dark as those incubated with crude antiserum, but they give less background and the localization will be better. Monitor the reaction with a dissecting microscope. Some experience is required to know when the sections are dark enough. In the beginning, however, it is better to err on the side of excess so that there is some kind of signal. Therefore, react several sections until they are noticeably dark, and several others for a shorter period of time.

IMMUNOPEROXIDASE STAINING OF CULTURED CELLS

BASIC PROTOCOL 1

This protocol describes a method for the localization of antigens in cultured cells at the EM level by the immunoperoxidase staining procedure. The protocol has been specifically developed for adherent, monolayer cell cultures of established cell lines. However, cultured cells that grow in suspension can, in many cases, be attached to poly-L-lysine-coated dishes and then processed as described in this section. The protocol can also be easily applied to primary cells. In either case, specific conditions for culturing cells are left to the investigator (see *UNIT 1.1*).

Many of these steps can be modified to meet specific experimental needs (e.g., dish size and incubation times). All steps are conducted at room temperature unless otherwise indicated. This protocol describes one of three methods for removing the cells from the culture dishes. Selection of the appropriate method depends on whether (1) the antigen is located anywhere within the cell except the ER (this protocol), (2) the antigen is located in the ER (see Alternate Protocol 1), or (3) sections through the intact monolayer are desired (see Alternate Protocol 2).

Materials

Cells of interest
PLP fixative (see recipe), freshly prepared
PBS, pH 7.4 (*APPENDIX 2A*)
0.005% to 0.05% (w/v) saponin (Sigma)/PBS
Primary antibody in saponin/PBS with 0.1% (w/v) ovalbumin
HRP-conjugated secondary antibody: horseradish peroxidase–conjugated Fab fragments of anti–rabbit IgG or anti–mouse IgG (Biosys) in saponin/PBS with 0.1% (w/v) ovalbumin
Glutaraldehyde fixative: 1.5% (v/v) glutaraldehyde in 5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
7.5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
0.2% (w/v) DAB solution (see recipe), freshly prepared
3% (v/v) H_2O_2, freshly prepared from 30% H_2O_2 stock solution
1% (w/v) reduced OsO_4 solution (see recipe), freshly prepared and ice cold
100 mM sodium cacodylate, pH 7.4, ice cold
70%, 95%, and 100% (v/v) ethanol
100% propylene oxide, EM grade
1:1 (v/v) propylene oxide/plastic embedding resin (e.g., Spurr's)
100% plastic embedding resin (e.g., Spurr's)

35-mm disposable plastic petri dishes
Horizontal reciprocating shaker

NOTE: The propylene oxide detachment method of embedding can be somewhat tricky. It is advisable to practice on cells before attempting the immunoperoxidase staining (see step 18).

Prepare cells

1. Grow cells of interest to ~75% confluency in 35-mm disposable plastic petri dishes. Prepare two to three dishes for each experimental condition (see Strategic Planning and step 13).

   It seems best to not do the immunoperoxidase protocol soon after trypsinization and replating. It is better to plate the cells at lower density and wait 2 to 4 days for them to repopulate the dish before immunoperoxidase staining.

   Other dishes can be used for this procedure, but 35-mm dishes yield a reasonable number of cells for thin-sectioning, and larger dishes require more antibody.

2. Fix cells on the dish by replacing medium with 1 ml PLP fixative, taking care to prevent cells from drying out. Incubate 2 to 3 hr with gentle shaking on a horizontal reciprocating shaker.

   Rotary shakers are acceptable; however, they may cause the center of the dish to dry out if small volumes of fluid are used (e.g., during antibody incubations).

3. Remove fixative and wash cells three times, 5 min each, with 1 to 2 ml PBS.

4. Permeabilize cells by removing PBS and adding 1 to 2 ml of 0.005% to 0.05% saponin/PBS. Incubate for 5 min with gentle shaking.

   The minimal concentration of saponin required to allow antibody penetration should be determined by preliminary immunofluorescence experiments (see Strategic Planning). This concentration may vary depending upon the source of saponin and the type of cells. Nearly all cell types can be rendered optimally permeable using a concentration between 0.005% and 0.05%.

   NOTE: *The saponin concentration used here should also be used in all subsequent antibody incubations and washes.*

Expose cells to primary antibody

5. Remove saponin/PBS and apply ≥0.5 ml primary antibody in saponin/PBS. Incubate ≥l hr with gentle shaking.

   For 35-mm dishes, ~0.5 ml is the minimal volume needed to cover and keep the cells from drying out. An appropriate antibody concentration should be determined by preliminary immunofluorescence experiments (see Strategic Planning).

   Alternatively, cells can incubated in primary antibody for ~30 min at room temperature, and then shifted to 4°C for overnight incubation. Dishes should be covered and placed in a sealed, humidified chamber (e.g., a Tupperware-type container). Overnight incubation is especially useful if the day has been long. The peroxidase signal also tends to be improved, but sometimes with increased background.

6. Wash cells three times, 5 min each, with 1 to 2 ml saponin/PBS.

Expose cells to secondary antibody

7. Remove wash solution and apply 0.5 to 0.7 ml HRP-conjugated secondary antibody. Incubate ≥1 hr with gentle shaking.

   An appropriate dilution of the secondary antibody should be determined by preliminary immunoperoxidase staining experiments (see Strategic Planning). For the Biosys secon-

dary antibody, a good starting point is a 1:400 to 1:800 dilution. The Fab-HRP antibodies from Biosys are particularly good reagents. Their small size, relative to whole IgG-HRP or F(ab′)$_2$-HRP conjugates, allow lower concentrations of saponin to be used for membrane permeabilization.

If importing the Biosys antibody into the U.S., it is necessary to obtain a Permit for Importation and Transportation of Controlled Materials and Organisms and Vectors (VS Form 16-6A) from the USDA. To obtain this permit, an Application for Permit to Import Controlled Material or Import or Transport Organisms and Vectors must be submitted. This application can be obtained from and submitted to USDA, APHIS, VS, Federal Building, Hyattsville, MD 20782 (Fax no. 301-436-8226).

8. Wash cells three times, 5 min each, with 1 to 2 ml saponin/PBS.

9. Wash cells three times over a period of 5 to 10 min with 1 to 2 ml PBS (each wash) to rinse out the saponin.

   This is an important step because removal of saponin lessens the amount of DAB diffusion during the peroxidase reaction.

10. Fix cells by removing PBS, applying 1 ml glutaraldehyde fixative, and incubating for 30 to 60 min with gentle shaking.

11. Wash cells three times, 5 min each, with 1 to 2 ml of 7.5% sucrose/100 mM sodium cacodylate, pH 7.4.

Perform DAB reaction

12. Remove wash solution and apply 1 ml of 0.2% DAB solution.

    CAUTION: *DAB is carcinogenic. Wear gloves and work in fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.*

13. Start the DAB reaction by adding 3.3 µl of 3% H_2O_2 to the dish. Quickly swirl to mix the H_2O_2 and DAB. Start timing and monitoring the reaction using an upright or inverted light microscope (see Strategic Planning). React one dish until the reaction product is robust and obvious, one dish until the brown/black DAB reaction product is just visible, and one to a point somewhere in between.

    Start with a reaction taken to yield a robust product, and then scale back the time to give lesser amounts. The optimal length of time for the DAB reaction can very greatly from 2 to 30 min. A nearly-optimal reaction time can be established by preliminary immunoperoxidase staining experiments (see Strategic Planning). With experience, this procedure can be reduced to two dishes/experimental condition.

14. Stop the reaction by removing DAB solution and quickly rinsing cells three times with 1 to 2 ml of 7.5% sucrose/100 mM sodium cacodylate, pH 7.4.

Osmicate the cells

15. In a fume hood, remove wash solution and osmicate cells with 1 ml ice-cold, 1% reduced OsO_4 solution. Cover petri dishes and place in a Tupperware-type container. Seal well and incubate for 1 hr at 4°C with gentle shaking.

    CAUTION: *Wear gloves when handling osmium. Perform this step in a fume hood to avoid osmium vapors. Be sure that the Tupperware-type container is well sealed—use Parafilm if unsure. Dispose of used osmium according to institutional guidelines for hazardous chemicals.*

16. Return cells to the fume hood, remove osmium solution, and rinse cells quickly three times with 1 to 2 ml ice-cold 100 mM sodium cacodylate, pH 7.4. Allow cells to reach room temperature.

17. Dehydrate cells with a graded series of ethanol (70%, 95%, 100%) as typically done for conventional plastic embedding. Perform three or four quick rinses with ~2 ml of each ethanol solution.

Detach cells and embed in plastic

18. Remove ethanol and quickly add ~0.5 to 1.0 ml of 100% propylene oxide with a glass Pasteur pipet.

    CAUTION: *Perform embedding procedure in the fume hood.*

19. Quickly score the bottom of the dish in a crude checkerboard pattern with the tip of the pipet. Immediately pipet the propylene oxide up and down to dislodge cells from the dish as the plastic dissolves.

    The bottom of the dish starts to turn mottled in appearance and then turns white, and sheets of cells should be seen detaching from the dish.

20. Collect cells and place in a microcentrifuge tube.

    If desired, the cells can be pooled from the dishes that were subjected to varying DAB incubation times to yield a single cell pellet. The advantage of combining cells is that only one pellet needs to be sectioned, giving a sampling of all reaction times in a single section. To identify which DAB reaction time gave the best EM-level staining, the cells from each dish should be placed in separate microcentrifuge tubes.

21. Microcentrifuge to obtain a tight pellet, using a horizontal-type rotor (e.g., Beckman model 11 microcentrifuge) for 2 to 3 min at the highest setting.

    CAUTION: *Be sure that the tubes are tightly capped because propylene oxide fumes are explosive.*

22. Remove propylene oxide and wash the cell pellet twice with ~1 ml of 100% propylene oxide. Microcentrifuge again if pellet becomes disrupted.

23. Remove propylene oxide and replace with ~1 ml of 1:1 propylene oxide/plastic embedding resin. Incubate with gentle rotation for 30 min.

24. Remove 1:1 mixture and wash pellet twice with ~1 ml of 100% plastic embedding resin. Incubate overnight in 100% plastic embedding resin with gentle rotation.

25. Replace with fresh resin mix and polymerize according to manufacturer's specifications for the plastic.

Section and view the cells

26. Cut silver/gold sections by standard ultramicrotomy methods.

27. Stain thin sections with lead citrate only. Omit uranyl acetate to generate a good ratio of signal (DAB-OsO_4 deposits) to noise (rest of the cytoplasm). For details, see Brown et al. (1984).

28. Visualize the sectioned material by standard transmission EM.

    Contrast of the electron-dense product can also be slightly increased by operating the EM at lower accelerating voltages (e.g., 60 versus 80 kV).

DETACHMENT AND EMBEDDING OF CULTURED CELLS FOR ANALYSIS OF ER ANTIGENS

ALTERNATE PROTOCOL 1

This protocol is specifically designed to remove dehydrated cells from the dishes and embed them in plastic when antigens are localized to the ER. It avoids the use of propylene oxide, which tends to extract the DAB reaction product from the ER.

1. Expose the cells to primary and secondary antibody, fix the cells, stain them with OsO_4, and dehydrate them (see Basic Protocol 1, steps 1 through 17).
2. With 100% ethanol still in the dishes, scrape cells from the dish with a small rubber policeman or cell scraper. Collect the detached cells and transfer them to a microcentrifuge tube.

   If desired, the cells from each of the two to three DAB treatment times can be combined in one tube to prepare a single pellet containing cells from each of the treatment groups. To identify which DAB reaction time gave the best EM-level staining, keep the cells from each dish in separate microcentrifuge tubes.
3. Microcentrifuge the cells to obtain a tight pellet, using a horizontal-type rotor (e.g., Beckman model 11 microcentrifuge) for 2 to 3 min at the highest setting.
4. Remove the ethanol and embed directly in 100% plastic embedding resin with several changes of resin, including one overnight incubation. Polymerize the plastic according to the supplier's instructions.

   CAUTION: *Perform embedding procedure in a fume hood.*
5. Cut silver/gold sections by ultramicrotomy, stain with lead citrate, and visualize the sections (see Basic Protocol 1, steps 26 through 28).

EMBEDDING OF CULTURED CELLS FOR ANALYSIS OF MONOLAYER SECTIONS

ALTERNATE PROTOCOL 2

This protocol is used to prepare sections that span the full depth of the monolayer, allowing cells to be embedded, and thus antigens to be localized, while maintaining the attached cellular morphology. This method can be used whether or not the antigen is located in the ER.

Additional Materials *(also see Basic Protocol 1)*

Epon 812 resin
Embedding molds

1. Expose the cells to primary and secondary antibody, fix the cells, stain them with OsO_4, and dehydrate them (see Basic Protocol 1, steps 1 through 17).
2. Rinse the cells twice more with 100% ethanol.
3. Remove the ethanol and rinse the cells three times with 100% Epon 812 resin.

   CAUTION: *Perform embedding procedure in fume hood.*

   For this method, Spurr's formulation should not be used because it is difficult to separate from the petri dish. The Epon 812 equivalent resin is recommended.
4. Incubate cells overnight in unpolymerized plastic resin.
5. Remove the resin, apply a thin layer (1 to 2 mm) of fresh 100% resin, and polymerize the resin according to the supplier's instructions.
6. Separate the hardened plastic resin from the dish using pliers or a hammer.

7. Use a razor blade to cut small squares (~5-mm^2) of the plastic resin.

   The squares should be small enough to fit in the embedding mold.

8. Place a square of the polymerized plastic into an embedding mold with the cell side facing upward.

9. Cover the square with a thin layer of unpolymerized plastic resin. Place another square with the cell side facing downward to make a sandwich.

   If the polymerized plastic is thin enough, three to four layers can be sandwiched together.

10. Carefully overlay the sandwich with unpolymerized plastic resin to fill the mold and polymerize the resin according to the supplier's instructions.

11. Cut silver/gold sections by standard ultramicrotomy methods, orienting the resin blocks so the sections are cut perpendicular to the plane of the monolayer. Stain with lead citrate and visualize the sections (see Basic Protocol 1, steps 27 and 28).

BASIC PROTOCOL 2

IMMUNOPEROXIDASE STAINING OF TISSUE SECTIONS

This protocol describes a method for immunoperoxidase staining of cells in tissue sections, and is useful for resolving both intracellular and extracellular antigens. Localizing antigens in cells within tissues can provide a valuable complementary data set to that obtained by immunoperoxidase localization in cultured cells (see Basic Protocol 1), because often a large number of different cell types can be examined. However, this method differs in several significant ways from that applied to cultured cells due to the inaccessibility of cells contained within tissues. A primary difference is that tissues must be sectioned prior to labeling, in order to allow antibody penetration into the cells. All steps are done at room temperature unless otherwise noted. This protocol requires expertise with a Cryostat-type tissue sectioner.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Animal tissue of interest
PLP fixative (see recipe), freshly prepared
PBS, pH 7.4 (*APPENDIX 2A*)
10% (v/v) dimethyl sulfoxide (DMSO) in PBS, 4°C
Isopentane (2-methylbutane)
Liquid nitrogen
Tissue-Tek OCT compound (Baxter)
0.1% (w/v) ovalbumin/PBS
Primary antibody in 0.1% ovalbumin/PBS with 0.02% (w/v) sodium azide (NaN_3)
HRP-conjugated secondary antibody: horseradish peroxidase–conjugated Fab fragments of anti–rabbit IgG or anti–mouse IgG (Biosys) in 0.1% ovalbumin/PBS
Glutaraldehyde fixative: 1.5% (v/v) glutaraldehyde in 5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
7.5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
0.2% (w/v) DAB solution (see recipe), freshly prepared
3% (v/v) H_2O_2, freshly prepared from 30% H_2O_2 stock solution
1% (w/v) reduced OsO_4 solution (see recipe), freshly prepared and ice cold
100 mM sodium cacodylate, pH 7.4, ice cold

70%, 95%, and 100% (v/v) ethanol
100% propylene oxide, EM grade
1:1 (v/v) propylene oxide/plastic embedding resin (e.g., Spurr's)
100% plastic embedding resin (e.g., Spurr's)

Large styrofoam box or container
Cryostat-type tissue sectioner
10 × 75–mm test tubes
Slow-moving shaker or mixer
Flat embedding molds

Fix tissue

1. Fix animal tissues by perfusion (if possible) with PLP fixative for 5 min.
2. Remove tissues from animal, immerse in ~5 ml PLP fixative, and carefully slice into small pieces of workable size (~2 mm^3). Prepare extra pieces of tissue (at least three or four per tissue sample).

   If desired, obtain and fix a variety of tissues from the animal to establish a tissue bank. Once they are frozen in liquid nitrogen (see step 15), tissues can be stored almost indefinitely and used when needed.
3. Continue to fix tissue pieces by immersion for 4 to 6 hr.
4. Wash tissue pieces four times, 15 min each, in ~5 ml PBS.

Freeze tissue

5. Cryoprotect the tissue pieces by placing in ~5 ml cold 10% DMSO in PBS for 1 hr at 4°C.
6. Fill a small (~50-ml) metal beaker or cup ~¾ full with isopentane.
7. Bore a "seat" slightly less than the diameter of the cup in the bottom of a large styrofoam box or container. Place the cup in the container so that it fits snugly in the seat.
8. Fill the container with liquid nitrogen just up to the level of the isopentane and wait for the isopentane to freeze (~10 to 20 min).
9. With a small metal probe (e.g., spatula blade), melt a small well in the frozen isopentane until it is large enough to hold a piece of tissue.
10. Wait a few seconds for the isopentane to cool (but not freeze). During this time, place a piece of tissue on the end of a wooden applicator stick.
11. Rapidly plunge the end of the stick with tissue into the well of liquid isopentane.

    Within seconds, the tissue will turn white and be frozen throughout.
12. Leave in the isopentane for 5 to 10 sec.

    Do not leave the tissue in longer or the isopentane will freeze with the tissue in it.
13. Remove the stick and place in the liquid nitrogen bath.

    Tissues can be left there until all of the pieces have been frozen in a similar fashion. It may be necessary to occasionally remelt the frozen isopentane if doing several pieces.
14. With a scalpel equilibrated in liquid nitrogen, cut the tissue off the stick while immersed in liquid nitrogen being careful to keep fingers out of the liquid nitrogen.
15. Punch several small holes in a cryovial so that liquid nitrogen will bathe the tissue during storage.

16. With cold forceps, pick up the frozen tissue pieces and place in the cryovial. Store in liquid nitrogen.

    Tissue frozen in this manner can be stored indefinitely in liquid nitrogen. This is a handy stopping point.

Cut sections on a Cryostat

17. Retrieve frozen tissue and keep in a liquid nitrogen bath until sectioned.

18. Remove one piece of tissue, mount in Tissue-Tek OCT compound, and assemble the chuck of a Cryostat-type tissue sectioner. Equilibrate the tissue to −18° to −20°C.

19. Cut ~10- to 20-μm-thick sections according to manufacturer's instructions.

    Thinner sections provide better antibody penetration, but very thin ones are difficult to handle and tend to curl. For most tissues, 10 to 20 μm seems to be fine. Thicker sections are required for some tissues. For example, glomeruli tend to fall out of thin kidney sections, so kidneys should be cut at ~30 μm.

    For the subsequent immunoreactions, three to five good sections per tissue sample are normally sufficient; however, the beginner might want to cut seven to nine because some may be lost along the way.

20. Place sections into 10 × 75–mm test tubes (two to four per tube) containing 2 to 3 ml of 0.1% ovalbumin/PBS, and allow sections to come to room temperature.

Carry out antibody incubations

21. Allow the sections to sink to the bottom of the tube, carefully aspirate the ovalbumin/PBS, and gently add 200 to 300 μl primary antibody, avoiding any physical damage to the sections.

    The approximate starting antibody concentration can be determined by preliminary immunofluorescene experiments using cultured cells (see Strategic Planning) or on 2- to 4-μm-thick cryostat sections. Good immunoperoxidase signals can be achieved in sectioned material by doubling the antibody concentration that gives a good immunofluorescence signal.

22. Seal tube with Parafilm and incubate overnight with gentle shaking or mixing.

    Care must be taken to ensure that all sections remain immersed in the fluid and do not dry out.

23. Wash sections six times, 15 min each, with 1 ml of 0.1% ovalbumin/PBS with gentle shaking.

24. Remove wash solution and apply 200 to 300 μl HRP-conjugated secondary antibody. Incubate for 2 hr with gentle shaking.

    An appropriate dilution of the secondary antibody should be determined by preliminary immunoperoxidase staining experiments (see Strategic Planning). For the Biosys secondary antibody, a starting point is a 1:400 to 1:800 dilution. The Fab-HRP antibodies from Biosys are particularly good reagents (for import information for this antibody, see Basic Protocol 1, step 7).

25. Wash sections four times, 15 min each, with 1 ml of 0.1% ovalbumin/PBS with gentle shaking.

Fix sections

26. Fix sections by removing ovalbumin/PBS, applying 1 to 2 ml glutaraldehyde fixative, and incubating for 60 min with gentle shaking.

27. Wash sections four times, 10 min each, with 1 ml of 7.5% sucrose/100 mM sodium cacodylate, pH 7.4, with gentle shaking.

Carry out DAB reaction

28. Remove wash solution and apply 1 ml of 0.2% DAB solution. Incubate for 1 min.

    CAUTION: *DAB is carcinogenic. Wear gloves and work in fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.*

29. Start the DAB reaction by adding 3.3 µl of 3% H_2O_2. Quickly swirl to mix the H_2O_2 and DAB. Start timing and monitor the reaction using a dissecting microscope. React several sections until they are noticeably dark, and several other sections for a shorter period of time.

    The reactions can be carried out in the tubes and inspected with a dissecting microscope, or the sections can be transferred to a dish for viewing with an inverted microscope.

    The optimal length of time for the DAB reaction should be between 5 and 30 min for tissue sections. A nearly-optimal reaction time can be established by preliminary immunoperoxidase staining experiments (see Strategic Planning).

30. Stop the reaction by removing DAB solution and rinsing sections four times quickly with 7.5% sucrose/100 mM sodium cacodylate, pH 7.4.

Osmicate the tissue

31. Place tubes on ice in a fume hood. Remove wash solution and osmicate tissue sections with 200 to 300 µl of ice-cold, 1% reduced OsO_4 solution. Seal tubes with Parafilm and incubate for 1 hr at 4°C with gentle shaking.

    CAUTION: *Wear gloves when handling OsO_4. This step must be done in a fume hood to avoid osmium vapors. Use a small volume of osmium to reduce osmium waste. Dispose of used osmium according to institutional guidelines for hazardous chemicals.*

32. Return tubes to the fume hood, remove osmium solution, and wash sections three times quickly with 1 to 2 ml ice-cold 100 mM sodium cacodylate, pH 7.4. Allow sections to reach room temperature.

33. Dehydrate sections with a graded series of ethanol (70%, 95%, 100%) as typically done for conventional plastic embedding. Perform three or four quick rinses with 2 to 3 ml of each ethanol solution.

Embed sections

34. Replace ethanol with two 1-vol changes of 100% propylene oxide.

    CAUTION: *Perform embedding procedure in a fume hood.*

35. Replace propylene oxide with 1 vol of 1:1 propylene oxide/plastic embedding resin (e.g., Spurr's). Incubate with gentle mixing for 30 min.

36. Remove 1:1 mixture, add 1 vol of 100% unpolymerized plastic embedding resin, and gently mix overnight.

37. Replace resin with at least two 1-vol changes of fresh resin.

38. Transfer sections to flat embedding molds. Fill mold with resin and polymerize according to manufacturer's specifications for the resin.

    Several sections can be stacked in one mold and then thin sections can be made through the entire depth of all sections for greater sample size.

39. Cut silver/gold sections, stain with lead citrate, and visualize the sections (see Basic Protocol 1, steps 26 to 28).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers see* ***SUPPLIERS APPENDIX****.*

Diaminobenzidine (DAB) solution, 0.2% (w/v)

0.2% (w/v) 3,3′-diaminobenzidine tetrahydrochloride
7.5% (w/v) sucrose in 100 mM sodium cacodylate buffer, pH 7.4

Dissolve the appropriate amount of DAB in ~90% of the final desired volume of sodium cacodylate/sucrose buffer. Mix vigorously on a magnetic stirring plate in the dark (put a box over the stirring plate). Use 1 N NaOH to readjust the pH to ~7.2 to 7.4, checking by continuous monitoring with a pH meter. Bring to the final desired volume with sodium cacodylate/sucrose buffer. Filter by passing through a 0.2-µm filter. Keep in the dark until use.

The DAB should dissolve in several minutes and the solution should appear clear if slightly brownish. The pH will drop to ~6.5.

CAUTION: *DAB is carcinogenic. Wear gloves and work in fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.*

Periodate/lysine/paraformaldehyde (PLP) fixative

Solution A: 0.2 M lysine·HCl/phosphate buffer

Dissolve 1.83 g lysine·HCl in 50 ml H_2O. With continuous monitoring with a pH meter, add 0.1 M Na_2HPO_4 dropwise until pH 7.4 is reached. Bring solution to 100 ml with 0.1 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*). Store up to several weeks at 4°C.

Solution B: 8% (w/v) paraformaldehyde

Add 8 g paraformaldehyde to 80 ml H_2O while mixing on a magnetic stirring plate. Heat slowly with stirring until temperature reaches 60°C. Add 1 N NaOH dropwise until solution clears (~3 to 5 drops/100 ml). Bring to 100 ml with H_2O. After cooling, filter with Whatman no. 1 paper. Store up to several weeks at 4°C.

Fixative

Combine 3 parts solution A with 1 part solution B. Add sodium periodate ($NaIO_4$) to a final concentration of 0.01 M (21.3 mg/10 ml). Use the complete fixative at room temperature within 1 to 2 hr.

CAUTION: *Prepare the paraformaldehyde in a fume hood and wear protective gloves.*

Reduced osmium tetroxide (OsO_4) solution, 1% (w/v)

4% stock solution: Prepare 4% (w/v) OsO_4 in water and place in a glass container free of any organic contaminants. Store indefinitely at 4°C.

Working solution: Prepare a solution of 1% (w/v) OsO_4 in 100 mM sodium cacodylate, pH 7.4, by mixing one part 4% (w/v) OsO_4, two parts 200 mM sodium cacodylate, pH 7.4, and one part H_2O in a disposable plastic centrifuge tube. Chill on ice. Add solid $K_4Fe(CN)_6$ (potassium ferrocyanide) to a final concentration of 1% (w/v) and mix vigorously until it is dissolved and the solution has turned brown/black. Prepare fresh before use.

CAUTION: *Osmium solutions must be contained in a properly vented fume hood, and protective gloves should be worn. Dispose of osmium and contaminated supplies according to institutional guidelines for hazardous chemicals.*

COMMENTARY

Background Information

The ability to localize antigens, usually proteins and glycoproteins, at the EM level by immunocytochemical labeling methods has had a tremendous impact on our understanding of the subcellular and molecular organization of cells and on the dynamic processes carried out within them. Moreover, understanding the function of gene products is often aided by, and in many cases dependent on, knowing the subcellular location of the RNA or protein of interest. Not surprisingly, numerous immunocytochemical methodologies have been developed to couple the tremendous specificity of antibodies with staining methods that render antigen-antibody complexes detectable by either light or electron microscopy.

In order to be generally applicable for cell biological studies, all immunocytochemical protocols must overcome three common challenges: (1) allowing antibodies access to the intracellular environment, (2) preserving antigenicity, and (3) maintaining cellular morphology. Frustratingly, it is usually the case that solving one of these challenges only leads to problems with the others. For example, methods for maintaining excellent cellular morphology (e.g., glutaraldehyde fixation) are often counter-productive for preserving antigenicity. The protocols detailed in this unit strike a compromise that combines excellent morphological preservation with the power of antibody specificity and the signal amplification provided by enzyme cytochemistry via the peroxidase reaction (Graham and Karnovsky, 1966; Novikoff and Goldfischer, 1969; Novikoff, 1980).

Immunoperoxidase labeling at both the LM and EM levels has evolved over many years with important advances made by many laboratories (e.g., McLean and Nakane, 1974; Broadwell et al., 1979; Novikoff et al., 1979; Courtoy et al., 1980, 1982; Tougard et al., 1980; Louvard et al., 1982; Brown and Farquhar, 1984). The protocols detailed here derive from these efforts, and are useful for quite a variety of antigens. Not surprisingly, however, there are many variations of the immunoperoxidase labeling method, and it may be that some variations work better for particular antigen/antibody combinations. A few alternative procedures that readers might find useful or interesting are found in Brown and Farquhar (1989), Norgren and Lehman (1989), Saraste et al. (1995), Li et al. (1997), and Todd (1997). Conversely, DAB cytochemistry remains little changed since its early development and modification (Graham and Karnovsky, 1966; Novikoff and Goldfischer, 1969; Novikoff, 1980) and the application of ferrocyanide-reduced osmium to enhance electron density (Karnovsky, 1971).

Immunoperoxidase labeling at the EM level provides several important advantages over other methods. First, it is highly sensitive because the immunolabeled signal can be enzymatically amplified during the DAB reaction, and therefore it is quite useful for the detection of sparse antigens such as membrane receptors. Second, the morphological preservation of intracellular organelles and structures by PLP fixation (McLean and Nakane, 1974) can reach that of conventional glutaraldehyde-fixed samples. In this regard, the PLP fixative is particularly versatile for immunoperoxidase labeling at the EM level. Third, the method is fairly simple and does not require any special equipment or skills beyond those needed for standard transmission EM (TEM) of plastic-embedded samples. These three characteristics are in contrast to immunogold labeling of cryosections, which requires a special and expensive cryoultramicrotome and a high degree of specialized skill and training to generate frozen thin sections, does not provide for amplification of the immunolabeling signal, and typically generates samples in which membranes are negative images, making interpretation of results sometimes difficult.

Nevertheless, immunoperoxidase labeling has three important disadvantages when compared to immunogold. First, immunoperoxidase labeling is, by its nature, not quantitative. It gives only a qualitative picture (like immunofluorescence) of where an antigen is located in the cell. Immunogold labeling can, by rigorous statistical methods, provide important quantitative information about antigen distribution. Second, immunoperoxidase suffers from potential diffusion of the DAB reaction product away from the site of antigen-antibody binding. This diffusion leads to lower resolution than with immunogold labeling, possibly leading to erroneous conclusions about the actual site of antigen location. Third, double immunolabeling by immunoperoxidase is not generally possible, although one method for doing this has been described (Norgren and Lehman, 1989). For discussions of the limita-

tions of DAB cytochemistry see Novikoff (1973, 1980) and Courtoy et al. (1983).

Given its attractions and limitations, immunoperoxidase labeling at the EM level can be particularly useful for localizing molecules contained within intracellular membrane-bounded organelles, where antigens might be sparse (e.g., membrane receptors), but where they are sequestered within a compartment that prevents diffusion of the DAB reaction product into the cytoplasm. However, immunoperoxidase labeling can also be productively applied to antigens not contained within membranes, including both extracellular antigens (such as those in the extracellular matrix; Courtoy et al., 1980, 1982), and cell-surface antigens (Kerjaschki and Farquhar, 1983; Brown et al., 1984). In addition, immunoperoxidase can be useful for localizing certain cytoplasmic antigens, especially if they are localized to a specific region of the cell or if a low level of resolution is required (for example, when attempting to identify a particular cell type in a complex tissue or organ).

Critical Parameters and Troubleshooting

The most common problems with immunoperoxidase staining are the lack of a DAB reaction product signal or a reaction that leads to inappropriate diffusion of reaction product away from the sites of antigen-antibody binding. Without question, the DAB reaction is the most tricky step in this procedure. For this reason, two to three dishes of cultured cells (or multiple tissue sections) should be processed per experimental condition, using a range of DAB reaction times within each sample. However, with a little experience the reaction can become quite reliable and reproducible. A lack of signal at the EM level can virtually always be avoided by conducting the preliminary experiments for LM and fluorescence microscopy (see Strategic Planning). It cannot be over-emphasized how much time can be saved by spending a week or two on these preliminary experiments to establish important initial parameters.

Nevertheless, a weak or undetectable signal at the LM or EM level may result from one or more problems listed in Table 4.6.1. These problems are typically easy to diagnose and rectify by LM-level screening. When testing a new antibody for immunoperoxidase staining, be sure to include a few dishes for a known positive control antibody to distinguish between potential problems with the new primary antibody and other subsequent steps. This positive control is especially important because occasionally an antibody may work particularly well by immunofluorescence on methanol- or formalin-fixed cells but will not give an immunoperoxidase signal on cells fixed with PLP.

Inappropriate diffusion of the DAB reaction product may be harder to avoid in the beginning because it is usually only detected by EM (Fig. 4.6.1). If the preliminary experiments indicate that the antigen under investigation is contained within a membrane-bounded organelle, then all the reaction product should be sequestered within that compartment. Diffusion of reaction product out of the organelle and into the cytoplasm may indicate one of several possible problems detailed in Table 4.6.1.

Once good reaction product is obtained at the EM level, photographic documentation should be obtained. Although EM film is relatively expensive, do not cut corners here. Expose a number of negatives to have many good choices when it comes time to picking the best, most-representative image. Also, if there is a particularly good image that really demonstrates the point you want to make, take several pictures, perhaps slightly altering the focus on a few, in case something happens to one of the negatives. It is usually much easier to find an EM negative than it is to find that same cell again.

Anticipated Results

At the EM level, the DAB reaction product should be noticeably electron dense—more so than any cellular structure including membranes, ribosomes, or heterochromatin (Fig. 4.6.1). The reaction product may have a slightly mottled appearance if filling a large membrane-bounded compartment. Oxidized DAB has an affinity for membranes, so even if an antigen is present throughout the lumen of a vacuolar organelle, the reaction product may not be uniformly distributed within that lumen. Rather, the DAB reaction product may diffuse and bind to the inner surface of the limiting membrane yielding a dark rim of staining. At the LM level, the DAB reaction product appears a brown/black color.

Time Considerations

Immunoperoxidase staining of cultured cells

The labeling protocol from the beginning of fixation to the start of embedding can be done in a single day; however, it will take at least 8

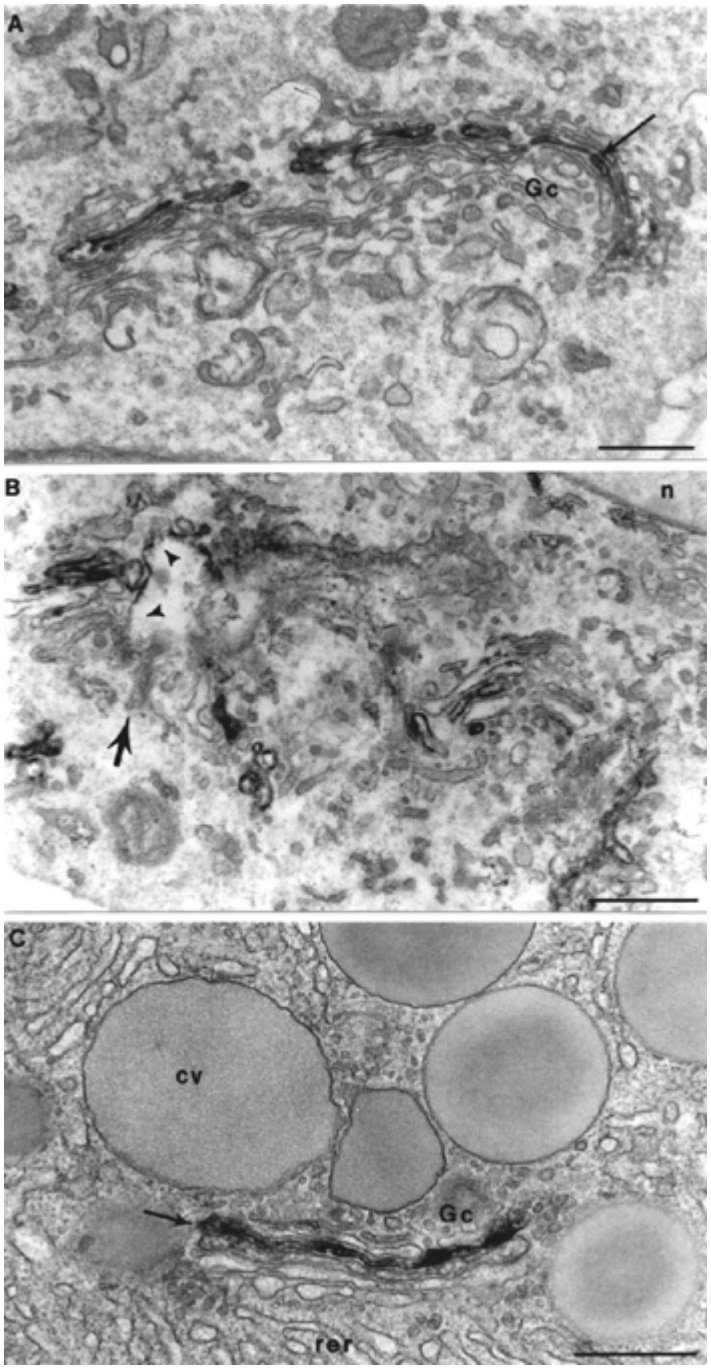


Figure 4.6.1 Immunoperoxidase localization of resident Golgi-complex enzymes in cultured cells and cells from tissue sections. (**A** and **B**) Rat clone 9 hepatocytes stained by the methods described in Basic Protocol 1 for cultured cells with antibodies to the medial Golgi enzyme α-mannosidase II. In (**A**), the very electron-dense DAB reaction product (arrow) stands out in sharp contrast to the rest of the cytoplasm and fills mainly one or two cisternal elements of the Golgi stack. This example illustrates a well-preserved, immunolabeled cell whose reacted organelle membranes are morphologically intact. Note that the DAB reaction product remains sequestered within the lumen of the cisternae. In (**B**), another clone 9 cell, reacted with the same antibody, shows significant damage resulting from cisternal elements that have become extremely dilated ("exploded") and broken (arrowheads). As a consequence, the DAB reaction product leaks into the cytoplasm (arrow). In (**C**), a portion of a rat pancreatic acinar cell is shown. The cell was labeled with antibodies against sialyltransferase, a *trans* Golgi enzyme, by the methods described in Basic Protocol 2. In this cell, the DAB reaction product also stands in sharp contrast to the cytoplasm and is sequestered within an intact cisternae (arrow). Abbreviations: cv, condensing vacuole; Gc, Golgi cisternae; n, nucleus; rer, rough endoplasmic reticulum. Scale bars, 0.5 μm.

Table 4.6.1 Troubleshooting Guide for Immunoperoxidase Localization Protocol

Problem	Possible cause	Solution
Poor general morphology	Inadequate fixation time	Fix longer
	Fixative made incorrectly	Follow recipe carefully; ensure that paraformaldehyde goes into solution
Organelles appear to be exploded, leaving holes in the cytoplasm (Fig. 4.6.1B)	DAB reaction too long or rapid	React for shorter period of time and/or slow the reaction down on ice
Weak or no DAB reaction product	Antibody titer and/or affinity low	Prepare IgG fraction or affinity purify antibodies
	Cells not well permeabilized	Increase saponin concentration or incubation time
	HRP-conjugated antibody no longer active	Check HRP activity by testing with small amount of DAB and H_2O_2
	H_2O_2 not good	Use fresh H_2O_2; check H_2O_2 in a test tube with DAB solution and 1-2 µl HRP-conjugated antibody to see if substrate turns dark brown/black
DAB reaction product diffused away from primary site of reaction and/or from membrane-bounded organelles that appear to have exploded (see above)	Samples over-reacted for DAB deposition	React for shorter period of time; lower concentration of primary or secondary antibody
	Saponin not sufficiently washed out	Increase number of washes in PBS
Immunofluorescence indicates that antigen is a membrane protein, but immunoperoxidase results in a diffuse cytoplasmic stain near an organelle but not within the lumen	DAB has possibly leaked out through a compromised membrane (see above), or worse, the epitope recognized is on the cytoplasmic face of membranes	Not much can be done if the epitope is cytoplasmically oriented; try reacting for a shorter period of time and/or on ice
DAB reaction product seen by LM but not EM	OsO_4 not properly reduced	Follow recipe for reduced osmium
General background staining	Used antiserum or antibody that is not specific	Use IgG fraction or affinity-purified antibodies; try increasing ovalbumin concentration to 1% when diluting antibody to reduce nonspecific binding
DAB not soluble	Used 3,3′-DAB free base	Use 3, 3′-DAB tetrahydrochloride
Cells will not detach from dish	Inferior quality propylene oxide	Use EM-grade propylene oxide

to 9 hr even in experienced hands and can be quite fatiguing. Also, keep in mind that this timeframe does not account for any experimental manipulations that might have been done to the cells before fixation. Therefore, the author recommends that on day 1 (following any experimental procedures) cells be fixed and processed up to and including incubation in the primary antibody, at which point the cells can be kept overnight as described in the protocol. The time from fixation to addition of the primary antibody will take minimally ~3 to 4 hr. The author does not recommend incubating cells overnight in the secondary antibody, because this gives a slightly higher nonspecific background labeling. Thus, on day 2, cells are processed through incubation with the secondary antibody, the DAB reaction, osmication, and the start of embedding. This process will take minimally ~4 to 5 hr. Infiltration of embedding plastic will proceed overnight and all the next day (day 3), and another overnight incubation is required for heat-activated polymerization. Thus, cells are ready for thin sectioning, staining, and visualization on day 4.

Immunoperoxidase staining of tissue sections

Immunoperoxidase labeling of tissue sections can conveniently be done in five days.

Day 1: Fix and freeze tissues. This will take anywhere from 6 to 8 hr depending on how many tissue samples are being processed. Tissues can be stored frozen indefinitely, until used to complete the protocol.

Day 2: In the afternoon, cut the cryostat sections. Apply the first antibody and incubate overnight. These steps can take anywhere from 1 to 4 hr depending on the level of experience with microtomes and the number of samples being processed. With experience, days 1 and 2 can be combined.

Day 3: Complete the procedure from application of secondary antibody to start of embedding sections (4 to 6 hr). Allow embedding to proceed overnight.

Day 4: Continue embedding during the day and perform heat-activated polymerization overnight.

Day 5: Thin section and stain tissue for visualization.

Literature Cited

Broadwell, R.D., Oliver, C., and Brightman, M.W. 1979. Localization of neurophysin within organelles associated with protein synthesis and packaging in the hypothalamoneurophysial system: An immunocytochemical study. *Proc. Natl. Acad. Sci. U.S.A.* 76:5999-6003.

Brown, W.J. and Farquhar, M.G. 1984. The mannose-6-phosphate receptor for lysosomal enzymes is localized in the cis Golgi cisternae. *Cell* 36:295-307.

Brown, W.J. and Farquhar, M.G. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. *Methods Cell Biol.* 31:553-569.

Brown, W.J., Constantinescu, E., and Farquhar, M.G. 1984. Redistribution of mannose-6 phosphate receptors induced by tunicamycin and chloroquine. *J. Cell Biol.* 99:320-326.

Courtoy, P.J., Kanwar, Y.S., Hynes, R.O., and Farquhar, M.G. 1980. Fibronectin localization in the rat glomerulus. *J. Cell Biol.* 87:691-696.

Courtoy, P.J., Timpl, R., and Farquhar, M.G. 1982. Comparative distribution of laminin, type IV collagen, and fibronectin in the rat glomerulus. *J. Histochem. Cytochem.* 30:874-886.

Courtoy, P.J., Picton, D.H., and Farquhar, M.G. 1983. Resolution and limitations of the immunoperoxidase procedure in the localization of extracellular matrix antigens. *J. Histochem. Cytochem.* 31:945-951.

Graham, R.C. and Karnovsky, M.J. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291-302.

Karnovsky, M.J. 1971. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. *J. Cell Biol.* 51:146a.

Kerjaschki, D. and Farquhar, M.G. 1983. Immunocytochemical localization of the Heyman's nephritis antigen (gp330) in glomerular epithelial cells of normal Lewis rats. *J. Exp. Med.* 157:667-686.

Li, J.Y., De Camilli, P., and Dahlstrom, A. 1997. Intraneuronal trafficking and distribution of amphiphysin and synaptojanin in the rat peripheral nervous system and the spinal cord. *Eur. J. Neurosci.* 9:1864-1874.

Louvard, D., Reggio, H., and Warren, G. 1982. Antibodies to the Golgi complex and the rough endoplasmic reticulum. *J. Cell Biol.* 92:92-107.

McLean, I.W. and Nakane, P.K. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077-1083.

Norgren, R.B. and Lehman, M.N. 1989. A double-label pre-embedding immunoperoxidase technique for electron microscopy using diaminobenzidine and tetramethylbenzidine as markers. *J. Histochem. Cytochem.* 37:1283-1289.

Novikoff, A.B. 1973. Studies on the structure and function of cell organelles: 3,3′-Diaminobenzidine cytochemistry. *In* Electron Microscopy and Cytochemistry (E. Wisse, W. Daems, I. Molenaar, and P. Van Duijn, eds.) pp. 89-109. North-Holland Publishing, New York.

Novikoff, A.B. 1980. DAB cytochemistry: Artifact problems in its current use. *J. Histochem. Cytochem.* 28:1036-1038.

Novikoff, A.B. and Goldfischer, S. 1969. Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. *J. Histochem. Cytochem.* 17:675-680.

Novikoff, A.B., Novikoff, P.M., Stockert, R.J., Becker, F.F., Yam, A., Poruchynsky, M.S., Levin, W., and Thomas, P.E. 1979. Immunocytochemical localization of epoxide hydrase in hyperplastic nodules induced in rat liver by 2-acetylaminofluorene. *Proc. Natl. Acad. Sci. U.S.A.* 76:5207-5211.

Saraste, J., Lahtinen, U., and Goud, B. 1995. Localization of the small GTP-binding protein rab1p to early compartments of the secretory pathway. *J. Cell Sci.* 108:1541-1552.

Todd, A.J. 1997. A method for combining confocal and electron microscopic examination of sections processed for double or triple labelling immunocytochemistry. *J. Neurosci. Methods* 73:149-157.

Tougard, C., Picart, R., and Tixier-Vidal, A. 1980. Electron microscopic studies on the secretory process in rat prolactin cells in primary culture. *Am. J. Anat.* 158:471-490.

Contributed by William J. Brown
Cornell University
Ithaca, New York

Cryo-Immunogold Electron Microscopy

INTRODUCTION

Subcellular localization of gene products, such as mRNA, miRNA, proteins, as well as (glyco)lipids, and other antigens using high-resolution cryo-immunogold electron microscopy allows for topological biochemistry at the ultrastructural level (Basic Protocol). This is the most sensitive procedure for immunodetection of several antigens simultaneously on ultrathin sections prepared from chemically fixed cells or tissues, because aldehyde fixation (Support Protocols 1 and 2) is the only denaturation step. The omission of harsh organic solvents (such as those used for plastic embedding) ensures better preservation of antigenicity. The fixed material is embedded in gelatin (Support Protocol 3), cryosectioned (Support Protocol 4), and mounted on Formvar-coated grids (Support Protocol 5). The basic principles and variations are described in the protocols below and illustrated in the accompanying videos.

IMMUNOGOLD LABELING

BASIC PROTOCOL

The precise reaction conditions used for immunogold labeling may vary depending on the antibody, and this is determined by trial and error (a useful reference is Raposo et al., 1997). This is a typical labeling protocol during which the sectioned material is exposed first to antibodies and then to protein A–gold particles. If the primary antibody does not bind to protein A, incubation with a secondary antibody that does bind protein A can be added. All procedures are carried out at room temperature. Note that for murine tissue, a secondary anti-mouse antibody can cause false positive artifacts. This antibody will also detect endogenous Ig of the mouse if present in the tissue. An alternative would be to use primary antibodies or Fab's directly coupled to ultrasmall gold and silver enhancement (see Alternate Protocol) (see *http://www.aurion.nl*) or to biotin with a rabbit anti biotin followed by protein A gold.

Materials

Cells or tissue of interest, fixed (Support Protocol 1 or 2), embedded (Support Protocol 3), cryo-sectioned (Support Protocol 4), and mounted on Formvar-coated grids (Support Protocol 5)
2% (w/v) gelatin-coated 2.5-cm Petri dishes
PBS (*APPENDIX 2A*) containing 0.15 M glycine
PBS containing 1% (w/v) BSA
Primary antibody diluted in PBS/1% BSA
PBS/0.1% (w/v) BSA
Secondary antibody in PBS/1% BSA (optional)
10-nm protein A–gold particles at OD 0.1 (commercially available from Cell Biology, Medical School, Utrecht University, The Netherlands) in PBS/1% BSA
PBS containing 1% (v/v) glutaraldehyde
Uranyl oxalate solution (see recipe; optional)
Methyl cellulose/uranyl acetate solution (see recipe)
Parafilm M
Whatman no. 50 filter paper
37°C incubator
Forceps
Stainless steel loop slightly larger than grids attached to P1000 (blue, 1000-μl) pipet tips (see Video 1 at *http://www.currentprotocols.com*).

Contributed by Peter J. Peters, Erik Bos, and Alexander Griekspoor
Current Protocols in Cell Biology (2006) 4.7.1-4.7.19

Prepare the sample

1. Place the grid containing cryosections on cold 2% gelatin plates and leave for 30 min at 37°C.

   The 2% gelatin will melt and the grids will float on top of the liquified gelatin. The 12% gelatin that was introduced into the sample during the embedding step is melted away at this point into the 2% gelatin.

2. Lift the floating grid with a pair of fine forceps and transfer it onto 100-µl droplets of PBS/0.15 M glycine laid out on a sheet of Parafilm M, keeping the upper part of the grid dry at all times (see Video 2 at *http://www.currentprotocols.com*).

   The bottom part of the grid should always remain wet during transfer to the next droplet.

3. Wash five times, each time by floating the grid for 2 min on fresh 100-µl droplets of PBS/0.15 M glycine.

   All subsequent treatments (washings and incubations) involve the transfer of the grid onto fresh droplets of medium that are laid out on Parafilm M. About 100-µl droplets should be used for all solutions except the antibody and protein A–gold, for which 5-µl droplets are used. Grids receiving the same antibody treatments can be washed together on a single drop of washing solution.

4. Wash grid once, by floating for 3 min on a 100-µl droplet of PBS/1% BSA.

Expose the sample to antibody

5. Incubate grid for the appropriate period of time at room temperature on a 5-µl droplet of primary antibody diluted to an appropriate concentration in PBS/1% BSA.

   The appropriate dilution and incubation times vary with the antibody used. Incubation time ranges from 30 to 100 min. Antibody titer is usually in the range of 0.2 µg/ml but must be tittered for each antibody in order to find the best specific signal-to-background signal ratio.

6. Wash the grid five times, each time by floating for 3 min on 100-µl droplets of PBS/0.15 M glycine, and then perform a final wash on PBS/0.1% BSA.

7. *Optional:* When a primary antibody is used that does not react with protein A (Table 7.2.1), incubate grid with a secondary (bridging) antibody that binds protein A, diluted in PBS/1% BSA, for 20 min and rinse five times for 2 min on PBS/0.15 M glycine.

Expose the sample to protein A gold

8. Incubate grid for 20 min on 10-µl droplets of 10-nm protein A–gold particles (at an OD of 0.1) in PBS/1% BSA.

9. Wash grid three times with rapid changes and then with six 3-min changes, each time by floating on 100-µl droplets of PBS.

Fix the sample

10. Incubate grid for 5 min on 100-µl droplets of PBS/1% glutaraldehyde in chemical hood.

    For double immunogold labeling, after this step wash the grids with PBS/0.15 M glycine (step 2) and repeat the entire procedure from that point on with the second primary antibody. For double-labeling experiments, use protein A with different sizes of gold particles (e.g., 10 and 15 nm).

11. Wash grid ten times, each time by floating for 2 min on 100-µl droplets of distilled water.

12. *Optional:* Incubate grid for 5 min on 100-µl droplets of uranyl oxalate solution and rinse by floating for 1 min on a 100-µl droplet of water to prevent possible artifacts introduced by uranyl acetate.

Embed the sample

13. Quickly wash grid by floating for 1 min on 100-µl droplets of methyl cellulose/uranyl acetate solution, then floating 5 min on fresh 100-µl droplets of methyl cellulose/uranyl acetate solution on ice.

14. Retrieve the grid with a stainless steel loop of diameter slightly larger than the grid, glued into a P1000 pipet tip.

15. Drain away excess methyl cellulose/uranyl acetate solution by touching the loop at an angle of 90° to a filter paper (Whatman no. 50) and tracking the loop from left to right for 2 cm. Stop and lift the loop from the paper just before the remaining volume gets absorbed by the paper (see Video 3 at *http://www.currentprotocols.com*).

16. Air dry grid at room temperature by placing the pipet tips upside-down in a pipet tip rack.

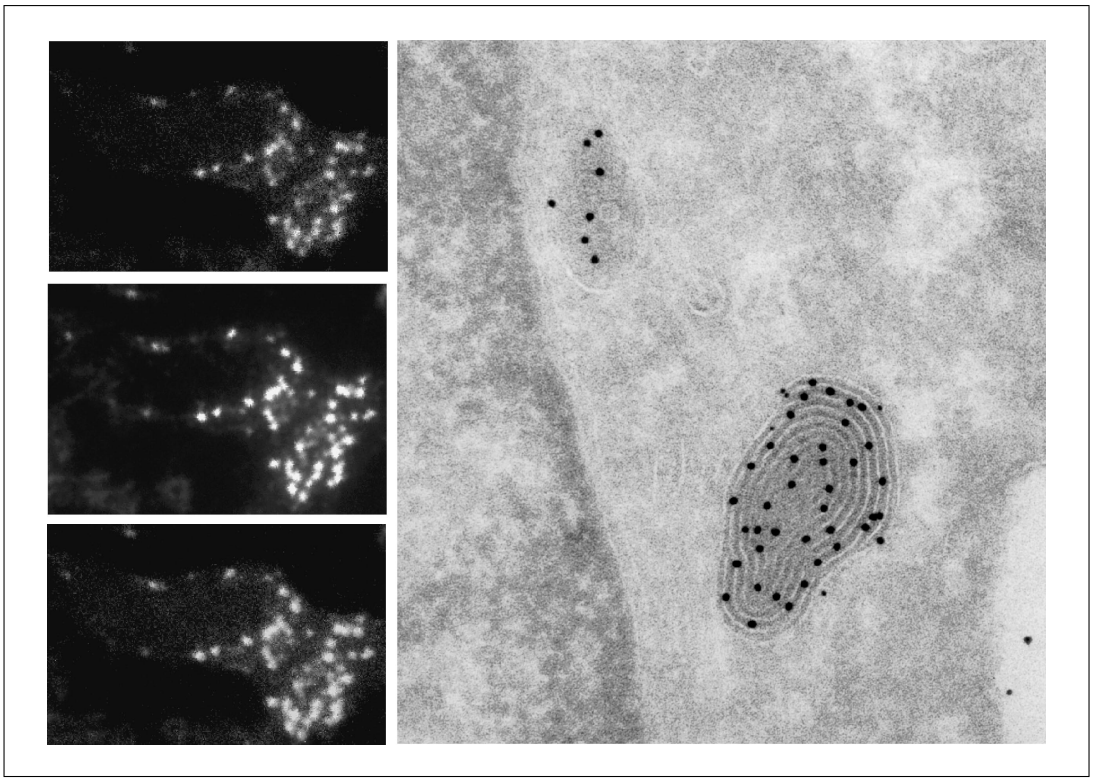


Figure 4.7.1 Light microscope and electron microscope micrographs of two adjacent ultrathin cryosection of a dentritic cell. **LM** MHC class II was detected together with CD1b by immunofluorescence on semithin cryosections of 280 nm. The CD1b is presented in the upper left panel; MHC class II in the lower left panel and a merge of the pictures is shown in the left bottom panel. CD1b and MHC class II molecules are located intracellular in the lysosomal MHC class II compartments (MIIC; see Peters et al., 1991a). Note that details of immunofluorescence pattern is comparable if not better then confocal laser scan microscopy. **EM** micrograph (right panel) of a 50-nm section. Immunogold labeling of CD1b (small gold) and MHC class II (large gold) within MIIC. Please note that by EM one can see a differential distribution of CD1b versus MHC class II within the MIIC not seen by LM. The CD1b molecules reside on the outer membrane while class II molecules are on the internal membranes of the MIIC. For the color version of this figure go to *http://www.currentprotocols.com*.

A film of methyl cellulose/uranyl acetate is left on the grid after drying. This is important to give optimal contrast and to preserve the integrity of membrane structures. The film should be slightly thicker than the sections and can be assessed by the interference colors viewed under a stereomicroscope. A gold-colored film is optimal for 50- to 70–nm sections. Currently the authors prefer one-slot grids for creating uniform thickness of cellulose and evaluating serial sections.

Examine the sample

17. Examine grid by electron microscopy at 80 kV with a small aperture.

    See Figure 4.7.1 for an example of results.

ALTERNATE PROTOCOL

IMMUNOGOLD LABELING WITH SILVER ENHANCEMENT

When ultrasmall gold–coupled antibodies or protein A is used as a reagent, silver enhancement is employed to make the ultrasmall gold particles visible in the electron microscope.

Additional Materials *(also see Basic Protocol)*

Gelatin storage plates containing grids with cryosections
PBS containing 0.5% (w/v) cold fish skin gelatin (CFG)
Fab fragments of the primary antibody conjugated to Aurion ultrasmall gold particles
Ultrasmall gold silver enhancement kit (Aurion)

Prepare the sample

1. Place the gelatin storage plates containing the inverted grids with the cryosections in an incubator maintained at 37°C for 20 min.

   The gelatin will melt and the grids that are on top will float as the gelatin changes from a solid underground to a liquid. Also, residual 10% gelatin that was introduced into the sample during the embedding step is melted away at this point. The upper part of the grid should always remain dry.

2. Lift the floating grid with a pair of forceps and transfer onto 100-µl droplets of PBS/0.15 M glycine laid out on a sheet of Parafilm M.

3. Wash five times, each time by floating the grid for 3 min on fresh 100-µl droplets of PBS/0.15 M glycine.

   All subsequent treatments (washings and incubations) involve the transfer of the grids onto fresh droplets of medium that are laid out on Parafilm. 100-µl droplets should be used for all solutions except the antibody and gold, for which 5- and 7-µl droplets are used, respectively.

4. Wash grid by floating for 3 min on 100-µl droplets of PBS/1% BSA or PBS/0.5% CFG.

Expose the sample to antibody conjugated to gold

5. Incubate grid for the appropriate period of time at room temperature on 5-µl droplets of antibody Fab fragments conjugated to Aurion ultrasmall gold diluted to an appropriate concentration in PBS/1% BSA.

   The appropriate dilution and incubation times vary with the antibody used. Incubation time ranges from 30 to 100 min. Antibody titer is usually in the range of 0.2 µg/ml but must be titered for each antibody, at the EM level, in pilot experiments.

   One can use any Fab for proper custom service coupling. See Mironov et al. (2003) for good results.

6. Wash grid six times, each time by floating for 3 min on 100-µl droplets of PBS.

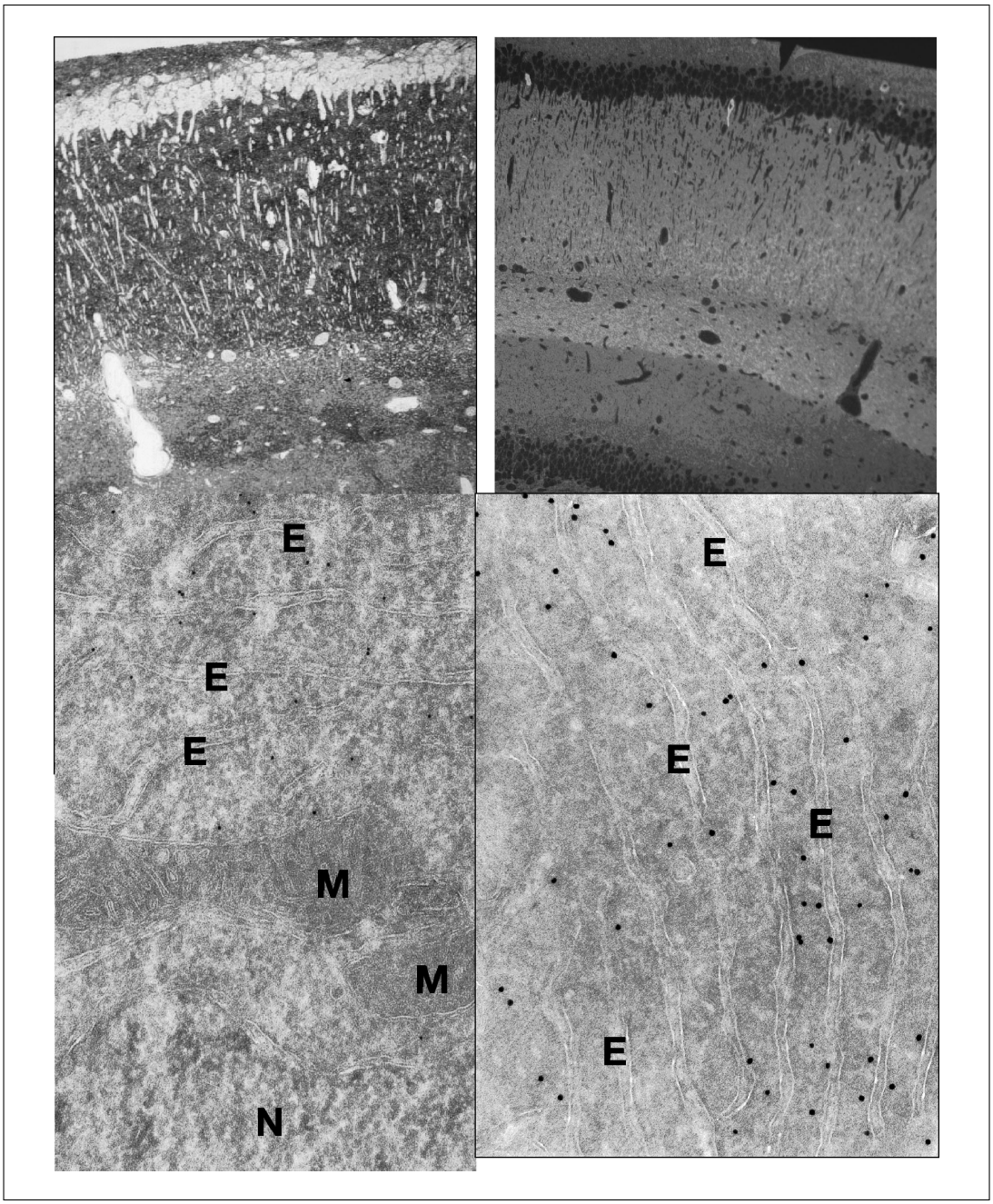


Figure 4.7.2 **LM** Hippocampal semithin cryosections labeled for PrP^C with Fab D18. Labeling is concentrated in the stratum oriens (o), stratum radiatum (r) and lacunosum-moleculare (lm); cell bodies of pyramidal (p) and granule (g) layers are free of labeling with the exception of a few cells. Upper left: Fab D18 was used with a secondary polyclonal antibody and protein A–gold (5 nm) that was subsequently visualized by silver enhancement (Aurion). Upper right: Fluorescent labeling shows a labeling pattern similar to panel A. **EM** PrP is concentrated in the cytosol in a subpopulation of neurons. Lower left: protein–A gold particles label PrP. Lower right: Direct immunolabeling with Fab D18-ultrasmall gold (Aurion) and silver enhancement demonstrates the same cytPrP abundance. For the color version of this figure go to *http://www.currentprotocols.com*.

Fix the sample

7. Incubate grid for 5 min on 100-µl droplets of PBS/1% glutaraldehyde.
8. Wash grid ten times, each time by floating for 2 min on 100-µl droplets of distilled water.

Enhance the staining

9. Prepare the enhancement mixture by adding 20 drops of the silver enhancer solution to 1 drop of the developer solution. Incubate grid for 30 min at room temperature on 100-µl droplets of the enhancement mixture.

   IMPORTANT NOTE: *This incubation procedure is temperature dependent. If the temperature is increased, then the incubation time must be decreased!*

10. Wash grid ten times, each time by floating for 2 min on 100-µl droplets of distilled water.
11. Incubate grid for a maximum of 2 min on 100-µl droplets of uranyl oxalate solution.

    Incubation >2 min will interfere with the silver deposits!

Embed the sample

12. Quickly wash grid on one 100-µl droplet of distilled water and one 100-µl droplet of methyl cellulose/uranyl acetate solution, then float the grid 2 min in a fresh 100-µl droplets of methyl cellulose/uranyl acetate solution on ice.
13. Retrieve the grid with a stainless steel loop of diameter slightly larger than the grid, attached to a P1000 pipet tip.
14. Drain away the excess of methyl cellulose/uranyl acetate solution by touching the loop at an angle of 90° to a filter paper (see Video 3).
15. Air dry grid at room temperature by placing the pipet tips upside-down in a pipet tip rack.

    A film of methyl cellulose/uranyl acetate is left on the grid after drying. This is important to give optimal contrast and to preserve the integrity of membrane structures.

Examine the sample

16. Examine grid by electron microscopy at 80 kV with a small aperture.

    See Figure 4.7.2 for an example of material stained with Aurion ultra small gold silver enhancement kit.

SUPPORT PROTOCOL 1

ALDEHYDE FIXATION OF CELLS FOR CRYO-IMMUNOGOLD LABELING

The chemical fixation step is critical for cryo-immunogold electron microscopy. The purpose of fixation is to cross-link and immobilize all subcellular structures and antigens in their most native form and maintain maximum antigenicity. Two different fixatives are commonly used: formaldehyde (FA), or a combination of FA and glutaraldehyde (GA). It is good practice to use these fixatives side by side so that later it can be determined which one is most suitable for the antigens that are being studied. (See Video 4 at *http://www.currentprotocols.com*.)

Materials

Cultures of adherent cells (from a 10-cm dish) or cells in suspension
Culture medium appropriate to the cell type
2× fixative, pre-warmed to 37°C: 2× FA (see recipe) *or* 2× FA/GA (see recipe)
Storage solution (see recipe)
PBS containing 0.15 M glycine, 37°C
PBS containing 1% (w/v) gelatin

Cell scraper
Glass Pasteur pipets (do not use plastic since several cell types may adhere to plastic)
Table-top centrifuge
1.5-ml microcentrifuge tubes
15-ml tubes (Falcon)

1. Add fresh medium to cells the day before the experiment.

   Cells should have been growing for more then 1 day (routinely 3 days) on the dish.

   At the time of fixation adherent cells should be ~ 75% confluent; the density of cells in suspension should be ~1 × 10^6 cells/ml. The total volume of growth medium should be 5 ml.

   Alternatively, the cells can be grown on an EM grid to permit analysis of the living cells by light and/or epifluorescence microscopy, microinjection of the cells, and then sectioning of the grid for EM analysis. To grow cell on an EM grid, place Formvar-coated golden grids on the bottom of a plastic Petri dish with the film side up. Add prewarmed PBS/1% gelatin to cover the entire dish. Let stand for 10 min at 37°C. Rinse with prewarmed PBS and add PBS/1% glutaraldehyde to cover the dish and allow to stand for 10 min. Rinse with sterile PBS/0.15 M glycine and then air dry. Place a droplet of cell suspension on top of each grid without floating the grids. Allow the cells to settle for 15 min and then add culture medium. Incubate for 12 to 36 hr at 37°C and evaluate the cell density by light microscopy (Stoorvogel et al., 1996).

2. Add 5 ml of 2× fixative (pre-warmed) to 5 ml of a suspension of cells that have just come out of the incubator or to a 10-cm plate of adherent cells. Incubate the cells horizontally for 24 hr (FA) or 2 hr (FA/GA) at room temperature. Do not disturb (shake or vortex) cells during fixation period.

   CAUTION: *Fixatives are corrosive to the skin, nose, and eyes. Therefore, it is advisable to work in the chemical hood with a double set of gloves and safety goggles.*

   Don't wash or centrifuge cells in PBS or any other medium before fixation, in order to avoid artifacts.

For adherent cells

3a. Replace the fixative with PBS/0.15 M glycine and wash 2 times with PBS/0.15 M glycine. Replace PBS/0.15 M glycine with 5 ml of pre-warmed PBS/1% gelatin and leave for 30 min at 37°C.

   Adherent cells can become severely damaged by air-drying. Wash cell cultures one by one and replace solutions quickly. Always leave the plates in a horizontal position!

   Using gelatin to scrape the cells produces a greater yield of cells and protects the cells from mechanical damage.

   Cells may detach after fixation. Therefore, keep the fixative and rinsing solutions, centrifuge the floating cells from these solutions and resuspend them replacing the fixative with PBS/0.15 M glycine. Pool these recovered cells later with the scraped cells. Under several experimental conditions (e.g., transfection, RNAi) cells may detach from the surface of the culture dish, go into suspension during culture, and may be missed. In this case, add an equal volume of 2× fixative to the collected medium and pellet the cells. Add the pelleted cells to the other fixed cells.

   Resuspend cells gently with a Pasteur pipet (do not use a vortexer, because this may damage ultrastructural details). Take up small volumes in the pipet when resuspending, so that cells do not get trapped on glass surface.

4a. Scrape the cells with a cell scraper. Transfer to a 1-ml tube.

5a. Centrifuge the cells 3 min at 1000× *g*, room temperature. Resuspend cells in 1 ml PBS and transfer to 1.5-ml microcentrifuge tube.

For some cells higher centrifugation speed is required.

If the samples are to be shipped to another laboratory by air, ship them in a room-temperature Styrofoam storage box to prevent freezing of the samples during flight. Frozen samples are useless!

For suspension cells

3b. Transfer suspension of fixed cells to 15-ml tube and centrifuge 3 min at 1000× *g*, room temperature.

For some cells a higher centrifugation speed is required.

4b. Resuspend cells in 1 ml PBS/0.15 M glycine and transfer to 1.5-ml microcentrifuge tube. Rinse twice in PBS/0.15 M glycine.

If fixed cells have to be stored, replace fixative with storage buffer. Make sure that the tube is filled to the top with storage buffer; this will prevent drying of cells in the tube. Cells scraped in gelatin should be rinsed in PBS/0.15 M glycine before storage buffer is added (aldehyde fixative also cross-links gelatin). Store microcentrifuge tubes that contain the fixed cells indefinitely in storage solution at 4°C in an airtight container (e.g., a 50-ml centrifuge tube).

If the samples are to be shipped to another laboratory by air, ship them in a room-temperature Styrofoam storage box to prevent freezing of the samples during flight. Frozen samples are useless!

SUPPORT PROTOCOL 2

FIXATION OF TISSUE FOR CRYO-IMMUNOGOLD LABELING

Pieces of tissue biopsy, dissected organs, or embryos can be fixed for cryo-immunogold labeling.

Materials

Tissue of interest
1x fixative: 1× FA (see recipe), *or* 1× FA/GA (see recipe)
Storage solution (see recipe)
Razor blades, acetone cleaned
Glass vials with screw caps
Fine forceps

1. Add one piece of freshly dissected tissue from an anesthetized mouse, biopsy tissue, dissected organ, or embryo immediately to 2 ml of 1× FA fixative and one to 2 ml of 1× FA/GA fixative in a glass vials with screw caps. For perfusion fixation, use a very short perfusion pulse with warm culture medium (e.g., DMEM or PBS) before perfusing with fixative.

Within 1 min after oxygen deprivation or temperature loss, cells alter dramatically at the ultrastructural level and in antigen distribution!

CAUTION: *Fixatives are corrosive to the skin, nose, and eyes; therefore it is advisable to work in the chemical hood with a double set of gloves and safety goggles.*

Perfusion fixation of tissue is preferable for achieving good morphology. The authors routinely use this for mice.

2. Cut tissue into 3-mm thick pieces using clean acetone-rinsed, air-dried razor blades and fine forceps.

Use of a dissecting microscope aids careful handling and reduces mechanical damage to the tissue. Perform this operation in a chemical hood.

3. Fix FA-fixed tissue at room temperature for 24 hr and GA/FA-fixed tissue for 2 hr, except fix GA/FA perfusion–fixed brains overnight.

   Fixing longer will result in difficulties when sectioning.

   For studies on GPI-anchored proteins that only label on FA-fixed tissue, the authors prefer to fix cells in the cold to avoid redistribution of the protein.

4. After fixation, transfer tissue to storage solution in glass vials. Store at 4°C in an airtight container.

   Once in storage solution, in preparation for embedding, the authors routinely place brain and other tissue in PBS and cut 0.5-mm slices with a vibratome for proper micro-anatomical examination of the tissue used for EM. For further storage, slices are then transferred back into storage solution.

EMBEDDING SAMPLES FOR CRYO-IMMUNOGOLD LABELING

SUPPORT PROTOCOL 3

Cells and tissues are embedded in gelatin to facilitate easy handling during the rest of the procedure. It is advisable to have a dissecting microscope with cold-light optics permanently present in a cold room. Care should be taken to prevent the samples from drying out. Gelatin allows solidification of a cell suspension and loose tissue at low temperature, so that it becomes a piece of material that can be handled relatively easily. The gelatin will melt once the ultrathin cryosections are prepared for immunolabeling. Sucrose infiltration prevents the material from ice-crystal damage during freezing in liquid nitrogen.

Materials

Fixed cells (see Support Protocol 1) or tissue (see Support Protocol 2) in storage solution
PBS
PBS containing 0.15 M glycine
12% (w/v) gelatin in 0.1 M sodium phosphate buffer (see recipe), 37°C
2% and 5% (w/v) gelatin in 0.1 M sodium phosphate buffer (see recipe), 37°C (for tissue)
2.3 M sucrose in 0.1 M sodium phosphate buffer, pH 7.4
Parafilm M
Glass Pasteur pipets
Razor blades, acetone-rinsed and air-dried
Dissecting microscope with cold-light optics
1-ml plastic vials
10-ml vials
Table-top centrifuge
End-over-end rotator (~10 sec per rotation)
Fine forceps
Greiner analyzer cup 14/24-mm, 1.5-ml, and caps (Greiner Bio-One)
Aluminum specimen holders, or steel pins, roughened with sandpaper and acetone-cleaned, dust-free
15-ml aluminum cryo tubes with two holes punched in the top
Liquid nitrogen

For fixed cells

1a. Centrifuge fixed cells 3 min at 1000 × *g*, room temperature. Remove supernatant. Resuspend cells in PBS/0.15 M glycine and centrifuge again. Repeat once more.

2a. Resuspend cells in 1 ml of 12%, 37°C gelatin, then incubate 5 min at 37°C.

3a. Centrifuge cells for 3 min at 11,180 × *g*, room temperature. Let stand on ice for 10 min to allow the gelatin to solidify.

4a. Cut the microcentrifuge tubes just above the cell pellet and cut in halves. Transfer to a 10-ml vial containing pre-cooled PBS, and keep on ice (see Video 5 at *http://www.currentprotocols.com*).

The microcentrifuge tube should be opened before cutting to avoid pressure inside the tube which will squeeze out the gelatin from the cut end.

Cold PBS prevents the gelatin from melting.

These steps are best carried out in a cold room, to ensure that the gelatin does not melt and that the sample does not dry out, as well as for easy handling with sucrose.

5a. Transfer the pellet halves to a droplet of cold PBS using forceps, but without exerting any pressure on the cells, and cut the cells/gelatin into slices of 0.5 mm. Then cut the slices into 0.5-mm cubic blocks, using clean (acetone rinsed, air dried) razor blades, under a dissecting microscope.

For fixed tissues

1b. Wash tissue two times, each time for 5 min, in PBS/0.15 M glycine.

2b. Incubate 30 min each in 2%, 5% gelatin, and twice, 30 min each, in 12% gelatin in an end-over-end rotator (13 rotations/min) at 37°C.

3b. Place the tissue in a small drop of gelatin on top of a piece of Parafim M and remove excess gelatin by pushing the piece of tissue along the Parafilm surface when the gelatin is still liquid. Put the Parafilm on ice so the remaining gelatin covering the tissue solidifies. Cover the gelatin-infiltrated piece of tissue with a drop of cold PBS. Then, proceed to step 6.

6. Transfer the cubic blocks to a Greiner cup with a cap and containing 2.3 M sucrose.

7. Rotate sample in sucrose for at least 4 hr in an end-over-end rotator in the cold room.

8. Working under the dissecting microscope and using fine tweezers with a minimum of force, transfer the sample, along with a minimum of sucrose, from the vial to very clean aluminum specimen holder that has been roughened with sandpaper or scratched with a sharp steel pin.

Roughening or scratching increases the surface of the specimen holder, which ensures better attachment of the sucrose.

9. Remove excess sucrose using clean filter paper, and transfer holders to small aluminum cryo containers filled with liquid nitrogen (see Video 6 at *http://www.currentprotocols.com*). Store in liquid nitrogen.

Sample blocks can be stored in liquid nitrogen for years and are ready for ultrathin cryosectioning repeatedly (see Support Protocol 4).

SUPPORT PROTOCOL 4

CRYOSECTIONING FOR CRYO-IMMUNOGOLD LABELING

The procedure for ultrathin cryosectioning is demanding, it requires training and practice; learning this skill is comparable to learning a musical instrument for professional performance.

Materials

Sample blocks that are stored in liquid nitrogen (see Support Protocol 3)
Section retrieval solution (see recipe), freshly prepared

Ultramicrotome (Leica) with cryochamber and antistatic devices (Diatome)
Diamond knife (Diatome; cryo-immuno 35°)
Trimming knife (Diatome; cryo-trim 45° or 20°)

Eyelash; undamaged, clean and mounted on a wooden stick (see Video 7 at *http://www.currentprotocols.com*).

3-mm diameter loop made of twisted 0.3-mm romanium wire (Winkelstroeter Dentaurum) on a 15-cm wooden stick (see Video 1 at *http://www.currentprotocols.com*)

Carbon- and formvar-coated copper grids (see Support Protocol 5)

1. Cool the ultramicrotome to −100°C. Insert and secure specimen, trimming knife, and knife for ultrathin sectioning in the ultramicrotome as described by the manufacturer.

2. Trim the front face of the block with a trimming knife by making sections of 200-nm thickness, at a speed of 100 mm/sec.

   Trimming of the sample is a prerequisite for making ribbons of ultrathin sections. The end result is a perfectly trimmed rectangular block from which ultrathin cryosections are made.

3. Trim the edges of the block by making sections from the short side of the sample block, and stop sectioning when the counter on the control unit of the microtome indicates 100 to 120 μm. Leave the block <0.25 mm wide.

4. Rotate the sample at 90° angles and trim along each side to end up with a shiny rectangular block of 0.25 × 0.50–mm or smaller. Rotate the sample back to the original position.

   At this step, it is advisable to check whether the trimmed block is suitable for further sectioning, by cutting a few 0.25-μm thin cryosections. These can then be picked up (step 6) and evaluated by light microscopy. Methylene blue staining or immunocytochemistry (UNITS 4.3 & 4.6) may be performed on these sections. If necessary, the specimen can be trimmed further after the light microscope examination. Also, sections for LM evaluation can be used to chose the proper area of interest for further trimming. This works best with the 20° Diatome trimming knife.

5. For ultrathin sectioning, set and maintain the temperature of the cryochamber, knife holder, and specimen holder of the microtome at −120°C.

6. Cut ultrathin sections of 70-nm thickness with a gold color (for FA-fixed material) or 50-nm with a light yellow color (for FA/GA-fixed material) at a speed of 1 to 2 mm/sec using a clean diamond knife (see Video 8 at *http://www.currentprotocols.com*).

   The Cryo Immuno diamond knife is preferred to the Ultrathin 35 diamond knife because it makes section manipulation easier and reduces the potential for damage to the sections. Sections are less likely to curl or fly away as they move from the diamond knife to a diamond platform, and they are less likely to twist or crumple on transfer to the pick up drop. Also it is easier to clear frost and ice crystals from the diamond surface, again reducing the potential for section damage.

   Usually an antistatic device in the chamber is used to facilitate sectioning.

   Sections should be lined up adjacent to each other so as to form a ribbon 0. 25-mm wide made up of 7 to 10 sections (depending on compression). During sectioning, sections should be manipulated with an eyelash mounted on a wooden stick in order to carefully bring the ribbon down the knife as sections are added, and to flatten out any curled up or wrinkled sections if needed.

7. Retrieve sections from the cryo chamber using a droplet of pick-up solution created on a 3-mm stainless steel loop attached to a 15-cm wooden stick. Press the droplet onto the face of the ribbon of sections and quickly remove the loop carrying the sections from the cryo chamber (see Videos 9 and 10 at *http://www.currentprotocols.com*).

   Keep the retrieval solution cup always covered with a cap to avoid evaporation and place the retrieval solution cup (Greiner Bio-One) on ice. The curvature of the droplets used for picking up should not be too great.

Avoid contact of the metal loop with the knife-edge, because this can severely damage the edge.

Successful pick-up depends on correct timing (i.e., the droplet should be applied to the section when the pick-up solution is cold but before ice crystals appear in the droplet), which is difficult to indicate and should be found by experience. Once the correct timing is found, perfectly flat sections can be obtained.

8. As soon as the sections are thawed, place them immediately onto Formvar- and carbon-coated copper grids, ensuring that the entire usable surface of the grid is covered by methyl cellulose/sucrose. Store sections up to several weeks in a closed box at 4°C. See Griffiths and Posthuma, 2002 for a study of storage conditions.

9. Before finishing the cryo-microtomy session, clean the diamond knives before the sections dry on the knife. See the Diatome webpage for instructions on cleaning diamond knives (Handling and Use Manual at *http://www.emsdiasum.com/diatome/diamond%5Fknives).*

SUPPORT PROTOCOL 5

PREPARATION OF CARBON- AND FORMVAR-COATED COPPER GRIDS

Formvar- and carbon-coated copper grids are used for mounting sections.

Materials

6% (w/v) ammonium hydroxide solution
Acetone
Formvar (Merck)
Chloroform, analytical grade

100-mesh copper grids or one-hole grids
100-ml glass-stoppered Erlenmeyer flask
Glass microscope slides
Coplin jar
Adhesive paper, e.g., address labels for envelopes
Formvar/carbon-coating device (BOC Edwards or equivalent)

Prepare the grids

1. Add 1 ml of 6% ammonium hydroxide solution to 200 copper grids (100 mesh) in a glass tube. Stir on a vortex and stop the reaction when the ammonium hydroxide solution appears slightly green when held next to a white surface. Rinse grids exhaustively in tap water (at least 10 rinses) and subsequently dry in acetone.

Prepare the Formvar solution

2. Dissolve 1.1 g Formvar in 100 ml chloroform in a glass-stoppered Erlenmeyer flask with stirring. When Formvar is dissolved stop stirring and let the solution stand overnight.

3. Rub the surface of a glass microscope slide with a soft cotton cloth and then with lens paper. Make sure that the slide is very clean and dust free.

4. Transfer the Formvar solution gently into a separating funnel with straight sides.

Prepare the Formvar film

5. Place the slide for 70% of its length into Formvar solution for 1 min (see Video 11 at *http://www.currentprotocols.com*).

6. Let the Formvar drain by opening the valve and then allow the slide to air dry in the funnel for 1 min.

The rate at which the Formvar solution drains from the container (e.g., ~5mm/sec) will determine the thickness of the Formvar coat (optimum thickness ~50 nm). A regulator can be used to control the flow rate.

7. Rub the edges of the glass slide with lens paper and scrape a thin strip of Formvar from the bottom side with a razor blade to split the film.
8. Fill a glass container completely with distilled water and level off the surface with a glass pipet. Breathe on the slide, then immediately hold the slide vertically and insert it slowly into the water, allowing the film to float on the surface.

   Formvar film will float on the surface and should have a grayish (interference color) appearance (due to the 50-nm thickness).

Coat the grids with Formvar

9. Using fine forceps place up to about 40 clean 100-mesh copper grids onto the Formvar film, with the rough surface of the grid facing the film. Avoid the edges of the film and any areas that do not appear regular.
10. Remove the Formvar film plus grids from the water surface using an ethanol-cleaned glass slide covered with a white address label on one side. Put the slide onto the Formvar film and push it into the water so that the grids adhere to the paper on the slide.
11. Let the grids air dry.
12. Evaluate the quality of the Formvar-coated grids by viewing one or two in an electron microscope.

    The film should be dust free, without significant crevices, holes, or tears, and should not break under a high-energy beam (80 kV without objective aperture).

Carbon coat the grids

13. Following the equipment manufacturer's instructions, coat the grids at least 1 day later with a layer of evaporated carbon under high vacuum. Store coated grids for up to 2 months and if needed, glow discharge them if they repel water.

    The desired thickness is critical and can be judged by evaluating the color of the address label relative to a noncoated address label. There should be only a minor change in color (the coated address label should be slightly darker in color).

    If the retrieval solution does not completely cover the grid when the loop is removed, it is necessary to glow discharge the grid. Freshly made carbon support films tend to have a hydrophobic surface which inhibits the spreading of solutions. After glow discharge treatment with air, the carbon film becomes hydrophilic and negatively charged, thus allowing easy spreading of aqueous droplets (Aebi and Pollard, 1987).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BSA stock solution, 10% (w/v)

Prepare 10% (w/v) bovine serum albumin (BSA, fraction V; Sigma) in 0.1 M sodium phosphate buffer containing 0.02% (w/v) sodium azide. Stir slowly at 4°C until BSA dissolves. Adjust pH to 7.4 and centrifuge 1 hr at 100,000 × *g*, 4°C. Retain supernatant and store in 1-ml aliquots at 4°C in closed tubes.

The BSA fraction V is prepared by heat treatment and organic solvent precipitation.

Formaldehyde (FA) fixative, 2×

Thaw a 5-ml aliquot of 16% (w/v) formaldehyde (FA) stock solution (see recipe) in 60°C water. When solution is very clear and particles of FA are dissolved completely, mix the following:

10 ml 0.4 M PHEM buffer (see recipe; 0.2 M final)
5 ml 16% FA stock (see recipe; 4% w/v final)
5 ml H_2O
Prepare immediately before use

For fixation of tissues, this solution should be diluted further (1:1) to 0.1 M PHEM buffer and 2% PFA (1×)

Formaldehyde (FA) stock solution, 16% (w/v)

Dissolve 32 g paraformaldehyde in 200 ml water. To help dissolve the FA, gradually add about 3 drops of 10 N NaOH and stir on a hotplate until the solution is clear. Do not heat above 60°C. Divide formaldehyde solution into aliquots in 10-ml tubes. Store up to several years at −20°C in a sealed box.

CAUTION: *FA is toxic, so it is necessary to prepare the solution in a fume hood.*

Once dissolved, paraformaldehyde becomes formaldehyde.

Never use formalin (37% formaldehyde in a solution that contains 10% methyl alcohol) to prepare this fixative. The methyl alcohol in formalin destroys cell ultrastructure.

Formaldehyde/glutaraldehyde (FA/GA) fixative, 2×

Thaw a 5-ml aliquot of 16% formaldehyde stock solution (see recipe) in 60°C water. When solution is very clear and particles of FA are dissolved completely, mix the following:

10 ml 0.4 M PHEM buffer (see recipe; 0.2 M final)
5 ml 16% (w/v) FA stock solution (see recipe; 4% w/v final)
1 ml 8% (v/v) glutaraldehyde (GA; 0.4% v/v final)
4 ml H_2O
Prepare immediately before use

For fixation of tissues, this solution should be diluted further (1:1) to 0.1 M PHEM, 2% FA and 0.2% GA (1×).

Gelatin, 1%, 2%, 5%, and 12% (w/v)

Prepare gelatin (Merck) solutions in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.02% (w/v) azide. Store months at 4°C in aliquots of 2 to 10 ml. Store 2% gelatin to be used for labeling in small petri dishes in a closed box at 4°C to avoid drying.

Methyl cellulose/uranyl acetate

Methyl cellulose stock: Add 2 g methyl cellulose (Sigma, 25 centipoise), while stirring, to 98 ml distilled water prewarmed to 90°C. Cool on ice while stirring, until temperature has dropped to 10°C. Stir overnight at low speed in the cold room. Let the solution stand for 3 days in the cold room. Centrifuge 95 min at 97,000 × *g*, 4°C, then divide the supernatant into 10-ml aliquots and store for up to 2 months at 4°C.

To prepare methyl cellulose/uranyl acetate: Carefully mix 1 ml of 4% uranyl acetate (see recipe) and 9 ml of methyl cellulose stock. Store up to a few weeks at 4°C in a dark container.

CAUTION: *When working with radioactivity and heavy metal, take appropriate precautions to avoid contamination of yourself and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer.*

PHEM buffer, 0.4 M

Dissolve 72.5 g PIPES (240 mM final) in 600 ml 0.375 N NaOH with stirring. Then, still stirring, gradually add 15.2 g EGTA (40 mM final). Dissolve 23.8 g HEPES (100 mM final) in 200 ml water and add this to the other mixture. Add 1.63 g $MgCl_2$ (8 mM final). Adjust pH to 6.9 with 1 N NaOH and adjust volume to 1000 ml. Store up to several years at −20°C.

Section retrieval solution

Prepare a 1:1 mixture of 2.3 M sucrose in 0.1 M sodium phosphate buffer, pH 7.4, and 2% (v/v) methyl cellulose in distilled water. Mix on an end-over-end rotator in the cold for 20 min. Prepare fresh.

Storage solution

Thaw a 5-ml aliquot of 16% formaldehyde stock solution (see recipe) in 60°C water. When solution is clear, mix the following:

5 ml 0.4 M PHEM buffer (see recipe)
0.6 ml 16% FA stock solution (see recipe), thawed and clear
14 ml H_2O
Store months at 4°C

The final concentrations are 0.1 M PHEM and 0.5% FA.

Uranyl acetate, 4% (v/v)

Prepare a 4% stock of uranyl acetate in distilled water and adjust pH to 4 with 0.1 N HCl if necessary. Store in the dark for up to 1 month at 4°C; filter through 0.22-μm Millipore filter before use.

CAUTION: *When working with radioactivity, take appropriate precautions to avoid contamination of yourself and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer.*

Uranyl oxalate solution

Mix 1 vol of 4% uranyl acetate (see recipe) and 1 vol of 0.15 M oxalic acid. Carefully adjust pH to 7 with 25% (v/v) ammonium hydroxide, stirring well after adding each drop to ensure that any precipitate that forms can dissolve. Store up to several months in the dark at 4°C.

COMMENTARY

Background Information

The pioneers in ulltrathin cryosectioning were Fernandez-Moran, Bernad, Luduc, Appleton, and Christensen. Kent Christensen was the first to devise a cryosectioning bowl attachable to a conventional ultramicrotome (Christensen, 1971). These pioneers were able to show some structural details in ultrathin cryosections.

However, rapid strides in the field of ultrathin cryosectioning came from the work of K. Tokuyasu, who developed a cryosectioning technique that is also referred to as the Tokuyasu method (Tokuyasu, 1973). He introduced a high-molar sucrose solution to infiltrate aldehyde-fixed specimens as a way to prevent ice-crystal formation and to provide the frozen specimens plasticity suitable for ultrathin sectioning. In addition, he introduced a new method of retrieving ultrathin cryosections lying on a dry knife. This was done by approaching the section with a droplet of high-molar sucrose that was about to freeze, and then quickly attaching the sections on the droplet to retrieve them from the cryochamber.

Tokuyasu faced still another problem: negative staining was effective in protecting cryosections from air drying damages, but the background was often too dense to allow recognition of immunolabeling markers, particularly ferritin which was the only marker available then. One of his solutions

was to embed the sections in a mixture of a heavy metal compound and an oganic material (Tokuyasu and Singer, 1976; Tokuyasu, 1978). This procedure is now widely applied to examination of cryosections.

The pioneers in immunogold labeling were Romano and Romano (1977), Roth et al. (1978), and Batten and Hopkins (1979). They developed procedures to couple *Staphylococcal* protein A–coated colloidal gold particles for use in localization of antigens at the EM level.

Slot and Geuze (1981) devised a simple procedure to produce a wide range of sizes of gold particles, which allowed detection of more than one signal. A comprehensive review on the history of colloidal gold in EM is the article by J. Roth (1996).

Quantitative EM immunocytochemistry was first carried out on plastic sections by Roth et al. (1978). In EM immunocytochemistry, plastic sections are advantageous over cryosections in some aspects (e.g., Roth et al., 1981; Roth and Berger, 1982) but generally it is a disadvantage because antigenicity is not as well preserved and detection is not as sensitive as with cryosections.

It was Slot and Geuze (1981) who introduced the immunogold labeling procedure on ultrathin cryosections and laid the basis for the procedure outlined in this unit. The use of different sizes of gold particles made the technique very powerful for localizing multiple antigens at the EM level (Slot et al., 1991). Griffiths et al. (1982) used a uranyl acetate and methyl cellulose mixture to stain and embed cryosections to improve the delineation of cellular structures. Major improvements in ultrothin cryosectionin were brought about by the commercial development of a high-quality cryo-ultramicrotome and cryospecific diamond knives. Cryosectioning of cells and tissues was simplified by embedding them in gelatin and cutting them into blocks after solidifying them in the cold room before sucrose infiltration (Peters et al., 1991b). Today, it is possible to localize three different antigens on ultrathin cryosections using glutaraldehyde as a blocking step between labeling procedures (Slot et al., 1991). More recently, Liou et al. (1996) showed that the structural integrity of cryosections was greatly improved by substituting a mixture of methyl cellulose and sucrose for the sucrose initially introduced by Tokuyasu. This procedure has now become first choice for high-resolution detection of antigens at the subcellular level. The methodology of amtigen localization at the EM level in epithelial cells grown on polycarbonate filters has been described (Peters and Hunziker, 2001). Recently, improved methods for quantitative immunogold EM have been formalized. Terry Mayhew and colleagues (Mayhew et al., 2002) described very useful methods for random sampling, stereological estimation, and inferential statistics that underlie them. For current immunogold EM this methodology is a must for tackling many questions.

Critical Parameters and Troubleshooting

In immunogold staining of ultrathin cryosections, there are many steps where things can go wrong. Unfortunately, whether this has happened can be determined only at the end of the procedure, by electron microscopy. The most commonly encountered problems are: low-quality sections, absence of specific labeling, high background labeling, co-label artifacts, dirty grids, and weak contrast.

Poor sections are often due to unsuitable preparation of the specimen, bad quality of knife, or instability in the cryomicrotome. Dilutions of fixatives must be prepared just before they are used for specimen fixation. Specimens that have dried out during preparation, or the presence of residual sucrose around the gelatin block, may cause sectioning problems. Also, loose attachment of the specimen holder or knife holder to the cryomicrotome will cause problems. Contamination of specimen and knife, which can result from condensation of ice crystals, must be avoided.

Nonspecific labeling is encountered mainly because the antibody does not recognize the antigen after aldehyde fixation. This can be tested by evaluating the antibody on aldehyde-fixed material by FACS (fluorescence cell sorter analysis) or immunofluorescence microscopy (*UNIT 4.3*), prior to testing the antibody at the EM level.

Overexpression of the antigen (protein) in a control cell that does not express the antigen endogenously or the use of RNAi or knockout mice are the best quality controls for specificity.

Background labeling is often the result of an excessive concentration of protein A gold or because the antibody titer was too high. For the protein A gold concentration, it is best to follow the instructions of the manufacturer. For most applications, the antibody titer is used in the range of 0.2 to 2 μg/ml.

It is best to try different dilutions and evaluate the specific signal-to-background ratio. Polyclonal, monoclonal, and recombinant Fab antibodies can be successfully employed. It is important to use a bridging antibody that binds specifically and efficiently to protein A gold as an intermediate step if the primary antibody does not bind protein A efficiently.

When double-labeling procedures are carried out, co-label artifacts are often encountered. In this case, the two sizes of gold particles are always adjacent to each other, at ~20 nm apart, and this is often mistaken for colocalization. The cause of colabeling is not understood, but often the antibody that is used for the detection of the second antigen binds to the antibody that was used to detect the first antigen. Also, protein A gold that is used in the second step may still bind to the antibody used in the first step. Glutaraldehyde incubation after the first immunolabeling should overcome the later problem. It is important to always check using a single label first.

Dirt on the grid is usually caused by phosphate contamination of the upper surface of grids or if phosphate becomes mixed with uranyl solutions before drying. Also, partial drying out of droplets during the immunolabeling incubation can result in dirty grids. Always keep the grids under a Petri dish during incubation and never touch the Parafilm where droplets are being applied.

A screening of antibodies by FACS or light microscopy before and after aldehyde fixation, and an evaluation of antibody specificity by immunoblots (*UNIT 6.2*) and immunoprecipitation (*UNIT 7.2*) may speed successful labeling on cryosections. Control cells that are transfected with or without the specific protein are very instrumental as a positive control.

Protein A gold particles of 10 and 15 nm are routinely used. The authors do not favor Ig-coated gold particles. Detection of antigen on murine tissue with monoclonal antibodies results in false positive label if a mouse Ig secondary antibody is used as a bridging step. In that case the use of monoclonal antibodies or Fab's coupled to ultrasmall gold (Aurion) with subsequent silver enhancement (kit from Aurion) is the best option (Mironov et al., 2003). The authors have recently discovered that protein A gold binds directly to prion proteins on live but not on fixed cells (Peters et al., 2003).

Transmission electron microscopy is carried out at 80 kV with the smallest objective aperture. Exposure time of film is between 0.5 and 1 sec. Damage to sections can be reduced by never evaluating sections without objective aperture.

Anticipated Results

Successful immunogold labeling should allow the detection of specific antigens at the subcellular level. Figure 4.7.1 shows light and electron micrographs of a dendritic cell labeled with anti-MHC and CD1b. Both LM and EM studies of two adjacent sections can easily be applied (van der Wel et al., 2003, 2005; Swanson and Peters, 2005).

Quantitative analysis

This aspect, which is very critical for electron microscopy work, is beyond the scope of this protocol. For a recent review see Mayhew et al., 2002. For an application of their methodology see Mironov et al., 2003; Touret et al., 2005.

Electron tomography of cryosections

The next great challenge will clearly be to understand the three-dimensional architecture of the cell in terms of the spatial arrangement of its protein components, and to describe quantitatively how this arrangement alters as the cells respond to stimuli.

Electron tomography is a powerful method to determine the three-dimensional structures of large macromolecular assemblies in cells where crystallographic methods are not applicable. The essence of this approach is to obtain a series of projection images in an electron microscope by tilting the specimen relative to the electron beam and combining the images computationally to reconstruct the three-dimensional structure. The authors have recently, in collaboration with Sriram Subramaniam (NCI) and Helmut Gnaegi (Diatome), used electron tomographic imaging to determine the molecular architecture of *E.coli* cells. By placing individual molecules into the density map derived from tomography, it was possible to construct a three-dimensional molecular model for a membrane protein network. The current excitement in this field arises from the expectation that the ongoing advances in cryo-sectioning, diamond knife developments, microscope instrumentation and computational methods may ultimately make it possible to routinely obtain resolutions in the range of 20Å, potentially good enough to locate individual proteins in a cell. The method used builds from the methodology described here, but the details are beyond the scope of this unit. For further reading see Lefman et al. (2004).

Recently the authors have developed a new "sandwich" pick-up technique that allows better preservation and less extraction of the cryosection. In addition cryo-sections are vitrified after pickup and thawing in a "vitroblot" to optimize vitrification to permit cryo-electron tomography (P.J. Peters, unpub.observ.).

Time Considerations

On average, it takes ~1 week to test and evaluate newly fixed cells with a new antibody. Often, however, multiple antibodies need to be tested at varying dilutions and under varying experimental conditions.

Acknowledgement

The methods outlined in this protocol could not have been developed without the input of former colleagues at the EM Laboratory in Utrecht. The authors are also grateful for the outstanding help of Elly van Donselaar, Nicole van der Wel, Diane Houben, and Sue Godsave.

Literature Cited

Aebi, U. and Pollard, T.D. 1987. A glow discharge unit to render microscope grids and other surfaces hydrophilic. *J. Electron Microsc. Tech.* 7:29-33.

Batten, T.F. and Hopkins, C.R. 1979. Use of protein A-coated colloidal gold particles for immunoelectronmicroscopic localization of ACTH on ultrathin sections. *Histochemistry* 60:317-220.

Christensen, A.K. 1971. Frozen thin sections of fresh tissue for electron microscopy, with a description of pancreas and liver. *J. Cell Biol.* 51:772-804.

Griffiths, G. and Posthuma, J. 2002. A reliable and convenient method to store ultrathin thawed cryoscetions prior to immunolabeling. *J. Histochem. Cytochem.* 50:57-62.

Griffiths, G., Brands, R., Burke, B., Louvard, D., and Warren, G. 1982. Viral membrane proteins acquire galactose in trans Golgi cisternae during intracellular transport. *J. Cell Biol.* 95:781-792.

Lefman, J., Zhang, P., Hirai, T., Juliani, J., Bliss, D., Kessel, M., Bos, E., Weis, R., Peters, P.J., and Subramaniam, S. 2004. Three-dimensional imaging of chemotaxis receptor networks in a bacterial cell. *J. Bacteriol.* 186:5052-5061.

Liou, W., Geuze, H.J., and Slot, J.W. 1996. Improving structural integrity of cryosections for immunogold labeling. *Histochem. Cell Biol.* 106:41-58.

Mayhew, T.M., Lucocq, J.M., and Griffiths, G. 2002. Relative labeling index: A novel stereological approach to test for non-random immunogold labelling of organelles and membranes on transmission electron microscopy thin sections. *J. Microsc.* 205:153-164.

Mironov, A. Jr., Latawiec, D., Wille, H., Bouzamondo-Bernstein, E., Legname, G., Williamson, R.A., Burton, D., DeArmond, S.J., Prusiner, S.B., and Peters, P.J. 2003 Cytosolic prion protein in neurons. *J. Neurosci.* 23:7183-7193.

Peters, P.J. and Hunziker, W. 2001. Subcellular localization of Rab17 by cryo-immunogold electron microscopy in epithelial cells grown on polycarbonate filters. *Methods Enzymol.* 329:210-225.

Peters, P.J., Neefjes, J.J., Oorschot, V., Ploegh, H.L., and Geuze, H.J. 1991a. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349:669-676.

Peters, P.J., Borst, J., Oorschot, V., Fukuda, M., Krahenbuhl, O., Tschopp, J., Slot, J.W., and Geuze, H.J. 1991b. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* 173:1099-1109.

Peters, P.J., Mironov, A. Jr., Peretz, D., van Donselaar, E., Leclerc, E., Erpel, S., DeArmond, S.J., Burton, D.R., Williamson, R.A., Vey, M., and Prusiner, S.B. 2003. Trafficking of prion proteins through a caveolae-mediated endosomal pathway. *J. Cell Biol.* 162:703-717.

Raposo, G., Kleijmeer, M.J., Posthuma, G., Slot, J.W., and Geuze, H.J. 1997. Immunogold labeling of ultrathin cryosections: Application in immunology. *In* Handbook of Experimental Immunology. Vol 4, 5th ed. (M.A. Cambridge, L.A. Herzenberg, D. Weir, L.A. Herzenberg, and C. Blackwell, eds.) pp. 208:1-11. Blackwell Scientific, Oxford.

Romano, E.L. and Romano, M. 1977. Staphylococcal protein A bound to colloidal gold: A useful reagent to label antigen-antibody sites in electron microscopy. *Immunochemistry* 14:711-715.

Roth, J. 1996. The silver anniversary of gold: 25 years of the colloidal gold marker system for immunocytochemistry and histochemistry. *Histochem. Cell Biol.* 106:1-8.

Roth, J. and Berger, E.G., 1982. Immunocytochemical localization of galactosyltransferase in HeLa cells: Codistribution with thiamine pyrophosphatase in trans Golgi cisternae. *J. Cell Biol.* 93:223-229.

Roth, J., Bendayan, M., and Orci, L. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* 26:1074-1081.

Roth, J., Thorens, B., Hunziker, W., Norman, A.W., and Orci, L. 1981. Vitamin D–dependent calcium binding protein: Immunocytochemical localization in chick kidney. *Science* 214:197-200.

Slot, J.W. and Geuze, H.J. 1981. Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* 90:533-536.

Slot, J.W., Geuze, H.J., Gigengack, S., Lienhard, G.E., and James, D.E. 1991. Immunolocalization of the insulin regulatable glucose transport in brown adipose tissue of rat. *J. Cell Biol.* 113:123-135.

Stoorvogel, W., Oorschot, V., and Geuze, H.J. 1996. A novel class of clarithin-coated vesicles budding from endosomes. *J. Cell. Biol.* 132:21–33.

Swanson, J.A. and Peters, P.J. 2005. Subcellular imaging techniques—microscopic visual imaging. *Curr. Opin. Microbiol.* 8:313-315.

Tokuyasu, K.T. 1973. A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.* 57:551-565.

Tokuyasu, K.T. 1978. A study of positive staining of ultrathin frozen sections. *J. Ultrstruct. Res* 63:287-307.

Tokuyasu, K.T. and Singer, S.J. 1976. Improved procedures for immunoferritin of ultrathin frozen sections. *J. Cell Biol.* 71:894-906.

Touret, N., Paroutis, P., Terebiznik, S., Pypaert, M., Chow, A., Jiang, A., Ghaio, J., Yip, C., Moore, H., van der Wel, N., Houben, D., Peters, P., de Chastellier, C., Mellman, I., and Grinstein, S. 2005. Quantitative and dynamic assessment of the contribution of the endoplasmic reticulum to phagosome formation. *Curr. Opin. Microbiol.* 8:151-170.

van der Wel, N.N., Sugita, M., Fluitsma, D.M., Cao, X., Schreibelt, G., Brenner, M.B., and Peters, P.J. 2003. CD1 and major histocompatibility complex II molecules follow a different course during dendritic cell maturation. *Mol. Biol. Cell* 14:3378-3388.

van der Wel, N.N., Fluitsma, D.M., Dascher, C.C., Brenner, M.B., and Peters, P.J., 2005. Subcellular localization of mycobacteria in tissues and detection of lipid antigens in organelles using cryo-techniques for light and electron microscopy. *Curr. Opin. Microbiol.* 8:323-330.

Internet Resources

http://www.nki.nl/nkidep/h4/peters/peters.htm

The website for more information about the methods described in this unit and related techniques. For more information contact Peter J. Peters at p.peters@nki.nl.

Contributed by Peter J. Peters, Erik Bos, and Alexander Griekspoor (camera and video editing)
Netherlands Cancer Institute
Amsterdam, The Netherlands

Correlative Video Light/Electron Microscopy

BASIC PROTOCOL

This unit describes newly developed methods that allow the examination of living cells by time-lapse analysis with the subsequent identification of the just-observed organelle under an electron microscope.

Many cellular functions, such as intracellular traffic, cytokinesis, and cell migration crucially depend on rapid translocations and/or shape changes of specific intracellular organelles. To understand how such functions are organized and executed in vivo, it is important to observe in real time in living cells such dynamic structures as a budding transport carrier, an elongating microtubule, or a developing mitotic spindle, but to have the degree of spatial resolution afforded by electron microscopy (EM). Most suitable for this is a conceptually simple, yet powerful, method called correlative video light/electron microscopy (CVLEM), by which observations of the in vivo dynamics and the ultrastructure of intracellular objects can indeed be combined to achieve the above-mentioned result. This unit describes this methodology, illustrates the type of questions that the CVLEM approach was designed to address, and discusses the expertise required for successful application of the technique.

The CVLEM procedure includes several stages: (1) transfection of living cells with an appropriate green fluorescent protein (GFP) fusion protein, (2) observation of structures labeled with GFP in living cells, (3) fixation, (4) immunolabeling for EM, (5) embedding, (6) identification of the cell in the resin block, (7) sectioning, and (8) EM analysis. During the first step, cells have to be transfected with the cDNA encoding the GFP fusion protein whereby the structure of interest can be discovered in living cells (Lippincott-Schwartz and Smith, 1997). In this way, it is possible to gain information about the structure, including its dynamic properties (i.e., motility, speed and direction, changes in size and shape) and life cycle. At the end of this stage, it is necessary to stabilize the cell structure by addition of a fixative, allowing one to capture the fluorescent object at the moment of interest. As GFP is not visible under an electron microscope, immunostaining is required to identify the GFP-labeled structure at an EM level. This protocol uses immunogold and immunoperoxidase protocols to perform staining for EM. Usually the immunogold protocol (Burry et al., 1992) is suitable for labeling the vast majority of antigens; the immunoperoxidase protocol should be used only to label antigens residing within small membrane-enclosed compartments, because the electron-dense products of the peroxidase reaction tend to diffuse from the place of antibody binding (Brown and Farquhar, 1989; Deerinck et al., 1994). Once stained, the cells must be prepared for EM by traditional epoxy (or other) embedding techniques, and the cell and the structure of interest must be identified in EM sections. Finding an individual subcellular structure in a single thin section can be complex, and sometimes impossible, simply because most of the cellular organelles are bigger than the thickness of a routine EM section and may lie in a plane different from the plane of a random section. Analysis of serial EM sections of the whole cell is thus required to identify the structure previously observed in vivo. An example of such an identification is shown in Figure 4.8.1. Finally, EM analysis of serial sections can be supported by digital three-dimensional serial reconstruction or high-voltage EM tomography.

Contributed by Roman S. Polishchuk and Alexander A. Mironov

Current Protocols in Cell Biology (2001) 4.8.1-4.8.9

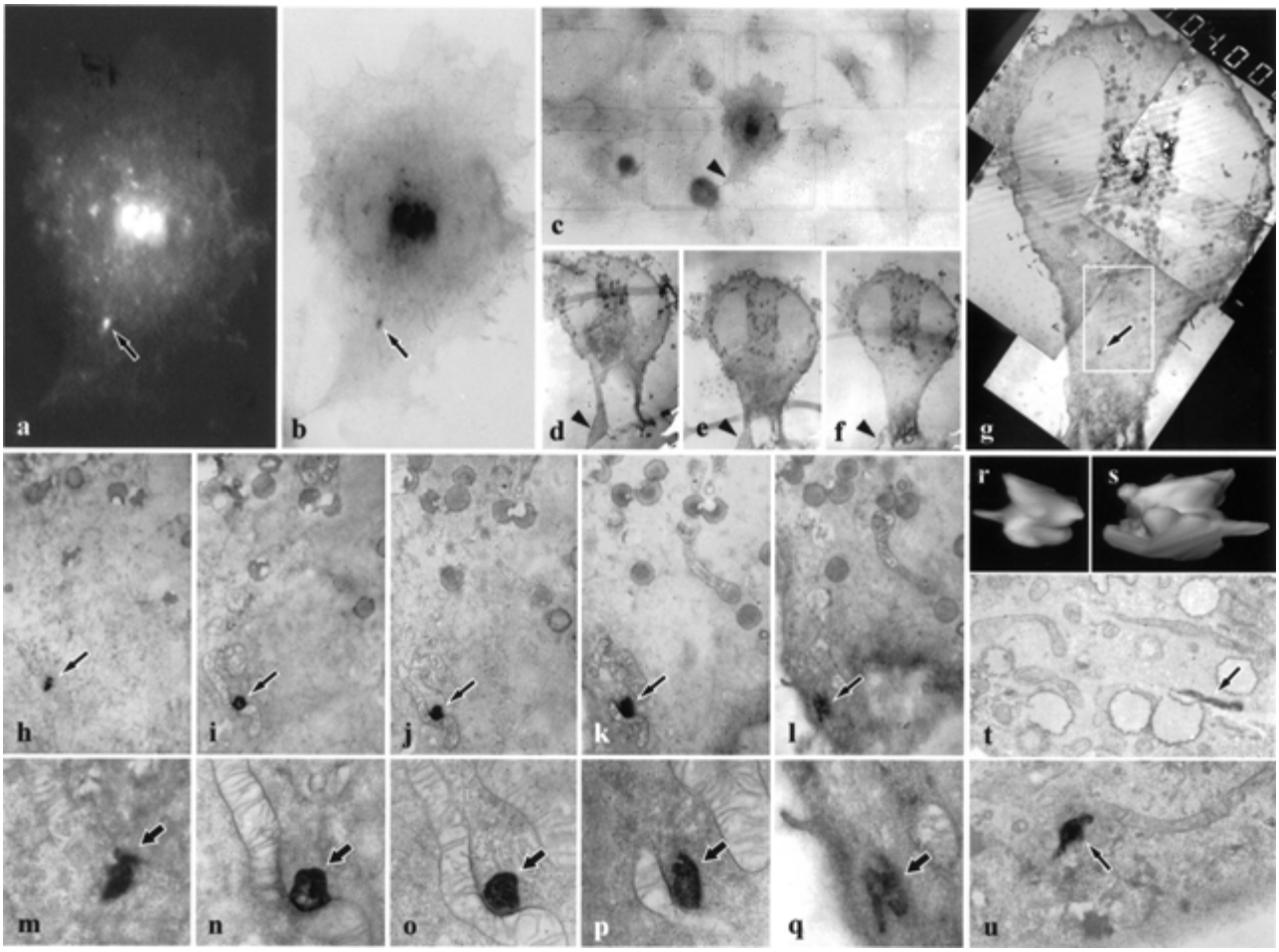


Figure 4.8.1 In vivo dynamics and ultrastructure of an individual Golgi-to-plasmalemma carrier (GPC) studied using CVLEM. The GPC in (**a**) (arrow) was observed in vivo using time-lapse confocal microscopy. After fixation, the cells were immunoperoxidase labeled and embedded in epoxy resin. The structure in (**b**) (arrow) was identified as the fluorescent GPC shown in panel **a** by the computer-aided superimposition of the two images using the CELLocate coordinates [an example of which is shown in (**c**)]. The general pattern of immunoperoxidase labeling in (**b**) coincided with the fluorescent pattern of vesicular stomatitis virus glycoprotein–GFP (VSVG-GFP) in (**a**). Serial sections of the cell were then produced (**d** through **f**), and the first section displaying the transport intermediate [(**g**); arrow] was captured at low magnification (**f**, **g**). Note that the sections contain structures that are helpful for identification [e.g., protrusion in (**c** through **f**); arrowheads]. (**h** through **q**) represent a series of consecutive 200-nm sections containing the GPC (arrow). The field shown in (**h** through **l**) is the area of the cell identified by a white box in (**g**). Three-dimensional reconstruction characterizes the GPC as an elongated saccules with a short tubular protrusion (**r**, **s**). Other GPCs identified using the same approach appeared either as tubules [(**t**); arrow] or saccules with short tubules [(**u**); arrow]. The bar in panel **u** represents the following lengths in the other panels: 9 μm (*a*, *b*), 31 μm (*c*), 12.2 μm (*d-f*), 6 μm (*g*), 2.1 μm (*h-l*), 700 nm (*m-q*), 320 nm (*r*, s), 1.2 μm (*t*, *u*).

Materials

Cells of interests
cDNA encoding an appropriate GFP fusion protein
Fixative A (see recipe)
Fixative B (see recipe)
PBS (*APPENDIX 2A*)
Blocking solution (see recipe)
Primary antibody to label structure of interest
Monovalent Fab fragments of secondary antibody conjugated with horseradish peroxidase (HRP; Rockland) or Nanogold (Nanoprobes)
1% (w/v) glutaraldehyde in 0.2 M HEPES buffer, pH 7.3

Gold enhancement mixture (see recipe)
DAB solution (see recipe)
2% (w/v) OsO_4 (Electron Microscopy Sciences) in water
3% (w/v) potassium ferrocyanide in 0.2 M cacodylate buffer
0.2 M cacodylate buffer, pH 7.4 (see recipe)
50%, 70%, 90%, and 100% (v/v) ethanol
Epoxy resin (see recipe)
8-mm resin cylinder prepared in advance from a cylindrical mold

35-mm MatTek petri dishes with CELLocate coverslip and map of CELLocate grid (MatTek)
Inverted fluorescence microscope
Multiphoton microscope, laser-scanning confocal microscope, or digitalized fluorescence inverted microscope capable of acquiring a time-lapse series of images by computer
60°C oven
Ultramicrotome with sample holder, glass knife, and diamond knife
Eyelashes
Pick-up loop (Agar)
Adjustable-angle laboratory clamps
Slot grids covered with carbon-formvar supporting film (Electron Microscopy Sciences, Agar)
Electron microscope
Software for three-dimensional reconstruction from serial sections

Additional reagents and equipment for transfection (*APPENDIX 3A*)

Transfect cells

1. Plate cells of interest at 50% to 60% confluence on a 35-mm MatTek petri dish with the CELLocate coverslip attached to its bottom.

   The CELLocate coverslip contains an etched grid with coordinates that allow the localization of the cell of interest at any step in the preparation.

   To facilitate the process of locating the visualized cell of interest in the resin block, the cells should be plated at 50% to 60% confluence.

2. Transfect cells with cDNA encoding an appropriate GFP fusion protein using any method described in *APPENDIX 3A*.

   If electroporation is used, steps 1 and 2 should be reversed.

   The authors have successfully used procollagen I–mannosidase II–, and vesicular stomatitis virus glycoprotein (VSVG)–GFP constructs for transfection.

Observe labeled structures in living cells

3. Place the dish under an inverted fluorescence microscope, select a transfected cell of interest, and identify its position relative to the coordinates of the CELLocate grid.

4. Draw (or photograph) the position of the cell on the map of the CELLocate grid.

5. Observe the dynamics of the GFP-labeled structures in the selected living cell using a multiphoton microscope, laser-scanning confocal microscope, or digitalized fluorescence inverted microscope that allows acquisition of a time-lapse series of images by a computer.

Fix cells

6. At the moment of interest, while still acquiring images, add fixative A to the cell culture medium at a ratio of 1:1 fixative/medium.

   Fixation usually induces the fast fading of GFP fluorescence and blocks the motion of labeled structures in the cell.

7. Stop acquiring images and keep cells in fixative for 5 to 10 min.

   During this time it is useful to acquire a Z series of images of the cell.

8. Wash with 2 ml fixative B and leave the cells in this fixative for 30 min.

Immunolabel cells for EM

For EM with Nanogold:

9a. Wash cells three times for 5 min with 2 ml PBS.

10a. Incubate cells 30 min in 2 ml blocking solution.

11a. Incubate cells overnight with primary antibody diluted in blocking solution.

   The dilution depends on the antibody; in general, the concentration of antibodies for EM should be 5- to 10-fold higher than for immunofluorescence.

12a. Wash cells six times for 2 min with 2 ml PBS.

13a. Dilute Nanogold-conjugated Fab fragments of the secondary antibody 1:50 (v/v) in blocking solution and add it to the cells; incubate 2 hr.

14a. Wash cells six times for 2 min with 2 ml PBS.

15a. Fix cells 5 min with 1 ml of 1% glutaraldehyde in 0.2 M HEPES buffer, pH 7.3.

16a. Wash cells three times for 5 min with 2 ml PBS and then again three times with distilled water.

17a. Incubate cells 6 to 10 min with 0.5 ml gold enhancement mixture.

18a. Wash cells three times for 5 min with 2 ml distilled water.

For EM with HRP:

9b. Wash cells three times for 2 min with 2 ml PBS.

10b. Incubate cells 30 min in 1 ml blocking solution.

11b. Incubate cells overnight with primary antibody diluted in blocking solution.

   The dilution depends on the antibody; in general, the concentration of antibodies for EM should be 5- to 10-fold higher than for immunofluorescence.

12b. Wash cells six times for 2 min with 2 ml PBS.

13b. Dilute HRP-conjugated Fab fragments of the secondary antibody 1:50 (v/v) in blocking solution and add it to the cells; incubate 2 hr.

14b. Wash cells six times for 2 min with 2 ml PBS.

15b. Fix cells 5 min with 1 ml of 1% glutaraldehyde in 0.2 M HEPES buffer, pH 7.3.

16b. Wash cells three times for 5 min with 2 ml PBS.

17b. Incubate the cells with 1 ml DAB solution.

18b. Wash cells three times for 2 min with 2 ml PBS.

Embed cells

19. Incubate cells in 0.5 ml of a 1:1 (v/v) mixture of 2% OsO_4 and 3% potassium ferrocyanide in 0.1 M cacodylate buffer on ice for 1 hr.
20. Wash cells with 2 ml distilled water.
21. Dehydrate cells in a series of ethanol solutions: 50% (10 min), 70% (10 min), 90% (10 min), and three times in 100% (10 min each).
22. Incubate cells 1 to 2 hr in 1:1 (v/v) epoxy resin/ethanol, room temperature.
23. Incubate cells in pure epoxy resin for 1 hr at room temperature and then overnight (≥12 hr) in a 60°C oven.

Isolate cell of interest in the resin block

24. Put a droplet of fresh epoxy resin on the block where the examined cell is located. Use the fresh resin as glue to attach the block of embedded cells to the flat base of an 8-mm resin cylinder. Return the assembly to the 60°C oven for an additional 16 hr.
25. Carefully lift the resin from the petri dish and cover glass.

    It is easiest to remove the resin by gently bending the cylinder to and fro.

    If the CELLocate cover glass cannot be detached from the embedded cells, incubate the sample in a plastic tube with hydrofluoric acid (HF; Sigma) for 30 to 60 min to dissolve the glass. Do not use a glass tube, as it wll be dissolved by the HF. Wash with water and monitor the completeness of glass dissolution under a stereomicroscope. If glass is not dissolved, repeat treatment with HF and wash again with water. Incubate sample 60 min in 0.1 M PBS or 0.2 M HEPES buffer, pH 7.3, to neutralize the HF, wash with water, and dry.

    If desired, embedded specimens can be stored for an unlimited time at room temperature before sectioning and analysis.

Prepare EM sections

26. Trim the resin block to give a pyramid of ~3 × 3 mm size with the cell of interest in its center.
27. Put a sample holder into an ultramicrotome so that the segment arc of the ultramicrotome is in the vertical position. Secure a glass knife in place.
28. Bring the sample as close to the knife as possible.
29. Rotate the knife stage to align the bottom edge of the pyramid parallel to the knife edge.
30. Tilt the segment arc and the knife to adjust the gap between the knife edge and the surface of the sample.

    The gap is visible as a bright band if all three lamps of the ultramicrotome are switched on. It must be identical in width during the up and down movements of the resin block to ensure that every point of the sample surface containing the cell of interest is at the same distance from the knife edge.

31. Turn the specimen holder 90° to the left or to the right and trim the edges of the resin block to leave a narrow pyramid (≤100 µm wide) with its long axes parallel to the knife edge.

    The pyramid should be as narrow as possible and the cell of interest should be in its center. An experienced person can trim a pyramid directly with a razor blade.

32. Turn the specimen holder 90° back and lock it in exactly the same position as before.

    This is very important.

33. Replace the glass knife with a diamond knife and position the latter towards the pyramid.

34. Make serial sections according to the instructions for the ultramicrotome.

35. Stop the motor. Use two eyelashes to divide the band of the sections into pieces suitable in size for collection with a pick-up loop.

    Each piece should be small enough to fit completely inside the inner circle of the loop without touching it.

36. Pick up a band of sections by touching the pick-up loop to the surface of water containing the band, making sure the band is completely inside the inner circle of the loop and does not touch it.

37. Raise the loop with the sections on it, and fix the loop inside the lab clamps near the stereomicroscope of the microtome.

    The loop should be visible under the stereomicroscope of the ultramicrotome.

38. Take a formvar-coated slot grid and gently touch sections on the water (do not touch the loop) with the carbon-coated surface of the grid.

39. Very slowly move the slot grid away from the loop.

    If the movement is slow enough, the water is eliminated from the surface of the supporting film, and only a very small droplet of water remains on the grid, which does not hinder the placement of the grid directly into the grid container. Grids can be stored an unlimited time at room temperature.

Perform EM analysis

40. Place the slot grid under the electron microscope and identify the cell, using the traces of the coordinated grid filled with the resin on the first few sections.

41. Take consecutive photographs (or acquire the images with a computer using a video camera) from the serial sections until the organelle of interest (just observed by light microscopy) is no longer seen.

42. Using software designed for three-dimensional reconstruction, align the images and make a three-dimensional model according to the instructions for the software.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Blocking solution

100 ml PBS (*APPENDIX 2A*) containing:
0.5 g BSA
0.1 g saponin
0.27 g NH_4Cl
Store up to 3 months at 4°C

Cacodylate buffer, 0. 2 M

2.12 g sodium cacodylate
100 ml water
Adjust to pH 7.4 with 1 N HCl
Store up to 6 months at 4°C

Diaminobenzidine (DAB) solution

10 mg DAB
20 ml 0.1 M Tris·Cl, pH ~7.6 (*APPENDIX 2A*)
Prepare fresh
Just before use add 13.3 µl of 30% H_2O_2

Epoxy resin

Combine 20 g epoxy embedding resin (Fluka), 13.0 g dodecenylsuccinic acid (DDSA), and 11.5 g methylnadic anhydride (MNA) in a test tube. Heat in a 60°C oven for 2 to 3 min and then vortex well to mix the components. Add 0.9 g 2,4,6 Tris (dimethylaminomethyl) phenol (DMP-30) and immediately vortex again. Store 1-ml aliquots up to 6 months at –20°C.

Fixative A (0.1% glutaraldehyde, 4% paraformaldehyde)

Add 1.25 ml of 8% (w/v) glutaraldehyde to 100 ml of fixative B (see recipe). Store up to 2 to 3 days at 4°C.

Fixative B (4% paraformaldehyde)

Dissolve 4 g paraformaldehyde powder in 100 ml 0.2 M HEPES buffer, pH 7.4, while stirring and heat to 60°C. Add a few droplets of 1 N NaOH to form a clean solution. Store up to 3 months at 4°C.

Gold enhancement mixture

Mix equal volumes of solutions A (enhancer; green cap) and B (activator; yellow cap) of a Gold-enhance kit (Nanoprobes) and wait 5 min. Add equal volumes of solution C (initiator; purple cap) and then solution D (buffer; white cap) and mix. Prepare ~400 µl final reagent per dish.

A convenient method is to use equal numbers of droplets from each bottle.

COMMENTARY

Background Information

Correlative light/electron microscopy (CLEM) was developed several years ago, and has been used when the analysis of immunofluorescently labeled structures required higher resolution than can be achieved using light microscopy (Tokuyasu and Maher, 1987; Powell et al., 1998). In spite of its potential, CLEM has not been used very often, probably because the possibility of correlating two static images–one fluorescent and one electron microscopical–is of interest only in a limited number of situations. The real gains from CLEM come from its combination with the kind of dynamic observations obtainable from GFP video microscopy in living cells (i.e., from its use in CVLEM). The CVLEM approach is potentially valuable in any area of cell biology where elucidating the three-dimensional ultrastructure of individual dynamic cellular objects at times of choice can be informative (Mironov et al., 2000). For example, the growth of a subset of microtubules can be visualized in vivo (Perez et al., 1999). By CVLEM, it is now possible to study at EM resolution the environment in which they are growing, as well as the interactions of the microtubule tips with other cytoskeletal elements or intracellular organelles, at various stages of tip growth. In addition to cytoskeletal dynamics, CVLEM could also be useful for studying cell division and cell-cell interactions (although the specific questions to be addressed are best left to the specialists). CVLEM has been applied successfully to characterize the ultrastructure of membrane carriers transporting secretory proteins from the Golgi complex to the cell surface (Polishchuk et al., 2000).

One limitation, and, at the same time, attraction, of CVLEM is its complexity. The use of this technique is demanding, and to master its various steps requires a whole array of skills. However, microscopy is developing fast both in the field of live-cell imaging and in the field of EM, and new powerful technologies are rapidly becoming available in user-friendly versions. This should make the use of CVLEM appealing to a number of cell biologists. Through the use of fluorescent proteins of different colors, two or more marker proteins can

be observed simultaneously (Ellenberg et al., 1999). This will allow analysis of the interactions between different organelles and organelle subdomains (Ellenberg et al., 1999; Pollok and Heim, 1999). Combining these and other methods with the quickly developing electron microscope tomography (Ladinsky et al., 1994) will increase the subtlety and range of the questions that can be answered by CVLEM. While we wait for the microscopy of the future, endowed with the magical power to show cellular structures with EM resolution in vivo in real time, CVLEM offers a useful chance to look deeper inside living cells.

Critical Parameters

Taken together, all the steps of CVLEM represent quite a long procedure (see Time Considerations) and require significant effort by a researcher or technician. Hence, it would be especially disappointing to loose such tour-de-force experiments due to small problems in specimen handling. To apply CVLEM successfully, several important parameters should always be taken into account by the experimenter.

Fixation/labeling protocol. Preliminary experiments should be carried out to understand whether the antibodies selected for labeling of a GFP–fusion protein work with the immuno-EM protocol. Many antibodies that give perfect results for immunofluorescence do not work for immuno-EM staining, because the glutaraldehyde used in most EM fixatives tends to cross-link amino groups of antigen epitopes, therefore decreasing the antigenicity of the target protein and restricting the penetration of antibodies across the cytosol. However, a decrease or an absence of glutaraldehyde in the fixative can result in poor preservation of ultrastructure of the intracellular organelles. If immuno-EM labeling is not successful, it may be possible to optimize the concentration of glutaraldehyde in the fixative, or to use the periodate/lysine/paraformaldehyde fixative described by Brown and Farquhar (1989). In addition, it is important to select an immunoperoxidase or immunogold protocol to label the structure of interest. To label epitopes of a GFP–fusion protein located in the cytosol, only the immunogold protocol should be used; for other epitopes, HRP labeling is also suitable.

Locating the cell of interest. It is extremely important to be able to find the cell of interest at any step in the CVLEM procedure. Only cells located on the grid of the MatTek petri dish can be selected for time-lapse observations. The position of the cell of interest on the grid should be noted, otherwise the cell will be difficult to locate again. Low-magnification images showing the field surrounding the cell of interest can greatly help when trimming the resin block around the cell and when locating the cell under the electron microscope. In this case, neighboring cells can be used as landmarks to identify the cell of interest. It is more convenient to work with cells that are plated at a lower confluence (e.g., 50% to 60%). It is also useful to have fluorescent and phase-contrast images of the target cell, because particular structures (e.g., microvilli, pseudopodia, inclusions) can also be used to help find both the cell and the structure of interest.

Section thickness. The thickness of serial sections should be ~50 nm (or less) for very precise three-dimensional reconstruction, ~80 nm for routine work, or ~250 nm for electron microscope tomography.

Anticipated Results

Once considerations for specific localization of the cell and the organelle have been optimized, the precise three-dimensional structure of the organelle and its connections with other organelles at different moments of its life cycle can be identified.

Time Considerations

The entire CVLEM procedure requires 4 to 5 days. During the first day, cells must be transfected with cDNA. The next day, it is possible to make observations in living cells, fix them, and start the immunolabeling. The third day is required to complete the immunolabeling and resin embedding of the cells. During the fourth experimental day, the cell of interest is identified in the resin block and cut into serial sections. On the fifth day, EM analysis is performed. If desired, it is possible to store embedded specimens indefinitely before sectioning and analysis.

Literature Cited

Brown, W.J. and Farquhar, M.G. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. *Methods Cell Biol.* 31:553-569.

Burry, R.W., Vandre, D.D., and Hayes, D.M. 1992. Silver enhancement of gold antibody probes in pre-embedding electron microscopic immunocytochemistry. *J. Histochem. Cytochem.* 40:1849-1856.

Deerinck, T.J., Martone, M.E., Lev-Ram, V., Green, D.P., Tsien, R.Y., Spector, D.L., Huang, S., and Ellisman, M.H. 1994. Fluorescence photooxidation with eosin: A method for high resolution

immunolocalization and in situ hybridization detection for light and electron microscopy. *J. Cell Biol.* 126:901-910.

Ellenberg, J., Lippincott-Schwartz, J., and Presley, J.F. 1999. Dual-colour imaging with GFP variants. *Trends Cell Biol.* 9:52-56.

Ladinsky, M.S., Kremer, J.R., Furcinitti, P.S., McIntosh, J.R., and Howell, K.E. 1994. HVEM tomography of the trans-Golgi network: Structural insights and identification of a lace-like vesicle coat. *J. Cell Biol.* 127:29-38.

Lippincott-Schwartz, J. and Smith, C.L. 1997. Insights into secretory and endocytic membrane traffic using green fluorescent protein chimeras. *Curr. Opin. Neurobiol.* 7:631-639.

Mironov, A.A., Polishchuk, R.S., and Luini, A. 2000. Visualising membrane traffic in vivo by combined video fluorescence and 3-D-electron microscopy. *Trends Cell Biol.* 10:349-353.

Perez, F., Diamantopoulos, G.S., Stalder, R., and Kreis, T.E. 1999. CLIP-170 highlights growing microtubule ends in vivo. *Cell* 96:517-527.

Polishchuk, R.S., Polishchuk, E.V., Marra, P., Buccione, R., Alberti, S., Luini, A., and Mironov, A.A. 2000. GFP-based correlative light-electron microscopy reveals the saccular-tubular ultrastructure of carriers in transit from the Golgi apparatus to the plasma membrane. *J. Cell Biol.* 148:45-58.

Pollok, B.A. and Heim, R. 1999. Using GFP in FRET-based applications. *Trends Cell Biol.* 9:57-60.

Powell, R.D., Halsey, C.M., and Hainfeld, J.F. 1998. Combined fluorescent and gold immunoprobes: Reagents and methods for correlative light and electron microscopy. *Microsc. Res. Tech.* 42:2-12.

Tokuyasu, K.T. and Maher, P.A. 1987. Immunocytochemical studies of cardiac myofibrillogenesis in early chick embryos. II. Generation of α-actinin dots within titin spots at the time of the first myofibril formation. *J. Cell Biol.* 105:2795-2801.

Contributed by Roman S. Polishchuk and Alexander A. Mironov
Consorzio Mario Negri Sud
S. Maria Imbaro (Chieti), Italy

Polarization Microscopy

INTRODUCTION

Polarization microscopy, also called polarized light microscopy, allows one to nondestructively follow dynamic, anisotropic organization of living cells and tissues at the microscopic as well as submicroscopic levels.

Since, in polarization microscopy, light waves are used to probe the specimen, spatial resolution of the image is limited to ~0.2 µm, even using objective and condenser lenses with numerical apertures approaching 1.4. Nevertheless, the brightness of each resolved area in the image measures the polarization optical property, most commonly birefringence, of the corresponding minute area in the specimen. The birefringence directly reflects the anisotropic organization of the microscopically unresolvable, fine structure—namely, the submicroscopic, molecular, and atomic lattice arrangements of the specimen within each diffraction-limited image area.

Thus, e.g., the assembly and disassembly dynamics of spindle microtubules and the developmental changes in sperm chromatin could be revealed, and the arrangement of the atomic lattice in developing biocrystalline spicules, as well as the packing arrangement of DNA molecules in sperm chromosomes, could be unveiled with polarization microscopy at levels even finer than those conventionally seen with electron microscopy.

Examples in this unit were chosen mostly from those particularly familiar to the author and which have been used successfully by many generations of students and co-workers. Beyond the biological material mentioned below, or those that are commonly used for studying living cell behavior and fine structure of their organelles, a treasure trove of suggestions for other living cells appropriate for study can be found throughout an article by Belar (1928).

The first part of this unit touches on some general procedures, and outlines the background optical theory and relevant literature sources for polarization microscopy. The unit then examines several basic examples for using the polarizing microscope, emphasizing the study of living cells or products, starting with those that can be carried out with relatively simple equipment and proceeding to examples that require more advanced instruments. For instructions on setting up and operating a polarization microscope see Murphy (2001), Oldenbourg (1999), and manufacturer's instructions.

For more comprehensive discussions and an annotated list of references on polarized light and birefringence phenomena, see Inoué (1986, especially Appendix 3). For additional information, see Bennett (1950), Jenkins and White (1957), Hartshorne and Stuart (1960), and Hecht (1998).

Types and Transmission Axis of Polars

Polarizers, or polars, range from plastic-sheet "Polaroids" to calcite polarizers of many designs. Although very expensive and fragile, the calcite polarizers provide the theoretically maximum transmission (i.e., nearly 50% of the incoming nonpolarized light) over a wide wavelength range. Their extinction factor (EF; see in Example 5 below) also tends to be an order of magnitude better than the sheet Polaroids. On the other hand, light transmitted through the calcite polars has to be collimated into parallel beams in order to avoid astigmatism. They are used in expensive polarization optical systems requiring the highest EF and minimum loss of light. Some new sheet polarizers such as "Polacolor" and those available from Spindler and Hoyer are reported to have both high transmission for white light and high EFs.

Sheet Polaroids generally contain a stretched sheet of polyvinyl alcohol, a long-chain polymer whose backbone can be aligned by heating and stretching the polymer sheet that has been cast from an aqueous solution. Commonly they are stained with polyiodide micelles, whose conjugated backbones lie parallel to the aligned polyvinyl alcohol chains. It is the conjugated backbone of the polyiodide that gives rise to the *dichroism* of these Polaroid sheets (Inoué, 1986). They can be made into large sheets and can easily be cut into various shapes with a pair of scissors. This is the material used for Polaroid sunglasses. As polars in microscopes, their soft and somewhat wavy surfaces are protected by mounting them with index-matched cement between antireflection-coated glass cover plates.

The transmission axis (E-vector direction of the transmitted polarized light) for Polaroid sunglasses is oriented vertically in order to reduce the glare from horizontal surfaces, such as the road, water, or hood of a car. Light

Contributed by Shinya Inoué

Current Protocols in Cell Biology (2002) 4.9.1-4.9.27

reflected from such (nonmetallic) horizontal surfaces contains a high proportion of polarized light whose E- (electric-) wave oscillates horizontally and hence is cut out by the Polaroid sunglasses. Thus, the Polaroid sunglasses become convenient, quick references for establishing the E-vector transmission direction of other polars.

Birefringence

Light traveling inside a birefringent material is split into two waves that travel at different speeds. The slower wave suffers greater refraction than the faster wave since the refractive index experienced by each wave is inversely proportional to its velocity of propagation.

The two waves vibrate in planes that lie perpendicular to each other, as dictated by the material's optical axes, and to the direction of propagation of the light ray. For a ray traveling in a particular direction through a birefringent material, the electric (E-) vector direction encountered by the slow wave is called the slow axis, and that of the fast wave the fast axis.

Optical Axes and Optic Axis

Broadly speaking, crystals can be classified into six groups depending on their axes of symmetry. All crystals, except those in the cubic group (e.g., NaCl, KCl), are birefringent, that is, they transmit light whose E-vectors, oscillating in different planes, travel at different velocities (Hartshorne and Stuart, 1960; Inoué, 1986). However, each crystal type has a unique single axis, or two axes, along which light is transmitted at equal speeds regardless of the direction of the E-vector. In other words, light propagating along such an axis does not suffer birefringence. Such an axis is called the optic axis of the crystal or the birefringent material.

In addition to the optic axis, a crystal generally has three principal optical axes (one of which may correspond to the optic axis). Between crossed polars, when the optical axes of a crystal come to lie parallel to the polarizer or analyzer, the entering polarized light is not split into two components, thus encountering no birefringence, and so the crystal appears dark. This extinction is seen intermittently in a swimming pluteus larva of a sea urchin, when the long axis of one of the biocrystalline, skeletal spicules comes to lie along the polarizer or analyzer transmission direction (see Example 1; Fig. 4.9.1).

In the younger, gastrula-stage sea urchin embryo, the triradiate precursor of the spicule may appear not to be birefringent unless viewed from its side. That is because the optic axis of these biocrystals, made of calcite, lies perpendicular to the plane of the triradiate spicule.

With the background between crossed polars made gray by turning a compensator from its extinction orientation, regions of the birefringent specimen whose axes coincide with the transmission axes of the polars appear with similar brightness to the background, i.e., neither brighter nor darker than the background. Four such regions are commonly seen, e.g., on the fertilization envelope, asters at the spindle poles, and starch grains (see Examples 3 and 4 and Fig. 4.9.4).

Sign of Birefringence and Coefficient of Birefringence

Depending on the crystal type, light may travel faster or slower along the optic axis compared to other axes. The former type of crystal is known to be positively birefringent (e.g., quartz, collagen fiber), and the latter negatively birefringent (e.g., calcite, DNA thread; Inoué, 1986). In addition to its sign, each crystal type exhibits a unique amount of birefringence, the coefficient of birefringence, which is the *retardance*-per-unit thickness of crystal (see discussion of Retardance, below). In fact, the signs of birefringence and refractive indices for light traveling along the axes of pure crystals are so unique that they are used in crystallography for identifying their composition and lattice type.

Traditionally, in biological polarization microscopy, the expressions positive and negative birefringence are used relative to some obvious frame of reference (see Examples 3 and 4) rather than to the crystal axis of the object. For example, the fertilization envelope is said to be positively birefringent relative to its tangent, meaning the E-wave oscillating parallel to the tangent of the fertilization envelope (the obvious outline seen outside the equator of the egg; Fig. 4.9.4) is retarded (refracted) more than the E-wave oscillating perpendicular to the envelope. Since the fertilization envelope is a hollow sphere, the axis of symmetry (and, hence, optic axis for any unit area of the envelope) should be its radius rather than its tangent. So, according to the crystallographic definition, the envelope has a negative birefringence. On the other hand, if one were to make a thread of the material that makes up the envelope, the axis of symmetry would be along the length of the thread, so that the traditional nomenclature commonly used in biology does have its utility.

Retardance

Light waves traveling through a birefringent specimen are split into two orthogonally polarized components, one being refracted more, or traveling slower, than the other (Bennett, 1950; Inoué, 1986). The phase difference between these two waves as they emerge from the specimen is known as their retardance, or birefringence retardation. The retardance (Δ) is, in fact, the difference in refractive indices ($n_e - n_o$) experienced by the "extraordinary" and "ordinary" waves multiplied by the thickness (d) of the specimen. Thus $\Delta = (n_e - n_o) \times d$.

The retardance can be expressed in fractions of wavelengths or in nanometer units; e.g., $\lambda/4$, or 136.5 nm (= 546 nm/4), for a quarter-wave plate for the mercury green line. A mitotic spindle may show a retardance of 2.7 nm, or only 1/200 wavelength for the mercury green line.

Intrinsic, Form, and "Accidental" Birefringence

Birefringence of a specimen may arise from anisotropy (difference in physical property exhibited for different directions in the same material) of structural organization at various submicroscopic levels that are all too fine to be resolved with the light microscope. If the ultrastructure of the specimen is anisotropic at a level usually detectable with the electron microscope, but not with the light microscope (e.g., stacks of cell membranes, such as in the Schwann sheaths of nerve or the outer segments of the retina, parallel-oriented filament systems such as the F-actin thin filaments in muscle, microtubules in flagellar axonemes, and spindle fibers), they exhibit an optical anisotropy known as form birefringence. Form birefringence arises from the parallel array of filaments or membranes whose refractive index differs from that of the imbibing medium and therefore, disappears when the refractive indexes of the two match. For filaments, the sign of form birefringence is positive, whereas for membranes it is negative.

When the birefringence arises from anisotropy within the molecules themselves, it is not altered by the refractive index of the imbibing medium, and the material is said to possess intrinsic birefringence. The regularly aligned polypeptide chain in collagen fibers and in the myosin filaments of muscle A-bands, the regularly aligned DNA protein in mature sperm head, and the biocrystalline calcite in the skeletal spicules of sea urchin embryos are some examples of material that exhibit intrinsic birefringence.

While intrinsic birefringence tends to be considerably stronger than form birefringence, the two may contribute more or less equally in some biological structures, either with the same or opposite signs of birefringence. For example, the positive intrinsic birefringence of lipid molecules, or of membrane proteins, may overcome the negative form birefringence introduced by the lipid bilayers when the refractive index of the imbibing medium approaches that of the bilayers (Ambronn and Frey, 1926; Schmidt, 1935, 1937; Frey-Wyssling, 1953).

In addition to the naturally observed intrinsic and form birefringence, one may encounter birefringence induced by flow or by external forces such as stretch or compression or by the application of electrical, magnetic, or other fields. The induced birefringence, in turn, may reflect the intrinsic or form birefringence of the aligned molecules or submicroscopic particles, or may reflect distortion of the electron orbits within the substance itself. In this last regard, strain birefringence can be induced even in naturally isotropic substances such as glass, isotropic crystals, or well annealed plastics. In fact, the strain birefringence in the objective and condenser lenses can be high enough to interfere with observation of small specimen retardances, and special strain-free objectives (designated with P or Pol) are sometimes supplied to minimize such effects.

Edge Birefringence

The edges of objects, viewed with polarization microscopy using high-NA objective lenses, may display what appears to be a double birefringent layer. While in some cases these may represent the birefringence due to membrane fine structure or its molecular organization, in other cases they can be caused merely by the presence of a sharp refractive index gradient at the edges of the object. The object can be purely isotropic; the only condition required is that there exist a sharp gradient of refractive index at the boundary between the object and its surrounding. Such birefringence is called "edge birefringence."

As detailed by Oldenbourg (1991), with edge birefringence the inner, thin "birefringent layer" shows a slow axis (axis with greater refractive index) parallel to the boundary and an outer thin "birefringent layer" with its slow axis perpendicular to the boundary when the refractive index of the object is greater than its surroundings. When the refractive index of the object becomes equal to its surroundings, the edge birefringence disappears. When the re-

fractive index of the object becomes less than its surroundings, the directions of the slow axes are reversed.

As with edge birefringence, form birefringence also disappears when the refractive index of the rodlets or platelets matches that of the imbibing medium. But the sign of form birefringence does not change below and above the matched index. The slow axis direction is unchanged (relative to the specimen axis) above and below index match point. In contrast, with edge birefringence the signs of birefringence (in the two layers on both sides of the optical boundary) are reversed in media whose refractive index is greater than the index match point compared to the situation in media below the match point. In contrast to both edge and form birefringence, the retardance due to intrinsic birefringence is unaffected by the refractive index of the imbibing medium.

It is important to distinguish edge birefringence from form or intrinsic birefringence since edge birefringence reflects an optical effect that has nothing to do with the molecular or fine structural anisotropy that we are probing.

COLOR IN POLARIZED LIGHT

Interference Colors

Many crystals that are normally colorless, including the biocrystalline spicules of sea urchin plutei, show in vivid color when observed in white light between crossed polars (Fig. 4.9.2). The color reflects the birefringence retardation (or retardance, i.e., the phase difference) that the two components of the orthogonally polarized light components suffered in passing through the crystal. For example, if the retardance were 550 nm, or a full wavelength for green light, the two orthogonal components would add back together to form the initial state (i.e., of plane polarized light as before entering the crystal) upon exiting the crystal. Thus, green light is extinguished by the analyzer. Wavelengths other than 550 nm are elliptically polarized and are partially transmitted by the analyzer. Thus, the complementary color of green, i.e., red, is seen through the analyzer (Inoué, 1986).

Similarly, for an object giving a retardance of 490 nm, full wavelength for blue light, blue is extinguished and its complementary color yellow is seen through the analyzer. For a retardance of 568 nm, full wavelength for yellow light, yellow is extinguished and its complementary color blue is seen through the analyzer. For a retardance of 600 nm, full wavelength for red light, red is extinguished and its complementary color green is seen through the analyzer. These interference colors thus measure the retardance of the specimen.

For a cylindrical object like the pluteus skeletal spicule, light would travel the furthest distance across its middle and suffer the most retardation, so that the axis of the spicule would appear with the highest-order interference color. Thus, the axis may appear green, surrounded by blue, then red, then yellow, then gray, etc., as light travels through decreasing thickness towards the outer margin of the cylindrical biocrystal and is thus retarded, proportionately, less and less.

Dichroism

In addition to optical anisotropy that is manifested as birefringence (difference of refractive index for light waves whose E-waves are oscillating in different planes), one may also encounter dichroism (difference of absorbance, or of absorption spectrum, for light waves whose E-waves are oscillating in different planes). Whereas a birefringent material simply changes the phase of polarized light, a dichroic material transmits light to different degrees, depending on the wavelengths and the angle between its axes and the plane of the entering polarized light.

Polaroid films serve as effective polars by taking advantage of the aligned polyiodide chains that show a strong dichroism for most wavelengths in the visible spectrum. The conjugated electrons that make up the backbone of the polyiodide chains strongly absorb the (E-) component of the visible light waves that are polarized parallel to that direction, whereas they absorb little of the component that is polarized at right angles to the backbone.

The dichroism of Polaroid sheets that extends over much of the visible wavelength range is, however, an exception. Most dichroic materials (or pleochroic materials, i.e., many-colored instead of showing two different colors, depending on the vibration direction of the polarization of light) exhibit two different absorption curves depending on the orientation of their chromophores relative to the plane (of the E-wave) of polarized light.

DNA in mature sperm is a biological material that shows prominent dichroism in ultraviolet (UV) light. The conjugated bonds in the purine and pyrimidine bases of B-form DNA, regularly oriented at right angles to the DNA backbone as in steps of a spiral staircase, give

rise to a UV dichroism with a dichroic ratio of 4, spanning the wavelength range of ~250 to 380 nm. This dichroism is present because the conjugated bonds in the bases (that resonate in the UV range) lie at right angles to the B-form DNA backbone. So the UV polarized at right angles to the backbone is absorbed four times more effectively than UV that is polarized parallel to the DNA backbone (Wilkins, 1951; Inoué and Sato, 1966). This gives rise to the UV dichroism that has a negative sign (i.e., greater absorbance perpendicular to the backbone compared to parallel to the backbone).

The same conjugated bonds give rise to the much greater polarizability of the electrons, and, hence, to higher refractive indexes for visible light waves whose E-fields lie in the plane of the bases compared to fields along the backbone of DNA. That gives rise to the strong negative birefringence of B-form DNA in the visible wavelength range (Inoué and Sato, 1966; Inoué, 1986). The author took advantage of this dichroism and birefringence, which are both abolished upon polarized UV microbeam irradiation (possibly by dimerization of the thymidine bases), to analyze the detailed packing arrangement of DNA and to uniquely visualize chromosomes, taking advantage of a needle-shaped head of an insect sperm (Fig. 4.9.7; Inoué and Sato, 1966).

Chromophores with such high dichroic ratios can be recognized by a change in light transmittance or absorbance, or of color, as the orientation of a single polar (a polarizer in the absence of an analyzer, or an analyzer in the absence of a polarizer) is changed.

Cell inclusions with regularly oriented natural chromophores—e.g., retinal rods and cones (Schmidt, 1935), sickled blood cells (Perutz and Mitchison, 1950; Hárosi, 1981), and chloroplasts (Breton et al., 1973; Hsu and Lee, 1987)—may also show some degree of dichroism. Cell inclusions, or products with regularly oriented fine structure that have been stained with dyes, may likewise be dichroic.

When the dichroic ratio is low, as in these latter examples, it may require a sensitive spectrophotometer to detect a change in color, or wavelength dependence of light transmittance, as the specimen orientation is changed relative to the orientation of the polarized light (Hárosi and MacNichol, 1974). In such cases, the dichroism may still be detected visually if one uses both a polarizer and an analyzer and orients one of the two a few degrees from the crossed position. The complementary dichroic colors can then be seen visually by turning the polar or compensator in opposite directions from the extinction orientation.

Using the dichroic fluorescence of tetramethylrhodamine that was used to sparsely decorate actin filaments, Kinosita and coworkers were able to demonstrate, and measure, the speed of rotation of individual actin filaments around their own axis as the filaments are propelled by myosin molecules coated on the surface of a microscope coverslip. Through video contrast enhancement of the fluorescence microscope images taken in polarized light, they were able to show the cyclic brightness change in the dichroic fluorescent dye as the single actin filament, <10 nm in diameter, glided along the lawn of myosin heads (Kinosita, 1999).

The sensitivity for measuring the orientation of a dichroic fluorescent molecule, such as green fluorescent protein, can be increased several fold by using parallel polars instead of a single polarizer or analyzer (Inoué and Goda, 2001).

Colloidal Gold, Pinholes

Spindle microtubules in *Haemanthus* endosperm cells decorated with 20 nm-diameter colloidal gold appear as dark black lines when observed in a compensated field between crossed polars in monochromic light. These same microtubules show in striking colors ranging from red to purple, green, orange, and gold when they are observed in white light with the polars off-crossed by a few degrees and the compensator orientated to different directions (Inoué and Spring, 1997, Color Plate III). These colors are attributed to anisotropy of Tyndall light scattering (or di-Tyndallism) by the linear array of colloidal gold particles that are arranged quite densely (not contacting each other, yet acting as an effective conductor, or antenna, for the high-frequency electromagnetic light waves) along the lengths of each microtubule.

Examined at high magnification, pinholes in thin metal film, evaporated on coverslips that are used to test the performance of microscope objective lenses, can also each appear with striking saturated colors when examined in polarized white light. Light from the pinholes becomes alternately extinguished as the microscope stage is rotated. In other words, each pinhole is acting as a polarizer that appears to have a narrow transmission wavelength. No doubt, each pinhole is not completely circular but must be elongated, since they are generally produced by some random dust particle or impediment to sputtering or high-vacuum depo-

sition of the metal film. Thus, each "pinhole" acts as an anisotropic nonconductor in the thin metal film.

In fact, a very narrow crack in a thin metal film preferentially transmits light that is polarized with its E-vector oriented perpendicular to the narrow crack and whose wavelength reflects the local width of the submicroscopic crack (S. Inoué, unpub. observ.). The situation is somewhat the converse of the aligned polyiodide chains or of the colloidal gold-decorated microtubules, in that instead of a submicroscopic thread-shaped conductor, one has a thread-shaped non-conductor surrounded by a conducting film.

Anomalous Birefringence

Stained or colored biological tissue and cell components often exhibit "anomalous birefringence," i.e., the color observed in white light between crossed polars actually changes with adjustment of the compensator. In other words, the birefringence, or the retardance observed, is a function of the wavelength of light. Such a color change, or "anomalous birefringence," is observed even when the retardance of the object is a small fraction of the wavelength of light (Perutz and Mitchison, 1950).

Colorless objects, on the other hand, generally show birefringence that changes only slightly with variation of wavelengths. What color is observed for a colorless object is primarily due to interference colors when the retardance approaches a wavelength or more, as described earlier (Fig. 4.9.2).

On the other hand, with strongly absorbing materials such as a dye or stain, the refractive index drops sharply on the shorter wavelength side and rises precipitously on the longer wavelength side of the absorption peak ("anomalous dispersion"; Jenkins and White, 1957; Hecht, 1998). Since the wavelength-specific absorption of light in a dye or stain is commonly brought about by conjugated ring structures, the birefringence associated with such an anisotropic structure would also be expected to show a strong dispersive character (wavelength dependence). The anisotropic dispersive character would then give rise to anomalous birefringence. Objects exhibiting anomalous birefringence would also be expected to show some dichroism as well.

These several wavelength-dependent polarization optical effects (as with polarization of fluorescence, a topic not covered here) are of considerable potential interest as potent tools for analyzing dynamic changes within, or interactions among, biological molecules and fine structures. Further studies on the physical optical bases of these phenomena should lead to their wider application in cell biology.

OPTICAL ROTATION

Optical rotation (also known as circular birefringence) is a polarization optical effect that may (rarely under the microscope) induce an object to become bright between crossed polars. Unlike (linear) birefringence, the brightness due to optical rotation does not depend on the regular alignment of the molecules but on their internal asymmetry and thus is observed even in solutions of the particular molecules. Optical rotation may also be seen in certain crystalline substances, such as quartz and other material viewed along the helical axes of the molecules that make up the crystal.

An object exhibiting optical rotation does not undergo extinction between crossed polars as the microscope stage revolves. Instead, the object can be made darker by turning the polarizer or analyzer away from their crossed orientation. Except in pure monochromatic light, as a polar is turned, these "optically active" objects or solutions acquire a brownish or bluish hue instead of undergoing complete extinction. The color reflects a high degree of wavelength dependence that is characteristic of optical rotation. Such wavelength dependence of optical rotation is known as optical rotatory dispersion. The infrared optical rotatory dispersion, e.g., of myosin and related proteins in solution, has been used to determine the degree of helix content of protein molecules (Szent-Györgyi et al., 1960).

VIDEO MICROSCOPY

As in many other modes of microscopy, the capabilities of polarization microscopy have been substantially enhanced by the application of video and electronic image processing, analysis, and device control. As described in Allen et al. (1981) and Inoué (1981b) and summarized, e.g., in Shotten (1993), and Inoué and Spring (1997), video microscopy can effectively enhance image contrast, subtract background, reduce noise, and allow image capture with better corrected higher-NA objective lenses and even with relatively short exposures.

The new LC PolScope introduced by Oldenbourg, provides striking, high-contrast images of weakly birefringent cellular regions and does so independently of specimen axis orientation. The recent system introduced by N. Allen and co-workers selectively enhances (or

suppresses) contrast in differential interference contrast (DIC) microscopy and should also be applicable for bringing out birefringent regions of the specimen.

Oldenbourg's LC PolScope

Oldenbourg's LC PolScope (which can be adapted to most research-grade microscopes outfitted for polarization microscopy) provides striking, high-contrast images of weakly birefringent objects. Furthermore, the system has the unique attribute of providing maps of birefringence distribution that is independent of orientation of the specimen axis within the focal plane.

The new system uses circularly, instead of linearly polarized light and two electronically controlled liquid crystal compensators instead of a mechanically driven conventional compensator (Oldenbourg and Mei, 1995; Oldenbourg, 1996). Four video images are acquired into computer memory in rapid succession. In acquiring each of these four images, the computer provides appropriate voltages that adjust the retardances of the two compensators. The LC PolScope uses no mechanically moving compensators and records the images in perfect register. From the set of four images, the retardance map is immediately calculated by the computer and displayed on the monitor.

The calculated image generated with the LC PolScope is, in fact, a map of birefringence distribution whose intensity is directly proportional to the retardance of each specimen point. In contrast, with images obtained by standard polarization microscopy, the intensity of each image point is proportional to the square of the retardance of the specimen point. The LC PolScope can also display a map of slow-axis orientations for pixels for each image point.

The LC PolScope has been used to display the dynamic distribution of microtubules in newt lung epithelial cells undergoing mitosis (see on-line video accompanying Inoué and Oldenbourg, 1998) and the highly dynamic behavior of actin filaments and network in cultured growth cones of *Aplysia* neurons (Katoh et al., 1999) with unprecedented clarity.

The LC PolScope system is available from CRI (Cambridge Research and Instrumentation).

Nina Allen's Compensator

In another recent development, Holzwarth et al. (1997, 2000) also use a voltage-driven liquid-crystal compensator for DIC observations with video microscopy. They use crossed linear polars rather than circular polarizers as in Oldenbourg's system. Appropriate voltages, applied by a computer to the compensator, generate an additive and a subtractive shift to the phase difference introduced by the Wollaston or Nomarsky prism. Each of these images is stored in computer memory and subtracted from each other. The difference image displays the DIC effect alone, while subtracting away stationary background noise and image features that did not originate from interference between the two sheared rays.

Although designed for use in DIC and still retaining the orientation-dependent contrast characteristic of images observed between crossed linear polarizers, Allen's new compensator applied to polarization microscopy should also effectively bring forth contrast in weakly birefringent regions of the specimen. Conversely, the modified system could selectively suppress contrast due to birefringence by adding (rather than subtracting) the two images stored in computer memory and selectively bring forth image features not based on optical anisotropy.

Centrifuge Polarizing Microscope

Living cells centrifuged on an isopycnic gradient at several thousand $\times g$ (earth's gravitational field) tend to stratify with their lighter components packed at the centripetal pole and heavier inclusions packed at the centrifugal pole of the cell (Harvey, 1940; Zalokar, 1960). In the process, cytoskeletal elements, membranes, and organelles tend to become aligned or deformed. To study the fine structure and function of stratified cell components and to learn about the mechanical linkages of the organelles to the membranes and cytoskeletal elements, the author of this unit has developed a centrifuge polarizing microscope (CPM; Inoué et al., 1998, 2001a).

The CPM allows video-rate polarization microscope observations of the spinning specimen, with image resolution of 1 μm, in living cells suspended in an isopycnic solution or crawling on a glass substrate. Birefringence retardations can be detected and measured down to 1 nm in cells that are exposed to up to $10{,}500 \times g$.

With the CPM, the author has found striking changes in the conformation of endoplasmic reticulum immediately following fertilization of sea urchin eggs, has followed the process of Ca^{2+}-induced nuclear-envelope breakdown and meiotic spindle formation in a marine annelid egg, measured the climbing forces generated

by myosin mutants of slime mold amoebae, and observed the enigmatic formation and rapid precipitation of dense "comets" in a concentrated solution of a thread-shaped virus (Inoué et al., 2001b).

APPLICATIONS OF POLARIZATION MICROSCOPY

Example 1: Examining Biocrystals

Hard tissue generated in living organisms often contains, or is made up of, biocrystals. Biocrystals are indistinguishable (in terms of their atomic lattice and, hence, intrinsic optical properties) from inorganic crystals, yet their morphology is primarily determined by their biological function and seldom reflects the habits (e.g., cleavage faces) of the natural crystal.

A common form of biocrystal, such as the skeletal spicules found in embryonic sea urchins (or the plates of their adult forms), is based on calcite, a highly birefringent, negative uniaxial material that is the main ingredient of chalk.

The birefringence, growth, and morphogenesis of calcareous biocrystals, such as the skeletal spicules generated by the mesenchyme cells in the gastrula- and pluteus-stage sea urchin larvae, can be studied with a simple polarizing microscope that can be easily assembled.

All one needs is a polarizer, inserted in the illumination path before the condenser of the microscope, and an analyzer (crossed relative to the polarizer) placed after a low (10× or 20×) power objective lens. In fact, both of these "polars" may already be present on the microscope. If the microscope is equipped for DIC, simply slide out the Nomarsky prism and set the condenser turret to the bright-field position. Close down the condenser iris so that the condenser NA becomes just below half that of the objective NA. Make sure the polars are carefully crossed so that, with the lamp brightness turned up, the blank field becomes completely (or very) dark in Köhler illumination (see *UNIT 4.1*).

When live gastrula or pluteus larvae are observed between crossed polars, the pair of triradiate or skeletal spicules should alternately twinkle like stars as the ciliated embryo swims about (see Fig. 4.9.1). The thicker parts of the skeletal spicule may show brilliant interference colors when observed in white light (see Fig. 4.9.2).

One can even make these observations using a dissecting microscope of the type that allows transillumination of the specimen. Simply attach a polarizer below the specimen-support glass plate and an analyzer below the objective lenses. The polars can be sheet Polaroids, perhaps those cut to appropriate shape from plastic Polaroid sunglasses. With the analyzer transmission axis oriented horizontally (i.e., turned 90° with respect to the way Polaroid sunglasses would normally be oriented), the polarizer mounted below the glass specimen support plate can be oriented vertically (north-south) by rotating the support plate to achieve extinction. Note that it is important to observe the specimen in a glass container, not in a plastic Petri dish, since most plastic lab ware exhibits very strong birefringence that can overwhelm the specimen birefringence.

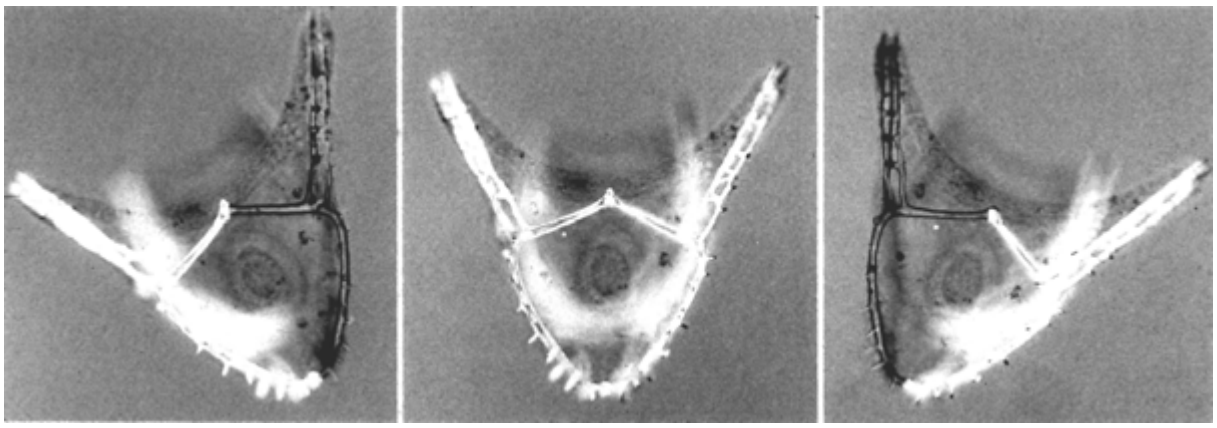


Figure 4.9.1 Pair of skeletal spicules in sea urchin pluteus larva. As the ciliated pluteus swims about in the field of the polarizing microscope, the skeletal spicules alternately shine and become extinguished. Despite its complex shape, the whole of each spicule is extinguished at once when its (calcite) crystal axes come to lie parallel to the axes of the crossed polars. Such extinction pattern is characteristic of biocrystals (from Okazaki et al., 1980).

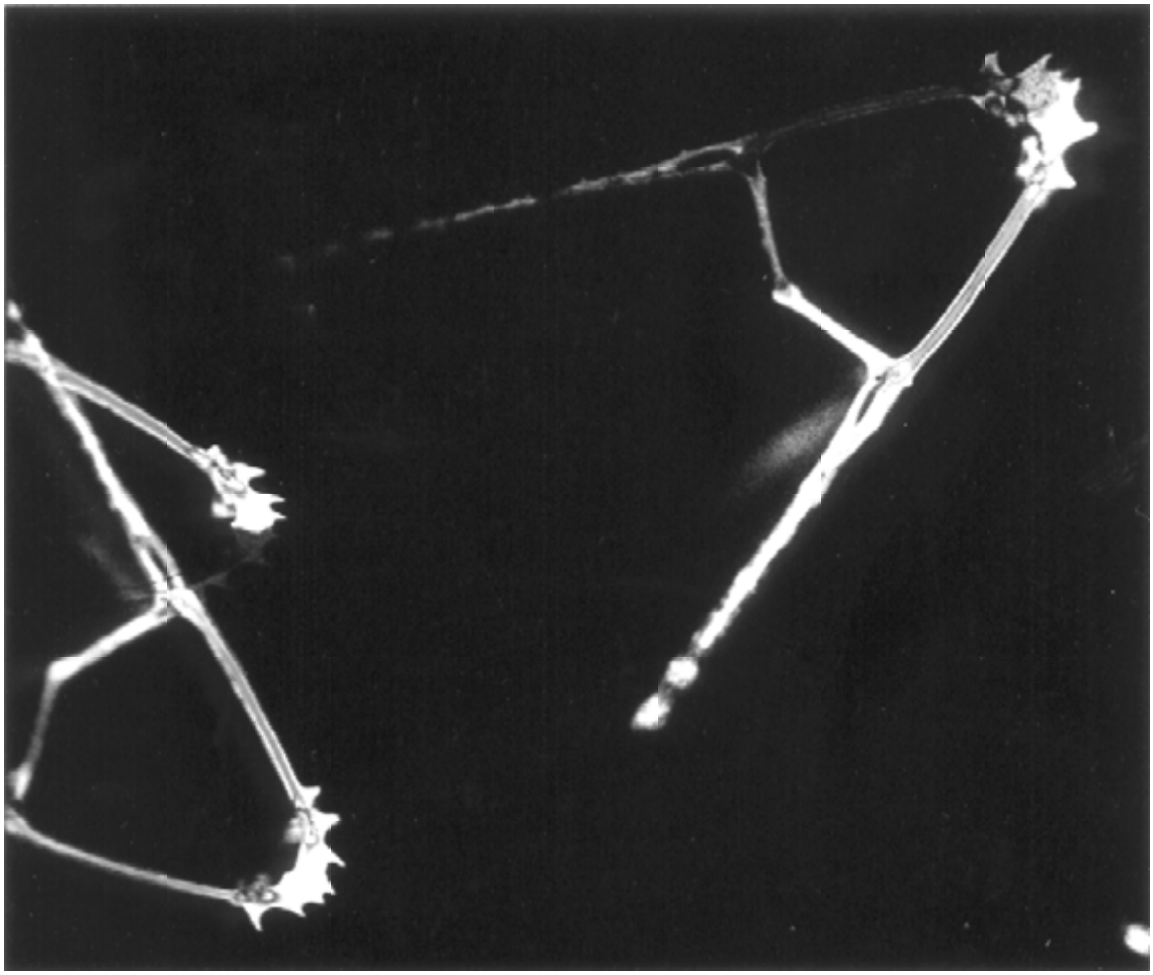


Figure 4.9.2 Skeletal spicules isolated from sea urchin pluteus larvae observed between crossed polars in white light. The calcite spicule is so strongly birefringent (coefficient of birefringence = –0.172) that a region just over 3-μm thick already introduces a 580-nm retardation. In that region, yellow light exiting the spicule is extinguished by the analyzer so the spicule takes on a bluish hue. Figure courtesy of Jan Hinsch, Leica. **See color plate.**

While appearing to be a rudimentary exercise, consideration of the polarization optics and close observations of the image of the specimen can, in fact, reveal the directions and homogeneity of optical axes of the biocrystals, yield their sign and coefficient of birefringence, relate the growth of the biocrystal to the activity of the mesenchyme cells, and produce other useful information (Okazaki and Inoué, 1976).

In addition to the calcareous skeletal spicules that consist of a single biocrystal in echinoderm embryos, most bone and teeth contain an array of biocrystals (Schmidt, 1924).

Example 2: Examining Collagen, Muscle, and Histological Preparations

Many transparent organisms and tissue sections exhibit birefringent regions that can be seen with simple polarizing microscopes. The wavy collagen fibers and cross-striated muscle in animal tissues (see Fig. 4.9.3), and cellulose fibrils in the walls of plant cells (e.g., see Green, 1963), stand out prominently sometimes amidst bright crystalline inclusions. Likewise, the keratin layers in hair (which can vary with animal species) and synthetic fibers show strong birefringence.

Some of the crystalline inclusions seen in the background were formed in life, while other randomly scattered crystals could have developed in the medium in which a fixed specimen is embedded. Others, which at first glance may appear to be simple crystals (with simple optical axes as in biocrystals), upon closer inspection may turn out to have complex (more or less radially symmetric) extinction patterns. These may be starch grains with concentric lamellar structures that show characteristic extinction

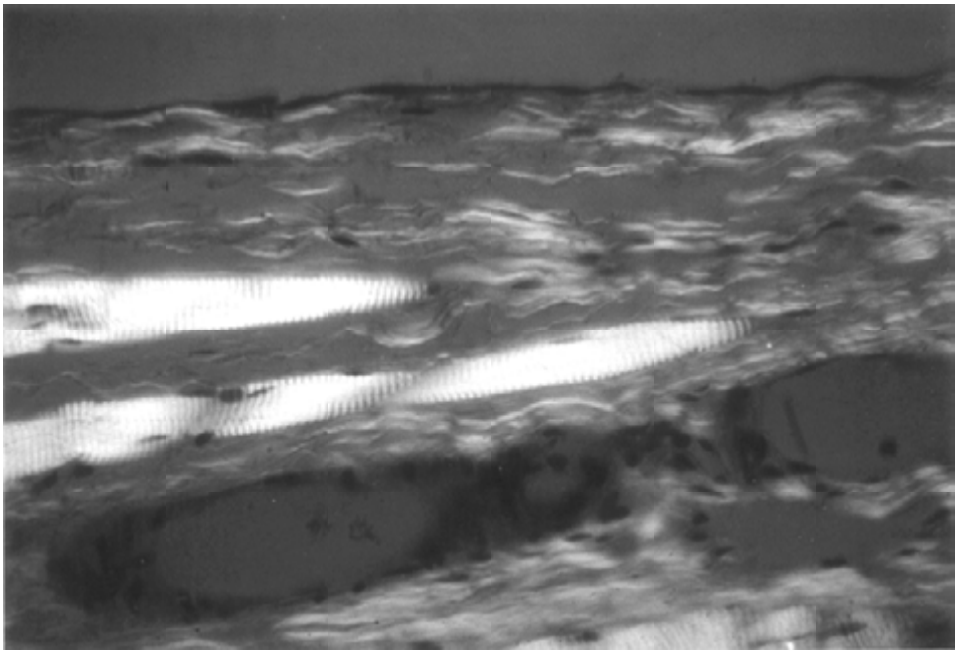


Figure 4.9.3 Tissue section showing birefringent cross-striated muscle and collagen fibers. A typical H & E–stained tissue section observed between crossed polars in the presence of a Brace-Köhler compensator. The birefringent A-bands of the cross-striated muscle fibrils and the wavy axes of the birefringent collagen fibers stand out. **See color plate.**

patterns (see Frey-Wyssling, 1953). On a larger scale, a more or less similar pattern can be seen in the shells of embryonic gastropods (Schmidt, 1924).

Example 3: Preparing to Study Spindles and Asters

In contrast to the structures described above, which may show interference colors between crossed polars and tend to show retardances of the order of many hundreds of nanometers, other structures in living cells display a very weak birefringence retardation that amounts to only a few to a fraction of a nanometer. These include the arrays of microtubules that make up the spindle fibers, astral rays, and flagellar and ciliary axonemes, the arrays of actin and intermediate filaments, various membrane systems, and chromatin in sperm heads.

Unlike the examples mentioned above, whose birefringence is mostly based on the anisotropy of their intramolecular bonds (intrinsic birefringence), many of the intracellular filament and membrane arrays exhibit form birefringence that is based on their ultrastructural geometry.

In order to detect and study the weak birefringence common to intracellular structures, one needs a polarizing microscope equipped with polars yielding a high extinction factor (see Example 4), strain-free optics, a Brace-Köhler compensator, and a bright light source (Swann and Mitchison, 1950; Inoué and Dan, 1951).

While the test specimen and protocol described below are useful for checking the performance of a polarizing microscope equipped with the highest-NA lenses, in this protocol for observing fertilization and spindle and aster formation in marine eggs, it is recommended that one use a 10× to 20× objective lens with the condenser iris partly closed so that $NA_{cond} = 0.3 \times NA_{obj}$.

To see whether a microscope has been adequately equipped and adjusted for studying weak birefringence of intracellular structures, one can try out a convenient test specimen prepared as follows. On a clean microscope slide place a few drops of saliva. Place a clean coverslip on top and observe in Köhler illumination (see *UNIT 4.1*) using a bright light source. Make sure the polars are fully crossed and that the microscope is equipped with a Brace-Köhler compensator.

If the microscope is adequately equipped and adjusted, the buccal epithelial cells (the so-called "spit cells" that are present in the saliva) should appear brighter or darker than the gray background depending on the orientations of the cell and the compensator. The gray back-

ground is introduced by the compensator and varies with the orientation of its axes between the crossed polars, as described below.

If the spit cells show up in this fashion, both brighter and darker than the gray background depending on their orientation, the polarizing microscope should be ready for other applications. However, before removing the test slide, go through the following exercises, which will give further insight into the submicroscopic structure of the cell structure that is being examined.

When a spit cell in focus is brighter than the background, the retardances of the cell and the compensator are added together and introduce a greater phase difference, thus making the specimen appear brighter. Since the slow axis of the birefringent spit cells (i.e., the long axis of their keratin fibrils) is generally oriented along the long axis of the cell, they appear bright when that axis lies in the same quadrant as the slow axis of the compensator.

When the slow axes of the specimen and compensator lie in opposite quadrants, their retardances cancel (subtract from) each other, so that the specimen appears darker than the background field.

Using these criteria, record which quadrant of the compensator contains its slow axis. Some cells appear bright in some regions and dark in others. There the keratin fibrils are not uniformly aligned parallel to the long axis of the cell, but in different directions depending on the part of the cell.

Note also that the cells (or cell regions) in certain orientations appear neither brighter nor darker than the field. With the compensator removed (or brought to its extinction orientation), those cell regions should appear as dark as the extinguished background. Those are cells, or cell regions, in which the keratin fibrils are oriented exactly parallel or perpendicular to the polarizer and analyzer axes. Verify this explanation by revolving the microscope stage or by observing a cell that is flowing and changing its orientation slowly.

Just as the specimen becomes dark when its optical axes, in the absence of a compensator, coincide with those of the polars, the background for the whole field becomes dark when the compensator optical axes are oriented parallel to the axes of the polars. These extinction orientations show the optical axes, respectively of the compensator or specimen, which relate directly to the orientations of their atomic bonds or fine structure.

In testing for the adequacy of the polarizing microscope through these exercises, the apparatus is also set up to establish the optical axes and signs of birefringence of the weekly birefringent specimen. Through such an exercise, we can interpret the nature and alignment of the submicroscopic molecular or fine-structural arrays that are themselves much too small to be resolved with the light microscope.

Example 4: Examining Fertilization, and Spindle and Aster Formation, in Egg Cells

Elevation and hardening of fertilization envelope

Collect eggs and sperm from appropriate species of gravid sea urchins or sand dollars (Costello et al., 1957; Schroeder, 1986; Lutz and Inoué, 1986). Use species with optically clear eggs such as Lytechinus variegatus, *Lytechinus pictus*, or *Echinarchnius parma*.

Wash the eggs in filtered seawater three times, each time by allowing the eggs to settle, changing the seawater above the layered eggs, and again letting the eggs settle. Add a dilute suspension of sperm to a portion of the washed, unfertilized eggs (save some unfertilized eggs in a larger dish). Then, quickly make a preparation using a clean slide and coverslip (with a coverslip fragment or small piece of Scotch tape placed under one edge to prevent squashing the egg). Observe the elevation of the fertilization envelope with the polarizing microscope. In the first 3 to 5 min after fertilization, note how the fertilization envelope elevates and becomes increasingly birefringent.

Note also that the parts of the envelope that lie in the same quadrants as the slow axis of the compensator become quite bright while parts of the envelope in the other quadrants become darker than the gray background (Fig. 4.9.4). In other words, the slow axis for the envelope lies parallel to its surface, or the envelope shows a "tangentially positive birefringence." As the birefringence of the envelope rises, the middle (crescent-shaped) region of the darker part of the envelope may become brighter. For that region, the birefringence retardation of the envelope has become greater than that of the compensator. This effect is more pronounced the smaller the retardance introduced by the compensator. In fact, with the compensator oriented in its extinction orientation, all four quadrants of the envelope will shine equally brightly.

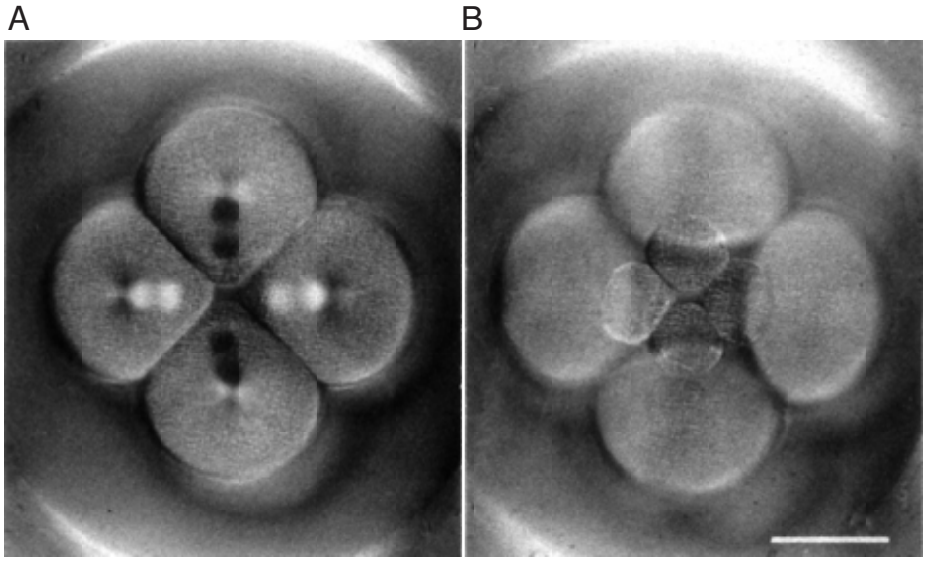


Figure 4.9.4 Birefringence of fertilization envelope and fourth-division mitotic spindles in a sand dollar egg. (**A**) The tangentially positive birefringence of the fertilization envelope and the radially positive birefringence of the mitotic spindle and aster microtubules show clearly in this transparent cleavage stage egg of *Echinarchnius parma.* The egg is viewed near its vegetal pole, to which the vegetal fourth-division spindles have converged. (**B**) The asymmetric location of the vegetal pole spindles has given rise to four small micromeres, whose descendants give rise to the gametes and the mesenchyme cells that pattern the pair of skeletal spicules. Compensator slow axis is oriented horizontally.

This rise in positive birefringence of the fertilization envelope reflects the deposition of a fibrous inner layer that strengthens the envelope. In fact, it is possible to strip off the envelope by passing the fertilized eggs through a fine-meshed bolting cloth, within 3 to 4 min after fertilization, after the fertilization envelope has risen sufficiently, but before it has hardened (as judged by its strong birefringence).

Mitotic spindle and asters: Assembly and disassembly of mitotic microtubules

Make a fresh preparation ~40 min to 2 hr after fertilization (~15 to 20 min before the onset of first cleavage, depending on the species and temperature). In this preparation made at the appropriate time, one should see the birefringent asters and the mitotic spindle gradually appearing.

Note how both the asters and spindle initially appear as small, weakly birefringent structures that may be barely detectable.

Depending on the orientation of their fibrillar components (microtubules) and the compensator setting, portions of the asters and spindle may appear bright or dark. In fact, each aster will generally appear as a radial structure with two dark and two bright quadrants since the microtubules radiate out from the centrosome.

Over the next several minutes, the asters and spindle gradually grow in size and birefringence, until the spindle reaches full metaphase. At anaphase, the clear gap in birefringence that develops between the two half spindles by full metaphase gradually increases in length as the chromosomes are moved polewards.

Starting with the first appearance of the tiny spindle and asters, make time-lapse video records of the events every 5 to 15 sec apart, making sure that a time-of-day signal and audio records of any comments are included. Keep a record of room temperature near the microscope. If possible, record also the orientation of the compensator that just extinguishes the darkest part of the spindle. Choose spindles whose long axes lie in the plane of focus and that are oriented at 45° to the transmission axes of the polars. This exercise may be easier if observations are made on eggs in their second division where two spindles should be oriented parallel to the first cleavage plane.

Depending on the species being used and on the microscope, the chromosomes themselves may or may not be visible. Switching to DIC or phase contrast may help see the chromosomes in favorable cases (see Figure 1 in Salmon and Segal, 1980).

As the spindle approaches telophase and the half-spindle birefringence diminishes, the asters

generally grow to their maximum size and birefringence. Shortly thereafter, the cell cleaves in a plane bisecting the anaphase spindle.

The astral rays and fibers in the spindle display a positive birefringence along the length of their fibrils or of their microtubules. Since virtually all of their birefringence can be accounted for by the form birefringence of their constituent microtubules (Sato et al., 1975), the rise and fall and distribution of their birefringence directly reveals the change in concentration and distribution of oriented microtubules, which themselves are too small to be resolved with the light microscope.

Observation with a polarizing microscope of changes in the spindle and astral birefringence, associated with the natural progress of cell division or in cells exposed to cold or mitosis-inhibiting chemicals such as colchicine, have laid an important foundation for our present understanding of the submicroscopic organization and events in mitosis, as well as the assembly properties of the mitotic, and other labile, microtubules (Inoué, 1952a, 1952b, 1964, 1981a; Inoué and Sato, 1967; Inoué and Salmon, 1995).

Example 5: Using Polarization Microscopy at Maximum Resolution

So far, these examples have dealt with observations and measurements of weak birefringence that could be made with lower-power microscope objective lenses. In order to gain more image resolution, one must not only use higher-power objective lenses, but objective and condenser lenses with higher numerical apertures (NAs). That is because the minimum distance (d) between two small points that can just be resolved is given by the following relationship (see *UNIT 4.1* for further detail):

$d = 1.22 \times$ (wavelength of light)/(NA of objective + working NA of condenser).

While the exercises described in Examples 1 to 4 can be carried out with a simple polarizing microscope equipped with objective lenses that have relatively low NAs, they become difficult or impossible when one attempts the same procedures with lenses that have NAs of >0.5. That is because a considerable amount of background light leaks through the crossed polars at high NAs, no matter how carefully the system is adjusted. The extinction factor {EF = (Intensity with parallel polars)/(Intensity with crossed polars)} that may be as high as 10^4 for low-power objectives which are clean and free of strain birefringence, may drop to as low as 10^2 for some highest-NA objectives. It takes place even with carefully selected strain-free lenses and is generally caused by two factors: the differential transmission of the polarized components of light in and perpendicular to the plane of incidence and their phase differences, both introduced at oblique optical interfaces that lie between the polarizer and analyzer.

In simple terms, light must be refracted at high angles of incidence by the objective and condenser lenses, and the slide and coverslip surfaces, in order to gain the high NA needed for obtaining the high image resolution. In polarization microscopy, that very fact makes the EF drop precipitously at higher NAs. In other words, without some special remedies as described below, the detection of very weak birefringence and generation of very high image resolution are mutually incompatible.

Fortunately, there are a few remedies, although these are somewhat difficult to implement, that can help alleviate these problems. To understand these remedies, first observe the appearance of the back aperture of the objective lens. This can be done by replacing the ocular of the microscope with a telescope used for adjusting a phase-contrast system. Or, if your microscope is equipped with a Bertrand lens, insert and focus the Bertrand lens so that it, together with the ocular, makes up a telescope that images the back aperture of the objective lens. Alternatively, remove the ocular and peek down directly at the back aperture of the objective lens.

Confirm that you have indeed focused on the back aperture plane. There you should see the image of the condenser iris, whose size changes as the condenser iris is opened and closed. With proper Köhler illumination, if you open and close the field diaphragm rather than the condenser iris, only the brightness of the back aperture and not the size of the iris image should change.

In the absence of a compensator, observe the objective lens back aperture as the polarizer and analyzer are crossed and slightly off-crossed. If the lenses are free of strain birefringence and birefringent dirt or dust particles, the objective back aperture should show a dark cross flanked by brighter quadrants between the arms of the cross when the polars are exactly crossed. The arms of the dark cross lie along the transmission axes of the polars (Fig. 4.9.5A).

When the polarizer or analyzer is slightly off-crossed, the dark arms of the cross turn into two dark V shapes located in opposing quad-

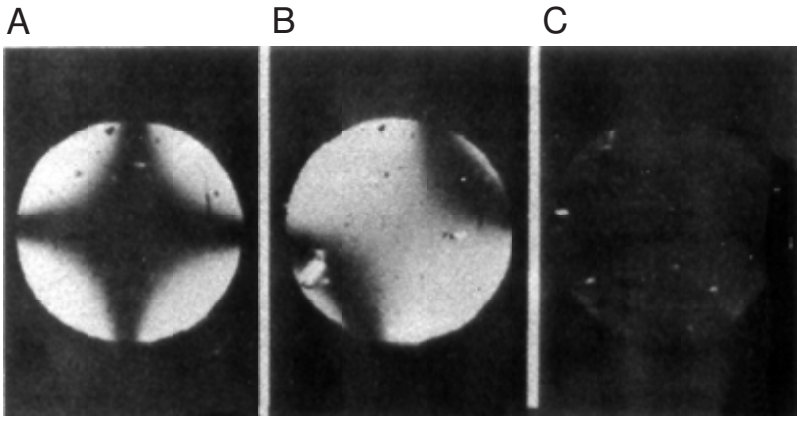


Figure 4.9.5 Patterns observed at the back aperture of polarizing microscope equipped with high-NA (×97/1.25) strain-free, matched condenser and objective lenses. (**A**) The back aperture shows a dark cross between crossed polars in the absence of a birefringent specimen or a compensator, if the objective and condenser lenses are free from *lateral* strain birefringence. The lenses may, however, still be introducing *radial* birefringence! (**B**) As the analyzer (or polarizer) is turned, the cross opens up into two dark Vs that should remain dark up to the edge of the aperture if there is no contributing birefringence. Turning the polar in the opposite direction reverses the quadrant in which the Vs appear. (If the Vs do not move out to the periphery of the aperture but fade rapidly as a polar is turned, the contrast of the Vs can generally be restored by adjusting a compensator. That region of the aperture is then introducing elliptically polarized light, either because a lens suffers radial strain birefringence or because of the nature of the anti-reflection coating used on the lens.) (**C**) Extinction pattern of the same lens pair shown in A and B, but equipped with a polarization rectifier. The whole back aperture (up to the maximum NA), as well as the microscope field, is now well extinguished. (From Inoué and Hyde, 1957.)

rants of the back aperture (see Fig. 4.9.5B). Those V-shaped regions are where the plane of the incident polarized light has been rotated (by lower transmission of the polarized light vector vibrating normal to the plane of incidence compared to that vibrating parallel to the plane of incidence).

As the polars are further off-crossed, the V-shaped regions move further out, but may become faint and no longer be very dark. At this point a Brace-Köhler compensator inserted at the appropriate orientation can restore the darkness of the Vs. In other words, light rays traversing the objective and condenser lenses (and slide and coverslip), at angles and directions corresponding to those regions that have now become darkened at the back aperture of the objective lens, act as though those particular rays experienced birefringence or had suffered a phase difference and become elliptically polarized. This elliptical polarization is commonly due to the anti-reflection coating applied to the lens surfaces. As with the rotation of polarized light, it also increases with the angle of incidence and the departure of the plane of incidence from the vibration plane of the polarized light incident at each air-glass interface.

The net result of all of these polarization-altering effects at the lens (and slide and coverslip) surfaces is the dark cross at extinction and the two sets of Vs that move further out into opposing quadrants.

The polarized light modified in this fashion (in the four quadrants between the arms of the dark cross) is transmitted by the analyzer crossed with the polarizer and, hence, gives rise to the large amount of background "stray light" found with high-NA lenses.

To minimize this stray light that drowns the low-contrast image of weakly birefringent specimens, a simple, quick fix is to close down the condenser iris and use an objective lens with a lower NA. However, one loses image resolution as well as the amount of image brightness needed to see or record the images. Also, the depth of field can become unacceptably deep and make for a confusing image.

Oil-immersion objective and condenser lenses tend to depolarize the light less and hence, introduce less stray light compared to "high dry" objective lenses that have the same NA. That is because up to four air-glass interfaces, where the rays suffer the steepest angle of incidence, are essentially eliminated by oil immersion between the objective lens and coverslip, and condenser lens and slide.

Video enhancement can also help to a certain degree. By analog or digital processing, the

unwanted background stray light can be offset (suppressed) and the image contrast can be raised so that the optical problems can be overcome to some degree (Allen et al., 1981; Inoué, 1981b; Inoué and Spring, 1997).

A proper remedy for the depolarization of light is accomplished by using objective and condenser lenses equipped with polarization rectifiers. By using "rectified" lenses, one can, in fact, restore much of the polarization states of the light passing each region of the objective lens back aperture (Fig. 4.9.5C) and thus achieve high extinction for the full aperture even when using high-NA lenses (Inoué and Hyde, 1957).

In addition to allowing the detection and measurement of very weakly birefringent minute objects, rectification gets rid of the diffraction anomaly that is introduced in images of weakly birefringent objects. The diffraction anomaly, which arises from the different states of polarization in different quadrants of the aperture plane, can even reverse the contrast of weakly birefringent minute objects and prevent proper interpretation of the specimen's fine structure (Inoué and Kubota, 1958; Inoué and Spring, 1997).

Unfortunately, rectified lenses, which used to be available from American Optical, and from Nikon, are currently not commercially available. However, the following two alternative approaches can provide some relief of the problems discussed.

The first uses two opposite-handed circular polarizers instead of crossed linear polarizers to extinguish the field (Huxley, 1960). An advanced version of this type of polarizing microscope is Oldenbourg's "LC PolScope."

As a final alternative, one can gain some improvement of image resolution combined with elevated extinction by using an appropriate mask at the aperture plane. The mask, placed at the back aperture of the objective lens and/or at the plane of the condenser iris diaphragm, would be shaped so as to transmit light along the dark arms of the cross, while cutting out the modified polarized light that would have passed through the rest of the aperture.

Such masks would definitely raise the EF, making the background much darker and allowing detection of very low birefringence retardances. Such masks would also effectively provide high NAs in two orthogonal directions, thus yielding high resolution for objects that diffract light in those directions. The accompanying modifications of the point-spread function may be a slight penalty to pay in order to gain high extinction and freedom from anomalous diffraction at very high NAs.

Example 6: High-Resolution Polarization Microscopy of Meiotic Mitosis and Spermiogenesis

Using a polarizing microscope as described in Example 5, it is possible to use high-NA objective lenses (combined with high effective NA of the condenser) to examine the detailed distribution of birefringence as in the mitotic spindle of a dividing cell or the coil-of-a-coil packing arrangement of DNA in certain chromosomes.

Some cell types are more suited for such observations: such as single-layered tissue cells that remain flat instead of rounding up during mitosis (McIntosh et al., 1975), cells without excessive light scattering or birefringent inclusions, cells with large and clearly visible chromosomes, and cells in which different mitotic and developmental stages are well synchronized. Some examples include: newt lung epithelial cells (Rieder and Hard, 1990; Oldenbourg, 1999), endosperm cells of the African blood lily (Inoué and Bajer, 1961), some symbiotic protozoa in the wood-eating cockroach (Inoué and Ritter, 1978), spermatocytes of crane flies (Forer, 1965), grasshopper spermatocytes (Fig. 4.9.6 and Zhang and Nicklas, 1996), and maturing spermatids (Fig. 4.9.7 and Inoué and Sato, 1966). The extensive article (in German) by Belar (1928) is a treasure trove of cell sources available for observing various features of cells in the living state as well as after fixation and staining.

The following describes a method for preparing grasshopper spermatocytes and spermatids for high-resolution polarization microscopy. The following materials are required:

Healthy male grasshoppers, preferably of *Dissosteira*, *Chortophaga*, or related species that fly well for several meters at a time, rather than those that only hop or fly for short distances (for some reason, the spermatocyte mitochondria, which are birefringent, are better aligned in the former group of grasshoppers and interfere less with detailed observation of spindle birefringence);

Grasshopper Ringer's solution (see Table 4.9.2 and preparation instructions below);

Clean Syracuse watch glasses;

Bio-cleaned slide and coverslips (see Table 4.9.1 and instructions for bio-cleaning below);

A pair of sharp iridectomy scissors;

A sharp pair of stainless-steel watchmaker's forceps;

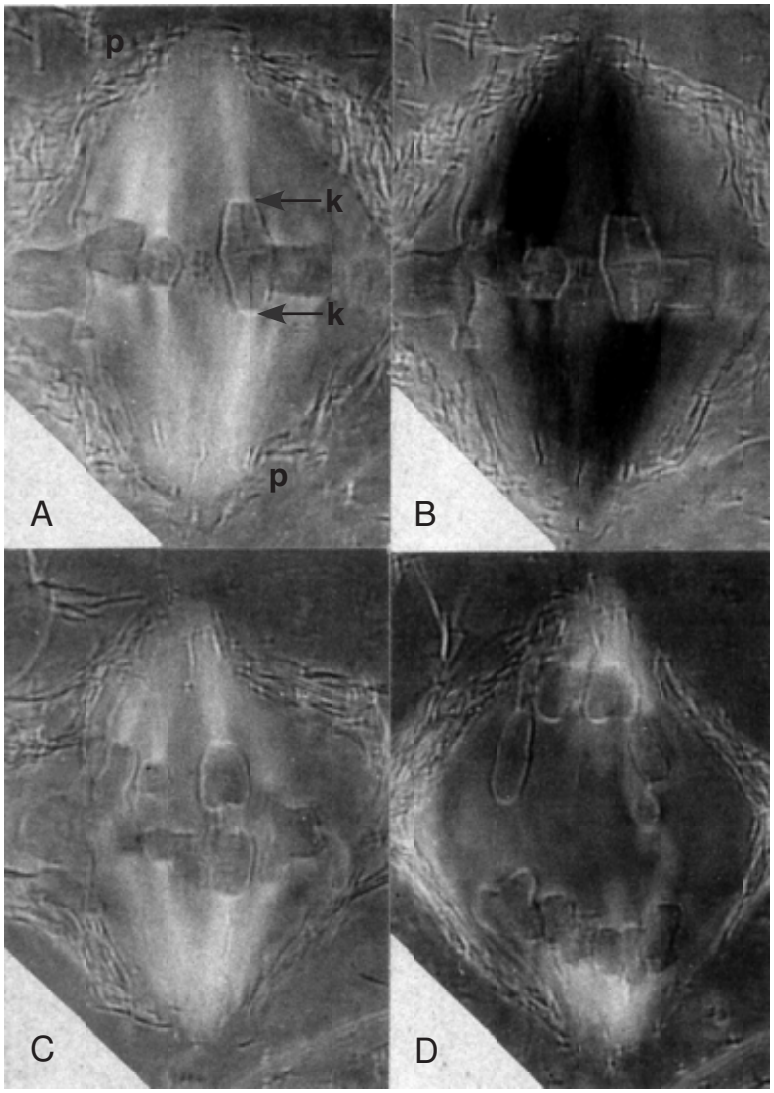


Figure 4.9.6 Meiosis-1 spindle of grasshopper spermatocyte observed with rectified optics. The positive birefringence of the bundle of microtubules that make up the chromosomal spindle fibers shows prominently. The kinetochores (k) also appear to show the same sign of birefringence as the microtubules, but it has not yet been established whether this reflects fine-structural anisotropy of the kinetochore, or primarily the edge birefringence of the chromosome at the kinetochore. The mitochondria also show a longitudinally positive birefringence with overlapping edge birefringence. The compensator slow axis lies parallel to the spindle fibers in panels (**A**), (**C**), and (**D**), so that the positively birefringent microtubule bundles appear brighter than the background. (**C**) The compensator orientation is reversed so that the microtubule bundles appear in reverse contrast. (From Nicklas, 1971.) For online version of time-lapse series of this and other cells in mitosis, see *http://www.molbiolcell.org* and Inoué and Oldenbourg (1998).

Sharp, stainless-steel dissecting needles;
A sharp hypodermic needle mounted on a wooden handle;
Pasteur pipets with rubber bulbs;
Small balls of absorbent cotton;
6- to 8-mm-wide strips of filter paper;
Mineral oil or other innocuous oils such as Halocarbon oil 27;
Valap (see *UNIT 13.1*);
70% ethanol;
Dissecting microscope with trans-illumination.

Preparation instructions for several of these items, along with some parameters critical to performing the experiments described here, are included in the discussion of Ensuring that Cells are "Happy" While They Are Being Observed Under the Microscope, below.

Let the male grasshopper hold onto a small wad of absorbent cotton or a small piece of tissue, so as to absorb the brown fluid expelled

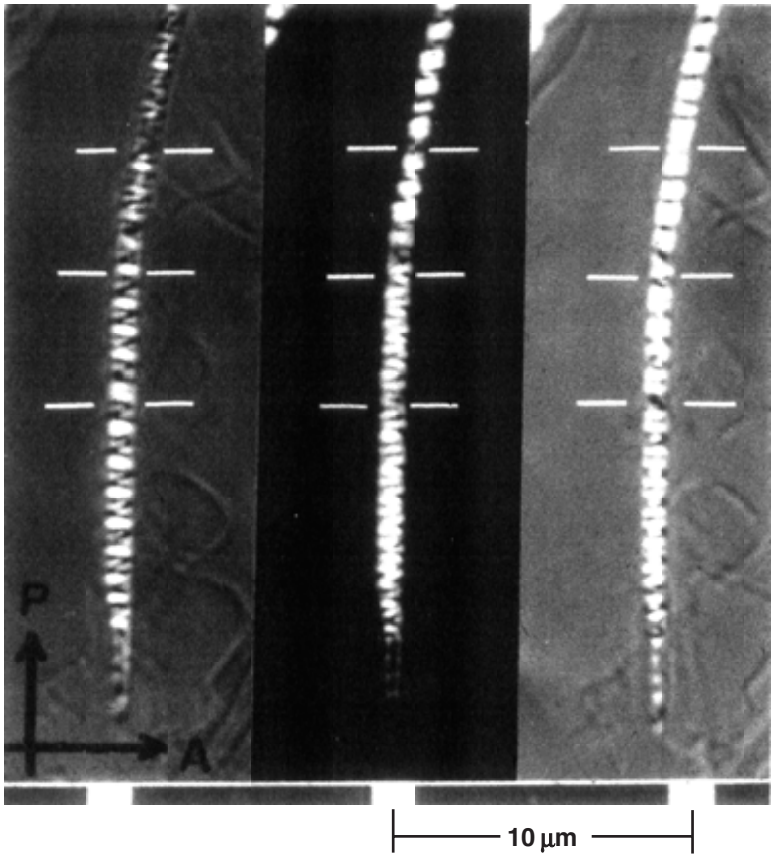


Figure 4.9.7 Live sperm head of cave cricket viewed with a high-resolution polarizing microscope. The sperm head immersed in DMSO (dimethyl sulfoxide) is viewed at three compensator settings using rectified optics (×97/1.25 NA). P, A: transmission axes of the polarizer and analyzer. The compensator slow axis is oriented perpendicular to the sperm axis in the middle panel, turned ~5° clockwise in the left panel and ~4° counterclockwise in the right panel. The helical regions of the DNA, wound in a coil of coil within the chromosomes, appear bright or dark depending on their slow axis orientations relative to that of the compensator. Bars indicate junctions of chromosomes that are packed in tandem in the needle-shaped sperm head. This is the first (and virtually only) mode of microscopy by which the packing arrangement of DNA and the chromosomes have been clearly imaged in live sperm of any species. From Inoué and Sato (1966).

from its mouth. Lift and clip off the wings at their bases. Then, holding onto the grasshopper with his hind legs folded under the abdomen, insert one blade of the iridectomy scissors pointing up and forward at around the third or fourth from the last abdominal segment as shown in Figure 4.9.8. Make an incision that covers four or five segments along the back of the abdomen. Then gently squeeze the lower part of the abdomen until the pair of testes (Fig. 4.9.9; usually colored bright yellow, orange, or red) pop out from the incision. Cut the ducts and place the testes in the saline (grasshopper Ringer's) solution in a watch glass.

Under the dissecting microscope, use a watchmaker's forceps and sharp needle to peel off the colored layer of fat and the Malpigian tubules that surround the testes. They can be toxic to the isolated spermatocytes if not removed from the final preparation. Freed from the covering material, the whitish lobes of the testes should now look like a bundle of bananas. With a piece of absorbent cotton moistened with 70% ethanol, wipe clean the iridectomy scissors, forceps, and needle used, and then transfer the lobe bundles of the testes to a clean Syracuse watch glass containing fresh saline solution.

Again, under the dissecting microscope, use sharp dissecting needles to separate a testis into small bundles containing three to four intact lobes. Cut apart the bundles where they bunch together, i.e., at the proximal end of the lobes where they share a common sperm duct.

Using the Pasteur pipet, transfer one small bundle of lobes together with a small drop of

saline onto a clean slide. Place a clean coverslip on top and remove excess solution with a small piece of filter paper or tissue paper until the lobes are just starting to be flattened.

Observe the preparation under the polarizing microscope using a low-power (10×) objective lens. In each intact lobe, there should be located, from the duct end towards the distal end, more or less in the following order, bundles of mature sperm heads, immature sperm heads, and sperm tails, then follicles containing spermatids, secondary spermatocytes, primary spermatocytes, and, finally, spermatogonial cells (Fig. 4.9.10). The birefringence of the heads of the mature and immature sperm, as well as of some of the spermatid, and perhaps of the bundle of sperm tail, should be quite prominent.

By using a Brace-Köhler compensator, establish the sign of birefringence (direction of

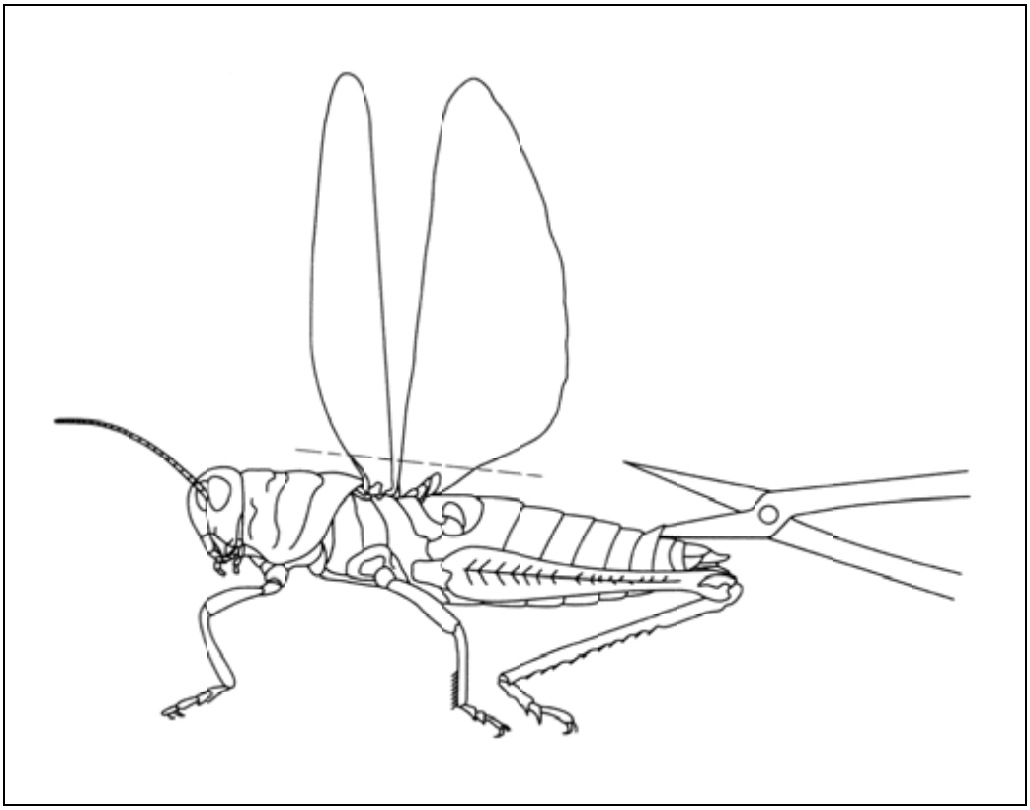


Figure 4.9.8 Dissecting a male grasshopper to expose testes (also see Fig. 4.9.9 and instructions in text).

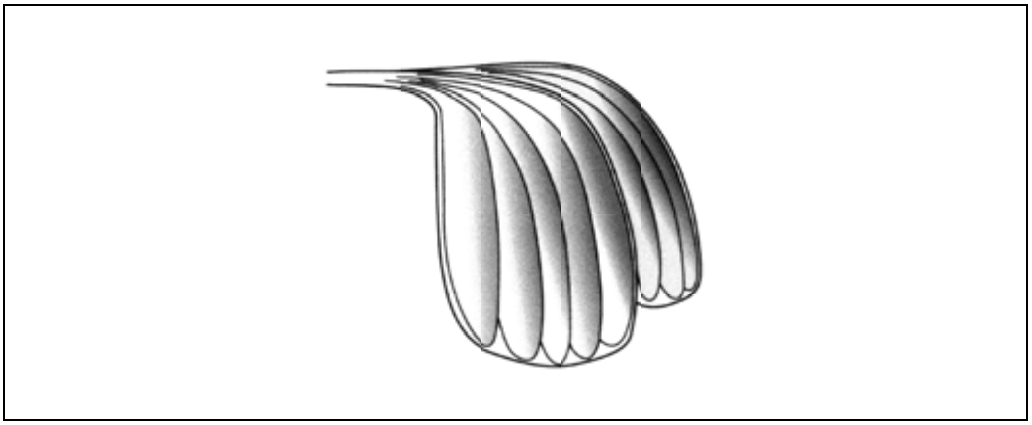


Figure 4.9.9 The pair of testes extracted from a grasshopper. The bundle of lobes in each testis, made like a bunch of bananas and held together where they share a common sperm duct, are covered by a brightly colored sheath. The distal ends of the lobes splay apart when the testicular sheath is peeled off.

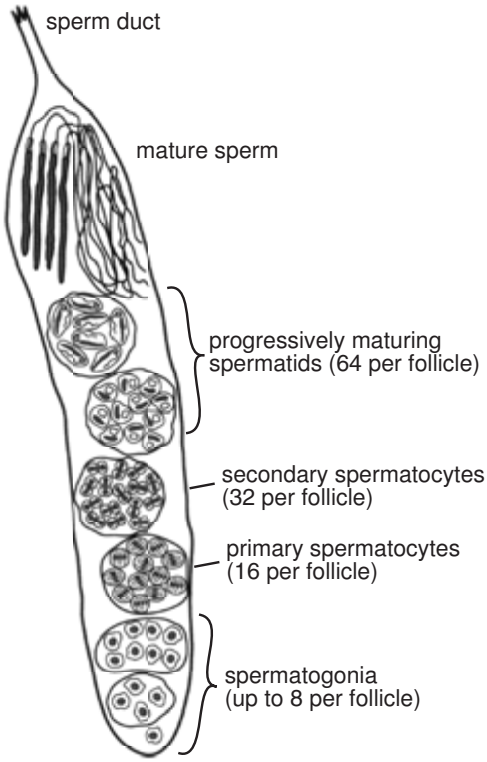


Figure 4.9.10 Arrangement of follicles and cells (not to scale) in the lobe of a grasshopper testis. This highly schematic diagram illustrates one of the many lobes, which are arranged like a bunch of bananas, in a grasshopper testis. The cells, developing synchronously within each follicle in the lobe, are progressively more mature from the distal end of the lobe to the proximal end where the lobes are joined by the sperm ducts. For a photograph of section of fixed lobes, see Figure 1 in Belar (1929).

the slow axes of these structures relative to their long axes). Note that the contrast due to sperm tail birefringence, which is mostly due to the form birefringence of their axonemal microtubules, is reversed from that of the sperm head, which is almost exclusively due to the intrinsic birefringence of the DNA molecules aligned parallel to the length of the sperm head.

Next, make a fresh preparation for observing the spindle fibers and chromosomes and individual sperm with greater resolution. This exercise may require some practice, since a combination of care and speed of preparation is important. Also, do not be frustrated if, at first try, primary spermatocytes at just the right stage of meiotic mitosis are not found. There are a number of follicles at different stages of spermiogenesis in each testicular lobe, and the divisions of the 16 primary spermatocytes in each follicle are synchronized, but you may not come across a lobe that contains a follicle at the right stage. Usually, it is not necessary to try more than two or three lobes.

Using a pair of watchmaker's forceps, transfer a bundle of three to four testicular lobes onto a bio-cleaned microscope coverslip. The coverslip should be resting on a clean microscope slide or some other appropriate holder that lifts it off from the lab bench or other surfaces, which may be a source of lint or other birefringent dust. In making the transfer, be sure to hold onto the duct ends of the lobes and not the other, free end. During the transfer, touch the lobes gently to the clean, dry rim of the watch glass to remove much of the saline solution.

Using a hypodermic needle with a very sharp tip as a knife, cut the lobes (now resting on the coverslip) near their free ends. Then, holding onto the duct end of the lobes with a watchmaker's forceps, draw the lobes in a spiral a few millimeters in radius, making sure that their free, cut ends trail on the surface of the coverslip. As you draw the spiral, cells should flow out together with the testicular fluid, from the cut ends of the lobes onto the surface of the

coverslip. Those near the cut end flow out first, then those closer to the ducts that you are holding onto. Pick up and discard the residue of the lobes. The result is a spiral-shaped thin film of cells, more or less arranged in order from least differentiated cells to mature sperm (see Fig. 4.9.10).

Quickly place a drop of oil (Halocarbon 27 or light mineral oil) on the spiral, invert the coverslip, and cover the preparation by contacting onto a clean slide. Do not use any spacers. Remove the excess oil by contacting a strip of filter paper to the rim of the coverslip, then seal the preparation with Valap.

The slide is now ready for observation of the spindle and chromosomes in meiotic divisions and different stages of sperm maturation. The observations can be carried out on the same live cells for many hours. Not only are the cells arranged and clustered, more or less in the order of stages of spermiogenesis, but they should also lie in reasonably flat monolayers making them suitable for observations with high-NA lenses using polarization microscopy, phase contrast, or DIC.

Note, however, that the testicular lobes, from the time they are removed from the saline medium, and especially the very thin spread of cells in the spiral, could almost instantly lose water by evaporation before being covered by the Halocarbon oil. Thus, some practice and speed of operation becomes important for the last phases of the preparation described above.

In successful preparations observed in high-extinction polarization microscopy, one finds meiosis-I spindles (which are considerably larger than those in meiosis-II) with a moderate degree of positive birefringence, somewhat rounded spindle poles (in contrast to being very pointed), and chromosomal spindle fibers that appear somewhat fluffy (rather than too sharp as though drawn as a line). Such cells can be expected to complete meiosis-I as well as meiosis-II in several hours (see Inoué, 1964).

The process of sperm maturation, on the other hand, is very slow, so that one is not likely to see the actual transition of single spermatids from one stage to the next. The successive stages of sperm maturation, including the rise of negative birefringence in the spermatid nucleus associated with their dehydration and shape changes, should, however, be neatly displayed in chronological order on the slide, so that it should not be difficult to visualize the sequence of events.

Example 7: Examining Meiotic Spindles and Chromosomes

Note that in grasshopper spermatocytes, it can take several hours between the time when an apparently mature spindle is formed and the beginning of anaphase. During that interval, known as prometaphase, chromosomes oscillate back and forth to both sides of the metaphase plate (Deitz, 1969). Concurrently, the associated spindle fibers change the strength of their birefringence. With time-lapse cinematography or video, one can also observe considerable fluctuation of the spindle birefringence (the so-called "Northern Lights Phenomenon"; Inoué, 1964), reflecting the dynamic growth and shortening of their constituent microtubules (Mitchison and Kirschner, 1984; Walker et al., 1988).

The sex chromosomes, in particular, may be found to travel all the way form one spindle pole to another. The oscillatory behavior of these prometaphase chromosomes, and the associated stretching and contraction of their kinetochores to which the chromosomal spindle fibers are attached, may be observed more clearly by switching from polarization to DIC or phase-contrast microscopy (Salmon and Segal, 1980; Nicklas et al., 1995).

Once full metaphase is established, and all of the chromosomes have lined up on the metaphase plate, a signal is released and the chromosomes synchronously enter anaphase (Nicklas et al., 1995). Note the change in distribution and strength of birefringence of the spindle fibers and astral rays as anaphase progresses. What can one infer about the behavior of the microtubules from these observations?

Note also, as anaphase progresses, that another bundle of thread-shaped, birefringent elements lines up along the long axis and surrounding the spindle, to be pinched apart into two as the cell cleaves. Note the sign of birefringence of these mitochondrial threads.

In each daughter cell that results from the division of the spermatocytes, the mitochondria come together to form a dense, round body, the Nebenkern, which has a diameter similar to the reconstituted daughter nucleus and is often mistaken for the latter. The Nebenkern shows a higher refractive index than their surrounding cytoplasm, whereas the daughter nuclei themselves tend to have a lower refractive index than the surrounding cytoplasm. Using DIC optics, verify these refractive index differences by observing the shadow-cast appearances at the edges of these structures.

Example 8: Examining The Sperm and Maturing Spermatids

The needle-shaped, mature sperm heads in grasshoppers show a very strong birefringence whose character is reversed from that of the sperm tail and the acrosome at the tip of the head. Establish the sign of birefringence of these three regions.

Also note that, unlike the nuclei in spermatocytes (and many other cells), the refractive index of the sperm head is very high, reflecting the loss of water and condensation of its chromatin during spermatid maturation.

Choose a series of spermatids starting with those that are nearly mature to very young ones. As maturation progresses, the sperm nucleus becomes more and more elongated, while its birefringence increases. The coefficient of birefringence of the mature sperm is, in fact, extremely high, amounting to -2×10^{-2}, approximately that of a thread of pure DNA (Schmidt, 1941; Wilkins, 1951; Inoué and Sato, 1966).

Note that before the spermatid starts to lose water and become elongated, the nucleus is almost nonbirefringent. Most cell nuclei show very little birefringence (with the exception of those in sperm and in certain Dinoflagellates; Schmidt, 1932, 1937; Cachon et al., 1989), despite the fact that they contain a large quantity of DNA.

In maturing spermatids, note also that the axis of birefringence of the sperm head is tipped this way and that and is not totally parallel to the elongated axis of the head, as in the fully mature sperm. In some insects, such as the cave cricket, the axes of negative birefringence, even of the mature sperm head, can still be resolved and seen not to be uniform. Instead, they are disposed more or less in a zigzag fashion (Fig. 4.9.7), reflecting the packing arrangement of their DNA backbone and chromosomes. The detailed packing arrangement of the DNA bases, including at levels far smaller than the resolution limit of the polarizing microscope, was determined by irradiation of the live sperm head with a microbeam of polarized UV and by quantitatively measuring changes in the birefringence and their axes for each resolvable area (Inoué and Sato, 1966).

Ensuring that Cells Are "Happy" While They Are Being Observed Under the Microscope

To observe the behavior or fine structure in living cells under a microscope, there are several factors that demand special attention. This is especially true when observations are to be made for extended periods of time and under conditions that require brilliant illumination, as in polarization microscopy, to visualize weakly birefringent objects. On the other hand, birefringence can be an especially effective monitor for the physiological state, and "happiness," of the cell.

Biological material

Start with healthy organisms and cells. Clearly, one cannot expect to gain healthy cells from diseased or necrotic plants or animals or gain normal fertilization and development when the gametes are not fully ripe.

Culture medium

Be alert to toxic contaminants, especially those that can affect the cells at low concentrations. Heavy metals such as copper, zinc, and silver, which can be present in trace amounts even in conventional reagent-grade sodium chloride, can be sources of trouble since NaCl is used in high concentrations in artificial seawater and physiological saline. Likewise, for making culture media, distilled water made in glass or quartz stills (with adequate vapor, i.e., water droplet, suppression) is preferred over that from metal stills. Deionizers remove trace metals, but one should be careful that the water is not contaminated by mold or bacteria (which can grow in the columns or tubing) and their products including surfactants (which show up as long-lasting foam after the water is shaken or bubbled).

Slide and coverslips

Cells prepared between a slide and coverslip are exposed to a large area of the glass surface relative to the volume of the medium bathing the cell. In addition, the cells are likely to be contacting the glass surface directly. Thus, slight contaminants on the glass surface can disproportionately affect the cells being observed. Eliminate surfactants and heavy metals that may affect the well-being of the cells as well as birefringent contaminants that may interfere with observations by polarization microscopy (see below).

Oxygen supply

In addition to changes in pH of the medium, anoxia (and hyperoxia in the case of some obligatory anaerobes; Ritter et al., 1978) can interfere with proper health and division of cells grown in the confined space between the slide and coverslip. Tissue culture cells are commonly observed through special flow-through microscope chambers where the culture me-

dium is appropriately oxygenated and its pH and temperature are monitored or controlled. Alternatively, developing marine embryos and dividing protists can be observed continuously for many hours using a micro-chamber containing an equilibrated gas phase formed between a slide and coverslip (see Inoué and Spring, 1997). Microscope preparation of cells covered with a layer of nontoxic oil (even under the coverslip) can survive for many hours since many oils, especially silicone and fluorocarbon oils, dissolve gases, including oxygen, very effectively. Halocarbon oils, such as Kel F-10, FC-47, and Voltalef that previously were used to cover cultured cell smears, e.g., of insect spermatocytes, are no longer available. Instead, the Halocarbon oils 27 or 200 (Halocarbon Products; see *SUPPLIERS APPENDIX*), used for immersing developing *Drosophila* embryos, would appear to be reasonable, currently available alternatives. The manufacturer recommends that fresh oils be bubbled with air before use for a day or two to replenish the oxygen that was evacuated during manufacturing.

Temperature

While slides containing cultured cells of mammalian origin generally require heating, most invertebrate cells may need to be kept cool, i.e., no warmer than moderate room temperature. For observing such cells, be especially careful with the illumination used for polarization microscopy. A source with high brightness is needed in order to see or record the weak birefringence in the cell. But most high-brightness sources (not just quartz halogen or other tungsten filament lamps, but also mercury and xenon arc lamps) produce a huge quantity of infrared. Be sure to use a good quality heat-cut filter, and, if practical, a high-transmission (>65%) narrow-band-pass (±15 nm) green interference filter. That is because the preparation can be heated up with the infrared and far red portions of the spectrum, and cells can react adversely to the blue end of the spectrum (Langford and Inoué, 1979).

In addition, use Köhler illumination and keep the illuminated area to the smallest practical size by adjusting the opening of the field diaphragm. Even using a laser beam, it is not easy to heat up a portion of the specimen with a diffraction-limited small spot of light because water carries away the heat very effectively. On the other hand, the light from an unfiltered incandescent or arc source can rapidly heat up the specimen under the microscope if the illumination covers a large area of the specimen.

The birefringence of the mitotic spindle (as well as its shape) can be a sensitive thermometer that reflects the temperature of the specimen, which is otherwise quite difficult to monitor closely. At lower temperatures, the spindle birefringence drops, while at higher (physiologically compatible) temperatures the birefringence rises (showing enhanced polymerization of tubulin) following a remarkably reproducible curve (Inoué et al., 1975; Nicklas, 1979).

Drying

With cells mounted between slide and coverslip in an aqueous medium be especially alert to drying and increased tonicity of the medium. Valap (see *UNIT 13.1*) is an effective material that can be applied to seal cells mounted in aqueous solutions between the slide and coverslip. The distribution of birefringence in the spindle can indicate the tonicity of the medium. In hypertonic media the chromosomal spindle fibers tend to appear as sharp pencil-drawn lines that converge sharply to the spindle poles (a sign of poor health, although partially reversible), while in less concentrated media, they appear fluffier.

Bio-clean slides and coverslips

In preparing cells for long-term observations under a microscope, one needs to be especially careful to prepare media with the appropriate pH and degree of oxygenation, free of heavy metals. It is especially important to use glassware that is "biologically clean." Since the cells being observed under a microscope are bathed in a very small volume of medium and their surfaces contact a disproportionately large area of the glass surface (and the concentration of any contaminant can become exceptionally high), the slide and coverslips in particular must be bio-clean.

Bio-clean glassware should be free from soap and detergent in addition to even minute amounts of heavy metal, fixatives, and other toxic contaminants or residues that may have been left or adsorbed onto the glass surface. Glassware (including slides and coverslips) and plasticware that have been acquired new from suppliers (except specially packaged pipet tips and capped centrifuge tubes, etc.) should not be assumed to be bio-clean. Table 4.9.1 shows the sequence of procedures used in the author's laboratory for preparing bio-clean glassware and slides and coverslips (Lutz and Inoué, 1986).

Glassware once used with fixatives should be separated by marking with clear, indelible F

Table 4.9.1 Procedures for Bio-Cleaning Glassware (after Lutz and Inoué, 1986)

Step	Procedure
1	Fill 500-ml beaker with hot tap water and enough detergent to make the solution sudsy.
2	Drop 30 to 50 slides or coverslips *individually* into the beaker. Try to position the slides in a criss-cross arrangement in the beaker.
3	Soak for 15 to 30 min.
4	Place beaker in ultrasonic washer for 3 to 5 min.
5	Rinse many times in tap water to remove the majority of the detergent.
6	Using stainless-steel forceps, transfer slides or coverslips individually into another beaker containing tap-distilled water. Repeat this individual transfer/rinse ten times. Sonicate beaker 3 to 5 min before the last transfer.
7	Individually transfer slides or coverslips into a beaker containing glass-distilled water. Repeat this ten times. Sonicate beaker for 3 to 5 min before the last transfer.
8	Individually transfer slides or coverslips into bioclean storage jars filled with 80% ethanol for storage.
9[a]	Before use, remove slide or coverslip from alcohol using clean forceps and wipe dry with a Kimwipe folded twice (four thicknesses). Do not rub.

[a]If possible, use a centrifuge ("Spin Dry" available from Technical Video) to avoid the possibility of lint associated with Step 9.

marks, and not mixed with bio-clean glassware, since it may be very difficult to get rid of some ingredients of fixatives.

In addition to preparing bio-clean slides and coverslips, keep their surfaces, as well as optical surfaces of the microscope that lie between the polarizer and analyzer, free from birefringent dirt and dust particles. In particular, be careful to prevent pieces of lint (including cellulose fibers, which often have birefringence retardations close to a wavelength or more) from falling on to, or becoming attached to, the surfaces of slides and coverslips. Such fibers can drastically lower the EF of the whole polarization optics and completely interfere with observation of weak birefringence of intracellular structures. Since clean glass surfaces tend to attract lint particles electrostatically, avoid laying your slide or coverslip directly on a bench top, or on the surface of the glass plate that supports the specimen on a dissecting microscope. Instead, place the slide and coverslip on a spacer, such as a fresh applicator stick bent into a V shape, or prop it up by supporting one end by a couple of millimeters off the surface. Note that Kleenex and lens tissue used to wipe the slide or coverslip can also be a source of cellulose fibers. To avoid wiping, use a centrifuge to spin off the alcohol used for the final rinse of the slide and coverslip (see Figures 3 to 11 in Inoué and Spring, 1997).

Grasshopper Ringer's solution

Table 4.9.2 provides the composition of saline solutions that can be used for preparing the grasshopper testes and for observing meiosis in their spermatocytes for many hours (Nicklas et al., 1979). Note that the final tonicity of the medium (plus testicular fluid) may vary somewhat, depending in part on the atmospheric humidity at the time the contents of the testicular lobe are streaked onto the coverslip. Thus, Belar advises either breathing onto the preparation with one's mouth wide open or blowing with lips nearly closed, depending on whether the preparation should be made more or less dilute before sealing the preparation. As noted before, the morphology and birefringence distribution in the spindles are, in fact, sensitive indicators of the cell's health and whether the preparation is or is not appropriate for long-term observation of the division events.

Valap

Valap is a low-melting-point, nontoxic, waxy material used for sealing coverslips. Valap is made by simmering a 1:1:1 mixture of vaseline, lanolin, and paraffin (using indirect heat to prevent overheating or catching on fire) for several hours (also see *UNIT 13.1*). Use of beeswax instead of paraffin, or Valab, yields a somewhat more adhesive sealant. A major advantage of these sealants is that they can be applied directly to seal the edges of coverslips covering wet specimens.

Valap and Valab can be applied either from a melt using a small brush or with a warm spatula or heating wire made by passing current (e.g., from a low-voltage-microscope lamp transformer) through an ~5-inch length of U-

shaped nichrome wire fastened to an insulated holder such as an electric plug fixture (Fig. 4.9.11). Be careful not to pass too much current through the heating wire (if it smokes, it is too hot) since the sealant can spit, and small droplets of this highly birefringent aggregate that land on the coverslip can ruin the extinction of the polarization optical system.

After applying the sealant, examine all four sides of the coverslip with a dissecting microscope or magnifying glass to make sure that there are no imperfect seals or pinholes, since water evaporates rapidly from even a very small hole in the seal.

Effect of fixation

Polarization microscopy, carried out on living cells that continue to divide, develop, or otherwise undergo normal physiological or developmental activities, provides a good

Table 4.9.2 Composition of Insect Ringer Solution[a]

Stock solution[b]
500 mM PIPES
27 mM KCl
67.5 mM $CaCl_2$
2.5 mM $MgCl_2$
Adjust pH to 6.8 to 6.9 with 10 N NaOH[c]

[a]This is the solution currently recommended by Nicklas et al. (1979) and supercedes the earlier formula of Niklas (Nicklas and Staehly, 1967) as well as those of Belar (1929, footnotes on pp. 364 and 387). In his 1929 artcile, Belar describes and illustrates with photographs of live cells the appearance of pseudopodia, chromosomes, and mitochondria of spermatocytes exposed to media with different degrees of tonicity. He also warns that the preparation be kept from being heated by (the infrared rays from) the light source. See Inoué, 1952b, for effect of temperature on the birefringence of spindle fibers (polymerization of spindle microtubules), which serves as a sensitive intracellular thermometer.

[b]Prepare fresh in glass-distilled water each month and store in refrigerator.

[c]On day of use, dilute 5 ml of stock solution to between 27 and 38 ml (start with 32 ml) with glass-distilled water. The final optimum concentration depends on the extent of evaporation during culture preparation and the physiological state of the animals; the required dilution usually does not change over a period of a week or two.

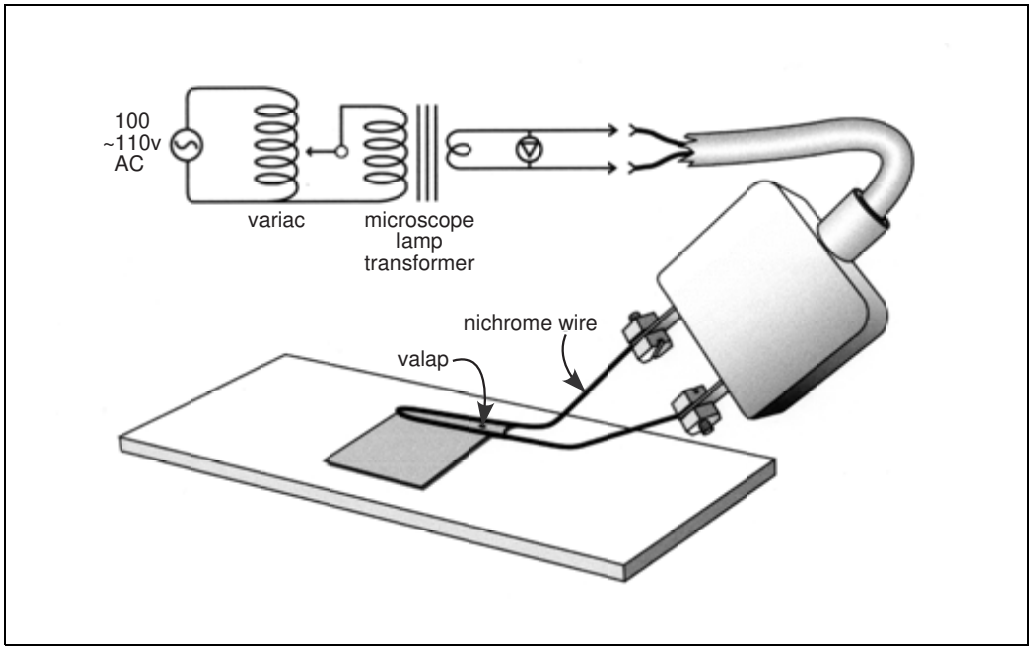


Figure 4.9.11 Electric heater for sealing coverslips with Valap. A 5-in.-long piece of ~20-G nichrome wire is bent as shown and attached, via a convenient handle (e.g., a two-pronged AC plug), to a variable voltage transformer (e.g., one used to power a low voltage microscope lamp). The melted Valap, that has formed a bridge by capillarity between the arms of the nichrome wire loop, is transferred to and painted along the edges of the cover slip.

baseline for understanding the submicroscopic organization and its changes in these cells. That baseline is an important yardstick to heed in considering whether the fine structure of the cell has remained intact when one fixes a cell, or tissue, in preparation for electron microscopy, cytochemistry, or other technique.

When the same cell loses, gains, or changes its birefringence appreciably upon fixation, one needs to wonder whether the fixation itself may not have abolished, created, or altered the fine structure of the cell component. Note that the refractive index of the medium in which the fine structure is immersed also affects the form birefringence of the structure.

For example, prior to introduction of glutaraldehyde as a fixative, spindle and other labile microtubules tended to disappear with standard osmium fixation (Inoué, 1993). Even with glutaraldehyde fixation, there is the possibility that not all of the labile microtubules are preserved (Sato et al., 1975). Given the preparative techniques used for electron microscopy today, we may still be unaware of the loss in fine structural organization encountered by some of the cell's labile filament or membrane systems (e.g., see Burgos et al., 2000).

Acknowledgments

The author is grateful to: Dr. Bruce Nicklas of Duke University for providing updated information on grasshopper Ringer's solutions and on Halocarbon oils, Jan Hinsch of Leica for providing the original for Fig. 4.9.2 and for help with locating references, Drs. Rudolf Oldenbourg and Michael Shribak of the Marine Biological Laboratory for comments on a draft version of the article, Drs. Edward D. Salmon, Nina Allen, and Sid Shaw for help in locating references, the Woods Hole Oceanographic Institution Graphics Services Group for expert help in preparing the figures, and Jane MacNeil of the Marine Biological Laboratory for her tireless help in preparing this article.

LITERATURE CITED

Allen, R.D., Travis, J.L., Allen, N.S., and Yilmaz, H. 1981. Video-enhanced contrast polarization (AVEC-POL) microscopy: A new method applied to the detection of birefringence in the motile reticulopodial network of *Allogromia laticollaris*. *Cell Motil.* 1:275-289.

Ambronn, H. and Frey, A. 1926. Das Polarisationsmikroskop, seine Anwedung in der Kolloidforschung in der Färbarei. Akademische Verlag, Leipzig, Germany.

Belar, K. 1928. Die Technik der Deskriptiven Cytologie. *In* Methodik der Wissenschaftlichen Biologie, Vol. 1 (T. Péterfi, ed.), pp. 638-735. Julius Springer, Berlin.

Belar, K. 1929. Beiträge zur Kausalanalyse der Mitose. II. Untersuchungen an den Spermatocyten von *Chorthippus* (*Stenobothrus*) *lineatus* Panz. *Arch. Entwicklungsmech. Organismen* 118:359-484, plus 8 plates.

Bennett, H.S. 1950. The microscopical investigation of biological materials with polarized light. *In* Handbook of Microscopical Technique (C.E. McClung, ed.) pp. 591-677. Harper & Row (Hoeber), New York.

Breton, J., Michel-Villaz, M., and Paillotin, G. 1973. Orientation and structural proteins in the photosynthetic membrane of spinach chloroplasts: A linear dichroism study. *Biochim. Biophys. Acta.* 314:42-56.

Burgos, M.H., Goda, M., and Inoué, S. 2000. Fertilization-induced changes in the fine structure of stratified *Arbacia* eggs. II. Observations with electron microscopy. *Biol. Bull.* 199:213-214.

Cachon, J., Sato, H., Cachon, M., and Sato, Y. 1989. Analysis by polarizing microscopy of chromosomal structure among dinoflagellates and its phylogenetic involvement. *Biol. Cell* 65:51-60.

Costello, D.P., Davidson, M.E., Eggers, A., Fox, M.H., and Henley, C. 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Lancaster Press, Lancaster, Pa.

Deitz, R. 1969. Bau und Funktion des Spindelapparats. *Naturwissenschaften* 56:237-248.

Forer, A. 1965. Local reduction of spindle fiber birefringence in living *Nephrotoma suturalis* (Loew) spermatocytes induced by ultraviolet microbeam irradiation. *J. Cell Biol.* 25:95-117.

Frey-Wyssling, A. 1953. Fine structure of protoplasmic derivatives. *In* Submicroscopic Morphology of Protoplasm, Chapter 3, pp. 279-370. Elsevier/North-Holland, Amsterdam.

Green, P.B. 1963. On mechanisms of elongation. *In* Cytodifferential and Macromolecular Synthesis (M. Locke, ed.) pp. 203-234. Academic Press, New York.

Hárosi, F.I. 1981. Microspectrophotometry and optical phenomena: Birefringence, dichroism, and anomalous dispersion. *In* Springer Series in Optical Sciences, Vol. 23: Vertebrate Photoreceptor Optics (J.M. Enoch and F.L. Tobey, Jr., eds.) pp. 337-399. Springer-Verlag, Berlin.

Hárosi, F.I. and MacNichol, Jr., E.F. 1974. Dichroic microspectrophotometer: A computer-assisted, rapid, wavelength-scanning photometer for measuring linear dichroism in single cells. *J. Opt. Soc. Am.* 64:903-913.

Hartshorne, N.H. and Stuart, A. 1960. Crystals and the Polarising Microscope: A Handbook for Chemists and Others, 3rd Ed. Arnold, London.

Harvey, E.B. 1940. A comparison of the development of nucleate and non-nucleate eggs of *Arbacia punctulata*. *Biol. Bull.* 79:166-187.

Hecht, E. 1998. Polarization. *In* Optics, 3rd ed., Chapter 8, pp. 319-377. Addison-Wesley, Reading, Mass.

Holzwarth, G., Webb, S.C., Kubinski, D.J., and Allen, N.S. 1997. Improving DIC microscopy with polarization modulation. *J. Microscopy* 188:249-254.

Holzwarth, G.M., Hill, D.B., and McLaughlin, E.B. 2000. Polarization-modulated differential-interference contrast microscopy with a variable retarder. *Applied Optics* 39:6288-6294.

Hsu, B.-D. and Lee, Y.-Y. 1987. Orientation of pigments and pigment-protein complexes in the diatom *Cylindrotheca fusiformis*. A linear dichroism study. *Biochim. Biophys. Acta* 893:572-577.

Huxley, A.F. 1960. British patent specification 856,621. Improvements in or relating to polarizing microscopes (applied July 20, 1956).

Inoué, S. 1952a. The effect of colchicine on the microscopic and submicroscopic structure of the mitotic spindle. *Exp. Cell Res. Suppl.* 2:305-318.

Inoué, S. 1952b. Effect of temperature on the birefringence of the mitotic spindle. *Biol. Bull.* 103:316.

Inoué, S. 1964. Organization and function of the mitotic spindle. *In* Primitive Motile Systems in Cell Biology (R.D. Allen and N. Kamiya, eds.) pp. 579-598. Academic Press, New York.

Inoué, S. 1981a. Cell division and the mitotic spindle. *J. Cell Biol.* 91:131s-147s.

Inoué, S. 1981b. Video image processing greatly enhances contrast, quality, and speed in polarization based microscopy. *J. Cell Biol.* 89:346-356.

Inoué, S. 1986. Video Microscopy. Plenum, New York.

Inoué, S. 1993. Porter and the fine architecture of dividing cells. *In* The Biological Century: Friday Evening Talks at the Marine Biological Laboratory (R.B. Barlow, Jr., J.E. Dowling, and G. Weissmann, eds.) pp. 100-115. Harvard University Press, Boston.

Inoué, S. and Dan, K. 1951. Birefringence of the dividing cell. *J. Morphol.* 89:423-456.

Inoué, S. and Goda, M. 2001. Fluorescence polarization of GFP crystals. *Biol. Bull.* 201:231-233.

Inoué, S. and Hyde, W.L. 1957. Studies on depolarization of light at microscope lens surfaces. II. The simultaneous realization of high resolution and high sensitivity with the polarizing microscope. *J. Biophys. and Biochem. Cytol.* 3:831-838.

Inoué, S. and Kubota, H. 1958. Diffraction anomaly in polarizing microscopes. *Nature* 182:1725-1726.

Inoué, S. and Bajer, A. 1961. Birefringence in endosperm mitosis. *Chromosoma* (Berl.) 12:48-63.

Inoué, S. and Sato, H. 1966. Deoxyribonucleic acid arrangement in living sperm. *In* Molecular Architecture in Cell Physiology (T. Haysahi and A.G. Szent-Györgyi, eds.) pp. 209-248. Prentice Hall, New York.

Inoué, S. and Sato, H. 1967. Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. *J. Gen. Physiol.* 50:259-292.

Inoué, S. and Ritter, H. 1978. Mitosis in *Barbulanympha*. II. Dynamics of a two-stage anaphase, nuclear morphogenesis, and cytokinesis. *J. Cell Biol.* 77:655-684.

Inoué, S. and Salmon, E.D. 1995. Force generation by microtubule assembly/disassembly in mitosis and related movements. *Mol. Biol. Cell* 6:1619-1640.

Inoué, S. and Spring, K.R. 1997. Environmental considerations. *In* Video Microscopy: The Fundamentals, 2nd ed., Section 13.5, pp. 595-603. Plenum, New York.

Inoué, S. and Oldenbourg, R. 1998. Video essay: Microtubule dynamics in mitotic spindle displayed by polarized light microscopy. *Mol. Biol. Cell* 9:1603-1607.

Inoué, S., Fuseler, J., Salmon, E.D., and Ellis, G.W. 1975. Functional organization of mitotic microtubules: Physical chemistry of the in vivo equilibrium system. *Biophys. J.* 15:725-744.

Inoué, S., Knudson, R.A., Suzuki, K., Okada, N., Takahashi, H., Iida, M., and Yamanaka, K. 1998. Centrifuge polarizing microscope. *Microsc. Microanal.* 4 (Suppl. 2):36-37.

Inoué, S., Knudson, R.A., Goda, M., Suzuki, K., Nagano, C., Okada, N., Takahashi, H., Ichie, K., Iida, M., and Yamanaka, K. 2001a. Centrifuge polarizing microscope. I. Rationale, design, and instrument performance. *J. Microscopy* 201:341-356.

Inoué, S., Goda, M., and Knudson, R.A. 2001b. Centrifuge polarizing microscope. II. Sample biological applications. *J. Microscopy* 201:357-367.

Jenkins, F.A. and White, H.E. 1957. Fundamentals of Optics, 3rd ed., pp. 20-29, 407-606. McGraw-Hill, New York.

Katoh, K., Hammar, K., Smith, P.J.S., and Oldenbourg, R. 1999. Birefringence imaging directly reveals architectural dynamics of filamentous actin in living growth cones. *Mol. Biol. Cell* 10:197-210.

Kinosita, K. 1999. Real time imaging of rotating molecular machines. *In* A Half Century of Advances in Microscopy (R.B. Silver, ed.). *FASEB J.* 13:s201-s208.

Langford, G.M. and Inoué, S. 1979. Motility of the microtubular axostyle in *Pyrsonympha*. *J. Cell Biol.* 80:521-538.

Lutz, D.A. and Inoué, S. 1986. Techniques for observing living gametes and embryos. *In* Methods in Cell Biology, Vol. 27 (T. Schroeder, ed.) pp. 89-110. Academic Press, New York.

McIntosh, J.R., Cande, W.Z., and Snyder, J.A. 1975. Structure and physiology of the mammalian mitotic spindle. *In* Molecules and Cell

Movement (S. Inoué and R.E. Stephens, eds.) pp. 31-76. Raven Press, New York.

Mitchison, T. and Kirschner, M. 1984. Dynamic instability of microtubule growth. *Nature* 312:237-242.

Murphy, D.B. 2001. Polarization microscopy. *In* Fundamentals of Light Microscopy and Electronic Imaging, pp. 135-151. Wiley-Liss, New York.

Nicklas, R.B. 1971. Mitosis *In* Advances in Cell Biology, Vol. 2 (D.M. Prescott, L. Goldstein, and E. McConkey, eds.) pp. 225-289. Appleton-Century-Crofts, New York.

Nicklas, R.B. 1979. Chromosome movement and spindle birefringence in locally heated cells: Interaction versus local control. *Chromosoma* (Berl.) 74:1-37.

Nicklas, R.B. and Staehly, C.A. 1967. Chromosome micromanipulation. I. The mechanics of chromosome attachment to the spindle. *Chromosoma* 21:1-16.

Nicklas, R.B., Brinkley, B.R., Pepper, D.A., Kubai, D.F., and Rickards, G.K. 1979. Electron microscopy of spermatocytes previously studied in life: Methods and some observations on micromanipulated chromosomes. *J. Cell Sci.* 35:87-104.

Nicklas, R.B., Ward, S.C., and Gorbsky, G.J. 1995. Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J. Cell Biol.* 130:929-939.

Okazaki, K. and Inoué, S. 1976. Crystal property of the larval sea urchin spicule. *Development, Growth and Differentiation* 18:413-434.

Okazaki, K., McDonald, K., and Inoué, S. 1980. Sea urchin larval spicule observed with the scanning electron microscope. *In* The Mechanisms of Biomineralization in Animals and Plants (M. Omori and N. Watabe, eds.) pp. 159-168. Tokai University Press, Tokyo.

Oldenbourg, R. 1991. Analysis of edge birefringence. *Biophys. J.* 60:629-641.

Oldenbourg, R. 1996. A new view on polarization microscopy. *Nature* 381:811-812.

Oldenbourg, R. 1999. Polarized light microscopy of spindles. *In* Methods in Cell Biology, Vol. 61 (C.L. Rieder, ed.) pp. 175-208. Academic Press, New York.

Oldenbourg, R. and Mei, G. 1995. New polarized light microscope with precision universal compensator. *J. Microsc*. 180:140-147.

Perutz, M.F. and Mitchison, J.M. 1950. State of hemoglobin in sickle-cell anemia. *Nature* 166:677.

Rieder, C. and Hard, R. 1990. Newt lung epithelial cells: Cultivation, use, and advantages for biomedical research. *Int. Rev. Cytol.* 122:153-220.

Ritter, H., Inoué, S., and Kubai, D. 1978. Mitosis in *Barbulanympha*. I. Spindle structure, formation and kinetochore engagement. *J. Cell Biol.* 77:638-654.

Salmon, E.D. and Segal, R.R. 1980. Calcium labile mitotic spindles isolated from sea urchin eggs (*Lytechinus variegatus*). *J. Cell Biol.* 89:355-365.

Sato, H., Ellis, G.W., and Inoué, S. 1975. Microtubular origin of mitotic spindle form birefringence: Demonstration of the applicability of Wiener's equation. *J. Cell Biol.* 67:501-517.

Schmidt, W.J. 1924. Die Bausteine des Tierkörpers in Polarisiertem Lichte. Cohen, Bonn.

Schmidt, W.J. 1932. Der submikroskopische Bau des Chromatins. III. Mitteilung: Über die Doppelbrechung der Isosporenkerne von Thalassicolla. *Arch. Protkde* 78:613-627.

Schmidt, W.J. 1935. Doppelbrechung, Dichroismus und Feinbau des Außengliedes der Sehzellen vom Frosch. *Z. Zellforschg.* 22:485-522.

Schmidt, W.J. 1937. Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma. *In* Protoplasma-Monographien, Vol. 11. Borntraeger, Berlin.

Schmidt, W.J. 1941. Einiges über optische Anisotropie und Feinbau von Chromatin und Chromosomen. *Chromosoma* 2:86-110.

Schroeder, T.E., ed. 1986. Echinoderm Gametes and Embryos. Methods in Cell Biology, Vol. 27. Academic Press, New York.

Shotten, D.M. (ed.) 1993. Electronic Light Microscopy. Wiley-Liss, New York.

Swann, M.M. and Mitchison, J.M. 1950. Refinements in polarized light microscopy. *J. Exp. Biol.* 27:226-237.

Szent-Györgyi, A., Cohen, C. and Philpott, D.E. 1960. Light meromyosin fraction I: A helical molecule from myosin. *J. Mol. Biol.* 2:133-142.

Walker, R.A., O'Brien, E.T., Pryer, N.K., Soboeiro, M.F., Voter, W.A., Erickson, H.P., and Salmon, E.D. 1988. Dynamic instability of individual microtubules analyzed by video light microscopy: Rate constants and transition frequencies. *J. Cell Biol.* 107:1437-1448.

Wilkins, M.H.F. 1951. Ultraviolet dichroism and molecular structure in living cells. II. Electron microscopy of nuclear membrane. *Publ. Staz. Zool. Napoli* 23 (Suppl.):104-114.

Zalokar, M. 1960. Cytochemistry of centrifuged hyphae of *Neurospora*. *Exp. Cell Res.* 19:114-132.

Zhang, D. and Nicklas, R.B. 1996. "Anaphase" and cytokinesis in the absence of chromosomes. *Nature* 382:466-468.

Contributed by Shinya Inoué
Marine Biological Laboratory
Woods Hole, Massachusetts

Fluorescent Speckle Microscopy (FSM) of Microtubules and Actin in Living Cells

This unit describes the execution of an epifluorescent microscopic method, fluorescent speckle microscopy (FSM), that allows visualization of assembly/disassembly dynamics, movement, and turnover of macromolecular assemblies in vivo and in vitro. Microinjection or expression in living cells of fluorophore-conjugated proteins followed by their incorporation into cellular structures and visualization by fluorescence microscopy has yielded much information about protein localization and dynamics (Wang, 1989; Prasher, 1995). However, this method has been limited by high-background fluorescence from unincorporated and out-of-focus incorporated fluorescent proteins and difficulty in detecting movement of fluorescent structures because of their uniform labeling. These problems have been partially alleviated by use of more cumbersome methods of laser photobleaching and photoactivation of fluorescence in which a portion of molecules in a structure is marked and the behavior of the molecules in the marked region is monitored (Wolf, 1989; Mitchison et al., 1998). In contrast, FSM uses a very low concentration of fluorescent subunits that co-assemble with endogenous unlabeled subunits giving a structure with a speckled appearance in high-resolution fluorescence images (Waterman-Storer et al., 1998; Fig. 4.10.1). The appearance, disappearance, and movement of speckles stand out to the eye in time-lapse FSM. These changes in fluorescent speckle pattern correspond to the assembly, disassembly, and movement of the structure and can be quantitated (Waterman-Storer et al., 1999). FSM images are captured using conventional wide-field fluorescence light microscopy and digital imaging with a low-noise, cooled charge-coupled device (CCD) camera. FSM provides information about protein dynamics throughout the field of view, as opposed to within a small marked region of the cell as is the case for laser photobleaching and photoactivation of fluorescence techniques. FSM also significantly reduces out-of-focus fluorescence and greatly improves visibility of fluorescently-labeled structures and their dynamics in thick regions of living cells (Waterman-Storer et al., 1998). Finally, the low level of fluorescent protein used in FSM is much less likely to perturb cellular protein balance thereby reducing the chances of artifactual changes in cell behavior due to the effects of protein "overexpression."

This unit will focus on the utilization of FSM for observing microtubule and actin cytoskeletal dynamics in vivo. For microtubules, which can easily be distinguished in fluorescence light microscopy as single filaments both in vivo and in vitro, the fiduciary marked filaments seen in FSM will allow one to unequivocally distinguish between microtubule translocation as powered by motor proteins and microtubule treadmilling, in which one end of the polymer assembles concomitant with polymer disassembly at the other end (Waterman-Storer and Salmon, 1997). Microtubule FSM also allows observation of microtubule behavior within mitotic spindles containing many hundreds of microtubules and in the central regions of interphase cells. For actin, FSM can be used for studying the assembly dynamics and retrograde movement of actin meshworks in the leading lamella of migrating cells (Waterman-Storer et al., 1998).

STRATEGIC PLANNING

General

Successful FSM requires knowledge and experience in high-resolution epifluorescence microscopy, basic tissue culture and biochemistry skills, and considerable skill in single-cell microinjection and handling of cells for live-cell imaging. This unit provides detailed information in basic protocols for designing a digital microscope system for obtaining

Contributed by Clare Waterman-Storer
Current Protocols in Cell Biology (2002) 4.10.1-4.10.26

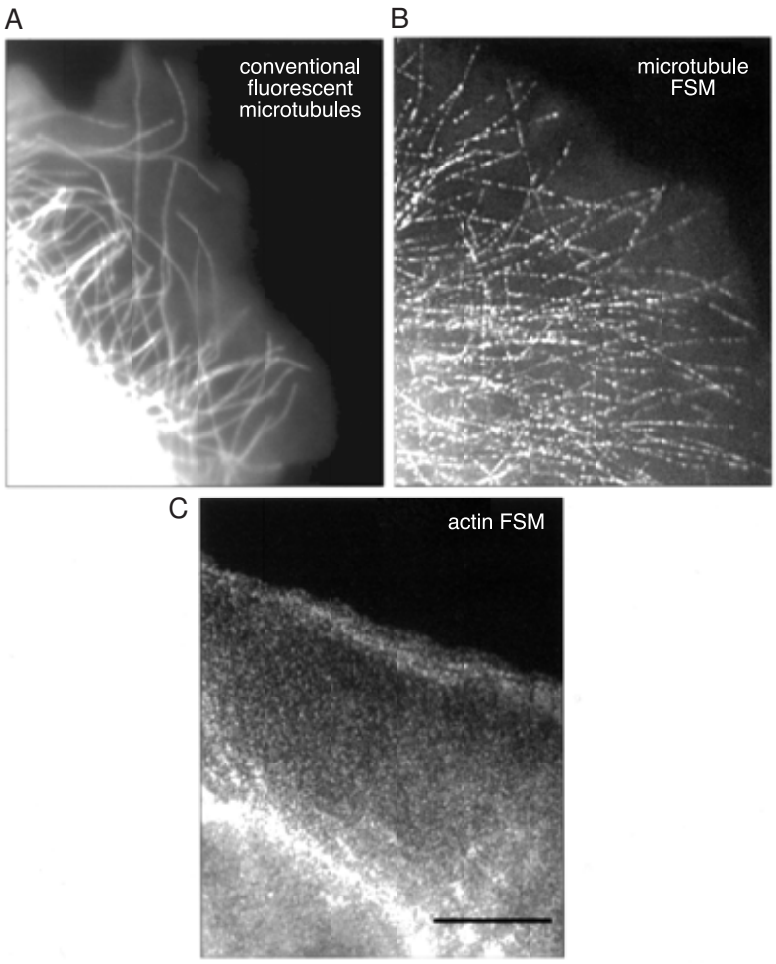


Figure 4.10.1 FSM images of actin (**C**) and microtubules (**B**) in the lamellae of living epithelial cells. A comparison of conventional image of fluorescent microtubules (**A**), labeled with X-rhodamine tubulin, in which labeled tubulin makes up ~10% of the cellular tubulin pool with microtubule FSM (**B**), X-rhodamine tubulin, in which labeled tubulin makes up 0.5% of the tubulin pool. In the conventional image (**A**), there is a high background fluorescence that masks details in the central regions of the cell, and the microtubules are continuously labeled along their lengths, devoid of fiduciary marks. In the FSM image (**B**), the background fluorescence is low, there is no loss in the ability to see the full length of the individual microtubules, and they are covered with fiduciary marks. The images were taken on the same imaging system with the same objective lens. The exposure time for (**A**) was ~200 msec while that for (**B**) was 1500 msec. (**C**) FSM image of a cell injected with low level of X-rhodamine labeled actin. Note the speckled appearance of the lamella, which is made up of a very dense meshwork of cross-linked actin filaments. Bar = 10 μm, all images are at the same magnification.

time-lapse FSM images (see Basic Protocol 1), tips for success in live-cell fluorescence microscopy (see Basic Protocol 2), and basic quantitative analysis of FSM images (see Basic Protocol 3); and support protocols for fluorescent labeling of tubulin (see Support Protocol 1) and actin (see Support Protocol 2). Please refer to *UNIT 4.2* for information on basic fluorescence microscopy, *UNIT 1.1* for cell and tissue culturing, and details of how to carry out single-cell microinjection.

Prior to performing FSM imaging, good quality fluorescently labeled protein must be prepared. Labeled proteins that have given good results in FSM applications can be obtained commercially from Cytoskeleton, Inc.. However, if imaging cytoskeletal dynamics is going to be ongoing in the laboratory, it is much more economical to prepare the proteins oneself. For making fluorescent tubulin, unlabeled tubulin must first be made.

Tubulin purification requires a source of fresh pig or cow brains, and can be prepared by the method described in *UNIT 13.1*, Support Protocol 3. Fluorescent labeling of actin requires a source for muscle acetone powder. Although the powder is available from Sigma, better quality acetone powder can be obtained from a laboratory that specializes in actin biochemistry (generally, these laboratories store large quantities in freezers), or alternatively, it can be prepared by the method of Pardee and Spudich (1982).

Choice of Fluorophore

Support Protocols 1 and 2 describe preparation of tubulin and actin, respectively, that is covalently bound to a succinimidyl ester derivative of the fluorophore of choice. The Molecular Probes catalog is an excellent source of information about the chemistry of fluorophores. Succinimidyl ester derivatives react with lysine residues that are accessible on the surface of the protein. For both tubulin and actin, there is more than one lysine available for reaction, thus, there is the opportunity for higher dye-to-protein ratios, and therefore, brighter fluorescence. For tubulin labeling, succinimidyl esters have, historically, given the greatest success in yield and functional product capable of polymerization and depolymerization (Hyman et al., 1991). Actin labeled with 5-iodoacetamide, which reacts with a single cysteine residue (cys374) on actin, has been well characterized (Wang and Taylor, 1980), however, the dye-to-protein ratio is lower than labeling on lysines.

In choosing the "color" (excitation and emission spectrum) of the fluorophore for FSM imaging, there are two things to keep in mind—the phototoxicity effects on cells of particular wavelengths and endogenous cellular auto-fluorescence. Cells are sensitive to and damaged by short wavelengths; these effects are manifested as retraction of the cell edge, rounding up, and eventually death. Thus, fluorophores that excite at <450 nm (e.g., Coumarin, pyrene) should not be used. Even wavelengths between 450 and 500 nm that excite fluorescein-like dyes (e.g., FITC, Oregon green, Bodipy FL, Alexa 488, or Cy-2) can produce phototoxicity. However, excitation wavelengths beyond the visible (>700 nm, infrared) should be avoided, as well, due to heating effects. The best choice in terms of minimizing phototoxic effects are yellow/orange (570 to 600 nm, tetramethylrhodamine, TAMRA, Cy-3, Bodipy TMR, Alexa 546), orange/red (600 to 630 nm, Lissamine rhodamine, X-rhodamine, Alexa 568, Texas red), or far-red emitting dyes (>630 nm, Cy-5). Of these, the longer wavelength exciters are better, although emission of dyes such as Cy-5 are very difficult, if not impossible, to see by eye, and thus, are problematic to work with. The author's best success with FSM has come from using the X-rhodamine, Alexa 568, or Texas red fluorophores.

Cellular auto-fluorescence is caused by flavo-proteins that excite in the UV and blue, and emit in blue and green (450 to 550 nm) wavelengths. This auto-fluorescence contributes to background when using fluorescein (FITC) or fluorescein-like dyes, and it "dilutes" the speckle contrast. The author has found this effect to be particularly problematic for actin FSM, in regions of the cell where actin filaments make up cross-linked meshworks that appear as uniform, dense fields of speckles in FSM images (Fig. 4.10.1). If the use of green emitters is unavoidable, as is the case in the author's laboratory where microtubules and actin are labeled with spectrally distinct fluorophores and monitored by dual wavelength FSM, green emitters can be used with some success for microtubule FSM.

From this discussion, it is apparent that low-level expression of green fluorescent protein (GFP) fusion proteins are not optimal for FSM. First, there is the problem of cellular autofluorescence. Second, the large size (23 kDa) of the GFP moiety, often results in a sizable proportion of the expressed fusion protein losing its ability to functionally incorporate into the cytoskeleton, contributing to background fluorescence. A third problem is the lack of a suitable method for preventing photobleaching of GFP. Finally,

the brightness (quantum efficiency) of the GFP fluorophore is much lower than chemical fluorophores. For studying a microtubule-binding protein, the author has alleviated this last problem to a large degree by fusing multiple (3 to 5) GFP molecules in tandem, joined by short flexible linkers, to the microtubule binding domain of the protein and selecting cells for imaging that express very low levels of the fusion protein (Faire et al., 1999). However, this approach would be unadvisable for actin or tubulin, which would surely be rendered dysfunctional by the incorporation of a 75- to 125-kDa attachment.

BASIC PROTOCOL 1

DESIGNING A MICROSCOPE SYSTEM FOR TIME-LAPSE DIGITAL FSM

As opposed to a step-by-step protocol, in this section, the basic components needed to set up an FSM system are discussed, giving the recommendations for critical elements required in each type of component (Fig. 4.10.2). It is *imperative* that one reads the Background Information on the theory of FSM image formation before one attempts to understand how and why particular components are chosen for the imaging system.

After reading the discussion of FSM image formation (see Background Information), it is clear that there are three key elements of the microscope imaging system to ensure successful FSM imaging. First, the epifluorescent microscope should be as efficient as possible at collecting photons and should be capable of high-resolution, high-magnification imaging. This is achieved with high-quality, 1.4-NA oil-immersion objective lenses with minimal lens elements. Additionally, this requires a simple optical path between the specimen and camera with as few intervening components as possible. Second, the camera should be highly sensitive, low noise, and should have high enough spatial resolution to match the resolution of the microscope. These properties are met by several cooled CCDs available on the market today. Finally, since fluorophores are subject to photobleaching, light exposure to the specimen should be kept to a minimum. This is accomplished through the use of an electronically controlled shutter on the epi-illuminator that only opens during the time of camera exposure. In addition, a computer with a digital image acquisition board and software for controlling the shutter and image acquisition timing are needed to integrate the system.

Materials

Upright or inverted epi-fluorescent microscope and optics including:
- Epi-illuminator
- High-magnification objective lens (e.g., 60×, 63×, or 100×)
- Excitation filter, emission filter, and dichromatic mirror
- Electronically controlled shutter
- Cooled CCD camera
- Computer, digital image acquisition board, and software for control of shutter and image acquisition

Microscope stand

Upright or inverted epi-fluorescent microscope and optics

1. The microscope stand should be of biological research quality, with a substantial mass that resists vibration and is not subject to temperature-induced expansion and contraction.

   If possible, the microscope should be mounted on a vibration isolation table. The focusing system should be of high quality, with a means by which to lock focus during long-term time-lapse imaging, since slight shifts in focus result in changes in speckle intensity that can be artifactually interpreted as cytoskeletal dynamics.

2. The microscope should be equipped with a high quality epi-illuminator including a 100-W HBO mercury arc lamp, and a lamp housing with a parabolic mirror that

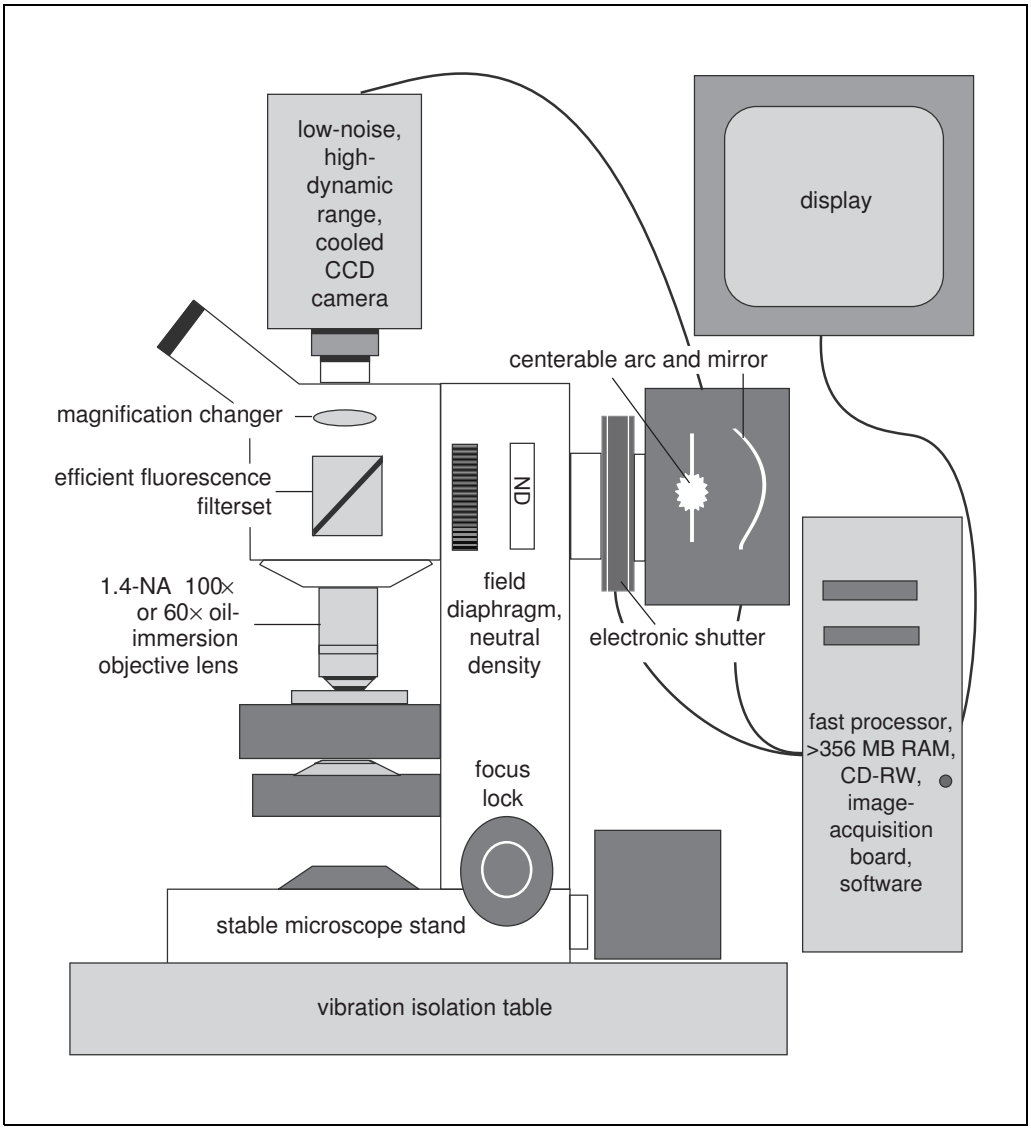


Figure 4.10.2 Diagrammatic view of the components of an upright FSM imaging system. See Basic Protocol 1 for details.

allows manual control of bulb and mirror centration for proper alignment of Köehler epi-illumination.

It is very helpful, though not necessary, to have manual control of centration and size of the field diaphragm of the epi-illuminator. Closing down the field diaphragm to just the area of the specimen being imaged reduces photo-damage to the whole specimen and reduces out-of-focus fluorescent flare in the image. Similarly, it is helpful if the epi-illuminator is equipped with slots in which to insert neutral density filters to control specimen illumination. An infrared blocking filter can also be included in the illumination path to minimize exposure of the specimen to damaging heat.

3. The objective lens (which also acts as the condenser in epi-illumination) should allow the highest resolution possible, thus, one with the highest numerical aperture available, i.e., 1.4-NA oil-immersion; and magnification of 60×, 63×, or 100×, the choice being dependent on the spatial resolution of the camera detector (see Matching Microscope and Detector Resolution below).

Keep in mind that 60× or 63× lenses are often more efficient at transmitting light than 100× lenses. However, never sacrifice system resolution for this small gain in light detection. The

lens should not contain contrast-forming elements such as phase rings that block transmission of photons. The lens should be corrected for chromatic aberration (apochromatic) and should be highly efficient at passing the wavelengths of light being imaged, which depends on the choice of fluorophore. Ask the manufacturer for information on the spectral efficiency of the lens.

When imaging a small specimen, it is not necessary that the lens be flat-field corrected (plan-corrected). Indeed, often objectives specifically designed for efficient epi-fluorescence (Super Fluor-, Nikon; Fluor-, or Fluar- lenses) are not plan-corrected because this correction adds glass elements to the lens, and the more glass elements, the more places to lose photons. However, for a flat, wide–field view, the author has had good luck using DIC-grade plan apochromat objectives.

4. The excitation and emission filters and a dichromatic mirror should be as efficient as possible for exciting the fluorophore, separating the excitation from emission, and collecting the emission of the fluorophore of choice. The use of long-pass filters rather than band-pass filters may maximize this efficiency.

5. The path from the objective to the detector should be simple and contain as few intervening components as possible to allow maximum photon collection. Remove analyzers, wave plates, and Wollaston prisms that are used for various modes of polarization microscopy. Optovars or magnification changers should be optimally removed; however, if needed to match the microscope resolution to the detector resolution (see below), this is a source of light loss that will have to be tolerated.

6. The camera port should utilize the most direct path from the specimen. For an upright microscope, this would be the one port directly over the objective, and for an inverted configuration, a bottom or "Keller" port underneath the microscope is best. Remove prisms from the light path that split the image between the ocular and camera port.

   The bottom port requires a hole in the table upon which the microscope is seated, in order to accommodate the camera. If this is not possible, a side camera port, which requires one mirror to direct the image to the camera is better than a front port that requires at least two mirrors.

Electronically-controlled shutter

7. The electronically-controlled shutter should be mounted with proper adapters in the light path between the lamp-house and the epi-illuminator.

8. The shutter should be mirrored on the surface facing the lamp to reflect heat away from the specimen.

9. The shutter should operate quietly, quickly, reliably, and without excessive vibration. Obtain a shutter that can be actuated from a software triggered pulse via the serial or parallel port of the computer.

Cooled CCD camera

10. The camera should be a scientific-grade slow-scan cooled CCD camera; a video-rate CCD camera equipped with a cooling device cannot be substituted. The choice of camera will be discussed in terms of the choice of the CCD chip itself and then the electronics of the camera in which the chip is housed. Various combinations of the two are available from different camera manufacturers. For a general introduction and in-depth discussion of CCD cameras, see Inoué and Spring (1997).

The CCD chip

11. *Spatial resolution.* The spatial resolution is determined by the physical size of the silicon photodiodes or "pixels" on the CCD chip. These currently range in size from ~6 × 6– to 30 × 30–μm. The larger the pixel size, the more magnification from the

microscope will be needed to ensure resolution-limited images. Thus, smaller pixel size is better for FSM as it will not require photon-robbing magnification changers or Optovars in the light path. The total number of pixels making up the CCD and the pixel size will determine the imaging area.

12. *Spectral sensitivity.* Different types of CCD's have specific probabilities at any given wavelength of converting a photon to a photoelectron that is counted as signal by the camera, i.e., quantum efficiency (QE). For example, a Sony HyperHad CCD has ~45% chance of converting a green photon to a photoelectron (45% QE for green light) and a ~25% QE for red light. Thus, this CCD is much more efficient at detecting green fluorescence than red. Manufacturers supply graphs of the wavelength versus QE for their available CCDs. A CCD should be chosen that has spectral sensitivity for the fluorophore of choice.

13. *Illumination geometry.* CCDs can be illuminated from their front or back sides. Illumination from the front requires that the light pass through substrate materials to reach the photosensitive area, reducing QE. Back-illuminated CCDs are physically thinned to allow illumination directly on the back side of the photosensitive surface, making them much more sensitive (also much more fragile and expensive). However, because of the thinning process, there are limits to the size of the pixels, usually around ~25 × 25–μm, with the smallest currently available at 13 × 13–μm. Thus, one has to weigh whether the increased sensitivity is worth the loss in having to put an Optovar in the image path, as well as whether one can afford the expense. For FSM applications, sensitive front-illuminated CCDs have worked quite well.

14. *Readout geometry*. Once photons are converted to charge in the array of pixels, the charges must be read out to an image acquisition board so that the image can be reconstructed in the computer by assigning a gray value to the relative charge at each pixel position. Charges are transferred out of the CCD in three basic ways. Full-frame readout occurs as each row of pixel charges is transferred serially out of the CCD one row after another. This type is the slowest and introduces the most noise into the image, although, this type of readout is still acceptable for FSM if other camera electronics do not introduce sources of noise. In contrast, in frame-transfer and interline-transfer CCDs, either the entire pixel charge array or whole rows of pixels are rapidly transferred to an array of pixels that are masked from light. The charges are then read out from the masked area while the imaging area is being exposed to light again. These types are much faster and less noisy than the full-frame readout. Frame-transfer CCDs tend to be more expensive and interline-transfer CCDs suffer from lower resolution, although this problem has been overcome in recent years. Thus, all three geometries are acceptable for FSM, although the increased speed of the interline-transfer CCD may offer an advantage for increasing image-acquisition rate to observe actively ruffling cells or microtubule motor activity.

Camera electronics

15. A CCD with the lowest temperature cooling system within the means of one's budget should be chosen. Heat on the CCD can be interpreted by the photodiodes as light, thus, contributing to image noise. Different camera manufacturers will house the same CCD in cameras with different degrees of cooling ranging from 20°C below room temperature to −50° or −60°C. Do not choose the least expensive CCD because heat is an avoidable source for noise that can easily mask the very faint FSM signal.

16. *Readout speed.* In general, the faster the readout speed, the more error is introduced during charge transfer, which translates to noise in the image. Speeds in modern cameras range from 100 kHz in some low-noise back-illuminated cameras, to 14 to

15 MHz in interline- and frame-transfer cameras. For quantitative FSM imaging of cytoskeletal dynamics, image acquisition rates of 1 to 2 images/sec may be required, which cannot be accomplished by the slower cameras. Here, a reasonable compromise of speed and low noise must be sought, but it is recommended not to buy a camera much slower than 1 mHz.

17. *Dynamic range.* Although the total number of photons that can be absorbed by each pixel before it is saturated with charge is set for a given CCD, the number of gray levels this amount of charge is divided up into for display is not fixed. It can be represented by 8, 10, 12, 14, or 16 bits of information per pixel, corresponding to 256, 1024, 4096, 16,384, or 65,536 (2^8, 2^{10}, 2^{12}, 2^{14}, or 2^{16}) gray levels, respectively. For FSM imaging of very dim specimens, it is important to have the biggest dynamic range possible (again, within a reasonable budget). This is required so that there will be finer gradations of gray within the very small portion of the dynamic range that is utilized. Successful FSM has been achieved using 10-bit cameras, although ≥12 bits/pixel is recommended.

18. *Subarraying and binning.* Being able to read out only a specified portion of the CCD (subarraying) can increase image acquisition speed for imaging small areas of a cell, but it is not necessary. Binning, in which the charges in a group of pixels are combined and read out as a single pixel to increase sensitivity should not be done in FSM, as this effectively increases pixel size and decreases CCD resolution.

Computer, digital image acquisition board, and software for control of shutter and image acquisition

19. A computer with the fastest processor and most random access memory (RAM) affordable should be used. Time-lapse FSM image series are large files often on the order of ≥100 MB, and computer "horsepower" is necessary to view and manipulate these.

    The author recommends ≥356 MB of RAM.

20. *File storage.* A compact disc read/write (CD R/W) device is the most economical choice recommended to archive the large files generated by time-lapse FSM. The fastest write speed available within budget should be chosen.

21. Use the image acquisition board recommended by the camera and software manufacturer, making sure that the board can handle the bit depth of the camera. Many cameras come with their own boards.

22. Software should be capable of time-lapse digital image acquisition and triggering the shutter during camera exposure. The software should allow easy viewing of time-lapse series as movies, with control of play-back rate and adjustment of brightness and contrast in the entire image series. Basic image processing including the ability to perform low-pass filtering and image arithmetic (subtraction, multiplication, etc.) is required. The software should provide the ability to perform quantitative analysis of intensity, position, and distance.

    The author has used MetaMorph (Universal Imaging) with outstanding success. However, NIH-Image freeware (http://rsb.info.nih.gov/nih-image/) is also very versatile and many free macros are available.

Matching microscope and detector resolution

23. The key to actually achieving resolution-limited images of fluorescent speckles is matching microscope and detector resolution. Magnifying the diffraction-limited spot to the size of 3 pixels on the CCD is required so that the CCD does not limit

imaging system resolution or produce aliasing between pixels. This is called the Nyquist sampling criterion. Any magnification over this value does not contain any more information and simply reduces the area of the specimen that is imaged. The magnification (M) required to achieve this is given by

$$M = 3(P_{width})/r$$

where P_{width} is the width of a pixel on the CCD and r is the size of the diffraction-limited spot. Thus, for red fluorescence with a resolution limit of 0.27 μm (for red fluorescence, see Background Information) and a camera with 6.7-μm pixels, the magnification required to satisfy the Nyquist criterion is 74.4×. Thus, either a 100× objective or a 60× with a 1.25× Optovar should be used, whichever transmits more light.

TIME-LAPSE FSM IMAGING OF THE CYTOSKELETON IN LIVING CELLS

BASIC PROTOCOL 2

This protocol describes how to handle living cells for FSM imaging. Included are basic recommendations for microinjection, detailed instructions for prevention of photobleaching, and image acquisition. Photobleaching of chemical fluorophores (not GFP) is dependent on dissolved oxygen in the medium. Thus, photobleaching can be substantially prevented by removal of dissolved oxygen, in this case, with a commercial product, Oxyrase, and by then sealing the imaging chamber from contact with air (Waterman-Storer et al., 1993).

Materials

~0.5 mg/ml labeled cytoskeletal protein (tubulin or actin, see Support Protocols 1 or 2, respectively)

Cultured tissue cells grown on 22 × 22–mm glass coverslips in small plastic petri dishes

Valap (*UNIT 13.1*)

Buffered filming medium (see recipe)

Oxyrase EC (Oxyrase Inc.)

Microultracentrifuge (Optima TL or TLX, Beckman Instruments; or Discovery M120, Sorvall)

0.6-ml microcentrifuge tubes

Swinging bucket rotor for microultracentrifuge with adapters for holding 0.6-ml microcentrifuge tubes (for the Beckman TLS-55 rotor, the standard thick-walled 1.4-ml polycarbonate tubes are used; and for the Sorvall, custom adapters are needed)

Microinjection needles (see recipe)

Microloader pipet tips (Eppendorf or equivalent) or narrow-gauge syringe (narrow enough to fit in the bore of the microinjection needle, Hamilton Company)

Single-cell microinjection system capable of controlled backpressure of 0.1 to 20 psi and with a precision micromanipulator for injection of single cells, mounted on an inverted microscope equipped with a long-working-distance phase-contrast condenser and a 40× dry phase-contrast objective lens with a working distance long enough to focus through the plastic petri dish and coverslip.

Microscope stage incubator (optional)

1 × 3–in. glass microscope slides

Scotch double-stick tape (3M)

Cotton swabs

FSM imaging system (see Basic Protocol 1)

Prepare labeled cytoskeletal protein

1. Cool the microultracentrifuge and rotor to 4°C.
2. Rapidly thaw and immediately place on ice a 3-µl aliquot of ~0.5 mg/ml labeled cytoskeletal protein.
3. Pellet insoluble protein in the sample. Cut off the lid of the 0.6-ml microcentrifuge tube containing the labeled protein and place the tube in the polycarbonate adapter for the swinging bucket rotor. Make sure to place a balance tube in the opposite bucket. Microultracentrifuge 20 min at 77,000 × *g* (Beckman TLS-55 rotor at 30,000 rpm), 4°C.

   Do not microultracentrifuge any faster, as this will crush the microcentrifuge tube.
4. During the microultracentrifugation, locate the cultured tissue cells to be injected on the microscope stage using a 40× phase-contrast objective lens.

   All manufacturers correct their high-resolution optics for the thickness of a no. 1.5 coverslip.

   Cell choice is obviously dependent on the biological question; however, it is advisable to choose flat, large, well-spread cells as these are better for microinjecting and imaging. PtK1 are a favorite for mitosis studies, while Swiss 3T3 are a favorite for cell locomotion studies.
5. Following microultracentrifugation, promptly remove the sample from the microultracentrifuge and transfer to ice.

Microinject cells with labeled protein

6. Backload a microinjection needle with ~0.5 µl of protein solution using either a microloader pipet tip or a narrow-gauge syringe.

   Be careful not to touch the insoluble pellet when retrieving the solution from the microcentrifuge tube.
7. Microinject several cells in the center of the coverslip using ~0.5 to 1.5 psi constant pressure.

   The constant flow from the needle helps to keep it from clogging.

   It is not recommended to transiently increase the pressure during microinjection, as this will result in too much fluorescent protein in the cells, ruining the speckle effect.
8. Return the cells to a 37°C, 5% CO_2 incubator for 1 hr to recover from microinjection and to allow incorporation of labeled protein into the cytoskeleton.
9. During this time, place filming medium in a 37°C water bath, melt Valap on a hot plate set on low; and turn on the microscope, camera, and stage incubator to allow temperatures to equilibrate.

   A microscope stage incubator is only required for cells from warm-blooded species. An incubator can be as simple as a home hair blow dryer custom-fitted with a rheostat for control of the heating coils and a thermometer taped to the stage.

Mount cells for imaging

10. Just prior to mounting the specimen, prepare 1 ml of buffered filming medium containing 20 to 30 µl Oxyrase EC.

   The amount of Oxyrase the specific cell type can tolerate must be determined empirically. Some cells are more sensitive to oxygen deprivation than others. For sensitive cells (such as PtK1), make sure the filming medium is prepared with high glucose medium, so that extra glucose will be available for anaerobic metabolism.

11. Prepare a filming chamber by placing two 3 × 30–mm strips of double-stick tape side by side 1 cm apart and parallel with the long axis onto a 1 × 3–in. glass microscope slide to form a channel where the cells will be.

12. Retrieve the coverslip containing the injected cells from the dish of medium, dry the back side and the edges that will be placed on the tape strips, and place the coverslip, cell-side down onto the tape so that the area containing the injected cells lies between the strips of tape.

    Press the coverslip into place to form a good seal with the tape.

13. Add Oxyrase-containing filming medium to the cell "chamber" by pipetting it against the edge of the coverslip between the strips of tape. Exchange the medium several times by wicking away medium with filter paper from the opposite open end of the chamber while pipetting.

    Be sure not to introduce any bubbles into the chamber during pipetting as the presence of air in the chamber will cause problems with photobleaching.

14. Carefully dry the edges of the specimen chamber (do not introduce bubbles) and use a cotton swab to apply melted Valap along all edges of the coverslip to seal it completely to the slide.

Observe mounted cells

15. Allow the slide to equilibrate to the proper temperature on the microscope stage for 10 to 15 min. Turn the room lights off to allow one's eyes to adapt to the dark.

16. Use epifluorescent illumination to locate the fluorescent-injected cells.

    The cells should be quite dimly fluorescent and may not be visible with a low-power, low-NA objective lens, therefore, one may have to search with an oil-immersion lens. After locating the cells, switch to high-magnification, high-NA objective lens. At this point, be careful about illuminating the specimen and causing photobleaching—look only as long as needed to get a cell in focus and centered in the field of view then quickly shutter the light. Use of neutral density filters to attenuate illumination during focus and centration may also be helpful in preserving the fluorescence in the specimen.

    Cytoskeletal polymers are excluded from the nucleus; cells with fluorescent nuclei are likely dead. Dimly fluorescent individual microtubules should be visible by eye, while fluorescent actin should be rather diffuse throughout the cell and perhaps brighter in stress fibers.

17. Pick a dim cell to start with because microinjection will result in cells of varying fluorescent brightness.

    With practice, one will be able to judge by eye the brightness required for good FSM.

18. Illuminate only the area of interest by adjusting the field diaphragm of the epi-illuminator.

Optimize camera conditions and acquire time-lapse images

19. Optimize exposure time. First, make sure the camera is set to the maximal dynamic range possible and set the image display to "autoscale." Without autoscaling, the image will likely appear black since the FSM image is expected to use a very small portion of the low end of the dynamic range of the camera. Then, take a 500-msec "dark image" image with the illuminating light shuttered to get a measurement of the background gray level of the camera. Next, be sure the illumination shutter is set to synchronize with the camera exposure. Then, take images of the fully illuminated specimen (no neutral density filters), trying to get the specimen signal to ~10% higher in gray level value than the background.

For example, if the background gray level in a 14-bit (16,384 gray level) camera is 500, try for an exposure that will give the specimen a gray level of ~550. The sample should be dim enough that this very small amount of signal above the noise should require a rather long exposure time. A good starting point is 500- to 1000-msec exposures, but upwards of 2000 msec may be required.

The time of exposure will vary depending on the camera used, the brightness of the sample, the age of the arc lamp, and the tolerance of the specimen to light. Signs of photo-damage include cessation of cell motility, retraction of cell lamellipodia, or blockage of mitotic progression.

20. Optimize focus. Take exposures while slightly adjusting the fine focus until the best focused–fluorescent speckles can be seen. Then, lock the focus.

    If the actin network does not appear speckly, or if individual microtubules do not appear discontinuously labeled along their lengths, there is too much labeled protein in the cell. Find another, more dimly labeled cell.

21. Acquire time-lapse image series. For following actin movement, acquire images every 10 to 30 sec. For following microtubule dynamics and movement, acquire images at 3- to 10-sec intervals. Image as long as is needed or until the sample is photobleached.

BASIC PROTOCOL 3

QUALITATIVE AND QUANTITATIVE ANALYSIS OF TIME-LAPSE FSM IMAGE SERIES

Methods are presented to extract measurements of speckle movement and lifetime to determine polymer velocities and turnover times.

Materials

Stage micrometer (Fisher)
Image analysis software

1. Calibrate the pixel-to-distance conversion factor. Take an image of a 10–μm stage micrometer using the objective lens used for image acquisition.

   The number of pixels in 10–μm is used to calculate the pixel-to-distance conversion factor. Most software packages will allow this factor to be input so that measurements made are automatically reported in micrometers as opposed to pixels.

2. Adjust the brightness and contrast of the image series to maximize the speckle appearance.

3. Play the time-lapse series as a movie, paying attention to the movement, appearance, and disappearance of fluorescent speckles.

4. To enhance FSM images, perform an "unsharp mask" filter on each image in the time-lapse series (Waterman-Storer et al., 1999).

   This consists of performing a 9 × 9– or 10 × 10–low-pass filter on the image and then multiplying by a constant of 0.5. This low-passed, scaled image is then subtracted from the original image, and the result is multiplied by a constant to scale it to the same average gray level as the original image. This series of operations greatly enhances speckle appearance.

5. To measure speckle movement rates in complex fields of speckles such as the mitotic spindle or the actin-rich lamella of a migrating cell, perform kymograph analysis (Waterman-Storer et al., 1999; Fig. 4.10.3).

   There are two ways to make kymographs of speckle motion in an image series. In the first method, a narrow rectangular cursor is drawn on the image stack, with the long axis of the

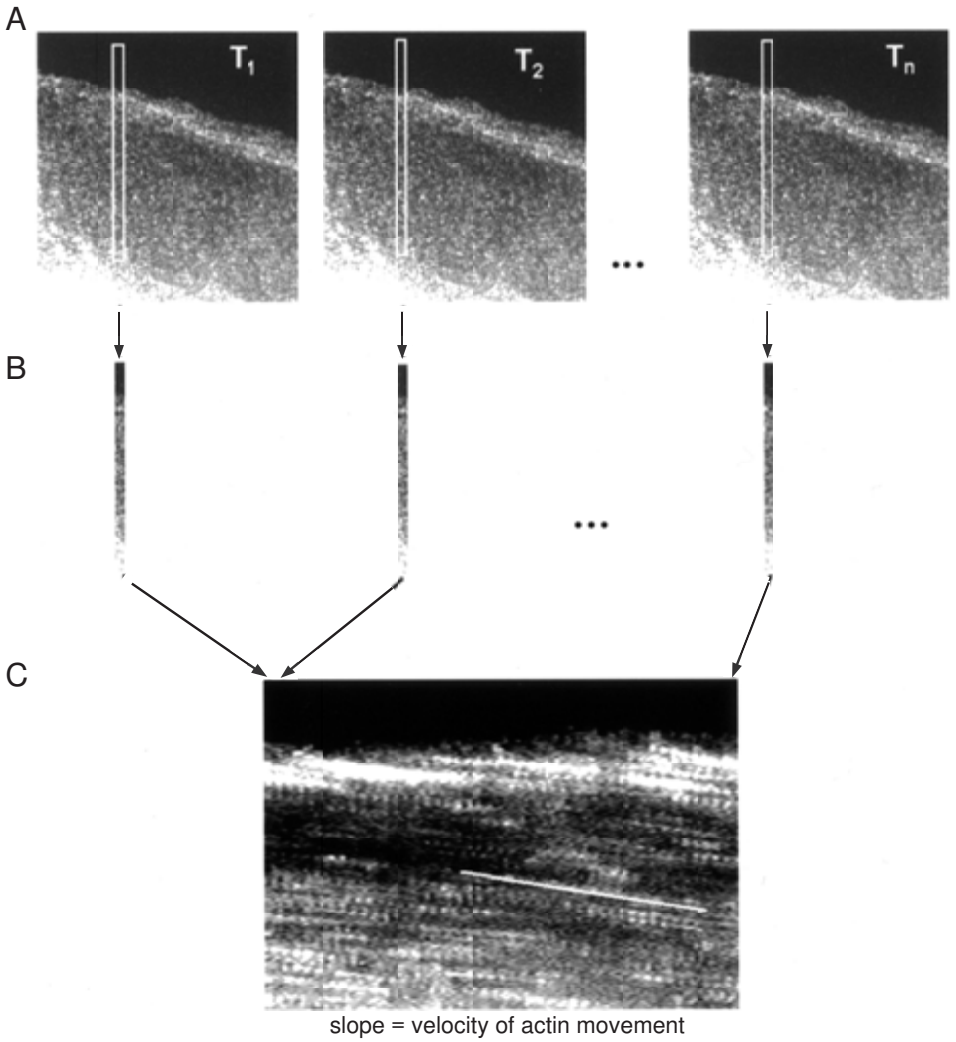


Figure 4.10.3 Construction of a kymograph. (**A**) A long thin rectangular region whose long axis is parallel to the direction of speckle movement is selected in each image in a time-lapse series (T_1- T_n). (**B**) This region is extracted from each image and pasted side-by-side in a montage to form the kymograph (**C**). As speckles move along the axis of the rectangular region in the original image series over time, they appear as oblique streaks on the kymograph, the slope of which correspond to the velocity of speckle movement (see Basic Protocol 3).

rectangle aligned with the direction of speckle movement determined by viewing the series as a movie. The rectangle should be 2 to 4 pixels in width and the length of the area desired for motion analysis. This region of interest is then copied from each image in the time-lapse series, and pasted side-by-side in a montage to form the kymograph picture. By eye, the speckles within the rectangular region that are moving along its long axis produce oblique white lines in the kymograph, the slope of which yields the velocity of motion. The disadvantage of this method is that it only measures speckle movements in either horizontal or vertical directions.

The second method requires the image processing software to possess a "kymograph function." The MetaMorph system is used as an example. The point-to-point line drawing function in MetaMorph is used to draw a line in any direction along the direction of movement for motion analysis on the first image in the time-lapse series. This has the advantage that the line can curve with the curvature of motion. The "kymograph" routine in MetaMorph samples pixel intensity values along this line for each image in a series and

montages these values into an image where the horizontal direction is the pixel values measured along the line, and the vertical direction is lines from subsequent images in the time series. Measurements of distance in the horizontal direction are calibrated in micrometer units. Measurements of distance in the vertical direction must be calibrated to the total time of the number of images in the series. For example, if the images are recorded at 10-sec intervals and there are 50 images in the series, then the vertical distance of the kymograph corresponds to 500 sec. Velocities are determined by the slopes of the oblique trajectories of speckle movements in the kymographs.

6. Use single-speckle motion tracking to track the movement of single cytoskeletal polymers.

   Currently, this can only be done by hand, but software for automated tracking is being developed. Commercially available automated tracking software is incapable of tracking single speckles in a dense field of similar speckles, which in addition are often fuzzy, ill-defined, tiny, and subject to intensity changes due to slight shifts in focus. Hand tracking is done by measuring the distance of the speckle from its origin at time zero in each frame of the time-lapse series. This can often be done semi-automatically using software functions such as "track points" in MetaMorph. The distance over time can be used to calculate instantaneous velocities (change in distance from frame n to frame n + 1) or average velocities by performing regression analysis of the distance versus time data.

7. Measure speckle lifetime to give a value for the lifetime of the cytoskeletal filament, i.e., its rate of turnover.

   In theory, the intensity value of a single pixel at the center of a fluorescent speckle could be recorded at every point in time and the lifetime of the speckle determined from this "fluorescent life history." However, this is currently very difficult in practice because of problems with photobleaching, shifts in focus that affect speckle intensity, and minute movement of speckles in living specimens. Again, image analysis software that takes these problems into consideration is currently being developed.

SUPPORT PROTOCOL 1

PREPARATION OF FLUORESCENTLY LABELED TUBULIN FOR FSM

This is a modification of a protocol developed by Hyman et al. (1991; also see the Mitchison laboratory Web page, *http://iccbweb.med.harvard.edu/mitchisonlab/Pages/label.html*). In this method, tubulin is first polymerized to bury the sites of tubulin dimer-dimer interactions to mask them from being labeled, then polymerized tubulin is reacted with fluorophore. The succinimidyl ester derivative of the fluorescent dye reacts with lysine residues at a high pH. Tubulin is readily denatured at high pH, so the longer the labeling reaction is allowed to proceed, the lower the yield, therefore, do not exceed the recommended reaction time. The reaction is then quenched by lowering the pH, and the tubulin is cycled for a temperature-dependent round of depolymerization and repolymerization (see *UNIT 13.1* for more information on the biochemistry of tubulin) to select for the assembly competent labeled tubulin dimers before finally being depolymerized in microinjection buffer and frozen for storage. This method takes ~7 hr and should yield ~10 mg of labeled tubulin, which should be useful for 6 to 8 months frozen at −80°C.

Materials

10-ml aliquots of phosphocellulose-purified tubulin in column buffer (CB; totaling 40 to 60 mg of tubulin; *UNIT 13.1*)
CB/BRB-80 conversion buffer (see recipe)
100 mM GTP (see recipe)
Glycerol
Labeling buffer (see recipe)
High-pH cushion (see recipe)
Quench (see recipe)

Low-pH cushion (see recipe)
Succinimidyl-ester derivative of fluorescent probe of choice
Anhydrous DMSO
Injection buffer (IB; see recipe)
1× BRB-80 (see recipe for 10×)
1 M $MgCl_2$ (*APPENDIX 2A*)

37°C water bath
100- and 10-ml graduated cylinder
Parafilm
Ultracentrifuge (with a Beckman 70.1 Ti rotor; or equivalent)
13.5-ml ultracentrifuge screw-cap polycarbonate tubes
Manual pipet-pump type pipettor (VWR or equivalent)
1.6-ml microcentrifuge tubes
Microultracentrifuge (Beckman Optima TLX with TLA-100.4 rotor or Sorvall RC M120 GX with S100AT4 rotor)
5.1-ml microultracentrifuge polycarbonate tubes
7-ml dounce homogenizer with "B" pestle
Spectrophotometer with a small-volume quartz cuvette
0.6-ml UV-impermeant microcentrifuge tubes

Prepare tubulin

1. Thaw three to four 10-ml aliquots of phosphocellulose-purified tubulin (frozen off the column in CB; *UNIT 13.1*) by immersing tubes in a 37°C water bath and agitating gently and continuously until they are fully thawed. As soon as the solutions are fully thawed, transfer tubes to ice and pool the aliquots into a 100-ml graduated cylinder on ice.

2. Add 1/20 vol CB/BRB-80 conversion buffer and add 100 mM GTP for a final concentration of 1 mM. Allow tubulin to bind GTP for 5 min on ice.

3. Add 1/3 vol glycerol, cover the cylinder with Parafilm, and mix very well by gentle inversion.

4. Incubate 40 min in a 37°C water bath to allow tubulin to polymerize, gently mixing every 10 min.

   Make sure the water level in the bath is as deep as the liquid level in the cylinder so that the temperature is constant throughout the whole solution.

5. During this incubation prewarm the ultracentrifuge with a Beckman 70.1 Ti rotor, labeling buffer, high-pH cushion, Quench, low-pH cushion, and several 13.5-ml ultracentrifuge screw-cap polycarbonate tubes to 37°C.

   The rotor can be prewarmed by putting it in a watertight plastic bag and submersing it in warm water (in a separate 37°C water bath from the polymerization reaction). The centrifuge may only warm up to ambient temperature. If this is the case, so long as the rotor is at the proper temperature, the microtubules should be intact.

Isolate microtubules

6. At the end of the incubation, add 3 ml warm high-pH cushion to each warm 13.5-ml ultracentrifuge tube. Slowly layer the polymerized tubulin onto the cushion by using a manual pipet-pump to slowly drip the tubulin solution down the side of the ultracentrifuge tube, being careful not to mix the tubulin with the dense cushion. Divide the microtubule solution evenly among the ultracentrifuge tubes. Balance pairs of filled ultracentrifuge tubes to within 0.01 g, and remember to include the centrifuge cap.

7. Ultracentrifuge the microtubules 1 hr at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 37°C.

8. During the ultracentrifugation, make up 100 mM succinimidyl-ester derivative of fluorescent dye of choice in anhydrous DMSO in a 1.6-ml microcentrifuge tube. Warm to 37°C and vortex it well to get as much as possible into solution.

9. Just before microtubule ultracentrifugation is over (step 7), microcentrifuge the insoluble fluorescent dye solution for a few minutes at maximum speed, room temperature. Promptly collect the supernatant and transfer it to a new 1.6-ml microcentrifuge tube.

10. After the ultracentrifugation, note the position of the clear/pearlescent pellet in each tube. Aspirate the supernatant to half the height of the cushion, and wash the cushion with 1 to 2 ml of warm labeling buffer. Then, completely aspirate the supernatant off, rinse the pellet gently with 1 ml warm labeling buffer, and aspirate again. Immediately add 500 µl warm labeling buffer to each tube, and return the tubes to the 37°C water bath.

11. Resuspend each pellet by pipetting up and down in warm labeling buffer. While resuspending one pellet, keep the remaining tubes in the 37°C water bath, and use the same pipet tip for all tubes.

    Careful and thorough resuspension is key to high labeling efficiency.

    The pellets are dense and sticky. Use a cut-off tip (cut ~5 mm off the end) on a 1-ml pipetman, starting with the 500 µl in each tube. Dial the pipetman in to 300 µl, and pipet liquid up and down over the pellet until it is fully resuspended with no chunks present. Be patient, avoid frothing the solution, and do not poke the tip into the pellet as chunks of the pellet will get stuck inside the tip and will be lost.

12. After the first 500 µl, pool the resuspended pellets in one tube in the 37°C water bath, and go back and rinse each tube thoroughly with another 300 µl of warm labeling buffer. Pool all together, a total of 2 to 3 ml, and keep in the 37°C water bath.

Label microtubules

13. Add fluorophore dye solution (step 9) to the resuspended microtubules to 10 mM final concentration, mix well by gentle vortexing, and incubate 30 min at 37°C. Gently vortex every few minutes throughout the labeling reaction, being careful not to froth the solution.

14. During the labeling incubation, warm up the microultracentrifuge with rotor and several 5.1-ml microultracentrifuge polycarbonate tubes to 37°C.

15. Stop the labeling reaction by adding an equal volume of Quench.

16. Put 1 ml of low-pH cushion into each 5.1-ml microultracentrifuge tube, and layer 2 ml of the labeled polymer on top. Microultracentrifuge microtubules 30 min at 200,000 × *g* (Beckman TLA-100.4 rotor at 70,000 rpm), 37°C.

17. During the centrifugation, cool a 7-ml dounce homogenizer, pestle, and 10 ml of injection buffer (IB) on ice and warm up a few milliliters of distilled water to 37°C.

18. After centrifugation, aspirate the supernatant to 1/2 cushion volume, wash the cushion with a few drops of warm distilled water, and then aspirate the remaining cushion.

Depolymerize labeled microtubules

19. Immediately add 500 µl cold IB to each tube. Incubate 10 min on ice to let depolymerization initiate before beginning to resuspend the pellets. Pool resuspended

pellets into the dounce homogenizer on ice, rinse each tube with an additional 200 µl of cold IB, pool all.

This pellet will be horribly sticky. Do not attempt to fully resuspend the pellet in the microultracentrifuge tube, but instead try to partially resuspend as much solids as possible and transfer them into the homogenizer. As always, be patient and do not introduce bubbles into the solution.

20. Allow the microtubules to depolymerize for 30 min on ice, slowly raising and lowering the pestel in the homogenizer the entire time, being careful not to introduce bubbles and keeping the homogenizer immersed in ice.

21. During depolymerization, chill the microultracentrifuge, a few 5.1-ml microultracentrifuge tubes, and the rotor to 2°C.

22. Transfer the resuspended pellets to cold 5.1-ml microultracentrifuge tubes and clarify the depolymerized tubulin by microultracentrifuging 10 min at 415,000 × *g* (Beckman TLA-100.4 rotor at 100,000 rpm), 2°C.

Polymerize labeled microtubules

23. Collect the supernatants and pool in a 10-ml graduated cylinder at room temperature. Add 1/10 vol of 10× BRB-80, 1 M $MgCl_2$ to a final concentration of 4 mM, 100 mM GTP to a final concentration of 1 mM, and finally 1/3 vol glycerol. Mix well by inversion.

24. Polymerize microtubules by incubating the graduated cylinder, covered with Parafilm, for 45 min in a 37°C water bath, with occasional mixing.

25. During the incubation, warm up the rotor, microultracentrifuge, and two 5.1-ml microultracentrifuge tubes to 37°C.

26. Just prior to termination of incubation, put 0.5 ml of low-pH cushion in each warm 5.1-ml microultracentrifuge tube. After the incubation, layer the polymerized microtubules on each low-pH cushion.

27. Microultracentrifuge microtubules 30 min at 200,000 × *g* (in the TLA-100.4 rotor at 70,000 rpm), 37°C.

28. During this microultracentrifugation, chill 5 ml IB on ice and warm 5 ml of 1× BRB-80 to 37°C.

29. After the microultracentrifugation, aspirate the supernatant to 1/2 cushion volume, wash the cushion two times with a few drops of warm 1× BRB-80, then aspirate the supernatant completely, wash the pellet very gently two times with warm 1× BRB-80 to remove all residual glycerol (which is toxic if microinjected into cells).

30. Cover pellets with 200 µl of cold IB and incubate 5 min on ice before resuspending the pellets. During this time, chill the microultracentrifuge and rotor to 4°C.

31. Resuspend the pellets in a total of 300 µl per tube by pipetting, using the second 100 µl as a rinse. Pool the resuspended pellets in a clean, chilled microultracentrifuge tube and cover it with Parafilm.

These pellets should not be as sticky as the last ones and should be fairly easy to resuspend by pipetting.

Depolymerize microtubules

32. Depolymerize the resuspended microtubules by incubating 30 min on ice.

33. Clarify the depolymerized microtubules by microultracentrifuging for 5 min at 415,000 × *g* (in the TLA-100.4 rotor at 100,000 rpm), 4°C.

34. After microultracentrifugation, transfer the supernatant to a new 1.6-ml microcentrifuge tube on ice.

Calculate concentration of tubulin and fluorophore

35. Calculate concentration of tubulin, the fluorescent dye-to-protein ratio, and the percent yield of the preparation. Measure the absorbance of a 1:100 dilution (in water) of the tubulin solution at 278 nm (A_{278}) and at the excitation maximum of the fluorophore in a quartz cuvette, using water to blank the spectrophotometer. Calculate the concentration of tubulin using the extinction coefficient for tubulin ε = 115,000 M^{-1} cm^{-1} at 278 nm and Beer's law:

$$\text{Concentration} = \text{dilution factor} \times A_{278}/\varepsilon$$

36. Calculate the concentration of fluorescent dye similarly, using the extinction coefficient supplied by the fluorescent dye manufacturer (contact technical service for information).

The fluorescent dye-to-protein ratio is simply the ratio of the molar concentrations. For calculation of the percent yield, determine the total amount of labeled tubulin obtained from the concentration and volume and use the molecular weight of the dimer, 110 kDa to convert to milligrams to compare to the original amount of tubulin labeled.

37. Adjust the concentration of labeled tubulin to 20 to 30 mg/ml with IB, and drop freeze 3-µl aliquots in 0.6-ml UV-impermeant microcentrifuge tubes in liquid nitrogen for storage at –80°C.

38. For use for microinjection, rapidly thaw an aliquot of concentrated labeled tubulin and transfer immediately to ice. Dilute to 0.5 mg/ml with cold IB, and drop freeze 3-µl aliquots for use within 2 weeks.

SUPPORT PROTOCOL 2

PREPARATION OF FLUORESCENTLY LABELED ACTIN FOR FSM

This is a modification of a protocol developed by J.S. Sanger and J.W. Sanger (Turnacioglu et al., 1998). In this protocol, actin is labeled on lysines with a succinimidyl ester derivative of the fluorescent dye of choice. The labeling is carried out on actin filaments, so that the dye does not bind to regions of the actin monomer that are involved in filament formation, and then the labeled actin is taken through rounds of depolymerization, polymerization, and depolymerization to select for functional labeled molecules, similar to the protocol for labeling tubulin. However, actin polymerization is not temperature dependent like tubulin, but salt dependent with polymerization driven by high-salt concentrations and depolymerization occurring at low-salt concentrations (Pardee and Spudich, 1982). Thus, the depolymerization portion of the cycle occurs very slowly (i.e., over 3 days) by dialysis to remove salt, and should not be rushed. This protocol takes a total of 7 days and should yield 20 to 30 mg of labeled actin, which is good for >1 year when stored at −80°C.

Materials

Muscle (rabbit psoas or chicken breast) acetone powder
1 M KCl (*APPENDIX 2A*)
1 M $MgCl_2$ (*APPENDIX 2A*)
100 mM ATP (see recipe)
Succinimidyl ester derivative of the fluorescent probe of choice, pre-equilibrated to room temperature
Anhydrous DMSO

1 M sodium bicarbonate (see recipe), freshly prepared
1 M NH_4Cl (see recipe)
G-buffer, 4°C (see recipe; ATP should be added just prior to use)

50-ml beaker
Refrigerated ultracentrifuge with rotor (e.g., Beckman 70.1 Ti rotor)
13.5-ml screw-cap ultracentrifuge tubes
50-ml and 1-liter graduated cylinder
Spectrophotometer and quartz cuvette
1.6-ml microcentrifuge tubes
Aluminum foil
Refrigerated centrifuge with rotor (e.g., Sorvall SS-34 rotor)
50-ml open-top polycarbonate centrifuge tubes (or equivalent)
7-ml dounce homogenizer with type "B" pestle
Pretreated glycerol-free cellulose dialysis tubing (see recipe), ~2 cm diameter, 20,000 MWCO (i.e., Spectra/Por 6, Fisher)
Dialysis tubing clips
0.6-ml UV-impermeant microcentrifuge tubes

Extract and polymerize actin monomers from muscle

1. Add 1 g of muscle acetone powder to 25 ml chilled deionized, distilled water in a 50-ml beaker. Extract the actin monomers by mixing 30 min at 4°C. During extraction, cool the ultracentrifuge and rotor to 4°C.

   Use a magnetic stirrer and stir plate in the cold room.

   Muscle acetone powder is commercially available from Sigma, but actin preparations from commercial sources will not give the best yields. A better source is from a laboratory that purifies actin regularly (generally, these laboratories store large frozen quantities) or to prepare the powder oneself using the method of Pardee and Spudich (1982).

2. Transfer to 13.5-ml ultracentrifuge tubes and clarify the extract by ultracentrifuging 1 hr at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

   Be sure to balance paired tubes by weight to within 0.01 g.

3. After the centrifugation, carefully collect supernatant in a 50-ml graduated cylinder. Determine the initial amount of actin monomers by measuring the absorbance at 290 nm (A_{290}) of a 1:10 dilution and using the extinction coefficient of actin $\varepsilon = 0.62\ M^{-1}\ cm^{-1}$ in the equation for Beer's law (see Support Protocol 1, step 35).

4. Polymerize the actin by adding 1 M KCl (final 0.1 M), 1 M $MgCl_2$ (final 2 mM), and 100 mM ATP (final 1 mM). Transfer to a 50-ml beaker and stir 30 min at room temperature.

5. During the polymerization, dissolve the fluorophore in anhydrous DMSO to a concentration of 100 mM in a 1.6-ml microcentrifuge tube. Warm fluorescent dye solution for 10 min to 37°C and vortex vigorously.

6. Clarify the dye solution by microcentrifuging 5 min at maximum speed, room temperature. Promptly transfer the supernatant to a fresh 1.6-ml microcentrifuge tube and keep at room temperature.

7. After polymerization, raise the pH of the actin solution to ~pH 9 for the labeling reaction by adding 1/5 vol of 1 M sodium bicarbonate.

Label actin

8. Add fluorescent dye solution to the protein solution at a ratio of 5 mole fluorophore to 1 mole actin. Add a stir bar to the beaker, cover the beaker completely with aluminum foil, and stir 45 min at room temperature.

9. After 45 min, add a second aliquot of fluorophore, equal to the amount of the first addition, re-cover the beaker, and continue stirring for an additional 45 min, room temperature.

   During this time, chill the super-speed centrifuge and rotor to 4°C.

10. Following labeling, quench the reaction by adding 1 M NH_4Cl to a final concentration of 50 mM. Stir 10 min, 4°C.

11. Transfer the solution to 50-ml super-speed centrifuge tubes and pellet large aggregated material by centrifuging 10 min at 12,000 × *g* (Sorvall SS-34 rotor at 10,000 rpm), 4°C.

12. Collect the supernatant and transfer to new 50-ml screw-cap ultracentrifuge tubes. Balance the tubes to within 0.01 g and ultracentrifuge the F-actin 60 min at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

13. Note the position of the pellet in the ultracentrifuge tube and aspirate the supernatant. Resuspend pellet in 5 ml of G-buffer.

14. Pool pellets in a 7-ml dounce homogenizer and homogenize slowly on ice to resuspend, being careful not to introduce bubbles; then thoroughly rinse the ultracentrifuge tubes with an additional 1 ml of G-buffer.

Depolymerize the F-actin

15. Transfer the resuspended pellets into pretreated dialysis tubing (clipped at one end to form a bag) that has been rinsed with G-buffer. Squeeze out the bubbles and seal the opposite end with another clip. Place the dialysis bag in a 1-liter graduated cylinder containing 1 liter G-buffer and a magnetic stir bar. Cover the entire cylinder with aluminum foil, and stir for 3 days in a cold room, changing the buffer once every day.

16. Collect the dialyzed actin, transfer it to 13.5-ml ultracentrifuge tubes, and clarify the actin solution by ultracentrifuging 1 hr at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

17. Collect the supernatant containing G-actin into a small beaker and discard the pellet.

Polymerize G-actin to F-actin

18. Polymerize G-actin into F-actin by addition of 1 M KCl (final 0.1 M), 1 M $MgCl_2$ (final 2 mM), and 100 mM ATP (final 1 mM); and mix 60 min at 4°C.

19. Transfer the solution to an ultracentrifuge tube and pellet the F-actin by ultracentrifuging 60 min at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

20. Aspirate the supernatant and resuspend the pellet in 1 to 2 ml of cold G-buffer in the dounce homogenizer. Transfer to a dialysis bag.

21. Dialyze the resuspended pellet in 1 liter G-buffer in a graduated cylinder covered with aluminum foil for 3 days, 4°C, changing the buffer once every day.

    Add ATP fresh to each change of dialysis buffer

22. Following dialysis, transfer the dialysate to an 13.5-ml ultracentrifuge tubes and clarify the G-actin by ultracentrifuging 1 hr at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

Analyze actin

23. Collect the supernatant and determine the concentration, total protein, percent yield, and fluorophore-to-actin ratio of the final product (see Support Protocol 1, step 35 for tubulin labeling).

    The extinction coefficient for actin at 290 nm is 0.62 M^{-1} cm^{-1}, the molecular mass of actin monomer is 45 kDa, and the extinction coefficient for the fluorophore is supplied by the manufacturer.

24. Drop-freeze the labeled actin in 10-µl aliquots in 0.6-ml UV-impermeant microcentrifuge tubes and store at −80°C.

25. For use for microinjection, rapidly thaw an aliquot of concentrated labeled actin and transfer immediately to ice. Dilute to 0.5 mg/ml with cold IB, and drop freeze 3-µl aliquots for use within 2 weeks.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ammonium chloride (NH_4Cl), 1 M

5.35 g NH_4Cl
100 ml distilled H_2O
Store up to 6 months at room temperature

ATP, 100 mM

8.5 ml H_2O
0.55 g Na_2ATP
pH to ~7.0 with NaOH
Bring to final volume to 10 ml with H_2O
Dispense into 200-µl aliquots
Store up to 1 year at −20°C

BRB-80, 10×

400 mM K PIPES, pH 6.8 (see recipe)
5 mM $MgCl_2$ (*APPENDIX 2A* for 1 M)
5 mM EGTA (*UNIT 13.1*)
Store up to 1 year at 4°C

Buffered filming medium

14.8 g/liter Leibovitz L-15 medium powder lacking phenol red (Sigma)
7 mM HEPES, sodium salt
1× antibiotic/antimycotic (100×, Life Technologies; penicillin/streptomycin/amphotericin B)
10% (w/v) fetal bovine serum (FBS, *APPENDIX 2A*)
Adjust pH to 7.2 and sterile filter
Store up to 3 months at 4°C

CB/BRB-80 conversion buffer

1.18 M K PIPES, pH 6.8 (see recipe)
11 mM $MgCl_2$ (*APPENDIX 2A* for 1 M)
Store up to 6 months at room temperature

G-buffer

2 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
0.2 mM $CaCl_2$ (*APPENDIX 2A*)
Add just before use:
0.2 mM ATP (see recipe for 100 mM)
0.5 mM 2-mercaptoethanol
Store up to 1 day at 4°C

Glycerol-free cellulose dialysis tubing, pretreated

Boil 10 min in 10 mM EDTA. Store in sealed jar for up to 1 year at 4°C. Rinse well with water before use.

GTP, 100 mM

Check molecular weight of GTP lot to determine the amount required for 10 ml of a 100 mM solution
8.5 ml distilled H_2O
1 ml of 1 M $MgSO_4$ (*APPENDIX 2A*)
Adjust pH to 7.0
Distilled H_2O to 10 ml
Dispense into 200-µl aliquots, store up to 1 year at −20°C

HEPES, 1 M (pH 8.6)

119.15 g HEPES (free acid)
Distilled H_2O to 400 ml
Add solid KOH a few pellets at a time while mixing until the pH is ~8.4
Add concentrated KOH dropwise to achieve pH 8.6
Distilled H_2O to 500 ml
Sterile filter and store up to 1 year at 4°C

High-pH cushion

0.1 M HEPES, sodium salt, pH 8.6 (add from 1 M stock; see recipe)
1 mM $MgCl_2$ (*APPENDIX 2A*)
1 mM EGTA (*UNIT 13.1*)
60% (w/v) glycerol
Store up to 6 months at room temperature

Injection buffer (IB)

50 mM potassium glutamate
0.5 M $MgCl_2$ (*APPENDIX 2A*)
Store up to 2 years at −20°C

Labeling buffer

0.1 M HEPES, sodium salt, pH 8.6
1 mM $MgCl_2$ (*APPENDIX 2A*)
1 mM EGTA (*UNIT 13.1*)
40% (w/v) glycerol
Store up to 6 months at room temperature

Low-pH cushion

1× BRB-80 (see recipe for 10×)
60% (w/v) glycerol
Store up to 6 months at room temperature

Microinjection needles

For Eppendorf systems, standard femtotip microinjection needles work well. For home-pulled needles, a blunt-tipped needle with a ~0.5- to 1.0-μm i.d. tip opening should be used. Either needle type should be pre-coated inside and out with hexamethyldisilazane (HMDS, Pierce Chemical). This is done by propping the needles on a piece of modeling clay in a petri dish, putting a couple of drops of HMDS on the bottom of the dish, quickly covering the top, and putting the dish in the fume hood overnight to allow the HMDS to vaporize and coat the needles. This treatment greatly reduces needle-clogging problems. Needles may be tested by watching flow of a 1 mg/ml fluorescent dextran solution from a needle immersed in culture medium using a 40× lens and epifluorescent illumination. At 1- to 2-psi needle pressure, the solution should flow *very* slowly from the needle, i.e., a small "cloud" of fluorescence as big as ~1/4 the field of view should take 2 to 4 sec to form.

PIPES, 1.5 M (pH 6.8)

226.8 g PIPES (free acid)
H_2O to 400 ml
Add solid KOH a few pellets at a time while mixing until the pH is ~6.6
Add concentrated KOH dropwise to achieve pH 6.8
Distilled H_2O to 500 ml
Sterile filter and store up to 1 year at 4°C

Quench

2× BRB-80 (see recipe for 10×)
100 mM potassium glutamate
40% (w/v) glycerol
Store up to 6 months at room temperature

Sodium bicarbonate, 1 M

8.40 g $NaHCO_3$
100 ml distilled H_2O
Make up fresh and keep at room temperature

COMMENTARY

Background Information

Principles of FSM image formation

FSM was originally characterized for imaging fluorescent microtubules, thus it is important to understand the basics of microtubule assembly and structure in order to understand FSM images of microtubules. Similar principles apply to FSM images of actin.

Microtubules dynamically assemble in cells from a cytoplasmic pool of α/β tubulin dimers (reviewed by Desai and Mitchison, 1997). Dimers of 8 × 5–nm are oriented head-to-tail along the 13 protofilaments that comprise the 25-nm diameter cylindrical wall of a microtubule (Fig. 4.10.4). There are 1625 dimers in 1 μm of microtubule length. Microtubules grow by dimer association with their ends. Thus, microtubules can be fluorescently labeled by attachment of chemical fluorophores to purified tubulin dimers, then incorporation of the fluorescent dimers into the ends of a microtubule polymer. As seen in Figure 4.10.1, microtubules assembled from a low fraction (~0.5%) of fluorescent tubulin appear speckled in high-resolution images, while microtubules assembled from higher fractions (~10%) of labeled tubulin appear continuously labeled along their lengths.

The resolution of fluorescent microtubules in the light microscope depends on the emission wavelength of the fluorophore (Inoué and Spring, 1997). Resolution limit in wide-field fluorescence microscopy is given by

$$r = 0.61\lambda/NA_{obj}$$

where λ is the wavelength of emission light and NA_{obj} is the numerical aperture of the objective lens. In practice, this equation means that the image of a single fluorophore will be the same

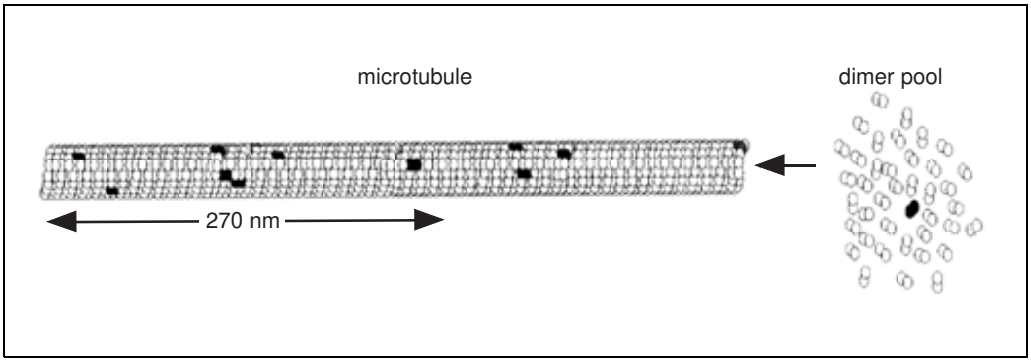


Figure 4.10.4 Stochastic growth model for how microtubules get fluorescent speckles. See Background Information. R (red fluorescence) = $0.61\lambda/NA_{obj}$ = 270 nm = 440 tubulin dimers; $M = FN = 4.4$ for 1% labeled tubulin; SD = $[Nf(1-f)]^{0.5} = 2.15$; C = SD/M = 0.48; as mean, M, decreases, standard deviation, SD, increases, and speckle contrast, C, increases.

size as the image of however many fluorophores that can fit in a resolution-limited spot, only the single fluorophore will be much dimmer than the group. For example, resolution is ~270 nm for the 620-nm fluorescence from tubulins conjugated to the X-rhodamine fluorophore. Thus, the image of a linear, 25-nm wide, red fluorescent microtubule is a linear series of resolution-limited 270-nm-wide fluorescent spots along the microtubule, with the intensity of the resolution-limited spots related to the number of fluorophores within each spot.

Analysis of the assembly of purified tubulins in vitro (Waterman-Storer and Salmon, 1998) has shown that fluorescent speckles in microtubule FSM images are generated by the normal stochastic association of fluorescently labeled and unlabeled tubulin dimers with growing microtubule ends (Fig. 4.10.4). Each time a dimer is added to the microtubule end, the probability that it will be a dimer with a fluorophore depends on the fraction (f) of labeled dimers in the tubulin pool. If, for example, f = 1%, then each time a dimer is added to a microtubule end, there is a 1 in 100 chance it will have bound fluorophore. Over many microns of microtubule length (1625 dimers/μm), the mean number of fluorescent dimers (M), in 270 nm, the size of the resolution-limited spot, is:

$$M = fN$$

where f is the fraction of labeled fluorophores and N is the number of dimers in 270 nm of microtubule, or 440 dimers. For a situation in which f = 1%, the average number of fluorescent dimer per 270 nm is M = 4.4. In FSM images, the speckle pattern along the microtubule is produced by variations from the mean in the number of fluorophores per resolution-limited spot. The standard deviation (SD) for a stochastic process is

$$SD = (Nf(1\text{-}f))^{0.5}$$

which is approximately the square root of the mean for small values of the fraction of labeled tubulin. For a mean value of 4.4, SD = 2.1. This high standard deviation relative to the mean explains how microtubules get fluorescent speckles as a result of a high variability in the number of fluorescent tubulin subunits per resolvable unit distance along the microtubule. The author defines speckle contrast (C) as:

$$C = SD/M$$

At low fractions of fluorescent dimer, the standard deviation is high relative to the mean, producing more contrasting speckles. For the microtubule FSM image seen in Figure 4.10.1, the fraction of labeled tubulin of ~0.5% or less is estimated. At this concentration, the number of fluorophores per diffraction-limited spot has been calculated to be between 0 and 7 (Waterman-Storer and Salmon, 1999).

The above analysis also does not consider the effects of background fluorescence on speckle contrast. As the fraction, f, of fluorescently labeled tubulin decreases, background fluorescence also decreases, giving less out-of-focus fluorescence and higher speckle contrast in the FSM images.

Critical Parameters and Troubleshooting

The critical parameters for setting up a time-lapse FSM imaging system will not be discussed here as Basic Protocol 1 dealt with this in detail. Similarly, troubleshooting hardware and software problems is virtually impossible without knowledge of the specific components

of the imaging system. These problems are best dealt with by consultation with the manufacturer as problems arise.

For successful live-cell FSM imaging, the most critical components are proper fluorescent protein concentration, successful suppression of photobleaching, and high quality, well labeled, fully functional protein. Fluorescent speckles cannot be detected if the concentration of fluorescent protein in the cell is too high. Although, the needle concentrations recommended have been used successfully, the amount of protein that gets incorporated into the cell depends on the microinjection technique. Tubulin can be diluted with IB and actin can be diluted with G-buffer to alleviate problems of overly bright cells. Conversely, if there is not enough labeled protein, the camera exposures required to capture an image will be excessively long and may result in motion artifacts in the image. This can easily be solved by increasing the needle concentration.

Photobleaching problems can occur if the imaging chamber is not fully sealed, if there are air bubbles present in the chamber, or if the potency of the Oxyrase is wearing out. Make sure the edges of the coverslip are dry when attaching it to the tape and use Oxyrase that is stored for <1 year at −80°C. Labeled tubulin or actin that is incompetent for polymerization due to denaturation or blockage of protein-protein interaction sites by dye binding will not contribute to forming fluorescent speckles by incorporating into microtubules or actin filaments, but it will surely contribute to soluble background fluorescence, making the dimly fluorescent speckled structure difficult to detect above the background. If the background is too high and the protein is not incorporating into polymers, the labeling will have to be carried out again.

The points to pay attention to during labeling of fluorescent tubulin to increase yield and produce well-labeled, functional protein is to not over-label the protein by extending the labeling reaction time, to save time by having solutions and centrifuges pre-equilibrated to the proper temperatures, and to be meticulous in resuspending pellets and collecting supernatants. After the first microtubule polymerization and centrifugation, when resuspending the microtubules for labeling, keeping the pellets warm and using warm buffer for resuspension is essential. This is a step where much tubulin can be lost if it is allowed to depolymerize prior to labeling. After centrifuging the labeled microtubules, resuspension of the labeled microtubule pellet and depolymerization is another step where yield can be reduced. If the pellet is not completely resuspended, the microtubules will not depolymerize and will be lost in the subsequent clarification step—thus, be patient and homogenize slowly during the entire depolymerization incubation. A final key point to note is that small amounts (<15 mg) of starting material when carrying out these protocols should not be used. Yield for the protocols is knowingly very low, and if small amounts are used to start with, one may end with no labeled product.

Actin labeling is quite easy because one begins with such a large amount of material and actin is such a stable protein that it is difficult to be unsuccessful. One key parameter is the quality of the acetone powder. Actin will not be readily extracted from poor powder. Use the most finely powdered or "fluffiest" powder within the supply. Do not include hard chunks or dark-colored particles in the starting material for the prep as these will not have been fully acetone extracted. It is not recommended to speed the depolymerization steps by decreasing dialysis time. This will cause large losses of protein, which will fail to depolymerize and subsequently be lost in the pellet of the clarification centrifugation. One may change dialysis buffers more often, but do not decrease dialysis time. In fact, one may increase the dialysis for up to 5 days if necessary. When changing dialysis buffers, always add the ATP fresh to the buffer.

The labeling efficiency of tubulin or actin should not have dramatic effects on imaging. However if a very poor labeling is achieved (<0.5 dye per protein), the large amount of protein microinjected to get good images may have some effects on the balance of polymer/monomer in the cell.

Anticipated Results

When live cells are injected with low levels of well-prepared fluorescently labeled cytoskeletal proteins and imaged on an efficient epifluorescent microscope at high resolution with a sensitive, low-noise cooled CCD that is matched in resolution to the microscope optics, FSM images like those in Figure 4.10.1 should be obtained. In time lapse, microtubule FSM should reveal the assembly dynamics of speckled microtubule ends at the cell periphery as microtubules undergo dynamic instability, growing at 1 to 10 μm/min and shortening at 5 to 20 μm/min, with frequent switching between these states. The speckles on microtubules

should remain constant in pattern and only change after depolymerization and repolymerization. In this regard, keep in mind that very slight shifts in focus can change the intensity of speckles dramatically. For time-lapse actin FSM, actin speckles should appear along the leading edges of migrating cells and move towards the cell center at 0.1 to 2 μm/min. In addition, within the lamella actin network, fluorescent speckles should appear and disappear as actin within the meshwork turns over. Actin in stress fibers and bundles will appear as linear arrays of speckles, and contractility can be seen as speckles move within these bundles.

Time Considerations

Relative to preparing fluorescent proteins, performing time-lapse FSM of living cells requires little time and is dependent on the biological process of interest. The cells will need to be microinjected, to recover for ~1 hr after microinjection, to be mounted for live-cell microscopy, and then to be imaged on the microscope for as long as is required by the biological process of interest. Fluorescent labeling of tubulin requires 1 full day, provided tubulin has already been purified according to *UNIT 13.1*, Support Protocol 3. Fluorescent labeling of actin takes 7 days with the first day, in which the labeling is carried out, being the most labor intensive, with ~6 hr required. Day 2 and 3 need 10 min each, 4 hr on day 4, 10 min on both days 5 and 6, and 2 to 3 hr on day 7. The method should yield 20 to 30 mg of labeled actin. Both of these fluorescent proteins are good stored at −80°C for a reasonably long time, although labeled actin appears to be more stable (>1 year) than tubulin (6 to 8 months).

Literature Cited

Desai, A. and Mitchison, T.J. 1997. Microtubule polymerization dynamics. *Ann. Rev. Cell Dev. Biol.* 13:83-117.

Faire, K., Waterman-Storer, C.M., Gruber, D., Masson, D., Salmon, E.D., and Bulinski, J.C. 1999. Dynamic behavior of GFP-labeled E-MAP-115 (ensconsin) in cultured cells. *J. Cell Sci.* 112:4243-4255.

Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L., and Mitchison, T. 1991. Preparation of modified tubulins. *Methods Enzymol.* 196:478-485.

Inoué, S. and Spring, K. 1997. Video Microscopy: The Fundamentals (2nd Ed.) Plenum, New York.

Mitchison, T.J., Sawin, K.E., Theriot, J.A., Gee, K., and Mallavarapu, A. 1998. Caged fluorescent probes. *Methods in Enzymol.* 291:63-78.

Pardee, J.D. and Spudich, J.A. 1982. Purification of muscle actin. *Methods Enzymol.* 85:164-181.

Prasher, D.C. 1995. Using GFP to see the light. *Trends Genet.* 11:320-323.

Turnacioglu, K.K., Sanger, J.W., and Sanger, J.M. 1998. Sites of monomeric actin incorporation in living PrK2 and REF-52 cells. *Cell Motil. Cytoskel.* 40:59-70.

Wang, Y.L. 1989. Fluorescent analog cytochemistry: Tracing functional protein components in living cells. *Methods Cell Biol.* 29:1-12.

Wang, Y.L. and Taylor, D.L. 1980. Preparation and characterization of a new molecular cytochemical probe: 5-Iodoacetamidofluorescein-labeled actin. *J. Histochem. Cytochem.* 28:1198-206.

Waterman-Storer, C.M. and Salmon, E.D. 1997. Actomyosin-based retrograde flow of microtubules in the lamella of migrating epithelial cells influences microtubule dynamic instability and turnover and is associated with microtubule breakage and treadmilling. *J. Cell Biol.* 139:417-434.

Waterman-Storer, C.M. and Salmon, E.D. 1998. How microtubules get fluorescent speckles. *Biophys. J.* 75:2059-2069.

Waterman-Storer, C.M. and Salmon, E.D. 1999. Fluorescent speckle microscopy of microtubules: How low can you go? *FASEB J.* 13:S225-S230.

Waterman-Storer, C.M., Sanger, J.W., and Sanger, J.M. 1993. Dynamics of organelles in the mitotic spindles of living cells: Membrane and microtubule interactions. *Cell Motil. Cytoskel.* 26:19-39.

Waterman-Storer, C.M., Desai, A., Bulinski, J.C., and Salmon, E.D. 1998. Fluorescent speckle microscopy: Visualizing the movement, assembly, and turnover of macromolecular assemblies in living cells. *Curr. Biol.* 8:1227-1230.

Waterman-Storer, C.M., Desai, A., and Salmon, E.D. 1999. Fluorescent speckle microscopy of spindle microtubule assembly and motility in living cells. *Methods Cell Biol.* 61:155-173.

Wolf, D.E. 1989. Designing, building, and using a fluorescence recovery after photobleaching instrument. *Methods Cell Biol.* 30:271-306.

Contributed by Clare Waterman-Storer
The Scripps Research Institute
La Jolla, California

Two-Photon Excitation Microscopy for the Study of Living Cells and Tissues

The effective sensitivity of fluorescence microscopy, especially with thick samples, is limited by out-of-focus flare. This limitation is greatly reduced in a confocal microscope (*UNIT 4.5*; White et al., 1987), which uses a confocal pinhole to reject out-of-focus background and produce unblurred imaging of thin (<1 μm) optical sections. Alternatively, deconvolution microscopy, using a conventional microscope, digitally reconstructs an image using the measured point spread function of the optics (Agard et al., 1989; Carrington et al., 1990; Holmes et al., 1995; McNally et al., 1999). Two-photon excitation microscopy (also known as nonlinear optical microscopy or two-photon laser scanning microscopy; Denk et al., 1990) is another alternative to confocal and deconvolution microscopy that provides clear advantages for three-dimensional imaging. In particular, two-photon excitation excels at imaging of living cells, especially within intact tissues such as brain slices, embryos, whole organs, and even animals. This unit will describe the basic physical principles of two-photon excitation and discuss the advantages and limitations of its use in laser-scanning microscopy. Practical considerations for this technique will be highlighted in order to illustrate the utility of this technique and demonstrate some of its physical limitations. Finally, selected applications of two-photon excitation microscopy will be discussed, in order to illustrate how this technique has made possible experiments that could not have been performed otherwise.

Before performing any optical-sectioning experiment, careful consideration should be given to selecting the technique that is best suited to answer the biological question. For fluorescence microscopy on relatively thick samples, two-photon excitation often provides the most attractive solution, although complementary three-dimensional fluorescence microscopy methods each have particular benefits that make them better suited for certain experiments.

Confocal microscopy uses a pinhole to reject out-of-focus background fluorescence. Thus, this technique allows three-dimensional sectioning into thicker tissues. However, the excitation light generates fluorescence, and thus photobleaching and phototoxicity, throughout the specimen, but it only collects signal from within the plane of focus. This can cause significant photobleaching and phototoxicity problems, especially in live samples. Also, the penetration depth in confocal microscopy is limited by absorption of excitation energy throughout the beam path and by sample scattering of both the excitation and emission photons.

Deconvolution techniques often provide the best solution for samples with relatively low out-of-focus background or for samples with low signal levels (McNally et al., 1999). Since deconvolution methods use conventional wide-field microscopes for image acquisition, the excitation intensity is generally kept low. Thus, deconvolution is usually effective for imaging monolayers of living cells. It is important to realize, though, that many so-called deconvolution methods are simply nonlinear data filters that do not generate quantitative data. Only true, constrained, iterative deconvolution methods provide quantitative data that can be used for further analysis. However, deconvolution on wide-field fluorescence microscopes provides limited penetration into thick samples, as a result of increased out-of-focus background and light scattering. Also, because of the heavy computation required, the deconvolved images cannot give immediate feedback during the experiment.

Two-photon excitation provides three-dimensional sectioning without absorption (and thus photobleaching and phototoxicity) above and below the plane of focus. Consequently, it offers increased depth penetration over confocal microscopy and can be less phototoxic to live samples. Thus, two-photon excitation microscopy is used, in preference to these other techniques, for experiments that require deep penetration into live tissue or intact animal samples. However, because the photophysics involved with two-photon excitation is different from conventional fluorescence excitation, deleterious effects are occasionally observed with two-photon excitation of certain fluorophores, which in turn limits the applicability of this method for optical sectioning in thin samples.

Contributed by Jonathan V. Rocheleau and David W. Piston

Current Protocols in Cell Biology (2003) 4.11.1-4.11.15

TWO-PHOTON EXCITATION

Principles of Two-Photon Excitation

Two-photon excitation is a relatively old concept in quantum optics. It was first proposed theoretically by Maria Göppert-Mayer in her doctoral dissertation (Göppert-Mayer, 1931) and observed experimentally shortly after the invention of the laser (Kaiser and Garrett, 1961). Thus, much of the theoretical and experimental background is well understood. Two-photon excitation arises from the simultaneous absorption of two photons in a single quantitized event. Since the energy of a photon is inversely proportional to its wavelength, the two photons should be about twice the wavelength required for one-photon excitation. For example, a fluorophore that normally absorbs ultraviolet light (~350 nm) can also be excited by two red photons (~700 nm) if they reach the fluorophore at the same time (Fig. 4.11.1). In this case, "the same time" means within ~10^{-18} sec. Because two-photon excitation depends on simultaneous absorption, the resulting fluorescence emission depends on the square of the excitation intensity. This quadratic dependence gives rise to many of the significant advantages associated with two-photon excitation microscopy (see Two-Photon Excitation in Laser-Scanning Microscopy). To obtain a significant number of two-photon absorption events (where both photons interact with the fluorophore at the same time), the photon density must be approximately a million times what is required to generate the same number of one-photon absorptions. This means that extremely high laser powers are required to generate significant two-photon-excited fluorescence. These powers are easily achieved by focusing mode-locked (pulsed) lasers, where the power during the peak of the pulse is high enough to generate significant two-photon excitation, but the average laser power is fairly low (Denk et al., 1995). In this case, the resulting two-photon-excited state from which emission occurs is the same singlet state that is populated during a conventional fluorescence experiment. Thus, fluorescent emission after two-photon excitation is exactly the same as generated in normal one-photon excitation (Denk et al., 1995).

Another nonlinear optical process, three-photon excitation, may also prove useful for biological experiments (Maiti et al., 1997). Three-photon excitation works in much the same way as two-photon excitation, except that three photons must interact with the fluorophore at the same time. Because of the quantum-mechanical properties of fluorescence absorption, the photon density required for three-photon excitation is only about ten-fold greater than what is needed for two-photon absorption (rather than another million-fold greater). This makes three-photon excitation attractive for some experiments. For instance, an infrared laser (~1050 nm) can bring about three-photon excitation of an ultraviolet-absorbing fluorophore (~350 nm) and simultaneously produce two-photon excitation of a green-absorbing fluorophore (~525 nm). Three-photon excitation can also be used to extend the region of useful imaging into the deep ultraviolet (i.e., by use of 720-nm light to excite a fluorophore that normally absorbs at 240 nm). This can be useful, since ultraviolet wavelengths below ~300 nm are very problematic for regular microscope optics. Higher-order nonlinear effects are also possible and have been experimentally demonstrated (e.g., four-photon absorption), although it is unlikely that they will find any immediate application in biological research.

Two-Photon Excitation in Laser-Scanning Microscopy

The powerful advantages of using two-photon excitation in laser-scanning microscopy arise from the basic physical principle that the absorption depends on the square of the excitation intensity. In practice, focusing a single

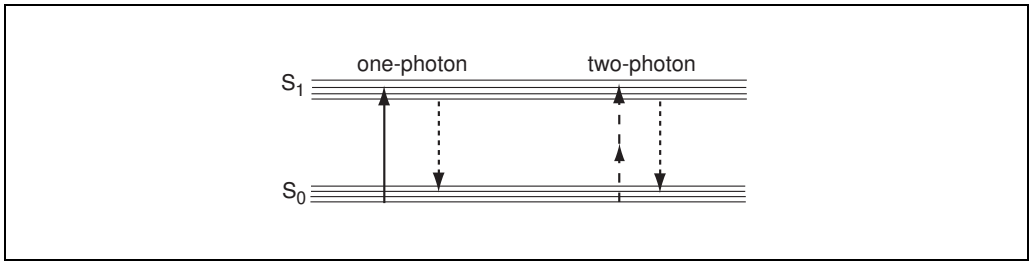


Figure 4.11.1 Jablonski diagram of a single light blue photon (solid black arrow) being absorbed to generate an excited state (left). Simultaneous absorption of two photons of red light (dashed arrows) can produce the identical excited state.

pulsed laser through the microscope optics generates two-photon excitation. As the laser beam is focused, the photons become more crowded, and the chance of two of them interacting simultaneously with a single fluorophore increases. In fact, the focal point is the only place where the photons are crowded enough to generate an appreciable amount of two-photon excitation. This is illustrated in Figure 4.11.2. Above the focal point, the photon density is not high enough for two photons to be within the absorption cross-section of a single fluorophore at the same time. However, at the focal point, the photons are so crowded that it is possible to find two of them within the absorption cross-section of a single fluorophore simultaneously. In practice, two-photon excitation microscopy is made possible not only by crowding the photons spatially (by focusing in the microscope), but also by concentrating the photons in time (by using the pulses from a mode-locked laser). This gives the needed excitation intensities for two-photon excitation, but the pulse duty cycle (the duration of the pulse divided by the time between pulses) of 10^{-5} limits the average input power to <10 mW, which is just slightly greater than what is used in confocal microscopy. The pulse durations are considered ultrashort, typically from ~100 femtoseconds to ~1 picosecond (10^{-13} to 10^{-12} sec), but from the perspective of the fluorophore (where the absorption event is on the 10^{-18} sec time scale), they appear to be quite long in duration.

The localization of two-photon excitation to the focal point provides most of the advantages over confocal microscopy. In a confocal microscope, fluorescence is excited throughout the sample, but only signal from the focal plane passes through the confocal pinhole, so background-free data can be collected. By contrast, two-photon excitation only generates fluorescence at the focal plane, so there is no background, and no pinhole is required. This dramatic difference between confocal and two-photon excitation microscopy can be demon-

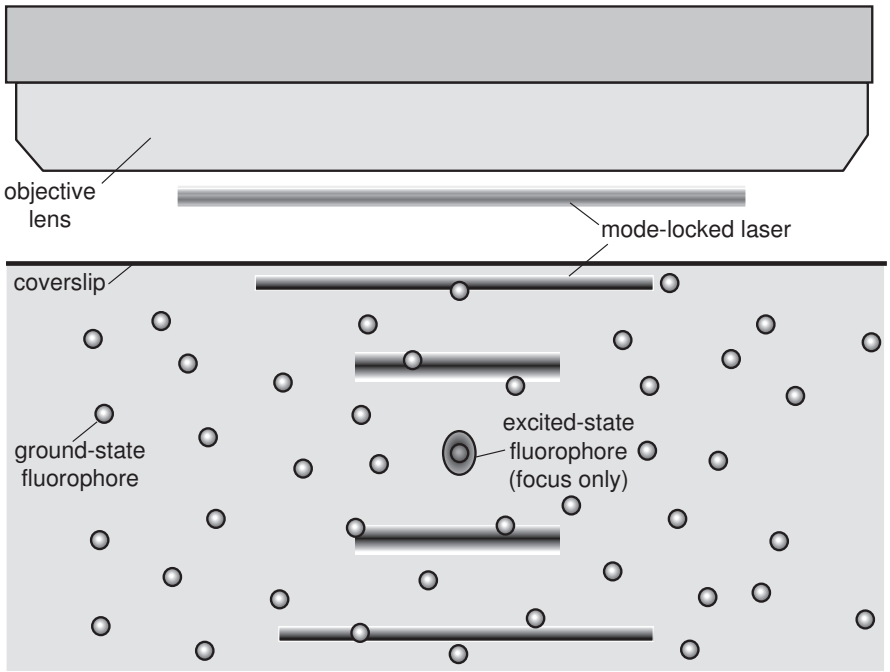


Figure 4.11.2 Demonstration of photon crowding that occurs at the focus of a microscope. As the pulses of red laser exit the objective lens and pass through a sample containing fluorophores (small circles), there is nearly zero probability of two photons passing simultaneously within the cross-section of a single fluorophore located outside the focal point. This is true even during the peak of each laser pulse. However, because of the extremely high photon density at the focal point, it is possible for two photons to interact simultaneously with a fluorophore, which becomes excited. To reach a sufficiently high concentration, the light is crowded both spatially (to the focus of the objective lens) and temporally (through the pulses of the mode-locked laser).

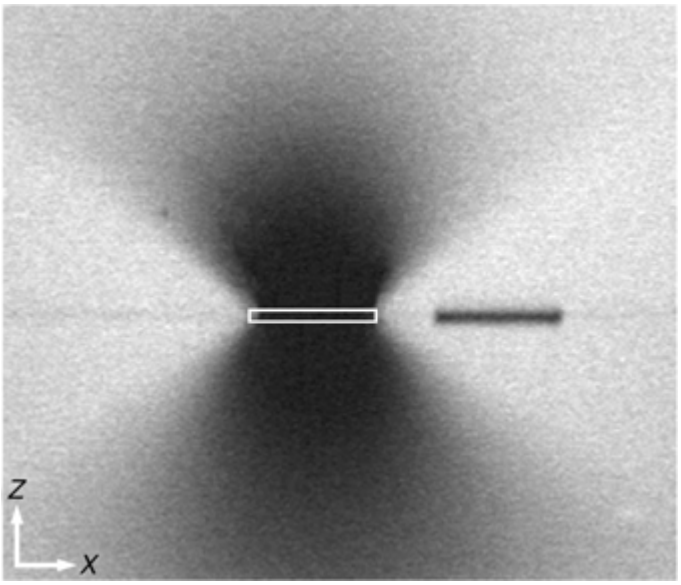


Figure 4.11.3 Photobleaching (*x-z*) profiles caused by one-photon and two-photon excitation. The *x-z* profile of the excitation pattern in a confocal microscope (left side pattern) formed by repeatedly scanning a single *x-y* optical section in a thick film of fluorescein-formvar until fluorescence was completely bleached. The white box represents the focal plane or the region from which data was collected by the confocal microscope. Nearly uniform bleaching occurred both above and below the focal plane. The same excitation pattern is shown for two-photon excitation (right side pattern). Two-photon excitation causes no photobleaching (excitation) outside the focal plane.

strated by imaging the photobleaching patterns of each method. Figure 4.11.3 shows the *x-z* photobleaching pattern that arises from repeated scanning of a single *x-y* plane (or the image plane) in a fluorescein-stained formvar film. The confocal microscope laser excites fluorophores above and below the focal plane (shown by the white box); hence bleaching is observed in these areas (Fig. 4.11.3; left). In contrast, two-photon excitation only occurs at the focal plane and confines bleaching to this area (Fig. 4.11.3; right).

The localization of excitation yields many advantageous effects. First, the three-dimensional resolution of a two-photon excitation microscope is identical to that of an ideal confocal microscope. Second, because there is no out-of-focus absorption, more of the excitation light penetrates through the sample to the plane of focus. This can lead to greatly increased sample penetration—generally at least two to three times deeper than is possible with confocal microscopy (Centonze and White, 1998). Third, as shown in Figure 4.11.3, use of two-photon excitation minimizes photobleaching and photodamage, two of the most important limitations in fluorescence microscopy of living cells and tissues. Although damage to cells owing to interactions with light is poorly understood, decreasing photodamage will lead to extended viability of the biological samples under investigation. Since practical experience indicates that the red excitation light alone does not affect cell viability, it is likely that most of the photodamage is associated with two-photon absorption and thus occurs only at the focal plane.

Two-photon excitation microscopy does not require a pinhole to obtain three-dimensional resolution, which allows flexible detection geometries. The geometries for both descanned- and nondescanned-detection two-photon excitation are shown in Figure 4.11.4. In the descanned geometry, the emitted light (represented by the dotted pattern) returns through the same path as the excitation light hitting the scanning mirrors before passing through the confocal pinhole to the detector. In confocal microscopy, this geometry is necessary to block the detection of out-of-focus emission. Nondescanned beam paths provide more alternatives: (1) a dichroic mirror, located directly after the objective lens, reflects the emitted light through a transfer lens to a detector placed in a plane conjugate to the objective rear aperture ("conjugate plane detector" in Fig. 4.11.4); (2) the

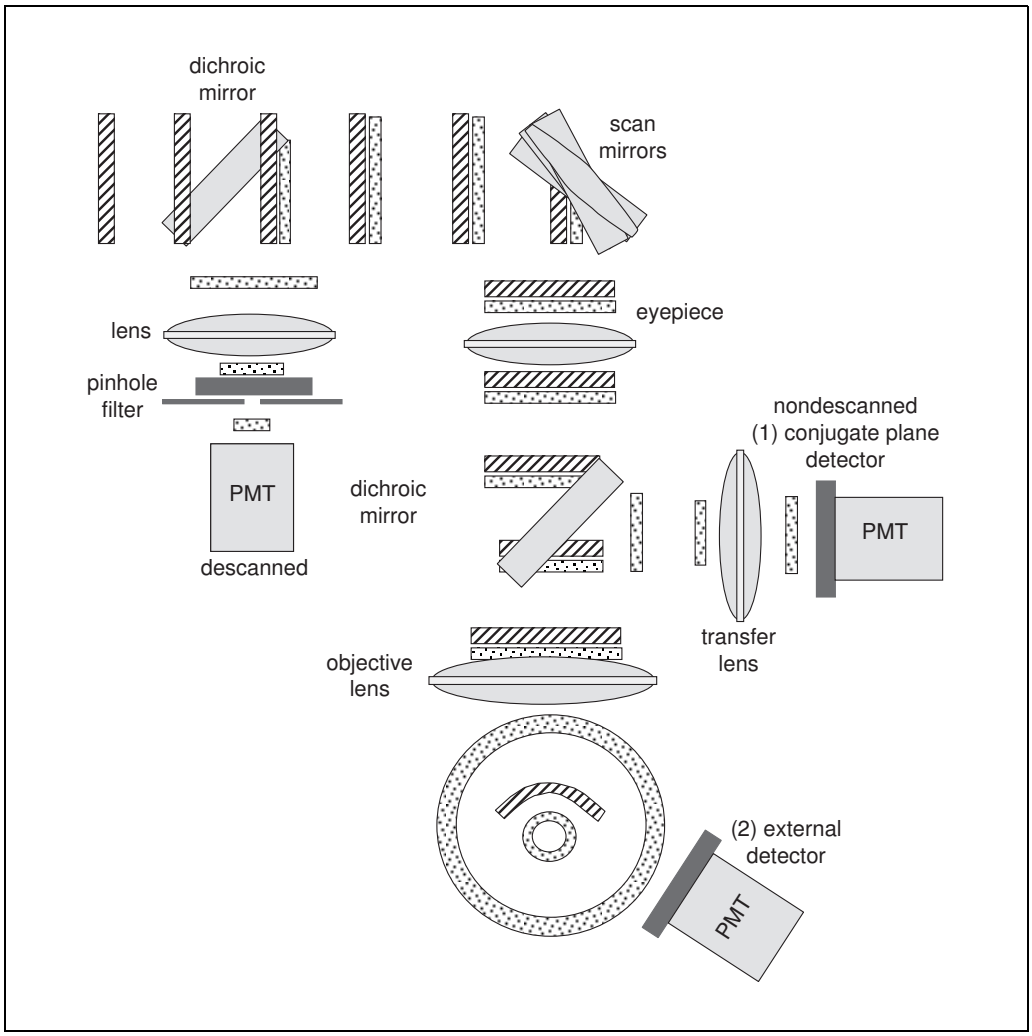


Figure 4.11.4 Descanned and nondescanned detection geometries used with two-photon excitation microscope. The incoming laser light is raster scanned (*x* and *y* scan mirrors), and is focused onto the sample by the objective lens. The descanned detection path returns through the excitation beam path, passing back through the scanning mirrors, and it is focused through the confocal pinhole to the photomultiplier tube (PMT). With nondescanned detection, the fluorescence: (1) returns through the objective lens and then is reflected by a dichroic mirror through a transfer lens that focuses the light so that the back aperture of the objective is conjugate to the front face of the PMT, or (2) is detected directly from the sample using an external PMT.

emitted light is collected, without passing through the objective lens, directly from the sample using an external detector ("external detector" in Fig. 4.11.4); or (3) the emitted light is reflected by a dichroic mirror to a charged-coupled device (CCD) camera at the intermediate image plane, in order to collect the wide-field image (not shown in Fig. 4.11.4). This last type of geometry is used for fast data acquisition systems using two-photon excitation. Although it is possible to use descanned detection for two-photon excitation, using a nondescanned alternative (listed as 2 above) is recommended to fully utilize the depth penetration of this technique. The nondescanned path allows for collection of more scattered photons, requires fewer optical elements (e.g., mirrors, lens), and reduces the distance over which dust particles in the air will interfere with the fluorescence signal. Therefore, using nondescanned detection with two-photon excitation dramatically increases collection efficiency and is a must for maximal depth penetration into living tissue.

PRACTICAL CONSIDERATIONS FOR TWO-PHOTON EXCITATION MICROSCOPY

Mechanism of Deep Sectioning

As mentioned above, the most powerful advantage of two-photon excitation microscopy

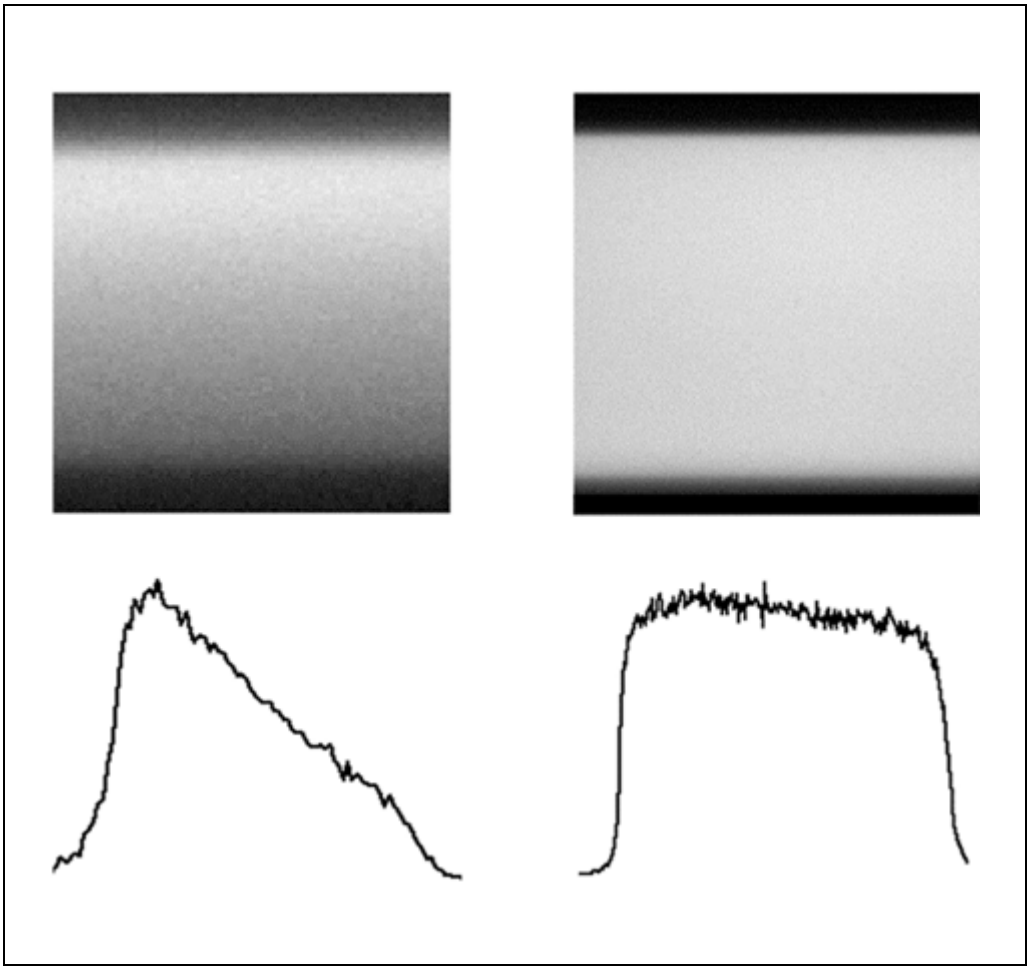


Figure 4.11.5 Top: *x-z* images of fluorescence and *z*-profiles from a rhodamine-stained polymer film (no scattering). Bottom: Intensity profiles from *z*-scan of *x-z* scan. Because there is no out-of-focus absorption in two-photon excitation, much more excitation light reaches deep into the sample so the signal is brighter and the imaging depth larger.

is its ability to provide superior optical sectioning deep into thick samples. Thus, it is important to understand how this increased depth of penetration is achieved. There are three physical mechanisms that combine to allow this increased effectiveness in thick samples: (1) lack of out-of-focus absorption allows more of the excitation light to reach the sample; (2) the red and infrared light used in two-photon excitation is scattered less than bluer light; and (3) the effects of light scattering are less detrimental to two-photon microscopy than confocal microscopy. These three points can be considered separately.

The first point is that the lack of out-of-focus absorption in two-photon excitation microscopy allows more of the excitation light to reach the focal plane. In a confocal microscope, excitation photons are absorbed by any fluorophores along the excitation light path, so fewer photons reach the focus, thus decreasing the signal. This effect becomes worse if the sample contains fluorophores throughout, as shown in Figure 4.11.5. The polymer sample shown in this figure is nonscattering but contains a uniform distribution of a high fluorophore concentration. In this figure, the top of the *x-z* scan is closest to the objective lens, and the fluorescence intensity is plotted as a function of depth into the sample (*z* distance) for each *x-z* scan. In the case of one-photon excitation (confocal microscopy), the intensity shows a steady decrease with penetration depth as the excitation light is absorbed before it reaches the deeper focal planes. In contrast, two-photon absorption only occurs in the focal plane, so there is no absorption of excitation light by fluorophores between the objective lens and the focal plane. Thus, all of the excitation reaches the focal plane, which keeps the fluorescence signal constant throughout the depth of the polymer. This dramatic difference between confocal and two-photon excitation is shown on the right in Figure 4.11.5, where the intensity is relatively stable with penetration depth.

The second point is that the redder excitation light used in two-photon excitation microscopy is less scattered by the sample than is bluer excitation light used in conventional excitation. Biological tissue can be considered as a medium with a nonuniform index of refraction. Light traveling through such a medium is scattered off in various directions. In fluorescence microscopy, the incoming excitation light can be scattered away before it reaches the focal plane, and the resulting fluorescence can also be scattered as it passes back through the sample before it reaches the detector. Both of these effects will reduce the collected fluorescence signal. Because of the irregular distribution of material within biological samples, it is not possible to calculate the scattering behavior precisely. However, the simplest approximation, Rayleigh scattering, gives a minimal estimate of the fraction of light scattered in such a system. For this case, the amount of scattered light is inversely proportional to the fourth power of the light's wavelength ($1/\lambda^4$). Using this estimate, 488-nm (one-photon) light would be expected to scatter ~7-fold more than 800-nm (two-photon) light. Thus, even more of the two-photon excitation laser light can reach the focal plane, which further increases the depth of penetration into the sample. In practice, scattering from tissue structures is always greater than is predicted by the Rayleigh approximation, but it is always the case that longer (redder) wavelengths are scattered less than shorter (bluer) wavelengths. On the detection arm of the microscope, the emitted fluorescence is identical regardless of whether it was generated using one- or two-photon excitation, so scattering of fluorescence emission affects both methods equally.

The third point is that any scattering of the excitation or fluorescence light does not affect signal collection as significantly as it does in confocal microscopy. This is due simply to the physics of image formation in two-photon excitation microscopy. While the lack of out-of-focus absorption and the differences in scattering both allow for increased excitation light to reach the focal plane deep within intact tissue, this third point actually allows for increased image contrast with two-photon excitation. The

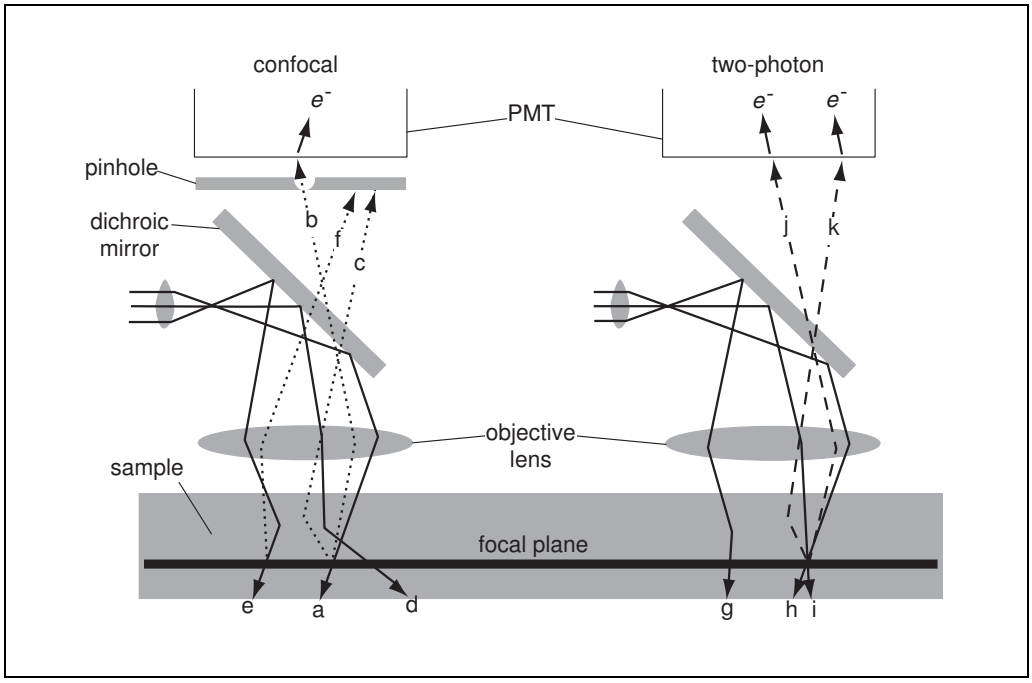


Figure 4.11.6 In confocal microscopy (shown on the left), excitation light reaches the focus (a), and fluorescence from the focus is collected and passes through a pinhole (b). Fluorescence that is scattered (c) does not pass through the pinhole, thus reducing signal. Any scattering of the excitation beam (d and e) can cause fluorescence (f), which may add background to the image. This fluorescence is inefficiently collected but can cause a background fog. For two-photon excitation microscopy (shown on the right), any scattering of a single excitation photon (g) will not cause background, since the chance of two photons scattering to the same place at the same time is essentially zero. Scattering and out-of-focus absorption are reduced with two-photon excitation, resulting in an increase in the amount of excitation light reaching the focus (h and i). Unscattered fluorescence is collected by the PMT (j). Further, because no pinhole is needed, any scattered fluorescence photons can still be collected, thus greatly increasing the collected signal (k).

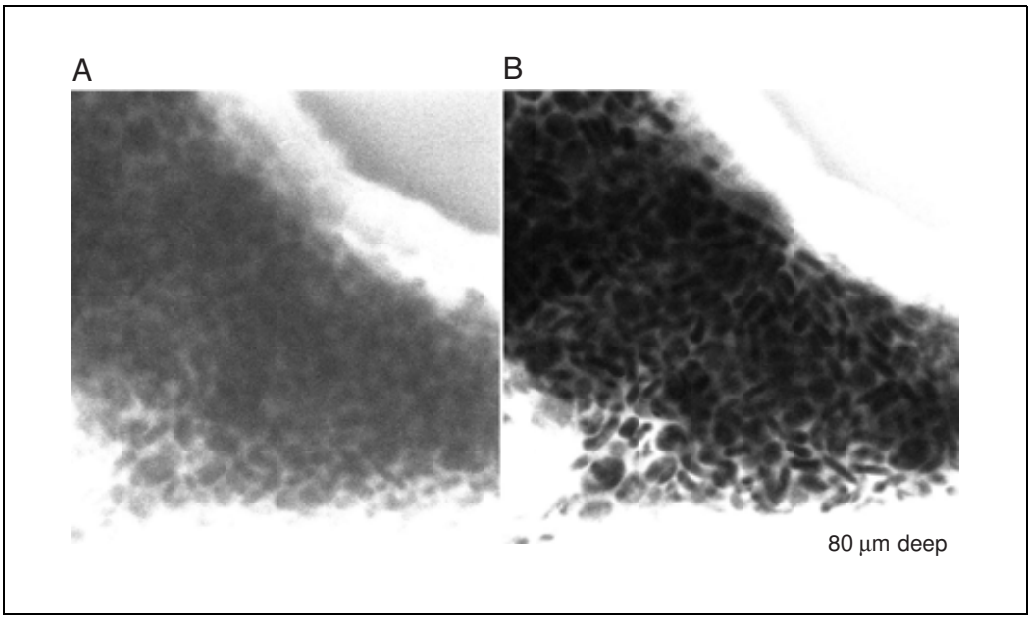


Figure 4.11.7 A shark choroid plexus stained with fluorescein. These images were collected 80 mm into the sample, where the image quality of the one-photon microscope is minimally acceptable. In contrast, using two-photon excitation at this same focal plane allows the collection of an image with excellent intensity contrast.

reasons behind this point are illustrated in Figure 4.11.6 (Denk and Svoboda, 1997).

In a confocal microscope, the excitation light (blue) is focused into the sample (a, in Fig. 4.11.6), and the fluorescence (green; represented by dotted lines) from that focal spot is captured by the objective lens, passes cleanly through the pinhole, and reaches the detector (b). This fluorescence light is the desired signal, but some of it can be scattered as it passes back through the sample (c). This scattered fluorescence does not pass through the pinhole and is therefore lost and not detected. These losses greatly reduce the detected fluorescence signal. As the excitation light passes through the sample, it may be absorbed (d) or scattered before it reaches the focus (e). If it is absorbed, it can generate fluorescence. Since this fluorescence does not arise from the focal spot, it does not pass through the pinhole, so it is not efficiently detected. However, a small portion of out-of-focus fluorescence can be scattered into the pinhole and then be detected. This fluorescence will create a background fog that will be roughly constant across the image, as shown in the examples below. This fog reduces the dynamic range of the image, thus reducing the image contrast. Likewise, the scattered excitation can generate fluorescence (e), and this fluorescence can also contribute to the background fog (f).

In the case of two-photon excitation, the excitation photons (red; represented by dashed lines in Fig. 4.11.6) can also be scattered (g). However, the chance of two photons being scattered simultaneously to the same place is essentially zero, so that the background fog that plagues confocal microscopy in thick samples is not generated in two-photon excitation. Also, more of the excitation light reaches the focal plane (h and i) due to the two points discussed above—i.e., the reduced out-of-focus absorption and the decreased scattering of the redder two-photon excitation light. Importantly, the generated fluorescence (green) has an increased chance of being detected by the photomultiplier tube (PMT) even if scattered (j) because no pinhole is present to block it (k). This insensitivity to scattering effects and lack of out-of-focus absorption allow for the preservation of the full image contrast from deep within samples.

A comparison of confocal and two-photon microscopy imaging quality is shown in Figure 4.11.7 of a shark choroid plexus stained with fluorescein. These images were collected 80 μm into the sample, which is the maximal depth for sufficient image contrast from confocal microscopy for this sample. While the signal level of the brightest features can easily be matched between the two methods, the background fog seen in the confocal image greatly reduces the image contrast. The two-photon excitation image, on the other hand, shows excellent intensity contrast. However, because scattering of the fluorescence is significant in thick biologi-

cal samples, the use of descanned detection and an open pinhole is not enough to gain the advantages of two-photon excitation (Centonze and White, 1998). To gain the full advantage, a nondescanned detection scheme (Fig. 4.11.4, where the fluorescence does not pass back through the scanning system as it must in a confocal microscope) must be used to increase the fluorescence collection efficiency (Piston et al., 1994). Using the same shark sample shown in Figure 4.11.7, a comparison of imaging using descanned (pinhole-open) and nondescanned detection is shown in Figure 4.11.8. In each detection geometry, the same imaging optics (i.e., dichroic mirror, barrier filter, and PMT detector) were used. The sample was first imaged using descanned detection at approximately the largest depth (140 μm) that allowed some intensity contrast (Fig. 4.11.8A). Keeping all settings constant and switching to nondescanned detection, the image shown in Figure 4.11.8B was collected. This image is clearly saturated in many regions of the image, demonstrating the improved signal collection with this detection geometry. Because the excitation was identical in both cases, this 8-fold increase in signal is due solely to the collection of scattered fluorescence photons. To obtain a nonsaturated image, the PMT voltage was reduced from 1000 to 750 V (Fig. 4.11.8C). Clearly, this sample can now be scanned even more deeply using the nondescanned detection. In fact, the depth of penetration in this sample was not limited by the tissue but by the working distance of the objective lens.

Image Resolution

The resolution of two-photon excitation is no better than that achieved with a well-aligned confocal microscope. In fact, the use of longer excitation wavelengths (i.e., red or infrared, instead of ultraviolet or blue) results in a larger resolution spot for two-photon excitation (Sheppard and Gu, 1990). If a biological structure cannot be resolved in the confocal microscope, it will not be resolved in a two-photon excitation laser-scanning microscope either. This point is well understood by experts in the field, but it is often missed by prospective users in the biomedical research community.

Imaging Thick Samples

As mentioned above, there are three reasons why two-photon excitation is more effective in thick samples: lack of out-of-focus absorption allows more of the excitation light to reach the sample, the red exciting light is less scattered, and the effects of fluorescence scattering are less detrimental to two-photon microscopy than to confocal microscopy. When utilizing long-working-distance optics and a nondescanned detection mechanism, the depth of penetration and image quality is often limited by the ability to effectively label the tissue—i.e., with increasing depth it becomes increasingly difficult to introduce fluorescent labels into tissues. The expression of green fluorescent protein (GFP) in transgenic animals is likely to enhance the usefulness of two-photon excitation in vivo imaging. Transgenic animals promise enormous opportunity for the ability to fluorescently label specific organs and pro-

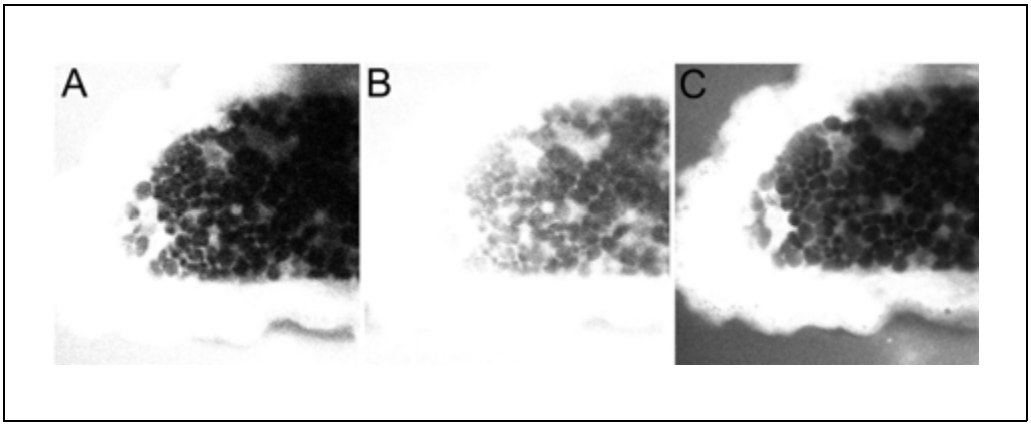


Figure 4.11.8 A comparison of descanned and nondescanned two-photon scanning depth. The sample was first imaged using descanned detection at approximately the longest depth (140 mm) that allowed achieving some intensity contrast (**A**). Keeping all settings constant and switching to nondescanned detection, the image shown in (**B**) was collected. This image is clearly saturated in many regions of the image demonstrating the improved signal collection with this collection geometry. In order to obtain a non-saturated image, the photomultiplier tube gain was changed from 1000 to 750 V (**C**).

teins of living animals for detection using two-photon excitation. Alternatively, tissue characteristics may also limit depth of the penetration. This is especially a concern in either heavily pigmented tissue, such as liver, or highly scattering tissue, such as skin.

Imaging Thin Samples

For imaging of thin samples, two-photon excitation might not offer significant advantages over conventional confocal microscopy. This is because of slightly increased photobleaching in the focal plane (the total photobleaching in a thick sample is still much reduced, though; see Fig. 4.11.3; Patterson and Piston, 2000). One example where two-photon excitation is beneficial, even for thin preparations, is the imaging of ultraviolet-excited fluorophores such as NADH (see Examples of Two-Photon Excitation Microscopy). In such experiments, ultraviolet light appears to be more harmful than the two-photon induced photobleaching. To determine if two-photon excitation would be beneficial, it is always worthwhile attempting the experiments on a confocal microscope first. Once it is known what the limitations of the confocal microscope are for the measurements, it can easily be determined whether the use of two-photon excitation would be advantageous for completing the experiments.

Absorption Spectrum

Two-photon absorption spectra can bear little resemblance to the corresponding single-photon spectra (Birge, 1986). Experience so far indicates that most fluorophores work pretty well with two-photon excitation at twice the wavelength of their one-photon absorption peak. For reasons beyond the scope of this unit, fluorophores with a nonsymmetric chemical structure tend to follow this rule more closely than symmetric ones. For example, fluorescent proteins (e.g., CFP, GFP, and YFP) have a nonsymmetric fluorophore and absorb well at twice their one-photon excitation. However, to take full advantage of two-photon excitation microscopy, these absorption spectra must be measured, which is considerably more difficult than measuring conventional one-photon absorption spectra. Only a few sources for this information exist (Birge, 1986; Xu et al., 1995, 1996; Albota et al., 1998; Neu et al., 2002), although, as this technique becomes more widespread, it is likely that two-photon absorption spectra will become more generally available.

Localized Photochemistry

Two-photon excitation also allows the initiation of photochemical reactions in the focal region. These chemistries involve ultraviolet light–induced reactions for which two-photon excitation can be substituted. For example, the uncaging of a dye, photochemically inducing a nonfluorescent molecule to become fluorescent, can be initiated in individual cells of a tissue using two-photon excitation. Alternatively, biological stimulators or suppressants can also be uncaged. Methods for two-photon-excited uncaging have not been fully developed, largely because the photoreactions can be quite slow (from milliseconds to seconds). Thus, the compound might diffuse over several micrometers within the sample between the time when it is excited and the time it becomes active. Nonetheless, this technique holds the promise of interesting biological applications, some of which will be discussed below.

Laser Sources

While the instrumentation requirements for two-photon excitation microscopy are largely indistinguishable from those for confocal microscopy, the laser excitation source is considerably different. Two types of ultrafast mode–locked laser systems are in general use today: Ti-sapphire lasers and Nd-YLF lasers. These systems do not require water cooling and plug in to regular electrical outlets, but they are considerably more expensive than the small air-cooled lasers used in confocal microscopy. The wavelength tunability of the Ti-sapphire laser (700 to 1100 nm) makes it more versatile than the single-wavelength Nd-YLF laser (1047 nm). Currently, computer-controlled Ti-sapphire lasers cover the wavelength range of 720 to 900 nm in an automated fashion. Further improvements in the ease of use and versatility of these systems are likely to continue in the near future.

Laser Power

The power necessary to excite a fluorescent sample has an optimal limit. Fluorescence intensity increases with increasing power, but the fluorophore eventually becomes saturated. Saturation occurs at laser powers that cause a significant proportion of the fluorescent molecules to exist in their excited rather than ground state (~1 mW at the sample for one-photon excitation, ~50 mW at the sample for two-photon excitation; Denk et al., 1990); therefore, more photons simply are unable to excite more molecules. Any additional excitation energy

beyond saturation contributes to increased photodamage and photobleaching. For each experimental setup, one needs to assess the damage imposed during beam scanning; it should be noted that trivial cell-viability tests (such as esterase activity or dye exclusion) do not always accurately reflect cellular photodamage. Often, a more rigorous functional test is more informative. For example, the viability of hamster embryos was confirmed by their continued development (Squirrell et al., 1999), and the viability of pancreatic islets was confirmed by their maintenance of normal glucose-stimulated NAD(P)H response (Bennett et al., 1996).

EXAMPLES OF TWO-PHOTON EXCITATION MICROSCOPY

This section will highlight some of the available literature to illustrate common situations where two-photon excitation is preferable to confocal imaging. These examples will focus on the benefits of two-photon excitation due to its reduced phototoxicity, increased tissue imaging depth, and ability to initiate localized photochemistry. The details of these experiments will be left to the specified references.

Two-photon excitation is generally less phototoxic than confocal microscopy. This is well demonstrated by time-lapse imaging of hamster embryo development (Squirrell et al., 1999). In this study, the embryo's development was monitored continuously for more than 10 hr using two-photon excitation of a vital mitochondrial dye. Conversely, normal embryo development ceased after only a few minutes of confocal laser exposure. It was thought that the two-photon excitation laser (1047 nm) greatly increased the embryo's viability. These researchers also used two-photon excitation microscopy to assess the effects of inorganic phosphate on hamster embryo development (Ludwig et al., 2001). In this study, the mitochondrial distribution of living hamster embryos cultured in varying amounts of inorganic phosphate was imaged at 6 hr of culture using two-photon excitation microscopy. The embryos were developed further prior to morphological assessment at 27 and 51 hr of culture. It was clear from these studies that the two-photon illumination was nonperturbing to the development of these embryos, while parallel confocal imaging damaged the embyros.

The nontoxic nature of two-photon excitation has allowed in vivo imaging of human skin (Masters et al., 1997). This work involved detailed spectroscopy of the autofluorescent signals from skin at various depths (0 to 50 and 100 to 15 μm) using excitation wavelengths ranging from 730 to 960 nm. When used in conjunction with reflected-light confocal microscopy, two-photon excitation microscopy nondestructively provides detailed white light and autofluorescent images of skin layers from the same region of skin (Masters and So, 1999).

Two-photon excitation is also used to avoid the phototoxic effects of ultraviolet irradiation. This feature is especially useful for the imaging of the naturally occurring reduced pyridine nucleotides—NAD(P)H—as an indicator of cellular respiration. NAD(P)H has a small absorption cross-section and a low quantum yield and absorbs in the ultraviolet. Thus, it is difficult to measure, and its imaging has the potential to cause considerable photodamage. NAD(P)H imaging has proven useful for studying the pathophysiology of cultured partially differentiated L6 myotube cells (Perriott et al., 2001). In cellular NAD(P)H images, the autofluorescence pattern reflects primarily NADH in mitochondria as spotted regions over a diffuse cytoplasmic signal. In differentiated cells, the fluorescence is evident as columns of mitochondria between muscle fiber striations. With increasing glucose concentration, an increase in fluorescence was readily apparent. Overall, this study showed that the kinetics of glucose utilization in real time can be defined in a single cell or averaged over several cells, and that there is homogeneity in glucose metabolism.

Quantitative two-photon imaging of NAD(P)H has also been done on individual β cells within the pancreatic islet (a quasi-spherical micro-organ consisting of ~1000 cells; Bennett et al., 1996). Furthermore, the spatial resolution of this technique allows the separation of both the cytoplasmic and mitochondrial NAD(P)H signals (Patterson et al., 2000). Figure 4.11.9 is a typical image of β cell NAD(P)H autofluorescence within an intact islet, displaying a signal from both the cytoplasm and mitochondria. The outlines of single cells are visible, as are the nuclei, both of which appear dark. The separation of cytoplasmic and mitochondrial signals allowed detailed examination of the metabolism of glucose and pyruvate in these regions of pancreatic islet β cells. Current models for glucose-stimulated insulin secretion (GSIS) suggest that metabolites further along the signal-transduction pathway should cause a similar cascade of signaling events and lead to insulin secretion. However, pyruvate potentiates GSIS but does not induce insulin secretion on its own. Using two-photon imaging of NAD(P)H and separation of cyto-

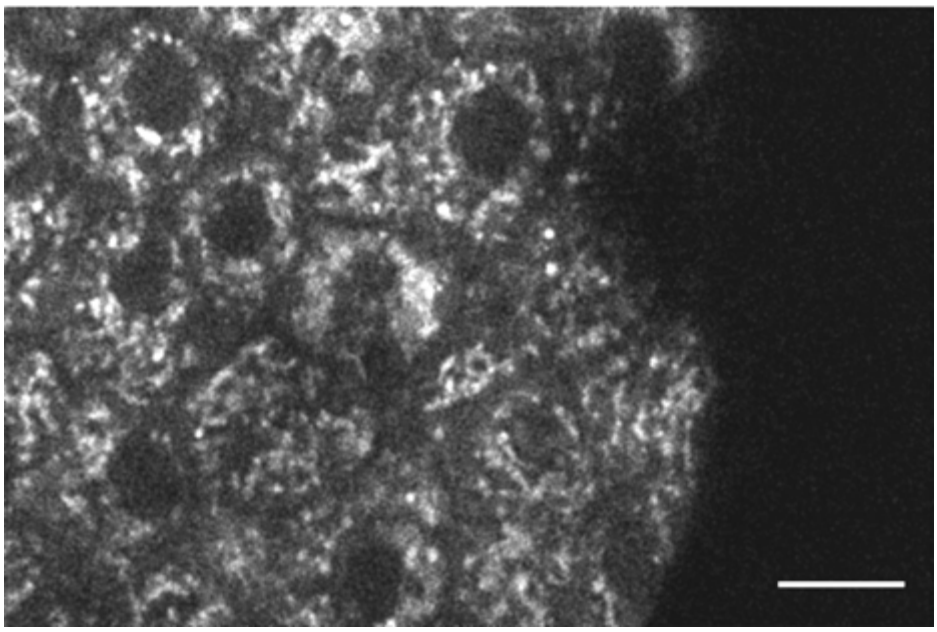


Figure 4.11.9 Optical section of NAD(P)H autofluorescence from an intact pancreatic islet. NAD(P)H signal arises from both the cytoplasm and mitochondria, the latter being brighter and somewhat punctate. Cell outlines and nuclei—where there is little or no NAD(P)H—appear dark. Bar, 10 μm.

plasmic and mitochondrial signals, it was shown that β cells metabolize pyruvate, although transiently (Rocheleau et al., 2002). This transient mitochondrial response suggests two separate models, which are currently under study: either mitochondrial pyruvate transport is inhibited during late pyruvate metabolism or the tricarboxylic cycle is inhibited during late pyruvate metabolism. In these studies, living pancreatic islets were repetitively scanned at sampling rates that are unobtainable by biochemical methods. Such repetitive imaging simply cannot be performed using confocal microscopy, owing to the limitations imposed by photobleaching and ultraviolet-induced photodamage.

Because two-photon microscopy uses mode-locked (pulsed) lasers, it is ideal for extension into fluorescence lifetime imaging (Piston et al., 1992; French et al., 1998). Imaging of the nanosecond fluorescence decay times gives information that is independent of fluorophore concentration. For instance, lifetime imaging can give an unambiguous value for fluorescence resonance energy transfer (FRET) efficiency between two probes (Harpur et al., 2001). Recently, two-photon-excited lifetime imaging microscopy of NAD(P)H has been used to quantify the NAD(P)H concentration in different subcellular compartments. Free NADH levels in the nucleus regulate the corepressor CtBP, involved in cell-cycle regulation and transformation transcriptional pathways. Using two-photon NAD(P)H microscopy and lifetime imaging, it was shown that the free NADH levels in the nucleus closely correspond to the half-maximal concentration for CtBP binding (Zhang et al, 2002).

The technique of two-photon excitation is also useful with other established biophysical techniques, such as fluorescence correlation spectroscopy (FCS; Schwille et al., 1999) and fluorescence recovery after photobleaching (FRAP; *UNITS 13.5 & 21.1*; Brown et al., 1999). Both techniques generally utilize stationary one-photon (continuous-wave) lasers. FCS determines the occupation number and diffusion characteristics of fluorescent probes inside the focal volume of the stationary beam, and it has proven useful in molecule-interaction and diffusion studies. FRAP, through photobleaching of fluorescence in the focal area and observation of fluorescence recovery, has been used to study macroscopic diffusion of fluorescent molecules. These techniques have both been used extensively to examine the diffusion char-

acteristics of fluorescent probes on cultured cell membranes. Currently, the complexity of these two techniques has limited the utility of these techniques to in vitro systems and cell culture models. The well defined excitation volume in two-photon microscopy is useful for quantitative FCS and FRAP. Furthermore, these techniques are likely to show great utility in studying bimolecular dynamics in thick living tissues using two-photon rather than one-photon excitation.

Deep penetration with two-photon excitation allows in vivo imaging, although a number of complexities need to be overcome when imaging living animals. In vivo fluorescent imaging involves two-photon excitation imaging of a living animal through the skin, through surgical openings, or through coverslip windows placed onto the animal. When working with live animals, it is often more difficult to fluorescently label the sample. Specific labeling of neurons in living mice, using a Ca^{2+} indicator, has been done to monitor neural function using two-photon excitation (Svoboda et al., 1997). The expression of green fluorescent proteins (GFP) in transgenic animals to fluorescently label specific organs and proteins is likely to permit new applications of two-photon excitation in vivo imaging (Potter et al., 2001). Another complexity when working with living animals is that the samples can move during the imaging process. Currently, in vivo studies are done with anesthetized animals, and imaging rates are increased in order to limit the effect of this movement. In the future, it is likely that technological advances such as the miniaturization of two-photon microscopes for attachment directly to the animal (Helmchen et al., 2001) will allow in vivo imaging of freely moving animals.

The microcircuitry of neurons in brain slices has been mapped using two-photon imaging (Kozloski et al., 2001; Peterlin et al., 2001). These researchers used bulk loading of calcium indicator combined with two-photon imaging. They triggered a signal neuron and then mapped the calcium signal initiated in connecting (follower) neurons. It was determined that the neocortex is composed of precise circuits. The followers belonged to a few selective anatomical classes and their positions were determined between animals (Kozloski et al., 2001).

A final and potentially very powerful application of two-photon excitation microscopy is three-dimensionally resolved photorelease of caged compounds (uncaging). For example, techniques have been developed for quantitative two-photon uncaging of calcium (Brown et al., 1999). As discussed previously, the photochemical reactions involved are generally quite slow (from milliseconds to seconds), which allows the compound to diffuse over several micrometers within the sample between the time when it is excited and the time it becomes active (Kiskin et al., 2002). Diffusion is not a problem when this technique is used to mark cells by uncaging membrane-impermeant fluorescent molecules. This technique has been successfully used to track the development of sea urchin embryo cell lineages (Piston et al., 1998). Faster-uncaging molecules have been successfully used in studies of uncaging stimulants using two-photon excitation to map neuron receptors (Denk, 1994; Matsuzaki et al., 2001). These studies utilized the three-dimensional nature of two-photon excitation to photochemically uncage stimulants in the imaging medium. When the stimulant was uncaged near the membrane, it stimulated receptors in close proximity, which was detected through a patch clamp of the cell. In this type of imaging, excitatory response, rather than photons, maps the image. In particular, a study utilizing uncaging of MNI-glutamate in conjunction with a whole-cell clamp for detection of signal was successfully used to map glutamate receptors on cultured hippocampal neurons and hippocampal CA1 pyramidal neurons in acute slice preparations (Matsuzaki et al., 2001). These researchers were able to obtain excellent lateral and axial FWHM diameters of 0.6 and 1.4 μm, respectively, in the slice preparations, indicating fast uncaging chemistries. These researchers determined that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors are abundant in mushroom spines and that the distribution of these receptors is tightly correlated with spine geometry.

CONCLUSIONS

Two-photon excitation microscopy shows great utility for dynamic imaging of living cells in thick samples, such as intact tissue. This often makes possible experiments where conventional imaging cannot be performed. Using a mode-locked (pulsed) laser and photon crowding at the focal point, two-photon excitation only occurs at the focal plane. Localized excitation results in emission from only the focal region, providing sectioning ability without the use of a pinhole, and it reduces phototoxicity because photodamage is largely limited to the focal volume. Although two-photon

excitation microscopy does not generate images with higher resolution than confocal microscopy, it does allow for increased depth penetration into samples. This increased depth penetration is possible due in part to the open pinhole geometry of the two-photon microscope, lack of out-of-focus absorption of the excitation light, and decreased scattering of the excitation light. In order to take full advantage of the depth of penetration, it is necessary to use nondescanned detection geometries because of the dramatic increase in collection efficiency of scattered photons. Clearly, the advantages of two-photon excitation have allowed for experiments that would not be possible using confocal microscopy, and, as this technique gains popularity and the technology gets less expensive, it is expected that more and more exciting experiments will be achieved.

LITERATURE CITED

Agard, D.A., Hiraoka, Y., Shaw P., and Sedat, J.W. 1989. Fluorescence microscopy in three dimensions. *Methods Cell Biol.* 30:353-377.

Albota, M.A., Xu, C., and Webb, W.W. 1998. Two-photon fluorescence excitation cross sections of biomolecular probes from 690 to 960 nm. *Appl. Optics* 37:7352.

Bennett, B.D., Jetton, T.L., Ying, G., Magnuson, M.A., and Piston, D.W. 1996. Quantitative subcellular imaging of glucose metabolism within intact pancreatic islets. *J. Biol. Chem.* 271:3647-3651.

Birge, R.R. 1986. Two-photon spectroscopy of protein-bound chromophores. *Acc. Chem. Res.* 19:138-146.

Brown, E.B., Shear, J.B., Adams, S.R., Tsien, R.Y., and Webb, W.W. 1999. Photolysis of caged calcium in femtoliter volumes using two-photon excitation. *Biophys. J.* 76:489-499.

Carrington, W.A., Fogarty, K.E., and Fay, F.S. 1990. 3-D fluorescence imaging of single cells using image restoration. *In* Non-Invasive Techniques in Cell Biology. (J.K. Foskett, and S. Grinstein, eds.) pp. 53-72. Wiley-Liss, New York.

Centonze, V.E. and White, J.G. 1998. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. *Biophys. J.* 75:2015-2024.

Denk, W. 1994. Two-photon scanning photochemical microscopy: Mapping ligand-gated ion channel distributions. *Proc. Natl. Acad. Sci. U.S.A.* 91:6629-6633.

Denk, W. and Svoboda, K. 1997. Photon upmanship: Why multiphoton imaging is more than a gimmick. *Neuron.* 18:351-357.

Denk, W., Strickler, J.H., and Webb, W.W. 1990. Two-photon laser scanning fluorescence microscopy. *Science* 248:73-76.

Denk, W., Piston, D.W., and Webb, W.W. 1995. Two-photon excitation in laser scanning microscopy. *In* The Handbook of Biological Confocal Microscopy. 2nd ed. (J. Pawley, ed.) pp. 445-458. Plenum, New York.

French, T., So, P.T., Dong, C.Y., Berland, K.M., and Gratton, E. 1998. Fluorescence lifetime imaging techniques for microscopy. *Methods Cell Biol.* 56:277-304.

Göppert-Mayer, M. 1931. Über Elementarakte mit zwei Quantensprüngen. *Ann. Physik. (Berlin)* 9:273-294.

Harpur, A.G., Wouters, F.S., and Bastiaens, P.I. 2001. Imaging FRET between spectrally similar GFP molecules in single cells. *Nat. Biotechnol.* 19:167-169.

Helmchen, F., Fee, M.S., Tank, D.W, and Denk, W. 2001. A miniature head-mounted two-photon microscope: High-resolution brain imaging in freely moving animals. *Neuron.* 31:903-912.

Holmes, T.J., Bhattacharyya, S., Cooper, J.A., Hanzel, D., Krishnamurthi, V., Lin, W., Roysam, B., Szarowski, D.H., and Turner, J.N. 1995. Light microscopic images reconstructed by maximum likelihood deconvolution. *In* The Handbook of Biological Confocal Microscopy. (J. Pawley, ed.) pp. 389-402. Plenum, New York.

Kaiser, W. and Garrett, C.G.B. 1961. Two-photon excitation in $CaF_2:Eu^{2+}$. *Phys. Rev. Lett.* 7:229-231.

Kiskin, N.I, Chillingworth, R., McCray, J.A., Piston D., and Ogden, D. 2002. The efficiency of two-photon photolysis of a "caged" fluorophore, *o*-1-(2-nitrophenyl)ethylpyranine, in relation to photodamage of synaptic terminals. *Eur. Biophys. J.* 30:588-604.

Kozloski, J., Hamzei-Sichani, F., and Yuste, R. 2001. Stereotyped position of local synaptic targets in neocortex. *Science* 293:868-872.

Ludwig, T.E., Squirrell, J.M., Palmenberg, A.C., and Bavister, B.D. 2001. Relationship between development, metabolism, and mitochondrial organization in 2-cell hamster embryos in the presence of low levels of phosphate. *Biol. Reprod.* 65:1648-1654.

Maiti, S., Shear, J.B., Williams, R.M., Zipfel, W.R., and Webb, W.W. 1997. Measuring serotonin distribution in live cells with three-photon excitation. *Science* 275:530-532.

Masters, B.R. and So, P.T. 1999. Multi-photon excitation microscopy and confocal microscopy imaging of in vivo human skin: A comparison. *Microsc. Microanal.* 5:282-289.

Masters, B.R., So, P.T., and Gratton, E. 1997. Multiphoton excitation fluorescence microscopy and spectroscopy of in vivo human skin. *Biophys. J.* 72:2405-2412.

Matsuzaki, M., Ellis-Davies, G.C., Nemoto, T., Miyashita, Y., Iino, M., and Kasai, H. 2001. Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. *Nat. Neurosci.* 4:1086-1092.

McNally, J.G, Karpova, T., Cooper, J., and Conchello, J.A. 1999. Three-dimensional imaging

by deconvolution microscopy. *Methods* 19:373-385.

Neu, T.R., Kuhlicke, U., and Lawrence, J.R. 2002. Assessment of fluorochromes for two-photon laser scanning microscopy of biofilms. *Appl. Environ. Microbiol.* 68:901-909.

Patterson, G.H. and Piston, D.W. 2000. Photobleaching in two-photon excitation microscopy. *Biophys. J.* 78:2159-2162.

Patterson, G.H., Knobel, S.M., Arkhammer, P.O.G., Thastrup, O., and Piston, D.W. 2000. Separation of the glucose-stimulated cytoplasmic and mitochondrial NAD(P)H responses in pancreatic islet β cells. *Proc. Nat. Acad. Sci. U.S.A.* 97:5203-5207.

Perriott, L.M., Kono, T., Whitesell, R.R., Knobel, S.M., Piston, D.W., Granner, D.K., Powers, A.C., and May, J.M. 2001. Glucose uptake and metabolism by cultured human skeletal muscle cells: Rate-limiting steps. *Am. J. Phys: Endo. Metab.* 281:E72-E80.

Peterlin, Z.A., Kozloski, J., Mao, B.Q., Tsiola, A., and Yuste, R. 2000. Optical probing of neuronal circuits with calcium indicators. *Proc. Natl. Acad. Sci. U.S.A.* 97:3619-3624.

Piston, D.W., Sandison, D.R., and Webb, W.W. 1992. Time-resolved imaging and background rejection by two-photon excitation in laser scanning microscopy. *SPIE* 1640:379-389.

Piston, D.W., Kirby, M.S., Cheng, H., Lederer, W.J., and Webb, W.W. 1994. Two-photon excitation fluorescence imaging of 3-dimensional calcium-ion activity. *Appl. Optics* 33:662-669.

Piston, D.W., Summers, R.G., Knobel, S.M., and Morrill, J.B. 1998. Characterization of involution during sea urchin gastrulation using two-photon excited photorelease and confocal microscopy. *Microsc. and Microanal.* 4:404-414.

Potter, S.M., Zheng, C., Koos, D.S., Feinstein, P., Fraser, S.E., and Mombaerts, P. 2001. Structure and emergence of specific olfactory glomeruli in the mouse. *J. Neurosci.* 21:9713-9723.

Rocheleau, J.V., Head, W.S., Nicolson, W.E., Powers, A.C., and Piston, D.W. 2002. Pancreatic islet beta cells transiently metabolize pyruvate. *J. Biol. Chem.* 277:30914-30920.

Schwille, P., Haupts, U., Maiti, S., and Webb, W.W. 1999. Molecular dynamics in living cells observed by fluorescence correlation spectroscopy with one- and two-photon excitation. *Biophys J.* 77:2251-2265.

Sheppard, C.J.R. and Gu, M. 1990. Image formation in two-photon excitation microscopy. *Optik* 86:104-106.

Squirrell, J.M., Wokosin, D.L., White, J.G., and Bavister, B.D. 1999. Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. *Nat. Biotechnol.* 17:763-767.

Svoboda, K., Tank, D., Kleinfeld, D., and Denk, W. 1997. In vivo dendritic calcium dynamics in neocortical pyramidal neurons. *Nature* 385:161-165.

White, J.B., Amos, W.B., and Fordham, M. 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J. Cell Biol.* 105:41-48.

Xu, C., Guild, J., Webb, W.W., and Denk, W. 1995. Determination of absolute two-photon excitation cross sections by in situ second-order autocorrelation. *Optics Lett.* 20:2372.

Xu, C., Zipfel W., Shear, J.B., Williams, R.M., and Webb, W.W. 1996. Multiphoton fluorescence excitation: New spectral windows for biological nonlinear microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 93:10763-10768.

Zhang, Q., Piston, D.W., and Goodman, R.H. 2002. Regulation of corepressor function by nuclear NADH. *Science* 295:1895-1897.

Contributed by Jonathan V. Rocheleau and David W. Piston
Vanderbilt University
Nashville, Tennessee

Total Internal Reflection Fluorescence Microscopy for High-Resolution Imaging of Cell-Surface Events

The wavelength of light imposes a physical limit of ~400 nm on the maximum resolution that can be achieved using light microscopy. This unit will describe the use of total internal reflection fluorescence microscopy (TIR-FM), or evanescent wave microscopy, an approach that partially overcomes this physical limit and permits one to selectively image just those fluorophores in the optical plane (along the *z* axis) within 50 nm of the cell surface. TIR-FM works by means of limiting the depth of penetration of the excitation light within this narrow region. This narrow excitatory plane not only provides a high signal-to-noise ratio but also minimizes the photodamage to the cell.

Basic through-the-objective TIR-FM setups are now commercially available from Olympus and Nikon, and these can be used for most generic applications. The Basic Protocol, however, outlines a procedure for setting up a TIR-FM microscope in the laboratory using a through-the-prism configuration, and the Alternate Protocol outlines a modification of this procedure for a through-the-objective configuration. These outlines are intended to serve as a starting point; the exact details of any home-made setup will ultimately be decided based on the exact application.

SETTING UP A THROUGH-THE-PRISM TIR-FM SYSTEM

BASIC PROTOCOL

A prism can be used to direct the excitation beam to the samples above the critical angle for total internal reflection. Based on the relative position of the prism, coverslip and the objective there are various ways to set up the prism based TIR setup. These variants are described in the Background Information and in Figure 4.12.3. This section describes the protocol used for setting up the basic prism based setup in which the prism is adjacent to the objective (Fig. 4.12.3C).

Materials

40- to 100-nm fluorescent polystyrene beads (Molecular Probes)
0.05× phosphate-buffered saline (PBS; *APPENDIX 2A*)
Immersion oil appropriate for objective (see Critical Parameters)

Wide-field epifluorescence microscope, upright or inverted (*UNIT 4.1*) with appropriate excitation and emission filters
Prism (see Background Information; Edmund Scientific)
Laser (see Critical Parameters; Spectrum Physics, Coherent, Melles-Griot, and others), with single-mode laser optical fiber or system of mirrors and focusing lens
Sykes-Moore chamber (Bellco Glass) or other coverslip mounting chamber
Laser-safety goggles

Set up microscope, prism, and laser

1. Use a conventional wide-field fluorescence microscope (upright or inverted). Mount the prism either on the holder for the condenser or build a stable mounting that has the option of vertical motion.

   The choice of prism shape (e.g., cubic, rectangular, equilateral, or trapezoidal) depends on the desired configuration of the setup—i.e., whether the prism is adjacent or opposite

Contributed by Jyoti K. Jaiswal and Sanford M. Simon
Current Protocols in Cell Biology (2003) 4.12.1-4.12.15

to the objective. In this protocol, a through-the-prism system where the prism is adjacent to the objective is described (see Background Information and Fig. 4.12.3C).

The prism should have the freedom to move parallel to the optical axis with its surface in contact with the coverslip. For a microscope that focuses by moving the stage up and down, a micromanipulator that can hold a prism could be fixed to the microscope stage.

2. Direct the laser light to the prism by using either a single-mode laser fiber or a system of mirrors and focusing lens with adjustable angle mounts fixed to the table. Include a suitable shutter in the laser light path to permit switching between the epifluorescent illumination from the arc lamp and the TIR-FM illumination.

Establish area of TIR using fluorescent beads

3. Prepare a 1:1000 dilution of 40- to 100-nm fluorescent polystyrene beads in 0.05× PBS. Mount a coverslip on the microscope in the same manner as for the actual experiment (e.g., mounted using a Sykes-Moore chamber or any other coverslip mounting chamber). Add sufficient volume of solution to cover the entire coverslip.

4. Use the arc lamp illumination to focus on the fluorescent beads that are immotile (attached to the coverslip).

5. Place a small droplet of immersion oil directly on the prism. Carefully translate the prism vertically so that it touches and spreads the oil, but does not inhibit the lateral sliding motion.

   Beading up of the oil around the edges of the prism should be prevented by using little oil and by wicking away the excess, as it could interfere with the illumination path.

6. Wearing the laser safety goggles, with the help of mirrors (and without any focusing lens in place), adjust the collimated laser beam position so that it is directly in line with the objective's optical axis (see Background Information).

   The beam can be observed by the scattering of the laser light as it traverses the prism, oil, and oil-glass interface.

7. Insert the focusing lens and focus the beam roughly at the region of the interface in line with the objective. Using the focusing lens mount, adjust the lateral position of the lens (not the mirrors controlling the raw laser beam) so that the laser beam hits the interface between the coverslip and the aqueous solution directly in line with the objective. To guide this adjustment, look directly at the beam path for three closely aligned spots of scattered light, corresponding to where the focused beam first crosses the immersion oil layer, where it totally reflects off the sample surface, and where it exits by crossing the oil again.

8. Using the epifluorescence excitation, ensure that the immotile fluorescent beads are still in focus.

   In epifluorescence, the motile beads will appear as a diffuse fluorescent blur. If TIR is achieved, the immotile beads (attached to the glass) should always be visible, but the motile fluorescent beads will appear to be constantly bouncing in and out of the evanescent field and hence in and out of the field of view. If this "bouncing" of beads is not clearly visible, then readjust the incident angle of the laser beam.

9. Once the conditions described under step 8 are obtained, use the focusing lens to center the area of TIR within the field of view.

   The focusing lens can be moved forward or backward along the laser optical path to achieve the desired size of the total internal reflection area on the coverslip.

Examine specimen

10. Once the optics are correctly aligned for total internal reflection, translate the prism vertically to remove the sample with fluorescent beads and replace it with the experimental sample. Translate the prism back to the same position to make optical contact.

    Although the total internal reflection region will not be in exactly the same spot because of irreproducibility in the prism height, it will be close enough to make final adjustments with the focusing lens while observing fluorescence from the new sample.

SETTING UP A THROUGH-THE-OBJECTIVE TIR-FM SYSTEM

ALTERNATE PROTOCOL

An objective with a high numerical aperature (>1.45 NA, see Table 4.12.1) can be used to bring the excitation beam to the sample above the critical angle for total internal reflection. The same objective can be used to collect the resulting emission from the sample (Fig. 4.13.4A). This section will describe how this can be achieved. See Background Information for further details and Critical Parameters for choosing the proper objective for your purpose.

Additional Materials *(also see Basic Protocol)*

Optical mounts including mirrors, optical fiber couplers, and fiber optics or lenses for coupling the laser beam expander to the microscope port (Olympus, TILL Photonics)
Plano-convex lens of short radius of curvature *or* hemispherical or triangular prism and converging lens with focal length of several centimeters
High-numerical-aperture objective (see Critical Parameters)

1. Set up microscope and laser, and prepare bead suspension (see Basic Protocol).

   For ease of switching back and forth between TIR-FM and epifluorescence illumination, the optics used to combine the arc lamp and TIR light sources should include a movable mirror that is easily reached and that can be used to switch between the two light paths. This can be achieved by making the laser beam perpendicular to the arc-lamp beam, and inserting a retractable mirror at an angle of 45° to both the beams at the place where the two meet. Depending on whether the mirror is inserted or not, one can choose between the arc lamp and the laser beam.

Steer the laser beam into the center of the objective

2. Place a triangular or hemispherical prism or a plano-convex lens (flat side down) on a coverslip and place this assembly on the microscope stage.

3. Add immersion oil between the objective and the coverslip and focus on the upper surface of the coverslip using the halogen lamp illumination.

   Usually dust and defects on the coverslip can be easily seen, to assess the focus.

4. Allow the raw collimated laser beam to enter the standard epi-illumination port and field diaphragm along the optical axis. Steer the beam such that removing all the obstructions between the coverslip sample and the room ceiling produces a large area of laser illumination on the ceiling, roughly straight up.

Obtain and confirm TIR illumination

5. Reposition the beam so that it enters the center of the field diaphragm at an angle that is no longer parallel to the optical axis. Slowly increase the angle until the laser illumination on the ceiling, moving to an ever-lower position on the side wall, just disappears (at this angle, the beam is just blocked by the internal aperture of the objective). Reduce the entrance angle so that half the illuminated area is visible.

6. In order to focus the raw beam at the back focal plane of the objective place the converging lens about 20 cm up-beam from the field diaphragm. This will cause the illuminated region on the wall to become smaller.

7. Move the converging lens longitudinally (along the axis of the laser beam) towards the field diaphragm.

   The back-focal plane can be seen by removing the eyepiece. Some microscopes (e.g., Olympus 1 × 70) have a toggle switch near the eyepiece that permits visualizing the back focal plane without removing the eyepiece.

   This will continuously minimize the illuminated region on the wall until the focal point of the lens falls exactly at the objective's back focal plane. At this position, the beam emerging from the objective lens is roughly collimated and would be propagating upward along the inner periphery of the objective.

8. Verify that total internal reflection is achieved by moving the coverslip horizontally to a new spot that is not under the prism.

   Almost no light should emerge, except for some scattering, because of total internal reflection at the glass coverslip–air interface.

9. Replace the bare coverslip with an identical one containing a solution of fluorescent beads and again verify that no light emerges, even at the glass-water interface.

   If the light does emerge, then change the angle of the incident beam using the steering mirrors just until the point when no light emerges.

10. Confirm that TIR excitation is indeed achieved by focusing the epifluorescence excitation on the fluorescent beads, then switching to TIR illumination.

    When TIR is achieved, the diffuse background fluorescence from the beads will be lost. Instead there will be two populations of beads. A small number of immotile beads (attached to the glass) will always be visible and a larger population will bounce in and out of view. If this bouncing of beads is not apparent, adjust the incident angle of the laser beam until it is.

11. Fine tune the mirrors and converging lens to center the total internal reflection field.

    The size of the total internal reflection fluorescence area on the sample is directly proportional to the laser-beam size at the field diaphragm. To change this size, replace the converging lens with another one of different focal length but always keep its focal point at the objective's back focal plane, which is at a fixed position up-beam from the microscope.

Examine specimen

12. Replace the fluorescent beads with the actual sample.

    When the cells are in focus, the total internal reflection optics should be perfectly aligned without need for further adjustment.

SUPPORT PROTOCOL

MEASURING THE INCIDENT ANGLE OF THE EXCITATORY BEAM

While for the prism-based setup the angle of the incident beam can be measured directly. However, the following indirect method can be used to measure the angle of incidence for both through-the-prism and through-the-objective types of setup.

For materials, see Alternate Protocol.

1. Follow steps 1 to 9 of the Basic Protocol or 1 to 11 of the Alternate Protocol to obtain TIR.
2. Without disturbing the optical setup of the excitation beam, replace the coverslip containing the beads with a coverslip containing a hemispherical prism or a plano-convex lens (see Alternate Protocol, step 2).
3. Place a screen on the wall adjacent to the microscope to mark the position of the beam exiting the prism.
4. Carefully mark the position on the screen that corresponds to the vertical position of the coverslip on the stage.
5. Measure the horizontal positions of these two spots with relation to their points of exit from the coverslip.
6. Using the above values draw a triangle connecting these three points, and calculate the angle between the TIR beam and the base (abscissa) of the triangle.
7. Determine the angle of incidence of the excitatory beam by subtracting the calculated angle from 90°.

COMMENTARY

Background Information

The earliest recorded demonstration of the phenomena of total internal reflection was by John Tyndall on May 19, 1854. Tyndall, who is credited for explaining why the sky is blue, presented a demonstration entitled "On Some Phenomena Connected with the Motion of Liquids." His lecture covered the properties of thin streams of water. At the end, he presented a demonstration in which light, leaving a water tank through a hole in its side, would follow the water as it fell to the ground (Fig. 4.12.1). The light hit the interior sides of the water stream at an angle steep enough that it was completely reflected. No light was observed to leave the water stream and, instead, the water served as a light pipe (Pepper, 1860; Greenslade, 1997).

The fundamental principle of TIR-FM is based on one additional observation. Total internal reflection of the light means that there is no energy propagated on the opposite side of the interface. However, a standing field is generated at the interface, into the medium of lower refractive index on the side opposite the incident wave. This field, called the evanescent field or evanescent wave, decays exponentially with increasing distance from the interface (Fig. 4.12.2A). The characteristic distance for decay of the evanescent-wave intensity is a function of the incident angle of the light beam, its wavelength, and the difference in the refractive index of media on either side of the interface. For most biological applications, altering these parameters the field of illumination can be adjusted to any depth between 50 and 200 nm. Thus, the predominant excitation of fluorophores is within this distance from the interface.

In 1965, Hirschfeld developed this as a method for selectively illuminating a surface at a solid-liquid interface. However, it was not until 1981 that this approach was adopted for use in biology to image cell-substrate contact (Axelrod, 1981, 1983; Axelrod et al., 1981; Gingell, 1981). Although TIR-FM has been applied for a variety of biological applications over the past two decades, very recent technical advances in fast imaging and image analysis, as well as the availability of commercial TIR fluorescence microscopes, are opening up possibilities for wider and easier application of this technique (Axelrod, 1989, 2001). As a result, TIR-FM has been used for a variety of live-cell applications, including study of cell-surface contact, diffusion of proteins and lipids in the plasma membrane, submembrane actin dynamics, and vesicular exocytosis (Schmoranzer et al., 2000; Sund and Axelrod, 2000; Wagner and Tamm, 2000; Amann and Pollard, 2001; Steyer and Almers, 2001; Toomre and Manstein, 2001).

Theory of TIR-FM

When a beam of light encounters a medium with lower refractive index, it changes its path such that the resulting beam is refracted (bent away) from the path of the incident beam. For TIR-FM, the incident angle, θ, (as measured from the plane normal to the incident surface) has to be large enough for the beam to totally internally reflect back into the medium with the higher refractive index (Fig. 4.12.2B). This con-

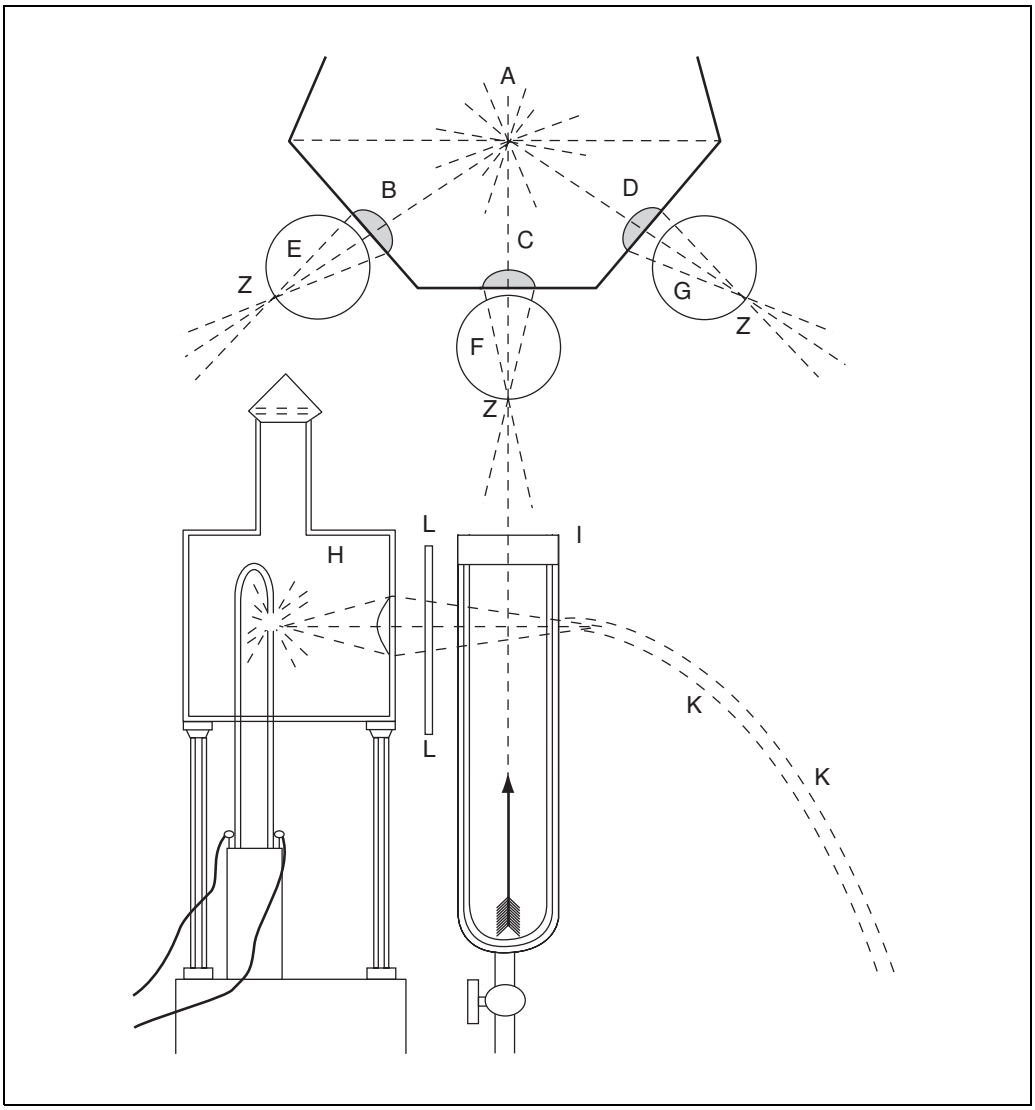


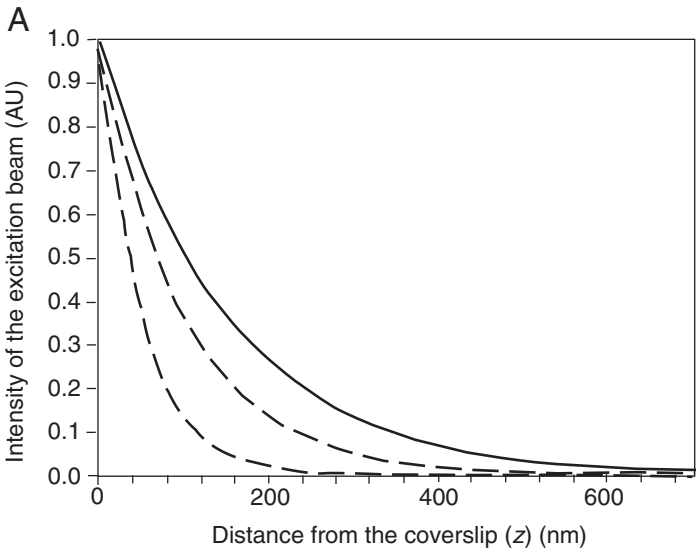
Figure 4.12.1 Illustration of the classic experiment ("The Liquid Vein") demonstrating total internal reflection. The apparatus (viewed from the top in the upper part of the figure and from the side in the lower) consists of a Duboscq lantern with an electric arc light (A), three sides of which are furnished with lenses (E, F, and G). Outside the lantern are three cylinders of water, each with a circular glass window and orifices at (Z) from which the water and the rays of light pass out. In the lower diagram, a section is seen of one side of the Dubosoq lantern (H) and one cylinder of water (I), with the water entering from below through the stopcock. The stream of illuminated water is labeled K, and L represents a bit of colored glass held between the lantern and the cylinder of water to impart color to the light. From Pepper (1860).

dition occurs above a critical angle. For a light beam that is traveling from a medium with high refractive index (n1), such as oil, glass, or silica, into a medium with lower refractive index (n2), such as water or air, the critical angle (qc) is:

$$\theta_c = \sin^{-1}\frac{n_2}{n_1}$$

Equation 4.12.1

For TIR to occur, the ratio n2/n1 must be <1, and the value of q should be > qc. For all values of q < qc, the light beam propagates through the interface, leading to a wide-field illumination. Glass coverslips typically used to grow tissue culture cells have a refractive index (n1) of 1.515, while the cytosol and the medium surrounding the cells have refractive indices (n2) ranging from 1.33 to 1.38. To obtain total internal reflection at the higher end of the cellular refractive index (1.38), the angle of incidence must be larger than the critical angle of [sin-1 (1.38/1.515) = 65.64°].



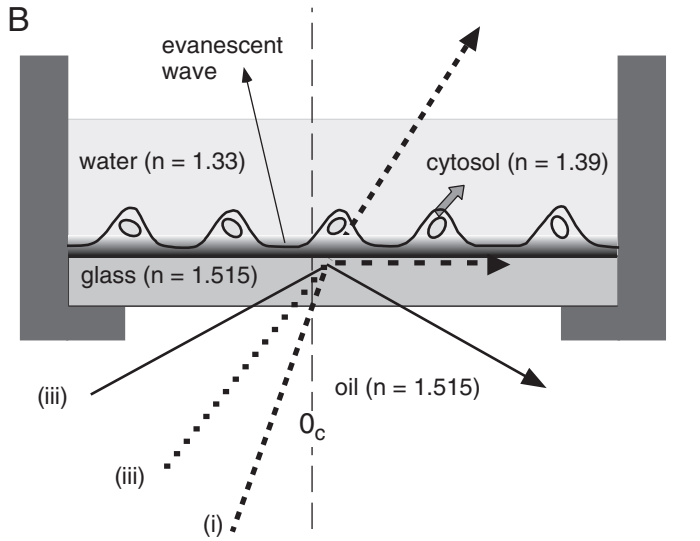


Figure 4.12.2 (**A**) Exponential decay of evanescent field. The intensity of the evanescent field in the aqueous medium is plotted over the distance from the coverslip/water interface for typical decay lengths (dotted line, $d = 50$ nm; dashed line, $d = 100$ nm; and solid line, $d = 150$ nm) used in most experiments. (**B**) Illustration of Snell's law, refraction, and total internal reflection. Beams that are (i) refracted, (ii) incident at the critical angle, and (iii) totally internally reflected are illustrated at a glass-water interface. The evanescent field is indicated as a gradient of gray at the interface that falls exponentially with distance from the interface.

If, instead of intact cells, permeabilized or fixed cells are used, the effective refractive index is that of aqueous buffer (n2 = 1.33), and the critical incidence angle is reduced to 61.39°.

For values of q > qc, the resulting electromagnetic evanescent field has the same frequency, wavelength, and polarity as the incident light. For an infinitely wide beam, the intensity of the evanescent wave (measured in units of energy/area/sec) decays exponentially with perpendicular distance from the interface between the two media (Fig. 4.12.2A). The intensity (I) at any given point z is related to the intensity at the interface [$I(0)$] by:

$$I(z) = I(0)e^{-\frac{z}{d}}$$

Equation 4.12.2

where for a light of wavelength λ, which is incident at the interface at an angle θ, the depth of penetration (*d*) is given by:

$$d = \frac{\lambda}{4\pi}\left(n_1^2 \sin^2 \theta - n_2^2\right)^{-\frac{1}{2}}$$

Equation 4.12.3

At $\theta > \theta_c$ evanescent wave intensity is equal to the intensity of the incident beam, but the intensity approaches zero as θ approaches 90°.

Methods for achieving total internal reflection

The necessary and sufficient criterion for obtaining total internal reflection is that the excitation light be introduced on the surface of the coverslip at an angle greater than the critical angle. This can be achieved in a variety of different ways, and, as detailed below, each of these have their advantages and limitations.

Excitation through a prism: In the prism-type TIR-FM setup, the excitatory beam is coupled into the coverslip via a prism (Fig. 4.12.3A, B, and C). At the first interface between the air and the prism, the angle of the excitatory beam is narrow enough that most of it enters the prism with minimal refraction. The prism and the coverslip are in contact with the oil, whose refractive index matches that of the glass. Thus, the excitation beam passes through this interface with minimal reflection or refraction. However, changing the angle of incidence of the beam on the prism allows alteration of the angle at which the beam hits the interface of the coverslip and the cell. At a steep enough angle, the excitation beam is totally internally reflected at this interface, resulting in excitation

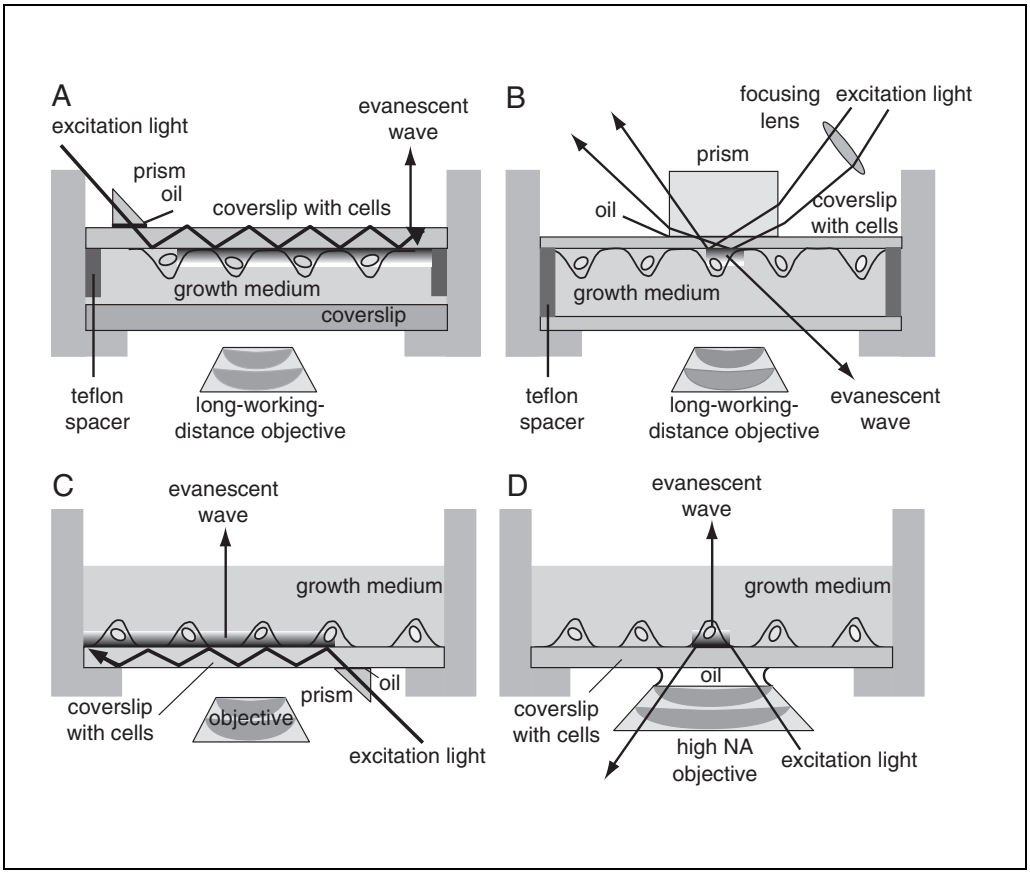


Figure 4.12.3 Possible arrangements for setting up total internal reflection fluorescence microscope. In (**A**) and (**B**), the excitation beam is passed through the prism opposite the objective. Unlike (A), a narrow field of TIR illumination is achieved in (B) with the use of a bigger prism that allows the internally reflected light to pass through the prism instead of being reflected back into the coverslip. In (**C**), the excitation beam passes though the prism adjacent to the objective, and in (**D**), excitation takes place through the objective.

of a narrow region of the cell near the coverslip. The resulting fluorescence from the cell is collected by an objective lens.

An advantage of the prism-based approach is that it allows separation of the excitation and emission light paths. This reduces the background fluorescence caused by scattering of the excitation light in the objective. This setup also reduces the need for elaborate optics necessary to prevent the bleed-through of the excitation light into the emission channel. However, there are many limitations to this approach. First, once the sample has been focused, the prism must be carefully positioned on the coverslip and maintained such that it does not lose the coupling with the coverslip. Second, the oil used to couple the prism to the coverslip must be kept very clean throughout an experiment; any dust will cause scattering of the excitation light. Third, during positioning of the prism on the surface, the oil must not be allowed to spread on the coverslip, since TIR will be lost from places where the oil spreads on the surface of the glass.

Depending on the position of the prism used to target the excitation light, with respect to the objective lens, there are two basic types of prism-based setup, as described in the following paragraphs.

Excitation through the prism opposite to the objective: As described in Figure 4.12.3A and B, in this setup the fluorescence emission is collected through an objective from the side opposite to that of the prism. Depending on the prism used, cells on the entire coverslip can be illuminated (Fig. 4.12.3A) or the excitation light can be limited to a small region under observation (Fig. 4.12.3B). The relatively large distance between sample and objective limits this application to an objective with a long working distance and a low NA (typically 0.75 or less). Thus, this technique is less sensitive and not suitable for low-light applications.

Excitation through the prism adjacent to the objective: In this setup, the prism is on the same side of the coverslip as the objective, while the specimen is on the opposite side (Fig. 4.12.3C). The specimen can be translated while the prism remains laterally fixed. The advantage of this approach is that it allows the use of objectives with higher NA than can be used with the prism on the opposite side of the objective. A disadvantage of this configuration is that oil- or glycerol-immersion objectives cannot be used. If oil/glycerol were used at the interface of the prism and coverslip and any of it were to smear, it would lead to loss of total internal reflection. Thus, the choice of objectives is limited to air- or water-immersion objectives. This puts an upper limit to the numerical aperture of the objective, thus reducing the sensitivity of the detection. Depending upon how the coupling is done, this could lead to a fairly wide field of excitation which could be an advantage or disadvantage depending upon the experimental interest.

Excitation through the glass support setup: This approach, which is useful for large-scale evanescent wave excitation, is a slight variation on the excitation-through-the-prism approach. Here the excitation beam is directly coupled to a thin glass surface with the specimen on it. The laser has to be introduced at an angle such that, after refraction in the glass support, the light impinges upon the sample at an angle > qc, thus generating an evanescent wave. This can be done by making an appropriate angle at the edge of the glass surface. This allows the use of any objective of choice; at the same time it does not restrict the accessibility to the sample. However, due to the larger field of evanescent illumination, it leads to photobleaching even outside the field of view. Also, if the coupling between the laser and the coverslip is not robust, it could lead to fluctuations or even a total loss of evanescent field.

Excitation through the objective: In the objective-type TIR-FM setup, the supercritical angle on the coverslip-cell interface is reached by focusing the excitatory beam on the periphery of the back focal plane of the objective (Fig. 4.12.4A). In this way, the beam is refracted by the objective and directed through the refractive index–matched media: the objective, the immersion liquid, and the coverslip. The objective thus functions both as the prism, delivering the exciting beam, and as the optics, collecting the emission. The main advantage is that objective-type TIR-FM employs high-numerical-aperture, short-working-distance objectives, resulting in collection of much higher fluorescence emission compared to the prism-type approach. This results from the relationship whereby light collection is approximately equal to the square of the NA; thus, a 1.65 NA objective used in the objective-type TIR will collect 90% more light than a 1.2 NA water objective required for the prism-type TIR. Moreover, such a setup makes the sample fully accessible for other manipulations such as microinjection, patch

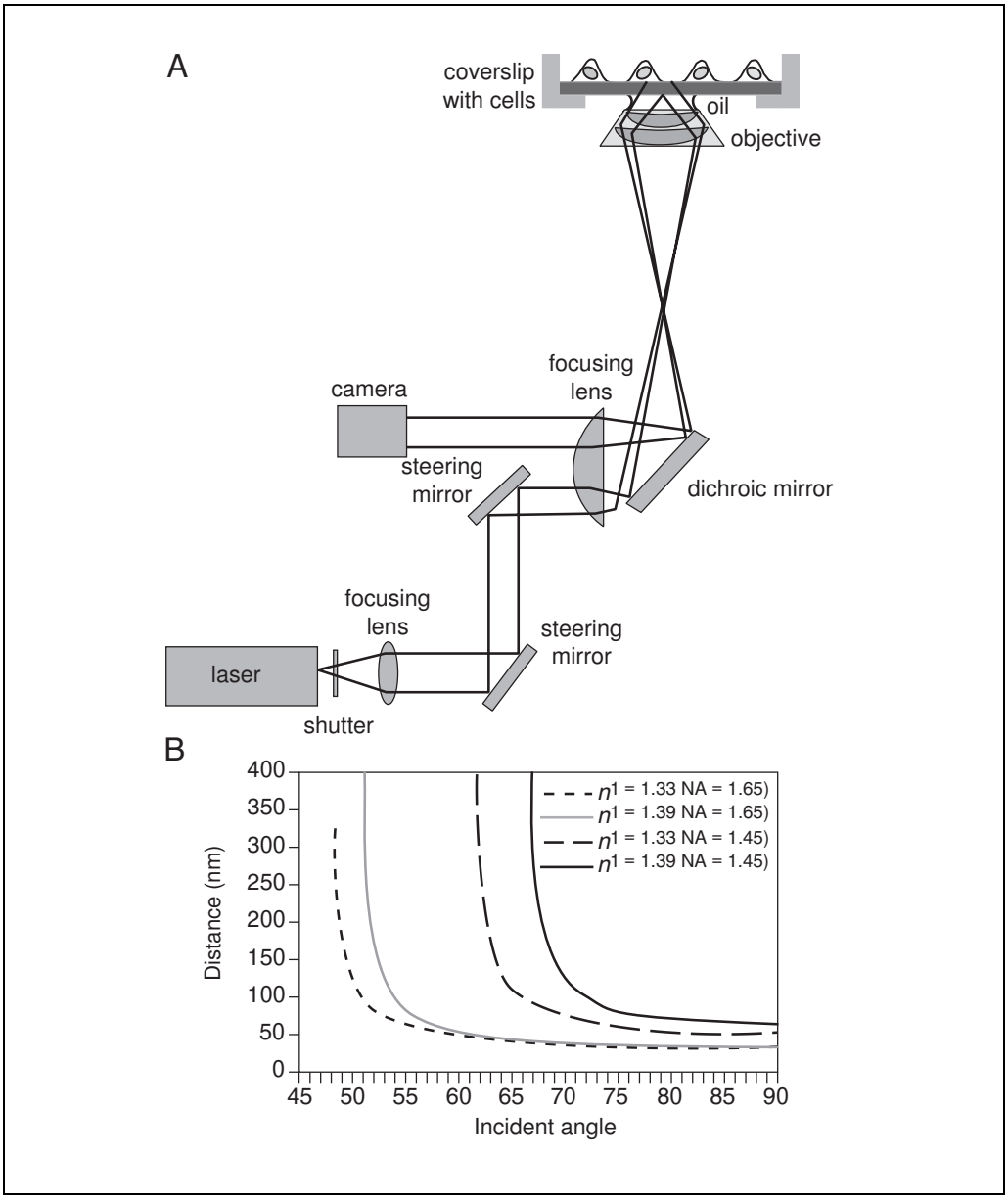


Figure 4.12.4 Through-the-objective TIR-FM setup. (**A**) Excitation and emission light path. (**B**) Relationship between incident angle of the beam and its evanescent depth. The spatial decay constant for the evanescent field is plotted for both the NA 1.45 and 1.65 objectives according to Equation 4.12.2 for refractive indices (n_1) of 1.33 and 1.39, typical of water and cytosol, respectively.

clamping, and atomic force microscopy. Another advantage of this approach is that it permits the use of both a laser-based and an arc lamp–based excitation light source for TIR. Finally this approach is easier and more reproducible in terms of setup: it does not require oil to couple the prism to the objective; it does not require a careful positioning of the prism on the coverslip; and from experiment to experiment, there is less variability in alignment of the excitatory beam. This approach has some limitations. First, since the excitation light has to pass through the same optics as are used to collect the emission, dichroics and filters must be used to minimize inadvertent detection of the excitation light. Second, if the excitation is not properly aligned, it is possible to get scattering within the objective, which would lead to loss of TIR-FM.

Critical Parameters

Depending on which of the methods is used to achieve TIR, the optics used (prism or high-NA objective, or edged glass) to target the

excitation light need to be altered, but the rest of the setup is similar in all cases.

Epifluorescence microscope

Both an upright or an inverted epifluorescence microscope can be used for an TIRF microscope. For applications that require access to the sample for other manipulations such as microinjection, atomic force microscopy, patch clamping etc. use of inverted microscope with the objective-type setup (Fig. 4.12.3D) or with a "prism adjacent to the objective" setup (Fig 4.12.3C) is recommended. However, an upright microscope with long working distance objective can also be used in conjunction with "prism opposite to the objective" setup to provide a limited access to the sample.

Light source

In principle, it is possible to employ conventional tungsten halogen or mercury/xenon arc lamps to conduct TIR-FM experiments. However, using an arc lamp requires generation of a sharp-edged shadow image of an opaque circular disc at the back focal plane of the objective. Although the arc lamp provides the convenience of selecting any excitation wavelength from the UV to the visible spectrum just by changing the excitation filters, a majority of the investigations reported in the literature are performed using laser illumination. The reason for this is that laser light is brighter, coherent, polarized, and well collimated. Hence, it is easier to direct into the objective or prism using standard beam expanders, mirrors, and focusing lenses. Collimation of the light emitted by the arc lamp results in severe (>90%) reduction of intensity, making it difficult to use the commercial arc lamps as light sources for TIR-FM. Another advantage of the laser source is that it can be used to illuminate an area of a defined geometry that can be easily adjusted to accommodate experimental variations. However, a limitation of laser illumination is the unavoidable interference fringing on the specimen. These fringes can be somewhat reduced by meticulously cleaning the optical surfaces. Additionally, a field with a uniform concentration of fluorophore can be used to generate an image that can then be used to normalize for the spatial inhomogenities in illumination. Alternatively, for experiments requiring highly uniform illumination, the fringing can be eliminated by using a commercially available laser fiber phase scrambler.

For most applications, a laser with a single-wavelength output of ≥20 mW should be adequate. However, for applications requiring short exposure time, e.g., FRAP (*UNITS 13.5 & 21.1*), lasers with output of 100 mW should be used. In either case, the laser should provide an option for regulating its output externally. If the laser source does not provide the means to regulate the power output, then an acousto-optic modulator (AOM) or a set of neutral-density filters should be introduced in the laser light path. Note that AOMs provide a continuous control of the intensity from zero to the maximum, but they tend to lose up to 50% of the input light. Hence, use of AOMs necessitates lasers with higher power.

For any of the types of TIR-FM setup described above, excitation light from a laser source can be introduced either through an optical fiber or as a free-standing beam. However, the exact optical arrangement is dependent upon the setup used to obtain TIR-FM. For through-the-prism setup, use of optical fiber minimizes the risk of accidental direct exposure to the laser beam. Depending on the position of the prism (with respect to the sample), the fiber has to be placed either above or below the microscope stage and positioned to target the beam into the prism at an appropriate angle for TIR. Similarly a fiber-based setup should be used for the through-the-glass-support-based setup. For the objective-based setup, the laser light could be introduced using a fiber in the same port as the epifluorescence light from the arc lamp; the two light sources can thus be coupled. There are commercially available adaptors from Olympus, Nikon, and TILL Photonics (see *SUPPLIERS APPENDIX*) that permit coupling the light sources for a variety of microscopes. Alternatively the free-standing laser light can also be steered into the microscope using mirrors and focused on the back focal plane of the objective using a set of focusing lens (Fig. 4.12.4A).

Objectives

As described in Equation 4.12.1, in order to collect maximum amount of light emitted by the sample (and hence obtain brighter images) it is necessary to use an objective with as large a numerical aperture (NA) as possible. Any long-working-distance objective can be used for a prism-based TIR-FM setup with the prism on the opposite side (Fig. 4.12.3A and B). However, for a setup with the prism on the same

side of the sample as the objective (Fig. 4.12.3C), the choice is an air objective (preferably with a high NA). The choice of objective for through-the-objective TIR-FM setup is crucial. This can be achieved reliably only with oil-immersion objectives having an NA of 1.45 or larger.

As can be deduced from Equation 4.12.1, for an objective lens with refractive index of 1.515 and a NA of 1.40, the maximum angle at which the light can be incident on the sample is [$\sin^{-1}(1.4/1.515) = 67.53°$]. For viewing live cells using an objective with n = 1.515, the critical angle is 65.63° (Table 4.12.1; Fig. 4.12.4B). This leaves a very small region of the objective that can be used to obtain TIR. Because the refractive index of the cell is not uniform along all of its surface, and because even a small extent of scattering of excitation light could lead to a loss of total internal reflection, use of objectives with NA of 1.4 or lower is not practical for obtaining through-the-objective TIR. Development of objective lenses with NA of 1.45 by Olympus, Nikon, and Zeiss, and with NA of 1.65 by Olympus, has made it possible to reliably obtain TIR through the objective (Table 4.12.1).

The 1.65 NA objectives: In contrast to all other objectives that make use of glass with a refractive index of 1.515, the 1.65 NA Apo 100× objective by Olympus uses glass with a refractive index of 1.788. The higher refractive index of the glass, together with the high numerical aperture of the objective, has a few consequences for TIR microscopy. First, there is a relatively wide range of angles over which TIR can be achieved (Terakawa et al., 1997; Kawano et al., 2000; Table 4.12.1, Fig. 4.12.4B). Second, the higher refractive index makes the TIR field much less sensitive to variations in the refractive index of the cell, resulting in a uniform evanescent field all along the cell surface. At an angle of 63° (typical for this objective) the space constant ranges from 44 nm (n_2 = 1.33) to 48 nm (n_2 = 1.37). As the angle is varied for n_2 = 1.33, the space constant for this field ranges from 49 nm (for 60°) to 42 nm (for 66°). The main disadvantages of this objective are the requirement for a special high-refractive-index immersion liquid (n = 1.78) and high-refractive-index coverslips (n = 1.788). The currently available immersion liquid is volatile and slightly toxic, leaving a crystalline remnant on the objective after a few hours in a heated environment. The high-refractive-index cover slips supplied by Olympus (composed of Ohara glass LAH64, thickness 0.150 mm) are very expensive, fragile, and acid- and base-labile, and hence difficult to reuse. An acid-resistant alternative for this coverslip is the Schott-glass type SF11, available in 0.150 mm thickness for one-third the price of the Olympus coverslips from VA Optical (see *SUPPLIERS APPENDIX*). However, SF11 is slightly more autofluorescent than LAH64 and causes higher chromatic error. It is therefore only usable for bright and single-color fluorescent samples.

The 1.45 NA objectives: Most of the problems associated with the 1.65 NA objective are overcome by the recent introduction of 60× 1.45 NA objective (Olympus; Nikon) and the 100× 1.45 NA objective (Zeiss; Nikon), which use regular immersion oil and coverslips. The decay constant of the evanescent field for the 60× 1.45 NA objective is also larger (d = 90 to 200 nm) than for the 100× 1.65 NA objective (d = 50 to 100 nm). The range of incident angles for TIR is smaller for the 1.45 NA objectives compared to the 1.65 NA objective (Table 4.12.1). Also, since the 1.45 NA objective uses standard glass with a refractive index of 1.515, the decay distance is more sensitive to local variations in the refractive index of the cell (Table 4.12.1).

Immersion oil

For the objective-type TIR-FM setup it is crucial that the immersion oil being used is refractive index matched with the objective and the cover glass. While use of low autofluorescence immersion oil is desired, most commercially available oil do not have refractive indices suitable for use with the available high NA objectives. Thus the refractive index of the immersion oil should be carefully examined before its use in TIR-FM.

Filters and mirrors

Special excitation and emission filters and dichoric mirrors need to be used with the TIR setup. Due to the higher intensity of the laser light sources, instead of the conventional excitation filters (which are appropriate for blocking light from an arc lamp), excitation filters with higher optical density (>4) should be used. The emission filters must also be carefully chosen so that they are blocked for light outside of the desired wavelength range. Also, the higher laser intensity necessitates use of di-

Table 4.12.1 Total Reflection Angles and Maximum Angles for Objective Lenses

NA of objective	n_1	n_2	Critical angle (θ_c)	Maximum angle of incidence (θ)	Least depth of penetration, d (nm)
1.40	1.515	1.38	65.63° (NA = 1.38)	67.53° (NA = 1.40)	168
1.45	1.515	1.38	65.63° (NA = 1.38)	73.15° (NA = 1.45)	89
1.65	1.788	1.38	50.51° (NA = 1.38)	67.34° (NA = 1.65)	55

chroic mirrors with better antireflective coating, so as to minimize the reflection off the back surface of the mirror. These measures are necessary because leakage of even small amounts of scattered excitation light of different wavelength or of light reflected from the back surface of the dichroic mirror (hence traveling at an angle different from that of the primary beam) would decrease the signal-to-noise ratio.

Troubleshooting

Beam path

If total internal reflection cannot be achieved, make sure that the beam path is not obstructed and that it is still being targeted to the sample. Follow the steps in the Basic and Alternate Protocols to achieve TIR and confirm the existence of TIR using the fluorescent beads. An alternative approach to confirm the existence of TIR involves the use of two dyes, e.g., Fluorescein (green) and Dil (red). A procedure for using these dyes to test for the TIR is as follows.

1. Treat the surface of the cover glass for 10 min with a solution of DiI dissolved at 0.5 mg/ml in ethanol.
2. Rinse off the coverslip with water and add a 0.1mg/ml solution of carboxyfluorescein.
3. Illuminate the sample with 488 nm epifluorescent light. This should lead to both red and green emission.
4. Now switch to TIR illumination. If the TIR setup is not optimal, fluorescence emission of both the dyes will still be visible. Adjust the angle of the incident beam to the point where most of the emission is red, while the green emission is minimal. By shifting the excitatory beam back and forth, one can identify the critical point at which the emission sharply shifts from green (epi) to red (TIR); this will be the point at which the beam is incident on the sample at the critical angle.

Image homogeneity

Often the TIR-FM image of cells that appear homogeneous in epi-illumination appear heterogeneous in TIR illumination. This heterogeneity could be due to dirt and defects on the surface of the coverslip or to the fact that the cells have not adhered well enough to the coverslip. Either of these factors would cause the cell to be illuminated nonuniformly all along the bottom surface of the cell. Since TIR excitation decays exponentially with distance from the coverslip, even slight undulations in the cell membrane due to improper adherence or due to dirt and defects on the coverslip could cause illumination artifacts. To ascertain whether the heterogeneity is due to the illumination artifact of TIR, label the cell membrane using a membrane-specific dye (e.g., DiI) and observe using TIR. If the cell is not attached uniformly to the glass surface, then the fluorescence will be nonuniform. This problem can be remedied by coating the surface of the coverslip with suitable matrix molecules, such as fibronectin or collagen, that will promote better adherence of cells to the coverslip surface. However, cells that do not adhere well to the coverslips might not be suitable for carrying out TIR-FM.

Anticipated Results

As with any form of optical sectioning microscopy, TIR-FM minimizes the out-of-focus fluorescence, and hence it has a high signal-to-noise ratio for the images. This is essential for imaging fluorescently tagged molecules that are present throughout the cell but that carry out their function only in a very narrow region near the cell membrane. For example, actin bundles present near the cell surface are those that are involved in cell adhesion and migration. While wide-field fluorescence imaging of cells expressing fluorescently tagged actin collects fluorescence from actin both near the cell sur-

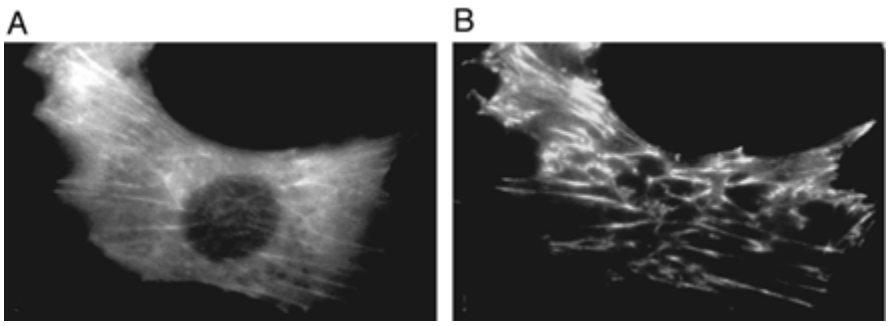


Figure 4.12.5 Imaging the distribution of GFP-labeled actin in live cells. Basal surface of normal rabbit kidney (NRK) cells stably expressing the actin-EGFP was imaged using (**A**) epifluorescence illumination and (**B**) TIR-FM illumination. While the fluorescence of G actin from the entire cell body makes it difficult to resolve the presence of the F actin bundles using epifluorescence, TIR-FM allows visualizing these bundles by illuminating only a narrow region of the cell adjacent to the membrane-coverslip interface.

face and deeper inside the cell, TIR-FM allows imaging of only the actin near the cell surface (Fig. 4.12.5). This allows visualization of changes in actin dynamics as the cells attach and migrate on the coverslip.

Time Considerations

The sample preparation procedure for TIR-FM is just the same as for conventional fluorescence imaging. Hence, once the microscope is set up for operating in the TIR-FM mode, the only additional time needed for carrying out TIR-FM imaging is that involved in ensuring that the TIR beam is properly aligned (<15 min).

Literature Cited

Amann, K.J. and Pollard, T.D. 2001. Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 98:15009-15013.

Axelrod, D. 1981. Cell-substrate contacts illuminated by total internal reflection fluorescence. *J. Cell Biol.* 89:141-145.

Axelrod, D. 1983. Lateral motion of membrane proteins and biological function. *J. Membrane Biol.* 75:1-10.

Axelrod, D. 1989. Total internal reflection fluorescence microscopy. *Methods Cell. Biol.* 30:245-270.

Axelrod, D. 2001. Selective imaging of surface fluorescence with very high aperture microscope objectives. *J. Biomed. Opt.* 6:6-13.

Axelrod, D., Bauer, H.C., Stya, M., and Christian, C.N. 1981. A factor from neurons induces partial immobilization of nonclustered acetylcholine receptors on cultured muscle cells. *J. Cell Biol.* 88:459-462.

Gingell, D. 1981. The interpretation of interference-reflection images of spread cells: Significant contributions from thin peripheral cytoplasm. *J. Cell Sci.* 49:237-247.

Greenslade, T.B. Jr. 1997. The Liquid Vein: Nineteenth Century Illustrations (LVII). *Phys. Teach.* 35:207.

Kawano, Y., Abe, C., Kaneda, T., Aono, Y., Abe, K., Tamura, K., and Terakawa, S. 2000. High numerical aperature objective lenses and optical system improved objective type total internal reflection fluorescence microscopy. *Proceedings SPIE* 4098:142-151.

Pepper, J.H. 1860. The boy's playbook of science: Including the various manipulations and arrangements of chemical and philosophical apparatus required for the successful performance of scientific experiments. *In* Illustration of the Elementary Branches of Chemistry and Natural Philosophy. Routledge, Warne, and Routledge, London.

Schmoranzer, J., Goulian, M., Axelrod, D., and Simon, S.M. 2000. Imaging constitutive exocytosis with total internal reflection fluorescence microscopy. *J. Cell Biol.* 149:23-32.

Steyer, J.A. and Almers, W. 2001. A real-time view of life within 100 nm of the plasma membrane. *Nat. Rev. Mol. Cell Biol.* 2:268-275.

Sund, S.E. and Axelrod, D. 2000. Actin dynamics at the living cell submembrane imaged by total internal reflection fluorescence photobleaching. *Biophys. J.* 79:1655-1669.

Terakawa, S., Sakurai, T., and Abe, K. 1997. Development of an objective lens with a high numerical aperature for light microscopy. *Bioimages* 5:24.

Toomre, D. and Manstein, D.J. 2001. Lighting up the cell surface with evanescent wave microscopy. *Trends Cell Biol.* 11:298-303.

Wagner, M.L. and Tamm, L.K. 2000. Tethered polymer-supported planar lipid bilayers for reconstitution of integral membrane proteins: Silane-polyethyleneglycol-lipid as a cushion and covalent linker. *Biophys. J.* 79:1400-1414.

Contributed by Jyoti K. Jaiswal and
Sanford M. Simon
Rockefeller University
New York, New York

Fluorescent Labeling of Yeast

Fluorescence microscopy techniques for yeast cells are fairly similar to those used with mammalian tissue culture cells. As with mammalian cells, fluorescent dyes can be used to identify organelles, GFP fusion proteins can be used to track the localization and dynamics of a specific protein, and membrane trafficking pathways can be evaluated using pulse-chase methods with fluorescent markers. This unit is designed to present a range of fluorescent techniques commonly used for the baker's yeast *Saccharomyces cerevisiae*.

One major difference between fluorescent labeling in mammalian cells versus yeast cells is size. Mammalian cells can range from 10 to 100 μm, compared to ~3 to 5 μm for a yeast cell, making identification of some subcellular structures and clear colocalization determinations somewhat challenging. Using larger, diploid cells for imaging can sometimes help with this problem. In addition, newer confocal microscopes (*UNIT 4.5*) have been designed with more precise control over pinhole size and *z*-axis movements, allowing slices as thin as 0.4 μm, which is thin enough to image multiple planes inside a yeast cell. For reference, most publications involving yeast cell light microscopy use a range of confocal, deconvolution (McNally et al., 1999) and standard fluorescence microscopy and are presented at ~1000× magnification.

The focus of this unit is fluorescence labeling for localization of a specific protein as well as techniques for tracing the movement of molecules through the endocytic pathway. The protocols begin by describing a few of the basic techniques commonly used to label the vacuole (see Basic Protocol 1 and Alternate Protocol 1), which is the degradative compartment of a yeast cell (equivalent to the lysosome of mammalian cells) and is among the more easily identified organelles in yeast. Following this method are assays of the endocytic pathway, which generally leads to delivery to the vacuole, using fluorescent labels (see Basic Protocols 2 and 3, and Alternate Protocol 2). The unit also describes both live and fixed cell imaging techniques for specific proteins as well as techniques to label the filamentous actin cytoskeleton in yeast cells (see Basic Protocols 4 to 6, and Alternate Protocols 3 and 4). Finally, the authors have included a simple technique for identifying budding patterns in haploid and diploid yeast cells (see Basic Protocol 7).

This is by no means a comprehensive list of the methods available for fluorescent techniques in yeast cells. In this unit, the authors have tried to focus on methods that have been tested repeatedly in their laboratory to provide as much insight as possible. The internet is a great resource for additional and alternate yeast protocols that laboratories are willing to share. Entering "yeast protocol" in most search engine queries will provide numerous options. In addition, methods describing flow cytometry/cell sorting of fluorescently labeled yeast (Vida and Wendland, 2002), as well as vital fluorescent dyes to label mitochondria and other structures (Erdmann and Gould, 2002; Nunnari et al., 2002) are also available. The Molecular Probes catalog is another great resource for many useful reagents.

STRATEGIC PLANNING

For each of the protocols, the first step requires the ability to prepare growing yeast cells in medium. See *UNIT 1.6* for a description of liquid culture growth protocols for yeast cells (also see *UNIT 3.8*). Unless otherwise stated, either rich or selective/minimal medium is acceptable. Measure the concentration of yeast cells by an OD_{600} reading in a spectrophotometer expressed as an OD_{600} per milliliter culture. For *most* of the protocols (i.e., unless specifically stated otherwise), it is acceptable to dilute cells back from a semisatu-

Contributed by J.J. Baggett, J.D. Shaw, C.J. Sciambi, H.A. Watson, and B. Wendland
Current Protocols in Cell Biology (2003) 4.13.1-4.13.28

rated/saturated culture in the morning as long as the cells double in concentration at least once (after the dilution in the morning) before beginning the assay.

When pelleting yeast cells, it is important to centrifuge at low speeds to preserve the shape and structure of the yeast cells and organelles. For small volumes (≤1.5 ml), use a microcentrifuge and pellet cells at 300 to 800 × *g* for 1 to 5 min. For larger volumes, use a clinical centrifuge and 15- to 50-ml tubes, but pellet cells for 5 to 10 min at 300 to 800 × *g*. If the cells are deformed in any way, decrease the centrifugation speeds. Also, cells in minimal/synthetic medium often do not pellet as well as cells in rich medium (e.g., YEPD). To improve recovery, it may be necessary to pellet the cells, remove ~80% to 90% of the medium, and then centrifuge again before removing the remaining medium. As noted in each protocol, safe centrifugation speeds are normally in the range of 300 to 800 × *g* for live yeast cells.

Most of the methods prescribe the use of concanavalin A (con A)–coated coverslips (22 × 22–mm) to help immobilize yeast cells. The production of con A–coated coverslips is described in Reagents and Solutions.

Yeast strains that are *ade2* mutants (grow as pink or red colonies) accumulate a fluorescent intermediate in the vacuole. If an ADE2$^+$ strain cannot be used, it will be necessary to take special measures to reduce or prevent accumulation of this interfering signal. To do so, add adenine (from a concentrated stock) to a final concentration that is five times normal (i.e., 0.1 mg/ml) to the medium at all times (even to rich/YEPD medium), including the overnight growth medium.

BASIC PROTOCOL 1

LABELING THE VACUOLE IN LIVE CELLS WITH CDCFDA

5(6)-Carboxy-2′-7′-dichlorofluorescein diacetate (CDCFDA, Molecular Probes) is a vacuole-specific vital dye. Briefly, when incubated with cells, it passively diffuses through membranes because of its neutral state. When the dye reaches the vacuole, esterases cleave CDCFDA to form a charged, fluorescent molecule. The charge prevents subsequent diffusion of the fluorescent label from the vacuole. Cells can be viewed by fluorescence microscopy under a FITC filter.

Materials

Yeast cells (see Strategic Planning)
0.5 M sodium citrate, pH 4.0 (see recipe)
10 mM 5(6)-carboxy-2′-7′-dichlorofluorescein diacetate (CDCFDA; Molecular Probes) in anhydrous DMSO (store in the dark for 3 months to 1 year at −20°C)
PBS (pH 7.4)/2% (w/v) glucose (see recipe)

Shaking incubator set to appropriate growth temperature
Con A–coated coverslips (see recipe)
FITC filter

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

1. Culture yeast cells overnight to log phase (~0.3 to 1.0 OD_{600}/ml; *UNIT 1.6*) and transfer 900 µl into a room temperature 1.5-ml microcentrifuge tube.
2. Add 100 µl of 0.5 M sodium citrate, pH 4.0, and 1 µl of 10 mM CDCFDA in anhydrous DMSO.
3. Incubate 15 min at growth temperature with shaking.
4. Centrifuge gently 3 to 5 min at 300 × *g*, room temperature. Aspirate supernatant.

5. Resuspend in 100 µl PBS (pH 7.4)/2% (w/v) glucose.
6. Mount 2 to 3 ml onto con A–coated coverslips and observe using a FITC filter (*UNIT 4.2*).

LABELING ACIDIC ORGANELLES WITH QUINACRINE IN LIVE CELLS

ALTERNATE PROTOCOL 1

6-Chloro-9(4-diethylamino-1-methylbutylamino)-2-methoxyacridine dihydrochloride (quinacrine) is a vital dye that can be used to label acidic compartments in cells (i.e., it gives more general information about the state and function of the vacuole). The unprotonated form can diffuse across cell membranes, allowing labeling of intact live cells. Upon encountering an acidic environment, the molecule becomes protonated, restricting its ability to diffuse across membranes and enhancing its fluorescent signal, making quinacrine an effective tool for specific labeling of acidic organelles. When the dye is used in excess, the quinacrine signal is quenched, so if the fluorescent signal is low, try decreasing the amount of quinacrine used as well as increasing it. The labeled cells are viewed by fluorescence microscopy under a FITC filter.

Additional Materials *(also see Basic Protocol 1)*

Rich medium (*UNIT 1.6*)
1 M HEPES, pH 7.6: adjust to pH 7.6 with KOH (store up to 1 year at room temperature)
20 µM quinacrine (Sigma) in 1 M HEPES, pH 7.6 (store in the dark for 3 months to 1 year at −20°C)
100 mM HEPES (pH 7.6)/2% (w/v) glucose (see recipe), ice cold

1. Culture yeast cells overnight to log phase (0.3 to 1.0 OD_{600}/ml; *UNIT 1.6*). Transfer 1 ml cells to a 1.5-ml microcentrifuge tube and centrifuge 3 to 5 min at 300 × *g*, room temperature. Aspirate medium and cool pellets on ice (~ 2 to 5 min).
2. Resuspend in 0.9 ml rich medium. Add 100 µl of 1 M HEPES, pH 7.6, and 10 µl of 20 µM quinacrine in 1 M HEPES, pH 7.6.
3. Incubate 5 min at growth temperature with shaking.
4. Gently pellet cells by centrifuging 3 to 5 min at 300 × *g*, room temperature. Remove medium and cool pellets on ice (~ 2 to 5 min).
5. Resuspend in 50 µl ice-cold 100 mM HEPES (pH 7.6)/2% (w/v) glucose. Keep on ice.
6. Mount 2 to 3 ml onto con A–coated coverslips and observe using a FITC filter (*UNIT 4.2*).

The quinacrine signal is often faint, so be prepared to take images for up to 10 sec.

LABELING THE ENDOCYTIC PATHWAY IN LIVE CELLS WITH FM4-64

BASIC PROTOCOL 2

N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64; Molecular Probes), is an amphipathic fluorescent molecule that inserts into lipid membranes, fluorescing only when in this hydrophobic environment. Its charged polar head prevents passive diffusion through membranes, thus it labels only the exoplasmic leaflet of the bilayer. This property makes FM4-64 an excellent marker of the endocytic pathway. Briefly, cells are labeled with dye for a short pulse, then uninternalized dye, including that inserted into the plasma membrane, is washed away. The dye is then chased in live cells for 40 to 60 min, allowing it to reach a steady state localization. In wild-type cells, the FM4-64 accumulates at the vacuolar membrane. The total amount of cell-associated fluorescence represents the approximate rate of internalization over the pulse time, such that differences in intensity can reflect differences in internalization rates.

If cells labeled with this method exhibit an altered fluorescent pattern, this suggests a block or kinetic delay in transit through the intermediate compartments of the endocytic pathway. Cells are viewed by fluorescence microscopy using a rhodamine/TRITC filter.

Materials

Yeast cells (see Strategic Planning)
Rich medium (*UNIT 1.6*) at growth temperature
FM4-64 (see recipe)

Heat block at assay temperature
Water bath with shaker set at assay temperature
Con A–coated slides (see recipe)
Rhodamine/TRITC filter

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

1. Culture yeast cells overnight to log phase (~0.3 to 1.0 OD_{600}/ml *UNIT 1.6*). If cells are not already in rich medium, transfer them into rich medium at growth temperature by centrifuging 3 to 5 min at ~300 to 800 × *g*, room temperature, pouring off the supernatant, and resuspending in rich medium.

2. *Optional:* If the assay temperature is different than the growth temperature (e.g., when testing a temperature sensitive mutant at its restrictive temperature), shift cells to the assay temperature for 10 min to 1 hr.

   From this point on, keep all medium and cells at the assay temperature as much as possible (use a heat block at assay temperature to hold microcentrifuge tubes while aspirating and washing).

3. Transfer 1 ml cells to a 1.5-ml microcentrifuge tube and centrifuge gently 0.5 to 5 min at 300 × *g*, room temperature. Aspirate medium and add 50 µl FM4-64.

   Using shorter centrifugation times to pellet the cells will reduce the time they are not at the assay temperature.

4. Incubate cells 15 to 20 min at growth temperature with gentle shaking.

5. Add 1 ml prewarmed medium to each tube, then centrifuge gently as described in step 3. Remove supernatant and add 1 ml prewarmed medium. Repeat this wash two more times (for a total of three), incubating cells in medium for 15 to 60 sec between centrifugations.

   These wash steps are necessary to extract uninternalized dye from the exoplasmic leaflet of the plasma membrane. FM4-64 has an off rate of ~2 sec.

6. Incubate 30 to 60 min in shaking water bath at assay temperature.

   Use longer chase times for lower assay temperatures.

7. Pellet cells gently as described in step 3 and aspirate medium. Gently resuspend pellets in 50 to 100 µl medium. If testing a temperature sensitive (ts) allele, store samples on ice until mounted on coverslips.

   Storage on ice will prevent further trafficking of the dye in the nonrestrictive environment, particularly in the case of ts alleles that recover at permissive temperature.

8. Mount 2 to 3 µl onto con A–coated coverslips and observe immediately under a rhodamine/TRITC filter (*UNIT 4.2*).

INTERNALIZATION TIME-COURSE ASSAY USING FM4-64

ALTERNATE PROTOCOL 2

In order to better evaluate the location of a block or decreases in the rate of endocytosis through endosomes, a time-course assay can be used. Briefly, cells are labeled on ice rather than at growth temperatures, washed quickly to remove uninternalized dye from the medium while leaving the plasma membrane labeling in place, and then allowed to internalize dye for 0 to 20 min (or longer) in a chase at assay temperatures. Cells are collected and kept cold until placed on coverslips for viewing. See Figure 4.13.1 for an example of wild-type endocytosis patterns over 0 to 20 min chase time.

Additional Materials *(also see Basic Protocol 2)*

FM4-64 (see recipe), ice cold
Rich medium with and without a carbon source (*UNIT 1.6*), ice cold

1. Culture yeast cells and incubate at restrictive temperatures (if appropriate) as described (see Basic Protocol 2, steps 1 and 2). Gently pellet four or more separate 1-ml aliquots of each strain in microcentrifuge tubes (see Basic Protocol 2, step 1), aspirate, and cool the pellets on ice. Resuspend each tube in 50 µl FM4-64, prechilled on ice.

   Use a 1:50 dilution of stock FM4-64 (see recipe).

2. Label the plasma membrane by incubating tubes 15 to 20 min in an ice bath with sufficient water to make a slush.

3. To one tube from each strain (zero time point), add 1 ml ice-cold rich medium without a carbon source and centrifuge 3 min at ~300 × *g*, 4°C. Aspirate and repeat this wash once more. Resuspend pellets in 50 to 100 µl rich medium with no carbon source. Keep these cells on ice at all times from this point forward.

4. Wash the remaining three (or more) tubes of each sample two times with cold rich medium containing a carbon source as described in step 3, but do not incubate between washes. Resuspend in 1 ml rich medium containing a carbon source.

   Do not wash more than this, otherwise it is possible to lose too much plasma membrane labeling.

5. Place the tubes into a water bath at assay temperature with shaking. After 5 min, remove 1 tube for each sample and wash cells twice with ice-cold rich medium without a carbon source. Resuspend as in step 3 (keep as the 5 min time point).

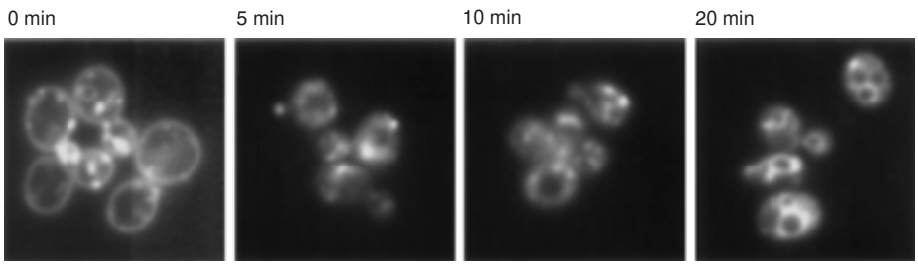


Figure 4.13.1 Wild-type behavior in an FM4-64 time-course assay. Cells were treated as described (see Alternate Protocol 2) with an assay temperature of 30°C and imaged using a Zeiss fluorescence microscope and 100× objective with a rhodamine/TRITC filter. Shown are wild-type images from time points 0, 5, 10, and 20 min. Note that at the zero time point, labeling is almost entirely on the plasma membrane, and by the 20 min time point, the label is mostly on the vacuolar membranes (one to five circular organelles that are ~1/5 to 1/2 of the yeast cells).

Continue to incubate remaining tubes, collecting time points as described at 10, 15, and 20 min.

Longer time points can be collected as well by starting with more tubes for each sample in step 1 and continuing the assay. For lower assay temperatures, it may be necessary to collect chase time points for as long as 90 min.

6. Mount 2 to 3 µl onto con A–coated coverslips and observe with a rhodamine/TRITC filter (*UNIT 4.2*).

BASIC PROTOCOL 3

ENDOCYTIC RATE MEASUREMENT BY LUCIFER YELLOW UPTAKE IN LIVE CELLS

Lucifer yellow (LY) is a fluorescent fluid-phase dye that does not pass through membranes. It is taken into cells by endocytosis of bulk fluid-phase material from the extracellular space. It is delivered to the vacuole, where its accumulation is an indicator of general rates of internalization. This technique requires a few tricks, so on the first attempt be sure to follow the method and troubleshooting notes carefully. Note that it is unclear whether LY is completely stable for long-term storage. If the experiment doesn't work, try using a fresh batch of LY.

Materials

Yeast cells (see Strategic Planning)
Culture medium, preferably rich (*UNIT 1.6*)
Rich medium (*UNIT 1.6*)
40 mg/ml lucifer yellow (see recipe)
Phosphate/azide/fluoride buffer (see recipe), ice cold

16-G needle
Incubator with orbital shaker set at assay temperature
Con A–coated coverslips (see recipe)
FITC filter

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

1. Culture yeast cells in culture medium (preferably rich) overnight to 1×10^7 cells/ml (~0.1 OD_{600}/ml depending on strain) at growth temperature (*UNIT 1.6*).

   The overnight culture should be sufficiently dilute such that the density never exceeds this value—i.e., if the cells grow to a density greater than ~0.1 OD_{600}/ml, it is recommended that a new culture be started for this assay. The cells should not be diluted to a lower density and regrown until they have doubled.

   For some strains, it is very important that the cells have never exceeded this density in liquid culture (see Critical Parameters); however, some labs have reported using cells that are ~0.5 OD_{600}/ml with success. The variability may be dependent on strain and LY batch/lot. The authors have found that SEY6210 does not work at all for LY-uptake experiments; however, the Research Genetics wild-type strain BY4741 has worked very well in the authors' experience.

2. Gently pellet 1 ml cells by centrifuging 2 min at 300 to 800 × *g*, room temperature. Aspirate the medium.

3. Resuspend cells in 90 µl rich medium and add 10 µl of 40 mg/ml lucifer yellow. Mix well by pipetting carefully. Do not vortex.

4. Close the tube and poke a hole in the lid with a 16-G needle to allow gas exchange.

   Gas exchange is very important.

5. Incubate tubes at growth or assay temperature with orbital mixing for 1.5 to 2 hr in the dark (e.g., covered with aluminum foil). Try to use an air incubator to avoid contamination of the sample through the hole in the lid.

   It is important that cells be continuously aerated during this process through the hole in the lid. Also, mixing the tubes by snapping or flicking the tube periodically may help maintain proper aeration.

6. Add 1 ml ice-cold phosphate/azide/fluoride buffer and centrifuge as in step 2. Repeat this wash three times, resuspending the pellet between washes.

   If cells are not forming tight pellets, remove only 80% to 90% of the medium from the pellet, then centrifuge again and remove the remaining medium for each wash.

7. Resuspend the final pellet in 30 µl phosphate/azide/fluoride buffer and keep cells on ice.

8. Mount cells on con A–coated coverslips and view by fluorescence microscopy using a FITC filter (*UNIT 4.2*).

   LY is stable for up to 4 hr in cells if kept cold, wet, and dark, so it is best to store cells in the ventilated tubes used for the assay on slushy ice that is kept covered. The signal may be faint, so be prepared to take images for up to 10 sec under the FITC filter.

   Very bright vacuoles indicate cells that are dead or are about to die. This accumulation is due to a transporter in the vacuolar membrane that concentrates LY by a nonendocytic mechanism after the plasma membrane has become permeable.

LOCALIZATION OF PROTEINS IN LIVE YEAST CELLS WITH GFP-FUSION PROTEINS

BASIC PROTOCOL 4

A common technique for in vivo localization of a specific protein in any cell type is to create a chimera between the protein and the fluorescent protein GFP, or any of its variants. Described below is a method for viewing these fusion proteins in live yeast cells. This method was developed for use of fusion proteins with EGFP (Tsien, 1998), so keep in mind that use of different mutant forms of GFP may have slight differences in pretreatment of cells. In this assay, Tris buffer is added and incubated with cells briefly to achieve a slightly basic pH, which increases fluorescence of EGFP. In addition to this protocol, an alternate method (see Alternate Protocol 3) is described for viewing GFP fusion proteins in cells labeled with a rhodamine-phalloidin-labeled actin cytoskeleton.

Materials

Yeast cells expressing GFP-protein chimeras (*APPENDIX 3A*; also see Strategic Planning)
1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
PBS (pH 7.4)/2% (w/v) glucose (see recipe)

Incubator set to appropriate growth temperature
Incubator set to assay temperature
Con A–coated coverslips (see recipe)
FITC filter

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

1. Culture yeast cells expressing GFP-protein chimeras overnight to mid-log phase (~0.3 to 0.8 OD_{600}/ml) at growth temperature (*UNIT 1.6*). If doing a temperature shift assay, incubate (i.e., shift) cells 15 to 60 min at assay temperature.

   Use selective medium if the fusion protein is carried on a plasmid.

2. Add 1 M Tris·Cl, pH 7.5, to cultures to a final concentration of 10 mM.
3. Incubate cells ~5 min at assay temperature with shaking.
4. Centrifuge 1 ml cells in a 1.5-ml microcentrifuge tube 3 to 5 min at 300 to 800 × *g*, room temperature. Aspirate medium.
5. Resuspend cells in 50 to 200 µl PBS (pH 7.4)/2% (w/v) glucose.
6. Mount cells on con A–coated coverslips and view by fluorescence microscopy under a FITC filter (*UNIT 4.2*).

BASIC PROTOCOL 5

LABELING THE ACTIN CYTOSKELETON WITH RHODAMINE-PHALLOIDIN IN FIXED CELLS

The filamentous actin cytoskeletal network in yeast cells displays a dynamic polarized pattern throughout the cell cycle (Amberg, 1998). In cells about to bud and in small budded cells, filamentous actin cortical patches are polarized to the site of the newly emerging or emerged bud. Cytoplasmic filamentous actin cables are aligned along the mother-bud axis. As the yeast cell switches from polarized to isotropic growth, the actin patches and cables reorient throughout the mother and bud. At cytokinesis, the actin patches recongregate to the mother-bud neck and the cables orient in both mother and bud towards the neck as well. Mushroom toxins, such as phalloidin, bind filamentous actin with high specificity. Attachment of fluorescent tags, such as rhodamine, to phalloidin allows the use of this toxin as a label in yeast cells for the filamentous actin cytoskeleton. Briefly, cells are fixed in mid-log phase to ensure a mixed population of early budded, late budded, and cytokinesing cells. The cells are then permeabilized and incubated with toxin. DAPI (Molecular Probes) is included in the toxin incubation to stain nucleic acids. This assists in determining cell cycle stage (see Lew and Reed, 1995). Cells are washed to remove unbound dye and viewed under fluorescence microscopy with rhodamine/TRITC and UV/DAPI filters. See Figure 4.13.2 for an example of F-actin patterns in early budded cells as well as those undergoing cytokinesis. In addition, see Figure 4.13.3 for the depolarized, chunky F-actin pattern in mutant cells.

Materials

Yeast cells (see Strategic Planning)
Rich or selective medium (*UNIT 1.6*)
In-medium fixation cocktail (see recipe)
Overnight fixation cocktail (see recipe)
PBS, pH 7.4 (see recipe)
0.2% (v/v) Triton X-100 in SHA buffer (see recipe)
PBS containing phalloidin and DAPI (see recipe)
DABCO antifade (see recipe)

Water bath with shaker set at assay temperature
End-over-end rotator
Con A–coated coverslips (see recipe)
Rhodamine/TRITC and UV/DAPI filters

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

Prepare cells

1. Incubate 10 ml yeast cell cultures overnight to early- to mid-log phase (0.3 to 0.7 OD_{600}/ml) in rich or selective medium (*UNIT 1.6*).

Try to keep the final concentration (step 3) below 0.7 OD_{600}/ml.

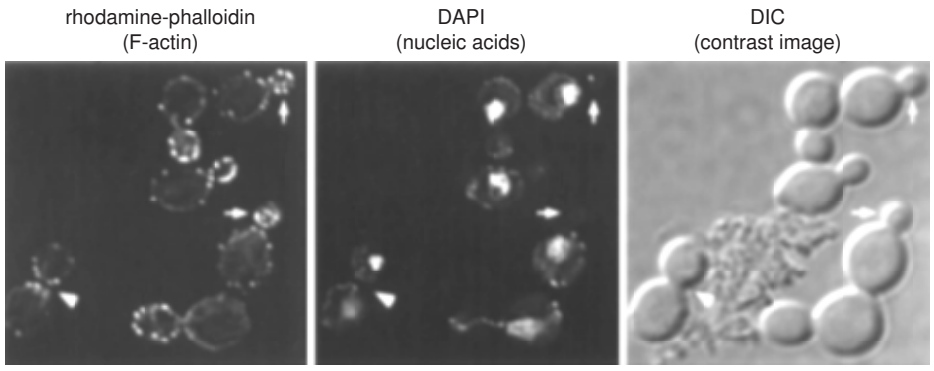


Figure 4.13.2 Filamentous actin in fixed wild-type yeast cells. Cells were treated as described (see Basic Protocol 5) with an assay temperature of 37°C and a temperature shift to 37°C 90 min before fixing. Cells were imaged using a Zeiss fluorescence microscope and 100× objective with a rhodamine/TRITC filter, DAPI optics, and differential interference contrast (DIC). The F-actin and nucleic acid images were deconvolved using a Deltavision deconvolution microscopy program. Arrows in all three panels show polarized F-actin patches in the growing bud of yeast cells in the early bud stage. The small number of patches in the mother cell are sometimes seen but indicate a slight depolarization, which may be due to incomplete recovery from the temperature shift. Arrow heads in all three panels delineate the mother-bud neck in cells undergoing cytokinesis. The large spots in the DAPI images are nuclei, and the smaller spots are nucleoids in mitochondria.

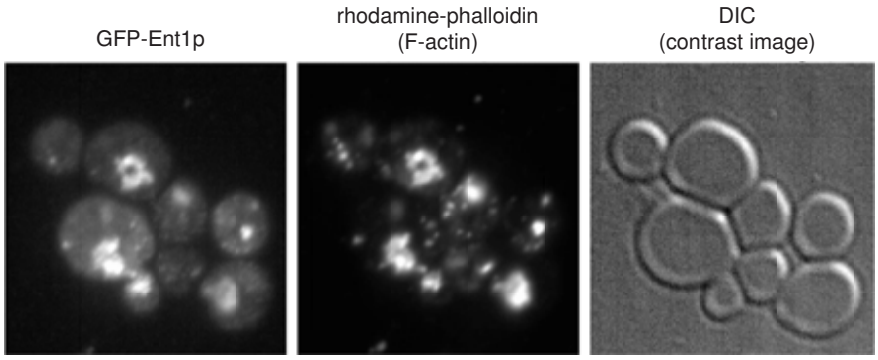


Figure 4.13.3 GFP-Ent1p and F-actin colocalization in mutant cells. Cells were treated as described (see Alternate Protocol 3) with an assay temperature of 30°C. Cells were imaged using a Zeiss fluorescence microscope and 100× objective with a FITC filter, a rhodamine/TRITC filter, and DIC. Note that the F-actin is both depolarized (not localized to growing small buds) and found in large clumps rather than small punctae. The GFP-Ent1p is localizing to these mislocalized F-actin clumps. Reprinted from Watson et al. (2001) with permission by the American Society for Cell Biology.

2. If the assay temperature is different than the growth temperature, shift the cells to the assay temperature when their concentration is in early-log phase (0.2 to 0.4 OD_{600}/ml), so that by the end of the shift (≥90 min) they will still be in the correct range.

   Even wild-type yeast cells respond to heat shock by temporarily reorganizing their actin cytoskeleton to a depolarized state. A prolonged exposure (90 min) to the elevated temperature is required to allow cells to re-establish their normal actin polarity; therefore, cells must be shifted for ≥90 min prior to fixation.

Fix cells

3. Record the final concentration (OD_{600}/ml) of each sample. Then, in a ventilated hood, add 2.5 ml in-medium fixation cocktail to each 10 ml cell culture, adding directly to the culture (i.e., do not pellet cells).

4a. *For nonshifted cells:* Place cells back into a water bath with shaker and incubate 1 to 2 hr.

4b. *For temperature-shifted cells:* Keep cells in fix at the elevated temperature for at least the first 20 min out of a total 1 to 2 hr fixation period (see step 4a).

   Once cells have begun to fix, temperature should no longer matter.

5. After at least 1 hr of fixation, prepare cells for further fixing overnight. Pellet cells (i.e., centrifuge 5 min at 1500 ×*g*, room temperature), aspirate, and resuspend in 1 ml overnight fixation cocktail per sample. Transfer to a 1.5-ml microcentrifuge tube.

6. Incubate overnight at room temperature, with agitation (end-over-end rotator), at room temperature.

7. Centrifuge 5 min at 1500 to 2000 × *g*, room temperature. Wash once by resuspending in 1 ml water (use this resuspension to transfer cells to 1.5-ml microcentrifuge tubes if they are not in them already), then pelleting cells and aspirating. Wash twice with 1 ml PBS, pH 7.4.

   Because cells are fixed, faster centrifugation (as denoted above) is okay.

Permeabilize cells

8. Resuspend cells in 1.0 ml of 0.2% (v/v) Triton X-100 in SHA buffer and incubate 10 to 15 min at room temperature.

   There is no need to agitate.

9. Wash three times in 1 ml PBS to remove detergent. Resuspend cells to 10 OD_{600}/ml in PBS (using final OD_{600}/ml from step 3). Remove 100 µl (i.e., 1 OD_{600}) to a new tube, pellet cells and aspirate liquid.

   It is important to wash out the detergent well. Remaining detergent will increase the level of background signal.

Label cells

10. Label cells by resuspending in 30 to 50 µl PBS containing rhodamine-phalloidin and DAPI. Incubate in the dark 1 hr at room temperature.

    Alternative fluorescent labels conjugated to phalloidin are available and should work with this method.

    It is also possible to label cells overnight at 4°C in the cold room. This works quite well. If this is done, wash at least two additional times.

11. Wash 3 times in 0.5 ml PBS.

    To avoid losing the pellet in this step, remove the PBS from the pellet during each wash by pipetting rather than aspirating.

12. Resuspend final pellet in 30 to 50 ml DABCO antifade. Mount 2 to 3 ml of cells on con A–coated coverslips and view with rhodamine/TRITC and UV/DAPI filters (*UNIT 4.2*).

LABELING OF LIVE CELLS WITH RHODAMINE-PHALLOIDIN FOR COLOCALIZATION WITH GFP FUSION PROTEIN

ALTERNATE PROTOCOL 3

Fixation is sometimes deleterious to GFP localization, in particular for some peripherally associated membrane proteins. The following method is used to label unfixed cells with rhodamine-phalloidin, which allows GFP localization to be maintained. Instead of fixing cells, they are simultaneously permeabilized and labeled with rhodamine-phalloidin. It is assumed that the actin cytoskeleton and associated proteins are immobilized during the process of labeling due to the ability of phalloidin to stabilize actin filaments (Cooper, 1987). With this method, GFP localization is often preserved; however, this should be confirmed by comparison to a control in the same experiment. This control should consist of nonpermeabilized live cells containing the same GFP-fusion protein but not stained with phalloidin (see Basic Protocol 4). See Figure 4.13.3 for an example of a GFP-tagged protein that colocalizes with F-actin in mutant cells.

Additional Materials *(also see Basic Protocol 5)*

Yeast cells expressing GFP-fusion constructs (*APPENDIX 3A*; also see Strategic Planning)
Permeabilization/staining solution (see recipe)
FITC and rhodamine/TRITC filters

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

1. Culture yeast cells expressing GFP-fusion constructs overnight to early- to mid-log phase (~0.3 to 0.8 OD_{600}/ml; *UNIT 1.6*).

   Use selective medium if the fusion protein is carried on a plasmid.

2. Harvest 1 to 5 ml cells (less if the OD_{600} is at the upper value). Centrifuge 5 min at 300 to 600 × *g*, room temperature. Resuspend in 50 to 100 µl permeabilization/staining solution.

3. Incubate 10 min in the dark, then place on ice an additional 10 to 15 min, also in the dark.

4. Mount 2 to 3 µl cells on con A–coated coverslips and view under FITC and rhodamine/TRITC filters (*UNIT 4.2*).

   For unknown reasons, many cells will exhibit either one fluorescent label or the other, so be patient in looking for cells with both labels. It is generally better to use the FITC channel to choose an area of cells, then switch to the rhodamine channel to check for phalloidin labeling.

BASIC PROTOCOL 6

LOCALIZATION OF A PROTEIN IN FIXED YEAST CELLS WITH IMMUNOFLUORESCENCE

Methods for the use of immunofluorescence in yeast are very similar to methods for labeling mammalian cells, although the small size of yeast cells can make detailed localization slightly more difficult. However, many organelles have characteristic localization or available marker proteins so that double-labeling experiments with a specific protein can be effective. For choosing and pretesting antibodies for immunofluorescence, refer to *UNIT 4.3* on mammalian cell immunofluorescence. In brief, yeast cells are fixed, made into spheroplasts to remove the cell wall (which is necessary for antibody access to intracellular compartments), and permeabilized before they are attached to coverslips or slides using polylysine. The cells are then blocked, labeled with primary antibody, labeled again with fluorescently conjugated secondary antibodies, and viewed using a fluorescence microscope.

Materials

Yeast cell cultures (see Strategic Planning)
1× and 5× fixation cocktail (see recipe)
SHA buffer (see recipe)
Spheroplasting solution (see recipe)
1% (v/v) Triton X-100 in SHA buffer
2 mg/ml poly-L-lysine (Sigma; store up to 3 months to 1 year at −20°C)
WT buffer (see recipe)
Primary and secondary antibodies
Fixed or spheroplasted cells from a strain with the target antigen deleted (optional)
1 mg/ml DABCO antifade (see recipe)
Nail polish

Incubators set at growth temperature and 30°C
Standard slides or slides coated with Teflon to form wells (Polysciences)
Pipet tip connected to an aspirator
Moisture chamber (see recipe)
Weight (e.g., book)
Fluorescence filters

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

NOTE: It is recommended that 1× AEBSF or similar protease inhibitors be added to all prepared solutions used in this protocol. This reduces loss of rare antigens due to activity remaining from vacuolar proteases.

Fix cells

1. Incubate 10 ml yeast cell cultures overnight to mid- to late-log phase (~0.5 to 1.0 OD_{600}/ml; *UNIT 1.6*).
2. Add 2.5 ml of 5× fixation cocktail to each 10 ml of culture. Shake for 1 to 2 hr at growth temperature.
3. Centrifuge 5 min at 300 to 800 × *g*, room temperature. Pour off medium and resuspend in 1 ml of 1× fixation cocktail. Transfer to 1.5-ml microcentrifuge tubes. Fix cells for 12 to 24 hr with gentle mixing at room temperature.
4. Pellet cells as described in step 3 and aspirate the supernatant. Resuspend in a small amount of SHA buffer for storage at 4°C (up to 1 to 2 days) or continue directly to step 6 for immediate use.

Make spheroplasts and permeablize cells

5. If cells have been stored, pellet as described in step 3. Aspirate the supernatant.

6. Resuspend in 1 ml spheroplasting solution. Incubate 30 min at 30°C (or lower temperature if assay/growth temperature was below 30°C, but increase time of incubation by 5 to 10 min for every 2°C less) flicking tubes every 10 min or so to mix.

7. Centrifuge 5 min at 300 × *g*, room temperature. Aspirate supernatant and resuspend in 0.5 ml of 1% (v/v) Triton X-100 in SHA buffer. Incubate ~10 min at room temperature.

   As an alternative, 1% (w/v) SDS can also be used to permeabilize, but since it is a harsher detergent, it may damage cells.

8. Wash twice in 1 ml SHA buffer and resuspend in SHA buffer to ~1.2 OD_{600} per 50 µl (~24 OD_{600}/ml).

Prepare for incubation on slides

9. Prepare slides by applying 10 to 20 µl of 2 mg/ml poly-L-lysine. Spread the solution with a pipet tip over the area of the slide where the cells should stick (usually a ~3- to 4-mm diameter circle). Incubate 15 min at room temperature

   There are two types of slides that can be used. The first alternative is to use standard slides. After the cells settle, the area of interest will be visible on the slide. The second alternative is to use slides precoated with Teflon to form wells (e.g., Polysciences slides). This nicely contains the liquid on the slide during incubations and doesn't put pressure on the cells when the coverslips are applied, but it can lead to unflattened cells with multiple out-of-focus layers.

10. Aspirate polylysine by placing a pipet tip connected to an aspirator at one edge of the puddle to draw off the fluid. Wash three times by dripping water over the area and aspirating as with the polylysine.

11. Add 15 µl fixed spheroplasts (step 8) dropwise as in step 10 to each slide and let settle 15 to 30 min in a moisture chamber. Aspirate excess liquid as described above (step 10).

    From now until mounting coverslips, be careful not to let the cells dry out on the slide and keep the slides inside the moisture chamber. Keep liquid on the cells at all times, aspirating just before adding a new solution.

12. Block cells by dropwise addition of 15 to 30 µl of WT buffer and incubation for at least 15 min at room temperature. Aspirate immediately before antibody treatment.

13. *Optional:* Incubate primary and secondary antibodies with fixed or spheroplasted cells from a strain with the target antigen deleted. Pellet the deletion cells out of the blocked antibody solutions (i.e., 5 min at ≥10,0000 × *g*, room temperature) and use the supernatant (which will already be diluted) for steps 14 (primary) and/or 17 (secondary).

    Try to use the same dilutions and incubation times as will be used in the assay (it is often convenient to set this up during step 12). This can help reduce background, if needed. In addition, microcentrifuging all antibodies (even those not preincubated with deletion cells) can help by removing precipitates. After diluting the antibodies in WT buffer (both primary and secondary), pellet precipitates by microcentrifuging 5 min at maximum speed, room temperature.

Incubate in primary antibody

14. Add 15 µl primary antibody diluted in WT buffer to the blocked cells on the slide. Incubate 60 to 90 min at room temperature or overnight at 4°C in the moisture chamber.

    The dilution concentration depends on the antibody but typically ranges from 1:10 to 1:500. A good rule of thumb is to use ten times the concentration used for successful immunoblotting.

15. Wash five times with 15 to 30 µl WT buffer by dripping liquid over the cells and then aspirating for each wash.

Incubate in secondary antibody

16. Add 15 µl secondary antibody diluted in WT buffer. Incubate 45 to 90 min in the dark at room temperature or overnight at 4°C in the moisture chamber.

    Longer incubations will give higher backgrounds.

    Signal can be increased by using a nonconjugated secondary antibody and a fluorescently conjugated tertiary antibody. For example, use primary antibody raised in a rabbit that recognizes the protein, followed by secondary antibody goat anti-rabbit IgG, then tertiary antibody FITC-conjugated donkey anti-goat IgG. For each antibody after the primary, follow the secondary antibody incubation times and wash conditions in this step.

17. Wash five times as described in step 15.

Mount the samples

18. Aspirate the liquid and add 3 to 5 µl of 1 mg/ml DABCO antifade to the cells. Place the coverslip over the cells on the slide (or overturn the coverslip with the cells attached onto a slide). For gentle flattening of the cells for viewing, cover the entire slide (with coverslip already present) with a folded Kimwipe (to absorb excess mounting solution) and press down firmly and evenly with a weight (e.g., a book) for 1 to 2 min.

    Paraphenylenediamine (PPD) is sometimes used for antifade, but it has been found to cleave some fluorophores off the antibody. DABCO does not cause this problem.

19. To seal the coverslip and prevent drying of cells, paint the edges of with nail polish. Let dry in the dark.

    Be careful not to use too much or the polish will seep under the coverslips and obscure the cells.

20. View cells under the appropriate fluorescent filters, which depends on the fluorophore conjugated to the final antibody (*UNIT 4.2*). To store cells for viewing on another day, keep the already sealed slides at 4°C (or −20°C) in the dark (up to 2 weeks).

ALTERNATE PROTOCOL 4

LOCALIZATION IN SPHEROPLASTED THEN FIXED CELLS WITH IMMUNOFLUORESCENCE

Although the method described above (see Basic Protocol 6; i.e., fixation followed by spheroplasting cells) generally works well, this alternative method works better for labeling some proteins. The best method may depend on the antigen or antibody, and needs to be determined empirically; however, for proteins localized to cortical actin patches, the cells need to be fixed before spheroplasting to maintain polarized localization.

Materials

Yeast cell cultures (see Strategic Planning)
Softening medium: 0.1 M Tris·Cl, pH 9.4 (*APPENDIX 2A*)/10 mM DTT (freshly prepared)
Spheroplasting medium (see recipe)
37% (v/v) formaldehyde
SHA buffer (see recipe)
SHA buffer/1% LDAO (see recipe)

Incubator set to growth temperature
30°C incubator with orbital shaker

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and permeablizing and staining cells for proteins (see Basic Protocol 6, steps 9 to 20)

NOTE: It is recommended that 1× AEBSF or similar protease inhibitors be added to all solutions prepared for this protocol. This reduces loss of rare antigens due to activity remaining from vacuolar proteases.

1. Incubate 10 ml yeast cell cultures overnight to mid- to late-log phase (~0.5 to 1.0 OD_{600}/ml).
2. Centrifuge an amount of cells equivalent to an OD_{600} of 10 for 5 min at 300 to 800 × *g*, room temperature. Aspirate and resuspend in 2 ml softening medium. Incubate 10 min at room temperature.
3. Pellet cells gently as described in step 2, aspirate, and resuspend in 2 ml spheroplasting medium. Mix by vortexing on low. Incubate 10 min with orbital shaking at 30°C.
4. To fix cells, add 37% formaldehyde to a final concentration of 4%. Incubate 1 hr at 30°C.
5. Pellet cells gently as described in step 2, aspirate, and resuspend in 1 ml SHA buffer. Transfer to a 1.5-ml microcentrifuge tube.
6. Pellet cells gently as described in step 2, aspirate, and resuspend in 0.5 ml SHA buffer/1% LDAO to permeabilize. Incubate 10 min at room temperature.
7. Wash cells twice with 1 ml SHA buffer, pelleting, aspirating, and resuspending each time.
8. Resuspend final pellet in 400 µl SHA buffer. Visualize proteins as described (see Basic Protocol 6, steps 9 to 20).

LABELING CELL WALL CHITIN DEPOSITION WITH CALCOFLUOR WHITE

BASIC PROTOCOL 7

Bud scars are enriched in the polysaccharide chitin and can be observed as rings on the cell walls of yeast. In haploid cells, the normal pattern of budding is axial (the next bud forms adjacent to the site of the previous bud), whereas in diploid cells it is bipolar (occurs from either end of the elongated cell). When cells are defective for processes such as endocytosis or for actin cytoskeletal structure, budding patterns often become random in the diploid cell, resulting in rounder, less football-shaped diploids. By labeling cells with calcofluor white (CfW), which binds irreversibly to chitin in the cell wall, improper budding can be seen as nonbipolar patterns of rings on diploid cells (e.g., bud scars are found at the equator). See Figure 4.13.4 for an example of normal and mutant diploid bud scars stained with calcofluor white.

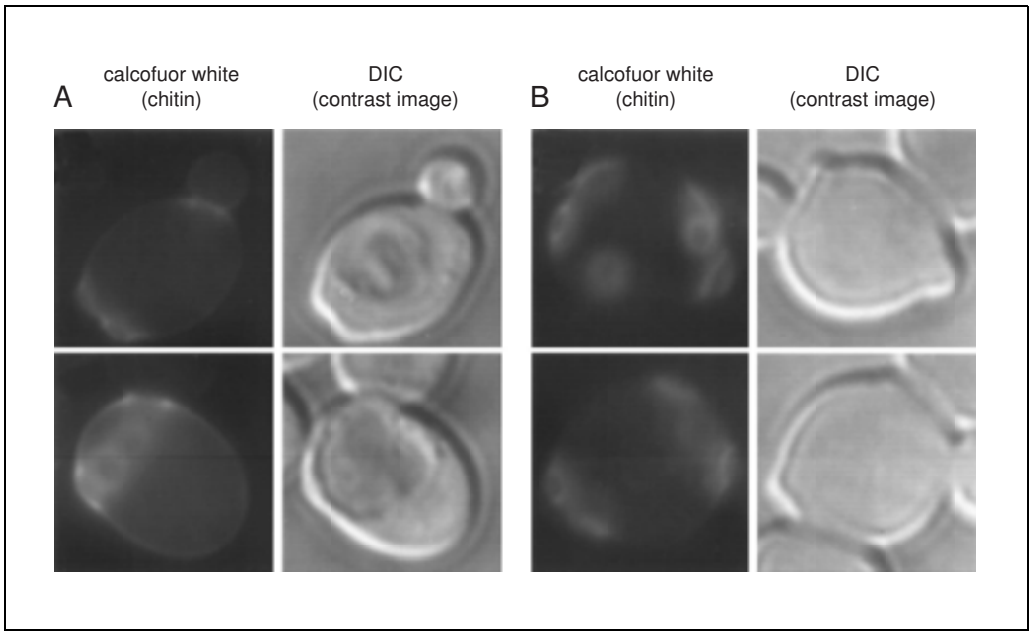


Figure 4.13.4 Bud scars in wild-type and mutant cells. Diploid cells were treated as described (see Basic Protocol 7). Cells were imaged using a Zeiss fluorescence microscope and 100× objective with DAPI optics and DIC. (**A**) Two cells exhibiting normal bud scars at each of the elongated ends of these diploid cells. (**B**) Two mutant cells, exhibiting randomly arranged bud scars. It is also clear that these diploid cells do not have a normal elongated shape.

Materials

Yeast cells (see Strategic Planning)
1 mg/ml CfW (see recipe)

Incubator set to growth temperature
Con A–coated coverslips (see recipe)
UV/DAPI filter

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

1. Culture yeast cells overnight to a high density (>1.0 OD_{600}/ml; *UNIT 1.6*).

   This increases the population of cells that are old enough to have budded multiple times.

2. Using an amount of cells equivalent to an OD_{600} of 2 to 5, centrifuge 5 min at 300 to 800 × *g*, room temperature. Aspirate medium.

3. Resuspend cells to 5 OD_{600}/ml in 1 mg/ml CfW solution.

4. Incubate 5 min at room temperature with gentle agitation.

5. Centrifuge as described in step 2 and aspirate medium. Resuspend in 1 ml water, centrifuge again, and aspirate medium. Repeat this wash twice more.

6. Resuspend final pellet in 100 to 500 µl water.

   Use more for a larger cell pellet.

7. Mount cells on con A–coated coverslips and view under a UV/DAPI filter (*UNIT 4.2*).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CfW, 1 mg/ml

Dissolve calcofluor white (CfW; Sigma) to 1 mg/ml in water. CfW takes a long time to dissolve; however, dissolution may be aided by the addition of 0.1 N sodium hydroxide dropwise with mixing until the solution clears. Store up to 3 months to 1 year in the dark at 4°C.

CfW is also known as Fluorescent Brightener 28.

Con A–coated slides

Prepare a 2 mg/ml stock solution of concanavalin A (con A; Sigma) in water (store up to 1 year at −20°C). Pipet 5 µl con A onto each 22 × 22–mm coverslip, spread with the side of a clean pipet tip, and allow to air dry in a dust-free room (if possible). Con A–coated coverslips can be made a few days in advance, but it is preferable to make them the same day of use. Store at room temperature.

Poly-L-lysine can also be used to immobilize yeast cells for microscopy (see Basic Protocol 6). Be sure to keep track of which side of the coverslip is coated.

DABCO antifade

Dissolve 1,4-diazabicyclo[2.2.2]octane (DABCO; Molecular Probes) in 90% glycerol to 1 mg/ml. Protect from light as much as possible during preparation. Store in the dark at −80°C for long term storage (i.e., up to 1 year), or store smaller aliquots at −20°C for short term use (i.e., up to 3 months).

PPD (paraphenylenediamine) is sometimes used for antifade, but it has been found to cleave some fluorophores off the antibody. DABCO does not cause this problem.

DAPI, 1 mg/ml

Dissolve sufficient 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) in water to prepare a 1 mg/ml (1000×) solution. Store in the dark for up to 3 months to 1 year at −20°C.

Fixation cocktail, 5×

0.5 M potassium phosphate, pH 6.5 (see recipe)
20% (v/v) formaldehyde (from 37% stock)
Prepare fresh

All 37% (v/v) formaldehyde should be used within 3 months of opening.

FM4-64

Stock solution: Prepare a 1 mg/ml solution of FM4-64 (Molecular Probes) in DMSO. Store in small aliquots up to a few months at 20°C.

Working solution: Freshly dilute 1 mg/ml FM4-64 stock (see above) 1:50 for assay temperatures ≤30°C or 1:100 for assay temperatures >30°C in rich medium (*UNIT 1.6*). Once the dye is diluted in rich medium, prewarm to assay temperature.

IMPORTANT NOTE: *Do not prepare diluted FM4-64 more than 30 min prior to use.*

Repeated freeze thaws may decrease lifetime (see Critical Parameters).

HEPES/100 mM/ 2% (w/v) glucose

Dilute 1 M HEPES, adjusted to pH 7.6 with potassium hydroxide, 1:10 in water. Freshly add glucose from a concentrated stock (i.e., 50% w/v) to 2% (w/v). Store up to 24 hr at room temperature.

In-medium fixation cocktail

Combine 1.15 ml of 1 M potassium phosphate buffer, pH 6.5 (see recipe), and 1.35 ml of 37% (v/v) formaldehyde for every 10 ml cells to be fixed. Prepare fresh.

All 37% (v/v) formaldehyde should be used within 3 months of opening.

Lucifer yellow, 40 mg/ml

Dissolve the dilithium salt of lucifer yellow CH (LY; Molecular Probes or Sigma) to 40 mg/ml in water. Store at 4°C for short periods (weeks) or –20°C for long periods (months).

It is unclear whether LY is completely stable for long-term storage (see Critical Parameters). If the experiment doesn't work, try using a fresh batch of LY.

Moisture chamber

A moisture chamber is used for keeping the cells on the slides/coverslips from drying out during incubations; they should be kept moist and in the dark. One recommended method is to use a tissue culture dish (15 cm or 6 well) with a damp paper towel pressed into the lid (it shouldn't fall if it is damp, but not soaked, and pressed firmly) and aluminum foil covering the entire lid and base.

Overnight fixation cocktail

For every 10 ml needed, mix 7.9 ml water, 1.0 ml of 1 M potassium phosphate, pH 6.5 (see recipe), and 1.1 ml of 37% formaldehyde. Prepare fresh.

All 37% (v/v) formaldehyde should be used within 3 months of opening.

PBS, pH 7.4

10 mM sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*)
150 mM NaCl
Filter, sterilize, and store up to 6 months at room temperature.

PBS containing phalloidin and DAPI

Dilute 200 U/ml rhodamine-phalloidin stock (see recipe) 1:10 and 1 mg/ml DAPI (see recipe) 1:1000 in PBS, pH 7.4 (see recipe). Make fresh within 1 hr of use and keep dark.

PBS (pH 7.4)/2% (w/v) glucose

Add glucose fresh from a concentrated stock (i.e., 50% w/v) to PBS, pH 7.4 (see recipe), at a final concentration of 2% (w/v). Prepare fresh.

Permeabilization solution

In PBS, pH 7.4 (see recipe) prepare:
1 M sorbitol
0.1% (w/v) saponin
0.1 mg/ml RNase
5 mM $MgCl_2$
Prepare fresh

Permeabilization/staining solution

Add 50 µl of 200 U/ml rhodamine-phalloidin stock (see recipe) per 1 ml permeabilization solution (see recipe). Make fresh within 1 hr of use and keep dark.

Phosphate/azide/fluoride buffer

50 mM potassium phosphate buffer, pH 7.5 (*APPENDIX 2A*)
10 mM NaN_3 (add fresh from concentrated stock)
10 mM NaF (add fresh from concentrated stock)
Prepare fresh

Some methods use sodium rather than potassium phosphate buffer.

Potassium phosphate, 0.04, 0.5, and 1 M (pH 6.5)

For a 1 M solution, mix 2 parts 1 M dibasic potassium phosphate (K_2HPO_4) with 1 part of 1 M monobasic potassium phosphate (KH_2PO_4). Store up to 1 year at room temperature. To prepare a 0.5 M solution, dilute 1:1 with water. For a 0.04 M solution, dilute 1:25 in water.

Rhodamine- or Texas red–phalloidin, 200 U/ml

Dissolve sufficient rhodamine- or Texas red–phalloidin in 1.5 ml methanol to prepare 200 U/ml as directed by manufacturer (see instructions on tube). Store in the dark for 3 to 6 months at −20°C.

Alternative fluorescent labels conjugated to phalloidin are available and should also work.

SHA buffer

1 M sorbitol
0.1 M HEPES, pH 7.5 (adjust pH with NaOH)
5 mM sodium azide
Prepare fresh from stock solutions

SHA buffer/LDAO, 1% (w/v)

Freshly prepare 1% (w/v) lauryldimethylamine oxide from a concentrated (i.e., 10% w/v) stock solution in SHA buffer (see recipe). Prepare fresh.

Sodium citrate, 0.5 M (pH 4.0)

Prepare a 0.5 M solution of citric acid and adjust the pH to 4.0 with sodium hydroxide. Store up to 1 year at room temperature.

Spheroplasting medium

Prepare the following 10 to 50 ml (depending on the number of samples) synthetic growth medium (i.e.,YNB/SD) containing amino acids and 2% glucose:
1 M sorbitol
0.04 M potassium phosphate buffer, pH 6.5 (see recipe)
10 µg/ml zymolyase
Make fresh within 1 hr of use.

Spheroplasting solution

In SHA buffer (see recipe) prepare:
0.2% (v/v) β-mercaptoethanol
45 µg/ml zymolyase (Seikagaku Corp.)
Make fresh within 1 hr of use.

WT buffer

50 mM HEPES, pH 7.5 (adjust pH with NaOH)
150 mM NaCl
0.1% (v/v) Tween-20
1 mM sodium azide
5% (w/v) IgG-free BSA
Prepare fresh if possible, but can be stored up to 2 days at 4°C during the course of assay.

COMMENTARY

Background Information

Imaging in yeast cells is constantly improving due to advances in microscopy techniques, and also as a result of the development of new and better microscopes and organelle-specific labels (Kohlwein, 2000).

Elucidating pathways

Saccharomyces cerevisiae is best known for its genetic malleability, allowing easy creation and identification of mutant phenotypes. The membrane trafficking pathways in yeast cells have been extensively investigated and characterized over the last few decades, leading to identification of the molecular components of the secretory pathway (Novick et al., 1980), the endocytic pathway (Chvatchko et al., 1986; Raths et al., 1993; Munn and Riezman, 1994; Wendland et al., 1996), and the organelles involved in these two important transport pathways and movement between them (Wendland et al., 1998; D'Hondt et al., 2000; Antonny and Schekman, 2001). This unit provides a few of the methods that have been developed to easily, and sometimes quickly, identify defects in endocytic and vacuolar sorting pathways. In addition to these vital microscopic techniques are, of course, a complementary set of biochemical techniques to study many of these same molecular structures (Conibear and Stevens, 2002; Sipos and Fuller, 2002).

GFP-fusion proteins

The use of GFP-fusion proteins in both mammalian and yeast cells has exploded in the last decade or so. Roger Tsien provides a detailed and careful review of the many achievements since the discovery of this useful protein and its applications (Tsien, 1998). In genetically malleable yeast cells, creation and introduction of GFP-fusion proteins is fairly straightforward. Shuttle vectors are available that allow simple cloning of a specific gene directly into promoter- and GFP-containing vectors at polylinkers designed to allow in-frame insertions. In addition, GFP tags can be fused to the C terminus of a protein behind its endogenous promoter. Reviews on the creation and use of GFP-fusion proteins are also available (Rines et al., 2002; Tatchell and Robinson, 2002).

Labeling cells using rhodamine-phallodin

Early work on yeast actin and tubulin led to the discovery of a distinct pattern in the distribution and structure of these two cytoskeletal elements (Adams and Pringle, 1984). In *Saccharomyces cerevisiae*, the actin cytoskeleton can be recognized in two distinct forms, actin cortical patches and actin cables. Each structure corresponds to bundles of filamentous actin, but they are structurally distinct and believed to perform different functions. The stereotyped localization pattern of actin cytoskeletal elements during the cell cycle is required for normal polarized bud growth and cytokinesis. Early methods for visualization of filamentous actin in yeast cells using the mushroom toxin phalloidin are still very similar to those used today (Adams and Pringle, 1991), although the protocol described in this unit includes a permeabilization step that the authors have found increases the percentage of labeled cells dramatically.

Immunofluorescence

Immunofluorescence techniques have been applied in yeast cells with much success, and several suggestions and methodologies have been described previously (Pringle et al., 1989, 1991). The development of confocal microscopes that allow optical sections thinner than 0.4 μm have aided in the creation of better, more precise immunofluorescence images. In addition, colocalization images can be more definitive. The methods described here are only two of many possible immunofluorescence preparations available, although most methods used are simply variations on the two basic methods provided. Removal of the cell wall to allow antibody access to internal structures is an essential step to any immunofluorescence protocol using yeast, but because spheroplasting (cell wall removal) can be stressful to living yeast cells, many methods include this step after fixation is completed.

Labeling chitin in the yeast cell wall

Chitin is a minor component of the yeast cell wall in content, but it is essential for yeast cell survival. Its role in septum formation at the mother-bud neck in cytokinesis is believed to be this essential function (Cabib et al., 1982, 2001). Chitin is deposited in a ring at the site where a bud emerges. These chitin rings represent the major concentration of chitin in the cell wall, although minor amounts of chitin are distributed throughout to provide strength and rigidity. The action of three chitin synthases, Chs1p, Chs2p, and Chs3p, are required for

different functions in the synthesis and deposition of chitin during the cell cycle. Chs3p exhibits a unique form of regulation that requires the recycling endocytic pathway for delivery to sites of chitin synthesis (Ziman et al., 1996; Holthuis et al., 1998). In this assay, deposition of chitin in rings is visualized to examine cells for a polarized pattern of bud site selection. Loss of this polarized pattern correlates with defects in polarized cell growth in general and actin cytoskeletal defects (Casamayor and Snyder, 2002).

Critical Parameters

Labeling the vacuole in live cells with CDCFDA

CDCFDA must be made in anhydrous DMSO to prevent premature hydrolysis and maintain the neutral, diffusible form. For this assay (see Basic Protocol 1), the cells do not need to be at a precise concentration, but using cells in early- to mid-log phase at the time of assay and centrifuging as gently as possible will increase the likelihood of visualizing pretty yeast cells with vacuoles that are larger and fewer in number. Haploid yeast cells that are rounded rather than elongated or bent, with vacuoles visible as solid circles are desired.

Labeling acidic organelles with quinacrine in live cells

This is a fairly simple method (see Alternate Protocol 1), but the signal is often faint. As with labeling vacuoles (see Basic Protocol 1), it is preferable to have the yeast cells in early- to mid-log phase, but it is not required for the assay to work.

Labeling the endocytic pathway in live cells with FM4-64

FM4-64 is fairly stable in DMSO, but the authors have found that repeated freeze-thawing cycles will decrease its fluorescence. It is best to dissolve the compound in DMSO and aliquot small amounts for storage at −20°C. In addition, once the FM4-64 is diluted into medium, it will lose activity slowly, so do not prepare diluted FM4-64 too far in advance (see Reagents and Solutions). In this assay (see Basic Protocol 2), it is *very* important to treat the cells gently, because this will increase the likelihood of obtaining pretty yeast cells and vacuoles; therefore, pellet cells as gently as possible and resuspend them by pipetting or inverting tubes gently, never vortex. It is also easier to measure pixel intensity of larger vacuolar membranes because there are fewer overlapping membranes through the yeast cell, so use cells that are closer to early- to mid-log if possible. Finally, it is important to wash the cells in medium thoroughly after the pulse incubation to make sure that all plasma membrane labeling is removed. Leftover FM4-64 on the plasma membrane may mask any dim phenotypes because the cells will have plenty of time during the chase to internalize this label.

Internalization time course with FM4-64

Follow many of the critical parameters described for labeling the endocytic pathway (see Basic Protocol 2) for this method (see Alternate Protocol 2) as well; however, it is important *not* to wash cells thoroughly after labeling. In this method, plasma membrane labeling is necessary since no internalization should have occurred during the label period on ice. Wash cells gently and briefly by spinning immediately after resuspension, keeping them cold. For better temperature control during the labeling and washing process, keep microcentrifuge tubes sitting in slushy ice. Finally, keep in mind that chase time assay temperatures <30°C will require longer time points than 20 min, and may take up to 60 to 90 min before cells exhibiting a kinetic delay (i.e., mutants) will show vacuolar labeling.

Endocytic rate measurement by LY uptake in live cells

Endocytic measurement by lucifer yellow (LY) uptake (see Basic Protocol 3) often requires strict adherence to several critical parameters, and in some strains lucifer yellow uptake will not work at all. In particular, for this fluid to be internalized successfully in many strains, the cells must be grown overnight from inoculation to very early- or early-log phase only. To achieve this most conveniently, it is often easier to inoculate a dilution series of the cells for overnight cultures, allowing use of whichever culture is at the best concentration when ready to begin the assay. Some other laboratories, however, have found that their strains can be at mid-log concentrations and still internalize lucifer yellow at high rates. Thus, after the described method has been found to work, it may be desirable to experiment with different growth concentrations to see if mid-log phase cells work for the strain in question.

While cells are incubated with the lucifer yellow label, it is *essential* that they be allowed proper gas exchange with the outside air. Closed microcentrifuge tubes do not allow this, so it is best to use a needle (~16 to 18 G) to poke a hole in the top of each of the sample tubes. Do not invert the tubes after this. It is also very important that the lucifer yellow be kept in the dark at all times. Cover the tubes with aluminum foil whenever possible and keep them in the dark on ice until they are mounted onto slides. Finally, lucifer yellow has been reported to work after storage at both 4° and −20°C. Once the assay is working with fresh stocks, it may be a good idea to try several different storage conditions to see which one works best for the particular lucifer yellow stock being used.

Localization of proteins in live yeast cells with GFP fusion proteins

Viewing GFP-fusion proteins can be very easy, or a terrible ordeal, but this depends much more on the fusion protein than on the GFP viewing method. The method described in this unit (see Basic Protocol 4) is fairly simple, and the only critical parameter is that if the GFP-fusion protein is not integrated into the genome of the yeast strain being used, selection for the plasmid carrying it must be maintained at all times. Also, if the medium is already neutral/slightly basic, it is not necessary to add the Tris buffer. The best way to determine this is a side-by-side comparison.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

There are several critical parameters for labeling the actin cytoskeleton properly, but most of them involve proper preparation of cells before fixation. In order to achieve a nice mix of cell cycle stages at the end of this assay (see Basic Protocol 5), it is vital that the cells be either in early- or mid-log phase. Even late-log phase will result in a much lower percentage of early budded and cytokinesing cells. To this end, if cells are diluted in the morning, make sure that they have doubled *more* than one time to get all of the cells back into the early- to mid-log stages. In addition, even in wild-type cells, heat shock (i.e., a temperature shift from the overnight growth temperature), will result in complete depolarization of the actin cytoskeleton (as does centrifugation at excessive speeds), followed by a repolarization. Repolarization is a 90-min process, so if cells are shifted, they need to be at the assay temperature for ≥90 min before fixation. During this shift, keep in mind that the concentration is still important because the cells will continue to grow during this shift, and often grow faster at the higher temperature. During the assay, it is important to wash the cells well between each major step (fixation to permeabilization, permeabilization to dye labeling, dye labeling to viewing) to prevent the accumulation of background fluorescence.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion proteins

This is a pretty simple method (see Alternate Protocol 3) that, if it works, can be very informative. The main critical parameter is to prepare GFP-labeled only cells (see Basic Protocol 4) alongside this assay to make sure that permeabilization or treatment with the toxin does not affect the GFP pattern.

Localization of a protein in fixed yeast cells with immunofluorescence

Immunofluorescence techniques are all fairly similar and have a few standard critical parameters. The most important is to use a very specific primary antibody. Polyclonal antibodies are often used, but they need to be tested thoroughly for their specificity. This is usually done by immunoblot, which is described in *UNIT 6.2*. Washes between antibody incubations are necessary to remove nonspecifically bound antibodies, and it is also important to make sure the cells are kept moist at all times. In addition, it is important to make sure that the protocol instructions (see Basic Protocol 6) and incubation times are followed carefully. Finally, when doing colocalization, always test the fluorescent tags for bleed-through in both fluorescent channels/filter sets by preparing two extra sets of cells, each with one of the two labels being used.

Localization in spheroplasted, then fixed cells with immunofluorescence

The same critical parameters apply for this protocol (see Alternate Protocol 4) as for localization of proteins in fixed yeast cells with immunofluorescence (see Basic Protocol 6). If it is not known what localization pattern is expected, it is a good idea to try both this protocol and Basic Protocol 6, to determine which label works best, as well as to verify that the pattern of fluorescence is the same with both methods.

Labeling cell wall chitin deposition with calcofluor white

This method (see Basic Protocol 7) is fairly simple. To make sure that most cells will be old (i.e., have undergone multiple divisions), it is necessary to have a cell population that is saturated. This decreases the percentage of newly formed daughter cells that will have only the birth scar.

Troubleshooting

With all fluorescent methods, the first thing to try if there is little to no fluorescent signal is a fresh batch of the fluorescent compound. Also, *always* include a wild-type control in all assays in case the lack of signal is from a mutant strain. Finally, make sure that the correct filter sets for the compound being visualized are being used on the microscope (spectra for dyes are usually available from the suppliers).

Labeling the vacuole in live cells with CDCFDA

If a fresh batch of CDCFDA is being used and a good signal is still not achieved, try adjusting the concentration of CDCFDA in the incubation. Too much may be causing quenching of the signal, so try starting with a range from ~5 μM to ~20 μM to check for improvement in signal. Also, try adjusting the pH or concentration of sodium citrate in case the content of the medium is affecting the assay.

Labeling acidic organelles with quinacrine in live cells

If a fresh batch of quinacrine is being used and a good signal is still not achieved, try decreasing the concentration of quinacrine by setting up four tubes of the same strain and using final concentrations of 1, 1.5, 2, and 2.5 μM. If a better signal is obtained, it may be desirable to try a series of concentrations around the best signal until the optimum concentration for the strain is obtained. If the signal is still too low, try increasing the incubation time with the dye.

Labeling the endocytic pathway in live cells with FM4-64

If a fresh batch of FM4-64 is being used and a good signal is still not achieved, try using the FM4-64 at a higher concentration/lower dilution (i.e., 1:50 or 1:75 if 1:100 is currently being used). If a higher concentration of FM4-64 is already being used, try using a lower concentration (more dilute), because high concentrations of FM4-64 can quench the signal. If the amount of fluorescent signal is high enough, but the wild-type yeast vacuoles are unusually fragmented, try using cells closer to early log phase. If the cells are not rounded and pretty, centrifuge at lower speeds and resuspend as gently as possible to improve their structure.

Internalization time course with FM4-64

If the fluorescence is too dim in the wild-type cells in this assay, troubleshooting will depend on which time point is giving the low signal. If the zero time point is missing plasma membrane labeling, most likely the washing is too thorough. Decrease the number, time of incubation, and/or volume of washes for all of the samples (so that later time points also maintain this PM label to have dye to internalize), and try to resuspend cells more gently. If the first time point after zero is too faint, but the zero time point and later times were okay, it may be that the assay temperature and strain being used require more time to internalize before the first time point is taken. If all of the time points after zero are too faint, these samples may be being washed more thoroughly than the zero time point. Try to make washes as close to equal as possible. Finally, if the final time point does not show mostly vacuolar staining in at least the wild-type cells, it is necessary to extend the assay to later time points until it does. Keep in mind that the number of samples to view under the microscope later increase rapidly with this assay, so rather than including additional time points, it may be desirable to spread the time points evenly over the extended time.

Endocytic rate measurement by LY uptake in live cells

If a fresh batch of lucifer yellow is being used, and a good signal is still not achieved, first try borrowing or getting a few different background strains and testing them out. As a positive control, the Research Genetics strain, BY4741 has worked most robustly and reliably for the authors. If none of the other wild-type strains give a visible/measurable signal, then start troubleshooting the method. If some of these strains give a signal, but the laboratory background strain does not, it may be that the strain will not work with this assay (for example, the authors have never seen lucifer yellow uptake in the wild-type strain SEY6210). To troubleshoot the method, start by making sure that all of the details of the protocol are being

followed, then try some of these suggested modifications to improve the signal.

1. Use one or two strains at several different starting concentrations and see if any one concentration works better.
2. Try altering the lucifer yellow concentration during the internalization incubation.
3. Try increasing the size of the gas exchange hole in the microcentrifuge tube, or increasing or decreasing the agitation rate during the dye incubation.

Keep in mind that this signal is often very low even when the assay works, so if vacuolar labeling is visible in the wild-type at all, this may be the maximal signal. Also remember that an extremely brightly stained vacuole, as noted in the method, is a sign of a dying yeast cell and is not due to endocytosis.

Localization of proteins in live yeast cells with GFP fusion proteins

If a good signal is not being achieved for the fusion protein, start by trying a GFP-fusion protein that is known to work (e.g., borrow from another laboratory or obtain one for which details on construction and strain are published so it will be known that nothing is being missed). If neither works, try changing to a different medium in case the experimental medium is too acidic or contains fluorescence quenchers. If the tester GFP-fusion protein works, then it is likely to be a problem with the fusion protein. First confirm that the fusion protein is stably produced by immunoblotting (*UNIT 6.2*). Also, many GFP-fusion proteins give a faint signal when a non-GFP version of the same protein is already available in the cell. Try integrating the GFP fusion in place of the genomic copy (or deleting the genomic copy and using the GFP fusion as the only copy on a plasmid). If this doesn't work, try using a high-copy-number vector, but keep in mind that increasing the levels of the fusion protein can harm the cell and/or result in altered localization due to high protein levels. Sometimes fusing GFP to the protein affects its folding/fluorescence, localization, or function, so it is possible to try creating an N-terminal fusion if previously a C-terminal fusion was created. Keep in mind that any fusion proteins used should be tested for functionality by rescue of mutant phenotypes wherever possible.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

If a fresh batch of rhodamine-phalloidin is being used and a good signal is still not achieved, it may be that the cells are not being sufficiently permeabilized. Try using about half the total OD_{600} amount of cells and make sure to use a full 1 ml of 0.2% Triton X-100 for the permeabilization step. If the actin is not properly polarized, the cells may be becoming distressed before or at the beginning of fixation. Be extra careful to treat cells gently until they have been in the fixation solution for at least 1 hr. If the cells are deformed or otherwise ugly in structure, the formaldehyde being used during fixation may be bad or the centrifugation used during preparation may be too fast. Try using fresh formaldehyde first. Finally, if a high background level of fluorescence is being observed, try adding extra washing steps after both the permeabilization (high background often comes from residual detergent when phalloidin is added) and phalloidin incubation steps. All of these washes make it easy to lose the pellet; remember that it is better to remove wash solutions with a pipet than an aspirator.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion proteins

If a fresh batch of rhodamine-phalloidin is being used and a good signal for the actin is still not being achieved, try decreasing the total amount of cells taken and increasing the volume of permeabilization/staining solution to give each individual cell better access to both detergent and toxin label. If the GFP signal is too low in the actin-labeled cells, but not in the nonpermeabilized GFP-labeled-only cells, check the pH of the permeabilization solution and make sure that it is still around 7.4 (acidic pH decreases EGFP signal).

Localization of a protein in fixed yeast cells and in spheroplasted, then fixed cells with immunofluorescence

If the fluorescent signal is too low, try increasing the primary antibody concentration (it may be desirable to do a series of dilutions), or the time or temperature used when incubating with primary antibody. If doing colocalizations and both fluorescent signals are low, try adjusting the permeabilization or spheroplasting methods to give the antibodies better access to their targets. If the background signal is too high, try decreasing the secondary antibody incubation time or concentration, increasing the number of washes between antibody treatments, or changing the primary antibody.

Labeling cell wall chitin deposition with calcofluor white

Calcofluor white staining is usually very bright. If this signal is low, make sure the calcofluor white has dissolved into solution before use. If too much background is present, decrease the concentration of CfW. If not enough bud scars per cell are being achieved to assess budding patterns, try adjusting the growth conditions used in preparing the cells for the assay.

Anticipated Results

Labeling the vacuole in live cells with CDCFDA

CDCFDA evenly labels the entire lumen of any compartment exhibiting esterase activity. In wild-type cells one to five filled circles of fluorescence should be observed, taking up ~1/3 to 1/2 of the total cell volume. Mutant cells with defective delivery of hydrolases to the vacuole could exhibit normal structure but severely decreased fluorescence or abnormal structure including fragmented vacuoles and/or a Class E compartment, an enlarged late endosome–like structure adjacent to the vacuole (Raymond et al., 1992).

Labeling acidic organelles with quinacrine in live cells

Quinacrine labels the entire lumen of acidic membrane-enclosed compartments. In wild-type cells one to five filled circles or fluorescence should be observed, taking up ~1/3 to 1/2 of the total cell volume. Mutant cells may exhibit many of the defects described for CDCFDA.

Labeling the endocytic pathway in live cells with FM4-64

FM4-64 labels membranes only, and in wild-type cells should accumulate on the vacuolar membrane after the chase. In wild-type cells approximately one to five rings of fluorescence should be observed, each with a dark interior, taking up ~1/3 to 1/2 of the total cell volume. Mutant cells with a defect in endocytosis could exhibit abnormal fluorescence patterns such as endosomal labeling (punctate spots around the vacuole or adjacent to the plasma membrane) as well as decreased total cell-associated fluorescence. This can be measured by a cell sorter or approximated by measuring pixel intensity of vacuolar membranes from microscopy images taken under the same exposure conditions.

Internalization time course with FM4-64

In an internalization time course, the zero time point should exhibit a single ring of fluorescence for each cell corresponding to the plasma membrane. If the zero time point cells show some endosomal label, they may be getting a little warmer during the labeling or washing steps than they should. Make sure the centrifugation is performed at 4°C. When looking through time points after zero, endosomal membrane labeling should be visible (several punctate spots near the plasma membrane), then late endosomal labeling (punctate spots farther in the interior of the cell), then finally vacuolar labeling (one to five rings of fluorescence, taking up ~1/3 to 1/2 of the cell interior). See Figure 4.13.1 for an example of wild-type internalization at 30°C. Mutant cells defective for endocytosis could exhibit a delay in the progression of the dye through these stages, as well as decreased signal in the time points after zero (where all samples should have approximately equal label) due to removal of plasma membrane label in the postchase washes.

Endocytic rate measurement by lucifer yellow uptake in live cells

Lucifer yellow is a fluid-phase dye; therefore, in wild-type cells it should accumulate in approximately one to five filled circles of fluorescence, taking up ~1/3 to 1/2 of the interior of the cell. Mutant cells defective for endocytosis should exhibit decreased or absent fluorescence signal.

Localization of proteins in live yeast cells with GFP fusion proteins

The anticipated localization pattern for this assay will depend completely on the GFP-fusion protein being observed.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

Filamentous actin will be found in two forms, cortical actin patches and extended actin cables. As described, these two structures exhibit a distinct pattern throughout the cell cycle. In cells with small buds under DIC, ~90% of the cortical actin patches should be in the small daughter bud. Actin cables should extend along the axis from the bud into the mother cell, perpendicular to the bud neck. In cells with large buds, if DAPI staining shows the nucleus near the mother-bud neck, the nucleus is about to divide and the actin cytoskeleton should be randomly distributed in both cells. If the DAPI staining shows two separate nuclei, one in each

cell, then the actin cortical patches should be at the mother-bud neck and cables should extend outward from this neck in both directions. Figure 4.13.2 shows an example of these wild-type patterns. Mutants may be defective for polarization, for formation of cables or actin patches, or may have defects in regulating actin dynamics—e.g., few large actin clumps rather than several small patches, which may be depolarized. See Figure 4.13.3.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion protein

Filamentous actin should follow the same patterns described for Basic Protocol 5, but again, GFP-fusion protein patterns will depend on the fusion protein used. Keep in mind that the protein might localize to cortical patches that are distinct from cortical actin patches. Figure 4.13.3 shows an example of GFP-Ent1p, which does localize to actin patches, but in these mutant cells actin patches are chunky and depolarized.

Localization of a protein in fixed yeast cells and in spheroplasted then fixed cells with immunofluorescence

The anticipated localization pattern for these assays will depend completely on the protein being observed.

Labeling cell wall chitin deposition with calcofluor white

Calcofluor white binds to chitin deposits in the yeast cell wall. Because there are low levels of chitin throughout the cell wall, with heavier deposits at scars formed at previous bud sites, the CfW pattern is usually characterized by a barely visible outline of the yeast cell, with several small rings of bright fluorescence on the outside edges/surface of the cell. Each individual ring is usually <1/10 the size of the yeast cell, and in wild-type diploid cells, one to several rings will usually be found on each end of the elongated cell. The middle third of each diploid yeast cell should be dark (devoid of chitin rings) in wild-type cells. In cells defective for recycling endocytosis, chitin deposition is decreased and rings will be absent or barely visible. In mutant cells defective for polarized cell growth, including actin cytoskeletal defects, the chitin rings will be found randomly over the surface of the diploid cells, and the cells themselves are usually not elongated but instead are round. Examples of both wild-type and depolarized chitin deposition are shown in Figure 4.13.4.

Time Considerations

For all of the protocols, there is an initial time investment required to prepare the cells for the assay. Inoculation of a culture the day before the assay is required to obtain a liquid culture ready for staining. Most of these assays require log-phase cells which may take up to 5 hr the next day to prepare if cells are saturated in the morning and must be diluted to bring the cells back to log phase. Remember that not all assays can use the cells diluted in the morning.

Labeling the vacuole in live cells with CDCFDA

Preparation of CDCFDA-labeled cells should take <1 hr once cells are in log phase. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling acidic organelles with quinacrine in live cells

Preparation of quinacrine labeled cells should take <30 min once cells are in log phase. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling the endocytic pathway in live cells with FM4-64

The FM4-64 assay should take anywhere from 1 to 3 hr once cells are in log phase. Once cells are ready for viewing, they should be observed as soon as possible but preferably not more than 1 hr after preparation. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample.

Internalization time course with FM4-64

The FM4-64 time course should take anywhere from 1 to 2 hr once cells are in log phase. Prepare the solutions just before harvesting the cells, making sure that all media are completely prechilled. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample (remember that this is per strain multiplied by the number of time points).

Endocytic rate measurement by lucifer yellow uptake in live cells

The lucifer yellow assay should take ~3 hr once cells are in early log phase. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample.

Localization of proteins in live yeast cells with GFP-fusion proteins

Preparation of GFP-fusion protein cells for viewing is very simple. Other than additional time for a temperature shift, once cells are in log phase they can be ready for viewing in as little as 5 min. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

Preparation of F-actin labeled cells could take as little as 4 to 5 hr once cells are in log phase, but it can also be stretched out over 3 days. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion protein

Preparation of colabeled F-actin and GFP cells should take <1 hr once cells are in log phase. For an experienced fluorescence microscopist, viewing time should take 15 to 25 min per sample because it may take more time to find cells with both labels.

Localization of a protein in fixed yeast cells with immunofluorescence

Preparation of yeast cells with immunofluorescence requires an overnight fixation step after cells are in log phase. Once cells are fixed, the preparation could take as little as 5 hr, but often requires additional overnight incubation with the primary antibody as well. It is best to plan well and plan ahead for this assay, reading the method thoroughly before beginning the preparation. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Localization in spheroplasted, then fixed, cells with immunofluorescence

This method for preparation of yeast cells with immunofluorescence can be done in 1 long day (~7 hr of preparation once cells are in log phase) but is likely to require at least 2 days for proper preparation of cells. As with Basic Protocol 6, make sure to read the method thoroughly before beginning the assay. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling cell wall chitin deposition with calcofluor white

Preparation of calcofluor white labeled cells should take <15 min starting from a dense overnight culture. Prepare the CfW solution a few days prior to beginning the assay since it may take over 24 hr to dissolve in solution. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Literature Cited

Adams, A.E. and Pringle, J.R. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant saccharomyces cerevisiae. *J Cell Biol.* 98:934-945.

Adams, A.E. and Pringle, J.R. 1991. Staining of actin with fluorochrome-conjugated phalloidin. *Methods Enzymol.* 194:729-731.

Amberg, D.C. 1998. Three-dimensional imaging of the yeast actin cytoskeleton through the budding cell cycle. *Mol. Biol. Cell* 9:3259-3262.

Antonny, B. and Schekman, R. 2001. Er export: Public transportation by the copii coach. *Curr. Opin. Cell Biol.* 13:438-443.

Cabib, E., Roberts, R., and Bowers, B. 1982. Synthesis of the yeast cell wall and its regulation. *Annu. Rev. Biochem.* 51:763-793.

Cabib, E., Roh, D.H., Schmidt, M., Crotti, L.B., and Varma, A. 2001. The yeast cell wall and septum as paradigms of cell growth and morphogenesis. *J. Biol. Chem.* 276:19679-19682.

Casamayor, A. and Snyder, M. 2002. Bud-site selection and cell polarity in budding yeast. *Curr. Opin. Microbiol.* 5:179-186.

Chvatchko, Y., Howald, I., and Riezman, H. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell* 46:355-364.

Conibear, E. and Stevens, T.H. 2002. Studying yeast vacuoles. *Methods Enzymol* 351:408-432.

Cooper, J.A. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell. Biol.* 105:1473-1478.

D'Hondt, K., Heese-Peck, A., and Riezman, H. 2000. Protein and lipid requirements for endocytosis. *Annu. Rev. Genet.* 34:255-295.

Erdmann, R. and Gould, S.J. 2002. Visualization and purification of yeast peroxisomes. *Methods Enzymol.* 351:365-381.

Holthuis, J.C., Nichols, B.J., and Pelham, H.R. 1998. The syntaxin Tlg1p mediates trafficking of chitin synthase III to polarized growth sites in yeast. *Mol. Biol. Cell* 9:3383-3397.

Kohlwein, S.D. 2000. The beauty of the yeast: Live cell microscopy at the limits of optical resolution. *Microsc. Res. Tech.* 51:511-529.

Lew, D.J. and Reed, S.I. 1995. Cell cycle control of morphogenesis in budding yeast. *Curr. Opin. Genet. Dev.* 5:17-23.

McNally, J.G., Karpova, T., Cooper, J., and Conchello, J.A. 1999. Three-dimensional imaging by deconvolution microscopy. *Methods* 19:373-385.

Munn, A.L. and Riezman, H. 1994. Endocytosis is required for the growth of vacuolar h(+)-atpase-defective yeast: Identification of six new end genes. *J. Cell Biol.* 127:373-386.

Novick, P., Field, C., and Schekman, R. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21:205-215.

Nunnari, J., Wong, E.D., Meeusen, S., and Wagner, J.A. 2002. Studying the behavior of mitochondria. *Methods Enzymol.* 351:381-393.

Pringle, J.R., Preston, R.A., Adams, A.E., Stearns, T., Drubin, D.G., Haarer, B.K., and Jones, E.W. 1989. Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31:357-435.

Pringle, J.R., Adams, A.E., Drubin, D.G., and Haarer, B.K. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* 194:565-602.

Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. 1993. End3 and end4: Two mutants defective in receptor-mediated and fluid-phase endocytosis in saccharomyces cerevisiae. *J. Cell Biol.* 120:55-65.

Raymond, C.K., Howald-Stevenson, I., Vater, C.A., and Stevens, T.H. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: Evidence for a prevacuolar compartment in the class E vps mutants. *Mol. Biol. Cell.* 3:1389-1402.

Rines, D.R., He, X., and Sorger, P.K. 2002. Quantitative microscopy of green fluorescent protein-labeled yeast. *Methods Enzymol.* 351:16-34.

Sipos, G. and Fuller, R.S. 2002. Separation of golgi and endosomal compartments. *Methods Enzymol.* 351:351-365.

Tatchell, K. and Robinson, L.C. 2002. Use of green fluorescent protein in living yeast cells. *Methods Enzymol.* 351:661-683.

Tsien, R.Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 67:509-544.

Vida, T. and Wendland, B. 2002. Flow cytometry/cell sorting for isolating membrane trafficking mutants in yeast. *Methods Enzymol.* 351:623-631.

Watson, H.A., Cope, M.J., Groen, A.C., Drubin, D.G., and Wendland, B. 2001. In vivo role for actin-regulating kinases in endocytosis and yeast epsin phosphorylation. *Mol. Bio. Cell* 12:3668-3679.

Wendland, B., McCaffery, J.M., Xiao, Q., and Emr, S.D. 1996. A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. *J. Cell Biol.* 135:1485-1500.

Wendland, B., Emr, S.D., and Riezman, H. 1998. Protein traffic in the yeast endocytic and vacuolar protein sorting pathways. *Curr. Opin. Cell Biol.* 10:513-522.

Ziman, M., Chuang, J.S., and Schekman, R.W. 1996. Chs1p and chs3p, two proteins involved in chitin synthesis, populate a compartment of the saccharomyces cerevisiae endocytic pathway. *Mol. Biol. Cell* 7:1909-1919.

Contributed by J.J. Baggett, J.D. Shaw, C.J. Sciambi, H.A. Watson, and B. Wendland
Johns Hopkins University
Baltimore, Maryland

Fluorescence Lifetime Imaging Microscopy

Nowadays, fluorescence microscopy plays a central role in the fields of biology, biophysics, and in the life sciences in general. Its utility for imaging tissues, subcellular compartments, and even single molecules with high contrast is expanded by the possibility to investigate different properties of fluorescence like intensity, polarization, lifetime, and other spectroscopic parameters. The fluorescence lifetime in particular is a molecular property that offers a clearer fluorochrome signature than intensity spectra, and it contains information on the local molecular environment and chromophore photophysics of fluorescent labels. For these reasons, fluorescence lifetime imaging microscopy (FLIM) is used to enhance the image contrast of biological samples and to study molecular interactions of fluorescently labeled biomolecules by Förster resonance energy transfer (FRET). This unit will mainly focus on the second possibility. FRET is a photophysical phenomenon that allows the investigation of biochemical reactions in living cells. Under physiological conditions, FRET occurs exclusively between interacting molecules labeled with suitable fluorescent dyes with proper optical characteristics.

FRET imaged by FLIM exhibits the normal diffraction-limited spatial resolution offered by the light microscopical system used, but it maps interaction events that are on the 10- to 100-Å scale. Fluorescence microscopy flourished in the last decade with the advent of different detection techniques and the discovery of the green fluorescent protein and its different spectral variants. FRET imaging by FLIM contributed to the recent increase in sensing possibilities in cells.

Both the phenomena of energy transfer and lifetime are described theoretically and practically in this unit. Theoretical aspects that are usually neglected but are connected with the practical limitations of these techniques are also discussed. The limitations, pitfalls, and crucial considerations for FLIM and FRET will be demonstrated using experimental and simplified theoretical frameworks.

STRATEGIC PLANNING

FLIM—Fluorescence Lifetime Imaging Microscopy

Fluorescence emission is a property of molecules that can absorb photons and re-emit the accepted energy as photons with lower energy, i.e., at higher wavelengths. This process is described by the energy level (Jabłoñksy) diagram presented in Figure 4.14.1. Thicker horizontal lines represent different electronic energy levels with their associated vibrational states (thinner lines). These are ordered with energy increasing from bottom to top of the graph. Transitions are plotted with straight lines for radiative processes and with wavy lines for non-radiative phenomena.

Only considering the ground state, the first two excited states, and the first triplet state, the number of possible transitions between different electronic states is quite variable:

(1) photon absorption: $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_0 \rightarrow S_2$

(2) radiative de-excitation between singlet states (fluorescence processes)

(3) non-radiative de-excitations (quenching)

(4) radiative transition $T_1 \rightarrow S_0$ (phosphorescence).

Each transition is associated with a rate constant, a molecular property that could eventually be perturbed by the environment. In fluorescence microscopy, usually, interest is in the fluorescence transition $S_1 \rightarrow S_0$.

Contributed by Alessandro Esposito and Fred S. Wouters

Current Protocols in Cell Biology (2004) 4.14.1-4.14.30

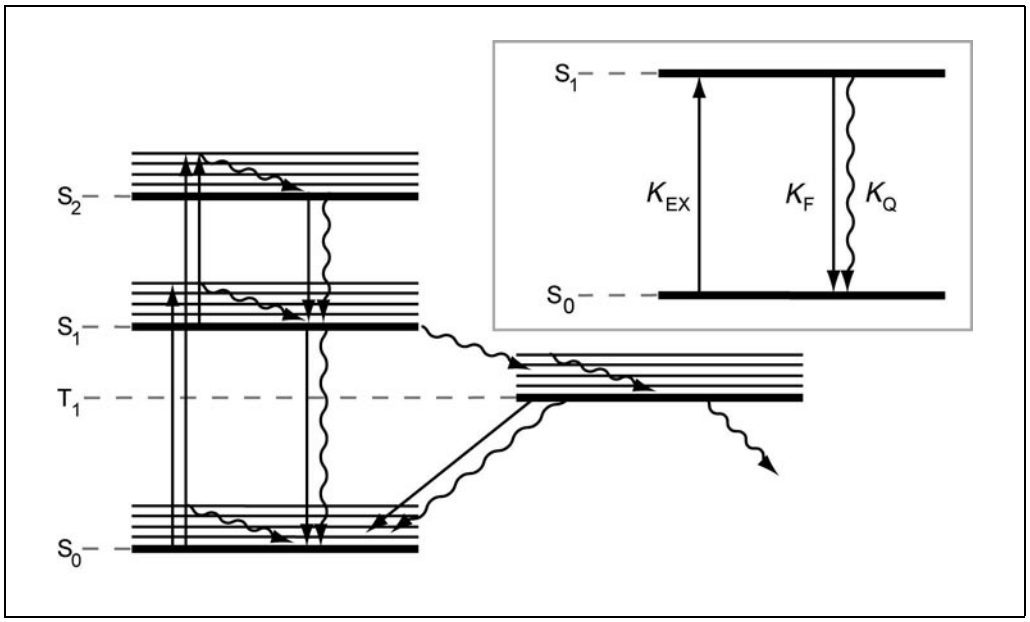


Figure 4.14.1 Typical depiction of fluorochrome photophysics (Jabłoñksy diagram). Plotted are the first three singlet and the first triplet electronic states (a more detailed description can be found in Strategic Planning). Top inset: simplified Jabłoñksy diagram, with photon absorption (K_{EX}), fluorescence (K_F), and internal conversion (K_Q, quenching) transitions indicated. Straight lines represent radiative transitions, wavy lines indicate non-radiative transitions.

The excited state S_1 has a defined lifetime, which depends on the rate constants of the different de-excitation pathways. After a photon is absorbed, a fluorescence photon is emitted after a certain delay. Thus, one definition of lifetime is the average time that a molecule spends in its excited state.

Average time means that the time decay is stochastic and follows a distribution that, in general, is exponential:

$$p(t) = \frac{1}{\tau} e^{-\frac{t}{\tau}}$$

Equation 4.14.1

The exponential decay constant τ of this probability distribution $p(t)$ also represents the lifetime and it is easy to find that the two definitions match when one considers the *average* lifetime.

The lifetime depends on equilibrium of the different rate constants:

$$\tau = \frac{1}{K_F + K_{NR}}$$

Equation 4.14.2

where K_F is the rate of photon emission, while K_{NR} is the rate of non-radiative de-excitation that could represent collisional quenching and/or energy transfer. An increase of these non-radiative processes due to interaction with the environment thus reduces the lifetime of a fluorochrome, which could be used by FLIM to probe these environmental influences. If the interactions with the environment are negligible or invariant, the lifetime represents a more reliable marker for a fluorescent species than its spectra. In fact, lifetime distributions are considerably narrower than intensity spectra.

FLIM has been used successfully for the separation of different fluorophores in multiple-stained samples (Figure 4.14.5; Pepperkok et al., 1999; Neher and Neher, 2004) to map endogenous autofluorescent cellular metabolites like NADH (Lakowicz et al., 1992) or to measure ratiometric probes like the high-affinity calcium indicator dye Indo-1 (Szmacinski et al., 1993). FLIM, performed on tissues, presents a growing and interesting diagnostic tool in the medical sciences (Tadrous et al., 2003). A recent investigation (Eliceiri et al., 2003) using FLIM on tissues stained with conventional histological dyes also demonstrated improved contrast and information content. In conclusion, FLIM is a well-established microscopy technique in the fields of biology, physics, and medicine. This unit will mainly focus on the lifetime-based detection of FRET because of its direct relevance for, and promising applicability in, the rapidly developing and converging fields of modern cell biology and biophysics.

FRET—Förster Resonance Energy Transfer

FRET is the phenomenon by which a donor fluorochrome transfers energy to an acceptor molecule in a non-radiative manner (Förster, 1965; Clegg, 1996). This phenomenon can be better understood by a quantum physical model but is easier explained by the classical concept of coulombic dipole-dipole interactions, although without emission and consequent re-absorption of a real photon. The simplified Jabłoñksy diagram in Figure 4.14.2 presents the transitions between the donor and acceptor ground and excited states. The energy transfer transition is given by:

$$(D,a) \overset{K_{ET}}{\rightarrow} (d,A)$$

Equation 4.14.3

where D, A denote excited states and d, a ground states of donor and acceptor, respectively. Consider the FRETting donor-acceptor pair as a system with four quantum states (Fig. 4.14.2):

1. *da*, both donor and acceptor, respectively, are in the ground state;
2. *Da*, only the donor is excited;
3. *DA*, both donor and acceptor are excited;
4. *dA*, only the acceptor is excited.

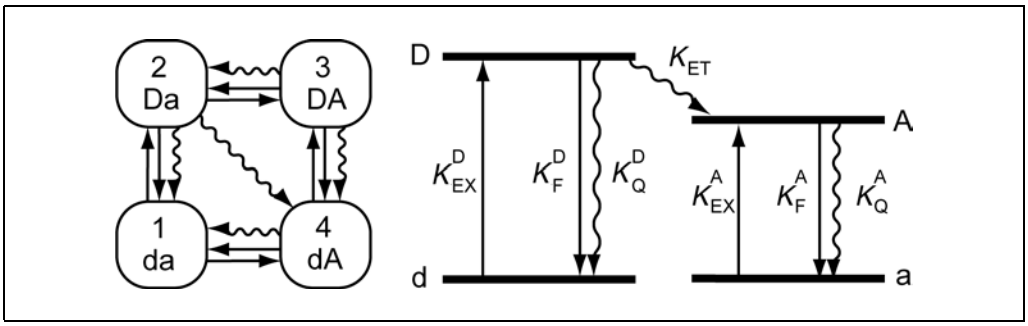


Figure 4.14.2 Four-state transition diagram. The simplified energy level diagram (right) illustrates transitions between ground and excited state of donor (*D*) and acceptor (*A*) fluorophores. The donor-acceptor pair can be considered as a unique photophysical system with four states (left) in which the diagonal radiation-less transition represents energy transfer. Lower-case letters indicate ground state and capital letters indicate excited state.

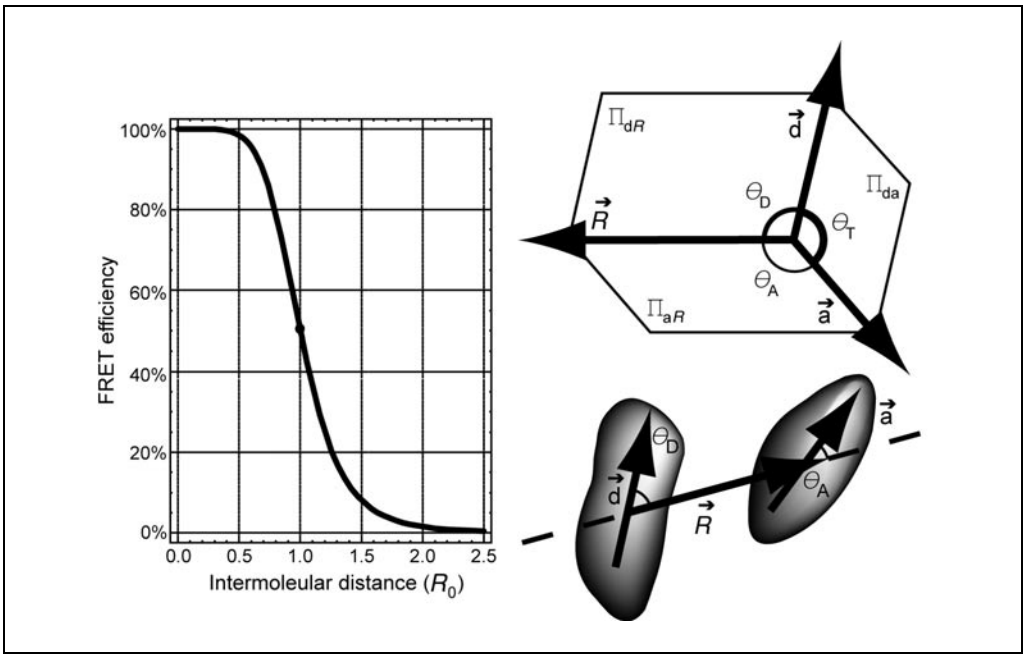


Figure 4.14.3 Spatial degrees of freedom in a FRET pair. The energy transfer phenomenon is a dipole-dipole interaction that depends on the intermolecular distance (R) and on the relative orientation of the two molecular dipole moments. The FRET efficiency increases with the sixth power of the intermolecular distance (left graph). The Förster radius, the distance at which the donor transfers energy to the acceptor with 50% efficiency, is a characteristic of the pair. Two interacting molecules are depicted (bottom-right) with their electrical dipole moment and the intermolecular distance vectors. The angle definitions of the three vectors are drawn in the upper-right scheme. Π indicates the plane defined by the different vectors and θ represents the angles (defined in Strategic Planning) that are most commonly used as coordinates for the orientational factors.

The energy or excitation transfer (ET) transition adds a de-excitation pathway to the single fluorochrome diagram (Figure 4.14.1 inset) and hence reduces the lifetime of the donor excited state in accordance with Equation 4.14.2, as it increases the probability per time unit of excited state depopulation. The quantum yield of this transition is called the FRET efficiency (ε). When the FRET efficiency is maximal (100%), i.e., when K_{ET} is much greater than the other rate constants, all absorbed energy is transferred from donor to acceptor.

FRET is often called fluorescence resonance energy transfer. Because this name is ambiguous and could lead to the assumption that there is emission of a fluorescence photon by the donor or that the acceptor should be a fluorescent molecule, it is preferable to associate the first letter with the name Förster, the discoverer of this phenomenon. For the same reason, FRET is also referred to simply as RET or ET (energy transfer or excitation transfer) in literature.

One of the most interesting aspects of ET is its extreme dependence on the intermolecular distance. An ET event occurs typically between distances of 10 and 100 Å, in the order of magnitude of protein size. Molar concentrations are required to reach these intermolecular distances in the absence of a specific interaction mechanism, a meaningless range under physiological conditions. This implies that if two molecules are FRETting in a biological system, they are also interacting. The second interesting aspect is that the FRET efficiency falls with the sixth power of the intermolecular distance (Figure 4.14.3):

$$K_{ET} = \frac{1}{\tau_D}\frac{R_0^6}{R^6};\ R_0^6 = \frac{\kappa^2}{n^4}Q_D J;\ \varepsilon = \frac{R_0^6}{R_0^6 + R^6}$$

Equation 4.14.4

Equation 4.14.4 illustrates the parameters of interest in this unit. R_0 is called the critical or Förster distance (or Förster radius). This parameter is a property of the FRET pair and is linked to the overlap integral J of the donor emission and acceptor absorption spectra, the donor quantum efficiency Q_D, the refractive index of the medium n and an orientational factor κ. R_0 is the distance at which the two molecules transfer energy with 50% efficiency. The sixth-power dependence of FRET on a fluorochrome separation distance causes FRET to sense only short (<10 nm) distance interactions, which prevents false-positive detection of interactions. It also allows high sensitivity for intermolecular distance variations over a scale of a couple of nanometers where the efficiency goes from all to none.

κ is an orientational parameter whose most common definition is shown in Equation 4.14.5:

$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$

Equation 4.14.5

As represented in Figure 4.14.3, θ_T, θ_D, and θ_A are the angles between donor-acceptor dipole moments, the donor dipole moment–intermolecular distance vector, and the acceptor dipole moment–intermolecular distance vector, respectively. Another useful definition is:

$$\kappa^2 = \cos^2\omega(1 + 3\cos^2\theta_D)$$

Equation 4.14.6

in which ω is the angle between the acceptor dipole moment and the electric field originating from the donor transition moment. This second relation illustrates that the FRET efficiency depends on two angular degrees of freedom and an intermolecular distance degree of freedom. κ^2 assumes a maximum value of 4 only when the donor and acceptor dipoles and the intermolecular distance vector are parallel or antiparallel as shown in Table 4.14.1. This happens when the acceptor transition dipole is parallel or

Table 4.14.1 Extreme Orientational Parameter Configurations[a]

κ^2	θ_T	θ_D	θ_A
4	0	π	0
	0	0	π
	π	0	0
	π	π	π
0	$\pi/2$	$\pi/2$	θ_A
	$\pi/2$	$3\pi/2$	θ_A
	$3\pi/2$	$\pi/2$	θ_A
	$3\pi/2$	$3\pi/2$	θ_A
	$\pi/2$	θ_D	$\pi/2$
	$\pi/2$	θ_D	$3\pi/2$
	$3\pi/2$	θ_D	$\pi/2$
	$3\pi/2$	θ_D	$3\pi/2$

[a]The orientational parameter κ^2 can be represented as a function of three angles that define the relative orientation between the donor and the acceptor as shown in Figure 4.14.3. There are only four discrete values at which κ^2 is maximal while there are continuous distributions of angles for which κ^2 is zero. In the latter case, it is sufficient that the donor *or* the acceptor dipole is orthogonal to the intermolecular distance while orthogonal to each other. For this reason, the probability to obtein high κ^2 values is very much lower than for lower values.

antiparallel ($\omega = 0, \pi$) to the donor electrical field. κ^2 assumes the minimum value of zero in more configurations, namely when these two vectors are orthogonal ($\omega = \pi/2, 3\pi/2$) and at least one is perpendicular to the intermolecular distance vector. For this reason, the relative orientation of two fluorochromes plays a crucial role in the yield of energy transfer.

A complete description of this aspect of ET is given by Lakowicz (1999) and van der Meer (1999). The latter work shows that the probability to obtain a maximum value for κ^2 is almost negligible compared to other, lower values. In any case, the probability on a particular value of κ^2 decreases with increasing values. The FRET efficiency between two rigidly connected chromophores will most probably be reduced by an unfavorable orientation of the two dipole moments. κ^2 is equal to 3/2 when complete rotational freedom between the two molecules can be assumed. This might hold true for small, conjugated organic fluorophores, but it is likely not the case for genetic fusions with the visible fluorescent proteins (VFPs). In this case, the chromophore mobility is restricted inside the β-barrel of the VFP structure and the VFPs are likely to bury into the surface of the fused protein. The orientation factor must therefore be optimized in the design of FRET-based biosensors by linker mutagenesis strategies to avoid the most probable but less optimal relative donor-acceptor orientation outcome.

Another important parameter is the refractive index of the medium. The energy transfer efficiency depends on the inverse of the fourth power of the refractive index. Some problems can arise when changing the mounting medium or buffer solution. Mounting cells in mowiol or glycerol, for example, causes an increase of the refractive index up to 1.48 to 1.49, which is considerably higher than the refractive index of cytoplasm, which is estimated to be 1.38. At very low FRET efficiency, this can cause a significant reduction of energy transfer. The possible effect of the refractive index should be considered critically under experimental conditions where the refractive index is varied over a broader range.

Theory: Energy Transfer, Energy Migration, and Lifetimes

Energy migration (EM) is the radiative migration of energy between molecules, i.e., the re-absorption of emitted photons. This phenomenon could dominate over longer distances where EM is relevant because it only falls with the second power of the intermolecular distance. Although EM can be considered as a distinct phenomenon, there are theoretical frameworks in which the two processes are explained as different manifestations of the same phenomenon (Juzeliûnas and Andrews, 1999). As only the non-radiative energy transfer couples the donor-acceptor photophysics such that the donor lifetime is reduced, EM does not affect FLIM-based FRET measurements.

For these reasons, a lifetime map obtained by fluorescence lifetime imaging microscopy represents the distribution of Förster resonance energy transfer. By application of Equations 4.14.2, 4.14.3, and 4.14.4, a quantitative FRET image can be retrieved from the following relation:

$$\varepsilon = 1 - \frac{\tau}{\tau_0}$$

Equation 4.14.7

in which τ is the observed reduced lifetime of the donor while τ_0 is the non-FRETting lifetime that, as reported before, is a donor property.

FRET, FLIM, and molecular biological tools together offer a powerful toolset for the investigation of protein function in the physiological setting of the intact living cell or tissue. In the current proteomics era, there is a demand for identification of molecular activity of identified gene products and elucidation of their interplay in the signalling network of the cell. These questions demand a screening approach to obtain a proteome-wide diagram of protein-protein interactions, modifications, and activities, all of which can be addressed by FRET. This static picture of interactions needs to be complemented with spatiotemporal information, thus requiring measurements in living cells.

Time and Frequency Domain (TD/FD) FLIM

Fluorescence lifetime imaging microscopes are classified into two main categories depending on the detection technique used. Time domain FLIM uses a pulsed-light source and detects the time decay distribution of the emitted fluorescence. Frequency domain FLIM typically uses sinusoidally modulated excitation light and senses the lifetime-dependent demodulation and phase-delay of the emission signal. Pulsed excitation can also be applied in the frequency domain. Both kinds of detections can be implemented on wide-field or scanning microscopes, but presently, the most common setups are wide-field FD-FLIM and laser-scanning TD-FLIM.

TD-FLIM: Excitation Light

A flash of light is required to time resolve the sample lifetime. This flash causes the transient population of the first excited state of the fluorochrome, and the detectors will follow the decay distribution of the emitted fluorescence. Pulsed lasers with pulse widths in the pico- or femtosecond range are the best light sources for the time domain. These lasers provide delta-like excitation, causing the time response of the system to depend primarily on the fluorescence characteristic of the fluorochrome and from the timing properties of detectors. A typical TD-FLIM takes advantage of a Ti:Sapphire-pulsed laser usually employed for two-photon (TP) microscopy (*UNIT 4.11*). The TP excitation regime can be used conveniently to generate lifetime images with the optical sectioning capabilities and the typical resolution of this technique. Single-photon excitation (SP) is usually achieved by regeneration of the near infrared (NIR) Ti:Sapphire emission using crystals that double the light frequency by second harmonic generation. Today, picosecond solid-state lasers are also available that allow a simpler setup to be built and that eliminate the need for expensive, yet more flexible and tuneable lasers like those used for TP excitation.

TD-FLIM: Common Scanning Setup

A common setup consists of a two-photon laser scanning microscope (TPLSM) or confocal laser scanning microscope (CLSM) configuration because the most suitable detectors capable of retrieving the fast decay distributions are point-detectors like photo-multiplier tubes (PMTs), multi-channel plate photo-multiplier tubes (MCP-PMTs), and single-photon avalanche diodes (SPADs). The laser beam is scanned over the sample and the sub-nanosecond light pulses transiently populate fluorochromes, which immediately relax to the ground state with a time-decay distribution that is typical for each fluorochrome. The distribution of the decay times is sampled at each pixel location.

A TD-CLSM has some advantages over the TD-TPLSM FLIM configuration. It is well known from literature that the confocal microscope has an intrinsically higher resolution than a TP microscope (Denk et al., 1995). Moreover, the pinhole could be removed to use the microscope in normal scanning mode to allow the acquisition of more photons from a thicker focal plane. This is sometimes beneficial to collect sufficient photons under demanding biological applications albeit at the cost of spatial resolution. When exciting specific fluorochromes, SP excitation exhibits a clear advantage over TP as

the absorption spectra of the latter are considerably broader. It is possible to excite multiple fluorochromes simultaneously in a sample by TP excitation and this is usually an advantage (Esposito et al., 2004), but it could present a problem in FRET measurements because direct excitation of the acceptor should generally be avoided. In any case, if sectioning is required or when lifetime detection is used for contrast enhancement between different fluorochromes, TPLSM regains its advantages.

TD-FLIM: Detection with TCSPC or TGSPC

Time-correlated single photon counting (TCSPC) and time-gated single photon counting (TGSPC) are the two most frequently used techniques for time-domain detection. TCSPC (O'Connor and Phillips, 1984; Becker et al., 2004) detects the time-delay between a single laser pulse and the first photon emitted from the sample. The presence of detector dead time prevents the detection of further photons that are emitted after the ones that have been counted, which can cause a loss of detectable signal at high-emission rates. A time-correlated system builds the distribution of arrival times from the first emitted photon per pulse from which, through pixel-by-pixel fitting routines, a lifetime image is retrieved. Usually, the reversed start-stop configuration is applied with high-repetition rate pulses. In this case, the time-delay between a detected photon and the next laser pulse is measured to avoid pulse-pileup. Time-gated detectors perform single photon counting by integration of fluorescent signals in two (Wang et al., 1991; Buurman et al., 1992; van der Oord et al., 2001) or more time windows (de Grauw and Gerritsen, 2001). These detectors collect almost all photons that emerge from the sample and can be gated after a sub-nanosecond initial delay to suppress the contribution of short-lifetime background signals to the measurement. Data collected in two windows can be simply analyzed according to Equation 4.14.8 to obtain an average lifetime estimator without the need for time-consuming and computationally expensive fitting procedures. Gating systems with multiple time windows can also resolve multi-exponential decays.

TD-FLIM: Wide-Field Microscopes

Typical time-domain FLIM setups are based on scanning microscopes but wide-field versions (Wang et al., 1991; Siegel et al., 2001b; Webb et al., 2002) are also interesting because they can be operated faster. The classical TD wide-field FLIM is built around a multi-channel plate with gated sampling of decay fluorescence intensities at defined time-delays. A minimum of two images acquired at different time delays are needed to retrieve a lifetime image. Recently, a more efficient wide-field gated system has been developed (Agronskaia et al., 2003) that projects two time-delayed images of the sample side-by-side onto the same multi-channel plate (MCP). The emitted fluorescence is split into two separate optical paths with different lengths, corrected for different magnification factors and focussed on the gated device. The two recorded images can be spatially matched and their ratio is a good lifetime estimator for mono-exponential decays. The rapid lifetime determination (RLD) formula (Ballew and Demas, 1989) can be used to retrieve sample lifetime:

$$\tau = \frac{\Delta\tau}{\ln(I_1/I_0)}$$

Equation 4.14.8

where I_0, I_1 are the non-delayed and delayed image, respectively, and $\Delta\tau$ is the gating time width. This is the same formalism that is also used for two-window time-gated scanning microscopes. This wide-field setup was reported to reach a maximum acquisition speed of 100 Hz, compared to other systems where speed seems to be limited to 1 Hz.

FD-FLIM: Excitation Light

A frequency domain microscope commonly uses a sinusoidally modulated light source whose intensity is described by the following equation:

$$I = I_0\left[1 + m_0 \cos(\omega t + \varphi_0)\right]$$

Equation 4.14.9

where I_0 is average light intensity, m is modulation depth, ω is circular frequency, and φ_0 is a phase bias. The donor emission follows this sine wave but, if periodicity is in a time scale comparable to donor lifetime, emitted fluorescence is demodulated and delayed by the lifetime itself. After proper calibration of the system with a reference sample, it is possible to determine these two parameters which are linked to the sample lifetimes with the following equation:

$$m = \sqrt{\frac{1}{1 + \omega^2 \tau^2}};\ \varphi = \arctan(\omega\tau)$$

Equation 4.14.10

Because typical biologically interesting fluorescent probes exhibit nanosecond-lifetimes, the excitation intensity is modulated at several tens of megahertz. This is achieved by use of acousto-optic modulators (AOM), transparent crystals in which an ultrasonic wave field creates a refraction index grid (Piston et al., 1989). A coherent beam passing through such a crystal is diffracted and the first-order diffraction spot is almost completely modulated. Pulsed-light sources can also be used in the frequency domain (Gratton et al., 2003).

FD-FLIM: Wide-Field Detection

The most common setup for frequency domain imaging is based on a wide-field microscope. The coherence of the modulated laser beam is removed by a spinning ground-glass disc or a continuously vibrated optical fiber to suppress speckle noise and then expanded on the input port of a conventional fluorescent microscope.

To detect demodulation and phase delay of fluorescence emission, fluorescence signal is cross-correlated with the excitation signal by homodyning or heterodyning techniques (Spencer and Weber, 1969; Gadella et al., 1994; also see Lakowicz, 1999). The former technique is simpler and current electronics are sufficiently fast to perform homodyming. Whatever solution is adopted, a device with spatial resolution that can multiply an electronical reference signal with a light signal is needed because it is currently not possible to directly multiply (cross-correlate) two light signals. These devices are the multi-channel plates that are also used for gating in wide-field time-domain implementations. They can be gated to $<$100 psec, which is more than sufficient for typical systems that are operated in the 50- to 100-MHz region. The fluorescence photons arrive at the photocathode of the MCP and generate photoelectrons. The probability that a photon is detected, i.e., a photoelectron is emitted, is proportional to the voltage on the photocathode. This is modulated with voltages proportional to the excitation signal to achieve cross-correlation in the homodyning technique. The number of photoelectrons is subsequently amplified and imaged onto a phosphor screen. The response of the former is slow (milliseconds) and integrates the signal. The resulting image is projected onto a CCD camera. The MCP-CCD ensemble behaves as a phase-sensitive camera (Lakowicz and Berndt, 1991). If the frequency of the gain-modulation is different from the modulated excitation, cross-correlation is performed in heterodyning mode. Here, phase shift and modulation are transferred to a signal at a difference frequency. This passes through the low-pass filter (slow response)

phosphor screen to be sampled by a CCD camera in time. In the homodyning technique, several images are acquired at different, relative phase delays injected into the MCP gain. This image phase stack is compared with the reference measurement and then analyzed by a pixel-by-pixel Fourier analysis algorithm or sine function fitting routine. The demodulation and phase-lag maps are inverted to obtain two lifetime estimations. Multi-component lifetime systems are usually considered non-resolvable with frequency domain techniques, but they can be resolved in the time-domain by multi-exponential fitting routines. Recent works show how at least a two-component lifetime system can also be resolved in the frequency domain by global analysis (Verveer and Bastiaens, 2003). Global analysis has been previously applied to investigate multi-component lifetime systems (Verveer et al., 2000; Squire et al., 2000) using multi-frequency FD-FLIM data.

The authors recently developed an alternative analysis method for resolving lifetime heterogeneity in general and for a two-component (FRETing) system in particular (Esposito et al., unpub. observ.).

FD-FLIM: Phase-Fluorimeters and IMS

The principle by which lifetimes are measured with an FD-FLIM microscope has a history in cuvette-based phase fluorimeters. These systems are commercially available and some solutions are very simple and compact. They are fast and precise and their only limitation is that they perform single-spot measurements. This implies that only an average lifetime can be obtained from a sample. A simple and inexpensive compromise between microscopes and phase fluorimeters is possible and suitable for a large number of biological questions that do not strictly rely on spatial resolution; such a system has been reported (Herman et al., 2001). Here, a LED was driven by a phase fluorimeter to excite a sample in a region of interest. The emitted fluorescence, collected by the microscope, is focussed on the phase-fluorimeter PMT to allow detection of an average lifetime over a whole cell. The intensity modulated multiple-wavelength scanning (IMS) microscopy technique is equivalent to phase fluorimeters, which rely on lock-in amplification. Lifetime imaging by IMS has been reported (Carlsson and Liljeborg, 1998). The main parts of the system are a lock-in amplifier and a scanning head. Scanning is performed at a frequency that is typically several orders of magnitude slower than the frequency of the modulated light. Therefore, a lock-in amplifier sees the scanning mirrors of the microscope frozen in time, enabling it to perform a lifetime estimation. This adaptation of phase fluorimetry for use in microscopy, although interesting, did not find considerable application in the literature.

LEDs and New-Generation Detectors

Although there is no real commercial effort to produce new light sources and detectors specifically for lifetime detection, technology is certainly advanced enough to deliver new potential tools for this field. Both light-emitting diodes (LED) and photodiode detectors have seen tremendous improvements in the past few years thanks to intense industrial research for the purpose of optical data transmission. Very high brightness (up to 10 cd) and rapidly modulatable (up to 200 MHz) LEDs are currently available (Harms et al., 1999; Herman et al., 2001; Landgraf, 2001; O'Hagan et al., 2002). For detectors, there is no immediate commercial perspective for bi-dimensional integration of fast detectors that could store phase- or time-delayed images onto the same chip and with higher resolution than an MCP. However, current state-of-the-art technology did produce interesting detectors like SPDA sensors (Mitic et al., 2003) or CMOS and CCD cameras with ever increasing yield and speed. CCDs were also recently used as fast-gating devices by directly modulating their gain (Mitchell et al., 2002a,b). The production of all-solid-state detectors that store gated images at different time or phase delays on the same chip is realistic and eagerly awaited in the field. The authors are currently exploring the applicability of one such experimental design for lifetime detection.

Other FLIM Techniques

In recent years, several new FLIM techniques were designed. ϕFLIM (van Munster and Gadella, 2004a) is a slightly modified FD-FLIM setup where one of two signal generators is phase modulated, and a different acquisition protocol is adopted. In this setup, steady-state, cosine and sine transforms that are required for Fourier analysis are directly grabbed (see Basic Protocol 1 and Anticipated Results; Eq. 4.14.13) rather than measuring an image phase stack. This speeds up the standard FD-FLIM method and avoids systematic errors that could occur when only a few images are acquired per phase stack. Although ϕFLIM presents a sensible and potentially useful variation on the frequency-domain theme, it is currently more sensitive to noise and further development is required before it becomes a practical alternative.

A standard FD-FLIM setup can also be modified by use of polarized excitation light and detection through parallel or orthogonal polarization analyzers. This technique, called "dynamic fluorescence anisotropy imaging microscopy in the frequency-domain" or rFLIM (Clayton et al., 2002), allows determination of rotational correlation time and fluorochrome lifetime on a pixel-by-pixel basis. rFLIM provides access to information on the environment of the fluorochrome and allows for detection of FRET between spectrally identical FRET pairs, called homoFRET. When energy is transferred from a donor to an identical acceptor species, the depolarization that usually occurs due to rotation of the fluorochrome is emphasized because of the different relative orientations of two homo-transferring fluorescent molecules.

A standard TD-FLIM can be modified with the addition of a second TCSPC detector spectrally separated from the first one. Two acquisitions can be used to determine both lifetime and a spectral maximum estimator at each sample location. This technique is called "spectrally resolved fluorescence lifetime imaging microscopy" or sFLIM and has been successfully used for the detection of single RNA molecules in living cells (Knemeyer et al., 2003).

An FD-FLIM can be operated with more than one acousto-optic modulator (AOM), injecting more than one harmonic component into the fluorescence signal. This system called multi-frequency FLIM or mfFLIM has been developed (Squire et al., 2000) to resolve multi-lifetime samples without the need for taking sequential lifetime images at different modulation frequencies. The same investigation can be performed by injecting a broader harmonic content in the emitted-fluorescence signal by pulsed-laser excitation.

Finally, a pump-probe microscope with lifetime imaging capabilities has been developed (Dong et al., 1995). A pump-laser pulse populates the donor excited state while a probe-laser pulse delayed with respect to the pump pulse, stimulates photon emission. By sampling the emitted fluorescence at different pump-probe time delays, sample lifetimes can be estimated.

Choosing a FLIM Setup

A theoretical figure of merit has been derived (Draaijer et al., 1995; Gerritsen et al., 2002; Carlsson and Philip, 2002; Philip and Carlsson, 2003), which allows comparison between different lifetime detection methods. It is clear that frequency- and time-domain methods converge to ideal performances under optimal conditions. In a recent study (Gratton et al., 2003), frequency-domain and time-domain FLIM were compared on the same scanning setup. It was shown that FD-FLIM performance is better with realistic biological samples. However, TD-TCSPC-lifetime imaging can achieve a time resolution close to 25 psec and exhibits an ideal photon collection efficiency at low photon-counting rates, although under these conditions, acquisition times are very long (1 to 30 min). TD-TGSPC allows increased acquisition and post-processing speeds, above all with the use of two-gate

systems, albeit at the cost of lower photon efficiencies. The IMS frequency–domain technique compares with time-domain systems. All these techniques are mostly performed with two-photon excitation and using confocal, laser-scanning microscopes. Wide-field detection requires use of gated image intensifiers and consequently suffers from a lower spatial resolution and overall photon collection efficiency. However, poor photon counts usually generated by biological samples sometimes require a non-confocal light collection scheme, thereby sacrificing spatial resolution to obtain sufficient fluorescence detection. For these reasons and the simpler hardware required, wide-field FD-FLIM represents a good compromise for lifetime imaging.

The selection of the FLIM system is also strictly dependent on its application. TD-TCSPC can give the most accurate results, and it excels when detailed investigation of photophysical processes is required. TD-TGSPC can be more efficient for biological applications, and a wide-field FD-FLIMs can, in general, be faster and easier to operate but with lower spatial resolution. Specific lifetime detection variants, briefly described previously, can also offer particular advantages.

Commercially available TD and FD systems, also in their simplified versions, can give sufficiently reliable lifetime estimations to be used as quantification instruments for detection of molecular interactions and protein biochemical states by FRET. These systems also offer less-expensive and easier-to-use solutions when compared with the typical custom-built FLIM setups.

In the following section two protocols are described for the operation of wide-field frequency-domain and a laser-scanning two-photon TCSPC time-domain lifetime microscope. A more detailed description of the hardware and some typical results are in Anticipated Results. With information given in the first section and the Literature Cited, the adaptation of these protocols to wide-field TD and scanning FD is straight-forward.

Sample preparation protocols are available in other Current Protocols books, in this chapter, and regarding FRET application, specifically *UNIT 17.1*.

BASIC PROTOCOL 1

OPERATING A WIDE-FIELD FD-FLIM SETUP

Proper calibration of the microscope setup is required to obtain reliable quantitative lifetime estimations. The optical path, hosted on an adequately vibration-isolated optical table, should be aligned and maintained periodically and checked before each use. Without a proper calibration, FD-FLIM systems are vulnerable to systematic errors. This does not affect the qualitative meaning of the measurements, but a fine calibration can be necessary for more quantitative estimations (Hanley et al., 2001). The present protocol describes the standard alignment and calibration procedure for a frequency-domain wide-field system. Furthermore, the acquisition of an image phase stack and its post-processing for estimation of sample lifetime are illustrated. Although there are several possibilities for building and operating a frequency-domain lifetime microscope, this protocol describes the most common and robust setup.

Materials

Single-exponential decaying fluorochrome with a well-defined lifetime (e.g., Rhodamine-6G)
Basic components of a wide-field FD-FLIM (see Commentary)
Positive-intrinsic-negative (PIN) diode connected to an oscilloscope (bandwidth up to 100 MHz)
Mirror, reflective foil, homogenous flat fluorescent or scattering sample

CAUTION: Protective goggles matching the used laser wavelength and power are recommended when aligning the optical path. Lasers can be harmful and standard laser safety rules should always be followed. Moreover, handle the optics with cotton gloves to avoid dirty or scratched optical surfaces.

Tune and align laser

1. Tune and peak laser at the desired frequency.

   This is straightforward with lasers typically used in the frequency domain. Every time the light wavelength is changed, the optical path requires subtle re-alignment because the optics focus light in slightly different positions.

2. Align acousto-optic modulator (AOM) to obtain a diffraction pattern (see Figure 4.14.4, top panel). Drive AOM with proper frequency.

   The AOM modulates the laser beam when it passes almost longitudinally, but it is slightly tilted through the radio frequency (RF)-driven crystal.

3. Get the laser beam to pass longitudinally through its optical window. Slightly tilt the AOM.

   If no diffraction pattern is detected, change the driving frequency and iteratively adjust the crystal position until a clear diffraction pattern appears.

4. Align and tune the AOM to reach the best modulation performance. Monitor light intensity of the first-order spot of the diffraction pattern with the PIN diode connected to the oscilloscope. Sweep frequency to obtain maximum light modulation. Adjust voltage on the AOM to reach maximum modulation with minimum possible distortion. Re-align the AOM in a fine manner to optimize light modulation.

5. Select the first-order spot, attenuate, and de-speckle the laser light.

   The first-order spot can be selected by an iris. A neutral density filter wheel can be used to attenuate the laser light when required. The laser beam, before being expanded, can be de-speckled by focusing it on a multi-mode fiber optic cable that is continuously vibrated (50 Hz provided by the mains are enough). Speckle noise can also be removed by a spinning ground-glass disk, but a vibrating optical fiber is the best choice for simplicity and user safety.

6. Couple laser beam with microscope.

   The laser beam can be expanded by a lens of proper focal length onto one of the input ports of the microscope.

7. Align laser beam with microscope optics by imaging a flat and homogeneous sample like a reflective foil, a mirror, a scattering or a fluorescent solution. Move the coupling optics to try to obtain the most homogeneous and bright field. Close, if available, the field iris aperture of the microscope and, with a focussed sample, move optics so that the iris shape is focussed together with sample. Repeat all previous adjustments iteratively to find the best compromise between brightness, homogeneity, and focus of the excitation light source.

8. Calibrate microscope with a reference lifetime by placing a reference sample like a zero-lifetime sample (reflective foil or scatterer) or a solution of a well-known and characterized single-exponential lifetime fluorochrome on the stage. Measure modulation and phase-lag of the reference according to steps 9 to 15 and use these parameters to calibrate a lifetime imaging (Equations 4.14.13 to 4.14.15).

   A pixel-by-pixel calibration can be necessary if the detector response is not spatially homogeneous.

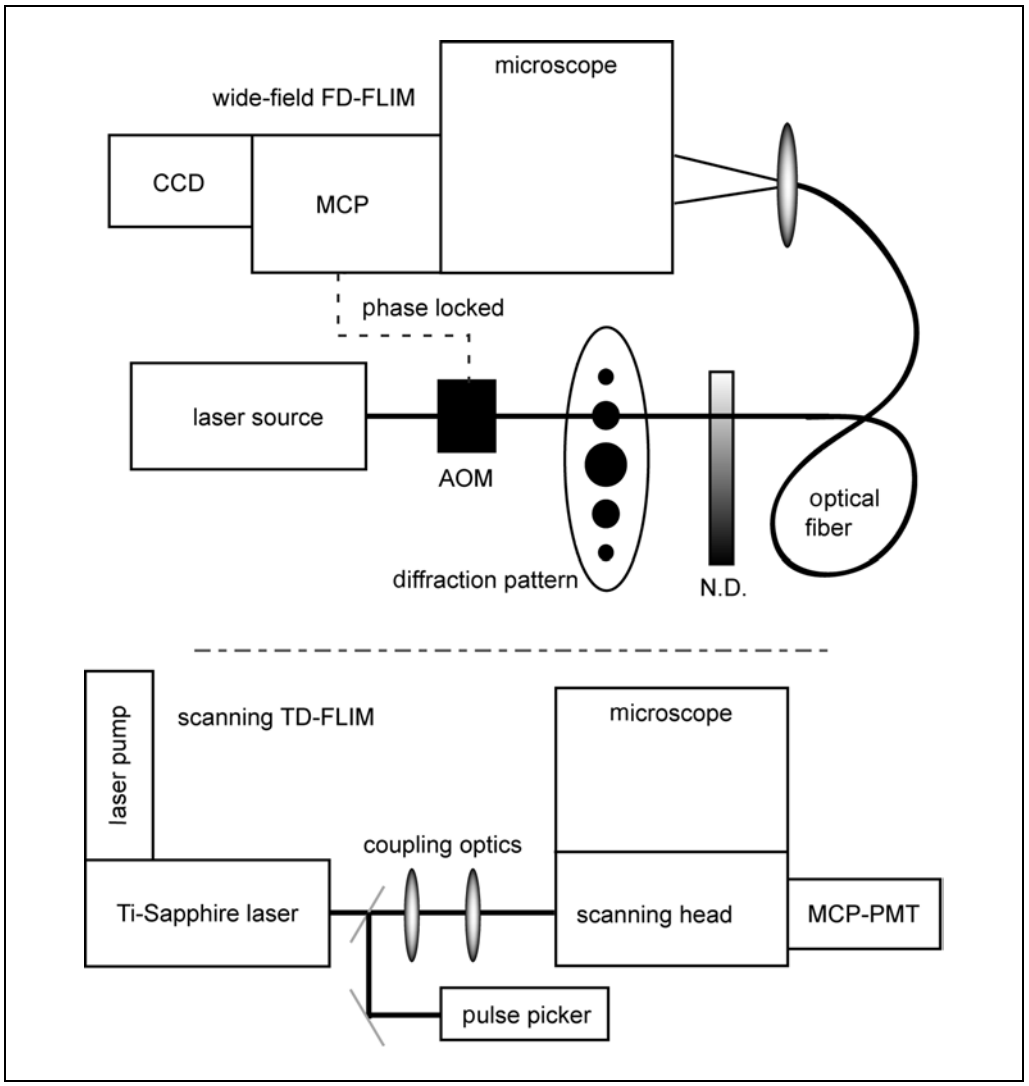


Figure 4.14.4 Typical setup diagrams of wide-field FD-FLIM and two-photon laser scanning TD-FLIM. In wide-field frequency-domain lifetime microscopy, a continuous-wave laser is usually sinusoidally modulated by an acousto-optic modulator. The first order spot is selected, attenuated, and subsequently focussed into a continuously vibrated optical fiber. The laser beam is then expanded onto an input port of a wide-field microscope. The fluorescence emission is focussed on the MCP photocathode and its phosphor screen output is imaged by a CCD camera. In laser scanning TCSPC time-domain FLIM, a pulsed-light source, like a green laser, pumps a Ti:Sapphire NIR laser coupled to the scanning head of a microscope. The laser beam is split and monitored by a photodiode to provide the trigger for the TCSPC timing. The emission fluorescence is focussed on the single-photon counting detector. A detailed description of the setup is given in Anticipated Results.

Acquire a lifetime image

9. Initialize imaging parameters. Place sample on the stage. Once the field-of-view to be imaged is selected, set exposure time, gain, and laser intensity of the system avoiding saturation of the detector during phase stack acquisition.

   This can be done by adjusting the phase delay to get maximal fluorescence emission from the focussed sample and increasing gain or laser power below the detector saturation level.

10. Set the microscope to zero-phase delay.

    To speed-up phase stack post-processing, it is useful to remove system phase bias so that acquisition of images starts at the phase at which a zero-lifetime reference is in phase with the source light. The images need to be recorded at equal phase-delay steps over the complete period of excitation light modulation in a number greater than or equal to three.

For fast measurement, a two-image counter-phase acquisition schema can also be used as proposed by Schneider and Clegg (1997).

11. Acquire phase stack by initializing the system and opening the excitation light shutter. Grab an image and close the excitation light shutter. Change the relative phase of the detector and complete phase stack acquisition.

    Appropriate time-delays in the acquisition protocol could be necessary. The use of motorized shutters avoids photobleaching during dead time of phase stack acquisition. Their use can be limited to the start and end of acquisition to decrease acquisition time.

12. (Optional) Correct phase stack for photobleaching. Acquire a second image phase stack with a reverse-phase protocol.

    The two stacks can be averaged to compensate for photobleaching. In this manner, all the images are acquired with the same cumulative light dose and the phase stack is corrected at the first-order approximation allowing lifetime estimation also in presence of significant photobleaching (in practice, up to 50%).

13. Compute Fourier coefficients (Equation 4.14.13).

    The image phase stack can be summed to obtain a standard fluorescent image (F_0) and summed after the multiplication of each image by the cosine (F_{cos}) or sine (F_{sin}) of the phase at which they are acquired (see Anticipated Results: Wide-field FD-FLIM). The resulting images are the sine and cosine transforms of the phase stack and together with the stack average, they represent the zeroth (steady-state fluorescence image) and first Fourier coefficients.

14. Compute a mask for lifetime images by applying a threshold to the DC image. Compute the lifetime using the mask to avoid unnecessary computation and possible numerical errors.

15. (Optional) Compute phase delay and demodulation factors according to Equation 4.14.14.

    This step is necessary during the acquisition of a reference. Skipping this step for the phase lifetime estimation saves a significant amount of computational time.

16. Compute sample lifetimes by Equation 4.14.15.

    The algorithm implementation of Equations 4.14.13 through 4.14.15 is used to retrieve two lifetime estimations of the sample. Step 15 can be skipped by substituting Equation 4.14.14 in 4.14.15. The images should always be masked to remove background and possibly computed only on unmasked pixels to reduce processing time.

17. Analyze lifetime estimations.

    Controls with single fluorochromes without interaction with the environment should appear flat in contrast to a typical steady-state fluorescence image. Lifetime distributions can be analyzed to detect lifetime subpopulations and bi-dimensional histograms of phase lifetime versus modulation lifetime (or vice-versa) allows the detection of lifetime subpopulations and heterogeneities.

OPERATING A TD-FLIM BASED ON TPLSM

BASIC PROTOCOL 2

A time-domain FLIM based on a two-photon microscope requires certain know-how for daily maintenance and regular realignment of the system. Although significant advances were made in recent years in production of user-friendly systems, like picosecond all-solid-state lasers and self-tuneable Ti:Sapphire light sources, use of laser sources like the Tsunami (Spectra Physics) and the Mira (Coherent) is still more common in most microscopy facilities. This protocol describes alignment of a typical scanning microscope with a manually operated Ti:Sapphire light source. These near infrared (NIR) lasers possess emission powers that are harmful to the eyes and, depending on the tuned wavelength,

are not visible to the naked eye. For this reason, the following protocol needs to be followed with particular care and respect to user safety regulations for systems with open optical paths. The present protocol describes how to align and perform daily maintenance of a two-photon system and explains its use for lifetime imaging.

Materials

Homogenous flat fluorescent sample
Basic components of a scanning TD-FLIM (see Strategic Planning and Critical Parameters)
Infrared viewer

CAUTION: Wear protective goggles matching near-infrared radiation when aligning the optical path. Lasers can be harmful and standard laser safety rules should always be followed. Moreover, handle the optics with cotton gloves to avoid dirty or scratched optical surfaces.

Tune and align laser

1. Check the laser pump alignment.

   A Ti:Sapphire laser is pumped by a green laser. This pump should be optically aligned. Its alignment is not part of daily maintenance but a lasing failure of the NIR laser could be caused by loss of proper positioning and focusing of the pump beam on the Ti:Sapphire crystal.

2. Tune and peak Ti:Sapphire laser at the desired frequency. Peak the power of laser by iteratively moving main mirror adjustment controls and adjust positioning of the cavity prism or prisms to obtain laser mode locked. Tune light wavelength at 10- to 20-nm steps adjusting peak power and prism position at each step. Once operational wavelength is reached, fine peak the power and check for mode-locking.

   A manually operated two-photon laser cavity needs to be properly tuned and aligned before every use. If lasing fails, it might be necessary to clean the optical components from dust. Non-vacuum cavities can require opening, adhere to the user manual of the laser supplier in this case.

3. Align optics for coupling of laser with microscope scanning head. Couple a femtosecond-pulsed laser with the scanning head of a microscope by air because fiber optics ruin the pulse-shape and consequently the efficiency of the TP excitation. Align all optics, especially after changing the wavelength over broad ranges. Align mirrors from the laser source towards the scanning head, centering each optic element with iterative use of positioning controls. When the scanning head is reached, center the laser on the scanning mirrors through the input optics.

4. Fine align microscope scanning head by placing a homogeneous and bright fluorescent sample on the microscope stage and then scanning the sample with sufficiently high power, under fast scanning conditions and without zoom.

   Increasing power, gain, integration time, and sweeping the focus position could be necessary to detect a fluorescent signal. If no signal is detected, coarsely reposition the laser beam at the scanning head input until a signal appears, avoiding sudden and long saturation of detectors. Find a compromise between homogeneity and brightness of the field of view by iteratively adjusting the last mirror of coupling and positioning of the scanning head. For rough alignment, it is sometimes necessary to remove the objective. It is relatively easy to detect the presence of a misaligned laser beam by placing a white paper in the microscope optical path. It is now straightforward to center the beam and start with fine alignment.

5. Operate the microscope.

   Once a laser source is focussed on the sample, FLIM is ready for operation. Usually a lifetime reference is not required because time response of the system can be retrieved directly from the data fit, but particular systems could require further calibrations. For instance, use of a picosecond—rather than a femtosecond—pulsed laser requires measurement of the laser pulse shape to deconvolve time distributions and retrieve more quantitative and reliable measurements. If time-domain electronics lose synchronization with laser pulses, check (if available to inspection) the position of the photo-diode that provides the timing trigger.

Acquire a lifetime image

6. Initialize imaging parameters. Set sample on stage and once the field of view is selected and focussed, select an adequate image pixel resolution.

   Imaging with more than 512 × 512 pixels is not recommended because of poor photon counts that can be reached. The expert user will find a good compromise in choosing a proper image dimension. In any case, the image can be spatially binned in the post-processing stage. Find a balance between laser power and detector gain. Increasing light intensity will cause more photodamage and photobleaching of the fluorescent dye, but a low excitation rate requires longer integration times to reach suitable photon counts. Two-photon excitation, although reducing photobleaching over three dimensions, increases loss of fluorescent probes and sample damage in the single focal plane with second- or higher-order intensity dependence. In this case, but not for single-photon excitation, lower laser power and longer integration times reduce photobleaching.

7. Acquire an image by starting acquisition and allowing the electronics to store a time stack for an appropriate integration time.

   It is not rare to collect photons for ≥1 min although only experience can suggest a correct time window.

8. Analyze the time stack by fitting the time-decay distribution with the most appropriate model. Fit pixel by pixel the time stack with a simpler function that models the investigated photophysical system (commonly, a single-exponential fit). Analyze residuals and check for particular correlations or trends.

   After a good fit, the residuals represent only noise and are randomly distributed around zero. If this is not the case, a different model is required.

9. Analyze lifetime image.

   The simpler fit procedure obtained by a mono-exponential decay results in an intensity image, a lifetime map, and a χ^2 output that represents, pixel by pixel, the goodness of fit. The lifetime map needs to be masked based on tresholded intensity and χ^2 images. Pixels with a total amount of photons <100 have an uncertainty >10%. An intensity threshold minimly between 10 (>30% uncertainty) and 100 counted photons is suggested. Also, mask out pixels that exhibit high χ^2 values.

10. (Optional) Fit more complex decay distributions.

    If analysis of residuals or χ^2 values suggests fitting with more complex functions, repeat step 8 with a different model. Addition of more fit parameters also allows the fit to adapt better to a noisy distribution. Only apply different models if χ^2 values reduce consistently. A multi-exponential decay fit typically results in images representing the population size of each time component, the lifetime images, and a χ^2 map.

11. Analyze lifetime distributions.

    Once the time stack is processed and converted into lifetime maps, analysis of the lifetime distribution allows detection of different lifetime populations. Inspection of the intensity image segmented by the corresponding lifetime values allows detection of possible different populations or presence of artifacts.

COMMENTARY

Background Information

Fluorescence lifetime detection has been available from the 1920s when E. Gaviola built the first phase fluorimeter. The main developments and biological applications of lifetime detection were carried out by Gregorio Weber and colleagues. It should be noted that fluorescence lifetime measurements and FRET, although known for a long time, could only recently be implemented in microscopy by development of technologies that were necessary to construct the first FLIM systems capable of quantitative FRET determinations. In the 1980s, significant advances in both phase-sensitive detection (Gratton and Limkeman, 1983; Gratton et al., 1984) and time domain (O'Connor and Phillips, 1984) led to the implementation of lifetime detection in microscopy. The first FLIM microscopes were wide-field frequency (Wang et al., 1989; Morgan et al., 1990; Lakowicz and Berndt, 1991; Gadella et al., 1993) and time-domain (Wang et al., 1991) setups. Soon after, time-correlated (Ghiggino et al., 1992) and time-gated (Buurman et al., 1992) single photon counting techniques were adapted to scanning microscopes, followed by the combination of FLIM and two-photon excitation (So et al., 1995). A third class of lifetime detection was introduced later (Dong et al., 1995) based on stimulated emission imaging induced by pump and probe excitation.

Due to its high value for biology, FRET imaging by FLIM took only a few years to be applied (Oida et al., 1993; Gadella et al., 1995). Especially together with advances in molecular biology and the availability of visible fluorescent proteins (Lippincott-Schwartz et al., 2001; Zhang et al., 2002), FLIM became one of the most quantitative tools available for detection of protein-protein interactions and protein biochemical status inside cells (Bastiaens and Pepperkok, 2000; Wouters et al., 2001; Elangovan et al., 2002; Bunt and Wouters, 2004).

In less than two decades, FLIM has been continuously improved and today presents a mature technique that, thanks to burgeoning technologies, is continuously developing into more sensitive and user-friendly systems.

Critical Parameters and Troubleshooting

FRET efficiency

Several aspects should be considered in the selection of a FRET pair. First, the donor needs to be a fluorescent (or luminescent) molecule with a high quantum yield and should be excitable in the range of available excitation wavelengths. The acceptor absorption spectrum should overlap significantly with the donor emission spectrum, and its extinction coefficient should be high in order to increase the Förster distance. There are no particular requirements for the quantum yield of the acceptor that could also be a non-fluorescent molecule. The FRET efficiency can also be increased by optimizing the rotational parameter κ^2 by modification of the linker between the fusion protein and the donor/acceptor fluorescent protein. The contribution of a rotational effect to FRET in a biosensor construct can be evaluated by swapping donor and acceptor. If FRET efficiencies change, κ^2 optimization should be considered to increase the energy transfer efficiency.

Spectra

Narrow spectra are commonly required for multi-staining experiments. When using FLIM to image FRET, it is preferable or necessary to avoid spectral contamination. Even though lifetime measurements are less susceptible to spectral contamination and multi-component lifetime systems can, in principle, be resolved by multi-exponential fitting in the time domain or by multi-frequency measurements in the frequency domain, acceptor fluorescence bleed-through can inject artifacts in FRET estimation, and direct acceptor excitation can reduce or suppress the energy transfer process. Nevertheless, the strictest spectral requirement for FRET is to maximize donor emission and acceptor absorption spectral overlap, but not at the cost of significant spectral contamination.

If acceptor bleed-through in the donor channel is impossible to remove, the acceptor lifetime can be detected as signature of sensitized emission. This technique has been used in the frequency domain (Harpur et al., 2001) and can probably also be implemented in the time domain. In fact, although time domain measurements resolve multi-exponential decays, low photon counts or the use of a two-window time-gated system could require such detection when non-negligible spectral contamination is present. The use of a low-quantum-yield (or dark) acceptor completely eliminates the spectral contamination problem in FRET experiments.

Photobleaching

Photobleaching is the photo-inactivation of a chromophore and can affect both donor and

acceptor. Both phenomena can, in fact, be exploited in specific intensity based FRET detection methods (Wouters et al., 1998). In all other FRET detection measurements, photobleaching should be minimized. Even though lifetimes are intensity independent, lifetime imaging could also suffer from photobleaching artifacts. The most evident problem is degradation of the signal-to-noise ratio. In scanning time domain, which usually requires longer acquisition times, a good compromise is needed between excitation light intensities and acquisition times. Furthermore, TCSPC is less efficient at very high photon counts (Gratton et al., 2003). Reducing laser power, i.e., emitted photons, while increasing acquisition time, avoids saturation effects. This is also effective to reduce photobleaching when using two-photon excitation. TPE, in fact, reduces bulk photobleaching, but photobleaching in the focal plane is increased and usually even exceeds the excitation rate because of the intrinsic non-linear photophysics of TP absorption (Patterson and Piston, 2000). Prolonged exposure times also increase the collection of autofluorescence especially when high photobleaching occurs. Autofluorescence can thus inject more heterogeneity in the measurement, preventing adequate data fitting and typically reducing measured lifetimes. Time-gated systems can cancel out short-lifetime autofluorescence contribution by use of an initial gate-off time. More photostable fluorochromes, higher concentrations of fluorophores, and anti-bleaching agents can be used to reduce this problem.

The wide-field frequency domain lifetime imaging (and wide-field time domain) method is based on the sequential acquisition of images to create an image phase stack (time stack). Photobleaching can modify the phase time stack, injecting artifacts in the lifetime estimation. Up to 15% of photobleaching can be tolerated. Photobleaching tolerance can be further increased by correcting the phase stack by acquisition of a second phase stack with a reverse phase delay protocol. More recently, the randomization of phase-image acquisition was shown to significantly reduce photobleaching artifacts without the need of the reverse-phase decay protocol (van Munster and Gadella, 2004b). The average of the forward and reverse phase stacks is then used for a lifetime estimation. This correction relies on a first-order approximation of photobleaching which holds up to 50% of signal loss (Gadella et al., 1994). Furthermore, the phase-shift and demodulation lifetime estimations diverge due to photobleaching. Their averaged value is, therefore, also quite stable at non-negligible levels of photobleaching.

In conclusion, donor photobleaching can be afforded both in time domain and frequency domain by wide-field and by scanning setups. In contrast, FLIM estimations of FRET can seriously suffer from acceptor photo inactivation. Acceptor photobleaching can be caused by direct excitation and by energy transfer itself. The result is a loss of FRET and concomitant recovery of donor fluorescence. Therefore, when the energy transfer is reduced by acceptor bleaching, FLIM detects increasing amounts of non-FRETting donor lifetimes. In this case, increased exposure times cause a dilution, and eventually a loss, of the FRET signal.

This problem can be minimized by avoiding direct excitation, e.g., by the choice of adequate FRET pairs, use of fluorophores that exhibit a large Stoke's shift, and use of an acceptor with considerably lower photobleaching susceptibility than the donor, e.g., photostable and/or low-quantum-yield dyes.

The commonly used FRET pairs GFP-YFP and Cy3-Cy5 suffer from such an unfavorable bleaching susceptibility. Also, the new combination of GFP and monomeric RFP is affected. Good combinations are GFP-Cy3 and pairs that use the photostable Alexa dyes as acceptor.

Furthermore, it should be noted that for donor-acceptor systems that are free to diffuse and contain an excess of acceptor molecules, photobleaching by FRET generally does not present a problem as bleached acceptors are quickly replenished.

Fluorochrome lifetimes

Contrast enhancement by lifetime imaging is also applicable when the fluorochromes are spectrally similar but exhibit separable lifetimes. This method is very useful as lifetime distributions are generally sharper than spectra, allowing experimental designs where spectrally mixed emissions are resolved by lifetime (Pepperkok et al., 1999). Furthermore, it is possible to combine lifetime and spectra data (Siegel et al., 2001b) to unmixed emissions from multiple fluorescent species (Carlsson and Liljeborg, 1997; Neher and Neher, 2004).

Fluorochromes can have multi-exponential or complex fluorescence decays. CFP, for instance, exhibits a double-exponential decay. Furthermore, lifetimes of this GFP mutant are dependent on excitation wavelength. Lifetime heterogeneities, whenever possible, should

be avoided because these decays require more photons to be resolved, and the lifetime distributions will be broader. The recently published Cerulean CFP mutant (Rizzo et al., 2004) was reported to exhibit improved lifetime characteristic and a 2.5-fold increase in brightness, which could potentially alleviate this problem associated with the use of CFP in FLIM.

In a FRET experiment, it is advisable to select longer lifetime donor fluorophores as the determination of FRET using subnanosecond lifetime dyes becomes more difficult and uncertain. With respect to FRET efficiency, there are no particular requirements to the acceptor lifetime other than the practical consideration of acceptor photobleaching prevention by selection of short lifetime dyes. The latter also carry the advantage that the generation of a considerable acceptor-excited population is avoided. As acceptor fluorophores in the excited state cannot accept another quantum of energy from a nearby excited donor molecule, an excited population represents a FRET-incompetent fraction that frustrates the FRET phenomenon (Schönle et al., 1999; Jares-Erijman and Jovin, 2003.).

False negatives and false positives

With the exception of instrumental errors and improper use, FRET imaged by FLIM cannot practically detect false-positive interactions. In contrast, false-negative detection can be caused when the distance or orientation between fluorophores on interacting molecules is beyond the limit for efficient FRET detection. For this reason, FRET-resolved interactions are reliable but absence of FRET only carries information in a very limited number of cases like in biosensors based on linked FRET pairs. The two rotational and one distance degrees of freedom play a crucial role in the detection of FRET. Both factors can be optimized by finding the proper location for positioning of chromophore conjugation or by optimizing linkers. Without detailed structural information, however, this optimization will have to be performed empirically.

Acquisition times and signal-to-noise ratio

The acquisition time of a lifetime image can vary over a broad range from fractions of seconds up to several minutes depending on the adopted system and on the sample brightness. A high-speed time-gated time-domain FLIM has been developed (Agronskaia et al., 2003) that reaches a sampling frequency of 100 Hz. Similar implementations can probably be designed for frequency domain setups. In general, standard FD and TD setups usually acquire images at ~1 Hz. The most sensitive TCSPC-based TD-FLIM, using a MCP-PMT detector, can rarely be operated with exposure times <1 min for a reasonably sized image, and when multi-exponential decays or FRET are detected, the acquisition times can increase up to 30 min (Becker et al., 2004). Other time-domain systems can limit the integration time to some seconds (Gerritsen et al., 2002). A standard wide-field FD-FLIM is usually operated on time scales well under 1 min and can easily approach 1-Hz acquisition rates independent of image size. These systems also exhibit shorter processing times thanks to the very efficient Fourier analysis algorithm implementation. TCSPC is computationally expensive because of the fitting procedure while a two-window time-gated system also reaches fast post-processing speeds due to the rapid RLD algorithm. The use of faster acquisition protocols and algorithms has been described; these allow a wide-field FD-FLIM (Schneider and Clegg, 1997) and a scanning TD-FLIM (Trabesinger et al., 2002) to operate in real-time.

Acquisition time is a crucial parameter, not only for the detection of short-lived events or motile structures in living cells, but also in connection with problems described for photobleaching. The lower limit of exposure times is set by the requirement for sufficient signals for a reliable lifetime determination. For single photon counting, in which poissonian noise dominates, error in the intensity image is equal to the inverse of the square root of the photon count. The error in lifetime estimation is larger. For time-domain detection, depending on the system, the error can become twice as large. Therefore, care should be taken that acquisition times always produce photon counts that exceed a tolerable uncertainty threshold. More than 200 counts, for example, are required for a reliable mono-exponential decay fit and 1000 counts give ~5% of statistical error in the lifetime estimation, while a bi-exponential fit requires at least 10,000 counts (Köllner and Wolfrum, 1992).

The selection of acquisition times in other FLIM systems relies more on the experience of the user. In any case, the error in a lifetime estimation for different intensities can be verified by the analysis of the standard deviation of the retrieved lifetime in samples with homogeneous lifetimes, e.g., fluorophore solutions.

Anticipated Results

Scanning TD-FLIM

The time-domain FLIM setup that was used (Figs. 4.14.5 and 4.14.7) is an upgraded TSC-SP2 AOBS laser scanning confocal microscope (Leica Microsystems) equipped with a Ti:Sapphire Mira900 two-photon laser pumped by a Verdi-V8 laser (both from Coherent) in the mode-locked femtosecond-pulsed regime (Fig. 4.14.4, bottom). The laser was tuned at 820 nm or 900 nm for proper TP excitation of CFP and GFP, respectively (Chen and Periasamy, 2004). A custom-made emission filter wheel is placed between the output port of the scanning-head and the TD-FLIM detector, an MCP-PMT (R3809U-50 by Hamamatsu Photonics) that allows high sensitivity and sub-nanosecond time resolution. Fluorescence emission of CFP without YFP contamination was detected using a band-pass filter centered at 480 ± 15 nm and a 520 ± 15 nm filter was used for GFP detection. The time-resolved fluorescence decays are reconstructed by TCSPC. The acquisition board (SPC830) and software (SPCImage) were from Becker & Hickl.

Figure 4.14.5 shows a TD-TCSPC image of two constructs that were expressed simultaneously in HeLa cells. This preparation shows how FRET reduces donor lifetimes and how lifetimes enhance contrast. The first construct consists of a tandem fusion of CFP and YFP that exhibits efficient FRET. This construct is too large to enter the nucleus passively and, accordingly, the signature of ET, i.e., reduced CFP lifetime, is detected in the cytoplasm. The average lifetime in this region is 1.75 ± 0.13 nsec (mean ± standard deviation), which is significantly reduced compared with 2.28 ± 0.05 nsec reported for CFP in the literature (Elangovan et al., 2002; Becker et al., 2004). In the spectral window used, the CFP-YFP construct is spectrally completely equivalent to the second construct, CFP fused to a nucleolar targeting signal. The lifetime measurement shows a clear contrast between the nucleoli and the cytoplasm that identifies the two different constructs. This is shown in Figure 4.14.5 where the intensity image (Fig. 4.14.5A) is segmented according to the lifetime map (Fig. 4.14.5B). A lifetime threshold of 2 nsec allows the clean discrimination between two differently stained areas (Fig. 4.14.5C,D). The mean lifetime values over the nucleoli were equal to 2.20 ± 0.07 nsec, in full agreement with the reported lifetime of CFP and equal to measurements on cytoplasmically targeted CFP alone (not shown). The nucleolar CFP signal presents an intrinsic non-FRETting lifetime reference that allows the quantification of the FRET efficiency: 20 ± 6% (errors computed by error propagation). This reference can also be created by photobleaching YFP in a defined region of the cell (not shown).

The CFP exhibits a bi-exponential excitation wavelength-dependent lifetime by itself. The use of this fluorescent protein for FLIM is possible but its lifetime heterogeneity can limit its quantitative use. This is more relevant to FRET analysis because the number of photons that should be collected to quantify the multi-exponential decay of CFP FRETting and non-FRETting fractions is difficult to achieve in biological samples.

In general, the analysis of the χ^2 values (Fig. 4.14.5E,F) is crucial for data interpretation because they represent goodness of fit. In particular, the time stack needs to be fitted pixel by pixel with the proper model. Very often, it is necessary to spatially bin images in order to achieve suitable counts for either mono- or bi-exponential decay fits. For these reasons, it is necessary to fit only those pixels where an adequate number of photons are collected. At the end of the fitting procedure, the χ^2 (the authors always refer to χ^2 normalized to degrees of freedom) should be close to unity. A detailed overview of χ^2 analysis is presented by Lackowicz (1999). The χ^2 maps for single and bi-exponential fits of this data set are shown in Figure 4.14.5E,F. It is clear that the bi-exponential fit, although not ideal, is more reliable than the single-exponential fit. Furthermore, the average χ^2 value in nucleolar areas is 1.05 and 1.02 for mono- and double-exponential decay but 2.01 and 1.26, respectively, for cytoplasmic regions. Fits are less precise in these areas because of increased lifetime heterogeneity due to some conformational freedom in the CFP-YFP construct. This also increases the heterogeneity in FRET efficiencies, i.e., measured lifetimes. Another important consideration is demonstrated in Figure 4.14.5E, where the χ^2 values in dimmer regions are lower. This is a misleading artifact caused by the fact that the fitting algorithm (Eq. 4.14.11) tolerates more noise in regions with low intensity:

$$\chi^2 = \sum_i \frac{\left(N_i^{(\mathrm{fit})} - N_i^{(\mathrm{obs})}\right)^2}{N_i^{(\mathrm{obs})}}$$

Equation 4.14.11

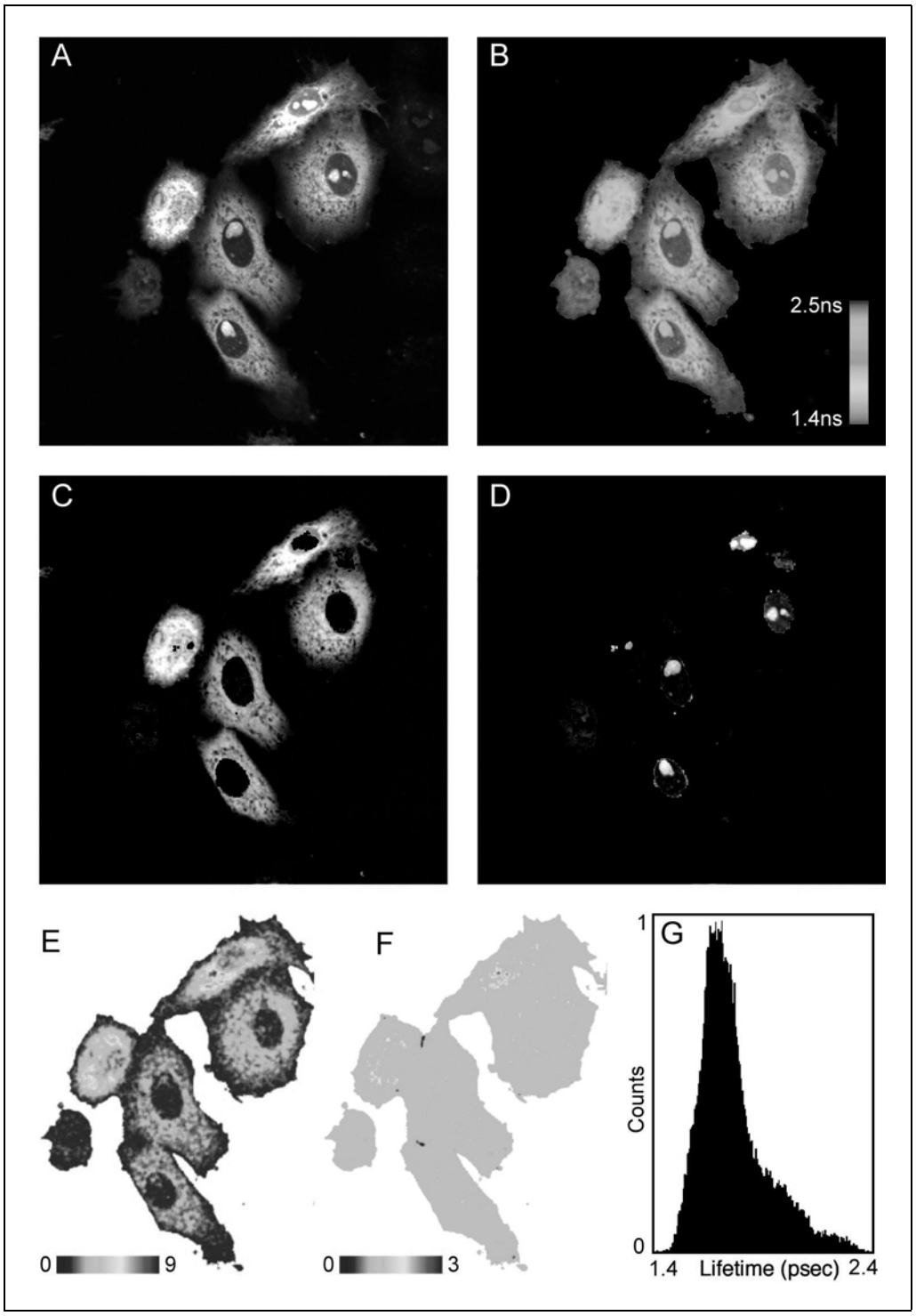


Figure 4.14.5 HeLa cells expressing nucleolar CFP and a cytosolic CFP-YFP (Venus) fusion construct. The two-photon laser scanning single-photon counting image (**A**) is acquired through a band-pass filter centered at 480 ± 15 nm in the CFP spectral band. The spectrally undistinguishable emission from the two cyan constructs is adequately segmented in a cytoplasm (**C**) and nucleolar (**D**) staining by tresholding the lifetime map (**B**) at 2 nsec. Chi square analysis is required to judge the reliability of the lifetime estimations and the fitting model (**E,F**). (**G**) shows the sample lifetime distribution. The long-lifetime shoulder corresponds to the non-FRETting CFP in the nucleoli. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.interscience.wiley.com/c_p/colorfigures.htm.*

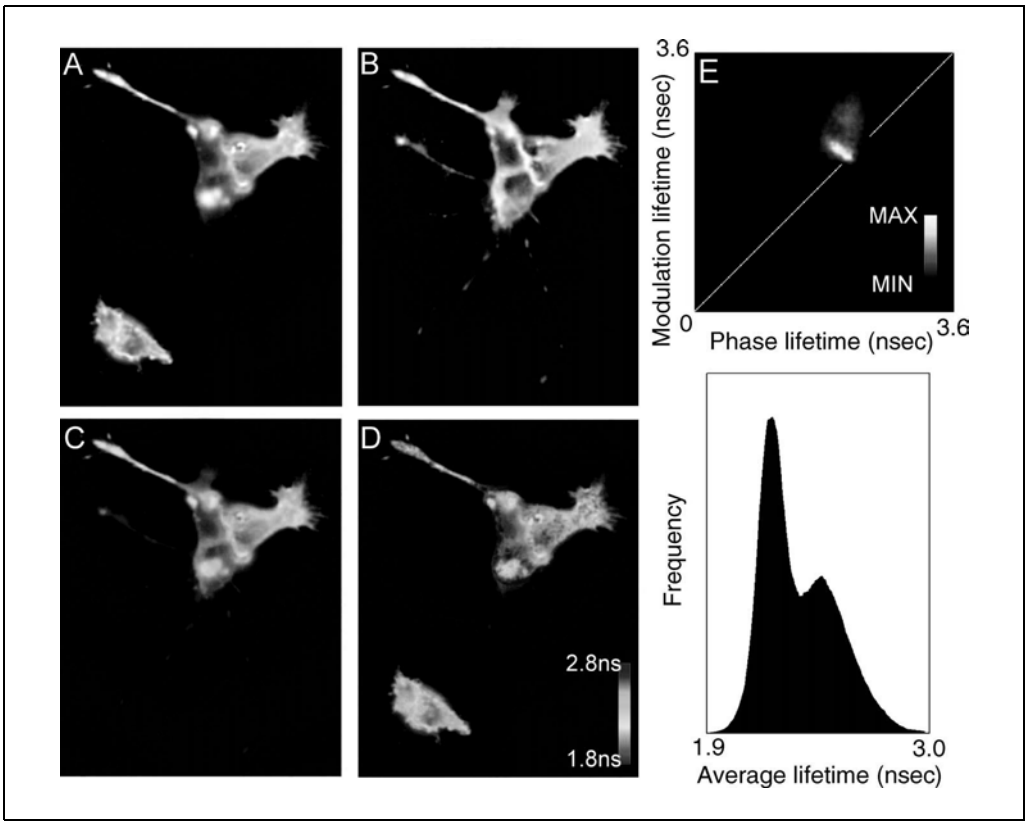


Figure 4.14.6 Human SHSY-5Y neuroblastoma cells expressing a GPI-GFP construct (**A**) simultaneously stained with cholera toxin–Alexa594 (**B**). The images are acquired by a wide-field microscope and the overlay (**C**) of the GFP and Alexa594 reveal most extensive colocalization in the top right cell that extends one process to the upper left of the image. The lifetime map (**D**), acquired by wide-field FD-FLIM, reveals the locations where the donor and acceptor are interacting. A control cell is shown in the inset in **A** and **D**. The bi-dimensional histogram (**E**) shows the two populations of pixels corresponding to the non-FRETting (upper right) and FRETting (lower left) fractions. The bottom-right panel shows the bi-dimensional distribution of the average lifetimes. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.interscience.wiley.com/c_p/colorfigures.htm.*

In fact, each squared difference between the experimental data points and the fit (squared residuals) is normalized to the variance of the measured (obs = observed) number of photon that, for the photon counting technique, is equal to the observed number of photons itself. In dimmer regions, an inappropriate model can fit a noisy measurement and the χ^2 determination tolerates larger errors. Since it is not always possible to fit the right model with adequate photon counting statistics and because systematic errors can increase the χ^2 value, χ^2 analysis is always required but should not be over-interpreted. Analysis of the residuals can help to judge the reliability of the hypothesized model. Residuals should be distributed around zero, representing only noise. Non-random residual distributions are an indication to change the fit model. The use of a stretched exponential fit (Eq. 4.14.12) seems to be a good compromise for analysis of time-domain data (Siegel et al., 2001a):

$$I_0 e^{-\left(\frac{t}{\tau}\right)^{\frac{1}{h}}}$$

Equation 4.14.12

with the addition of only one parameter (h) to the single exponential decay, heterogeneous distributions can be suitably fitted. This is because stretched exponential fits consider a continuous distribution, rather than discrete lifetimes.

Wide-field FD-FLIM

The frequency domain FLIM (Fig. 4.14.4, top) is custom-built around a fully automated Axiovert200M inverted fluorescence microscope (Zeiss) and an LSTEP (Märzhäuser

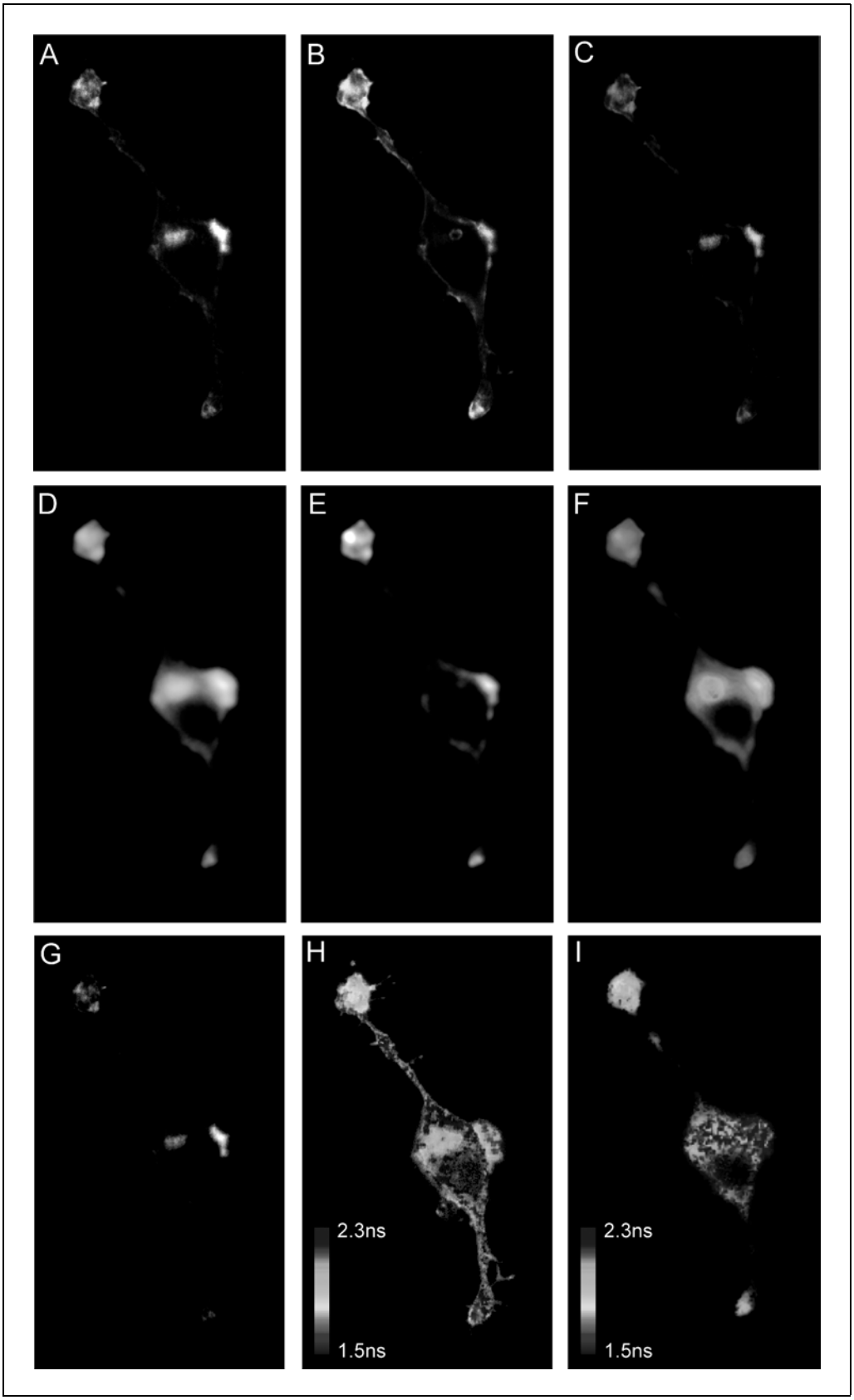


Figure 4.14.7 Legend at right.

Wetzlar) x-y stage equipped with a customized controller. The microscope is completely remote-controlled to allow automatic positioning of sample, shutter operation, and selection of focus, filter, and light source. The excitation light is provided by an Innova 300C Argon laser (Coherent). The 488-nm laser line was selected for excitation of the GFP. The laser beam height is maintained by beam-steering optomechanics and is directed through the crystal of a standing-wave acousto-optic modulator ME405 (IntrAction). The first-order sine-like-modulated spot of the diffraction pattern emerging from the AOM is selected by an iris and is attenuated by a variable neutral density filter wheel. The beam is then focussed onto a multi-mode optical fiber that is continuously vibrated at 50 Hz. The emanating laser beam is expanded by a concave lens onto the back input port of the microscope. Fluorescence emission of the sample is detected by an ImagerQE CCD connected to the output phosphor screen of an HRI multi-channel plate intensifier, both by Lavision. Two phase-locked signal generators IFR2023A (IFR System) provide the electrical signals for the MCP reference (~80 MHz in the presented measurements) and to drive the AOM.

Figure 4.14.6 shows a typical image set acquired by this microscope. SHSY5Y human neuroblastoma cells were transfected with GPI-GFP and were incubated with Alexa594-labeled cholera toxin B subunit (CTB). The GPI-GFP locates to lipid microdomains, called rafts, by virtue of its acylation, the CTB binds to raft-resident GM1 gangliosides. Both raft markers are expected to interact and give rise to FRET when recruited to the same type of raft. Figure 4.14.6A and B show the GPI-GFP donor and CTB acceptor images, respectively. A phase stack of 16 images was collected with forward and reverse phase delays, and averaged to correct for photobleaching (Gadella et al., 1994). The zeroth and first two Fourier coefficients were computed for each pixel. They can be calculated by Equation 4.14.13 (Clegg and Schneider, 1996):

$$F_0 = K^{-1} \sum_{k=1}^{K} D_k$$

$$F_{\cos} = \sum_{k=1}^{K} D_k \cos\theta_k$$

$$F_{\sin} = \sum_{k=1}^{K} D_k \sin\theta_k$$

Equation 4.14.13

where D_k are single images of the phase stack, K is the number of images, and θ_k the phase delays. F_0 corresponds to a normal steady-state fluorescence image (also called the DC image) and $F_{\cos}$, $F_{\sin}$ are the cosine- and sine-transforms, respectively, of the stack by which, using Equation 4.14.14, the pixel-by-pixel modulation and phase of the emitted fluorescence is retrieved:

$$m = \frac{\sqrt{F_{\sin}^2 + F_{\cos}^2}}{F_0}$$

$$\varphi = \arctan\left(\frac{F_{\sin}}{F_{s\cos}}\right)$$

Equation 4.14.14

Figure 4.14.7 *(at left)* Human SHSY-5Y neuroblastoma cell expressing GPI-GFP and stained with cholera toxin–Alexa594 (see Fig. 4.14.6). The same cell was imaged by a laser scanning time-domain (**A,B,C,G,H**) and a wide-field frequency domain (**D,E,F,I**) FLIM. **A** and **D** show GFP staining, **B** and **E** the Alexa594, and **C** and **F** the respective overlays and colocalizations. The comparison between the confocal (upper row) and the wide-field (middle row) images reveals the intrinsic difference in resolution between a CLSM and an image intensifier (MCP). For the same reason, the time-domain lifetime map (**H**), overlaid with the intensity two-photon TCSPC image, shows a better spatial resolution compared to the lifetime distributions imaged by the wide-field microscope in the frequency-domain setup. The main advantage of the latter technique is its speed in imaging of dim sample (<30 sec) compared to longer time exposure (20 min) required by a sensitive TCSPC system equipped with an MCP-PMT detector. The differences in lifetime are explained by different imaged focal plane position and thickness, but both systems detect molecular interactions between markers (25% and 35% FRET maximum for FD and TD, respectively). Note that interactions are found in the cellular extremities and part of the somal plasma membrane. Intracellular GPI-GFP, which was not exposed to the CTB does not give rise to FRET. Even though significant colocalization can be seen in a membrane patch to the right of the cellular soma, the markers there do not physically interact. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.interscience.wiley.com/c_p/colorfigures.htm.*

To retrieve the phase- and modulation-lifetime estimation using Equation 4.14.10, the microscope was previously calibrated with a reflecting foil in the sample focal plane as a zero-lifetime reference. This allows the computation of the absolute phase delay and the demodulation factor between the excitation light and emitted fluorescence for each pixel. The lifetime images were computed according to Equation 4.14.15:

$$\tau_\phi = \omega^{-1} \tan(\phi - \phi_0)$$

$$\tau_m = \omega^{-1} \sqrt{\left(\frac{m_0}{m}\right)^2 - 1}$$

Equation 4.14.15

where the subscript 0 denotes a zero-lifetime reference phase and modulation.

Figure 4.14.6D shows an overlay of the average lifetime and fluorescence image of the donor. Modulation lifetime is always greater than the phase estimation and the true lifetime lies between these retrieved values. The average of the apparent lifetimes (Lakowicz, 1999) is the best estimator for the true sample lifetime. FD-data can be masked according to χ^2 values if a fitting routine is applied instead of the Fourier analysis or if a statistical estimator is available (Gadella et al., 1994).

This experiment was selected to demonstrate the detection of FRET in a demanding research sample. The choice for membrane-bound components limits the fluorescence emitted from the sample and is not optimal for wide-field detection. From the overlay of the donor and acceptor intensities (Fig. 4.14.6C), it is clear that the two signals correlate more in the cell at the top-right whose processes extend to the top-left corner. The lifetime map reveals a clear FRET signature in only this cell. In contrast to the colocalization information, the FRET analysis shows interaction between the two raft markers in defined regions of the cell. This finding reflects the presence of rafts containing only one of the markers in the entire cell, but rafts containing both only in select areas of the cell. Colocalization of the two fluorochromes is not sufficient to demonstrate their interaction. The analysis of the bi-dimensional histogram of phase- versus modulation-lifetime estimations, color-coded by the frequency of their occurrence and the distribution of average lifetimes, discriminates two populations of pixels correlating to FRETting and non-FRETting donors. In general, the analysis of the lifetime distributions offers an index to identify sample heterogeneity. The two apparent lifetimes, in fact, should be equal for single exponential decaying fluorochromes, but they tend to diverge at increasing sample heterogeneity (Lacowicz, 1999; Murata et al., 2000), e.g., caused by FRET.

Wide-field and scanning detection

The presented Anticipated Results offer more than a comparison between frequency- and time-domain configurations, it also allows comparison between wide-field and scanning detection.

Figure 4.14.7 shows images of the same cell acquired by two different microscopes that were previously described. The images in the top row show the GPI-GFP (Fig. 4.14.7A) and the cholera toxin–Alexa594 (Fig. 4.14.7B) fluorescence emission acquired by the confocal microscope and their overlay (Fig. 4.14.7D). These images represent the practical limits of spatial resolution in these samples. The middle row shows the same signals acquired by a wide-field microscope with an image intensifier. The difference in spatial resolution caused by the resolution limit of the MCP is evident. As a consequence, the lifetime map obtained with the two-photon laser scanning microscope (Fig. 4.14.7F,G) has an intrinsically higher spatial resolution than the lifetime image acquired by wide-field detection. Both systems reveal FRET but the latter method detects lower overall energy transfer efficiencies because the reduced lifetime FRET signal is diluted by donor fluorescence emanating from components in the intrinsically thicker focal plane and out-of-focus volume that have not been exposed to extracellularly applied CTB.

The advantage of wide-field detection arises when faster acquisition times are needed. This particular sample was imaged with an integration time of 25 min in the scanning microscope while the phase stack was built in <30 sec by the wide-field microscope. This sample was quite demanding and required the upper limit of acquisition time that is used in practice for scanning and wide-field microscopes.

Conclusions

Lifetime imaging presents an important and powerful tool for cell biology and biophysics. Its use will certainly spread as more user-friendly and less-expensive commercial solutions become available. Lifetimes can be used to enhance the contrast in biological and medical imaging and can be used to screen for

intermolecular interactions and molecular activities in living cells. Lifetime images are quantitative and intuitive, but particular attention should be given to data analysis and interpretation. Even though false-positive interactions are practically impossible to obtain by lifetime imaging of FRET, over-interpretation of data and instrumental errors are always possible. Proper reliability analysis is required to certify FLIM findings and to estimate their noise content. Nowadays, optical techniques present a pillar of cellular biophysics. With the advent of quantitative techniques like FLIM and FRET, it is revolutionizing the life sciences. FLIM by itself, but above all its capability to measure FRET, will play a fundamental role as a medical diagnostic tool, a sensitive instrument to probe the photophysics behavior of optically active molecules, a tool for studying the behavior of biomolecules, and it will provide support in the proteomics challenge to understand cellular proteins performing their tasks in the cell. This demands an interdisciplinary effort of cell biology, microscopy, and physics.

Time Considerations

The limiting step in any FLIM experiment is the preparation of the biological sample. Time considerations for expression of fluorescent fusion proteins and immunocytochemistry can be found in *UNIT 17.1.* The practical time aspects of FLIM measurements are not different from those of any other fluorescence microscopy experiments, with the exception of the necessity to acquire multiple images (see Acquisition Times and Signal-to-Noise Ratio).

Acknowledgments

The authors would like to express their gratitude to Mika Ruonala and Mišo Mitkovski for providing the biological samples.

Literature Cited

Agronskaia, A.V., Tertoolen, L., and Gerritsen, H.C. 2003. High frame rate fluorescence lifetime imaging. *J. Phys. D. Appl. Phys.* 36:1655-1662.

Ballew, R.M. and Demas, J.N. 1989. An error analysis of the rapid lifetime determination method for the evaluation of single exponential decays. *Anal. Chem.* 61:30-33.

Bastiaens, P.I. and Pepperkok, R. 2000. Observing proteins in their natural habitat: The living cell. *Trends Biochem. Sci.* 25:631-637.

Becker, W., Bergmann, A., Hinkl, M.A., König, K., Benndorf, K., and Biskup, C. 2004. Fluorescence lifetime imaging by time-correlated single-photon counting. *Microsc. Res. Tech.* 63:58-66.

Bunt, G. and Wouters, F.S. 2004. Visualization of molecular activities inside living cells with fluorescent labels. *Int. Rev. Cytol.* 237:205-277.

Buurman, E.P., Sanders, R., Draaijer, A., Gerritsen, H.C., van Veen, J.J.F., Houpt, P.M., and Levine, Y.K. 1992. Fluorescence lifetime imaging using a confocal laser scanning microscope. *Scanning* 14:155-159.

Carlsson, K. and Liljeborg, A. 1997. Confocal fluorescence microscopy using spectral and lifetime information to simultaneously record four fluorophores with high channel separation. *J. Microsc.* 185:37-46.

Carlsson, K. and Liljeborg, A. 1998. Simultaneous confocal lifetime imaging of multiple fluorophores using the intensity-modulated multiple-wavelength scanning (IMS) technique. *J. Microsc.* 191:119-127.

Carlsson, K. and Philip, J.P. 2002. Theoretical investigation of the signal-to-noise ratio for different fluorescence lifetime imaging techniques. *Proc. SPIE* 4622:70-78.

Chen, Y. and Periasamy, A. 2004. Characterization of two-photon excitation fluorescence lifetime imaging microscopy for protein localization. *Microsc. Res. Tech.* 63:72-80.

Clayton, A.H.A., Hanley, Q.S., Arndt-Jovin, D.J., Subramaniam, V., and Jovin, T.M. 2002. Dynamic fluorescence anisotropy imaging microscopy in the frequency domain (rFLIM). *Biophys. J.* 83:1631-1649.

Clegg, R.M. 1996. Fluorescence resonance energy transfer. *In* Fluorescence Imaging Spectroscopy and Microscopy, vol. 137 (X.F. Wang and B. Herman, eds.) pp. 180-252. John Wiley & Sons, London.

Clegg, R.M. and Schneider, P.C. 1996. Fluorescence lifetime-resolved imaging microscopy: A general description of lifetime-resolved imaging measurements. *In* Fluorescence Microscopy and Fluorescence Probes (J. Slavík, ed.) pp. 15-33. Plenum Press, New York.

de Grauw, C.J. and Gerritsen, H.C. 2001. Multiple time-gate module for fluorescence lifetime imaging. *Appl. Spectrosc.* 55:670-678.

Denk, W., Piston, D.W., and Webb, W.W. 1995. Two-photon molecular excitation in laser-scanning microscopy. *In* Handbook of Biological Confocal Microscopy (J.B. Pawley, ed.) pp. 445-458. Plenum Press, New York.

Dong, C.Y., So, P.T.C., French, T., and Gratton, E. 1995. Fluorescence lifetime imaging by asynchronous pump-probe microscopy. *Biophys. J.* 69:2234-2242.

Draaijer, A., Sanders, R., and Gerritsen, H.C. 1995. Fluorescent lifetime imaging: A new tool in confocal microscopy. *In* Handbook of Biological Confocal Microscopy (J.B. Pawley, ed.) pp. 491-505. Plenum Press, New York.

Elangovan, M., Day, R.N., and Periasamy, A. 2002. Nanosecond fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy to localize the protein interactions in a single living cell. *J. Microsc.* 205:3-14.

Eliceiri, K.W., Fan, C.H., Lyons, G.E., and White, J.G. 2003. Analysis of histology specimens using lifetime multiphoton microscopy. *J. Biomed. Opt.* 8:376-380.

Esposito, A., Federici, F., Usai, C., Cannone, F., Chirico, G., Collini, M., and Diaspro, A. 2004. Notes on theory and experimental conditions behind two-photon excitation microscopy. *Microsc. Res. Tech.* 63:12-17.

Esposito, A., Gerritsen, H.C., and Wouters, F.S. In press. Fluorescence lifetime heterogeneity resolution in the frequency-domain by lifetime moments analysis (LiMA).

Förster, T. 1965. Delocalized excitation and excitation transfer. *In* Modern Quantum Chemistry—Istanbul Lectures, Part III (O. Sinanoğlu, ed.) pp. 93-137. Academic Press, New York.

Gadella, T.W.J. and Jovin, T.M. 1995. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J. Cell Biol.* 129:1543-1558.

Gadella, T.W.J., Jovin, T.M., and Clegg, R.M. 1993. Fluorescence lifetime imaging microscopy (FLIM): Spatial resolution of microstructures on the nanosecond time scale. *Biophys. Chem.* 48:221-239.

Gadella, T.W.J., Clegg, R.M., and Jovin, T.M. 1994. Fluorescence lifetime imaging microscopy: Pixel-by-pixel analysis of phase-modulation data. *Bioimaging* 2:139-159.

Gerritsen, H.C., Asselbergs, M.A., Agronskaia, A.V., and Van Sark, W.G. 2002. Fluorescence lifetime imaging in scanning microscopes: Acquisition speed, photon economy and lifetime resolution. *J. Microsc.* 206:218-224.

Ghiggino, K.P., Harris, M.R., and Spizzirri, P.G. 1992. Fluorescence lifetime measurements using a novel fiber-optic laser scanning confocal microscope. *Rev. Sci. Instrum.* 63:2999-3002.

Gratton, E. and Limkeman, M. 1983. A continuously variable frequency cross-correlation phase fluorometer with picosecond resolution. *Biophys. J.* 44:315-324.

Gratton, E., Limkeman, M., Lakowicz, J.R., Maliwal, B.P., Cherek, H., and Laczko, G. 1984. Resolution of mixtures of fluorophores using variable-frequency phase and modulation data. *Biophys. J.* 46:479-486.

Gratton, E., Breusegem, S., Sutin, J., Ruan, Q., and Barry, N. 2003. Fluorescence lifetime imaging for the two-photon microscope: Time-domain and frequency-domain methods. *J. Biomed. Opt.* 8:381-390.

Hanley, Q.S., Subramaniam, V., Arndt-Jovin, D.J., and Jovin, T.M. 2001. Fluorescence lifetime imaging: Multi-point calibration, minimum resolvable differences, and artifact suppression. *Cytometry* 43:248-260.

Harms, P., Sipior, J., Ram, N., Carter, G.M., and Rao, G. 1999. Low cost phase-modulation measurements of nanosecond fluorescence lifetimes using a lock-in amplifier. *Rev. Sci. Instrum.* 70:1535-1539.

Harpur, A.G., Wouters, F.S., and Bastiaens, P.I. 2001. Imaging FRET between spectrally similar GFP molecules in single cells. *Nat. Biotechnol.* 19:167-169.

Herman, P., Maliwal, B.P., Lin, H.-J., and Lakowicz, J.R. 2001. Frequency-domain fluorescence microscopy with the LED as a light source. *J. Microsc.* 203:176-181.

Jares-Erijman, E.A. and Jovin, T.M. 2003. FRET imaging. *Nat. Biotechnol.* 21:1387-1395.

Juzeliûnas, G. and Andrews, D.L. 1999. Unified theory of radiative and radiationless energy transfer. *In* Resonance Energy Transfer (D.L. Andrews and A.A. Demidov, eds.) pp. 65-110. John Wiley & Sons, London.

Knemeyer, J.P., Herten, D.P., and Sauer, M. 2003. Detection and identification of single molecules in living cells using spectrally resolved fluorescence lifetime imaging microscopy. *Anal. Chem.* 75:2147-2153.

Köllner, M. and Wolfrum, J. 1992. How many photons are necessary for fluorescence-lifetime measurements? *Chem. Phys. Lett.* 200:199-204.

Lakowicz, J.R. 1999. Principles of Fluorescence Spectroscopy, 2nd ed. Plenum Press, New York.

Lakowicz, J.R. and Berndt, K.W. 1991. Lifetime-selective fluorescence imaging using an rf phase-sensitive camera. *Rev. Sci. Intrum.* 62:1727-1734.

Lakowicz, J.R., Szmacinski, H., Nowaczyk, K., and Johnson, M.L. 1992. Fluorescence lifetime imaging of free and protein-bound NADH. *Proc. Natl. Acad. Sci. U.S.A.* 89:1271-1275.

Landgraf, S. 2001. Application of semiconductor light sources for investigations of photochemical reactions. *Spectrochim. Acta A* 57:2029-2048.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell. Biol.* 2:444-456.

Mitchell, A.C., Wall, J.E., Murray, J.G., and Morgan, C.G. 2002a. Direct modulation of the effective sensitivity of a CCD detector: A new approach to time-resolved fluorescence imaging. *J. Microsc.* 206:225-232.

Mitchell, A.C., Wall, J.E., Murray, J.G., and Morgan, C.G. 2002b. Measurement of nanosecond time-resolved fluorescence with a directly gated interline CCD camera. *J. Microsc.* 206:233-238.

Mitić, J., Anhut, T., Meier, M., Ducros, M., Serov, A., and Lasser, T. 2003. Optical sectioning in wide-field microscopy obtained by dynamic structured light illumination and detection based on a smart pixel detector array. *Opt. Lett.* 28:698-700.

Morgan, C.G., Mitchell, A.C., and Murray, J.G. 1990. Nanosecond time-resolved fluorescence microscopy: Principles and practice. *Trans. Roy. Microsc. Soc.* 1:463-466.

Murata, S., Herman, P., Lin, H.J., and Lakowicz, J.R. 2000. Fluorescence lifetime imaging of

nuclear DNA: Effect of fluorescence resonance energy transfer. *Cytometry* 41:178-185.

Neher, R. and Neher, E. 2004. Optimizing imaging parameters for the separation of multiple labels in a fluorescence image. *J. Microsc.* 213:46-62.

O'Connor, D.V. and Phillips, D. 1984. Time Correlated Single Photon Counting. Academic Press, London.

O'Hagan, W.J., McKenna, M., Sherrington, D.C., Rolinski, O.J., and Birch, D.J.S. 2002. MHz LED source for nanosecond fluorescence sensing. *Meas. Sci. Technol.* 13:84-91.

Oida, T., Sako, T., and Kusumi, A. 1993. Fluorescence lifetime imaging microscopy (flimscopy). Methodology development and application to studies of endosome fusion in single cells. *Biophys. J.* 64:676-685.

Patterson, G.H. and Piston, D.W. 2000. Photobleaching in two-photon excitation microscopy. *Biophys. J.* 78:2159-2162.

Pepperkok, R., Squire, A., Galey, S., and Bastiaens, P.I.H. 1999. Simultaneous detection of multiple green fluorescent proteins in live cells by fluorescence lifetime imaging microscopy. *Curr. Biol.* 9:269-272.

Philip, J. and Carlsson, K. 2003. Theoretical investigation of the signal-to-noise ratio in fluorescence lifetime imaging. *J. Opt. Soc. Am. A* 20:368-379.

Piston, D.W., Marriott, G., Radiovoyevich, T., Clegg, R.M., Jovin, T.M., and Gratton, E. 1989. Wide-band acousto-optic light modulator for frequency domain fluorimetry and phosphorimetry. *Rev. Sci. Instrum.* 60:2596-2600.

Rizzo, M.A., Springer, G.H., Granada, B., and Piston, D.W. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* 22:1-5.

Schneider, P.C. and Clegg, R.M. 1997. Rapid acquisition, analysis, and display of fluorescence lifetime-resolved images for real-time applications. *Rev. Sci. Instrum.* 68:4107-4119.

Schönle, A., Hänninen, P.E., and Hell, S.W. 1999. Nonlinear fluorescence through intermolecular energy transfer and resolution increase in fluorescence microscopy. *Ann. Phys.* 8:115-133.

Siegel, J., Benny Lee, K.C., Webb, S.E.D., Lévêque-Fort, S., Cole, M.J., Jones, R., Dowling, K., French, P.M.W., and Lever, M.J. 2001a. Application of the stretched exponential function to fluorescence lifetime imaging of biological tissue. *Biophys. J.* 81:1265-1274.

Siegel, J., Elson, D.S., Webb, S.E.D., Parsons-Karavassilis, D., Lévêque-Fort, S., Cole, M.J., Lever, M.J., French, P.M.W., Neil, M.A.A., Jukaitis, R., Sucharov, L.O., and Wilson, T. 2001b. Whole-field five-dimensional fluorescence microscopy combining lifetime and spectral resolution with optical sectioning. *Opt. Lett.* 26:1338-1340.

So, P.T.C., French, T., Yu, W.M., Berland, K.M., Dong, C.Y., and Gratton, E. 1995. Time-resolved fluorescence microscopy using two-photon excitation. *Bioimaging* 3:49-63.

Spencer, R.D. and Weber, G. 1969. Measurements of subnanosecond fluorescence lifetimes with a cross-correlation phase fluorimeter. *Ann. N.Y. Acad. Sci.* 158:361-376.

Squire, A., Verveer, P.J., and Bastiaens, P.I. 2000. Multiple frequency fluorescence lifetime imaging microscopy. *J. Microsc.* 197:136-149.

Szmacinski, H., Gryczynski, I., and Lakowicz, J.R. 1993. Calcium-dependent fluorescence lifetimes of Indo-1 for one- and two-photon excitation of fluorescence. *Photochem. Photobiol.* 58:341-345.

Tadrous, P.J., Siegel, J., French, P.M., Shousha, S., Lalani, El-N., and Stamp, G.W. 2003. Fluorescence lifetime imaging of unstained tissues: Early results in human breast cancer. *J. Pathol.* 199:309-317.

Trabesinger, W., Hübner, C.G., Hecht, B., and Wild, U.P. 2002. Continuous real-time measurement of fluorescence lifetimes. *Rev. Sci. Instrum.* 73:3122-3124.

van der Meer, B.W. 1999. Orientational aspects in pair energy transfer. *In* Resonance Energy Transfer (D.L. Andrews and A.A. Demidov, eds.) pp. 151-172. John Wiley & Sons, London.

van der Oord, C.J.R., de Grauw, C.J., and Gerritsen, H.C. 2001. Fluorescence lifetime imaging module LIMO for CLSM. *Proc. SPIE* 4252:119-123.

van Munster, E.B. and Gadella, T.W.J. Jr. 2004a. ϕFLIM: A new method to avoid aliasing in frequency-domain fluorescence lifetime imaging microscopy. *J. Microsc.* 213:29-38.

van Munster, E.B. and Gadella, T.W.J. Jr. 2004b. Suppression of photobleaching-induced artifacts in frequency-domain FLIM by permutation of the recording order. *Cytometry* 58:185-194.

Verveer, P.J. and Bastiaens, P.I.H. 2003. Evaluation of global analysis algorithms for single frequency fluorescence lifetime imaging microscopy data. *J. Microsc.* 209:1-7.

Verveer, P.J., Squire, A., and Bastiaens, P.I.H. 2000. Global analysis of fluorescence lifetime imaging microscopy data. *Biophys. J.* 78:2127-2137.

Wang, X.F., Uchida, T., and Minami, S. 1989. A fluorescence lifetime distribution measurement system based on phase-resolved detection using an image dissector tube. *Appl. Spectr.* 43:840-845.

Wang, X.F., Uchida, T., Coleman, D.M., and Minami, S. 1991. A two-dimensional fluorescence lifetime imaging system using a gated image intensifier. *Appl. Spectr.* 45:360-366.

Webb, S.E.D., Gu, Y., Lévêque-Fort, S., Siegel, J., Cole, M.J., Dowling, K., Jones, R., French, P.M.W., Neil, M.A.A., Ju kaitis, R., Sucharov, L.O.D., Wilson, T., and Lever, M.J. 2002. A wide-field time-domain fluorescence lifetime imaging microscope with optical sectioning. *Rev. Sci. Instrum.* 73:1898-1907.

Wouters, F.S., Bastiaens, P.I.H., Wirtz, K.W.A., and Jovin, T.M. 1998. FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nsL-TP) with fatty acids

oxidation enzymes in peroxisomes. *EMBO J.* 17:7179-7189.

Wouters, F.S., Verveer, P.J., and Bastiaens, P.I.H. 2001. Imaging biochemistry inside cells. *Trends Cell Bio.* 11:203-211.

Zhang, J., Campbell, R.E., Ting, A.Y., and Tsien, R.Y. 2002. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell. Biol.* 3:906-918.

Internet Resources

http://www.lavision.de/index.shtml

http://www.becker-hickl.de/

The microscopes that were described in this unit were built by assembling standard wide-field or scanning microscopes with detectors purchased from LaVision and Becker&Hickl. LaVision offers MCP-CCD cameras suitable for wide-field imaging, while Becker&Hickl sells electronics and detectors for TCSPC, compatible also with FCS and other techniques.

http://www.lavisionbiotec.com/

http://www.tautec.com/

A complete wide-field system, except light sources, can be acquired from LaVision BioTec or from its U.S. reseller TauTec.

http://www.lambert-instruments.com/

Lambert-Instruments offers a setup with a LED as a light source with the possibility to drive other light sources like lasers.

http://www.nikon-instruments.com/

A TGSPC system (LIMO) is available from Nikon Instech.

http://www.picoquant.com/

PicoQuant sells a stage scanning TCSPC microscope and a broad range of lifetime products.

http://www.jobinyvon.co.uk/

Jobin-Yvon sells a frequency-domain confocal system and a broad range of lifetime products.

http://www.iss.com/index.html

http://www.edinst.com/

A broad range of lifetime products is available from ISS and Edinburgh Instruments.

http://www.kentech.co.uk/

http://www.hamamatsu.com/

http://www.coherentinc.com/

http://www.spectra-physics.com/sp/cda/home

http://www.mellesgriot.com/

Other manufacturers or resellers for lasers and detectors that are of interest for FLIM are Kentech Instruments, Hamamatsu Photonics K.K., Coherent, Spectra Physics, and Melles-Griot.

Contributed by Alessandro Esposito and Fred S. Wouters
European Neuroscience Institute-Goetingen
Goetingen, Germany

Biological Second and Third Harmonic Generation Microscopy

This unit describes how higher harmonic generation microscopy (HHGM) is applied to detect native, nonstained cell and tissue structures that were previously only accessible after immunohistochemical or immunofluorescent labeling.

Nonlinear optical microscopy by multiphoton excitation has developed into a popular, and powerful approach that combines different excitation and emission techniques for the three-dimensional (3-D) reconstruction of biological specimens (Denk et al., 1990; Konig, 2000). The specific approaches include detection of two- and three-photon excited fluorescence, two-photon excited fluorescence lifetime (FLIM), and the second and third harmonic generation. Specimens amenable to multiphoton microscopy include fixed and live samples, thin and thick slices or samples with transparent or relatively opaque properties, as well as intravital microscopy in anesthetized animals (see *UNIT 4.11*). The most obvious advantages of multiphoton microscopy include: (1) greater tissue penetration up to the millimeter range due to near-infrared excitation and consequently, less scatter; (2) reduced phototoxicity imposed onto cells and tissues due to the inherent confocality exciting only fluorophores in the focal plane and the lack of a detector pinhole resulting in improved optical detection efficiency; (3) the broader excitation range of fluorophores, compared to single photon excitation, allowing multicolor imaging using the same excitation wavelength; and (4) the possibility of combining fluorescence microscopy with other detection modes, including HHGM and FLIM. Therefore, nonlinear imaging is being used for nearly any application in cell biology that requires multicolor imaging, deep tissue penetration, low phototoxicity, and high resolution, similar to confocal microscopy.

Two-photon or multiphoton excited HHGM has become one of the most popular and powerful techniques for the reconstruction of intrinsic structural and molecular properties of cells, extracellular matrix, and bone without the use of dyes or stains (Campagnola et al., 2002; Friedl, 2004). Two modalities of HHGM are presently being used for biomedical applications, the second (SHG) and third (THG) harmonic generation imaging (the principle and theoretical basis for these imaging modalities are detailed in the Commentary). SHG and THG imaging provides structural and molecular information that is complementary to two-photon fluorescence (TPF) imaging and can be simultaneously combined with other two-photon excited modalities, including two-photon photoactivation, two-photon correlated spectroscopy, two-photon single-particle tracking, and two-photon lifetime microscopy. HHGM is most useful in the imaging of connective tissue, collagen fibers and fascia, as well as striated muscle, inflammatory cells, blood vessels, and hair at a resolution near the diffraction limit of visible light.

This unit will provide details on the experimental setup and some biological applications of HHGM, including 3-D connective tissue reconstruction and dynamic imaging of cell motility in 3-D tissues. In the commentary, the physical basics of HHGM are described, additional examples for HHGM are given, and there is a discussion on how to detect and minimize multiphoton-excited phototoxicity.

STRATEGIC PLANNING

Materials detectable by SHG and THG

The first step is to consider whether the molecular structure of interest can be detected by HHGM. Because this is a young technique, progressive use of this technology will lead

Contributed by Peter Friedl, Katarina Wolf, Ulrich H. von Andrian, and Gregory Harms
Current Protocols in Cell Biology (2007) 4.15.1-4.15.21

Table 4.15.1 Second and Third Harmonic Imaging of Cell and Tissue Structures

Compound	Excitation wavelength (nm)[a]	Molecular property	Reference	Technical comments; substrates
SHG				
Fibrillar collagen	700–1064	Triple coil of coiled α-helices	Stoller et al., 2002; Cox et al., 2003; Yasui et al., 2004	Dermis, vessel walls, peritumoral stroma; diminished signal during proteolytic degradation
Tubulin	760–880	Coil of whole protein units forming tube with polarized ends	Dombeck et al., 2003	Brain slices; dynamic imaging of mitotic spindles at 880 nm
Myosin		Two α-helix polymer proteins coiled in a left hand	Both et al., 2004; Plotnikov et al., 2006	
Styryl dyes FM4-64 RH 237	 1064 880	Forms anisotropic scaffold along plasma membrane the orientation of which is voltage-dependent	Dombeck et al., 2005; Sacconi et al., 2005	Suitable for backward detection; scanning in the msec range; no photobleaching
Cellulose	840–880; 1230	Structural symmetry of β 1:4-linked glycans	Cox et al., 2005	Low S/N[b]
Starch (in chloroplasts)	840–880; 1230	Structural symmetry of α 1:4-linked glycans	Cox et al., 2005	High S/N[b]
DNA	730–800	Unknown	Gauderon et al., 2001	
THG				
Lipids and lipid bodies	1050–1250	High comparative third order susceptibility, $\chi^{(3)}$	Debarre et al., 2006	Good backward S/N[b] THG in weakly absorbing tissue
Myosin	1230	High comparative third order susceptibility, $\chi^{(3)}$	Chu et al., 2004	
Collagen	1050–1300	Unknown	Kao, 2004	
Hydroxyapatite crystals in tooth enamel	1050–1300	Unknown	Kao, 2004	
Inorganic crystals ($CaCO_3$) in cytolyths, mouse bone, and spincules	1500	High comparative third order susceptibility, $\chi^{(3)}$	Oron et al., 2004	
Oxyhemoglobin	1260–1470	High comparative third order susceptibility, $\chi^{(3)}$	Schaller et al., 2000	Low S/N[b]

[a]Detection at $\lambda_{ex}/2$ for SHG and $\lambda_{ex}/3$ for THG.
[b]Abbreviation: S/N, signal-to-noise ratio.

to rapid growth of the materials list, therefore a database search on molecular structures and optimum wavelength characteristics should be performed before starting a higher harmonic generation imaging (HHGI) project.

Biological Second and Third Harmonic Generation Microscopy

Structures to be detected: Second harmonic generation is caused by materials that are structured in crystalline-like lattices that are noncentrosymmetric on length scales on the order of the excitation light wavelength called the coherence length (Chu et al., 2002; Mohler et al., 2003; see Table 4.15.1). Besides metal and ion crystals, several

biological materials contain repetitive or symmetric structural units, including coiled-coil structures and polymeric proteins (Table 4.15.1). Third harmonic generation is a much weaker process, however elicited by different types of materials, particularly phase transitions, such as water-lipid interfaces (Table 4.15.1).

Combination with fluorescence: Because HHGI can be combined with fluorescence microscopy and other imaging modalities, it is necessary to consider which excitation and detection wavelengths are required to excite the fluorophores together with SHG or THG (Gauderon et al., 2001; Mempel et al., 2004b). The combination of higher harmonic and fluorescence microscopy has allowed for versatile combinations of cell and tissue imaging in cell biology, histology, and tissue engineering (Table 4.15.1).

Sample type: For the imaging of live thick tissue samples (>100 μm thick) of strong light-scattering properties, including connective tissues and lymph nodes, near-infrared excitation offers increased penetration depth in thicker light-scattering samples at a relatively low degree of cellular phototoxicity and thus, is superior to single-photon excitation. The properties of HHGM to reconstruct 3-D ECM structures and cell membranes together with fluorescence show particular promise in the assessment of tissue morphogenesis during development and pathological remodeling, including wound repair and neoplasia.

DESIGNING A MICROSCOPE SYSTEM FOR HHGM

BASIC PROTOCOL 1

The hardware setup for HHGM (Fig. 4.15.1) follows, in principle, that of a laser-scanning microscope for visible wavelength emission and detection (see *UNIT 4.5*) and a two-photon excitation microscope using pulsed near-infrared laser sources (see *UNIT 4.11*). Any two-photon microscope already includes the capability of HHGI, therefore the setup description here will address specific features only. Besides higher harmonic generation imaging, the setup allows the excitation and detection of fluorophores (see *UNIT 4.11*), and using additional laser and detector hardware, coherent anti-Stokes Raman scattering (CARS) microscopy, fluorescence lifetime imaging (FLIM; see *UNIT 4.14*) in parallel and without crosstalk.

The laser beam is generated by a titanium-sapphire laser, either alone or in combination with a synchronously pumped optical parametric oscillator (OPO; Table 4.15.2). Using a cascade of mirrors, the laser beam is directed into the microscope by a computer-controlled *xy*-scanner consisting of one or two galvanometer-driven mirrors (Table 4.15.3) coupled to an upright or inverted microscope stage. The reconstruction of 3-D specimens requires serial *z*-sectioning and thus, a computerized *z*-positioning of sample stage or

Table 4.15.2 Laser Sources and Wave-lengths

Laser type	Wave length (nm)	Comments
Titanium:Sapphire	700–1040	Popular and broadly used laser type, but cost-intense; modern systems include computer-automated wavelength tuning and user-friendly maintenance
Fianium fiber	1064	Useful for measurements of membrane potential
Chromium fosterite	1230	Greatly reduced tissue autofluorescence at very long excitation wave length; low photodamage
Optical parametric oscillator (OPO)	1000–1600	Requires a Ti:Sa pump laser at fixed wavelength (e.g., 775 nm); computer-automated tuning available

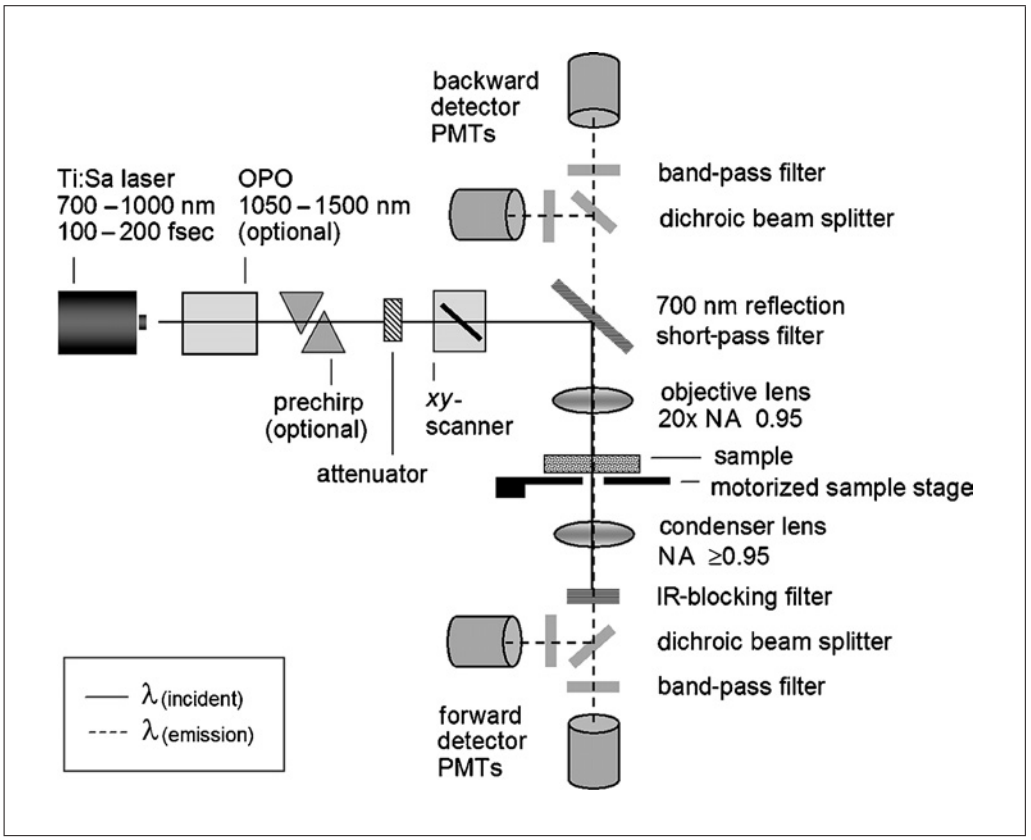


Figure 4.15.1 Set-up of a photomultiplier-based multiphoton microscope for higher harmonic generation microscopy. The beam of a mode-locked Ti:Sa laser is passed through an optional prechirp path consisting of two prisms to compensate for shifts in pulse width, followed by an attenuator of the beam intensity, and one or two galvanomirrors for scanning. For tuning of the incident beam above 1 μm, an optical parametric oscillator (OPO) pumped by the Ti:Sa laser is required. After reflection by a dichroic mirror, the beam is focused by the objective lens into the sample. Emission signals elicited in the focal plane of the sample are detected in forward direction by the condenser lens and in backward direction through the objective lens, spectrally separated by a dichroic filter and passed through a band-pass filter for each individual signal-adapted bandwidth before the detector photomultipliers (PMTs). In both directions, the transmitted or reflected laser light is blocked by band pass filters.

Table 4.15.3 Commercially Available Multiphoton Systems

System	Website	Technical details
Zeiss HLSM 510 MP	*http://www.zeiss.de*	4 non-descanned PMTs (2 forward, 2 backward), up to 6 descanned PMTs; includes 3-D and 4-D reconstruction software
Leica TCS MP	*http://www.leica-microsystems.com*	4 non-descanned PMTs (2 forward, 2 (4) backward), up to 6 descanned PMTs; includes 3-D and 4-D reconstruction software
LaVision Biotech	*http://www.lavisionbiotec.de/start_product.html*	8 non-descanned PMTs (4 forward, 5 backward); includes optional multibeam CCD camera-based version
Prairie Technologies	*http://www.prairie-technologies.com/ultima.htm*	4 non-descanned PMTs in the backward direction

Table 4.15.4 Software Packages for 3-D and 4-D Data Reconstruction and Analysis

System	Website	Technical details	Comments
Imaris (Bitplane)	*http://www.bitplane.com*	3-D and 4-D multicolor reconstruction, cell tracking, colocalization, microfilament tracing	Fast reconstruction; user-friendly software handling
Volocity (Improvision)	*https://www.improvision.com/*	3-D and 4-D multicolor reconstruction, cell tracking, colocalization, angiogenesis tool	64 bit version available, user-friendly platform; limitations with large datasets
Amira	*http://www.amiravis.com/*	3-D multicolor reconstruction, volume rendering	Not able to reconstruct 4-D stacks, limited export functions, limitations with large data stacks
NIH Image	*http://rsb.info.nih.gov/nih-image/Default.html*	Mac and PC version available, fast and powerful tool for data handling and manual tracking; good video manipulation tools	Open-access shareware platform; large number of plug-ins generated by users available; no automated tracking or 3-D surface rendering

objective, as is available for most high-quality microscopes. The detection unit includes beam splitters and band-pass filters designed for the wavelength-resolved detection of emitted light using photomultipliers (PMTs).

The operation of the system including scan-head control and image acquisition is controlled by acquisition routines provided by the manufacturer of the scanning system. Because image acquisition will generate large amounts of data, the hardware and software design should be optimized for maximum throughput and storage capability as well as fast processor speed. These rules are described in detail in *UNIT 4.10*. However, appropriate image reconstruction and analysis often require the use of additional software designed to handle large data stacks (Table 4.15.4). These data analysis software packages can retrieve primary files generated by most major confocal and multiphoton systems and if required, custom-designed input can be obtained from the manufacturer on demand.

Materials

Upright or inverted microscope and optics suitable for epifluorescence detection including:
- Epi-illuminator (bright field)
- High numeric aperture objective (NA > 0.7)
- Detector unit using photomultipliers, beam splitters and band-pass filters
- Sample stage including computer-driven *z*-position control (alternatively *xyz*-position control)
- Heating system to maintain 37°C (optional)

Multiphoton laser including:
- Diode-pump laser coupled to titanium-sapphire laser (Table 4.15.2)
- Incoupling of the laser beam into the scan head via mirrors (beam path)

Laser scanning unit including computer-controlled operation system for scanning conditions, signal acquisition, primary image reconstruction and storage (Table 4.15.3)

Software for 3-D and 4-D image reconstruction (Table 4.15.4)

Vibration isolation table designed for stable mounting of optical instruments

Powermeter to measure laser light intensities along the beam path, including infra-objective level

Adjust beam path and optics

1. Place all optical and electronic components including the microscope, except for the computer, on a vibration isolation table.

   All optical and electronic components including the microscope need to be of the highest quality.

2. Obtain specific information from the microscope and scan-head manufacturers, which should include the parameters of all mirrors, lenses, and the objective. To reduce absorption or scattering of light, use silver coatings for mirrors.

   To provide sufficient sensitivity for weak signals, the optical transparency of the total beam path (from laser source to the location of the sample) should be >30%.

   For excitation above 1200 nm, the optical elements of most systems (mirrors, tube lens, and scan lens) must be custom-designed for increased infrared transparency.

   Upon request, most manufacturers will provide an appropriate alternative system design for microscope, scan head, and detectors.

3. Set up epifluorescence detection, which is not mandatory, but it is useful for initial sample screening and positioning.

4. Select objectives that allow high resolution (indicated by high NA) and maximum transparency.

   For intravital microscopy in living animals, long working-distance objectives of NA 0.80 to 0.95 (usually 20× magnification) are popular because of their versatility and deep tissue penetration capabilities (up to 2 mm) and high light sensitivity; however they often do not provide sufficient detailed resolution for cytoskeletal structures or the subcellular distribution of membrane components. Objectives of higher NA are usually limited by their short working distance (≤250 μm); however they provide better subcellular detail, which can be important for imaging of cell and tissue cultures. The choice for oil- versus water- or multi-immersion objective depends upon the type of sample used. For intravital and live-cell microscopy, water-immersion dipping objectives are preferred because they can be directly introduced into the sample medium. For further details on objectives see UNIT 4.11.

5. Detect HHG using photomultipliers in either forward/transmission or backward direction. To avoid loss of signal, use a collection lens in the forward direction with a NA the same or higher, compared to the illumination NA determined by the objective (Fig. 4.15.1).

   Whereas fluorescence intensities detected in the backward or forward direction are often equal, SHG transmission often shows a 5- to 10-fold stronger intensity, compared to the backward detection, yet using a sensitive objective, the backward detection will still deliver sufficient signal for the detection of collagen and myosin in native state (Fig. 4.15.2).

   Because of the inherent confocality achieved by multiphoton excitation, the detectors do not require a pinhole, thus are nondescanned (i.e., do not go again through the scanhead). Besides PMTs, a CCD camera may be placed to detect higher harmonic signals in the forward direction, but not in the backward direction, because the reflected signal mostly comprises scattered photons and consequently, gives low signal-to-noise ratio at the focal plane of the CCD chip. For third harmonic imaging, at least in theory, emission is nonisotropic and might be detected by CCD camera-based systems in the forward and backward direction.

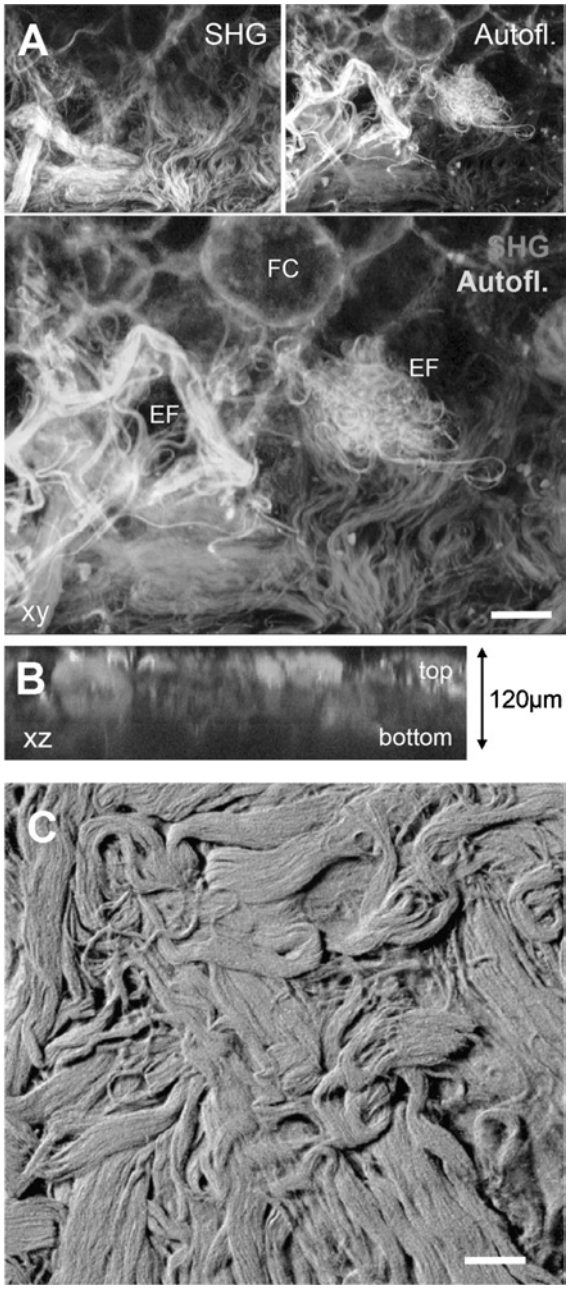


Figure 4.15.2 Three-dimensional reconstruction of collagen bundles from human dermis. Non-labeled 3-mm thick samples from human dermis were fixed in 4% buffered paraformaldehyde and sliced tangentially. The wavelength of the incident beam was 880 nm, the polarization vertical and horizontal, and the band-pass filters 440/20 (SHG) and 515/30 (autofluorescence). Images were acquired using a 20× N.A. 0.95 water immersion objective, resulting in an optical transverse resolution of 500 nm and a pixel resolution of ∼400 nm. The axial resolution was 4 to 5 μm. Images show (**A**) SHG together with autofluorescence in *x-y*, (**B**) the reconstructed *x-z* projection, and (**C**) topographic reconstruction of SHG only. EF, elastic fiber; FC, fat cell. Bar = 20 μm. For color version of this figure see *http://www.currentprotocols.com*.

Select lasers

6. As a laser source, select a tunable titanium-sapphire laser generating femtosecond pulses of laser light to provide maximum flexibility for HHGI in biological applications (Table 4.15.2). Adjust the wavelength within a tuning range from 700 to ∼1000 nm. Alternatively, use lasers generating a fixed wavelength (Table 4.15.2).

In general, short-pulse lasers allow smaller spot size and higher resolution. For excitation above 1000 nm, fixed-stage lasers for specific wavelength or a tunable OPO deliver wavelengths up to 1600 nm (Table 4.15.2).

Select detectors and filters

7. In contrast to fluorescence emission, the SHG (THG) emission is near exactly half (one-third) the introduced wave-length. Set conditions so that SHG and THG are spectrally separated from other spectra of the emission signal by dichroic mirrors and relatively narrow band-pass filters spanning 10 to 20 nm. The aim is to block the incident laser beam at least twice at the level of the high-pass and band-pass optical filters. All filters and dichroic mirrors should block the wavelengths at which the laser operates (i.e., up to 1000 nm for Ti:Sapphire laser; up to 1500 nm for an OPO-based system). Further set up details are described in *UNIT 4.11*. As alternative to a cascade of dichroic filters, spectral separation can be achieved by a prism and dynamic range mirrors or an optical array detector, as used in the descanned beam path of Leica or Zeiss confocal systems, respectively (Table 4.15.4). As detectors, very high sensitivity PMTs that have a narrow dynamic range or PMTs with lower sensitivity but higher dynamic range may be used, depending on the application.

   For most applications in ex vivo samples and intravital microscopy, an overlapping spectrum of emitted HHG and fluorescence spectra at different intensity will be present, which requires a broad dynamic range of the PMT detectors at the expense of sensitivity at the ultra-low signal strength. For applications that include very low SHG and THG and relatively limited intensity, such as the detection of small numbers of multimers, a high sensitivity PMT or avalanche photodiodes to count single photons might be required.

BASIC PROTOCOL 2

DETECTION OF FIBRILLAR COLLAGEN IN CONNECTIVE TISSUE EX VIVO

Fibrillar collagen is a very bright second harmonic generator at excitation range above 800 nm; it is suitable for the quantitative reconstruction of ex vivo and in vivo tissue samples (Fig. 4.15.2). Vertically oriented collagen fibers scatter mostly in the forward direction, whereas horizontally oriented fibers scatter bidirectionally (Zipfel et al., 2003), thus, imaging of horizontally positioned fibers should be attempted by adjusting the orientation of the sample.

For 3-D reconstruction of thick specimens of low optical transparency detection of the backward scatter is required, such as thick slices of human skin ex vivo (Fig. 4.15.2) or murine skin during intravital microscopy (Fig. 4.15.3). Other collagenous tissues that require SHG backward detection due to low transparency are bone and parenchymatous organs.

Materials

Freshly excised piece of tissue or organ
Medium of choice (e.g., RPMI, DMEM, or PBS; *APPENDIX 2A*)

Additional reagents and equipment for designing a microscope system for HHGM (Basic Protocol 1)

1. Place freshly excised piece of tissue or organ directly into liquid nitrogen (−196°C).
2. Retrieve the sample and position it onto a microscope slide and add 200 to 1000 µl of medium compatible with the cell or tissue type being used (e.g., RPMI, DMEM, or PBS).
3. Position sample at the microscope and dip the objective directly into the medium.

   If the interface between sample and objective loses water, the immersion depth can be increased by using a polyethylene ring or placing the sample within a petri dish.

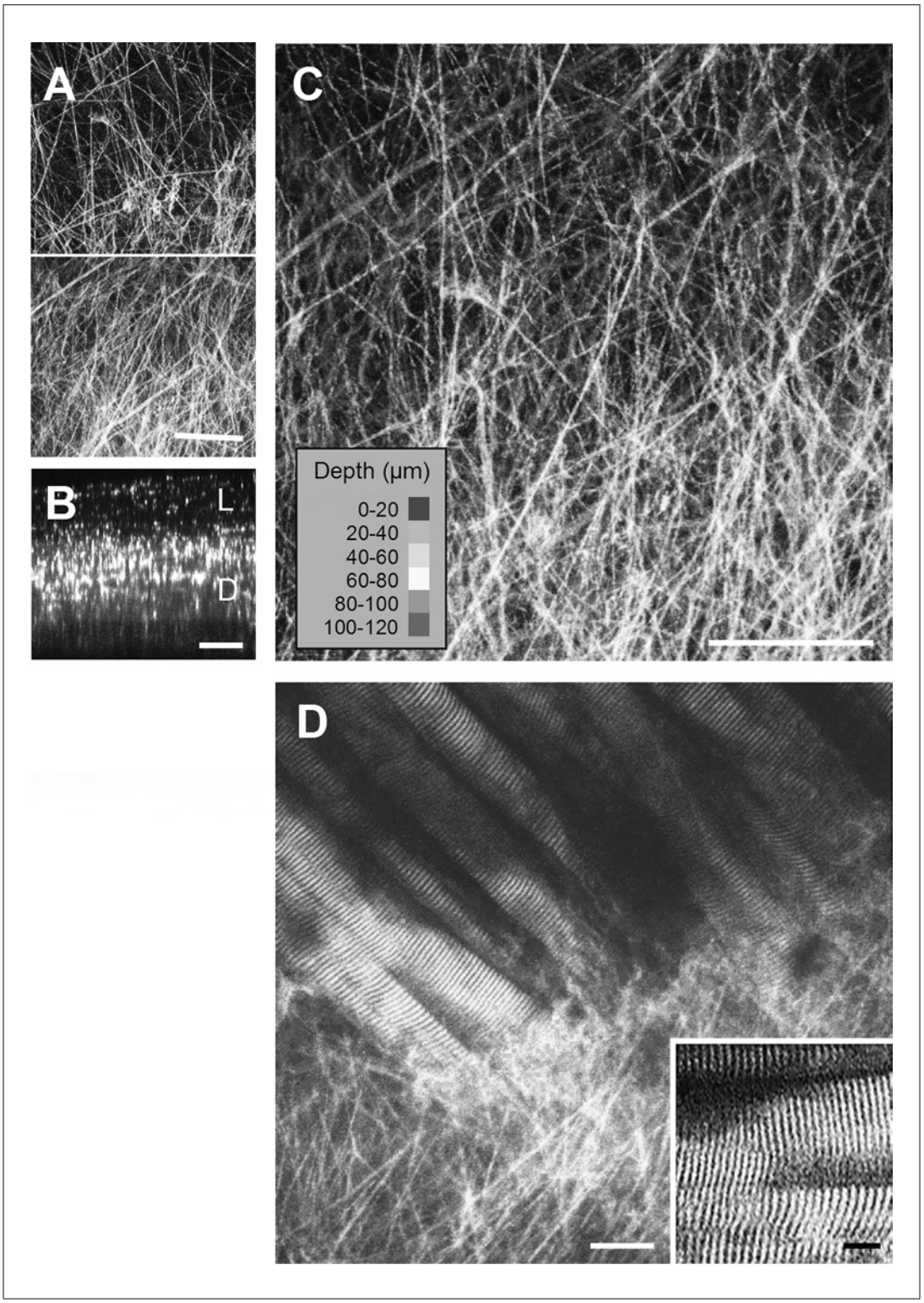


Figure 4.15.3 Intravital SHG microscopy of individual collagen fibers and striated muscle in the mouse dermis. The epidermis of live anesthetized BL6 mice was horizontally removed and *z*-sectioning up to 400 nm in depth was performed using 800-μm excitation and a 20× N.A. 0.75 water immersion objective, as described (Wolf et al., 2003). SHG was detected using an upright microscope at 400/20 nm in backward direction for both *x-y* (**A, C**) and *x-z* sectioning (**B**). (**A**) Depth-encoded reconstruction of collagen fibers. (**B**) Vertical reconstruction showing the loose ("L") and dense ("D") dermal connective tissue, and (**C**) false-color encoded horizontal representation of penetration depth. (**D**) Intravital SHG detection of striated muscle and collagen-rich fascia. Bars = 50 μm (A to C), 30 μm (D) and 10 μm (D, inset). For color version of this figure see *http://www.currentprotocols.com*.

4. Scan sample at 880 nm. To separate SHG at 440 nm from other signals, use a 440/15 or 440/20 band-pass filter before the PMT.

   The autofluorescence emission of native tissues covers the ranges from 480 to 650 nm, thus a dichroic 480-nm beam splitter or below will be useful. Whereas the emission peak of SHG signal shifts with the wavelength of the incident beam, the fluorescence spectrum remains fairly stable.

   For the multimodal reconstruction of human skin, SHG is readily combined with green or red autofluorescence, representing mostly elastic fibers, elastolytic (denatured) collagen fibers, and fat cells (Konig et al., 2005). The combined detection of SHG and autofluorescence was used in the reconstruction of human and mouse skin (Campagnola et al., 2002; Wolf et al., 2003), native arterial vessels (Zoumi et al., 2004; Boulesteix et al., 2006), heart valves (Konig et al., 2005), native mouse lymph node (Mempel et al., 2004a), and lung (Debarre et al., 2006).

5. If necessary, increase the intensity of the SHG signal by altering the polarization of the incident laser beam. Alternatively, try rotating the sample. If signal intensity is very low, increase laser power to 50 to 100 mW to lead to sufficient SHG excitation.

   The SHG signal elicited by collagen stems from the crystalline-like triple-helical structure whose cross-section has rotation symmetry but not inversion symmetry. The signal stems from surface layers of the collagen fibril but not from inner structures (Williams et al., 2005), and its intensity strongly depends on the tertiary and quaternary fibril structure, as well as ionic strength of the surrounding medium. Dissociation of the fibrillar structure and thus second order susceptibility tensor χ by a hyperosmotic agent, such as glycerin which is used as transparency-enhancing clearing agent in transcutaneous microscopy, leads to the denaturation and hence reduction of SHG signal of fibrillar collagen which is reversible upon rehydration (Yeh et al., 2003). Structural changes caused by air-drying leads to a 4-fold reduction of SHG (Yasui et al., 2004), denaturation by heating to 50° to 75°C increases the SHG signal by up to 2.5-fold, whereas higher temperature causes complete disintegration and loss of signal (Tan et al., 2005). Conversely, chemical cross-linking reduces effects caused by denaturation (Yeh et al., 2003). For future work, the susceptibility of structural changes in collagen to SHG imaging will be useful to study collagen assembly and remodeling in morphogenesis and disease state.

6. To calibrate the penetration depth of the system, define the top and bottom of the tissue volume and reconstruct the volume by acquiring sequential scans at different *z*-positions.

   In human skin using 880-nm excitation, which has a lower absorbance and scattering than lower wavelengths, sensitive reconstruction of autofluorescence is achieved up to a depth of 120 μm (Fig. 4.15.2B), whereas the decay of the SHG signal is apparent earlier. In contrast to fibrillar collagens, nonfibrillar collagen, basement membranes, epidermal keratin, and fibrin are not known to elicit SHG (Yasui et al., 2004).

BASIC PROTOCOL 3

DETECTION OF SHG IN MOUSE TISSUES BY INTRAVITAL MICROSCOPY

Whereas in histology and most immunofluorescence applications, samples are processed by fixation and slicing, intravital microscopy in small animal models is based upon minimum change to the specimen observed. Emission thus is preferably endogenous and includes SHG, autofluorescence, and light emitted by endogenously expressed fluorophores in transgenic cells or animals. Here, SHG detection results in an anatomically correct reconstruction of extracellular matrix and certain cell types, such as striated muscle cells.

Materials

Mouse sample

Additional reagents and equipment for preparing mouse samples for intravital microscopy (*UNIT 4.11*), acquiring a *z*-stack (Basic Protocol 2) and, designing a microscope system for HHGM (Basic Protocol 1)

1. Prepare the mouse sample for intravital microscopy, as described in *UNIT 4.11*. The location of interest needs to be stably positioned and fixed on the microscope stage, e.g., using needles or custom-designed holders.
2. Acquire a *z*-stack as described in Basic Protocol 2.
3. Reconstruct the 3-D stack from several individual scans by optical superimposition to generate an extended focus in depth representation. Alternatively, use *x*-*z*-reconstruction to obtain information about the total depth and signal intensity changes relative to penetration depth.

   In contrast to human skin, mouse dermis is less densely textured and consists of loosely oriented individual rather than densely packed collagen fibers (Fig.4.15.3A, B). Particularly due to the low scattering of light, high resolution reconstruction of individual fibers by SHG is achieved up to a depth of 400 μm (Fig. 4.15.3).

4. Using the same setup, detect striated muscle cells in vivo, using SHG. If the signal intensity is low, try rotating the sample slightly around the vertical axis to increase signal strength. For maximum SHG, orient the polarization of the incident beam to 45°, not 0° or 180° to the length orientation of the myosin filaments.

   The sarcomeres are 2- to 3-μm long contractile actomyosin units in striated muscle and cardiomyocytes that generate strong SHG (Fig. 4.15.3D; Both et al., 2004) and moderate THG (Chu et al., 2004). The signal is induced by the α-helical structure of the coiled rod region of the myosin filament, not by myosin heads and actin filaments (Plotnikov et al., 2006). In contrast to SHG, THG is much less angle-dependent (Chu et al., 2004).

SIMULTANEOUS DETECTION OF CELLS AND COLLAGEN FIBERS IN VITRO AND IN VIVO

BASIC PROTOCOL 4

Dynamic imaging of cell-ECM interactions in 3-D in vitro collagen lattices represent a useful, in vivo-like model to reconstruct molecular cell functions in the 3-D tissue-like context (Abbott, 2003). To simultaneously visualize the collagen fibers and the shape and position of live cells, SHG is combined with two-photon excited fluorescence microscopy (Figs. 4.15.4 and 4.15.5).

Materials

Cells expressing GFP or labeled with fluorescent dye (Support Protocol 1)
Collagen lattice containing fluorescent cells (Support Protocol 2)

Additional reagents and equipment for designing a microscope system for HHGM (Basic Protocol 1)

1. No specific cell label is required if the cells endogenously express GFP and thereby provide an intrinsic fluorescent signal for detecting their morphology. Alternatively, the cytoplasm or compartment of interest may be labeled with a fluorescent dye, such as Calcein AM or CFSE which label the cytoplasm (see Support Protocol 1).
2. Incorporate the cells within a 3-D collagen lattice (see Support Protocol 2).
3. Select a wavelength between 800 and 880 nm for excitation and 400 and 440 nm (λ/2) for detection of SHG of collagen fibers (e.g., 440/10). For fluorescence, use a dichroic filter below 510 nm and a band-pass filter suitable for the spectrum of FITC to detect the cells (e.g., 515/30) or a long-pass filter (e.g., LP515).

   Because absorption of light by 3-D collagen lattices is much weaker than by dense human tissues, the excitation wavelength of 800 nm has the advantage of strong excitation and emission strength.

4. To detect SHG and TPF in live samples, use simultaneous two-channel scanning. To determine the number of adjacent sections required for complete volume

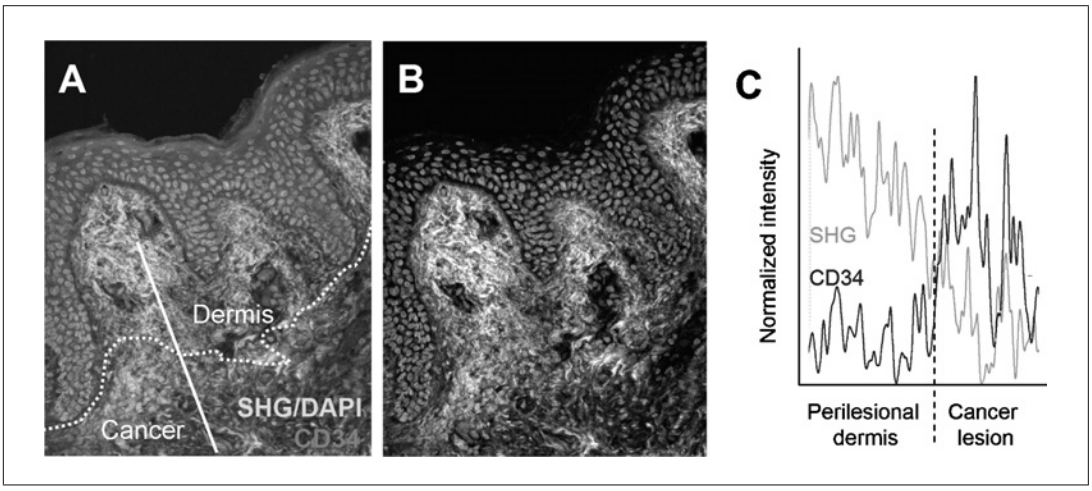


Figure 4.15.4 Multimodal SHG and fluorescence microscopy of invasive cancer lesion. Frozen section (10 μm) of human dermatofibrosarcoma protuberans was stained with anti-CD34 antibody (staining cancer cells) and secondary Alexa 543–conjugated secondary antibody and DAPI and analyzed as described in Figure 4.15.3. The emission of SHG and DAPI was detected at 440/20 and Alexa 543 was detected at 620/40. (**A**) Two-color representation of SHG and fluorescence in epidermis, dermis, and cancer lesion. (**B**) SHG of collagen and DAPI signal alone, showing strongly reduced SHG in the cancer lesion. (**C**) Normalized intensity curves for normal and cancerous tissue show the loss of SHG at the border and within the cancer lesion, as reconstructed along the solid line in (A). For color version of this figure see *http://www.currentprotocols.com*.

reconstruction, run a preliminary *z*-stack and perform a frame-to-frame review of the images. If the same horizontal collagen fiber is present in at least one but no more than two adjacent sections and if diagonally sectioned fibers continuously "run" from frame to frame without jumps, sufficient overlap is present to achieve loss-less 3-D image acquisition. If the axial resolution is known, calculate the number of sections required. A stack depth of 20 μm requires at least five consecutive *z*-sections for an axial resolution of 5 μm (20× objective) or 11 for an axial resolution of 2 μm (63× objective) for loss-less reconstruction.

The aspect and resolution of SHG from collagen matches to great extent the reconstruction using confocal reflection microscopy (Fig. 4.15.5; see UNIT 4.5; Wolf et al., 2003). Using intravital microscopy in anesthetized mice, the combination of fluorescence and SHG allows the dynamic reconstruction of lymphocyte migration and interaction with antigen-presenting cells in the lymph node (Mempel et al., 2004b) and the dissemination of cancer cells in the mammary fat pad towards blood vessels and the immigration therein (Wang et al., 2002).

Quantitative reconstruction of the data set by xyz tracking shows both the fate of migrating cells in cell-cell communication and reverse transmigration in vivo and its relation to existing tissue structures, such as migration in between and along collagen fibers (contact guidance; Wang et al., 2002; Mempel et al., 2004a). Other approaches to image live cells include the tracking of intracellular vesicles in hepatocytes using THG (Debarre et al., 2006) and time-lapse reconstruction of the sarcomere shortening in striated muscle cells in vitro and in vivo (Plotnikov et al., 2006). In all cases, a fast enough acquisition of 3-D data stacks (up to 100 individual slices) requires relatively strong SHG and fluorescence signals in the backward direction and 4-D image reconstruction by a software independent of the scanning routine. Presently available shareware and commercially available software packages for 3-D reconstruction and data analysis are shown in Table 4.15.4.

5. Run a time series by serially acquiring *z*-stacks at defined time intervals, such as one frame per 15 sec for fast leukocytes or one frame per 5 min for slow tumor cells.

Observation periods may vary from a few minutes to several days, depending on the magnitude of migration dynamics.

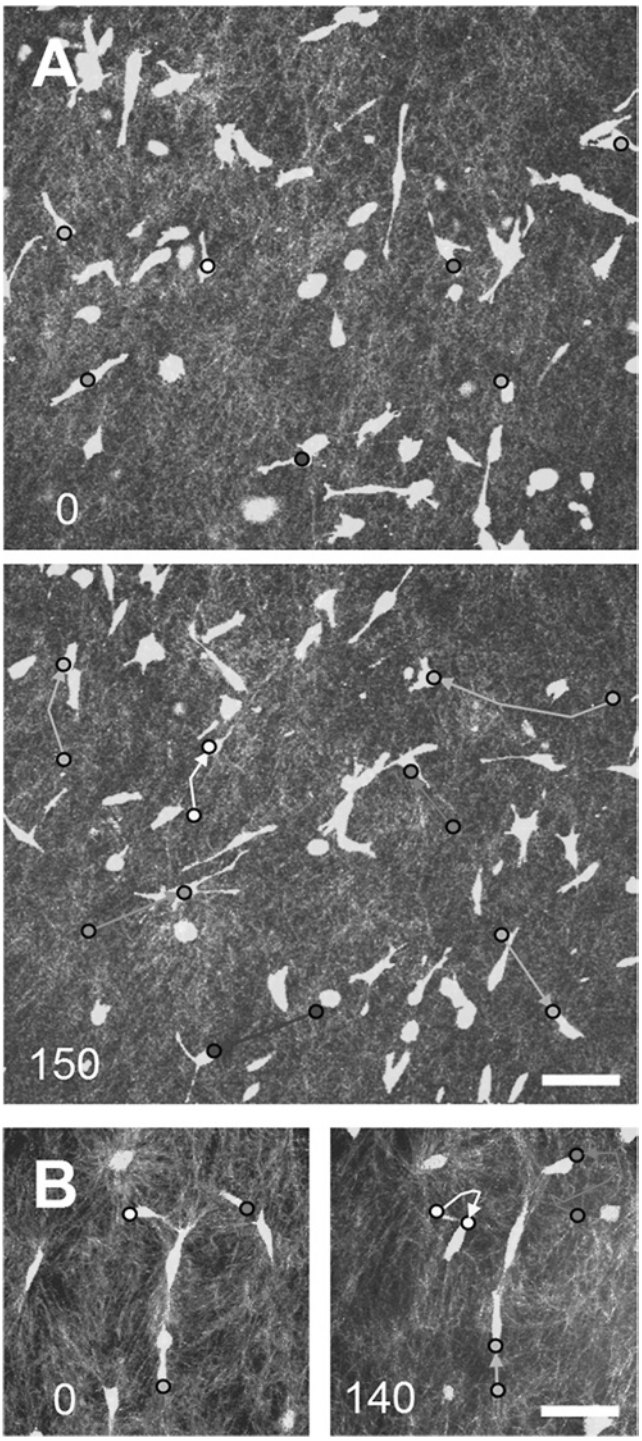


Figure 4.15.5 Real-time reconstruction of cancer cell migration in 3-D collagen lattices using simultaneous multiphoton SHG and fluorescence detection (**A**), compared to confocal reflection and fluorescence microscopy (**B**). HT-1080 fibrosarcoma cells were labeled with Calcein-AM (green), incorporated into 3-D collagen lattices and monitored by either multiphoton (A) or single-photon excited confocal microscopy (B). *Z*-stacks of 16-μm scanning depth (4 sections) were obtained at 15 (A) and 5 (B) min time intervals and superimposed. Position changes of selected cells during the observation interval are highlighted by start and end point as well as migration path. Collagen is equally well reconstructed by SHG, compared to confocal reflection microscopy (Friedl et al., 1997). Preserved cell elongation and position change during the entire observation indicate the absence of photodamage in both settings (incident light power in (A) was 15 mW). Bars = 80 μm. Time (min) is indicated in the lower left corner. For color version of this figure see *http://www.currentprotocols.com*.

SUPPORT PROTOCOL 1

CYTOPLASMIC STAINING OF LIVE CELLS

This procedure is used to stain the cells in 3-D collagen cultures to differentiate the cells from the collagen fibrils.

Materials

Cultured cells in culture flask or petri dish
Calcein-AM (Molecular Probes)
DMSO (Invitrogen)
Dulbecco's modified Eagle's medium (DMEM) *or* other medium (e.g., PBS; *APPENDIX 2A*); may contain serum and antibiotics

1. Add a small aliquot of Calcein-AM from stock solution (1 mM; in DMSO) to the medium of the culture in a collagen lattice to obtain a final concentration of 1 μM.
2. Incubate the culture for 60 min at 37°C.
3. Remove the supernatant and wash the culture twice using medium or PBS.
4. Proceed with Basic Protocol 4.

SUPPORT PROTOCOL 2

ESTABLISHMENT OF 3-D COLLAGEN CULTURES

3-D collagen lattices represent a simple yet biologically meaningful alternative to 2-D liquid culture approaches to measure cell functions, such as proliferation, survival, differentiation, and migration (Friedl et al., 1997; Friedl, 2004). This procedure describes how cells are incorporated into the 3-D lattices so they are embedded from the onset of the culture.

Materials

Adherent cells
2 mM EDTA solution in PBS, pH 7.4
Dulbecco's modified Eagle's medium (DMEM) or other medium; may contain serum and antibiotics (*APPENDIX 2A*)
10× concentrated minimal essential Eagle's medium (MEM; Flow Laboratories) or other 10× concentrated medium
7.5% (w/v) sodium bicarbonate solution (Life Technologies)
3.0 mg/ml collagen stock solution, acidified (e.g., bovine dermal collagen; Vitrogen; Nutacon BV or rat tail collagen; Becton Dickinson)
Migration chamber (see recipe)

1. Detach adherent cells (e.g., 2×10^5) by incubating in 2 mM EDTA for 2 to 5 min, 37°C. Wash twice with 1 to 10 ml of medium, centrifuging 10 min at 300 × *g*, 4°C each time. Decant the supernatant and resuspend the cells in 500 μL medium.

   EDTA is preferred over trypsin/EDTA because it does not lead to alterations in surface protein composition caused by the enzymatic removal of adhesion receptors.

2. Add 100 μl of 10× MEM to 50 μl sodium bicarbonate in a small sterile tube. Check that the indicator color changes from yellow (acidic) to purple (basic).
3. Add 750 μl cell-free collagen stock solution and mix until homogenous slightly purple color is obtained.

   Avoid air bubbles while mixing.

4. Add 450 μl cell suspension in medium and mix until homogenous bright pink color is reached. Again, avoid generating air bubbles.

   The pH should now be 7.8 to 8.0. To verify the pH use a pH indicator strip.

5. Add the cell-containing collagen solution into the incubation chamber.
6. Place the incubation chamber into the incubator (37°C, 5% CO_2) for 15 to 30 min for collagen polymerization and equilibration of the gas conditions. The final pH should be 7.4 to 7.5.

   If a commercially available climatized incubator is used with the video microscope, steps 1 to 6 can be modified for ECM culture in conventional petri dishes or microtiter plates.
7. Fill the chamber with warm and previously equilibrated medium.

   This medium can contain chemokines or other factors, such as antibody or pharmacological inhibitors.
8. Close the migration chamber, place it on the microscope stage and proceed with Basic Protocol 4.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Migration chamber

A migration chamber is a cell incubation chamber, either from commercial sources or self-constructed (Friedl and Bröcker, 2004). Most microscope manufacturers provide small chambers for a small petri dish or specialized incubation chambers for extra small volumes. Alternatively, an incubation chamber can be self-constructed. An example for a self-constructed migration chamber is detailed in Friedl and Bröcker (2004).

COMMENTARY

Background Information

Physical principle of two-photon and multiphoton effects

Two-photon excitation occurs by the near-simultaneous absorption of two or more photons by a molecule normally excitable by one photon of twice the energy and occurs the moment two lower energy photons are simultaneously absorbed by the same molecule (Fig. 4.15.6A). The first photon generates a virtual state of the electron and the second must be absorbed before the virtual state is de-excited (i.e., within the Heissenberg approximation of 10^{-15} sec). Together, both photons result in an excitation of nearly the same impact as a single higher energy photon excitation of the same molecule. In order to achieve a high enough photon density for two-photon excitation, the irradiant laser light needs to be phase-(mode)-locked and pulsed.

The pulse width of the irradiant beam is determined from an autocorrelation (in time) experiment in which the full-width at half-maximum of the pulse is in the range of 2 to 10 psec (for picosecond lasers) or 100 to 300 fsec (for femtosecond lasers) interrupted by an illumination-free interval of several nanoseconds, which results in a repetition rate of 76 to 96 MHz (Fig. 4.15.6A). Because of the high photon density during the pulse, high probabilities for two-photons or multiple photons to become near-simultaneously absorbed by a molecule is high, provided minimum of tens of milliwatts (for fsec pulses) are present in the focal plane under the objective (corresponding to an overall intensity ranging from MW/cm^2 up to GW/cm^2).

Two-photon excitation is quadratically related to the illumination intensity at the focal point, and only at the focal point is a significant number of photons achieved. Consequently, signal arises only from the scanned focal volume, and thin optical sections are being generated in 3-D samples, whereas little excitation and photodamage occurs outside the focal plane. Therefore, multiphoton excitation lacks photobleaching and phototoxicity in regions above or below the focal plane.

Higher harmonic generation (HHG)

HHG includes SHG and THG (Fig. 4.15.6B). SHG results from the sum frequency doubling to produce a narrow band of half the excitation wavelength and twice the energy (Fig. 4.15.6B). THG results from the sum frequency tripling to produce emission at a third of the excitation wavelength and triple

the energy (Fig. 4.15.6B). HHG occurs only in the focal plane providing sufficiently high photon density and, similar to multiphoton fluorescence microscopy, represents an inherently confocal approach allowing 3-D reconstruction from *z*-slices (Fig. 4.15.6C). Emitted HHG signals are commonly detected by photomultipliers (Fig. 4.15.6C). In contrast to fluorescence, most cases of HHG do not involve the absorption of light, thus relatively little heat is produced. Therefore, pure harmonic generation imaging tends to be much less phototoxic than two-photon or multiphoton fluorescence microscopy.

SHG

SHG is a nonlinear coherent scattering process that conserves energy. The summation of the light rays into the second harmonic frequency results from phase matching of the incident light with an environment that encourages summation of frequencies. The resulting signal

$$\mathrm{SHG} \propto \frac{(p \cdot \chi^{(2)})^2}{\tau}$$

is dependent on the square of the input laser power, p, a susceptibility coefficient, χ, and the width of the laser pulse, τ. As the light rays pass through this environment the electric field of the light exerts forces on the electron charge distribution and consecutive changes in a dipole moment, referred to as the electric polarization. The electric polarization (P) is linear, but for high-energy electric fields, such as an incident laser pulse (E) of high energy, P becomes a summation of linear and nonlinear terms. SHG is sensitive to structural molecular alterations caused by external factors such as temperature, folding, osmolarity, and ionic strength; biological structures may vary in their proportion of linear and nonlinear properties. Therefore, SHG should be tried out for each sample and contains an empiric component. The electric polarization is further determined by a susceptibility factor (χ) which depends on the environmental and molecular structure and polarization angle with respect to the light rays, as defined by

$$P^{(2)}_{\mathrm{SHG}} = \chi^{(2)} E \cdot E$$

The intensity of emitted light is nonlinearly proportional to the input light frequency but has a quadratic dependence, which means that below a certain threshold at low excitation energy no signal will be obtained whereas a near linear emission is achieved above the threshold. In the linear range, intensity changes can thus be used for quantitative image analysis.

The strength of SHG is further dependent on the size scale to which the noncentrosymmetric (NCS) medium is effective, called the coherence length, L_c, $\Delta k \cdot L_c \approx \pi$, where Δk is the difference in wavevectors,

$$k = \frac{2\pi}{\lambda}$$

between the fundamental and second harmonic waves of wavelength, λ. The coherence length is dependent on the incident wavelength and must be in the order of the NCS period size.

SHG is an anisotropic process and mainly propagates in the same direction of the incident light, whereas only a minor fraction is backscattered (Fig. 4.15.6C). The ratio of SHG signal for forward to backward direction often ranges above 5:1 (tubulin, fibrillar collagen; Dombeck et al., 2003), thus forward detection is required for low S/N ratio.

THG

THG is based on the "third order susceptibility" which means that its signal has a cubic dependence on input intensity:

$$P^{(3)}_{\mathrm{THG}} = \chi^{(3)} E \cdot E \cdot E$$

Unlike the physical and geometrical rules of SHG, all materials potentially possess properties to elicit THG to a varying degree, but the THG signal intensity is more sensitive to and requires high average powers and small pulsewidths,

$$\mathrm{THG} \propto \frac{(p \cdot \chi^{(3)})^3}{\tau^2}$$

Similar to SHG, the susceptibility factor, $\chi^{(3)}$ in THG is variable and also changes vastly from material to material, thus extensive spectral examination is required for each individual sample type. Near-infrared input elicits third harmonic generation of ultraviolet light of relatively lower penetration depths in scattering samples, whereas the use of excitation above 1200 nm reaches TGH signals in the visible range. THG is an isotropic process, therefore emission signal intensities are equally strong in forward and backward directions.

Although the above theoretical considerations predict ubiquitous THG for most biological samples, empirical evidence suggests that TGH signals, which are intrinsically weak,

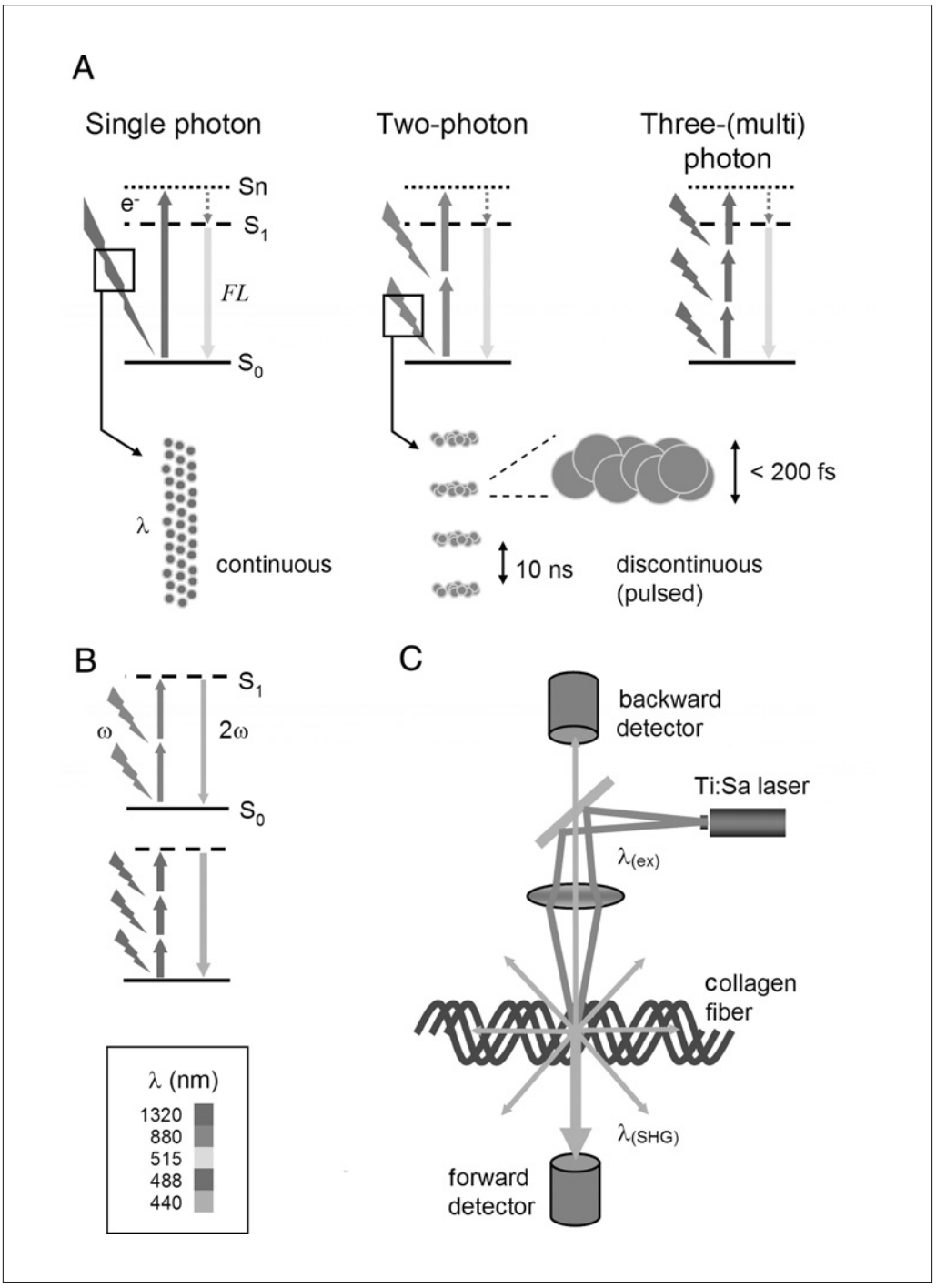


Figure 4.15.6 Principles of single-, two-, and three-photon excited fluorescence (**A**), compared to second and third harmonic generation (**B, C**). (**A**) Excitation of fluorescence. Single-photon excitation results from a continuous photon flux, generating continuous emission. Two- and three-photon excitation are caused by high-energy pulses of light of longer wavelengths. In both cases, the emission spectrum is near identical. (**B**) Excitation of second and third harmonic generation. Two (top) or three photons (bottom) of pulsed high-energy light are converted to one photon of two- or three-fold energy, respectively, corresponding to half or a third of the wavelength. (**C**) Beam path of forward and backward SHG induced by triple-helical collagen. Abbreviations: *FL*, fluorescence emission; S_0, nonexcited state of an electron; S_1, excited state of an electron; S_n, short-lived excited state of higher energy level; ω, frequency of the light; $\lambda_{(ex)}$, wavelength of excitation beam; $\lambda_{(SHG)}$, wavelength resulting from SHG. For color version of this figure see *http://www.currentprotocols.com*.

are further dampened by homogeneous out-of-focus regions just above and below the focal point that interfere destructively and delete the net signal.

Critical Parameters and Troubleshooting

Photodamage in live samples

The maximum photon flux and thus signal-to-noise ratio is limited by photodamage which varies for different sample types. Even though the use of (near-)infrared light causes less photodamage to live cells and tissues than visible or UV light excitation, the use of too high peak power required for enhancing weak two-photon and multiphoton effects can severely compromise cell function and integrity. The mechanisms of laser-induced cell damage are not resolved, however a combination of local heat, absorption of incident illumination, and toxic photoproducts of chromophores are plausible explanations. A reliable net indicator of photodamage is the generation of gas bubbles in the sample causing the loss of specific signal of both SHG and fluorescence (Fig. 4.15.7).

As a rule of thumb for the tuning range from 700 to 1000 nm, intensities of up to 7 mW, as measured by a powermeter below the objective, will not cause photodamage and intensities up to 15 mW can be tolerated by live cells over several hours if the lag phase between scanning is sufficiently long (Fig. 4.15.6). Much higher excitation intensities up to 100 mW or more in the focal plane can be used for detecting weak signals of the tissue microarchitecture without signs of photodamage or denaturation, such as collagen and elastin in fixed samples (Dombeck et al., 2005; Boulesteix et al., 2006; Debarre et al., 2006). At illumination >1000 nm, higher intensities are well tolerated. Long-term continuous-time scanning in living *Xenopus* embryos using excitation at 1280 nm and a power of 75 mW is required to generate strong SHG and THG signals yet lacks apparent toxicity (Sun, 2005). Likewise, excitation of membrane potential dyes at 1064 nm up to 80 mW is tolerated by neuronal cells, suggesting that long-term real-time microscopy by multiphoton excitation will develop towards better tolerability using excitation wavelengths above 1 μm.

Anticipated Results

Other structures detected by HHGI

Microtubules In cells, polymerized tubulin forms the filamentous microtubule network important in cell polarity, vesicle trafficking, and mitosis. Microtubules in the polymerized state, but not after depolymerization by nocodazole, generate relatively weak SHG signals in brain slices (Dombeck et al., 2003). Mitotic spindles can be detected in living cultured cells (Dombeck et al., 2003) or intact embryos (Sun, 2005).

DNA. Condensed chromosomes during mitosis, but not interphase DNA, after extraction and fixation and mounting on a glass slide can be detected by SHGI, using 800-nm excitation wavelength (Gauderon et al., 2001). By contrast, no published example has monitored SHG or THG of nonlabeled DNA in live cells and tissues, suggesting that the sample preparation contributes to the noncentrosymmetry and spatial arrangement of the chromosome structure.

Bone and teeth. Mineralized bone matrix and developing central bone marrow cavity in the mouse embryo is detected by THG up to penetration depths of several hundred microns (Oron et al., 2004). Despite progressive mineralization, both loose and compact bone regions are resolved in detail. In sections from teeth, linear SHG and even stronger THG signal is generated by dentin, most likely originating from the underlying collagen fiber scaffold (Kao, 2004). Particularly inorganic crystals and inorganic complexes in tissue display an abrupt change in refractive index at their surfaces, relative to the surrounding tissue, which is strongly susceptible to THG.

Membrane potential. Styryl dyes are elongated molecules with large hyper-Rayleigh scattering efficiency that get aligned in roughly perpendicular order along the plasma membrane to form anisotropic scaffolds, the orientation of which is sensitive to the transmembrane electric field. Alterations of the membrane potential, as during depolarization, cause electro-optic realignment and thus, anisotropy that is exploited as increased SHG for membrane potential measurements (Pons et al., 2003; Dombeck et al., 2004; Millard et al., 2004; Sacconi et al., 2005). Live-cell studies indicate that the dyes RH 237, FM4-64, and JPW1114 show sufficient signal-to-noise ratio and little toxicity (Dombeck et al., 2005) and can be combined with conventional fluorescent indicators, including Ca^{2+}- and Na^{+}-sensitive dyes. The absolute change in signal intensity amounts to 5% to 20% per 100 mV which is linear to the amount of depolarization and can be calibrated by voltage-gated patch clamp technique (Dombeck et al., 2005; Sacconi et al., 2005). Differences of absolute

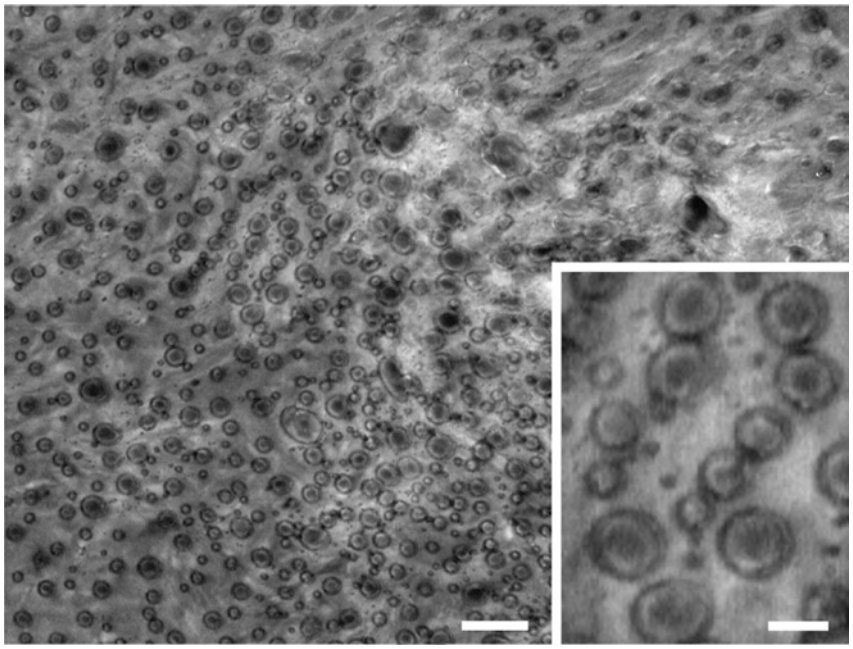


Figure 4.15.7 Multiphoton-induced photodamage and loss of SHG signal in native section of the dermis. Excitation was obtained at 880 nm, the incident light power was 160 mW (~10 scans). Photodamage was apparent from gas bubbles and the loss of both SHG (blue) and autofluorescence (green and red). Bars = 20 μm and 10 μm (inset). For color version of this figure see *http://www.currentprotocols.com*.

SHG sensitivity are specific for each dye, cell type, and subcellular location of the region of interest, and transformed cells appear to develop stronger signals than nontransformed cells (Dombeck et al., 2005; Sacconi et al., 2005). Forward detection in transparent cell culture as well as backward detection (at 80% lower efficiency) from thick tissue slices at high temporal (<1 msec) and spatial resolution can be achieved (Dombeck et al., 2005). Thus, SHG imaging will be an important approach to intravital reconstruction of fast-voltage transients in cell membranes during cell-cell signaling at minimum interference with the electrophysiological properties of the membrane.

Plants. Cellulose and starch are both polymers of glucose. Cellulose is composed of a polymer of β-1,4 linkages and starch of α-1,4 linkages. Individual polymer chains are cross-linked and arrange in a crystal-like order to form fibrils of high tensile strength that are susceptible to SHG. Starch, because of its open helical structure, causes higher SHG intensity than cellulose, which is more linear in structure (Cox et al., 2005).

Lipid bilayers. Although all materials have the susceptibility for THG, lipid membranes contained in aqueous medium have a comparatively high third-order susceptibility due to the formation of water-lipid interfaces. Strong THG signals are generated by lipid membrane and lipid bodies in tissues and in vitro systems, including lipid bodies and vesicles in live hepatocytes (Debarre et al., 2006).

SHG and THG probes. Several extrinsically applied labels exhibit strong SHG signals, either based on their own structure, or after their interaction with cell and tissue components. These include gold nanoparticles for immunological staining (Jumah et al., 2004; Lippitz et al., 2005) and polyhydroxybutyrate (PHB) polymers that generate biodegradable fibrillar structures used for tissue engineering (Deng et al., 2003).

Time Considerations

The acquisition of a single image frame takes 0.2 to 5 sec, depending on the scanning frequency and resolution. Image stack acquisition from the reconstruction of a 3-D volume requires a few up to several hundred images, thus may take several seconds up to minutes for a single data point. Whereas a single microscopy experiment is complete within a few minutes to hours, sample preparation, as well as image reconstruction and data analysis, may take up to days and weeks.

Acknowledgements

The authors would like to thank Volker Andresen for expert system design and helpful discussions and Matthias Gunzer for providing information on software use.

Literature Cited

Abbott, A. 2003. Cell culture: Biology's new dimension. *Nature*. 424:870-872.

Both, M., Vogel, M., Friedrich, O., von Wegner, F., Kunsting, T., Fink, R.H., and Uttenweiler, D. 2004. Second harmonic imaging of intrinsic signals in muscle fibers in situ. *J. Biomed. Opt*. 9:882-892.

Boulesteix, T., Pena, A.M., Pages, N., Godeau, G., Sauviat, M.P., Beaurepaire, E., and Schanne-Klein, M.C. 2006. Micrometer scale Ex Vivo multiphoton imaging of unstained arterial wall structure. *Cytometry A*. 69:20-26.

Campagnola, P.J., Millard, A.C., Terasaki, M., Hoppe, P.E., Malone, C.J., and Mohler, W.A. 2002. Three-dimensional high-resolution second-harmonic generation imaging of endogenous structural proteins in biological tissues. *Biophys. J*. 82:493-508.

Chu, S.W., Chen, I.H., Liu, T.M., Sun, C.K., Lee, S.P., Lin, B.L., Cheng, P.C., Kuo, M.X., Lin, D.J., and Liu, H.L. 2002. Nonlinear biophotonic crystal effects revealed with multimodal nonlinear microscopy. *J. Microsc*. 208:190-200.

Chu, S.W., Chen, S.Y., Chern, G.W., Tsai, T.H., Chen, Y.C., Lin, B.L., and Sun, C.K. 2004. Studies of chi(2)/chi(3) tensors in submicron-scaled bio-tissues by polarization harmonics optical microscopy. *Biophys. J*. 86:3914-3922.

Cox, G., Kable, E., Jones, A., Fraser, I., Manconi, F., and Gorrell, M.D. 2003. 3-dimensional imaging of collagen using second harmonic generation. *J. Struct. Biol*. 141:53-62.

Cox, G., Moreno, N., and Feijo, J. 2005. Second-harmonic imaging of plant polysaccharides. *J. Biomed. Opt*. 10:024013.

Debarre, D., Supatto, W., Pena, A.M., Fabre, M., Tordjmann, T., Combettes, L., Schanne-Klein, M.C., and Beaurepaire, E. 2006. Imaging lipid bodies in cells and tissues using third-harmonic generation microscopy. *Nat. Methods*. 3:47-53.

Deng, Y., Miranda, P., Pajares, A., Guiberteau, F., and Lawn, B.R. 2003. Fracture of ceramic/ceramic/polymer trilayers for biomechanical applications. *J. Biomed. Mater. Res. A*. 67:828-833.

Denk, W., Strickler, J.H., and Webb, W.W. 1990. Two-photon laser scanning fluorescence microscopy. *Science* 248:73-76.

Dombeck, D.A., Kasischke, K.A., Vishwasrao, H.D., Ingelsson, M., Hyman, B.T., and Webb, W.W. 2003. Uniform polarity microtubule assemblies imaged in native brain tissue by second-harmonic generation microscopy. *Proc. Natl. Acad. Sci. U.S.A*. 100:7081-7086.

Dombeck, D.A., Blanchard-Desce, M., and Webb, W.W. 2004. Optical recording of action potentials with second-harmonic generation microscopy. *J. Neurosci*. 24:999-1003.

Dombeck, D.A., Sacconi, L., Blanchard-Desce, M., and Webb, W.W. 2005. Optical recording of fast neuronal membrane potential transients in acute mammalian brain slices by second-harmonic generation microscopy. *J. Neurophysiol*. 94:3628-3636.

Friedl, P. 2004. Dynamic imaging of cellular interactions with extracellular matrix. *Histochem. Cell Biol*. 183:183-190.

Friedl, P. and Bröcker, E.B. 2004. Reconstructing leukocyte migration in 3D extracellular matrix by time-lapse videomicroscopy and computer-assisted cell tracking. *Methods Mol. Biol*. 239:77-90.

Friedl, P., Maaser, K., Klein, C.E., Niggemann, B., Krohne, G., and Zanker, K.S. 1997. Migration of highly aggressive MV3 melanoma cells in 3-dimensional collagen lattices results in local matrix reorganization and shedding of alpha2 and beta1 integrins and CD44. *Cancer Res*. 57:2061-2070.

Gauderon, R., Lukins, P.B., and Sheppard, C.J. 2001. Simultaneous multichannel nonlinear imaging: Combined two-photon excited fluorescence and second-harmonic generation microscopy. *Micron*. 32:685-689.

Jumah, N.A., Ameer-Beg, S.M., White, N.S., Prasad, K.V., and Bellhouse, B.J. 2004. Identification of second harmonic optical effects from vaccine coated gold microparticles. *Phys. Med. Biol*. 49:3603-3612.

Kao, F.J. 2004. The use of optical parametric oscillator for harmonic generation and two-photon UV fluorescence microscopy. *Microsc. Res. Tech*. 63:175-181.

Konig, K. 2000. Multiphoton microscopy in life sciences. *J. Microsc*. 200:83-104.

Konig, K., Schenke-Layland, K., Riemann, I., and Stock, U.A. 2005. Multiphoton autofluorescence imaging of intratissue elastic fibers. *Biomaterials*. 26:495-500.

Lippitz, M., van Dijk, M.A., and Orrit, M. 2005. Third-harmonic generation from single gold nanoparticles. *Nano Lett*. 5:799-802.

Mempel, T.R., Henrickson, S.E., and von Andrian, U.H. 2004a. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*. 427:154-159.

Mempel, T.R., Scimone, M.L., Mora, J.R., and von Andrian, U.H. 2004b. In vivo imaging of leukocyte trafficking in blood vessels and tissues. *Curr. Opin. Immunol*. 16:406-417.

Millard, A.C., Jin, L., Wei, M.D., Wuskell, J.P., Lewis, A., and Loew, L.M. 2004. Sensitivity of second harmonic generation from styryl dyes to transmembrane potential. *Biophys. J*. 86:1169-1176.

Mohler, W., Millard, A.C., and Campagnola, P.J. 2003. Second harmonic generation imaging of

endogenous structural proteins. *Methods* 29:97-109.

Oron, D., Yelin, D., Tal, E., Raz, S., Fachima, R., and Silberberg, Y. 2004. Depth-resolved structural imaging by third-harmonic generation microscopy. *J. Struct. Biol.* 147:3-11.

Plotnikov, S.V., Millard, A.C., Campagnola, P.J., and Mohler, W.A. 2006. Characterization of the myosin-based source for second-harmonic generation from muscle sarcomeres. *Biophys. J.* 90:693-703.

Pons, T., Moreaux, L., Mongin, O., Blanchard-Desce, M., and Mertz, J. 2003. Mechanisms of membrane potential sensing with second-harmonic generation microscopy. *J. Biomed. Opt.* 8:428-431.

Sacconi, L., D'Amico, M., Vanzi, F., Biagiotti, T., Antolini, T., Olivotto, M., and Pavone, F.S. 2005. Second-harmonic generation sensitivity to transmembrane potential in normal and tumor cells. *J. Biomed. Opt.* 10:024014.

Schaller, R.D., Johnson, J.C., and Saykally, R.J. 2000. Nonlinear chemical imaging microscopy: Near-field third harmonic generation imaging of human red blood cells. *Anal. Chem.* 72:5361-5364.

Stoller, P., Kim, B.M., Rubenchik, A.M., Reiser, K.M., and Da Silva, L.B. 2002. Polarization-dependent optical second-harmonic imaging of a rat-tail tendon. *J. Biomed. Opt.* 7:205-214.

Sun, C.K. 2005. Higher harmonic generation microscopy. *Adv. Biochem. Eng. Biotechnol.* 95:17-56.

Tan, H.Y., Teng, S.W., Lo, W., Lin, W.C., Lin, S.J., Jee, S.H., and Dong, C.Y. 2005. Characterizing the thermally induced structural changes to intact porcine eye, part 1: Second harmonic generation imaging of cornea stroma. *J. Biomed. Opt.* 10:54019.

Wang, W., Wyckoff, J.B., Frohlich, V.C., Oleynikov, Y., Huttelmaier, S., Zavadil, J., Cermak, L., Bottinger, E.P., Singer, R.H., White, J.G., Segall, J.E., and Condeelis, J.S. 2002. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res.* 62:6278-6288.

Williams, R.M., Zipfel, W.R., and Webb, W.W. 2005. Interpreting second-harmonic generation images of collagen I fibrils. *Biophys. J.* 88:1377-1386.

Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U.H., Deryugina, E.I., Strongin, A.Y., Brocker, E.B., and Friedl, P. 2003. Compensation mechanism in tumor cell migration: Mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J. Cell Biol.* 160:267-277.

Yasui, T., Tohno, Y., and Araki, T. 2004. Characterization of collagen orientation in human dermis by two-dimensional second-harmonic-generation polarimetry. *J. Biomed. Opt.* 9:259-264.

Yeh, A.T., Choi, B., Nelson, J.S., and Tromberg, B.J. 2003. Reversible dissociation of collagen in tissues. *J. Invest. Dermatol.* 121:1332-1335.

Zipfel, W.R., Williams, R.M., Christie, R., Nikitin, A.Y., Hyman, B.T., and Webb, W.W. 2003. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc. Natl. Acad. Sci. U.S.A.* 100:7075-7080.

Zoumi, A., Lu, X., Kassab, G.S., and Tromberg, B.J. 2004. Imaging coronary artery microstructure using second-harmonic and two-photon fluorescence microscopy. *Biophys. J.* 87:2778-2786.

Contributed by Peter Friedl, Katarina Wolf, and Gregory Harms
University of Würzburg
Würzburg, Germany

Ulrich H. von Andrian
Harvard Medical School
Boston, Massachusetts

Analyzing Real-Time Video Microscopy: The Dynamics and Geometry of Vesicles and Tubules in Endocytosis

In recent years there has been a rapid increase in the use of real-time video microscopy of cellular processes. Typically, a protein of interest is fluorescently tagged, and a sequence of images is obtained at regular time intervals. To date, the analysis of the movies has been largely qualitative, with a protein of interest being observed to be transported between locations, noting general changes in morphology over time, and occasionally making dimensional measurements. But real-time imaging offers many more opportunities to make systematic measurement of quantities such as volume, surface area, and flux, and their changes over time. As well as allowing quantitative comparisons between systems when, for instance, a protein is modified, this approach can lead to a better qualitative understanding by refining thinking about the systems observed. With the use of three-dimensional real-time video microscopy becoming more common (Rietdorf and Zimmermann, 2000), quantitative geometric analysis will only increase in importance.

One approach to analyzing real-time cellular processes is to model the structures as geometric objects in order to estimate quantities such as surface area and volume, and their changes over time. A fundamental assumption is that membrane is conserved, i.e., any membrane entering or leaving a system can be accounted for. For example, in the case of the vesicle-tubule systems this corresponds to the assumption that for any decrease in the membrane area of a vesicle there is a corresponding increase in membrane in a tubule. Whether membrane area conservation holds in an experiment depends strongly on the cellular system and the observation methods, but once the assumption is made or has been shown experimentally to be true, it is possible to infer many quantities not directly observable in the images.

Generally, it may be true that there are membrane transport events that are not being observed in individual live-cell video microscopy experiments. But, by making assumptions or estimations about them, mathematically testable hypotheses can be generated. The measurements obtained from the experimental data will then either confirm what is expected, or the hypotheses will have to be revisited.

The following sections demonstrate how to apply geometric and conservation principles to experimental data using endosomal membrane tubulation following endocytosis as an example. Each section first describes a general principle, shows how the principle has been applied to an example, and concludes with the observations and conjectures that the analysis has yielded.

DATA AND MEASUREMENT

The following example uses tubulation of a vesicle during endocytosis. Some typical images are shown in Figure 4.16.1 from a sequence of 90 images taken at 10-sec intervals, giving a total time span of 900 seconds (15 min; Kerr et al., 2006). For the purposes of this example, this sequence will be referred to as The Movie. In The Movie (see Video 1 at *http://www.currentprotocols.com*) the vesicle is initially irregular in shape (Fig. 4.16.1A), but it is observed to rapidly become circular as tubulation is initiated (Fig. 4.16.1B). In the center left of Figure 4.16.1B a roughly spherical vesicle with multiple tubules radiating out from it can be seen. Over 900 sec the vesicle decreases in radius from ~2.5 μm to 1.25 μm. There is an initial burst of tubule activity with many long and branched tubules formed. After ~250 sec (25 frames), tubule activity significantly decreases, with only a few short tubules observed and little or no branching (Fig. 4.16.1C). Faster frame rate movies of up to 1 frame per second have been taken, and in these the tubules are observed to grow, break off, and be transported away from the vesicle (Kerr et al., 2006).

The real-time video microscopy images are recorded as a sequence of TIFF format images with a fixed time interval between each frame. Using software such as ImageJ (see Internet Resources), each frame may be examined individually and measurements obtained. ImageJ enables the user to measure common geometric properties such as length, fit circles and ellipses to structure in the images, and record measurements over a

Contributed by Nicholas Hamilton, Markus C. Kerr, Kevin Burrage, and Rohan D. Teasdale
Current Protocols in Cell Biology (2007) 4.16.1-4.16.11

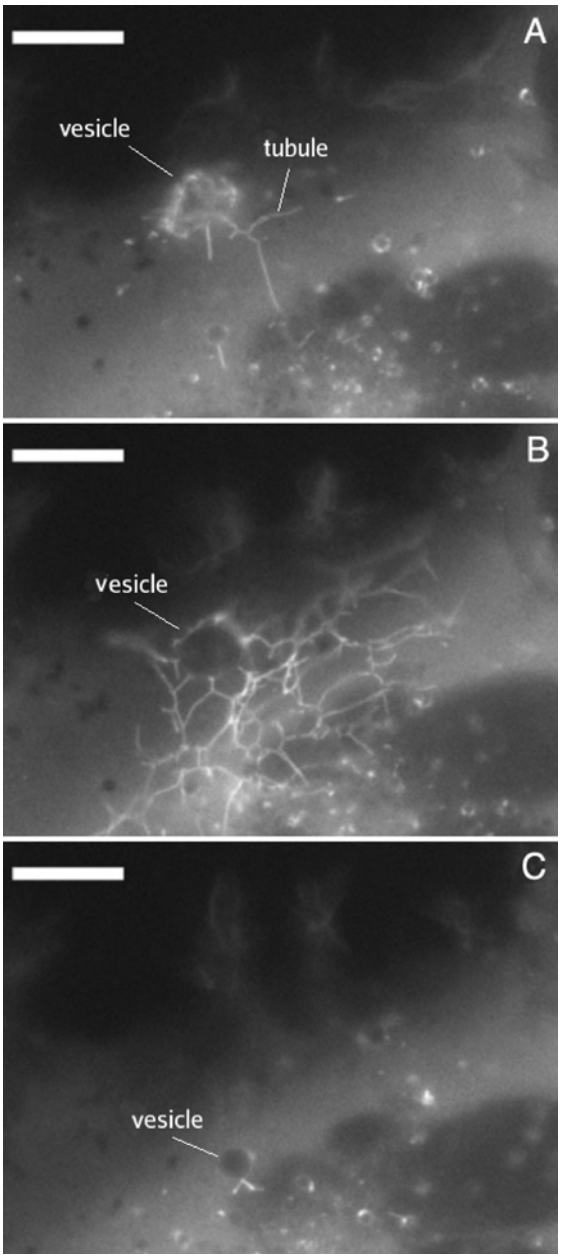


Figure 4.16.1 Tubule formation on a vesicle during endocytosis. Flp-In HEK293 cells stably expressing GFP-SNX5 were generated as described in Merino-Trigo et al. (2004). HEK-GFP-SNX5 cell monolayers were serum-starved overnight before being exposed to 100 ng/ml recombinant EGF ~1 min before the movie was started. Time-lapse movies were recorded using an Olympus IX71 inverted microscope equipped with an Olympus 60X oil objective. Images were captured with an IMAGO Super VGA 12 bit 1280 × 1024 pixel CCD camera (T.I.L.L. Photonics). Imaging control and post-capture image analysis are performed using TILLvisION software and ImageJ v1.31 (see Internet Resources). The delay between image capture was 10 sec. A single macropinocytic event heavily decorated with GFP-SNX5 shortly after its formation at the plasma membrane is presented. GFP-SNX5 is evident on tubular extensions of the limiting membrane of the macropinosome, within the cytosol and on smaller perinuclear puncta reminiscent of early endosomes. Image (**A**) shows the irregularly shaped vesicle and tubules at time 0. Image (**B**) is 210 sec later. Significant tubulation has occurred: many long and branched tubules are apparent, and the vesicle appears circular. Image (**C**) is taken at 780 sec. The vesicle has decreased significantly in area and only a few short tubules are apparent. Bars represents 10 μm. Time-lapse movie available as Video 1 at *http://www.currentprotocols.com*.

sequence of frames. Knowing the time interval between frames, the rates of change of measured quantities over time may then be calculated. Measurements of vesicle surface area over time in The Movie are shown in Figure 4.16.2. Measurements from other movies show that the tubules range in length from 1 μm to 12 μm, and a typical value for rate of longitudinal growth is 0.5 μm/sec (Kerr et al., 2006). Fixed cell electron micrographs show the tubules having average radii of ~18.36±4.42 nm (M. Lindsay and R. Parton, unpub. observ.).

The question arises of whether the geometric and membrane conservation assumptions are reasonable. Because only the proteins that are marked are captured in the images, it is possible that membrane is being added or removed from the vesicle system unobserved. However, experiments using nocodazole, which blocks tubule growth, show the vesicles exhibiting relatively little decrease in size (Kerr et al., 2006); this suggests that the majority of membrane being transported into or out of the vesicle is via tubule growth. Further, cell membranes can typically stretch only 1% to 4% before rupturing (Lipowsky and Sackmann, 1995); hence, membrane surface areas observed are conserved in the sense that for any reduction in the surface area of the vesicle there must be a corresponding increase in surface area of tubule. As better imaging techniques such as real-time three-dimensional video microscopy become more common, it will be possible to measure surface areas more directly and account for all the membrane being trafficked.

CONSERVATION OF SURFACE AREA

In many systems it is desirable to be able to quantify how membrane is trafficked between locations. Here a geometric model of a vesicle/tubule system is constructed under the assumptions that membrane is conserved, the vesicle is a sphere, and a tubule is a cylinder. The aim is to attempt to quantify how membrane, and hence receptors, are trafficked during tubulation of a vesicle.

In Figure 4.16.3, at time 0 there is a spherical vesicle of radius r_{V0}. At time t a tubule of length $L(t)$ and radius r_T has grown causing the vesicle to decrease in radius to $r_V(t)$. The surface area of the vesicle is initially $4\pi {r_{V0}}^2$ and is $4\pi r_V(t)^2$ at time t. The loss in the surface area of the vesicle is given by $4\pi[{r_{V0}}^2 - r_V(t)^2]$. The curved surface of the tubule at time t has area $2\pi r_T L(t)$. If surface area is conserved it must then be that the loss in surface area of the vesicle equals the gain in that of the tubule, i.e., $4\pi[{r_{V0}}^2 - r_V(t)^2] = 2\pi r_T L(t)$.

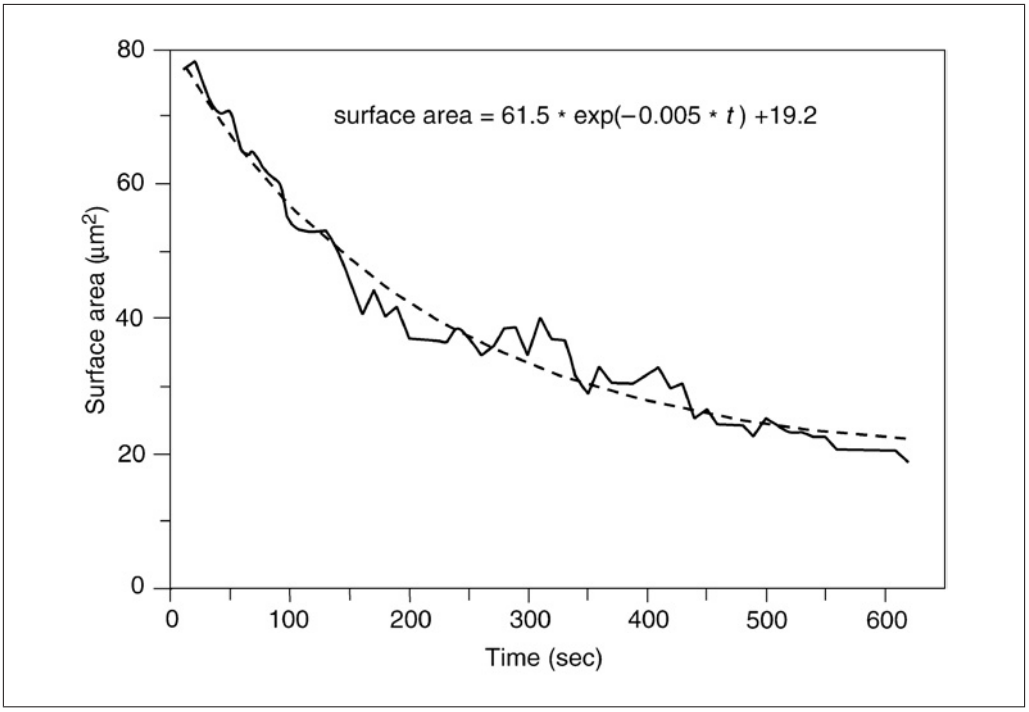


Figure 4.16.2 The rate of decrease of surface area of the vesicle in The Movie (frames 22 to 83), with an exponential best fit curve (dotted). Surface area is given as four times the area of an ellipse fitted to the vesicle membrane surface.

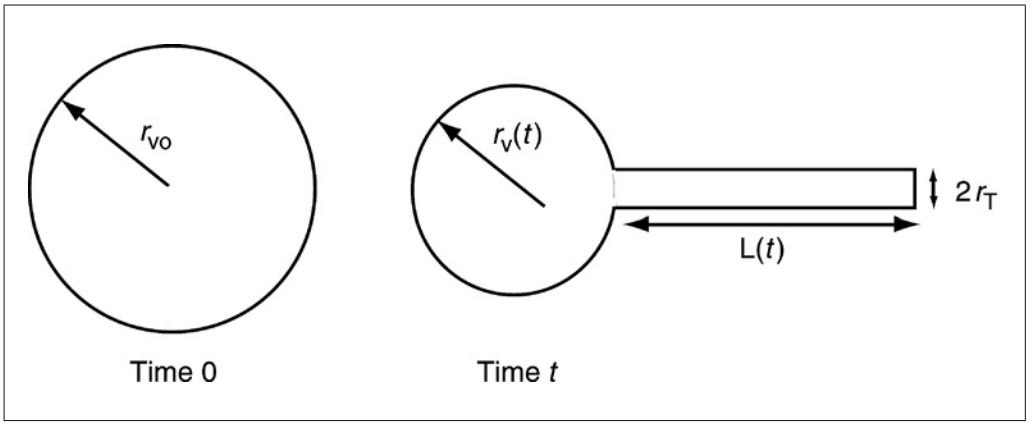


Figure 4.16.3 Change in a vesicles' surface area and volume as a tubule grows. At time 0 the spherical vesicle has radius r_{V0}. At time t, a cylindrical tubule of length $L(t)$ and radius r_T has grown, decreasing the radius of the vesicle to $r_V(t)$. If surface area is conserved, then the decrease in surface area of the vesicle will equal the increase in surface area of the tubule, i.e., $4\pi[r^2_{V0} - r^2_V(t)] = 2\pi r_T L(t)$. The volume contained in the vesicle is initially $4/3\pi r^3_{V0}$. The total volume contained with the vesicle and tubule at time t is $4/3\pi r^3_V(t) + \pi r^2_T L(t)$.

Rearranging terms gives the expression:

$$r_V(t) = \left(r_{V0}^2 - \frac{r_T L(t)}{2} \right)^{1/2}$$

Equation 4.16.1

Alternatively, the terms can be rearranged to express the amount of tubule formed for a given radius change:

$$L(t) = \frac{2\left[r_{V0}^2 - r_V^2(t) \right]}{r_T}$$

Equation 4.16.2

A typical value for the rate of growth of a tubule is 0.5 μm/sec (measurements obtained from separate movies to The Movie; data not shown), and so setting $L(t) = 0.5t$ in Equation 4.16.1 gives a complete description of how the radius of a vesicle would be expected to change as a tubule grows, under the assumption of observed surface area conservation. Further, Equation 4.16.2 can be used to estimate the total length of tubule that formed for a given observed vesicular radius decrease. For instance, in The Movie the radius changes from ~2.5 μm to 1.25 μm. Setting $r_{V0} = 2.5$ μm, $r_V(t) = 1.25$ μm, and a value of 18.4 nm for r_T, Equation 4.16.2 estimates the total length of tubule formed over the period of observation to be 510 μm. Also, while decreasing in radius from 2.5 μm to 1.25 μm, one-quarter of the membrane of the vesicle is retained by the vesicle while the remainder is transferred into the tubules.

This section has shown how a simple geometric model can be used to estimate the length of tubule and amount of membrane being trafficked. Now attention is turned to the volume contained within the systems.

MEASUREMENT OF VOLUME

Because liquids are essentially incompressible, principles of volume conservation may be applied to the movies of cellular processes and used to estimate how solutes are trafficked. Again, the approach is to assume surface area is conserved and model the membrane structures as geometric objects.

But there is a problem: a sphere is the unique surface that contains maximum volume for a given surface area. When tubules form, the total surface area of the vesicle together with the tubules remains the same, but less volume may be contained within the system. The amount of volume lost is readily calculated for a given change in radius as follows. If, as in the previous section, the initial radius of the vesicle when no tubulation has occurred is r_{V0}, and after time t the radius has decreased to $r_V(t)$ (see Fig. 4.16.3), with a total length $L(t)$ of tubule formed, then the total volume of the vesicle-tubule system at time t is

$$\frac{4}{3}\pi r_V^3(t) + \pi r_T^2 L(t)$$

Equation 4.16.3

Using the expression for $L(t)$ given in Equation 4.16.2, the difference of the initial volume, $4/3\pi r_{V0}{}^3$, and the final volume of the

vesicle-tubule system is calculated to be

$$\Delta V = \frac{4}{3}\pi\left[r_{V0}^3 - r_V^3(t)\right] - 2\pi r_T\left[r_{V0}^2 - r_V^2(t)\right]$$

Equation 4.16.4

Note this *is not* volume that is contained in the tubules; it is bulk volume that must be moving from the interior to the exterior of the vesicle by passing through the vesicle membrane. In the case of The Movie, taking $r_{V0} = 2.5$ μm, $r_V(t) = 1.25$ μm, and $r_T = 0.0184$ μm, the volume no longer contained within the vesicle-tubule system is $\Delta V = 56.73$ μm^3. This compares to an initial volume contained in the vesicle of 65.45 μm^3, a final volume in the vesicle of 8.72 μm^3, and a volume of 0.54 μm^3 that has gone into tubules. Hence approximately seven-eighths of the volume is lost. Even if nontubular (unobserved) membrane, e.g., budding vesicles, were being transported out of the system, similar volume loss problems would still occur. What happens to the lost volume is considered in the next section. It is likely that water is being transported out of the vesicle system via aquaporin channels (Murata et al., 2000). If this is indeed the case, and the solute is retained within the vesicle/tubules system, then the majority of solute remains within the vesicle, and the volume of solute transported from the vesicle via tubules is trivial (0.54 μm^3) in comparison.

FLUX ACROSS A MEMBRANE, SOLUTE CONCENTRATION, AND pH CHANGE

Whenever the volume of an enclosed system such as a vesicle is not conserved, it follows that there must be a flux into or out of the system. Depending on what is being transported, this may have consequences for concentration of solutes and pH. In this section it is shown how geometry may be used to estimate flux, concentration, and pH change.

Suppose, as in Figure 4.16.4, that initially a vesicle has radius $r + \delta r$, and at a time δt later the radius has decreased by an amount δr. For small δr, the volume contained "between" these vesicles is the surface area of the "inner" vesicle multiplied by the thickness δr, and so is given by $4\pi r^2\delta r$. The *volume flow*, i.e., the volume crossing the membrane per unit time, is then given (as δt approaches 0) by

$$4\pi r^2\frac{\delta r}{\delta t} \rightarrow -4\pi r^2\frac{dr}{dt}$$

Equation 4.16.5

The minus sign occurs because the radius is decreasing. The *volume flux*, defined to be the volume flow *per unit area* of the vesicle surface, is then just dr/dt. (Flux is sometimes defined as the number of molecules transported per unit area per unit time, rather than the volume of molecules per unit area per unit

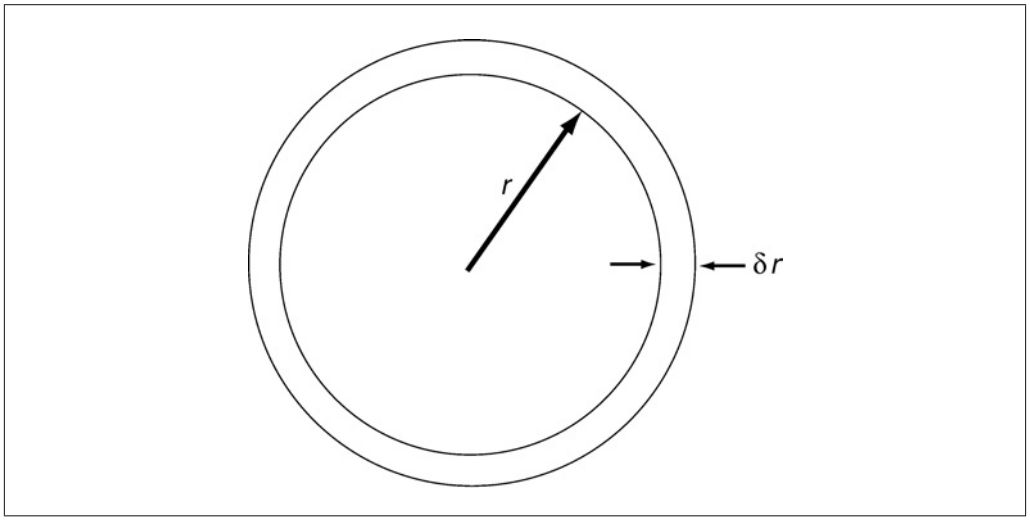


Figure 4.16.4 Flux across the membrane. Initially the spherical vesicle has radius $r + \delta r$. At a time δt later, the radius has decreased by δr. The volume "between" the vesicle is then $4\pi r^2\delta r$, and the volume crossing the membrane per unit time is $4\pi r^2\delta r/\delta t$, which becomes $-4\pi r^2 dr/dt$ as $\delta t \rightarrow 0$. Hence the volume flux, the volume flow per unit surface area of the vesicle, is $-dr/dt$.

time). But for homogenous liquids, volume and amount are essentially interchangeable given sufficient numbers of molecules. Hence to calculate the flux across the membrane only the rate of decrease of the radius of the vesicle over time needs to be known.

In Figure 4.16.2, measurements of the surface area of the vesicle over time in The Movie are shown. The radius at any time is readily calculated as $r = [A(t)/4\pi]^{1/2}$, where $A(t)$ is the surface area at that time, and plotting the radius against time for the first 200 sec gives a graph with slope of approximately -0.0041 μm/sec. Hence in The Movie there is a flux of ~ 0.0041 $\mu m^3/\mu m^2$/sec across the membrane. It may be that some volume is being lost by being transported through the membrane of the tubules. However, at any instant in time the surface area of the tubules is much smaller than that of the vesicle, and given the relatively small diameter of a tubule, the effect is likely to be trivial. Another possibility is that the vesicle ruptures allowing the content of the lumen to escape. However, separate experiments marking the contents of the vesicle have enabled the concentration of the contents of a vesicle to be observed directly. Dextran was fluorescently marked and introduced into the intercellular environment, entering the interior of each vesicle as it forms. As tubulation occurred and the area of the vesicle decreased from 7.3 μm^2 to 5.3 μm^2, a marked increase in average intensity by a factor of 1.66 was observed in the central region of the vesicle. If the vesicle membranes were ruptured the intensity would be expected to remain constant or decrease.

It seems likely that the flux across the membrane is mostly in the form of water being transported through aquaporin channels (de Baey and Lanzavecchia, 2000). A flux of 0.0041 $\mu m^3/\mu m^2$/sec is orders of magnitude slower than water fluxes across lipid bilayers that have been observed previously (see Borgnia et al., 1999 and references included there) and so is biologically feasible. In the next section it will be shown how pressure and tension considerations may be used to explain the flux.

If indeed it is water that is being transported across the vesicle membrane, then the solute in the vesicle is being significantly concentrated by the tubule formation and associated vesicle reduction. As seen above, the volume entering the tubules over the lifetime of the vesicle is small compared to the volume contained in the vesicle. Hence, in The Movie, where the radius decreases by a factor of two, the final volume of the vesicle is eight times smaller than that of the initial vesicle, and so an 8-fold increase in solute concentration is predicted. It may be that such an increase in concentration is biologically significant and that the formation of tubules is not only to enable efficient recycling of receptors, as is commonly thought, but also to increase solute concentration.

Another point of interest is that if it is indeed water that is being selectively removed, then there may be an associated pH change in the vesicle. Under the hypothesis that water and not hydrogen ions are selectively crossing the membrane, an 8-fold reduction in vesicle size could lead to an 8-fold concentration in hydrogen ions and so to a change in pH of ~ 0.9 ($\log_{10} 8$). Aquaporin channels are known to facilitate the transport of water at the expense of ions such as protons (Murata et al., 2000), and so they provide a mechanism by which such a pH change could occur. It is also known that pH facilitates ligand-receptor disassociation (Johnson et al., 1993; Presley et al., 1997), one pathway to acidification being via proton-translocating ATPases (Forgac, 1989); it may be that vesicle reduction is another pathway that affects the outcomes for trafficking of the ligand (Lindermann and Lauffenburger, 1986). Further, in Johnson et al. (1993), it was found that receptor return to the plasma membrane is slowed when the endosome is not properly acidified.

PRESSURE, TENSION, AND MORPHOLOGY

Large changes in membrane morphology are sometimes observed in real-time cellular movies, and these can often be understood in terms of pressure, tension, and flux. Whenever there is a flux though a membrane there is a pressure difference across the membrane. The difference may be osmotic in nature due to a difference in the solute concentrations in the interior and exterior (Zhu et al., 2002), or it may be hydrostatic due to some force being applied. In the hydrostatic case the flux across the membrane is proportional to the pressure difference (Zhu et al., 2002), and so the flux through the membrane over time provides a measure of the relative value of the pressure difference over time. The hydrostatic pressure difference is also proportional to the tension in the membrane (Lipowsky and Sackmann, 1995). Such a tension will often alter the shape of a membrane to a more regular shape such as a sphere. Another point of interest is that virtually all cell membranes contain ion

channels that may be opened or closed by mechanical transduction (Sachs, 1991); so it may be that different molecules are being transported across the membrane when it is under differing tensions. This section shows how proportionality principles between pressure, tension, and flux may be applied to understand how the morphology of the vesicle changes over time and to calculate relative values of pressure and tension in the vesicle membrane.

In The Movie, the vesicles is observed to be initially irregular in shape, but within a few tens of seconds of tubulation being initiated it appears circular. In the previous section it was seen that there is a flux across the vesicle membrane. The associated pressure difference might be due to differing solute concentrations between the interior and exterior of the vesicle. However, the more likely explanation is that as tubules form, the membrane of the vesicle tightens, and the tension in the surface induces a hydrostatic pressure difference. In this case, the flux across the membrane is proportional to the pressure difference, and so the value of the volume flux (or, indeed, the rate of change of radius as shown above) over time provides a measure of the relative value of the pressure difference over time. Further, because the hydrostatic pressure difference is also proportional to the tension in the membrane, the rate of change of radius over time also gives a measure of the relative tension in the membrane.

Using the fitted curve to The Movie data given in Figure 4.16.2 (the fitted curve is discussed in more detail later), an expression for the rate of change of radius can be obtained. For a given surface area $S(t)$ at time t, the radius $r(t) = [S(t)/4\pi]^{1/2}$, and taking the derivative of this with respect to time gives

$$\frac{dr}{dt} = \frac{-0.01084e^{-0.005t}}{61.5e^{-0.005t} + 19.2}$$

Equation 4.16.6

This equation has value -0.000134 at $t = 0$ and changes to -0.0000242 at $t = 600$, and so the ratio of $-0.000134/-0.0000242 = 5.5$ gives the estimate that the pressure difference and tension in the vesicle membrane were around five times larger initially than they were 600 sec later. Whether solutes are being trafficked across the membrane at all or at different rates as the tension in vesicle membrane changes, is an open question.

VESICLE FUSION

Another area open to geometric analysis is vesicle fusion. In these events, two vesicles join together to form a single vesicle. If the vesicles are spherical, then the conservation or otherwise of surface area and volume may be examined.

Suppose two vesicles of radius r_1 and r_2 fuse to form a single vesicle of radius r_3 (see Fig. 4.16.5). If surface area is conserved, the equality $4\pi {r_1}^2 + 4\pi {r_2}^2 = 4\pi {r_3}^2$ holds, i.e.,

$$r_1^2 + r_2^2 = r_3^2$$

Equation 4.16.7

As was noted previously, conservation of surface area is in conflict with conservation of volume. If the vesicles have volumes V_1, V_2 and V_3, respectively, the fractional increase in volume of the vesicle system is also readily

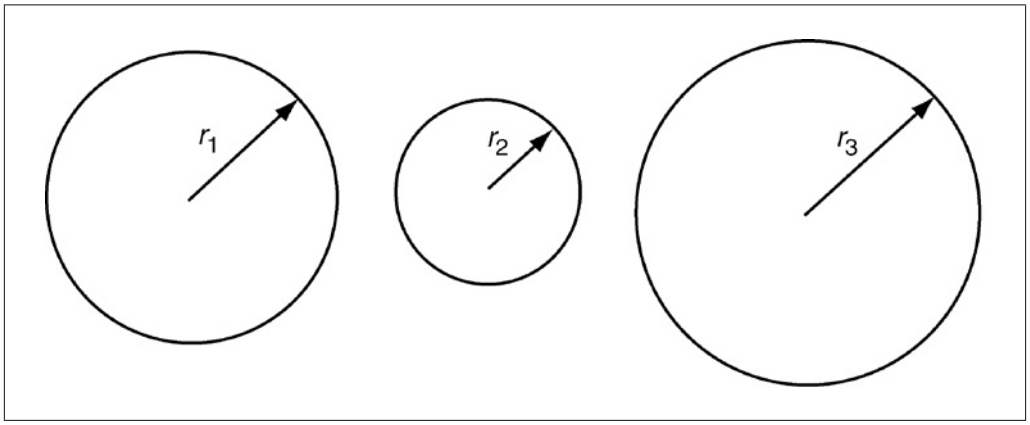


Figure 4.16.5 Fusion of vesicles. Two spherical vesicles of radius r_1 and r_2 merge to form a single vesicle of radius r_3. If surface area is conserved then the relation $4/3\pi {r_1}^2 + 4/3\pi {r_2}^2 \neq 4/3\pi {r_3}^2$ holds. However, if surface area is conserved volume can not be, i.e., $4/3\pi {r_1}^3 + 4/3\pi {r_2}^3 \neq 4/3\pi {r_3}^3$.

calculated as

$$\frac{V_3-(V_1+V_2)}{V_1+V_2}=\frac{\frac{4}{3}\pi\left[r_3^3-(r_1^3+r_2^3)\right]}{\frac{4}{3}\pi(r_1^3+r_2^3)}$$

Equation 4.16.8

In the particular case that the initial vesicles have the same radius (i.e., $r_1 = r_2$), Equation 4.16.7 shows that if surface area is conserved one would expect to observe the radius of the postfusion vesicle to be $\sqrt{2}$ times that of the prefusion vesicles. In this case, Equation 4.16.8 shows that, under the assumptions that the vesicles are all spheres and surface area is conserved, the fused vesicle will have 41.4% greater volume than the prefusion vesicles. In general, for vesicles of any radii fusing, if surface area is conserved and the vesicles are all spheres, then because a sphere is the unique shape that maximizes volume for a given surface area, there will be an increase in volume contained in the postfusion vesicle.

In Roberts et al. (1999), several high-quality real-time movies of vesicle fusion are presented, in particular figure 4 in that paper provides a sequence amenable to measurement where the vesicles are apparently circular. Taking measurements of area in units of pixels (1 μm is ∼120 pixels in the movie); in frame 29 the vesicles have areas of 2032 and 2166, while in frame 52 the fused vesicle has an area of 4181. The corresponding radii are then 50.9, 51.9, and 73.0 pixels. Under the assumption that the three vesicles are spherical, the surface area of each vesicle is then four times the area observed, giving areas of 8128, 8464, and 16724, respectively. Hence surface area appears to be approximately conserved since $8128 + 8464 = 16529 \approx 16724$. Also notice that the postfusion radius is almost exactly $\sqrt{2}$ times that of the prefusion vesicles, giving some confidence that the conservations of membrane and sphericality hypotheses are true. In contrast, the volumes of the vesicles are respectively 551238, 585770, and 1626950, giving the result that the fused vesicle appears to contain 43% more volume than the two vesicles before fusion and that a substantial volume is entering the system during fusion.

Another movie where two large vesicles fuse has been obtained by Richard et al. (2004). Taking measurements from the first and final frames in units of pixels, the prefused vesicles and the fused vesicle have measured areas of 18996, 16509 and 34106, respectively. These correspond to radii of 77.8, 72.5, and 104.5 pixels; surface areas of 75984, 66036, and 136424; and volumes of 1969505, 1595677, and 4738164. Again, the radius of the fused vesicle is $\sim\sqrt{2}$ times that of the prefusion vesicles. The surface area appears to be approximately conserved, with the fused vesicle having surface area only 4% smaller than the sum of the surface areas of the prefusion vesicles. But the volume of the fused vesicle is 33% larger than volume contained within the prefusion vesicles. It appears that as vesicles fuse there is a rapid uptake of volume, though the mechanism is not clear.

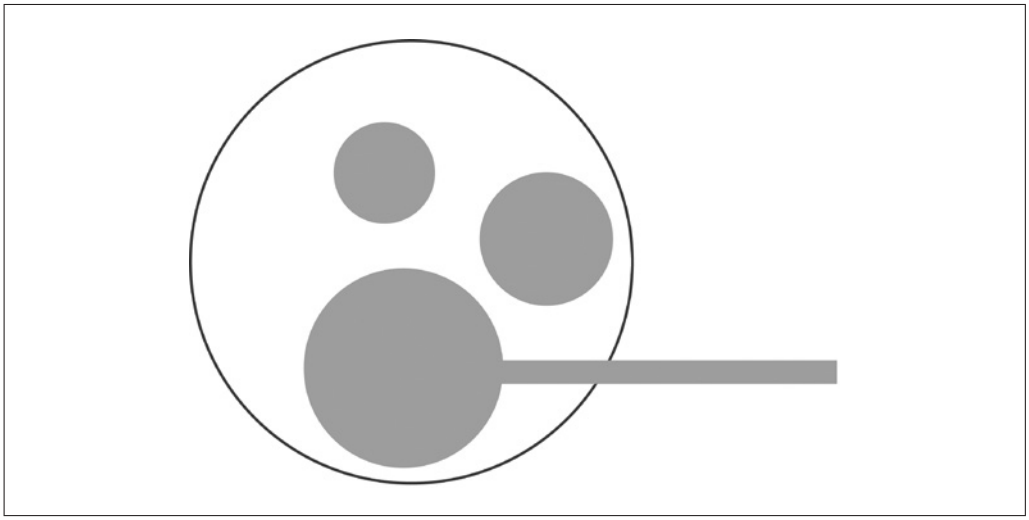


Figure 4.16.6 Vesicle membrane subdomains or patches. The vesicle membrane contains subdomains (shown in grey) on which tubulation is initiated. As the tubule grows the subdomain membrane is drawn into the tubule and the subdomain decreases in size.

PROPORTIONALITY AND SURFACE AREA

Proportionality is a common principle when describing changes in physical and biological systems: the rate at which a collection of radioactive particles decays is proportional to the number of particles, or in a self-inhibiting chemical reaction the rate of change of product may be proportional to the amount of product. The *rate equation* for some changing quantity $N(t)$ of such a process will be

$$\frac{dN(t)}{dt} = -kN(t)$$

Equation 4.16.9

where k is a constant that governs the speed with which $N(t)$ changes. It states that at time t, the rate at which $N(t)$ changes is proportional to $N(t)$ itself.

The solution to this rate equation is

$$N(t) = N_0 e^{-kt}$$

Equation 4.16.10

where N_0 is the initial quantity $N(0)$. Depending on the sign of k, $N(t)$ will either exponentially increase or decrease. Here it is shown how proportionality may be applied to describe the reduction in surface area measured in the vesicular systems.

The vesicle membrane surface is not homogeneous and contains subdomains or patches of membrane on which tubulation is initiated (Mukherjee and Maxfield, 2000; Baumgart et al., 2003; Kerr et al., 2006). When a tubule is initiated, the membrane from a patch is drawn into the tubule as it extends, and the patch reduces in size (see Fig. 4.16.6). If there were large areas of patches on the vesicle surface, it seems likely that there would be a higher probability that in a given time interval a tubule will begin to form on one of the patches than if there were only a few small patches. Hence a reasonable hypothesis is that the larger the total surface area of the patches on the vesicle surface, the more tubules that will form in a given time interval, and so the total surface area of the vesicle given over to patches will decrease at a greater rate. In other words the rate of decrease of the total patch area on the vesicle surface will be proportional to the patch area at that time. This can be formalized as a rate equation for the total patch area $S_p(t)$ of the vesicle at time t as

$$\frac{dS_p(t)}{dt} = -kS_p(t)$$

Equation 4.16.11

where k is a rate constant.

The solution to this equation is

$$S_p(t) = S_{p0} e^{-kt}$$

Equation 4.16.12

and describes how one would expect the total patch area to change over time. The constant S_{p0} then is the total area of patches at time 0.

Because the non-patch membrane area of the vesicle is not expected to decrease, the *total* surface area of the vesicle would be expected to be of the form

$$S(t) = S_{p0} e^{-kt} + S_{np}$$

Equation 4.16.13

where S_{np} is the surface area of the vesicle not able to be incorporated into tubules. Fitting a curve of the above form to The Movie data in Figure 4.16.2 gives the equation

$$S(t) = 61.5e^{-0.005t} + 19.2$$

Equation 4.16.14

A plot of Equation 4.16.14 is shown as a dotted line in Figure 4.16.2, and it appears to fit the data very well. Note that since the decrease in patch area is an exponential, the total patch area is being halved every $\ln(2)/0.005 \approx 141$ seconds. The area of the initial vesicle that was in patches at time zero is predicted as 61.6 μm^2, with the remaining surface area of the vesicle being 19.2 μm^2.

While the predicted proportion of surface area given over to patches in The Movie is no surprise since the lack of tubule activity later in the movie already suggested that the majority of the patches had tubulated, what has been gained by the proportionality model is the ability to predict given only partial data. If, instead of 600 sec of data shown in Figure 4.16.2, only the first 300 sec had been obtained, it would still be possible to create the above model and predict the final size of the vesicle even if these were not observed experimentally.

It would be appealing to be able to relate the surface area decrease measured and modeled above directly to the individual patches on which tubulation is initiated. However, detailed observations of the areas of all of the patches on a given vesicle have yet to be made, although qualitative insight can still be gained by considering the proportionality hypothesis for patch area decrease and what it would imply.

It would be expected that a larger patch will begin to tubulate more quickly than a smaller one because there is more area for tubulation to be initiated; so a likely scenario is that the

probability of a patch forming a tubule in a given time interval is proportional to the area of the patch. Hence, a patch that is twice the size of another will be twice as likely to begin tubulation in a given time interval and so will have a mean lifetime half that of the smaller one. If this is correct, then one would expect the mean lifetimes of the larger patches to be much shorter than that of the smaller ones. Indeed, in The Movie and other movies of tubulation, an explosion of growth of long tubules (large patches) is observed early on, while later only a few short tubules are seen.

Another feature that might be expected is for multiple tubules to begin growth on a single patch. If a patch is large and a tubule begins to form, it may last for several tens of seconds before the surface area is completely drawn out into the tubule. Thus, there is a probability that a second tubule will begin to form on the patch in this time. The points at which the two tubules initiated on a single patch are attached to the vesicle will become closer as the patch is depleted, until they merge and branching occurs: a single tubule is attached to the vesicle, and that tubule then splits into two branches. Again, almost all of the tubules at the beginning of The Movie are long and have multiple branches, while later the tubules are short and exhibit no branching, as predicted.

CONCLUSIONS

The authors have outlined how simple geometric and conservation principles can be applied to the analysis of real-time movies of cellular processes to make predictions and generate ideas for further experiments. The main principles are:

Surface Area. Membrane area should be conserved; if not, membrane is entering or leaving the system, the amount of which may be quantifiable.

Volume. Volume should be conserved, and if not, volume is entering or leaving the system; this may have consequences such as pH change or solute concentration.

Pressure. Flux across a membrane is associated with a pressure difference and tension in the membrane; this can lead to changes in morphology.

Proportionality. Many processes occur at rates proportional to the area available for them to occur on.

Using these principles, it has been possible to get a better qualitative understanding of the systems observed in The Movie and related experiments: why there is very rapid tubulation initially, why tubules later in the movies are of shorter length, why tubules often exhibit branching early in the movies but not later on, why vesicles are initially morphologically irregular but rapidly become regular, and what may govern the rate of decrease in vesicle size. On the quantitative side, under the hypothesis of observed surface area conservation, measurement and models allowed prediction of the surface area of a vesicle available to form tubules, the lifetime of an average patch, the total length of tubule formed, relative changes in pressure and tension across and in the vesicle membrane, volume flux across the vesicle membrane, pH change, and solute/disassociated ligand concentration. The latter is particularly interesting because it may be that one of the goals of tubulation is not just to efficiently recycle receptors but also to concentrate the solute.

It should be emphasized that the aim of the endocytosis example presented has not necessarily been to develop a complete mathematical model of the system in the mode of, for instance, the famous Hodgkin-Huxley squid axon equations. There may be membrane transport events that are not being observed in the live cell video microscopy experiments, though the experiments using nocodazole suggest that few, if any, such events are occurring, and real vesicles and tubules certainly deviate to some degree from spheres and cylinders. However, by making reasonable assumptions, attempting to capture mathematically the essence of what is being observed, and doing "back of the envelope calculations" allow refinement in thinking about the systems, investigation of whether the conclusions reached are biologically reasonable, and generation of experimentally testable hypotheses. The approach is towards an experimental mathematics in which as much as is known about a system is geometrically modeled in order to ascertain the consequences and further experiments that the model suggests.

ACKNOWLEDGEMENTS

The research of N.H. and K.B. is partially supported through the Australian Research Council's award of a Federation Fellowship to K.B. R.D.T. is supported by a National Health and Medical Research Council of Australia R. Douglas Wright Career Development Award. M.C.K. is supported by an ARC Australian Post-Graduate Award. The authors would also like to thank Prof. Robert G. Parton and Prof. John Hancock for careful reading and invaluable suggestions during the preparation of this manuscript.

LITERATURE CITED

Baumgart, T., Hess, S.T., and Web, W.W. 2003. Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension. *Nature* 425:821-824.

Borgnia, M., Nielsen, S., Engel, A., and Agre, P. 1999. Cellular and molecular biology of the aquaporin water channels. *Rev. Biochem.* 68:425-458.

de Baey, A. and Lanzavecchia, A. 2000. The role of aquaporins in dendritic cell macropinocytosis. *J. Exp. Med.* 191:743-747.

Forgac, M. 1989. Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol. Rev.* 69:765-96.

Johnson, L.S., Dunn, K.W., Pytowski, B., and McGraw, T.E. 1993. Endosome acidification and receptor trafficking: Bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. *Mol. Biol. Cell.* 4:1251-66.

Kerr, M.C., Lindsay, M.R., Luetterforst, R., Hamilton, N., Simpson, F., Parton, R.G., Gleeson, P.A., and Teasdale, R.D. 2006. Visualisation of macropinosome maturation by the recruitment of sorting nexins. *J. Cell Sci.* 119:3967-3980.

Lindermann, J.J. and Lauffenburger, D.A. 1986. Analysis of intracellular receptor/ligand sorting. *Biophys. J.* 50:295-305.

Lipowsky, R. and Sackmann, E. (eds.) 1995. Handbook of Biological Physics, Vol. 1: Structure and Dynamics of Membranes. Elsevier, North-Holland.

Merino-Trigo, A., Kerr, M.C., Houghton, F., Lindberg, A., Mitchell, C., Teasdale, R.D., and Goleason, P.A. 2004. Sorting nexin 5 is localized to a subdomain of the early endosomes and is recruited to the plasma membrane following EGR stimulation. *J. Cell. Sci.* 117:6413-6424.

Mukherjee, S. and Maxfield, F.R. 2000. Role of membrane organization and membrane domains in endocytotic lipid trafficking. *Traffic* 1:203-211.

Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J.B., Engel, A, and Fujiyoshi, Y. 2000. Structural determinants of water permeation through aquaporin-1. *Nature* 407:599-605.

Presley, J.F., Mayor, S., Mcgraw, T.E., Dunn, K.W., and Maxfield, F.R. 1997. Bafilomycin A1 treatment retards transferrin receptor recycling more than bulk membrane recycling. *J. Biol. Chem.* 272:13929-13936.

Richard, A., Marchi-Artzner, V., Lalloz, M.N., Brienne, M.J., Artzner, F., Gulik-Krzywicki, T. Guedeau-Boudevillek, M.A., and Lehn, J.M. 2004. Fusogenic supramolecular vesicle systems induced by metal ion binding to amphiphilic ligands. *Proc. Natl. Acad. Sci. U.S.A.* 101:15279-15284.

Rietdorf, J. and Zimmermann, T. 2000. 4D Microscopy: Exploring Time and Space. *G.I.T. Imaging & Microscopy* 2:44-46.

Roberts, R.L., Barbieri, M.A., Pryse, K.M., Chua, M., Morisaki, J.H., and Stahl, P.D. 1999. Endosome fusion in living cells overexpressing GFP-rab5. *J. Cell Sci.* 112:3667-3675.

Sachs, F. 1991. Mechanical transduction by membrane ion channels: A mini review. *Mol. Cell Biochem.* 104:57-60.

Zhu, F., Tajkhorshid, E., and Schulten, K. 2002. Pressure-induced water transport in membrane channels studied by molecular dynamics. *Biophys. J.* 83:154-160.

INTERNET RESOURCES

http://rsb.info.nih.gov/ij

The U.S. National Institutes of Health Web site for ImageJ written by W.S. Rasband and updated from 1997 to 2007.

http://www.till-photonics.com/Products/software.php

The T.I.L.L. Photonics Web site for the TILLvisION image analysis software.

Contributed by Nicholas Hamilton, Markus C. Kerr, Kevin Burrage, and Rohan D. Teasdale

ARC Centre in Bioinformatics Institute for Molecular Bioscience
The University of Queensland
St. Lucia, Australia

Scanning Electron Microscopy of Cell Surface Morphology

Samantha Passey,[1] Stéphanie Pellegrin,[1] and Harry Mellor[1]

[1]Mammalian Cell Biology Laboratory, Department of Biochemistry, School of Medical Sciences, University of Bristol, United Kingdom

ABSTRACT

The surface of metazoan cells is a landscape not clearly visualized by light microscopy. Many cells elaborate protrusive structures such as microvilli, filopodia, lamellipodia, and surface ruffles that play important roles in the interaction between the cell and its environment. The high resolution of scanning electron microscopy makes it an ideal technique for studies of the cell surface; however, preservation of fine surface structure can be problematic. Here we highlight the critical factors in sample preparation to ensure optimal high-resolution imaging of the surface of mammalian cells and tissues. *Curr. Protoc. Cell Biol.* 37:4.17.1-4.17.13. © 2007 by John Wiley & Sons, Inc.

Keywords: filopodia • microvilli • lamellepodia • SEM • actin

INTRODUCTION

Many cells make protrusive surface structures including lamellipodia, filopodia, and microvilli, and the fine detail of such structures is often beyond the limits of resolution of conventional light microscopy. The higher resolution afforded by scanning electron microscopy (SEM) allows detailed examination of the three-dimensional morphology of such structures. Indeed, depending on the instrument, the scanning electron microscope has a magnification range from 15× to 200,000× with a resolution between 1 and 20 nm.

In order to be visualized by SEM, biological specimens have to be prepared carefully (i.e., dried) to withstand the vacuum inside the microscope. They also have to be made conductive, usually by being coated with a very thin layer of gold. The image is generated by an electron beam which is focused and scanned over the specimen. As the electron beam hits the sample, secondary electrons are knocked loose from its surface. These secondary electrons are detected and the final image is built up from the number of electrons emitted from each spot on the sample. The brightness of the signal depends on the number of secondary electrons reaching the detector.

This unit describes the preparation of cells for scanning electron microscopy examination. The Basic Protocol describes the preparation of nonadherent lymphocytes for SEM, with the Alternate Protocols 2 and 3 being suitable for the preparation of adherent cells.

PREPARATION OF LYMPHOCYTES FOR SCANNING ELECTRON MICROSCOPY

BASIC PROTOCOL

This protocol assumes the cells of interest are already available either as continuously growing cell lines or as recently isolated primary peripheral blood mononuclear cells (PBMC), isolated by density gradient centrifugation using a suitable reagent such as Histopaque 1077 (Sigma-Aldrich). Specific leukocyte and lymphocyte subsets can be purified from PBMC using commercially available separation technologies such as the magnetic MACS separation reagents (Miltenyi Biotec).

Current Protocols in Cell Biology 4.17.1-4.17.13, December 2007
Published online December 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0417s37

CAUTION: Nitric acid, glutaraldehyde, hexamethyldisilazane, and osmium tetroxide are hazardous chemicals; ensure appropriate safety, handling, storage, and disposal procedures are followed.

NOTE: Cells should be maintained at 37°C in a humidified 5% (v/v) CO_2 atmosphere unless otherwise specified. We would recommend allowing an overnight incubation of primary lymphocytes immediately following isolation to allow cells to recover their normal morphology following the isolation and purification procedures.

NOTE: All chemicals that come into contact with live cells must be sterile and aseptic technique used to prevent contamination. SEM is a good way to discover poor sterile technique.

Materials

5 M nitric acid
Methanol
Poly-L-lysine (0.1% w/v, Sigma-Aldrich)
Phosphate-buffered saline (*APPENDIX 2A*)
Cells of interest (this procedure has been used for SEM examination of primary human lymphocytes and also mouse and human lymphocyte cell lines)
RPMI 1640 medium (Sigma-Aldrich)
2.5% glutaraldehyde (see recipe)
Sodium phosphate buffer, pH 7.4 (see recipe)
Sodium phosphate buffer, pH 6.0 (see recipe)
0.5% osmium tetroxide (see recipe)
25%, 50%, 75%, 90%, and 100% ethanol
Liquid CO_2
High-purity argon

13-mm round glass coverslips
Glass petri dish
80°C oven
12-well tissue culture plates
1.5-ml microcentrifuge tube
Parafilm
Shaking incubator with variable speed
Benchtop microcentrifuge
Critical point drying apparatus (e.g., Tousimis Samdri; *http://www.tousimis.com*)
Aluminum sample stubs (12-mm, Agar Scientific)
Adhesive sample mounting pads (Adhesive Carbon Tabs, 12-mm, Agar Scientific)
Gold sputter coater
Vacuum
Dehumidified environment (e.g., sealed plastic container in the presence of silica gel)

Additional reagents and equipment for counting cells using a hemacytometer (*UNIT 1.1*)

Prepare poly-L-lysine coated coverslips

1. Immerse 13-mm round glass coverslips in 5 M nitric acid for 15 min at room temperature in a fume hood.

   Nitric acid removes traces of oil, dust, etc. from the coverslip surface and allows an even coating of poly-L-lysine.

2. Carefully decant the nitric acid in the fume hood and wash the coverslips ~10 times with copious amounts of double-distilled water. Decant all remaining water after final wash.

   Take care when adding water to any residual acid.

3. Wash the coverslips twice in methanol.
4. Spread the coverslips in a glass petri dish and dry in a hot oven at 80°C for at least 4 hr until the coverslips are completely dry.

   The acid-washed coverslips are treated as being sterile, but for more stringent sterilization they could be autoclaved.

   The acid-washed coverslips can be stored in the glass petri dish at room temperature indefinitely, but the dish should be opened only under sterile conditions to prevent contamination.
5. Transfer the required number of coverslips to wells of a 12-well plate, one per well for each sample to be prepared.
6. Immerse each coverslip in 0.5 to 1 ml poly-L-lysine and incubate for 30 min at room temperature.
7. Aspirate the poly-L-lysine and wash coverslips three times, each time with 1 ml PBS. Allow to dry (~30 min to 1 hr).

   Poly-L-lysine coated coverslips can be stored up to 1 month at 4°C, allow to warm to room temperature before use.

Fix cells with glutaraldehyde

8. Count cells (*UNIT 1.1*). Suspend cells at 4×10^7 cells/ml in prewarmed RPMI 1640 medium.

   Prewarming the medium to 37°C reduces the temperature shock when resuspending cells and helps to minimize morphological changes associated with changes in temperature. Centrifugation steps should be performed at room temperature or warmer.
9. Add 150 µl cell suspension to 1.35 ml of 2.5% glutaraldehyde at 37°C, in a 1.5-ml microcentrifuge tube. Mix gently by inversion.

   It is preferable that suspension cells be fixed in suspension prior to attachment to glass coverslips as morphological changes occur during the attachment of unfixed cells to surfaces. Fresh EM-grade glutaraldehyde must be used as older or less-pure grades may contain contaminants and breakdown products that will lead to fixation artifacts.
10. Seal tubes with Parafilm and incubate for 2 hr at 37°C, with gentle shaking (50 to 100 rpm).

    Shaking the cells minimizes cell clumping that can occur due to cross-linking of cell-surface components by the glutaraldehyde fixative. The cells should not be agitated too vigorously (100 rpm maximum) as vigorous agitation may damage fine surface structures.
11. Pellet cells by centrifuging 5 min at $300 \times g$, room temperature (or warmer) using a microcentrifuge. Remove the supernatant carefully with a pipet and resuspend the cell pellet very carefully in 1 ml sodium phosphate buffer, pH 7.4, to wash the cell. Repeat once.

    Care should be taken when resuspending cells; we recommend using a 1-ml pipet tip with the end cut to widen the tip and reduce shear forces that could result in cell damage.
12. After the second wash, remove the supernatant and carefully resuspend the cells in 20 µl sodium phosphate buffer, pH 6.0.

    The washing steps are very important to remove all traces of glutaraldehyde, which would otherwise cause reduced adhesion of fixed cells to poly-L-lysine. The lower-pH phosphate buffer ensures that the poly-L-lysine side chains are fully protonated-aiding adhesion of cells to the coverslip.

Fix the cells with osmium tetroxide

13. Spot the 20 µl of cell suspension (~6×10^6 cells) onto the corresponding poly-L-lysine-coated coverslip in the well of the 12-well plate. Repeat for each cell sample/coverslip to be prepared. Incubate the coverslips in the plate 30 min at room temperature.

    The spot may not cover the whole coverslip and will settle as a thick drop due to the high surface tension of the drop in contact with the poly-L-lysine on the coverslip surface.

14. Add 0.75 ml of 2.5% glutaraldehyde to each coverslip and incubate 30 min at room temperature.

15. Wash coverslips twice in sodium phosphate buffer, pH 7.4.

    Care should be taken when performing washes not to detach cells from the coverslip surface. Keep the pipet tip away from the coverslip and when adding fresh liquid take care to avoid directing the liquid at the coverslip surface, drip it down the side of the well to gently fill the well and prevent unwanted cell loss from the coverslips.

16. Immerse each coverslip in 0.5 ml 0.5% osmium tetroxide in sodium phosphate buffer, pH 7.4, for 1 hr at room temperature.

    Osmium tetroxide fixation is not essential for SEM but helps to preserve membrane appearance by reducing the elution of membrane lipids during the later dehydration steps (Newell, 1980). The plastic will darken. Osmium tetroxide is toxic and highly volatile—use in the fume hood at all times. Make up osmium solutions in a glass bottle as they will react with plastic.

17. Wash coverslips three times in sodium phosphate buffer, pH 7.4.

Dry fixed samples

We dry samples by critical point drying after dehydration in ethanol (Boyde and Wood, 1969). This gives the most reproducible drying without artifacts. A procedure is given for the Tousimis Samdri 780 dryer, which illustrates the main points. An alternative solvent replacement protocol is given for those without a critical point dryer.

18. Dehydrate the samples through a series of graded ethanol washes (25%, 50%, 75%, 90%, 100%), incubating for 20 min at room temperature in each ethanol grade.

    Samples can be left in the 90% wash overnight if necessary, but must then be taken through the 100% wash immediately before drying. Samples left in 100% ethanol will absorb water from the air and so become partially hydrated.

19. Place the coverslips in the chamber of the critical point dryer.

    It is essential that the samples remain submerged in ethanol—any drying before critical point drying will destroy ultrastructure.

20. Cool the chamber to ~0°C and then open the inlet vent to allow liquid CO_2 to enter the chamber for ~15 min.

    Our critical point dryer has a jacketed chamber and flushing this outer jacket with liquid CO_2 cools the sample. Other models may use refrigeration or Peltier cooling to achieve this. The aim is to replace the ethanol in the sample with a liquid transition fluid—in this case CO_2. Nitrous oxide can also be used. You should be able to see the two liquids mixing in the chamber and then becoming uniform when all of the ethanol is removed. At this point, you should also no longer be able to smell ethanol in the gas emerging from the waste vent. It is important that ALL traces of ethanol be removed before proceeding to the next step.

21. Close the chamber and heat the chamber to just over 31°C. Open the vent to allow the CO_2 to escape slowly—over ~15 min.

    The critical point of liquid CO_2 is 31.1°C at a pressure of 780 kPa. At this temperature (or above) the meniscus between the liquid and gas phase will disappear, avoiding distortion of fine surface structures by reducing surface tension forces. The pressure in the chamber will be ~8 MPa during venting.

22. Remove the dry samples and mount the coverslips on aluminum sample stubs using the adhesive pads.

Coat dried samples

The dried samples need to be given a thin coating of a conductive element before SEM to increase the surface conductance of the sample and so avoid surface charging of the sample under the beam. We use sputter coating with gold. Because of the high density of gold atoms, this treatment also decreases the penetration of the beam into the sample and so improves the resolution of surface edges. Instructions will vary between models of coater—a brief technique is given below.

23. Place samples in a sputter coater and apply a vacuum of ~1.33 Pa (10^{-2} Torr). Flush the system with high-purity argon to a pressure of ~13.3 Pa (10^{-1} Torr).

    The chamber must be clean to ensure good coating—free from residues of vacuum pump oil. Similarly, the argon should be free of contaminants.

24. Apply the current for between 1 to 5 min. The argon will glow purple.

    Under glow discharge, ions bombarding the gold cathode cause discharge of gold ions—plasma sputtering. The sample is attached to the anode and so becomes coated in a thin layer of gold. Coating is from many directions and so is relatively even over complicated three-dimensional surfaces. A metal coating of 5 to 15 nm is optimal for good image quality whilst avoiding electrical charging (Brunk et al., 1981). Thicker coats may be required if osmium fixation has not been used.

    Gold-palladium can be used to coat samples and is reported to give a less granular coating than gold alone (Brunk et al., 1981); however, at the resolution for imaging microvilli and other surface structures we see no texture to the surface coat when using gold alone. The alternative coating method to sputter coating is thermal evaporation—although this can give a less even coat over intricate three-dimensional surfaces.

25. Store samples in a dehumidified environment (i.e., in a sealed plastic container in the presence of silica gel).

    Samples can be stored for extended periods of time provided the low-humidity environment is maintained.

ALTERNATIVE DRYING PROTOCOL—HMDS REPLACEMENT

ALTERNATE PROTOCOL 1

With HMDS replacement, ethanol is replaced with the volatile solvent hexamethyldisilazane, which is then removed by evaporation (Braet et al., 1997). The technique is simpler than critical point drying, and requires no specialized equipment. HMDS has a low surface tension and also helps preserve surface structure by cross-linking proteins. In our experience the results are far more variable than critical point drying and often delicate surface structures are lost—the technique is great when it works, but it does not work reliably.

Additional Materials *(also see Basic Protocol)*

Hexamethyldisilazane (HMDS, Sigma)
Filter paper
Desiccator

1. Perform steps 1 to 18 of the Basic Protocol to prepare the sample.
2. In the fume hood, immerse samples in 100% HMDS for 3 min.
3. Blot excess HMDS with filter paper and dry samples in a desiccator for 30 min.

   Drying in an anhydrous environment is important to avoid possible contamination of the samples with water.

4. Continue with steps 22 to 25 of the Basic Protocol.

ALTERNATE PROTOCOL 2

PREPARATION OF INTESTINAL EPITHELIAL CELLS FOR SEM

The Caco-2 cell line, derived from a human colon adenocarcinoma, can assemble a microvilli-rich brush border upon reaching confluence and has been used extensively as an in vitro model for human intestinal enterocytes (Pinto et al., 1983; Grasset et al., 1984; Peterson and Mooseker, 1992; Dean and Kenny, 2004). In this protocol, Caco-2 cells are cultured for 14 days on permeable supports to allow the cells to feed basolaterally and to differentiate fully (see Fig. 4.17.1). Prior to fixation, transepithelial electrical resistance (TER) is measured with a voltohmmeter to assess monolayer differentiation and integrity. The cells are then fixed and processed according to the method described in the Basic Protocol, with only a few alterations indicated below. The permeable membranes on which the cells are grown can easily curl up when removed from their plastic support so the membranes are cut only just before gold coating (see Fig. 4.17.2).

Additional Materials *(see also Basic Protocol)*

Caco-2 cell line from ECACC or ATCC
Cell culture medium (see recipe)
1% osmium tetroxide (see recipe)

Transwell polycarbonate membrane inserts (1.1-cm^2 surface area; 0.4-μm pore; Corning, no. 3401) placed in a 12-well tissue culture dish
Humidified 37°C, 5% CO_2 incubator
Voltohmmeter
Sharp blade

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

Prepare cells

1. After trypsinization (*UNIT 1.1*), resuspend the Caco-2 cells in cell culture medium at a concentration of 10^6 cells/ml.

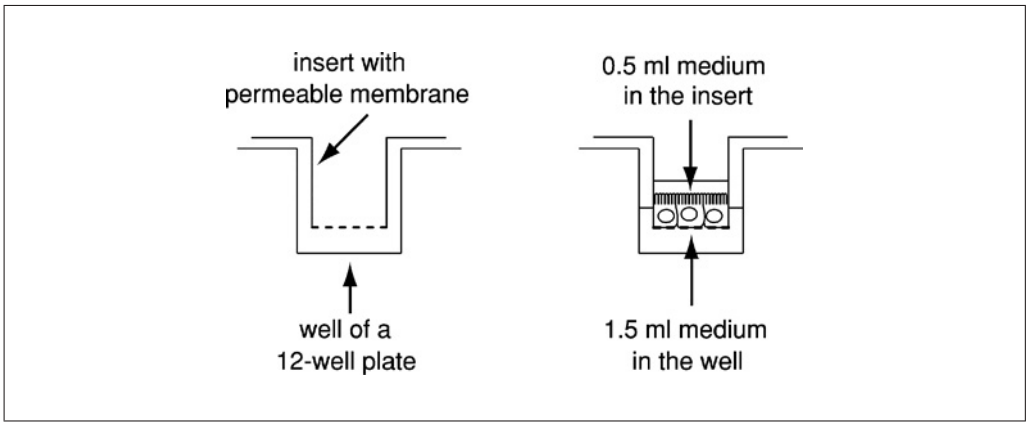


Figure 4.17.1 Culturing Caco-2 cells on permeable inserts.

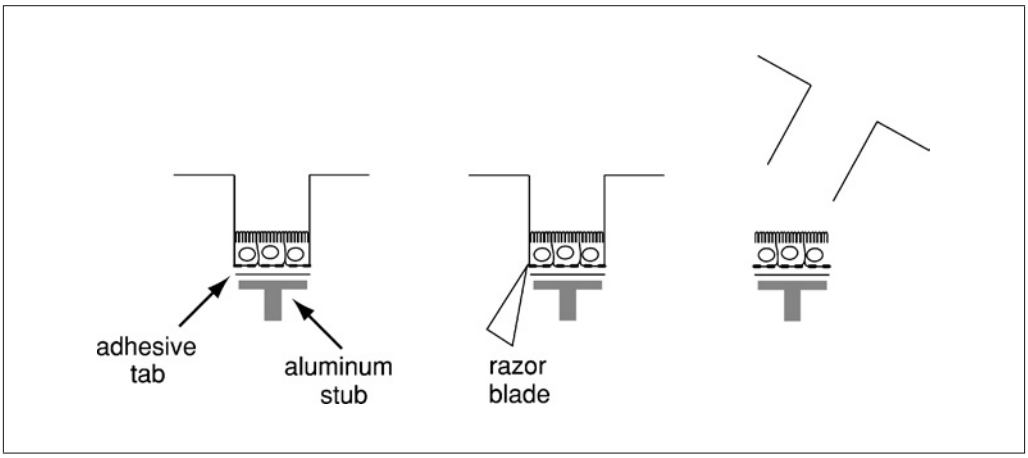


Figure 4.17.2 Cutting the insert membrane before gold coating.

2. Seed 5×10^5 Caco-2 cells (i.e., 0.5 ml) onto each 1.1-cm^2 Transwell polycarbonate membrane insert placed in a 12-well tissue culture dish. Carefully dispense 1.5 ml culture medium in the well (i.e., outside the Transwell).

   Figure 4.17.1 illustrates how the cells are cultured on permeable inserts.

3. Incubate the cells at 37°C in a humidified atmosphere of 5% CO_2 for 14 days. Give fresh culture medium daily by gently replacing both the 0.5 ml medium above the cells (i.e., inside the insert) and the 1.5 ml in the well (i.e., outside the insert).

Fix the cells

4. Before fixing the cells, measure the TER of the Caco-2 cells monolayer. Also measure the TER of a blank insert placed in tissue culture medium.

   The TER of the Caco-2 monolayer should be at least 250 Ω cm^2 above that of a blank filter. If the TER is not at the desired level, the cells are probably not confluent and not polarized, and would not have assembled a microvilli-rich brush border. The cultures with low TER can be grown for longer, to give the cells a chance to divide and become confluent. If they have been grown for 20 days or so they might never become fully confluent and polarized; in this case, they can be discarded.

   It can be very difficult to see the cells cultured on permeable filters. Measuring TER is important as it gives an idea of monolayer integrity.

5. Replace the culture medium with prewarmed 2.5% glutaraldehyde in sodium phosphate buffer, pH 7.4, at 37°C (described in the Basic Protocol). Place the fixative both above the cells (0.5 ml) and around the Transwell (1.5 ml).

6. Seal the plate with Parafilm and incubate for 2 hr at 37°C.

7. Wash three times in fresh sodium phosphate buffer, pH 7.4. Carry out all the washes in the 12-well plate by carefully removing the solutions from above and below the cells and gently adding fresh buffer to both the insert and the well.

8. In the fume hood, add 1% (w/v) osmium tetroxide in sodium phosphate buffer, pH 7.4, for 1hr.

   Osmium tetroxide is very toxic and highly volatile—use in the fume hood at all times. Make up solutions in a glass bottle as they react with plastic.

9. Wash three times in sodium phosphate buffer, pH 7.4.

   If necessary, following secondary fixation, samples can be stored at 4°C for indefinite length of time. The sodium phosphate buffer should be changed several times during the first few days to remove any unreacted osmium and the samples should not be allowed to dry. Samples should be warmed to room temperature before the ethanol dehydration procedure.

Dry the samples

10. Dehydrate the fixed samples through a series of graded ethanol washes (see Basic Protocol, step 18).
11. Dry the samples by placing the whole insert in the chamber of the critical point dryer (see Basic Protocol, steps 19 to 22).
12. Mount the whole insert onto an aluminum sample stub using a carbon adhesive pad (see Fig. 4.17.2). Be careful not to stretch the membrane.
13. Using a sharp blade, cut the membrane around the stub to remove the plastic support (see Fig. 4.17.2).

Coat the samples

14. Coat the samples with gold as described in the Basic Protocol, steps 23 to 25.

ALTERNATE PROTOCOL 3

PREPARATION OF OTHER ADHERENT CELL TYPES FOR SEM

Clearly, a modified version of the Caco-2 method (Alternate Protocol 2) can be used for any adherent cell line grown on glass coverslips. Place the coverslips in a 6- or 12-well tissue culture dish, fix the cells as described in step 5 of Alternate Protocol 2 and proceed through the remaining steps of Alternate Protocol 2

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cell culture medium

Dulbecco's modified Eagles medium (DMEM) supplemented with:
10% (v/v) heat-inactivated fetal bovine serum
2 mM L-glutamine
1% (v/v) non-essential amino acids
100 μg/ml streptomycin and 100 U/ml penicillin
Store up to manufacturer's recommended shelf life at 4°C

Glutaraldehyde, 2.5%

For 20 ml of buffer combine:
2 ml 25% (v/v) EM-grade glutaraldehyde
2 ml 1 M sodium phosphate buffer, pH 7.4 (see recipe)
16 ml ultrapure water (resistivity > 18 MΩcm^{-1}; e.g., Milli-Q grade)
Prepare fresh

CAUTION: *Glutaraldehyde is toxic and waste should be kept to a minimum.*

This recipe can be scaled up or down depending on the number of coverslips to be processed.

Different working strengths of glutaraldehyde fixative can be prepared by varying the amount of 25% stock glutaraldehyde. Concentrations below 2% are not recommended as fixation in low concentrations of glutaraldehyde can allow changes in morphology after fixation (Brunk et al., 1981).

Isoosmotic sodium phosphate buffers

The sodium phosphate buffer used is a mixture of Na_2HPO_4 and NaH_2PO_4 and the ratio of the two forms determines the final pH (Sambrook et al., 1989). Prepare the following stock solutions in ultrapure water:

1 M Na_2HPO_4
1 M NaH_2PO_4

The critical factor with the choice of buffer is that it is of the same osmotic strength as the tissue. Hypotonic or hypertonic buffers will lead to swelling or shrinking of fine surface structures, respectively. The two different buffer components will dissociate in solution as follows (the dissociation of hydrogen ions is slight):

$Na_2HPO_4 \rightarrow 2Na^+ + {}^-HPO_4$ (3 ions)
$NaH_2PO_4 \rightarrow Na^+ + {}^-H_2PO_4$ (2 ions)

For 100 ml of 1 M sodium phosphate buffer, see Table 4.17.1.
Make any necessary minor adjustments to pH using NaOH or HCl as appropriate.

The final concentration of the pH 7.4 buffer will be 100 mM sodium phosphate, and 130 mM for the pH 6.0 buffer—both will have an osmotic strength of ~280 mOsm.

Table 4.17.1 Phosphate Buffer Recipes and Osmolarity

	pH 7.4 phosphate buffer			pH 6.0 phosphate buffer		
	vol (ml)	moles/liter	Osm	vol (ml)	moles/liter	Osm
1 M Na_2HPO_4	77.4	0.774	2.32	12	0.12	0.36
1 M NaH_2PO_4	22.6	0.226	0.45	88	0.88	1.76
Total	100	1	2.77	100	1	2.12

Osmium tetroxide, 0.5%

For 8 ml of buffer combine:
1 ml 4% (w/v) osmium tetroxide solution (Sigma)
0.8 ml 1 M sodium phosphate buffer, pH 7.4 (see recipe)
6.2 ml ultrapure water (resistivity > 18 $M\Omega cm^{-1}$; e.g., Milli-Q grade)
Prepare fresh

CAUTION: *Osmium tetroxide is toxic and waste should be kept to a minimum.*

This recipe can be scaled up or down depending on the number of coverslips to be processed.

Osmium tetroxide, 1%

For 8 ml of buffer combine:
2 ml 4% (w/v) osmium tetroxide solution (Sigma)
0.8 ml 1 M sodium phosphate buffer, pH 7.4 (see recipe)
5.2 ml ultrapure water (resistivity > 18 $M\Omega cm^{-1}$; e.g., Milli-Q grade
Prepare fresh

CAUTION: *Osmium tetroxide is toxic and waste should be kept to a minimum.*

This recipe can be scaled up or down depending on the number of coverslips to be processed.

COMMENTARY

Background Information

Cells have numerous processes on their surfaces that play various roles in specialized cell functions. For example, lymphocytes circulating in the blood circulation possess thin microvilli that are important for the interaction between these cells and the endothelial cells lining the blood vessel walls, allowing the lymphocytes to exit the blood stream and migrate to lymph nodes or nonlymphoid sites in order to perform their varied roles in the immune response (Abitorabi et al., 1997). Lymphocyte microvilli are highly dynamic, extending in response to stimulation with certain cytokines, such as IL-4, or bacterial proteins including LPS (Davey et al., 1998), and being lost in response to other stimuli such as the chemokine SDF-1α (Brown et al., 2003). The ability to

regulate the lengths of their surface protrusions and the arrangement of their cytoskeleton is essential for many lymphocyte functions including rolling and tethering to allow them to exit the bloodstream and in migration within tissues in response to chemokine stimulation.

Adherent cells such as polarized epithelial cells also possess microvilli although they appear to be much more stable and long-lived structures in contrast to the dynamic microvilli seen on lymphocyte surfaces. The apical microvilli observed on intestinal epithelial cells form the brush border and are involved in increasing the surface area of the apical surface for increased absorption.

Such fine protrusive structures are very sensitive to changes in osmolarity, mechanical damage, and other conditions that affect cell behavior in living cells such as temperature and pH. The successful preservation of such delicate structures is a challenge for the preparation of cells for examination by SEM, particularly nonadherent cells such as lymphocytes that constantly collide with other cells during processing. When preparing such cells for SEM, the conditions both prior to and during the preparation process must be carefully considered to maximize the preservation of cell morphology and reduce the likelihood of introducing processing artifacts including cracking, shrinkage, and swelling of fine surface structures.

Although at first daunting, SEM provides unparalleled resolution for the examination of surface morphology in a range of cell types. When combined with light microscopy studies in fixed or live cells, SEM can greatly enhance our understanding of events that happen at an ultrastructural level that is beyond the scope of light microscopy alone.

Critical Parameters and Troubleshooting

The main problem associated with fixation of cells for SEM is the generation of fixation and processing artifacts including cracking, shrinkage, or osmotic swelling. Fine structures such as lymphocyte microvilli and membrane protrusions are particularly susceptible and various steps can be taken to minimize the introduction of artifacts during processing. Examples of such processing artifacts are shown in Figures 4.17.3, 4.17.4, and 4.17.5.

Osmolarity. Glutaraldehyde does not destroy membrane semi-permeability and cells fixed in glutaraldehyde are sensitive to changes in osmolarity during processing until they have undergone secondary fixation in OsO_4 (Brunk et al., 1981). Therefore it is important to fix and process cells using a buffer of comparable osmolarity to the cell culture medium, typically between 280 to 300 mOsm. When considering the osmolarity of the fixation buffer it has been reported that glutaraldehyde does not contribute to the effective osmotic pressure of the fixative, and it is the osmolarity of the fixative vehicle that must be equal to the osmolarity of the cell culture medium (Brunk et al., 1981). Previous workers have distinguished between the absolute osmolarity of fixative solutions, and the effective osmolarity—what the solution does to your sample. Ultimately, the ideal osmotic support can sometimes only be derived empirically, and if you see evidence of osmotic damage, you will need to adjust the osmolarity of your fixative to optimize it to your particular sample. This protocol describes the use of sodium phosphate as the fixative vehicle, other vehicles including 0.1 M Na-Cacodylate supplemented with 0.1 M sucrose have also been used successfully for fixation of cells and preservation of

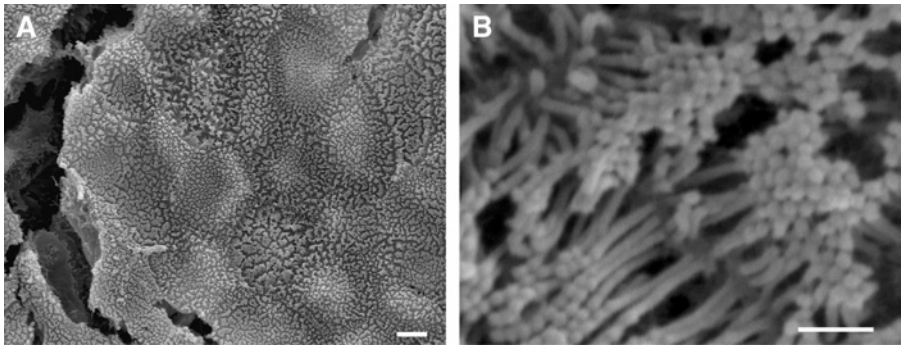


Figure 4.17.3 Artifactual cracking (**A**) and swelling of microvilli tips (**B**) in Caco-2 cells grown on permeable filters. Scale bars: 5 μm (A) and 500 nm (B).

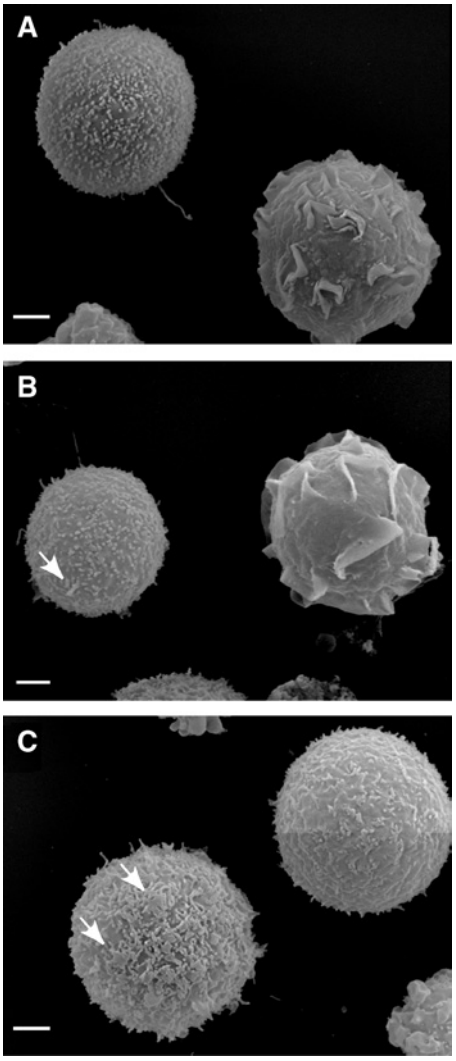


Figure 4.17.4 Varying the glutaraldehyde fixative concentration affected lymphocyte microvilli morphology. Mouse pre-B cells 300.19 were fixed in sodium phosphate buffer containing (**A**) 2.5%, (**B**) 3.5%, or (**C**) 4.5% glutaraldehyde and processed for SEM. Merging or aggregation of surface protrusions can be observed at 3.5% and 4.5% glutaraldehyde concentrations (arrows). Scale bar = 2 μm.

fine surface structures for SEM (Brunk et al., 1981).

Glutaraldehyde concentration. While glutaraldehyde concentrations of up to 6% have been reported to not affect the preservation of surface structures, we have noticed an apparent merging or aggregation of cell surface microvilli at concentrations exceeding 3%, as shown in Figure 4.17.4. We interpret this observation as being an artifact of high glutaraldehyde concentrations, potentially due to cross-linking of cell surface factors by the fixative. Ideally, optimal glutaraldehyde concentration would be determined empirically for the cells being studied.

Temperature. Temperature is an essential factor to consider when minimizing potential processing artifacts, as changes in temperature in living cells can result in altered morphology and the loss of motile surface structures (Newell, 1980; Brunk et al., 1981). All steps up to and including the primary fixation in glutaraldehyde should be performed at 37°C, and the fixative should be warmed to 37°C before use.

Dehydration. Artifacts are inevitably introduced during the dehydration process, whether dehydration is carried out by freeze-drying or by a series of graded ethanol washes followed by critical point drying or HMDS solvent

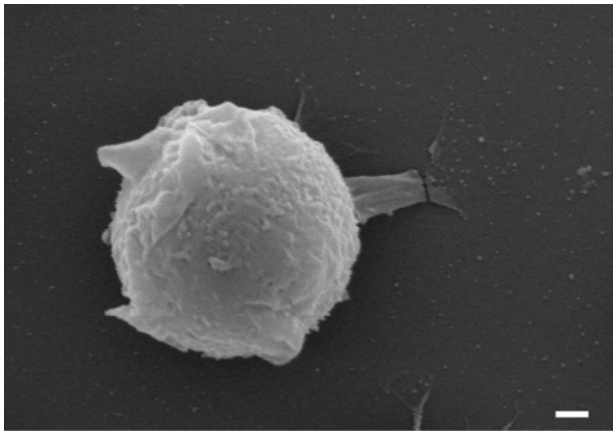


Figure 4.17.5 Artifactual cracking of attached cell protrusions and loss of surface structures in fixed lymphocytes. Mouse 300.19 pre-B cells were fixed in 2.5% glutaraldehyde and processed for SEM. Scale bar = 1 μm.

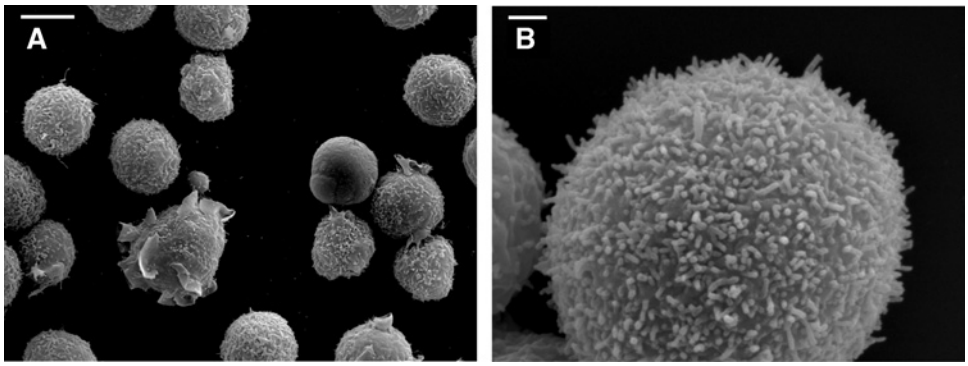


Figure 4.17.6 Surface morphology of lymphocytes. Mouse 300.19 pre-B cells were processed for SEM using the described protocol. Surface morphology has been preserved with the absence of apparent cracks and osmotic swelling, particularly in thin microvilli structures. Panel (**A**) shows lower magnification (scale bar: 5 μm); panel (**B**) shows higher magnification (scale bar: 1 μm).

dehydration as described here. Critical point drying gives rise to significantly fewer dehydration artifacts than other methods. Shrinkage often occurs during dehydration and may be apparent as large cracks in cell monolayers and as breaks in adherent protrusive structures such as retraction fibers (Brunk et al., 1981). Examples of dehydration artifacts are given in Figures 4.17.3 and 4.17.5.

Anticipated Results

Cells prepared using this protocol should possess well-preserved surface structures, as shown in Figure 4.17.6, with no evidence of swelling or distortion or artifacts including cracking of cell processes.

Time Considerations

From start to finish the preparation procedure takes ~8 hr. If necessary the fixed and dehydrated coverslips can be stored overnight in 90% ethanol prior to the critical point drying step; in this case we would recommend an additional 20 min wash in 100% ethanol before proceeding to critical point drying to ensure any residual moisture is removed. Coating takes ~5 min, but this may vary from machine to machine.

Literature Cited

Abitorabi, M.A., Pachynski, R.K., Ferrando, R.E., Tidswell, M., and Erle, D.J. 1997. Presentation of integrins on leukocyte microvilli: A role for the extracellular domain in determining membrane localization. *J. Cell Biol.* 139:563-571.

Boyde, A. and Wood, C. 1969. Preparation of animal tissues for surface-scanning electron microscopy. *J. Microsc.* 90:221-249.

Braet, F., De Zanger, R., and Wisse, E. 1997. Drying cells for SEM, AFM and TEM by hexamethyldisilazane: A study on hepatic endothelial cells. *J. Microsc.* 186:84-87.

Brown, M. J., Nijhara, R., Hallam, J. A., Gignac, M., Yamada, K. M., Erlandsen, S. L., Delon, J., Kruhlak, M., and Shaw, S. 2003. Chemokine stimulation of human peripheral blood T lymphocytes induces rapid dephosphorylation of ERM proteins, which facilitates loss of microvilli and polarization. *Blood* 102:3890-3899.

Brunk, U., Collins, V. P, and Arro, E. 1981. The fixation, dehydration, drying and coating of cultured cells for SEM. *J. Microsc.* 123:121-131.

Davey, E.J., Thyberg, J., Conrad, D.H., and Severinson, E. 1998. Regulation of Cell Morphology in B Lymphocytes by IL-4: Evidence for Induced Cytoskeletal Changes. *J. Immunol.* 160:5366-5373.

Dean, P. and Kenny, B. 2004. Intestinal barrier dysfunction by enteropathogenic *Escherichia coli* is mediated by two effector molecules and a bacterial surface protein. *Mol. Microbiol.* 54:665-675.

Grasset, E., Pinto, M., Dussaulx, E., Zweibaum, A., and Desjeux, J.F. 1984. Epithelial properties of human colonic carcinoma cell line Caco-2: Electrical parameters. *Am. J. Physiol.* 247:C260-267.

Newell, D.G. 1980. The white cell system. *In* Biomedical Research Applications of Scanning Electron Microscopy (G.M. Hodges and R.C. Hallowes, eds.) pp. 219-305. Academic Press, London.

Peterson, M.D. and Mooseker, M.S. 1992. Characterization of the enterocyte-like brush border cytoskeleton of the C2BBe clones of the human intestinal cell line, Caco-2. *J. Cell Sci.* 102:581-600.

Pinto, M., Robine-Leon, S., Appay, M-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J., and Zweibaum, A. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* 47:323-330.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, N.Y.

Fluorescence Imaging Techniques for Studying *Drosophila* Embryo Development

Manos Mavrakis,[1] Richa Rikhy,[2] Mary Lilly,[2] and Jennifer Lippincott-Schwartz[2]

[1]Institute of Developmental Biology of Marseille-Luminy, UMR6216 CNRS-Université de la Méditerranée, Marseille, France
[2]Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

ABSTRACT

This unit describes fluorescence-based techniques for noninvasive imaging of development in living *Drosophila* embryos, discussing considerations for fluorescent imaging within living embryos and providing protocols for generation of flies expressing fluorescently tagged proteins and for preparation of embryos for fluorescent imaging. The unit details time-lapse confocal imaging of live embryos and discusses optimizing image acquisition and performing three-dimensional imaging. Finally, the unit provides a variety of specific methods for optical highlighting of specific subsets of fluorescently tagged proteins and organelles in the embryo, including fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), and photoactivation techniques, permitting analysis of specific movements of fluorescently tagged proteins within cells. These protocols, together with the relative ease of generating transgenic animals and the ability to express tagged proteins in specific tissues or at specific developmental times, provide powerful means for examining in vivo behavior of any tagged protein in embryos in myriad mutant backgrounds. *Curr. Protoc. Cell Biol.* 39:4.18.1-4.18.43. © 2008 by John Wiley & Sons, Inc.

Keywords: *Drosophila* • embryo • fluorescence • imaging • photobleaching • photoactivation

INTRODUCTION

The *Drosophila* embryo has served as a powerful model for defining the pathways and organizational principles that guide embryogenesis in a wide array of animals. Key to this success has been the development of molecular genetics tools that facilitate the rapid molecular, as well as functional, characterization of genes that act to build the embryo. But only recently, due to advances in live-cell imaging of fluorescently tagged fusion proteins, has it become possible to investigate how proteins responsible for embryonic development are dynamically expressed and distributed in living embryos. In *Drosophila*, live-cell imaging studies are greatly facilitated by the relative ease of generating transgenic animals that express fluorescently tagged proteins, as well as the ability to express tagged proteins in a specific tissue or at specific developmental times. Finally, the wealth of genetic resources available in *Drosophila* provides the unique opportunity to examine the in vivo behavior of a tagged protein in the embryo in myriad mutant backgrounds.

This unit describes several fluorescence-based techniques for noninvasive imaging of *Drosophila* development in the living embryo. It begins with a discussion of the challenges and considerations in fluorescent imaging within an embryo, which produces

Current Protocols in Cell Biology 4.18.1-4.18.43, June 2008
Published online June 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0418s39

poor images under conventional wide-field fluorescence microscopy because of its thickness and the resulting autofluorescence and out-of-focus fluorescence. The unit then proceeds to Basic Protocol 1 for generating fluorescently tagged proteins within the fly. The method of choice—the Gal4/UAS system—permits expression of fluorescently tagged proteins-of-interest in a spatially and temporally controlled manner. Next, Basic Protocol 2 discusses how to prepare embryos for fluorescent imaging, including dechorionation and mounting on a coverslip in a way that allows the embryo to continue to develop normally to the first instar larval stage. Basic Protocols 3 and 4 then focus on how to perform noninvasive time-lapse confocal imaging of live fly embryos using confocal laser scanning microscopy (CLSM) and two-photon laser scanning microscopy (2PLSM), respectively. Among the issues discussed are optimizing image acquisition and performing three-dimensional (3D) imaging. The unit goes on to describe a variety of specific techniques that permit optical highlighting of specific subsets of fluorescently tagged proteins and organelles in the embryo, including fluorescence recovery after photobleaching (FRAP, Basic Protocol 5), fluorescence loss in photobleaching (FLIP, Basic Protocol 6), and photoactivation techniques (Basic Protocol 7). These techniques allow the specific movements of fluorescently tagged proteins within cells to be monitored and analyzed. Specific examples of the techniques are illustrated and discussed.

STRATEGIC PLANNING

Working Knowledge of *Drosophila* Embryonic Development

High-resolution fluorescence microscopy of living *Drosophila* embryos presents a challenge because of the optical characteristics of the embryo, as well as the rapid, dramatic changes in tissue morphology that take place during the development of the fertilized egg into a multicellular animal (first instar larva). A working knowledge of embryonic development is required for proper understanding of the optical properties of the tissue to be imaged, as well as other elements important for imaging applications (e.g., effect of temperature on development or rapid tissue movements). Morphogenesis during *Drosophila* embryogenesis has been studied in great detail, and an atlas of *Drosophila* embryo development depicting the different morphogenetic phenomena, as well as their timing, can be found on the Web site *http://www.sdbonline.org/fly/atlas/00atlas.htm*. The Interactive Fly Web site (*http://www.sdbonline.org/fly/aimain/1aahome.htm*) and the FlyMove Web site (*http://flymove.uni-muenster.de*) further provide images, movies, and detailed descriptions of tissue and organ development during embryogenesis.

Briefly, during *Drosophila* fertilization, sperm penetrate through the micropyle at the egg's anterior tip while the egg is still in the uterus. Females can retain fertilized eggs in the uterus for variable periods of time before deposition. The *Drosophila* embryo is, for the first 2 hr, a syncytium, i.e., the nuclei divide and migrate in a common cytoplasm. During the first hour after fertilization, nuclei lie and divide in the interior of the embryo (preblastoderm stage). Most nuclei then migrate synchronously to the periphery of the embryo and undergo four more divisions in a common cytoplasm (syncytial blastoderm stage). After the fourth division, ~2 hr after fertilization, membranes synchronously invaginate to encase individual nuclei and produce a layer of epithelial cells at the embryo periphery within ~1 hr. This cellularization event occurs at a time in development referred to as midblastula transition, whereby nuclear division slows down and RNA transcription from the nuclei is greatly enhanced. Foe and Alberts (1983) and Foe et al. (1993) provide excellent, in-depth descriptions of the events taking place from fertilization of the egg to cellularization in the embryo.

Cellularization is immediately followed by gastrulation (~3 hr after fertilization), a stage where dramatic cell rearrangements generate the germ layers (endoderm, mesoderm, and ectoderm), the specific regions of the embryo that will give rise to the different organ

systems. Costa et al. (1993) describe in detail the morphogenetic phenomena during this stage. During the hours that follow gastrulation, a complex series of morphogenetic processes occur, with cells in each of the germ layers differentiating to form the organs (e.g., midgut, tracheae, salivary glands). Segmentation of the embryo also occurs during this period and serves to generate the body plan of *Drosophila*, with distinct head and tail ends, as well as segments that will form the thorax and abdomen. During segmentation, the segregation of the imaginal disks (sacs of cells that will give rise to adult structures during pupal metamorphosis) also takes place. First instar larvae hatch at ~21 to 22 hr after fertilization at 25°C. For a review of embryonic development of insects, see Anderson (1972).

Practical Considerations for Fluorescence Microscopy of Living *Drosophila* Embryos

The physical properties of the *Drosophila* embryo (size, thickness, opacity, autofluorescence), properties of the developing tissues (geometry, movements), as well as imaging temperature have to be carefully considered before attempting any high-resolution imaging application. Considerations relating to these properties are discussed in the rest of this section.

Size of embryos

Wild-type *Drosophila* embryos are 511±21 μm in length and 184±5 μm in width (n = 100 Canton S embryos). If high spatial resolution is not required, a low magnification (16× or 20×), low-numerical-aperture (NA) objective can be used to look at whole embryos (e.g., a Zeiss Achroplan 20×/0.45 NA or an Olympus UPlanApo 20×/0.70 NA lens). This is particularly useful for scoring embryos expressing a specific fluorescently tagged protein or identifying fluorescent embryos at a specific stage in development. However, high-resolution imaging applications necessitate the use of higher-magnification, high-NA objectives (discussed below).

The volume of an embryo can be calculated by considering the volume of an ellipsoid of minor axes $2a = 2b$ and major axis $2c$ (Fig. 4.18.1A):

$$V = 4/3\ \pi\ \times a \times b \times c = 9.0 \pm 0.6 \text{ nl } (n = 100 \text{ Canton S embryos})$$

This information can be useful when calculating dilution factors for microinjecting drugs or fluorescent molecules into the embryo with automatic microinjectors.

Thickness of embryos

The *Drosophila* embryo is a thick specimen for optical fluorescence microscopy. One major problem encountered in imaging thick specimens is high background and emitted fluorescence from out-of-focus regions, which result in low-contrast images when using conventional (wide-field) fluorescence microscopy. Confocal laser scanning microscopy (CLSM, reviewed in *UNIT 4.5*), which we discuss in this unit, addresses this problem with the use of a pinhole to reject out-of-focus light and a point-scanning light source (laser), resulting in high-contrast images; it is the method of choice for high-resolution four-dimensional (4D; 3D over time) imaging of living *Drosophila* embryos. Multiphoton excitation microscopy (reviewed in *UNIT 4.11*) has been successfully used for deep-tissue imaging in thick living specimens. We discuss the potential of two-photon excitation for imaging living *Drosophila* embryos below (Light scattering and out-of-focus absorption) and in Basic Protocol 4.

Several powerful approaches, which are based on illumination through multiple objectives or use spatially structured illumination, have been developed to extend resolution (reviewed in Gustafsson, 1999), but they are not yet compatible with the image acquisition

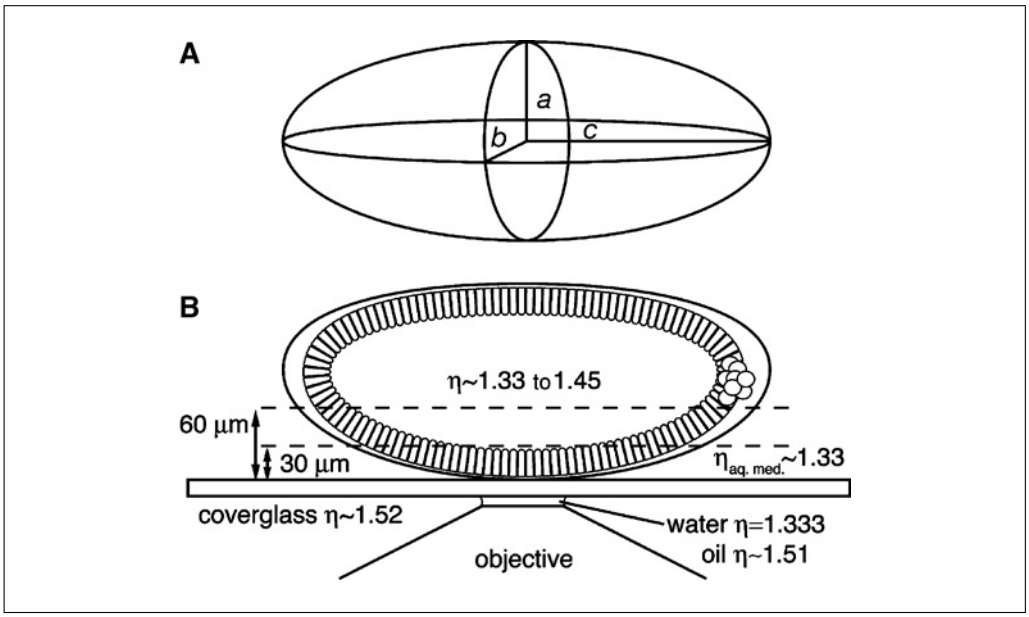


Figure 4.18.1 Size and thickness of the *Drosophila* embryo. (**A**) Schematic representation of the *Drosophila* embryo as an ellipsoid of minor axes $2a = 2b$ and major axis $2c$. (**B**) Schematic representation of a cellularizing embryo showing that imaging of the invaginating plasma membrane requires focal planes >30 μm deep in the embryo. The differences in the refractive indices of the tissue (η_{tissue} ~1.33 to 1.45), the aqueous medium (aq. med.) in which the embryo is mounted (η ~1.33), the coverglass ($\eta_{coverglass}$ ~1.52), and the immersion medium result in a distortion of the light path and severe spherical aberrations in focal planes deep in the embryo when oil is used (η_{oil} ~1.51). The use of water lenses ($\eta_{water} = 1.33$) significantly reduces these aberrations.

speed required for live imaging, and they require sophisticated equipment and alignment procedures. Of particular interest is selective plane illumination microscopy (SPIM; Huisken et al., 2004), a recently developed technique that uses a thin sheet of light to illuminate (from the side) only the focal plane of the detection lens. SPIM provides optical sectioning and large penetration depth, induces less photodamage, and further, achieves isotropic spatial resolution when images are acquired along multiple orientations of the sample. SPIM has been used for imaging deep in living *Drosophila* embryos (Huisken et al., 2004) and is particularly suited for low-NA systems (NA < 0.8), achieving a substantially increased axial resolution compared to CLSM (Engelbrecht and Stelzer, 2006).

The major considerations related to the thickness of living *Drosophila* tissue are the following:

1. *Light scattering and out-of-focus absorption*. When the excitation (illumination) or imaging (emitted fluorescence) path intersects regions with differing refractive indices (e.g., organelles, lipid droplets, yolk particles, or tissue in the embryo interior), their curved surfaces act as microlenses to scatter light in all directions. Some of the excitation light will be scattered enroute to the focal plane. Excitation photons are further absorbed by any fluorophores along the excitation light path, so overall fewer photons reach the focal plane, thus decreasing excitation intensity with increasing depth in the specimen. At the same time, scattered excitation light will be absorbed and will generate fluorescence at sites outside the imaging plane, thereby increasing the background that needs to be removed by the pinhole. Photobleaching and phototoxicity will also be induced in regions of the sample that are not contributing to the image. Emitted light from the focal spot will also be scattered, preventing it from passing through the pinhole, even though it arose from the correct focal plane. Inevitably, some of the light arising from regions outside the

imaging plane, which should be blocked at the pinhole, will be scattered so that it passes through and contributes noise. Overall, light scattering and out-of-focus absorption limit the maximum imaging depth in CLSM to <40 μm deep in the *Drosophila* embryo. The signal decreases dramatically with increasing depth (see Fig. 4.18.2), compromising resolution and quantitative measurements. Regions closest to the surface do not suffer as much from scattering and out-of-focus absorption, and they can be readily imaged up to 60 μm away from the coverglass in sagittal sections (Fig. 4.18.2, arrows).

The limited penetration depth due to scattering and out-of-focus absorption can be most efficiently addressed in two-photon laser scanning microscopy (*UNIT 4.11*). Two-photon excitation occurs only in the focal plane, so there is no absorption of excitation light (and consequently no photobleaching or photodamage) in out-of-focus regions between the objective lens and the focal plane. Furthermore, longer wavelengths used in two-photon excitation are scattered less than shorter wavelengths. Thus, much more of the two-photon excitation light penetrates through the sample to reach the focal plane, increasing the depth of penetration into the tissue to >100 μm (see Fig. 4.18.2). Finally, a pinhole is not required in two-photon excitation, and multiply-scattered photons arising from the focal plane can be collected in a nondescanned (see Basic Protocol 4, step 4 annotation) detection geometry. The net result is that the signal-to-noise ratio decreases less rapidly with imaging depth.

2. *Spherical aberrations*. The highest-NA oil-immersion objectives commonly used for high-resolution fluorescence microscopy are designed for observing specimens directly in touch with or within a few micrometers of the coverglass, which is in contact with the lens through immersion oil. The *Drosophila* embryo is mounted on a coverglass in an aqueous solution (Fig. 4.18.1B; Basic Protocol 2). Due to the thickness and ellipsoid geometry of the embryo, imaging subcellular processes during development often requires focal planes deep into the specimen. For example, imaging cytoplasmic organelles or invaginating plasma membrane during cellularization typically requires focal planes at ~40 to 60 μm away from the coverglass (Fig. 4.18.1B). As the focal plane is set deeper into the specimen (already 10 μm away from the coverglass), differences in the refractive indices of the aqueous medium in which the embryo is mounted ($\eta_{\text{aq. med.}} \sim 1.33$), interior of the embryo (cellular material has a refractive index $\eta_{\text{cell.}} \sim 1.33$ to 1.45; Beuthan et al., 1996; Johnsen and Widder, 1999; Dirckx et al., 2005), coverglass ($\eta_{\text{coverglass}} \sim 1.52$), and immersion oil ($\eta_{\text{oil}} \sim 1.51$; Fig. 4.18.1B) result in distortion of the imaging light path.

This causes severe spherical aberrations, observed as blurring, loss of contrast, and a reduced axial resolution, leading to misinterpretation of morphology and geometry of structures residing deep in the embryo. This problem is greatly ameliorated by the use of water-immersion objectives. When water immersion is used ($\eta_{\text{water}} = 1.333$), spherical aberrations are significantly reduced and images can theoretically be collected as deeply as the working distance of the lens permits. In practice, scattering and out-of-focus absorption limit the achieved penetration depth. For example, the Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens (working distance of 280 μm at a 0.17-mm coverglass thickness) enables imaging up to ~40 μm into the embryo with one-photon excitation (CLSM), ~60 μm with two-photon excitation and descanned detection, and ~100 μm with two-photon excitation and a nondescanned detection geometry (see Fig. 4.18.2). Since the correction for spherical aberration depends on the length of the optical path through the coverglass, it is critically important to adjust the correction collar on the water-immersion objective to the thickness of the coverglass used. Failure to adjust the collar to the coverglass thickness will result in severe spherical aberrations and will greatly reduce axial resolution.

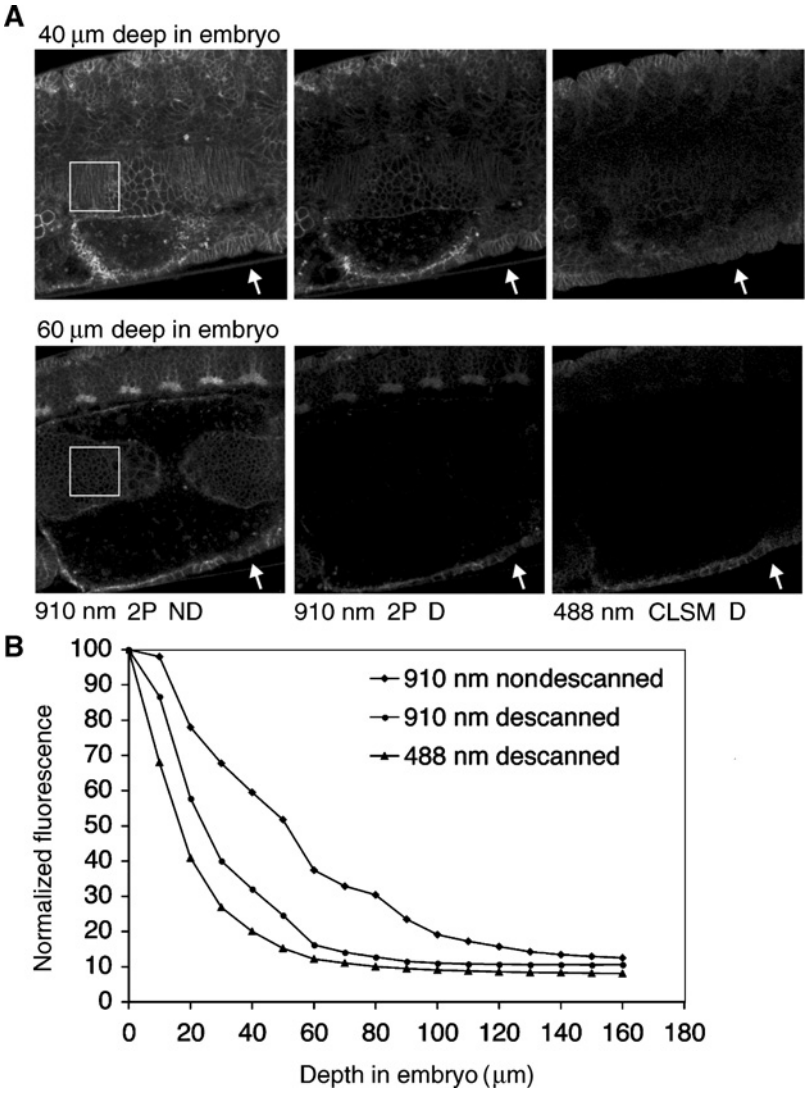


Figure 4.18.2 Fluorescence decreases dramatically with focal planes deep in the embryo in confocal laser scanning microscopy (CLSM) but less so in two-photon (2P) excitation. (**A**) Optical sections of living fluorescent embryos during late gastrulation, in focal planes 40 and 60 μm deep in the embryo. Images were acquired with two-photon excitation at 910 nm and a nondescanned (ND) detection light path (left panels), two-photon excitation at 910 nm and descanned (D) detection (middle panels), or 488 nm in CLSM (right panels). (**B**) Plot of fluorescence intensity in a region of interest (white outlined square in panel A) as a function of depth in the embryo. Fluorescence is normalized to 100% at the embryo surface. In focal planes 40 μm deep in the embryo, the signal decreased to 20% with 488-nm excitation, 30% with two-photon excitation at 910 nm, but only 60% when using two-photon excitation with a nondescanned detection light path. In focal planes 60 μm deep in the embryo, no structure is discernible with 488-nm excitation in the embryo interior. The outline of cells is visible with two-photon excitation at 910 nm and descanned detection, but much more pronounced when a nondescanned light path is used (signal decreases to 40% as opposed to <20% with descanned detection). Note that the signal decreases much less with depth in regions closest to the surface (arrows) because the light path does not intersect deep tissue that causes scattering and out-of-focus absorption.

3. *Chromatic aberrations*. Chromatic aberrations are common in multicolor confocal microscopy. These aberrations result in different excitation wavelengths being focused at different points and different emitted wavelengths being collected from different points in the tissue. Horizontal displacements in the image plane (lateral chromatic aberrations), resulting in different magnification of different colors, can be minimized by moving the region of interest (ROI) to the center of the microscope field. However, vertical color displacements along the focal axis (axial chromatic aberration) are present throughout the microscope field, and this is a serious problem when performing colocalization studies or live multifluorophore imaging (see Basic Protocol 3).

Microscope objectives for high-resolution fluorescence microscopy of living embryos must be corrected for chromatic aberrations. Commercially available lenses that are corrected to the highest degree for chromatic aberrations are named *Apochromat* or *Apo*. However, the optics design in the corrected lenses is specific for a particular arrangement of refractive indices for different wavelengths. The heterogeneity of refractive indices in the embryo interior will most likely not match the design specifications and will lead to chromatic aberrations of varying magnitudes. Chromatic aberrations are not dependent on imaging depth. However, failure to correct for spherical aberrations (e.g., by misadjusting the correction collar) will result in complicated artifactual color shifts in focal planes deep into the sample. Thus, interpretation of slight differences in localization demands careful control experiments to rule out chromatic aberrations.

4. *Choice of objective*. Long-working-distance, high-NA, water-immersion objectives corrected for coverglass thickness, field curvature and chromatic aberrations (e.g., the Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens or the Olympus UPLAPO 60×/1.2 NA water lens) are the best choices for high-resolution live 4D imaging of thick *Drosophila* embryos with CLSM. Magnification, NA, and transmittance of the lens to specific wavelengths need to be carefully chosen based on the application (see Basic Protocols 3 to 7).

Opacity of embryos

The outermost shell of the embryo, the chorion, is a specialized layer secreted by follicle cells during oogenesis. It has two dorsal appendages that facilitate gas exchange in the developing embryo (Fig. 4.18.3B, arrow). The presence of the chorion renders the embryo opaque for transillumination and epifluorescence. However, it is easily removed (dechorionation) by immersing the embryos in a sodium hypochlorite solution (bleach; see Basic Protocol 2 and Fig. 4.18.3C), making the embryos transparent for imaging purposes. Dechorionated embryos mounted in water or phosphate-buffered saline solution develop normally to first-instar larvae.

Autofluorescence of embryos

The major sources of autofluorescence in a dechorionated *Drosophila* embryo are yolk granules and the vitelline membrane. The autofluorescence emission spectra for yolk and vitelline membrane (VM) at excitation wavelengths routinely used for common fluorophores are depicted in Figure 4.18.4. The emission spectra of ECFP, EGFP, EYFP, and mRFP1 and the respective emission detection windows for common bandpass emission filters used for these fluorescent protein (FP) variants are superimposed on the autofluorescence spectra for reference. It is evident that both yolk and VM are highly autofluorescent at violet (820 nm, two-photon) and blue (458 nm, 488 nm) wavelengths, and their emission maxima interfere with the emission detection for ECFP and EGFP. Yolk and VM autofluorescence are much less pronounced at yellow (514 nm) and minimal at red and far-red (543 nm, 633 nm) wavelengths. The problem of autofluorescence is illustrated in Figure 4.18.5A, where confocal images of wild-type (Canton S) embryos

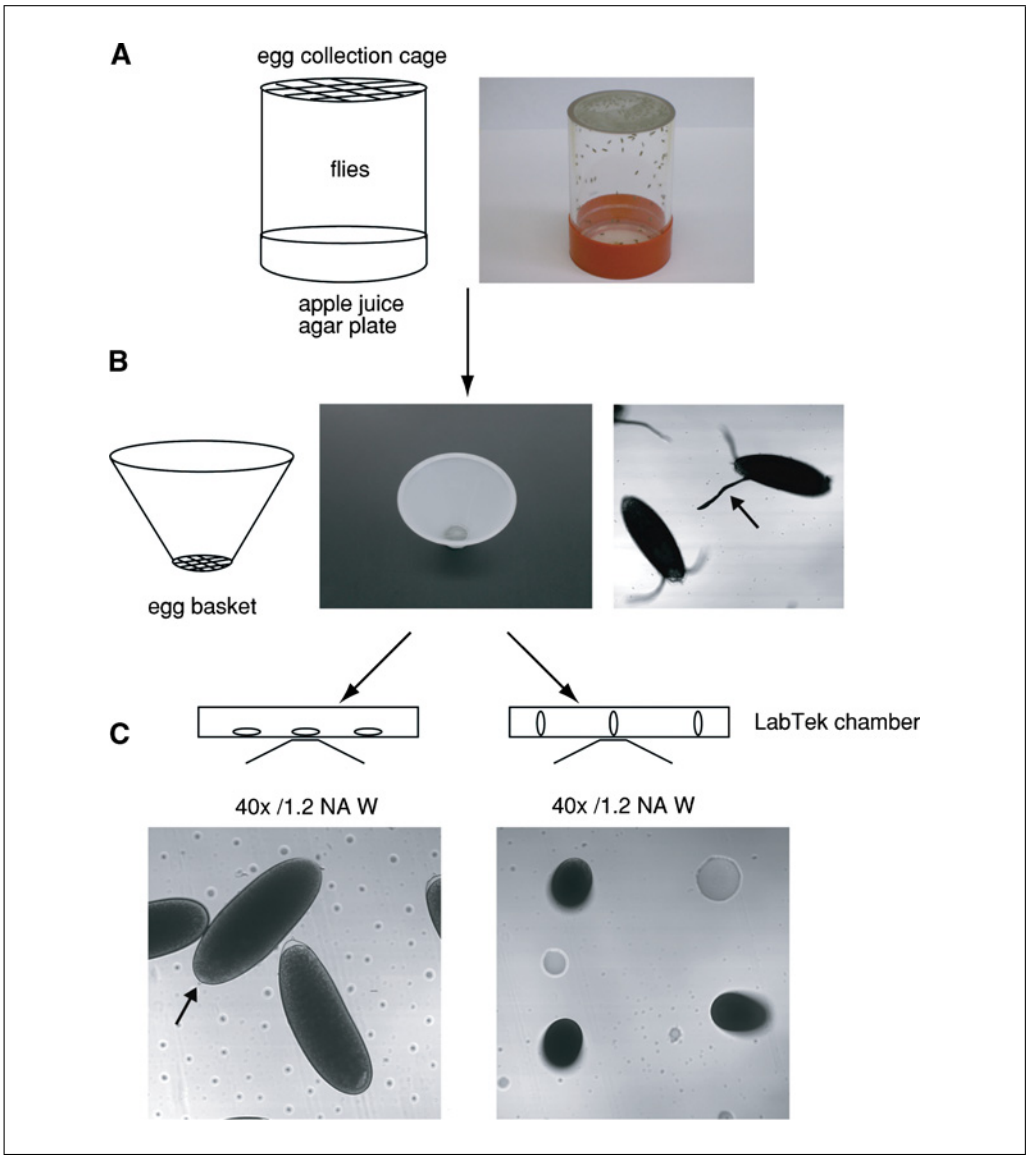


Figure 4.18.3 Preparation of *Drosophila* embryos for fluorescence microscopy. (**A**) The fly embryo collection cage with an apple juice agar plate. The left image is a schematic of the fly cage. The right image shows a cage from Genesee Scientific (which fits 60-mm petri dishes) containing adult flies over a yeasted apple juice agar plate. (**B**) The modified funnel with a 100-μm stainless steel mesh for embryo collection and dechorionation. The left image shows a schematic and the middle image is a photograph of a funnel with a mesh. The right image shows chorionated *Drosophila* embryos imaged with DIC optics on a confocal microscope. Note that the chorionated embryos appear opaque and are not suitable for imaging. The arrows point to the dorsal appendages of the embryo chorion. (**C**) Mounting of embryos in LabTek chambers for imaging. The embryos are dechorionated by treatment with diluted bleach for 1 min (see Basic Protocol 2) and then mounted on either the dorsolateral/ventrolateral side (left schematic) or on their posterior (right schematic). The left image shows dechorionated *Drosophila* embryos with DIC optics mounted on their dorsolateral/ventrolateral side. The arrow points to the micropyle on the anterior of the embryo. The right image shows dechorionated *Drosophila* embryos mounted on their posterior.

are shown, acquired with excitation/emission settings routinely used for live imaging of common fluorophores.

Although autofluorescence can be greatly reduced by imaging at longer wavelengths, live multifluorophore imaging (e.g., Cerulean/Venus, mGFP/mRFP1; see Basic Protocol 3) typically requires at least one fluorescent variant in the blue region. Because autofluorescence is not diffuse but confined to specific structures (yolk, VM), the optical

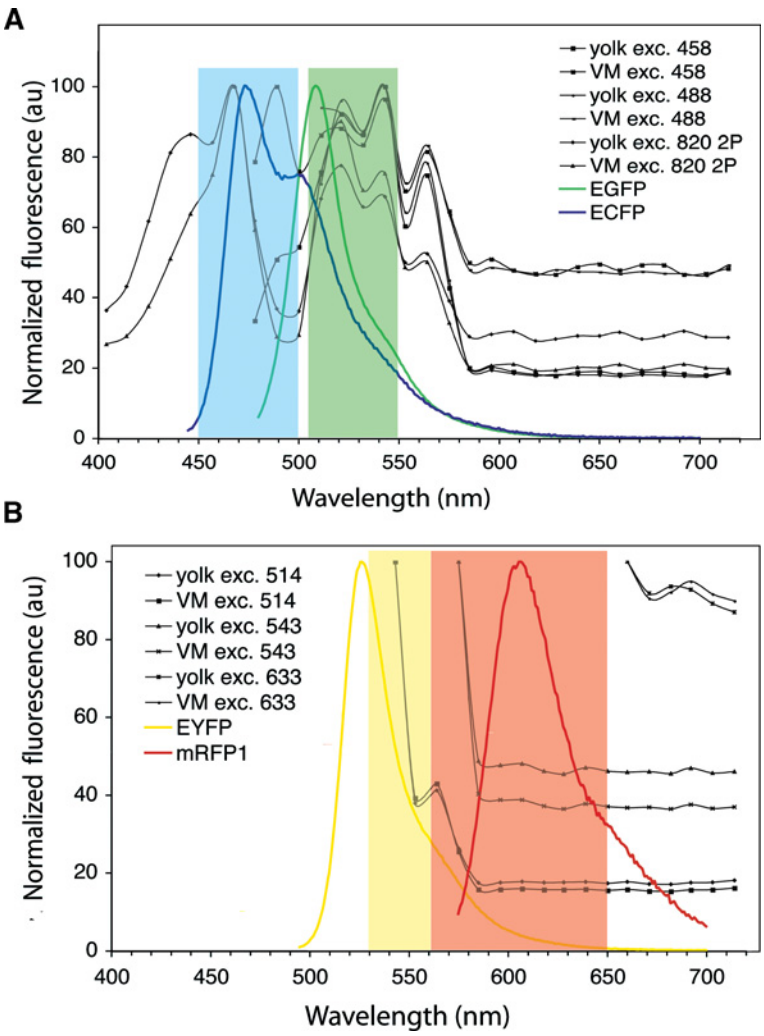


Figure 4.18.4 Yolk and vitelline membrane (VM) autofluorescence interferes with the fluorescence of common fluorophores at violet and blue visible wavelengths. The autofluorescence emission spectra for yolk and VM are shown at excitation wavelengths (exc.) routinely used to excite common fluorophores. The emission spectra of ECFP, EGFP, EYFP and mRFP1 are superimposed on the autofluorescence spectra, as are the emission detection windows commonly used for the above variants. (**A**) Yolk and VM are highly autofluorescent (two-photon excitation at 820 nm) in violet and blue (458 and 488 nm), but less pronounced (**B**) in red (543 nm) and far-red (633 nm): Abbreviation: au, arbitrary units; 2P, two-photon. For the color version of this figure go to *http://www.currentprotocols.com*.

sectioning capability of the CLSM helps to greatly reduce, if not to eliminate, this problem. This, in turn, depends on the depth of the focal plane used, as well as the stage of development of the embryo, because yolk distribution changes during embryogenesis. In focal planes closer to the embryo surface, using a narrow pinhole will eliminate VM autofluorescence. Interference from yolk will depend on the stage of the embryo. For example, in early embryos yolk is enriched in the cortical cytoplasm and interferes with ECFP/EGFP fluorescence (Fig. 4.18.5C, arrows), whereas in cellularizing embryos yolk is displaced below the nuclei and is not a problem (Fig. 4.18.5B, star). In contrast, sagittal views always suffer from VM autofluorescence when plasma membrane fluorescence directly beneath the VM is imaged (Fig. 4.18.5B, left panel). One way to distinguish

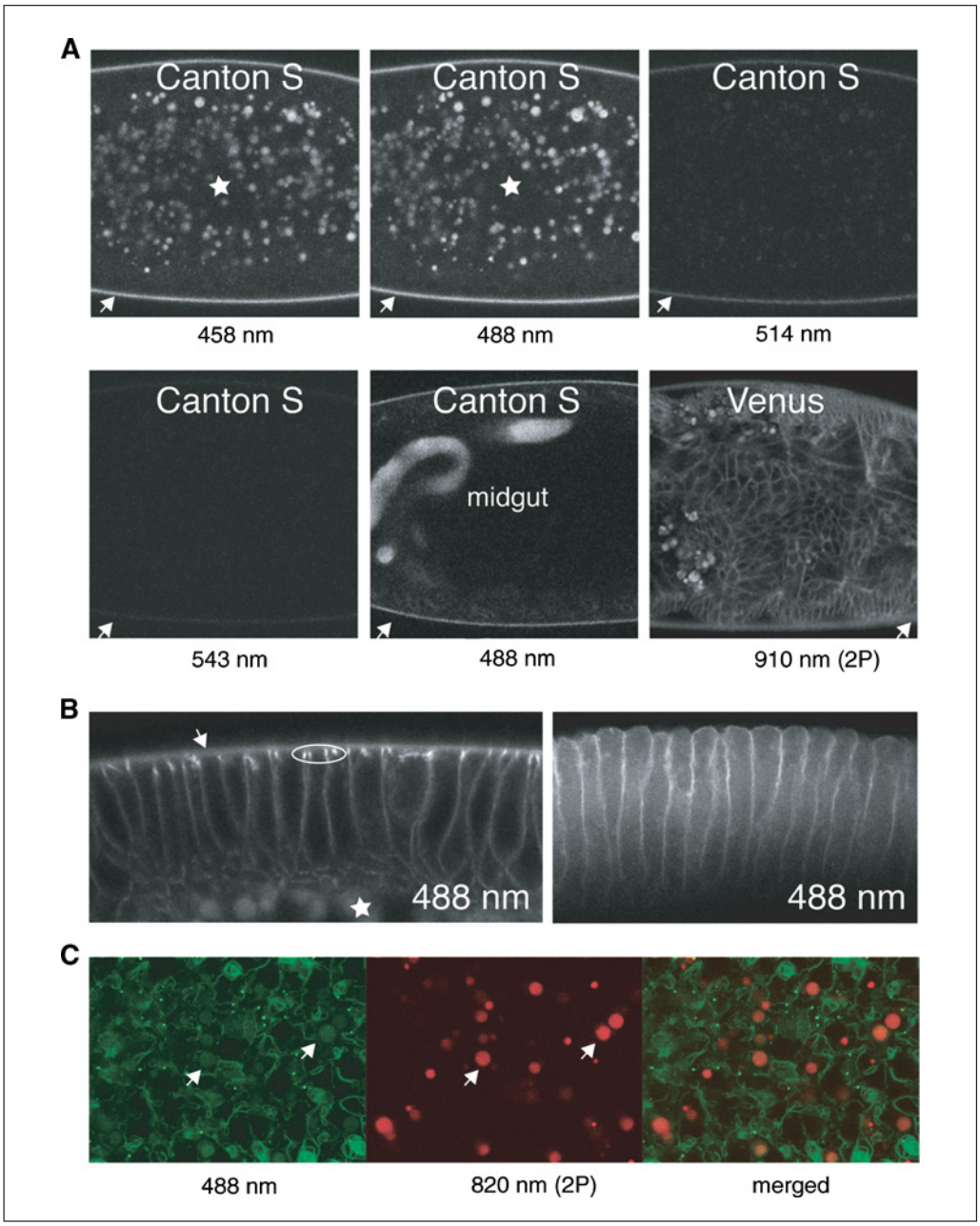


Figure 4.18.5 Interference from yolk and vitelline membrane (VM) autofluorescence depends on the depth of the focal plane, the brightness of the fluorophores, and the stage of development. (**A**) Confocal images of sagittal sections in wild-type (Canton S) embryos and a Venus-expressing embryo at wavelengths routinely used to excite common fluorophores. VM autofluorescence (arrows) is pronounced in all sagittal sections, whereas yolk autofluorescence (star) is confined to yolk granules in early embryos and the midgut in late embryos (458 and 488 nm). (**B**, left panel) Confocal image of an embryo expressing E-cadherin-GFP during early gastrulation. VM autofluorescence (arrow) interferes with green fluorescence (white outlined region) adjacent to the plasma membrane. Yolk autofluorescence does not interfere since yolk (star) is displaced below cells at this stage. (**B**, right panel) Confocal image of an embryo expressing a brighter plasma membrane fluorescent protein during the same stage. Because of the stronger signal, lower excitation levels are used, and VM and yolk autofluorescence are not observed. (**C**) Confocal image of an embryo expressing a GFP-tagged ER lumenal protein. Excitation at 488 nm (green) excites both GFP and yolk autofluorescence (green, arrows). Yolk autofluorescence (red, arrows) can be distinguished from green fluorescence by two-photon (2P) excitation at 820 nm (also see Fig. 4.18.4). For the color version of this figure go to *http://www.currentprotocols.com*.

yolk and VM autofluorescence from fluorescence is to use two-photon excitation at 820 nm. As shown in Figure 4.18.5C, a detection window of 430 to 470 nm will detect only autofluorescence (Fig. 4.18.4A and Fig. 4.18.5C, 820 nm). This is an easy way to distinguish autofluorescence from true signal, but it cannot be used to quantitatively subtract autofluorescence because the intensities of yolk/VM excited with one- versus two-photon excitation are not comparable. Alternatively, 488 nm can be used to excite both yolk autofluorescence and green fluorescence, and a linear unmixing procedure can be applied to separate the signals, based on the different emission spectra of yolk and EGFP at 488 nm (Fig. 4.18.4A).

Yolk and VM autofluorescence are particularly useful in photoactivation experiments, when the photoactivatable protein is the only protein expressed in the embryo and when it does not fluoresce at the scanning wavelength (e.g., in PA-GFP expressing embryos; see Basic Protocol 7). In these embryos, autofluorescence can be used as a guide for locating the focal plane of interest for photoactivation, since yolk and VM will be the only visible structures prior to photoactivation (see Basic Protocol 7).

Geometry

Optical sectioning in CLSM is either perpendicular (*xy*) or parallel (*xz* or *yz*) to the optical axis (*z*). Mammalian tissue culture cells grown in standard medium form thin, flat monolayers (~5 to 10 μm thick, with epithelial cells up to ~20 μm thick), and the architecture of a cell region can easily be deduced from acquired optical sections. Optical sectioning in the *Drosophila* embryo or any other living multicellular tissue presents a challenge for correlating the acquired images with the precise architecture of the corresponding region.

This complication is illustrated in Figure 4.18.6, in *xy* optical sections taken in a cellularizing embryo. Cells are organized in a predominantly hexagonal pattern, as is the case in most epithelia in the multicellular embryo (Gibson et al., 2006). Surface cross-sections (Fig. 4.18.6A) reveal the characteristic honeycomb pattern of this organization, with *xy* (the imaging focal plane) parallel to the plasma membrane closest to the coverglass, and *z* (the optical axis) parallel to the invaginating plasma membrane during cellularization. This hexagonal pattern is much less obvious as one focuses deeper, in parasagittal cross-sections (Fig. 4.18.6B). Even though cells appear to be adjacent to one another, their precise spacing and organization in the focal plane depends on the orientation of the hexagonal pattern (Fig. 4.18.6B, left versus right panel), exact position of the focal plane (Fig. 4.18.6B, focal plane α versus β, γ, δ), distribution of polygonal cell types (a mixture of pentagons, hexagons, heptagons, etc.; Gibson et al., 2006), and stage of development (e.g., cell intercalation during germband elongation in gastrulating embryos gives rise to a highly heterogeneous organization at a given time point).

Understanding the geometry of the ROI is extremely important for appropriately selecting and optimizing the image acquisition parameters (e.g., pinhole opening and interval for *z*-sectioning; see Basic Protocols 3 to 6), as well as for quantitative measurements. The use of image processing software packages with the capability of reconstructing and rendering from 3D and 4D datasets is extremely useful, both for better visualization and understanding of the biological processes observed and for quantitation. Examples of image analysis and processing software packages routinely used are ImageJ (free from the National Institutes of Health; download at *http://rsb.info.nih.gov/ij*), Volocity (Improvision), Imaris (Bitplane), and MetaMorph (Molecular Devices).

When mounted embryos are not strictly parallel or perpendicular to the coverglass, optical sections can be more complicated to interpret. Therefore, take care to mount embryos as parallel or as perpendicular as possible to the surface of the coverglass (see Basic Protocol 2).

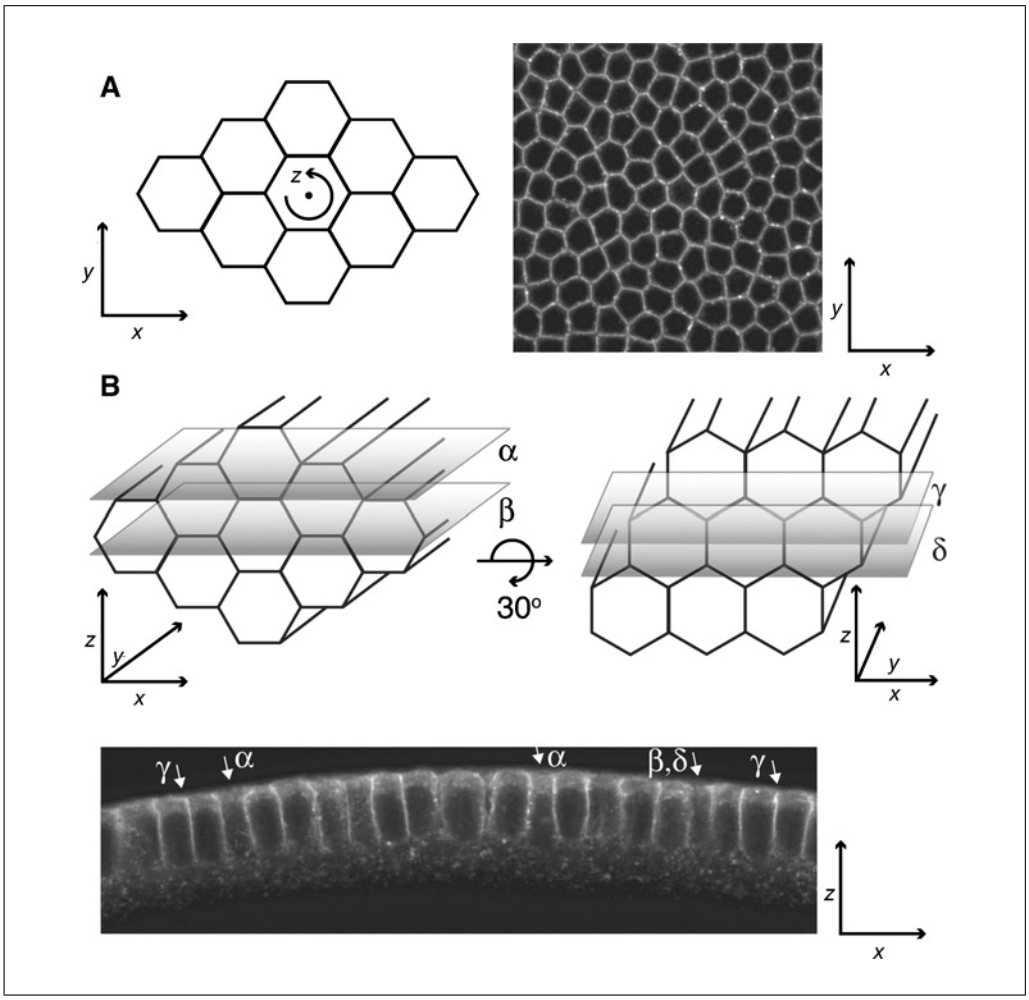


Figure 4.18.6 Tissue geometry and image acquisition. (**A**) Surface optical sections in a cellularizing embryo reveal the characteristic honeycomb pattern of the forming epithelial cells. The imaging focal plane (*xy*) is parallel to the PM closest to the coverglass, and the optical axis (*z*) is parallel to the invaginating plasma membrane. (**B**) The hexagonal pattern of the forming cells is less obvious in focal planes deeper in the embryo. Even though cells appear to be adjacent to one another, their precise architecture in the focal plane depends on the orientation of the hexagonal pattern (left versus right panel) and the exact position of the focal plane (focal plane α versus β, γ, δ), as well as the distribution of polygonal cell types (see mixture of pentagons and hexagons in A, right panel).

Movement

Large-scale movement within the developing embryo is a major problem in time-lapse imaging and photobleaching experiments. Periodic yolk contractions and mitotic waves in the early embryo or migration and rearrangement of whole epithelia in a gastrulating embryo are examples of processes that contribute to movement. These movements often result in a vertical or lateral shift of the ROI altogether. This poses a problem not only in general image acquisition; it is particularly significant in quantitative photobleaching experiments. Movement of the tissue during a FRAP protocol or movement of the bleach ROI in a FLIP protocol will compromise quantitation, and the acquired datasets will be unusable. Being aware of the speed and duration of such movements (see *http://flymove.uni-muenster.de*) will help the user to decide a good time frame for the designed experiment with minimal interference from movements. The speed of image acquisition is ultimately the limiting factor for temporal resolution and should be chosen and optimized depending on the application and question of interest.

Lowering the temperature to 18°C can be very useful to slow down overall development (see below). If temperature-sensitive measurements are made (e.g., diffusion coefficients)

Table 4.18.1 The Effect of Temperature on *Drosophila* Embryo Cellularization

Temperature (°C)	Time to complete cellularization (min)
18	67 ± 6
24	53 ± 7
28	39 ±2
32	40 ± 4

or temperature-sensitive processes are observed, control experiments at physiological temperature should be performed.

Temperature

Drosophila is an ectothermic (cold-blooded) organism and thus the speed of development is dependent on temperature. As an example, measurements of the time required for completion of cellularization at different temperatures show that there is an increase in the speed of this process with increasing temperature (see Table 4.18.1). Cellularization in the embryo is severely affected at temperatures above 34°C. The permissive or physiological temperatures for various stages of development can be different and require experimental standardization. Lowering the temperature is a useful manipulation for slowing down development, especially in the study of fast processes such as calcium transients from the endoplasmic reticulum (Parry et al., 2005).

In addition, genetic and biochemical analysis of various biological processes has been greatly facilitated by the use of temperature-sensitive mutants, which have been isolated in various mutagenesis screens in *Drosophila* (Suzuki et al., 1967). These temperature-sensitive mutants have also proven useful for blocking processes by increasing the temperature at the desired time point during development. Thus, a combination of temperature control at desired time points during imaging may be useful for the study of various mutants and processes.

All microscopes may be equipped with a temperature controlled stage, maintained at the desired temperature with the help of circulating water from a water bath. Alternatively, microscopes may be equipped with a closed controlled environment chamber around the stage, which isolates the sample from the outside world, allowing for temperature control and perfusion of desired liquids.

GENERATION OF TRANSGENIC *DROSOPHILA* FOR LIVE FLUORESCENCE MICROSCOPY USING THE Gal4/UAS SYSTEM

BASIC PROTOCOL 1

This protocol describes how to generate transgenic *Drosophila* lines to enable single- or multifluorophore imaging in living tissue. To express fluorescently tagged proteins of interest in a spatially and temporally controlled manner, we specifically employ the Gal4/UAS system. This system has been extensively used for directed gene expression in *Drosophila* (Brand and Perrimon, 1993). Briefly, Gal4 is a yeast transcription factor that was shown to activate transcription in *Drosophila* (Fischer et al., 1988). *Drosophila* lines expressing the Gal4 protein are crossed to lines carrying the target gene of interest, which is genetically fused to a fluorescent protein (FP) variant and contains Gal4-binding sites (the UAS, or upstream activation sequence) within its promoter. The tagged gene of interest will be transcribed only in those cells and tissues where Gal4 is expressed. The effect of directed misexpression of any cloned gene on development can be then studied in living *Drosophila* tissue by fluorescence microscopy.

Materials

Gene of interest cDNA: e.g., from *Drosophila* Genomics Research Center (DGRC), *http://dgrc.cgb.indiana.edu*

Vector carrying genetically encoded fluorescent protein (FP) variant (e.g., mCherry, Clontech; EGFP and Cerulean, Addgene)

pUASP or pUAST vector for subcloning FP-tagged gene of interest and for transforming *Drosophila* (DGRC)

Drosophila Gal4 line for driving expression in tissue of interest (see FlyBase, *http://flystocks.bio.indiana.edu/Browse/misc-browse/gal4.htm*)

Drosophila balancer stock for generating flies for dual fluorescent imaging: Sp/SM6a; MKRS, Sb/TM6, Tb (see FlyBase, *http://flystocks.bio.indiana.edu/Browse/balancers/bala ncers.htm*)

Stereomicroscope with epifluorescence illumination (e.g., Zeiss Stemi SV11 or Leica MZ FLIII, with 1.0 or 1.6 objectives and 10 eyepiece)

Additional reagents and equipment for cloning genes (see *APPENDIX 3A*)

Generate fluorescently tagged construct of gene of interest

1. Fuse the gene of interest cDNA to the desired fluorescent tag.

   We suggest the use of the latest generation of FP spectral variants, which have been improved for folding, chromophore maturation, brightness, photostability, and minimal self-association (see UNIT 21.5). Cerulean (Rizzo et al., 2004), Venus (Nagai et al., 2002), and mCherry (Shaner et al., 2004) are optimized versions of previously used ECFP, EYFP, and DsRed, respectively. The monomerizing mutation A206K (Zacharias et al., 2002) can be applied to virtually any Aequorea victoria variant in order to generate a true monomer (m; e.g., mEGFP, mCerulean, mVenus). PA-GFP and mEosFP are currently the photoactivatable variants with the most potential for optical pulse-chase experiments in living Drosophila (see Basic Protocol 7). Choose the fluorescent variant or combination of variants based on the intended application requirements (see Basic Protocols 3 to 6).

   Most FPs have some acid sensitivity, and their fluorescence is quenched when targeted to acidic compartments such as the lumen of lysosomes or secretory granules. Avoid using acid-sensitive FPs (pK_a>6.0; e.g., mOrange, GFPs, and YFPs) when the proteins are targeted to compartments of low or unstable pH. Instead, use FPs with excellent pH resistance (pK_a<5.0; e.g., CopGFP, Cerulean, mKO, mRFP1, or mCherry) to avoid artifacts in quantitative measurements caused by acid quenching.

2. Subclone the FP-tagged gene into the pUASP or pUAST vector.

   The pUAST vector contains five Gal4/UAS sites and a minimal hsp70 promoter upstream of the cloning site for the gene of interest (Brand and Perrimon, 1993). The original pUAST vector was ineffective for expression of genes in the female germline during oogenesis. A modified vector (pUASP) was made (Rorth, 1998), which contains fourteen Gal4/UAS sites, a minimal transposase promoter upstream of the cloning site for the gene of interest, as well as the 3'UTR from a maternally expressed gene.

   If you are studying processes during oogenesis or very early embryogenesis (first 3 hr), clone the FP-tagged gene of interest into pUASP and not pUAST. Expression of the UASP-FP-tagged protein will occur in the female germline and the FP will be loaded into very early embryos when present in trans with the appropriate Gal4 driver.

   pUASP and pUAST are ampicillin-resistant vectors available at DGRC, together with information for cloning (http://dgrc.cgb.indiana.edu/vectors/store/vectors.html). Alternatively, genes of interest may be directly cloned in frame with fluorescent tags already encoded in the pUASP vector, also available at DGRC (http://dgrc.cgb.indiana.edu/vectors/store/vectors.html).

   We suggest that the expression of the FP-tagged proteins be verified either in mammalian cells or in Drosophila S2 cells. For S2 cells it will be necessary to cotransfect pActGal4 and pUASP-FP to drive the expression of the fluorescent protein.

Generate transgenic Drosophila carrying the UAS-FP-tagged transgene

3. Generate transgenic fly lines by injecting the UAS-FP-transgene vector into embryos and selecting transformed flies that carry the transgenes on different chromosomes.

   Detailed protocols for these steps are out of the scope of this chapter. Excellent protocols for these steps are available in other works (Santamaria and Gans, 1980; Rubin and Spradling, 1983; Kiehart et al., 2000).

   Alternatively, there are various academic institutions or companies (e.g., BestGene, Genetic Services) which perform embryo injections to generate transgenic fly lines. The fly lines obtained should be mapped to identify the chromosome in which the transgene is inserted.

   Both pUASP and pUAST contain a mini white gene. This is very useful for identifying the presence of the transgene in transformed progeny, if the plasmid is injected into embryos of white-eyed flies. Depending upon where the transgene is inserted into the fly genome, the eye color of the transgenic fly can range from light orange to red.

Carry out genetic crosses to express fluorescently tagged proteins of interest

4. Perform appropriate genetic crosses to bring the enhancer Gal4 and the pUASP constructs together for expression in the progeny (see Fig. 4.18.7).

   A scheme of genetic crosses is detailed in Figure 4.18.7 for the generation of embryos expressing a single (Fig. 4.18.7A) or multiple (Fig. 4.18.7B) FP-tagged proteins. Balancer chromosomes carry genetic markers that enable the selection of flies carrying both the nanos-Gal4 and pUASP-FP(s) in the F_1 generation. Alternatively, flies carrying both the nanos-Gal4 and pUASP-FP(s) may be selected by observing females under a stereomicroscope with epifluorescence illumination.

   Examples of adult females containing nanos-Gal4; pUASP-tubulin-GFP and maternal-α4-tubulin-Gal4; pUASP-mRFP are shown in Figure 4.18.7C.

   To express Gal4 in a tissue-specific manner, an enhancerless Gal4 was inserted randomly into the Drosophila genome (Brand and Perrimon, 1993). Depending on its site of integration, hundreds of lines were generated expressing Gal4 in distinct spatiotemporal patterns, each reflecting the native activity of the trapped enhancer. This has given the Drosophila community several enhancer-Gal4s that allow tissue-specific expression of Gal4. A

Figure 4.18.7 *(on next page)* Generation of flies expressing the fluorescent transgene of interest. (**A**) Example of genetic crosses showing the generation of flies expressing a single fluorescent transgene in early embryos. In the F_0 generation, flies carrying the Gal4 driver are crossed to those carrying the fluorescent transgene (pUASP-FP). F_1 females that carry both the nanos-Gal4 and the pUASP-FP are selected based on genetic markers or by observing females under a stereomicroscope with epifluorescence illumination. (These females express the protein of interest in the germline and thus will load the protein in early F_2 embryos.) pUASP-FP; nanos-Gal4 females may be crossed with sibling males of the same genotype or any other males of choice depending upon the genotype of the embryos that need to be imaged. (**B**) Example of genetic crosses showing the generation of flies expressing two fluorescent transgenes in early embryos. In the F_0 generation, flies carrying the fluorescent transgenes (pUASP-FP1 on the second chromosome and pUASP-FP2 on the third chromosome) are crossed to those carrying balancer chromosomes. Balancer chromosomes enable the selection of flies carrying both the pUASP-FP1 and pUASP-FP2 in the F_1 generation. F_2 generation flies containing the two transgenes are crossed to those carrying nanos-Gal4. In the F_3 generation, female flies carrying both pUASP transgenes and the Gal4 driver will express FP_1 and FP_2 during oogenesis, and the embryos produced by these flies are used for imaging. The females carrying the two transgenes and the Gal4 driver may be crossed with sibling males of the same genotype or any other males of choice depending upon the genotype of the embryos that need to be imaged. (**C**) Selection of adult flies expressing the fluorescent protein of interest in the female germline viewed using a stereomicroscope with epifluorescence illumination. Note the green fluorescent signal in the abdomen of flies expressing tubulin-GFP and the red fluorescent signal in flies expressing mRFP1. Control nanos-Gal4 flies show no fluorescent signal using the same green and red emission filters. The flies expressing the fluorescent transgenes may be selected and used for embryo collection. For the color version of this figure go to *http://www.currentprotocols.com*.

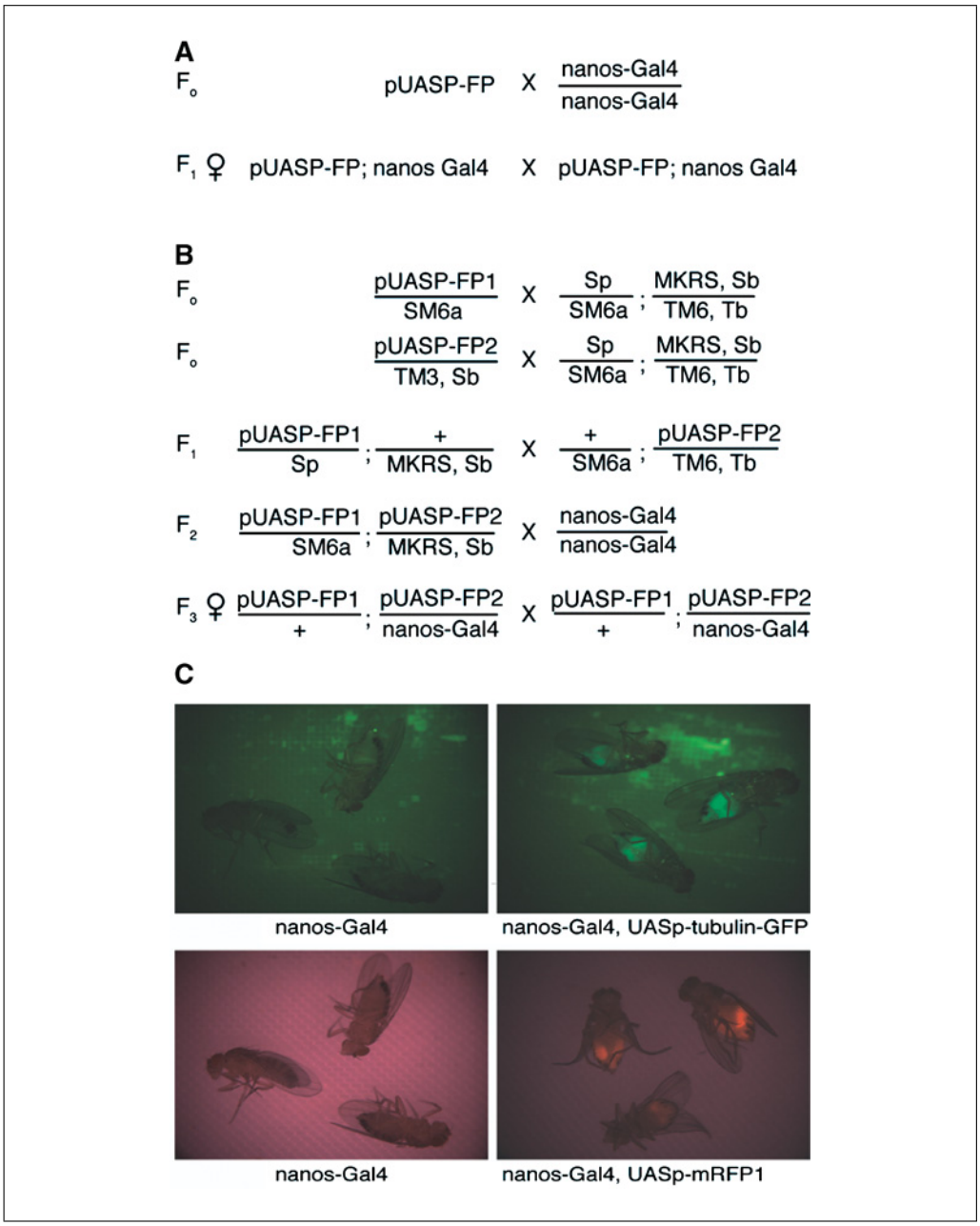


Figure 4.18.7 *(Legend on previous page)*

catalog of Gal4 lines is available from FlyBase at http://flystocks.bio.indiana.edu/Browse/misc-browse/gal4.htm.

Alternatively, when a heat-shock inducible hsp70-Gal4 is used, a temporal control of Gal4-driven expression during development is possible. In this case the target gene will be expressed in all tissues.

The enhancer Gal4 lines commonly used for expression of target genes during oogenesis are maternal-α4-tubulin-Gal4 and nanos-Gal4. If maternal-α4-tubulin-Gal4 or nanos-Gal4 females are crossed to males carrying UAST/UASP-transgenes, expression of fluorescently tagged proteins in F_1 embryos is not observed until early- to mid-gastrulation.

For imaging fluorescently tagged proteins in very early embryos, cross maternal-α4-tubulin-Gal4 or nanos-Gal4 lines to UASP-FP lines (Hacker and Perrimon, 1998; Van Doren et al., 1998). Emerging F_1 females will express the proteins of interest in the germline and thus will load these proteins in very early F_2 embryos.

It should be noted that nanos-Gal4 often appears to have variegated expression resulting in differential reporter/target transcript amounts in the ovaries and embryo.

PREPARATION OF *DROSOPHILA* EMBRYOS FOR FLUORESCENCE MICROSCOPY

Most protocols for preparing *Drosophila* embryos for live imaging involve dechorionation and mounting of embryos on a coverglass covered with halocarbon oil to prevent the embryos from drying out (Hazelrigg, 2000). Here, we present a protocol where embryos are mounted in phosphate-buffered saline or distilled water in a chambered coverglass. This protocol presents the following advantages: (1) embryos do not suffer from anoxia during long-term imaging (which is the case when embryos are covered with halocarbon oil), (2) it allows for quick mounting and screening of many embryos, and (3) water-immersion objectives can be used for imaging deeper into the embryo with minimal spherical aberrations (see Strategic Planning). Embryos mounted in this manner develop normally to first-instar larvae. Embryos may be mounted either on their posterior or on their dorsolateral/ventrolateral side depending upon the nature of the experiment.

Materials

Embryo collection cages that fit 60-mm petri dishes (Genesee Scientific)
Apple or grape juice agar plates (see recipe)
Yeast paste: prepared by mixing 1 g dry yeast with 1.8 ml distilled H_2O to make a thick paste
50% bleach: bleach (4% to 6% available chlorine) diluted 1:1 with distilled H_2O just prior to use
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Dichlorodimethylsilane (Fluka)
Egg basket (Fig. 4.18.3B): prepared by removing the stem of a plastic funnel (~60 mm top internal diameter) with a blade, slightly melting the small diameter opening, and attaching to a piece of 100-μm stainless steel wire or 100- or 120-μm nylon mesh (Genesee Scientific 57-102 *or* BD Falcon 352360)
Squirt bottle with distilled H_2O
Dissecting microscope (e.g., Zeiss Stemi SV6/11 or Stemi 2000)
Fine paintbrush (No. 1) for transferring and handling embryos
Imaging chamber: No. 1.0 Borosilicate LabTek Chambered Coverglass (Nunc) *or* No. 1.0 Borosilicate MatTek Glass-Bottom Dishes (MatTek)

Collect embryos

1. Take a freshly prepared apple juice agar plate and add a dab of thick yeast paste in the center of the plate.
2. Place 50 to 100, 2- to 3-day-old adult flies expressing the fluorescently tagged protein of interest in a cage and cover with a yeasted apple juice agar plate (Fig. 4.18.3A). Maintain flies in an incubator at 25°C.

   Collection plates must be at room temperature before use. Females will not lay eggs on cold plates!

3. Replace with a fresh apple juice agar plate at the same time every day for 2 to 3 days to synchronize egg laying.

   Change plates daily and monitor humidity. If agar plates are drying out, place a paper towel in a beaker containing water and put it close to the cage.

4. On the day of the imaging experiment, replace the fresh juice agar plate 1 hr before the actual egg collection. Incubate the cage at 25°C for 1 hr and discard this "prelay" plate.

   Females can retain fertilized eggs in the uterus for variable periods of time, so the prelay plate contains embryos at different stages.

5. Place a fresh apple juice agar plate and incubate for the appropriate time to obtain embryos at the desired stage of development prior to imaging.

   The same population of flies may be used for embryo collection in cages for ~10 days if apple juice agar plates are changed daily.

Dechorionate embryos

6. Add some distilled water in the apple juice agar plate and brush the embryos gently into suspension. Pour the water containing the embryos into the egg basket (Fig. 4.18.3B). Wash with copious amounts of distilled water, using a tap or squirt bottle, to get rid of the yeast paste.

7. Place the funnel containing the embryos in freshly prepared 50% bleach and agitate gently so the bleach solution disperses the embryos. Incubate 1 min with periodic gentle agitation.

   This treatment will remove the chorion, which makes the embryo opaque for imaging (Fig. 4.18.3B).

   Always prepare the 50% bleach solution fresh. Bleach loses its potency after dilution and with aging.

   When embryos are dechorionated, their hydrophobic vitelline membranes tend to stick to the sides of the funnel, and embryos also clump together. If dorsal appendages are still observed under the dissecting microscope just prior to mounting (see Fig. 4.18.3B), place embryos back in bleach solution for 5 to 10 sec, rinse thoroughly with distilled water, and check again. Do not overexpose embryos to bleach, or they will be damaged.

8. Immediately wash the embryos thoroughly with distilled water, using a tap or squirt bottle, to get rid of any residual bleach. Squirt distilled water on the inner sides of the funnel to bring embryos that stick to the sides onto the mesh.

Mount embryos on their dorsolateral/ventrolateral sides

9a. Blot the mesh containing the embryos on filter paper. Working rapidly under a dissection microscope, use a fine paintbrush to pick the dechorionated embryos from the mesh and mount them on a chambered coverglass on their dorsolateral/ventrolateral sides (Fig. 4.18.3C). Work rapidly to mount embryos so that they are not crowded and so that they do not touch each other, to avoid anoxia and defects in development.

10a. Slowly add PBS with a pipet from the side of the chamber in order to avoid air bubbles. Add enough PBS to cover the embryos and to compensate for evaporation during imaging (e.g., 2 ml for a two-chambered LabTek coverglass). If air bubbles form while mounting, use the paintbrush to gently move the embryo around to remove the air bubbles. Do not dislodge embryos from the coverglass, or they will float.

   Air bubbles will effectively block the light path and are deleterious for imaging—they appear as dark spheres or shadows. If the embryos are not dry enough, air bubbles will appear on the sides of the embryos upon addition of PBS.

   Distilled water may be used as an alternative to PBS. This is especially useful when imaging with an upright microscope and a water dipping objective (e.g., a Zeiss Plan-Apochromat 63×/1.0 VIS-IR water lens).

   Microscope objectives for high-resolution fluorescence microscopy have a correction collar to compensate for coverglass thicknesses between 0.14 and 0.18 mm. We recommend the use of No. 1.0 (0.13 to 0.16 mm) LabTek or MatTek chambered coverglasses. The use of thicker No. 1.5 (0.16 to 0.18 mm) coverglasses is not recommended because it will compromise working distance. The coverglass thickness can easily be measured on a laser scanning microscope, and the correction collar can be set to a high precision.

Mount embryos on their posterior sides

9b. To mount embryos on their posterior, coat the coverglass with dichlorodimethylsilane to render the glass surface hydrophobic. Add silane to cover the coverglass, let sit for 3 hr, then remove and wash thoroughly with distilled water.

Silane vapors are extremely volatile and toxic and therefore addition of silane to the chamber must be done in a fume hood. Silanized coverglasses are good to use for a week.

10b. Mount dechorionated embryos on the coverglass on their dorsolateral/ventrolateral sides as described in step 9a and add PBS. With the help of the paintbrush slowly move the embryos to an upright position so that they sit on their posterior side (Fig. 4.18.3C).

The hydrophobic vitelline membrane interacts with the hydrophobic silanized surface of the coverglass and prevents the embryo from floating during this manipulation.

The embryos must be mounted on the posterior side and not the anterior side because the micropyle (see arrow in Fig. 4.18.3C) present on the anterior side is very sensitive to manipulations.

TIME-LAPSE CONFOCAL IMAGING OF LIVING *DROSOPHILA* EMBRYOS

BASIC PROTOCOL 3

Time-lapse observation of developing embryos expressing fluorescent proteins (FPs) targeted to specific tissues and/or subcellular compartments can yield important information about the dynamics of cell rearrangements and/or changes in the spatial distribution of different proteins within single cells in the course of development. Moreover, it enables the investigator to simultaneously track multiple cellular events. This protocol focuses on noninvasive, time-lapse, confocal microscopic observation of fluorescently tagged proteins in living *Drosophila* embryos. It is assumed that the investigator is familiar with the basic operation of a confocal microscope. However, guidelines are given throughout the protocol to help in the determination of the optimal imaging conditions for living embryos expressing one or more FPs.

Materials

Drosophila embryos expressing fluorescent proteins (FPs) of interest (Basic Protocol 1), mounted in LabTek or MatTek imaging chamber (Basic Protocol 2)
Confocal laser scanning microscope (CLSM; e.g., Zeiss LSM 510 or Olympus FluoView FV1000) equipped with:
Laser lines and filter sets appropriate for the FPs of interest
High-numerical-aperture (NA), water-immersion objective (e.g., Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens or Olympus UPLAPO 60×/1.2 NA water lens)

Set up the imaging system

1. Set up the confocal laser scanning microscope and its associated hardware. Use a high-NA objective for maximal signal collection and spatial resolution.

The brightness (fluorescence intensity) of the acquired image is proportional to NA^4/$magnification^2$. This means that a 40× objective is superior to higher-magnification objectives (60× or 100×) of identical NA values because it will yield the brightest images with the same resolving power. A 40× lens yields a field of view of 230 μm × 230 μm at zoom 1.0 on a Zeiss LSM 510, or 318 μm × 318 μm at zoom 1.0 on an Olympus FluoView FV1000.

We recommend the use of optical zoom during image acquisition with a 40× lens to yield images equivalent in size to using a 100× lens without compromising brightness or resolution. Higher magnification (60× or 100×) is required for imaging very small structures (e.g., transport intermediates and secretory granules).

If fluorescence is very weak (e.g., embryos with low expression levels of the FP), consider using less corrected high-NA oil lenses (e.g., a Fluar 40×/1.3 NA oil lens), which have fewer optical elements and higher light transmission. Use oil lenses only for imaging at the embryo surface closest to the coverslip to minimize spherical aberrations.

2. Turn on the appropriate excitation laser lines at least 15 min before starting image acquisition.

   This step will minimize power fluctuations that will compromise fluorescence intensity measurements during subsequent time-lapse imaging.

3. Configure the light path for optimal excitation and emission detection of the fluorophores expressed in the embryo.

   We recommend that the investigator closely examine the spectral profiles of the FPs of interest to ensure an optimal excitation and emission filter combination.

   Most software packages provide the user with a list of preset light path configurations for combinations of common fluorophores. However, the investigator should determine if the preset configuration is indeed optimal for the specific FP(s) of interest and modify the configuration, if needed. UNIT 21.5 provides an excellent overview of the latest generation of FPs and the optimal excitation and emission settings.

   The choice of filters is critical for achieving high signal-to-noise levels and minimizing spectral bleedthrough at the same time, and it should be based on the application. For example, if GFP is the only FP in the embryo, use a longpass filter (e.g., LP505) instead of a bandpass emission filter (e.g., BP505-530) to detect most of the GFP fluorescence.

Locate the embryos of interest

4. Place the imaging chamber containing the mounted embryos on the microscope stage.

5. Use bright-field (transmitted light) or/and epifluorescence illumination to locate the embryos of interest, and focus on the vitelline membrane, with the following considerations:

   Make sure the area of interest is within the working distance of the objective lens!

   If the area of interest is >60 μm deep in the embryo, consider using two-photon excitation with nondescanned detectors (see Fig. 4.18.2) in combination with a long-working-distance, high-NA water lens (e.g. a Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens).

   Take care to avoid photobleaching the fluorescence in the embryo.

   If all embryos express the fluorophores of interest, do not use epifluorescence illumination to locate the embryos. Instead, use bright field illumination to locate the stages of development desired. If only a population of embryos is fluorescent or contains all fluorophores of interest, then use epifluorescence; minimize exposure to get the embryo in focus and centered in the field of view, and then quickly shutter the light. Use neutral density filters to attenuate illumination during focus and centering to preserve the fluorescence in the embryo.

6. Visually inspect the embryos to select normally developing embryos that have not been damaged during dechorionation. Avoid using embryos that have associated air bubbles or that are not completely dechorionated.

   Damaged embryos exude cytoplasm at the micropyle.

   Air bubbles effectively block light transmission and appear as dark areas in an otherwise fluorescent region, whereas pieces of chorion appear as bright autofluorescent structures. As a result, both air bubbles and pieces of chorion will compromise quantitative measurements of fluorescence if they are located in regions of interest.

7. Before starting the laser scanning, set the laser power down, the detector gain up, choose a fast scan time (typically 1 sec per scan), zoom 1.0, and adjust the pinhole to 1 to 2 Airy units.

   For imaging GFP-expressing embryos on a Zeiss LSM 510, start with 1.0% transmission of laser light (for the 488-nm line of a 25-mW argon laser with a tube current of 6A), PMT gain 800, 1 sec per scan, and a pinhole of 1.5 Airy units (1.3 μm optical slice for the Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens).

8. Start fast laser scanning, bring the embryo to the desired focal plane, and move the region of interest (ROI) to the center of the field of view. Take a single scan.

   Most software packages have a low-resolution or/and rapid-scanning mode (Fast XY on Zeiss software or Focus×2 and Focus×4 on Olympus software) that makes preliminary scanning more efficient in rapidly selecting the focal plane and ROI with minimal exposure to laser light.

Set parameters and acquire images for single fluorophore imaging

9a. Determine the optimal instrument parameters for image acquisition in embryos expressing one FP. Optimize the parameters of percent transmission of excitation light, pinhole size, detector gain and offset, scan speed, averaging, zoom factor, and frame size based on the application requirements:

Use the lowest percent transmission of excitation light that will give a good signal-to-noise ratio but will also minimize photobleaching of the fluorophore and will not damage the embryo.

The optimal percent transmission of excitation light will depend on the laser output used (e.g., 40-mW and 25-mW lasers versus 1-mW or 5-mW lasers), the expression levels, brightness and photostability of the FPs, as well as the physical characteristics of the sample (e.g., imaging deeper in the embryo will require an increase in excitation light to compensate for loss of light due to scattering and out-of-focus absorption from surrounding tissue).

Adjust the pinhole size based on the experimental requirements.

Opening the pinhole to 1.5 to 2 Airy units (1.3-μm to 1.7-μm optical slice for the Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens) will produce a thicker optical section compared to a pinhole of 1 Airy unit (0.9-μm optical slice for the same lens) and slightly reduce resolution, but it will significantly improve signal intensity and yield more specimen detail. This is particularly useful for dim samples.

Adjust the gain and black level (offset) of the PMT detector to take advantage of the full dynamic range of the detector. Scan the specimen, adjust the gain so that the brightest pixel in the image is slightly below white (gray level = 255), and adjust the black level so that the dimmest pixel is slightly above black (gray level = 0) in an 8-bit system.

This will ensure that all signals fall within the dynamic range of the PMT, and it is essential for quantitative measurements. All microscope software packages include a pseudocolor image display mode (also known as lookup tables, LUT) that facilitates adjustment of the dynamic range.

Take longer exposures to improve the signal-to-noise ratio, either by using longer scan times or/and averaging the signal from multiple scans (line by line or frame by frame).

We suggest the use of line averaging over frame averaging (which averages full frames), as it yields sharper images because there is less blurring due to movements in the embryo or slight changes in the focal plane. For dim specimens, summing the signal from multiple scans (line by line) will enable better signal detection.

Use a pixel size small enough to take advantage of the resolving capability of the lens but large enough to avoid oversampling.

The number of pixels and zoom control provide the capability to match the pixel size to the optical characteristics. A general guideline for selecting an appropriate zoom factor, based on the Nyquist Sampling Theorem, states that pixel size should be equal to the optical resolution divided by 2.3 (Webb and Dorey, 1995).

For example, for the Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens, lateral resolution is 0.17 μm and the optimal pixel size would be 0.07 μm (which is met, e.g., with a 1024 × 1024 pixel frame and a zoom 3.0).

Making the pixel size too small will subject the embryo to more irradiation than necessary, with increased photobleaching. Because signal-to-noise is often more critical than optical resolution, in most cases sampling with a pixel size half the resolution is most useful without noticeable image degradation.

Determine the optimal zoom factor in order to visualize the structures of interest.

The rate of photobleaching is proportional to the square of the zoom factor. Thus, zooming in will increase the risk for photobleaching the fluorophores.

The best trade-off between signal intensity and resolution will depend on the characteristics of the sample and aims of the experiments.

10a. Once the appropriate instrument parameters have been determined, acquire time-lapse images:

Carefully monitor the first few images for correct detector gain and black level (offset) and adjust accordingly in order to work in the dynamic range of the detector.

If the image is saturated, try turning down the detector gain or lower the excitation light levels and restart the time-lapse sequence. To identify unwanted photobleaching of the fluorophore, measure the loss of fluorescence during acquisition of the time series. To minimize photobleaching the fluorophore(s), turn down the excitation levels, reduce light exposure by decreasing the scan speed and/or signal averaging, or zoom out (photobleaching is proportional to the square of the zoom factor).

Improve temporal resolution by reducing the number of pixels per frame, zooming in to image a smaller area, increasing scan speed, or reducing signal averaging.

You need to find a trade-off between the desired spatial resolution, the signal-to-noise ratio, and the speed of image acquisition based on the experimental requirements. Most commercial confocal microscopes enable the investigator to scan only a selected ROI or multiple ROIs without having to zoom in, thus preserving spatial resolution while minimizing exposure of the rest of the sample to illumination.

For acquiring >10 frames per sec, specialized systems with fast scanning capabilities and/or a very sensitive CCD camera for detection are necessary. Examples of such commercial systems are the Zeiss LSM 5 LIVE (up to 120 frames per sec at 512 × 512 pixels) and the spinning-disk confocal PerkinElmer UltraviewRS system (15 frames per sec at 672 × 512 pixels).

The Zeiss CLSM software offers an advanced time series macro called Multitime for imaging multiple samples in the same chamber. This enables the imaging of multiple embryos in tandem and at multiple stage locations defined by the user. It also includes an autofocus function, which compensates for the effects of mechanical and thermal focus drifts during long-term experiments. The user can define stage locations, focus, and time intervals for the individual acquisitions.

Set parameters and acquire images for multiple fluorophore imaging

9b. Determine the optimal instrument parameters for image acquisition in embryos expressing two or more FPs (multicolor imaging). Optimize the parameters of percent transmission of excitation light, pinhole size, detector gain and offset, scan speed,

averaging, zoom factor, and frame size based on the application requirements, with the following considerations:

Determine the capability of your microscope setup to minimize spectral bleedthrough (also referred to as cross-talk) resulting from excitation or/and emission spectral overlap exhibited by FP combinations, based on the spectral profiles of the FPs to be imaged, the excitation laser lines and emission filters available, as well as the potential use of software packages for spectral unmixing.

The primary concern when choosing fluorophores for simultaneous imaging is potential spectral bleedthrough. The most useful combinations that can be readily separated are cyan and either green or yellow (e.g., Cerulean and Venus, or Cerulean and mGFP), and red variants with either green or yellow FPs (e.g., EGFP and mCherry, or Venus and mRFP1). For triple imaging, the combination of cyan, yellow (or green), and red offers an excellent solution (e.g., Cerulean, Venus, and mCherry).

Spectral unmixing involves the collection of spectra during image acquisition in the FP-expressing embryo and a post-acquisition mathematical unmixing algorithm, and this is available in some setups (e.g., Zeiss LSM 510 META). The green and yellow combination (e.g., EGFP and Venus) is not often used together because of significant cross-talk, but spectral unmixing can be used to efficiently separate the signals.

Balance the desired parameters (fast image acquisition versus the optimal separation and signal-to-noise ratio for each fluorophore) by appropriately choosing the optimal parameters for each fluorophore (excitation levels, pinhole size, detector gain and offset, scan speed, and averaging).

The FPs vary in relative brightness and photostability (see UNIT 21.5) and may have different expression levels in the embryo, so that each color may require different image collection conditions. The rate of data acquisition slows with the addition of each color, due to the requirement for different image collection conditions.

Always use sequential line scanning (also referred to as multitrack line scanning) to rapidly switch between laser lines and collect the image for each channel one line at a time as the frame is being recorded.

This sequential scanning avoids exciting all fluorophores at the same time and is very effective in reducing or eliminating spectral bleedthrough. We do not recommend multitrack frame scanning because even small movements of subcellular structures or cells in the embryo will compromise the image and also decrease the accuracy of colocalization studies.

10b. Once the appropriate instrument parameters have been determined, acquire images of successive optical slices through the sample (*z*-sectioning) over time (*t*; 4D imaging):

Use an interfocal plane interval equal to half the optical slice thickness (which is determined by the NA of the lens and the pinhole size) to adequately sample the specimen in the *z*-axis.

For example, for the Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens and a pinhole of 1 Airy unit (which corresponds to a 0.9-μm thick optical slice), use an interval of 0.45 μm for z-sectioning. Do not collect images at shorter intervals because this will result in oversampling with an increased risk of photobleaching.

Use image analysis software (e.g., ImageJ, free software from the National Institutes of Health, downloadable at http://rsb.info.nih.gov/ij; Volocity, Improvision; Imaris, Bitplane; or MetaMorph, Molecular Devices) to analyze the acquired 4D datasets (*xyzt*).

z-stacks (the whole stack or selected slices) can be projected or rendered in three dimensions for better visualization, and quantitative measurements of volume changes and fluorescence intensity changes, as well as particle tracking analysis, are possible.

11. After a series of images or *z*-stacks has been collected, immediately save them to disk.

Develop a strategy for labeling the image files before beginning to image. This will save time between successive imaging sessions in living, developing embryos. Always save raw unprocessed datasets and make sure they contain all the information that might be required later for image processing, measurements, and presentation purposes or for reusing the same parameters (e.g., pinhole size, excitation levels, lens magnification, and zoom factor, pixel dwell time) for imaging other samples.

BASIC PROTOCOL 4

TIME-LAPSE IMAGING OF LIVING *DROSOPHILA* EMBRYOS WITH TWO-PHOTON LASER SCANNING MICROSCOPY

Confocal laser-scanning microscopy (CLSM, Basic Protocol 3) is the method of choice for routine high-resolution 4D imaging up to ~40 μm deep in living *Drosophila* tissue. This protocol focuses on the use of two-photon excitation laser scanning microscopy (2PLSM) to image fluorescently tagged proteins up to ~100 μm deep in living *Drosophila* embryos (see Strategic Planning and Fig. 4.18.2). The larger penetration depth with two-photon excitation (*UNIT 4.11*) is due to (1) the lack of out-of-focus absorption of the excitation light, (2) significantly less scattering of the longer wavelengths used in two-photon excitation, and (3) a detection geometry that enables high-efficiency collection of multiply-scattered emission photons. The lack of out-of-focus excitation further reduces photodamage and thus increases tissue viability, which is crucial for long-term imaging. Finally, two-photon excitation allows imaging and photomanipulation (e.g., photobleaching) in a highly localized fashion. The major drawback of 2PLSM is local heating effects caused by the high concentrations of near infrared (NIR) photons required for two-photon excitation. Thus, the investigator needs to carefully optimize conditions for deep imaging in order to minimize such effects.

The major differences between a two-photon and a confocal microscope setup are the excitation laser and the detection pathway (*UNIT 4.11*). Two-photon excitation requires ultrafast pulsed lasers (now commercially available) and can be coupled with a laser scanning microscope setup (e.g., Zeiss LSM 5 META NLO). However, ultrafast pulsed lasers for 2PLSM are much more expensive than the visible light lasers commonly used for CLSM. Furthermore, a deeper understanding of the physics and more technical effort are required to optimize deep imaging with 2PLSM without damaging the living tissue. As a result, 2PLSM is not yet as widely applied as CLSM, but it is the method of choice for routine high-resolution imaging ~60 to 100 μm deep in living *Drosophila* embryos. Technical details on how to optimize the laser source for 2PLSM (beam size, pulse width, etc.) are beyond the scope of this protocol and are available in specialized reviews (Dickinson, 2005; Helmchen and Denk, 2005). The investigator needs to be familiar with time-lapse confocal imaging (Basic Protocol 3) before attempting 2PLSM.

Materials

Drosophila embryos expressing fluorescent proteins (FPs) of interest (Basic Protocol 1), mounted in LabTek or MatTek imaging chamber (Basic Protocol 2)
Zeiss LSM 510 META equipped with:
Coherent Chameleon titanium/sapphire laser, tunable from 720 nm to 930 nm
Filter sets appropriate for the FPs of interest
High-numerical-aperture (NA) water-immersion objective (e.g., Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens)

Set up the imaging system

1. Set up the confocal laser scanning microscope and its associated hardware. Use long-working-distance, high-NA water-immersion objectives with high transmission to near infrared (NIR) and visible (VIS) wavelengths.

   The most widely used lasers for 2PLSM are titanium/sapphire (Ti:sapphire) lasers. Commercially available Ti:sapphire lasers provide computer-controlled wavelength tuning over a broad range (∼700 to 1000 nm) in an automated fashion. The average power (∼1 W over the central portion of the tuning range) provided by these commercial systems is sufficient for most applications.

   Most highly corrected high-NA objectives have >80% transmittance at visible wavelengths but low transmittance at NIR light. Long-working-distance lenses with a high NA are favored for deep imaging so that the beam can be focused deep into the tissue and the emission photons can be collected efficiently. Furthermore, high-NA lenses increase the two-photon excitation probability by increasing the density of the photon flux at the focal plane (the two-photon excitation probability is directly proportional to NA^4).

   For example, a Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens is very effective for 2PLSM because it has a large working distance (280 μm at a 0.17-mm coverglass thickness) and enables deep imaging with minimal spherical aberrations; it has ∼85% transmittance at 500 nm and ∼75% transmittance at 820 nm, and a high collection efficiency.

2. Turn on the Ti:sapphire laser at least 30 min before starting to image. Mode-lock the laser at the desired wavelength.

3. Configure the light path for optimal excitation and emission detection of the fluorophores expressed in the embryo.

   Configure the dichroic mirrors in the light path so as to reflect the infrared excitation wavelengths to the sample. Two-photon excitation spectra of common FP variants have been recorded (Spiess et al., 2004): optimal excitation of ECFP occurs at ∼850 nm, EGFP at ∼950 to 970 nm, and EYFP at ∼960 to 970 nm. Suboptimal excitation wavelengths are routinely used and result in the same emission profiles and emission maxima (Spiess et al., 2004). Two-photon emission spectra are identical with one-photon emission spectra of the same FP variant. Choose appropriate emission filters with an IR block (e.g., a BP500-550 IR filter for two-photon excited EGFP).

4. Make the choice between descanned and nondescanned detection based on the imaging depth and the signal brightness.

   There are two major detection modes in 2PLSM. The first one uses exactly the same optical pathway as in CLSM, i.e., the emitted light is captured (descanned) using galvanometric scanning mirrors before passing through the pinhole to the detector. The pinhole in this case is set to its maximum aperture. This is called the descanned mode.

   The other detection mode is called the nondescanned mode. In nondescanned mode, the emitted light does not pass through the galvanometric scanning mirrors, and no pinhole is used. Instead, the emitted light is collected on an external detector outside the scanning module. Hence, a nondescanned detection light path allows collection of as many scattered fluorescence photons as possible and thus increases the signal-to- noise ratio when imaging deep in living tissue (see Fig. 4.18.2).

Locate the embryos of interest

5. Place the imaging chamber containing the mounted embryos on the microscope stage.

6. Use bright-field (transmitted light) or/and epifluorescence illumination to locate the embryos of interest, and focus on the vitelline membrane (see Basic Protocol 3, steps 5 and 6).

7. Before starting the laser scanning, set the laser power down, the detector gain up, choose a fast scan time (typically 1 sec per scan) and zoom 1.0.

   For imaging Venus-expressing embryos on a Zeiss LSM 510 META coupled with a Coherent Chameleon Ti:sapphire laser, start with 20% transmission of a 900-mW, 910-nm laser light, PMT gain 900, 1 sec per scan, and zoom 1.0 for a Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens.

8. Start fast laser scanning, bring the embryo to the desired focal plane and move the region of interest (ROI) to the center of the field of view. Take a single scan.

   Most software has a rapid scanning mode ("Fast XY" W on Zeiss software) that makes preliminary scanning more efficient in rapidly selecting the focal plane and ROI with minimal exposure to laser light.

Establish conditions for maximal two-photon excitation and optimal image-acquisition

9. Determine the optimal instrument parameters for two-photon excitation and image acquisition. Optimize the following parameters based on the application requirements: percent transmission of excitation light, detector gain and offset, scan speed, averaging, zoom factor, and frame size. The guidelines provided for optimizing image acquisition in CLSM (Basic Protocol 3, steps 9a and 9b) also apply to 2PLSM.

   The probability of two-photon excitation is proportional to the incident photon flux density squared. Thus, the simplest way to increase two-photon excitation is by increasing the average power of the laser. However, this increases the heat produced and can dramatically affect viability and development or disrupt cellular structures.

   Use transmission images of the observed tissue and look for dramatic morphological changes due to photodamage during the experiment. Use any observations related to the question asked for assessing viability (cell cycle duration, cell cycle progression, etc.). For example, living embryos imaged with 20 mW of 910-nm laser light at the back aperture of a Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens progress normally through syncytial nuclear cycles and cellularization.

   Poor alignment of the two-photon laser into the microscope will have a profound effect on excitation efficiency. Technical details regarding laser alignment and other parameters that affect two-photon excitation (e.g., beam size at the back aperture and pulse length) are available in specialized reviews (Dickinson, 2005; Helmchen and Denk, 2005).

Acquire time-lapse images: single focal plane or z-stack over time

10. Once the appropriate instrument parameters have been determined, acquire time-lapse images of one or more optical slices through the sample (*z*-sectioning) over time (see Basic Protocol 3, steps 10a and 10b).

11. After a series of images has been collected, immediately save them to disk.

    Develop a strategy for labeling the image files before beginning to image. This will save time between successive imaging sessions in living, developing embryos. Always save raw unprocessed datasets and make sure that they contain all the information that might be required later for image processing, measurements, and presentation purposes or for reusing the same parameters (e.g., excitation levels, lens magnification and zoom factor, pixel dwell time) for imaging other samples.

BASIC PROTOCOL 5

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING IN LIVING *DROSOPHILA* EMBRYOS USING A LASER SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE PHOTOBLEACHING

This protocol focuses on the application of the fluorescence recovery after photobleaching (FRAP) technique in living *Drosophila* embryos. Photobleaching is the photo-induced alteration of a fluorophore that extinguishes its fluorescence. In a typical FRAP experiment, fluorescence in a selected region of interest (ROI) within a cell is photobleached

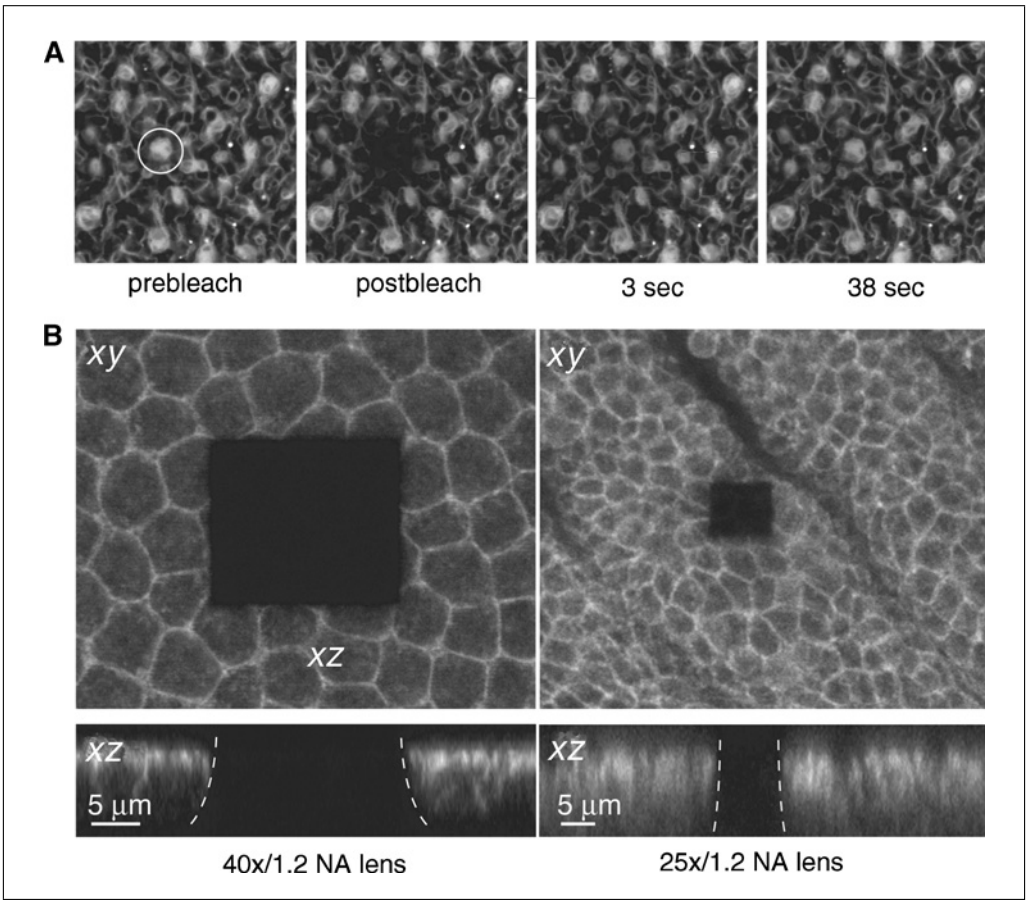


Figure 4.18.8 Example of FRAP and effects of lens magnification and NA in bleaching depth. (**A**) A region of interest (white outlined circle) is photobleached in a syncytial embryo expressing an ER lumenal protein. FRAP (see Basic Protocol 5) revealed that this protein is highly mobile in the ER lumen, and fluorescence recovery occurred in less than a minute. (**B**) A fluorescent embryo was fixed, and a region of interest was photobleached with lenses of different magnification and numerical aperture (NA). A high-resolution *z*-stack was then acquired to image the bleached volume. High-NA lenses (left panel) focus the laser beam best and bleach more efficiently in the focal plane, and the conical shape of the illumination pattern is obvious in *xz* sections. Conversely, low-NA lenses (right panel) bleach more uniformly along the optical axis, consistent with a more cylindrical shape of illumination.

once with a high-intensity laser beam, and fluorescence recovery in the bleached ROI is monitored over time with low-intensity laser light, as nonbleached molecules move into the bleached region (see Fig. 4.18.8). Quantitative measurements of the fluorescence recovery kinetics provide insights into protein and organelle mobility and dynamics, including (1) the estimation of the diffusion coefficient of molecules, (2) the fraction of mobile molecules under specific experimental conditions, and (3) the assessment of continuity or discontinuity in an organelle (for reviews, see Klonis et al., 2002; Carrero et al., 2003).

UNIT 21.1 describes in detail how to perform and analyze FRAP experiments in mammalian cells, and that unit should be consulted in addition to the protocol described here. Performing FRAP in living *Drosophila* embryos involves essentially the same principles as in a mammalian cell, but there are additional considerations specific to the thickness, geometry, and scattering properties of the tissue that should be taken into account when working with *Drosophila*. There is no universal protocol for FRAP experiments. Every photobleaching experiment is performed in a unique way, depending on the photobleaching characteristics of the fluorescent proteins (FPs) used, recovery kinetics of the process

under study, geometry of the ROI, and how the images will be analyzed. This protocol provides general guidelines that should be considered when performing photobleaching experiments in living embryos. The investigator needs to be familiar with time-lapse confocal imaging (Basic Protocol 3) before attempting photobleaching experiments.

Materials

Drosophila embryos expressing fluorescent proteins (FPs) of interest (Basic Protocol 1), mounted in LabTek or MatTek imaging chamber (Basic Protocol 2)

Confocal laser scanning microscope (CLSM) capable of selective photobleaching (e.g., Zeiss LSM 510 or Olympus FluoView FV1000), equipped with laser lines and filter sets appropriate for the FPs of interest

Set up the imaging system

1. Set up the microscope and prepare embryos for time-lapse confocal imaging as described in Basic Protocol 3 (steps 1 to 3) with the following considerations:

   Choose an FP that is bright and photostable under low-intensity illumination conditions, but photobleaches fast and irreversibly under high-intensity illumination.

   EGFP, Venus, or mCherry are examples of variants suitable for photobleaching experiments in embryos expressing a single FP.

   When designing photobleaching experiments in embryos expressing two FPs (e.g., Venus and mCherry), tag the protein of interest for photobleaching to the red-shifted variant (mCherry in this case) and not vice versa. This will eliminate cross-excitation of the blue-shifted variant (Venus) during photobleaching.

   Alternatively, suboptimal excitation of the blue-shifted variant can be used. For example, in embryos expressing EGFP and mCherry, use 458 nm to excite and photobleach EGFP (instead of the more optimal 488 nm) in order to minimize cross-exciting mCherry.

   Make sure that you have the appropriate laser power output needed for rapid and efficient photobleaching.

   Bleaching is achieved at the same wavelength as used for excitation, but with a typically 100- to 1000-fold higher intensity. For mGFP bleaching, a 25-mW or 30-mW, 488-nm Ar ion laser is sufficient for most applications. 100-mW 488-nm diode lasers are available for applications requiring rapid bleaching times. Most CLSMs use relatively weak HeNe lasers (1 mW) for green wavelengths. For routine photobleaching experiments with red variants, configure your system with stronger green lasers such as a 20- or 50-mW diode-pumped solid-state 532-nm laser or a 10-mW or 25-mW, 561-nm laser diode.

   Choose an appropriate objective lens.

   Water-immersion objectives used for high-resolution time-lapse confocal imaging of living Drosophila can also be used for photobleaching experiments (e.g., a Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR lens is sufficient for most applications). The NA of the objective is the property that most influences the depth and efficiency of photobleaching. High-NA lenses focus the laser beam best and bleach more efficiently in the focal plane, but they do not bleach out-of-focus regions as efficiently. Conversely, low-NA lenses do not focus the laser beam as well and bleach less efficiently, but they do so more uniformly along the optical axis in out-of-focus regions.

   The choice of the objective will depend on the thickness of the structure to be photobleached and the biological question asked. A 40×/1.20 NA water lens is sufficient for most applications while allowing for high spatial resolution, maximal signal collection, and minimal aberrations. If a larger depth of bleaching (i.e., bleach volume along the optical axis) is required (e.g., to bleach throughout the cytoplasm of epithelial cells) a 25×/ 0.8 NA is preferable (Fig. 4.18.8).

Establish FRAP conditions

2. Identify the embryo of interest, bring it to the desired focus, and move the ROI (cell or field of cells) to the center of the field of view (see Basic Protocol 3, steps 4 to 6).

3. Take a single scan and optimize the following parameters based on the application requirements: laser power and percent transmission of excitation light, pinhole size, detector gain and offset, scan speed, averaging, zoom factor, frame size, and acquisition frequency.

   Minimize bleaching during acquisition of the prebleach and postbleach series by using the minimum laser transmission to yield an acceptable image.

   Recommended conditions for a 25-mW, 488-nm argon laser are 0.1% to 1.0% transmission at a tube current of 6A.

   Adjust the pinhole size based on the experimental requirements.

   If photobleaching of the structure of interest in z is homogeneous, then an open pinhole is preferable in order to collect as much signal as possible for accurate fluorescence quantitation. However, when the ROI is thin or masked by a large diffuse pool of fluorescent molecules, a closed pinhole is preferable to image only the ROI.

   For example, when the protein of interest localizes both to the cytoplasm and to the plasma membrane, only a thin optical section will reveal the plasma membrane pool, and a closed pinhole should be used to measure plasma membrane–associated kinetics.

   Adjust the gain and black level (offset) of the PMT detector in order to acquire images using the full dynamic range of the detector (see Basic Protocol 3, step 9a).

   This is absolutely required for quantitative measurements of fluorescence intensity changes during the photobleaching experiment. Acquire 12-bit images rather than 8-bit images in order to increase the dynamic range of the measurements and enable detection of small changes in fluorescence intensity.

   Use zooms that include the whole cell containing the bleach ROI in order to correct the post-bleach series for the photobleached fraction.

   Zooms that include neighboring cells or other regions in the tissue in the same field as the bleach ROI are preferable in order to correct for acquisition photobleaching and laser fluctuations. Higher zooms are in general beneficial because they increase spatial and temporal resolution. Furthermore, the rate of photobleaching is proportional to the square of the zoom factor. Thus, zooming in will favor rapid photobleaching (but may also result in undesired bleaching during postbleach image acquisition).

   Adjust the time resolution (often more critical in photobleaching experiments than spatial resolution).

   To increase time resolution for the fluorescence recovery time series, minimize or avoid averaging and use a fast scan speed to yield an acceptable image. In cases of very fast recovery kinetics, reduce the number of pixels (frame size) to increase even more the acquisition frequency.

   For example, scanning a 512 × 512 frame at zoom 2.0 using the fastest scan speed on a Zeiss LSM 510 META yields a scan time of 786 msec and reducing the frame to 512 × 150 using the same zoom and scan speed yields a scan time of 230 msec. Most commercial confocal microscopes enable the investigator to scan only a selected ROI without having to zoom in, thus increasing time resolution, preserving spatial resolution, and minimizing exposure of the rest of the sample to illumination.

   For acquiring >10 frames per sec or simultaneously bleaching and acquiring images (required in the case of very rapid recovery kinetics), specialized systems are necessary. Examples of such commercial systems are the Zeiss 5 DUO system (which combines the ultrafast scanning and detection of a Zeiss LSM 5 LIVE with the photobleaching capability

of a Zeiss LSM 510 META), the Olympus FluoView FV1000 system with an SIM scanner (a synchronized laser scanning system that allows one to simultaneously image and bleach), and the PerkinElmer spinning disk UltraVIEW ERS with a PhotoKinesis unit (that enables bleaching selective targets as they are moving, after image acquisition has started).

These imaging parameters will be used for the prebleach series and for the fluorescence recovery time series.

4. Define an ROI for the photobleach.

   Most bleaching functions in commercial microscope operating software applications allow for the arbitrary selection of the bleach ROI shape and size. Using a larger ROI will result in longer bleaching times and longer fluorescence recovery times, but this may be preferable in the case of rapidly diffusing molecules.

5. Determine photobleaching conditions at the same scan speed and zoom used for image acquisition.

   The illuminating beam has a 3D profile (i.e., it bleaches and records fluorescence in a 3D volume). We recommend measuring the depth and efficiency of bleaching in the specific ROI in fixed tissue, and then using the same instrument parameters (objective, lasers, and imaging settings) for the actual experiment. Perform a bleach and then acquire a high-resolution z-stack of the bleached region to accurately calculate the dimensions of the bleached volume and the completeness of bleaching (Fig. 4.18.8). This information will be useful when assessing or generating models for fitting the FRAP data (see discussion in step 13).

   Photobleaching should be as rapid and as complete as possible (see discussion in step 13). To neglect the exchange of bleached and nonbleached molecules during bleaching, the total bleaching time should be at least 15 times smaller than the recovery half time (Meyvis et al., 1999).

   At a given scan speed and zoom, photobleaching efficiency will be determined by the laser power output, transmission of the laser used, number of bleach iterations, as well as abundance and diffusive properties of the FP under study. We recommend a series of pilot experiments in fixed tissue (if possible) to determine the minimum number of iterations needed for complete bleaching of the fluorophore under the same imaging settings used for image acquisition. If the bleach is not complete, increase the number of iterations, increase the laser output (useful for very dynamic processes), or zoom in.

   Alternatively, you can optimize photobleaching time and efficiency in living tissue in neighboring cells of the primary ROI.

   Recommended conditions for photobleaching with a 25-mW, 488-nm argon laser are 100% transmission at a tube current of 6 A.

Collect FRAP data

6. Once the appropriate instrument parameters for prebleach and postbleach image acquisition (step 3) and photobleaching (steps 4 and 5) have been determined, perform the actual photobleaching experiment by integrating steps 7, 8, and 9 in a time-lapse function.

   Most microscope operating software applications offer a bleaching function or a macro interface that enables the investigator to set up the parameters for the photobleaching experiment and integrate these in a time-lapse function: area to be bleached, number of prebleach images, laser transmission and iterations for bleaching, number of postbleach images, and interval between acquired images.

7. Acquire a series of five to 10 prebleach images to measure the total steady-state fluorescence intensity before photobleaching.

   This prebleach series will be used to correct the post-bleach series for the photobleached fraction, as well as the whole experiment for acquisition photobleaching and laser intensity fluctuations.

8. Selectively photobleach the ROI, using the conditions established in step 5.

9. Acquire a series of postbleach images until the fluorescence recovery process has reached a steady state.

   Adjust the image acquisition frequency to yield as many data points as possible during the recovery process, in particular during the recovery half time. Acquire postbleach images for 10 to 50 times longer than the recovery half time of the bleached protein of interest (Axelrod et al., 1976; Gordon et al., 1995).

10. After the end of the photobleaching experiment, immediately save the dataset to disk.

    If the bleach ROI is not automatically saved with the images, save a prebleach image with an overlay of the bleach ROI used. Knowledge of the precise position, size, and shape of the bleach ROI is absolutely required for quantitative measurements in this region. Do not rely on visually assessing the position of the bleach box based on the first postbleach images.

    Develop a strategy for labeling the image files before beginning to image. This will save time between successive imaging sessions in living, developing embryos. Always save raw unprocessed datasets and make sure that they contain all the information that might be required later for image processing, measurements, and presentation purposes or for reusing the same parameters e.g., (pinhole size, excitation levels, lens magnification and zoom factor, pixel dwell time) for imaging other samples.

11. Collect more than five FRAP datasets for statistical analysis for each condition using the exact same instrument parameters.

Process FRAP data

12. Perform basic FRAP data analysis in four steps:

    a. Determine raw fluorescence intensity measurements in ROIs of the cell.

    b. Subtract background and correct for the bleached fraction.

    c. Correct for laser fluctuations and photobleaching during acquisition.

    d. Normalize data to compare different experiments.

13. Use this initial image analysis to generate a plot displaying fluorescence changes in the bleached ROI over time.

    From this plot, a number of parameters can be extracted, including the half time for recovery ($t_{1/2}$) and the mobile and immobile fractions of the fluorescent molecules under study. For basic image analysis and kinetic modeling of FRAP data, consult UNIT 21.1 and Rabut and Ellenberg (2005).

    Despite the widespread use of photobleaching microscopy, few models exist for quantitative analysis of FRAP data acquired with CLSM. FRAP theory was initially developed for nonscanning microscopes, and many of the assumptions that were initially formulated are still widely used in quantitative FRAP models. However, bleaching and fluorescence recovery monitoring are fundamentally different in CLSM from FRAP with nonscanning microscopes (for a theoretical analysis of the bleaching process in CLSM, see Braeckmans et al., 2003).

    The validity of existing quantitative FRAP models or the need to generate a new model to analyze FRAP data obtained with CLSM depend ultimately on the process under study (e.g., 2D versus 3D diffusion), recovery kinetics, instrument parameters used for bleaching and acquisition of fluorescence recovery (which influence the depth and efficiency of bleaching), as well as the scanning and detection speed of the CLSM system used.

    An example of where the choice of the quantitative FRAP model is of great importance is the estimation of diffusion coefficients for rapidly diffusing molecules in the cytoplasm or nucleoplasm of the cell. To date, most FRAP models assume that both bleaching and image acquisition are sufficiently fast to neglect diffusion during those periods (Axelrod et al., 1976; Braeckmans et al., 2003). As a rule, the total bleaching time should be at least 15 times smaller than the recovery half time to neglect diffusion during bleaching (Meyvis

et al., 1999). However, in FRAP experiments with standard commercial CLSM systems, pixels are acquired sequentially, so the bleached region is imaged a few milliseconds after the start of image acquisition.

Furthermore, most applications require much longer bleaching times than theoretically required to neglect diffusion. Before the first postbleach image is acquired, significant diffusion takes place (demonstrated experimentally in Braga et al., 2004), and calculated diffusion coefficients with widely used FRAP models are significantly underestimated. Recently, a 3D FRAP model was developed that takes into account diffusion during the bleach period (Braga et al., 2004) and can be readily applied on most commercial CLSMs. Quantitative FRAP models have also been developed to address the interplay between diffusion and binding events (Sprague et al., 2004).

BASIC PROTOCOL 6

FLUORESCENCE LOSS IN PHOTOBLEACHING IN LIVING *DROSOPHILA* EMBRYOS USING A LASER SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE PHOTOBLEACHING

This protocol focuses on the application of the fluorescence loss in photobleaching (FLIP) technique in living *Drosophila* embryos. In a typical FLIP experiment, fluorescence in a selected region of interest (ROI) within a cell is repeatedly photobleached over time, and loss of fluorescence in nonbleached regions of the cell is monitored as nonbleached molecules move into the bleached region and get bleached. Measurements of the fluorescence loss kinetics provide insights into the exchange of components between compartments, including (1) the extent of continuity or discontinuity in an organelle and (2) exchange of fluorescent pools between different compartments under specific experimental conditions. The same guidelines we provide for FRAP experiments (Basic Protocol 5) should be considered when performing FLIP in living embryos. The investigator needs to be familiar with time-lapse confocal imaging (Basic Protocol 3) before attempting photobleaching experiments.

Materials

Drosophila embryos expressing fluorescent proteins (FPs) of interest (Basic Protocol 1), mounted in LabTek or MatTek imaging chamber (Basic Protocol 2)

Confocal laser scanning microscope capable of selective photobleaching (e.g., Zeiss LSM 510 or Olympus FluoView FV1000), equipped with laser lines and filter sets appropriate for the FPs of interest

Set up the imaging system

1. Set up the microscope and prepare embryos as described for FRAP in Basic Protocol 5, step 1).

Establish FLIP conditions

2. Identify the embryo of interest, bring it to the desired focus, and move the ROI (cell or field of cells) to the center of the field of view.

3. Take a single scan and optimize the following parameters based on the application requirements: laser power and percent transmission of excitation light, pinhole size, detector gain and offset, scan speed, averaging, zoom factor, frame size, and acquisition frequency (see Basic Protocol 5, step 3).

   These imaging parameters will be used for the prebleach series and for the images collected between successive bleaches.

   Include at least one neighboring unbleached cell (or group of cells) in the field in addition to the cell containing the bleach ROI. Fluorescence intensity changes in the control cells will be used to correct for acquisition photobleaching and laser fluctuations during the FLIP protocol.

4. Define an ROI for the photobleach (see Basic Protocol 5, step 4).

5. Determine photobleaching conditions at the same scan speed and zoom used for image acquisition (see Basic Protocol 5, step 5).

Collect FLIP data

6. Once the appropriate instrument parameters for image acquisition and photobleaching have been determined, perform the actual photobleaching experiment by integrating steps 7 to 9 in a time-lapse function.

   Most microscope operating software applications offer a bleaching function or a macro interface that enables the investigator to set up the parameters for the photobleaching experiment and integrate these in a time-lapse function: area to be bleached, number of prebleach images, laser transmission and iterations for bleaching, number of images between successive bleaches, and interval between acquired images.

7. Acquire a series of five to ten prebleach images to measure the total steady-state fluorescence intensity before photobleaching.

   This prebleach series can be used to correct the whole experiment for acquisition photobleaching and laser intensity fluctuations.

8. Selectively photobleach the ROI, using the conditions established in step 5.

9. Acquire one image of the whole cell and photobleach the ROI again. Repeat the process until the fluorescence intensity of the structure of interest is similar to background.

   If the proteins are mobile and the fluorescent structure of interest is continuous, the total fluorescence of the structure will be depleted over time. The time delay between successive bleaches should allow a significant fraction of molecules in unbleached regions to move into the bleach ROI. Collecting more than one image between successive bleaches is very useful for monitoring shifts in the focal plane. If the bleach ROI moves laterally during acquisition, the experiment should be aborted.

10. After the end of the photobleaching experiment, immediately save the dataset to disk.

    If the bleach ROI is not automatically saved with the images, also save a prebleach image with an overlay of the bleach ROI used. Knowledge of the precise position, size, and shape of the bleach ROI is absolutely required for quantitative measurements in regions outside the FLIP ROI. Do not rely on visually assessing the position of the bleach box based on the first postbleach images.

    Develop a strategy for labeling the image files before beginning to image. This will save time between successive imaging sessions in living, developing embryos. Always save raw unprocessed datasets and make sure that they contain all the information that might be required later for image processing, measurements, and presentation purposes or for reusing the same parameters (e.g., pinhole size, excitation levels, lens magnification and zoom factor, pixel dwell time) for imaging other samples.

11. Collect more than five FLIP datasets for statistical analysis for each condition, using the exact same instrument parameters.

Process FLIP data

12. Perform basic FLIP data analysis in four steps:

    a. Determine raw fluorescence intensity measurements in ROIs of the cell.

    b. Subtract background.

    c. Correct for laser fluctuations and photobleaching during acquisition.

    d. Normalize data to compare different experiments.

13. Use this initial image analysis to generate a plot displaying fluorescence changes in the bleached/unbleached ROIs over time.

From this plot, a number of parameters can be extracted, including the half time for equilibration and the mobile and immobile fractions of the fluorescent molecules under study. For basic image analysis and kinetic modeling using FLIP data, consult UNIT 21.1 *and Rabut and Ellenberg (2005).*

BASIC PROTOCOL 7

PHOTOACTIVATION IN LIVING *DROSOPHILA* EMBRYOS USING A LASER SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE PHOTOBLEACHING

Photoactivatable fluorescent proteins (PAFPs) present a powerful tool for the noninvasive optical labeling and tracking of proteins, organelles, and cells in living *Drosophila* tissues (Lippincott-Schwartz and Patterson, 2008). PAFPs undergo a change in their spectral properties upon irradiation with light of a specific wavelength and intensity. Some PAFPs (e.g., PA-GFP) convert from a dark to a bright fluorescent state upon photoactivation, whereas others (e.g., mEosFP) undergo photoconversion from a green to a red fluorescent form. PAFP-tagged proteins that undergo irreversible photoconversion can be used to monitor the movement of the tagged molecules within a steady-state population without interference from new protein synthesis, as newly synthesized molecules will not be fluorescent at the imaging wavelengths of the photoconverted species.

PA-GFP (Patterson and Lippincott-Schwartz, 2002) and mEos-FP (Wiedenmann et al., 2004) have the most potential among the available PAFPs for long-term protein tracking experiments, since they are both monomeric, undergo irreversible photoconversion, and exhibit high contrast between their pre- and post-activated states (i.e., from dark to green for PA-GFP or from green to red for mEosFP). Both PA-GFP and mEosFP have been successfully used in living *Xenopus* and chick embryos (Stark and Kulesa, 2005; Nienhaus et al., 2006). This protocol focuses on the photoactivation of PA-GFP- or mEos-FP-tagged proteins in living *Drosophila* embryos. While the protocol we describe may be adapted for other photoactivatable proteins, the various technical aspects of each protein must be considered. The investigator needs to be familiar with time-lapse confocal imaging and FRAP (Basic Protocols 3 and 5) before attempting photoactivation experiments. UNIT 21.6 describes in detail how to perform and optimize photoactivation experiments and should be also be consulted when using the protocol below.

Materials

Drosophila embryos expressing PA-GFP- or mEosFP-tagged proteins of interest (see Basic Protocol 1), mounted in LabTek or MatTek imaging chamber (see Basic Protocol 2)

Confocal laser scanning microscope capable of selective photobleaching (e.g., Zeiss LSM 510 or Olympus FluoView FV1000), equipped with a violet light laser line (e.g., a 405-nm diode laser or a 413-nm krypton laser), as well as visible light lasers appropriate for imaging PA-GFP or mEosFP

Set up the imaging system

1. Set up the microscope and prepare embryos for time-lapse confocal imaging as described in Basic Protocol 3, steps 1 to 4, with the following considerations:

   Configure the dichroic mirrors in the light path to accommodate both excitation sources: 405 or 413 nm for photoactivation, and 488 nm for imaging of photoconverted PA-GFP.

   Irradiation of PA-GFP with intense violet light (~400 nm) causes irreversible photoconversion of PA-GFP from a dark state (that does not fluoresce when excited at 488 nm)

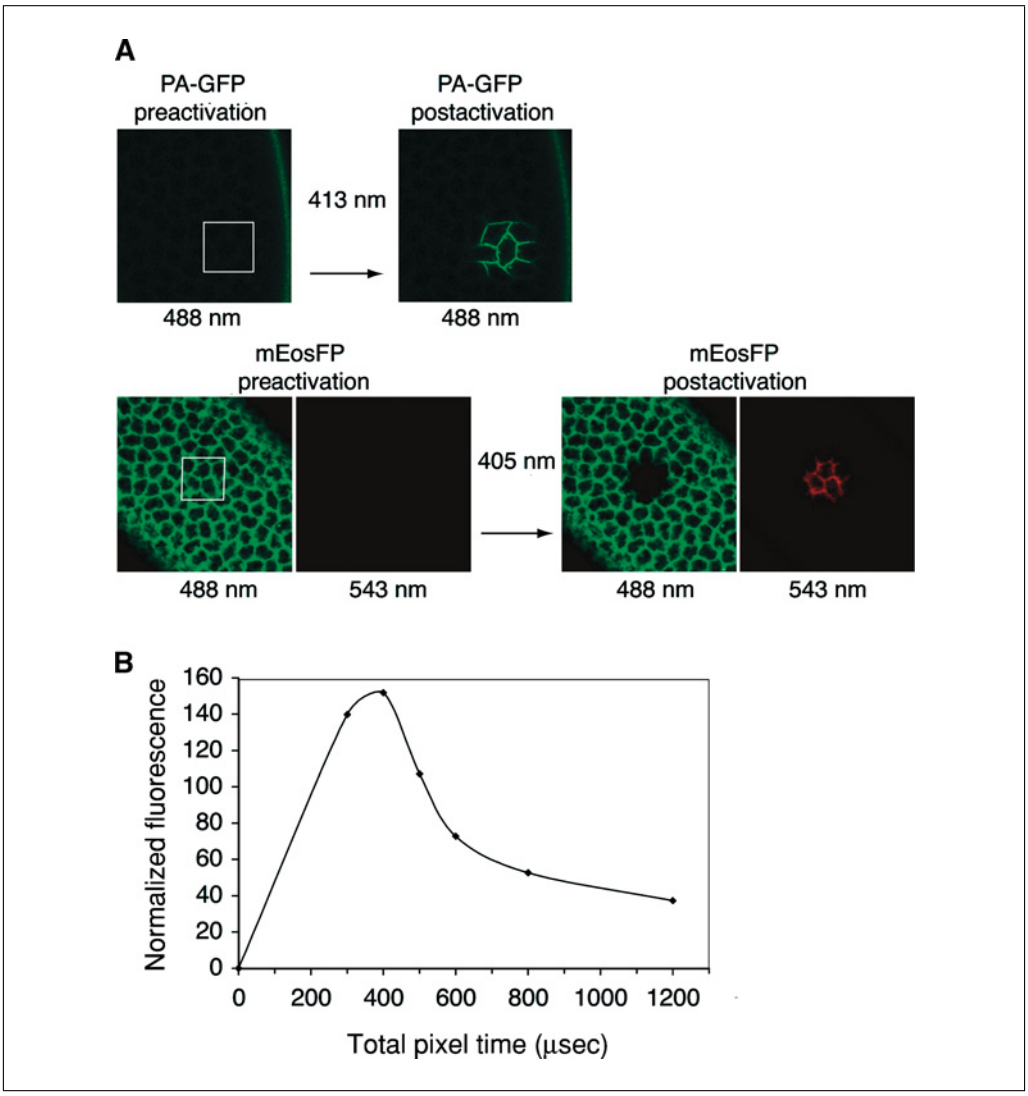


Figure 4.18.9 Example of photoactivation of PA-GFP and mEosFP in living embryos and optimization of total pixel time for maximal photoactivation (see Basic Protocol 7). (**A**) Irradiation of PA-GFP with 413 nm in a region of interest (white outlined square) in a living embryo causes photoconversion of PA-GFP from a dark state (that does not fluoresce when excited at 488 nm) to a bright green fluorescent state (that fluoresces at 488 nm). Bottom panel, Irradiation of mEos-FP with 405 nm in a region of interest (white outlined square) in a living embryo causes photoconversion of mEosFP from a green to a red fluorescent form. (**B**) Determination of the total pixel time required for optimal photoactivation, i.e., for the highest possible photoactivation with the least amount of bleaching. 0.68 mW of 405-nm laser power was used at the back aperture of an Olympus UPlanFl 40×/1.30 oil objective to photoactivate a region of interest in a living mEosFP-expressing embryo at zoom 3.0. Fluorescence was measured and plotted for different total pixel times. For 400 μsec total photoactivation pixel time (200 iterations at 2 μsec/pixel), full activation could be obtained. Fluorescence decreased over longer total pixel times due to photobleaching. For the color version of this figure go to *http://www.currentprotocols.com*.

to a bright green fluorescent state (that fluoresces at 488 nm; see Fig. 4.18.9). The excitation and emission spectra of photoactivated PA-GFP are slightly red-shifted compared with EGFP, so long-pass emission filters (LP505) or band-pass filters (BP505-530 or BP505-550) commonly used for EGFP imaging can be used for imaging PA-GFP.

Configure the dichroic mirrors in the light path to accommodate all excitation sources: 405 or 413 nm for photoactivation, 488 nm for imaging the green form before photoconversion and 543 nm (or 561 nm) for imaging the red form of mEosFP after photoconversion.

Irradiation of mEos-FP with violet light (~400 nm) causes irreversible photoconversion of mEosFP from a green to a red fluorescent form (see Fig. 4.18.9). Standard emission filters used for green/red imaging (see Basic Protocol 3, step 9b) can be used for imaging mEosFP.

Make sure that you have the appropriate violet light laser lines and laser power output needed for rapid and efficient photoactivation.

Both PA-GFP and mEosFP can be efficiently photoconverted with either a 25 to 50-mW, 405-nm diode laser or a 25-mW, 413-nm krypton laser.

Establish which lens available in your lab is most efficient in transmitting violet light, to optimize photoactivation.

Most highly corrected, high-NA objectives have the >80% transmittance to visible wavelengths but low transmittance to violet wavelengths (405 nm, 413 nm) required for photoactivation. For example, a Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR lens has 75% transmittance to 405 or 413 nm, compared to 80% to 85% transmittance for a Zeiss Fluar 40×/1.30 oil lens and 65% for a Zeiss C-Apochromat 63×/1.20 W Corr UV-VIS-IR for the same wavelengths. Also, high-NA lenses focus the laser beam best and photoactivate more efficiently in the focal plane compared to low-NA lenses. Lower-magnification lenses are recommended over higher-magnification lenses with an identical NA (e.g., a 40×/1.2 over a 63×/1.2 NA lens) because they often have a larger back aperture diameter and allow more light to reach the focal plane.

Establish photoactivation conditions

2. Identify the embryo of interest, bring it to the desired focus, and move the ROI (region of interest; cell or field of cells) to the center of the field of view (see Basic Protocol 3, steps 5 and 6).

3. Take a single scan and optimize the following parameters based on the application requirements: laser power and percent transmission of excitation light, pinhole size, detector gain and offset, scan speed, averaging, zoom factor, frame size, and acquisition frequency (see Basic Protocol 3, steps 9a and 9b, and Basic Protocol 5, step 3).

   PA-GFP fluoresces very little when excited at 488 nm (see Fig. 4.18.9). Unless a transgenic line has been generated, finding a embryo expressing PA-GFP can be problematic. Furthermore, finding the focal plane of interest before photoactivation can also be difficult in the absence of other fluorescent markers. Autofluorescence of the vitelline membrane and yolk can be used as a rough guide in pilot experiments to determine the focal plane in embryos that express PA-GFP (Fig. 4.18.9).

   Alternatively, co-expression of a cyan or red fluorescent marker in the same tissue that contains the ROI can be used as a guide to identify PA-GFP-positive cells.

   The level of PA-GFP or mEosFP fluorescence after photoactivation is not always easy to estimate. Some trial and error with various excitation power and detector gain settings will be required initially in order that fluorescence intensities in pre- and post-activation images are in the dynamic range of the detector.

   Slow scan speeds and/or high zooms are preferable because they increase the excitation power per unit area and thus result in shorter photoactivation times. However, they must be chosen so as not to cause undesired bleaching during pre- and post-activation image acquisition.

   These imaging parameters will be used for the pre- and post-activation time series.

4. Define an ROI for the photoactivation. Use the bleaching function available in commercial microscope operating software packages to arbitrarily select the photoactivation ROI shape and size.

   The size of the ROI will affect photoactivation time and depends on the biological process under study. For example, photoactivation time is not a critical factor when

photoactivation (typically in the range of msec to sec) is used to highlight a cell population that migrates in a period of tens of minutes. However, photoactivation time might be critical in the case of rapidly diffusing molecules.

5. Determine optimal photoactivation conditions at the same scan speed and zoom used for image acquisition, with the following considerations:

   Optimize the power and duration of the photoactivation so as to obtain the highest possible photoactivation with the least amount of bleaching.

   Photoactivation of PA-GFP and mEosFP is dependent on the duration of excitation power per unit area. This means that the level of photoactivation depends on the total time the photoactivation laser (405 or 413 nm) will spend per pixel. For longer than optimal total pixel times, the level of photoactivated fluorescence will decrease due to photobleaching by the repeated exposure to the 405-nm or 413-nm laser light.

   Conduct a series of pilot experiments (recommended) in fixed tissue (if possible) to determine the total pixel time required for optimal photoactivation (see Fig. 4.18.9).

   For a given objective, scan speed, zoom factor, and excitation power level (determined by the percent laser transmission used) measure the increase in fluorescence after photoactivation for different total pixel times. Increase the total pixel time by increasing the number of excitation scans (iterations) with the 405-nm or 413-nm laser.

   Fluorescence will increase to an apex (defined as the optimal total pixel time for photoactivation) and then decrease over longer total pixel times due to photobleaching. In the example in Figure 4.18.9, ~0.68 mW of 405-nm laser power was used at the back aperture of an Olympus UPlanFl 40×/1.30 oil objective. At zoom 3.0 and 400 μsec total photoactivation pixel time (200 iterations at 2 μsec/pixel), full activation could be obtained (Fig. 4.18.9B). At longer pixel times, photobleaching outbalances photoactivation and fluorescence decreases. In this case, photoactivated fluorescence appears depleted within the photoactivation ROI, which is surrounded by a fluorescent rim.

   Repeat the above measurements for a different excitation power and also for a different zoom factor.

   Decreasing the excitation power at the back aperture of the lens will require longer total pixel times. Higher zoom factors increase the excitation power per unit area and will shift the total pixel time and power required for optimal photoactivation to lower values. This optimization routine will be enough to determine the optimal parameters (objective, zoom factor, laser transmission, and total pixel time) for experiments on a daily basis, assuming minimal fluctuations in laser power and optical alignment.

   Use a handheld laser power meter to measure the excitation power at the back aperture of the objective. Simply remove the objective and measure the 405-nm or 413-nm laser power at the nosepiece at several transmission levels.

   This routine takes a few minutes, and knowledge of the power at the back aperture can be used to adjust photoactivation parameters in case of laser power or alignment fluctuations.

   Control for cytotoxicity when performing photoactivation experiments using short wavelengths (405-nm or 413-nm lasers).

   This step is imperative! Use any observations of photoactivation artifacts due to cytotoxicity related to the question asked (e.g., cell cycle duration, cell cycle progression).

Perform photoactivation experiment

6. Once the appropriate instrument parameters for pre- and post-activation image acquisition and photoactivation (steps 2 to 5) have been determined, perform the actual photoactivation experiment by integrating steps 7, 8, and 9 in a time-lapse function.

Most microscope operating software packages offer a bleaching function or a macro interface that enables the investigator to set up the parameters for the photoactivation experiment and integrate these in a time-lapse function: area to be photoactivated, number of preactivation images, laser transmission and iterations for activation, number of postactivation images and interval between acquired images.

7. Acquire a series of one to five pre-activation images. Use 488-nm excitation for PA-GFP or image simultaneously at 488 and 543 nm (or 561 nm) for mEosFP.
8. Selectively photoactivate the ROI, using the 405-nm or 413-nm laser and the conditions established in step 5.
9. Acquire a series of post-activation images using the excitation settings used for the pre-activation images in step 7.
10. After the end of the experiment, immediately save the dataset to disk. If the photoactivation ROI is not automatically saved with the images, also save a pre-activation image with an overlay of the ROI used.

Knowledge of the precise position, size, and shape of the bleach ROI might be required for quantitative measurements in this region, depending on the application.

Develop a strategy for labeling the image files before beginning to image. This will save time between successive imaging sessions in living, developing embryos. Always save raw unprocessed datasets and make sure that they contain all the information that might be required later for image processing, measurements, and presentation purpose or for reusing the same parameters (e.g., pinhole size, excitation levels, lens magnification and zoom factor, pixel dwell time) for imaging other samples.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Apple or grape juice agar plates

For 500 ml of apple or grape juice agar medium:
15 g agar
8 g sucrose
165 ml apple or grape juice
0.1 g methyl *p*-hydroxybenzoate (methyl paraben; Sigma)

Add water to the agar to a final volume of 335 ml and microwave 5 min to dissolve the agar. Dissolve the sucrose and methyl paraben in the juice by microwaving 5 min. Combine both microwaved mixtures and mix thoroughly. Pour ~8 ml per 60 × 15–mm petri dish (Genesee Scientific *or* BD). Once the juice agar plates have cooled and hardened, store up to ~1.5 month at 4°C in a sealed box (to prevent drying out).

COMMENTARY

Background Information

Advances in the development of user-friendly commercially available laser scanning microscopes and the generation of improved fluorescent protein (FP) variants that cover the entire visible spectrum have made fluorescence microscopy an enormously powerful tool in the biological community. The combination of these advances with *Drosophila* genetics, which enables the investigator to control the spatial and temporal expression of any protein of interest throughout development, has made it possible to gain insights into protein, organellar, and cellular dynamics in living tissues in a noninvasive way and with unprecedented resolution.

The generation of transgenic *Drosophila* has become a standard technique for the investigation of a wide variety of biological questions (Fujioka et al., 2000). Virtually any cloned gene can be inserted into the genome using the P transposable element system. A

large collection of *Drosophila* cDNAs is available at the *Drosophila* Genomics Research Center (DGRC; *http://dgrc.cgb.indiana.edu*). For a catalog of Gal4 transgenic lines that can be used to express the protein of interest in specific tissues or temporal patterns, the reader is referred to the FlyBase Web site at *http://flystocks.bio.indiana.edu/Browse/misc-browse/gal4.htm*. A catalog of vectors for subcloning fluorescently tagged genes of interest and for transforming *Drosophila* is available at DGRC (*http://dgrc.cgb.indiana.edu/vectors/store/vectors.html*).

Background information on microscopy and the use of FPs is available from several sources. For an excellent review on the latest generation of FP variants, the reader is referred to *UNIT 21.5*. The use of FPs to study protein dynamics has been reviewed (Lippincott-Schwartz et al., 2001; Lippincott-Schwartz and Patterson, 2003). The Molecular Expressions Web site (*http://micro.magnet.fsu.edu/primer*) is one of the best microscopy Web sites. It is an extremely resourceful and up-to-date Web site, covering topics from image formation and microscope basics to imaging parameters for FPs. It further features interactive tutorials that help the investigator understand in depth the different parameters involved in image acquisition (e.g., effect of pinhole size and lens NA on resolution, spectral bleedthrough in multicolor imaging). Basic confocal microscopy is reviewed in *UNIT 4.5*. For more detailed reviews of confocal laser scanning microscopy (CLSM), see Murray (2005) and *http://www.olympusconfocal.com/theory/LSCMIntro.pdf*. *UNIT 4.11* gives an introduction to two-photon excitation microscopy. For more detailed reviews of two-photon laser scanning microscopy (2PLSM) and deep-tissue imaging using 2PLSM, see Dickinson (2005) and Helmchen and Denk (2005).

The advantages and considerations of using CLSM and 2PLSM for live *Drosophila* tissue imaging are discussed at the beginning of this unit and in Basic Protocol 4. CLSM (Basic Protocol 3) is currently the method of choice for routine high-resolution imaging of living *Drosophila* tissue. The major drawback of CLSM is limited penetration depth due to scattering and out-of-focus absorption, which limit imaging to <40 μm deep in a living *Drosophila* embryo (see Fig. 4.18.2). For routine imaging at 40 to 100 μm deep in the embryo, 2PLSM (Basic Protocol 4) is the method of choice. The major drawback of 2PLSM is local heating effects caused by the high powers of near infrared (NIR) photons required for two-photon excitation. Furthermore, ultrafast pulsed lasers for 2PLSM are much more expensive than the visible light lasers commonly used for CLSM. Finally, more technical effort is required for the optimization of the laser source for two-photon excitation.

Photobleaching techniques applied to live cell imaging have transformed our understanding of cellular organization and protein dynamics. The mobility of virtually any molecule can be measured in any location in living cells, and the spatial and temporal dynamics of subcellular processes can be monitored with unprecedented resolution. For reviews of FRAP theory and applications, see Klonis et al. (2002), Carrero et al. (2003), and Lippincott-Schwartz et al. (2003). FRAP theory was initially developed for nonscanning microscopes, but many of the assumptions that were initially formulated are still widely used in quantitative FRAP models (Axelrod et al., 1976; Gordon et al., 1995). However, bleaching and fluorescence recovery monitoring are fundamentally different in CLSM and FRAP with nonscanning microscopes. Braeckmans et al. (2003) provides a thorough theoretical explanation of the bleaching process in CLSM. A 3D FRAP model was recently reported that can be easily applied to any commercial CLSM setup (Braeckmans et al., 2003). Braga et al. (2004) further developed a 3D FRAP model that takes into account diffusion during the bleach period. For basic image analysis and kinetic modeling of photobleaching data, see *UNIT 21.1*, Rabut and Ellenberg (2005), and Phair and Misteli (2001). Quantitative FRAP models have also been developed to address the interplay between diffusion and binding events (Sprague et al., 2004; Sprague and McNally, 2005).

Photoactivatable FPs enable the investigator to track individual cells or cell populations in developing tissues, probe organelle continuity, or monitor protein and organelle trafficking and turnover in living cells in a noninvasive way. For reviews of photoactivatable FPs and applications, refer to Lukyanov et al. (2005), Nienhaus et al. (2006), and Lippincott-Schwartz and Patterson (2008). Patterson and Lippincott-Schwartz (2004) and *UNIT 21.6* discuss the technical aspects of photoactivation using PA-GFP in a CLSM setup, PA-GFP in a CLSM setup. PA-GFP and mEos-FP have the most potential among the available PAFPs for long-term protein tracking experiments because they are both monomeric, undergo irreversible photoconversion, and

exhibit high contrast between their pre- and post-activated states. Both PA-GFP and mEosFP have been successfully used in living *Xenopus* and chick embryos (Stark and Kulesa, 2005; Nienhaus et al., 2006), and the use of PA-GFP has also been reported in the fly (Post et al., 2005).

Tvaruskó et al. (2005) provide an overview of image analysis tools for studying dynamic processes in living cells. ImageJ (previously known as NIH Image) is a free, powerful image analysis program developed at the National Institutes of Health, and it has many useful tools for analysis of confocal images. It can be downloaded from *http://rsb.info.nih.gov/ij*. Other widely used image analysis software packages are Volocity (Improvision), Imaris (Bitplane), or MetaMorph (Molecular Devices).

Critical Parameters and Troubleshooting

The critical parameters for setting up time-lapse imaging (Basic Protocol 3 and 4), photobleaching (Basic Protocols 5 and 6), or photoactivation (Basic Protocol 7) experiments in living *Drosophila* tissue and optimization of data collection are described in great detail in the respective protocols. We emphasize below some of the parameters that are critical for the success of imaging experiments in living tissue using CLSM and 2PLSM.

Choice of fluorescent protein

The investigator should use the latest generation of spectral variants, which have been improved for folding, chromophore maturation, brightness, photostability, and minimal self-association (see *UNIT 21.5*). However, the choice of a specific FP or combination of FPs depends exclusively on the application requirements. In all cases, the investigator needs to minimize undesired photobleaching of the FPs during image acquisition. For long-term protein tracking experiments, bright and photostable FPs are preferable. For multi-fluorophore imaging, the investigator should closely examine the spectral profiles of the FPs of interest, as well as the excitation and emission filters available in the CLSM setup, in order to optimize the light path and obtain maximal signal without spectral bleedthrough. Some effort is required to optimize signal collection for multiple fluorophores because FPs vary in relative brightness and photostability.

For photobleaching experiments, the FP should be bright and photostable under low-intensity illumination conditions, but photobleach fast and irreversibly under high-intensity illumination. The most desirable features for photoactivatable proteins are that they undergo irreversible photoconversion and that they exhibit high contrast between their pre- and post-activated states. PA-GFP and mEosFP fulfill these criteria and thus currently have the most potential.

Choice of objectives

Due to the thickness and geometry of the tissue, imaging subcellular processes during development often requires focal planes deep into the tissue. Because of the differences in refractive indices in the embryo interior and the immersion medium, severe spherical aberrations arise when focal planes are set >10 μm deep, and oil is used as an immersion medium. Unless optical sections are collected at the surface of the tissue closest to the coverglass, the investigator should always use water-immersion objectives to minimize spherical aberrations. Long-working-distance, high-NA water-immersion objectives corrected for coverglass thickness, field curvature, and chromatic aberrations (e.g., the Zeiss C-Apochromat 40×/1.20 W Corr UV-VIS-IR water lens or the Olympus UPLAPO 60×/1.2 NA water lens) are the best choice for high-resolution live 4D imaging of thick *Drosophila* tissue with CLSM.

Magnification, NA, and transmittance of the objective to specific wavelengths need to be carefully chosen based on the application. A high-NA objective for maximal signal collection and spatial resolution is generally preferable for high-resolution time-lapse imaging applications. Magnifications higher than 40× are not recommended because they yield less bright images and increase the risk for photobleaching. However, 60× or 100× lenses might be required for imaging very small structures (e.g., peroxisomes). Water-immersion objectives used for high-resolution time-lapse imaging can also be used for photobleaching experiments. The choice of the NA of the objective (which affects the depth of bleaching) will depend on the thickness of the structure to be photobleached. For photoactivation experiments, use high-NA objectives with high transmittance to violet wavelengths (405 nm, 413 nm). For two-photon excitation, use long-working-distance, high-NA water-immersion objectives with high transmission to near infrared (NIR) and visible (VIS) wavelengths.

Choice of microscope setup

Today, the investigator is confronted with the difficult task of choosing among a wide variety of user-friendly, commercially available laser scanning microscopes. The choice of the CLSM setup depends on the application requirements. High spatial resolution can be easily obtained with all modern commercial CLSM systems. Time resolution is often much more critical in photobleaching and photoactivation experiments than spatial resolution. If there is a need for fast image acquisition or simultaneous bleaching and image acquisition (required in the case of very rapid recovery kinetics), specialized systems are necessary. Examples of such commercial systems are the Zeiss 5 DUO system (which combines the ultrafast scanning and detection of a Zeiss LSM 5 LIVE with the photobleaching capability of a Zeiss LSM 510 META), the Olympus FluoView FV1000 system with a SIM scanner (a synchronized laser scanning system that allows to simultaneously image and bleach), and the PerkinElmer spinning disk UltraVIEW ERS with a PhotoKinesis unit (that enables bleaching selective targets as they are moving, after image acquisition has started).

Phototoxicity and photodamage

It is imperative to control for cytotoxicity when performing photoactivation experiments using short wavelengths (405-nm or 413-nm lasers) or two-photon excitation microscopy using high powers of near infrared (NIR) photons. Use transmission images of the observed tissue and look for morphological changes due to toxicity and photodamage during the experiment. Any observations related to the question asked (e.g., cell cycle duration, cell cycle progression) should be carefully examined for photoactivation or two-photon excitation artifacts.

Anticipated Results

The protocols described in this unit provide guidance to the investigator about various parameters that need to be considered while designing a live imaging experiment in the *Drosophila* embryo. The correct choice of objectives, choice of fluorophores, and choice of CLSM versus 2PLSM is imperative for a successful experiment. By following the suggestions given in every protocol, the investigator should be able to select embryos expressing the fluorescently tagged protein of interest and to perform time-lapse imaging in a desired tissue. The photobleaching protocols should enable the investigator to follow dynamics of protein molecules, organelles, or subsets of cells in the living *Drosophila* embryo. With the data obtained, the investigator can calculate the diffusion coefficient of a molecule and the mobile and immobile fractions of the molecules. Using the photoactivation protocol, the investigator should be able to perform optical pulse-chase experiments to study the spatiotemporal dynamics of proteins, organelles, and cells in the developmental context of choice.

Time Considerations

The generation of transgenic flies involves procurement of the cDNA of interest and cloning into the appropriate vector for injections. The injection of DNA into embryos, selection of the transformed flies, chromosome mapping, and checking for expression may take as long as 3 months. Genetic crosses performed to obtain flies that will express the fluorescent transgene will take ~15 days for one transgene and 30 days for two transgenes, at 25°C. Finally, the *Drosophila* embryos have to be aged to the desired stage of development prior to imaging. For example, imaging the early syncytial divisions (nuclear cycle 11 to 13) involves 1 hr for the prelay and an additional hour to age the embryo. The preparation and mounting of embryos in the LabTek/MatTek chamber take ~10 min.

The optimization of photobleaching and photoactivation may take a few hours, depending upon the prior experience of the investigator with CLSM. The actual time of the experiments depends on the application and the dynamics of the process under study. For example, the photobleaching experiment involving an endoplasmic reticulum lumenal protein (Figure 4.18.8A) has taken less than 1 min. This experiment was performed in early stages of the embryo before nuclear migration to the periphery. To perform multiple experiments of this type, the investigator will need to organize additional egg collections to obtain the same stage of development for further photobleaching experiments.

Literature Cited

Anderson, D.T., 1972. The development of hemimetabolous insects. *In* Developmental Systems: Insects, Vol. 1. pp. 95-163. (S.J. Counce and C.H. Waddington, eds.) Academic Press, London.

Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E., and Webb, W.W. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055-1069.

Beuthan, J., Minet, O., Helfmann, J., Herrig, M., and Muller, G. 1996. The spatial variation of the

refractive index in biological cells. *Phys. Med. Biol.* 41:369-382.

Braeckmans, K., Peeters, L., Sanders, N.N., De Smedt, S.C., and Demeester, J. 2003. Three-dimensional fluorescence recovery after photobleaching with the confocal scanning laser microscope. *Biophys. J.* 85:2240-2252.

Braga, J., Desterro, J.M., and Carmo-Fonseca, M. 2004. Intracellular macromolecular mobility measured by fluorescence recovery after photobleaching with confocal laser scanning microscopes. *Mol. Biol. Cell.* 15:4749-60.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 118:401-415.

Carrero, G., McDonald, D., Crawford, E., de Vries, G., and Hendzel, M.J. 2003. Using FRAP and mathematical modeling to determine the in vivo kinetics of nuclear proteins. *Methods*. 29:14-28.

Costa, M., Sweeton, D., and Wieschaus, E. 1993. Gastrulation in *Drosophila:* Cellular mechanisms of morphogenetic movements. *In* The Development of *Drosophila melanogaster* (M. Bate and A. Martinez-Arias, eds.) pp. 425-465. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Dickinson, M.E. 2005. Multiphoton and multispectral laser-scanning microscopy. *In* Live Cell Imaging: A Laboratory Manual. (R.D. Goldman and D.L. Spector, eds.) pp. 281-301. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Dirckx, J.J., Kuypers, L.C., and Decraemer, W.F. 2005. Refractive index of tissue measured with confocal microscopy. *J. Biomed. Opt.* 10:44014.

Engelbrecht, C.J. and Stelzer, E.H. 2006. Resolution enhancement in a light-sheet-based microscope (SPIM). *Opt. Lett.* 31:1477-1479.

Fischer, J.A., Giniger, E., Maniatis, T., and Ptashne, M. 1988. Gal4 activates transcription in *Drosophila*. *Nature* 332:853-856.

Foe, V.E. and Alberts, B.M. 1983. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci* 61:31-70.

Foe, V.E., Odell, G.M., and Edgar, B.A. 1993. Mitosis and morphogenesis in the *Drosophila* embryo: Point and counterpoint. *In* The Development of *Drosophila melanogaster* (M. Bate and A. Martinez-Arias, eds.) pp. 149-300. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Fujioka, M., Jaynes, J.B., Bejsovec, A., and Weir, M. 2000. Production of transgenic *Drosophila*. *Methods Mol. Biol.* 136:353-363.

Gibson, M.C., Patel, A.B., Nagpal, R., and Perrimon, N. 2006. The emergence of geometric order in proliferating metazoan epithelia. *Nature* 442:1038-1041.

Gordon, G.W., Chazotte, B., Wang, X.F., and Herman, B. 1995. Analysis of simulated and experimental fluorescence recovery after photobleaching. Data for two diffusing components. *Biophys. J.* 68:766-778.

Gustafsson, M.G. 1999. Extended resolution fluorescence microscopy. *Curr. Opin. Struct. Biol.* 9:627-634.

Hacker, U. and Perrimon, N. 1998. DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in *Drosophila*. *Genes Dev*. 12:274-284.

Hazelrigg, T. 2000. GFP and other reporters. *In Drosophila* Protocols. (W. Sullivan, M. Ashburner, and R.S. Hawley, eds.) pp. 313-344. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Helmchen, F. and Denk, W. 2005. Deep tissue two-photon microscopy. *Nat. Methods.* 2:932-940.

Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J., and Stelzer, E.H. 2004. Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305:1007-1009.

Johnsen, S. and Widder, E.A. 1999. The physical basis of transparency in biological tissue: Ultrastructure and the minimization of light scattering. *J. Theor. Biol.* 199:181-198.

Kiehart, D.P., Crawford, J.M., and Montague, R.A. 2000. Quantitative microinjection of *Drosophila* embryos. *In Drosophila* Protocols. (W. Sullivan, M. Ashburner, and R.S. Hawley, eds.) pp. 345-360. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Klonis, N., Rug, M., Harper, I., Wickham, M., Cowman, A., and Tilley, L. 2002. Fluorescence photobleaching analysis for the study of cellular dynamics. *Eur. Biophys. J.* 31:36-51.

Lippincott-Schwartz, J. and Patterson, G.H. 2003. Development and use of fluorescent protein markers in living cells. *Science* 300:87-91.

Lippincott-Schwartz, J., and Patterson, G.H. 2008. Fluorescent proteins for photoactivation experiments. *Methods in cell biology* 85:45-61.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* 2:444-456.

Lippincott-Schwartz, J., Altan-Bonnet, N., and Patterson, G.H. 2003. Photobleaching and photoactivation: Following protein dynamics in living cells. *Nat. Cell Biol.* Suppl:S7-14.

Lukyanov, K.A., Chudakov, D.M., Lukyanov, S., and Verkhusha, V.V. 2005. Innovation: Photoactivatable fluorescent proteins. *Nat. Rev. Mol. Cell Biol.* 6:885-891.

Meyvis, T.K., De Smedt, S.C., Van Oostveldt, P., and Demeester, J. 1999. Fluorescence recovery after photobleaching: A versatile tool for mobility and interaction measurements in pharmaceutical research. *Pharm. Res.* 16:1153-1162.

Murray, J.M. 2005. Confocal microscopy, deconvolution, and structured illumination methods. *In* Live Cell Imaging: A Laboratory Manual.

(R.D. Goldman and D.L. Spector, eds.) pp. 239-279. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., and Miyawaki, A. 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* 20:87-90.

Nienhaus, G.U., Nienhaus, K., Holzle, A., Ivanchenko, S., Renzi, F., Oswald, F., Wolff, M., Schmitt, F., Rocker, C., Vallone, B., Weidemann, W., Heilker, R., Nar, H., and Wiedenmann, J. 2006. Photoconvertible fluorescent protein EosFP: Biophysical properties and cell biology applications. *Photochem. Photobiol.* 82:351-358.

Parry, H., McDougall, A., and Whitaker, M. 2005. Microdomains bounded by endoplasmic reticulum segregate cell cycle calcium transients in syncytial *Drosophila* embryos. *J. Cell Biol.* 171:47-59.

Patterson, G.H. and Lippincott-Schwartz, J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297:1873-1877.

Patterson, G.H. and Lippincott-Schwartz, J. 2004. Selective photolabeling of proteins using photoactivatable GFP. *Methods* 32:445-450.

Phair, R.D. and Misteli, T. 2001. Kinetic modelling approaches to in vivo imaging. *Nat. Rev. Mol. Cell Biol.* 2:898-907.

Post, J.N., Lidke, K.A., Rieger, B., and Arndt-Jovin, D.J. 2005. One- and two-photon photoactivation of a paGFP-fusion protein in live *Drosophila* embryos. *FEBS Lett*. 579:325-330.

Rabut, G. and Ellenberg, J. 2005. Photobleaching techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, iFRAP, and FLIP. *In* Live Cell Imaging: A Laboratory Manual. (R.D. Goldman and D.L. Spector, eds.) pp. 101-126. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Rizzo, M.A., Springer, G.H., Granada, B., and Piston, D.W. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* 22:445-449.

Rorth, P. 1998. Gal4 in the *Drosophila* female germline. *Mech. Dev.* 78:113-118.

Rubin, G.M. and Spradling, A.C. 1983. Vectors for P element-mediated gene transfer in *Drosophila*. *Nucleic Acids Res*. 11:6341-6351.

Santamaria, P. and Gans, M. 1980. Chimaeras of *Drosophila melanogaster* obtained by injection of haploid nuclei. *Nature* 287:143-144.

Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N., Palmer, A.E., and Tsien, R.Y. 2004. Improved monomeric red, orange, and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* 22:1567-1572.

Spiess, E., Bestvater, F., Heckel-Pompey, A., Toth, K., Hacker, M., Stobrawa, G., Feurer, T., Wotzlaw, C., Berchner-Pfannschmidt, U., Porwol, T., and Acker, H. 2004. Two-photon excitation and emission spectra of the green fluorescent protein variants ECFP, EGFP and EYFP *J. Microsc.* 217:200-204.

Sprague, B.L. and McNally, J.G. 2005. FRAP analysis of binding: Proper and fitting. *Trends Cell Biol*. 15:84-91.

Sprague, B.L., Pego, R.L., Stavreva, D.A., and McNally, J.G. 2004. Analysis of binding reactions by fluorescence recovery after photobleaching. *Biophys. J.* 86:3473-3495.

Stark, D.A. and Kulesa, P.M. 2005. Photoactivatable green fluorescent protein as a single-cell marker in living embryos. *Dev. Dyn.* 233:983-992.

Suzuki, D.T., Piternick, L.K., Hayashi, S., Tarasoff, M., Baillie, D., and Erasmus, U. 1967. Temperature-sensitive mutations in *Drosophila melanogaster* I. Relative frequencies among gamma-ray and chemically induced sex-linked recessive lethals and semilethals. *Proc. Natl. Acad. Sci. U.S.A.* 57:907-912.

Tvaruskó, W., Mattes, J., and Eils, R. 2005. Analyzing live cell data and tracking dynamic movements. *In* Live Cell Imaging: A Laboratory Manual. (R.D. Goldman and D.L. Spector, eds.) pp. 302-326. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Van Doren, M., Williamson, A.L., and Lehmann, R. 1998. Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr. Biol.* 8:243-246.

Webb, R.H. and Dorey, C.K. 1995. The pixelated image. *In* Handbook of Biological Confocal Microscopy. (J.B. Pawley, ed.) pp. 55-67. Plenum Press, New York.

Wiedenmann, J., Ivanchenko, S., Oswald, F., Schmitt, F., Rocker, C., Salih, A., Spindler, K.D., and Nienhaus, G.U. 2004. EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proc. Natl. Acad. Sci. U.S.A.* 101:15905-15910.

Zacharias, D.A., Violin, J.D., Newton, A.C., and Tsien, R.Y. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* 296:913-916.

Quantitative Colocalization Analysis of Confocal Fluorescence Microscopy Images

Vadim Zinchuk[1] and Olga Zinchuk[2]

[1]Department of Anatomy and Cell Biology, Kochi University, Faculty of Medicine, Japan
[2]Institute of Anatomy, University of Berne, CH-3000, Berne, Switzerland

ABSTRACT

Colocalization is an important finding in many cell biological studies. This unit describes a protocol for quantitative evaluation of images with colocalization based on the calculation of a number of specialized coefficients. First, images of double-stained sections are subjected to background correction. Then, various coefficients are calculated. Meanings of the coefficients and a guide to interpretation of their results indicating either presence or absence of colocalization are given. Success in colocalization studies depends on the quality of analyzed images, proper preparation of them for coefficients calculations, and correct interpretation of obtained results. This protocol helps to ensure reliability of colocalization coefficients calculations. *Curr. Protoc. Cell Biol.* 39:4.19.1-4.19.16. © 2008 by John Wiley & Sons, Inc.

Keywords: quantitative colocalization • confocal fluorescence microscopy • image analysis

INTRODUCTION

Quantitative colocalization analysis (QCA) is a method to estimate the degree of colocalization of antigens in multicolor confocal immunofluorescence microscopy images. Colocalization is observed when staining of two or more antigens in the same section, labeled by corresponding antibodies with different excitation spectra and therefore visualized in different colors, overlaps. Although colocalization is a mere coexistence of molecules in a very close physical location, it can provide valuable clues clarifying their common characteristics. Scientific applicability of colocalization observed per se is, however, limited because it is perceived differently by the eye and thus can frequently be misleading. Thus, the method described here evaluates colocalization objectively by estimating its quantitative characteristics.

The method is applicable to dual-color confocal images of sections stained with antibodies with well-separated excitation spectra, acquired using proper set up of confocal microscopes, and stored in the image file format that preserves image data necessary for quantification. It relies on the use of specialized software that calculates a number of colocalization coefficients to understand the observed colocalization in greater detail. Prior to calculating the coefficients, the software performs background correction of the images. This important step is required to avoid obtaining false-positive results and is not available with other tools.

The protocol described in this unit demonstrates the use of QCA for studying functionally synergetic proteins. As an example, the use of the method is illustrated by quantifying colocalization of bile salt export pump (Bsep) and multidrug resistance protein 2 (Mrp2) in the liver. The background correction procedure is performed using different methods and is accompanied by the respective scatter grams. The meanings of coefficients and

Current Protocols in Cell Biology 4.19.1-4.19.16, June 2008
Published online June 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0419s39

their importance is explained. Finally, the interpretation of the results of coefficients calculations in terms of their biological significance is given.

STRATEGIC PLANNING

General

Successful QCA requires good knowledge and experience in basic immunohistochemical techniques (*UNIT 4.3*), confocal fluorescence microscopy (*UNIT 4.5*), and handling and processing of digital images. This unit provides practical information about quantitative colocalization and coefficients used to estimate it, gives tips for success in background correction procedure, and describes applicability of calculations results. Please refer to *UNITS 4.2* and *4.3* for basic information on basic fluorescence microscopy and immunofluorescence staining, respectively.

Steps of QCA

1. Ensure that image is properly prepared, acquired, and processed.

2. Open image in colocalization analysis software and perform background correction.

3. Calculate colocalization coefficients.

4. Save obtained results for further reference.

See also Table 4.19.1 for comments and tips.

Importance of Using Properly Prepared Images

Although QCA is the most important step in colocalization studies, it is just the final step. Its results are fully dependent on all previous steps of sample preparation, microscope set up, and image acquisition and handling. To emphasize the importance of image suitability to ensure reliable results of coefficients calculations, it is presented as a first step of QCA. The most important rules which should be followed to obtain confocal images useable for quantification are as follows:

Table 4.19.1 Steps of QCA

Step	Comments and tips
1. Ensure suitability of images	Make sure to use proper microscope set up and keep images unedited to prevent the loss of original information
2. Open image and correct background	Correct background in either Auto or Manual mode. Try several background correction settings to find out the best set up for your images. Once determined, keep them the same for all images in a study to ensure compatibility of calculations results.
3. Calculate coefficients	As a rule, calculate at least Pearson's correlation coefficient (R_r) and overlap coefficient according to Manders (R). Determine other coefficients as well if these two do not provide conclusive information and/or if you are interested in individual contribution of the antigens to the observed colocalization. Make sure that results of coefficients calculations fall within their values and pay attention to their dynamical changes. Use the opportunity provided by the software to view actually colocalized pixels in their image location.
4. Save obtained results for further reference	Save as much information about the analyzed image as is allowed by the software. You may find valuable information when examining image data in relation with coefficients calculations later on.

Sample preparation

1. Ensure that the antibodies you are using are specific and do not cross react.
2. Make the proper choice of fluorophores by selecting ones with well-separated excitation and emission spectra.
3. Use the same mounting medium for all of the samples that you will be performing calculations on.
4. As antifading reagents tend to increase background fluorescence, try to avoid using them.
5. Always use unstained control samples to check for autofluorescence.

Microscope set up

1. Reduce chromatic shift by using plan apochromatic lenses.
2. Maximize emission collection while avoiding bleed-through effect by using optimized emission filters.

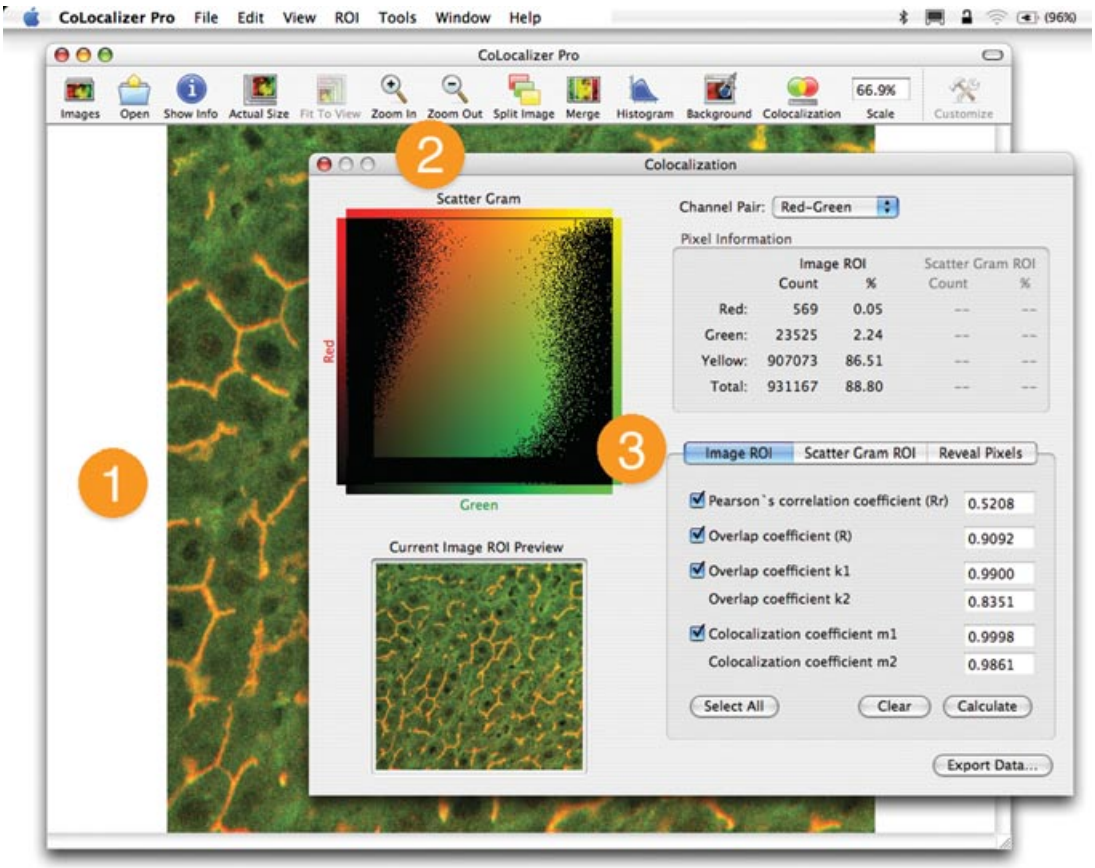


Figure 4.19.1 Main and Colocalization windows of CoLocalizer Pro software used for quantitative colocalization analysis. The image to be examined opens in the main window (1) and can be magnified to view areas with colocalization in greater detail (2). The ROI selection tool can be used for background correction in Manual mode and for selecting image area for coefficients calculations. After background correction procedure, image opens in a Colocalization window (3), where all the necessary calculations according to the selected pair of channels can be performed. For color version of this figure see *http://www.currentprotocols.com*.

3. Make sure you are using the same objective lens for obtaining all images you are planning to compare.

4. Ensure proper set up of the size of microscope pinhole.

Image acquisition and handling

1. Remember to acquire images sequentially to minimize bleed-through effect.

2. Avoid acquiring images that are too bright and images with too much contrast, as it may result in image saturation.

3. Never resave image files in any other graphic format than TIFF, as it may result in the loss of original data necessary for quantification.

4. Remember that any adjustments of brightness, contrast, etc. of your images using graphics-editing software may prevent further use of them for quantification purposes.

5. After making sure your images are suitable for colocalization studies, open them in QCA software (Fig. 4.19.1) and perform quantitative analysis according to the protocol below.

As an example of colocalized proteins, colocalization of bile salt export pump (Bsep) and multidrug resistance protein 2 (Mrp2) in the liver is shown (Fig. 4.19.2A). These proteins work in concert to regulate transport of bile salt and organic anions, respectively (Gerloff et al., 1998; Konig et al., 1999), but they are regulated differently and change their location and colocalization in response to various stimuli (Zinchuk et al., 2002, 2005b; Dombrowski et al., 2006). Bsep is stained in red, while Mrp2 is stained in green. The areas of location of both proteins are seen in yellow color. A respective scatter gram (Fig. 4.19.2B) reveals distribution of pixels according to the selected pair of channels.

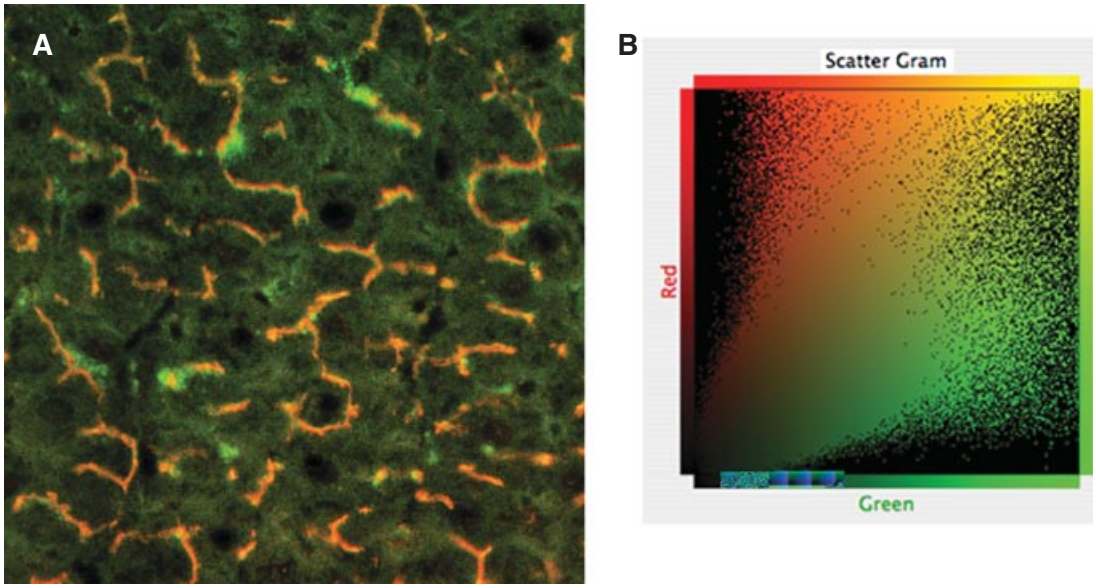


Figure 4.19.2 Confocal immunofluorescence micrograph of colocalized Bsep (red fluorescence) and Mrp2 (green fluorescence) proteins in the rat liver (**A**) alongside with its corresponding scatter gram (**B**). Note a significant number of pixels along the diagonal of Scatter Gram, a typical pixel representation of the image with colocalization. The scale bar indicates 10 μm. For color version of this figure see *http://www.currentprotocols.com*.

In the scatter gram, colocalized pixels are located along the diagonal, while those with no colocalization occupy left and bottom portions. As can be seen in the figure, the scatter gram represents unique information about an image file and is useful for viewing colocalized pixels in the image.

QUANTIATIVE COLOCALIZATION ANALYSIS

BASIC PROTOCOL

The present protocol is used to quantify colocalization in dual-color confocal microscopy images of sections stained with fluorescence-labeled antibodies with well-separated excitation spectra, obtained using correct microscope set up, and saved in the image file format that ensures preservation of image data required for quantification. It relies on the use of specialized software that enables determination of colocalization coefficients within a single session of image analysis, making calculation results not only highly relevant and comparable, but also easily reproducible. The validation of the results of quantitative colocalization experiments and their applicability are presented.

Materials

Cryostat-cut 6- to 8-μm thick sections of rat liver
Acetone for tissue fixation
Blocking solution: 10% (v/v) goat serum in 0.1 M Tris-buffered saline (TBS) containing 0.1% Triton X-100
Primary antibodies against Bsep and Mrp2 proteins [Santa Cruz Biotechnology; anti-Bsep antibody was donated by Dr. Bruno Stieger (Department of Medicine, University of Zurich, Switzerland)] or other proteins of interest (primary antibodies should be raised in different species)
Non-immune IgG to control the specificity of immunostaining
0.1 M Tris-buffered saline (TBS; *APPENDIX 2A*)
Corresponding secondary antibodies with different excitation spectra, e.g., Alexa 488 and Alexa 594 from Molecular Probes (antibodies should not be cross-reacting)
Glycerol

Poly-L-lysine-coated glass slides
Cover glass for mounting sections
Confocal microscope for image acquisition (any brand)
Argon-krypton laser (Siemens)
Software: either CoLocalizer Pro or CoLocalizer Express (CoLocalization Research Software; *http://homepage.mac.com/colocalizerpro/*) for QCA

Additional reagents and equipment for immunofluorescence staining (*UNIT 4.3*) and fluorescence microscopy (*UNIT 4.2*)

Perform immunostaining

1. Pick up a 6- to 8-μm thick section of liver on poly-L-lysine-coated glass slides, air dry for 1 hr, and fix in acetone for 30 min at −20°C.
2. Apply 500 μl blocking solution for 1 hr at room temperature to block nonspecific binding.
3. Incubate with 500 μl anti-Bsep antibody diluted 1:100 for 1 hr at room temperature.

   In parallel, perform the same procedure using non-immune IgG instead of primary antibodies to control specificity of immunostaining.
4. Rinse three times with 10 ml of 0.1 M TBS.
5. Incubate with 500 μl anti-Mrp2 antibody diluted 1:100 for another 1 hour at room temperature.

6. Rinse three times with 10 ml 0.1 M TBS.
7. Incubate with a mixture of corresponding secondary antibodies conjugated with Alexa 488 and Alexa 594, respectively, for 1 hr at room temperature. Dilute both antibodies 1:400.
8. Wash three times with 10 ml 0.1 M TBS.
9. Mount sections with glycerol and apply a coverglass.

Perform microscopy

10. Use immersion lens (e.g., Plan-Neofluar 40 /0.75 mm) of confocal microscope to examine sections.
11. Check samples for autofluorescence. If autofluorescence is detected, discard samples. If not, continue examining them.
12. Use an argon-krypton laser at the wavelength of 488 and 543 nm to visualize double fluorescence for green and red channels, respectively.
13. Keep the pinhole of the microscope at the same position when examining samples.
14. After finding satisfactory staining (not too bright and not too contrasty), perform sequential scanning for each channel to minimize the crosstalk of fluorophores.
15. Save images according to each channel (in this case you will need to merge them into one image for quantification purposes later on) or overlayed in TIFF format with maximum possible image resolution.

Transfer images from microscope and perform quantitative colocalization analysis

16. Transfer obtained images to a computer for analysis.

    We use a Mac Pro computer operated under Mac OS X 10.4.10, but even a less powerful computer is fully satisfactory. The minimum system requirement for the software is Mac OS X 10.2.8.

17. Import image to CoLocalizer Pro software for analysis and perform background correction on the imported image. Choose suitable background correction settings for your images by trying Auto mode first.

Correct background

Note the following: (1) The software will issue a warning if user attempts to perform coefficients calculations with no background corrected. (2) Low Contrast and Weak Fluorescence presets (Auto mode) may be too rough in some cases as the number of removed pixels can be excessive. (3) Successful background correction of the image with colocalization will show pixels concentrating along the diagonal of the scatter gram, while the majority of pixels at the right and bottom portions of it will be removed. The areas at right and bottom should however not be too wide. (4) For beginners, we recommend resetting the image to its original state every time when trying another background correction mode. With some experience, this may become unnecessary. (5) Once determined, background correction settings should be used consistently for all images you intend to compare. (6) Background correction will have an impact on coefficients calculations. Unsatisfactory corrected background can result in erroneous coefficients readings.

For automatic correction of background

18a. Correct background in Auto mode. After opening image (Fig. 4.19.3A) in the main window of the software, choose Background Correction under the Tools in the application menu bar or click Background icon.

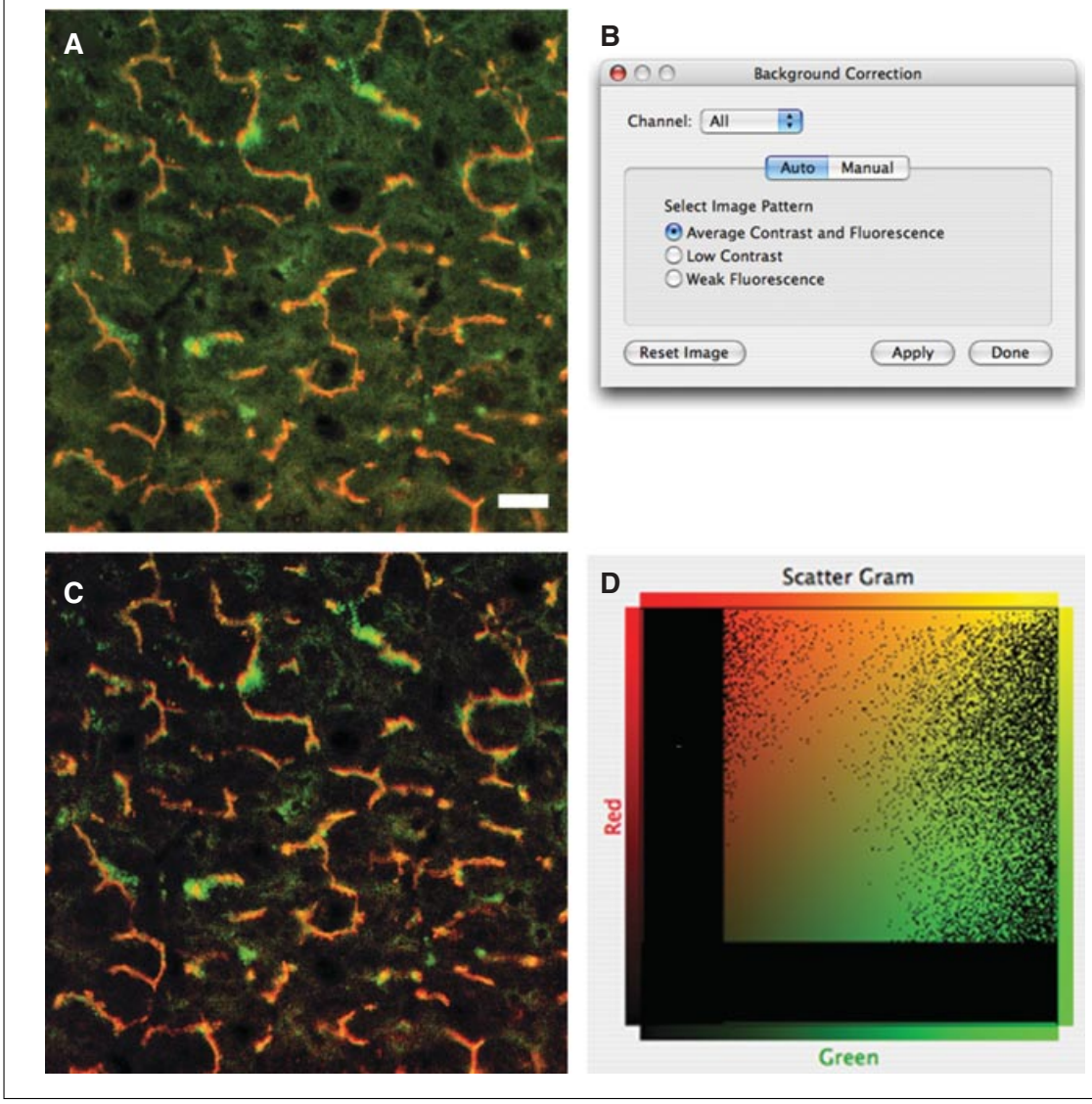


Figure 4.19.3 Background correction in Auto mode is straightforward and done by selecting one of the presets reflecting the most common image patterns. Image of colocalization of Bsep and Mrp2 proteins before (**A**) and after (**C**) correction. Background correction window (**B**) and the respective scatter gram following background correction using the Average Contrast and Fluorescence preset (**D**) are shown. Black areas at the right and the bottom of scatter gram indicate removed pixels (D). The areas are relatively narrow, which means that the number of removed pixels is acceptable. The scale bar indicates 10 μm. For color version of this figure see *http://www.currentprotocols.com*.

19a. Select Auto in the Background Correction window (Fig. 4.19.3B) and click Apply. Then, dismiss the Background Correction window by clicking Done.

Following background correction the image appears slightly changed due to the absence of the portion of pixels (Fig. 4.19.3C). The corresponding scatter gram reveals the extent of background correction (Fig. 4.19.3D).

20a. If the scatter gram shows a relatively small number of removed pixels (Fig. 4.19.3D), process the image for coefficients calculations.

If the background is more than shown, reset image and try correcting background in manual mode using Selected ROI.

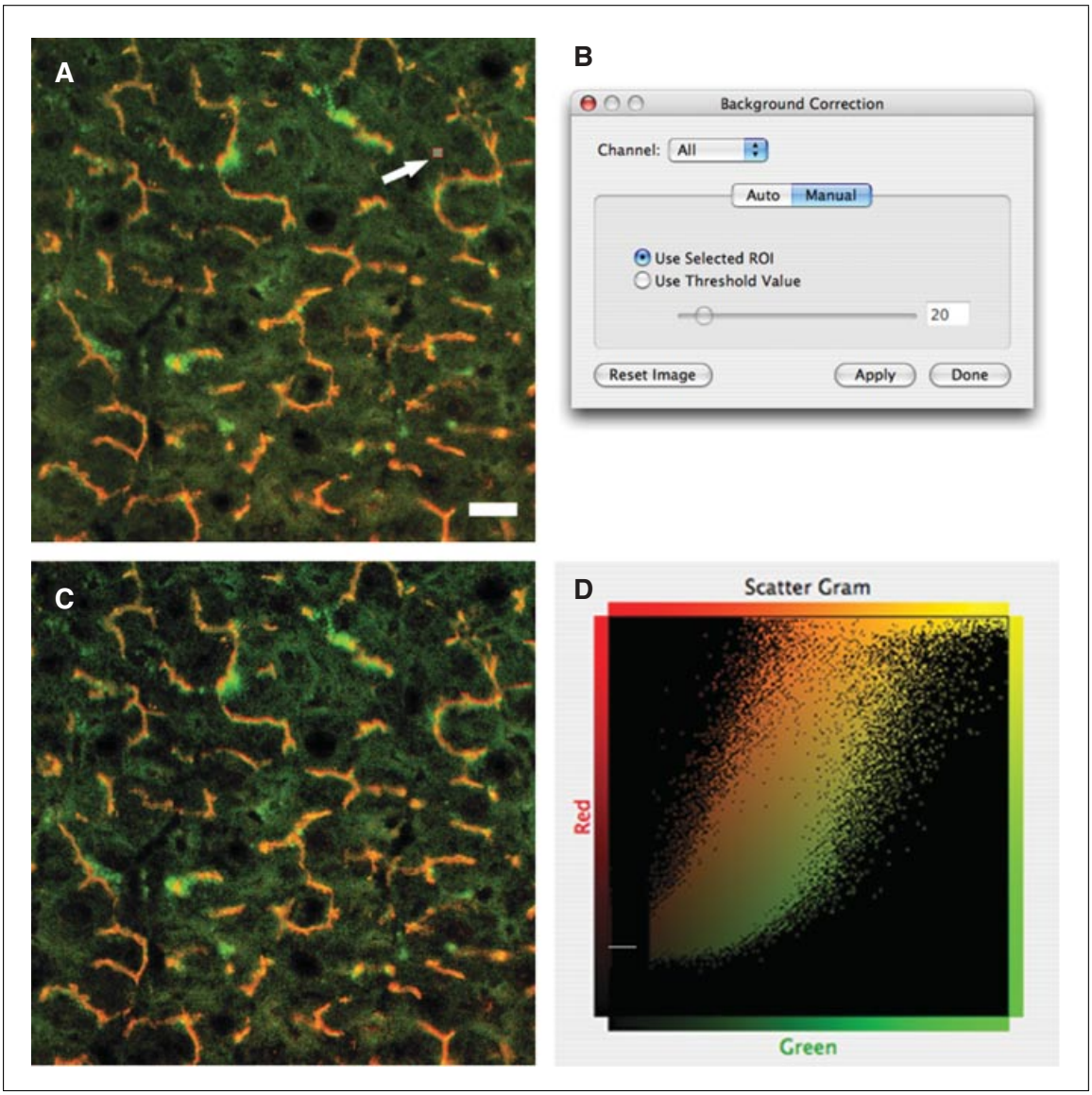


Figure 4.19.4 Background correction in Manual mode using selected ROI on the same test image before (**A**) and after (**C**) correction. Area at image background used for selecting pixel values for background correction is indicated by an arrow (A). Background correction window (**B**) and the respective scatter gram after background was corrected (**D**) are presented. Remaining pixels (yellow) tend to concentrate along the diagonal of the Scatter Gram, while the majority of pixels with Red (right portion) and Green (bottom portion) values are removed (D). The scale bar indicates 10 μm. For color version of this figure see *http://www.currentprotocols.com*.

For manual correction of background

18b. Correct background in manual mode (this is the mode we recommend to use) using Selected ROI. After opening image (Fig. 4.19.4A) in the main window of the software, choose Background Correction under the Tools in the application menu bar or click Background icon. Select Manual in the Background Correction window (Fig. 4.19.4B).

19b. Using rectangular or oval ROI selection tool, select a small area, e.g., 10 × 10 pixel size, in the image background.

If you are examining cells in culture, it is best to select any area outside of the cells. If you are examining tissue sections, select an area in the tissue background which is dark, but not completely black, i.e., that absorbed some fluorescence. Such areas can be considered as the areas containing only pixels of the background level.

20b. Then, click Apply and dismiss Background Correction window by clicking Done.

Following background correction the image will appear changed depending on the number of removed pixels (Fig. 4.19.4C). The corresponding scatter gram reveals the extent of background correction (Fig. 4.19.4D). Figure 4.19.4D shows what the scatter gram of a properly corrected image should look like: remaining pixels concentrate mainly along the diagonal of the scatter gram and the areas of removed red (at right) and green (at left) pixels are not too wide.

You may also try correcting background in Manual mode using Threshold Value. We find it useful for images that are more bright and contrasty than the average. It works similarly to Auto mode, with the exception that you are free to choose the exact number of pixels to be removed. It can provide good correction results, but, in comparison to the selected ROI option, does not give the important advantage of being able to tailor correction to the unique pixel profile of analyzed images.

21. After background correction step, perform coefficients calculations.

Calculate coefficients and view colocalized pixels

22. Using the whole image as a ROI, open Colocalization window, and proceed as described below.

23. Depending on the shape appearance of areas with colocalization, use a suitable ROI tool in the menu bar to select image areas containing predominantly colocalization.

In the test image used in this protocol, we used the Lasso tool.

24. Repeat calculations for at least three different areas with colocalization.

Thus, you will have at least four sets of data: one for the whole image and the other three for each of the selected ROIs. The resulting coefficients values will be the average of these four.

25. Select a pair of channels according to which coefficients will be calculated, for example red-green.

After selection is made, respective pixel information will be shown in the Pixel Information section of the Colocalization window (Fig. 4.19.5).

The total number of pixels shown in the Pixel Information section of the Colocalization window may not necessarily be 100% and this is correct, because pixels for colocalization analysis are counted according to channel pairs, taking into consideration color components. If, for example, a red-green pair of channels is selected, pixels with some levels of blue component may not be included to the count.

26. In the colocalization window, select coefficients you would like to calculate.

Pearson's coefficient is a default coefficient.

27. Calculate all coefficients paying particular attention to the results of Pearson's and Manders' coefficients.

28. After calculating coefficients for image ROI, perform calculations of coefficients for scatter gram ROI (M_1 and M_2 coefficients) by clicking Scatter Gram ROI tab in the Coefficients section of the Colocalization window.

29. Then, click Reveal Pixels tab in the same window section and view colocalized pixels in the image by clicking Colocalized button.

Under this tab, you may also select pixels of interest in the scatter gram and then view them in their image location by clicking Selected button. Highlight pixels by using any color you like using color picker.

30. Export calculations results as Text and Microsoft Excel files for further statistical analysis.

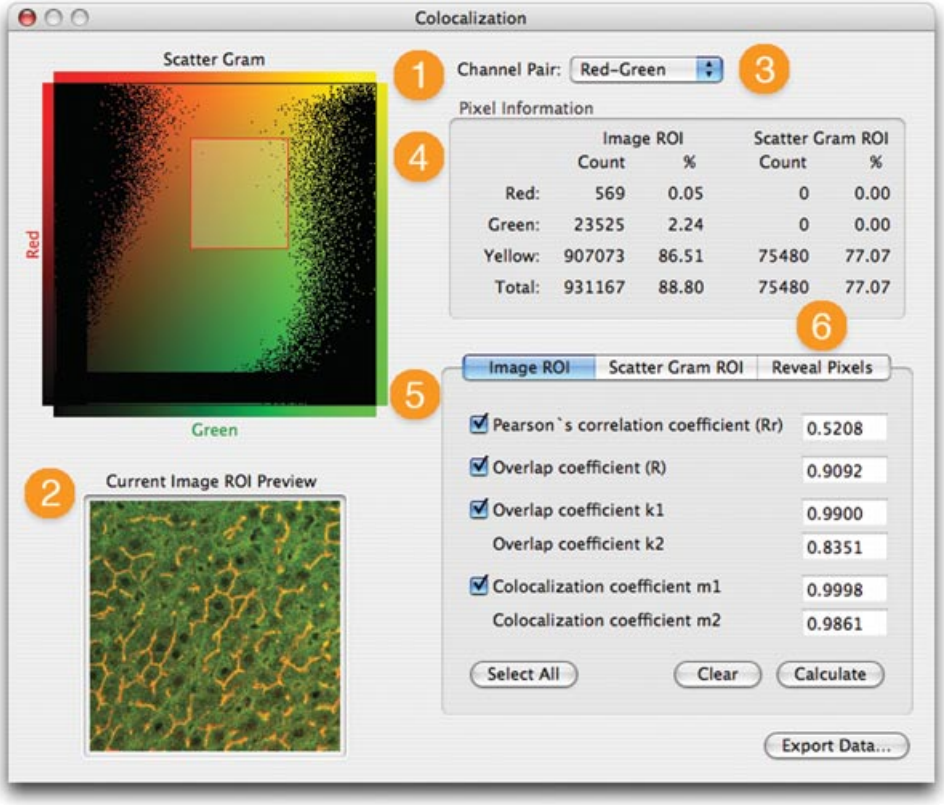


Figure 4.19.5 Colocalization window of the software shows scatter gram (1) of the selected ROI, the ROI itself (2), a pair of channels according to which the coefficients can be calculated (3), pixel information of the analyzed ROI (4), and the options to perform calculations of the coefficients (5). The important option to view exclusively colocalized pixels in the image is also given (6). All calculations results can be exported as Text and Microsoft Excel files. All data used for calculations can be saved in PDF and HTML formats and presented as session reports. For color version of this figure see *http://www.currentprotocols.com*.

Table 4.19.2 Comparison of Coefficients Obtained without Background Correction and with Background Corrected in Auto and Manual Modes[a]

Coefficients	Background not corrected	Background corrected in Auto mode	Background corrected using Selected ROI
Pearson's correlation coefficient (R_r)	0.91±0.1	0.86±0.1	0.74±0.08
Overlap coefficient according to Manders (R)	0.76±0.06	0.71±0.1	0.69±0.1
Overlap coefficients k_1 and k_2	0.99±0.1 and 0.83±0.1	0.97±0.08 and 0.73±0.07	0.91±0.07 and 0.70±0.1
Colocalization coefficients m_1 and m_2	1.0±0.1 and 1.0±0.1	0.99±0.1 and 0.98±0.09	0.99±0.09 and 0.94±0.08

[a]Calculation of coefficients with background intact results in obtaining ~20% of false-positive results. Results obtained after background corrected using Selected ROI appear to be the most applicable. An average of coefficients of five examined areas is shown. $p < 0.05$.

31. Make a report of the calculations session in either PDF or HTML format.

In addition to the analyzed image and calculations results, include in the report the image with scatter gram inserted in it. This is a very handy way to present the analyzed image together with its unique colocalization profile for publishing.

Results of coefficients calculations on the test image without and with background correction are presented in Table 4.19.2.

COMMENTARY

Background Information

The word "colocalization" is one of the most frequently used words in modern cell and molecular biological studies. It is used to describe the existence of two or more different molecules in a very close spatial position in the cell (Smallcombe, 2001). The molecules are visualized when examining images according to two or more fluorescence channels generated by corresponding fluorophores and observing the same specimen region. At the same time, the phenomenon behind it is perhaps one of the most misrepresented and misunderstood. The most common misconception is that colocalization of antigens equals sharing of their functional characteristics (North, 2006).

The theoretical basis of quantitative colocalization has been available for some time, but its adoption in practical studies has been slow. As a result, even in the current publications proteins continue to be described as "more" or "less" colocalized with no quantitative justification of any kind. Importantly, the lack of quantitative assessment does not allow researchers to extend their observations of colocalization to analyzing important changes of proteins in dynamics, as well as in association with other proteins (Lippincott-Schwartz et al., 2001).

In this protocol, we gathered in one unit all the necessary information required for performing reliable quantitative colocalization studies.

Background correction

Although confocal fluorescence microscopy allows demonstration of the precise location of fluorophores, confocal images are not usable for quantification right away because they have low signal-to-noise ratio. This means that they have high levels of background noise, which not only negatively affects image resolution by hiding structural details, but can also introduce false-positive results. It was estimated that these levels of noise can reach as high as 30% of maximum intensity of fluorescence (Landmann and Marbet, 2004). Thus, it can be approximately said that if QCA is performed on "raw" (with background intact) confocal images, the results will be 30% wrong. Therefore, prior to performing calculations of coefficients, background noise in confocal images must be removed, i.e., the background needs to be corrected. The background correction procedure, as well as all other steps, including coefficients calculations described in this protocol, are performed with the help of CoLocalizer Pro 2.0 software. The software is now widely used in various colocalization studies (Zinchuk et al., 2004, 2005a,b; Criscuoli et al., 2005; Kato et al., 2005; Cario et al., 2006; Head et al., 2006; Patel et al., 2006; Rey et al., 2006; Swaney et al., 2006; Tsutsumi et al., 2006; Berg et al., 2007; Clizbe et al., 2007; Desplanques et al., 2007; Mutch et al., 2007; Rocker et al., 2007; Van Acker et al., 2007; Watanabe et al., 2007). Although other software options to estimate colocalization exist as well, an important advantage of CoLocalizer Pro is that it is a stand-alone application not bundled with the microscopes and not tied to any proprietary image file format. The software has background correction functionality built-in, so that moving from the background correction step to calculation of colocalization coefficients is done in just a single button click. It should be mentioned that several image restoration techniques were recently introduced to improve the signal-to-noise ratio in confocal images by applying complex image restoration algorithms, but using them results in a significant image transformation. The approach based on the mentioned software, on the contrary, is quick, simple, and uses original image data. Importantly, background correction settings can be reused, thus ensuring that all images in a study are prepared in the same way, and thus the results of coefficients calculations are easily compatible and comparable. There are several options to perform the background correction procedure.

Background correction in Auto mode (option 1)

A simple and efficient way to correct background is by using presets options according to the most common image patterns. Software then uses special formulas to remove a predefined number of pixels according to these patterns, such as: (1) Average Contrast and Fluorescence, (2) Low Contrast, and (3) Weak Fluorescence.

According to their names, Average Contrast and Fluorescence, the default preset, should be used for images with average contrast and fluorescence intensity and should be suitable in the most cases. Low-contrast images should be corrected using the Low Contrast preset. If the image has weak fluorescence, the Weak Fluorescence preset should be chosen.

Background correction in Manual mode (option 2)

However, Auto mode may be too straightforward in some cases and remove too many pixels. Manual mode is much more sensitive and customizable. More importantly, it allows performance of background correction using the unique pixel profile of analyzed images. In this mode, background can be corrected using either: (1) Selected ROI or (2) Threshold Value.

Using these options, background can be corrected either for one or for all channels. Correcting for all channels is advantageous, because it is often difficult to predict what particular channel needs to be corrected. Selecting All channels guarantees that no "background noise" pixels will be left in the image. Viewing the corresponding scatter gram in the software colocalization window helps to determine the extent of pixel removal. If too many pixels are removed, the image can be reset and background correction repeated again by selecting a different area. Although this procedure requires some experience, you will become an expert in the background correction fairly quickly. When using Threshold Value, it is possible to select the exact extent of pixel removal.

Colocalization coefficients

Colocalization is determined by calculating a number of specialized values representing the proportion of colocalized pixels. These values are estimated according to colocalization coefficients (Fig. 4.19.5). The following coefficients are used.

Pearson's correlation coefficient (R_r)

$$R_r = \frac{\sum_i (S1_i - S1_{aver}) \cdot (S2_i - S2_{aver})}{\sqrt{\sum_i (S1_i - S1_{aver})^2 \cdot \sum_i (S2_i - S2_{aver})^2}}$$

where $S1$ represents signal intensity of pixels in channel 1 and $S2$ represents signal intensity of pixels in channel 2; $S1_{aver}$ and $S2_{aver}$ shows the average intensities of these respective channels. This coefficient, being one of standard measures in pattern recognition, was first employed to estimate colocalization and is used for describing the correlation of the intensity distributions between channels. It takes into consideration only similarity between shapes, while ignoring the intensities of signals. Its values range between -1.0 and 1.0, where 0 indicates no significant correlation and -1.0 indicates complete negative correlation.

Overlap coefficient according to Manders (R)

$$R = \frac{\sum_i S1_i \cdot S2_1}{\sqrt{\sum_i (S1_i)^2 \cdot \sum_i (S2_1)^2}}$$

where $S1$ represents signal intensity of pixels in channel 1 and $S2$ represents signal intensity of pixels in channel 2. This coefficient was developed specifically for estimating colocalization (Manders et al., 1993). Its advantage is that it is insensitive to the limitations of typical fluorescence imaging, such as efficiency of hybridization, sample photobleaching, and camera quantum efficiency. The values of this coefficient are in the range from 0 to 1.0. If the image has an overlap coefficient 0.5, it implies that 50% of both its objects, i.e., pixels, overlap. A value of zero means that there are no overlapping pixels.

Overlap coefficients k_1 and k_2

$$k_1 = \frac{\sum_i S1_i \cdot S2_i}{\sum_i (S1_i)^2}$$

and

$$k_2 = \frac{\sum_i S1_i \cdot S2_i}{\sum_i (S2_i)^2}$$

where $S1$ represents signal intensity of pixels in channel 1 and $S2$ represents signal intensity of pixels in channel 2. These coefficients

split the value of colocalization into a pair of separate parameters. They depend on the sum of the products of the intensities of two channels and are sensitive to the differences in the intensities of signals.

Colocalization coefficients m_1 and m_2

$$m_1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i}$$

Table 4.19.3 Coefficients Used to Estimate Colocalization with Their Meanings, Values, and Use

Coefficient	Meaning	Values	Usability
Pearson's correlation coefficient (R_r)	Describes the correlation of the intensity distribution between channels	From −1.0 to 1.0; 0 indicates no significant correlation and −1.0 indicates complete negative correlation	Useable in any colocalization experiment
Overlap coefficient according to Manders (R)	Indicates an actual overlap of the signals, is considered to represent the true degree of colocalization	From 0 to 1.0; 0.4 implies that 40% of both selected channels colocalize	Can be used in any colocalization experiment, especially applicable when the intensities of fluorescence of detected antigens differ
Overlap coefficients k_1 and k_2	Split the value of colocalization into the two separate parameters, allows determination of the contribution of each antigen to the areas with colocalization	Vary	Useable in any colocalization experiment
Colocalization coefficients m_1 and m_2	Describe the contribution of each one from two selected channels to the pixels of interest	From 0 to 1.0; m_1 and m_2 of 1.0 and 0.3 for red-green pair imply that all red pixels colocalize with green, but only 30% of green pixels colocalize with red	Useable in any colocalization experiment
Colocalization coefficients M_1 and M_2	Identical to m_1 and m_2, but applied to analyzing scatter gram ROI	From 0 to 1.0; M_1 and M_2 of 1.0 and 0.3 for red-green pair imply that all red pixels colocalize with green, but only 30% of green pixels	Useable in any colocalization experiment

Table 4.19.4 Troubleshooting Guide to QCA

Problem	Possible cause	Solution
Scatter gram shows too many pixels removed following background correction	Wrong choice of background correction method	If background was corrected in Auto mode, try using Manual mode. In Manual mode, use the Selected ROI preset and try selecting different image areas.
Scatter gram shows too few pixels removed following background correction	Background was not corrected enough	If background was corrected using Selected ROI, select different ROI. If Threshold Value was employed, use slider to increase the number of pixels to be removed.
Results of coefficients calculations do not fall within their standard values	Mistake(s) in image acquisition and handling	Obtain another image

Table 4.19.5 Summary of the Values of Coefficients Calculations Indicating Ranges When Antigens Can Be Interpreted as Colocalized and When Not[a]

Coefficient	Values indicating colocalization	Values indicating absence of colocalization
Pearson's correlation coefficient (R_r)	From 0.5 to 1.0	From −1.0 to 0.5
Overlap coefficient according to Manders (R)	From 0.6 to 1.0	From 0 to 0.6
Overlap coefficients k_1 and k_2	Any close values, like 0.5 and 0.6 or 0.8 and 0.9	Any distant values, like 0.5 and 0.9 or 0.2 and 0.7
Colocalization coefficients m_1 and m_2	More than 0.5	Less than 0.5
Colocalization coefficients M_1 and M_2	More than 0.5	Less than 0.5

[a]The table is based on a large pool of statistically significant coefficients results obtained when analyzing colocalization of various antigens in brain, heart, liver, kidney, muscle, and blood labeled by FITC, Texas Red, and various Alexa dyes.

and

$$m_2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}$$

where $S1_{i,coloc} = S1_i$ if $S2_i > 0$ and $S2_{i,coloc} = S2_i$ if $S1_i > 0$. For example, if the red-green pair of channels is selected and m_1 and m_2 are 1.0 and 0.5, respectively, this means that all red pixels colocalize with green pixels, but only half of green pixels colocalize with red ones. The value of 1.0 for both channels indicates perfect colocalization.

Colocalization coefficients M_1 and M_2

$$m1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i}$$

and

$$m2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}$$

These coefficients are identical to m_1 and m_2, but used for analyzing scatter gram ROI.

All coefficients are applied to examining selected ROI. ROI can represent the whole image or be just a small part.

For convenience, all described coefficients are also presented in Table 4.19.3.

Troubleshooting

See Table 4.19.4 for troubleshooting guide.

Anticipated Results

Table 4.19.5 gives interpretation of coefficients calculations results specifying when antigens can be considered as colocalized and when not.

Limitations

Reliable QCA is limited only by the suitability of images to be analyzed and their pixel resolution. Requirements for the suitability of images are described in the protocol in detail. Microscope-dependent suitability is constantly improving, as is the pixel resolution, together with the advancements of the quality of confocal microscopes.

Time Considerations

In comparison with performing immunohistochemical staining and confocal microscope observations, QCA requires very little time and is dependent solely on the results of immunofluorescence staining. The most time-consuming part can be the background correction step, but only until the proper setup is determined. Calculations of coefficients are usually done in a matter of minutes.

Literature Cited

Berg, K.A., Zardeneta, G., Hargreaves, K.M., Clarke, W.P., and Milam, S.B. 2007. Integrins regulate opioid receptor signaling in trigeminal ganglion neurons. *Neuroscience* 144:889-897.

Cario, E., Golenbock, D.T., Visintin, A., Runzi, M., Gerken, G., and Podolsky, D.K. 2006. Trypsin-sensitive modulation of intestinal epithelial MD-2 as mechanism of lipopolysaccharide tolerance. *J. Immunol.* 176:4258-4266.

Clizbe, D.B., Owens, M.L., Masuda, K.R., Shackelford, J.E., and Krisans, S.K. 2007. IDI2, a second isopentenyl diphosphate isomerase in mammals. *J. Biol. Chem.* 282:6668-6676.

Criscuoli, M.L., Nguyen, M., and Eliceiri, B.P. 2005. Tumor metastasis but not tumor growth is dependent on Src-mediated vascular permeability. *Blood* 105:1508-1514.

Desplanques, A.S., Nauwynck, H.J., Tilleman, K., Deforce, D., and Favoreel, H.W. 2007. Tyrosine phosphorylation and lipid raft association of pseudorabies virus glycoprotein E during antibody-mediated capping. *Virology* 362:60-66.

Dombrowski, F., Stieger, B., and Beuers, U. 2006. Tauroursodeoxycholic acid inserts the bile salt export pump into canalicular membranes of cholestatic rat liver. *Lab. Invest.* 86:166-174.

Gerloff, T., Stieger, B., Hagenbuch, B., Madon, J., Landmann, L., Roth, J., Hofmann, A.F., and Meier, P.J. 1998. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J. Biol. Chem.* 273:10046-10050.

Head, B.P., Patel, H.H, Roth, D.M., Murray, F., Swaney, J.S., Niesman, I.R., Farquhar, M.G., and Insel, P.A. 2006. Microtubules and actin microfilaments regulate lipid raft/caveolae localization of adenylyl cyclase signaling components. *J. Biol. Chem.* 281:26391-26399.

Kato, T., Muraski, J., Chen, Y., Tsujita, Y., Wall, J., Glembotski, C.C., Schaefer, E., Beckerle, M., and Sussman, M.A. 2005. Atrial natriuretic peptide promotes cardiomyocyte survival by cGMP-dependent nuclear accumulation of zyxin and Akt. *J. Clin. Invest.* 115:2716-2730.

Konig, J., Nies, A.T., Cui, Y., Leier, I., and Keppler, D. 1999. Conjugate export pumps of the multidrug resistance protein (MRP) family: Localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim. Biophys. Acta* 1461:377-394.

Landmann, L. and Marbet, P. 2004. Colocalization analysis yields superior results after image restoration. *Microsc. Res. Tech.* 64:103-112.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* 2:444-456.

Manders, E.M.M., Verbeek, F.J., and Aten, J.A. 1993. Measurement of co-localization of objects in dual-colour confocal images. *J. Microsc.* 169:375-382.

Mutch, C.M., Sanyal, R., Unruh, T.L., Grigoriou, L., Zhu, M., Zhang, W., and Deans, J.P. 2007. Activation-induced endocytosis of the raft-associated transmembrane adaptor protein LAB/NTAL in B lymphocytes: Evidence for a role in internalization of the B cell receptor. *Int. Immunol.* 19:19-30.

North, A.J. 2006. Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. *J. Cell Biol.* 172:9-18.

Patel, H.H., Head, B.P., Petersen, H.N., Niesman, I.R., Huang, D., Gross, G.J., Insel, P.A., and Roth, D.M. 2006. Protection of adult rat cardiac myocytes from ischemic cell death: Role of caveolar microdomains and delta-opioid receptors. *Am. J. Physiol. Heart Circ. Physiol.* 291:H344-H350.

Rey, O., Young, S.H., Papazyan, R., Shapiro, M.S., and Rozengurt, E. 2006. Requirement of the TRPC1 cation channel in the generation of transient Ca2+ oscillations by the calcium-sensing receptor. *J. Biol. Chem.* 281:38730-38737.

Rocker, C., Manolov, D.E., Kuzmenkina, E.V., Tron, K., Slatosch, H., Torzewski, J., and Nienhaus, G.U. 2007. Affinity of C-reactive protein toward FcgammaRI is strongly enhanced by the gamma-chain. *Am. J. Pathol.* 170:755-763.

Smallcombe, A. 2001. Multicolor imaging: The important question of co-localization. *Biotechniques* 30:1240-1246.

Swaney, J.S., Patel, H.H., Yokoyama, U., Head, B.P., Roth, D.M., and Insel, P.A. 2006. Focal adhesions in (myo)fibroblasts scaffold adenylyl cyclase with phosphorylated caveolin. *J. Biol. Chem.* 281:17173-17179.

Tsutsumi, Y.M., Patel, H.H., Huang, D., and Roth, D.M. 2006. Role of 12-lipoxygenase in volatile anesthetic-induced delayed preconditioning in mice. *Am. J. Physiol. Heart Circ. Physiol.* 291:H979-H983.

Van Acker, G.J., Weiss, E., Steer, M.L., and Perides, G. 2007. Cause-effect relationships between zymogen activation and other early events in secretagogue-induced acute pancreatitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292:G1738-G1746.

Watanabe, T., Sorensen, E.M., Naito, A., Schott, M., Kim, S., and Ahlquist, P. 2007. Involvement of host cellular multivesicular body functions in hepatitis B virus budding. *Proc. Natl. Acad. Sci. U. S. A.* 104:10205-10210.

Zinchuk, V.S., Okada, T., Akimaru, K., and Seguchi, H. 2002. Asynchronous expression and colocalization of Bsep and Mrp2 during development of rat liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 282:G540-G548.

Zinchuk, O., Fukushima, A., Hangstefer, E., and Ueno, H. 2004. Dynamics of PAF-induced conjunctivitis reveals differential expression of PAF receptor by macrophages and eosinophils in the rat. *Cell Tissue Res*. 317:265-277.

Zinchuk, O., Fukushima, A., Zinchuk, V., Fukata, K., and Ueno, H. 2005a. Direct action of platelet activating factor (PAF) induces eosinophil accumulation and enhances expression of PAF receptors in conjunctivitis. *Mol. Vis.* 11:114-123.

Zinchuk, V., Zinchuk, O., and Okada, T. 2005b. Experimental LPS-induced cholestasis alters subcellular distribution and affects colocalization of Mrp2 and Bsep proteins: A quantitative colocalization study. *Microsc. Res. Tech.* 67:65-70.

Key References

Manders et al., 1993. See above.

First description of the correlation coefficient and examples of its use.

Smallcombe, 2003. See above.

Practical look at colocalization, some critical views and advice for performing colocalization experiments.

North, 2006. See above.
Review with focus on proper interpretation of the results of fluorescence microscopy studies, drawbacks and limitations of biological imagery.

Internet Resources

http://homepage.mac.com/colocalizerpro/
Web site of CoLocalization Research Software, creators of CoLocalizer Pro and CoLocalizer Express software applications used for quantitative estimation of colocalization.

Visualizing Protease Activity in Living Cells: From Two Dimensions to Four Dimensions

Christopher Jedeszko,[1] Mansoureh Sameni,[1] Mary B. Olive,[1] Kamiar Moin,[1] and Bonnie F. Sloane[1]

[1]Department of Pharmacology and Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan

ABSTRACT

Proteolytic degradation of extracellular matrix (ECM) components by cells is an important metabolic activity as cells grow, remodel, and migrate through the ECM. The ability to analyze ECM degradation can be valuable in the study of developmental processes as well as pathologies, such as cancer. In this unit we describe an in vitro live cell–based method to image and quantitatively measure the degradation of ECM components by live cells. Cells are grown in the presence of fluorescent dye-quenched protein substrates (DQ-gelatin, DQ-collagen I, and DQ-collagen IV) that are mixed with protein matrices. Upon proteolytic cleavage, fluorescence is released that directly reflects the level of proteolysis by the cells. Using confocal microscopy and advanced imaging software, the fluorescence is detected and accurate measurements of proteolytic degradation in three and four dimensions can be assessed. *Curr. Protoc. Cell Biol.* 39:4.20.1-4.20.15. © 2008 by John Wiley & Sons, Inc.

Keywords: 3-D culture • recombinant basement membrane • DQ-substrates • proteases • live cell imaging • ECM

INTRODUCTION

In vivo, cellular microenvironments are constructed of complex combinations of extracellular matrix (ECM) proteins that are under constant turnover and modification. During most forms of cellular motility, cells remodel their immediate microenvironment via proteolytic degradation of ECM (Friedl and Wolf, 2003). Thus, the ability to examine the proteolytic degradation of ECM by living cells is crucial for understanding many aspects of cellular motility in normal processes and in pathologies, such as cancer. The assay described here allows one to quantitatively analyze extracellular and intracellular proteolysis associated with degradation of ECM surrounding the cells. This is a microscopy-based assay in which proteolysis of fluorogenic dye-quenched protein substrates (DQ-substrates) by live cells is imaged. The cells are grown as either two-dimensional (2-D) or three-dimensional (3-D) cultures on ECM or embedded in ECM, respectively. The DQ-substrates are mixed into the ECM and provide a protein substrate that can be cleaved by many proteases. This differs from the use of small molecule synthetic substrates that are cleaved selectively by a single protease or protease class.

Originally, the DQ-substrates were developed as nonselective, broad-spectrum substrates for in vitro applications, such as analysis of protease activity in solution (Menges et al., 1997). We have adapted their use for live-cell confocal imaging in order to investigate the contribution of multiple classes of proteases to ECM degradation by migrating or invading cells. Visualization and accurate quantitative analysis of ECM proteolysis relies on the fluorescent characteristics of the DQ-substrates. The native, undegraded DQ-substrates are heavily conjugated with the fluorogenic dye, fluorescein. The close

Current Protocols in Cell Biology 4.20.1-4.20.15, June 2008
Published online June 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0420s39

proximity of the dye molecules to each other renders native DQ-substrates fluorescently quenched due to a Fluorescence Resonance Energy Transfer (also known as Forster Resonance Energy Transfer or FRET) effect. Upon proteolytic hydrolysis of the DQ-substrate into smaller fragments, the fluorogenic dye molecules are separated, self-quenching is lost, and a strong fluorescence is emitted. The fluorescent signal is proportional to the proteolytic activity, and the pattern depends on the DQ-substrate itself. There is little background fluorescence in the absence of cells for either DQ-collagen I (Fig. 4.20.1A) or DQ-collagen IV (Fig. 4.20.1C). The cellular cleavage of DQ-collagen I (Fig. 4.20.1B) or DQ-collagen IV (Fig. 4.20.1D) differs, with the former appearing as fluorescent dots along the collagen I fibrils and the latter as diffuse fluorescence. Depending on the cell lines and ECM, cells will grow as either 2-D monolayers, e.g., on gelatin and collagen I, or as 3-D structures on complex matrices, such as reconstituted basement membrane (rBM; Fig. 4.20.2). The ECM can be mixed with any one of an assortment of DQ-substrates (e.g., DQ-albumin, DQ-gelatin, DQ-collagen I, or DQ-collagen IV) available through Invitrogen, thus creating customized combinations through which to analyze proteolysis in the context of a defined ECM. Recent studies from our laboratory and others employing this assay have shown that normal and tumor cells use several classes of proteases to actively degrade collagen IV and collagen I and, depending on the cell type, the degradation products are found intracellularly and/or extracellularly (Sameni et al., 2003; Cavallo-Medved et al., 2005; Podgorski et al., 2005; Tsai et al., 2005; Urbich et al., 2005; Sloane et al., 2006). We describe here the use of three combinations of matrices:

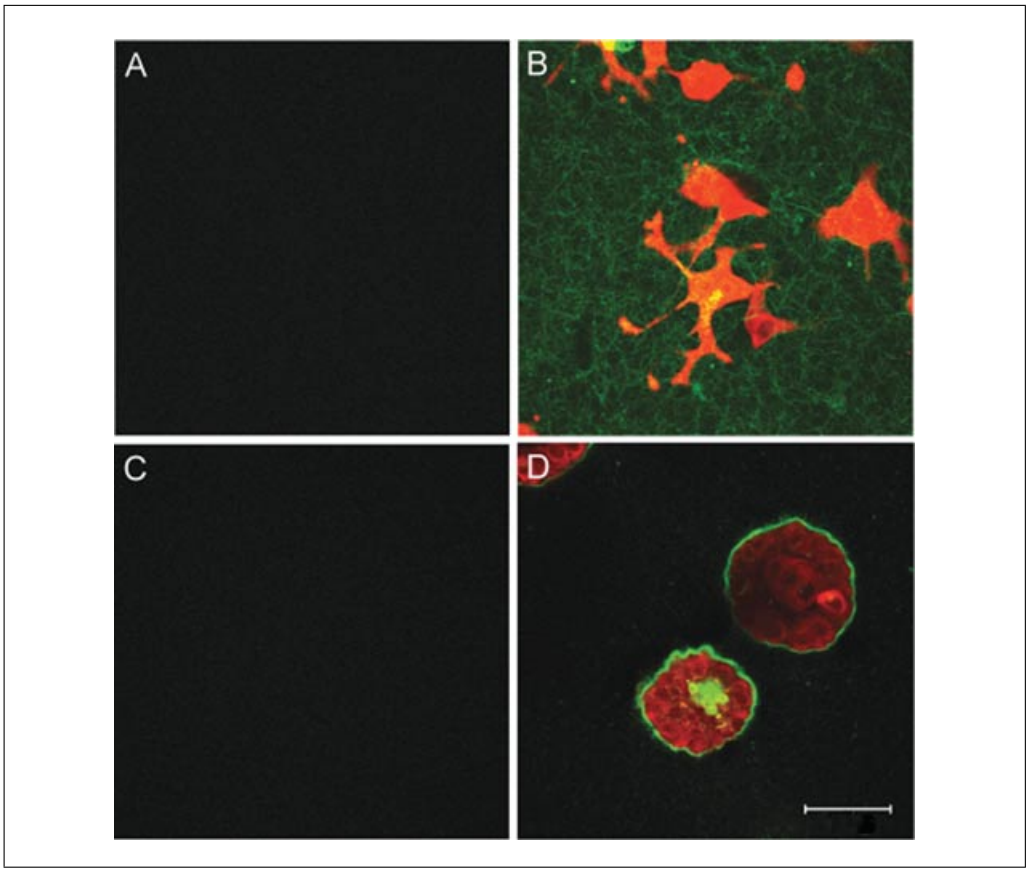


Figure 4.20.1 Culture with live cells is required to cause release of green fluorescence from DQ-substrates. Confocal images of green fluorescent channel at a *z*-level below the ECM-medium border. Cultures containing either collagen I with DQ-collagen I (**A,B**) or rBM containing DQ-collagen IV (**C,D**) were imaged after 3 days of culture without cells (A,C), with human breast cancer cells (B) or with human breast epithelial cells (D). Cells were prelabeled with CellTracker Orange. Bar, 50 μm. For color version of this figure see *http://www.currentprotocols.com*.

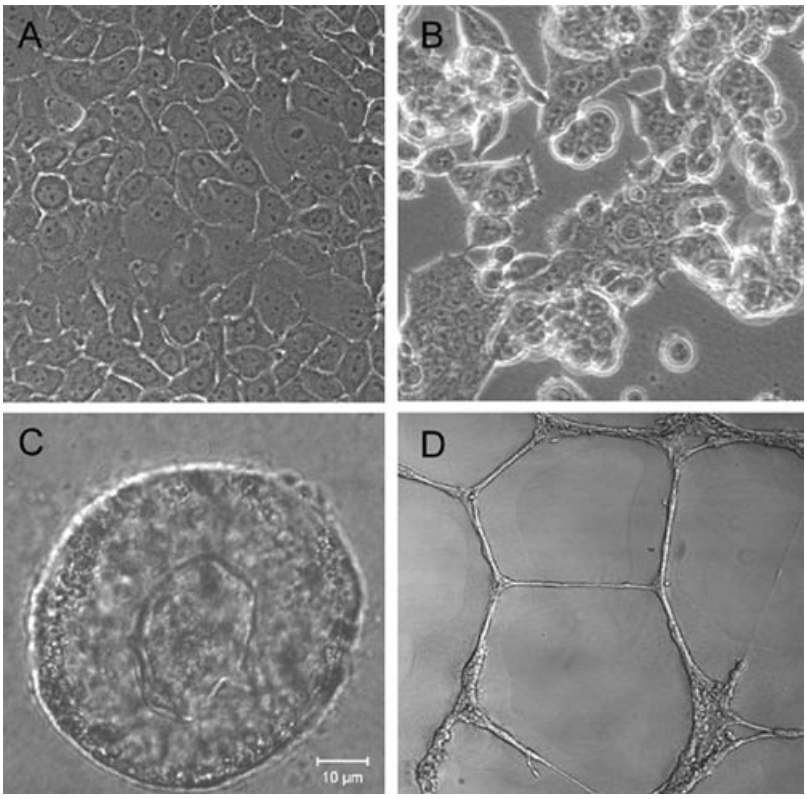


Figure 4.20.2 Examples of cellular morphologies observed on different ECM. Phase contrast or DIC images of: (**A**) human breast cancer cells forming a simple monolayer on gelatin; (**B**) human prostate cancer cells forming clusters and sheets on collagen type I; (**C**) human colon carcinoma cells forming a 3-D spheroid of polarized cells surrounding a hollow lumen in rBM; and (**D**) human umbilical vein endothelial cells forming a tubular network of cellular cords on rBM.

(1) DQ-collagen I in collagen I; (2) DQ-gelatin in gelatin; and (3) DQ-collagen IV in recombinant basement membrane (rBM; Support Protocol 1).

QUANTITATIVE MEASUREMENT OF PROTEOLYTIC DEGRADATION OF DQ-SUBSTRATES IN LIVE-CELL CULTURES USING A LASER SCANNING CONFOCAL MICROSCOPE

BASIC PROTOCOL 1

Cells are grown on ECM containing DQ-substrates as described in Support Protocol 1. Although either an inverted or an upright laser scanning confocal microscope can be used for imaging live cells, this assay is most readily performed using an upright microscope. An upright microscope is ideally suited due to the inherent thickness (1 to 3 mm) of the culture sample (DQ-substrate in ECM + cells). Regardless of the microscope, the sample thickness requires the use of objectives with long working distances. For live-cell imaging at high magnification on an upright microscope, we routinely use water-immersion dipping objectives (40× and 63× Achroplan) that can be immersed in the cell culture medium. Note that these are different than the normal high-power Apochromat water-immersion objectives. The latter have very-short working distances (10 to 15 µM) and cannot be used with thick specimens to obtain a sharp focus through the entire sample. For imaging with an inverted microscope, one will use either a lower magnification lens or a long-working-distance lens (see Commentary). In our laboratory, we routinely use a Zeiss upright microscope equipped with Zeiss LSM 510 META confocal optics and argon, HeNe, and two-photon lasers. Together with a microscope stage incubator, this setup allows a high level of flexibility in regard to sample dimensions, imaging timeframes, and fluorogenic dyes.

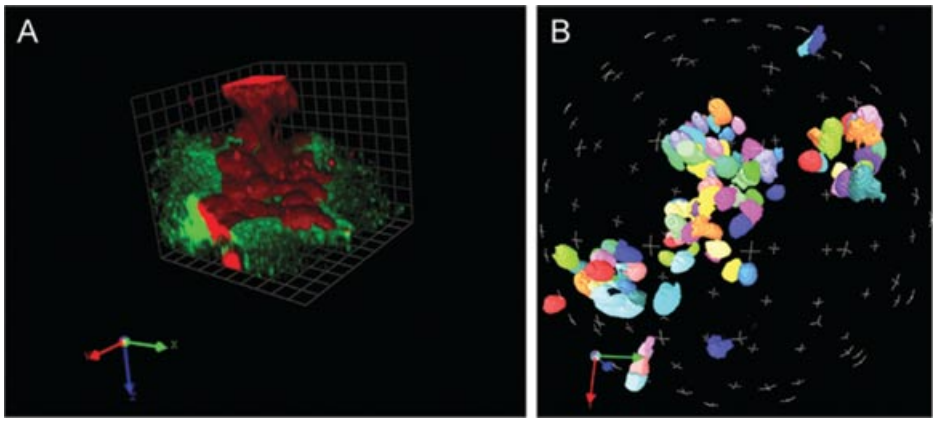


Figure 4.20.3 Frame captures of 3-D reconstructions of human breast cancer cells grown in rBM containing DQ-collagen IV. Image stacks containing the DQ-substrate channel (green), the nuclei channel (blue), and the CellTracker channel (red) were used to create graphical 3-D reconstructions/projections using the QVTR feature of the Volocity imaging software suite. The 3-D image can be rotated 360° in order to (**A**) view proteolysis of DQ-substrates (green) in relation to the cells (red) or (**B**) view only the nuclei for the purpose of counting cells. For color version of this figure see *http://www.currentprotocols.com*.

The key to quantitative imaging of proteolysis in three dimensions is the ability to acquire fluorescence without pixel saturation from single planes of focus encompassing the entire specimen. Confocal microscopy allows us to image sequentially above, through, and below the objects of interest. This optical *z*-sectioning and imaging of accumulated fluorescence gives a spatial 3-D output that represents proteolysis of DQ-substrates. Furthermore, the same field of view can be imaged repeatedly over a period of time to collect data in four dimensions (4-D), i.e., spatially and temporally. Regardless of whether data collection is over time or just a snapshot of one time point, accurate determination of proteolytic activity and localization of such activity requires the collection of data from all the cells and their surrounding 3-D volume (*x,y,z*-limits). As some cell types aggressively invade into the ECM, the optical sections may need to span several hundred micrometers in the *z*-direction. Therefore, prelabeling the cells (Support Protocol 1) and their nuclei (Basic Protocol 1) with fluorescent dyes will enable the user to not only define the entire relevant 3-D volume, but also to relate total DQ-substrate hydrolysis to cell number. In addition, prelabeling the cells with cell tracking dyes that occupy the entire cell enables differentiation of intracellular from extracellular proteolysis and their quantification (Basic Protocol 2). The additional data from nuclear and cytoplasmic staining allows normalization of proteolysis to cell number across samples. Visually, most advanced imaging applications are capable of transforming the collection of 2-D images from optical *z*-sections to graphical 3-D reconstructions of cells and areas of DQ-substrate degradation (Figure 4.20.3).

Materials

Cell-permeable DNA-binding dye [Hoechst 33342 (Invitrogen) or DRAQ5 (Biostatus Ltd)]

Cell culture medium

Prelabeled cell cultures established in ECM containing DQ-substrates (Support Protocol 1), grown on coverslips in 35-mm cell culture petri dishes

Upright laser scanning confocal microscope equipped with a water-immersion lens and appropriate filter sets and lasers

Microscope stage incubator with humidity, temperature, and CO_2 control

Prepare for imaging

1. Set up the microscope and stage incubator.

   In order to avoid power fluctuations during early imaging, turn on the microscope lasers for at least 5 min prior to imaging. When imaging over extended periods of time it is important to equilibrate the stage incubator to 37°C and 5% CO_2 and 40% to 50% noncondensing humidity.

2. To the 35-mm cell culture petri dishes containing prelabeled cell cultures, add DNA-binding fluorescent dye (Hoechst 33342) to a final concentration of 5 μg/ml in culture medium.

   The culture medium is unspecified as medium is user defined for the individual cell line(s) to be studied.

   When performing live-cell imaging over extended periods of time, the time to add the DNA dye is an important consideration. Some DNA binding agents like Hoechst 33342 and Draq5 are not toxic over short periods of time, yet may have adverse effects on the cells over longer times (hours). Therefore, it is recommended that these dyes be added to the culture medium 10 min before short-term imaging (<1 hr) or 1 hr before the end of long-term imaging. Alternatively, cells can be engineered to express a nuclear-targeted fluorescent protein.

3. Place the 35-mm cell culture dish on the microscope stage.

   Ensure that the coverslip is located in the center of the culture dish. This will allow ample room for the objective to navigate the entire surface of the coverslip.

4. Identify the structures of interest.

   Choose structures that are representative of the population being analyzed. The thickness of structures being imaged should not exceed the focal limit of the microscope.

Establish imaging parameters

5. Using a fast-scanning mode on the green channel (excitation 488 nm, emission 521 nm), bring structures into the focal plane with the brightest fluorescence.

6. Scan an image of the structure at the desired excitation light intensity, line averaging, zoom, etc.

7. Adjust the detector pinhole diameter to one airy unit.

8. Modify detector gain for maximal fluorescence signal without pixel saturation (pixel intensities that exceed the detector scale, i.e., >255 for an 8-bit image).

   Saturated pixels only register as the maximum detector value, 255, so the true pixel intensity cannot be calculated under these conditions. Detector gain and offset will vary depending on the DQ-substrate used (type/lot number) and the laser power. The imaging parameters should be held constant for all z-sections through the 3-D structures.

9. Repeat the same adjustments (steps 5 through 8) for the other channels used.

10. Define the *z* distance limits, including the first and last optical sections of a stack as well as the *z*-stack interval distance between optical sections.

    Use a fast-scan mode along with the manual focus adjustment to determine the focal plane in which the fluorescence signal from the DQ-substrate begins and ends. This is usually near the top and bottom of the cellular structures. These positions will be different for each structure imaged and will have to be determined for each structure. Larger structures and highly motile cells will require a greater number of sections to image the entire volume (x,y,z). Although the number of sections will change from structure to structure, the thickness of each section and distance between z-sections must be kept consistent in order to accurately compare structures.

Collect fluorescence data

11. Create a *z*-stack of images including scans for the DQ-substrate, DNA binding dye, and cell tracking dye.

    At each focal plane the fluorescence corresponding to all channels is collected. The green fluorescence represents cleaved DQ-substrate. The fluorescence from cytoplasmic cell tracking dyes, such as CellTracker, will be used to identify cellular boundaries. The fluorescence from DNA-binding dyes will define the nuclear area in all cells not in late mitosis and allow determination of cell number.

12. Collect at least two to four different *z*-stacks of representative cellular structures and surrounding matrix from each experimental condition and treatment.

BASIC PROTOCOL 2

QUANTITATIVE ANALYSIS OF CONFOCAL IMAGE *Z*-STACKS

Following the acquisition of data in the form of *z*-stacks, image analysis software is used for quantification. The fluorescence data acquired due to the proteolytic cleavage of DQ-substrates can be used to visualize DQ-substrate proteolysis and quantify proteolysis in particular areas or entire volumes (Fig. 4.20.4).

Materials

Z-stacks (Basic Protocol 1)

Computer system capable of processing large image files: 350 mHz or faster processor, large storage capacity, and at least 256 MB video RAM and minimum 1 GB system RAM

Advanced image processing/analysis software capable of applying thresholds and measuring pixel intensities [e.g., ImageJ (Open Source, Public Domain), MetaMorph (Molecular Devices), and Volocity (Improvision)]

Process the confocal image stacks for analysis

1. Export confocal *z*-stacks.

   This is performed only if the microscope software saves image stacks in a format that cannot be read by the available image analysis software. It is important to export in a format that retains all image information. We recommend that images be exported as uncompressed Tagged Image File Format (TIFF). This file format will be accepted by all image analysis software.

2. Open an image stack in its original file format or reassemble the exported TIFF files as an image stack.

   Almost all advanced image processing software packages can assemble a series of two-dimensional images into a three-dimensional image stack, provided the image names end in sequential numbers designating their positions in the stack.

3. Separate the DQ-substrate channel, the cell tracker channel, and the DNA-binding dye channel.

   The two-dimensional images containing data from DQ-substrate cleavage, cell tracking, and DNA-binding dyes are all captured on different channels. Fluorescence resulting from the cleavage of DQ-substrates is captured on the green channel due to the emission spectrum of the fluorochrome (FITC). In order to quantify the image information from this channel, it must first be separated from all other channels and processed for analysis. The result of separation will be three 8-bit gray-scale image stacks. The green channel stack will yield the total (extracellular and intracellular) degradation information. The red channel will be used to delineate the intracellular cytoplasmic area of cells and be used to separate intracellular from extracellular areas in the green channel (step 7). The blue channel will be used to determine the number of cells as long as each cell contains only one nucleus (step 10).

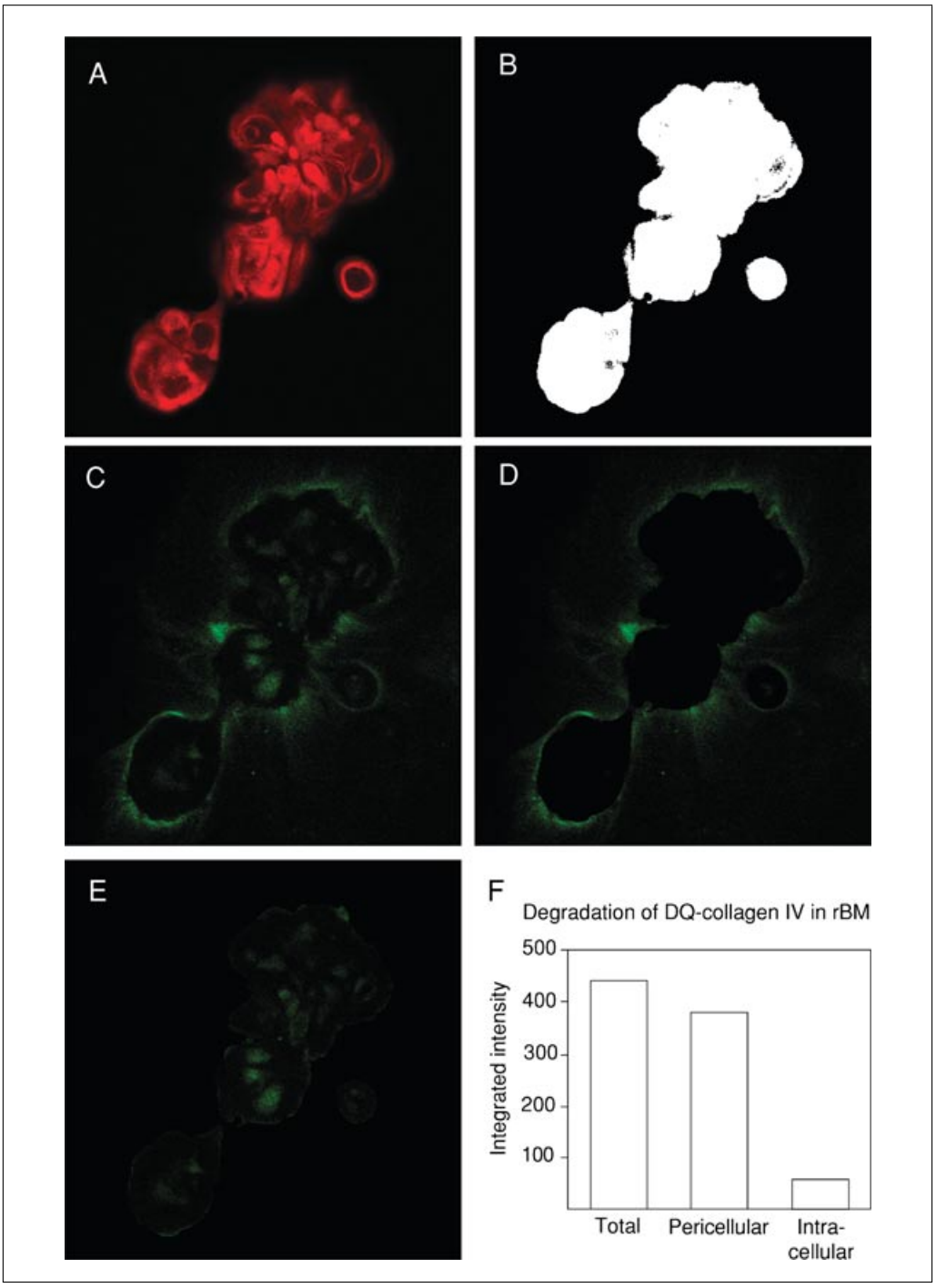


Figure 4.20.4 Quantification of proteolysis and discrimination of intracellular and extracellular localization of degradation fragments in human breast epithelial cells grown in rBM containing DQ-collagen IV. (**A**) Single optical section at equatorial plane showing fluorescence of the CellTracker Orange channel. (**B**) Same optical section but a binarized image of the CellTracker Orange and DNA dye channels. (**C**) Total (extracellular and intracellular) degradation fragments of DQ-collagen IV in same optical section. (**D**) Extracellular degradation fragments of DQ-collagen IV, obtained by masking image of panel C with the image from panel B to eliminate all signal from intracellular areas. (**E**) Intracellular degradation fragments of DQ-collagen IV. (**F**) Total degradation fragments of DQ-collagen IV in all optical sections were quantified as normalized integrated intensity per cell. Total fluorescence separated into intracellular and pericellular components using image arithmetic in MetaMorph software. For color version of this figure see *http://www.currentprotocols.com*.

4. Apply an inclusive intensity threshold to the DQ-substrate channel.

   Prior to measuring pixel intensities, most imaging programs require that the image data that constitutes signal be in some way distinguished from nonsignal, or background, image data. This is commonly referred to as thresholding or segmentation and is usually applied on the basis of gray-scale levels. Such a threshold defines the range of gray-scale values that describe the regions of interest. Subsequent measurements are applied only to pixels that fall within this range. Although the selection is arbitrary, it must be held constant for all image stacks used for comparison. It is a good idea to record these threshold ranges for later reference. Use image stacks that represent the most intense and least intense signals when determining threshold range

5. Measure the intensities of all pixels under the threshold and log data to a spreadsheet.

   Many advanced imaging software applications have several measures of intensity. It is best to measure integrated intensity rather than average intensity, as this will incorporate the threshold range applied earlier. Some software packages output one integrated intensity value for each x,y image plane in the stack. In this case, sum this value across image planes and export data to a spreadsheet. This value will represent total degradation and later be averaged by the number of cells.

Separate intracellular from extracellular degradation

6. Combine the cell tracking and DNA-binding dye channels using an arithmetic addition function.

   In order to completely mask all intracellular degradation, the fluorescence intensity data from the cell tracking and DNA-binding dye channels need to be combined to make a new stack.

7. Binarize the stack created in step 6.

   The fluorescence intensity data from the new stack need to be maximized or saturated at all pixels representing intracellular areas. This is done by converting the 8-bit image stack into a binary image stack. The result of the binerization is an image stack in which all pixels that contained cell tracking and DNA binding dye fluorescence are now at maximum intensity.

8. Determine pericellular degradation by applying an arithmetic subtraction of the binerized data obtained in step 7 from the DQ-substrate channel image stack.

   To calculate extracellular degradation (Degradation$_P$), the fluorescence information (gray value) from areas considered intracellular must be removed using an image arithmetic subtract function. The binarized stack (C) from step 7 provides the spatial information (x,y coordinates) needed for the imaging software to subtract the gray-value data from corresponding x,y coordinates in the total DQ-substrate channel stack (Degradation$_T$). The result of this function is the creation of a new stack (Degradation$_P$) containing only fluorescence data from extracellular regions. Measure and record the intensities of all pixels in the image stack.

$$\text{Degradation}_\text{P} = \text{Degradation}_\text{T} - \text{C}$$

9. Determine intracellular degradation by applying an arithmetic subtraction of the pericellular degradation stack created in step 8 from the total DQ-substrate channel stack (Degradation$_\text{T}$).

   To calculate intracellular degradation, the fluorescence information (gray value) from the extracellular degradation stack (Degradation$_P$) must be subtracted from the total DQ-substrate channel (Degradation$_T$). The result of this operation is the creation of a new stack (Degradation$_I$) containing only fluorescence data from intracellular regions. Measure and record the intensities of all pixels of the image stack.

$$\text{Degradation}_\text{I} = \text{Degradation}_\text{T} - \text{Degradation}_\text{P}$$

Determine the cell number in image stack

10. Create a 3-D reconstruction using the fluorescence from the DNA-binding-dye channel.

    Due to inherent differences in the sizes and shapes of cellular structures within an imaged area, degradation information (total, intracellular, pericellular) is normalized to the number of cells represented by the number of nuclei present. Accurate determination of nuclei requires the software to render nuclei in three dimensions and display a visual representation of the rendering that can be manipulated in three dimensions (x,y,z-directions) by the user.

11. Use a classifier to define all nuclei as objects and segment them from one another.

    Most advanced image analysis software allows classification of objects based on intensity thresholds and size constraints. Many also have functions like noise reduction and separation of touching objects via an edge detection algorithm and shape constraints. Some software packages even allow classification of objects based on morphometric parameters, such as X and Y centroid, radial dispersion, texture moment difference, etc. These additional parameters are not generally necessary for the discrimination of ovoid objects, such as nuclei from the background and from each other. Create a custom classifier combining as many classification criteria as needed to define nuclei as discrete objects in three dimensions. For the first image, it may be necessary to apply the classifier more than once, evaluating its ability to successfully describe the nuclei. When satisfied, save the classifier. It should apply to the other images as well, provided the same settings were used to acquire the images.

12. Count the objects.

    If a counting option does not exist in the software, manually count the objects classified. Each measurement represents one object. Advanced imaging software will generally apply a unique color overlay to each identified object to assist the user in visualization of the distinct objects.

13. Evaluate the classifier in 3-D rendered mode.

    Evaluate the classifier once again by rotating the 3-D structure and viewing the overlay resulting from the measurement. If satisfied that the classifier has correctly segregated individual nuclei, record the count in the spreadsheet. Because this is highly subjective, more than one observer should evaluate.

14. Divide the desired intensity measure from the DQ-substrate channel by the total number of nuclei to obtain degradation per cell.

PREPARE CELL CULTURES ON ECM CONTAINING DQ-SUBSTRATES

SUPPORT PROTOCOL 1

The cell cultures being imaged must be grown on or within ECM containing the desired DQ-substrate. The ECM can exert a dramatically different effect on cellular morphology and physiology. The formation of unique structures such as monolayers, spheroids, acini, tubes, and branching clusters may depend on the composition and stiffness of ECM, media supplements, densities of cell seeding, etc. We have found that on gelatin and collagen type I, cells primarily grow as 2-D monolayers like those seen on uncoated plastic and glass. In contrast, in complex matrices they form 3-D structures (e.g., see Fig. 4.20.2).

Prior to setting up cell cultures on ECM containing DQ-substrates, cells should first be prelabeled with a fluorescent cell tracking dye. Alternatively, cells can be transfected or transduced to drive expression of a fluorescent protein, such as monomeric Red Fluorescent Protein (mRFP), which we have found to remain cytosolic. There are two reasons to use fluorescently labeled cells: (1) to delineate the intracellular area taken up by cells in order to separate intracellular from extracellular degradation using imaging analysis software; and (2) to distinguish among the different cell types in cocultures. Invitrogen

provides several cell tracking fluorescent dyes with distinct excitation/emission spectra. This allows the user to label different cell types with unique colors, thus distinguishing each cell type in cocultures. The investigator must be aware of the limitation of the microscope for handling multiple colors and availability of excitation and emission bandwidths. After establishing cell cultures on ECM containing a DQ-substrate, the user can begin imaging immediately or at a later time. Cells will begin to degrade DQ-substrates shortly after seeding; however, sufficient hydrolysis for detection may require hours to accumulate. If long periods of culture time are required, as when the cells are slow to form 3-D structures or when degradation needs to be monitored in later stages of culture, the 3-D structures may need to be grown in the absence of DQ-substrates, harvested, and then reseeded onto coverslips coated with ECM containing DQ-substrate. Please see Support Protocol 2 for indications when reseeding should be considered.

Materials

DQ-substrates: DQ-collagen IV, DQ-collagen I, DQ-gelatin (Invitrogen)
Cell line of interest
CellTracker Orange CMTMR Dye (Invitrogen)
Powdered bovine skin gelatin (Sigma)
Sucrose (Sigma)
1× and 10× phosphate-buffered saline, sterile (PBS; *APPENDIX 2A*)
Collagen I (Cohesion Laboratories)
NaOH
Recombinant basement membrane (rBM): Matrigel (BD) or Cultrex (Trevigen)
Cell culture medium, phenol red–free

56°C water bath
0.22-μm filter
12-mm no.1 round glass coverslips, acid-washed and sterilized by baking
35-mm cell culture petri dishes
100-μl pipettor
37°C humidified incubator

Additional reagents and equipment for trypsinizing and counting cells (*UNIT 1.1*)

1. Reconstitute DQ-substrate in water.

   All DQ-substrates are shipped from the manufacturer as a lyophilized powder requiring reconstitution. This is accomplished at room temperature with sterile distilled water for at least 1 hr. We recommend making a stock solution of 1 mg/ml. Once reconstituted, 10- to 20-μl aliquots should be made and stored at 4°C.

2. Preload cells with membrane permeable cell tracking dye.

   Subconfluent cells should be prelabeled 2 to 3 hr before establishing cultures on ECM containing DQ-substrate. The chemistries of the individual dyes differ and their distribution within the cell can vary among cell types. Invitrogen has developed several thiol-reactive Cell Tracker probes that are nontoxic and yield fluorescent products that are retained inside live cells through several population doublings. These membrane permeable dyes undergo a GST-mediated reaction to produce membrane-impermeant glutathione–fluorescent dye adducts. We recommend that one test several dyes and determine concentrations and incubation times for each cell type to ensure dye distribution throughout cytoplasm. The duration of culture time before and during imaging must be considered since the dye is diluted as cells divide. We have found that Cell Tracker Orange CMTMR works well and will detail its use here (see below).

Dilute DQ-substrates in ECM

DQ-substrates need to be diluted in an appropriate ECM depending on your experimental requirements. There are several different commercially available ECM (e.g., gelatin,

collagen I, and rBM), which complement the different DQ-substrates. In our laboratory we dilute DQ-substrates with their constituent matched ECM (i.e., DQ-gelatin with gelatin, DQ-collagen I with collagen I, DQ-collagen IV with rBM). The rationale for diluting DQ-collagen IV in rBM is that collagen IV is a major component of basement membranes in vivo and in commercially available rBMs from EHS tumors. Each ECM requires slightly different handling techniques and therefore each will be discussed separately. Regardless of the DQ-substrate or the ECM used, we have found that the ideal final concentration of DQ-substrate is 25 μg/ml. Because there are lot-to-lot variations in both the DQ-substrates and the ECM, we recommend that all experiments be repeated with the same lot.

Prepare a gelatin + DQ-gelatin substrate

3a. Add 2% powered bovine skin gelatin and 2% sucrose to sterile PBS and heat to 56°C in a water bath until both gelatin and sucrose dissolve.

4a. Filter solution using a sterile 0.22-μm syringe filter.

5a. Allow to cool slightly before adding DQ-substrate at 25 μg/ml.

Avoid cooling to room temperature because gelatin will begin to solidify as it cools.

Prepare a collagen I + DQ-collagen I substrate

3b. Purchase collagen I and store at 4°C as a liquid.

4b. Prior to mixing with DQ-substrates, convert collagen I into a state in which it will solidify. To do this make a solution containing 80% (w/v) collagen I, 10% (v/v) 10× PBS, and 10% (v/v) 0.1 N NaOH (pH 7.4).

5b. At this point mix the collagen I solution with DQ-substrate to a final concentration of 25 μg/ml. Gently pipet to ensure thorough mixing.

Prepare recombinant basement membrane + DQ collagen IV substrate

3c. Thaw rBM on ice overnight at 4°C.

The rBM remains a liquid on ice but solidifies rapidly when warmed so it should be handled on ice at all times.

4c. Dilute the appropriate amount of DQ-substrate with an appropriate amount of rBM in a prechilled container to a final concentration of 25 μg/ml.

5c. Mix on ice using gentle pipetting to avoid creating bubbles. Keep on ice.

Take extra precautions during pipetting as errors may occur due to the viscosity of the rBM.

Prepare cultures

6. Coat glass coverslips with ECM containing DQ-substrate. Place two round coverslips in a 35-mm cell culture dish. Using a 100-μl micropipettor, carefully pipet and spread 50 μl of the solution made above over the entire surface of each coverslip.

Take care not generate air bubbles and stay within the dimensions of the coverslip.

7. Promptly place in either a humidified incubator at 37°C without CO_2 for 15 min (rBM) or 30 min (collagen I), or at 4°C for 10 min (gelatin).

The coated coverslips must be promptly placed in a humidified incubator to prevent dehydration/shrinking of the ECM. Include control coverslips that will be cultured without cells.

Alternatively, the investigator may choose to directly coat the bottom of a 35-mm dish.

8. Seed cells onto coated coverslips. While the rBM is solidifying, trypsinize and count the prelabeled cells (*UNIT 1.1*).

   Be sure that cells are in a single-cell suspension.

9. Centrifuge the cells 5 min at 80 to 100 × *g*, room temperature, and resuspend in culture medium so that the desired number of cells per coverslip is contained in a 50-μl volume.

   The culture medium is unspecified as medium is user defined for the individual cell line(s) to be studied.

   If doing a coculture experiment, a mixture of cell types should be made with the appropriate cell numbers mixed in a 50-μl volume of medium.

10. Place 50 μl suspension of cells onto each coated coverslips. Carefully place the 35-mm dish containing the cells on coverslips into a 37°C incubator. Allow 30 to 60 min for the cells to attach to the ECM.

    If performing coculture keep in mind that one cell line may require longer times to adhere to the ECM.

11. Fill the 35-mm culture dish with 2 ml of culture medium. If using assay supplements, these should be added at this time.

12. Incubate for desired time before imaging.

    The time period between seeding cells and imaging must be determined by the investigator and depends on the experimental design and cell types. Imaging can commence as soon as cells have attached to the ECM. We have imaged up to 3 weeks after seeding with negligible loss of fluorescence intensity.

SUPPORT PROTOCOL 2

HARVESTING 3-D SPHEROIDS OF CELLS GROWN IN rBM: AN ALTERNATIVE TO DIRECTLY GROWING ON ECM CONTAINING DQ-SUBSTRATES

When cells in rBM need to be grown over long periods of time before imaging, the cultures may first be grown without DQ-substrates and then transplanted into fresh ECM that has been mixed with the DQ-substrate. Harvesting the initial cultures from rBM requires gentle enzymatic digestion of the rBM so that the cellular structures can be removed with minimal disruption. Therefore, harvesting is only recommended for cell types that grow as tight spheroids or aggregates. Harvesting will significantly alter the 3-D morphology of structures composed of tubes, networks, and branches.

Materials

Cell culture grown on rBM within a 60-mm cell culture dish
1× phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile
6 U/mg lyophilized dispase (Roche)
Cell culture medium, phenol red–free

37°C incubator
Microscope
5-ml pipet
15-ml conical tube
Centrifuge

Additional reagents and equipment for preparing cultures on freshly coated coverslips (Support Protocol 1)

1. Aspirate medium from the cell culture and wash cells twice, each time with 5 ml PBS.

2. Dilute the lyophilized dispase to 1.5 U/ml using sterile PBS. Add 2 ml dispase solution to completely cover the dish. Place culture dish into a 37°C incubator. After 15 min, begin to periodically monitor dish with a standard microscope for the release of cellular structures.

   Complete digestion should occur within 60 minutes.

3. Add 1 ml fresh PBS. Use gentle pipetting with 5-ml pipet to complete the release of cellular structures from the rBM and culture dish. Use additional PBS if needed. Collect cellular structures with pipet into a 15-ml conical tube.

4. Gently centrifuge structures 5 min at 80 × *g*, room temperature.

   Spinning cells at 80 × g for 5 min will be sufficient to pellet even the smallest clusters of cells. If structures are large (>200 cells), we recommend that structures be allowed to sediment to the bottom of a 15-ml conical tube instead of centrifuging.

5. Aspirate the PBS and resuspend structures in appropriate volume of fresh cell culture medium.

   The culture medium is unspecified as medium is user defined for the individual cell line(s) to be studied.

   The volume used for resuspension will depend on the initial number of structures in the original dish and the number of structures desired per coverslip. As in Support Protocol 1, ~50 μl of medium can be pipetted onto a coated coverslip

6. Gently pipet cellular structures onto freshly coated coverslips from Support Protocol 1 and incubate.

7. Image the cultures at the appropriate time.

COMMENTARY

Background Information

The ability of cells to degrade ECM was formerly imaged by growing cells on FITC-labeled ECM and looking for a loss in fluorescence as a result of ECM degradation. With this method it is often difficult to assess loss of fluorescence, in particular small losses, because the background fluorescence is so intense. As a result the areas of fluorescence loss may not be associated with cells. For example, we have found tracks in which there is loss of fluorescence when tumor cells are grown on FITC-labeled ECM (Sloane, 1996). These tracks suggest that the cells are migrating and degrading the ECM as they migrate. We developed the DQ-substrate assay described here so that proteolysis by live cells could be imaged in real-time.

The fluorescence of the DQ-substrates is quenched as a result of extensive labeling, the close association of FITC molecules, and a transfer of nonradiative energy, i.e., a FRET effect. Upon proteolytic cleavage, the distance between the FITC molecules increases, resulting in emission of fluorescence (see Fig. 4.20.1B and D). This increase in fluorescence occurs on a nonfluorescent background (see Fig. 4.29.1A and C) and thus is readily observed. Furthermore, the gain in fluorescence can be imaged without fixation. When we use DQ-substrates for imaging of proteolysis by live cells, we see fluorescent fragments of DQ-substrates both extracellularly and intracellularly. At least some of the intracellular fluorescence represents intracellular degradation as cell-permeable inhibitors of lysosomal cysteine cathepsins reduce the amount of intracellular fluorescence (Sameni et al., 2000, 2003). The presence intracellularly of fluorescent fragments of DQ-substrates requires endocytosis by live cells. This may be due to endocytosis of intact DQ-substrate and its degradation intracellularly or endocytosis of fluorescent fragments of DQ-substrate that had been degraded extracellularly. In either case, the intracellular fluorescence could not be visualized unless the cells were alive, a clear advantage of the DQ-substrate assay. A potential disadvantage is that the DQ-substrates are proteins, so they do not allow one to directly assess the activity of any individual protease or protease class. As some DQ-substrates are proteins that would be encountered in vivo by migrating or invading cells, e.g., collagen IV or I, analyzing their degradation could allow one to identify proteases that are involved

in normal developmental or pathological processes. Furthermore, more than one protease or protease class can degrade these protein substrates, and thus their use should allow one to identify multiple proteases and potentially proteolytic pathways that are involved in these processes.

Another advantage of DQ-collagen substrates is that they allow one to study proteolysis in cells growing in a 3-D context that mimics the in vivo environment. There are several excellent reviews on the value of 3-D in vitro models (Schmeichel and Bissell, 2003; Debnath and Brugge, 2005; Yamada and Cukierman, 2007).

Critical Parameters

These protocols describe methods to set up and image cell cultures using an upright microscope equipped with long-working-distance dipping objectives. Although an upright microscope is ideal for the sample thicknesses of 3-D cultures, inverted microscopes can be used. Several microscope companies manufacture long-working-distance (2 to 3 mm) high-power dry objectives, such as the Zeiss 40× LD and 63× LD Plan-neofluar, which can be used in inverted microscopes. Along with long-working-distance dry objectives, growing cells on glass-bottom culture dishes (coverslip thickness) will minimize the distance between the objective and the cells within the ECM. Furthermore, microscope-grade glass has optical characteristics that are optimal for high-quality imaging.

Troubleshooting

Fluorescence from DQ-substrates appears as large, amorphous deposits that are not associated with cells. The DQ-substrates may have been handled improperly following solubilization. Once in solution the DQ-substrates should not be frozen. Do not vortex any solution containing DQ-substrates and avoid excessive pipetting. Also, do not let either the substrate or the ECM dry out. Make sure that the lot number of DQ-substrate has not expired or been recalled.

Collagen I does not adhere well to glass. We recommend establishing cell cultures on collagen I polymerized within a 35-mm plastic culture dish or on plastic coverslips. Note that the increased surface area of a 35-mm dish will also mean that higher cell numbers must be seeded.

Anticipated Results

Specific results will depend on the cells analyzed, the use of monolayer or three-dimensional cultures, and the DQ-substrates and ECM used for the assay. Degradation of the DQ-substrates can be seen within hours after plating and can be imaged in real-time in cultures growing in an incubator on the microscope stage. If stability of the setup can be maintained, cultures can be imaged in real-time for long time periods; we have imaged for as long as 24 hr. This requires a stage incubator that can maintain temperature, humidity, oxygen tension, and CO_2. Degradation of the DQ-substrates is seen within hours after plating. Not surprisingly, the denatured substrate DQ-gelatin is degraded faster than is either DQ-collagen I or DQ-collagen IV. Whether the DQ-substrates are degraded extracellularly, intracellularly or both may depend on the ECM in which they are mixed. The ECM can affect the morphology of the cells and whether they grow as two-dimensional monolayers or three-dimensional structures (e.g., see Fig. 4.20.2) and in addition can influence the ability of the DQ-substrates to be endocytosized.

Time Considerations

3-D cellular structures and cocultures require long imaging times. The thicker a 3-D structure is, the more optical sections needed to image. It may take minutes to record an optical section into each fluorescent channel; this is particularly true for high-resolution images requiring long scan times. If one is imaging cocultures in which each cell type is labeled with a fluorescent dye/marker, then each color must be scanned into a separate fluorescent channel. Another consideration is that variability among structures requires imaging of a greater number of structures if the data are to be statistically significant.

Time at which one should perform fluorescent labeling. Since cytoplasmic dyes that can be used to prelabel cells will be diluted out with each cell division, these dyes can only be used to label short-term cultures, or need to be added shortly before imaging, e.g., 2 hr (Support Protocol 1). The timing of staining with DNA binding dyes, such as Hoechst 33342 may affect cell viability. Due to their interaction with DNA, these dyes will induce apoptosis of most cells within 12 hr.

Acknowledgement

This work was supported by National Institutes of Health (NIH) grant CA 56586, an NIH National Technology Center for Networks and Pathways grant U54 RR020843, a Department of Defense (DOD) predoctoral traineeship award to CAJ (BC051230), and a DOD Breast Cancer Center of Excellence Award (DAMD1702-1-0693). The Microscopy and Imaging Resource Center is supported, in part,

by NIH Center Grants U54 RR020843, P30 CA 22453, and P30 ES 06639. We thank Dora Cavallo-Medved and Izabela Podgorski for providing images for Figure 4.20.2.

Literature Cited

Cavallo-Medved, D., Mai, J., Dosescu, J., Sameni, M., and Sloane, B.F. 2005. Caveolin-1 mediates the expression and localization of cathepsin B, pro-urokinase plasminogen activator and their cell-surface receptors in human colorectal carcinoma cells. *J. Cell Sci.* 118:1493-1503.

Debnath, J. and Brugge, J.S. 2005. Modelling glandular epithelial cancers in three-dimensional cultures. *Nat. Rev. Cancer* 5:675-688.

Friedl, P. and Wolf, K. 2003. Proteolytic and non-proteolytic migration of tumour cells and leucocytes. *Biochem. Soc. Symp.* 70:277-285.

Menges, D.A., Ternullo, D.L., Tan-Wilson, A.L., and Gal, S. 1997. Continuous assay of proteases using a microtiter plate fluorescence reader. *Anal. Biochem.* 254:144-147.

Podgorski, I., Linebaugh, B.E., Sameni, M., Jedeszko, C., Bhagat, S., Cher, M.L., and Sloane, B.F. 2005. Bone microenvironment modulates expression and activity of cathepsin B in prostate cancer. *Neoplasia* 7:207-223.

Sameni, M., Moin, K., and Sloane, B.F. 2000. Imaging proteolysis by living human breast cancer cells. *Neoplasia* 2:496-504.

Sameni, M., Dosescu, J. Moin, K., and Sloane, B.F. 2003. Functional imaging of proteolysis: Stromal and inflammatory cells increase tumor proteolysis. *Mol. Imaging* 2:159-175.

Schmeichel, K.L. and Bissell, M.J. 2003. Modeling tissue-specific signaling and organ function in three dimensions. *J. Cell Sci.* 116:2377-2388.

Sloane, B.F. 1996. Suicidal tumor proteases. *Nat. Biotechnol.* 14:826-827.

Sloane, B.F., Sameni, M., Podgorski, I., Cavallo-Medved, D., and Moin, K. 2006. Functional imaging of tumor proteolysis. *Annu. Rev. Pharmacol. Toxicol.* 46:301-315.

Tsai, K.K., Chuang, E.Y., Little, J.B., and Yuan, Z.M. 2005. Cellular mechanisms for low-dose ionizing radiation-induced perturbation of the breast tissue microenvironment. *Cancer Res*. 65:6734-6744.

Urbich, C., Heeschen, C., Aicher, A., Sasaki, K., Bruhl, T., Farhadi, M.R., Vajkoczy, P., Hofmann, W.K., Peters, C., Pennacchio, L.A., Amolmaali, N.D., Chavakis, E., Reinheckel, T., Zeiher, A.M., and Dimmler, S. 2005. Cathepsin L is required for endothelial progenitor cell-induced neovascularization. *Nat. Med.* 11:206-213.

Yamada, K.M. and Cukierman, E. 2007. Modeling tissue morphogenesis and cancer in 3D. *Cell* 130:601-610.

Photoactivated Localization Microscopy (PALM) of Adhesion Complexes

Hari Shroff,[1] Helen White,[1] and Eric Betzig[1]

[1]Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, Virginia

ABSTRACT

Key to understanding a protein's biological function is the accurate determination of its spatial distribution inside a cell. Although fluorescent protein markers allow the targeting of specific proteins with molecular precision, much of this information is lost when the resultant fusion proteins are imaged with conventional, diffraction-limited optics. In response, several imaging modalities that are capable of resolution below the diffraction limit (~200 nm) have emerged. Here, both single- and dual-color superresolution imaging of biological structures using photoactivated localization microscopy (PALM) are described. The examples discussed focus on adhesion complexes: dense, protein-filled assemblies that form at the interface between cells and their substrata. A particular emphasis is placed on the instrumentation and photoactivatable fluorescent protein (PA-FP) tags necessary to achieve PALM images at ~20 nm resolution in 5 to 30 min in fixed cells. *Curr. Protoc. Cell Biol.* 41:4.21.1-4.21.27. © 2008 by John Wiley & Sons, Inc.

Keywords: PALM • superresolution • adhesion complex • fluorescent proteins

INTRODUCTION

The accurate determination of a protein's spatial distribution inside a cell is often intimately related to its function. Fluorescence microscopy allows the imaging and subsequent analysis of protein distributions inside cellular specimens, and fluorescent proteins allow an investigator to genetically target a protein with molecular precision. Unfortunately, valuable information is lost when conventional fluorescence microscopy is used to image the resulting protein fusions, because diffraction limits the smallest features that can be resolved with an optical microscope to ~200 nm.

In response, a number of far-field superresolution imaging modalities that are capable of breaking the diffraction barrier have emerged (Gustafsson, 2000, 2005; Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006; Willig et al., 2006). Photoactivated localization microscopy (PALM; Betzig et al., 2006) relies on the stochastic activation, localization, and bleaching of single photoswitchable molecules, and initially used photoactivatable fluorescent proteins (PA-FPs, reviewed in Wiedenmann and Nienhaus, 2006; also see *UNIT 21.6*) at the high density and localization precision necessary to provide images at $>10\times$ higher spatial resolution than corresponding diffraction-limited images (Fig. 4.21.1). Several other methods (Hess et al., 2006; Rust et al., 2006) have relied upon similar characteristics of photoswitchable molecules to provide superresolution, albeit at lower density and spatial resolution.

This unit describes the application of PALM to the imaging of adhesion complexes, transmembrane protein assemblies that form attachment points between the cytoskeleton and substratum and that are critical in cell migration (Zamir and Geiger, 2001). Adhesion complexes represent a particularly good test system for PALM: as many types of proteins (>90) are concentrated in structures as small as 0.5 μm (Zaidel-Bar et al., 2007), conventional fluorescence imaging is clearly inadequate to resolve internal adhesion complex structure (Fig. 4.21.1).

Current Protocols in Cell Biology 4.21.1-4.21.27, December 2008
Published online December 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0421s41

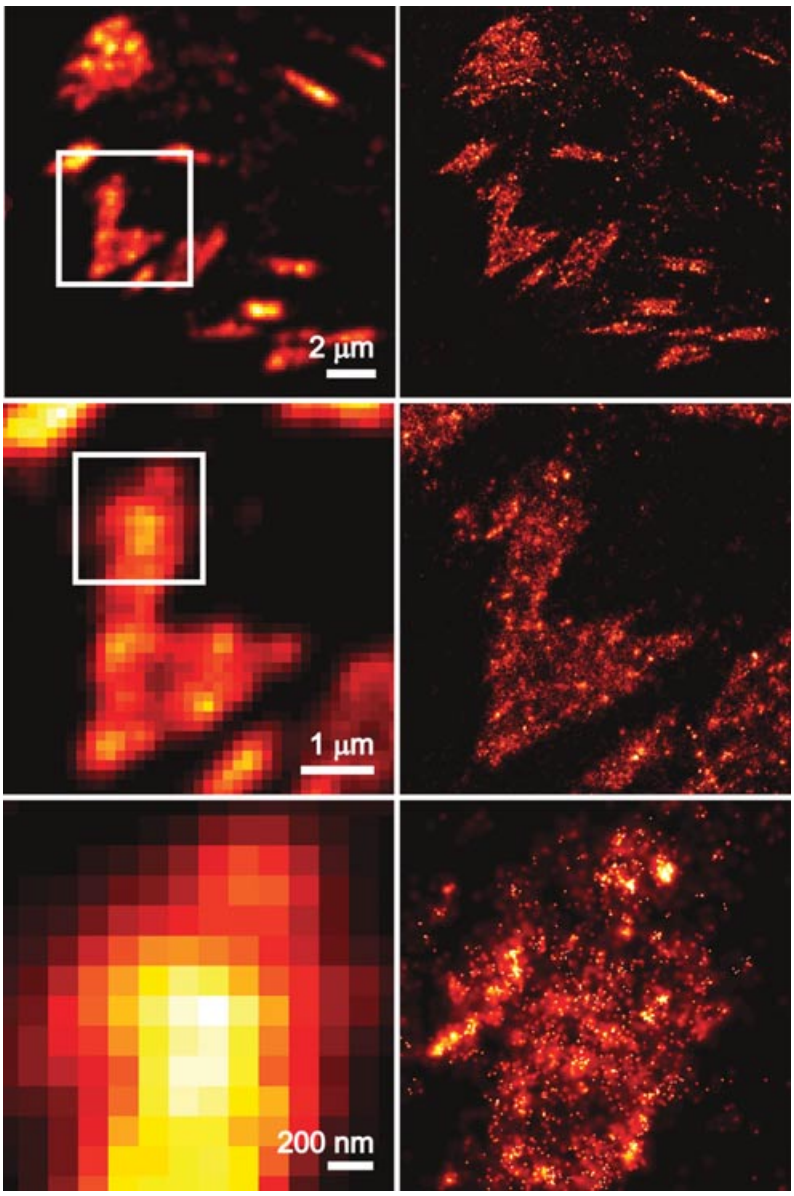


Figure 4.21.1 Comparison between diffraction-limited, summed TIRF (left) and PALM (right) images of tdEos/vinculin distributions in adhesion complexes present at the surface of a fixed HFF-1 fibroblast. Middle/bottom panels show higher magnification views of the boxed regions in top/middle panels. Note the greatly increased resolution and dynamic range evident with PALM. Displayed images were rendered from 40,000 single-molecule images; >100,000 molecules are plotted in each PALM image. For color version of this figure see *http://www.currentprotocols.com*.

Several considerations are important in achieving the best PALM images of adhesion complexes. First, the choice of photoactivatable tag dictates the maximum achievable resolution; this consideration is discussed in Strategic Planning. Second although PALM instrumentation is relatively straightforward, some additional knowledge in designing a PALM system is helpful and is covered in Basic Protocol 1. A method that uses PALM instrumentation in combination with appropriate sample preparation techniques to provide superresolution images of the adhesion protein paxillin is described in Basic Protocol 2. Finally, Basic Protocol 3 describes the additional steps necessary to undertake dual-color PALM-imaging of two adhesion complex proteins (vinculin and α-actinin).

STRATEGIC PLANNING

PALM is a single-molecule technique: the raw data consist of a stack of thousands of individual frames, each containing diffraction-limited fluorescence images of single photoswitchable molecules present in the sample. The fluorescence images of each molecule are analyzed to determine their centers with high precision, and the resultant information is used to generate a high-resolution PALM image of the positions of the molecules. The resolution of PALM is thus dependent on *localization precision*: how well the position of each single molecule can be determined from its diffraction-limited image. Perhaps more subtle but equally important in determining the ultimate PALM resolution is the *density* of molecules present in the sample (see Background Information for a detailed discussion of factors influencing PALM resolution). The localization precision depends on maximizing the signal-to-noise ratio in each image, a characteristic that in turn depends on maximizing the collected photons from each photoswitchable molecule and minimizing the background fluorescence. These instrumentation considerations are discussed in Basic Protocol 1. However, the localization precision also depends on the total number of photons emitted by each molecule before it bleaches, an intrinsic property of the molecule. Furthermore, taking full advantage of the molecular density in each sample is contingent on the *contrast ratio* of the molecule, another intrinsic property (see Background Information). It is the authors' belief that the correct choice of photoswitchable molecule is the single most important factor in obtaining high-quality PALM images—that choice is the subject of this section.

All PALM experiments currently conducted by the authors use PA-FPs as the photoswitchable molecules, instead of exogenously introduced caged dyes—such as caged fluorescein, resorufin, or rhodamine (Mitchison et al., 1998; Betzig et al., 2006)—that are conjugated to antibodies or proteins via small-molecule labeling strategies (Chen and Ting, 2005; Giepmans et al., 2006). PA-FPs have the tremendous advantage of being exquisitely specific; by genetically fusing a PA-FP to a target protein and transfecting the cellular sample, the investigator is assured that there is a 1:1 correspondence between a detected molecule and its target, and thus avoids the nonspecific binding that arises with dye-conjugated antibodies or proteins. PA-FPs also better approximate the size of a target protein, whereas antibodies can introduce considerable uncertainty due to their size (10 to 20 nm, depending on whether both primary and secondary antibodies are used). Due to their small size, PA-FPs can achieve higher densities in principle than antibodies, and thus offer increased PALM resolution. Finally, PA-FP genes are readily available as plasmid DNAs and are easily ligated to DNA coding for the protein target, whereas at the present time, caged dyes are not commercially available and must be custom synthesized. Some of the potential downsides of PA-FPs are mentioned in Critical Parameters and Troubleshooting.

Most PA-FPs can be broken up into two classes: those that switch from a green fluorescent state to an orange state (e.g., EosFP, Wiedenmann et al., 2004; KikGR, Tsutsui et al., 2005; and Kaede, Ando et al., 2002) upon photoactivation, and those that switch from a dark state to a green fluorescent state (e.g., PA-GFP, Patterson and Lippincott-Schwartz, 2002; or Dronpa, Ando et al., 2004) upon photoactivation. After testing candidates from both classes, the authors have concluded that EosFP is the best choice for PALM, due both to the high number of emitted photons before bleaching (>1000 photons, providing localization precision to $\sim$10 nm; Shroff et al., 2007) and the greatly increased contrast ratio ($>10^3$, providing both increased localization precision and enabling PALM-imaging of densely-labeled specimens) relative to other PA-FPs (see Fig. 4.21.2 and Background Information). Also, as the activated state of Eos has a fluorescence emission spectrum that peaks at 580 nm, emission filters can be chosen to minimize cellular autofluorescence (more prevalent in the 450 to 550 nm region, overlapping with the emission spectrum of

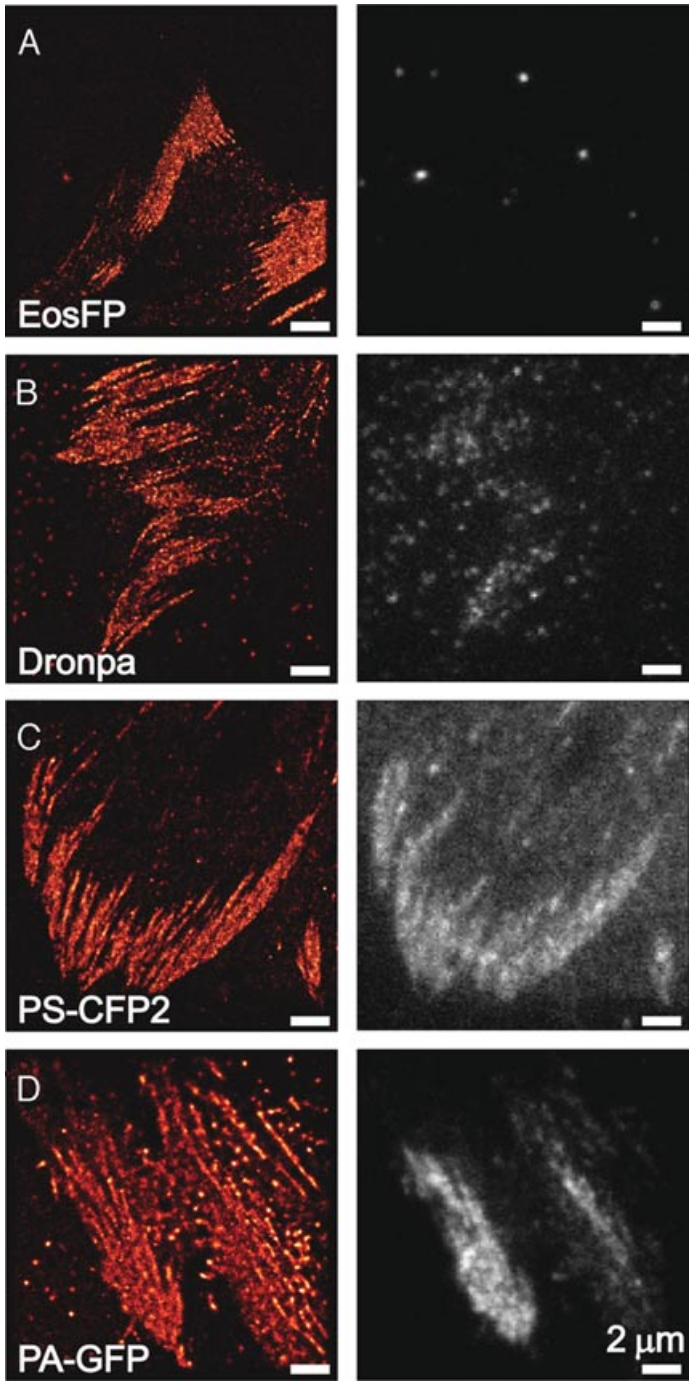


Figure 4.21.2 Comparison of contrast ratios for four different PA-FPs, each fused to the adhesion protein paxillin. Left column tabulates PALM images obtained in each case: (**A**) mEosFP; (**B**) Dronpa; (**C**) PS-CFP2; (**D**) PA-GFP. Right column contains, for each PA-FP, frame 1000 of the stack of single-molecule images used to generate the corresponding PALM image on the left. Background largely reflects emission from the large pool of inactive PA-FPs. Note the correlation between low background (i.e., high contrast ratio between the active and inactive states) and crispness of the PALM image (i.e., localization precision). Reproduced from Shroff et al., 2007. For color version of this figure see *http://www.currentprotocols.com*.

PA-GFP and Dronpa). Finally, unlike KikGR, EosFP can be completely photoconverted from its green state to the orange state, a characteristic that is essential for the serial dual-label PALM procedure described in Basic Protocol 3.

Despite these factors, there may be situations where it is advantageous to use other PA-FPs as the PALM label. For example, in dual-label PALM-imaging (Shroff et al., 2007), it is necessary to use EosFP in conjunction with another PA-FP (either Dronpa or PS-CFP2; Chudakov et al., 2004, 2007). If the investigator must use an FP or fluorophore as a conventional, diffraction-limited label, and the label has significant spectral overlap with EosFP, it may be necessary to use a different PA-FP as the PALM label (although it is better to first pick the PALM label and then the diffraction-limited label, as the choice of PA-FPs is considerably more limited). Finally, any FP-labeling strategy is prone to aggregation artifacts, and these problems can be exacerbated if the label is present in nonmonomeric form. EosFP is currently available in tetrameric, dimeric, tandem-dimeric (commonly used by the authors and available from Michael Davidson, Florida State University), and monomeric forms, but only the first three forms fold efficiently at 37°C. The development of a monomeric Eos that folds at 37°C is ongoing, but in the interim there may be some applications that demand the existing monomeric PA-FPs—Dronpa, PA-GFP, PS-CFP2, or Dendra2 (Chudakov et al., 2007).

PREPARING PALM INSTRUMENTATION

BASIC PROTOCOL 1

Instead of a conventional step-by-step protocol, this section describes the instrumentation necessary for PALM. As PALM is inherently a single-molecule technique, standard methods for suppressing background fluorescence and for detecting relatively faint single-molecule emissions apply (Weiss, 1999). In particular, total internal reflection (TIRF) microscopy (Axelrod, 2001; *UNIT 4.12*) is advantageous for PALM, because the evanescent excitation wave penetrates <200 nm into the sample, leading to extreme rejection of the background fluorescence common in cellular samples, thus facilitating the detection of single fluorescent molecules. TIRF is also a widefield technique, implying that many molecules per frame can be imaged simultaneously, thus vastly increasing the acquisition speed. TIRF is particularly appropriate for imaging adhesion complexes, as these structures are located close (within 100 nm) to the substrate and are thus within the TIRF excitation region. Note that TIRF is not an absolute requirement for PALM, as certain specimens (such as bacteria) are sufficiently thin that autofluorescence does not impede the localization of single molecules illuminated in epifluorescence. Also, other studies (Hess et al., 2006; Egner et al., 2007) have reported epifluorescence PALM imaging, albeit with lower resolution images than those reported here.

With some minor modifications and additions to the existing hardware, it is possible to adapt an existing commercial TIRF microscope for PALM (Shroff et al., 2007; Fig. 4.21.3). Free-space coupling (i.e., without an optical fiber) of appropriate excitation and activation lasers into the microscope ensures that enough power is delivered to the sample, and permits fast PALM imaging. Using a microscope objective with as high a numerical aperture (NA) as possible allows high photon collection efficiency and facilitates TIRF. Choosing an appropriate electron-multiplying charge-coupled device (EMCCD) camera is an important factor in imaging the faint emissions from single fluorescent molecules, as is choosing the best available excitation, emission, and dichroic filters. Finally, a computer with acquisition software, image acquisition board, and a large hard drive is important for acquiring and storing the large PALM acquisition stacks. The authors have tried to be as general as possible in describing the major components, but have given specific recommendations when relevant. Note that Carl Zeiss has recently licensed the PALM technology, with plans to develop commercial PALM microscopes.

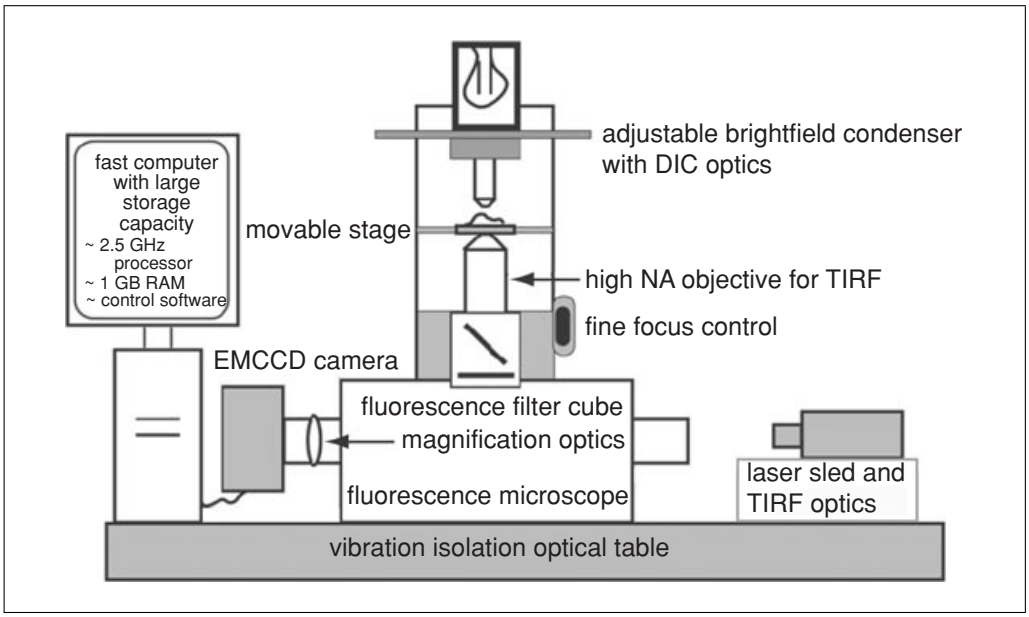


Figure 4.21.3 Cartoon view of the main instrumentation components necessary for PALM. See the text for a detailed discussion of various components, and note that Figure 4.21.6 and Figure 4.21.7 show a detailed view of the sled and TIRF optics for coupling the excitation and activation lasers into the microscope body, while Figure 4.21.4 and Figure 4.21.5 show the sample holder and sample chamber (not shown in cartoon) in detail. The figure is not drawn to scale.

Materials

Optical table (Technical Manufacturing Corporation)
Inverted fluorescence microscope (e.g., Olympus IX-81) assembly, including:
- Brightfield and DIC optics
- Capability for TIRF illumination
- High NA objective lenses (1.45 NA, 1.49 NA, or 1.65 NA) with matching coverslips, and immersion media
- Internal magnification lens (optional, depending on objective magnification)
- Additional magnification before camera (optional, depending on objective magnification)

Mercury *or* xenon lamp
Mechanically stable stage and sample mount
Appropriate excitation and activation lasers and optics (beam expanders, neutral density filters, half-wave plates, and dichroic beamsplitters) for coupling lasers into the TIRF microscope path
Appropriate excitation, emission, and dichroic filters
Acousto-optic tunable filter (AA Opto-Electronic, AA-AOTFnC-VIS)
EMCCD camera (see step 12 for details)
Computer with appropriate control software (see step 14 for details)
Computer with appropriate analysis software (see step 16 for details)

Set up inverted fluorescence microscope with associated optics and stage

1. Bolt a research-quality fluorescence microscope to an optical table in order to minimize unwanted vibrations and to prevent relative motion between the microscope and table. Use a microscope with ports for epi- and TIRF-illumination, and for mounting an EMCCD camera for detection. Use a brightfield condenser and differential interference contrast (DIC) optics for imaging samples conventionally before PALM-imaging, and a mercury or xenon lamp to aid in initial examination

of the specimen with diffraction-limited epifluorescence imaging prior to PALM-imaging.

Some of these components are illustrated in Fig.4.21.3.

The authors use an Olympus IX-81 as the base microscope for PALM. The microscope is bolted to a 4 × 8–ft optical table, large enough to comfortably fit both the microscope and the associated lasers and TIRF optics described below. The back-port of the microscope is fitted with the Olympus U-DP dual-port tube to switch between widefield epi-illumination (with a mercury or xenon lamp) and laser illumination (for TIRF). The microscope is also fitted with a polarizer, analyzer, and Nomarski DIC prisms for transmitted DIC imaging. Given the sample chamber design described below, a long working-distance condenser is necessary to prevent the condenser from physically colliding with the sample chamber. The authors use an NA 0.55, 27-mm working-distance condenser (Olympus, IX2-LWUCD). Finally, the authors recommend using an adjustable, focusable lamp housing (Olympus, IX-HLSH100), as they have obtained more uniform DIC illumination with this device than with the standard lamp housing (U-LH100L-3-5), which cannot be focused or adjusted.

2. Minimize sample drift by bolting a mechanically stable, rigid stage to the microscope frame.

   If a motorized stage with linear encoders is used, it is easy to repeatedly return to the same position on the sample, a feature that is useful for marking appropriately transfected cells.

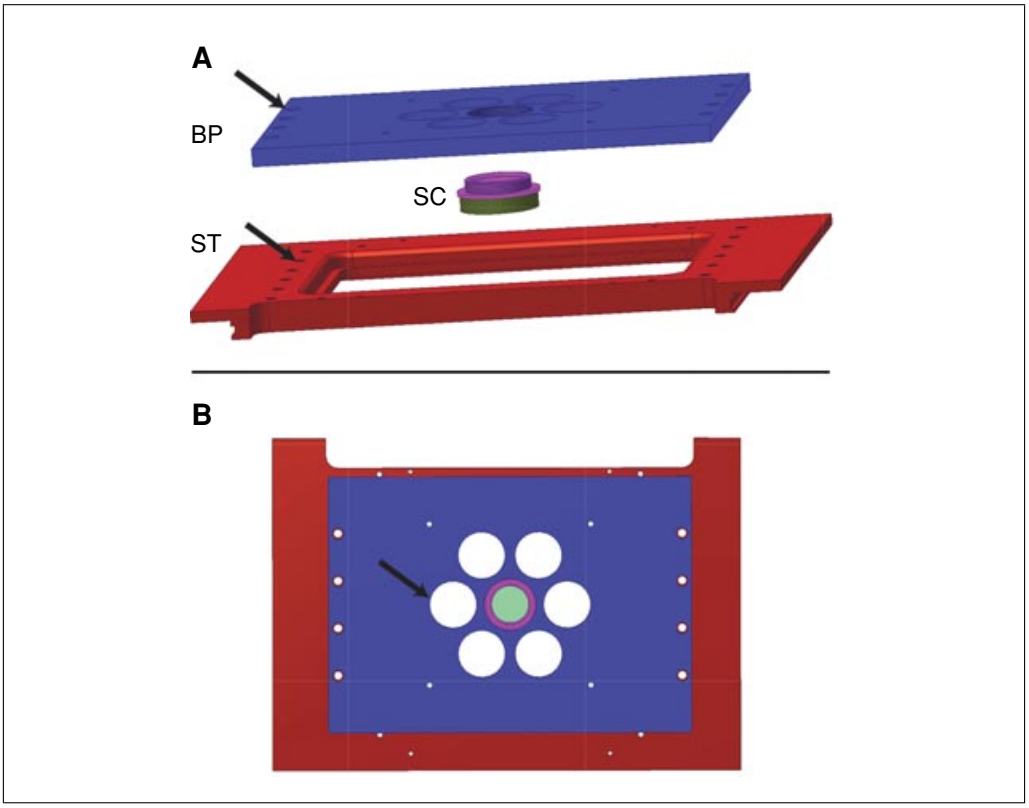


Figure 4.21.4 Sample holder assembly. (**A**) The sample holder in PALM consists of a sample chamber assembly (SC, shown in more detail in Figure 4.21.5), that is threaded and fits into a top, steel baseplate (BP). The BP contains holes that are sized appropriately so that it can be bolted into the threaded holes of the ASI stage (ST) with appropriately threaded screws. Holes for bolting BP to ST are shown with arrows. (**B**) Bird's eye view of the sample holder. Additional access holes in BP (arrows) are useful for cleaning out old immersion oil from the bottom surface of SC (as might accumulate with the high-index oil used with the 1.65 NA objective), while keeping the BP bolted to ST.

Although most stages come with a variety of inserts for convenient sample loading, use of clips to hold down the sample should be avoided. A more stable option is to design a baseplate that can be bolted to the stage (Fig. 4.21.4), and a threaded coverslip holder that contains the sample (Fig. 4.21.5) and that can be attached firmly to the baseplate.

The authors use an MS-2000 XY stage with linear encoders (ASI). The stage is bolted to the IX-81 frame directly above the objective, and a baseplate holding the sample chamber (that holds the sample-containing coverslip) is bolted to the stage.

TIRF excitation scheme

3. Select appropriate laser wavelengths for activating and exciting the PA-FP chosen as the PALM-label. Use solid-state diode lasers to provide ample power at a reasonable cost.

   Almost all available PA-FPs are activated efficiently at 405 nm; thus a laser with this wavelength is required. For PALM-imaging EosFP, a 561-nm laser is also advised, as it can be used for excitation of the activated (orange) state (an Argon/Krypton mixed gas laser has a 568-nm laser line, which is closer to the 580-nm absorption peak of EosFP, but this laser is also considerably more expensive than a 561-nm diode laser).

   A 488-nm laser is also very useful for imaging the inactive state of EosFP (i.e., for imaging the distribution of EosFP at the diffraction limit before PALM-imaging and determining which cells are expressing the fusion protein at the desired levels), and is essential for exciting the activated (green) state of Dronpa or PA-GFP.

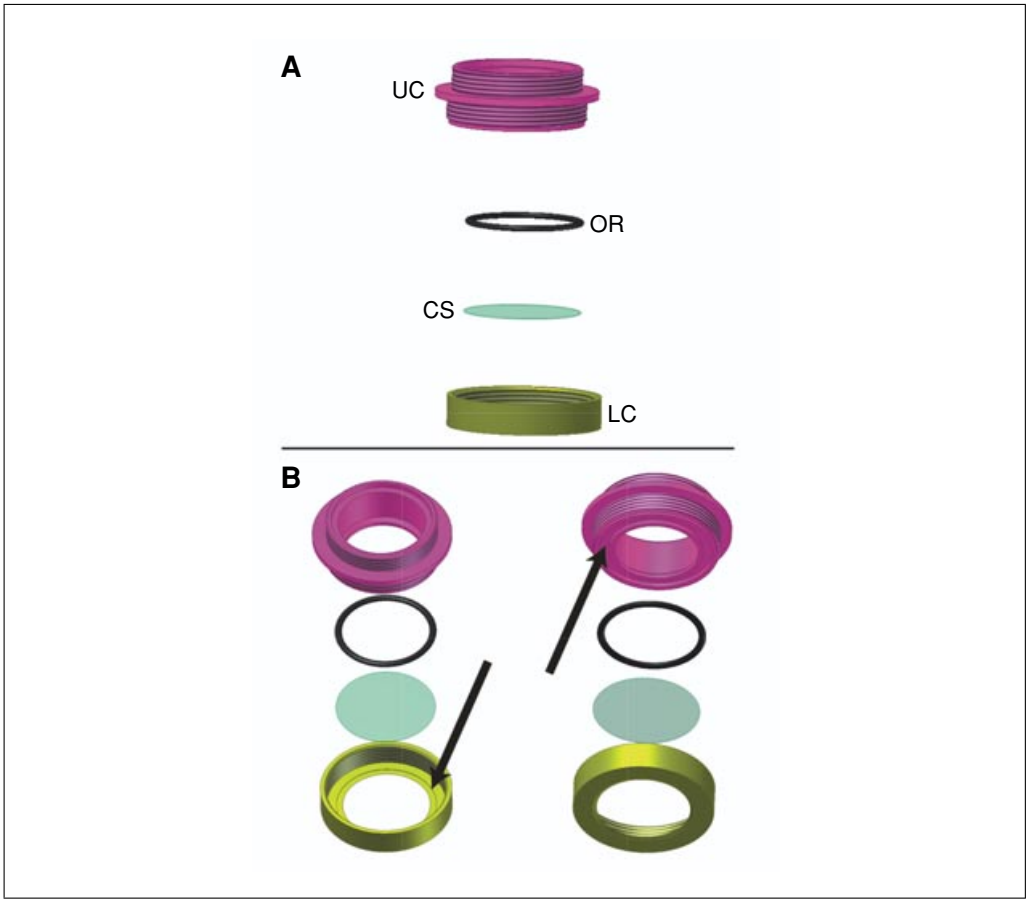


Figure 4.21.5 Sample chamber assembly. (**A**) The sample chamber used in PALM consists of upper (UC) and lower (LC) steel assemblies that thread together. Between these two components are sandwiched the coverslip (CS) containing the sample, and an O-Ring (OR). OR prevents leaks when CS is immersed in buffer. (**B**) Different perspective view of the sample chamber assembly, with grooves in UC and LC for OR and CS indicated with arrows.

The authors use a 50-mW 405-nm laser (Coherent, 405-50C) for activation, a 150-mW 561-nm laser (Crystalaser, GCL-150-561) to excite the active state of EosFP, and a 50-mW 488-nm laser (Newport Spectra-Physics Cyan Scientific Laser) for imaging the inactive state of EosFP or the activated state of Dronpa. The characteristics from these diode lasers are not always as advertised: the mode quality may be poor and the intensity may drift slightly. For PALM, these characteristics are not critical, as long as enough power is available to excite or activate molecules in the desired region at the sample. However, a particularly poor beam profile may be difficult to focus at the back focal plane (BFP) of the objective without clipping, may be hard to co-align with the other laser beams, and may thus impede successful TIRF illumination of the specimen. The authors have noticed that the beam quality from the 405-nm laser is somewhat poor; utilizing a spatial filter (a telescope system consisting of 12-mm and 50-mm focal length lenses separated by the sum of their focal lengths, and a 10-μm pinhole positioned at the intermediate image plane between the two lenses) immediately after the laser output cleans up the beam profile considerably and improves the ease with which the sample is TIRF-activated.

4. Free-space couple all lasers into a TIRF-capable illumination microscope port. Use neutral density filters and half-wave plates (FIL) to control the intensity and polarization [important for maximum transmission through the acousto-optic tunable filter (AOTF) element discussed below] of each beam. In addition, use a narrow bandpass excitation filter (Semrock LL01-488-25) with the 488-nm laser to reduce emission noise. Use beam expanders (BE) after each laser to yield a common beam diameter (important for establishing an excitation/activation region of common size at the sample).

   One possible coupling scheme is shown in Figure 4.21.6 (reproduced from the supporting information in Shroff et al., 2007). Activation and excitation lasers are indicated by their respective wavelengths. Beam expanders are made from achromatic lenses (Edmund Scientific) mounted in $^1/_2$-in. diameter lens tubes (Thorlabs).

5. Combine the 488-nm and 561-nm excitation beams with a dichroic beamsplitter (DC) prior to passage through an acousto-optic tunable filter (AOTF), then combine excitation beams with the 405-nm activation beam with a second dichroic beamsplitter (DC).

   An FF-506-Di02-25 × 36 (Semrock) dichroic beamsplitter is used to combine 488-nm and 561-nm beams before the AOTF.

   The AOTF serves as a rapid switch to select between wavelengths, and can also be synchronized to the frame acquisition cycle of the detection EMCCD (discussed further below) in order to minimize photobleaching during the read-out period between frames.

   Synchronization can be easily achieved by connecting the Fire output signal from the EMCCD detector to the AOTF input. The AOTF output beam in turn is combined with the 405-nm activation beam using a second dichroic beamsplitter (Semrock, FF458-Di02-25×36). The reason for combining the activation beam with the excitation beams in a position downstream of the AOTF is that the AOTF used does not transmit wavelengths <450 nm efficiently. Goniometer (Thorlabs, cat. no. GN05)–mounted glass windows (CVI Inc.; 2 windows/goniometer, each 12.5-mm diameter, 5-mm thick) are used to fine-adjust the position of each beam (TX, TY), thereby facilitating their mutual overlap, and the assorted dichroic beamsplitters and mirrors in the system provide further adjustment of the position and direction of propagation.

   Although it is also possible to couple all three lasers into a commercial TIRF illumination unit using an appropriate multi-wavelength fiber (e.g., the PointSource kineFlex fiber), these fibers transmit at best 50% to 60% of the incident laser light, and typically <50% due to the poor mode quality of the beams from the diode lasers. This results in excitation intensities that are too low for the fastest PALM acquisitions.

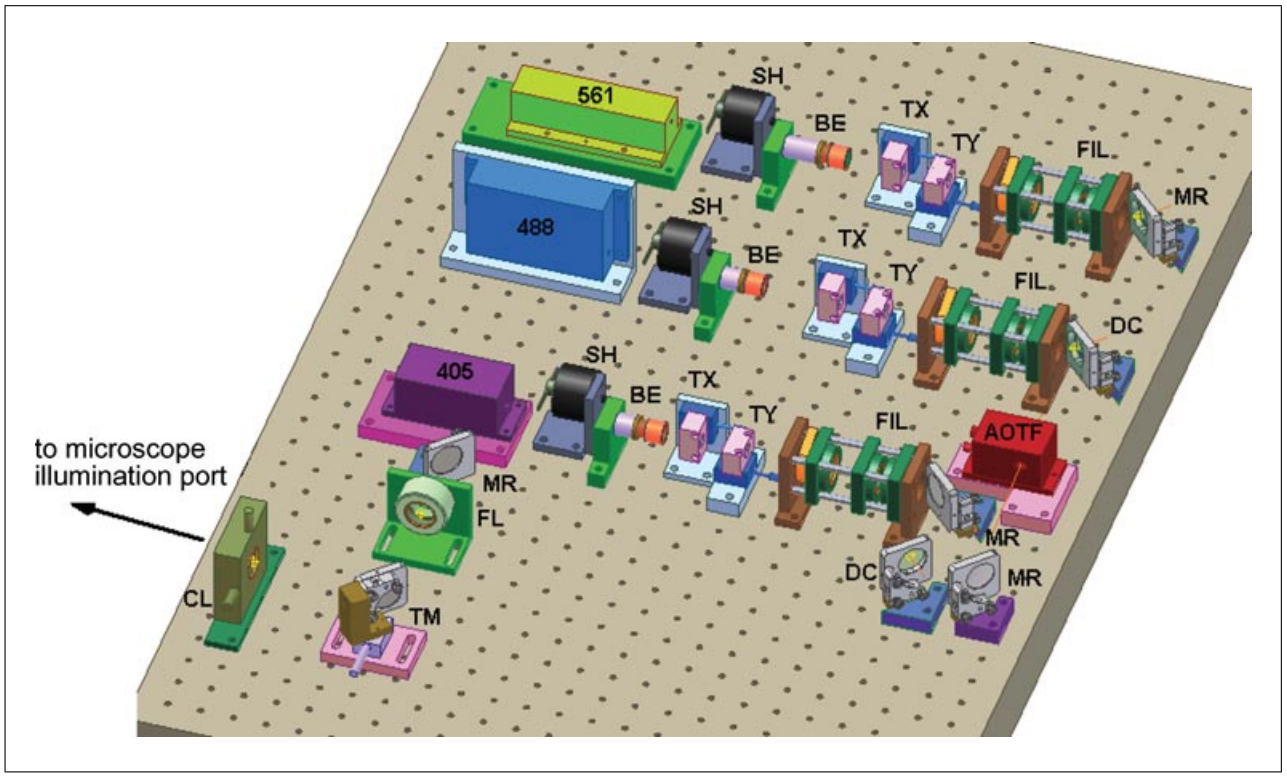


Figure 4.21.6 Scheme for coupling excitation and activation light into the microscope for PALM. Lasers 488 and 561 excite the active forms of Dronpa and EosFP, respectively, and laser 405 converts each of these PA-FPs to the active form. The remaining elements are used to overlap the three lasers beams, adjust their diameters, and ensure their mutual propagation in a common direction (see text for a more detailed discussion). Symbol key: SH = shutter; BE = beam expander; TX = x direction beam translator; TY = y direction beam translator; FIL = filter group (wavelength, intensity, and polarization); MR = mirror; DC = dichroic beamsplitter; AOTF = acousto-optic tunable filter; FL = focusing lens; TM = translating mirror; CL = collimating lens. See the text in Basic Protocol 1 for discussion. Reproduced from Shroff et al., 2007.

6. Once excitation and activation beams are co-aligned, bring them to a focus at the back focal plane (BFP) of the objective used for TIRF excitation and activation.

   This can be done with the aid of two lenses, FL (focusing lens) and CL (collimating lens; Fig. 4.21.6 and Fig. 4.21.7).

7. Position the entire laser illumination system relative to the imaging microscope such that the BFP of lens CL is coincident with the front focal plane of a 200-mm relay lens internal to the microscope (Fig. 4.21.7). By doing so, the focus created by lens FL is imaged at the BFP of the microscope objective.

   With this geometry, the image of the focus can be moved within the objective BFP using the translating mirror (TM), thereby switching between epi and TIRF modes of laser excitation without affecting the position of the spot at the sample. Lastly, by switching between lenses CL of differing focal length, and by changing the magnification of the BEs, the diameter of the beam launched into the microscope can be changed, resulting in a change in the excitation/activation spot size, and thus the laser intensity, at the sample. Small spots can be used to achieve fast single-molecule frame rates (~10 to 20 msec), or large spots can be used to image at slower rates but over larger fields of view.

   The commercial Olympus IX-81 TIRF Illuminator can be conveniently modified and stripped down to conform with the guidelines presented above. This system is usually attached to the rear port of the IX-81. The authors use the U-DP attachment (Olympus) to switch between arc lamp and TIRF illumination (Fig. 4.21.7), but only the 200 mm relay lens in the TIRF illuminator is necessary—the entire fiber-coupling assembly can be removed.

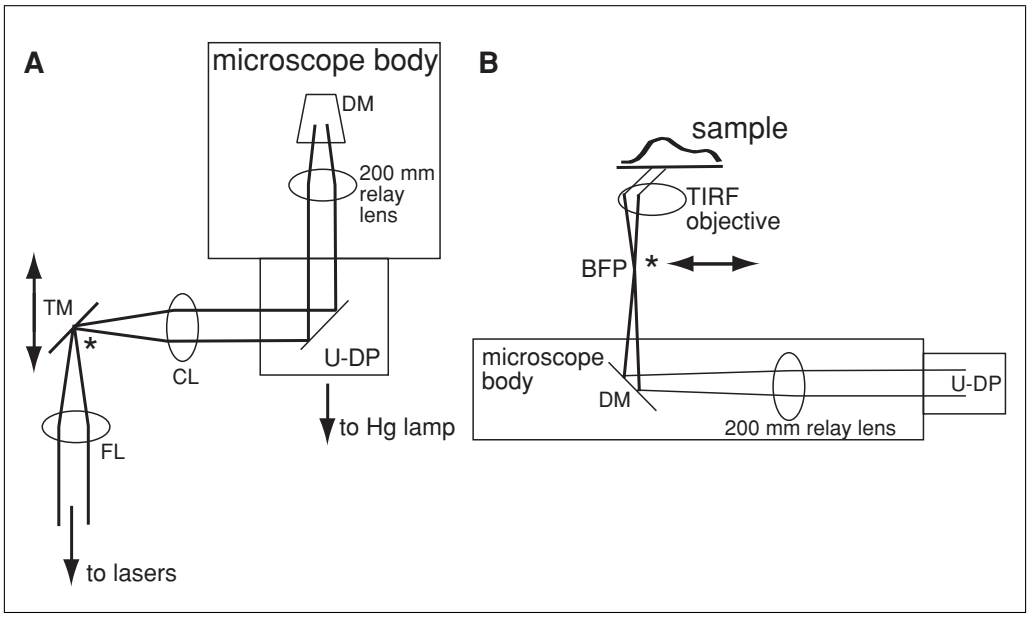


Figure 4.21.7 Cartoon view of the optical path used for TIRF illumination. (**A**) Bird's eye view. Laser light is focused by lens FL, reflected from mirror TM, and recollimated by lens CL (note that these 3 optical elements are shown also in Figure 4.21.6). It enters the microscope back port via the Olympus U-DP attachment (used for switching between TIRF and mercury or xenon lamp illumination), whereby a single 200-mm relay lens (internal to the microscope body) focuses the light onto the back focal plane (BFP) of the objective, via the dichroic mirror (DM) in the filter cube assembly (not shown for clarity). (**B**) Side view of the light path after the U-DP. Light focused at the objective BFP emerges from the objective (shown as a single lens for clarity) as parallel rays, illuminating the sample at a critical angle in TIRF. Note that the focus at the BFP of the objective and the focus created between CL and FL are located in conjugate planes (indicated by the asterisks in the cartoon); translating TM thus serves to translate the focus at the BFP, allowing easy switching between epi- and TIRF configurations without changing the location of the illumination spot at the sample. See the text in Basic Protocol 1 for discussion.

Excitation, dichroic, and emission filters

8. Carefully choose excitation, dichroic, and emission filters to maximize the fluorescence signal while rejecting excitation light.

   The use of lasers can sometimes preclude the use of excitation filters, as the excitation light is already quasi-monochromatic. However, occasionally, broadband emission noise generated in the laser cavity or autofluorescence generated elsewhere in the optical path necessitates the use of narrowband excitation filters.

9. Select the dichroic filter to simultaneously reflect both activation and excitation wavelengths (so that time is not wasted in switching dichroics between activation and excitation steps during the PALM-imaging), while transmitting the much weaker fluorescent emissions with high efficiency.

10. Finally, select the emission filter to provide >6 OD suppression of excitation light and activation light, while maximally transmitting the fluorescence emission.

    Bandpass emission filters have been found to better suppress the excitation light than longpass filters, such as Raman edge filters. Dichroic and emission filters can be conveniently combined in a fluorescence filter cube holder and placed within the microscope in a standard filter cube turret, but excitation filters must be placed upstream of the dichroic.

    For PALM-imaging the activated state of EosFP, the authors use the dichroic FF562-Di02-25×36 (Semrock) and the bandpass emission filter FF01-617/73-25 (Semrock). For imaging the inactive state of EosFP or the activated state of Dronpa, the dichroic T495lp (Chroma) and the bandpass emission filter ET525/50 (Chroma) are used; equivalent results can be obtained with a standard GFP filter cube set.

High NA objectives

11. To achieve TIRF, the NA of the objective lens must be greater than the index of refraction *n* of the sample (typically 1.38 for cellular components). The number of objectives that satisfy this criterion are limited, and practically the investigator is limited to objectives of NA 1.45, 1.49, and 1.65 (the last manufactured exclusively by Olympus).

    Higher NAs imply both better photon collection efficiency and an increased range of angles over which TIRF is achieved (wider TIRF annulus). Better photon collection efficiency results in a greater signal-to-noise ratio and thus higher localization precision of individual molecules. A wider TIRF annulus leads to a greater range of adjustment of the evanescent wave decay length, and makes achieving TIRF easier than with a more limited annulus, as it is easier to focus a spot at the BFP of the objective without clipping the beam. The investigator is thus urged to purchase the objective with the highest possible NA, subject to the constraints discussed below.

    It is important to note that the 1.65 NA objective, while providing substantially better TIRF than other objectives, requires expensive high-index ($n = 1.78$) coverslips (APO100X-CG, Olympus), and high-index ($n = 1.78$) immersion oil (Series M, Cargille). The oil is toxic to work with, requiring gloves and added safety precautions, and the coverslips are expensive and difficult to reclean. The oil also absorbs in the visible spectrum, particularly at the 405-nm activation wavelength. It thus degrades over time and may need to be replaced during an experiment. Degradation is exacerbated at higher laser powers and elevated temperatures, making this objective unsuitable for experiments on living cells and for the fastest PALM imaging (where increased power is necessary to bleach activated molecules as fast as possible). Obtaining high-quality DIC images with this objective is problematic, as it is not strain-free. Furthermore, it is not a plan objective, so care must be taken to image the specimen near the center of the field-of-view; otherwise aberrations degrade the quality of the point spread function (PSF) and thus limit the localization precision. Despite these caveats, the authors have observed >1.5× photon collection efficiency of the 1.65 NA objective compared to a 1.49 NA objective and significantly lower autofluorescence of the high-index coverslips compared to the more conventional glass coverslips used with the lower NA objective. The authors thus recommend the 1.65 NA objective for PALM-imaging fixed samples when the PALM image quality is of paramount importance and when acquisition time is not critical. For applications when high-quality DIC is important, on living cells that require 37°C, or when speed is critical, the 1.49 NA objective is the better choice.

    When using 1.45 or 1.49 NA objectives, it is important to use the correct immersion oil and coverslip thickness in order to minimize image aberrations (especially spherical aberration). Most high NA oil objectives are designed to work with no. 1.5 thickness glass coverslips; the authors use 25-mm no. 1.5 coverslips from Warner Instruments (cat. no. 64-0715). Index-matched immersion oils can be obtained from Cargille Laboratories—the authors recommend using type DF immersion oil, as it has very low autofluorescence and minimizes aberrations (type FF oil is marginally less autofluorescent, but introduces slight aberrations in the PSF).

EMCCD camera

12. To take full advantage of the wide-field TIRF geometry in PALM and to amplify the single-molecule fluorescence emissions from PA-FPs, use an electron-multiplying cooled CCD (EMCCD) camera as the detector.

    These cameras are capable of single-photon detection, as they employ an on-chip multiplication gain that boosts weak signals above the readout noise of the camera. Furthermore, fast (up to 10 MHz/pixel) readout and the ability to operate the camera in frame-transfer mode (spooling acquired data to disk while simultaneously acquiring new data) allow rapid PALM-imaging. The ability to cool the chip to −50°C or less ensures that dark noise (thermal motion of charges in the sensor that can be spuriously counted as photons) is negligible. Most chips can also be purchased with a back-illuminated option (light impinges on the back side of a thinned CCD instead of the front side), leading to a quantum efficiency (the ratio of incident to detected photons) of ∼90% across the visible spectrum.

Many single-molecule fluorescence laboratories use EMCCD cameras from Andor Technology. Several options exist, with different pixel sizes, chip sizes, and readout speeds. The investigator should consider the total system magnification (important in optimizing localization accuracy, discussed below) and desired imaging area before deciding what chip to purchase.

The authors use a DV887ECS-BV (Andor Technology) as the EMCCD, operating in frame-transfer mode and with 10 MHz A/D readout speed. A 512 × 512 pixel chip with 16-μm pixels and a system magnification of 120 offers a total field-of-view of ~70 μm. In practice, a smaller region of the chip is used for imaging (typically 128 × 128 or 256 × 256 pixels) for decreased acquisition time and decreased file sizes.

Optimizing system magnification

13. When localizing single molecules, it is important to match the pixel size of the EMCCD camera to the size of the point spread function (PSF, the diffraction-limited image of a point emitter). If the pixel size is too large, the molecule's PSF will be concentrated within too few pixels (undersampled), and there will not be sufficient spatial resolution to determine the center of the PSF precisely. Conversely, if the pixel size is too small, the PSF will be spread out across too many pixels, and the signal/noise per pixel will be too low to precisely determine the PSF center. The best choice is a pixel size that is approximately equal to the standard deviation in the PSF—see Thompson et al. (2002) for a more complete discussion.

    Given the different magnifications of the high NA objectives mentioned above (typically 60× or 100×), it is thus often necessary to increase the total magnification of the imaging system to meet the above criterion. This is accomplished either by the use of internal magnification lenses in the microscope system (usually either a 1.6× or 2× system), or a magnification system placed immediately between the camera and microscope port.

    When imaging with the 100×, 1.65 NA objective, the authors employ a 1.2× C-mount adaptor (Diagnostic Instruments, DD12NLC) before the EMCCD for a total system magnification of 120×. When using the 60×, 1.49 NA objective, the authors employ a 2× internal magnification lens and a 1.2× C-mount adaptor for a total magnification of 144×. The effective pixel size when using the camera mentioned above is thus 133 or 111 nm depending on the objective used.

Computer hardware and software requirements

14. A computer with sufficient RAM, processor power, and hard drive space to process and store the stack of single-molecule frames that make up an eventual PALM image is essential. Additionally, the computer must have a slot for a digital acquisition card—the Andor EMCCD cameras typically come with their own card (PCI format). Minimum requirements for running the Andor EMCCD are a 2.4 GHz Pentium processor, 1 GB of RAM, and Microsoft Windows 2000 or XP. An analog output board and/or USB interface may be useful for controlling the power of activation and excitation lasers.

    The authors use an industrial computer (Advantech) with 2 GB RAM, a dual-core 3 GHz processor, and a 320 GB hard drive. As PALM images are typically composed of tens of thousands of single-molecule frames (corresponding file sizes can easily be >5 GB, especially if large fields of view are imaged), large hard drives are necessary for initial storage of data—the authors thus recommend purchasing as large a hard drive as possible. For long-term storage of PALM data, a server with larger storage capacity is recommended. A fast connection to archival storage is also helpful, to prevent long delays while transferring data.

15. As PALM imaging is expedited by simultaneously activating the PA-FPs and exciting them, in principle no sophisticated software is needed to independently control or shutter the lasers. The Andor EMCCD cameras come with software [Andor Solis (i)] that is sufficient for acquiring PALM single-molecule image stacks, but in practice it may be desirable to develop integrated software that allows the user to control

the laser power (via analog or TTL control), camera acquisition parameters (EM gain, exposure time, and total number of recorded frames), and shuttering for the lasers.

16. As recording a long series of single-molecule frames can be relatively taxing on a computer, the authors perform the localization analysis and image rendering post acquisition using a different computer and appropriate analysis software. With efficient coding, it may be possible to perform the analysis in real-time, or close to real-time, thus eliminating the need for transporting large data files to a different analysis computer. The basic strategy in rendering PALM images is to replot each molecule *m* as a Gaussian centered at coordinates $\mathbf{x}_m$, $\mathbf{y}_m$ and of width $\boldsymbol{\sigma}_m$ (the latter represents the positional uncertainty, or standard error on the mean position of the molecule), where $\boldsymbol{\sigma}_m$ is typically much less than the positional standard deviation in the original diffraction-limited PSF, $\mathbf{s}_m$. The relevant analysis can be broken down into several steps. First, intensity peaks in the raw data that correspond to individual molecules (or fiducial markers, see Basic Protocol 2 below) are identified. This identification allows image data corresponding to a molecule to be summed across all frames and pixels in which the molecule appears. Second, $\mathbf{x}_m$, $\mathbf{y}_m$ and $\boldsymbol{\sigma}_m$ are determined by fitting the summed intensity data to a Gaussian mask, according to the theory described in Thompson et al. (2002). If fiducial markers are used in the experiment, these parameters can be corrected for sample drift during the acquisition. Finally, the molecules are rendered as Gaussians whose brightness indicates the probability that the molecule can be found at a given location. A more complete description of the analysis software is beyond the scope of this unit, but the reader is directed to the supplementary information in Betzig et al. (2006) for more detailed information.

BASIC PROTOCOL 2

PALM-IMAGING tdEos/PAXILLIN DISTRIBUTIONS IN FIXED CELLS

This protocol describes the PALM imaging of tdEos/paxillin distributions in fixed fibroblast cells. The protocol can be broken up into several steps. First, the cells must be transiently transfected with the tdEos/paxillin plasmid and plated onto appropriately cleaned coverslips. Although the cells can be transfected with common chemical reagents—e.g., Lipofectamine (Invitrogen), Effectene (Qiagen), FuGENE (Roche)—the authors have had far better success with the Nucleofection kit from Amaxa Biosystems, routinely achieving transfection efficiencies of >70% with high cell viability. Cleaning coverslips, plating cells, and subsequent transfection are covered in Support Protocols 1 and 2. Second, the transfected cells must be chemically fixed under conditions that preserve as much of the native cellular structure and EosFP fluorescence as possible. The fixation procedure described below is a modification of the protocol described in Galbraith et al. (1998). Finally, the fixed, transfected cells are PALM-imaged with a microscope similar to that described in Basic Protocol 1.

Although the example described here is the imaging of tdEos/paxillin in HFF-1 cells, the protocol is general: the authors have successfully adapted it for PALM-imaging vinculin, actin, α-actinin, and zyxin in CHO and NIH 3T3 cells.

Materials

EM grade paraformaldehyde
Clean water (filtered through a Millipore system)
10 N NaOH solution
2× PHEM buffer (see recipe)
Cleaned coverslips (Support Protocol 1) plated with HFF-1 cells transfected with tdEos/paxillin, in 35-mm plastic dishes (Support Protocol 2)

Immersion oil
100-nm and 40-nm Au particles (Microspheres-Nanospheres, cat. nos. 790114-010 and 790122-010)

Chemical hood
1-liter glass beaker
Hotplate with magnetic stirring capability
Magnetic stir bar
0.2-μm filter
37°C warm room or equivalent heating system
Fine steel forceps
Microscope set up (as described in Basic Protocol 1)
Lens paper
EMCCD camera (see Basic Protocol 1)
Benchtop vortexer/sonicator

Fix transfected cells

1. Prepare 4% paraformaldehyde (PF) solution by adding 4 g PF to 100 ml clean water in a glass beaker. Place the beaker (with a magnetic stir bar) on a hotplate, and heat the solution to ~80°C while stirring. Carefully add 80 μl of 10 N NaOH (this is necessary to fully dissolve the PF) to the mixture. When the PF appears dissolved, filter the solution using a 0.2-μm filter to remove any residual solid particulates.

   The investigator should wear gloves, and this procedure should be conducted in a fume hood, as paraformaldehyde is quite toxic. Monitor the solution carefully, as it should be kept from boiling.

2. Prepare 2% (v/v) PF/1× PHEM buffer (fixative) by combining the 4% PF and 2× PHEM solutions in equal volumes. Also prepare 1× PHEM by combining 2× PHEM and clean water in equal volumes. Heat the fixative and 1× PHEM to 37°C.

   Fresh fixative should be prepared daily for best morphological preservation of the cells.

3. Transfer the 35-mm dish containing the transfected sample (Support Protocol 2) from the incubator to the warm room. Gently pipet the growth medium off the cells and replace it with 1 ml prewarmed fixative. Incubate for 15 min at 37°C, then wash the coverslips three times, each time with 1 ml 1× PHEM.

   Pipetting/removing solutions should be done gently to ensure that cells are not ripped from the coverslip. Care should be also taken to ensure that the coverslip is completely immersed in liquid, so that it does not dry out.

Identify transfected cells that are suitable for PALM

4. With forceps, carefully lift and transfer the fixed sample from the 35-mm plastic dish to the sample holder and microscope stage. Immerse the sample in 1× PHEM once the sample is placed in its holder (to prevent it from drying out) and wipe off the bottom surface of the coverslip (the side that will face the objective) with lens paper (it is essential that water and immersion oil do not mix during the imaging).

5. Apply the appropriate immersion oil to the chosen TIRF objective.

6. Focus on the cells with the microscope eyepiece, using either brightfield or DIC optics.

7. Start the acquisition software and cool the EMCCD camera to −50°C or lower.

   The authors have not noticed a significant decrease in dark noise below −50°C.

8. At the maximum gain of the EMCCD, use the 488-nm laser at low power in combination with the GFP filter cube to scan the sample in TIRF and identify cells that express tdEos/paxillin fusion proteins. If a stage with linear encoders is used, note the positions of suitable cells so that they can be easily found later.

   As paxillin forms distinctive finger-like structures at the cell surface, identification of transfected cells should be straightforward.

   Note that because EosFP switches its fluorescence from green to orange upon photoactivation, the green fluorescence (excited by 488-nm light) provides a convenient monitor of protein expression at the diffraction-limited level, and can be used to screen cells to decide if they are suitably transfected for PALM.

   The determination of the 488-nm power to use in this step is empirical and depends on the particular experimental configuration. Generally, the authors use as low a power as possible in illuminating the sample (10 to 30 μW over an excitation region $\sim$30 μm in diameter, for intensities of $\sim$1 W/cm^2 at the sample) so as not to bleach the inactive EosFP molecules before PALM-imaging. A power meter is useful for measuring the laser power; the authors use a FieldMaxII-TO digital power meter (Coherent).

   With experience, the investigator can use the real-time intensities displayed by the camera acquisition software to determine what an acceptable level of transfection is, how much power to apply, and what exposure time should be used for minimal bleaching of the sample. Finally, note that there is often an inverse correlation between the cell health and the level of transfection; brightly fluorescent cells may not necessarily have the best morphology and vice versa. The investigator should find a cell that is sufficiently transfected but also appears to have good morphology in DIC.

9. If a suitable cell is found, prepare a 10× dilution of Au particles in 1× PHEM. Vortex the suspension until it is well-mixed (it may be necessary to also vortex the stock solution of fiducials if they have settled to the bottom of the container).

   Sonicating the mixture can also aid in breaking up Au aggregates.

   Because the Au particles are fluorescent but do not bleach during the acquisition period, they function as fiducial markers, and are important in correcting for mechanical drift during the acquisition period (Betzig et al., 2006). The basic strategy is to determine the position of the fiducials over the entire image acquisition, and to subtract these positions from the coordinates of the localized molecules when reconstructing a PALM image.

   The authors have found that 100-nm Au particles are best when imaging the orange, activated state of EosFP, whereas 40-nm Au particles are better when imaging the green, activated state of Dronpa.

   The concentration of Au fiducials (and incubation time) should be determined empirically. Generally it is best to have 2 to 5 fiducials visible in the imaging field of view when PALM-imaging—their positions can be averaged and used to remove drift more accurately than if only one fiducial is found. Conversely, too many fiducials impede the isolation of single molecules, as it is difficult to identify molecules located near a fiducial.

10. Carefully pipet off the 1× PHEM solution covering the coverslip, and replace it with 1 ml of the Au suspension. Incubate 5 to 10 min, or long enough for the Au particles to settle and stick to the coverslip surface, at 25°C.

11. Once a suitable density of Au fiducials is achieved, rinse the sample once with 1 ml 1× PHEM.

PALM-image a suitable cell

12. Switch to the appropriate filter cube for imaging the activated state of EosFP, and illuminate the sample with the 561-nm laser. Adjust the laser power until it is sufficient for achieving good signal-to-noise (S/N) images of single activated molecules and fiducials at the desired exposure time.

The laser power should be increased until single molecules can be visualized at high S/N at a minimal exposure time. The intensity of fiducials should be comparable to single-molecule fluorescence; occasionally, it is possible that fiducials will be too dim or too bright (the latter will saturate the EMCCD, and are usually indicative of Au aggregates), in which case the investigator is advised to add more fiducials. Also, in the case of the 1.65 NA objective, a practical limit on the maximum laser power is set by the absorption in the immersion oil. The final power and exposure time are dependent on the details of the investigator's setup, and are again best determined empirically. The authors typically use ~10 mW of 561-nm power (over an excitation region 30 μm in diameter, for intensities of ~ 1 kW/cm^2) and 20 to 50 msec exposure times.

13. With the laser power and exposure time adjusted, start the PALM acquisition. Illuminate the sample initially only with the 561-nm laser beam, to bleach the small population of EosFP molecules that appears fluorescent at the start of the acquisition.

    There is a population of EosFP that is spontaneously activated (and is thus excited) at the start of any PALM acquisition. These molecules must be bleached before activating further molecules, otherwise too many molecules will be activated at once, impeding the isolation of individual molecules and degrading the localization precision (Fig. 4.21.8A).

14. Illuminate the sample with the 405-nm activation laser at low power (it may be necessary to insert neutral density filters into the beam path to reduce the laser power). As the acquisition proceeds and the pool of unactivated molecules is depleted, increase the activation power in order to keep the number of activated molecules roughly constant per frame.

    Care must be taken to ensure that the optimal number of molecules is activated per frame. If too few molecules are activated at a given time, the data acquisition is slowed down and it will take longer to acquire the complete PALM dataset (Fig. 4.21.8B). The 405-nm laser power should be increased until the molecular density per frame increases. If too many molecules are activated, the image will appear diffraction-limited, retarding localization accuracy (Fig. 4.21.8A). The 405-nm laser power should be decreased until single molecules are easily discerned. From a speed perspective, the optimal activation power occurs when molecules appear "bumper to bumper," separated just enough to be spatially resolved (Fig. 4.21.8C).

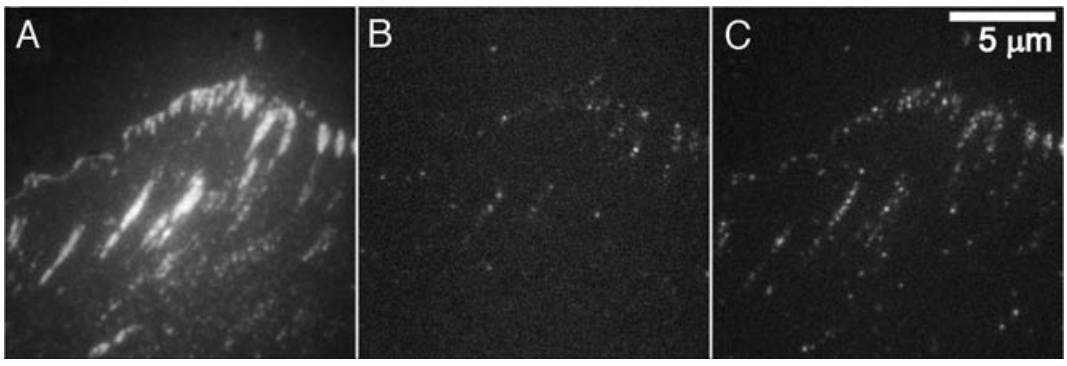


Figure 4.21.8 The effect of different levels of activation power on single-molecule frames in a PALM acquisition. Images are of tdEos/paxillin molecules in adhesion complexes, and were taken with identical excitation power and exposure time, but differing levels of activation power. In (**A**), the activation power was too high: too many molecules were activated and thus it is difficult to discern individual molecules. The image appears diffraction limited, impeding the localization of single-molecules. In contrast, (**B**) displays a frame where the activation power was too low; consequently few molecules are excited and it will take longer than necessary to excite and bleach all tdEos molecules. The optimal activation power is illustrated in (**C**), where single-molecules are in close proximity but still resolvable. For color version of this figure see *http://www.currentprotocols.com*.

15. Proceed with data acquisition, collecting frames until the population of single activated molecules becomes insignificant even at high activation laser power.

For long acquisitions, and depending on the mechanical stability of the instrument, it may be necessary to manually adjust the focus of the objective during PALM-imaging. Although several commercial auto-focus systems exist for correcting objective focus drift during data acquisition, there is always a slight time lag between when the auto-focus system is engaged and when the focus is reset to the original position. A simpler (and less expensive) alternative is to manually adjust the focus of the microscope during the PALM acquisition. A microscope system with an ultra-fine focus is useful for this purpose.

BASIC PROTOCOL 3

DUAL-COLOR PALM-IMAGING OF tdEos/VINCULIN AND DRONPA α-ACTININ IN FIXED CELLS

Single-color PALM can reveal the spatial distribution of a target protein at >10× resolution than with conventional fluorescence microscopy. While informative, cellular function is often driven by the interaction of two or more proteins. It would thus be valuable to accurately determine the spatial distribution of several interacting proteins using a multicolor superresolution technique. As described in Shroff et al. (2007), it is possible to serially PALM-image two adhesion complex proteins by tagging the first with EosFP and the second with Dronpa. This protocol shows that, with relatively few modifications, it is possible to adapt Basic Protocol 2 for the PALM-imaging of vinculin (Ziegler et al., 2006) and α-actinin (Otey and Carpen, 2004; Fig. 4.21.9).

Materials

HFF-1 cells cotransfected with tdEos/vinculin and Dronpa/α-actinin plasmid constructs (these plasmids are made in-house by the authors, but can also be obtained from Mike Davidson, Florida State University)

100-nm and 40-nm Au particles (Microspheres-Nanospheres, cat. nos. 790114-010 and 790122-010)

Additional reagents and equipment for cleaning coverslips (Support Protocol 1), transfecting cells (Support Protocol 2), and PALM-imaging both Eos (Basic Protocol 2) and Dronpa

Prepare sample

1. Follow Support Protocols 1 and 2 for cleaning coverslips and transfecting cells. Instead of using the amaxa system to nucleofect cells with only a single plasmid, cotransfect cells with both plasmid constructs.

As transiently transfecting cells with more than one expression plasmid stresses the cellular machinery more than if a single plasmid is transfected, some optimization of DNA concentration may be necessary to obtain optimal cell viability and expression level. The investigator is recommended to start with equal concentrations of both plasmid DNAs, and increase/decrease the concentration of either or both until a high percentage of cells survive the nucleofection and express enough of both proteins for good dual-color PALM-imaging.

2. Follow steps 4 to 8 in Basic Protocol 2 to identify transfected cells suitable for PALM.

As α-actinin cross-links actin stress fibers, and vinculin localizes to adhesion complexes at the termini of stress fibers, cells that are doubly-transfected should exhibit both morphologies (compare Fig. 4.21.9A and Fig. 4.21.9B). It should thus be possible to identify appropriately transfected cells by scanning the sample in TIRF, using the 488-nm laser.

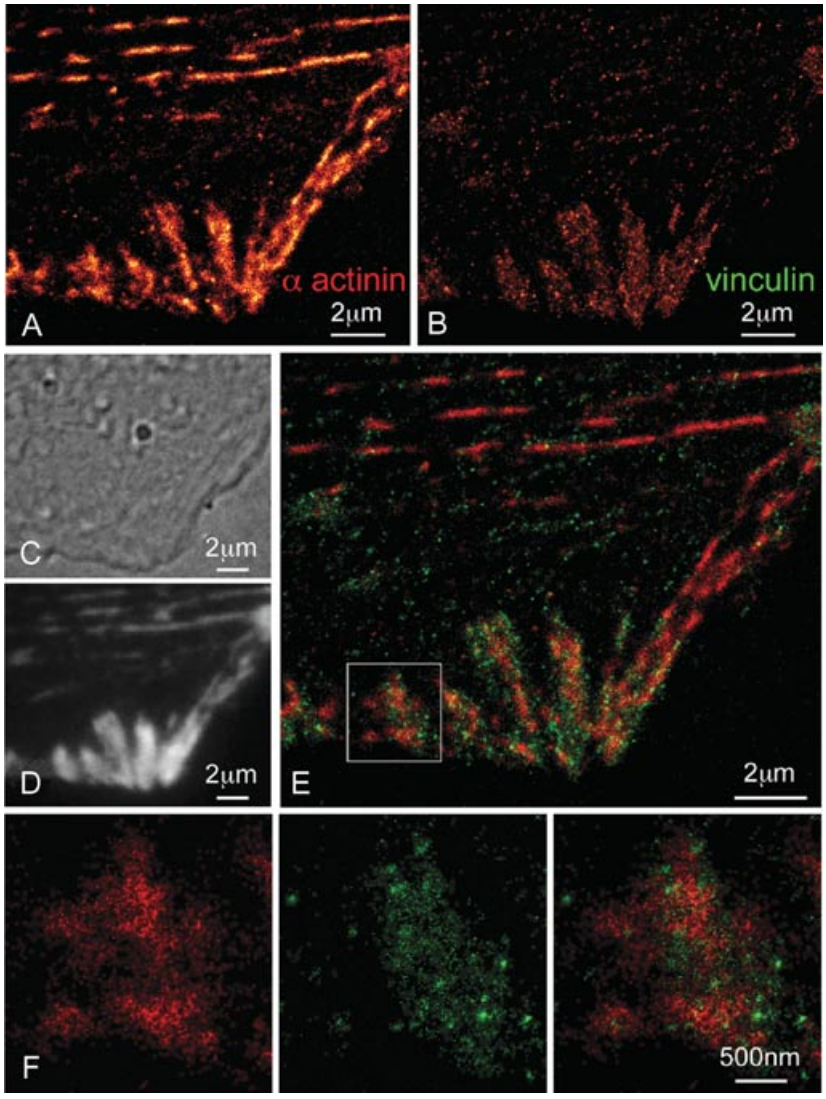


Figure 4.21.9 Dual-color PALM image of tdEos/vinculin and Dronpa/α-actinin in a fixed HFF-1 cell: (**A**) PALM image of Dronpa-tagged α-actinin; (**B**) PALM image of tdEos-tagged vinculin; (**C**) DIC image revealing morphology; (**D**) TIRF image of combined tdEos and Dronpa emission (note the distinct morphologies of α-actinin and vinculin); (**E**) dual-color PALM overlay of α-actinin (red) and vinculin (green); (**F**) α-actinin, vinculin, and overlaid PALM images within the single adhesion shown in the box in (E). Panels (E) and (F) reveal that α-actinin and vinculin only partially co-localize within each adhesion complex, with α-actinin existing in large patches emanating from stress fibers and vinculin coalescing in small, dense clusters scattered across each adhesion. Reproduced from Shroff et al., 2007. For color version of this figure see *http://www.currentprotocols.com*.

3. Prepare a 1:1 mixture of 40 nm Au particles and 100 nm Au particles, and incubate the sample with a 10× dilution as described in steps 9 to 11 of Basic Protocol 2.

   As both Eos and Dronpa are to be imaged, it is useful to include both kinds of fiducials. For registering both Eos and Dronpa images post-acquisition, it is essential that some fiducials show up in both Eos and Dronpa channels.

PALM-image the Eos channel

4. Follow steps 12 to 15 in Basic Protocol 2 to PALM-image the Eos channel. Ensure that all Eos molecules are bleached before proceeding.

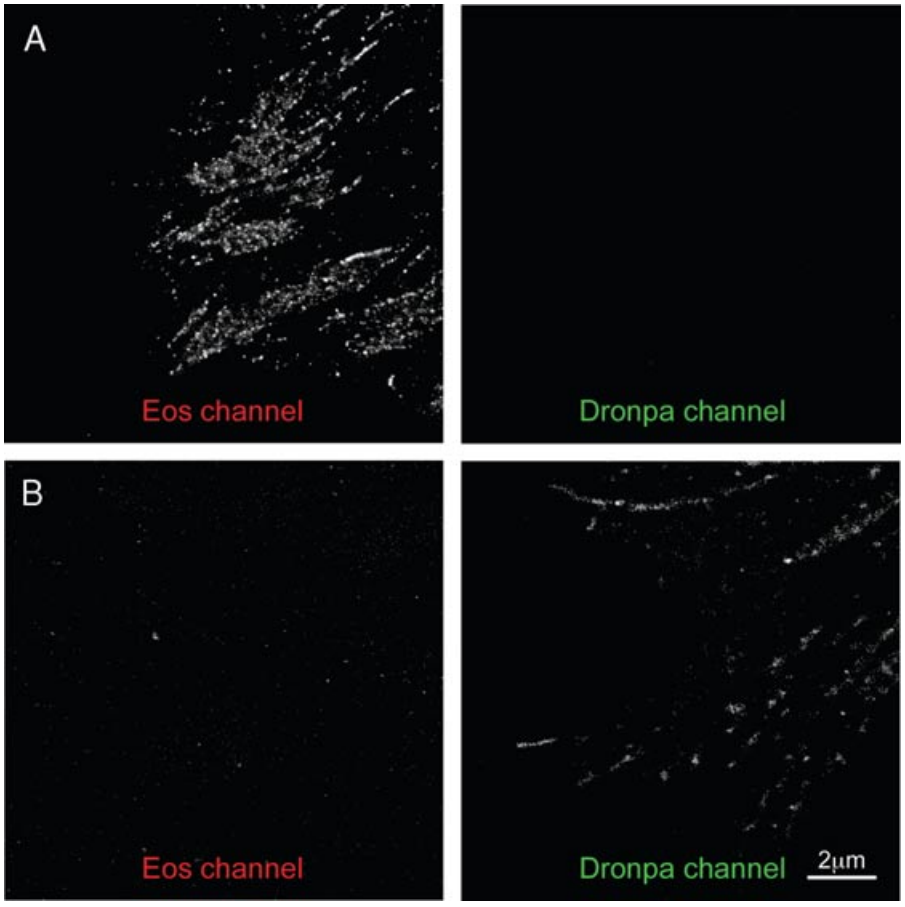


Figure 4.21.10 Crosstalk control experiments. (**A**) Left: Eos channel PALM image of tdEos-tagged paxillin in an HFF-1 cell. Over 221,000 molecules were localized over 100,000 frames. Right: Dronpa channel PALM image of the same cell. 8900 molecules were localized over 50,000 frames. Note the negligible misidentification of Eos as Dronpa. (**B**) Left: Eos channel PALM image of Dronpa-tagged paxillin in an HFF-1 cell. 7800 molecules were localized over 50,000 frames. Right: Dronpa channel PALM image of the same cell. Over 22,000 molecules were localized over 8200 frames. The number of detected molecules in the Eos channel in (B) is comparable to that found in untransfected control cells. In all cases, the Dronpa channel image was obtained after the EosFP channel image, and the images from both channels are plotted at identical brightness and contrast. Reproduced from Shroff et al., 2007.

As the technique described in Shroff et al. (2007) is serial, it is important to thoroughly activate and bleach the tdEos/vinculin molecules before attempting to PALM-image the Dronpa/α-actinin. Otherwise, residual Eos molecules that fluoresce in the green channel will be incorrectly identified as Dronpa molecules. The authors recommend doing control experiments akin to those described in Figure 4.21.10, to gain an intuition for what the background should look like in the Eos channel after all Eos molecules are bleached, and before PALM-imaging the Dronpa channel. Such crosstalk control experiments are also a good check to ensure that fluorescence emission filters are optimized and that bleed-through of one fluorescent tag onto the other channel is negligible.

PALM-image the Dronpa channel

5. Turn off the 405-nm and 561-nm lasers. Switch to the GFP filter cube for imaging Dronpa. Start the acquisition software and illuminate the sample with the 488-nm laser.

Initially, the pool of Dronpa molecules that were activated during the course of PALM-imaging Eos will fluoresce, contributing a bright green background and making it difficult

to discern single Dronpa molecules. Within several hundred frames (depending on the exposure time and laser power used), this background should die down, as Dronpa molecules are driven into a dark state with prolonged exposure to 488 nm. Ensure that the imaging area appears mostly dark (there are always some Dronpa molecules that are spontaneously active and appear to blink during the acquisition) before proceeding to the next step.

The high-index immersion oil used with the 1.65 NA objective is considerably less tolerant to 488 nm than 561 nm, as the absorption is greater in the bluer portion of the spectrum. Consequently, it is recommended to use a lower laser power with 488 nm than with 561 nm. The choice of how much power to use is best determined by the investigator. The authors use 3 to 5 mW (measured prior to the entrance of the objective) and 50 to 100 msec exposure time.

6. After the bright background of activated Dronpa molecules dies down, turn on the 405-nm laser at lower power. Follow steps 14 and 15 of Basic Protocol 2, gradually ramping the 405-nm laser power until all Dronpa molecules have been activated and bleached.

PREPARING CLEAN COVERSLIPS

SUPPORT PROTOCOL 1

For PALM experiments, the coverslips upon which the cells are grown should be cleaned as thoroughly as possible. This is because debris deposited on the coverslips is often excited by the activation and excitation lasers, and will contribute either to elevated background counts in each frame or as spurious single-molecule fluorescence. This protocol describes a chemical cleaning method based on the industry standard for removing contaminants from semiconductor wafers. It is considerably less reactive (and hazardous) than other cleaning methods, but still adequate for PALM-imaging. Although 1% hydrofluoric acid (HF) is suitable for cleaning conventional glass coverslips in ~5 min (see supporting information in Betzig et al., 2006), it reacts with the high-index coverslips used with the 1.65 NA objective, rendering them opaque and unsuitable for imaging.

Materials

Ammonium hydroxide
Hydrogen peroxide
Clean water (filtered through a Millipore system)
Methanol (spectroscopic grade)
Clean air supply (preferably filtered through a 0.2-μm pore-size filter)
RBS-35 liquid detergent concentrate (Pierce, cat. no. 27950)
Acetone (spectroscopic grade)

Chemical fume hood
100-ml graduated cylinder
250-ml glass beaker
25-mm diameter glass coverslips, no. 1.5 (Warner Instruments, cat. no. 64-0715; for use with 1.45 NA or 1.49 NA objectives)
High-index 20-mm diameter coverslips (Olympus, cat. no. APO100X-CG; for use with 1.65 NA objective)
Hotplate with magnetic stirring capability
Magnetic stir bar
Corrosion-resistant staining rack for holding coverslips (Thomas Scientific, cat. no. 8542E40)
Metal tongs for holding staining rack
Fine steel forceps
Compressed butane/natural gas, burner, and lighter

1. In a chemical fume hood, use a 100-ml graduated cylinder to prepare the etch solution by combining 125 ml clean water, 25 ml ammonium hydroxide, and 25 ml hydrogen peroxide in a 250-ml beaker. Heat the mixture to 80°C, and add a bar for stirring.

   The investigator should wear gloves, and take care to avoid exposing the skin to the corrosive solvents used in the cleaning solution.

2. Load the staining rack with coverslips and place it into the etch solution with the metal tongs. Let the coverslips incubate at least 3 hr (overnight is preferred for high-index coverslips) while stirring.

3. Fill another 250-ml beaker with clean water and carefully transfer the rack into the new beaker. Rinse the rack with copious amounts of clean water (at least 10 beaker volumes). Dispose of the cleaning solution appropriately.

4. Carefully remove a cleaned coverslip from the rack with the steel forceps (holding the coverslip near the edge) and rinse both sides with methanol.

   The methanol can be conveniently stored in a squeeze bottle and squirted onto the coverslip.

5. Rapidly remove excess methanol from the coverslip by blowing it away with the filtered air.

   A pipet tip can be connected with tubing to the air supply and used as an aid in blowing away the methanol.

6. Connect the burner to the butane (or natural gas), and light. Pass the coverslip quickly through the topmost portion of the flame, and repeat this process three times.

   The coverslip should be dry (or almost).

   Care must be taken not to hold the coverslip too long (more than a second) in the flame, otherwise it may crack due to thermal shock.

7. Store the cleaned coverslip dry in a clean staining rack. Repeat steps 4 to 6 with the rest of the coverslips. Cover the filled staining rack to prevent dust from settling on the coverslips, and store dry until the coverslips are ready for use.

8. The high-index coverslips are expensive, but can be recleaned and reused several times. Store used coverslips in a 2% solution of RBS detergent solution, and rinse with copious amounts of clean water before repeating steps 1 to 4. Before repeating steps 5 to 7, sonicate the coverslip for 5 min in acetone and rinse well with clean water.

SUPPORT PROTOCOL 2

TRANSFECTION OF tdEos/PAXILLIN INTO HFF-1 CELLS

Using the Nucleofector 96-well shuttle system (amaxa biosystems), HFF-1 cells are nucleofected with plasmid DNA in accordance to the manufacturer's guidelines for optimal viability and transfection efficiency. Nucleofection of lower passage cells harvested while in log growth, with highly purified DNA, results in transfection efficiencies >70%.

NOTE: All solutions and materials coming into direct contact with live cells must be sterile and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 tissue culture incubator unless otherwise specified.

Materials

70% ethanol
Human plasma fibronectin diluted in 1× PBS (without divalent cations)
1% (w/v) BSA/DMEM HG, heat-inactivated (see recipe)

HFF-1 growth medium (see recipe)
0.05% (w/v) trypsin-0.53mM EDTA
Cell Line 96-well Nucleofector Kit SE (amaxa, VHCA-1001) containing:
SE solution
Supplement
Plasmid: tdEos/paxillin (~0.5 to 1 μg/μl)
Normal human foreskin fibroblast (HFF-1) cells (ATCC cat. no. SCRC-1041) grown in 75-cm^2 cell culture flasks with 0.2-μm vent caps for 2 to 3 days before transfection

Compressed butane/natural gas, burner, and lighter
Fine steel forceps
Cleaned coverslips (see Support Protocol 1)
35 × 10–mm cell culture dishes
37°C water bath
1.5-ml microcentrifuge tube *or* 15-ml conical centrifuge tubes
Centrifuge capable of 90 × *g* (Eppendorf 5810 or equivalent)
Centrifuge rotor (Eppendorf 5810 A-4-62 or equivalent)
Rainin LTS pipets (0.1 to 200 μl)
Rainin LTS tips (RT-L10F and RT-L200F)
Nucleofector 96-well shuttle system (amaxa biosystems)

Additional reagents and equipment for performing a viable cell count (*UNIT 1.1*)

Pre-coat coverslips with 10 μg/ml fibronectin (day 1)

1. Spray forceps with 70% ethanol and pass through flame of ignited butane gas to sterilize. Use forceps to transfer each cover slip to a 35 × 10–mm dish. Add ~200 μl of 10 μg/ml fibronectin-1× PBS solution to coverslip surface and store overnight at 4°C.

Transfect cells (day 2)

2. Remove fibronectin solution and block coverslips with 200 μl heat-inactivated 1% BSA-DMEM. Store 1 to 2 hr at room temperature.

3. Remove BSA solution and add 1.5 to 2.0 ml HFF-1 growth medium per dish; incubate 30 to 60 min at 37°C in humidified 5% CO_2 to equilibrate dishes to culture conditions prior to plating cells.

4. Prewarm the HFF-1 growth medium and trypsin-EDTA in a 37°C water bath.

5. Acclimate the amaxa Nucleofector Kit SE and plasmid to room temperature.

6. Mix the volume of Nucleofector SE solution required by experiment. Use a mix ratio of 162.5 μl SE solution to 36 μl 96-well supplement. A total of 20 μl is required per shuttle well.

7. Use 5.0 ml trypsin-EDTA to detach HFF-1 cells per 75-cm^2 tissue culture flask. Neutralize cell suspension with an equal volume of HFF-1 growth medium.

   Before harvest, cells should be ~70% confluent.

8. Count an aliquot of the cells (*UNIT 1.1*) and calculate the volume sufficient to nucleofect 4 to 5 × 10^5 live cells per shuttle well. Transfer cell suspension to microcentrifuge tube(s) and centrifuge 7 min at 90 × *g*, room temperature.

   If a larger volume of cell suspension is required, a larger 15- ml conical centrifuge tube may be used to centrifuge cells.

   Per manufacturer's recommendation, one spin-down is preferred.

9. Carefully, remove all fluid from the pellet and gently resuspend the cells in 20 µl Nucleofector SE solution (prepared in step 6) per 4 to 5×10^5 live cells. Add ~1 µg plasmid DNA per 20 µl of Nucleofector SE solution. Gently mix cells, plasmid, and nucleofector solution. Load 20 µl per shuttle well.

   Per manufacturer recommendation, the volume of plasmid solution should not exceed 2 µl per shuttle well.

   Rainin RT-L10F tips are recommended by manufacturer for loading shuttle wells. Avoid creating air bubbles.

10. Load plate in amaxa apparatus, select program DS 137, and initiate nucleofection.

11. Upon completion of nucleofection, remove shuttle plate and add 80 µl HFF-1 growth medium (from dishes prepared in step 3) per shuttle well. Place shuttle plate inside incubator. After 10 min, transfer contents of each shuttle well to a pre-equilibrated 35-mm dish (prepared in step 3) and gently dispense cell suspension on top of coverslip surface.

12. Incubate plates at 37°C in 5% humidified CO_2.

    Cells are imaged ~24 to 36 hr post-nucleofection.

REAGENTS AND SOLUTIONS

Use ultrapure water purified by reverse osmosis and Millipore Milli-Q system in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BSA, 1% (w/v)/DMEM HG (heat-inactivated)

Prepare 1% (w/v) BSA fraction V (EMD OmniPur 2930) in high glucose DMEM (DMEM HG) without phenol red (Cellgro, 17-205-CV) supplemented with 4 mM L-glutamine. Heat for 30 min at 56°C and filter with a 0.2-µm cellulose acetate filter. Store up to 1 month at 4°C.

HFF-1 growth medium

High glucose DMEM without phenol red (Cellgro, 17-205-CV)
15% (v/v) FBS
4 mM L-glutamine
Store up to 1 month at 4°C

PHEM buffer, 1×

60 mM PIPES
25 mM HEPES
10 mM EGTA
2 mM $MgCl_2$
Adjust pH to 6.9 using NaOH
Store up to 1 year at 4°C

COMMENTARY

Background Information

Resolution considerations in PALM

The ultimate resolution achievable in PALM (Betzig et al., 2006), stochastic optical reconstruction microscopy (STORM; Rust et al., 2006), and fPALM (Hess et al., 2006) is dependent on two criteria: the localization precision (how well the center of each PSF can be determined) and the density of available photoactivatable molecules.

In the case of negligible background, it can be shown (Thompson et al., 2002) that the error in the PSF center determination is inversely proportional to the square root of the number of collected photons. Thus, a photoactivatable tag that emits 100-fold more photons

than another would give a 10-fold increase in localization precision. Indeed, synthetic fluorophores such as Cy3 yield $>10^6$ collected photons and enable localization down to ~1.5 nm (Yildiz et al., 2003) in ideal conditions. Maximizing the collection efficiency of the instrument (with high NA objectives and appropriate filters and camera) while rejecting background (with TIRF) is thus critical for high localization precision.

Although a high number of collected photons are desirable from a localization standpoint, in practice other factors (errors in the PSF fit, drift correction, background due to other fluorescent tags present at high density, or autofluorescence of the sample) limit the localization precision to ~10 nm for the majority of photoactivatable molecules. Furthermore, from the perspective of PALM-imaging as fast as possible, it is undesirable to use a tag that emits too many photons, as it takes a longer time to bleach the tag. Molecular bleaching is in fact the rate-limiting step in acquiring more data, as a given tag should be bleached before another can be activated and imaged in the same diffraction-limited region (DLR).

EosFP is well-matched to PALM needs: using the 1.65 NA objective and TIRF, >1000 photons are collected on average per molecule. This is enough photons to localize most molecules to within 20 nm (>85% of the molecules in Figure 4.21.1 are localized to within this precision), but not so many photons that molecules persist over too many frames. In contrast, Dronpa emits too few photons per switching cycle, and the localization precision and thus resolution are compromised as a result [compare Dronpa and Eos images in Shroff et al. (2007)].

Besides localization precision, the other key determinant of resolution is the density of molecules. According to the Nyquist-Shannon sampling theorem (Shannon, 1949), the sampling interval (mean distance between neighboring localized molecules) must be at least twice as fine as the desired resolution; otherwise, the feature of interest will be undersampled and unresolved. For example, in two dimensions (applicable for PALM-imaging adhesion complex systems), to achieve 10-nm resolution, molecules must be spaced 5 nm apart in each dimension, for a minimum density of 4×10^4 molecules/μm^2 or ~2000 molecules in a DLR of 250-nm in diameter.

As mentioned in the introduction, PA-FPs are better suited to achieving these densities than caged dye conjugated antibodies, as although caged dyes are often smaller than PA-FPs, the large size of antibodies still limits the densities that can be practically achieved. This situation may change if small-molecule labeling strategies (Chen and Ting, 2005; Giepmans et al., 2006) improve to the point where caged dyes can be routinely attached to a target protein with very high specificity.

Regardless of the labeling strategy (endogenous PA-FPs, exogenous caged dyes, or the cyanine dyes used in STORM), another factor that influences both the maximum achievable density and the localization precision is the contrast ratio, which is the ratio in fluorescence intensity between the inactive and activated states of the photoswitchable molecule. If the contrast ratio is less than the density of the probe in a given diffraction-limited region, the weak intensity from the inactive molecules will overwhelm the intensity from a single activated molecule, confounding both localization and the measurement of densely labeled samples. Figure 4.21.2 illustrates the contrast ratio for four common PA-FPs, showing that the large contrast ratio of Eos, which has been shown to be capable of measuring densities as high as 10^5 molecules/μm^2 (Betzig et al., 2006), or sufficient for 10-nm resolution, results in superior images, both at the single-molecule and PALM levels. The contrast ratio has not been measured for most photoswitchable molecules, but based on the reports so far, EosFP is the only PA-FP able to achieve imaging resolutions <20 nm. Note that the synthetic caged dye Q-rhodamine has a very high contrast ratio (see supporting material in Betzig et al., 2006), and that a new rhodamine-amide with very high contrast ratio has been recently reported (Fölling et al., 2007).

Critical Parameters and Troubleshooting

Although increasing the density of expressed PA-FPs is desirable from the standpoint of the Nyquist criterion and to increase spatial resolution, the investigator should be wary of overexpression artifacts. In some cases, the size of the PA-FP tag may affect the function of the target protein deleteriously. Controls should be done to ensure that the morphological and kinetic characteristics (shape, growth, and motility) of the transfected cells used for PALM-imaging are as close as possible to their untransfected neighbors. As mentioned in Strategic Planning, expressing non-monomeric PA-FPs at high density may also lead to increased aggregation, so the investigator is urged to interpret all PALM results with care.

Transfected cells should be imaged as soon as possible after fixation. The authors have noticed a pronounced decrease in sample fluorescence several hours post-fixation, presumably due to degradation of the PA-FPs. As a decreased number of detected molecules leads to a decrease in the final resolution, the authors fix samples immediately prior to imaging. Also, morphology of the cells is better preserved if fresh fixative is made daily.

During the acquisition period, the most critical parameter to control is the degree of photoactivation at the sample. If too many molecules are photoactivated simultaneously (Fig. 4.21.8A), it will be difficult to localize each one precisely, leading to decreased resolution in the final PALM image. This problem is easy to diagnose by carefully monitoring the acquisition as it proceeds: if the sample is diffraction-limited in appearance, and the investigator cannot make out the blinking and bleaching that is characteristic of single-molecule fluorescence, the activation power is likely too high and needs to be reduced until single-molecule fluorescence is visible (Fig. 4.21.8C). The authors avoid this problem by starting each PALM acquisition with the activation laser off; only after most molecules are bleached is the activation laser turned on, and at a very low power.

Another important consideration that has been neglected in several recent reports is the importance of the drift correction procedure. Uncorrected drift manifests itself as a blur in the final PALM image. Especially for longer acquisitions, and depending on the mechanical stability of the system, mechanical drift can be >100 nm. It is thus critical to measure and then correct the drift if the desired spatial resolution is to be better than this.

Both the degree of photoactivation and drift correction are important when performing dual-color PALM, but now the investigator must also ensure that the population of Eos-tagged molecules are completely bleached before PALM-imaging the Dronpa channel, or else the Eos molecules will be spuriously identified as Dronpa molecules. As illustrated in Figure 4.21.10, after bleaching out the Eos channel, the level of background counts in the Dronpa channel should be comparable to the background measured in an untransfected cell.

Anticipated Results

When a TIRF microscope is adapted for PALM as described in Basic Protocol 1, EosFP is used as the photoactivatable tag, and the guidelines in Basic Protocol 2 followed, the investigator can expect PALM images similar to Figure 4.21.1, with high (~20 nm) spatial resolution. Following Basic Protocol 3 leads to dual-color superresolution imaging of adhesion complexes, and images akin to Figure 4.21.9. If laser power is not limiting, single-color PALM experiments on living systems are feasible, because molecules can be activated and bleached fast enough to track slowly evolving processes (e.g., adhesion complex dynamics that evolve on the timescale of tens of seconds) at superresolution. Dual-color, superresolution imaging of live cells awaits the development of new, spectrally distinct PA-FPs; because the approach outlined in Basic Protocol 3 is serial, it cannot be used in simultaneous two-color imaging. The authors expect that, in time, PALM will yield new biological insights, especially for those systems that can be densely labeled and are easily amenable to TIRF. For thicker specimens such as tissues, methods will need to be developed for sectioning (either optical or physical) of the sample, PALM-imaging the individual sections, and reassembling the three-dimensional PALM image.

Time Considerations

Clean coverslips are prepared in ~12 hr (Support Protocol 1). It takes an additional 12 hr to coat the cleaned coverslips with fibronectin. Once cells are ready for transfection (see Support Protocol 2), it takes ~1 hr to perform the amaxa transfection and then a further 24 to 48 hr before expression levels are suitable for PALM. Imaging takes between 5 to 30 min if high laser powers are used to bleach the activated molecules, although this time depends on the size of the desired field of view, and the density and number of expressed PA-FPs. Sample preparation thus constitutes the bulk of the preparation time in PALM.

Acknowledgements

The authors thank Jim and Cathy Galbraith (National Institutes of Health) and Na Ji and Tim Brown (Janelia Farm Research Campus) for their feedback and helpful suggestions on the manuscript.

Literature Cited

Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. 2002. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99:12651-12656.

Ando, R., Mizuno, H., and Miyawaki, A. 2004. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science* 306:1370-1373.

Axelrod, D. 2001. Total internal reflection fluorescence microscopy in cell biology. *Traffic* 2:764-774.

Betzig, E., Patterson, G.H., Sougrat, R., Lindwasser, O.W., Olenych, S., Bonifacino, J.S., Davidson, M.W., Lippincott-Schwartz, J., and Hess, H.F. 2006. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313:1642-1645.

Chen, I. and Ting, A.Y. 2005. Site-specific labeling of proteins with small molecules in live cells. *Curr. Opin. Biotechnol.* 16:35-40.

Chudakov, D.M., Verkhusha, V.V., Staroverov, D.B., Souslova, E.A., Lukyanov, S., and Lukyanov, K.A. 2004. Photoswitchable cyan fluorescent protein for protein tracking. *Nat. Biotechnol.* 22:1435-1439.

Chudakov, D.M., Lukyanov, S., and Lukyanov, K.A. 2007. Tracking intracellular protein movements using photoswitchable fluorescent proteins PS-CFP2 and Dendra2. *Nat. Protocols* 2:2024-2032.

Egner, A., Geisler, C., Middendorff, C., Bock, H., Wenzel, D., Medda, R., Andresen, M., Stiel, A.C., Jakobs, S., Eggeling, C., Schönle, A., and Hell, S.W. 2007. Fluorescence nanoscopy in whole cells by asynchronous localization of photoswitching emitters. *Biophys. J.* 93:3285-3290.

Fölling, J., Belov, V., Kunetsky, R., Medda, R., Schönle, A., Egner, A., Eggeling, C., Bossi, M., and Hell, S.W. Photochromic rhodamines provide nanoscopy with optical sectioning. *Angew. Chem. Int. Ed.* 46:6266-6270.

Galbraith, C.G., Skalak, R., and Chien, S. 1998. Shear stress induces spatial reorganization of the endothelial cell cytoskeleton. *Cell Motil. Cytoskeleton* 40:317-330.

Giepmans, B.N.G., Adams, S.R., Ellisman, M.H., and Tsien, R.Y. 2006. The fluorescent toolbox for assessing protein location and function. *Science* 312:217-224.

Gustafsson, M.G.L. 2000. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* 198:82-87.

Gustafsson, M.G.L. 2005. Nonlinear structured-illumination microscopy: Wide-field fluorescence imaging with theoretically unlimited resolution. *Proc. Natl. Acad. Sci. U.S.A.* 102:13081-13086.

Hess, S.T., Girirajan, T.P.K., and Mason, M.D. 2006. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys. J.* 91:4258-4272.

Mitchison, T.J., Sawin, K.E., Theriot, J.A., Gee, K., and Mallavarapu, A. 1998. Caged fluorescent probes. *Methods Enzymol.* 291:63-78.

Otey, C.A. and Carpen, O. 2004. Alpha-actinin revisited: A fresh look at an old player. *Cell Mot. Cytoskel.* 58:104-111.

Patterson, G.H. and Lippincott-Schwartz, J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297:1873-1877.

Rust, M.J., Bates, M., and Zhuang, X. 2006. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM) *Nat. Methods* 3:793-796.

Shannon, C.E. 1949. Communication in the presence of noise. *Proc. IRE* 37:10-21.

Shroff, H., Galbraith, C.G., Galbraith, J.A., White, H., Gillette, J., Olenych, S., Davidson, M.W., and Betzig, E. 2007. Dual-color superresolution imaging of genetically expressed probes within individual adhesion complexes. *Proc. Natl. Acad. Sci. U.S.A.* 104:20308-20313.

Thompson, R.E., Larson, D.R., and Webb, W.W. 2002. Precise nanometer localization analysis for individual fluorescent probes. *Biophys. J.* 82:2775-2783.

Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N., and Miywaki, A. 2005. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep.* 6:233-238.

Weiss, S. 1999. Fluorescence spectroscopy of single biomolecules. *Science* 283:1676-1683.

Wiedenmann, J. and Nienhaus, G.U. 2006. Live-cell imaging with EosFP and other photoactivatable marker proteins of the GFP family. *Expert Rev. Proteomics* 3:361-374.

Wiedenmann, J., Ivanchenko, S., Oswald, F., Schmitt, F., Rocker, C., Salih, A., Spindler, K.-D., and Nienhaus, G.U. 2004. EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proc. Natl. Acad. Sci. U.S.A.* 101:15905-15910.

Willig, K.I., Rizzoli, S.O., Westphal, V., Jahn, R., and Hell, S.W. 2006. STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. *Nature* 440:935-939.

Yildiz, A., Forkey, J.N., McKinney, S.A., Ha, T., Goldman, Y.E., and Selvin, P.R. 2003. Myosin V walks hand-over-hand: Single fluorophore imaging with 1.5-nm localization. *Science* 300:2061-2065.

Zaidel-Bar, R., Itzkovitz, S., Ma'ayan, A., Iyengar, R., and Geiger, B. 2007. Functional atlas of the integrin adhesome. *Nat. Cell Biol.* 9:858-867.

Zamir, E. and Geiger, B. 2001. Molecular complexity and dynamics of cell-matrix adhesions. *J. Cell Sci.* 114:3583-3590.

Ziegler, W.H., Liddington, R.C., and Critchley, D.R. 2006. The structure and regulation of vinculin. *Trends Cell Biol.* 16:453-460.

Culturing MDCK Cells in Three Dimensions for Analyzing Intracellular Dynamics

Natalie Elia[1] and Jennifer Lippincott-Schwartz[1]

[1]Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

ABSTRACT

Epithelial cells grown in three-dimensional (3-D) cultures of extracellular matrix differentiate into a multicellular structure of polarized cells. This process shares many characteristics with the physiological development of an epithelial tissue and the formation of polarity in epithelial cells. Imaging 3-D cultures of polarized epithelial cells is therefore a powerful tool to study epithelial architecture and morphogenesis under close-to-physiological conditions. The new generation of confocal microscopes allows live-cell imaging of fluorescently tagged molecules in these cultures. This opens up new opportunities for studying how molecules behave and are distinguished asymmetrically within a 3-D setting. This unit discusses technical aspects for culturing and imaging MDCK 3-D culture for both fixed 3-D cultures and live-cell imaging. *Curr. Protoc. Cell Biol.* 43:4.22.1-4.22.18. © 2009 by John Wiley & Sons, Inc.

Keywords: 3-D cultures • MDCK cells • live-cell imaging • MDCK cyst • epithelial cyst • 3-D culture imaging

INTRODUCTION

Madin-Darby canine kidney (MDCK) cells embedded in extracellular matrix (ECM) form three-dimensional (3-D) cultures. These cultures are spherical cysts characterized by a hollow lumen surrounded by one layer of polarized cells (Fig. 4.22.2C). 3-D MDCK cultures recapitulate numerous features of epithelial tissues in vivo (O'Brien et al., 2002; Debanath and Brugge, 2005) and therefore provide an ideal model system to study epithelial morphogenesis and polarity under physiological conditions in a tissue culture dish.

Live-cell imaging of 3-D MDCK cultures using fluorescently tagged proteins provides a unique opportunity to study the intracellular dynamics of proteins in these cells.

This unit describes fluorescence-based techniques to image intracellular proteins in fixed and live 3-D cultures using confocal fluorescent microscopy. It begins by describing the general requirements for fluorescent imaging of these cultures. Then, a protocol for plating and growing MDCK 3-D cultures for both fixed and live-cell imaging is provided. Next, an indirect immunostaining protocol is described. A live-cell imaging protocol is then discussed, including the optimal settings for imaging different fluorescently tagged proteins and the more advanced live-cell imaging techniques of fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP).

STRATEGIC PLANNING

Culture Conditions

Conditions for growing MDCKII cells (herein referred to as MDCK) in 3-D cultures are described in this unit. Other MDCK clones may require different conditions for 3-D culture formation.

Current Protocols in Cell Biology 4.22.1-4.22.18, June 2009
Published online June 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0422s43

Because MDCK cells may lose their ability to form a 3-D culture when they are subjected to repeated trypsinization cycles, it is important to use low-passage cells and to have a large number of frozen aliquots for each cell type.

Fluorescent Tags

Imaging a fluorescently tagged protein in live 3-D cultures usually requires that the protein already be stably expressed in the MDCK cells. This is because it is very difficult to transfect 3-D cultures with the conventional transfection reagents (i.e., Lipofectamine, Fugen6) after the cultures are formed. In addition, transiently transfecting the cells before plating them in collagen is very inefficient because the level of the over-expressed protein usually drops by the time the 3-D cultures are formed and ready to be imaged (4 to 9 days later). To obtain the best high-quality images, the protein of interest should be tagged with a bright fluorescent protein that is not sensitive for photobleaching, such as EGFP. Moreover, it is best to use a clonal line strongly expressing the fluorescent protein of interest. If this is impossible, FACS sorting of the cells after transfection to enrich the population of expressing cells is recommended.

BASIC PROTOCOL 1

GROWING MDCK THREE-DIMENSIONAL CULTURES

To induce 3-D culture formation of MDCK cells, extracellular matrix components need to be provided. This protocol will describe plating of MDCK cells in a collagen I matrix, which is a major component of the extracellular matrix. Plated in collagen I, MDCK cells form 3-D cultures that further differentiate into tubes in the presence of the hepatocyte growth factor, HGF (O'Brien, 2002). This protocol describes plating of MDCK 3-D cultures in membrane filter inserts (Fig. 4.22.1B), which is best suited for immunofluorescence, or in chamber slides (Fig. 4.22.1A), which are required for live-cell imaging. For immunofluorescence, MDCK 3-D cultures may also be plated in chamber slides; however, the quality of the images will be best when the cells are plated in membrane filters due to the smaller distance of the cultures from the coverslip.

Since both protocols are very similar, they will be described as one with specific instructions for each method indicated at the necessary steps.

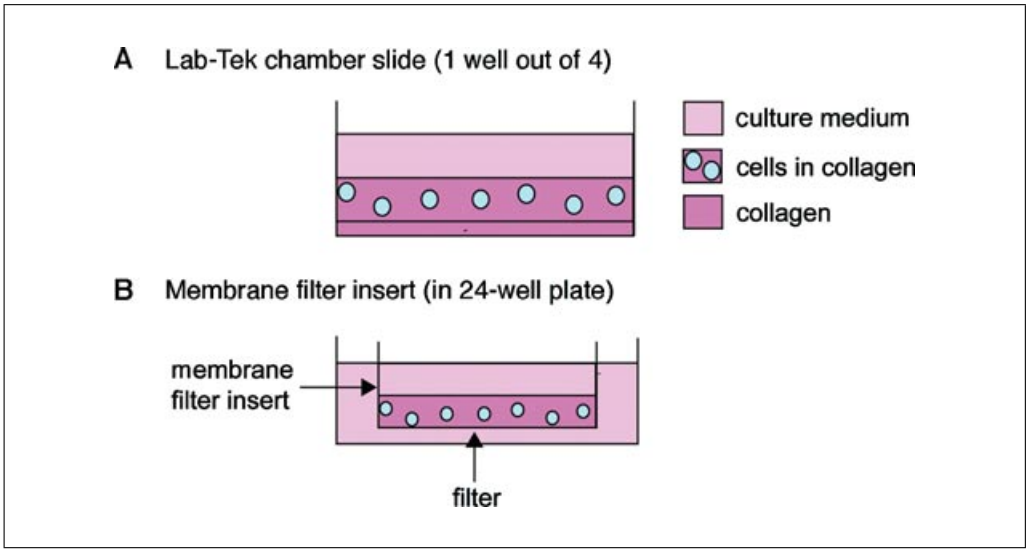


Figure 4.22.1 Schematic representations of 3-D culturing of MDCK cells plated in a chamber slide (**A**) or in a membrane filter insert (**B**).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

MDCK II cells
MEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin
1 mg/ml of G418, optional
0.25% (w/v) trypsin/1 mM EDTA
200 mM GlutaMAX (GIBCO, Invitrogen) stock (or 200 mM glutamine)
280 mM $NaHCO_3$ in water (store at 4°C)
10× MEM without glutamine (store at 4°C)
1 M HEPES, pH 7.6
3 mg/ml PureCol purified collagen I (INAMED Biomaterials) or any highly purified bovine collagen I (store at 4°C)

Sterile tissue culture hood
10-cm tissue culture dishes
15-ml conical tube
pH indicator strips
4-well Lab-Tek chamber slide (Nalge Nunc cat. no. 155383)
37°C oven
Nunc Anapore membrane inserts (0.2-μm pore, 10-mm; Nalge Nunc cat. no. 13935)
Hemacytometer

Prepare cells

1. Grow MDCK II cells in MEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. When using a stable cell line, add the selection antibiotic (e.g., G418 1 mg/ml).

   For stable cell lines the medium should contain 1 mg/ml of G418 or any other antibiotic that was used to generate the cell line.

2. Maintain cells by splitting every 3 to 4 days with 0.25% trypsin/EDTA (*UNIT 1.1*).

   MDCK cells should be split 1 day post-confluency (100%), after they have formed a tightly packed layer of hexagonal cells.

3. Split a post-confluent dish of MDCK II cells 1:10 to a 10-cm dish 1 day prior to plating day.

   This step is required to make sure that the cells are in their growing and proliferating stage, which is crucial for 3-D culture formation.

4. Prepare collagen I solution by combining the following in a 15-ml conical tube:

   750 μl 200 mM GlutaMAX (final 24 mM)
   625 μl 280 mM $NaHCO_3$ (final 2.8 mM)
   625 μl 10× MEM (final 1×)
   125 μl 1 M HEPES, pH 7.6 (final 20 mM)
   4.13 ml collagen I (final 2 mg/ml)
   Total volume 6.255 ml

   Mix by pipetting up and down. Verify that the pH is neutral by applying 100 μl of the collagen I solution onto a pH indicator strip. If the pH is not neutral, make a fresh

solution. Use a fresh HEPES stock. Do not try to neutralize pH because this will change the concentration of collagen in solution. Store the solution at 4°C during plating procedure to avoid premature polymerization of the collagen. Do not keep for more than a few hours.

Make a fresh collagen I solution for every experiment.

Prepare plates

5a. *For live-cell imaging.* Precoat the chamber slide with 75 to 100 µl of collagen I solution for each well of a 4-well chamber slide. Make sure the collagen solution completely covers the bottom of the chamber by tapping on the chamber. If tapping does not help, use a pipet tip to spread the solution throughout the well surface. Incubate chamber for 30 min in a 37°C oven (without CO_2). Gently tilt the dish to make sure the collagen has polymerized. If not, incubate for a longer period of time.

Precoating is a critical step for live-cell imaging because it reduces the number of cells that adhere to the coverslips and thus do not form a 3-D culture. However, precoating with too much collagen solution will increase the distance of the 3-D cultures from the coverslip, which will dramatically reduce imaging quality due to the thickness of the sample.

5b. *For immunostaining*. Place Nunc membrane filter inserts into each well of a 24-well plate.

Precoating is not necessary for this procedure.

Prepare cells

6. Trypsinize cells with 1 ml of 0.25% trypsin/EDTA for ~10 min in a cell incubator. After cells fully detached from the dish, add ~10 ml MEM growth medium.

7. Pipet cells up and down to obtain a single-cell suspension.

MDCK cells tend to form cell clumps and therefore it is often necessary to pipet them vigorously to obtain a single-cell suspension.

8. Pellet cells by centrifuging 4 min at 400 × *g*, room temperature.

9. Remove supernatant and wash cell pellet with ~5 ml medium. Repeat centrifugation in step 8.

10. Remove supernatant and resuspend cells in 2 ml medium. Pipet up and down to avoid cell clumps. Avoid bubbles.

This step is critical for obtaining uniform cultures with a single central lumen.

11. Determine cell concentration (cells/ml) using a hemacytometer (*UNIT 1.1*).

12. Add cells to the collagen solution to a final concentration of 3×10^4 cells/ml.

For live cell imaging, cells can be plated at higher concentrations (up to 6×10^4 cells/ml).

When adding the cells, make sure that the volume of cells does not exceed 10% of the collagen solution volume because the diluted collagen solution will not polymerize properly into a matrix.

Plate cells

13a. *For live-cell imaging.* Add 300 µl of collagen/cell mixture to a precoated single well of a 4-well chamber slide. Make sure that that the collagen/cell solution is evenly distributed by gently shaking the dish and avoiding bubble formation.

13b. *For immunostaining*. Apply 150 µl of collagen/cell mixture to the center of a membrane filter insert. Make sure that the collagen/cell solution is evenly distributed by gently shaking the dish and avoiding bubble formation.

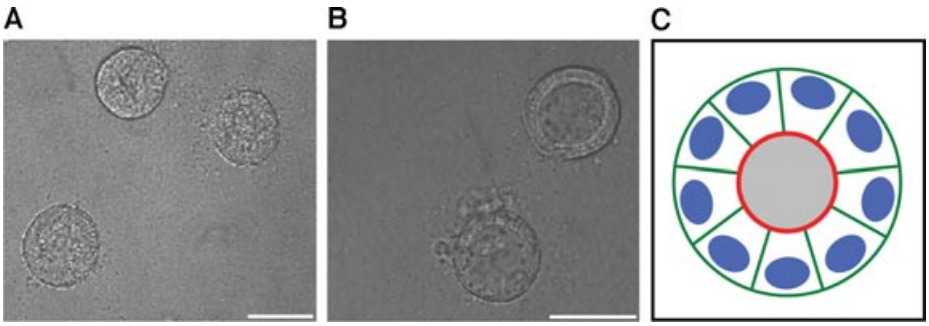


Figure 4.22.2 A bright field image of 3-D cultures of MDCK cells plated in a Lab-tek chamber slide at day 4 (**A**) and day 7 (**B**). Scale bar = 50 μm. (**C**) A schematic representation of a 3-D culture. Red, apical membrane; green, basolateral membrane; blue, nucleus; gray, lumen. For color version of this figure go to *http://www.currentprotocols.com.*

14. Incubate 30 to 45 min in a 37°C oven without CO_2.

    Collagen I polymerizes best under these conditions. Different temperatures or CO_2 levels may cause uneven polymerization that can cause variability in 3-D culture morphology in the same dish. It is therefore recommended to place the dish in the 37°C oven as soon as possible and to avoid CO_2.

    The time required for polymerization may differ between different batches of purified collagen I.

15. Gently tap the dish to make sure that the collagen has solidified and formed a gel texture.

16. After the collagen has polymerized, add culture medium to the wells and place in a 37°C, 5% CO_2 incubator.

    For chamber slides, add 500 μl to each well of a 4-well slide.

    For membrane filter inserts, add 500 μl to the inside of the insert and 1 ml to the outside (between the insert and the 24-well plate well; see Fig 4.22.1).

Maintain 3-D cultures

17. For chamber slides, change the medium every 1 to 2 days. For membrane filter inserts, change the medium every 2 to 3 days.

18. Observe the cultures for formation and development of the lumen.

    A lumen is already observed at day 4 (Fig 4.22.2) and cultures continue to develop until day 12. For a more extensive description of culture morphology, see Anticipated Results.

INDIRECT IMMUNOFLUORESCENCE OF MDCK 3-D CULTURES

BASIC PROTOCOL 2

The intracellular localization of proteins in 3-D culture may be different than that found in other systems due to differences in environmental cues and morphogenetic responses. It is therefore crucial to study the localization of endogenous proteins via indirect immunofluorescence (*UNIT 4.3*). Indirect immunofluorescence also allows for the assessment of correct cell polarity and other morphological features of the 3-D cultures prior to performing live-cell imaging experiments. Common polarity markers that specifically label certain domains in polarized cells (apical membrane, basolateral membrane, tight-junctions) should be used in indirect immunofluorescence analysis (Figs. 4.22.3 and 4.22.4). This permits detection of morphological phenotypes that may have been induced by overexpression of the protein of interest and evaluation of the quality of the

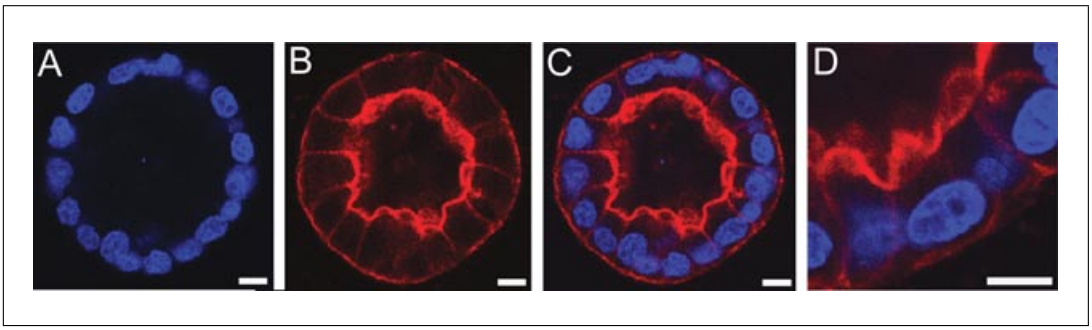


Figure 4.22.3 An immunofluorescent image of a fixed MDCK cyst in a 3-D culture at day 5. Nuclei were stained with Hoechst 33258 (blue) (**A**) and actin was stained with Texas-red phalloidin (red) (**B**). Images were taken using a 60× oil objective NA 1.4. (**C**) Merged image. (**D**) A zoomed-in image of polarized cells in the culture. Scale bar = 10 μm. For color version of this figure go to *http://www.currentprotocols.com.*

cultures. Importantly, it permits determination of whether the 3-D culture is fully polarized. Live-cell imaging of 3-D cultures that are not fully polarized may lead to misleading results.

The most commonly used method for indirect immunofluorescence of 3-D cultures involves whole-culture fixation. This approach is less time consuming and avoids extensive culture extraction that may affect culture morphology. This method is described in detail and includes recommended staining for culture evaluation.

Materials

3-D MDCK cultures on membrane inserts (see Basic Protocol 1)
1000 U/ml collagenase-1 (type CVII, Sigma cat. no. C-2799) in PBS+ stock (store in aliquots at −80°C)
PBS+: 1× PBS supplemented with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} (store at room temperature)
4% (w/v) paraformaldehyde (PFA)
Permeabilization solution: 10% FBS, 0.5% Triton X-100 in PBS (make fresh for every experiment, store at 4°C during the experiment)
Primary antibody against protein of interest
Secondary antibody tagged with fluorophore (e.g., Alexa and Cy dyes)
Hoechst 33258 nuclear dye (Molecular Probes cat. no. H-3569)
Fluorescently tagged phalloidin (Molecular Probes; Invitrogen)
Fluoromount-G mounting solution (SouthernBiotech cat. no. 0100-01)
Clear nail polish

37°C incubator
Platform shaker
Microscope slides
Carbon steel blades
Fine forceps
Circular coverslips (18 to 25 mm)

Treat cultures with collagenase

1. To improve the permeability of the antibody through the collagen matrix, treat 3-D cultures with collagenase. Dilute a 1000-U/ml aliquot of collagenase-1 stock solution 1:10 with PBS+.
2. Wash cultures quickly three times with PBS+ (250 μl to the inside of the membrane insert and 500 μl to the outside).

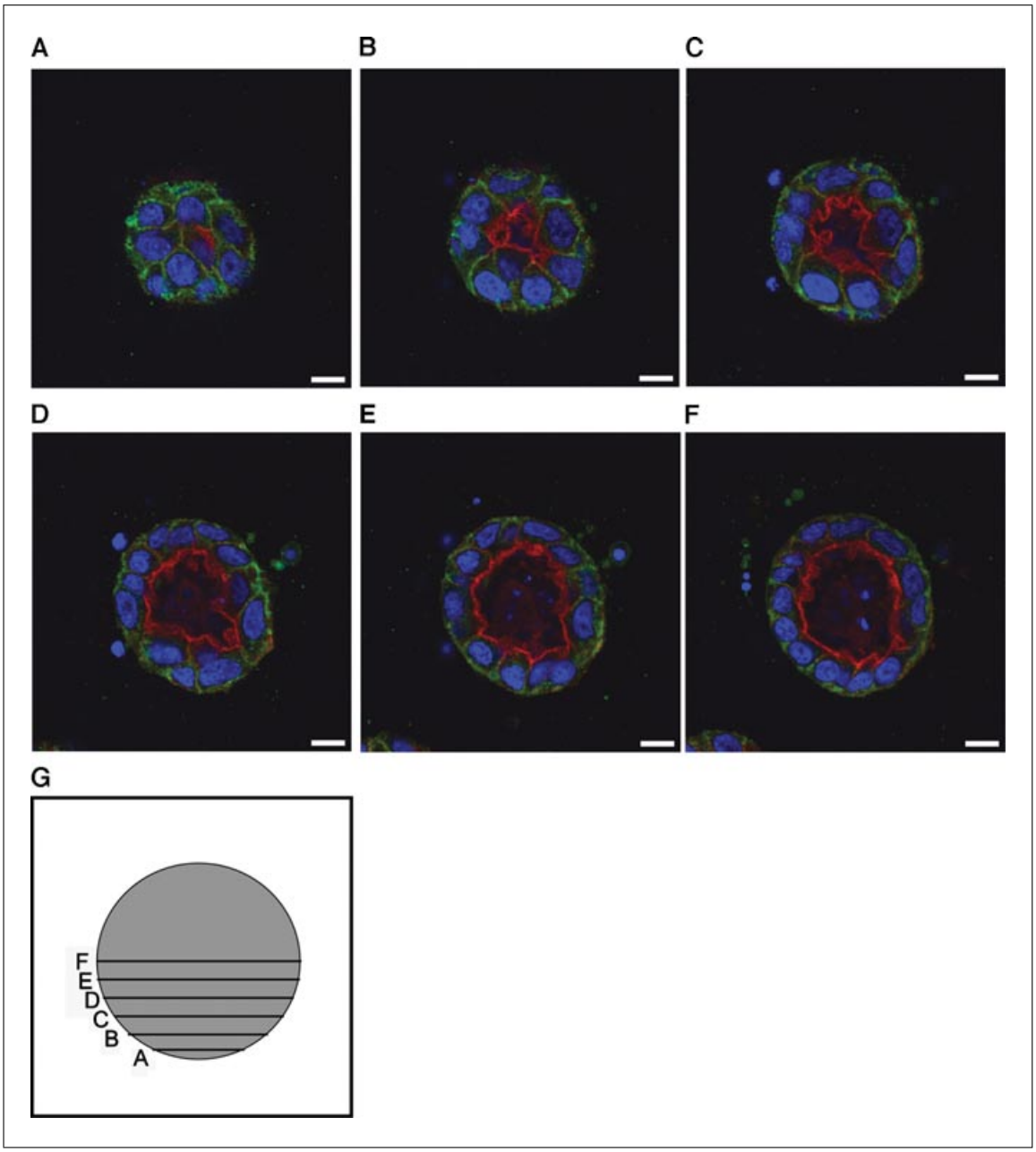


Figure 4.22.4 (**A-F**) Different optical *z* sections of an MDCK cyst in a 3-D culture from the bottom (A) to the middle (F) of the culture. Culture was fixed at day 5 and stained with Hoechst 33258 (blue), Phalloidin (red), and E-cadherin (green). (**G**) A schematic view of the different optical *z* sections shown in (A-F). Scale bar = 10 μm. For color version of this figure go to *http://www.currentprotocols.com.*

3. Apply 250 μl of collagenase-1 solution to the inside of the membrane insert and 500 μl to the outside. Incubate 10 min in a 37°C incubator. Do not incubate for longer times.

   Extensive collagenase treatment will solubilize the collagen matrix, which will make the sample difficult to work with and may affect culture morphology.

4. Wash cultures quickly three times with PBS+ (250 μl to the inside and 500 μl to the outside).

Fix cultures

5. Add 4% PFA (250 μl to the inside and 500 μl to the outside). Shake slowly for 30 min at room temperature.

6. Wash quickly three times with PBS+ followed by three 10-min washes (apply 250 µl to the inside and 500 µl to the outside).

Fixed cultures can be stored for up to 2 weeks at 4°C in 0.1% PFA.

Permeabilize and block

7. Add permeabilization solution (250 µl to the inside and 500 µl to the outside) and shake slowly for 30 min at room temperature.

Stain with antibody

8. Dilute primary antibody to the desired working dilution in permeabilization solution.

Use at least two times the concentration as that for adherent cells on a coverslip or according to manufacturer's instructions.

9. Add 200 µl diluted primary antibody to the inside of a membrane insert and 250 µl to the outside. Incubate overnight at 4°C.

Overnight incubation is usually recommended to maximize antibody permeability. Shorter incubation times (shaking for 3 to 4 hr at room temperature) may be possible in some cases.

Antibody permeability is a potential problem in whole-mount immunofluorescence of 3-D cultures since the antibody needs to penetrate the collagen polymers, the cell membrane, and the lumen in some cases. It is therefore important to use high antibody concentrations and long incubation times to increase the probability for maximal antibody staining.

10. Wash six times—three quick washes followed by three 10-min washes—with permeabilization solution (apply 250 µl to the inside of the membrane filter and 500 µl to the outside).

11. Dilute secondary antibody tagged with fluorophore to the desired working dilution in permeabilization solution.

For the Alexa and Cy dyes, a 1:400 dilution usually gives a strong fluorescence signal that is easily detectible through the collagen layer.

12. For nuclear staining, add Hoechst 33258 at a 1:1000 dilution to the secondary antibody mix.

Nuclear staining is recommended as an indicator for culture morphology.

13. For actin staining, add a fluorescently tagged phalloidin (Molecular Probes; Invitrogen) at a 1:300 dilution to the secondary antibody mixture.

Cytoskeleton staining is also recommended as a routine staining at this stage.

14. Add 200 µl secondary antibody solution to the inside of a membrane insert and 250 µl to the outside of the insert. Incubate 2 to 3 hr with shaking at room temperature.

15. Wash six times with permeabilization solution—three quick washes followed by three 15-min washes (250 µl to the inside and 500 µl to the outside).

Mount samples

16. Apply a large drop of Fluoromount-G mounting solution onto a microscope slide.

17. Cut the bottom of the membrane insert with a carbon steel blade and lift the gel carefully with fine forceps.

The membrane filters are rigid and therefore cutting them without breaking them can be difficult. Even if the filter breaks, the gel can still be lifted off the filter. It is important not to damage the collagen gel.

18. Place the gel (with or without the glass filter) onto the Fluoromount-G mounting solution drop on the microscope slide. Make sure that the top of the gel is facing up.
19. Cover the gel with a circular coverslip (18 to 25 mm). Make sure the culture is completely covered with mounting solution. If not, apply extra mounting solution to the edge of the coverslip. Avoid bubbles at all times. Avoid pressing the coverslip toward the gel.
20. Wait a few minutes to allow the mounting solution to spread and remove excess mounting solution, but be careful not to remove too much.

    If unsure, it is preferable to have some excess left.
21. Place the sample in a dark place overnight at room temperature to allow the mounting solution to dry.
22. Seal the sample with clear nail polish.

    Store samples for up to 6 months at 4°C in the dark.

IMAGING IMMUNOSTAINED 3-D CULTURES

BASIC PROTOCOL 3

MDCK 3-D cultures form a three-dimensional hollow sphere composed of a single cell layer. The average diameter of the sphere is 50 to 60 μm and, therefore, images from different focal planes should be taken and combined together to image the entire structure. This can be done by taking a series of optical sections using confocal microscopy. *Z* sectioning through a 3-D culture also allows examination of the intracellular localization of proteins in single cells. For this purpose, it is best to take an optical *z* section from the middle of the cyst (where the lumen is the largest). This optical section provides a nice cross section of the polarized cells surrounding the lumen (Fig. 4.22.4F). In this view it easy to determine whether a protein is localized to the apical, basal, or lateral membrane.

One of the difficulties in imaging whole-mount 3-D cultures is the thickness of the sample and the distance of the cells from the coverslip. This means that the imaging parameters should be adjusted very carefully to acquire the best possible images. However, even when the imaging parameters are optimized, the image quality will still be lower than imaging adherent cells on a coverslip.

Materials

Immunostained 3-D culture sample
High-resolution confocal microscope (e.g., either the Olympus F1000 or the Zeiss 510)
Oil-immersion objective: 40× NA 1.3 Plan Neofluar or UPlanFI or 60×/63× NA 1.4 Plan Apochromat oil objective
Three-dimensional rendering software for image analysis of 3-D culture cysts (e.g., Volocity software from Improvision)

Set up microscope

1. Use a high-resolution confocal microscope for imaging immunostained 3-D cultures.
2. Image immunostained 3-D cultures using an oil objective.

   A 40× NA 1.3 objective allows imaging deeper into the collagen and is therefore recommended for imaging cysts that are buried deep in the collagen.
3. To improve image quality, use a 1 airy unit pinhole size, image at low speed, average each pixel at least two times, and increase pixel resolution when necessary (>512 × 512).

4. When detailed intracellular localization is required, zoom in and focus on three to four cells of the culture and acquire thinner *z* sections (up to 50% overlap between two *z* sections).

   Imaging using these parameters can be time consuming. To save time, it is usually enough to image only one half of the cyst. If imaging half the cyst, make sure to image the half that is closer to the coverslip.

5. (Optional) Use a spinning disk confocal microscope, which enables high-speed image acquisition.

   A spinning disk confocal microscope is also recommended for imaging fine intracellular structures such as microtubules, which can be resolved better in this system.

Analyze data

6. Use a three-dimensional reconstruction of a 3-D culture cyst to provide detailed information that is not readily detectible from looking at the *z* sections separately. It is therefore recommended to use a three-dimensional rendering software for image analysis of 3-D culture cysts (e.g., Volocity software from Improvision).

ALTERNATE PROTOCOL

LIVE-CELL IMAGING OF MDCK 3-D CULTURES

Live-cell imaging of 3-D cultures is a powerful tool to study both the dynamics of cells in a culture during culture morphogenesis and the dynamics of intracellular proteins in a single cell in real time. Live-cell imaging can also be used as a more direct way to study protein localization because it gets around the need to use antibodies, which sometimes have permeability and specificity issues. This protocol details the culture conditions and the microscopy setups that are best suited for live-cell imaging of 3-D cultures.

Materials

Fluorescently labeled 3-D MDCK cultures (see Basic Protocol 1)
Collagen-coated 4-well chamber slides (Nunc; see Basic Protocol 1)
FM4-64 membrane dye (Invitrogen)

37°C incubator
37°C, 5% CO_2 incubator
Confocal microscope (for high-speed acquisition, spinning disc confocal or the Zeiss LSM live duo system)
Water objective (40× or 60× NA1.2)

Prepare cultures

1. Plate 300 µl collagen solution of fluorescently labeled MDCK cells in a precoated 4-well chamber slide as described in Basic Protocol 1. Incubate 30 to 45 min at 37°C without CO_2.

   Cultures can be imaged from day 1 to day 12 depending on the desired experiment.

2. To fluorescently label the plasma membrane of live cells in 3-D culture, apply 4 µM of FM4-64 dye to the culture medium and incubate 1 hr in a 37°C 5% CO_2 tissue culture incubator.

   Imaging 3-D cultures in bright field gives poor images (Fig. 4.22.5). It is therefore difficult to determine the cell boundaries in a 3-D culture and the subcellular localization of the protein of interest in a single cell. Fluorescence labeling of the plasma membrane outlines the boundaries of all cells in the sample and makes it easy to detect 3-D culture cysts, to distinguish between different cells in a cyst and to outline the different membranes of a single cell (apical, basolateral).

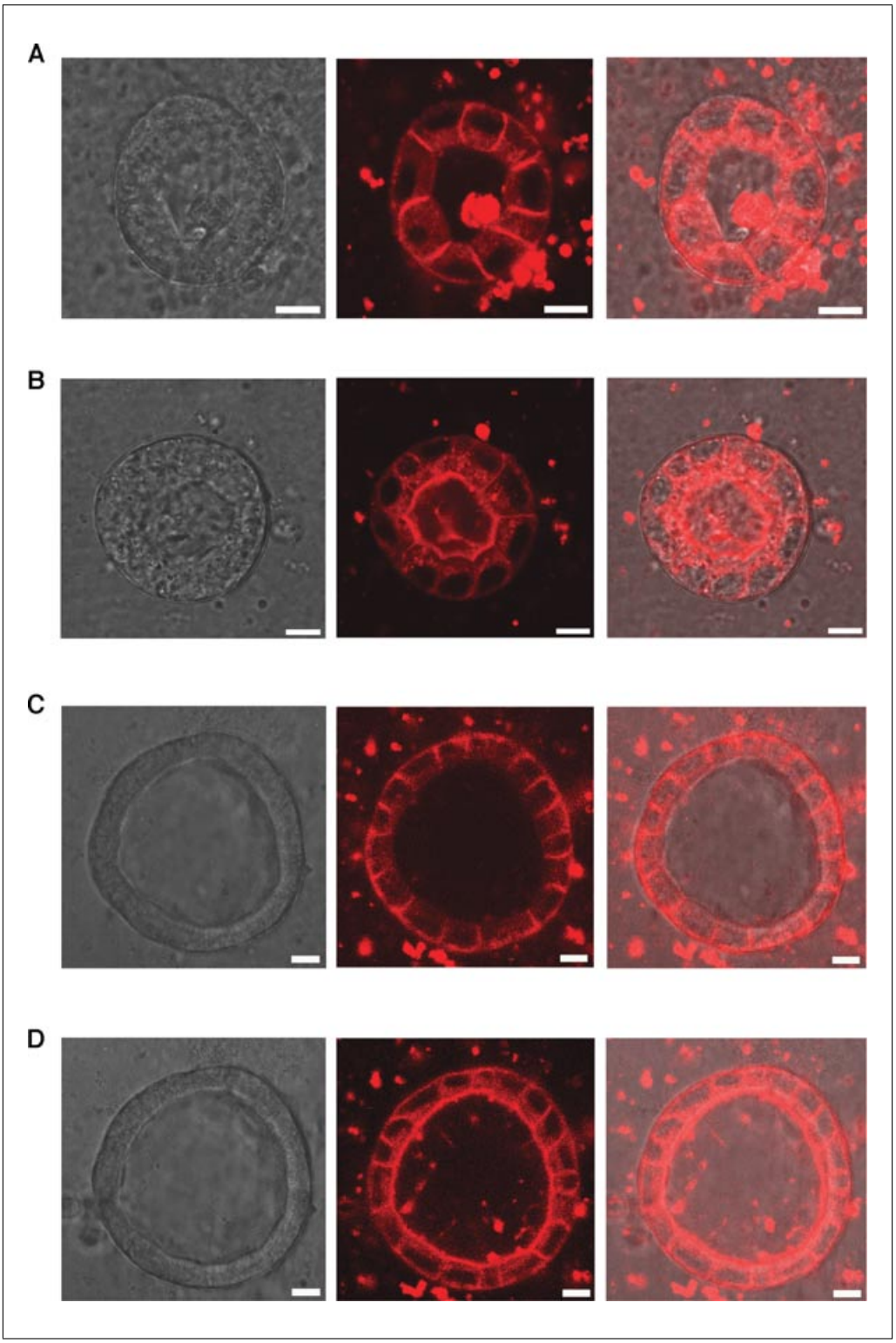


Figure 4.22.5 Live-cell imaging of MDCK cysts in 3-D cultures using the membrane dye FM4-64. (**A,B**) cultures at day 4. (**C,D**) cultures at day 6. (**A,C**) 30 min after addition of the dye the basolateral membrane is labeled. (**B,D**) 2 hr post-dye addition the apical membrane is labeled as well as intracellular endocytic structures. Scale bar = 10 μm. For color version of this figure go to *http://www.currentprotocols.com.*

The dye will initially label the basolateral membrane and than the apical membrane (60 min later; Fig. 4.22.4). During this time, the FM4-64 dye will also endocytose. The intracellular staining of the dye is stable for days and therefore this dye can be used for extensive live imaging experiments (up to several days).

Set up microscope

Live-cell imaging of collagen-embedded 3-D cultures is somewhat challenging. Both the thickness of the sample and the collagen itself lead to fluorescence light scattering and therefore high laser intensity is required to acquire an image. Imaging under these conditions makes the specimen sensitive to photobleaching of the fluorescence signal and to culture photodamage. It is therefore very important to optimize the fluorescence parameters prior to performing a live-cell imaging experiment. These parameters should be adjusted for every experiment depending on the intensity of the fluorescence signal and the condition of the cultures. There are, however, a few general guidelines that can be followed.

3. Use a confocal microscope to image live 3-D cultures. For high-speed image acquisition it is best to use either a spinning disc confocal or the Zeiss LSM live duo system.
4. Select the culture. Image only the cysts that are closest to the coverslip and have the brightest fluorescence.

   Increasing the imaging distance will greatly reduce image quality and will require higher laser intensities, which will lead to extensive photobleaching and photodamage.

   Using the cultures with the brightest fluorescence will reduce the laser intensity required for imaging and will therefore minimize photobleaching and culture photodamage.
5. Use a water objective (40× or 60× NA1.2) to maximize depth of field and minimize photobleaching.

   However, in some cases, a 63× NA 1.4 or a 40× NA 1.3 are also applicable.
6. Use line scanning, scan at low speed, average each pixel at least twice, and increase pixel resolution when necessary (>512 × 512).
7. Set up the pinhole between 1 and 1.5 airy units to give the best *z*-sectioning results. When taking a series of *z* sections, make sure the sections overlap.
8. For extended imaging (more than a few hours), use an auto-focus or a perfect-focus module to avoid focus shift in time.

   This will prevent focus drift in time and will allow detection of whole culture movement along the z axis in time.

BASIC PROTOCOL 4

FRAP AND FLIP IN MDCK 3-D CULTURES

Photobleaching techniques are highly useful to study the intracellular dynamics of proteins. In fluorescence recovery after photobleaching (FRAP), a specific region of interest (ROI) is bleached once with a high-laser intensity and the diffusion rate of the protein of interest to this region can be determined. In fluorescence loss in photobleaching (FLIP), the depicted region of interest is repeatedly bleached and the kinetics of fluorescence signal decay from different domains throughout the cell can be measured. The rate of recovery in FRAP and the fluorescence decay pattern in FLIP reflect the subcellular dynamics of the protein of interest, therefore providing a direct tool to study protein dynamics in real time (for more information, see *UNIT 21.1*). There are two critical points that should be taken under consideration when applying these techniques to 3-D cultures. First, these techniques require exposure of the sample to high laser power for long durations, which can cause extensive photodamage to the cells in the culture and extensive photobleaching of the sample. Second, the 3-D culture is a thick specimen and the time it takes to image throughout the *z* axis greatly reduces time resolution.

To get around these difficulties, it is best to use the Zeiss live duo line scanner, which is capable of taking hyperfast z sections, or a spinning disk confocal. The scanning and photobleaching parameters should also be adjusted to enable efficient photobleaching of the ROI with minimal photobleaching of the overall sample. Extensive photobleaching of the specimen will greatly affect the results of both FRAP and FLIP experiments. It is therefore very important to measure the change in fluorescence in different parts of the photobleached cells as well as in neighboring cells in every photobleaching experiment (see Figs. 4.22.6 and 4.22.7).

In general, all of the suggestions regarding culture condition, culture preparation, and imaging setups listed for live-cell imaging holds here but with some modifications.

Suitable scanning and bleaching parameters for EGFP using the Zeiss live duo line scanner are listed below:

1. Objective: The C-Apochromat 63× water objective NA 1.2 is recommended.
2. Scanning parameters: Frame size 1024 × 1024, scan speed between 1 to 2 frames/sec, frame mean 1, pinhole size up to 2 μm.
3. Bleaching parameters: Scan speed 6 μsec/pixel, iteration 3×. Low iteration number is crucial to avoid culture photodamage.

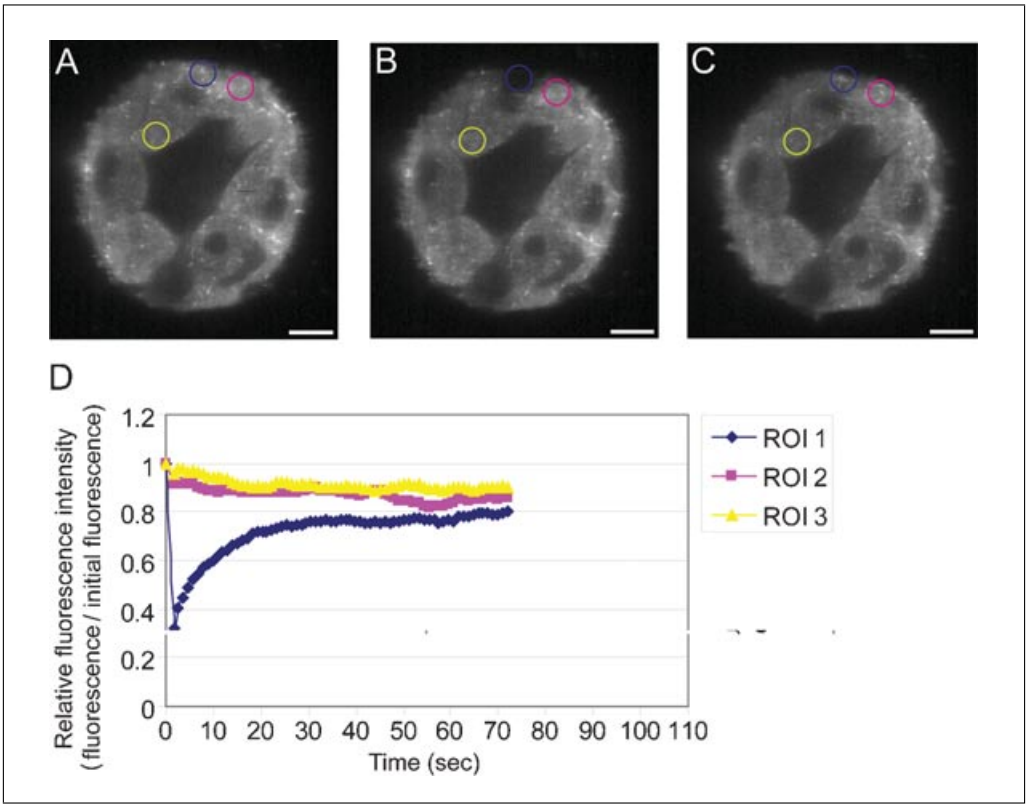


Figure 4.22.6 FRAP analysis of MDCK cells stably expressing the plus end binding protein clip170-GFP in 3-D cultures. (**A**) Before bleaching, (**B**) the first image post bleaching. (**C**) 25 sec post bleaching the bleached area has recovered. (**D**) The change in fluorescence intensities over time. ROI 1: the bleached zone; ROI 2: a different region of the same cell; ROI 3: a region from a neighboring cell. The ROIs are depicted in (A-C) and are color coded according to the graph (**D**). Cultures are in day 4. Scale bar = 10 μm. For color version of this figure go to *http://www.currentprotocols.com.*

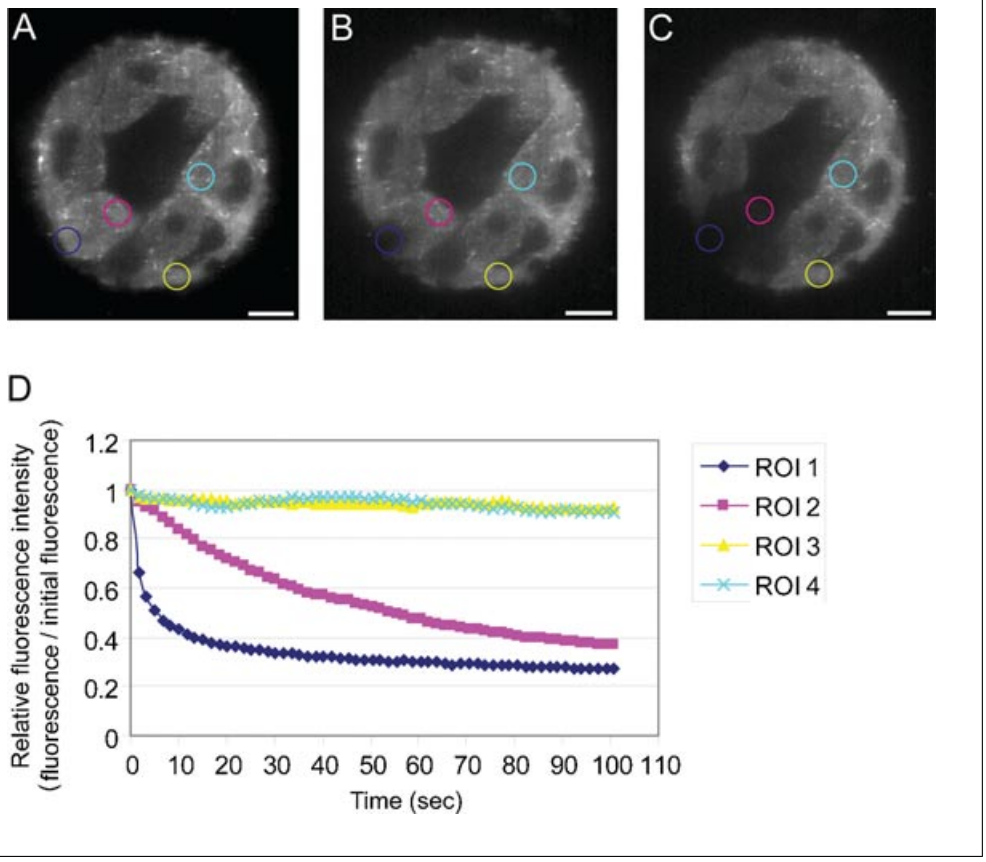


Figure 4.22.7 FLIP experiment of a 4-day-old 3-D culture expressing Clip170-GFP. (**A**) Before bleaching. (**B**) After the first bleaching cycle. (**C**) After 60 bleaching cycles. (**D**) The change in fluorescence intensities over time. ROI 1: the bleached zone; ROI 2: a different region of the same cell; ROI 3 and 4: regions from neighboring cells. The ROIs are depicted in (A-C) and are color coded according to the graph (**D**). Scale bar = 10 μm. For color version of this figure go to *http://www.currentprotocols.com.*

COMMENTARY

Background Information

Epithelial cells serve as a barrier between the interior of the body and the outside world. To accomplish this barrier function, epithelial tissues have developed special characteristics such as polarized morphology, unique cell-cell contacts, and intimate interactions with the extracellular matrix (ECM). MDCK cells have been extensively studied as a model system for epithelial polarity mainly due to their ability to form apical-basolateral polarity in a tissue culture dish. When plated on a permeable filter, MDCK cells form a monolayer of polarized cells in which the basal membrane is attached to the filter and the apical membrane faces the culture medium (Simons and Fuller, 1985) Although much of the knowledge regarding cell polarity came from these studies, this system is somewhat artificial since it provides a strong cue for polarization, which also determines directionality. Plated in collagen, or any other extracellular matrix, MDCK cells form a three-dimensional cyst composed of polarized cells. In this system, MDCK cells embedded in extracellular matrix organize in a multicellular structure that creates its own lumen. This process recapitulates many of the physiological characteristics of lumen formation during epithelial development and share many morphological similarities to epithelial tissue (McAteer et al., 1987; O'Brien et al., 2002; Martin-Belmonte and Mostov, 2008). This system therefore provides a unique opportunity to study epithelial morphogenesis in a tissue culture dish. Moreover, MDCK 3-D cultures also provide a more physiological model system for cell polarity, its regulation, and its relationships with the extracellular matrix. Using this system, it was shown that Rac1 determines apical membrane orientation

through an effect on the basolateral membrane (O'Brien et al., 2001) and that CDC42 and aPKC (atypical protein kinase c) are essential for the formation of both the apical membrane and the inner lumen (Martin-Belmonte et al., 2007)

Plated in 3-D cultures, epithelial cells form cysts that are characterized by a spherical epithelial monolayer with a central single hollow lumen (Fig. 4.22.2C). The epithelial cells surrounding the lumen are polarized with the apical membrane facing the inner lumen. The apical membrane is separated from the basolateral membrane by the tight junction complex (TJ), which also creates a diffusion barrier to the inner lumen (Martin-Belmonte and Mostov, 2008).

3-D cultures are also used in cancer research as a tissue culture tool for tumor progression and reversion and many of the current knowledge on 3-D cultures came from these studies (Schmeichel and Bissell, 2003; Debnath and Brugge, 2005). 3-D culture development and lumen formation was of focus in these studies and several distinctive stages in culture formation were specified. First, apicobasal polarization is observed and the lumen is filled with unpolarized cells. Second, the nonpolarized cells trapped inside the layer of polarized cells undergo apoptosis. This leads to the formation of a hollow lumen, which is maintained throughout the lifetime of the culture. Apoptosis has also been shown to occur during lumen formation in MDCK cultures plated on collagen (Kim et al., 2007; Martin-Belmonte et al., 2008). However, the preliminary steps of 3-D culture formation and the role of apoptosis in 3-D culture development are not fully understood.

The protocols described in this unit are optimized for growing MDCK cells in a collagen I matrix. Other extracellular matrixes (such as Matrigel) and other cell lines (such as mammary epithelial cells) can also be used as a 3-D culture model system. The advantage of using a collagen matrix is the simplicity of the matrix components. However, for some applications, using a more complex extracellular matrix is required. A protocol for growing 3-D cultures of breast cancer cell lines in Matrigel was recently published by Mina Bissell's group (Lee, 2007). This protocol is highly recommended when these parameters are desired.

3-D cultures can also be used for biochemical analysis. Those protocols are however outside the scope of this unit. An extensively detailed protocol for biochemical analysis of MDCK cells in 3-D cultures is described by Keith Mostov's group (O'Brien et al., 2006); Mostov is one of the pioneers in studying MDCK cells in 3-D cultures. It also includes plating and immunostaining protocols for MDCK cells in collagen and is recommended as another source of information for issues that might not be covered in this unit.

Live-cell imaging is a powerful technique to study cellular morphogenesis in real time. These techniques, however, have not been applied for 3-D culture formation in the past, mainly due to technical difficulties. 3-D cultures are a relatively challenging specimen for live-cell imaging for several reasons. First, the cultures are embedded in matrix and therefore the distance between the cells and the objective is substantially larger than in adherent cells. Second, the matrix that surrounds the cells scatters the fluorescent light. Third, the culture is a complex and thick organization of cells in all directions (x, y, z) and imaging it in all dimensions is time consuming and therefore gives poor resolution in time. The new generation of high-speed confocal microscopes provides new possibilities for live-cell imaging that can be optimized for 3-D culture imaging. Imaging those cultures live can shed light on epithelial morphogenesis, the involvement of different proteins in these stages, and the intracellular dynamics of proteins during this process.

Critical Parameters

There are a few parameters that need to be considered before starting a 3-D culture experiment.

Before plating cells for 3-D culturing, the type of desired experiment should be determined. For an immunostaining experiment, it is better to plate the cultures on membrane filter inserts, and for live-cell imaging, the culture should be plated on a chamber slide.

For live-cell imaging, the cells should stably express a fluorescent-tagged version of the protein of interest. Moreover, the fluorescent signal should be as strong as possible to overcome the difficulties of imaging these samples.

The general morphology of the cultures should be estimated before any live-cell imaging experiment. This could be done using the FM4-64 membrane dye or by fixing and immunostaining the culture after the imaging experiment. It is critical to determine if the culture has normal morphology (a single hollow lumen surrounded by a monolayer of polarized cells) because altered morphology may affect intracellular dynamics.

Table 4.22.1 Troubleshooting Guide for Growing and Imaging 3-D Cultures

Protocol	Problem	Possible cause	Solution
Growing MDCK 3-D cultures (see Basic Protocol 1)	Collagen I does not polymerize	pH is not neutral	Check the pH of the collagen I solution before adding the cells. pH should be neutral. Lower pH inhibits polymerization.
		Insufficient incubation time	Increase incubation time in 37°C oven. Different batches of purified collagen I often require different polymerization time.
	No 3-D culture cyst formation or altered 3-D culture cyst morphology (see Anticipated Results)	Cell concentration	Plate cells again and make sure to use the recommended cell concentration. A very low concentration will decrease the probability for 3-D culture formation. A too high concentration will lead to cell aggregation and to defects in lumen formation.
		Cells are not in their proliferative state	Make sure the cells being used for plating 3-D cultures do not exceed 70% confluency
			Thaw a new aliquot of MDCK cells with a low passage number
		Cells were not in a single-cell suspension when plated	MDCK cells tend to form cell clumps. Pipet the cells up and down throughout all cell preparation stages. When counting the cells, make sure that they are not in clumps.
		Cells were over trypsinized	Trypsinize cells for the minimum required time. Add an extra wash with culture medium before plating the cells to efficiently remove trypsin.
		For cells plated in chamber slides: chamber slide was not coated properly	Increase the volume of collagen I solution used for pre-coating
			Make sure the collagen solution covers the entire surface of the chamber slide
			Make sure the collagen solution used for precoating has completely polymerized before adding the collgen/cell mixture
Immunofluorescence (see Basic Protocol 2)	No antibody staining	Permeability of the antibody	Antibody permeabiliztion problems can be caused by insufficient breakdown of the collagen matrix by the collagenase that is used to permeabilize the collagen matrix. Make sure to use a fresh aliquot of collagenase solution at the specified concentration. Try increasing the incubation time by 1 to 2 min.
			Incubate with primary antibody overnight
			Increase secondary antibody incubation time
	High background	Insufficient blocking	Increase incubation time with permeabilization solution
			Make sure the antibodies are diluted in permeabilization solution
			Replace 10% FBS with fish skin gelatin (7 mg/ml) in permeabilization solution

continued

Table 4.22.1 Troubleshooting Guide for Growing and Imaging 3-D Cultures, *continued*

Protocol	Problem	Possible cause	Solution
	Antibody staining is not as expected	Detergent in permeabilization solution distorts protein localization	Use saponin (0.025%) instead of Triton X-100 in permeabilization solution
Live-cell imaging (see Basic Protocol 3)	Unable to focus on cultures in bright field	Cells are too far from coverslip	Change to a water objective or to an objective with slightly lower NA
			Reduce the volume used for precoating of the chamber slide
	No or very little fluorescence signal	Cells are too far from slide/coverslip	Try finding a 3-D culture cyst that is closer to the slide/cover slip. Change to a 40× NA 1.3 objective. When using a point scanner, zoom in to achieve the same magnification.
		Fluorescence signal is quickly bleached	Switch to a water objective
			Minimize exposure to light before image acquisition
		Fluorescent signal is masked	Clean both the objective and the slide/coverslip thoroughly before imaging. Make sure to use the immersion oil recommended by the manufacturer.
		Expression level of the fluorescently tagged protein is too low	FACS sort the cells to enrich the population of high fluorescent cells

The overall condition of the cells in the chamber slide well should also be taken under consideration before starting an experiment. Extensive death of neighboring cells, for example, can affect the physiology of a culture with normal morphology in ways yet to be evaluated. This is especially critical in 3-D cultures because diffusion inside the collagen polymer is limited and therefore the different cellular factors that are released during cell death will remain in the surrounding area for a long time and will not be washed out easily. It is therefore recommended to image only 3-D cultures that are part of a chamber slide culture that is in good condition.

Troubleshooting

See Table 4.22.1 for troubleshooting imaging of 3-D cultures.

Anticipated Results

3-D culture morphology

The overall morphology of 3-D culture cysts can be observed by bright field. In the authors' experience, at day 4 a small lumen surrounded by few cells is observed (Fig. 4.22.2A). At day 7, cultures continue to develop and the lumen grows bigger (Fig. 4.22.2B).

Immunostaining

The following are recommended immunostainings for evaluating culture morphology and polarization.

Nuclear staining. If cultures develop normally, expect a single circular layer of nuclei (Fig. 4.22.3A). Looking for these unique circles is very useful when screening for cultures with normal morphology.

Phalloidin staining. Actin is highly accumulated on the apical surface of the polarized cells surrounding the lumen (Fig. 4.22.3B). In addition, a weaker actin staining can also be observed throughout the outline of the cells. Therefore, phalloidin staining is recommended to evaluate cell polarity and to determine the boundaries of the cells surrounding the lumen.

A 3-D culture is a three-dimensional sample that is composed of many cells. Therefore, different optical slices through the z axis will

show different populations of cells in the 3-D culture and different areas of the lumen. A series of different optical sections of a single 3-D culture cyst is presented in Figure 4.22.4 to demonstrate the different appearances of the cyst in the *z* dimension.

Live-cell imaging

The membrane dye FM4-64 can be used to label the membranes of individual cells in live 3-D cultures. This dye can be used at any time point between 30 min and several days depending on the desired experiment. The dye changes its staining over time and it is therefore important to distinguish between the different labeling patterns. At the first stage, the basolateral membrane is stained (~30 min after adding the dye; Fig. 4.22.5A,C). Later on, this staining is accompanied by an apical membrane staining and an intracellular pool of endocytosed dye (~1.5 hr after adding the dye; Fig. 4.22.5B,D). At steady state, the apical membrane staining usually remains, the endocytosed pool increases, and the basolateral staining disappears.

FRAP of intracellular proteins

After photobleaching, a specific ROI, the specified area should be completely dark while the rest of the fluorescence in the culture should not be affected. During recovery (if any) the bleached box should be gradually filled with fluorescence. Figure 4.22.6 demonstrates a FRAP experiment of the microtubule plus end binding protein Clip170.

FLIP of intracellular proteins

In FLIP experiments, the area of the ROI is continuously bleached and the total decay of fluorescence in the cell is monitored. In these experiments, the overall fluorescence signal of a protein that has a cytosolic pool should be decreased in time. However, the fluorescence in neighboring cells should remain the same and this should be used as a control. Figure 4.22.7 represents a FLIP experiment of Clip170.

Time Considerations

Plating 3-D cultures require 2 to 4 hr. Growing cultures and developing them take 4 to 12 days. Immunostaining requires 2 days.

Literature Cited

Debnath, J. and Brugge, J.S. 2005. Modeling glandular epithelial cancers in three-dimensional cultures. *Nat. Rev. Cancer.* 5:675-688.

Kim, M., Datta, A., Brakeman, P., Yu, W., and Mostov, K.E. 2007. Polarity proteins PAR6 and aPKC regulate cell death through GSK-3beta in 3D epithelial morphogenesis. *J. Cell Sci.* 120:2309-2317.

Lee, G.Y., Kenny, P.A., Lee, E.H., and Bissell, M.J. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat. Methods* 4:359-365.

Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V., and Mostov, K. 2007. PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. *Cell* 128:383-397.

Martin-Belmonte, F. and Mostov, K. 2008. Regulation of cell polarity during epithelial morphogenesis. *Curr. Opin. Cell Biol.* 20:227-234.

Martín-Belmonte, F., Yu, W., Rodríguez-Fraticelli, A.E., Ewald, A., Werb, Z., Alonso, M.A., and Mostov, K. 2008. Cell-polarity dynamics control the mechanism of lumen formation in epithelial morphogenesis. *Curr. Biol.* 18:507-513.

McAteer, J.A., Evan, A.P., and Gardner, K.D. 1987. Morphogenetic clonal growth of kidney epithelial cell line MDCK. *Anat. Rec.* 217:229-239.

O'Brien, L.E., Jou, T.S., Pollack, A.L., Zhang, Q., Hansen, S.H., Yurchenco, P., and Mostov, K.E. 2001. Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat. Cell Biol.* 3:831-838.

O'Brien, L.E., Zegers, M.M.P., and Mostov, K.E. 2002. Building epithelial architecture: Insights from three-dimensional culture models. *Nat. Rev. Mol. Cell Biol.* 3:531-537.

O'Brien, L.E., Yu, W., Tang, K., Jou, T.S., Zegers, M.M., and Mostov, K.E. 2006. Morphological and biochemical analysis of Rac1 in three-dimensional epithelial cell cultures. *Methods Enzymol.* 406:676-691.

Schmeichel, K.L. and Bissell, M.J. 2003. Modeling tissue-specific signaling and organ function in three dimensions. *J. Cell Sci.* 116:2377-2388.

Simons, K. and Fuller, S.D. 1985. Cell surface polarity in epithelia. *Annu. Rev. Cell Biol.* 1:243-288.

Key Reference

O'Brien et al., 2006. See above.

This method paper describes in detail a protocol for plating and growing MDCK cells for immunostaining as well as for biochemical analysis.

Interference Reflection Microscopy

Valarie A. Barr[1] and Stephen C. Bunnell[2]

[1]National Cancer Institute, National Institutes of Health, Bethesda, Maryland
[2]Sackler School of Graduate Biomedical Science, Tufts University, Boston, Massachusetts

ABSTRACT

Interference reflection microscopy (IRM) is an optical technique used to study cell adhesion or cell mobility on a glass coverslip. The interference of reflected light waves generates images with high contrast and definition. IRM can be used to examine almost any cell that will rest upon a glass surface, although it is most useful in examining sites of close contact between a cell and substratum. This unit presents methods for obtaining IRM images of cells with particular emphasis on IRM imaging with a laser scanning confocal microscope (LSCM), as most LSCM are already capable of recording these images without any modification of the instrument. Techniques are presented for imaging fixed and live cells, as well as simultaneous multi-channel capture of fluorescence and reflection images. *Curr. Protoc. Cell Biol.* 45:4.23.1-4.23.19. © 2009 by John Wiley & Sons, Inc.

Keywords: reflection contrast microscopy • confocal microscopy • contrast enhancement • refractive index • cell adhesion

INTRODUCTION

This unit describes a microscopic technique that uses reflected light to visualize structures at or near a glass coverslip. Interference reflection microscopy (IRM), also known as interference contrast, interference reflection contrast, and surface contrast microscopy, has been used to study a wide range of cellular behaviors including cell adhesion, motility, exocytosis, and endocytosis. This technique relies on reflections from an incident beam of light as it passes through materials of different refractive indices. In the case of a cell on a glass coverslip, reflected beams are generated as light passes from the coverslip to aqueous medium, and again as light goes from the aqueous medium to the cell membrane. These reflected beams interfere, producing either constructive or destructive interference depending on the thickness of the layer of aqueous medium between the cell membrane and the glass surface. For monochromatic light, intensity in the IRM image is a function of the distance between the cell and coverslip and can indicate the strength of adhesion to the surface.

This unit includes protocols for fixed and live-cell imaging IRM, as well as protocols for coating coverslips for use with this technique. A very wide variety of samples can be imaged by IRM, including embedded material. In fact, the technique was originally used to image mineral samples. However, in this unit, we are concerned with IRM of cells and in particular IRM using a laser scanning confocal microscope. Moreover, a detailed description of IRM of fixed embedded samples using epi-illumination is available (Prins et al., 2005). Specific examples are included with each protocol, but the techniques can be applied very broadly.

STRATEGIC PLANNING

Obtaining images with the IRM technique requires using a microscope with a suitable light path for collecting reflected light, samples with a different refractive index than the embedding medium, and an interface between the objective and sample made of material

Current Protocols in Cell Biology 4.23.1-4.23.19, December 2009
Published online December 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0423s45

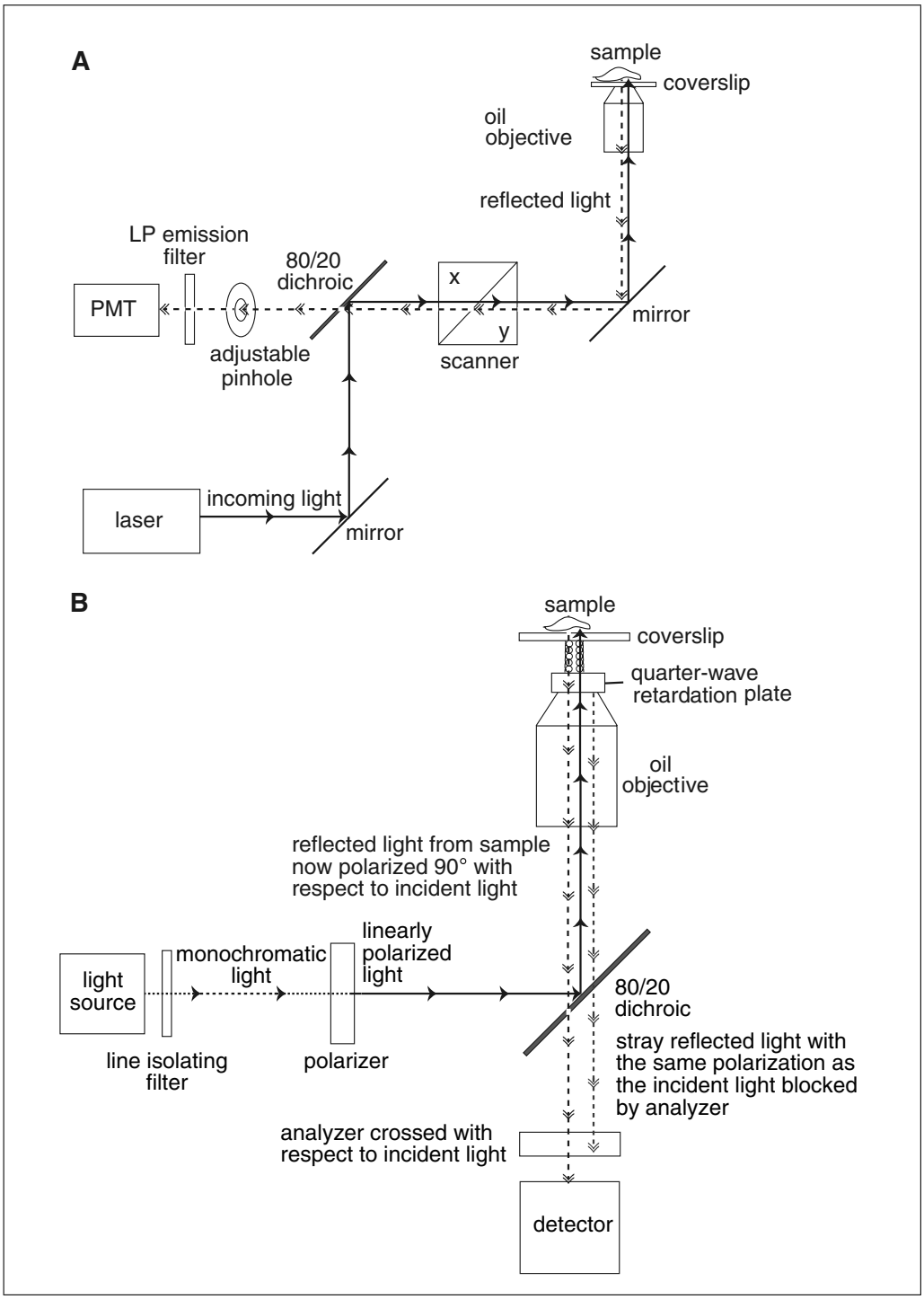


Figure 4.23.1 *(legend appears on following next page)*

with yet another refractive index, usually glass. Oil immersion objectives must be used and the mounting medium should have a lower refractive index than the immersion oil so that the first reflected beam is generated at the coverslip/medium or coverslip/cell interface. Polychromatic light will produce colored interference fringes while monochromatic light produces quantifiable gray-scale interference images.

Laser scanning microscopes already possess a suitable light path (Fig. 4.23.1A) and require only a configuration where the reflected laser light is able to reach a detector through an emission filter that allows the incident light to pass through. Since reflected

light is abundant compared to the amount of light emitted by most fluorescent molecules, the laser intensity and detector gain can be reduced for IRM imaging. Simply focusing at the coverslip with a sample containing cells that have adhered to the slide should generate an IRM image. With a slightly different configuration, IRM images can also be collected simultaneously with fluorescent images in systems with multiple detectors. Most spinning disk confocal microscopes do not have a light path that is easily adapted to IRM imaging.

Nonconfocal epi-illumination microscopes can also be used to collect IRM images, but some modification of the light path is usually needed to enhance contrast and reduce stray reflections that might otherwise overwhelm the IRM image (Fig. 4.23.1B). Stray reflections are removed by ensuring that the reflected light coming from the sample is polarized 90° relative to the incident light and then allowing only light reflected from the sample to pass through an appropriately oriented polarizer in front of the detector. The first optical element needed for this transition is a polarizer that converts the incoming beam to linearly polarized light. This polarized light then passes through a quarter-wave retardation plate placed on the objective lens with the fast axis set at 45° relative to the axis of the polarizer. This converts the linearly polarized light into circularly polarized light. When the circularly polarized light is reflected from the sample it changes handedness, that is right-handed circularly polarized light becomes left-handed circularly polarized light. As it passes back through the lambda quarter plate again, the circularly polarized light is converted back to linearly polarized light now polarized at 90° relative to the incident beam. Finally, another polarizer, the analyzer, is set in front of the detector at an orientation 90° relative to the first polarizer. Thus, only the rotated beam reflected from the sample reaches the detector (Ploem, 1975; Dorogi and Keller, 1993).

Other devices can be added to further improve the image. The contrast of the image can be enhanced by adding a central stop and field diaphragm before the first polarizer. In addition, an aperture that allows variation of the illuminating numerical aperture (NA) can also be useful for some studies (Izzard and Lochner, 1976). To obtain quantifiable gray-scale images, a line-isolating filter is needed to produce monochromatic light. Although this sounds complicated, most microscope manufacturers can supply and install all the optical elements needed for IRM imaging on an epi-illumination microscope.

It is often necessary to add a layer of material to the coverslip for adhesion or activation of the cell being imaged. This does not usually present a problem in obtaining good IRM images. IRM images have been taken with coverslips coated with antibodies, integrin

Figure 4.23.1 *(appears on previous page)* Suitable light paths for IRM imaging (**A**) Light path in a laser scanning confocal microscope. The laser, usually 543 nm or 633 nm, is reflected by the main beamsplitter into the microscope, through the objective where it reflects from the sample. The galvanometric mirrors in the scanner deflect the laser, scanning across the entire field to produce a full image. The reflected beams pass back through the neutral dichroic, 80/20 in this diagram, and are sent to the detector. The emission filter is chosen to allow the incident light to reach the detector. The pinhole can be either completely open or closed down to one Airy unit. (**B**) Light path in an epi-illumination microscope. White light from a high-pressure mercury or xenon lamp passes through a line-isolating filter and a polarizer to produce monochromatic, linearly polarized incident light. The main beamsplitter reflects this light into the microscope, through the objective and then through a quarter-wave retardation plate. The emerging light is now circularly polarized. The reflected light is circularly polarized but with the opposite handedness from the incoming beam. After passing back through the quarter-wave plate, the reflected light is now linearly polarized with an orientation 90° with respect to the incident light. The reflected light passes through the neutral dichroic, 80/20 in this diagram, and through the analyzer, a second polarizing filter oriented 90° relative to the first polarizer, to reach the detector. Stray light from internal reflections in the microscope will be linearly polarized with the original orientation and will not pass through the analyzer to the detector.

ligands, or other thin layers of protein. IRM can even be performed on cells migrating on polymer films (Csaderova et al., 2002).

If long-term imaging is desired, precautions must be taken to ensure the viability of the samples as is necessary for all live-cell imaging techniques. This usually means using an incubation system on the microscope stage to control heat, humidity, and CO_2 needed to maintain the pH of the culture medium. A complete discussion of stage incubators is beyond the scope of this unit but a page is listed under Internet Resources containing links to many manufacturers of these devices.

IRM is quite sensitive to the plane of focus, so refocusing will usually be needed during longer experiments. Most microscope manufacturers now produce microscopes with built-in autofocusing modules. When the module is activated, it maintains a defined focal plane throughout the experiment. Almost all confocal systems, even those without a built-in focusing module, also have software that either allows scripting of a time series to include an autofocus step or have subroutines that allow the user to assemble a series of microscope commands so that an autofocus routine can be run at discrete intervals during a time series. However, some autofocus routines are very slow and add considerable time between images. It is often possible to manually refocus during image acquisition, although this can be quite tedious. This method is often the fastest and may be required if the cells themselves are moving. Another approach is to collect a short *z*-stack around the plane of focus and select the best focal plane after the time series ends.

BASIC PROTOCOL

INTERFERENCE REFLECTION IMAGING OF PARAFORMALDEHYDE-FIXED NIH 3T3 CELLS WITH A LASER SCANNING CONFOCAL MICROSCOPE

This protocol consists simply of placing a sample with cells either resting on or attached to a glass coverslip onto a microscope equipped with a suitable light path, and then focusing on the coverslip through an oil immersion objective. Either live or fixed samples can be used. In contrast to fluorescent imaging of fixed samples, the refractive index of the mounting medium should be different from that of the immersion oil and coverslip in order to generate reflected beams.

Materials

Samples (e.g., fixed NIH 3T3 cells)
DMEM cell culture medium (see recipe) or other suitable cell culture medium (with refraction index different from immersion oil)
Phosphate-buffered saline (PBS; see recipe)
2.5% (w/v) paraformaldehyde (see recipe)

Culture flasks for maintaining cell cultures before imaging
Lab-Tek II Chambered Cover Glasses, two-well (Nalge Nunc, cat. no. 155379) or other no. 1 or no. 1.5 coverslips (glass-bottom 96- or 384-well plates may be used)
37°C, 5% CO_2, humidified incubator
Laser Scanning Confocal Microscope equipped with oil immersion objective

Prepare the cells

1. Prepare samples (e.g., fixed NIH 3T3 cells). Plate cells in DMEM culture medium directly into the Lab-Tek II chambered coverslip 10^4 cells/cm^2; for fibroblasts, the 2-well size is usually best. For a 2-well chamber, use 4×10^4 cells in 2 ml of culture medium.

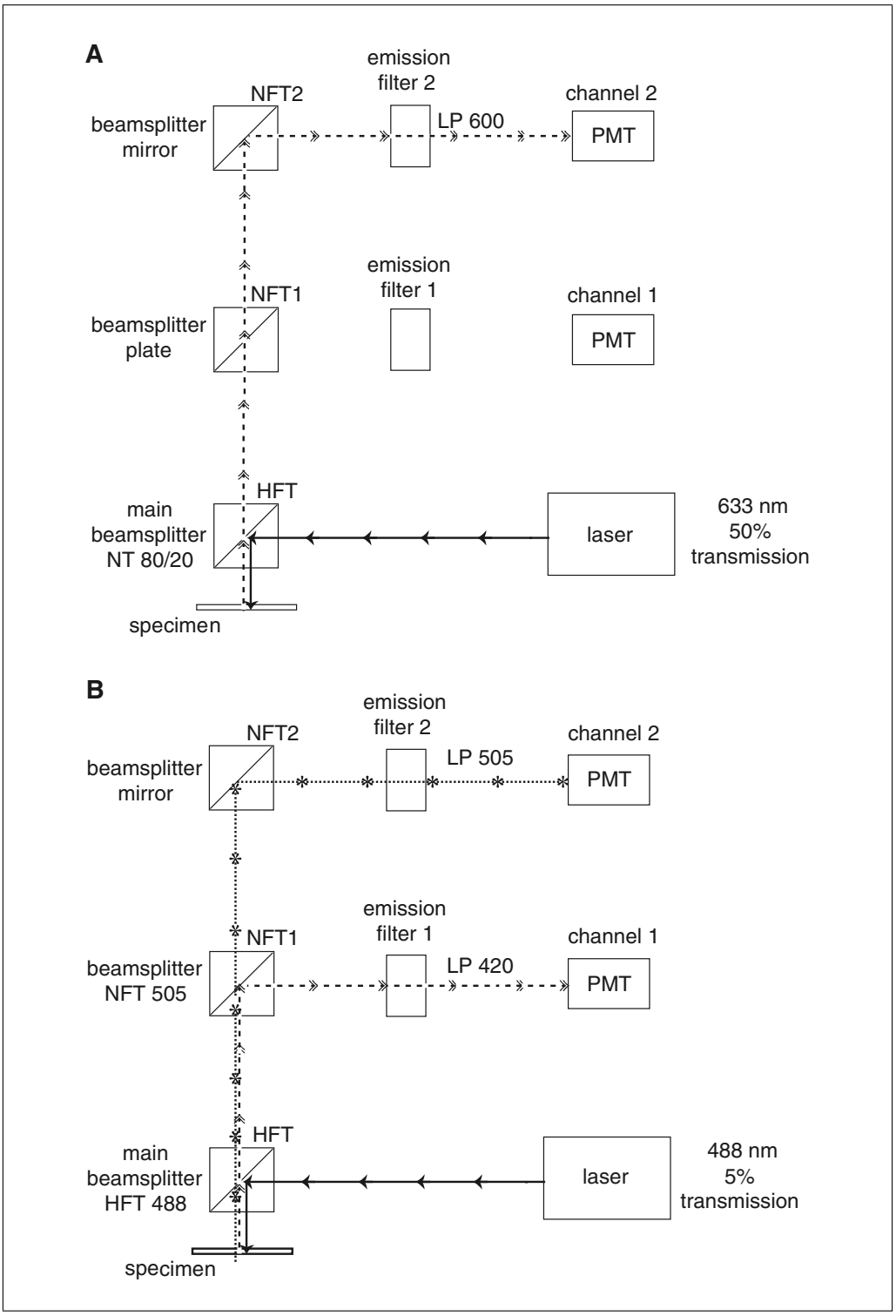


Figure 4.23.2 *(legend appears on following next page)*

2. After growing the cells overnight in a standard incubator (37°C, 5% CO_2, and 95% humidity), rinse them twice in 1.5 ml PBS and fix them in 1.5 ml 2.5% paraformaldehyde for 15 min at room temperature (~21°C).
3. Rinse the fixed cells three times, each time in 1.5 ml PBS.
4. Image the cells in PBS.

For other kinds of cells, different culture media could be used, other coverslips could be used for cell adhesion, and the fixation conditions could be varied to suit the experiment.

Set up a configuration

5. For the most commonly used configuration for a laser scanning microscope, use either a red (543 or 568 nm) or far red (633 or 647 nm) laser line, although any laser line can be used to generate a reflection image.
6. As the main beamsplitter, usually use a neutral dichroic (80/20 or 70/30).
7. Select the secondary beamsplitters to direct the reflected light to an emission filter that allows the incident laser light to pass to the detector (Fig. 4.23.2A).
8. An example of a standard IRM configuration is the one used for focusing on a slide prior to running an automated pinhole adjustment routine; it usually provides a suitable IRM configuration.

DIC images can also be obtained using a transmitted light detector, but the focal plane for IRM and DIC images is usually different.

9. An easy way to obtain both a DIC and an IRM image is to use the same software that is used for obtaining *z*-stacks during fluorescence imaging. Then set the two parameters to obtain an IRM image near the coverslip followed by a DIC image at a higher focal plane.

Figure 4.23.2 *(appears on previous page)* Suitable configurations for obtaining IRM images with a laser scanning confocal microscope. (**A**) Detection of an IRM image alone. Any laser line can be used but usually a red or far-red line is used. This example shows a configuration using a 633-nm laser. The main beamsplitter is a neutral 80/20 or 70/30 dichroic depending on which is available in the microscope. This will direct the incoming laser to the sample but will still allow the reflected light to reach the detector. The reflected light could be sent to either of the two detectors in this diagram, as long as the emission filter will allow the light to pass. Here, the LP 600-nm filter will pass the 633-nm reflected light, but almost any LP filter would be suitable. Therefore, any detector in the system can usually be used to capture an IRM image. (**B**) Simultaneous detection of fluorescent and IRM images. In this case, the configuration settings will be based primarily on those needed to detect the emitted fluorescence and the IRM image will be captured with whatever detector is free and has a suitable emission filter. This example shows simultaneous imaging of green fluorescence (505 to 530 nm) and reflected light. The laser line, 488 nm, and the main beamsplitter, NFT 488, are chosen to fit the characteristics of the fluorophore. The IRM image will be formed with the reflected light that passes through the main dichroic because it is not a perfect optical device. If the main dichroic is so efficient that no incident light is allowed through, it will be necessary to use a neutral dichroic for the IRM image. The emission side beamsplitters are chosen to send the emitted fluorescence to one detector and the incident wavelength to another. In this diagram, all light longer than 505 nm is gathered for the fluorescence image and all shorter wavelengths, including 488 nm, are used for the IRM image. If the fluorescence is very weak, this beamsplitter can even be set at 470 nm, to collect as much light as possible for the fluorescent image. The remaining reflected light will be sufficient to produce an IRM image. While the emission filter for the fluorescence image should match the emitted fluorescence, almost any long-pass filter will allow the reflected light to reach the detector.

Focus on the sample

10. Use DIC or other transmitted light mode to find and focus on cells.
11. Turn on the laser, adjust focus while in scanning mode to find the bright reflection image, which will only appear over a very narrow range of focal planes.
12. Determine if open or narrow pinhole is optimal for the desired IRM images.

    Decreasing the size of the pinhole increases the sharpness of the IRM image; however, small variations in focus will produce an IRM image containing interference fringes. Taking an IRM image with an open pinhole will produce an image that is less sensitive to focus drift (see Anticipated Results for illustrations). Therefore, the experimenter needs to decide which parameter is more important, the sharpness of the image or insensitivity to focus drift.

13. Adjust detector gains for high-contrast image.
14. Readjust focus to obtain a zero-order interference image and then readjust the detectors for best contrast.

TIME-LAPSE IRM IMAGING OF LIVE MIGRATING *DICYTOSTELIUM DISCOIDEUM* AMOEBA

ALTERNATE PROTOCOL 1

Most confocal systems contain software that allows repeated image acquisition. Activating the appropriate imaging subroutine while using an IRM configuration allows the acquisition of IRM images over time. This allows imaging of many kinds of motile cells ranging from migrating *Dicytostelium discoideum* amoeba to activated T cells spreading on a stimulatory surface. If mammalian cells are being used, a microscope stage incubator may be needed. The exact kind of stage incubator will depend on the kind of microscope being used, the kind of cells being imaged, and the length of the experiment. A Web site is given at the end of this unit with links to numerous manufacturers of these devices. In addition, the microscope manufacturers themselves also produce stage incubators.

***Additional Materials** (also see the Basic Protocol)*

PB buffer for *Dicytostelium discoideum* (see recipe) or culture medium without phenol red for other cells
Dicytostelium discoideum amoeba developed according to standard protocols (Parent, 2001) or other live cells
Lab-Tek II one-well chambers (Nalge Nunc, cat. no. 155360)

1. Plate living *D. discoideum* cells into Lab-Tek II chambered coverslips.

   Developed cells should be diluted in PB buffer to 2×10^6 cells/ml and 5 μl drops should be placed in the chamber. Several drops can be placed in a single well.

   For Dicytostelium discoideum, either the one- or two-chamber size is usually best.

2. Allow amoebas to adhere for 5 min and then cover with 3 ml PB buffer for a one-well chamber.

   Other cells should be plated and covered with an appropriate culture medium.

3. Set up an incubation system if needed and allow the system to come to equilibrium. Put the chambers with cells onto the microscope stage.

   For Dicytostelium discoideum, no environmental controls are needed.

4. Proceed according to the Basic Protocol (steps 5 to 8 and 10 to 14) to obtain an IRM image of a live cell. If desired, add a transmitted light detector to the configuration and set a *z*-stack that will capture an IRM image in focus near the bottom of the

z-stack and a transmitted light image at the top of the z-stack (see Basic Protocol step 9).

5. Run a test series with time-lapse acquisition software to determine if refocusing will be needed. If so, add an autofocusing subroutine.

   The exact nature of the autofocusing will depend on the microscope being used and the desired interval between images.

6. Run full time-lapse image acquisition.

7. Select the best images from the z-stack if capturing multiple focal planes.

ALTERNATE PROTOCOL 2

MULTI-CHANNEL FLUORESCENCE AND IRM IMAGING

This protocol describes collecting both IRM and fluorescence images simultaneously using two photomultiplier detectors. If live cells are to be imaged over time, the same considerations for refocusing and incubation from Alternate Protocol 1 will apply.

Additional Materials *(also see the Basic Protocol and Alternate Protocol 1)*

Live sample cells expressing a fluorescent marker [e.g., transfected Jurkat T cells, clone E6.1 (ATCC # TIB-152) expressing a ZAP70 tagged with YFP and spreading on a stimulatory surface]
Imaging buffer (see recipe)

Stage incubator
Hot air blower (Nevtek) and objective heater (Bioptechs) for temperature control on the microscope stage for the Jurkat T cells
Lab-Tek II four-well chambered coverslips (Nalge Nunc, cat. No. 155382) coated with murine IgG1 to CDε clone Hit3a (BD Pharmingen) to activate the Jurkat T cells (see Support Protocol)
Laser Scanning Confocal Microscope equipped with at least two photomultipliers

1. Prepare cells. For Jurkat T cells, centrifuge the suspended cells from an actively growing culture for 5 min at 200 × g, 23°C. Resuspend the cells in imaging buffer at a density of 2×10^6 cells/ml.

   Fixed cells can also be used.

2. Set up the stage incubator.

3. For Jurkat T cells, turn on the objective heater and hot air blower, then place an antibody-coated coverslip on the microscope stage. Remove the PBS that has been stored in the chamber and fill the chamber about half full with warm imaging buffer. Then allow the system to come to thermal equilibrium.

4. Set up configuration to collect two channels simultaneously.

5. Select the laser to be used based on the excitation of the fluorescent probe being monitored.

6. In most cases, the main beamsplitter can be the optimal dichroic for fluorescence imaging because enough reflected light will pass through to generate an image. Since the IRM image will be collected at a shorter wavelength than the emitted fluorescence, select the secondary beamsplitters to send the reflected light through an emission filter that allows the incident light to reach the detector.

   As much emitted light as possible is sent to a detector through an emission filter suitable for the emitted fluorescence. Figure 4.23.2B shows a configuration suitable for collection of FITC, GFP, or YFP while simultaneously collecting IRM images.

7. Add the 3 to 5 μl resuspended Jurkat T cells (6000 to 10,000 cells) to the equilibrated chamber on the microscope stage.

   Alternatively, cells could be added to a chamber before placing it on the stage. For some adhesion assays, it may be necessary to add the cells several hours before imaging begins.

8. Proceed according to the Basic Protocol (steps 5 to 8 and 10 to 14). In this case, it is possible to use fluorescence for the initial focusing step.

   For Jurkat T cells, focusing is done quickly so that cells can be visualized soon after first contact with the coverslip.

   For time-lapse and live cell imaging, use the conditions in Alternate Protocol 1.

PREPARING COATED COVERSLIPS FOR IRM IMAGING

SUPPORT PROTOCOL

Coverslips may be coated with a large number of different substances to promote adhesion or activation of the cells being imaged. This protocol gives basic instructions for cleaning and coating coverslips.

Materials

Coverslip cleaning solution (see recipe)
Poly-L-lysine solution (Sigma-Aldrich, cat. No. P8920)
Distilled water
Fibronectin coating solution (see recipe)
Antibody coating solution (see recipe)
Phosphate-buffered saline (PBS; see recipe)

Coverslips [e.g., Lab-Tek II 4-well chambered coverslips; Nalge Nunc, cat. no. 155382 (but any chamber with a no. 1 or no. 1.5 coverslip bottom can be used)]
Drying oven

Prepare the coverslips

1. Clean the coverslips with cleaning solution by filling the chambered coverslip halfway and incubating 15 min at room temperature.
2. Completely remove cleaning solution by aspiration.
3. Dry the chambers in a drying oven set at 45°C for at least 30 min.
4. Dilute a commercially available poly-L-lysine solution to 0.01% in distilled H_2O.

   Poly-L-lysine solution can be stored up to 6 months at room temperature.

5. Fill the clean chambers halfway with the 0.01% poly-L-lysine and incubate 15 min at room temperature.

   In many cases, the desired coating will not adhere directly to a glass surface, so a preliminary coating of poly-L-lysine is applied first.

6. Completely remove the solution by aspiration.
7. Dry the chambers at 45°C for at least 30 min.

   These chambers can be stored for several weeks.

8. Add the appropriate coating solution either to cleaned or cleaned and poly-L-lysine–coated chambered coverslips.

For fibronectin coating

9a. Dilute 10 to 25 μg/ml fibronectin in PBS and add to a cleaned coverslip so that the coverslip is barely covered (e.g., 400 μl in one well of a four-well chambered coverslip).

10a. Incubate the coverslip for 1 to 2 hr at 37°C.

11a. Aspirate the solution.

12a. Plate the cells on the coated coverslip.

For antibody coatings

9b. Dilute 10 μg/ml antibody in PBS.

10b. Add antibody solution to the poly-L-lysine–coated coverslips so that the coverslip is barely covered (400 μl in one well of a four-well chambered coverslip).

11b. Incubate the coverslips overnight at 4°C.

12b. Rinse three times, each time with 1 ml PBS.

13b. Store filled with PBS.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antibody coating solution (10 μg/ml in PBS)

20 μl anti-human CD3ε antibody clone Hit 3a 200 μg/ml (BD Pharmingen, cat. no. P555336)
380 μl phosphate-buffered saline (PBS; see recipe)
Use immediately, store coated chamber <1 week at 4°C

Cleaning solution

70 ml of 100% (v/v) ethanol (Sigma-Aldrich, cat. no. E7023)
20 ml distilled water
10 ml of 10 M HCl (Sigma-Aldrich, cat. no. H7020)
Store <1 year at room temperature

DMEM culture medium

50 ml fetal bovine serum (FBS; Invitrogen, cat. no. 10437-028)
5 ml glutamine (from a 100× stock from Invitrogen, cat. no. 25030-081)
5 ml penicillin/streptomycin (from a 100× from Invitrogen, cat. no. 15140-122)
DMEM medium (Invitrogen, cat. no. 11995) to 500 ml
Filter sterilize using a 0.22-μm filter
Store <1 month at 4°C

Fibronectin coating solution (10 to 25 μg/ml in PBS)

25 μl fibronectin (1 mg/ml solution; Sigma-Aldrich, cat. no. F1141)
975 μl phosphate-buffered saline (PBS; see recipe)
Use immediately

Imaging buffer

5 ml fetal bovine serum (FBS; Invitrogen, cat. no. 10437-028)
0.125 ml of 1 M HEPES, pH 7.0 (Invitrogen, cat. no. 15630-130)
RPMI medium, without phenol red (Invitrogen, cat. no. 11835) to 50 ml
Filter sterilize using a 0.22-μm filter
Store <1 month at 4°C

Paraformaldehyde, 2.5% (w/v)

2.5 g paraformaldehyde (Ted Pella, cat. no. 18501)
100 ml phosphate-buffered saline (PBS; see recipe)
Prewarm PBS to 65°C and dissolve paraformaldehyde while heating to 80°
Store <6 months at −20°C

PB buffer (5 mM potassium phosphate pH 6.2)

0.68 g KH_2PO_4 (JT Baker, cat. no. 3246-01)
1.34 g $Na_2HPO_4 \cdot 7H_2O$ (Mallinckrodt Chemicals, cat. no. 7914-04)
Distilled H_2O to 1 liter
Store <6 months at room temperature

Phosphate-buffered saline (PBS)

1× phosphate-buffered saline without calcium or magnesium (Invitrogen, cat. no. 10010-031)
Store <6 months at room temperature

COMMENTARY

Background Information

Most of the analytical work on IRM comes from epi-illumination microscopes, not from IRM performed with a confocal microscope; however, the physical interactions are the same. In 1964, Curtis first applied the optical methods previously used to examine reflections from thin films to cells grown on glass coverslips (Curtis, 1964). Curtis analyzed his images in terms of reflections from the glass/medium and the medium/cell interface (Fig. 4.23.3A). The first reflection, r_1, is generated as light travels from the glass coverslip (η_1=1.515) to the culture medium ($\eta_2 \sim 1.34$) and another reflection, r_2, is generated as the light crosses from the medium into the cell ($\eta_3 \sim 1.37$). When the two reflected beams meet at a detector, they can interfere, producing an interference image.

The strength of a reflected beam at a single interface is proportional to the difference in refractive indices. In this system, these differences are relatively small (Izzard and Lochner, 1976), so only the primary reflections contribute to the IRM image. The greatest difference in refractive indices occurs at the glass/medium interface (1.515 − 1.34). Thus, in the absence of interference, the brightest reflection will come from the cell-free areas of the coverslip. Any areas in the IRM image that are brighter than the cell-free background must be due to positive interference, while darker areas are due to negative interference.

While light is a complex quantum phenomenon that can be viewed in several different ways, to understand the generation of the interference image it is easiest to think of the reflected light as two waves, r_1 and r_2. The superposition of the two reflected light waves at the detector generates the interference image. Because the two reflected waves have gone different distances through different materials, the two reflected waves may reach the detector with different phases. Constructive interference occurs if the waves arrive at the detector in phase, that is aligned so the amplitudes of the peaks add together and the superimposed wave is larger than the two incoming waves. Alternatively, destructive interference occurs if the waves are aligned peak to trough, so the superimposed wave is smaller than either incoming wave.

The alignment of the two reflected waves can be predicted by calculating the optical path difference (OPD), that is, the difference between the optical paths traveled by the two waves. The length of an optical path is defined as the product of physical path length and refractive index of the material through which the light wave travels. Two major factors affect the OPD; the difference in the physical paths traveled by the reflected beams and a phase reversal that affects beams reflected from light passing from material with lower refractive index into material with a higher refractive index. In our example (Fig. 4.34.3A), reflected wave r_2 has traveled farther than wave r_1 by going through the thin film of medium with a thickness of d twice. Considering only light that arrives at normal incidence (when θ; the refraction angle = 0), this gives a physical path difference of $2d$ and an OPD of $2d\eta_2$. In addition, there is the phase shift at the medium/cell interface and the sign of the electric field of the

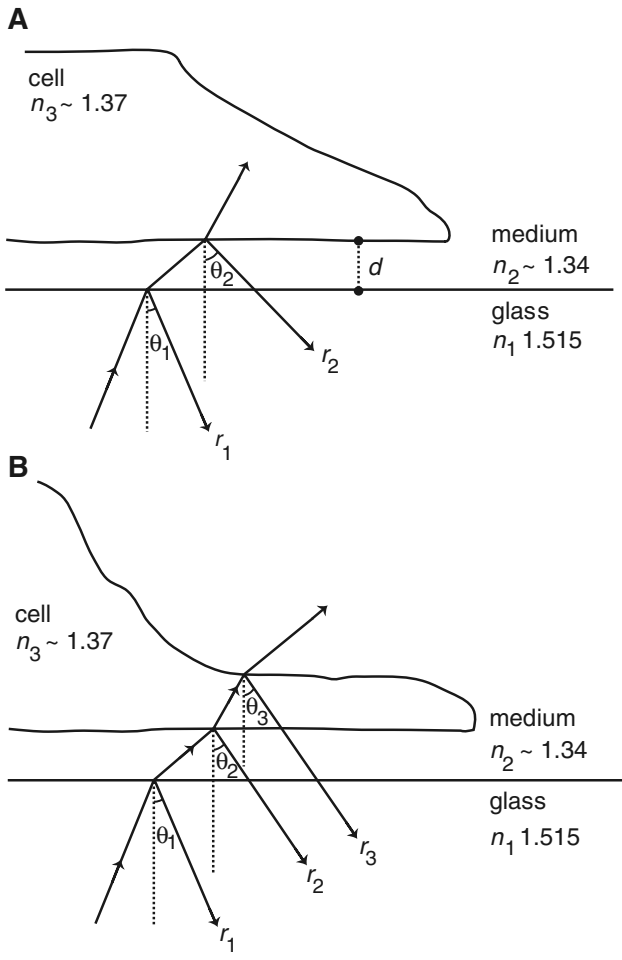


Figure 4.23.3 Diagrams representing the reflections generated by a cell on a coverslip. (**A**) Cell separated from the coverslip by a thin layer of thickness (*d*). The first reflection, r_1, is generated at the glass/medium interface with incident angle θ_1. The refracted beam continues through the medium and generates another reflection, r_2, with reflection angle θ_2 at the medium/cell interface. r_2 will undergo a phase reversal because it occurs at a transition from lower to higher refractive index. The light that continues through the cell can be used to form a transmitted light image if an appropriate detector is available. (**B**) An additional reflection from the top of the cell may also contribute to the IRM image. When higher-order interference fringes contribute to the image, the top of the cell will also contribute a third reflection, r_3.

reflected light is inverted. This can be represented in the OPD calculation as an additional path length of $\lambda/2$, so the OPD between r_1 and r_2 is $2d\eta_2 + \lambda/2$. If the light approaches the surface obliquely with a refraction angle θ, the OPD is $2d\eta_2\cos\theta + \lambda/2$.

It is possible to understand how an IRM image is produced by a cell on a coverslip by examining the OPD between the reflected waves as the distance between the cell and the coverslip varies. If the OPD is an integer multiple of the wavelength of the light, $m\lambda$, the waves align exactly peak-to-peak, generating maxima in the inference image. The integer m is called as the order of interference. Given that the OPD between r_1 and r_2 is $2d\eta_2 + \lambda/2$, the first maximum occurs in the image when $d = \lambda/4\eta_2$. As the cell gets closer to the coverslip and d gets smaller, the superimposed waves become more out of phase and the reflected intensity diminishes. When the OPD is $(m + {}^1/_2)\,\lambda$, the waves align exactly peak to trough, generating minima in the image. The first minimum is generated as d approaches 0, giving an OPD near $\lambda/2$, where there is maximum destructive interference. So in a zero-order image ($m = 0$), the OPD is smaller than λ and the darkest areas correspond to places where the cell touches the coverslip while the brightest areas correspond to places where the

distance between the cell and coverslip is $\sim^1/_4$ of the wavelength of the incident light (Beck and Bereiter-Hahn, 1981; Verschueren, 1985).

However, it is clear that a full quantitative analysis of the interference pattern that would allow calculation of d or η from reflection images is much more complicated. In 1976, Izzard and Lochner determined that reflections from further away also contribute to the interference image, producing images with interference fringes inside the cell (Izzard and Lochner, 1976). In this case, the cell acts as a second thin film (Fig. 4.23.3B) and reflections from the top membrane are seen in the IRM images. These higher-order patterns can be eliminated by increasing the illuminating numerical aperture of the system, which allows light entering at more oblique angles to contribute to the image. When the illuminating numerical aperture was >1, their IRM image only contained reflections from areas where the layer of medium was thin, generally <100 to 150 nm. This effect was attributed to a decrease in contrast in the interference fringes produced at greater distances from the coverslip because in the higher order fringes light reflecting at more oblique angles produce maxima in the interference pattern that overlap with minima from light entering at sharper angles. Gringell and Todd challenged this conclusion by showing that the loss of contrast could occur because increasing the illuminating numerical aperture strongly damped the maxima and minima of the higher-ordered fringes (Gingell and Todd, 1979). However, they agreed that at higher illuminating numerical aperture, it is not necessary to consider the reflections from the top of the cell if thickness of the cytoplasm is >1 μm and the distance between the cell and coverslip <250 nm. Their calculations also showed that IRM images from areas of very thin cytoplasm, such as spreading edges, would be affected by reflections from the top of the cell even at high illuminating numerical aperture (Gingell, 1981).

Later, Thorell and his colleagues suggested that the darkest areas of IRM images were due to weak reflections from areas of direct contact between the cell and coverslip (Bereiter-Hahn et al., 1979). They argued that if the darkest areas were interference minima where d is near to but not yet zero, the intensity seen in the image should be affected by changes in the incident wavelength or refractive index of the medium. Since neither perturbation had any effect, they concluded that these areas were not interference minima. However, as interference images from small gaps ($d < 5$ nm) are also relatively insensitive to changes in wavelength and refractive index, the exact nature of the darkest area remains controversial (Gingell and Vince, 1982).

Overall, there is general agreement that if the distance between the cell and coverslip is between 0 and about 100 nm, the intensity in the reflected light pattern increases with distance (Abercrombie and Dunn, 1975; Verschueren, 1985; Opas, 1990). In the IRM image, whites occur where the thickness of the layer of medium is ~100 nm, grays are generated at intermediate distances, while blacks occur at distances <15 nm and are an indication of strong adhesion (Fig. 4.23.4). If the IRM image contains areas where the thickness of the cell is <1 μm, reflections from the top of the cell will contribute significantly to the image and even semi-quantitative interpretation will be difficult unless the thickness of the cytoplasm is known.

It should be clear from this discussion, that reflection interference contrast is quite different from differential interference contrast (DIC) despite the similarities in the two names. DIC employs polarized light and special Wollaston prisms to convert differences in refractive indices into gray level differences, producing pseudo-three-dimensional images (Keller, 1997). Moreover, IRM is also different from total internal reflection fluorescence or TIRF microscopy. The latter uses a laser turned to a very oblique angle to generate an evanescent wave that is used to excite fluorophores near the coverslip (Toomre and Manstein, 2001).

Critical Parameters and Troubleshooting

The most critical parameter is to use a microscope with a suitable light path. It should be possible to see a very bright reflection from the coverslip when the focus is moved slightly below the focal plane of cells adhering to the coverslip. The exact z position is also critical; it is very easy for the focus to shift to a position where no image is visible.

The refractive index of the mounting medium is important, as a larger difference in refractive index generates brighter reflections. It is best to avoid alcohol- and acetone-based fixatives, as these will induce shrinkage in the samples that can affect contacts with the coverslip.

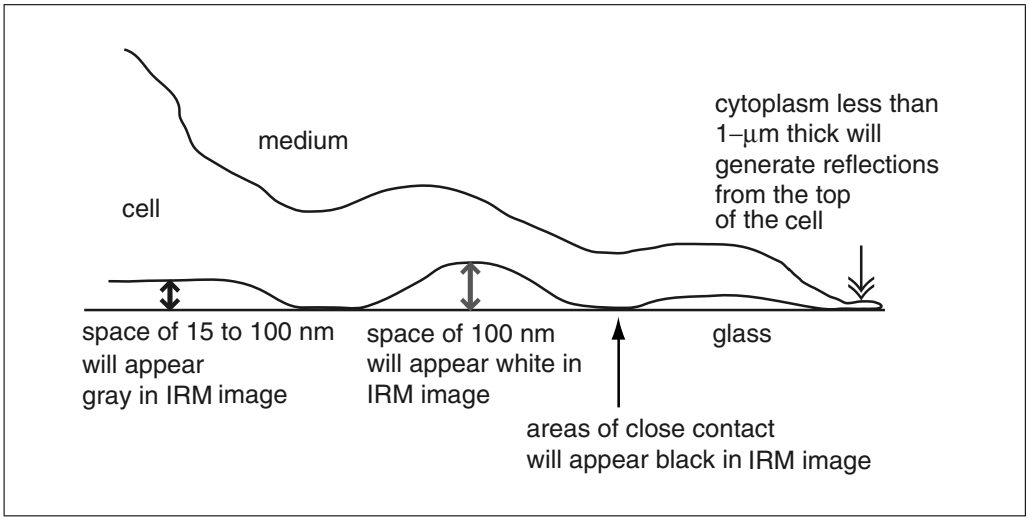


Figure 4.23.4 Gray-level intensities observed in a zero-order IRM images. The size of the gaps between the cell and coverslip have been exaggerated. A cell adhering to the coverslip will generate black areas on an IRM image in areas of close contact where the gap between the cell and coverslip is <15 nm (black arrow). If the gap is between 15 and 100 nm, the IRM image will show shades of gray (double-headed arrow). The brightest areas on the IRM image will come from areas where the cell is ~100 nm from the coverslip (gray double-headed arrow). If the cytoplasm is <1-μm thick (feathered arrow), reflections from the top of the cell will affect the intensity of the IRM image, so it will not be possible to approximate the distance between the cell and the coverslip unless the thickness of the layer of cytoplasm is known.

Anticipated Results

This technique should produce a gray-scale image of approximately the same shape as the cell being viewed. Figure 4.23.5A shows a DIC image of a NIH 3T3 fibroblast in the left panel and an IRM image of the same cell taken with a Zeiss 510 LSCM with the pinhole completely open in the middle panel. The right panel shows another IRM image taken with a 1.0-μm optical slice to reduce the field of focus. Because these are very flat cells, the DIC image has very low contrast, particularly when compared to the dramatic IRM image. As explained in the Commentary, in this zero-order IRM image, the darkest areas come from the regions of closest contact, where the cell is either adhered to the coverslip or is no more than 15 nm from the coverslip. The intensity of the reflected light increases with distance from the coverslip and the brightest areas in the image come from regions that are ~100 nm ($\lambda/4d\eta_{medium}$) from

Figure 4.23.5 *(appears on following next page)* IRM images of fixed NIH 3T3 cells. Images were obtained with a Zeiss LSCM 510 using a 63× oil objective NA 1.4. The configuration shown in Figure 4.23.2A was used for (A) and (B). A similar configuration using a 543-nm laser line was used for the last part of (C). (**A**) Comparison of DIC and IRM images of the same cell. Left panel: low-contrast DIC image showing intracellular organelles and details of the cell shape, but containing little information about the adherent surface. Bar = 10 μm. Middle panel: High-contrast IRM image taken with an open pinhole. Right panel: IRM image taken with the pinhole set a 1 Airy unit (1 AU). This is similar to the other IRM image, but the smaller pinhole gives a sharper image. (**B**) Effects of focal plane in images taken with narrow or open pinhole. Top panels: Images of the same field taken at successively higher focal planes with the pinhole set to 1 AU. The higher-order interference fringes are evident with little change in focus. Bottom panels: A similar series of images taken at successively higher focal planes with the pinhole completely open. These images are primarily formed from the zero-order interference fringe. Bar = 5 μm. (**C**) The zero-order interference image is similar when taken with light of different wavelengths. Top panels: The zero-order (left side) and higher-order (right side) images taken with a 633-nm laser. Bottom panels: The zero-order (left side) and higher-order (right side) images taken with a 543-nm laser. Bar = 20 μm. The dark areas of close contact indicated with black arrowheads outlined in white remain fairly constant through this set of images. While there is some variation in the remaining gray level intensities in the two zero-order images, the overall appearance of the images is very similar. In contrast, the higher-order interference images are shifted substantially by changes in incident wavelength.

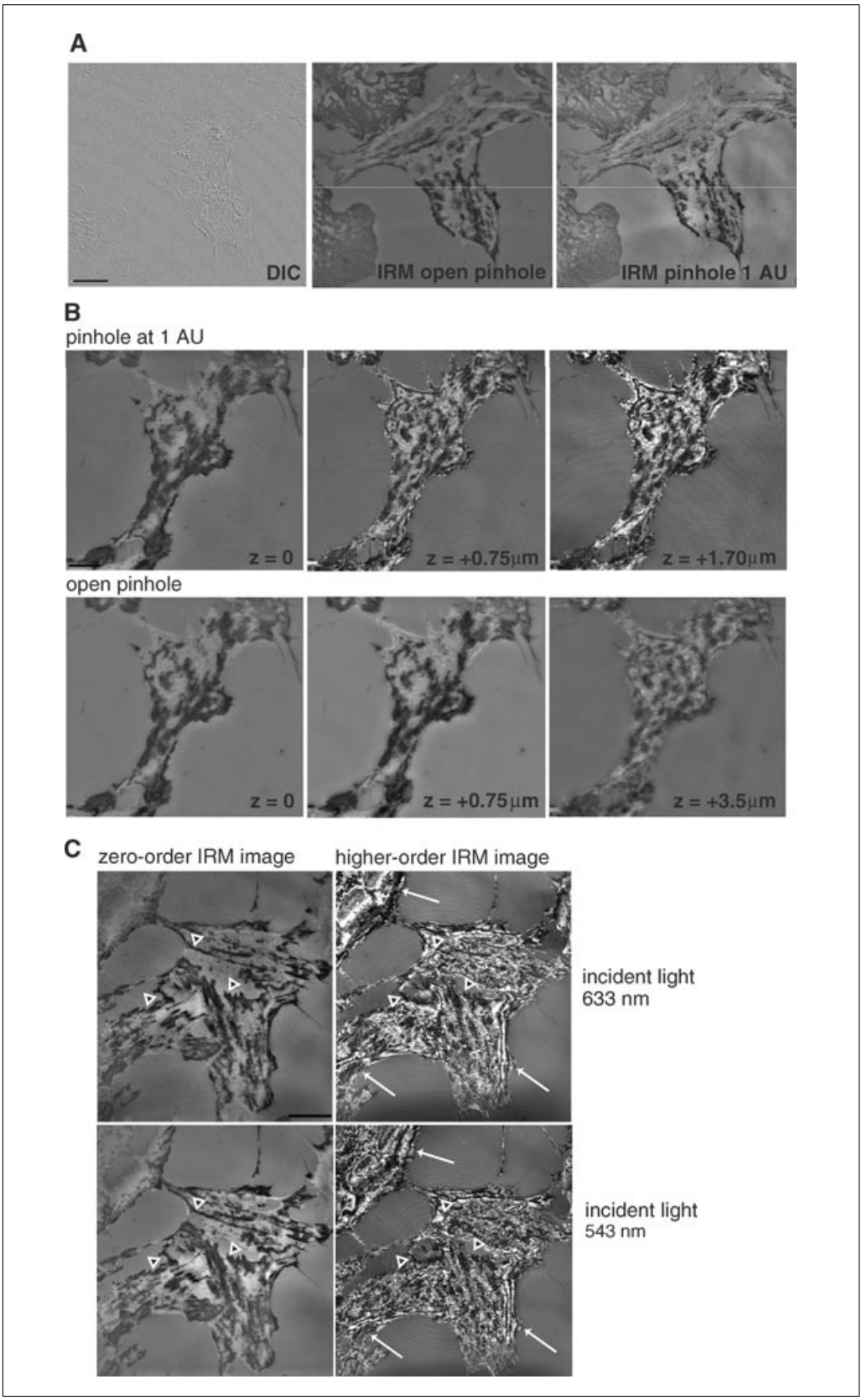


Figure 4.23.5 *(legend appears on previous page)*

the coverslip. The information in the DIC image is quite different, showing finer details at the edge of the cell, as well as showing structures inside the cell itself. The two IRM images look very similar but reducing the pinhole often produces sharper images. However, using a reduced pinhole can introduce other problems, as shown in the next set of images (Fig. 4.23.5B). The first panel shows an IRM image with a 1.0-μm optical slice as before, while the middle panel shows an image taken at a slightly higher focal plane. In the second image, more interference fringes are beginning to show up and complicate the zero-order image. The right panel shows an IRM image from a still higher focal plane that is completely dominated by the interference fringe pattern. Since the smaller pinhole restricts the focal plane, more light is gathered from higher in the cell as the focal plane is moved up. This allows imaging of the reflections from either the top of the cell or the nuclear membrane generating higher-order reflection fringes. The lower panels show equivalent images taken with the pinhole open. In these images, the higher-order fringe pattern is less visible and the image is out of focus before the interference fringes contribute significantly to the image. Information about the OPD at the different areas can be obtained from the fringe pattern; however, for most applications the zero-order image is more useful. The last set of images demonstrates the effects of changing the laser wavelength (Fig. 4.23.5C). The zero-order images shown in the left panels are similar in images whether 633-nm light or 543-nm light is used. In particular, the dark areas of close contact are virtually unchanged, although the gray levels in areas of intermediate distance show some variation. In contrast, the right panels contain images showing the higher-order interference fringes, where the pattern itself is a function of wavelength. In these images, fringe patterns change substantially as the wavelength changes.

Figure 4.23.6 shows a time series of IRM images from migrating *Dicytostelium discoideum* and DIC images captured in the same

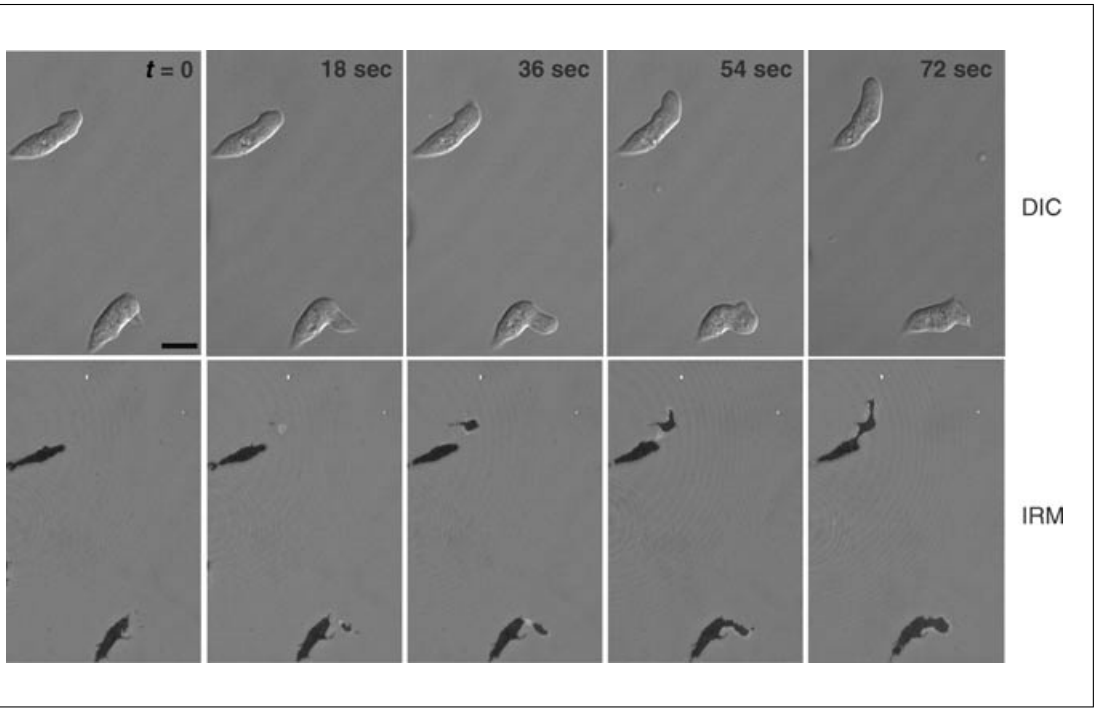


Figure 4.23.6 Application of IRM to migrating *Dicytostelium discoideum* cells. *Dicytostelium discoideum* amoeba were developed according to standard protocols to produce mobile cells. The images were taken using the same settings as Figure 4.23.5 with the addition of a transmitted light detector to the track. DIC optics installed on the microscope were used for the transmitted light image. Instead of single images, *z* stacks were collected at each time point and different focal planes were chosen to show the DIC or IRM images. Top panels: Time series of DIC image from the top of the *z* stack. The cells appear to glide over the coverslip. Bottom panels: IRM images from the bottom of the *z* stack in the same time series. Bar = 10 μm. These images clearly show that the amoeba move by extending a pseudopod that is not in contact with the coverslip and then forming a new contact site some distance from the cell body.

z-stack. The DIC images appear to show an amoeba gliding continuously over the coverslip, while the IRM image clearly shows a new contact site forming at a discrete distance from the current adhesion site. The IRM image also shows streaks of material left behind as the amoeba moves along. Thus, the IRM image contains information about cell adhesion during migration that cannot be obtained from the DIC image.

Figure 4.23.7 shows a time series of a spreading T cell along with simultaneous fluorescent imaging of an YFP-tagged protein. The YFP-tagged protein is a proximal kinase, ZAP-70, that is required for signal transduction downstream of the T cell receptor. These images show the immediate recruitment of the kinase to areas where the cell touches a stimulatory coverslip that is coated with anti-T cell receptor antibodies.

The high-contrast IRM images are useful for quantifying the extent and area of cell-surface contact. For example, to find the areas of activated T cells, an IRM image of a field of spread T cells was taken (Fig. 4.23.8; left panel). This image is easy to threshold using automated routines (middle panel). In this case, the built-in thresholding algorithm from

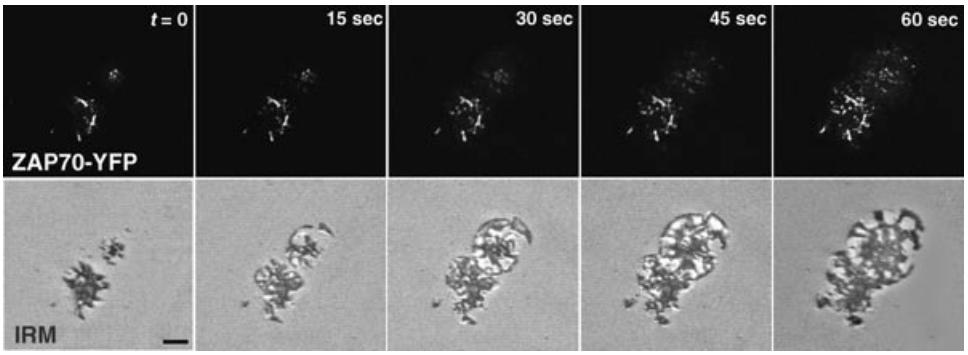


Figure 4.23.7 Simultaneous capture of fluorescence and interference reflection images of transfected Jurkat T cells expressing ZAP70-YFP. The configuration shown in Figure 4.23.2B was used to obtain these images. Top panels: Time series of YFP fluorescent images. The ZAP70-YFP clusters as the T cell spreads on the stimulatory coverslip. Bottom images: IRM images captured in the same track clearly show the T cell spreading across the coverslip. Bar = 5 μm. A comparison of the two images shows that ZAP70 clusters at the contact sites.

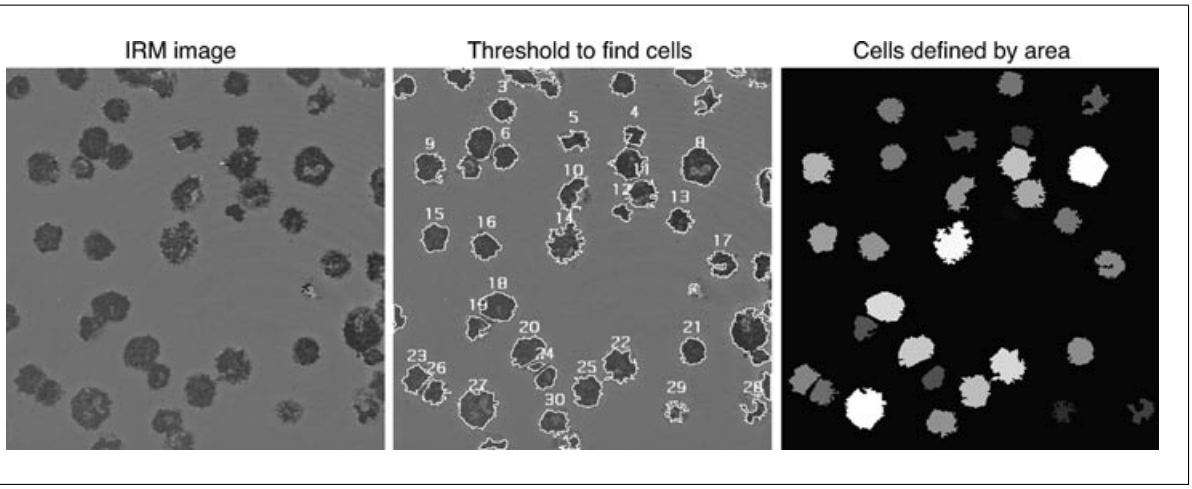


Figure 4.23.8 Quantification of an IRM image. Left panel: IRM image of fixed mouse T cells obtained using the same conditions as the cells in Figure 4.23.5. The T cells were allowed to spread on a stimulatory coverslip and were then fixed in 2.5% (w/v) paraformaldehyde. Middle panel: An auto-thresholding algorithm was used to outline the T cells based on the gray-level contrast and object shape. Some touching cells were improperly outlined together and were then separated by hand, for example no. 23 and no. 26. Right panels: The same field of cells visualized with an LUT where the gray level of each cell is proportional to its area. Lighter colors indicate cells with larger areas.

the software package iVision (BioVision) was used to outline the individual cells and identify each outlined cell with a number. The left panel shows an image where the gray level of each cell is proportional to the area outlined in the IRM image.

Time Considerations

The time frame for completing the protocols will vary tremendously depending on the kind of experiments to be performed and the samples being imaged. At one end of the spectrum, T cells could be resuspended in sample buffer, followed by live imaging of <3 min so the entire protocol could be completed in <15 min. Alternatively, if the experiments require precoating coverslips, culturing cells for days before beginning imaging, and conclude with days of imaging, the entire protocol may encompass an entire week.

Literature Cited

Abercrombie, M. and Dunn, G.A. 1975. Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. *Exp. Cell Res.* 92:57-62.

Beck, K. and Bereiter-Hahn, J. 1981. Evaluation of reflection interference contrast microscope images of living cells. *Microsc. Acta* 84:153-178.

Bereiter-Hahn, J., Fox, C.H., and Thorell, B. 1979. Quantitative reflection contrast microscopy of living cells. *J. Cell Biol.* 82:767-779.

Csaderova, L., Riehle, M., and Curtis, A. 2002. Detection of cell forces by measuring deformation of polymer films using interference reflection microscopy. *Eur. Cells Mater.* 4:64-65.

Curtis, A.S. 1964. The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. *J. Cell Biol.* 20:199-215.

Dorogi, P.L. and Keller, H.E. 1993. Antiflex microscopy of cell adhesion. *J. N.I.H. Res.* 5:79.

Gingell, D. 1981. The interpretation of interference-reflection images of spread cells: Significant contributions from thin peripheral cytoplasm. *J. Cell Sci.* 49:237-247.

Gingell, D. and Todd, I. 1979. Interference reflection microscopy. A quantitative theory for image interpretation and its application to cell-substratum separation measurement. *Biophys. J.* 26:507-526.

Gingell, D. and Vince, S. 1982. Cell-glass separation depends on salt concentration and valency: Measurements on Dictyostelium amoeba by finite apertrue interferometry. *J. Cell Sci.* 54:299-310.

Izzard, C.S. and Lochner, L.R. 1976. Cell-to-substrate contacts in living fibroblasts: An interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129-159.

Keller, H.E. 1997. Contrast enhancement in light microscopy. *Curr. Protoc. Cytom.* 0:2.1.1-2.1.11.

Opas, M. 1990. Biomedical applications of interference reflection microscopy. *Proc. SPIE.* 1121:351-356.

Parent, C.A. 2001. Dictyostelium cell dynamics. *Curr. Protoc. Cell Biol.* 9:12.5.1-12.5.19.

Ploem, J.S. 1975. Reflection-contrast microscopy as a tool for investigation of the attachment of living cells to a glass surface. *In* Mononuclear Phagocytes in Immunity, Infection and Pathology. (R. von Furth, ed.) pp.405-421. Blackwell Scientific Publications, London.

Prins, F.A., Cornelese-ten Velde, I., and Heer, E. 2005. Reflection contrast microscopy; The bridge between light and electron microscopy. *In* Cell Imaging Techniques: Methods and Protocols. Vol. 319. (B.T. Mossman, ed.) pp. 363-401. Humana Press, Totowa, N.J.

Toomre, D. and Manstein, D.J. 2001. Lighting up the cell surface with evanescent wave microscopy. *Trends Cell Biol.* 11:298-303.

Verschueren, H. 1985. Interference reflection microscopy in cell biology: Methodology and applications. *J. Cell Sci.* 75:279-301.

Key References

Beck and Bereiter-Hahn, 1981. See above.
Very thorough analysis of IRM images and the quantitative aspects of thin-film optics

Cornelese-ten Velde, I., Bonnet, J., Tanke, H.J., and Ploem, J.S. 1990. Reflection contrast microscopy performed on epi-illumination microscope stands: Comparison of reflection contrast- and epi-polarization microscopy. *J. Microsc.* 159:1-13.
Descriptions of epi-illumination microscopes configured for IRM imaging.

Curtis, A.S. 1964. See above.
First application of IRM to biological samples and quantitative analysis of the images.

Gingell, D. 1981. See above.
Analysis showing it is necessary to consider the thickness of the cell cytoplasm when analyzing an IRM image.

Izzard and Lochner, 1976. See above.
Description of the role of higher-order interference fringes in IRM images.

Ploem, 1975. See above.
Classic work on applying IRM imaging to biological samples using epi-illumination.

Verschueren, 1985. See above.
Very clear review of IRM imaging in biology that discusses many of the seminal papers.

Internet Resources

http://www.microscopyu.com/articles/confocal/reflectedconfocalintro.html
A brief description of confocal reflection imaging.

http://www.olympusfluoview.com/resources/specimenchambers.html
Vendors of incubation systems for microscopes.

Laser scanning confocal microscope manufacturers

http://www.leica-microsystems.com/
Leica Microsystem, Bannockburn, IL.

http://www.zeiss.com/
Carl Zeiss MicroImaging Inc. Thornwood, NY.

http://www.nikon.com/index.htm
Nikon Inc., Melville, NY.

http://www.olympusamerica.com
Olympus America Inc., Center Valley, PA.

Microscope manufacturers with IRM modules for epifluorescence microscopes

http://www.zeiss.com/
Carl Zeiss MicroImaging Inc., Thornwood, NY.

http://www.leica-microsystems.com/
Leica Microsystems, Bannockburn, IL.

http://www.nikon.com/index.htm
Nikon Inc., Melville NY.

http://www.olympusamerica.com
Olympus America Inc., Center Valley, PA.

http://www.biovis.com/
BioVision Technologies, Inc., Exton, PA.

Fluorescence Correlation Spectroscopy in Living Cells: A Practical Approach

Nihal Altan-Bonnet[1] and Grégoire Altan-Bonnet[2]

[1]Department of Biology, Rutgers University, Newark, New Jersey
[2]Program in Computational Biology and Immunology, Memorial Sloan Kettering Cancer Center, New York, New York

ABSTRACT

Fluorescence correlation spectroscopy (FCS) is a single-molecule fluorescence technique used to monitor molecular dynamics. It can be applied to living cells expressing fluorescently labeled proteins and lipids to determine the diffusion timescales and the total number of diffusing fluorescent molecules in the cell. A practical step-by-step approach to performing FCS with a commercial spectroscopy/microscopy system, the Zeiss Confocor 3, how to set up live-cell FCS experiments, acquire reliable data, and finally how to analyze the data acquired, are all described in this unit. *Curr. Protoc. Cell Biol.* 45:4.24.1-4.24.14. © 2009 by John Wiley & Sons, Inc.

Keywords: fluorescence correlation spectroscopy • FCS • live-cell imaging • green fluorescent protein • GFP • diffusion

INTRODUCTION

Fluorescence correlation spectroscopy (FCS) is a single-molecule fluorescence technique used to monitor molecular dynamics. When applied to living cells, FCS has been used to decipher the diffusion times of protein and lipid molecules, to determine the concentrations of a particular molecule at a specific cellular location, and to determine kinetic parameters such as on/off rates, constants, and binding coefficients for protein/protein interactions (Schwille, 2001).

The basic principle of FCS is that a fluorescent molecule emits photons while moving through a confocal volume illuminated by a laser (Fig. 4.24.1). The number of photons that can be collected from the confocal volume depends on the diffusion time of the fluorescent molecules (which is a function of the mass of the molecule), the concentration of molecules, the quantum yield of the fluorophore attached to the molecule, and finally the size of the illuminated volume, which is dependent on the instrument (Krichevsky and Bonnet, 2002). Typically, a small diffraction-limited confocal volume of $(0.3)^2 \times 1\text{–}\mu m^3$ (about the size of an *E. coli*) is excited with a laser beam, and then the emitted photons are collected and counted on a photodiode detector. These measurements are then used to calculate and display autocorrelation and cross-correlation functions, coincidence diagrams, photon count histograms, count rate diagrams, and pulse density histograms, from which diffusion times, on/off rates, etc., can be extracted using data-fitting and -modeling software (Bacia and Schwille, 2003).

In this protocol, the basic design of an FCS measurement in living cells is presented, and critical issues in experimental design (from the choice of dyes or cells to data analysis) are addressed. The user must be aware that FCS requires subsequent adjustments in the experimental design (e.g., choice of fluorophore, derivation of analytical fitting equations, etc.) to successfully address specific biological questions.

Current Protocols in Cell Biology 4.24.1-4.24.14, December 2009
Published online December 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0424s45

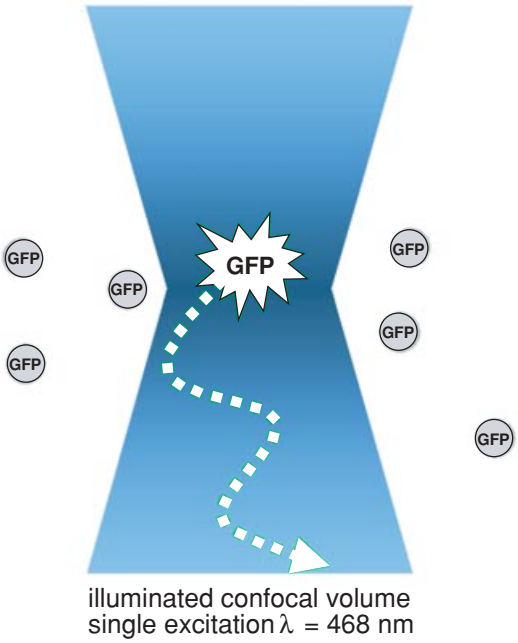


Figure 4.24.1 Sketch of the optical setting for an FCS measurement.

STRATEGIC PLANNING

Equipment and Materials

The ConfoCor 3/LSM 510 system is a commercially available system from Carl Zeiss Microimaging, which combines an LSM 510 META laser scanning microscope with an FCS module to acquire high-resolution images and sensitive fluctuation analysis. The ConfoCor 3 detection allows one to quantitatively analyze the concentration, position, interaction, and mobility of molecules. The new experimental capabilities provided by the ConfoCor 3 take effect in the investigation of molecule distribution in cells, of diffusion processes by fluorescence correlation spectroscopy, and of protein complex formation in the detection of common movements and the quantification of receptor/ligand interactions.

The software controls the detection module and allows convenient analysis of single measurements or software-controlled multi-measurements. Autocorrelations are calculated in real time, i.e., at the same time as the current measurement. And there is always the choice of using the raw data for individual analysis.

Other options include advanced software modules that incorporate the most common methods, free diffusion, anomalous diffusion (Wachsmuth et al., 2000), and flow (Gosch et al., 2000), provide for user-defined models, allow for global and interactive fitting with the possibility of defining start values and boundaries, and make photon counting histograms possible (Chen et al., 1999, 2000).

Nunc Lab-Tek (*http://www.nuncbrand.com/en/page.aspx?id=2298*), Mat-tek chambers (*http://www.glass-bottom-dishes.com*) are deep-welled chambers with cover-glass bottoms onto which cells are plated. Many cells stick directly onto glass but the surfaces can also be coated with poly-lysine to facilitate sticking. The cover-glass bottoms of the chambers allow high-magnification, high-resolution microscopy with water-immersion objectives. Cells should be plated sufficiently in advance such that they have adhered and acclimated to culture conditions. For FCS experiments, the cell medium should be one without phenol red dye, to limit background fluorescence noise. Additionally, if carbon

dioxide levels on the microscope stage cannot be controlled, supplement the medium with HEPES buffer, pH 7.2, to 25 to 50 mM final concentration.

High numerical aperture (NA) water-immersion objectives are critical components of an FCS system. The signal-to-noise ratio for an FCS measurement is proportional to the square of the collected fluorescence per fluorescent object, hence the need to maximize NA (Krichevsky and Bonnet, 2002). Living cells are imaged in water-based media; therefore it is best to use water-immersion objectives whose limited spherical aberrations ensure a more accurate definition of the confocal volume from which FCS measurements are made. Typically, a 40×/1.2-NA water-immersion objective with collar adjustment for the thickness of the coverslip is used; this minimizes aberrations in the *z*-axis and provides a tight diffraction-limited confocal volume of illumination inside the primarily aqueous cytoplasm of a living cell.

Fluorescent dyes are used for calibrating an FCS instrument. Fluorescent dyes (e.g., Oregon Green or FITC), fluorescently labeled dextran, or Fluospheres (Invitrogen) can all be used for instrument calibration prior to acquisition from cells. Typically, a single concentration of Oregon Green (100 nM dilution) in phosphate-buffered saline (PBS) is placed into an 8-well LabTek chamber with no. 1.5 coverslip bottom. Calibration should be done daily for every experiment to align pinhole and the detectors and maximize the number of collected photons.

Choice of labeling for molecule of study must be determined, e.g., GFP, YFP, CFP, and mRFP are used for FCS measurements. XFP-tagged proteins can be exogenously expressed in cells by transfecting a plasmid encoding the chimera ≥8 hr prior to FCS. Alternatively, the fluorophores Oregon Green, FITC, Rhodamine 6G, etc. can be covalently linked to the protein or lipid molecule under study and microinjected into the cells. Special care in the choice of organic fluorophores should be taken to maximize the signal-to-noise ratio for FCS analysis. Practically, this implies maximizing the number of photons that are collectable from one individual fluorophore (Koppel, 1974). In particular, when studying internal conformational changes, e.g., rotation, intramolecular quenching (Bonnet et al., 1998; Haupts et al., 1998), one should avoid fluorophores with large singlet-triplet rate of conversion (e.g., use Oregon Green over triplet-prone FITC). Furthermore, one should always prefer dyes that minimize photobleaching. This is especially relevant when performing FCS on slow-diffusing molecules such as membrane proteins.

Any cell type (flat cells like fibroblasts or rounded cells like lymphocytes) can be used for FCS measurements as long as cells are adherent to or can be tethered on the glass coverslip (e.g., by cross-linking onto poly-L-lysine-coated coverslips or by adsorption onto antibody-coated coverslips) to minimize movement during fluorescence acquisition (in the latter case, proper control experiments must be performed to validate that the tethering does not perturb biological functions).

For transfection, any nucleic acid transfection agent can be used. Fugene (Roche Diagnostics) or Lipofectamine (Invitrogen) is usually used. The protocols for transfection can be obtained from the manufacturers' Web sites.

The level of fluorescent protein expression is an important parameter because too high levels of expression force the experimenter to decrease laser excitation to avoid detector saturation with the fluorescence collected from fluorescent objects. This decreased laser excitation in turn would substantially decrease the number of emitted photons that can be collected per fluorescent object and, therefore, the FCS measurements. One solution is to photobleach a large fraction of the fluorescence from the cell before making FCS measurements. In the ConfoCor 3 Acquisition menu, one can set the FCS protocol to bleach for a user-defined amount of time at a high laser intensity setting before switching to a lower laser power for the actual FCS acquisition.

SETTING UP FLUORESCENCE CORRELATION SPECTROSCOPY FOR LIVING-CELL MEASUREMENTS

This protocol describes the steps for setting up the Zeiss LSM 510 ConfoCor 3 FCS system, one of the more popular turnkey FCS systems commercially available. For other setups, the step-by-step instructions may vary.

Materials

Cells growing on a glass-bottomed culture dish (Mat-Tek) or chambered slide (Lab-Tek, Nunc) with lid at 50% confluency
Medium for incubation on microscope stage (culture medium with serum but without phenol red, supplemented with 25 to 50 mM HEPES)
Petroleum jelly (e.g., Vaseline, Cheeseborough)
100 mM Oregon Green in PBS or other fluorescently labeled molecule for calibration

FCS machine, e.g., Zeiss LSM 510 ConfoCor 3 FCS system
8-well Nunc-Lab Tek chambers

Prepare cells

1. One hour prior to FCS measurements, change the medium of the cells at 50% confluency into a medium that is optimal for keeping cells on the microscope stage.

   This medium is typically the tissue culture medium that the cells are normally maintained in (containing serum), buffered with 20 to 50 mM HEPES, pH 7.3, but without phenol red.

   The absence of phenol red pH indicator (commonly used in tissue culture media) helps to minimize the levels of background fluorescence during FCS.

2. Fill the Nunc Lab-Tek chambers as high as possible with the medium to ensure a large volume above the cells that will not evaporate over the course of the measurement time. Moreover, to minimize evaporation, smear the inside of the chamber lid with a coat of petroleum jelly and fit the lid onto the chamber.

   Sealing the lid with petroleum jelly allows the user to open the lid for adding various agents to the cell medium and will limit evaporation, which can cause changes in the salt concentrations and pH of the medium. Alternatively, if cells are left alone for long periods of time or if no additions are necessary, the outside edges of the lid can be glued to the chamber.

   There are several options for temperature control of the microscope stage. An airstream incubator ASI 400 (Nevtek) in combination with a digital thermometer with a thermocoupler (Omega Instruments) whose temperature sensor is attached to the head of the condenser or to a site on the stage adjacent to the cell chamber is a convenient set-up for holding steady temperatures (±0.5°C) in most applications requiring imaging up to 12 hr (Rabut and Ellenberg 2004; Altan-Bonnet et al., 2006). It also allows easy access to the cell chamber for adding reagents to the cells during the course of the experiment while on the stage without shaking the cell chamber or stage, both of which could result in the loss of the position of the cell being imaged. The drawbacks are exposure to ambient CO_2 and humidity, the usual degradation of the buffer, and the fact that over time the medium will gradually evaporate and become basic in pH. If long-term maintenance of cells is necessary, then it is recommended to invest in a closed chamber set-up that encloses the microscope stage and optics.

Set up for microscopy

3. Turn on the FCS machine following the manufacturer's instructions. Turn on the stage temperature control and set it to the desired temperature. Wait at least 30 min before FCS acquisition.

4. Turn on all needed lasers. Wait at least 30 min before starting measurements to allow stabilization and to minimize fluctuations in laser output.

   Typically, the argon laser should be run at 50% of maximum output. This setting maximizes the stability of laser emission.

5. Go to ConfoCor mode, and set up the optical path—this includes choosing the laser line for excitation, choosing the laser line intensity, selecting the dichroic mirrors that will allow both the 488-nm excitation and emission >500 nm, and selecting the emission band-pass filters and the detector.

   For example, for Oregon Green or GFP, excitation is 488 nm at ~1% intensity, a 488-nm dichroic mirror, and a band-pass filter of 500 to 550 nm.

6. First, place the 8-well Nunc-Lab Tek chamber containing 100 nM Oregon Green (or another fluorescently labeled molecule) in PBS onto the stage.

7. Go to the Sample Carrier menu and select the correct sample carrier, i.e., chamber type (in this case, a Nunc 8-well chamber).

8. Select the exact chamber position from which the Oregon Green dye acquisition will be made. Focus 200 μm above the glass coverslip into the solution.

   One easy way to determine if you are above the glass coverslip is to run a line scan from the confocal imaging mode. Start out the line scan with the pinhole completely open and the objective far away from the bottom of the glass coverslip. As you are running the line scans, move the focus wheel of the microscope such that the objective will move closer to the glass coverslip. While moving up the objective, pay attention to the line scan window, which will output a line with a hill, which is the reflected excitation light not yet focused on a glass coverslip surface. As the objective hits the bottom glass coverslip surface, the line will increase in amplitude, then decrease, and finally hit a maximum again when the objective reaches the top surface of the glass coverslip. At this point, one can use the objective control stepper (present on most confocal systems) to go 200 μm above the top glass coverslip.

Align and optimize system

9. Set the Pinhole, from the ConfoCor 3 Adjust menu item, to 70 μm and run Pinhole Alignment. Adjust the pinhole using the Oregon Green dye solution. During pinhole alignment, move the pinhole along the *x* and *y* axis until the maximum intensity of emission is found.

10. Once the pinhole is aligned, take a Count Rate of the solution (this is, the number of photons emitted by individual molecules per second).

    At a concentration of 100 nM Oregon Green, the minimal count per molecule should be at least ~5000 counts/sec. If lower than this, realign pinholes either through the software for a minimal alignment or manually for more significant alignment issues (this procedure usually requires service by a professional contractor).

Optimize image

11. Switch to the chamber with cells once the system is aligned and optimized with the Oregon Green sample. Switch to confocal imaging mode (i.e., go out of FCS menu). Perform a single *x-y* scan of the cells (in confocal imaging mode; Fig. 4.24.2A). When appropriate cells have been identified for imaging, acquire quick scans while adjusting the focus, the pinhole, laser intensity, detector gain, and amplifier gain, to determine the optimal image.

    The quality of this image will effect where you spot the beam for collecting FCS data.

    The x-y scan pinhole should be adjusted to give a high-resolution image, typically ~1 Airy unit. To be <1 Airy unit is usually not recommended since there is very little resolution gained to compensate for the significant loss in light collection from the sample.

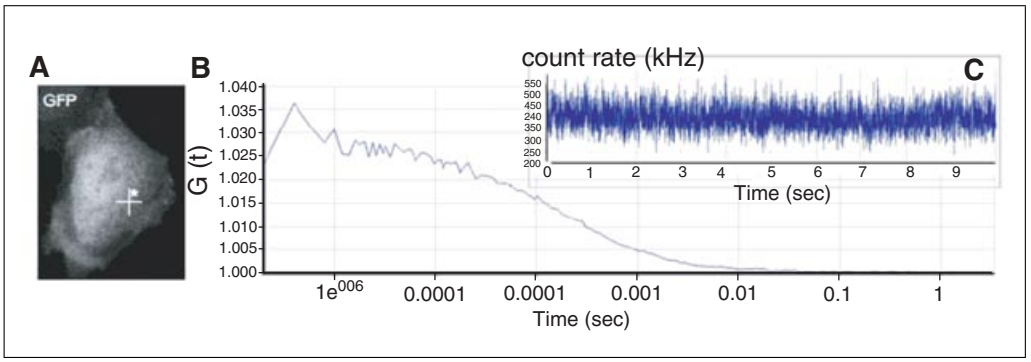


Figure 4.24.2 Autocorrelation on cytosolic GFP. Autocorrelation function is collected from a confocal volume chosen in the cell cytoplasm where soluble GFP is being expressed (**A**). Note how the baseline for the autocorrelation function is flat (between 100 msec and 1 sec) yielding a good fit and diffusion estimate (**B**). The count rate is stable indicating the necessary lack of photobleaching (Inset **C**).

12. When the object is to collect correlation spectra from the plasma membrane or any other membrane, acquire a z-stack of 1 μm and determine the z-position where the fluorescence intensity is at its peak. It is best to limit the laser excitation intensity and keep the number of z slices to a minimum to minimize bleaching the sample.

13. Save the image in a database and keep a copy of it on the screen.

14. Switch back to the FCS (or ConfoCor) mode.

Select the method

15. Go to the Methods menu item. Select from either of the preexisting methods or set up a new method.

16. To set up a new method, go to Acquisition Setup under the Measure menu, and then change the laser excitation, dichroic, and band-pass filter settings to the appropriate settings for the fluorophore inside the cells.

    For GFP-expressing cells, these settings will be similar to those used to obtain count rates for Oregon Green dye.

17. Save these settings under a new method name.

18. Keep laser excitation intensity low such that the Triplet Fraction is <10% of the molecules.

    The triplet state of the molecule is when it is in an excited but dark state—where it has given off only a portion of the absorbed energy as photons. To determine the triplet fraction at a particular laser intensity, pay attention to the Triplet Fraction column of the data table generated during FCS acquisition.

19. Go to the Measure menu option. Switch carrier from 8-well Nunc to LSM Image. Then mark positions in the cell where data collection is desired (Fig. 4.24.2A). Pick several positions within the cell and one position outside the cell for background measurement. Do not forget to hit the Mark positions button for this will number each position.

20. Go to Acquisition under the Measure menu, and set the acquisition time from each site in the cell.

    Typically, this time should be ~10 to 15 sec or 1000-fold the diffusion time of the molecule being investigated.

21. Set the Repeat count (this is the number of readings taken from each site).

    For good data fitting, take at least ten measurements from each site.

Acquire data

22. Start acquisition.

    During acquisition, fluorescence read out will be taken from each site for a pre-defined amount of time (~10 sec). A typical read out for GFP expressed in the cytoplasm is presented in Fig. 4.24.2 including the Correlation function G(t) versus lagtime (B) and the count rate over time (C).

    Longer acquisitions from a single marked site will allow for more statistical information for long lagtimes (t >1 sec). This will benefit curve fitting but it may also result in increased bleaching of the sample. Typical acquisition times are 10 sec. In the acquisition menu, one can also introduce a pre-bleach time prior to FCS acquisition. Here the sample will be photobleached for a discrete amount of time before recording correlation. This is useful if the sample fluorescence is too high. Always perform measurements a second time with five times higher power to check consistency with power amplitude.

23. Save the correlation graphs generated by the Zeiss LSM software. Additionally, save the raw acquired data and export as a .txt file to an analysis software (e.g., Prism, Kaleidagraph, or Matlab) for further custom modeling and fitting.

CHECKING THE CONFOCAL AND FCS ALIGNMENT

SUPPORT PROTOCOL

This is a simple way to check the ConfoCor and FCS *x-y* alignment.

Materials

1 μmol FITC solution in DMSO
No. 1 coverslips
ConfoCor FCS system (Zeiss LSM 510)

1. Place a drop of FITC solution onto a no.1 coverslip.
2. Dry in the dark 30 min in ambient air.
3. Set up the ConfoCor FCS system for confocal imaging (see Basic Protocol 1).
4. Using LSM mode, image the surface of the coverslip in the confocal setup. Leave the LSM image window and control screen on the computer screen.
5. Go back to the ConfoCor Measurement menu and mark positions on the recorded LSM image.
6. Set up a long-time exposure (10 min) for FCS measurement with maximal laser power (100% of 488-nm line) to bleach the marked sites.
7. Re-image the surface of the coverslip using LSM mode and measure by drawing a line using the overlay option in the ConfoCor image display menu, the shift between the targeted FCS site (under the arrow), and the actual measurement site (bleach spot).
8. Adjust the *x* and *y* registries under the Settings in the LSM+ConfoCor menu item.
9. Repeat steps 4 to 6 to ensure the accurate registry of confocal and FCS volumes.

ANALYZING FCS DATA

BASIC PROTOCOL 2

The most difficult task in a successful FCS measurement is the fitting and interpretation of the collected data. Although these are common tasks of any experimental measurement, there are a few issues that are specific for FCS and these will be addressed in this section.

FCS monitors any fluctuation of fluorescence within the confocal volume. Typically, any variation of fluorescence on time-scales from 1 μsec to 1 sec will generate a relaxation in

the autocorrelation curve that must be fitted and interpreted. To successfully process the data, the experimenter must derive an analytical formula accounting for all fluctuations. For FCS in living cells, these analytical fits may be hard to derive, which leaves the experimenter with hard-to-interpret phenomenological time-scales that are still sufficient for an analytical assay in perturbation experiment (comparison of wild-type and drug-treated or gene-perturbed cells).

Simple two-dimensional diffusion model for a single species

$$G(\tau)=\left[1+\frac{1}{N}\right]\left[\frac{1}{1+\frac{\tau}{\tau_D}}\right]$$

Equation 4.24.1

Where N is the average number of fluorescent objects within the confocal volume, τ_D the correlation time $\tau_D = \omega^2/4/D$, where D is the diffusion coefficient of the fluorescent object and ω is the radial diameter of the confocal volume. The LSR510-ConfoCor software has the capacity to use a theoretically more accurate diffusion fit including the longitudinal diffusion. But, a three-dimensional formula does not improve the statistical accuracy of the fit, and it requires careful calibration of the longitudinal dimension of the confocal volume to be valuable. A typical curve for soluble GFP diffusing with a cell cytoplasm is presented in Fig. 4.24.2.

Simple two-dimensional diffusion model for multi-species

$$G(\tau)=\left[1+\sum_i \frac{1}{N_i}\right]\left[\frac{1}{1+\frac{\tau}{\tau_i}}\right]$$

Equation 4.24.2

Where N_i is the average number of fluorescent objects labeled i within the confocal volume, $\tau_0 = \omega^2/4/D_i$, where D_i is the diffusion coefficient of the fluorescent object labeled i and ω is the radial diameter of the confocal volume. Note that the more difference there is between the diffusion of the fluorescent objects, the better the fit of the data.

Abnormal diffusion

In many living cell settings, the diffusion of fluorescent objects encompasses multi-scale, multi-time-scale moves. An explicit description of such a convoluted process is formally impossible, and experimenters routinely use a phenomenological "stretched diffusion" formula to fit the FCS data. An example of such a fit was used by Weiss et al. (2003):

$$G(\tau)=\left[1+\frac{1}{N}\right]\left[\frac{1}{1+\left(\frac{\tau}{\tau_D}\right)^{\alpha}}\right]$$

Equation 4.24.3

where N is the average number of fluorescent objects within the confocal volume, τ_D is a characteristic diffusion time-scale, and α is the stretching parameter (with $0<\alpha<1$). The smaller the value of α, the more convoluted the diffusion is.

Relaxation model with two-dimensional diffusion

When the fluorescent object under study fluctuates between quenched and fluorescent states, FCS can monitor the chemical relaxation of this process (Haupts et al., 1998; Altan-Bonnet et al., 2003):

$$G(\tau)=1+\left[\frac{1}{N}\right]\left[\frac{1}{1+\frac{\tau}{\tau_0}}\right]\left(1+\beta e^{-\frac{\tau}{\tau_R}}\right)$$

Equation 4.24.4

where N is the average number of fluorescent objects within the confocal volume, τ_0 is the characteristic diffusion time-scale, and τ_R is the characteristic chemical-relaxation time-scale. For a simple on-off with characteristic chemical rates k_{on} and k_{off}:

$$\tau_R=(k_{on}+k_{off})^{-1} \text{ and } \beta=K_{equilibrium}=\frac{k_{on}}{k_{off}}$$

Equation 4.24.5

In practice, the dynamic range for the FCS autocorrelation amplitude β is too small to yield an accurate estimate of K—a separate measurement is then required.

More generally, the analytical fits used to analyze FCS measurements can become complicated by the rich dynamics of a system (e.g., Altan-Bonnet et al., 2003). There are reviews documenting different applications with different fitting functions for FCS measurements in living cells (Krichevsky and Bonnet, 2002; Carl Zeiss Microimaging, 2006).

FCS output parameters

A typical FCS curve for diffusing fluorescently tagged molecules within the cytoplasm of a living cell and the associated fit to help FCS users understand what each parameter means (see Fig. 4.24.3) is described on a mixture of two species (component no. 1 and 2):

$$G(\tau)=1+\left(\left[\frac{1}{N_1}\right]\left[\frac{1}{1+\frac{\tau}{\tau_1}}\right]+\left[\frac{1}{N_2}\right]\left[\frac{1}{1+\frac{\tau}{\tau_2}}\right]\right)\left(1+\left[\frac{p}{1-p}\right]e^{\frac{-\tau}{\tau_T}}\right)$$

Equation 4.24.6

Diffusion times are $\tau_1 = 100$ μsec and $\tau_2 = 10$ msec, with $N_1 = 2$ and $N_2 = 2$ particles/confocal volume. The dye has a triplet time τ_T of 1 μsec and a triplet fraction at 10% ($p = 0.1$). Note how the two diffusion times yield two relaxations that can hardly be separated.

Typically, the fit is:

$$G(\tau) = 1 + \left(\sum_i \frac{1}{N_i} \frac{1}{1 + \frac{\tau}{\tau_i}} \right) \left(1 + \left[\frac{p}{1-p} \right] e^{\frac{-\tau}{\tau_T}} \right) = 1 + \left(\sum_i \beta_i \frac{1}{1 + \frac{\tau}{\tau_i}} \right) \left(1 + \beta_{\text{triplet}} e \frac{-\tau}{\tau_T} \right)$$

Equation 4.24.7

ConfoCor fitting software generates a table with the following parameters:

a. Count rate [kHz] is the total number of photons collected by a detector per second.
b. Correlation is the amplitude of the correlation function $G(0)$.
c. Counts per molecule [kHz] is computed as (Count rate)$(G(0) - 1)$. This parameter can be very useful to estimate the state of aggregation of a fluorescent protein.
d. Amplitude Number particles $= N_i$ (note that

$$G(0) - 1 = \sum_i \frac{1}{N_i}$$

Equation 4.24.7A

when the triplet fraction p is negligible).
e. Triplet state fraction [%] is $p(100)$.
f. Triplet state relaxation time [μsec] is τ_T.
g. Component i fraction [%] is

$$\text{Component } i \text{ Fraction } [\%] = 100 \left[\frac{N_i}{\sum_i N_i} \right]$$

Equation 4.24.7B

h. Component i diffusion time [μsec]: τ_i,
i. Translation structural parameter should be fixed upon calibration of the optical setup. To calibrate the size of the confocal volume, perform an FCS measurement on a solution of fluorescent dye (e.g., Oregon Green) at a known concentration c. Measure the amplitude number of particles (N) in the FCS measurement. Then,

$$V_{\text{confocal}} = \frac{N}{cN_a}$$

Equation 4.24.8

where $N_a = 6.02 \times 10^{23}$ is the Avogadro number.

In particular, this will enable you to calibrate the translational structure parameter (TSM) as:

$$\text{TSM} = \frac{V_{\text{confocal}}}{\pi \omega^2}, \text{where } \omega = \sqrt{4D\tau_D}$$

Equation 4.24.9

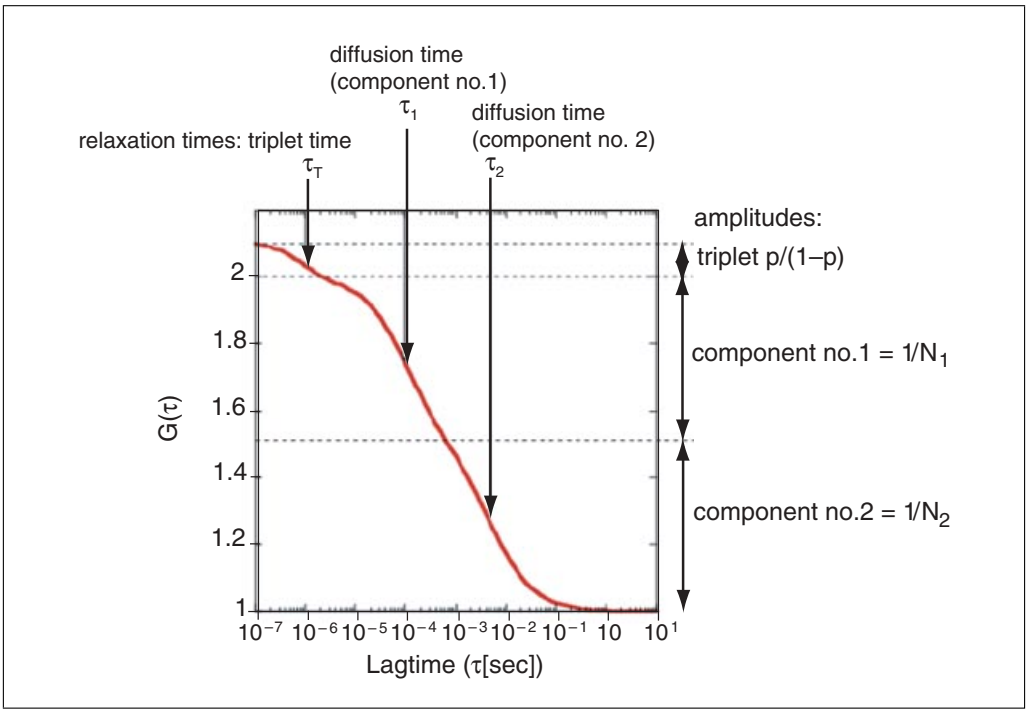


Figure 4.24.3 Typical FCS curve and relevant parameters.

It corresponds to the aspect ratio of the confocal volume, defined as the ratio of its longitudinal direction with the transversal dimension. Typically, this parameter should be set ~10.

COMMENTARY

Background Information

Fluorescence correlation spectroscopy (FCS) is a technique to monitor the relaxation dynamics of a biomolecular system. It relies on fluctuation in the rate of photon emission for fluorescent objects; these fluctuations classically arise from diffusion in and out of a collection volume or via quenching/dequenching of fluorophores upon conformational changes. FCS was originally designed as a spectroscopy technique to monitor kinetic relaxation in bulk solution, e.g., the kinetics of reversible binding of ethidium bromide onto double-stranded DNA (Magde et al., 1972). Since the 1990s and the reliance on confocal microscopy setup to optimize fluorescence collection (Rigler et al., 1993), FCS has become more practical and moved towards more applications in living cells—for a review, see Schwille (2001). The introduction of FCS capabilities onto commercial confocal microscopes was a key step towards making this technique accessible to the cell biology community.

FCS offers many advantages compared to other techniques that have been used to monitor biomolecular events by fluorescence, e.g., fluorescence recovery after photobleaching (FRAP) or fluorescence resonance energy transfer (FRET). FCS in living cells achieves single molecule resolution by minimizing the background fluorescence (confocal setup typically limits the collection volume to a 0.3 × 0.3 × 1–μm^3 volume) and optimizing the photon collection efficiency (the introduction of reliable single-photon avalanche photodiodes was critical to make FCS accessible beyond Optics). FCS is particularly useful for application in living cells, as it yields complex measurements to match molecular complexity: using FRET, one may estimate the complexity of a mixture of fluorophores (e.g., aggregation, stoichiometry, etc.); using FRAP, one could monitor biomolecular dynamics (e.g., estimation of diffusion coefficients, estimation of mobile versus immobile fractions); FCS can achieve all these measurements at once (Hess et al., 2002), and yield additional information (in particular, an absolute estimate of concentrations of fluorescent molecules).

Critical Parameters and Troubleshooting

Optimizing the signal-to-noise ratio of FCS measurements

Optimizing an FCS measurement implies optimizing the actual number of photons collected from individual fluorescent objects. This parameter can be obtained from the count rate/molecule in the LSM 510-ConfoCor software.

Typically, for GFP in living cells, with 1% of the 488-nm lane of a 40-Watt argon laser, and a 70-μm collection pinhole, one should obtain typically 15,000 photons per sec (15 kHz/molecule). Once the experimental system (with a chosen fluorophore) has been configured, only three parameters—the optical alignment, the laser excitation, and the acquisition time—need to be optimized by the experimenter. LSM 510 ConfoCor 3 allows the experimenter to automatically align the acquisition pinhole (in *x* and *y* directions) based on maximization of collected fluorescence. The *z*-alignment is more difficult to optimize but factory settings are usually sufficient. The experimenter should then increase the laser intensity until fluorescence photobleaching becomes prevalent (this can be detected by seeing a non-flat baseline at long lag times, Fig. 4.24.4). Finally, the experimenter should then increase the percentage of laser power to P_b when fluorescence photobleaching becomes prevalent (this can be detected by seeing a non-flat baseline at long lag times, Fig. 4.24.4). The authors usually set back the laser power to $P_b/10$ to minimize photobleaching during actual data acquisition. Finally, the experimenter should optimize the acquisition time to allow sufficient photon collection and achieve statistical significance for the autocorrelation function while minimizing photobleaching in the spot of interest.

Note that the number of photons collected from individual fluorophores may be limited by the saturation of the photoreceptors (theoretically 5 million counts/sec, practically 1 million photons/sec). Thus, there are intrinsic limitations on the concentration of fluorescent objects per confocal volume that is compatible with sufficient photon collection for FCS. Typically, if one would like to collect at a count rate of 5000/sec/molecule, there will be at most 200 molecules in a confocal volume of typically 1fl, and the maximum concentration of fluorescent objects in this experiment would be 0.4 μM. Hence the need to select lower-expressing cells or pre-bleach the samples.

Photobleaching

Photobleaching of the fluorophore under consideration should not be a concern in FCS experiments when relaxation time-scales <50 msec are under consideration (typical for proteins diffusing in the cytoplasm). However, for membrane proteins or for proteins bound to large biological objects (vesicles, mitochondria, etc.), residency time within every confocal volume gets larger than 50 msec, and one should pay special attention to the issue of photobleaching.

In a typical FCS application in living cells, photobleaching appears as a large decay in the autocorrelation function with typical time-scales between 100 msec and 10 sec (Fig. 4.24.4). To maintain the statistical relevance of data fitting, one must make sure that the autocorrelation baseline at long lag times is flat. The rule of thumb is then to obtain at least half a decade of flat baseline at

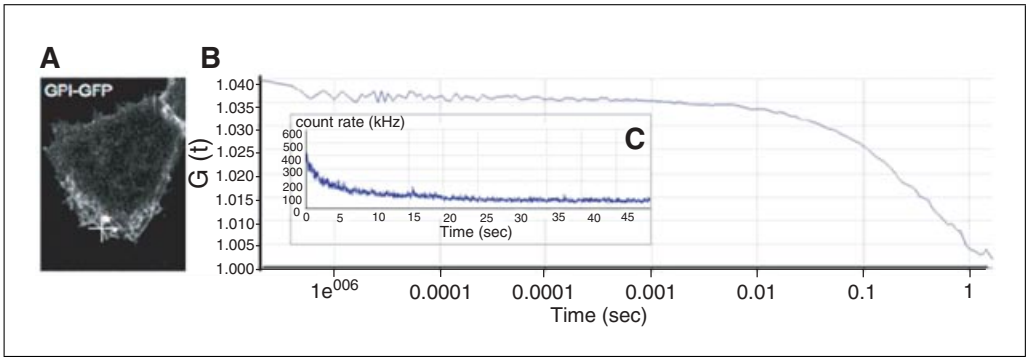


Figure 4.24.4 Autocorrelation on plasma membrane–associated GPI-GFP. Autocorrelation function is collected from in a confocal volume chosen on the plasma membrane, where GFP is anchored via a GPI domain (**A**). Note how the baseline for the autocorrelation function is not flat (between 100 msec and 1 sec) because of photobleaching of the plasma membrane–anchored GFP (**B**). Photobleaching is apparent as an exponential decay in the countrate (Inset **C**).

1.0 to be secure that photobleaching will not be an issue in the analysis of the autocorrelation function. A second rule to experimentally control for photobleaching consists in repeating every FCS measurement with increased laser excitation; if photobleaching is irrelevant, the overall shape of the autocorrelation function should not be affected by the change in laser intensity.

Triplet formation and environmental variation of fluorescence

In some experiments, one would like to gain information about faster fluorescence fluctuations (with time-scales <100 μsec compared to typical diffusion events >100 μsec), in particular, to study GFP dynamics and gain information on pH environment in living cells, or to study small fluorophore diffusion. In that range of time-scales, one must pay attention to potential artifacts due to the fluorophore internal dynamics or detector limitations.

Fluorophore internal dynamics in the 10- to 100-μsec range as measured by FCS can be informative of the biological environment (e.g., monitoring GFP quenching at low pH). On the other hand, triplet formation (a situation where the fluorophore goes from a singlet-excitable state to a triplet-quenched state) occurs with time-scales between 1 μsec and 10 μsec (Fig. 4.24.5). An easy solution to control for potential excitation-induced dynamics is to check the consistency of the FCS measurements for different laser intensities. Note that diffusion time-scales will increase slightly as laser intensity is increased because of an increase of excitation volume.

Triplet formation is a problem encountered mostly with inorganic dyes (e.g., FITC) at high laser intensity. GFP and other naturally fluorescent proteins have very low triplet conversion probability (Widengren and Rigler, 1998). It is not recommended to use the LSM 510-ConfoCor to decipher sub-microsecond fluctuation dynamics as detector after pulsing, because it introduces a highly correlated signal in the autocorrelation curve in that time-range (Krichevsky and Bonnet, 2002).

Aligning confocal and FCS volumes

In the Zeiss ConfoCor 3 as well as the Leica FCS2, scanner mirrors of the confocal microscope are used for precise positioning, ensuring that the correlation spectra are recorded from the exact locations marked on the images scanned. However, it is still good practice to check for any offset between the scanners and the FCS stage positioning, especially if precision is needed <100 nm in x and y. To determine the offset, one can use a macro (available on the Zeiss or Leica instruments) or do it manually with an 'ad hoc' procedure to ensure that the two confocal volumes from which fluorescence is collected are exactly aligned, such that confocal imaging and FCS measurements are in registry. Note that misregistry along the z axis is unlikely as this optical alignment does not vary much from the factory-settings; only x and y alignments need to be performed before every FCS session (see Support Protocol).

To perform offset compensation with a macro, follow instrument manufacturer's instructions.

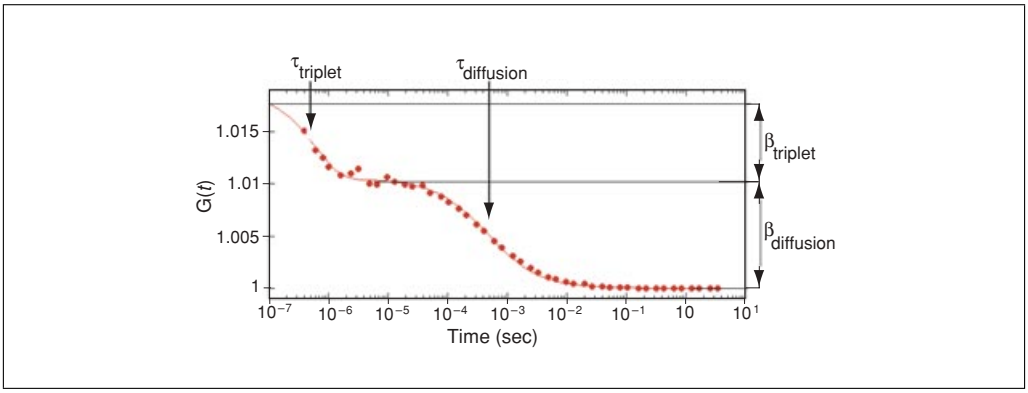


Figure 4.24.5 Autocorrelation function (circle) and associated fit (continuous line) for a solution of FITC-labeled IgG. The fit corresponds to a single component two-dimensional diffusion with a triplet relaxation equation (Equation 4.24.5). The two relaxations can easily be distinguished: the slow one is associated with diffusion ($\tau_{diffusion} = 500$ μsec) and the fast one with triplet relaxation ($\tau_{triplet} = 0.6$ μsec). Their respective amplitudes are $\beta_{diffusion} = 0.01$ (hence there are $1/\beta_{diffusion} = 100$ fluorescent objects per confocal volume) and $\beta_{triplet} = 0.82$ (hence, $p_{triplet} = 0.45$ as $\beta_{triplet} = p_{triplet}/(1 - p_{triplet})$.

Anticipated Results

From millisecond-resolution FCS measurements of fluorescently labeled objects, one can determine how many species of molecules may be diffusing through the confocal volume; measure the diffusion coefficient of the molecules; measure the actual number of molecules diffusing within the confocal volume; and from there calculate the total number of molecules within the cell. One can also determine the fraction of triplet state molecules within the fluorescing population. The measurements of the fluorescence per object can yield an estimate of the number of aggregated molecules, if the fluorescence per molecule has been properly calibrated. For cytosolic soluble GFP, a typical diffusion time scale is ~100 to 250 μsec, triplet state fraction is ~10% to 20%, counts per molecule is ~10 kHz.

Time Considerations

From turning on the instrument to aligning and calibrating the instrument with dye molecules until it is ready for recording correlation spectra from experimental samples takes 30 min up to 2 hr. Once the instrument is aligned, actual recordings from sites within a cell can take from a few seconds to a few minutes, depending on the number of sites and the number of recordings obtained from each site. Data analysis can take several hours or more depending on the experimenter's knowledge of statistics.

Literature Cited

Altan-Bonnet, G., Libchaber, A., and Krichevsky, O. 2003. Bubble dynamics in double-stranded DNA. *Phys. Rev. Lett.* 90: 138101.

Altan-Bonnet, N., Sougrat, R., Liu, W., Snapp, E.L., Ward, T., and Lippincott-Schwartz, J. 2006. Golgi inheritance in mammalian cells is mediated through endoplasmic reticulum export activities. *Mol. Biol. Cell* 17:990-1005.

Bacia, K. and Schwille, P. 2003. A dynamic view of cellular processes by in vivo fluorescence auto- and cross-correlation spectroscopy. *Methods* 29:74-85.

Bonnet, G., Krichevsky, O., and Libchaber, A. 1998. Kinetics of conformational fluctuations in DNA hairpin-loops. *Proc. Natl. Acad. Sci. U.S.A.* 95:8602-8606.

Carl Zeiss Microimaging, 2006. ConfoCor 3 Operating Manual, Release 4.0.

Chen, Y., Müller, J.D., So, P.T., and Gratton, E. 1999. The photon counting histogram in fluorescence fluctuation spectroscopy. *Biophys. J.* 77:553-567.

Chen, Y., Müller, J.D., Ruan, Q., and Gratton, E. 2002. Molecular brightness characterization of EGFP in vivo by fluorescence fluctuation spectroscopy. *Biophys. J.* 82:133-144.

Gosch, M., Blom, H., Holm, J., Heino, T., and Rigler, R. 2000. Hydrodynamic flow profiling in microchannel structures by single molecule fluorescence correlation spectroscopy. *Anal. Chem.* 72:3260-3265.

Haupts, U., Maiti, S., Schwille, P., and Webb, W.W. 1998. Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* 95:13573-13578.

Hess, S.T., Huang, S., Heikal, A.A., and Webb, W.W. 2002. Biological and chemical applications of fluorescence correlation spectroscopy: A review. *Biochemistry* 41:697-705.

Koppel, D.E. 1974. Statistical accuracy in fluorescence correlation spectroscopy. *Phys. Rev. A* 10:1938-1945.

Krichevsky, O. and Bonnet, G. 2002. Fluorescence correlation spectroscopy: The technique and its applications. *Rep. Progr. Physics* 65:251-297.

Magde, D., Elson, E., and Webb, W.W. 1972. Thermodynamic fluctuations in a reacting system—Measurement by fluorescence correlation spectroscopy. *Phys. Rev. Lett.* 29:705-708.

Rabut, G. and Ellenberg, J. 2004. Automatic real-time three-dimensional cell tracking by fluorescence microscopy. *J. Microsc.* 216:131-137.

Rigler, R., Mets, Ü., Widengren, J., and Kask, P. 1993. Fluorescence correlation spectroscopy with high count rate and low background: Analysis of translational diffusion. *Eur. J. Biophys.* 22:169-175.

Schwille, P. 2001. Fluorescence correlation spectroscopy and its potential for intracellular applications. *Cell Biochem. Biophys.* 34:383-408.

Wachsmuth, M., Waldeck, W., and Langowski, J. 2000. Anomalous diffusion of fluorescent probes inside living cell nuclei investigated by spatially-resolved fluorescence correlation spectroscopy. *J. Mol. Biol.* 298:677-689.

Weiss, M., Hashimoto, H., and Nilsson, T. 2003. Anomalous protein diffusion in living cells as seen by fluorescence correlation spectroscopy. *Biophys. J.* 84:4043-4052.

Widengren, J. and Rigler, R. 1998. Fluorescence correlation spectroscopy as a tool to investigate chemical reactions in solutions and on cell surfaces. *Cell Mol. Biol.* 44:857-879.

Analysis of Mitochondrial Dynamics and Functions Using Imaging Approaches

Kasturi Mitra[1] and Jennifer Lippincott-Schwartz[1]

[1]Cell Biology and Metabolism Program (CBMP), National Institute of Child Health and Human Development (NICHD), NIH, Bethesda, Maryland

ABSTRACT

Mitochondria are organelles that have been primarily known as the powerhouse of the cell. However, recent advances in the field have revealed that mitochondria are also involved in many other cellular activities like lipid modifications, redox balance, calcium balance, and even controlled cell death. These multifunctional organelles are motile and highly dynamic in shapes and forms; the dynamism is brought about by the mitochondria's ability to undergo fission and fusion with each other. Therefore, it is very important to be able to image mitochondrial shape changes to relate to the variety of cellular functions these organelles have to accomplish. The protocols described here will enable researchers to perform steady-state and time-lapse imaging of mitochondria in live cells by using confocal microscopy. High-resolution three-dimensional imaging of mitochondria will not only be helpful in understanding mitochondrial structure in detail but it also could be used to analyze their structural relationships with other organelles in the cell. FRAP (fluorescence recovery after photobleaching) studies can be performed to understand mitochondrial dynamics or dynamics of any mitochondrial molecule within the organelle. The microirradiation assay can be performed to study functional continuity between mitochondria. A protocol for measuring mitochondrial potential has also been included in this unit. In conclusion, the protocols described here will aid the understanding of mitochondrial structure-function relationship. *Curr. Protoc. Cell Biol.* 46:4.25.1-4.25.21.

Keywords: mitochondria • imaging • live cell

INTRODUCTION

Mitochondria are double membrane-bound organelles harboring their own DNA; they have been classically thought to be the powerhouse of the cell. Recent studies of mitochondria in the cellular context have revealed several other functional contributions of the organelles, including redox homeostasis, lipid modification, calcium homeostasis, and cell death processes (Scheffler, 2001). The inner membrane of mitochondria maintains a transmembrane gradient of ions that is known as mitochondrial potential. Measuring mitochondrial potential, which drives mitochondrial ATP synthesis (Nicholls and Budd, 2000), can assess mitochondrial function and integrity. Although early studies with isolated mitochondria have elaborately described mitochondrial properties, today's challenge is to understand the widespread roles of mitochondria and their interplay with other intracellular organelles. Live cell imaging of mitochondria, more than a decade ago, revealed these organelles are highly dynamic. Since then many researchers have focused on understanding the dynamism of these organelles (Detmer and Chan, 2007), as this property might be coupled to how mitochondria perform their many functions inside the cells. Therefore, techniques for imaging mitochondria are continually being improved to help further understand the structure/function relationship of these dynamic organelles.

Mitochondria are distributed throughout the cell and they exist in different sizes and forms that range from small granular to highly filamentous shapes. Although lack of

Current Protocols in Cell Biology 4.25.1-4.25.21, March 2010
Published online March 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0425s46

mitochondrial dynamism is deleterious to cells, it is not understood how changes in mitochondrial morphology bring about change in mitochondrial function (reviewed in Detmer and Chan, 2007). The dynamics of mitochondrial forms within cells is controlled by specific molecules involved in mitochondrial fission and fusion, as well as those controlling the cytoskeletal structure, most importantly microtubules (De Vos et al., 2005). The steady-state fission-fusion events of mitochondria together with the motility of individual mitochondria pose serious challenges for high-resolution live cell imaging of mitochondria; this challenge has only been recently met with the advent of confocal microscopes with high-speed acquisition. This unit describes fluorescence-based confocal imaging approaches to study mitochondrial dynamics and function in live mammalian cells.

STRATEGIC PLANNING

Live cell imaging of mitochondria can be performed by tagging resident mitochondrial proteins with fluorescent proteins or by adding specific mitochondrial targeting sequences to fluorescent proteins (Okamoto et al., 2001). Commercially available dyes can also be used, although with limitations (Chen, 1988). Depending on the researcher's interest, there are various approaches for imaging mitochondria. For example, high-resolution three-dimensional imaging can provide details about mitochondrial structures and their association with organelles like the endoplasmic reticulum. Time-lapse imaging techniques allow aspects of mitochondrial dynamics to be studied quantitatively and qualitatively. It can also be used to visualize exchange of molecules, like mitochondrial DNA, between mitochondria undergoing fission and fusion events (Detmer and Chan, 2007). The physical and electrical continuity between mitochondria undergoing fission and fusion can also be studied using live cell imaging approaches employing FRAP and microirradiation protocols. Finally, mitochondrial potential can be quantified on a single cell or single mitochondrion based on incorporation of specific fluorescent dyes.

As mitochondria are an integral part of the cell death signaling machinery, their morphology is highly sensitive to the status of cellular health. A great deal of caution, therefore, is required to avoid causing any perturbations to this organelle during imaging. The points discussed below should always be considered when preparing samples for imaging mitochondria in order to optimize imaging conditions and to avoid artifacts. The protocols described here are optimized for adherent cells with 75% confluency. However, they can be modified as per the requirements for suspended cells.

Sample Preparation

1. When working with mitochondrial markers tagged to fluorescent proteins (FP), it is recommended to first make a stable cell line expressing the molecule of interest. This is because transient expression itself is a stress to cells, which might affect mitochondrial morphology. For example, we have found that the DsRed molecule targeted to the mitochondrial matrix causes mitochondrial fragmentation after 48 hr of transient transfection. For primary cultures where markers cannot be stably expressed, transient expression systems can be used to express molecules at low levels for short period of time. One can also choose to use fluorescent dyes to stain mitochondria for short-term experiments.

In the case of overexpression systems, transient or stable, precautions have to be taken to ensure that expression of the fluorescent molecule of interest is not altering mitochondrial morphology. This can be done by comparing mitochondrial morphology in the stable line and its parental line. The steps include: (1) staining the parental line (that is not expressing the mitochondrial FP) with a fluorescent mitochondrial dye (Mitotracker green etc.); (2) counting cells according to mitochondrial morphology as fragmented/intermediate/tubular (Karbowski et al., 2006); (3) counting cells according to

mitochondrial morphology as fragmented/ intermediate/tubular in the stable line expressing mitochondrial fluorescent protein; (4) comparing the distribution of mitochondrial morphology of the parental line to that of the cell population expressing the fluorescent mitochondrial molecule.

2. The medium used for imaging should always be pre-incubated in a 37°C incubator with 5% CO_2 for 30 to 60 min.

3. The pre-incubated medium should be used to replace the medium in which cells were grown in the imaging chambers. This has to be done immediately after the plated cells have been brought out of the incubator. Next, the medium in the imaging chamber has to be carefully overlayed with mineral oil that would form a meniscus on the medium. The level of the medium has to be high enough so that the oil meniscus is not close to the bottom of the chamber where cells are plated, which might kill the cells. The actual purpose of the mineral oil is to prevent CO_2 from escaping the medium, which would change the pH of the medium. CO_2-independent medium can be used to avoid use of mineral oil. However, this is not recommended for experiments demanding more than 1 hr. In this time, evaporation of water from the medium would concentrate the components of the medium, which in turn might affect mitochondrial morphology and function.

4. The stage warmer should be preset 30 to 45 min before commencement of the experiment. A properly calibrated temperature probe is mandatory. The calibration of the temperature probe should be checked by dipping the probe in two water baths maintained at different temperatures. This ensures that the monitored temperature at the stage is correct. To monitor the stage temperature, the temperature probe should be put at the top of the objective (but not touching it), exactly at the point where the specimen in the stage holder to be imaged will be held during imaging.

Choice of Mitochondrial Marker

As mitochondria are double membrane-bounded organelles, one needs to have knowledge about the localization of the molecule/dye of interest in the mitochondria for proper interpretation of imaging results. At the resolution of light microscopy, one cannot distinguish between the outer and inner membranes but can distinguish between the (outer/inner) membranes and the matrix. Therefore, the final outcome of experiments might differ with different choices of molecules to image mitochondria.

Although high-resolution *z* stacks can be acquired by using fluorescent dyes, time-lapse imaging for assessment of mitochondrial dynamics could be erroneous when performed with fluorescent dyes. This is because dye addition has the possibility of affecting mitochondrial behavior. The best and reproducible result is obtained by using fluorescent mitochondrial markers like mitoRFP/mitoGFP/mitoYFP. Use fluorescent molecules with the excitation peak at longer wavelengths to avoid the possibility of mitochondrial damage from more energetic shorter wavelengths. Only the stable lines expressing mitochondrial markers can be imaged for very long time periods, even more than 24 hr. By contrast, experiments using fluorescent dyes need to be performed within 30 to 45 min, after which the mitochondria usually fragment in response to the foreign chemical.

For photobleaching experiments, the mitochondrial fluorescent tag should be chosen such that it is bright and photostable under low illumination but photobleaches fast and almost irreversibly. The irreversibility of photo bleaching is very important because if the bleached molecule reverts to its fluorescent state it will contribute to the recovery. This would lead to erroneous conclusion about the physical mitochondrial process under investigation. This can be checked by performing the experiment, with the exact same configuration as chosen, but in a fixed sample. If the reversion of the bleached molecule is minimal there should be no recovery in the fixed sample.

BASIC PROTOCOL 1

HIGH-RESOLUTION *z*-STACK AND TIME-LAPSE IMAGING OF MITOCHONDRIA

Analyzing a *z*-stack across the depth of the cell provides steady-state three-dimensional information about mitochondrial morphology while time-lapse imaging allows changes in mitochondrial morphology (dynamics) to be studied over time. Figure 4.25.1 depicts a representation of a projection of *z*-stacks of mitochondria in a single cell.

Materials

Stably transfected cells expressing fluorescent-tagged molecule of interest *or* mitochondrial fluorescent dyes of interest
Chosen dye (see the Support Protocol)
Immersion oil

Labtek or Matek chambers
CO_2 incubator at 37°C
Microscope stage heater and temperature probe (e.g., Oka-lab)
Confocal laser scanning microscope, like Zeiss LSM510 or similar, with appropriate laser and filter sets required for imaging
63× Plan-Neofluar oil objective with high NA (1.4)

1. Plate stably transfected cells in Labtek/Matek chambers at least 10 to 16 hr before imaging.
2. Stain the cells with the chosen dye, if required (see the Support Protocol).
3. Prepare the sample (see Sample Preparation in Strategic Planning).
4. Preheat the microscope stage to 37°C. Turn on the appropriate laser at least 15 min prior to the experiment to give it time to stabilize.
5. Put a drop of immersion oil on the objective and fix the chamber to be used to the stage holder properly.

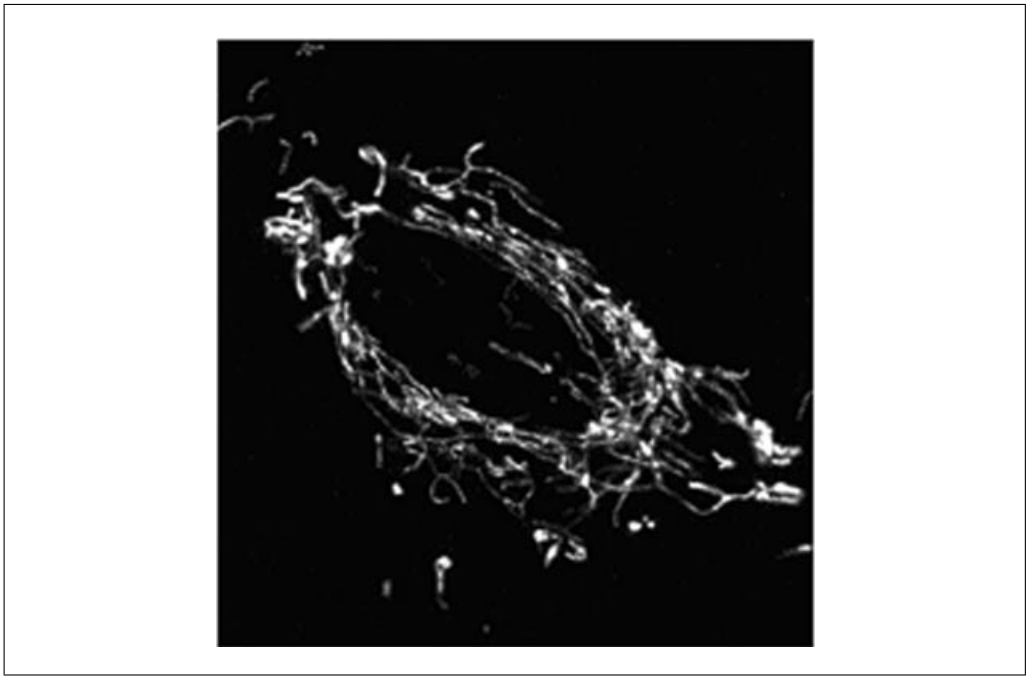


Figure 4.25.1 High-resolution image of mitochondria in a stable fibroblast cell line expressing mitoRFP. The image is a projection of *z*-stacks of mitochondria. A 543-nm HeNe laser was used for imaging and *z*-stacks were acquired following Basic Protocol 1. Imaging settings are listed in Table 4.25.1. The raw image has been converted into gray scale.

Table 4.25.1 Scanning Parameters for Acquiring High-Resolution *z*-Stacks of Mitochondria

Parameter	Value
Zoom[a]	2.5-3
Pixel size	1024 × 1024
Scan speed	7
Scan number for averaging	2
Pinhole	1 airy unit
Laser intensity	1%-20%
Detector gain	600

[a]High-resolution imaging of mitochondria would limit the investigator to scan one cell at a time, or part of a cell or even individual mitochondria. To image a whole cell the zoom factor (digital magnification) will have to be decided based on the size of the cell. A zoom factor of more than 5 is not recommended as this would cause the image to be pixilated, but this value can be used, if absolutely necessary, by adjusting other parameters.

6. Choose the appropriate configuration for the fluorophore to be imaged.

   To be able to do so one needs prior knowledge of the excitation and emission properties of the fluorophore (UNIT 21.5)

7. Choose a cell to be imaged by using the FAST scan button in minimum zoom.

   One can also quickly select a cell by looking through the eyepiece of the microscope with the proper filter. Selecting a cell, if not accomplished within few seconds, might cause bleaching of the fluorophore or photodamage to the mitochondria.

8. Adjust the laser intensity and detector gain according to the brightness of the sample in such a way that the signal from any pixel is not saturated (as checked in the palette in the LSM 510 systems).

   For quantitative imaging, this is essential, but for qualitative purposes some pixels may be allowed to cross the threshold to be able to detect other very faint pixels. This is because the fluorescent signal might not be distributed to all the mitochondria homogeneously, especially in certain conditions where normal mitochondrial morphology is perturbed.

9. Set the scanning parameters including zoom, pixel size, scan speed, scan number for averaging, pinhole, laser intensity, detector gain, etc. and scan a single section.

   Table 4.25.1 provides values for these parameters for proper imaging of mitochondria of a whole cell. If necessary, the parameters can be changed to yield better results.

10. If satisfied with the image, check for minimal bleaching.

    This is very important for time-lapse imaging. With laser power and scanning parameters being fixed, bleaching would depend on:

    (1) Scanning frequency: As mitochondrial movement per second is below the resolution limit of light microscope (as measured in neurons; De Vos et al., 2003), an imaging speed of 1 frame per second serves the purpose. One can further reduce the speed to 0.2 frames per second or 1 frame every 5 sec.

    (2) Number of scans: The total number of scans in a time series would depend on the duration of the experiment. For minimal bleaching, it is recommended to give more time between scans when intended to perform a longer time series experiment.

    The scanning parameters can be altered to reduce bleaching by enhancing scan speed and reducing averaging. Doing so will compromise the resolution. Therefore, a degree of trade

off between resolution and dynamics of mitochondrial structure has to be considered; the choice will be dependent on the goal of the experiment being performed.

Bleaching could arise during acquisition of z-stacks. This can be assessed by performing a time series where the time interval will be the same as the scan time of a single z-section. If there is no reduction in signal in this time, the parameters are suitable for z-sectioning. If not, the laser intensity and detector gain have to be adjusted according to the brightness of the sample.

11. Acquire the time series or *z*-stacks and save images.

    For acquiring overlapping z-stacks, the size of the optical slice should be twice than that of the z-interval, while for non-overlapping z-stacks, the optical slice should be close to that of the z-interval. For example, if pinhole size allows light from a 1-μm optical slice, the z-interval should be 0.5 μm for overlapping z-stacks and 1 μm for non-overlapping z-stacks. Oversampling might scan the same portion of the image more than once while undersampling might miss scanning portions. This could introduce errors in quantitative measurements like mitochondrial size, especially in case of tubular mitochondria.

    For long-term time series experiments, one should be aware of the drift in focus (due to thermal or other fluctuations) in the imaging setup and include autofocusing modules, like Multitime (Zeiss) for correcting the drift.

ALTERNATE PROTOCOL

IMAGING MITOCHONDRIAL MORPHOLOGY ALTERATIONS

The morphology of the mitochondria is influenced directly and indirectly by many factors. For example, nearly all the agents causing mitochondria-dependent cell death have been found to cause mitochondrial fragmentation (Karbowski and Youle, 2003). Recent studies are also being focused on identifying drugs that would directly impact the mitochondrial fission-fusion machinery and alter mitochondrial morphology (Cassidy-Stone et al., 2008). To image the effect of drugs or other chemicals on mitochondrial morphology/function, include the following steps for modifying acquisition of time series as mentioned in Basic Protocol 1. An example is illustrated in Figure 4.25.2, where time-lapse images have been acquired immediately after addition of microtubule depolarizing drug, nocodazole (5 μg/ml) to investigate the change in mitochondrial morphology after disrupting microtubules. Note the alteration in mitochondrial morphology from time 0 (before addition) to 30 min after addition.

Additional Materials *(also see Basic Protocol 1)*

Drug of choice (e.g., 5 μg/ml nocodazole)

1. Prepare the sample as in Basic Protocol 1 (or as in the Sample Preparation section of Strategic Planning), but do not overlay the sample medium with mineral oil.
2. Acquire 2 to 5 images of the chosen field.
3. Stop scanning and carefully remove the lid of the chamber.
4. Carefully aspirate 50% of the medium from the chamber using a pipet.
5. Add a volume equal to the volume removed of a 2× concentration of the drug to the chamber.

   The drug is added at twice the working concentration for the drug.

6. Carefully overlay with mineral oil.
7. Put the lid of the chamber back and quickly resume imaging.

   Care should be taken not to alter the plane of focus during the procedure of addition of drug. If the focus appears to be shifted, one has to quickly reset the focus keeping the last scanned image of the field as standard. The time period between stopping and resuming scanning has to be taken into account during the final analysis of the time series.

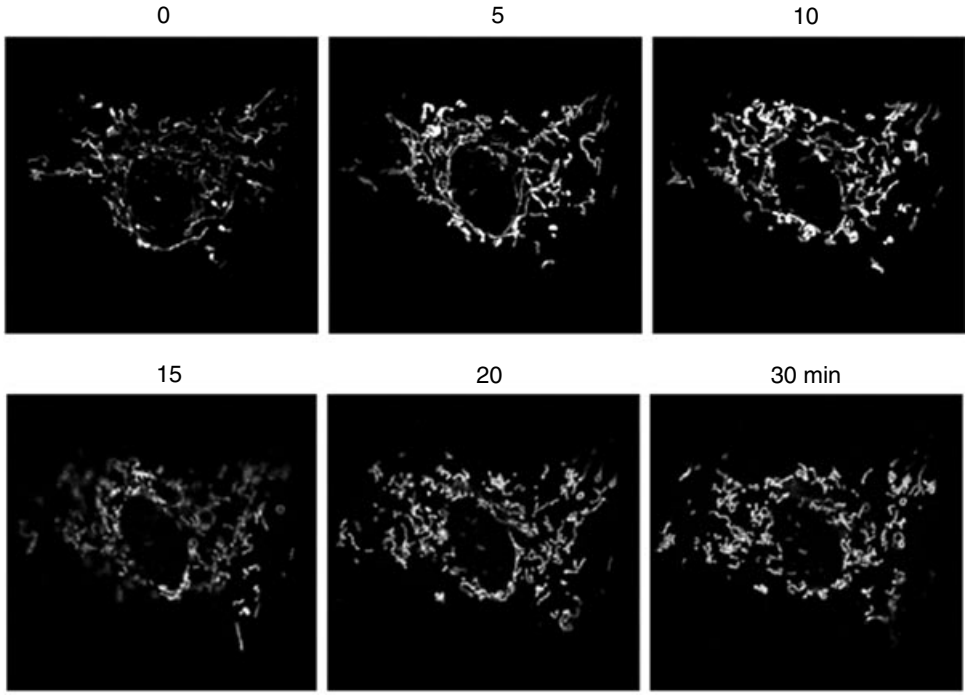


Figure 4.25.2 Time series of mitochondria after addition of nocodazole to depolymerize microtubules. Experiment was performed in a fibroblast line stably expressing mitoRFP. Time-lapse images were acquired following Basic Protocol 1 and the Alternate Protocol. The numbers represent time (in min) after addition of nocodazole. A 543-nm HeNe laser was used. The raw image has been converted into gray scale. Images were acquired using a 63× objective in zoom 2. Pinhole size was 2.5 airy units.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING ON MITOCHONDRIA

BASIC PROTOCOL 2

Fluorescence recovery after photobleaching (FRAP) is a powerful biophysical method that is used to understand the dynamics of molecules or structures in live cells. This method involves photobleaching of fluorescent molecules within a certain area of a cell and then monitoring recovery of fluorescence in the bleached zone by movement of molecules/organellar structures from the surrounding unbleached zone. For more detailed discussion of the theory of FRAP, please see *UNIT 21.1*. This experiment involves time-lapse imaging as it depends on the kinetics of mitochondria per se or that of a mitochondrial protein. Three types of information related to mitochondrial dynamics can be obtained from FRAP experiments depending on the time scale of recovery: (1) mobility of the fluorescent molecule—the time scale of recovery is in milliseconds and the recovery kinetics is a measure of diffusion of the fluorescent molecule of interest; (2) mitochondrial continuity: the time scale of recovery is from few seconds to 2 to 3 min depending on the area of bleach; this gives a measure of continuity of the mitochondrial compartment where the fluorescent molecule resides (please refer to Mitra et. al., 2009 for a specific example); (3) mitochondrial movement/dynamics (fission-fusion): the time scale is between 2 to 3 min to ∼1 hr; this recovery is due to the combination of mitochondrial motility and fission-fusion dynamics. The time scale of the FRAP experiment has to be chosen depending on the investigator's goal. The protocol here uses a microscope with faster scanning speed for measuring molecular mobility. Since the protocols are the same for all three options, they are mentioned under the same heading.

Materials

Confocal laser scanning microscope, like Zeiss LSM510 or similar, with appropriate laser and filter sets
63× Plan-Neofluar oil objective with high NA (1.4) is best for imaging mitochondria in tissue culture cells
Microscope stage heater and temperature probe

1. Repeat steps 1 to 7 from Basic Protocol 1.
2. Set the scanning parameters including mode, zoom, pixel size, scan speed, scan number for averaging, pinhole, laser intensity, detector gain, etc. Verify the set of parameters by doing a single scan each time after adjusting a parameter. Table 4.25.2 provides FRAP parameters for all the three options.

   Settings may be altered to improve imaging depending on the system.

3. *Define region of interest (ROI):* Bleaching will be performed and recovery will be followed in the specified ROI.

   Two important criteria about selection of an ROI should be considered.

   Size: One should have an expectation regarding the source of recovery of fluorescence (for all options) in the ROI before setting this parameter. The size of the ROI should not be more than 30% to 50% of the total signal area, so that the recovery of fluorescence in a given time from the unbleached zone (70% to 50%) can be appreciably detected. The time to reach maximal recovery by movement of molecule inside mitochondria (molecular mobility, mitochondrial continuity) or that of mitochondria themselves (mitochondrial mobility/dynamics) would depend on the available unbleached fluorescence that would contribute to the recovery. The ROI size would be dependent on which option of the FRAP one chooses to do. For molecular mobility, the ROI should be only around 0.5 × 0.5 μm; just enough to cover a small zone on an individual mitochondria (Table 4.25.2,

Table 4.25.2 Scanning Parameters for Performing FRAP Analysis on Mitochondria

Parameter	Molecular mobility	Mitochondrial continuity	Mitochondrial mobility/dynamics
Objective	63×	63×	63×
Zoom	1	2	2-3
Pixel size	512 × 80	512 × 512	512 × 512
Scan speed	9	8	7-8
Scan number for averaging	1	1	1
Pinhole[a]	Open	Open	Open
Laser intensity	0.5%-10%	0.5%-10%	0.5%-10%
Detector gain	600	600	600
Size of ROI	0.5 × 0.5 μm	Variable[b]	Variable[b]
Time interval	5 msec	1 sec	5 min
Bleaching time	7 μsec	>1 msec <1 sec	>1 msec <1 sec
Bleach iterations	2	5	5
Time-lapse imaging duration	500 msec	1-5 min	30 min to 1 hr

[a]The pinhole should be kept open to be able to collect light from the whole depth of the cell. If the pinhole were narrow, then movement of mitochondria in and out of the optical plane would introduce fluctuations in the reading. However, an open pinhole would compromise the resolution to a certain extent. Reduction of laser intensity can sometimes help to avoid out-of-focus signal, which, however, would depend on organization of mitochondria in the cell of interest.
[b]Refer to the text on ROIs.

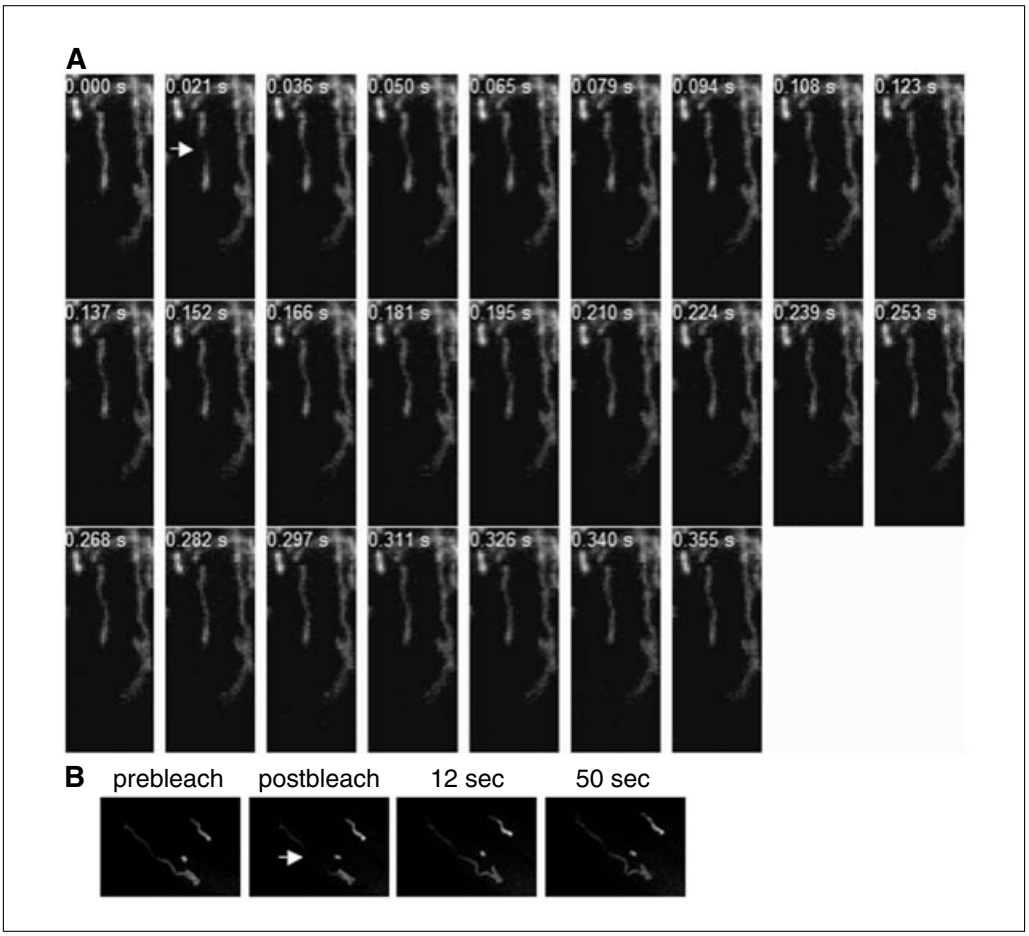


Figure 4.25.3 Fluorescence recovery after photobleaching on single mitochondria. Experiment was performed in a fibroblast line stably expressing mitoRFP following Basic Protocol 2. The time scale of recovery is in milliseconds. The arrow points to the bleached zone. Another mitochondrion in the field of view gives an assessment of overall bleaching during the recovery period. High-speed laser scanning confocal microscope (Zeiss LSM5 Duo) was used in **A** while a similar but slower microscope (Zeiss 510) was used in **B**. Prebleach and postbleach images depict mitochondria before and after the single bleaching pulse, respectively. A 543-nm HeNe laser line was used for imaging and other imaging settings used are mentioned in Table 4.25.2.

Fig. 4.25.4). For mitochondrial continuity and mobility/dynamics, one can decide to have the ROI spanning 5% to 2% of the cell either across the cell or locally (Fig. 4.25.3).

Placement: For continuity and mobility/dynamics measurements, the ROI should be placed in a region where the mitochondrial population is roughly uniformly distributed. In all the options, the ROI should be placed close to the center of the area of the total distributed signal. This means close to the center of the cell for continuity and dynamic measurements (Fig. 4.25.4) and the center of the mitochondrion of interest for molecular mobility studies (Fig. 4.25.3). This allows for movement of the fluorescence from all directions of the surrounding unbleached zone into the bleached zone. Wrong placement of the ROI, for example ROI placed to one side, might introduce errors in analyses and interpretation of the results.

4. *Standardize bleaching conditions:* Photobleaching is achieved by using maximum power of the of the same laser that is used for scanning. If the fluorescent molecule of choice is RFP, then it is recommended to use 458-, 488-, and 514-nm Argon lasers in addition to the less powerful 543-nm HeNe laser. As the RFP excitation spectrum with the peak at 543 nm also includes these wavelengths, the other lasers will enhance bleaching. For powerful 543-nm lasers, this might not be necessary.

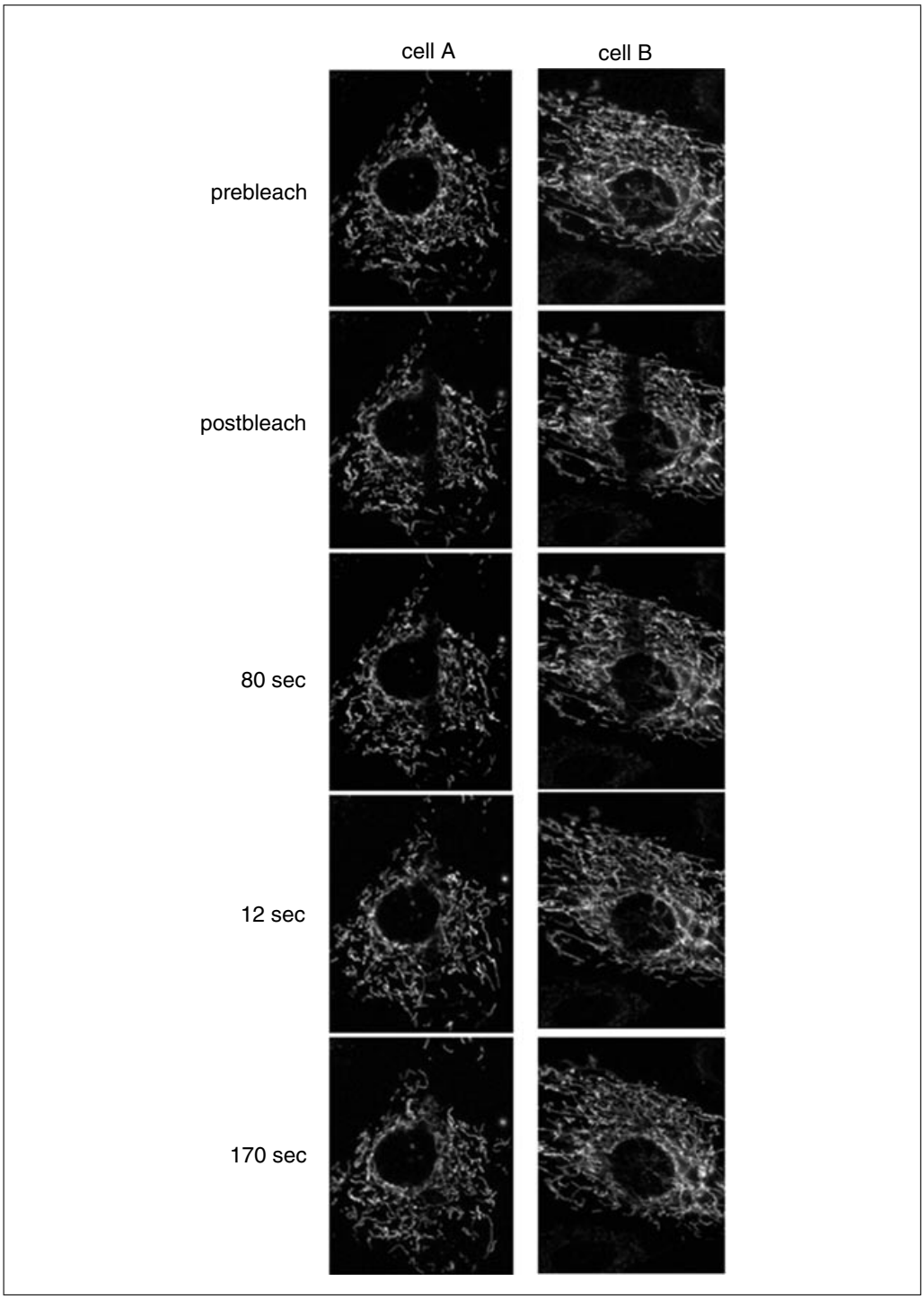


Figure 4.25.4 Fluorescence recovery after photobleaching on a mitochondrial population. Experiment was performed in a fibroblast line stably expressing mitoRFP following Basic Protocol 2. Prebleach and postbleach images depict mitochondria before and after the single bleaching pulse, respectively. The numbers represent time after post bleach in seconds. Two cells, A and B, have been shown to compare different recovery kinetics. A 543-nm HeNe laser line was used for imaging and other imaging settings used is mentioned in Table 4.25.2.

In continuity and mobility/dynamics measurements, bleaching should be as fast and as complete as possible; this can be increased by increasing the number of bleaching iterations; for molecular mobility measurements where the recovery kinetics is in milliseconds, bleaching should be faster and restricted to 20% to 40% of the initial fluorescence intensity. However, one should consider a trade off between the number of bleaching iterations and bleaching time. Bleaching for longer than necessary could damage mitochondria.

5. *Set the time interval and total imaging time:* These parameters depend on the option chosen.

   Scanning in short intervals would necessitate faster scanning. This may be achieved by restricting the scanning area to the zone of interest (Fig. 4.25.3A).

6. Acquire and save the time series to be analyzed when required.

   The quantitative analysis can be performed according to Goodwin and Kenworthy (2005). The number of recovery curves required for statistically significant analyses should be between 10 to 20. The data may be used to calculate half-time of recovery (t1/2), mobile and immobile fractions of the fluorescent molecule (see UNIT 21.1), diffusion coefficients (Partikian et al., 1998), and continuity parameters (Karbowski et al., 2006). An example of analysis of FRAP data has been provided in Figures 4.25.4 and 4.25.5. The figures depict analyses of mitochondrial connectivity in each of the two cells; bleaching was performed in the long ROI and the recovery in the small ROIs in each cell is compared to the recovery in the long ROI of the same cell. In cell A, individual ROIs appear to have different recovery kinetics while in cell B, individual ROIs have similar kinetics.

   The analysis will include correction for overall photobleaching that might have occurred during acquisition of the time series and total fluorescence that could contribute to the recovery. As mitochondria usually form small or large networks sharing their contents and continuously rearrange the branching points of the networks, it is very difficult to

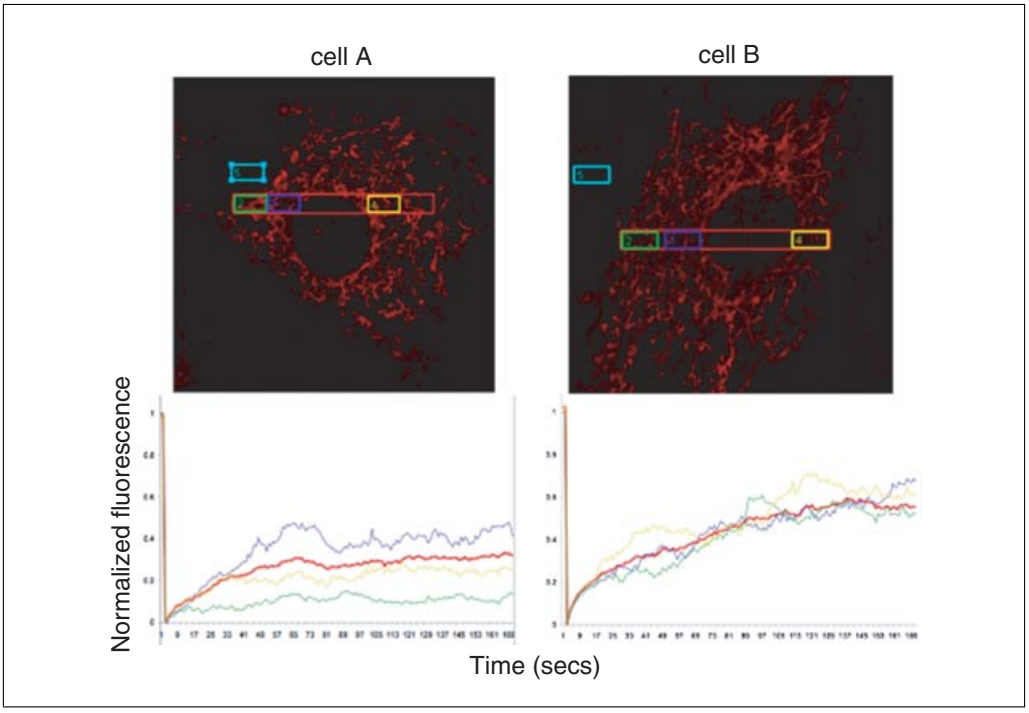


Figure 4.25.5 Analysis of connectivity by FRAP on a mitochondrial population. Images of cell A/B of Figure 4.25.4 were analyzed according to Goodwin and Kenworthy (2005). Different ROIs are color-coded corresponding to the associated graph showing recovery kinetics. The signal in the blue ROI was used to subtract the background signal. Signal was bleached in the red ROIs and recovery was monitored in all the ROIs. The analyses here do not include correction for bleaching and are normalized by the initial fluorescence. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0425*.

have a knowledge of the total source of unbleached fluorescence that could contribute to the recovery. Depending on the analysis performed, the total source of unbleached fluorescence factor might be considered constant or ignored. For example in molecular mobility, if the individual mitochondrion appears to form a part of the network, then it is recommended not to consider the factor. However, it is recommended that this kind of an analysis be performed on mitochondria near the edge of the cell where there will very few separated mitochondrial elements (Fig. 4.25.3B). In the example of connectivity analysis provided in Figure 4.25.5, the normalization by the total fluorescence has been ignored. In this case, the alternative is to consider the total mitochondrial fluorescence as the source of recovery (which may not be the case in reality).

FRAP on mitochondria to analyze mitochondrial connectivity or diffusion of mitochondrial molecule is complicated by the motility of the organelles. This problem cannot be avoided, but some verification has to be done before proceeding for image analyses. Careful examination of the time series images is required for any of the three possible errors that could have been introduced during image acquisition: (1) sudden appearance of one or more motile mitochondria in the ROI considered; (2) mitochondria shifting or moving away, thus making the signal in the ROI unrepresentative of the experimental result for molecular mobility measurement; (3) the whole cell of interest moving during acquiring the time series for continuity and mobility/dynamics measurements. Any time series having one or more of these errors have to be discarded from the analyses.

BASIC PROTOCOL 3

MICROIRRADIATION ASSAY TO ASSESS ELECTRICAL CONTINUITY IN MITOCHONDRIA

Knowledge of mitochondrial biochemistry and energetic properties has been used to design fluorescent dyes (commercially available from Molecular Probes) to stain mitochondria in live cells (*UNIT 4.4* and Support Protocol 1 covers staining of mitochondria with these dyes). The fluorescent derivatives of certain cationic lipophilic compounds (i.e., TMRE) concentrate inside the mitochondria that maintain inner membrane potential. Therefore, uptake of TMRE can be used to quantitatively determine mitochondrial transmembrane potential. TMRE-loaded mitochondria are highly sensitive to laser scanning, which is reflected in the spontaneous depolarization that can happen when imaging TMRE loaded mitochondria in cells (Bunting, 1992). This phenomenon can be manipulated in a controlled fashion using a high-intensity laser to irradiate and thus depolarize TMRE-loaded mitochondria, resulting in immediate loss of TMRE from the irradiated mitochondria (Amchenkova et al., 1988). Directed irradiation in an ROI can cause controlled depolarization very locally in an individual mitochondrion in a cell. This local depolarization will rapidly spread from the irradiated point to mitochondrial elements in electrical continuity to the irradiated point (Fig. 4.25.6). Therefore, one can assess the extent of electrical continuity of mitochondria in an individual by micro-irradiating one point in a mitochondrion and monitoring, by time-lapse imaging, the spread of depolarization to other electrically continuous mitochondria.

Materials

Microscope stage heater and temperature probe
Laser scanning confocal microscope with (1) a 543-nm laser line and appropriate filter sets for imaging rhodamine and (2) a 2-photon chameleon laser
63× plan neofluar objective
Power meter (from Coherent)

Additional reagents and equipment for staining with TMRE (Support Protocol)

1. Preheat the microscope stage to 37°C and turn on the 543-nm HeNe laser and the 2-photon chameleon laser.
2. Stain with TMRE as in the Support Protocol.

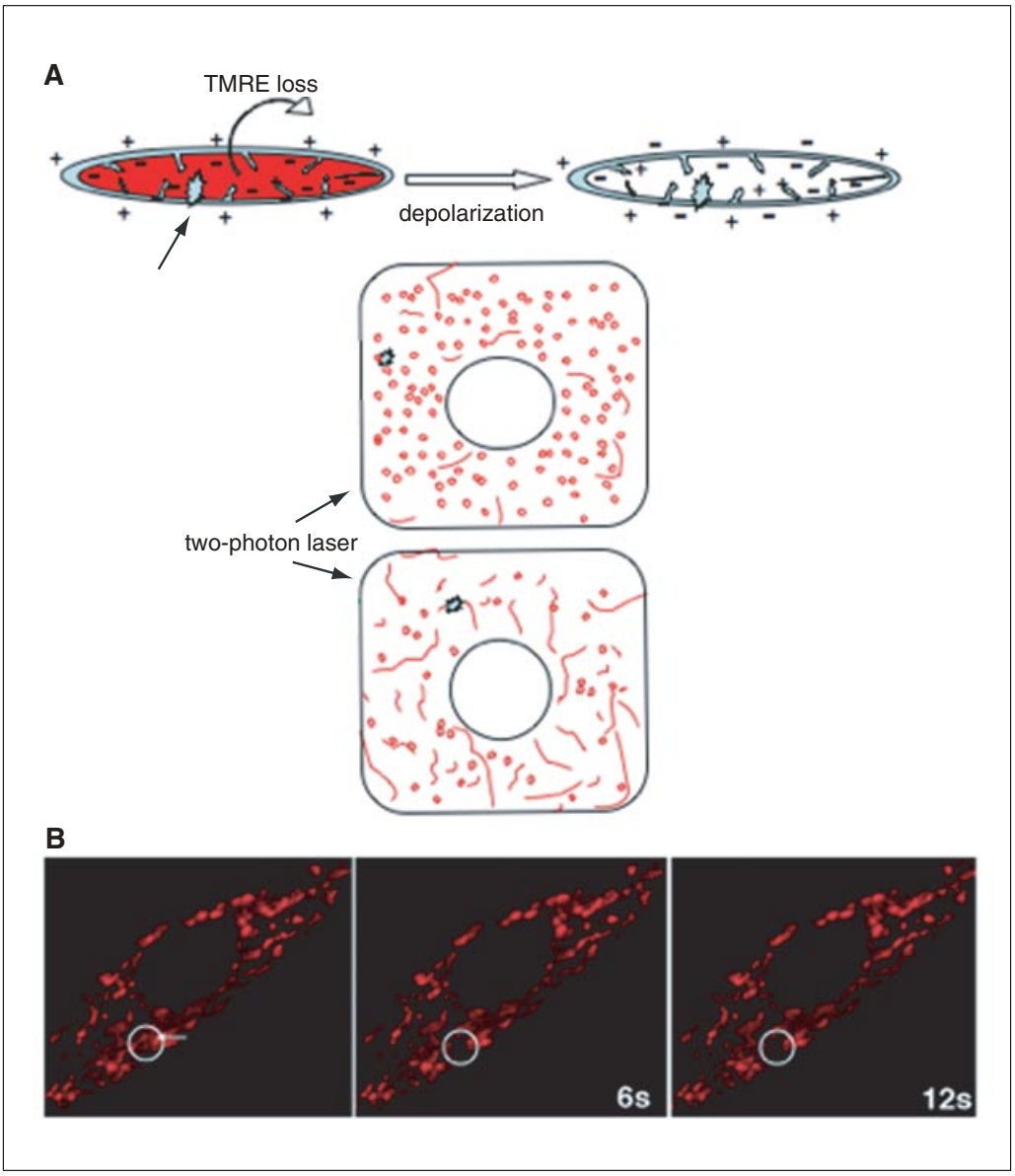


Figure 4.25.6 Microirradiation of TMRE-loaded mitochondria. (**A**) Depicts schematic representation of the microirradiation protocol. Mitochondria maintaining transmembrane potential (of charge) incorporate TMRE in the matrix. After being irradiated (green arrows) by the 2-photon laser locally, loss of TMRE occurs from the whole mitochondria due to depolarization triggered by the laser (as depicted for a single mitochondria in the upper panel and inside a cell in the lower panels). When this experiment is performed in cells, only single mitochondria are targeted for irradiation. (**B**) Shows images of a microirradiation experiment in a fibroblast where the arrow points to the site of irradiation. Images were acquired following Basic Protocol 3. Loss of TMRE signal is seen in the circled area in the time mentioned in seconds. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0425*.

3. Repeat steps 1 to 7 from Basic Protocol 1.
4. *Set up imaging settings:* Use the 543-nm laser line for imaging. To image TMRE, use settings for Rhodamine. An open pinhole is used to collect signal from the whole cell. Choose zoom factor 4 for high-resolution imaging. Scanning speed and averaging can be decided by the investigator to obtain best possible resolution.
5. *Define ROI:* To be able to cause microirradiation for assessing electrical continuity one should choose an ROI covering only a small area of the mitochondrial population. The ROI should not exceed an area of 1 × 1–μm.

6. *Set up microirradiation settings*: Chose the 2-photon chameleon laser as the bleaching laser for causing microirradiation. Tune the laser to 800 nm. Use 10% to 20% of the laser power and appropriate filters to cut off longer wavelengths. Set number of iterations to 5. Set optimum imaging speed to one frame per second to be able to monitor spread of depolarization.

   All parameters, including the objective, should be kept constant for reproducible microirradiation, as each will affect the net power delivered to unit area of the sample. The choice of the microirradiating laser depends on the power of the laser. As the physics behind 2-photon excitement requires the laser to be highly powerful (Piston, 1999), this type of laser has been used in this protocol. Proper alignment of the 2-photon laser is critical to achieve the required power for microirradiation (see Dickinson, 2005 for details about 2-photon microscopy). The power at the rear end of the objective should be optimally ~60 mW, as measured with the power meter. Any other laser achieving the power requirement can also be used to microirradiate.

7. *Assess damage to the cell and mitochondria:* The optimal microirradiation settings should aim at causing mitochondrial depolarization and loss of TMRE within the ROI but not damage the cell or fragment the irradiated mitochondrion.

   The damage to the cell can be assessed by performing time-lapse imaging with the transmitted light/DIC, with the microirradiation setup. This time series has to be examined carefully to confirm that no unwanted formation has occurred at the point of irradiation and the cell has not swelled in the time scale of the experiment, both of which would indicate cell damage.

   Mitochondrial damage can be assessed by performing the microirradiation time-lapse experiment on cells expressing a mitochondrial fluorescent protein marker. Observing the mitochondria at the irradiated zone carefully, in the microirradiation time series, one should confirm that the microirradiation does not fragment the mitochondrion in the time scale of the experiment, which could be an indication of damage.

   If there is any indication of cellular or mitochondrial damage, the power of microirradiation has to be minimized by reducing the bleaching iterations or the power of the laser.

8. Acquire the microirradiation time series and save images to be analyzed when required.

SUPPORT PROTOCOL

STAINING MITOCHONDRIA IN LIVE CELLS TO ASSESS MITOCHONDRIAL FUNCTION BY IMAGING

Knowledge of the mitochondrial lipid environment has been used to synthesize fluorescent compounds that stain mitochondria in live cells. This protocol describes staining and imaging of live tissue culture cells using the mitochondrial labels TMRE, JC-1, mitotracker green, or Picogreen. Mitotracker Green and Non Acridyl Orange are such compounds that can be used to measure mitochondrial mass. Therefore, from the same cells stained with both Mitotracker green and TMRE (for mitochondrial potential, see Basic Protocol 3), one can assess mitochondrial potential per unit of mitochondrial mass on a single-cell basis or in single mitochondria. Compounds like JC-1 are also taken up by the mitochondria by the virtue of their transmembrane potential. This dye, which gets enriched in the high potential zones of mitochondria, can form aggregates that have altered emission spectra (Smiley et al., 1991). Moreover, mitochondrial DNA can be visualized with the DNA binding dye PicoGreen (Ashley et al., 2005; Fig. 4.25.7).

Additional Materials *(also see Basic Protocol 1)*

Appropriate growth medium
Respective dye: e.g., TMRE/Mitotracker Green/JC-1/Picogreen (Molecular Probes)
DMSO
Mineral oil

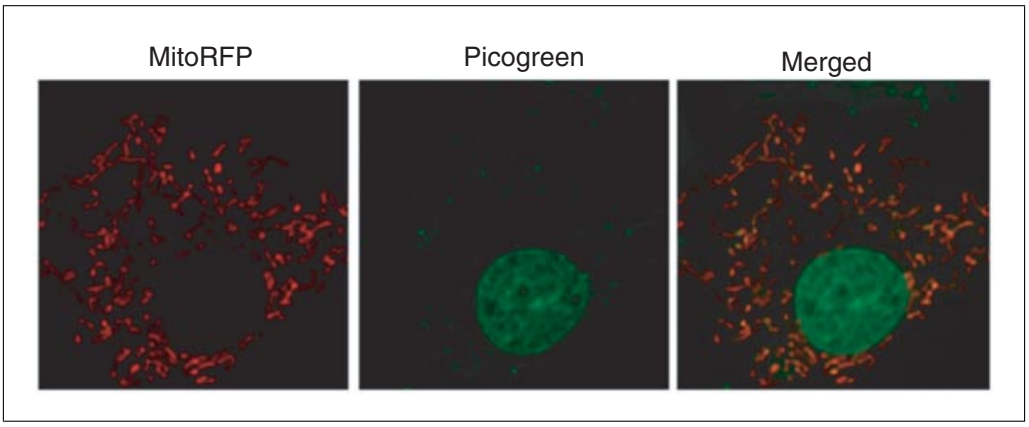


Figure 4.25.7 Picogreen staining of mitochondrial DNA. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb0425*.

37°C CO_2 incubator
Fluorescence microscope with proper filters for Rhodamine and Fluorescein

1. Plate cells in Labtek or Matek chambers with 75% confluency and perform the experimental procedure as required on the cells at least 16 hr after plating.
2. Incubate required volume of growth medium in the CO_2 incubator 1 hr at 37°C.
3. Thaw an aliquot of the respective dye and make the staining solution by diluting in the pre-incubated medium. Follow Table 4.25.3 for working concentrations.

   Each dye solution should be made in DMSO and maintained as small aliquots at −20°C. Freezing and thawing more than 5 to 6 times is not recommended.
4. Add a generous volume of the staining solution to the cells and incubate for 15 min in 37°C incubator.
5. Wash the cells three times, each time with pre-incubated medium in the same volume as that of the added staining solution (from step 4).
6. For double staining of Mitotracker Green and TMRE (to assess mitochondrial potential per unit mass), add diluted TMRE solution to cells already stained with Mitotracker green at this point and incubate for 15 min more.

   The following criteria have to be confirmed:

   (1) TMRE loading is below the quenching limits: When mitochondria are overloaded with TMRE a phenomenon of self-quenching occurs between TMRE molecules (Ward et al., 2000). This reduces the net TMRE signal obtained from mitochondria and thus will produce erroneous results. The concentration of TMRE mentioned here is below quenching limits but one should check this for every experimental set up as mentioned below.

   (2) No FRET occurs between Mitotracker Green and TMRE: fluorescence resonance energy transfer is a process by which energy is transferred from a donor molecule to an acceptor molecule with an overlapping excitation spectrum with the donor emission spectrum (UNIT 17.1). It has been reported that at certain concentrations FRET occurs from Mitotracker Green to TMRE molecules inside the mitochondria (Elmore et al., 2004). This will reduce the green signal in the presence of TMRE. Although the protocol involves much lower concentrations of both the dyes than necessary to undergo FRET, it is recommended this be verified in every experimental set up.

   Both of these caveats can be easily verified by performing time-lapse imaging of a double-stained sample after adding FCCP as mentioned in the Alternate Protocol. Addition of

Table 4.25.3 Working Concentration for Various Mitochondrial Fluorescent Dyes

Fluorescent dye	Working concentrations
TMRE	25-50 nM
Mitotracker Green	50-100 mM
Mitotracker red 633i[a,b]	250 nM
JC-1[c]	5 nM
PicoGreen[d]	1:500 dilution

[a]Overstaining results in the incorporation even in the endoplasmic reticulum.
[b]This dye and some others not mentioned here, are dependent on the mitochondrial potential to variable extents. Please refer to the relevant literature for the correct choice of dyes.
[c]Although the dye JC-1 can be used for qualitative purposes to assess heterogeneity in mitochondrial potential, it does not yield quantitative results and also often causes distortion in mitochondrial morphology. The dye forms particulate structures in solution; therefore, it is recommended to do a serial dilution after spinning down the solution each time. Otherwise, the particulate structure will deposit down on the bottom of the chamber during staining and the cells close to the particulate matter will incorporate more of the dye, yielding very erroneous results.
[d]Dilution is from a stock bought from Molecular Probes (P7581). Mitochondrial DNA would appear as cytoplasmic dots if optimally stained with this stain as seen in Figure 4.25.5. It is always recommended to perform dual staining with another mitochondrial marker or dye as cytoplasmic dots could also be due to mycoplasma infections.

FCCP would cause mitochondrial depolarization and loss of TMRE from the mitochondria. Therefore, if depolarization by FCCP reduces the mitochondrial red signal, the TMRE used is below the quenching level. On the other hand, if depolarization by FCCP increases the intensity of TMRE signal from mitochondria, it is an indication that TMRE loading is beyond the quenching levels. In this case, the working TMRE concentration has to be lowered and the test has to be repeated further. If the loss of TMRE signal by FCCP does not enhance the green signal from Mitotracker Green, then it is an indication of lack of energy transfer between the two fluorescent dyes. If addition of FCCP increases the signal from Mitotracker Green (indicative of FRET), then the working concentrations of the dyes have to be reduced and the test has to be repeated further.

In immortalized cells, an alternative for normalizing the mitochondrial mass could be replacing Mitotracker Green with stably expressing mitoGFP. This fluorescent molecule has a similar spectrum to that of Mitotracker Green and resides in the mitochondrial matrix. For this purpose, one needs to transfect mitoGFP into the cell line and select a stable clone that will be expressing mitoGFP equally in all the cells derived from the clone.

It is recommended to always include a control for hyperpolarization and depolarization in this analysis. Oligomycin would cause hyperpolarization by reducing mitochondrial ATP synthase activity and the ionophore FCCP would cause depolarization (Nicholls and Ward, 2000). As the assessment of mitochondrial potential by this kind of an analysis is done on a comparative scale, the samples treated with oligomycin and FCCP would serve to define the uppermost and lowermost limits in the scale of detection.

7. Repeat step 5.

8. Add pre-incubated medium and layer with mineral oil on top of the medium.

Add enough medium to ensure that the mineral oil meniscus does not touch the bottom of the chamber where cells are coated.

Table 4.25.4 Imaging Parameters for Measuring Mitochondrial Potential per Unit Mass

Parameter	Individual cell	Individual mitochondria
Objective	40×	63×
Zoom	0.7	2-3
Scan speed	8	8
Averaging	2	2
Optical slice	Open	1 μm
z-section	———	0.5 μm
Laser power[a]	1%-10%	1%-10%

[a]The TMRE imaging of mitochondria at zoom 2 or 3 should be done very cautiously, as high laser power could quickly photodamage TMRE-loaded mitochondria (Bunting, 1992). Therefore, for optimum results, scanning laser intensity of the both the lasers should be kept as low as possible.

9. Choose the appropriate parameters for imaging, as is listed in Table 4.25.4.

   For assessing mitochondrial potential per unit mass, the configuration of filters for imaging should be chosen so that they restrict bleed through from one channel to the other in cells stained with both Mitotracker Green and TMRE. Adjust the laser intensity and detector gain according to the brightness of the sample in such a way so that the signal from any pixel is not saturated. Follow Table 4.25.4 for setting imaging parameters depending on whether the aim is to detect total mitochondrial potential per cell or that of individual mitochondria in single cells. Parameters can be altered for better imaging depending on the system.

10. Perform imaging as soon as possible according to the relevant set of imaging parameters.

    Beyond a period of 30 to 45 min, the mitochondria might start fragmenting leading to erroneous results.

    There could be variations in the sensitivity level according to the cell type and one should make a note of that. Generally, more sensitive cells would allow less time before mitochondria fragment and alter potential as an indication of a death trigger. Therefore, everything should be ready before the experiment and one should be quick in finishing all the scans.

11. Save the images and analyze the images at your convenience.

    The ratio of TMRE/Mitotracker Green is an assessment of mitochondrial potential in arbitrary units.

COMMENTARY

Background Information

The name mitochondria is a fusion of the Greek words "mito" meaning filaments and "chondro" meaning grains. The length of each mitochondrion can extend from 500 nm to 1 mm or more, the average diameter being 500 nm (Detmer and Chan, 2007). Contributions of electron microscopists aided in the detailed understanding of mitochondrial structure while biochemical analyses of isolated mitochondria identified the organelle as a seat of intermediate metabolism. In this era of live cell imaging, mitochondrial biologists are motivated to understand the structure-function relationship of mitochondria in live cells.

Imaging of mitochondria in live yeast and mammalial cells has been made possible by designing vectors expressing fluorescent proteins that are tagged to either a resident mitochondrial protein or any particular mitochondrial targeting sequence, targeting the fluorescent protein into any of the three mitochondrial compartments. Detailed strategy to that end in

yeast can be found in Okamoto et al. (2001) and mammals in Rizzuto et al. (1996). The latest generation of fluorescent protein variants is discussed in *UNIT 21.5*. The targeted fluorescent protein essentially enables the researchers to visualize the behavior of mitochondria within cells and/or study the behavior of the particular fluorescently tagged mitochondrial protein.

High-resolution live cell imaging of mitochondria aided in understanding the correlation of mitochondrial fragmentation and physiological properties of apoptotic cytochrome *c* release (Frank et al., 2001) or ROS production (You et al., 2006). The varied mitochondrial morphology in a population of cells can be quantitated from static images as fragmented, intermediate, or tubular according to the predominant mitochondrial morphology in an individual cell. However, in some extreme instances when mitochondria could also appear as clumps, it is erroneous to include them in any of the above three categories of mitochondrial morphology. These clumps could arise from clumping of fragmented mitochondria or collapse of a fused form of mitochondria. Therefore, in these situations, it is critical to perform FRAP analysis of any mitochondrial matrix molecule. This assay provides important measurements for mitochondrial continuity that would arise in fused mitochondrial structures. The assay could also be modified in a variety of aspects to understand mitochondrial dynamism. The interpretation of the recovery after photobleaching has to be carefully performed where the time scale of recovery is crucial for highly mobile organelles like mitochondria; failing to appreciate this property might yield confusing results. Furthermore, the alteration of recovery kinetics in a FRAP study for mitochondrial connectivity or motility might be contributed to by altered diffusing properties of the matrix molecule of interest. This has to be verified by performing FRAPs in individual mitochondria and any statistically significant change has to be included in the analysis of matrix continuity. To capture the complete kinetics of the recovery of the matrix molecule special microscopes are required with super fast scanning modules, like LSM5 Duo or Nikon epifluorescence microscope with super-fast integration time of the camera and photomultipliers attached to the microscope (Partikian et al., 1998). This will allow very fast photobleaching on the order of microseconds (Fig. 4.25.4A) that is required for molecules with diffusion coefficient in the order of 2–3 $\times$ $10^{(-7)}$ cm^2/sec, as in mitochondrial matrix. Slower microscopes, with longer bleaching time, will spread the bleaching beyond the bleach box, therefore increasing the recovery time (Fig. 4.25.4B). This kind of analysis might be used in a comparative scale but the data should not be used to determine diffusion coefficient.

The microirradiation protocol of TMRE-loaded mitochondria was first designed by Amchenkova et al (1988), and a similar technique has been also used to study various other organelles (Berns, 2007). High laser power has been shown to introduce holes in mitochondria (Khodjakov et al., 2004), thus disrupting mitochondrial potential across the inner membrane. Laser-induced depolarization of TMRE-loaded mitochondria has also been shown to increase localized ROS production (O'Reilly et al., 2003). This experiment has to be performed with caution about the cellular damage during the experimentation (as discussed before) and controls have to be included each day of experimentation. When dealing with more than one cell type, proper controls have to be included for each cell type under examination. It has to be very well appreciated that in a controlled setup the irradiated cell, behaving normally within the small time span of the experiment, might eventually die. Therefore, long-term experimentation may not be performed on such a cell. The microirradiation experiment is important from the mitochondrial bioenergetics point of view, as this qualitatively addresses the electrical continuity of the mitochondria at any given time point that might have impact on the total ATP output from the mitochondrial system (Skulachev, 2001).

The staining of mitochondria with different commercially available dyes is routinely practiced and the background information may be obtained from *UNIT 4.4*.

Post-processing of image analysis is a critical step for proper interpretation of imaging data. Although this unit does not cover image analysis in detail, mention may be made of some software that will be helpful for the purpose. Among the proprietary software, Zeiss' own Image Examiner can be used to analyze laser scanning confocal images. A free downloadable version that can be used as a viewer is available on Zeiss' Web site (*http://www.zeiss.de/C12567BE0045ACF1/Contents-Frame/CAA2EF638EC5F0D3C1256ADF0050E2F1*). Volocity and Metamorph can also be used. An open source software called Image J (*http://rsbweb.nih.gov/ij/download.html*) is very helpful for all basic purposes. Relevant plugins can be included for advanced purposes.

Table 4.25.5 Troubleshooting Guide to Live Cell Mitochondrial Imaging

Problems	Possible causes[a]
z-stacks and time lapse	
Fragmented/clumped mitochondria	Cells are not healthy pH or temperature shock Mineral oil touching the cells High amount of lipid-based transfecting agents used Transient transfection of the mitochondrial marker Effects of over-expression of the protein Immensely high scanning laser power Use of high-energy/low-wavelength laser (413/405-nm etc) or any other UV laser
Immotile mitochondria	Cells are not healthy for any of the reasons mentioned above High speed of imaging
Projection of *z*-stacks is blurry	Oversampling along *z*-axis Wide pinhole High scanning speed Saturated signals Low power objective
FRAP	
No recovery	Dead cells Fragmented mitochondria Immotile mitochondria Bleach box covers more than 30% of the total signal Inappropriately low pinhole size Bleaching caused by high laser power
Bleached zone is more than the bleach box	Misalignment of the laser head and bleach box Slow bleaching
Not enough bleaching	Low laser power chosen for bleaching For RFP, 488-, 458-, and 514-nm lasers not chosen
Poor recovery curve	Very long time intervals set for monitoring recovery phase Signal saturated in the beginning Scanning laser causing bleaching Not enough bleaching by the bleaching laser(s) Bleached molecule reverting to the fluorescent stage Movement of mitochondria interfering with recovery
Microirradiation	
No loss of TMRE after microirradiation	No irradiation due to application of lower laser power Misalignment of the 2-photon laser Low iterations chosen for irradiation Low zoom factor chosen
Mitochondria shifting away during imaging *or* mitochondria fragmenting	Cell is swelling due to more than optimum irradiation
Increase in TMRE signal after microirradiation	TMRE loading is beyond quenching levels

continued

Table 4.25.5 Troubleshooting Guide to Live Cell Mitochondrial Imaging

Problems	Possible causes[a]
Staining for potential	
No staining	Mitochondria are absolutely depolarized because cells are not healthy (as mentioned above)
Poor resolution	Overstaining Inappropriate pinhole size Lack of proper washing after staining
The oligomycin/FCCP-treated samples not producing expected results as controls	Overload of TMRE beyond the quenching levels FRET between Mitotracker Green and TMRE Bleed through from detector channel for Mitotracker Green to that of TMRE
Loss of TMRE while scanning	Power of the scanning laser power is more than optimum
Fragmented mitochondria	Stained sample left for more than 30 to 45 min

[a]For solutions please refer to the corresponding sections.

One could also include self-written macros in the list of Image J plugins.

Troubleshooting

This section discusses the problems that might arise while or before imaging mitochondria in particular. The general troubleshooting for imaging per se will not be discussed here. The possible causes to any problem are very crisply mentioned here and are discussed in more detail in the relevant protocol. See Table 4.25.5 for a troubleshooting guide.

Anticipated Results

The protocols described in this unit will help the researcher to understand mitochondrial function by imaging. The precautions mentioned here for imaging mitochondria are very critical to be able to achieve the goal of establishing structure/function relationships of mitochondria. By the correct use of imaging parameters and choice of fluorophore, the investigator can not only acquire static mitochondrial images but also perform time-lapse imaging on mitochondria. Live cell imaging of mitochondria including the FRAP and microirradiation protocols can be used by investigators to study the dynamics of mitochondria in quantitative terms and can also be used to understand dynamics of individual proteins in mitochondria. For example, study of the fluorescently tagged intermembrane space protein cytochrome *c* by live cell imaging has uncovered many important aspects of apoptotic release of this protein into the cytosol (Goldstein et al., 2000). Live cell imaging studies of mitochondria imply that imaging of fluorescent-tagged versions of mitochondrial proteins have tremendous potential of unraveling many unexplored aspects of mitochondrial physiology and its crosstalk with other cellular compartments.

Time Considerations

Generation of a stably expressing cell line takes 1 to 2 months depending on the cell type. Staining with fluorescent dyes and sample preparation takes from 30 min to 2 hr. Stained samples should be imaged within 45 min. Acquiring each *z*-stack will take <1 min; it takes a little longer for very slow scan speeds. The total time for a time-lapse experiment would depend on the kinetics of the phenomenon to be observed. To observe mitochondrial dynamism, a time lapse of 2 to 3 min should be sufficient. Each FRAP assay will take from less than half a minute to an hour, depending on the goal of the experiment. Extent of electrical continuity can be captured within 15 sec or less.

Literature Cited

Amchenkova, A.A., Bakeeva, L.E., Chentsov, Y.S., Skulachev, V.P., and Zorov, D.B. 1988. Coupling membranes as energy-transmitting cables. I. Filamentous mitochondria in fibroblasts and mitochondrial clusters in cardiomyocytes. *J. Cell Biol.* 107:481-495.

Ashley, N., Harris, D., and Poulton, J. 2005. Detection of mitochondrial DNA depletion in living human cells using PicoGreen staining. *Exp. Cell Res.* 303:432-446.

Berns, M.W. 2007. A history of laser scissors (microbeams). *Methods Cell Biol.* 82:1-58.

Bunting, J.R. 1992. A test of the singlet oxygen mechanism of cationic dye photosensitization of mitochondrial damage. *Photochem. Photobiol.* 55:81-87.

Cassidy-Stone, A., Chipuk, J.E., Ingerman, E., Song, C., Yoo, C., Kuwana, T., Kurth, M.J., Shaw, J.T., Hinshaw, J.E., Green, D.R., and Nunnan, J. 2008. Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. *Dev. Cell* 14:193-204.

Chen, L.B. 1988. Mitochondrial membrane potential in living cells. *Annu. Rev. Cell Biol.* 4:155-181.

Detmer, S.A. and Chan, D.C. 2007. Functions and dysfunctions of mitochondrial dynamics. *Nat. Rev. Mol. Cell Biol.* 8:870-879.

De Vos, K.J., Sable, J., Miller, K.E., and Sheetz, M.P. 2003. Expression of phosphatidylinositol (4,5) bisphosphate-specific pleckstrin homology domains alters direction but not the level of axonal transport of mitochondria. *Mol. Biol. Cell* 14:3636-3649.

De Vos, K.J., Allan, V.J., Grierson, A.J., and Sheetz, M.P. 2005. Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. *Curr. Biol.* 15:678-683.

Dickinson, M.E 2005. Multiphoton and multispectral laser scanning microscopy. *In* Live Cell Imaging: A Laboratory Manual. (R.D. Goldman and D.L. Spector, eds) pp. 281-301. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Elmore, S.P., Nishimura, Y., Qian, T., Herman, B., and Lemasters, J.J. 2004. Discrimination of depolarized from polarized mitochondria by confocal fluorescence resonance energy transfer. *Arch. Biochem. Biophys.* 422:145-152.

Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., and Youle, R.J. 2001. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev. Cell* 1:515-525.

Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I., and Green, D.R. 2000. The coordinate release of cytochrome *c* during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* 2:156-162.

Goodwin, J.S. and Kenworthy, A.K. 2005. Photobleaching approaches to investigate diffusional mobility and trafficking of Ras in living cells. *Methods* 37:154-164.

Karbowski, M. and Youle, R.J. 2003. Dynamics of mitochondrial morphology in healthy cells and during apoptosis. *Cell Death Differ.* 10:870-880.

Karbowski, M., Norris, K.L., Cleland, M.M., Jeong, S.Y., and Youle, R.J. 2006. Role of Bax and Bak in mitochondrial morphogenesis. *Nature* 443:658-662.

Khodjakov, A., Rieder, C., Mannella, C.A., and Kinnally, K.W. 2004. Laser micro-irradiation of mitochondria: Is there an amplified mitochondrial death signal in neural cells? *Mitochondrion* 3:217-227.

Mitra, K., Wunder, C., Roysam, B., Lin, G., and Lippincott-Schwartz, J. 2009. A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proc. Natl. Acad. Sci. U.S.A.* 106:11960-11965.

Nicholls, D.G. and Budd, S.L. 2000. Mitochondria and neuronal survival. *Physiol. Rev.* 80:315-360.

Nicholls, D.G. and Ward, M.W. 2000. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: Mortality and millivolts. *Trends Neurosci.* 23:166-174.

Okamoto, K., Perlman, P.S., and Butow, R.A. 2001. Targeting of green fluorescent protein to mitochondria. *Methods Cell Biol.* 65:277-283.

O'Reilly, C.M., Fogarty, K.E., Drummond, R.M., Tuft, R.A., and Walsh J.V. Jr. 2003. Quantitative analysis of spontaneous mitochondrial depolarizations. *Biophys. J.* 85:3350-3357.

Partikian, A., Olveczky, B., Swaminathan, R., Li, Y., and Verkman, A.S. 1998. Rapid diffusion of green fluorescent protein in the mitochondrial matrix. *J. Cell Biol.* 140:821-829.

Piston, D.W. 1999. Imaging living cells and tissues by two-photon excitation microscopy. *Trends Cell Biol.* 9:66-69.

Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R.Y., and Pozzan, T. 1996. Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo. *Curr. Biol.* 6:183-188.

Scheffler, I.E. 2001. A century of mitochondrial research: Achievements and perspectives. *Mitochondrion* 1:3-31.

Skulachev, V.P. 2001. Mitochondrial filaments and clusters as intracellular power-transmitting cables. *Trends Biochem. Sci.* 26:23-29.

Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D. Jr., and Chen, L.B. 1991. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc. Natl. Acad. Sci. U.S.A.* 88:3671-3675.

Ward, M.W., Rego, A.C., Frenguelli, B.G., and Nicholls, D.G. 2000. Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. *J. Neurosci.* 20:7208-7219.

You, T., Robotham, J.L., and Yoon, Y. 2006. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proc. Natl. Acad. Sci. U.S.A.* 103:2653-2658.

Organelle Atlas

APPENDIX TO CHAPTER 4

This atlas contains images of cellular organelles visualized using a wide variety of reagents and techniques and demonstrates the diversity of methods for exploring the cell. The atlas consists of the figures listed in Table 4A.1.

Table 4A.1 Figures in Organelle Atlas[a]

Figure	Organelle(s)/structure(s)	Visualized using:
Fig. 4A.1	Mitotic spindle and chromosomes	γ-tubulin-GFP, anti-β-tubulin antibody, Hoechst 33342
Fig. 4A.2	DNA and β-tubulin	Hoechst 33342, anti-β-tubulin antibody
Fig. 4A.3	Nuclear pore complexes	POM121-GFP
Fig. 4A.4	Nuclear envelope	Lamin B receptor–GFP
Fig. 4A.5	Myosin, actin stress fibers	Anti-myosin antibody, rhodamine-conjugated phalloidin
Fig. 4A.6	Focal adhesions	Anti-vinculin antibody
Fig. 4A.7	Intermediate filaments	Anti-vimentin antibody
Fig. 4A.8	Microtubules and Golgi complex	Anti-tubulin and anti-galactosyltransferase antibodies
Fig. 4A.9	Endoplasmic reticulum	$DiOC_6$
Fig. 4A.10	Endoplasmic reticulum	Anti-ER-resident protein antibodies
Fig. 4A.11	Lysosomes	Rhodamine-ovalbumin
Fig. 4A.12	Lysosomes	Anti-LEP100 antibodies
Fig. 4A.13	Lysosomes, endosomes	Rhodamine-conjugated wheat germ agglutinin
Fig. 4A.14	Golgi complex	Anti–mannosidase II antibodies, brefeldin A, nocodazole
Fig. 4A.15	Mitochondria	TMRE, calcium green AM
Fig. 4A.16	Endosomes	Anti-transferrin antibodies
Fig. 4A.17	Trans-Golgi network	Anti-TGN38 antibodies
Fig. 4A.18	Trans-Golgi network, endosomes	Monoclonal anti-AP1 antibodies (100/3)
Fig. 4A.19	Trans-Golgi network, endosomes	Anti-TGN46 and anti-AP3 antibodies
Fig. 4A.20	Plasma membrane clathrin-coated pits, clathrin-coated vesicles	Anti-AP2 antibodies
Fig. 4A.21	Plasma membrane clathrin-coated pits, trans-Golgi network, endosomes, clathrin-coated vesicles	Monoclonal anti-clathrin antibodies (X22)
Fig. 4A.22	Fibronectin (an extracellular matrix protein), nuclei	Rhodamine-conjugated anti-fibronectin antibody, DAPI

[a]Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; $DiOC_6$, dicarbocyanine dye; GFP, green fluorescent protein; TGN, trans-Golgi network; TMRE, tetramethylrhodamine ethylester.

Current Protocols in Cell Biology (1998) 4A.1-4A.12

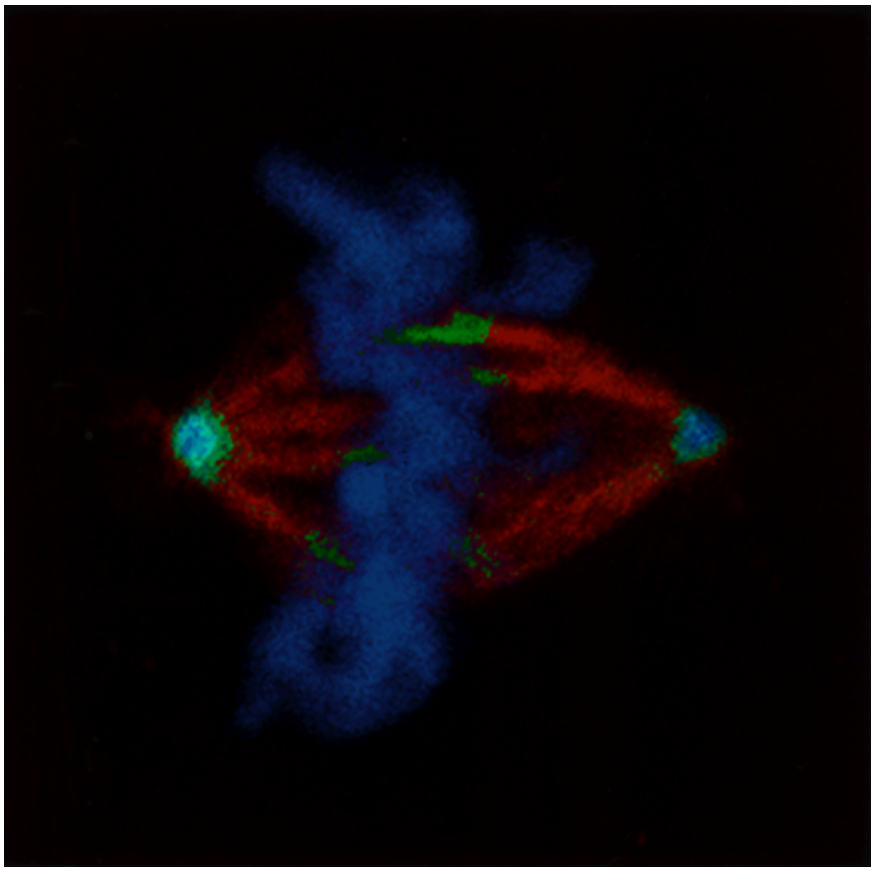


Figure 4A.1 Mitotic NRK cell in metaphase. Red, mouse anti-β-tubulin antibody; green, γ-tubulin-GFP; blue, Hoechst 33342. Figure provided by Jan Ellenberg, NICHD/NIH.

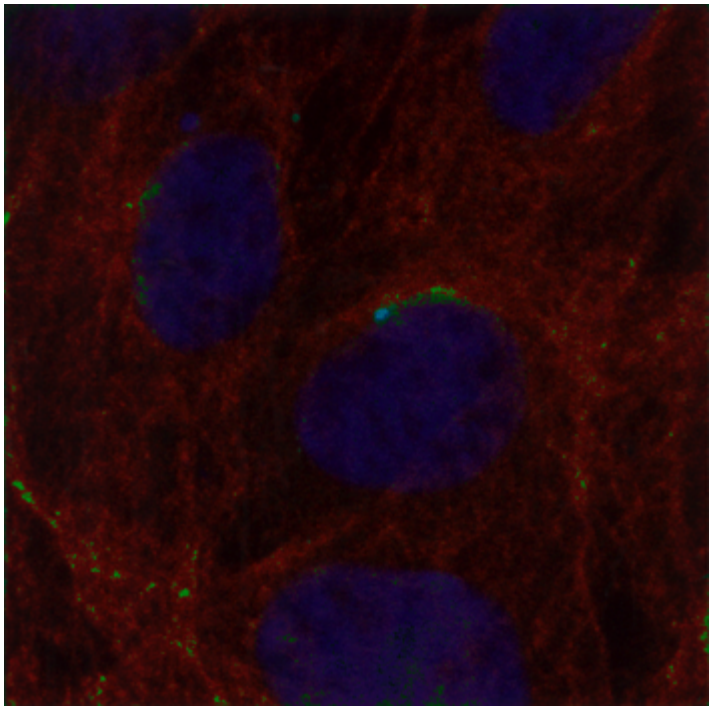


Figure 4A.2 Distribution of DNA (Hoechst 33342, blue) and β-tubulin (anti-β-tubulin antibody, red) in interphase NRK cells. Figure provided by Jan Ellenberg, NICHD/NIH.

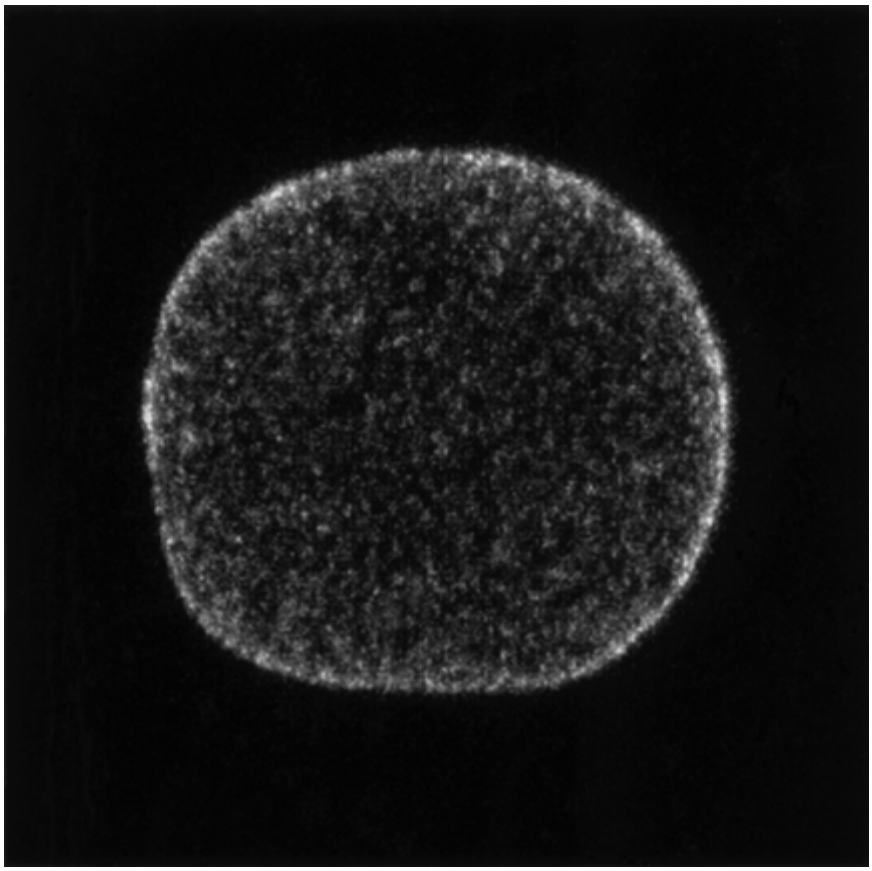


Figure 4A.3 Nuclear pore complexes in PTK_2 cell stained with POM121 tagged with green fluorescent protein (GFP). Figure provided by Jan Ellenberg, NICHD/NIH.

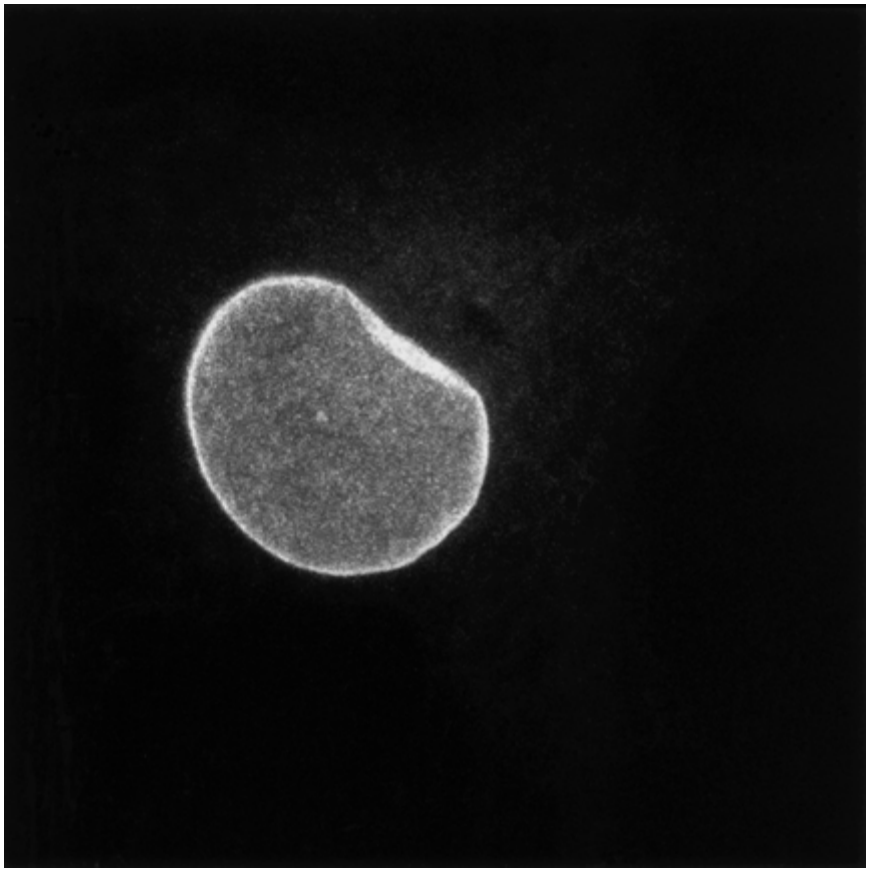


Figure 4A.4 Nuclear envelope in COS cell labeled with lamin B receptor tagged with green fluorescent protein (GFP). Figure provided by Jan Ellenberg, NICHD/NIH.

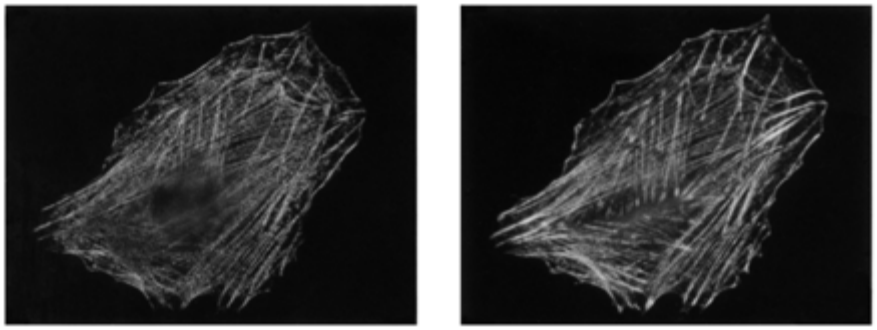


Figure 4A.5 (**A**) Myosin localization in formaldehyde-fixed NRK cell labeled with mouse anti-myosin antibodies. (**B**) Stress fibers in the same formaldehyde-fixed NRK cell labeled with rhodamine-conjugated phalloidin. Figure provided by Julie Donaldson, NHBLI/NIH.

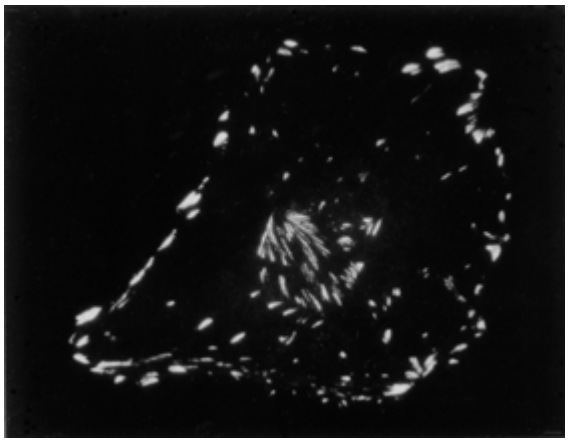


Figure 4A.6 Focal adhesions in formaldehyde-fixed NRK cell labeled with mouse anti-vinculin antibodies. Figure provided by Julie Donaldson, NHBLI/NIH.

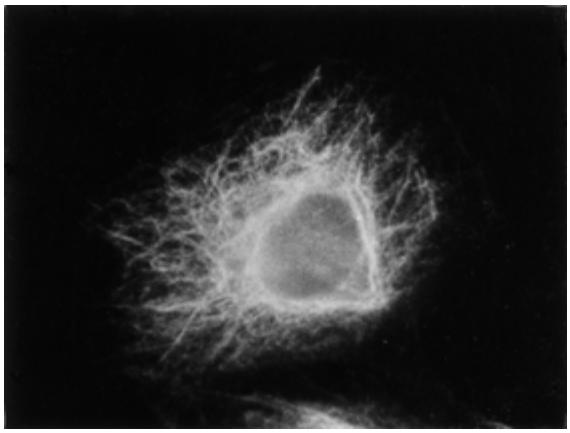


Figure 4A.7 Intermediate filaments in formaldehyde-fixed NRK cell labeled with rabbit anti-vimentin antibodies. Figure provided by Julie Donaldson, NHBLI/NIH.

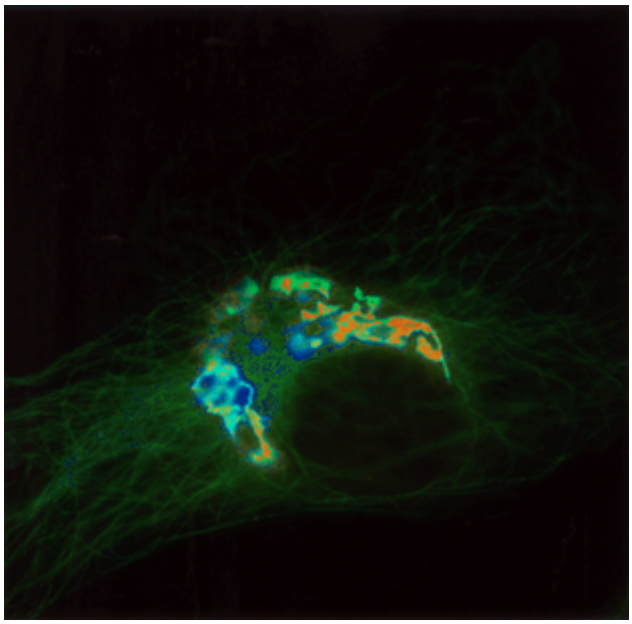


Figure 4A.8 Microtubules and Golgi complex in HeLa cell stained with anti-tubulin antibodies (green) and antibodies to the Golgi complex enzyme galactosyl transferase (yellow-orange). Figure provided by Jennifer Lippincott-Schwartz, NICHD/NIH.

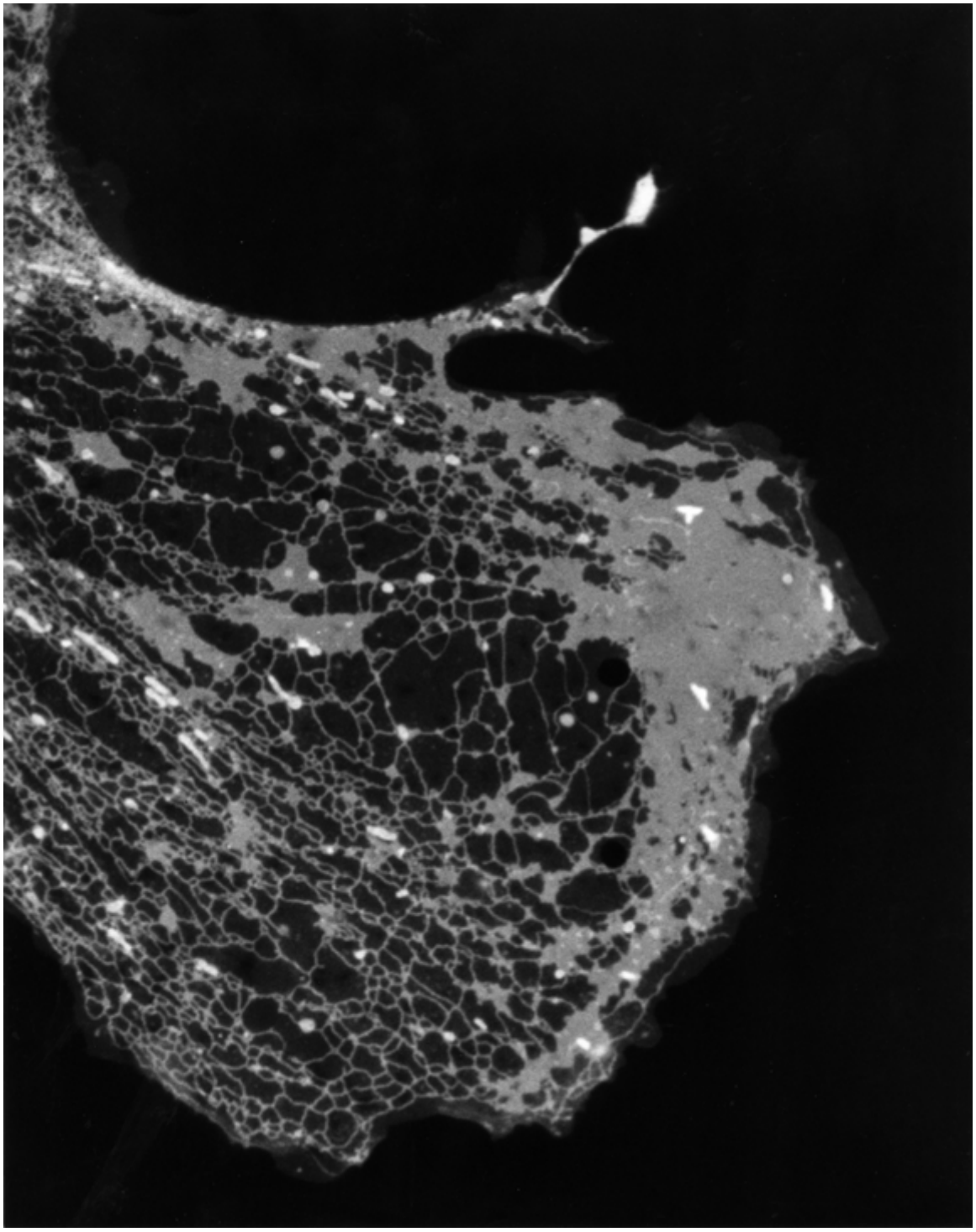


Figure 4A.9 Endoplasmic reticulum (ER) in frog skin cell labeled with $DiOC_6$. Figure provided by Mark Terasaki, University of Connecticut Health Center.

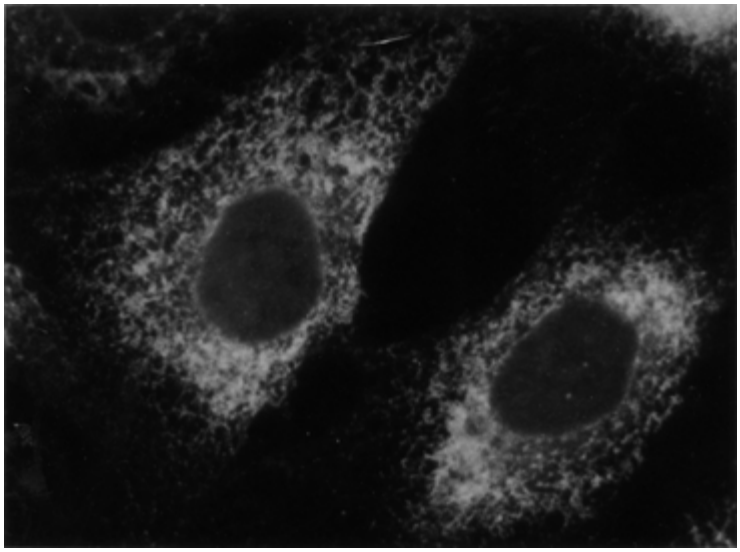


Figure 4A.10 Endoplasmic reticulum (ER) in NRK cell labeled with antibodies to ER-resident proteins (from D. Louvard). Figure provided by Jennifer Lippincott-Schwartz, NICHD/NIH.

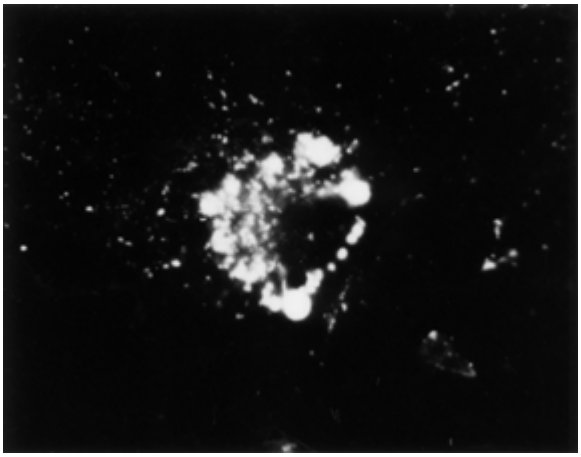


Figure 4A.11 Lysosomes in chicken fibroblast labeled with rhodamine-conjugated ovalbumin taken up by the cells over 4 hr. Figure provided by Jennitfer Lippincott-Schwartz, NICHD/NIH.

Figure 4A.12 Lysosomes in chicken fibroblast labeled with antibodies to LEP100, a 100-kDa lysosomal membrane protein. Figure provided by Jennifer Lippincott-Schwartz, NICHD/NIH.

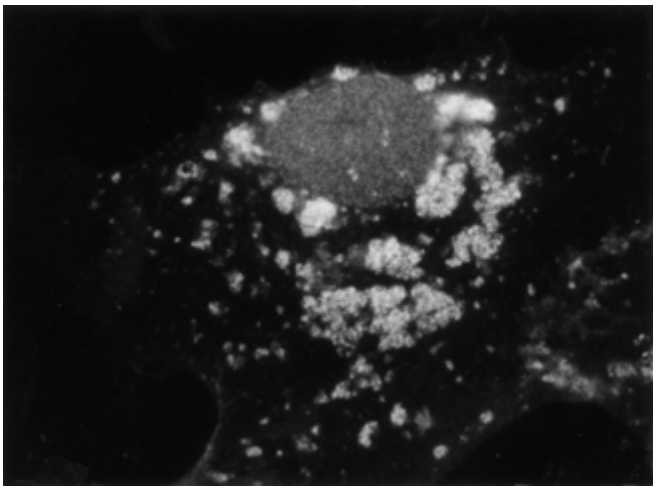


Figure 4A.13 HeLa cell stained with rhodamine-conjugated wheat germ agglutinin (WGA), which binds to sialic acid and *N*-acetylglucosaminyl residues on glycoproteins. Glycoproteins enriched in these residues are found in lysosomes and endosomes. Figure provided by Jennifer Lippincott-Schwartz, NICHD/NIH.

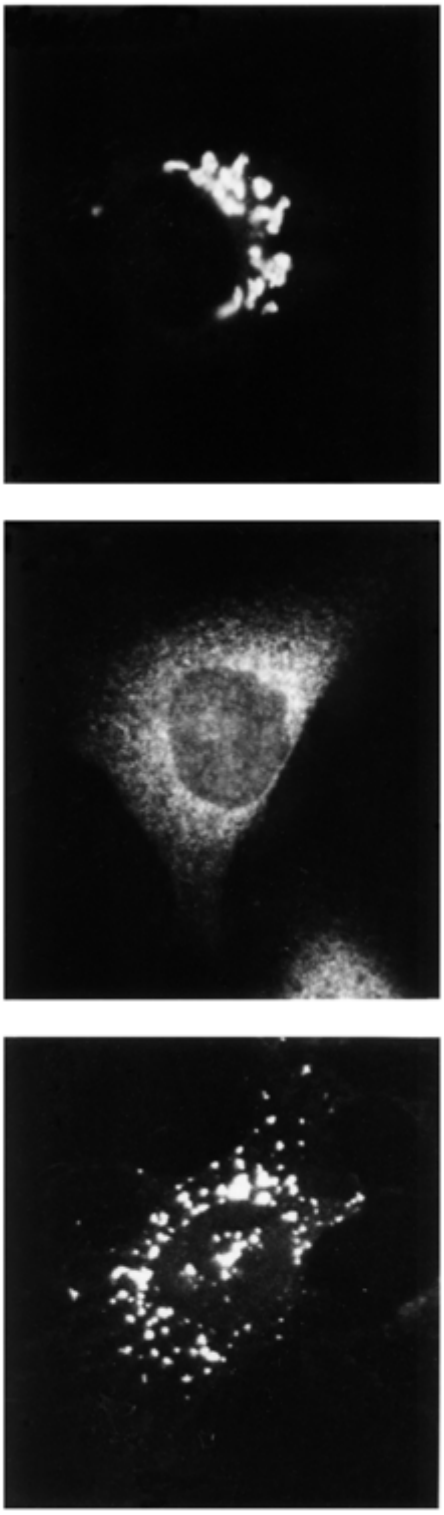


Figure 4A.14 Distribution of antibodies to the Golgi enzyme mannosidase II in fixed and permeabilized HeLa cells (**A**) untreated; (**B)** treated with brefeldin A; or (**C**) treated with nocodazole, which depolymerizes microtubules. Figure provided by Jennifer Lippincott-Schwartz, NICHD/NIH.

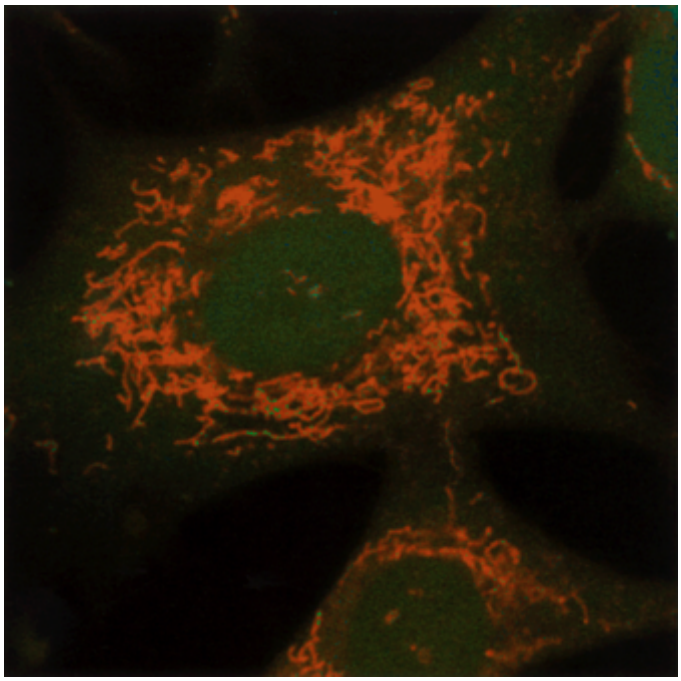


Figure 4A.15 Neuroblastoma cell stained with TMRE to label mitochondria (orange) and with calcium green AM to monitor calcium. Figure provided by Ion Moran, University of Connecticut Health Center.

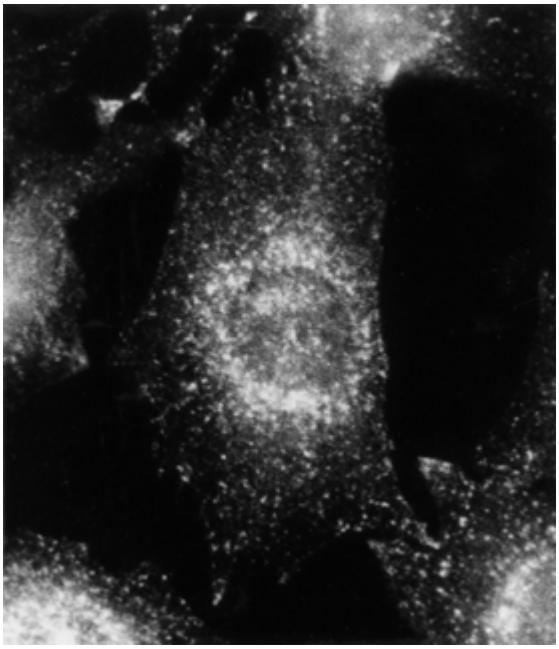


Figure 4A.16 Distribution of endosomes in NRK cell incubated in the presence of iron-saturated transferrin and labeled with fluorescent antibodies to transferrin. Figure provided by Jennifer Lippincott-Schwartz, NICHD/NIH.

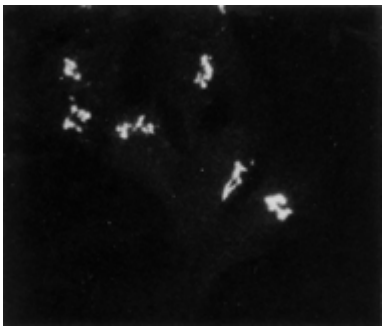


Figure 4A.17 Trans-Golgi network (TGN) in NRK cell labeled with antibodies to TGN-38, a membrane protein. Figure provided by Jennifer Lippincott-Schwartz, NICHD/NIH.

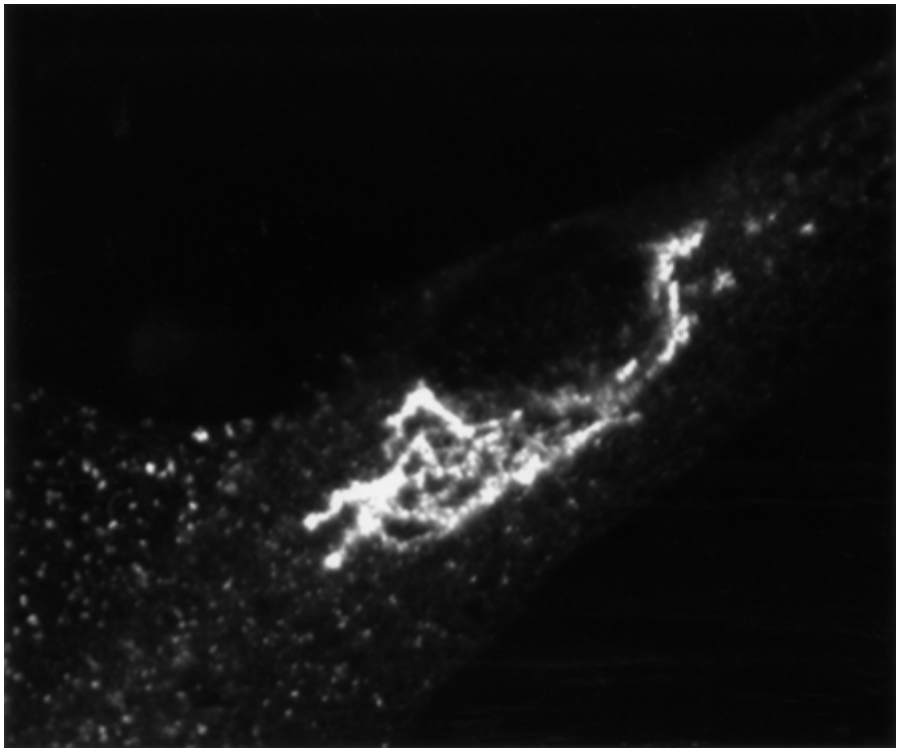


Figure 4A.18 Distribution of the adaptor complex AP1 in human fibroblasts stained with monoclonal 100/3 in the trans-Golgi network and endosomal structures. Figure provided by Juan Bonifacino, NICHD/NIH.

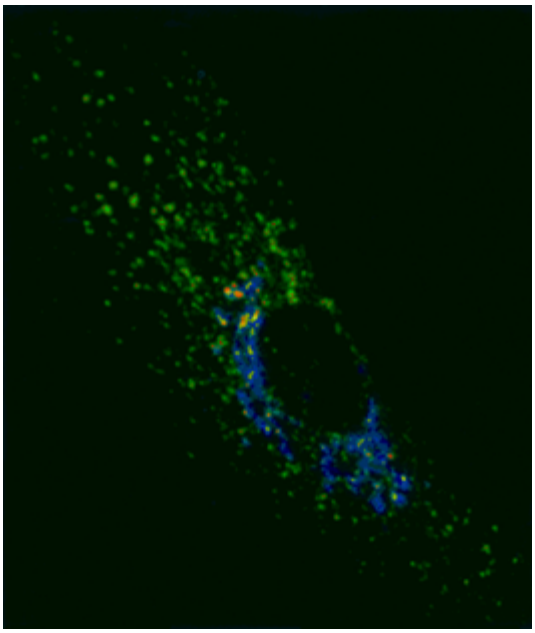


Figure 4A.19 Distribution of the trans-Golgi network and the adaptor complex AP3 in human fibroblasts labeled with antibodies to TGN46 (green) and AP3 (red). Figure provided by Juan Bonifacino, NICHD/NIH.

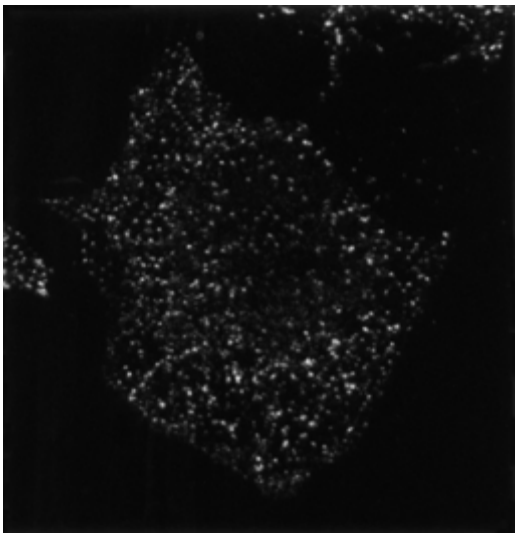


Figure 4A.20 Localization of adaptor complex AP2 to plasma membrane clathrin-coated pits and clathrin-coated vesicles in HeLa cells labeled with anti-AP2 antibodies. Figure provided by Juan Bonifacino, NICHD/NIH.

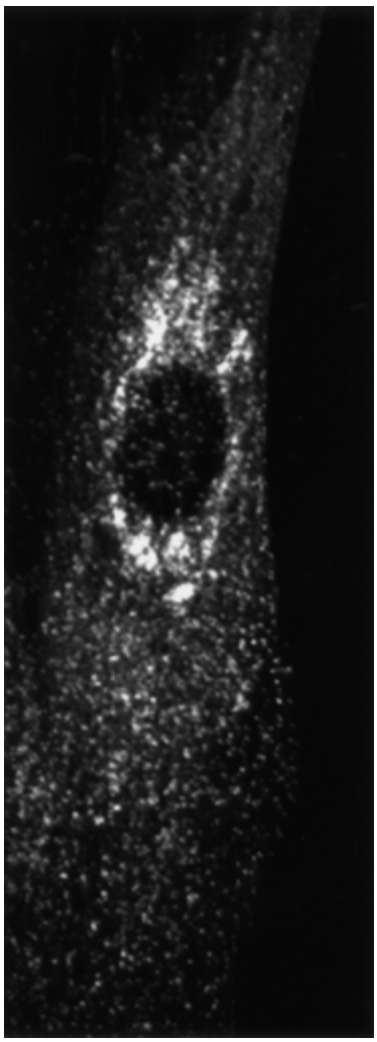


Figure 4A.21 Clathrin distribution in human fibroblasts labeled with monoclonal antibody X22 (Affinity Bioreagents). Figure provided by Juan Bonifacino, NICHD/NIH.

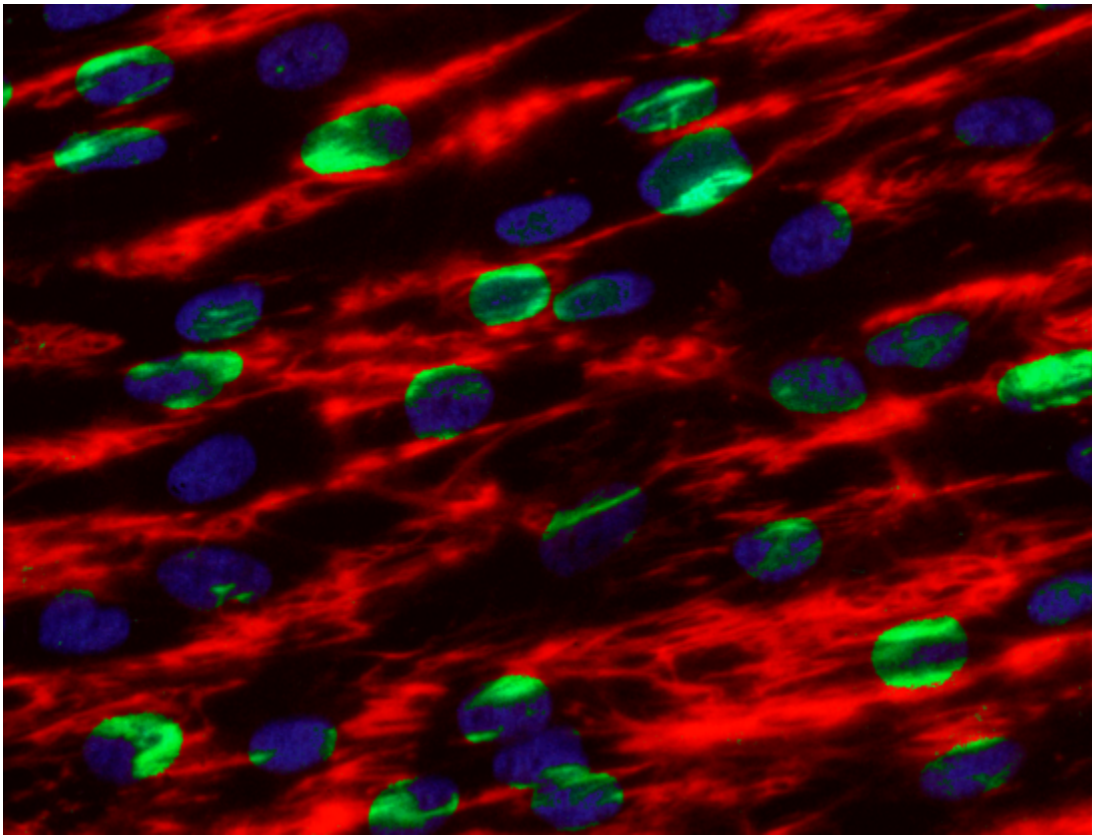


Figure 4A.22 Distribution of the extracellular matrix protein fibronectin in cells labeled with rhodamine-conjugated antibody to fibronectin (red) and DAPI (blue). Figure provided by Kenneth Yamada, NIDR/NIH.

CHAPTER 5
Characterization of Cellular Proteins

INTRODUCTION

Proteins are the most diverse, versatile components of living organisms. They are widely variable in size, shape, charge, stability, solubility, and other physical-chemical properties. This diversity derives from their being composed of combinations of about 20 distinct amino acids arranged in any number and sequence. The polypeptide chain folds into a particular conformation which is largely determined by the sequence of amino acids. The virtually limitless number of possible conformations allows proteins to perform a variety of cellular functions. Proteins can act as catalysts, receptors, hormones, carriers, membrane channels, molecular motors, cytoskeletal elements, protective shells, regulators of gene expression, components of the translation and trafficking machineries, and so on. They are found in every subcellular compartment and some are secreted into the extracellular space, where they modify the surroundings of the cell or exert effects on other cells.

The structural and functional plasticity of proteins, and their critical roles in most cellular processes, make them prime subjects of interest to cell biologists. There are many excellent methodologies to characterize the structural features of purified proteins. These methodologies have been covered in several books. Most often, however, cell biologists are confronted with the task of characterizing specific proteins in mixtures with other proteins, as is the case for whole cells, subcellular fractions, cell extracts, and partially purified proteins. In many cases, the protein of interest is a minor component of these mixtures. Over the years, scientists have learned how to take advantage of the structural and functional diversity of proteins to devise methods that allow the characterization of specific proteins in complex mixtures. These methods consist of separating proteins according to their physical-chemical properties using techniques such as ultracentrifugal sedimentation on sucrose gradients or gel filtration, followed by detection of the proteins by some specific enzymatic activity or, more often, by their reactivity with specific antibody reagents. This type of approach is the focus of the protocols presented in this chapter.

The chapter begins with an overview of the physical state of proteins within cells (*UNIT 5.1*). The overview discusses the classification of proteins into globular, fibrous, and transmembrane proteins. This is followed by a description of the modes of association of proteins with membranes and the determinants of topology of transmembrane proteins. *UNIT 5.1* then discusses the factors that determine the physical heterogeneity of proteins, including size, charge, chemical modifications, and oligomeric or multimeric assembly. This leads to a description of some of the methods that are used to characterize the properties of proteins in complex mixtures.

Transmembrane proteins can assume a number of different topologies. For example, the amino and carboxy termini of the polypeptide chains can be exposed to either the lumenal/extracellular or cytosolic aspects of intracellular organelles. The simplest integral membrane proteins have only one membrane-spanning segment. However, many polypeptide chains span the membrane two or more times. Although the topology of transmembrane proteins can, in many instances, be inferred from amino acid sequence

Contributed by Juan S. Bonifacino
Current Protocols in Cell Biology (2000) 5.0.1-5.0.2

information, experimental verification is always required. *UNIT 5.2* includes a set of protocols designed to assess the topologies of proteins within membranes. The first is based on the accessibility of proteins within intact cells or membrane-bound organelles to externally added proteases. The effects of proteolysis are then generally evaluated by one-dimensional polyacrylamide gel electrophoresis (*UNIT 6.1*), often in combination with immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*). Another protocol in *UNIT 5.2* is based on the accessibility of protein epitopes to antibody reagents, using immunofluorescence microscopy as a means of detecting the antibody (also see *UNIT 4.3*). Methods for marking proteins with epitope tags are presented as a complement to the accessibility-based protocols. *UNIT 5.2* ends with a method to assess topology using reporter fusions in yeast cells.

Sizing of a protein in its native state can be accomplished by determining its hydrodynamic parameters, such as the sedimentation coefficient and Stokes' radius. Knowledge of these parameters in many cases allows calculation of the molecular masses of the native protein or protein complexes. *UNIT 5.3* contains a detailed description of one of the most commonly used methods for estimating the size of cellular proteins: sedimentation-velocity analysis on sucrose gradients. This method consists of analyzing the migration of proteins on a continuous gradient of sucrose upon application of a centrifugal field. After fractionation of the gradient, proteins are detected by measurement of an intrinsic activity or by polyacrylamide gel electrophoresis (*UNIT 6.1*) in combination with immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*). *UNIT 5.3* also describes the use of equations to calculate the hydrodynamic parameters of proteins from sedimentation experiments.

UNIT 5.4 presents several methods for determining the nature of protein-membrane associations (i.e., distinguishing peripheral versus integral membrane proteins). Although most integral membrane proteins are associated with membranes via hydrophobic polypeptide segments, a subset are tethered to the membranes by glycosyl phosphatidylinositol (GPI) groups. This unit presents a method for examining membrane association through GPI groups, based on hydrolysis with phosphatidylinositol-specific phospholipase C (PI-PLC). GPI-anchored proteins and some transmembrane proteins are resistant to solubilization by certain nonionic detergents such as Triton X-100. For some, this is due to their association with plasma membrane domains enriched in glycosphingolipids and cholesterol. These domains are sometimes contained within invaginations of the plasma membrane known as caveolae. This unit provides a protocol for solubilization of Triton-insoluble plasma membrane proteins using detergents such as octylglucoside or CHAPS. Also included are procedures for isolation of caveolar membrane domains.

In addition to sedimentation-velocity analysis (*UNIT 5.3*), another commonly used method for estimating the size of cellular proteins in their native state is size exclusion chromatography (also known as gel filtration; *UNIT 5.5*). In this method, a solution of proteins (the "mobile phase") is passed over a column containing a matrix of porous particles (the "stationary phase"). Proteins partition according to their ability to penetrate the interior of the particles, such that larger proteins elute earlier than smaller ones. The size of the proteins can be estimated by comparison with the behavior of protein standards of known molecular size. The parameter that best characterizes the behavior of proteins on size exclusion chromatography is the Stokes radius, R_s. Knowledge of this parameter, and of parameters derived from sedimentation-velocity analysis (*UNIT 5.3*), allows calculation of the molecular weights of proteins.

Juan S. Bonifacino

Overview of the Physical State of Proteins Within Cells

The word protein comes from the Greek word *proteios*, meaning primary. And, indeed, proteins are of primary importance in the study of cell function. It is difficult to imagine a cellular function not linked with proteins. Almost all biochemical catalysis is carried out by protein enzymes. Proteins participate in gene regulation, transcription, and translation. Intracellular filaments give shape to a cell while extracellular proteins hold cells together to form organs. Proteins transport other molecules, such as oxygen, to tissues. Antibody molecules contribute to host defense against infections. Protein hormones relay information between cells. Moreover, protein machines, such as actin-myosin complexes, can perform useful work including cell movement. Thus, studying proteins is a prerequisite in understanding cell structure and function.

The physical characterization of proteins began well over 150 years ago with Mulder's characterization of the atomic composition of proteins. In the latter half of the nineteenth century Hoppe-Seyler (1864) crystallized hemoglobin and Kühn (1876) purified trypsin. A variety of physical methods have been developed over the years to increase convenience and precision in the characterization and isolation of proteins. These include ultracentrifugation, chromatography, electrophoresis, and others. In many instances our understanding of cell proteins parallels the introduction and use of new techniques to examine their structure and function.

PROTEIN CLASSIFICATIONS

All proteins are constructed as a linear sequence(s) of various numbers and combinations of ~20 α-amino acids joined by peptide bonds to form structures from thousands to millions of daltons in size. Proteins are the most complex and heterogeneous molecules found in cells, where they account for >50% of the dry weight of cells and ~75% of tissues.

Proteins can be classified into three broad groups: globular, fibrous, and transmembrane (Fig. 5.1.1; Table 5.1.1). Globular proteins are, by definition, globe-shaped, although in prac-

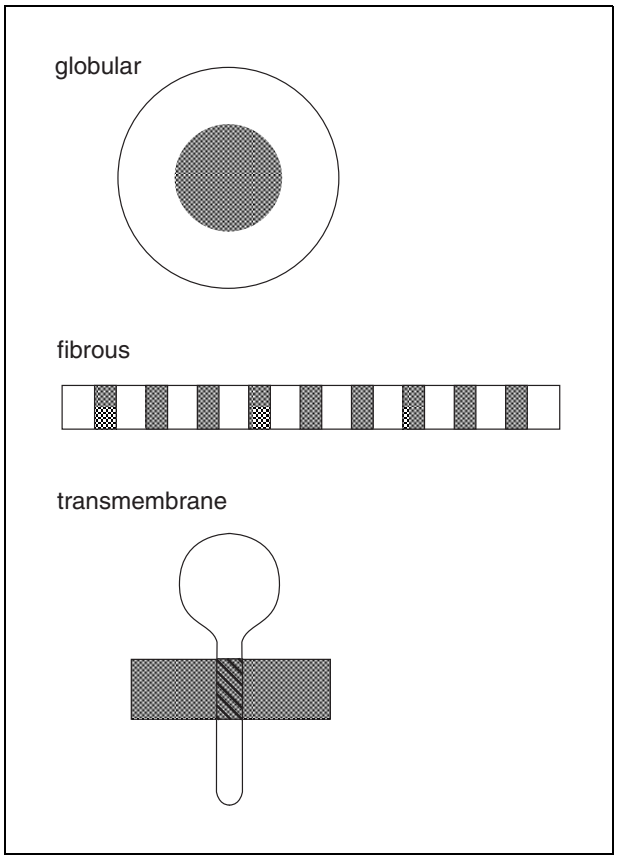


Figure 5.1.1 General classifications of proteins. In these schematic representations of globular, fibrous, and transmembrane proteins, hydrophobic regions are shaded. Note that the disposition of hydrophobic residues often reflects the protein class.

Contributed by Howard R. Petty
Current Protocols in Cell Biology (1998) 5.1.1-5.1.10

Table 5.1.1 Broad Classifications for Proteins[a]

Type	Location/type	Examples
Globular	Intracellular	Hemoglobin, lactate dehydrogenase, cytochrome *c*
	Extracellular	Serum albumin, immunoglobulins, lysozyme
Fibrous	Intracellular	Intermediate filaments, tropomyosin, lamins
	Extracellular	Collagen, keratin, elastins
Transmembrane	Single pass	Insulin receptor, glycophorin, HLAs[b]
	Multipass	Glucose transporter, rhodopsin, acetylcholine receptor

[a]Additional information regarding fibrous and transmembrane proteins can be found in Squire and Vibert (1987) and Petty (1993). Information concerning globular proteins can be found in numerous books on proteins and enzymes such as Schultz and Schirmer (1979).

[b]Human histocompatibility leukocyte antigens.

tice they can be spherical or ellipsoidal. Globular proteins are generally soluble in aqueous environments. Examples of globular proteins are hemoglobin, serum albumin, and most enzymes. Fibrous proteins are elongated linear molecules that are generally insoluble in water and resist applied stresses and strains. Collagen is a physically tough molecule of connective tissue. Just as collagen gives strength to connective tissues, intermediate filaments linked to desmosomes give strength to cells in tissues. The third general class of proteins, transmembrane proteins, contain a hydrophobic sequence buried within the membrane; these proteins are discussed more fully below (see Membrane Proteins).

These protein categories are not mutually exclusive. For example, the nominally fibrous intermediate filament proteins also have globular domains. Similarly, transmembrane proteins almost always possess globular domains. Thus, these definitions serve as a useful guide but should not be rigidly applied.

HYDROPATHY PATTERNS OFTEN REFLECT A PROTEIN'S CLASSIFICATION

A key physical feature of proteins is their hydropathy pattern (i.e., the distribution of hydrophobic and hydrophilic amino acid residues). Indeed, hydrophobic interactions provide the primary net free energy required for protein folding. Figure 5.1.1 illustrates the disposition of hydrophobic amino acids in proteins. In an intact globular protein, hydrophobic amino acids are generally shielded from the aqueous environment by coalescing at the center of the molecule, with the more hydrophilic residues exposed at its surface. However, the linear arrangement of hydrophobic residues fluctuates in an apparently random fashion. The α helices within globular proteins may express a hydrophobic face oriented toward the center of the protein. (Within these helices hydrophobic residues are nonrandomly positioned every three or four amino acids to yield a hydrophobic face.) For coiled-coil α helix–containing fibrous proteins, such as tropomyosin and α-keratin, hydrophobic residues at periodic intervals allow close van der Waals contact of the chains and potentiate assembly as hydrophobic residues are removed from the aqueous environment (Schulz and Schirmer, 1979; Parry, 1987). Secondarily, regularly spaced charged groups can also contribute to the shape of fibrous proteins (Schulz and Schirmer, 1979; Parry, 1987). Transmembrane proteins provide a rather different physical arrangement of hydrophobic residues in which hydrophobic residues are collected primarily into a series of amino acids that is embedded within a cell membrane.

One important means of analyzing the hydropathy of a sequenced protein is a hydropathy plot (Kyte and Doolittle, 1982). In this method, each amino acid residue is assigned a hydropathy value, an ad hoc measure that largely reflects its relative aqueous solubility; these values are plotted after being averaged. The successful interpretation of hydropathy plots

depends on the parameters chosen for averaging. The parameters are the number of residues averaged (amino acid interval or "window") and how many amino acids are skipped when calculating the next average (step size). Using this approach with a window of ~10 residues, it is often possible to find the positions of hydrophobic residues coalescing near the interior of globular proteins. The method is particularly useful in predicting transmembrane domains of proteins, generally with a window of ~20 amino acids. To detect the repetitious pattern of coiled-coil fibrous proteins, however, windows smaller than the repeat length would be required.

MEMBRANE PROTEINS

In addition to their presence in the extracellular and intracellular milieus, proteins are also found in association with biological membranes. Proteins constitute one-half to three-quarters of the dry weight of membranes. Membrane proteins perform a broad variety of functions including intermembrane and intercellular recognition, transmembrane signaling, most energy-harvesting processes, and biosynthesis in the endoplasmic reticulum (ER) and Golgi complex.

Membrane proteins have been traditionally characterized as integral (or intrinsic) or peripheral (or extrinsic) on the basis of operational criteria. Peripheral membrane proteins are associated with membrane surfaces and can be dislodged from membranes using hypotonic or hypertonic solutions, pH changes, or chelation of divalent cations. Components of the erythrocyte membrane skeleton, for example, are peripheral membrane proteins. Although most peripheral proteins are removed by washing a sample with buffers, integral proteins cannot be removed by such treatments. To isolate integral membrane proteins, which are embedded within the lipid bilayer, one must use detergents that disrupt the bilayer and bind to the proteins, thus solubilizing them. In general, integral membrane proteins have a portion of their peptide sequence buried in the lipid bilayer whereas peripheral proteins do not. However, the discovery of glycosylphosphatidylinositol (GPI)-linked membrane proteins added to the ambiguity of the situation. GPI-linked proteins are globular proteins with no membrane-associated peptide sequence, yet they require harsh conditions for solubilization.

As the technology for studying membrane proteins improved, it became necessary to develop a more precise vocabulary to describe membrane proteins. Transmembrane integral membrane proteins have at least one stretch of amino acids spanning a membrane. Membrane proteins are classified as type I, II, III, or IV depending on the nature of their biosynthesis and topology in membranes (Spiess, 1995; Table 5.1.2 and Fig. 5.1.2). The biosynthetic insertion of these proteins in membranes is, in turn, dependent on the presence or absence of a cleavable signal peptide, the relative positions of the hydrophobic transmembrane domain and positively charged topogenic signals, and/or

Table 5.1.2 Definitions of Integral Transmembrane Proteins

Type	Definition	Examples
I	An N-terminal–cleavable signal peptide is removed at the luminal face yielding a luminal N terminal during biosynthesis. (Positive charges are found on C-terminal side of first long hydrophobic sequence after the signal peptide.)	LDL receptor, insulin receptor, glycophorin A, thrombin receptor
II	An N-terminal–uncleaved signal peptide leads to a cytoplasmic N terminus. (Positive charges are generally found on N-terminal side of first long hydrophobic sequence.)	Transferrin receptor, sucrase/isomaltase, band 3
III	A long N-terminal hydrophobic sequence is followed by a sequence of positive charges. This leads to a luminal N terminus in the absence of a cleavable signal peptide.	β-Adrenergic receptor, cytochrome P450
IV	A short C terminus is present at the luminal side of membrane. A large N terminus is exposed at the cytoplasmic face.	Synaptobrevin, UBC6

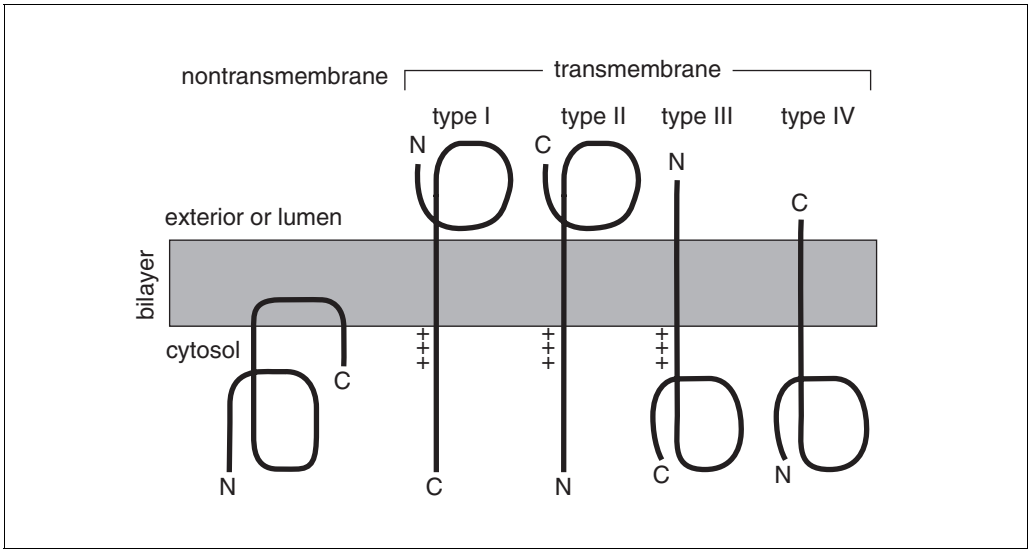


Figure 5.1.2 Membrane proteins containing hydrophobic anchors. A nontransmembrane or monotopic membrane protein is anchored to the membrane via a hydrophobic amino acid sequence. Transmembrane proteins are classified as types I, II, III, and IV (Table 5.1.2). The first transmembrane segment of a multispanning membrane protein can be inserted as in type I, II, or III proteins. This segment functions as a start-transfer peptide. Subsequent transmembrane segments will function as stop-transfer and start-transfer sequences, resulting in a multispanning membrane topology (see Fig. 5.2.1).

the mechanism of nascent protein delivery to the ER.

Type I membrane proteins are synthesized with an amino-terminal signal sequence that is inserted into the ER membrane. When the signal sequence is proteolytically removed in the ER lumen, a new luminal amino terminus is exposed. A series of positively charged residues at the C-terminal side of the first hydrophobic transmembrane domain following the signal sequence generally denotes the end of the first transmembrane domain (von Heijne and Gavel, 1988). Although a hydrophobic transmembrane domain followed by a positive sequence of amino acids is sufficient to act as a stop-transfer signal, this motif is not required for stop-transfer events and other, less well-understood regulatory mechanisms are also involved (Andrews and Johnson, 1996).

Membrane proteins types I, II, and III are delivered to the ER membrane via a signal recognition particle (SRP)-dependent mechanism. In contrast to type I proteins, type II and III membrane proteins do not have a cleavable N-terminal signal sequence. Instead, they have an internal hydrophobic signal that acts as both a signal sequence for ER delivery and a transmembrane domain in the mature protein. Type II proteins have a cytoplasmic amino terminus and a luminal (or extracellular) carboxyl terminus. In this case a positively charged sequence of amino acids at the N-terminal side of the first hydrophobic sequence causes the amino terminus to be retained at the cytoplasmic face of the ER membrane. Thus the internal uncleaved signal peptide becomes the transmembrane domain of the mature protein.

Type III membrane proteins have the same overall topology as type I proteins, but they are inserted into membranes by a different mechanism. In type III proteins the first hydrophobic sequence of amino acids is immediately followed by a series of positively charged amino acids. Thus, the first hydrophobic sequence becomes the transmembrane domain of the protein, with the amino terminus at the luminal face of the membrane.

Type IV membrane proteins are characterized by a large, cytoplasmically exposed amino-terminal domain and a short carboxyl-terminal domain facing the lumen. Importantly, these proteins are delivered to the ER by an unknown SRP-independent mechanism.

In addition to the single-pass membrane proteins just described, integral membrane proteins can display zero, two, three, or more transmembrane domains. Some membrane proteins, such as cytochrome b_5, have protein segments buried in the hydrophobic core of membranes but do not cross the membrane. Membrane proteins with multiple membrane-spanning domains are classified as type I, II, or

III depending on the topogenic signals in the first transmembrane domain. For example, a multispan membrane protein with a cleavable signal sequence, luminal amino terminal, and a positively charged sequence following the first transmembrane domain from the amino terminal, such as the thrombin receptor, is a type I membrane protein. The remaining transmembrane domains are inserted into the bilayer depending on the orientation of the first transmembrane domain. Multispanning type II and III proteins are similarly defined according to the properties of their single-spanning counterparts.

In addition to hydrophobic protein sequences acting as membrane anchors, membrane proteins may also carry bilayer-associated hydrophobic lipid components. These hydrophobic lipid anchors define three broad groups of lipid-modified proteins: fatty acylated, isoprenoid-linked, and GPI-linked (Fig. 5.1.3). Several cytosolic transmembrane proteins have been identified that contain a covalently attached hydrophobic fatty acyl residue. For example, fatty acids, including palmitic, palmitoleic, *cis*-vaccenic, and cyclopropylene-hexadecanoic, are covalently linked to the amino terminus and the amino-terminal glycerylcysteine of *E. coli* lipoprotein. Moreover, palmitate- and myristate-labeled transmembrane proteins have been observed in eukaryotic cells (e.g., Schlesinger et al., 1980).

In both isoprenoid-linked and GPI-linked proteins, globular proteins become membrane-bound due to the addition of a hydrophobic lipid moiety. Certain proteins containing conserved cysteine residues at or near the C-terminus are modified by prenylation, in which a farnesyl or geranylgeranyl isoprenoid tail is added (Zhang and Casey, 1996). This hydrophobic moiety promotes protein association with the cytoplasmic face of cell membranes. Notably, cytosolic G proteins and protein kinases that participate in signal transduction are prenylated.

GPI-linked proteins are a major class of membrane proteins (Cardoso de Almeida, 1992; Englund, 1993). In contrast to isoprenoid-modified proteins, GPI-linked proteins are attached to the luminal or extracellular face of membranes via a glycosylphosphatidylinositol anchor of variable structure (e.g.,

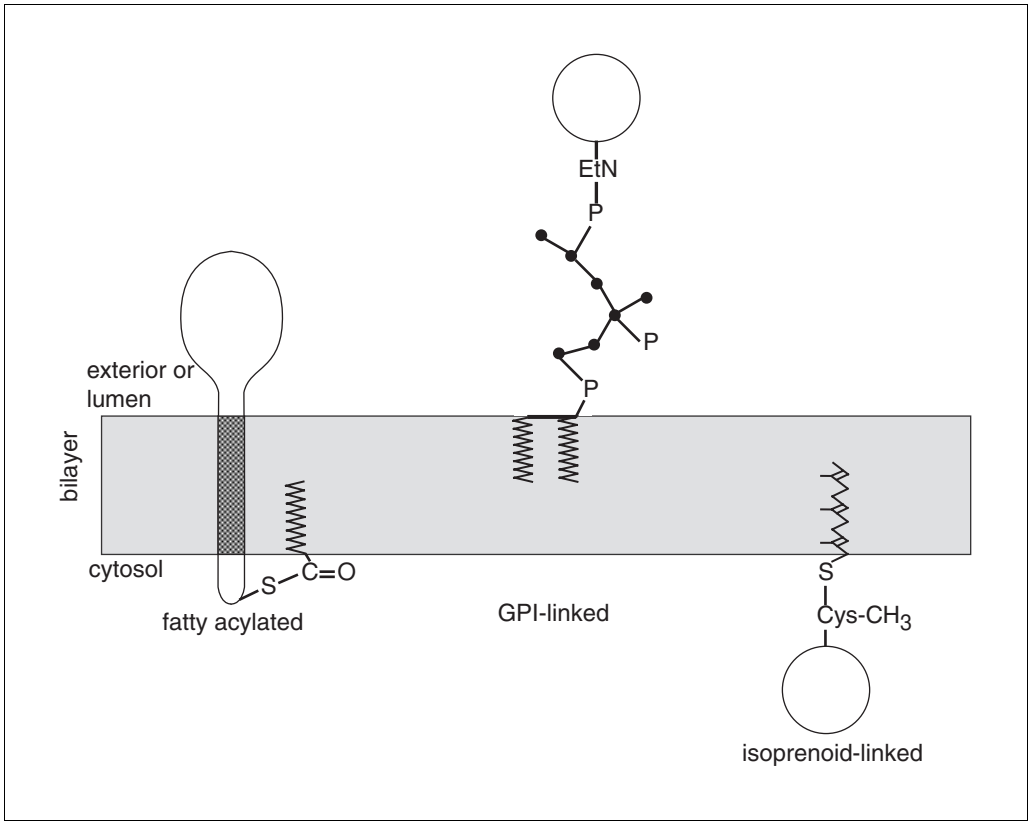


Figure 5.1.3 Membrane proteins containing lipid moieties. In the simplest case, fatty acids can be covalently attached to transmembrane proteins. Hydrophobic tails are also attached to proteins to form isoprenoid-linked proteins. A third class of lipid-attached proteins are the GPI-linked proteins. Hydrophobic regions are shaded.

Fig. 5.1.3). Well over 100 GPI-linked proteins have been identified in cells, where they perform numerous functions including acting as enzymes and receptors. The ability of GPI-linked proteins, which possess no transmembrane or cytosolic sequences, to elicit transmembrane signals seems paradoxical. However, studies have suggested that interactions with other proteins, including transmembrane integrins (Petty and Todd, 1996), contribute to transmembrane signaling of these proteins. Although GPI-linked proteins must collaborate with other membrane proteins to elicit signals, they do possess certain functional advantages. First, GPI-linked proteins (and isoprenoid-linked proteins as well) diffuse in membranes much faster than transmembrane proteins and thus relay information faster. Second, certain cells, such as leukocytes, can rapidly shed their GPI-linked proteins, thus altering their functional properties in seconds. Although the importance of lipid-linked membrane proteins has only recently been appreciated, the impact of these structures on our understanding of cell properties is growing rapidly.

ADDITIONAL FACTORS AFFECTING THE PHYSICAL HETEROGENEITY OF PROTEINS

Additional factors contributing to the physical-chemical heterogeneity of proteins are size, charge, chemical modifications, and assembly. A typical amino acid has a molecular mass of ~110 Da, and a small protein has a molecular mass of a few thousand daltons (e.g., for insulin, M_r = 5733). Large proteins have molecular masses of several hundred thousand daltons. When proteins are assembled to form large multiprotein complexes such as ribosomes, molecular masses are well into the millions. The diameters of these structures range from 4 Å for an individual amino acid to ~30 nm for a ribosome.

Electrostatic charge is of major importance in protein structure and function. Charged proteins are more soluble than uncharged proteins. The large number of positive charges on histones allow them to bind DNA. The spatial arrangement of charges on cytochrome *c* allows it to bind the complementary charges of its oxidase and reductase, thereby orienting the proteins prior to electron transfer. Similarly, the arrangement of charges on the apoprotein and receptor for low-density lipoprotein (LDL) allows for lock-and-key–like interactions (Petty, 1993). In addition to structural and binding considerations, electrostatic interactions play a regulatory role. For example, the phosphorylation and dephosphorylation of insulin receptors alter electrostatic interations between the active site and a regulatory loop of the kinase domain, thereby changing its three-dimensional shape (Hubbard et al., 1994). This changes the V_{max} of the kinase, thus triggering intracellular signals.

In addition to the types of physical heterogeneity listed above, >100 distinct chemical modifications of proteins have been observed. These include, for example, glycosylation, ubiquitin attachment, phosphorylation, acetylation, and hydroxylation (Table 5.1.3). Thus, proteins undergo extensive physical-chemical modification.

PROTEIN ASSEMBLIES

Proteins can be assembled in a variety of states in both aqueous media and within membranes. Protein assembly into complex supramolecular structures plays vital roles in enzyme regulation, cell skeleton formation, and transmembrane signaling. Both covalent bonds and noncovalent bonds participate in protein assembly. One frequently encountered covalent mechanism of protein assembly is the formation of disulfide bonds. These covalent linkages often form during protein maturation. They can link two separate proteins together or two portions of the same protein. For example, the two chains of insulin molecules are held together by disulfides, as are the two chains of its membrane receptor. However, disulfide bond formation is mostly limited to oxidative environments such as the ER lumen and the exterior face of the cell surface.

One of the best-known examples of noncovalent assembly is the formation of hemoglobin tetramers. Polymerization is another frequently encountered mechanism for protein assembly in cells. The globular protein actin polymerizes to form microfilaments in the absence of covalent bond formation. Intermediate filaments are formed by the polymerization of fibrous proteins. Under certain circumstances transmembrane proteins polymerize as well; bacteriorhodopsin, for example, forms two-dimensional pseudocrystals called purple membranes. Protein assemblies formed from various numbers of similar units are homodimers, homooligomers, and homopolymers.

Assembly of protein structures from dissimilar subunits is more common than assembly from identical subunits. For example, heterodimers are formed from the α and β chains of integrins within cell membranes. Complex

Table 5.1.3 Common Physical-Chemical Modifications of Proteins[a]

Modification	Example
Homodimerization	Transferrin receptor
Homooligomerization	*S. typhimurium* glutamine synthetase
Homopolymerization	Actin
Heterodimerization	Integrins
Heterooligomerization	Histones, proteasomes
Heteropolymerization	Ribosomes
Proteolytic cleavage	Signal peptide cleavage in ER
Prosthetic group addition	Heme addition to cytochromes and hemoglobin
Oxidation-reduction	Disulfide bond formation in ER
Glycosylation	Glycoprotein maturation
Phosphorylation	Regulation of protein function, such as the tyrosine kinase activity of insulin receptors
Acetylation	Blockage of N-termini of certain membrane proteins
Ubiquitination	Ubiquitin-dependent proteolysis via proteasomes, histones
Hydroxylation	Proline hydroxylation on collagen
Fatty acylation	Insulin receptors, *E. coli* lipoprotein
Isoprenylation	G proteins
GPI addition	Alkaline phosphatase, urokinase receptors

[a]For details, see Freedman and Hawkins (1980, 1985), Schlesinger et al. (1980), Englund (1993), and Zhang and Casey (1996).

heterooligomeric and heteropolymeric structures vary from relatively small structures such as histone octamers, which bind to DNA in the nucleus, to large particles such as ribosomes, found both in the cytosol and attached to nuclear and ER membranes. The signal recognition particle is a relatively small heterooligomeric structure, composed of one RNA subunit and six proteins, that potentiates the delivery of secretory and most membrane proteins to the ER membrane. Membrane-associated heterooligomeric structures have also been observed. One of the best examples of such structures is the components of the electron transport systems in chloroplasts and mitochondria (Petty, 1993). For example, the ubiquinone-cytochrome *c* reductase is composed of eleven different subunits. Thus, proteins can be assembled in a variety of manners within cells.

Although some protein assemblies, such as intermediate filaments, are static structures, many are dynamic structures which provide functional flexibility. For example, microfilaments can rapidly assemble and disassemble. In addition to the physical changes in assembly state, compositional dynamics is also observed. For example, interferon γ treatment alters the composition of proteasomes. Developmental changes in protein composition are also observed. As an example, fetal and newborn forms of a component of cytochrome *c* reductase are expressed in humans. Thus, protein assemblies can be characterized by both physical and compositional dynamics.

ALTERING THE SOLUBILITY OF PROTEINS: PROTEIN EXTRACTION

The in vitro characterization of cellular proteins begins with their extraction from tissues or cells into a buffer. With the exception of globular secretory proteins, such as those found in plasma, proteins are generally not easily accessible for experimental manipulation. For example, many fibrous proteins are not soluble in aqueous buffers. Cellular proteins are entrapped within or on a cell and therefore must be extracted from the cell in a soluble form.

A variety of methods including osmotic lysis, enzyme digestion, homogenization using a blender or mortar and pestle, and disruption by French press and sonication have been employed to disrupt cells. For a cytosolic protein such as hemoglobin, no further extraction from the sample is necessary. However, many important cellular proteins, such as those associated with membranes, cytoskeletal components, and DNA, remain insoluble. To further solubilize cell proteins, both nonionic (e.g., Triton

X-100) and ionic (e.g., sodium dodecyl sulfate) detergents are often employed. Detergents are small amphipathic molecules that interact with both nonpolar and polar environments. Detergents disrupt membranes. They also bind to hydrophobic regions of proteins, such as their transmembrane domains, thereby replacing the unfavorable contacts between hydrophobic protein regions and water with the more favorable hydrophilic domains of the detergent. Thus, instead of the hydrophobic regions of the insoluble protein forming an aggregate in the bottom of a test tube, the protein becomes soluble and can be employed in most in vitro analyses.

In addition to detergents, several other solubilization strategies are useful for the extraction and in vitro characterization of proteins (Table 5.1.4). Chaotropic agents enhance the transfer of nonpolar molecules to aqueous environments by their disrupting influence on water structure. Chaotropic agents are generally large molecular ions such as thiocyanate (SCN^-), perchlorate (ClO_4^-), and trichloroacetate (CCl_3COO^-). Hydrophobic interactions are also reduced by exposure to organic solvents and low salt concentrations. Electrostatic interactions are reduced by high salt conditions; this decreases the Debye-Hückel screening length and coulombic attraction. To disrupt hydrogen bonds, high concentrations of urea or guanidine are often employed. More vigorous methods of sample denaturation using very low pH or harsh detergents such as sodium dodecyl sulfate are also used to diminish intermolecular contacts.

Once proteins are extracted, their size can be characterized by ultracentrifugation on sucrose gradients (*UNIT 5.3*), gel filtration chromatography, SDS-PAGE (*UNIT 6.1*), and other methods (Table 5.1.5). The charge characteristics of proteins can be assessed using isoelectric focusing and ion-exchange chromatography. Specific interactions, such as antigen-antibody and biotin-avidin interactions, can also be employed in the characterization and isolation of proteins. These are useful in immunoblotting (*UNIT 6.2*) and affinity chromatography methods.

LIMITATIONS OF THE IN VITRO MANIPULATION OF PROTEINS

The very act of isolating proteins perturbs their physical environment. Although this is not

Table 5.1.4 Physical Bases of Common Protein Extraction and/or Elution Methods

Physical property perturbed	Agents
Hydrogen bonds	Urea or guanidine·HCl, pH changes
Ion pair interactions	High salt, pH changes
Hydrophobic interactions	Detergents, chaotropic agents, organic solvents, low salt

Table 5.1.5 Physical Bases of Common Protein Characterization and Isolation Methods

Physical property	Method	References to other units[a]
Solubility	Extraction with salts, detergents, and enzymes	Racker (1985)
Size	Ultracentrifugation on sucrose gradients	*CPCB 5.3*
	Gel filtration	*CPMB 10.9*
	SDS-PAGE	*CPCB 6.1*
Charge	Isoelectric focusing	*CPMB 10.3*
	Ion-exchange chromatography	*CPMB 10.10 & 10.13*
Biospecific interaction	Immunoblotting	*CPCB 6.2*
	Immunoaffinity chromatography	*CPMB 10.11A*
Hydrophobicity	Hydrophobic chromatography	*CPMB 10.15*
	Reversed-phase HPLC	*CPMB 10.12*

[a]For references in CPMB see Ausubel et al. (eds.) 1998.

often a major problem, a few cautionary notes should be made. The most primitive compartment of a cell, the cytosol, is a chemically reducing environment. Consequently, free sulfhydryl groups are observed in the cytosol; in fact, multiple cytosolic pathways help in preserving the proper redox conditions. On the other hand, the extracellular milieu and the luminal side of the ER are oxidative environments. The oxidizing condition within the ER is presumably due to the unidirectional transport of glutathione and cystine. Consequently, disulfides are frequently observed in the ER and extracellular environments. Thus, to prevent disulfide formation during manipulation, sulfhydryl blocking reagents such as iodoacetamide are included in extraction buffers (see *UNIT 7.2*). The cytosol is also a K^+-rich and Ca^{2+}-poor solution. These parameters should be considered in designing physiologically relevant experiments.

The experimental manipulation of membrane proteins is decidedly more difficult. The exterior face exists in a high Na^+ and Ca^{2+} solution that is oxidative; just the opposite is true for the cytoplasmic face. Since no appropriate solvent exists for such isolated proteins, experimental questions can be directed at properties associated with just one face of the molecule. A second limitation common to all in vitro studies of transmembrane proteins is that they must be solubilized using detergents. In addition to solubilizing a transmembrane protein, detergents can also bind to hydrophobic regions in the globular domain(s) of the protein, thus affecting the properties under study. One means of countering this problem is to test several detergents in the hope of finding one that retains the full biological activity of the purified protein.

Protein solubilization can also lead to loss of physiologically relevant protein-protein interactions. This can occur by simple dilution or by disruption of noncovalent interactions among proteins. For example, hemoglobin exists as a supersaturated solution in vivo which cannot be duplicated in vitro. Furthermore, protein-protein associations are generally stronger in the restricted confines of membranes than alter solubilization into a buffer. Thus, protein assemblies found in cells may disappear during solubilization. One means of countering these potential difficulties is to covalently cross-link protein assemblies prior to disruption and to solubilize proteins using mild detergents (e.g., Brij-58).

CONCLUSIONS

Structural motifs, especially stretches of hydrophobic amino acids, contribute to the shape of a protein and its classification as globular, fibrous, or transmembrane. Proteins are heterogeneous at many different levels including physical attributes, covalent modifications, and supramolecular assembly. The physical properties of proteins are used to characterize and isolate these molecules. For example, the size of a protein is examined by sedimentation on sucrose gradients (*UNIT 5.3*), gel filtration, and polyacrylamide gel electrophoresis (*UNIT 6.1*). Its charge is the key physical parameter in isoelectric focusing and ion-exchange chromatography. The units that follow contain detailed protocols describing the characterization of cellular proteins.

LITERATURE CITED

Andrews, D.W. and Johnson, A.E. 1996. The translocon: More than a hole in the ER membrane? *Trends Biochem. Sci.* 21:365-369.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 1998. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Cardoso de Almeida, M.L. 1992. GPI Membrane Anchors. Academic Press, New York.

Englund, P.T. 1993. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annu. Rev. Biochem.* 62:121-138.

Freedman, R.B. and Hawkins, H.C. 1980. The Enzymology of Post-Translational Modifications of Proteins, Vol. 1. Academic Press, New York.

Freedman, R.B. and Hawkins, H.C. 1985. The Enzymology of Post-Translational Modifications of Proteins, Vol. 2. Academic Press, New York.

Hoppe-Seyler, F. 1864. Über die chemischen und optischen eigenschaften des blutfarbstoffs. *Virchows Arch.* 29:233-235.

Hubbard, S.R., Wei, L., Ellis, L., and Hendrickson, W.A. 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* 372:746-754.

Kühn, W. 1876. Über das verhalten verschiedner organisirter und sogenannter ungeformter fermete. Über das trypsin (enzym des pankreas) [Reprint, *FEBS Lett.* 62:E3-E7 (1976).].

Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* 157:105-132.

Parry, D.A.D. 1987. Fibrous protein structure and sequence analysis. *In* Fibrous Protein Structure (J.M. Squire and P.J. Vibert, eds.) pp. 141-171. Academic Press, New York.

Petty, H.R. 1993. Molecular Biology of Membranes: Structure and Function. Plenum, New York.

Petty, H.R. and Todd III, R.F. 1996. Integrins as promiscuous signal transduction elements. *Immunol. Today* 17:209-212.

Racker, E. 1985. Reconstitutions of Transporters, Receptors, and Pathological States. Academic Press, New York.

Schlesinger, M.J., Magee, A.I., and Schmidt, M.F.G. 1980. Fatty acylation of proteins in cultured cells. *J. Biol. Chem.* 255:10021-10024.

Schultz, G.E. and Schirmer, R.M. 1979. Principles of Protein Structure. Springer-Verlag, New York.

Spiess, M. 1995. Heads or tails—what determines the orientation of proteins in the membrane. *FEBS Lett.* 369:76-79.

Squire, J.M. and Vibert, P.J. (eds.) 1987. Fibrous Protein Structure. Academic Press, New York.

von Heijne, G. and Gavel, Y. 1988. Topogenic signals in integral membrane proteins. *Eur. J. Biochem.* 174:671-678.

Zhang, F.L. and Casey, P.J. 1996. Protein prenylation: Molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* 65:241-269.

KEY REFERENCES

Racker, 1985. See above.

A wonderful little book on membrane protein manipulation which disproves the hypothesis that scientists can't write.

Tanford, C. 1961. Physical Chemistry of Macromolecules. Academic Press, New York.

A rigorous introduction to the physical properties of proteins, which remains useful several decades later.

Tanford, C. 1980. The Hydrophobic Effect. John Wiley & Sons, New York.

A very readable introduction to the hydrophobic effect.

INTERNET RESOURCES

http://www.expasy.ch

A user-friendly protein database including two-dimensional PAGE data and 3D protein structures.

ftp://ftp.pdb.bnl.gov/

Contains protein crystallography data.

Contributed by Howard R. Petty
Wayne State University
Detroit, Michigan

Determining the Topology of an Integral Membrane Protein

A variety of approaches have been developed for assigning the aqueous domains of integral membrane proteins to either side of a biological membrane. This unit describes three general methods that can be used to solve the topologies of eukaryotic membrane proteins whose primary sequences have been deduced from their corresponding DNA sequences: (1) testing of water-soluble domains for their accessibility to a reagent added to membranes exhibiting a defined orientation (see Basic Protocols 1 and 2), (2) epitope tagging (see Support Protocol), and (3) a genetic approach in which a reporter enzyme fused to various truncated fragments of a membrane protein acts as a sensor of sequences disposed intra- or extracytoplasmically (see Basic Protocol 3).

STRATEGIC PLANNING

Before initiating an experimental analysis, it is helpful to build a conceptual model of a protein's topology (see Fig. 5.1.2 for a representation of different types of integral membrane proteins). Inspection of the primary sequences of integral membrane proteins usually reveals a pattern of alternating stretches of hydrophilic (water-soluble) and hydrophobic (water-insoluble) amino acids. Very good computer-based approaches are used to illuminate these patterns (Kyte and Doolittle, 1982; Engelman et al., 1986). The results are presented as a graph, in which each amino acid is compared to the hydrophobicity of the amino acids immediately surrounding it in the linear sequence. When visual inspection of the graph reveals a hydrophobic stretch containing at least 18 to 20 amino acids, a segment is identified that is long enough to potentially span the membrane in an α-helical configuration (Deisenhofer et al., 1985). To build this conceptual model, the authors use MacVector software available form Oxford Molecular.

A representative hydropathy analysis of signal peptidase subunit SPC12 (Kalies and Hartmann, 1996) is shown in Figure 5.2.1A. Since this graph reveals an even number of putative transmembrane segments (two) for SPC12, the resulting model predicts that the N and C termini localize to the same side of the membrane. It should be noted that intra- and extracytoplasmic domains are not distinguishable using this analysis; thus, two opposing models for SPC12 topology are compatible with the analysis performed (see Fig. 5.2.1B). A hydropathy plot alone is, therefore, not a predictor of transmembrane orientation, nor is it assured that such a plot will identify all membrane-spanning segments. Experimental evidence is necessary to establish the orientations of integral membrane proteins within the lipid bilayer.

Protease Digestion

In this experimental approach (see Basic Protocol 1), a protease exhibiting a broad substrate specificity is used as a probe of membrane protein topology. When the enzyme is added to only one side of the target membrane or membrane vesicle, the size of the proteolytic fragment(s) generated reveals the accessible site(s) and hence the orientation across the membrane of the protein segment(s) containing this site or sites. A proteolytic fragment is detected using a specific antibody. Usually, a series of antipeptide antibodies are prepared, such that each hydrophilic domain is recognized by a specific antibody. The number of hydrophilic domains is usually greater than the number of hydrophobic domains by one. For example, SPC12 has two hydrophobic domains and three hydrophilic domains. Thus, a minimum of three antipeptide antibody preparations are needed to probe the hydrophilic domains of SPC12. Sometimes, however, an antipeptide antibody does

Contributed by Neil Green, Hong Fang, Kai-Uwe Kalies, and Victor Canfield
Current Protocols in Cell Biology (1998) 5.2.1-5.2.27

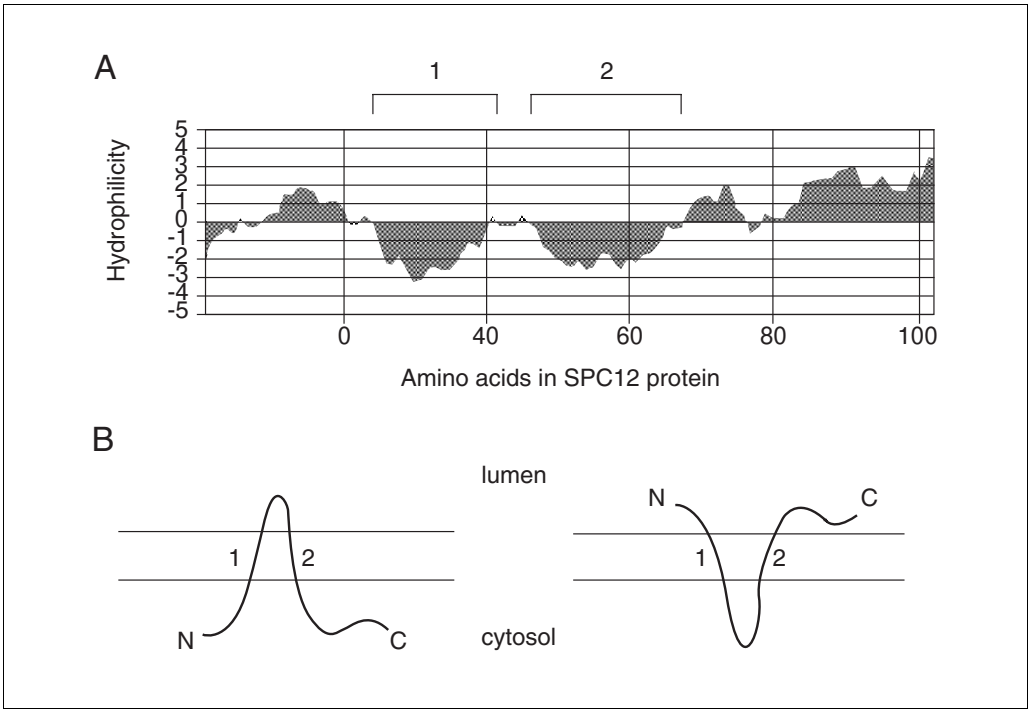


Figure 5.2.1 (**A**) Hydropathy analysis of SPC12 protein (Kalies and Hartmann, 1996). The data were obtained using the method of Kyte and Doolittle (1982). Hydrophilicity was examined using a window of seven amino acids. The positions of two potential transmembrane segments are shown. (**B**) Conceptualized topologies of SPC12. The data from (A) were used to construct two possible topologies. Hydrophobic segments 1 and 2 are shown spanning the membrane.

not recognize the corresponding peptide sequence in the intact protein; consequently, two or more antipeptide antibody preparations directed against a single hydrophilic domain may be needed.

In addition to this series of antipeptide antibodies, a control antibody is needed that recognizes a luminal protein of the closed membrane system to be analyzed. The orientation of a hydrophilic domain proteolyzed on addition of a protease can then be determined with confidence, providing the luminal protein control is resistant under this condition. A second control uses a mild detergent to solubilize the membranes. Protein sequences localized to both membrane surfaces and to the luminal space are exposed to the protease after detergent addition (Fig. 5.2.2 illustrates these points).

Unusually small hydrophilic domains may be resistant to proteolysis, even if they are exposed to the protease in the absence of detergent. Since addition of detergent often renders the entire protein susceptible, it is not always clear whether a domain that is resistant to proteolytic attack in the absence of detergent is localized to the lumen of the vesicle or just naturally resistant. As discussed below, the accessibility of such domains may be enhanced by using a foreign epitope (or multiple epitopes) inserted into the sequence of a small hydrophilic domain, thereby increasing its size (see Epitope Tagging and Reporter Gene Fusions).

The major advantage of using protease digestion to determine membrane protein topology is that proteins can be examined in their native form without any alterations to their primary sequences, such as those resulting from epitope additions and gene fusions (see Epitope Tagging and Reporter Gene Fusions). The picture derived from protease digestion should therefore reflect the true state of the protein in the cell, especially when the protein is examined in an in vivo expressed form (such as in whole cells or a specific cellular

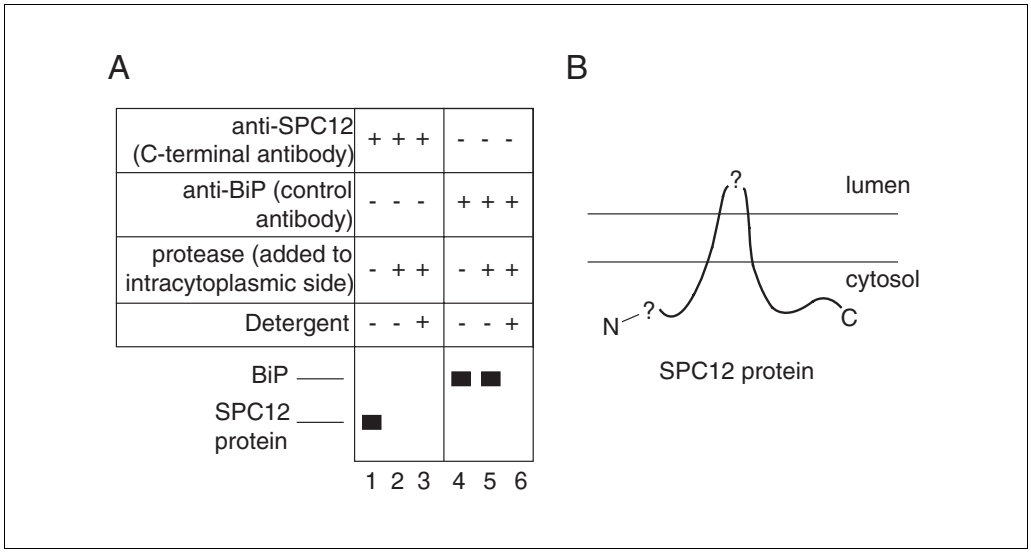


Figure 5.2.2 (**A**) Idealized immunoblot. The cartoon depicts an immunoblot of proteolyzed microsomes probed with an antibody directed against a C-terminal peptide of SPC12 or control antibodies directed against BiP, a luminal binding protein present in microsomal vesicles (Haas and Wabl, 1984; Bole et al., 1986). Addition or omission of a reagent from the mixture is indicated by + and –, respectively. The positions of BiP and SPC12 are indicated. For further discussion of (A), see Anticipated Results. (**B**) Deduced topology of SPC12's C terminus. The C terminus of SPC12 is presumed to localize to the cytosolic side of the membrane on the basis of the idealized results depicted in (A). The locations of the N terminus and presumed luminal domain of SPC12 cannot be assigned from these data and are therefore indicated with question marks.

organelle). Since membrane proteins are present both at the cell surface and at the surface of intracellular organelles, the literature should be reviewed to pick a procedure for enrichment of the desired membrane from a particular cell type. Examples of protease-digestion studies of membrane proteins residing within isolated organelles and organelle-derived vesicles are described by Graham et al. (1994), Kalies and Hartmann (1996), Kalish et al. (1995), and Li and Shore (1992). Regardless of the procedure chosen, it should produce membranes having a defined orientation, such that polypeptide domains exposed to the lumen, which corresponds to the extracytoplasmic side of the membrane for enriched organelle preparations and microsomal vesicles, are insensitive to the added protease in the absence of detergents.

A disadvantage of the protease digestion approach is that protein fragments must sometimes be identified to assess the topology of a particular region within a membrane protein. Since protein fragments are usually observed by SDS-PAGE (see Fig. 5.2.2) using the protease digestion protocol, small fragments (<2 kDa) may be difficult to detect.

While it is preferable to examine a protein in the membrane in which it resides normally, a procedure may not be available for enrichment of the desired membrane. As an alternative, an in vitro system of enriched microsomal membranes incubated cotranslationally with a membrane protein can be used (Walter and Blobel, 1983; Mize et al., 1986; see *UNIT 11.4*). This in vitro system is useful for examining proteins residing within the endoplasmic reticulum (ER) membrane and proteins transported from the ER to either the lysosome or another compartment of the secretory pathway (such as the cell surface).

Immunofluorescence Staining

An immunofluorescence staining technique is presented in this unit (see Basic Protocol 2) as a second experimental approach for determining membrane protein topology. In principle, this technique is similar to protease digestion, except that a fluorophore-con-

jugated secondary antibody is employed, instead of a protease, as a probe. A series of antipeptide antibodies is prepared, each recognizing a specific hydrophilic domain of the membrane protein under study. The antipeptide antibodies used for the protease digestion approach may be useful in the immunofluorescence staining approach provided that the antibodies recognize their corresponding epitopes in the membrane protein embedded within the lipid bilayer.

For the immunofluorescence staining protocol, the antibodies are added individually to the surface of an appropriate membrane, which must be closed and exhibit a defined orientation. When the antibody binds to an exposed hydrophilic domain, the antibody can be visualized using a fluorophore-conjugated secondary antibody that emits light detectable by confocal laser scanning microscopy or epifluorescence (see *UNIT 4.3* for a description of these microscopic techniques). Light detected from a bound antibody is evidence that the corresponding hydrophilic domain localizes to the exterior surface of the membrane examined. Protein domains exposed to the inside surface of the plasma membrane can also be probed after membrane permeabilization (Canfield and Levenson, 1993).

As with protease digestion, a major advantage of using the immunofluorescence staining approach is that a protein can be examined in its native form. A further advantage not available using protease digestion is that the orientation of a domain is determined using a microscope instead of detections of proteolytic fragments that may be very small. On the other hand, both immunofluorescence staining and protease digestion approaches suffer from the problem that particularly small hydrophilic domains may be inaccessible to the antibody or protease added. Thus, it may be possible to assign only a subset of the hydrophilic domains to a particular side of the membrane using either approach. Another potential problem with the immunofluorescence staining procedure is that the detergent used to permeabilize the membrane may expose an extracellular domain that is buried within a protein or protein complex. An extracellular domain inaccessible to the antibody in the absence of detergent could therefore be interpreted falsely to localize to the intracellular surface of the membrane.

Epitope Tagging

In determining membrane protein topology using epitope tagging (see Support Protocol), a foreign epitope is placed within the sequence of a membrane protein. The topology of the domain containing the inserted epitope can then be determined using protease digestion or immunofluorescence staining. The advantage of using a foreign epitope to assess topology is that a single antibody preparation available commercially is employed in lieu of a series of antipeptide antibodies, which can be expensive, time-consuming to prepare, and may not always recognize the intact membrane protein. Furthermore, a small epitope is inserted into a protein's sequence as opposed to fusion of a longer reporter domain, such as in construction of gene fusions (see Reporter Gene Fusion). This small epitope is less likely to alter the native orientation of the protein under study. In addition, multiple epitopes can be inserted into a single hydrophilic domain. The addition of multiple epitopes increases the likelihood that small hydrophilic domains will be accessible to the protease or antibody added. A foreign epitope may, however, inhibit the function of the protein into which it is placed. Since it may be unclear whether epitope insertion disrupts amino acids critical for activity or alters a protein's topology, the results obtained are more reliable when the epitope-tagged protein is shown to be functional in cells or in an in vitro assay.

A general guideline for minimizing interference with the topology of the target protein is to place the epitope ≥15 amino acids from the nearby transmembrane segment(s). This avoids interfering with charged residues that may be important for determining the

orientation of a transmembrane segment (Hartmann et al., 1989). In addition, tagging the N- and C-terminal ends of some membrane proteins should be avoided, since their termini often contain compartment-localization signals (for example, see von Heijne, 1984; Nilsson et al., 1989; Swinkels et al., 1991). However, if it is determined that such signals do not exist at the termini of the target protein, the N and/or C termini are often good sites for epitope tagging, because placement of the epitope at either end does not interrupt the linear sequence of the target protein internally. If the hydrophilic domain to be probed is very small, it may be impossible to place a foreign epitope >15 amino acids from the upstream and/or downstream transmembrane segment. In such instances, one can still place the epitope within the hydrophilic domain; however, caution should be used when interpreting the topological picture derived from study of a very small domain tagged with a foreign epitope.

A variety of epitope tags are available (see Background Information); however, the HA epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984) is recommended because it contains only two nonclustered charges and has been used previously in topological studies (e.g., Canfield and Levenson, 1993). Clusters of negatively charged amino acids have been shown to interfere with the integration of membrane proteins into the ER membrane, especially when the charge cluster is located near the luminal side of a transmembrane segment (Green and Walter, 1992).

Representative DNA fragments used to tag membrane proteins internally and at the N- and C-terminal ends are shown in Figure 5.2.3. These fragments can be synthesized (complementary strands must be prepared) and then inserted into naturally occurring restriction sites or into sites constructed by site-directed mutagenesis. The restriction sites used should be located in sequences corresponding to the protein's hydrophilic domains. To insert these DNA fragments, blunt-end restriction sites should be used when possible, or sites producing sticky ends can be made blunt-ended by standard molecular biological techniques. In addition, the DNA fragments shown in Figure 5.2.3 contain a blunt-end restriction site (*Pvu*II). This restriction site provides a means to introduce a second fragment encoding the HA epitope. Such double-tagged proteins may allow the target protein to be better recognized by anti-HA antibodies than their corresponding single-tagged versions (Canfield et al., 1996). Repetitive insertion of DNA fragments encoding the HA epitope into each new *Pvu*II site makes it possible to create proteins tagged multiple times within a single hydrophilic domain. It should be noted, however, that increasing the size of a particular domain significantly may render the protein nonfunctional.

Reporter Gene Fusions

A genetic approach for determining membrane protein topology is also available (see Basic Protocol 3). A series of gene fusions are constructed in which an enzyme (reporter moiety) is joined to various truncated fragments of an integral membrane protein (Fig. 5.2.4). The enzyme used for a reporter moiety is chosen because it exhibits activity only when placed on one side of the membrane. On expression of the fusions in vivo, measurements of enzyme activity indicate the orientation of the domains that contain the fusion joints (Fig. 5.2.5). This approach has been developed to examine proteins inserted into the membrane of the ER in the yeast *Saccharomyces cerevisiae* (Sengstag et al., 1990). However, numerous membrane proteins located throughout the secretory pathway and on the surfaces of cells contain signals for targeting to the ER membrane. These proteins are therefore amenable to topological analysis on integration within the ER membrane prior to trafficking to their ultimate destinations.

To avoid interfering with integration of the target protein, the joint connecting the membrane protein fragment and the reporter moiety should be placed ≥15 amino acids

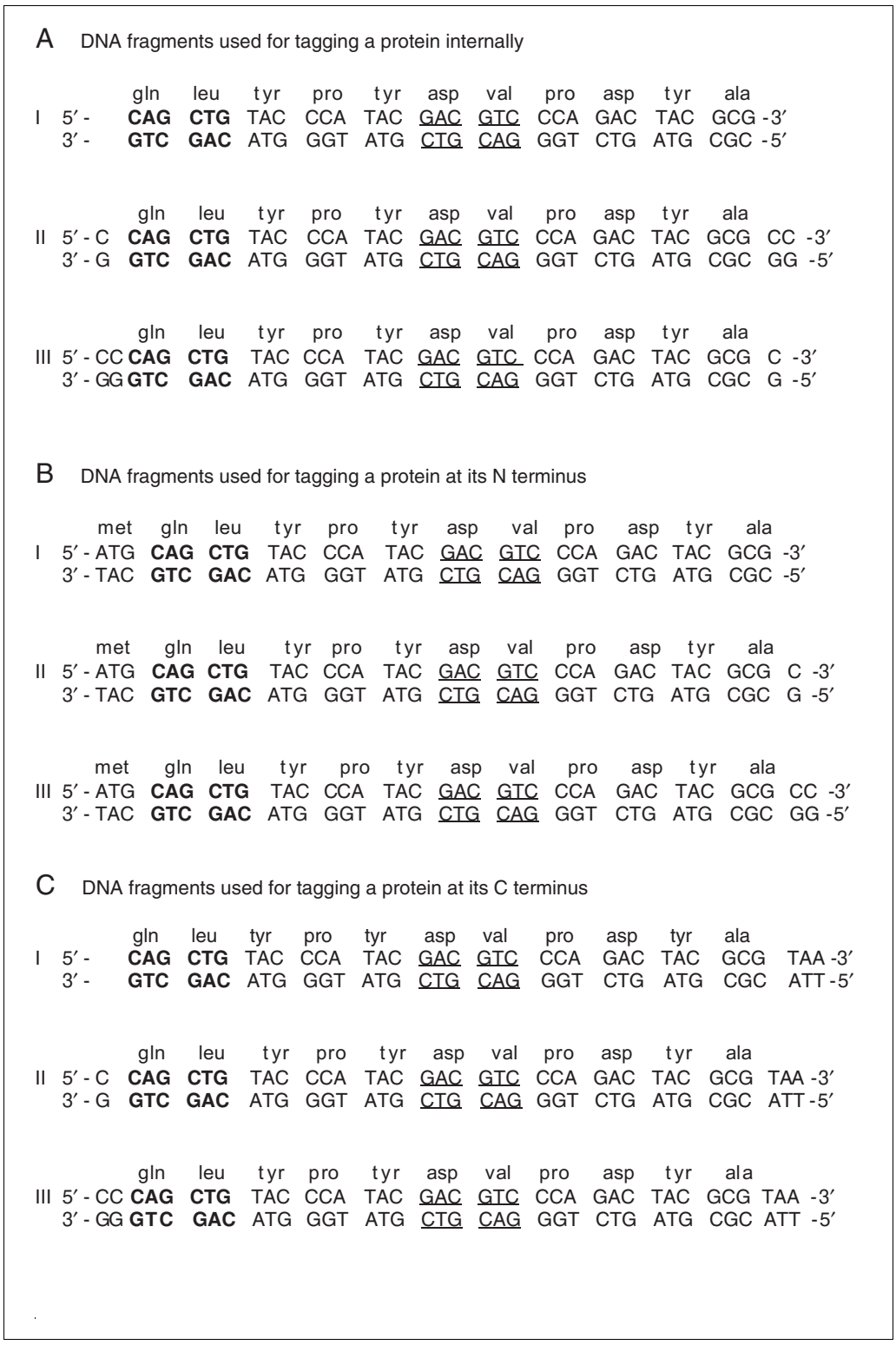


Figure 5.2.3 Examples of DNA fragments used for tagging proteins with the HA epitope. Use of fragments I, II, and III allow in-frame fusions to any reading frame internally (**A**), at the N terminus (**B**), or at the C terminus (**C**). If the translation initiation and termination codons endogenous to the gene of interest are used, the ATG codons shown in (B) and TAA codons shown in (C) can be eliminated from the fragments depicted here. *Pvu*II (bold) and *Aat*II (underlined) restriction sites are indicated.

from the upstream transmembrane segment. When probing a hydrophilic domain containing <15 amino acids, it is acceptable to place the fusion joint after the first 3 to 5 amino acids of the following hydrophobic stretch, if one exists, in order to avoid disrupting charged residues that may be critical for orienting the upstream transmembrane segment (an example of this placement within a downstream transmembrane segment is shown in Figure 5.2.6). If, on the other hand, the small domain being probed lies at the protein's C terminus, the fusion joint should be placed at the C-terminal end of the protein.

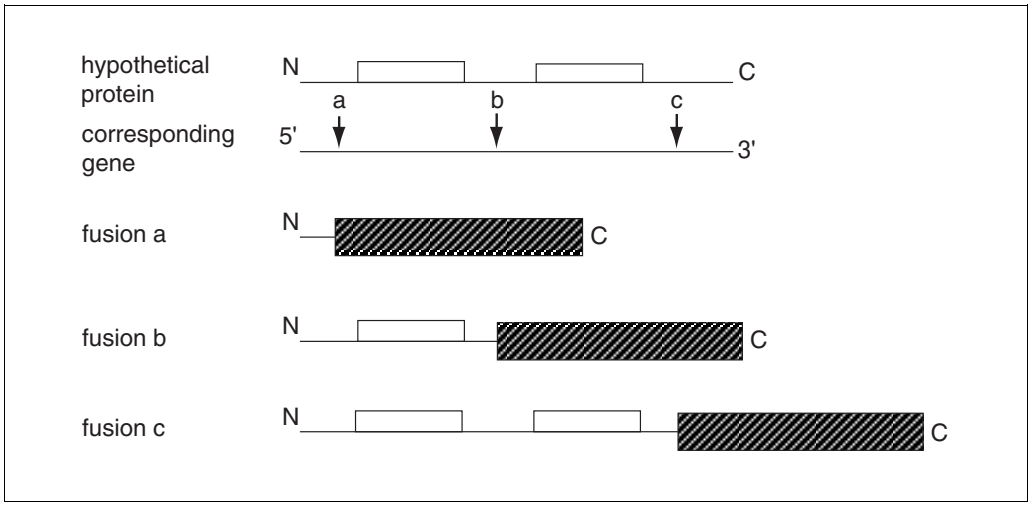


Figure 5.2.4 Placement of fusion joints in an integral membrane protein. Putative transmembrane segments are indicated by open boxes. The positions of the fusion joints to be constructed in the corresponding gene are indicated by arrows and denoted a, b, and c. A series of fusions of the invertase fragment-histidinol dehydrogenase (inv-HD) sequence (striped box) to N-terminal fragments of the hypothetical protein are indicated as fusions a, b, and c.

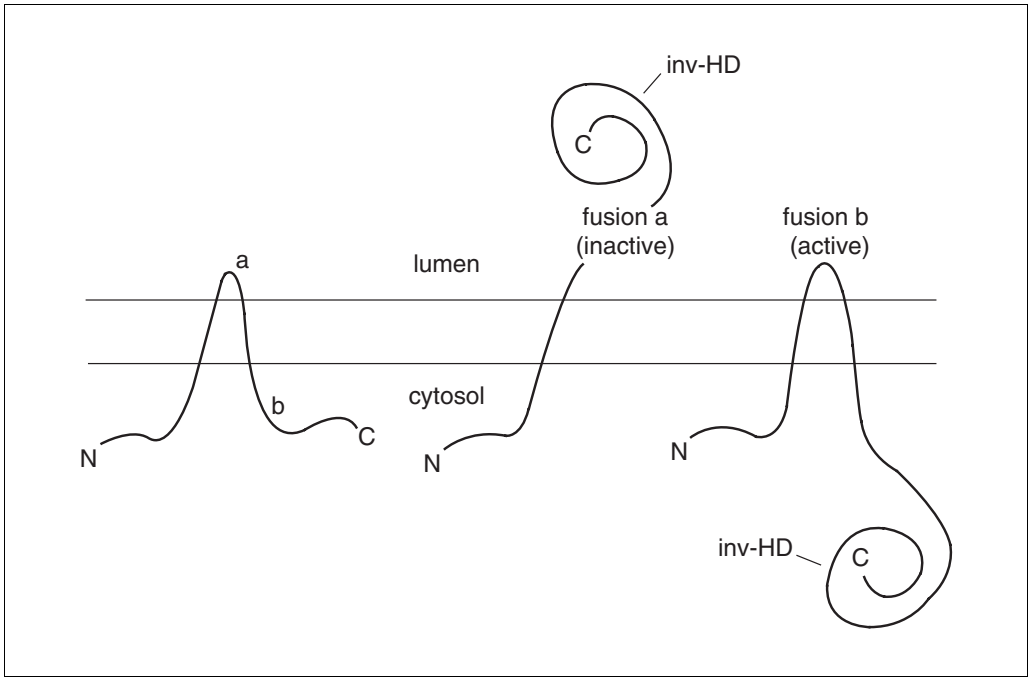


Figure 5.2.5 Fusion approach to determining membrane protein topology. The fusion of inv-HD at positions a and b in a hypothetical protein are indicated. Fusion a lacks HD activity due to the translocation of HD to the luminal side of the membrane. Fusion b exhibits HD activity due to the presence of HD in the cytosol.

Selecting a Method

A number of factors govern the selection of a protocol best suited for assessing the topology of a particular protein. Since protease digestion and immunofluorescence staining are used to probe a full-length protein, as opposed to truncated fragments, results obtained from these approaches may be more reliable, although problems associated with accessibility to the protease or antibody used can occur. Use of a foreign epitope to assess membrane protein topology can also yield very reliable results, provided the inserted epitope does not inhibit function of the target protein. Because of its simplicity, however, the reporter fusion approach is often employed as a way to get a general picture. As this approach involves fusion of a large foreign domain to N-terminal fragments, the topological assignments should be augmented with measurements of at least a few hydrophilic regions in the intact functional protein to determine whether different approaches yield the same topological picture. By complementing the reporter fusion analysis with one of the first two approaches, the derived map should provide a reasonably accurate assessment of the protein's topology. An example of this complementary approach is described by Lai et al. (1996).

BASIC PROTOCOL 1

PROTEASE DIGESTION

This protocol uses ER-derived vesicles from canine pancreas. These microsomes are purified according to the method of Walter and Blobel (1983) and exhibit a cytoplasmic-side-out orientation. Digestion of a hydrophilic domain present on the surface of microsomal vesicles therefore indicates that the exposed region is disposed to the cytosolic side of the membrane. This protocol uses immunoblotting to examine unlabeled proteins present in these membranes, although radiolabeled proteins integrated in vitro can also

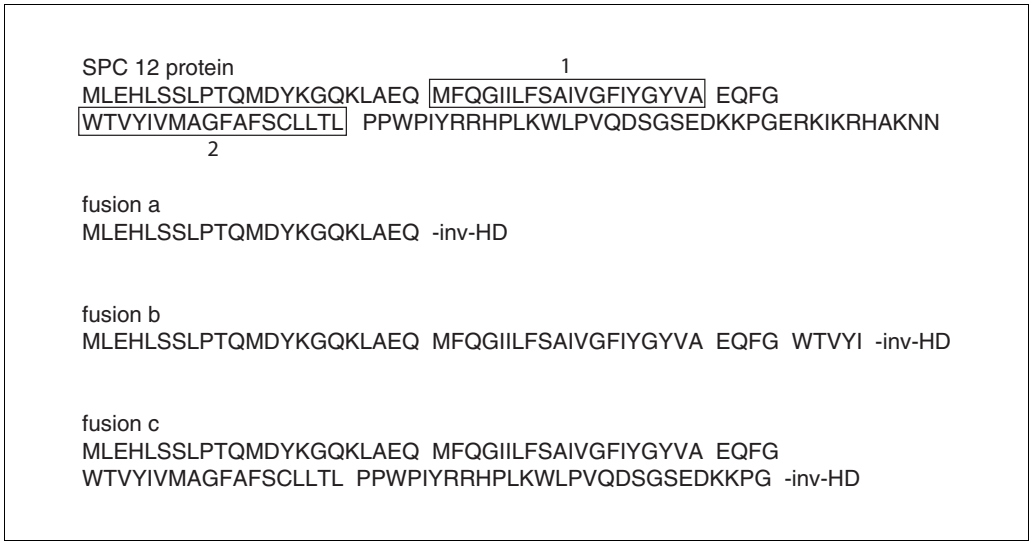


Figure 5.2.6 Positioning of fusion joints in SPC12 protein. The inv-HD reporter was placed at three positions in the SPC12 protein to discern the orientation of the three hydrophilic domains flanking hydrophobic segments 1 and 2 (open boxes). Note that the fusion joint of fusion c is placed >15 amino acids from the upstream hydrophobic segment. However, since the hydrophilic domain located between hydrophobic segments 1 and 2 consists of only 4 amino acids (EQFG), it is not possible to place the fusion joint more than 15 amino acids from hydrophobic segment 1 without capturing most of hydrophobic segment 2 in fusion b. Therefore, the fusion joint is positioned such that only 5 amino acids of hydrophobic segment 2 are present in fusion b. In placing the fusion joint at this position, the charged residues immediately following hydrophobic segment 1 are left undisturbed. Fusion a lacks a putative transmembrane segment and will remain cytosolic regardless of the topology of the N terminus of intact SPC12 protein. One exception to this is that, if the polypeptide chain contains an N-terminal signal sequence (von Heijne, 1984), fusion a would probably be transported to the ER lumen.

be studied (Walter and Blobel, 1983; Mize et al., 1986; see *UNIT 11.4* for analysis of protein translocation into canine microsomes). The protocol can also be adapted to microsomes prepared from metabolically labeled cells (*UNIT 7.1*; Bonnerot et al., 1994). As a control to determine whether the target protein is inherently sensitive to the protease used, proteolyzed membranes are compared to membranes solubilized in a mild detergent, such as Triton X-100, prior to protease addition.

In this protocol, proteinase K is employed as a representative protease due to its broad substrate specificity. In addition, proteinase K is active under the buffer conditions described (as are many other proteases), and proteinase K retains activity in the presence of Triton X-100. The buffer also contains sucrose to help maintain the integrity of membrane vesicles. Another control, which tests for the presence of intact membranes, examines a luminal protein native to the membrane vesicles used. Provided the membranes are intact, the luminal protein control should be resistant to proteinase K in the absence of detergent. Examples of ER proteins used for control purposes are TRAPβ (Kalies and Hartmann, 1996) and BiP (Mullins et al., 1995).

Materials

Canine pancreatic microsomal membranes (see *UNIT 11.4*)
Magnesium/sucrose/BSA (MSB) buffer (see recipe)
20% (w/v) Triton X-100
10 mg/ml proteinase K (see recipe)
100% (w/v) trichloroacetic acid (TCA) (see recipe)
2× SDS sample buffer (*APPENDIX 2A*)
Antibodies directed against a series of peptides corresponding to specific hydrophilic regions of the target protein
Control antibodies directed against a luminal protein or a luminal domain of a membrane protein in the membrane system to be analyzed

Additional reagents and equipment for preparing canine pancreatic microsomes (*UNIT 11.4*), separating proteins by SDS-PAGE (*UNIT 6.1*), and detecting proteins by immunoblotting (*UNIT 6.2*)

Prepare microsomal membranes

1. Purify microsomal membranes according to the method described in *UNIT 11.4*.

   Microsomes are stored at −80°C at a final concentration of 50 A_{280} units per ml. One equivalent is defined as 1 μl of this mixture. When stored under these conditions, microsomes are stable ≥1 year.

   Be sure that inhibitors of the protease to be used are omitted from the original membrane preparation (such as PMSF in the case of proteinase K; see APPENDIX 1B). If protease inhibitor is present, wash the membranes by centrifuging 20 min at ~100,000 × g, 4°C. Suspend the pellet in MSB buffer at the same concentration by pipetting up and down in a plastic tip.

2. Slowly thaw ~50 μl of microsomes by placing a tube containing the microsomes in ice.

   This amount of membranes is needed for examinations that use one antipeptide antibody and a control antibody. An additional 50-μl aliquot is needed for each additional examination.

3. Prepare ten samples, each containing 5 μl (5 equivalents) rough microsomes diluted with MSB buffer to a final volume of 20 μl in microcentrifuge tubes.

   Steps 3 to 8 should be performed using tubes kept in ice. Polypropylene microcentrifuge tubes have been found to work satisfactorily.

4. Add 1 µl of 20% (w/v) Triton X-100 to five of the tubes and mix by pipetting the solution up and down.

   These detergent-treated aliquots serve as controls to demonstrate that the target protein is inherently sensitive to the protease used. The five tubes lacking Triton X-100 are sample tubes and the five tubes containing Triton X-100 are control tubes.

Perform protease digestion

5. Add 10 mg/ml proteinase K to four of the sample tubes and four of the control tubes to yield final concentrations of 1, 20, 100, and 500 µg/ml.

   The amount of protease is varied to find the concentration which allows good digestion of the target protein yet is low enough that the protease does not destroy the membrane structure (thus making the membrane permeable to the protease added). Protease should be omitted from the fifth tube in the sample and control sets.

6. Mix samples by pipetting the solutions up and down a few times and incubate 30 min.

7. Stop proteolysis by adding 100% (w/v) TCA to a final concentration of 15%.

Analyze protein digests

8. Incubate all ten tubes 15 min on ice and collect TCA pellets by microcentrifuging 5 min at ~10,000 rpm (8,000 × *g*), room temperature.

9. Remove supernatant and suspend the pellets in 10 µl of 2× SDS sample buffer and 10 µl H_2O by vortex mixing at room temperature.

   Be sure that all of the pellet has been suspended. The mixtures should be blue because of bromphenol blue, which is present in the sample buffer. If the tubes are yellow (because of high acidity), 1-µl aliquots of 1 M Tris base can be added successively and then mixed until the mixture turns blue.

10. Load 10 µl from each tube into sample wells of a SDS-PAGE gel.

    The lanes should be organized as follows. The first five lanes contain one-half of the mixture from the five sample tubes, and the next five lanes contain one-half of the mixture from the five control tubes. The next five lanes contain one-half of the mixture from the five sample tubes, and the final five lanes contain one-half of the mixture from the five control tubes.

    A 12.5% polyacrylamide gel can be used to identify protein fragments of ~2 to 30 kDa, whereas a 7% acrylamide gel can be used to identify protein fragments of 30 to 100 kDa.

11. Analyze samples using SDS-PAGE (*UNIT 6.1*).

12. Examine the separated protein fragments by immunoblotting (*UNIT 6.2*), using one of the antipeptide antibodies and the control antibody directed against a luminal protein or a luminal domain of a membrane protein in the membrane system to be analyzed.

    The blot should be cut in half. The gel portion containing the first ten lanes is to be immunoblotted with an antibody directed against the target protein. The gel portion containing the second group of ten lanes should be subjected to immunoblotting using an antibody directed against the luminal protein control. The data should be interpreted by following examples described in Figure 5.2.2A and Anticipated Results.

13. Repeat steps 3 to 12 using each of the antipeptide antibodies and the control antibody.

IMMUNOFLUORESCENCE STAINING

This protocol illustrates use of an immunofluorescence technique to probe the topology of membrane proteins. The protocol is designed specifically to examine proteins confined to the plasma membranes of cells lacking an outer cell wall, such as cultured mammalian cells. This protocol has been optimized for using human embryonic kidney (HEK) 293 cells, although other cultured cell lines can be used with only minor modifications (noted below). These cells can be grown on glass coverslips and permeabilized with low concentrations of a mild detergent (Nonidet P-40 is used here), which allows antibodies access to the cytoplasmic compartment. The permeabilized cells and a second nonpermeabilized cell preparation are incubated with an antipeptide antibody followed by incubation with a fluorophore-conjugated secondary antibody. Cells are then examined by fluorescence microscopy (*UNIT 4.2*). As a control, cells expressing a plasma membrane protein of known topology should be examined using antibodies directed to a cytosolic domain and an extracytoplasmic domain.

This approach can employ primary antibodies directed against peptides corresponding to the membrane protein under study, such as the antipeptide antibodies described in Basic Protocol 1. However, antipeptide antibodies are found often not to recognize their corresponding epitopes within a native membrane protein (Carrasco et al., 1986). The procedure described here therefore uses a membrane protein that has been tagged with the HA epitope (see Support Protocol for methods regarding epitope tagging). Use of the HA epitope can minimize cost and time associated with the preparation of antipeptide antibodies. The primary and secondary antibodies used in this protocol are the anti-HA epitope mouse monoclonal antibody 12CA5 and a rhodamine-conjugated rabbit anti-mouse IgG, respectively, but other combinations of antibodies can be used as well (Canfield and Levenson, 1993).

Materials

HEK 293 cells (ATCC #CRL 1573)
4% (w/v) paraformaldehyde (see recipe)
Nonidet P-40/goat serum/BSA (NGB) solution (see recipe)
Anti-HA mouse monoclonal antibody 12CA5 (Boehringer Mannheim)
Rhodamine-conjugated rabbit anti-mouse immunoglobulin G (IgG)
DMEM/FBS/HEPES (DFH) solution (see recipe)
Fluoromount G mounting medium (Fisher)

6-well tissue culture plates
Glass coverslips, 22-mm diameter

Additional reagents and equipment for immunofluorescence staining of fixed mammalian cells (*UNIT 4.3*), epifluorescence (*UNIT 4.2*) or confocal laser microscopy, and growing cultured mammalian cells (*UNIT 1.1*)

1. Seed HEK 293 cells stably expressing the polypeptide of interest on glass coverslips and grow 2 to 3 days at 37°C in 6-well plates before processing for immunofluorescence.

   Alternatively, transient transfections can be performed on cells grown on glass coverslips in 6-well plates (UNIT 1.1). Cells should be handled using typical aseptic technique required for growing cells in culture (UNIT 1.3).

   The glass coverslips should be cleaned thoroughly using a strong detergent and washed extensively with distilled water.

   Duplicate cultures are needed in order to provide sets to be examined in the absence and presence of permeabilizing agents and in the absence or presence of the polypeptide of

interest. A single 6-well plate thus provides a triplicate analysis of cells to be treated with permeabilizing agents and containing the polypeptide of interest and a triplicate analysis of cells treated with permeabilizing agents and lacking the polypeptide of interest. A distinct plate provides a triplicate analysis for the set not treated with permeabilizing agents and either containing or not containing the polypeptide of interest (see below).

All steps except the antibody incubations can easily be carried out in 6-well tissue culture plates, using volumes of 2 to 3 ml per well for incubations and washes.

Methods for HA tagging, and testing for function of tagged proteins in transiently transfected HEK 293 cells are described in the Support Protocol.

2. For the permeabilized set, aspirate culture medium and wash coverslips once in PBS. Replace PBS with 4% paraformaldehyde (2 to 3 ml) and fix 10 min at room temperature.
3. Aspirate fixing reagent and rinse cells three times with PBS. Permeabilize and block cells in NGB solution (2 to 3 ml) for 15 min at room temperature.
4. Dilute monoclonal antibody 12CA5 to 1 µg/ml in NGB. Centrifuge antibody dilution 2 min at 8000 × *g* to remove aggregates.

   Although this antibody is supplied at a specified concentration, different batches appear to have different characteristics, and it may be necessary for individual users to optimize their antibody dilutions.

5. Place coverslips cell-side-up on Parafilm and immediately add primary antibody (100 µl per coverslip). Incubate 1 hr at room temperature in a covered, humidified chamber.

   A small petri dish or any small covered container will serve as a humidified chamber.

   For more tightly adherent cells, the volume of antibody used can be reduced further as follows. Pipet antibody (50 µl) directly onto Parafilm. Place coverslips on droplet, cell-side-down.

6. Return coverslips to NGB (2 to 3 ml) in the 6-well plate. Wash three times with PBS (10 min each wash) and once with NGB.
7. Incubate cells 1 hr at room temperature in NGB solution (2 to 3 ml) containing a rhodamine-conjugated rabbit anti-mouse IgG.

   The secondary antibody should be diluted according to the supplier's recommendations. Optimization of antibody dilution may be necessary.

8. Return coverslips to NGB solution and wash three times with PBS.
9. Dip coverslips in distilled water and mount on slides using Fluoromount-G (15 µl per slide).
10. For the nonpermeabilized set, replace growth medium with DFH solution and chill 15 min to 4°C.

    These cells provide the set that is nonpermeable in the presence of primary antibody. These steps can most conveniently be performed concurrently with processing of permeabilized cells.

11. Incubate with monoclonal antibody 12CA5 (2 µg/ml) for 1 hr in a humidified chamber at 4°C as described in step 5.

    Note that a higher concentration of antibody is used for this low-temperature incubation.

12. Wash three times with cold DFH and once with cold PBS.

13. Fix, permeabilize, and incubate "nonpermeabilized" cells with secondary antibody, as described in steps 2 to 3 and 7 to 9.

    The nonpermeabilized set is permeabilized immediately before addition of secondary antibody. This step serves not only to bind the secondary antibody to the primary antibody but also to determine whether the secondary antibody binds nonspecifically to either surface of the plasma membrane.

14. Examine permeabilized and nonpermeabilized cells using epifluorescence or confocal laser scanning microscopy.

    Fluorescence staining of only the permeabilized set is suggestive of an antibody bound to a cytosolic domain, whereas fluorescence appearing in both the permeabilized and nonpermeabilized sets reveals a domain placed at the extracytoplasmic side of the plasma membrane. A range from 5% to 25% of the cells on a particular coverslip may be stained well.

    The control cells lacking the polypeptide of interest are needed because one difficulty associated with the use of 12CA5 is that it is not absolutely specific for the HA epitope. Cross-reactivity is seen both on immunoblots and by immunofluorescence in nontransfected or nontagged cells.The resulting background may decrease the sensitivity with which the transfected, tagged polypeptide can be visualized. If the background signal is too high to detect the protein of interest, even after diluting the primary and secondary antibodies further than that described above, try other anti-HA antibody preparations such as those available from BAbCO. Note, however, that some of these antibodies may not recognize HA tags placed internally within the protein sequence.

EPITOPE TAGGING

SUPPORT PROTOCOL

In this protocol, a series of molecular biological manipulations are used to place a foreign epitope into a hydrophilic region of a membrane protein. Integration of the expressed protein leads to placement of the epitope on one side of the membrane. The orientation of the tagged domain can be assessed by monitoring the accessibility of the epitope to a protease (see Basic Protocol 1) or to its cognate antibody (see Basic Protocol 2).

The HA epitope, YPYDVPDYA, is recommended for use in this protocol because it contains only two charged amino acids. As shown in Figure 5.2.3, a set of three DNA fragments (differing by addition of 0, 1, or 2 base pairs at one or both ends) can be used to place the HA epitope internally within the sequence of a protein, N-terminally, or C-terminally. The fragments are inserted into a blunt-end restriction site occurring naturally within the protein's gene or into a restriction site constructed by site-directed mutagenesis (see *APPENDIX 3*). Depending on the reading frame of the site into which the epitope is inserted, only one of the three DNA fragments is needed to permit in-frame fusion between the membrane protein sequence and the epitope at its N and/or C termini. These fragments also contain the blunt-end *Pvu*II restriction site. This site provides a convenient place to insert a second fragment encoding the HA epitope, which is used if the single tag is found not to present the membrane protein adequately to the added antibody. Alternatively, DNA fragments containing a blunt-end restriction site other than *Pvu*II, such as *Eco*RV, can be synthesized if this *Pvu*II site is not unique on the plasmid containing the gene of interest. The DNA sequence encoding the HA epitope depicted in Figure 5.2.3 also contains an *Aat*II restriction site that can be used to verify insertion of the fragment into the plasmid used. It is usually necessary to verify orientation of the inserted fragment by DNA sequencing. This is not the only way to introduce an epitope into a membrane protein. A DNA sequence encoding the epitope can be introduced by site-directed mutagenesis.

Having introduced a DNA fragment encoding the HA epitope into a cloned gene, the tagged protein should be tested for function. If the tagged protein exhibits normal functional properties, then it is likely that the epitope introduced has not altered the overall

structure of the protein. To test for function, many types of assays can be performed. The type of assay used is dependent on the particular protein under study. For example, the HA-tagged rodent Na,K-ATPase α subunit can be tested for its ability to confer ouabain resistance to HEK 293 cells transfected with the tagged DNA construct (see Basic Protocol 2; Canfield et al., 1996). A tagged protein can be introduced into yeast cells, testing for complementation of a mutant lacking that protein or an evolutionarily conserved protein (Kurihara and Silver, 1993). Alternatively, a tagged protein may be expressed to high levels, enriched, then tested for activity using an in vitro assay.

Materials

TE buffer (*APPENDIX 2A*)
Plasmid DNA encoding the protein of interest
E. coli cells to be transformed

Additional reagents and equipment for synthesizing oligonucleotides (*APPENDIX 3*), ligating DNA (*APPENDIX 3*), transforming *E. coli* (*APPENDIX 3*), isolating plasmid DNA from *E. coli* (*APPENDIX 3*), identifying plasmids by restriction endonuclease digestion (*APPENDIX 3*), and sequencing oligonucleotides (*APPENDIX 3*)

1. Determine whether fragment I, II, or III (Fig. 5.2.3) is needed to produce an in-frame fusion between the N- and/or C-terminal ends of the HA epitope and the target protein.

2. Synthesize the appropriate DNA fragment(s) identified from step 1.

   Complementary oligonucleotides should be synthesized.

3. Mix two complementary DNA strands together, each to a final concentration of 1 A_{260} unit per 50 µl in TE buffer.

   Oligonucleotides are usually synthesized with their 5′ ends unphosphorylated. The oligonucleotides used should be left unphosphorylated to prevent insertion of multiple oligonucleotides into the linearized plasmid.

4. Anneal the strands by heating the solution 5 min at 70°C and then letting the solution cool in air at room temperature.

5. Mix 5 µl of the oligonucleotide solution with 0.1 µg of linearized plasmid DNA encoding the protein of interest, ligate the mixture in 10 µl of ligation buffer, and transform *E. coli* with the ligation mixture (*APPENDIX 3*).

   The DNA should be linearized at a restriction site contained within a sequence corresponding to the hydrophilic domain to be probed. If such a restriction site does not exist, one can be introduced by site-directed mutagenesis (as illustrated below, blunt-end sites are preferable). If possible, the epitope should be placed ≥15 amino acids from the nearby transmembrane segment(s). If the restriction site used produces sticky ends, the site should be made blunt-ended using standard molecular biological manipulations. To minimize religation of the plasmid without the DNA fragment to be inserted, the ligation mixture should be treated with the restriction enzyme used to produce the blunt end, provided a restriction enzyme producing blunt ends was used.

   The above-described ratio of fragment and linear plasmid is acceptable in many instances. However, sometimes this ratio may need to be varied in order to achieve efficient ligation of the fragment to the plasmid. Try increasing the concentration of oligonucleotides from 1 A_{260} unit to 2, 5, and 10 A_{260} units per 50 µl in TE buffer (step 3).

   Any of the E. coli strains commonly used for plasmid transformations is suitable, such as HB101 and MC1061.

6. Isolate plasmids from individual *E. coli* transformants and identify plasmids containing the insert using restriction analysis (*APPENDIX 3*).

If a fragment shown in Figure 5.2.3 is synthesized, restriction enzymes AatII and PvuII are useful for identifying the desired construct.

7. Determine the DNA sequence of the fragment inserted and residues surrounding this fragment to ensure correct orientation and in-frame fusion.

8. Express the epitope-tagged protein in an appropriate cell and test for its function. Examine its topology using Basic Protocols 1 or 2.

   If the tagged protein is not recognized by antibodies directed against the HA epitope, double-HA-tagged proteins can be constructed by inserting the appropriate DNA fragment (Fig. 5.2.3) into the PvuII site of the construct tagged with only one HA epitope. Successive insertions into each new PvuII site will produce proteins tagged with increasing numbers of HA epitopes.

   Plasmid DNA can be stored indefinitely in TE buffer at −20°C and should be introduced into the cell examined prior to use.

REPORTER GENE FUSIONS

BASIC PROTOCOL 3

In this protocol, a series of gene fusions is constructed in which a reporter moiety is fused to various truncated fragments of a target membrane protein. To construct these fusions, DNA fragments encoding a series of N-terminal fragments of a membrane protein should be synthesized by the polymerase chain reaction (PCR; see *APPENDIX 3*). For each PCR amplification, two primers are required: one corresponding to a sequence upstream of the promoter of the relevant gene and one corresponding to sequences at the desired fusion joint. Only one upstream primer is required, whereas a downstream primer corresponding to each of the fusion joints is usually needed. For construction of PCR fragments, the upstream primer contains a *Bam*HI site or a site compatible with the *Bam*HI sticky end, such as *Bgl*II or *Bcl*I. The downstream primer contains a *Xho*I site or a site compatible with *Xho*I, such as *Sal*I. Compatible sites are needed when the DNA fragment to be inserted contains an internal *Bam*HI or *Xho*I site. To ensure that the reading frame is maintained across the fusion joint, the *Xho*I site in the downstream primer is placed in the following reading frame: C TCG AG (where TCG encodes an in-frame serine).

A vector that can be used for expression of fusions in yeast is pA189invHD (Green and Walter, 1992; Fig. 5.2.7). pA189invHD is used for construction of gene fusions encoding the C-terminal moiety histidinol dehydrogenase (HD). HD is a cytoplasmic enzyme that is enzymatically inactive when fused to a luminal domain of a membrane protein inserted into the ER membrane. This shuttle vector contains the 2μm DNA fragment for replication in yeast and the ColE1 replication origin for replication in *E. coli*. pA189invHD can be selected in yeast cells containing a mutation in the *URA3* gene due to the presence of plasmid-borne *URA3*. The vector can also be maintained in *E. coli*, as it confers ampicillin resistance. pA189invHD contains a single *Bam*HI site and a single *Xho*I site. Restriction of pA189invHD with *Bam*HI and *Xho*I generates two DNA fragments (13 kb and 0.8 kb). The larger of these fragments contains the selectable markers and origins of replication described above. The smaller fragment encodes a portion of arginine permease, whose topology was analyzed previously using this vector system (Green and Walter, 1992). The fragment encoding arginine permease is therefore replaced with PCR-amplified fragments encoding N-terminal truncations of the membrane protein to be examined. For examining the topology of a protein without its native promoter, the yeast *ADH1* promoter (Bennetzen and Hall, 1982) is included on pA189invHD. When using the *ADH1* promoter, the upstream primer should contain a *Bam*HI site in front of the initiation codon for the membrane protein. For efficient expression from the *ADH1* promoter in pA189invHD, it is important that no ATG codon is present between the *Bam*HI site and the initiation codon.

Materials

Reporter plasmid (Fig. 5.2.7): pA189invHD (available from Neil Green, Vanderbilt University)

S. cerevisiae strain FC2-12B (*MATα trp1-1 leu2-1 ura3-52 his4-401 HOL1-1 can1-1*; available from Neil Green, Vanderbilt University)

SD +HIS agar plates (see recipe)

SD +HOL agar plates (see recipe)

Thermocycler

Additional reagents and equipment for the polymerase chain reaction (PCR; *APPENDIX 3*), agarose gel electrophoresis (*APPENDIX 3*), restriction endonuclease digestion (*APPENDIX 3*), and transformation of *E. coli* and *S. cerevisiae* (*APPENDIX 3*)

Construct gene fusions

1. Amplify by PCR a series of DNA fragments encoding truncations of a membrane protein, using appropriate primers.

   The DNA primers used in the PCR amplification can be synthesized by one of a number of companies offering services in oligonucleotide synthesis. The upstream primer should

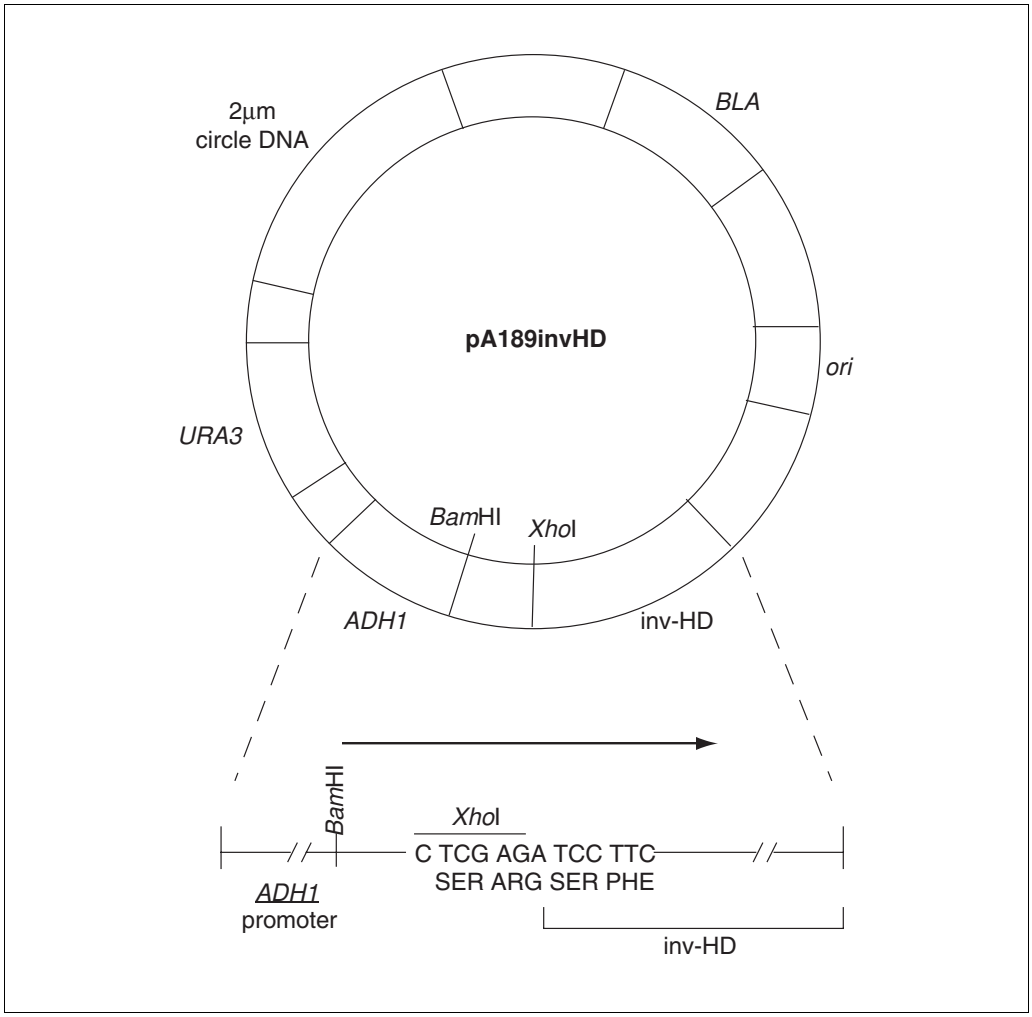


Figure 5.2.7 Gene fusion vector pA189invHD. The direction of transcription is indicated by an arrow. Abbreviations: *BLA*, β-lactamase gene; *ADH1*, alcohol dehydrogenase gene promoter; *URA3*, orotidine-5′-phosphate decarboxylase gene; ori, ColE1 replication origin; inv-HD, fusion of invertase fragment-histidinol dehydrogenase genes.

contain a 5′ BamHI site (or compatible site) followed by at least 20 nucleotides complementary to the DNA sequence to be amplified. The downstream primer should contain a 5′ XhoI site (or compatible site) followed by at least 20 nucleotides from the reverse strand of the DNA sequence to be amplified. The BamHI site should be placed immediately before the initiation codon if the ADH1 promoter of pA189invHD is to be used or before the promoter of the gene to be studied. The XhoI site should correspond to a site in a hydrophilic domain of the membrane protein studied. It is also recommended that the primers contain 2 to 3 nucleotides at the 5′ end of the restriction site for efficient cutting by the restriction enzyme after PCR amplification.

2. Purify the amplified fragments by agarose gel electrophoresis and digest with *Bam*HI and *Xho*I.

   Overnight restrictions are sometimes needed for efficient cutting of XhoI sites located near the ends of DNA fragments.

3. Digest the reporter plasmid pA189invHD with *Bam*HI and *Xho*I.

4. Purify the larger, 13-kb DNA fragment produced from the restriction digestion by agarose gel electrophoresis (see *APPENDIX 3*). Ligate the 13-kb fragment to each of the fragments produced in step 2.

   The smaller fragment (0.8 kb) encodes a part of arginine permease and should be discarded.

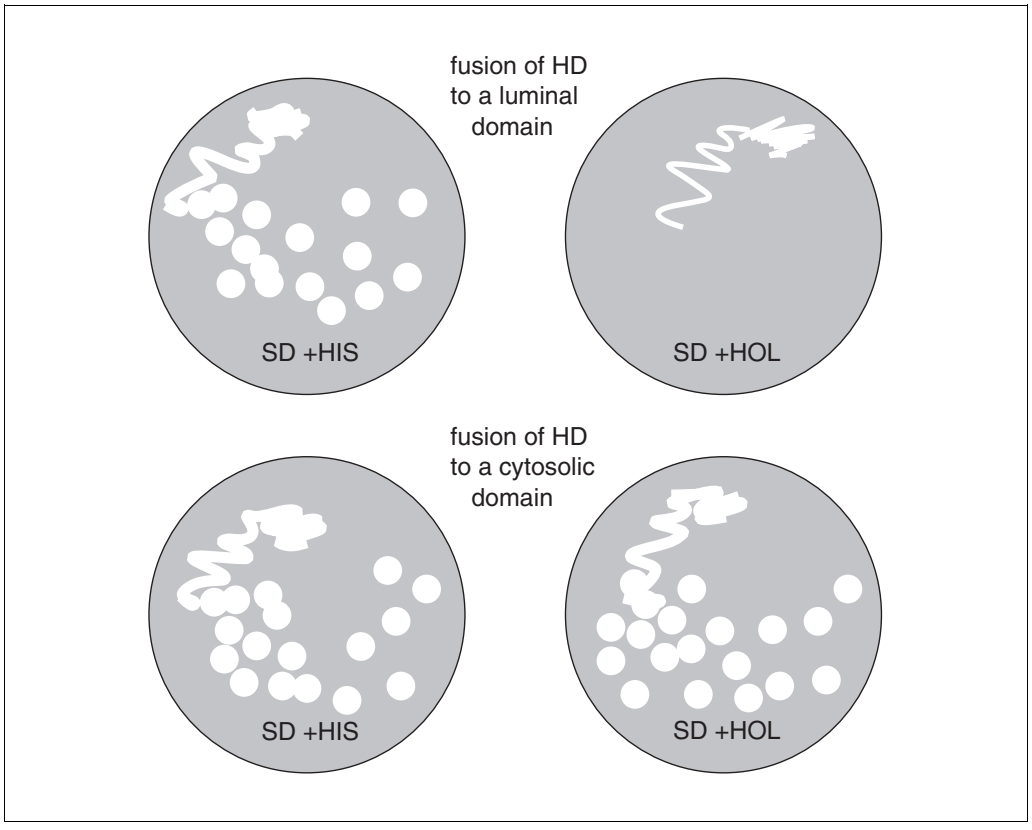


Figure 5.2.8 Cell growth assay for determining membrane protein topology. Cells of yeast strain FC2-12B bearing a fusion protein that fuses the HD moiety to a luminal domain of an integral membrane protein or bearing a fusion protein that fuses the HD moiety to a cytosolic domain of an integral membrane protein are streaked for single colonies on SD +HIS agar plates and SD +HOL agar plates, then incubated 4 to 5 days at 30°C. When large colonies appear on SD +HIS agar plates and little growth is detected on SD +HOL agar plates, the reporter is interpreted to be fused to a luminal domain of the membrane protein under study. On the other hand, when large colonies appear on both the SD +HIS and SD +HOL agar plates, the reporter is interpreted to be fused to a cytosolic domain.

5. Transform a standard Amp^s *E. coli* strain and isolate the desired plasmid construct.

   A variety of E. coli strains can be used, such as strains HB101 and MC1061.

Perform genetic assay

6. Transform the construct isolated from *E. coli* into *S. cerevisiae* strain FC2-12B and select transformants on SD +HIS agar plates.

   Yeast cells should be handled using aseptic technique.

   Strain FC2-12B contains a ura3 mutation that permits selection for pA189invHD-derived plasmids. Transformants should appear as individual colonies after 4 days on agar plates incubated at 30°C.

7. Test transformed yeast cells for growth by streaking cells on SD +HOL agar plates and SD +HIS agar plates.

   Individual colonies should appear after 4 to 5 days at 30°C. An example of the results expected is shown in Figure 5.2.8. As shown in this figure, fusion of HD to a luminal or cytosolic domain does not affect cell growth on SD +HIS agar plates. However, fusion of HD to a luminal domain inhibits growth on SD +HOL agar plates. Fusion of HD to a cytosolic domain does not inhibit cell growth on either type of agar plate.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DMEM/FBS/HEPES (DFH) solution

10% (v/v) fetal bovine serum
20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4
Prepare in supplemented Dulbecco's modified Eagle medium (DMEM; *APPENDIX 2A*)
Prepare fresh

The fetal bovine serum should not be heat-inactivated.

Magnesium/sucrose/BSA (MSB) buffer

150 mM potassium acetate
5 mM magnesium acetate
50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.6
200 mM sucrose
1 mM dithiothreitol (*APPENDIX 2A*)
Prepare fresh

Nonidet P-40/goat serum/BSA (NGB) solution

0.05% (v/v) Nonidet P-40 (Igepal AC-630)
5% (v/v) goat serum
1% (w/v) BSA
Prepare in PBS (*APPENDIX 2A*), pH 7.4
Prepare fresh

Paraformaldehyde, 4%

Heat 900 ml water to 55° to 60°C on a stirring hot plate in a fume hood. Add 40 g paraformaldehyde powder and stir 30 min. If powder has not dissolved, add a few NaOH pellets one at a time (waiting a few minutes between pellets) until the paraformaldehyde dissolves. Add 100 ml of 10× PBS (*APPENDIX 2A*), filter, cool to room temperature, and adjust to pH 7.4 with HCl. Store up to 1 week at 4°C.

CAUTION: *The fume hood is used because paraformaldehyde fumes are toxic. The "Prill" form of paraformaldehyde from EM Sciences is safest to use, as it pours without creating a cloud.*

Proteinase K, 10 mg/ml

10 mg proteinase K (lyophilized powder, ~80% protein, 10 to 20 U/mg protein; Sigma)
1 ml 50 mM HEPES, pH 7.6
Prepare fresh on ice

No activation of the proteinase K is necessary; the purchased enzyme is ready to use.

SD +HIS agar plates

0.7 g/liter yeast extract (without amino acids; Difco)
20 g/liter glucose
20 g/liter agar
0.1 mg/ml L-tryptophan
0.1 mg/ml L-leucine
0.1 mg/ml L-histidine

Mix the ingredients together before autoclaving. Pour the autoclaved solution into 100 × 15–mm petri dishes. Store plates <1 month at 4°C.

SD +HOL agar plates

0.7 g/liter yeast extract (without amino acids; Difco)
20 g/liter glucose
20 g/liter agar
0.1 mg/ml L-tryptophan
0.1 mg/ml L-leucine
0.1 mg/ml L-histidinol dihydrochloride (Sigma)

Filter-sterilize the histidinol solution and add to the remainder after autoclaving. Store plates <1 month at 4°C.

TCA, 100%

250 g solid trichloroacetic acid (TCA)
141 ml water
Store <6 months at 4°C

COMMENTARY

Background Information

Integral membrane proteins can often be distinguished from peripherally bound proteins in that the latter are extractable from the membrane under conditions of high pH (Steck and Yu, 1973; Fujiki et al., 1982; Russel and Model, 1982). Resistance of integral membrane proteins to extraction is attributed to the presence of transmembrane segments which help anchor proteins to the lipid bilayer. Visual and computer-based inspections of the primary sequences of integral membrane proteins usually reveal one or more hydrophobic amino acid stretches interspersed among hydrophilic stretches in an alternating pattern. This pattern suggests a topology in which neighboring hydrophilic domains are positioned on opposite sides of a membrane through integration of hydrophobic transmembrane regions. These topological predictions have been generally confirmed through an emerging number of crystal structures of integral membrane proteins (Deisenhofer et al., 1985; Xia et al., 1997). Despite the availability of methods for X-ray diffraction analyses of membrane proteins, topological studies still occupy an important place in protein structural studies. This is due to the fact that structures of membrane proteins are much less easily obtained than those of water-soluble proteins because of difficulties in obtaining crystals. As most integral membrane proteins require a specific topology for function, determining the orientations of hydrophilic domains may provide significant insight into the proteins' roles in vivo.

Protease digestion

Protease digestion is one of the oldest methods for determining membrane protein topology. This approach relies on the principle that the lipid bilayer is generally impermeable to proteases. Therefore, addition of a protease to the outside surface of a membrane vesicle results in degradation of protein sequences lo-

cated on the exterior surface, thereby revealing the luminal content (Gerber et al., 1977). It is best to examine a protein in its native membrane environment. The protease digestion protocol described in this unit (see Basic Protocol 1) is useful for examining proteins residing within isolated ER membrane vesicles by immunoblotting, although this procedure can be adapted easily to the study of proteins present in the plasma membrane and in other membrane-bound organelles. The primary difference is in the enrichment procedure used to isolate the desired membrane preparation (Li and Shore, 1992; Graham et al., 1994; Kalish et al., 1995).

Alternatively, one may examine the topologies of proteins integrated into microsomal vesicles in vitro. Such proteins are found to assume a specific topology that can be probed by the addition of a protease (Mize et al., 1986). In vitro–expressed proteins can, however, suffer the problem of being integrated inefficiently (Skach et al., 1994), and in vitro systems may not offer a functional assay assuring that the membrane protein has achieved its native structure. However, the membrane integration machinery in the ER is believed to perform accurately in vitro, at least with native membrane proteins (see *UNIT 11.4*).

In order to gain a complete picture of a protein's topology using the protease digestion method, each hydrophilic domain must be inherently sensitive to the protease used. That is, accurate pictures are obtained only when each domain is digestible either at the surface of the vesicle or in detergent-solubilized solutions. To ensure that the hydrophilic domain being probed can be proteolytically cleaved, it should contain a site recognized by the protease used. Proteases exhibiting a broad substrate specificity, such as proteinase K and trypsin, should therefore be used.

For particularly small domains, accessibility to a protease may be a problem. Furthermore, since digested regions are probed with antibodies directed against a series of peptides corresponding to various hydrophilic regions within the membrane protein, there is no guarantee that all of the antibody preparations will recognize the intact protein (Seckler et al., 1986). Despite these potential problems, the protease digestion approach is a proven method, particularly as it has been used to probe large hydrophilic domains.

Immunofluorescence staining

Immunofluorescence staining has been used widely to analyze the topology of proteins localized to the plasma membrane (Canfield and Levenson, 1993; Canfield et al., 1996). This method (see Basic Protocol 2) has the advantage that domains present on either side of the membrane can be visualized with a microscope, as opposed to detection of proteolytic fragments which may be small and difficult to find by SDS-PAGE. Compared to the protease digestion approach, immunofluorescence staining has not been used generally to study membrane protein topology in organelle-based systems. It should be possible, however, to identify hydrophilic domains located at the surface of peroxisomes in mammalian cells by taking advantage of the fact that the plasma membrane, but not the peroxisomal membrane, is permeabilized with the detergent digitonin (Swinkels et al., 1991). Since both plasma and peroxisomal membranes are permeabilized after addition of Triton X-100, use of these detergent combinations should permit a topological analysis of peroxisomal proteins.

Epitope tagging

Preparation of antipeptide antibodies needed for Basic Protocols 1 and 2 is costly and time-consuming. These problems are exacerbated by the fact that many antipeptide antibodies fail to recognize their corresponding epitope within the intact membrane protein. An alternative approach, epitope tagging, is therefore presented in this unit (see Support Protocol). A DNA fragment encoding a foreign epitope recognized by a commercially available antibody is introduced into the gene encoding a membrane protein. A tagged domain can then be identified using protease digestion (see Basic Protocol 1) or immunofluorescence staining (see Basic Protocol 2; Canfield and Levenson, 1993; Skach et al., 1994; Canfield et al., 1996).

There are, however, disadvantages that may result from the addition of an epitope. The epitope may render the membrane protein into which it has been inserted nonfunctional (Canfield et al., 1996). In such cases, it is not always clear whether the topology has been disrupted or whether the epitope has been inserted into a site critical for activity. In either event, results derived from studies of a nonfunctional protein are unreliable in the absence of supportive topological data. Even if the tagged protein is functional, the possibility remains that the foreign

epitope has disrupted the topology of a region unimportant to the protein's activity under the conditions measured.

Although the HA epitope has been used successfully for determining membrane protein topology (Canfield and Levenson, 1993), it contains two charged amino acids (YPYDVPDYA; Wilson et al., 1984). A number of studies have shown that charged residues located near membrane-spanning sequences play a role in determining transmembrane orientation in eukaryotic cells. Indeed, introduction of clusters of negatively charged amino acids proximal to transmembrane segments can inhibit their integration (Green and Walter, 1992). However, the HA epitope contains fewer charged amino acids than FLAG (DYKDDDDK; Hopp et al., 1988), c-myc (EQKLISEEDL; Evan et al., 1985), and VSV-G (YTDIEMNRLGK; Kreis, 1986) epitopes and is therefore recommended for use. The AU1 epitope (DTYRYI) derived from the papillomavirus major capsid protein also contains only two charged amino acids (Lim et al., 1990); however, it has not yet been utilized for topological analysis to the extent that the HA epitope has.

To avoid interfering further with integration of the target protein, the foreign epitope should be placed ≥15 amino acids from the neighboring transmembrane segment(s) without disrupting membrane targeting signals which are commonly present at the N- and/or C-terminal ends of the polypeptide chain. Another concern is that addition of artificial sequences into a membrane protein may affect its stability or localization to a compartment within the cell. Indeed, retention of mutant proteins in the secretory pathway is well documented (Gething et al., 1986; Kreis and Lodish, 1986). Loss of stability or missorting may be evidenced through failure to find the tagged protein in its normal cellular location.

Reporter gene fusions

Since the mid-1980s, a genetic approach has been used to solve membrane protein topologies (see Basic Protocol 3). The reporter gene fusion method was developed originally to study the topology of plasma membrane proteins in bacterial systems (Manoil and Beckwith, 1986; Froshauer et al., 1988) and then adapted for the study of proteins integrated into the ER membrane in the yeast *Saccharomyces cerevisiae* (Sengstag et al., 1990). More recently, the reporter enzyme β-galactosidase has been used for topological studies in *Caenorhabditis elegans* (Doan et al., 1996; Lai et al., 1996). Reporter fusion approaches developed for analyses in yeast and *C. elegans* examine membrane proteins integrated into the ER and either residing within the ER or transported to a compartment of the secretory pathway or to the lysosome. Due to conservation of the ER membrane integration machinery in diverse eukaryotic organisms (Hartmann et al., 1994), cross-species analyses of eukaryotic proteins using the simple yeast system are probably valid. Recently, a gene fusion system has been used to study the topology of a protein localized to the peroxisomal membrane (Elgersma et al., 1997), and it is likely that over the next few years the reporter gene fusion method will be extended to other organelles and eukaryotic cell types.

The cytoplasmic enzyme histidinol dehydrogenase (HD) is well suited for use as a reporter of membrane protein topology in yeast cells (Sengstag et al., 1990). HD is encoded by the *HIS4* gene in *Saccharomyces cerevisiae* (Donahue et al., 1982). When expressed on the cytosolic side of the membrane, HD converts histidinol to histidine, an activity that permits yeast that are *his4*$^{-}$ to grow on agar plates supplemented with histidinol instead of histidine. On the extracytoplasmic side, however, HD is enzymatically inactive in vivo, presumably due to its sequestration from the histidinol substrate, glycosylation of the HD moiety, and/or degradation of HD within the ER lumen (Green and Walter, 1992; Mullins et al., 1995).

To construct gene fusions in pA189invHD, the 800-bp *Bam*HI-*Xho*I fragment can be replaced with a DNA fragment encoding an N-terminal truncation of the target membrane protein. This leads to the production of fusions between the upstream membrane protein fragment and the C-terminal inv-HD sequence. In pA189invHD, the inv sequence (an internal fragment of the yeast invertase protein; Taussig and Carlson, 1983) serves as a spacer that separates HD from the membrane-spanning segments of the upstream membrane protein (Green and Walter, 1992).

The main advantage of the reporter gene fusion approach described here is its simplicity. The cell growth assay provides a quick and reliable means of assessing the location of a hydrophilic domain within a membrane protein examined in yeast. Furthermore, a fusion joint can be constructed anywhere along the primary sequence using standard molecular biological protocols. This means that all hydrophilic domains within a multispanning protein, includ-

ing small domains, can, in principle, be examined by constructing a series of gene fusions. However, such fusions eliminate from the construct all sequences in the membrane protein located downstream of the fusion joint. This approach therefore makes the assumption that membrane proteins integrate into the bilayer in a linear fashion, from the N to the C terminus. This idea is in accord with the model that membrane proteins contain alternating signal and stop transfer signals positioned linearly along the primary sequence (Friedlander and Blobel, 1985). While this assumption may be accurate in some instances, the fusion approach cannot identify transmembrane domains that insert into the membrane with the help of C-terminal sequences (Finer-Moore and Stroud, 1984).

In light of current models suggesting that multiple transmembrane domains accumulate within the translocation channel before their entry into the lipid bilayer (Hegde and Lingappa, 1997), caution should be used when interpreting results obtained using only the reporter fusion approach. For this reason, combining the gene fusion approach with one of the other approaches described in this unit will not only yield a more reliable topological picture but may also provide information about the mechanism of integration of the protein examined.

Critical Parameters

Protease digestion

Several factors are important for interpreting membrane protein topology using the protease digestion method. Determining the location of a hydrophilic domain that is at the side of the membrane to which the protease has not been added can be difficult, if the domain in question is small. This is due to the fact that digestion of those portions exposed to the protease may leave a fragment too small to detect using SDS-PAGE. Thus, the failure to detect a protein fragment may be due to a detection problem, not to the susceptibility of the domain to the protease added (see Anticipated Results). On the other hand, a domain exposed to the protease may be resistant or inaccessible if the domain is small. Therefore, it is critical to interpret carefully results regarding the disposition of a small domain. To assign a small hydrophilic domain to the correct side of the membrane, it is best to examine the topology over the entire protein, then determine whether the results obtained are consistent with the deduced assignment of the domain(s) in question. One way to enhance the chance that a small domain is susceptible is to use a protease that has a broad substrate specificity, such as proteinase K or trypsin. The use of a control protein or protein domain that faces the luminal side of the membrane system to be probed is essential to the analysis, since the membranes examined must be closed and impermeable to the protease added in the absence of detergent.

Immunofluorescence staining

There are several technical features of the immunofluorescence staining protocol that are critical to a successful outcome. The use of very clean coverslips is necessary. Coverslips should be washed in a strong detergent and rinsed thoroughly to remove all traces of the detergent. The adherence of tissue culture cells to the coverslips can be a problem sometimes. The protocol described here works well for HEK 293 cells, but when other cells are used, different types of coverslips can be tried. For example, Lab-Tek Chamber Slides (Nunc) provide a surface to which many cell types attach well. When using HEK 293 cells, it is observed that cells which are about 70% confluent yield the best staining results. Using the procedures outlined here, it takes about 3 days following seeding on coverslips to achieve this confluency; however, different times may be necessary in different experiments. In addition, these conditions may need to be varied for cell types other than HEK 293 cells.

The most important control is to examine cells lacking the protein of interest. This control detects the background staining that may appear in the cells under study. If the cells containing the protein of interest give a much more intense staining than cells lacking the protein, then the results should be reasonably reliable. The protocol listed here calls for a membrane protein control to be analyzed. This control is very useful, provided that antibodies directed against this control protein are available. If not, then it is essential that at least one hydrophilic domain located on the intracytoplasmic side of the membrane be probed, as such a domain controls for the possible disruption of the membrane prior to addition of the primary antibody.

Epitope tagging

Epitope tagging offers an inexpensive shortcut to analyzing membrane protein topology using either the protease digestion or the immunofluorescence staining protocol. However, there is one issue that is important in achieving the reliability necessary. The results are more

certain to be valid if the epitope does not inhibit function of the target protein. It is therefore important to examine the function of the epitope-tagged protein using an in vivo or in vitro assay. To improve the chance that the epitope does not alter the topology of a protein (and thus its function), an epitope lacking clusters of charged amino acids, such as the HA epitope, should be used. Furthermore, the epitope should be placed >15 amino acids away from the upstream and/or downstream transmembrane segments, in order to avoid disrupting the content of charged amino acids already existing near the transmembrane segments. If it is not possible to obey this 15 amino acid restriction (because the domain probed is small) then place the epitope as far away as possible from the transmembrane segment(s).

Reporter gene fusions

When membrane protein topology is assessed using an in vivo assay (such as the one described here), the reporter gene fusion approach is the quickest way to determine membrane protein topology. However, the fusions constructed usually render the target protein nonfunctional. Topological assignments are therefore best served by using this method to gain only a general picture of a protein's topology and then complementing the data with at least a partial analysis using one of the above-listed approaches. Since a functional assay is not always practical, there are some issues to consider when constructing the fusions in order to minimize the chance that the reporter affects the topology of the protein being probed. The fusion joint should be constructed >15 amino acids away from the upstream transmembrane segment. For small hydrophilic domains, the fusion joint can be placed after the first few amino acids of the downstream transmembrane segment if one exists (see Fig. 5.2.6). Another factor that is important is the amino-terminal sequence of the reporter itself. When the reporter contains clusters of charged amino acids at its amino terminus, the reporter may inhibit the integration of the upstream transmembrane segments. Indeed, the His4p reporter appears to suffer this problem (Green and Walter, 1992). For this reason, the reporter construct described in this protocol contains a spacer sequence consisting of a fragment of invertase (Fig. 5.2.7).

Troubleshooting

Refer to Table 5.2.1 for a troubleshooting guide.

Anticipated Results

A typical result expected from the protease digestion method (see Basic Protocol 1) is shown in Figure 5.2.2A. Note that no apparent fragment is detected from proteolysis of the representative protein SPC12 in the absence of detergent (lane 2). If a fragment had been detected, the result would have suggested that the C terminus localizes to the luminal side of the membrane. However, such a fragment could have been quite small (as few as 20 to 30 amino acids) if the C-terminal hydrophilic domain contained only a few amino acids. A clue as to the expected size of a particular fragment can be obtained by examining a hydropathy plot of the protein. Data similar to those shown in Figure 5.2.2A are expected from the analysis of epitope-tagged proteins (see Support Protocol), except that instead of antipeptide antibodies, antibodies directed against the epitope employed should be used. Results expected from immunofluorescence analyses (see Basic Protocol 2) of epitope-tagged proteins have been reported (Canfield and Levenson, 1993; Canfield et al., 1996). Results expected from reporter fusion analyses (see Basic Protocol 3) in yeast (Sengstag et al., 1990) and *C. elegans* (Doan et al., 1996; Lai et al., 1996) have also been reported.

Time Considerations

The time frames given below are for procedures that work well in the first few attempts. Longer times will, of course, be needed when significant troubleshooting is required.

Protease digestion

Antipeptide antibodies directed against each hydrophilic domain of the target protein can take 3 months or longer to prepare. This time can be spent working out the conditions for preparing enriched membranes containing the protein of interest. If control antibodies are available, they can be used to verify the integrity of the membranes being prepared. It usually takes no more than 2 to 3 days to obtain most membrane preparations. The protease treatments and SDS-PAGE analyses also take 2 to 3 days (see Basic Protocol 1).

Immunofluorescence staining

As with the protease digestion technique, most of the time is spent preparing a series of antipeptide antibodies. This time is lessened if commercially available epitope tags are used (see Support Protocol). When examining cells grown in tissue culture medium (see Basic

Table 5.2.1 Troubleshooting Guide for Problems Encountered in Determining Membrane Protein Topology

Problem	Possible cause	Solution
Protease digestion		
Target protein is not degraded in detergent.	Protein is resistant to protease used.	Try another protease.
	Protein is shielded by other proteins.	Treat membrane with buffer containing 500 mM potassium acetate and add 500 mM potassium acetate to the detergent control. To release ribosomes from ER microsomes, treat with MSB containing 500 mM potassium acetate, 0.2 mM GTP, 1 mM puromycin.
	Protein is part of a protease-resistant complex.	Preincubate membranes 10 min at 40°-60°C. Monitor membrane integrity using a control membrane protein.
The target protein is digested in the detergent but not in intact vesicles.	The protein may lack a cytoplasmic domain or is shielded.	Treat membranes with 500 mM potassium acetate as described above before adding the protease.
Poor proteolysis of target and control proteins is found.	Protease inhibitors or nonfunctional protease is present.	Ensure inhibitors are absent from membrane preparation by washing membranes by high-speed centrifugation (see Basic Protocol 1, step 1). Be sure protease is active by examining proteolyzed and unproteolyzed membranes using SDS-PAGE (*UNIT 6.1*).
Degradation of a luminal protein control occurs even without addition of detergent.	Too much protease is used.	Titrate protease to lower concentrations than those indicated in Basic Protocol 1.
	The membrane is leaky.	Change the cell fractionation protocol (Chapter 3).
Immunofluorescence staining		
The antibody used does not recognize the target protein in the permeabilized and nonpermeabilized sets.	The antibody used binds the target protein inefficiently in the context of the membrane.	Test whether antibody binds the protein by immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*) from detergent-solubilized membranes.
		If the hydrophilic domain being probed is small, try inserting one or more epitopes into the domain (see Support Protocol) and repeat analysis.
Hydrophilic domains presumed to be located on opposite sides of the membrane are detected in both the permeabilized and nonpermeabilized sets.	The nonpermeabilized cells were leaky at the time primary antibody was added.	Be sure that detergent was not added to DFH solution. Examine a control protein with known topology.
Control cells lacking the polypeptide of interest are stained strongly by the secondary antibody.	The concentrations of primary and/or secondary antibodies are tood high.	Test whether dilution of the antibodies decreases the background signal. Try using a different primary antibody preparation.
Epitope tagging		
The tagged protein is not functional.	The protein is degraded, mislocalized, or out of frame.	Test whether protein is present in its native membrane by immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*). Sequence the gene segment encoding the epitope and flanking amino acids.

continued

Table 5.2.1 Troubleshooting Guide for Problems Encountered in Determining Membrane Protein Topology, continued

Problem	Possible cause	Solution
	The epitope inhibits the active site or topology of the protein.	Place the epitope at a different site in the protein domain, including sites further away from the transmembrane stretches. Alternatively, proceed with topology assays, but corroborate results using a different method.
The antibody does not recognize an epitope-tagged protein even though the protein is functional.	The epitope is inaccessible.	Introduce multiple epitopes into the site.
Reporter gene fusions		
Fusions to hydrophilic domains on both sides of a putative transmembrane segment possess HD enzymatic activity.	The L-histidinol reagent is contaminated with histidine.	Test whether strain FC2-12B grows on SC +HOL agar plates. If it does, try a different batch of histidinol or a different supplier.
	The hydrophobic segment identified does not actually span the membrane, or the transmembrane segment inserts into the bilayer aided by downstream sequences not contained in the fusion protein.	Reassess whether the hydrophobic segment is a transmembrane segment. Monitor topology of the region in question using a different approach.
None of the fusions possess enzymatic activity.	The fusions are not made.	Sequence fusion joints to ensure reading frame is maintained. Ensure that no ATG codons are present between the promoter and the initiation codon. Use the strong *ADH1* promoter on pA189invHD.
Fusions expected to be inactive are active.	The fusion joint interferes with integration of the upstream sequences.	Position the joint further away from the upstream transmembrane segment. Corroborate results using a different method.

Protocol 2), it takes about 3 days to grow cells on coverslips before the immunofluorescence analysis. The analysis takes about 1 day, which includes fixing the cells, incubating with primary and secondary antibodies, and examining the cells by microscopy.

Epitope tagging

The time required to perform the molecular biological manipulations required to introduce a foreign epitope into each hydrophilic domain of a membrane protein (see Support Protocol) depends on the number of distinct hydrophilic domains present in the protein and whether single, double, or multiple tags are introduced into each domain. A person skilled in the use of molecular biological techniques could introduce a set of single and double tags into a type I integral membrane protein (Fig. 5.1.2) in <1 month, test the function of the various tagged proteins in vivo or in vitro in a few weeks, and localize the epitopes with respect to the membrane using protease digestion (see Basic Protocol 1) or immunofluorescence staining (see Basic Protocol 2) in ~1 week using either technique. Thus, in ~2 months, one could tag such a protein and examine its topology. Longer periods (up to 6 months) may be required to examine proteins containing several transmembrane segments.

Reporter gene fusions

Using the described systematic approach for constructing gene fusions (see Basic Protocol 3), a series of constructs can be made in <1 month by a person experienced in using molecular biological techniques. Analyses of the fusions require ~1 week using the yeast system or a few weeks using a higher eukaryotic system.

Literature Cited

Bennetzen, J.L. and Hall, B.D. 1982. The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase I. *J. Biol. Chem.* 257:3018-3025.

Bole, D.G., Hendershot, L.M., and Kearney, J.F. 1986. Posttranslational association of immunoglobulin heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.

Bonnerot, C., Marks, M.S., Cosson, P., Robertson, E.J., Bikoff, E.K., Germain, R.N., and Bonifacino, J.S. 1994. Association with BiP and aggregation of class II MHC molecules synthesized in the absence of invariant chain. *EMBO J.* 13:934-944.

Canfield, V.A. and Levenson, R. 1993. Transmembrane organization of the Na,K-ATPase determined by epitope addition. *Biochemistry* 32:13782-13786.

Canfield, V.A., Norbeck, L., and Levenson, R. 1996. Localization of cytoplasmic and extracellular domains of Na,K-ATPase by epitope tag insertion. *Biochemistry* 35:14165-14172.

Carrasco, N., Herzlinger, D., Danho, W., and Kaback, H.R. 1986. Preparation of monoclonal antibodies and site-directed polyclonal antibodies against the *lac* permease of *Escherichia coli*. *Methods Enzymol.* 125:453-467.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. 1985. Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3Å resolution. *Nature* 318:618-624.

Doan, A., Thinakaran, G., Borchelt, D.R., Slunt, H.H., Ratovitsky, R., Podlisny, M., Selkoe, D.J., Seeger, M., Gandy, S.E., Price, D.L., and Sisodia, S.S. 1996. Protein topology of presenilin 1. *Neuron* 17:1023-1030.

Donahue, T.F., Farabaugh, P.J., and Fink, G.R. 1982. The nucleotide sequence of the *HIS4* region of yeast. *Gene* 18:47-59.

Elgersma, Y., Kwast, L., van den Berg, M., Snyder, W.B., Distel, B., Subramani, S., and Tabak, H.F. 1997. Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in *S. cerevisiae*, causes proliferation of the endoplasmic reticulum membrane. *EMBO J.* 16:7326-7341.

Engelman, D., Steitz, T., and Goldman, A. 1986. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Chem.* 15:321-353.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610-3616.

Finer-Moore, J. and Stroud, R. 1984. Amphipathic analysis and possible formation of the ion channel in an acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.* 81:155-159.

Friedlander, M. and Blobel, G. 1985. Bovine opsin has more than one signal sequence. *Nature* 318:338-343.

Froshauer, S., Green, N., Boyd, D., McGovern, K., and Beckwith, J. 1988. Genetic analysis of the membrane insertion and topology of MalF, a cytoplasmic membrane protein of *Escherichia coli. J. Mol. Biol.* 200:501-511.

Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment. Application to endoplasmic reticulum. *J. Cell Biol.* 93:97-102.

Gerber, G., Gray, C., Wildenauer, D., and Khorana, G. 1977. Orientation of bacteriorhodopsin in *Halobacterium halobium* as studied by selective proteolysis. *Proc. Natl. Acad. Sci. U.S.A.* 81:155-159.

Gething, M.-J., McCammon, K., and Sambrook, J. 1986. Expression of wild type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell* 46:939-950.

Graham, T.R., Seeger, M., Payne, G., Mackay, V.L., and Emr, S.D. 1994. Clathrin-dependent localization of α1,3 mannosyltransferase to the Golgi complex of *Saccharomyces cerevisiae. J. Cell Biol.* 127: 667-678.

Green, N. and Walter, P. 1992. C-terminal sequences can inhibit the insertion of membrane proteins into the endoplasmic reticulum of *Saccharomyces cerevisiae. Mol. Cell. Biol.* 12:276-282.

Haas, I.G. and Wabl, M. 1984. Immunoglobulin heavy chain binding protein. *Nature* 306:387-389.

Hartmann, E., Rapoport, T.A., and Lodish, H.F. 1989. Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc. Natl. Acad. Sci. U.S.A.* 86:5786-5790.

Hartmann, E., Sommer, T., Prehn, S., Gorlich, D., Jentsch, S., and Rapoport, T.A. 1994. Evolutionary conservation of components of the protein translocation complex. *Nature* 367:654-657.

Hegde, R.S. and Lingappa, V.R. 1997. Membrane protein biogenesis: Regulated complexity at the endoplasmic reticulum. *Cell* 91:575-582.

Hopp, T.P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L., and Conlon, P.J. 1988. A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio/Technology* 6:1204-1210.

Kalies, K.-U. and Hartmann, E. 1996. Membrane topology of the 12- and the 25-kDa subunits of the mammalian signal peptidase complex. *J. Biol. Chem.* 271:3925-3929.

Kalish, J.E., Theda, C., Morrell, J.C., Berg, J.M., and Gould, S.J. 1995. Formation of the peroxisome lumen is abolished by loss of *Pichia pastoris* Pas7p, a zinc-binding integral membrane protein of the peroxisome. *Mol. Cell. Biol.* 15:6406-6419.

Kreis, T.E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO J*. 5:931-941.

Kreis, T.E. and Lodish, H.F. 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. *Cell* 46:929-937.

Kurihara, T. and Silver, P. 1993. Suppression of *sec63* mutation identifies a novel component of the yeast endoplasmic reticulum translocation apparatus. *Mol. Biol. Cell* 4:919-930.

Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol*. 157:105-132.

Lai, C.-C., Hong, K., Kinnell, M., Chalfie, M., and Driscoll, M. 1996. Sequence and transmembrane topology of MEC-4, an ion channel subunit required for mechanotransduction in *Caenorhabditis elegans. J. Cell Biol*. 133:1071-1081.

Li, J.-M. and Shore, G.C. 1992. Reversal of the orientation of an integral protein of the mitochondrial outer membrane. *Science* 256:1815-1817.

Lim, P.S., Jensen, A.B., Cowsert, L., Nakai, Y., Lim, L.Y., Jin, X.W., and Sundberg, J.P. 1990. Distribution and specific identification of papillomavirus major capsid protein epitopes by immunocytochemistry and epitope scanning of synthetic peptides. *J. Infect. Dis*. 162:1263-1269.

Manoil, C. and Beckwith, J. 1986. A genetic approach to analyzing membrane protein topology. *Science* 233:1403-1408.

Mize, N.K., Andrews, D.W., and Lingappa, V.R. 1986. A stop transfer sequence recognizes receptors for nascent chain translocation across the endoplasmic reticulum membrane. *Cell* 47:711-719.

Mullins, C., Lu, Y., Campbell, A., Fang, H., and Green, N. 1995. A mutation affecting signal peptidase inhibits degradation of an abnormal membrane protein in *Saccharomyces cerevisiae*. *J. Biol. Chem*. 270:17139-17147.

Nilsson, T., Jackson, M.R., and Peterson, P.A. 1989. Short cytoplasmic sequences serve as retention signal for transmembrane proteins in the endoplasmic reticulum. *Cell* 58:707-718.

Russel, M. and Model, P. 1982. Filamentous phage pre-coat is an integral membrane protein: Analysis by a new method of membrane preparation. *Cell* 28:177-184.

Seckler, R., Moroy, T., Wright, J.K., and Overath, P. 1986. Anti-peptide antibodies and proteases as structural probes for the lactose/H^+ transporter of *Escherichia coli*: A loop around amino acid residue 130 faces the cytoplasmic side of the membrane. *Biochemistry* 25:2403-2409.

Sengstag, C., Stirling, C., Schekman, R., and Rine, J. 1990. Genetic and biochemical evaluation of eukaryotic membrane protein topology: Multiple transmembrane domains of *Saccharomyces cerevisiae* 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Mol. Cell. Biol*. 10:672-680.

Skach, W.R., Shi, L., Dalayag, M.C., Frigeri, A., Lingappa, V.R., and Verkman, A.S. 1994. Biogenesis and transmembrane topology of the CHIP28 water channel at the endoplasmic reticulum. *J. Cell Biol*. 125:803-815.

Steck, T.L. and Yu, J. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. *J. Supramol. Struct*. 1:220-248.

Swinkels, B.W., Gould, S.J., Bodnar, A.G., Rachubinski, R.A., and Subramani, S. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J*. 10:3255-3262.

Taussig, R. and Carlson, M. 1983. Nucleotide sequence of the yeast *SUC2* gene for invertase. *Nucl. Acids Res*. 11:1943-1954.

von Heijne, G. 1984. Analysis of the distribution of charged residues in the N-terminal region of signal sequences: Implications for protein export in prokaryotic and eukaryotic cells. *EMBO J*. 3:2315-2318.

Walter, P. and Blobel, G. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol*. 96:84-93.

Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenson, A.R., Connolly, M.L., and Lerner, R.A. 1984. The structure of an antigenic determinant in a protein. *Cell* 37:767-778.

Xia, D., Yu, C.-A., Kim, H., Xia, J.-Z., Kachurin, A.M., Zhang, L., Yu, L., and Deisenhofer, J. 1997. Crystal structure of the cytochrome bc_1 complex from bovine heart mitochondria. *Science* 277:60-66.

Contributed by Neil Green and Hong Fang
Vanderbilt University School of Medicine
Nashville, Tennessee

Kai-Uwe Kalies
Max Delbruck Center for Molecular Medicine
Berlin, Germany

Victor Canfield
Pennsylvania State University College of Medicine
Hershey, Pennsylvania

Determination of Molecular Size by Zonal Sedimentation Analysis on Sucrose Density Gradients

This unit describes one of best methods for estimating molecular size of proteins or protein complexes under nondenaturing conditions, namely, zonal sedimentation analysis on sucrose gradients. This method separates macromolecules on the basis of sedimentation in a centrifugal field. In simplest terms, larger molecules will be subjected to a greater force in a given time of centrifugation at a constant speed, and will therefore migrate farther into a gradient than smaller molecules. Migration is a property of the sedimentation coefficient of a given macromolecule and reflects its mass, shape, density, and hydration state. Thus, given appropriate controls and the value of the partial specific volume (a measure of the density of a macromolecule), mass can be either approximated or, given the value of the Stokes radius, calculated.

Because separations are performed under nondenaturing conditions, sedimentation analysis is suitable for determination of the molecular size of multisubunit protein complexes or of the oligomerization state of a homooligomer. The method, first developed by Martin and Ames (1961), is particularly suitable for globular proteins and can be used with some limitations for mass approximation of integral membrane proteins in detergent solutions. Most importantly, proteins or protein complexes in heterogeneous mixtures, such as cell lysates and homogenates, cell supernatants, and sera, can be analyzed by this assay if an appropriate activity (enzyme activity, antibody binding, or spectrophotometric property) can be measured. During separation, a sucrose gradient provides stability for sedimentation of macromolecules against diffusion and mechanical disturbance while permitting sedimentation at constant velocity.

Traditional methods of sedimentation analysis have provided relatively broad fractionation of protein complexes, particularly integral membrane proteins in detergent solutions, permitting only rough estimations of molecular mass. Nevertheless, these determinations have consistently approximated the actual molecular mass of globular soluble proteins. Furthermore, recent advances in gradient formation and fractionation may provide for more precise analyses. Although the protocols in this unit focus on proteins, zonal centrifugation is also suitable for size determination of nucleoprotein complexes, including viruses, and small nucleic acid species.

Protocols are provided for the preparation of sucrose gradients, the separation of cell lysates on the gradients (see Basic Protocol 1 and Alternate Protocol 1), and fractionation of the sucrose gradients themselves to provide molecular size determinations over narrow or broad ranges (see Basic Protocol 2 and Alternate Protocols 2 and 3). Support protocols are provided for choosing molecular size markers to use in estimating sedimentation coefficients and molecular weights (see Support Protocol 1), for calculating sedimentation coefficients (see Support Protocol 2), and for calculating molecular mass based on sedimentation coefficient and other parameters (see Support Protocols 3, 4, and 5). An additional protocol is provided for differential sedimentation in sucrose gradients made with either water or deuterium oxide (heavy water; D_2O), which allows for the direct determination of partial specific volumes and thus circumvents some of the assumptions implicit in molecular weight estimations based on migration relative to marker proteins (see Basic Protocol 3).

Contributed by Michael S. Marks

Current Protocols in Cell Biology (1998) 5.3.1-5.3.33

STRATEGIC PLANNING

In zonal centrifugation an initial preparation, for example, a cell lysate, is subjected to centrifugation through a sucrose gradient in an ultracentrifuge, and then the gradient is eluted into distinct fractions. Analysis is therefore limited by (1) the method for detection of the protein of interest within the fractions and (2) the method for calculating the sedimentation coefficient itself and the corresponding molecular weight. These accompanying methods must be thought out and prepared in advance if a meaningful analysis is to be obtained. Furthermore, consideration needs to be given to the range of separation and to the resolution needed in order to determine (1) the slope of the gradient, (2) the size of tube for ultracentrifugation, and (3) the length of ultracentrifugation. Finally, one must consider the variety of available methods for formation and fractionation of the gradients and choose those most appropriate for the anticipated needs and/or equipment availability.

Detection of Proteins within Fractions

The primary assays for detecting a given protein during the development of the zonal centrifugation technique were spectrophotometric identification and enzymatic activity measurement. Spectrophotometric detection is limited either to analysis of purified proteins or relatively simple mixtures of a few proteins by detection at 280 nm, or to analysis of proteins with unique spectral properties within a mixture (such as heme-containing or chromogenically tagged proteins). Conventional protein detection at 280 nm requires at least microgram quantities for any reasonable analysis. Similar nonspecific protein detection methods can make use of protein assays such as the Lowry and Bradford assays; while perhaps more sensitive, these assays are also limited to analysis of only purified or partially purified proteins. Simple gel electrophoresis (*UNIT 6.1*) and staining with Coomassie blue or silver may provide useful analyses of somewhat more complex mixtures and more limiting (submicrogram) quantities. This method requires knowledge of the molecular mass of at least one subunit of a protein complex.

Enzymatic activity is still a useful parameter for assaying either purified proteins or proteins within a mixture; however, it is useful within a mixture only if a unique activity can be ascribed to a single protein. Furthermore, activity assays are variably sensitive to the concentration of the enzyme, and any assay must take into account the buffer components of the gradient fractions and any effect of sucrose concentration.

Today, most methods of detection rely on antibodies specific for the protein in question. Since antibodies can be exquisitely specific, immunodetection can be performed on fractions of the most crude samples, including cell and tissue homogenates and lysates. Furthermore, most immunodetection methods are unaffected by common components in the gradients themselves, and they are extremely sensitive, requiring only minute quantities of protein that would be undetectable by other means. Applicable immunodetection methods include enzyme-linked immunosorbent assays (ELISAs), SDS gel electrophoresis and immunoblotting (*UNITS 6.1 & 6.2*), and immunoprecipitation of metabolically labeled or surface radioiodinated proteins within a cell lysate (*UNIT 7.2*). When using radiolabeled source material, one must take particular care to follow typical precautions to avoid contamination of the experimenter and surroundings during the fractionation of the sucrose gradients (see *APPENDIX 1D*).

Methods for Formation of Gradients

One of the most critical points for the success of the zonal centrifugation experiment is preparation of the sucrose gradients. For accurate analyses, all gradients within a given experiment must be prepared to be as nearly identical as possible. Furthermore, the

gradient should be as linear as possible in order for macromolecules to migrate consistently. Therefore, the choice of method for formation of gradients is a critical one. This unit describes two of the most frequently used methods for formation of sucrose density gradients. The first method, using the Gradient Master (Biocomp), is clearly superior in that multiple gradients can be prepared identically, consistently, and simultaneously with minimal time and effort. However, the Gradient Master is not inexpensive and may not be available to all laboratories; furthermore, it requires extra accessories for each different sized centrifuge tube in which gradients are required to be made. Alternate Protocol 1 describes how to make gradients using a mixing chamber, a more common, versatile, and inexpensive device available in many laboratories and from many vendors. This method is more time-consuming and has the potential to provide less consistent and nonlinear gradients. However, if proper care is taken, mixing chamber devices can provide gradients of sufficient quality for most experimental needs. Other instruments not described here may be available from other manufacturers.

Sedimentation Conditions

Variables in the sedimentation conditions include length of time of centrifugation, size of the tube, and slope of the sucrose density gradient. All of these variables affect the migration and resolution of proteins and should be considered prior to the experiment. The appropriate length of time of centrifugation is dependent on the range of fractionation desired. Shorter runs will provide a greater range of fractionation, but the resolution will be small. Longer runs may result in precipitation of high-molecular-weight material, but they will provide greater resolution in lower-molecular-weight ranges. In general, maximal resolution is obtained if the protein(s) of interest migrates near the middle of the gradient after the run. If the approximate molecular weight and corresponding expected sedimentation coefficient are known, then the time required for the protein to migrate halfway through the gradient can be estimated from the equation $T = k'/2S$, where T is the time in hours, k' is a constant for any given rotor at a given speed (335 for the SW41 Ti rotor at 39,000 rpm used in the examples in this protocol), and S is the sedimentation coefficient of the protein. Obviously, changing the rotor used and the corresponding size of the tube will affect the length of the centrifuge run required for sedimentation of a given protein complex; it should take roughly half as long for a protein of a given size to migrate to the middle of a 60-mm tube than it would for the same protein to migrate to the middle of a 120-mm tube under the same centrifugal force. The concentration of sucrose in the gradient can also affect sedimentation. An increased concentration of sucrose in the gradient can allow for greater resolution in higher-molecular-weight ranges. A 5% to 20% sucrose gradient permits a relatively constant velocity of particle movement and so is a good starting point for samples of unknown characteristics.

Methods for Fractionation of Gradients

Fractionation of the gradient is the most important step in determining the resolution of the sedimentation experiment. Care must be taken to minimize the amount of turbulence during elution to avoid disturbing the gradient and causing band spreading. Although microbore tubing may be used during elution, for example, to elute material through a peristaltic pump and/or to a fraction collector, be aware that the tubing introduces mixing caused by laminar capillary flow; fluid contacting the wall of the tubing moves at a slower rate than fluid in the middle of the tubing. Traditional methods of elution introduce a significant degree of mixing due to turbulence and/or laminar capillary flow. These methods have therefore provided relatively low resolution for the sedimentation experiment. In most cases, however, this low resolution has been sufficient to provide information on the relative mass of a protein or protein complex.

There are many methods of gradient elution, and numerous commercial devices are available to perform them. Generally speaking, there are two ways of eluting the gradient: from the bottom and from the top. Elution from the top requires suction, either mechanically from a pump or manually from a pipettor. Among the most popular instruments for this type of elution is the Büchler Auto Densi-Flow system (Labconco) which uses a stepper motor to continually lower a suction head; the suction head contains an electronic eye that detects the top of the gradient and controls the stepper motor. Another popular instrument, available from ISCO, uses a syringe pump to dispense a dense solution through the bottom of the tube, pushing up on the gradient and into a suction head at the top of the gradient. The suction process with both instruments can result in significant turbulence and band spreading due to the nature of hydrodynamic forces and capillary action. A recent theoretical advance involves use of a stepper motor and a trumpet-shaped tip to elute the gradient from the top. The trumpet tip is designed to minimize the effects of capillary flow and turbulence in the suction. The manufacturers (Biocomp) claim that this allows for high resolution of bands separated by as little as 1.5 mm. All of these instruments are quite costly and are variably available in different laboratories that routinely perform sedimentation analyses. To use them, the reader is referred to the manufacturer's instructions.

Elution from the bottom eliminates the requirement for suction during the elution, but it is limited in that any pellet that forms at the bottom of the tube during centrifugation may interfere with elution, causing turbulence and mixing of material from the pellet with material contacting the pellet. This problem can be largely avoided by first clearing the sample prior to fractionation by centrifugation at 100,000 × *g*. Nevertheless, given the length of centrifugation and the constant subjection to high *g* forces, it is likely that some pellet will form in most samples during an experiment.

This unit describes low-cost, simple protocols of gradient elution that use common equipment available in most laboratories. All of these protocols are more cumbersome and less efficient than the automated elution methods using the instruments described above. Nevertheless, they can provide fractionation with sufficient resolution for most needs.

Calculation of Sedimentation Coefficient and Molecular Weight

Once the samples have been sedimented through gradients, fractionated, and analyzed for the presence of the protein of interest and standard proteins, sedimentation coefficients can be estimated or calculated. The sedimentation coefficient, *S*, is expressed in units called the Svedberg (S); one Svedberg is equivalent to 10^{-13} sec. The value of *S* can be calculated directly from the results of the experiment: the time of centrifugation, the angular velocity of the rotor, and the position of migration of the sample. However, this value is not in itself particularly meaningful. Reported values of sedimentation coefficients are under the "standard state" of water at 20°C ($S_{20,w}$). Therefore, calculated *S* values must be converted to $S_{20,w}$ values for any meaningful analysis. Methods for determination of these parameters are discussed in detail in Support Protocols 2, 3, 4, and 5. However, consideration of *S* values must be given in the planning stages of the experiment, as these values can be used to estimate the molecular weight or the oligomerization state of macromolecules.

In most cases, experiments will include a sample in which protein standards of known sedimentation coefficient are fractionated in parallel or in the same tube as experimental samples. The choice of standard proteins and the method chosen to detect them must be considered in planning the experiment, including determining the number of centrifuge tubes used. In some cases, such as analyses of integral membrane proteins, it may be

desirable to use intrinsic standards with characteristics that are as similar as possible to the sample in question. In other cases, such as analyses of soluble globular protein complexes, purified proteins available from commercial sources may suffice as size standards. A list of some typical standard proteins along with their sedimentation coefficients, partial specific volumes, and molecular weights is provided in Table 5.3.2 in Support Protocol 1.

Additional pieces of information and some assumptions will be required. In some cases, it is possible to estimate molecular mass by assuming a globular conformation with typical hydration and density, similar to standard proteins used in the analysis. If it is comfortable to assume that the properties of the standard proteins and the unknown sample are similar, then relative sedimentation coefficients can be estimated directly by comparing the migration of standard proteins to that of the sample. This is described in Support Protocol 2. However, for samples with unknown properties, and particularly for integral membrane protein complexes in detergent solutions, these assumptions and estimations may not be appropriate. The difficulties are amplified for integral membrane protein complexes by the typically high partial specific volumes of some common detergents, such as Triton X-100 and NP-40, relative to that of spherical proteins.

For more precise analyses, as described in Support Protocols 3, 4, and 5, other parameters will be needed. The concentration of sucrose in each fraction must be determined by refractometry (described in Basic Protocol 1) in order to extrapolate the values of density and viscosity of the medium, which in turn are parameters necessary to convert values of S to $S_{20,w}$. A value for partial specific volume (υ) must be included as well and can be attained in one of three ways. If the sample is known to be globular and water soluble, then one can use a "typical" value of υ as listed in Table 5.3.2 in Support Protocol 1. If the contents of the sample are known or can be estimated (such as in analysis of the oligomeric state of a purified complex), including the amino acid, sugar, and detergent composition of each component, then one can calculate a value for υ by summing the individual υ values of each component as described in Support Protocol 4. Finally, υ and $S_{20,w}$ can be measured directly by monitoring the sedimentation through two different solutions of distinct densities; in practice, this can be done by performing two parallel sedimentation experiments in which one gradient solution is made with water and the other with D_2O, as described in Basic Protocol 3. The methods used should be considered carefully based on the degree of precision required in the analysis and on the available information regarding the sample under analysis.

ZONAL SEDIMENTATION USING SUCROSE GRADIENTS FORMED BY A GRADIENT MASTER

BASIC PROTOCOL 1

This protocol first describes one of the best methods for preparing sucrose density gradients, and then describes the use of these gradients for sedimenting protein mixtures in a preparative ultracentrifuge. The proteins are recovered by fractionation (for hole puncture and elution, see Basic Protocol 2; for peristaltic elution, see Alternate Protocol 2; for manual fractionation from the top of the gradient, see Alternate Protocol 3) and detected by an appropriate method (see Strategic Planning). An alternative way of making sucrose gradients using a mixing chamber is given in Alternate Protocol 1.

Multiple gradients can be reliably and reproducibly prepared in a single step using the Gradient Master, an instrument designed by Coombs and Watts (1985) and marketed by Biocomp Instruments (see *SUPPLIERS APPENDIX*). The basis of the protocol is to create each gradient in a single tube, using solutions corresponding to the beginning and endpoint concentrations of sucrose. The more dense solution is placed at the bottom, and the less dense solution is placed at the top. The tube is then capped, tilted at a sharp angle, and

rotated about its longitudinal axis at a predetermined speed. If the angle and speed of rotation are appropriate, this procedure mixes the contents in a reproducible and precise fashion to form a linear gradient from the most to the least dense sucrose concentration. The manufacturer provides explicit protocols for the formation of gradients of various concentrations of sucrose and other media in tubes of various sizes. The protocol below describes preparation of a typical gradient of 5% to 20% sucrose in 14 × 89–mm tubes, which are used in the Beckman SW41 Ti swinging-bucket rotor or equivalent for the preparative ultracentrifuge. The protocol can be adapted for sucrose gradients of different concentrations or for different sized tubes according to the manufacturer's instructions.

Once the gradients are formed and cooled to 4°C, samples are loaded onto the gradients and subjected to ultracentrifugation. For analysis of results, standard proteins are also sedimented under the same conditions (for use of molecular size markers, see Support Protocol 1). Centrifugation is typically performed at the maximum safe speed for a particular rotor (in this example, 39,000 rpm in the SW41 Ti rotor, with an average force of 188,000 × *g*). As discussed in Strategic Planning, the length of time of centrifugation and the size of the ultracentrifuge tube and corresponding rotor can be varied and depend on the range of fractionation desired. The concentration of sucrose in the gradient can also be varied; it has been clearly demonstrated that particles move at constant velocity through a 5% to 20% sucrose gradient, and thus this is a good starting point. The example here uses a 5% to 20% gradient in centrifuge tubes designed for the SW41 Ti swinging-bucket rotor.

Materials

Buffer ingredients for stock solutions (e.g., HEPES, NaCl, Triton X-100, protease inhibitors, sodium azide)
Sucrose, ultrapure
Sample in 100 to 250 µl volume (e.g., cell lysate)

Whatman no. 1 filter paper or 0.22-µm filter
Refractometer
14 × 89–mm centrifuge tubes for SW41 Ti rotor
Gradient Master (Biocomp), base unit, marker block, and holder for six 14 × 89–mm tubes
10-ml syringe fitted with a 6-inch wide-bore cannula
SW41 Ti rotor (Beckman) or equivalent
Ultracentrifuge (Beckman LS-70M or equivalent)

Prepare sucrose stock solutions

1. Prepare stocks for the buffers to be used for the sucrose gradients.

   All stocks should be filter sterilized, using a 0.22- or 0.45-µm filter for long-term storage, except for detergent stocks. A typical buffer for fractionation of cell lysates and analysis of integral membrane proteins by immunodetection methods would contain:

   0.02 M HEPES, pH 7.4 (from a 1 M stock)
   0.15 M sodium chloride (from a 5 M stock)
   0.05% (w/v) Triton X-100 (from a 20% stock)
   Protease inhibitors (from individual stocks as needed)
   0.02% (w/v) sodium azide (from a 2% stock).

   In this example, the buffer is at physiological ionic strength and pH. Triton X-100 is included as a nonionic detergent to maintain solubility of integral membrane proteins; it could be omitted for analysis of soluble proteins in membrane-depleted cell homogenates, or substituted by another detergent as appropriate. Optimally, several runs in different detergents with distinct properties would be performed to ensure that physiological

complexes are observed. Other detergents of choice might include the zwitterionic detergent CHAPS or the neutral detergent n-octylglucoside, either of which may be removed from samples later by dialysis. Sodium azide is included as a preservative and may be omitted without serious consequences if the gradients are poured and used in a timely fashion. In most cases, protease inhibitors are not necessary at this stage if they were included in cell lysates; however, maintenance of some labile proteins may require the continued presence of inhibitors that block proteases by noncovalent interactions.

Different applications will require different buffers. For example, nucleotides, calcium, magnesium, or other ions may be required to maintain the integrity of some protein complexes. Finally, buffers of different pH or ionic strength may be required for some applications, which may complicate analysis of the sedimentation coefficient.

2. Prepare a 60% (w/v) stock solution of ultrapure sucrose in doubly deionized or distilled water with 0.02% (w/v) sodium azide. Filter the stocks at least through Whatman no. 1 filter paper, and preferably through a 0.22-µm filter, to remove any particulates.

   Use only Ultrapure sources of sucrose; contaminants present in reagent grade sucrose may disrupt protein complexes and interfere with preparation of stocks. When preparing the sucrose stock solution, assume that the sucrose is not 100% pure. Even ultrapure sources may not be 100% sucrose on a per weight basis. Do not bring the solution to final volume until you have checked the concentration of an initial solution by refractometry.

3. Check the concentration of the sucrose stock solution using a refractometer. Place a drop of the solution on the refractometer window and read the refractive index. Calculate the true sucrose concentration based on the refractive index and adjust the concentration such that the refractive index reads 1.4418.

   The refractive index, a measure of the viscosity of the solution, provides the most accurate way to determine sucrose concentration. The refractive indices for given concentrations of sucrose at 20°C have been predetermined and are listed in Table 5.3.1. A 60% sucrose solution at 20°C should have a refractive index of 1.4418. Temperature affects the refractive index, so readings of solutions at other temperatures must take into account a correction factor. Correction factors and additional data are available in the CRC Handbook of Chemistry and Physics (Lide, 1997).

Table 5.3.1 Refractive Index of Standard Aqueous Sucrose Solutions[a]

Sucrose concentration (%)	Refractive index
0	1.3330
3	1.3374
5	1.3403
7	1.3433
10	1.3479
12	1.3510
15	1.3557
20	1.3639
25	1.3723
30	1.3811
35	1.3902
40	1.3997
50	1.4200
60	1.4418

[a]Data from Lide (1997).

4. Using the buffer and sucrose stock solutions and doubly deionized or distilled water, prepare working solutions of 5% (w/v) sucrose and 20% (w/v) sucrose in the appropriate buffer.

   When sodium azide is used as a preservative, large quantities of stock solutions can be prepared and stored at 4°C. Otherwise, enough should be prepared for all of the samples in the experiment.

   In this example, there will be six tubes for the SW41 Ti rotor. Each tube has a capacity of 12.6 ml and will receive 6.3 ml of each solution. Thus, ≥38 ml of each working solution is required, and more should be prepared to be safe. Working solutions should be checked for sucrose concentration by refractometry.

Prepare gradients

5. Prepare centrifuge tubes by marking the half-full line. Place each tube into the appropriate marker block of the Gradient Master and draw a line on the tube just above the level of the block. Label each tube for sample identification.

6. Using a pipet, fill each tube to ~2 mm above the half-full line with the less dense endpoint solution—in this example, the 5% sucrose working solution. Be careful not to make any bubbles while filling the tube.

   For the SW41 Ti rotor, this should take ~6.3 ml per tube.

7. Using a 10-ml syringe fitted with a 6-inch wide-bore cannula, underlay each tube with the heavier endpoint solution—in this example, the 20% sucrose working solution. To do this, carefully lower the cannula to the bottom of the tube, and slowly push the plunger of the syringe to release the contents. Keep the end of the cannula close to but below the interface and slowly fill the tube. Continue filling until the interface lies exactly at the half-full line. Carefully remove the cannula, and cap the tube with the short caps provided with the Gradient Master. Remove any air bubbles. Repeat this procedure for all tubes to be used in the experiment (at least two to provide a balance tube for a single run).

   The interface between the light and dense solutions will be visible as a sharp line. Be careful not to disturb the upper layer by pushing too fast or by shaking the cannula while dispensing the heavy solution. Placement of the cap should remove any air space in the tube; be sure that there are no bubbles.

8. Prepare the Gradient Master for forming the gradients. First, level the Gradient Master base unit according to the manufacturer's instructions. Then, set the time, angle, and rpm of rotation appropriate for the gradient being prepared.

   The manufacturer provides a table with optimal parameters for common gradients, and provides a protocol to determine the optimal parameters for less common gradients.

   In this case, a 5% to 20% gradient in 14 × 89–mm tubes requires 2 min 25 sec of rotation at an angle of 81.5° from the vertical at 15 rpm.

9. Finally, place tubes into the tube holder and start the rotation by pressing run.

   The Gradient Master will tilt the tubes at the appropriate angle, rotate at the desired speed for the desired amount of time, and then return the tubes to the vertical position.

10. Uncap the tubes, balance the tubes by weighing them on a laboratory balance and removing any excess from the very top, and store the tubes at 4°C until ready for the samples.

    The gradients are cooled to 4°C before samples are loaded. It is convenient to store the gradients, covered with plastic wrap, in a cold room. Gradients are stable for several days, but it is usually best to prepare them a few hours before use. Preparation of samples may

proceed or conclude during cooling of the gradients. Be sure that your colleagues are aware not to bang into the tubes—this will obviously disturb the gradients!

Load and centrifuge samples

11. Before loading the samples, be sure that the ultracentrifuge has been turned on and is cooled to the appropriate temperature.

    Most often, it is convenient to perform the ultracentrifugation at 4°C. The ultracentrifuge should be turned on with the vacuum running if it is cooling or cooled to prevent condensation within the chamber.

12. Carefully remove and discard material from the top of the gradient equivalent to the volume of sample that you wish to load.

    After separation, the width of the ultimate bands will be at least the same as the width of the sample loaded. Therefore, the sample volume should be minimal compared to the volume of the tube. For the 14 × 89–mm tubes of the SW41 Ti rotor, the volume loaded should be ≤250 µl for efficient resolution, and a smaller volume (~100 µl) is preferable. The sample must be in a solution that is less dense than the lower endpoint sucrose solution (e.g., 5% sucrose) such that it remains at the top of the gradient. It is a good idea to prefilter the sample or clear it by ultracentrifugation at high g forces. Be sure to include enough tubes to balance the SW41 Ti rotor; if an odd number of samples is required, prepare a balance tube with an equivalent gradient and an equivalent volume of sample.

13. Carefully and very slowly load the sample onto the gradient, taking care to avoid disturbing the top of the gradient.

14. Place all gradients into the buckets of the rotor and carefully transport them to the ultracentrifuge. Place the buckets into the rotor head. Turn off the vacuum to the ultracentrifuge, and open the ultracentrifuge door. Carefully lower the rotor into the ultracentrifuge chamber. Close the door and start the vacuum. Set the speed, time of centrifugation, acceleration rate (as slow as possible), and deceleration rate (brake off). Begin centrifugation by pressing start.

    Speed and time should be determined for each experiment. Generally, each rotor is run at maximum speed, but slower speeds may permit resolution of lower-molecular-weight material in a convenient time frame. Time of centrifugation will depend on the resolution and fractionation range desired. Generally, times start at ~16 hr and go up to 48 hr. To achieve equivalent separations in shorter times, use a smaller tube and sample size.

End the run

15. Be sure that the ultracentrifuge has stopped rotating. Turn off the vacuum, open the chamber door, and remove the rotor from the ultracentrifuge. Close the door to the ultracentrifuge and restart the vacuum. If another run is not planned, allow the ultracentrifuge to return to room temperature before turning off the vacuum; ensure that the vacuum is released (open the chamber door and reclose it), and turn off the power.

16. Remove sample buckets from the rotor and place them in the carrying tray. Carefully store the buckets briefly at 4°C until elution.

    Samples should be eluted as soon as possible (within a few hours) to avoid band spreading and loss of resolution. Following elution (for details on fractionation of gradients, see Basic Protocol 2 and Alternate Protocols 2 and 3), the fractions are analyzed using the appropriate, predetermined method (see Detection of Proteins).

ALTERNATE PROTOCOL 1

ZONAL SEDIMENTATION USING SUCROSE GRADIENTS FORMED BY A GRADIENT MAKER

The Biocomp Gradient Master used in Basic Protocol 1 is a relatively expensive instrument and may not be universally available. However, it is possible to create individual sucrose gradients with significant reproducibility using a standard polycarbonate mixing chamber, which is a much less expensive item. The basic device, available from numerous sources, contains two separate cylindrical chambers connected by a small tube at the bottom of each chamber. It is critical that the dimensions of the two chambers are identical. Typically, a stopcock is placed within the connecting tube to prevent contact between the chambers until desired. An outlet tube emanates from the bottom of one of the chambers (chamber 1) and is usually also connected with a stopcock to prevent flow until desired. A stir bar is placed in chamber 1, and the device is placed on a stir plate. A typical design is outlined in Figure 5.3.1.

The operation of the mixing device is theoretically simple. Equal volumes of the endpoint sucrose solutions are placed in each chamber, and the connection between the two chambers is opened. If the heights of the liquids in the two chambers are the same, then there should be no flow from one chamber to the other. If the outlet tube is now opened, however, fluid will flow out of chamber 1. This will create an imbalance in the hydrostatic pressure in the two chambers; as a result, fluid from chamber 2 will flow into chamber 1 such that the height of the liquids in the two chambers remains the same. If the less dense sucrose solution is placed in chamber 1, this means that the more dense sucrose solution will flow into chamber 1 as fluid exits the device. If the outflow is slow, and the solution in chamber 1 at any given time is kept well mixed by the action of the stir bar, then the result will be a linear gradient of sucrose concentration, from the least dense to the most

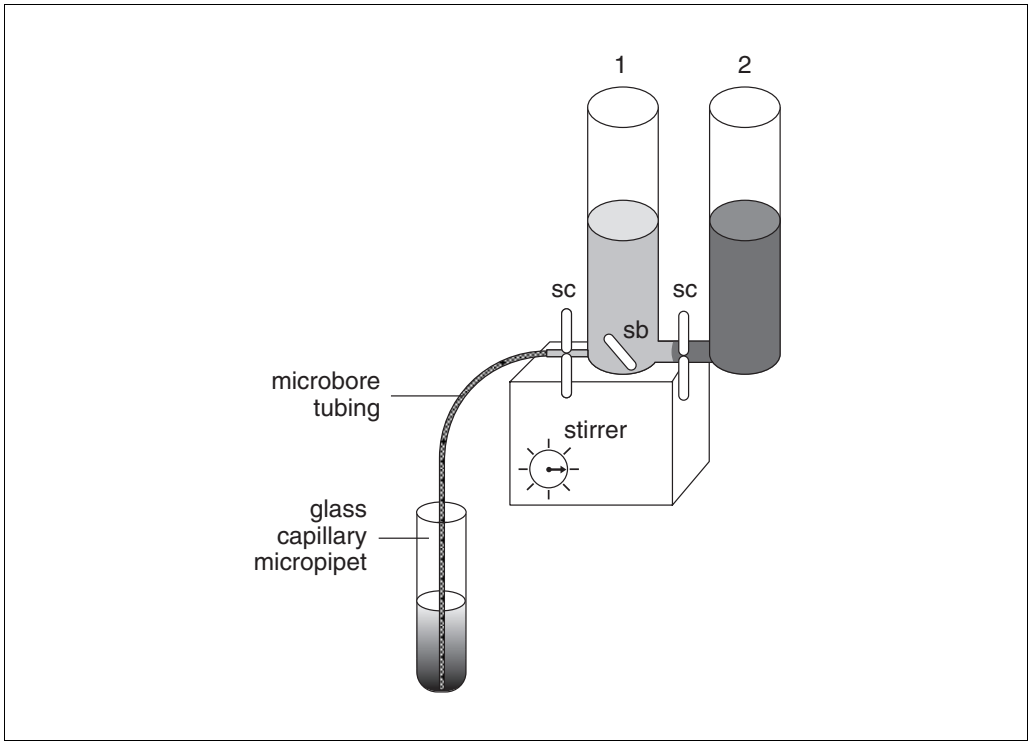


Figure 5.3.1 Formation of sucrose gradients with manual gradient maker. A typical apparatus is diagrammed. The dense (darker) material is in chamber 2 (2), and the less dense material is in chamber 1 (1). Material is kept constantly stirred by adding a stir bar (sb) in chamber 1 and placing chamber 1 over an electric stirrer as indicated. Stopcocks (sc) regulate the flow between the chambers and out of chamber 1. Microbore tubing and a glass capillary micropipet carry the material into the gradient tube.

dense, exiting the device. For preparation of sucrose gradients, the outflow will enter the bottom of a centrifuge tube, such that the increasingly dense solution will continually underlie the fluid already present in the tube.

The mixing chamber device can be connected through any of a variety of apparatuses to increase the efficiency and speed with which gradients are formed, including a peristaltic pump, a specialized flow sensor/delivery system (such as the Auto Densi-Flow system from Labconco), or a system to deliver the gradient solution to multiple tubes at once (such as the Büchler Density Gradient Forming System from Labconco). All of these require specialized equipment that may be quite costly but which may be available in laboratories that routinely perform these procedures.

The protocol below describes the basic procedure for gradient formation using gravity flow and the 5% to 20% sucrose gradient and 14 × 89–mm ultracentrifuge tubes as an example. As with the Gradient Master, different endpoint sucrose solutions and different sized tubes can be easily accommodated.

Additional Materials *(also see Basic Protocol 1)*

Gradient maker, small volume (e.g., 15 ml)
Microbore tubing
Glass capillary micropipet
Peristaltic pump or other pumping device (optional)

Prepare sucrose solutions and set up gradient maker

1. Prepare 5% and 20% sucrose working solutions (see Basic Protocol 1, steps 1 to 4), allowing a slight excess (perhaps 1 ml extra per sample) of each final sucrose solution to ensure sufficient filling of each tube by this method. Be sure to prepare enough.

2. Place the gradient maker on a stir plate on a shelf or attach to a ring stand to provide a sufficient flow rate to the tube on the bench. Level the gradient maker using a bubble level. Attach a sufficient length of microbore tubing to the outflow connector of the gradient maker so as to reach the tubes in a rack on the bench. Finally, attach a glass capillary micropipet to the end of the tubing.

   The glass capillary micropipet should fit snugly into the microbore tubing. The inner diameter of the microbore tubing and capillary micropipet should be as similar as possible to that of the outflow connector of the gradient maker in order to minimize turbulence during the preparation of the gradient.

3. Place the glass capillary pipet into a centrifuge tube in a rack on the bench such that the open end of the capillary pipet is at the bottom of the tube. Test flow rates from the gradient maker using water and ensure that the rate is ≤0.5 to 1 ml/min. Adjust the rate by moving the gradient maker up or down relative to the bench or alternatively by adjusting a peristaltic pump.

   It is easiest to use gravity to elute the gradient from the gradient maker. Alternatively, the outflow tubing can be connected through a peristaltic pump to provide even flow at a slow rate. However, care should be taken to minimize the length of tubing through which the sucrose solution will flow. Passage through the tubing creates turbulence, which may disrupt the linearity and reproducibility of the gradient. The use of a peristaltic pump may provide additional turbulence. In addition, care should be taken to minimize the speed at which the gradient is formed, because too great a speed of flow will create additional turbulence. A flow rate of ≤0.5 to 1 ml/min allows for sufficient mixing and ensures a linear gradient. The capillary micropipet can be held in place over the centrifuge tube using a cap with a small hole pierced through it, through which the tubing and capillary micropipet are passed. Care should be taken such that the cap does not contact the contents of the tube.

Load sucrose solutions in the gradient maker

4. Make sure that all stopcocks are in the vertical (closed) position. Place 6.4 ml of 5% sucrose solution in chamber 1 (from which the outflow connector emanates). Open the stopcock between the two chambers briefly to allow for the tube connecting them to fill with the solution (this ensures that there are no air bubbles blocking flow between the two chambers). Transfer any solution that accumulated in chamber 2 back to chamber 1, but make sure that the connecting tube between the chambers stays full of solution.

   Entrapment of air bubbles is the biggest problem with this technique; any air bubbles in the connecting tube or the outflow tubing will slow the movement of the solutions, cause turbulence, and prevent efficient formation of linear gradients. Care must be taken to avoid trapping air bubbles. It may be necessary to carefully tap on the chamber device to get full flow between the two chambers.

5. Place 6.4 ml of 20% sucrose solution in chamber 2. Place a small flea stir bar in chamber 1. Engage the stir plate at a speed sufficient to mix the entire contents of chamber 1 without creating bubbles or a large vortex in the solution.

6. Check the height of the solutions in the two chambers. If necessary, carefully add enough sucrose solution to the appropriate chamber to bring the solutions in the two chambers to exactly the same height.

7. Open the stopcock between the two solutions.

   If the height of the solutions in the two chambers was the same, there should be no flow between them at this point.

Form the gradients

8. Carefully open the stopcock to the outflow tubing to begin emptying the contents of chamber 1.

   As the contents empty, the gradient should be eluted into the centrifuge tube, and the height of the solution in both chambers 1 and 2 should fall evenly. If necessary, adjust the height of the device and the stir plate to ensure a slow, even flow rate.

   If necessary, carefully tap the outflow tube to begin the flow.

9. When both chambers and the outflow tubing are empty, and the gradient is completely formed, carefully and slowly remove the glass micropipet from the tube. If the volume of liquid in the tube is too high (overflowed the centrifuge tube) or too low (did not fill the centrifuge tube completely), then adjust the volume for each solution for the next tube. Rinse and dry the gradient maker and repeat steps 3 to 8 for each tube in the experiment. Store the gradients in a rack at 4°C until the experiment is performed.

Perform ultracentrifugation

10. Load and centrifuge samples (see Basic Protocol 1, steps 10 to 16).

SUPPORT PROTOCOL 1

USE AND PREPARATION OF COMMON MOLECULAR SIZE MARKERS

For most purposes, standard size markers should be included either in a separate tube or mixed with the sample for determination of sedimentation coefficient and molecular size of the sample of interest. It is critical to understand the limitations of the use of these markers. Sedimentation coefficients are affected by molecular mass, density, shape, and hydration; density and hydration are considered in the value of the partial specific volume (υ). Soluble globular proteins are roughly spherical in shape, tightly packed, and have similar degrees of hydration. They therefore have similar partial specific volumes. Thus, by comparing the sedimentation rates of soluble globular proteins, it is possible to infer molecular mass using the following formula, as described by Martin and Ames (1961):

$S_1/S_2 = (M_{r1}/M_{r2})^{2/3}$, in which S_1 and S_2 are the sedimentation coefficients of two proteins, and M_{r1} and M_{r2} are the apparent molecular weights of these proteins.

Table 5.3.2 provides a list of some common soluble proteins, their sedimentation coefficients, and molecular weights. It is apparent from the data that the equation above holds rather well for this set of proteins, primarily because the υ values are similar. For a given sedimentation experiment, it is best to choose two or three different proteins as standards covering a range of sedimentation coefficients. Try to choose standards that will be in the range of molecular weight expected for the particular sample. Then the equation above can be used with values determined from the standard migrating closest to the sample, or an average of M_r values calculated from the migration of each of several standards can be chosen as the final value.

These standards are not appropriate for calculation of the sedimentation coefficients of proteins with dissimilar υ values. Examples are proteins with extended rather than globular conformations, denatured proteins, and integral membrane proteins in detergent solutions. Nevertheless, the soluble standards are useful for comparing different runs and ensuring reproducibility of gradients. Furthermore, relative sedimentation coefficients can be determined, and these may be useful in determining the oligomeric state of a given protein subunit, regardless of its conformation. Finally, molecular mass can be estimated by comparison to a more relevant standard protein. For example, the size of an integral membrane protein with immunoglobulin-like domains can be compared with the known size of an immunoglobulin superfamily member, such as surface IgG (155 kDa), in the same detergent solution. Similarly, extended soluble molecules, like myosins, could be compared with the migration of different myosins of known sedimentation behavior and aggregation number. So long as antibodies to similar molecules as well as sources of these molecules are available, these analyses are possible and may yield more relevant information concerning relative molecular mass. It should be made clear, however, that this type of analysis is subject to limitations based on the assumption that the partial specific volume and/or detergent binding of the test sample is similar to that of the standard.

Table 5.3.2 Sedimentation Coefficient, Partial Specific Volume, and Corresponding Molecular Weight of Typical Protein Standards[a]

Protein	$S_{20,w}$ (S)	Molecular weight	υ (ml/g)
Bovine heart cytochrome *c*	1.7	12,300	0.728
Lysozyme	1.9	14,400	0.726
Sperm whale myoglobin	2.0	17,200	0.741
Chicken ovalbumin	3.6	42,881	0.748
Bovine serum albumin	4.6	66,000	0.734
Human transferrin	4.9	77,049	0.765
Human serum IgG	7.1	155,000	0.735
Rabbit aldolase	7.3	158,000	0.742
Bovine catalase	11.3	247,000	0.730
Rat β-glucuronidase	12.5	288,000	0.731
Horse heart ferritin	16.5	460,000	0.738
E. coli β-galactosidase	15.9	540,000	0.730
Thyroglobulin	19.3	667,000	0.720
Human serum α-macroglobulin	19.6	720,000	0.731

[a]Values from GenBank Sequence Database, National Center for Biotechnology Information; Sober (1970), Lundh (1973), Keller and Touster (1975), Millero et al. (1976), Reisler et al. (1977), Hall and Roberts (1978), deHaen (1987), and Buchner et al. (1991).

Additional Materials *(also see Basic Protocol 1 and Alternate Protocol 1)*

Protein standards (e.g., Sigma)

1. Weigh out relevant standard proteins and suspend in a buffer identical to that in which samples are prepared.

   The amount used will depend on the method of analysis. Initially, it may be best to identify standards by SDS polyacrylamide gel electrophoresis (UNIT 6.1) and staining with Coomassie blue (APPENDIX 3). In this case, it would be optimal to use ~100 μg of each standard protein in the mix. Subsequent studies can be done with more limited numbers of standards, and analysis may be by absorbance at 280 nm or protein assay with less protein once the general elution position is known.

2. Remove insoluble material by microcentrifugation for 15 min at maximum speed, 4°C. Discard the pellet.

3. Load the standards on a separate gradient from the sample and fractionate by ultracentrifugation in the same manner as the sample (see Basic Protocol 1, steps 11 to 16).

   Following elution (see Basic Protocol 2 or Alternate Protocol 2 or 3), the fractions can be analyzed by a convenient assay, such as SDS-PAGE (UNIT 6.1) and staining with Coomassie blue (APPENDIX 3). If space is limited, it may be possible to analyze the standards in the same tube as the sample either by mixing with a concentrated stock solution or by dissolving standards in the sample tube. While this may minimize the number of tubes used, it has disadvantages in that unknown potential interactions between the standards and the sample may affect sedimentation properties, and different analyses may be required to assay for standards and sample.

BASIC PROTOCOL 2

FRACTIONATION BY PUNCTURE AND ELUTION FROM THE BOTTOM OF THE GRADIENT

As described in Strategic Planning, fractionation of the gradient is the most critical step in determining the resolution of the sedimentation experiment. Numerous commercial instruments are available to remove fractions from either the top or the bottom of the gradient tube, and each has its advantages and disadvantages. Because these instruments are costly and not uniformly available, this unit provides protocols for simple methods of gradient elution that use common and/or inexpensive equipment (this protocol covers hole puncture and elution; for peristaltic elution, see Alternate Protocol 2, and for manual elution from the top of the gradient, see Alternate Protocol 3). While these methods are more cumbersome and less efficient than the automated elution methods, they nonetheless provide sufficient resolution to allow for reproducible sedimentation analyses under most conditions at low cost and without specialized equipment.

Hole puncture and elution from the bottom of the gradient is a simple and useful technique and was the first used for gradient fractionation. A hole is introduced in the bottom of the ultracentrifuge tube, and the gradient is then eluted from the bottom in drops, collecting drops counted manually or with a fraction collector. Although the hole may be created by hand with a needle, this creates the risk of skewing the placement of the hole (if it is not directly at the bottom, the gradient will not be eluted linearly), of inserting the needle too far into the gradient (eliminating the most dense fractions), and of disturbing the gradient by placement of the needle. A number of inexpensive devices are commercially available for holding the centrifuge tube and creating a hole at the bottom in a standardized way. One of the most popular units is made by Büchler. Nevertheless, puncturing with a common needle is sufficient for most purposes.

Materials

Gradients to be eluted (see Basic Protocol 1 or Alternate Protocol 1)
Sample tubes for fraction collector (e.g., 12 × 75–mm or microcentrifuge tubes)
Ring stand and clamp for tubes
27-G needle or commercial puncture device (e.g., Büchler)
Microbore tubing
Fraction collector (optional)

1. Perform a test elution using a gradient prepared in the same type of tube as the sample(s). Prepare sample tubes to collect the material eluted from the gradient. Pierce the bottom of the centrifuge tube with a 27-G needle. Leaving the needle in place, collect drops such that fractions of the desired volume are collected, and determine the number of fractions of the same drop number required to completely elute the gradient.

   Piercing the centrifuge tube with a 27-G needle should make a hole small enough such that the gradient elutes slowly by dripping from the bottom. The number of fractions desired will vary depending on the needs of the experiment. Higher resolution will obviously require analysis of more fractions. Fraction size should be no less than the volume of sample initially loaded onto the gradient; the final band of separated material cannot occupy less volume than it did when it started. It should also be recognized that the volume of a drop will increase as the gradient is eluted and the sucrose concentration in the fractions decreases. Thus, fractions collected by drops will not be of consistent volume. Although the changes in volume are relatively minor and will be consistent across different samples if the hole size is consistent, they may affect the results in the analyses that follow.

2. Place the first gradient to be eluted in a clamp attached to a ring stand. Be sure that the centrifuge tube is firmly held in place.

3. Prepare the required number of sample tubes for collection of fractions and number them.

   In this procedure, the most dense fractions are eluted first and the least dense fractions are eluted last. For the purposes of the calculations described in the support protocols, fractions should be numbered backwards—that is, the least dense (last collected) fraction should be numbered 1, and the most dense fraction (first collected) should be given the last number.

4. Place the collection tubes directly under the centrifuge tube, leaving enough room for the needle and for some manipulation.

5. Pierce the bottom of the centrifuge tube with the needle. Leaving the needle in place, collect fractions as drops from the needle.

   The Büchler apparatus uses a screw pin to pierce the tube under controlled conditions, and then remains in place for the gradient material to pass through. This makes the hole size more consistent, allows for a more controlled formation of drops, and permits attachment of microbore tubing to a fraction collector.

6. Store sample tubes at 4°C until ready to assay fractions for the presence of the sample of interest and/or for marker proteins.

   Detection of proteins within fractions can be done by any of a variety of methods (see Strategic Planning).

ALTERNATE PROTOCOL 2

FRACTIONATION BY PERISTALTIC ELUTION FROM THE BOTTOM OF THE GRADIENT

Despite the spreading of bands due to extensive laminar capillary flow, the technique of peristaltic elution is popular because of its full automation, its use of simple, common equipment, its speed, and its relative ease to set up. A capillary micropipet is attached to a peristaltic pump and then placed in the gradient tube. The gradient is then eluted from the bottom through the capillary micropipet and into a fraction collector by the action of the peristaltic pump. This can be done in the cold room to avoid thermal inactivation of the sample.

Additional Materials *(also see Basic Protocol 2)*

Gradients to be eluted (see Basic Protocol 1 or Alternate Protocol 1)
Mock gradient (same gradient as used for samples)

Peristaltic pump
Fraction collector with appropriate tubes (12 × 75–mm tubes or 1.5-ml microcentrifuge tubes)
Glass microbore capillary pipet
Microbore tubing
Cap for centrifuge tubes with hole in the middle for microbore tubing
Rack for centrifuge tubes

1. Set up a peristaltic pump in line with a fraction collector. Using a mock gradient of identical composition to that used in the experiments, determine the pump speed to provide a slow, even flow of material in the direction out of the sample side (0.5 to 1 ml/min). Determine the fraction volume required to elute the entire gradient into the designated number of fractions. Adjust the fraction collector to collect fractions of the appropriate size. Prepare the fraction collector with the appropriate number of tubes and include extra tubes in case there are problems with the fraction collector.

   In this case, the fraction collector must be integrated with the peristaltic pump to determine the time required for the appropriate collection volume. Try to minimize the amount of tubing used to make the connection between sample, peristaltic pump, and fraction collector.

   It is best to collect on the basis of time rather than drop size, because the drop size will change as the sucrose concentration changes through the gradient. To determine the speed of the peristaltic pump and the time or drop number required to collect fractions of a certain size, perform a "dry run," first with water and then with a mock sucrose gradient. A collection rate of ~0.5 to 1 ml/min is sufficient for adequate resolution under most circumstances using the 14 × 89–mm tubes. Choose the number of tubes and the fraction size relevant for the level of resolution required. The fraction size should not be smaller than the volume of the initial sample applied to the sucrose gradient.

2. Place a glass microbore capillary pipet on the end of the microbore tubing from the peristaltic pump that is to come from the sample.

3. Prepare a cap for the gradient centrifuge tube with a small hole in the center so the cap sits outside of the lip of the tube, and the capillary pipet fits through the hole.

   An old bottle cap, the cap from a 17 × 100–mm tissue culture tube, or even a thick layer of Parafilm will be sufficient.

4. Place the first gradient sample in a rack close to the peristaltic pump and make sure the sample is held firmly in place. Place the cap over the tube. Slowly place the microbore capillary pipet through the hole in the cap and into the sample tube, being careful not to disturb the gradient. Be sure that the capillary pipet is resting directly at the bottom of the tube and is supported by the cap.

   The hole should have been placed such that the capillary pipet sits in the center of the tube.

5. Turn on the peristaltic pump and the fraction collector simultaneously.

   The gradient should be eluted into fractions of a constant volume. In this procedure, the most dense fractions are eluted first and the least dense fractions are eluted last. For the purposes of the calculations described in the support protocols, fractions should be numbered backwards—that is, the least dense (last collected) fraction should be numbered 1, and the most dense fraction (first collected) should be given the last number.

6. Store sample tubes at 4°C until ready to assay fractions for the presence of the sample of interest and/or for marker proteins.

   Detection of proteins within fractions can be done by any of a variety of methods, as outlined under Strategic Planning.

MANUAL FRACTIONATION BY REMOVAL OF MATERIAL FROM THE TOP OF THE GRADIENT

ALTERNATE PROTOCOL 3

Manual fractionation provides relatively low resolution and may be cumbersome or tiresome for the operator. Nevertheless, it requires no special equipment and can provide reproducible results.

Additional Materials *(also see Basic Protocol 2)*

Manual pipettor and tips

1. Prepare the required number of sample tubes for each gradient and number them. Determine the fraction volume required to elute the entire gradient into the designated number of fractions.

   For the 14 × 89–mm tubes of the SW41 Ti rotor, separation into twelve fractions requires a fraction size of ~1 ml, whereas separation into fifteen fractions requires a fraction size of ~0.8 ml.

2. Place each gradient tube in a tightly fitted rack so that movement is minimized.

3. Set a pipettor to the appropriate volume. Steadying your arm against a firm support, carefully place the tip of the pipettor just below the top of the gradient. Slowly take up the appropriate volume from the top of the gradient, continually lowering the pipet tip such that it stays in the same position relative to the top of the gradient. Dispense into the first sample tube. Repeat this process until the gradient is completely eluted.

   In this procedure, the least dense fractions are eluted first and the most dense fractions are eluted last. For the purposes of the calculations described in the support protocols, fractions should be numbered starting with number 1.

   If desired, suspend any pellet that has formed at the bottom of the gradient using an appropriate buffer and transfer to a new sample tube. For some purposes it may be of interest to analyze the pelleted material, for example, to determine whether any of the protein of interest has aggregated, resulting in an insoluble mass. In this case, it may be necessary to use denaturing conditions, such as 0.1% SDS, 6 M guanidine·HCl, or 8 M urea, to solubilize the pellet.

4. Store sample tubes at 4°C until ready to assay fractions for the presence of the sample of interest and/or for marker proteins.

   Detection of proteins within fractions can be done by any of a variety of methods as outlined under Strategic Planning.

SUPPORT PROTOCOL 2

DETERMINATION OF SEDIMENTATION COEFFICIENTS BY EXTRAPOLATION FROM MIGRATION OF STANDARD PROTEINS

As described in Strategic Planning, the sedimentation coefficient, *S*, of an unknown sample can be calculated directly from the parameters of the fractionation and the migration of the sample. However, *S* values will vary depending on the temperature and composition of the medium through which the sample is sedimenting, and they must be converted to a sedimentation coefficient under the standard state of water at 20°C ($S_{20,w}$) for there to be any meaningful relationship to molecular weight. If the properties of the standard proteins and the sample are similar, then relative sedimentation coefficients can be easily estimated directly by comparing the migration of standard proteins to that of the sample, as described in this support protocol. A more precise analysis is based on other measurable or calculable parameters (see Support Protocols 3, 4, and 5).

This protocol can be used if the experiment included standard proteins with known $S_{20,w}$ values and aqueous soluble, globular test samples. If the migration of the standards is determined, then the sedimentation coefficient can be calculated by assuming that the sample and the standard have similar partial specific volumes.

Materials

Results from analysis of fractions for presence of sample of interest
Results from analysis of fractions for presence of standards
Calculator

1. Calculate a ratio, *R*, based on the distance traveled by the standard and the experimental sample from the meniscus. According to the equation from Martin and Ames (1961),

$$R = \frac{\text{distance traveled from meniscus by sample}}{\text{distance traveled from meniscus by standard}}$$

   Assuming that fractions of equal size were taken, substitute the distance traveled by the quantity [peak fraction # − 1].

   The value of [peak fraction # − 1] is taken because elution with fraction 1 is equivalent to no migration (this should be approximately the volume loaded onto the gradient).

2. Using the value of *R* and the known $S_{20,w}$ value of the standard, compute the $S_{20,w}$ value of the sample.

   Since macromolecules move linearly through the gradient over time, the ratio R also represents the ratio of sedimentation values, such that

$$R = \frac{S_{20,w} \text{ of sample}}{S_{20,w} \text{ of standard}}$$

   Therefore, $S_{20,w}$ of sample = $R \times S_{20,w}$ of standard.

   These calculations assume that the S value observed is proportional to that under standard conditions of water at 20°C. If the sedimentation buffer has physiological levels of salt and is near neutral pH, this assumption is a reasonable one.

DETERMINATION OF SEDIMENTATION COEFFICIENTS BY CONVERSION OF $S_{T,m}$ TO $S_{20,K}$ VALUES

A more accurate assessment of sedimentation coefficient can be made if one eliminates assumptions regarding any similarity between sample and standard regarding shape and the value of the partial specific volume. This method uses first a calculation of a

sedimentation coefficient $S_{T,m}$ for the conditions of the experiment (see Support Protocol 3), and then conversion to a standard sedimentation coefficient $S_{20,w}$ (see Support Protocol 5). These calculations require knowledge of a number of measured and extrapolated parameters. The measured parameters are (1) the distance migrated by the sample in the experiment (described below), (2) the sucrose concentration of the medium at the sample elution position (by refractometry of fractions), and (3) the time (t) of centrifugation. Using these measurements, one can extrapolate the values of other parameters, such as (1) density (ρ) and (2) viscosity (η) of the medium (from standard tables for sucrose solutions of various concentrations and at various temperatures) and (3) the distance from the center of rotation to the sample after centrifugation (manufacturer's information + distance migrated). Standard tables of density and viscosity of sucrose solutions are available (Lide, 1997), and these values are quite valid for aqueous solutions in the absence of detergent. For analyses of integral membrane proteins in detergent solutions, these values will be altered by the detergent, making the calculations less reliable. However, for most purposes, it will be sufficient to use these published values in estimating $S_{20,w}$ values. The final parameter is the partial specific volume (υ) of the sample protein or protein complex. This value can be either roughly estimated for a water soluble structure of presumably spherical shape, more closely approximated by summing the partial specific volumes of known components (see Support Protocol 4), or measured directly (see Basic Protocol 3).

Determination of the Value of $S_{T,m}$

SUPPORT PROTOCOL 3

As described by Martin and Ames (1961), the sedimentation coefficient S at a given temperature T in a solvent m is defined as $S_{T,m} = (dr/dt)/\omega^2 r$, in which dr/dt is the distance traveled over time or velocity in mm/sec (constant in sucrose gradients), r is the distance from the axis of rotation in mm, and ω is the angular velocity of the rotor in radians/second. These parameters can be determined relatively easily from the measurements of the experiment.

Materials

Results from analysis of fractions for presence of sample of interest
Results from analysis of fractions for presence of standard
Metric ruler
Calculator

1. Calculate the value of dr/dt, as described in Clarke (1975), by measuring the distance traveled by the molecule in the gradient during the centrifugation: $dr/dt = (R - r_0)/t$, in which r_0 is the position of the sample when applied at the top of the gradient, R is the position of the sample at the end of the run, and t is the time of centrifugation in seconds.

   The value of $(R - r_0)$ is the distance that the band has migrated in the tube. To calculate this, measure the length of the tube and multiply by the relative position of the sample after centrifugation. For example, if a 14 × 89–mm tube is used and the sample elutes in the middle fraction of the gradient, then the distance traveled would be 89 mm × 1/2 or ~45 mm.

2. Calculate the angular velocity, expressed in radians/second, as $\omega = 2\pi$ rpm/60, in which rpm is the speed of rotation of the rotor during the experiment.

   Using the SW41 Ti rotor at 39,000 rpm, $\omega = (2\pi)(39{,}000)/60 = 4082$ radians/sec.

3. Calculate the average distance r_{avg}, taken as $r_{avg} = r_{min} + (R - r_0)/2$, in which r_{min} is the distance from the center of the rotor to the top of the tube (which should be provided by the manufacturer of the rotor).

The distance traveled by the macromolecule will have constantly changed during the course of the experiment. However, one can calculate the average S value by using the average value of r during the course of the experiment, r_{avg}, which is defined as the position halfway between the starting point (r_0) and ending point (R) of travel for the sample. The value of r_{min} for the SW41 Ti rotor is 67.4 mm. Thus, for a band migrating in the center of the tube, r_{avg} = 67.4 mm + (45 mm/2) = 89.9 mm.

4. Using these values, calculate $S_{T,m}$ as

$$S_{T,m} = \frac{dr/dt}{\omega^2 r_{\text{avg}}} = \frac{(R - r_0)/t}{(2\pi \text{ rpm}/60)^2 [r_{\text{min}} + (R - r_0/2)]}$$

Using the figures above for a band migrating in the center of the tube in a SW41 Ti rotor centrifuged 16 hr at 39,000 rpm, the corresponding $S_{T,m}$ value is 5.2×10^{-13} sec or 5.2 S.

The difficulties in this calculation lie in the measurement of R. Typically, the zonal sedimentation experiment results in broad bands of protein, and so the measurement of R is approximated as the center of migration. Both the calculation and the measurements, and therefore the value for S at the end, are inexact. Finally, for determination of a typical sedimentation coefficient under standard conditions ($S_{20,w}$), this calculation is only the beginning. To use the value of $S_{T,m}$ to calculate $S_{20,w}$, see Support Protocols 4 and 5.

SUPPORT PROTOCOL 4

Determination of the Value of Partial Specific Volume (υ)

The partial specific volume is the most thorny value in determining the sedimentation coefficient of a protein or protein complex. Partial specific volume υ is defined as the increase in volume of an infinite volume of solvent by dissolving 1 g of solute, and it is expressed in liter/g or ml/mg. It is essentially a measure of the packing or density of the protein. Standard pycnometric methods for measuring partial specific volumes are impractical for all but the most readily available purified proteins and are impossible for proteins in complex mixtures, such as cell lysates, or for integral membrane protein complexes. One tenable method for determining partial specific volume of a protein in a mixture is parallel sedimentation in media with different densities (see Basic Protocol 3). However, information regarding the sedimentation coefficient of some proteins can be inferred by making certain assumptions even if partial specific volumes are not measured.

First, most soluble globular proteins or protein complexes have partial specific volumes that fit within the range of 0.72 to 0.75 ml/mg. If a protein or protein complex can be expected to fit this description, then start by assuming a similar value for its υ and enter it into the equation given below for $S_{20,w}$ values. This is only valid for proteins that lack glycosylation and associated lipids/detergents.

Alternatively, if the composition of the protein or protein complex is known (e.g., for determining the oligomerization state of a protein with one known subunit), the value of υ can be estimated by summing the υ values of each component using the following equation from Cohn and Edsall (1943): $\upsilon = \Sigma\, \upsilon_i w_i$, in which υ_i is the partial specific volume of each component and w_i is the percent mass of each component in the final complex. If the amino acid sequence of the protein component is known, this equation can be used to compute the value of υ for the protein component by summing the known partial specific volumes (υ_i) of individual amino acids on a weighted basis (w_i). If the sample is a glycoprotein with a known number of N- and O-linked glycans, the contribution of these glycans to the total value of υ can be included by summing the υ_i values of each individual sugar residue within the glycan (based on the composition of a standard N- or O-linked glycan; see below) and then factoring in the relative mass contribution of the glycan to the molecular weight of the glycoprotein.

Table 5.3.3 Partial Specific Volume of Amino Acids[a]

Amino acid	υ (ml/mg)	Amino acid	υ (ml/mg)	Amino acid	υ (ml/mg)
Alanine	0.74	Glycine	0.64	Proline	0.76
Arginine	0.70	Histidine	0.67	Serine	0.63
Asparagine	0.62	Isoleucine	0.90	Threonine	0.70
Aspartic acid	0.60	Leucine	0.90	Tryptophan	0.74
Cysteine	0.61	Lysine	0.82	Tyrosine	0.71
Glutamic acid	0.66	Methionine	0.75	Valine	0.86
Glutamine	0.67	Phenylalanine	0.77		

[a]Values from Cohn and Edsall (1943).

Table 5.3.4 Partial Specific Volume of Sugar Residues[a]

Residue	υ (ml/mg)
Hexoses (mannose, glucose, galactose, fucose)	0.613
N-Acetylhexosamines (e.g., GlcNAc, GalNAc)	0.666
N-Acetylneuraminic acid (sialic acid)	0.584
N-Glycolylneuraminic acid (sialic acid)	0.557

[a]Values from Gibbons (1972). A typical high mannose N-linked oligosaccharide would consist of 2 *N*-acetylglucosamine residues (GlcNAc) and 5 to 8 mannose residues. A typical complex N-linked oligosaccharide would consist of 3 to 5 GlcNAc residues, 3 to 5 mannose residues, a fucose residue, 1 to 3 galactose residues, and 1 to 3 sialic acid residues (primarily *N*-acetylneuraminic acid in humans). The composition of O-linked glycans in glycoproteins within the secretory pathway and nucleus varies, and usually begins with addition of *N*-acetylgalactosamine (GalNAc).

Table 5.3.5 Partial Specific Volume, Aggregation Number, Monomer Molecular Weight, and Critical Micelle Concentration of Typical Detergents in Biological Buffers[a]

Detergent	υ (ml/mg)	Aggregation number[b]	Molecular weight (g/mol)[c]	Critical micelle concentration (mM)[d]
Ionic				
Sodium deoxycholate	0.76	12	415	4
Nonionic				
$C_{12}E_8$	0.97	120	540	0.09
NP-40	0.94	140	603	0.3
Octyl-β-D-glucoside	0.85	84	292	25
Triton X-100	0.94	100-155	625	0.29
Tween 80	0.896	58	1310	0.012
Zwitterionic				
CHAPS	0.81	10	615	6.5
Surfactant-like				
Digitonin	0.74	60	1229	—

[a]Values from Clarke (1975), Watts et al. (1982), Horne et al. (1986), and Neugebauer (1994).

[b]Aggregation number is the number of monomers included in a typical micelle.

[c]Molecular weight of the monomer. Micelle molecular weight is computed as the product of the aggregation number and the monomer molecular weight.

[d]Critical micelle concentration (CMC) is the concentration above which the detergent monomers are primarily incorporated into micelles. Below the CMC, the detergent behaves primarily as monomers. Values are for detergent in aqueous solution with >0.05 M salt at 20°C.

Finally, if the sample is an integral membrane protein or protein complex dissolved in a detergent solution, one must factor in the relative mass contribution and the υ value of the detergent. It is possible to directly measure detergent binding using radiolabeled detergent and a quantitative assay for the protein in question (e.g., see Clarke, 1975). Otherwise, assumptions concerning the amount of detergent bound must be made. For proteins with single membrane-spanning regions, assume that the protein is bound by its transmembrane domain to a single detergent micelle. In this case, mass contributions by the detergent must consider the aggregation number for each detergent (the number of monomers included within a typical micelle) and the monomer molecular weight. Proteins with multiple membrane-spanning regions or additional hydrophobic domains may bind more detergent per complex, however, and so this assumption may not be valid. It should be clear that calculation of the partial specific volumes in this manner is considered an estimate and is based on numerous assumptions (composition of the complex, composition of oligosaccharides, binding of detergent) that may not be accurate.

Tables 5.3.3, 5.3.4, and 5.3.5 provide relevant data for partial specific volumes of amino acids, sugar residues, and common detergents, along with relevant properties of some of the detergents necessary for calculating percent mass contributions. Note that addition of glycans will decrease the value of υ, whereas incorporation into typical nonionic detergents will tend to increase the value of υ. The opposite effect will be apparent for the sedimentation coefficient.

SUPPORT PROTOCOL 5

Determination of the Value of $S_{20,w}$

Once the value of $S_{T,m}$ has been calculated (see Support Protocol 3) and the value of υ has been either determined experimentally or estimated (see Support Protocol 4), the value of $S_{T,m}$ must be corrected to the value of $S_{20,w}$ at the "standard state" of 20°C in water. This correction, as described by Martin and Ames (1961), relies not only on the partial specific volume (υ) of the protein but also on the viscosity (η) and density (ρ) of the medium used in the experiment. The correction is applied as

$$S_{20,w} = S_{T,m} \frac{\eta_{T,m}}{\eta_{20,w}} \frac{(1/\upsilon - \rho_{20,w})}{(1/\upsilon - \rho_{T,m})}$$

in which $\eta_{T,m}$ and $\rho_{T,m}$ are the viscosity and density, respectively, of the medium m at the temperature T of the experiment, and $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20°C. For a sample at the position r_{avg}, halfway between the starting point and endpoint of migration, one can determine the values of $\eta_{T,m}/\eta_{20,w}$ and $\rho_{T,m}$ from published tables on the basis of the experimentally determined densities of sucrose (measured by refractometry) at each eluted fraction.

Materials

Fractions eluted from sucrose gradients (see Basic Protocol 2 or Alternate Protocol 2 or 3)
Refractometer

1. Using a refractometer, determine the refractive index of each fraction from the origin to the peak position in which the sample eluted. From the refractive index, calculate the concentration of sucrose in each fraction using the values in Table 5.3.1 (or the more comprehensive published values in Lide, 1997).

   If the experiment was successful, the sucrose concentration should increase linearly from the origin to the last sample when plotted on a graph.

2. Determine the fraction corresponding to the average distance traveled by the sample, r_{avg}, which is the fraction halfway between the origin (fraction 1) and the endpoint of migration for the sample, keeping in mind that fraction 1 should be considered equivalent to no migration.

   For example, if the sample migrated to the halfway point in the 5% to 20% sucrose gradient and the gradient was eluted into fifteen tubes of equal volume, then the sample would have eluted with a peak at fraction 8, and r_{avg} would have been obtained in fractions [(8 − 1)/2 + 1] or fraction 4.5—this is halfway between fractions 4 and 5.

3. Using the sucrose concentrations determined in step 1 and the value of r_{avg} determined in step 2, calculate the concentration of sucrose at r_{avg}.

   In this example, a linear gradient would predict that fractions 4 and 5 would have sucrose concentrations of ~6.7% and ~7.8%, respectively; therefore, at r_{avg} the sucrose concentration would be 7.25%.

4. From published tables, determine the values of $\eta_{T,m}/\eta_{20,w}$ and $\rho_{T,m}$ corresponding to the sucrose concentration at r_{avg}.

   For the example described, $\eta_{T,m}/\eta_{20,w}$ at r_{avg} would be 1.222 and $\rho_{T,m}$ at r_{avg} would be 1.0269.

   Note that the use of these values assumes that other components of the sample buffer, such as salts and detergent, have no effect on viscosity or density. This may not be valid when using detergents in the analysis of integral membrane proteins.

5. Use the values of $\eta_{T,m}/\eta_{20,w}$, $\rho_{T,m}$, and $S_{T,m}$ at r_{avg} and the value of υ to calculate $S_{20,w}$ according to the equation above.

   In this example, assuming a "typical" υ of 0.74 liter/g, the value of $S_{20,w}$ is computed as

$$S_{20,w} = (5.2S)(1.222)\frac{(1/0.74 - 1.00)}{(1/0.74 - 1.0269)} = 6.9S$$

   From this sample calculation, it is clear how critically the values of the viscosity and density of the medium and the partial specific volume impact on the final value of the sedimentation coefficient.

DETERMINATION OF PARTIAL SPECIFIC VOLUME AND SEDIMENTATION COEFFICIENT BY PARALLEL SEDIMENTATION IN MEDIA WITH DIFFERENT DENSITIES

BASIC PROTOCOL 3

As discussed above, one of the major limitations to interpreting the data from a sedimentation experiment is the inaccessibility of the value of the partial specific volume (υ) for the sample in question. It would therefore be desirable to have a means to measure both partial specific volume and sedimentation coefficient in a single experiment without any assumptions regarding the structure of the sample under study. This protocol describes a rather simple technique based on a method described by Edelstein and Schachman (1967) for sedimentation equilibrium experiments and later adapted for zonal sedimentation through sucrose gradients by Meunier et al. (1972) and Clarke (1975). In this protocol, sedimentation is performed simultaneously in two tubes with media of different density. All other components being equal, the density of the media is altered by using either water (H_2O) or deuterium oxide (D_2O) as the solvent. Because these two solvents are chemically similar, it can be assumed that the values of both $S_{20,w}$ and υ will be the same in the two gradients. Therefore, using sedimentation data from both gradients and solving two equations with two variables, one at a time, it is possible to determine both $S_{20,w}$ and υ. The method is based on the correction formulas described above (see Support Protocol

5), in which $S_{20,w}$ is dependent on the density of the medium. The use of D_2O raises the basal density from 1.0 g/liter to 1.11 g/liter. The technique is enhanced through the use of $D_2{}^{18}O$, if available, which changes the density even more and allows for more reliable calculations.

Materials

Deuterium oxide (heavy water, D_2O)
Additional reagents and equipment for zonal sedimentation using sucrose gradients (see Basic Protocol 1 or Alternate Protocol 1), fractionation of gradients by elution (see Basic Protocol 2 or Alternate Protocols 2 or 3), and detection of proteins within fractions (see Strategic Planning)

Perform zonal sedimentation analysis

1. Prepare sucrose stock solutions and sedimentation buffers in 90% D_2O. Prepare a fresh stock solution of 60% sucrose using 90% D_2O as the solvent, and measure sucrose concentration by refractometry. Prepare final working solutions using this sucrose stock and the regular buffer and salt stock solutions made in H_2O, bringing the final working solution to a total of 90% (v/v) D_2O.

   All of the solutions should be made with 90% D_2O replacing H_2O. D_2O can be purchased from typical chemical vendors (e.g., Sigma, Fisher, Aldrich) at 99% purity, which is sufficient for these experiments. Using 90% D_2O rather than 100% simplifies making the gradient solutions such that stock salt solutions in H_2O can still be used.

2. Using Basic Protocol 1 or Alternate Protocol 1, prepare parallel gradients using the working solutions made in H_2O and in D_2O.

   There should be enough gradients to fractionate both the experimental sample and the standards (see Support Protocol 1) in both H_2O and D_2O.

3. Apply the samples to the gradients and subject them to ultracentrifugation.

4. Using Basic Protocol 2 or Alternate Protocol 2 or 3, elute fractions for each gradient.

5. Test for the presence of the sample and the standard proteins using the appropriate assays, and determine the distribution in each gradient. Measure the refractive index of each fraction from each gradient, and determine the corresponding sucrose concentration.

Determine partial specific volume and sedimentation coefficient

6. Determine the values for R (distance traveled from the meniscus) and r_{avg} for the sample and for standards in each gradient as described for a single gradient (see Support Protocols 2 and 3). Using the value of r_{avg} and the measurements from refractometry of the fractions, determine the sucrose concentration and the corresponding medium density (ρ_i) at r_{avg} from standard tables.

   A given protein would be expected to migrate a shorter distance in the gradient prepared in 90% D_2O than in the gradient prepared in H_2O.

7. Choose a standard σ that migrates similarly to the unknown sample U in each gradient for further analysis. Use the migration of the standard to solve for K_i in the equation $R_{i\sigma} = K_i S_{20,w\sigma}(1 - \upsilon_\sigma \rho_i)$, in which $R_{i\sigma}$ is the distance traveled from the meniscus for the standard, $S_{20,w\sigma}$ is the sedimentation coefficient for the standard, υ_σ is the partial specific volume of the standard, and ρ_i is the density of the medium at r_{avg}. Determine K_i for both media, resulting in a K_1 and a K_2 value (where $i = 1$ is H_2O and $i = 2$ is D_2O).

   These values will be constant for a given medium and a given range of migration, and they will allow determination of the sedimentation coefficient and partial specific volume of the

unknown sample. The value of K_i includes the parameters of viscosity, density of water under standard conditions, angular velocity, and speed of migration.

8. Using the values of R_i, ρ_i, and K_i determined above, solve for the partial specific volume υ_U of the unknown sample:

$$\upsilon_U = \frac{R_1K_2 - R_2K_1}{R_1K_2\rho_2 - R_2K_1\rho_1}$$

Since the value of $S_{20,w}$ does not change in different media, then the equation for migration in two different media is expressed as

$$\frac{R_{1U}}{R_{2U}} = \frac{K_1(1-\upsilon\rho_1)}{K_2(1-\upsilon\rho_2)}$$

Thus, knowing the values of R_i, ρ_i, and K_i in H_2O and D_2O allows for calculation of υ_U by rearrangement to the equation above.

9. Finally, use the value of υ_U to determine the value of $S_{20,wU}$ using the original formula: $R_{iU} = K_iS_{20,wU}(1 - \upsilon_U\rho_i)$, or any of the formulas described for single gradients (see Support Protocol 5).

It should be noted that for integral membrane proteins in detergent solutions, the values of υ and $S_{20,w}$ calculated here include the contributions from the detergent. Determination of the contribution of the detergent to these parameters requires knowledge of the mass of detergent bound relative to the mass of the protein complex (see Support Protocol 4). Clarke (1975) includes formulas to calculate these values for the protein alone using this information.

COMMENTARY

Background Information

Molecular mass of individual polypeptide chains is easily estimated by gel electrophoresis techniques using charged denaturing reagents, such as sodium dodecyl sulfate, to maintain the polypeptides in an extended conformation and neutralize charge effects (*UNIT 6.1*). However, determining the molecular mass of a multisubunit protein complex is a more difficult undertaking. Denaturation nearly always results in disruption of the complex, making size analysis under denaturing conditions impossible. Fortunately, there are a variety of techniques available to determine characteristics that correspond to the size of protein complexes under nondenaturing conditions. The two most widely used and applicable techniques are gel-filtration (size exclusion) chromatography and sedimentation analysis on sucrose density gradients. Both techniques are advantageous in that they are applicable to analysis of individual complexes within heterogeneous mixtures of proteins, such as cell lysates, given an appropriate specific analytical assay for the particular protein complex of interest. Both can also be used to estimate the size of integral membrane protein complexes that are solubilized in detergents, albeit with a number of limitations. Neither technique allows direct measurement of molecular weight, but rather provides parameters that correlate with molecular weight for particles with a given shape. However, each is useful for determining relative molecular size or oligomerization state. Furthermore, given several other parameters, molecular weight can be directly calculated from combined data obtained from both sedimentation and gel-filtration analyses.

Gel-filtration chromatography permits determination of the Stokes radius of a given molecule, which is roughly proportional to molecular mass for a globular protein or protein complex. It can be a very high resolution technique for determining the molecular mass of soluble proteins, particularly when coupled to a high-performance liquid chromatography system. However, it has a number of drawbacks for analysis of integral membrane protein complexes due to the contribution of molecular mass of detergent micelles used to solubilize these complexes, although the use of detergents with small micelle sizes can minimize this difficulty. Furthermore, one is limited to analysis of a single sample at a time unless multiple chromatographic columns are set up simultaneously. Altering the size discrimination of the

column is difficult in that it requires changing the gel matrix.

Sedimentation analysis circumvents some of these difficulties and provides a different measure of molecular size—a sedimentation coefficient. Multiple samples can be analyzed in a single experiment, limited only by the number of sample positions in an ultracentrifuge rotor. Size discrimination can be altered by simply changing the sucrose concentration within the gradient or the time of ultracentrifugation. Although detergents do affect the sedimentation rate, the inclusion of proper controls or knowledge of the physical characteristics of the detergent can permit accurate analyses of integral membrane protein complexes. One drawback of this type of analysis is that there are a number of assumptions implicit in interpreting most simple sedimentation experiments; although protocols provided in this unit permit the elimination of some of these assumptions, others must be made in most cases. Another drawback is the limited resolution of the technique using traditional methods. Even this drawback, however, has been addressed in recent advances.

Theory

The theoretical basis for the zonal sedimentation experiment is in the definition of the sedimentation coefficient, *S*. The sedimentation coefficient in a given medium is defined by the equation $S = (dr/dt)/\omega^2 r$, in which r is the distance traveled by a macromolecule, ω is the angular velocity of the rotor, and dr/dt is the distance traveled over time (for complete discussion of theory, see Tanford, 1961; Williams, 1972). Classical experiments by Svedberg and others established the techniques of sedimentation equilibrium and sedimentation velocity to analyze the properties of purified macromolecules in solution under standard conditions (water at 20°C). These techniques begin with a uniform distribution of macromolecules in solution, and then follow either the shape of the curve of a distribution of macromolecules throughout the gradient after equilibrium has been reached between sedimentation and flotation (sedimentation equilibrium) or the velocity of movement of the leading edge of sedimenting material (sedimentation velocity). These classical techniques require a specialized analytical ultracentrifuge and relatively large quantities of purified material for analysis (for complete discussion, see Van Holde, 1975). They are thus limited in applicability, particularly for unpurified proteins.

Martin and Ames (1961) modified the velocity sedimentation technique by following the movement of a band or zone of macromolecules through a sucrose gradient. The beauty of the sucrose gradient is that dr/dt is nearly constant—that is, there is little variability in the speed with which the molecule moves as it passes through the gradient. Thus, at a given angular velocity and time, S is inversely proportional to r, allowing for direct determination of sedimentation coefficients from migration at a single time point. This avoided the difficulties of the sedimentation velocity experiment, which required measurement of position at various times during the experiment to establish a rate of movement through the medium. Furthermore, it permitted identification of proteins within a complex mixture by analysis subsequent to centrifugation.

Classical sedimentation coefficients, as described in published tables, are corrected to the standard state of water at 20°C. Therefore, observed sedimentation coefficients must be corrected for the properties of the medium to determine the standard sedimentation coefficient, $S_{20,w}$. The sedimentation coefficient is a property of the size and density of a macromolecule. Therefore, the $S_{20,w}$ value is affected by the density and viscosity of the medium relative to that of the macromolecule in question. Higher density will provide a greater buoyancy, and higher viscosity will deter the movement of the macromolecule through the gradient.

The value of $S_{20,w}$ can be expressed as

$$S_{20,w} = \frac{dr}{dt}\frac{\eta_{T,m}}{\eta_{20,w}}\frac{(1/\upsilon - \rho_{20,w})}{(1/\upsilon - \rho_{T,m})}\frac{1}{\omega^2 r}$$

in which $\rho_{T,m}$ and $\rho_{20,w}$ are the density of the medium m at temperature T and of water at 20°C, respectively, and $\eta_{T,m}$ and $\eta_{20,w}$ are the viscosity of the medium m at temperature T and water at 20°C, respectively. The value υ is the partial specific volume, which is defined as the inverse of the density of the macromolecule and is expressed as volume per mass. For most globular proteins with a spherical shape, partial specific volumes fall within the range of 0.72 to 0.75 ml/mg, and there is therefore a roughly linear relationship between sedimentation coefficient and molecular weight. Thus, for most globular proteins in neutral aqueous solutions, the value of the sedimentation coefficient can be extrapolated directly to molecular weight.

Other variables, however, may influence the sedimentation coefficient such that it may not be an accurate measure of molecular weight. For example, a protein that is less densely

packed than the typical globular protein, such as those with a toroid shape or a high proportion of polar, solute-exposed amino acids, will have a higher partial specific volume and higher sedimentation coefficient for a given molecular mass than a densely packed, globular protein. Similarly, asymmetry will have a dramatic effect on the sedimentation coefficient; thus, proteins with elongated structures, such as myosins and integral membrane proteins in detergents, will have a reduced sedimentation coefficient for a given molecular mass. Thus, sedimentation coefficient alone cannot be used as a direct measure of molecular weight unless the properties of the macromolecule are known.

A protocol is provided to determine the partial specific volume of a protein complex using sedimentation in media with different densities (see Basic Protocol 3). This protocol uses two parallel gradients in which the solute is either water or deuterium oxide. Since each of these solvents has a distinct density but otherwise are chemically similar, migration in each gradient will differ solely by virtue of the density of the medium. However, the values of $S_{20,w}$ and υ are constant, regardless of the density of the media. Therefore, if one first calculates a constant for each medium [which includes the parameters $(dr/dt)/\omega^2 r$, $\rho_{T,m}$, and $\eta_{T,m}$] by using values for a standard protein with similar migration to the unknown sample, then one can solve for υ of the sample and use this value to calculate $S_{20,w}$. For a protein complex soluble in aqueous medium, such as a nuclear or cytosolic protein complex, these values represent the final goal of the experiment. For integral membrane proteins or protein complexes solubilized in detergents, however, it is necessary to subtract from these values the contribution from the detergent.

Assuming that the sedimentation coefficient $S_{20,w}$ and partial specific volume υ are accurately determined, the molecular weight M of a macromolecular complex can be calculated by providing the value of the Stokes radius, a, determined by gel-filtration analysis under similar buffer conditions:

$$M = \frac{6\pi N a S_{20,w} \eta_{20,w}}{1 - \upsilon \rho_{20,w}}$$

in which N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20°C, and $\rho_{20,w}$ is the density of water at 20°C. This is the only valid way to accurately determine molecular weight from either sedimentation or gel-filtration experiments without making a number of assumptions regarding the nature of the protein complex.

Practical considerations

The sedimentation of a macromolecule is affected by the medium in which the experiment is done. The density and viscosity of the medium will affect the rate of sedimentation and are included in standard equations for determining the sedimentation coefficient. For accurate determinations, most experiments should be done at near neutral pH and physiological ionic strength, because interactions with buffer components may affect the degree of hydration or intermolecular interactions and thereby alter the partial specific volume of the macromolecule under study.

One of the most important buffer components for integral membrane proteins are detergents. Detergents are required to solubilize most integral membrane proteins (Helenius and Simons, 1975; Tanford and Reynolds, 1976), but the properties of the detergent, particularly its partial specific volume and aggregation number for association with micelles, will affect the migration of the protein-detergent complex during fractionation (Doms, 1990). In addition, some detergents may disrupt particularly labile protein complexes. Detergents tend to have high partial specific volume values and therefore will drastically alter the sedimentation of a bound protein complex.

Detergents with high aggregation numbers, such as the common nonionic detergents Triton X-100 and NP-40, will have high detergent:protein mass ratios in a complex and will thus contribute largely to the sedimentation properties and partial specific volumes of the complex. The zwitterionic detergent CHAPS and the nonionic detergent *n*-octylglucoside tend to be favorable for both sedimentation and gel-filtration experiments because of their small micelle size. The surfactant-like detergent digitonin is also useful to solubilize labile protein complexes. To extract the maximal amount of information from a sedimentation experiment, it is necessary to know the amount of detergent bound to a protein complex. This value is often difficult to determine, particularly for proteins within a complex mixture, and usually requires the use of radioactive detergent and quantitation of the protein sample (Clarke, 1975). It should be mentioned that many water-soluble and peripheral membrane proteins will bind detergent as well, often in a less predictable manner (Helenius and Simons, 1975).

Regardless of the caveats, there are several ways of extracting information from sedimentation experiments even in the absence of an exact molecular weight determination. For ex-

ample, the oligomerization or aggregation state of a single subunit can be inferred by comparing the migration of samples known to exist in monomeric versus oligomeric conformation. This may be useful, for example, in following the changes in oligomeric state over time by pulse-chase metabolic labeling experiments. Even without knowledge of different conformations, some assumptions can be made. For example, regardless of the caveats described here, a 50-kDa subunit that migrates with a sedimentation coefficient of ~15 S is unlikely to exist in solution as a monomer. Furthermore, for proteins that do not interact with detergents, one can determine both sedimentation coefficients and partial specific volumes by differential sedimentation in H_2O and D_2O (see Basic Protocol 3). By making a number of assumptions regarding composition of a complex, amount of detergent bound (number of detergent micelles bound), and values provided in this unit for partial specific volumes of detergents, glycan moieties, and amino acids, these experiments can allow one to estimate molecular size even for an integral membrane glycoprotein.

There are numerous ways of performing the sedimentation experiment, mostly based on the different means of creating and eluting the gradient. To form the gradient, the method of choice employs the Biocomp Gradient Master (see Basic Protocol 1). The Gradient Master produces reliable, consistent gradients with minimal effort. Other protocols, including Alternate Protocol 1 using a manual gradient maker, are trickier to master, more time-consuming, and offer less reproducibility because each gradient is made separately. Nevertheless, the manual method may be more attractive to a laboratory with a limited budget. Other commercial devices are available for making gradients, but for the money, the design of the Gradient Master is superior.

For elution of the gradients, there are many more methods, and each laboratory has its favorite. The three presented in this unit all use inexpensive and/or common equipment that is likely to be available in most laboratories. Elution from the bottom through hole puncture (see Basic Protocol 2) is probably the best choice for optimal resolution. However, unless one of the commercial piercing devices is available (such as the Büchler apparatus), it may be difficult to master optimal placement of the hole and to control the rate of flow. Furthermore, the method is cumbersome because the drops are usually collected manually. Finally, since all of the material passes through the bottom, any precipitated material that accumulated at the bottom will contact the remaining contents of the tube and may interact with this material, block the flow, or cause turbulence, all of which will affect the resolution of the experiment.

The technique of elution from the bottom by action of a peristaltic pump (see Alternate Protocol 2), which is one of the most widely used methods, has the advantage that it can be set up to work automatically in a cold room without constant supervision. However, it suffers the greatest amount of laminar capillary flow turbulence of any of the methods and has the same difficulties with a pellet as the hole puncture method. The final method, pipetting from the top (see Alternate Protocol 3), is cumbersome, suffers from variable suction-induced turbulence, and may frustrate the technologically inclined investigator. However, it is the least expensive, offers the least disturbance of the pellet, and can provide reproducible results. All of the commercial devices mentioned, particularly the trumpet-tip eluter from Biocomp, are likely to provide superior resolution to these more simple methods. However, the devices are all quite costly and may limit the adaptability of the technique to different sized ultracentrifuge tubes and rotors.

For computational analysis, the choice of protocols is dependent on the needs of the experiment. If relative migration is the only concern—for example, if the experiment is to follow the oligomerization state of a polypeptide over a time course or series of different treatments or to merely separate known complexes for analysis—then assignment of an absolute value for $S_{20,w}$ may not be necessary, and the experiment can be done using markers simply as a reference point (for preparation and analysis of molecular size markers, see Support Protocols 1 and 2). For more accurate determinations of $S_{20,w}$ values, clearly the superior method would eliminate any assumptions regarding partial specific volume. Thus, it would be optimal to use Basic Protocol 3 in which sedimentation is done in parallel gradients with H_2O and D_2O. On the other hand, if the composition of the complex is known definitively, then either an assumed average υ value of 0.74 ml/mg or υ values estimated from summation of the weighted contribution of υ_i values may be sufficient (see Support Protocols 3 through 5).

Critical Parameters and Troubleshooting

The critical parameters of the zonal sedimentation experiment fall into two categories: those that affect the experimental outcome and those that affect the interpretation. These are discussed separately.

Experimental factors

The critical parameters for the success of the fractionation of a protein sample are (1) the method of detection of the protein sample, (2) the preparation of the sample for sedimentation, (3) the linearity and reproducibility of the sucrose gradients, (4) care in ultracentrifugation, and (5) the ability to elute the gradients with the least amount of turbulence and disturbance.

1. The method of detection must be sufficiently worked out beforehand to ensure that the sedimentation experiment can be analyzed properly. The detection method must be sensitive enough to detect a sample after fractionation, assuming distribution and dilution over several fractions. On the other hand, the method should also provide linear (or at least nearly linear) detection of sample over the range of concentrations used in order to be able to detect a peak of migration among several fractions. Furthermore, the method must not be sensitive to components of the sucrose gradient itself; otherwise, these components must be removed by a dialysis or precipitation step prior to analysis. For these reasons, immunologic detection techniques, such as immunoblotting (*UNIT 6.2*), immunoprecipitation of radiolabeled material (*UNIT 7.2*), and ELISAs, are typically the methods of choice. Even in these cases, however, the sucrose gradient solution may interfere. For example, large amounts of sucrose may affect the integrity of a lane in a polyacrylamide gel. The volume of the fraction needs to be considered for application to the well of a gel or a 96-well plate; if the fraction size is 0.8 ml but the well only holds 40 µl, only 5% of the fraction can be assayed without a concentration step. Be certain that the details of the analysis and compatibility with the fractionation are worked out well before starting the density gradient experiment. Failure to do so may mean that a fractionation is done with no ability to detect the desired component.

2. Sample preparation should also be resolved before attempting the sedimentation experiment. The volume of the sample should be minimized in order to maximize the resolution of the sucrose gradient fractionation. Bands are not concentrated during centrifugation, and therefore the volume of the sample will be the smallest volume in which the desired component will ultimately elute after fractionation. If particulates are not removed, they may disrupt the gradient during the run, resulting in band spreading and loss of resolution. Furthermore, they will contribute to a pellet during the centrifugation, which may interfere with the elution method and cause additional band spreading. The sample as prepared must be less dense than the least dense endpoint sucrose solution (5% in the example given). The concentration of the sample and the buffer in which it is prepared must be considered; a highly concentrated cell lysate, for example, may be dense due to the contents of the lysate rather than the buffer, and may sink in 5% sucrose. This will be apparent on loading of the sample. It is important that the buffer of the sucrose gradient closely approximates that of the lysate. Otherwise, material from the sample may precipitate on contact with the sucrose gradient solution during loading. Lastly, the stability of the sample should be considered prior to the experiment for timing of preparation of the sample and the gradients. If the protein or enzyme under analysis is unstable in solution, it may not survive the long centrifugation required in the sedimentation experiment or a prolonged period between sample preparation and loading.

3. The linearity and reproducibility of gradient formation are necessary for two reasons. First and most important, changes in the slope of the gradient will result in changes in the velocity of the sample components as they migrate through the tube during centrifugation. Since the basis of the use of the sucrose gradient is to maintain a constant velocity (allowing for computation of rate of movement by measuring distance traveled at one time point), any alterations in velocity completely invalidate subsequent computation of sedimentation coefficient. Thus, unless the sample has a sedimentation coefficient and partial specific volume identical to that of one of the standards, it will not be possible to determine even a relative sedimentation coefficient if the gradient is not linear. The other critical aspect of gradient linearity is the physical stability of the sample maintained by the gradient during handling of the ultracentrifuge tube. If the sucrose concentration is uniform or nearly uniform for any length of the tube, the sample is more likely to

be disrupted during loading, eluting, or other handling. This will result in an erratic and heterogeneous distribution.

If using the Gradient Master (see Basic Protocol 1), a lack of linearity of the gradient may result if the angle, speed, or time of rotation is not optimal for the particular gradient being made. Check the manufacturer's recommendations and recheck the procedure for programming the Gradient Master for the desired parameters. If using a manual gradient maker (see Alternate Protocol 1), there may be numerous causes for a lack of linearity. Turbulence and inappropriate mixing of samples may occur if any bubbles are present in any of the connections between chambers or to the sample, if there is too rapid a flow from the gradient maker, if the microbore tubing is too long, or if the glass micropipet is not sturdily held in place or is not resting firmly at the bottom of the tube. Too much or too little volume will also disrupt the experiment, particularly if volume is not reproducible from tube to tube. It may be helpful to prepare some mock gradients with a dye in one of the solutions to test for linearity using either method. Linearity can be determined after elution by refractometry of each fraction. Finally, any inconsistency in preparation of the sucrose stock solutions may cause loss of reproducibility of migration from one experiment to the next; samples will move faster in a 4% to 16% gradient than in a 5% to 20% gradient because of lower density and viscosity. Inconsistencies will result in altered migration in different experiments. Thus, it is important to check the sucrose concentrations of the stock solutions by refractometry.

4. Care must be taken during the beginning and end stages of centrifugation such that the tubes are not disrupted by mishandling or by too rapid acceleration or braking in the ultracentrifuge. Each of these mishaps may disrupt the gradients, causing mixing and eventual loss of resolution. The simplest way to avoid this is to take appropriate care in loading the centrifuge rotor in the ultracentrifuge and then program the ultracentrifuge with the slowest possible acceleration rate and no brake at the end of the run; although it will take a long time for the rotor to reach full speed (typically ~15 min for the SW41 Ti rotor) and to come to a stop (usually ~30 min for the SW41 Ti rotor), it is worth it to ensure the proper outcome of the experiment.

5. The final and perhaps most critical feature of the experimental manipulation is the elution. All of the described methods provide some degree of turbulence or laminar capillary flow during the elution, which will affect the resolution of the experiment. Too much turbulence will disrupt the gradient and result in broad bands after analysis or in irregular, nonsymmetrical elution profiles. Most of the automated methods also introduce some turbulence, albeit less than the manual methods. The best way to reduce turbulence is to elute as slowly as possible. If using the hole puncture method (see Basic Protocol 2), be sure that the hole is at the very bottom and that the hole is not clogged by any precipitate (this can be avoided by preclearing the sample of particulates). Try to use a needle that produces the smallest possible drop volume. It may be helpful to attach the needle to some tubing or a drop former to standardize the size of the drop for a given viscosity. If using the peristaltic pump (see Alternate Protocol 2), be sure that the capillary micropipet is firmly placed within the tube, that the pump flow is constant (some pumps introduce a certain degree of backflow and should be avoided), and that the amount of microbore tubing between sample and fraction collector is minimal. If an overly large backflow is observed, check to make sure that the pump is set up appropriately. If using the manual pipetting method (see Alternate Protocol 3), be sure that the pipettor remains as close to the top of the tube as possible as material is withdrawn from the gradient. If using the automated instruments, be sure to follow the manufacturer's instructions carefully!

Interpretation of data

Interpreting the data from the sedimentation experiment is also subject to a number of critical parameters (see Support Protocols 1 through 5). It is most important to choose as standards molecules that resemble the test sample as closely as possible. If partial specific volumes of the test sample and standards are similar, then it is likely that sedimentation coefficients will be accurately measured and molecular weights accurately estimated based on migration of the standards. The use of standards with dissimilar partial specific volumes will reduce the accuracy of these determinations. Even when using the parallel D_2O/H_2O system (see Basic Protocol 3), standards chosen for determination of the K_i values must have similar values for sedimentation coefficient and partial specific volume. Otherwise, there will be a significant degree of error in the calculations, because the K_i constants include partial specific volume. Most importantly, it is critical not to

make the mistake that the sedimentation properties correspond precisely to molecular weight; these properties may reflect molecular weight but cannot be extrapolated directly without information concerning the Stokes radius or frictional coefficient.

The use of detergents to solubilize integral membrane proteins introduces a major hindrance to interpretation of sedimentation experiments. Calculated values will always include contributions from the detergent, and it is difficult to accurately assess experimentally what these contributions are. Perhaps the best way to derive meaningful information is to perform the sedimentation analysis in several different detergents with distinct properties. Combined data from these different analyses, interpreted with the known properties of the detergents (aggregation number, molecular weight, and partial specific volume), may permit determination of the contribution of the detergent to the size of the protein complex. Such an analysis may also reveal any differences in stability of protein complexes in different detergents by dramatic changes in size of the complex. Be careful in this interpretation, however; large increases in sedimentation coefficient, partial specific volume, and molecular weight may also reflect a high degree of binding to detergents, such as Triton X-100, with high values of micelle molecular weight and partial specific volume. It is always wise to perform the analysis in different detergents to strengthen interpretation of the size of the protein complex.

Anticipated Results

The expected results will obviously vary depending on the nature of the sample tested, the number of fractions collected, and the particular method used. If one of the traditional methods, as outlined here, is used for eluting the gradient and fractions are collected that are equal in volume to the preparation loaded onto the gradient, then one might expect a single, discrete molecular species to elute in a symmetrical distribution over fractions corresponding to three to five volumes of loaded sample. The center of the peak would correspond to R for the purposes of calculation. Calculations of $S_{20,w}$ (and υ, if the double gradient system is used) would be expected to have an intrinsic error of ~5% due to the turbulence and mixing of most of these methods and the imprecision of the measurements. The more fractions that are collected (within reason), the higher the precision will be. If one of the automated elution methods is used, resolution may be greatly improved, with distribution over one to two low-volume fractions and a lower intrinsic error under the best circumstances.

On the other hand, many macromolecules may not elute as a single, discrete species. For example, many proteins that are misfolded, unassembled, or in the process of folding or assembly tend to form heterogeneous aggregates and/or associate with varying numbers of chaperone proteins (Gething and Sambrook, 1992; Hartl, 1996). This heterogeneity will be apparent as a broad distribution over many fractions of the gradient. In these cases, the material with the lowest sedimentation coefficient (earliest fraction) may contain monomers. A protein would also have a heterogeneous distribution if it exists in the cell in multiple oligomeric forms. An example would be major histocompatibility complex (MHC) class II molecules, which are incorporated into a nine-subunit complex during assembly and early biosynthetic transport, but which are eventually incorporated into the plasma membrane as heterodimers associated with a small peptide. The steady-state distribution of one of the subunits of a MHC class II molecule would therefore appear as two peaks in a sedimentation experiment, one corresponding to the nonamer and the other to the dimer (Roche et al., 1991). A protein that exists with multiple partners in the cell, such as many well-described transcription factors and cytoskeletal elements, may also have a heterogeneous distribution due to the formation of such multiple complexes.

Lastly, other biophysical features, such as instability of a protein complex or heterogeneity in detergent binding, may result in the lack of a discrete peak of elution. In many of these examples, the use of specific antibodies that recognize only a single conformation of a protein complex, and/or the use of pulse-chase metabolic labeling combined with immunoprecipitation from individual fractions, may reduce or eliminate heterogeneity by limiting analysis to individual complexes or particular stages of biosynthetic assembly and molecular lifetime. Migration of standard proteins in a discrete peak would serve to distinguish between a biologically relevant heterogeneous distribution and a poor experiment.

Time Considerations

The time required to complete the technical aspects of the sedimentation experiment will vary depending on the preparation time for the sample to be analyzed, the method of gradient

preparation, the time required for centrifugation, and the method of assaying for the sample. Typically, the gradients can be prepared, loaded, and started in the ultracentrifuge in a single day. This is particularly true when using the Gradient Master to prepare the gradients—gradient preparation can take <1 hr, and sample loading takes only a few minutes per gradient. The manual method, on the other hand, requires at least 35 to 45 min to make each gradient including preparation time; if the sample needs to be freshly prepared before loading, it may be a long day. A typical gradient run would be 16 hr (overnight if the sample is loaded into the ultracentrifuge in the evening). Elution can take anywhere from 15 to 45 min per sample, depending on the protocol used; the automated methods are faster than the manual methods. If the assay is rapid, the sample preparation is not complicated, and the sedimentation is performed for 16 to 20 hr, the entire experiment can be finished in 2 days. A typical experiment using cell lysates and assay by immunoblotting is more realistically completed in 3 to 4 days.

Literature Cited

Buchner, J., Renner, M., Lilie, H., Hinz, H.-J., Jaeniche, R., Kiefhaler, T., and Rudolph, R. 1991. Alternatively folded states of an immunoglobulin. *Biochemistry* 30:6922-6929.

Clarke, S. 1975. The size and detergent binding of membrane proteins. *J. Biol. Chem.* 250:5459-5469.

Cohn, E.J. and Edsall, J.T. 1943. Density and apparent specific volume of proteins. *In* Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions (E.J. Cohn and J.T. Edsall, eds.) pp. 370-381. Harvard Publishing, New York.

Coombs, D.H. and Watts, N.R. 1985. Generating sucrose gradients in three minutes by tilted tube rotation. *Anal. Biochem.* 148:254-259.

de Haen, C. 1987. Molecular weight standards for calibration of gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis: ferritin and apoferritin. *Anal. Biochem.* 166:235-245.

Doms, R.W. 1990. Oligomerization and protein transport. *Methods Enzymol.* 191:841-854.

Edelstein, S.J. and Schachman, H.K. 1967. The simultaneous determination of partial specific volumes and molecular weights with microgram quantities. *J. Biol. Chem.* 242:306-311.

Gething, M.J. and Sambrook, J. 1992. Protein folding in the cell. *Nature* 355:33-45.

Gibbons, R.A. 1972. Physico-chemical methods for the determination of the purity, molecular size and shape of glycoproteins. *In* Glycoproteins: Their Composition, Structure, and Function (A. Gottschalk, ed.) pp. 31-128. Elsevier/North Holland, Amsterdam.

Hall, P.K. and Roberts, R.C. 1978. Physical and chemical properties of human plasma α_2-macroglobulin. *Biochem. J.* 171:27-38.

Hartl, U.F. 1996. Molecular chaperones in cellular protein folding. *Nature* 381:571-579.

Helenius, A. and Simons, K. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta* 415:29-79.

Horne, W.A., Weiland, G.A., and Oswald, R.E. 1986. Solubilization and hydrodynamic characterization of the dihydropyridine receptor from rat ventricular muscle. *J. Biol. Chem.* 261:3588-3594.

Keller, R.K. and Touster, O. 1975. Physical and chemical properties of β-glucuronidase from the preputial gland of the female rat. *J. Biol. Chem.* 250:4765-4769.

Lide, D.R. 1997. CRC Handbook of Chemistry and Physics, 78th ed. CRC Press, Boca Raton, Fla.

Lundh, S. 1973. Determination of the molecular weight and partial specific volume of human transferrin by means of a new two-stage density gradient equilibrium method. *Int. J. Peptide Protein Res.* 5:304-325.

Martin, R.G. and Ames, B.N. 1961. A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. *J. Biol. Chem.* 236:1372-1379.

Meunier, J.C., Olsen, R.W., and Changeux, J.P. 1972. Studies on the cholinergic receptor protein from *Electrophorus electricus*. Effect of detergents on some hydrodynamic properties of the receptor protein in solution. *FEBS Lett.* 24:63-68.

Millero, F.J., Ward, G.K., and Chetirkin, P. 1976. Partial specific volume, expansibility, compressibility, and heat capacity of aqueous lysozyme solutions. *J. Biol. Chem.* 251:4001-4004.

Neugebauer, J. 1994. A Guide to the Properties and Uses of Detergents in Biology and Biochemistry. Calbiochem-Novabiochem International, La Jolla, Calif.

Reisler, E., Haik, Y., and Eisenberg, H. 1977. Bovine serum albumin and aqueous guanidine hydrochloride solutions. Preferential and absolute interactions and comparison with other systems. *Biochemistry* 16:197-203.

Roche, P.A., Marks, M.S., and Cresswell, P. 1991. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature* 354:392-394.

Sober, H.A. 1970. CRC Handbook of Biochemistry. Selected Data for Molecular Biology, 2nd ed. CRC Press, Boca Raton, Fla.

Tanford, C. 1961. Physical Chemistry of Macromolecules. John Wiley & Sons, New York.

Tanford, C. and Reynolds, J.A. 1976. Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta* 457:133-170.

Van Holde, K.E. 1975. Sedimentation analysis of proteins. *In* The Proteins, 3rd ed., Vol. 1 (H. Neurath and R.L. Hill, eds.) pp. 225-291. Academic Press, New York.

Watts, T.H., Kay, C.M., and Paranchych, W. 1982. Dissociation and characterization of pilin isolated from *Pseudomonas aeruginosa* strains PAK and PAO. *Can. J. Biochem.* 60:867-872.

Williams, J.W. 1972. Ultracentrifugation of Macromolecules. Academic Press, New York.

Key References

Helenius and Simons, 1975. See above.

This is an excellent review covering the properties of detergents and how they bind to integral membrane proteins. It should be used as a guide for interpreting sedimentation experiments of integral membrane proteins in detergent solutions.

Martin and Ames, 1961. See above.

This is the seminal paper describing the technique of zonal sedimentation in sucrose gradients and supplies most of the basic background for understanding and performing the technique.

Van Holde, 1975. See above.

This is an excellent review article covering the theoretical aspects of all of the major methods for sedimentation analysis of macromolecules.

Internet Resource

http://131.202.97.21

The Biocomp home page describes the Biocomp Gradient Master and Piston Gradient Fractionator with trumpet tip in detail.

Contributed by Michael S. Marks
University of Pennsylvania
School of Medicine
Philadelphia, Pennsylvania

Analysis of the Association of Proteins with Membranes

This unit contains several protocols for analyzing the association of proteins with membranes. These include complementary protocols for determining if a given protein is an integral or peripheral membrane protein–i.e., alkaline carbonate extraction (see Basic Protocol 1), urea extraction (see Alternate Protocol 1), high-salt extraction (see Alternate Protocol 2), and Triton X-114 phase separation (see Alternate Protocol 3).

In addition, protocols are included for assessing whether a protein is associated with membranes via a C-terminal glycophospholipid anchor, termed glycosyl phosphatidylinositol (GPI). Here, a phosphoinositol (PI)–specific phospholipase is used to enzymatically release the hydrophobic "greasy foot" from this class of membrane proteins (see Basic Protocol 2). Some membrane components are selectively resistant to solubilization by Triton X-100 at 4°C; these proteins can be solubilized using other detergents (see Basic Protocol 3).

Finally, there are two protocols for purifying caveolae-derived plasma membrane domains from cultured cells (see Basic Protocol 4). One uses Triton X-100; the other is a detergent-free method (see Alternate Protocol 4).

The unit also contains protocols for Triton X-114 precondensation (see Support Protocol 1) and preparation of a protease inhibitor stock solution (see Support Protocol 2) to be used in the corresponding basic protocols.

ALKALINE CARBONATE EXTRACTION

BASIC PROTOCOL 1

This method developed by Fujiki et al. (1982a) uses alkaline pH to disrupt membranes. Under these conditions, membrane vesicles are converted to open membrane sheets that can be recovered with an ultracentrifugation step. The soluble contents of the vesicles are released into the supernatant fraction. Proteins peripherally associated with the membrane are also found in the supernatant. Only integral membrane proteins remain associated with the membrane under those conditions. This method can be used on membrane proteins from all intracellular membranes. Examples include but are not limited to membranes isolated from the endoplasmic reticulum (Fujiki et al., 1982a,b), peroxisomes (Fujiki et al., 1982a,b), mitochondria (Fujiki et al., 1982a,b; Scherer et al., 1992), Golgi (Scherer et al., 1996) and plasma membrane (James et al., 1989).

The identical procedure can be performed with cells radiolabeled as described in *UNIT 7.1*. If an immunoprecipitation of the respective fraction is desired rather than a direct application to SDS-PAGE, the samples should be treated as described in steps 1 to 4; however, instead of adding an equal volume of 2× SDS-PAGE sample buffer in step 5a, the samples should be treated with nonionic detergents as in steps 5b and 6b (also see *UNIT 7.2*)

Materials

Cells of interest
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
100 mM NaCl
100 mM sodium carbonate, pH 11.5, ice cold
2× SDS-PAGE sample buffer (*APPENDIX 2A*)
Triton X-100

Contributed by R.B. Schwab, T. Okamoto, P.E. Scherer, and M.P. Lisanti
Current Protocols in Cell Biology (2000) 5.4.1-5.4.17

N-octyl glucoside
Tris·Cl, pH 7.5 (*APPENDIX 2A*)
NaCl

2-ml Dounce homogenizer
Beckman TL-100 centrifuge with TLA 100.2 rotor
26-G needles and 1-ml syringes
95°C water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and immunoprecipitation (*UNIT 7.2*)

1a. *For adherent cells:* Wash ~1×10^7 cells three times with ice-cold 20 ml PBS, then once with 100 mM NaCl.

1b. *For nonadherent cells:* Wash ~1×10^7 cells as in step 1a, but isolate cells between washes by gentle microcentrifugation (2 min at a maximum of $1000 \times g$, 4°C).

2. Scrape (adherent) or resuspend (nonadherent) cells into 1 ml of ice-cold 100 mM sodium carbonate, pH 11.5, and homogenize with five strokes in a 2-ml Dounce homogenizer.

3. Incubate homogenate 30 min at 4°C, then centrifuge 60 min at 150,000 to 200,000 × *g* (100,000 rpm in a TLA 100.2 rotor), 4°C.

4. Transfer the supernatant to a fresh tube and resuspend pellet in 1.0 ml of 100 mM sodium carbonate, pH 11.5.

To facilitate the resuspension of the pellet, the pellet fraction can be homogenized in the Dounce homogenizer.

For SDS-PAGE and/or immunoblotting

5a. Immediately mix pellet and supernatant fractions with an equal volume of 2× SDS-PAGE sample buffer. Shear genomic DNA in pellet sample by passing through a 26-G needle several times.

If necessary, samples can be neutralized by adding 5 to 10 µl of 1 M Tris·Cl, pH 7.0. The shearing will decrease the sample's viscosity.

6a. Heat samples at 95°C for 5 min.

The samples can now be used directly for SDS-PAGE (UNIT 6.1) and immunoblot analysis (UNIT 6.2). Load equal volumes of both supernatant and pellet fraction. Alternatively, samples can be stored frozen at –20° or –80°C for weeks.

For immunoprecipitation

5b. Add Triton X-100 to a final concentration of 1% (w/v), *N*-octyl glucoside to a final concentration of 60 mM, Tris·Cl, pH 7.5, to a final concentration of 50 mM, and NaCl to a final concentration of 300 mM.

The pH of the final solution should not exceed 8.0. If necessary, add more Tris·Cl, pH 7.5, to lower the pH.

6b. Incubate sample on ice for 5 min, then microcentrifuge 10 min at $15,000 \times g$, 4°C, to remove insoluble debris. Immunoprecipitate using a standard reaction (*UNIT 7.2*).

UREA EXTRACTION

ALTERNATE PROTOCOL 1

This procedure is identical to the alkaline carbonate extraction (see Basic Protocol 1), except that 100 mM sodium carbonate, pH 11.5, is replaced with a solution containing 2 M urea and 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5. Moderately high concentrations of urea (2 M) are commonly used to extract peripheral and soluble proteins from vesicles (Schook et al., 1979). Urea diminishes the hydrophobic interactions and thereby removes these proteins from the membrane. It is important to note that cyanate is formed spontaneously from urea. The cyanate ion can react with both amino and sulfhydryl groups of amino acids causing such problems as irreversible inactivation of enzymes and altered retention times on reversed-phase HPLC after Edman degradation of proteins. Therefore, solutions should be prepared fresh from high-quality urea, or the solution should be deionized prior to use. Store urea-containing solutions up to 1 week at 4°C.

HIGH-SALT EXTRACTION

ALTERNATE PROTOCOL 2

At high concentrations, salt exerts effects on proteins that depend both on the concentration and the nature of the salt. In this context, NaCl and KCl are primarily used to disrupt polar interactions between peripheral membrane proteins and integral membrane proteins. Once again this procedure is identical to the alkaline carbonate extraction (see Basic Protocol 1), except in this case 100 mM sodium carbonate is replaced with a solution containing 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/0.5 M NaCl. KCl may be used as an alternative to NaCl. Salt solutions can be stored at room temperature, but must be chilled well prior to use.

TRITON X-114 PHASE SEPARATION

ALTERNATE PROTOCOL 3

A solution of the nonionic detergent Triton X-114 is homogeneous at 0°C but separates into an aqueous phase and a detergent phase above 20°C. The extent of this detergent-phase separation increases with temperature. When membrane fractions are solubilized with a Triton X-114 solution on ice and then placed at 37°C, hydrophilic proteins are found in the aqueous phase, whereas integral membrane proteins are recovered in the detergent phase. Integral membrane proteins can thus be separated from soluble and peripheral proteins.

Materials

Cells of interest
Cell lysis buffer (see recipe)
Tris/NaCl/EDTA buffer (see recipe)
TNET-OG buffer (see recipe)
2× SDS-PAGE sample buffer (*APPENDIX 2A*)

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

1. Add 1 ml ice-cold cell lysis buffer to 2–5 × 10^6 cells and lyse cells by incubating 45 to 60 min at 4°C.
2. Transfer solution to a microcentrifuge tube and microcentrifuge 15 min at 10,000 × *g*, 4°C.
3. Transfer supernatant to a fresh microcentrifuge tube and incubate at 37°C for 3 min.
4. Centrifuge solution 1 min at 10,000 × *g*, room temperature. Transfer the upper (aqueous) phase, which should represent ~95% of the total volume, to a fresh microcentrifuge tube. Retain the detergent phase for step 6.

5. Re-extract the aqueous phase from step 4 by adding 100 µl cell lysis buffer and repeating steps 3 and 4. Discard the lower detergent phase from this step.

6. Re-extract the detergent phase from step 4 by adding 10 vol Tris/NaCl/EDTA buffer. Repeat steps 3 and 4 then discard the upper, aqueous phase.

7a. *For aqueous phase*: Use the aqueous phase (from step 5) directly for immunoprecipitation reactions (*UNIT 7.2*).

7b. *For detergent phase*: Dilute the detergent phase (from step 6) to 1 ml with TNET-OG buffer and use for immunoprecipitation (*UNIT 7.2*).

7c. *For direct analysis of the extracted samples by SDS-PAGE:* Mix 5% of both aqueous (approx. 50 µl) and detergent phases (2.5 µl) with 50 µl 2× SDS-PAGE sample buffer.

If not to be analyzed immediately, samples can be stored at −20° or −80°C for 1 to 2 weeks.

SUPPORT PROTOCOL 1

TRITON X-114 PRECONDENSATION

The purpose of this support protocol is to prepare a precondensed stock solution of Triton X-114 (Bordier, 1981). Precondensation is required to remove certain impurities that may interfere with the phase separation properties of Triton X-114.

Materials

Triton X-114
Tris-buffered saline (TBS): 10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/150 mM NaCl
Sodium dodecyl sulfate (SDS)
Triton X-100

1. Add 20 g Triton X-114 to 980 ml Tris-buffered saline.

2. Allow to dissolve at 4°C for ~1 hr with stirring or until solution is clear, then incubate at 30°C overnight with stirring.

3. Discard the large aqueous phase (upper phase) and replace with fresh Tris-buffered saline. Once again dissolve at 4°C and incubate overnight at 30°C.

4. Repeat step 3 two more times.

The last detergent phase (lower phase) is the stock solution to use for experiments.

5. *Optional:* Determine the concentration of the last detergent phase by measuring the absorbance of a 1:1000 dilution in the presence of 1% SDS at 275 nm and compare to the absorbance of a set of standard solutions of Triton X-100 at 275 nm to determine the final concentration.

The extinction coefficient of Triton X-100 is virtually identical to that of Triton X-114.

There is actually no real need to determine the exact concentration of the final stock as it varies between 9.5% and 10.5% Triton X-114 and can simply be used at a dilution of 1:10. See recipe for cell lysis buffer in Reagents and Solutions.

6. Store the final stock solution up to 1 week at 4°C.

PREPARATION OF 100× PROTEASE INHIBITORS STOCK SOLUTION

SUPPORT PROTOCOL 2

Protease inhibitors are used to prevent proteolysis during extraction. This stock mixture inhibits most common proteases (also see *APPENDIX 1B*).

Materials

Antipain
Pepstatin A
Leupeptin
Dimethylsulfoxide (DMSO)

1. Make separate 1000× solutions of antipain, pepstatin A, and leupeptin by dissolving 5 mg of each in 500 µl DMSO.
2. Combine 50 µl of each 1000× solution and add 350 µl DMSO.
3. Divide this combined 100× stock solution into small aliquots (i.e., 100 µl) and store at –20°C.
4. Use 10 µl/ml for a final concentration of 10 µg/ml of each protease inhibitor. Do not exceed 10 µl/ml (final DMSO concentration should be <1%).

PI-PLC CLEAVAGE OF GPI-LINKED PROTEINS

BASIC PROTOCOL 2

This procedure is used to detect glycosyl phosphatidylinositol (GPI)-linked proteins by converting them from a hydrophobic to a hydrophilic state. The procedure consists of the enzymatic removal of the GPI anchor using phosphatidylinositol-specific phospholipase C (PI-PLC). The removal of the GPI anchor causes the protein to shift from the detergent-rich phase to the detergent-poor phase of Triton X-114 extracts.

Materials

Cells of interest
Tris/NaCl/EDTA buffer (see recipe)
PI-PLC incubation buffer (see recipe)
1000 U/ml PI-PLC (phosphatidylinositol-specific phospholipase C; Boehringer Mannheim)
Phenyl-Sepharose 4B (Pharmacia Biotech)
Tris-buffered saline (TBS): 20 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/150 mM NaCl
12.5 mg/ml (100×) sodium deoxycholate
Trichloracetic acid
2× SDS-PAGE sample buffer (*APPENDIX 2A*)
1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1% (w/v) SDS/200 mM Tris·Cl, pH 8.0 (see *APPENDIX 2A* for Tris·Cl)
TNET buffer (see recipe)

Eppendorf vortex mixer
Boiling water bath

Additional reagents and equipment for Triton X-114 phase separation (see Alternate Protocol 3), SDS-PAGE (*UNIT 6.1*), and immunoprecipitation (*UNIT 7.2*).

1. Using ~1×10^7 cells, follow the Triton X-114 phase separation protocol (see Alternate Protocol 3) with the following variations:
   a. In step 3 of Alternate Protocol 3, incubate samples at 37°C for 20 min instead of 3 min.
   b. In step 6 of Alternate Protocol 3, re-extract samples twice against fresh Tris/NaCl/EDTA buffer, keeping the detergent phases.

2. Dilute the detergent-rich phases 10-fold with PI-PLC incubation buffer and add PI-PLC to a concentration of 4 to 8 U/ml.
3. Mix continuously on a vortex mixer at 37°C for 1 hr.
4. Repartition samples by microcentrifuging 1 min at 10,000 × *g*, room temperature. Collect the upper (aqueous) phases.
5. Prepare a 50% slurry of phenyl-Sepharose 4B in Tris-buffered saline. Add 200 µl of the slurry per ml aqueous phase and incubate samples overnight at 4°C to remove trace amounts of hydrophobic membrane proteins.
6. Microcentrifuge samples 10 sec at ≤2000 × *g*, 4°C, and transfer supernatants to fresh microcentrifuge tubes. Repeat to clear all beads.
7. Precipitate by adding sodium deoxycholate (as 100× stock) to 125 µg/ml and trichloracetic acid to 6% (w/v). Incubate on ice for 10 min.
8. Microcentrifuge samples 4 min at 10,000 × *g*, 4°C, and remove supernatant.

9a. *For SDS-PAGE*: Resuspend each pellet in an equal volume of of 2× SDS-PAGE sample buffer and neutralize by adding small amounts (~5 µl) of 1 M Tris·Cl, pH 8.0, and use for SDS-PAGE (*UNIT 6.1*).

9b. *For immunoprecipitation*: Resuspend each pellet in 100 µl of 1% (w/v) SDS/200 mM Tris·Cl pH 8.0. Boil 5 min, then dilute samples in 10 vol TNET buffer. Proceed with immunoprecipitation (*UNIT 7.2*).

BASIC PROTOCOL 3

DETERGENT SOLUBILIZATION OF TRITON X-100 INSOLUBLE INTEGRAL MEMBRANE AND GPI-LINKED PROTEINS

Several caveolar membrane components are selectively resistant to solubilization by Triton X-100 at 4°C, which is the most commonly used protocol for the solubilization of integral membrane proteins. The unusual Triton insolubility of these caveolar domains has been attributed to their high glycosphingolipid content, as glycosphingolipids are intrinsically Triton-insoluble. They can, however, be solubilized in other detergents, such as octylglucoside or CHAPS. Solubilization can also be performed using SDS under experimental conditions that permit denaturation of protein or protein complexes.

Materials

Cells of interest
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Detergent solution—one of the following:
60 mM *N*-octylglucoside in either TBS (20mM Tris·Cl, pH 8.0/150 mM NaCl) or MES-buffered saline (25 mM MES, pH 6.5/150 mM NaCl)
30 mM CHAPS in either TBS (20mM Tris·Cl, pH 8.0/150 mM NaCl) or MES-buffered saline (25 mM MES, pH 6.5/150 mM NaCl)
Wash buffer containing Triton X-100 (*UNIT 7.2*)

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2*)

1. Wash 1×10^6 to 1×10^7 cells twice in PBS (see Basic Protocol 1, steps 1a and 1b).
2. Add 1 ml of 60 mM *N*-octylglucoside or 30 mM CHAPS in TBS or MES-buffered saline. Vortex, then incubate at 4°C for 30 min to 1 hr.

   60 mM N-octylglucoside is most frequently used for solubilizing caveolae because it is the most efficient detergent for this purpose.

3. Centrifuge 10 min at 15,000 × *g*, 4°C, to remove insoluble debris.
4. Use the supernatant for a standard immunoprecipitation reaction (*UNIT 7.2*).

5. Wash immunoprecipitates with Triton X-100–containing wash buffer as described in *UNIT 7.2*.

   This alternative wash buffer is used to minimize the amount of N-octylglucoside needed, as that detergent is rather expensive. After washing, the packed beads can be stored frozen for weeks.

TRITON-BASED PURIFICATION OF CAVEOLAE-DERIVED MEMBRANES

BASIC PROTOCOL 4

Caveolae are vesicular organelles attached to the plasma membrane that have a characteristic lipid and protein composition (Sargiacomo et al., 1993; Lisanti et al., 1994). Caveolae membranes are highly enriched in glycosphingolipids and cholesterol, making them extremely light and therefore buoyant in sucrose density gradients. This unique lipid composition also confers resistance to solubilization by nonionic detergents such as Triton X-100 and NP-40 at low temperatures. For example, when intact cells were fixed in paraformaldehyde, extracted with Triton X-100, and then examined by electron microscopy, the insoluble membranes that remained were found to be caveolae (Moldovan et al., 1995). These special properties allow the isolation of caveolae from other cell organelles as described below and in Alternate Protocol 4.

Materials

MDCK (Madin-Darby canine kidney) cells (or virtually any other nontransformed cell line)
DMEM medium containing 5% FBS (*UNIT 1.2*) with 100 IU/ml penicillin and 50 µg/ml streptomycin
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
Lysis buffer for caveolae (see recipe)
5%, 30%, and 80% sucrose solutions (see recipe)
MES-buffered saline: 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/150 mM NaCl

75-cm^3 tissue culture flasks
150-mm tissue culture dishes
Cell scrapers
15-ml tubes
Dounce homogenizer
Beckman L8 ultracentrifuge with SW 41 rotor and corresponding ultraclear centrifuge tubes

Additional reagents and equipment for cell culture and trypsinization of cells (*UNIT 1.1*), preparation of serum-containing tissue culture medium (*UNIT 1.2*), and preparation of sucrose gradients (*UNIT 5.3*)

1. Split MDCK cells by trypsinization (*UNIT 1.1*) from one 75-cm^2 flask into three 75-cm^2 flasks, and grow to confluence in DMEM/5% FBS . Split cells from the flasks into six 150-mm dishes. Let cells grow for at least 2 days (to confluence) before harvesting.

   Omit any antifungal agents such as Nystatin or Fungizone, as these are cholesterol-binding antibiotics that will disrupt caveolae architecture.

2. Rinse the cells in each 150-mm dish three times, each time with 20 ml ice-cold PBS. Thoroughly remove PBS and add 1 ml of ice-cold lysis buffer for caveolae. Collect cells with a cell scraper and place in a 15-ml tube on ice. Rinse dish with an additional 1 ml of lysis buffer for caveolae to collect remaining cells and pool with the first 1 ml in the tube.

   Use each plate for a separate gradient.

3. Homogenize with 8 to 10 strokes of a Dounce homogenizer. Adjust to 40% (w/v) sucrose by adding an equal volume (2 ml) of ice-cold 80% sucrose. Vortex briefly until homogeneous.
4. Place homogenate at the bottom of an ultraclear Beckman ultracentrifuge tube and form a linear gradient atop it, using 4 ml each of 5% sucrose and 30% sucrose, at a flow rate of not more than 0.5 ml per min (*UNIT 5.3*). Adjust the weight of each gradient to within 0.1 g.
5. Centrifuge 12 to 16 hr (overnight) at 200,000 × *g* (39,000 rpm in an SW 41 rotor), 4°C.
6. Collect twelve 1-ml fractions from the top (*UNIT 5.3*). Snap freeze in liquid nitrogen and store frozen at −80°C.

   Fraction 13 is the insoluble pellet.

   Subsequent analysis of the total fractions can be done without pelleting the membranes.
7. *Alternatively:* Harvest the opaque band (migrating at ~10% to 20% w/v sucrose), dilute with MES-buffered saline, and microcentrifuge 60 min at 15,000 × *g*, 4°C. Snap freeze the pellet in liquid nitrogen and store at −80°C.

   A 150-mm dish of MDCK cells representing 9 to 11 mg protein yields ~4 to 6 μg of caveolae-enriched membrane domains—i.e., ~0.05 % of the initial homogenate. Triton X-100 extraction solubilizes ~85% of the protein (8.5 mg), the majority of which remains in the 40% (w/v) sucrose layer, while ~1.5 mg forms an insoluble pellet below the 40% sucrose.

   Organelle-specific membrane marker assays can be performed (Sargiacomo et al., 1993; Lisanti et al., 1994). These assay systems are not affected by the presence or absence of 1% Triton X-100 in the initial homogenate.

   The purified caveolar fractions can be combined.
8. Store frozen at −80°C for months, until further use in immunoblotting or immunoprecipitation.

ALTERNATE PROTOCOL 4

DETERGENT-FREE PURIFICATION OF CAVEOLAE-DERIVED MEMBRANES

Recently, it has been suggested that the inclusion of detergent in the initial homogenization step results in the loss of resident prenylated caveolin-associated proteins, such as $G_{\beta\gamma}$ subunits. To preserve these interactions, a detergent-free method for the purification of caveolin-rich membrane domains has been developed. This slightly modified scheme replaces the detergent Triton X-100 with sodium carbonate. Sodium carbonate extraction is routinely used to determine if proteins are firmly attached to membranes and caveolin is not solubilized by sodium carbonate. Using this modified scheme, endogenous caveolin and recombinant caveolin have been shown to be recovered almost quantitatively in fractions 5 and 6 while excluding most cellular proteins. In addition, caveolin has been shown to be separated from the GPI-linked plasma membrane marker, carbonic anhydrase IV. This is consistent with recent observations that GPI-linked proteins are not concentrated directly within caveolae but may reside in close proximity to the ''neck regions'' of caveolae within intact cells. This method is adapted from Song et al. (1996).

Materials

MDCK (Madin-Darby canine kidney) cells or any other cell
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice cold
500 mM sodium carbonate, pH 11, ice cold

MES-buffered saline: 25 mM MES, pH 6.5/0.15 M NaCl
MES-buffered saline plus 1% (w/v) Triton X-100 and PMSF
5%, 35%, and 90% (w/v) sucrose (see recipe)
3× SDS-PAGE sample buffer (see recipe)

Cell scrapers
Polytron tissue grinder (Kinematica GmbH, Brinkmann Instruments)
Branson Sonifier 250 (Branson Ultrasonic)
Beckman ultracentrifuge with SW 41 rotor and corresponding (14 × 89–mm) ultraclear tubes

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2*) and SDS-PAGE (*UNIT 6.1*)

1. Culture cells to produce six 150-mm plates at confluence (see Basic Protocol 4, step 1). Wash cells three times with ice-cold PBS (see Basic Protocol 4, step 2).
2. Add 1 ml ice-cold 500 mM sodium carbonate, pH 11.0 to one plate of cells, scrape the cells into the liquid and transfer to the second plate. Continue in this manner for all six plates. Repeat with a second 1-ml aliquot of sodium carbonate and combine.
3. Place cells on ice. Disrupt the cells using a Polytron tissue grinder at maximum setting for 30 sec.
4. Sonicate cells constantly for 30 sec at setting 2 on the Branson Sonifier 250. Next, sonicate cells with 30-sec bursts at a setting of 4 or 5. Look at the lysate. If it is relatively clear and free of clumps, then it is ready to use. If not, sonicate an additional 30 sec at setting 2.
5. Add the 2 ml of cell lysate to 2 ml of 90% (w/v) sucrose in a 14 × 89–mm Beckman centrifuge ultraclear tube. Vortex until homogeneous.

   The volume of tube is a little more than 12 ml.

   After mixing with the cell lysate the final concentration of sucrose is 45% (w/v).
6. Atop the 45% layer, add 4 ml of 35% sucrose, then 4 ml of 5% sucrose, to generate a discontinuous sucrose gradient.

   Be careful when adding successive layers so as to not disrupt the layer beneath.
7. Centrifuge 12 to 16 hr (overnight) at 200,000 × *g* (39,000 rpm in an SW 41 rotor), 4°C.
8. Collect twelve 1-ml fractions from the top (*UNIT 5.3*). Snap freeze all fractions in liquid nitrogen and store frozen at −80°C.

   Fraction 13 is the insoluble pellet.

   A light-scattering band confined to the 5%/35% sucrose interface contains caveolin, a protein concentrated in caveolae, but excludes most other cellular proteins. Analyze total fractions (do not pellet).

   The purified caveolar fractions can be stored frozen at −80°C for months until further use in immunoblotting (UNIT 6.2) or immunoprecipitation (UNIT 7.2).
9. Mix with an equal volume of 3× SDS-PAGE sample buffer that contains 100 mM NaOH to disrupt SDS-resistant caveolin complexes. Analyze by SDS-PAGE (*UNIT 6.1*).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cell lysis buffer

1% (w/v) Triton X-114 (from precondensed stock; see Support Protocol 1)
10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
150 mM NaCl
1 mM EDTA
Store up to 1 month at 4°C
10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2; final concentration 10 µg/ml each of antipain, pepstatin, and leupeptin), added immediately before use

Lysis buffer for caveolae

1% (w/v) Triton X-100
25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)
150 mM NaCl
Store up to 1 month at 4°C
Add 100 µl of saturated ice-cold ethanol solution of PMSF per 10 ml of buffer immediately before use.

PI-PLC incubation buffer

100 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
50 mM NaCl
1 mM EDTA
0.5 M methyl-α-D-mannopyranoside
Prepare fresh

SDS sample buffer with NaOH, 3×

150 mM Tris·Cl, pH 6.8
300 mM dithiothreitol
6% (w/v) SDS
0.3% (w/v) bromophenol blue
30% (w/v) glycerol
30 mM NaOH
Add 1 M NaOH to a final concentration of 100 mM just before use
Store up to several months at −20°C

Sucrose solutions for Basic Protocol 4 (80%, 30%, and 5% w/v)

80% (w/v): Prepare an 80% (w/v) solution of sucrose in MES-buffered saline [25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5/150 mM NaCl, without PMSF or Triton X-100].

30% (w/v): Dilute 37.5 ml of 80% (w/v) sucrose to 100 ml with MES-buffered saline.

5% (w/v): Dilute 6.25 ml of 80% (w/v) sucrose to 100 ml with MES-buffered saline.

Prepare fresh each time

Sucrose solutions for Alternate Protocol 4 (90%, 35%, and 5% w/v)

90% (w/v): Prepare 90% (w/v) sucrose in MES-buffered saline (final concentration of MES is 10 mM, pH 6.5, and that of NaCl is 150 mM; the rest of the volume is water). Since 90% (w/v) sucrose is hard to dissolve, it is generally heated. First, heat a beaker of water until it boils. Next, place the 90% sucrose mixture, contained in a smaller uncapped beaker/bottle, in the boiling water. Stir the sucrose until it completely dissolves. Store 90% (w/v) sucrose at room temperature for up to one

year (it crystallizes at colder temperatures). Prechill only the amount to be used, just before use.

35% (w/v): Dilute 90% (w/v) sucrose to 35% (w/v) in MES-buffered saline containing 250 mM sodium carbonate, pH 11.0.

5% (w/v): Dilute 90% (w/v) sucrose to 5% (w/v) in MES-buffered saline containing 250 mM sodium carbonate, pH 11.0.

Store the diluted sucrose solutions up to 6 months at 4°C.

A stock solution of 500 mM sodium carbonate is made by simply dissolving sodium carbonate in water. The pH of the solution is about 11. The pH does not need to be adjusted. This solution can be stored at 4°C up to one year.

TNET buffer

1% (w/v) Triton X-100
150 mM NaCl
5 mM EDTA
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Store up to 1 month at 4°C
10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2), added immediately before use

TNET-OG buffer

1% (w/v) Triton X-100
150 mM NaCl
5 mM EDTA
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
60 mM *N*-octylglucoside
Store up to 1 month at 4°C
10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2), added immediately before use

Tris/NaCl/EDTA buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
150 mM NaCl
1 mM EDTA
Store up to 1 month at 4°C
10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2), added immediately before use

COMMENTARY

Background information

From a practical point of view, it is essential to isolate membranes that preserve their in vivo physical properties in order to identify proteins associated with specific membrane domains. The membrane isolation method that uses high pH sodium carbonate buffer efficiently removes peripherally associated proteins (>90%), releases <10% of the integral membrane proteins in caveolae, and preserves the original membrane properties. Thus this method is useful for preparing membranes for further isolation of specific domains. Integral proteins are also not removed using high salt buffer (1 M or 3 M NaCl or 0.6 M KCl) or urea, treatments which efficiently remove peripheral proteins. Extraction with urea and high salt are alternative methods for isolating membranes to identify integral membrane components.

Caveolae

Caveolae are vesicular organelles attached to the plasma membrane, with a diameter of ~50 to 100 nm. The principal protein components of caveolae are members of the caveolin family of proteins, termed caveolins 1, 2, and 3. Caveolins 1 and 2 are most abundantly expressed in adipocytes, endothelial cells, and fibroblastic cell types, while the expression of

caveolin 3 is muscle-specific. Caveolins interact directly with a number of caveolae-associated signaling molecules, such as H-Ras, heterotrimeric G-proteins, epidermal growth factor receptor, protein kinase C, Src-family tyrosine kinases, and nitric oxide synthase (NOS) isoforms.

In addition to specific proteins, caveolae are enriched in certain lipids such as glycosphingolipids and cholesterol. Using liposomal systems, it has been well established that lipid microdomains consisting of glycosphingolipids and cholesterol can be formed when a certain ratio of glycosphingolipids, cholesterol, and phospholipids are mixed, even in the absence of protein components. It has been proposed that glycosphingolipids can be tightly packed with cholesterol because of their saturated fatty acid composition. These lipids are exclusively localized in the outer leaflet of the lipid bilayer. Phospholipids with saturated fatty acids and cholesterol constitute the inner leaflet of the microdomains. Caveolin associates with these microdomains by direct interaction with cholesterol and glycosphingolipids.

Caveolae purification

Several methods have been developed for purifying caveolae (Chang et al., 1994; Lisanti et al., 1994; Sargiacomo et al., 1993; Schnitzer et al., 1995a,b; Smart et al., 1995; Song et al., 1996). Initially, these methods took advantage of the natural detergent-insolubility of caveolae membranes. More recently, detergent-free methods have become available that are dependent on the intrinsic light buoyant density of caveolae membranes (Smart et al., 1995; Song et al., 1996). Purification of caveolae by all of these methods reveals the dramatic enrichment of caveolins and signaling molecules relative to plasma membrane. Signaling molecules appear to form a tight complex with caveolin 1.

Caveolae have also been shown to be detergent-resistant structures in vivo. For example, when intact cells were fixed with paraformaldehyde, extracted with cold Triton X-100, and then examined by electron microscopy, the insoluble membranes that remained were found to be caveolae (Moldovan et al., 1995). In support of these morphological findings, caveolins are Triton X-100–insoluble in caveolae (Sargiacomo et al., 1993; Lisanti et al., 1994), but Triton X-100 soluble in the Golgi (Smart et al., 1994). This compartment-specific Triton X-100 insolubility is most likely due to the association of caveolin with cholesterol and sphingolipids, which form a Triton X-100–insoluble microenvironment, termed a liquid-ordered phase.

Biochemical isolation of caveolae membrane domains takes advantage of the unique temperature dependence of solubilization with Triton X-100. Caveolae microdomains are solubilized by Triton X-100 at temperatures >8°C, but not at 4°C. Therefore, caveolae microdomains can be isolated biochemically from cultured cells or tissues as insoluble material after solubilizing the cells with Triton X-100 at 4°C. Since caveolae membrane domains are light because of their lipid composition, caveolae microdomains are purified more than 2000-fold with a single sucrose gradient ultracentrifugation. Caveolae microdomains are recovered as a fine dense band at the border of 5% (w/v) and 35% (w/v) sucrose after ultracentrifugation at 200,000 × *g* overnight. Caveolin-1 is used as a marker protein for caveolae organelles. The amount of caveolae protein isolated by this procedure is typically 5 μg out of 10 mg starting material (equivalent to six 150-mm culture dishes). Caveolae structure is still preserved in caveolae isolated by this procedure. As octylglucoside possesses a similar structure to glycosphingolipids, this nonionic detergent is used for solubilization of caveolae microdomains. Although urea, as well as high salt, is frequently used for solubilization of membrane-bound proteins, these methodologies do not solubilize caveolae microdomains.

Although the use of Triton X-100 for caveolae isolation is straightforward and well established, not all caveolae protein components are recovered in the caveolae fraction by this procedure. For example, the H-Ras protein was initially proposed not to be a caveolar component. Triton X-100 is able to dissociate H-Ras and other prenylated proteins from caveolae microdomains. The same is true for Gβγ subunits. The sodium carbonate method has been developed to alleviate this problem. Because of its high pH, sodium carbonate solubilizes proteins peripherally associated with the membrane. In contrast, integral membrane proteins are not solubilized by this method. This method also allows purification of caveolae microdomains by more than 2000-fold. Caveolin-1 is again recovered within caveolae microdomains. The purity of caveolae microdomains obtained with this methodology is therefore comparable to that with the Triton X-100 method. The caveolae purification approach with sodium carbonate is termed the "detergent-free caveolae purification method." With this method, the H-Ras protein has been reproduc-

ibly recovered in caveolae microdomains. As caveolae residents are more broadly purified with detergent-free approaches, the authors recommend the detergent-free method as the first methodology to be employed for caveolae purification. Another potential problem in using Triton X-100 for caveolae isolation is a possible contamination of artificial lipid membrane domains for which lipid composition is similar to that of caveolae. Solubilized lipids released from membranes treated with Triton X-100 may form artificial lipid domains like caveolae, since glycosphingolipids have a high affinity for association with cholesterol. The detergent-free method has an advantage over the Triton X-100 method in this regard. The authors recommend using both of these methodologies to localize the protein of one's interest in caveolae.

Caveolae-related domains or pre-caveolae or both?

A number of investigators have purified "caveolae" from cells and tissues that lack apparent expression of caveolin (Kubler et al., 1996; Parolini et al., 1996; Brown and London, 1997; Simons and Ikonen, 1997). These domains have been termed detergent-insoluble glycolipid membrane complexes (DIGs), Triton-insoluble complexes (TICs), detergent-resistant membranes (DRMs), and low-density membranes (LDMs). The authors suggest that the term caveolae-related domains (CRDs) be used to describe these structures. Like caveolae, these microdomains are dramatically enriched in cholesterol, sphingolipids, and lipid-modified signaling molecules (Brown and London, 1997; Simons and Ikonen, 1997). The existence of "caveolae-related domains" or CRDs that do not contain caveolin has caused considerable confusion (Simons and Ikonen, 1997). However, this was at a time when only one caveolin gene was known to exist, i.e., caveolin (now termed caveolin-1). It is now known that there are other caveolin genes, such as caveolin-2 and -3. In addition, it has been shown that caveolin-1 and caveolae are down-regulated in response to cell transformation, while caveolin-2 levels remain constant (Scherer et al., 1997). As a consequence, many commonly used cell lines lack caveolin-1 protein expression and visible caveolae, because they are immortalized or transformed. Also, other detergent-insoluble membrane proteins have recently been cloned, and one or more of them may represent functional homologs of the caveolins (Bickel et al., 1997; Puertollano et al., 1997; Simons and Ikonen, 1997).

CRDs can also be produced in vitro simply by mixing cholesterol, sphingolipids, and phospholipids in the appropriate ratio (Brown and London, 1997). Their Triton insolubility is a physical property of their molecular organization that produces a liquid-ordered membrane domain (rather than fluid or liquid-crystalline). Caveolin-1 is found associated with glycosphingolipids in vivo as shown using chemical cross-linking with a radiolabeled glycosphingolipid GM1 (Fra et al., 1995a). It binds cholesterol directly (Murata et al., 1995) and requires a high local concentration of cholesterol (>30%) to insert into model lipid membranes (Murata et al., 1995; Li et al., 1996a). These findings suggest that a true functional relationship exists between CRDs and mature caveolae. For example, during the biogenesis of mature caveolae, CRDs would need to exist as precursors for proper insertion of caveolins into membranes. Thus, in cells that express caveolins, these CRDs may represent "pre-caveolae" that simply lack caveolins. In support of this reductionist model, recombinant expression of caveolin-1 in cells that lack morphologically detectable caveolae is sufficient to drive the formation of mature invaginated caveolae (Fra et al., 1995b; Li et al., 1996b; Engelman et al., 1997). This indicates that cells normally make the ingredients that are necessary for the formation of mature caveolae, and insertion of caveolin proteins may only be a late phase in this process.

GPI-anchored proteins and their association with caveolae

The GPI anchor contains two fatty acyl groups and several sugars, including glucosamine, mannose, and inositol (Fig. 5.4.1). Various experiments have shown that the phospholipid anchor is both necessary and sufficient for binding these cell-surface proteins to the plasma membrane. The enzyme PI-specific phospholipase C cleaves the phosphate-glycerol bond in phospholipids as well as in glycosyl phosphatidylinositol anchors. Treatment of cells with phospholipase C releases glycosyl phosphatidylinositol-anchored proteins such as the Thy-1 protein and alkaline phosphatase from the cell surface. Experimentally, the PI-PLC mediated cleavage can be performed in intact cells, extracted membranes, and purified proteins reconstituted in liposomes.

GPI-linked proteins can be associated with CRDs and caveolae in the outer leaflets of lipid bilayers by using their saturated fatty acid moiety directly inserted into the unique lipid environment.

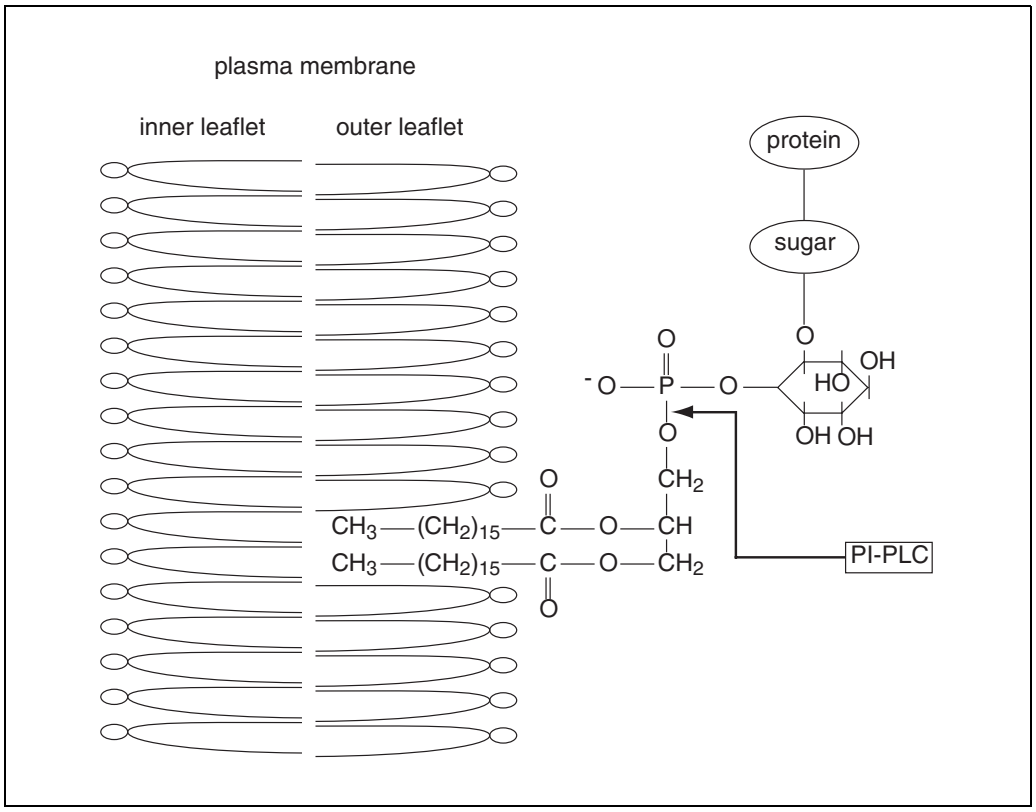


Figure 5.4.1 The glycosyl phosphoinositol (GPI) anchor. PI-PLC, phosphatidylinositol-specific phospholipase C.

It has been debated whether GPI-anchored proteins are constitutively associated with caveolae or are only associated with caveolae upon cross-linking with antibody probes. In the case of the tissue factor (TF) receptor system, this GPI-anchored protein is only associated with caveolae upon binding its natural ligand (Sevinsky et al., 1996). Thus, antibody probes may mimic natural endogenous ligands and target clustered GPI-anchored proteins to caveolae.

GPI-anchored molecules are recovered associated with membrane fractions that are resistant to solubilization to 1% Triton X-100 or 1% Triton X-114 at 4°C. It is, therefore, reasonable to speculate that GPI molecules are also associated with membrane domains with basic biochemical characteristics similar to those of caveolae, irrespective of the argument whether GPI molecules are in fact localized in caveolae. This assumption is further strengthened by the fact that the membrane fraction which contains GPI molecules is solubilized by 60 mM *N*-octylglucoside in a highly efficient manner. Of note, 20 mM CHAPS is less effective in its solubilization. At 37°C, however, 1% Triton X-100 solubilizes GPI-containing membrane with a similar efficiency to that of *N*-octylglucoside. At this temperature, 1% Triton X 114 solubilizes the membrane with 60% efficiency compared to Triton X-100. Since Triton X-114 partitions GPI molecules to the aqueous phase with PI-PLC treatment, Triton X 114 phase separation is used for the detection of GPI molecules in membranes. This method also makes it possible to identify membranes which have a similar biochemical property to that of caveolae.

Critical Parameters

Whether a given protein is an integral or peripheral membrane protein can be determined by alkaline carbonate extraction, urea extraction, high-salt extraction, and Triton X-114 phase separation protocols. The following parameters should be kept in mind while performing experiments.

In order to avoid protein degradation, experiments should be performed at 4°C. Solutions should also be chilled before use. For Triton X-114 phase separation, samples should be incubated at 37°C after solubilization, then centrifuged at room temperature.

In order to achieve optimal solubilization, the authors recommend that solubilization be performed from 30 min to 1 hr. For Triton

X-114 phase separation, samples should be kept at 37°C for 3 min after solubilization. This 3-min incubation should be strictly observed for achieving optimal phase separation and avoiding protein degradation.

Membrane proteins have ionic as well as hydrophobic interactions, and for this reason 150 mM NaCl is included in solubilization solutions for Triton X-114 phase separation protocol. As the salt concentration is already high, 100 mM NaCl is sufficient for the alkaline carbonate protocol. In urea and high-salt protocols, no additional NaCl is necessary.

The efficiency of solubilization is directly influenced by protein-salt extraction methods, and 1 ml of the Triton X-114-containing lysis buffer per 2–5 × 10^7 cells should be strictly observed for Triton X-114 phase separation protocols (see Basic Protocol 4, step 1).

In Basic Protocols 1 and 3, the criterion for solubilization is retention in the supernatant after centrifugation of solutions for 1 hr at 105,000 × *g*. The authors recommend centrifugation at 150,000 to 200,000 × *g* for 1 hr in order to recover completely solubilized proteins.

In order to avoid cyanate formation from urea, it is important to prepare urea solution just before use. For Triton X-114 stock solution, precondensation is required to remove impurities. Protease inhibitors can be kept as 100× condensed solutions in freezer.

PI-PLC cleavage releases GPI-linked proteins from membranes. In the method described here (Basic Protocol 2), PI-PLC cleavage should be performed at 37°C in Triton X-114-containing solution for at least 1 hr.

Proteins in caveolae or CRD, including GPI-anchored proteins, are solubilized in 60 mM *N*-octylglucoside or 30 mM CHAPS at 4°C. The following parameters should be kept in mind while performing these experiments.

Detergent solubility is strictly dependent upon the temperature. Temperature should be kept below 4°C in order to assess the Triton X-100 insolubility of the protein in CRD. All the experiments should be performed on ice. At 8°C, most of the proteins in CRD can be solubilized even by buffers containing 1% Triton X-100 as the detergent. This should be kept in mind especially when purifying caveolae using sucrose density gradient centrifugation. Cell extraction with Triton X-100 should also be performed at 4°C. It is recommended that the experiment be performed in the cold room, with the temperature kept below 4°C. Solutions should also be kept at 4°C before use.

Perform solubilization for 30 min to 1 hr in order to avoid protein degradation by proteases and insufficient solubilization.

The authors recommend detergent solubilization of proteins in CRD at concentrations of 60 mM *N*-octylglucoside or 30 mM CHAPS. These concentrations were determined by the critical micellar concentration (CMC) of these detergents. In order to achieve complete solubilization of proteins localized in CRDs, it is important to use detergents at twice the concentration of CMC value.

Add protease inhibitors to the solubilization buffer to a final 1× concentration in order to prevent protease-mediated degradation of proteins during solubilization. This is especially important when whole animal tissues are used as a source of protein for extraction. The protease inhibitors (Support Protocol 2) should be used in TNET-OG buffer, TNET buffer, and cell lysis buffer. 1 mM PMSF alone is sufficient for lysis for caveolae.

NaCl at 150 mM should be added to the solubilization buffer in order to maintain proteins under physiological conditions.

Caveolins are the principal protein component of caveolae. It is therefore of essence to probe the blot of fractionated samples with anti-caveolin antibodies, which are available commercially from Transduction Laboratories (see *SUPPLIERS APPENDIX*) to track the position of caveolae-derived membranes in these sucrose density gradients.

Anticipated Results

Integral membrane proteins can be separated from soluble and peripherally associated membrane proteins using alkaline carbonate extraction, urea extraction, or high-salt extraction protocols. In the Triton X-114 phase separation protocol, the aqueous phase contains peripherally associated membrane proteins and the detergent phase contains integral membrane proteins.

Proteins within CRDs can be separated from non-CRD proteins, as long as 1% Triton-X 100 is used at temperatures below 4°C. More than 99.95% of the CRD protein can be separated from non-CRD proteins following ultracentrifugation on a sucrose gradient.

Time Considerations

For alkaline carbonate extraction, urea extraction, and high-salt extraction protocols, it takes 1 hr for cell preparation and solubilization, 1 hr for centrifugation, 1 hr for SDS-PAGE, and 24 hr for immunoblotting. For Tri-

ton X-114 phase separation, it takes 3 hr for cell preparation and solubilization, 1 hr for SDS-PAGE, and 24 hr for immunoblotting.

For purification of caveolae, detergent solubilization takes 1 hr and ultracentrifugation requires 24 hr. SDS-PAGE takes one hr and immunoblotting takes another 24 hr. Results will be available within 2 days.

Literature Cited

Bickel, P.E., Scherer, P.E., Schnitzer, J.E., Oh, P., Lisanti, M.P., and Lodish, H.F. 1997. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. *J. Biol. Chem.* 272:13793-13802.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604-1607.

Brown, D.A., and London, E. 1997. Structure of detergent-resistant membrane domains: Does phase separation occur in biological membranes? *Biochem Biophys Res Commun.* 240:1-7.

Chang, W.J., Ying, Y., Rothberg, K., Hooper, N., Turner, A., Gambliel, H., De Gunzburg, J., Mumby, S., Gilman, A., and Anderson, R.G.W. 1994. Purification and characterization of smooth muscle cell caveolae. *J. Cell Biol.* 126:127-138.

Engelman, J.A., Wycoff, C.C., Yasuhara, S., Song, K.S., Okamoto, T., and Lisanti, M.P. 1997. Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J. Biol. Chem.* 272:16374-16381.

Fra, A.M., Masserini, M., Palestini, P., Sonnino, S., and Simons, K. 1995a. A photo-reactive derivative of ganglioside GM1 specifically cross-links VIP21-caveolin on the cell surface. *FEBS Lett.* 375:11-14.

Fra, A.M., Williamson, E., Simons, K., and Parton, R.G. 1995b. De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc. Natl. Acad. Sci. U.S.A.* 92:8655-8659.

Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. 1982a. Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. *J. Cell Biol.* 93:97-102.

Fujiki, Y., Fowler, S., Shio, H., Hubbard, A.L., and Lazarow, P.B. 1982b. Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: Comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* 93:103-110.

James, G. and Olson, E.N. 1989. Identification of a novel fatty acylated protein that partitions between the plasma membrane and cytosol and is deacylated in response to serum and growth factor stimulation. *J. Biol. Chem.* 264:20998-21006.

Kubler, E., Dohlman, H.G., and Lisanti, M.P. 1996. Identification of Triton-X100 insoluble membrane domains in the yeast *Saccharomyces cerevisiae*: A model system for the molecular evolution of mammalian caveolin genes. *J. Biol. Chem.* 271:32975-32980.

Li, S., Song, K.S., and Lisanti, M.P. 1996a. Expression and characterization of recombinant caveolin: Purification by poly-histidine tagging and cholesterol-dependent incorporation into defined lipid membranes. *J. Biol. Chem.* 271:568-573.

Li, S., Song, K.S., Koh, S., and Lisanti, M.P. 1996b. Baculovirus-based expression of mammalian caveolin in Sf21 insect cells: A model system for the biochemical and morphological study of caveolae biogenesis. *J. Biol. Chem.* 271:28647-28654.

Lisanti, M.P., Scherer, P.E., Vidugiriene, J., Tang, Z.-L., Hermanoski-Vosatka, A., Tu, Y.-H., Cook, R.F., and Sargiacomo, M. 1994. Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: Implications for human disease. *J. Cell Biol.* 126:111-126.

Moldovan, N.I., Heltianu, C., Simionescu, N., and Simionescu, M. 1995. Ultrastructural evidence of differential solubility in Triton X-100 of endothelial vesicles and plasma membrane. *Exp. Cell Res.* 219:309-313.

Murata, M., Peranen, J., Schreiner, R., Weiland, F., Kurzchalia, T., and Simons, K. 1995. VIP21/caveolin is a cholesterol-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 92:10339-10343.

Parolini, I., Sargiacomo, M., Lisanti, M.P., and Peschle, C. 1996. Signal transduction and GPI-linked proteins (Lyn, Lck, CD4, CD45, G proteins, CD 55) selectively localize in Triton-insoluble plasma membrane domains of human leukemic cell lines and normal granulocytes. *Blood* 87:3783-3794.

Puertollano, R., Li, S., Lisanti, M.P., and Alonso, M.A. 1997. Recombinant expression of the MAL proteolipid, a component of glycolipid-enriched membrane microdomains, induces the formation of vesicular structures in insect cells. *J. Biol. Chem.* 272:18311-18357.

Sargiacomo, M., Sudol, M., Tang, Z.-L., and Lisanti, M.P. 1993. Signal transducing molecules and GPI-linked proteins form a caveolin- rich insoluble complex in MDCK cells. *J. Cell Biol.* 122:789-807.

Scherer, P.E., Manning-Krieg, U.C., Jeno, P., Schatz, G., and Horst, M. 1992. Identification of a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane. *Proc. Natl. Acad. Sci. U.S.A.* 89:11930-11934.

Scherer, P.E., Lederkremer, G.Z., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. 1996. Cab45, a novel (Ca^{2+})-binding protein localized to the Golgi lumen. *J. Cell Biol.* 133:257-268.

Scherer, P.E., Lewis, R.Y., Volonte, D., Engelman, J.A., Galbiati, F., Couet, J., Kohtz, D.S., van

Donselaar, E., Peters, P., and Lisanti, M.P. 1997. Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. *J. Biol. Chem.* 272:29337-29346.

Schnitzer, J.E., Liu, J., and Oh, P. 1995a. Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases. *J. Biol. Chem.* 270:14399-14404.

Schnitzer, J.E., Oh, P., Jacobson, B.S., and Dvorak, A.M. 1995b. Caveolae from luminal plasmalemma of rat lung endothelium: Microdomains enriched in caveolin, $Ca2^{+}$-ATPase, and inositol triphosphate receptor. *Proc. Natl. Acad. Sci. U.S.A.* 92:1759-1763.

Schook, W., Puszkin, S., Bloom, W., Ores, C., and Kochwa, S. 1979. Mechanochemical properties of brain clathrin: Interactions with actin and alpha-actinin and polymerization into basketlike structures or filaments. *Proc Natl Acad Sci U.S.A.* 76:116-120.

Sevinsky, J.R., Rao, L.V.M., and Ruf, W. 1996. Ligand-induced protease receptor translocation into caveolae: A mechanism for regulating cell surface proteolysis of the tissue factor-dependent coagulation pathway. *J. Cell Biol.* 133:293-304.

Simons, K. and Ikonen, E. 1997. Functional rafts in cell membranes. *Nature* 387:569-572.

Smart, E., Ying, Y.-S., Conrad, P., and Anderson, R.G.W. 1994. Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. *J. Cell Biol.* 127:1185-1197.

Smart, E.J., Ying, Y., Mineo, C., and Anderson, R.G.W. 1995. A detergent free method for purifying caveolae membrane from tissue cultured cells. *Proc. Natl. Acad. Sci. U.S.A.* 92:10104-10108.

Song, K.S., Li, S., Okamoto, T., Quilliam, L., Sargiacomo, M., and Lisanti, M.P. 1996. Copurification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent free purification of caveolae membranes. *J. Biol. Chem.* 271:9690-9697.

Contributed by T. Okamoto
Cleveland Clinic Foundation
Cleveland, Ohio

R.B. Schwab, P.E. Scherer, and M.P. Lisanti
Albert Einstein College of Medicine
Bronx, New York

Determination of Molecular Size by Size-Exclusion Chromatography (Gel Filtration)

One of the most popular methods for measuring the size of proteins is size-exclusion chromatography (SEC). Gel filtration is a synonymous term for SEC, except that the former indicates an aqueous mobile phase whereas the latter is more general. The principle underlying separation is as follows: the chromatographic support, or gel, consists of particles containing pores that can be penetrated by solute molecules to a degree dependent on their molecular size. Hence solute molecules partition, as a function of size, between mobile phase contained inside and outside the support particles. When a column is packed with such a support and a mobile phase is passed through it, larger molecules emerge earlier than smaller ones because the latter have more mobile phase available to them through increased access to the interior of the beads.

SEC is widely used for separation and characterization of polymers, including biological macromolecules, especially proteins. In addition to giving information on protein size, SEC can also be used for purification, analysis of purity, and for study of protein interactions, either self-association or with other molecules. This unit is concerned with the application of SEC for investigating molecular size of cellular proteins in their native (non-denatured) state. Sample, such as tissue extract, is applied to the column and mobile phase (aqueous buffer) is pumped through the column. Protein peaks in the eluate are visualized by on-line spectrophotometric detection, and fractions are collected for further analysis by specific assays to identify the elution volume, V_e, of each protein. Using this value and two additional parameters, V_o, the void volume, and V_t, the total volume, a distribution coefficient, K_d, for each protein can be calculated. The size of each protein can be then determined provided that the SEC column has been previously calibrated with suitable protein standards. Size is most commonly expressed as Stokes radius, R_S, a parameter that is known for many proteins. Some proteins that are recommended for use as calibration standards are listed in Table 5.5.3. If additional parameters gained from sedimentation analysis are available, the molecular weight may also be calculated (see *UNIT 5.3*).

Gel filtration is the term originally applied during development of the technique in the 1950s, and continues to be widely used by biochemists, and indeed by manufacturers, for SEC in an aqueous mobile phase. Conventional gel filtration is carried out on soft gels, such as Sephacryl, in glass or plastic columns with flow controlled by peristaltic pumps or gravity, and run times on the order of several hours. Improvements in technology have led to the introduction of new supports with decreased particle size and increased efficiencies, though requiring higher operating pressures. These size-exclusion high-performance liquid chromatography (SE-HPLC) systems have enabled purification and analysis of proteins to be carried out ten to a hundred times faster than by conventional gel filtration. A medium-pressure high-performance system called fast protein liquid chromatography (FPLC) that avoids the use of stainless steel components has been developed by one major supplier (Amersham Pharmacia Biotech). Columns developed for this system can also be operated on ordinary HPLC systems. In this unit protocols are provided for SE-HPLC (Basic Protocol 1) and for conventional gel filtration (Basic Protocol 2) including calibration of columns in terms of Stokes radius, R_S, using protein standards.

Contributed by G. Brent Irvine
Current Protocols in Cell Biology (2000) 5.5.1-5.5.16

STRATEGIC PLANNING

Conventional SEC or SE-HPLC

This initial decision on whether to use a conventional or high-performance system is largely dictated by availability of equipment. Workers in laboratories with easy access to an HPLC or FPLC system and prepacked high-performance columns will benefit from much faster run times. However, if such equipment is unavailable then an empty column can be packed with gel and operated on a conventional chromatography system. This latter option clearly requires more time, not only for actual runs but also for conditioning the gel, packing the column, and checking its performance (see Basic Protocol 2). Its major advantage is that much larger volumes of sample can be processed.

Fractionation Range and Selectivity

Selection of the most appropriate prepacked column or packing material is crucial for success, since the support pore size controls the solute size range in which fractionation occurs. Different supports contain beads whose pores range in size from ~3 nm to 100 nm. Manufacturers usually specify pore size and indicate the fractionation range in terms of molecular weights of polymer types, with polypeptides and dextrans often being listed as examples. For example, Zorbax GF-250 prepacked columns, with 15-nm pore size, are stated as having a fractionation range for proteins from 4,000 to 400,000 daltons (Da). Fractionation ranges, pore size, particle size, and suppliers are listed in Table 5.5.1 for a selection of prepacked columns, and in Table 5.5.2 for a selection of packing materials. More detailed information is available on products from a wide variety of manufacturers listed in Wu (1999). Manufacturers' catalogues often show plots Kd against log molecular weight. For most globular proteins, this plot is a sigmoidal curve that is approximately linear in the middle section, where

$$K_d = a - b \log M$$

Table 5.5.1 Prepacked Columns for HPLC or FPLC[a]

Column	Pore size (nm)	Fractionation range for globular proteins (Da)	Particle size (μm)	Dimensions (diameter × length, mm)	Company[b]
Superdex 75 HR 10/30	6	3000-70,000	13	10 × 300	APB
Superdex 200 HR 10/30	13	10,000-600,000	13	10 × 300	APB
Superose 12HR 10/30	13	1000-300,000	10	10 × 300	APB
Superose 6 HR 10/30	25	5000-5,000,000	13	10 × 300	APB
TSK-G2000SW	12.5	5000-100,000	10	7.5 × 30 7.5 × 60	TH
TSK-G2000SW$_{XL}$	12.5	5000-150,000	5	7.5 × 30	TH
TSK-G3000SW	25	10,000-500,000	10	7.5 × 30 7.5 × 60	TH
TSK-G3000SW$_{XL}$	25	10,000-500,000	5	7.5 × 30	TH
TSK-G4000SW	45	20,000-7,000,000	13	7.5 × 30	TH
TSK-G4000SW$_{XL}$	45	20,000-10,000,000	8	7.5 × 30 7.5 × 60	TH
Zorbax GF-250	15	4,000-400,000	4	7.6 × 250	HP
Zorbax GF-450	30	10,000-1,000,000	6	9.4 × 250	HP

[a]FPLC compatible columns are produced mainly by APB for their own chromatography system. However, other supports, such as the TSK G series, are also available in glass columns suitable for FPLC as well as in stainless steel columns for HPLC.

[b]Abbreviations: APB, Amersham Pharmacia Biotech; HP, Hewlett Packard; TH, TosoHaas.

Table 5.5.2 Packing Materials for Conventional Chromatography

Support	Pore size (nm)	Fractionation range for globular proteins (Da)	Particle size (μm)	Supplier[a]
Sephacryl S100 HR	6.6	1000-100,000	25-75	APB
Sephacryl S200 HR	7.7	5000-250,000	25-75	APB
Sephacryl S300 HR	13	10,000-1,500,000	25-75	APB
Sephacryl S400 HR	31	20,000-8,000,000	25-75	APB
Superdex 75 prep grade		3000-70,000	24-44	APB
Superdex 200 prep grade		10,000-600,000	24-44	APB
Superose 6 prep grade	21	5000-500,000	34	APB
Toyopearl HW-50S	12.5	500-80,000	20-40	TH
Toyopearl HW-55S	50	10,000-700,000	20-40	TH
Toyopearl HW-65S	100	40,000-5,000,000	20-40	TH

[a]Abbreviations: APB, Amersham Pharmacia Biotech; TH, TosoHaas.

where a is the intercept on the ordinal axis and b is the slope.

From such plots one can estimate fractionation range (the working range lies in the linear portion, between $\sim 0.9 > K_d > 0.1$) and the selectivity. This latter parameter depends on the slope of the plot and is a function of the pore size distribution. A support with average pore size distribution in a narrow band gives high selectivity (large value of b) but a restricted separation range (the larger the value of b, the lower the range of values for M). If no estimate is available for the size of the protein under investigation, it is better to use a support with a broad fractionation range. A support of higher selectivity in a more restricted fractionation range can then be used later to give a more precise value for solute size.

Selecting a Column for Conventional SEC

For conventional SEC a suitable empty column must be chosen for packing. Dimensions are typically 1 to 1.6 cm internal diameter and 50 to 100 cm long. Suitable columns include the C and XK series from Amersham Pharmacia Biotech, available in lengths up to 100 cm. These columns can be equipped with adaptors that enable the eluant to be applied directly to the bed surface. Other accessories include packing reservoirs so that the total gel bed can be poured in a single operation. For the highest degree of reproducibility, a thermostatic jacketed column may be used to allow precise temperature control. Alternatively, the column may be operated in a cold room. This may be particularly important for samples that must be kept at low temperatures. For many purposes, however, operation at room temperature, often in the presence of antimicrobial agents is adequate. In the case of SE-HPLC the short run times (10 to 15 min) generally mean that low temperatures are not necessary.

Identifying Peaks

Identifying the elution volume of each protein peak during calibration of a column with protein standards is carried out by on-line detection, usually with a UV monitor measuring absorbance at 210 to 220 or at 280 nm. However, when a sample of cell extract is subjected to chromatography there will be many proteins present and baseline resolution between them is unlikely to occur, so that peak identification is impossible. Identification of the elution volume of the protein of interest is then dependent on collecting the eluate using a fraction collector and assaying each fraction. Possible assays include electrophoresis and immunoblotting (Chapter 6), measurement of enzyme activity, and ligand-binding.

It is important that sufficient material is present in each fraction to facilitate the assay, but otherwise fraction volume should be minimized to reduce error in estimating elution volume. For example, fraction volume of 0.05 to 0.2 ml would be appropriate for SE-HPLC columns that have V_i values of 5 to 10 ml, whereas fraction volume of 1 ml would be sufficient to reduce error in estimating V_e to ~1% for conventional columns with V_i values of ~100 ml.

BASIC PROTOCOL 1

SE-HPLC

This protocol describes separation on a prepacked column using an HPLC system. It is the protocol of choice unless large sample volumes or lack of equipment require the use of a conventional chromatography system. A prepacked column is connected to an HPLC system and is equilibrated with mobile phase. Elution volumes are then determined for standards and sample. FPLC gives similar results (see Table 5.5.1 for FPLC-compatible columns).

Materials

Mobile phase (see recipe)
Total volume (V_t) marker (see recipe)
Void volume (V_o) marker (see recipe)
Protein calibration standards (see recipe)
Solution in mobile phase of protein whose size is to be determined

0.22-µm filters (protein-compatible type for solutions containing protein)
HPLC system, comprising injector, one pump, UV-detector, and chart recorder or integrator
Prepacked SE-HPLC column (Table 5.5.1) with appropriate fractionation range (see Strategic Planning)
Guard column
10- to 100-µl syringe compatible with injector used
Filtration apparatus
Vacuum pump
Fraction collector

Chromatographic procedure

1. Prepare mobile phase and filter through a 0.22-µm filter under vacuum.

   This removes dust particles and so improves column life. It also degasses the mobile phase, which helps prevent air bubbles forming in the detector; air bubbles can give rise to spurious peaks. However, it is less crucial to degas mobile phase for SE-HPLC than for reversed phase HPLC because, unlike the latter mode, there is no mixing of aqueous and organic solvents, a process that produces bubbles.

2. Prime HPLC pump and injector with mobile phase.

3. Connect the outlet of the injector to the inlet of the guard column. Connect the outlet of the guard column to the inlet of the main column. Connect the outlet of the main column to the inlet of the detector.

   Most manufacturers sell guard columns appropriate for use with their size-exclusion columns. In order to protect the expensive size-exclusion column it is strongly recommended that a guard column be used to remove particle contaminants and protein aggregates.

4. Allow the guard column and column to equilibrate in the mobile phase at the flow rate to be used for chromatography until the absorbance at the chosen wavelength for detection is constant.

   Columns are usually stored in 0.02% sodium azide or ethanol/water mixtures to prevent bacterial growth. When changing to the mobile phase required, some manufacturers

recommend that the flow rate should not exceed half the normal maximum flow rate. The flow rate to be used depends on the column/packing combination, and manufacturers' recommendations should be consulted so that maximum pressures are not exceeded. Typical flow rates are 1 ml/min, but sharper peaks may be obtained at lower flow rates of 0.5 or even 0.1 ml/min. Run times will, of course, be correspondingly longer, and for size estimation there is little advantage to be gained in very low flow rates (see Commentary, Resolution).

Proteins can be detected at various wavelengths in the UV range. Detection at 214 nm is more sensitive than at 280 nm (Shaw, 1997).

5. Using the syringe compatible with the injector, inject 20 µl of void volume marker to determine V_o.

   Because of the high reproducibility of flow rate given by HPLC pumps, time rather than volume is measured and volumes are calculated using the flow rate.

6. Inject 20 µl of total volume marker to determine V_t.

7. Inject a solution (20 µl) of one of the protein calibration standards and determine its elution volume, V_e, from the time at which the absorbance peak is at a maximum. Repeat this procedure until all the standards have been injected.

   A chromatogram showing the separation of seven solutes during a single run on a Zorbax Bio-series GF-250 column is shown in Fig. 5.5.1. One would not normally attempt to run

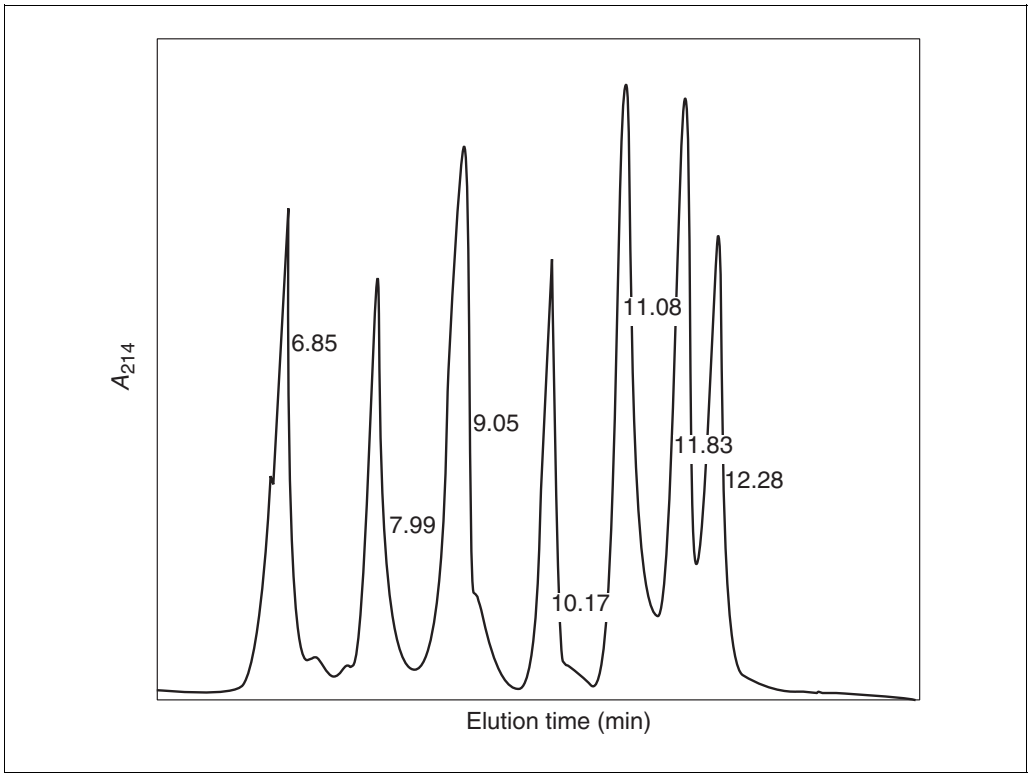


Figure 5.5.1 Separation of a mixture of seven solutes on a Zorbax Bio-series GF-250 column. 20 µl of a mixture containing ~1.5 µg of each protein was injected. The solutes were, in order of elution, thyroglobulin, alcohol dehydrogenase, ovalbumin, myoglobin, insulin, glucagon and sodium azide. The number beside each peak is the elution time in minutes. The absorbance of the highest peak, insulin, was 0.105. The equipment was a model 501 pump, a 441 absorbance detector operating at 214 nm, a 746 data module, all from Waters and a Rheodyne model 7125 injector with 20-µl loop. The column was a Zorbax Bio-Series GF-250 with guard column (Dupont). The mobile phase was 0.2 M sodium phosphate buffer, pH 7.0, and the flow rate was 1 ml/min. This figure is reproduced from Irvine (1996) with permission from Humana Press, Totowa, N.J.

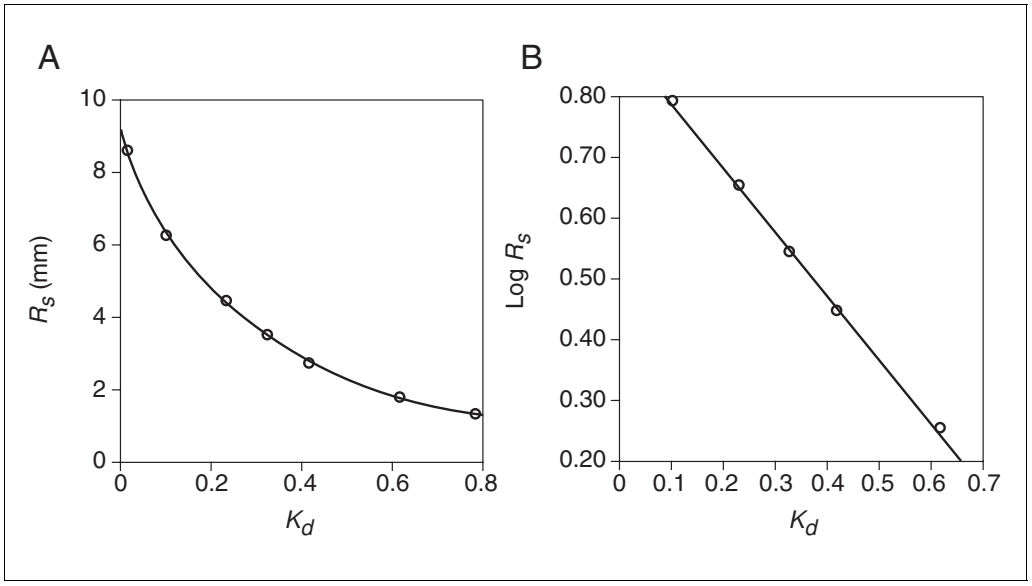


Figure 5.5.2 Plots of calibration curves for standard proteins. The data were obtained on the column and under the conditions described in Figure 5.5.1. The protein standards were thyroglobulin, apoferritin, yeast alcohol dehydrogenase, serum albumin, ovalbumin, myoglobin, and insulin. Stokes radii for these proteins are listed in Table 5.5.3 (except for alcohol dehydrogenase, with R_S = 4.55 nm). (**A**) A plot of R_S versus K_d in which all the data were used. (**B**) A plot of log R_S versus K_d. In the latter plot the top and bottom values of K_d (thyroglobulin and insulin) were omitted. V_o was determined to be 6.67 ml from the elution peak of blue dextran. V_t was determined to be 12.28 ml from the elution peak of sodium azide.

this number of standards at one time. The example does, however, show the resolution possible with SE-HPLC.

8. Inject a solution (20 µl) of the protein of unknown size and determine its elution volume, V_e, from the absorbance profile. If the sample contains more than one protein, and the peaks cannot be assigned with certainty, collect fractions and assay each fraction for the relevant activity.

   If a volume greater than 20 µl is required to facilitate assay, use this injection volume when measuring V_o and V_t and calibration standards. However, do not exceed 100 µl for a 7.5 × 300–mm column (see Commentary, Resolution).

9. If it is not to be used for several days, store the column in an appropriate antimicrobial solvent at room temperature.

Construction of calibration plots

10. Plot R_S versus K_d, and log R_S versus K_d.

    If the log plot is linear for the column used, it may be preferable to use this graph for estimating the size of the unknown protein.

    Once V_t, V_o, and the V_e values for a range of calibration standards are known for a column, it is possible to calculate K_d for each standard and construct a plot relating K_d and RS (see Background Information).

    Examples of such plots for protein standards on a Zorbax GF-250 column are shown in Figure 5.5.2.

    The R_S values for a number of proteins that have been shown to be suitable for column calibration are given in Table 5.5.3.

Table 5.5.3 Stokes Radii and Molecular Weights for Recommended Protein Standards[a]

Protein	Source	Molecular weight (Da)	Stokes radius (nm)
Cytochrome *c*	Horse heart	11,700	1.7
Ribonuclease A	Bovine pancreas	13,700	1.75
Myoglobin	Horse muscle	16,950	1.9
Trypsin inhibitor	Soybean	22,100	2.2
β-lactoglobulin	Bovine milk	35,000	2.75
Ovalbumin	Chicken egg	43,000	2.8
Alkaline phosphatase	*E. coli*	86,000	3.3
Serum albumin	Bovine serum	66,000	3.5
Transferrin	Human	81,000	3.6
Aldolase	Rabbit muscle	158,000	4.6
Catalase	Bovine liver	220,000	5.2
Aspartate transcarbamylase	*E.coli*	306,000	6.0
Apoferritin	Horse spleen	475,000	6.3
β-Galactosidase	*E.coli*	465,000	6.9
Thyroglobulin	Bovine thyroid	669,000	8.6

[a]Proteins found to be suitable for use as calibration standards as reported by Le Maire et al. (1986, 1987).

CONVENTIONAL SEC

BASIC PROTOCOL 2

This protocol describes how to pack a column and use it to carry out chromatography of proteins (see Table 5.5.2). Although acceptable results are obtained, the method is much more time consuming than using a prepacked column. However, larger sample volumes can be accommodated.

Materials

Mobile phase for conventional SEC (see recipe)
Gel filtration support (Table 5.5.2) with appropriate fractionation range (see Strategic Planning)
Colored marker (see recipe)
Total volume (V_t) marker (see recipe)
Protein calibration standards (see recipe)
Solution in mobile phase of protein whose size is to be determined

0.22-μm filters (protein-compatible type for solutions containing protein)
Buchner funnel and flask
Vacuum pump
Empty column (see Strategic Planning) with column fittings and packing reservoir
10-ml syringe
Peristaltic pump
UV-detector
Chart recorder or integrator
Filtration apparatus
Fraction collector
Sample applicator

Prepare the gel

1. Prepare mobile phase solution and filter through a 0.22-µm filter under vacuum.

   This removes dust particles and so improves column life. It also degasses the mobile phase, which helps prevent air bubbles forming in the column after it has been packed.

2a. *If the gel is supplied in dry form:* Weigh out the quantity of dry gel that will be required for the desired bed volume as calculated from the swelling factor given by the manufacturer. Add a volume of mobile phase equal to twice the final bed volume. Stir the slurry gently with a glass rod and allow the gel to swell for 24 hr.

   Stirring with a magnetic stir bar is not recommended as this can break the gel particles causing the formation of fines.

2b. *If the gel is supplied in pre-swollen form:* Wash the required quantity of swollen gel on a Buchner funnel to remove the storage buffer. Replace the storage buffer with a volume of mobile phase equal to twice the final bed volume.

3. Stir the slurry and then allow it to settle. Decant the liquid above the gel to remove fines. Replace the lost liquid with fresh mobile phase.

   Fines are damaged or smaller than average gel particles that can clog the gel bed or the nets at the column end. They can be detected as cloudiness in the top layer of liquid formed when the slurry is stirred and then allowed to stand. Since they settle more slowly than normal particles, they can be largely eliminated by repeatedly decanting.

4. Repeat step 3 until the liquid phase is clear. Adjust the volume of liquid so that the settled gel bed accounts for about half of the total volume.

Pour the column

5. Mount the column vertically (use a plumb line) on a laboratory stand.

   If the column has been used previously, make sure that it, and all fittings and nets, have been thoroughly cleaned. The column should be kept at a constant temperature and not placed in direct sunlight.

6. If the gel bed will occupy more than half the column volume, add a packing reservoir so that packing can be carried out in one operation.

   The packing reservoir is an extension that fits onto the top of the column and allows the whole gel slurry to be poured in one operation. It is required if the bed volume will be greater than half the volume of the empty column.

7. Add, via a syringe, a few milliliters of mobile phase through the outlet to flush back any air trapped in the outlet net. Close the outlet tubing.

   Ensure that the nets at the column ends are clean and have not been clogged by fines.

8. Repeat step 7 for the inlet adaptor and place it in a beaker of mobile phase to prevent air from entering.

   The adaptor is an adjustable column end piece. Columns are usually set up with one adaptor at the top and a fixed end piece at the bottom. The adaptor that will form the upper seal to the gel bed is slowly pushed down the inside of the column until it makes contact with the bed. Sample application is then much easier than to an unprotected layer of gel, where it is very easy to disturb the surface.

9. Prime the peristaltic pump with mobile phase and adjust the flow rate to the required value. Attach to the inlet of the column or packing reservoir. Stop the pump.

   Use a flow rate recommended by the manufacturer for the gel/column size combination (see, for example, Pharmacia Biotech, 1996). If the flow exceeds the recommended rate, the column bed may be compressed and flow rates will decrease.

10. Gently mix the gel slurry by swirling. In a single operation, pour the gel along a tilted glass rod into the column or reservoir.

Pack the column

11. Close the column using either reservoir lid or top adaptor, open the outlet and turn on the pump to start pumping mobile phase through the column. Check the flow rate because the back pressure from the column may alter the initial value.

12. Continue flow until the bed height is constant.

13. Stop the pump, remove packing reservoir if attached, and gently move the top adaptor flush onto the bed surface.

    Care must be exercised to keep the top of the bed layer even.

14. Resume flow for 1 hr to stabilize the gel bed height. Recheck the flow rate and readjust the adaptor flush onto the bed surface if necessary.

    For some gels the manufacturer recommends a higher flow rate at this stage than was used for packing the column. Otherwise use the same flow rate. After packing is complete there must be no space between the adaptor and gel bed as this would allow mixing to occur, especially during sample application, and this would adversely affect resolution.

Check the column

15. Inspect the column to ensure there are no cracks or air bubbles in the gel bed.

16. Inject an aliquot (~1% to 2% of the bed volume) of the colored marker using the sample applicator. Carry out chromatographic separation and determine the void volume from the blue dextran peak.

    Sample may be applied from a syringe via a valve system onto the column or into a loading loop. Sample may also be pumped directly onto the column. Sample loops have the advantage of giving reproducible sample volumes. For a well-packed column the colored marker should separate into sharp bands of color.

    V_o, or indeed the elution volume of any peak, requires accurate measurement of the volume of fractions collected. This can be done by weighing, although using a graduated cylinder may be acceptable. The elution volume will be the sum of the volumes of fractions prior to the fraction containing the peak apex, plus half the volume of that fraction.

17. Inject an aliquot of total volume marker and measure V_t.

    If it is desired to check the efficiency of the column, it is possible to calculate the number of theoretical plates from the equation

    $N = 5.54(V_e/W_h)^2$

    where N is the number of theoretical plates, V_e is the elution volume of peak, and W_h is the peak width at half the peak height.

    If efficiency is to be estimated from this step, the volume of sample loaded should not exceed 0.5% to 1% of V_t, otherwise peak width may be affected. For supports of small particle size (10-µm) use 0.5% V_t, whereas for 1000-µm particle size, 1% V_t is acceptable (Hagel, 1989). For most supports, V_t is between 80% and 95% of the bed volume.

18. Inject a solution (~1% to 2% of the bed volume) of one of the protein calibration standards and determine its elution volume, V_e, from the absorbance profile. Repeat this procedure until all the standards have been injected.

19. Inject a solution of the protein of unknown size and determine its elution volume, V_e, from the absorbance profile. If the sample contains more than one protein, and the peaks cannot be assigned with certainty, collect fractions and assay each fraction for the relevant activity (see Strategic Planning).

20. If it is not to be used for several days, store the column in an appropriate antimicrobial solvent at room temperature.

Construction of calibration plots

21. Plot R_S versus K_d, and log R_S versus K_d.

If the log plot is linear for the column used it may be preferable to use this graph for estimating the size of the unknown protein.

Once V_t, V_o, and the V_e values for a range of calibration standards are known for a column it is possible to calculate K_d for each standard and construct a plot relating K_d and R_S (see Background Information).

Examples of such plots for protein standards on a Zorbax GF-250 column are shown in Figure 5.5.2.

The R_S values for a number of proteins that have been shown to be suitable for column calibration are given in Table 5.5.3.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent for preparation of recipes. Filter all solutions through a 0.22-µm filter. Use a protein-compatible filter for sample solutions. Antimicrobial agents are often added to mobile phases, especially those in which columns are to be stored. 20% (v/v) ethanol is suitable for addition to columns and many gels for storage, but not during chromatography. Chlorhexidine (0.002% w/v) and sodium azide (0.02% w/v) are often used for the latter purpose. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CAUTION: *Sodium azide is believed to be a mutagen. It should be handled with care (see suppliers' safety advice) and measures should be taken to avoid contact with solutions. It can also lead to explosions when disposed of via lead pipes. Solutions should be collected in waste bottles.*

Colored marker

Sequentially dissolve blue dextran (average molecular weight 2,000,000 Da) at 1 mg/ml, cytochrome *c* (molecular weight of 11,900 Da) at 4 mg/ml, and potassium dichromate (formula weight of 294 Da) at 1 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*) containing 0.1 M NaCl. Filter through a 0.22-µm filter. Store for 1 week at 4°C.

For a gel of large pore size (fractionation range lower limit 10,000 Da or more) omit the cytochrome c.

Mobile phase for conventional SEC

0.1 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*) containing 0.1 M NaCl. Filter through a 0.22-µm filter. Store filtered buffer for 1 week at 4°C.

If antimicrobial agent, such as azide, has been added it can be stored for several months at 4°C.

The use of high ionic strength buffer is required to prevent ionic interactions, especially with silica-based columns (see Critical Parameters, Nonideal SEC). Sodoim chloride at 0.1 to 0.2 M is often added to SEC buffers for this purpose. It is advisable to consult the manufacturer's technical literature for advice on each individual packing material.

If antimicrobial agent is added it must be compatible with the assay or detection system used. For example, solutions of 0.02% (w/v) sodium azide have a high absorbance at low UV wavelengths, making detection at 214 nm impossible.

Mobile phase for SE-HPLC

0.2 M sodium phosphate buffer, pH 7.0. Filter through a 0.22-µm filter. Store filtered buffer for 1 week at 4°C.

If antimicrobial agent, such as azide, has been added it can be stored for several months at 4°C.

The use of high ionic strength buffer is required to prevent ionic interactions, especially with silica-based columns (see Critical Parameters, Nonideal SEC). Sodium chloride at 0.1 to 0.2 M is often added to SEC buffers for this purpose. However, if stainless steel systems are being used, it is better to avoid the use of chloride to prevent corrosion. It is advisable to consult the manufacturer's technical literature for advice on each individual column.

If antimicrobial agent is added it must be compatible with the assay or detection system used. For example, solutions of 0.02% w/v sodium azide have a high absorbance at low UV wavelengths, making detection at 214 nm impossible.

Protein calibration standards

Proteins suitable for use as standards are listed in Table 5.5.3. Dissolve each protein in the appropriate mobile phase at a concentration of ~0.5 mg/ml. Filter through a 0.22-µm filter.

Some proteins in solution can be stored for several months at −20°C, but others are unstable and must be freshly prepared. This must be determined by trial and error.

To save time, it is possible to mix two or more standards in the same run, provided the peaks are well separated and the proteins are homogeneous.

Total volume (V_t) marker

Dissolve acetone at a concentration of 5 mg/ml or sodium azide at a concentration of 10 mg/ml in the appropriate mobile phase. Filter through a 0.22-µm filter.

Water or deuterium oxide can also be used.

Detection of both acetone and azide is possible at 214 nm. Acetone can also be detected at 280 nm. Water gives a negative peak on spectrophotometric detectors. Deuterium oxide requires a refractive index detector.

Void volume (V_o) marker

Dissolve blue dextran (average molecular weight 2,000,000) at a concentration of 1 mg/ml in the appropriate mobile phase. Filter through a 0.22-µm filter. Store for 1 week at 4°C.

Blue dextran is adsorbed by some supports, for example, TSK G3000SW (Himmel and Squire, 1981). Calf thymus DNA has been reported to be a suitable alternative for supports with very large pore size (Pfannkoch et al., 1980).

COMMENTARY

Background Information

The chromatographic separation of proteins from small molecules on the basis of size was first described by Porath and Flodin (1959) who called the process "gel filtration." The column packing, or gel, was dextran cross-linked with epichlorohydrin and sold by Pharmacia under the trade name Sephadex. Moore (1964) applied a similar principle to the separation of polymers on cross-linked polystyrene gels in organic solvents, but named this "gel permeation chromatography." Although the terms gel filtration and gel permeation chromatography originally connoted aqueous and organic eluants respectively, both terms came to be used by manufacturers of such supports for the separation of peptides and proteins. Additional names, such as molecular sieve chromatography, steric exclusion, and gel chromatography have also been used, leading to some confusion. The term size exclusion chromatography (SEC) is more descriptive of the principle on which separation is based and is slowly replacing the older names. The molecular size of a solute molecule determines the degree to which it can penetrate the pores of the support. Molecules that are wholly excluded emerge from the column first, at the void volume, V_o. This represents the volume in the interstitial space (outside the support particles) and is measured by chromatography of very large molecules. Molecules that can enter the pores freely have full access to an additional space, the internal pore volume, V_i. Such molecules emerge at the total volume, V_t, which corresponds to the elution volume of molecules that are small enough to be distributed freely both inside and outside the pores and so have access to the total volume available to the mobile phase. Hence

$$V_t = V_o + V_i.$$

A solute molecule that is partially restricted from the pores will emerge with elution volume, V_e, between the two extremes, V_o and V_t. The distribution coefficient, K_d, for such a molecule represents the fraction of V_i available to it for diffusion. Hence

$$V_e = V_o + K_d V_i$$

and so

$$K_d = (V_e - V_o)/V_i$$

V_i is experimentally determined from the equation

$$V_i = V_t - V_o$$

Hence

$$K_d = (V_e - V_o)/(V_t - V_o)$$

Measurement of V_t is not trivial because many small molecules may experience ionic or hydrophobic interactions with the support (see Critical Parameters, Nonideal SEC). In addition, for supports with very small pore size, size exclusion effects may still operate even in the very-low-molecular-weight range (near a few hundred Da), restricting the possibilities for suitable markers. Deuterium oxide has been shown to be a reliable marker for V_t, but it requires a refractive index detector. In some cases it may be simpler to measure the geometric volume of the packed column bed, or bed volume, V_c. This is the sum of V_t and the solid support volume, V_s, which is not accessible to solvent. V_c (in ml) can be calculated from the equation for the volume of a cylinder, $\pi r^2 h$, where r is the radius of the column and h is the column length, both expressed in cm. The parameter K_{av} can then be used instead of K_d, where

$$K_{av} = (V_e - V_o)/(V_c - V_o)$$

Since

$$K_{av} = K_d V_i/(V_i + V_s)$$

K_{av} is directly proportional to K_d, as indeed is V_e. For comparison of results between different columns packed with the same support, K_d or K_{av} have obvious advantages since they are independent of column length.

Parameters descriptive of molecular size

Since the physical basis for discrimination in SEC is size, one would expect that there would be some parameter related to the dimensions and shape of the solute molecule that would determine its K_d value. All molecules with the same value for this parameter should have identical K_d values on a SEC column. Dimensional parameters that have been suggested for such a "universal calibration" include two terms related to the hydrodynamic properties of proteins, namely Stokes radius (R_S) and viscosity radius (R_η). In order to explain these terms it is necessary to consider briefly the hydrodynamic properties of proteins, particularly their diffusion coefficients and intrinsic viscosities. More detailed treatment can be found in Tanford (1961).

Stokes radius. Stokes law defines the frictional coefficient, f_0, for a spherical particle of radius, r_o, moving through a liquid of viscosity, η, by the following equation:

$$f_0 = 6\pi\eta r_o$$

If the frictional coefficient, f, were to be experimentally determined for a protein, using diffusion experiments or dynamic light scattering, one could make the assumption that the protein was a spherical particle and so calculate a value for its radius from Stokes equation. This is called the Stokes radius, R_S, and is the radius of the sphere that would have the frictional coefficient of the protein under consideration.

Now the radius, R, of a spherical particle is related to its volume, V, by the equation

$$V = (4/3)\pi R^3$$

and molecular volume is given by the equation

$$V = M\nu/N$$

where M is molecular weight, ν is partial specific volume and N is Avogadro's number. Hence

$$4/3\pi R^3 = M\nu/N$$

Partial specific volume can be calculated for a protein of known amino acid composition by summation of amino acid residue volumes (see *UNIT 5.3*). Hence, if the molecular weight and amino acid composition of any protein is known (say, from mass spectrometry) this enables values for M and ν, and hence its theoretical radius, R, to be calculated. Invariably we find that $R_S > R$. In other words, proteins behave like larger spheres than would be predicted from their mass and molecular volume. This is the consequence of proteins having shapes that are not perfect spheres and/or of hydration by solvent molecules, so the Stokes radius has no exact physical meaning for most proteins.

Combining the terms representing theoretical radius, R, with the Stokes equation gives a theoretical frictional coefficient, f_0, where:

$$f_0 = 6\pi\eta(3M\nu/4\pi N)^{1/3}$$

Since $R_S > R$, the ratio f/f_0 is >1; it is not possible for f/f_0 to have a value <1 since, for a given particle volume, a perfect sphere has the lowest possible diffusion coefficient. Most globular proteins are found to have f/f_0 values between 1 and 1.4, indicating neither a high degree of solvation nor asymmetry. If the f/f_0 ratio is much larger than unity this indicates that the hydrodynamic behavior of the protein is very different from that of an unsolvated sphere. For example, myosin, a protein consisting of two globular heads connected to a long coiled tail, has a value of 3.65 for f/f_0.

Viscosity radius. An alternative to the Stokes radius comes from employing hydrodynamic volumes derived from the Einstein viscosity law for spherical solute molecules

$$[\eta] = 2.5(NV_h/M)$$

where $[\eta]$ is the intrinsic viscosity of the solute molecule and V_h is its hydrodynamic volume (Nave et al., 1993).

The radius, R_η relating to this hydrodynamic volume can be calculated using the equation:

$$R_\eta = (3[\eta]M/10\pi N)^{1/3}$$

R_η is usually referred to as the viscosity radius, but is sometimes called the viscosity-based Stokes radius. This can lead to considerable confusion in the literature because many authors do not distinguish between Stokes radius derived using diffusion data from that derived using viscosity data. For nearly spherical globular proteins the two are virtually identical. However, for elongated shapes $R_\eta > R_S$, for example by ~15% for random coil polymers, and by even greater values for long fibrous molecules (Tanford et al., 1974).

Current evidence appears to indicate that R_η is more closely correlated to SEC behavior than is R_S (Horiike et al., 1983; Potschka, 1987). However, even this parameter is not suitable for a universal calibration that includes globular proteins, random coils, and rods (Dubin and Principi, 1989).

Several mathematical models that relate SEC to the geometries of protein solutes and support pores have been proposed. These models give rise to various plots that should result in a linear relationship between a function of solute radius, usually R_S, and some SEC parameter, usually K_d (Ackers, 1970). However, none has proven totally satisfactory. A more general treatment based on a Gaussian distribution of pore size was used to develop a statistical model that gave rise to the widely quoted equation:

$$R_S = a + b\ \mathrm{erfc}^{-1}K_d$$

where a and b are constants and erfc^{-1} is the inverse error function complement (available from statistical tables; Ackers, 1970).

This equation continues to be used by many workers, but it has been criticized on theoretical and experimental grounds (Nozaki et al., 1976; Le Maire et al., 1987). Le Maire et al. (1987) advocate plotting K_d directly against R_S. Such a plot is non-linear and sufficient data points must be included to adequately cover the separation range. However, for some supports, though not for others, a plot of ln R_S versus K_d gives a straight line (Le Maire et al., 1989). Examples of both a direct plot and a log plot are shown in Figure 5.5.2.

For random coiled polymers, molecular weight varies in a predictable manner with Stokes radius. For reduced proteins in a denaturing solvent, such as guanidinium hydrochloride, it is thus possible to relate SEC behavior directly to molecular weight (Fish et al., 1970). Usually K_d versus log molecular weight is plotted, giving a sigmoidal calibration curve with an approximately linear portion between K_d = 0.1 and K_d = 0.8. Since the K_d is proportional to K_{av} or V_e, plots of these parameters against log molecular weight are also used. For native proteins, however, since there is no direct relationship between molecular weight and R_S, calculation of molecular weight from SEC data requires knowledge of the sedimentation coefficient. Stokes radii of standard proteins and their K_d values are used to produce a calibration curve (see above) from which the Stokes radius of the unknown can be determined. Since the product of sedimentation coefficient and Stokes radius is related to molecular weight, as described in *UNIT 5.3*, the equation on page 5.3.27 can be used to calculate accurate molecular weights for proteins of known sedimentation coefficient.

Critical Parameters

Nonideal SEC

Ideal SEC separates solutes only on the basis of size. This will only occur when the support is neutral and of similar polarity to the mobile phase. If the support is more hydrophobic than

the mobile phase, then hydrophobic interactions with solute molecules will delay their elution. If there are charges present on the support, then ion-exchange can occur with oppositely charged solute molecules, delaying their elution. In extreme cases, solute molecules can emerge well after V_t. Solute molecules with the same charge as that of the support will experience electrostatic repulsion from the pores, referred to as "ion exclusion," and emerge earlier than expected on the basis of size. Silica to which a hydrophilic phase such as a diol has been bonded still contains underivatized silanol groups. Above pH 3 these are largely anionic and will interact with ionic solutes. Depending on the value of its isoelectric point, a protein can be cationic or anionic at pH 7. Proteins that are positively charged will undergo ion exchange, causing them to be retarded. Conversely, anionic proteins will experience ion exclusion, and will be eluted earlier than expected on the basis of size alone (Pfannkoch et al., 1980; Irvine and Shaw, 1986). Silica-based packings give rise to a greater or lesser degree to nonideal SEC. The nonsilica based supports, such as Superose, with a lower surface density of ionizable groups and low hydrophobicity, might be expected to produce more nearly ideal SEC (Dubin and Principi, 1989). In order to reduce ionic interactions with either type of support, it is necessary to use a mobile phase of high ionic strength. On the other hand, an increase in ionic strength promotes the formation of hydrophobic interactions. To minimize both ionic and hydrophobic interactions, the mobile phase should have an ionic strength between 0.2 and 0.5 M (Regnier, 1983).

Detection

By far the most common technique applied for the detection of polypeptides is spectrophotometry. Peptide bonds absorb UV light with a maximum ~187 nm, but wavelengths below 200 nm are seldom used because of interference from buffer components; in practice 210, 214, or 220 nm are the most frequently reported wavelengths. In mobile phases that absorb strongly in the far UV range, monitoring the absorbance in the most sensitive region for proteins (200 to 220 nm) is no longer possible, and a longer wavelength, such as 280 nm, must be used. Only proteins that contain tyrosine or tryptophan will absorb light at 280 nm. With a 280-nm detector set at ten times the sensitivity of a 214-nm detector, a single tyrosyl residue produces a similar deflection at 280 nm to five peptide bonds at 214 nm; a single tryptophanyl residue produces a similar deflection to fifteen peptide bonds (Shaw, 1997).

Solubilization of membrane proteins is often carried out in nonionic detergent. However, some of the commonly used detergents, such as Triton X-100, contain aromatic groups that absorb in the UV range and so interfere with detection at 280 nm. This difficulty can be avoided by using detergents such as octylglucoside or the alkyl polyethers for solubilization.

On-line light scattering detectors give direct information on molecular weight that is independent of elution position and have been applied to detection of proteins and their aggregates (Wen et al., 1996). Electrospray ionization mass spectrometry (EI-MS) requires the use of volatile solvents, and so its application to SEC of native proteins has been restricted due to prevalence of mobile phases of high ionic strength.

Resolution

Chromatographic modes such as ion exchange or reversed phase require a change in mobile phase composition during the run. In such gradient-based modes, volumes that greatly exceed the geometric column volume, V_c, are often used. SEC operates by isocratic elution, i.e., with a mobile phase of fixed composition. This has the advantage that there is no need to re-equilibrate the column between runs, so that the next sample can be injected immediately after a volume of eluant equal to V_t has passed, assuming that ideal SEC is operating. However, from consideration of the equation $V_e = V_o + K_d V_i$, it is clear that in SEC all separations must occur within a relatively small volume equal to the pore volume, V_i. For the most frequently used HPLC column sizes, this value ranges from ~5 ml (for columns of dimensions 7.5×300 mm) to 12 ml (for columns of dimensions 10×300–mm). All the components of the mixture to be separated must be resolved within this volume. The practical peak capacity, n (the maximum number of peaks that can be separated on a given column), may be estimated from the equation

$$n \approx 1 + (V_i / V_t) N^{1/2} / 4 R_{es}$$

in terms of pore fraction, V_i/V_t, plate number, N, and resolution, R_{es} (Hagel, 1992). From this equation it can be estimated that practical peak capacities for SEC columns will be ~13 completely resolved peaks on well-packed columns. Hagel (1989) has discussed several parameters than can be altered in order to increase

resolution. These include maximizing pore volume, number of theoretical plates, column length, permeability (V_i/V_o), and decreasing particle size and flow rate. Although lower flow rates generally lead to an increase in resolution, this is not usually a critical factor in determining protein size, so that in practice relatively high flow rates can be used. Typical flow rates are 0.5 to 1.0 ml/min for a 7.5 × 300–mm prepacked SE-HPLC column to 5 to 40 cm/hr, depending on the support, for a conventional SEC column.

Loading is a function of volume and concentration of sample applied, and both of these will affect resolution (Regnier, 1983). Solutions containing high sample concentrations can lead to increased viscosity and hence decreased diffusion coefficient of solutes, leading to decrease in resolution. This is more of a problem for preparative than analytical SEC, where protein concentrations are usually <1 mg/ml. For maximum resolution the volume in which sample is dissolved should be <0.5% of the column volume (~50 µl for a 7.5 × 300–mm column with 10-µm particles; Hagel, 1989). In general, however, achieving baseline resolution in SEC is less critical for estimating protein size than for other applications, such as purification.

Troubleshooting

In SE-HPLC especially, but also in conventional SEC, it is most important to filter solutions to prevent clogging of the column with insoluble material. The use of a guard column is very beneficial in this respect. It needs to be replaced periodically, as soon as any increase in the usual operating pressure is observed.

In conventional SEC, decreased flow rates can result from clogging of column ends with fines. Reversing the flow may help, but otherwise the column will need repacking. Decreased flow can also arise because the column bed may have become compressed, usually due to flow rates that are too high. When repacking a column, nets need cleaning and the gel should be treated to remove fines (see Basic Protocol 2, step 3).

Spurious peaks in the UV detector are often due to air bubbles forming as eluate emerges at the low pressure end of the column. Degassing the mobile phase will overcome this problem.

Anticipated Results

Running standard proteins gives a very good indication that the technique is working normally, since the calibration curve should be smooth if R_S is plotted against K_d (see Fig 5.5.2A) or approximately linear if log R_S is plotted against K_d (Fig. 5.5.2B). If a standard runs abnormally, nonideal SEC may be operating (see Critical Parameters). In such a case, changes in the ionic strength or pH of the mobile phase should markedly affect the K_d value obtained.

Time Considerations

For SE-HPLC equilibrating the column may take ~30 to 6 min. Each run takes ~15 min. For conventional SEC packing, swelling the gel takes a day and packing the column takes several hours. Each run takes 5 to 10 hr, depending on flow rate used.

Literature Cited

Ackers, G.K. 1970. Analytical gel chromatography of proteins. *Adv. Protein Chem.* 24:343-446.

Dubin, P.L and Principi, J.M. 1989. Failure of universal calibration for size exclusion chromatography of rodlike macromolecules versus random coils and globular proteins. *Macromolecule*s 22:1891-1896.

Fish, W.W., Reynolds, J.A., and Tanford, C. 1970. Gel chromatography of proteins in denaturing solvents. *J. Biol. Chem*. 245:5166-5168.

Hagel, L. 1989. Gel filtration. *In* Protein Purification: Principles, High Resolution Methods and Applications (J.C. Janson and L. Ryden, eds.) pp. 63-106. VCH Publishers, New York.

Hagel, L. 1992. Peak capacity of columns for size-exclusion chromatography. *J. Chromatogr.* 591:47-54.

Himmel, M.E. and Squire, P.G. 1981. High pressure gel permeation chromatography of native proteins on TSK-SW columns. *Int. J. Pept. Protein Res.* 17:365-373.

Horiike, K., Tojo, H., Yamano, T., and Nozaki, M. 1983. Interpretation of Stokes radius of macromolecules determined by gel filtration chromatography. *J. Biochem*. 93:99-106.

Irvine, G.B. 1996. Molecular weight estimation for native proteins using size-exclusion high-performance chromatography. *In* The Protein Protocols Handbook (J.M. Walker, ed.) pp. 473-478. Humana Press, Totowa, N.J.

Irvine, G.B and Shaw, C. 1986. High performance gel permeation chromatography of proteins and peptides on columns of TSK-G2000-SW and TSK-G3000-SW. *Anal. Biochem.* 155:141-148.

Le Maire, M., Aggerbeck, L.P., Monteilhet, C., Andersen, J.P., and Moller, J.V. 1986. The use of high-performance liquid chromatography for the determination of size and molecular weight of proteins: A caution and a list of membrane proteins suitable as standards. *Anal. Biochem.* 154:525-535.

Le Maire, M., Ghazi, A., Moller, J.V., and Aggerbeck, L.P. 1987. The use of gel chromatography for the determination of sizes and relative mo-

lecular masses of proteins. *Biochem. J.* 243:399-404.

Le Maire, M., Ghazi, A., Martin, M., and Brochard, F. 1989. Calibration curves for size-exclusion chromatography: Description of HPLC gels in terms of porous fractals. *J. Biochem.* 106:814-817.

Moore, J.C. 1964. Gel permeation chromatography. I. A new method for molecular weight distribution of high polymers. *J. Polymer Sci.* A2:835-843.

Nave, R., Weber, K., and Potschka, M. 1993. Universal calibration of size-exclusion chromatography for proteins in guanidinium hydrochloride including the high-molecular-mass proteins titin and nebulin. *J. Chromatogr.* 654:229-246.

Nozaki, Y., Schechter, N.M., Reynolds, J.A., and Tanford, C. 1976. Use of gel chromatography for the determination of the Stokes radii of proteins in the presence and absence of detergents. A reexamination. *Biochemistry* 15:3884-3890.

Pfannkoch, E., Lu, K.C., Regnier, F.E., and Barth, H.G. 1980. Characterization of some commercial high performance size-exclusion chromatography columns for water-soluble polymers. *J. Chromatogr. Sci.* 18:430-441.

Pharmacia Biotech. 1996. Gel Filtration Principles and Methods, 6th ed. Lund, Sweden.

Porath, J. and Flodin, P. 1959. Gel filtration: A method for desalting and group separation. *Nature* 183:1657-1659.

Potschka, M. 1987. Universal calibration of gel permeation chromatography and determination of molecular shape in solution. *Anal. Biochem.* 162:47-64.

Regnier, F.E. 1983. High-performance liquid chromatography of proteins. *Methods Enzymol.* 91:137-190.

Shaw, C. 1997. Purification of extracted peptides for structural analysis. *In* Neuropeptide Protocols; Methods in Molecular Biology, Vol. 73 (G.B. Irvine and C.H. Williams, eds.) pp. 9-16. Humana Press, Totowa, N.J.

Tanford, C. 1961. Physical Chemistry of Macromolecules. John Wiley & Sons, New York.

Tanford, C., Nozaki, Y., Reynolds, J.A., and Makino, S. 1974. Molecular characterization of proteins in detergent solutions. *Biochemistry* 13:2369-2374.

Wen, J., Arakawa, T., and Philo, J.S. 1996. Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. *Anal. Biochem.* 240:155-166.

Wu, C.-S. 1999. Column Handbook for Size Exclusion Chromatography. Academic Press, San Diego.

Key References

Ackers, 1970. See above.

A detailed and rigorous treatment of theory.

Hagel, 1989. See above.

A comprehensive review with emphasis on protein SEC.

Pharmacia Biotech. 1996. See above.

Of most use for the manufacturer's own products, but has much good general practical advice on packing columns and is illustrated with many diagrams and photographs.

Contributed by G. Brent Irvine
Queen's University Belfast
Medical Biology Centre
Belfast, U.K.

Identification of Proteins in Complex Mixtures Using Liquid Chromatography and Mass Spectrometry

Most cellular processes are executed and regulated by multiple proteins that act as part of a complex mixture. The identification of protein interactions in complex mixtures can provide fundamental information about the cellular processes being studied. Attempts to study protein interactions have traditionally focused on the isolation and identification of individual proteins. Understanding the role of a protein in a complex mixture, however, may depend not only on identifying an individual protein but also on identifying other proteins in the mixture and the effect of their association on the cellular system. Protein mixtures can be composed of proteins acting as tightly bound complexes, or they may include loosely bound proteins whose activity depends on self-assembly into a larger protein structure. Additionally, protein mixtures may include proteins that interact in a transient association, including protein-modifying enzymes such as proteases and kinases. This unit describes the current state of technologies available for directly identifying proteins in complex mixtures using liquid chromatography (LC) and mass spectrometry (MS). Two basic approaches are typically used. In one, proteins are separated by polyacrylamide gel electrophoresis (PAGE), isolated, digested, and analyzed by MS. In the second, LC and MS are directly coupled, allowing identification of proteins in complex mixtures without prior separation. For a detailed discussion of these approaches, see Background Information.

This unit focuses on the direct LC-MS methods. Samples are prepared from mixtures of proteins by proteolytic digestion without separation (see Basic Protocol 1). The sample is loaded onto a microcapillary column (see Basic Protocol 2) and analyzed on-line using a combined method of LC and electrospray–ionization tandem mass spectrometry (ESI-MS/MS; see Basic Protocol 3). Additional protocols are given for data analysis (see Basic Protocol 4) and pulling and packing the microcapillary column (see Support Protocol). Finally, to allow MS analysis of proteins separated by PAGE, a protocol is given for preparing digested proteins in slices from one- or two-dimensional polyacrylamide gels (see Alternate Protocol).

NOTE: To avoid contamination of the proteins, wear gloves while working with the protein sample and avoid exposing the sample to dust.

SAMPLE PREPARATION FOR DIRECT ANALYSIS OF PEPTIDES IN MIXTURES

BASIC PROTOCOL 1

This protocol describes the preparation of samples to be analyzed directly in a one- or two-dimensional liquid chromatography–tandem mass spectrometry (LC-MS/MS) experiment, and it is based on the method described by Link et al. (1999). The protocol is described for a sample containing 1 mg of protein, although samples with as little as 100 µg can be routinely analyzed. The insoluble fraction is treated with cyanogen bromide to digest proteins into long peptides which can be further digested by Lys-C and trypsin. Formic acid is used to increase solubility.

Contributed by Claire Delahunty and John R. Yates III
Current Protocols in Cell Biology (2003) 5.6.1-5.6.17

Materials

Total cell lysates, insoluble fractions or membrane proteins, soluble fractions, or unknown sample
Cyanogen bromide (e.g., Aldrich), for insoluble fractions or membrane proteins only
96% (v/v) formic acid (e.g., Aldrich), for insoluble fractions or membrane proteins only
Ammonium bicarbonate (e.g., J.T. Baker), for insoluble fractions only
100 mM Tris, pH 8.5/8 M urea (e.g., Sigma)
1 M Tris (2-carboxyethyl)phosphine hydrochloride (TCEP; e.g., Pierce)/100 mM ammonium bicarbonate
500 mM iodoacetamide
Endoproteinase Lys-C (Roche Diagnostics)
100 mM $CaCl_2$ (e.g., Mallinckrodt Baker; *APPENDIX 2A*)
Porozyme bulk immobilized trypsin beads (Applied Biosystems)
88% (v/v) formic acid (e.g., Aldrich)

Additional reagents and equipment for trichloroacetic acid (TCA) precipitation (*UNIT 7.1*)

CAUTION: Cyanogen bromide is highly toxic and volatile; carry out all work with this compound in a well-ventilated fume hood. TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions

Prepare sample fractions (total cell lysates or unknown samples only)

1. Place a total cell lysate or unknown sample containing ~1 to 5 mg protein in a 1.5-ml microcentrifuge tube and separate the soluble and insoluble fractions by microcentrifuging 30 min at 4000 rpm. Transfer the soluble fraction to a clean 1.5-ml microcentrifuge tube and proceed to step 2. Keep the insoluble fraction in the original microcentrifuge tube and pretreat with cyanogen bromide (steps 3 to 5).
2. Precipitate a volume of the soluble fraction equivalent to 500 µg protein using TCA (*UNIT 7.1*). Proceed to step 6.

   Starting amounts can be 100 µg to 2 mg. Adjust reagents accordingly.

Pretreat with cyanogen bromide (insoluble fractions or membrane proteins only)

3. Prepare a solution of 500 mg/ml cyanogen bromide in 96% formic acid in a chemical fume hood.
4. Add 100 µl of the cyanogen bromide solution to the insoluble pellet and incubate overnight at room temperature in the dark.
5. Add 100 µl water and carefully adjust the pH to 8 to 8.5 using solid ammonium bicarbonate. Reduce volume almost to dryness in a Speedvac evaporator. Proceed to step 6.

Reduce and carboxyamidate proteins

6. Resuspend sample (step 2 or step 5) in 30 µl of 100 mM Tris·Cl, pH 8.5/8 M urea.
7. Add 0.15 µl of 1 M TCEP (5 mM final concentration) and incubate 10 min at room temperature.
8. Add 1.2 µl of 500 mM iodoacetamide (20 mM final concentration) and incubate 20 min in the dark.

Perform enzyme digestion

9. Add 5 µl of 1.0 µg/µl endoproteinase Lys-C and incubate 4 to 8 hr at 37°C in the dark.

   A 1:100 ratio of enzyme to sample concentration is used.

10. Add 82.5 µl water to dilute solution to 2 M.

11. Add 2.4 µl of 100 mM $CaCl_2$ (2 mM final concentration) and 10 µl Porozyme bulk immobilized trypsin beads. Incubate with shaking 12 hr at 37°C in the dark.

12. Quench by adding 5.8 µl of 88% formic acid and microcentrifuge the samples 10 min at 14,000 rpm (maximum speed). Transfer supernatant to a clean 1.5-ml microcentrifuge tube and discard the pellet.

    The sample is now ready to be loaded onto the LC column (see Basic Protocol 2). It should be loaded within a few hours, or can be stored indefinitely at –80°C.

SAMPLE PREPARATION BY IN-GEL DIGESTION OF SILVER- OR COOMASSIE-STAINED SPOTS FOLLOWING PAGE

ALTERNATE PROTOCOL

This protocol describes the technique used to isolate peptides from a protein purified by one- or two-dimensional PAGE, and is based on the method by Jensen et al. (1999). Gel slices of widely varying acrylamide content (≤18%) and size can be processed in this way. A gel spot of interest is excised from the polyacrylamide gel and subjected to destaining. The gel piece is then dried and rehydrated with a reducing and alkylating procedure. The alkylated proteins are digested to peptides in-gel, extracted, lyophilized, and resuspended in 5% formic acid. Samples are then ready for analysis.

Materials

Coomassie- or silver-stained (*UNIT 6.6*) one- or two-dimensional acrylamide gel (*UNITS 6.1 & 6.4*) containing proteins of interest
30 mM potassium ferricyanide
100 mM sodium thiosulfate
Acetonitrile (e.g., Fisher), HPLC grade
25 and 100 mM ammonium bicarbonate (J.T. Baker)
10 mM Tris(2-carboxyethyl)phosphine (TCEP; e.g., Pierce)/100 mM ammonium bicarbonate
Iodoacetamide/ammonium bicarbonate solution (see recipe)
Trypsin digestion solution (see recipe), 4°C
50 mM ammonium bicarbonate/5 mM $CaCl_2$ (e.g., Mallinckrodt Baker)
5% (v/v) formic acid (e.g., Aldrich)

Glass plate, cleaned with methanol or ethanol
Scalpel or razor blades, cleaned with methanol or ethanol
Gel-loading pipet tips

Excise protein band

1. Wash a Coomassie- or silver-stained polyacrylamide gel containing proteins of interest two times with water for 10 min each.

2. Transfer gel to a glass plate and use a scalpel or razor blade cleaned with methanol or ethanol to excise the band of interest. Cut as close to the protein as possible to reduce the amount of background gel. Excise a second gel piece of approximately the same size from a nonprotein region to use as a control.

   The gel should be kept moist with water while the bands are excised.

3. Cut the gel piece into 1 × 1–mm squares (gel thickness will vary). If the gel stain is dark, place two to three 1 × 1–mm squares into a 1.5-ml microcentrifuge tube. If it is faint, use up to ten pieces.

   Volumes in subsequent steps are based on a starting gel volume of ~100 µl and should be scaled as appropriate.

4. Microcentrifuge 10 sec at 14,000 rpm (maximum speed) so that the gel pieces settle to the bottom of the tube.

Destain gel

5. *For silver stain only:* Prepare a fresh 1:1 (v/v) mixture of 30 mM potassium ferricyanide/100 mM sodium thiosulfate. Cover silver-stained gel cubes with 100 µl of this solution and incubate 5 min at room temperature. Discard liquid and wash gel pieces three times with 100 µl water to quench the reaction. Discard water and proceed to step 6.
6. Wash gel pieces (silver-stained from step 5 or Coomassie-stained from step 4) with 2 vol (200 µl) water for 15 min. Shake well or vortex lightly.
7. Remove water with a gel-loading pipet tip, being very careful not to remove any portion of the gel.
8. Add 100 µl water followed by 100 µl acetonitrile and wash gel pieces 15 min.
9. Remove liquid and add 100 µl acetonitrile to cover the gel pieces. Tap tube with fingers to mix.
10. Allow the pieces to shrink (they will turn sticky white) and then remove the acetonitrile.
11. Rehydrate with 100 µl of 100 mM ammonium bicarbonate for 5 min.
12. Add 100 µl acetonitrile and incubate 15 min. If a strong blue color persists, incubate again for 15 min with constant vortexing.
13. Remove liquid and dry ~15 min in a Speedvac evaporator. If blue color persists, repeat wash starting with step 9.

Reduce and alkylate proteins

14. Rehydrate gel pieces with 100 µl of 10 mM TCEP/100 mM ammonium bicarbonate to cover the gel. Tap tube with fingers to mix and incubate 20 to 30 min at room temperature.
15. Remove liquid and quickly add 100 µl freshly prepared iodoacetamide/ammonium bicarbonate solution. Incubate 30 min at room temperature in the dark.
16. Remove liquid and add 100 µl acetonitrile to cover gel pieces.
17. Allow the pieces to shrink (again, they will turn sticky white) and remove acetonitrile.
18. Add 100 µl of 100 mM ammonium bicarbonate and rehydrate 5 min.
19. Add 100 µl acetonitrile and incubate 15 min.

    All the Coomassie stain should be removed at this point. If a blue color still persists, steps 9 to 19 should be repeated.

20. Remove liquid and dry down completely in the Speedvac evaporator (≥30 min).

    The actual drying time will depend on the original volume of gel.

Perform in-gel digestion

21. Add 50 µl prechilled (4°C) trypsin digestion solution to just cover the gel pieces and incubate 45 min on ice. Check gel pieces every 15 min and add more digestion solution if they are not covered.
22. Remove supernatant and add 50 µl of 50 mM ammonium bicarbonate/5 mM $CaCl_2$. Incubate overnight at 37°C.

Extract peptides

23. Microcentrifuge tubes for ~30 sec at maximum speed, transfer buffer to a 1.5-ml microcentrifuge tube, and keep gel pieces.
24. Add 100 µl of 25 mM ammonium bicarbonate to cover the gel pieces and incubate 15 min at room temperature.

    The volume should not exceed 100 µl.

25. Add 100 µl acetonitrile and incubate 15 min at room temperature while vortexing. Transfer supernatant to the 1.5-ml tube in step 23 and keep gel pieces.
26. Add 100 µl of 5% formic acid to the gel pieces and incubate 15 min at room temperature.
27. Add 100 µl acetonitrile and incubate 15 min at room temperature while vortexing. Transfer supernatant to the 1.5-ml tube in step 23 and keep gel pieces.
28. Repeat steps 26 to 27, but discard gel pieces.
29. Dry sample in the Speedvac evaporator for ~2 hr.
30. Resuspend peptides in 10 to 30 µl of 5% formic acid.

    The peptides can be stored indefinitely at −80°C before being analyzed (see Basic Protocol 3).

LOADING A PROTEIN SAMPLE FOR MICROCAPILLARY COLUMN LIQUID CHROMATOGRAPHY

BASIC PROTOCOL 2

The digested protein sample is loaded onto the column in a method similar to column packing (see Support Protocol).

Materials

Digested protein sample (see Basic Protocol 1 or Alternate Protocol)
Pre-equilibrated microcapillary column (see Support Protocol)
High-pressure bomb designed for pneumatically loading microcapillaries (see Dongré et al., 1997) connected to helium gas tank

1. Place microcentrifuge tube containing acidified peptides in the center of a high-pressure bomb connected to a helium gas tank.
2. Close the bomb lid and tighten the screws.
3. Insert a pre-equilibrated microcapillary column into the bomb (point up) until the flat end of the column is immersed in the sample and is touching the bottom of the tube. Tighten the screw that holds the microcapillary.
4. Turn the helium gas tank on at 300 to 700 psi to pressurize the bomb and thus load the sample.

Loading progress can be monitored by measuring the solvent displaced from the column. The volume of the eluent solvent should be equal to the volume of the sample being loaded.

CAUTION: *The bomb is under high pressure. Make sure a safe and pressure-tested device is used.*

5. After loading is complete, slowly depressurize the bomb to avoid disrupting the packing material, and securely grasp the column while opening the bomb.

Sample is now loaded onto the strong cation exchange portion of the column and is ready for analysis (see Basic Protocol 3). The loaded column should be used immediately. If not, the column should be stored tip down in buffer A (see Basic Protocol 3) and used as soon as possible. Column tips are very fragile.

SUPPORT PROTOCOL

PREPARING MICROCAPILLARY COLUMNS FOR LIQUID CHROMATOGRAPHY

Microcapillary columns (Kennedy and Jorgenson, 1989) for one- or two-dimensional LC-MS/MS are created from fused-silica microcapillaries by using a laser puller. The laser puller is critical for creating capillary columns, and attempts to manually pull the capillaries can give unsatisfactory and irreproducible results. The pulled capillary (column) is then packed using a protocol that is similar for single and biphasic columns.

Additional materials *(also see Basic Protocol 2)*

Methanol (e.g., Fisher), HPLC grade
C_{18} packing material
Partisphere strong cation exchange (SCX; Whatman; for biphasic columns only)
Buffer A: 5% (v/v) acetonitrile/0.1% (v/v) formic acid

Laser puller (model P-2000; Sutter Instruments)
Ceramic scribe
100 × 365–µm (i.d. × o.d.) fused-silica capillary (J&W Scientific)
Alcohol burner
PEEK microcross splitter (Upchurch)
Quaternary high-performance liquid chromatography (HPLC) pump

Pull microcapillary column

1. Set the program of a laser puller for three cycles as follows:

Heat	270°C
Filament	0
Velocity	30
Delay	128 msec
Pull	0.

These values are specifically for a 100 × 365–µm microcapillary in the Sutter Instruments laser puller. They are unitless (except the delay) and are preset in this instrument. If another puller is used, the values will have to be determined empirically.

2. Use a ceramic scribe to cut a 50- to 54-cm length of 100 × 365–µm fused-silica capillary from the bulk. Use an alcohol burner to burn off the coating covering ~3 cm in the center part of the microcapillary. Clean the capillary using a methanol-soaked lint-free tissue.

3. Place the microcapillary in the puller, run the program and form two pulled columns. Store the pulled columns at room temperature, free of dust, with special care to protect tip.

The column tip will have an internal diameter of ~2 to 5 μm and each column will be ~25 to 27 cm long.

Pack column

4. Measure ~500 μg C_{18} packing material into a 1.5-ml microcentrifuge tube and suspend it in 1 ml methanol.
5. Place the microcentrifuge tube in the center of a high-pressure bomb connected to a helium gas tank. Position the lid and make sure all screws are secure.
6. Insert the column (step 3) into the bomb (point up) until the flat end of the column is immersed in the packing material. Gently tap the end of the column against the bottom of the tube and then raise it ~0.5 cm from the bottom. Tighten the screw that holds the microcapillary.
7. Turn the helium on at 300 to 700 psi and fill column to a length of 7 to 10 cm.

   Packing material should be seen moving up the microcapillary. As loading progresses, methanol accumulating at the tip of the column can be removed with a lint-free tissue. If the packing material slows too much before the desired packing length is achieved, the capillary can be loosened and tapped gently on the bottom of the tube to reactivate the process.

   CAUTION: *The bomb is under high pressure. Make sure a safe and pressure-tested device is used.*

8. Slowly depressurize the bomb to avoid disrupting the packing material and securely grasp the column while opening the bomb.

9a. *For a one-dimensional reversed-phase column:* Remove the column from the bomb and store in methanol up to 1 week at 4°C until pre-equilibration.

9b. *For a two-dimensional (biphasic) reversed-phase column:* Remove the column from the bomb and replace the microcentrifuge tube with a second tube containing 500 μg Partisphere SCX in 1 ml methanol. Repeat column packing as described (steps 6 to 8). Store packed column in methanol up to 1 week at 4°C until pre-equilibration.

   Because the SCX resin remains suspended in the methanol slurry, tapping the column on the bottom of the tube is not always necessary.

Pre-equilibrate column

10. Place the column in a PEEK microcross splitter.

    The splitter is used to interface the microcapillary column with the mass spectrometer.

11. Place a quaternary HPLC pump in-line with the splitter and equilibrate the microcapillary column for 30 min using buffer A at a flow rate of 100 μl/min.
12. Store equilibrated column up to 24 hr at 4°C or room temperature.

MULTIDIMENSIONAL PROTEIN IDENTIFICATION TECHNOLOGY FOR ANALYZING COMPLEX MIXTURES

BASIC PROTOCOL 3

Combining liquid chromatography (LC) with electrospray ionization–tandem mass spectrometry (ESI-MS/MS) allows on-line analysis of complex protein mixtures. This protocol describes separation by multidimensional protein identification technology (MudPIT). In this technique, a pulled microcapillary column is packed with two independent chromatography phases. In the first dimension, peptides are separated by strong cation exchange and are eluted into the second phase by a step gradient of increasing salt concentration. The second phase consists of reversed-phase material, which separates the

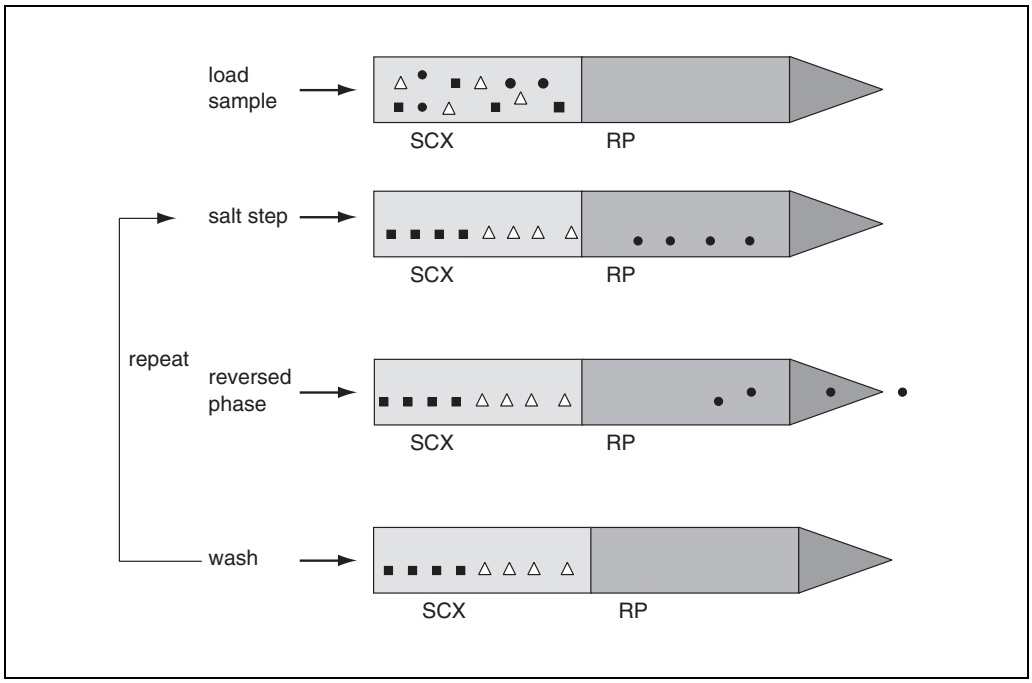


Figure 5.6.1 Integrated two-dimensional liquid chromatography. Peptides are separated in the first dimension by strong cation exchange (SCX) followed by reversed-phase (RP) separation and elution into the mass spectrometer. Triangles, circles, and squares represent different peptides.

peptides on the basis of hydrophobicity before they are eluted directly into the mass spectrometer (Fig. 5.6.1). The method described is for a six-step multidimensional separation. For highly complex or concentrated samples, twelve-step programs are frequently used and simply require smaller increments in the proportion of buffer C used in step 5. If one-dimensional separation is used, the salt steps in the chromatography are eliminated. For two-dimensional LC, a quaternary HPLC pump is required to deliver the three different buffers necessary for two-dimensional separations.

Materials

Microcapillary column loaded with protein sample (see Basic Protocol 2)
Buffer A: 5% (v/v) acetonitrile/0.1% (v/v) formic acid
Buffer B: 80% (v/v) acetonitrile/0.1% (v/v) formic acid
Buffer C: 500 mM ammonium acetate (e.g., Sigma)/5% (v/v) acetonitrile/0.1% (v/v) formic acid

PEEK microcross splitter (Upchurch)
LCQ ion trap mass spectrometer (ThermoFinnigan) including custom platform for nanoelectrospray, or equivalent tandem mass spectrometer
XYZ translational positioner (Newport)
HPLC system, including quaternary HPLC pump (e.g., Agilent, ThermoFinnigan, Waters; for two-dimensional LC only) and nitrogen gas source

Set up HPLC and MS system

1. Place a microcapillary column loaded with a protein sample in a PEEK microcross splitter and mount it on the custom platform of an LCQ ion trap mass spectrometer.

   The platform replaces the original electrospray source and is described in Yates et al. (2000). Plans for the platform may be available from the author by a material transfer agreement.

2. Align the column with the mass spectrometer orifice using an XYZ translational positioner.

The column should be directly in line with the mass spectrometer at a distance of 2 to 3 mm.

3. Set up an HPLC system, including a quaternary HPLC pump for two-dimensional LC. Split the flow from the HPLC with the PEEK microcross splitter so that effective flow rate from the HPLC is 0.15 to 0.25 µl/min and spray voltage is 2.4 kV. Set the nitrogen gas (sheath gas) flow rate to 0.2 liter/min.

   For one-dimensional LC, a binary HPLC pump can be used.

Perform chromatography

4. Run a 100-min gradient of 0% to 100% buffer B in buffer A. Hold 10 min at 100% buffer B.

5. Run the following 110-min program.

   a. 3 min with 100% buffer A
   b. 2 min with 10% buffer C in buffer A
   c. 5 min with 100% buffer A
   d. 10-min gradient from 0% to 15% buffer B in buffer A
   e. 90-min gradient from 15% to 55% buffer B in buffer A.

   Repeat this program four more times, but increase the concentration of buffer C (substep b) in each repetition until 80% is obtained (e.g., 10%, 25%, 50%, and 80%).

6. Run the following 130-min program.

   a. 5 min with 100% buffer A
   b. 20 min with 80% buffer C
   c. 5 min with 100% buffer A
   d. 10-min gradient from 0% to 10% buffer B in buffer A
   e. 90-min gradient from 10% to 45% buffer B in buffer A.

Set up and run mass spectrometer

7. Set the LCQ ion trap mass spectrometer to acquire a full MS scan over the *m/z* range of 400 to 1400, with an electron multiplier voltage of 1000 V.

   The LCQ is operated via an Instrument Methods file in the Sequence Setup window of Xcalibur.

8. Select for the top three ions from the MS scan to be analyzed automatically in a tandem MS/MS experiment.

   Dynamic exclusion is enabled with a repeat count of 1, a duration of 0.5 min, and a 30- to 45-sec exclusion duration window.

9. Set relative collision energy to 35% with a 30-msec activation time.

10. Run sample and collect data.

    As the peptide mixture elutes into the mass spectrometer, data are acquired in a data-dependent manner.

BASIC PROTOCOL 4

ANALYSIS OF LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY DATA

The great quantity of data generated in a two-dimensional LC-MS/MS experiment is most efficiently analyzed by algorithms that search the experimental data against known sequences in DNA or protein databases (see Internet Resources). The most effective algorithms for searching databases with MS/MS data require no interpretation of the raw data. In these algorithms, the data are directly correlated with protein or DNA data contained in the database. Several algorithms exist to analyze MS/MS data. The SEQUEST algorithm (Thermo Finnigan) can run directly on the LCQ ion trap mass spectrometer. Alternatively, Web-based search tools are available (see Internet Resources).

SEQUEST matches a peptide sequence in the database with MS/MS spectra by first identifying those peptides with matching molecular weight. The specificity of the enzyme used to digest the mixture significantly reduces the number of possible match candidates. From this list of candidates, predicted fragment ions for each of the peptides are generated and the similarity of the reconstructed spectra to the experimental data is determined. The cross-correlation value is a measure of the quality of the match between the sequence and the spectrum. The difference between the correlation score for the top-scoring match candidate and the candidate ranked second is a measure of the quality of the match versus other possible sequences.

In general, spectra of modified peptides can be searched through available databases. The mass increment of the modification is added to the mass of the unmodified amino acid and this new mass is used in the database search. For example, to search for phosphorylated residues, the program is told to look for mass changes of 80 to Ser, Thr, and Tyr residues. This search is usually done in a differential manner by checking to see if each occurrence of Ser, Thr, or Tyr is modified. To search different types of modifications, the increase in molecular weight expected for the residue is input into the program. Some types of modifications (e.g., methionine oxidation, phosphorylation) may not occur at all possible locations, and thus the possibility of modification must be considered separately for each occurrence of the amino acid.

Database searching can be performed with peptides obtained from organisms without completed genome sequences if a genome sequence is available for a related organism. For example, to analyze peptides from a rat protein, one would create a database of all mammalian protein sequences for the search. Databases are large text files of sequences; consequently the sequences available for related organisms (e.g., human, mouse, and rat) are combined into a single file for the search.

REAGENTS AND SOLUTIONS

Use HPLC-grade water and solvents in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Iodoacetamide/ammonium bicarbonate solution

100 µl 1 M ammonium bicarbonate (J.T. Baker; 100 mM final)
900 µl H_2O
10 mg iodoacetamide (Sigma; 55 mM final)
Always make fresh

Trypsin digestion solution

5 µl 1 M $CaCl_2$ (e.g., Mallinckrodt Baker; *APPENDIX 2A*; 5 mM final)
50 µl 1 M ammonium bicarbonate (50 mM final)
945 µl H_2O
Store up to 1 week at 4°C
Just before use, add 2.5 µl of 0.5 µg/µl trypsin (Promega) per 100 µl total volume (12.5 ng/µl final)

COMMENTARY

Background Information

The development of methods to separate complex mixtures of proteins using two-dimensional gel electrophoresis led to large-scale studies of global protein expression, which is now referred to as proteomics. Using two-dimensional electrophoresis, hundreds of proteins could be separated and visualized, but identification of the individual proteins using Edman sequencing methods was very difficult and time consuming (Aebersold, 1986; Matsudaira, 1987). Advances in techniques to sequence proteins using mass spectrometry (MS; Hunt, 1986) soon replaced the slower and less-sensitive chemical degradation methods.

MS techniques have been most frequently coupled with two-dimensional polyacrylamide gel electrophoresis (PAGE; Jensen et al., 1999b). Proteins separated by two-dimensional PAGE are identified by MS after a sequential analysis of tryptic digests of the individual gel spots. As powerful as this integrated method is, it is limited in its sensitivity and its ability to analyze specific classes of proteins (Gygi et al., 2000; Santoni et al., 2000), and it does not have a broad dynamic range. Two-dimensional PAGE is also a time-consuming and labor-intensive technique that is inherently limited for high-throughput analysis because it requires individual proteins to be excised and extracted from the gel prior to mass analysis.

As an alternative to gel-based methods, liquid chromatographic techniques have now been coupled directly with the mass spectrometer, enabling identification of proteins in complex mixtures through chromatographic separation based on chemical properties followed by separation based on mass-to-charge ratios (m/z) and subsequent structural characterization (Eng et al., 1994; McCormack et al., 1997; Gatlin et al., 1998; Link et al., 1999; Washburn et al., 2001; Wolters et al., 2001; MacCoss et al., 2002).

Mass spectrometry

MS has proven to be a valuable technique for protein mixture analysis (Yates, 1998a; Washburn and Yates, 2000; Yates et al., 2000; Peng and Gygi, 2001). The design of a mass spectrometer makes it particularly well suited for this purpose. All mass spectrometers are composed of three fundamental parts (Fig. 5.6.2). The first segment is the ionization source where the sample molecules are converted into gas phase ions and are introduced into the mass spectrometer. The ions enter the second segment, the mass analyzer, where they are separated on the basis of their m/z values before passing into the third segment, the ion detector. Until the mid-1980s, MS was most commonly used for the characterization of small molecules because there was no way to convert larger biological molecules to gas phase ions. The advent of MS as a technique for the analysis of biological molecules was greatly expedited by the development of important ionization techniques that allowed the transfer of large, biologically important molecules into the gas phase with little or no fragmentation (Fenn et al., 1989). For protein analysis, the two most common ionization techniques are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI).

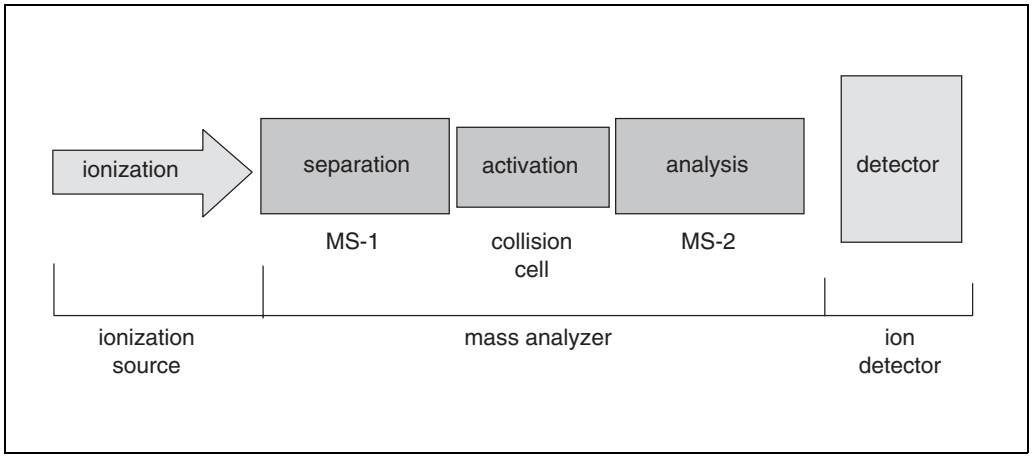


Figure 5.6.2 All mass spectrometers are composed of three basic parts: an ionization source, a mass analyzer, and an ion detector. The mass analyzer depicted in this figure is from a tandem mass spectrometer. MS-1 and MS-2 indicate the tandem mass spectrometers.

Matrix-assisted laser desorption/ionization

In the MALDI technique, analyte is co-crystallized with an acidified matrix and deposited on a polished stainless steel plate. The matrix is a small molecule that absorbs energy from a pulsed laser of specific wavelength. The energy absorbed from the laser is thermally dissipated, resulting in rapid dissociation of the matrix and analyte from the plate surface. The matrix and analyte are both transferred to the gas phase as ions. MALDI is typically coupled with a time-of-flight (TOF) mass analyzer. TOF analyzers separate ions based on the time it takes them to travel the length of the flight tube. Analytes are introduced into the TOF tube when the MALDI plate containing the co-crystallized analyte and matrix is placed in the vacuum chamber of the TOF instrument. High voltage is applied to the MALDI plate, generating a strong electric field between the plate and the entrance to the TOF analyzer. Ions of smaller mass are accelerated toward the flight tube entrance faster than those of larger mass and, once inside the TOF tube, smaller ions traverse the flight path and reach the detector more quickly. The detector records the time and intensity of ions and, once flight time is correlated with mass, generates a spectrum of observed *m*/*z* values. Because the charge of most ions generated in MALDI is 1, the spectrum is usually a straightforward account of ion masses.

Electrospray ionization

In the ESI technique, ionization is achieved when an electric field is applied to a flowing analyte solution. The applied potential imparts a charge to the liquid, creating small droplets of analyte-containing solvent that move toward the entrance to the mass spectrometer. As the solvent particles move into the mass spectrometer, they are repeatedly desolvated and fragmented into smaller particles in a process that results in multiply charged ions.

Unlike MALDI, ESI is performed at atmospheric pressure and can easily be coupled to separation methods such high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) to allow continuous infusion of samples. By utilizing microbore capillaries and extremely low flow rates for introduction of the sample, detection limits at the femtomole level are possible with excellent signal-to-noise ratios (Gatlin et al., 1998). ESI is most frequently coupled with a triple quadrupole, quadrupole-time-of-flight (QTOF), or ion trap mass spectrometer. Triple quadrupole mass analyzers are comprised of three sets of four parallel metal rods. When direct current and radio frequency voltage is applied to these rods, an electric field is created, which directs the trajectory of ions as they progress down the axis between the rods. If an ion meets the *m*/z requirements determined by the voltage to the quadrupole, it will pass through the quadrupole; all other ions will be rejected. In the triple quadrupole instrument, electric fields are generated in the first and third set of quadrupoles, whereas radio frequency voltages are applied to the second quadrupole. The second quadrupole serves as a collision cell where ions collide with atoms of neutral gases to form peptide fragments (Hunt et al., 1986). In the QTOF instrument, the third quadrupole is replaced by a TOF mass analyzer, resulting in a hybrid instrument that has the high mass accuracy of a TOF instrument coupled with the resolution of a quadrupole instrument.

An ion trap instrument operates by collecting and storing ions coming from the source in a trap, scanning the collection of ions for their *m*/*z* values, and sequentially ejecting ions from the trap for MS/MS analysis. Ions are maintained in the trap by the application of direct current and radio frequency voltages; they are ejected from the trap by stepping the radio frequency. For the analysis of proteins and peptides, as well as other biological molecules, the mass analyzer portion of the mass spectrometer typically includes two mass analyzers separated by an ion-activation device (Fig. 5.6.2). In this tandem arrangement of mass analyzers (MS/MS), a mixture of tryptic peptides is introduced into the first mass analyzer where they are separated by their *m*/*z* values. After separation, selected ions are advanced to the ion-activation device. While numerous ion-activation techniques are available, the one most commonly used is collision-induced dissociation (CID). In CID, slow-moving peptide ions collide with inert gas molecules with sufficient kinetic energy to cause peptide bonds to break. The charged fragments from this dissociation are advanced to the second mass spectrometer where they are separated on the basis of their *m*/*z* values before progressing to the ion detector (Fig. 5.6.3). These low-energy collisions result in fragmentation primarily at the amide bond of the peptide, creating a ladder of sequencing ions that differ by one amino acid. By assembling the collection of ions and determining the mass difference between fragments, the amino acid sequence of the peptide can be determined (Hunt et al., 1986).

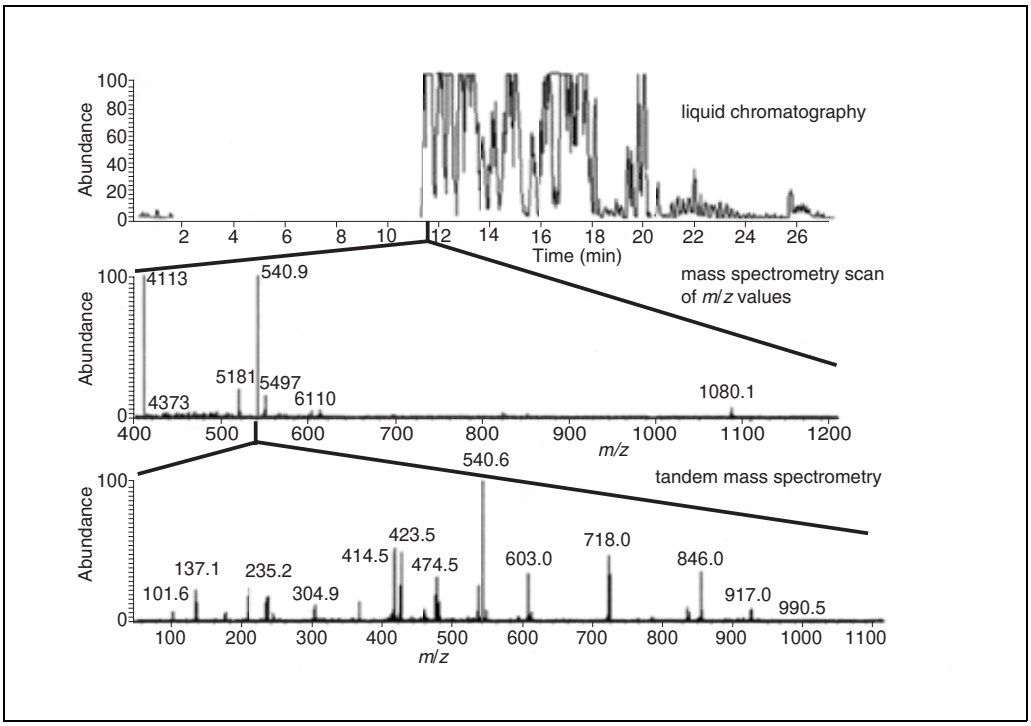


Figure 5.6.3 In the LC-MS/MS technique, peptides are first separated in a liquid chromatographic step. Fractions from the chromatography are subjected to MS and selected ions from the MS experiment are further fragmented in an MS/MS experiment.

Protein mixture analysis: MALDI versus ESI

Even using MS techniques, high-throughput analysis of protein mixtures was not feasible until large databases of protein and DNA sequence information became available and search algorithms were developed that could correlate MS data to the information in the databases (Eng et al., 1994; Yates, 1998b). The information generated by both MALDI-TOF-MS and ESI-MS/MS experiments has been used to identify proteins in complex mixtures.

The high accuracy of peptide mass measurement using a MALDI-TOF instrument allows elucidation of the amino acid composition of the peptide, and computerized search algorithms allow matching of the observed data with proteins in a database. In this method, a collection of peptides generated from tryptic digestion of a mixture of proteins is analyzed with the MALDI-TOF-MS. The masses of the individual peptides can be used to search against databases of known proteins to identify theoretical peptides of matching masses and their respective protein sources. Because mass alone is not always unique for peptide identification, it is typical that a number of peptides from a protein would be needed for identification.

Unlike MALDI, the peptide fragmentation data collected from the ESI-MS/MS experiment are specific for a unique amino acid sequence. Because fragmentation patterns of peptides in a CID experiment are predictable, theoretical fragmentation patterns can be generated from sequences in a database, and the observed spectra can be searched against these (see Basic Protocol 4). Because the spectral data are sequence specific, only a single peptide spectrum is needed for protein identification. The ESI-MS/MS method requires no pre-interpretation of spectra prior to a database search. Algorithms automatically search uninterpreted MS/MS spectra against protein and DNA databases (Fig. 5.6.4). Sensitivity for peptide detection using either MALDI or ESI is 0.5 to 10 fmol.

Sample preparation: PAGE versus LC

When analyzing samples of highly complex mixtures, sample complexity must usually be reduced before the sample is introduced into the mass spectrometer. As discussed above, one method of achieving this is through separation of the proteins by gel electrophoresis. For protein mixtures of low complexity, one-dimensional SDS-PAGE (*UNIT 6.1*) has been used. Using this technique, proteins can be separated on the basis of molecular weight, but the degree of resolution is modest. For more complex mixtures, a two-dimensional SDS-PAGE sepa-

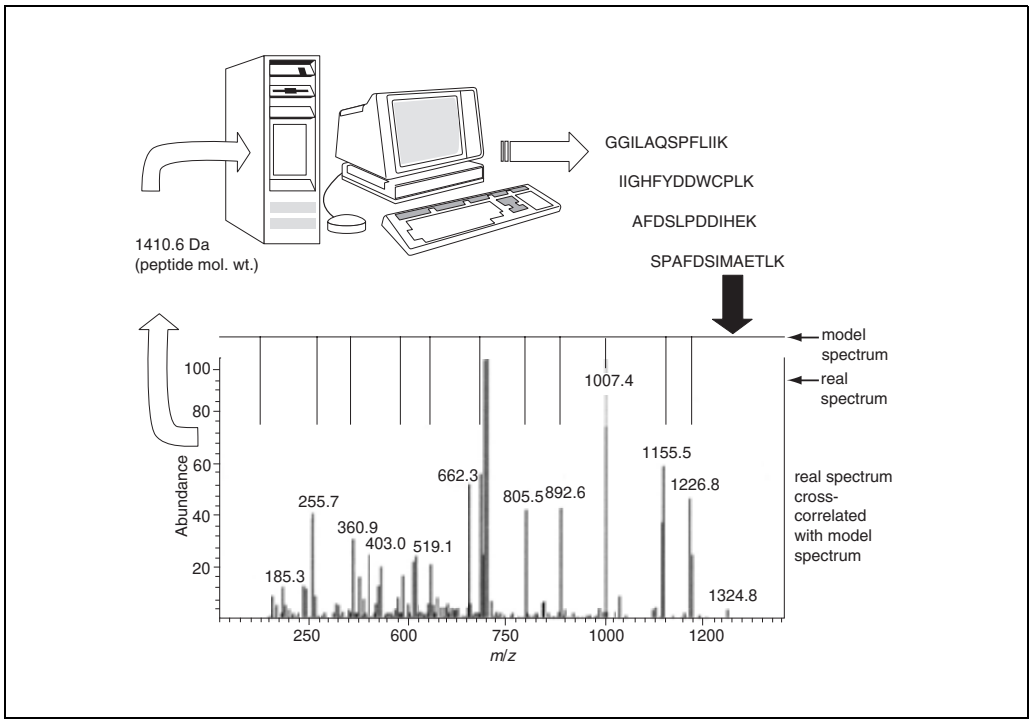


Figure 5.6.4 Search algorithms employed for LC-MS/MS data interpretation search uninterpreted MS/MS spectra against protein and DNA databases. Results are scored on the cross-correlation fit between a theoretical (or model) spectrum and the tandem mass spectra obtained from the experiment (real spectrum).

ration method (*UNIT 6.4*) has been widely adopted. In this technique, proteins are separated on the basis of isoelectric point in the first dimension and by molecular weight in the second. The resolution capability of two-dimensional SDS-PAGE is excellent, and it is theoretically capable of detecting up to 10,000 protein spots. Nevertheless, the method lacks sufficient dynamic range to identify low-abundance proteins in complex mixtures and has been shown to be incompatible with certain classes of proteins (Gygi et al., 2000; Santoni et al., 2000). To mitigate some of the problems of identifying minor protein constituents from a two-dimensional gel, an enrichment step is possible prior to electrophoresis. However, enrichment techniques require some knowledge of biological or chemical activity and are thus often not possible when working with unknown proteins.

Alternatively, mixtures of proteins can be proteolytically digested without separation and directly analyzed by microcolumn-LC-MS/MS. By combining liquid chromatography with ESI-MS/MS, on-line analysis of complex mixtures is possible. In this method, a pulled microcapillary column is packed with a reversed-phase packing material and molecules are separated on the basis of their molecular weight in the reversed-phase chromatography step and then separated according to their *m/z* value in the mass spectrometer. After the mixture is loaded on the chromatography column, no further sample handling is necessary. When even greater peptide separation is desired, multidimensional protein identification technology (MudPIT) can be employed (Link et al., 1999; Washburn and Yates, 2000; Washburn et al., 2001; Wolters et al., 2001). In this technique, peptides are first separated by strong cation exchange, and are eluted onto a second phase by a step gradient of increasing salt concentration. The second phase consists of reversed-phase material that separates on the basis of hydrophobicity. Peptides are then eluted directly onto the MS. Methods for direct analysis of protein mixtures have been applied to a number of systems and have been proven useful over a large dynamic range and for all classes of proteins, including membrane proteins.

Critical Parameters

The success of protein mixture analysis by MS is largely dependent on the success of the methods used to isolate and purify the proteins to be analyzed. For gel-based methods, analysis is usually performed on peptides obtained by enzymatic digestion after separation of the proteins on a gel. Ideally, each band excised from the gel represents a single protein. Prefractiona-

tion of complex samples prior to gel electrophoresis might be required to obtain clean separation. Sample handling steps should be minimized to avoid unnecessary sample loss and to reduce the risk of contamination of the sample with keratin, which can easily obscure the sample protein in the mass spectrum. It is therefore critical to wear gloves when working with the proteins and to avoid exposure to dust. Care must also be taken to eliminate contaminants from glassware and plasticware. Proteins purified on a two-dimensional gel can be extracted from the gel and digested with trypsin prior to analysis on the mass spectrometer, or they can be digested in the gel prior to extraction. The protocol for in-gel digestion of proteins described here (Basic Protocol 1) is a widely used and very effective method. The authors have been able to reduce the time required for this protocol, however, by reducing or eliminating some of the wash steps.

For direct analysis of proteins using LC-MS/MS, low–flow rate LC (200 to 300 nl/min) is necessary to achieve sensitivity at the femtomole scale. Flow rates should not, however, drop below 100 nl/min, or the flow may stop completely. Optimal flow rates can be achieved using a standard LC setup equipped with a precolumn splitting device. Successful chromatography is also dependent on producing, packing, and loading a high-quality microcapillary column. Microcapillaries are most reproducibly constructed using a commercial laser puller, but in some labs they are produced manually by weighting the end of the capillary while heating the center. Microcapillary columns should have an internal diameter of 50 to 100 μm, with a tip internal diameter of ~5 μm. Microcapillaries with smaller diameters can clog easily; larger diameters result in less-efficient electrospray. Frequently, spray problems that arise from larger column diameters can be offset by increasing the voltage.

Columns should be packed with packing material that is 3 to 10 μm in size. A number of different packing materials are suitable for proteomics applications including Aqua (Phenomex), Betabasic (Thermo Hypersil-Keystone), Targa (Bodman Industries), and Polaris (Metachem). Zorbax (Hewlett-Packard) has also been used but requires the use of an ion-pairing solvent (i.e., 0.02% heptaflourobutryic acid added to HPLC solvents), which may cause signal suppression in the mass spectrometer.

Troubleshooting

For two-dimensional LC-MS/MS analysis, the production of the microcapillary column is critical for success. If the column is not properly pulled, then packing material will not load smoothly and chromatography will be poor. If no solvent flow is seen at the tip of the column during column loading, attempts can be made to slightly open the tip by gently scoring it with a ceramic scribe. If the solvent flow does not improve after a few attempts to score the column tip, it should be abandoned, as it usually proves problematic throughout the rest of the experiment. If the column becomes plugged during loading of the sample, it is frequently a sign that the sample has not been properly centrifuged prior to loading to ensure that particulate in the sample does not enter the column. Often, clogged tips can be opened by immersing the column in hot water. If the column tip breaks after the sample has been loaded, it is possible to pack another microcapillary column with 4 cm of the reversed-phase material and attach the original column with a PEEK connector.

It is important to use clean, fresh solvents for chromatography on the microcapillary column because impure HPLC solvents can result in high background noise. Finally, the mass spectrometer needs to be cleaned and tuned approximately every 10 to 15 days, depending on use. The loss of calibration can usually be detected by a blank MS/MS spectrum or a marked decrease in the size of the raw file in the data output after acquisition. If this happens, the instrument needs to be recalibrated using commercial standards.

Anticipated Results

Identification of proteins by LC-MS/MS or two-dimensional electrophoresis followed by MS/MS is made by the identification of peptides generated by enzymatic digestion of the protein. The fraction of the intact protein sequence represented by the identified peptides is referred to as sequence coverage. Reliable identification of a protein is possible with as little as 5% to 10% coverage. Experimental results depend on the complexity of the sample, the enzyme used to digest the sample, and the source of the sample. In a recent publication, MacCoss et al. (2002) showed sequence coverage for five proteins (Cdc2, Cdc13, Cig 1, Crystallin α A chain, and Crystallin α B chain). Trypsin digestion resulted in sequence coverage ranging from 28.9% to 74.5%. Combined results from digestion with three separate en-

zymes increased the sequence coverage to 57.8% to 94.9%.

Using these methods, all post-translational modifications—including phosphorylation, methylation, and acylation—can be identified. Attachment of carbohydrates and lipids is possible but more complex as these modifications are more labile.

Time Considerations

The time required for LC-MS/MS analysis is dependent on the complexity of the sample and the method used to analyze it. For trypsin-digested, gel-purified proteins, a one-dimensional column with a 20- to 50-min gradient is sufficient, whereas a one-dimensional separation of simple mixtures will usually require a 2- to 3-hr gradient. The time required for a multidimensional protein identification technology (MuDPIT) run is determined by the number of salt bumps incorporated in the experiment. A six-step run lasts ~12 hr and a twelve-step run lasts ~24 hr. Additionally, pulling columns will take less than 5 min and column packing will take 15 to 90 min depending on how many phases are being loaded. Sample loading will take anywhere from 15 min to 5 hr depending on the volume and viscosity of the sample and on the size of the column being loaded.

Literature Cited

Aebersold, R.H., Pipes, G., Hood, L.E., and Kent, S.B. 1986. Electroblotting onto activated glass. High efficiency preparation of proteins from analytical sodium dodecyl sulfate-polyacrylamide gels for direct sequence analysis. *J. Biol. Chem.* 261:4229-4238.

Dongré, A.R., Eng, J.K., and Yates, J.R. III. 1997. Emerging tandem-mass-spectrometry techniques for the rapid identification of proteins. *Trends Biotechnol.* 15:418-425.

Eng, J., McCormack, A.L., and Yates, J. R. III. 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* 5:976-989.

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., and Whitehouse, C.M. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246:64-71.

Gatlin, C.L., Kleeman, G.R., Hays, L.G., Link, A.J., and Yates, J.R. III. 1998. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. *Anal. Biochem.* 263:93-101.

Gygi, S., Corthals, G.L., Zhang, Y., Rochon, Y., and Aebersold, R. 2000. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc. Natl. Acad. Sci. U.S.A.* 97:9390-9395.

Hunt, D.F., Yates, J.R. III, Shabanowitz, J., Winston, S., and Hauer, C.R. 1986. Protein sequencing by mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* 83:6233-6237.

Jensen, O.N., Wilm, M., Shevchenko, A., and Mann, M. 1999a. Peptide sequencing of 2-DE gel-isolated proteins by nanospray tandem mass spectrometry. *In* 2-D Proteome Analysis Protocols (A.J. Link, ed.) pp. 571-578. Humana Press, Totowa, N.J.

Jensen, O.N., Wilm, M., Shevchenko, A., and Mann, M. 1999b. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. *In* 2-D Proteome Analysis Protocols (A.J. Link, ed.) pp. 513-530. Humana Press, Totowa, N.J.

Kennedy, R.T. and Jorgenson, J.W. 1989. Quantitative analysis of individual neurons by open tubular liquid chromatography with voltammetric detection. *Anal. Chem.* 61:1128-1135.

Link, A.J., Eng, J., Schieltz, D.M., Carmack, E., Mize, G.J., Morris, D.R., Garvik, B.M., and Yates, J.R. III. 1999. Direct analysis of protein complexes using mass spectrometry. *Nature Biotechnol.* 17:676-682.

MacCoss, M.J., McDonald, W.H., Saraf, A., Sadygov, R., Clark, J.M., Tasto, J.J., Gould, K.L., Wolters, D., Washburn, M., Weiss, A., Clark, J.I., and Yates, J.R. III. 2002. Shotgun identification of protein modifications from protein complexes and lens tissue. *Proc. Natl. Acad. Sci. U.S.A.* 99:7900-7905.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262:10035-10038.

McCormack, A.L., Schieltz, D.M., Goode, B., Yang, S., Barnes, G., Drubin, D., and Yates, J.R. III. 1997. Direct analysis and identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. *Anal. Chem.* 69:767-776.

Peng, J. and Gygi, S.P. 2001. Proteomics: The move to mixtures. *J. Mass Spectrom.* 36:1083-1091.

Santoni, V., Malloy, M., and Rabilloud, T. 2000. Membrane proteins and proteomics: Un amour impossible? *Electrophoresis* 21:1054-1076.

Washburn, M.P. and Yates, J.R. III. 2000. New methods of proteome analysis: Multidimensional chromatography and mass spectrometry. *In* Proteomics: A Trends Guide (W. Blackstock and M. Mann, eds.) pp. 27-31. Elsevier Science, London, U.K.

Washburn, M.P., Wolters, D., and Yates, J.R. III. 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnol.* 19:242-247.

Wolters, D.A., Washburn, M.P., and Yates, J.R. III. 2001. An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem.* 73:5683-5690.

Yates, J.R. III. 1998a. Mass spectrometry and the age of the proteome. *J. Mass Spectrom.* 33:1-19.

Yates, J.R. III. 1998b. Database searching using mass spectrometry data. *Electrophoresis* 19:893-900.

Yates, J.R. III. 2000. Mass spectrometry from genomics to proteomics. *Trends Genet.* 16:5-8.

Internet Resources

ncbi.nlm.nih.gov/Ftp/index.html

National Center for Biotechnology Information's FTP site, from which genome databases can be downloaded.

genome-www.stanford.edu

Web site for Stanford Genomic Resources, including genome databases.

www.tigr.org

Web site for The Institute for Genome Research, including genome databases.

prospector.ucsf.edu

www.matrixscience.com

prowl.rockefeller.edu/PROWL/pepfragch.html

Sites for Web-based search tools for analyzing MS/MS data.

Contributed by Claire Delahunty and John R. Yates III
The Scripps Research Institute
La Jolla, California

Determining Membrane Protein Topologies in Single Cells and High-Throughput Screening Applications

Christian Wunder,[1,2] Jennifer Lippincott-Schwartz,[1] and Holger Lorenz[3]

[1]National Institute of Child Health and Human Development, National Institutes of Health, Bethesda Maryland
[2]Institut Curie, Centre de Recherche, Traffic, Signaling and Delivery Group; CNRS UMR144, Paris, France
[3]Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), Heidelberg, Germany

ABSTRACT

Correct localization and topology are crucial for a protein's cellular function. To determine topologies of membrane proteins, a new technique, called fluorescence protease protection (FPP) assay, has recently been established. The sole requirements for FPP are the expression of fluorescent-protein fusion proteins and the selective permeabilization of the plasma membrane, permitting a wide range of cell types and organelles to be investigated. Proteins topologies in organelles like endoplasmic reticulum, Golgi apparatus, mitochondria, peroxisomes, and autophagosomes have already been determined by FPP. Here, two different step-by-step protocols of the FPP assay are provided. First, we describe the FPP assay using fluorescence microscopy for single adherent cells, and second, we outline the FPP assay for high-throughput screening applications. *Curr. Protoc. Cell Biol.* 49:5.7.1-5.7.12. © 2010 by John Wiley & Sons, Inc.

Keywords: protein topology • fluorescence microscopy • high-throughput screening

INTRODUCTION

This unit describes a fluorescence-based imaging technology, which combines live-cell microscopy with a biochemical approach to assess transmembrane protein topology (Lorenz et al., 2006). The technique uses fluorescent protein (FP) fusion proteins. Our approach provides information about the position of a FP tag relative to a lipid membrane. The FPP assay requires no additional design beyond the construction of a FP fusion protein expressed in either tissue culture or primary cells. The assay is based upon the inaccessibility of proteases to protected intracellular regions of permeabilized cells. To permeabilize cells, we take advantage of the sharp gradient of cholesterol in the lipid bilayers of cells. Relative to other intracellular membranes, the lipid bilayer of the plasma membrane contains by far the most cholesterol, with ~65% to 80% of the free cellular cholesterol (Liscum and Munn, 1999). In this assay, we use the cholesterol-binding drug digitonin to selectively permeabilize the plasma membrane.

Permeabilization allows the protease trypsin to enter the cell from the extracellular environment. As a consequence, not only proteins facing the environment, but also intracellular membrane protein domains facing the cytosol are degraded by the protease. Trypsin-mediated destruction of FP fusions with proteins-of-interest (e.g., N- or C-terminal tags) will thus shed light on the orientations of the target proteins (Fig. 5.7.1).

In this unit, two FPP protocols are described to visualize and quantitate protein topology: FPP for fluorescence microscopes (see Basic Protocol 1) and FPP for high-throughput screening using a fluorescence microplate reader (see Basic Protocol 2). This unit also

Current Protocols in Cell Biology 5.7.1-5.7.12, December 2010
Published online December 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb0507s49

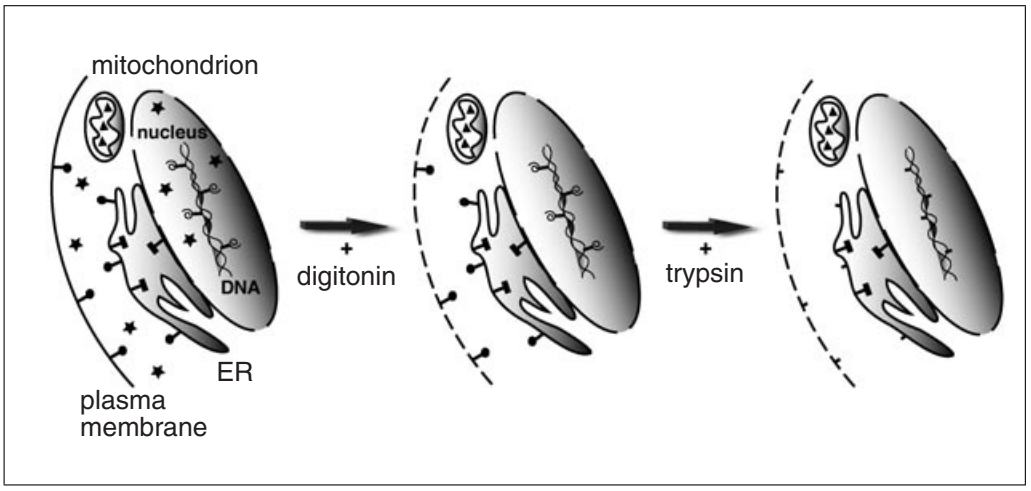


Figure 5.7.1 Mechanism and possible applications of the FPP assay. Cartoon of the FPP assay illustrates localization of different fluorescent protein–tagged proteins, soluble or membrane bound. Addition of digitonin permeabilizes the plasma membrane and causes release of unbound cytosolic and nuclear molecules (star). Membrane- (disc) or DNA-bound (spiral) molecules will stay. Incubation with the protease trypsin digests all fluorescent proteins that are not protected by a lipid membrane. Luminal proteins of the ER (rectangle) or mitochondrion (triangle) are protected from proteases by a lipid membrane. These shielded fluorescence signals will remain.

describes a support protocol for optimizing permeabilization and trypsin treatment of cells.

STRATEGIC PLANNING

Fluorescent Protein Tag

The method requires the construction of a fusion between the coding sequence of a FP and the gene-of-interest. We recommend creating different sets of expression plasmids encoding FP-fusion proteins. For single-spanning membrane proteins, at least two versions of the protein should be created, an amino- and a carboxy-terminal fusion with a FP. For multi-spanning membrane proteins, in addition to N- and C-terminal fusions, either a fusion with an FP inserted within the protein-of-interest or a truncated fusion should be made. Special care must be taken when genes of transmembrane proteins with a targeting sequence or signal sequences are fused to a FP at the amino-terminus. The targeting sequence or signal sequence is often removed by proteolytic cleavage following protein translocation. In such cases, the FP must be inserted after the cleavage site of the signal sequence to generate a mature fusion protein-of-interest harboring an amino-terminal FP tag.

BASIC PROTOCOL 1

FPP ASSAY FOR FLUORESCENCE MICROSCOPES

This protocol describes, first, the plating and transfection of cells in chamber slides, which is required for live-cell imaging, and second, the use of digitonin and trypsin to determine membrane topology of the protein-of-interest. To optimize the FPP assay with untested proteins-of-interest, please pay special attention to the Support Protocol.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile techniques should be applied accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator, unless otherwise specified.

CAUTION: Digitonin is toxic by inhalation, by skin contact, and if swallowed. Wear suitable protective clothing and gloves.

Materials

Adherent cells (e.g., HeLa, COS-7, NRK, HEK293) to be transfected with a soluble FP and/or a FP fusion plasmid
Cell culture medium for cells of interest (e.g., RPMI 1640 or DMEM, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin)
FP expression plasmid (e.g., Clontech) encoding the fluorescent protein-of-interest
Transfection reagent (e.g., FuGENE 6; Roche)
FP (soluble) expression plasmid, e.g., mCFP, EGFP (Clontech)
KHM buffer (see recipe) or cell culture medium without FBS and without phenol red
Protease solution (see recipe)
Digitonin (see recipe)

Sterile tissue culture hood
8-well Lab-Tek chamber slide (Nalge Nunc, cat. no. 155411)
Inverted fluorescence microscope (either widefield or confocal), capable of time lapse recording

Additional reagents and equipment for transfection of cells (*UNIT 20.6*)

Set up the imaging chamber

1. To image adherent cells on chambered slides, seed cells on Lab-Tek chambered coverglass in 0.2 ml cell culture medium.

   The cell density for imaging depends on cell type, cell size, and experimental design. Confluency of cells should be 60% to 90% at the time of the FPP assay.

   These chambers consist of wells with a cover glass bottom, which permits the use of high-numerical-aperture oil objectives for viewing.

Transfect the cells

2. To enhance the probability of having multiple cells to choose from, transfect the cells with a FP chimeric protein vector using a high-efficiency transfection method, such as a lipid transfection reagent with low toxicity (e.g., FuGENE6; *UNIT 20.6*). To control for plasma membrane permeabilization, use double-transfected cells: cells co-expressing the FP-tagged protein-of-interest together with a spectral different soluble FP with non-overlapping spectral properties, e.g., mCFP, EGFP (Clontech).

   Before performing an FPP assay protocol, the investigator must ensure that there is sufficient FP fluorescence in the expressing cells to maintain a significant fluorescent signal relative to background noise during image acquisition. Most standard transfection protocols are sufficient to provide bright specimens. Stable transfectants express lower levels of protein. Transient transfectants usually express higher levels of proteins; this sometimes results in overexpression artifacts, such as protein aggregation or saturation of protein targeting machinery, which lead to inappropriate localization. Immunofluorescence staining of the endogenous protein with specific antibodies should always confirm proper localization of the overexpressed FP-tagged protein.

   Adherent cells should be transiently transfected 6 to 24 hr prior to the experiment. Most commercially available FP expression vectors are under the control of a very strong promoter, e.g., CMV promoter.

Set up the imaging system

3. Set up the fluorescence microscope and its associated hardware.

   It is assumed that the investigator is familiar with the basic operation of the microscope.

   Use a high-NA objective for maximal signal collection and spatial resolution.

Configure the light path for optimal excitation and emission detection of the fluorophores expressed in the transfected cells.

We recommend that the investigator closely examine the spectral profiles of the FPs of interest to ensure an optimal excitation and emission filter combination.

Most software packages provide the user with a list of preset light path configurations for combinations of common fluorophores. However, the investigator should determine if the preset configuration is indeed optimal for the specific FP of interest and modify the configuration, if needed.

The choice of filters is critical for achieving high signal-to-noise levels and minimizing spectral bleed-through at the same time.

Optional: Pretrypsinization for plasma membrane localized FP

If you are investigating the topology of a plasma membrane protein, these steps are required, otherwise, continue with step 11.

4. Remove the cell culture medium from the cells co-expressing the FP-tagged protein-of-interest and a soluble FP.
5. Wash the cells three times, for 1 min each time in 0.2 ml KHM buffer (or alternatively with serum-free medium), at a temperature that is appropriate for the experiment.

   In our hands, temperatures of 20° to 37°C were suitable for the protocol.

6. Place the chamber containing the cells in KHM buffer on the fluorescence microscope stage.
7. Record the images, which represent the pretrypsinization and prepermeabilization situation.
8. Add 0.2 ml 4 to 8 mM of the protease solution (in KHM buffer) directly onto the cells.

   Use the effective trypsin concentration determined according to the Support Protocol.

9. Immediately, start taking images on the fluorescence microscope to record how fast fluorescent signals of the protein-of-interest tagged with a FP disappear.
10. Wash the cells three times, for 1 min each time in 0.2 ml cell culture medium containing serum, at a temperature that is appropriate for the experiment.

    This step is required to inhibit the protease activity of trypsin before permeabilizing the plasma membrane.

Permeabilize the plasma membrane

11. Remove the cell culture medium from the cells co-expressing the FP-tagged protein-of-interest and a soluble FP. Wash the cells three times, for 1 min each time in 0.2 ml KHM buffer (or alternatively with serum-free medium), at a temperature that is appropriate for the experiment.
12. Place the chamber containing the cells in KHM buffer on the fluorescence microscope stage.
13. Record the first images, which represent the prepermeabilization situation.
14. To permeabilize the plasma membrane, add the same volume of KHM buffer containing the (previously determined) effective digitonin concentration to the cells (see the Support Protocol).

    The overexpressed soluble FP diffuses freely in the cytosol and nucleoplasm. Effective permeabilization of the plasma membrane by digitonin results in the disappearance of the FP signal within 10 to 60 sec.

15. Take images of the cells after the digitonin application to capture the post-permeabilization situation.

    The fluorescence signal of the membrane-anchored FP will remain and data acquisition should be reduced to few frames to prevent bleaching of the FP.

Disrupt fluorescence signal

16. Wash the cells in 0.2 ml KHM buffer (optional) and then add 0.2 ml 4 to 8 mM of the protease solution (in KHM buffer) directly onto the cells.

    Use the effective trypsin concentration determined according to the Support Protocol.

17. Immediately, start taking images on the fluorescence microscope to record how fast fluorescent signals of the protein-of-interest tagged with a FP disappear or persist.

    Duration of imaging should be adapted according to the Support Protocol.

Quantify fluorescence signal-intensities

18. Assess recorded images and quantify signal intensities to determine the subcellular localization and topology of the protein.

    As the FPP assay provides a visual screen for determining protein orientations within membranes, an unaffected FP signal will indicate a protected protein domain (i.e., not exposed to the cytosol), while the disappearance of the FP signal will clearly indicate the exposure of the FP-containing protein domain to the cytosolic side. Hence, the persistence or disappearance of the fluorescence signal upon the FPP assay will give information about the protein's localization and topology.

    Freely available image analysis software (e.g., ImageJ, NIH) or software on existing microscope platforms (e.g., Zeiss LSM Image Examiner, Zeiss) can be used to measure fluorescence intensities.

ESTABLISH CONDITIONS FOR OPTIMAL PLASMA MEMBRANE PERMEABILIZATION AND DESTRUCTION OF FPs

SUPPORT PROTOCOL

This protocol describes how to optimize plasma-membrane permeabilization using digitonin, followed by the determination of effective trypsin concentration, to analyze FP localization.

Additional Materials *(also see Basic Protocol 1)*

FP (soluble) expression plasmid, e.g., mCFP, EGFP (Clontech)
Membrane-anchored, but cytosol-facing FP, e.g., Caveolin 1-YFP (Tagawa et al., 2005) *or* VSVG-YFP (Presley et al., 1997)

NOTE: Soluble- and membrane-anchored FPs must have non-overlapping spectral properties.

Set up the imaging chamber

1. To visualize adherent cells on chambered slides, seed cells on Lab-Tek chambered coverglass.

Transfect the cells

2. To enhance the probability of having multiple cells to choose from, transfect the cells with soluble- and membrane-anchored FP vectors using a high-efficiency transfection method, such as a lipid transfection reagent with low toxicity (e.g., FuGENE6; *UNIT 20.6*).

3. Determine the optimal expression level of the FPs and the time requirements for imaging empirically for each sample and condition.

Set up the imaging system

4. Set up the fluorescence microscope and its associated hardware.

Permeabilize the plasma membrane

5. Remove the cell culture medium from the cells expressing a soluble FP only. Wash the cells three times, each time for 1 min in 0.2 ml KHM buffer (or alternatively with serum-free medium), at a temperature that is appropriate for the experiment.
6. Place the chamber-containing cells in KHM buffer on the fluorescence microscope stage.
7. Record the first images, which represent the prepermeabilization situation.
8. To permeabilize the plasma membrane, add the same volume of KHM buffer containing digitonin to the cells. Determine the effective digitonin concentration by applying increasing concentrations of digitonin.

   Effective permeabilization of the plasma membrane using digitonin results in the complete disappearance of the fluorescence signal within 10 to 60 sec.

   A good starting concentration for most cell lines tested (COS-7, NRK, HeLa, BHK, N2a) is 20 μM digitonin. If 20 μM digitonin is not sufficient to permeabilize the cells, increase the digitonin concentration in 20 μM increments. Use the lowest possible digitonin concentration that provides efficient plasma membrane permeabilization.

Permeabilize the plasma membrane and disrupt FP

9. To permeabilize the plasma membrane of cells that express a soluble FP and a membrane-anchored FP, wash cells in 0.2 ml KHM buffer.
10. Record the first images, which represent the prepermeabilization situation.
11. Incubate the cells with KHM buffer containing the (previously determined) effective digitonin concentration.
12. Take images of the cells after the digitonin application to capture the post-permeabilization situation.
13. Wash cells in 0.2 ml KHM buffer (optional) and add 0.2 ml 4 to 8 mM of the protease solution (in KHM buffer) directly onto the cells.
14. Immediately, start taking images on the fluorescence microscope to record how fast fluorescent signals disappear or persist.

    The effective protease concentration results in the disappearance of the FP signal within 30 to 90 sec.

    Under special circumstances in which the sensitivity of the protein-of-interest to trypsin or its accessibility is under question, the use of an alternative protease (e.g., 50 μg/ml proteinase K), or a combination of proteases, is recommended.

BASIC PROTOCOL 2

FPP ASSAY FOR HIGH-THROUGHPUT SCREENING (HTS)

This protocol describes, first, the preparation of cells for the HTS FPP assay, and second, a step-by-step guide to determine the membrane localization of the protein-of-choice under a variety of conditions. It is recommended to determine the required concentrations of digitonin and trypsin, timing of the permeabilization step, and FP destruction according to Basic Protocol 1 and the Support Protocol prior to performing the following high-throughput protocol.

Materials

Adherent cells to be transfected with a soluble FP and/or a FP fusion plasmid
Transfection reagent (e.g., FuGENE 6; Roche)
Cell culture medium for cells of interest (e.g., RPMI 1640 or DMEM, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin)
FP (soluble) expression plasmid, e.g., mCherry (Clontech)
FP expression plasmid containing DNA encoding the protein-of-interest
0.05% (w/v) trypsin/0.53 mM EDTA (Invitrogen)
Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS)
KHM buffer (see recipe) *or* cell culture medium without FBS and without phenol red
Digitonin (see recipe)
Protease solution (see recipe)

Sterile tissue culture hood
10-cm cell culture dish
Hemacytometer
96-well tissue culture plates (Special Optics Low Fluorescence Assay Plates; Sigma-Aldrich: Corning CLS3720)
Microplate fluorescence reader [e.g., from Varian (Cary Eclipse), BioTek, Tecan)]
Microplate washer and dispenser (e.g., EL406, BioTek; multichannel pipets may be used for initial trial experiments)

Transfect the cells

1. To facilitate robust expression levels of the FPs in cells, transfect cells in a large tissue culture dish (e.g., 10-cm tissue culture dish) using a high-efficiency, nontoxic transfection method, such as a lipid transfection reagent with low toxicity (e.g., FuGENE6; *UNIT 20.6*).

   Adherent cells should be transiently transfected 18 to 24 hr prior to the FPP assay.

   To control each step of the experiment, it is advised to use four different cells. First, cells transfected with a soluble FP only; second, cells transfected with the protein-of-interest tagged with a FP; third, cells transfected with both plasmids; and fourth, nontransfected cells. The two FPs should have non-overlapping spectral properties.

Set up the imaging chamber

2. Trypsinize the transfected cells as follows:
   a. At a time point 5 to 6 hr post-transfection, wash the cells with 10 ml CMF-PBS and remove the PBS.
   b. Add 2 ml trypsin/EDTA and wait for 3 to 5 min or until the cells are easily detached by slightly knocking on the tissue culture dish (time depends on the cell line used).
   c. Stop the trypsinization by adding 8 ml of cell culture medium containing 10% FBS.
   d. To break up the cell clumps, in order to obtain a single-cell suspension, pipet the cell sample up and down several times.
3. Count the cells with a hemacytometer and transfer them into 96-well tissue culture plates equipped with transparent bottoms.

   These chambers consist of white or black wells with a cover glass bottom, which permits the use of a fluorescence reader.

   The cell density for fluorescence scanning depends on cell type, cell size, and experimental design. Confluency of cells should be 70% to 80% at the time of the FPP assay.
4. Leave the cells for at least 12 hr in a tissue culture incubator.

Modulate cell culture conditions

5. Add certain drugs or inhibitors, infect with viruses or bacteria, etc. according to the experimental design.

Set up the fluorescence reader

6. Set up the fluorescence reader and the acquisition software.

   Fluorescence readers with a bottom-reading mode are preferred.

   We recommend that the investigator closely examine the spectral profiles of the FPs of interest to ensure an optimal excitation and emission filter combination.

   Most software packages provide the user with a list of preset light path configurations for combinations of common fluorophores. However, the investigator should determine if the preset configuration is indeed optimal for the specific FP of interest and modify the configuration, if needed.

   The choice of filters (bandwidth of fluorescence detection) and time of acquisition is critical for achieving high signal-to-noise levels and minimizing spectral bleed-through at the same time.

   The bottom-reading mode should be preferred over top reading, as it provides better signal-to-noise ratios for adherent cells.

Permeabilize the plasma membrane

7. Place the 96-well plate with the transfected cells in a microplate washer and dispenser.

8. Remove the cell culture medium from the cells and wash the cells three times, each time for 1 min in 0.15 ml KHM buffer (or alternatively with serum-free and phenol-red free medium), at a temperature that is appropriate for the experiment.

   In our hands, temperatures of 20° to 37°C were suitable for the protocol.

9. Place the 96-well plates with cells in KHM buffer on the fluorescence reader.

10. Record the first dataset, which represents the prepermeabilization situation.

11. Place the 96-well plate in a microplate washer and dispenser.

12. Add the same volume of KHM buffer containing the (previously determined) effective digitonin concentration to the cells (see the Support Protocol).

    The soluble FP diffuses freely in the cytosol and nucleoplasm. Effective permeabilization of the plasma membrane by digitonin results in the diffusion of soluble FPs outside the cell and consequently the disappearance of the FP-signal within 10 to 60 sec.

13. Place the 96-well plates in digitonin/KHM buffer on the fluorescence reader.

14. Measure the fluorescence of the cells after the digitonin application to capture the post-permeabilization state. Use the end-point or single-point acquisition mode to prevent bleaching of the fluorescence signal.

Destroy fluorescence signal

15. Place the 96-well plate with the permeabilized cells in the microplate washer and dispenser.

16. Wash cells in 0.15 ml KHM buffer (optional) and then add 4 to 8 mM of the protease solution (in KHM buffer) directly onto the cells.

17. Place the 96-well plate with the cells in trypsin/KHM buffer on the fluorescence reader.

18. Use the kinetic-fluorescence acquisition mode to record how fast fluorescent signals of the protein-of-interest tagged with a FP disappear or persist. Measure the signals

in intervals to prevent bleaching of the fluorescence signal [e.g., every 1 min, depending on the previously determined timing of trypsin-mediated disruption of the fluorescence signal (see the Support Protocol)].

Quantify fluorescence signal intensities

19. Assess acquired fluorescence data. Normalize the data using the fluorescence signal of non-transfected cells (blank). Use signal intensities of replicates to calculate the median and standard deviation of the samples, and determine loss of fluorescence signal dependent on different treatments or concentrations.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Digitonin stock solution

Dissolve digitonin (Calbiochem, cat. no. 300410) in water (24.58 mg/ml, 20 mM) by shaking in a thermomixer at 100°C until a clear solution is obtained.

Commercial digitonin sometimes has very low solubility because of purity problems. Always choose the highest-purity or purify digitonin according to a protocol by Janski and Cornell (1980).

Solubilized digitonin remains stable in solution for at least 1 week at room temperature. The capability of digitonin to permeabilize membranes may differ from batch to batch from all suppliers because of differences in purity.

KHM buffer

110 mM potassium acetate
20 mM HEPES, pH 7.4
2 mM $MgCl_2$
Store up to 6 months at 4°C

Protease solution

Dissolve trypsin in KHM buffer to get an 8 mM solution. Make aliquots and store up to 12 months at −20°C.

Alternatively, 50 μg/ml proteinase K can be used for the FPP assay.

COMMENTARY

Background Information

The FPP assay utilizes specific permeabilization of the cholesterol-rich plasma membrane using digitonin and the resulting accessibility of intracellular compartments to nonspecific proteases. Cholesterol is the prevalent sterol in vertebrates, and the intercalation of digitonin into cholesterol-rich membranes leads to their leakiness. Digitonin forms a complex with unesterified 3-β-hydroxysterols (Takagi et al., 1982). The extent of permeabilization is sufficient to allow cytosolic contents to diffuse across the plasma membrane. However, intracellular organelles and the cytoskeletal system are retained in cells permeabilized with digitonin (Plutner et al., 1992; Wilson et al., 1995). In addition to movement of cytosolic contents out of the cell, permeabilization allows relatively small molecules, like the protease trypsin, to enter the cell from the extracellular environment. Digitonin is unable to permeabilize lipid membranes with low cholesterol content efficiently (e.g., endoplasmic reticulum, Golgi, peroxisomes, mitochondria, and autophagosomes). Hence, lipid membrane–enclosed organelles are not accessible to trypsin. Trypsin will exclusively destroy the FP if it is facing the cytosol or exoplasmic environment. Therefore, the FPP assay provides information about the localization and membrane orientation of a fluorescence-tagged protein.

Critical Parameters

The critical parameters for setting up time-lapse imaging (Basic Protocol 1) and

high-throughput screening (Basic Protocol 2) experiments are described in great detail in the respective protocols. There are several considerations for microscopy imaging and high-throughput screening experiments in living cells that require additional emphasis.

Choice of fluorescent protein

The investigator should use the latest generation of spectral variants, which have been improved for folding, chromophore maturation, brightness, and photostability. In all cases, the investigator needs to minimize undesired

Table 5.7.1 Troubleshooting Guide for Fluorescence Protease Protection (FPP) Assay

Problem	Possible cause	Solution
Fluorescence signal of soluble FP does not disappear after digitonin application.	Inefficient digitonin concentration for plasma membrane permeabilization.	Increase digitonin concentration.
The effective digitonin concentration for cell permeabilization does not work in every cell culture well.	Difference in cell confluencies lead to altered plasma membrane permeabilization.	Keep cell confluencies constant in different cell chambers.
	Remaining medium with FBS (contains cholesterol) in the cell culture chamber captures digitonin.	Be sure to wash the samples three times with KHM buffer before the application of digitonin.
Digitonin permeabilizes intracellular membranes.	Digitonin concentration that is too high may permeabilize membranes with low cholesterol content.	Reduce and optimize digitonin concentration.
Fluorophore signal on either side of an organelle membrane is gone after addition of trypsin.	Both fluorophores were bleached.	Decrease excitation light intensity or reduce acquisition time per frame.
	Digitonin concentration is too high.	Reduce and optimize digitonin concentration.
Intracellular organelle morphology changes dramatically upon prolonged digitonin incubation.	Organelle morphology of different cell types is variably affected by digitonin.	Keep digitonin incubation time as short as possible and wash cells in KHM buffer immediately after permeabilization.
	Change of milieu for very sensitive organelles, like mitochondria.	Replace KHM buffer with a more complex, cytosol-like buffer (Kuznetsov et al., 2008).
Digitonin permeabilization and protease digestion vary significantly between cells in the same chamber.	The distribution of digitonin and protease is restricted in the cell chamber.	Ensure efficient blending of digitonin and protease by adding sufficient volumes to the chamber.
Cells are mostly on the edge of the tissue culture well.	Convex meniscus of culture medium. Plates were swirled for seeding.	Use a small volume for cell seeding and add more medium later.
Cells do not adhere to the tissue culture well with the glass bottom for imaging or HTS.	Cells adhere better to plastic than to glass.	Try fibronectin (5 $\mu g/cm^2$), laminin (2 $\mu g/cm^2$), or collagen (5 $\mu g/cm^2$) coating before seeding.
There is no difference in the fluorescence signal before and after digitonin treatment with a soluble FP in the HTS-protocol.	Bottom reader detects FP, which was released from cells into the supernatant.	Add one additional wash step between the digitonin treatment and scanning with fluorescence reader.
High standard deviation of the HTS-data.	Nonuniform expression of FP.	Generation of stable cell lines.
Very low signal with fluorescence reader.	You are using a top reader.	Use the bottom reader mode. Use a well-established FP-tagged protein to verify sensitivity of the fluorescence reader.
	FP expression is very low.	Choose a higher expressing clone of the stable transfected cell line.

photobleaching of the FPs during data acquisition. Bright and photostable FPs are preferable. For multifluorophore imaging, the investigator should closely examine the spectral profiles of the FPs of interest, as well as the excitation and emission filters available in the microscope and fluorescence reader setup, in order to optimize the light path and obtain maximal signal without spectral bleedthrough. A further problem is the potential aggregation of some fluorescent proteins, which impedes any cellular application and the FPP assay especially.

Choice of objectives

High numerical aperture (NA) oil-immersion objectives corrected for coverglass thickness, field curvature, and chromatic aberrations (e.g., Zeiss Plan-Apochromat 63×/ 1.4 NA oil lens or the Olympus PLAN APO 60×/1.4 NA oil lens) are good choices for high-resolution imaging. However, high magnification and high numerical aperture objectives may increase the risk of photobleaching.

Troubleshooting

Table 5.7.1 lists some problems that may be encountered in fluorescence imaging and HTS of the FPP assay along with some possible causes and solutions.

Anticipated Results

By following the suggestions given in every protocol, the investigator should be able to select cells expressing the fluorescent-tagged protein-of-interest. Plasma membrane permeabilization upon digitonin treatment immediately provides data on whether the protein-of-interest is stably bound to any cellular structure or is freely diffusing in the cytosol or nucleoplasm (the fluorescence signal disappears). Such a complete disappearance of signal from the cells is also observed for soluble FP alone. As outlined in the Support Protocol, a soluble FP alone can be used as the control molecule to determine the appropriate digitonin concentration to permeabilize the plasma membrane.

Addition of trypsin to digitonin-permeabilized cells will provide further information about the subcellular localization and topology of the protein-of-interest. The exact position of the FP tag within the fusion protein sequence is important for topology determination using the FPP assay. If the FP tag is fused to the terminus of a protein, which is enclosed within a protected subcellular environment like the lumen of intracellular organelles, the FP signal will be resistant to protease addition. In contrast, if the protein-of-interest spans the membrane of an intracellular organelle and the FP-tagged domain is exposed to the cytosol, the FP tag will be affected by the addition of protease.

Time Considerations

Basic Protocol 1

Steps 1 and 2 require 30 min each for cell plating and transfection, plus 6 to 24 hr to detect fluorescent protein expression in transfected cells. Steps 3 to 17 require ~20 to 30 min for sample preparation and data acquisition to carry out the FPP assay on one sample. Analyzing the images and quantifying the signal intensities (step 18) requires an additional 10 to 20 min per sample.

Basic Protocol 2

Steps 1, 2, and 3 require 30 min each for cell plating and transfection, plus 12 to 24 hr to detect fluorescent protein expression in transfected cells (step 4). Steps 5 to 18 require about 30 to 40 min for sample preparation and data acquisition to carry out the FPP assay on one 96-well plate. Analyzing the images and quantifying the signal intensities (step 19) require an additional 10 to 20 min per plate.

Literature Cited

Janski, A.M. and Cornell, N.W. 1980. Subcellular distribution of enzymes determined by rapid digitonin fractionation of isolated hepatocytes. *Biochem. J.* 186:423-429.

Kuznetsov, A.V., Veksler, V., Gellerich, F.N., Saks, V., Margreiter, R., and Kunz, W.S. 2008. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.* 3:965-976.

Liscum, L. and Munn, N.J. 1999. Intracellular cholesterol transport. *Biochim. Biophys. Acta* 1438:19-37.

Lorenz, H., Hailey, D.W., and Lippincott-Schwartz, J. 2006. Fluorescence protease protection of GFP chimeras to reveal protein topology and subcellular localization. *Nat. Methods* 3:205-210.

Plutner, H., Davidson, H.W., Saraste, J., and Balch, W.E. 1992. Morphological analysis of protein transport from the ER to Golgi membranes in digitonin-permeabilized cells: Role of the P58 containing compartment. *J. Cell Biol.* 119:1097-1116.

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J., and Lippincott-Schwartz, J. 1997. ER-to-Golgi transport visualized in living cells. *Nature* 389:81-85.

Tagawa, A., Mezzacasa, A., Hayer, A., Longatti, A., Pelkmans, L., and Helenius, A. 2005. Assembly and trafficking of caveolar domains in the

cell: Caveolae as stable, cargo-triggered, vesicular transporters. *J. Cell Biol.* 170:769-779.

Takagi, S., Otsuka, H., Akiyama, T., and Sankawa, U. 1982. Digitonin-cholesterol complex formation—effects of varying the length of the side chain. *Chem. Pharm. Bull.* 30:3485-3492.

Wilson, R., Allen, A.J., Oliver, J., Brookman, J.L., High, S., and Bulleid, N.J. 1995. The translocation, folding, assembly and redox-dependent degradation of secretory and membrane proteins in semi-permeabilized mammalian cells. *Biochem. J.* 307:679-687.

CHAPTER 6
Electrophoresis and Immunoblotting

INTRODUCTION

The development of powerful new technologies is a major driving force of scientific progress. A good example of this is the role that electrophoretic techniques have played in the evolution of modern cell biology. Electrophoresis and related applications have contributed greatly to the understanding of the molecular bases of cell structure and function. The combination of high resolution, ease of use, speed, low cost, and versatility of electrophoretic techniques is unmatched by any other method used to separate proteins. It is for this reason that electrophoresis is an indispensable tool in any cell biology laboratory and that papers describing basic techniques of protein electrophoresis (e.g., Laemmli, 1970, and O'Farrell, 1975, to name just a couple) are among the most cited articles in this field. Laemmli's technique of discontinuous gel electrophoresis in the presence of SDS, for example, continues to be widely used and referenced almost 30 years after publication. Thus, no book of techniques in cell biology would be complete without a detailed description of electrophoretic techniques.

Chapter 6 begins with *UNIT 6.1*, which is a collection of state-of-the-art protocols for analyzing proteins by one-dimensional electrophoresis under denaturing conditions on polyacrylamide gels. Sodium dodecyl sulfate (SDS), in combination with a reducing agent and heat, is most often used as a denaturant. This type of electrophoresis is thus referred to as SDS-polyacylamide gel electrophoresis (SDS-PAGE). Denaturation of the proteins prior to electrophoresis allows for enhanced resolution and discrimination of proteins on the basis of molecular size rather than charge or shape. Utilization of a discontinuous system (i.e., the apposition of "stacking" and "separating" gels) results in concentration of dilute samples and enhanced band sharpness. *UNIT 6.1* presents an overview of electricity and electrophoresis, followed by detailed protocols for SDS-PAGE using either Laemmli's buffers and gel system or modifications of this system (i.e., use of Tris-tricine buffers, higher concentrations of buffers, gradient gels, single-concentration gels, and minigels). The unit also explains how to calculate the apparent molecular weights of proteins from SDS-PAGE data.

The next unit in the chapter, *UNIT 6.2*, describes protocols for immunoblotting (also referred to as western blotting). In this technique, proteins separated by any of the electrophoretic techniques described in *UNIT 6.1* are electrophoretically transferred ("electroblotted") onto a membrane. The membrane, which thus becomes a replica of the polyacrylamide gel, is subsequently probed with antibodies to specific proteins. The primary antibodies can be revealed by an additional incubation with ^{125}I-labeled secondary antibodies or protein A, followed by autoradiography (*UNIT 6.3*). In recent years, however, the use of radioiodinated antibodies has been progressively replaced by nonradioactive detection with antibodies coupled to enzymes such as alkaline phosphatase or horseradish peroxidase (see *UNIT 16.5*). The enzymes act on substrates which are converted to colored, luminescent, or fluorescent products. Nonradioactive methods are just as sensitive as radioactive methods, with the added advantage that they do not require the special precautions associated with the use of radioactivity. Nonradioactive detection is nowadays the method of choice for visualizing immunoblotted proteins. A disadvantage of nonradioactive methods is that they have a narrower linear range of detection, which can be a problem in experiments that require accurate quantitation of protein levels.

Contributed by Juan S. Bonifacino
Current Protocols in Cell Biology (2002) 6.0.1-6.0.3

Radiolabeled proteins separated by electrophoresis or proteins detected by immunoblotting with radioiodinated antibodies or protein A can be visualized by autoradiography, as described in *UNIT 6.3*. In this technique, ionizing radiation emanating from the radionuclides impresses a photographic film. The technique can be made more sensitive by the use of intensifying screens or scintillating compounds, which emit light upon radiation absorption, which then impresses the film. The unit contains several protocols for autoradiographic detection of various radionuclides, including methods for enhancing the signal with intensifying screens or by fluorography. Also included in *UNIT 6.3* are discussions of the quantification of film images by densitometry and the direct detection and quantification of radioactive samples in gels by phosphor imaging.

The resolution of electrophoretic techniques can be enormously enhanced by combining two different electrophoretic procedures performed successively in perpendicular directions (i.e., two-dimensional gel electrophoresis). The most common type of two-dimensional gel electrophoresis is based on separation of proteins by isoelectric focusing on a tube gel (first dimension) followed by SDS-PAGE on a slab gel (second dimension). The two processes separate proteins on the basis of charge and size, respectively, allowing resolution of up to several thousand proteins on a single two-dimensional gel. *UNIT 6.4* describes several methods for separating proteins by two-dimensional isoelectric focusing/SDS-PAGE. In addition, this unit presents a protocol for two-dimensional nonreducing/reducing electrophoresis in which proteins are separated by SDS-PAGE under nonreducing conditions in the first dimension and under reducing conditions in the second dimension. This type of two-dimensional gel electrophoresis allows analysis of intersubunit disulfide bonds in multiprotein complexes and, in some cases, of intrasubunit disulfide bonds. Both types of two-dimensional gel electrophoresis can be used for either analytical or preparative purposes.

Another useful method for electrophoretic separation of cellular proteins is one-dimensional electrophoresis under nondenaturing conditions. Two protocols describing variations of this method are included in *UNIT 6.5*. What distinguishes this method from those described in *UNIT 6.1* and *UNIT 6.4* is that protein samples are not exposed to denaturing agents (i.e., SDS or urea) either prior to or during electrophoresis. Thus, proteins migrate according to their native properties, such as size, shape, and charge. This allows analysis of the oligomeric state of proteins, conformational changes, charge heterogeneity, and post-translational modifications that affect conformation or charge while having minimal effects on the molecular weights of the proteins. In many cases, this method preserves the intrinsic function of the proteins, which allows their detection with specific activity or binding assays. The first protocol describes continuous electrophoresis on nondenaturing polyacrylamide gels. This system involves electrophoresis on a single separating gel and uses the same buffer in the chambers and the gel. The second protocol, discontinuous electrophoresis on nondenaturing polyacrylamide gels, is a variation of SDS-PAGE in which SDS and reducing agents are omitted from all the solutions. Determination of the migration of proteins on parallel gels made up of different concentrations of acrylamide and bisacrylamide allows calculation of their molecular weights using Ferguson plots.

Proteins separated by electrophoresis can be visualized by direct staining of the gels. Four procedures for staining proteins in gels based on different principles are presented in *UNIT 6.6*. These procedures involve staining with Coomassie blue, silver, SYPRO ruby or zinc ions. The unit describes the basic protocols and provides guidelines for the selection of a specific protocol.

The separation of proteins by polyacrylamide gel electrophoresis is limited to proteins with molecular weights less than ~300,000. The electrophoretic separation of larger proteins or multiprotein complexes requires the use of other matrix materials. A suitable material is agarose, which is most commonly used for the separation of DNA. *UNIT 6.7* presents protocols for the electrophoretic separation of proteins on agarose gels. In

addition to permitting the separation of very large proteins, these protocols allow the analysis of multimerization or aggregation. Although these processes can also be analyzed by sedimentation on sucrose gradients (*UNIT 6.3*) or gel filtration (*UNIT 6.4*), agarose gel electrophoresis is more convenient for analysis of large numbers of samples.

LITERATURE CITED

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

O'Farrell, P.H. 1975. High resolution two-dimensional polyacrylamide gel electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.

Juan S. Bonifacino

One-Dimensional SDS Gel Electrophoresis of Proteins

Sean R. Gallagher[1]

[1]UVP, Inc., Upland, California

ABSTRACT

Electrophoresis is used to separate complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates), to investigate subunit compositions, and to verify homogeneity of protein samples. It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis, proteins migrate in response to an electrical field through pores in a polyacrylamide gel matrix; pore size decreases with increasing acrylamide concentration. The combination of pore size and protein charge, size, and shape determines the migration rate of the protein. In this unit, the standard Laemmli method is described for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS). Both full-size and minigel formats are detailed. Several modifications are provided for specific applications. For separation of peptides and small proteins, the standard buffers are replaced with either a Tris-tricine buffer system or a modified Tris buffer in the absence of urea. Continuous SDS-PAGE is a simplified method in which the same buffer is used for both the gel and the electrode solutions and the stacking gel is omitted. Other protocols cover the preparation and use of ultrathin gels and gradient gels, and the simultaneous preparation of multiple gels. *Curr. Protoc. Cell Biol.* 37:6.1.1-6.1.38. © 2007 by John Wiley & Sons, Inc.

Keywords: protein • electrophoresis • separation • polyacrylamide • SDS-PAGE

INTRODUCTION

Electrophoresis is used to separate complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates), to investigate subunit compositions, and to verify homogeneity of protein samples. It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis (PAGE), proteins migrate in response to an electrical field through pores in the gel matrix; pore size decreases with higher acrylamide concentrations. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein.

The standard Laemmli method (see Basic Protocol 1) is used for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS). The standard method for full-size gels (e.g., 14 × 14 cm) can be adapted for the minigel format (e.g., 7.3 × 8.3 cm; see Basic Protocol 2). Minigels provide rapid separation but give lower resolution.

Several alternate protocols are provided for specific applications. The first two alternate protocols cover electrophoresis of peptides and small proteins, separations that require modification of standard buffers: either a Tris-tricine buffer system (see Alternate Protocol 1) or a modified Tris buffer in the absence of urea (see Alternate Protocol 2). Continuous SDS-PAGE is a simplified method in which the same buffer is used for both gel and electrode solutions and the stacking gel is omitted (see Alternate Protocol 3). Other protocols cover the preparation and electrophoresis of various types of gels:

Current Protocols in Cell Biology 6.1.1-6.1.38, December 2007
Published online December 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0601s37

ultrathin gels (see Alternate Protocol 4), multiple single-concentration gels (see Support Protocol 1), gradient gels (see Alternate Protocol 5), multiple gradient gels (see Support Protocol 2), and multiple gradient minigels (see Support Protocol 3). Proteins separated on gels can be subsequently analyzed by immunoblotting (*UNIT 6.2*), autoradiography or phosphor imaging (*UNIT 6.3*), or staining with protein dyes (*UNIT 6.6*). Protein size is determined by comparing the mobility of the protein band to the mobility of the dye front (see Support Protocol 4).

CAUTION: Before any protocols are used, it is extremely important to read the following section about electricity and electrophoresis.

ELECTRICITY AND ELECTROPHORESIS

Many researchers are poorly informed concerning the electrical parameters of running a gel. It is important to note that the voltages and currents used during electrophoresis are dangerous and potentially lethal. Thus, safety should be an overriding concern. A working knowledge of electricity is an asset in determining what conditions to use and in troubleshooting the electrophoretic separation, if necessary. For example, an unusually high or low voltage for a given current (milliampere) might indicate an improperly made buffer or an electrical leak in the chamber.

Safety Considerations

1. Never remove or insert high-voltage leads unless the power supply voltage is turned down to zero and the power supply is turned off. Always grasp high-voltage leads one at a time with one hand only. Never insert or remove high-voltage leads with both hands. This can shunt potentially lethal electricity through the chest and heart should electrical contact be made between a hand and a bare wire. On older or homemade instruments, the banana plugs may not be shielded and can still be connected to the power supply at the same time they make contact with a hand. With commercial modern power supplies, this is less of an issue. However, with age and use, wires may become exposed through cracks in the insulation or poor connections. Carefully inspect all cables and connections and replace frayed or exposed wires immediately.

2. Always start with the power supply turned off. Have the power supply controls turned all the way down to zero. Then hook up the gel apparatus: generally, connect the red high-voltage lead to the red outlet and the black high-voltage lead to the black outlet. Turn the power supply on with the controls set at zero and the high-voltage leads connected. Then turn up the voltage, current, or power to the desired level. Reverse the process when the power supply is turned off: i.e., to disconnect the gel, turn the power supply down to zero, wait for the meters to read zero, turn off the power supply, and then disconnect the gel apparatus one lead at a time.

   CAUTION: *If the gel is first disconnected and then the power supply turned off, a considerable amount of electrical charge is stored internally. The charge will stay in the power supply over a long time. This will discharge through the outlets even though the power supply is turned off and can deliver an electrical shock.*

Ohm's Law and Electrophoresis

Understanding how a gel apparatus is connected to the power supply requires a basic understanding of Ohm's law: voltage = current × resistance, or $V = IR$. A gel can be viewed as a resistor and the power supply as the voltage and current source. Most power supplies deliver constant current or constant voltage. Some will also deliver constant power: power = voltage × current, or $VI = I^2R$. The discussion below focuses on constant current because this is the most common mode in vertical SDS-PAGE.

Most modern commercial equipment is color-coded so that the red or positive terminal of the power supply can simply be connected to the red lead of the gel apparatus, which goes to the lower buffer chamber. The black lead is connected to the black or negative terminal and goes to the upper buffer chamber. This configuration is designed to work with vertical slab gel electrophoreses in which negatively charged proteins or nucleic acids move to the positive electrode in the lower buffer chamber (an anionic system).

When a single gel is attached to a power supply, the negative charges flow from the negative cathode (black) terminal into the upper buffer chamber, through the gel, and into the lower buffer chamber. The lower buffer chamber is connected to the positive anode (red) terminal to complete the circuit. Thus, negatively charged molecules, such as SDS-coated proteins and nucleic acids, move from the negative cathode attached to the upper buffer chamber toward the positive anode attached to the lower chamber. SDS-PAGE is an anionic system because of the negatively charged SDS.

Occasionally, proteins are separated in cationic systems. In these gels, the proteins are positively charged because of the very low pH of the gel buffers (e.g., acetic acid/urea gels for histone separations) or the presence of a cationic detergent (e.g., cetyltrimethyl-ammonium bromide, CTAB). Proteins move toward the negative electrode (cathode) in cationic gel systems, and the polarity is reversed compared to SDS-PAGE: the red lead from the lower buffer chamber is attached to the black outlet of the power supply, and the black lead from the upper buffer chamber is attached to the red outlet of the power supply.

Most SDS-PAGE separations are performed under constant current (consult instructions from the manufacturer to set the power supply for constant current operation). The resistance of the gel will increase during SDS-PAGE in the standard Laemmli system. If the current is constant, then the voltage will increase during the run as the resistance goes up.

Power supplies usually have more than one pair of outlets. The pairs are connected in parallel with one another internally. If more than one gel is connected directly to the outlets of a power supply, then these gels are connected in parallel (Fig. 6.1.1). In a parallel circuit, the voltage is the same across each gel. In other words, if the power supply reads 100 V, then each gel has 100 V across its electrodes. The total current, however, is the sum of the individual currents going through each gel. Therefore, under constant current it is necessary to increase the current for each additional gel that is connected to the power supply. Two identical gels require double the current to achieve the same starting voltages and electrophoresis separation times.

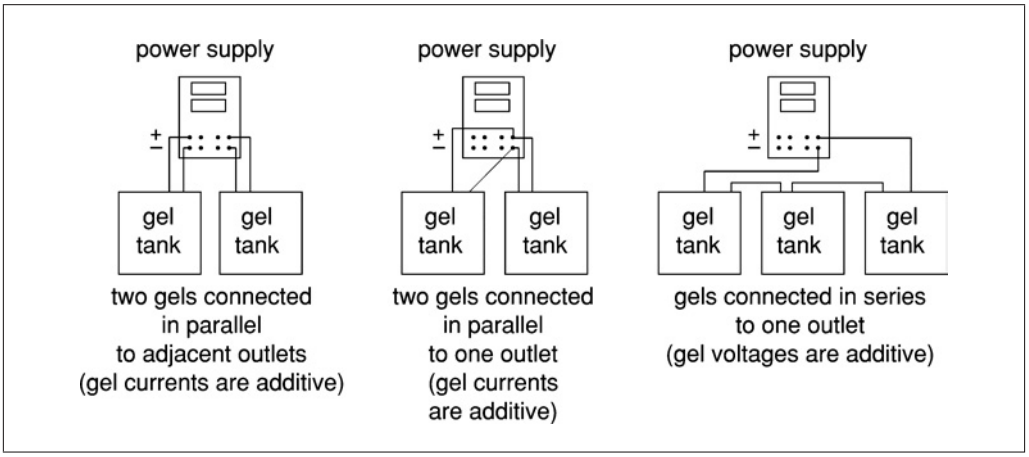


Figure 6.1.1 Series and parallel connections of gel tanks to power supply.

Multiple gel apparatuses can also be connected to one pair of outlets on a power supply. This is useful with older power supplies that have a limited number of outlets. When connecting several gel units to one outlet, make certain the connections between the units are shielded and protected from moisture. The gels can be connected in parallel or in series (Fig. 6.1.1). When gels are connected through the same outlet in parallel, the principle of additive currents is the same as for gels connected through different outlets in parallel. In the case of two or more gels running off the same outlet in series, the current is the same for every gel. If 10 mA is displayed by the power supply meter, for example, each gel in series will experience 10 mA. The voltage, however, is additive for each gel. If one gel at a constant 10 mA produces 100 V, then two identical gels in series will produce 200 V (100 V each) and so on. Thus, the voltage can limit the number of units connected in series on low-voltage power supplies.

Gel thickness affects the above relationships. A 1.5-mm gel can be thought of as two 0.75-mm-thick gels run in parallel. Because currents are additive in parallel circuits, a 0.75-mm gel will require half the current of the 1.5-mm gel to achieve the same starting voltage and separation time. If gel thickness is doubled, then the current must also be doubled. There are limits to the amount of current that can be applied. Thicker gels require more current, generating more heat that must be dissipated. Unless temperature control is available in the gel unit, a thick gel should be run more slowly than a thin gel.

NOTE: Milli-Q-purified water or equivalent should be used throughout the protocols.

BASIC PROTOCOL 1

DENATURING (SDS) DISCONTINUOUS GEL ELECTROPHORESIS: LAEMMLI GEL METHOD

One-dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel (sometimes called resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus. After sample proteins are solubilized by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. The stacking gel, through a combination of low porosity and a lower buffer concentration and pH, concentrates proteins into a thin stack before they enter the resolving gel. 2-Mercaptoethanol (2-ME) or dithiothreitol (DTT) is added during solubilization to reduce disulfide bonds.

This protocol is designed for a vertical slab gel with a maximum size of 0.75 mm × 14 cm × 14 cm. For thicker gels or minigels (see Basic Protocol 2 and Support Protocol 3), the volumes of stacking and separating gels and the operating current must be adjusted. Additional protocols describe the preparation of ultrathin gels (see Alternate Protocol 4) and gradient gels (see Alternate Protocol 5), as well as the use of gel casters to make multiple gels, both single-concentration gels (see Support Protocol 1) and gradient gels (see Support Protocol 2).

Materials

Separating and stacking gel solutions (Table 6.1.1)
H_2O-saturated isobutyl alcohol
1× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 6.1.1)
Protein sample, on ice
2× and 1× SDS sample buffer (see recipe)
Protein molecular weight standards (Tables 6.1.2 and 6.1.3)
6× SDS sample buffer (see recipe; optional)
1× SDS electrophoresis buffer (see recipe)

Electrophoresis apparatus: e.g., Protean II 16-cm cell (Bio-Rad) or SE 600/400 16-cm unit (Hoefer) with clamps, glass plates, casting stand, and buffer chambers
0.75-mm spacers
0.45-μm filters (used in stock solution preparation)
25-ml Erlenmeyer side-arm flasks
Vacuum pump with cold trap
0.75-mm Teflon comb with 1, 3, 5, 10, 15, or 20 teeth
Screw-top microcentrifuge tubes (recommended)
25- or 100-μl syringe with flat-tipped needle
Constant-current power supply (see Electricity and Electrophoresis above)

Pour the separating gel

1. Assemble the glass-plate sandwich of the electrophoresis apparatus according to manufacturer's instructions using two clean glass plates and two 0.75-mm spacers.

   If needed, clean the glass plates in liquid Alconox or RBS-35 (Pierce). These aqueous-based solutions are compatible with silver and Coomassie blue staining procedures.

2. Lock the sandwich to the casting stand.

3. Prepare the separating gel solution as directed in Table 6.1.1, degassing using a rubber-stoppered 25-ml Erlenmeyer side-arm flask connected with vacuum tubing to a vacuum pump with a cold trap. After adding the specified amount of 10% ammonium persulfate and TEMED to the degassed solution, stir gently to mix.

   The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. Generally, use 5% gels for SDS-denatured proteins of 60 to 200 kDa, 10% gels for SDS-denatured proteins of 16 to 70 kDa, and 15% gels for SDS-denatured proteins of 12 to 45 kDa (Table 6.1.1).

   The stacking gel is the same regardless of the separating gel used.

4. Using a Pasteur pipet, apply the separating gel solution to the sandwich along an edge of one of the spacers until the height of the solution between the glass plates is ~11 cm.

   Use the solution immediately; otherwise it will polymerize in the flask.

   Sample volumes <10 μl do not require a stacking gel. In this case, cast the resolving gel as usual, but extend the resolving gel into the comb (step 10) to form the wells. The proteins are then separated under the same conditions as used when a stacking gel is present. Although this protocol works well with single-concentration gels, a gradient gel is recommended for maximum resolution (see Alternate Protocol 5).

5. Using another Pasteur pipet, slowly cover the top of the gel with a layer (~1 cm thick) of H_2O-saturated isobutyl alcohol, by gently layering the isobutyl alcohol against the edge of one and then the other of the spacers.

   Be careful not to disturb the gel surface. The overlay provides a barrier to oxygen, which inhibits polymerization, and allows a flat interface to form during gel formation.

   The H_2O-saturated isobutyl alcohol is prepared by shaking isobutyl alcohol and H_2O in a separatory funnel. The aqueous (lower) phase is removed. This procedure is repeated several times. The final upper phase is H_2O-saturated isobutyl alcohol.

6. Allow the gel to polymerize 30 to 60 min at room temperature.

   A sharp optical discontinuity at the overlay/gel interface will be visible on polymerization. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both. Ammonium persulfate solution should be made fresh before use. Ammonium persulfate should "crackle" when added to the water. If not, fresh ammonium persulfate should be purchased. Purchase TEMED in small bottles so, if necessary, a new previously unopened source can be tried.

Table 6.1.1 Recipes for Polyacrylamide Separating and Stacking Gels[a]

SEPARATING GEL

Stock solution[b]	Final acrylamide concentration in separating gel (%)[c]									
	5	6	7	7.5	8	9	10	12	13	15
30% (w/v) acrylamide/ 0.8% (w/v) bisacrylamide	2.50	3.00	3.50	3.75	4.00	4.50	5.00	6.00	6.50	7.50
4× Tris·Cl/SDS, pH 8.8	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.50	7.25	6.75	6.25	5.25	4.75	3.75
10% (w/v) ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED[e]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution, 4× Tris·Cl/SDS, pH 8.8 (see reagents, below), and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

STACKING GEL (*3.9% w/v acrylamide*)

In a 25-ml side-arm flask, mix 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4× Tris·Cl/SDS, pH 6.8 (see reagents, below), and 3.05 ml H_2O. Degas under vacuum 10 to 15 min. Add 25 µl of 10% ammonium persulfate and 5 µl TEMED. Swirl gently to mix. Use immediately.

REAGENTS USED IN GELS

30% (w/v) acrylamide/0.8% (w/v) bisacrylamide

Mix 30.0 g acrylamide and 0.8 g *N,N′*-methylenebisacrylamide with H_2O in a total volume of 100 ml. Filter the solution through a 0.45-µm filter and store at 4°C in the dark. The 2× crystallized grades of acrylamide and bisacrylamide are recommended. Discard after 30 days, as acrylamide gradually hydrolyzes to acrylic acid and ammonia.

CAUTION: Acrylamide monomer is neurotoxic. A mask should be worn when weighing acrylamide powder. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth.

4× Tris·Cl/SDS, pH 6.8 *(0.5 M Tris·Cl containing 0.4% w/v SDS)*

Dissolve 6.05 g Tris base in 40 ml H_2O. Adjust to pH 6.8 with 1 N HCl. Add H_2O to 100 ml total volume. Filter the solution through a 0.45-µm filter, add 0.4 g SDS, and store at 4°C up to 1 month.

4× Tris·Cl/SDS, pH 8.8 *(1.5 M Tris·Cl containing 0.4% w/v SDS)*

Dissolve 91 g Tris base in 300 ml H_2O. Adjust to pH 8.8 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter, add 2 g SDS, and store at 4°C up to 1 month.

[a]The recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for a gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Volumes are in milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See annotation to step 3, Basic Protocol 1.

[d]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

[e]TEMED, *N,N,N,N*-tetramethylethylenediamine.

Table 6.1.2 Molecular Weights of Protein Standards for Polyacrylamide Gel Electrophoresis[a]

Protein	Molecular weight (Da)
Cytochrome *c*	11,700
α-Lactalbumin	14,200
Lysozyme (hen egg white)	14,300
Myoglobin (sperm whale)	16,800
β-Lactoglobulin	18,400
Trypsin inhibitor (soybean)	20,100
Trypsinogen, PMSF treated	24,000
Carbonic anhydrase (bovine erythrocytes)	29,000
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	36,000
Lactate dehydrogenase (porcine heart)	36,000
Aldolase	40,000
Ovalbumin	45,000
Catalase	57,000
Bovine serum albumin	66,000
Phosphorylase *b* (rabbit muscle)	97,400
β-Galactosidase	116,000
RNA polymerase, *E. coli*	160,000
Myosin, heavy chain (rabbit muscle)	205,000

[a]Protein standards are commercially available as prepared mixtures (see Table 6.1.3).

Pour the stacking gel

7. Pour off the layer of H_2O-saturated isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8.

   Residual isobutyl alcohol can reduce resolution of the protein bands; therefore, it must be completely removed. The isobutyl alcohol overlay should not be left on the gel longer than 2 hr.

8. Prepare the stacking gel solution as directed in Table 6.1.1.

   Use the solution immediately to keep it from polymerizing in the flask.

9. Using a Pasteur pipet, allow the stacking gel solution to trickle slowly into the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is ~1 cm from the top of the plates.

   Be careful not to introduce air bubbles into the stacking gel.

10. Insert a 0.75-mm Teflon comb into the layer of stacking gel solution. If necessary, add additional stacking gel to fill the spaces in the comb completely.

    Again, be careful not to trap air bubbles in the tooth edges of the comb; they will cause small circular depressions in the well after polymerization that will lead to distortion in the protein bands during separation.

11. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

    A sharp optical discontinuity will be visible around the wells on polymerization. Again, failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

Table 6.1.3 Protein Standard Mixtures Available from Selected Suppliers

Applications[a]	1-D	2-D[b]	Im	Pre[c]	Fluor	Gly	Phos	Bio	Tag	IEF	Nat
Bio-Rad	X	X		X				X	X	X	
CalBiochem	X						X				
Cell Signaling Technology	X			X				X			
Favorgen	X			X							
GE Healthcare	X		X	X				X	X	X	
Invitrogen	X		X	X	X	X	X		X	X	
NEB	X			X							
Norgen Biotek	X		X								
Novagen	X		X	X					X		
PerkinElmer	X			X							
Pierce	X	X		X	X						
Promega	X										
Qiagen	X		X						X		
R & D Systems	X			X				X			
Roche Applied Science	X								X		
Sigma-Aldrich	X	X	X	X	X	X	X	X		X	X
Upstate	X		X				X				
USB	X		X							X	

[a]Abbreviations: 1-D, one-dimensional gels; 2-D, two-dimensional gels; Im, immunoblotting; Pre, prestained; Fluor, fluorescent; Gly, glycoprotein; Phos, phosphoprotein; Bio, biotinylated; Tag, tagged; IEF, isoelectic focusing; Nat, native.

[b]2-D standards are useful as independently characterized internal controls or reference standards for 2-D SDS-PAGE. Many investigators simply use an internally characterized test sample as a reference set.

[c]Prestained standards, while not as sharply delineated as unstained standards, can be used to monitor progress of the separation since the bands are visible through the gel cassette during electrophoresis. They are also useful for marking the position of a band after electroblotting to a nitrocellulose or PVDF membrane prior to immunoassay or analysis by mass spectrometry.

Prepare the sample and load the gel

12. Dilute a portion of the protein sample to be analyzed 1:1 (v/v) with 2× SDS sample buffer and heat 3 to 5 min at 100°C in a sealed screw-cap microcentrifuge tube. If the sample is a precipitated protein pellet, dissolve the protein in 50 to 100 µl of 1× SDS sample buffer and boil 3 to 5 min at 100°C. Dissolve protein molecular weight standards in 1× SDS sample buffer according to supplier's instructions; use these standards as a control (Tables 6.1.2 and 6.1.3).

    For dilute protein solutions, consider using 5:1 protein solution/6× SDS sample buffer to increase the amount of protein loaded. Proteins can also be concentrated by precipitation in acetone, ethanol, or trichloroacetic acid (TCA), but losses will occur.

    For a 0.8-cm-wide well, 25 to 50 µg total protein in <20 µl is recommended for a complex mixture when staining with Coomassie blue, and 1 to 10 µg total protein is needed for samples containing one or a few proteins. If silver staining is used, 10- to 100-fold less protein can be applied (0.01 to 5 µg in <20 µl depending on sample complexity).

    To achieve the highest resolution possible, the following precautions are recommended. Prior to adding the sample buffer, keep samples at 0°C. Add the SDS sample buffer (room temperature) directly to the 0°C sample (still on ice) in a screw-top microcentrifuge tube.

Cap the tube to prevent evaporation, vortex, and transfer directly to a 100°C water bath for 3 to 5 min. Let immunoprecipitates dissolve for 1 hr at 56°C in 1× SDS sample buffer prior to boiling. DO NOT leave the sample in SDS sample buffer at room temperature without first heating to 100°C to inactivate proteases (see Critical Parameters and Troubleshooting). Endogenous proteases are very active in SDS sample buffer and will cause severe degradation of the sample proteins after even a few minutes at room temperature. To test for possible proteases, mix the sample with SDS sample buffer without heating and leave at room temperature for 1 to 3 hr. A loss of high-molecular-weight bands and a general smearing of the banding pattern indicate a protease problem. Once heated, the samples can sit at room temperature for the time it takes to load samples.

13. Carefully remove the Teflon comb without tearing the edges of the polyacrylamide wells. After the comb is removed, rinse wells with 1× SDS electrophoresis buffer.

    The rinse removes unpolymerized monomer; otherwise, the monomer will continue to polymerize after the comb is removed, creating uneven wells that will interfere with sample loading and subsequent separation.

14. Using a Pasteur pipet, fill the wells with 1× SDS electrophoresis buffer.

    If well walls are not upright, they can be manipulated with a flat-tipped needle attached to a syringe.

15. Attach gel sandwich to upper buffer chamber following manufacturer's instructions.

16. Fill lower buffer chamber with the recommended amount of 1× SDS electrophoresis buffer.

17. Place sandwich attached to upper buffer chamber into lower buffer chamber.

18. Partially fill the upper buffer chamber with 1× SDS electrophoresis buffer so that the sample wells of the stacking gel are filled with buffer.

    Monitor the upper buffer chamber for leaks and, if necessary, reassemble the unit. A slow leak in the upper buffer chamber may cause arcing around the upper electrode and damage the upper buffer chamber.

19. Using a 25- or 100-µl syringe with a flat-tipped needle, load the protein sample(s) into one or more wells by carefully applying the sample as a thin layer at the bottom of the wells. Load control wells with molecular weight standards. Add an equal volume of 1× SDS sample buffer to any empty wells to prevent spreading of adjoining lanes.

    Disposable loading tips can be used with automatic pipettors to simplify loading.

    Preparing the samples at approximately the same concentration and loading an equal volume to each well will ensure that all lanes are the same width and that the proteins run evenly. If unequal volumes of sample buffer are added to wells, the lane with the larger volume will spread during electrophoresis and constrict the adjacent lanes, causing distortions.

    The samples will layer on the bottom of the wells because the glycerol added to the sample buffer gives the solution a greater density than the electrophoresis buffer. To keep bands tight, hold the tip of the needle near the bottom of the well and load the samples slowly. The bromphenol blue in the sample buffer makes sample application easy to follow visually.

20. Fill the remainder of the upper buffer chamber with additional 1× SDS electrophoresis buffer so that the upper platinum electrode is completely covered. Do this slowly so that samples are not swept into adjacent wells.

Run the gel

21. Connect the power supply to the cell and run at 10 mA of constant current for a slab gel 0.75 mm thick, until the bromphenol blue tracking dye enters the separating gel. Then increase the current to 15 mA.

    For a standard 16-cm gel sandwich, 4 mA per 0.75-mm-thick gel will run ~15 hr (i.e., overnight); 15 mA per 0.75-mm gel will take 4 to 5 hr. To run two gels or a 1.5-mm-thick gel, simply double the current. When running a 1.5-mm gel at 30 mA, the temperature must be controlled (10° to 20°C) with a circulating constant-temperature water bath to prevent "smiling" (curvature in the migratory band). Temperatures <5°C should not be used because SDS in the running buffer will precipitate.

    If the level of buffer in the upper chamber decreases, a leak has occurred.

22. After the bromphenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

    Refer to Safety Considerations under Electricity and Electrophoresis.

Disassemble the gel

23. Discard electrophoresis buffer and remove the upper buffer chamber with the attached gel sandwich.

24. Orient the gel so that the order of the sample wells is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels.

25. Carefully slide one of the spacers halfway from the edge of the sandwich along its entire length. Use the exposed spacer as a lever to pry open the glass plate, exposing the gel.

26. Carefully remove the gel from the lower plate. Cut a small triangle off one corner of the gel so the lane orientation is not lost during staining and drying. Proceed with protein detection.

    Gradient gels are most easily picked up without tearing from the high-concentration end of the gel using gloved fingers. Single-concentration gels <10% can be picked up and placed in fixative, but are more easily removed if first immersed in fixative while left on the plate, allowing the gel to float off.

    The gel can be stained with Coomassie blue or silver (UNIT 6.6), or proteins can be electroeluted, electroblotted onto a polyvinylidene difluoride (PVDF) membrane for subsequent staining or sequence analysis, or transferred to a membrane for immunoblotting (UNIT 6.2). If the proteins are radiolabeled, they can be detected by autoradiography (UNIT 6.3).

ALTERNATE PROTOCOL 1

ELECTROPHORESIS IN TRIS-TRICINE BUFFER SYSTEMS

Separation of peptides and proteins under 10 to 15 kDa is not possible in the traditional Laemmli discontinuous gel system (see Basic Protocol 1). This is due to the comigration of SDS and smaller proteins, obscuring the resolution. Two approaches to obtain the separation of small proteins and peptides in the range of 5 to 20 kDa are presented: the following Tris-tricine method and a system using increased buffer concentrations (see Alternate Protocol 2). The Tris-tricine system uses a modified buffer to separate the SDS and peptides, thus improving resolution. Several precast gels are available for use with the tricine formulations (Table 6.1.4).

Table 6.1.4 Precast Gels Available from Selected Suppliers

	Format		Application				Instrument Compatibility			
	Large	Mini	2-D[a]	Native	Peptide	SDS	Bio-Rad	Cambrex	Hoefer	Invitrogen
Bio-Rad	X	X	X	X	X	X	X			
Cambrex		X	X	X	X	X	X	X	X	X
Jule	X	X	X	X	X	X	X	X	X	X
Invitrogen		X	X	X	X	X	X	X	X	X

[a]Two-dimensional analysis.

Table 6.1.5 Recipes for Tricine Peptide Separating and Stacking Gels[a]

SEPARATING AND STACKING GELS

Stock solution[b]	Separating gel	Stacking gel
30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	9.80 ml	1.62 ml
Tris·Cl/SDS, pH 8.45	10.00 ml	3.10 ml
H_2O	7.03 ml	7.78 ml
Glycerol	4.00 g (3.17 ml)	—
10% (w/v) ammonium persulfate[c]	50 µl	25 µl
TEMED	10 µl	5 µl

Prepare separating and stacking gel solutions separately.

In a 50-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (Table 6.1.1), Tris·Cl/SDS, pH 8.45 (see reagents, below), and H_2O. Add glycerol to separating gel only. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix; use immediately.

ADDITIONAL REAGENTS USED IN GELS

Tris·Cl/SDS, pH 8.45 *(3.0 M Tris·Cl containing 0.3% w/v SDS)*

Dissolve 182 g Tris base in 300 ml H_2O. Adjust to pH 8.45 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter, add 1.5 g SDS, and store at 4°C up to 1 month.

[a]The recipes produce 30 ml of separating gel and 12.5 ml of stacking gel, which are adequate for two gels of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the Tris-tricine buffer system of Schagger and von Jagow (1987).
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

Additional Materials *(also see Basic Protocol 1)*

Separating and stacking gel solutions (Table 6.1.5)
2× tricine sample buffer (see recipe)
Peptide molecular weight standards (Table 6.1.6)
Cathode buffer (see recipe)
Anode buffer (see recipe)
Coomassie blue G-250 staining solution (see recipe)
10% (v/v) acetic acid
50-ml Erlenmeyer side-arm flasks

Table 6.1.6 Molecular Weights of Peptide Standards for Polyacrylamide Gel Electrophoresis[a]

Peptide	Molecular weight (Da)
Myoglobin (polypeptide backbone)	16,950
Myoglobin 1-131	14,440
Myoglobin 56-153	10,600
Myoglobin 56-131	8,160
Myoglobin 1-55	6,210
Glucagon	3,480
Myoglobin 132-153	2,510

[a]Peptide standards are commercially available from Sigma-Aldrich. See Sigma-Aldrich Technical Bulletin MWSDS70-L for molecular weight markers for proteins.

1. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 1 to 11) using the recipes in Table 6.1.5.
2. Prepare the sample (see Basic Protocol 1, step 12), but substitute 2× tricine sample buffer for the 2× SDS sample buffer, and heat the sample at 40°C for 30 to 60 min to improve solubilization prior to loading. Use peptide molecular weight standards (Table 6.1.6).

   If proteolytic activity is a problem (see Basic Protocol 1, step 12), heating samples to 100°C for 3 to 5 min may be required.
3. Set up the electrophoresis apparatus and load the gel (see Basic Protocol 1, steps 13 to 20), but use cathode buffer or water to rinse the wells, use cathode buffer in the upper buffer chamber, and use anode buffer in the lower buffer chamber.

   The cathode buffer contains the tricine.
4. Connect the power supply to the cell and run 1 hr at 30 V (constant voltage) followed by 4 to 5 hr at 150 V (constant voltage). Use a heat exchanger to keep the electrophoresis chamber at room temperature.
5. After the tracking dye has reached the bottom of the separating gel, disconnect the power supply.

   Refer to Safety Considerations under Electricity and Electrophoresis.

   Coomassie blue G-250 is used as a tracking dye instead of bromphenol blue because it moves ahead of the smallest peptides.
6. Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Stain proteins in the gel for 1 to 2 hr in Coomassie blue G-250 staining solution. Follow by destaining with 10% acetic acid, changing the solution every 30 min until background is clear (3 to 5 changes). For higher sensitivity, use silver staining as a recommended alternative.

   Prolonged staining and destaining will result in the loss of resolution of the smaller proteins (<10 kDa). Proteins diffuse within the gel and out of the gel, resulting in a loss of staining intensity and resolution.

ALTERNATE PROTOCOL 2

NONUREA PEPTIDE SEPARATIONS WITH TRIS BUFFERS

A simple modification of the traditional Laemmli buffer system presented in Basic Protocol 1, in which the increased concentration of buffers provides better separation between the stacked peptides and the SDS micelles, permits reasonable separation of peptides as small as 5 kDa.

Additional Materials *(also see Basic Protocol 1)*

Separating and stacking gel solutions (Table 6.1.7)
2× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 6.1.1)
2× SDS electrophoresis buffer (see recipe)

1. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 1 to 11), using the modified recipes in Table 6.1.7. After removing the isobutyl alcohol overlay from the separating gel, rinse with 2× Tris·Cl/SDS, pH 8.8, rather than 1× Tris·Cl/SDS.

2. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20), but substitute 2× SDS electrophoresis buffer for the 1× SDS electrophoresis buffer.

Table 6.1.6 lists the standards for small protein separations.

Table 6.1.7 Recipes for Modified Laemmli Peptide Separating and Stacking Gels[a]

SEPARATING AND STACKING GELS

Stock solution[b]	Separating gel	Stacking gel
30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	10.00 ml	0.65 ml
8× Tris·Cl, pH 8.8	3.75 ml	—
4× Tris·Cl, pH 6.8	—	1.25 ml
10% (w/v) SDS[c]	0.15 ml	50 µl
H_2O	1.00 ml	3.00 ml
10% (w/v) ammonium persulfate[c]	50 µl	25 µl
TEMED	10 µl	5 µl

Prepare separating and stacking gel solutions separately.

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 8× Tris·Cl, pH 8.8, or 4× Tris·Cl, pH 6.8 (see reagents below), 10% SDS, and H_2O. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

ADDITIONAL REAGENTS USED IN GELS

4× Tris·Cl, pH 6.8 *(0.5 M Tris·Cl)*

Dissolve 6.05 g Tris base in 40 ml H_2O. Adjust to pH 6.8 with 1 N HCl. Add H_2O to 100 ml total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at 4°C.

8× Tris·Cl, pH 8.8 *(3.0 M Tris·Cl)*

Dissolve 182 g Tris base in 300 ml H_2O. Adjust to pH 8.8 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at 4°C.

[a]The recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the modified Laemmli peptide separation system of Okajima et al. (1993).
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

3. Run the gel (see Basic Protocol 1, steps 21 and 22).

   Note that the separations will take ~25% longer than those using Basic Protocol 1. The increased buffer concentrations lead to faster transit through the stacking gel but lower mobility in the resolving gel.

4. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

   Proteins in the gel may now be stained.

ALTERNATE PROTOCOL 3

CONTINUOUS SDS-PAGE

With continuous SDS-PAGE, the same buffer is used for both the gel and electrode solutions. Although continuous gels lack the resolution of the discontinuous systems, they are extremely versatile, less prone to mobility artifacts, and much easier to prepare. The stacking gel is omitted.

Additional Materials *(also see Basic Protocol 1)*

Separating gel solution (Table 6.1.8)
2× and 1× phosphate/SDS sample buffer (see recipe)
1× phosphate/SDS electrophoresis buffer (see recipe)

Table 6.1.8 Recipes for Separating Gels for Continuous SDS-PAGE[a]

SEPARATING GEL

	Final acrylamide concentration in the separating gel (%)[c]										
Stock solution[b]	5	6	7	8	9	10	11	12	13	14	15
30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	2.5	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50	7.00	7.50
4× phosphate/SDS, pH 7.2	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25	3.75
10% (w/v) ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× phosphate/SDS, pH 7.2, and H_2O. Degas under vacuum about 5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

ADDITIONAL REAGENTS USED IN GELS

4× phosphate/SDS, pH 7.2 *(0.4 M sodium phosphate/0.4% w/v SDS)*

Mix 46.8 g $NaH_2PO_4 \cdot H_2O$, 231.6 g $Na_2HPO_4 \cdot 7H_2O$, and 12 g SDS in 3 liters H_2O. Store at 4°C for up to 3 months.

[a] The recipes produce 15 ml of separating gel, which is adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the original continuous phosphate buffer system of Weber et al. (1972).
[b] All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c] Volumes are in milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See Basic Protocol 1, annotation to step 3.
[d] Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

1. Prepare and pour a single separating gel (see Basic Protocol 1, steps 1 to 4), except use the recipe in Table 6.1.8 and fill the gel sandwich to the top. Omit the stacking gel. Insert the comb (see Basic Protocol 1, step 10) and allow the gel to polymerize 30 to 60 min at room temperature.

2. Mix the protein sample 1:1 with 2× phosphate/SDS sample buffer and heat to 100°C for 2 min.

   For large sample volumes or samples suspended in high-ionic-strength buffers (>50 mM), dialyze against 1× sample buffer prior to electrophoresis. Note that the precautions about proteases (see Basic Protocol 1, step 12) apply here.

3. Assemble the electrophoresis apparatus and load the sample (see Basic Protocol 1, steps 13 to 20) using the phosphate/SDS electrophoresis buffer. Load empty wells with 1× phosphate/SDS sample buffer.

4. Connect the power supply and start the run with 15 mA per 0.75-mm-thick gel until the tracking dye has entered the gel. Continue electrophoresis at 30 mA for 3 hr (5% gel), 5 hr (10% gel), 8 hr (15% gel), or until the dye reaches the bottom of the gel.

   Use temperature control if available to maintain the gel at 15° to 20°C. SDS will precipitate below 15°C in this system.

5. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

   See Safety Considerations in introduction. Proteins in the gel may now be stained.

CASTING AND RUNNING ULTRATHIN GELS

ALTERNATE PROTOCOL 4

Ultrathin gels provide superb resolution but are difficult to handle. In this application, gels are cast on GelBond, a Mylar support material. Silver staining is recommended for the best resolution. Combs and spacers for gels <0.5 mm thick are not readily available for most protein electrophoresis units. However, by adapting combs and spacers used for DNA sequencing, casting gels from 0.2 to 0.5 mm thick is straightforward.

Additional Materials *(also see Basic Protocol 1)*

95% (v/v) ethanol
GelBond (Lonza) cut to a size slightly smaller than the gel plate dimensions
Glue stick
Ink roller (available from art supply stores)
Combs and spacers (0.19 to 0.5 mm; sequencing gel spacers and combs can be cut to fit)

1. Wash gel plates with water-based laboratory detergent followed by successive rinses with hot tap water, deionized water, and finally 95% ethanol. Allow to air dry.

   Gel plates must be extremely clean for casting thin gels. Gloves are needed throughout these procedures to prevent contamination by proteins on the surface of skin.

2. Apply a streak of adhesive from a glue stick to the bottom edge of the glass plate. Quickly position the GelBond with the hydrophobic side down (a drop of water will bead up on the hydrophobic surface). Apply pressure with Kimwipe tissue to attach the GelBond firmly to the plate. Finally, pull the top portion of the GelBond back, place a few drops of water underneath, and roll flat with an ink roller.

   Make sure the GelBond does not extend beyond the edges of the upper and lower sealing surface of the plate. This will cause it to buckle on sealing. Reposition the GelBond if needed to prevent it from extending beyond the glass plate. Material may also be trimmed to fit flush with the plate edge.

3. Assemble the gel cassette according to the manufacturer's instructions (also see Basic Protocol 1, steps 1 and 2). Just prior to assembly, blow air over the surface of both the GelBond and the opposing glass surface to remove any particulate material (e.g., dust).

   Sequencing gel spacers can be easily adapted. First, cut the spacers slightly longer than the length of the gel plate. Position a spacer along each edge of the glass plate and assemble the gel sandwich, clamping in place. With a razor blade, trim the excess spacer at top and bottom to get a reusable spacer exactly the size of the plate.

4. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 3 to 9). In place of the Teflon comb, insert a square well sequencing comb cut to fit within the gel sandwich. Allow the stacking gel to polymerize 30 to 45 min at room temperature.

   Less solution is needed for ultrathin gels. For example, a 0.5-mm-thick gel requires 33% less gel solution than a 0.75-mm gel.

5. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20).

   When preparing protein samples for ultrathin gels, 3 to 4 μl at 5 μg protein/μl is required for Coomassie blue R-250 staining, whereas 10-fold less is needed for silver staining.

6. Run the gel (see Basic Protocol 1, steps 21 and 22), except conduct the electrophoresis at 7 mA/gel (0.25-mm-thick gels) or 14 mA/gel (0.5-mm-thick gels) for 4 to 5 hr.

7. When the separation is complete, disassemble the unit and remove the gel (see Basic Protocol 1, steps 23 to 26) with the GelBond still attached. With a gloved hand, wash away the adhesive material from the back of the GelBond under a stream of water before proceeding to protein detection.

   Either Coomassie blue or silver staining may be used, but silver staining produces particularly fine resolution with thin GelBond-backed gels. Compared to staining thicker (>0.75 mm) gels, thin (<0.75 mm) gels stain and destain more quickly. Although the optimum staining times must be empirically determined, all steps in Coomassie blue and silver staining procedures are generally reduced by half.

SUPPORT PROTOCOL 1

CASTING MULTIPLE SINGLE-CONCENTRATION GELS

Casting multiple gels at one time has several advantages. All the gels are identical, so sample separation is not affected by gel-to-gel variation. Furthermore, casting ten gels is only slightly more difficult than casting two gels. Once cast, gels can be stored for several days in a refrigerator.

Additional Materials *(also see Basic Protocol 1)*

Separating and stacking gels for single-concentration gels (Table 6.1.9)
Multiple gel caster (Bio-Rad, Hoefer)
100-ml disposable syringe and flat-tipped needle
Extra plates and spacers
14 × 14–cm acrylic blocks or polycarbonate sheets
250- and 500-ml side-arm flasks (used in gel preparation)
Long razor blade *or* plastic wedge (Wonder Wedge, Hoefer)
Resealable plastic bags

Pour the separating gel

1. Assemble the multiple gel caster according to the manufacturer's instructions.

   With the Hoefer unit, make sure to insert the large triangular space filler plug in the base of the caster. The plug is removed when casting gradient gels (see Support Protocol 2).

Table 6.1.9 Recipes for Multiple Single-Concentration Polyacrylamide Gels[a]

SEPARATING GEL

Stock solution[b]	Final acrylamide concentration in the separating gel (%)[c]										
	5	6	7	8	9	10	11	12	13	14	15
30% (w/v) acrylamide/ 0.8% (w/v) bisacrylamide	52	62	72	83	93	103	114	124	134	145	155
4× Tris·Cl/SDS, pH 8.8	78	78	78	78	78	78	78	78	78	78	78
H_2O	181	171	160	150	140	129	119	109	98	88	78
10% (w/v) ammonium persulfate[d]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TEMED	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21

Preparation of separating gel

In a 500-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× Tris·Cl/SDS, pH 8.8 (Table 6.1.1), and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

STACKING GEL

In a 250-ml side-arm flask, mix 13.0 ml 30% acrylamide/0.8% bisacrylamide solution, 25 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1), and 61 ml H_2O. Degas under vacuum ~5 min. Add 0.25 ml 10% ammonium persulfate and 50 µl TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce about 300 ml of separating gel and 100 ml of stacking gel, which are adequate for ten gels of dimensions 1.5 mm × 14 cm × 14 cm, with extra solution should there be a leak or spill while casting the gels. For thinner spacers or fewer gels, calculate volumes using the equation in the annotation to step 4. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Volumes in table body are in milliliters. The desired percentage of acrylamide in separating gel depends on the molecular size of the protein being separated. See Basic Protocol 1, annotation to step 3.
[d]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

2. Assemble glass sandwiches and stack them in the casting chamber. Stack up to ten 1.5-mm gels and fill in extra space with acrylic blocks or polycarbonate sheets to hold the sandwiches tightly in place. Make sure the spacers are straight along the top, right, and left edges of the glass plates and that all edges of the stack are flush.

   The presence of loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis. Polycarbonate inhibits gel polymerization. Therefore, if polycarbonate sheets are placed in the caster before and after the set of glass sandwiches, the entire set will slide out as one block after polymerization. Placing polycarbonate sheets between each gel sandwich makes them easier to separate from one another after polymerization.

3. Place the front sealing plate on the casting chamber, making sure the stack fits snugly. Secure the plate with four spring clamps and tighten the bottom thumb screws.

4. Prepare the separating (resolving) gel solution (Table 6.1.9).

   A 12-cm separating gel with a 4-cm stacking gel is recommended.

   If fewer than ten gels are prepared (Table 6.1.9), use the following formula to estimate the amount of separating gel volume needed:

   volume = gel no. × height (cm) × width (cm) × thickness (cm) + 4 × gel no. + 10 ml.

5. Using a 100-ml disposable syringe with flat-tipped needle, inject the resolving gel solution down the side of one spacer into the multiple caster. A channel in the silicone plug distributes the solution throughout the whole caster. Avoid introducing bubbles by giving the caster a quick tap on the benchtop once the caster is filled.
6. Overlay the center of each gel with 100 μl H_2O-saturated isobutyl alcohol and let polymerize for 1 to 2 hr.
7. Drain off the overlay and rinse the surface with 1× Tris·Cl/SDS, pH 8.8. If the gels will not be used immediately, skip to step 12.

Pour the stacking gel

8. Prepare the stacking gel solution either singly (see Basic Protocol 1, step 8) or for all the gels at once (Table 6.1.9).

   The stacking gel solution should be prepared just before it is poured.

9. Fill each sandwich in the caster with stacking gel solution.
10. Insert a comb into each sandwich and let the gel polymerize for 2 hr.

    Insert the combs at a 45° angle to avoid trapping air underneath the comb teeth. Air bubbles will inhibit polymerization and cause dents in the wells and a distorted pattern of protein bands.

11. Remove the combs and rinse wells with 1× SDS electrophoresis buffer.

Remove the gels from the caster

12. Remove the gels from the caster and separate by carefully inserting a long razor blade or knife between the gel sandwiches.

    A plastic wedge (Hoefer's Wonder Wedge) also works well.

13. Clean the outside of each gel plate with running water to remove the residual polymerized and unpolymerized acrylamide.
14. Overlay gels to be stored with 1× Tris·Cl/SDS, pH 8.8, place in a resealable plastic bag, and store at 4°C until needed (up to 1 week).

ALTERNATE PROTOCOL 5

SEPARATION OF PROTEINS ON GRADIENT GELS

Gels that consist of a gradient of increasing polyacrylamide concentration resolve a much wider size range of proteins than standard single-concentration gels (see Critical Parameters and Troubleshooting). The protein bands are also much sharper, particularly in the low-molecular-weight range. Unlike single-concentration gels, gradient gels separate proteins in a way that can be represented easily to give a linear plot from 10 to 200 kDa. This facilitates molecular weight estimations.

The quantities given below provide separating gel solution sufficient for two 0.75-mm gels (~7 ml of each concentration) or one 1.5-mm gel (~14 ml of each concentration). Volumes can be adjusted to accommodate gel sandwiches of different dimensions.

Additional Materials *(also see Basic Protocol 1)*

Light and heavy acrylamide gel solutions (Table 6.1.10)
Bromphenol blue (optional; for checking practice gradient)
10% ammonium persulfate (prepare fresh)
TEMED

Gradient maker (30 to 50 ml, Hoefer SG30 or SG50; *or* 30 to 100 ml, Bio-Rad 385)
Tygon tubing with micropipet tip
Peristaltic pump (optional; e.g., Markson A-13002, A-34040, or A-34105 minipump)
Whatman 3MM filter paper

Set up the gradient maker and prepare the gel solutions

1. Assemble the magnetic stirrer and gradient maker on a ring stand as shown in Figure 6.1.2. Connect the outlet valve of the gradient maker to Tygon tubing attached to a micropipet tip that is placed over the vertical gel sandwich. If desired, place a peristaltic pump in line between the gradient maker and the gel sandwich.

   A peristaltic pump will simplify casting by providing a smooth flow rate.

2. Place a small stir-bar into the mixing chamber of the gradient maker (i.e., the chamber connected to the outlet).

Table 6.1.10 Light and Heavy Acrylamide Gel Solutions for Gradient Gels

	Acrylamide concentration in light gel solution (%)[a,b]									
Stock solution	5	6	7	8	9	10	11	12	13	14
30% acrylamide/0.8% bisacrylamide[c]	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0
4× Tris·Cl/SDS, pH 8.8[c]	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25
10% ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED[d]	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005

	Acrylamide concentration in heavy gel solution (%)[a,b]										
Stock solution	10	11	12	13	14	15	16	17	18	19	20
30% acrylamide/0.8% bisacrylamide[c]	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
4× Tris·Cl/SDS, pH 8.8[c]	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0
Sucrose (g)	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25
10% ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED[d]	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005

[a] To survey proteins ≥10 kDa, 5% to 20% gradient gels are recommended. To expand the range between 10 and 200 kDa, a 10% to 20% gradient gel is recommended.
[b] Volumes are in milliliters (sucrose is in grams). Keep light gel solution at room temperature prior to use (no longer than 1 hr). Keep heavy solution on ice.
[c] See Table 6.1.1 for preparation.
[d] Ammonium persulfate and TEMED are added directly to the gradient chambers immediately before the gel is poured. It is best to prepare ammonium persulfate fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

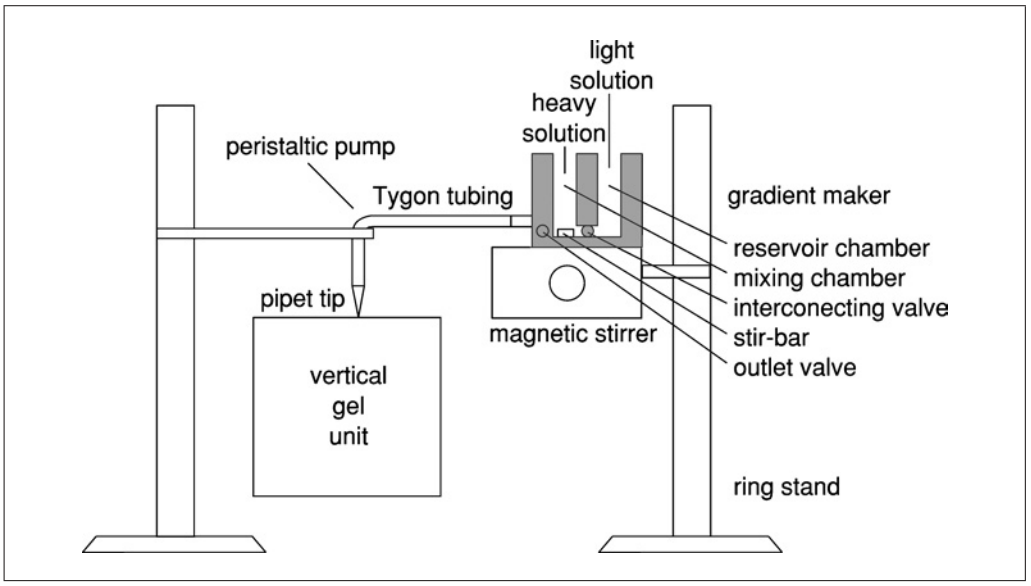


Figure 6.1.2 Gradient gel setup. A peristaltic pump, though not required, will provide better control.

3. Using the recipes in Table 6.1.10, prepare light and heavy acrylamide gel solutions *without* ammonium persulfate or TEMED.

   Recommended gradient ranges are 5% to 20% for most applications (to separate proteins of 10 to several hundred kilodaltons).

   Deaeration is not recommended for either the light or heavy solution. Omitting the deaeration will allow polymerization to proceed more slowly, letting the gradient establish itself in the gel sandwich before polymerization takes place.

   Keep the heavy solution on ice until use. Once the ammonium persulfate is added to the heavy solution, it will polymerize without TEMED, albeit more slowly; keeping the solution on ice prevents this. The gel solution will come to room temperature during casting. The higher the percentage of acrylamide, the more severe the problem of premature polymerization.

4. With the outlet port and interconnecting valve between the two chambers closed, pipet 7 ml of light (low-concentration) acrylamide gel solution into the reservoir chamber for one 0.75-mm-thick gradient gel.

   A practice run with heavy and light solutions is recommended. Bromphenol blue should be added to the heavy solution to demonstrate linearity of the practice gradient.

5. Open the interconnecting valve briefly to allow a small amount (~200 µl) of light solution to flow through the valve and into the mixing chamber.

   The presence of air bubbles in the interconnecting valve may obstruct the flow between chambers during casting.

6. Add 7 ml of heavy (high-concentration) acrylamide gel solution to the mixing chamber.

7. Add 23 µl of 10% ammonium persulfate and ~2.3 µl TEMED per 7 ml acrylamide solution to each chamber. Mix the solutions in each chamber with a disposable pipet.

Form the gradient and cast the gel

8. Open the interconnecting valve completely.

   Some of the heavy solution will flow back into the reservoir chamber containing light solution as the two chambers equilibrate. This will not affect the formation of the gradient.

9. Turn on the magnetic stirrer and adjust the rate to produce a slight vortex in the mixing chamber.

10. Open the outlet of the gradient maker slowly. Adjust the outlet valve to a flow rate of 2 ml/min. If using a peristaltic pump, calibrate the flow rate with a graduated cylinder prior to casting the gel.

    Some adjustment of the flow rate may be necessary during casting. If the light solution is not flowing into the mixing chamber, a bubble may be caught in the interconnecting valve. Quickly close the outlet and cover the top of the reservoir chamber with a gloved thumb. Push down with the thumb to increase the pressure in the chamber and force the air bubble out of the center valve.

11. Fill the gel sandwich from the top. Place the pipet tip against one side of the sandwich so the solution flows down one plate only. The heavy solution will flow into the sandwich first, followed by progressively lighter solution.

12. Watch as the last of the light solution drains into the outlet tube and adjust the flow rate to ensure that the last few milliliters of solution do not flow quickly into the gel sandwich and disturb the gradient.

13. Overlay the gradient gel with H_2O-saturated isobutyl alcohol. Allow the gel to polymerize ~1 hr.

    In this gel system, the gel will polymerize from the bottom (i.e., heavy solution) up. Because polymerization is an exothermic reaction, heat can be felt evolving from the bottom of the gel sandwich during polymerization. A sharp optical discontinuity at the gel-overlay interface indicates that polymerization has occurred. In general, 1 hr is adequate for polymerization.

14. Remove the H_2O-saturated isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8. Cast the stacking gel (see Basic Protocol 1, steps 8 to 11).

    The gel can be covered with 1× Tris·Cl/SDS, pH 8.8, sealed in a plastic bag, and stored for up to 1 week.

Load and run the gel

15. Prepare the protein sample and protein molecular-weight-standards mixture. Load and run the gel (see Basic Protocol 1, steps 13 to 26).

    The gel can be stained with Coomassie blue or silver (UNIT 6.6).

16. After staining, dry the gels onto Whatman 3MM or equivalent filter paper.

    Gradient gels >0.75 mm thick require special handling during drying to prevent cracking. The simplest approach to drying gradient gels is to use thin gels; ≤0.75-mm gradient gels with ≤20% acrylamide solutions will dry without cracking as long as the vacuum pump is working properly and the cold trap is dry at the onset of drying. For gradient gels >0.75 mm thick, add 3% (w/v) glycerol to the final destaining solution to help prevent cracking. Another method is to dehydrate and shrink the gel in 30% methanol for up to 3 hr prior to drying. Then place the gel in distilled water for 5 min before drying.

CASTING MULTIPLE GRADIENT GELS

SUPPORT PROTOCOL 2

Casting gradient gels in a multiple gel caster has several advantages. In addition to the time savings, batch casting produces gels that are essentially identical. This is particularly important for gradient gels, where slight variations in casting technique can cause variations in protein mobility. The gels may be stored for up to 1 week after casting to ensure internal consistency from run to run during the week. Furthermore, gels with several ranges of concentrations (e.g., 5% to 20% and 10% to 20% acrylamide) can be cast and stored, giving much more flexibility to optimize separations.

Additional Materials *(also see Alternate Protocol 5)*

Plug solution (see recipe)
Light and heavy acrylamide gel solutions for multiple gradient gels (Table 6.1.11)
TEMED
H_2O-saturated isobutyl alcohol

Multiple gel caster (Bio-Rad, Hoefer)
Peristaltic pump (25 ml/min)
500- or 1000-ml gradient maker (Bio-Rad, Hoefer)
Tygon tubing

Set up system and pour separating gel

1. Assemble the multiple caster as in casting multiple single-concentration gels (see Support Protocol 1, steps 1 to 3), making sure to remove the triangular space filler plugs in the bottom of the caster.

   The plug is used only when casting single-concentration gels.

Table 6.1.11 Light and Heavy Acrylamide Gel Solutions for Casting Multiple Gradient Gels

	Acrylamide concentration in light gel solution (%)[a,b]									
Stock solution	5	6	7	8	9	10	11	12	13	14
30% acrylamide/0.8% bisacrylamide[c]	28	33	39	44	50	55	61	66	72	77
4× Tris·Cl/SDS, pH 8.8[c]	41	41	41	41	41	41	41	41	41	41
H_2O	96	91	85	80	74	69	63	58	52	47
10% ammonium persulfate[d]	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
TEMED	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054

	Acrylamide concentration in heavy gel solution (%)[a,b]										
Stock solution	10	11	12	13	14	15	16	17	18	19	20
30% acrylamide/0.8% bisacrylamide[c]	55	61	66	72	77	83	88	94	99	105	110
4× Tris·Cl/SDS, pH 8.8[c]	41	41	41	41	41	41	41	41	41	41	41
H_2O	55	50	44	39	33	28	22	17	11	5.5	0
Sucrose (g)	25	25	25	25	25	25	25	25	25	25	25
10% ammonium persulfate[d]	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
TEMED	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054

[a]To survey proteins ≥10 kDa, 5% to 20% gradient gels are recommended. To expand the range between 10 and 200 kDa, a 10% to 20% gradient gel is recommended.
[b]Volumes are in milliliters (sucrose is in grams). Recipes produce ten 1.5-mm-thick gradient gels with 10 ml extra solution to account for losses in tubing. Keep light gel solution at room temperature prior to use (no longer than 1 hr). Keep heavy solution on ice.
[c]See Table 6.1.1 for preparation.
[d]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

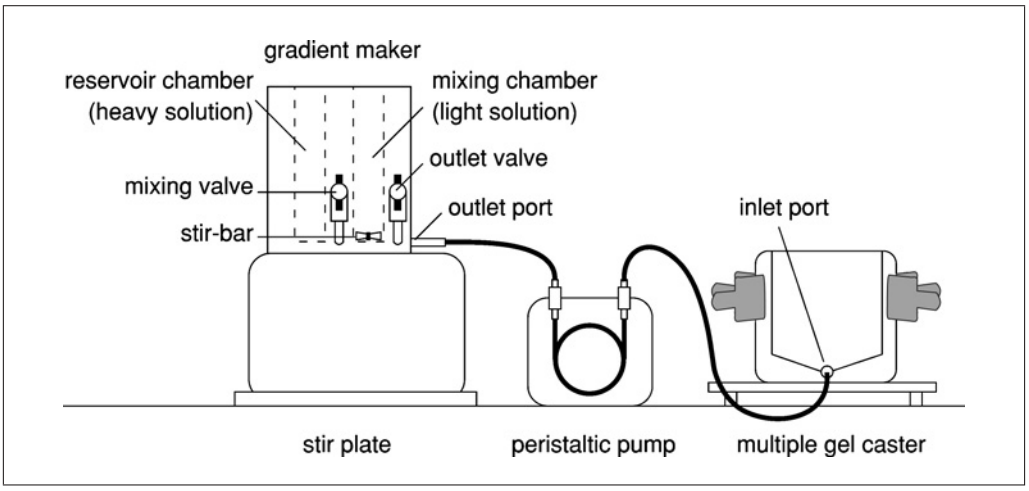


Figure 6.1.3 Setup for casting multiple gradient gels. Casting multiple gradient gels requires a peristaltic pump and a multiple gel caster. Gel solution is introduced through the bottom of the multiple caster.

2. Set up the peristaltic pump (Fig. 6.1.3). Using a graduated cylinder and water, adjust the flow rate so that the volume of the gradient solution plus volume of plug solution is poured in ~15 to 18 min (~25 ml/min).

3. Set up the gradient maker. Close all valves and place a stir-bar in the mixing chamber, which is the one with the outlet port. Attach one end of a piece of Tygon tubing to the outlet of the gradient maker. Run the other end of the tubing through the peristaltic pump and attach it to the red inlet port at the bottom of the caster.

   Choose a gradient maker that holds no more than four times the total volume of the gradient solution to be poured (i.e., a 1000- or 500-ml gradient maker).

4. Prepare solutions for the gradient maker (Table 6.1.11).

   Deaeration is not recommended for gradient gels.

5. *Immediately* after adding TEMED to the gel solutions, pour the light (low-concentration) solution into the mixing chamber (the one with the port). Open the mixing valve slightly to allow the tunnel to fill and to avoid air bubbles. Close the valve again and pour the heavy (high-concentration) acrylamide solution into the reservoir chamber.

6. Start the magnetic stirrer and open the outlet valve; then start the pump and open the mixing valve.

   In units for casting multiple gels, acrylamide solution flows in from the bottom. To use a multiple casting unit, the light solution is placed in the mixing chamber and the heavy solution in the reservoir. This is the reverse of casting a single gel (see Alternate Protocol 5). Thus, light solution enters the multiple caster first, followed by progressively heavier solution. Finally, the acrylamide solution is stabilized in the multiple caster with a heavy plug solution and allowed to polymerize (see step 8 and manufacturer's instructions).

7. When almost all the acrylamide solution is gone from the gradient maker, stop the pump and close the mixing valve. Tilt the gradient maker toward the outlet side and remove the last milliliters of the mix. Do not allow air bubbles to enter the tubing.

8. Add the plug solution to the mixing chamber and start the pump. Make sure that no bubbles are introduced. Continue pumping until the bottom of the caster is filled with plug solution to just below the glass plates; then turn off the pump. Clamp the tubing close to the red port of the casting chamber.

9. Quickly overlay each separate gel sandwich with 100 µl H_2O-saturated isobutyl alcohol. Use the same amount on each sandwich. Allow the gels to polymerize for ~1 hr.

   Failure to use the same amount of overlay solution will cause the gel sandwiches to polymerize at different heights.

10. Drain off the overlay and rinse the surface of the gels with 1× Tris·Cl/SDS, pH 8.8.

Pour stacking gel and remove gels from caster

11. Prepare and cast the stacking gel as in casting multiple single-concentration gels (see Support Protocol 1, steps 8 to 11).

12. Remove gels from the caster and clean the gel sandwiches (see Support Protocol 1, steps 12 and 13). Store gels, if necessary, according to the instructions for multiple single-concentration gels (see Support Protocol 1, step 14).

BASIC PROTOCOL 2

ELECTROPHORESIS IN SINGLE-CONCENTRATION MINIGELS

Separation of proteins in a small-gel format is becoming increasingly popular for applications that range from isolating material for peptide sequencing to performing routine protein separations. The unique combination of speed and high resolution is the foremost advantage of small gels. Additionally, small gels are easily adapted to single-concentration, gradient, and two-dimensional SDS-PAGE procedures. The minigel procedures described are adaptations of larger gel systems.

This protocol describes the use of a multiple gel caster. The caster is simple to use, and up to five gels can be prepared at one time with this procedure. Single gels can be prepared using adaptations in the manufacturer's instructions. A multiple gel caster is the only practical way to produce small linear polyacrylamide gradient gels (see Support Protocol 3).

Materials

Minigel vertical gel unit (Hoefer Mighty Small SE 250/280 *or* Bio-Rad Mini-Protean II) with glass plates, clamps, and buffer chambers
0.75-mm spacers
Multiple gel caster (Hoefer SE-275/295 *or* Bio-Rad Mini-Protean II multicasting chamber)
Acrylic plate (Hoefer SE-217 *or* Bio-Rad 165-1957) or polycarbonate separation sheet (Hoefer SE-213 *or* Bio-Rad 165-1958)
10- and 50-ml syringes
Combs (Teflon, Hoefer SE-211A series or Bio-Rad Mini-Protean II)
Long razor blade
Micropipet
Additional reagents and equipment for standard denaturing SDS-PAGE (see Basic Protocol 1)

Pour the separating gel

1. Assemble each gel sandwich by stacking, in order, the notched (Hoefer) or small rectangular (Bio-Rad) plate, 0.75-mm spacers, and the larger rectangular plate. Be sure to align the spacers properly, with the ends flush with the top and bottom edge of the two plates, when positioning the sandwiches in the multiple gel caster (Fig. 6.1.4).

   The protocol described is basically for the Hoefer system. For other systems, make adjustments according to the manufacturer's instructions. Alternatively, precast minigels can be purchased from a number of suppliers (see Table 6.1.4).

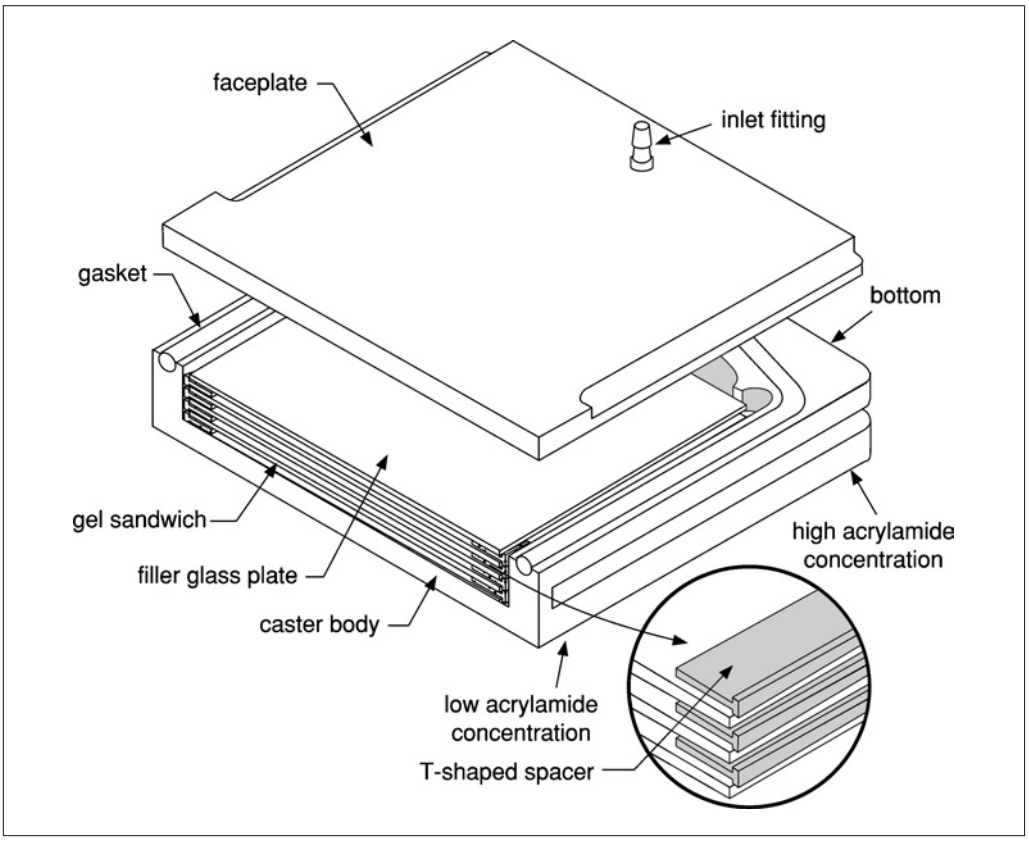


Figure 6.1.4 Minigel sandwiches positioned in the multiple gel caster. Extra glass or acrylic plates or polycarbonate sheets are used to fill any free space in the caster and to ensure that the gel sandwiches are held firmly in place.

The multiple casters from Hoefer have a notch in the base designed for casting gradient gels. A silicone rubber insert fills up this space when casting single-concentration gels. The Hoefer spacers are T-shaped to prevent slipping. The flanged edge of the spacer must be positioned against the outside edge of the glass plate. Placing a sheet of wax paper between the gel sandwiches will help separate the sandwiches after polymerization.

2. Fit the gel sandwiches tightly in the multiple gel caster. Use an acrylic plate or polycarbonate separation sheet to eliminate any slack in the chamber.

   Loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis.

3. Place the front faceplate on the caster, clamp it in place against the silicone gasket, and verify alignment of the glass plates and spacers.

4. Prepare the separating gel solution as directed in Table 6.1.1. For five 0.75-mm-thick gels, prepare ~30 ml solution (i.e., double the volumes listed).

   To compute the total gel volume needed, multiply the area of the gel (e.g., 7.3 × 8.3 cm) by the thickness of the gel (e.g., 0.75 mm) and then by the number of gels in the caster. If needed, add ~4 to 5 ml of extra gel solution to account for the space around the outside of the gel sandwiches.

   Do not add TEMED and ammonium persulfate until just before use.

5. Fill a 50-ml syringe with the separating gel solution and slowly inject it into the caster until the gels are 6 cm high, allowing 1.5 cm for the stacking gel.

6. Overlay each gel with 100 µl H_2O-saturated isobutyl alcohol. Allow the gels to polymerize for ~1 hr.

Pour the stacking gel

7. Remove the isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8.

   Stacking gels can be cast one at a time with the gel mounted on the electrophoresis unit, or all at once in the multiple caster.

8. Practice placing a comb in the gel sandwiches before preparing the stacking gel solution. Press the comb against the rectangular or taller plate so that all teeth of the comb are aligned with the opening in the gel sandwich, then insert into the sandwich. Remove combs after practicing.

9. Prepare the stacking gel solution (2 ml per gel) as directed in Table 6.1.1. Fill a 10-ml syringe with stacking gel solution and inject the solution into each gel sandwich.

10. Insert combs, taking care not to trap bubbles. Allow the gels to polymerize 1 hr.

11. Remove the front faceplate. Carefully pull the gels out of the caster, using a long razor blade to separate the sandwiches.

    If the gels are left to polymerize for prolonged periods, they will be difficult to remove from the caster.

    The gels can be stored tightly wrapped in plastic wrap with the combs left in place inside a sealable bag to prevent drying for ∼1 week. Without the stacking gel, the separating gel can be stored for 2 to 3 weeks. Keep gels moist with 1× Tris·Cl/SDS, pH 8.8, at 4°C. Do not store gels in the multiple caster.

Prepare the sample, load the gel, and conduct electrophoresis

12. Remove the combs and rinse the sample wells with 1× SDS electrophoresis buffer. Place a line indicating the bottom of each well on the front glass plate with a marker.

13. Fill the upper and lower buffer chambers with 1× SDS electrophoresis buffer. The upper chamber should be filled to 1 to 2 cm over the notched plate.

14. Prepare the protein sample and protein standards mixture (see Basic Protocol 1, step 12).

15. Load the sample using a micropipet. Insert the pipet tip through the upper buffer and into the well. The mark on the glass plate will act as a guide. Dispense the sample into the well.

    For a complex mixture, 20 to 25 μg protein in 10 μl SDS sample buffer will give a strongly stained Coomassie blue pattern. Much smaller amounts (1 to 5 μg) are required for highly purified proteins, and a 10- to 100-fold smaller amount of protein in the same volume (e.g., 10 μl) is required for silver staining.

16. Electrophorese samples at 10 to 15 mA per 0.75-mm gel until the dye front reaches the bottom of the gel (∼1 to 1.5 hr).

17. Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Proceed with detection of proteins.

SUPPORT PROTOCOL 3

PREPARING MULTIPLE GRADIENT MINIGELS

Polyacrylamide gradients not only enhance the resolution of larger-format gels but also greatly improve protein separation in the small format. Casting gradient minigels one at a time is not generally feasible because of the small volumes used, but multiple gel casters make it easy to cast several small gradient gels at one time. The gels are cast from the bottom in multiple casters, with the light acrylamide solution entering first. This is the opposite of casting one gel at a time, in which the heavy solution enters from the top of the gel sandwich and flows down to the bottom.

Additional Materials *(also see Basic Protocol 2)*

Plug solution (see recipe)

Additional reagents and equipment for preparing gradient gels (see Alternate Protocol 5)

Set up the system and prepare the gel solutions

1. Assemble minigel sandwiches in the multiple gel caster as described for single-concentration minigels (see Basic Protocol 2, steps 1 to 3).

2. Set up the 30-ml gradient maker, magnetic stirrer, peristaltic pump (optional), and Tygon tubing as in Figure 6.1.3. Connect the outlet of the 30-ml gradient maker to the inlet at the base of the front faceplate of the caster.

   The monomer solution will be introduced through the inlet at the bottom of the front faceplate of the caster first, followed by progressively heavier solution.

3. Prepare light and heavy acrylamide gel solutions (Table 6.1.10). Use ~12 ml of each solution for five 0.75-mm-thick minigels.

   Adjust volumes if a different thickness or number of gels is needed. Do not add ammonium persulfate until just before use. Deaeration is not recommended for gradient gels.

4. With the outlet and interconnecting valve closed, add the heavy solution to the reservoir chamber. Briefly open the interconnecting valve to let a small amount of heavy solution through to the mixing chamber, clearing the valve of air.

5. Fill the mixing chamber with light solution. Add 4 µl TEMED per 12 ml acrylamide solution to each chamber and mix with a disposable pipet.

Form the gradient and cast the gels

6. Turn on the magnetic stirrer. Open the interconnecting valve and allow the chambers to equilibrate. Then slowly open the outlet port to allow the solution to flow from the gradient maker to the multiple caster by gravity (a peristaltic pump may be used for better control). Adjust the flow rate to 3 to 4 ml/min.

   Faster flow rates are possible and will also produce good gradients. However, a fast flow increases the potential for introduction of bubbles into the caster.

7. Close the outlet port as the last of the gradient solution leaves the mixing chamber, just before air enters the outlet tube. Fill the two chambers with plug solution and slowly open the outlet once again.

8. Allow the plug solution to push the acrylamide in the caster up into the plates. Close the outlet when the plug solution reaches the bottom of the plates.

   A discontinuity between the bottom of the gels and the plug solution will be obvious.

9. Quickly add 100 µl H_2O-saturated isobutyl alcohol to each gel sandwich. Let the gels polymerize undisturbed for ~1 hr.

10. Prepare and pour the stacking gel (see Basic Protocol 2, steps 9 and 10).

Disassemble the system

11. Disconnect the gradient maker, place the caster in a sink, and remove the front faceplate. The plug solution will drain out from the bottom of the caster.

12. Remove the gels (see Basic Protocol 2, step 11).

    Gradient minigels can be stored as described for single-concentration minigels (see Basic Protocol 2, step 11 annotation). For instructions on preparing, loading, and running the gels, see Basic Protocol 2, steps 12 to 17.

SUPPORT PROTOCOL 4

CALCULATING MOLECULAR MASS

Determining the molecular mass of an unknown protein or nucleic acid fragment is straightforward given the use of calibration size standards in the same gel. Typically, a tracking dye such as bromphenol blue is added to the sample prior to loading on the gel (e.g., see recipe for SDS sample buffer). The tracking dye moves ahead of the proteins and serves as a relative mobility marker. A set of protein standards is separated in the same gel as the protein sample containing the unknown. The standards are used to create a standard curve of relative mobility versus size or molecular mass. Although digital image analysis has greatly simplified calculating the mass of an unknown protein separated by electrophoresis, manual assessments of molecular mass are useful and use the same basic calculations.

1. Calculate the relative mobility (R_f) using following formula:

   R_f = distance migrated by protein/distance migrated by marker.

   Placing a molecular mass/R_f mobility acetate overlay calculator (Fig. 6.1.5) on the gel is a quick way to determine R_f. Simply align the top and bottom of the overlay with the top of the gel and the dye front, respectively, to get a read out of R_f.

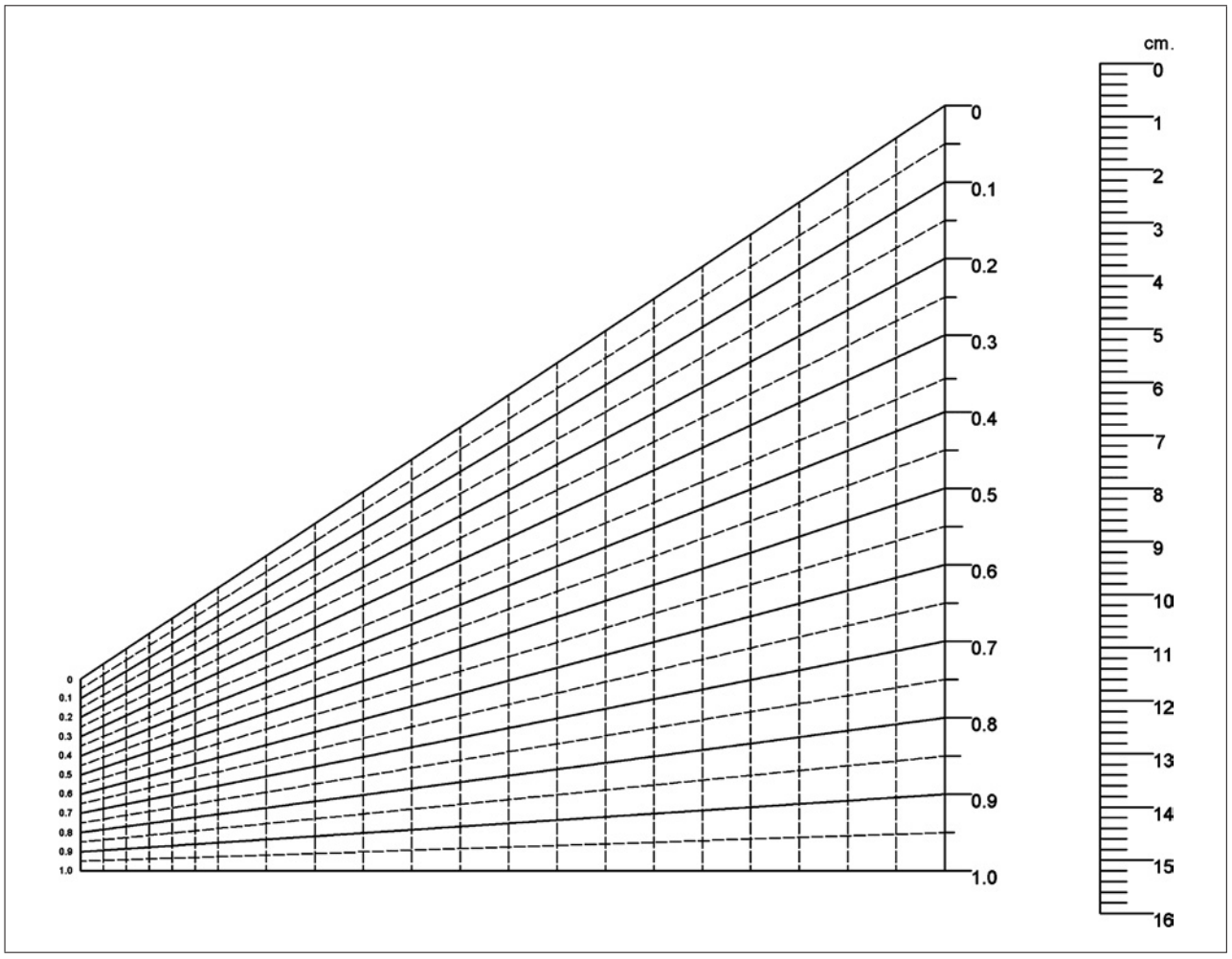


Figure 6.1.5 Example of an R_f calculator. This sheet is copied to transparency film using a paper copier and used as an overlay on the gel. When the transparency is placed on top of the gel, so that the top of the gel aligns with the top of the calculator and the dye front aligns with the bottom of the calculator, the R_f can be read directly off the overlay. Note that the calculator accommodates a range of gel lengths. The overlay should be copied at a 1:1 ratio so that the centimeter scale remains accurate. However, as long as the overlay can fit the top and bottom of the gel, the R_f numbers will be accurate.

2. Plot log protein mass on the *y* axis versus relative mobility of the standards on the *x* axis (Fig. 6.1.6).
3. Perform linear regression using a calculator or analysis program.
4. Use the linear regression equation ($y = mx + b$) to estimate the mass of the unknown:

   Log molecular weight = (slope)(mobility of unknown) + *y* intercept.

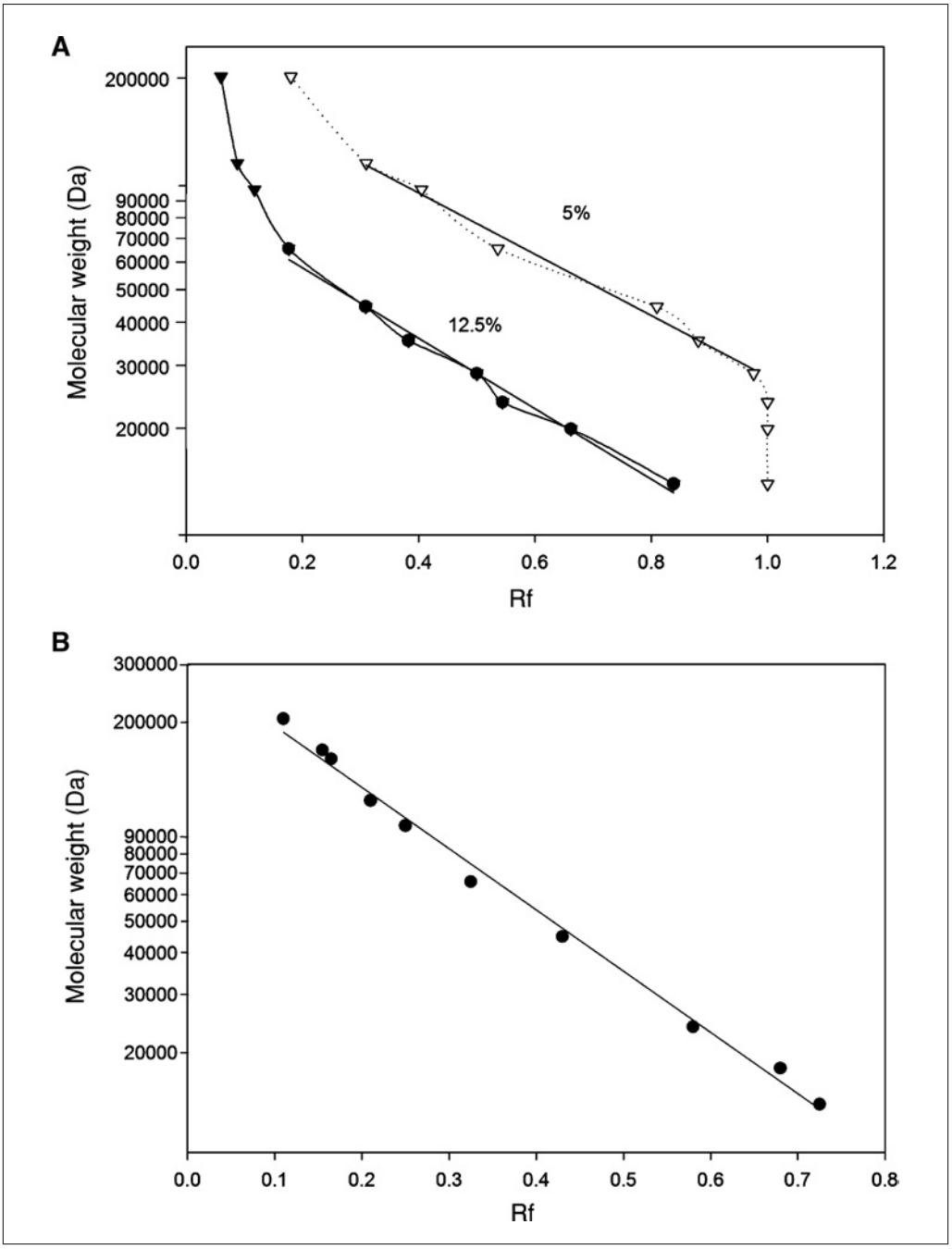


Figure 6.1.6 Standard protein molecular weight curves for **(A)** single concentration (5% and 12.5%) and **(B)** gradient (5% to 20%) gels. Protein standards are separated via SDS-PAGE, visualized by staining with Coomassie blue (*UNIT 6.6*), and measured relative to the dye front to give the relative mobility (R_f). Note the single-concentration gel has a more limited range of linearity than the gradient gel. The standard curve permits the calculation of the molecular weight of an unknown by using the R_f of the unknown to predict the molecular weight.

REAGENTS AND SOLUTIONS

Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Anode buffer

121.1 g Tris base (0.2 M final)
500 ml H_2O
Adjust to pH 8.9 with concentrated HCl
Dilute to 5 liters with H_2O
Store at 4°C up to 1 month

Final concentration is 0.2 M Tris·Cl, pH 8.9.

Cathode buffer

12.11 g Tris base (0.1 M final)
17.92 g tricine (0.1 M final)
1 g SDS [0.1% w/v final; recrystallization (see recipe) optional]
Dilute to 1 liter with H_2O
Do not adjust pH
Store at 4°C up to 1 month

Coomassie blue G-250 staining solution

200 ml acetic acid (20% v/v final)
1800 ml H_2O
0.5 g Coomassie blue G-250 (0.025% w/v final)
Mix 1 hr and filter (Whatman no. 1 paper)
Store at room temperature indefinitely

Phosphate/SDS electrophoresis buffer

Dilute 500 ml of 4× phosphate/SDS, pH 7.2 (Table 6.1.8) with H_2O to 2 liters. Store at 4°C up to 1 month.

Final concentrations are 0.1 M sodium phosphate (pH 7.2)/0.1% (w/v) SDS.

Phosphate/SDS sample buffer, 2× (for continuous systems)

0.5 ml 4× phosphate/SDS, pH 7.2 (Table 6.1.8; 20 mM sodium phosphate final)
0.2 g SDS [2% w/v final; recrystallization (see recipe) optional]
0.1 mg bromphenol blue (0.001% w/v final)
0.31 g DTT (0.2 M final)
2.0 ml glycerol (20% v/v final)
Add H_2O to 10 ml and mix

Plug solution

0.125 M Tris·Cl, pH 8.8 (*APPENDIX 2A*)
50% (w/v) sucrose
0.001% (w/v) bromphenol blue
Store at 4°C up to 1 month

Recrystallized SDS (optional)

High-purity SDS is available from several suppliers, but for some sensitive applications (e.g., protein sequencing) recrystallization is useful. Commercially available electrophoresis-grade SDS is usually of sufficient purity for most applications.

continued

Add 100 g SDS to 450 ml ethanol and heat to 55°C. While stirring, gradually add 50 to 75 ml hot H_2O until all SDS dissolves. Add 10 g activated charcoal (Norit 1, Sigma) to solution. After 10 min, filter solution through Whatman no. 5 paper on a Buchner funnel to remove charcoal. Chill filtrate 24 hr at 4°C and 24 hr at −20°C. Collect crystalline SDS on a coarse-frit (porosity A) sintered-glass funnel and wash with 800 ml −20°C ethanol (reagent grade). Repeat crystallization without adding activated charcoal. Dry recrystallized SDS under vacuum overnight at room temperature. Store in a desiccator over phosphorous pentoxide (P_2O_5) in a dark bottle.

If proteins will be electroeluted or electroblotted for protein sequence analysis, it may be desirable to crystallize the SDS twice from ethanol/H_2O (Hunkapiller et al., 1983).

SDS electrophoresis buffer, 5×

15.1 g Tris base (0.125 M final)
72.0 g glycine (0.96 M final)
5.0 g SDS [0.5% w/v final; recrystallization (see recipe) optional]
H_2O to 1000 ml
Dilute to 1× or 2× for working solution, as appropriate

Do not adjust the pH of the stock solution, as the solution is pH 8.3 when diluted. Store at 0° to 4°C until use (up to 1 month).

SDS sample buffer, 2× (for discontinuous systems)

25 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1)
20 ml glycerol (20% final)
4 g SDS [4% w/v final; recrystallization (see recipe) optional]
2 ml 2-ME *or* 3.1 g DTT (0.2% v/v 2-ME or 0.2 M DTT final)
1 mg bromphenol blue (0.001% w/v final)
Add H_2O to 100 ml and mix
Store in 1-ml aliquots at −70°C

To avoid reducing proteins to subunits (if desired), omit 2-ME or DTT (reducing agent) and add 10 mM iodoacetamide to prevent disulfide interchange.

SDS sample buffer, 6× (for discontinuous systems)

7 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1)
3.0 ml glycerol (30% v/v final)
1 g SDS [10% w/v final; recrystallization (see recipe) optional]
0.93 g DTT (0.6 M final)
1.2 mg bromphenol blue (0.012% w/v final)
Add H_2O to 10 ml (if needed)
Store in 0.5-ml aliquots at −70°C

Tricine sample buffer, 2×

2 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1; 0.1 M)
2.4 ml (3.0 g) glycerol (24% v/v final)
0.8 g SDS [8% w/v final; recrystallization (see recipe) optional]
0.31 g DTT (0.2 M final)
2 mg Coomassie blue G-250 (0.02% w/v final)
Add H_2O to 10 ml and mix

COMMENTARY

Background Information

Although electrophoresis has been studied for over two centuries, Arne Tiselius (University of Uppsala, Sweden) put the technique on the map with his Ph.D. thesis in 1930, which demonstrated moving zones of serum proteins in a special square-shaped, free-solution electrophoresis cell. His original work showed for the first time that protein components in serum could be separated, giving the now familiar designations for α, β, and γ globulin. Arne Tiselius was awarded the Nobel Prize in Chemistry in 1948 (*http://nobelprize.org/chemistry/laureates/1948/index.html*) "... for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins."

Electrophoresis is the movement of a charged particle, including large molecules such as DNA and proteins, in a liquid medium under the influence of an electric field. The driving force (QE) on the charged molecule is a product of the charge (Q) and the electric field (E) across the separation gel. Larger proteins move more slowly, as do proteins with a lower net charge. Furthermore, if a protein is in native (compact) form it will migrate more quickly than the same protein fully denatured and extended, where it experiences more frictional resistance with the surrounding medium. Resistance in electrophoresis is defined as $f = 6\pi rv\eta$, where f is the resistance of the medium to electrophoretic movement, r the radius of the protein (assumed to be a sphere), v the electrophoretic velocity, and η the viscosity of the fluid. The elements contributing to driving force and resistance together indicate that protein charge, size, shape, solution viscosity, and applied voltage are key factors influencing electrophoretic separation.

Electrophoresis is used to separate complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates), investigate subunit compositions, and verify homogeneity of protein samples (Table 6.1.12). It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis (PAGE), proteins migrate in response to an electrical field through pores in a gel matrix consisting of polymers of cross-linked acrylamide. The pore size is determined by acrylamide concentration. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein.

Polyacrylamide gels form after polymerization of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by *N,N′*-methylenebisacrylamide (Fig. 6.1.7). The polymerization reaction is initiated by the addition of ammonium persulfate, and the reaction is accelerated by TEMED, which catalyzes the formation of free radicals from ammonium persulfate. Because oxygen inhibits the polymerization process, deaerating the gel solution before the polymerization catalysts are added will speed up polymerization; deaeration is not recommended for the gradient gel protocols because slower polymerization facilitates casting of gradient gels.

Precast gels for commonly used vertical minigel and standard-sized SDS-PAGE apparatuses are available from several manufacturers (Table 6.1.4). Flatbed (horizontal) isoelectric focusing (IEF) and SDS-PAGE gels are not listed. Hoefer supplies a range of horizontal gels for a variety of applications and should be consulted for further information. When using precast gels, pay strict attention to shelf life. In general, manufacturers overrate the shelf life, and the sooner the gels are used, the better. When reasonably fresh, precast gels provide excellent resolution that is as good or better than a typical gel cast in the laboratory.

The most widely used method for discontinuous gel electrophoresis is the system described by Laemmli (1970). This is the denaturing (SDS) discontinuous method used in Basic Protocol 1. A discontinuous buffer system uses buffers of different pH and composition to generate a discontinuous pH and voltage gradient in the gel. Because the discontinuous gel system concentrates the proteins in each sample into narrow bands, the applied sample may be more dilute than that used for continuous electrophoresis.

In the discontinuous system the sample first passes through a stacking gel, which has large pores. The stacking gel buffer contains chloride ions (called the leading ions) whose electrophoretic mobility is greater than the mobility of the proteins in the sample. The electrophoresis buffer contains glycine ions (called the trailing ions) whose electrophoretic mobility is less than the mobility of the proteins in the sample. The net result is that the faster migrating ions leave a zone of lower conductivity between themselves and the migrating protein. The higher voltage gradient in this zone allows the proteins to move faster and to "stack" in the zone between the leading

Table 6.1.12 Key Applications of Protein Electrophoresis[a]

Technique	Separation principle	Application	For more information
Native PAGE	Native charge, size, and shape	Purification, determination of native protein size, and identification of protein complexes	*UNIT 6.5*
1-D SDS-PAGE	Size dependent: SDS imparts a negative charge to proteins, giving a constant charge to mass ratio	Size estimation, purity check, purification, subunit composition, protein expression	*UNIT 6.1*
IEF	Intrinsic charge with both native and denatured proteins	Purification, purity check, isoenzyme analysis	Ploegh (1995)
2-D SDS-PAGE	Isoelectric point in the first dimension, size in the second	Protein expression, purification, posttranslational analysis, proteomics	*UNIT 6.4*

[a]Abbreviations: 1-D, one-dimensional; 2-D, two-dimensional; IEF, isoelectric focusing.

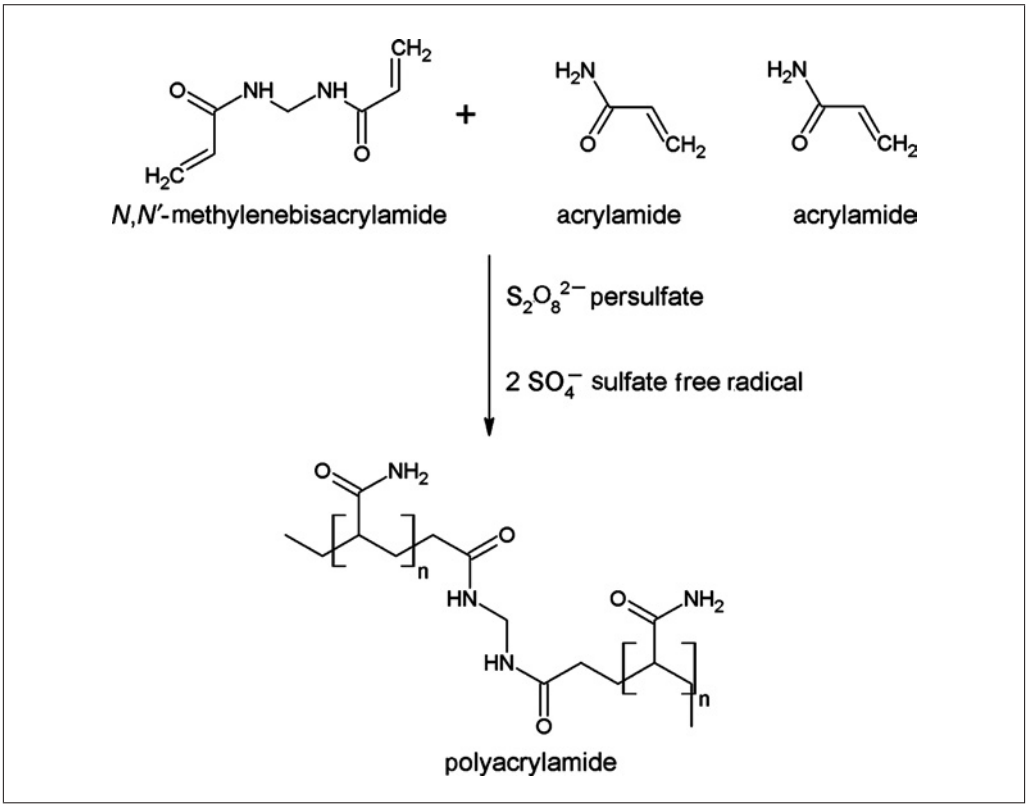


Figure 6.1.7 Structures of acrylamide and bisacrylamide and the associated reaction producing the polyacrylamide matrix used for protein separation.

and trailing ions. After leaving the stacking gel, the protein enters the separating gel. The separating gel has a smaller pore size, a higher salt concentration, and higher pH compared to the stacking gel. In the separating gel, the glycine ions migrate past the proteins, and the proteins are separated according to either molecular size in a denaturing gel (containing SDS) or molecular shape, size, and charge in a nondenaturing gel.

Proteins are denatured by heating in the presence of a low-molecular-weight thiol

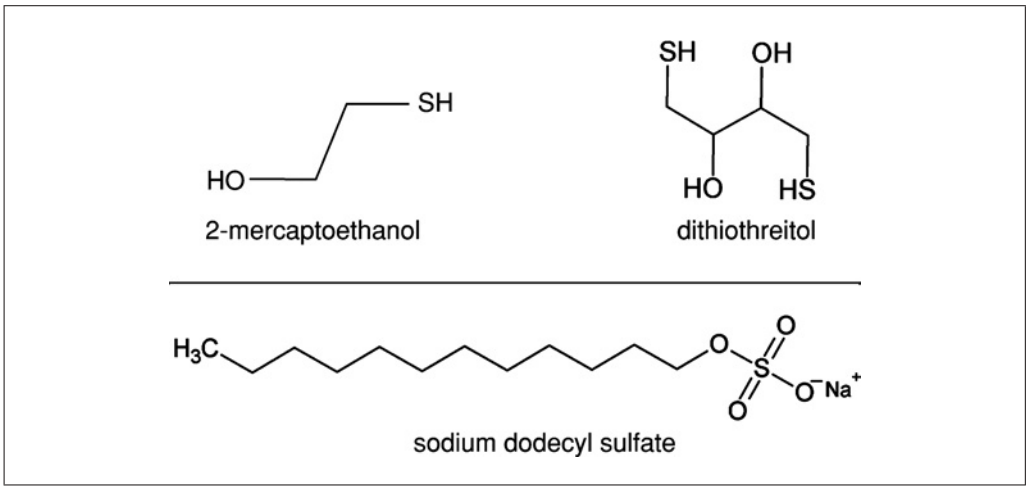


Figure 6.1.8 Structures of sodium dodecyl sulfate, dithiothreitol, and 2-mercaptoethanol: used to break disulfide bonds in proteins so they are fully denatured.

(2-ME or DTT) and SDS (Fig. 6.1.8). Most proteins bind SDS in a constant-weight ratio, leading to identical charge densities for the denatured proteins. Thus, the SDS-protein complexes migrate in the polyacrylamide gel according to size, not charge. Most proteins are resolved on polyacrylamide gels containing from 5% to 15% acrylamide and 0.2% to 0.5% bisacrylamide (see Table 6.1.1). The relationship between the relative mobility and log molecular weight is linear over these ranges (Fig. 6.1.6). With the use of plots like those shown in Figure 6.1.6, the molecular weight of an unknown protein (or its subunits) may be determined by comparison with known protein standards (Table 6.1.2). In general, all of the procedures in this unit are suitable for radiolabeled and biotinylated proteins without modification.

Basic Protocol 1 relies on denaturing proteins in the presence of SDS and 2-ME or DTT. Under these conditions, the subunits of proteins are dissociated and their biological activities are lost. A true estimate of a protein's molecular size can be made by comparing the relative mobility of the unknown protein to proteins in a calibration mixture (Support Protocol 4). Gradient gels (Alternate Protocol 5) simplify molecular-weight determinations by producing a linear relationship between log molecular weight of the protein and log % T over a much wider size range than single-concentration gels. Although percent acrylamide monomer is a more common measure of gel concentration, % T, the percentage of total monomer (acrylamide plus bisacrylamide) in the solution or gel, is used for molecular weight calculations in gradient gels. The % T of a stained protein is estimated assuming the acrylamide gradient is linear. For example, proteins in the gel shown in Figure 6.1.9 were separated in a 5.1% to 20.5% T acrylamide gradient. The % T of the point halfway through the resolving gel is 12.5% T. Simply plotting log molecular mass versus distance moved into the gel (or R_f) also produces a relatively linear standard curve over a fairly wide size range.

If two proteins have identical molecular sizes, they more than likely will not be resolved with one-dimensional SDS-PAGE, and two-dimensional SDS-PAGE should be considered. Unusual protein compositions can cause anomalous mobilities during electrophoresis (see Critical Parameters and Troubleshooting), but similar-sized proteins of widely different amino acid composition or structure may still be resolved from one another using one-dimensional SDS-PAGE. Purified protein complexes or multimeric proteins consisting of subunits of different molecular size will be resolved into constituent polypeptides. Comparison of the protein bands obtained under nonreducing and reducing conditions provides information about the molecular size of disulfide cross-linked component subunits. The individual polypeptides can be isolated by electroelution or electroblotting, and the amino acid sequences can be determined.

Both the tricine (Schagger and von Jagow, 1987) and the modified Laemmli (Okajima et al., 1993) peptide separation procedures presented here (Alternate Protocols 1 and 2) are simple to set up and provide resolution down to 5 kDa. To separate peptides below 5 kDa, the tricine procedure must be modified by preparing a 16.5% T, 2.7% C resolving gel that uses a 10% T spacer gel between the stacking

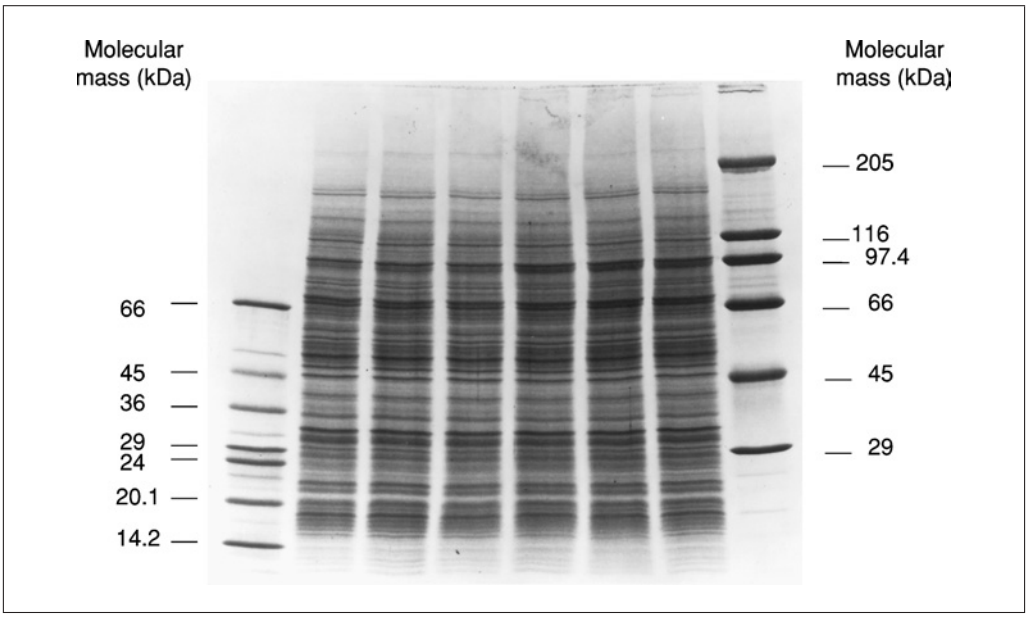


Figure 6.1.9 Separation of membrane proteins by 5.1% to 20.5% T polyacrylamide gradient SDS-PAGE. Approximately 30 μl of 1× SDS sample buffer containing 30 μg of Alaskan pea (*Pisum sativum*) membrane proteins was loaded in wells of a 14 × 14–cm, 0.75-mm-thick gel. Standard proteins were included in the outside lanes. The gel was run at 4 mA for ≈15 hr.

and resolving gel (Schagger and von Jagow, 1987). The % C is the percentage of cross-linker (bisacrylamide) in the total monomer (acrylamide plus bisacrylamide).

Continuous electrophoresis, where the same buffer is used throughout the tank and gel, is popular because of its versatility and simplicity. The phosphate system described in Alternate Protocol 3 is based on that of Weber et al. (1972). Although unable to produce the high-resolution separations of the discontinuous SDS-PAGE procedures, continuous SDS-PAGE uses fewer solutions with one basic buffer and no stacking gel. Artifacts are also less likely to occur in continuous systems. Pepsin, for example, migrates anomalously on Laemmli-based discontinuous SDS-PAGE but has the expected mobility after electrophoresis in the phosphate-based continuous system described here. This is also true of cross-linked proteins.

Multiple gel casting (Support Protocols 1 to 3) is appropriate when gel-to-gel consistency is paramount or when the number of gels processed exceeds five a week. The variety of multiple gel casters, gradient makers, and inexpensive pumps available from major suppliers simplifies the process of casting gels in the laboratory. Alternatively, precast gradient gels are available for most major brands of gel apparatuses (Table 6.1.4).

Minigels (Basic Protocol 2) are generally considered to be in the 8 × 10–cm size range, although there is considerable variation in exact size. Every technique that is used on larger systems can be translated with little difficulty into the minigel format. This includes standard and gradient SDS-PAGE and separations for immunoblotting and peptide sequencing. Two-dimensional SDS-PAGE electrophoresis also adapts well, but here the limitation of separation area becomes apparent; for high-resolution separations, large-format gels are required. Gradient minigels (Support Protocol 3) are popular due to the combination of separation range and resolution (Matsudaira and Burgess, 1978). They are particularly useful for separation of proteins prior to peptide sequencing.

Mylar support (GelBond) provides a practical way of casting, running, and, staining extremely thin gels. When gels <0.75 mm thick are used, reagents have much better access both into and out of the gel, reducing staining time in both Coomassie blue and silver staining. Double and broadened images caused by differential migration of the protein across the thickness of the gel are minimized, improving resolution.

Critical Parameters and Troubleshooting

If an electrophoretically separated protein will be electroeluted or electroblotted for sequence analysis, the highest-purity reagents available should be used. If necessary, SDS

can be purified by recrystallization following the procedure given in Reagents and Solutions.

If the gels polymerize too fast, the amount of ammonium persulfate should be reduced by one-third to one-half. If the gels polymerize too slowly or fail to polymerize all the way to the top, use fresh ammonium persulfate or increase the amount of ammonium persulfate by one-third to one-half. The overlay should be added slowly down the spacer edge to prevent the overlay solution from crashing down and disturbing the gel interface.

After a separating gel is poured, it may be stored with an overlay of the same buffer used in the gel. Immediately prior to use, the stacking gel should be poured; otherwise, there will be a gradual diffusion-driven mixing of buffers between the two gels, which will cause a loss of resolution.

The protein of interest should be present in 0.2- to 1-μg amounts in a complex mixture of proteins if the gel will be stained by Coomassie blue (*UNIT 6.6*). Typically, 30 to 50 μg of a complex protein mixture in a total volume of <20 μl is loaded on a 0.75-mm-thick slab gel (16 cm, 10 wells).

When casting multiple gradient gels, eliminate all bubbles in the outlet tubing of the gradient maker. If air bubbles get into the outlet tube, they may flow into the caster and then up through the gradient being poured, causing an area of distortion in the polymerized gel. Air bubbles are not so great a problem when casting single gradient gels from the top. As the gels are cast, the stirrer must be slowed so that the vortex in the mixing chamber does not allow air to enter the outlet.

Uneven heating of the gel causes differential migration of proteins, with the outer lanes moving more slowly than the center lanes (called smiling). Increased heat transfer eliminates smiling and can be achieved by filling the lower buffer chamber with buffer all the way to the level of the sample wells, by maintaining a constant temperature between 10° to 20°C, and by stirring the lower buffer with a magnetic stirrer. Alternatively, decrease the heat load by running at a lower current.

If the tracking dye band is diffuse, prepare fresh buffer and acrylamide monomer stocks. If the protein bands are diffuse, increase the current by 25% to 50% to complete the run more quickly and minimize band diffusion, use a higher percentage of acrylamide, or try a gradient gel. Lengthy separations using gradient gels generally produce good results (Fig. 6.1.9). Check for possible proteolytic degradation that may cause loss of high-molecular-weight bands and create a smeared banding pattern.

If there is vertical streaking of protein bands, decrease the amount of sample loaded on the gel, further purify the protein of interest to reduce the amount of contaminating protein applied to the gel, or reduce the current by 25%. Another cause of vertical streaking of protein bands is precipitation, which can sometimes be eliminated by centrifuging the sample or by reducing the percentage of acrylamide in the gel.

Proteins can migrate faster or slower than their actual molecular weight would indicate. Abnormal migration is usually associated with a high proportion of basic or charged amino acids (Takano et al., 1988). Other problems can occur during isolation and preparation of the protein sample for electrophoresis. Proteolysis of proteins during cell fractionation by endogenous proteases can cause subtle band splitting and smearing in the resulting electrophoretogram (electrophoresis pattern). Many endogenous proteases are very active in SDS sample buffers and will rapidly degrade the sample; thus, first heating the samples to 70° to 100°C for 3 min is recommended.

In some cases, heating to 100°C in sample buffer will cause selective aggregation of proteins, creating a smeared layer of Coomassie blue–stained material at the top of the gel (Gallagher and Leonard, 1987). To avoid heating artifacts and also prevent proteolysis, the use of specific protease inhibitors during protein isolation and/or lower heating temperatures (70° to 80°C) have been effective (Dhugga et al., 1988).

Although continuous gels suffer from poor band sharpness, they are less prone to artifacts caused by aggregation and protein cross-linking. If streaking or aggregation appear to be a problem with the Laemmli system, then the same sample should be subjected to continuous SDS-PAGE to see if the problem is intrinsic to the Laemmli gel or the sample.

If the protein bands spread laterally from gel lanes, the time between applying the sample and running the gel should be reduced in order to decrease the diffusion of sample out of the wells. Alternatively, the acrylamide percentage should be increased in the stacking gels from 4% to 4.5% or 5% acrylamide, or the operating current should be increased by 25% to decrease diffusion in the stacking gel. Use caution when adding 1× SDS electrophoresis buffer to the upper buffer chamber. Samples

can get swept into adjacent wells and onto the top of the well arm.

If the protein bands are uneven, the stacking gel may not have been adequately polymerized. This can be corrected by deaerating the stacking gel solution thoroughly or by increasing the ammonium persulfate and TEMED concentrations by one-third to one-half. Another cause of distorted bands is salt in the protein sample, which can be removed by dialysis, gel filtration, or precipitation. Skewed protein bands can be caused by an uneven interface between the stacking and separating gels, which can be corrected by starting over and being careful not to disturb the separating gel while overlaying with isobutyl alcohol.

If a run takes too long, the buffers may be too concentrated or the operating current too low. If the run is too short, the buffers may be too dilute or the operating current too high.

If double bands are observed, the protein may be partially oxidized or partially degraded. Oxidation can be minimized by increasing the 2-ME concentration in the sample buffer or by preparing a fresh protein sample. If fewer bands than expected are observed and there is a heavy protein band at the dye front, increase the acrylamide percentage in the gel.

Anticipated Results

Polyacrylamide gel electrophoresis done under denaturing and reducing conditions should resolve any two proteins, except two of identical size. Resolution of proteins in the presence of SDS is a function of gel concentration and the size of the proteins being separated. Under nondenaturing conditions, the biological activity of a protein will be maintained.

Comparison of reducing and nonreducing denaturing gels can also provide valuable information about the number of disulfide cross-linked subunits in a protein complex. If the subunits are held together by disulfide linkages, the protein will separate in denaturing gels as a complex or as smaller-sized subunits under nonreducing or reducing conditions, respectively. However, proteins separated on nonreducing denaturing gels appear more diffuse and exhibit less overall resolution than those separated on reducing gels.

Gradient gels provide superior protein-band sharpness and resolve a larger size range of proteins, making them ideal for most types of experiments in spite of being more difficult to prepare. Molecular-weight calculations are simplified because of the extended linear relationship between size and protein position in the gel. Increased band sharpness of both high- and low-molecular-weight proteins on the same gel greatly simplifies survey experiments, such as gene expression studies where the characteristics of the responsive protein are not known. Furthermore, the increased resolution dramatically improves autoradiographic analysis. Preparation of gradient gels is straightforward, although practice with gradient solutions containing dye is recommended. The gradient gels can be stored for several days at 0° to 4°C before casting the stacking gel.

Time Considerations

Preparation of separating and stacking gels requires 2 to 3 hr. Gradient gels generally take 5 min to cast singly. Casting multiple single-concentration gels requires an additional 10 min for assembly. Casting multiple gradient gels takes 15 to 20 min plus assembly time. It takes 4 to 5 hr to run a 14 × 14–cm, 0.75-mm gel at 15 mA (70 to 150 V), and 3 to 4 hr to run a 0.75-mm gel at 20 mA (80 to 200 V). Overnight separations of ~12 hr require 4 mA per 0.75-mm gel. It takes 4 to 5 hr to run a 1.5-mm gel at 30 mA. Electrophoresis is normally performed at 15° to 20°C, with the temperature held constant using a circulating water bath. For air-cooled electrophoresis units, lower currents and thus longer run times are recommended.

It takes ~1 hr to run a 0.75-mm minigel at 20 mA (100 to 120 V). Separation times are not significantly different for gradient minigels.

Literature Cited

Dhugga, K.S., Waines, J.G., and Leonard, R.T. 1988. Correlated induction of nitrate uptake and membrane polypeptides in corn roots. *Plant Physiol.* 87:120-125.

Gallagher, S.R. and Leonard, R.T. 1987. Electrophoretic characterization of a detergent-treated plasma membrane fraction from corn roots. *Plant Physiol.* 83:265-271.

Hunkapiller, M.W., Lujan, E., Ostrander, F., and Hood, L.E. 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. *Methods Enzymol.* 91:227-236.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Matsudaira, P.T. and Burgess, D.R. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386-396.

Okajima, T., Tanabe, T., and Yasuda, T. 1993. Nonurea sodium dodecyl sulfate-polyacrylamide gel electrophoresis with

high-molarity buffers for the separation of proteins and peptides. *Anal. Biochem.* 211:293-300.

Ploegh, H.L. 1995. One-Dimensional Isoelectric Focusing of Proteins in Slab Gels. *In* Current Protocols in Protein Science (J.E. Coligan, B.M. Dunn, D.W. Speicher, and P.T. Wingfield, eds.) pp. 10.2.1-10.2.8. John Wiley & Sons, Hoboken, N.J.

Schagger, H. and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379.

Takano, E., Maki, M., Mori, H., Hatanaka, N., Marti, T., Titani, K., Kannagi, R., Ooi, T., and Murachi, T. 1988. Pig heart calpastatin: Identification of repetitive domain structures and anomalous behavior in polyacrylamide gel electrophoresis. *Biochemistry* 27:1964-1972.

Weber, K., Pringle, J.R., and Osborn, M. 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. *Methods Enzymol.* 26:3-27.

Key Reference

Hames, B.D. and Rickwood, D. (eds.) 1990. Gel Electrophoresis of Proteins: A Practical Approach, 2nd ed. Oxford University Press, New York.

An excellent book describing gel electrophoresis of proteins.

Immunoblotting and Immunodetection

Immunoblotting (often referred to as western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). Following solubilization, the material is separated by SDS-PAGE (*UNIT 6.1*). The antigens are then electrophoretically transferred in a tank (see Basic Protocol 1) or a semidry transfer apparatus (see Alternate Protocol 1) to a nitrocellulose, PVDF, or nylon membrane, a process that can be monitored by reversible staining (see Support Protocol 1) or by Ponceau S staining (see Support Protocol 2). Previously stained gels may also be blotted (see Alternate Protocol 2).

The transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing either a protein or detergent blocking agent. After probing with the primary antibody, the membrane is washed and the antibody-antigen complexes are identified with horseradish peroxidase (HRPO) or alkaline phosphatase enzymes coupled to the secondary anti-IgG antibody (e.g., goat anti–rabbit IgG). The enzymes are attached directly (see Basic Protocol 2) or via an avidin-biotin bridge (see Alternate Protocol 3) to the secondary antibody. Chromogenic or luminescent substrates (see Basic Protocol 3 and Alternate Protocol 4) are then used to visualize the activity. Finally, membranes may be stripped and reprobed (see Support Protocol 3).

NOTE: When handling gels and membranes wear powder-free gloves.

PROTEIN BLOTTING WITH TANK TRANSFER SYSTEMS

BASIC PROTOCOL 1

In this procedure, blotting is performed in a tank of buffer with the gel in a vertical orientation, completely submerged between two large electrode panels. In some systems up to four gels can be transferred at one time. For difficult-to-transfer proteins (>100 kDa or hydrophobic; e.g., myosin), tank blotting is preferable to semidry systems (see Basic Protocol 2) because prolonged transfers are possible without buffer depletion. However, transfers >1 hr at high power require cooling using a heat exchanger and a circulating water bath that can maintain a constant transfer temperature of 10° to 20°C.

Materials

Samples for analysis
Protein molecular weight standards (*UNIT 6.1*), prestained (Sigma or Bio-Rad) or biotinylated (Vector Labs or Sigma)
Transfer buffer (see recipe)

Powder-free gloves
Scotch-Brite pads (3M) or equivalent sponge
Whatman 3MM filter paper or equivalent
Transfer membrane: 0.45-µm nitrocellulose (Millipore or Schleicher & Schuell), PVDF (Millipore Immobilon P), neutral nylon (Pall Biodyne A), *or* positively charged nylon (Pall Biodyne B; Bio-Rad Zetabind) membrane
Electroblotting apparatus (EC Apparatus, Bio-Rad, or Amersham Pharmacia Biotech)
Indelible pen (e.g., Paper-Mate ballpoint) or soft lead pencil

Additional reagents and equipment for gel electrophoresis (*UNIT 6.1*) and staining proteins in gels and on membranes (see Support Protocol 1)

NOTE: Deionized, distilled water should be used throughout this protocol.

Contributed by Sean Gallagher, Scott E. Winston, Steven A. Fuller, and John G.R. Hurrell
Current Protocols in Cell Biology (1998) 6.2.1-6.2.20

Electrophorese the protein sample

1. Prepare antigenic samples and separate proteins using small or standard-sized one- or two-dimensional gels (*UNIT 6.1*). Include prestained or biotinylated protein molecular weight standards in one or more gel lanes.

   The protein markers will transfer to the membrane and conveniently indicate membrane orientation and sizes of proteins after immunostaining.

   A variety of gel sizes and percentages of acrylamide can be used (UNIT 6.1). Most routinely used are either 14 cm × 14 cm × 0.75–mm gels or 8 cm × 10 cm × 0.75–mm minigels. Acrylamide concentrations vary from 5% to 20%, but are usually in the 10% to 15% range.

Assemble the immunoblot sandwich

2. When electrophoresis is complete, disassemble gel sandwich and remove stacking gel. Equilibrate gel 30 min at room temperature in transfer buffer.

   Oil from hands blocks the transfer.

   Match the appropriate transfer buffer to the membrane (see Reagents and Solutions).

   Gel equilibration is required to prevent a change in the size of the gel during transfer. Any shift in gel dimension will result in a blurred transfer pattern.

3. Assemble transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with transfer buffer so that cassette is covered.

   The transfer cassette should be assembled under buffer to minimize trapping of air bubbles. Use Figure 6.2.1 as a guide to assembly.

4. On bottom half of plastic transfer cassette, place Scotch-Brite pad or sponge, followed by a sheet of filter paper cut to same size as gel and prewet with transfer buffer.

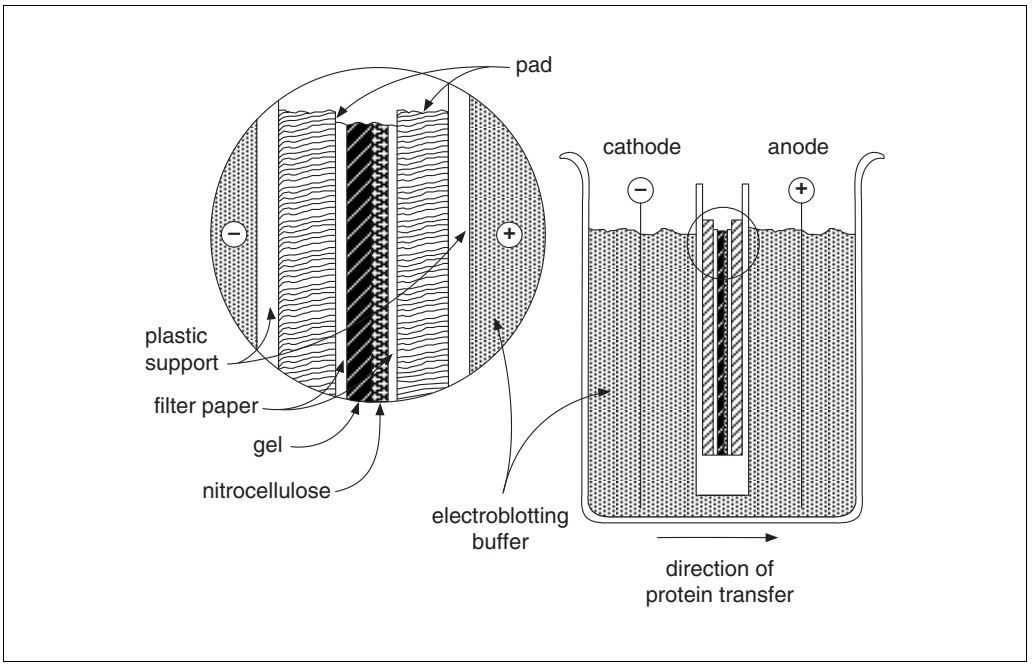


Figure 6.2.1 Immunoblotting with a tank blotting unit. The polyacrylamide gel containing the protein is laid on a sheet of filter paper. The uncovered side of the gel is overlaid with a sheet of membrane precut to the size of the gel plus 1 to 2 mm on each edge, then this membrane is overlaid with another sheet of filter paper. The filter paper containing the gel and membrane is sandwiched between Scotch-Brite pads. This sandwich is placed in a plastic support, and the entire assembly is placed in a tank containing transfer buffer. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Charged proteins are transferred electrophoretically from the gel onto the membrane. Transfer is achieved by applying a voltage of 100 V for 1 to 2 hr (with cooling) or 14 V overnight.

5. Place gel on top of filter paper. The side of the gel touching the paper arbitrarily becomes the cathode side of the gel (i.e., ultimately toward the negative electrode when positioned in the tank). Remove any air bubbles between gel and filter paper by gently rolling a test tube or glass rod over surface of gel.

   Any bubbles between the filter paper, gel, and membrane will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

   Proteins are usually negatively charged in transfer buffer and move toward the positive anode. However, some proteins may be positively charged. An additional membrane placed on the cathode side of the gel will bind these proteins.

6. Prepare transfer membrane. Cut membrane to same size as gel plus 1 to 2 mm on each edge. Place into distilled water slowly, with one edge at a 45° angle. Equilibrate 10 to 15 min in transfer buffer.

   The water will wick up into the membrane, wetting the entire surface. If it is inserted too quickly into the water, air gets trapped and will appear as white blotches in the membrane. Protein will not transfer onto these areas.

   This wetting procedure works for nitrocellulose and nylon membranes only. PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer. For these membranes, first immerse 1 to 2 sec in 100% methanol, then equilibrate 10 to 15 min with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet once again with methanol and transfer buffer as described above.

7. Moisten surface of gel with transfer buffer. Place prewetted membrane directly on top side of gel (i.e., anode side) and remove all air bubbles as in step 5.

   Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

   The use of 0.2-μm membranes may improve retention of smaller-molecular-weight proteins.

8. Wet another piece of Whatman 3MM filter paper, place on anode side of membrane, and remove all air bubbles. Place another Scotch-Brite pad or sponge on top of this filter paper.

9. Complete assembly by locking top half of the transfer cassette into place (Fig. 6.2.1).

   It is important to orient the sandwich so that the membrane faces the anode (positively charged) side of the tank.

Transfer proteins from gel to membrane

10. Fill tank with transfer buffer and place transfer cassette containing sandwich into electroblotting apparatus in correct orientation. Connect leads of power supply to corresponding anode and cathode sides of electroblotting apparatus.

    Transfer buffer should cover the electrode panels but should not touch the base of the banana plug.

11. Electrophoretically transfer proteins from gel to membrane for 30 min to 1 hr at 100 V with cooling or overnight at 14 V (constant voltage), in a cold room.

    Transfer time is dependent on the thickness and the percent acrylamide of the gel, as well as the size of the protein being transferred. In general, proteins are transferred within 1 to 6 hr, but high-molecular-weight molecules may take longer. Overnight transfer at low voltage is reliable and convenient. Cooling (at 10° to 20°C) is required for transfers >1 hr at high power. Heat exchanger cooling cores using a circulating water bath are placed into the transfer unit for cooling.

12. Turn off the power supply and disassemble the apparatus. Remove membrane from blotting apparatus and note orientation by cutting a corner or marking with a soft lead pencil or Paper-Mate ballpoint pen.

    Many ballpoint inks come off, but Paper-Mate stays on the membrane.

    Membranes can be dried and stored in resealable plastic bags at 4°C for 1 year or longer at this point. Prior to further processing, dried PVDF membranes must be placed into a small amount of 100% methanol to wet the membrane, then in distilled water to remove the methanol.

13. Stain gel for total protein with Coomassie blue to verify transfer efficiency. If desired, stain membrane reversibly to visualize transferred proteins (see Support Protocol 1), or irreversibly with Coomassie blue, India ink, naphthol blue, or colloidal gold.

    These staining procedures are incompatible with nylon membranes.

    If membrane shows significant staining on the backside, either the gel was heavily overloaded or the membrane has poor protein-binding capacity (see Troubleshooting). In either case, protein-binding sites on the side facing the gel are saturated, allowing protein to migrate to the other side of the membrane. Nitrocellulose in particular will show diminished binding capacity with age or poor storage conditions (e.g., high temperature and humidity). In addition, some proteins simply do not bind well to a particular matrix. By using several membrane sheets in place of one, the protein can be detected as it passes through each consecutive sheet. This will give an indication of how efficiently the membrane binds to a particular protein.

14. Proceed with immunoprobing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 3 and 4).

ALTERNATE PROTOCOL 1

PROTEIN BLOTTING WITH SEMIDRY SYSTEMS

Even and efficient transfer of most proteins is also possible with semidry blotting, a convenient alternative to tank transfer systems. Instead of being placed vertically into a tank filled with transfer buffer, the gel is held horizontally between buffer-saturated blotting paper that is in contact with the electrodes (Fig. 6.2.2), greatly reducing the amount of buffer required. The electrodes are close together, giving high field strengths and rapid transfer with a standard electrophoresis power supply. Prolonged transfers (>1 hr) are not recommended; tank blotting (see Basic Protocol 1) should be used for proteins that require long blotting times for efficient transfer.

Additional Materials *(also see Basic Protocol 1)*

Six sheets of Whatman 3MM filter paper or equivalent, cut to size of gel and saturated with transfer buffer
Semidry transfer unit (Amersham Pharmacia Biotech, Bio-Rad, or Sartorius)

1. Prepare samples and separate proteins using small or standard-sized one- or two-dimensional gels (*UNIT 6.1*).

   Because transfer efficiency depends on many factors (e.g., gel concentration and thickness, protein size, shape, and net charge) results may vary. Below is a guideline for 0.75–mm–thick SDS-PAGE gels transferred by semidry blotting.

Percent acrylamide (resolving gel)	Size range transferred (~100% efficiency)
5–7	29–150 kDa
8–10	14–66 kDa
13–15	<36 kDa
18–20	<20 kDa

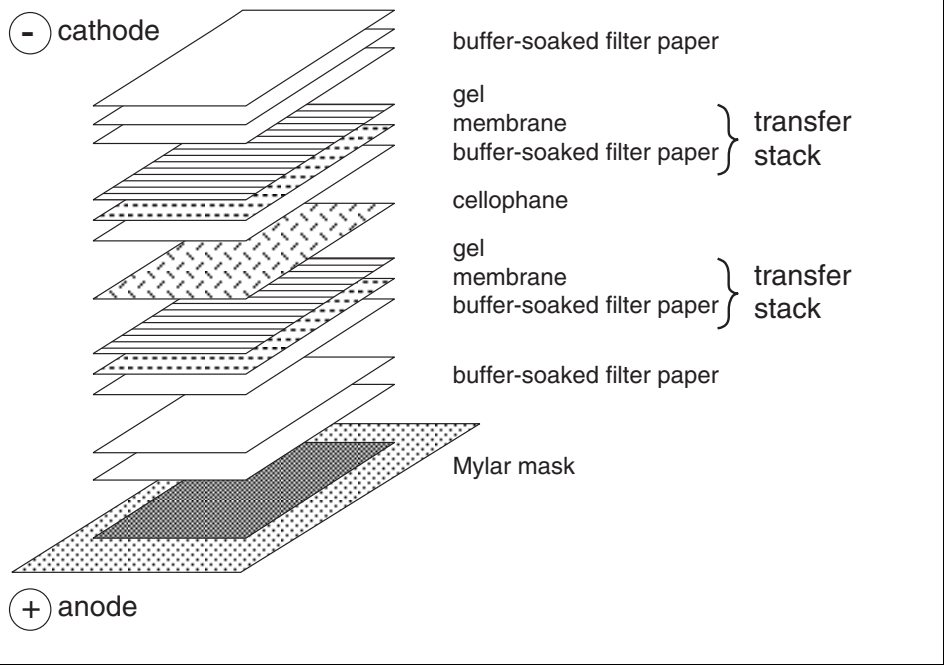


Figure 6.2.2 Immunoblotting with a semidry transfer unit. Generally, the lower electrode is the anode, and one gel is transferred at a time. A Mylar mask (optional in some units) is put in place on the anode. This is followed by three sheets of transfer buffer–soaked filter paper, the membrane, the gel, and finally, three more sheets of buffer-soaked filter paper. To transfer multiple gels, construct transfer stacks as illustrated, and separate each with a sheet of porous cellophane. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Transfer is achieved by applying a maximum current of 0.8 mA/cm^2 of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm), this means 60 and 200 mA, respectively.

2. Prepare transfer membrane (see Basic Protocol 1, step 6).
3. Disassemble gel sandwich. Remove and discard stacking gel.

   Equilibration of the separating gel with transfer buffer is not normally required for semidry blotting, but it may improve transfer in some cases.

4. Place three sheets of filter paper saturated with transfer buffer on the anode (Fig. 6.2.2).

   Most transfer units are designed so that negatively charged proteins move downward toward either a platinum or graphite positive electrode (anode).

   CAPS transfer buffer, pH 10.5 (see recipe for transfer buffer) can be used in place of the Tris/glycine/methanol transfer buffer of Basic Protocol 1. CAPS buffer should be used if the protein is to be sequenced right on the membrane (Moos, 1992), as glycine will interfere with this procedure.

   The filter paper should be cut to the exact size of the gel. This forces the current to flow only through the gel and not through overlapping filter paper. Some manufacturers (e.g., Amersham Pharmacia Biotech) recommend placing a Mylar mask on the lower platinum anode. With an opening that is slightly less than the size of the gel, the mask forces the current to flow through the gel and not the surrounding electrode area during transfer.

5. Place equilibrated transfer membrane on top of filter paper stack. Remove all bubbles between membrane and filter paper by rolling a test tube over surface of membrane.

   Any bubbles in the filter paper stack or between the filter paper, membrane, and gel will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

6. Place gel on top of membrane. Gently roll a test tube over surface of gel to ensure intimate contact between gel and membrane and to remove any interfering bubbles.

   Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

7. Complete the transfer stack by putting the three remaining sheets of filter paper on top of gel. Roll out bubbles as described above.

   Multiple gels can be transferred using semidry blotting. Simply put a sheet of porous cellophane (Amersham Pharmacia Biotech) or dialysis membrane (Bio-Rad or Sartorius) equilibrated with transfer buffer between each transfer stack (Fig. 6.2.2). Transfer efficiency is dependent on the position of the transfer stack in the blotting unit and for critical applications transferring one gel at a time is recommended. The gel next to the anode tends to be more efficiently transferred when blotting more than one gel at a time.

Transfer proteins from gel to membrane

8. Place top electrode onto transfer stack.

   Most units have safety-interlock features and can only be assembled one way. Consult manufacturer's instructions for details.

   Once assembled, do not move the top electrode. This can shift the transfer stack and move the gel relative to the membrane. Some transfer will occur as soon as the gel contacts the membrane, and any shifting of the transfer stack after assembly will distort the transfer pattern.

9. Carefully connect high-voltage leads to the power supply (see *UNIT 6.1* for safety precautions). Apply constant current to initiate protein transfer. Transfers of 1 hr are generally sufficient.

   In general, do not exceed 0.8 mA/cm^2 of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm) this means ~60 and 200 mA, respectively.

   Monitor the temperature of the transfer unit directly above the gel by touch. The unit should not exceed 45°C. If the outside of the unit is warm, too much current is being applied. Note that units with graphite electrodes are more prone to heating, because graphite has much more resistance to current flow than platinum or steel electrodes.

10. After transfer, turn off power supply and disassemble unit. Remove membrane from transfer stack, marking orientation as in step 12 of Basic Protocol 1. Proceed with staining and immunoprobing (see Basic Protocol 1, steps 13 and 14).

ALTERNATE PROTOCOL 2

BLOTTING OF STAINED GELS

Gels stained with Coomassie blue R250 can be effectively immunoblotted by the following procedure, based on Perides et al. (1986) and Dionisi et al. (1995). Briefly, the stained gel is soaked in a series of solutions designed to increase the solubility of the proteins after staining and fixation. After transfer, the membranes are treated with 45% or 100% methanol to decrease the Coomassie blue bound to the membrane prior to processing for chromogenic development. For chemiluminescent development, removal of the Coomassie blue is generally not needed.

Materials

Destained gel containing proteins of interest
25 mM Tris base/192 mM glycine/1% SDS
25 mM Tris base/192 mM glycine/0.1% SDS

1. Soak destained gel containing proteins of interest in distilled water for 15 min.
2. Equilibrate gel with 25 mM Tris base/192 mM glycine/1% SDS for 1 hr with gentle agitation.
3. Transfer gel to 25 mM Tris base/192 mM glycine/0.1% SDS and equilibrate 30 min with gentle agitation.

   To increase transfer efficiency of larger proteins, the gel should be transferred to the above solution with 6 M urea for an additional 30 min.

4. Proceed with transfer (see Basic Protocol 1, steps 2 to 12).

   For the most efficient transfer and binding to the membrane, the transfer buffer should contain SDS.

5. After transfer, soak membranes for 10 to 30 min in 45% methanol (nitrocellulose) or 100% methanol (nylon or PVDF) to remove the bound Coomassie blue.

   This step is not needed if using chemiluminescent reactions or radiolabeled protein A for immunodevelopment. Destaining of the nitrocellulose membrane is enhanced by adding a small ball of laboratory tissue to the methanol to absorb the Coomassie blue.

6. Proceed with immunoprobing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 3 and 4).

REVERSIBLE STAINING OF TRANSFERRED PROTEINS

SUPPORT PROTOCOL 1

To verify transfer efficiency, nitrocellulose and PVDF membranes can be reversibly stained. This method will not work on nylon membranes.

Additional Materials *(also see Basic Protocol 1)*

Ponceau S solution (see recipe)

Additional reagents and equipment for photographing membranes

1. Following protein transfer to nitrocellulose or PVDF (see Basic Protocol 1 or Alternate Protocol 1), place membrane in Ponceau S solution 5 min at room temperature.
2. Destain 2 min in water. Photograph membrane if required and mark the molecular-weight-standard band locations with indelible ink.
3. Completely destain membrane by soaking an additional 10 min in water.

QUANTITATION OF PROTEIN WITH PONCEAU S

SUPPORT PROTOCOL 2

In addition to qualitatively visualizing proteins on membranes after blotting, Ponceau S provides a convenient method for quantifying the amount of protein in a given lane. By eluting the dye off the strip and reading in a spectrophotometer (A_{525}), an internal control value of protein on a lane is obtained. This value is used to correct for any differences in protein loading from lane to lane. Comparison of the Ponceau S value to the chemiluminescent or chromogenic immunodetection value determined by densitometry provides a straightforward correction for lane-to-lane variation. This method works best for complex mixtures where the immunodetected protein represents a small proportion of the total protein (Klein et al., 1995).

Additional Materials *(also see Basic Protocol 1)*

Spectrophotometer and 2-ml cuvette

1. Following protein transfer to nitrocellulose, PVDF, or nylon (see Basic Protocol 1 or Alternate Protocol 1), stain membrane, photograph, and destain (see Support Protocol 1).

   Membranes should be destained until the background becomes white.

2. Mark lanes with a soft pencil and cut lanes into strips.
3. Place each strip into 7 ml of distilled water for 7 min and remove the resulting solution. If any particulates are visible, centrifuge 30 min at 2000 rpm to remove them.
4. Read A_{525} in a 2-ml cuvette.

   Any variation in gel-to-gel sample loading and blotting efficiency will be reflected in a change in A_{525} of the sample lanes when compared to the control. The change in A_{525} can be calibrated to a known amount of protein loaded on the control lane. This will be a relative value, however, since the transfer out of the gel and binding to the membrane is rarely 100%.

BASIC PROTOCOL 2

IMMUNOPROBING WITH DIRECTLY CONJUGATED SECONDARY ANTIBODY

Immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is immersed in blocking buffer to fill all protein-binding sites with a nonreactive protein or detergent. Next, it is placed in a solution containing the antibody directed against the antigen (primary antibody). The blot is washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (secondary antibody; e.g., goat anti-rabbit IgG). Antigens are identified by chromogenic or luminescent visualization (see Basic Protocol 3 and Alternate Protocol 4) of the antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane. Tween 20 is a common alternative to protein-blocking agents when using nitrocellulose or PVDF filters.

Materials

Membrane with transferred proteins (see Basic Protocol 1 or Alternate Protocol 1)
Blocking buffer appropriate for membrane and detection protocol (see recipe)
Primary antibody specific for protein of interest
TTBS (nitrocellulose or PVDF) or TBS (nylon; see *APPENDIX 2A* for recipes)
Secondary antibody conjugate: horseradish peroxidase (HRPO)–or alkaline phosphatase (AP)–anti-Ig conjugate (Cappel, Vector Labs, Kirkegaard & Perry, or Sigma; dilute as indicated by manufacturer and store frozen in 25-µl aliquots until use)
Heat-sealable plastic bag
Powder-free gloves
Plastic box

1. Place membrane in heat-sealable plastic bag with 5 ml blocking buffer and seal bag. Incubate 30 min to 1 hr at room temperature with agitation on an orbital shaker or rocking platform.

   Usually 5 ml buffer is sufficient for two to three membranes (14 × 14–cm size). If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Dilute primary antibody in blocking buffer.

   Primary antibody dilution is determined empirically but is typically 1/100 to 1/1000 for a polyclonal antibody (Fig. 6.2.3), 1/10 to 1/100 for hybridoma supernatants, and ≥1/1000

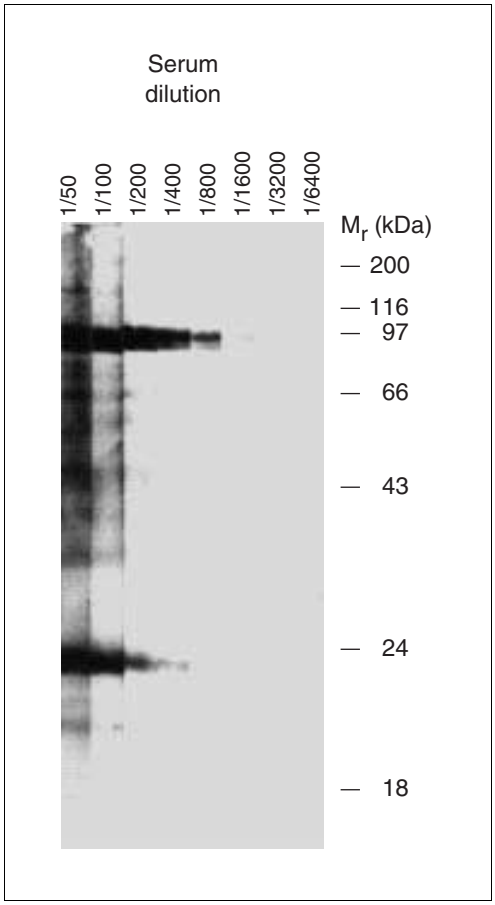


Figure 6.2.3 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. Blot was developed with HRPO-coupled avidin-biotin reagents according to the second alternate protocol and visualized with 4-chloro-1-naphthol (4CN). Note how background improves with dilution.

for murine ascites fluid containing monoclonal antibodies. Ten- to one-hundred-fold higher dilutions can be used with alkaline phosphatase– or luminescence–based detection systems. Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., >2 days at 4°C) is not recommended.

3. Open bag and pour out blocking buffer. Replace with diluted primary antibody and incubate 30 min to 1 hr at room temperature with constant agitation.

   Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on conjugate used.

   When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from plastic bag with gloved hand. Place in plastic box and wash four times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10 to 15 min each time.

5. Dilute secondary antibody HRPO- or AP-anti-Ig conjugate in blocking buffer.

   Commercially available enzyme–conjugated secondary antibody is usually diluted 1/200 to 1/2000 prior to use (Harlow and Lane, 1988).

6. Place membrane in new heat-sealable plastic bag, add diluted HRPO- or AP-anti-Ig conjugate, and incubate 30 min to 1 hr at room temperature with constant agitation.

   When using plastic incubation trays, see step 3 annotation for proper antibody solution volumes.

7. Remove membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 4).

ALTERNATE PROTOCOL 3

IMMUNOPROBING WITH AVIDIN-BIOTIN COUPLING TO SECONDARY ANTIBODY

The following procedure is based on the Vectastain ABC kit from Vector Labs (see *SUPPLIERS APPENDIX*). It uses an avidin-biotin complex to attach horseradish peroxidase (HRPO) or alkaline phosphatase (AP) to the biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity due to the multiple reporter enzymes bound to each secondary antibody. In addition, the detergent Tween 20 is a popular alternative to protein-blocking agents when using nitrocellulose or PVDF filters.

Additional Materials *(also see Basic Protocol 2)*

Blocking buffer appropriate for membrane and detection protocol (see recipe)
TTBS (nitrocellulose or PVDF) *or* TBS (neutral or positively charged nylon; see *APPENDIX 2A* for recipes)
Vectastain ABC (HRPO) or ABC-AP (AP) kit (Vector Labs) containing the following: reagent A (avidin), reagent B (biotinylated HRPO or AP), and biotinylated secondary antibody (request membrane immunodetection protocols when ordering)

1. Equilibrate membrane in appropriate blocking buffer in heat-sealed plastic bag with constant agitation using an orbital shaker or rocking platform. For nitrocellulose and PVDF, incubate 30 to 60 min at room temperature. For nylon, incubate ≥2 hr at 37°C.

   TTBS is well suited for avidin-biotin systems. For nylon, protein-binding agents are recommended. Because nonfat dry milk contains residual biotin, which will interfere with the immunoassay, it must be used in the blocking step only. If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Prepare primary antibody solution in TTBS (nitrocellulose or PVDF) or TBS (nylon).

   Dilutions of sera containing primary antibody generally range from 1/100 to 1/100,000. This depends in large part on the sensitivity of the detection system. With high-sensitivity avidin-biotin systems, dilutions from 1/1000 to 1/100,000 are common. Higher dilutions can be used with AP- or luminescence-based detection systems. To determine the appropriate concentration of the primary antibody, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Schleicher and Schuell; Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. 6.2.3).

3. Open bag, remove blocking buffer, and add enough primary antibody solution to cover membrane. Incubate 30 min at room temperature with gentle rocking.

   Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on conjugate used.

   When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from bag and place in plastic box. Wash membrane three times over a 15-min span in TTBS (nitrocellulose or PVDF) or TBS (nylon). Add enough TTBS or TBS to fully cover the membrane (e.g., 5 to 10 ml/strip or 25 to 50 ml/whole membrane).

5. Prepare biotinylated secondary antibody solution by diluting two drops biotinylated antibody with 50 to 100 ml TTBS (nitrocellulose or PVDF) or TBS (nylon).

   This dilution gives both high sensitivity and enough volume to easily cover a large 14 × 14–cm membrane.

6. Transfer membrane to fresh plastic bag containing secondary antibody solution. Incubate 30 min at room temperature with slow rocking, then wash as in step 4.

   When using plastic incubation trays, see step 3 annotations for proper antibody solution volumes.

7. While membrane is being incubated with secondary antibody, prepare avidin-biotin-HRPO or -AP complex. Mix two drops Vectastain reagent A and two drops reagent B into 10 ml TTBS (nitrocellulose or PVDF) or TBS (nylon). Incubate 30 min at room temperature, then further dilute to 50 ml with TTBS or TBS.

   Diluting the A and B reagents to 50 ml expands the amount of membrane that can be probed without greatly affecting sensitivity. Sodium azide is a peroxidase inhibitor and should not be used as a preservative. Casein, nonfat dry milk, serum, and some grades of BSA may interfere with the formation of the avidin-biotin complex and should not be used in the presence of avidin or biotin reagents (Gillespie and Hudspeth, 1991; Vector Labs).

8. Transfer membrane to avidin-biotin-enzyme solution. Incubate 30 min at room temperature with slow rocking, then wash over a 30-min span as in step 4.

9. Develop membrane according to the appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 4).

VISUALIZATION WITH CHROMOGENIC SUBSTRATES

BASIC PROTOCOL 3

Bound antigens are typically visualized with chromogenic substrates. The substrates 4CN, DAB/$NiCl_2$, and TMB are commonly used with horseradish peroxidase (HRPO)–based immunodetection procedures, while BCIP/NBT is recommended for alkaline phosphatase (AP)–based procedures (see Table 6.2.1). After incubation with primary and secondary antibodies, the membrane is placed in the appropriate substrate solution. Protein bands usually appear within a few minutes.

Materials

Membrane with transferred proteins and probed with antibody-enzyme complex (see Basic Protocol 2 or Alternate Protocol 3)
TBS (*APPENDIX 2A*)
Chromogenic visualization solution (Table 6.2.1)

Additional reagents and equipment for gel photography

1. If final membrane wash (see Basic Protocol 2, step 7, or see Alternate Protocol 3, step 8) was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

   The Tween 20 in TTBS interferes with 4CN development (Bjerrum et al., 1988).

2. Place membrane into chromogenic visualization solution. Bands should appear in 10 to 30 min.

3. Terminate reaction by washing membrane in distilled water. Air dry and photograph for a permanent record.

Table 6.2.1 Chromogenic and Luminescent Visualization Systems[a]

System	Reagent[b]	Reaction/Detection	Comments[c]
Chromogenic			
HRPO-based	4CN	Oxidized products form purple precipitate	Not very sensitive (Tween 20 inhibits reaction); fades rapidly upon exposure to light
	DAB/$NiCl_2$[d]	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane easily scanned
	TMB[e]	Forms dark purple stain	More stable, less toxic than DAB/$NiCl_2$; may be somewhat more sensitive[e]; can be used with all membrane types; kits available from Kirkegaard & Perry, TSI, Moss, and Vector Labs
AP-based	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results	More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity
Luminescent			
HRPO-based	Luminol/H_2O_2/ *p*-iodophenol	Oxidized luminol substrate gives off blue light; *p*-iodophenol increases light output	Very convenient, sensitive system; reaction detected within a few seconds to 1 hr
AP-based	Substituted 1,2-dioxetane-phosphates (e.g., AMPPD, CSPD, Lumigen-PPD, Lumi-Phos 530[f])	Dephosphorylated substrate gives off light	Protocol described gives reasonable sensitivity on all membrane types; consult instructions of reagent manufacturer for maximum sensitivity and minimum background (see Troubleshooting)

[a]Abbreviations: AMPPD or Lumigen-PPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-napthol; CSPD, AMPPD with substituted chlorine moiety on adamantine ring; DAB, 3,3′-diaminobenzidine; HRPO, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3′,5,5′-tetramethylbenzidine.

[b]Recipes and suppliers are listed in Reagents and Solutions except for TMP, for which use of a kit is recommended.

[c]See Commentary for further details.

[d]DAB/$NiCl_2$ can be used without the nickel enhancement, but it is much less sensitive.

[e]McKimm-Breschkin (1990) reported that if nitrocellulose filters are first treated with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0), TMB precipitates onto the membrane with a sensitivity much greater than 4CN or DAB, and equal to or better than that of BCIP/NBT.

[f]Lumi-Phos 530 contains dioxetane phosphate, $MgCl_2$, CTAB (cetyltrimethylammonium bromide), and fluorescent enhancer in a pH 9.6 buffer.

ALTERNATE PROTOCOL 4

VISUALIZATION WITH LUMINESCENT SUBSTRATES

Antigens can also be visualized with luminescent substrates. Detection with light offers both speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for horseradish peroxidase (HRPO) systems or dioxetane phosphate for alkaline phosphatase (AP) systems, sealed in thin plastic wrap, and placed firmly against film. Exposures range from a few seconds to several hours, although typically strong signals appear within a few seconds or minutes.

Additional Materials *(also see Basic Protocol 3)*

Luminescent substrate buffer: 50 mM Tris·Cl, pH 7.5 (HRPO; *APPENDIX 2A*) *or* dioxetane phosphate substrate buffer (alkaline phosphatase; see recipe)
Nitro-Block solution (AP reactions only): 5% (v/v) Nitro-Block (Tropix) in dioxetane phosphate substrate buffer, prepared just before use
Luminescent visualization solution (Table 6.2.1)
Clear plastic wrap

Additional reagents and equipment for autoradiography (*UNIT 6.3*)

NOTE: See Troubleshooting section for suggestions concerning optimization of this protocol, particularly when employing AP-based systems.

1. Equilibrate membrane in two 15-min washes with 50 ml substrate buffer.

   For blots of whole gels, use 50 ml substrate buffer; for strips, use 5 to 10 ml/strip.

2. *For AP reactions using nitrocellulose or PVDF membranes:* Incubate 5 min in Nitro-Block solution, followed by 5 min in substrate buffer (volumes as in step 1).

   Nitro-Block enhances light output from the dioxetane substrate in reactions using AMPPD, CSPD, or Lumigen-PPD concentrate. It is required for nitrocellulose and recommended for PVDF membranes. It is not needed for Lumi-Phos 530, AP reactions on nylon membranes, or HRPO-based reactions on any type of membrane. Lumi-Phos 530 is not recommended for nitrocellulose membranes.

3. Transfer membrane to visualization solution. Soak 30 sec (HRPO reactions) to 5 min (AP reactions; volumes as in step 1).

   Alternatively, lay out a square of plastic wrap and pipet 1 to 2 ml visualization solution into the middle. Place membrane on the plastic so that the visualization solution spreads out evenly from edge to edge. Fold wrap back onto membrane, seal, and proceed to step 5.

4. Remove membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane to form a liquid-tight enclosure.

   To ensure an optimal image, only one layer of plastic should be between the membrane and film. Sealable bags are an effective alternative. Moisture must not come in contact with the X-ray film.

5. In a darkroom, place membrane face down onto film.

   Do this quickly and do not reposition; a double image will be formed if the membrane is moved while in contact with the film. A blurred image is usually caused by poor contact between membrane and film; use a film cassette that ensures a tight fit.

6. Expose film for a few seconds to several hours.

   Typically, immunoblots produce very strong signals within a few seconds or minutes. However, weak signals may require several hours to an overnight exposure. If no image is detected, expose film 30 min to 1 hr, and if needed, overnight (see Troubleshooting).

7. If desired, wash membrane in two 15-min washes of 50 ml TBS and process for chromogenic development (see Basic Protocol 3).

   Chemiluminescent and chromogenic immunoblotting can be easily combined on a single blot to provide a permanent visual marker of a known protein. First probe membrane with the chemiluminescent reactions to record on film. If stripping and reprobing is needed, then process by wetting and NaOH treatment (see Support Protocol 3). For the last reaction, use chromogenic development to produce a permanent visual record of the blot. Alternatively, once the film record of the chemiluminescent blot is recorded, the blot can be rinsed briefly with distilled water and placed in the appropriate chromogenic solution for chromogenic development of the blot. This results in a permanent reference stain on the blot for comparison to the more easily scanned and quantitated film record.

SUPPORT PROTOCOL 3

STRIPPING AND REUSING MEMBRANES

This stripping procedure works with blotted membranes from one- and two-dimensional gels as well as with proteins blotted from previously stained gels (Suck and Krupinska, 1996). Reprobing PVDF membranes that have been developed with chemiluminescent reagents is simple and straightforward. All residual antibodies are removed from the membrane by first rewetting it in water and then briefly treating it with NaOH. Although repeated reprobing can lead to loss of signal, up to five reprobings generally are feasible. The blot should have been previously blocked with 5% nonfat dry milk prior to treatment.

Materials

0.2 M NaOH

1. Wash blot 5 min in distilled water.

   In order to effectively reprobe the membranes, casein (for AP systems) or nonfat dry milk must be used as the blocking agent. Chromogenic development leaves a permanent stain on the membrane that is difficult to remove, and should not be used when reprobing. The stain can interfere with subsequent analysis if reactive bands from sequential immunostainings are close together.

2. Transfer to 0.2 M NaOH and wash 5 min.
3. Wash blot 5 min in distilled water.
4. Proceed with immunoprobing procedure (see Basic Protocol 2 and Alternate Protocol 3).

   Casein or nonfat dry milk is recommended as blocking agent when reprobing membranes.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**. For selection of appropriate chromogenic or luminescent solutions, and for definition of abbreviations, see Table 6.2.1.*

Alkaline phosphate substrate buffer

100 mM Tris·Cl, pH 9.5
100 mM NaCl
5 mM $MgCl_2$

BCIP/NBT visualization solution

Mix 33 µl NBT stock (100 mg NBT in 2 ml at 70% DMF, stored <1 year at 4°C) and 5 ml alkaline phosphate substrate buffer (see recipe). Add 17 µl BCIP stock (100 mg BCIP in 2 ml of 100% DMF, stored <1 year at 4°C) and mix. Stable 1 hr at room temperature.

Recipe is from Harlow and Lane (1988). Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, Moss, and Vector Labs.

Blocking buffer

Colorimetric detection:

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in TBS (TTBS; *APPENDIX 2A*).

For neutral and positively charged nylon: Tris-buffered saline (TBS; *APPENDIX 2A*) containing 10% (w/v) nonfat dry milk. Prepare just before use.

TTBS can be stored ≤1 week at 4°C.

Luminescence detection:

For nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A): 0.2% casein (e.g., Hammarsten grade or I-Block; Tropix) in TTBS (*APPENDIX 2A*). Prepare just before use.

continued

For positively charged nylon: 6% (w/v) casein/1% (v/v) polyvinyl pyrrolidone (PVP) in TTBS (*APPENDIX 2A*). With constant mixing, add casein and PVP to warm (65°C) TTBS. Stir for 5 min. Cool before use. Prepare just before use.

4CN visualization solution

Mix 20 ml ice-cold methanol with 60 mg 4CN. Separately mix 60 µl of 30% H_2O_2 with 100 ml TBS (*APPENDIX 2A*) at room temperature. Rapidly mix the two solutions and use immediately.

DAB/$NiCl_2$ visualization solution

5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 µl DAB stock (40 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
15 µl 3% H_2O_2
Mix just before use

CAUTION: *Handle DAB carefully, wearing gloves and mask; it is a carcinogen.*

Suppliers of peroxidase substrates are Sigma, Kirkegaard & Perry, Moss, and Vector Labs.

Dioxetane phosphate substrate buffer

1 mM $MgCl_2$
0.1 M diethanolamine
0.02% sodium azide (optional)
Adjust to pH 10 with HCl and use fresh

Traditionally, the AMPPD substrate buffer has been a solution containing 1 mM $MgCl_2$ and 50 mM sodium carbonate/bicarbonate, pH 9.6 (Gillespie and Hudspeth, 1991). The use of diethanolamine results in better light output (Tropix Western Light instructions).

Alternatively, 100 mM Tris·Cl (pH 9.5)/100 mM NaCl/5 mM $MgCl_2$ can be used (Sandhu et al., 1991).

Dioxetane phosphate visualization solution

Prepare 0.1 mg/ml AMPPD or CSPD (Tropix) *or* Lumigen-PPD (Lumigen; see Table 6.2.1) substrate in dioxetane phosphate substrate buffer (see recipe). Prepare just before use. Lumi-Phos 530 (Boehringer Mannheim or Lumigen) is a ready-to-use solution and can be applied directly to the membrane.

This concentration of AMPPD substrate (240 µM) is the minimum recommended by Tropix Western Light. Ten-fold lower concentrations can be used but require longer exposures.

Luminol visualization solution

0.5 ml 10× luminol stock [40 mg luminol (Sigma) in 10 ml DMSO]
0.5 ml 10× *p*-iodophenol stock [optional; 10 mg (Aldrich) in 10 ml DMSO]
2.5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
25 µl 3% H_2O_2
H_2O to 5 ml
Prepare just before use

Recipe is from Schneppenheim et al. (1991). Premixed luminol substrate mix (Mast Immunosystems; Amersham ECL; Du Pont NEN Renaissance; Kirkegaard & Perry LumiGLO) may also be used. p-iodophenol is an optional enhancing agent that increases light output. Luminol and p-iodophenol stocks can be stored for ≤6 months at −20°C.

Ponceau S solution

Dissolve 0.5 g Ponceau S in 1 ml glacial acetic acid. Bring to 100 ml with water. Prepare just before use.

Transfer buffer

Add 18.2 g Tris base and 86.5 g glycine to 4 liters of water. Add 1200 ml methanol and bring to 6 liters with water. The pH of the solution is ~8.3 to 8.4. For use with PVDF filters, decrease methanol concentration to 15%; for nylon filters, omit methanol altogether.

CAPS transfer buffer can also be used. Add 2.21 g cyclohexylaminopropane sulfonic acid (CAPS; free acid), 0.5 g DTT, 150 ml methanol, and water to 1 liter. Adjust to pH 10.5 with NaOH and chill to 4°C. For proteins >60 kDa, reduce methanol content to 1% (Moos, 1992).

COMMENTARY

Background Information

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal (*UNIT 7.2*). However, there are several problems inherent with this method, including the requirement for radiolabeling of antigen, co-precipitation of tightly associated macromolecules, occasional difficulty in obtaining precipitating antibodies, and insolubility of various antigens (Talbot et al., 1984).

To circumvent these problems, electroblotting (Towbin et al., 1979)—subsequently popularized as western blotting or immunoblotting (Burnette, 1981)—was conceived. Immunoblotting is a rapid and sensitive assay for the detection and characterization of proteins that works by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization and electrophoretic separation of proteins, glycoproteins, or lipopolysaccharides by SDS-PAGE (*UNIT 6.1*) or urea-PAGE, followed by quantitative transfer and irreversible binding to nitrocellulose, PVDF, or nylon. This technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies and is highly sensitive (1 ng of antigen can be detected).

Electroblotting of previously stained gels is a convenient way to visualize and document the gel prior to immunoblotting. Transfer efficiencies at all molecular weights will be lower with fixed and stained gels. This is particularly true of proteins >50 kDa (Perides et al., 1986). The additional incubation in 6 M urea will significantly increase transfer efficiency of all proteins and is required for proteins >50 kDa.

Ponceau S staining provides an easy method for calibrating and quantitating the amount of material on a nitrocellulose or PVDF blot. An alternative to this method is to use an internal protein control with a separate antibody probe, but these tend to be expensive and time-consuming to use. Other applications for Ponceau S calibration include monitoring transfer efficiency under varied conditions for optimization of tank and semidry blotting.

Immunoblotted proteins can be detected by chromogenic or luminescent assays (see Table 6.2.1 for a description of the reagents available for each system, their reactions, and a comparison of their advantages and disadvantages). Luminescent detection methods offer several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both HRPO and phosphatase systems without the need for radioisotopes. Substrates for the latter have only recently been applied to protein blotting (see Gillespie and Hudspeth, 1991; Sandhu et al., 1991; Bronstein et al., 1992). Luminescent detection can be completed in as little as a few seconds; exposures rarely go more than 1 hr. Depending on the system, the luminescence can last for 3 days, permitting multiple exposures of the same blot. Furthermore, the signal is detected by film, and varying the exposure can result in more or less sensitivity. Luminescent blots can be easily erased and reprobed because the reaction products are soluble and do not deposit on the membrane (see below). Compared to chromogenic development, the luminescent image recorded on film is easier to photograph and to quantitate by densitometry.

Alkaline phosphatase–based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted (see Reagents and Solutions). The procedure described in Alternate Protocol 4 gives reasonable sensitivity on nitrocellulose, PVDF, and nylon membranes with a minimum of steps.

Critical Parameters

First and foremost, the antibody being used should recognize denatured antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always be run in parallel. Time of transfer and primary anti-

body and conjugate dilutions should always be optimized.

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1988). These include Tween 20, PVP, nonfat dry milk, casein, BSA, and serum. A 0.1% (v/v) solution of Tween 20 in TBS (TTBS), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of nitrocellulose and PVDF membranes (Blake et al., 1984). In contrast to dry milk/TBS blocking solution (BLOTTO), TTBS is stable and has a long shelf life at 4°C. Furthermore, TTBS generally produces a clean background and permits subsequent staining with India ink. However, even with the application of such standard blocking procedures as 5% to 10% milk protein or 0.05% to 0.1% Tween 20, background can still be a significant problem. If this happens, using a blocking protein (e.g., goat, horse, or rabbit normal serum) from the same species as the primary antibody can reduce the background, presumably by reducing cross-reactivity between the primary antibodies and the blocking agent. Combinations of blocking agents can also be effective. Thus, 0.1% human serum albumin (HSA) and 0.05% Tween 20 in TBS is recommended when probing Immobilon-P membranes with human serum (Craig et al., 1993). However, this can also lead to overall loss of antigen signal, requiring a ten-fold increase in the primary antibody (serum) concentration to achieve an adequate background free antigen signal.

When using chemiluminescent detection for immunoblotting, high background frequently occurs, particularly for strong signals (Pampori et al., 1995). Several methods are available for reducing the background from chemiluminescent reactions. These include changing the type and concentration of blocking agents (see above), optimizing antibody concentrations, letting the reaction proceed for several minutes before exposing to film, or simply limiting the exposure time of the film on the blot. These procedures are not always successful, however, and can lead to inconsistent results. An alternative approach is to reduce the concentration of reagents ten-fold. This effectively removes the background and has a number of advantages which include lower cost, increased signal-to-noise ratio, and reduced detection of cross-reacting species.

Two types of nylon membrane are used for western transfer—neutral (e.g., Pall Biodyne A) and positively charged (e.g., Pall Biodyne B). Although the positively charged membranes have very good protein-binding characteristics, they tend to give a higher background. These membranes remain positively charged from pH 3 to pH 10. Neutral nylon membranes are also charged, having a mix of amino and carboxyl groups that give an isoelectric point of 6.5. Because of their high binding capacity, positively charged membranes are popular for protein applications using luminescence.

Nylon membranes require more stringent blocking steps. Here 10% nonfat dry milk in TBS is recommended for chromogenic development. During luminescence development, however, background is a more significant problem. Compared to dry milk, purified casein has minimal endogenous alkaline phosphatase activity (AP activity leads to high background) and is therefore recommended as a blocking agent for nitrocellulose, PVDF, and nylon membranes. Positively charged nylon requires much more stringent blocking with 6% (w/v) casein and 1% (v/v) polyvinylpyrrolidone (PVP). Because nonfat dry milk and casein may contain biotin that will interfere with avidin-biotin reactions, subsequent steps are done without protein-blocking agents when using these systems. If background is a problem, highly purified casein (0.2% to 6%) added to the antibody incubation buffers may help.

If reprobing is desired, blots can be air dried and stored at 4°C for 3 months after chemiluminescence detection. After drying, store in a sealed freezer bag until use. Repeated probing will lead to a gradual loss of signal and increased background. However, this will depend in part on the properties of the sample.

If the primary procedure is problematic due to loss of sensitivity or an increase in the background, then two possible alternative procedures for stripping membranes are recommended. The first uses 2-mercaptoethanol and SDS (Kaufmann et al., 1987; Tesfaigzi et al., 1994). Briefly, the membranes are incubated in 2% SDS/100 mM Tris·Cl, pH 7.4/100 mM 2-mercaptoethanol for 30 min at 70°C, effectively removing primary and secondary antibodies. As with the primary procedure recommended above, the repeated probing should be done with caution due to the potential loss of detection signal, and 5% nonfat dry milk is required as a blocking agent. The milk blocking agent facilitates antibody removal from the blot (Kaufmann et al., 1987). The second uses guanidine·HCl. For nylon and PVDF membranes (do *not* use with nitrocellulose), incubate the immunoblot in 7 M guanidine·HCl for

10 min at room temperature. (The short wash time is critical, as guanidine·HCl is a very strong denaturant, so do not leave the filter in this solution >15 min.) Pour off excess guanidine·HCl and then rinse the membrane several times in 1× TTBS. Reblock the membrane and proceed with the standard immunoblotting procedure. Membranes stripped using this procedure can generally be reused three or four times.

Troubleshooting

There are several problems associated with immunoblotting. The antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS or urea), and some antibodies may not recognize the denatured form of the antigen transferred to the membrane. The results observed may be entirely dependent on the denaturation and transfer system used. For example, zwitterionic detergents have been shown to restore the antigenicity of outer membrane proteins in immunoblotting (Mandrell and Zollinger, 1984). Gel electrophoresis under nondenaturing conditions can also be performed.

Other potential problems include high background, nonspecific or weak cross-reactivity of antibodies, poor protein transfer or membrane binding efficiency, and insufficient sensitivity. For an extensive survey and discussion of immunoblotting problems and artifacts, see Bjerrum et al. (1988).

If no transfer of protein has occurred, check the power supply and electroblot apparatus to make sure that the proper electrical connections were made and that power was delivered during transfer. In addition, check that the correct orientation of filter and gel relative to the anode and cathode electrodes was used.

If the transfer efficiency using the tank system appears to be low, increase the transfer time or power. Cooling (using the unit's built-in cooling cores) is generally required for transfers >1 hr. At no time should the buffer temperature go above 45°C. Prolonged transfers (>1 hr) are not possible in semidry transfer units due to rapid buffer depletion.

Alternatively, the transfer buffer can be modified to increase efficiency. Adding SDS at a concentration of 0.1% to the transfer buffer improves the transfer of all proteins out of the gel, particularly those above 60 to 90 kD in size. Lowering the concentration of methanol will also improve the recovery of proteins from the gel. These procedures are tradeoffs. Methanol improves the binding of proteins to PVDF and nitrocellulose, but at the same time hinders transfer. With SDS present, transfer efficiency is improved, but the SDS can interfere with protein binding to the membrane. Nylon and PVDF membranes are particularly sensitive to SDS interference. If needed, 0.01% to 0.02% SDS may be used in PVDF membrane transfer buffers (Millipore, 1990). SDS and methanol should not be used in the transfer buffer for nylon (Peluso and Rosenberg, 1987).

Gel cross-linking and thickness also have a profound effect on the transfer efficiency. In general, 0.5- to 0.75-mm-thick gels will transfer much more efficiently than thicker gels (e.g., 1.5 mm thick). Gels with a higher acrylamide percentage will also transfer less efficiently. Proteins can be particularly difficult to transfer from gradient gels, and a combination of longer transfer times, thin gels, and the addition of SDS to the transfer buffer may be needed.

If the protein bands are diffuse, check the transfer cassette. The gel must be held firmly against the membrane during transfer. If the transfer sandwich is loose in the cassette, add another thin sponge or more blotter paper to both sides.

Occasionally, a grid pattern will be apparent on the membrane after tank transfer. This is caused by having either the gel or the membrane too close to the sides of the cassette. Correct this by adding more layers of filter paper to diffuse the current flowing through the gel and membrane. Use a thinner sponge and more filter paper if necessary.

If air bubbles are trapped between the filter and the gel, they will appear as clear white areas on the filter after blotting and staining. Take extra care to make sure that all bubbles are removed.

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. A control using preimmune serum or only the secondary antibody will determine if these problems are due to the primary antibody. Try switching to another blocking agent; protein blocking agents may weakly cross-react. Lowering the concentration of primary antibody should decrease background and improve specificity (Fig. 6.2.3).

Due to the nature of light and the method of detection, certain precautions are warranted when using luminescent visualization (e.g., Harper and Murphy, 1991). Very strong signals can overshadow nearby weaker signals on the membrane. Because light will pipe through the membrane and the surrounding plastic wrap, overexposure will produce a broad diffuse im-

age on the film. The signal can also saturate the film, exposing the film to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

With the alkaline phosphatase substrate AMPPD, nitrocellulose, PVDF, and nylon membranes require 2, 4, and 8 to 12 hr, respectively, to reach maximum light emission. In addition, PVDF is reported to give a stronger signal than nitrocellulose (Tropix Western Light instructions). Positively charged nylon requires special blocking procedures to minimize background (Gillespie and Hudspeth, 1991). These procedures include using a blocking and primary antibody solution containing 6% casein, 1% polyvinylpyrrolidone-40 (PVP-40), 3 mM NaN_3, 10 mM EDTA, and PBS, pH 6.8. Prior to use, the casein must be heated to 65°C to reduce alkaline phosphatase activity in the casein itself. In addition, maximum sensitivity has been observed when free biotin or biotinylated proteins are removed by pretreating the casein with avidin-agarose (Sigma).

Anticipated Results

Immunoblotting should result in the detection of one or more bands. Although antibodies directed against a single protein should produce a single band, degradation of the sample (e.g., via endogenous proteolytic activity) may cause visualization of multiple bands of slightly different size. Multimers will also form spontaneously, causing higher-molecular-weight bands on the blot. If simultaneously testing multiple antibodies directed against a complex protein mixture (e.g., using patient sera against SDS-PAGE-separated viral proteins in AIDS western blot test), multiple bands will be visualized. Typically, picogram to nanogram sensitivities are common in protein blotting and immunodetection procedures.

Time Considerations

The entire immunoblotting procedure can be completed in 1 to 2 days, depending on transfer time and type of gel. Gel electrophoresis requires 4 to 6 hr on a regular gel and 1 hr on a minigel. Transfer time can be 1 hr (high-power transfer) to overnight. Blocking, conjugate incubation, and washing each take 30 min to 1 hr. Finally, substrate incubation requires 10 to 30 min (chromogen) and a few seconds to several hours (luminescence).

Literature Cited

Bjerrum, O.J., Larsen, K.P., and Heegaard, N.H.H. 1988. Nonspecific binding and artifacts-specificity problems and troubleshooting with an atlas of immunoblotting artifacts. *In* CRC Handbook of Immunoblotting of Proteins, Vol. I: Technical Descriptions (O.J. Bjerrum and N.H.H. Heegaard, eds.) pp. 227-254. CRC Press, Boca Raton, Fla.

Blake, M.S., Johnston, K.H., Russell-Jones, G.J., and Gotschlich, E.C. 1984. A rapid, sensitive method for detection of alkaline phosphatase–conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175-179.

Bronstein, I., Voyta, J.C., Murphy, O.J., Bresnick, L., and Kricka, L.J. 1992. Improved chemiluminescent Western blotting procedure. *BioTechniques* 12:748-753.

Burnette, W.N. 1981. Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.

Craig, W.Y., Poulin, S.E., Collins, M.F., Ledue, T.B., and Ritchie, R.F. 1993. Background staining in immunoblot assays. Reduction of signal caused by cross-reactivity with blocking agents. *J. Immunol. Methods* 158:67-76.

Dionisi, H.M., Checa, S.K., and Viale, A.M. 1995. Protein immunoblotting of stained gels. *BioTechniques* 19:348-350.

Gillespie, P.G. and Hudspeth, A.J. 1991. Chemiluminescence detection of proteins from single cells. *Proc. Natl. Acad. Sci. U.S.A.* 88:2563-2567.

Harlow, E. and Lane, D. 1988. Immunoblotting. *In* Antibodies: A Laboratory Manual, pp. 471-510. CSH Laboratory, Cold Spring Harbor, N.Y.

Harper, D.R. and Murphy, G. 1991. Nonuniform variation in band pattern with luminol/horseradish peroxidase Western blotting. *Anal. Biochem.* 192:59-63.

Kaufmann, S.H., Ewing, C.M., and Shaper, J.H. 1987. The erasable Western blot. *Anal. Biochem.* 161:89-95.

Klein, D., Kern, R.M., and Sokol, R.Z. 1995. A method for quantification and correction of proteins after transfer to immobilization membranes. *Biochem. Mol. Biol. Int.* 36:59-66.

Mandrell, R.E. and Zollinger, W.D. 1984. Use of zwitterionic detergent for the restoration of antibody-binding capacity of electroblotted meningococcal outer membrane proteins. *J. Immunol. Methods* 67:1-11.

McKimm-Breschkin, J.L. 1990. The use of tetramethylbenzidine for solid phase immunoassays. *J. Immunological Methods* 135:277-280.

Millipore. 1990. Protein blotting protocols for Immobilon-P transfer membrane. Bedford, Mass.

Moos, M. 1992. Isolation of proteins for microsequence analysis *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 8.7.1-8.7.12. Greene Publishing Associates and John Wiley & Sons, New York.

Pampori, N.A., Pampori, M.K., and Shapiro, B.H. 1995. Dilution of the chemiluminescence reagents reduces the background noise on Western blots. *BioTechniques* 18:588-590.

Peluso, R.W. and Rosenberg, G.H. 1987. Quantitative electrotransfer of proteins from sodium dodecyl sulfate polyacrylamide gels onto positively charged nylon membranes. *Anal. Biochem.* 162:389-398.

Perides, G., Plagens, U., and Traub, P. 1986. Protein transfer from fixed, stained, and dried polyacrylamide gels and immunoblot with protein A–gold. *Anal. Biochem.* 152:94-99.

Sandhu, G.S., Eckloff, B.W., and Kline, B.C. 1991. Chemiluminescent substrates increase sensitivity of antigen detection in Western blots. *BioTechniques* 11:14-16.

Schneppenheim, R., Budde, U., Dahlmann, N. and Rautenberg, P. 1991. Luminography—a new, highly sensitive visualization method for electrophoresis. *Electrophoresis* 12:367-372.

Suck, R.W.L. and Krupinska, K. 1996. Repeated probing of Western blots obtained from Coomassie Brilliant Blue–stained or unstained polyacrylamide gels. *BioTechniques* 21:418-422.

Talbot, P.V., Knobler, R.L., and Buchmeier, M. 1984. Western and dot immunoblotting analysis of viral antigens and antibodies: Application to murine hepatitis virus. *J. Immunol. Methods* 73:177-188.

Tesfaigzi, J., Smith-Harrison, W., and Carlson, D.M. 1994. A simple method for reusing western blots on PVDF membranes. *BioTechniques* 17:268-269.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76:4350-4354.

Key References

Bjerrum, O.J. and Schafer-Nielsen, C. 1986. Buffer systems and transfer parameters for semidry electroblotting with a horizontal apparatus. *In* Electrophoresis '86 (M.J. Dunn, ed.) pp. 315-327. VCH Publishers, Deerfield Beach, Fla.

Describes the semidry blotting system.

Gillespie and Hudspeth, 1991. See above.

Describes alkaline phosphatase–luminescent detection methods.

Harlow and Lane, 1988. See above.

Details alternative detection methods.

Salinovich, O. and Montelaro, R.C. 1986. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* 156:341-347.

Describes the use of Ponceau S staining for immunoblotting.

Schneppenheim et al., 1991. See above.

Details peroxidase-based luminescent detection methods.

Contributed by Sean R. Gallagher
Motorola Corporation
Tempe, Arizona

Scott E. Winston and Steven A. Fuller (tank transfer systems)
Univax Biologics
Rockville, Maryland

John G.R. Hurrell (tank transfer systems; reversible staining of proteins)
Boehringer Mannheim Biochemicals
Indianapolis, Indiana

Detection and Quantitation of Radiolabeled Proteins in Gels and Blots

This unit presents procedures for visualizing and quantitating radiolabeled proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*) or affixed to filter membranes. Autoradiography (see Basic Protocol) is the most common method by which this is accomplished, and X-ray film is the traditional recording medium. The use of autoradiography with gels requires that the gel be dried prior to being placed in contact with the film (see Support Protocol 1). The decay of radioactive materials within the dried gel or filter leaves an image on the film that reflects its distribution in the sample. Film images can be quantified by densitometry (see Support Protocol 4) to obtain a relative measure of the amount of radioactivity in the sample.

The use of X-ray films for autoradiography, however, suffers from two drawbacks: lack of sensitivity and a limited linear range over which the image density reflects the amount of radioactivity. Lack of sensitivity can be overcome by fluorography (see Alternate Protocol 1) or by the use of intensifying screens (see Support Protocol 2), both of which enhance the radioactive signal. Ensuring that the exposure is within a linear range requires careful controls; film is often preflashed (see Support Protocol 3) to increase the linear measurement range for weakly radioactive samples, and it is important to ensure that the film not be saturated to attain strong radioactive signals. Sensitivity and linear ranges of measurement can be greatly extended by using a phosphor imaging system (see Alternate Protocol 2). Phosphor imaging also makes it much faster and easier to quantify radioactive samples.

To enhance radioactive signals, solid-state scintillation is frequently employed to convert the energy released by radioactive molecules to visible light. This is accomplished in several different ways. In fluorography (see Alternate Protocol 1) organic scintillants are incorporated into the sample to increase the proportion of emitted energy detected from low-energy β particles (e.g., from ^{3}H, ^{14}C, and ^{35}S). Another method uses high-density, fluorescent "intensifying screens" (see Support Protocol 2), which are placed next to the sample and used to capture the excess energy of γ rays (e.g., those produced by ^{125}I) and high-energy β particles (e.g., from ^{32}P).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

AUTORADIOGRAPHY

BASIC PROTOCOL

Autoradiography uses X-ray film to visualize and quantitate radioactive molecules that have been electrophoresed through agarose or polyacrylamide gels (*UNIT 6.1*), hybridized to filters (e.g., immunoblots; *UNIT 6.2*), or chromatographed through paper or thin-layer plates. A photon of light or the β particles and γ rays released from radioactive molecules "activate" silver bromide crystals on the film emulsion. This renders them capable of being reduced through the developing process to form silver metal (a "grain"). The silver grains on the film form the image.

The choice of film is critical for autoradiography. Double-coated films (e.g., Kodak X-Omat AR and Fuji RX) contain two emulsion layers on either side of a polyester support and are most commonly used for autoradiography (Laskey and Mills, 1977). Double-coated films are ideal for detecting the high-energy β particles emitted by ^{32}P and ^{125}I,

Contributed by Daniel Voytas and Ning Ke
Current Protocols in Cell Biology (1998) 6.3.1-6.3.10

since they can penetrate the polyester support and expose both emulsion layers. These films are normally used with calcium tungstate ($CaWO_4$) intensifying screens at reduced temperature (−70°C); they are highly sensitive to the blue light emitted by these screens. The green-light-sensitive BioMax MS film (Kodak) is a double-coated film spectrally matched to the blue- and green-light-emitting BioMax MS intensifying screen. The BioMax MS film/BioMax MS intensifying screen system normally gives greatest sensitivity to ^{32}P (four times greater than that of X-Omat AR film with $CaWO_4$ screens).

Single-coated films, containing one emulsion layer (e.g., Kodak BioMax MR), are optimized for direct-exposure techniques with medium-energy radioisotopes (e.g., ^{14}C, ^{35}S, and ^{33}P, but not ^{3}H). The majority of the β particles emitted by these isotopes cannot pass through the polyester support of double-coated films, and therefore the emulsion layer on the other side of the film is useless. Even though direct exposure with single-coated films gives better clarity for medium-energy isotopes, single-coated films often require longer exposure times. Fluorography, therefore, is often used to enhance sensitivity. The blue-light-sensitive double-coated X-Omat AR film is generally used for fluorography with 2,5-diphenyloxazole (PPO; which emits at 388 nm), sodium salicylate (which emits at 420 nm), and commercial fluorographic solutions and sprays (e.g., from Amersham and DuPont NEN; which emit light in the blue end of the spectrum).

Materials

Fixed and dried gel (see Support Protocol 1) or filter (e.g., from immunoblotting; *UNIT 6.2*)
Developer: Kodak developer and replenisher, prepared according to the manufacturer's instructions, 18° to 20°C
Fixer: Kodak fixer and replenisher, prepared according to the manufacturer's instructions, 18° to 20°C
Metal film cassette or paper film cassette with particle-board supports and metal binder clips
Plastic wrap (e.g., Saran Wrap)
X-ray film
Trays to hold film processing solutions
Clips for hanging film

1. In a darkroom illuminated with a safelight, place the sample (e.g., dried gel or filter) in the film cassette. Cover the sample with plastic wrap to prevent it from sticking to the film and contaminating the cassette with radioactivity.

   The safelight should be a bulb of <15 W that is equipped with a Kodak GBX-2 red filter (or equivalent).

   Fluorescent glow-in-the-dark ink (available at craft stores) is a convenient way to mark samples exposed to film. The ink can be spread on adhesive labels, which in turn are placed on the plastic wrap around the edge of the sample. If exposed to light prior to autoradiography, the ink will fluoresce and expose the film, making it possible to orient the film image on the dried gel after developing.

2. Place a sheet of X-ray film on top of the sample, then close and secure the film cassette (see Fig. 6.3.1).

   If preflashed film is used for direct exposure (see Support Protocol 3), the exposed side should face the sample. Preflashed film should be used if sample is weakly radioactive or if quantitation of the radioactivity is desired. For single-coated film, the emulsion layer should face the sample.

   If a paper cassette is used, particle-board supports the same size as the cassette should be placed on either side and secured with the metal binder clips. This will ensure that the sample and film are held in contact and do not shift during exposure.

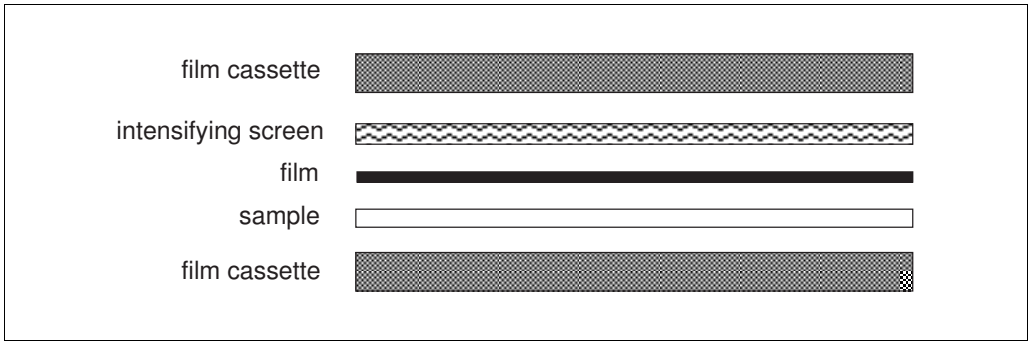


Figure 6.3.1 Autoradiography setup: intensifying screen, film, and sample in film cassette.

3. Expose the film for the desired length of time and at the appropriate temperature.

   Time of exposure will depend on the strength of the radioactivity in the sample and, in most cases, will have to be determined empirically by making multiple exposures for different lengths of time. To help estimate exposure time, a Geiger counter can often be used to detect the relative amount of radioactivity in the sample. With experience, this can help alleviate the trial and error often associated with obtaining the optimum exposure. Time of exposure and use of internal controls are particularly important if quantitative comparisons between experiments are desired.

4. After exposure, return cassette to the darkroom and remove film for developing.

   If the film was exposed at −70°C, allow the cassette to come to room temperature before developing. This will avoid static discharge, which can cause black dots or stripes on the autoradiogram.

   Automated film developers are also available and can be used to develop the film.

5. Immerse the film for 5 min in 18° to 20°C developer, then wash 1 min in running water at room temperature.

   Shorter periods of time in developer will yield a lighter image. The amount of time in developer, therefore, can be used to roughly control intensity of the image.

6. Immerse the film for 5 min in 18° to 20°C fixer, then wash for 15 min in running water.

7. Hang the film to dry.

   The orientation of the film with respect to the gel can be determined by the images of the fluorescent markers.

FIXING AND DRYING GELS FOR AUTORADIOGRAPHY

SUPPORT PROTOCOL 1

SDS-PAGE gels containing radiolabeled proteins should be fixed and dried before exposure to film. This will prevent the gel from sticking to the film, improve the sharpness of the image, and increase sensitivity slightly. However, if the specific activity of the sample is high or the detection method is sensitive (e.g., where a phosphor imager is used; see Alternate Protocol 2), then fixing and drying the gel may not be necessary. Gel dryers are available from a number of manufacturers (e.g., Bio-Rad), most of which use heat and a vacuum to accelerate the drying process.

Materials

Gel from SDS-PAGE (*UNIT 6.1*)
Fixing solution: 10% (v/v) glacial acetic acid/20% (v/v) methanol in H_2O
Alternative fixing solution (for gels with ≥15% acrylamide or thicker than 1.5 mm): 3% (v/v) glycerol/10% (v/v) glacial acetic acid/20% (v/v) methanol in H_2O

Glass dish
Rotary shaker
Filter paper (Whatman 3MM) in sheets at least 1 to 2 cm larger than gel
Plastic wrap (e.g., Saran Wrap)
Gel dryer with vacuum pump

1. After electrophoresis, remove the gel and the supporting glass plates from the electrophoresis apparatus and place in a glass dish. Carefully remove the upper glass plate by gently prying apart the corners with a metal spatula. Make a notch in the upper right hand corner of the gel for orientation.

   Since the gel contains radiolabeled proteins, be sure to follow the necessary guidelines for handling radioactivity. Everything that comes in contact with the gel is potentially radioactive.

2a. *For gels with <15% acrylamide and <1.5 mm thick:* Place the glass dish in a fume hood and pour enough fixing solution into the dish to cover the gel. Place the dish on a rotary shaker and gently rotate until 5 min after all of the blue color from the bromphenol blue in the sample buffer (if used) has disappeared (~30 min total).

   The bromphenol blue typically used in SDS-PAGE sample buffer will turn yellow as the acidic fixing solution diffuses into the gel.

   During fixing, the gel will typically slide off the lower glass plate, which can be removed.

2b. *For gels with ≥15% acrylamide or >1.5 mm thick:* Fix gel as in step 2a, but soak 1 hr in alternative fixing solution.

   The glycerol in the alternative fixing solution should help prevent cracking during drying.

3. Pour off the fixing solution and rinse the gel for a few minutes with deionized water.

   CAUTION: *Remember that solutions that come in contact with the gel are potentially radioactive.*

4. Carefully pour off the water and position the gel in the center of the glass dish. Be sure that any excess water is drained. Place a sheet of Whatman 3MM filter paper, at least 1 to 2 cm larger than the gel, over the gel.

   The gel will stick to the filter paper, which will allow it to be lifted and turned over with the gel side facing up.

5. Cover gel with plastic wrap. Smooth the wrap with a piece of tissue paper to remove any air bubbles or wrinkles.

6. Place a piece of filter paper on the gel support of the gel dryer to prevent contamination of the dryer by radioactivity.

7. Place the filter paper/gel/plastic wrap sandwich on the filter paper in the gel dryer with the plastic sheet facing up.

8. Position the rubber sealing gasket of the gel dryer over the gel. Set the appropriate heat setting on the gel dryer (normally 80°C; 60°C if the gel contains a fluor). Apply the vacuum and allow the gel to dry (typically 2 hr for a gel of 1 mm thickness).

Removing the gel before it is completely dry can lead to cracking; it is therefore not a good idea to rush the drying process. A rough indication of whether the gel is dry can be obtained by feeling the gel under the sealing gasket. If the gel is dry, it should be warm over the entire surface.

9. Remove gel from dryer and proceed with autoradiography (see Basic Protocol).

USE OF INTENSIFYING SCREENS

SUPPORT PROTOCOL 2

Intensifying screens are used to enhance the film image generated by radioactive molecules (Laskey and Mills, 1977; Laskey, 1980). They are used strictly in conjunction with strong β-emitting isotopes such as ^{32}P or γ-emitting isotopes such as ^{125}I. Emissions from these forms of radiation will frequently pass completely through a film, but they can be absorbed by an intensifying screen which fluoresces and exposes the film with multiple photons of light. While an intensifying screen will substantially enhance the film image as compared with direct exposure (Table 6.3.1), some loss of image resolution will occur due to light scatter. Intensifying screens are distributed by most laboratory supply companies (e.g., Fisher, Sigma, and Kodak).

As shown in Figure 6.3.1, the film should be placed between the sample and the intensifying screen. Preflashed film (see Support Protocol 3) should be used if the sample is weakly radioactive or if quantitation of the radioactivity is desired. The preflashed side of the film should be placed adjacent to the intensifying screen. For very weakly radioactive samples, a second screen can be placed on the other side of the radioactive sample (i.e., screen, then sample, then film, then screen), but this causes further loss in resolution due to light scatter. Also, the sample and sample support must be sufficiently transparent to allow light from the second screen to reach the film. The film should be exposed at −70°C to stabilize the silver bromide crystals activated by the radioactivity or the light emitted from the screen.

Table 6.3.1 Different Methods for Isotope Detection and Their Sensitivities[a]

Isotope	Method[b]	Sensitivity[c]	Enhancement over direct autoradiography[d]
^{125}I	S	100	16
^{32}P	S	50	10.5
^{14}C	F	400	15
^{35}S	F	400	15
^{3}H	F	8000	>100

[a]Exposures conducted at −70°C using preexposed film.

[b]S, intensifying screen; F, fluorography using PPO.

[c]Defined as dpm/cm^2 required for detectable image (A_{540} = 0.02) in 24 hr.

[d]Direct autoradiography for comparison was performed on Kodirex film (Laskey, 1980).

PREFLASHING (PREEXPOSING) FILM

Silver bromide crystals that are activated by light, β particles, or γ rays are highly unstable and quickly revert back to their stable form. The absorption of several photons increases their stability but does not ensure development; approximately five photons of light are required to obtain a 50% probability that any single silver bromide crystal will be developed during film processing. This inefficiency means that film images produced by very low levels of exposure will be disproportionately faint. However, two measures can be taken to maximize efficiency and linearity of exposure at the low levels commonly encountered in ordinary use. First, the film should be preexposed to a hypersensitizing flash of light, which provides several photons per silver bromide crystal and stably activates them without providing enough exposure to cause them to become developed. This allows a linear relationship to be drawn between the film image and the amount of radioactivity in the sample. Second, film exposure should be conducted at low temperatures (−70°C) to slow the reversal of activated silver bromide crystals to their stable form (Laskey and Mills, 1975).

Film can be hypersensitized by exposure to a flash of light (<1 msec) provided by a photographic flash unit or a stroboscope before being placed onto the radioactive sample for exposure of the autoradiogram (Laskey and Mills, 1975, 1977). As the optimal light intensity required for preexposure varies with the type of film and the flash unit being used, the ideal exposure is best determined empirically as described below.

Materials

Stroboscope or flash unit (e.g., Auto 22 Electronic Flash from Vivitar or Sensitize Pre-Flash from Amersham Pharmacia Biotech)
Neutral-density filter (Kodak)
Orange filter (Wratten 22; Kodak)
X-ray film
Spectrophotometer

1. Cover the stroboscope or flash unit with the neutral-density and orange filter.

   This serves to decrease the intensity of emitted light, particularly the blue wavelengths to which X-ray films are most sensitive. Filters are not required for the Amersham flash unit.

2. Place the film perpendicular to the light source at a distance of ≥50 cm to ensure uniform illumination.

3. Expose a series of test films for different flash lengths, then develop them (see Basic Protocol).

   An uneven fog level on film can be remedied by placing a porous paper diffuser, such as Whatman no. 1 filter paper, between the film and the light source.

4. Cut the films into pieces that fit into a cuvette holder of a spectrophotometer and measure the absorbance at 540 nm.

   Choose an exposure time that causes the absorbance of the preexposed film to increase by 0.15 with respect to film that was not preexposed.

FLUOROGRAPHY

ALTERNATE PROTOCOL 1

Organic scintillants can be included in radioactive samples to obtain autoradiograms of weak β-emitting isotopes such as ^{3}H, ^{14}C, and ^{35}S. The scintillant fluoresces upon absorption of β particles from these isotopes, facilitating film exposure. Fluorographs of radioactive molecules in polyacrylamide gels have traditionally used the scintillant PPO (2,5-diphenyloxazole; Laskey and Mills, 1975). PPO, however, has largely been replaced with commercial scintillation formulations that reduce the amount of preparation time and are considerably safer to use. These scintillants (e.g., Enhance from NEN Life Science) come with complete instructions for their use. In addition, spray applicators are also available that can be used on filters or thin-layer plates. The expected levels of image enhancement obtained through fluorography are listed in Table 6.3.1. Sodium salicylate can also be used for fluorography as described below (Chamberlain, 1979). It yields levels of image enhancement comparable to organic scintillants, although it sometimes causes a more diffuse film image. The conditions should work for most standard sizes and thicknesses of gels.

CAUTION: Gloves should be worn at all times; sodium salicylate can elicit allergic reactions and is readily absorbed through the skin.

Materials

Polyacrylamide gel
1 M sodium salicylate, pH 5 to 7, freshly prepared

Additional reagents and equipment for fixing and drying gels (see Support Protocol 1)

1. If gel is acid-fixed, soak for 1 to 5 hr in ~20 vol water to prevent precipitation of salicylic acid from the sodium salicylate.
2. Soak gel 30 min in 10 vol of 1 M sodium salicylate, pH 5 to 7.

   To prevent cracking of gels with >15% acrylamide or thicker than 1.5 mm, 2% (v/v) glycerol can be added to the 1 M sodium salicylate.
3. Dry the gel (see Support Protocol 1) and proceed with autoradiography (see Basic Protocol).

DENSITOMETRY

SUPPORT PROTOCOL 4

Film images obtained by autoradiographic methods can be quantified by densitometry. Densitometers work by comparing the intensity of light transmitted through a sample with the intensity of the incident light. The amount of light transmitted will be proportional to the amount of radioactivity in the gel, provided that the film has been properly preexposed (see Support Protocol 3). The linear range of correctly preexposed film is 0.1 to 1.0 absorbance units. However, if the preexposure is excessive—i.e., an increase of >0.2 absorbance units (A_{540}) treated film/untreated film—smaller amounts of radioactivity will produce disproportionately dense images. Autoradiograms that exceed an absorbance of 1.4 absorbance units (A_{540}) have saturated all available silver bromide crystals and also cannot be evaluated quantitatively.

Densitometers are available from several manufacturers (e.g., Molecular Dynamics, Bio-Rad, and UVP). Most models come with software that facilitates calculations and allows the user to define the region of the film to be measured. Procedures for the use of these machines vary and instructions are provided by the manufacturer. Densitometers are also available that measure light reflected from a sample. Reflectance densitometers are useful in instances where the sample medium is completely opaque—e.g., filters that have been probed using nonradioactive colorimetric detection assays.

ALTERNATE PROTOCOL 2

PHOSPHOR IMAGING

Phosphor imaging screens can be used as an alternative to film for recording and quantifying autoradiographic images (Johnston et al., 1990). They can detect radioisotopes such as ^{32}P, ^{125}I, ^{14}C, ^{35}S, and ^{3}H. There are several advantages of phosphor imaging over film: (1) linear dynamic ranges are 5 orders of magnitude, compared to ~1.5 orders of magnitude for film (Fig. 6.3.2); (2) exposure times are 10 to 250 times faster than with film; (3) quantification is much easier and faster, and most imagers come with software to directly analyze data; (4) fluorography and gel drying are often unnecessary because of the sensitivity of phosphor imaging; and (5) phosphor screens can be reused indefinitely if handled carefully.

Phosphor imaging screens are composed of crystals of $BaFBr:Eu^{+2}$. When the screen is exposed to ionizing radiation such as α, β, or γ radiation, or wavelengths of light shorter than 380 nm, the electrons from Eu^{+2} are excited and then trapped in an "F-center" of the $BaFBr^{-}$ complex; this results in the oxidation of Eu^{+2} to Eu^{+3}, which forms the latent image on the screen. After exposure, the latent image is released by scanning the screen with a laser (633 nm). During scanning, Eu^{3+} reverts back to Eu^{+2}, releasing a photon at 390 nm. The luminescence can then be collected and measured in relation to the position of the scanning laser beam. The result is a representation of the latent image on the storage phosphor imaging plates. The image can then be viewed on a video monitor and analyzed with the aid of appropriate software.

Some companies (e.g., Bio-Rad) offer different screens for use with different isotopes. They vary principally in the protective coating on the screen, which is optimized for low- or high-energy β particles or γ rays. No coating is typically used for weak β emitters such as tritium. More recently, screens have also been developed that measure chemiluminescence. Such screens are particularly valuable for use with many nonradioactive labeling protocols.

The protocol below is for the PhosphorImager system from Molecular Dynamics; other phosphor imaging systems are available from Bio-Rad, Imaging Research, and National Diagnostics.

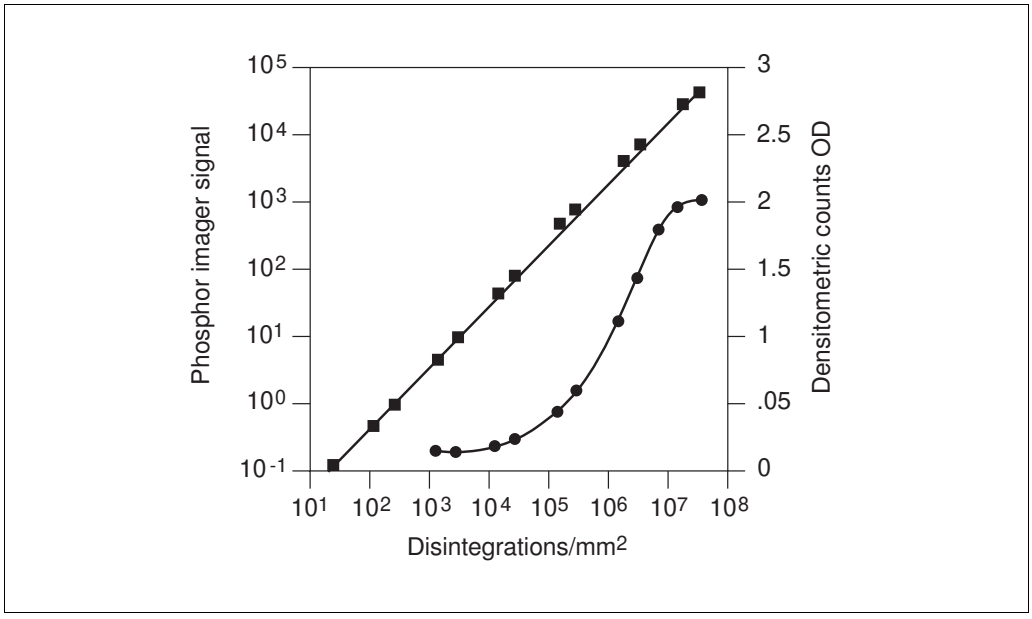


Figure 6.3.2 ^{32}P dilution series quantified on Model GS-525 phosphor imager (squares), compared to film (circles). Image courtesy of Bio-Rad, Hercules, Calif.

Materials

Gel or filter (e.g., from immunoblotting; *UNIT 6.2*)
PhosphorImager system (Molecular Dynamics) including:
ImageEraser light box
Exposure cassette with phosphor screen
Scanning software

1. Erase any latent image on the phosphor screen left by a previous user, or caused by background radiation, by exposure to visible light.

   The PhosphorImager system comes with an extra-bright light box (ImageEraser) for this purpose. Standard laboratory light boxes may also be used.

2. Cover gel or filter with plastic wrap to protect the exposure cassette. Place wrapped gel or filter in the PhosphorImager cassette and close to begin exposure.

   The gel does not have to be dried for this procedure. The phosphor screen is affixed to the lid of the cassette. Exposure times are typically one-tenth of the time required for film exposure.

3. After exposure, slide the screen face down into the PhosphorImager system.

4. Select the scanning area using the software supplied with the PhosphorImager and start scanning.

   The blue light emitted during scanning is collected to produce the latent image.

5. Analyze and quantitate the image using the software provided.

6. Erase the phosphor screen by exposing it to visible light as in step 1.

COMMENTARY

Background Information

The ability to detect radiolabeled proteins is critical to many studies in cell biology. A variety of labeling methods are described throughout this manual, many of which are used to follow protein purification, protein processing, or the movement of proteins within the cell. More often than not, detection of radiolabeled proteins is coupled with the resolving power of SDS polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*). Radiolabeled proteins separated on gels can be used directly to obtain an autoradiographic image. Alternatively, proteins separated by SDS-PAGE are frequently transferred to membranes (*UNIT 6.2*) and detected using radiolabeled probes such as antibodies and ^{125}I-labeled protein A. The autoradiographic image, whether generated on film or a phosphor screen, reflects the distribution of the radioactive proteins on the two-dimensional surface of the gel or filter. Molecular sizes of radiolabeled proteins, therefore, can be determined by correlating their positions with molecular markers. Also, the density of the band images can be used to determine the relative quantities of the radiolabeled proteins in the sample.

Critical Parameters

The sensitivity of the detection device and the strength of the radioactive signal are the two most important parameters for autoradiography. Sensitivity can be enhanced by treating samples with fluors or by using intensifying screens (Table 6.3.2). Because phosphor imaging is 10 to 250 times more sensitive than film (Johnston et al., 1990), this technology makes it possible to monitor radioactive samples that would previously have gone undetected with film.

A second important parameter is the range over which the measurement device is linear. Film requires preflashing in order for the intensity of the image to be linear with respect to the amount of radioactivity, particularly for weakly radioactive samples (Laskey and Mills, 1975, 1977). Phosphor imaging offers a much wider linear range of measurement (5 orders of magnitude compared to 1.5 for film; Johnston et al., 1990). This makes it possible to accurately

Table 6.3.2 Film Choice and Exposure Temperature for Autoradiography

Isotope	Enhancement method	Film	Exposure temperature
^{3}H	Fluorography	Double-coated	−70°C
^{35}S, ^{14}C, ^{32}P	None	Single-coated	Room temperature
^{35}S, ^{14}C, ^{32}P	Fluorography	Double-coated	−70°C
^{32}P, ^{125}I	$CaWO_4$ intensifying screens	Double-coated	−70°C

quantitate very weak or very strong radioactive samples.

Troubleshooting

Cracking is one of the most common problems encountered when drying gels. This often occurs if the gel is removed from the dryer before it has adequately dried or if drying temperatures are too high. To overcome this problem, drying times should be extended and the performance of the vacuum pump and heater unit should be checked. For many gels, particularly for those with high percentages of polyacrylamide or >1.5 mm thick, cracking can be reduced by using an alternative fixing solution containing glycerol (3% glycerol/10% glacial acetic acid/20% methanol; see Support Protocol 1).

Among the biggest problems encountered in autoradiography are images that are either too weak or too intense. Such problems can be solved by varying the exposure time. Estimating initial exposure time is difficult, since the amount of radioactivity in the sample is often unknown. A Geiger counter can offer some guidance with certain isotopes. For highly exposed film, the length of time in developer can be reduced to produce a lighter image. It is particularly important to remember that if accurate quantification of the film image is desired, film must be preflashed so that there is a linear relationship between the amount of radioactivity in the sample and the image intensity.

Artifacts, such as black spots and stripes, can be avoided during developing by making sure that no moisture comes in contact with the film and that films exposed at −70°C are brought to room temperature before developing. Also, it must be noted that β particles from weak isotopes such as ^{3}H cannot penetrate plastic wrap, and plastic wraps can attenuate signals from ^{35}S and ^{14}C up to two-fold.

Anticipated Results

The protocols described here should yield a film image of a gel that can be quantified, stored, and photographed for publication.

Time Considerations

Fixing a gel will require ~45 min. Drying will take an additional 2 hr for a gel 1 mm in thickness. Incorporation of a fluor will add ~45 min to the processing time.

For gels >1.5 mm thick or with >15% acrylamide, an additional 30 min will be required for fixing and ~30 additional minutes will be required for drying.

The length of exposure for films in autoradiography can range from a few minutes to a few weeks, depending on the strength of the radioactivity in the sample. Most exposures last from several hours to a few days. Exposure time can be reduced more than 10-fold with a phosphor imager.

Literature Cited

Chamberlain, J.P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132-135.

Johnston, R.F., Pickett, S.C., and Barker, D.L. 1990. Autoradiography using storage phosphor technology. *Electrophoresis* 11:355-360.

Laskey, R.A. 1980. The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. *Methods Enzymol.* 65:363-371.

Laskey, R.A. and Mills, A.D. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335-341.

Laskey, R.A. and Mills, A.D. 1977. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. *FEBS Lett.* 82:314-316.

Contributed by Daniel Voytas and Ning Ke
Iowa State University
Ames, Iowa

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis combines two different electrophoretic separating techniques in perpendicular directions to provide a much greater separation of complex protein mixtures than either of the individual procedures. The most common two-dimensional technique uses isoelectric focusing (IEF) (see Basic Protocols 1 and 2) followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a perpendicular direction (see Basic Protocol 3). This combination of isoelectric point (pI) and size separation is the most powerful tool for protein separations currently available. After staining, proteins appear on the final two-dimensional gel as round or elliptical spots instead of the rectangular bands observed on one-dimensional gels. Although the total separating power of large-format two-dimensional gels is estimated to be >5000 spots per gel, in practice a single two-dimensional separation of a complex mixture such as a whole-cell or tissue extract may produce 1000 to 2000 well-resolved spots when a sensitive detection method is used.

Until recently, the most common IEF procedures were based on the use of soluble ampholytes, relatively small organic molecules with various isoelectric points and buffering capacities. The pH gradient for IEF gels is produced when the soluble ampholytes migrate in the gel matrix until they reach their isoelectric point. Because stable pH gradients outside the pH 4.0 to 8.0 range are difficult to create with soluble ampholytes, alternative protocols using nonequilibrium conditions are required to resolve proteins with pI values below 4.0 (see Alternate Protocol 1 for acidic proteins) or above 8.0 (see Alternate Protocol 2 for basic proteins). One of the more important limitations of soluble ampholytes is the difficulty in obtaining highly reproducible pH profiles, especially when very narrow pH ranges are needed.

An increasingly attractive alternative to soluble ampholytes is the use of immobilized pH gradient (IPG) gels (see Basic Protocol 2). In this system, the buffering side chains are covalently incorporated into the acrylamide matrix, and any pH range and curve shape can be generated by pouring a gradient gel using two solutions that differ in ampholyte composition rather than acrylamide concentration. As with tube gels, the initial electrophoresis is followed by a second separation using SDS-PAGE in a perpendicular direction (see Basic Protocol 4). The use of IPG gels has recently increased, for at least three major reasons: many of the technical problems associated with their use have been solved or substantially minimized, reproducible premade IPG gels are now commercially available, and lately strong interest has arisen in using two-dimensional gels for proteome analysis studies (analyzing and comparing the complete protein profiles of cell lines, tissue samples, or single-celled organisms).

Another common two-dimensional electrophoresis format is a nonreducing/reducing electrophoretic separation (see Alternate Protocol 3), which provides useful information about intersubunit disulfides or protein-protein complexes that have been cross-linked using a bifunctional chemical cross-linker containing a disulfide bond within the linker region.

This unit also includes support protocols describing pI standards and pH profile measurements (see Support Protocol 1), electrophoresis of immobilized pH gradient gels (see Support Protocol 2), casting Immobiline gels (see Support Protocol 3), preparation of tissue culture cells and solid tissues for isoelectric focusing (see Support Protocols 4 and 5), preparation of molecular weight standards for two-dimensional gels (see Support Protocol 6), and two-dimensional protein databases (see Support Protocol 7).

Contributed by Sandra Harper, Jacek Mozdzanowski., and David Speicher

Current Protocols in Cell Biology (1999) 6.4.1-6.4.36

NOTE: High-purity water (e.g., Milli-Q water or equivalent) is essential for all solutions. For cautions relating to electricity and electrophoresis, see Safety Considerations in the introduction to *UNIT 6.1*.

BASIC PROTOCOL 1

HIGH-RESOLUTION EQUILIBRIUM ISOELECTRIC FOCUSING IN TUBE GELS

This protocol describes the preparation of broad-range first-dimension gels using soluble ampholytes that resolve proteins with pI values between approximately 4.0 and 8.0, and is based on the original procedure described by O'Farrell (1975). The procedure presented here refers specifically to 3-mm IEF tube gels (first-dimension) combined with 1.5-mm-thick 16 × 16–cm (size of separating gel) second-dimension gels (see Basic Protocol 3) and may be easily adapted to a variety of different gel sizes (see Table 6.4.1). A 3-mm IEF gel has a total protein capacity of ~500 µg for complex protein mixtures such as whole-cell extracts. The maximum capacity of any single protein spot is ~0.5 to 5 µg, depending on the solubility of the protein near its isoelectric point and the separation distance from any near neighbors.

In this protocol, gels are cast and prefocused before the sample is loaded. The proteins are then separated according to isoelectric point, and the gels are extruded from the tubes and stored. Measuring pH profiles in IEF gels is a convenient and accurate method for determining pI (see Support Protocol 1). To provide optimal reproducibility, multiple gels should be cast and run simultaneously. This is especially important for comparative studies involving complex mixtures of proteins.

The IEF gels may be cast either by pouring the gel solution into the gel tubes (steps 3a to 7a) or by using hydrostatic pressure (steps 3b to 7b). Pouring the gel solution into the gel tubes is convenient for 3-mm-diameter IEF gels and requires only a minimal excess of reagents. Because the gels are cast using a long needle and syringe, for narrower gels, where the needle does not fit inside the gel tube, casting using hydrostatic pressure is more appropriate. This method requires a larger excess of reagents and special casting cylinders. Many types of ampholytes are readily available from different suppliers to form the desired pH profiles. As ampholytes may vary significantly in their performance, careful selection of the appropriate ampholytes is usually necessary (see Commentary).

Materials

Chromic acid, in acid-resistant container
Urea (ultrapure)
30% acrylamide/0.8% bisacrylamide (see recipe)
20% (w/v) Triton X-100 (see recipe)
Ampholytes (e.g., pH 3-10/2D; ESA)
TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine)
2.5% (w/v) ammonium persulfate (see recipe; prepare immediately before use)
8 M urea (see recipe; prepare immediately before use)
0.1 M orthophosphoric acid (H_3PO_4; see recipe)
0.1 M NaOH (make fresh daily)
Lysis buffer (see recipe)
Protein samples to be analyzed
Equilibration buffer (see recipe)
2-Mercaptoethanol

Isoelectric focusing apparatus (e.g., Protean II xi 2D from Bio-Rad or equivalent) with glass tubes, casting stand, buffer chambers, rubber grommets, and plugs
37°C water bath

continued

110°C oven
10-ml syringe equipped with filter capsule (0.22 or 0.45 µm, e.g., Costar µStar LB)
10-ml syringe equipped with blunt needle [e.g., 20-G × 6 in. (15 cm) or 18-G × 6 in. (15 cm)]
Large glass cylinder sealed at bottom with Parafilm (optional, for hydrostatic pressure casting method only)
2000-V power supply
60-ml syringe
Metal or plastic scoop
Dry ice pellets

Wash tubes and prepare the gel mixture

1. Remove the glass tubes from a chromic acid–filled container. Extensively wash the tubes with water, using high-purity water for the last wash. Dry the tubes at least 1 hr in an oven at 110°C and store them at room temperature, covered with aluminum foil.

   To prevent gels from sticking to the glass tubes, gel tubes have to be very clean. Satisfactory results are obtained by storing the tubes in chromic acid between uses and washing them shortly before use. Because drying the tubes requires at least 1 hr, cleaning steps should be performed the day before gels will be cast.

   CAUTION: *Chromic acid is highly corrosive; follow supplier's precautions carefully.*

2. Prepare the gel solution by mixing:

   16.9 g urea
   4.0 ml of 30% acrylamide/0.8% bisacrylamide
   3.0 ml of 20% (w/v) Triton X-100
   7.5 ml water
   3.0 ml ampholytes.

   Briefly warm the mixture in a 37°C water bath to solubilize urea if needed.

   To minimize decomposition of urea, never warm any solutions containing urea above 37°C, use ultrapure urea, and prepare solutions immediately before use.

   Choice of ampholyte composition is one of the key factors determining the quality of isoelectric focusing separations. Substantial differences in performance, resolution, and shape of the pH gradient formed may be observed with different combinations of ampholytes and with ampholytes from different suppliers. ESA's ampholytes (pH 3-10/2D) are suited for most applications and give reproducible results.

   Although purity of all reagents is important, the purity of urea and choice of ampholytes are among the most critical factors for the quality and performance of isoelectric focusing. Most commercially available reagents marketed specifically for two-dimensional gel electrophoresis should be suitable, although individual lots of reagents from any supplier may provide variability and/or unacceptable results.

Cast gels by pouring

3a. Wrap one end of each glass tube with Parafilm and mount the tube in a casting stand. Mark all the tubes to indicate the desired gel height.

   For reproducible results, all gels should be the same height.

4a. Filter the gel solution using a 10-ml syringe equipped with a syringe-tip filter capsule. Briefly degas the gel solution (~5 min) either by sonication or under vacuum. Then add 42.5 µl TEMED and 187.5 µl of 2.5% (w/v) ammonium persulfate solution to the filtered gel mixture and swirl gently to mix.

5a. Using a 10-ml syringe with a blunt needle, fill each glass tube with gel solution to the desired height. Make sure there are no air bubbles trapped in the gel.

A needle is the best choice for casting gels if tubes of 3-mm inner diameter are used. For narrower tubes, the use of hydrostatic pressure is more appropriate (see steps 3b to 7b, below). For long gels the needle can be extended by inserting a piece of capillary polyethylene tubing over the needle tip. The amount of gel solution described in step 2 is sufficient for sixteen 3-mm tube gels that are 16 cm long.

6a. Immediately overlay each gel with ~50 µl of 8 M urea.

A pipettor with a capillary pipet tip is a convenient tool for overlaying with urea. Avoid mixing the overlay and gel solutions. Polymerization starts to occur ~15 min after the addition of TEMED and ammonium persulfate. It is essential that the gels be poured and overlaid before significant polymerization has occurred.

7a. Let the gels polymerize at least 3 hr prior to use.

Urea decomposes at a substantial rate at room temperature; therefore, the gels should be used the same day they are cast.

Cast gels using hydrostatic pressure

3b. Place a rubber band around the gel tubes so they form a tight bundle. Place the bundle inside a larger glass cylinder that is sealed at the bottom with several layers of Parafilm. All tubes must be precisely vertical.

The dimensions of the larger cylinder depend on the dimensions and number of gel tubes. Excessive space will require more gel solution to cast the gels.

4b. Filter the gel solution using a 10-ml syringe and filter capsule. Degas the gel solution briefly (~5 min) either with sonication or under vacuum. Add 42.5 µl TEMED and 187.5 µl of 2.5% ammonium persulfate solution and swirl.

5b. Pipet the gel solution into the bottom of the glass cylinder. Gently run water down the outside of the tube bundle using a wash bottle. Keep adding water until the gel mix reaches the desired height.

Hydrostatic pressure will force the gel solution into the tubes. Sufficient gel solution must be used to obtain the desired gel height while avoiding forcing any water into the tubes. The volume of gel solution required can be estimated as follows: number of gels × 3.14 × (tube internal radius in cm)^2 × height in cm + ~10 ml to keep a safe level of gel mix at the bottom of the casting cylinder. As water is less dense than the gel solution, the water level will be slightly higher than the level of gel solution inside the tubes.

6b. Overlay the gels with 8 M urea.

Urea decomposes at a substantial rate at room temperature; therefore, the gels should be used the same day they are cast.

7b. Let the gels polymerize at least 3 hr prior to use.

Mount the gels in the electrophoresis unit

8. Prepare the lower electrode solution by degassing the proper amount of 0.1 M H_3PO_4 under vacuum with stirring for at least 5 min. Fill the bottom electrophoresis chamber.

The amount of phosphoric acid depends on the length of the gel tubes and the type of electrophoresis unit. The solution should cover the entire gel for good heat dissipation. Approximately 3 liters are required for Protean II xi 2D electrophoresis units.

9. Remove the gel tubes from the casting stand, remove the Parafilm from the tube bottoms, and inspect gels for irregularities or trapped air bubbles. Discard imperfect gels. If using gels cast with hydrostatic pressure, remove the bundle of tubes en bloc, cut off excess acrylamide with a razor blade, and then rinse away remaining acrylamide particles from the outside of each tube.

10. Place a rubber grommet on the top of the tube. Approximately 5 mm of the tube should be visible above the upper edge of the grommet.

11. Mount the tube with the grommet in the upper reservoir and plug any unused holes.

    After the tube is seated, its lower end must be submerged in the lower electrode solution. Be sure to remove any air bubbles trapped at the bottom of the tube by shaking or tapping the tube gently. Alternatively, with some units bubbles can be dislodged by raising and lowering the tubes or by using a long curved needle and syringe.

Prefocus the gels

12. Prepare the 0.1 M NaOH upper electrode solution by degassing under vacuum with stirring for at least 5 min.

    The amount of upper electrode solution necessary depends on the type of electrophoresis chamber. If a Bio-Rad Protean II xi 2D apparatus is used, 1 liter of 0.1 M NaOH is sufficient for both prefocusing and the separation.

13. Remove the 8 M urea overlay from the top of the gels using a Pasteur pipet and place ~50 µl lysis buffer on the top of each gel.

14. Overlay lysis buffer with the degassed 0.1 M NaOH to fill the gel tubes. Avoid mixing of NaOH with the lysis buffer.

15. Pour the degassed 0.1 M NaOH into the upper chamber, making sure that all the gel tubes are covered with the electrode solution. Check carefully for leaks and air bubbles, then place lid on apparatus.

16. Connect the electrodes to a power supply by the red (+) lead to the lower chamber and the black (–) lead to the upper chamber.

    The voltages and currents used during electrophoresis are dangerous and potentially lethal. Safety considerations are given in the Electricity and Electrophoresis section of UNIT 6.1.

17. Prefocus for 30 min using 500 V constant voltage.

Load the samples

18. Turn off power supply (see Safety Considerations in *UNIT 6.1*), disconnect leads, and remove lid. Using a 60-ml syringe, remove the electrode solution (0.1 M NaOH) from the upper chamber.

19. Remove the electrode solution and the overlay solution from each tube. Be careful not to damage the gel surface.

20. Place ~50 µl lysis buffer on the top of each gel. Wait at least 2 min.

21. Remove the lysis buffer from the tubes.

    Rinsing the gels with lysis buffer removes any residual NaOH and protects the samples against exposure to high pH.

22. Load protein samples to be analyzed and carefully overlay each sample with ~50 µl lysis buffer diluted with water 8:2 (v/v). Avoid mixing the buffer with the sample.

    The overlay solution protects samples from direct contact with the strong base used as an upper electrode solution. Dilution of the lysis buffer with water is necessary to decrease the density so the overlay does not mix with the sample.

    A 3-mm-i.d. × 16-cm-long IEF gel has a total protein capacity of ~500 µg for whole-cell extracts and other complex protein mixtures. The maximum capacity for any single protein spot is ~0.5 to 5 µg, depending on its solubility near its isoelectric point and the separation distance from any near neighbors. Preparation of relatively pure protein samples for

isoelectric focusing is generally straightforward. The sample usually may be prepared in one of the following ways: dialyze into any compatible low-ionic-strength buffer, lyophilize in a volatile or compatible low-ionic-strength buffer and dissolve in lysis buffer, or precipitate the protein using trichloroacetic acid (TCA) and redissolve in lysis buffer. For preparing extracts from cultured cells and from tissue samples, see Support Protocol 4 and Support Protocol 5, respectively.

The minimum sample concentration of protein or radioactivity has to be sufficient for the desired detection method. For complex protein mixtures such as tissue or cell extracts, a 500-µg total load is recommended for Coomassie blue staining or electroblotting (UNIT 6.2) for subsequent structural analysis, a 50-µg total protein load should be sufficient for silver staining or immunoblotting, and no less than 100,000 counts/gel is recommended for proteins labeled with ^{3}H, ^{14}C, or ^{35}S for autoradiography purposes. Sample volumes should be <150 µl for 3-mm gels and <40 µl for 1.5-mm gels. This implies at least a 5 µg/µl protein concentration in the sample for gels to be stained with Coomassie blue.

23. Carefully fill all tubes with 0.1 M NaOH. Avoid mixing the NaOH solution with the overlay solution and the sample.

24. Fill the upper reservoir with 0.1 M NaOH. Be sure that all gel tubes are covered with the solution.

Run the gels

25. Connect the electrodes to a power supply with red (+) to the lower chamber and black (−) to the upper chamber.

26. Focus for a total of 12,000 Vhr.

Unlike other electrophoretic techniques, in IEF the volt-hour is the most common unit describing the "time" of isoelectric focusing. The initial voltage is usually set according to the desired number of volt-hours in a way that is convenient for the operator (i.e., so that the separation will run overnight), but it should not be <400 V. The upper voltage limit is restricted by heat released in the gels during isoelectric focusing. At constant voltage the current will be the highest during the first hour of separation. The initial current will be strongly influenced by the ionic strength of the samples loaded onto the gels. An initial voltage of <800 V is recommended for 3-mm gels loaded with samples containing less than 100 mM salts/buffers; the voltage could be increased to 1200 V after ~1 hr, if cooling is used. The current is a derivative of voltage and is never preset for isoelectric focusing purposes. Some power supplies allow preprogramming the desired number of volt-hours and continuously adjust voltage and current during the isoelectric focusing procedure (constant power). The total number of volt-hours is a major factor that affects separation in the first dimension. Optimal focusing time will vary for different ampholyte combinations, but 12,000 Vhr is a reasonable value for most systems. To achieve a total of 12,000 Vhr set the power supply to 667 V for 18 hr. These conditions are convenient for an overnight separation and do not require use of a cooling unit. Higher voltages can be used but may cause overheating of gels unless a highly efficient cooling system is employed. The maximum practical voltage decreases with increased gel tube inner diameter. Focusing for too long may cause cathodic drift and result in a shifted pH profile in the gel, whereas focusing for a short time will decrease resolution.

Extrude and store gels

27. Turn off power supply and carefully disconnect leads. Detach the lid and remove the NaOH solution from the upper reservoir of the electrophoresis chamber using a 60-ml disposable plastic syringe.

28. Remove one gel tube at a time from the chamber.

29. Using a 10-ml syringe equipped with a blunt needle, slowly and carefully inject water between the gel and glass tube. Start from the bottom of the tube, then repeat the procedure from the top. The gel should slide out of the glass tube.

It is convenient to let the gel slide from the glass tube onto a metal or plastic scoop, which facilitates transfer of the gel into a storage vial. It is relatively easy to break the gel during extrusion, and practicing on several unused gels is recommended. To extrude smaller-diameter gels, use water pressure generated by a syringe connected to the gel tube with Tygon tubing. If clean, unscratched glass tubes are used, extrusion should be easy.

30. Using the scoop, slide the gel into a 4.5-ml cryovial containing 3 ml equilibration buffer and 50 µl 2-mercaptoethanol. Close the vial, incubate exactly 5 min at room temperature, then freeze by placing the tube horizontally on top of dry ice pellets. Do not move or agitate the tube while the sample is freezing.

    The IEF gels may be run on a second-dimension gel immediately (see Basic Protocol 3), or can be stored at −80°C for many weeks. Even when the second-dimension is to be run immediately, extruded gels should be frozen after a carefully controlled incubation time at room temperature, such as the 5 min cited above for 3-mm-i.d. gels, to minimize diffusion of proteins out of the IEF gel. This short incubation before freezing will allow glycerol to diffuse into the gel. Too short an incubation or agitation during freezing can result in gel breakage. The total incubation time in equilibration buffer (sum of the time prior to freezing and after thawing) is critical and should be carefully controlled. Insufficient incubation time in equilibration buffer will not allow sufficient time for SDS to diffuse into the gel and saturate sites on the proteins. Excessive incubation times can result in appreciable protein losses due to diffusion out of the highly porous IEF gel.

CONDUCTING pH PROFILE MEASUREMENTS

SUPPORT PROTOCOL 1

Standards with different isoelectric points can help in evaluating the performance of a specific system and determining the effective pH range in the isoelectric focusing gel. Many pI standards are commercially available from different suppliers. It is most useful to separate a mixture of standard proteins that is prepared from several individual proteins or purchased as a preformulated kit. This mixture should be run in parallel with experimental samples on a separate reference gel. It is generally not recommended to run pI standards together in the same gel with samples because of possible interference with migration and identification of proteins of interest. Instead of analyzing standard proteins, a more precise evaluation of the pH profile can be made by directly measuring the pH throughout the gel using either a surface pH electrode or the following procedure.

1. Prepare and focus one or two gels (see Basic Protocol 1, steps 1 to 26) without any sample in parallel with experimental samples.
2. Prepare 20 to 40 glass test tubes each containing 1 ml high-purity, degassed water for each gel that will be used to measure the pH gradient (measurements on duplicate gels are recommended).

    The number of tubes required per gel equals twice the gel length (in cm).

3. After electric focusing is completed, extrude the blank gels (see Basic Protocol 1, steps 27 to 29). Briefly rinse the gels with water.

    After extrusion, gel surfaces may be contaminated with electrode solutions. Rinsing with water is essential for obtaining reliable pH profiles.

4. Place the gel on a glass plate with a plastic ruler below the plate. Cut the gel into 0.5-cm pieces using a sharp razor blade.
5. Place each gel piece in a test tube containing 1 ml water.

    Do not mix the order of samples because each gel piece represents a single pH profile data point.

6. Place all test tubes on a shaker and shake gently for 1 hr at room temperature.

7. Read the pH of each solution and plot the pH profile as a function of the distance from the top of the gel.

ALTERNATE PROTOCOL 1

NONEQUILIBRIUM ISOELECTRIC FOCUSING OF VERY ACIDIC PROTEINS

Basic Protocol 1 is sufficient for separating proteins with isoelectric points greater than ~3.5 to 4.0. For very acidic proteins, however, a nonequilibrium system is needed. The major features of this method are utilization of a shorter focusing time (without reaching equilibrium), a modified ampholyte mixture, and different electrode solutions.

Additional Materials *(also see Basic Protocol 1)*

10% (w/v) ammonium persulfate (prepare immediately before use)
Concentrated sulfuric acid (used in lower chamber electrode solution)
Ampholytes, pH 2-11 (used in upper chamber electrode solution)

To analyze very acidic proteins, follow Basic Protocol 1 with these exceptions in the indicated steps:

2. When preparing the gel solution, use the following mixture of ampholytes: 2.4 ml ampholytes pH 2.5-4 and 0.6 ml ampholytes pH 2-11.

4. Following the procedure for casting gels by pouring, add 100 µl of 10% ammonium persulfate solution, swirl, add 42.5 µl TEMED, and swirl again.

   Gel mixtures containing entirely or predominantly very acidic or very basic ampholytes are generally difficult to polymerize. Use of an increased ammonium persulfate concentration and adherence to the proper order of adding the reagents should ensure polymerization.

8. Prepare the bottom chamber electrode solution by adding 4.5 ml concentrated sulfuric acid to 3 liters water. Degas at least 5 min.

Omit steps 12 to 19 (do not prefocus the gels).

20. Remove the 8 M urea (polymerization overlay solution) and place ~50 µl lysis buffer on top of each gel. Wait at least 2 min, then remove the lysis buffer.

23. Carefully fill all tubes with the upper chamber electrode (anode) solution prepared by mixing pH 2-11 ampholytes with water in a 1:40 ratio.

24. Fill the upper buffer chamber (anode) with the solution described in step 23.

    Iminodiacetic acid (10 mM) may be a more economical alternative anode solution.

26. Focus for a total of 4000 Vhr.

ALTERNATE PROTOCOL 2

NONEQUILIBRIUM ISOELECTRIC FOCUSING OF BASIC PROTEINS

In general, most equilibrium IEF gel systems using soluble ampholytes produce pH gradients that do not exceed pH 8.0 on the basic end, yet many proteins have higher pI values. For this reason samples containing very basic proteins are usually focused using a nonequilibrium system. In an equilibrium system, proteins are loaded on the basic end of the gel and migrate toward the acidic end until they reach a pH equal to their pI. In nonequilibrium systems, the sample is loaded on the acidic end of the gel, and focusing is terminated after a relatively short time (fewer volt-hours).

To run nonequilibrium IEF gels, follow the procedure previously described (see Basic Protocol 1) with these alterations in the indicated steps:

8. Use 0.1 M NaOH as the lower electrode solution.

   Electrode solutions and electrodes are reversed in this procedure relative to equilibrium isoelectric focusing.

Omit steps 12 to 19 (do not prefocus the gels).

20. Remove the 8 M urea (polymerization overlay solution) and place 50 µl lysis buffer on top of each gel. Wait at least 2 min, then remove the lysis buffer.

23. After loading the samples and overlaying with lysis buffer diluted with water 8:2 (v/v) as in Basic Protocol 1, use 0.1 M H_3PO_4 instead of NaOH to fill all gel tubes.

24. Use 0.1 M H_3PO_4 as the upper electrode solution.

25. Reverse the connection of electrodes—i.e., connect the red (+) lead to the upper chamber and the black (−) lead to the lower chamber.

26. Focus for a total of 3000 to 5000 Vhr.

    The optimal number of volt-hours depends on the nature of the sample and the ampholytes used. The values recommended above may need to be adjusted empirically.

ISOELECTRIC FOCUSING USING IMMOBILIZED pH GRADIENT GEL STRIPS

BASIC PROTOCOL 2

In immobilized pH gradient (IPG) gels, the ampholytes are covalently linked to the acrylamide matrix, which facilitates production of highly reproducible gradients as well as very narrow pH gradients for optimal resolution of minor charge differences. A variety of precast gels and all the necessary equipment are commercially available from either Amersham Pharmacia Biotech or Bio-Rad. Equipment and chemicals are also available for the user to cast gels in the laboratory (see Support Protocol 3), although precast gels are likely to suffice for the majority of applications. Narrow strips of precast IEF gels (Immobiline DryStrips or Ready Strip IPG strips) may be used to achieve a first-dimension separation for two-dimensional gel electrophoresis, and broader precast slab gels (Immobiline DryPlates) can be used to compare multiple samples after IEF separation only (see Support Protocol 2 and Table 6.4.3). In this protocol, precast Immobiline DryStrips from Amersham Pharmacia Biotech are rehydrated overnight using the reswelling cassette (one to twelve sample strips may be handled at a time); samples are applied using sample cup holders and gel strips are isoelectric focused overnight. This procedure has been adapted from instruction booklets provided by Amersham Pharmacia Biotech with Immobiline Dry Strip Kits and with the Immobiline DryStrip reswelling tray. Recently, both Amersham Pharmacia Biotech and Bio-Rad have developed newer IEF systems, the IPGphor Isoelectric Focusing System and the Protean IEF Cell, respectively. These systems simplify IPG strip handling and the overall isoelectric focusing procedure. The IPGphor system also integrates IPG strip reswelling and electrophoresis steps in a single strip holder. These systems include very high-voltage power supplies integrated with efficient cooling units to permit more rapid isoelectric focusing. In general, premade commercial IPG strips from any supplier can be used with any isoelectric focusing device that can physically accommodate the strip. The appropriate user manuals should be consulted for specific details.

Since there is a greater selection of pH ranges for premade Immobiline DryPlates than DryStrips, it is sometimes convenient to cut DryPlates into strips prior to rehydrating the gel to obtain narrower pH ranges where needed. See Basic Protocol 4 for details concerning preparing and running the second-dimension gel.

Wear gloves throughout the procedure and handle the Immobiline DryStrips with forceps where feasible to prevent extraneous protein contamination of the gels and gel solutions.

Table 6.4.1 Rehydration Solutions for Immobiline DryStrips[a]

Component	Final conc.	DryStrip type		
		3-10L	3-10NL	4-7L
Ultrapure urea[b]	7 M	2.1 g	2.1 g	2.1 g
Thiourea[b]	2 M	0.76 g	0.76 g	0.76 g
CHAPS[c]	2%	0.1 g	0.1 g	0.1 g
Pharmalyte pH 3-10	1:50[d]	100 µl		
Pharmalyte pH 4-6.5			50 µl	100 µl
Pharmalyte pH 8-10.5			25 µl	
Ampholine pH 6-8			25 µl	
DTT	0.3%	75 mg	75 mg	75 mg
Bromphenol blue	Trace	A few grains	A few grains	A few grains
Milli-Q water		To 5 ml	To 5 ml	To 5 ml

[a]Rehydration solutions should be prepared fresh immediately before use or stored as frozen aliquots and should be filtered using a 0.2-µm filter. Minimize total time the solution is at room temperature prior to use to minimize decomposition of urea. If the reswelling tray is used, ~250 or 400 µl rehydration solution is required per 11- or 18-cm DryStrip, respectively.

[b]A "total urea" concentration of 9 M is typically used. Thiourea is more effective than urea for minimizing protein precipitation during isoelectric focusing, but its solubility is lower. The combination of 7 M urea/2 M thiourea usually results in superior sample solubilization and isoelectric focusing as compared with 9 M urea alone.

[c]The optimal detergent and detergent concentration should be empirically determined. Other common alternatives are Triton X-100 and octyl-glucoside. The detergent used must be nonionic or zwitterionic to avoid high current and consequent overheating during isoelectrofocusing.

[d]Alternatively, soluble ampholytes are available as preformulated "IPC buffer" concentrates for each type of IPG strip.

Thoroughly clean all equipment with a mild laboratory detergent solution, rinse well with Milli-Q water, and allow to dry before using. Solutions containing 10 M urea may be heated *briefly* to 30° to 40°C to aid in solubilization.

Materials

Urea (ultrapure)
CHAPS or Triton X-100
Pharmalyte 3-10, 4-6.5, and/or 8-10.5 soluble ampholytes (see Table 6.4.1; Amersham Pharmacia Biotech)
Ampholine pH 6-8 (Amersham Pharmacia Biotech)
DTT (dithiothreitol)
Bromphenol blue
Precast Immobiline DryStrips (Amersham Pharmacia Biotech)
DryStrip cover fluid (Amersham Pharmacia Biotech)
Immobiline DryStrip kit (Amersham Pharmacia Biotech) including:
  Cathode electrode
  Anode electrode
  Sample cup bar
  Tray
  Sample cups
  Immobiline strip aligner
  IEF electrode strips
  Sample application pieces
  Instruction manual

continued

Protein sample to be analyzed
Lysis buffer (see recipe)

Immobiline DryStrip reswelling tray (Amersham Pharmacia Biotech)
Forceps
Filter paper
Glass plate
Flatbed electrophoresis unit (Amersham Pharmacia Biotech Multiphor II or equivalent)
Recirculating cooling water bath
Power supply (minimum capacity of 3000 to 3500 V)
Petri dishes

Additional reagents and equipment for protein detection by staining (*APPENDIX 3*) and/or for electroblotting (*UNIT 6.2*, optional)

Rehydrate the Immobiline DryStrip(s)

1. Prepare an appropriate rehydration solution for the type of DryStrips to be used as described in Table 6.4.1 (~400 µl rehydration solution per 18-cm DryStrip).

   The rehydration solution should contain 7 M urea/2 M thiourea, and 2% CHAPS or another appropriate detergent (zwitterionic or nonionic) such as Triton X-100, NP-40, or n-octyl-glucoside should be included in the rehydration solution to aid in sample solubility. The optimal detergent and detergent concentration may vary with type of sample and should be determined empirically.

   One possible method of loading large sample volumes onto IPG gels is to add the sample directly to the rehydration solution. Sample loading during rehydration is preferred when using the IPGphor system with its single strip holder for both rehydration and isoelectric focusing.

   Solutions containing urea should be filtered using a 0.2-µm filter before use.

2. Slide the protective lid off the reswelling tray and level the tray by adjusting the leveling feet until the leveling bubble is centered.

3. For an 18-cm gel, pipet 350 to 400 µl of rehydration solution into a slot of the reswelling tray. Move the pipet along the length of the well while adding the solution to spread it evenly throughout the length of the slot. Avoid excessive air bubble formation while pipetting this solution.

   If the IPGphor system is used, pipet the rehydration solution containing the protein sample into each cleaned ceramic holder.

4. Remove the protective cover from the Immobiline DryStrips and gently place them, gel side down, into the prepared slot.

   To facilitate their removal after rehydration, the strips should be oriented with their pointed ends at the sloped end of the slots in the rehydration tray. Be careful not to trap any air bubbles under the gel strips.

5. Overlay each strip with 2 to 3 ml of DryStrip cover fluid to prevent evaporation and urea crystallization. Slide the protective lid into place and allow gels to rehydrate overnight (~16 hours) at room temperature.

   Shorter rehydration times can be used, although a minimum of 6 to 8 hr is usually needed to completely and reproducibly rehydrate the gels. Do not substantially exceed 16 hr as extensive incubation, especially rehydrating gels over a weekend, increases potential problems due to evaporation and subsequent urea crystallization. In addition, long incubation times increase the extent of urea decomposition, which will increase the risk of amino group modification on proteins by the cyanate produced from urea decomposition.

   If the IPGphor system is used, a low voltage (30 to 40 V) can be applied during rehydration, which improves isoelectric focusing and protein yields of some samples. With this device,

protocols can be preprogrammed so as to allow isoelectric focusing to immediately follow rehydration. Hence, both rehydration with low voltage and isoelectric focusing can be completed overnight, since ~8 hr is sufficient for rehydration, and up to 8000 V can be used in later stages of isoelectric focusing to shorten the total focusing time.

6. After the overnight rehydration, slide the lid off the reswelling tray. Place a forceps tip into the slight depression under each strip and remove the strip. Gently blot any excess oil or moisture from the plastic backing of the rehydrated strips with filter paper. A damp piece of filter paper may also be used to blot the surface of the gel. Some of the paper may adhere to the gel and should be gently peeled away. The gel is now ready to be placed in the strip aligner on the cooling plate of the electrophoresis unit.

   Do not allow the gel to dehydrate prior to placing it on the cooling plate in step 11. (Steps 7 to 10 should be completed prior to removing the strips from the reswelling tray.)

Run the first dimension

7. Level the Multiphor II electrophoresis unit, then connect it to a circulating cooling water bath. Allow it to cool to 15°C for 1 to 2 hr to ensure even cooling. Do not cool below 15°C to prevent precipitation of urea in the gels.
8. Pipet ~5 ml DryStrip cover fluid onto the surface of the Multiphor II cooling plate. Position the Immobiline DryStrip tray on the cooling plate oriented with the red (+, anodic) electrode at the top, near the cooling tubes.

   Avoid large air bubbles between the cooling plate and the tray (small bubbles should not cause a problem).

9. Connect the red and black electrode leads on the tray to their respective positions on the Multiphor II unit. Pour 10 ml of DryStrip cover fluid into the tray. Place the Immobiline DryStrip aligner on top of the oil, groove side up.

   Avoid getting oil on top of the strip aligner. The possible presence of small air bubbles under the strip aligner is not important.

10. Cut two electrode strips to a length of 11 cm (regardless of the number of DryStrips used). Place the electrode strips onto a clean glass plate and soak each one with 0.5 ml Milli-Q water. Blot with a Kimwipe or tissue paper to remove excess water.

    The electrode strips should be evenly soaked and just damp after blotting. Excessive water could cause sample streaking.

11. Transfer the strips from step 6 to adjacent grooves in the aligner tray. Position the rounded (acidic) end of each strip near the top of the tray at the red electrode (anode) near the cooling tubes, and the square end at the bottom of the tray near the black electrode (cathode). Be sure that the edges of all gel strips at the anode end are lined up evenly.
12. Place the blotted electrode strips from step 9 on top of the gel surface of the DryStrips near the anode and cathode ends of the gel. Position the red (anode) and black (cathode) electrodes on top of the electrode strips at their respective ends.

    After the electrodes have been pressed down on top of the electrode strips, check that the gel strips have not shifted position.

13. Push the sample cups onto the sample cup bar. Place the sample cup bar near the anode end of the gel so that the small spacer arm just touches the electrode and the sample cups are nearest to the electrode, but do not allow the cups to touch the gel.

    The sample cups should face the nearest electrode. The acidic end of the gel can usually be used for sample application; however, the optimal loading position may need to be determined empirically for different types of samples. At high protein concentrations and/or at non-optimal pH, samples may precipitate in the gel at the loading position.

14. Position one sample cup above each gel strip and push down to ensure good contact between the bottom of the sample cup and the gel strip. Make sure the gel strips have not shifted position.

15. Pour 70 to 80 ml of DryStrip cover fluid into the tray (it will cover the gels). If oil leaks into the sample cups, adjust the cups to stop leakage. When there is no leakage into the sample cups, add enough cover fluid to the tray to completely cover the sample cups (~150 ml).

16. Pipet protein samples (in lysis buffer) into the sample cups by underlaying. The sample should sink to the bottom of the cup. Check for leakage of the sample out of the sample cup.

    Samples should either be lyophilized and then solubilized in lysis buffer, or diluted 9 parts lysis buffer to 1 part sample. The maximum volume each sample cup holds is 100 µl. The complexity of the sample, the sample solubility at the loading concentration and pH used, the thickness of the second-dimension gel, and the detection method to be employed should be considered when deciding how much protein to load. As a starting reference, typical loading ranges for 1.0- to 1.5-mm-thick 18-cm × 18-cm gels would be ~5 to 20 ng per major spot for silver staining and ~1 to 5 µg per major spot for Coomassie blue staining. When very complex samples are used such as whole cell extracts, total protein loads are likely to be ~20 to 100 µg for silver staining and ~200 to 1000 µg for Coomassie blue staining. The salt concentration in samples should be kept <50 mM and, if the sample contains SDS, the final SDS concentration should be <0.25%.

17. Place the lid on the Multiphor II unit and connect the leads to a power supply. Focus the gels with constant voltage for 2 to 3 hr at 500 V followed by 12 to 16 hr at 3500 V for a total of 40 to 60 kVhr. Refer to the user manual for exact recommended voltage conditions for each type of Immobiline DryStrip.

    The optimal number of Vhr will depend upon the pH range of the Immobiline Strip used, the type of sample, and the sample load and volume; therefore, the optimal Vhr should be empirically determined for different applications.

18. When isoelectric focusing is complete, disconnect the power supply and remove the cover from the Multiphor II unit. Remove the electrodes, electrode strips, and sample cup bar from the tray.

    If gels are to be run in the second dimension immediately after isofocusing, steps 1 to 3 of Basic Protocol 4 should be completed prior to terminating isofocusing.

19. Using forceps, remove the DryStrips from the tray. If the gels are to be run in the second dimension immediately, place in a petri dish with the support film along the wall of the dish and proceed directly to equilibration of the gel (see Basic Protocol 4, step 4). Alternatively, gels may be stored sealed in a plastic bag at −80°C until ready to run the second-dimension gel.

    Gels may be stored at least 2 to 3 months at −80°C. Do not place in the equilibration buffers required for the second dimension prior to storage.

ELECTROPHORESIS ON IMMOBILIZED pH GRADIENT GELS

SUPPORT PROTOCOL 2

In this protocol, after precast IEF gels (Immobiline DryPlates) from Amersham Pharmacia Biotech are rehydrated, samples are loaded and subjected to isoelectric focusing. Gels are typically run at 2500 to 3500 V and require focusing times of 2 to 7 hr. Protein samples may be detected by conventional methods such as Coomassie blue or silver staining. Isoelectric points can be determined with the use of pI calibration proteins; alternatively, because the gradient is linear, one can measure the migration distance across the gel and estimate the pI at each location. As noted in Basic Protocol 2, Immobiline DryPlates can be cut into 3-mm-wide strips to use as the first dimension of two-dimensional gels, since

DryPlates are available in narrower pH ranges than DryStrips. DryPlates can also be used as described in this protocol to simultaneously separate multiple samples in a single dimension. Applications of this method include initial screening of samples to determine the optimal pH gradient prior to running more time-consuming two-dimensional gels, prescreening fractions from a chromatographic purification step prior to running two-dimensional gels, and evaluation of charge heterogeneity of purified proteins.

Additional Materials *(also see Basic Protocol 2)*

Precast DryPlate gel (Amersham Pharmacia Biotech)
Repel-Silane (Amersham Pharmacia Biotech)
Paraffin oil
Protein samples to be analyzed
Reswelling Cassette kit (Amersham Pharmacia Biotech) including:
  125 × 260 × 3–mm glass plate with 0.5-mm U frame
  125 × 260 × 3–mm glass plate
  Silicone tubing
  Pinchcock
  Clamps
  20-ml syringe
Roller (Amersham Pharmacia Biotech)
Whatman no. 1 filter paper

Flatbed electrophoresis unit (Amersham Pharmacia Biotech Multiphor II)
10° *or* 15°C cooling water bath
Electrode strips
Sample applicator strip or sample application pieces
Power supply (minimum capacity 3000 to 3500 V)

Additional reagents and equipment for protein detection by staining (*APPENDIX 3*) and for electroblotting (*UNIT 6.2*; optional)

Rehydrate the gel

1. Remove precast gel from packaging. If the entire gel is not needed, cut off the required number of lanes and reseal the unused gel. Mark the polarity of the gel section to be used by cutting a small triangle off the anode corner. Handle the gel by the support film only.

   It is critical that the lanes are cut from the gel in the proper orientation to preserve the pH gradient (see Fig. 6.4.1), and polarity must be indicated for proper orientation of electrodes later in the procedure.

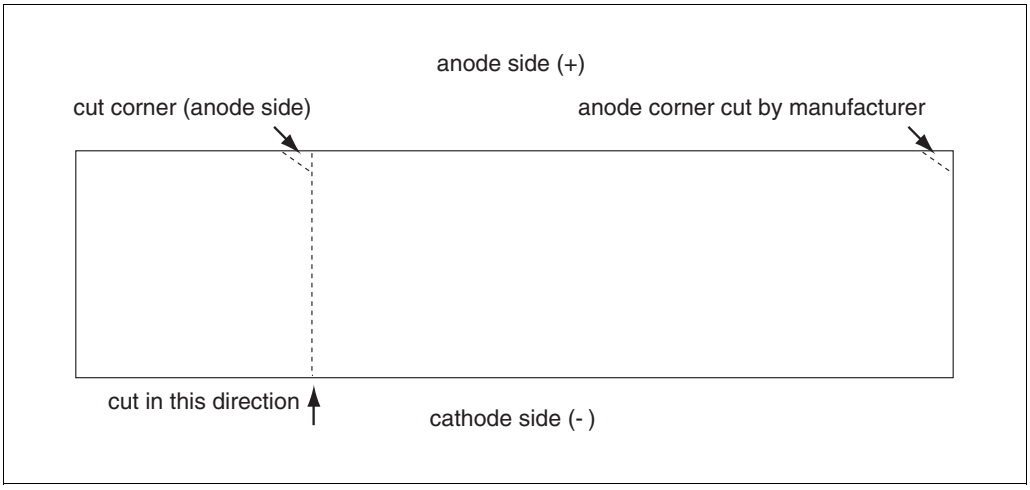


Figure 6.4.1 Marking orientation of a precast IPG gel when only a portion of the gel is used.

2. Use the Reswelling Cassette to rehydrate the gel. Connect silicone tubing through hole in the bottom corner of the U-frame plate, seal with silicone glue, and connect the pinchcock to the other end of the tubing. Place a glass plate on a clean flat surface and wet with a few drops of water. Place the gel on the plate, gel side up. Gently roll with a clean rubber roller to remove any air bubbles.

3. Cover the plate and gel with the plate fitted with the U frame.

   The U-frame plate should be coated with a thin layer of Repel-Silane to prevent the gel from sticking to the plate.

4. Place clamps around the edges of the plates, making sure the seal is tight.

5. Slowly fill the cassette with the desired rehydration solution using a 20-ml syringe connected to the silicone tubing and let stand for the recommended amount of time. Precool electrophoresis unit 1 to 2 hr prior to electrophoresis (see step 9).

   Reswelling with water for 2 to 3 hr is normally sufficient. If using additives such as urea, Triton, glycerol, or reducing agents, allow the gel to rehydrate overnight. Additives can be used to improve solubility of proteins near their isoelectric point. Reducing reagents such as DTT are used to reduce disulfide bonds.

6. When gel has been allowed to rehydrate completely, remove the clamps and gently pry the plates apart.

7. Moisten a piece of filter paper with water and place on top of the gel, then layer with a piece of dry filter paper.

8. Gently blot the gel by rolling over the dry filter paper with the rubber roller to remove excess water. The gel is now ready to be placed on the cooling plate.

   Do not let the gel dehydrate prior to placing it on the cooling plate in step 11.

Run the gel

9. Connect the flatbed electrophoresis unit to a recirculating cooling water bath. Allow to cool to 10°C for 1 to 2 hr to ensure even cooling. If the gel has been rehydrated in the presence of urea, do not cool below 15°C so that urea does not precipitate.

10. Pipet 2 to 3 ml paraffin oil onto the surface of the cooling plate.

11. Position the gel on the cooling plate, being careful not to trap air bubbles between the gel and the plate. Orient the gel so that the polarity of the gel matches the polarity of the cooling plate.

12. Soak two electrode strips with ~3 ml water, then blot to remove excess water.

13. Lay a blotted electrode strip along each long edge of the gel. Cut off the ends of the electrode strip so that it does not extend beyond the edge of the gel.

14. Load protein samples to be analyzed onto the gel. Use an applicator strip for sample volumes between 5 and 20 µl (make sure contact between the strip and the gel is uniform). Use sample application pieces for sample volumes >20 µl. Remove the application pieces halfway through focusing. For sample volumes of 2 to 10 µl, samples may be spotted directly on the gel without using applicator strips. See manufacturer's instructions for further details.

    An important experimental consideration is the position in the pH gradient where the sample is applied. The acidic end of the gel can usually be used for sample application; however, the optimal loading position may need to be determined empirically for different types of samples. At high protein concentrations and/or at nonoptimal pHs, samples may precipitate in the gel at the loading position.

Samples should contain <50 mM salt or buffer components; greater concentrations will cause local overheating of the gel. If possible, salt-free samples should be solubilized or dialyzed in the rehydration buffer.

15. Align the electrodes with the electrode strips, put the safety lid in place, and connect the apparatus to the power supply. Conduct electrophoresis at 3000 V.

    Broad-range IPG gels, such as Immobiline DryPlate, pH 3-10, should be run at ~3000 V for 2 to 4 hr. Narrower-range gels are also run at 3000 V but may require a focusing time of 4 to 7 hr.

16. After removing gels from the electrophoresis apparatus, detect proteins using any conventional staining technique such as Coomassie blue or silver staining.

17. Preserve the gels by sealing in a plastic bag or by drying for a permanent record. Alternatively, electrotransfer the proteins on the gel to a membrane.

    To dry a gel, presoak it first in a preservation solution. For silver-stained gels, use a solution of 5% to 10% (w/v) glycerol/30% (v/v) ethanol; for Coomassie blue–stained gels, use a solution of 5% to 10% (w/v) glycerol/16% (v/v) ethanol/8% (w/v) acetic acid. After soaking the gel, place it on a glass plate gel side up, cover with a cellophane sheet soaked in preservation solution, and allow to dry at room temperature.

    For electrotransfer (UNIT 6.2), use film remover to remove the plastic support film from the gel. Electrotransfer of proteins to a polyvinylidene difluoride (PVDF) membrane using a Multiphor II NovaBlot transfer kit (Amersham Pharmacia Biotech) is recommended. Transferring IPG gels requires special procedures; see the transfer kit manual for instructions.

SUPPORT PROTOCOL 3

CASTING AN IMMOBILINE GEL

An alternative to precast IPG gels is the use of Amersham Pharmacia Biotech Immobilines to cast immobilized pH gradient gels with customized pH gradients and ranges, including very narrow pH ranges, to improve separation of proteins with small charge differences. This protocol describes the general procedure of casting custom-made Immobiline gels. The Reswelling Cassette used in Basic Protocol 2 for rehydrating gels is employed for casting the gels, which are polyacrylamide gels poured with a gradient of Immobilines following instructions provided by Amersham Pharmacia Biotech application note 324.

Additional Materials *(also see Support Protocol 2)*

GelBond PAG film (Amersham Pharmacia Biotech)
Immobiline solutions (Amersham Pharmacia Biotech)
2.5% (v/v) glycerol

Gradient maker
Orbital shaker

Additional materials and equipment for rehydrating immobilized pH gradient gels (see Basic Protocol 2)

Cast the gel

1. Coat the plate with the U frame with Repel-Silane to prevent the gel from sticking to the glass plate.

2. Place a glass plate on a clean, flat surface and wet with several drops of water. Cover with a sheet of GelBond PAG film, hydrophilic side up. Use the roller to remove any air bubbles trapped between the film and the glass plate.

3. Place the plate with the U frame on top of the GelBond PAG film. Clamp the plates together on three sides.

4. Mix the Immobiline solutions following the instructions provided by Amersham Pharmacia Biotech application note 324 to prepare the desired pH range. Cast the pH gradient gel using a gradient maker.

   Once the catalysts have been added to the gel solution, it is important to work quickly to ensure that the gradient is poured before polymerization occurs. See UNIT 6.1 for instructions for using gradient makers.

5. Do not disturb the gel during the first 10 min to allow the gradient to stabilize. Allow the gel to polymerize 1 hr in a 50°C oven.

Dry and store the gel

6. Allow the gel to cool to room temperature, then disassemble the cassette. Cut off a small corner to label the anode end of the gel.

7. Wash the gel 2 to 3 hr with 200 to 300 ml water. Use an orbital shaker and change the water two or three times.

   At this stage the gel may be used immediately (if no additives are needed) or dried as described in steps 8 to 10.

8. Wash the gel 30 min to 1 hr with 200 to 300 ml of 2.5% (v/v) glycerol.

9. Place the gel on a glass plate (gel side up) in a dust-free environment and allow to dry at room temperature overnight.

10. Store the dried gel in a sealed plastic bag at −20°C for up to 2 months.

    The gels may be rehydrated when needed (see Support Protocol 2).

PREPARING TISSUE CULTURE CELL EXTRACTS FOR ISOELECTRIC FOCUSING

SUPPORT PROTOCOL 4

Preparation of samples containing relatively pure proteins for isoelectric focusing is generally straightforward (see Basic Protocol 1, step 22). In contrast, complex samples such as whole-cell extracts, tissue extracts, or subcellular fractions are more difficult to prepare for successful isoelectric focusing. Solubility limitations both prior to isoelectrofocusing and during focusing restrict analysis of these complex samples to protocols that include nonionic detergents and urea (see Basic Protocol 1). In addition, the presence of DNA and RNA in crude cell extracts further complicates isoelectric focusing. The protocol presented below is suitable for preparing samples from cell cultures and is based on quantities compatible with silver staining or Coomassie blue staining. If smaller cell numbers and high-sensitivity detection methods such as autoradiography are used, volumes and quantities should be adjusted as needed.

In this protocol the cells are harvested and washed in phosphate-buffered saline (PBS) with proteolysis inhibitors, then lysed in Tris/SDS buffer using sonication, after which the total protein concentration is determined in the lysate. The lysate is further treated with a mixture of DNase and RNase, and additional SDS and reducing agent are added. At this stage, the samples can be stored at −80°C for an extended time or, after addition of urea and lysis buffer, may be loaded directly onto prefocused IEF gels. Filtration of the final sample prior to loading onto the IEF gel is essential for quality of isoelectric focusing.

Materials

Cell culture flasks containing cells of interest
PBS with proteolysis inhibitors (PBS/I buffer; see recipe)
Dry ice/ethanol (optional, for freezing samples)
Tris/SDS buffer (see recipe)
BCA protein assay kit (Pierce)
DNase and RNase solution (see recipe)
20% (w/v) SDS (*APPENDIX 2E*)
2-Mercaptoethanol
Urea (ultrapure)
Lysis buffer (see recipe)

50-ml centrifuge tube
Centrifuge with rotor (e.g., Beckman JS-4.2), 4°C
1- to 2-ml cryovials
Microcentrifuge, 4°C
Sonicator with microtip
0.2-µm microcentrifuge filter units (e.g., Millipore Ultrafree-MC filter units)

Harvest and wash the cells

1. Place cell culture flasks containing cells of interest on ice.
2. Rapidly wash cells three times with 2 to 6 ml PBS/I buffer. Keep the flasks on ice.

   The required volume of PBS/I buffer depends on the flask size. For example, use 2 ml for a 25-cm^2 tissue culture flask and 6 ml for a 75-cm^2 tissue culture flask.

3. Add 2 to 6 ml PBS/I buffer to the flask, scrape the cells using a cell scraper, and transfer the suspension to a 50-ml centrifuge tube. Repeat this step with another 2 to 6 ml buffer to ensure complete transfer of the cells.

   Cells grown in suspension are washed in an analogous manner using repetitive centrifugation.

4. Collect the cells by centrifuging 15 min at 2600 × *g* (3000 rpm in Beckman JS-4.2 rotor), 4°C.
5. Discard the supernatant and resuspend the cells in a small volume of PBS/I buffer.
6. Transfer the cell suspension to a labeled cryovial.

   The weight of the empty cryovial can be determined prior to use if the wet weight of the cell pellet is desired as a reference value rather than cell number, radioactivity, or another criterion.

7. Microcentrifuge the cells 15 min at maximum speed, 4°C.
8. Remove and discard the supernatant using a pipettor or Pasteur pipet. Weigh the vial containing the cell pellet. Record the wet weight of the cell pellet (in mg).
9. Freeze the cell pellet in a dry ice/ethanol mixture (optional).

   Frozen cells can be stored at −80°C for at least several months.

Prepare the cell pellets for isoelectric focusing

10. Retrieve cell pellets from −80°C storage if samples were frozen.
11. Add 400 µl Tris/SDS buffer per 50 to 100 mg cell pellet wet weight. Keep the cells on ice at all times.

    The total amount of protein in the pellet is roughly 5% of the wet pellet weight.

12. Sonicate the sample three times for 3 sec using a sonicator with a microtip at medium power. Keep the samples on ice during sonication.

    Use pulse sonication or wait at least 5 min between sonications. Minimizing heat generation is essential because substantial proteolysis can occur if the sample warms appreciably. If additional sonication is necessary (i.e., if the sample is not homogeneous), let the sample cool down on ice before the next series of sonications.

13. Run a BCA protein assay to determine the protein concentration if sample will be loaded on that basis. For maximum accuracy use the same amount of Tris/SDS buffer in standards as in experimental samples.

14. Prepare labeled cryovials to store aliquots of the sample, if desired. Precool vials on ice prior to making aliquots.

    The amount of protein per aliquot depends on the anticipated future uses of the sample, as repeated freezing and thawing should be avoided. About 500 µg/gel whole-cell extract is a maximum load for preparative purposes using 3-mm gels (i.e., isolation of proteins for sequencing or other structural work). Approximately 50 µg/gel is an appropriate load for silver staining. The final protein concentration after completion of the protocol (steps 15 to 20) will equal the concentration found by protein assay divided by 1.1, owing to the addition of reagents after the protein assay step.

15. Add 20 µl DNase and RNase solution per 400 µl Tris/SDS buffer used for sonication (step 11). Incubate 10 min on ice.

16. Add 20 µl of 20% SDS solution and 5 µl of 2-mercaptoethanol per 400 µl Tris/SDS buffer used in step 11. Incubate 5 min at 37°C.

17. Quickly divide samples into previously prepared cryovials and immediately freeze aliquots using a dry ice/ethanol bath. Store at −80°C. Samples stored at −80°C are stable ≥1 year.

    Work quickly to minimize potential proteolysis.

    This step may be omitted if the samples are to be loaded on IEF gels immediately. Generally, the total amount of sample greatly exceeds the amount required for an IEF gel, and freezing aliquots is beneficial. To avoid potential reproducibility problems, all samples should be processed identically.

Prepare the samples for isoelectric focusing

18. If cell extracts were frozen, thaw samples and immediately add dry urea to 9 M final concentration.

    The amount of urea (in mg) equals 0.83 times the sample volume (in µl). For example, use 83 mg urea per 100 µl sample. The final volume of the sample (with urea added) equals 1.6 times the initial volume (160 µl in the same example).

19. Add an equal volume of lysis buffer (160 µl in the above example) and warm briefly if necessary to dissolve urea.

20. Filter samples using a 0.2-µm microcentrifuge filter unit by microcentrifuging at maximum speed, room temperature, until the entire sample has passed through the filter. Load the desired volume onto the IEF gel.

    If a 500-µg total protein load per 3-mm gel is desired (a practical maximum load for most whole-cell extracts), the protein concentration determined during the protein assay has to be ≥5 µg/µl. If the sample is less concentrated, the sample volume required will be too large for a 3-mm IEF gel. Alternatively, sample loads can be based on cell numbers, radioactivity, or any other appropriate reference (see Basic Protocol 1, step 22).

SUPPORT PROTOCOL 5

PREPARING PROTEINS IN TISSUE SAMPLES

Tissue samples are usually solubilized in lysis buffer using homogenization. After centrifugation, the protein sample can be loaded onto the first-dimension gel. In general, much higher sample-to-sample variability is expected when tissue samples are analyzed.

Materials

Tissue samples
Lysis buffer (see recipe)

Dounce homogenizer or equivalent
Ultracentrifuge and rotor (e.g., Beckman Ti70), 2°C

1. Place tissue sample in a Dounce homogenizer, add ~2 ml lysis buffer per 100 mg tissue, and homogenize the sample on ice (e.g., 3 to 5 strokes).
2. Let the mixture stand a few minutes, then transfer to an appropriately sized ultracentrifuge tube depending on total sample volume.
3. Centrifuge 2 hr at 100,000 × *g* (e.g., 33,000 rpm in a Beckman Ti70 rotor for 100,000 × *g*), or 1 hr at 200,000 × *g*, 2°C.
4. Divide the supernatant into aliquots and freeze at −80°C or immediately load an appropriate volume onto the IEF gel.

BASIC PROTOCOL 3

SECOND-DIMENSION ELECTROPHORESIS OF IEF TUBE GELS

Second-dimension gels are identical to those described in *UNIT 6.1* except for sample loading, which requires a broad, flat well. A broad well can be cast using an appropriate two-dimensional comb if the second-dimension gel thickness is slightly larger than that of the first-dimension gel. Alternatively, when the second-dimension gel is being cast, water can be layered over the entire surface of the gel to produce a flat surface that will accommodate the first-dimension gel.

Narrow analytical isoelectric focusing gels (≤1.5 mm) that fit between the glass plates of the second-dimension gel do not generally require a stacking gel, although a stacking gel may improve resolution under some circumstances. Stacking gels are essential when first-dimension gels >1.5 mm are loaded on reduced-thickness second-dimension gels, for example, when 3-mm first-dimension gels are loaded on 1.5-mm second-dimension gels. To ensure the best reproducibility, casting multiple second-dimension gels in a multigel casting stand is strongly recommended. This is especially important when gradient gels are used for the second-dimension and/or critical comparisons of multiple samples are planned.

This protocol describes all the specific steps required for successfully casting and running the second-dimension gel. The use of beveled plates and an agarose overlay is especially important when 3-mm IEF gels are loaded onto 1.5-mm second-dimension gels.

Materials

2% (w/v) agarose (see recipe)
Equilibration buffer (see recipe)
Isoelectric focusing gels containing protein samples to be analyzed (see Basic Protocol 1)
Piece of agarose containing molecular weight standards (see Support Protocol 6)

Beveled glass plates
Boiling water bath
Metal or plastic scoop

Additional reagents and equipment for linear and gradient Laemmli gels (*UNIT 6.1*)

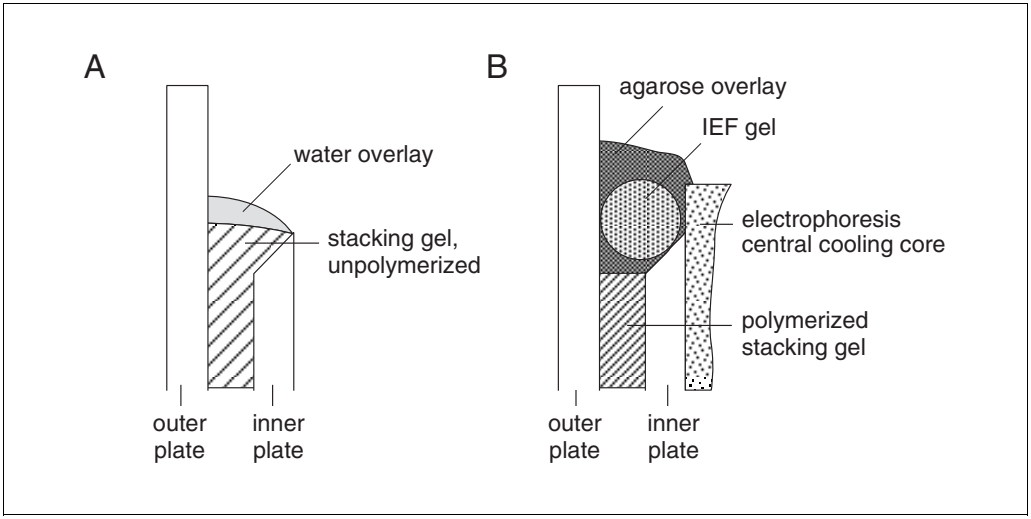


Figure 6.4.2 Casting the second-dimension gel and loading the IEF gel. (**A**) The stacking gel solution should reach to the upper edge of the beveled plate, and then the gel solution has to be overlaid with a minimum volume of water. The water will stay on the surface because of surface tension. (**B**) After polymerization, the gel is mounted on the central cooling core of the electrophoresis unit, and the equilibrated IEF gel is placed on top of the polymerized stacking gel. Excess buffer is removed, and the IEF gel is overlaid with hot agarose/equilibration buffer mixture. After the agarose solidifies, the upper electrophoresis chamber is filled with buffer.

Cast the second-dimension gels

1. Assemble the glass-plate sandwich of an electrophoresis apparatus, using a beveled plate for the shorter side of the gel sandwich.

   A beveled plate provides more space for a thicker IEF gel and will accommodate a first-dimension gel that is at least 1 to 2 mm larger than the thickness of the second-dimension gel.

2. If the thickness of the first-dimension gel exceeds that of the second-dimension gel, pour a separating gel of the desired acrylamide concentration and immediately overlay with water to produce a smooth surface. The separating gel height should be a minimum of 2 cm below the top of the beveled plate to accommodate the stacking gel.

3. After the separating gel has polymerized (a sharp interface between the polymerized gel and the water overlay will reappear), remove the overlay, rinse the gel surface with water, and pour the stacking gel. The stacking gel solution should reach to the top of the bevel. Immediately overlay the stacking gel solution with a minimum amount of water, which will adhere owing to the surface tension (see Fig. 6.4.2A).

   A water overlay of the stacking gel provides a smooth surface and better contact between the IEF gel and second-dimension gel. A small volume of water has to be used to avoid lowering the upper edge of the stacking gel below the edge of the beveled plate. The stacking gel height must be between 1.5 and 2 cm. The solution is filled to the top of the bevel so that after the slight shrinkage that occurs during polymerization the top of the polymerized gel will be near the bottom of the bevel (see Fig. 6.4.2B).

Load the isoelectric focusing gels onto the second-dimension gels

4. Assemble second-dimension gels in an electrophoresis chamber. Do not pour electrophoresis buffer into the upper chamber.

5. Melt 2% (w/v) agarose in a boiling water bath and add an equal volume of equilibration buffer for use in step 11. Keep agarose/equilibration buffer in the boiling water bath until step 11 is completed.

6. Retrieve isoelectric focusing gels containing protein samples to be analyzed from storage. Incubate cryotubes containing frozen IEF gels in a 37°C water bath for 15 min for a 3-mm tube gel. A 5- to 7-min incubation is sufficient for 1.5-mm or thinner IEF gels. Do not agitate during thawing, as vigorous agitation of a partially thawed gel can break the gel.

   During this thawing/equilibration step, SDS in the equilibration solution in which the gels were frozen diffuses into the gel matrix and binds to proteins in the IEF gel. The length of incubation in the equilibration buffer is critical because insufficient saturation of proteins with SDS will contribute to vertical streaks on staining. On the other hand, extended incubation in equilibration buffer will result in excessive loss of proteins owing to diffusion of protein out of the gel, which is especially critical for thin IEF gels. For this reason, it is recommended that after extrusion from the IEF tube IEF gels be initially incubated for 5 min to allow adequate diffusion of glycerol into the gel to minimize gel breakage, followed by freezing on dry ice (see Basic Protocol 1, step 30). This is desirable even if the second-dimension gel will be run directly after isoelectric focusing, as it is the most feasible way of precisely controlling the equilibration time while the remaining gels in the IEF run are extruded.

7. Pour the gel and equilibration solution out of the cryovial onto a metal or plastic scoop. Carefully remove excess equilibration buffer with a pipet.
8. Place a few milliliters of electrophoresis buffer on the top of the second-dimension gel.
9. Slowly slide the IEF gel off the scoop and onto the top of the second-dimension gel. Remove all air bubbles trapped between the gels. Remove excess electrophoresis buffer from the top of the second-dimension gel.

   The basic end of the gel may be placed on either the left or right side of the second-dimension gel. However, once a convention is established, all gels should be oriented the same way. The acidic end of the IEF gel can be recognized in two ways: the bromphenol blue will usually be yellow, and a bulge (increased gel diameter) will be present.

10. Place a piece of agarose containing molecular weight standards (see Support Protocol 6) beside the basic side of the IEF gel (optional).

    Note that when molecular weight standards are used, the isoelectric focusing gel has to be shorter than the width of the second-dimension gel.

11. Carefully overlay the IEF gel (and the gel piece with standard proteins) with the hot agarose/equilibration buffer mixture (~2 ml/gel) prepared in step 5. Let the agarose solidify.

    The agarose prevents the IEF gel from shifting position and ensures good contact between the IEF and second-dimension gels.

12. Carefully pour electrophoresis buffer into the upper reservoir, taking care to avoid disturbing the agarose-covered IEF gel.
13. Connect electrodes and run the gels.

    See UNIT 6.1 for electrophoresis conditions.

BASIC PROTOCOL 4

SECOND-DIMENSION ELECTROPHORESIS OF IPG GELS

In this protocol, vertical gel electrophoresis is used as the second dimension for IPG gels in an analogous manner to the protocol described for the second dimension of IEF tube gels (see Basic Protocol 3). One difference is the use of second-dimension gel spacers or gel apparatus that will accommodate an 18-cm-long Immobiline DryStrip; Bio-Rad offers a conversion kit to increase the gel width from 16 cm to 18 cm, and Amersham Pharmacia Biotech offers the Iso-Dalt gel system. The use of beveled plates is not necessary as the 0.5-mm strips are narrower than the second-dimension gel (1.0 or 1.5 mm thick). Another change involves a two-step equilibration of the strips prior to electrophoresis.

Additional Materials *(also see Basic Protocol 3)*

DryStrip equilibration solutions 1 and 2 (see recipes; prepare fresh in step 4)
Immobiline IPG DryStrip with focused protein (see Basic Protocol 2)
Platform shaker

Cast the second-dimension gel

1. Assemble the glass-plate sandwich of an electrophoresis apparatus, using gel plates wide enough to accommodate an 18-cm-long DryStrip gel.

   Beveled plates are not necessary. If the spacers are not wide enough to accommodate an 18 cm gel, the ends of the gel strip may be trimmed away from the IPG gel so that it will fit on top of the second dimension; however, some very basic or acidic proteins may be lost.

2. Pour a separating gel of the desired acrylamide concentration and immediately overlay with water to produce a smooth surface.

   The separating gel should be a minimum of ~2.5 cm below the top of the inner plate to accommodate a 2-cm stacking gel.

3. After the separating gel has polymerized, remove the water overlay, rinse the gel surface with water to remove any unpolymerized acrylamide, and pour the stacking gel to a height of 0.5 cm from the top of the plate. Overlay with water to produce a smooth surface.

   A water overlay provides a smooth surface for better contact between the Immobiline DryStrip and the second dimension gel. The stacking gel height should be ~2 cm.

Load the Immobiline IPG DryStrip gel onto the second-dimension gel

4. Prepare Immobiline DryStrip equilibration solutions 1 and 2 (see recipe).

5. Assemble the second-dimension gels in a electrophoresis chamber. Do not pour electrophoresis buffer into the upper chamber.

6. Melt 2% (w/v) agarose in a boiling water bath. Mix a solution of 1 part 2% agarose to 2 parts equilibration solution 2.

   Keep agarose/equilibration buffer mixture in boiling water bath until step 11 is completed.

   The agarose prevents the IPG DryStrip from shifting position and ensures good contact between the IEF and second-dimension gels.

7. Using forceps, remove the IPG gels from the electrophoresis tray after isoelectric focusing is complete or from the −80°C freezer (see Basic Protocol 2, step 19) and place each strip in a separate petri dish with the support film side of the strip facing the petri dish wall. Add 15 ml of DryStrip equilibration buffer 1. Cover and place on a platform shaker for 10 min.

   Strips may be run in the second dimension immediately after isofocusing or after storage at −80°C. If the strips have been stored at −80°C, remove them from the freezer, then place in petri dish as stated and continue with the equilibration procedure.

8. Discard equilibration buffer 1 and add 15 ml of equilibration buffer 2. Cover and place on a platform shaker for 10 min.

9. Dampen a piece of filter paper and place on a glass plate. Remove the DryStrips from equilibration buffer 2. Place each strip on its edge on the filter paper to remove any excess buffer.

   Strips should not be left in this position for >10 min, or spot sharpness may be affected.

10. Add a small amount of SDS electrode buffer along the glass plate above the second-dimension gel. Place the DryStrip gel in the well with the gel facing out and the basic side to the left. Push the DryStrip down so that it is firmly in contact with the stacking gel of the second-dimension gel. Remove excess running buffer.
11. Overlay the IPG gel strip with the agarose/equilibration buffer (from step 6) and allow agarose to solidify.
12. Carefully pour electrophoresis buffer into the upper reservoir, taking care to avoid disturbing the agarose-embedded IPG DryStrip.
13. Connect electrodes and run the gels.

    See UNIT 6.1 for electrophoresis conditions.

SUPPORT PROTOCOL 6

PREPARING MOLECULAR WEIGHT STANDARDS FOR TWO-DIMENSIONAL GELS

Molecular weight markers are usually necessary for the identification of proteins or as references to describe experimental proteins on two-dimensional gels. In many cases, molecular weight markers are required only at the beginning of a project. Once the system is established, common proteins in the sample (e.g., actin or tubulin) provide sufficient references for molecular weight identification on subsequent gels. To minimize any differences in migration of the molecular weight standards and isoelectric focused proteins, the standard proteins should be loaded on the second-dimension gel in the same manner as the IEF gel. This protocol describes the preparation of standards in solidified agarose. The agarose pieces may be stored at −80°C for at least a year and provide a convenient source of standards for the second-dimension gel. The procedure described is recommended for 3-mm IEF gels. Narrow standards in solidified agarose (made in tubes ≤1.5 mm in diameter) can be prepared by the same method, but extrusion of the thinner agarose gel without breaking is more difficult. The protocol supplies molecular weight markers containing ~2.5 µg of each standard suitable for Coomassie blue staining or 0.25 µg of each standard for silver staining.

Materials

Molecular weight standards (Table 6.1.2)
1× SDS sample buffer (*UNIT 6.1*)
2% (w/v) agarose (see recipe)

Boiling water bath
Glass tubes (3-mm inner diameter)
Plastic or metal tray

1. Prepare 3 ml molecular weight standards in 1× SDS sample buffer using 250 µg of each standard.

   The stated amount is appropriate for Coomassie blue staining of gels. If silver staining is planned, use 25 µg of each standard.
2. Mix the standards with 2 ml of 2% (w/v) agarose melted in a boiling water bath.
3. Prepare clean glass tubes by wrapping one end with Parafilm. Pour the hot mixture into the tubes and let the agarose solidify.
4. Carefully extrude the agarose from the tubes.
5. Cut agarose rods into 5-mm pieces using a razor blade.
6. Freeze all pieces separately on a plastic or metal tray using dry ice.
7. Collect frozen pieces in a plastic bottle and store at −80°C. The standards may be stored ≥1 year.

DIAGONAL GEL ELECTROPHORESIS (NONREDUCING/ REDUCING GELS)

ALTERNATE PROTOCOL 3

Protein subunit compositions and cross-linked protein complexes can be analyzed by two-dimensional gel electrophoresis using separation under nonreducing conditions in the first dimension followed by reduction of disulfide bonds and separation under reducing conditions in the second dimension. Most proteins will migrate equal distances in both dimensions, forming a diagonal pattern. Proteins containing interchain disulfide bonds will be dissociated into individual subunits and can be resolved in the second-dimension gel.

The approach is similar to that described for two-dimensional gel electrophoresis (see Basic Protocol 3) except, in this protocol, the first-dimension gels are nonreducing (i.e., 2-mercaptoethanol or dithiothreitol is omitted from sample buffer) SDS-denaturing gels instead of isoelectric focusing gels. Use of 1.2-mm tube gels for the first-dimension separation and 1.5-mm slab gels for the second-dimension run is recommended.

Additional Materials *(also see Basic Protocol 3)*

Separating and stacking gel solutions (see Table 6.1.1)
1× SDS sample buffer *without* reducing agents (*UNIT 6.1*)
Reducing buffer (see recipe)
1.5% (w/v) agarose in reducing buffer (see recipe; optional, for securing first-dimension gel on second-dimension gel)
Two-dimensional comb (optional)

Additional reagents and equipment for casting tube gels (see Basic Protocol 1), SDS-PAGE (*UNIT 6.1*), and protein staining (*APPENDIX 3*)

Pour and run the first-dimension gel

1. Clean and dry 1.2-mm glass gel tubes for the first-dimension gel (see Basic Protocol 1, step 1).

2. Prepare a separating gel solution with the desired percentage acrylamide (Table 6.1.1); omit the stacking gel for the first dimension.

   Stacking gels can usually be avoided in the first dimension by keeping sample volumes small (i.e., ≤10 µl).

   Less than 200 µl of gel solution is required to cast a single 1.2-mm tube gel 12 cm in length. Adjust the amounts from Table 6.1.1 accordingly.

3. Cast the first-dimension polyacrylamide gels in 1.2-mm tubes using a syringe with a long needle (see Basic Protocol 1, step 5a). Overlay with water and allow the gels to polymerize.

4. Prepare samples in 1× SDS sample buffer without any reducing reagents (i.e., no 2-mercaptoethanol or DTT). Load the samples and electrophorese until the tracking dye is ~1 cm from the bottom of the tube.

Reduce sample and run the second-dimension gel

5. Extrude the gel from the tube (see Basic Protocol 1, steps 27 to 29).

6. Place the extruded gel in a test tube containing 5 ml reducing buffer. Equilibrate 15 min at 37°C with gentle agitation.

7. Cast the second-dimension separating and stacking gels (see Basic Protocol 3, steps 1 to 3), making sure that the top of the stacking gel is at least 5 mm below the top of

the short glass plate. Layer water across the entire stacking gel or use a two-dimensional comb.

Most two-dimensional gel combs have a separate small well for a standard or reference sample.

The use of beveled plates (see Basic Protocol 3, steps 1 to 3) is not essential but is still preferred because it will facilitate loading of the first-dimension gel. In this procedure, the first-dimension gel will fit between the glass plates if 1.2-mm tubes are used for the first dimension and 1.5-mm gels are used for the second dimension.

8. Load the first-dimension gel onto the second-dimension gel. Remove any air bubbles trapped between the gels.

   If the first-dimension gel does not remain securely in place, it can be embedded using 1.5% (w/v) agarose in reducing buffer.

9. Carefully pour electrophoresis buffer into the upper electrophoresis chamber and electrophorese using voltages and times appropriate for the gel type selected.

   Parameters for electrophoresis are given in UNIT 6.1.

SUPPORT PROTOCOL 7

USING TWO-DIMENSIONAL PROTEIN DATABASES

Computerized image acquisition and manipulation constitute the only practical method for systematic qualitative and quantitative evaluation of complex protein patterns from different samples that are to be compared by high-resolution two-dimensional gel analysis. Examples of experimental applications include comparisons of tumor cells or tissues with appropriate normal controls and comparisons of a single cell line under different experimental conditions.

There are currently a number of commercially available image acquisition/computer systems specifically designed for comparing two-dimensional gels and storing associated information in a database. The systems include both hardware and the necessary software for comparing different gels and producing databases containing the two-dimensional protein patterns, with options for annotating specific spots and producing quantitative comparisons among large numbers of different samples. With most systems, images can be acquired from either stained gels or autoradiographs. The equipment used to obtain two-dimensional gel images includes laser scanners, video cameras, and phosphoimagers. After image acquisition, software running on a microcomputer or workstation is used to refine the image, detect spots, and match spots between different gels.

It is essential that very high-quality, reproducible gels be used for computerized comparisons. The greatest dynamic range in protein abundance for a single two-dimensional gel can be obtained using autoradiography or phosphoimaging (*UNIT 6.3*). With these methods, up to several thousand spots can be compared and tracked. A representative reference gel or a composite image can be stored and used as a reference for future experiments.

Information related to each spot on the two-dimensional pattern, including the quantity of protein in the indicated spot on different gels used in the comparison, can be archived and updated. Other known information related to a specific spot can also be added to the investigator-built database, including the pI, molecular weight, amino acid composition, sequence, and/or identity of the protein and any other important attributes correlated with the indicated spot. A number of research groups, including those of Garrels and Celis (Garrels, 1989; Garrels and Franza, 1989; Celis et al., 1991), have extensively characterized hundreds of spots from specific cell lines and have used multiple methods to characterize proteins of interest. The most definitive methods for establishing the identi-

ties for proteins of interest detected by computer-assisted comparisons are protein sequence analysis and, more recently, mass spectrometry of tryptic fragments. Both methods are compatible with the quantities of protein that can be recovered from two-dimensional gels.

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

30% acrylamide/0.8% bisacrylamide

30 g acrylamide
0.8 g bisacrylamide
H_2O to 100 ml

Filter solution through 0.2- to 0.45-µm filter (e.g., Micro Filtration Systems, cellulose nitrate, 0.2 µm). Store at 4°C (stable at least 3 months).

CAUTION: *Acrylamide is a neurotoxin. Wear gloves and a dust mask when handling solid acrylamide. Wear gloves when working with acrylamide solution. Never pipet acrylamide solutions by mouth.*

Agarose in reducing buffer, 1.5% (w/v)

Mix 0.15 g agarose and 10 ml reducing buffer (see recipe). Heat in boiling water bath until dissolved. Prepare immediately before use.

Agarose, 2% (w/v)

Mix 2 g agarose and 100 ml water. Stir on a hot plate until dissolved. Keeping the solution near 100°C, divide by placing 5-ml aliquots in 25-ml glass screw-cap tubes. Let the aliquots solidify. Store at 4°C (stable at least 3 months).

Ammonium persulfate, 2.5% (w/v)

0.25 g ammonium persulfate
H_2O to 10 ml
Prepare immediately before use

DNase and RNase solution

Mix 2.50 ml of 1 M Tris·Cl, pH 7.0 (*APPENDIX 2A*), 250 µl of 1 M $MgCl_2$, (*APPENDIX 2A*), and 2.2 ml water. Add to 5 mg DNase (Worthington) and dissolve DNase. Add 2.5 mg RNase (in solution; Worthington). Mix well, divide into 50- or 100-µl aliquots, and store at −80°C (stable at least 1 year).

The volume of RNase solution needed is variable and depends on the protein concentration, which is reported on the vial label (in mg/ml). The volume of RNase added should be <200 µl; if a larger volume is used, the amount of H_2O should be decreased proportionally.

Final concentrations are 0.5 M Tris·Cl, pH 7.0, 0.1 M $MgCl_2$, 0.1% (w/v) DNase, and 0.05% RNase.

DryStrip equilibration solutions

20 ml 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
72 g ultrapure urea
60 ml glycerol
2 g sodium dodecyl sulfate (SDS)
67 ml Milli-Q-purified water
For solution 1: Add 50 mg DTT per 10 ml of equilibration buffer
For solution 2: Add 0.45 g iodoacetamide and a few grains bromphenol blue per 10 ml of equilibration buffer

Make fresh immediately before use

Final concentrations are 50 mM Tris·Cl, pH 6.8; 6 M urea; 30% glycerol; and 1% SDS in a final volume of 200 ml.

EDTA, 2% (w/v)

2 g Na_2EDTA
H_2O to 100 ml
Adjust to pH 7.0 with NaOH
Store at room temperature (stable several months)

Titrate while dissolving. EDTA is difficult to dissolve without addition of NaOH even when the disodium salt is used.

Equilibration buffer

3 g SDS
7.4 ml 2% (w/v) EDTA, pH 7.0 (see recipe)
10 ml glycerol
2 ml 1.0 M Tris·Cl, pH 8.65 (*APPENDIX 2A*)
0.3 ml bromphenol blue (saturated solution in H_2O)
H_2O to 100 ml
Store at room temperature (stable for several weeks)

Final concentrations are 3% (w/v) SDS, 0.4 mM EDTA, 10% (v/v) glycerol, and 20 mM Tris·Cl, pH 8.65.

Leupeptin, 2 mg/ml

20 mg leupeptin
10 ml water
Divide into convenient volumes
Store at −20°C (stable at least 1 year)

Lysis buffer

2.59 g urea (ultrapure)
1.6 ml H_2O
0.25 ml 2-mercaptoethanol
0.3 ml ampholytes
1.0 ml 20% (w/v) Triton X-100 solution (see recipe)
Prepare immediately before use

Use same ampholytes as for the IEF gel formulation. To dissolve urea, warm the mixture in a 30°C water bath if necessary.

Orthophosphoric acid (H_3PO_4), 0.1 M

13.7 ml 85% phosphoric acid
Water to 2 liters
Make fresh daily

Must be degassed prior to use.

PBS, 10×

152 g NaCl
24 g monobasic sodium phosphate, anhydrous
1600 ml H_2O
Adjust pH to ~6.7 with NaOH
Add H_2O to 2 liters
Store at room temperature (stable at least 1 month)

The 1× solution should be pH 7.3 to 7.5. Final concentrations are 130 mM NaCl and 10 mM sodium phosphate.

PBS with proteolysis inhibitors (PBS/I buffer)

20 ml 10× PBS (see recipe)
20 ml 2% (w/v) EDTA, pH 7.0 (see recipe)

continued

200 µl 0.15 M phenylmethylsulfonyl fluoride (PMSF) in 2-propanol
100 µl 2 mg/ml leupeptin (see recipe)
200 µl 1 mg/ml pepstatin (see recipe)
Adjust to pH 7.2 with HCl
Add H_2O to 200 ml
Prepare immediately before use

Diisopropyl fluorophosphate (DFP) is a better serine protease inhibitor than PMSF at lower temperatures (0° to 4°C); however, although both compounds are toxic, exceptional caution must be exercised with DFP owing to its volatility. If DFP is used, work in a chemical fume hood and carefully follow the supplier's precautions. DFP and PMSF have half-lives on the order of hours in aqueous neutral solutions, and the degradation rate increases rapidly as the pH is increased above neutral. Make aqueous solutions immediately before use. Use of 1 M NaOH is convenient to inactivate residual DFP or PMSF.

Final concentrations are 10 mM sodium phosphate, 130 mM NaCl, 0.2% EDTA, 0.15 mM PMSF, 1µg/ml leupeptin, and 1 µg/ml pepstatin.

Pepstatin, 1 mg/ml

10 mg pepstatin
10 ml anhydrous ethanol
Divide into convenient volumes
Store at −20°C (stable at least 1 year)

Thaw aliquots and mix well immediately before use.

Reducing buffer

0.5 g dithiothreitol (DTT)
0.1 g SDS
1.51 g Tris base
Adjust to pH 6.8 with HCl
Add H_2O to 100 ml
Prepare fresh every time

Final concentrations are 0.5% (w/v) DTT, 0.1% (w/v) SDS, and 125 mM Tris·Cl, pH 6.8.

Tris/SDS buffer

0.3 g SDS
0.6 g Tris base
Adjust to pH 8.0 with HCl
Add H_2O to 100 ml
Divide into 5-ml aliquots
Store at −80°C (stable at least 1 year)

Final concentrations are 0.3% (w/v) SDS and 50 mM Tris·Cl, pH 8.0.

Triton X-100 solution, 20% (w/v)

3 g Triton X-100
12 ml H_2O
Warm in 37°C water bath to dissolve Triton X-100
Store at 4°C (stable ~2 weeks)

Urea, 8 M

0.75 g ultrapure urea
1.0 ml H_2O
Prepare immediately before use

Avoid heating above room temperature.

COMMENTARY

Background Information

Two-dimensional gel electrophoresis, using isoelectric focusing followed by SDS-PAGE, is the single most powerful analytical method currently available for separating complex protein mixtures such as whole-cell or tissue extracts. It is therefore a valuable method for following disease-related changes or for detecting changes in protein expression under diverse experimental conditions. To achieve maximum reproducibility between samples to be compared, multiple gels should be cast and run simultaneously (Anderson and Anderson, 1978a,b).

Despite the exceptionally high resolving power of the method, the total number of proteins that can be resolved in a single two-dimensional gel is only ~1000 to 2000, whereas the total number of proteins in a single mammalian cell type is likely to be at least 10 to 20 times higher. Therefore, the old guideline that a single spot on a two-dimensional gel is a single protein needs to be revised as analytical detection methods improve. Conversely, a single protein (single gene product) can produce multiple, usually adjacent spots in the isoelectric focusing dimension owing to variable degrees of chemical or posttranslational modification. Common examples of variable posttranslational modifications that can usually be detected on two-dimensional gels include phosphorylation, glycosylation, and acetylation. Examples of chemical modifications that cause charge heterogeneity include deamidation of side-chain amines (usually asparagines), oxidation of sensitive side chains, and modification of lysines. Potential modification of lysines by urea is particularly important because urea rapidly decomposes to form cyanate, which readily reacts with amino groups, especially above pH 7. Despite potential side reactions, urea is the most useful IEF additive for maintaining solubility of proteins near their isoelectric points.

Two-dimensional PAGE has become a valuable preparative tool for protein isolation in addition to its historical role as an analytical method. The sensitivity of many protein analysis methods has improved to the point where one or several spots from two-dimensional gels are sufficient for protein identification using mass spectrometry methods.

Commercially available equipment for running two-dimensional gels can be divided into four groups based on size: microgels (Amersham Pharmacia Biotech Phast system), minigels (e.g., Bio-Rad or Amersham Pharmacia Biotech), standard or full-sized gels (e.g., Bio-Rad or Amersham Pharmacia Biotech), and large or "giant" gels (ESA Investigator 2D gel system or Amersham Pharmacia Biotech Iso-Dalt gel system). In general, the larger the gel, the better the final resolution, but as gel size increases so do costs, difficulty of gel handling, and time requirements.

Standard-size gels provide adequate resolution for most applications and are relatively easy to handle. A 3-mm soluble ampholyte tube IEF gel or a 0.5 mm × 3 mm × 18-cm Immobiline gel has a total protein capacity of ~500 µg for complex protein mixtures such as whole-cell extracts. The maximum capacity for any single protein spot is ~0.5 to 5 µg, depending on the solubility of the protein near its isoelectric point and the separation distance from any near neighbors. A variety of alternative gel sizes, their limits, and their advantages are summarized in Table 6.4.2. The lower protein limit for any of the systems is determined strictly by the available detection methods.

Proteins can be detected in two-dimensional gels by the same wide range of techniques used for one-dimensional gels. Autoradiography (*UNIT 6.3*), silver staining, and electroblotting to PVDF membranes (*UNIT 6.2*) followed by colloidal gold or colloidal silver staining or immunodetection (*UNIT 6.2*) are among the most sensitive techniques available. If a larger amount of protein is available, Coomassie blue staining of the gel or amido black staining of a PVDF membrane after electrotransfer (*UNIT 6.2*) would be the detection methods of choice.

The major technical limitation in two-dimensional gel electrophoresis is gel-to-gel variation. Even when extreme care is exercised to produce highly reproducible first- and second-dimension gels, some gel-related variability among gels cast at the same time is likely to persist. Another source of variability includes differences in extraction or recovery of proteins during sample solubilization and handling. Maximizing resolution and reproducibility is especially important if computerized comparisons of two-dimensional gels of complex protein mixtures such as cell or tissue extracts are being attempted.

Isoelectric focusing using soluble ampholytes

Soluble ampholytes are mixtures of low-molecular-weight organic compounds with differing side-chain pK_a values that provide buffering capacity. In an IEF gel, the ampholytes migrate to their isoelectric point, where they provide buffer-

Table 6.4.2 Size Options for Two-Dimensional Gel Electrophoresis

Gel type	First-dimension gel		Second-dimension gel[a]		Purpose	Comments[b]
	Diameter *D* (mm)	Length *L* (cm)	Thickness *T* (mm)	Height *H* (cm)		
Microgels/minigels[c]	<1.5	<10	<*D*	<10	Analytical	1-4
	<1.5	<10	>*D*	<10	Analytical	3-6
	>1.5	<10	<*D*[d]	<10	Analytical/preparative	1,2,7,8
Full-size gels[e]	<1.5	12-18	<*D*	12-18	Analytical	1-4
	<1.5	12-18	>*D*	12-18	Analytical	3-6
	>1.5	12-18	<*D*[d]	12-18	Analytical/preparative	1,2,7,8
Giant gels[f]	<1.5	>20	>*D*	>20	Analytical	4-6,9
	>1.5	>20	<*D*[d]	>20	Analytical/preparative	1-3,7

[a]The second-dimension gel width has to be at least equal to the IEF tube gel height.

[b]Key to comments: (1) tube gel cannot be placed directly on top of second-dimension gel, and use of agarose is recommended; (2) use of stacking gel is recommended; (3) extrusion and handling are relatively difficult; (4) total protein load is limited to usually ≤50 µg for whole-cell or tissue extracts; (5) tube gel can be placed directly on top of second-dimension gel, and use of agarose is not necessary; (6) use of a stacking gel is not necessary; (7) total protein load capacity is relatively large; (8) extrusion and handling are relatively simple; (9) extrusion and handling are very difficult.

[c]Minigel systems provide rapid separations with moderate resolution. Microgels (Phastgels) are precast gels that are slightly smaller than most minigels.

[d]Use of second-dimension gels thicker than 1.5 mm is generally not recommended owing to difficulty with either efficient staining or efficient electroblotting.

[e]Full-size gels provide resolution satisfactory for most applications.

[f]Giant gels provide very good resolution. Specialized equipment is required, such as Investigator 2D (ESA), Iso-Dalt (Hoeffer Pharmacia), or homemade giant-size gel systems.

ing capacity and hence produce stable pH gradients. In theory, any desired pH gradient could be produced by blending ampholytes with appropriate pK_a values. In practice, it is relatively easy to produce pH gradients from ~pH 3.5 or 4.0 to pH 8.0, but stable soluble gradients outside this range are usually not technically feasible. Within these pH limits, some manipulation of the gradient shape and pH range is possible by blending different amounts of specific pH range ampholytes. For example, 0.50 ml of pH 5-7 ampholytes plus 0.25 ml of pH 4-8 ampholytes can be used instead of 0.75 ml of pH 4-8 ampholytes alone to increase the separation distance of proteins in the pH 5.0 to 7.0 range.

Basic Protocol 1 is based on use of 3-mm first-dimension isoelectric focusing gels and 1.5-mm second-dimension gels using the Bio-Rad two-dimensional gel apparatus (Protean II xi 2D). The method can be easily adapted to equipment from other suppliers or to different-sized gels by adjusting the quantities of reagents used. The protocol uses 8 M urea and Triton X-100 as solubilizing agents. Solubilization of the protein sample applied to the gel as well as maintenance of solubility during electric focusing are the most critical factors influencing the quality of separation in the first dimension. The most common modification to Triton X-100–based procedures is addition of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) to the gel and solubilizing buffer mixtures. Addition of SDS to complex samples such as tissue or cell extracts can also enhance reproducible solubilization of the largest possible subset of proteins. Although SDS is charged, in the presence of Triton X-100 it is separated from proteins during focusing and migrates to the acidic end of the gel. Regardless of the method used to maintain solubility, some proteins (especially those >100 kDa) tend to precipitate at pH values approaching their isoelectric point and thus produce horizontal smears on the final second-dimension gel.

Basic Protocol 1 requires several modifications for successful separation of very acidic (Alternate Protocol 1) or very basic (Alternate Protocol 2) proteins. In both cases, prefocusing of the gels must be avoided, and the isoelectrofocusing time has to be reduced. A short separation time does not allow the system to reach equilibrium and is used to establish the desired

Table 6.4.3 Commercially Available Precast IPG Gels and Immobiline Chemicals

Name	Use	Available pH range
Immobiline DryPlate[a]	Running one-dimensional immobilized pH gradient gels	pH 4.0-7.0, pH 4.2-4.9, pH 4.5-5.4, pH 5.0-6.0, pH 5.6-6.6
Immobiline DryStrip[a]	Running first dimension in two-dimensional gels	
110 mm		pH 4-7L, pH 3-10L
180 mm		pH 4-7L, pH 3-10L[c], pH 3-10NL[d]
IPG Ready Strips[b]	Running first dimension in two-dimensional gels	
7 cm		pH 3-10, pH 4-7, pH 3-6, pH 5-8, pH 7-10
11 cm		pH 3-10, pH 4-7, pH 3-6, pH 5-8, pH 7-10
Immobiline II[a]	Creating custom gradient immobilized pHgradient gels	pK 3.6, pK 4.6, pK 6.2, pK 7.0, pK 8.5, pK 9.3

[a]From Amersham Pharmacia Biotech.

[b]From Bio-Rad.

[c]A linear gradient with maximum resolution above pH 7.0.

[d]A nonlinear gradient with best resolution at pH 5.0-7.0.

pH gradient. Separation of very acidic proteins requires modifications of gel and electrode solutions. Separation of very basic proteins has to be performed with the positions of electrodes and electrode solutions reversed in the electrophoresis chamber.

The total protein load per gel depends on the complexity of the sample, the solubility of proteins in the sample, and the diameter of the first-dimension gel. Approximately 4 times as much total sample can be applied to a 3-mm gel compared with a 1.5-mm gel. Another advantage of a larger-diameter isoelectric focusing gel is that extrusion and gel handling are easier owing to improved strength of the gel. If care is exercised in loading the first-dimension gel onto the 1.5-mm second-dimension gel, the final resolution will be similar to that obtained using smaller-diameter IEF gels. The separating or resolving power of a system is dependent on the quality of ampholytes used, the slope of the pH gradient, and the lengths of both first- and second-dimension gels.

Immobilized pH gradient gels

In immobilized pH gradient (IPG) gels (Basic Protocol 2), the pH gradient is an integral part of the polyacrylamide matrix (Strahler and Hanash, 1991). Because the pH gradient is covalently associated with the polyacrylamide gel matrix, precise, reproducible, and very high-resolution separations can be achieved. A variety of precast gels and all the necessary equipment are commercially available from either Amersham Pharmacia Biotech or Bio-Rad. Reproducible two-dimensional gels can be obtained by running a sample on a narrow strip of immobilized pH gradient gel and then running it in a vertical SDS second-dimension gel. Isoelectrofocusing is performed in a horizontal electrophoresis unit in which multiple gel strips may be run simultaneously. Equipment and reagents are also available for the user to cast custom Immobiline gels in the laboratory.

Some of the major advantages of using precast IPG gels are their ease of use and high reproducibility, the time savings realized, and the fact that precision narrow-range gradients can be used to resolve small charge differences. Because the pH gradient is covalently coupled to the polyacrylamide gel matrix, the pH gradient remains stable and linear during prolonged electrophoresis, thus ensuring reproducibility. This is in contrast to conventional IEF gels, where gradient drift occurs during prolonged electrophoresis. Additionally, the precast gels can be rehydrated in water or in solutions with one or more additives such as urea, CHAPS or Triton X-100, carrier ampholytes, glycerol, and reducing agents, which may help to increase protein solubility. Precast gel strips are available with pH ranges such as pH 3.0 to 10.0 or pH 4.0 to 7.0 as well as narrower ranges (as DryPlates). In addition, a variety of Immobilines permits the user to cast IPG gels with customized pH gradients of any gradient range and shape between pH 3.0 and pH 10.0.

Table 6.4.3 lists types of commercially available gels and Immobilines. With the Immobiline

system, the apparent pI of a given protein may be slightly different from that determined by other methods. Therefore, it is recommended that a broad-range gradient be tried initially, followed by a narrower-range gradient, if needed.

Diagonal gel electrophoresis

Diagonal gel electrophoresis is a form of two-dimensional analysis useful for investigating the subunit composition of multisubunit proteins containing interchain disulfide bonds (Goverman and Lewis, 1991). Proteins are electrophoresed in the first-dimension in a tube gel (or a slab gel) under nonreducing conditions. The proteins are then reduced in situ, and the first gel (or a strip thereof) is layered onto a second gel and electrophoresed. In the second gel, the proteins migrate at right angles to the original, first-dimension migration. Most cellular proteins are not disulfide-linked and will fall on the "diagonal" in this system; that is, they migrate approximately equal distances in both directions during electrophoresis and lie approximately on the diagonal line connecting opposite corners of the gel. On reduction, component subunits of proteins connected by interchain disulfide bonds will resolve below the diagonal because the individual subunits migrate faster than the disulfide-linked complex during the second electrophoresis. Some proteins with internal disulfide bonds, but no interchain disulfides, may migrate slightly above the diagonal because internal disulfides can produce a more compact molecular shape (causing faster migration in the first dimension).

Investigator 2D gel system

The Investigator 2D gel system was introduced in 1990 by Millipore as the first commercial "large" or "giant" format two-dimensional gel system designed for analytical purposes, although analogous homemade units had been reported earlier (Garrels, 1979; Young et al., 1983). This product line, including precast first- and second-dimension gels, can now be purchased from ESA. The Investigator 2D gel system uses larger gel sizes than the gels described in this unit as well as a number of novel approaches and reagents designed to enhance resolution and gel-to-gel reproducibility. The major features of this system include the following: an increased length of the first-dimension gel (20 cm), an increased length and width of the second-dimension gel (20 $\times$ 22 cm), a thread reinforcement of the isoelectric focusing gel, temperature control during electrophoresis of the second-dimension gel, and use of a special high-tensile-strength acrylamide to facilitate handling of the large, thin second-dimension gel. Narrow gel tubes (1.2 mm) for the first-dimension are standard, but 3-mm-inner-diameter tubes are available to accommodate larger protein loads. The 3-mm IEF gels do not have the thread reinforcement. One modification related to the above protocol includes addition of 0.3% (w/v) CHAPS in the gel solution, which is intended to improve the solubility of proteins and their migration and separation in the gel. The system is supplied with a manual that adequately describes the method.

Critical Parameters and Troubleshooting

Although no individual steps in two-dimensional gel analysis are exceptionally difficult, the large number of steps involved increase the likelihood and possible severity of errors or problems. Several steps are especially critical and may require optimization. The first is sample preparation. Proteins applied to IEF gels have to be completely solubilized. Residual precipitate or even soluble aggregates are likely to cause artifacts on the end of the tube gel or position on the IPG strip where the sample is loaded. Precipitated or aggregated proteins may also interact with soluble proteins, causing components with normally good solubility to coprecipitate or migrate anomalously.

Two general rules of thumb apply to sample solubility: (1) the more complex the sample or the more crude the extract, the more likely problems will be encountered with sample solubility, and (2) the higher the protein load applied to the gel, the more likely solubility problems will arise. Care must also be taken to avoid proteolysis during sample preparation, especially when complex, impure samples are used. If samples are frozen either before or after solubilization, they should be stored at −80°C and should not be subjected to repeated freeze-thawing. Addition of SDS to the solubilization solution increases the solubilization of some proteins; however, SDS should only be used when the IEF gels contain urea and Triton X-100, as the solubilizing agents are required for effective separation of the strongly anionic SDS molecules from the proteins during isoelectric focusing. Heating of samples in urea-containing solutions must be avoided because urea readily decomposes to cyanate, which reacts with amino groups and causes charge heterogeneity. High concentrations of salts and buffers in the sample should be avoided. Ionic compounds increase the conductivity of the sample and can result in localized overheating, especially for Immobiline gels.

Samples analyzed on immobilized pH gradient gels should not contain precipitates. The concentration of salts and buffer ions in the sample should be kept to a minimum (<50 mM) to avoid local overheating of the gel during electrophoresis. If the sample forms aggregates or precipitates at the point of application, apply the sample to a different location (different pH) on the gel, or load the sample during the rehydration step.

Early decisions that must be made include the size of the gels required and whether a soluble ampholyte system or an Immobiline gel will be used. The major consideration affecting appropriate gel size is the degree of resolution needed. In general, the smallest gel format should be selected that will provide the needed degree of resolution, because smaller gels are easier, less expensive, and faster to run. Therefore, quick screening of samples or analysis of relatively simple samples can easily be accomplished with microgels or minigels. In contrast, if detailed qualitative or quantitative comparisons of cell or tissue extracts are planned, standard-size or large gels are indicated. Similarly, 3-mm or larger first-dimension tube gels followed by 1.5-mm second-dimension gels in the standard or large format are indicated if the two-dimensional gels will be used for preparative isolation of a protein for applications such as raising antibodies or conducting structural analysis. Immobilized pH gradient gels should very seriously be considered as an alternative to soluble ampholyte gels for most separations owing to their stable and reproducible pH profiles. The IPG gels are particularly appropriate when a narrow pH range is required.

Immobilized pH gradient gels are typically run at 2500 to 3500 V and typically require a focusing time of 16 to 18 hr. Use of high voltage (8,000 to 10,000 V) integrated power supplies/cooling units can shorten focusing times to <4 hr. Optimal focusing conditions may be experimentally determined by applying the sample to different positions on the gel and estimating the time for the migration patterns to coincide. Some proteins may require longer run times to reach their pI. Because there is no gradient drift, the potential problems with longer run times are limited to sample modifications or drying out of the gel. These problems usually can be minimized by including a reducing agent in the rehydration solution and coating the top surface of the gel with paraffin oil.

The quality of the first-dimensional separation is strongly dependent on the purity of the reagents used, especially the urea and ampholytes. One fairly commonly encountered frustration, when soluble ampholyte gels are used, is that different batches of ampholytes from the same supplier will sometimes produce markedly different two-dimensional gel patterns. Therefore, it is advisable to purchase an adequate supply of a single lot of ampholytes to meet anticipated needs for an entire study where such an approach is feasible. However, whereas ampholytes usually have a reasonably long shelf life at 4°C (usually up to a year), shelf life as well as total ampholyte requirements often cannot be predicted with much certainty.

When any doubt arises about the purity or quality of ampholytes or any other reagent, the reagent should be replaced immediately. Constant monitoring of the system performance, especially when changing lots of ampholytes, urea, or acrylamide, can help minimize potential reagent-associated problems. In most cases, the best standard for a given two-dimensional gel system is an experimental sample or control that is available in sufficient quantity so that many replicate aliquots can be frozen and stored for an extended time at −80°C; alternatively, a sample that can be reproducibly prepared over a long time frame would make an acceptable standard. Such an experimental standard or reference is more likely to detect subtle, but experimentally important, changes in the two-dimensional gel system than commercially available IEF or SDS gel standard mixes.

Another critical factor is equilibration of the first-dimension gel in the second-dimension equilibration buffer. During this step, urea diffuses out of the IEF gel while SDS and reducing reagent diffuse into the gel. If the gel is inadequately saturated with SDS, vertical streaking will result. However, if the gel is incubated in the equilibration buffer for an extended time, a substantial amount of the protein can rapidly diffuse out of the large-pore IEF gel. Losses arising from diffusion can be critical for any experiment because different proteins will diffuse at different rates, but rigorous control of the incubation step is especially important if quantitative comparisons among gels are planned. The simplest method of controlling the incubation time is to freeze the extruded IEF gel after a carefully controlled 5-min incubation in the equilibration buffer; any additional equilibration incubation time required can then be incorporated and carefully controlled when the sample is thawed for loading onto the second-dimension gel.

Another crucial step is loading of the equilibrated IEF gel onto the top of the second-dimension gel. Any irregularity or obstruction between the two gels, including particles of dirt or air bubbles, will affect the flow of current and disrupt

the resolution of proteins in the final gel. Similarly, poor contact or any movement of the IEF gel during electrophoresis of the proteins out of the IEF gel into the second-dimension gel will lead to artifacts. Therefore, it is advisable to embed the IEF gel in a buffered agarose matrix to ensure good electrical contact between the gels and to prevent gel movement after electrophoresis is initiated.

Finally, the choice of second-dimension gel composition and separation conditions can influence the quality of results. A proper percentage of acrylamide should be selected to optimize resolution within the desired molecular mass range. If gradient gels are needed, use of a multiple gel casting stand is the best way to ensure reproducibility among samples within a single experiment. Further details on optimization of two-dimensional gel systems are presented by Hochstrasser et al. (1988).

When no technical, sample-related, or reagent-related problems are encountered, the final stained two-dimensional gels should contain numerous rounded or slightly elliptical spots. Typically, more than 1000 spots can be detected on a standard 16 × 14–cm gel when using a sensitive staining protocol such as silver staining or autoradiography and a whole-cell extract as a sample. Some horizontal streaks for most high-molecular-mass proteins (proteins exceeding ~100 kDa) are common owing to the decreased solubility of larger proteins near their isoelectric points, even in the presence of urea and nonionic detergent. However, excessive horizontal smearing of proteins smaller than 100 kDa indicates poor isoelectric focusing, which could be related to one or more of the following factors: sample improperly solubilized or contaminated with interfering substances such as large nucleic acid molecules, poor purity of reagents (check the urea first), poor-quality ampholytes, or insufficient isoelectric focusing (total volt-hours too low). It is important to note that in general the solubility of any protein is the lowest near its isoelectric point, but there are vast differences among proteins in terms of both the minimum concentration where precipitation becomes a problem and the degree to which precipitation can be prevented by adding different solubilization agents. The best conditions for maintaining solubility during isoelectric focusing for a given sample type must be determined empirically, although the most universal conditions are those described in the protocols in this unit. In contrast, IEF systems that do not use any detergents or denaturants are limited to that fairly small percentage of proteins which maintain good solubility near their isoelectric point.

If high-molecular-weight proteins are expected but are not present in the final two-dimensional gel, check the sample preparation protocol as well as the sample storage conditions. The most likely problem is proteolysis during sample preparation. Multiple freeze-thawing cycles could contribute to this problem. Vertical smears on the two-dimensional gel suggest (1) insufficient equilibration of the IEF gel (not enough SDS bound to the proteins), (2) poor contact between the IEF and second-dimension gels, or (3) problems related to the stacking gel (too short or wrong buffer). Use of a stacking gel is especially important when large-diameter IEF gels are loaded onto smaller second-dimension gels.

Omission of Triton X-100 or other nonionic or zwitterionic detergent from the final sample loaded on the gel can yield poor results, especially for samples containing SDS, because the amount of detergent in the IEF gels alone may be insufficient to remove bound SDS from proteins. The presence of Triton X-100 in the sample is especially important when SDS sample buffer is used to solubilize protein samples (i.e., after immunoprecipitation). The amount of Triton X-100 in the lysis buffer is normally sufficient for effective dissociation of SDS from proteins. If poor results are encountered with SDS-containing samples, try decreasing the final SDS concentration and/or increasing the final Triton X-100 concentration in the sample.

If no proteins are detected on the gel, check whether (1) the total protein load is appropriate for the detection method used, (2) the orientation of electrical connections is wrong or electrical connection during isoelectric focusing is poor (all gels from that run will be blank), (3) an air bubble obstructs current in a single IEF tube, or (4) the electrical connection is incorrect or is poor during the second-dimension gel separation. Careful monitoring of current and voltage at the beginning, during, and at the end of electrophoretic separations is strongly recommended. Recording the initial and final current and voltage will also facilitate troubleshooting. Additional guidelines for troubleshooting and evaluating artifacts in two-dimensional gel electrophoresis are described by Dunbar (1987).

Anticipated Results

A two-dimensional electrophoretic separation of proteins should produce a pattern of round or elliptical spots separated from one another. The pI range of the separated proteins as well as the observed molecular weight range depend on the first-dimension isoelectric focusing protocol and the percentage of acrylamide used for the second-

dimension gel. A complex protein mixture such as a whole-cell extract should produce more than 1000 silver-stained spots distributed over most of the gel area. Fewer spots will be detected with less sensitive detection techniques such as Coomassie blue staining. On the other hand, separation of radiolabeled proteins and use of multiple exposures permit detection of many low-abundance proteins.

Time Considerations

Time requirements are very dependent on gel size and whether an external cooling unit is used to permit faster separations. Isoelectric focusing using the standard-size gel format described in Basic Protocol 1 with 3-mm tubes is most conveniently done in an overnight run of ~16 to 18 hr. This separation time can be decreased to ~5 to 6 hr using higher voltages and an external cooling device. Isoelectric focusing of 18-cm-long IPG gels requires ~16 to 18 hr. Extruding a set of sixteen IEF tube gels and freezing them in equilibration buffer takes 1 to 2 hr, including setup time. Preparing and running SDS gels is described in UNIT 6.1. It takes ~30 min to thaw, equilibrate, and load two second-dimension gels.

Overall, if standard-size gels are used without external cooling, it will take ~3 working days before the results of two-dimensional electrophoresis are obtained. A single person can conveniently run about 16 two-dimensional gels in one week, depending on the amount of electrophoresis equipment available. The rate-limiting step in most laboratories is running the second-dimension gels because 16 soluble ampholyte IEF gels or 12 IPG gels can be focused in one run, but loading, running, and detecting results from 12 to 16 second-dimension gels requires substantial operator time and electrophoresis equipment.

Literature Cited

Anderson, N.G. and Anderson, N.L. 1978a. Two-dimensional analysis of serum and tissue proteins: Multiple isoelectric focusing. *Anal. Biochem.* 85:331-340.

Anderson, N.L. and Anderson, N.G. 1978b. Two-dimensional analysis of serum and tissue proteins: Multiple gradient-slab gel electrophoresis. *Anal. Biochem.* 85:341-354.

Celis, J.E., Rasmussen, H.H., Leffers, H., Madsen, P., Honore, B., Gesser, B., Dejgaard, K., and Vandekerckhove, J. 1991. Human cellular protein patterns and their link to genome DNA sequence data: Usefulness of two-dimensional gel electrophoresis and microsequencing. *FASEB J.* 5:2200-2208.

Dunbar, B. 1987. Troubleshooting and artifacts in two-dimensional polyacrylamide gel electrophoresis. *In* Two-Dimensional Electrophoresis and Immunological Techniques (B.S. Dunbar, ed.) pp. 173-195. Plenum, New York.

Garrels, J.I. 1979. Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by cloned cell lines. *J. Biol. Chem.* 54:7961-7977.

Garrels, J.I. 1989. The QUEST system for quantitative analysis of two-dimensional gels. *J. Biol. Chem.* 264:5269-5282.

Garrels, J.I. and Franza, Jr., B.R. 1989. The REF52 protein database: Methods of database construction and analysis using the QUEST system and characterizations of protein patterns from proliferating and quiescent REF52 cells. *J. Biol. Chem.* 264:5283-5298.

Goverman, J. and Lewis, K. 1991. Separation of disulfide-bonded polypeptides using two-dimensional diagonal gel electrophoresis. *Methods* 3:125-127.

Hochstrasser, D.F., Harrington, M.C., Hochstrasser, A.C., Miller, M.J., and Merril, C.R. 1988. Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal. Biochem.* 173:424-435.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.

Strahler, J.R. and Hanash, S.M. 1991. Immobilized pH gradients: Analytical and preparative use. *Methods* 3:109-114.

Young, D.A., Voris, B.P., Maytin, E.V., and Colbert, R.A. 1983. Very high resolution two-dimensional electrophoretic separation of proteins on giant gels. *Methods Enzymol.* 91:190-214.

Key Reference

Hochstrasser et al., 1988. See above.

Discusses methods for improving and troubleshooting two-dimensional separation.

Contributed by Sandra Harper, Jacek Mozdzanowski, and David Speicher
The Wistar Institute
Philadelphia, Pennsylvania

One-Dimensional Electrophoresis Using Nondenaturing Conditions

Nondenaturing or "native" electrophoresis—i.e., electrophoresis in the absence of denaturants such as detergents and urea—is an often-overlooked technique for determining the native size, subunit structure, and optimal separation of a protein. Because mobility depends on the size, shape, and intrinsic charge of the protein, nondenaturing electrophoresis provides a set of separation parameters distinctly different from mainly size-dependent denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; *UNITS 6.1*) and charge-dependent isoelectric focusing (IEF; *UNIT 6.4*). Two protocols are presented below. Continuous PAGE (see Basic Protocol) is highly flexible, permitting cationic and anionic electrophoresis over a full range of pH. The discontinuous procedure (see Alternate Protocol) is limited to proteins negatively charged at neutral pH but provides high resolution for accurate size calibration.

CONTINUOUS ELECTROPHORESIS IN NONDENATURING POLYACRYLAMIDE GELS

BASIC PROTOCOL

Separation of proteins by nondenaturing electrophoresis requires the same type of equipment used for denaturing slab gels (*UNIT 6.1*) and is adaptable to a range of gel sizes (e.g., from 7.3 × 8.3–cm minigels to 14 × 16–cm full-size gels) and matrix types (e.g., single-concentration and gradient gels). This protocol outlines straightforward procedures for making acrylamide solutions, casting separating gels (stacking gels are omitted), loading samples, and conducting electrophoresis. Continuous systems, although flexible, do not give the high-resolution separation found in discontinuous systems (see Alternate Protocol).

Separation in a continuous system (i.e., in which the same buffer is used for preparing acrylamide solutions and filling electrophoresis chambers) is governed by pH, and this protocol describes four types of buffers useful over discrete ranges from pH 3.7 to pH 10.6. Use of unadjusted acetic acid gel buffer can extend the range to pH 2.0. The choice of pH and thus the buffer system depends on the protein being studied (i.e., its isoelectric point) and often must be determined empirically. In general, the system should be between pH 5.0 and 8.0 for optimal results. Extremes of pH can lead to precipitation or denaturation of the protein. Acrylamide concentrations are empirically determined, but the higher the percent acrylamide, the sharper the protein bands.

It is important to include native protein standards in the electrophoresis runs. Several manufacturers supply standards for isoelectric focusing that are also suitable for native electrophoresis. The standards have a range of isoelectric points and will carry a net positive, negative, or zero charge depending on the pH of the gel system. Alternatively, Sigma supplies a standard kit that is useful for calculating molecular weights under neutral pH, nondenaturing conditions. The samples and standard proteins, should be used at concentrations of ~1 to 2 μg/μl.

Materials

4× acetic acid gel buffer (200 mM acetic acid, pH 3.7 to 5.6; see recipe)
4× phosphate gel buffer (400 mM sodium phosphate, pH 5.8 to 8.0; see recipe)
4× Tris gel buffer (200 mM Tris·Cl, pH 7.1 to 8.9; see recipe)
4× glycine gel buffer (200 mM glycine, pH 8.6 to 10.6; see recipe)
300 mM sodium sulfite (0.38 g in 10 ml H_2O; used in acetic acid gel preparation)
Protein samples to be analyzed

Contributed by Sean R. Gallagher
Current Protocols in Cell Biology (2000) 6.5.1-6.5.11

Native protein standards
Electrophoresis buffer: appropriate 4× gel buffer diluted to 1× with H_2O
75-ml side-arm flask (used in gel preparation)

Additional reagents and equipment for gel electrophoresis (*UNIT 6.1*) and staining proteins in gels (*APPENDIX 3*)

Prepare the gel

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and secure it to the casting stand.

   Either single-concentration or gradient gels can be used in the minigel or standard-size format. Gradient gels will enhance the band sharpness of the separated proteins.

2. Prepare acrylamide solutions according to the recipes in Table 6.5.1, Table 6.5.2, Table 6.5.3, or Table 6.5.4, adding the ammonium persulfate and TEMED just before use.

   Deaeration of the solution before the polymerization catalysts are added will speed polymerization by removing inhibitory oxygen, but this is not generally required. The pH used depends on many factors. The most important are the pI values of both the protein of interest and any contaminants, as well as protein mobility and protein solubility. Determining which pH and thus which buffer system to use is largely empirical. However, extremes of pH (<4.0 and >9.0) can lead to denaturation and should be avoided. Prior knowledge of the pI of a protein (UNITS 6.4) allows determination of the net charge under

Table 6.5.1 Recipes for Acetic Acid Nondenaturing Polyacrylamide Gels[a]: pH Range 3.7 to 5.6[b]

Stock solution[c]	Final acrylamide concentration in gel (%)[d]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
300 mM sodium sulfite[e]	0.4	0.4	0.4	0.4	0.4	0.4	0.4
4× acetic acid gel buffer	10	10	10	10	10	10	10
H_2O	22.58	19.28	15.98	12.48	9.28	5.96	2.68
10% (w/v) ammonium persulfate[e,f]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
TEMED[f]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 300 mM sodium sulfite, 4× acetic acid gel buffer (see Reagents and Solutions), and H_2O. If desired to speed polymerization, degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]The pH range can be extended to ~2.0 (the pH of acetic acid) by using unadjusted acetic acid in place of 4× acetic acid gel buffer, although there is little buffering capacity at this pH.

[c]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[d]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[e]Must be freshly made. Sodium sulfite is needed for efficient polymerization at acid pH.

[f]Added just before polymerization.

Table 6.5.2 Recipes for Phosphate Nondenaturing Polyacrylamide Gels[a]: pH Range 5.8 to 8.0

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× phosphate gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d,e]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× phosphate gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.
[d]Must be freshly made.
[e]Added just before polymerization.

the separation conditions (i.e., if gel pH < protein pI, the protein will have a net positive charge; if gel pH > protein pI, the protein will be negatively charged).

3. Pour gel to 2 cm from the top of the gel mold and insert the comb. Avoid trapping air bubbles under the comb teeth.

   Air bubbles will cause small semicircular depressions in the well and lead to distortions in the protein banding pattern.

4. Allow gel solution to polymerize 1 to 2 hr.

   Polymerization is indicated by a sharp optical discontinuity around the wells.

Prepare samples and load the wells

5. Solubilize the protein sample to be analyzed using 5% (w/v) sucrose in water or dilute (1 to 5 mM) gel buffer if possible. Also prepare native protein standards.

   The concentration of protein will vary depending on the sample complexity and detection method. For Coomassie blue staining of highly enriched samples such as the standards, use 1 to 2 mg/ml (1 to 2 μg/μl). For more complex mixtures, use 5 to 10 mg/ml (5 to 10 μg/μl). Load 10- to 100-fold less for silver staining. In general, samples should be loaded in a minimum volume, preferably 10 to 20 μl for 0.75- and 1.5-mm-thick gels, respectively. With thin gels, this means using a more concentrated protein sample.

6. Remove comb carefully and rinse wells with electrophoresis buffer (appropriate 4× gel buffer diluted to 1×).

   Rinsing with electrophoresis buffer is needed to remove residual unpolymerized acrylamide monomer, which will continue to polymerize after comb removal, creating uneven wells that may interfere with sample loading.

Table 6.5.3 Recipes for Tris Nondenaturing Polyacrylamide Gels[a]: pH Range 7.1 to 8.9

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× Tris gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d,e]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× Tris gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[d]Must be freshly made.

[e]Added just before polymerization.

7. Fill wells with electrophoresis buffer. If desired, prerun gel.

The gel can be prerun at this point to remove any charged material such as ammonium persulfate from the gel prior to loading the sample. Assemble the electrophoresis unit and fill the buffer chambers with electrophoresis buffer. Run the gel at 300 V until the current no longer drops. This should take ~30 min. Disassemble the unit, discard the buffer, and proceed to the next step.

8. Carefully load up to 10 µl (0.75-mm gels) or 20 µl (1.5-mm gels) sample per lane as a thin layer at the bottom of the wells. Load control wells with native protein standards. Add an equal volume of electrophoresis buffer to any empty wells to prevent spreading of adjoining lanes.

Mobility (R_f) markers require special consideration in nondenaturing gel systems. For cationic systems, cytochrome c (pI ~9 to 10, 5 to 10 µg/lane) works well as an R_f marker. Bromphenol blue (10 µg/ml) is a suitable marker for anionic systems. The marker should be included in the solubilization buffer with the sample.

Perform the separation

9. Assemble the gel unit, fill the upper and lower buffer chambers with electrophoresis buffer, and connect the unit to the power supply. Set current to 30 mA for a 1.5-mm-thick gel (15 mA for a 0.75-mm-thick gel).

If the protein is negatively charged under the separation conditions, then the standard SDS-PAGE electrode polarity should be used (proteins will migrate to the anode or positive electrode; see UNIT 6.1). If the protein is positively charged, then the electrodes should be reversed at the power supply (i.e., red high-voltage cable to the black output and black high-voltage lead to the red output) so the positively charged protein migrates to the negative cathode.

Table 6.5.4 Recipes for Glycine Nondenaturing Polyacrylamide Gels[a]: pH Range 8.6 to 10.6

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× glycine gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d,e]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× glycine gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[d]Must be freshly made.

[e]Added just before polymerization.

10. Continue electrophoresis until the R_f marker reaches the bottom of the gel.

    For minigels, electrophoresis will take 1 to 2 hr. Standard gels require 4 to 6 hr runs.

11. Turn off power supply, disassemble the unit, and remove gel from sandwich.

12. Stain the gel according to *APPENDIX 3*.

NATIVE DISCONTINUOUS ELECTROPHORESIS AND GENERATION OF MOLECULAR WEIGHT STANDARD CURVES (FERGUSON PLOTS)

ALTERNATE PROTOCOL

One straightforward approach to discontinuous native electrophoresis is to leave out the SDS and reducing agent (DTT) from the standard Laemmli SDS-PAGE protocol (*UNIT 6.1*). The gels are prepared as described in *UNIT 6.5* except that the sample buffer contains no SDS or DTT (samples are not heated), and the gel and electrophoresis solutions are prepared without SDS. This protocol illustrates the separation of standard proteins at four different concentrations of acrylamide and how the results are used to construct a molecular weight standard curve (Ferguson plot) without the need for SDS. By plotting relative mobility against %T (percentage weight per volume of acrylamide plus bisacrylamide in the gel), the presence of isoforms and multimeric proteins can also be detected.

Materials

4× Tris·Cl, pH 8.8 (1.5 M Tris·Cl; *APPENDIX 2A*)
4× Tris·Cl, pH 6.8 (0.5 M Tris·Cl)
Protein sample of interest
2× Tris/glycerol sample buffer (see recipe)

Native protein standards (e.g., Sigma nondenatured protein molecular weight kit)
Tris/glycine electrophoresis buffer (see recipe)

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and place it in the casting stand.
2. Prepare and cast the gels, using 4× Tris·Cl, pH 8.8, for the separating gel and 4× Tris·Cl, pH 6.8, for the stacking gel instead of the SDS-containing counterparts (Table 6.1.1). Prepare a minimum of four separate gels at different acrylamide concentrations.

   A typical range of concentrations is from 5% to 12.5% (e.g., 5%, 7.5%, 10%, 12.5% acrylamide). As with SDS-PAGE, typical gel thickness ranges from 0.75 to 1.5 mm. The 0.75-mm-thick gels are recommended because they offer a combination of fast staining and high resolution.

3. Mix protein sample of interest 1:1 with 2× Tris/glycerol sample buffer to attain a 1 to 2 µg/µl final concentration. Also prepare native protein standards. Remove comb, rinse wells, and load 10 to 20 µl per well for Coomassie brilliant blue staining, and 1 to 2 µl for silver staining.

   Some proteins must be dissolved in 50 mM NaCl or water to become fully solubilized prior to mixing with the sample buffer (Sigma, 1986).

4. Assemble gel electrophoresis unit, using Tris/glycine electrophoresis buffer to fill both lower and upper buffer chambers. Connect power supply and conduct electrophoresis.

   Conditions for separation are the same as for discontinuous SDS-PAGE (i.e., 30 mA for 1.5-mm-thick gels, 15 mA for 0.75-mm-thick gels). For standard-size gels the separation takes 4 to 5 hr; for minigels, 1 to 2 hr is required. Alternatively, standard gels can be run at 4 to 6 mA/gel overnight.

5. After the bromphenol blue R_f marker has reached the bottom of the gel, fix and stain the proteins in the gels according to *APPENDIX 3*. Estimate relative mobilities of the proteins.

   An example of a stained gel is shown in Figure 6.5.1. A minimum of four gel concentrations is recommended. In Figure 6.5.1, Sigma native molecular weight standards were separated on 5%, 7.5%, 10%, and 12.5% acrylamide gels (5.1%, 7.7%, 10.3%, and 12.8% T, respectively).

6. Plot log R_f against gel concentration (% T) (Fig. 6.5.2). Determine the slope of K_r using linear regression.
7. Plot $-\log K_r$ of the curves from step 6 against log molecular weight of the standards (Fig. 6.5.3). Determine the slope using linear regression.
8. Estimate the size of the standards and unknowns from the generated curve (Ferguson plot).

   Use the curve generated by linear regression to estimate the predicted size of the standard for comparison to the actual size stated by the supplier. This indicates the accuracy of the curve. The $-\log K<D_r$ value (y) of the unknown is then used to predict the molecular weight (x).

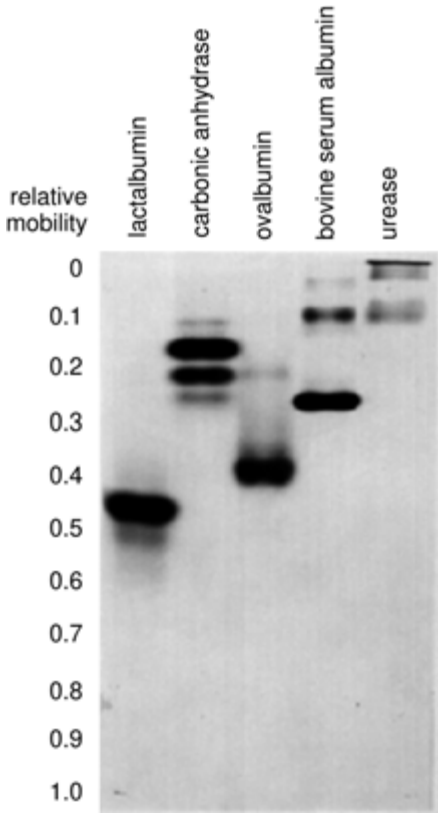


Figure 6.5.1 Separation of native protein standards under nondenaturing conditions by discontinuous polyacrylamide gel electrophoresis at 12.8% T. Approximately 20 μg protein was loaded per lane on a 1.5-mm-thick, 16-cm-long gel. The gel and samples were prepared according to the Alternate Protocol and were electrophoresed 16 hr at 6 mA. Proteins were stained with Coomassie blue.

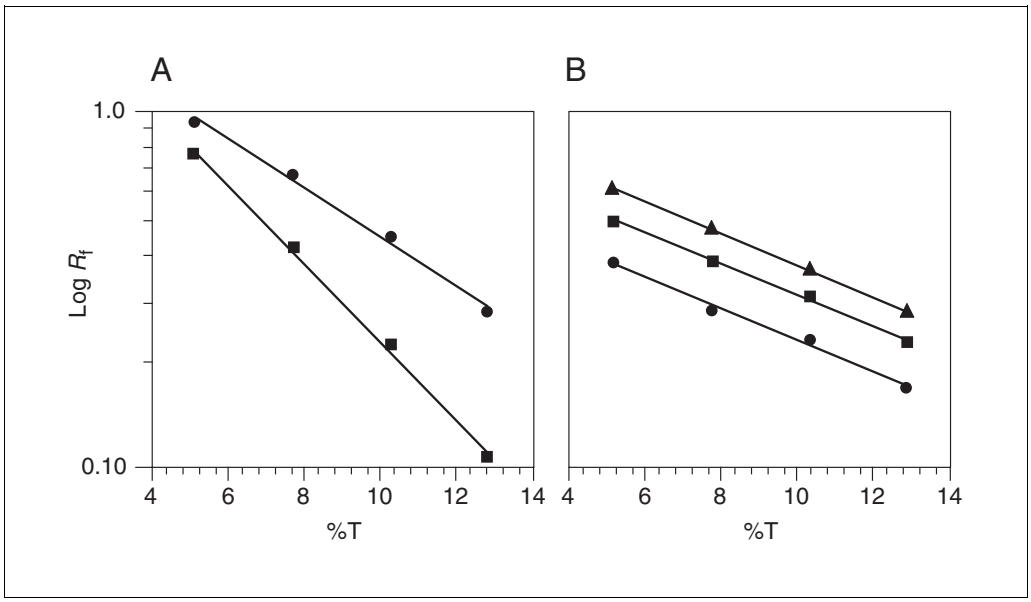


Figure 6.5.2 Effect of %T on the relative mobility of several native proteins. The relative mobility (R_f) of the standard proteins shown in Figure 6.5.1 was determined at four different gel concentrations and plotted as log R_f against %T. See text for details. (**A**) BSA monomer (squares) and dimer (circles); (**B**) carbonic anhydrase isoforms.

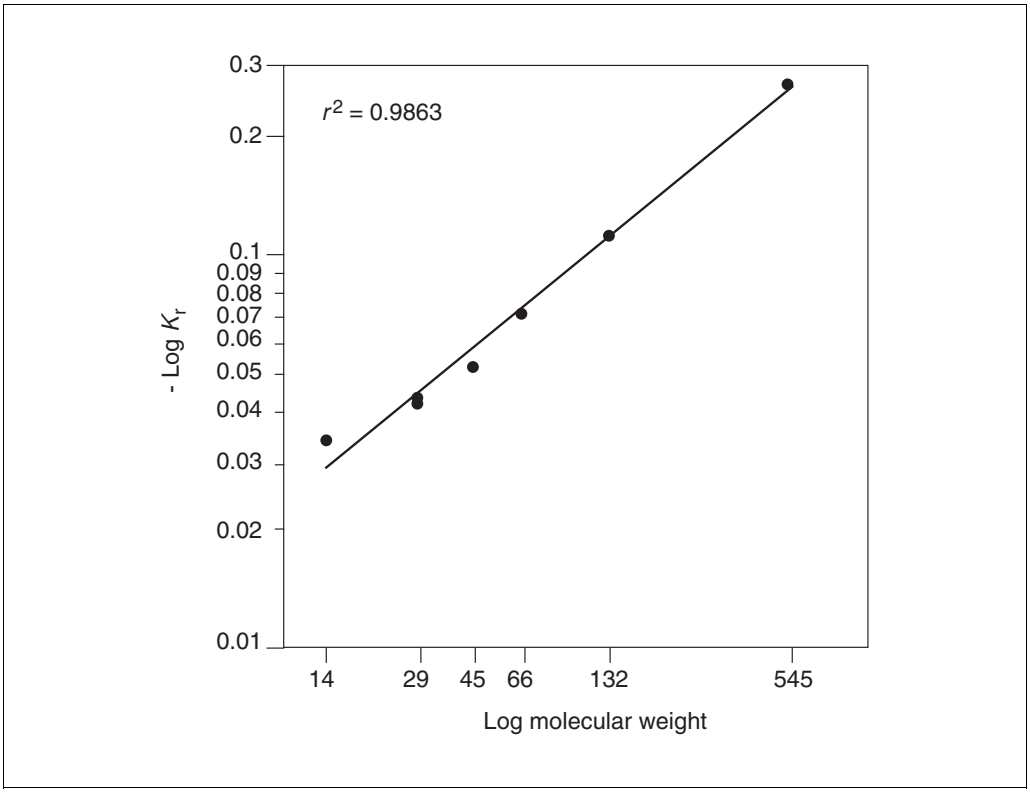


Figure 6.5.3 Native molecular weight standard curve. The –log slope of the line (K_r) from Figure 6.5.2 is plotted against log molecular weight of the standards.

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acetic acid gel buffer, 4× (200 mM acetic acid, pH 3.7 to 5.6)

11.49 ml glacial acetic acid
Add to 500 ml H_2O
Adjust to pH 3.7 to 5.6 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Glycine gel buffer, 4× (200 mM glycine, pH 8.6 to 10.6)

15.01 g glycine
Add to 500 ml H_2O
Adjust to pH 8.6 to 10.6 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Phosphate gel buffer, 4× (400 mM sodium phosphate, pH 5.8 to 8.0)

55.2 g $NaH_2PO_4 \cdot H_2O$
Add to 500 ml H_2O
Adjust to pH 5.8 to 8.0 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Tris gel buffer, 4× (200 mM Tris·Cl, pH 7.1 to 8.9)

24.23 g Tris base
Add to 500 ml H_2O
Adjust to pH 7.1 to 8.9 with 1 M HCl
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Tris/glycerol sample buffer, 2×

25 ml 0.5 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
20 ml glycerol
1 mg bromphenol blue
Add H_2O to 100 ml and mix
Store in 1-ml aliquots up to 6 months at −70°C

Tris/glycine electrophoresis buffer

15.1 g Tris base
72.0 g glycine
H_2O to 5000 ml
Store up to 1 month at 4°C

COMMENTARY

Background Information

Under nondenaturing conditions, in which protein activity, native charge, and conformation are sustained, electrophoretic separation depends on many factors, including size, shape, and charge. Characteristics such as intrinsic molecular weight (i.e., in the absence of denaturation), the number of isoforms, and the presence of multimeric proteins can be determined with nondenaturing electrophoresis (often called native electrophoresis).

The most important application of nondenaturing electrophoresis is in the determination of native protein size (see Alternate Protocol). Ferguson plots (reviewed by Andrews, 1986) were first described for starch gels (Ferguson, 1964) and then for polyacrylamide gels (Hedrick and Smith, 1968). Ferguson plots are prepared by separating proteins under nondenaturing conditions at several different gel concentrations. As the acrylamide concentration (%T) is increased, the relative mobility (R_f) of the protein decreases. This is plotted as log relative mobility (on the *y* axis) versus %T (on the *x* axis) to produce a straight line. The slope of this line is referred to as the retardation coefficient (K_r) and measures how effectively a protein is slowed by the increase in %T. Large proteins will be retarded much more significantly than small proteins with increasing gel concentration, with the size of the protein being proportional to the slope of the curve. Once the K_r plots for several size standards are generated (Fig. 6.5.2), the K_r values are plotted against the molecular weight of the standard proteins using a log-log graph (Fig. 6.5.3). The retardation coefficient also depends on a large number of other variables including temperature, pH, buffer type, ionic strength, and %C (percent bisacrylamide cross-linker). All these factors should be kept constant for a given experiment.

In addition to estimated size, other types of information are available from the Ferguson plots (Rodbard and Chrambach, 1971; Andrews, 1986). For example, if two components differ in size but have the same charge per unit size (e.g., for a multimeric protein with identical subunits), curves similar to those illustrated by BSA monomer and dimer (Fig. 6.5.2A) will result. Note that when the curve is extrapolated back to 0% T, it is evident that the monomer and the dimer have similar free solution mobilities. Furthermore, as the acrylamide concentration is increased, the separation between the two also increases. However, if two proteins have similar sizes but different amounts of charge, the curves will be parallel on the log plot. This is illustrated by the carbonic anhydrase isoforms (Fig. 6.5.2B). In this example, optimal separation of the isoforms occurs at the lower concentrations of acrylamide as this is a log plot.

Further applications of nondenaturing electrophoresis include preparative purification. The pH of the gel determines the net charge on the protein. Below its isoelectric point (pI) a protein will have a net positive charge, whereas above its pI it will have a net negative charge.

In general, most proteins will be positively charged at pH 2.0 to 4.0; above pH 8.0, most proteins will be negatively charged. As these general guidelines imply, the majority of proteins have isoelectric points between pH 4.0 and 8.0. There are, however, many exceptions. A protein with a highly acidic isoelectric point (e.g., pepsin, with a pI of 2.2) will remain negatively charged at a pH down to its pI. Although a full range of pH options are given, extremes of pH (<4.0 and >9.0) should be avoided, if possible, to minimize denaturation or inactivation. By picking an appropriate electrophoresis pH, it is possible to ensure that the protein of interest will be either positively or negatively charged so that it can be selectively run into the gel, excluding a large proportion of contaminants that have the opposite or no charge. Furthermore, the pH conditions determine the mobility and can be adjusted to ensure a difference in mobility between the protein of interest and contaminants.

Continuous gel systems (see Basic Protocol) offer the most flexibility in terms of separation design. The pH can be tailored so that a given protein has a net positive, neutral, or negative charge. Depending on the polarity of the gel, the protein can then be excluded from or electrophoresed into the gel. Discontinuous gels have a fixed pH and gel polarity. For the nondenaturing Laemmli gel presented in the Alternate Protocol, the proteins of interest should have an isoelectric point of ≤7.0 in order to be negatively charged so that they move into the gel. Other more basic and more acidic discontinuous gel systems can be found in Hames (1990) and Schägger (1994).

Critical Parameters

The success of a gel separation under nondenaturing conditions depends on many factors, and two of the most important are protein solubility and isoelectric point. The protein must be soluble at the pH and the ionic strength of the gel, and it must be charged at that pH in order to move into the gel. If the protein experiences a pH below its isoelectric point, then it will have a net positive charge and will move to the negative electrode. Note that this is the reverse of typical SDS-PAGE. If the protein experiences a pH above its isoelectric point, it will have a net negative charge and will migrate to the positive electrode.

Solubility is a complex issue. Membrane-associated and other hydrophobic proteins are difficult to separate by nondenaturing electrophoresis (Schägger, 1994). Nonionic detergents at concentrations up to 1% and solubilizing reagents such as urea (4 to 8 M) can be used, but these reagents, especially urea, are likely to alter the protein's conformation and most likely the isoelectric point by exposing previously hidden charged groups. If detergent or urea must be included for solubilization, the minimum required to solubilize the protein should be used. Schägger (1994) lists several nonionic detergents suitable for solubilization. Among the more popular are octylglucoside and CHAPS. In general, detergents should be used near the critical micelle concentration (CMC; 0.001% to 1%, depending on the detergent).

The gel concentration has a dramatic effect on resolution and should be optimized in order to achieve the best separation and band sharpness. In general, increasing the %T will improve band sharpness.

Troubleshooting

Gel polymerization at acid pH can be problematic, and sodium sulfite is needed for efficient polymerization (Andrews, 1986). Both the ammonium persulfate and the sodium sulfite must be freshly made, and the highest quality reagents available should be used. Furthermore, the gel solutions should be at room temperature for effective polymerization.

If the protein does not enter the gel and no stained material is present at the well surface, try reversing the polarity of the electrode. If material concentrates at the top of the gel, try lowering the acrylamide concentration. Stained material at the top of the gel may also indicate poor solubilization, and increasing the ionic strength of the solubilization buffer or adding a small amount of urea and/or nonionic detergent may be required.

Anticipated Results

Proteins will resolve depending on their solubility and native charge at the chosen pH. Ideally, a distinct band representing the protein of interest will be visible. If the band is diffuse, then increasing the gel concentration or using a gradient gel will improve resolution. If the band is not visible, then the protein may be at its isoelectric point or may have moved out of the gel because it had the wrong charge. Continuous gel systems, although more versatile, will give lower resolution than discontinuous gels. Detergents or other solubilizing agents such as urea may be needed to fully solubilize and resolve the protein. Once the conditions that resolve the protein are determined, Ferguson plots will give indications of multiple

subunit structure, native size, and potential isoform relationships.

Time Considerations

Separations will be complete when the tracking dye or protein reaches the bottom of the gel. For minigels, this generally takes 1 to 2 hr, using 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels require 4 to 5 hr at 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels can also run overnight at 4 to 6 or 8 to 12 mA for 0.75- or 1.5-mm-thick gels, respectively.

Literature Cited

Andrews, A.T. 1986. Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications, 2nd ed. Oxford University Press, New York.

Ferguson, K.A. 1964. Starch-gel electrophoresis—application to the classification of pituitary proteins and polypeptides. *Metabolism* 13:985-1002.

Hames, D. 1990. One-dimensional polyacrylamide gel electrophoresis. *In* Gel Electrophoresis of Proteins: A Practical Approach, 2nd ed. (B.D. Hames and D. Rickwood, eds.) pp. 1-147. Oxford University Press, New York.

Hedrick, J.L. and Smith, A.J. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.* 126:155-164.

Rodbard, D. and Chrambach, A. 1971. Estimation of molecular radius, free mobility, and valence using polyacrylamide gel electrophoresis. *Anal. Biochem.* 40:95-134.

Schägger, H. 1994. Native gel electrophoresis. *In* A Practical Guide to Membrane Protein Purification (G. Von Jagow and H. Schägger, eds.) pp. 81-104. Academic Press, San Diego.

Sigma. 1986. Nondenatured protein molecular weight marker kit (Technical Bulletin No. MKR-137). Sigma Chemical Company, St. Louis, Mo.

Key Reference

Andrews, 1986. See above.

Covers a variety of electrophoretic techniques, including nondenaturing electrophoresis and Ferguson plots.

Contributed by Sean R. Gallagher
Motorola Corporation BioChip Systems
Tempe, Arizona

Staining Proteins in Gels

Polyacrylamide gel electrophoresis (PAGE) is a powerful method for the resolution of protein mixtures according to the proteins' charge-to-mass ratio (nondenaturing-PAGE; *UNIT 6.5*), mass (SDS-PAGE; *UNIT 6.1*), isoelectric point (isoelectric focusing) or combinations of these properties (two-dimensional PAGE; *UNIT 6.4*). A number of protocols have been developed to visualize the separated protein bands following electrophoresis. In this unit, four different methods, which are widely used in cell biology laboratories, are described. These are: staining with the dye Coomassie blue (Basic Protocol 1 and Alternate Protocol 1), silver staining (Basic Protocol 2), fluorescent staining with SYPRO Ruby (Basic Protocol 3), and negative staining with zinc (Basic Protocol 4).

Staining with Coomassie blue (Basic Protocol 1) is a relatively simple and very popular method, albeit less sensitive than other procedures. A variation of this protocol, suitable for staining proteins in isoelectric focusing gels, is described under Alternate Protocol 1. Staining with silver (Basic Protocol 2) is probably the most sensitive method, although it is time-consuming and incompatible with some downstream applications (see Commentary). The remaining two methods (Basic Protocols 3 and 4) are more sensitive that Coomassie blue staining and recommended for downstream applications where protein fixation needs to be avoided (e.g., analysis of biological activity). Some of the protocols can be combined. For instance, some researchers prefer to stain gels first with Coomassie blue (Basic Protocol 1) or zinc (Basic Protocol 4) and, if higher sensitivity is necessary, subsequently with silver (Basic Protocol 2). Further details on the differences between the methods, as well as guidelines for their selection according to specific applications, are discussed in the Commentary.

In the following protocols, the volumes of solutions are indicated in "gel volumes." When staining slab mini-gels, 10 gel vol correspond to ~50 to 80 ml.

STAINING PROTEIN GELS WITH COOMASSIE BLUE

BASIC PROTOCOL 1

Coomassie brilliant blue R-250, a triphenylmethane anionic dye, binds avidly to almost all proteins in either native or denatured states and is widely used for detection of proteins in polyacrylamide gels. In this protocol, proteins separated by either nondenaturing- or SDS-PAGE (one- or two-dimensional) are fixed and stained by soaking the gel in a Coomassie blue staining solution; the unbound dye is subsequently removed by washing with destaining solution to yield blue protein bands on a clear background.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNIT 6.1*)
Coomassie blue staining solution (see recipe)
Destaining solution: 30% (v/v) methanol/10% (v/v) acetic acid in distilled water (store up to 4 months at room temperature)
Storage solution: 7% (v/v) acetic acid/5% (v/v) methanol in distilled water (store up to 4 months at room temperature)
Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Platform shaker (optional)

CAUTION: Glacial acetic acid and methanol are volatile and toxic. The destaining and storage solutions should be prepared in a chemical fume hood, and gloves should be worn throughout the staining procedure.

Contributed by Esteban C. Dell'Angelica and Juan S. Bonifacino
Current Protocols in Cell Biology (2000) 6.6.1-6.6.14

NOTE: All steps should be performed at room temperature.

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of Coomassie blue staining solution.
2. Incubate with gentle agitation for ≥20 min for a gel ≤1 mm thick or ≥1 hr for a gel >1 mm thick.

   The use of a platform shaker is recommended. Most proteins are fixed due to the presence of methanol in the staining solution; gels can therefore be left in staining solution for many hours (i.e., overnight) without any adverse effect, unless the protein(s) of interest is very small (<5 kDa) and can be lost by diffusion due to incomplete fixation.
3. Remove the Coomassie blue staining solution and rinse the gel briefly with distilled water.

   The Coomassie blue staining solution can be reused several times.
4. Add ~10 gel vol of destaining solution and incubate with gentle agitation until the solution becomes as dark as the gel matrix.
5. Discard the destaining solution.
6. Repeat steps 4 and 5 several times until a clear background is obtained.

   Both the total amount of destaining solution and the destaining time can be reduced by placing a piece of adsorbent material with affinity for the Coomassie dye (e.g., Whatman 3MM filter paper) inside the container having the gel in destaining solution.

   NOTE: *Avoid excessive incubation of the gel with destaining solution as it can result in decreased sensitivity due to dissociation of protein-dye complexes.*
7. Add ~10 gel vol storage solution and incubate for 10 to 15 min with gentle agitation.

   At this point, the gel can be stored at 4°C for several months in a plastic container or sealed plastic bag containing storage solution. Alternatively, it can be soaked in 2% (v/v) glycerol for 15 to 30 min, placed onto Whatman 3MM filter paper, and subsequently dried on a vacuum system.

ALTERNATE PROTOCOL 1

STAINING PROTEIN GELS WITH COOMASSIE BLUE AFTER ISOELECTRIC FOCUSING

In polyacrylamide gel isoelectric focusing, proteins are resolved based on their isoelectric points on a pH gradient generated by a mixture of ampholytes. Many commercially available ampholytes bind Coomassie blue and, therefore, interfere with protein detection. To overcome this problem, the following procedure involves treatment with trichloroacetic acid (TCA) to fix proteins while removing ampholytes and uses $CuSO_4$ in the staining solution to help reduce the background (Righetti and Drysdale, 1974).

Additional Materials *(see also Basic Protocol 1)*

Polyacrylamide isoelectric focusing gel with protein(s) of interest (e.g., *UNITS 6.4 & 15.4*)
20% (w/v) TCA solution in water
IEF Coomassie blue stock solution (see recipe)
10% (w/v) $CuSO_4$ in distilled water (store for up to 4 months at room temperature)

CAUTION: TCA is extremely caustic. Protect eyes and wear gloves when preparing and handling TCA solutions.

1. Make fresh staining solution by mixing 90 ml of destaining solution, 10 ml of IEF Coomassie blue stock solution and 1 ml of 10% (w/v) $CuSO_4$ in distilled water.
2. Remove polyacrylamide gel from isoelectric focusing assembly and place it in a plastic container with lid containing 5 to 10 gel vol of 20% (w/v) TCA solution in water.
3. Incubate for 30 to 60 min with gentle agitation.

   The use of a platform shaker is recommended. In this step, proteins are selectively fixed while ampholytes are removed from the gel.
4. Remove the TCA solution and add ~10 gel vol destaining solution.

   Do not reuse the TCA solution; dispose of it following applicable safety regulations for chemical waste. In this step, the TCA is removed from the gel.
5. Incubate 15 min with gentle agitation.
6. Discard the destaining solution and add freshly prepared staining solution (from step 1).
7. Incubate with gentle agitation for ≥20 min for a gel ≤1 mm thick or ≥1 hr for a gel >1 mm thick.
8. Remove the staining solution and rinse the gel briefly with distilled water.

   Do not reuse the staining solution.
9. Destain the gel (see Basic Protocol 1, steps 4 to 7).

STAINING PROTEIN GELS WITH SILVER

BASIC PROTOCOL 2

Silver staining is based on the selective reduction of silver ions at sites of the gel that contain proteins and other macromolecules. The following protocol is adapted from that of Blum et al. (1987), and involves pretreatment of a fixed polyacrylamide gel with thiosulfate (step 6) followed by impregnation with silver nitrate (step 9) and color development with formaldehyde at a high pH (steps 12 and 13). Pretreatment with thiosulfate significantly increases the sensitivity and improves the contrast of protein staining. In addition, the presence of low concentrations of thiosulfate during color development reduces nonspecific staining of the gel surface. The procedure is applicable for both nondenaturing- (*UNIT 6.5*) and SDS-PAGE (one- or two-dimensional; *UNIT 6.1* and *UNIT 6.4*) and yields dark (brown to black) protein bands on a clear background.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNITS 6.1, 6.4 & 6.5*), either unfixed or fixed and stained with Coomassie blue (see Basic Protocol 1 and Alternate Protocol 1)
Deionized water (HPLC grade or Milli-Q)
50% (v/v) ethanol in deionized water
Fixative solution (see recipe)
Thiosulfate solution (see recipe)
Silver nitrate solution (see recipe)
Developer solution (see recipe)
50% (v/v) methanol/12% (v/v) acetic acid in deionized water
50% (v/v) methanol in water

continued

Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Clean plastic containers (at least six; lids of pipet tip containers are appropriate for staining mini-gels)
Aluminum foil
Platform shaker (optional)

CAUTION: Glacial acetic acid and methanol are volatile and toxic; solutions containing these solvents should be prepared in a chemical fume hood and handled with care.

NOTE: All solutions should be prepared in deionized water (Milli-Q or HPLC grade). At all times wear gloves that have been rinsed extensively with distilled water. Handle the gel by using clean forceps having blunt tips or by touching the corners with clean, powder-free gloves. All steps should be performed at room temperature.

Fix the gel

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of fixative solution.

   Steps 1 and 2 are for unfixed gels only. If the polyacrylamide gel was previously fixed and/or stained with Coomassie blue (Basic Protocol 1 or Alternative Protocol 1), proceed directly to step 3.

2. Incubate for ≥30 min with gentle agitation.

   The use of a platform shaker is recommended.

Pretreat the gel

3. Transfer the gel to a clean plastic container having ~10 gel vol of 50% ethanol in deionized water. Incubate 20 min with gentle agitation.
4. Replace the solution with another ~10 gel vol of 50% (v/v) ethanol in deionized water. Incubate 20 min with gentle agitation. Repeat this step once (total of three pretreatment steps).

   The plastic container used in steps 3 and 4 can be reused in step 16. Discard the ethanol solution and rinse the container briefly with deionized water.

5. Transfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate 5 to 10 min with gentle agitation.
6. Discard the water and add ~10 gel vol thiosulfate solution. Incubate for exactly 1 min with gentle agitation.

   Incubations longer than 1 min will result in increased background staining.

7. Tranfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate exactly 20 sec.
8. Change the water in the container and incubate exactly 20 sec. Perform this step twice (for a total of three).

   The plastic container used in steps 7 and 8 can be reused in steps 10 and 11 but should first be rinsed thoroughly with deionized water.

Stain the gel

9. Transfer the gel to a clean plastic container containing 5 to 10 gel vol silver nitrate solution. Cover the container with aluminum foil and incubate 20 min with gentle agitation.

   Light exposure causes the reduction of silver ions and may therefore result in increased background staining.

10. Transfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate exactly 20 sec.

11. Change the water in the container and incubate again exactly 20 sec.

The plastic container used in steps 10 and 11 can be reused in steps 14 and 15 but should first be rinsed with deionized water.

Develop the gel stain

12. Transfer the gel to a clean plastic container having ~10 gel vol developer solution.

13. Incubate with gentle agitation until the protein band(s) of interest becomes visible (or until the gel matrix begins to get too dark).

This step lasts a few minutes (≤10 min) and requires continuous visual inspection as the protein bands may develop rapidly, and long incubations may result in high background staining. Since some developing will still occur during steps 14 and 15, it is advisable to proceed to the next step as soon as the staining is judged to be optimal.

14. Transfer the gel to a clean plastic container having ~10 gel vol deionized water. Incubate 30 sec.

15. Change the water in the container and incubate an additional 30 sec.

16. Transfer the gel to a clean plastic container having ~10 gel vol 50% methanol/12% acetic acid in deionized water. Incubate 10 min with gentle agitation.

17. Remove the solution from the container and add ~10 gel vol of 50% methanol.

The gel can be stored in this solution for several months at 4°C. Alternatively, the gel can be rehydrated by soaking in water for 5 to 10 min, soaked in 2% (v/v) glycerol for another 15 to 30 min, placed onto Whatman 3MM filter paper, and then dried on a vacuum system.

FLUORESCENCE DETECTION OF PROTEINS IN GELS

BASIC PROTOCOL 3

In this method, proteins separated on polyacrylamide gels [either nondenaturing- (*UNIT 6.5*) or SDS-PAGE, one- or two-dimensional (*UNIT 6.1* or *UNIT 6.4*)] are incubated with SYPRO Ruby, a commercially available fluorescent compound that interacts specifically with proteins. Following incubation, protein bands can be readily visualized using standard 300-nm transillumination.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNIT 6.1*, *UNIT 6.4*, or *UNIT 6.5*)
SYPRO Ruby protein gel stain (Molecular Probes)
Distilled water

Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Platform shaker (optional)
300-nm UV transilluminator
Photographic camera or CCD camera (optional)

CAUTION: The potential toxicity of the SYPRO Ruby dye, which comprises an organic component and ruthenium, has not been fully evaluated. Gloves should be worn throughout the staining procedure. For disposal, the stain solution should be poured through activated charcoal, and the dye adsorbed to activated charcoal destroyed in a chemical incinerator following local environmental regulations.

NOTE: Protect SYPRO Ruby protein gel stain from light. If the plastic container with lid used for staining is transparent, cover it completely with aluminum foil.

NOTE: All steps should be performed at room temperature.

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of SYPRO Ruby protein gel stain.
2. Incubate 3 hr with gentle agitation.

   The use of a platform shaker is recommended.
3. Remove the SYPRO Ruby protein gel stain and rinse the gel briefly with distilled water.

   Do not reuse the stain solution. Follow local recommendations for the disposal of potentially toxic organic compounds.
4. Add ~10 gel vol distilled water and incubate 10 min with gentle agitation.
5. Change the distilled water in the container and incubate another 10 min with gentle agitation.
6. Visualize protein bands by fluorescence on a 300-nm UV transillumination unit.

   Images can be acquired using a photographic camera with appropriate film (e.g., Polaroid 667 black-and-white print film) or a CCD camera.

   CAUTION: *Use UV-rated eye protection to avoid direct exposure of the eyes to the UV light.*

   At this point, the gel is ready for downstream applications (e.g., electroelution and immunoblotting).

BASIC PROTOCOL 4

REVERSIBLE PROTEIN STAINING WITH ZINC

In this method, protein bands separated on SDS-PAGE gels [one- or two-dimensional (*UNIT 6.1* or *UNIT 6.4*)] are visualized as clear spots on an opaque white background, the latter being generated by precipitation of SDS with zinc ions. This negative staining procedure is sensitive, fast, and completely reversible upon removal of zinc with a chelating agent.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNIT 6.1*)
GelCode E-Zinc Reversible Stain Kit (Pierce), containing:
  E-Zinc Stain
  E-Zinc Developer
  E-Zinc Eraser
Distilled water
Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Platform shaker (optional)

NOTE: All steps should be performed at room temperature.

Stain the gel

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing ~10 gel vol E-Zinc Stain solution.
2. Incubate 10 min with gentle agitation.

   The use of a platform shaker is recommended.

3. Remove the E-Zinc Stain solution from the container and replace it with ~10 gel vol E-Zinc Developer solution.

   Do not reuse the stain solution.

4. Incubate 1 to 2 min with gentle agitation. Proceed to the next step as soon as the staining is judged to be optimal.

5. Remove the E-Zinc Developer solution from the container and replace it with ~10 gel vol distilled water.

   Do not reuse the developer solution.

Wash the gel

6. Incubate 1 min with gentle agitation.

7. Change the water in the container.

8. Visualize the protein bands by placing the polyacrylamide gel on a dark (black or blue) surface.

   The stained gel can be kept in water for several weeks at 4°C.

Solubilize the SDS/zinc precipitate (optional)

9. Incubate the gel with ~10 gel vol E-Zinc Eraser with gentle agitation until the gel matrix is completely clear (~5 to 10 min).

   These two steps involve solubilization of the SDS/zinc precipitate to completely destain the gel, thus allowing further analysis of the protein band(s) of interest.

10. Rinse the gel with distilled water.

    At this point, the gel is ready for downstream applications (e.g., electroelution, immunoblotting, etc.).

REAGENTS AND SOLUTIONS

*Use HPLC-grade or Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Coomassie blue staining solution

For 100 ml: Dissolve 0.1 g Coomassie brilliant blue R-250 in a mixture of 30 ml methanol and 20 ml water. Subsequently, add 40 ml of water and 10 ml of glacial acetic acid. Store for up to 4 months at room temperature.

CAUTION: *Glacial acetic acid and methanol are volatile and toxic and should be handled in a chemical fume hood.*

This solution is 0.1% (w/v) Coomassie brilliant blue R-250 in methanol/acetic acid/water (3:1:6 v/v/v).

Developer solution

Dissolve 6 g Na_2CO_3 in 98 ml water. Add 50 µl of 37% formaldehyde and 2 ml of thiosulfate solution (see recipe). Prepare fresh before use.

CAUTION: *Formaldehyde is toxic and carcinogenic. Wear gloves and handle the concentrated (37%) solution in a chemical hood.*

Fixative solution

Mix 50 ml of methanol with 12 ml glacial acetic acid and add water to 100 ml. Add 50 µl of 37% formaldehyde. Prepare fresh before use.

CAUTION: *Glacial acetic acid and methanol are volatile and toxic. Formaldehyde is toxic and carcinogenic. Wear gloves and prepare the solution in a chemical fume hood.*

IEF Coomassie blue stock solution

Dissolve 0.2% (w/v) Coomassie brilliant blue R-250 and 60% (v/v) methanol in water. Store for up to 4 months at room temperature.

Silver nitrate solution

Dissolve 0.2 g $AgNO_3$ in 100 ml water. Add 75 µl of 37% formaldehyde. Protect the solution from light. Prepare fresh before use.

CAUTION: *Formaldehyde is toxic and carcinogenic. Wear gloves and handle the concentrated (37%) solution in a chemical fume hood.*

Discard the solution if it becomes cloudy.

Thiosulfate solution

Dissolve 20 mg $Na_2SO_4 \cdot 5H_2O$ in 100 ml water. Prepare fresh before use.

COMMENTARY

Background Information

The first protocols developed for the visualization of proteins bands after electrophoresis in polyacrylamide or other gel matrices relied on the use of dyes with selective avidity for polypeptides. Among these dyes, which included amido black 10B and fast green FCF, the Coomassie blue dyes gave higher sensitivity (Wilson, 1979) and became routinely used. A large number of staining protocols using Coomassie brilliant blue R-250 as a dye have been published (see Neuhoff et al., 1985, and references therein). The protocol described in this unit (Basic Protocol 1) involves simultaneous protein fixation and staining in a solution containing the dye in methanol/acetic acid/water, followed by removal of the unbound dye by washing with methanol/acetic acid/water. This is the most commonly used protocol for protein staining in gels. Proposed variations to this procedure include using no methanol to retain small polypeptides (Schägger and von Jagow, 1987), decreasing the concentrations of both methanol and acetic acid to limit protein fixation (Rosenfeld et al., 1992), and using ion-pairing agents to reduce background (Choi et al., 1996). Protocols using a colloidal form of Coomassie brilliant blue G-250, which rival in sensitivity those using Coomassie brilliant blue R-250, have also been described (see for example: Neuhoff et al., 1985; DeSilva, 1995).

A dramatic increase in sensitivity was achieved with the introduction of protein staining methods based on the selective reduction of silver ions to form metallic silver images (Switzer et al., 1979). The method relies on the autocatalytic reduction of silver, a key phenomenon of the photographic process, and differences in reduction potential of silver at the sites occupied by macromolecules relative to that at the rest of the gel matrix. The differences in reduction potentials can be manipulated to obtain either a positive or negative image of protein bands. Positive silver staining protocols comprise the following stages: (1) fixation of proteins and elimination of interfering substances (e.g., amino acids, Tris, SDS), (2) sensitization with an agent to increase sensitivity and/or contrast, (3) impregnation with silver solution (silver nitrate, basic silver ammonia or silver-diamine complex), (4) controlled rinse to remove silver ions not associated with proteins, (5) image development, and (6) image development stopping (reviewed by Rabilloud et al., 1994). The procedure described in Basic Protocol 2 (Blum et al., 1987) involves sensitization with thiosulfate, impregnation with silver nitrate, and image development using a dilute formaldehyde solution at high pH. Small amounts of thiosulfate included in the developer solution help to keep the background low by complexing silver ions that otherwise could form spurious silver deposits in the gel matrix. This procedure is highly sensitive and compatible with virtually all types of PAGE systems. Unlike other silver staining protocols, which yield a mixture of positive and negative bands or different band colors, the images of the protein bands are rather uniform. A drawback of this and other silver staining methods is that they can also stain other macromolecules, namely DNA, RNA and bacterial lipopolysaccharides.

Procedures for the fluorescent staining of proteins in polyacrylamide gels include covalent modification with fluorescamine or fluorescein isothiocyanate, and noncovalent staining with the hydrophobic probes 1-anilinonaphthalene-8-sulfonic acid or nile red (see

Steinberg et al., 1996, and references therein). A family of fluorescent protein stain reagents, including SYPRO Orange, SYPRO Red, and SYPRO Ruby, has recently been introduced and reported to be highly sensitive and protein specific (Steinberg et al., 1996). These reagents detect protein-SDS complexes, rather than protein functional groups, and are compatible with most downstream applications. Known interferences are colored stains or prosthetic groups, Triton X-100, and the excess of SDS at the migration front. A simple protocol for protein staining with SYPRO Ruby, which is commercially available and said to be the most sensitive dye within the SYPRO family, is described in Basic Protocol 3.

A different group of protein staining methods for SDS-PAGE are based on the reversible formation of SDS precipitates, thus allowing visualization of protein bands as clear areas on an opaque background. Although SDS precipitation can be simply achieved by lowering the temperature of the gel to 0° to 4°C for a few hours, faster and more sensitive methods involve the formation of insoluble complexes with heavy metal salts such as copper (Garfin, 1990) or zinc (Fernández-Patron et al., 1995a). A protocol for rapid, reversible staining with zinc is described in Basic Protocol 4. The protocol uses a kit that is commercially available at a reasonable price. If cost is a major issue, however, the reader is referred to the protocol described by Fernández-Patron et al. (1995a).

Additional staining methods not discussed in this unit include radiolabeling with [^{35}S]thiourea and silver bromide (Wallace and Saluz, 1992), combined Coomassie blue/zinc staining (Fernández-Patron et al., 1995b) and mixed Evans blue/rhodamine-B staining (Na et al., 1994).

Critical Parameters

Comparison of staining procedures

None of the staining methods is optimal for all types of applications. The choice of the most appropriate method for a particular experiment will depend on a number of considerations, including: sensitivity, simplicity, duration, long-term documentation, quantitation, and downstream applications. The following guidelines are restricted to the procedures described in this unit, namely staining with Coomassie blue (Basic Protocol 1 and Alternate Protocol 1), silver (Basic Protocol 2), SYPRO Ruby (Basic Protocol 3) and zinc (Basic Protocol 4).

Sensitivity

Figure 6.6.1 shows a comparative experiment in which serial dilutions of protein standards were run on SDS-PAGE (*UNIT 6.1*) and subsequently stained following the four Basic Protocols described in this unit. Among the four methods, silver (Fig. 6.6.1B) and Coomassie blue staining (Fig. 6.6.1A) gave the highest and lowest sensitivity, respectively. The detection limit of fluorescent staining with SYPRO Ruby could be greatly enhanced by the integration effect of a CCD or photographic camera. Thus, while by direct observation (on a UV transillumination unit) SYPRO Ruby staining was significantly less sensitive than negative zinc staining, both methods gave comparable sensitivities upon the use of a CCD camera to integrate the signal of SYPRO Ruby-stained bands (Fig. 6.6.1C and D).

Simplicity

While staining with Coomassie blue, SYPRO Ruby, or zinc are relatively simple methods, silver staining involves a number of steps that need to be carefully controlled, most notably sensitization with thiosulfate (Basic Protocol 2, step 6), rinsing with water before and after silver impregnation (Basic Protocol 2, steps 7 to 8 and 10 to 11, respectively) and image development (Basic Protocol 2, step 13). In fact, the duration of the image development step critically influences the sensitivity of silver staining, thus resulting in experiment-to-experiment variations in band color intensity. Staining with SYPRO Ruby requires a UV transilluminator to visualize the protein bands and the careful disposal of this potentially toxic dye (see Basic Protocol 3).

Documentation

Gels stained with Coomassie blue, silver, or zinc can be stored at 4°C for several weeks without significant decrease in sensitivity. For long-term storage, gels stained with Coomassie blue, silver, or SYPRO Ruby can be equilibrated in 2% (w/v) glycerol and dried on a gel dryer, although in the case of SYPRO Ruby–stained gels this can result in decreased sensitivity. For photography or digital image acquisition, the blue bands of Coomassie blue-stained gels have less contrast than the dark brown/black bands of silver-stained gels, and SYPRO Ruby–stained gels require the use of an appropriate photographic film (e.g., Polaroid 667 black and white film) or a sensitive CCD camera. Negatively zinc-stained gels can

be problematic for photography or imaging on a CCD camera.

Quantitative analysis

Scanning densitometric analyses of protein bands from stained polyacrylamide gels have been used to determine the relative abundance of proteins in complex mixtures, the purity of protein samples, and even the stoichiometry of multisubunit protein complexes. These quantitative analyses are performed assuming identical staining properties of the different proteins in the sample (i.e., that their staining results in the same band intensity per protein mass unit). This assumption is not necessarily correct, especially for Coomassie blue or silver staining where band intensities are known to be influenced by the proteins' amino acid compositions. Another limitation is that measurements should be made only within a linear range. Staining with SYPRO dyes, which interact with the protein-SDS complex rather than protein functional groups, has been reported to give relatively less protein-to-protein variability and

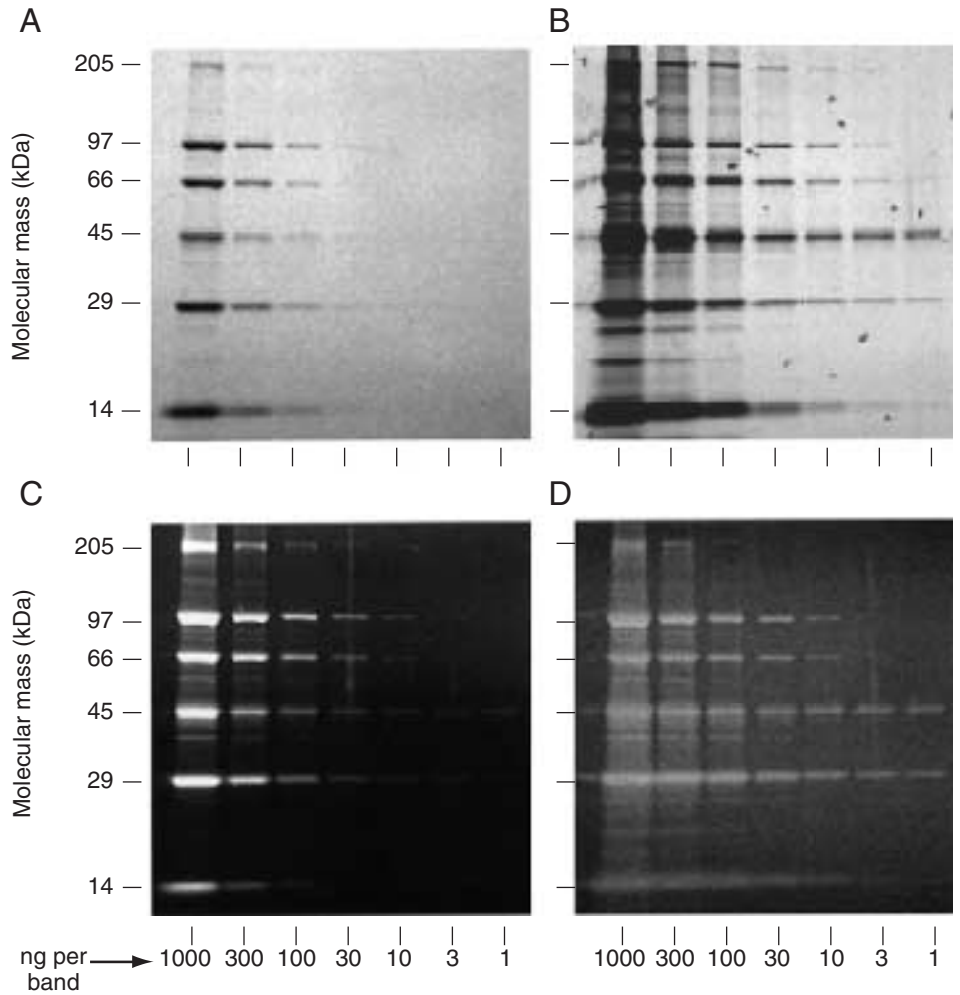


Figure 6.6.1 Comparison of the protein staining methods described in this unit. Serial dilutions of a mixture of rabbit muscle myosin (~205 kDa), rabbit muscle phosphorylase b (~97 kDa), bovine serum albumin (~66 kDa), chicken ovalbumin (~45 kDa), bovine erythrocyte carbonic anhydrase (~29 kDa) and chicken egg lysozyme (~14 kDa) were resolved by SDS-PAGE on a 4% to 20% T gradient gel and stained with: (**A**) Coomassie blue (Basic Protocol 1), (**B**) silver (Basic Protocol 2), (**C**) SYPRO Ruby (Basic Protocol 3), or (**D**) zinc (Basic Protocol 4). The amounts (in nanograms) per band loaded on each lane are indicated at the bottom of the figure. Images of stained gels were acquired on a Bio-Rad Gel Doc 1000 gel documentation system, using a standard UV (300 nm) transilluminator with (**A**, **B**, **D**) or without (**C**) a UV/white light conversion screen (Bio-Rad). The image shown in (**C**) was acquired using an integration time of 0.6 sec in order to optimize sensitivity.

Table 6.6.1 Troubleshooting Guide for Staining with Coomassie Blue (Basic Protocol 1)

Problem	Cause	Solution
No bands are detected		
Background is completely clear	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
Background is dark blue	Insufficient destaining	Continue to destain the gel until the background is clear (steps 4 to 6).
Protein bands are too faint		
	Staining solution is too old (i.e., methanol has evaporated)	Prepare new Coomassie blue staining solution.
	Insufficient staining time	Re-stain the gel using a longer staining time (step 2)
	Excessive destaining	Re-stain the gel monitoring band and background color during destaining (steps 4 to 6)
High background areas		
High background restricted to lanes with samples	Interfering compounds in the samples	Fix proteins with TCA before staining (see Alternate Protocol 1, steps 2 to 5).
Blue spots at the gel surface	Powder or dirt deposited on the surface during handling of the gel	Use clean, powder-free gloves. Remove powder or dirt by gently touching the surface with a powder-free glove.

Table 6.6.2 Troubleshooting Guide for Staining with Silver (Basic Protocol 2)

Problem	Cause	Solution
Protein bands are absent or too faint		
Background is clear	Amount of protein(s) below detection limit	Check protein concentration in original sample.
	Insufficient image development	Re-stain the gel extending the image development step (step 13) until background begins to get too dark.
	Formaldehyde is too old (white polymers in concentrated solution)	Use formaldehyde from a fresh concentrated solution.
	Silver nitrate solution is not fresh	Prepare fresh silver nitrate solution just before use. Discard the solution if it becomes cloudy.
Background is too dark	Gel has been overdeveloped	Monitor image development (step 13) continuously. Proceed to the next step as soon as the staining is considered satisfactory.
	Poor quality of reagents	Use reagents of the highest purity available. Prepare the solutions using HPLC-grade water.
Band images and background color develop too fast	Concentration of sodium thiosulfate ($Na_2S_2O_3$) in developer and thiosulfate solutions is not optimal	Increase sodium thiosulfate concentration in both solutions (e.g., 2- to 3-fold). Do not reuse the solutions.
Metallic silver is deposited on the gel surface	Powder or dirt deposited on the surface during gel handling	Handle the gels with clean forceps or gloves that have been rinsed with water.
	Metallic silver present in the silver nitrate solution	Protect the solution from light. Discard the solution if it gets cloudly.

Table 6.6.3 Troubleshooting Guide for Fluorescent Staining (Basic Protocol 3)

Problem	Cause	Solution
Protein bands are absent or too faint		
Clear background	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
	Direct eye visualization is not sensitive enough	Visualize the bands by using a CCD camera or by photography.
	Photographic or imaging conditions are not appropiate	Use sensitive photographic films (Polaroid 667 black-and-white or similar film) or appropiate CCD camera. Increase exposure (integration) time.
	Stain solution too old or inactivated by light	Use fresh stain solution. Protect it from the light. Do not reuse it.
	Protein(s) of interest pre-stained or containing colored prosthetic groups	If possible, remove prosthetic group(s) from proteins and avoid using pre-stained proteins. Otherwise, use an alternative staining method (e.g., Basic Protocol 2 or Basic Protocol 4).
Highly fluorescent background	Too much unbound dye remains in the gel matrix	Rinse the gel further with water (i.e., repeat steps 4 and 5).
	Gel is placed for visualization on an autofluorescent plastic surface (e.g., Saran Wrap)	Place the gel directly onto the surface of the UV transillumination unit.
	Gel is attached to a polyester surface with affinity for the dye (e.g., backing material of PhastGels)	Remove the gel from the polyester surface before visualization.
Heavy stain at the SDS-PAGE migration front	SYPRO dyes bind micellar SDS at the migration front	Allow the SDS front to migrate out of the gel.
Odd marks at the gel surface	Portions of the gel squeezed or have powder deposits due to handling	Handle the gel gently, wearing gloves that have been rinsed with water.

Table 6.6.4 Troubleshooting Guide for Staining with Zinc (Basic Protocol 4)

Problem	Cause	Solution
Protein bands are absent or too faint	Insufficient contrast due to the use of clear surfaces for visualization	Visualize the bands by placing the gel against a dark surface.
	Gel is overdeveloped	Destain the gel (steps 9 and 10) and stain again monitoring the appearance of bands against a dark surface.
	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
Blackground does not turn opaque	Stain solution or developer are too old	Use fresh stain and developer solutions (do not reuse them).

longer linear ranges than the other staining methods (Steinberg et al., 1996). Nevertheless, in order to check the validity of the measurements it is always advisable to load in the gel, together with the sample(s) to be quantitated, internal controls or a calibration curve with standard protein(s).

Downstream applications

Although PAGE was initially used only for analytical purposes, a large variety of microtechniques have been developed that use PAGE as a preparative step. Proteins separated by one- or two-dimensional PAGE can be treated in-gel with proteases (Rosenfeld et al., 1992) or cyanogen bromide (Córdoba et al., 1997) to obtain peptide fragments for microsequencing, electroeluted, or transferred to nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes (*UNIT 6.2*) for further analysis such as immunodetection or Edman degradation. Proteins separated by nondenaturing PAGE (*UNIT 6.5*), or sometimes even by SDS-PAGE, can be analyzed in-gel for enzymatic or ligand-binding activity. Both the SYPRO Ruby and negative zinc staining methods are compatible with virtually all of these applications. A notable exception is activity analysis of proteins separated by nondenaturing PAGE which are sensitive to SDS, as both staining procedures require the presence of SDS for band visualization. On the other hand, Coomassie blue and silver gel staining involve protein fixation and, therefore, are compatible with fewer downstream applications. It is worth mentioning, however, that Coomassie-stained proteins can be efficiently electrotransferred to PVDF membranes for Edman degradation sequencing (U. Hellman, pers. commun.), although for immunoblot analysis the Coomassie blue dye is a known interference. Coomassie blue staining has been widely used to visualize protein bands destined for in-gel generation of peptides for microsequencing, mainly because these downstream applications have not been sensitive enough to allow analysis of protein bands below the detection limit of the dye. Recent methodological improvements, however, have made it possible to obtain sequence information from as little as 5 ng of protein from polyacrylamide gels stained with silver (Wilm et al., 1996).

Troubleshooting

Tables 6.6.1 to 6.6.4 summarize common problems that may arise during staining of proteins in polyacrylamide gels. Before using any of the methods to analyze a "real" sample, it is advisable to test the method first on a gel containing known amounts of standard proteins (e.g., see Fig. 6.6.1).

Anticipated Results

The protocols described in this unit should allow detection of submicrogram amounts of protein separated by PAGE as exemplified by Figure 6.6.1.

Time Considerations

By far, negative staining with zinc is the fastest method among the ones described in this unit. Images can be obtained within 20 min after the polyacrylamide gel is removed from the electrophoresis unit. Silver staining requires 30 min for fixation and another ~2.5 hr to complete the procedure. Both the Coomassie blue and SYPRO Ruby procedures require ~3.5 hr for obtaining optimal protein-to-background staining ratio. Suboptimal staining with SYPRO Ruby can be obtained by incubating the gel with stain solution for 60 to 90 min and skipping the subsequent washing steps (Basic Protocol 3, steps 4 and 5). In the case of Coomassie blue staining, binding of the dye to proteins is a fast process that depends mainly on its rate of diffusion into the gel matrix (Hitchman and Ekstrom, 1994). Removal of the unbound Coomassie dye from the gel, which also relies on diffusion, can be accelerated by placing an adsorbent material with affinity for the dye (e.g., Whatman 3MM filter paper) into the destaining solution with the gel or by increasing the temperature of destaining (e.g., to 37°C). In both cases, however, caution should be taken to avoid excessive destaining that could decrease sensitivity.

Literature Cited

Blum, H., Beier, H., and Gross, H.J. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8:93-99.

Choi, J.K., Na, D.S., Hong, H.Y., Choi, D.K., Yoon, S.H., and Yoo, G.S. 1996. A fast and sensitive Coomassie blue staining for proteins in polyacrylamide gels using ion-pairing agent. *Anal. Lett.* 29:1517-1525.

Córdoba, O.L., Linskens, S.B., Dacci, E., and Santomé, J.A. 1997. "In gel" cleavage with cyanogen bromide for protein internal sequencing. *J. Biochem. Biophys. Methods* 35:1-10.

DeSilva, T.M. 1995. Protein detection in gels using fixation. *In* Current Protocols in Protein Science (J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, and P.T. Wingfield, eds.) pp. 10.5.1-10.5.12. John Wiley & Sons, New York.

Fernández-Patron, C., Calero, M., Collazo, P.R., Garc'a, J.R., Madrazo, J., Musacchio, A., Sori-

ano, F., Estrada, R., Frank, R., Castellanos-Serra, L.R., and Mendez, E. 1995a. Protein reverse staining—High efficiency microanalysis of unmodified proteins detected on electrophoresis gels. *Anal. Biochem.* 224:203-211.

Fernández-Patron, C., Hardy, E., Sosa, A. Seoane, J., and Castellanos-Serra, L.R. 1995b. Double staining of Coomassie blue-stained polyacrylamide gels by imidazole sodium dodecyl-sulfate zinc reverse staining—sensitive detection of Coomassie blue–undetected proteins. *Anal. Biochem.* 224:263-269.

Garfin, D.E. 1990. One-dimensional gel electrophoresis. *Methods Enzymol.* 182:425-441.

Hitchman, M.L. and Ekstrom, B. 1994. An investigation of the factors controlling the staining and destaining of electrophoresis gels. *Electrophoresis* 15:200-208.

Na, D.S., Hong, H.Y., Yoo, G.S., and Choi, J.K. 1994. Evans blue staining method for detection of proteins on polyacrylamide gels with rhodamine-B. *Anal. Lett.* 27:1265-1275.

Neuhoff, V., Stamm, R., and Eibl, H. 1985. Clear background and highly sensitive protein staining with Coomassie blue dyes in polyacrylamide gels: A systematic analysis. *Electrophoresis* 6:427-448.

Rabilloud, T., Vuillard, L., Gilly, C., and Lawrence J.-J. 1994. Silver-staining of proteins in polyacrylamide gels: A general overview. *Cell. Mol. Biol.* 40:57-75.

Righetti, P.G. and Drysdale, J.W. 1974. Isoelectric focusing in gels. *J. Chromatogr.* 98:271-321.

Rosenfeld, J., Capdevielle, J., Guillemot, J.C., and Ferrara, P. 1992. In-gel digestion of proteins for internal sequence analysis after 1-dimensional or 2-dimensional gel electrophoresis. *Anal. Biochem.* 203:173-179.

Schägger, H. and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379.

Steinberg, T.H., Jones, L.J., Haugland, R.P., and Singer, V.L. 1996. SYPRO Orange and SYPRO Red protein gel stains: One-step fluorescent staining of denaturing gels for detection of nanogram levels of protein. *Anal. Biochem.* 239:223-237.

Switzer, R.C., Merril, C.R., and Shifrin, S. 1979. A highly sensitive silver-stain for detecting protein and peptides in polyacrylamide gels. *Anal. Biochem.* 98:231-237.

Wallace, A. and Saluz, H.P. 1992. Ultramicrodetection of proteins in polyacrylamide gels. *Anal. Biochem.* 203:27-34.

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* 379:466-469.

Wilson C.M. 1979. Studies and critique of amido black 10B, Coomassie blue R, and fast green FCF as stains for proteins after polyacrylamide gel electrophoresis. *Anal. Biochem.* 96:263-278.

Contributed by Esteban C. Dell'Angelica and Juan S. Bonifacino
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

Agarose Gel Electrophoresis of Proteins

This unit describes the use of agarose gel as a matrix for the electrophoresis of proteins. Although agarose is widely used as a material for molecular sieving, it is not often used for the electrophoresis of proteins. When it is used for this purpose, it is generally employed for the electrophoresis of very large proteins. In other electrophoresis procedures it is part of a composite polyacrylamide/agarose system. The system described below utilizes agarose alone as the gel matrix. As with acrylamide gels, the proteins thus separated may be transferred to a membrane (immunoblotting) for further analysis, or the proteins may be identified directly in the gel using stains or labeled antibodies. A particular advantage of the method described in the Basic Protocol is the preparation of the gel in a horizontal electrophoresis bed which saves time and eliminates many of the difficulties commonly encountered in the preparation of acrylamide or composite gels in a vertical preparation apparatus.

These protocols describe methods for the separation and identification of von Willebrand factor (vWF), an extremely large plasma protein that is comprised of multimers ranging from 850,000 to 20,000,000 Da. The methods can be applied to other mixtures containing large proteins, multimeric proteins, and other large protein complexes such as fibrinogen and fibrin complexes (Shainoff, 1991). While the use of an SDS buffer system allows separation of proteins on the basis of size, nondenatured proteins can be separated in "native" agarose gels if their charge and configuration allow for satisfactory partitioning. Von Willebrand factor multimers are stable in SDS due to their disulfide linkages, and the protocols below utilize SDS. The agarose gel electrophoresis and blotting with immunodetection procedure (see Basic Protocol) utilizes identification of protein by a specific antibody followed by chemiluminescent detection methods. Major advantages of this method include the technical ease of preparing the gel, increased sensitivity, and a much shorter turn-around time than in-gel antibody analysis (see Alternate Protocol). It also eliminates the use of radioactive isotopes**.** In addition, the primary and secondary antibodies can be easily removed and the membrane (PVDF) probed again with a different detection antibody. This allows the laboratory the opportunity to detect a second protein or antigenic site on the same protein and also a "second chance" to correct an omission in the procedure, especially for samples in limited supply (*UNIT 6.2*); however, the immunoblotting step of such large proteins must be done with care in order to assure the adequate transfer of the largest proteins. The Alternate Protocol uses direct identification of the protein in the gel by a specific radiolabeled antibody, eliminating the immunoblotting step, but sacrificing sensitivity.

AGAROSE GEL ELECTROPHORESIS AND BLOTTING WITH IMMUNODETECTION

BASIC PROTOCOL

The following is a method for separating a complex mixture of human plasma proteins by continuous SDS horizontal (submerged) agarose gel electrophoresis. The very large multimers of circulating plasma von Willebrand factor (or more highly purified preparations of this protein) are identified using a specific antibody and visualized using a chemiluminescent reagent. This protocol utilizes a 20×25–cm horizontal gel apparatus. Dry agarose is weighed, mixed with electrophoresis buffer, and melted in a hot water bath. The agarose is allowed to partially cool, then is poured into a horizontal casting frame with a Teflon comb in place, and allowed to solidify. The gel is covered with 1 to 2 mm cold electrophoresis buffer and the comb is carefully removed. Prepared samples containing the proteins of interest are diluted with sample buffer and loaded into wells. Electrophoresis is carried out for 3 to 6 hours at 4°C. The gel is placed into a vertical tank transfer

Contributed by Dennis M. Krizek and Margaret E. Rick

Current Protocols in Cell Biology (2002) 6.7.1-6.7.13

apparatus (*UNIT 6.2*), and the proteins are transferred overnight onto an immobilization matrix (PVDF membrane). The membrane is blocked and probed with a polyclonal primary antibody specific for the antigen of interest and, after washing, a conjugated secondary antibody is introduced. After incubation the membrane is washed again, and the protein bands of interest are illuminated with a chemiluminescent detection system.

Materials

SeaKem HGT(P) agarose (Bio Whittaker or equivalent)
1× electrophoresis buffer, 4°C (see recipe)
Protein samples
2× sample buffer (see recipe)
0.25× transfer buffer without methanol (see recipe)
Blocking buffer (*UNIT 6.2*) containing 5% (w/v) nonfat dry milk, fresh
Antibodies:
  Primary: rabbit anti-vWF (Dako)
  Secondary: donkey horseradish peroxidase–linked anti-rabbit Ig (Amersham Pharmacia)
ECL Western Blotting Analysis system (Amersham Pharmacia)

Aluminum foil
Boiling waterbath (optional)
Horizon 20-25 horizontal electrophoresis apparatus (Life Technologies) or equivalent
Teflon comb (e.g., 1 × 9–mm, 20-well)
Pipet with fine tip or equivalent
0.45-µm Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore)
Additional reagents and equipment for protein transfer to membranes and immunoblotting (*UNIT 6.2*)

Cast agarose gels

1. Weigh 1.2 g SeaKem HGT(P) agarose and transfer to a 250-ml flask containing 200 ml of 1× electrophoresis buffer. Add a Teflon-coated magnetic stir bar and tightly cover the flask with aluminum foil.

2. Heat in a boiling water bath, with slow mixing to avoid bubbles, until clear. Alternatively, microwave until boiling

   CAUTION: *Whenever a solution is heated in a microwave the chance of superheating ("boil up") is always present. Protective gloves, gown, and eyewear should be worn at all times.*

3. Assemble Horizon 20-25 horizontal electrophoresis apparatus or equivalent according to manufacturer's recommendations.

   The wedge shaped casting dams of the Horizon (Life Technologies) horizontal apparatus are easily placed in backwards, resulting in leaking of liquid agarose. Care should be exercised so that the dams form a perpendicular angle with the UVT tray.

4. Cool agarose to 55° to 60°C and pour the molten agarose into the electrophoresis apparatus to a depth of 4 mm.

   It is often helpful to prewarm the electrophoresis apparatus with warm water or in an oven to avoid cooling the agarose and causing the agarose to solidify unevenly.

5. Carefully insert the desired (e.g., 1 × 9–mm, 20-well) Teflon comb with care to avoid bubbles.

Removing the comb while the agarose is still in a molten state and then reinserting often eliminates the formation of bubbles under the teeth of the comb.

6. After the agarose has solidified, place the apparatus at 4°C and allow the agarose to age 20 to 30 minutes.

   The electrophoresis is to be carried out at 4°C. The apparatus may either be placed in a cold room, the electrophoresis buffer can be circulated through a refrigeration unit, or the apparatus can be packed in wet ice.

7. Overlay the solidified gel with 2 to 3 mm electrophoresis buffer, 4° C.

8. Remove the sample comb by lifting vertically in one smooth motion.

   It is helpful to hold the gel down with the gloved fingers of the other hand to keep the gel from being pulled up when the comb is removed.

Prepare and load sample

9. Dilute the protein samples to twice the desired concentration using distilled water. Immediately add the diluted sample to an equal volume of 2× sample buffer.

10. Load a sample volume of 10 to 15 µl into the bottom of each sample well using a pipet with a fine tip or equivalent.

    Prior to sample loading examine each sample well to ascertain that air bubbles are not trapped in the well.

Electrophorese gel (also see UNIT 6.1)

11. Run samples into the gel matrix at a constant current of 25 mA for ~30 min or until the sample dye has completely entered the gel.

12. Pause the electrophoresis and decrease the level of the electrophoresis buffer to ~1 mm above the upper surface of the gel. Increase the constant current to 50 mA and run for an additional 3 to 4 hr, or until the marker dye has migrated at least 6 to 7 cm.

Blotting the gel

13. Perform immunoblotting to a 0.45-µm Immobilon-P polyvinylidene fluoride (PVDF) membrane in a tank transfer system as described (*UNIT 6.2*), with the exception of the following conditions:

    a. Electrophoretically transfer at a constant current of 100 mA at 4°C overnight.
    b. Use 0.25× transfer buffer without methanol.
    c. Use blocking buffer containing 5% (w/v) nonfat dry milk.

Immunodetect protein

14. Perform immunoprobing with directly conjugated secondary antibody as described (*UNIT 6.2*), except with the following variations which are specific for immunodetection of von Willebrand Factor protein:

    a. Dilute primary antibody, rabbit anti-vWF, to a concentration of 1:4000.
    b. Dilute secondary antibody, donkey horseradish peroxidase–linked anti-rabbit Ig, to a concentration of 1:2000.

Visualization

15. Visualize von Willebrand factor protein on the PVDF membrane by meticulously following the recommendations enclosed in the ECL Western Blotting Analysis system.

Visualization with Luminescent Substrates is discussed in UNIT 6.2. To determine the optimal concentration of antibody, run a preliminary gel followed by immunoblotting. Cut the blot into several vertical test strips, each containing 1 to 2 lanes. Use the supplier's recommended concentration of antibody as a starting point and process the test strips with 3- to 10- to 30-fold increased and decreased antibody concentrations in separate containers.

ALTERNATE PROTOCOL

AGARAOSE GEL ELECTROPHORESIS WITH IN-GEL ANTIBODY ANALYSIS

This alternative protocol describes a method for separating large plasma proteins using SDS agarose electrophoresis and visualizing the protein of interest directly in the gel with a ^{125}I-radiolabeled rabbit antibody, in this case, to human von Willebrand factor protein, and autoradiography (see also *UNIT 6.3*). The methodology applied to agarose electrophoresis of von Willebrand factor is outlined below. The gel preparation consists of mating two glass plates on a horizontal surface separated by a 0.5-mm spacer. A sheet of GelBond support film is fixed to the glass plate to provide support for the agarose. Agarose at a concentration of 1.35% (w/v) is poured between the glass plate and the spacer plate. After the gel is solidified, the apparatus is disassembled and 1.0 × 0.1–cm wells are punched into the gel. The gel is placed into a horizontal electrophoresis chamber, presoaked wicks are attached to the gel, and sample volumes of 8 µl are loaded. The prepared samples are electrophoresed until the sample dye has migrated 8 to 10 cm. The gel is immediately fixed with isopropanol/glacial acetic acid fixing solution. After the gel is washed, it is blocked with ethanolamine/BSA. The gel, after a second series of washes, is incubated with ^{125}I-labeled anti-vWF antibody for 10 to 24 hours at room temperature. After extensive washing and drying the gel is placed in a cassette with film and exposed 1 to 5 days.

Materials

In-gel sample buffer, fresh (see recipe)
Borate saline buffer (BSB; see recipe)
Sample
0.5% (w/v) bromphenol blue in H_2O
Isopropanol
Agarose gel buffer (see recipe)
SeaKem HGT(P) agarose (FMC/BioWhittaker Molecular)
Agarose running buffer (see recipe)
Fixing buffer (see recipe)
Blocking buffer, in-gel (see recipe)
^{125}I-labeled rabbit anti-human vWF polyclonal antibody (Dako #A0082): radiolabel using protocol of choice and immunopurify (Hoyer and Shainoff, 1980; also see *UNIT 7.10*)
2% (w/v) human IgG (see recipe)
High-salt wash buffer (see recipe)

12 × 75–mm polypropylene tube
12.5 × 26.0 × 0.3–cm glass plate (Amersham Pharamacia Biotech)
12.5 × 24.0–cm spacer plate with adherent 0.5-mm spacers (Amersham Pharmacia Biotech)
12.4 × 25.8–cm GelBond film (Amersham Pharmacia Biotech)
Flexiclamps (Amersham Pharmacia Biotech)
20-ml syringe
60°C oven
Aluminum foil
Gelman Delux electrophoresis chamber (Gelman Sciences) or equivalent

104 × 253–mm paper electrophoresis electrode wicks (Amersham Pharmacia Biotech)
Flattened no. 2 cork borer
Forceps, fine
Horizontal rotary mixer
Forced hot-air dryer (optional)
Kodak X-Omatic film cassette with Lanex screens and film

CAUTION: When working with radioactivity, take apptopriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by your local radiations safety officer (also see *APPENDIX 1D*).

Prepare sample

1. Prepare 3 ml fresh in-gel sample buffer.
2. Label a 12 × 75–mm polypropylene tube for each sample to be assayed. Add 20 µl borate saline buffer (BSB), 120 µl sample buffer, and 10 µl sample into each tube.
3. Cover each tube, vortex gently, and incubate 2 hr at 37°C.
4. Add 8 µl of 0.5% bromphenol blue to each sample and mix gently.

Prepare agarose gel

5. Prepare a boiling water bath by placing approximately 50 ml distilled water in a 250-ml beaker and incubating on a hot plate with magnetic stirring capabilities.
6. Use isopropanol to clean the 12.5 × 26.0 × 0.3–cm glass plate and 12.5 × 24.0–cm spacer plate with its adherent 0.5–mm spacers. Dry with a lint-free tissue (e.g., Kimwipe).

   It is advisable, because of the fragile nature of this gel, to prepare sufficient materials to pour a gel in reserve in the eventuality that one is rendered unusable.

7. Place a few milliliters of distilled water on the glass plate and adhere the hydrophilic side of a 12.4 × 25.8–cm GelBond film. Express any trapped air or excess water with a lint-free tissue.
8. Mate the glass plate with the adherent GelBond film and the spacer plate. Clamp with two flexiclamps.

   The gel-forming sandwich should consist of the glass plate (the larger of the two plates) with an adherent piece of GelBond film (hydrophilic side to the glass plate) and the spacer plate with its adherent spacer bars placed on top of the GelBond (Fig. 6.7.1).

9. Place the clamped plates and a 20-ml syringe in a 60°C oven for ~10 minutes to equilibrate.
10. Measure 40 ml agarose gel buffer into a 50-ml Erlenmeyer flask and add a Teflon-coated magnetic stir bar. Pour 0.54 g SeaKem HGT(P) agarose into the flask, tightly cover with aluminum foil, and place the flask into the boiling water bath (step 5). Dissolve the agarose with constant stirring. After the solution becomes clear, continue boiling an additional 10 min.
11. Remove the heated glass plate assembly (gel-forming sandwich) and the 20-ml syringe from the oven. Quickly fill the heated syringe with 20 ml hot agarose, and holding the plate assembly at an ~75° angle, fill the narrow space in the plate assembly using a back and forth motion to prevent bubbles from being trapped in the gel.

   Use of a needle with the syringe is optional.

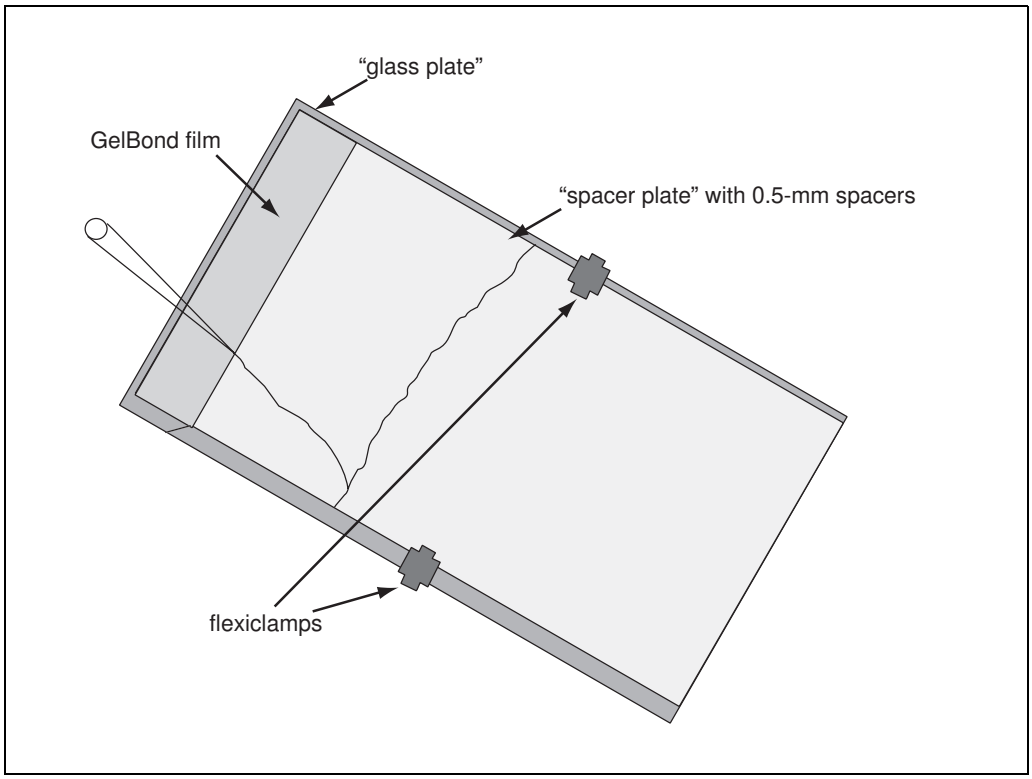


Figure 6.7.1 Diagram of casting gel in the Alternate Protocol. The layers include (back to front): the "glass plate" (the larger of the two plates), GelBond film (hydrophilic side adherent to the glass plate), and the "spacer plate" with attached 0.5-mm spacer bars. The agarose fills the narrow space between the GelBond and the "spacer plate."

12. After filling, lay the plate assembly flat and allow to cool and solidify ~45 min.

Electrophorese gel

13. Position the Gelman Delux electrophoresis chamber or equivalent electrophoresis apparatus on a flat surface and fill each electrode chamber with 400 to 450 ml agarose running buffer. Place 104 × 253–mm paper electrophoresis electrode wicks in each chamber to equilibrate in running buffer.

14. Remove the flexiclamps. Insert a thin spatula blade between the spacer plate and the gel attached to the glass plate. Carefully pry upward to separate the spacer plate. Examine the gel against a bright light for bubbles, thin areas, or areas of separation from the GelBond film.

    The agarose gel should now be attached to the GelBond support backing and be easily handled.

15. Punch the required number of 0.1 × 10-mm wells using a flattened no. 2 cork borer. Use a fine set of forceps to remove the agarose from the interior of each well.

    It is helpful to construct a template to place under the gel to facilitate punching the wells in an evenly spaced straight line. Also, it is necessary to work quickly because these gels are very thin and dry out rapidly.

16. Place a glass plate across the bridge on the electrophoresis chamber and place the gel attached to the GelBond on top of the plate. Affix the presoaked wicks (step 13) on each side of the gel so that there is continuity between the agarose running buffer in the electrode chambers and the gel.

17. Load 8 µl of sample into each well. Place the cover over the electrophoresis apparatus and connect the power supply.

18. Electrophorese at 25 V for ~30 min at room temperature to allow the samples to enter the gel matrix. Increase the power supply to 50 V and continue electrophoresis until the dye marker has migrated 8 to 10 cm from the wells (~3 to 5 hr).

Fix gel

21. Add 200 ml fixing buffer to a container appropriate to the size of the gel. Carefully remove the paper wicks and place the gel in fixing buffer 1 hr without agitation.

    The gel can also fix overnight in fixing buffer for a convenient stopping point.

22. Wash the gel 1 hr with 200 to 300 ml BSB with gentle mixing on a horizontal rotary mixer.

    A prerinse ~1 to 2 min before the wash with ~100 ml BSB is recommended

23. Prepare 250 ml fresh in-gel blocking buffer. Block gel 1 hr with gentle mixing.

24. Prerinse gel with ~100 ml BSB. Wash 1 hr with 200 to 300 ml BSB.

Immunodetect protein

25. Prepare a solution of dilute ^{125}I-labeled rabbit anti-human vWF polyclonal antibody in BSB to a concentration of ~2×10^6 cpm in a volume sufficient to cover the gel. Add 0.5 ml of 2% human IgG to the antibody solution.

    The majority of the gels can be covered with 50 to 70 ml.

    In an effort to minimize the volume of radioactive solutions, the authors' laboratory keeps a supply of dedicated plasticware to accommodate a wide variety of gel sizes. Strict laboratory precaution should be exercised in the preparation, handling, and disposal of radioactive materials.

26. Incubate the gel in the antibody solution at least 16 to 24 hr with gentle mixing.

27. Discard the antibody solution, adhering to standard radiation safety waste disposal protocols.

28. Wash the gel 1 hr in 200 ml high-salt wash buffer with gentle mixing using a horizontal rotary mixer.

29. Repeat the wash an additional three to four times.

    The washes should be monitored and continued until all excess ^{125}I is removed.

30. Wash 1 hr in 200 ml distilled water. Repeat once.

31. Dry the gel with a forced hot-air dryer directed on the gel, or air dry the gel.

Autoradiograph the gel

32. Place the gel in a Kodak X-Omatic film cassette with a Lanex screen. Under dark room conditions, place a piece of Kodak X-Omat film on the gel and then incubate the cassette at −70°C. Expose for an appropriate amount of time.

    Times vary from 1 to 5 days. It is helpful to secure the gel to the cassette with tape to keep it from changing position.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Agarose gel buffer

0.05 M Na_2HP0_4
0.1% (w/v) SDS
Adjust pH to 7.0 with concentrated HCl
Filter and store up to 3 months at room temperature

Agarose running buffer

0.1 M Na_2HP0_4
0.1% (w/v) SDS
Adjust pH to 7.0 with concentrated HCl
Store up to 3 months at room temperature

Blocking buffer, in-gel

15 ml 16.6 M ethanolamine (1.0 M)
250 mg fatty-acid-free Fraction V BSA
Add H_2O to ~200 ml
Adjust pH to 8.0 with concentrated HCl
Add H_2O to 250 ml
Prepare fresh on the day of use

Borate saline buffer (BSB)

15.4 g boric acid (36 mM)
65.06 g NaCl (143 mM)
1.40 g NaOH (0.005 N)
Add 1.56 ml concentrated HCl to adjust pH to 7.83
Adjust volume to 7 liters with H_2O
Store up to 3 months at room temperature

Electrophoresis buffer, 1×

Dilute 10× TAE (see recipe) 1/10 in water. Add 10 ml of 20% (w/v) SDS (*APPENDIX 2A*) per 2 liters. Final concentrations are 40 mM Tris-acetate, 1 mM EDTA, and 0.1% (w/v) SDS. Final pH is 7.8 to 8.3. Store up to 1 week at room temperature.

The solution is also referred to as 1× TAE-SDS. Two liters are required for the application.

Fixing buffer

50 ml isopropanol (25%)
20 ml glacial acetic acid (10%)
130 ml H_2O
Prepare fresh on the day of use

High-salt wash buffer

56.78 g Na_2HPO_4 (0.1 M final)
233.6 g NaCl (1 M final)
Adjust pH to 7.0 with concentrated HCl
Add H_2O to 4 liters
Store up to 3 months at room temperature

Human IgG, 2%

2.0 g human IgG in 100 ml BSB (see recipe)
Store in aliquots up to 1 year at −20°C

In-gel sample buffer

Stock solution
0.01M Na_2HP0_4
Adjust pH to 7.0 with HCl
Filter and store up to 3 months at room temperature
Working solution
To 3 ml stock solution add:
20.64 mg iodoacetamide
37.5 mg SDS
Prepare fresh

Sample buffer, 2×

20 ml 10× TAE (see recipe)
1 ml 20% (w/v) SDS (*APPENDIX 2A*)
20 ml glycerol
0.2 g bromphenol blue
Add H_2O to 200 ml
Store up to 1 year at room temperature

TAE, 10×

48.4 g Tris base (400 mM)
20 ml 0.5M EDTA (10 mM; *APPENDIX 2A*)
Adjust pH to 7.8 with glacial acetic acid
Adjust volume to 1 liter
Store up to 1 year at room temperature

Transfer buffer without methanol, 0.25× and 10×

For a 10× solution
250 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
1.92 M glycine
1.0% (w/v) SDS
Store up to 1 year at room temperature
This is the stock solution for transfer buffer and is also known as Tris/glycine/SDS (TG/SDS).

For a 0.25× solution
Dilute 10× transfer buffer 1/40 in distilled water. Final concentrations are 6.25 mM Tris·Cl, 48 mM glycine, and 0.025% (w/v) SDS. Final pH is 8.3. Store up to 1 week at room temperature.
Four liters are required for the application.

Table 6.7.1 Troubleshooting Guide for Agarose Electrophoresis and Immunoblotting

Problem	Possible cause	Solution
Agarose electrophoresis		
Run time too long or too short	Buffer concentration too high or too low	Verify buffer preparation
	Voltage too high or too low	Review and increase or decrease voltage or current settings
Band spreads into other lanes	Sample diffusing out of well or into surrounding gel	Minimize time for sample loading and start electrophoresis promptly
Samples leak underneath gel	Bottom of well torn when removing comb	Remove comb slowly
Bands migrate at different rates (visualized as "smiles," "frowns," and "sneers")	Gel cast unevenly	Use level to verify that the apparatus is level
	Uneven heat distribution	Decrease power settings. Cool buffer to 4°C. Circulate buffer.
Bands not in straight lines (visualized as a "wiggle")	Artifacts in wells	Flush wells with electrophoresis buffer
		Inspect wells for trapped air bubbles
Bromphenol turns yellow	pH change during electrophoresis	Verify pH of buffer
		Circulate buffer during run
Immunoblotting		
High background	Insufficient blocking	Increase concentration and/or time of blocking step
	Overdevelopment	Remove membrane from substrate after 1 min
	Protein contamination	Wash or replace fiber pads and clean apparatus
	Incomplete washing	Increase wash time and volume
		Review recipe, especially Tween 20
	Primary or secondary antibody too concentrated	Review supplier's recommendations
		Run test strips to optimize reactions
Weak signal or no reaction	Sample load insufficient	Increase amount of sample
	Low antibody specificity	Increase antibody concentration
	Antigen not transferred	Increase transfer time. Stain membrane for protein transfer.
	Conjugate not active	Mix more thoroughly

COMMENTARY

Background Information

The major use of agarose in protein analysis is its application as a matrix for molecular sieving; however, it is also widely used as a material that can be modified to form an affinity matrix for affinity chromatography. Because of its ability to separate proteins of very large size, it is also utilized for electrophoresis and preparation of very large proteins, ranging from several million to approximately fifty-thousand daltons. Agarose has the advantage of being nontoxic. In addition, it may be melted to allow recovery and further studies of the separated protein, and an excised band may be directly injected into an animal for immunization. Also, the use of native gels allows separation and recovery of nondenatured proteins for functional studies.

The electrophoresis procedure outlined above illustrates the ability of agarose to separate proteins of molecular weights that exceed

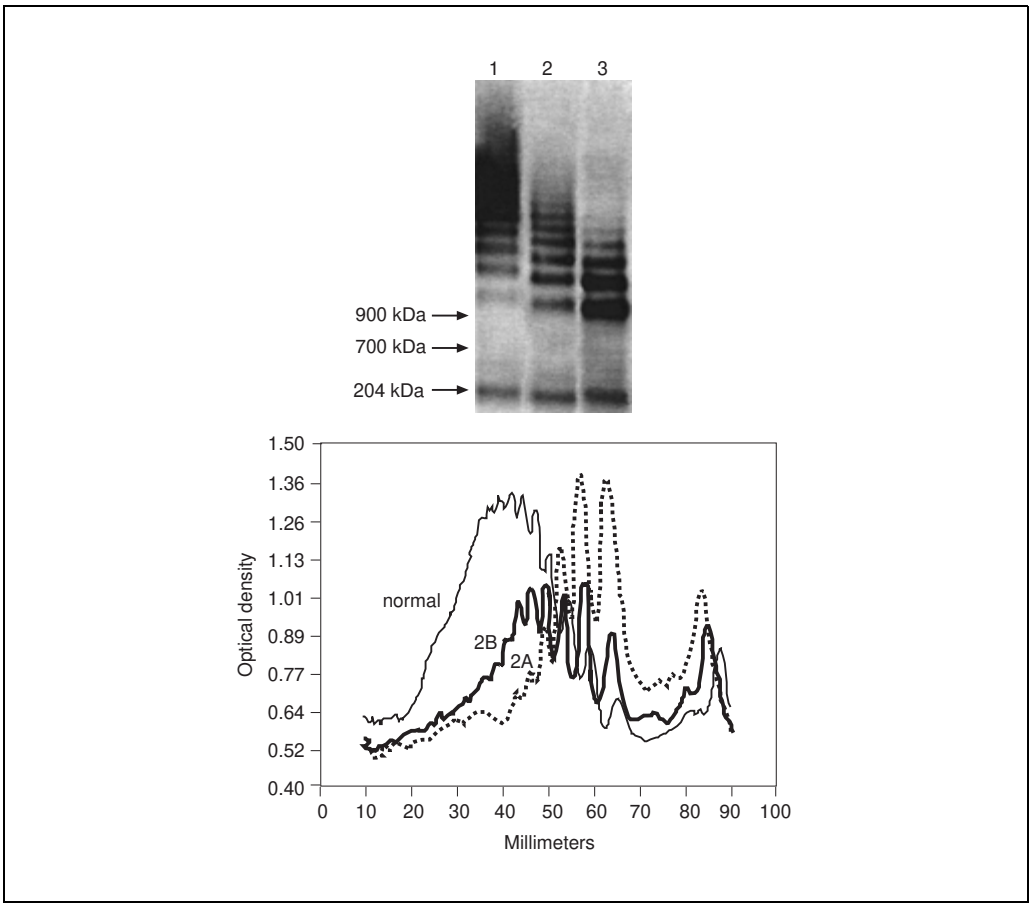


Figure 6.7.2 Luminograph of vWF multimers from normal plasma (lane 1), von Willebrand disease Type 2B plasma (lane 2), and von Willebrand disease Type 2A plasma (lane 3). A densitometric tracing is seen below. Reproduced with permission from Krizek and Rick (2000).

1×10^6 Da. For smaller proteins, higher concentrations of agarose (e.g., 3%) and shorter blotting times may be used. In instances where electroblotting cannot be carried out because of precipitation of the proteins during transfer due to separation from detergent, the proteins can be immobilized in the gel to prevent diffusion before immuno-identification. Immobilization of the proteins also allows for the use of sequential antibodies for identification of protein bands. Additionally, in-gel identification may also be important if there is uneven transfer of proteins due to dissimilar transfer characteristics. Immobilization of the proteins in the gel is accomplished with the use of an agarose gel that is modified by the addition of glycidol to yield a glyceryl agarose that contains aldehyde groups after oxidation by periodate. Proteins are covalently bound in the gel after electrophoresis by reaction of their amino groups with the aldehydes in the presence of the reducing agent, sodium cyanoborohydride. Further direct probing with antibodies can be carried out without the need for transfer to another support (Shainoff, 1993). Composite gels of agarose or glyoxal agarose have also been prepared to provide differing degrees of sieving (Peacock and Dingman, 1968; Shainoff, 1993).

Although few proteins are as large as vWF, the evaluation of von Willebrand factor multimers illustrates an important clinical application of the use of agarose as a medium for electrophoresis. Assessment for the presence of the largest multimers is physiologically important for the diagnosis of von Willebrand disease and for selection of the most appropriate treatment (Rick, 2001). The original procedure for this analysis (see Alternate Protocol, with minor modifications) included glycidol in the agarose which served to aid in the fixation of the protein bands while the further washing and antibody identification steps were accomplished (Hoyer and Shainoff, 1980). It was subsequently found that diffusion of protein bands was not a limiting factor with vWF, and this immobilization step was eliminated from the method.

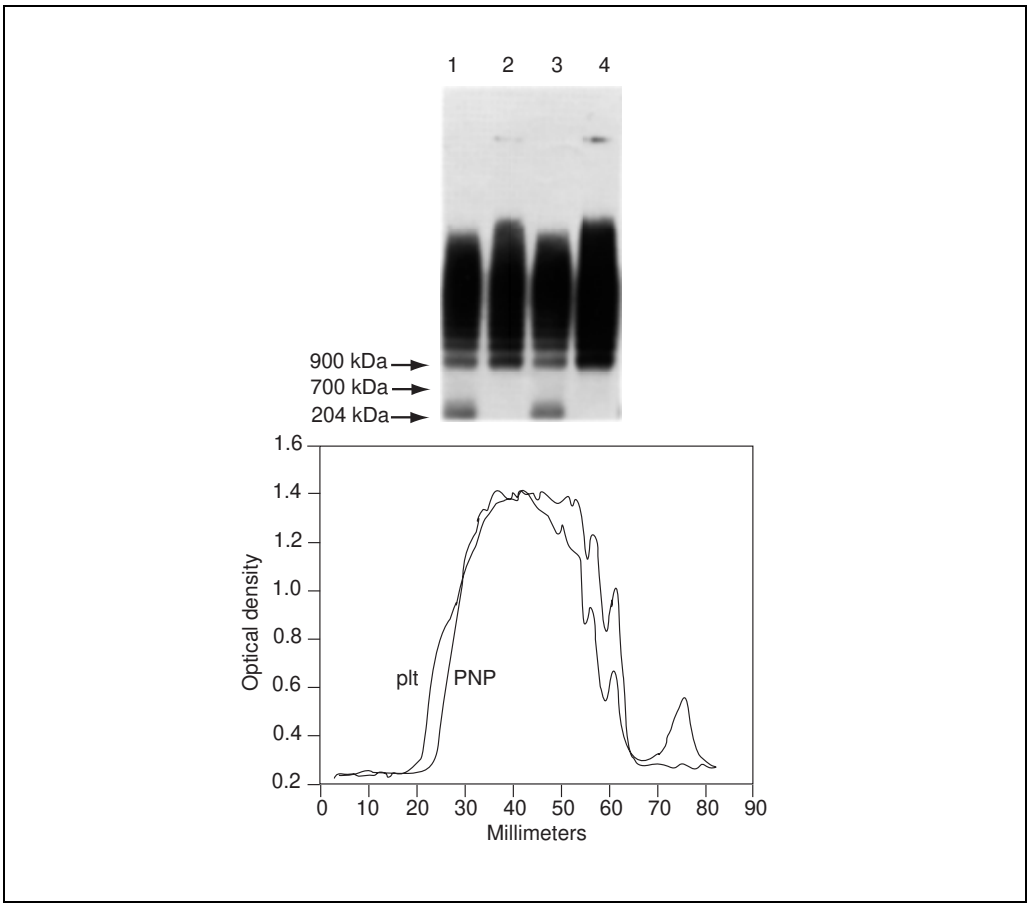


Figure 6.7.3 Luminograph of vWF multimers in pooled normal plasma (PNP; lanes 1 and 3) and vWF multimers extracted from platelets (plt; newly synthesized vWF; lanes 2 and 4). The densitometric tracing below shows the earlier "take-off" of the platelet vWF, indicating larger multimers. Adapted with permission from Krizek and Rick (2000).

Analysis of the distribution of von Willebrand factor multimers is also used to assess the function of an important protease that cleaves von Willebrand factor and decreases the prothrombotic "unusually high-molecular-weight" multimers of von Willebrand factor; these multimers are initially synthesized and secreted into the circulation, but are cleaved by the vWF protease (Krizek and Rick, 2001; Aronson, Krizek, and Rick, 2001).

Critical Parameters and Troubleshooting

It is important to maintain the temperature at 4°C during electrophoresis using the horizontal bed (see Basic Protocol). The blotting step must be carried out for a sufficient time to allow transfer of very high-molecular-weight proteins. Thorough washing after blocking buffer and antibody additions is important in both protocols. See Table 6.7.1 for troubleshooting agarose gel electrophoresis and immunoblotting.

Anticipated Results

The radiographs that result from the chemiluminescent and radioactive detection procedures show a wide distribution of multimer sizes of normal von Willebrand factor. In certain subtypes of von Willebrand disease (i.e., Type 2) there is a marked or modest decrease in the higher-molecular-weight multimers (Fig. 6.7.2). If newly synthesized von Willebrand factor is extracted from platelets, the unusually high-molecular-weight multimers are seen (Fig. 6.7.3). In samples that are incubated under conditions that activate the von Willebrand factor protease, a decrease in the high and intermediate sized multimers is seen (Fig. 6.7.4; Rick and Krizek, unpub. observ.).

Time Considerations

Both protocols should be started in the morning to allow sufficient time for electrophoresis. Horizontal electrophoresis and blotting can be completed within 48 hours: blotting is conveniently completed overnight and detec-

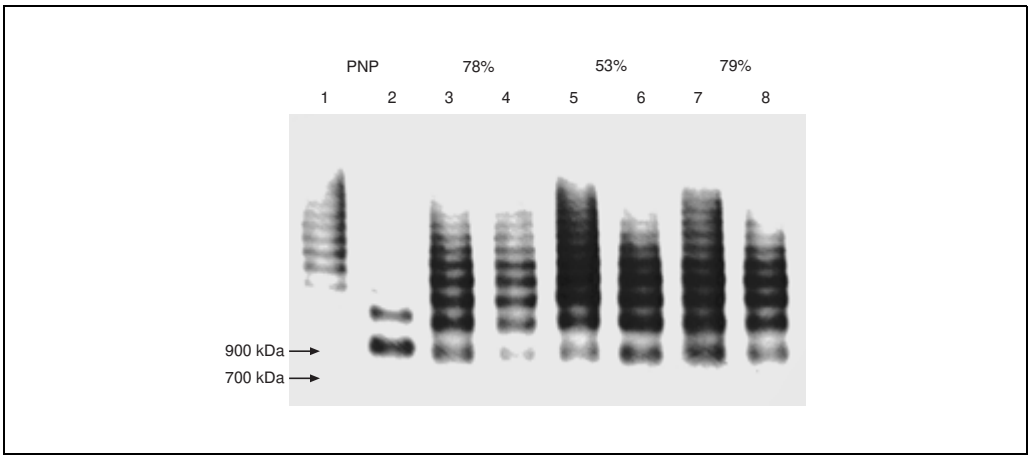


Figure 6.7.4 Luminograph of pooled normal plasma (PNP) showing normal vWF multimers (lane 1) and proteolysed multimers of normal vWF from PNP after exposure to conditions that activate the vWF protease (lane 2). Paired samples from patients with thrombotic thrombocytopenic purpura who have an inhibitor to the vWF protease are seen in lanes 3 to 8. Odd lanes contain plasma samples using conditions that do not activate the vWF protease, and even lanes contain the paired sample that was exposed to conditions that activate the protease. Very little proteolysis is observed in these patients' plasmas due to the presence of an inhibitor (even lanes). The numbers above the patient lanes indicate the retention of the high-molecular-weight multimers.

tion procedures can be completed the next day. The Alternate Protocol takes up to 5 or more days to obtain results, largely due to the time required for the incubation with antibody and development of the autoradiogram. Also, a radiolabeled antibody specific for the protein to be identified must be available.

Literature Cited

Aronson, D.L., Krizek, D.M., and Rick, M.E. 2001. A rapid assay for the vWF protease. *Thrombosis and Hemostasis* 85:184-185.

Hoyer, L.W. and Shainoff, J.R. 1980. Factor VIII-related protein circulates in normal human plasma as high molecular weight multimers. *Blood* 55:1056-1059.

Krizek, D.M and Rick, M.E. 2000. A rapid method to visualize von Willebrand factor multimers using agarose gel electrophoresis, immunolocalization, and luminographic detection. *Thrombosis Research* 97:457-62

Krizek, D.M. and Rick, M.E. 2001. Clinical application of a rapid method using agarose gel electrophoresis and western blotting to evaluate von Willebrand factor protease activity. *Electrophoresis* 22:946-949.

Peacock, A.C. and Dingman, W. C. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry* 7:668-674.

Rick, M.R. 2002. Hemophilia and von Willebrand disease. *In* UpToDate Clinical Reference Library, Release 9.1 (B.D. Rose, ed.) UpToDate, Wellesley, MA.

Shainoff, J.R. 1993. Electrophoresis and direct immunoprobing on glyoxal agarose. *In* Advances in Electrophoresis, Vol. 6 (A. Chrambach, M.J. Dunn, and B.J. Radola, eds.) pp. 65-176. VCH Publishers, New York.

Shainoff, J.R., Urbanic, D.A., and DiBello, P.M. 1991. Immunoelectrophoretic characterizations of the cross-linking of fibrinogen and fibrin by factor XIIIa and tissue transglutaminase. Identification of a rapid mode of hybrid alpha-/gamma-chain cross-linking that is promoted by the gamma-chain cross-linking. *J. Biol. Chem.* 266:6429-6437.

Key References

Krizek and Rick, 2000. See above.

This original paper of the procedure and use of immunoblotting and chemiluminescence for ararose gel electrophoresis provides the background and reasons for the development of this assay in the clinical laboratory setting.

Hoyer and Shainoff, 1980. See above.

This paper outlines the original "in-gel" procedure for the electrophoresis of very high molecular weight proteins and provides examples of its usefulness in understanding the structure of von Willebrand factor.

Shainoff, 1993. See above.

This reference provides general background and the rationale for the use of modified ararose for the separation and identification of (large) proteins by electrophoresis.

Contributed by Dennis M. Krizek and Margaret E. Rick
National Institutes of Health
Bethesda, Maryland

Fluorescence Detection of Glycoproteins in Gels and on Electroblots

The co-translational and post-translational covalent attachment of oligosaccharides to proteins is a common cellular event in eukaryotes, regulated by a variety of glycosidases and glycosyltransferases (Beeley, 1985; Reuter and Gabius, 1999; *UNIT 15.2*). Glycosylation profiles are dynamic, changing during development, differentiation, and disease. Glycosylation of proteins is critical to the adhesiveness of microorganisms and cells, cellular growth control, cell migration, tissue differentiation, and inflammatory reactions. Differences in glycosylation profiles are often used as a "barometer" to assess disease states. With the advent of proteomics, genome-wide protein analysis, there is renewed interest in the rapid and sensitive identification of glycoproteins by methods that do not require degradation of the protein component of the macromolecule (Packer et al., 1999; Hirabayashi et al., 2001). Until recently, there have been relatively few methods available for the direct analysis of glycans on proteins transferred to membranes and most especially of glycans on proteins within polyacrylamide gels (Packer et al., 1999; Koketsu and Linhardt, 2000; Raju, 2000) . Such methods could readily be incorporated into integrated proteomics platforms that utilize automated gel stainers, image analysis workstations, robotic spot excision instruments, protein digestion work stations, and mass spectrometers (Patton, 2000a,b).

There are two principal approaches to the detection of glycoproteins in gels and on blots; reacting carbohydrate groups by periodate/Schiff's base (PAS) chemistry and noncovalent binding of specific carbohydrate epitopes using lectin-based detection systems. The PAS method involves oxidation of carbohydrate groups, followed by conjugation with a chromogenic substrate (acid fuchsin, Alcian Blue), a fluorescent substrate (dansyl hydrazine, 8-aminonaphthalene-1, 3,6-trisulfonate, Pro-Q Emerald dye), biotin hydrazide, or digoxigenin hydrazide. Signal is detected directly in the case of the chromogenic or fluorescent conjugates and indirectly using enzyme conjugates of antibodies for bound digoxigenin or enzyme conjugates of streptavidin for bound biotin. Lectins permit detection of certain structural subclasses of glycoproteins by similar methods to those used in standard immunoblotting applications. Typically, lectin conjugates of biotin along with enzyme conjugates of streptavidin or direct conjugates of lectin and enzyme are utilized along with chromogenic, fluorogenic, or chemiluminescent substrates. Just as in immunoblotting, the most popular enzymes used to detect lectin or streptavidin are alkaline phosphatase and horseradish peroxidase.

This unit describes periodate/Schiff's base and lectin methods for the detection of glycoproteins. The Pro-Q Emerald 300 glycoprotein detection method permits fluorescent direct detection of glycoproteins in gels (see Basic Protocol 1) or on blots (see Alternate Protocol) without the use of enzyme amplification systems. The method may also be used to detect lipopolysaccharides, constituents of the outer membrane surrounding gram-negative bacteria. The Pro-Q glycoprotein blot stain protocol for concanavalin A (see Basic Protocol 2) is suitable for the detection of glycoproteins containing α-mannopyranosyl and α-glucopyranosyl residues on blots using an alkaline phosphatase–based signal amplification system. Using different enzyme-lectin conjugates, such as alkaline phosphatase conjugates of wheat germ agglutinin or *Griffonia simpliifolia* lectin II (GS-II), the method can be adapted to the detection of other glycan structures present in glycoproteins.

Contributed by Wayne F. Patton

Current Protocols in Cell Biology (2002) 6.8.1-6.8.15

BASIC PROTOCOL 1

FLUORESCENT DETECTION OF GLYCOPROTEINS IN POLYACRYLAMIDE GELS

Pro-Q Emerald 300 Glycoprotein Gel Stain Kit provides a robust method for differentially staining glycosylated and non-glycosylated proteins in the same gel. The technique combines the green fluorescent Pro-Q Emerald 300 glycoprotein stain with the orange-red fluorescent SYPRO Ruby total protein gel stain.

Pro-Q Emerald 300 glycoprotein stain reacts with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins. Using this stain allows detection of <1 ng glycoprotein/band, depending upon the nature and the degree of glycosylation, making it 100-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye (rosaniline). The green-fluorescent signal from the Pro-Q Emerald 300 stain can be visualized using a standard 300-nm UV (UV-B) illumination source. The Pro-Q Emerald 488 Glycoprotein Gel Stain Kit is quite similar to the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, but it is optimized for use with gel scanners equipped with 470- to 488-nm lasers. The Pro-Q Emerald dye staining method is more reliable than mobility-shift assays using glycosidases since even glycoproteins that are not susceptible to deglycosylation with specific enzymes may readily be identified as glycoproteins.

After detecting glycoproteins with Pro-Q Emerald 300 dye, total protein profiles may be detected using SYPRO Ruby protein gel stain. SYPRO Ruby protein gel stain interacts noncovalently with basic amino acid residues in proteins. The stain is capable of detecting <1 ng of protein/band, making it at least as sensitive as the best silver staining procedures available. The orange-red fluorescent signal from SYPRO Ruby protein gel stain can be visualized using a standard 300-nm UV (UV-B) illumination source, or alternatively may be excited using 470- to 488-nm laser, gas discharge, or xenon arc sources.

Materials

Protein sample of interest
Fix solution (see recipe)
Wash solution (see recipe)
Pro-Q Emerald 300 Glycoprotein Gel Stain Kit (Molecular Probes) containing:
- 50× Pro-Q Emerald 300 reagent, concentrate in DMF
- Pro-Q Emerald 300 dilution buffer
- Periodic acid (oxidizing reagent; see recipe)
- CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for approximately 20 gel lanes
- SYPRO Ruby protein gel stain

Deionized, high quality water
10% (v/v) methanol or ethanol, spectroscopy grade (optional)
7% (v/v) glacial acetic acid (optional)

Polystyrene staining dishes (e.g., a weighing boat for minigels or larger container for larger gels)
Orbital shaker
UV transilluminator
Photographic camera or CCD camera and appropriate filters

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis.

   Typically, the protein sample is diluted to ~10 to 100 µg/ml with 2× sample buffer, heated for 4 to 5 min to 95°C, and 5 to 10 µl of diluted sample is then applied per gel lane for 8 × 10–cm gels. Larger gels require proportionally more material.

   For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and provide alternating bands as positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (*UNIT 6.1*).

   The staining procedure is optimized for gels that are 0.5- to 1- mm thick.

Fix gel

3. After electrophoresis, fix the gel by immersing it in 75 to 100 ml of fix solution in a polystyrene staining dish and incubating with gentle agitation (e.g., on an orbital shaker at 50 rpm) for 45 min at room temperature.
4. Wash the gel by incubating it in 50 ml wash solution with gentle agitation for 10 min at room temperature. Repeat wash one additional time.
5. Oxidize the gel in 25 ml periodic acid solution with gentle agitation for 30 min at room temperature.
6. Wash the gel in 50 ml wash solution with gentle agitation for 5 to 10 min at room temperature. Repeat this washing step two additional times.

Stain gel for glycoproteins

7. Prepare fresh Pro-Q Emerald 300 staining solution by diluting the 50× Pro-Q Emerald 300 concentrate reagent 50-fold into Pro-Q Emerald 300 dilution buffer.

   For example, dilute 500 µl of 50× Pro-Q Emerald 300 reagent into 25 ml dilution buffer to make enough staining solution for one 8 × 10–cm gel.

8. Incubate the gel in subdued light in 25 ml of Pro-Q Emerald 300 staining solution (step 7) while gently agitating for 90 to 120 min.

   The signal can be seen after ~20 min and maximum sensitivity is reached at ~120 min. Staining overnight is not recommended.

9. Wash the gel with 50 ml wash solution for 15 min at room temperature. Repeat this wash one additional time. Do not leave the gel in wash solution for >2 hr, as the staining signal will start to decrease.
10. Visualize the stain using a standard UV transilluminator.

    The Pro-Q Emerald 300 stain has an excitation maximum at ~280 nm and an emission maximum near 530 nm. Stained glycoproteins can be visualized using a 300 nm UV-B transilluminator.

11. Document results before proceeding to the next step. Use a photographic camera or CCD camera and the appropriate filters to obtain the greatest sensitivity (see Fig. 6.8.1).

    Pro-Q Emerald dye signal will fade after SYPRO Ruby dye staining.

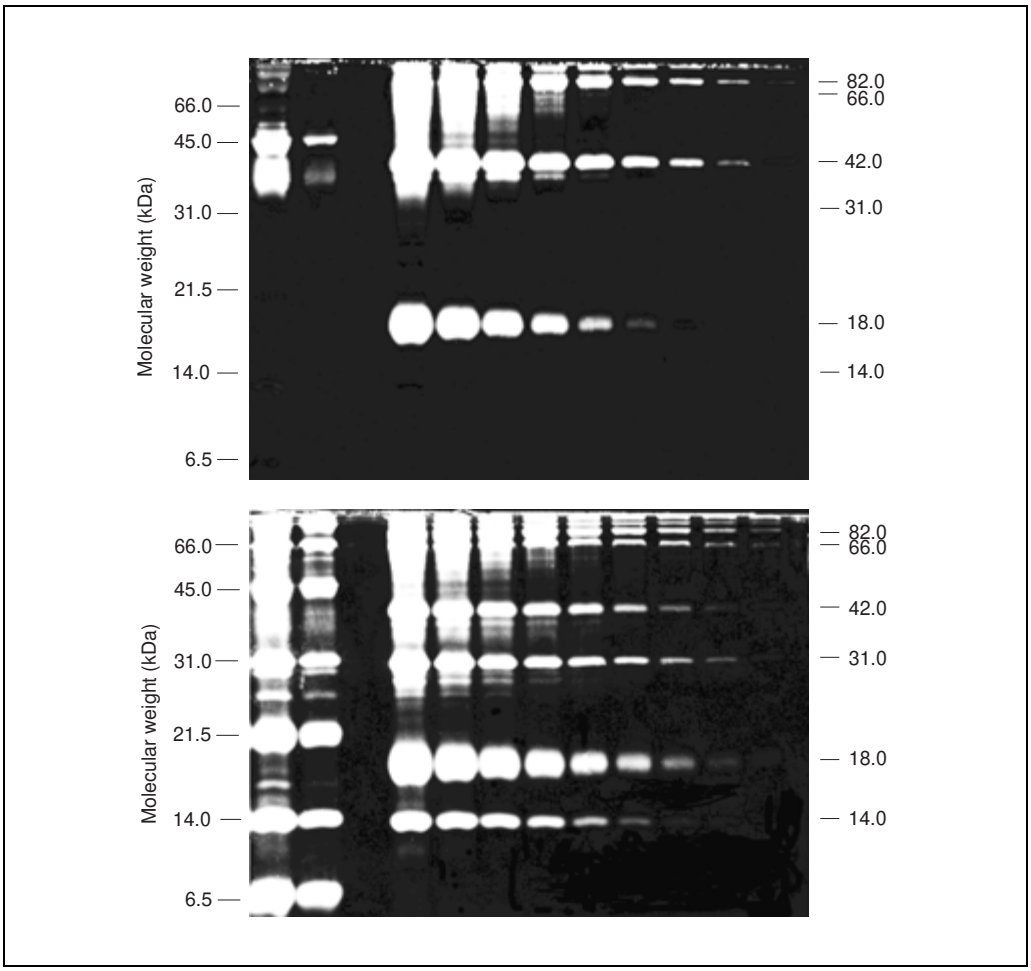


Figure 6.8.1 Sensitivity and specificity of glycoprotein detection in 13% SDS-polyacrylamide gels using Pro-Q Emerald 300 glycoprotein gel stain. (**A**) Detection of glycoproteins using Pro-Q Emerald 300 glycoprotein gel stain. (**B**) Detection of the total protein profile using SYPRO Ruby protein gel stain. Lanes 1 and 2, broad range molecular weight markers containing the 45-kDa glycoprotein ovalbumin, 1000 and 250 ng, respectively. Lane 3, blank. Lanes 4 to 12, CandyCane molecular-weight markers, a mixture of glycosylated and unglycosylated proteins (1000 to 3.9 ng, as two-fold serial dilutions). Gels were imaged using a Lumi-Imager F1 instrument (Roche Molecular Biochemicals). Both dyes were excited using the instrument's 300-nm UV-B transilluminator and images were captured using the instrument's cooled CCD camera. Pro-Q Emerald 300 dye signal was collected using the standard 520-nm band-pass emission filter. Gels were then stained with SYPRO Ruby protein gel stain and SYPRO Ruby dye signal was collected using the 600-nm band-pass emission filter provided with the instrument. Figure courtesy of Courtenay Hart, Molecular Probes.

Stain the gel for total protein

12. In order to counter-stain non-glycosylated proteins in the sample, pour the SYPRO Ruby protein gel staining solution into a small, clean plastic dish.

    For one or two standard-size mini-gels, use ~50 ml to 100 ml of staining solution; for larger gels, use 500 to 750 ml.

13. Place the gel into the staining solution and gently agitate (e.g., on an orbital shaker at 50 rpm) at room temperature.

    The staining time ranges from 90 min to 3 hr, depending upon the thickness and percentage of polyacrylamide in the gel. Specific staining can be seen in as little as 30 min. However, a minimum of 3 hr of staining is required for the maximum sensitivity and linearity. For convenience, gels may be left in the dye solution overnight or longer without over staining.

14. After staining, rinse the gel in water for 30 to 60 min to decrease background fluorescence.

    Alternatively, to further decrease background fluorescence, the gel can be washed in a mixture of 10% methanol (or ethanol) and 7% acetic acid for 30 min instead of water. The gel may be monitored periodically using UV illumination to determine the level of background fluorescence.

15. Visualize the stain using an appropriate method (see Fig. 6.8.1).

    The stained gel is best viewed on a standard 300-nm UV-B transilluminator, though stain will be visible using a 254-nm UV-C or 365-nm UV-A transilluminator. Gels may also be visualized using various laser scanners: 473-nm (SHG) laser, 488-nm argon-ion laser, or 532-nm (YAG) laser. Alternatively, use a xenon arc lamp, blue fluorescent light, or blue light–emitting diode (LED) source. Gels may be photographed by Polaroid or CCD camera. Use Polaroid 667 black-and-white print film and the SYPRO protein gel stain photographic filter (Molecular Probes).

FLUORESCENT DETECTION OF GLYCOPROTEINS ON ELECTROBLOT MEMBRANES

ALTERNATE PROTOCOL

Pro-Q Emerald 300 Glycoprotein Blot Stain Kit provides a robust method for differentially staining glycosylated and non-glycosylated proteins on the same electroblot. The technique combines the green fluorescent Pro-Q Emerald 300 glycoprotein stain with the orange-red fluorescent SYPRO Ruby total protein gel stain.

The Pro-Q Emerald 300 glycoprotein stain reacts with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins. Using this stain, allows detection of <1 ng glycoprotein/band, depending upon the nature and the degree of glycosylation, making it 100-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye (rosaniline). The green-fluorescent signal from Pro-Q Emerald 300 stain can be visualized using a standard 300-nm UV (UV-B) illumination source. The Pro-Q Emerald 488 Glycoprotein Blot Stain Kit is quite similar to the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, but is optimized for use with gel scanners equipped with 470- to 488-nm lasers. The staining method is more reliable than mobility-shift assays using glycosidases since even glycoproteins that are not susceptible to deglycosylation with specific enzymes may readily be identified as glycoproteins.

After detecting glycoproteins with Pro-Q Emerald 300 dye, total protein profiles may be detected using SYPRO Ruby protein blot stain. SYPRO Ruby protein blot stain interacts noncovalently with basic amino acid residues in proteins. The stain is capable of detecting <4 ng of protein/band, making it at least as sensitive as the best colloidal gold staining procedures available. The orange-red fluorescent signal from SYPRO Ruby protein blot stain can be visualized using a standard 300 nm UV (UV-B) illumination source or alternatively may be excited using 470- to 488-nm laser, gas discharge, or xenon arc sources.

Materials

Protein sample of interest
PVDF membrane
Fix solution (see recipe)
Wash solution (see recipe)
Pro-Q Emerald 300 Glycoprotein Blot Stain Kit (Molecular Probes) containing:
  50× Pro-Q Emerald 300 reagent, concentrate in DMF
  Pro-Q Emerald 300 dilution buffer
  Periodic acid (oxidizing solution; see recipe)

CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for ~20 gel lanes
SYPRO Ruby protein blot stain
Methanol, spectroscopy grade (optional)
Glacial acetic acid (optional)

95°C heat block
Polystyrene staining dishes (e.g., weighing boat for minigels or larger containers for larger gels)
Orbital shaker
UV epi-illuminator
Photographic camera or CCD camera and appropriate filters

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*) and electroblotting (*UNIT 6.2*)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis.

   Typically, the protein sample is diluted to ~10 to100 µg/ml with 2× sample buffer, heated for 4 to 5 min at 95°C, and then 5 to 10 µl of diluted sample is applied per gel lane for 8 × 10–cm gels. Larger gels require proportionally more material.

   For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and as alternate bands of positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (*UNIT 6.1*).

   The procedure is optimized for gels that are 0.5- to 1-mm thick.

Prepare blot

3. After electrophoresis, transfer the proteins to PVDF membrane using standard electroblotting procedures (*UNIT 6.2*).

   The use of nitrocellulose membranes is not recommended.

4. After transfer, fix the blot by immersing in 25 ml fix solution and incubate with gentle agitation (e.g., on an orbital shaker at 50 rpm) for 45 min at room temperature.

5. Wash the blot by incubating in 25 ml wash solution with gentle agitation for 10 min, room temperature. Repeat this wash step one additional time.

6. Oxidize the blot in 25 ml periodic acid solution with gentle agitation for 30 min.

7. Wash the blot in 25 ml wash solution with gentle agitation for 5 to 10 min. Repeat this washing step two additional times.

Visualize glycoproteins

8. Prepare fresh Pro-Q Emerald 300 staining solution by diluting the 50× Pro-Q Emerald 300 concentrate reagent 50-fold into Pro-Q Emerald 300 dilution buffer.

   For example, dilute 500 µl of 50× Pro-Q Emerald 300 concentrate reagent into 25 ml of dilution buffer to make enough staining solution for one 8 × 10–cm gel.

9. Incubate the blot in the dark in 25 ml Pro-Q Emerald 300 staining solution (step 8) while gently agitating for 90 to 120 min, room temperature.

   The signal can be seen after ~20 min and maximum sensitivity is reached at ~120 min. Staining overnight is not recommended.

10. Wash the blot with 25 ml wash solution for 15 min at room temperature. Repeat this wash one additional time. Do not leave the blot in wash solution for >2 hr, as the staining signal will start to decrease.

11. Allow the membrane to air dry.

12. Visualize the stain using a standard 300-nm UV epi-illuminator.

    The Pro-Q Emerald 300 stain has an excitation maximum at ~280 nm and an emission maximum near 530 nm.

    A UV transilluminator may also be used to visualize the glycoproteins, but this results in poorer detection sensitivity.

13. Document results before proceeding to the next step using a photographic camera or CCD camera with the appropriate filters to obtain the greatest sensitivity.

    Pro-Q Emerald dye signal will fade after SYPRO Ruby dye staining.

Visualize total protein

14. In order to counter-stain non-glycosylated proteins in the sample, pour the SYPRO Ruby protein blot stain solution into a small, clean plastic dish.

    For one or two standard-size mini-blots, use ~50 ml to 100 ml of staining solution; for larger blots, use 500 to 750 ml.

15. Place the air-dried blot face down onto the surface of the staining solution and gently agitate (e.g., on an orbital shaker at 50 rpm) for 15 min at room temperature.

16. After staining, rinse the blot in four changes of water for 1 min each to decrease background fluorescence.

17. Allow blots to air dry and visualize the stain using an appropriate method.

    The stained blot is best viewed on a standard 300-nm UV epi-illuminator, though stain will be visible using a 254-nm UV-C or 365-nm UV-A epi-illuminator. Blots may also be visualized using various laser scanners: 473-nm (SHG) laser, 488-nm argon-ion laser, or 532-nm (YAG) laser. Alternatively, use a xenon arc lamp, blue fluorescent light, or blue light–emitting diode (LED) source.

    Blots may be photographed by Polaroid or CCD camera. Use Polaroid 667 black-and-white print film and the SYPRO protein gel stain photographic filter (Molecular Probes). Exposure times vary with the intensity of the illumination source; for an f-stop of 4.5, ~1 to 3 sec should be required.

FLUORESCENT DETECTION OF GLYCOPROTEINS CONTAINING TERMINAL α-MANNOPYRANOSYL AND α-GLUCOPYRANOSYL RESIDUES ON ELECTROBLOT MEMBRANES

BASIC PROTOCOL 2

Lectins are sugar-binding proteins of nonimmune origin capable of agglutinating cells or precipitating glycoconjugates (Beeley, 1985). The specific interactions between labeled lectins and oligosaccharides form the basis of glycoprotein detection after separation by gel electrophoresis and transfer to membranes by electroblotting. Concanavalin A is a tetrameric protein, with each subunit containing a carbohydrate-binding site, a calcium ion–binding site, and a manganese–ion binding site. Concanavalin A binds specifically

to α-D-mannopyranosyl and α-D-glucopyranosyl residues, with substitutions or modifications at the C-3, C-4, or C-6 positions of the ring structure leading to greatly diminished binding (Beeley, 1985).

The Pro-Q Glycoprotein Blot Stain Kit with concanavalin A utilizes alkaline phosphatase-conjugated concanavalin A along with the fluorogenic substrate DDAO phosphate [9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate] to detect glycoproteins on nitrocellulose and poly(vinylidene difluoride) (PVDF) membranes. The detection procedure is similar to that of standard western (immuno)blotting. DDAO phosphate is rapidly converted to the long wavelength, red-fluorescent product, DDAO. DDAO absorbs maximally at either 275 nm or 646 nm and emits maximally at 659 nm. Consequently, the blots may be imaged using standard UV epi-illumination or with a laser-based gel scanner equipped with appropriate excitation source. The enzymatic amplification step greatly enhances the signal, allowing low nanogram detection of glycoproteins, a sensitivity on par with chemiluminescence detection methods. Pro-Q Glycoprotein Blot Stain kits with wheat germ agglutinin or with *Griffonia simpliifolia* lectin II (GS-II) allow detection of *N*-acetylglucosamine and sialic acid residues or terminal *N*-acetylglucosamine residues, respectively. The detection procedures for these lectins are quite similar to the concanavalin A method, and the same fluorogenic substrate, DDAO-phosphate is used in the kits.

Materials

Protein samples of interest
PVDF membrane
50% methanol
Wash solution II (see recipe)
Blocking solution (see recipe)
Pro-Q Glycoprotein Blot Stain Kit with Concanavalin A (Molecular Probes) containing:
  Concanavalin A, alkaline phosphatase conjugate (Con A-AP) stock solution (see recipe)
  DDAO phosphate stock solution (see recipe)
  Dimethylformamide (DMF)
  CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for ~20 gel lanes
Incubation buffer (see recipe)
10 mM Tris/1 mM $MgCl_2$, pH 9.5

Polystyrene staining dishes (e.g., weigh boat for minigel or larger container for larger gels)
Plastic wrap
UV epi-illumination and a digital or film camera, or a laser equipped with a 633-nm helium-neon laser or 635-nm diode laser source

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*), electroblotting procedures (*UNIT 6.2*), and SYPRO Ruby protein blot staining (see Alternate Protocol)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*).

   Typically, the protein sample is diluted to ~10 to 100 μg/ml with 2× sample buffer, heated for 4 to 5 min at 95°C, and 5 to 10 μl of diluted sample is then applied per gel lane for 8× 10–cm gels. Larger gels require proportionally more material.

For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and as alternating bands of positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (*UNIT 6.1*).

   The procedure is optimized for gels that are 0.5- to 1-mm thick.

Blot proteins

3. After electrophoresis, transfer the proteins to PVDF membrane using standard electroblotting procedures (*UNIT 6.2*).

   The use of nitrocellulose membranes is not recommended.

4. *Optional*: Stain blots with SYPRO Ruby protein blot stain at this point to visualize the total protein pattern and to verify that the blotting procedure was successful. Follow the staining procedure described in Alternate Protocol, steps 14 to 17.

   Total protein staining must be performed prior to lectin blotting as the blocking mixture will produce very high background on the blot. Since SYPRO Ruby protein blot stain is washed off during the subsequent lectin blotting process, it is important to document staining results before continuing with the procedure.

5. If the PVDF blot is dry, briefly hydrate in 50% methanol and incubate in wash solution II for 10 min at room temperature. Repeat the wash step for a total of three washes.

Visualize glycoproteins

6. Incubate the blot in blocking solution for 1 to 2 hr at room temperature.
7. Briefly pellet any potential protein aggregates in the Con A-AP stock solution by microcentrifugation. Using the supernatant only, dilute the Con A-AP stock solution 2000-fold by adding 5 µl to 10 ml of incubation buffer for a final concentration of 1 µg/ml. Remove the blocking solution that the blot is immersed in and incubate the blot with Con A-AP solution for 1 hr at room temperature.
8. Remove the diluted Con A-AP solution and wash the blot in blocking solution four times for 10 min each at room temperature.
9. Perform two final washes in wash solution II for 5 min each at room temperature.
10. Dilute the DDAO phosphate stock solution 1000-fold into 10 mM Tris/1 mM $MgCl_2$, pH 9.5, for a final concentration of 1.25 µM.

    Approximately 1 ml of the DDAO phosphate staining solution will be needed for an 8× 10–cm blot. Note that DDAO phosphate is unstable when stored at room temperature as an aqueous solution. Always make up the DDAO phosphate staining solution just prior to use.

11. Incubate the blot in freshly prepared DDAO phosphate staining solution.

    The staining step may be performed either face up or face down, depending on the configuration of the imaging instrumentation being used. If using UV epi-illumination or a laser scanner with a light source that illuminates from above the imaging bed, stain the blot face up. For laser scanners with light sources that illuminate the blot from below the imaging bed, stain the blot face down.

12. Using powder-free gloves, cut a piece of plastic wrap to the size of the blot. For face-up staining, place the blot on the plastic wrap and pipet 1 ml of DDAO phosphate staining solution onto the blot. For face-down staining, pipet 1 ml of the DDAO

phosphate staining solution onto the plastic wrap and lay the blot face down onto the solution, being careful not to trap air bubbles.

The time required for optimal staining must be determined empirically because the substrate turnover rate depends on the amount of glycoprotein on the blot. Generally, a 5- to 20-min incubation is sufficient, but overnight incubation is permissible. Do not wash the blot after staining as this will cause extensive loss of signal. The blot may be air-dried, however.

13. Visualize the fluorescent DDAO product using either UV epi-illumination and a digital or film camera, or using a laser equipped with a 633-nm helium-neon laser or 635-nm diode laser source. For UV epi-illumination, place the blot, signal side up, on a flat surface. For highest sensitivity and lowest background, use a UV-blocking filter, such as the SYPRO gel stain photographic filter. Long-pass filters with a cutoff at ~630 nm are ideal for CCD-cameras. For laser scanners, place the blot, signal side down, on the scanner bed. For highest sensitivity, match the light sources and filters of the instrument as closely as possible to the absorbance maximum (646 nm) and emission maximum (659 nm) of DDAO.

14. If desired, the Con A-AP complex can be stripped off of the blot and the blot reprobed with another lectin-AP complex or an antibody-AP complex. To strip, incubate the blot in stripping buffer for 40 min at 50°C with gentle agitation. Then, wash the blot in wash buffer two times for 5 min each at room temperature.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking solution

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
150 mM NaCl
0.2% (v/v) Tween 20
0.25% (w/v) Mowiol 4-88 (Calbiochem or VWR)
Store up to 6 months at room temperature

The use of Mowiol 4-88 in the Blocking buffer is not essential, but does decrease background staining and improves detection sensitivity. As an alternative to Mowiol 4-88, 0.5% (w/v) bovine serum albumin or 4% (w/v) gelatin (high purity, e.g., TopBlock from Juro Supply) may be used.

CandyCane molecular weight standards

For a standard lane on an 8 × 10–cm gel, dilute 0.5 µl of the standards with 7.5 µl of 2× sample buffer (see recipe) and vortex. This will result in ~250 ng of each protein per lane, a sufficient amount for detection of the glycoproteins by the Pro-Q Emerald 300 stain. For large 16 × 18–cm gels, double the amount of standard and buffer used. Store up to 6 months at room temperature.

Con A-AP stock solution

Prepare a 2 mg/ml stock solution of Con A-AP by dissolving the vial contents in 250 µl deionized water and add 2 mM sodium azide. The stock solution is stable for at least 6 months when stored undiluted at 4°C. Do not freeze.

DDAO phosphate stock solution

Add 200 µl DMF to the vial containing 250 µg DDAO phosphate {[9H-(1,3-dichloro-9, 9-dimethylacridin-2-one-7-yl] phosphate, diammonium salt} to make 1.25 mg/ml stock solution. Store the stock solution at –20°C, desiccated and protected from light. When properly stored, the stock solution should be stable for at least 6 months. When the solution turns a blue color, the substrate has broken down and is no longer usable. Prepare the working solution fresh.

Fix solution

Prepare a solution of 50% methanol and 50% deionized water. Store up to 6 months at room temperature.

One 8 × 10–cm gel will require ~100 ml of fix solution.

Incubation buffer

Prepare blocking solution (see recipe) with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Store up to 6 months at room temperature.

Periodic acid solution

Add 250 ml of 3% (v/v) acetic acid to the bottle containing the periodic acid (oxidizing solution) and mix until completely dissolved. Store up to 6 months at room temperature.

Sample buffer, 2×

100 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
20% (v/v) glycerol
4% (w/v) sodium dodecyl sulfate
0.1% (w/v) bromophenol blue
Store up to 6 months at room temperature

Wash solution

Prepare a solution of 3% (v/v) glacial acetic acid in water. Store up to 6 months at room temperature.

One 8 × 10–cm gel will require ~400 ml of wash solution.

Wash solution II

Prepare a solution of 50 mM Tris·Cl, pH 7.5/150 mM NaCl. Store up to 6 months at room temperature.

COMMENTARY

Background Information

The analysis of protein glycosylation is readily accomplished by polyacrylamide gel electrophoresis (Koch and Smith, 1990; Packer et al., 1997; Taverna et al., 1998; Koketsu and Linhardt, 2000). However, relatively few high-sensitivity methods have been developed to reliably detect oligosaccharide residues covalently attached to proteins for visualization in polyacrylamide gels or on electroblot membranes (Packer et al., 1997; Packer et al., 1999; Koketsu and Lindhardt, 2000). Perhaps the most common procedure to visualize glycoproteins reported in the literature entails detection by periodic acid/Schiff (PAS) staining using the colorimetric acid fuchsin dye. A major limitation of this method is that detection sensitivity is poorer than Coomassie Blue staining, rendering the technique obsolete for modern high-sensitivity proteomics investigations. Other methods in use include PAS-labeling with digoxigenin hydrazide followed by immunodetection with anti-digoxigenin antibody conjugated to alkaline phosphatase, or PAS-labeling with biotin hydrazide, followed by detection with horseradish peroxidase or alkaline phosphatase conjugated to streptavidin (Packer et al., 1995). Lectins are commonly employed to detect certain structural subclasses of glycoproteins by methods similar to those employed in immunoblotting (Koketsu and Lindhardt, 2000). All of these methods require that pro-

teins be electroblotted to membranes first and many glycoproteins transfer relatively poorly. In addition, detection of glycoproteins after electroblotting is very time consuming compared with direct detection in gels. A recently developed approach to the detection of glycoproteins relies upon the utilization of a new fluorescent hydrazide, Pro-Q Emerald 300 dye, that is affixed to glycoproteins using a standard PAS conjugation mechanism (Steinberg et al., 2001). The glycols present in glycoproteins are initially oxidized to aldehydes using periodic acid. The dye then reacts with the aldehydes on the glycoproteins to generate the fluorescent conjugate. A reduction step with sodium metabisulfite or sodium borohydride is not required to stabilize the resulting conjugate.

Critical Parameters

All stock solutions should be prepared using deionized water (dH_2O) having a resistance of at least 18 MΩ. All stock solutions may be stored for up to 6 months at room temperature, except when specifically indicated. Dilution of the DDAO phosphate stock solution or the Pro-Q Emerald 300 dye solution should be performed immediately prior to their use in the staining protocols. Both reagents are unstable when stored at room temperature as aqueous solutions. The staining methods outlined in this unit are highly sensitive and it is critical that all glassware and staining dishes be scrupulously clean. Gels and blots should never be touched or otherwise manipulated using bare hands. Always wear powder-free latex gloves when handling gels and blots during all staining procedures for the fluorescence detection of glycoproteins.

Troubleshooting

Should the detection sensitivity obtained using the cited fluorescence methods be suboptimal, there are two potential sources for the problem, either instrumental or chemical. With respect to the imaging instrument, it is important to clean the surface of the transilluminator after each use with deionized water and a soft cloth (e.g., cheesecloth). Otherwise, fluorescent dyes can accumulate on the glass surface and cause a high background fluorescence. The polyester backing on some pre-cast gels is highly fluorescent. For maximum sensitivity using a UV transilluminator, the gel should be placed polyacrylamide side down and an emission filter used to screen out the blue fluorescence of the plastic. For UV detection of fluorophores, a 300-nm UV-B transilluminator with six 15-watt bulbs is recommended. Excitation with different UV light sources, such as a simple hand held UV lamp will not provide the same level of detection sensitivity as a full-fledged transilluminator. For all three procedures described in this unit, using a Polaroid camera and Polaroid 667 black-and-white print film, the highest sensitivity is achieved with a 490-nm long-pass filter, such as the SYPRO protein gel stain photographic filter (S-6656; Molecular Probes). Gels are typically photographed using an f-stop of 4.5 for 2 to 4 sec, using multiple 1-sec exposures. Using a CCD camera, images are best obtained by digitizing at ~1024 × 1024–pixels resolution with 12-, 14-, or 16-bit gray-scale levels per pixel. A 520-nm long-pass filter is suitable for visualizing Pro-Q Emerald dye, while a 580-nm long-pass filter is appropriate for detection of DDAO. A CCD camera-based image analysis system can gather quantitative information that will allow comparison of fluorescence intensities between different bands or spots. Using such a system, the Pro-Q Emerald dye and the DDAO dye have a linear dynamic range of three orders of magnitude.

A potential problem associated with the Pro-Q Emerald 300 glycoprotein gel stain is nonspecific labeling of non-glycosylated proteins. The most common source of this problem is the presence of residual SDS in the polyacrylamide gel. Adding an extra fixation step to the procedure should prevent its occurrence. The author finds that an overnight fixation step for two-dimensional gels is advisable. When detecting glycans using Pro-Q Emerald 300 glycoprotein gel stain, it is prudent to run control gels in which the periodate oxidation step has been omitted. Similar precautions are advisable when evaluating results using other glycoprotein detection methods. This avoids erroneous interpretation of results arising from low levels of noncovalent binding of dye molecules or confusion arising from the inherent fluorescence of certain high-abundance proteins in the gel profile.

The most common problem encountered using the Pro-Q glycoprotein detection kit with concanavalin A is poor signal intensity. This is usually due to decomposition of the DDAO phosphate stock solution. When the stock solution appears by eye to be a blue color, the substrate has broken down and is no longer usable.

Table 6.8.1 Comparison of Commonly Used Glycoprotein Detection Methods for Polyacrylamide Gels and Electroblot Membranes

Detection method	Time required (hr)	Number of steps	Detection sensitivity (1) α1-acid glycoprotein (40% CHO)[a] (2) glucose oxidase (12% CHO)[a] (3) avidin (7% CHO)[a]		Assets (+) or liabilities (–)
			Gels	*Blots*	
Acid fuchsin sulfite (pararosaniline)	5-6	7	(1) 75 ng (2) 150 ng (3) 150 ng	20 ng 75 ng 75 ng	(+) short procedure (+) can use either on blots or in gels (–) poor sensitivity
Biotin hydrazide/ streptavidin-HRP Luminol detection reagents	6	11	(1) na (2) na (3) na	18-37 ng 37 ng 150 ng	(–) signal fades over time, optimal image, 20-30 min (–) cannot save and reimage blots
Biotin hydrazide/ streptavidin-alkaline phosphatase NBT/BCIP solution	5-6	11	(1) na (2) na (3) na	2 ng 5-9 ng 18-37 ng	(+) good sensitivity (+) can save and reimage blots (–) long procedure (–) cross reaction with carbonic anhydrase
Pro-Q Glycoprotein Detection Kit with Con-A alkaline phosphatase (see Basic Protocol 2)	4	5	(1) na (2) na (3) na	not detected <15.6 ng 15.6 ng	(+) can save and re-image blots (+) can strip and reprobe (+) can post-stain with total protein stains (+/–) stains specific subsets of glycoproteins (–) long procedure
Dansyl hydrazine	4	9	(1) 1.25-2.5 ng (2) 1.25-2.5 ng (3) 16-19 ng	not tested	(+) inexpensive (–) requires longer exposure for competitive brightness (–) low-level non-specific detection of unglycosylated proteins (–) requires hot, acidified DMSO
Digoxigenin-3-*O*-succinyl-ε-aminocaproic acid hydrazide/ Anti-digoxigenin-alkaline phosphatase. Stain with NBT/x-phosphate	5-6	11	(1) na (2) na (3) na	2 ng 5-9 ng 18-37 ng	(+) good sensitivity (+) can save and reimage blots (–) long procedure (–) cross reaction with carbonic anhydrase
Pro-Q Emerald 300 Dye (see Basic Protocol 1 and Alternate Protocol)	2 (blots) 4 (gels)	7	(1) 300 pg (2) 300 pg (3) 1-2 ng	2 ng 18 ng 9 ng	(+) can use either on blots or in gels (+) great sensitivity (+) can save and re-image blots (+) short procedure (+) can counterstain unglycosylated proteins with SYPRO Ruby dye

[a]Abbreviations: CHO, carbohydrate; na, not applicable.

Anticipated Results

The performance characteristics of the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, Pro-Q Emerald 300 Glycoprotein Blot Stain Kit, and Pro-Q Glycoprotein Detection Kit with Concanavalin A are summarized in Table 6.8.1 and contrasted with alternative glycoprotein detection technologies. The fluorescence-based methods permit detection of low-nanogram amounts of glycoprotein with a dynamic range of quantitation that encompasses three orders of magnitude of glycoprotein abundance. Pro-Q Emerald 300 dye may be used to detect a variety of glycoconjugates in addition to glycoproteins, such as bacterial lipopolysaccharides (LPS) and glycogen. Detection sensitivity for chondroitin 4-sulfate, however, is ~3000-fold poorer than glycogen or LPS, with limits of detection in the vicinity of 16 μg of applied material. This is not unexpected as glycosaminoglycans such as chondroitin sulfate, hyaluronic acid, and keratan sulfate are known to stain poorly by conventional PAS procedures. Concanavalin A specifically binds to nonsubstituted and 2-*O*-substituted α-mannosyl residues and thus detects fewer glycoproteins than the Pro-Q Emerald 300 dye. For example, α1-acid glycoprotein is not detected by concanavalin A. Similarly, glycoproteins such as ovomucoid (28 kDa) and ovotransferrin (76 kDa) are not effectively detected by concanavalin A. The differences in staining specificity between the Pro-Q glycoprotein detection kit with concanavalin A, the Pro-Q glycoprotein detection kit with wheat germ agglutinin and the Pro-Q Emerald 300 glycoprotein stain kits can be exploited in defining structural features of glycans on glycoproteins.

Time Considerations

The time considerations and number of steps required to detect glycoproteins using the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, Pro-Q Emerald 300 Glycoprotein Blot Stain Kit, and Pro-Q Glycoprotein Detection Kit with Concanavalin A are summarized in Table 6.8.1 and contrasted with alternative glycoprotein detection technologies. The methods can be completed in ~2 to 4 hr and require 5 to 7 steps to complete. This compares favorably with other methods that may require as much as 6 hr and 11 steps to complete.

Literature Cited

Beeley, J. 1985. Glycoproteins and proteoglycan techniques. *In* Laboratory Techniques in Biochemistry and Molecular Biology (R. Burdon and P. van Knippenberg, eds.) vol. 16, pp. 5-28. Elsevier Press, New York.

Hirabayashi, J., Arata, Y., and Kasai, K. 2001. Glycome project: Concept, strategy and preliminary application to *Caenorhabditis elegans*. *Proteomics* 1:285-294.

Koch, G. and Smith, M. 1990. The analysis of glycoproteins in cells and tissues by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* 11:213-219

Koketsu, M. and Linhardt, R. 2000. Electrophoresis for the analysis of acidic oligosaccharides. *Anal. Biochem*. 283:136-145.

Packer, N., Pawlak, A., Kett, W., Gooley, A., Redmond, J., and Williams, K. 1997. Proteome analysis of glycoforms: A review of strategies for the microcharacterization of glycoproteins separated by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* 18:452-460.

Packer, N., Ball, M., and Devine, P. 1999. Glycobiology and proteomics. *In* 2-D Proteome Analysis Protocols, Methods in Molecular Biology (A. Link, ed.) vol. 112, pp.341-352. Humana Press, Totowa, NJ.

Patton, W. 2000a. A thousand Points of light; The application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics. *Electrophoresis* 21:1123-1144.

Patton, W. 2000b. Making blind robots see; The synergy between fluorescent dyes and imaging devices in automated proteomics. *BioTechniques* 28:944-957.

Raju, T. 2000. Electrophoretic methods for the analysis of N-linked oligosaccharides. *Anal. Biochem.* 283:125-132.

Reuter, G. and Gabius, H. 1999. Eukaryotic glycosylation: Whim of nature or multipurpose tool? *Cell Mol. Life Sci.* 55:368-422.

Steinberg, T., Pretty On Top, K., Berggren, K., Kemper, C., Jones, L., Diwu, Z., Haugland, R., and Patton, W. 2001. Rapid and simple single nanogram detection of glycoproteins in polyacrylamide gels and on electroblots. *Proteomics* 1:841-855.

Taverna, M., Tran, N., Merry, T., Horvath, E., and Ferrier, D. 1998. Electrophoretic methods for process monitoring and the quality assessment of recombinant glycoproteins. *Electrophoresis* 19:2572-2594.

Key References

Steinberg et al., 2001. See above.

Describes detection of glycoproteins in gels and on blots using Pro-Q Emerald 300 Glycoprotein Detection Kits as well as the detection of concanavalin A–binding and wheat germ agglutinin–binding glycoproteins on blots using lectin–alkaline phosphatase conjugates and DDAO phosphate.

Berggren, K., Steinberg, T., Lauber, W., Carroll, J., Lopez, M., Chernokalskaya, E., Zieske, L., Diwu, Z., Haugland, R., and Patton, W. 1999. A luminescent ruthenium complex for ultrasensitive detection of proteins immobilized on membrane supports. *Anal. Biochem.* 276:129-143.

Describes counter-staining with SYPRO Ruby dye for the detection of total protein profiles on electroblot membranes.

Berggren, K., Chernokalskaya, E., Steinberg, T., Kemper, C., Lopez, M., Diwu, Z., Haugland, R., and Patton, W. 2000. Background-free, high-sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfate–polyacrylamide gels using a luminescent ruthenium complex. *Electrophoresis* 21:2509-2521.

Describes counter-staining with SYPRO Ruby dye for the detection of total protein profiles in polyacrylamide gels.

Lopez, M., Berggren, K., Chernokalskaya, E., Lazarev, A., Robinson, M., and Patton, W. 2000. A comparison of silver stain and SYPRO Ruby protein gel stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis* 21:3673-3683.

Describes optimized methods for protein identification by matrix-assisted laser desorption time-of-flight mass spectrometry after staining gels with SYPRO Ruby dye.

Internet Resources

http://www.cbs.dtu.dk/databases/OGLYCBASE/

O-GLYCBASE; a database of 198 glycoprotein entries with experimentally verified O-glycosylation site information.

http://www.glycosuite.com

GlycoSuite; a relational database that curates information from the scientific literature on glycoprotein derived glycan structures, their biological sources, the references in which the glycan was described, and the methods used to determine the glycan structure.

http://www.expasy.ch/tools/glycomod/

GlycoMod; a software tool designed to find all possible compositions of a glycan structure from its experimentally determined mass.

http://www.probes.com

Molecular Probes commercial Web site containing information about fluorescence detection technologies, including glycoprotein, total protein, lipopolysaccharides, and nucleic acids.

Contributed by Wayne F. Patton
Molecular Probes, Inc.
Eugene, Oregon

Digital Electrophoresis Analysis

Gel electrophoresis has become a ubiquitous method in molecular biology for separating biomolecules. This prominence is the result of several factors, including the robustness, speed, and potentially high throughput of the technique. The results of this method are traditionally documented using silver halide–based photography followed by manual interpretation. While this remains an excellent method for qualitative documentation of single-gel results, digital capture offers a number of significant advantages when documentation requires quantitation and sophisticated analysis. Digital images of gel electropherograms can be obtained rapidly using an image-capture device, and the images can be easily manipulated using image analysis software.

REASONS FOR DIGITAL DOCUMENTATION AND ANALYSIS

There are several reasons to consider digital documentation and analysis of electrophoresis results. These justifications can usually be categorized into issues of ease of handling, accuracy, reproducibility, and cost.

Ease of Handling

A major advantage of the digital revolution has been in storage and retrieval of information. Storage in notebooks and filing cabinets previously meant that searching for specific data or experiments was a tedious manual process. With digital information, modern search engines can quickly find specific information in a fraction of the time usually required for a manual search. Making backup copies of nondigital data can be difficult, expensive, and time-consuming since it requires copying, retyping, or photographic reproduction. Copies of digital data can be generated more easily and at reduced costs.

Manipulation of information is also easier when it is in a digital format. While the cut-and-paste analogy comes from physical documentation, it takes on a new perspective when applied digitally. Electrophoresis images can be resized, cropped, and inserted into reports. Data can be passed to spreadsheets and statistical packages for analysis and later insertion into notebooks and reports. These reports can be passed out via the Internet to colleagues throughout the world. A single individual can do all this in a few hours.

Digital analysis also provides an easier method for handling the data when comparing large numbers of results or large numbers of separate experiments. Research that requires comparing the banding patterns on 1000 gels containing 50 lanes each can be an undertaking of heroic proportions if the analysis is performed manually. Database software can dramatically speed the analysis and handle the more mundane tasks, leaving the researcher free to interpret the data.

Accuracy

The human eye is an extremely versatile measuring instrument. It can handle light intensities covering a range of nearly nine orders of magnitude and is sensitive to a fairly wide spectrum of light (Russ, 1995). Yet the eye cannot accurately and reproducibly quantitate density and patterns, nor can it deal with large numbers of bands or spots. Accuracy of measurement is a primary reason for using digital analysis on electrophoretically separated proteins and nucleic acids. Two categories of accuracy are key to digital analysis: positional accuracy, which is important for mobility determinations such as molecular weight, and quantitative accuracy.

Positional accuracy is based on both resolution of the recording medium and measuring accuracy. Silver halide–based recording has a theoretical resolution based on ~2000 imaging elements (silver grains) per inch. Measurement traditionally occurs using a ruler, with an accuracy of ~20 to 40 elements (50 to 100 elements per inch). In comparison, typical digital systems have 200 to 600 picture elements (pixels) per inch. The advantage that digital systems have is in measuring accuracy, which can occur at the level of a single imaging element.

Quantitative accuracy is also an issue. The amount of material represented by a band or spot is difficult to determine accurately from an image of a gel unless it is a digital image. On a digital image, the amount present is directly correlated with the derived volume of the band or spot—the volume is calculated using the intensity values of the pixels within the object.

Reproducibility

Any technique or measurement is only as good as its ability to be faithfully replicated. With software-defined routines, measurements

Contributed by Scott Medberry and Sean Gallagher
Current Protocols in Cell Biology (2002) 6.19.1-6.19.14

are performed in the same manner every time. Allowing the computer to do repetitive tasks and complicated calculations minimizes the chance for individual errors. This does not imply that such measurements are correct, just that they are reproducible. An incorrect routine or algorithm can also invalidate data.

Cost

A consideration when evaluating any laboratory method is cost. Digital electrophoresis analysis equipment can be expensive. In many cases, however, it offers the only method for achieving acceptable analysis performance. In other cases, equal performance can be achieved using silver halide technology. However, traditional photography can also be expensive when the costs of consumable supplies such as film and developers, as well as other expensive requirements such as developing tanks and dark rooms, are included. Often, digital methods can be a good choice when all costs are considered.

KEY TERMS FOR IMAGING

There are several specialized terms encountered during digital image analysis. The most commonly encountered are contrast, brightness, gamma, saturation, resolution, and dynamic range. They describe controls on how the light detectors report a range of light intensities. Below is a brief description of each.

Contrast

Contrast describes the slope of the light intensity response curve. An increase in the contrast increases the slope of the curve. The result is a more detailed display over a narrowed range of intensities with less detail in the remaining portions of the intensity range. This is depicted in Figure 6.9.1A and 6.9.1B, where a normal, unadjusted image and a contrast-adjusted image are displayed, respectively. The contrast was increased on midrange intensity values in Figure 6.9.1B to highlight band intensity differences at the expense of background information. Images with a narrow range of informative intensities can benefit from increasing the contrast, since that effectively increases the scale and improves detection of minor differences in intensity. Contrast settings should be lowered if information is being lost outside of the contrast range. For example, in Figure 6.9.1B, loss of background information between peaks indicates that this image should not be used for quantitation.

Brightness

While brightness can have many different definitions, only one will be considered here. Brightness shifts the light intensity response curve without changing its slope as is shown in Figure 6.9.1C. Another name for brightness is black level, since it is commonly used to control the number of black picture elements (pixels) in an image. Incorrect brightness levels can lead either to high background and potential image saturation or, as is illustrated in Figure 6.9.1C, to a total loss of background information and partial loss of band information.

Gamma

Nonlinear corrections are often applied to images to compensate for how the eye perceives changes in intensity, how display devices reproduce images, or both. The most common correction is an exponential one, with the exponent in the equation termed the gamma. A typical gamma value for camera-based systems is 0.45 to 0.50, and is illustrated in Figure 6.9.1D. This is a compromise value that compensates for the 2.2 to 2.5 gamma present in most video monitors and the print dynamics of most printers. Since it is a nonlinear correction, special care must be taken if quantitation is desired. Unless directed to by the manufacturer, gamma values other than 1.0 should be avoided when quantitating. More information on gamma correction can be found on Poynton's Gamma FAQ (*www.inforamp.net/~poynton/Poynton-color.html*).

Dynamic Range

Dynamic range describes the breadth of intensity values detectable by a system and is usually expressed in logarithmic terms such as orders of magnitude, decades, or optical density (OD) units. A large dynamic range is important when trying to quantitate over a wide range of concentrations. The most accurate quantitation occurs in the linear part of the dynamic range, which is usually not the complete dynamic range of the system. An additional consideration is the dynamic range of the visualization method. Many popular visualization methods have linear dynamic ranges of 1 to 2.5 orders of magnitude. An imaging system with greater dynamic range analyzing the results of such a visualization method will not improve the dynamic range.

Saturation

Saturation occurs when a detector or visualization method receives input levels beyond

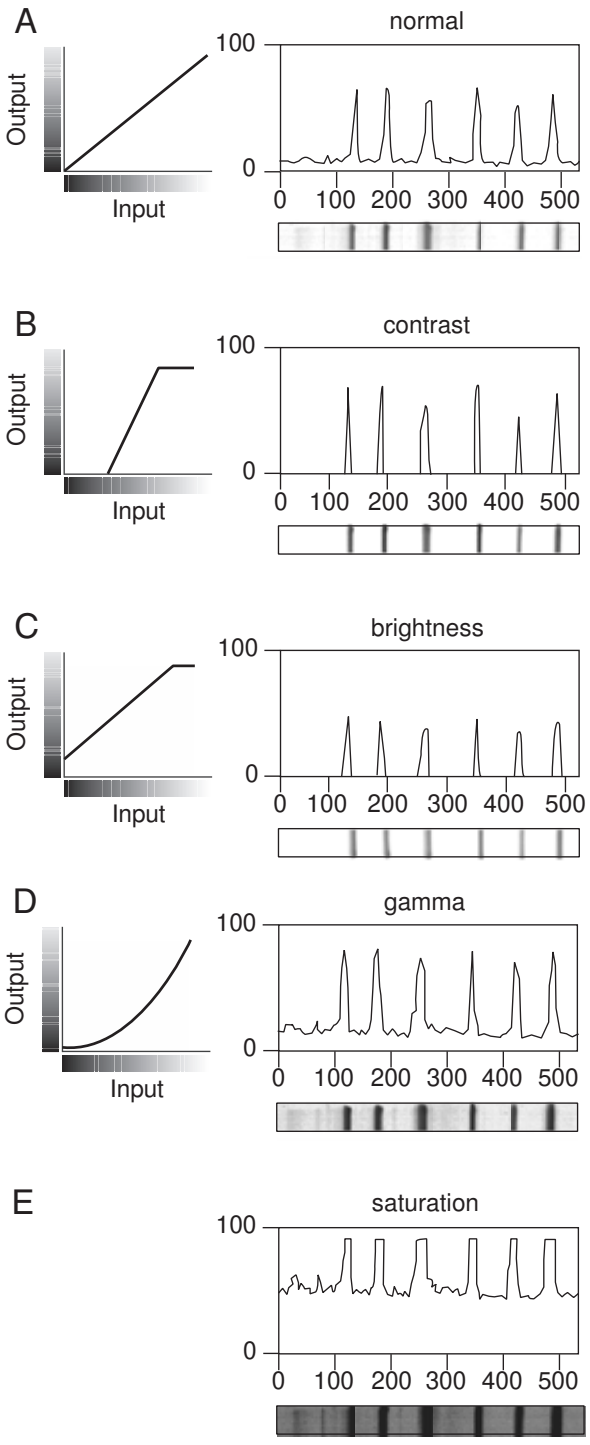


Figure 6.9.1 Examples of how altering image capture settings affects the image and the analysis. The graph on the left displays the light intensity response curve used for image capture while the image and resulting lane profile on the right display how the setting affects the image. The lane profile displays pixel position versus normalized pixel intensity (**A**). In this case, the output has not been altered, giving a straight line with a slope of 1 on the response curve. (**B**) The image acquisition was adjusted to increase the contrast of the displayed image. Although useful for images with a narrow range of informative intensity values, increasing the contrast can lead to a loss of low and high values. (**C**) Decreasing the brightness reduces peak values but also leads to a loss of the weak bands and original background. (**D**) Gamma adjusts raw data to appear more visually accurate. Note that this leads to a loss of fidelity between the adjusted image and the original. (**E**) Saturation indicates that the detector is reporting its maximum value or that the dynamic range for the visualization method has been exceeded.

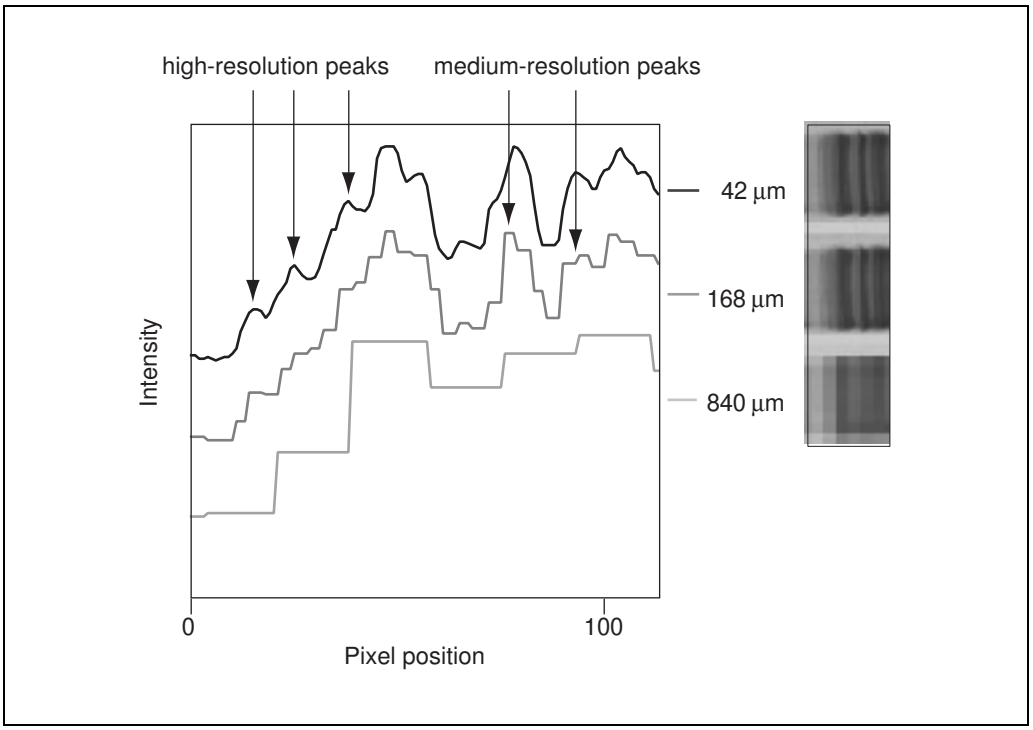


Figure 6.9.2 The effect of spatial resolution on the ability to detect closely spaced objects. Whole-cell protein lysates from *E. coli* were separated using SDS-PAGE and visualized with Coomassie blue staining. An image was captured at 42 μm (600 dpi), 168 μm (150 dpi), and 840 μm (30 dpi) from a segment of the lane, and a lane profile was generated for each image. The lane profiles have offset intensities to allow for comparison. Only major bands can be detected with the low-resolution image (840 μm); at higher resolutions more bands are detectable.

the maximum end of the dynamic range. This results in a loss of detail and quantitative information from those data points that are saturated. For fluorescent and luminescent samples, reduction in the sampling time can sometimes correct saturation problems. Optical density-based visualization techniques can also generate saturated images, as is illustrated in Figure 6.9.1E; this can sometimes be avoided with longer sampling times or increased detection source intensities. More often, it will be necessary to perform another electrophoresis with more dilute samples or to alter the visualization process to generate a less optically dense material.

Resolution

Resolution is the ability of a system to distinguish between two closely placed or similar objects. Three types of resolution are important for analysis—spatial resolution, intensity resolution, and technique-dependent resolution.

Spatial resolution is the ability to detect two closely placed objects in one-, two-, or three-dimensional space. It is most accurately described as the closest distance two objects can be placed and still be detected as separate objects. In practice, it is often defined nominally in terms of the number of detectors per unit area such as dots per inch (dpi) or the number of detectors present in total or in each dimension such as 512 × 512 (262,144 total detectors). Actual resolution is less than half the nominal resolution due to the need for two detectors for every resolvable object (one for the object and one for the separation space) and the effects of optical resolution. Figure 6.9.2 demonstrates how spatial resolution can affect detection of objects. The 42-μm resolution image allows detection of closely spaced bands, the 168-μm resolution image detects fewer bands, and the 840-μm resolution image detects only major bands. For instruments with on-line detection systems, a pseudo spatial resolution is often reported in units of time from the start of the separation or the time interval between two objects crossing the detection path.

Intensity resolution is the ability to identify small changes in intensity. It is a function of both the dynamic range of a detector and the number of potential values that detector can report. Greater dynamic range decreases the intensity resolution of a given detector. The number of potential values a detector reports is

described by its bit depth. An 8-bit detector can report 256 (2^8) different possible values, while a 12-bit detector can report 4,096 (2^{12}) values, and a 16-bit detector can report 65,536 (2^{16}) values. The higher the bit depth, the greater the intensity resolution.

Technique-dependent resolution directly affects the spatial and intensity resolution. Electrophoretic separation techniques that generate overlapping objects or that have object separation distances shorter than the spatial resolution will fail to provide reliable data. Many factors, including the amount of sample loaded, gel pore size, buffer constituents, and electrophoresis field strengths, can dramatically affect separation and resolution of biomolecules. Likewise, detection methods that can only generate a small range of discrete intensity values will not benefit from systems with improved intensity resolution.

IMAGE CAPTURE

Devices

Capturing digital images involves a detection beam or source, a sensor for that beam or source, and some method of assembling a two-dimensional image from the data generated. Most systems use a light source for detection. The light wavelengths used range from ultraviolet (UV) to infrared (IR) and can be broad spectrum or narrow wavelength. Broad-spectrum detection is more versatile since it can often be used for more than one detection wavelength. However, when compared to narrow-wavelength sources such as lasers, broad-spectrum detection suffers from reduced sensitivity and reduced dynamic range. While many types of light sensors have been used, including charge-coupled devices (CCDs), charge-injection devices (CIDs), and photon multiplier tubes (PMTs), technology advances in CCDs have led to their dominance. CCDs are semiconductor imaging devices that convert photons into charge. This charge is then read and converted into a digital format via an analog-to-digital converter (ADC).

The method of image assembly depends on the light source and detector geometry. One method is to capture the image all at once using a two-dimensionally arrayed CCD detector similar to the detectors found in digital and video cameras. Typically a camera-type sensor is paired with a light source that evenly illuminates the sample. This same sensor is often used with fluorescent and chemiluminescent detection methods, as its ability to detect light continuously over the entire sample reduces image capture times. Another method of image assembly is to capture the image a line at a time. This typically involves a linearly arrayed CCD scanning slowly across the sample in conjunction with the detection beam of light. The data from each line is then compiled into a composite image. Spatial resolution in this method can be significantly better on large-format samples than the resolution of a camera-based system. This method is also advantageous when OD-based detection is used, since the more focused light beam is usually of higher intensity and can penetrate denser material. A third method of image assembly is to use a point light source and single-element detector on each point on a sample. The image is then compiled from each point sampled. This method is slower than the others but can offer extremely high resolution and sensitivity. A fourth commonly encountered method is that of generating a pseudoimage of electrophoresis results through the use of a finish line type of detection system. This is comprised of a light source positioned at the bottom of the gel (i.e., the end opposite of the site of sample loading) and light detectors positioned next to each lane to detect the transmitted light or emitted fluorescence. A lane trace is generated using time on the *x* axis and light intensity on the *y* axis. The pseudoimage is then generated from this data (Sutherland et al., 1987).

Capture Process

Prior to image capture, electrophoretic separation and any visualization steps are performed. To calibrate the separation process, standards are usually run at the edges of the gel and often at internal positions. If quantitation of specific proteins or nucleic acids is to be performed, a dilution series of standards with similar properties to the experimental samples should also be included. After separation, the protein or nucleic acid is visualized if necessary. Visualization can include binding of a fluorochrome or chromophore such as Coomassie blue, precipitation of metal ions such as copper, silver, or gold, enzymatic reactions, and exposure of film or phosphor screens to radiant sources. These methods can be grouped based on the type of detection into optical density, fluorescence, chemiluminescence, and radioactivity. The suitability of popular detection devices with these methods is described in Table 6.9.1. Once visualization has occurred, image capture consists of the following steps: previewing the image while

Table 6.9.1 Compatability of Popular Image-Capture Devices with Common Visualization Methods[a]

Visualization method	Image-capture device				
	Silver halide photography	CCD camera	Desktop scanner	Storage phosphor	Fluorescent scanner
Optical density[b]	+	+	++	−	−
Fluorescence	+	+	−	−	++
Chemiluminescence	+	++	−	±	−
Radioactivity	+	−	−	++	−

[a]The device with the highest sensitivity and greatest dynamic range for a visualization method is marked with a ++, other devices that can detect this visualization method are indicated with a +, and devices that are not suitable for a visualization method are indicated with a −. A ± indicates that only some devices of this type can be used with this visualization method.

[b]Optical density methods include Coomassie blue staining.

adjusting capture parameters, capturing the image, and saving the image for later analysis.

During the preview process, capture parameters are optimized for data content and for ease and rapidity of later processing steps. Typically, the first step is to place the sample so that when the image is captured, the rectangular edges of the gel are horizontal and vertical on the monitor and any lanes are either horizontal or vertical. Since band and spot detection will be much easier if the image is properly oriented, this eliminates the need to later rotate the image digitally. Image rotation is time-consuming and can result in spatial linearity errors (a change in the size and shape of objects in image) caused by rectangular image-capture device geometries. The next step for camera-based systems is to adjust magnification and to focus the sample image. For thicker samples, it might be necessary to reduce the aperture on camera-based systems to get a sufficient depth of field to focus the entire sample. Often at this point image imperfections—e.g., dust, liquid, or other foreign objects that will detract from later analysis—are detected, and they need to be removed. Next, image intensity is set. Within the area of interest on the image, band or spot peaks should have values less than the maximum saturated value, and the background should have nonzero values. This is usually accomplished through adjustment of the light-source intensity or the sensor signal integration. If the device allows precapture optimization of other parameters such as spatial resolution, contrast, brightness, gain, or gamma, they are adjusted next. Note that this only applies to controls that affect the response of the sensor or processing of the image prior to a data reduction step and not to controls that affect the image at later stages. The latter process can enhance visualization of specific features but is best left to adjustments in look-up tables (LUTs) in later analysis steps rather than during image capture, since there is a risk of data loss during postacquisition image processing. LUTs are indexed palettes or tables where each index value corresponds to color or gray-scale intensity values present in an image. Many image analysis programs alter LUTs instead of image values directly, since it both is faster and does not change the original image data.

Once all the capture parameters are optimized, the image capture process is initiated. This might take less than a second for images captured with camera-based detectors and up to hours for scanning single-point detectors. When the image has been captured, it should be carefully examined for content. It should fully capture the area of interest and the parameters should have been set so that all necessary information is detectable. Furthermore, it should be in a form that will allow for easy analysis. Extra time spent optimizing the capture parameters will often result in a reduction in total image analysis time and in an increase in data quality. When the best possible image has been captured, it often contains information outside the area of interest. While this is unlikely to cause problems with later analysis, it is often advantageous to crop the image so that the only portion that is saved contains the area of interest. This reduces the amount of disk space necessary to store the image, and the image usually will load and analyze faster with the analysis software.

The last step in image capture is to save the image. Several options are available at this point, including choosing which location to

save the image at, what file type or format to use, and whether to use some form of compression.

The location where the image is saved is not as trivial a question as it might seem if the image will need to be transferred to another computer at some point. File sizes can easily exceed 15 megabytes on high-resolution images. This is a manageable size for hard drives but exceeds current floppy drive sizes by an order of magnitude. There are software utilities available that will subdivide files into disk-size chunks and then reassemble them at the next computer, but this is an inconvenient and slow method. If the computer used to help capture the image is connected to a network, the image files can easily be transferred this way or potentially saved on a central server. Alternatively, several types of high-capacity removable media are available (e.g., Zip or Jazz). This usually requires the installation of additional hardware onto two or more computers but does make backing up data easier.

Since image files can be very large, compression techniques are sometimes used to reduce disk space requirements. Compression algorithms use several methods, typically by replacing frequent or repetitive values or patterns with smaller reference values and by replacing pixel values with the smaller difference values describing the change in adjacent pixels. When the file is later decompressed, the compressed values are then replaced with the original information. Not all images compress equally, with simple images containing mostly repetitive motifs compressible by ≥90%, while complex images will benefit much less from compression. Because compression is a much slower method of saving files and will not benefit every file, compression is not used to save all files. Several different forms of compression are available but are separable into two main classes, lossless and lossy. Lossless methods faithfully and completely restore the image when it is decompressed (no loss of data) but offer only moderate file compression; compression values range from ~10% to 90%, depending on the image. Examples of lossless compression include Huffman coding (Huffman, 1952), RLE (Run Length Encoding), and LZW (Lempel, Ziv, and Welch; Welch, 1984). In comparison, lossy methods such as JPEG (Joint Photographic Experts Group), MPEG (Moving Picture Experts Group), or fractal compression schemes can reach compression values of ≥98% (Russ, 1995). The trade-off is that not all information from the original file is recovered during decompression. Lossy compression is sometimes necessary for applications with extremely large image files such as real time video capture, but it usually represents an unacceptable loss of data if used with electrophoresis image capture.

Many different file types have been developed to store digital images. Some of these file types are proprietary or hardware specific. For example, PICT is a Macintosh format and BMP is a PC-compatible format. Each file type has its own structure. Some types do not allow compression, for others it is optional, and for some it is mandatory. File types vary in the types of images they support, particularly in the number of colors or gray levels. Below is a brief description of a few of the more prevalent file types.

TIFF (Tagged-Image File Format) is one of the most commonly used formats. It is particularly versatile since it is an open format that can be modified for specific applications. One reason for its versatility is the ability to attach or tag data to the image. The tags can include information such as optical density calibration, resolution, experimenter, date of capture, and any other data that the application software supports. TIFF images can be monochrome, 4-, 8-, or 16-bit gray-scale, or one of many color-image formats. Compression is optional, with LZW, RLE, and JPEG often supported (Russ, 1995). Since TIFF is supported by both Macintosh and PC computers, it is a good choice for multiple-platform environments. The versatility of TIFF can also be a weakness. Since there are many different tagging schemes and since not all programs support all possible compression and color schemes, it is sometimes not possible for one program to access the information in a TIFF file generated by a different program.

GIF (Graphics Interchange Format) is a file format that is widely encountered on the Internet due to its compactness and standardization. Its compactness is attributable to a mandatory modified LZW compression. Another feature of GIF is the use of a LUT to index the values in the image. One interesting ability of GIF is that it supports storing multiple images within a single file. This can offer some advantages for applications such as time-lapse image capture. A GIF image can contain no more than 256 individual color or gray levels and does not support intensity resolutions higher than 8 bit. In addition, since the image

is implemented as a LUT, it also is not a true gray-scale image. Due to these limitations and others, alternative formats such as PNG have been developed to replace GIF.

PICT is a file format and graphics metafile language (it contains commands that can be played back to recreate an image) designed for the Apple Macintosh. It can contain both bitmap images and vector-based objects such as polygons and fonts. It supports a ≤256-gray-level LUT, and monochrome images can be RLE compressed. Because it only offers a 256-gray-level LUT, it has the same weaknesses that GIF does with true gray-scale and high-intensity-resolution images. In addition, any vector objects in the image are difficult to translate on a PC since they are designed to be interpreted by Macintosh QuickDraw routines.

BMP is the native bitmap file format present on Windows-based PCs. It supports 2-, 16-, 256-, or 16-million-level images. With images of ≤256 gray levels, it implements a LUT, while the highest-resolution image is implemented directly. RLE compression is optional for 16- and 256-gray-level images. Since compression is prohibited on 16 million-gray-level images and there is no intermediate level supported beyond 256 levels, BMP is not a good choice for images with high-intensity resolution requirements.

ANALYSIS

Once the image has been captured, the data needs to be analyzed and distilled into information about the results of the electrophoresis experiment. Through the use of standards and experimenter input, this software-driven process can estimate mass and quantity of objects in an image and detect relationships between objects within one image and between similar images. The type of software used depends on the analysis to be performed. Images from single electrophonetic separations are examined by one-dimensional analysis software optimized for lane-based band detection. Images from two-dimensional electrophoresis are best handled by specific programs designed to detect spots and to assign two mobility values and a quantity value to the spot. After the initial characterization of bands and spots, comparisons are often made between bands or spots from different experiments through the use of database programs and matching algorithms.

Software for One-Dimensional Analysis

Lane positioning

For one-dimensional analysis, the first activity is to detect the lanes on the image. One of three different methods is commonly employed for this. For images with straight, well-defined lanes with a large number of bands, automatic lane-detection algorithms can quickly and accurately place the lanes. On images with very well-defined lanes, such as pseudoimages from finish-line type electrophoresis equipment, automated lane calling based on image position is possible. For images with "smiling," bent, or irregular lanes, manual positioning of the lanes is often the fastest and most accurate method of lane definition. Regardless of the method of identifying the lanes, the lane boundaries need to be carefully set for accurate quantitation and mass determinations. Lane widths should be wide enough so that the entire area of all bands in that lane are included, but they should not be so wide as to include bands from adjacent lanes. To accomplish this, curved or bent lanes might need to be used in order to follow the electrophoresis lane pattern. Lane length and position also must be adjusted as necessary so that all bands of interest are included. If mass determinations are necessary, the sample loading point should probably also be included in the lane or be the start of the lane. At this point, lines of equal mobility (often called R_f or iso-molecular-weight lines) are added to the image as necessary. These lines allow for correction of lane-to-lane deviations in the mobility of reference bands and generate more accurate measurements of mass. A similar form of correction is also possible for within-lane correction of mobilities. This correction is important for accurate detection and quantitation of closely spaced bands.

Band detection

Once the lanes have been defined, the bands present in each lane need to be detected. There are many methods for detecting bands. One method is to systematically scan the lane profile from one end to the other, identifying regions of local maxima as bands. Another common method is to use first- and second-order derivatives of the lane image or lane profile in order to find inflection points in the change of slope in pixel intensity values (Patton, 1995). Regardless of the method used, it is often necessary to alter the search parameters so that they perform reliably under a given experimental condition.

Typical search parameters include ones for detection sensitivity, smoothing, minimum interband gaps, and minimum or maximum band peak size. Smoothing reduces the number of bands detected due to noise in the image. A minimum interband gap is often used to avoid detection of false secondary bands on the shoulders of primary bands. Limitations on peak sizes, especially for within-lane comparison to the largest band's peak, can be a useful way to allow sensitive detection of bands in underloaded lanes without detecting false bands in overloaded lanes.

Band edges are often detected in addition to band peaks in order to further define bands or to quantitate band amounts. This can be accomplished by using local minima, derivatives of the lane profile, or fixed parameters such as image distances or a percentage of band peak height. The band edges can be applied as edges perpendicular to the long axis of the lane or as a contour of equal intensity circling the band. The perpendicular method is advantageous for bands with uneven distribution of material across the face of the band, while the latter method is better for "smiling" or misshapen bands.

Background subtraction

With nearly all electrophoresis procedures, the most informative images have a low level of signal intensity at each pixel that does not result from protein or nucleic acid. Instead, this background intensity is attributable to the gel medium, the visualization method, electronic noise, and other factors. Since this background tends to be nonuniformly distributed throughout the image, failure to subtract it can make band detection and quantitation less accurate. Many methods of background subtraction are possible. Sometimes it is possible to generate a second image under conditions that do not detect the protein or nucleic acid. The second image is then digitally subtracted from the data-containing image to remove the background. More often, background information must be obtained from a single image. If the background varies uniformly across the image, a line that crosses the variation can be defined at a point where no bands are present. The intensity values at each point on the line can be used as the background value for the pixels perpendicular to the line at that point. Commonly, background is also present as variations in intensity along the long axis within each lane. One simple method is to take the lowest point in the lane profile as the background. Another method is to use an average value of the edge of each band as the background for that band. More complicated methods such as valley-to-valley and rolling-disk use local minima points in the lane profile to define a variable background along the length of the lane. Because there can be many different causes and distributions of background, no single method of background determination can be recommended for all experiments.

Characterization

Once lanes and bands have been detected, it is possible to interpret the mobility of the nucleic acid or protein bands. Depending on the method of electrophoretic separation, information on mass (length or size), pI, or relative mobility (R_f) can be inferred from mobility information. The mobility is characterized using a standard curve with internal standards of known properties. The type of curve depends on several parameters. By definition, with R_f-based separation, a linear first-order curve is used since it represents the linear relationship between mobility and R_f. Similarly, pI and mobility are generally linear in isoelectric focusing separations. For separations based on size, a curve generated from mobility versus the log of the molecular weight provides a relatively good fit as measured by the correlation coefficient (R^2). Several other curves have been suggested for size-based separations, including modified hyperbolic curves and curves of mobility versus (molecular weight)$^{2/3}$ that have good correlation coefficients (Plikaytis et al., 1986). In some cases, no single curve equation can adequately represent the data, and methods of fitting smooth contiguous curves using only neighboring points, such as a Lagrange or spline fit (described in Hamming, 1973), are necessary. This is most common for size separations with a very large range of separation sizes and with nonlinear gradient gels. Care must be taken with multiple-curve techniques since they rely on only a few data points for any one part of the composite curve, and outlying data points can drastically affect the outcome.

For size and R_f determination, a uniform position must be found in each lane as a point from which to measure the mobility of each band. Many software analysis packages use the end of the lane as the measuring start point, so for them it is important to position each lane start point at an iso-molecular-weight or iso-R_f point. A convenient point is the well or sample-loading position since it is usually easily de-

tectable and at an equal mobility position in each lane. A consistent point on each band must also be chosen to measure mobility. A band's peak is easily defined in digital image analysis and is commonly used. Since peak positions are harder to detect visually than edges on silver halide images, the leading band edge is sometimes used when comparing digital results with silver halide–based results.

Once lanes and bands have been detected, it is also possible to quantify the amount or at least relative amount of nucleic acid or protein present in each band. The amount in a band is related to the sum total of the intensity values of each pixel subtracted by the background value for each pixel in a band. For absorptively detected bands, intensity values are converted to OD values. The total value that is calculated is equivalent to the volume of the band and can be directly compared to other bands that are within the linear range for the visualization method. If standards of known amounts are loaded onto the same gel, they can be used to generate a standard curve that converts band volume into standard units such as micrograms. For greatest accuracy, it is important to be able to generate multiple standard curves when using visualization methods, such as Coomassie blue staining, that are affected by band or spot composition.

Quantitation becomes more complicated when bands are not fully resolved. In this case, material from one band is contributing to the volume measurement of an adjacent band and vice versa. The simplest method for handling this is to partition into each band only the volume within its edges. Alternatively, a Gaussian curve can be fitted to each band and the volume contained within the curve used to estimate the amount of the band. Since most electrophoresis bands have a pronounced skew towards the leading edge of the band, modified Gaussian curves have also been used (Smith and Thomas, 1990). In either case, the curve-fitting process is calculation intensive and can significantly increase analysis times for images with many bands.

Software for Two-Dimensional Analysis

In two-dimensional analysis, the first-dimension separation is performed in a single column or lane and then a second separation is performed perpendicular to the first. The result after visualization is a rectangular image of up to 10,000 spots. The most common two-dimensional gel type is one in which protein is separated first by apparent pI and second by molecular weight, although two-dimensional separation of nucleic acids is also possible. While many of the concepts and analysis techniques used with one-dimensional gels are applicable to two-dimensional gels, the complex nature of most two-dimensional gels requires somewhat different methodology. For example, spots are more difficult to detect since they are not conveniently arranged in lanes and can vary more in shape and overlap than bands. In addition, two-dimensional experiments usually require some method of comparing between two images, whereas one-dimensional images usually contain all of the information from an experiment.

Spot detection

Probably the most difficult aspect of two-dimensional analysis is efficient and accurate spot detection. If it is incorrectly done, it can lead to hours of manual editing. Due to the complexity and computational intensity of some algorithms, the detection process itself can last hours on relatively fast desktop computers. One theoretically effective but computationally intense method is to treat the image as essentially a three-dimensional image with spots treated as hills and background as valleys. A large number of Gaussian curves are then combined to describe the topology of the image. Many other methods make use of a digital-imaging technique known as filtering. In essence, filtering is a way to weight the value of a pixel and its neighbors in order to generate a new value for a pixel. By passing a filter across an image pixel by pixel, a secondary image is generated. Filters can be designed for many tasks, including sharpening an image or removing high-frequency noise. Filters can also be generated to help detect spots by making images that are first and second derivatives of the original image. The derivative images indicate inflection points in the intensity pattern and can be used to detect spot centers and edges. In a different method, called thresholding, filters can be used to detect the edges of objects. Instead of looking for inflection points, threshold filters identify intensities above a set level or at ratios between central and edge pixels above a set value. Since the edges on two-dimensional spots tend to be diffuse, sharpening filters are sometimes used prior to the thresholding filter. In some cases, multiple techniques are used to detect spots (Glasbey and Horgan, 1994).

Unlike one-dimensional detection, detection on images of two-dimensional experiments usually requires secondary processing to get acceptable performance. One example of a secondary process is to discard spots with sizes below a set minimum or above a set maximum. Another is to analyze spots that are oval for possible splitting into two spots. Even after secondary processing, it is likely that a small amount of manual editing will be necessary. When manually editing an image, care must be taken to use as objective criteria as possible, especially if two or more images are to be matched and spot volumes compared.

Characterization

In a two-dimensional system, determination of protein or nucleic acid mobility is complicated by the fact that there are two mobilities to account for and that the second-dimension separation tends to make estimation of the separation which occurred in the first dimension more difficult. One method for dealing with this is to have a series of markers in the sample that, after both separations are completed, are evenly distributed within the gel and image. It is also possible to estimate separation characteristics from calibration points located at the periphery of the gel. For example, distance measurements can be used to pass calibration data from the first dimension separation, and standards can be separated at the ends of the gel to calibrate for mobility in the second dimension. Regardless of the method used, in many instances, a series of related images will be examined and similar spots in each image will be matched. When this occurs, it is possible to calibrate one image and then pass the calibration information via the matches to the related images.

Quantity determination is similar in many regards to that which occurs in one-dimensional analysis, but there are some differences. If spot edges are detected, a simple method of determining spot volume is to take the sum of the intensity value of each pixel in the spot reduced by a background intensity value. Multiple Gaussian curves can also be fitted to the spot to approximate the volume (Garrels, 1989). More difficult is attempting to compensate for a skewed distribution in a size-separating dimension while trying to use a regular Gaussian fit for a pI separation, such as is encountered with the most common form of two-dimensional protein separations. The distribution of background makes quantitation more difficult in two-dimensional gels. There is no lane-dependent component, so it is necessary to use other methods such as image stripes, finding local minimum values or using values derived from the spot edges to determine background values.

Matching

Matching is the process in which proteins or nucleic acids with similar separation properties are linked or clustered together. Matching can occur within one image or between multiple images as long as a frame of reference is established. Matching allows for comparisons between samples. It also makes annotation and data entry easier, since if one spot or band is matched to others and is characterized or annotated, this information is easily passed to all the other matches. An underlying assumption of matching is that objects with similar separation properties are actually similar. Care must be taken to confirm the identity of matched spots or bands by other methods on critical experiments.

A simple form of matching is to link bands or spots at similar positions on the gel images. This works well when separation and imaging conditions are uniform. This is very seldom the case, since slight differences in the electrophoresis, visualization, and imaging conditions across a gel and between gels generates incorrect matching with this method. Since bands on one-dimensional gels are relatively easy to calibrate for mobility, matching can occur along contours of equal mobility. This dramatically decreases but does not eliminate the variability in detecting similar bands. Much of the remaining variability can be attributed to calibration errors. This error can often be compensated for by allowing a small tolerance in mobility values in determining whether a band is matched or not. Because of the difficulties in calibrating mobility in two-dimensional gels, it is often more practical to use matched spots for calibrating mobility than vice versa. Spot matching between two two-dimensional images starts with finding a small number of landmark spots that are used as seeds for subsequent matches (Appel et al., 1991; Monardo et al., 1994). There are many methods for finding the landmarks in both images, including finding the highest-intensity spots, finding spots in unique clusters, and manual positioning. The most common procedure from this point is to derive a vector that describes the direction and extent of the path from one matched spot to the other when the two images are superimposed. The vector is used as the basis for finding more matches near the landmark matches. To allow

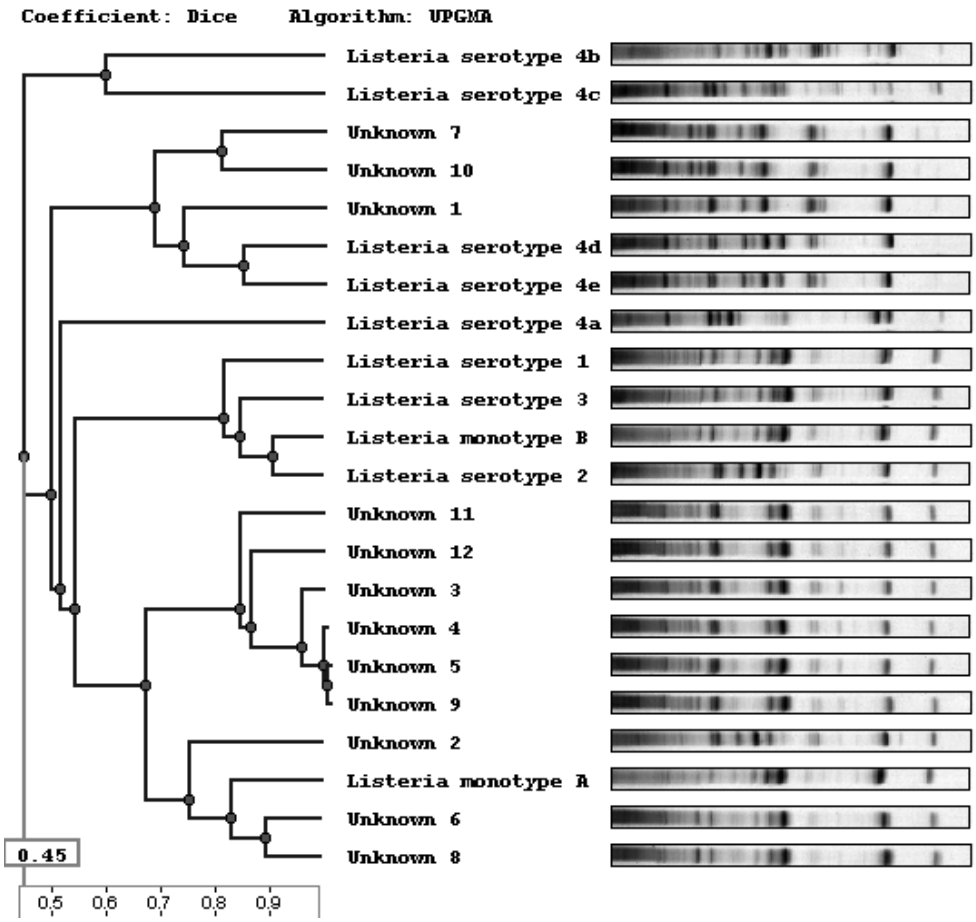


Figure 6.9.3 Example of a dendrogram generated from similarity data on band matching between lanes. DNA samples from 22 isolates of *Listeria* were subjected to Random Amplification of Polymorphic DNA (RAPD) analysis and the resulting electrophoresis image was analyzed with ImageMaster software (Amersham Pharmacia Biotech). Clustering was performed using the Dice coefficient with a tree structure based on the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA). Similarity values between isolates can be determined by locating the node that connects the isolates and reading the value from the scale on the lower left edge of the dendrogram.

for error, the area within a small radius is searched extending from the end of the vector. Once another match is found, its vector is computed and used as the starting point for finding neighboring matches. From this progression, the entire gel is matched. If all vectors are displayed graphically when matching is complete, questionable matches can often be identified as vectors that are significantly different from neighboring vectors.

One specialized use of matching is as an estimator of the similarity and potential genetic relatedness of organisms. For example, on a one-dimensional gel image, a ratio of the matched to unmatched bands for each pairwise combination of lanes can be calculated. This ratio can be used as an indicator of similarity, with values near 1 indicating a pair of highly similar lanes, and values near zero indicating very dissimilar lanes. Assuming that the contents of the lanes are valid samples of the originating organism's genetic makeup, the information on lane similarity can be converted to estimates of genetic similarity. A convenient way to display this similarity data graphically is to generate a dendrogram with similar objects close to each other and less similar objects more distantly placed. An example of such a dendrogram is presented in Figure 6.9.3, where samples from *Listeria* isolates are arranged based on banding pattern.

Databases

In many cases, image analysis is not the last step in the process. The image and analysis data need to be archived in a searchable format. There may be a need to analyze the data from multiple experiments conducted at different sites or in laboratories around the world. Bioinformatic links to diverse data sources might be desired to help develop a unified understanding of the biology behind particular phenomena. When these situations arise, database programs can be utilized to store, link, and search image analysis results.

As the number of images that are captured and analyzed grows, it becomes increasingly more difficult to find particular information from the large number of files that are stored. Relatively simple databases can be used if the major requirement is to find previously analyzed images and associated data. Such databases often display a miniaturized version of each image to aid in visual scanning for the file as well as simple searching for image-specific information such as date of analysis, file name, or other information that was entered at the time of image capture. More powerful database products are also available that can perform complex searches on data generated during the analysis. For an example, a search on a two-dimensional database might include finding proteins exhibiting a specific expression profile and having a molecular weight >20 kDa with a pI between 3 and 5 or 8 and 10 with an amount <50 ng in a series of experiments conducted <1 year ago. Such searches can quickly target potentially interesting molecules for further analysis.

With the increasing ease of transferring data through the Internet as well as local- and wide-area networks, it has become practical to quickly find and examine data from distant locations. Of course, great care must be taken to ensure that similar experimental conditions are employed, as otherwise the results will be difficult to compare. In this manner it is sometimes possible to dramatically increase the sample size and statistical accuracy as well as the probability of detecting rare events. In addition, if one data set is more completely characterized, this extra information can be extracted and applied to the other data set. For example if there is a band in common in two databases and there is sequence information for it in one database, that sequence information can be added to the other database. Currently most public electrophoresis database sites are two-dimensional protein databases. A list with links to many of these Internet database sites can be found at *http://www-lmmb.ncifcrf.gov/EP/table2Ddatabases.html*.

With biological questions becoming more complicated and the answers to the questions often requiring information from a variety of sources, it is becoming increasingly important to be able to move easily between information sources. A relational type of database can help achieve this. Unlike a conventional database with a fixed arrangement of data, relational databases have links between related files that allow for easy movement from one file to another. Another approach to interconnecting electrophoresis data with data from other sources is to generate a series of hypertext links between data sets, similar to what occurs on the Internet. Selecting a specific link moves the search to the related network site and the related information. Regardless of the method, the end goal is similar. An example of what is possible: a researcher selects a protein spot on a two-dimensional gel image, which triggers accessing of related information on this protein. The protein sequence is accessed from mass spectroscopy analysis of the spot on a separate gel. The sequence of the gene and the cDNA that generated the protein is retrieved. The expression pattern of the gene in various tissues and conditions, as well as information on similar genes in other organisms, is incorporated. Citations and annotations to this are retrieved as well. All of this information is compiled automatically into an interactive report about the protein. From this report, the researcher can formulate a more refined hypothesis and plan the most appropriate experiments to test it.

LITERATURE CITED

Appel, R.D., Hochstrasser, D.F., Funk, M., Vargas, J.R., Muller, A.F., and Scherrer, J.-R. 1991. The MELANIE project: From a biopsy to automatic protein map interpretation by computer. *Electrophoresis* 12:722-735.

Garrels, J.I. 1989. The QUEST system for quantitative analysis of two-dimensional gels. *J. Biol. Chem.* 264:5269-5282.

Glasbey, C.A. and Horgan, G.W. 1994. Image Analysis for the Biological Sciences. John Wiley & Sons, Chichester, England.

Hamming, R.W. 1973. Numerical methods for scientists and engineers, 2nd ed. Dover Publications, New York.

Huffman, D.A. 1952. A method for the construction of minimum-redundancy codes. *Proc. Inst. Elect. Radio Eng.* 40:9-12.

Monardo, P.J., Boutell, T., Garrels, J.I., and Latter, G.I. 1994. A distributed system for two-dimen-

sional gel analysis. *Comput. Appl. Biosci.* 10:137-143.

Patton, W.F. 1995. Biologist's perspective on analytical imaging systems as applied to protein gel electrophoresis. *J. Chromatogr. A.* 698:55-87.

Plikaytis, B.D., Carlone, G.M., Edmonds, P., and Mayer, L.W. 1986. Robust estimation of standard curves for protein molecular weight and linear-duplex DNA base-pair number after gel electrophoresis. *Anal. Biochem.* 152:346-364.

Russ, J.C. 1995. The Image Processing Handbook. CRC Press, Boca Raton, Fla.

Smith, J.M. and Thomas, D.J. 1990. Quantitative analysis of one-dimensional gel electrophoresis profiles. *Comput. Appl. Biosci.* 6:93-99.

Sutherland, J.C., Lin, B., Monteleone, D.C., Mugavero, J., Sutherland, B.M., and Trunk, J. 1987. Electronic imaging system for direct and rapid quantitation of fluorescence from electrophoretic gels: Application to ethidium bromide–stained DNA. *Anal. Biochem.* 163:446-457.

Welch, T.A. 1984. A technique for high performance data compression. *IEEE Computer.* 17:21-32.

KEY REFERENCES

Glasbey and Horgan, 1994. See above.

Describes general image-processing techniques as they are applied to biological images.

Russ, 1995. See above.

A general reference book on digital image capture and analysis.

Sutherland, J.C. 1993. Electronic imaging of electrophoretic gels and blots. *In* Advances in Electrophoresis, Vol. 6. (A. Chrambach, M.J. Dunn, and B.J. Radola, eds.) pp. 1-41. VCH Verlagsgesellschaft mbH, Weinheim, Germany.

Provides an overview of image capture with particular emphasis on types of capture equipment.

INTERNET RESOURCES

rsb.info.nih.gov/nih-image

NIH Image is free software that provides basic image analysis tools for the Macintosh.

http://www.inforamp.net/~poynton/Poynton-color.html

Contains an excellent description of gamma correction in the Gamma FAQ.

http://www-Immb.ncifcrf.gov/EP/table2Ddatabases.html

A list of links to many two-dimensional databases that are available via the Internet.

Contributed by Scott Medberry
Amersham Pharmacia Biotech
San Francisco, California

Sean Gallagher
Motorola Corporation
Tempe, Arizona

Two-Dimensional Blue Native Polyacrylamide Gel Electrophoresis

Wolfgang W.A. Schamel[1]

[1]Max Planck-Institut für Immunbiologie und Universität Freiburg, Biologie III, Freiburg, Germany

ABSTRACT

Multiprotein complexes play crucial roles in nearly all cell biological processes. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) is a powerful method to study these complexes. It is a native protein separation method that relies on the dye Coomassie blue to confer negative charge for separation. It has a higher resolution than gel filtration or sucrose density ultracentrifugation and can be used for protein complexes from 10 kDa to 10 MDa. If a second-dimension SDS-PAGE is applied (two-dimensional BN/SDS-PAGE), the size, subunit composition, and relative abundance of the different multiprotein complexes can be studied. In recent years, there has been a large increase in the number of publications where BN-PAGE was used to study protein-protein interactions. Here, we give detailed protocols for the separation of multiprotein complexes by two-dimensional BN/SDS-PAGE and for a related technique to determine the stoichiometry of these complexes. *Curr. Protoc. Cell Biol.* 38:6.10.1-6.10.21. © 2008 by John Wiley & Sons, Inc.

Keywords: multiprotein complex • native • gel electrophoresis • two-dimensional • Coomassie blue • protein-protein interaction

INTRODUCTION

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE; Basic Protocol 1) separates native proteins and protein complexes independently of their individual isoelectric points, with high-resolution capacity. This is done by conferring a negative charge on all proteins via the dye Coomassie blue. The separation depends mainly on the size of the protein complex. In combination with a second-dimension sodium dodecyl sulfate–PAGE (SDS-PAGE) in a perpendicular direction (Basic Protocol 2), it is an excellent choice to identify and characterize multiprotein complexes. It allows the determination of the size, subunit composition, and relative abundance of different multiprotein complexes from total cell and tissue homogenates, as well as from purified material. In addition, it is used for a one-step preparative purification of protein complexes.

One critical step in performing high-quality BN-PAGE is the preparation of the sample, which has to be devoid of potassium and divalent cations. The most useful source for protein complexes is a cellular lysate or tissue extract. Preparation of a cellular lysate suitable for separation by BN gels is done by dialysis to remove any cations and metabolites (see Support Protocol). A detailed protocol for pouring and running of the BN gradient gels (see Basic Protocol 1) and denaturation of the separated proteins followed by separation using a second dimension SDS-PAGE (see Basic Protocol 2) is given. Visualization of the resulting two-dimensional gel can be done according to standard protocols, including general protein stains (*UNIT 6.6*) and immunoblotting, often referred to as western blotting (*UNIT 6.2*).

A second dimension is not always required. Proteins can be detected after BN-PAGE by such standard procedures as general protein stains or transfer of the proteins to a

Current Protocols in Cell Biology 6.10.1-6.10.21, March 2008
Published online March 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0610s38

membrane under denaturing (see Alternate Protocol 1) or native (see Alternate Protocol 2) conditions followed by immunodetection. Using these protocols, there is a special requirement for the antibodies used to detect the proteins of interest, because Coomassie blue interferes with fluorescence-based visualization methods and because native proteins are often detected by different antibodies than those that detect denatured proteins.

A powerful method to determine the stoichiometry of multiprotein complexes is the Native Antibody-based MObility Shift (NAMOS) assay (see Basic Protocol 3). It is based on one-dimensional BN-PAGE and uses the fact that proteins migrate more slowly during the electrophoresis when an anti-subunit antibody is bound.

NOTE: High-purity water (e.g., Milli-Q or distilled water) should be used for all solutions. For cautions relating to electricity and electrophoresis, see Safety Considerations in the introduction to *UNIT 6.1*.

NOTE: Wear powder-free gloves throughout the procedure and work on ice or at 4°C whenever native proteins/protein complexes are present.

CAUTION: Acrylamide is hazardous; see *APPENDIX 2A* for guidelines on handling, storage, and disposal.

BASIC PROTOCOL 1

FIRST-DIMENSION BLUE NATIVE ELECTROPHORESIS

In this protocol, the pouring and running of vertical slab blue native (BN) gels is described. BN gels are gradient gels of low acrylamide percentage and strength. Thus, pouring and handling of the gels is not trivial. The gradient has to be even, in order to prevent any step that would result in the erroneous accumulation of proteins at a particular height of the gel. The BN gel solution with the higher acrylamide/bisacrylamide concentration is heavier than the low-percentage gel because of its high glycerol content. This density difference aids in establishment of a uniform gradient inside the glass plates. The same gel equipment that is used for normal SDS-PAGE can be used, but one has to be certain that no traces of SDS are present. To ensure absence of any detergent, the BN equipment should not be used for SDS-PAGE. BN gels are poured at room temperature and are cooled to 4°C before samples are loaded in the cold room. Alternatively, gel apparatuses that allow cooling can be used. Precast BN gels have recently been made commercially available, but in the author's experience, these are not as good as self-made gels.

It is strongly recommended to use multicasting equipment to pour several gels at once. This avoids steps of the gradient, saves time, and ensures best reproducibility for critical comparisons of multiple samples. Casting of multiple gradient gels is described in *UNIT 6.1*, Support Protocols 2 and 3, and Figures 6.1.3 and 6.1.4. Using these protocols, BN gels can be prepared using BN-specific solutions.

Materials

Low-percentage BN separating gel solution (see recipe)
High-percentage BN separating gel solution (see recipe)
Isobutyl alcohol
3× BN gel buffer (see recipe)
BN stacking gel solution (see recipe)
100× pervanadate solution (optional, if phosphorylation must be preserved; see recipe)
Sample: dialyzed cell lysate (Support Protocol), tissue homogenate, purified protein complex
Marker mixes 1 and 2 (see recipe)
BN anode buffer (see recipe)

BN cathode buffer (with 0.02% w/v Coomassie blue; see recipe)
BN cathode buffer (with low, 0.002% w/v Coomassie; see recipe)

Gel electrophoresis apparatus (see *UNIT 6.1*)
Gradient mixer (self-made or, e.g., from BioRad; Fig. 6.1.2)
Peristaltic pump (Fig. 6.1.2)
Power supply

Additional reagents and equipment for polyacrylamide gel electrophoresis (*UNIT 6.1*) and protein staining in gels (*UNIT 6.6*)

Set up the apparatus

1. Wash glass plates and pouring devices extensively with water.

   Do not use equipment that has been previously used for SDS-PAGE. No traces of detergent should be present on the glass plates

2. Assemble the glass plate sandwich of an electrophoresis apparatus.

   UNIT 6.1 describes these procedures in detail.

Prepare the gradient

3. Set up the equipment to pour gradient gels as shown in Figure 6.1.2 (*UNIT 6.1*).

   Gels will be poured at room temperature. For reproducibility, and to make the pouring more effective, it is recommended to pour at least 10 gels at once using multicasting equipment (e.g., BioRad).

4. Close the valves and place a magnetic stir bar into the mixing chamber of the gradient maker. Prepare low- and high-percentage BN separating gel solutions (see Reagents and Solution). Adjust the acrylamide/bisacrylamide concentration of the high-percentage BN separating gel solution according to your needs (Table 6.10.1). Add APS and TEMED only immediately before use.

   The volumes of the two solutions combined should be exactly equal to the volume required to fill the glass plate sandwich to the required height.

   When using multicasting equipment, the dead volume of the apparatus has to be determined empirically and added to the required volume of the gel solutions.

5. Pour the low-percentage (light) BN separating gel solution into the reservoir chambers and the high-percentage (heavy) BN separating gel solution into the mixing chamber of the gradient mixer (Fig. 6.1.2).

6. Open the interconnecting valve and force out the air bubble inside the connecting channel by pressing over the right cylinder with your thumb.

   CAUTION: *Make sure to wear gloves, in order to avoid contact with acrylamide.*

Cast the Blue Native separating gels

7. Switch on the peristaltic pump, open the outlet valve, and allow the gel to slowly enter between the glass plates. Ensure that the needle at the end of the Tygon tubing is always above the height of the liquid, so that the gradient is not disturbed.

   When using multicasting equipment, the gel solutions enter between the glass plates from the bottom. In this case, the low-percentage BN separating gel solution has to be placed into the mixing chamber and the high-percentage one into the reservoir chamber (Fig. 6.1.3). Figure 6.1.2 shows a pipet tip at the end of the Tygon tubing, but a needle may be more appropriate since a pipet tip might be too wide to fit between the glass plates.

8. Prepare water-saturated isobutyl alcohol by shaking isobutyl alcohol and water in a glass bottle. Using a Pasteur pipet, overlay the separating gel with water-saturated isobutyl alcohol (upper alcoholic phase from the mix) by gently layering the alcohol

Table 6.10.1 Gel Solutions for BN-PAGE[a]

	Low% BN separating gel solution	High% BN separating gel solution					Stacking gel solution
	4%	7%	10%	12%	16%	18%	3.2%
40% acrylamide/ bisacrylamide mix[b] (ml)	3.00	5.25	7.50	9.00	12.00	13.50	1.00
3× BN gel buffer[b] (ml)	10.00	10.00	10.00	10.00	10.00	10.00	4.17
dH_2O (ml)	17.00	—	—	—	—	—	7.33
70% (v/v) glycerol (ml)	—	14.75	12.50	11.00	8.00	6.50	—
10% ammonium persulfate (µl)	108	84	84	84	84	84	167
TEMED (µl)	11	8	8	8	8	8	17

[a]Numbers in the body of the table are milliliters of stock solution, except 10% APS and TEMED which are microliters and should be added only when the low- and high percentage BN gel solutions are already in the chambers of the mixing apparatus.
[b]See recipe in Reagents and Solutions.

against the edge of one and then the other of the spacers to produce a smooth surface.

In the multicasting equipment, all gels must be overlaid with the same volume of water-saturated isobutyl alcohol.

9. Clean pouring apparatus with water (do not use detergent).

10. Allow the gel to polymerize for at least 30 min at room temperature. Remove the isobutyl alcohol, wash twice with water, and overlay with 1× BN gel buffer. Store at 4°C (stable at least 1 year).

Pour the stacking gel

11. Just before use, pour the stacking gel using an appropriate comb and the BN stacking gel solution (see Reagents and Solutions).

Due to the low percentage of acrylamide/bisacrylamide, the comb might be difficult to remove without damaging the gel. Try to move the comb perpendicular to the plane of the glass plates while removing it to allow air to enter. Alternatively, the percentage of acrylamide/bisacrylamide can be increased to 3.5%.

Load the samples and run the Blue Native gels

All steps should be performed at 4°C (e.g., in a cold room).

12. Add 100× pervanadate solution to the dialysed lysate to a concentration of 1×, if phosphorylation needs to be preserved.

13. Mount the gel in the electrophoresis apparatus and load 5 to 30 µl dialysed cell lysate (prepared as in Support Protocol) in the dry wells of the mounted gel.

As control, an aliquot of the sample can be boiled with 1% SDS to destroy all multiprotein complexes. Leave one lane free between this control and the non-SDS samples.

14. Load 10 µl marker mix 1 and 10 µl marker mix 2 in two adjacent wells.

Only ferritin is seen during the electrophoresis, due to its brown color.

Alternatively, commercially available high-molecular-weight markers can be used (e.g., from Invitrogen or GE Healthcare).

15. Carefully overlay the samples in each well with the BN cathode buffer. Fill the upper/inner chamber with BN cathode buffer (containing 0.02% w/v Coomassie blue as described in Reagents and Solutions) and the lower/outer chamber with BN anode buffer.

16. Connect electrodes. If a small gel has been prepared (e.g., using BioRad Protean II or III), run at 100 V; if a large gel has been prepared (e.g., using BioRad Protean II xi), run at 150 V. Continue electrophoresis at the appropriate abovementioned voltage until the sample has entered the separating gel. At that point, increase voltage to 180 V for small gel or 400 V for large gel.

    Coomassie blue comes into contact with the samples inside the wells during the electrophoresis.

17. Remove the BN cathode buffer (especially from within the wells) after two-thirds of the gel run. Fill the upper/inner chamber with BN cathode buffer (low Coomassie blue) that contains only 0.002% (w/v) Coomassie blue. Continue electrophoresis at 180 V for small gel or 400 V for large gel.

    This procedure ensures that the individual lanes can be identified after the gel is run and can be omitted if a second-dimension gel is not to be applied (see, e.g., Alternate Protocols 1 and 2), or if precipitated material is visible between the stacking and separating gel.

18. Turn off power supply and remove the electrodes once the dye front has reached the bottom of the gel.

Extrude and store Blue Native gels

19. Remove the glass plates, with the gel in-between, from the electrophoresis apparatus.

20. Open the glass plates by lifting the smaller one and keeping the gel attached to the bigger, bottom plate. Remove the stacking gel with the smaller glass plate.

21. Cut away the lanes where the marker mixes were loaded from the rest of the gel using the smaller glass plate. Visualize the marker proteins by standard Coomassie blue or silver staining (*UNIT 6.6*).

    For some purposes, the ferritin marker (440 and 880 kDa), which is visible without staining, is sufficient. When assigning the positions of the marker, take into consideration that the gel might shrink upon staining.

    Alternatively, after the first-dimension BN-PAGE, proteins can be stained directly with silver or Coomassie brilliant blue (UNIT 6.6), or detected by immunoblotting (western blotting; Alternate Protocols 1 and 2). Optionally, stained spots can be cut out and the proteins identified by mass spectroscopy.

22. Stamp out each individual lane with the smaller glass plate. Either immediately run the second dimension SDS-PAGE (Basic Protocol 2), or freeze each lane wrapped in aluminum foil at −20°C (stable at least 1 year).

    Do not bend a frozen gel piece, since it breaks easily. Alternatively, the first-dimension BN gel can be transferred to a membrane followed by immunoblotting to visualize the proteins of interest (Alternate Protocols 1 and 2).

PREPARATION OF CELL LYSATES FOR BLUE NATIVE GEL ELECTROPHORESIS

SUPPORT PROTOCOL

Blue Native (BN) gel electrophoresis is suitable for the separation of pure proteins as well as complex protein mixtures such as subcellular fractions or cell lysates. Sample preparation is one of the critical steps in performing high-quality BN gels. First, the proteins have to be present in soluble and native form. Thus, the choice of detergent is important and one should start BN experiments by testing several nonionic detergents

for the protein/protein complex of interest (see Commentary). The detergent should be effective enough to extract the proteins from cellular membranes, but at the same time mild enough to keep multiprotein complexes intact. The most commonly used detergents are given in Reagents and Solutions. The proteins must be eluted in native form, if the purification procedure includes binding to a matrix, as e.g., in immuno- or affinity purifications (often referred to as immunoprecipitation; see *UNIT 7.2*). Second, the sample has to be prepared without potassium or divalent cations, since Coomassie blue and Coomassie blue–bound proteins precipitate with those ions and consequently do not enter the gel. Hence, cations that interact with Coomassie blue have to be removed and substituted by 6-aminohexanoic acid, in order to maintain a certain ionic strength of the solution, which is necessary for the solubility and stabilization of many protein complexes. Sodium ions are tolerated to a maximum concentration of 50 mM. Third, samples have to be loaded with detergent on BN gels. Otherwise, proteins aggregate during the stacking step of the electrophoresis.

Membrane and organelle fractions lysed in BN lysis buffer can be directly applied to BN gels. Cellular lysates have to be dialysed against BN dialysis buffer, in order to remove small cations and metabolites. Proteins bound to a matrix are washed and eluted in BN dialysis buffer.

This protocol describes the preparation of cell lysates. Cells are washed and lysed in any lysis buffer, although we recommend using the BN lysis buffer. After removal of insoluble material, the lysate is dialysed against BN dialysis buffer. In this protocol, a self-made dialysis setup utilizing dialysis membranes is described. Alternative desalting methods can be applied as well.

Materials

Cell culture dish (10 to 15 cm) containing cells of interest (80% confluent)
Phosphate-buffered saline (PBS; see recipe), ice cold
BN lysis buffer (see recipe)
BN dialysis buffer (see recipe)

Cell scrapers
10- to 50-ml centrifuge tubes
Refrigerated cell culture centrifuge (with adaptor cavities for microcentrifuge tubes in rotor accommodating 50-ml tube)
Dialysis membranes, MWCO 10 to 50 kDa (boiled and kept at 4°C in 0.001 M EDTA)
1-ml reaction tubes (e.g., microcentrifuge tubes)
Beaker (100-ml to 1-liter, depending on sample size)

Harvest and wash the cells

1. Place cell culture dish containing cells on ice.
2. For adherent cells, wash cells three times, each time with 2 to 10 ml ice-cold 1× PBS.

   Cells grown in suspension are washed three times with ice-cold 1× PBS by repeatedly pelleting the cells by centrifugation for 5 min at 350 × g, 4°C.

   The volume of PBS used for one washing step should be equal to the volume of medium in which the cells were cultivated.
3. Add 2 to 10 ml (i.e., volume in which cells were cultivated) of ice-cold 1× PBS to the dish, remove the cells using a cell scraper, and transfer the cell suspension to a 10- to 50-ml centrifuge tube.

   This step is omitted for cells grown in suspension.

4. Collect cells by centrifugation for 5 min at 350 × *g*, 4°C, and remove supernatant.
5. Resuspend cells in 1 ml cold 1× PBS, transfer cell suspension into a small reaction tube (e.g., microcentrifuge tube), and pellet cells by centrifugation for 5 min at 350 × *g*, 4°C.
6. Remove and discard the supernatant using a pipet.
7. Either move on to step 8 or freeze the cell pellet at −20°C.

   Frozen cells can be stored at −20°C for at least 6 months.

Prepare the cell lysates

8. Resuspend the cell pellet in ice-cold BN lysis buffer (or other lysis buffer that has been used successfully to extract your multiprotein complex of interest).

   The concentration of cells per volume lysis buffer depends on the amount of protein that should be loaded on the BN gel. As an approximation, use 10^6 cells per 250 to 500 µl lysis buffer.

9. Incubate on ice for 15 min to allow cell lysis.

   The lysis time can be prolonged, if it is convenient—e.g., in the case where many different samples are processed and some of them have to wait for the last ones.

10. Microcentrifuge 15 min at 13,000 × *g*, 4°C, to remove insoluble material.

Prepare to dialyze the lysate

11. Melt a hole in the cap of a microcentrifuge tube using the large-diameter end of a hot Pasteur pipet. Chill the tube on ice.
12. Transfer the supernatant from step 10 into the chilled tube from step 11.
13. Place a dialysis membrane over the opened tube and close the cap.

    Make sure that there are no folds or tears in the dialysis membrane.

14. Seal the cap at the edges with Parafilm.

    Make sure that the hole in the tube is not covered by the Parafilm.

15. Invert the tube and centrifuge upside-down at the lowest speed possible in the adaptor cavity for 50-ml tubes in a cell culture centrifuge for 10 sec at 4°C.
16. Prepare a 100-ml beaker with ice-cold BN dialysis buffer and a magnetic stirrer. Use at least 10 ml dialysis buffer per 100 µl sample.
17. Fix the tube upside down inside the beaker and remove air bubbles from the hole beneath the cap.

    Make sure that the dialysis membrane is not damaged.

Dialyze lysate

18. Switch on the magnetic stirrer and dialyze for 6 hr or overnight in a cold room.

    Make sure that stirring is not creating air bubbles at the dialysis membrane.

19. Collect the dialysed cell lysate in a new chilled microcentrifuge tube and analyze by BN-PAGE (Basic Protocol 1).

    If the sample will be subjected to two-dimensional analysis, reserve one-third of the sample to serve as a control in the second-dimension analysis.

    Freezing and thawing of cell lysates might lead to the aggregation of proteins. Therefore, the cell lysate should be separated immediately by BN-PAGE. For some proteins, freezing might be possible and has to be determined empirically.

BASIC PROTOCOL 2

SECOND-DIMENSION DENATURING ELECTROPHORESIS

A denaturing second-dimension gel is suitable to identify and characterize multiprotein complexes. It is recommended to use vertical denaturing (SDS) discontinuous gel electrophoresis (Laemlli method of SDS-PAGE; described in detail in *UNIT 6.1*, Basic Protocol 1). These gels can be linear or gradient slab gels run under reducing or nonreducing conditions. The only exception is that loading of the first-dimension BN gel strip requires a broad, flat well. The BN gel strip has to be fit between the glass plates of the second gel. The easiest way is to slightly increase the thickness of the second dimension compared to the first dimension. If this is done by putting cellophane tape on the spacers of the second dimension gel, the BN gel strip can be inserted without breaking and still sit firmly enough to prevent any movement during the electrophoresis. Alternatively, procedures as described in *UNIT 6.4* can be employed.

This protocol describes all the specific steps required for successfully casting and running the second-dimension SDS-PAGE, with extensive reference to *UNIT 6.1*. Typical expected results are depicted in Figure 6.10.1.

Materials

2× SDS sample buffer (*UNIT 6.1*)
1× SDS electrophoresis buffer (*UNIT 6.1*)
Lane from a BN-PAGE gel (Basic Protocol 1)

Thin adhesive tape (e.g., tesa tape; *http://www.tesatape.com*)
Platform shaker

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and two-dimensional gel electrophoresis (*UNIT 6.4*)

Cast the second-dimension SDS gels

1. Take spacers of the same thickness as the ones that were used for the first dimension (see Basic Protocol 1). Wrap thin adhesive tape once around them, in order to increase their thickness slightly.

2. Assemble the glass-plate sandwich using these spacers.

   A second-dimension gel that is slightly thicker than the first-dimension gel allows fitting in the first-dimension gel slice without problems, but still fixing it between the glass plates so that it cannot move.

3. Pour the separating SDS-PAGE gel as described in *UNIT 6.1*.

   For everyday purposes, degassing of the gel solution is not necessary. It is recommended to use multicasting equipment to pour several SDS gels at once. It saves time and ensures best reproducibility for comparisons of multiple samples. Casting of multiple gradient gels is described in UNIT 6.1.

4. Pour the stacking gel (*UNIT 6.1*) using combs that contain one large pocket that is wide enough to accommodate the first-dimension gel slice and one or two additional wells for marker and control samples (*UNIT 6.4*).

   After removing the comb, clean the large pocket of polymerized gel remnants using a spacer that has not been enlarged with a layer of tape.

Load the Blue Native gel strips onto the second-dimension SDS gels

5. Retrieve the first-dimension BN gel strips from storage. Incubate them for 10 min at room temperature in 2× SDS sample buffer by shaking in a small tray on a platform shaker.

   Make sure to work under a hood if working with a reducing sample buffer containing 2-mercaptoethanol.

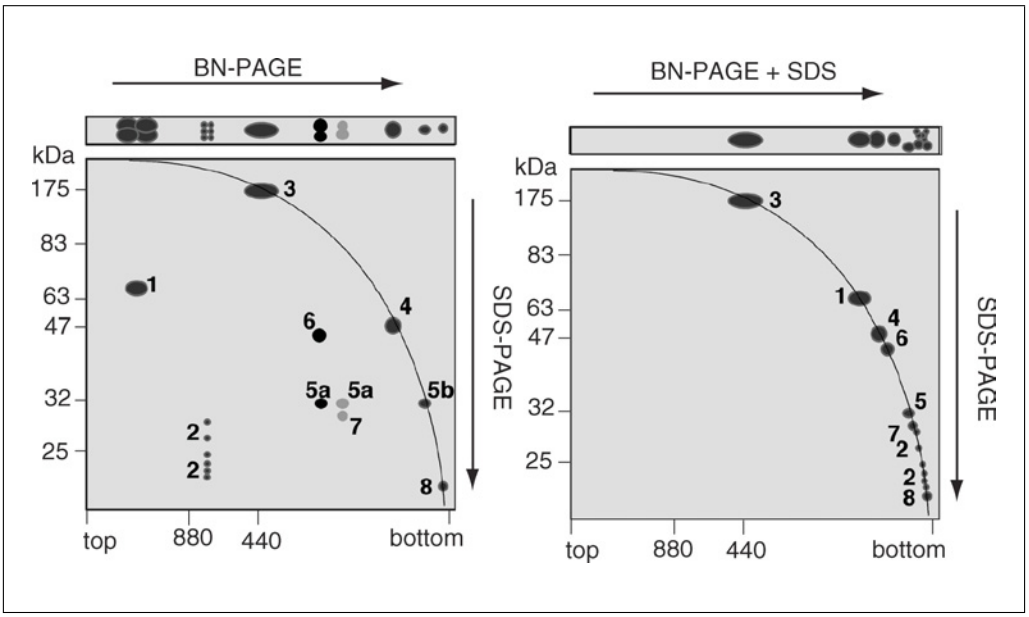


Figure 6.10.1 Two-dimensional BN/SDS-PAGE of cellular lysates. In this hypothetical experiment, total cellular lysates were prepared and dialyzed (Support Protocol). In (**A**), proteins were separated by BN-PAGE (Basic Protocol 1) and a subsequent second-dimensional SDS-PAGE (Basic Protocol 2). Visualization of the protein was done with silver staining. Monomeric proteins (#3, #4, and #5b) were localized to a hyperbolic-shaped diagonal, indicating that they had the same size in the first and in the second dimension. In contrast, proteins that were present in the same multiprotein complex were found as individual spots aligned in vertical columns. Complex #1 was a homotetrameric protein complex. Complex #2 was composed of six proteins of small individual sizes. Proteins #6 and #5 as well as #7 and #5 formed dimeric complexes. In addition, protein #5 was found as a monomer (#5b). Thus, protein #5 existed in three different forms. From the intensity of the spots, one could deduce that complex #6:#5 was more abundant than complex #7:#5. In (**B**), the dialysed lysate was boiled with 1% SDS before separation by BN-PAGE and SDS-PAGE. SDS destroys all multiprotein complexes; thus, the hyperbolic-shaped diagonal can identified. A similar wet experiment is described in Camacho-Carvajal et al. (2004).

6. Heat the strips with the sample buffer in a microwave oven at medium power until the solution boils. Let the gel strips shake for another 10 min at room temperature while cooling down.

   Heating the proteins in the presence of SDS leads to their unfolding and to the disruption of multiprotein complexes.

7. Fill the large pocket of the second-dimension gel with 1× SDS electrophoresis buffer, removing all air bubbles. Incline the gel with the large glass plate at the bottom. Let the first-dimension gel slice enter between the glass plates. Avoid any air bubbles and push carefully with a spacer without added tape.

8. Place the gel into the electrophoresis apparatus and load the markers and control samples into the small wells.

   It is recommended to keep one-third of the sample to be separated by the first dimension BN-PAGE (Support Protocol, step 19), boil it in 1× SDS electrophoresis buffer, and use it as a control sample in the second dimension.

9. Overlay the first-dimension gel strip with a 3-mm layer of 2× SDS sample buffer.

Run and analyze the second-dimension SDS gel

10. Run and disassemble the gel as described in *UNIT 6.1* (Basic Protocol 1).

    The dye front contains not only the bromphenol blue of the SDS sample buffer but also the Coomassie blue from the first dimension. The dye front of the first dimension will be visible as a dark blue spot at the bottom of the second dimension.

11. Visualize proteins using any method available.

The most commonly used methods are transfer to a membrane followed by immunodetection (western blotting; UNIT 6.2) or staining with silver or Coomassie brilliant blue (UNIT 6.6). Optionally, stained spots can be cut out and the protein identified by mass spectroscopy.

ALTERNATE PROTOCOL 1

DENATURING TRANSFER OF THE PROTEINS FROM THE FIRST-DIMENSION BN-PAGE GEL TO A MEMBRANE FOR IMMUNOBLOTTING

Protein complexes separated by BN gels can be visualized by immunoblotting (also referred to as western blotting) without a second-dimension separation. If a cell lysate is analyzed, a very specific antibody is necessary that does not cross-react with other proteins. It is recommended to first test the quality of the antibody by second-dimension BN/SDS-PAGE (see Commentary). In general, the same antibodies that are used for immunodetection after SDS-PAGE are potentially suitable.

In this protocol, the proteins/multiprotein complexes are first denatured within the BN gel by boiling in SDS. Subsequent steps, including the transfer to a membrane and the immunoblotting procedure, are similar to the standard protocol used after SDS-PAGE (*UNIT 6.2*). If PVDF membranes are used, the bound Coomassie blue can partially be removed. This is not the case for nitrocellulose membranes that can be used to immobilize the proteins after BN-PAGE as well.

Materials

First-dimension BN-PAGE gel (Basic Protocol 1)
Phosphate buffered saline (PBS; see recipe for 10×) containing 1% (w/v) SDS
Denaturing BN transfer buffer (see recipe)
Destaining solution (see recipe)

Platform shaker
PVDF (polyvinylidene fluoride) membrane (see *UNIT 6.2*)

Additional reagents and equipment for immunoblotting (*UNIT 6.2*)

1. Soak the first-dimension BN gel in 1× PBS with 1% SDS for 15 min by shaking in a small tray on a platform shaker.
2. Heat in a microwave at medium power until the solution boils. Let the gel shake for another 10 min at room temperature.
3. Transfer the denatured proteins to a PVDF membrane by wet or semi-dry blotting (*UNIT 6.2*). Use the denaturing BN transfer buffer, since it includes SDS.

   A protocol for wet transfer is described in UNIT 6.2 (Basic Protocol 1, including Fig. 6.2.1). Semi-dry transfer is described in UNIT 6.2 (Alternate Protocol 1, including Fig. 6.2.2).

   Before transfer, mark the ferritin marker with a pen on the membrane, since it is difficult to see afterwards.

4. *Optional:* Partially remove the transferred Coomassie blue from the PVDF membranes by incubation in destaining solution for 30 min.

   Immunodetection can also be performed without the destaining step. Coomassie blue cannot be removed completely and shows strong fluorescence at several wavelengths. Therefore, detection with fluorescently labeled antibodies cannot be done. In this case a second-dimension SDS-PAGE has to be used for immunoanalysis (Basic Protocol 2).

5. Block the membrane and immunodetect under standard conditions (*UNIT 6.2*).

NATIVE TRANSFER OF THE PROTEINS FROM THE FIRST-DIMENSION BN-PAGE GEL TO A MEMBRANE FOR IMMUNOBLOTTING

ALTERNATE PROTOCOL 2

Proteins are separated by BN-PAGE under native conditions. Transfer to a membrane and visualization by immunoblotting can also be performed under native conditions. If a cell lysate is analyzed, a very specific antibody that does not cross-react with other proteins is necessary. The antibodies that are used for immunodetection after SDS-PAGE might not be suitable, since they recognize unfolded proteins. Antibodies that recognize folded proteins and protein complexes, as used for immunoisolation (e.g., immunoprecipitation), flow cytometry, or immunofluorescence methods, should be tested for the application in this protocol.

In this protocol, the proteins/multiprotein complexes are transferred to a membrane in their native form. The following immunoblotting procedure is similar to the standard protocol used after SDS-PAGE (*UNIT 6.2*).

Materials

First-dimension BN-PAGE gel (Basic Protocol 1)
Native BN transfer buffer (see recipe)
Destaining solution (see recipe)
PVDF membrane (see *UNIT 6.2*)

Additional reagents and equipment for immunoblotting (*UNIT 6.2*)

1. Briefly rinse the first-dimension BN gel in native BN transfer buffer.
2. Transfer the native proteins to a PVDF membrane by semi-dry or wet blotting (*UNIT 6.2*). Use the native BN transfer buffer, since it does not contain SDS.

   Conditions for transfer are the same as those used for SDS-PAGE gels (UNIT 6.2).

   Before transfer, mark the ferritin marker with a pen on the membrane, since it is difficult to see afterwards.

3. *Optional:* Partially remove the transferred Coomassie blue from the PVDF membranes by incubation in destaining solution for 30 min.

   Immunodetection can also be performed without the destaining step. Coomassie blue cannot be removed completely and shows strong fluorescence at several wavelengths. Therefore, detection with fluorescently labeled antibodies cannot be accomplished. In this case a second-dimension SDS-PAGE has to be done (Basic Protocol 2).

4. Block the membrane and immunodetect under standard conditions (*UNIT 6.2*).

NATIVE ANTIBODY-BASED MOBILITY SHIFT (NAMOS) ASSAY

BASIC PROTOCOL 3

This protocol is a variant of Basic Protocol 1 and describes a method to determine the stoichiometry of multiprotein complexes based on BN-PAGE. It makes use of the fact that the proteins and protein complexes are separated in their native state. Antibodies that recognize a certain subunit of a protein complex are added to the sample before separation by BN-PAGE. This allows the formation of super-complexes comprising the protein complex and the antibody. Since the migration distance in BN-PAGE depends on the size of a protein complex, it directly reflects the number of bound antibodies and thus the copy number of the subunit they bind to. This method is called the Native Antibody-based MObility Shift (NAMOS) assay. With the NAMOS assay, it is even possible to determine the stoichiometry of a protein complex that is present in low amounts within a total cell lysate. Due to the large amount of added antibody, detection of the protein complex of interest has to be done by immunoblotting as described in Alternate Protocols 1 and 2, and *UNIT 6.2*.

Before performing the NAMOS assay, one has to set up the sample preparation (Support Protocol 1) and immunodetection (Alternate Protocols 1 and 2) conditions. It is required that the multiprotein complex of interest be separated as a single, clearly visible complex after BN-PAGE.

Typical expected results are displayed in Figure 6.10.2.

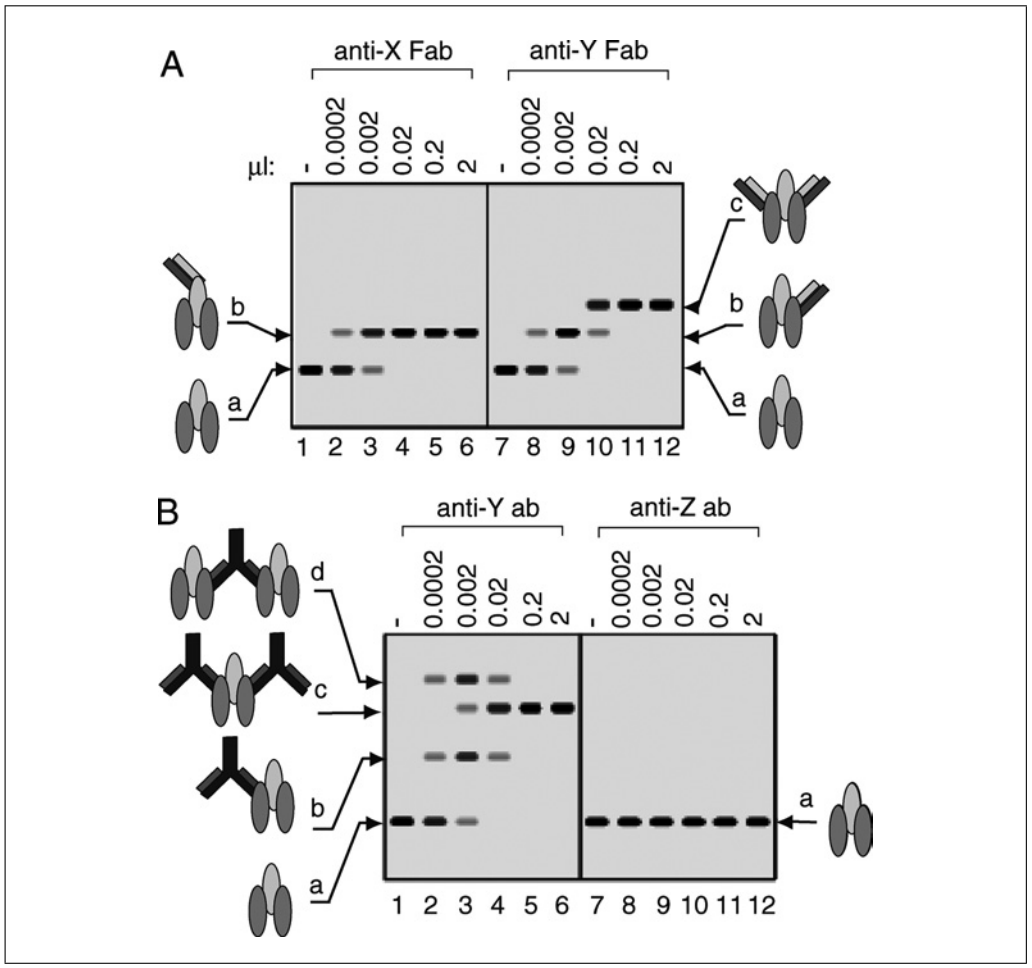


Figure 6.10.2 The Native Antibody-Based Mobility Shift (NAMOS) Assay. In this hypothetical experiment, the protein complex of interest comprises one X and two Y subunits (XY_2). Its stoichiometry is to be determined by the NAMOS assay (Basic Protocol 3). Cellular lysates containing complex XY_2 were separated by BN-PAGE and visualized by anti-XY_2 immunoblotting. Indeed, XY_2 was one defined complex (lanes 1 and 7, band a). In (**A**), increasing amounts of an anti-X Fab fragment were incubated with the lysate before separation. Consequently, a new band (band b) appeared with a concomitant loss of band a (lanes 2 to 6). Band b corresponded to a Fab-XY_2 complex, indicating that XY_2 contained one binding site for the anti-X Fab fragment and, thus, one copy of protein X. An anti-Y Fab fragment produced two bands (lanes 8 to 12, bands b and c), demonstrating that XY_2 had two copies of protein Y. In (**B**) complete antibodies were used for the NAMOS assay. In contrast to the Fab fragment, an antibody is able to simultaneously bind to two XY_2 complexes. Using anti-Y, one can see that these "super"-complexes (band d) disappeared with increasing concentrations of the antibody (lanes 2 to 5). At saturating concentrations, all XY_2 complexes bound to two antibodies (lanes 5 and 6, band c), indicative of two copies of protein Y in the complex. Thus, complete antibodies also allow the determination of stoichiometry. As a control, an irrelevant anti-Z antibody did not produce any shift of complex XY_2 (lanes 7 to 12). A similar wet experiment and possible difficulties in performing the NAMOS assay are described in Swamy et al. (2007).

Materials

Monoclonal antibodies that react with the proteins of interest
BN dialysis buffer (see recipe)
Sample for BN-PAGE
1-ml reaction tubes (e.g., microcentrifuge tubes)

Additional reagents and equipment for casting and running BN-PAGE gels (see Basic Protocol 1) and analysis of Blue Native gels by immunoblotting (Alternate Protocol 1 or 2)

Prepare the samples and load the Blue Native gel

1. Prepare a dilution series of the monoclonal antibodies of interest using the BN dialysis buffer containing the same detergent as the sample. Start with ~1 μg of antibody in 2 μl in the first reaction tube, then dilute 1:10, 1:100, 1:1,000, and 1:10,000 in another four tubes. Place the tubes on ice.

   Antibodies are mostly dissolved in PBS and, thus, the nondiluted antibody will be present with sodium chloride. If only 2 μl are used, the resulting salt concentration is tolerated by BN-PAGE. If, however, more than 2 μl have to be used, it is recommended to dialyze the antibody against BN dialysis buffer without any detergent (Support Protocol). Use fresh antibodies that have not been frozen and thawed too often, in order to avoid the presence of antibody aggregates. If possible, use Fab fragments of the antibodies.

2. Use any sample as described in the Support Protocol, e.g., a dialysed lysate or purified proteins. Add 10 to 20 μl of sample to each antibody-containing tube, mix, and incubate 20 min on ice.

3. Cast and pour the BN gel as in Basic Protocol 1. Use an acrylamide concentration (Table 6.10.1) in which the multiprotein complex of interest is located on the lower third of the gel after the electrophoresis.

   The reason for this is that all "antibody shifts" will be above the protein complex.

4. Load the BN gel at 4°C as in Basic Protocol 1. Load the samples in the order of the dilution (see Fig. 6.10.2). At the end, also load the solution containing the multiprotein complex (with no antibody) as reference.

   Do not load any sample in the well next to the sample with the highest concentration of antibody. In this well, the marker mix can be loaded. If a second series of antibody-added samples is loaded, use the reverse order (see Fig. 6.10.2).

Run and analyze the Blue Native gel

5. Run NAMOS BN gels as described in the Basic Protocol 1, except reduce the voltage at the beginning of the separation; if a small gel has been prepared (e.g., using BioRad Protean II or III), run at 50 V; if a large gel has been prepared (e.g., using BioRad Protean II xi), run at 75. Continue electrophoresis at the appropriate abovementioned voltage until the sample has entered the separating gel. At that point, increase voltage to 180 V for small gel or 400 V for large gel.

6. Analyze the gels by immunoblotting (Alternate Protocol 1 or 2) using only antibodies that recognize the protein/protein complex of interest.

   The detection method should not stain the monoclonal antibodies used at steps 1 and 2. For example, for detection, use directly labeled antibodies or polyclonal rabbit antiserum in combination with labeled anti–rabbit IgG antibodies that do not cross-react with the antibodies that were used for the shift. In order to control for the cross-reactivity, also load the highest concentration of the monoclonal antibody alone, i.e., without adding the protein/protein mixture of interest.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acrylamide/bisacrylamide mix

50 ml of 19:1 acrylamide/bisacrylamide solution
239 ml of 37.5:1 acrylamide/bisacrylamide solution
Store at room temperature (stable at least 1 year)

This will result in a ratio of 32:1 with 40% acrylamide.

BN anode buffer

Dilute 50 ml of 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl; store up to 1 year at room temperature) to 50 mM final by adding water to 1 liter.

BN cathode buffer

15 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (15 mM final)
50 ml 1 M tricine (50 mM final)
0.2 g Coomassie blue G250 (not other Coomassie blues) (0.02% w/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

BN cathode buffer (low Coomassie)

15 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (15 mM final)
50 ml 1 M tricine (50 mM final)
0.02 g Coomassie blue G250 (not other Coomassie blues) (0.002% w/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

BN dialysis buffer

Mix 2× BN lysis buffer stock solution (see recipe) and the detergent stock solution (see recipe) of choice so that the BN lysis buffer has a final concentration of 1× and the detergent solution has a final concentration of 0.1× (but for digitonin, use 0.3×, due to its low critical micelle concentration). Add PMSF and sodium orthovanadate (see recipe for protease and phosphatase inhibitor stock solutions) to a final concentration of 1×. Prepare fresh just before use and cool to 4°C.

Upon addition of orthovanadate, the buffer will assume a yellow color.

BN gel buffer, 3×

150 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (150 mM final)
200 ml 1 M 6-aminohexanoic acid (Sigma-Aldrich) stock solution (200 mM final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

BN lysis buffer

Mix 2× BN lysis buffer stock solution (see recipe) and the detergent stock solution (see recipe) of choice so that both have a final concentration of 1×. Add protease and phosphatase inhibitors (see recipe) to a final concentration of 1×. Prepare fresh just before use and cool to 4°C.

Upon addition of orthovanadate, the buffer will assume a yellow color.

BN lysis buffer stock solution (without detergent and inhibitors), 2×

40 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (20 mM final)
500 ml 1 M 6-aminohexanoic acid (Sigma-Aldrich) stock solution (500 mM final)
40 ml 1 M NaCl (20 mM final)
4 ml 0.5 M EDTA, pH 8.0 (*APPENDIX 2A*; 2 mM final)
200 ml glycerol (10% v/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

Preparation of an EDTA stock solution is described in UNIT 6.4.

BN stacking gel solution

3 ml 3× BN gel buffer (see recipe)
0.72 ml acrylamide/bisacrylamide mix (see recipe)
5.28 ml H_2O
120 µl 10% (w/v aqueous) ammonium persulfate (add immediately before pouring gel)
12 µl TEMED (add immediately before pouring gel)

This will result in a 3.2% solution. Adjust volumes as necessary.

Denaturing BN transfer buffer

5.81 g Tris base (48 mM final)
2.93 g glycine (39 mM final)
200 ml methanol (20% final)
1 g SDS (0.1% final)
H_2O to 1 liter
Store at 4°C (stable at least 1 year)

Destaining solution

450 ml methanol (45% v/v final)
100 ml acetic acid (10% v/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

Detergent stock solutions

2× digitonin:

Prepare a 2% (w/v) solution of digitonin (Sigma-Aldrich) in water by heating to 95°C. Store in 1- to 10-ml aliquots up to 5 years (possibly longer) at −20°C.

Thawed solutions are stable at room temperature for up to 1 week. If a precipitate forms, reheat the solution to 95°C until the digitonin redissolves.

10× Brij 96:

Prepare a 10% (w/v) solution of Brij 96 (e.g., Brij 96V) in water. Store up to 1 year (possibly longer) at room temperature.

10× Triton X-100:

Prepare a 10% (w/v) solution of Triton X-100 in water. Store up to 1 year (possibly longer) at room temperature.

10× NP-40:

Prepare a 10% (w/v) solution of Nonidet P-40 (NP-40) in water. Store up to 1 year (possibly longer) at room temperature.

10× dodecylmaltoside:

Prepare a 10% (w/v) solution of dodecylmaltoside (Sigma-Aldrich) in water. Store up to 1 year (possibly longer) at 4°C.

Any nonionic detergent that has been proven to be useful for the cells and proteins of interest can be used as well.

High-percentage BN separating gel solution

5 ml 3× BN gel buffer (see recipe)
5.63 ml acrylamide/bisacrylamide mix (see recipe)
4.38 ml 70% (v/v) glycerol
42 µl 10% (w/v aqueous) ammonium persulfate (add immediately before pouring gel)
4.2 µl TEMED (add immediately before pouring gel)

This will result in a 15% solution. Adjust volumes and acrylamide/bisacrylamide concentration as necessary. Useful concentrations range from 6% to 18%.

Low-percentage BN separating gel solution

5 ml 3× BN gel buffer (see recipe)
1.5 ml acrylamide/bisacrylamide mix (see recipe)
8.5 ml H_2O
54 µl 10% (w/v aqueous) ammonium persulfate (add immediately before pouring gel)
5.4 µl TEMED (add immediately before pouring gel)

This will result in a 4% solution. Adjust volumes as necessary.

Marker mix 1

20 µl 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl)
20 µl 1 M NaCl
143 µl 70% (v/v) glycerol
5 mg ferritin (440 kDa and 880 kDa)
5 mg catalase (232 kDa)
5 mg bovine serum albumin (66 kDa and 132 kDa)
H_2O to 1 ml
Store at 4°C (stable at least 1 year)

Marker mix 2

20 µl 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl)
20 µl 1 M NaCl
143 µl 70% (v/v) glycerol
5 mg thyroglobulin (670 kDa)
5 mg aldolase (158 kDa)
H_2O to 1 ml
Store at 4°C (stable at least 1 year)

Native BN transfer buffer

5.81 g Tris base (48 mM final)
2.93 g glycine (39 mM final)
200 ml methanol (20% final)
H_2O to 1 liter
Store at 4°C (stable at least 1 year)

Pervanadate, 100×

Mix, in the following order:
50 µl 50 mM sodium orthovanadate
57 µl H_2O
15 µl 30% H_2O_2
Incubate 5 to 30 min at room temperature (becomes brownish)
Prepare fresh

Phosphate-buffered saline (PBS), 10×

152 g NaCl (130 mM final)
24 g monobasic sodium phosphate, anhydrous (10 mM final)
1600 ml H_2O
Adjust pH to 7.4 with NaOH.
Add H_2O to 2 liters
Store at room temperature (stable at least 1 year)

PBS with 1% (w/v) SDS

100 ml of 10× PBS (see recipe; 1× final)
100 ml 10% (w/v) SDS stock solution (*APPENDIX 2A*; 1% final)
Add H_2O to 1 liter
Store at room temperature (stable at least 1 year)

Protease and phosphatase inhibitor stock solutions

1000× leupeptin:
Prepare a 10 mg/ml solution of leupeptin in water. Store in 1-ml aliquots up to 5 years (possibly longer) at −20°C.
1000× aprotinin:
Prepare a 10 mg/ml solution of aprotinin in water. Store in 1-ml aliquots up to 5 years (possibly longer) at −20°C.
100× PMSF:
Prepare a 100 mM solution of phenylmethylsulfonyl fluoride (PMSF) in ethanol. Store in 1-ml aliquots up to 5 years (possibly longer) at −20°C.
100× sodium orthovanadate:
Prepare a 50 mM solution of sodium orthovanadate in water. Store up to 1 year (possibly longer) at room temperature.
100× sodium fluoride:
Prepare 1 M solution of sodium fluoride in water. Store up to 5 years (possibly longer) at room temperature.

COMMENTARY

Background Information

Most if not all proteins require binding to other proteins in order to fulfill their function. Thus, they form multiprotein complexes (MPCs). Most proteins are part of several distinct complexes, as well as being present as monomers. The abundance of distinct complexes of which a certain protein is a subunit can vary enormously. Furthermore, complexes might have different stabilities and these can change over time and space. Therefore, identifying and analyzing complexes is a difficult task.

Common techniques to study complexes such as immuno-precipitation (*UNIT 7.2*) or two-hybrid (*UNIT 17.3*) methods allow the identification of binding partners of the protein of interest; they, however, do not yield any information about the size, number, composition, and relative abundance of MPCs. A high-resolution method that resolves these problems is Blue Native polyacrylamide gel electrophoresis (BN-PAGE; Schägger and von Jagow, 1991; Schägger et al., 1994). Originally it was developed by Hermann Schägger to separate mitochondrial membrane complexes in the mass range from 10 to 10,000 kDa (Schägger and von Jagow, 1991; Schägger et al., 1994). Later, the protocol was modified for general applicability (Camacho-Carvajal et al., 2004). Since the first description of BN-PAGE, descriptions of its use in publications has increased exponentially. It has been applied successfully in nearly every area of multiprotein research—e.g., purification of complexes, determination of their size (Schägger et al., 1994; Schägger, 1995; Model et al., 2001; Dudkina et al., 2005) and stoichiometry (Schamel et al., 2005; Swamy et al., 2007), protein complex assembly (Model et al., 2001), structure determination by two-dimensional crystallization and electron microscopy (Poetsch et al., 2000), identification of multiprotein complexes with mass spectroscopy (Rexroth et al., 2003; Camacho-Carvajal et al., 2004; Millar et al.,

2005), or clinical diagnostics of human disorders (Schägger, 1995; Schägger et al., 1996).

In principle, BN-PAGE can be used to identify protein complexes in a given biological sample or to further characterize known protein complexes (or protein-protein interactions). For the second application, it is recommended to first test a series of different nonionic detergents for their effects on the extractability and stability of the protein-protein interaction of interest by co-immunoprecipitation (*UNIT 7.2*) followed by SDS-PAGE (*UNIT 6.1*). Only a successful co-purification indicates that the complex of interest is stable and abundant enough to be detected by BN-PAGE.

In BN-PAGE, the dye Coomassie blue, which binds nonspecifically to proteins and is itself negatively charged, is used. Therefore, the electrophoretic mobility of a multiprotein complex is determined by the negative charge of the bound Coomassie blue dye and the size and shape of the complex. Coomassie blue does not act as a detergent and preserves the structure of protein complexes. In contrast to other native gel electrophoresis systems, protein complexes are separated independently of isoelectric point and, therefore, the size of a complex can be estimated. In addition, the binding of Coomassie blue to proteins reduces their tendency to aggregate during the stacking step of the electrophoresis.

Following the first-dimension BN-PAGE, a number of subsequent biochemical techniques can be applied. Although proteins are already visible as blue bands after BN-PAGE, it can be useful to stain again with Coomassie blue to reach a higher detection sensitivity. Alternatively, silver staining (*UNIT 6.6*) or immunoblotting (*UNIT 6.2*) are commonly used. The individual subunits of a complex can be identified by SDS-PAGE or the Native Antibody-Based Mobility Shift (NAMOS) assay (see Basic Protocol 3). The NAMOS assay is a variant of BN-PAGE in which the stoichiometry of multiprotein complexes can be determined without purification of the complex of interest (Swamy et al., 2007).

Critical Parameters and Troubleshooting

Potential problems in pouring and running acrylamide gels in general are described in *UNIT 6.1*. These include problems in the polymerization of the gel and critical factors when using multicasting equipment.

Removing the comb of the BN stacking gel might destroy the wells of the stacking gel, due to the low percentage of acrylamide. Try to remove the comb slowly, pulling it out slightly perpendicular to the plane of the gel. This allows air to enter the wells. If this does not help, increase the acrylamide/bisacrylamide concentration of the BN stacking gel by 0.3%. If the gel pieces that form the wells are not broken, but just displaced, try to fix them with a syringe needle. Unwanted air bubbles in the stacking gel can be aspirated using a syringe. Test for leakiness with the BN cathode buffer before loading your sample.

A proper acrylamide concentration for the BN gel and the subsequent second-dimension SDS gel should be selected to optimize resolution within the desired molecular weight range. Use 4% to 7% BN gels for protein complexes of more than 1000 kDa, 4% to 10% for 500 kDa, 4% to 12% for 250 kDa, and 4% to 18% for proteins smaller than 100 kDa.

The first critical step in BN-PAGE is the preparation of the sample, since potassium and divalent cations that interact with Coomassie blue have to be removed. They are substituted by 6-aminohexanoic acid in order to maintain a certain ionic strength of the solution, which increases the solubility of many proteins. Therefore, the lysate has to be dialysed against BN dialysis buffer.

One frequent problem is that Coomassie blue and Coomassie blue–bound proteins precipitate in the gel wells, and subsequently the sample does not enter the gel. In this case, either reduce the amount of sample or improve dialysis of the sample. Use a larger dialysis reservoir volume, remove all air bubbles at the dialysis membrane, or prolong dialysis time.

Depending on the detergent, the dye front might contain peaks of detergent that prevent proteins from entering below these areas. This is pronounced when using polyoxyethylene detergents such as Brij 96 and Triton X-100. If your multiprotein complex of interest runs at a higher position than these precipitates, then this might not be a problem. Otherwise reduce the amount of detergent in the BN dialysis buffer.

To detect the protein of interest by Coomassie brilliant blue staining, it should be present in 0.5 to 2 μg amounts. If silver staining is used, this amount can be reduced to 0.1 μg. 20 to 40 μg of a protein mixture is typically loaded into a well of 50-μl volume on a 1-mm slab BN gel (16 cm, 15 wells). For immunoblotting, much less protein is required, depending on the quality of the antibody used for detection.

Instead of giving a defined band in the first dimension or a circular spot in two-dimensional BN/SDS-PAGE, a smear that was generated in the first dimension may be seen. This could have several reasons. First, the electrophoresis might not have been optimal. Try to improve dialysis of the sample (see above). If the protein migrates much higher than the dye front, a shorter electrophoresis might help. Second, the protein might have aggregated. Decrease the amount of sample loaded. Do not freeze and thaw the sample. Try to further purify the protein of interest under native conditions. Third, the protein might be present in several overlapping complexes. A two-dimensional BN/BN-PAGE could prove whether several overlapping complexes coexist that contain the protein of interest. For details and other techniques to explore this possibility, see Schamel et al. (2005).

Sometimes the protein is detected in a defined complex, but not in a reproducible manner. In this case the complex might have been an artifact generated during the electrophoresis step. This could be due to a step in the gel gradient—i.e., the gradient was not continuous. When pouring the gradient, make sure that there is a continuous flow of liquid and also that the flow is continuous between the two cylinders of the gradient maker. While loading the first-dimension gel slice onto the second-dimension SDS-PAGE, there could have been a small air bubble under the slice. This prevents entry of protein along that vertical line, giving the impression that two MPCs exist, rather than one.

If a certain interaction between two proteins is not seen by co-immunopurifications, do not expect to detect it by BN-PAGE. If the protein of interest is only found as a monomer, although it should be present in a complex, the complex could have been disrupted by detergent or be of low abundance. Thus, the choice of detergent is important in extracting but not disrupting multiprotein complexes. Without detergent, proteins tend to aggregate during the stacking step of the electrophoresis and do not enter the separating gel properly. Thus, a certain concentration of detergent has to be present in the sample. Unfortunately, general rules for the choice of detergent do not exist. Compare several detergents of different classes and three different concentrations of each in preliminary experiments, including detergents that allow successful co-immunopurifications of the proteins expected to be present in a common complex.

In the BN lysis buffer, orthovanadate is included to inhibit phosphatases. Since orthovanadate is a small molecule, it is rapidly separated from the sample during the BN-PAGE run. Since it is a reversible inhibitor, phosphatases become active once orthovanadate is removed. Thus, phosphorylation of the proteins might be lost during the native electrophoresis, where active phosphatases might be present. Since pervanadate irreversibly inhibits phosphatases, it should not be omitted from the BN dialysis buffer when phosphorylation of proteins needs to be preserved.

One common surprise that is encountered in performing BN-PAGE is that the size of the protein complex of interest does not match the expected value. First, the detergent micelle around transmembrane regions adds to the size of the corresponding protein. Hence, the size of the protein or protein complex might be larger than that obtained by simply adding the molecular weights of the individual subunits. Thus, to estimate the size of transmembrane proteins (complexes), the mass calibration markers should also be transmembrane proteins solubilized with the same detergent. Some water-soluble proteins match the calibration curve of dodecylmaltoside-solubilized transmembrane proteins, and therefore might be used as standards (Schägger et al., 1994). Second, protein glycosylation and phosphorylation might alter the running behavior of proteins in BN-PAGE. Note that the marker proteins are usually non-transmembrane, non-glycosylated, and non-phosphorylated proteins. Especially if you work with transmembrane proteins, you cannot deduce the molecular weight of the proteins from BN-PAGE. Third, in addition to their molecular weight, proteins are also separated according to their shape and number of bound Coomassie blue molecules. Thus, proteins that deviate significantly from a ball-like shape and very basic proteins show reduced mobility. Lastly, it might be that the expected molecular weight value of the protein complex is wrong.

Many antibodies that work well for the immunodetection (western blotting) of your protein of interest after SDS-PAGE do not recognize the protein after the first-dimension BN-PAGE. In the author's experience, the removal of Coomassie blue from the blotting membrane does not help. Try both conditions for the transfer to the blotting membrane (Alternate Protocols 1 and 2). If this does

not help, then a two-dimensional BN/SDS-PAGE must be performed. In any case, one should verify immunoblotting results of a first-dimension BN-PAGE by two-dimensional BN/SDS-PAGE, in order to prove that the detected protein indeed is the protein of interest and not a cross-reactivity of the antibody.

Performing a successful NAMOS assay critically depends on the quality of the antibodies used to shift the protein complex of interest (Swamy et al., 2007). Several problems could arise. First, protein complexes clustered by antibodies can be very large; thus, use Alternate Protocol 1 to disassemble these aggregates to efficiently transfer them to the blotting membrane. Second, some antibodies aggregate with themselves, thus producing a ladder-like shift pattern (Swamy et al., 2007). With these antibodies, a continuous increase in antibody concentration leads to a constant generation of new larger bands while smaller ones disappear. Thus, the stoichiometry of a multiprotein complex cannot be determined with this type of antibody; however, it is possible to determine whether all complexes contain the subunit in question. Use a fresh preparation of antibody, prepare Fab fragments, or remove antibody aggregates by ultracentrifugation. Third, it is possible that a given antibody cannot bind to all identical subunits of a multiprotein complex, probably because the complex is not symmetric, rendering the epitope accessible in one copy of the subunit but not in its neighboring copy. Likewise, if the two epitopes are very close within the complex, two antibody molecules might not be able to bind simultaneously due to steric hindrance. This type of antibody gives an underestimate of the actual stoichiometry (Swamy et al., 2007). In conclusion, not all antibodies result in the number of shifts that correspond to the number of subunit copies present in the protein complex. Thus, it is recommended to use several independent antibodies per subunit, and the Fab fragments of those antibodies.

Anticipated Results

In BN-PAGE, native protein and multiprotein complexes are separated according to their size. Thus, using two-dimensional BN/SDS-PAGE, protein complexes can be identified and characterized in terms of their relative abundance, subunit composition, and size. Since BN-PAGE is only the separation technique, the sensitivity of detection depends on the detection method used. Furthermore, the NAMOS assay allows the determination of the stoichiometries even from nonpurified complexes. Typical results are illustrated in Figures 6.10.1 and 6.10.2. Comparison of two or more samples is possible if the pouring and running of the gels was done in similar way. In combination with modern mass spectroscopy, BN-PAGE, with its high-resolution properties, is an excellent choice to identify novel protein complexes from any biological source.

Time Considerations

Preparation of the sample as described in the Support Protocol takes 6 hr or overnight. If purified proteins or organelle fractions are used, the required time can be substantially longer. However, if utilizing desalting columns to obtain samples with low cation concentrations, the time can be shorter. Since the sample should be separated immediately by BN-PAGE (and cannot be frozen and stored), one should reserve some time for gel loading, which usually takes another 0.5 to 1 hr. Depending on the gel size, running of the BN-PAGE takes from 6 hr to overnight. Loading of two second-dimension SDS-PAGE gels takes ~1 to 1.5 hr. Running the SDS-PAGE takes between 1.5 hr and overnight, again depending on the size of the gels. Thus, two-dimensional BN/SDS-PAGE takes at least 1.5 days—not counting the visualization process of the proteins of interest.

One rate-limiting step is the running of the second-dimension gels. 30 to 40 lanes can easily be separated in parallel on two BN gels, but loading, running, and detection from 30 to 40 second-dimension gels requires substantial operator time and electrophoresis equipment. Therefore, whenever possible, one should try to use only first-dimension BN gels without the need for the second dimension.

Pouring gradient gels is another time-consuming process. To minimize the time requirement, it is strongly recommended to use multicasting equipment to pour several gels at once. This also ensures best reproducibility for critical comparisons of multiple samples.

Acknowledgements

I thank Michael Reth and Klaus Pfanner for initially introducing me to BN-PAGE and Hermann Schägger for his invaluable help in setting up the technique. I further thank Balbino Alarcón for his advice on establishing the NAMOS assay and members of my laboratory for constantly improving the various BN techniques: Margarita Camacho-Carvajal, Mahima Swamy, Thomas Bock, Eszter Molnar, Susana Minguet, Elaine Pashupati Dopfer, and Gabrielle Siegers.

I also acknowledge financial support by the European Union through an individual Marie Curie fellowship and the Deutsche Forschungsgemeinschaft through the Emmy Noether program (SCHA 976/1) and the SFB620.

Literature Cited

Camacho-Carvajal, M.M., Wollscheid, B., Aebersold, R., Steimle, V., and Schamel, W.W. 2004. Two-dimensional blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates: A proteomics approach. *Mol. Cell. Proteomics* 3:176-182.

Dudkina, N.V., Eubel, H., Keegstra, W., Boekema, E.J., and Braun, H.P. 2005. Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. *Proc. Natl. Acad. Sci. U.S.A.* 102:3225-3229.

Millar, A.H., Heazlewood, J.L., Kristensen, B.K., Braun, H.P., and Moller, I.M. 2005. The plant mitochondrial proteome. *Trends Plant Sci.* 10:36-43.

Model, K., Meisinger, C., Prinz, T., Wiedemann, N., Truscott, K.N., Pfanner, N., and Ryan, M.T. 2001. Multistep assembly of the protein import channel of the mitochondrial outer membrane. *Nat. Struct. Biol.* 8:361-370.

Poetsch, A., Neff, D., Seelert, H., Schägger, H., and Dencher, N.A. 2000. Dye removal, catalytic activity and 2D crystallization of chloroplast H(+)-ATP synthase purified by blue native electrophoresis. *Biochim. Biophys. Acta* 1466:339-349.

Rexroth, S., Meyer zu Tittingdorf, J.M., Krause, F., Dencher, N.A., and Seelert, H. 2003. Thylakoid membrane at altered metabolic state: Challenging the forgotten realms of the proteome. *Electrophoresis* 24:2814-2823.

Schamel, W.W., Arechaga, I., Risueno, R.M., van Santen, H.M., Cabezas, P., Risco, C., Valpuesta, J.M., and Alarcon, B. 2005. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J. Exp. Med.* 202:493-503.

Schägger, H. 1995. Quantification of oxidative phosphorylation enzymes after blue native electrophoresis and two-dimensional resolution: Normal complex I protein amounts in Parkinson's disease conflict with reduced catalytic activities. *Electrophoresis* 16:763-770.

Schägger, H. and von Jagow, G. 1991. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 199:223-231.

Schägger, H., Cramer, W.A., and von Jagow, G. 1994. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* 217:220-230.

Schägger, H., Bentlage, H., Ruitenbeek, W., Pfeiffer, K., Rotter, S., Rother, C., Bottcher-Purkl, A., and Lodemann, E. 1996. Electrophoretic separation of multiprotein complexes from blood platelets and cell lines: Technique for the analysis of diseases with defects in oxidative phosphorylation. *Electrophoresis* 17:709-714.

Swamy, M., Minguet, S., Siegers, G.M., Alarcon, B., and Schamel, W.W. 2007. A native antibody-based mobility-shift technique (NAMOS-assay) to determine the stoichiometry of multiprotein complexes. *J. Immunol. Methods* 324:74-83.

Key References

Camacho-Carvajal et al., 2004. See above.

Describes the separation of cellular lysates by BN-PAGE.

Schägger and von Jagow, 1991. See above.

Describes, for first time, BN-PAGE and second-dimension BN/SDS-PAGE using solubilized mitochondria.

Swamy et al., 2007. See above.

Details the NAMOS assay with an extensive discussion of anticipated results.

Measurement of Oxidatively-Induced Clustered DNA Lesions Using a Novel Adaptation of Single Cell Gel Electrophoresis (Comet Assay)

Alexandros G. Georgakilas,[1,2] Stewart M. Holt,[1,3] Jessica M. Hair,[1] and Charles W. Loftin[1]

[1]Thomas Harriot College of Arts and Sciences, East Carolina University, Greenville, North Carolina
[2]National Technical University of Athens, Zografou Campus, Athens, Greece
[3]Elon University, Elon, North Carolina

ABSTRACT

The two basic groups of complex DNA damage are double-strand breaks (DSBs) and non-DSB oxidatively-induced clustered DNA lesions (OCDLs). The single-cell gel electrophoresis (SCGE) or comet assay has been widely used for the detection of low levels of various types of DNA lesions including single-strand breaks (SSBs), DSBs, and oxidized bases per individual cell. There are limited data on the use of the comet assay for the detection of non-DSB clustered DNA lesions using different repair enzymes as enzymatic probes. This unit discusses a novel adaptation of the comet assay used to measure these unique types of lesions. Until now OCDL yields have been measured using primarily pulsed-field agarose gel electrophoresis. The advantages offered by the current approach are: (1) measurement of OCDL levels per individual cell; (2) use of a small number of cells (∼10,000) and relatively low doses of ionizing radiation (1 to 2 Gy) or low levels of oxidative stress, which are not compatible with standard agarose gel electrophoresis; and finally, (3) the assay is fast and allows direct comparison with pulsed-field gel electrophoresis results. *Curr. Protoc. Cell Biol.* 49:6.11.1-6.11.17.

Keywords: oxidatively-induced clustered DNA lesions • single-cell gel electrophoresis • repair enzymes • complex DNA damage

INTRODUCTION

This unit describes the analytical execution of a novel adaptation of the single-cell gel electrophoresis (SCGE) method using DNA agarose plugs. The standard comet assay uses a constant electric field, similar to any electrophoresis, to force migration of protein-free fragmented DNA embedded in a DNA/low-melting-point agarose mixture. During electrophoresis the fragmented DNA migrates out of the cell nucleus and into the gel matrix according to its length, forming a tail that resembles a comet tail, hence the name. The adaptation presented here allows measurement of bi-stranded non-double strand break (DSB) oxidatively-induced clustered DNA lesions (OCDLs) per individual cell. The novelty of this assay lies in the fact that DNA/agarose plug technology is used for measuring clusters per individual cell in the comet assay. This adaptation allows direct quantitative comparison with the most widely used independent method of pulsed-field gel electrophoresis (PFGE; Sutherland et al., 2003). The comet assay differs from PFGE in that it is a simpler and faster method already utilized in biomonitoring and genotoxicity assessment. Although both techniques involve the embedding of cells in a low-melting-point agarose suspension, lysis, and electrophoresis, comet assays offer a unique advantage since DNA fragmentation can be visualized under the

Current Protocols in Cell Biology 6.11.1-6.11.17, December 2010
Published online December 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb0611s49

microscope per individual cell using fluorescent DNA staining. The major experimental principles/methodology reside in the principle that supercoiled duplex DNA can be fragmented by DNA endonucleases, hydrogen peroxide, ionizing radiations, etc. The resulting DNA migration is directly related to the extent of DNA damage as evidenced by the formation of the comet tail. Therefore, DNA migration is a function of both size and amount of broken DNA fragments. The measurement of DNA damage is based on the percentage of fluorescence of the DNA in the tail rather than in the analysis of DNA fragment distribution. On the other hand, PFGE through the application of a pulsed and rotating electric field can be used for the analysis of DNA fragment distribution for lengths ranging from ~6 Mbp up to a few kilobasepairs.

This unit focuses on the utilization of SCGE for measurement of closely spaced (clustered) DNA lesions on opposing strands (bi-stranded; OCDLs) and on the same strand (uni-stranded or tandem; total sites) in human cells exposed to relatively low doses of ionizing radiation (1 to 10 Gy) or any other oxidizing agent capable of inducing equivalent levels of oxidative injury in the cell. This novel adaptation of SCGE uses DNA agarose plugs as in PFGE and has the following advantages compared with previous versions of SCGE: (1) the results are directly comparable with PFGE and other techniques measuring yields of clustered DNA damage; (2) the assay is practical, simple, and reproducible; and (3) the use of plugs allows for the production and extended storage of multiple samples whereas traditional SCGE methods did not. Earlier studies by Visvardis et al. (2000) have shown that the use of DNA agarose plugs can also be used in the comet assay for detection of strand breaks induced by the restriction enzyme *Not*I in human DNA. No studies exist on the use of this methodology in the detection of clustered DNA damage.

Successful SCGE requires basic knowledge of DNA agarose gel electrophoresis and fluorescence microscopy. Since it involves cell processing and enzymatic processing of DNA, knowledge and abilities in cell culture and basic enzymology are also required. This unit provides detailed methods for preparing cells for treatment with an oxidizing agent, creation of DNA agarose plugs, and repair enzyme treatment (see Basic Protocol 1), running an SCGE assay (see Basic Protocol 2), and proper quantitative analysis of the level of OCDLs and total sites per cell (see Support Protocol).

STRATEGIC PLANNING

Choice of Proper Repair Enzymes

The standard comet assay can be used for detection of frank single-strand breaks (SSBs) and double-strand breaks (DSBs) (denaturing and non-denaturing conditions, respectively) directly induced by any oxidizing agent. For the additional detection of OCDLs, the use of cellular repair enzymes is necessary. This unique approach for quantifying these types of bi-stranded oxidative DNA damage in human cells using DNA repair enzymes isolated from *E. coli* was initially developed by Sutherland et al. (2000a). Human or bacterial repair enzymes like formamidopyrimidine [fapy]-DNA glycosylase (Fpg), endonuclease III (*Endo*III), and endonuclease IV (*Endo*IV), with their appropriate buffers, can be used as enzymatic damage probes to cleave specific DNA lesions (Sutherland et al., 2003). The choice of enzyme is dependent on the type of oxidized base damage one aims to detect. For example, for the detection of abasic (AP) sites (DNA sites with a missing base), the human APE1 or bacterial *Nfo* can be used; in the detection of oxidized purines, the human OGG1 or bacterial Fpg can be used. The cleavage sites of different enzymes used for the detection of clusters are listed in Table 6.11.1. Although these enzymes are commercially available, the user must always check the specificity (Holt and Georgakilas, 2007) and, if possible, compare commercial enzymes with the same enzymes produced in the laboratory using bacterial cells (Georgakilas et al., 2004). Once a lesion is detected, as shown in Figure 6.11.1, in each cluster, repair enzymes will excise

Table 6.11.1 Bacterial or Human Repair Enzymes Used as Enzymatic Probes for the Detection of Oxidatively Clustered DNA Lesions (OCDLs) and their Expected Substrates

Repair enzyme used as damage probe[a]	Substrates
Human hAPE1 or *E. coli* Nfo protein (endonuclease IV)	Abasic: Several types of abasic sites including oxidized abasic sites, abasic sites modified with alkoxyamines and DNA containing urea residues; no activity is expected towards oxidized bases
Human OGG1 or *E.coli* Fpg protein (DNA glycosylase) Associated AP lyase activity	Oxidized purines (oxypurines): Majority of oxidized purines like FapyGua, FapyAde, C8-oxoGuanine, C8-oxoAdenine, and to a lesser extent, other modified purines and some abasic sites
Human NTH1 protein or *E.coli Endo*III (endonuclease III) Associated AP lyase activity	Oxidized pyrimidines (oxypyrimidines): Thymine residues damaged by ring saturation, fragmentation, or ring contraction including thymine glycol (Tg) and uracil residues (5-fo-Ura), FapyAde, 5-OH-Cyt and to a lesser extent FapyGua; some abasic sites

[a] All enzymes are commercially available from New England Biolabs or can be isolated using an approach similar to the one described by Georgakilas et al. (2004).

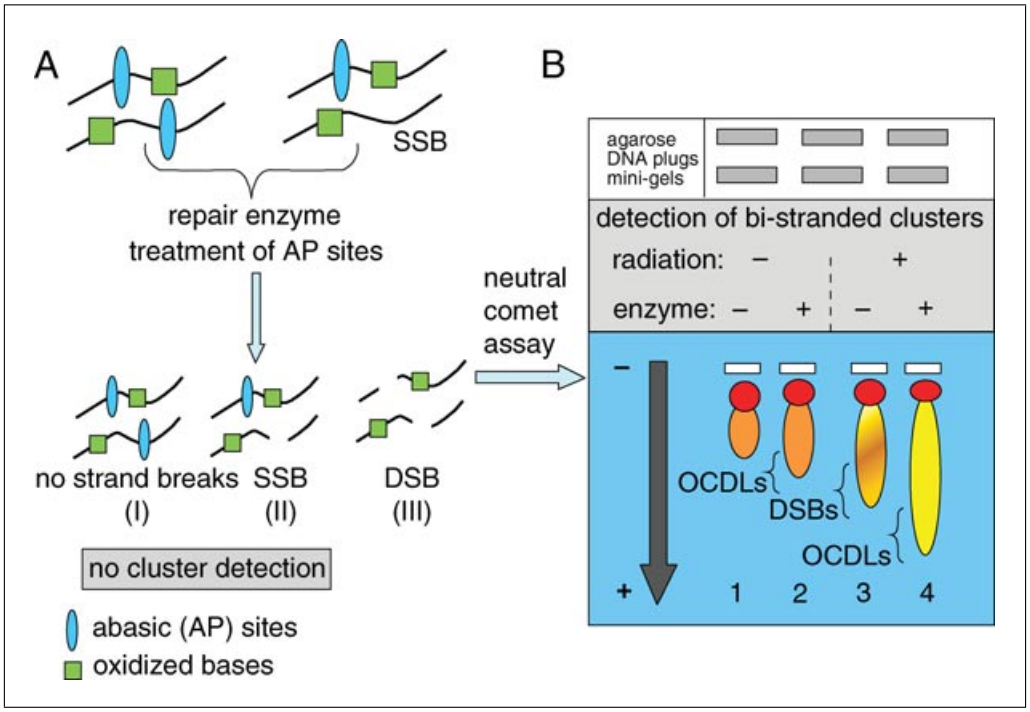


Figure 6.11.1 Detection of bi-stranded oxidatively induced clustered DNA lesions (OCDLs) using a modified neutral comet assay and agarose-DNA plugs as mini gels. (**A**) Principles of detection using a repair enzyme for a representative ideal type of a cluster consisting of a set of four bi-stranded base lesions (abasic-AP- sites and oxidized bases). Alternatively, one of the lesions can be a single-strand break, SSB. As shown in pathways I and II, no or incomplete cleavage of AP lesions by the repair enzyme AP endonuclease will lead to no detection of the cluster. In the case of cleavage of both lesions by the enzyme and induction of a DSB, detection of the cluster occurs (III). (**B**) Detection of clusters using neutral (non-denaturing) comet assay. Microscope slides containing human cells embedded in agarose plugs can be subjected to constant field electrophoresis and the double-strand breaks (DSBs) and OCDLs can be measured in the same run as the ones being induced by radiation. For lanes 1 and 2 (non-irradiated) low levels of endogenous OCDLs are expected. For lanes 3 and 4 (irradiated) higher levels of OCDLs are expected. Comparison of lane 3 and 1 will provide the yield of prompt DSBs induced by radiation. The measurement is based in the increase of comet tail moment (TM = %DNA in tail × tail length) and equations described in the Support Protocol.

the lesion and cleave the DNA strand by their intrinsic lyase activity (DNA glycosylases) or in the case of an AP endonuclease, it will cleave the DNA strand directly. In either case, an SSB is created in each strand or a DSB in the case of a cluster (Fig. 6.11.1). These de novo DSBs are equal to the clusters detected by the enzymatic probe (Sutherland et al., 2000b).

Choice of Neutral or Alkaline Running Conditions for the SCGE

The alternative uses of neutral or alkaline gel electrophoresis can provide the user the option of calculating bi-stranded or total lesions. Neutral (non-denaturing) conditions, described in this unit, can be used to detect and measure DSBs and OCDLs (non-DSB clusters) with the use of repair enzymes. With alkaline electrophoresis, one can detect all induced SSBs and alkali-labile or other enzyme-sensitive sites that are converted to breaks during alkaline electrophoresis (total sites). The term total sites refers to all the lesions existing in each DNA strand (Fig. 6.11.1). OCDLs only refer to the bi-stranded lesions located in opposing strands. In addition to normal and oxidized abasic sites, several oxidized bases including 5,6-dihydroxy-5,6-dihydrothymine, 5-formyluracil, and oxazolone may be converted into strand breaks under the alkaline conditions of single-cell gel electrophoresis analysis (Pouget et al., 2002; Douki et al., 2006). Although the novel adaptation of SCGE presented here is considered highly efficient and acceptable for the calculation of OCDL levels per cell, it does not directly calculate the exact number of sites per individual cell as does PFGE.

BASIC PROTOCOL 1

TREATMENT OF CELLS WITH AN OXIDIZING AGENT AND PREPARATION OF AGAROSE PLUGS

The concepts of DNA cluster quantification are simple, but execution is technically and analytically demanding. The major experimental principles and methodology underlying these measurements are discussed. In every experiment to calculate levels of bi-stranded clusters using neutral SCGE, the use of ionizing radiation (X- or γ-rays) as a standardization control is recommended. Even low doses of these types of radiations ($\sim$1 Gy) have been found to induce detectable levels of clusters in human cells (Sutherland et al., 2000a; Holt and Georgakilas, 2007). In addition, a linear response of cluster yields in response to increasing dose is expected for a wide range of doses (1 to 20 Gy). Separate samples can be prepared as a positive control using hydrogen peroxide, since it has been found that low-to-moderate concentrations of hydrogen peroxide ($\leq$100 μM) do not induce detectable levels of bi-stranded clusters (Holt and Georgakilas, 2007). In the case of tandem (total) lesions, both agents are expected to give detectable yields.

For treatment with the oxidizing agent, $\sim$10,000 cells in complete medium are exposed to this agent (similar to ionizing radiation) on ice. Cells must be kept on ice before and during the treatment period to inhibit any repair processes. Immediately following treatment and if no repair measurements are intended, EDTA is added to a final concentration of 50 mM and the cells are maintained in this solution until further processed. In the case of repair experiments for measuring processing efficiency, the cells are exposed to the agent at room temperature and are incubated at 37°C until the desired time-point and processed into sample plugs as described below. The non-treated (negative controls) must be processed in exactly the same manner as the the oxidizing agent–treated samples. To minimize oxidation artifacts during DNA isolation, all buffers must be freshly prepared, autoclaved, purged with argon or nitrogen for 15 min, and supplemented with 50 μM phenyl-tert-butyl nitrone in DMSO (PBN; Sigma), a non-toxic, free radical spin trap and scavenger (Lu et al., 2004; Tsao et al., 2007).

Materials

1% (w/v) molecular biology grade, normal-melting-point agarose in 1× PBS
Cells
Complete medium
Trypsin, optional
Phosphate buffered saline (PBS; *APPENDIX 2A*)
Trypan blue
Hydrogen peroxide (H_2O_2)
1 M Na_2EDTA
Low-melting-point agarose prepared in TE (molecular biology grade; Bio-Rad)
Plug lysis solution (see recipe)
1× TBE electrophoresis buffer (see recipe)
Appropriate repair enzymes and autoclaved and filtered buffers for enzymes (Fpg, *Endo*III, *Endo*IV; New England Biolabs)
1 M KCl, pH 7.8 (*APPENDIX 2A*), ice cold
Alkaline denaturation buffer (see recipe), ice cold

Autoclaved-sterile microscope slides (3 × 5–in.) and 18-mm^2 coverslips (Corning)
25-cm^2 cell culture flasks
Hemacytometer
^{137}Cs source
Refrigerated centrifuge
PFGE plug molds (BioRad)
37°C incubation oven or thermal cycler

Prepare slides

1. Prepare 3 × 5–in. microscope slides fully covered with 1 ml of 1% (w/v) molecular biology grade, normal-melting-point agarose in 1× PBS. Allow slides to dry overnight at room temperature.

   Make sure the agarose is maintained at 37°C to ensure solution consistency while applying. Using a Pasteur pipet, ensure that the agarose is uniformly applied on the surface of the slide and allow to dry at room temperature, then store the slides in a slide box in a dry environment.

   This should be done 24 hr prior to plug preparation to ensure complete drying of slides.

Prepare agarose/DNA plug

2. Grow cells in a 25-cm^2 cell culture flask in 5 ml complete medium at optimum conditions recommended by supplier.

   Cells can be grown in larger flasks if more sample plugs are desired.

   Cell concentration should not exceed 1 × 10^6 cells/ml. If cells are adherent, grow to ~95% confluence.

   Any type of cell line or even primary cells can be used.

3. Once cells reach appropriate confluence, trypsinize (if adherent; Francisco et al., 2008), resuspend in 1 to 2 ml PBS, and place on ice for 10 min.

4. Count cells after trypsinization.

   The expected yield of a 25-cm^2 flask if seeded at 50,000 cell/ml should be at least 25,000 cell/ml.

   The count will indicate how many sample plugs can be made from the contents of each flask. About 10,000 cells must be used per plug. Since preparation of a total number of ~10 to 20 plugs is recommended, a minimum of 100,000 to 200,000 cells is required for each treatment. Assuming one radiation dose, negative (untreated) control, and positive

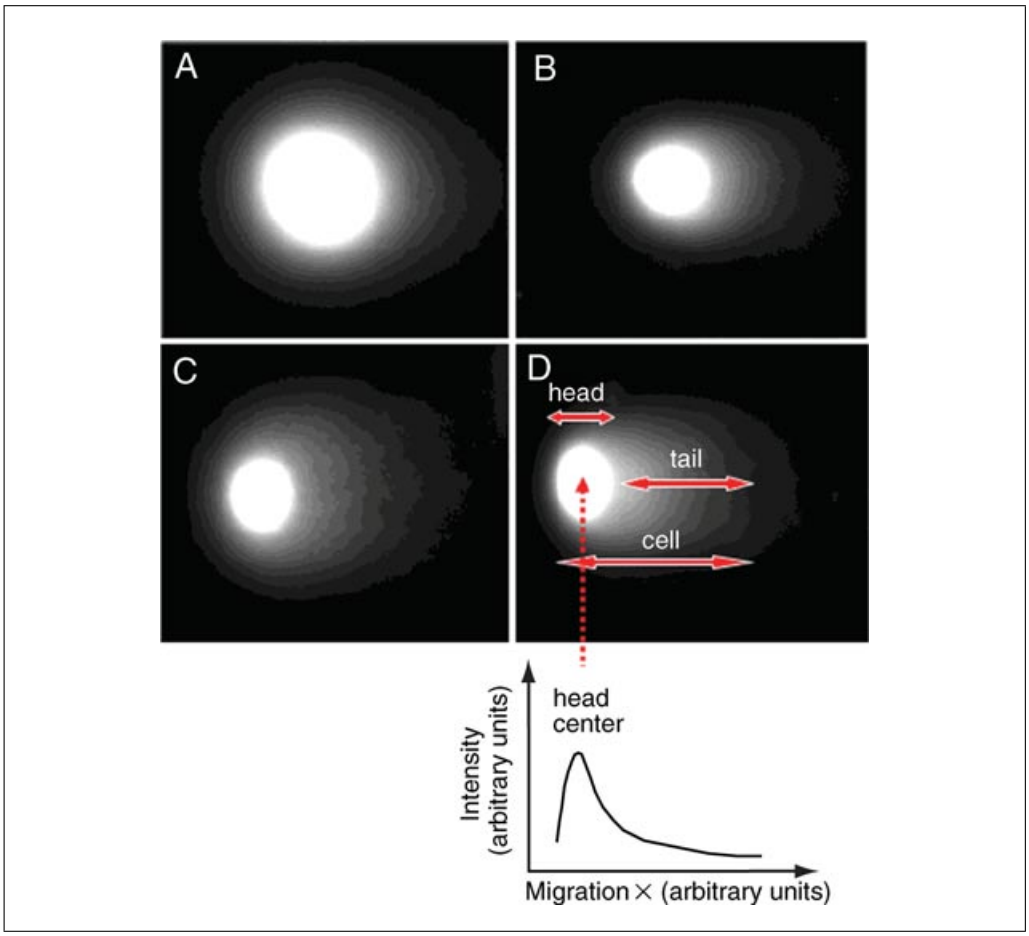


Figure 6.11.2 Detection of bi-stranded oxidatively induced clustered DNA lesions (OCDLs) induced by γ-irradiation in human pre-B acute lymphoblastic leukemia NALM-6 cells using single-cell gel electrophoresis (comet assay), non-denaturing conditions, and Fpg repair enzyme as a damage probe. (**A**) Upper left panel shows an unirradiated single cell (used as control) with a tail moment (TM) = ~6 μm. (**B**) Upper right panel shows an unirradiated cell treated with *E.coli* repair enzyme Fpg for the detection of oxypurine clusters with a TM = ~10 μm. Lower left panel (**C**) shows a single cell exposed to exposed to 5 Gy of γ-rays without repair enzyme treatment and with TM = ~ 20 μm. (**D**) Lower right panel shows cells a single exposed to 5 Gy of γ-rays treated with Fpg with a TM = ~ 35 μm. Tail moment = tail length (μm) × % tail DNA (intensity of stained DNA). An example of the suggested intensity profile (densitogram) is also shown in the specific comet (D). The increase in the tail moment for the enzyme-treated samples is attributed to the additional DNA fragmentation induced by recognition and cleavage of bi-stranded clustered lesions by the repair enzyme.

control (H_2O_2), a total minimum number of ~300,000 to 600,000 cells is suggested per experiment.

5. Determine the percent of viable cells using Trypan blue (Georgakilas et al., 2004) or other exclusion assay, e.g., erythrosin B(EB, Sigma).

6. Expose cells to low level γ-rays, for example, 3 to 5 Gy using a ^{137}Cs source. Use hydrogen peroxide (H_2O_2) as a positive control (50 to 100 μM, 15 min on ice).

   Exposure time will vary depending on the extent of DNA damage (oxidation) desired. At 5 Gy of γ-rays and under non-denaturating conditions, a tail moment (TM) of ~15 to 20 μm is expected (DSBs; Fig. 6.11.2).

7. Immediately after irradiation, add 1M Na_2EDTA to the medium to a final concentration of 50 mM. Maintain the cells in this medium on ice until further processed.

8. Perform a second Trypan blue exclusion assay after γ-ray exposure to assess cell viability and extent of cell death induced by γ-ray exposure.

   Viability directly after irradiation should be the same as prior to irradiation (≥90%). It should be measured immediately following irradiation; therefore, cell death should not be an issue if the procedure has not somehow compromised cell viability and initiated apoptosis or necrosis.

9. After irradiation and addition of Na_2EDTA, wash cells three times in 3 ml PBS each wash, centrifuging 5 min at 450–1000 × *g*, 4°C, each time.

10. Resuspend the cells in 1 to 2 ml PBS and count cells.

    The number of cells must be ~10,000 cells per plug. The cell count will establish the total number of cells available, which will determine the number of plugs that can be produced. If the percentage of dead cells does not exceed 5% to 10%, the presence of dead cells should not have a significant impact on the levels of DNA damage detected.

11. After centrifugation, embed the cells in an equal volume of low-melting-point agarose plugs at a final agarose concentration of 0.85% using plug molds similar to PFGE molds.

    PFGE plug molds can be purchased from various companies such as BioRad. Slight volume differences may exist between brands or type of plug molds. Typically, plug mold volume ranges from 80 to 100 μl. For example, if ten plugs are to be created, then 100,000 cells (500 μl in PBS) must be mixed with an equal volume of low-melting-point agarose and distributed equally to the ten molds.

    The formation of these DNA plugs allows direct comparison with PFGE.

12. Incubate the plugs (10 to 20 plugs) in 50 ml plug lysis solution for 1 hr on ice at 4°C in the dark.

13. After completion of lysis, wash plugs in 50 ml of 1× TBE electrophoresis buffer for 1 hr at 4°C in the dark with two buffer changes.

14. Mount up to six plugs per agarose-coated microscope (from step 1) slide using 0.85% low-melting-point agarose (Fig. 6.11.1).

15. Apply one to two 18-mm^2 coverslips over the plugs and allow the agarose to solidify 10 min at 4°C.

16a. *For neutral SCGE running conditions (detection of DSBs).* Wash the slides in 50 ml of 1× TBE electrophoresis buffer for 1 hr at 4°C in the dark with three buffer changes (Fig. 6.11.3).

16b. *For alkaline SCGE running conditions (detection of SSBs).* Wash plugs extensively in ice-cold alkaline denaturation buffer, and incubate in the same buffer for 45 min at 4°C in the dark (Fig. 6.11.3).

    For the detection of DSBs (neutral conditions) or SSBs (alkaline conditions), no further enzyme or other treatment is required, and therefore the slides with mounted plugs are subjected to electrophoresis.

17. For detection of DSB or SSB, proceed to Basic Protocol 2, step 1a or 1b (also see Fig. 6.11.3).

Repair enzyme treatment (detection of OCDLs and total enzyme sites)

Continuation from step 13 to detect OCDLs and total sites. At this point, neutral or alkaline conditions are being used; enzyme treatment for the detection of clustered sites is identical as shown in the flow chart of Figure 6.11.3. Differential handling occurs only after step 19 below. The steps of the following repair enzyme treatment are additional steps for OCDL detection. These steps are performed following lysing of plugs and prior to mounting on the slides.

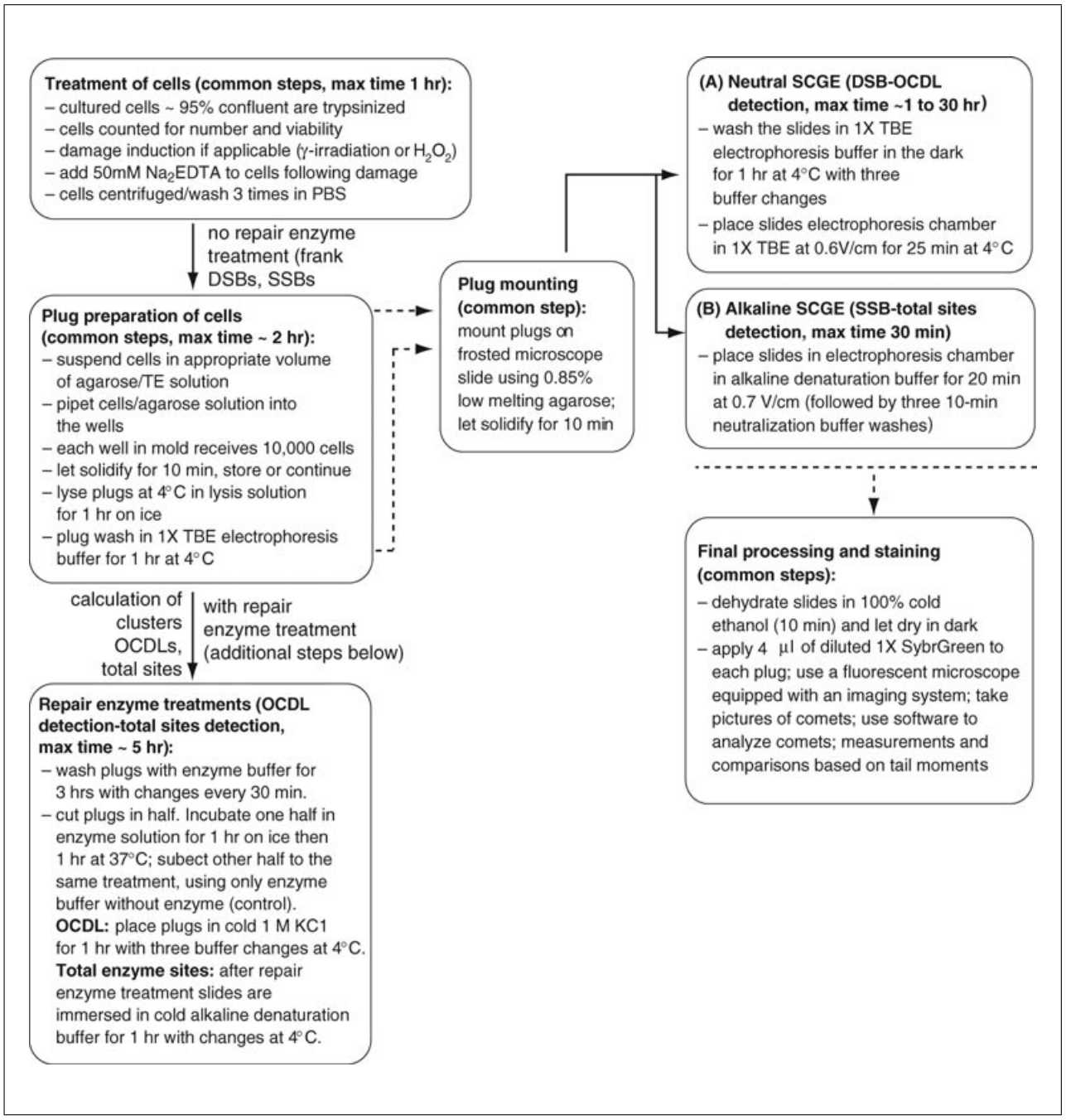


Figure 6.11.3 Overview (flow chart) of this adaptation of the single-cell gel electrophoresis (comet assay) used for the detection of bi-stranded oxidatively induced clustered DNA lesions (OCDLs) using low-melting-point agarose DNA plugs similar to pulsed-field gel electrophoresis (PFGE). In each step (box) the maximum recommended time is indicated along with the key steps followed.

18. To determine the ideal repair enzyme quantity, perform titration studies with the enzyme and the sample(s) with the highest expected level of DNA damage.

    For example in the case of radiation, the highest radiation dose, e.g., 5 Gy, should be used and titration curves should be calculated using increasing enzyme amounts (0 to 1 enzyme units/plug) in the appropriate enzyme buffer and incubated for 1 hr at 37°C. The optimum enzyme amount is calculated at the point when the titration curve reaches a plateau (Holt and Georgakilas, 2007).

    At the same time, the ideal enzyme amount must offer the minimum non-specific cleavage detected using a control sample (e.g., non-irradiated; Holt and Georgakilas, 2007).

19. To equilibrate the plug in enzyme buffer, after lysis, wash plugs with 1 ml/plug appropriate repair enzyme buffer as indicated by the manufacturer for 3 hr with six changes of buffer, 30 min each change.

    For example, for Fpg, use 10 mM Tris·Cl/10 mM $MgCl_2$, pH 7.0.

20. Cut each plug in half. Incubate one half of the plug (+) in 0.3 ml/plug enzyme solution (enzyme buffer plus enzyme amount), for 1 hr on ice followed by 1 hr in a 37°C incubation oven or thermal cycler. Subject the other half of the plug (−) to the same treatment, using just the enzyme buffer, i.e., without the enzyme.

    For example, the enzyme solution can consist of 0.5 U Fpg and 1 mM DTT in 300 μl of Fpg buffer.

For neutral conditions (OCDL detection)

21a. Following repair enzyme treatment, place plugs in 1 ml/plug ice-cold 1 M KCl (pH 7.8) for 1 hr with three buffer changes at 4°C to stop the enzyme reaction (KCl-treatment).

22a. Place slides in 1× TBE electrophoresis buffer for 1 hr with three buffer changes. Mount plugs on an agarose-coated microscope slide using 0.85% low-melting agarose and leave 10 min at 4°C. Once the agarose has cooled and the plug is affixed to the slide, proceed onto the electrophoresis portion of the experiment (see Basic Protocol 2, step 1b). Proceed to Basic Protocol 2, step 1a.

    As an additional test for the efficiency of KCl treatment to disrupt the DNA-protein complexes, a proteinase K treatment can be used. Following repair enzyme treatment, slides can be placed in a proteinase K solution (2 mg/ml proteinase K in plug lysis buffer, pH 7.8) for 1 hr at 37°C and then washed three times with TE buffer on ice (1 hr each wash).

For alkaline conditions (total sites detection)

21b. In the case of alkaline SCGE and after repair enzyme treatment, immerse plugs in 3 ml ice-cold alkaline denaturation buffer for 1 hr with three buffer changes at 4°C.

22b. Mount plugs on an agarose-coated microscope slide using 0.85% low melting agarose and left 10 min at 4°C. Once the agarose has cooled and the plug is affixed to the slide proceed onto the electrophoresis portion of the experiment (see Basic Protocol 2, step 1b).

RUNNING SINGLE-CELL GEL ELECTROPHORESIS (SCGE)

BASIC PROTOCOL 2

The SCGE in general is a rapid and sensitive technique for assessing low levels of DNA damage in human or plant cells. The use of repair enzymes significantly increases the sensitivity of the assay by a factor of 2 to 3 (Holt and Georgakilas, 2007). The actual electrophoresis part of the assay is considered very critical since slight changes in the electrophoretic conditions can induce a wide variability in results. In all cases, special attention should be given to three major parameters: the ionic strength of the electrophoretic buffer, the electric field strength, and the number of slides submerged in the electrophoretic tank. A higher ionic strength buffer will lead to an overestimation of real damage values appearing as longer comet tails. A conductivity meter can be used to ensure the same conductivity between experiments. At the same time, lower than needed ionic strength will result in low conductivity and underestimation of damage values. For this reason different slide numbers in the electrophoretic tank should be avoided since a high number can result in lower conductivity. Finally, all electrophoretic conditions including voltage and electric field strength (V/cm) should be standardized to controls, i.e., not to give significant yields of damage of non-damaged controls. The comet assay is in general a very versatile technique for detecting damage, and alterations to the electrophoresis running conditions can be used to quantify the level of a wide variety of DNA lesions.

Materials

Agarose-coated slides with treated DNA plugs (see Basic Protocol 1)
Neutral electrophoresis buffer (see recipe), ice cold
Alkaline (denaturation) electrophoresis running buffer (see recipe), ice cold
Neutralization buffer: 0.4 M Tris·Cl, pH 7.5 (store at 4°C)
100% ethanol, ice cold

30-cm horizontal constant-field gel electrophoresis chamber
Constant-field agarose gel electrophoresis apparatus with power supply
Storage box for slides under dark condition

1a. *Running SCGE under neutral conditions (DSB or OCDL detection)*. Place up to six slides (use always the same number) with mounted plugs into a 30-cm horizontal constant-field gel electrophoresis chamber containing 1× TBE and run for 25 min at 0.6 V/cm at 4°C. Proceed to step 3.

1b. *Running SCGE under alkaline conditions (SSBs or total enzyme sites detection).* Place up to six slides (use always the same number) with mounted plugs into a 30-cm horizontal constant-field gel electrophoresis chamber in ice-cold alkaline denaturation buffer for 20 min at 0.7 V/cm and at 4°C.

2b. To remove excess alkali, wash the slides three times for 10 min each wash with neutralization buffer. Proceed to step 3.

3. Dehydrate the slides in 100% ice-cold ethanol (10 min) and allow slides to dry in dark. Allow slides to dry at room temperature in the dark until plugs dehydrate and lay flush with the microscope slide, then cover box and store slides in a dry room until analysis.

The slides can be stored indefinitely in the dark after dehydration.

SUPPORT PROTOCOL

QUANTITATIVE ANALYSIS OF SCGE SLIDES

Quantitative analysis of SCGE slides assumes that undamaged DNA strands are too large and do not leave the nucleus, whereas the smaller the DNA fragments, the farther they are able to migrate within the gel matrix after the application of a constant electric field. Therefore, the amount of DNA that leaves the nucleus (head) is a measure of the amount of DNA damage in the cell (comet tail; Fig. 6.11.2). Image analysis measures the overall fluorescence intensity for the whole nucleus and the overall fluorescence intensity of tails, and compares the two signals. The stronger (brighter) the signal from the migrated tail DNA, the more damage has been induced by the oxidizing agent (Fig. 6.11.2).

Materials

SYBR Green DNA staining dye (Cambrex BioScience) diluted 1:10,000 (1×) in TE buffer (10,000× SybrGreen)
Fluorescence microscope (20×, 40×, or 60× objective lens) equipped with a monochrome CCD camera
Comet analysis software (e.g., CometScore, Tritek)

Stain slide and analyze

1. Rehydrate the slides 10 min in 5 ml deionized water at room temperature. Apply 40 µl of diluted SYBR Green (1×) to each plug on the slides.

To visualize and analyze the DNA damage, the slides should be stained with a DNA staining dye that efficiently stains both double- and single-stranded DNA like SYBR Green.

2. View individual (non-overlapping) cells, or comets, using a fluorescence microscope equipped with (at least) a monochrome charge-coupled device (CCD) imaging system.

   The type and objective is selected by the user.

   Each damaged cell within the plug will be seen as possessing a comet-like tail.

   The authors' laboratory uses a 20× objective to capture more comet cells per picture. Usually, in an asynchronous cell population, i.e., cells of different cell cycle phases, a relatively varying tail length (5% to 20% variation in length) will be observed. In synchronized cell populations, this phenomenon is expected to be minimized. If the exposure to the oxidizing agent (for example, ionizing radiation) induces random hits in the DNA, then a uniform distribution of damage (tail length) is to be expected in every cell (Fig. 6.11.2).

3. Conduct analysis with any appropriate comet software package measuring the Tail Moment (Olive et al., 1990). Measure 100 cells per plug with three replicates.

   The authors' laboratory uses CometScore (Tritek) as an effective, easy, and free package to score 100 cells per plug with three replicates (semi-automated).

   Tail moment (TM) is defined as the product of the tail length and the fraction of total DNA in the tail and it is calculated by multiplying the percent (%) DNA in the tail (which the software quantifies by dividing tail to total-head and tail-cell intensity) by the tail length (distance from the nucleus to the end of the tail). Thus, calculating the tail moment not only factors in the amount of DNA that migrates into the comet tail, but also the distance it migrates:

   tail moment (μm) = tail length × tail %DNA

   The tail moment can be explained as a true physical tail moment about the center of a comet head. Longer tails will differentiate cells with high damage compared to controls (very small tail; Fig. 6.11.2). The center of the comet head and the full extension of tail length are calculated by the user as shown in Figure 6.11.2 or automatically by the software. The software usually provides several scoring options like additional calculation of olive tail moment, tail length etc. Read software instructions carefully.

4. In all cases, calculate frank DSBs (directly induced by the oxidizing agent) as the tail moment increase (TMI) after irradiation:

$$\text{TMI (DSBs or SSBs)} = \text{TM}_{\text{IRR}} - \text{TM}_{\text{UNIRR}}$$

   where TM_{IRR} is the absolute TM value for the irradiated sample while TM_{UNIRR} is the corresponding TM for the unirradiated (control).

5. In the case of OCDLs (neutral conditions) or total sites (alkaline conditions), use the following equation:

$$\text{TMI (OCDLs or total enzyme sites)} = \text{TM}_{(+\text{enzyme})} - \text{TM}_{(-\text{enzyme})}$$

   where the $\text{TM}_{(+\text{enzyme})}$ is the absolute TM value after enzyme treatment while $\text{TM}_{(-\text{enzyme})}$ is the TM value for the non-enzyme treated sample (control).

   The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. In all cases, the increase in tail moment is attributed to additional DNA fragmentation induced by recognition of clustered lesions by repair enzyme (Fig. 6.11.1).

   Percent DNA in tail is directly related to amount of stain fluorescence in the tail. Special emphasis must be given to the minimization of light-exposure of cells during the duration of processing post-irradiation.

6. To correlate the TMI values with the values obtained from PFGE, which are usually expressed as number of lesions/Gbp/Gy in the case of radiation, assume an average number of 1000 SSBs/cell and 40 DSBs/cell at 1 Gy for X- or γ- rays (Pouget et al., 1999; Hada and Georgakilas, 2008). Based on the calculated rate of TMI/Gy plot, the SSB or DSB induction yield expressed as lesions/Gbp (assuming an average genome size of 6.4 Gbp for a diploid cell) as analytically described in Francisco et al., (2008). Apply the same calculations and plots for OCDLs using average yields obtained from the literature.

   For example, for Fpg-clusters, an average yield of 60 Fpg-clusters/cell at 1 Gy of γ-rays can be used for human cells (Hada and Georgakilas, 2008).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****. 50μM phenyl-t-butyl nitrone (PBN) should be used in all buffers except enzyme buffers used during plug treatment (see Critical Parameters).*

Alkaline denaturation/electrophoresis buffer

12 g NaOH (300 mM final)
0.29 g EDTA (1 mM final)
pH to 13 using 0.5 M NaOH
Bring to 1 liter with double-distilled H_2O
Store up to 2 weeks at 4°C

EndoIII buffer

3.15 g Tris·Cl (20 mM final)
0.29 g EDTA (1 mM final)
pH to 8.00 using 1 N NaOH or 1N HCl
Bring to 1 liter with double-distilled H_2O
Store up to 2 weeks at 4°C

Endo IV buffer

7.87 g Tris·Cl (50 mM final)
5.84 g/liter (100 mM NaCl final)
2.0331 g/liter $MgCl_2$ (10 mM final)
pH to 7.9 using 1 N NaOH or 1 N HCl
Store up to 2 weeks at 4°C

Fpg buffer

1.576 g Tris·Cl (10 mM final)
2.0331 g $MgCl_2$ (10 mM final)
pH to 7.00
Bring to 1 liter with double-distilled H_2O
Store up to 2 weeks at 4°C

KCl stop mix solution (for neutral assay)

16.681 g HEPES KOH (70 mM final)
7.456 g KCl (100 mM final)
29.225 g EDTA (100 mM final)
pH to 7.60
Bring to 1 liter with double-distilled H_2O
Store up to 8 weeks at 4°C

Neutral electrophoresis buffer, 1× TBE

89 mM Tris·Cl
89 mM boric acid
1 mM EDTA
pH to 8.5
Store up to 6 weeks at 4°C

Plug lysis solution

1.2114 g Tris·Cl (100 mM final)
29.225 g EDTA (100 mM final)
146.1 g NaCl (2.5 M final)
pH to 10.00
Bring to 1 liter with double-distilled H_2O
Store up to 6 weeks at room temperature
Immediately prior to use, add:
1% (v/v) Triton X100
10% (v/v) DMSO

TE buffer

1.576 g Tris·Cl (10 mM final)
29.25 g EDTA (1 mM final)
pH to 9
Bring to 1 liter with double-distilled H_2O
Store up to 6 weeks at room temperature

Tris-Borate-EDTA (TBE) buffer, 10×

108 g Tris base
55 g boric acid
9.3 g EDTA
pH to 8.30
Add double-distilled H_2O to bring to 1 liter
Store up to 6 weeks at room temperature
Dilute to 1× using double-distilled H_2O

COMMENTARY

Background Information

Principles of OCDL detection

There is an abundance of research dedicated to understanding the types and significance of DNA lesions induced in the cell endogenously or exogenously. Endogenous or exogenous oxidative stress is a major generator of protein and DNA damage (Franco et al., 2008). Examples of endogenous stress are various metabolic byproducts including hydroxyl radicals and hydrogen peroxide. Examples of exogenous oxidative stress are very low doses of ionizing radiation (X-rays, γ-rays, and α-particles) or various chemicals inducing a generation of free radicals (Hada and Georgakilas, 2008). In contrast to single isolated lesions, complex DNA damage, the signature of ionizing radiation and high levels of oxidative stress in cells and tissues, is comprised of closely spaced uni-stranded (tandem) or bi-stranded lesions forming a cluster of DNA damage (Fig. 6.11.1; Georgakilas, 2008). Numerous studies suggest the important role of oxidative DNA damage and its repair in cancer and aging (Hussain et al., 2003; Kastan and Bartek, 2004). This pool of oxidative DNA damage can contain a wide variety of single or clustered DNA lesions, including single-strand breaks (SSBs), oxidized bases and/or apurinic-apyrimidinic (abasic, AP) sites (Ward, 1994) in the form of isolated lesions as well as in the form of clustered ones (1 to 10 bp apart; Goodhead, 1994; Sutherland et al., 2000a). Bi-stranded or tandem DNA clusters can be resistant to processing by glycosylases or endonucleases, as shown in

in vitro studies (Chaudhry and Weinfeld, 1997; Harrison et al., 1999; McKenzie and Strauss, 2001; David-Cordonnier et al., 2002; Georgakilas et al., 2002; Eot-Houllier et al., 2005; Cunniffe et al., 2007). Such repair-resistant clusters can persist in cells or tissues for a substantial time after irradiation (up to several days or weeks; Georgakilas et al., 2004; Gollapalle et al., 2007).

One of the best known methods for the detection and measurement of clusters is a modified version of PFGE. As a second independent method, a few groups have developed a modified version of the neutral SCGE or comet assay again using repair enzymes as damage probes (Blaisdell and Wallace, 2001; Holt and Georgakilas, 2007). Since 1984 (Ostling and Johanson, 1984) and later in its alkaline version in 1988 (Singh et al., 1998), the SCGE, a modified version of microgel electrophoresis, has been widely used for the detection of low levels of various types of DNA lesions including SSBs, DSBs, and oxidized bases as reviewed in Collins (2004) and Olive and Banath (2006). The comet assay or microgel assay under neutral running conditions has been used for the detection of DSBs in a variety of cells including lymphocytes (Olive and Banath, 1993; Angelis et al., 1999; Yang et al., 2004; Trenz et al., 2005). There is very limited data on the use of the neutral comet assay (Angelis et al., 1999; Holt and Georgakilas, 2007) or microgel assay (Blaisdell and Wallace, 2001; Yang et al., 2006) for the detection of non-DSB clustered DNA lesions using different repair enzymes as enzymatic probes. In all cases, incomplete cleavage of lesions by the repair enzymes can lead to a detection of only a fraction of the clusters (Fig. 6.11.1; Hada and Georgakilas, 2008). This issue becomes very important especially in the case of high linear energy transfer (high-LET) radiations (damaging to DNA) where the density of the lesions is expected to be very high (Nikjoo et al., 2001). The clusters described in Figure 6.11.1 are in general an idealized form of a simple cluster. In reality and particularly in the case of high-LET, a complex DNA lesion would be expected, for example, an SSB or DSB with five to ten additional surrounding lesions (Georgakilas, 2008). Therefore, each cluster detected by the enzyme is only a fraction of the lesions participating in the cluster. The correction can be performed by using the alkaline version of the technique measuring the total number of lesions in the DNA (Francisco et al., 2008).

Critical Parameters

Buffers and solutions

All solutions and buffers should always be prepared with deionized, double-distilled, sterile water using sterile containers, and should be sterilized by appropriate means before use (e.g., autoclave). They should also be purged with argon or nitrogen and 50 μM phenyl-*tert*-butyl nitrone in DMSO (PBN; Sigma) should be added to minimize artifactual DNA oxidation that can give rise to increased background DNA damage. PBN should not be used for enzyme treatment buffers since it may inhibit enzyme activity. In addition, sterile reaction containers and measuring instruments should be used; gloves should always be worn to prevent any nuclease contamination. The use of a timed and thermoregulated instrument (i.e., PCR thermal cycler) for all enzyme assays rather than a water bath increases precision and reproducibility of reactions and minimizes introduction of nucleases or other contaminants resulting from immersion in a water bath. Fresh, filtered, and autoclaved solutions should be used, and solutions remaining after reactions are completed should be discarded (Sutherland et al., 2003).

Enzymes

Enzyme purification should always be performed using absolutely sterile solutions, equipment, and conditions. General purification procedures should be followed, modified, and updated as appropriate. The specific activity, specific cleavage, and non-specific cleavage are assessed by enzyme assays, and progress to protein purity is assessed by polyacrylamide gel electrophoresis at each step of the purification. The enzyme reactions must be terminated by use of a proper "stop mix" solution that halts enzyme action, disrupts DNA-protein interactions, but neither denatures the DNA nor interferes with subsequent gel electrophoresis (Sutherland et al., 2003). Enzyme–DNA complexes may interfere with DNA migration on the electrophoretic gel by inducing an artifactual decrease of the TM. Additional digestion with proteinase K removes residual enzymes and generally dissociates such resistant and strongly associated complexes, minimizes interference artifacts, and increases reproducibility.

Troubleshooting

Cloudy or white appearance of plugs

A cloudy or white appearance of the plugs can be caused by not properly dissolving the agarose or not removing/neutralizing all alkaline solution on the slides. Ensure that agarose is completely dissolved before using it. For incomplete removal of alkaline solution, longer and/or slightly more aggressive washes can be used.

Agarose plugs continually come off slide

Ensure that the agarose used to affix the plug to the slide has solidified before placing the slide in the electrophoresis chamber. Excessively aggressive washes can remove plugs as well. Decrease humidity by using low humidity conditions; for example, use a humidity-controlled chamber.

Tail moments differ despite same experimental conditions

Ensure all buffers are at correct pH and always apply the same duration and electric field (V/cm) for electrophoresis. Ensure cell viability of no less than 90%.

Comets are hard to score due to overlapping tails

Use a lower number of cells per plug.

Comets appear too bright

This can be due to overuse of dye or dye that is too concentrated. It may take a few minutes before the comets fluoresce under the microscope. Allow time for this to happen before adding additional dye.

Anticipated Results

When cells are exposed to an oxidizing agent like ionizing radiation and treated with a repair enzyme for the detection of clustered DNA lesions, an obvious difference in the comet tail should be detected between the enzyme-treated and non-treated samples as, in Figure 6.11.2. Increase in tail moment (TM) must be linear with dose at least up to ~10 Gy for the detection of non-DSB clusters and up to ~20 Gy for detection of DSBs. In many cases of exposing exponentially growing cells to an oxidizing agent, a great variation of TMs can be detected. This is due to cells with different DNA content and therefore users must try to score cells with similar DNA content (total intensity).

Time Considerations

Cell treatment, processing, DNA agarose plug creation, and electrophoresis can be completed in 4 to 5 hr. All steps must be performed within the exact time limits to avoid experimental variations and inconsistencies (see boxes of with maximum times allowed in Fig. 6.11.3). Special attention should be given to the time frame allowed for repair enzyme treatment since this can induce a great variability in the results. Therefore, use the same set time (1 hr at 37°C) for each experiment. Longer incubation times can result in increased comet tails and non-specific cleavage. In addition, special emphasis should be given to the time period from the moment slides with plugs are taken out of the electrophoresis, neutralized, and stained with a fluorescence dye. The most accurate and reliable results are obtained when scoring is performed immediately. Increased camera sensitivity will provide maximization of the acquisition of fluorescence from a cell and this will improve confidence in the metrics. To preserve fluorophore performance, illumination should be removed automatically when cells are not being imaged to prevent light-induced fluorescence quenching. If storage of plugs is needed, plugs can be stored in autoclaved TE solution purged with argon or nitrogen and added 50 μM phenyl-*tert*-butyl nitrone in DMSO (PBN; Sigma). For longer periods of storage (storage over 6 weeks), expect higher background damage. Storage up to 6 weeks under the described conditions is not expected to increase the background levels of DNA clusters significantly.

Acknowledgements

This work was supported by funds provided to Dr. Georgakilas by a 2009/2010 ECU Research/Creative Activity Award.

The authors declare no conflict of interest in this work.

Literature Cited

Angelis, K.J., Dusinska, M., and Collins, A.R. 1999. Single cell gel electrophoresis: Detection of DNA damage at different levels of sensitivity. *Electrophoresis* 20:2133-2138.

Blaisdell, J.O. and Wallace, S. 2001. Abortive base-excision repair of radiation-induced clustered DNA lesions in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 98:7426-7430.

Chaudhry, M.A. and Weinfeld, M. 1997. Reactivity of human apurinic/apyrimidinic endonuclease and *Escherichia coli* exonuclease III with bistranded abasic sites in DNA. *J. Biol. Chem.* 272:15650-15655.

Collins, A.R. 2004. The comet assay for DNA damage and repair: Principles, applications and limitations. *Mol. Biotechnol.* 26:249-261.

Cunniffe, S.M., Lomax, M.E., and O'Neill, P. 2007. An AP site can protect against the mutagenic potential of 8-oxoG when present within a tandem clustered site in E. coli. *DNA Repair* 6:1839-1849.

David-Cordonnier, M.H., Cunniffe, S.M.T., Hickson, I.D., and O'Neill, P. 2002. Efficiency of incision of an AP site within clustered DNA damage by the major human AP endonuclease. *Biochemistry* 41:634-642.

Douki, T., Ravanat, J.L., Pouget, J.P., Testard, I., and Cadet, J. 2006. Minor contribution of direct ionization to DNA base damage induced by heavy ions. *Int. J. Radiat. Biol.* 82:119-127.

Eot-Houllier, G., Eon-Marchais, S., Gasparutto, D., and Sage, E. 2005. Processing of a complex multiply damaged DNA site by human cell extracts and purified repair proteins. *Nucleic Acids Res.* 33:260-271.

Francisco, D.C., Peddi, P., Hair, J.M., Flood, B.A., Cecil, A.M., Kalogerinis, P.T., Sigounas, G., and Georgakilas, A.G. 2008. Induction and processing of complex DNA damage in human breast cancer cells MCF-7 and non-malignant MCF-10A cells. *Free Radic. Biol. Med.* 44:558-569.

Franco, R., Schoneveld, O., Georgakilas, A.G., and Panayiotidis, M.I. 2008. Oxidative stress, DNA methylation and carcinogenesis. *Cancer Lett*. 266:6-12.

Georgakilas, A.G. 2008. Processing of DNA damage clusters in human cells: Current status of knowledge. *Mol. Biosyst.* 4:30-35.

Georgakilas, A.G., Bennett, P.V., and Sutherland, B.M. 2002. High efficiency detection of bistranded abasic clusters in γ-irradiated DNA by putrescine. *Nucleic Acids Res.* 30:2800-2808.

Georgakilas, A.G., Bennett, P.V., Wilson, D.M. III, and Sutherland, B.M. 2004. Processing of bistranded abasic DNA clusters in gamma-irradiated human hematopoietic cells. *Nucleic Acids Res.* 32:5609-5620.

Gollapalle, E., Wong, R., Adetolu, R., Tsao, D., Francisco, D., Sigounas, G., and Georgakilas, A.G. 2007. Detection of oxidative clustered DNA lesions in X-irradiated mouse skin tissues and human MCF-7 breast cancer cells. *Radiat. Res.* 167:207-216.

Goodhead, D.T. 1994. Initial events in the cellular effects of ionizing radiations: Clustered damage in DNA. *Int. J. Radiat. Biol.* 65:7-17.

Hada, M. and Georgakilas, A.G. 2008. Formation of clustered DNA damage after high-LET irradiation: A review. *J. Radiat. Res.* 49:203-210.

Harrison, L., Hatahet, Z., and Wallace, S. 1999. In vitro repair of synthetic ionizing radiation-induced multiply damaged DNA sites. *J. Mol. Biol.* 290:667-684.

Holt, S.M. and Georgakilas, A.G. 2007. Detection of complex DNA damage in γ-irradiated acute lymphoblastic leukemia pre-B NALM-6 cells. *Radiat. Res.* 168:527-534.

Hussain, S.P., Hofseth, L.J., and Harris, C.C. 2003. Radical causes of cancer. *Nat. Rev. Cancer* 3:276-285.

Kastan, M.B. and Bartek, J. 2004. Cell-cycle checkpoints and cancer. *Nature* 432:316-323.

Lu, T., Pan, Y., Kao, S.Y., Li, C., Kohane, I., Chan, J., and Yankner, B.A. 2004. Gene regulation and DNA damage in the aging human brain. *Nature* 429:883-891.

McKenzie, A.A. and Strauss, P.R. 2001. Oligonucleotides with bistranded abasic sites interfere with substrate binding and catalysis by human apurinic/apyrimidinic endonuclease. *Biochemistry* 40:13254-13261.

Nikjoo, H., O'Neill, P., Wilson, E.W., and Goodhead, D. 2001. Computational approach for determing the spectrum of DNA damage induced by ionizing radiation. *Radiat. Res.* 156:577-583.

Olive, P.L. and Banath, J.P. 1993. Detection of DNA double-strand breaks through the cell cycle after exposure to X-rays, bleomycin, etoposide and 125IdUrd. *Int. J. Rad. Biol.* 64:349-358.

Olive, P.L. and Banath, J.P. 2006. The comet assay: A method to measure DNA damage in individual cells. *Nat. Protoc.* 1:23-29.

Olive, P.L., Banáth, J.P., and Durand, R.E. 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat. Res.* 122:86-94.

Ostling, O. and Johanson, K.J. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.* 123:291-298.

Pouget, J.-P., Ravanat, J.-L., Douki, T., Richard, M.-J., and Cadet, J. 1999. Measurement of DNA base damage in cells exposed to low doses of gamma-radiation: Comparison between the HPLC-EC and comet assays. *Int. J. Radiat. Biol.* 75:51-58.

Pouget, J.P., Frelon, S., Ravanat, J.L., Testard, I., Odin, F., and Cadet, J. 2002. Formation of modified DNA bases in cells exposed either to gamma radiation or to high-LET particles. *Radiat. Res.* 157:589-595.

Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. 1998. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175:184-191.

Sutherland, B., Bennett, P.V., Sidorkina, O., and Laval, J. 2000a. DNA damage clusters induced by ionizing radiation in isolated DNA and in human cells. *Proc. Natl. Acad. Sci. U.S.A.* 97:103-108.

Sutherland, B.M., Bennett, P.V., Sidorkina, O., and Laval, J. 2000b. Clustered damages and total lesions induced in DNA by ionizing radiation: Oxidized bases and strand breaks. *Biochemistry* 39:8026-8031.

Sutherland, B.M., Georgakilas, A.G., Bennett, P.V., Laval, J., and Sutherland, J.C. 2003. Quantifying clustered DNA damage induction and repair by gel electrophoresis, electronic imaging and number average length analysis. *Mutat. Res.* 531:93-107.

Trenz, K., Schutz, P., and Speit, G. 2005. Radiosensitivity of lymphoblastoid cell lines with a heterozygous BRCA1 mutation is not detected by the comet assay and pulsed field gel electrophoresis. *Mutagenesis* 20:131-137.

Tsao, D., Kalogerinis, P., Tabrizi, I., Dingfelder, M., Stewart, R.D., and Georgakilas, A.G. 2007. Induction and processing of clustered DNA lesions in human monocytes exposed to low doses of HZE ^{56}Fe particles. *Radiat. Res.* 168:87-97.

Visvardis, E.E., Haveles, K.S., Pataryas, T.A., Margaritis, L.H., Sophianopoulou, V., and Sideris, E.G. 2000. Diversity of peripheral blood mononuclear cells as revealed by a novel multiple microgel comet assay. *Environ. Molec. Mutagen.* 36:32-39.

Ward, J.F. 1994. The complexity of DNA damage: Relevance to biological consequences. *Int. J. Radiat. Biol.* 66:427-432.

Yang, N., Galick, H., and Wallace, S.S. 2004. Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks. *DNA Repair* 3:1323-1334.

Yang, N., Chaudhry, M.A., and Wallace, S.S. 2006. Base excision repair by hNTH1 and hOGG1: A two edged sword in the processing of DNA damage in gamma-irradiated human cells. *DNA Repair* 5:43-51.

CHAPTER 7
Protein Labeling and Immunoprecipitation

INTRODUCTION

The characterization of cellular proteins in complex mixtures, as well as analysis of their biosynthesis, processing, intracellular trafficking and degradation, generally requires that the proteins be labeled either intrinsically (*UNIT 7.1*) or extrinsically. The labeled proteins are then isolated by immunoprecipitation (*UNIT 7.2*) and analyzed using electrophoretic techniques (Chapter 6).

Intrinsic labeling refers to the incorporation of a labeled precursor into the protein during synthesis. The most common form of intrinsic labeling consists of supplying the labeled precursor to live cells so that it becomes incorporated into proteins by the cell's own biosynthetic machinery. This form of intrinsic labeling is referred to as metabolic or biosynthetic labeling. The complexity and fidelity of the cell's biosynthetic pathways does not allow much flexibility in the design of precursors suitable for metabolic labeling. For instance, chemical derivatization of amino acid precursors renders them extraneous to plasma membrane transporters and aminoacyl-tRNA synthetases. Incorporation of unnatural amino acids can also affect the folding and stability of the polypeptide chains. Thus, the choice of precursors is limited to those labeled with an uncommon isotope of one of its constituent atoms. Although stable heavy isotopes have been used to label precursors, by far the most common means of labeling amino acids entails substituting radionuclides such as ^{35}S, ^{3}H, or ^{14}C in place of their nonradioactive counterparts. Because isotopes have identical biochemical properties, these substitutions do not affect protein synthesis.

The first unit in this chapter (*UNIT 7.1*) presents a compilation of protocols for metabolic labeling of proteins in mammalian cells using radiolabeled amino acids. The preferred radiolabeled amino acid for this purpose is [^{35}S]methionine, although [^{35}S]cysteine or ^{3}H-labeled amino acids can also be used. One of the methods described in *UNIT 7.1* is referred to as pulse labeling, and consists of incubating the cells with the radiolabeled amino acid for short periods (10 to 30 min). Pulse labeling is used to label newly synthesized proteins. In many cases, the pulse is followed by a chase in which cells are further incubated with the unlabeled counterpart of the precursor used for labeling. Pulse-chase protocols allow study of the fate of proteins after synthesis. Another method described in this unit is referred to as long-term labeling, and consists of labeling cells for periods of 6 to 32 hr. Long-term labeling is generally used to study the properties of mature proteins or protein complexes, rather than their biogenesis.

Metabolically labeled proteins can be directly analyzed using electrophoretic techniques (*UNIT 6.1*). Most often, however, specific metabolically labeled proteins are isolated from other cellular proteins by immunoprecipitation. Even when metabolically labeled proteins are first separated by the physical methods described in Chapter 5, specific proteins ultimately have to be isolated by immunoprecipitation. Immunoprecipitation techniques exploit the ability of the immune system to produce specific antibodies to virtually any protein (see Chapter 16). In the most widely used immunoprecipitation protocols, antibodies are bound to protein A–agarose beads, after which the immobilized antibodies are incubated with a detergent extract of the cells. Specific proteins can then be rapidly separated from other proteins in the mixture by repeated washing of the beads. *UNIT 7.2*

Contributed by Juan S. Bonifacino
Current Protocols in Cell Biology (2002) 7.0.1-7.0.3

describes a series of simple protocols for immunoprecipitation of proteins from cell extracts. Included in this unit are protocols for extracting both adherent and suspended animal cells under either nondenaturing or denaturing conditions. There is also a protocol for lysing yeast cells, which requires mechanical breakage of the cell wall. *UNIT 7.2* also includes procedures for binding antibodies to protein-agarose beads, incubating the antibody-bound beads with cell extracts, and washing the beads. Finally, the unit describes an immunoprecipitation-recapture protocol in which proteins are immunoprecipitated twice sequentially with either the same antibody or different antibodies. This type of protocol can be used to reduce the nonspecific background of the first immunoprecipitation or to analyze the composition and assembly of multiprotein complexes.

Proteins can be metabolically labeled using precursors other than amino acids—e.g., sulfate, lipids, carbohydrates, and phosphate, provided that the proteins are modified by the addition of these groups. *UNIT 7.3* describes protocols for both long-term and pulse-chase metabolic labeling using [^{35}S]sulfate. Use of these protocols allows radiolabeling of proteins that are sulfated on either tyrosine residues or carbohydrate moieties. *UNIT 7.4* describes protocols for metabolic labeling fatty-acylated proteins with [^{3}H]palmitic acid or [^{3}H]myristic acid. This unit also includes protocols for determining the type of linkage in which the fatty acid is attached and the identity of the fatty acid. *UNIT 7.5* deals with another type of lipid modification of proteins, namely prenylation by addition of farnesyl or geranylgeranyl groups. Metabolic labeling of prenylated proteins can be achieved by incubation with [^{3}H]mevalonic acid, which is a precursor of prenyl groups. Prenylated proteins are often also carboxyl-methylated. This modification can be studied by metabolic labeling with [^{3}H-methyl]methionine, a protocol for which is also included in this unit.

The yeast, *Saccharomyces cerevisiae*, is widely used as a model organism for the study of cellular processes that are common to all eukaryotic cells. Critical to these studies is the ability to label yeast proteins metabolically. Metabolic labeling and immunoprecipitation of yeast proteins can be achieved by modification of methods described for mammalian proteins (*UNITS 7.1 & 7.2*). *UNIT 7.6* describes protocols for metabolic labeling yeast proteins with ^{35}S-labeled amino acid mixtures and for mechanical disruption of the labeled yeast cells with glass beads (a variation of this method of yeast cell lysis is described in *UNIT 7.2*). In addition, *UNIT 7.6* includes protocols for preparation of spheroplasts from the radiolabeled yeast cells by enzymatic lysis of the cell wall, and for treatment of immunoprecipitated yeast proteins with endoglycosidase H. This unit also discusses criteria for metabolic labeling of yeast cells with precursors other than ^{35}S-labeled amino acid mixtures.

Another organism that is commonly used in biological research is the fruit fly, *Drosophila melanogaster*. Unlike *Saccharomyces cerevisiae, Drosophila* is a multicellular organism and possesses a body organization and function that is in many ways similar to that of higher eukaryotes. Powerful genetic methodologies have been developed that allow detailed analyses of embryonic development and function of organ systems. Studies at the cellular level in *Drosophila*, however, have lagged behind. Methods for metabolically labeling *Drosophila* cells in culture, in particular, are hard to find. In *UNIT 7.7* we present a protocol for metabolic labeling and immunoprecipitation using *Drosophila* S2M3 cells. The unit also contains a protocol for the growth and maintenance of S2M3 cells. Finally the Commentary provides references for preparation of primary cultures of *Drosophila* cells. The ability to culture *Drosophila* cells from wild-type and mutant flies allows analysis of the cellular roles of specific gene products.

The protocols described thus far describe labeling of proteins and protein conjugates by incubation of cells with radiolabeled precursors (i.e., intrinsic labeling). However, proteins can also be labeled, either in purified form or in mixtures, after extraction from the cells (i.e., extrinsic labeling). *UNIT 7.9* presents the first set of protocols in this chapter

dealing with extrinsic labeling of proteins. In our aerobic world, cellular proteins are constantly suffering oxidative damage by reactive oxygen species. Amino acid residues, such as histidine, lysine, arginine, proline, threonine, cytosine, tyrosine and aspartate, can all undergo oxidative modification within cells. Upon protein extraction from cells, modified amino acid residues can be detected and quantified by reaction with specific chemical reagents. The products of these reactions can then be analyzed by gel electrophoresis, mass spectrometry, or ELISA, either directly (as described in this unit) or following isolation of specific proteins by immunoprecipitation (*UNIT 7.2*).

Most of the protocols discussed to this point are based on intrinsic labeling of cellular proteins or protein conjugates. However, proteins can also be labeled extrinsically by chemical modification of specific amino acid residues. A commonly used method is the chemical modification of tyrosine resides with ^{125}iodine. Radioiodination can be used to label proteins on the cell surface and in isolated organelles, detergent extract, or soluble fractions as described in *UNIT 7.9*. Proteins can be labeled to high specific activities, generally with little or no loss of function. The risk of contamination by inhalation of volatile radioiodine, however, has led to the replacement of radioiodination by safer extrinsic labeling techniques such as biotinylation.

Juan S. Bonifacino

Metabolic Labeling with Amino Acids

Metabolic labeling techniques are used to study biosynthesis, processing, intracellular transport, secretion, degradation and physical-chemical properties of proteins. In this unit, protocols are described for metabolically labeling mammalian cells with radiolabeled amino acids (Table 7.1.1). Cells labeled using these procedures are suitable for analysis by immunoprecipitation (*UNIT 7.2*), characterization of cellular proteins (see Chapter 5), analysis of protein trafficking, and one- and two-dimensional gel electrophoresis (see Chapter 6). The first protocols describe pulse-labeling (10 to 30 min) of mammalian cells in suspension with [^{35}S]methionine (see Basic Protocol), and necessary modifications for adherent mammalian cells (see Alternate Protocol 1). Alternate protocols are also presented for pulse-chase analysis (see Alternate Protocol 2) and long-term labeling ("steady state," 6 to 32 hr; see Alternate Protocol 3). This is followed by conditions for metabolic labeling of cells with radiolabeled amino acids other than [^{35}S]methionine (see Alternate Protocol 4). The degree of label incorporation can be determined by precipitation with trichloroacetic acid (TCA; see Support Protocol).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Table 7.1.1 Radiolabeled Amino Acids Used in Metabolic Labeling of Proteins

Amino acid[a]	Frequency (%)[b]	Radioisotope	Specific activity (Ci/mmol)	Comments[c]
Leucine	10.4	^{3}H	5-190	E
Serine	8.1	^{3}H	5-40	T, I
Glutamic acid	7.3	^{3}H	15-80	T, I
Lysine	7.0	^{3}H	40-110	E
Alanine	7.0	^{3}H	10-85	T
Valine	6.2	^{3}H	10-65	E
Glycine	5.7	^{3}H	10-60	T, I
Threonine	5.6	^{3}H	5-25	E
Arginine	5.0	^{3}H	30-70	E
Aspartic acid	4.9	^{3}H	10-50	T, I
Proline	4.9	^{3}H	15-130	—
Glutamine	4.5	^{3}H	20-60	T, I
Phenylalanine	4.5	^{3}H	15-140	E
Tyrosine	3.6	^{3}H	15-60	—
Asparagine[d]	3.5	—	—	—
Cysteine	3.4	^{35}S	>800	—
Isoleucine	2.9	^{3}H	30-140	E
Histidine	2.5	^{3}H	30-70	E
Methionine	1.8	^{35}S	>800	E
Tryptophan	1.3	^{3}H	20-30	E

[a]All amino acids in this table are in the L configuration.
[b]Frequency of amino acid residues in proteins, taken from Lathe (1985).
[c]E, essential amino acids; T, amino acids that are modified by transamination (Coligan et al., 1983); I, amino acids that are converted by cells to other amino acids.
[d]Asparagine is difficult to label (Coligan et al., 1983).

Contributed by Juan S. Bonifacino
Current Protocols in Cell Biology (1998) 7.1.1-7.1.10

SAFETY PRECAUTIONS FOR WORKING WITH ^{35}S-LABELED COMPOUNDS

When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Solutions containing ^{35}S-labeled compounds have been found to release volatile radioactive substances (Meisenhelder and Hunter, 1988). In addition to the usual safety practices followed when handling radioactive materials (see *APPENDIX 1D*), some extra precautions should be taken when using ^{35}S-labeled amino acids:

1. Vials containing ^{35}S-labeled compounds should always be handled in a designated fume hood equipped with an activated charcoal filter. This includes thawing the solution, opening the vial, and adding the radiolabeled amino acid to the labeling medium. Before opening, vials should be vented with a needle attached to a syringe packed with activated charcoal. Avoid using tissue culture hoods for this purpose as they are likely to become contaminated.
2. Minimize exposure of ^{35}S-containing solutions to the air.
3. Use baths, incubators, and centrifuges designated for work with radioactive materials. Place a tray containing a layer of activated charcoal in the CO_2 incubator to reduce the amount of ^{35}S-labeled compounds released to the air during cell labeling. Alternatively, filters impregnated with activated carbon (β-Safe, Schleicher & Schuell) can be attached to the covers of tissue culture dishes.
4. Monitor areas used during labeling by conducting wipe tests.
5. Dispose of solid and liquid ^{35}S waste quickly and with appropriate precautions.

BASIC PROTOCOL

PULSE-LABELING OF CELLS IN SUSPENSION WITH [^{35}S]METHIONINE

Pulse-labeling of proteins is performed by incubating cells for short periods (≤30 min) in culture medium containing a radiolabeled amino acid. The conditions described below are optimized for labeling times of 10 to 30 min. The same protocol is used for labeling cells for up to 6 hr, although a smaller number of cells and/or a larger amount of labeling medium should be used for optimal results. Very short pulses (<10 min) may require special conditions that are discussed elsewhere (see Critical Parameters). For labeling times >6 hr, see Alternate Protocol 3. [^{35}S]methionine is the radiolabeled amino acid of choice for metabolic labeling of proteins because of its high specific activity (>800 Ci/mmol) and ease of detection. A potential disadvantage of [^{35}S]methionine is its low abundance in proteins (~1.8% of the average amino acid composition; see Table 7.1.1). For proteins that contain little (e.g., only one) or no methionine, other radiolabeled amino acids should be used (see Alternate Protocol 4).

Materials

[^{35}S]L-Methionine (>800 Ci/mmol) or [^{35}S]-labeled protein hydrolyzate (>1000 Ci/mmol)
Pulse-labeling medium (see recipe), warmed to 37°C
Cell suspension (e.g., Jurkat, RBL, K562, BW5147, T and B cell hybridomas), grown in a humidified, 37°C, 5% CO_2 incubator or prepared from tissues (e.g., lymphocytes, *UNIT 2.2*)
PBS (*APPENDIX 2A*), ice cold
Vacuum aspirator with trap for liquid radioactive waste

Additional reagents and equipment for TCA precipitation (optional; see Support Protocol)

1. Thaw [^{35}S]methionine at room temperature and prepare a 0.1 to 0.2 mCi/ml working solution in prewarmed (37°C) pulse-labeling medium.

   CAUTION: *Volatile ^{35}S-containing compounds can be released during the labeling procedure (refer to Safety Precautions and APPENDIX 1D). Keep [^{35}S]methionine-containing medium in a tightly capped tube in a 37°C water bath until use. Do not let it sit >60 min at 37°C.*

2. Harvest 0.5–2 × 10^7 cells in suspension by centrifuging 5 min at 300 × *g*, room temperature.

3. Wash cells with ~10 ml prewarmed pulse-labeling medium. Centrifuge 5 min at 300 × *g*, room temperature, and aspirate supernatant. Resuspend cells by gently tapping the bottom of the tubes and repeat wash.

4. Resuspend cells at 5 × 10^6 cells/ml in prewarmed pulse-labeling medium and incubate 15 min in a 37°C water bath to deplete intracellular pools of methionine. Invert tubes periodically to resuspend cells.

5. Centrifuge cells 5 min at 300 × *g*, room temperature, and aspirate supernatant.

6. Resuspend cells in a fresh 15-ml centrifuge tube, using 2 ml [^{35}S]methionine working solution (step 1). Cap tubes tightly. Incubate cells 10 to 30 min (see Critical Parameters) in a 37°C water bath, resuspending frequently by gentle inversion of the tubes.

   Alternatively, use a rotator placed in a 37°C incubator.

7. Centrifuge cells 5 min at 300 × *g*, 4°C, and aspirate supernatant. Resuspend with gentle swirling in 10 ml ice-cold PBS and repeat centrifugation.

   CAUTION: *The medium and wash are radioactive—follow applicable safety regulations for disposal.*

   Because of the short times and high concentrations of radiolabeled amino acids employed, pulse-labeling may not result in complete depletion of label from the medium. If this is the case, the labeling mixture can be reused. Collect the labeling medium containing [^{35}S]methionine or ^{35}S-labeled protein hydrolyzate and filter carefully through a 0.45-µm filter unit. Estimate the percentage of unincorporated radioactivity by scintillation counting aliquots of the labeling mixture before and after labeling the cells. Store the labeling mixture up to 2 months frozen at −20°C.

8. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

   Labeled cells are now ready for the desired processing and analysis. If cell pellets cannot be processed immediately, they can be kept on ice for a few hours or frozen at −80°C for several days. Thaw frozen cell pellets on ice before analysis. In most cases, freezing cells will not affect the biochemical properties of the proteins, but freezing and thawing cell extracts after solubilization with detergents (UNIT 7.2) can cause dissociation of multisubunit complexes or proteolysis of labeled proteins.

ALTERNATE PROTOCOL 1

PULSE-LABELING OF ADHERENT CELLS WITH [^{35}S]METHIONINE

Labeling adherent cells is essentially the same as described for cells in suspension except that cells are labeled while attached to a dish. As with suspended cells, adherent cells are pulse-labeled 10 to 30 min (see Basic Protocol introduction). Alternatively, some adherent cells can be detached from plates by incubation for 10 min at 37°C with 10 mM EDTA in PBS (*APPENDIX 2A*) and labeled as a suspension (see Basic Protocol). This is particularly advantageous when labeling cells for pulse-chase experiments, because it simplifies handling of multiple samples and allows more uniform labeling of the cells.

Additional Materials *(also see Basic Protocol)*

Adherent cells (e.g., HeLa, NRK, M1, COS-1, CV-1, or fibroblasts or endothelial cells in primary culture; *UNITS 2.1 & 2.3*)
100-mm tissue culture dishes

1. Grow adherent cells to 80% to 90% confluency in 100-mm tissue culture dishes.

   Depending on the cell type, a confluent 100-mm dish will contain 0.5–2 × 10^7 cells.

2. Prepare [^{35}S]methionine working solution as described (see Basic Protocol, step 1).

3. Aspirate culture medium from the dishes and wash twice by gently swirling with 10 ml prewarmed (37°C) pulse-labeling medium, aspirating medium after each wash.

4. Add 5 ml prewarmed pulse-labeling medium and incubate 15 min in a humidified, 37°C, 5% CO_2 incubator to deplete intracellular pools of methionine.

5. Remove medium from cells, add 2 ml [^{35}S]methionine working solution (from step 2), and incubate 10 to 30 min in a CO_2 incubator.

   If necessary, as little as 1 ml of labeling medium per plate can be used. It is critical, however, that the plates sit on a perfectly horizontal surface during incubation to avoid drying of the cell monolayer.

6. Remove medium from cells. Wash once with 10 ml ice-cold PBS and remove PBS.

   CAUTION: *The medium and wash are radioactive—follow applicable safety regulations for disposal.*

   If significant detachment of cells occurs during labeling, it may be necessary to scrape the cells carefully in the [^{35}S]methionine-containing medium and transfer the suspension to a 15-ml centrifuge tube before centrifuging and washing with PBS.

7. Add 10 ml ice-cold PBS and scrape cells carefully with either a disposable plastic scraper or a rubber policeman.

8. Transfer the suspension to a 15-ml centrifuge tube, centrifuge 5 min at 300 × *g*, 4°C, and discard supernatant.

9. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

   Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

PULSE-CHASE LABELING OF CELLS WITH [^{35}S]METHIONINE

ALTERNATE PROTOCOL 2

Pulse-chase protocols are used to analyze time-dependent processes, such as posttranslational modification, transport, secretion, or degradation of newly synthesized proteins. Cells in suspension or adherent cells are pulse-labeled with [^{35}S]methionine (see Basic Protocol and Alternate Protocol 1), after which they are incubated (chased) in complete medium containing excess unlabeled methionine, as described below.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Chase medium (see recipe), 37°C

1. Prepare 0.5–2 × 10^7 cells per sample per time point, and pulse-label 10 to 30 min with 2 ml of 0.1 to 0.2 mCi/ml [^{35}S]methionine per 0.5–2 × 10^7 cells (see Basic Protocol, steps 1 to 6, or see Alternate Protocol 1, steps 1 to 5).
2. Remove the [^{35}S]methionine working solution, wash once with 10 ml 37°C chase medium, and add 10 ml 37°C chase medium.

   Rapid termination of the labeling reaction can be achieved by adding two times the volume of chase medium containing excess unlabeled methionine (15 mg/liter) directly to the labeling mixture.

   Final concentration of cells in suspension should be 2 × 10^6 cells/ml for chases ≤2 hr, or 0.5 × 10^6 cells/ml for chases >2 hr. For adherent cells, add 10 ml/100-mm dish.
3. Incubate for the desired time at 37°C. Incubate cell suspensions with rotation in tightly capped tubes. Incubate adherent cells in a CO_2 incubator.

4a. *For cells in suspension:* Collect cells by centrifuging 5 min at 300 × *g*, 4°C.

   The supernatant can be discarded or can be collected for analysis of proteins that are secreted or shed into the medium.

4b. *For adherent cells:* Scrape off adherent cells and transfer to 15-ml centrifuge tubes. Centrifuge 5 min at 300 × *g*, 4°C, and either save or discard the supernatant as in step 4a.

5. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

   Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

LONG-TERM LABELING OF CELLS WITH [^{35}S]METHIONINE

ALTERNATE PROTOCOL 3

Long-term labeling refers to continuous metabolic labeling of cells for periods of 6 to 32 hr. Long-term labeling is particularly advantageous when studying proteins that are synthesized at low rates. It is also used to accumulate mature labeled proteins, rather than biosynthetic precursors, for characterization of their properties. Steady-state labeling is a form of long-term labeling in which cells are incubated with the radiolabeled amino acid until the rates of synthesis and degradation of the radiolabeled proteins are equal. Steady-state labeling allows calculation of the stoichiometry of subunits within a protein complex, provided that the primary structure of the subunits is known. In these procedures, unlabeled methionine is added to the medium to maintain cell viability and to sustain incorporation of label for the duration of the experiment. The amount of unlabeled methionine added depends on factors such as the length of the labeling period and the cell density. Media containing between 5% and 20% the normal amount of unlabeled methionine are generally used. The conditions described below are suitable for overnight (~16 hr) labeling of cells in suspension or adherent cells.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Long-term labeling medium (see recipe), warmed to 37°C
75-cm^2 tissue culture flask

1. Prepare a 0.02 to 0.1 mCi/ml [^{35}S]methionine working solution in prewarmed (37°C) long-term labeling medium (see Basic Protocol, step 1).

For cell suspensions

2a. Prepare and wash cells once with prewarmed long-term labeling medium (see Basic Protocol, steps 2 and 3).

3a. Resuspend 0.5–2 × 10^7 cells in 25 ml of 0.02 to 0.1 mCi/ml [^{35}S]methionine working solution and transfer to a 75-cm^2 tissue culture flask.

For adherent cells

2b. Grow 0.5–2 × 10^7 adherent cells in a 75-cm^2 tissue culture flask (80% to 90% confluency) in a CO_2 incubator. Wash once with prewarmed long-term labeling medium (see Alternate Protocol 1, step 3).

3b. Add 25 ml of 0.02 to 0.1 mCi/ml [^{35}S]methionine working solution to each 75-cm^2 flask.

4. Tighten caps to prevent release of volatile ^{35}S-labeled compounds. Incubate 16 hr in a CO_2 incubator.

5. Wash cells and determine incorporation (see Basic Protocol, steps 7 and 8, or Alternate Protocol 1, steps 6 to 9).

Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

ALTERNATE PROTOCOL 4

METABOLIC LABELING WITH OTHER RADIOLABELED AMINO ACIDS

In some instances, it may be necessary to label proteins with radiolabeled amino acids other than [^{35}S]methionine—e.g., when the proteins have a low methionine content or have no methionine residues at all. In these cases, the best choices are [^{35}S]cysteine or [^{3}H]leucine.

Cysteine residues are more abundant in proteins than are methionine residues (3.4% versus 1.8%; Table 7.1.1), although cysteine is less stable. Formulations of [^{35}S]cysteine that have high specific activity (>800 Ci/mmol) can be obtained from several companies. Leucine has the advantage of being the most frequent amino acid in proteins (10.4%; Table 7.1.1) and specific activities of [^{3}H]leucine are the highest among ^{3}H-labeled amino acids (up to 190 Ci/mmol). If proteins are known to be rich in a particular amino acid or if special methods are to be performed (e.g., radiochemical sequencing or multiple labeling), ^{3}H-labeled amino acids other than [^{3}H]leucine can be used.

Label cells with these amino acids as described for [^{35}S]methionine in the previous protocols. Substitute [^{35}S]cysteine or ^{3}H-labeled amino acids in the labeling medium. Use labeling media lacking cysteine or any other respective amino acid.

CAUTION: Solutions containing [^{35}S]cysteine release volatile ^{35}S-labeled compounds (refer to Safety Precautions).

TCA PRECIPITATION TO DETERMINE LABEL INCORPORATION

SUPPORT PROTOCOL

In metabolic labeling experiments, it is often useful to monitor the incorporation of radioactivity into total cellular proteins. This can easily be achieved by precipitation with trichloroacetic acid (TCA) using BSA as a carrier protein. This protocol can be used at the end of the labeling procedures in each protocol.

Materials

Labeled cell suspension (see Basic Protocol or Alternate Protocols 1 to 4)
BSA/NaN_3: 1 mg/ml BSA containing 0.02% (w/v) sodium azide (NaN_3)
10% (w/v) TCA solution (see recipe), ice cold
Ethanol

Filtration apparatus attached to a vacuum line
2.5-cm glass microfiber filter disks (Whatman GF/C)

CAUTION: TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.

1. Add 10 to 20 µl of a labeled cell suspension to 0.1 ml BSA/NaN_3. Place on ice.
2. Add 1 ml ice-cold 10% (w/v) TCA solution. Vortex vigorously and incubate 30 min on ice.
3. Filter the suspension onto 2.5-cm glass microfiber filter disks in a filtration apparatus under vacuum.
4. Wash the disks twice with 5 ml ice-cold 10% (w/v) TCA solution and twice with ethanol. Air dry 30 min.

   CAUTION: *The wash fluids should be handled as mixed chemical/radioactive waste—follow applicable safety regulations for disposal (see APPENDIX 1D).*

5. Spot the same volume of the radiolabeled cell suspension used in step 1 (10 to 20 µl) on a glass microfiber disk. Air dry.

   This disk will be used to measure the total amount of radiolabeled amino acid in the cell suspension.

6. Transfer disks from steps 4 and 5 to 20-ml scintillation vials, add 5 ml scintillation fluid, and measure the radioactivity in a scintillation counter.
7. Calculate the ratio of TCA-precipitable label to total radioactivity (i.e., the ratio of sample radioactivity in step 4 to the total radioactivity in step 5).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

NOTE: Use sterile tissue culture technique to prepare these reagents.

Chase medium

Pulse-labeling medium (see recipe) containing 15 mg/liter unlabeled methionine or equivalent excess amount of other amino acid used for radiolabeling. Store up to 2 weeks at 4°C.

Long-term labeling medium (90% methionine-free medium)

Mix 9 vol pulse-labeling medium (see recipe), lacking methionine or other appropriate amino acid, with 1 vol chase medium (see recipe) containing the same amino acid. Store up to 2 weeks at 4°C.

Pulse-labeling medium

Use supplemented Dulbecco's modified Eagle medium (DMEM; see *APPENDIX 2A* but omit nonessential amino acids) or RPMI 1640, each lacking methionine or other specific amino acid, but containing 10% (v/v) FBS (dialyzed overnight against saline solution to remove unlabeled amino acids). Add 25 mM HEPES buffer (with pH adjusted to 7.4 with NaOH). Store up to 2 weeks at 4°C.

The amino acid used to label cells is omitted from this medium (e.g., if [^{35}S]methionine is employed, methionine-free DMEM or RPMI 1640 must be used). Specific amino acid–free media can be obtained from several tissue culture media suppliers, or can be prepared (see APPENDIX 2B) from amino acid–free medium by adding individual amino acid components, omitting the one that will be used to label. A kit for the preparation of such media is available (Select-Amine from Life Technologies).

Trichloroacetic acid (TCA) solution, 10% (w/v)

Prepare a 100% (w/v) TCA stock solution by dissolving the entire contents of a newly opened TCA bottle in water (e.g., dissolve the contents of a 500-g bottle of TCA in sufficient water to yield a final volume of 500 ml). Store up to 1 year at 4°C. Prepare 10% (w/v) TCA by dilution and store up to 3 months at 4°C.

CAUTION: *TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.*

COMMENTARY

Background Information

Metabolic labeling of cellular proteins is achieved by placing cells in a nutritional medium containing all components necessary for growth of cells in culture, except for one amino acid that is substituted by its radiolabeled form. The radiolabeled amino acids are transported across the plasma membrane by carrier-mediated systems and, once in the cytosol, are loaded onto tRNA molecules before being incorporated into newly synthesized proteins.

Because metabolic labeling techniques use the metabolic machinery of the cell to incorporate radiolabeled amino acids, there are limitations on the type of radiolabeled amino acids that can be employed. The list of potential precursors is restricted to L-amino acids normally found in proteins, in which one or more atoms are substituted by a radioisotope (Table 7.1.1). Sulfuric amino acids, such as methionine and cysteine, are conveniently labeled with ^{35}S. Most other amino acids can be obtained labeled with ^{3}H.

Critical Parameters

The protocols in this unit should be considered as models from which more specific methods can be designed. The conditions described here have been optimized for radiolabeling proteins that are expressed at low to moderate levels (10^4 to 10^5 copies per cell) and assume that the labeled cells will be used for immunoprecipitation (*UNIT 7.2*). This level of expression is characteristic of most endogenous membrane proteins, luminal organellar proteins, signal transduction proteins, and transcription factors. More abundant proteins, such as cytoskeletal proteins or proteins expressed by infection or transfection, can be labeled with less radiolabeled amino acid and/or fewer cells. The choice of a particular labeling protocol and its modification will be aided by careful consideration of a number of parameters that influence the incorporation of radiolabeled amino acids into proteins, as discussed below.

Selection of amino acid label

Purified [^{35}S]methionine of high specific activity (>800 Ci/mmol) can be obtained from several suppliers of radiolabeled amino acids. Protein hydrolyzates of *Escherichia coli* grown in the presence of $[^{35}SO_4]^{2-}$ (e.g., Tran^{35}S-label from ICN Biomedicals or Expre^{35}S^{35}S from NEN Life Sciences) can be used as substitutes for [^{35}S]methionine in metabolic labeling techniques. These preparations contain ^{35}S distributed among several different compounds. In a typical batch, ~70% of the radioactivity will be present as [^{35}S]methionine and ~15% as [^{35}S]cysteine; the remainder are other ^{35}S-labeled compounds. Labeling with these radioactive protein hydrolyzates in methionine-free medium will result in incorporation of label only in methionine residues; use of methionine- and cysteine-free medium will result in labeling of both methionine and cysteine residues.

Purified [^{35}S]methionine should be used whenever certainty of labeling with only [^{35}S]methionine is required. Another reason for using purified [^{35}S]methionine is that it tends to be more stable and emit less volatile decomposition products than ^{35}S-labeled protein hydrolyzates. In most cases, however, the relatively inexpensive radioactive protein hydrolyzates (~1/3 the cost of [^{35}S]methionine) can be used instead of purified [^{35}S]methionine. [^{35}S]methionine preparations should be stored frozen at −80°C. Under these conditions, they are stable for at least 1 month; the half-life of ^{35}S is 88 days.

For proteins with one or no methionine residues but several cysteine residues, labeling with [^{35}S]cysteine (available with specific activities >800 Ci/mmol) is a good option. ^{35}S-labeled protein hydrolyzates are not good sources of [^{35}S]cysteine for radiolabeling because only ~15% of the ^{35}S-labeled compounds are [^{35}S]cysteine.

Purified [^{3}H]leucine (available with specific activities of up to 190 Ci/mmol) is a good alternative to ^{35}S-labeled amino acids. The half-life of ^{3}H is ~12 years. The specific activities of other ^{3}H-labeled amino acids range between 5 and 140 Ci/mmol (Table 7.1.1). Several problems can arise when using certain ^{3}H-labeled amino acids due to their participation in metabolic pathways. The following problems must be considered when using tritiated amino acids for metabolic labeling of proteins.

Nonessential versus essential amino acids. Cells are able to synthesize nonessential amino acids from other compounds. If the radiolabeled amino acids used are nonessential, their specific activity will be reduced by dilution with the endogenously synthesized amino acids. Therefore, essential amino acids (E in Table 7.1.1) should be favored for metabolic labeling.

Transamination. The α-amino groups of many amino acids can be removed in reactions catalyzed by transaminases (T in Table 7.1.1). Deamination of the radiolabeled amino acids can be prevented by addition of the transaminase inhibitor (aminooxy)acetic acid during starvation and labeling of the cells (Coligan et al., 1983).

Interconversion. Certain radiolabeled amino acids can be converted by cells into other amino acids (I in Table 7.1.1). This problem is of particular importance in methods used to determine the amino acid composition or sequence of radiolabeled proteins.

Labeling time

The experimental purpose, the turnover rate of the protein of interest, and the viability of the cells should all be considered when determining the length of time for labeling. If the purpose of the experiment is to identify or characterize a protein precursor, pulse-labeling (10 to 30 min) must be used (see Basic Protocol and Alternate Protocol 1). If the protein of interest has a low turnover rate or if it is necessary to accumulate a labeled mature product, a protocol for long-term labeling (6 to 32 hr) is more appropriate (see Alternate Protocol 3). If the biosynthesis, posttranslational modification, intracellular transport, or fate of newly synthesized proteins is being analyzed, a pulse-chase protocol should be used (see Alternate Protocol 2).

The turnover rate of the protein of interest is an important parameter to be considered when determining the labeling time. Proteins with a high turnover rate are optimally labeled for short times. Longer times will result in increased labeling of other cellular proteins, causing a decrease in the relative abundance of the labeled protein of interest in the cell lysate. This could result in increased detection of nonspecific proteins in immunoprecipitates (see *UNIT 7.2*). Conversely, proteins that turn over slowly should be labeled for longer times.

Incorporation of radiolabeled amino acids into proteins is directly proportional to their length of labeling time for a certain period, after which it tends to plateau. When all the limiting amino acid is consumed, protein synthesis ceases. The length of the initial phase of linear incorporation will vary with the concentration of the labeling amino acid and the density and metabolic activity of the cells.

Concentration and specific activity of the radiolabeled amino acid

Because radiolabeled amino acids are used in limiting amounts, their incorporation into proteins is directly proportional to their concentration in the labeling medium. If very short pulses are required, the concentration of labeled amino acid can be increased to compensate for the shorter labeling time. The incorporation of radiolabeled amino acids is also directly proportional to their specific activity. Addition of unlabeled amino acids, as is required in long-term labeling protocols, will result in reduced incorporation over short periods.

Cell density

At low cell densities, the amount of labeled protein synthesized is directly proportional to the cell concentration. At high densities, however, incorporation will increase nonlinearly. Very concentrated cell samples (>5×10^7 cells/ml) can only be labeled for short periods (≤5 min) as rapid loss of metabolic activity and cell viability will occur due to acidification of the medium and accumulation of toxic metabolites.

Conditions for very short pulses

Very short pulses (2 to 10 min) are required to study posttranslational modifications that occur shortly after synthesis. This includes folding, disulfide bond formation, and early carbohydrate modifications of newly synthesized proteins. Proteins that are rapidly degraded after synthesis are also best studied with very short pulses. The protocol for very short pulses is similar to the Basic Protocol for pulse-labeling, with the following modifications: (1) the pulse-labeling medium contains higher concentration of radiolabeled amino acid (up to 1 mCi/ml), (2) labeling is stopped by addition of ice-cold PBS containing 20 mM freshly prepared *N*-ethylmaleimide (NEM) to prevent oxidation of free sulfhydryls, and (3) if a chase is necessary, 1 mM cycloheximide is added to the chase medium to quickly stop the elongation of nascent polypeptide chains. For additional details on very short pulses, see Braakman et al. (1991).

Temperature and pH

Unless otherwise required for special conditions (e.g., heat shock, temperature-sensitive mutants), metabolic labeling should be performed at 37°C. For this reason, it is important that the labeling medium be warmed to 37°C before adding it to the cells. A dramatic reduction in incorporation occurs at room temperature. It is also important that the pH of the labeling medium be ~7.4.

Anticipated Results

A typical incorporation of labeled amino acid precursor after a 30-min pulse under the conditions described in this unit (see Basic Protocol and Alternate Protocols 1 and 2) is 5% to 20%. In the case of long-term labeling, the incorporation typically reaches 30% to 60%. If the labeled cells are used for immunoprecipitation (*UNIT 7.2*), followed by SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*), specific bands should be visible within two hours to two months of exposure.

Time Considerations

It takes 1 to 2 hr to prepare cells and materials for labeling. The actual labeling time depends upon the protocol chosen—pulse-labeling takes 10 to 30 min and long-term labeling takes 6 to 32 hr. Washing and processing cells may take an additional 1.5 hr.

Literature Cited

Braakman, I., Hoover-Litty, H., Wagner, K.R., and Helenius, A. 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. *J. Cell Biol.* 114:401-411.

Coligan, J.E., Gates, F.T. III, Kimball, E.S., and Maloy, W.L. 1983. Radiochemical sequence analysis of metabolically labeled proteins. *Methods Enzymol.* 91:413-434.

Lathe, R. 1985. Synthetic oligonucleotide probes deduced from amino acid sequence data. Theoretical and practical considerations. *J. Mol. Biol.* 183:1-12.

Meisenhelder, J. and Hunter, T. 1988. Radioactive protein labelling techniques. *Nature* 335:120.

Key Reference

Coligan et al., 1983. See above.

Contains a detailed description of conditions used to metabolically label proteins with different radiolabeled amino acids.

Contributed by Juan S. Bonifacino
National Institute of Child Health
and Human Development/NIH
Bethesda, Maryland

Immunoprecipitation

Immunoprecipitation is a technique in which an antigen is isolated by binding to a specific antibody attached to a sedimentable matrix. The source of antigen for immunoprecipitation can be unlabeled cells or tissues, metabolically or extrinsically labeled cells (*UNIT 7.1*), subcellular fractions from either unlabeled or labeled cells (see Chapter 3), or in vitro–translated proteins (*UNIT 11.2*). Immunoprecipitation is also used to analyze protein fractions separated by other biochemical techniques such as gel filtration or sedimentation on density gradients (*UNIT 5.3*). Either polyclonal or monoclonal antibodies from various animal species can be used in immunoprecipitation protocols. Antibodies can be bound noncovalently to immunoadsorbents such as protein A– or protein G–agarose, or can be coupled covalently to a solid-phase matrix.

Immunoprecipitation protocols consist of several stages (Fig. 7.2.1; see Basic Protocol 1). In stage 1, the antigen is solubilized by one of several techniques for lysing cells. Soluble and membrane-associated antigens can be released from cells grown either in suspension culture (see Basic Protocol 1) or as a monolayer on tissue culture dishes (see Alternate Protocol 1) with nondenaturing detergents. Cells can also be lysed under denaturing conditions (see Alternate Protocol 2). Soluble antigens can also be extracted by mechanical disruption of cells in the absence of detergents (see Alternate Protocol 3). All of these lysis procedures are suitable for extracting antigens from animal cells. Yeast cells require disruption of their cell wall in order to allow extraction of the antigens (see Alternate Protocol 4). In stage 2, a specific antibody is attached, either noncovalently or covalently, to a sedimentable, solid-phase matrix to allow separation by low-speed centrifugation. This unit presents the noncovalent attachment of antibody to protein A– or protein G–agarose beads (see Basic Protocol 1). Stage 3 consists of incubating the solubilized antigen from stage 1 with the immobilized antibody from stage 2, followed by extensive washing to remove unbound proteins (see Basic Protocol 1). Immunoprecipitated antigens can be dissociated from antibodies and reprecipitated by a protocol referred to as "immunoprecipitation-recapture" (see Basic Protocol 2). This protocol can be used with the same antibody for further purification of the antigen, or with a second antibody to identify components of multisubunit complexes or to study protein-protein interactions (Fig. 7.2.2). Immunoprecipitated antigens can be analyzed by one-dimensional electrophoresis (*UNIT 6.1*), two-dimensional electrophoresis, or immunoblotting (*UNIT 6.2*). In some cases, immunoprecipitates can be used for structural or functional analyses of the isolated antigens. Immunoprecipitates can also be used as sources of immunogens for production of monoclonal or polyclonal antibodies.

IMMUNOPRECIPITATION USING CELLS IN SUSPENSION LYSED WITH A NONDENATURING DETERGENT SOLUTION

BASIC PROTOCOL 1

In this protocol, unlabeled or labeled cells in suspension are extracted by incubation in nondenaturing lysis buffer containing the nonionic detergent Triton X-100 (steps 1 to 7). This procedure results in the release of both soluble and membrane proteins; however, many cytoskeletal and nuclear proteins, as well as a fraction of membrane proteins, are not efficiently extracted under these conditions (see *UNIT 5.1*). The procedure allows immunoprecipitation with antibodies to epitopes that are exposed in native proteins.

For immunoprecipitation, a specific antibody is immobilized on a sedimentable, solid-phase matrix (steps 8 to 14). Although there are many ways to attach antibodies to matrices (see Commentary), the most commonly used methods rely on the property of immunoglobulins to bind *Staphylococcus aureus* protein A, or protein G from group G *Streptococcus* (Table 7.2.1). The best results are obtained by binding antibodies to protein A or

Contributed by Juan S. Bonifacino and Esteban C. Dell'Angelica
Current Protocols in Cell Biology (1998) 7.2.1-7.2.21

protein G that is covalently coupled to agarose beads. In this protocol, Sepharose beads are used (Sepharose is a more stable, cross-linked form of agarose). Immunoprecipitation is most often carried out using rabbit polyclonal or mouse monoclonal antibodies, which, with some exceptions (e.g., mouse IgG1), bind well to protein A (Table 7.2.1). Antibodies that do not bind to protein A–agarose can be adsorbed to protein G–agarose (Table 7.2.1) using exactly the same protocol. For optimal time management, incubation of antibodies with protein A–agarose can be carried out either before or during lysis of the cells.

The final stage in immunoprecipitation is combining the cell lysate with the antibody-conjugated beads and isolating the antigen (steps 18 to 26). This can be preceded by an optional preclearing step in which the lysate is absorbed with either "empty" protein A–agarose beads or with an irrelevant antibody bound to protein A–agarose (steps 15 to

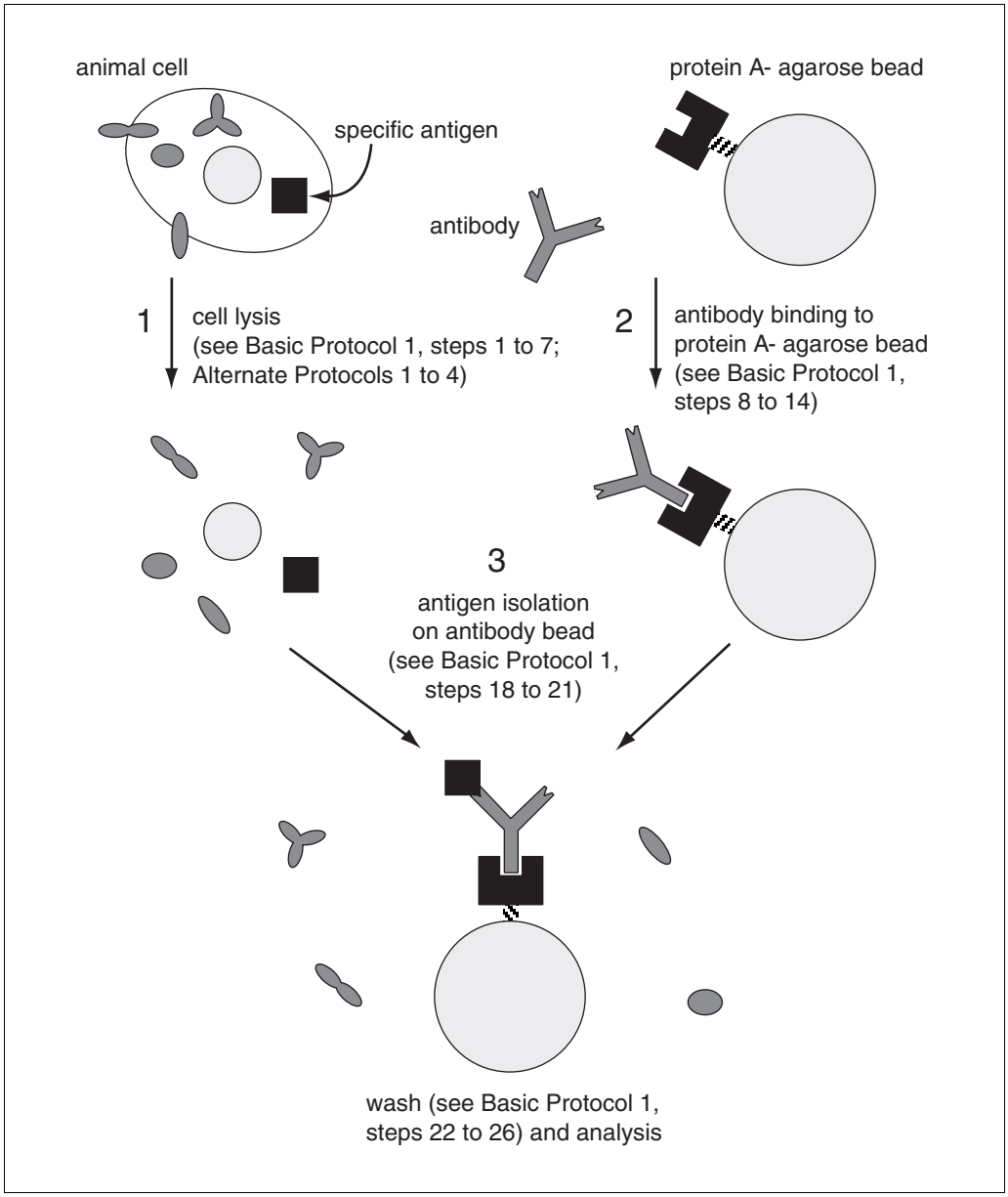


Figure 7.2.1 Schematic representation of the stages of a typical immunoprecipitation protocol. (**1**) Cell lysis: antigens are solubilized by extraction of the cells in the presence or absence of detergents. To increase specificity, the cell lysate can be precleared with protein A–agarose beads (steps 15 to 17, not shown). (**2**) Antibody immobilization: a specific antibody is bound to protein A–agarose beads. (**3**) Antigen capture: the solubilized antigen is isolated on antibody-conjugated beads.

17). The need for preclearing depends on the specific experimental system being studied and the quality of the antibody reagents. The protocol described below incorporates a preclearing step using protein A–agarose. Protein fractions separated by techniques such as gel filtration or sedimentation on sucrose gradients (*UNIT 5.3*) can be used in place of the cell lysate at this stage. After binding the antigen to the antibody-conjugated beads, the unbound proteins are removed by successive washing and sedimentation steps.

Materials

Unlabeled or labeled cells in suspension (*UNIT 7.1*)
PBS (*APPENDIX 2A*), ice cold
Nondenaturing lysis buffer (see recipe), ice cold
50% (v/v) protein A–Sepharose bead (Sigma, Pharmacia Biotech) slurry in PBS containing 0.1% (w/v) BSA and 0.01% (w/v) sodium azide (NaN_3)

Table 7.2.1 Binding of Antibodies to Protein A and Protein G[a,b,c]

Antibody	Protein A binding	Protein G binding[d]
Monoclonal antibodies[e]		
Human IgG1	++	++
Human IgG2	++	++
Human IgG3	–	++
Human IgG4	++	++
Mouse IgG1	+	++
Mouse IgG2a	++	++
Mouse IgG2b	++	++
Mouse IgG3	++	++
Rat IgG1	+	+
Rat IgG2a	–	++
Rat IgG2b	–	++
Rat IgG2c	++	++
Polyclonal antibodies		
Chicken	–	–
Donkey	–	++
Goat	+	++
Guinea pig	++	+
Hamster	+	++
Human	++	++
Monkey	++	++
Mouse	++	++
Rabbit	++	++
Rat	+	+
Sheep	+	++

[a] ++, moderate to strong binding; +, weak binding; –, no binding.

[b] A hybrid protein A/G molecule that combines the features of protein A and protein G, coupled to a solid-phase matrix, is available from Pierce.

[c] Information from Harlow and Lane (1988), and from Pharmacia Biotech, Pierce, and Jackson Immunoresearch.

[d] Native protein G binds albumin from several animal species. Recombinant variants of protein G have been engineered for better binding to rat, mouse, and guinea pig IgG, as well as for avoiding binding to serum albumin.

[e] Protein A binds some IgM, IgA, and IgE antibodies in addition to IgG, whereas protein G binds only IgG.

Specific polyclonal antibody (antiserum or affinity-purified immunoglobulin) or monoclonal antibody (ascites, culture supernatant, or purified immunoglobulin)
Control antibody of same type as specific antibody (e.g., preimmune serum or purified irrelevant immunoglobulin for specific polyclonal antibody; irrelevant ascites, culture supernatant, or purified immunoglobulin for specific monoclonal antibody; see Critical Parameters)
10% (w/v) BSA (*APPENDIX 2A*)
Wash buffer (see recipe), ice cold

Microcentrifuge with fixed-angle rotor (Eppendorf 5415C or equivalent)
Tube rotator (capable of end-over-end inversion)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

NOTE: All solutions should be ice cold and procedures should be carried out at 4°C or on ice.

Prepare cell lysate

1. Collect cells in suspension by centrifuging 5 min at 400 × *g*, 4°C, in a 15- or 50-ml capped conical tube. Place tube on ice.

   Approximately 0.5–2 × 10^7 cells are required to yield 1 ml lysate, which is generally used for each immunoprecipitation.

   Labeled cells are likely to have been pelleted earlier as part of the labeling procedure. If the cells are frozen, they should be thawed on ice before solubilization.

2. Aspirate supernatant with a Pasteur pipet attached to a vacuum trap.

   CAUTION: *Dispose of radioactive materials following applicable safety regulations (APPENDIX 1D).*

3. Resuspend cells gently by tapping the bottom of the tube. Rinse cells twice with ice-cold PBS as in steps 1 and 2, using the same volume of PBS as in the initial culture.

4. Add 1 ml ice-cold nondenaturing lysis buffer per ~0.5–2 × 10^7 cells and resuspend pellet by gentle agitation for 3 sec with a vortex mixer set at medium speed.

   Do not shake vigorously as this could result in loss of material or protein denaturation due to foaming.

5. Keep suspension on ice 15 to 30 min and transfer to a 1.5-ml conical microcentrifuge tube.

   Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.

6. Clear the lysate by microcentrifuging 15 min at 16,000 × *g* (maximum speed), 4°C.

   Centrifugation can be carried out in a microcentrifuge placed in a cold room or in a refrigerated microcentrifuge. Take precautions to ensure that the 4°C temperature is maintained during the spin (e.g., use a fixed-angle rotor with a lid, as the aerodynamics of this type of rotor reduces generation of heat by friction). If it is necessary to reduce background, the lysate can be spun for 1 hr at 100,000 × g in an ultracentrifuge.

7. Transfer the supernatant to a fresh microcentrifuge tube using an adjustable pipet fitted with a disposable tip. Do not disturb the pellet, and leave the last 20 to 40 µl of supernatant in the centrifuge tube. Keep the cleared lysate on ice until preclearing (step 15) or addition of antibody beads (step 18).

NOTE: *Resuspension of even a small amount of sedimented material will result in high nonspecific background due to carryover into the immunoprecipitation steps. A cloudy layer of lipids floating on top of the supernatant will not adversely affect the results of the immunoprecipitation.*

When the lysate is highly radioactive—as is the case for metabolically labeled cells—the use of tips with aerosol barriers is recommended to reduce the risk of contaminating internal components of the pipet.

Cell extracts can be frozen at −70°C until used for immunoprecipitation. However, it is preferable to lyse the cells immediately before immunoprecipitation in order to avoid protein degradation or dissociation of protein complexes. If possible, freeze the cell pellet from step 3 rather than the supernatant from step 7.

Prepare antibody-conjugated beads

8. In a 1.5-ml conical microcentrifuge tube, combine 30 µl of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and the following quantity of specific antibody (select one):

   1 to 5 µl polyclonal antiserum

   1 µg affinity-purified polyclonal antibody

   0.2 to 1 µl ascitic fluid containing monoclonal antibody

   1 µg purified monoclonal antibody

   20 to 100 µl hybridoma culture supernatant containing monoclonal antibody.

The quantities of antibody suggested are rough estimates based on the expected amount of specific antibodies in each preparation. Quantities can be increased or decreased, depending on the quality of the antibody preparation (see Commentary).

Substitute protein G for protein A if antibodies are of a species or subclass that does not bind to protein A (see Table 7.2.1).

If the same antibody will be used to immunoprecipitate multiple samples (e.g., samples from a pulse-chase experiment; UNIT 7.1), the quantities indicated above can be increased proportionally to the number of samples and incubated in a 15-ml capped conical tube. In this case, the beads should be divided into aliquots just prior to the addition of the cleared cell lysate (step 18).

Antibody-conjugated beads can be prepared prior to preparation of the cell lysate (steps 1 to 7), in order to minimize the time that the cell extract is kept on ice.

9. Set up a nonspecific immunoprecipitation control in a 1.5-ml conical microcentrifuge tube by incubating 30 µl of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and the appropiate control antibody (select one):

   1 to 5 µl preimmune serum as a control for a polyclonal antiserum

   1 µg purified irrelevant polyclonal antibody (an antibody to an epitope that is not present in the cell lysate) as a control for a purified polyclonal antibody

   0.2 to 1 µl ascitic fluid containing irrelevant monoclonal antibody (an antibody to an epitope that is not present in the cell lysate and of the same species and immunoglobulin subclass as the specific antibody) as a control for an ascitic fluid containing specific monoclonal antibody

1 µg purified irrelevant monoclonal antibody as a control for a purified monoclonal antibody

20 to 100 µl hybridoma culture supernatant containing irrelevant monoclonal antibody as a control for a hybridoma culture supernatant containing specific monoclonal antibody

The amount of irrelevant antibody should match that of the specific antibody and the antibody should be from the same species as the specific antibody.

10. Mix suspensions thoroughly. Tumble incubation mixtures end over end ≥1 hr at 4°C in a tube rotator.

    Addition of 0.01% (w/v) Triton X-100 may facilitate mixing of the suspension during tumbling. Incubations can be carried out for as long as 24 hr. This allows preparation of the antibody-conjugated beads prior to immunoprecipitation.

11. Microcentrifuge 2 sec at 16,000 × *g* (maximum speed), 4°C.

12. Aspirate the supernatant (containing unbound antibodies) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.

13. Add 1 ml nondenaturing lysis buffer and resuspend the beads by inverting the tube 3 or 4 times.

    For lysates prepared with detergents (this protocol and see Alternate Protocols 1 and 2), use 1 ml nondenaturing lysis buffer; for lysates prepared by mechanical disruption (see Alternate Protocol 3), use detergent-free lysis buffer (see recipe).

    Use of a repeat pipettor is recommended when processing multiple samples.

14. Wash by repeating steps 11 to 13, and then steps 11 and 12 once more.

    At this point the beads have been washed twice with lysis buffer and are ready to be used for immunoprecipitation. Antibody-bound beads can be stored up to 6 hr at 4°C until used.

Preclear lysate (optional)

15. In a microcentrifuge tube, combine 1 ml cell lysate (from step 7) and 30 µl of 50% protein A–Sepharose bead slurry.

    The purpose of this step is to remove from the lysate proteins that bind to protein A–Sepharose, as well as pieces of insoluble material that may have been carried over from previous steps. If the lysate was prepared from cells expressing immunoglobulins—such as spleen cells or cultured B cells—the preclearing step should be repeated at least 3 times to ensure complete removal of endogenous immunoglobulins.

    If cell lysates were frozen and thawed, they should be microcentrifuged 15 min at 16,000 × g (maximum speed), 4°C, before the preclearing step.

16. Tumble end over end 30 min at 4°C in a tube rotator.

17. Microcentrifuge 5 min at 16,000 × *g* (maximum speed), 4°C.

Immunoprecipitate

18. Add 10 µl of 10% BSA to the tube containing specific antibody bound to protein A–Sepharose beads (step 14), and transfer to this tube the entire volume of cleared lysate (from step 7 or 17). If a nonspecific immunoprecipitation control is performed, divide lysate in two ~0.4-ml aliquots, one for the specific antibody and the other for the nonspecific control.

    In order to avoid carryover of beads with precleared material, leave 20 to 40 µl of supernatant on top of the pellets in the preclearing tubes. Discard beads and remaining supernatant. The BSA quenches nonspecific binding to the antibody-conjugated beads during incubation with the cell lysate.

19. Incubate 1 to 2 hr at 4°C while mixing end over end in a tube rotator.

    Samples can be incubated overnight, although there is an increased risk of protein degradation, dissociation of multiprotein complexes, or formation of protein aggregates.

20. Microcentrifuge 5 sec at 16,000 × *g* (maximum speed), 4°C.

21. Aspirate the supernatant (containing unbound proteins) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.

    The supernatant can be kept up to 8 hr at 4°C or up to 1 month at −70°C for sequential immunoprecipitation of other antigens or for analysis of total proteins. To reutilize lysate, remove the supernatant carefully with an adjustable pipet fitted with a disposable tip. Before reprecipitation, preabsorb the lysate with protein A–Sepharose (as in steps 15 to 17) to remove antibodies that may have dissociated during the first immunoprecipitation.

    CAUTION: *Dispose of radioactive materials following applicable safety regulations.*

22. Add 1 ml ice-cold wash buffer, cap the tubes, and resuspend the beads by inverting the tube 3 or 4 times.

    Use of a repeat pipettor is recommended when processing multiple samples.

23. Microcentrifuge 2 sec at 16,000 × *g* (maximum speed), 4°C.

24. Aspirate the supernatant, leaving ~20 µl supernatant on top of the beads.

25. Wash beads three more times (steps 22 to 24).

    Total wash time (steps 22 to 26) should be ~30 min, keeping the samples on ice for 3 to 5 min between washes if necessary (see Critical Parameters).

26. Wash beads once more using 1 ml ice-cold PBS and aspirate supernatant completely with a drawn-out Pasteur pipet.

    The final product should be 15 µl of settled beads containing bound antigen.

    Immunoprecipitates can either be processed immediately or frozen at −20°C for later analysis. For subsequent analysis of the isolated proteins prior to electrophoresis (e.g., comparison of the electrophoretic mobility of the antigen with or without treatment with glycosidases), samples can be divided into two or more aliquots after addition of PBS. Transfer aliquots of the bead suspension to fresh tubes, centrifuge and aspirate as in the previous steps. Immunoprecipitates can be analyzed by one-dimensional electrophoresis (UNIT 6.1), two-dimensional electrophoresis, or immunoblotting (UNIT 6.2).

IMMUNOPRECIPITATION USING ADHERENT CELLS LYSED WITH A NONDENATURING DETERGENT SOLUTION

ALTERNATE PROTOCOL 1

Immunoprecipitation using adherent cells can be performed in the same manner as with nonadherent cells (see Basic Protocol 1). This protocol is essentially similar to steps 1 to 5 of Basic Protocol 1, but describes modifications necessary for using the same nondenaturing detergent solution to lyse cells attached to tissue culture plates. It is preferable to use cells grown on plates rather than in flasks, because the cell monolayer is more easily accessible.

Additional Materials *(also see Basic Protocol 1)*

Unlabeled or labeled cells grown as a monolayer on a tissue culture plate (*UNIT 7.1*)

NOTE: All solutions should be ice cold and procedures should be carried out at 4°C or on ice.

1. Rinse cells attached to a tissue culture plate twice with ice-cold PBS. Remove the PBS by aspiration with a Pasteur pipet attached to a vacuum trap.

   CAUTION: *Dispose of radioactive materials following applicable safety regulations.*

2. Place the tissue culture plate on ice.

3. Add ice-cold nondenaturing lysis buffer to the tissue culture plate.

   Use 1 ml lysis buffer for an 80% to 90% confluent 100-mm-diameter tissue culture plate. Depending on the cell type, a confluent 100-mm dish will contain 0.5–2 × 10^7 cells. For other plate sizes, adjust volume of lysis buffer according to the surface area of the plate.

4. Scrape the cells off the plate with a rubber policeman, and transfer the suspension to a 1.5-ml conical microcentrifuge tube using an adjustable pipettor fitted with a disposable tip. Vortex gently for 3 sec and keep tubes on ice for 15 to 30 min.

   Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.

5. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

ALTERNATE PROTOCOL 2

IMMUNOPRECIPITATION USING CELLS LYSED WITH DETERGENT UNDER DENATURING CONDITIONS

If epitopes of native proteins are not accessible to antibodies, or if the antigen cannot be extracted from the cell with nonionic detergents, cells should be solubilized under denaturing conditions. This protocol is based on that for nondenaturing conditions (see Basic Protocol 1, steps 1 to 7), with the following modifications. Denaturation is achieved by heating the cells in a denaturing lysis buffer that contains an ionic detergent such as SDS or Sarkosyl (*N*-lauroylsarcosine). The denaturing lysis buffer also contains DNase I to digest DNA released from the nucleus. Prior to immunoprecipitation, the denatured protein extract is diluted 10-fold with nondenaturing lysis buffer, which contains Triton X-100; this step protects the antigen-antibody interaction from interference by the ionic detergent. Immunoprecipitation is performed as described (see Basic Protocol 1).

The following protocol is described for cells in suspension culture, although it can be adapted for adherent cells (see Alternate Protocol 1). Only antibodies that react with denatured proteins can be used to immunoprecipitate proteins solubilized by this protocol.

Additional Materials *(also see Basic Protocol 1)*

Denaturing lysis buffer (see recipe)
Heating block set at 95°C (Eppendorf Thermomixer 5436 or equivalent)
25-G needle attached to 1-ml syringe

1. Collect cells in suspension culture (see Basic Protocol 1, steps 1 to 3). Place tubes on ice.

2. Add 100 µl denaturing lysis buffer per ~0.5–2 × 10^7 cells in the pellet.

3. Resuspend the cells by vortexing vigorously 2 to 3 sec at maximum speed. Transfer suspension to a 1.5-ml conical microcentrifuge tube.

   The suspension may be very viscous due to release of nuclear DNA.

   Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.

4. Heat samples 5 min at 95°C in a heating block.
5. Dilute the suspension with 0.9 ml nondenaturing lysis buffer. Mix gently.

   The excess 1% Triton X-100 in the nondenaturing lysis buffer sequesters SDS into Triton X-100 micelles.

6. Shear DNA by passing the suspension five to ten times through a 25-G needle attached to a 1-ml syringe.

   If the DNA is not digested by DNase I in the denaturing lysis buffer or thoroughly sheared mechanically, it will interfere with the separation of pellet and supernatant after centrifugation. Repeat mechanical disruption until the viscosity is reduced to manageable levels.

7. Incubate 5 min on ice.
8. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

IMMUNOPRECIPITATION USING CELLS LYSED WITHOUT DETERGENT

ALTERNATE PROTOCOL 3

Immunoprecipitation of proteins that are already soluble within cells (e.g., cytosolic or luminal organellar proteins) may not require the use of detergents. Instead, cells can be mechanically disrupted by repeated passage through a needle, and soluble proteins can be separated from insoluble material by centrifugation. The following protocol describes lysis of cells in a PBS-based detergent-free lysis buffer. Other buffer formulations may be used for specific proteins.

Additional Materials *(also see Basic Protocol 1)*

Detergent-free lysis buffer (see recipe)
25-G needle attached to 3-ml syringe

NOTE: All solutions should be ice-cold and procedures should be carried out at 4°C or on ice.

1. Collect and wash cells in suspension (see Basic Protocol 1, steps 1 to 3).
2. Add 1 ml of ice-cold detergent-free lysis buffer per ~0.5–2 × 10^7 cells in a pellet.
3. Resuspend the cells by gentle agitation for 3 sec with a vortex mixer set at medium speed.
4. Break cells by passing the suspension 15 to 20 times through a 25-G needle attached to a 3-ml syringe.

   Extrusion of the cell suspension from the syringe should be rapid, although care should be exercised to prevent splashing and excessive foaming. Cell breakage can be checked under a bright-field or phase-contrast microscope. Repeat procedure until >90% cells are broken.

   It is helpful to check ahead of time whether the cells can be broken in this way. If the cells are particularly resistant to mechanical breakage, they can be swollen for 10 min at 4°C with a hypotonic solution containing 10 mM Tris·Cl, pH 7.4 (APPENDIX 2A) before mechanical disruption.

5. Clear the lysate and perform immunoprecipitation (Basic Protocol 1, steps 6 to 26).

ALTERNATE PROTOCOL 4

IMMUNOPRECIPITATION USING YEAST CELLS DISRUPTED WITH GLASS BEADS

Unlike animal cells, yeast cells have an extremely resistant, detergent-insoluble cell wall. To allow extraction of cellular antigens, the cell wall needs to be broken by mechanical, enzymatic, or chemical means. The most commonly used procedure consists of vigorous vortexing of the yeast suspension with glass beads. The breakage can be done in the presence or absence of detergent, as previously described for animal cells (see Basic Protocol 1, Alternate Protocol 2, and Alternate Protocol 3). The protocol described below is suitable for mechanical disruption of most yeast species, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. A protocol for metabolic labeling for yeast has been described by Franzusoff et al. (1991).

Additional Materials *(also see Basic Protocol 1)*

Unlabeled or radiolabeled yeast cells
Lysis buffer, ice cold: nondenaturing, denaturing, or detergent-free lysis buffer (see recipes)
Glass beads (acid-washed, 425- to 600-μm diameter; Sigma)

NOTE: All solutions should be ice-cold and procedures should be carried out at 4°C or on ice.

1. Collect 10 ml of yeast culture at 1 OD_{600} per immunoprecipitation sample, and centrifuge 5 min at 4000 × *g*, 4°C. Place tube on ice.
2. Remove supernatant by aspiration with a Pasteur pipet attached to a vacuum trap.

   CAUTION: *Dispose of radioactive materials following applicable safety regulations.*
3. Loosen pellet by vortexing vigorously for 10 sec. Rinse cells twice with ice-cold distilled water as in steps 1 and 2.

   Radiolabeled yeast cells are likely to have been pelleted earlier as part of the labeling procedure. If the pellets are frozen, they should be thawed on ice prior to cell disruption.
4. Add 3 vol ice-cold lysis buffer and 3 vol at 1 OD_{600} glass beads per volume of pelleted yeast cells.

   Use nondenaturing lysis buffer or detergent-free lysis buffer as required for the antigen under study. If the experiment requires denaturation of the antigen, the procedure can be adapted (see Alternate Protocol 2 for higher eukaryotic cells); however, the yeast cells must be broken with glass beads before heating the sample at 95°C.
5. Shake cells by vortexing vigorously at maximum speed for four 30-sec periods, keeping the cells on ice for 30 sec between the periods.

   Check cell breakage under a bright-field or phase-contrast microscope. It is helpful to check ahead of time if the cells can be broken in this way.
6. Remove the yeast cell lysate from the beads using a pipettor with a disposable tip. Transfer to a fresh tube.
7. Add 4 vol (see step 4) lysis buffer to the glass beads, vortex for 2 sec, and combine this supernatant with the lysate from step 6.
8. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

IMMUNOPRECIPITATION-RECAPTURE

Once an antigen has been isolated by immunoprecipitation, it can be dissociated from the beads and reimmunoprecipitated ("recaptured") with either the same antibody used in the first immunoprecipitation or with a different antibody (Fig. 7.2.2). Immunoprecipitation-recapture with the same antibody allows identification of a specific antigen in cases where the first immunoprecipitation contains too many bands to allow unambiguous identifica-

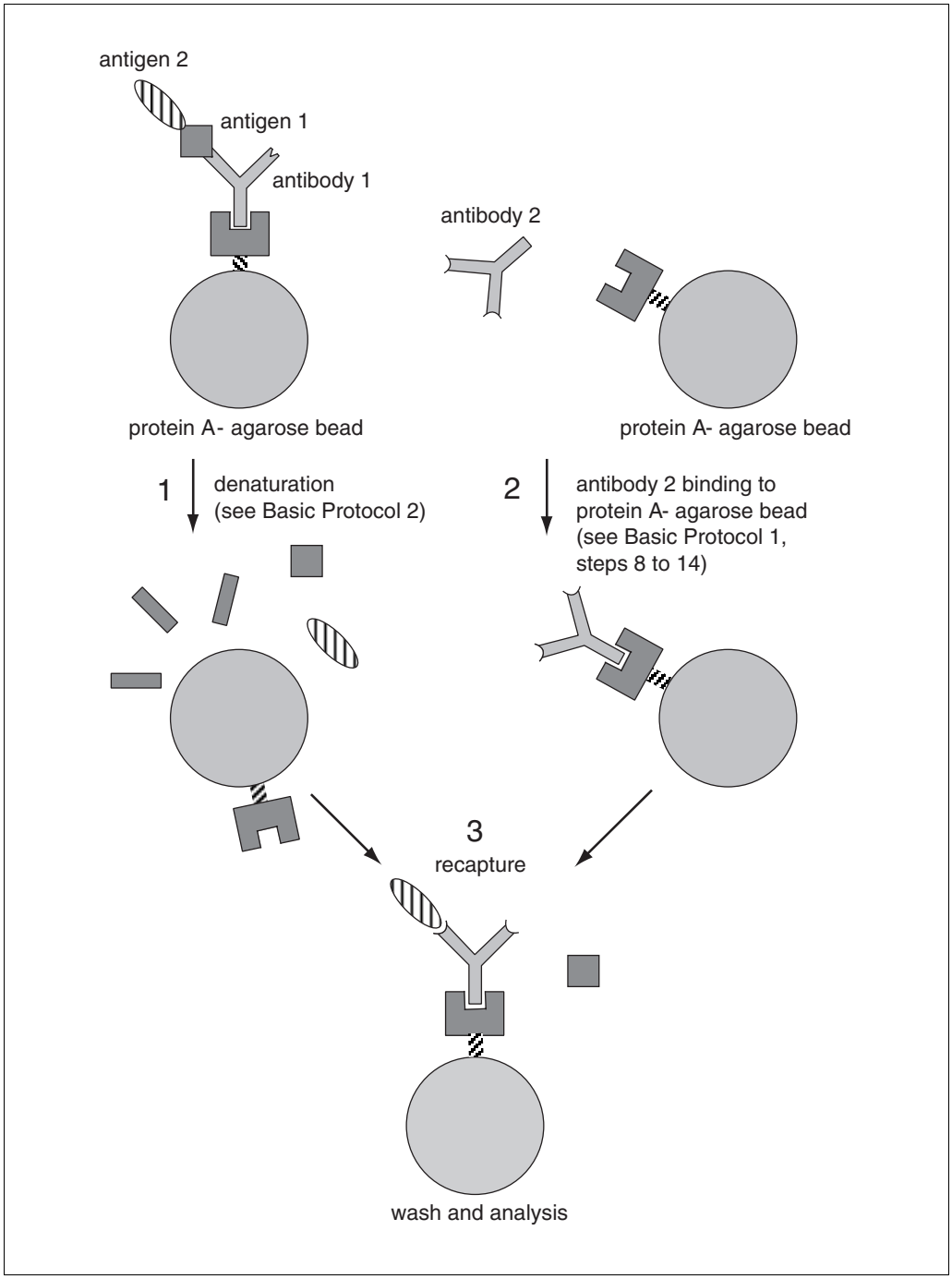


Figure 7.2.2 Scheme showing the stages of immunoprecipitation-recapture. (**1**) Dissociation and denaturation of the antigen: an antigen immunoprecipitated with antibody 1 bound to protein A–agarose beads is dissociated and denatured by heating in the presence of SDS and DTT. (**2**) Immobilization of the second antibody: antibody 2 is bound to protein A–agarose beads. (**3**) Recapture: the denatured antigen (striped oval) is recaptured on antibody 2 bound to protein A–agarose beads. Alternatively, antibody 1 can be used again for further purification of the original antigen (square).

tion. By using a different antibody in the second immunoprecipitation, immunoprecipitation-recapture can be used to analyze the subunit composition of multi-protein complexes (Fig. 7.2.3). The feasibility of this approach depends on the ability of the second antibody to recognize denatured antigens.

Dissociation of the antigen from the beads is achieved by denaturation of antigen-antibody-bead complexes at high temperature in the presence of SDS and DTT. Prior to recapture, the SDS is diluted in a solution containing Triton X-100, and the DTT is neutralized with excess iodoacetamide. Recapture is then performed as in the first immunoprecipitation (see Basic Protocol 1, step 26).

Materials

Elution buffer (see recipe)
Beads containing bound antigen (see Basic Protocol 1, step 26)
10% (w/v) BSA (*APPENDIX 2A*)
Nondenaturing lysis buffer (see recipe)
Heating block set at 95°C (Eppendorf Thermomixer 5436 or equivalent)

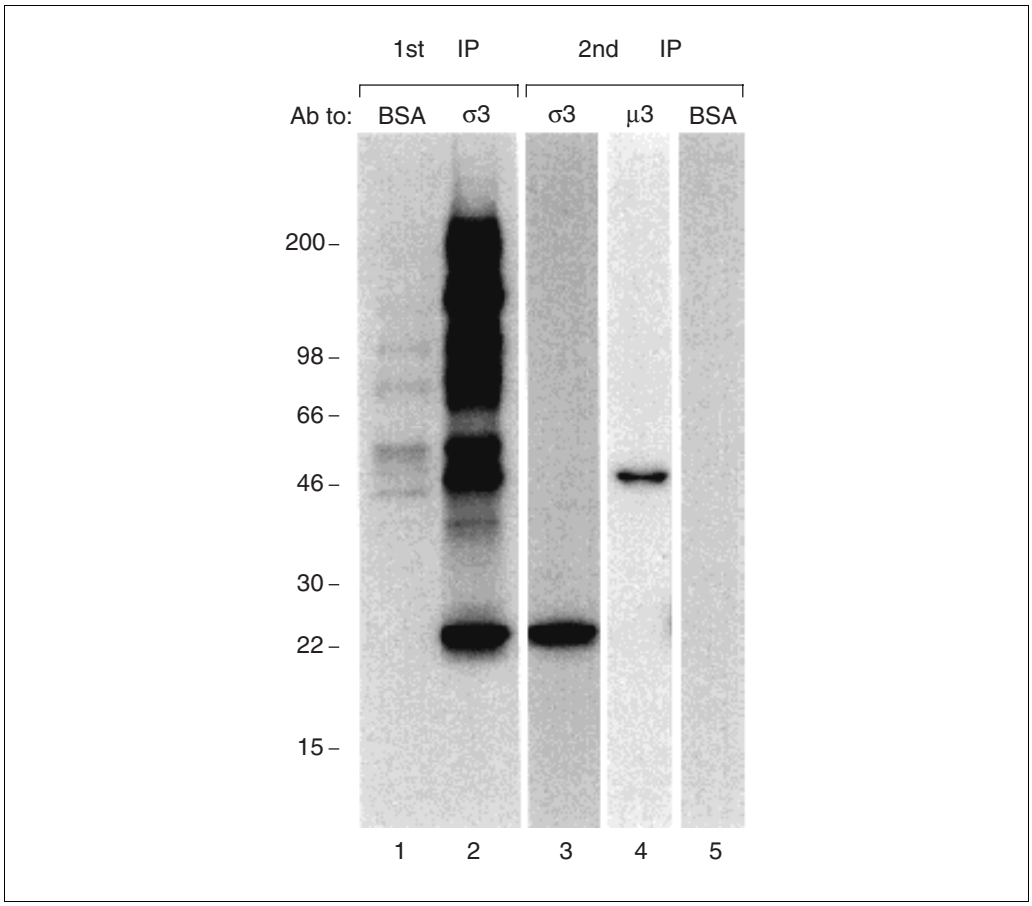


Figure 7.2.3 Example of an immunoprecipitation-recapture experiment. Human M1 fibroblasts were labeled overnight with [^{35}S]methionine (*UNIT 7.1*) and extracted with nondenaturing lysis buffer (see Basic Protocol 1). The cell extract was then subjected to immunoprecipitation with antibodies to BSA (irrelevant antibody control; lane 1) and to the AP-3 adaptor (σ3; lane 2), a protein complex involved in protein sorting. Notice the presence of several specific bands in lane 2. The AP-3 immunoprecipitate was denatured as described in Basic Protocol 2 and individual components of the AP-3 complex were recaptured with antibodies to two of its subunits: σ3 (M_r ~22,000; lane 3) and μ3 (M_r ~47,000; lane 4). An immunoprecipitation with an antibody to BSA was also performed as a nonspecific control (lane 5). The amount of immunoprecipitate loaded on lanes 1 and 2 is ~1/10 the amount loaded on lanes 3 to 5. Notice the presence of single bands in lanes 3 and 4. The positions of M_r standards (expressed as $10^{-3} \times M_r$) are shown at left. IP, immunoprecipitation.

1. Add 50 µl elution buffer to 15 µl beads containing bound antigen. Mix by vortexing.

   The DTT in the elution buffer reduces disulfide bonds in the antigen and the antibody, and the SDS contributes to the unfolding of polypeptide chains.

2. Incubate 5 min at room temperature and 5 min at 95°C in a heating block. Cool tubes to room temperature.

3. Add 10 µl of 10% BSA. Mix by gentle vortexing.

   BSA is added to prevent adsorption of antigen to the tube, and to quench nonspecific binding to antibody-conjugated beads.

4. Add 1 ml nondenaturing lysis buffer.

   The iodoacetamide in the nondenaturing lysis buffer reacts with the DTT and prevents it from reducing the antibody used in the recapture steps. The presence of PMSF and leupeptin in the buffer is not necessary at this step.

5. Incubate 10 min at room temperature.

6. Clear the lysate and perform second immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Denaturing lysis buffer

1% (w/v) SDS
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
5 mM EDTA (*APPENDIX 2A*)
Store up to 1 week at room temperature (SDS precipitates at 4°C)
Add the following fresh before use:
10 mM dithiothreitol (DTT, from powder)
1 mM PMSF (*APPENDIX 2A*)
2 µg/ml leupeptin (store 10 mg/ml stock in H_2O up to 6 months at −20°C)
15 U/ml DNase I (store 15,000 U/ml stock solution up to 2 years at −20°C)

1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H_2O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at −20°C.

Detergent-free lysis buffer

PBS (APPENDIX 2A) containing:
5 mM EDTA (*APPENDIX 2A*)
0.02% (w/v) sodium azide
Store up to 6 months at 4°C
Immediately before use add:
10 mM iodoacetamide (from powder)
1 mM PMSF (*APPENDIX 2A*)
2 µg/ml leupeptin (store 10 mg/ml stock in H_2O up to 6 months at −20°C)

1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H_2O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at −20°C.

Elution buffer

1% (w/v) SDS
100 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Store up to 1 week at room temperature
10 mM dithiothreitol (DTT, add fresh from powder before use)

Nondenaturing lysis buffer

1% (w/v) Triton X-100
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
300 mM NaCl
5 mM EDTA (*APPENDIX 2A*)
0.02% (w/v) sodium azide
Store up to 6 months at 4°C
Immediately before use add:
10 mM iodoacetamide (from powder)
1 mM PMSF (*APPENDIX 2A*)
2 μg/ml leupeptin (store 10 mg/ml stock in H_2O up to 6 months at –20°C)

1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H_2O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at –20°C.

Wash buffer

0.1% (w/v) Triton X-100
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
300 mM NaCl
5 mM EDTA (*APPENDIX 2A*)
0.02% (w/v) sodium azide
Store up to 6 months at 4°C

COMMENTARY

Background Information

The use of antibodies for immunoprecipitation has its origin in the *precipitin* reaction (Nisonoff, 1984). The term precipitin refers to the spontaneous precipitation of antigen-antibody complexes formed by interaction of certain polyclonal antibodies with their antigens. The precipitation arises from formation of large networks of antigen-antibody complexes, due to the bivalent or polyvalent nature of immunoglobulins and to the presence of two or more epitopes in some antigens. This phenomenon was quickly exploited to isolate antigens from protein mixtures; however, its use remained limited to antibodies and antigens that were capable of multivalent interaction. In addition, the efficiency of precipitate formation was highly dependent on the concentrations of antibody and antigen. Thus, the precipitin reaction was not generally applicable as a method for immunoprecipitation.

A significant improvement was the use of secondary anti-immunoglobulin reagents (generally anti-immunoglobulin serum) to cross-link the primary antibodies, thus promoting the formation of a precipitating network. Protocols based on the use of cross-linking secondary antibodies are still used in immunoprecipitation and are reputed to give very low backgrounds (Springer, 1996).

In the 1970s, immunoprecipitation became widely applicable to the study of cellular antigens as a result of several technological advances. A critical development was the introduction of methods for the production of monoclonal antibodies (Köhler and Milstein, 1975). The ability to produce unlimited amounts of antibodies with specificity against virtually any cellular antigen had a profound impact in many areas of biology and medicine. The fact that preparation of monoclonal antibodies did not require prior purification of the antigens accelerated the characterization of cellular proteins and organelles, a process in which immunoprecipitation protocols played a major role. To this day, monoclonal antibodies produced in mice or rats continue to be among the most useful tools in cell biology.

Another important development was the discovery of bacterial Fc receptors, proteins found

on the surface of bacteria that have the property of binding a wide range of immunoglobulins. Two of the most widely used bacterial Fc receptors are protein A from *Staphylococcus aureus* and protein G from group G streptococci. Protein A and protein G bind both polyclonal and monoclonal antibodies belonging to different subclasses and from different animal species (Table 7.2.1). Protein A was initially used to adsorb immunoglobulins as part of fixed, killed *Staphylococcus aureus* particles. Both protein A and protein G are now produced in large quantities by recombinant DNA procedures and are available coupled to solid-phase matrices such as agarose. In most cases, the binding of polyclonal or monoclonal antibodies to immobilized protein A (or G) avoids the need to use a secondary antibody to precipitate antigen-antibody complexes. Because of their broad specificity and ease of use, protein A–agarose and protein G–agarose (and related products) are the state-of-the-art reagents for the isolation of soluble antigen-antibody complexes in immunoprecipitation protocols.

Recent progress in the field of antibody engineering (reviewed by Rapley, 1995; Irving et al., 1996) promises to make antibody production a less time-consuming and haphazard process. Antibody fragments with high affinity for specific antigens can now be selected from phage display antibody libraries. Selected recombinant antibodies can then be produced in large quantities in *Escherichia coli*. Techniques have been developed for producing antibodies in soluble, secreted form. Affinity tags are added to the recombinant antibody molecules to facilitate purification, detection, and use in procedures such as immunoprecipitation. While attractive in principle, the production of recombinant antibodies has been plagued by technical difficulties that so far have limited their widespread use in cell biology. However, as technical problems are overcome, recombinant techniques will progressively replace immunization of animals as a way of producing antibodies for immunoprecipitation and for other applications.

Critical Parameters

Extraction of antigens

Isolation of cellular antigens by immunoprecipitation requires extraction of the cells so that the antigens are available for binding to specific antibodies, and are in a physical form that allows separation from other cellular components. Extraction with nondenaturing detergents such as Triton X-100 (see Basic Protocol 1 and Alternate Protocol 1) or in the absence of detergent (see Alternate Protocol 3) allows immunoprecipitation with antibodies to epitopes that are exposed on native proteins. Other nondenaturing detergents such as Nonidet P-40, CHAPS, digitonin, or octyl glucoside are also appropriate for extraction of native proteins (*UNIT 5.1*). Some of these detergents (e.g., digitonin) preserve weak protein-protein interactions better than Triton X-100. If the antigen is part of a complex that is insoluble in nondenaturing detergents (e.g., cytoskeletal structures, chromatin, membrane "rafts") or if the epitope is hidden within the folded structure of the protein, extraction under denaturing conditions is indicated (see Alternate Protocol 2).

The number of cells necessary to detect an immunoprecipitated antigen depends on the cellular abundance of the antigen and on the efficiency of radiolabeling. The protocols for radiolabeling (*UNIT 7.1*) and immunoprecipitation described in this book are appropriate for detection of antigens that are present at low to moderate levels (10,000 to 100,000 copies per cell), as is the case for most endogenous integral membrane proteins, signal transduction proteins, and transcription factors. For more abundant antigens, such as cytoskeletal and secretory proteins or proteins that are expressed by viral infection or transfection, the quantity of radiolabeled cells used in the immunoprecipitation can be reduced accordingly.

Production of antibodies

Immunoprecipitation can be carried out using either polyclonal or monoclonal antibodies (see discussion of selection below). Polyclonal antibodies are most often prepared by immunizing rabbits, although polyclonal antibodies produced in mice, guinea pigs, goats, sheep, and other animals, are also suitable for immunoprecipitation. Antigens used for polyclonal antibody production can be whole proteins purified from cells or tissues, or can be whole or partial proteins produced in bacteria or insect cells by recombinant DNA procedures. Another useful procedure is to immunize animals with peptides conjugated to a carrier protein. Production of polyclonal antibodies to recombinant proteins and peptides has become the most commonly used approach to obtain specific probes for immunoprecipitation and other immunochemical techniques, because it does not require purification of protein antigens from their native sources. The only requirement for making these antibodies is knowledge of the

sequence of a protein, information which is now relatively easy to obtain as a result of cDNA and genomic DNA sequencing projects. Polyclonal antibodies can be used for immunoprecipitation as whole serum, ammonium sulfate–precipitated immunoglobulin fractions, or affinity-purified immunoglobulins. Although all of these forms are suitable for immunoprecipitation, affinity-purified antibodies often give lower backgrounds and are more specific.

Most monoclonal antibodies are produced in mice or rats. The sources of antigen for monoclonal antibody production are the same as those for production of polyclonal antibodies, namely, proteins isolated from cells or tissues, recombinant proteins or protein fragments, and peptides. A significant advantage of monoclonal antibodies is that antigens do not need to be purified to serve as immunogens, as long as the screening method is specific for the antigen. Another advantage is the unlimited supply of monoclonal antibodies afforded by the ability to grow hybridomas in culture or in ascitic fluid. Many monoclonal antibodies can now be produced from hybridomas deposited in cell banks or are directly available from companies. Ascitic fluid, cell culture supernatant, and purified antibodies are all suitable sources of monoclonal antibodies for immunoprecipitation. Ascitic fluid and purified antibodies should be used when a high antibody titer is important. Cell culture supernatants have lower antibody titers, but tend to give cleaner immunoprecipitations than ascitic fluids due to the lack of contaminating antibodies.

Selection of antibodies: Polyclonal versus monoclonal

What type of antibody is best for immunoprecipitation? There is no simple answer to this question, as the outcome of both polyclonal and monoclonal antibody production protocols is still difficult to predict. Polyclonal antibodies to whole proteins (native or recombinant) have the advantage that they frequently recognize multiple epitopes on the target antigen, enabling them to generate large, multivalent immune complexes. Formation of these antigen-antibody networks enhances the avidity of the interactions and increases the efficiency of immunoprecipitation. Because these antibodies recognize several epitopes, there is a better chance that at least one epitope will be exposed on the surface of a solubilized protein and thus be available for interaction with antibodies. Thus, the likelihood of success is higher. These properties can be a disadvantage, though, as some polyvalent antibodies can cross-react with epitopes on other proteins, resulting in higher backgrounds and possible misidentification of antigens. By being directed to a short peptide sequence, anti-peptide polyclonal antibodies are less likely to cross-react with other proteins. However, their usefulness is dependent on whether the chosen sequence turns out to be a good immunogen in practice, as well as on whether this particular epitope is available for interaction with the antibody under the conditions used for immunoprecipitation.

Unfractionated antisera are often suitable for immunoprecipitation. However, there is a risk that serum proteins other than the antibody will bind nonspecifically to the immunoadsorbent, and in turn bind proteins in the lysate that are unrelated to the antigen. For instance, transferrin can bind nonspecifically to immunoadsorbents, potentially leading to the isolation of the transferrin receptor as a contaminant (Harford, 1984). Polyclonal antisera can also contain antibodies to other antigens (e.g., viruses, bacteria) to which the animal may have been exposed, and these antibodies can also cross-react with cellular proteins during immunoprecipitation. Affinity-purified antibodies are a better alternative when antisera do not yield clean immunoprecipitations. Affinity-purification can lead to loss of high-affinity or low-affinity antibodies; however, the higher specificity of affinity-purified antibodies generally makes them "cleaner" reagents for immunoprecipitation.

The specificity, high titer, and limitless supply of the best immunoprecipitating monoclonal antibodies are unmatched by those of polyclonal antibodies. However, not all monoclonal antibodies are useful for immunoprecipitation. Low-affinity monoclonal antibodies can perform acceptably in immunofluorescence microscopy protocols (*UNIT 4.3*) but may not be capable of holding on to the antigen during the repeated washes required in immunoprecipitation protocols. The use of ascitic fluid has the same potential pitfalls as the use of polyclonal antisera, as ascites may also contain endogenous antibodies to other antigens and proteins such as transferrin that can bind to other proteins in the lysate.

In conclusion, an informed empirical approach is recommended in order to select the best antibody for immunoprecipitation. In general, it is advisable to generate and/or test several antibodies to a particular antigen in order to find at least one that will perform well in immunoprecipitation protocols.

Antibody titer

The importance of using the right amount of antibody for immunoprecipitation cannot be overemphasized. This is especially the case for quantitative immunoprecipitation studies, in which the antibody should be in excess of the specific antigen. For instance, in pulse-chase analyses of protein degradation or secretion (*UNIT 7.1*), it is critical to use sufficient antibody to deplete the antigen from the cell lysate. This is particularly important for antigens that are expressed at high levels, a common occurrence with the growing use of high-yield protein expression systems such as vaccinia virus or replicating plasmids in COS cells. Consider for example a protein that is expressed at high levels inside the cell, and of which only a small fraction is secreted into the medium. If limiting amounts of antibody are used in a pulse-chase analysis of this protein, the proportion of protein secreted into the medium will be grossly overestimated, because the limiting antibody will bind only a small proportion of the cell-associated protein and a much higher proportion of the secreted protein. The same considerations apply to degradation studies. Thus, it is extremely important in quantitative studies to ensure that the antibody is in excess of the antigen in the cell samples. This can be ascertained by performing sequential immunoprecipitations of the samples (see Basic Protocol 1, annotation to step 21). If the second immunoprecipitation yields only a small amount of the antigen relative to that isolated in the first immunoprecipitation (<10%), then the antibody titer is appropriate. If, on the other hand, the amount of antigen isolated in the second immunoprecipitation is >10%, either more antibody or less antigen should be used.

Too much antibody can also be a problem, as nonspecific immunoprecipitation tends to increase with increasing amounts of immunoglobulins bound to the beads. Thus, titration of the antibody used for immunoprecipitation is strongly advised.

Immunoadsorbent

If cost is not an overriding issue, the use of protein A– or protein G–agarose is recommended for routine immunoprecipitation. Protein A– or protein G–agarose beads (or equivalent products) have a very high capacity for antibody binding (up to 10 to 20 mg of antibody per milliliter of gel). Both protein A and protein G bind a wide range of immunoglobulins (Table 7.2.1). Backgrounds from nonspecifically bound proteins are generally low. Protein A– and protein G–agarose beads are also stable and easy to sediment by low-speed centrifugation. A potential disadvantage, in addition to their cost, is that some polyclonal or monoclonal antibodies bind weakly or not at all to protein A or protein G (Table 7.2.1). This problem can be solved by using an intermediate rabbit antibody to the immunoglobulin of interest. For example, a goat polyclonal antibody can be indirectly bound to protein A–agarose by first incubating the protein A–agarose beads with a rabbit anti-goat immunoglobulin, and then incubating the beads with the goat polyclonal antibody. Anti-immunoglobulin antibodies (e.g., rabbit anti–goat immunoglobulins) coupled covalently to agarose can also be used for indirect immunoprecipitation in place of protein A– or protein G–agarose.

Fixed *Staphylococcus aureus* particles (Pansorbin) can be used as a less expensive alternative to protein A–agarose. They have a lower capacity, can give higher backgrounds, and take longer to sediment. However, they work quite well in many cases. In order to establish if they are appropriate for a particular experimental setup, conduct a preliminary comparison of the efficiency of protein A–agarose with *Staphylococcus aureus* particles as immunoadsorbent.

Specific antibodies coupled covalently to various affinity matrices can also be used for direct immunoprecipitation of antigens. After binding to protein A–agarose, antibodies can be cross-linked with dimethylpimelimidate (Gersten and Marchalonis, 1978). Purified antibodies can also be coupled directly to derivatized matrices such as CNBr-activated Sepharose (Springer, 1996). This latter approach avoids having to bind the antibody to protein A–agarose. Covalently bound antibodies should be used when elution of immunoglobulins from the beads complicates further analyses of the complexes. This is the case when proteins in immunoprecipitates are analyzed by one- or two-dimensional gel electrophoresis (*UNIT 6.1*) followed by Coomassie blue or silver staining, or are used for microsequencing. Also, the released immunoglobulins could interfere with detection of some antigens by immunoblotting (*UNIT 6.2*) following immunoprecipitation.

Nonspecific controls

For correct interpretation of immunoprecipitation results, it is critical to include appropriate nonspecific controls along with the specific samples. One type of control consists of setting up an incubation with an irrelevant antibody in the same biochemical form as the

experimental antibody (e.g., serum, ascites, affinity-purified immunoglobulin, antibody bound to protein A–agarose or directly conjugated to agarose), and belonging to the same species and immunoglobulin subclass as the experimental antibody (e.g., rabbit antiserum, mouse IgG2a). For an antiserum, the best control is preimmune serum (serum from the same animal obtained before immunization). Nonimmune serum from the same species is an acceptable substitute for preimmune serum in some cases. "No-antibody" controls are not appropriate because they do not account for nonspecific binding of proteins to immunoglobulins. In immunoprecipitation-recapture experiments, control immunoprecipitations with irrelevant antibodies should be performed for both the first and second immunoprecipitation steps (Fig. 7.2.3). Another type of control is to perform an immunoprecipitation from cells that do not express a specific antigen in parallel with immunoprecipitation of the antigen-expressing cells. For instance, untransfected cells are a perfect control for transfected cells. In yeast cells, null mutants that do not express a specific antigen are an ideal control for wild-type cells.

Order of stages

In the immunoprecipitation protocols described in this unit, the antibody is prebound to protein A–agarose before addition to the cell lysate containing the antigen. This differs from other protocols in which the free antibody is first added to the lysate and the antigen-antibody complexes are then collected by addition of the immunoadsorbent. Although both procedures can give good results, the authors prefer the protocols described here because this method allows better control of the amount of antibody bound to the immunoadsorbent. Prebinding antibodies to the immunoadsorbent beads allows removal of unbound antibodies. The presence of unbound antibodies in the incubation mixture could otherwise result in decreased recovery of the antigen on the immunoadsorbent beads. Another advantage of the prebinding procedure is that most proteins other than the immunoglobulin in the antibody sample (e.g., serum proteins) are removed from the beads and do not come in contact with the cell lysate. This eliminates potential adverse effects of these proteins on isolation of the antigen.

Washing

The five washes described (see Basic Protocol 1; four with wash buffer and one with PBS) are sufficient for maximal removal of unbound proteins; additional washes are unlikely to decrease the background any further. The last wash with PBS removes the Triton X-100 that can lead to decreased resolution on SDS-PAGE. It also removes other components of the wash buffer that could interfere with enzymatic treatment of immunoprecipitates. It is not advisable to complete all the washes quickly (e.g., in 5 min), because this may not allow enough time for included proteins to diffuse out of the gel matrix. Instead, beads should be washed over ~30 min, which may require keeping the samples on ice for periods of 3 to 5 min between washes. In order to reduce nonspecific bands, samples can be subjected to an additional wash with wash buffer containing 0.1% (w/v) SDS, or with a mixture of 0.1% (w/v) SDS and 0.1% (w/v) sodium deoxycholate (Fig. 7.2.4). This wash should be done between the fourth wash and the wash with PBS.

Troubleshooting

Two of the most common problems encountered in immunoprecipitation of metabolically labeled proteins are failure to detect specific antigens in the immunoprecipitates, and high background of nonspecifically bound proteins for antigens that were radiolabeled in vivo and analyzed by SDS-PAGE (*UNIT 6.1*) followed by autoradiography or fluorography (*UNIT 6.3*). When immunoprecipitates are analyzed by immunoblotting (*UNIT 6.2*), an additional problem may be the detection of immunoprecipitating antibody bands in the blots (Table 7.2.2).

Anticipated Results

For antigens that are present at >10,000 copies per cell, the radiolabeling and immunoprecipitation protocols described in this book would be expected to result in the detection of one or more bands corresponding to the specific antigen and associated proteins in the electrophoretograms. Specific bands should not be present in control immunoprecipitations done with irrelevant antibodies. If antigens are labeled with [^{35}S]methionine (*UNIT 7.1*), specific bands should be visible within 2 hr to 2 months of exposure. Due to the relatively low yield of the immunoprecipitation-recapture procedure (<10% of that of a single immunoprecipitation step), detection of specific bands is likely to require longer exposure times. This may turn out to be problematic due to the radioactive decay of ^{35}S (half-life = 88 days). In immunoprecipitation-recapture experiments in which the antibodies used for the first and second

immunoprecipitation steps recognize different antigens (e.g., for the study of protein-protein interactions; Fig. 7.2.2), it is advisable to include in the second immunoprecipitation step a positive control with either the antibody used for the first immunoprecipitation (if the antibody recognizes both the native and denatured forms of the antigen) or a different antibody with specificity for the same antigen (Fig. 7.2.3, lane 3).

Time Considerations

Preparation of cell extracts (Basic Protocol 1 and Alternate Protocols 1 to 4) takes 1 to 3 hr to complete, and isolation of the antigen on antibody-conjugated beads takes 2 to 3 hr. Binding antibodies to immunoadsorbent beads can be done prior to or simultaneously with preparation of the cell extracts and also takes 1 to 3 hr. Therefore, the whole immunoprecipitation procedure can be completed in 1 day. Immunoprecipitates can be analyzed immediately (e.g., resolved by SDS-PAGE) or frozen and analyzed another day. Immunoprecipitation-recapture experiments (Basic Protocol 2) require an additional 1 to 2 hr to denature and prepare the antigen for immunoprecipitation, and 2 to 3 hr to isolate the antigen. Completion

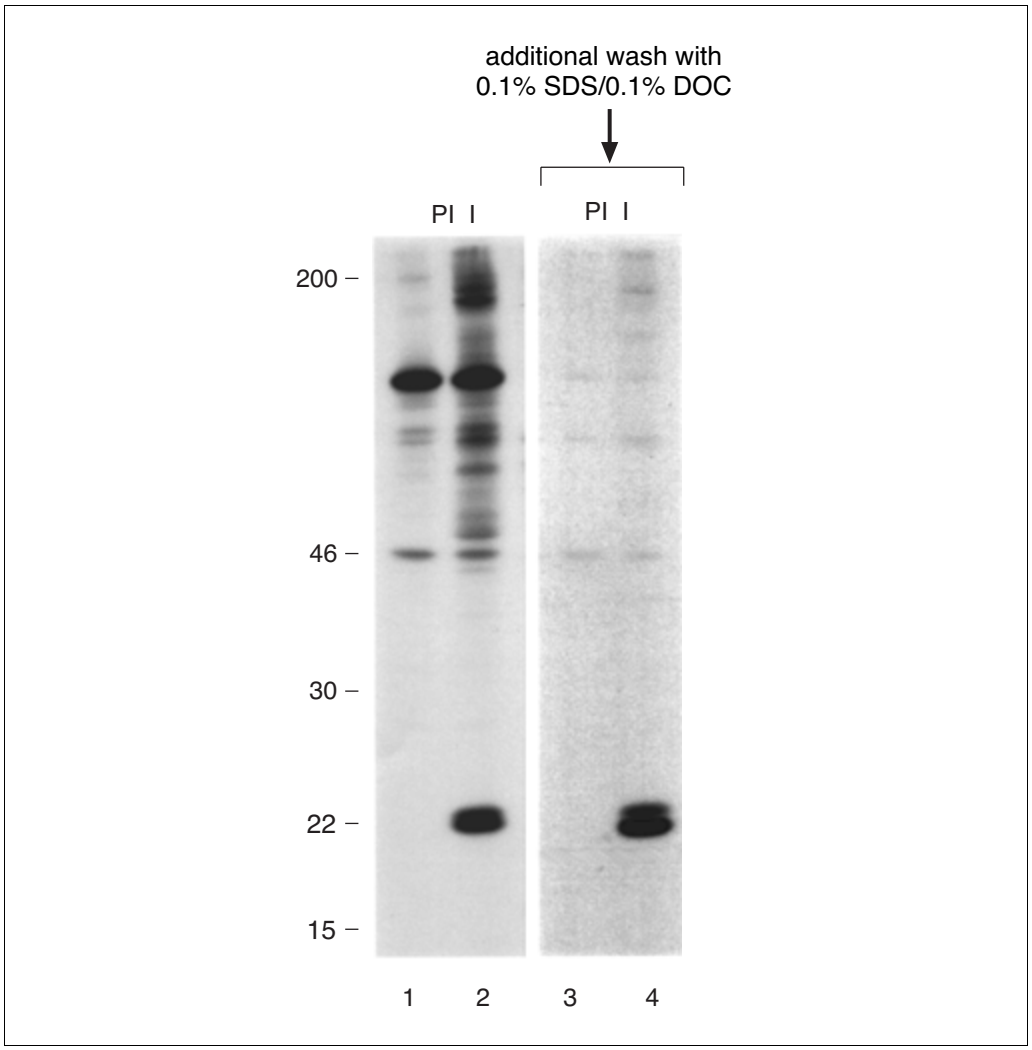


Figure 7.2.4 Lowering background by washing with SDS and sodium deoxycholate (DOC). In this experiment, BW5147 cells (mouse thymoma) labeled with [^{35}S]methionine for 1 hr were extracted with nondenaturing lysis buffer (see Basic Protocol 1). The extracts were subjected to immunoprecipitation with protein A–agarose beads incubated with either preimmune (PI) or immune (I) serum from a rabbit immunized with the ribosomal protein L17 (doublet at M_r ~22,000). Lanes 1 and 2 correspond to immunoprecipitates obtained using the protocols described in this unit. Notice the presence of nonspecific bands and/or associated proteins in lane 2. Lanes 3 and 4 correspond to beads that were washed an additional time with 0.1% (w/v) SDS and 0.1% (w/v) DOC. Notice the disappearance of most of the nonspecific bands and/or associated proteins. The positions of M_r standards (expressed as $10^{-3} \times M_r$) are shown at left.

Table 7.2.2 Troubleshooting Guide for Immunoprecipitation

Problem	Cause	Solution
No specific radiolabeled antigen band		
Gel is completely blank after prolonged autoradiographic exposure	Poorly labeled cells: too little radiolabeled precursor, too few cells labeled, lysis/loss of cells during labeling, too much cold amino acid in labeling mix, wrong labeling temperature	Check incorporation of label by TCA precipitation (*UNIT 7.1*); troubleshoot the labeling procedure
Only nonspecific bands present	Antigen does not contain the amino acid used for labeling	Label cells with another radiolabeled amino acid, or for glycoproteins, with tritiated sugar
	Antigen expressed at very low levels	Substitute cells known to express higher levels of antigens as detected by other methods; transfect cells for higher expression
	Protein has high turnover rate and is not well labeled by long-term labeling	Use pulse labeling
	Protein has a low turnover rate and is not well labeled by short-term labeling	Use long-term labeling
	Protein is not extracted by lysis buffer used to solubilize cells	Solublize with a different nondenaturing detergent or under denaturing conditions
	Antigen is not extracted with Triton X-100 at 4°C	Extract with Triton X-100 at 37°C or use another detergent
	Antibody is nonprecipitating	Identify and use antibody that precipitates antigen
	Epitope is not exposed in native antigen	Extract cells under denaturing conditions
	Antibody does not recognize denatured antigen	Extract cells under nondenaturing conditions
	Antibody does not bind to immunoadsorbent	Use a different immunoadsorbent (Table 7.2.1); use intermediate antibody
	Antigen is degraded during immunoprecipitation	Ensure that fresh protease inhibitors are present
High background of nonspecific bands		
Isolated lanes on gel with high background	Random carryover of detergent-insoluble proteins	Remove supernatant immediately after centrifugation, leaving a small amount with pellet; if resuspension occurs, recentrifuge
High background in all lanes	Incomplete washing	Cap tubes and invert several times during washes
	Poorly radiolabeled protein	Optimize duration of labeling to maximize signal-to-noise
	Incomplete removal of detergent-insoluble proteins	Centrifuge lysate 1 hr at 100,000 × *g*
	Insufficient unlabeled protein to quench nonspecific binding	Increase concentration of BSA

continued

Table 7.2.2 Troubleshooting Guide for Immunoprecipitation, continued

Problem	Cause	Solution
	Antibody contains aggregates	Microcentrifuge antibody 15 min at maximum speed before binding to beads
	Antibody solution contains nonspecific antibodies	Use affinity-purified antibodies; absorb antibody with acetone extract of cultured cells that do not express antigen; for yeast cells, absorb antibody with null mutant cells
	Too much antibody	Use less antibody
	Incomplete preclearing	Preclear with irrelevant antibody of same species of origin and immunoglobulin subclass bound to immunoadsorbent
	Nonspecifically immunoprecipitated proteins	Fractionate cell lysate (e.g, ammonium sulfate precipitation, lectin absorption, or gel filtration) prior to immunoprecipitation; after washes in wash buffer, wash beads once with 0.1% SDS in wash buffer or 0.1% SDS/0.1% sodium deoxycholate
Immunoprecipitating antibody detected in immunoblots		
Complete immunoglobulin or heavy and/or light chains visible in immunoblot	Protein A conjugate or secondary antibody recognizes immunoprecipitating antibody	Use antibody coupled covalently to solid-phase matrix for immunoprecipitation; probe blots with primary antibody from a different species and the appropriate secondary antibody specific for immunoblotting primary antibody

of an entire immunoprecipitation-recapture experiment requires a very long workday. Alternatively, samples can be frozen after the first immunoprecipitation, and the elution and recapture can be carried out another day.

Literature Cited

Franzusoff, A., Rothblatt, J., and Schekman, R. 1991. Analysis of polypeptide transit through yeast secretory pathway. *Methods Enzymol.* 194:662-674.

Gersten, D.M. and Marchalonis, J.J. 1978. A rapid, novel method for the solid-phase derivatization of IgG antibodies for immune-affinity chromatography. *J. Immunol. Methods* 24:305-309.

Harford, J. 1984. An artefact explains the apparent association of the transferrin receptor with a ras gene product. *Nature* 311:673-675.

Harlow, E. and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Irving, R.A., Hudson, P.J., and Goding, J.W. 1996. Construction, screening and expression of recombinant antibodies. *In* Monoclonal Antibodies: Principles and Practice (J.W. Goding, ed.). Academic Press, London.

Köhler, G. and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predetermined specificity. *Nature* 256:495-497.

Nisonoff, A. 1984. Introduction to Molecular Immunology. Sinauer Associates, Sunderland, Mass.

Rapley, R. 1995. The biotechnology and applications of antibody engineering. *Mol. Biotech.* 3:139-154.

Springer, T.A. 1996. Immunoprecipitation. *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeck, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 8.3.1-8.3.11. John Wiley & Sons, New York.

Contributed by Juan S. Bonifacino and Esteban C. Dell'Angelica
National Institute of Child Health and Human Development
Bethesda, Maryland

Metabolic Labeling with Sulfate

Many newly synthesized secretory or transmembrane proteins are posttranslationally modified as they traverse the secretory pathway (*UNIT 15.1*). Posttranslational modifications can be exploited to determine where a protein normally resides in a cell or to follow its transport through the cell. One of these modifications is the addition of sulfate either to the protein backbone on tyrosine residues or to carbohydrate side chains. Sulfation of proteins on tyrosine occurs in the lumen of the *trans*-Golgi network (TGN; Niehrs and Huttner, 1990) through the action of the enzyme tyrosylprotein sulfotransferase (TPST). Sulfation of carbohydrate side chains on proteins also occurs in the later compartments of the Golgi complex, mediated by a variety of sulfotransferases (for recent review see Bowman and Bertozzi, 1999). The xylose side chains of proteoglycans can also be sulfated. Thus, sulfation can potentially be used to study the transport of a variety of molecules as long as they (1) are substrates for the sulfotransferases and (2) are resident in or pass through the compartment(s) that contains the sulfotransferases.

Incorporation of radioactive sulfate into sulfated proteins and proteoglycans is a useful tool to study protein/proteoglycan transport from the Golgi complex. It provides greater resolution of the kinetics of transport than does metabolic labeling in the endoplasmic reticulum using radiolabeled amino acids (see *UNIT 7.1*). This unit describes how to label mammalian cells in culture with radioactive sulfate to determine whether the protein or proteoglycan of interest is a substrate for sulfotransferases and can be labeled with [^{35}S]sulfate.

This unit describes a long-term labeling protocol for adherent monolayer cells in culture (see Basic Protocol 1), which ensures that a high specific activity is achieved for subsequent analysis. Modification of this protocol to provide a pulse-chase protocol (see Basic Protocol 2) is achieved by shorter labeling times (the pulse), followed by a quenching of the label with excess cold sulfate (the chase).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*)

NOTE: All solutions used for the labeling protocols are tissue culture–grade reagents. Sterility of the media, label, and cultures is important for long-term labeling procedures.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

LONG-TERM [^{35}S]SULFATE LABELING OF MONOLAYER CELLS IN CULTURE

BASIC PROTOCOL 1

The most useful way to initially determine if the molecule of interest is sulfated is to label the cells to steady state using a lengthy labeling period—e.g., a time equivalent to at least one cell cycle period. This ensures that all the proteins within the cell that can be labeled with sulfate are labeled and provides the highest signal for starting the analysis. Cell cultures ~70% to 80% confluent are required along with the appropriate labeling medium and CO_2 incubators. All washing steps are performed at room temperature in a sterile cabinet.

Contributed by Sharon A. Tooze
Current Protocols in Cell Biology (1999) 7.3.1-7.3.7

Materials

Dialyzed serum (see recipe)
200 mM L-glutamine in H_2O
Sulfate-free DMEM (see recipe)
Sodium [^{35}S]sulfate (>1000 Ci/mmol)
Cells of interest, cultured to 70% to 80% confluence
DPBS (*APPENDIX 2A*), 4°C

Additional reagents and equipment for immunoprecipitation of proteins (*UNIT 7.2*) or for SDS-PAGE (*UNIT 6.1*) and fluorography (*UNIT 6.3*)

NOTE: The labeling medium is a modified formula for DMEM, which the author has found works best. Other media, such as RPMI, have not been used as successfully.

NOTE: All solutions for overnight labeling should be prepared and used under aseptic conditions.

NOTE: The size of the cultures used depends on the amount of the protein of interest present in the cells and the techniques used for subsequent analysis.

1. Prepare depletion medium by adding one-tenth the normal concentration of dialyzed serum and 4 mM glutamine to sulfate-free DMEM.

   Prepare enough medium to wash cultures once and for the addition of label. The amount of wash and labeling medium required depends on the size of the culture. Typically a 35-mm-diameter dish requires 2.0 ml and a 90-mm-diameter dish requires 10 ml.

   The addition of glutamine before each experiment allows the sulfate-free DMEM to be stored up to 6 months.

2. Prepare labeling medium by removing the appropriate amount of depletion medium to a plastic tube and adding sodium [^{35}S]sulfate to 0.2 to 0.5 mCi/ml.
3. Warm both media to 37°C in a water bath.
4. Gently wash cells once with depletion medium. Remove wash thoroughly and add labeling medium.
5. Ensure that cells are well covered, return them to the CO_2 incubator, and incubate overnight (≥16 hr).
6. Remove dishes from incubator and place on ice. Remove labeling medium and save.
7. Wash cells three times with ice-cold DPBS
8. Lyse in an appropriate solution and analyze by immunoprecipitation (*UNIT 7.2*) followed by SDS-PAGE (*UNIT 6.1*) and fluorography (*UNIT 6.3*). Use a lysis buffer that is optimal for solubilizing the cells and protein of interest.

   If the protein or proteoglycan of interest is secreted it maybe possible to detect it in the labeling medium. Adjust the medium with salts, buffer, and detergent to that present in the lysis conditions used for immunoprecipitation and proceed as in UNIT 7.2.

   After such a labeling experiment, it is worthwhile analyzing the total lysate and an aliquot of the medium by SDS-PAGE (UNIT 6.1). The molecule of interest may be highly sulfated and readily detectable without prior enrichment, even if it is a relatively minor fraction of the total protein.

PULSE-CHASE [^{35}S]SULFATE LABELING OF MONOLAYER CELLS IN CULTURE

Once the relative abundance of a protein is known and it can be easily detected after an overnight labeling with [^{35}S]sulfate, it is possible to use the radioactive sulfate in a pulse-chase protocol. This is done by labeling the protein for a short period of time (e.g., a 4- to 5-min pulse). The short time period is required to ensure that the labeled protein is still in the TGN. Further incubation (the chase) is done at 37°C after the addition of excess unlabeled sulfate to stop the incorporation of radioactive sulfate. The chase can be carried out for any length of time; the position of the radiolabeled proteins in the cell will be a function of the chase time. This procedure is invaluable if the protein or proteoglycan is thought to be secreted. If the protein or proteoglycan is thought to localize to a post-Golgi compartment, the protocol can be combined with cell fractionation to identify the intracellular compartment. The very short pulse provides a very accurate starting point for the analysis, and the kinetics of transport or secretion of the protein can be easily obtained.

Depending on the experiment it will be necessary to prepare several identical cultures: a dish of cells is required for each time point, including a zero time point, which is defined as the end of the pulse. A typical time course may have five or six chase time points (for example, 5, 10, 20, 40, and 80 min).

Materials

Cultured cells of interest, ~70% to 80% confluent
Dialyzed serum (see recipe)
200 mM L-glutamine in H_2O
Sulfate-free DMEM (see recipe)
Sodium [^{35}S]sulfate (>1000 Ci/mmol)
DPBS (*APPENDIX 2A*), 4°C
Chase medium (see recipe)
Platform rocker

Additional reagents and equipment for immunoprecipitation of proteins (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), and fluorography (*UNIT 6.3*)

1. Label dishes of cultured cells on bottom with appropriate time or sample number and organize them on an easily movable tray. Return to incubator.

   It is important to be organized before the experiment begins as the manipulations must be carried out as quickly as possible during the time course.

2. Prepare ~4 vol depletion medium for each culture dish by adding dialyzed serum to 1/100th the normal concentration and 4 mM glutamine to sulfate-free DMEM.

   For each time point, enough depletion medium will be needed to make the labeling medium, wash each culture twice, and incubate once for the depletion step. For washes and depletion, use 2.0 ml for a 35-mm-diameter dish and 10 ml for a 90-mm-diameter dish. For labeling medium, use 1.0 ml for a 35-mm dish and 2.5 ml for a 90-mm dish. A reduced volume of labeling medium is used to save on the cost of the label.

   The addition of glutamine before each experiment allows the sulfate-free DMEM to be stored up to 6 months.

3. Prepare the labeling medium required for all dishes as one batch. Transfer the amount required for each dish, multiplied by the number of dishes, to a plastic tube and add [^{35}S]sulfate to give 5 mCi/ml.

4. Warm both media to 37°C in a water bath.

5. Gently wash all dishes twice with depletion medium. Thoroughly remove second wash and add another aliquot of depletion medium. Incubate 20 min in the incubator.

   The depletion step reduces the intracellular pool of sulfate to allow the highest specific activity of incorporated radioactive sulfate to be achieved.

6. Remove depletion medium from all dishes and quickly add equal aliquots of labeling medium to each culture. Start timer. Ensure that cells are well covered, place on a platform rocker in the 37°C CO_2 incubator, and incubate for 4 min.

   The culture dishes should be rocked gently during the labeling period as the volume of medium used is not sufficient to cover cells.

   This 4-min incubation is effectively a 2-min pulse because 2 min is required for the [^{35}S]sulfate to be converted to the activated sulfate donor (PAPS, see Commentary).

7. Remove all dishes from incubator and quickly place the time-zero dish on ice. Remove labeling medium from the time-zero dish and save. Add ice-cold DPBS to that dish and keep on ice. Remove label from remaining dishes at room temperature, discard, add chase medium at twice the volume of the labeling medium, and return to the incubator but do not rock.

8. At predetermined time points, remove the appropriate dish from the incubator, place immediately on ice, remove and save medium, and add ice-cold DPBS. Keep dishes on ice. Repeat until all the dishes are on ice and the chase protocol is complete.

9. Wash all dishes three times with ice-cold DPBS.

10. Lyse in an appropriate solution and immunoprecipitate (*UNIT 7.2*). Use a lysis buffer that is optimal for solubilizing the cells and protein of interest.

11. If the protein/proteoglycan of interest is secreted, adjust the medium saved from the zero time point and each chase time point by adding salts, buffer, and detergent to the conditions required for immunoprecipitation, and proceed as in *UNIT 7.2*.

    CAUTION: *Remember that the time-zero medium contains the radioactive-sulfate solution and should be treated with appropriate precautions.*

12. Analyze immunoprecipitates by SDS-PAGE (*UNIT 6.1*) and fluorography (*UNIT 6.3*).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Chase medium

Use Dulbecco's minimal essential medium (DMEM; as purchased from supplier) or another growth medium. Use $^1/_{100}$th the normal serum concentrations to avoid problems with serum proteins during the analysis. Add 160 mM Na_2SO_4 (filter sterilized through a 0.2-µm filter) to a final concentration of 1.6 mM. Prepare fresh for each experiment.

Dialyzed serum

Dialyze serum overnight against DPBS (*APPENDIX 2A*) at 4°C. Use 100 times the serum volume for the dialysis, and change the solution once during dialysis. Sterile filter after dialysis and store in 1- or 2-ml aliquots in sterile tubes for up to 1 year at −20°C. After thawing, store dialyzed serum up to 1 month at 4°C.

Low–Met/Cys amino acid mixture, 10×

For 1 liter:

0.84 g L-arginine·HCl
0.063 g L-cystine
0.30 g glycine
0.42 g L-histidine·HCl
1.05 g L-isoleucine
1.05 g L-leucine
1.46 g L-lysine·HCl
0.03 g L-methionine
0.66 g L-phenylalanine
0.42 g L-serine
0.95 g L-threonine
0.16 g L-tryptophan
0.94 g L-valine
Mix into sterile Milli-Q-purified water and adjust volume to 1 liter
Store up to 6 months at −20°C

Having low methionine and cystine improves the sulfate incorporation by reducing the amount of sulfated essential amino acids that can be broken down, thereby diluting the radioactive pool of sulfate. Methionine and cystine at one-tenth the normal amount is usually sufficient to maintain protein synthesis and keep the cells healthy.

Tyrosine is added to the dilute sulfate-free DMEM because it is insoluble at 10× the normal concentration.

Do not add antibiotics as some (e.g., streptomycin) are sulfate salts.

Sulfate-free Dulbecco's minimal essential medium (DMEM)

For one liter:

265.00 mg $CaCl_2 \cdot 2H_2O$
0.10 mg $Fe(NO_3)_3 \cdot 9H_2O$
400.00 mg KCl
168.75 mg $MgCl_2 \cdot 6H_2O$
6400.00 mg NaCl
125.00 mg $NaH_2PO_4 \cdot H_2O$
5 ml 20% (w/v) glucose
10 ml 11.0 mg/ml sodium pyruvate, added dropwise
3 ml 0.5% (w/v) phenol red
49.3 ml 7.5% (w/v) $NaHCO_3$
72.0 mg tyrosine
40 ml 100× MEM vitamins (Sigma)
100 ml 10× low–Met/Cys amino acid mixture (see recipe)

Combine all ingredients except phenol red in 900 ml Milli-Q-purified water, cover, and stir to dissolve for several hours at room temperature or overnight at 4°C. Add phenol red and adjust pH to 7.2 with 1 M NaOH. Adjust volume to 1 liter. Sterile filter with 0.2-µm filter. Store up to 6 months at 4°C in the dark.

Use plastic containers or glassware that have not been washed with detergent to prepare media.

Sodium pyruvate is added dropwise to avoid precipitation.

COMMENTARY

Background Information

Posttranslational modifications of proteins and proteoglycans can be exploited to follow the route of the modified molecules through the secretory pathway. Sulfation on both carbohydrates and tyrosine residues is particularly useful for studying events in the later compartments of the secretory pathway—i.e., those after the *trans*-Golgi—and to study secretion. A short pulse of sulfate (5 min or less) can be used to label the proteins or proteoglycans in the compartment where the sulfotransferase is resident. Incubation with excess sulfate starts the chase of the sulfated molecules out of the Golgi complex to their final destination. Thus, the major advantage of using sulfation to follow proteins through the cell is the ability to label the molecules late in the secretory pathway, providing a sharply defined cohort of labeled molecules as the starting point for subsequent analysis.

Sulfation of carbohydrate chains on proteins and proteoglycans in the lumen of the Golgi complex is catalyzed by a variety of different sulfotransferases, some of which are only known as activities, while others have been extensively characterized, cloned, and sequenced. A co-substrate for the sulfotransferases is 3′-phosphoadenosine-5′-phosphosulfate (PAPS), which is synthesized in the cytosol and translocated across the Golgi membrane (for a comprehensive explanation see Huttner and Baeuerle, 1988). Sulfation of carbohydrates on proteins can occur on the N- or O-glycan chains (see *UNIT 15.2*), while sulfation of proteoglycans occurs on the glycosaminoglycan moiety. It is possible to determine the nature of the sulfated N- or O-glycan by using a variety of endoglycosidases that cleave the N- or O-linked oligosaccharide chains, such as endoglycosidase H (endo H; see *UNIT15.2*). There are several classes of sulfated proteoglycans, the most common being keratan sulfate, heparan sulfate, heparin, chrondroitin sulfate, and dermatan sulfate. Determining whether a sulfated molecule is a proteoglycan is more complex, as there is a limited range of available endoglycosidases, heparinase being the most commonly used. For the best methods to approach the analysis of more complex sulfated molecules, see Linhardt (1994).

The analysis of sulfated tyrosine residues in the protein backbone is more tractable. Posttranslational modification by sulfation on tyrosine residues is catalyzed by the enzyme tyrosylprotein sulfotransferase (Beisswanger et al., 1998) and occurs by transfer of the activated donor PAPS to the tyrosine residue. Sulfation of tyrosine residues, although widespread, is highly selective. The consensus features of the substrate recognition sequence in the protein backbone have been defined (Huttner and Baeuerle, 1988), allowing one to predict whether the protein of interest may be sulfated. If it is likely that the protein is sulfated on tyrosine residues, this can be confirmed by first demonstrating that the sulfated protein is resistant to endoglycosidases, and then chemically treating the protein with $Ba(OH)_2$, which allows the identification of Tyr-SO_4 by thin-layer chromatography (Huttner, 1984). A recent paper elegantly demonstrated just this approach (Farzan et al., 1999). This paper also demonstrated the usefulness of the drug chlorate, which inhibits the first enzyme (ATP-sulfurylase) in the biosynthesis of PAPS, and thereby inhibits the sulfation of both proteins and proteoglycans (Baeuerle and Huttner, 1986).

An interesting application of these approaches is to use the tyrosine sulfation consensus sequence to insert a sulfation site as a method of tagging secretory proteins to follow their movement out of the Golgi complex. This is particularly useful if the protein is not posttranslationally modified by N- or O-linked glycosylation. An example of this approach was pioneered by Niehrs et al. (1992).

Critical Parameters and Troubleshooting

The most important consideration is optimization of sulfate incorporation into the molecule to be analyzed. The efficiency of sulfation is the most variable parameter and relies on maintaining the cells in a medium that is compatible with continued protein synthesis and with sulfate labeling conditions. During the incubation with [^{35}S]sulfate, a series of reactions in the cytosol converts the [^{35}S]sulfate into [^{35}S]PAPS. Thus, it is important to reduce the concentration of endogenous PAPS to increase the specific activity of the radiolabeled PAPS. In some cell types this can be done easily by preincubation with sulfate-free medium, and is enhanced by reducing the methionine and cystine (Yanigashita et al., 1989).

If the suggested medium (DMEM) is not compatible with the cell types being used, it is possible to substitute other media prepared without sulfate, or even to substitute DPBS for

the labeling medium. However, if the suggested labeling medium is not suitable, pilot experiments should be done on total extracts to test the efficiency of [^{35}S]sulfate incorporation. It has been observed that some media (such as RPMI) do not allow efficient sulfate incorporation. Suitable media can be easily tested by incubating the cells for long-term labeling (see Basic Protocol 1) in the test medium, then measuring the incorporation of ^{35}S into proteins. One simple method to do this is to precipitate the proteins with trichloroacetic acid and then count the insoluble precipitate (see *UNIT 7.1*). The goal is to optimize the ^{35}S incorporation per microgram of protein.

Anticipated Results

The result of labeling with [^{35}S]sulfate should be a protein or proteoglycan that is radioactively labeled to allow easy detection.

Time Considerations

Four elements affect the time required to complete this type of experiment. These include the length of time required to (1) seed and culture cells to 70% to 80% confluence, (2) label overnight and prepare the sample (2 days total), (3) immunoprecipitate, and (4) expose the radioactive sample for detection. Parameters 1 through 3 should be known by the investigator before starting the experiment. Parameter 4 will depend on the specific activity of the [^{35}S]PAPS in the cell and the resulting incorporation of sulfate. Low levels of incorporation may result in exposure times exceeding a week.

Literature Cited

Baeuerle, P.A. and Huttner, W.B. 1986. Chlorate—A potent inhibitor of protein sulfation in intact cells. *Biochem. Biophys. Res. Commun.* 141:870-877.

Beisswanger, R., Corbeil, D., Vannier, C., Thiele, C., Dohrmann, U., Kellner, R., Ashman, K., Niehrs, C., and Huttner, W.B. 1998. Existence of distinct tyrosylprotein sulfotransferase genes: Molecular characterization of tyrosylprotein sulfotransferase-2. *Proc. Natl. Acad. Sci. U.S.A.* 95:11134-11139.

Bowman, K.G. and Bertozzi, C.R. 1999. Carbohydrate sulfotransferases: Mediators of extracellular communication. *Chem Biol*. 6:R9-R22.

Farzan, M., Mizabek, T., Kolchinsky, P., Wyatt, R., Cayabyab, M., Gerard, N.P., Gerard, C., Sodroski, J., and Choe, H. 1999. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell* 96:667-676.

Huttner, W.B. 1984. Determination and occurrence of tyrosine O-sulfate in proteins. *Methods Enzymol*. 107:200-223.

Huttner, W.B. and Baeuerle, P.A. 1988. Protein sulfation on tyrosine. *In* Modern Cell Biology, Vol. 6 (B. Satir, ed.) pp. 97-140. Alan R. Liss, New York.

Linhardt, R.J. 1994. Analysis of glycosaminoglycans with polysaccharide lyases. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.C. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 17.13.17-17.13.32. John Wiley & Sons, New York.

Niehrs, C. and Huttner, W.B. 1990. Purification and characterization of tyrosylprotein sulfotransferase. *EMBO J.* 9:35-42.

Niehrs, C., Huttner, W.B., and Rüther, U. 1992. In vivo expression and stoichiometric sulfation of the artificial protein sulfophilin, a polymer of tyrosine sulfation sites. *J. Biol. Chem.* 267:15938-15942.

Yanigashita, M., Salustri, A., and Hascall, V.C. 1989. Specific activity of radiolabeled hexosamines in metabolic labeling experiments. *Methods Enzymol.* 179:435-445.

Contributed by Sharon A. Tooze
Imperial Cancer Research Fund
London, United Kingdom

Metabolic Labeling with Fatty Acids

Protein acylation is the covalent attachment of fatty acids to a protein; the most commonly added fatty acids are myristate (14:0) and palmitate (16:0). First, radiolabeled fatty acids are used to label eukaryotic cells (see Basic Protocol 1). The radiolabeled material produced can then be analyzed by various methods: the type of fatty acid linkage can be determined (see Basic Protocol 2), the nature of the protein-bound label can be determined to check for interconversion (see Basic Protocol 3), and the protein-bound fatty acid can be identified (see Basic Protocol 4).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

BIOSYNTHETIC LABELING WITH FATTY ACIDS

BASIC PROTOCOL 1

To identify proteins that are modified with fatty acid groups, cultured cells are incubated first in medium containing sodium pyruvate, which acts as a source of acetyl-CoA and minimizes interconversion of the fatty acid to other metabolites, and then with [^{3}H]fatty acids. Fatty acids tritiated at positions 9 and 10 provide the best combination of high specific activity and detectability for in vitro labeling, and because the tritium label is far removed from the carboxyl end where β-oxidation occurs, reincorporation of label is minimized.

Materials

Cells for culture
Complete tissue culture medium appropriate for cells
Labeling medium: complete tissue culture medium containing the relevant dialyzed serum and 5 mM sodium pyruvate, 37°C
5 to 10 μCi/μl [9,10(*n*)-^{3}H]fatty acid, e.g., [9,10(*n*)-^{3}H]palmitic acid or [9,10(*n*)-^{3}H]myristic acid (30 to 60 Ci/mmol; Amersham International, American Radiolabeled Chemicals, or NEN Research Products) in ethanol
PBS, pH 7.2 (*APPENDIX 2A*), ice-cold
1% (w/v) SDS or SDS sample buffer (for SDS-PAGE, when using adherent or nonadherent cells respectively; *UNIT 6.1*) *or* RIPA lysis buffer (for immunoprecipitation; see recipe)
5× SDS sample buffer (see recipe)

Cell scrapers
Nitrogen gas

Additional reagents and equipment for culturing cells (*UNIT 1.1*), immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), treating a gel with sodium salicylate (*UNIT 6.3*), and fluorography (*UNIT 6.3*)

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Contributed by Caroline S. Jackson and Anthony I. Magee
Current Protocols in Cell Biology (2000) 7.4.1-7.4.10

1. On the day before the labeling experiment, split the cells into fresh complete tissue culture medium.

   Set up the cells at two split ratios; then choose the culture closest to 70% to 80% confluency for labeling.

2. The next day, replace the medium with a minimum volume of 37°C labeling medium. Incubate 1 hr.

   Cells in suspension should be used at a cell density of 10^6 to 10^7 cells/ml. For adherent cells that are 70% to 80% confluent, the minimum amount of medium necessary to cover the dish—e.g., 1.5 ml for 60-mm dishes and 3 ml for 100-mm dishes—should be used.

3. Add 5 to 10 µCi/µl [9,10(*n*)-^{3}H]fatty acid to a concentration of 50 to 500 µCi/ml. Incubate up to 24 hr.

   Cells vary in the rate and extent of incorporation (see Critical Parameters), so both the amount of label and the duration of incubation need to be optimized. Labeling cells overnight in the presence of 200 µCi/ml [^{3}H]fatty acid will maximize the chances of detecting labeled proteins. The amount of label and/or time of incubation can then be reduced if good incorporation of label is achieved, or increased if poor incorporation is attained.

   Short labeling times (e.g., pulses on the order of minutes up to 2 hr) require amounts of label at the higher end of the indicated range. In this case, uptake is relatively low and the medium plus label can be reused one or more times. The level of label in the medium can be monitored by scintillation counting. For longer incubations the interconversion of fatty acids becomes a greater problem, and the protein-bound fatty acid label should be analyzed (see Basic Protocols 3 and 4).

   If the [^{3}H]fatty acid is not supplied in ethanol or if the concentration is too low, remove the solvent by blowing nitrogen over the solution in its original container until dry and redissolve the label in ethanol at a concentration of 5 to 10 µCi/µl. Do not transfer into another container or evaporate the solvent in a plastic container, as this will cause a significant loss of label that will adhere to the side of the container.

For adherent cells:

4a. Place the dish on ice and aspirate the medium. Wash the cells twice with ice-cold PBS and lyse the cells by adding 1% SDS for SDS-PAGE (*UNIT 6.1*) *or* RIPA lysis buffer for immunoprecipitation (*UNIT 7.2*), using 100 µl of 1% SDS for a 60-mm dish or 300 µl for a 100-mm dish, or 1 ml RIPA lysis buffer.

   CAUTION: *Radioactive medium and washes must be disposed of appropriately.*

5a. Using a cell scraper, remove the lysed cells from the dish and transfer them to a 1.5-ml microcentrifuge tube. Add 20 µl lysate to 5 µl of 5× SDS-PAGE sample buffer. Use all of lysate for immunoprecipitation. Resuspend immunoprecipitate in 20 µl SDS sample buffer.

   For SDS-PAGE, use DTT at a final concentration ≤20 mM, and do not boil the samples, but incubate them only 3 min at 80°C; this is necessary because the thioester linkage of the fatty acid is susceptible to cleavage.

For nonadherent cells:

4b. Microcentrifuge the cell suspension 1 min at 6000 rpm, 4°C, to pellet the cells. Decant the supernatant and wash the cell pellet once by resuspending it in 1 ml ice-cold PBS and centrifuging again.

5b. Lyse the cells by resuspending the cell pellet in 100 µl SDS-PAGE sample buffer for discontinuous SDS-PAGE (*UNIT 6.1*) *or* 1 ml RIPA lysis buffer for immunoprecipitation (*UNIT 7.2*) for 10^6 to 10^7 cells. Resuspend immunoprecipitate in 20 µl SDS sample buffer.

CAUTION: *Radioactive medium and washes must be disposed of appropriately.*

For analysis of total protein-bound fatty acid label, lyse the cells in 100 µl 1% SDS.

For SDS-PAGE, use DTT at a final concentration ≤20 mM, and do not boil the samples, but incubate them only 3 min at 80°C; this is necessary because the thioester linkage of the fatty acid is susceptible to cleavage.

6. Analyze whole-cell lysate or immunoprecipitate on an SDS-PAGE minigel, using 20 µl lysate per lane. Store remaining lysate at −20°C.

7. Treat the gel with sodium salicylate (*UNIT 6.3*). Using preflashed film, fluorograph the gel (*UNIT 6.3*) at −70°C.

   Typical exposure times are overnight to 1 month.

   Alternatively, the gel may be treated with DMSO/2,5-diphenyloxazole (DMSO/PPO) instead of sodium salicylate (see Magee et al., 1995).

ANALYSIS OF FATTY ACID LINKAGE TO PROTEIN

BASIC PROTOCOL 2

To determine the type of linkage by which the [^{3}H]fatty acid is attached to the protein (i.e., thioester, oxyester, or amide linkage), the fatty acid is selectively cleaved from the protein. The most convenient method is to run replicate lanes on an SDS-PAGE gel, cut the lanes apart, and analyze each lane separately. Thioesters and amides are by far the most common types of linkage; oxyesters ar extremely rare.

Materials

Lysate or immunoprecipitate from [^{3}H]fatty acid–labeled cells (see Basic Protocol 1, step 6)
0.2 M potassium hydroxide (KOH) in methanol
Methanol
1 M hydroxylamine·HCl, titrated to pH 7.5 with NaOH
1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), treating a gel with sodium salicylate (*UNIT 6.3*), and fluorography (*UNIT 7.2*)

1. Run an SDS-PAGE gel (*UNIT 6.1*) using 20 µl lysate or immunoprecipitate from [^{3}H]fatty acid–labeled cells in each of four lanes.

2. Cut the four lanes apart and transfer each lane to a 15-ml tube containing one of the following solutions:

   0.2 M KOH in methanol
   Methanol
   1 M hydroxylamine·HCl
   1 M Tris·Cl, pH 7.5.

   Incubate 1 hr at room temperature with shaking.

   The 0.2 M KOH in methanol will cleave thio- and oxyesters, but not amides; 1 M hydroxylamine·HCl will rapidly cleave thioesters but will cleave oxyesters only poorly, and will not cleave amides. Methanol and 1 M Tris·Cl serve as controls.

3. Wash each gel strip three times, 5 min each time, with water. Treat the strips with sodium salicylate and fluorograph using preflashed film at −70°C (*UNIT 6.3*).

   Typical exposure times are overnight to 1 month.

Alternatively, the gel may be treated with DMSO/PPO instead of sodium salicylate (see Magee et al., 1995).

Cleavage is measured as a reduction in the fluorographic signal compared to those for controls, and can be quantitated by densitometric scanning of the lane or scintillation counting of excised bands. Bands with fatty acids linked to the protein by thioesters will be missing or greatly reduced in lanes treated with 0.2 M KOH in methanol and 1 M hydroxylamine·HCl; oxyesters will be greatly reduced or missing in the lane treated with 0.2 M KOH in methanol and may be slightly reduced in the lane treated with 1 M hydoxylamine·HCl; and amide linkages will not be affected by any of these treatments, so that proteins with amide-linked fatty acids will appear in all four lanes.

BASIC PROTOCOL 3

ANALYSIS OF TOTAL PROTEIN-BOUND FATTY ACID LABEL IN CELL EXTRACT

Due to problems of interconversion of fatty acids by β-oxidation and chain elongation and of reincorporation of label into other metabolic precursors, the protein-bound label derived from [^{3}H]fatty acids should be analyzed, especially for experiments with long labeling incubations. This protocol is used to determine how much of the label has been converted into other fatty acids or metabolites during the incubation; a different procedure must be used to determine whether the fatty acid on the protein of interest is different from that added during labeling (see Basic Protocol 4).

Materials

0.1 M HCl/acetone, −20°C
Lysate from [^{3}H]fatty acid–labeled cells in 1% SDS (see Basic Protocol 1, step 4a or 5b)
1% (w/v) SDS
2:1 (v/v) chloroform/methanol
Diethyl ether
6 M HCl (concentrated HCl diluted 1:1 with H_2O)
Hexane
5 to 10 µCi/µl [9,10(*n*)-^{3}H]fatty acid standards (30 to 60 Ci/mmol; Amersham International, American Radiolabeled Chemicals, or NEN Research Products) in ethanol
90:10 (v/v) acetonitrile/acetic acid
EN^{3}HANCE spray (NEN Research Products)

15-ml polypropylene centrifuge tubes
Mistral 3000i benchtop centrifuge with swing-out four-bucket rotor or equivalent
Nitrogen gas
30-ml thick-walled Teflon container with an air-tight screw top
110°C oven
Thin-layer chromatography tank
RP18 thin-layer chromatography plate (e.g., Merck)
Kodak XAR-5 film, preflashed

Precipitate protein

1. Add 5 vol of 0.1 M HCl/acetone to 100 µl lysate from [^{3}H]fatty acid–labeled cells in 1% SDS in a 15-ml polypropylene tube. Incubate ≥1 hr at −20°C.

   This will precipitate the protein.

2. Centrifuge 10 min at 1500 × *g* (1000 rpm in Mistral 3000i swing-out rotor), 4°C, to pellet the precipitate. Remove the supernatant and allow the pellet to air dry gently.

Remove free label

3. Dissolve the pellet in a minimum volume of 1% SDS and transfer to a 1.5-ml microcentrifuge tube. Add 5 vol of 0.1 M HCl/acetone. Incubate ≥1 hr at −20°C.

4. Repeat steps 2 and 3.

   These precipitation steps concentrate the protein and remove much of the SDS and free label.

5. Add 500 µl of 2:1 chloroform/methanol and vortex. Centrifuge 10 min at 1000 rpm, 4°C, and remove the supernatant. Repeat this step at least three times until no more free label is extracted into the organic solvent, as determined by scintillation counting of the supernatant.

6. Add 100 µl diethyl ether to the pellet and vortex. Centrifuge 10 min at 1000 rpm, 4°C, and decant the supernatant. Dry the pellet by placing the microcentrifuge tube under a gentle stream of nitrogen.

7. Place the tube into a 30-ml thick-walled Teflon container with an air-tight screw top containing 1 ml of 6 M HCl. Flush the tube and container with nitrogen. Close the lid tightly and incubate in an oven 16 hr at 110°C.

   This hydrolyzes the fatty acids from the protein.

Extract hydrolyzed fatty acids

8. Extract the contents of the tube twice with 0.5 ml hexane and pool the extracts. Dissolve the residue in 0.5 ml of 1% SDS. Determine the radioactivity in the hexane extracts and in the residue.

   Fatty acids will be extracted into hexane, while label incorporated into sugars and amino acids will be mainly in the hexane-insoluble residue.

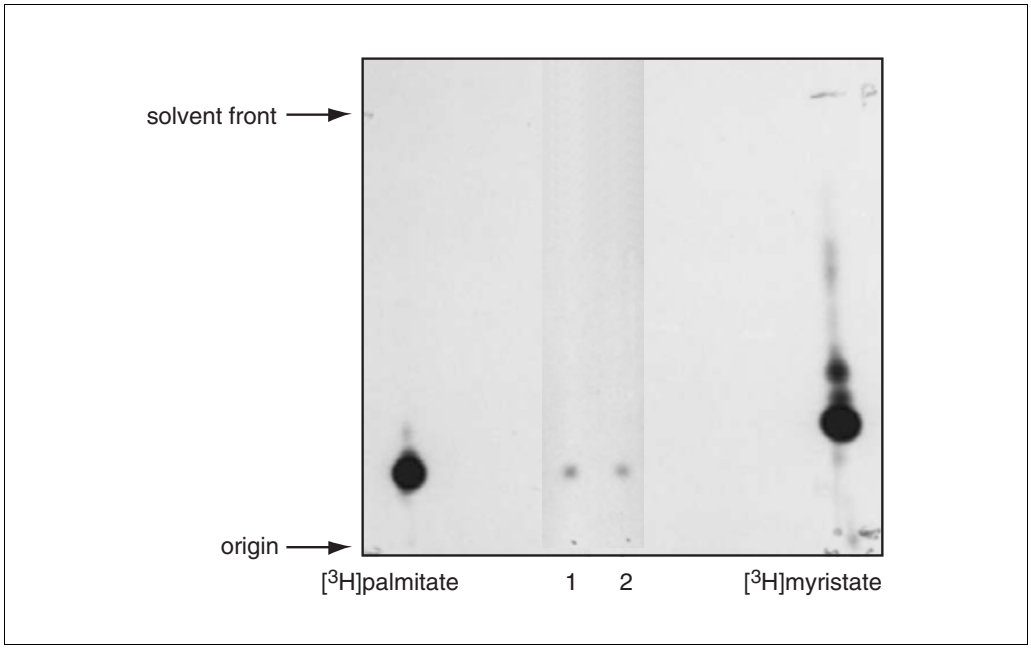


Figure 7.4.1 Fluorogram of thin-layer chromatography plate showing analysis of acylated nerve growth factor (NGF) receptor. Outside lanes, migration of 0.5 µCi [^{3}H]palmitate and [^{3}H]myristate standards. Lane 1, NGF receptor immunoprecipitated from cells labeled with [^{3}H]palmitic acid. Lane 2, NGF receptor immunoprecipitated from cells labeled with [^{3}H]myristic acid. Although the cells were labeled with different fatty acids, the protein was labeled with palmitic acid due to chain elongation of [^{3}H]myristic acid to [^{3}H]palmitic acid by the cells. Exposure for standards, 1 week; exposure for lanes 1 and 2, 1 month.

9. Evaporate the hexane extracts just to dryness with a gentle stream of nitrogen. Dissolve in 2 to 5 µl of 2:1 chloroform/methanol.

It is important not to overdry the sample because it may then be difficult to dissolve.

Identify fatty acids

10. Preequilibrate a thin-layer chromatography tank with 90:10 acetonitrile/acetic acid for 15 min.

11. Spot resuspended hexane extract onto an RP18 thin-layer chromatography plate. Dilute 1 µl [9,10(*n*)-^{3}H]fatty acid standards in ethanol to give 1 µCi/µl and spot 0.5 µl in parallel lanes. Develop the plate in 90:10 acetonitrile/acetic acid. Air dry the plate.

12. Detect the radioactivity by spraying the plate with EN^{3}HANCE spray and exposing it to preflashed Kodak XAR-5 film overnight at −70°C. Identify the fatty acids.

See Figure 7.4.1 for an example of a typical fluorogram.

BASIC PROTOCOL 4

ANALYSIS OF FATTY ACID LABEL IDENTITY

This protocol is used to identify the labeled fatty acid(s) associated with a specific protein band on an SDS-PAGE gel. Following electrophoresis, the band of interest is located either by comparison with molecular weight standards or more accurately by fluorography of a sodium salicylate–treated gel (see *UNIT 6.3*). DMSO/PPO–treated gels cannot be used. The labeled material is analyzed by thin-layer chromatography.

Materials

SDS-PAGE gel of lysate from [^{3}H]fatty acid–labeled cells
Additional reagents and equipment for analysis of protein-bound label (see Basic Protocol 3)

1. Excise the band(s) of interest from a wet or dried (fluorographed) SDS-PAGE gel. Wash three times with shaking, 5 min each, with 0.5 ml water.

If the gel is fluorographed it should be treated with sodium salicylate (UNIT 6.3), not DMSO/PPO solution. The dried gel piece will rehydrate and the salicylate will be washed out during the washes.

2. Place the gel piece in a 1.5-ml microcentrifuge tube and lyophilize.

3. Hydrolyze the fatty acids in the band and identify them by thin-layer chromatography (see Basic Protocol 3, steps 7 to 12).

REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

RIPA (RadioImmunoPrecipitation Assay) lysis buffer

1% (w/w) Nonidet P-40 (NP-40)
1% (w/v) sodium deoxycholate
0.1% (w/v) SDS
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2 (*APPENDIX 2A*)
2 mM EDTA (*APPENDIX 2A*)
10 mM sodium fluoride

continued

0.2 mM sodium vanadate added fresh from 0.2 M stock solution
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

SDS sample buffer (for discontinuous systems), 5×

3.125 ml 1 M Tris·Cl, pH 6.8 (0.313 M final)
1 g SDS (10% final)
5 mg bromphenol blue (0.05% final)
5 ml glycerol (50% final)
H_2O to 10 ml
Store at room temperature
Add DTT to appropriate concentration (see Basic Protocol 1, annotations under steps 5a and 5b) just before use

Warm the solution before use because it tends to solidify.

COMMENTARY

Background Information

The two most common acyl groups that modify proteins are 14-carbon myristic acid and 16-carbon palmitic acid (Fig. 7.4.2), and they occur both on different and on overlapping sets of proteins. By increasing the hydrophobicity of the protein, these fatty acid moieties can play a role in localization of the protein to the membrane and sometimes to specific types of membrane structures—e.g., glycolipid-enriched domains (lipid rafts) or caveolae (Rodgers et al., 1994). Identifying the type of acylation of a protein and determining whether the level of modification can be affected by stimuli can provide more information on the mechanisms of action of proteins involved in signaling pathways.

Fatty acids are used in labeling cells in vitro because they will diffuse across the plasma membrane and then be converted to acyl-CoA by the action of the enzyme acyl-CoA synthetase. This activated form of the fatty acid is the substrate for protein-acyl transferase activities that transfer the acyl group to the protein. Tritiated fatty acids are most commonly used in biosynthetic labeling of proteins, but fluorescent analogs of fatty acids have been used to study palmitoylation of rhodopsin (Moench et al., 1994a,b), and [ω-^{125}I]iodo–fatty acids have been used to study myristoylation of v-src (Peseckis et al., 1993).

A wide variety of proteins are myristoylated, including viral structural proteins and many proteins involved in cell signaling, such as the α subunits of trimeric G proteins and the Src family of tyrosine kinases (Resh, 1994; Wedegaertner et al., 1995). Myristoylation most commonly occurs cotranslationally via an amide linkage to an NH_2-terminal glycine residue, and is an irreversible modification. Myristoylation is dependent on the removal of the initiator methionine and has been shown to occur by the time the nascent polypeptide is 100 amino acids long. Inhibitors of protein synthesis will

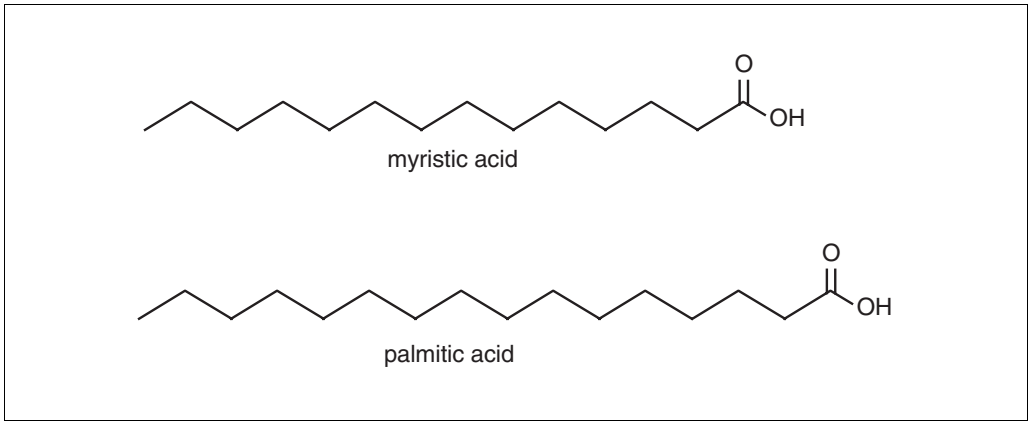


Figure 7.4.2 Structures of myristic and palmitic acids.

therefore block myristoylation. The enzyme responsible for NH_2-terminal myristoylation, *N*-myristoyl transferase (NMT), was first isolated from *Saccharomyces cerevisiae*; both the yeast and human homologs have been well characterized and show different protein substrate specificities (Gordon et al., 1991). Substrate specificity of yeast NMT is determined by recognition of a sterically unhindered NH_2-terminal glycine followed by an amino acid sequence that conforms to the following criteria: an uncharged residue or proline at position 2; any amino acid at positions 3 and 4; serine (more common), alanine, or threonine at position 5; no proline at position 6; and, preferably, basic residues at positions 7 and 8. Residues C-terminal to this region appear to be important in recognition of the α subunits of trimeric G proteins and the Src family of tyrosine kinases (Glover et al., 1988; Gordon et al., 1991).

The NMT enzymatic reaction proceeds by formation of a myristoyl-CoA-enzyme complex, subsequent binding of the peptide, transfer of the myristate moiety to the peptide, release of CoA, and release of the myristoylated peptide (Rudnick et al., 1991). Several assays have been developed for this enzyme (e.g., King and Sharma, 1991; Rudnick et al., 1992; French et al., 1994). Inhibitors of myristoylation include 2-hydroxymyristic acid, which is converted in vivo to 2-hydroxymyristoyl-CoA, a potent inhibitor of NMT. Other inhibitors of acylation are fatty acid analogs and other compounds that inhibit acyl CoA synthetase and therefore block the conversion of fatty acids to acyl CoA; the latter include α-bromopalmitate (DeGrella and Light, 1980) and the triacsin family of antibiotics (Tomoda et al., 1987). The endogenous pool of fatty acid can be depleted by incubation with cerulenin, an antibiotic that inhibits de novo fatty acid biosynthesis.

Myristoylation of a protein can be necessary for its activity, e.g., the transforming activity of Src (Kamps et al., 1986). Myristoylation alone may not be sufficient for a protein to be localized to the membrane; for this, further lipid modification, such as palmitoylation or cooperative interaction with protein sequences, is required (Resh, 1994).

Palmitoylation more accurately referred to as *S*-acylation, (with and without myristoylation) occurs on many signaling molecules, including rhodopsin, α-subunits of G proteins, and Src-family tyrosine kinases. *S*-acylation is a post-translational event occurring via a thioester linkage to a cysteine residue, and where it occurs with NH_2-terminal myristoylation, the myristoylation is usually a prerequisite for *S*-acylation. This may be because the enzyme responsible for *S*-acylation recognizes the myristoylated protein or, more likely, because myristoylation brings the protein to the correct cell location for *S*-acylation to occur. *S*-acylation is also found in conjunction with isoprenoid modification at the C-terminus of proteins belonging to the Ras superfamily; it is responsible for the localization of these proteins to the membrane (Newman and Magee, 1993). This *S*-acylation is dependent on prior modification of the protein by isoprenoid. G protein subunits such as the α_1 subfamily and members of the Src family of tyrosine kinases have an NH_2-terminal amino acid sequence of Met-Gly-Cys, where the initiator methionine is removed and replaced by myristate and the cysteine is *S*-acylated (Resh, 1994). *S*-acylation is a reversible modification and has been shown to be dynamic in vivo, with the level of *S*-acylation changing in response to various stimuli such as receptor activation, insulin, and growth factors (James and Olsen, 1989; Jochen et al., 1991; Wedegaertner et al., 1995). This phenomenon is thought to play a role in switching on or off signaling molecules by altering either the localization of the molecules or their presentation to other signaling molecules with which they interact. Deacylation of proteins is catalyzed by the activity of protein-*S*-acyl thioesterase (Camp and Hoffman, 1993). Protein-*S*-acyltransferase activities have been reported by several groups, but attempts to purify these to homogeneity have so far been unsuccessful. In fact, the possibility that *S*-acylation is nonenzymatic has recently been raised (Dunphy and Linder, 1998).

Analysis of the type of fatty acid linkage present should always be performed. Post-translational myristoylation via a thioester linkage has been found in platelets (Muszbek and Laposata, 1993). The term "palmitoylation" is not strictly accurate because other long-chain fatty acids, such as stearate (18:0) and oleate (18:1), can also be thioesterified to proteins; "*S*-acylation" is now becoming more commonly used to describe this modification. The acylating activity seems to be relatively unspecific for chain length and degree of unsaturation, and utilizes acyl-CoAs partly in proportion to their abundance in the cell—hence the predominance of palmitate. This and the potential for interconversion of labeled fatty acids necessitate analysis of the chain length of the attached label. Other methods can be used to identify the type of fatty acid attached to the

protein, depending on the availability of appropriate equipment; these include gas chromatography, reversed-phase HPLC, and mass spectroscopy (Aitken, 1992). Structural and sequence analysis of the acylated protein can be performed using mass spectrometry (Aitken, 1992).

Further information about the cellular localization of acylated proteins can be found by extraction of the cell lysate in the presence of either Triton X-114 or X-100. Extraction with Triton X-114, which has the property of phase separation at 30°C, can distinguish between hydrophobic and hydrophilic proteins (Aitken, 1992). Extraction of membranes with Triton X-100 at 4° and 37°C can identify proteins that are associated with glycolipid cholesterol–enriched lipid rafts (Rodgers et al., 1994).

Critical Parameters and Troubleshooting

Cells should be subconfluent; it is recommended that the cells be plated at two split ratios so the culture closest to 70% to 80% confluency can be selected to be used for labeling.

The pH of all solutions should be ≤7.5 in order to avoid hydrolysis of labile thioesters; the high pH of SDS-PAGE buffers does not seem to be a problem when using minigels, where the running times are relatively short. Dithiothreitol (DTT) or 2-mercaptoethanol should be used with care, as these can also cleave thioester bonds; for SDS-PAGE a maximum concentration of 20 mM DTT should be used and samples should not be boiled but incubated only 3 min at 80°C.

The use of short labeling times (especially for palmitate labeling) will reduce reincorporation of the label.

The ability to detect myristoylation and/or *S*-acylation of a protein using these methods will depend on the ability of the cells to take up radiolabeled compounds and incorporate them into metabolic precursors; the pool sizes of endogenous fatty acids and fatty acyl–CoA esters; the expression level of the protein-myristoyl transferase and supposed protein-*S*-acyl transferase; the abundance, rate of synthesis, and turnover of the protein(s) and modification(s) of interest; and the efficiency of antibodies for immunoprecipitation.

Anticipated Results

Typically, it is possible to detect myristoylated proteins using fluorographic exposure times ranging from <1 week for high-level expression of protein in transformed cells (e.g., Lck in LSTRA cells) to 1 to 4 weeks for a well-expressed endogenous protein.

Time Considerations

In vitro labeling experiments require 2 to 3 days for growing and labeling the cells. Harvesting the cells, preparing the lysate for SDS-PAGE (with or without prior immunoprecipitation) and SDS-PAGE require 1 to 2 days plus time for fluorography.

Linkage analysis of the labeled proteins takes 1 to 2 hr after the gel has been run. Analysis of the label in cell lysates requires 1 day to precipitate the protein, remove free label, and hydrolyze the sample. A second day is required to extract the label and perform thin-layer chromatography, and fluorography requires an overnight exposure. Analysis of the protein-bound label in gel bands takes ~3 days after fluorography (dried gel) and a similar amount of time from a wet gel, except the analysis begins after the gel has been run.

Literature Cited

Aitken, A. 1992. Structure determination of acylated proteins. *In* Lipid Modification of Proteins, A Practical Approach (N.M. Hooper and A.J. Turner, eds.) pp. 63-88. Oxford University Press, Oxford.

Camp, L.A. and Hoffmann, S.L. 1993. Purification and properties of a palmitoyl-protein thioesterase that cleaves palmitate from H-Ras. *J. Biol. Chem.* 268:22566-22574.

DeGrella, R.F. and Light, R.J. 1980. Uptake and metabolism of fatty acids by dispersed adult rat heart myocytes. *J. Biol. Chem.* 255:9739-9745.

Dunphy, J.T. and Linder, H.E. 1998. Signaling functions of protein palmitoylation. *Biochim. Biophys. Acta* 1436:245-261.

French, S.A., Christakis, H., O'Neill, R.R., and Miller, S.P.F. 1994. An assay for myristoyl-CoA:protein *N*-myristoyltransferase activity based on ion-exchange exclusion of [^{3}H]myristoyl peptide. *Anal. Biochem.* 220:115-121.

Glover, C.J., Goddard, C., and Felsted, R.L. 1988. *N*-Myristoylation of p60^{src}. *Biochem. J.* 250:485-491.

Gordon, J.I., Duronio, R.J., Rudnick, D.A., Adams, S.P., and Gokel, G.W. 1991. Protein *N*-myristoylation. *J. Biol. Chem.* 266:8647-8650.

James, G. and Olson, E.N. 1989. Identification of a novel fatty acylated protein that partitions between the plasma membrane and cytosol and is deacylated in response to serum and growth factor stimulation. *J. Biol. Chem.* 264:20988-21006.

Jochen, A., Hays, J., Lianos, E., and Hager, S. 1991. Insulin stimulates fatty acid acylation of adipocyte proteins. *Biochem. Biophys. Res. Commun.* 177:797-801.

Kamps, M.P., Buss, J.E., and Sefton, B.M. 1986. Rous sarcoma virus transforming protein lacking myristic acid phosphorylates known polypeptide substrates without inducing transformation. *Cell* 45:105-112.

King, M.J. and Sharma, R.K. 1991. *N*-Myristoyl transferase assay using phosphocellulose paper binding. *Anal. Biochem.* 199:149-153.

Magee, A.I., Wootton, J., and de Bony, J. (1995). Optimized methods for detecting radiolabeled lipid-modified proteins in polyacrylamide gels. *Methods in Enzymology* 250:330-336.

Moench, S.J., Terry, C.E., and Dewey, T.G. 1994a. Fluorescence labeling of the palmitoylation sites of rhodopsin. *Biochemistry* 33:5783-5790.

Moench, S.J., Moreland, J., Stewart, D.H., and Dewey, T.G. 1994b. Fluorescence studies of the location and membrane accessibility of the palmitoylation sites of rhodopsin. *Biochemistry* 33:5791-5796.

Muszbek, L. and Laposata, M. 1993. Myristoylation of proteins in platelets occurs predominantly through thioester linkages. *J. Biol. Chem.* 268:8251-8255.

Newman, C.M.H. and Magee, A.I. 1993. Post-translational processing of the ras superfamily of small GTP-binding proteins. *Biochim. Biophys. Acta* 1155:79-96.

Peseckis, S.M., Deichaite, I., and Resh, M.D. 1993. Iodinated fatty acids as probes for myristate processing and function. *J. Biol. Chem.* 268:5107-5114.

Resh, M.D. 1994. Myristoylation and palmitoylation of Src family members: The fats of the matter. *Cell* 76:411-413.

Rodgers, W., Crise, B., and Rose, J.K. 1994. Signals determining protein tyrosine kinase and glycosyl-phosphatidylinositol-anchored protein targeting to a glycolipid-enriched membrane fraction. *Mol. Cell. Biol.* 14:5384-5391.

Rudnick, D.A., McWherter, C.A., Rocque, W.J., Lennon, P.J., Getman, D.P., and Gordon, J.I. 1991. Kinetic and structural evidence for a sequential ordered bi bi mechanism of catalysis by *Saccharomyces cerevisiae* myristoyl-CoA:protein *N*-myristoyltransferase. *J. Biol. Chem.* 266:9732-9739.

Rudnick, D.A., Duronio, R.J., and Gordon, J.I. 1992. Methods for studying myristoyl-CoA:protein *N*-myristoyltransferase. *In* Lipid Modification of Proteins, A Practical Approach (N.M. Hooper and A.J. Turner, eds.) pp. 37-61. Oxford University Press, Oxford.

Tomoda, H., Igarashi, K., and Ømura, S. 1987. Inhibition of acyl-CoA synthetase by triacsins. *Biochim. Biophys. Acta* 921:595-598.

Wedegaertner, P.B., Wilson, P.T., and Bourne, H.B. 1995. Lipid modifications of trimeric G proteins. *J. Biol. Chem.* 270:503-506.

Key Reference

Casey, P.J. and Buss, J.E. 1995. Lipid modification of proteins. *Methods Enzymol.,* Vol. 250.

A compilation of methods used in studying lipid modification of proteins.

Contributed by Caroline S. Jackson and Anthony I. Magee
National Institute for Medical Research
London, United Kingdom

Metabolic Labeling of Prenyl and Carboxyl-Methyl Groups

In the last fifteen years or so, several post-translational modifications of proteins with lipid moieties have been discovered. Analysis of fatty acylation is covered in *UNIT 7.4*. This unit describes methods for analysis of prenylation and the carboxyl-methylation that often accompanies it. The two prenoid groups that have been found attached to proteins—farnesyl (C15) and geranylgeranyl (C20)—are both derived from intermediates in the isoprenoid biosynthetic pathway that utilizes mevalonic acid. In the protocols described here, radiolabeled mevalonate is used to label these intermediates in intact cells (see Basic Protocol 1); the labeled intermediates then become incorporated into proteins. Carboxyl-methylation of C-terminal prenylated cysteine residues often occurs subsequent to prenylation. Methods are given for radiolabeling of the methyl group with [^{3}H-methyl]-methionine, that is converted intracellularly into *S*-adenosylmethionine (see Basic Protocol 2). Three recent publications (Casey, 1990; Hooper and Turner, 1992; Casey and Buss, 1995) contain a number of methods related to analysis of lipid modification of proteins.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

PRENYLATION OF PROTEINS IN CULTURED CELLS

BASIC PROTOCOL 1

To radiolabel intermediates in the isoprenoid biosynthetic pathway, cultured cells are incubated with [^{3}H]mevalonic acid, which is the 6-carbon precursor to isopentenyl diphosphate. Isopentenyl diphosphate (5-carbon) is polymerized in a stepwise fashion to yield farnesyl (15-carbon) and geranylgeranyl (20-carbon) diphosphates, which are the known precursors of protein-bound prenyl groups. To maximize incorporation of exogenous [^{3}H]mevalonate, the endogenous pool of unlabeled mevalonate is usually depleted by preincubation with an inhibitor of hydroxymethyl glutaryl–coenzyme A (HMG-CoA) reductase, the enzyme responsible for production of mevalonate from hydroxymethyl glutarate. Medium containing dialyzed serum is also used to reduce the endogenous pool.

Materials

Cells
Complete tissue culture medium appropriate for the cells, 37°C
Complete tissue culture medium containing dialyzed serum, 37°C
10 mM mevinolin (see recipe)
1 μCi/μl *R*-[5-^{3}H]mevalonic acid (10 to 40 Ci/mmol; DuPont NEN or American Radiolabeled Chemicals)
PBS, pH 7.2 (*APPENDIX 2A*) ice-cold
1× SDS-PAGE sample buffer (*UNIT 6.1*) or RIPA lysis buffer for immunprecipitation (*UNIT 7.4*)
Nitrogen gas

Contributed by Anthony I. Magee
Current Protocols in Cell Biology (2000) 7.5.1-7.5.8

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), treating a gel with sodium salicylate (*UNIT 6.3*), and fluorography (*UNIT 6.3*)

1. On the day before the labeling experiment, split the cells into complete tissue culture medium.

   Set up cells at two split ratios and choose the culture closest to 70% to 80% confluency for labeling. The cultures should contain 10^6 to 10^7 cells.

2. On the day of the experiment, replace the culture medium with a minimum volume of prewarmed complete tissue culture medium containing dialyzed serum. Add 10 mM mevinolin to a final concentration of 50 µM. Preincubate the cells in this medium ≥60 min.

   Mevinolin is an inhibitor of HMG-CoA reductase. Alternative HMG-CoA reductase inhibitors are compactin/mevastatin (Sigma) and pravastatin (Pravachol, Bristol-Meyers Squibb).

3. Transfer enough 1 µCi/µl *R*-[5-^{3}H]mevalonic acid to label all samples at a concentration of 50 to 200 µCi/ml into a microcentrifuge tube. If necessary, remove organic solvent by exposing the tube to a gentle stream of nitrogen, but do not dry the mevalonic acid completely.

   The volume of the [^{3}H]mevalonic acid can be made up with sterile water to facilitate accurate pipetting.

   Alternatively, the organic solvent may be removed in a Speedvac evaporator.

   The mevalonic acid must not be heated above room temperature because it is unstable.

4. Add the partially dried [^{3}H]mevalonic acid at the desired concentration to the culture medium. Incubate up to 24 hr.

   The most appropriate concentration of label and duration of incubation should be experimentally determined for each cell type. Mevalonic acid and mevinolin tend to cyclize to form the acetone (see Troubleshooting). If this is suspected, the lactone can be hydrolyzed (see recipe for mevalonic acid in Reagents and Solutions).

5. Place the cells on ice and aspirate the medium. Wash the cells twice with ice-cold PBS.

   CAUTION*: Radioactive medium and washes must be disposed of appropriately.*

6. Lyse cells in the appropriate solution for further processing—e.g., for a 50-mm dish containing 4×10^6 cells, add 0.2 ml of 1× SDS-PAGE sample buffer (*UNIT 6.1*) or 1 ml RIPA lysis buffer for immunoprecipitation (*UNIT 7.2*).

   Use the entire lysate for immunoprecipitation.

7. Analyze the lysate or immunoprecipitate by SDS-PAGE (*UNIT 6.1*).

   Use 25 µl of the whole cell lysate for a minigel or 50 µl for a standard gel; load all of the immunoprecipitate after solubilizing it in 25 or 50 µl of 1× SDS-PAGE sample buffer.

8. Treat the gel with sodium salicylate and fluorograph the gels at −70°C using preflashed film (*UNIT 6.3*).

   Typical exposure times are 1 week to 1 month, although exposures up to 3 months may sometimes be required (see Time Considerations).

   Alternatively, the gel may be treated with DMSO/2,5-diphenyloxazole (DMSO/PPO) instead of sodium salicylate (see Magee et al., 1995), or with other commercially available water-soluble fluors such as Enlightening (DuPont NEN) or Amplify (Amersham).

CARBOXYL-METHYLATION OF PROTEINS IN CULTURED CELLS

BASIC PROTOCOL 2

Prenylation at C-terminal CXXX and CXC motifs is followed by carboxyl-methylation of the α-carboxyl group directly or after removal if XXX residues. This is performed by a membrane-associated methyl transferase that uses the universal methyl group donor *S*-adenosylmethionine (SAM). The methyl group of SAM can be radiolabeled in vitro by [^{3}H-methyl]methionine, and it is then transferred onto the α-carboxyl group as an alkali-labile ester. This can be distinguished from alkali-stable methylations such as lysyl N-methylation by treating the sample with sodium hydroxide, which releases [^{3}H]methanol. The released volatile [^{3}H]methanol is collected by distillation into scintillation fluid and counted (Chelsky et al., 1985; Gutierrez et al., 1989). Labeled methionine will also be incorporated into the peptide backbone and is alkali-stable, thus providing an internal standard that can be used to calculate the stoichiometry of carboxyl-methylation. The length of the labeling incubation required to get an acceptable signal depends inversely on the expression level of the protein under study.

Materials

Cells
Complete tissue culture medium (CM) appropriate for the cells
Methionine-free tissue culture medium containing dialyzed serum (MFM)
95:5 (v/v) MFM/complete culture medium with dialyzed serum (95:5 MFM/CM)
1 μCi/μl L-[^{3}H-methyl]methionine (~80 Ci/mmol, Amersham)
PBS, pH 7.2 (*APPENDIX 2A*), ice-cold
1× SDS-PAGE sample buffer (*UNIT 6.1*) or RIPA lysis buffer (*UNIT 7.4*)
1 M NaOH
1 M HCl

Warm room or incubator at 37°C
Preflashed film (*UNIT 6.3*)

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2*), treating a gel with sodium solicylate (*UNIT 6.3*), SDS-PAGE (*UNIT 6.1*), and fluorography (*UNIT 6.3*)

Label the cells

1. On the day before the labeling experiment, split the cells into fresh complete tissue culture medium.

   Set up cells at two split ratios and choose the culture closest to 70% to 80% confluency for labeling. The cultures should contain 10^6 to 10^7 cells.

For labeling times ≤8 hr

2a. On the day of the experiment, replace the culture medium with the minimum volume MFM, e.g., 3 ml for a 100-mm tissue culture dish. Incubate 1 hr.

3a. Add 200 μCi [^{3}H-methyl]methionine per milliliter MFM. Incubate up to 8 hr.

For labeling times of 8 to 24 hr

2b. On the day of the experiment, replace the culture medium with the minimum volume 95:5 MFM/CM, e.g., 3 ml for a 100-mm tissue culture dish. Incubate 1 hr.

3b. Add 50 µCi [^{3}H-methyl]methionine per milliliter 95:5 MFM/CM. Incubate 8 to 24 hr.

4. After labeling is complete, aspirate the medium and wash the cells twice with ice-cold PBS.

   CAUTION*: Labeling and wash media are radioactive.*

5. Lyse in the appropriate solution for further processing—e.g., for a 50-mm dish containing 4×10^6 cells, add 0.2 ml 1× SDS-PAGE sample buffer (*UNIT 6.1*) or 1 ml RIPA lysis buffer for immunoprecipitation (*UNIT 7.2*).

Separate and fluorograph the samples

6. Analyze the immunoprecipitate or lysate by SDS-PAGE (*UNIT 6.1*).

   Use 25 µl whole-cell lysate for a minigel or 50 µl for a standard gel. Use the whole immunoprecipitate solubilized in 25 or 50 µl of 1× SDS-PAGE sample buffer.

   The α-carboxyl-methyl esters are stable at the moderately alkaline pH of the standard Laemmli gel system, whereas methyl esters of the side-chain carboxyls of aspartate and glutamate are quite labile.

7. Treat the gel 20 min with sodium salicylate (*UNIT 6.3*) and immediately dry gel at 60°C on a backing of filter paper.

   The gel must not be dried at too high a temperature.

   Alternatively, the gel may be treated with DMSO/PPO instead of sodium salicylate (see Magee et al., 1995) or with other commercially available water-soluble fluors, such as Enlightening (Dupont NEN) or Amplify (Amersham).

8. Using radioactive ink, mark the filter paper to note the alignment of the gel. Fluorograph the gel at −70°C, using preflashed film. Expose the film for 1 week to 1 month.

   An inexpensive radioactive ink can made by washing out old radiolabel vials with a small volume (e.g., 0.1 ml) of an aqueous solution containing 0.01% (w/v) bromphenol blue.

9. Using the alignment marks, align the film exactly with the gel. Excise the radioactive band(s) of interest with a scalpel.

Release the alkali-labile label

10. Place each gel piece into a separate 1.5-ml microcentrifuge tube. Carefully lower the open tube into a scintillation vial that contains enough scintillation fluid to come about half way up the side of the tube.

11. Add enough 1 M NaOH into the tube to cover the gel piece (usually 100 to 200 µl). Immediately cap the scintillation vial, leaving the tube open inside. Incubate overnight in a 37°C warm room or 37°C incubator.

    During this incubation, radioactive methanol is hydrolytically released and distilled into the scintillation fluid.

12. The next day, uncap the vial and carefully remove the microcentrifuge tube, taking care not to spill any of the contents into the vial. Recap the vial.

    This scintillation vial contains the alkali-labile [^{3}H]methanol.

Analyze the sample for alkali-stable label

13. Neutralize the NaOH in the microcentrifuge tube by adding an equal volume of 1 M HCl. Transfer the contents of the tube to a new scintillation vial.

This scintillation vial contains the alkali-stable [^{3}H]label which represents [^{3}H]methionine incorporated into the peptide backbone. However, alkali-stable methyl groups cannot be distinguished from backbone methionine in this way, so it is essential to have good reason to believe that other methyl groups are not present before alkali-stable counts can be used to calculate stoichiometry of carboxyl-methylation.

14. Add a volume of scintillation fluid to this vial equal to that used for the alkali-labile sample (step 12).
15. Count both samples in a scintillation counter using the tritium channel.

Calculate the methylation stoichiometry

16. Calculate the methylation stoichiometry as:

$$\text{stoichiometry} = \frac{\text{alkali} - \text{labile cpm} \times \text{number of methionine residues in protein}}{\text{alkali} - \text{stable cpm.}}$$

This calculation can only be made if the methionine content of the protein is known. Also the possibility that the initiator methionine might be removed must be taken into account. This can be predicted from the primary sequence (Aitken, 1992).

REAGENTS AND SOLUTIONS

Use deionized or distilled in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Mevalonic acid, 100 mM

Hydrolyze mevalonic acid lactone (Sigma) to the free acid (Kita et al., 1980) by dissolving 30 mg mevalonic acid lactone in 1.8 ml ethanol at 55°C. Add 0.9 ml of 0.6 M NaOH and 18 ml water. Incubate 30 min at room temperature. Adjust to pH 8 with HCl and dilute to 100 mM. Store indefinitely at −20°C.

Mevinolin, 10 mM

Dissolve 90 mg mevinolin in 1.8 ml ethanol at 55°C. Add 0.9 ml of 0.6 M NaOH and 18 ml distilled water. Incubate 30 min at room temperature. Adjust to pH 8 with HCl and dilute to 10 mM. Store indefinitely at −20°C in 500-µl aliquots.

Mevinolin, also called Lovastatin and Mevacore, is available from Merck or Biomol Research Labs (see SUPPLIERS APPENDIX; Alberts, 1988). It is supplied as the lactone and must be hydrolyzed to the free acid as described in the recipe for mevalonic acid above (Kita et al., 1980).

COMMENTARY

Background Information

Prenylation of proteins is a widespread post-translational modification that occurs at C-terminal motifs of the type CXXX, CXC, or CC (where C is cysteine and X is any amino acid). Recent reviews give a thorough description of this field (Clarke, 1992; Giannakouros et al., 1993). Either the 15-carbon isoprenoid farnesol or the 20-carbon isoprenoid geranylgeraniol (see Fig. 7.5.1) can be added, depending on the exact sequence at the C-terminus. $CX_1X_2X_3$ motifs where X_3 is a small residue (e.g., methionine, serine, or alanine), are farnesylated; whereas if X_3 is large and hydrophobic (e.g., leucine or phenylalanine), they are geranylgeranylated. The nature of the X_1 and X_2 residues also influences the ability of the motif to become prenylated such that some apparent CXXX sequences are not modified. The CXC and CC motifs are also geranylgeranylated on both cysteines. The prenoid moiety is attached by a thioether linkage to the sulphydryl group of cysteine; this is an extremely stable linkage that is irreversible in vivo, but it can be chemically cleaved in vitro to allow analysis of the chain length of the attached isoprenoid (Farnsworth et al., 1990).

Prenylation occurs on otherwise cytosolic soluble proteins and is catalyzed by a ubiquitous family of at least three soluble heterodi-

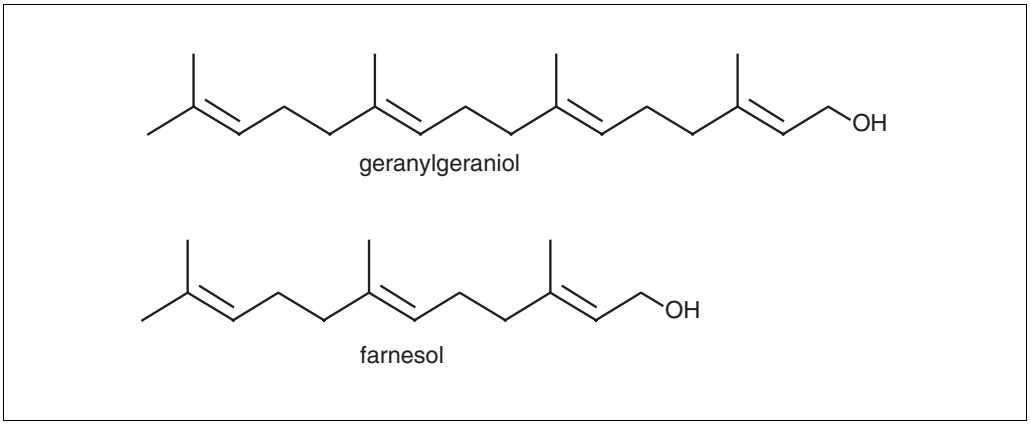


Figure 7.5.1 Structures of isoprenoid groups used in post-translational modification.

meric enzymes: farnesyl transferase, geranylgeranyl transferase I (active on CXXX motifs) and geranylgeranyl transferase II (active on CXC and CC motifs). In addition, geranylgeranyl transferase II has a third associated subunit called Rab escort protein that is required for activity. These enzymes utilize the activated diphosphates of farnesol and geranylgeraniol that are normal intermediates in the isoprenoid biosynthetic pathway, that leads to many important end products such as steroids, ubiquinone, and dolichols (Goldstein and Brown, 1990). Mevalonic acid is the precursor of these polyisoprenyl diphosphates and is readily taken up by most cells in culture. Hence radioactive mevalonate can be used to radiolabel prenyl chains biosynthetically. To maximize incorporation, endogenous mevalonate is usually depleted by preincubation of cells with an inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme responsible for mevalonate synthesis. Both the ability of cells to take up exogenous mevalonate and the sensitivity to HMG-CoA reductase inhibitors can vary enormously and needs to be experimentally tested (e.g., the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*; Giannakouros et al., 1992).

Subsequent to and dependent on prenylation, other modifications can take place. CXXX motifs are proteolyzed to remove the XXX residues by a membrane-associated endoprotease; then they are carboxyl-methylated on the free C-terminal α-carboxyl by a membrane-bound carboxyl-methyl transferase. This methyl ester group is chemically relatively labile; it is sensitive to mild alkali, although it is not as labile as the side-chain methyl esters of aspartate and glutamate, which can hydrolyze even during electrophoresis in mildly alkaline buffers such as are used in the standard Laemmli SDS-PAGE system (Laemmli, 1970). This lability is used experimentally as a test for the C-terminal α-carboxyl-methyl ester (Chelsky et al., 1985; Gutierrez et al., 1989). CXC motifs become doubly geranylgeranylated and are therefore also carboxyl-methylated because the methyl transferase recognizes a C-terminal prenylated cysteine. CC motifs, on the other hand, are not carboxyl-methylated. Many prenylated proteins also become palmitoylated (*S*-acylated) on nearby upstream cysteines. *S*-acylation is discussed in *UNIT 7.4*.

The primary function of prenylation is in membrane localization of substrate proteins that would otherwise be cytosolic because they are usually lacking in hydrophobic peptide sequences (Parenti and Magee, 1995). To perform this localization function, prenyl groups generally act in cooperation with themselves (e.g., for the doubly geranylgeranylated CXC proteins), with other modifications such palmitate moieties, or with peptide sequences. For example, the prenylated CXXX sequences of Ras proteins must cooperate with palmitoylation sites (in the case of H-ras, N-ras and K[A]-ras) or a polybasic region (as in K[B]-ras), which probably interacts with acidic membrane phospholipids (Hancock et al., 1991; Newman and Magee, 1993). These cooperating signals specify not only general membrane binding, as might be expected for a hydrophobic lipid moiety, but also targeting to specific subcellular destinations. The mechanism of this intracellular targeting is at present obscure.

Membrane association of prenylated proteins is frequently reversible in vivo. This is achieved in several ways (Newman et al., 1992). Both *S*-acylation and carboxyl-methylation can be dynamic in vivo, thus allowing modulation of the hydrophobicity and/or charge of the C-terminus. Phosphorylation of residues near the C-terminus will change the charge, reducing the interaction with acidic

phospholipid head groups. Thirdly, many prenylated proteins are removed from membranes by sequestration of their prenylated domains by binding proteins that mask the hydrophobic group and form soluble 1:1 complexes. These mechanisms allow prenylated proteins to move between subcellular sites, which is often an essential part of their function.

Critical Parameters and Troubleshooting

All of the labeling protocols in this unit that use cultured cells perform optimally when subconfluent cells are used. Certainly cells that are fully confluent should not be used. To ensure that cells at an appropriate density are available, it is helpful set up cultures at two different split ratios and use the ones that are most suitable. Occasionally a cell line is encountered that labels very poorly. Increasing the amount of radiolabel and/or the duration of labeling may lead to a satisfactory result. In the case of [^{3}H]mevalonic acid labeling, the amount of mevinolin used and the duration of pretreatment can be increased. [^{14}C]mevalonic acid has been used by some workers for radiolabeling prenyl moieties. Although tritium has a lower intrinsic detectability than ^{14}C due to its weaker emission, the author nevertheless finds ^{3}H is more sensitive because tritiated compounds can generally be obtained at much higher specific activities than ^{14}C derivatives, and that more than compensates for the weaker emission. For [^{3}H-methyl]methionine labeling, it may help to reduce the concentration of unlabeled methionine in the medium to zero and to increase the duration of pretreatment.

It is always useful to include an aliquot of the total cell lysate on the SDS-PAGE gel to assess the efficiency of labeling. Low levels of tritium require the highest sensitivity of fluorography for most rapid detection. The author has found that treating the gel with 2,5-diphenyloxazole in dimethylsulfoxide is the most sensitive method (Laskey, 1980; Magee et al., 1995). However, this method cannot be used if further processing of the gel for prenyl group analysis or alkaline release of carboxyl-methyl groups is to be performed. In these cases, fluorography is best performed after treating the gel with the water-soluble fluor salicylic acid (Chamberlain, 1979) or a commercial equivalent; this method is approximately half as sensitive.

Mevalonic acid and mevinolin tend to cyclize to form the lactone. If this is suspected (e.g., as indicated by a fall-off in labeling efficiency), the lactone can be hydrolyzed by the method of Kita et al. (1980) as described in the recipes (see Reagents and Solutions). Lipid substrates should never be dried to completion in plastic tubes because they are often irreversibly adsorbed to the surface.

The ability to detect prenylation and/or carboxyl-methylation of any given protein using these methods will depend on a number of factors: the ability of the cells to take up radiolabeled compounds and incorporate them into metabolic precursors; the sensitivity of the cells to hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors; the pool sizes of endogenous mevalonate, prenyl diphosphates, and *S*-adenosylmethionine (SAM); the expression level of the protein prenyl transferases and carboxyl-methyl transferase; the abundance, rate of synthesis, and turnover of the protein(s) and modification(s) of interest; and for immunoprecipitation, the efficiency of the antibodies.

Anticipated Results

Typically it is possible to detect prenylated proteins using fluorographic exposure times of one to a few days for transfected cDNAs expressed at high levels, e.g., in COS cells, or in vitro translated mRNAs, 2 or 3 weeks for well-expressed endogenous proteins, or up to 3 months for endogenous proteins expressed at low levels or in cells that incorporate mevalonate poorly.

Similar exposure times are required for carboxyl-methylated proteins. However, because much of the radiolabel incorporated into these proteins is in the form of backbone methionine, the exposure time to detect the protein by fluorography may not be a good guide to the sensitivity of detection for alkali-labile methyl groups. This can be predicted if the primary sequence of the protein and therefore, its methionine content is known; it clearly becomes more of a problem with larger proteins and those with many methionines.

Time Considerations

Labeling experiments using cultured cells typically require ~3 days to grow and label the cells. Harvesting and analyzing the cells by SDS-PAGE (with or without immunoprecipitation) take an additional 2 days, plus the time required for fluorography. After the fluorogram is developed, it takes 2 to 4 hr plus an overnight incubation to perform the analysis for alkali-labile and alkali-stable label, and the samples can be counted the next day.

Literature Cited

Aitken, A. 1992. Structure determination of acylated proteins. *In* Lipid Modifications of Proteins: A Practical Approach (N.M. Hooper and A.J. Turner. eds.) pp. 63-88. IRL Press, Oxford.

Alberts, A.W. 1988. Discovery, biochemistry and biology of lovastatin. *Am. J. Cardiol.* 62:10J-15J.

Casey, P. (ed.) 1990. Covalent Modification of Proteins by Lipids. *Methods*, Volume 1.

Casey, P., and Buss, J. (eds.) 1995. Lipid Modifications of Proteins. *Methods Enzymol*., Volume 250.

Chamberlain, J.P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132-135.

Chelsky, D., Ruskin, B., and Koshland, D.E. Jr. 1985. Methyl-esterified proteins in a mammalian cell line. *Biochemistry* 24:6651-6658.

Clarke, S. 1992. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu. Rev. Biochem*. 61:355-386.

Farnsworth, C.C., Casey, P.J., Howald, W.N., Glomset, J.A., and Gelb, M.H. 1990. Structural characterization of prenyl groups attached to proteins. *Methods* 1:231-240.

Giannakouros, T., Armstrong, J., and Magee, A.I. 1992. Protein prenylation in *Schizosaccharomyces pombe*. *FEBS Letts.* 297:103-106.

Giannakouros, T., Newman, C.M.H., Craighead, M.W., Armstrong, J., and Magee, A.I. 1993. Post-translational processing of *S. pombe* YPT5 protein: In vitro and in vivo analysis of processing mutants. *J. Biol. Chem.*, 268:24467-24474.

Goldstein, J.L. and Brown, M.S. 1990. Regulation of the mevalonate pathway. *Nature* 343:425-430.

Gutierrez, L., Magee, A.I., Marshall, C.J., and Hancock, J.F. 1989. Post-translational processing of $p21^{ras}$ is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J.* 8:1093-1098.

Hancock, J.F., Cadwallader, K., and Marshall, C.J. 1991. Methylation and proteolysis are essential for efficient membrane binding of prenylated $p21^{K\text{-}ras(B)}$. *EMBO J.* 10:641-646.

Hooper, N.M. and Turner, A.J. (eds.) 1992. Lipid Modification of Proteins: A Practical Approach. IRL Press, Oxford.

Kita, T., Brown, M.S., and Goldstein, J.L. 1980. Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase. *J. Clin. Invest.* 66:1094-1107.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Laskey, R.A. 1980. The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis *Methods Enzymol.* 65:363-369.

Magee, A.I., Wootton, J., and de Bony, J. 1995. Optimized methods for detecting radiolabeled lipid-modified proteins in polyacrylamide gels. *Methods Enzymol.* 250:330-336.

Newman, C.M.H. and Magee, A.I. 1993. Post-translational processing of the ras superfamily of small GTP-binding proteins. *Biochim. Biophys. Acta* 1155:79-96.

Newman, C.M.H., Giannakouros, T., Hancock, J.F., Fawell, E.H., Armstrong, J., and Magee, A.I. 1992. Post-translational processing of *Schizosaccharomyces pombe* YPT proteins. *J. Biol. Chem.* 267:11329-11336.

Parenti, M. and Magee, A.I. 1995. Fatty acid- and isoprenoid-linked membrane proteins. *In* Biomembranes, Vol. 1 (A.G. Lee, ed.) pp. 79-105. JAI Press, Greenwich, Connecticut.

Key References

Casey, P. (ed.) 1990. See above.

Casey, P. and Buss, J. (eds.) 1995. See above.

These references contain compilations of methods for studying lipid modifications of proteins.

Contributed by Anthony I. Magee
National Institute for Medical Research
London, United Kingdom

Metabolic Labeling and Immunoprecipitation of Yeast Proteins

Saccharomyces cerevisiae, hereafter referred to as yeast, has long been an important model system for studying basic cellular processes. An essential component of many of these studies has been the ability to pulse-label newly synthesized proteins and to follow the fate of a specific protein during a chase period by immunoprecipitation (*UNIT 7.2*) and SDS-PAGE (*UNIT 6.1*). Because yeast can assimilate inorganic sulfate into methionine and cysteine (Marzluf, 1997), it is possible to label yeast cells with $^{35}SO_4^{2-}$. However, for this purpose most researchers use a mixture of [^{35}S]methionine and [^{35}S]cysteine, which equilibrates more directly into the intracellular pools of charged aminoacyl-tRNAs. A cellular hydrolysate from *E. coli* labeled with $^{35}SO_4^{2-}$, marketed as TRAN^{35}S-LABEL (ICN) or EXPRE^{35}S^{35}S (NEN), works well as a source of [^{35}S] amino acids for metabolically labeling yeast proteins (see Basic Protocol).

Because yeast have a rigid cell wall, lysing the labeled cells in order to immunoprecipitate a specific protein poses an additional challenge. Mechanical shearing is the simplest and least expensive method for lysing yeast. This is done by vigorously vortexing samples in the presence of glass beads. Alternatively, one can remove the cell wall enzymatically using a β-glucanase preparation marketed as Zymolyase 100T (Seikagako Kogyo), Oxalyticase (Enzogenetics), or yeast lytic enzyme (ICN). The resulting spheroplasts can then be lysed by reducing the osmotic support (see Alternate Protocol). Both procedures are described below.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (see also *APPENDIX 1D*).

LABELING AND IMMUNOPRECIPITATING YEAST PROTEINS

BASIC PROTOCOL

The proteins of yeast are metabolically labeled using an *E. coli* hydrolysate as the source of [^{35}S]amino acids. After labeling, the cells are lysed using glass beads, and the protein(s) of interest are immunoprecipitated using specific antibody and protein A-Sepharose. Then the immunoprecipitates are analyzed by SDS-PAGE (*UNIT 6.1*) and fluorography (*UNIT 6.3*).

Materials

Yeast
Minimal medium containing 2% (w/v) glucose (SD medium), without agar (*UNIT 1.6*)
Methionine-free SD medium (*UNIT 1.6*)
[^{35}S]-protein hydrolyzate labeling mix (>1000 Ci/mmol)
50× chase solution (see recipe)
50% (w/v) trichloroacetic acid (TCA)
Acetone, ice cold
SDS/urea buffer (see recipe)
Detergent IP buffer (see recipe)
Antibody specific for protein of interest
Preimmune serum for a negative control
Protein A–Sepharose (see recipe)
Detergent/urea buffer (see recipe)

Contributed by Todd R. Graham
Current Protocols in Cell Biology (2000) 7.6.1-7.6.9

50 mM Tris·Cl, pH 7.5/1% (w/v) SDS
1× (w/v) SDS sample buffer (*APPENDIX 2A*)

125-ml culture flask
Disposable 1.7-ml centrifuge tubes
0.1- to 0.25-mm glass beads (Glen Mills)
Ready-Caps and vials (Beckman)
SpeedVac evaporator
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

Grow cells

1. Innoculate a single yeast colony from a plate into 25 ml of SD medium contained in a 125-ml culture flask. Use the appropriate medium supplements necessary to support growth of strains carrying auxotrophic mutations and to maintain selection for any plasmids. Shake vigorously (250 rpm) in a 30°C incubator overnight.

   It is usually best to innoculate the culture in the morning to ensure that there will be an adequate number of cells for the next day.

2. Use a spectrophotometer to measure the OD_{600} of a 1:4 or 1:10 dilution (in water) of the overnight culture.

   Since turbidity or light scattering is being measured in the spectrophotometer rather than absorbance, it is best to read in the 0.05 to 0.5 range to achieve a linear response to cell density. A culture with an OD_{600} reading of 1.0 (corrected for dilution) is considered to have a cell concentration of 1 OD_{600}/ml, which is equivalent to ~1×10^7 cells/ml for most strains.

3. Proceed to step 5 if the overnight culture is still in log phase (has not exceeded an OD_{600} of 1.0) and a sufficient number of cells are available for labeling.

   The number of cells required for a pulse-chase experiment depends on the expression level of the protein of interest and has to be determined empirically. However, a total of 8 OD_{600} are required for a typical pulse-chase experiment in which 2 OD_{600} of cells are harvested at each of four time points.

4. For overnight cultures exceeding an OD_{600}/ml of 1.0, dilute to 0.25 OD_{600} in the same medium (25 to 50 ml) and grow for 4 to 5 hr as described above.

   The cells will usually double only once in this time period when cultured in minimal medium.

5. Measure and record the OD_{600} of the culture and pellet the yeast by centrifuging 5 min at 5000 × *g*. Discard the spent medium and resuspend the cells in SD medium (methionine-free) to a concentration of 5 OD_{600}/ml.

6. Determine the number of cells required for the experiment and transfer an appropriate culture volume to a disposable centrifuge tube (i.e., Falcon 2059 or 2070).

   The culture should occupy <10% the total volume of the tube.

Label cells

7. Preincubate the cells at the desired temperature (typically 30°C) for 15 min with vigorous shaking (250 rpm). To initiate the labeling period, add [^{35}S]methionine and cysteine to a final concentration of 125 µCi/ml (or use 25 µCi/OD_{600} if a different concentration of cells is used). Incubate 5 to 15 min.

8. To initiate the chase, add 50× chase solution to a 1× final concentration. At the desired chase times (e.g., 0, 5, 15 and 45 min), transfer equal aliquots (100 to 400 µl) from

the culture to properly labeled microcentrifuge tubes sitting on ice containing 0.25 vol 50% (w/v) TCA (final TCA concentration equals 10%) to terminate the chase.

CAUTION: *TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.*

The cells can be stored in 10% TCA in a refrigerator for several days or processed immediately for immunoprecipitation.

Lyse cells

9. After at least 15 min in TCA, pellet the cells 10 min in a microcentrifuge at full speed. Aspirate the supernatant into a radioactive waste trap and wash the pellet with ~1.0 ml of ice-cold acetone. Microcentrifuge for 4 min at full speed and aspirate the acetone into the radioactive waste trap. Repeat the acetone wash.

   CAUTION: *The supernatants and washes should be handled as mixed chemical/radioactive wastes; follow applicable safety regulations for disposal (also see APPENDIX 1D).*

   Individually immersing the bottom of the tubes in a bath sonicator and then vortexing will help disperse the pellets.

10. Dry the acetone-washed TCA pellets in a SpeedVac evaporator, then add 100 µl of SDS/urea buffer and let sit for at least 15 min at room temperature.

11. Add glass beads (0.1- to 0.25-mm) to 80% to 90% (v/v) of the sample volume and vortex 1 min.

    Care should be taken here because glass beads adhering around the lip of the tube will prevent the cap from closing tightly. Use a razor blade to cut the bottom off of a microcentrifuge tube and use this as a "funnel" for adding the glass beads. A 0.1-ml capacity Coors porcelain spatula (Fisher, VWR) works well to deliver the appropriate volume of glass beads.

12. Heat the samples 4 min at 95°C, then vortex again for 15 to 30 sec.

13. Add 900 µl of detergent IP buffer, vortex and put the samples on ice for ≥10 min.

14. Microcentrifuge the samples 15 min at full speed and transfer 0.85 ml of the supernatants (lysate) to fresh tubes being careful not to disturb the glass beads or any insoluble material pelleted on top of the glass beads.

15. To determine the incorporation of ^{35}S into protein, spot 5 µl of each supernatant into a Beckman Ready-Cap, transfer the caps to vials and measure the radioactivity in a scintillation counter.

    Alternatively, measure incorporation of radioactivity by TCA precipitation (UNIT 7.1).

Immunoprecipitate protein

16. Add antiserum (usually 1 to 2 µl per OD of labeled cells) and 75 to 100 µl protein A–Sepharose suspension to the supernatants. Put the tubes in a rack and lay the rack on its side and rock the samples for 4 hr to overnight in a cold room. Agitate sufficiently to keep the protein A–Sepharose in suspension.

    Alternatively, use antibody pre-bound to protein A–Sepharose (see UNIT 7.2).

17. Pellet the immune complexes by microcentrifuging 30 sec at 3000 × g and transfer the supernatants to fresh tubes.

    Do not discard these supernatants as it is often desirable to immunoprecipitate a second protein from these lysates.

18. Wash the pellets twice with 1 ml of detergent/urea buffer, then once with detergent IP buffer. Aspirate each supernatant from the washes into the radioactive waste trap.
19. (*Optional*) To immunoprecipitate the lysates with a second antibody, clear any remaining antigen-antibody complexes by adding 75 µl of protein A–Sepharose and rocking the samples for 2 hr in the cold as above. Microcentrifuge 10 min at full speed and transfer the supernatants to fresh tubes. Use the cleared lysate to immunoprecipitate with a second antibody.
20. Resuspend the pellets in 100 µl of 50 mM Tris·Cl, pH 7.5/1% SDS and heat at 95°C for 4 min. Repeat steps 13 to 18, omitting step 15. Dry the final pellet for 5 to 10 min under vacuum.

    A second immunoprecipitation ("recapture") step is usually required to achieve a "clean" immunoprecipitation.
21. Resuspend the pellets in 50 µl of 1× SDS sample buffer and heat at 95°C for 4 min. Subject half of the sample to SDS-PAGE (*UNIT 6.1*). Save the remainder of the samples (at 4°C) in case the electrophoresis has to be done again. Process gels for fluorography or phosphor imaging (*UNIT 6.3*).

ALTERNATE PROTOCOL

MAKING YEAST SPHEROPLASTS

Proteins secreted from yeast are usually retained in the periplasmic space, which lies between the plasma membrane and the cell wall. The contents of the periplasmic space can be separated from the cells by enzymatically removing the cell wall and pelleting the resulting spheroplasts. Secreted proteins will remain in the supernatant. In addition, if one wishes to perform native immunoprecipitations or fractionate labeled yeast cells, it is necessary to remove the cell wall in order to gently lyse the cells by reducing the osmotic support.

Additional Materials *(also see Basic Protocol)*

Radiolabeled yeast cells
2× spheroplast/stop solution (see recipe)
Bovine serum albumin (BSA), fatty acid–free Fraction V
10 mg/ml Zymolyase 100T (Seikagako Kogyo)

1. Grow and label yeast cells (see Basic Protocol, steps 1 to 7).
2. Chase the cells, substituting 1 vol 2× spheroplast/stop solution for the 50% (w/v) TCA (see Basic Protocol, step 8). Add BSA to 1 mg/ml to act as a carrier if secreted proteins are going to be TCA precipitated from the periplasmic space fraction.
3. Keep samples on ice until all the chase points are collected.
4. Add 2 µl of 10 mg/ml Zymolyase 100T (ICN) per OD_{600} of cells in each sample and incubate 30 min at 30°C with gentle agitation.
5. Pellet cells by microcentrifuging 5 min at 5000 × *g* and transfer the supernatants to fresh tubes.
6. To monitor the spheroplasting reaction, take 200 µl of a 10 OD_{600}/ml suspension of unlabeled cells in 1× spheroplast/stop buffer, split the sample equally and add Zymolyase to one of the tubes. After incubating 30 min at 30°C, remove 25 µl from each tube and dilute to 1 ml with water. Read the OD_{600}.

    The untreated sample should give an OD_{600} reading of ~0.25, but the treated sample should lyse when diluted in water and give an OD_{600} reading that is 5% to 10% that of the untreated sample (~0.01 to 0.025). If the OD_{600} reading of the treated sample is ≥10% of the untreated sample, add more Zymolyase to the samples and continue the incubation.

Process yeast spheroplasts for immunoprecipitation or subcellular fractionation

7a. *For TCA precipitation followed by immunoprecipitation:* Add 0.25 vol of 50% TCA to the supernatants and 0.5 ml of 10% (w/v) TCA to the cell pellets to precipitate proteins. Proceed from this point to step 9 of the Basic Protocol.

7b. *For immunoprecipitation without TCA precipitation:* Dissolve cell pellets in 100 µl SDS-urea buffer, then heat at 95°C for 4 min to lyse cells and avoid TCA precipitation and glass-bead lysis steps. Proceed from this point to step 13 of the Basic Protocol.

7c. *For native immunoprecipitation:* Resuspend the cell pellets in 1 ml nondenaturing lysis buffer (*UNIT 7.2*). Perform native immunoprecipitation from the lysates as described in *UNIT 7.2*.

7d. *For subcellular frationation:* Lyse the spheroplasts by resuspending the cell pellet in low-osmotic-strength buffer (e.g., 25 mM Tris·Cl, pH 7.2/0.1 M sorbitol) and fractionate to enrich specific organelles (Graham et al., 1994).

ENDO H TREATMENT OF IMMUNOPRECIPITATES

SUPPORT PROTOCOL

N-linked oligosaccharides on yeast glycoproteins are sensitive to endoglycosidase H (endo H) regardless of the extent of Golgi modification. Thus, endo H treatment can be used to determine if an immunoprecipitated protein is modified with N-linked oligosaccharides (also see *UNIT 15.2*). Add 64 µl of freshly prepared SDS-2ME solution (0.2% w/v SDS/1% w/v 2-mercaptoethanol) to dried immunoprecipitates (Basic Protocol, step 17) and heat 4 min at 95°C. Add 16 µl of 250 mM sodium citrate pH 5.5 buffer to each sample. Mix and centrifuge briefly. Transfer 40 µl of each sample to a fresh tube, then add 0.5 mU of Endo H (0.5 µl) to one portion and incubate each pair of samples (+ and − endo H) overnight in a 37°C air incubator. Then add 13.3 µl of 4× SDS sample buffer (*APPENDIX 2A*) to each tube, heat 4 min at 95°C and electrophorese the samples in an SDS-polyacrylamide gel (*UNIT 6.1*).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Chase solution, 50×

50 mM methionine
5 mM cysteine
Filter sterilize and store up to 1 year at −20°C in small aliquots

Detergent IP buffer

50 mM Tris·Cl, pH 7.5
0.1 mM EDTA
150 mM NaCl
0.5% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20)
Store up to 3 months at 4°C

Detergent/urea buffer

100 mM Tris·Cl, pH 7.5
200 mM NaCl
0.5% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20)
2 M urea
Store up to 3 months at 4°C

Protein A–Sepharose

Swell 0.4 g protein A–Sepharose CL-4B (Pharmacia) in 11.2 ml of 10 mM Tris·Cl (pH 7.5)/1 mM NaN_3/1 mg/ml bovine serum albumin for 2 hr to overnight at 4°C. Aspirate the supernatant from the settled beads and add fresh buffer to the original volume. Store up to 3 months at 4°C.

SDS/urea buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EDTA
1% (w/v) SDS
6 M urea
Store up to 1 month at room temperature

Spheroplast/stop solution, 2×

2 M sorbitol
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
40 mM NaN_3
40 mM NaF
Store up to 6 months at room temperature
Add 20 mM DTT from 1 M stock solution just before use

COMMENTARY

Background Information

Radiolabeling of proteins, lipids, and carbohydrates

Incorporation of ^{35}S into newly synthesized yeast protein can be achieved by labeling cells with either $^{35}SO_4^{2-}$ or a mixture of [^{35}S]methionine and [^{35}S]cysteine. While $^{35}SO_4^{2-}$ is less expensive and is primarily incorporated into protein in yeast (and not other macromolecules such as polysaccharides), there are drawbacks to its use. For example, standard minimal medium contains a high concentration of cold sulfate, which obviously competes with the isotope for incorporation into protein and also represses the synthesis of the high-affinity sulfate permeases (Cherest et al., 1997). Thus, efficient uptake of $^{35}SO_4^{2-}$ requires growing the cells in a specially prepared low-sulfate medium (LSM) to induce expression of the permeases. LSM is SD medium where chloride salts are used in place of sulfate salts. LSM is supplemented with 100 µM $(NH_4)_2SO_4$ for growing cells and 25 µM $(NH_4)_2SO_4$ for labeling. Even with these precautions, a relatively high concentration of $^{35}SO_4^{2-}$ (250 to 500 µCi/ml) is needed to achieve an adequate incorporation of radiolabels into protein. Therefore, labeling cells with [^{35}S]amino acids has become the method of choice because standard minimal media (SD, methionine-free) can be used, and it is not necessary to starve the cells for sulfate prior to labeling. Care should be taken when working with small proteins or peptides to check if methionine or cysteine residues are present in the sequence. If not, it is possible to label cells with [^{3}H]leucine (or other amino acid) in minimal medium (SD, leucine-free).

The methods described in this unit can also be used to study the phosphorylation of proteins in vivo. Cells can be labeled with $^{32}PO_4^{3-}$ in a low-phosphate medium in which the phosphate salts in LSM are replaced with the appropriate chloride salts and then supplemented with 50 µM potassium phosphate. Cells are typically labeled 20 to 60 min at 100 µCi/OD_{600} (Reneke et al., 1988).

A substantial amount of $^{32}PO_4^{3-}$ is incorporated into phospholipid to the extent that cells can be labeled in standard minimal medium if one wishes to examine the bulk cellular phospholipid content. Cells are grown for six generations in minimal medium (supplemented with 0.1 mM inositol and 1 mM choline) containing 10 µCi/ml $^{32}PO_4^{3-}$ for steady-state determinations, or pulse-labeled 10 to 60 min at 50 µCi/ml (Klig et al., 1985). After labeling, the lipids are extracted in a polar extraction solvent and separated by two-dimensional paper chromatography (Steiner and Lester, 1972).

Yeast glycoproteins can also be radiolabeled with [2-^{3}H]mannose, which is specifically incorporated into O- and N-linked oli-

gosaccharides as well as a small percentage of lipid-linked products. The label is not incorporated into other compounds because catabolism of mannose, specifically the conversion of mannose-6-phosphate to fructose-6-phosphate, results in the removal of the label as tritiated water (Huffaker and Robbins, 1982). It is also helpful to grow and label the cells in minimal medium containing 1% galactose as the carbon source rather than glucose. Galactose will not compete with mannose for entry into the cell. Cells can be labeled continuously during growth at 0.1 to 10 μCi/ml or pulse-labeled at 100 to 500 μCi/ml with the cells at 20 OD_{600}/ml (Byrd et al., 1982).

Labeling conditions

The medium that yeast are grown in, as well as other conditions such as temperature and the stage of growth, can have a dramatic influence on the expression of specific genes. For example, if one wishes to examine the protein encoded by a galactose-regulated gene in a pulse-chase experiment, it is essential to culture the cells in galactose rather than glucose as the carbon source. In addition, many genes involved in fermenting carbon sources other than glucose are repressed when cells are grown in glucose. To derepress these genes, shift cells to medium containing 0.1% glucose for 1 hr prior to labeling or simply culture the cells using a different carbon source. However, labeling is typically done in synthetic minimal medium containing glucose, and it is preferable to grow the cells in the same medium used during the labeling period to avoid inducing changes in cellular metabolism. Another important consideration is that cells in the logarithmic stage of growth incorporate substantially more ^{35}S into protein than stationary phase cells.

One of the advantages of working with yeast is the availability of a large number of mutants that exhibit a defect in some cellular process. For temperature-sensitive mutants, it is often desirable to incubate cells at the nonpermissive temperature prior to labeling in order to establish the mutant phenotype. The length of this incubation period depends on the nature of the mutation carried by the strain and usually has to be determined empirically. In the case of the *sec18-1* mutant, for example, protein transport is blocked almost immediately upon shifting to the nonpermissive temperature and the cells remain viable for ≥2 hr (Graham and Emr, 1991). As a control, it is important to label wild-type cells that have been subjected to the same temperature shifts to be certain that the mutation and not the change in temperature is the cause of any effects being observed.

Critical Parameters and Troubleshooting

Yeast colonies used to innoculate the overnight culture should be <2 weeks old, particularly if they were grown on a minimal plate in order to select for a plasmid carried by the strain. It is preferable that the overnight culture does not grow to stationary phase because many strains can take several hours to start doubling again when diluted in minimal medium. However, 4 to 5 hr of incubation after diluting in fresh medium is usually sufficient for stationary phase cells to start growing. Poor growth of some strains in minimal medium can result in low incorporation of ^{35}S amino acids into protein ($<5 \times 10^6$ cpm/OD_{600}). In this case, add 0.2% (w/v) Difco yeast extract to the growth (steps 1 to 3) and chase (step 8) media. If yeast extract is used, it is essential to wash the yeast twice with sterile water (~30 ml) before resuspending the cells in labeling medium lacking yeast extract (steps 4 to 7). It is also very important to use clean glassware and centrifuge tubes that are free of detergent at all steps. Poor incorporation of ^{35}S in labeling experiments is usually solved by carefully remaking the medium. Old (>1 month) or improperly stored [^{35}S] amino acids can also contribute to labeling problems.

In some cases, a biological process may not go to completion during the chase period. For example, some fraction of a protein that is normally completely secreted in 15 min may remain inside the cell even after a 60-min chase. This may indicate that the cells are being depleted of energy or some other critical nutrient. To remedy this problem, add yeast extract and additional glucose to a final concentration of 0.2% and 2% (w/v), respectively, to the chase (step 8).

Some integral membrane proteins will aggregate when the cell lysates are heated to 95°C in SDS-urea buffer. In this case, the protein will migrate near the top of the resolving gel or even remain in the stacking gel during electrophoresis. To help prevent this from occurring, heat the samples at 50°C for 5 min or 37°C for 10 min (steps 12, 20, and 21). These conditions do not inactivate cellu-

lar proteases as efficiently as the 95°C incubation, so addition of protease inhibitors may be necessary if degradation products are apparent on the autoradiograms.

The quality of the data produced by the procedures described here is primarily dependent on the quality of the antiserum and the expression level of the proteins being examined. Perhaps the most critical parameter is at step 14 of the Basic Protocol, to carefully transfer the supernatant from the clearing centrifugation step without carrying over any insoluble material. In addition, the "double" immunoprecipitation protocol (step 20), while not essential, usually provides immunoprecipitated proteins that are free of background bands. However, there are additional steps that can be taken to "clean up" immunoprecipitations (*UNIT 7.2*).

1. After step 15, add 75 µl of protein A–Sepharose to the supernatants, rock the samples for 1 hr in the cold room and microcentrifuge 10 min at full speed. Then transfer the supernatants to fresh tubes and proceed to step 16.

2. If a strain is available that does not express the protein of interest (e.g., a strain carrying a complete disruption of the gene encoding the protein of interest), it is possible to prepare a cold-competing cell lysate to add to the immunoprecipitations. To do this, pellet the unlabeled cells and resuspend them at 250 OD_{600}/ml in SDS/urea buffer. Add glass beads until they occupy ~80% of the sample volume, vortex for 1 min and then heat 4 min at 95°C. Add 9 vol of detergent IP buffer, mix well by vortexing, and centrifuge 10 min at 10,000 × *g*. The cold-competing cell lysate can then be added in place of the detergent IP buffer at steps 13 and 20 of the Basic Protocol.

3. Several pathogenic fungi have cell wall carbohydrate structures that are antigenic and are similar to those of *Saccharomyces cerevisiae*. Thus, animal serum can carry antibodies that cross-react with yeast glycoproteins causing background problems in immunoprecipitates. This problem is usually apparent in pre-immune control immunoprecipitations. Yeast mannan (Sigma) can be added at 0.5 mg/ml at steps 13 and 20 to help reduce this background. Using affinity purified antibodies may also help to ameliorate this potential problem.

4. Some investigators wash the immunoprecipitates an additional time (step 17) in 1% (v/v) 2-mercaptoethanol to reduce background. Likewise, the concentration of detergent, SDS, or urea can be increased in the wash buffers to provide greater stringency. However, depending on the antibodies being used, these steps can cause significant loss of the protein of interest from the immunoprecipitates.

Anticipated Results

The basic labeling procedure should result in the incorporation of 0.5 to 4 × 10^7 cpm/OD_{600}. Specific bands should be visible anywhere from 2 hr to 2 weeks after exposure of dried gels to X-ray film. At this level of incorporation, a protein that constitutes ~0.1% of cellular protein should be visible in 5 to 40 hr on the autoradiogram.

Time Considerations

Typically, 15 to 30 min are required on day 1 for innoculation of cultures. Cell labeling and preparation of cell lysates for immunoprecipitation requires ~4 hr on day 2, and the initial antibody incubations are usually performed overnight (step 16). The first wash steps require 30 to 90 min on the morning of the third day (step 17), and the second antibody incubations are for 4 to 7 hr (step 20). The second wash steps and gel loading requires 30 to 90 min. With this regimen, samples are electrophoresed overnight and the gel is processed the morning of the fourth day. Depending on the extent of labeling and the expression of the target protein(s), fluorography can take hours or weeks.

Literature Cited

Byrd, J.C., Tarentino, A.L., Maley, F., Atkinson, P.H., and Trimble, R.B. 1982. Glycoprotein synthesis in yeast. Identification of Man8GlcNAc2 as an essential intermediate in oligosaccharide processing. *J. Biol. Chem.* 257:14657-14666.

Cherest, H., Davidian, J.C., Thomas, D., Benes, V., Ansorge, W., and Surdin-Kerjan, Y. 1997. Molecular characterization of two high-affinity sulfate transporters in *Saccharomyces cerevisiae*. *Genetics* 145:627-635.

Graham, T.R. and Emr, S.D. 1991. Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a *sec18*(NSF) mutant. *J. Cell Biol.* 114:207-218.

Graham, T.R., Seeger, M., Payne, G.S., MacKay, V., and Emr, S.D. 1994. Clathrin-dependent localization of α1,3 mannosyltransferase to the Golgi complex of *Saccharomyces cerevisiae*. *J. Cell Biol.* 127:667-678.

Huffaker, T. and Robbins, P. 1982. Temperature sensitive yeast mutants deficient in asparagine linked glycosylation. *J. Biol. Chem.* 257:3203-3210.

Klig, L.S., Homann, M.J., Carman, G.M., and Henry, S.A. 1985. Coordinate regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae:* Pleiotropically constitutive opi1 mutant. *J. Bacteriol.* 162:1135-1141.

Marzluf, G.A. 1997. Molecular genetics of sulfur assimilation in filamentous fungi and yeast. *Annu. Rev. Microbiol.* 51:73-96.

Reneke, J.E., Blumer, K.J., Courchesne, W.E., and Thorner, J. 1988. The carboxy-terminal segment of the yeast alpha-factor receptor is a regulatory domain. *Cell* 55:221-234.

Steiner, M.R. and Lester, R.L. 1972. In vitro studies of phospholipid biosynthesis in *Saccharomyces cerevisiae. Biochim. Biophys. Acta* 260:222-243.

Contributed by Todd R. Graham
Vanderbilt University
Nashville, Tennessee

Metabolic Labeling and Immunoprecipitation of *Drosophila* Proteins

Cultured cells derived from the fruit fly, *Drosophila melanogaster*, provide a powerful experimental system for the biochemical and cell biological study of protein function. Given the great availability of mutant flies, the ability to culture cells from such mutants permits large amounts of cellular material of homogeneous genotype to be obtained for investigation. This unit describes a method for metabolic labeling and immunoprecipitation of proteins from cultured *Drosophila* cells (see Basic Protocol). The specific methods presented here work well for the wild-type S2M3 cell line, which was adapted from Schneider S2 cells to grow in Shields and Sang M3 medium. The Support Protocol describes the procedure for growth and maintenance of these cells. When combined with pulse-chase approaches and subcellular fractionation, this method permits the investigation of such questions as the turnover and cellular localization of specific proteins.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

LABELING AND IMMUNOPRECIPITATION OF PROTEINS FROM *DROSOPHILA* S2M3 CELLS

BASIC PROTOCOL

Wild-type S2M3 cells can be labeled using either purified [^{35}S]methionine or an ^{35}S-labeled *E. coli* hydrolysate as a source for radioactive amino acids. Following metabolic labeling, the cells are gently solubilized with detergent and the protein of interest is immunoprecipitated using specific antibodies and protein A–Sepharose (*UNIT 7.2*). The precipitated proteins can then be analyzed by SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

Materials

Wild-type *Drosophila* S2M3 cells (ATCC CRL 1963) growing in culture in M3 medium (see Support Protocol)
M3 growth medium (see recipe)
M3 labeling medium (see recipe)
[^{35}S]L-Methionine (>800 Ci/mmol) or ^{35}S-labeled *E. coli* protein hydrolysate (e.g., TRAN^{35}S-LABEL, EXPRE^{35}S^{35}S; ICN Biomedicals; >1000 Ci/mmol)
Activated charcoal
PBS (*APPENDIX 2A*), ice-cold
Cell lysis buffer (see recipe)
1:1 (v/v) protein A–Sepharose bead slurry in PBS (store up to 24 hr at 4°C)
Specific antibody against protein of interest
IP wash buffer: 0.1% (v/v) Triton X-100 in PBS (*APPENDIX 2A*) 1× SDS sample buffer (*APPENDIX 2A*)
35-mm tissue culture dishes
Large sealed container (e.g., Tupperware)
Cell scrapers
End-over-end rotator
Additional reagents and equipment for culturing *Drosophila* cells (see Support Protocol)

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

Contributed by Harish Radhakrishna
Current Protocols in Cell Biology (2001) 7.7.1-7.7.6

Metabolically label cells

1. Grow cells to ~60% to 70% confluence in 35-mm tissue culture dishes at 25°C in *Drosophila* M3 growth medium (see Support Protocol).

   The doubling time for these cells is ~24 to 26 hr.

   These cells do not require a humidified CO_2 atmosphere for growth and can be cultured in a cool enclosed space such as a bench cabinet or drawer.

   If cells are to be transfected, adjust plating such that they are at the proper confluence at the time of labeling.

2. Rinse cells briefly with two changes of 1 ml each of M3 labeling medium. Then incubate cells with 1 ml fresh M3 labeling medium for 30 min to 1 hr to deplete intracellular methionine pools.

3. Thaw [^{35}S]methionine or ^{35}S-labeled *E. coli* hydrolysate and prepare a 100 to 200 µCi/ml labeling solution in M3 labeling medium.

   CAUTION: Open stock vials of ^{35}S-labeled amino acids in a fume hood, since volatile ^{35}S-containing compounds can be released.

4. Remove M3 labeling medium from cells and replace with 0.5 ml of [^{35}S]methionine labeling solution. Incubate cells 30 min to 2 hr at 25°C in a large sealed container containing a moistened paper towel. Place a 35-mm petri dish that contains a small amount of activated charcoal into the sealed container to absorb any released ^{35}S-containing compounds.

   Alternatively, a filter that is impregnated with activated charcoal can be used.

   The time of labeling can be shortened to 5 to 10 min to accommodate pulse-chase protocols. However, the concentration of radioactive amino acids should be increased to 0.5 to 1 mCi/ml and the chase medium should contain 1 g/liter yeast hydrolysate, a 10-fold excess of unlabeled methionine, and 10% undialyzed FBS.

5. Remove [^{35}S]methionine labeling solution and rinse cells three to four times with 1 ml of ice-cold PBS. Combine the labeling solution and the washes in a 10-ml screw-cap centrifuge tube and centrifuge 5 min at 300 × *g*, 4°C, to collect any cells that may have detached during the labeling period. Remove the supernatant.

   CAUTION: Discard the used labeling solution, all of the wash solutions, and all contaminated disposable material as radioactive waste following the established guidelines set by the local radiation safety officer.

6. Add 2 to 3 ml of ice-cold PBS and scrape cells with a disposable cell scraper. Transfer cell suspension to a 10-ml screw-cap conical centrifuge tube. Combine with the cells collected from the labeling solution.

7. Centrifuge pooled cells for 5 min at 300 × *g*, 4°C, and discard supernatant.

Lyse cells and perform immunoprecipitation

8. Add 0.5 ml of ice-cold cell lysis buffer to each tube and incubate in ice/water slurry for 20 min.

9. Microcentrifuge 1 min at maximum speed, 4°C, to bring down insoluble material.

10. Transfer supernatant to a fresh tube and add 0.5 ml of additional cell lysis buffer.

11. Add 50 µl of a 1:1 protein A–Sepharose/PBS slurry and place tubes at 4°C on an end-over-end rotator for 0.5 to 1 hr to help "preclear" the supernatant and minimize nonspecific binding of cellular proteins to the protein A–Sepharose beads.

The protein A–Sepharose should be washed prior to use by resuspending 50 µl, the beads should then be resuspended in PBS at 1:1, in 1 ml of PBS and centrifuging 1 min at maximum speed, 4°C.

12. Microcentrifuge 1 min at maximum speed, 4°C, to bring down the beads, and transfer the supernatant to a fresh tube.
13. Add 50 µl of fresh 1:1 protein A–Sepharose suspension and add the appropriate amount of specific antibody against the protein of interest.

    The amount of antibody used will vary for each specific antibody preparation, but a good rule of thumb is to use a small excess of antibody to ensure a more quantitative recovery of the desired protein.
14. Incubate the tube at 4°C on an end-over-end rotator for 1 to 2 hr and pellet the immune complexes by microcentrifuging 1 min at maximum speed, 4°C.
15. Discard supernatant and wash the immune complexes by resuspending the beads in 1 ml of IP wash buffer and microcentrifuging again at maximum speed, 4°C. Repeat two more times.
16. Resuspend the immune complexes in 50 µl of 1× SDS-PAGE sample buffer and process for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

GROWTH AND MAINTENANCE OF CULTURED *DROSOPHILA* S2M3 CELLS

SUPPPORT PROTOCOL

Most *Drosophila* cells, including S2M3 cells, are conveniently cultured at 22°C to 25°C in tightly capped 25-cm^2 culture flasks and do not require a regulated CO_2 atmosphere. Thus, these cells can be cultured in a dry, cool place (e.g., a bench cabinet), provided that the temperature remains stable. Subculturing is accomplished by dislodging the cells from the flask by passing a gentle stream of medium over the cells with a pipet and transferring the cells to a new flask at the desired split ratio.

Materials

Drosophila S2M3 cells (ATCC CRL 1963)
M3 growth medium (see recipe)
25-cm^2 (T-25) tissue culture flasks with plug-seal caps

1. Aspirate off the old medium from a culture grown in a 25-cm^2 flask in a sterile tissue culture hood.
2. Tilt the flask and pass a gentle stream of medium (~2 to 3 ml) over the cells using a sterile cotton-plugged 5¼-in. Pasteur pipet fitted with a rubber bulb.

   Most Drosophila cell lines adhere loosely to the growth substrate and thus do not require treatment with trypsin to dislodge them from the substrate. Indeed, the use of trypsin should be avoided since many lines are sensitive to trypsin.

   Cotton-plugged pipets can be sterilized by autoclaving them in square metal canisters and are an inexpensive alternative to using disposable plastic pipets.
3. Pipet the cells up and down, gently, to disperse them into a homogeneous suspension.
4. Transfer cells at a split ratio of 1:3 to 1:10 into a new 25-cm^2 flask that contains 3 ml of fresh M3 growth medium warmed to room temperature.

   S2M3 cells, like most Drosophila cells, should not be subcultured at high split ratios since they do not grow well when sparsely plated.
5. Tightly seal the flasks and place in a dry, cool place (e.g., a bench cabinet) where the temperature is fairly constant at 22° to 25°C.

Cells can also be plated into 35-mm-diameter dishes containing 1.5 to 2 ml of M3 medium for individual experiments; however, the outsides of these dishes should be sealed with Parafilm.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX.***

Cell lysis buffer

1% (v/v) Triton X-100
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
300 mM NaCl
5 mM EDTA
20 mM iodoacetamide
Store up to 6 months at 4°C
Just before use, add the following protease inhibitors:
100 µg/ml PMSF
1 µg/ml aprotinin

M3 growth medium

Shields and Sang M3 insect medium (Sigma-Aldrich) without *L*-glutamine and with potassium bicarbonate
10% (v/v) fetal bovine serum (FBS)
100 U/ml penicillin
100 µg/ml streptomycin
Store up to 2 to 3 months at 4°C

M3 labeling medium

Prepare Shields and Sang M3 insect medium without *L*-glutamine, with potassium bicarbonate lacking methionine, cysteine, and yeast hydrolysate (Sigma-Aldrich). Supplement the medium with 100 U/ml penicillin and 100 mg/ml streptomycin. This medium can be supplemented with 10% FBS that has been dialyzed overnight against saline to remove unlabeled amino acids. Store up to 6 months to 1 year at –20°C

COMMENTARY

Background Information

Since the late 1960s, roughly 100 continuous cell lines have been established from both wild-type and mutant *Drosophila* flies. Refer to Echalier (1997) for an excellent and comprehensive treatise on *Drosophila* cell culture and for specific methods to establish cell lines from *Drosophila* flies. Of the many wild-type cell lines described, the most commonly used lines are Echalier and Ohanessian's Kc cell line (Echalier and Ohanessian, 1970) and Schneider's S1, S2, and S3 cell lines (Schneider, 1972). Both the Kc line and the S2 line are available from ATCC. Both of these continuous cell lines were established from early *Drosophila* embryos, as were most continuous *Drosophila* cell lines described thus far. Both wild-type and mutant *Drosophila* cells have been used to investigate such processes as insect hormone responses, cell adhesion, and EGF signaling.

Metabolic labeling and immunoprecipitation methods have been used to study protein function in a variety of cultured *Drosophila* cell lines, often in conjunction with transfection studies. As an example, Zak and Shilo (1990) utilized pulse-chase labeling and immunoprecipitation to study biosynthesis of the *Drosophila* EGF receptor homolog, DER. Buzin and Petersen (1982) profiled the pattern of protein expression induced during heat shock using metabolic labeling with [^{35}S]methionine and two-dimensional electrophoresis in Kc cells.

Critical Parameters and Troubleshooting

Three important factors must be considered when designing metabolic labeling experi-

ments for cultured *Drosophila* cells: composition of culture medium, growth properties of the cells, and methods of transfection.

The three most commonly used media for culturing *Drosophila* cells are Echalier-Ohanessian's D22 medium (Kc cells), Schneider's medium (S1, S2, and S3 cells), and Shields and Sang M3 medium (S2M3 cells, Shields' G1 line, Sang's *shibire* S4 line). Both Schneider's medium and Shields and Sang M3 medium are commercially available from Sigma-Aldrich; Schneider's medium is also available from Life Technologies. A methionine-, leucine-, and yeast extract–free formulation of M3 medium is also available from Sigma-Aldrich, which is useful for metabolic labeling. Most *Drosophila* media are supplemented with 5% to 20% fetal bovine serum. Since most formulations of *Drosophila* culture media contain either yeast extract or lactalbumin hydrolysate, it is important to omit these during metabolic labeling since they provide a source of unlabeled amino acids that could reduce labeling efficiency. All of these media formulations can be conveniently made in the laboratory (see Echalier, 1997), and thus can be prepared with these supplements omitted in addition to the particular amino acid to be labeled.

Another important consideration is the growth conditions of *Drosophila* cells. Almost none of the established cell lines require a regulated CO_2 atmosphere, and almost all grow optimally at 22°C to 25°C. Thus, most *Drosophila* cells can be conveniently cultured literally on the bench top. However, caution must be exercised to ensure that the temperature does not rise much above 27°C, since the *Drosophila* heat-shock response is induced at temperatures of 30° to 37°C (see Echalier, 1997). During the heat-shock response, the synthesis of most proteins is repressed in favor of heat-shock proteins (Buzin and Petersen, 1982). Also, many *Drosophila* cells adhere loosely to the growth substrate and often detach and grow in suspension. Thus, any floating cells should also be collected from the labeling medium during metabolic labeling experiments. Finally, one should avoid sparse plating (e.g., >1:20 split ratio), since *Drosophila* cells do not grow well at low cell densities.

Drosophila cells can be transfected using a modified calcium phosphate coprecipitation method (*UNIT 15.5*), lipid-based transfection reagents, and electroporation. The most widely used method of transfection is a modified calcium phosphate method where *N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) is substituted for HEPES as a buffering agent (Chen and Okayama, 1987). 2× BES-buffered saline (2× BBS) contains 50 mM BES, pH 6.95, 280 mM NaCl, and 1.5 mM Na_2HPO_4. It is filter-sterilized and stored at −20°C).This change gives an ~10-fold higher transfection efficiency (Cherbas et al., 1994). Lipofectin-mediated transfection has also been shown to work well for cultured Kc and S2 cells (Echalier, 1997). Finally, electroporation works well for Kc167 cells (Cherbas et al., 1994). With regard to expression vectors, two commonly used promoters for efficient expression of proteins in *Drosophila* cells are the actin5C promoter (Jaynes and O'Farrell, 1988) and the metallothionein promoter (Bunch et al., 1988). Proteins are efficiently expressed in many cells when their transcripts are driven by the activity of these promoters. Three expression vectors, which utilize these promoters, are commercially available from Invitrogen. They are useful for expressing proteins in *Drosophila* cells; pMT/V5-His A,B,C (Metallothionein promoter); pMT/BiP/V5-His A,B,C (metallothionein promoter); pAc5.1/V5-His A,B,C (actin5C promoter).

Finally, proper controls should always be utilized to assess the specificity of each antibody used for immunoprecipitation. If using polyclonal antiserum, preimmune serum should be used on a duplicate sample to assess the specificity of immunoprecipitation. If using monoclonal antibodies, an irrelevant antibody should be used from the same species as the specific antibody, to assess nonspecific binding. Also, a mock immunoprecipitation, where the primary antibody is omitted, should be included to control for nonspecific binding to the protein A–Sepharose beads. The primary antibody should be titrated to optimize the amount used for immunoprecipitation such that it is present only in slight excess, to minimize nonspecific binding.

Anticipated Results

In a typical experiment, using a 30-min to 1-hr labeling period as described (see Basic Protocol), clearly visible bands result on X-ray film after 16 hr to 24 hr exposure following SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*). The relative transient transfection efficiency using the modified calcium phosphate method ranges from 10% to 20%. Thus, immunoprecipitation of transiently transfected proteins is variable and should be optimized for each specific case. This protocol should also

work equally well for labeling and immunoprecipitation of endogenous proteins.

Time Considerations

It usually requires 1.5 to 3 hr to prepare cells and perform the labeling, depending on the time used for labeling. Approximately 30 min is required to prepare cell extracts and 2 to 4 hr is required for immunoprecipitation. Washing and processing may require an additional 1 hr.

Literature Cited

Bunch, T., Grinblat, Y., and Goldstein, L.S. 1988. Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila* melanogaster cells. *Nucl. Acids Res.* 16:1043-1061.

Buzin, C.H. and Petersen, N.S. 1982. A comparison of the multiple heat-shock proteins in cell lines and larval salivary glands by two-dimensional gel electrophoresis. *J. Mol. Biol.* 158:181-201.

Chen, C. and Okayama, H. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745-2752.

Cherbas, L., Moss, R., and Cherbas, P. 1994. Transformation techniques for *Drosophila* cell lines. *Methods Cell Biol.* 44:161-179.

Echalier, G. 1997. *Drosophila* Cells in Culture. Academic Press, San Diego.

Echalier, G. and Ohanessian, A. 1970. In vitro culture of *Drosophila melanogaster* embryonic cells. *In Vitro* 6:162-172.

Jaynes, J.B. and O'Farrell, P.H. 1988. Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* 336:744-749.

Schneider, I. 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster. J. Embryol. Exp. Morphol.* 27:353-365.

Zak, N.B. and Shilo, B.Z. 1990. Biochemical properties of the *Drosophila* EGF receptor homolog (DER) protein. *Oncogene* 5:1589-1593.

Key Reference

Echalier, G. 1997. See above.

An excellent and comprehensive description of Drosophila cell culture protocols that includes methods for generating both primary and continuous cell lines, media formulations, and experimental uses of Drosophila cells.

Contributed by Harish Radhakrishna
Georgia Institute of Technology
Atlanta, Georgia

Metabolic Labeling of Glycoproteins with Radioactive Sugars

Most proteins synthesized in the secretory apparatus of mammalian cells are glycosylated. These glycoproteins are components of secretory and endocytic organelles, including the endoplasmic reticulum, Golgi complex, plasma membrane, endosomes, and lysosomes. These glycoproteins are also important constituents of the extracellular space. The glycans of these molecules include *N*-linked chains, which are linked to asparagine residues, and *O*-linked chains, which are linked to serine and threonine residues. The latter group includes short mucin-type chains and large glycosaminoglycan chains.

For glycoproteins from cultured cells, structural studies of glycoprotein glycans are best carried out on radioactive molecules prepared by metabolic labeling. This unit describes two methods for the preparation of metabolically labeled glycoconjugates from cultured cells. The first describes pulse-chase labeling (see Basic Protocol). Typically, cells are labeled in a pulse incubation of 5 to 30 min, followed by a chase incubation of up to several hours. Molecules labeled in this way are suitable for initial studies to characterize the types of glycans on a glycoprotein and to gather information about their biosynthesis. As an alternative, a method for long-term labeling is also described (see Alternate Protocol). Because the cells are labeled over one or more generations, uniform labeling of all the sugar residues in a molecule can be achieved, with each residue of a particular type being labeled to the same specific activity. This uniform labeling is important for detailed structural studies; however, only glycoproteins with mature glycans can be prepared using this protocol, so it provides little information about biosynthesis.

The unit also contains support protocols for the liberation of *N*- (see Support Protocol 1) and *O*-linked (see Support Protocol 2) glycans from glycoproteins. These protocols can be used to prepare labeled glycans for characterization and detailed structural analysis.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by your institution's Radiation Safety Officer (also see *UNIT 7.1* and *APPENDIX 1D*).

PULSE-CHASE LABELING WITH RADIOACTIVE PRECURSORS

BASIC PROTOCOL

It is important to optimize labeling conditions to obtain maximum incorporation of the radioactive precursor. The radioactive precursor must be chosen with care. First, only some sugars—e.g., galactose (Gal), glucose (Glc), glucosamine ($GlcNH_2$), and mannose (Man)—are incorporated with high efficiency. Others are incorporated poorly because cells do not have pathways for their efficient uptake and utilization. Second, many sugar precursors undergo extensive metabolism within cells. This metabolism may result in the loss of radioactivity from the precursor or conversion to molecules that cannot be incorporated into glycans. The precursor may also be converted into other sugars that are incorporated, resulting in glycans with several different types of radioactive residues. Consequently, choosing an appropriate radioactive precursor will help to ensure that glycans with the label in the desired sugar residues are obtained. In addition, the labeling conditions should be chosen to optimize precursor incorporation into the glycoproteins of interest. For sugars, this is usually achieved by labeling in medium with reduced glucose concentration. Similarly, for sulfate and phosphate labeling, medium deficient in these compounds is used. Finally, it is necessary to make sure that the precursor is

Contributed by Martin D. Snider
Current Protocols in Cell Biology (2002) 7.8.1-7.8.11

incorporated throughout the labeling period and to ensure that the cells remain healthy. These issues are discussed in detail elsewhere in the unit (see Commentary).

Metabolic labeling may be carried out either in a tissue culture incubator with a CO_2/air atmosphere or in an air atmosphere. In the former case, bicarbonate-buffered medium is used. In the latter, HEPES-buffered medium is employed, which is usually more convenient for short labelings. In addition, because incubations of radioactive samples do not need to be carried out in a tissue culture facility, use of HEPES-buffered medium limits the amount of lab space that must be certified for use with radioactive materials.

Materials

Growth medium: tissue culture medium supplemented with dialyzed serum (see recipe; use the serum concentration required for growth of the cultured cells being studied)

Tissue culture cells

Labeling medium (see recipe)

Radioactive precursor: [^{35}S]sulfate, [^{32}P]orthophosphate, or sugar labeled with either ^{3}H or ^{14}C

Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice cold

Additional reagents and equipment for experiments optimizing label incorporation (see Critical Parameters)

1. Perform small-scale preliminary experiments to determine incubation conditions that give optimal incorporation of label as described (see Critical Parameters). Based on these experiments, choose the appropriate: (1) radioactive precursor, (2) number of cells, (3) labeling medium, (4) volume of labeling medium, (5) concentration of radioactive precursor, and (6) labeling time.
2. Prepare tissue culture cells in either subconfluent monolayers by plating cells in growth medium at high density 1 to 2 days prior to the experiment, or suspensions.

   Suggested plating densities are given in Table 7.8.1.
3. Prepare labeling medium from sterile stocks and warm to 37°C.

   For most experiments with radioactive sugars, this will be Glc-free MEM with nonessential amino acids supplemented with ~0.1 mg/ml Glc and 2% to 5% (v/v) dialyzed serum. For labeling with sulfate and phosphate, use medium deficient in unlabeled precursor. Prepare enough medium for washing and labeling the cells with radioactive precursor (Table 7.8.1).

Table 7.8.1 Parameters for Plating and Labeling Cultured Cells

Culture	Cell number[a]	Wash	Label[b]	Chase[c]
Cell monolayers				
16 mm (24-well plate)	1.4×10^5	0.2 ml	0.1 ml	0.2 to 0.4 ml
35 mm	6×10^5	0.5 ml	0.3 ml	0.5 to 1.5 ml
60 mm	1.5×10^6	1 ml	0.7	1 to 3 ml
100 mm	4×10^6	3 ml	2 ml	3 to 10 ml
Cell suspensions[d]	Not applicable	5×10^6/ml	1.3×10^7/ml	0.5 to 2×10^6/ml

[a]For the Basic Protocol, cell monolayers are plated at the indicated densities 1 to 2 days prior to labeling. The density may have to be adjusted for individual cell lines. When long chases are used the cell density should be reduced. For the long labeling periods required for the Alternate Protocol, lower cell numbers should also be used.

[b]These volumes are suggested for experiments with labeling times of 1 hr or less, such as those of the Basic Protocol. Larger volumes must be used for longer pulse labelings and for the long incubations required in the Alternate Protocol.

[c]For chase incubations, the smaller volumes can be used for short chases. For longer incubations, larger volumes will be required to furnish enough nutrients for the cells.

[d]For cell suspensions, use the indicated cell densities to calculate the appropriate volumes.

4. Prepare a stock of radioactive precursor in warm labeling medium.

   Many sugars are supplied in ethanol solution. If this is the case, remove the appropriate volume of solution from the source vial and transfer to a test tube. Place the tube in a beaker of warm water (30° to 40°C) and dry the ethanol under a stream of nitrogen. Dissolve the radioactive sugar in the appropriate volume of labeling medium (Table 7.8.1) and warm to 37°C. For short labeling incubations, this solution need not be sterile. For longer incubations, filter sterilize using a disposable plastic syringe and filter.

   Best results are usually obtained with precursor of the highest available specific activity. For ^{3}H-labeled sugars, this is usually 30 to 60 Ci/mmol (1.1 to 2.2 TBq/mmol).

   See Critical Parameters, Optimizing labeling conditions, for a discussion of the rationale behind the choice of medium.

5a. *For cell monolayers:* Aspirate the medium and rinse the plate twice with labeling medium.

   Suggested volumes are provided in Table 7.8.1.

5b. *For suspension cells:* Centrifuge the cells 5 min at 200 × *g*, room temperature. Aspirate the supernatant and suspend in warm labeling medium (Table 7.8.1). Repeat once and centrifuge.

6. Add the labeling medium containing the radioactive precursor. For cell suspensions, suspend the cells in the medium. Incubate at the appropriate temperature in a CO_2/air or air atmosphere, depending whether the medium is buffered with bicarbonate or HEPES, respectively.

   The incubation time should have been determined previously from pilot experiments (see Commentary and step 1).

7. Remove the labeling medium, saving an aliquot for analysis of precursor utilization, if desired.

   It may be possible to reuse this solution to label another cell sample if the supply of nutrients and radioactive precursor are not exhausted during the incubation. In this case, the solution may be used immediately or stored frozen.

8. (*Optional*) If a chase incubation is to be performed, wash the cells once in warm growth medium containing normal levels of Glc. Add warm growth medium and incubate at 37°C for the desired time. Remove the medium, saving it for sample isolation if the glycoprotein of interest is secreted. Chill the cells on ice, wash them several times with ice-cold phosphate-buffered saline (PBS), and harvest.

   This growth medium can contain either HEPES or bicarbonate as a buffer, depending on whether or not the chase incubations will be done in a CO_2/air atmosphere. Table 7.8.1 lists suggested volumes.

   For studies of glycan biosynthesis samples will usually be collected at several chase times.

   In most cases, the glycoprotein of interest is isolated by immunoprecipitation (UNIT 7.2).

LONG-TERM LABELING WITH RADIOACTIVE PRECURSOR

ALTERNATE PROTOCOL

This protocol is useful for obtaining molecules with uniformly-labeled glycans, where all the sugar residues of a particular type are labeled to the same specific activity. This uniform labeling is crucial if the glycans are to be used for detailed structural studies. An additional advantage of this technique is that cells can be labeled with precursors that are taken up poorly by cultured cells, namely fucose (Fuc), mannosamine (ManN), xylose (Xyl), N-acetylglucosamine (GlcNAc), N-acetylmannosamine (ManNAc), and sialic acids. As described above (see Basic Protocol), it is important to optimize the labeling

conditions to obtain maximum incorporation and to ensure that the cells are healthy (see Commentary). The labeling may be carried out in either bicarbonate-buffered medium in a CO_2-containing atmosphere or in HEPES-buffered medium in air.

Additional Materials *(also see Basic Protocol)*

Disposable plastic syringe and 0.2-µm syringe filter

1. Carry out small-scale preliminary experiments to optimize the incorporation of label (see Critical Parameters; also see Basic Protocol, step 1).
2. In growth medium, prepare cell suspensions, or prepare subconfluent monolayers by plating cells 1 to 2 days prior to the experiment.

   The cells must be sparse enough that they will survive the entire labeling period. Suggested plating densities are given in Table 7.8.1.

3. Prepare a stock of radioactive precursor in warm growth medium as described (see Basic Protocol, step 4). Sterilize using a disposable plastic syringe and 0.2-µm syringe filter.

4a. *For monolayer cells:* Aspirate the growth medium and replace with medium containing radioactive precursor.

4b. *For suspension cells:* Centrifuge cells 5 min at 200 × *g*, room temperature. Aspirate the supernatant and suspend in labeling medium containing radioactive precursor.

   Suggested volumes are given in Table 7.8.1.

5. Incubate 12 to 48 hr at the appropriate temperature in a CO_2/air or air atmosphere, depending whether HEPES or bicarbonate is the buffer in the labeling medium.

   The incubation time should have been determined previously from pilot experiments (see Commentary and step 1).

6. Remove the labeling medium and save if the glycoprotein of interest is secreted. Otherwise, if required, save an aliquot for further analysis.
7. For isolation of cellular glycoproteins, chill the cells by placing on ice. Wash the cells several times with ice-cold PBS and harvest.

   In most cases the glycoprotein of interest will be isolated by immunoprecipitation (UNIT 7.2).

SUPPORT PROTOCOL 1

ENZYMATIC RELEASE OF *N*-LINKED GLYCANS FROM GLYCOPROTEINS

This protocol can be used to prepare *N*-linked glycans from labeled glycoproteins using peptide *N*-glycosidase F, an enzyme that cleaves intact *N*-linked glycans from polypeptides. All mammalian *N*-linked glycans are substrates for this enzyme (Tarentino et al., 1985; Chu, 1986). Since the enzyme will not use native glycoproteins as substrates, the glycoprotein substrates are first denatured with SDS. Excess nonionic detergent (e.g., NP-40) is added prior to digestion with the enzyme to prevent denaturation of the glycosidase.

Materials

Sample (e.g., see Basic Protocol or Alternate Protocol)
1% (w/v) SDS/0.1 M EDTA/0.5 M 2-mercaptoethanol
200 mM sodium phosphate, pH 8.6 (*APPENDIX 2A*)
10% (w/v) NP-40

Peptide *N*-glycosidase F (PNGase F, *N*-glycanase) from *F. meningosepticumi* or recombinant enzyme expressed in *E. coli* (Glyko or Roche Diagnostics)

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2*)

1. Immunoprecipitate the protein of interest from the sample (*UNIT 7.2*).

   The protein of interest may be collected on a solid support, such as agarose beads. Alternatively, proteins can be precipitated from medium or cell lysates as described below (see Support Protocol 2, steps 1 to 3).

   Lysate from 1×10^7 cells can be processed using this protocol.

2. Add 20 µl of 1% (w/v) SDS/0.1 M EDTA/0.5 M 2-mercaptoethanol to the immunoprecipitate, or to the pellet of precipitated protein, in microcentrifuge tubes. Mix well and heat 5 min at 95°C. Cool to room temperature and microcentrifuge briefly at maximum speed to collect the liquid at the bottom of the tube.

3. Add 20 µl of 200 mM sodium phosphate, pH 8.6, 15 µl of 10% (w/v) NP-40, and 25 µl water. Mix.

4. Add 2 U of peptide *N*-glycosidase F. Incubate 18 hr at 37°C.

5. Mix and microcentrifuge briefly at maximum speed to collect the liquid at the bottom of the tube.

   This solution contains free glycans. These can be characterized by chromatography or subjected to detailed structural analyses. The glycans may be frozen at −20°C and stored indefinitely; their lifetime is limited only by the decay of the radioisotope.

RELEASE OF *O*-LINKED GLYCANS FROM GLYCOPROTEINS

SUPPORT PROTOCOL 2

Incubation of glycoproteins with alkaline sodium borohydride cleaves *O*-linked glycans from glycoproteins and reduces them to the corresponding oligosaccharide alcohols (Carlson, 1968). These released glycans are suitable for structural analysis. The protocol can be performed on culture medium, cell lysates, or immunoprecipitated proteins attached to a solid support (e.g., agarose beads).

Materials

Sample (e.g., see Basic Protocol or Alternate Protocol)
20% (w/v) trichloroacetic acid (i.e., 20 g TCA in 89.4 ml H_2O), ice cold
80% (v/v) aqueous acetone, ice cold
1 M $NaBH_4$/50 mM NaOH
4 M acetic acid
Cation exchange resin: AG 50-X8 (Bio-Rad), H^+ form
Methanol
Nitrogen gas

Disposable chromatography column (e.g., Bio-Rad Poly-Prep columns)
Glass test tubes
Lyophilizer or SpeedVac evaporator

NOTE: Steps 1 to 3 apply only for whole cells or cell extracts. For immunoprecipitates on a solid support, begin at step 4.

1. Precipitate proteins from the sample in microcentrifuge tubes by adding an equal volume of ice-cold 20% (w/v) trichloroacetic acid and incubating 1 hr on ice. Microcentrifuge 10 min at 14,000 × *g*, 4°C. Discard the supernatant.

   If the sample contains a nonionic detergent such as NP-40, it will precipitate at this step.

2. Add 200 µl ice-cold 80% (v/v) aqueous acetone. Vortex, microcentrifuge, and discard the supernatant. Repeat.

   This step will extract the trichloroacetic acid and nonionic detergent from the sample.

   CAUTION: *The wastes from immunoprecipitation are mixed chemical wastes and should be handled appropriately (see Radiation Safety Officer and APPENDIX 1D).*

3. Let the tubes air dry at room temperature to remove most of the acetone. Take care not to let the sample dry completely.

4. Add 200 µl of 1 M $NaBH_4$/50 mM NaOH. Incubate 16 hr at 45°C. Vortex several times during the incubation.

   For immunoprecipitates, the $NaBH_4$ solution is added to the pellet of immunoprecipitated glycoprotein on a solid support.

5. Add 16.6 µl of 4 M acetic acid to destroy excess $NaBH_4$.

   The sample may foam as hydrogen gas is released from decomposition of the $NaBH_4$.

6. Prepare 0.9 ml cation exchange resin in disposable columns. Rinse with 3 ml water.

7. Load each sample onto a column and collect the flowthrough in glass test tubes. Rinse the column with 0.9 ml water, pooling this with the flowthrough.

8. Dry the samples using a lyophilizer or Speed-Vac evaporator.

9. Dissolve each residue in 50 µl methanol. Place the tubes in warm water and evaporate the methanol under a stream of nitrogen. Repeat this step 3 more times.

   This treatment converts the boric acid derived from the $NaBH_4$ to methyl borate, which is removed by evaporation.

10. Dissolve the glycans in water and transfer the liquid to a microcentrifuge tube.

    This solution contains free glycans. These can be characterized by chromatography or subjected to detailed structural analyses. The glycans may be frozen at −20°C and stored indefinitely. Their lifetime is limited by the decay of the radioisotope.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Dialyzed serum

Place serum in dialysis tubing and dialyze fetal bovine serum against a 20-fold excess of 0.15 M NaCl at 4°C with stirring (*APPENDIX 3D*). Change the dialysis buffer after 12 and 24 hr. Remove the serum from the dialysis bag and filter sterilize into sterile containers. Store up to 1 month at 4°C or up to 1 year at −80°C.

Labeling medium

Formulas for media deficient in glucose, sulfate, or phosphate are given in Table 7.8.2. These are used for labeling with radioactive sugars, sulfate, or phosphate, respectively. To prepare these media, add the liquid components to water (80% of the final volume). Add the dry components and stir until dissolved. Use either 25 mM sodium bicarbonate or 20 mM HEPES as a buffer, depending on whether or not the medium will be used in the presence of CO_2. Because the calcium and magnesium salts can form insoluble precipitates, add them last after the other salts have dissolved. Antibiotic and antimycotic agents (e.g., penicillin and streptomycin) can be added from liquid concentrates if desired. Adjust the pH to 7.1 to 7.2

Table 7.8.2 Deficient MEM with Nonessential Amino Acids for Incorporation of Sugar, Phosphate, or Sulfate Precursors

Ingredient	Glucose free	Phosphate free	Sulfate free
NaCl	3.4 g	3.4 g	3.4 g
KCl	0.2 g	0.2 g	0.2 g
$NaH_2PO_4 \cdot 2\ H_2O$	79 mg	—	79 mg
$CaCl_2 \cdot 2\ H_2O$	0.13 g	0.13 g	0.13 g
$MgSO_4 \cdot 7\ H_2O$	0.1 g	0.1 g	—
$MgCl_2 \cdot 6\ H_2O$	—	—	0.1 g
Phenol Red	5 mg	5 mg	5 mg
MEM 100× vitamins	5 ml	5 ml	5 ml
MEM 100 × nonessential amino acids	5 ml	5 ml	5 ml
MEM 50× amino acids (with L-glutamine)	10 ml	10 ml	10 ml
D-glucose	—	0.5 g	0.5 g
Buffer[a]			
$NaHCO_3$	1.1 g	1.1 g	1.1 g
HEPES	2.38 g	2.38 g	2.38 g

[a]Use sodium bicarbonate if incubations will be performed in the presence of CO_2; otherwise, use HEPES.

with 1 M HCl or 1 M NaOH and add water to bring the volume to 500 ml. Because this medium is prepared from separate components, the initial pH may be farther from the desired value than for most powdered media. Sterilize by filtration into sterile 100-ml bottles using a 0.2-μm bottletop filter. Store up to 3 months at 4°C.

Labeling with radioactive precursor is usually carried out in medium with a reduced concentration of the precursor and related compounds. Some deficient media can be purchased from suppliers. These media are also readily prepared from standard chemicals and liquid concentrates of MEM components, which are available from most suppliers of tissue culture media (e.g., Life Technologies, Sigma-Aldrich). Other reagents should be tissue-culture grade.

COMMENTARY

Background Information

The glycans are important constituents of glycoproteins and proteoglycans. They provide important functional groups of mature molecules. The glycans are also important in glycoprotein folding and in glycoprotein transport along the secretory and endocytic paths. Glycoprotein glycans are synthesized in a complex process that begins in the endoplasmic reticulum and ends in the Golgi complex (Kornfeld and Kornfeld, 1985; Lidholt, 1997; Perez-Vilar and Hill, 1999). The biosynthesis of these glycans is not only interesting in its own right, it has also been instrumental in understanding the compartmentation and function of the secretory pathway (see *UNITS 15.1 & 15.2*).

Initial studies of the glycans on a glycoprotein of interest are usually performed by studying molecules metabolically labeled in the protein backbone with radioactive amino acids (e.g., [^{35}S]methionine; *UNIT 7.1*). Typically a protein is labeled in a pulse-chase protocol. The protein is then immunoprecipitated (*UNIT 7.2*) and the size of the protein is monitored by SDS gel electrophoresis (*UNITS 6.1 & 15.2*). As the glycans are assembled during transport through the ER and Golgi, the apparent size of the glycoprotein increases on the gel. Additional information may be obtained using several modifications of this experiment.

First, treatment of cells with inhibitors of glycosylation provides information about the nature of the glycans. For example, tunicamycin, which inhibits the synthesis of *N*-linked glycans, has been widely used to test whether these glycans are added to proteins (Elbein, 1987).

Second, glycoproteins may be treated with specific endo- and exoglycosidases after they are isolated from the cells (*UNIT 15.2*). For example, the endoglycosidase peptide *N*-glycanase, which cleaves *N*-linked glycans from the peptide backbone can be used to test for the presence of these structures. Endoglycosidase H is also used widely because it cleaves immature N-glycans but not mature complex-type structures. Similarly, exoglycosidases, including neuraminidases, galactosidases, and mannosidases can be used both separately and in combination to test for the presence of sialic acid, Gal, and Man residues at the nonreducing ends of glycans. In all these cases, the effects of the treatments are assessed by comparing the mobility of treated and untreated samples by gel electrophoresis (*UNIT 15.2*). Some of these procedures can also be carried out on unlabeled proteins detected by immunoblotting with a specific antibody (*UNIT 6.2*).

Although much can be learned about protein glycosylation by this approach, these methods cannot provide information about the details of glycan structure. This analysis requires molecules with radioactively-labeled glycans.

Critical Parameters

Choosing a radioactive precursor

The choice of a radioactive label is determined by many issues. Obviously, the label must be a precursor of one of the sugar residues in the glycan of interest. The complex pathways of sugar uptake and metabolism and their relation to glycoprotein synthesis must also be considered (Fig. 7.8.1).

First, the position of the sugar in the glycan should be considered. If the goal is to study the maturation of a glycan during biosynthesis, sugars close to the reducing end of the structure are the desirable label, because these will be added first. All subsequent glycans in the pathway will be labeled. Conversely sugars that are added late in the pathway will only be found in mature structures.

Second, the ability of cells to take up and incorporate radioactive precursors must be considered. Some precursors are taken up rather poorly. These include fucose, ManN, Xyl, GlcNAc, ManNAc and sialic acids. Other sugars are taken up efficiently. This group includes Gal, Man, and GlcN; however, their uptake and incorporation is competitive with unlabeled Glc in the medium. Consequently, the incorporation of sugars in this group can be significantly improved by labeling in medium with reduced Glc. On the other hand, labeling in Glc-free medium must be avoided because Glc starvation can affect the synthesis of sugar precursors by the cells, which results in the synthesis of glycoproteins with altered glycans (Rearick et al., 1981).

Third, the conversion of intermediates within the cells must be considered carefully. This problem can be a serious one for sugar labeling because many precursors undergo extensive metabolism within mammalian cells. The metabolic pathways are summarized in Figure 7.8.1. For example, labeled GlcN is incorporated into glycoproteins as labeled GlcNAc, GalNAc, and sialic acids. This situation is different from the one encountered when cells are labeled with amino acids. In the latter case, the essential amino acids are effective precursors (e.g., ^{35}S-methionine) because these compounds cannot be synthesized and cells carry out only limited conversions. In some cases the problem of sugar metabolism can be overcome by choosing precursors whose radioactivity is lost upon conversion to other sugars. For example [2-^{3}H]Man is incorporated into glycans primarily as Man, with a small fraction as Fuc. The other pathway for metabolism of this sugar produces unlabeled fructose 6-phosphate, with loss of the radioactivity as $^{3}H_2O$. Specific labeling has also been obtained using cultured cell lines mutated in sugar metabolism. For example, the Chinese hamster ovary (CHO) cell mutant ldlD is defective in UDP-Gal epimerase (Kingsley et al., 1986), which interconverts both the UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc pairs. As a consequence, radioactive Gal and GalN are specific labels in these cells because they are not converted to other sugars and because de novo synthesis is blocked. They are incorporated with high specific activity as Gal and GalNAc, respectively.

Optimizing labeling conditions

Obtaining the maximum incorporation into the glycoprotein of interest requires optimizing the conditions used for metabolic labeling. The optimal conditions will vary depending on which protocol is used (see Basic Protocol and Alternate Protocol). The conditions used will depend on the abundance of the glycoprotein that is being studied. Labeled glycans from abundant glycoproteins can be prepared in adequate amounts from small cultures. Conversely, glycoproteins of low abundance will require larger cultures, larger amounts of label, and

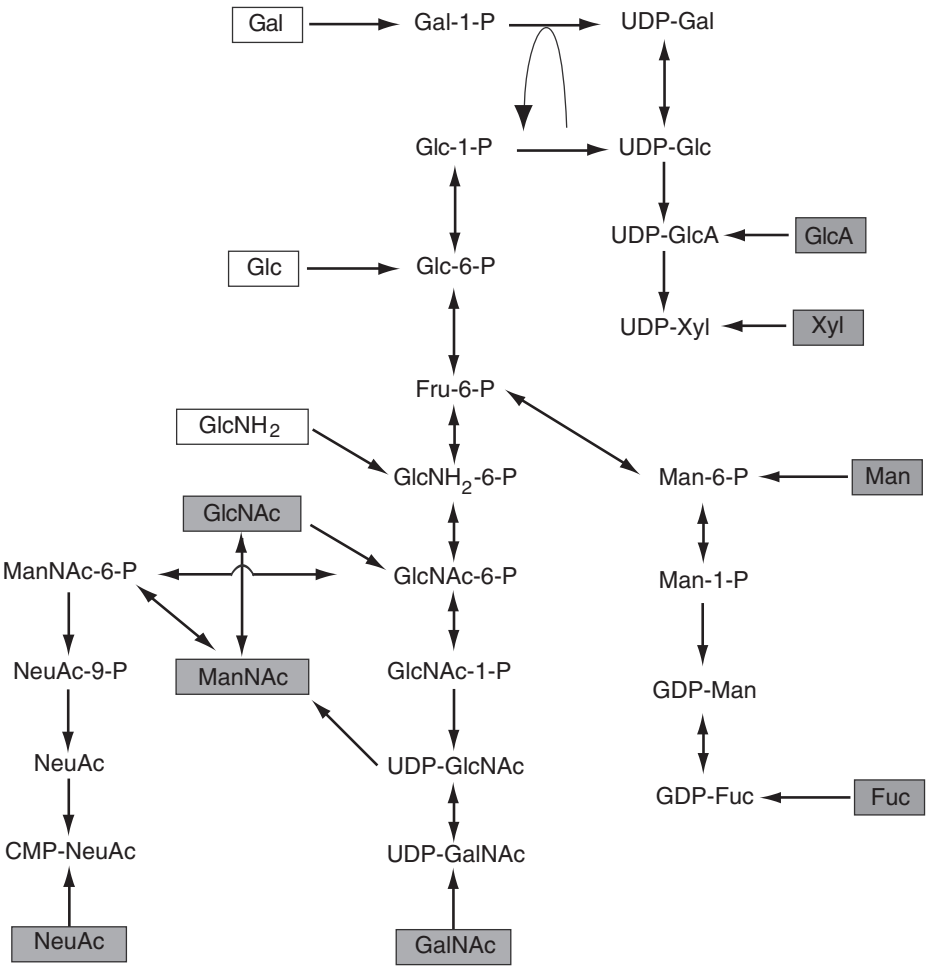


Figure 7.8.1 Pathways of sugar metabolism in mammalian cells. Compounds in boxes are precursors that can be taken up and incorporated into glycoconjugates. Pathways for conversion into the nucleotide sugar donors for biosynthesis are shown. Sugars in open boxes compete with Glc for uptake and incorporation. These are incorporated efficiently and can be used in the labeling procedures (see Basic Protocol and Alternate Protocol). Sugars in shaded boxes do not compete with Glc. They are incorporated less efficiently and should be used only in long-term labeling experiments (see Alternate Protocol). Abbreviations used: Fru, fructose; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; $GlcNH_2$, glucosamine; Man, mannose; ManNAc, N-acetylmannosamine; NeuAc, N-acetylneuraminic acid (a sialic acid); P, phosphate; UDP, uracil diphosphate; Xyl, xylose.

longer labeling periods. The following issues must be considered.

1. *Cell number*. It is advisable to label the largest number of cells possible. For cell monolayers, this is usually achieved with subconfluent cultures grown by plating cells at high density 1 to 2 days before use. For the Alternate Protocol, the cells must not become overgrown during the long labeling incubation. Suggested cell numbers are given in Table 7.8.1.

2. *Labeling volume*. Maximum incorporation is usually achieved when cells are incubated in the smallest volume possible. For pulse labeling (see Basic Protocol), the author usually labels cell suspensions at concentrations up to 1.3×10^7 cell/ml. For monolayer cells, the author uses minimum volumes of 0.3, 0.7, and 2 ml for 35-, 60-, and 100-mm dishes, respectively (Table 7.8.1). These volumes are appropriate for incubations of 1 hr or less. Larger volumes must be used for longer labelings.

3. *Medium*. As discussed above, Glc in the medium competes with the uptake and incorporation of many sugars. Consequently, for the

Basic Protocol, it is advisable to use these precursors in medium with reduced Glc (0.1 mg/ml). Lowering the Glc concentration further is not advisable because Glc starvation can affect glycan synthesis (Rearick et al., 1981). Serum that has been dialyzed to remove low-molecular-weight constituents should also be used. Preparation of dialyzed serum and medium with reduced Glc is described in Reagents and Solutions. The long-term labeling in the Alternate Protocol is usually carried out in complete medium to provide sufficient nutrients.

The cells must not exhaust the supply of Glc or other nutrients in the medium. As a rule of thumb for determining the nutrient supply in the medium, the author estimates that a confluent culture (~1×10^6 cells/ml) in normal medium (1 g/liter Glc) will exhaust its Glc in ~24 hr. This can be used to estimate the length of time available for cells under labeling conditions taking into account the increased cell concentration and reduced Glc concentration in the medium.

4. *Concentration of radioactive precursor*. Small-scale labeling can be carried out to determine the optimal concentration and specific activity (Diaz and Varki, 1995). Radioactive precursor is added to all cultures. In addition, cultures are supplemented with increasing concentrations of unlabeled precursor. The incorporation of radioactivity is determined and plotted against the total concentration of precursor. At low concentrations, incorporation will not depend strongly on concentration. At higher concentrations, incorporation will decrease linearly with concentration. The break point on the curve is the concentration that is saturating for uptake and incorporation. Choose a concentration 1.5- to 2-fold higher than this for incorporation.

5. *Labeling time*. Labeling time is determined by several factors. For pulse labeling (see Basic Protocol) the labeling time must be short enough to generate a homogeneous cohort of labeled molecules at the end of the pulse; however, longer labelings will yield greater incorporation. Consequently, the goal is to select the longest labeling time that still yields a set of molecules with similar glycans in a single cellular location. Most glycoproteins leave the ER within 10 to 40 min of synthesis and traverse the Golgi within a few minutes. The overall time required to traverse the secretory pathway is 30 to 90 min. These times can be used as a guide for choosing pulse labeling times. For long-term labeling (see Alternate Protocol), the turnover rate of the glycoprotein under study is a key factor. Since most cellular proteins have lifetimes of 12 to 24 hr, this limits the yield in long labelings, when the degradation of labeled protein approaches the synthesis of new molecules.

Small-scale pilot experiments should be performed to find optimal labeling conditions. The incorporation should increase with time, demonstrating an adequate supply of nutrients and radioactive precursor. For the Basic Protocol, glycan structure should be monitored to demonstrate that a homogeneous cohort of molecules is generated at the end of the pulse. Finally, the survival of the cells should be monitored by microscopy, trypan blue exclusion, and growth (*UNIT 1.1*) after returning to normal medium to ensure that the cells survive under the labeling conditions. Once optimal conditions have been defined, the amount of labeled glycoprotein recovered should be used to determine how to scale up conditions for preparative experiments.

Anticipated Results

The amount of labeled protein depends on the planned experiments. If the protein will be analyzed by electrophoresis, then only 200 to 1000 cpm/sample is required. If structural studies will be carried out, larger amounts (1×10^3 to 1×10^4 cpm) are required. The amount of labeled protein recovered will depend on the issues discussed in the preceding section. In addition to these factors, the choice of radioisotope is important. Because of their longer half lives, ^{3}H- and ^{14}C-labeled compounds have lower specific activity than ^{35}S and ^{32}P that are used to label proteins and nucleic acids. As an example, ~1×10^4 cpm were recovered in labeled transferrin receptor when 5×10^6 K562 human leukemia cells were labeled with 150 µCi [2-^{3}H]mannose for 45 min in 375 µl (Snider and Rogers, 1986).

As in all pulse-labeling experiments, the incorporation of radioactivity into products occurs with a lag time, while the radioactive label enters intracellular precursor pools. The turnover time of these pools determines the lag time that precedes incorporation into the product of interest. This turnover will also determine the continued incorporation at the beginning of the chase as radioactivity in the precursor pools is incorporated into product. This issue can be an important one for labeling glycans. For example, during the incorporation of [^{3}H]Man into *N*-linked glycans, the mannose is transferred via GDP-Man and dolichol-linked intermedi-

ates. Because the turnover time of these pools is 5 to 10 min in mammalian cells, there is a significant lag time before incorporation begins. In addition, incorporation continues to increase for 5 to 10 min after the beginning of a chase.

Time Considerations

Pulse-chase experiments take up to 24 hr, depending on the length of the chase. In a typical experiment, the pulse-chase labeling can be completed, cells can be lysed, and immunoprecipitation of the desired protein can be begun in a single day. Ten to twenty samples can be labeled and processed at once by one person. If very short pulses and chases are performed, it may not be possible to perform the required manipulation on large numbers of samples. In this case, it may be necessary to do the labeling on two smaller sets of samples. Long-term labeling experiments require 12 to 48 hr. Release of N- and O-linked glycans each requires ~24 hr. Groups of 10 to 20 samples can be handled easily by one person.

Literature Cited

Carlson, D.M. 1968. Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins. *J. Biol. Chem.* 243:616-626.

Chu, F.K. 1986. Requirements of cleavage of high mannose oligosaccharides in glycoproteins by peptide N-glycosidase F. *J. Biol. Chem.* 261:172-177.

Diaz, S. and Varki, A. 1995. Metabolic radiolabeling of animal cell glycoconjugates. *In* Current Protocols in Protein Science (J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, and P. Wingfield, eds.) pp. 12.2.1-12.2.15. John Wiley and Sons, New York.

Elbein, A. D. 1987. Glycosylation inhibitors for N-linked glycoproteins. *Methods Enzymol* 138:661-709.

Kingsley, D.M., Kozarsky, K.F., Hobbie, L., and Krieger, M. 1986. Reversible defects in O-linked glycosylation and LDL receptor expression in a UPD-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell* 44:749-759.

Kornfeld, R. and Kornfeld, S. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.

Lidholt, K. 1997. Biosynthesis of glycosaminoglycans in mammalian cells and in bacteria. *Biochem. Soc. Trans.* 25:866-870.

Perez-Vilar, J. and Hill, R.L. 1999. The structure and assembly of secreted mucins. *J. Biol. Chem.* 274:31751-31754.

Rearick, J.I., Chapman, A., and Kornfeld, S. 1981. Glucose starvation alters lipid-linked oligosaccharide biosynthesis in Chinese hamster ovary cells. *J. Biol. Chem.* 256:6255-6261.

Snider, M. D., and Rogers, O. C. 1986. Membrane traffic in animal cells: Cellular glycoproteins return to the site of Golgi mannosidase I. *J. Cell Biol.* 103:265-275.

Tarentino, A.L., Gomez, C.M., and Plummer, T.H., Jr. 1985. Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochemistry* 24:4665-4671.

Key References

Diaz and Varki, 1995. See above.

Methods for glycoprotein labeling in cultured cells.

Yurchenco, P.D., Ceccarini, C., and Atkinson, P.H. 1978. Labeling complex carbohydrates of animal cells with monosaccharides. *Methods Enzymol.* 50:175-204.

An excellent discussion of monosaccharide incorporation, with methods for determining the specific activity of incorporated label.

Contributed by Martin D. Snider
Case Western Reserve University
Cleveland, Ohio

Analysis of Oxidative Modification of Proteins

Reactions between protein molecules and reactive oxygen species (ROS) often lead to the modification of certain amino acid residues such as histidine, lysine, arginine, proline, and threonine, forming carbonyl derivatives. Carbonylation of proteins has thus often been employed for the quantification of generalized protein oxidation. Besides carbonylation, other types of oxidative damage that have been investigated in depth are the modifications of cysteine, tyrosine, and aspartate, or asparagine residues. Except for cysteine residues, whose oxidation is often determined by the loss of protein thiol groups, quantification of oxidative damage to tyrosine, and aspartate residues is usually carried out by the measurement of specific oxidation products such as dityrosine, nitrotyrosine (when nitrogen species are the oxidants), and isoaspartate.

This unit provides a variety of protocols for the determination of the protein oxidation products indicated above. A method for the quantification of protein carbonyls using spectrophotometric determination with a carbonyl specific reagent, 2,4-dinitrophenylhydrazine (DNPH), is described (see Basic Protocol 1), as are the details of immunoblot detection using anti-DNP antibodies to detect the carbonylation of specific proteins (see Support Protocol 1). Radiolabeling detection of total and specific proteins using tritiated sodium borohydride, another quantification method, is also included (see Basic Protocol 2 and Support Protocol 2) in this unit. Procedures are outlined for the selective determination of protein thiol groups radiolabeled with [^{14}C] iodoacetamide followed by gel electrophoresis (see Basic Protocol 3). Procedures for dityrosine measurement by GC/MS are described (see Basic Protocol 4), as are preparation of dityrosine standards and nitrotyrosine detection by competitive ELISA (see Support Protocols 3 and 4). Methods for the detection of isoaspartate formation in proteins/peptides, analyzed by a methyl transfer reaction catalyzed by protein-L-isoaspartyl methyltransferase (PIMT), using [^{3}H]methyl-*S*-adenosyl-L-methionine (SAM) as the methyl donor are given (see Basic Protocol 5), and the details of the different buffers needed for the electrophoretic separation of proteins containing isoaspartate residues are described (see Support Protocol 5).

NOTE: Tissue collection and preparation must be carried out in buffers supplemented with antioxidants, such as 100 μM diethylenetriaminepentaacetic acid (DTPA) and 1 mM butylated hydroxytoluene (BHT). The use of antioxidant buffers may be especially critical for the detection of trace amounts of oxidized products formed during aging or under oxidative stress. In addition, all buffers should be bubbled with nitrogen before use.

SPECTROPHOTOMETRIC QUANTITATION OF PROTEIN CARBONYLS USING 2,4-DINITROPHENYLHYDRAZINE

BASIC PROTOCOL 1

Protein carbonyl groups can specifically react with 2,4 dinitrophenylhydrazine (DNPH) to generate protein conjugated hydrazones (protein-DNP) which have a peak absorbance around 360 nm. The use of DNPH thus provides an index for the quantification of protein carbonyl content in protein mixtures or purified proteins.

Contributed by Liang-Jun Yan and Rajindar S. Sohal

Current Protocols in Cell Biology (2002) 7.9.1-7.9.25

Materials

DNPH solution (see recipe)
Protein solution
2 M HCl
20% (v/v) trichloroacetic acid solution, ice-cold (TCA; see recipe)
1:1 (v/v) ethanol/ethyl acetate
0.2% (w/v) SDS/20 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
Bicinchoninic acid protein assay kit (BCA; Pierce Co.)
Bovine serum albumin (BSA)

Benchtop centrifuge
Branson 2200 sonicator

1. Add 200 µl of DNPH solution to 1 ml of protein solution. Incubate the mixture at room temperature for 60 min. Prepare a blank by adding 200 µl of 2 M HCl without DNPH to one sample. Incubate under the same conditions.

   For tissue samples, start with 0.5 to 1.0 mg/ml protein. If higher protein concentrations are used for DNPH treatment, it will be very hard to dissolve the pellet after washing off the free DNPH (see Critical Parameters and Troubleshooting).

2. Add 1.2 ml of 20% trichloroacetic acid (TCA) solution to the DNPH-treated protein solution and blank, then incubate on ice 10 min. Centrifuge the sample in a benchtop centrifuge for 10 min at 750 to 1000 × *g*, room temperature.

3. Wash the pellet by adding 3 ml 1:1 (v/v) ethanol/ethyl acetate, followed by centrifugation in a benchtop centrifuge for 10 min at 750 to 1000 × *g* at room temperature. Sonicate at full power, room temperature, in a Branson 2200 sonicator until the pellet is completely broken up.

4. Repeat step 3 twice. Solubilize the final pellet in 1 ml 0.2% (w/v) SDS/20 mM Tris·Cl, pH 6.8.

   If the carbonyl content of lipoproteins is to be determined, use a denaturing buffer of 3% (w/v) SDS/150 mM sodium phosphate buffer, pH 6.8, to dissolve the final pellet (see Critical Parameters and Troubleshooting).

5. After the pellet is completely dissolved, pipet 100 µl of the protein solution for protein assay using the BCA kit. Use bovine serum albumin (BSA) as the protein standard.

   Initially, 6 M guanidine·HCl was used to dissolve the protein pellet, but samples dissolved in such a high concentration of guanidine·HCl are not suitable for further analysis by other techniques such as polyacrylamide gel electrophoresis (SDS-PAGE, UNIT 6.1).

6. Scan the sample in a spectrophotometer from 320 nm to 450 nm. Use the peak absorbency around 360 nm to calculate the carbonyl content. Use the protein samples treated with HCl, but not with DNPH, as blanks.

   The extinction coefficient (ε) for DNPH is 22,000 $M^{-1}cm^{-1}$. Protein carbonyl content (nmol/mg protein) = (absorbance × 10^6/22,000)/mg protein = absorbance × 45.45/mg protein.

7. Identify specific carbonylated proteins by SDS-PAGE (*UNIT 6.1*) and immunoblotting (see Support Protocol 1, also see *UNIT 6.2*).

IMMUNOBLOT DETECTION OF PROTEIN CARBONYLS

SUPPORT PROTOCOL 1

To identify specific proteins that are carbonylated, DNPH-treated proteins can be further separated by gel electrophoresis according to standard techniques. Once the proteins are electrotransferred from the gel to a PVDF membrane, protein-bound DNP in individual proteins can be probed by the use of anti-DNP antibodies (also see *UNIT 6.2*).

Materials

DNPH-treated proteins (Basic Protocol 1)
5% (w/v) nonfat dry milk in Tris-buffed saline with Tween-20 (TBST; see recipe)
Primary antibody (anti-DNP antibody; Sigma)
Secondary antibody: may be horseradish peroxidase–conjugated; select on the basis of nature of primary antibody
Tris-buffered saline with and without Tween-20 (TBS and TBST; see recipe)
ECL detection solution (Amersham)

Minigel electrophoresis unit and transfer unit (Bio-Rad; also see recipe for minigel recipes in *UNIT 6.1*)
Immobilon-P membranes (Millipore)
UV-transparent plastic wrap
X-ray film

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), staining gels with Coomassie blue (*UNIT 6.6*), and electroblotting proteins onto membranes (*UNIT 6.2*)

1. Separate DNPH-treated proteins by SDS-PAGE in a minigel electrophoresis unit (*UNIT 6.1*).

   DNPH-treated protein samples dissolved in 0.2% SDS/20 mM Tris buffer, pH 6.8 can be directly mixed with SDS-PAGE loading buffer and analyzed by SDS-PAGE.

   Two gels are usually run simultaneously, one for protein staining by Coomassie blue (UNIT 6.6), and the other for immunoblot detection.

2. Electroblot proteins from polyacrylamide gel to Immobilon-P membrane using a transfer unit per manufacturer's instructions.

   For an efficient transfer, perform tank transfer rather than semidry transfer (see UNIT 6.2 for additional details).

3. Block the membrane by immersing it in 5% (w/v) nonfat dry milk TBST for at least 1 hr at room temperature.

4. Wash the membrane with at least 50 ml TBST for 10 min. Repeat twice.

5. Immerse the membrane in primary antibody solution for at least 1 hr.

   Primary antibody solution is prepared by diluting the primary antibody in TBST solution containing 0.2% (w/v) BSA. Dilution of anti-DNP antibody is highly variable. A range of 1:1,000 to 1:10,000 is recommended (see Critical Parameters and Troubleshooting). Overnight incubation at 4°C usually gives better results. Do not reuse the antibody.

6. Wash the membrane with at least 50 ml TBST three times, each time for 10 min.

7. Incubate the membrane with secondary antibody at room temperature for at least 3 hr.

   Antibody is diluted in TBST solution containing 0.2% (w/v) BSA. Secondary antibody dilution usually ranges between 1:10,000 and 1:25,000 (see Critical Parameters and Troubleshooting). Do not reuse the antibody.

8. Wash the membrane three times with at least 50 ml TBST, each time for 10 min.

9. Wash the membrane twice with at least 50 ml TBS (no Tween 20), each time for 5 min.

10. Immerse the membrane with ECL detection solution for 1 min at room temperature.

    ECL detection solution comes with the enhanced chemiluminescence (ECL) detection kit from Amersham.

11. Drain off excess detection solution and wrap membrane in plastic wrap. Gently smooth out any air bubbles. Immediately expose the membrane to X-ray film.

    Film is usually exposed to the membrane for 0.5 to 2 min.

    During immunoblot detection of protein carbonyls, it is preferable to include both a positive and a negative control on the SDS-PAGE. A positive control can be BSA oxidized by an oxidant such as hypochlorite followed by DNPH treatment as described (see Basic Protocol 1). A negative control is usually the sample not treated with DNPH. The inclusion of a positive control will indicate whether the anti-DNP antibody reacts properly, while the inclusion of a negative control will identify any proteins that cross-react with anti-DNP antibody. Occurrence of a cross-reaction between a protein and the antibody indicates that immunoblotting is not DNP-dependent for this particular protein. In such a case, anti-DNP antibodies from a different source may have to be tried.

BASIC PROTOCOL 2

QUANTITATION OF PROTEIN CARBONYLS DERIVATIZED WITH TRITIATED SODIUM BOROHYDRIDE

When protein concentration is a limiting factor, or when the studied proteins, such as heme-containing proteins, have absorption maxima around 360 nm, protein carbonyls cannot be determined by the use of DNPH. In such cases, tritiated sodium borohydride, which is able to convert protein carbonyl groups into protein-bound ethanol groups, can be used. Because of the introduction of tritium onto oxidized proteins, the method provides a quantitative measurement of protein carbonyl content.

Materials

Protein solution
3 M Tris·Cl, pH 8.6 (*APPENDIX 2A*)
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
[^{3}H]$NaBH_4$ working solution (see recipe)
2 M HCl
20% (v/v) trichloroacetic acid solution, ice-cold (TCA; see recipe)
1:1 (v/v) ethanol/ethyl acetate
0.2% (w/v) SDS/20 mM Tris·Cl , pH 6.8 (*APPENDIX 2A)*
0.5% (w/v) SDS/0.1 M NaOH
BCA protein assay kit (Pierce)
Scintisafe Plus 50% cocktail (Fisher Scientific.)

Benchtop centrifuge
Scintillation vials

CAUTION: Perform all incubations in a hood as tritium gas may be released during the reaction. When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

1. Prepare a 1-ml reaction mixture containing 86 mM Tris·Cl, pH 8.6 (add from 3 M stock), 0.86 mM EDTA (add from 5 M stock), 50 mM tritiated sodium borohydride (add from working solution described in Reagents and Solutions), and 0.5 to 1.0 mg of protein.

    86 mM Tris buffer, pH 8.6 is used as the incubation buffer, as the authors have found that if phosphate buffer is used, proteins can be degraded during incubation.

EDTA is included in the reaction mixture so that any potential carbonyl-independent labeling can be prevented.

2. Incubate 30 min at 37°C. Add 200 µl of 2 M HCl to stop the reaction. Add an equal volume of 20% TCA (final concentration 10%) to precipitate the proteins.

   HCl also serves to render the alkaline reaction mixture acidic; otherwise, 10% TCA (final concentration) would not be sufficient to precipitate all proteins.

3. Keep the solution on ice for 10 min and then centrifuge 10 min at 750 to 1000 × *g*, 4°C, in a benchtop centrifuge.

4. Wash the protein pellet with 1:1 ethanol/ethyl acetate at least three times.

5. Dissolve the final pellet in 1 ml 0.5% SDS/0.1 M NaOH. Determine protein concentration for each sample using a BCA protein kit.

   Determination of protein concentration is necessary because the recovery after washing with ethanol/ethyl acetate is rarely 100% (see Critical Parameters and Troubleshooting).

6. Count the radioactivity of each sample by adding 4 ml Scintisafe Plus 50% cocktail to the sample and counting on a scintillation counter, or quantitate by electrophoretic separation and excision of a specific protein band (see Support Protocol 2).

   For the determination of the carbonyl content in a protein mixture, the sample can be counted right after the addition of scintillation cocktail. For the quantitation of the carbonyl content of a specific individual protein, the protein has to be separated from other proteins by means of SDS-PAGE, then processed as described in Support Protocol 2.

GEL ELECTROPHORETIC QUANTITATION OF PROTEIN CARBONYLS DERIVATIZED WITH TRITIATED SODIUM BOROHYDRIDE

SUPPORT PROTOCOL 2

To quantitate the carbonyl content of a specific protein in a protein mixture, as identified by immunoblot technique, protein samples derivatized with tritiated sodium borohydride can be separated by SDS-polyacrylamide gel electrophoresis (*UNIT 6.4*). The target protein band on the gel can then be excised and dissolved in 30% hydrogen peroxide, and incorporated radioactivity measured by the use of a scintillation counter.

Additional Materials *(also see Basic Protocol 2)*

Tritiated protein sample (see Basic Protocol 2)
30% (v/v) hydrogen peroxide

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and staining gels with Coomassie blue R-250 (*UNIT 6.6*).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

1. Label protein samples with [^{3}H]$NaBH_4$ (see Basic Protocol 2). Mix the labeled protein sample with SDS-PAGE loading buffer.

   TCA precipitation and washing with organic solvent is not needed.

2. Heat the sample 5 min at 95°C. Separate the tritiated protein sample by SDS-PAGE.

3. Stain the gel with Coomassie blue R-250 (*UNIT 6.6*) and destain until the bands can be clearly visualized.

4. Excise each band with a razor blade.

5. Put the excised gel bands in scintillation vials; incubate for 2 hr at 60°C to dry the gel slices.
6. Add 1 ml of 30% hydrogen peroxide to each vial and incubate for 24 hr at 60°C.
7. Add 4 ml Scintisafe Plus 50% cocktail and keep the vials at 4°C for at least 12 hr.

   Use Scintisafe Plus 50% cocktail because it is specifically designed for gel slices with solubilizers such as hydrogen peroxide. Other cocktails, such as Scintiverse BD, become emulsive after mixing with the solubilized gels, which can decrease counting efficiency.
8. Count radioactivity with a scintillation counter.

BASIC PROTOCOL 3

GEL ELECTROPHORETIC ANALYSIS OF PROTEIN THIOL GROUPS LABELED WITH [^{14}C] IODOACETAMIDE

To identify specific proteins in a tissue sample or a protein mixture exhibiting loss of thiol groups due to oxidative damage, the thiols can be labeled with [^{14}C] iodoacetamide and analyzed by gel electrophoresis. The gels, after being dried onto filter papers, can be autoradiographed. Loss of thiol groups is inversely correlated with radioactive intensity shown by autoradiography (*UNIT 6.3*). Oxidized proteins thus identified can be further quantitated by the use of liquid scintillation counting technique, exactly as described in Support Protocol 2 of this unit.

Materials

Protein sample
1% (w/v) SDS/0.6 mM Tris·Cl buffer, pH 8.6 (*APPENDIX 2A*)
2-mercaptoethanol, neat
Nitrogen gas
500 mM [^{14}C] iodoacetamide, 1 μCi/ml (Amersham)
500 mM nonradiolabeled iodoacetamide
SDS-PAGE gels for Bio-Rad Mini gel system (see recipe):
  10% resolving gel
  4% stacking gel
10% (v/v) trichloroacetic acid (see recipe)

Whatman 3MM filter paper
X-ray film

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and staining gels with Coomassie blue R-250 (*UNIT 6.1*).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

1. Dissolve protein sample (up to 1 mg) in 1 ml of 1% SDS/0.6 M Tris·Cl, pH 8.6.

   The inclusion of 1% SDS is meant to completely denature the protein and expose buried thiol groups.
2. Add 10 μl of 2-mercaptoethanol and incubate under nitrogen gas for 3 hr at room temperature.
3. Add 100 μl colorless 500 mM [^{14}C]iodoacetamide solution to a final concentration of 1 μCi/ml, using 500 mM freshly prepared, nonradiolabeled iodoacetamide, in distilled water, to dilute the [^{14}C]iodoacetamide to the proper level of radioactivity. Incubate at 37°C for 30 min with constant gentle agitation.

   CAUTION: *Iodoacetamide is intrinsically unstable in light, especially in solution; reactions should therefore be carried out in the dark.*

Iodoacetamide solutions must be colorless. A yellow color indicates the presence of iodine, which will quickly oxidize protein thiol groups and prevent labeling. Free iodine can also modify tyrosine residues.

The use of nonradiolabeled iodoacetamide will ensure an excess of the reagent over total thiol groups and a complete labeling.

4. Resolve proteins by SDS-PAGE with a 10% separating gel and 4% stacking gel using the Laemmli gel system (*UNIT 6.1*). After proteins are adequately separated, fix the gel in 10% TCA for 60 min. Stain with Coomassie blue R-250 and destain (*UNIT 6.6*).

   The sample incubation prior to loading should be done at 95°C for 5 min.

   With method described in this protocol, actual radioactivity counts can be obtained for any given samples, which can be used for statistical data analysis. A phosphor imager may not be appropriate in terms of quantitation, as it only gives the relative band intensity and thus may not be able to detect slight differences between samples.

5a. *For unknown protein samples:* Dry gels onto Whatman 3MM filter paper and expose to X-ray film for up to two weeks at −70°C.

   To identify target proteins, purify the protein using two-dimensional gel electrophoresis (UNIT 6.4) and perform N-terminal microsequencing, followed by a computer database search.

5b. *Given target proteins:* Proceed to Support Protocol 2, step 4.

QUANTIFICATION OF PROTEIN DITYROSINE RESIDUES BY MASS SPECTROMETRY

BASIC PROTOCOL 4

In this protocol, trace amounts of dityrosine residues formed in proteins due to oxidative damage are first released by acid hydrolysis. The released dityrosine residues are then analyzed by mass spectrometry following derivatization with heptaflurorbutyric anhydride/ethyl acetate. The assay entails the use of radiolabeled dityrosine as the internal standard in order to be quantitative.

Materials

Tissue sample
o,*o*′-dityrosine internal standards, labeled and unlabeled (see Support Protocol 3)
Nitrogen gas
6 M HCl/1% (v/v) benzoic acid/1% (v/v) phenol
Argon
10% and 0.1% (v/v) TCA solution (see recipe)
50 mM $NaHPO_4$/100 μM diethylenetriamine pentaacetic acid (DTPA), pH 7.4
25% methanol
1:3 (v/v) HCl/*n*-propyl alcohol
1:4 (v/v) pentafluoropropionic anhydride/ethyl acetate
Ethyl acetate
n-propanol
0.1% (w/v) trifluoroacetic acid (TFA)

Supelclean SPE reversed-phase C-18 column (Supelco)
Hewlett Packard 5890 gas chromatography equipped with a 12-m DB-1 capillary column interfaced with Hewlett-Packard 5988A mass spectrometer

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

1. Dry tissue sample under nitrogen and add 50 pmol ^{13}C-labeled *o,o′*-dityrosine as an internal standard.
2. Hydrolyze the sample in a tube flushed with an inert gas (i.e., argon) at 110°C for 24 hr in 0.5 ml of 6 M HCl/1% benzoic acid/1% phenol.
3. Supplement the sample with 50 µl 10% (v/v) TCA solution and pass over a reversed-phase C-18 column that has been previously washed with 12 ml of 50 mM $NaHPO_4$/100 µM diethylenetriamine pentaacetic acid (DTPA), pH 7.4, followed by 12 ml of 0.1% TFA.
4. Elute amino acids with 25% methanol and dry under vacuum for derivatization.

   Dityrosine is stable to acid hydrolysis and ~80% of the internal standard should be recovered from the C-18 column using this procedure.
5. Convert amino acids to carboxylic acid esters by the addition of 200 µl of 1:3 (v/v) concentrated HCl/*n*-propyl alcohol. Heat for 10 min at 65°C.
6. Add 50 µl of 1:4 (v/v) pentafluoropropionic anhydride/ethyl acetate to prepare pentafluoropropionyl derivatives of the amino acids. Heat at 65°C for 30 min.
7. Dry the derivatized samples under nitrogen and redissolve in 50 µl of ethyl acetate.
8. Analyze 1 µl of the sample using a gas chromatograph interfaced with a mass spectrometer with extended mass range. Set the injector and ion source temperature at 250°C and 150°C, respectively. Obtain full scan mass spectra and selected ion monitoring with the *n*-propyl heptafluorobutryl and the *n*-propyl pentafluoropropionyl derivatives of both authentic and isotopically labeled dityrosine in the negative-ion chemical ionization mode with methane as the reagent gas.

   The mass spectrum of the n-propyl heptafluorobutyryl derivative of dityrosine includes a small molecular ion at mass-to-charge (m/z) 1228 (M^-) and prominent ions at m/z 1208 (M^--HF) and 1030 (M^--$CF_3(CF_2)CHO$). Use m/z 1208 to quantify dityrosine.
9. For the analysis of tyrosine, dilute one aliquot of the derivatized amino acid (from step 7) 1:100 (v/v) with ethyl acetate. Inject 1 µl of the sample into the gas chromatograph with a 1:100 split prior to mass analysis. Maintain the initial column temperature of 120°C for 1 min and then increase to 220°C at 10°C/min.

   The mass spectrum of the n-propyl heptafluorobutyryl derivative of tyrosine reveals prominent ions at m/z 595 (M^--HF) and 417 [M^--$CF_3(CF_2)_2CHO$]. Use m/z 417 to quantify tyrosine.

   To ensure that interfering ions are not coeluting with the analyte, monitor the ratio of ion currents of the two most abundant ions of tyrosine and dityrosine in all analyses. Baseline-separate authentic and radiolabeled standards to exhibit retention times identical to those of analytes derived from tissue samples.

SUPPORT PROTOCOL 3

PREPARATION OF *o,o′*-DITYROSINE STANDARD

Dityrosine can be formed by horseradish peroxidase–catalyzed oxidation of tyrosine in the presence of hydrogen peroxide. Dityrosine produced in the mixture can then be purified by chromatographic methods. If radiolabeled dityrosine is to be made, radiolabeled tyrosine should be used as a starting material.

Materials

Horseradish peroxidase (grade I; Boehringer Mannheim)
0.1 M borate buffer, pH 9.1 (see recipe)
5 mM L-tyrosine (Sigma) or [$^{13}C_6$] L-tyrosine (Cambridge Isotope Laboratories) in 0.1 M borate buffer, pH 9.1
30% (v/v) H_2O_2
2-mercaptoethanol
0.01 M NaOH (*APPENDIX 2A*)
200 µM borate buffer, pH 8.8: diluted from 0.2 M borate buffer (see recipe) with H_2O
2.75 × 19.5–cm DEAE cellulose chromatography column (Bio-Rad)
20 µM $NaHCO_3$, pH 8.8 (see recipe)
Concentrated and 100 mM formic acid
100 mM NH_4HCO_3

Benchtop centrifuge
4 × 34.5–cm BioGel P-2 column (200-4-mesh; Bio-Rad)

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

1. Mix 10 mg horseradish peroxidase with 500 ml of 5 mM tyrosine solution prepared in 0.1 M borate buffer, pH 9.1.

   Use [$^{13}C_6$]tyrosine to prepare o,o′-[$^{13}C_{12}$]dityrosine if radiolabeled dityrosine is needed. Follow guidelines on the use and disposal of radioactive materials.

2. Add 142 µl of 30% H_2O_2 and swirl the solution briefly. After incubation at room temperature for 30 min, add 175 µl of 2-mercaptoethanol to the reaction mixture. Immediately freeze the solution in liquid nitrogen and lyophilize to dryness.

3. Dissolve the lyophilized substance in 250 ml of distilled water and adjust the pH to 8.8 with a few drops of 0.01 M NaOH. Load the solution onto a 2.75 × 19.75-cm DEAE column that has been pre-equilibrated with 20 µM $NaHCO_3$, pH 8.8. Elute the column with 200 µM borate buffer, pH 8.8.

   Under these conditions, tyrosine and dityrosine will elute in the breakthrough fractions.

4. Pool and lyophilize the dityrosine-containing solutions. Resuspend the lyophilized material in 20 ml of cold water and centrifuge for 15 min at 1000 × *g* in a benchtop centrifuge. Extract the pellet with 15 ml of water and combine the two supernatants. Adjust the pH to 7.0 with formic acid and incubate at 0°C overnight.

5. Remove any precipitate by centrifuging for 10 min at 1000 × *g* in a benchtop centrifuge, 4°C. Load the supernatant on a BioGel P-2 column equilibrated with 100 mM NH_4HCO_3. Elute the column with 100 mM NH_4HCO_3 with a flow rate 40 ml/hr. Monitor the elution of dityrosine at 370 nm.

   Under these conditions, dityrosine elutes earlier than tyrosine. Therefore, fractions constituting the first peak should be collected. Dityrosine elution can also be monitored by the use of a fluorometer (λ_{ex} = 280 nm; λ_{em} = 300 nm); however, the collected fractions need to be diluted before measurement.

6. Collect dityrosine fractions and lyophilize. Dissolve the lyophilized dityrosine in 20 ml of 100 mM formic acid. Adjust the pH to 2.5 by adding concentrated formic acid. Remove any precipitate that is formed during the pH adjustment by microcentrifuging

for 10 min at 1000 × *g*, room temperature, in a benchtop centrifuge. Load the solution to a BioGel P-2 column (same as above) equilibrated with 100 mM formic acid. Elute the column with 100 mM formic acid, lyophilize the dityrosine-containing solution, and store at −20°C.

The yield of dityrosine should be around 20%.

SUPPORT PROTOCOL 4

ANALYSIS OF PROTEIN-BOUND NITROTYROSINE BY A COMPETITIVE ELISA METHOD

The availability of anti-nitrotyrosine antibodies provides a very sensitive method for the detection of protein-bound nitrotyrosine in tissue samples, namely enzyme-linked immunosorbant assay (ELISA). In this protocol, nitro-BSA is coated onto ELISA plates, and nitrotyrosines are quantitated by the use of anti-nitrotyrosine antibodies. Competition is accomplished by adding either a potentially nitrated protein sample or a known amount of nitrotyrosine (in the form of nitro-BSA) as a standard. Each competes with the coated nitrated proteins for antibody binding. The amount of antibody that binds to the coated nitro-BSA is inversely proportional to the amount of nitrated protein (sample or standard) present in the solution added to the well of the plate.

Materials

10 µg/ml nitro-bovine serum albumin (nitro-BSA; Alexis Biochemicals) in plate coating buffer
Nitro-BSA standard (see recipe)
ELISA buffers (see recipe):
  Plate coating buffer
  1× phosphate-buffered saline/Tween 20 (PBST)
  Blocking buffer
  1× diethanolamine (DEA) buffer
Protein sample
Primary antibody: mouse anti-nitrotyrosine antibodies (Upstate Biotechnology)
Secondary antibody: rabbit anti-mouse IgG conjugated with alkaline phosphatase
Tris-buffered saline/Tween-20 (TBST; see recipe)
1 mg/ml *p*-nitrophenyl phosphate (5-mg tablets; Sigma) in DEA buffer (see recipe for ELISA buffer)
96-well ELISA plates
Plastic wrap
Plate reader

Prepare the ELISA plate

1. The day before the assay, coat each of the wells of a 96-well ELISA plate that are to be used to assay protein samples with 100 µl of 10 µg/ml nitro-BSA in plate coating buffer (1 µg nitro-BSA/well). Leave the wells to be used for blanks empty.

   Plate coating may have to be optimized (see Critical Parameters and Troubleshooting).

2. Wrap the plate with plastic wrap and incubate at 4°C overnight.

3. Add 100 µl of each protein sample (1 to 10 µg/ml) to be assayed to 100 µl primary antibody diluted 1:500 with blocking buffer, and incubate at 4°C overnight. Add 100 µl of this solution to the designated well. Prepare nitro-BSA competitor the same way for a standard curve.

   The nitrotyrosine in BSA and the protein sample will compete with the coating antigen (nitro-BSA in this case) for antibody binding.

The standard curve range is determined with serial dilutions of nitro-BSA between 10 µg/ml to 1 µg/ml. This will give a curve whose middle part is linear and flanked by a plateau curve on either side. The points that signal the start and the end of the linear part should be taken as the range for the standard curve.

A series of dilutions of the protein sample may need to be tried in order to have readings that will fall within the linear range of nitro-BSA competition curve (see Critical Parameters and Troubleshooting).

Perform ELISA

4. The day of the assay, rinse the plate three times with 1× PBST, add 300 µl blocking buffer to each well, including blanks, and incubate for 60 min at room temperature.
5. Remove the blocking buffer from the plate and discard. Add 100 µl of a serial dilution of each of the protein samples prepared in primary antibody solution (step 3) to each sample well. Add 100 µl of a serial dilution of nitro-BSA prepared with primary antibody to the designated wells on the same plate to generate a standard curve. Add 100 µl 1× PBST, without antibody, to blank wells.
6. Incubate at room temperature for 2 hr.
7. Rinse three times with TBST. Add 100 µl of secondary antibody diluted 1:1000 with blocking buffer to each sample and standard well. Add 100 µl PBST to blank wells.
8. Incubate at room temperature for 2 hr.

Detect nitrotyrosine

9. Discard reaction solutions from the wells and wash three times with PBST. Add 100 µl of 1 mg/ml *p*-nitrophenyl phosphate to all wells including the blanks.

   Remove any air bubbles with a clean needle.

   p-Nitrophenyl phosphate is a substrate for alkaline phosphatase and will provide a colorimetric group upon cleavage.

10. After 30 min, clean the bottom of the plate with a Kimwipe and read at 450 nm on a microtiter plate reader. Estimate nitrotyrosine concentration using the nitro-BSA standard curve. Express nitrotyrosine concentration in the protein sample as an equivalent of nitrotyrosine in nitro-BSA.

ENZYMATIC ANALYSIS OF ISOASPARTATE FORMATION

BASIC PROTOCOL 5

A novel technique for the determination of isoaspartate formation involves the use of protein-L-isoaspartyl methyltransferase (PIMT). PIMT catalyzes methyl transfer reactions using methyl-*S*-adenosyl-L-methionine (SAM) as the methyl donor. Because PIMT is highly specific for protein isoaspartate residues, the use of tritiated SAM, where the methyl group is radiolabeled, provides a quantitative method for the determination of isoaspartate residues formed in proteins/peptides.

Materials

0.2 M Bis-Tris buffer, pH 6.0 (see recipe)
10 µM [^{3}H]methyl-S-adenosyl-L-methionine (5 to 15 Ci/mmol; [^{3}H] SAM; NEN)
Protein-L-isoaspartyl methyltransferase (PIMT; Promega Corporation or purified from a known source)
0.2 M NaOH (*APPENDIX 2A*)
Safety-Solve II counting fluor (Research Products International)

Sponge plugs (Jaece Industries): cut into small pieces
Scintillation vials with extra caps

CAUTION: [^{3}H]methanol is volatile at room temperature. Perform all reactions under a hood. When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

1. Add [^{3}H]SAM to a final concentration of 10 µM to a protein sample solution in 0.2 M Bis-Tris buffer, pH 6.0, containing 40 U of PIMT in a microcentrifuge tube.

   The sample protein amount is usually between 0.5 and 1 mg and the total reaction volume is 50 µl.

   Blanks that do not have the protein to be analyzed and standards that have known concentrations of isoaspartate should be carried out under the same conditions at the same time.

   The Promega version of the enzyme comes included in the ISOQUANT Isoaspartate Detection kit.

2. Incubate the reactions for 30 min at 30°C.
3. Place the tubes on ice for 15 min to stop the PIMT-catalyzed methyl transfer reaction.
4. Microcentrifuge the tubes at 4°C for 2 min to bring all of the liquid to the bottom of the tube. Place on ice.
5. Add 50 µl of 0.2 M NaOH to hydrolyze the methyl esters so that [^{3}H] methanol can be released from the assayed proteins.

   Tubes may be vortexed briefly to collect liquid.

6. Immediately transfer 50 µl of each reaction solution to a sponge insert in a scintillation vial cap and place the cap on a vial containing 4 ml Safety-Solve scintillation cocktail.
7. Incubate the capped vials at 37°C for 60 min.

   Diffusion of methanol into the scintillation liquid is time-dependent. To obtain reproducible results, it is critical to incubate all reactions for the same length of time.

8. Remove the caps and replace with new caps that do not contain sponge inserts.
9. Count the samples in a scintillation counter.

SUPPORT PROTOCOL 5

GEL ELECTROPHORETIC ANALYSIS OF ISOASPARTATE FORMATION

To identify which proteins in a tissue sample or a protein mixture contain isoaspartate residues, the protein sample, incubated with [^{3}H]SAM and PIMT (see Basic Protocol 5), can be further analyzed by SDS-PAGE (*UNIT 6.1*) after mixing with acid SDS-PAGE sample buffer (see recipes for acid SDS-PAGE buffers). Protein bands can then be excised from the gel and solubilized in 30% hydrogen peroxide, exactly as described in Support Protocol 2 of this unit; however, for the measurement of isoaspartate formation, acid SDS-PAGE (pH 2.4), rather than the Laemmli gel system (pH 8.3), should be performed (see recipes for acid SDS-PAGE). This is necessary, as high pH running buffer will hydrolyze the base-labile isoaspartate methyl esters, leading to the release of [^{3}H]methanol into the running buffer. Do not use sodium hydroxide to stop the PIMT-catalyzed reaction when the samples are to be analyzed by acid SDS-PAGE.

REAGENTS AND SOLUTIONS

*Use deionized distilled water for the preparation of all buffers. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acid SDS-PAGE buffers (pH 2.4)

2× resolving gel buffer:
0.1 M H_2NaPO_4
2% (w/v) SDS
6 M urea
Adjust pH to 2.4 with HCl
Store up to several months at 4°C

1× running buffer:
0.03 M NaH_2PO_4
0.1% (w/v) SDS
0.2 M acetate
Adjust pH to 2.4 with HCl
Store up to 1 year at room temperature

2× sample buffer:
2% (w/v) SDS
6 M urea
10% (v/v) glycerol
0.01% (w/v) pyronin Y dye
16.5 mM NaCl
Adjust pH to 1.4 with HCl
Store up to 1 year at −20°C

2× stacking gel buffer:
2% (w/v) SDS
6 M urea
0.033 M NaCl
Adjust pH to 1.4 with HCl
Store up to 2 months at 4°C

Bis-Tris buffer, 0.2 M, pH 6.0

41.84 g bis-Tris (Sigma)
Adjust volume to 1 liter with H_2O
Adjust pH with concentrated HCl
Store up to 6 months at 4°C

Borate buffer, 0.1 M, pH 9.1

6.184 g boric acid
Adjust volume to 1 liter with H_2O
Adjust pH with 1 M NaOH
Store up to 2 months at room temperature

Borate buffer, 0.2 M, pH 8.8

12.37 g boric acid
Adjust volume to 1 liter with H_2O
Adjust pH with 1 M NaOH
Store up to 2 months at room temperature

DNPH solution

Dissolve 198 mg 2,4-dinitrophenylhydrazine (DNPH; mol. wt. 198.1) in 100 ml 2 M HCl (10 mM DNPH final). Store up to 1 year at room temperature in the dark since DNPH can be destroyed by light.

DNPH may require 1 to 2 hrs of constant stirring for complete solubilization.

ELISA buffers

Blocking buffer: Prepare 0.2% (w/v) gamma globulin (Sigma) in PBST (see below). Filter this buffer through 0.45 µm filter and store up to 1 week at 4°C.

1× diethanolamine (DEA) buffer:
24.25 ml 98% diethanolamine
200 ml H_2O
0.25 ml 20% NaN_3
25 mg $MgCl_2 \cdot 6H_2O$
Adjust pH to 9.8 with concentrated HCl
Bring to 250 ml with H_2O

Use Milli-Q purified water or equivalent.

10× phosphate-buffered saline/Tween 20 (PBST):
80 g NaC1
2 g KH_2PO_4
11.5 g Na_2HPO_4
2 g KC1
5 ml Tween 20
10 ml 20% (w/v) NaN_3
Dilute to a total volume of 2 liters with with H_2O
Store up to 2 months at room temperature

Plate coating buffer:
0.795 g Na_2CO_3
1.465 g $NaHCO_3$
0.5 ml 20% (w/v) NaN_3
Bring to 500 ml with H_2O
If necessary, adjust the pH with 1 M NaOH to ensure it is ~9.6

Use Milli-Q purified water or equivalent.

[^{3}H]Sodium borohydride ([^{3}H]$NaBH_4$) working solution

Add 5 ml 1 M nonradiolabeled sodium borohydride dissolved in 0.1 M NaOH to the original bottle in which tritiated sodium borohydride (activity 222.3 mCi/mmol; NEN Life Science Products) was received. Divide the solution into 100-µl aliquots in microcentrifuge tubes (0.5 ml/tube) and store up to 2 years at −80°C.

$NaHCO_3$, 20 µM, pH 8.8

16.8 mg $NaHCO_3$
Adjust volume to 1 liter with H_2O
Adjust pH with 1 M NaOH
Dilute 1:10 just before use
Store up to 2 months at room temperature

Nitro-BSA standard

Adjust the pH of a 5 mg/ml BSA solution (in water) to 3.5 with acetic acid. Add concentrated sodium nitrite (200 mM) to the BSA solution to obtain a final concentration of 1 mM. Incubate the reaction on a rotator at 37°C for 24 hr. The color change of the solution from colorless to yellow after the incubation usually indicates the presence of nitrotyrosine. Dialyze the nitrated BSA solution overnight against ELISA coating buffer. Determine nitrotyrosine concentration at 430 nm using a spectrophotometer ($\varepsilon = 4100\ M^{-1}cm^{-1}$, pH > 8.5). Store up to 2 months at 4°C.

SDS-PAGE gels (for Bio-Rad Mini gel system)

10% resolving gel:

6.1 ml 2× resolving buffer (Bio-Rad)
2.5 ml 40% 29:1 acrylamide/bisacrylamide
0.1 ml 0.06%, w/v $FeSO_4$
0.1 ml 1% (w/v) ascorbic acid
1.1 ml distilled water
0.1 ml 0.3% H_2O_2
Store up to 6 months at 4°C

4% stacking gel:

4.0 ml 2× stacking gel buffer (Bio-Rad)
0.5 ml 40% 29:1 acrylamide/bisacrylamide
0.065 ml 0.06%, w/v $FeSO_4$
0.065 ml 1%, w/v ascorbic acid
0.305 ml distilled water
0.065 ml 0.3% H_2O_2
Store up to 6 months at 4°C

IMPORTANT NOTE: *$FeSO_4$, ascorbic acid, and H_2O_2 are used as gel polymerization catalysts; prepare fresh.*

Trichloroacetic acid (TCA) solution, 20%, 10%, and 0.1% (v/v)

Prepare TCA stock solution by adding 227 ml water to a 500-g bottle of TCA. Add 10 ml of TCA stock solution to 40 ml of water to prepare 20% (v/v) TCA. Dilute to appropriate percentage with water. Store up to 1 year at room temperature.

Tris-buffered saline with and without Tween-20 (TBST and TBS)

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.9% (w/v) NaCl
0.1% (v/v) Tween-20 (omit for TBS)
Store up to 2 months at 4°C

COMMENTARY

Background Information

Proteins are acknowledged to be one of the major targets of endogenously generated reactive oxygen species (ROS; Stadtman, 1992). The study of protein oxidative modifications not only contributes to the elucidation of the mechanisms of ROS-mediated damage, but may also aid in understanding how protein oxidation is linked to losses in physiological functions under pathological processes or during aging. Furthermore, the identification of the protein targets of oxidative damage may potentially lead to the development of strategies that may ameliorate oxidative stress.

Several factors seem to be involved in intracellular accumulation of oxidized proteins. One major factor is the cellular level of oxidative stress, determined by the balance between the rate of ROS generation and the efficiency of antioxidative defenses (Stadtman and Berlett, 1997). The other factor determining the steady-state amount of oxidized proteins is the activity of proteolytic enzymes (Stadtman and Berlett, 1997). It is now firmly established that mild oxidation of proteins

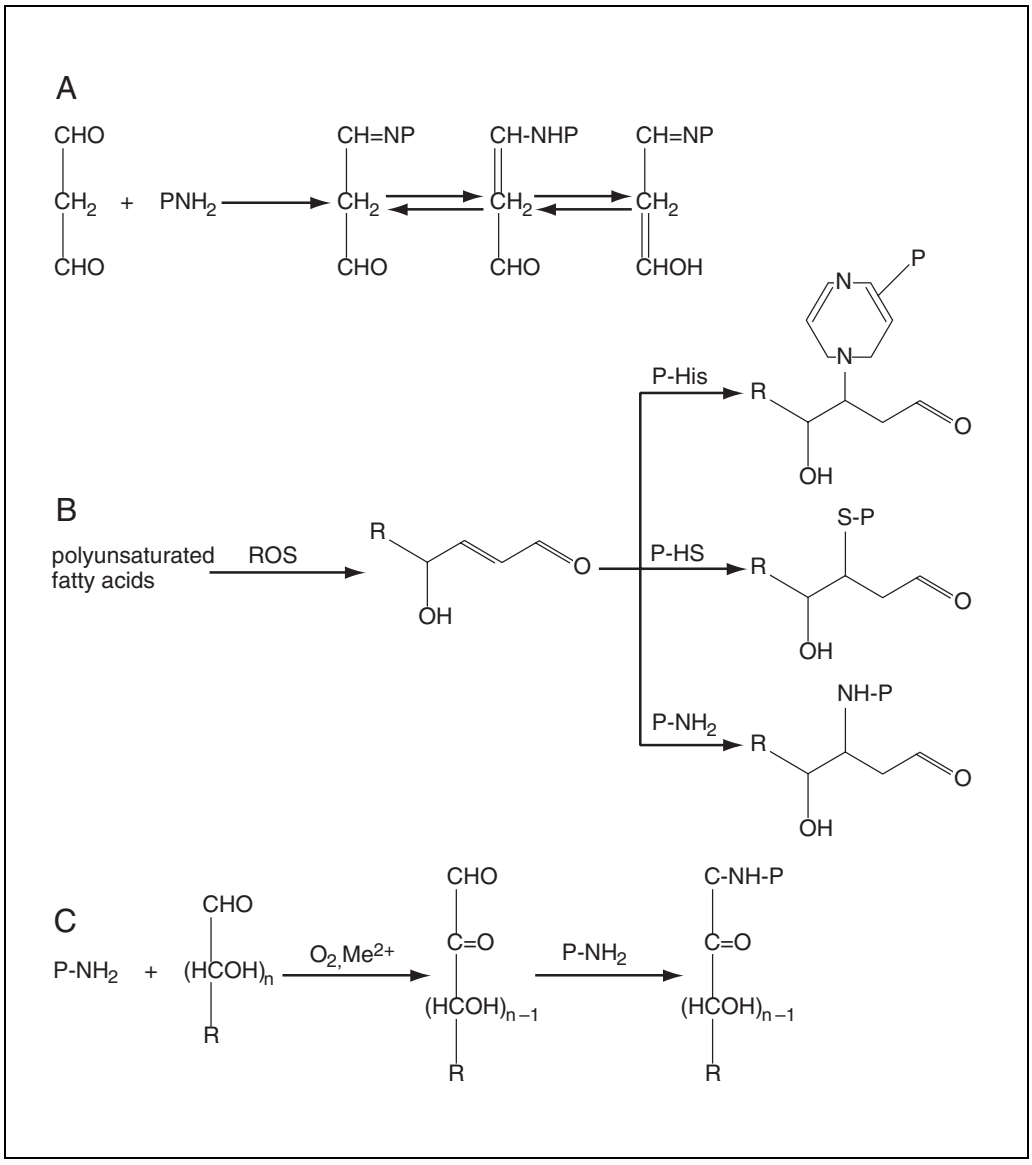


Figure 7.9.1 Generation of protein carbonyls by glycation and glycoxidation and by reactions with lipid peroxidation products of polyunsaturated fatty acids. (**A**) Reactions of protein amino groups (PNH_2) with the lipid peroxidation product, malondialdehyde. (**B**) Michael addition of 4-hydroxy-2-nonenal to protein lysine (P-NH_2), histidine (P-His), or cysteine (PSH) residues. (**C**) Reactions of sugars with protein lysyl amino groups (P-NH_2). "Me" represents "metal ions." Abbreviation: ROS, reactive oxygen species.

facilitates their eventual degradation by proteases, while heavily oxidized proteins in which cross-links usually occur are resistant to proteolysis (Davies et al., 1987). Oxidative damage to proteases themselves can further attenuate cellular ability to degrade oxidized proteins (Agarwal and Sohal, 1994).

Analysis of protein carbonylation

Some known mechanisms by which protein carbonylation occurs are shown in Figure 7.9.1. The reaction between DNPH and protein carbonyls, shown in Figure 7.9.2, was initially used for the measurement of metal catalyzed oxidative damage to glutamine synthase (Levine, 1983). The procedure, although very useful for the determination of protein carbonyl content in tissue samples, is not selective and is subject to interference by proteins containing chromophores that have absorbance near 360 nm. In contrast, immunoblotting is highly selective and can detect carbonylation of individual proteins, but the method is not meant to provide quantitative information. The borohydride reduction method, when used in conjunction with gel electrophoresis, is both selective and quantitative (Yan and Sohal, 1998a). Using protein carbonyls as an index, oxidative dam-

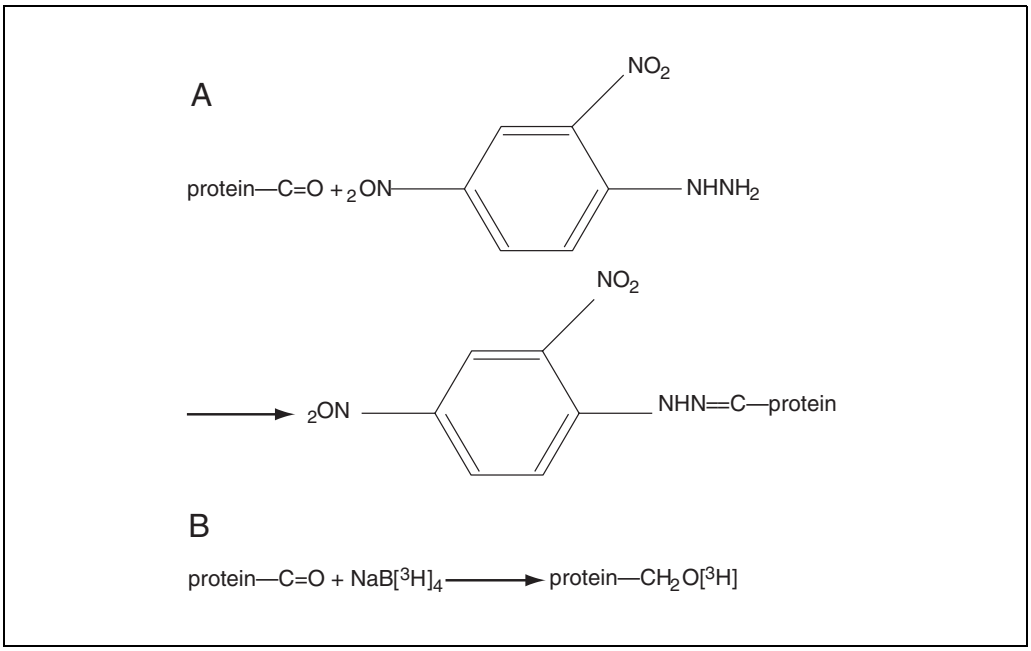


Figure 7.9.2 Methods for labeling protein carbonyls: (1) Derivatization of protein carbonyls with 2,4- dinitrophenylhydrozine (DNPH), forming protein conjugated dinitrophenylhydrozones. (2) Derivatization of protein carbonyls with tritiated sodium borohydride.

age to proteins has been found to be associated with aging and oxidative stress as well as a number of pathologies (Stadtman, 1992). Moreover, protein oxidation during aging has been recently established to be a highly selective phenomenon. Using the housefly as a model system, the authors' laboratory has demonstrated that in flight-muscle mitochondria, only aconitase and adenine nucleotide translocase exhibited a discernible age-dependent increase in protein carbonylation and a corresponding loss in their activities (Yan et al., 1997; Yan and Sohal, 1998b).

The four protocols for the measurement of protein carbonyls described in this unit are relatively simple and easy to perform, without the requirement of elaborate instrumentation. Analysis of protein carbonylation by methods other than those described here may also be used, should the relevant equipment be readily accessible. One such procedure requires HPLC separation, which has the advantage that the removal of free DNPH is unnecessary because the HPLC column can separate proteins from small molecules (Levine et al., 1994). HPLC requires a very small amount of proteins and can monitor both protein and protein-bound DNP absorption at two wavelengths simultaneously. The derivatization of protein carbonyls by DNPH, however, needs to be carried out in solutions that are compatible with HPLC columns. Another method, which also involves the use of anti-DNP antibodies, is the ELISA measurement of protein carbonyls (Buss et al., 1997). This method is quite sensitive and may be used for determination of whole tissue carbonyls, but it is only semiquantitative and lacks selectivity.

Recently, a fairly new method, the MALDI-MS technique, has been applied for the determination of protein carbonyls. The method works very well for small peptides or proteins such as cytochrome c, but for large peptides, the proteins need to be digested with proteases. The resulting small peptides are then separated by HPLC. A potential problem for large proteins with this method is that if a large number of small peptides are generated after protease digestion, HPLC separation of these peptides can become very tedious.

Analysis of protein thiol groups

Protein thiol groups (mainly cysteine residues) not only stabilize and maintain the three-dimensional conformation of many proteins but are also involved in biological redox couplings. Due to their high reactivity, protein thiol groups are very sensitive to oxidative damage induced by a variety of oxidation systems. Loss of protein thiol groups has been demonstrated during aging and in disease (Hughes et al., 1980; McKenzie et al., 1996).

Most of the currently available methods for quantitative analysis of protein thiol groups depend upon the formation of chemical derivatives. Due to the nucleophilic property of the –SH group, alkylation of cysteine thiol groups by

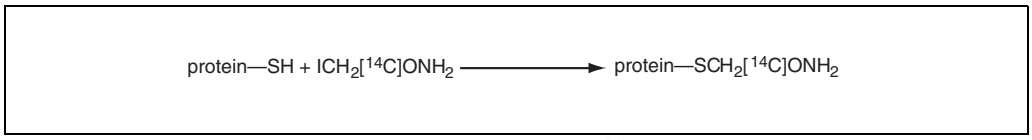


Figure 7.9.3 Radiolabeling of protein thiol groups with [^{14}C]iodoacetamide.

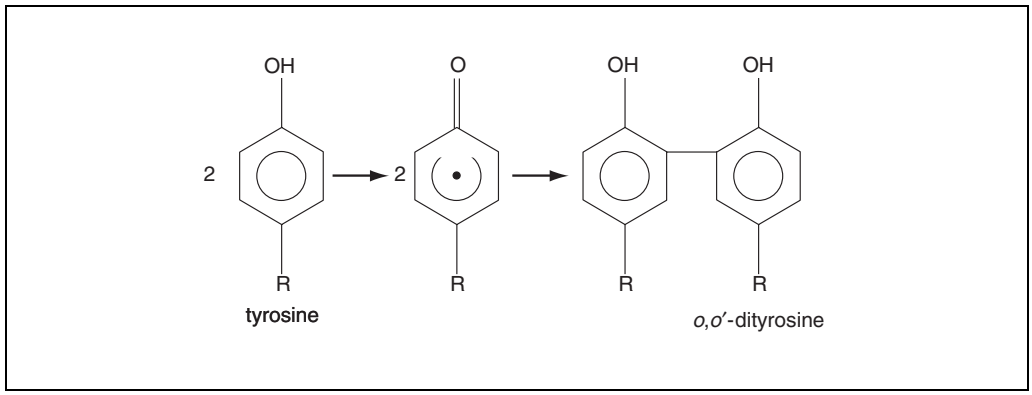


Figure 7.9.4 Formation of one molecule of *o,o′*-dityrosine from two molecules of tyrosine via tyrosyl radical intermediates.

alkylators such as iodoacetamide is widely used for either blocking or quantification of protein-bound thiols. There are two advantages when iodoacetamide is used. First, iodoacetamide does not create a carboxylate functional group; therefore, no new negative charges are introduced into the protein (Fig. 7.9.3). This may be necessary when the alkylated protein is to be analyzed by isoelectric focusing gel electrophoresis. Second, the bond formed from the reaction of iodoacetamide and a thiol group is a stable thioether linkage that is irreversible under normal gel electrophoretic conditions. Furthermore, when radiolabeled iodoacetamide is used, the reaction provides a highly quantitative method for the measurement of protein thiol groups. The use of iodoacetamide, together with gel electrophoresis, has led to a successful identification of glyceraldehyde-3-phosphate dehydrogenase as the enzyme that exhibits the most loss of protein thiol groups during inflammatory bowel disease (McKenzie et al., 1996).

Another classical method for the determination of protein thiol groups involves the use of 5,5′-dithiobis (2-nitrobenzoic acid) also known as DTNB or Ellman's reagent (Ellman, 1959). DTNB undergoes disulfide interchange with the release of 5-thio-2-nitrobenzoic anion (TNB), which has an absorption maximum at 412 nm and can be quantitated spectrophotometrically. The disadvantage of the DTNB method is that TNB is highly subject to reoxidation in an alkaline solution in the presence of oxygen and metal ions and its molar extinction coefficient changes slightly as a function of the solvent system used. In addition, DTNB itself can undergo hydrolysis of its disulfide bond at a pH higher than 9. Moreover, without prior protein purification, DTNB cannot be used for the quantification of individual proteins that exist in tissue homogenates and may have undergone the most loss of thiol groups caused by oxidative damage during aging or in diseases.

Analysis of tyrosine modification

Dityrosine formation. o,o′-Dityrosine is formed either when a hydroxyl radical cross-links tyrosines or by the reaction between protein bound tyrosine and a tyrosyl radical (Fig. 7.9.4). Dityrosine exhibits intense fluorescence at ~400 nm and thus can be measured with an excitation wavelength of ~315 nm in alkaline solutions or ~284 nm in acidic solutions. Based on this property, dityrosine, formed in proteins oxidized in vitro, have been frequently measured by HPLC equipped with a fluorometer as the detection device (Giulivi and Davies, 1993). A disadvantage is that molecules that co-elute, but structurally differ from dityrosine may confound the procedure. Moreover, the HPLC fluorometric method has not yet been successfully used for the detection of dityrosine formation during natural aging, suggesting that the amount of dityrosine in proteins, without challenge by severe oxidative stress, is below HPLC detection limit.

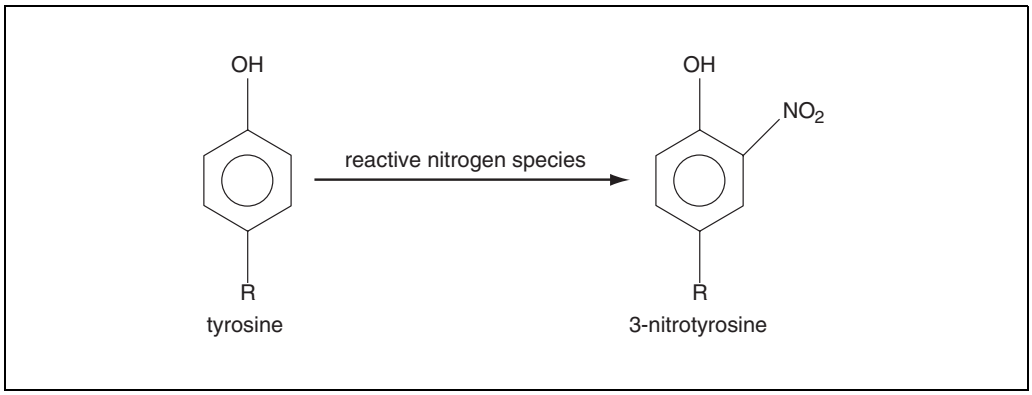


Figure 7.9.5 Formation of nitrotyrosine via reactive nitrogen species-mediated nitration of tyrosine.

In contrast, the GC/MS method described in this unit for dityrosine detection is highly sensitive and can structurally distinguish dityrosine from other tyrosine-modified derivatives, thereby reducing potential confusions caused by compounds that coelute with dityrosine during chromatography. GC/MS analysis also permits the use of a stable, isotopically labeled internal standard which, other than its heavy isotope, is structurally identical to dityrosine, and therefore behaves identically during sample preparation and analysis. Inclusion of such a standard also corrects for the dityrosine loss during processing and thus increases the precision of quantitative measurements. Using the GC/MS method, dityrosine concentrations in various mouse tissues have been found to be elevated during aging and are significantly decreased by caloric restriction (Leeuwenburgh et al., 1997). However, this method lacks selectivity when tissue samples are used as experimental materials, since no distinction can be made in the degree of dityrosine content among different protein species.

Nitrotyrosine formation. Nitrotyrosine is formed by reactions between tyrosine and reactive nitrogen intermediates (Fig. 7.9.5). Its level is elevated, especially during inflammation. Peroxynitrite, produced by the reaction between the nitrogen monoxide radical and superoxide, has been proposed to be the major oxidant that causes nitrotyrosine formation in living systems (Ischiropoulos et al., 1992). Nitrotyrosine can be easily determined spectrophotometrically as its maximum absorbance ranges from 350 nm to 450 nm, shifting from 365 nm (colorless) at pH < 3 to 428 nm (yellow) at pH > 9. However, the amount contained in tissue samples without oxidative challenge is far below the detection limit of a spectrophotometer. The ELISA method described in this unit, although only semiquantitative, is very sensitive for the measurement of nitrotyrosine in tissue samples. This method is especially useful when individual proteins in tissue samples need not be analyzed. For identification of specific proteins that might exhibit selective nitration during aging or under oxidative stress, immunoblot detection using anti-nitrotyrosine antibodies may be preferred.

Analysis of isoaspartate formation

Isoaspartate residues are usually formed from the deamidation of asparaginyl residues or isomerization of aspartyl residues (Fig. 7.9.6). Formation of isoaspartate residues in a protein not only alters the protein's structure, but may also cause the loss of the protein's activity. Although isoaspartate formation was initially found in in vitro aging of proteins stored at neutral pH (Graf et al., 1971), the discovery of the enzyme protein-L-isoaspartyl methyltransferase (PIMT) suggests that isoaspartate formation in proteins is physiologically relevant (Clark, 1985). Indeed, it has been found that a variety of tissue samples are able to be tritiated when incubated in the presence of PIMT and [^{3}H]*S*-adenosyl methionine (SAM; Aswad, 1995), demonstrating the occurrence of isoaspartate formation in vivo. Therefore, methods for the detection and quantification of isoaspartate formation in proteins/peptides are of interest to investigators studying post-translational modification of proteins.

The discovery of PIMT also provides a useful tool for the quantification of isoaspartate formation in proteins (Fig. 7.9.7), as described in this unit. The only limitation of the method, however, is that PIMT, as a single enzyme, is not commercially available. Isoaspartate formation can also be measured by isoelectric focusing gel analysis or proteolytic digestion of the protein followed by mass spectral analysis of the peptides resolved by reversed-phase HPLC. The isoelectric focus-

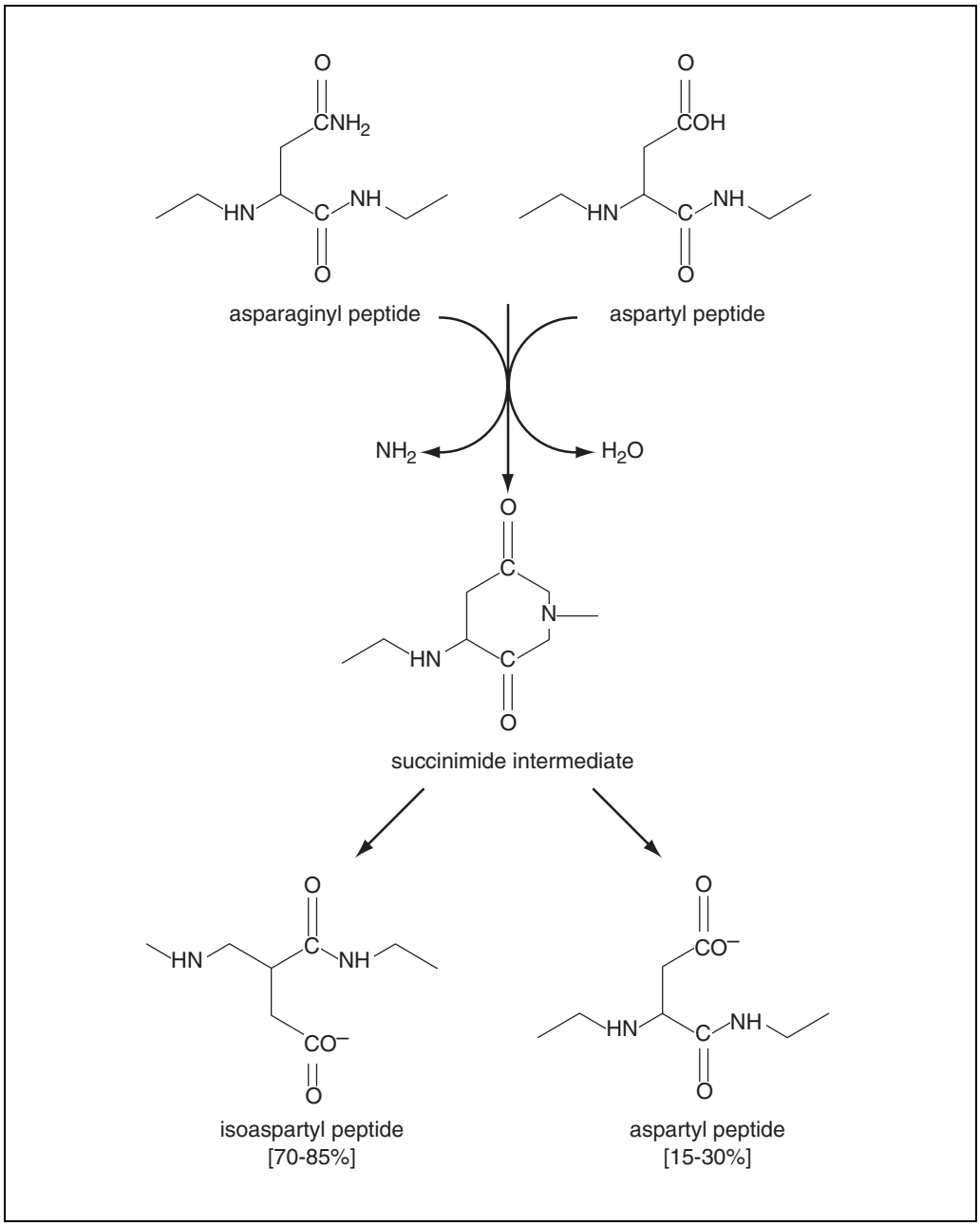


Figure 7.9.6 Formation of isoaspartate by the deamidation of asparagine or the isomerization of aspartate.

ing method is based on the fact that isoaspartate formation in a protein results in a net change of the protein's charge. However, the method cannot detect the formation of isoaspartate residues derived from aspartic acid which do not alter the net charge of the protein (i.e., aspartyl isomerization). In addition, changes in the isoelectric focusing pattern of a protein may also result from the modification of other amino acid residues, such as methionine or lysine oxidation; therefore, the isoelectric focusing method is not specific. Proteolytic digestion of a protein followed by HPLC and mass spectral analysis is capable of detecting the presence of isoaspartate residues in the protein; however, the HPLC method, based on the retention time of the digested peptides, may not be able to resolve subtle changes in the peptide that arise from the deamidation of a particular peptide. Moreover, many changes may affect the retention time of a particular peptide, which could coelute with the isoaspartate-containing peptide and therefore does not necessarily indicate the presence of isoaspartate residues.

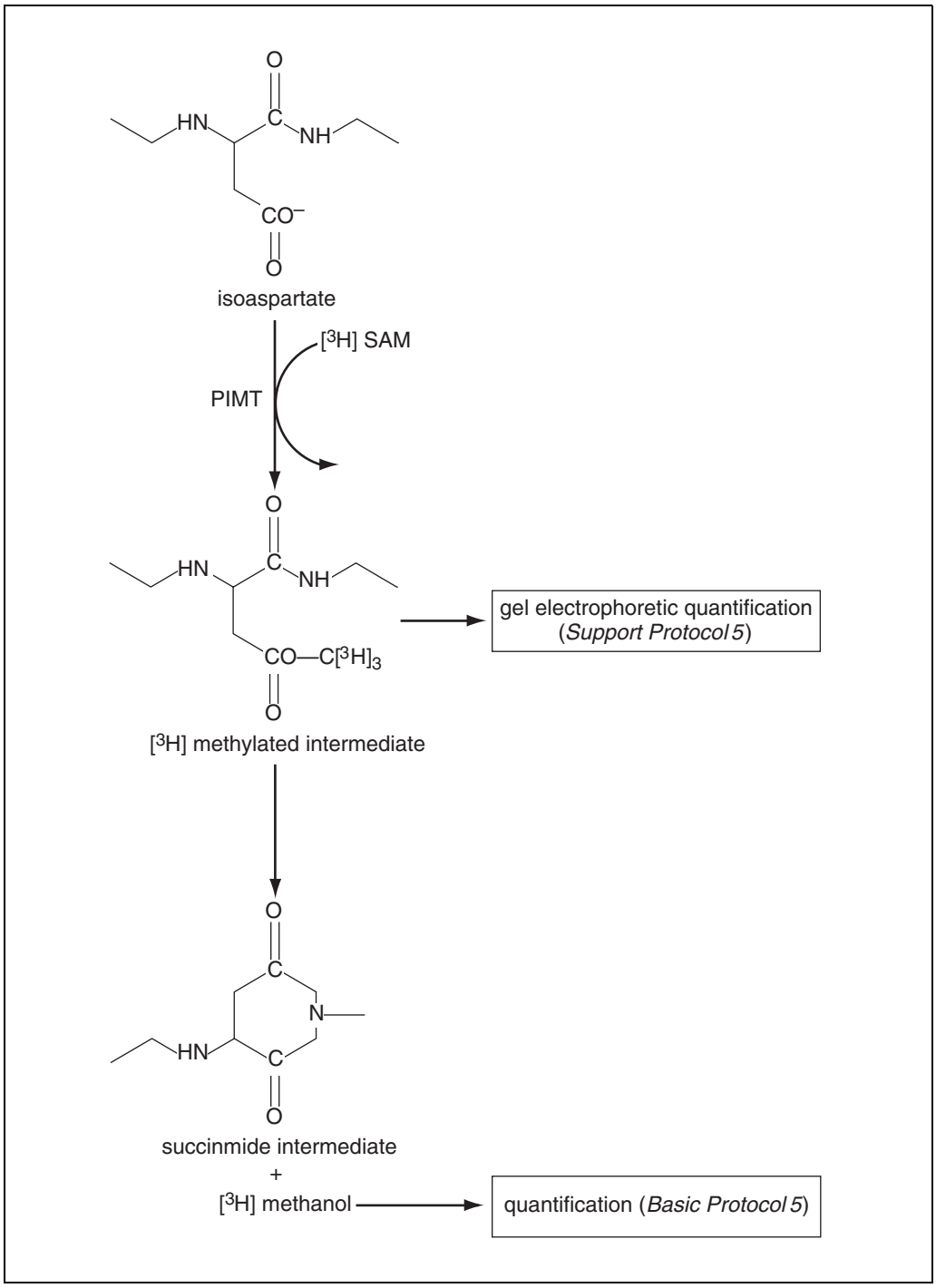


Figure 7.9.7 Pathway for the PIMT-catalyzed methylation of isoaspartate used for quantitation.

Critical Parameters and Troubleshooting

In all studies concerning oxidative damage to proteins, auto-oxidation of the tissues by ambient oxygen, following tissue isolation, should be minimized. Accordingly, tissue collection and preparation must be carried out in buffers supplemented with antioxidants, such as diethylenetriaminepentaacetic acid (DTPA) and butylated hydroxytoluene (BHT). The use of antioxidant buffers may be especially critical for the detection of trace amounts of oxidized products formed during aging or under oxidative stress. In addition, all buffers should be bubbled with nitrogen before use.

Analysis of protein carbonylation

Depending upon the method used, protein carbonyl content can be expressed as either nmol/mg protein or cpm/mg protein for the

DNPH (see Basic Protocol 1) and $NaBH_4$ (see Basic Protocol 2) method respectively. Therefore, the only critical parameter for protein carbonyl quantification is the protein concentration. As a rule, protein concentration after washing with ethanol/ethyl acetate, should always be determined. In addition, it should be noted that a high concentration of protein-conjugated DNP interferes with the BCA protein assay; therefore, for heavily oxidized proteins that have extremely high carbonyl content determined by DNPH, protein concentration should be assayed by UV absorption at 280 nm using BSA as a standard .

Two potential problems may arise when protein carbonyl content is measured spectrophotometrically using DNPH. First, absorption of protein-bound DNP in some experiments may be lower than that of a blank, which is the same protein sample treated with HCl (2 M) rather than DNPH. This problem may be caused by chromophores of certain proteins in the sample. If this is indeed the case, protein carbonyl content may have to be determined by other methods, such as the use of tritiated sodium borohydride (see Basic Protocol 2). The problem can also be caused by the difference in protein concentrations between a blank and a sample due to loss of proteins during washing. Therefore, care should be taken to minimize protein loss during washing steps, and protein concentration should always be determined after the removal of free DNPH (see Basic Protocol 2, step 4). Second, for tissue samples, it may be very difficult to dissolve the final protein pellet after washing. This problem is most likely caused by the use of high concentration of proteins in the initial step where protein samples are treated with DNPH. The authors have found that starting with 1 mg/ml protein will render the final pellet readily soluble. The solubility of a protein pellet will also depend on the nature of the protein; for example, lipoproteins and collagen are always quite difficult to dissolve after treatment with DNPH. In such a case, a denaturing buffer containing 150 mM phosphate and 3% SDS, pH 6.8 should be used.

For immunoblot detection of protein carbonyls, a major concern is the dilution of antibodies. If overdiluted, the immunostaining signal may be very weak; if underdiluted, the background may be too dark. Therefore, it is critical to optimize each antibody's dilution so that the immunostain signal is maximized while background staining is minimized. In addition, the amount of protein loaded onto SDS-PAGE should not be too high. Otherwise, nonspecific immune reactions may occur. Generally, 5 μg of a protein mixture is enough and less may be used for a purified protein.

When protein carbonyl content is determined by tritiated sodium borohydride (see Basic Protocol 2), protein concentrations should always be determined after the removal of free tritium because protein recovery after washing is rarely 100%. An 80% to 90% recovery for mitochondrial proteins has been routinely obtained in the authors' laboratory. When tritiated proteins are analyzed by SDS-PAGE, solubilization of more than one band of the same protein in one vial may be necessary to obtain enough radioactivity counts.

Analysis of protein thiol oxidation

When protein thiol groups are determined by radiolabeling with [^{14}C] iodoacetamide in conjunction with gel electrophoresis, it is necessary to include a final concentration of 1% SDS in the reaction mixture so that complete labeling can be ensured. For some proteins, the nucleophilicity of the thiolate (S^-) anion may be lower than potentially competing alcohol and amino groups in the same proteins; therefore, the iodoacetamide reaction may not be absolutely specific. In such cases, experimental conditions may need to be optimized by varying temperature, pH, and duration of the reactions so that the labeling for cysteine residues will be specific.

It should be noted that iodoacetamide is intrinsically unstable in light, especially in solution; reactions should therefore be carried out in the dark. Adding cysteine, glutathione, or mercaptosuccinic acid to the reaction mixture will quench thiol-reactive species, forming highly water-soluble adducts that are easily separated from proteins during gel electrophoresis.

Analysis of tyrosine oxidative modification

For GC/MS measurement of protein dityrosine residues, dityrosine always needs to be released by acid hydrolysis. Although it has been demonstrated by other investigators that the procedure of acid hydrolysis does not induce more oxidation of tyrosine, which can lead to additional dityrosine formation, care should be taken to ensure no further oxidation indeed takes place for a given sample under given experimental conditions by adding antioxidants to buffers. For such purposes, pure tyrosine should be used as a control during the acid hydrolysis process. In addition, for tissue samples (protein mixtures), the dityrosine level is usually above the detection limit of GC/MS method; however, this may not be the case for an individual protein. Therefore, it may be advisable to determine whether the

protein of interest has a dityrosine level that is above the limit of detection. To achieve this, analyze the protein at two widely different concentrations, 50- to 100-fold. If no dityrosine is detectable in the concentrated sample, it is most likely that the protein does not contain a significant amount of dityrosine residues.

For the measurement of protein nitrotyrosine by competitive ELISA, antibody dilution and the amount of nitro-BSA used for plate coating may have to be optimized. To achieve this, first titrate the amount of nitro-BSA needed to coat the plate versus a constant, high concentration of anti-nitrotyrosine antibody. Plot the absorbance values and select the lowest level that yields a strong signal. Next, coat the plate with this amount of nitro-BSA and vary the concentration of antibodies. Plot the obtained values versus antibody dilution and select a level of the antibody that is within the linear portion of the curve. Optimal dilution for the secondary antibody can also be obtained by similar titration. In addition, in most of the competitive ELISA assays, the first antibody (here anti-nitrotyrosine) may bind preferentially to the coating antigen because the latter on the plate is highly localized. Therefore, to ensure a thorough competition, samples to be analyzed should always be premixed with the first antibody and incubated overnight before the actual ELISA measurement.

Analysis of isoaspartate formation

When protein isoaspartate is measured by PIMT-catalyzed methyl transfer reaction, the protein solution must not contain any detergent such as SDS or chaotropic reagents such as guanidine-HCl, as these will denature the PIMT enzyme. Moreover, for an unknown protein or peptide, as a first step, it is useful to determine whether the protein has any isoaspartate residues. The protein can be diluted to several different concentrations and then analyzed. If the concentration of isoaspartic acid is not protein concentration-dependent, it is most likely that there is little or no isoaspartic acid present in the protein or peptide.

Some proteins, when not denatured, may contain isoaspartate residues in domains that make them poor substrates for PIMT. In such cases, the PIMT methylation reaction may not be complete and isoaspartate concentration may be underestimated. If so, proteins may need to be fragmented by proteolysis before they are used for isoaspartate determination. To determine if proteolysis is necessary, process a sample of undigested protein along with a digested sample. If the amount of isoaspartate measured in the intact protein is similar to that in the digested protein, the protein need not be fragmented for isoaspartate quantification. If a protein should require digestion by protease, the sample buffer used for blank reactions should contain the same protease and buffer components existing in the digestion, but without the protein sample. This will exclude any isoaspartate residues that may be present in the protease.

Anticipated Results

When performed correctly, protein carbonyl content of native proteins, such as BSA and human plasma, should be around 1 nmol/mg protein (usually 0.5 to 1.5 nmol/mg protein). Literature values for carbonyl content in normal tissues are usually between 1.5 and 2.0 nmol/mg protein (Reanick and Packer, 1994). If immunoblot is performed, immunostaining of protein carbonyls should be differential, which means protein oxidative damage does not occur equally in all proteins. Protein carbonylation analyzed by the use of tritiated borohydride is highly dependent on the source of tritiated borohydride, but for the same batch of the chemical with the same concentration, results are generally reproducible. If protein carbonyls derivatized with tritiated sodium borohydride are to be quantitated by a gel electrophoretic technique (see Support Protocol 2), radioactivity counts for each individual band in a protein mixture (<20 μg loaded), are usually less than 300 cpm if no more than one band is solubilized in a single vial.

For protein thiol groups measured by gel electrophoresis of iodoacetamide-treated proteins (see Basic Protocol 3), results of autoradiographic intensities should show a highly differential pattern for each individual protein. If a band exhibits the highest intensity among all the proteins resolved by SDS-PAGE, the protein would have the least loss in its thiol groups. In contrast, if a protein band exhibits a very weak intensity following autoradiography, it should have a maximal loss in its thiol groups. Depending on what proteins are being studied, results of liquid scintillation counting of an excised individual band may range from 500 cpm to 2000 cpm.

For mass spectrometric quantification of dityrosine (see Basic Protocol 4), the concentration of protein dityrosine is usually expressed as the ratio of dityrosine to tyrosine. In mouse, dityrosine concentration has been found to be around 0.3 to 0.6 mmol/mol for heart, 0.1 to 0.3 mmol/mol for skeletal muscle and brain, and 0.2 mmol/mol for liver (ter Steege et al., 1998). Nitrotyrosine, when determined by competitive ELISA and expressed as nitro-BSA equivalents, is about 0.12 μM in human plasma.

Isoaspartate concentration in proteins, usually expressed as pmol/mg protein, is 200 pmol/mg protein in murine brain and heart, and <50 pmol/mg protein in liver with no detectable isoaspartate residues in the plasma (Kim et al., 1997).

Time Considerations

Without the consideration of preparation of protein samples, for a small number of samples (<20), spectrophotometric quantitation (see Basic Protocol 1) can be finished within one day after the DNPH treatment. If a large number of samples are involved, the washing step may take a longer time, but one can always leave the protein pellet in the ethanol/ethyl acetate solution with the tubes capped lightly and stored under a hood.

Immunoblot detection of protein carbonyls (see Support Protocol 1), starting from SDS-PAGE, can be finished within 48 hr. The time can be shortened to 12 hr if the PVDF membrane is incubated for 1 hr each, with both primary and secondary antibodies.

The quantitation of protein carbonyls by electrophoresis (see Support Protocol 2) may require one week. Most of the time is spent on gel solubilization and liquid scintillation counting.

Gel electrophoretic analysis of protein thiol groups labeled with [^{14}C]iodoacetamide (see Basic Protocol 3) will take at least 2 weeks. Generally, radiolabeling of the protein samples and gel analysis can be finished within 2 days. The rest of the time is used for the exposure of X-ray film to the Whatman 3MM filter paper onto which gels have been dried.

Quantification of protein carbonyls by tritiated borohydride labeling (see Support Protocol 2) typical requires 2 days for ~20 samples. Labeling is generally accomplished in the first day, while the second day is left for scintillation counting.

Quantitation of protein dityrosine by mass spectrometry (see Basic Protocol 4) usually takes up to 1 week. Sample preparation, including tissue hydrolysis, can take 3 days; the actual quantitation by mass spectrometry can be finished within 2 days. Preparation of dityrosine standard (see Support Protocol 3) may take up to 1 week and the time used for lyophilization steps is flexible. In addition, the lyophilized samples can be stored at −80°C for several months before further analysis.

Competition ELISA measurement of nitrotyrosine (see Support Protocol 4) usually takes 2 days. Day one is allotted for sample preparation, plate coating and preincubation of the samples or nitro-BSA with anti-nitrotyrosine antibodies. Day two is used for antibody incubation, plate washing and data collection.

Enzymatic analysis of isoaspartate formation (see Basic Protocol 5), up to the step of liquid scintillation counting, can be finished in one day. After the addition of scintillation cocktail, the samples can be counted at any time. Gel electrophoretic analysis of isoaspartate formation (see Support Protocol 5) may require a week with most of the time spent on gel solubilization and liquid scintillation counting.

ACKNOWLEDGEMENTS

The authors thank Dr. Christiaan Leeuwenburgh for his help on the procedure for dityrosine quantification by GC/MS.

LITERATURE CITED

Agarwal, S., and Sohal, R.S. 1994. Aging and proteolysis of oxidized proteins. *Arch. Biochem. Biophys.* 309:24-28.

Aswad, D.W. (ed.). 1995. Deamidation and isoaspartate formation in peptides and proteins. CRC, Boca Raton, Fla.

Buss, H., Chan, T.P., Sluis, K.B., Domigan, N.M., and Winterbourn, C.C. 1997. Protein carbonyl measurement by a sensitive ELISA method [published errata appear in *Free Radic Biol Med* 1998 May; 24:1352]. *Free Radic. Biol. Med.* 23: 361-366.

Clark, S. 1985. Protein carboxyl methyltransferase: Two distinct classes of enzymes. *Annu. Rev. Biochem*. 54:479-506.

Davies, K.J., Lin, S.W., and Pacifici, R.E. 1987. Protein damage and degradation by oxygen radicals. IV. Degradation of denatured protein. *J. Biol. Chem*. 262:9914-9920.

Ellman, G.L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys*. 82:70-77.

Giulivi, C., and Davies, K.J. 1993. Dityrosine and tyrosine oxidation products are endogenous markers for the selective proteolysis of oxidatively modified red blood cell hemoglobin by (the 19 S) proteasome. *J. Biol. Chem*. 268:8752-8759.

Graf, L., Bajusz, S., Patthy, A., Barat, E., and Cseh, G. 1971. Revised amide location for porcine and human adrenocorticotropic hormone. *Acta Biochim. Biophys. Acad. Sci. Hung*. 6:415-418.

Hughes, B.A., Roth, G.S., and Pitha, J. 1980. Age-related decrease in repair of oxidative damage to surface sulfhydryl groups on rat adipocytes. *J. Cell. Physiol.* 103:349-33.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J.C., Smith, C.D., and Beckman, J.S. 1992. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys*. 298:431-437.

Kim, E., Lowenson, J.D., MacLaren, D.C., Clarke, S., and Young, S.G. 1997. Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. *Proc. Natl. Acad. Sci. U.S.A*. 94:6132-6137.

Leeuwenburgh, C., Wagner, P., Holloszy, J.O., Sohal, R.S., and Heinecke, J.W. 1997. Caloric restriction attenuates dityrosine cross-linking of cardiac and skeletal muscle proteins in aging mice. *Arch. Biochem. Biophys.* 346:74-80.

Levine, R.L. 1983. Oxidative modification of glutamine synthetase. *J. Biol. Chem.* 258:11823-11827.

Levine, R.L., Williams, J.A., Stadtman, E.R., and Shacter, E. 1994. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* 233:346-357.

McKenzie, S.J., Baker, M.S., Buffinton, G.D., and Doe, W.F. 1996. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J. Clin. Invest.* 98:136-141.

Reanick, A.Z. and Packer, L. 1994. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. *Methods Enzymol.* 233:357-363.

Stadtman, E.R. 1992. Protein oxidation and aging. *Science.* 257:1220-1224.

Stadtman, E.R., and Berlett, B.S. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem. Res. Toxicol.* 10:485-494.

ter Steege, J.C., Koster-Kamphuis, L., van Straaten, E.A., Forget, P.P., and Buurman, W.A. 1998. Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA. *Free Radic. Biol. Med.* 25:953-963.

Yan, L.J., Levine, R.L., and Sohal, R.S. 1997. Oxidative damage during aging targets mitochondrial aconitase. *Proc. Natl. Acad. Sci. USA.* 94:11168-11172.

Yan, L.J., and Sohal, R.S. 1998a. Gel electrophoretic quantitation of protein carbonyls derivatized with tritiated sodium borohydride. *Anal. Biochem.* 265:176-82.

Yan, L.J., and Sohal, R.S. 1998b. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proc. Natl. Acad. Sci U.S.A.* 95:12896-12901.

Key References

Berlett, B.S., and Stadtman, E.R. 1997. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* 272:20313-20316.

Detailed biochemical mechanisms of protein oxidation, general principles of intracellular accumulation of oxidized proteins and implication of oxidized proteins in aging and disease.

Heinecke, J.W, Hsu, F.F, Crowley, J.R, Hazen, S.L., Leeuwenburgh, C., Mueller, D.M., Rasmussen, J.E. and Turk, J. 1999. Detecting oxidative modification of biomolecules with isotope dilution mass spectrometry: Sensitive and quantitative assays for oxidized amino acids in proteins and tissues. *Methods Enzymol.* 300:124-144.

Extensive description of tyrosine modified products, including dityrosine, nitrotyrosine, and chlorotyrosine, by GC/MS method.

Aswad, 1995. See above.

Extensive coverage of methods for the quantification of isoaspartate formation in proteins/peptides. A useful collection of examples of isoaspartate formation in individual proteins.

Contributed by Liang-Jun Yan and
Rajindar S. Sohal
Southern Methodist University
Dallas, Texas

Radioiodination of Cellular Proteins

Radioiodination with ^{125}I is a useful method for the labeling of cellular proteins. Protocols describing the radioiodination of proteins expressed on the surface of live cells (Basic Protocol 1), proteins in subcellular fractions including membrane-solubilized proteins (Basic Protocol 2), and soluble proteins (Basic Protocol 3 and Alternate Protocol) are described below. Support Protocols 1 and 2 describe methods for preparing solubilized membrane proteins.

SAFETY PRECAUTIONS FOR WORKING WITH ^{125}I-LABELED COMPOUNDS

As with all radioactive isotopes, the experimenter should work only in areas designated for radioactive materials and should follow the guidelines set by the local radiation safety officer. Because ^{125}I is avidly taken up by the thyroid gland, safety guidelines often include a urine test and/or thyroid scan after the procedure.

Because of the risk of exposure to γ radiation, special care should be taken whenever ^{125}I is used. Therefore, a number of extra precautions need to be followed:

1. It is strongly recommended that this procedure be performed by two researchers. This allows one experimenter to do the labeling while the other provides the equipment needed and continually surveys for contamination with the appropriate sodium iodide crystal detector.

2. Vials containing unbound ^{125}I should always be opened in a fume hood designated for use with radioactive materials and equipped with an activated charcoal filter. This should protect the experimenter from the ^{125}I, which is extremely volatile and particularly hazardous to the thyroid gland.

3. All work should be carried out behind a set of lead bricks to block the γ radiation. A double row of standard 4-cm-thick bricks should be sufficient to provide protection from the ^{125}I.

4. When ^{125}I or radioiodinated cells are moved from the protective lead bricks to the fume hood, they should be encased behind lead shields to limit exposure of fellow researchers. Moreover, ^{125}I-labeled cells should be kept within a lead box in a designated incubator.

5. All subsequent processing with radioiodinated cells or proteins should be carried out behind lead shields while wearing protective clothing and gloves, using air monitoring devices, and working with extreme caution.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

CELL SURFACE LABELING WITH ^{125}I USING LACTOPEROXIDASE

BASIC PROTOCOL 1

In this procedure, cell surface proteins are labeled by adding Na[^{125}I] to cells in suspension. The cells are then pulsed with hydrogen peroxide and lactoperoxidase, which catalyzes the oxidation of ^{125}I and converts it to a reactive species. Care should be given to the use of proper concentrations of cells, radioiodine, and lactoperoxidase. The cells must be checked for viability after labeling to ensure that they have tolerated the procedure.

Contributed by Steve Caplan and Michal Baniyash
Current Protocols in Cell Biology (2002) 7.10.1-7.10.13

Materials

$0.5–1 \times 10^8$ cells in suspension
PBS (*APPENDIX 2A*), azide free and ice cold
Na[^{125}I] (0.1 mCi/µl; NEN Life Science Products, Amersham Pharmacia Biotech, or ICN Biomedicals)
Lactoperoxidase enzyme solution (see recipe)
30% (v/v) hydrogen peroxide stock solution
Lactoperoxidase buffer (see recipe), ice cold
10 mM NaI in PBS (*APPENDIX 2A*), ice cold

Fume hood with double row of 4-cm-thick lead bricks
γ counter and appropriate counting vials

Prepare cells and reagents

1. Centrifuge $0.5–1 \times 10^8$ cells in suspension 5 min at $500 \times g$, 4°C, to pellet cells.
2. Wash cells three times with 25 ml ice-cold PBS by centrifuging as above.

   It is critical that there be no sodium azide in the PBS, as this impairs peroxidase activity.
3. While centrifuging, thaw Na[^{125}I] and lactoperoxidase enzyme solution. Dilute 30% hydrogen peroxide stock solution 1:1000 in ice-cold lactoperoxidase buffer. Keep all reagents and solutions on ice.
4. Resuspend cells in 1 ml ice-cold lactoperoxidase buffer. Keep suspended cells on ice.

Radiolabel cells

5. While working in a fume hood, add 20 µl Na[^{125}I] to the cells (2 mCi/ml cell suspension). Mix gently with pipet tip. Keep cells on ice throughout the entire reaction.
6. Add 100 µl lactoperoxidase enzyme solution to cells. Mix gently with pipet tip.
7. Add 25 µl diluted hydrogen peroxidase solution to the cells. Mix gently with pipet tip. Incubate cells 3 min on ice.
8. Add 50 µl lactoperoxidase enzyme solution to cells and mix gently with pipet tip.
9. Add 25 µl diluted hydrogen peroxidase and mix gently with pipet tip. Incubate cells 3 min on ice.
10. Pulse cells with an additional 50 µl diluted hydrogen peroxidase and incubate 3 min on ice. Repeat this step three times, incubating cells for 3 min on ice after each addition.
11. Wash cells three times with 10 ml ice-cold 10 mM NaI in PBS.

    This will terminate the incorporation of ^{125}I.

    These washes will contain extremely high levels of unincorporated ^{125}I, so great care must be taken to avoid contamination.

Determine ^{125}I incorporation

12. Suspend cells in 10 ml of 10 mM NaI in PBS and transfer a 10-µl aliquot to a counting vial and count the γ radiation using a γ counter. Pellet the cells.

    Whereas this reading is not a measure of specific activity, it does give a preliminary indication of the intensity of the label and can be used for comparisons with subsequent labeling experiments. It is also possible to estimate the general specific activity by first lysing the cells and then precipitating a sample of the protein by trichloroacetate. The

amount of protein and γ radiation can then be measured to attain the radioactivity per milligram protein.

It is also possible to count the number and percentage of live cells after the label with trypan blue staining (UNIT 1.1), in which unstained cells (i.e., those that exclude trypan blue) are counted as live cells. However, extreme caution must be exercised to avoid contamination.

13. Process and analyze pelleted cells.

It is also possible to freeze cell pellets at −70°C. However, the experimenter should bear in mind that the half-life of ^{125}I is ~60 days and some radiolysis may occur upon prolonged storage. Iodianted cell surface proteins are useful when there are no available antibodies directed at the extracellular regions of the proteins of interest. They can also be used to determine surface expression and topology of iodinated proteins by SDS-PAGE analysis. Peptide mapping can also be performed on cell surface–iodinated protein.

RADIOIODINATION OF MEMBRANE-SOLUBILIZED PROTEINS

BASIC PROTOCOL 2

This protocol uses lactoperoxidase beads to catalyze the iodination reaction. The entire radioiodination procedure should be carried out in a fume hood located in a room designated for radioactive work with ^{125}I.

Materials

1 ml membrane-solubilized proteins from 2×10^8 cells (see Support Protocols 1 and 2)
Lactoperoxidase beads in 50% suspension (Worthington Biochemical)
Na[^{125}I] (0.1 mCi/µl; NEN Life Science Products, Amersham Pharmacia Biotech, or ICN Biomedicals)
3% (w/v) dextrose
Triton X-100 lysis buffer (see recipe) with 0.02% (w/v) BSA

PD-10 columns (10-ml columns prepacked with Sephadex G-25 resin; Amersham Pharmacia Biotech)
γ counter and appropriate counting vials

1. Add the following to 1 ml membrane-solubilized proteins from 2×10^8 cells:

20 µl lactoperoxidase beads in 50% suspension (or follow manufacturer's instructions)
20 µl Na[^{125}I] (2 mCi)
150 µl of 3% dextrose.

Using immobilized lactoperoxidase prevents radioiodination of the enzyme and contamination of the desired labeled proteins with labeled lactoperoxidase. Dextrose is added for the generation of hydrogen peroxide in vitro.

To later estimate the specific activity of the iodinated membrane-solubilized proteins, it is advisable to calculate the protein concentration prior to the labeling procedure. This may be done using the Bradford assay or any other method (APPENDIX 3B).

2. Incubate 30 min on ice, mixing well every 10 min.

3. Wash a PD-10 column three times with 10 ml Triton X-100 lysis buffer with 0.02% BSA.

BSA saturates the column so that the small amount of radiolabeled material is not lost nonspecifically on the column.

4. Add the contents of the labeling tube to the column and collect 1-ml fractions until no more fluid drains out.

The labeled sample may be spun down prior to its loading on the column to separate the lactoperoxidase-coated beads. Alternatively, the entire sample may be applied to the column, and the lactoperoxidase-coated beads will be stacked at the top of the column, without interfering with the separation.

5. Add 9 ml Triton X-100 lysis buffer with 0.02% BSA and continue collecting 1-ml fractions until eight fractions are collected.

   It is important to add the lysis buffer when there is no more sample above the Sephadex resin to avoid dilution of the labeled sample.

6. Transfer a 1- to 10-µl aliquot from each fraction to a counting vial and measure its radioactivity using a γ counter.

7. Estimate the specific activity (cpm/mg protein) of the peaks.

   Usually the main fractions containing the labeled proteins are tubes 3, 4, and 5, and free Na[^{125}I]–containing fractions are in tubes 7 and 8. Based on the protein concentration obtained for the nonlabeled membrane-solubilized proteins, the specific activity may be estimated. This assumes a similar loss of protein by the end of the time the labeling is carried out, but the method is useful for comparing the equality of the labeling reaction from one experiment to the next.

8. Pool the desired fractions in a 15-ml conical centrifuge tube.

   The radiolabeled sample can be used for immunoprecipitation, as a probe for far western analysis (UNIT 17.2), and other applications. It can be stored in 0.05% (w/v) sodium azide at 4°C or in 50% (v/v) glycerol at −20°C for ~2 months, taking into consideration the 60-day half-life of ^{125}I.

MEMBRANE PREPARATION

The isolation of membranes serves as an important purification step because it removes cytosolic proteins and nuclei prior to solubilization of membrane proteins. This procedure enables the efficient solubilization of membranes and cytoskeleton-associated proteins without causing high levels of viscosity as a result of chromatin release from the cell nucleus.

NOTE: All steps in these protocols should be carried out at 4°C or on ice.

SUPPORT PROTOCOL 1

Membrane Preparation by Homogenization

This protocol utilizes a glass-glass (Dounce) homogenizer, which has proven useful for a number of different cell types. The exact conditions, including the number of strokes required for optimal cell disruption without breakage of nuclei, must be determined for each cell type. This can generally be accomplished by monitoring the procedure with the use of a phase-contrast microscope.

Materials

Cells grown in culture or from dissected organs
PBS (*APPENDIX 2A*), ice cold
Dounce buffer (see recipe)
10× phosphatase inhibitors (see recipe), optional
Tonicity restoration buffer (see recipe), ice cold
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
Triton X-100 lysis buffer (see recipe)

Phase-contrast microscope
Glass-glass (Dounce) homogenizer with pestle B, ice cold

10-ml conical centrifuge tube, prechilled
Ultracentrifuge, Beckman TLA100.3 rotor or equivalent, and ultracentrifuge tubes, 4°C

Additional reagents and equipment for cell counting (*UNIT 1.1*)

Prepare cells

1. Harvest the required number of cells.

   Cells may be harvested from either tissue culture (UNIT 1.1) or organs by standard procedures. Cells in suspension are centrifuged, whereas it is necessary to collect adherent cells with a cell scraper prior to centrifuging. The number of cells to harvest depends on both the application and the cell type used. Typically, solubilized membranes from 2×10^8 cells are labeled. However, for particularly small cells, such as splenocytes or thymocytes, more cells may be used.

2. Wash cells twice with ice-cold PBS.

3. Count the cells (*UNIT 1.1*), keeping a sample of the intact cells to compare with the ruptured cells using a phase-contrast microscope.

4. Resuspend cells at $1–5 \times 10^8$ cells/ml in Dounce buffer with 1× phosphatase inhibitors if needed.

   Cell concentration is generally $1–5 \times 10^8$/ml, depending on the cell size. The Dounce buffer induces cell swelling and facilitates homogenization. The Mg^{2+} concentration in the buffer stabilizes the nuclear envelope, but is also a co-factor for a number of proteases. The lowest Mg^{2+} concentration at which nuclear integrity is maintained during the homogenization process is recommended. Nuclear integrity can be monitored using a phase-contrast microscope.

   If phosphorylated proteins are studied, tyrosine phosphatase inhibitors should be included in all solutions containing protease inhibitors.

5. Incubate cells 10 to 15 min on ice.

   The cells swell during this incubation.

Homogenize cells

6. Transfer cells to an ice-cold, tight-fitting glass-glass (Dounce) homogenizer.

7. Apply 30 to 50 strokes with pestle B in a straight up and down position.

   The number of strokes varies, depending on the lability of the cell membrane. This will have to be determined for each cell type by monitoring membrane disruption using the phase-contrast microscope.

8. Add ice-cold tonicity restoration buffer to a final concentration of 150 mM.

   Check to ensure that the cell membranes are ruptured using the phase-contrast microscope and compare them to the sample of intact cells. Tonicity restoration will maintain the integrity of the nuclei.

   An additional method for cell disruption is the shearing of cells through a 25-G needle. Whereas this also generally works well, the needle has a tendency to clog, and care must be taken to avoid contamination.

Collect membranes

9. Transfer cells to a prechilled 10-ml conical centrifuge tube and centrifuge 5 min at $500 \times g$, 4°C, to spin out nuclei. Save the supernatant, which contains the membranes, and use the cell pellet to check the results of the Dounce homogenization under the microscope.

The membrane fraction will appear turbid and the nuclear pellet will be white when compared to an intact cell pellet.

10. Add 0.5 M EDTA to supernatant to a final concentration of 5 mM.

    EDTA is added to chelate Mg^{2+} and subsequently decrease the activity of magnesium-dependent proteases.

11. Transfer supernatant to an ultracentrifuge tube and ultracentrifuge 1 hr at 100,000 × *g* (~50,000 rpm), 4°C, in a Beckman TLA100.3 rotor or equivalent.

12. Transfer the pellet, which contains the membranes, to a 1.5-ml microcentrifuge tube.

    After ultracentrifugation, a pellet containing the membranes will be seen whereas the supernatant should remain clear. The supernatant obtained after ultracentrifugation contains the cytosol and free lipids. Free lipids will float near the top of the tube.

Solubilize membranes

13. Resuspend membranes at 2×10^8 cell equivalents/ml in Triton X-100 lysis buffer for 20 to 45 min at 4°C with frequent, gentle vortexing.

    If a large amount of membrane is prepared (e.g., from 5×10^8 small cells, such as lymphocytes), resuspend the membranes first in lysis buffer without Triton X-100 and then add Triton X-100 to a final concentration of 0.5% (v/v).

14. Centrifuge 15 min at 12,000 × *g*, 4°C, to pellet insoluble material.

15. Save the soluble membrane extract in a small microcentrifuge tube.

    Membrane extracts can be frozen at –70°C until needed. However, freezing and thawing extracts may affect protein-protein interactions.

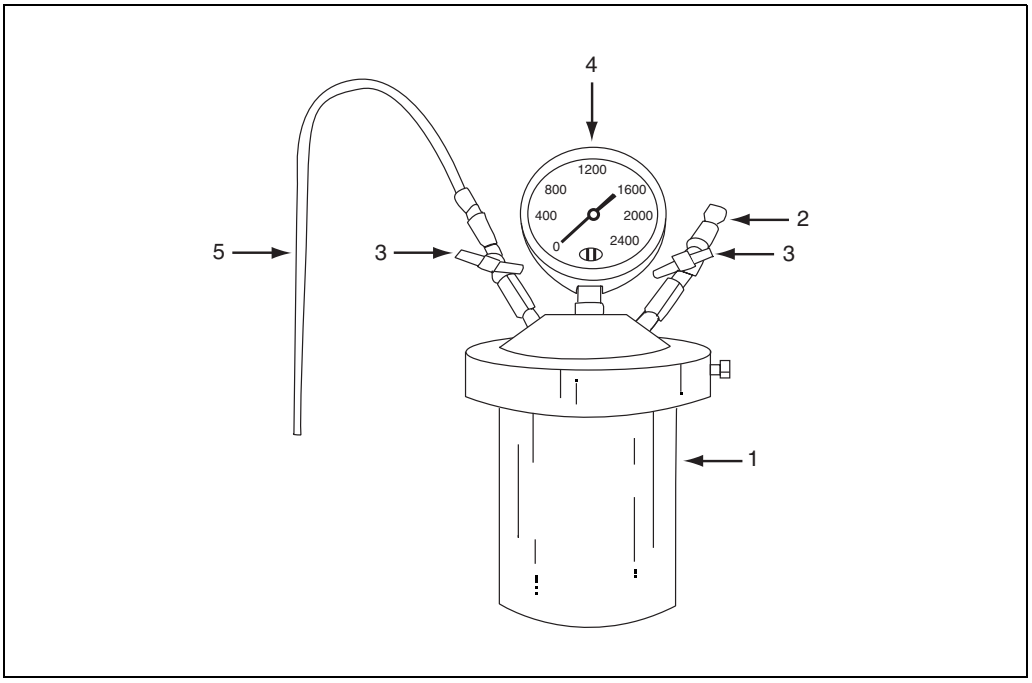


Figure 7.10.1 Nitrogen cavitation device. 1, High-pressure vessel (nitrogen bomb); 2, nitrogen filling tube; 3, valves regulating the flow of nitrogen to and from the vessel; 4, pressure gauge; 5, discharge tube.

Membrane Preparation by Nitrogen Cavitation

SUPPORT PROTOCOL 2

This is an alternative approach for membrane preparation. Nitrogen gas is dissolved in a high-pressure vessel (Fig. 7.10.1). Upon decompression, bubbles form and burst the cells. This technique is fast, easy, uniform, and reproducible. The efficiency should be determined by monitoring the samples with a phase-contrast microscope. A nitrogen cavitation device with a nitrogen gas tank is required, however, and this equipment is not inexpensive.

Additional Materials *(also see Support Protocol 1)*

Nitrogen cavitation device (e.g., Cell Disruption Bomb, Parr Instrument; Fig. 7.10.1)
Nitrogen gas tank

1. Harvest and swell cells as described (see Support Protocol 1, steps 1 to 5).
2. Place cells in a nitrogen cavitation device attached to a nitrogen gas tank and pressurize to 100 psi for 10 min at 4°C.

   Before the cavitation device is used, it should be pressurized and checked for leaks.

   This level of pressure is good for many cell types; however, the pressure, time of incubation, and rate of release from the nitrogen cavitation device should be optimized for each cell type.
3. Transfer cells to a prechilled 10-ml conical centrifuge tube.
4. Restore tonicity and collect membranes as described (see Support Protocol 1, steps 8 to 15).

RADIOIODINATION OF SOLUBLE PROTEINS

Raadioiodinated soluble proteins have been used extensively in cell biology and biochemistry, particularly for receptor binding and internalization assays. The high specificity by which radioiodination targets tyrosine residues (3.6% of the total amino acids in proteins) renders this technique less likely to impair antibody function than nonradioactive methods, such as biotinylation.

Lactoperoxidase-Catalyzed Radioiodination of Soluble Proteins

BASIC PROTOCOL 3

Soluble proteins can be radioiodinated by the lactoperoxidase-catalyzed or chloramine-T methods. The lactoperoxidase-catalyzed reaction is milder than the chloramine-T method and causes less protein denaturation. However, the specific activity of the radiolabeled proteins obtained by the lactoperoxidase-catalyzed method will be lower than that obtained with the chloramine-T method. Soluble proteins, such as immunoglobulins and protein A, can be radiolabeled using this protocol.

Materials

10 mg/ml BSA in PBS
PBS (*APPENDIX 2A*), ice cold
30% (v/v) hydrogen peroxide stock solution
0.025 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*), ice cold
1 mg/ml protein sample in PBS
Na[^{125}I] (~0.1 mCi/µl; NEN Life Science Products, Amersham Pharmacia Biotech, or ICN Biomedicals)
1.5 mg/ml lactoperoxidase enzyme (Sigma or Calbiochem) in PBS (*APPENDIX 2A*), stored in aliquots at −70°C or prepared fresh
15 mM NaI in PBS, prepared fresh and ice cold

PD-10 columns (10-ml columns prepacked with Sephadex G-25 resin; Amersham Pharmacia Biotech)
γ counter and appropriate counting vials

Set up column

1. Load a PD-10 column with 1 ml of 10 mg/ml BSA in PBS and then wash the column with 20 to 30 ml ice-cold PBS.

   The BSA saturates the column so that the small amount of radiolabeled material will not be absorbed nonspecifically to the column, which reduces recovery yields. Other carrier proteins such as fetal calf serum or ovalbumin can be used at the same concentration.

Label proteins

2. Immediately before use, dilute 30% hydrogen peroxide stock solution 1:1000 in ice-cold 0.025 M sodium phosphate buffer to a final concentration of 0.03%.

3. To 10 µl of 1 mg/ml protein sample, add the following:

   0.5 mCi Na[^{125}I]
   10 µl of 1.5 mg/ml lactoperoxidase enzyme
   2 µl of 0.03% hydrogen peroxide.

   Incubate 1 min at room temperature with continuous mixing.

   Because lactoperoxidase activity is inhibited by azide and sulfhydryl reagents, these should be avoided when the sample is prepared for labeling.

4. Repeat the addition of 2 µl of 0.03% hydrogen peroxide three more times at 1-min intervals.

5. Add 0.5 ml of 15 mM NaI in PBS.

   This serves to compete with the labeled NaI and terminate the reaction.

Isolate labeled proteins

6. Use a disposable pipet to layer the sample onto the PD-10 column (step 1) at room temperature and collect 1-ml fractions until no more fluid drains out.

7. Add 10 ml PBS and continue collecting 1-ml fractions.

   It is important to add the PBS when there is no more sample above the Sephadex resin to avoid dilution of the labeled sample.

8. Transfer a 1- to 10-µl aliquot from each fraction to a counting vial and measure its radioactivity using a γ counter.

9. Determine the specific activity (cpm/mg protein) of the peaks.

   The first peak should contain radioiodinated proteins, whereas the second should have unincorporated (free) radioiodide.

10. Pool the desired fractions in a 15-ml conical centrifuge tube.

    The radiolabeled sample can be used for immunoprecipitation (UNIT 7.2), as a probe for far western analysis (UNIT 17.2), and other applications. It can be stored in 0.05% (w/v) sodium azide at 4°C or in 50% (v/v) glycerol at −20°C for up to 2 months, taking into consideration the 60-day half-life of ^{125}I.

Chloramine-T-Mediated Radioiodination of Soluble Proteins

ALTERNATE PROTOCOL

As described above (see Basic Protocol 3), the chloramine-T method is not as mild as lactoperoxidase-catalyzed radioiodination. It does, however, result in radiolabeled proteins with a higher specific activity.

Additional Materials *(also see Basic Protocol 3)*

0.5 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*)
Chloramine-T (Sigma)
Sodium metabisulfite (Sigma)

1. Prepare a PD-10 column as described (see Basic Protocol 3, step 1).
2. To 10 µl of 1 mg/ml protein sample add 10 µl of 0.5 M sodium phosphate buffer.

   The phosphate buffer provides additional buffering capacity required to neutralize the Na[^{125}I], which is supplied in 0.1 M NaOH.
3. Immediately before use, prepare 2.25 mg/ml chloramine-T and 7 mg/ml sodium metabisulfite each in 0.5 M sodium phosphate buffer.

   The chloramine-T and sodium metabisulfite should be weighed in separate tubes and then dissolved in the proper volume of phosphate buffer immediately before use.

   CAUTION*: Wear gloves when handling these reagents because they are both toxic.*
4. Add 0.5 mCi Na[^{125}I] and 10 µl of 2.25 mg/ml chloramine-T to the protein sample (step 2). Incubate 1 min at room temperature.

   Chloramine-T is a potent oxidant that converts iodine into its reactive I^+ state.
5. Add 10 µl of 7 mg/ml sodium metabisulfite (step 3) to the mixture to stop the radioiodination reaction. Wait 1 min and then add 50 µl of 15 mM NaI in PBS.
6. Layer the sample onto the PD-10 column and isolate the labeled proteins as described (see Basic Protocol 3, steps 6 to 10).

REAGENTS AND SOLUTIONS

Use deionized water, preferably filtered, in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers see* ***SUPPLIERS APPENDIX****. It is recommended that all solutions be prepared using sterile reagents.*

Dounce buffer

10 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.5 mM $MgCl_2$ (*APPENDIX 2A*)
Store up to 1 year at 4°C
Immediately before use add:
10 mg/ml aprotinin (Boehringer Mannheim–Roche)
10 mg/ml leupeptin (Sigma)
1 mM PMSF (*APPENDIX 2A*; Sigma)
1.8 mg/ml iodoacetamide (Sigma)

PMSF should be added immediately prior to use, as it undergoes hydrolysis in aqueous solutions.

Lactoperoxidase buffer

Mix 16.8 ml PBS (*APPENDIX 2A*); 1 ml 0.5 M NaH_2PO_4, pH 7.6 (see recipe); and 2.2 ml water. Store indefinitely at 4°C, but ensure it is not contaminated and check the pH after prolonged storage and adjust as needed.

This buffer is a PBS-based buffer with additional sodium phosphate.

Lactoperoxidase enzyme solution

Dissolve 1.5 mg lactoperoxidase (Sigma or Calbiochem) in 1 ml lactoperoxidase buffer (see recipe). Store in 50- and 250-µl aliquots at −70°C and thaw only once before use

NaH_2PO_4, 0.5 M, pH 7.6

Add 6.9 g NaH_2PO_4 to 100 ml water. Adjust pH to 7.6 with 0.5 M $NaHPO_4$. Store indefinitely at 4°C.

Phosphatase inhibitors, 10×

4 mM sodium orthovanadate
4 mM EDTA, pH 8.0 (*APPENDIX 2A*)
100 mM NaF
100 mM sodium pyrophosphate
Adjust pH to 7.6 and freeze aliquots at −20°C

Tonicity restoration buffer

10 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.5 mM $MgCl_2$ (*APPENDIX 2A*)
0.6 M NaCl (*APPENDIX 2A*)
Store up to 1 year at 4°C
Immediately before use add:
10 mg/ml aprotinin (Boehringer Mannheim–Roche)
10 mg/ml leupeptin (Sigma)
1 mM PMSF (*APPENDIX 2A*; Sigma)
1.8 mg/ml iodoacetamide (Sigma)

PMSF should be added immediately prior to use, as it undergoes hydrolysis in aqueous solutions.

Triton X-100 lysis buffer

150 mM NaCl (*APPENDIX 2A*)
50 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.5% (v/v) Triton X-100
Store up to 1 year at 4°C
Immediately before use add:
10 mg/ml aprotinin (Boehringer Mannheim–Roche)
10 mg/ml leupeptin (Sigma)
1 mM PMSF (*APPENDIX 2A*; Sigma)
1.8 mg/ml iodoacetamide (Sigma)

PMSF should be added immediately prior to use, as it undergoes hydrolysis in aqueous solutions.

COMMENTARY

Background Information

The process of radioiodination occurs when ^{125}I (or ^{131}I) is introduced at the positions *ortho* to the hydroxyl group on tyrosine residues. This reaction may be carried out by either chemical or enzymatic oxidation activity. In most cases, ^{125}I is chosen for radioiodination; despite ^{131}I having about 7-fold greater specific radioactivity, the isotopic abundance of ^{131}I is usually <20% (due to ^{127}I contaminants). Moreover, the half-life of ^{131}I is only 8 days as compared to 60 days for ^{125}I.

Cell surface radioiodination

Cell surface labeling is an important tool to detect the presence of proteins on the cell surface. This technique is particularly useful for labeling surface membrane proteins for which there are no available antibodies directed against their extracellular domain. Such pro-

teins cannot be detected by simpler methods, such as the sorting of fluorescence-labeled cells or immunostaining. It can also be utilized to determine whether an unidentified protein that co-immunoprecipitates with a known protein is expressed on the cell surface membrane. Another application of cell surface labeling is to follow the kinetics of cell surface–expressed proteins. For example, cells can be stimulated and then surface labeled at various time points. Alternatively, cell surface proteins can be labeled, and then the labeled cells can be cultured without further labeling (i.e., chased) to determine the kinetics of the loss of labeled proteins from the cell surface.

Two commonly used cell surface labeling procedures are radioiodination and biotinylation. Lactoperoxidase-catalyzed radioiodination (Basic Protocol 1) almost exclusively labels tyrosine residues, whereas most methods of protein biotinylation primarily target lysine residues. This is an important consideration in choosing which type of cell surface label to utilize. Whereas the average frequency of tyrosine in proteins is only 3.6% of the total amino acids, lysine occurs with a frequency of 7%. However, one advantage of radioiodination is that it is unlikely to impair the ability of the protein to be recognized by a specific antibody during immunoprecipitation or immunoblot analysis. In contrast, conjugating proteins with biotin has been known to impede antibody binding if the biotinylated lysine residue happens to be within the epitope recognized by the antibody. Moreover, due to the high frequency of lysines in proteins, multiple biotinylation often occurs within the same protein. Each biotin that is conjugated adds an additional 500 Da to the molecular weight of the protein, and as a result, the resolved protein product often displays a range of molecular weights, depending on the number of conjugated biotin moieties. Interpreting these results can be confusing, especially when combined with assays to detect phosphorylated proteins, which often display an upshift in their apparent molecular weight.

Cell surface labeling of proteins by a lactoperoxidase-catalyzed reaction results in the covalent attachment of ^{125}I to accessible tyrosine residues (reviewed by Morrison, 1980). Because of the size of the enzyme, this reaction occurs exclusively on the outside of the plasma membrane. As the reaction and all reagents are kept on ice, limited internalization of radioiodinated surface proteins is expected.

There are various other methods for iodinating cell surface proteins, including the IODO-Beads reagent (Pierce) and the use of lactoperoxidase-coupled beads (rather than free lactoperoxidase), which can reportedly reduce background for certain applications (Koch and Haustein, 1976).

Preparation of membranes

Membrane preparation must precede iodination of membrane-solubilized proteins (Basic Protocol 2). Membrane preparation by homogenization (Support Protocol 1) utilizes one type of homogenizer that consists of a cylindrical glass homogenization vessel, in which either a rod-shaped Teflon-coated pestle or a glass pestle with a ball-shaped tip is inserted. Differences between the use of the two are summarized by Kinne-Saffran and Kinne (1989). The Teflon-glass homogenizer is advantageous for the homogenization of tissue fragments with a lower content of connective tissue, whereas the glass-glass homogenizer is more appropriate for tissue with a higher content of connective tissue and for single cells.

Membrane preparation by nitrogen cavitation (Support Protocol 2) allows quantitative disruption of cells in an inert atmosphere without danger of local heating. This method is mainly used for single cells in suspension or for cells grown in culture.

Radioiodination of soluble proteins

Labeling soluble proteins with radioiodine has been a standard laboratory procedure for many years. The techniques are simple, inexpensive, and reliable. Although different labeling techniques have been developed over the years, such as biotinylation and enzyme- or fluorescein-coupling, the use of radioiodinated proteins has some advantages when compared to these other labeling procedures. For example, samples containing radioiodinated proteins can be exposed to films for a longer time so that the signals emitted accumulate over time on autoradiographic film. (The ^{125}I half-life is 60 days.) Proteins labeled by other methods may provide limited levels of signal. This is particularly important when small amounts of a specific protein are tested. The radioiodination protocols presented here are those that are most commonly used (Morrison 1980). They depend on the availability of tyrosine residues, but in most cases, some histidine residues are also radiolabeled. Other labeling techniques are reviewed in Hubbard and Cohn (1976).

The use of immobilized lactoperoxidase as the catalyst for oxidation allows the easy removal of the enzyme itself, which can often

cause much background due to its own radioiodination. The column allows the removal of free radioiodine.

Critical Parameters

Safety precautions for working with ^{125}I are described at the beginning of this unit.

Cell surface radioiodination

The conditions suggested for this protocol have been optimized for a system in which immunoprecipitation is commonly the method of analysis. The number of cells used in this protocol is appropriate for cells of relatively small size, such as lymphocytes. For larger cells, such as fibroblasts, it may be advisable to radioiodinate $1–2 \times 10^7$ cells. It is important to test the viability of the cells after the procedure; if the cells have not tolerated the [^{125}I]Na labeling, there is likely to be a higher level of background (i.e., ^{125}I-labeled intracellular proteins). Typically, 95% viability should be anticipated. If lower levels of viability are encountered, the amounts of diluted hydrogen peroxide solution may be lowered.

Preparation of membranes

Success of membrane preparation depends upon the careful optimization of the conditions used for each cell type. For Dounce homogenization, the exact conditions, including swelling time and the number of strokes required for optimal cellular disruption without breakage of nuclei, must be determined. This can generally be accomplished by monitoring the disruption using a phase-contrast microscope. In the case of nitrogen cavitation, shearing and breakage of intracellular organelles is minimal if the pressure and time of equilibration are properly adjusted. It is important that the lysis is relatively rapid (to preserve transient associations of proteins), and that the pressure is high enough to ensure complete disruption of the cells and thereby prevent contamination of the nuclear fraction with intact cells. However, the pressure must be low enough to prevent excessive fragmentation of cell membranes.

Protease inhibitors should be used throughout the procedure and if phosphorylated proteins are of interest, phosphatase inhibitors should be used as well. Iodoacetamide is included with the protease inhibitors but it is added primarily because it blocks free sulfhydryls. This is required because after solubilization, disulfide bonds may be formed between polypeptides that are not normally bound covalently, leading to artifactual results.

Magnesium salts are used during cell breakage to stabilize the nuclear envelope; however, as magnesium is a co-factor for many proteases, these should be removed by chelation with EDTA after removal of nuclei.

Radioiodination of soluble proteins

Radioiodination of proteins may induce protein denaturation and inactivation, and therefore the labeling conditions for a specific protein should be determined. The use of lactoperoxidase beads for labeling membrane proteins, which are solubilized in lysis buffer, enables the researcher to perform the radioiodination by adding a minimal volume of beads and dextrose instead of the addition of lactoperoxidase solution and hydrogen peroxide periodically. Thus lysis buffer is not diluted and ratios between the Triton X-100 and the membrane lipids are maintained throughout the entire labeling procedure. Solubilized membrane proteins can be labeled in a manner similar to soluble proteins, utilizing lactoperoxidase and hydrogen peroxide. For the success of the labeling procedure, care should be taken to use proper concentrations of proteins (soluble or membrane-bound), radioiodine, and catalysts. For sensitive proteins such as IgE for example, a lower concentration of lactoperoxidase or chloramine-T and metabisulfite should be used.

Iodinated proteins should be stored in 0.05% (w/v) sodium azide at 4°C to inhibit bacterial contamination. Alternatively, aliquots may be stored at −20°C with 50% (v/v) glycerol. The presence of a carrier protein such as serum or albumin also helps prevent breakdown of protein due to radiation damage during storage.

Anticipated Results

Cell surface radioiodination

A typical cell surface radioiodination experiment should yield ~95% viable cells. A moderately expressed surface protein (with accessible tyrosines) should be detectable by immunoprecipitation and autoradiographic exposure of dried SDS-PAGE gels or of nitrocellulose membranes in 1 to 4 days. Highly expressed surface proteins, or proteins with multiple tyrosines may be detectable in exposure times of hours. An enhancing screen in the exposure cassette and storage of the cassette at −70°C will expedite results.

For many applications, an important control for surface-labeling experiments is testing the level of leakiness, or the level of intracellular proteins labeled by this procedure. One possi-

bility is to immunoprecipitate an entirely intracellular tyrosine-containing protein, such as actin or tubulin. Although these are highly abundant proteins, little or no label should be expected.

Preparation of membranes

Purification of membranes prior to solubilization results in a 5- to 10-fold purification over total cell extracts, but also in a variable decrease in yield. This will vary depending on the procedure used for cell breakage. In preparing membranes, as the efficiency of cellular disruptions increases, so does the degree of nuclear damage.

Membranes may also be isolated from specific intracellular organelles. Isolation of intracellular organelles is described in Chapter 3 of this book.

Radioiodination of soluble proteins

Radiolabeled soluble proteins can be used for immunoprecipitation (*UNIT 7.2*), immunoblotting (*UNIT 6.2*) and far western analysis (*UNIT 17.2*). Autoradiographic exposure time of dried SDS-PAGE gels or of nitrocellulose membranes can range from 1 to 7 days, depending on the levels of the labeled proteins. An enhancing screen in the exposure cassette and storage of the cassette at −70°C will expedite results.

Time Considerations

Once the cells have been harvested, the procedures described here should take <3 to 4 hr. For radioiodination of solubilized membrane proteins, membrane preparation takes an additional 2 to 3 hr.

Literature Cited

Hubbard, A.L. and Cohn, Z.A. 1976. Specific labels for cell surfaces. *In* Biochemical Analysis of Membranes (A.H. Maddy, ed.) pp. 427-501. Wiley & Sons, London.

Kinne-Saffran, E. and Kinne, R.K. 1989. Membrane isolation: Strategy, technique, markers. *Methods Enzymol.* 172:3-17.

Koch, N. and Haustein, D. 1981. Radioiodination of surface proteins and glycoproteins of lymphocytes by immobilized lactoperoxidase. *J. Immunol. Methods* 41:163-171.

Morrison, M. 1980. Lactoperoxidase-catalyzed radioiodination as a tool for investigation of proteins. *Methods Enzymol.* 70(A):214-220.

Key Reference

Bailey, G.S. 1994. Labeling of peptides and proteins by radioiodination. *In* Methods in Molecular Biology, Vol. 32 (J.M. Walker, ed.) pp. 441-448. Humana Press, Totowa, N.J.

Contains background information and standard protocols for radioiodination by chloramine-T- and lactoperoxidase-catalyzed radioiodination.

Contributed by Steve Caplan
NICHD, National Institutes of Health
Bethesda, Maryland

Michal Baniyash
Hebrew University-Hadassah Medical School
Jerusalem, Israel

CHAPTER 8
Cell Cycle Analysis

INTRODUCTION

The cell cycle is the ordered set of processes through which cells divide, producing two daughter cells possessing the same set of genetic information (Murray and Hunt, 1993). To pass successfully through the cell cycle, all cells must complete three essential tasks: replicate their DNA, faithfully segregate their chromosomes to the daughter cells, and separate the daughter cells from each other. In most cases, cells must also grow during each cell cycle so that the daughters are of sufficient size. One of the most fundamental decisions cells make is whether to progress through the cell cycle. Frequently, this decision is closely linked with choices of cell fate, such as whether to undergo differentiation or programmed cell death.

The last ten years has witnessed a dramatic growth in our understanding of the fundamental mechanisms that regulate the cell cycle. It has become clear that the cell cycles of all eukaryotes are regulated by a family of cyclin-dependent kinases (CDKs). As described in *UNIT 8.1*, these kinases drive both chromosomal DNA replication (S phase) and mitosis (M phase). These kinases are in turn controlled by phosphorylation and by the regulated proteolysis of their cyclin subunits. Orderly progression through the cell cycle is assured by regulatory mechanisms (frequently called checkpoints; Hartwell and Weinert, 1989) that inhibit advancement to the next phase of the cell cycle until necessary functions in the current phase are complete—e.g., by blocking the activation of mitotic CDKs and chromosome condensation until DNA replication is complete.

To address questions related to the cell cycle, it is essential to have clear markers for the cell-cycle state of cells. While mitosis is accompanied by clear morphological changes, biochemical markers are required to determine the state of cells during interphase. Since chromosomal DNA replication occurs during S phase, it is possible to assay directly whether individual cells are in S phase by measuring their capacity to incorporate nucleotide analogs, such as bromodeoxyuridine (BrDU), into DNA (*UNIT 8.2*). DNA content is a general diagnostic marker for whether cells have undergone DNA replication in S phase (thereby doubling their DNA content) or cell division in M phase (thereby reducing their DNA content by half). DNA content can also be monitored by flow cytometric analysis (*UNIT 8.4*). Finally, since different CDKs are active at different phases of the cell cycle, the activities of members of the CDK family also serve as a clear diagnostic marker for the cell-cycle state of cells (*UNIT 8.2*). A combined approach may be used for a more detailed description of the cell-cycle state, monitoring both the abundance of cyclin subunits and the DNA content of cells by flow cytometric analysis (*UNIT 8.4*).

The capacity to obtain populations of cells that are uniform with respect to their cell-cycle state is also essential for cell-cycle studies. Cultured cells can be arrested at various stages of the cell cycle through manipulation of their nutritional status or through treatment with inhibitors that block their progression through S phase or mitosis (*UNIT 8.3*). Alternatively, M phase cells can be obtained from adherent cultures through "mitotic shakeoff", taking advantage of the decreased attachment of cells to the culture vessel during mitosis (*UNIT 8.3*). Since a relatively small proportion of cells in an exponentially growing culture are in mitosis at any given time, mitotic shakeoff is frequently combined with some

Contributed by Mary Dasso
Current Protocols in Cell Biology (1999) 8.0.1-8.0.2

inhibitor treatment or nutrient manipulation. Prolonged arrest in response to nutritional manipulation or drug treatments can frequently perturb cell metabolism. In cases where this is undesirable, it is possible to make use of the fact that cell size is directly correlated with cell cycle stage. Centrifugal clutriation (*UNIT 8.5*), which separates cells on the basis of size, can be employed to obtain synchronous populations of cells from different cell cycle phases without drug treatments.

LITERATURE CITED

Hartwell, L.H. and Weinert, T.A. 1989. Checkpoints: Controls that ensure the order of cell cycle events. *Science* 246:629-634.

Murray, A.W. and Hunt, R.T. 1993. The Cell Cycle. W.H. Freeman, New York.

Mary Dasso

Overview of the Cell Cycle

Inductive signals from the environment and a variety of intracellular signals determine whether a cell enters the mitotic cell cycle and proliferates or withdraws from the mitotic cycle to meet with a nonproliferative fate. These signals influence the activation of cyclin-dependent kinases (CDKs), which lie at the heart of the intrinsic cell cycle machinery responsible for integrating cell division with developmental and environmental stimuli. A better understanding of these CDKs is expected to provide insight into the diverse processes that involve signal transduction, differentiation, senescence, apoptosis, development, and tumorigenesis.

THE BIOLOGY OF THE CELL CYCLE

A typical cell cycle proceeds with alternating rounds of DNA replication, which takes place during S phase, and nuclear and cytoplasmic division, which take place during M phase. DNA synthesis and mitotic division are separated by the intervening gap phases, G_1 and G_2, during which checkpoint controls are superimposed to preserve the accurate transmission of genetic information. Checkpoints achieve this by monitoring cell cycle events, such as DNA replication and spindle assembly, and generating signals in response to errors in these processes so that cell cycle progression can be halted until repairs are complete (Murray, 1994). These controls ensure that cell cycle events occur in the appropriate sequence and that cell cycle arrest will occur if these events are not carried out in the correct order. Checkpoint controls are responsible, therefore, for ensuring that cells enter S phase only after successful completion of the previous mitosis, attainment of adequate cell size, and repair of any spontaneous or environmentally induced DNA damage. Alternatively, checkpoints induce apoptotic pathways for damaged cells as a way of preventing them from giving rise to mutant progeny. For example, the tumor suppressor protein p53 plays an important role in DNA damage–induced cell death (Lowe et al., 1993) and G_1 phase arrest (Kuerbitz et al., 1992). Signals regulating checkpoints and controlling cell proliferation interact very closely with each other.

After each mitotic cycle, cells traversing G_1 phase receive signals from the extracellular environment, as well as intracellular cues dictated in part by checkpoints, before continuing in the cell cycle to replicate their DNA and divide once again. Extracellular signals that promote entry into S phase or the beginning of a new mitotic cycle include growth factors, mitogens, nutrients, and cell-substratum interactions. Alternatively, in the absence of mitogenic signals or in the presence of mitogenic antagonists, differentiation inducers, and certain spatial cues, cells withdraw from the cell cycle with unduplicated DNA and meet with alternate, nonproliferative fates, such as quiescence (G_0 phase) or differentiation. Some cell types may transiently undergo cell cycle arrest and then undergo apoptosis because the growth factors required for their proliferation may also be necessary for their continued survival.

A cell's "decision" either to commit to or withdraw from the mitotic cycle occurs at the restriction point late in G_1 phase, after which mitogenic growth factors are no longer required for cells to complete division (Pardee, 1989). In other words, cells that pass the restriction point, which occurs 1 to 3 hr prior to the onset of replication, proceed through the remainder of the cell cycle independently of environmental signals. The restriction point encompasses the critical regulatory mechanism that enables a cell to monitor its external and internal environment as well as the integration of these environmental cues to control the switch between alternative cellular fates, such as proliferation, temporal cell cycle arrest, quiescence, and differentiation. Passage through the restriction point and entry into S phase is determined by the phosphorylation status of the retinoblastoma tumor suppressor protein Rb, which is essentially governed by the activities of the CDKs.

THE CELL CYCLE ENGINE

The CDKs

The division cycle of a eukaryotic cell is currently understood (Morgan, 1995) to be a series of phases or transitions in which temporal order is imposed first by the sequential assembly and activation of the CDKs, and second by their proteolytic inactivation (Fig. 8.1.1). CDKs are enzymes composed of a catalytic subunit (the CDK) and an essential activator subunit (the cyclin). In mammalian cells,

Contributed by Michele S. Park and Andrew Koff

Current Protocols in Cell Biology (1998) 8.1.1-8.1.9

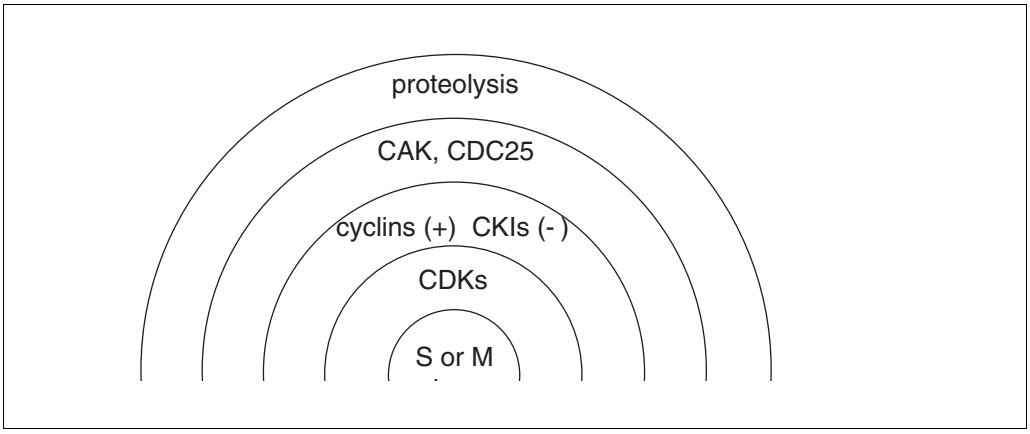


Figure 8.1.1 Peeling back the layers of proliferation control. This "onion" model of proliferation indicates the relationship of each cell cycle component to proliferation or cell division. Cyclin-dependent kinase (CDK) activity, which is absolutely required, depends upon physical interaction with positive regulatory subunits (cyclins) and can be prevented by physical interaction with negative regulatory subunits, called CDK-inhibitory proteins (CKIs). The activation of CDKs also requires phosphorylation by an activating kinase (CAK) and dephosphorylation of phosphates in the ATP-binding motif by members of the Cdc25 family. The entire mechanism can be regulated by proteolysis, because many cyclins and CKIs are targeted by ubiquitin-dependent degradation.

a succession of kinase subunits (CDK4, CDK6, CDK2, and Cdc2) is expressed along with a succession of cyclins (D, E, A, and B) as cells progress from G_1 to M. Cdc2 forms complexes with cyclins A and B to regulate entry into the M phase of the cell cycle.

CDK4/6 and CDK2, whose regulatory partners are the D-type cyclins (D1, D2, and D3) and cyclin E, respectively, represent two distinct classes of G_1 phase–specific CDKs whose activation is required for entry into S phase (Koff et al., 1992; Lew et al., 1991; Xiong et al., 1991). Cyclin D–dependent kinase activity is first detected in mid-G_1 phase as cells approach the G_1/S boundary. Cyclin D–dependent kinases have a distinct substrate preference for the retinoblastoma protein Rb (Matsushime et al., 1992, 1994; Meyerson and Harlow, 1994). Rb binds to and negatively regulates the activities of transcription factors such as E2F-DP1 heterodimers, whose functions are critical for the G_1/S phase transition (reviewed in Nevins, 1992). Under conditions favoring proliferation, the inactivating phosphorylation of Rb by the CDKs in mid- to late-G_1 phase results in liberation of E2F and other Rb-bound transcription factors, which then activate the transcription of S-phase genes. The phosphorylation of Rb is thereby modulated during the cell cycle, in that Rb is present in a hypophosphorylated, active state in G_0 and early G_1 and becomes hyperphosphorylated, and thereby inactive, in late G_1.

Cyclin E–associated kinase activity, although not required for phosphorylation of Rb, is rate limiting for entry into S phase. Cyclin E is expressed periodically at maximum levels near the G_1/S phase transition (Dulic et al., 1992; Koff et al., 1992) and is essential in *Drosophila* for completion of G_1 and progression into S phase (Knoblich et al., 1994). Cyclin E apparently regulates a transition different from that promoted by the D-type cyclins, because cyclin E, but not cyclin D1, is essential for entry into S phase in mammalian cells lacking functional Rb. Inactivation of any of the G_1 CDKs leads to cessation of proliferation and withdrawal from the mitotic cycle.

The activation status of the CDKs is regulated by many mechanisms reflecting both the diversity of the signals they integrate and the central importance of their roles in cell cycle control (Fig. 8.1.2). In general, these regulatory mechanisms act on the synthesis and degradation of the cyclin proteins, formation of cyclin-CDK complexes, positive and negative modification of kinase subunits by phosphorylation, and inhibitory constraints on kinase activity exerted by at least two families of CDK inhibitory proteins (CKIs).

Regulation of CDK activity by cyclins

The primary modulator of CDK activity is the cyclin subunit. Cyclin function is primarily controlled by changes in cyclin levels, which oscillate characteristically throughout the cell

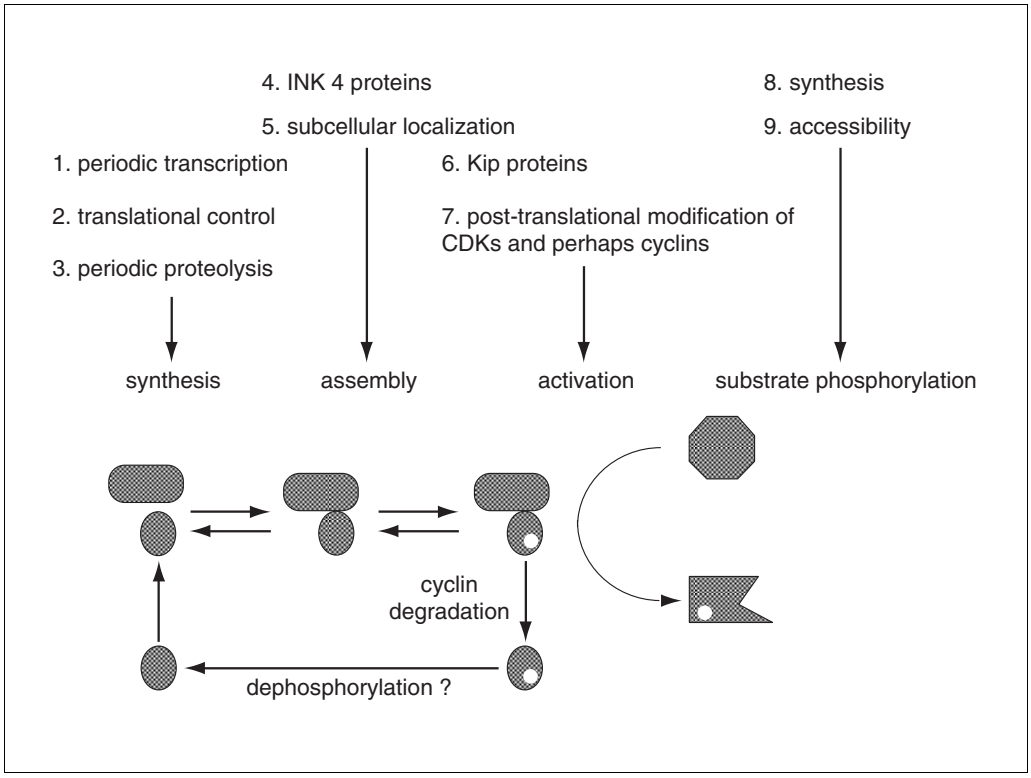


Figure 8.1.2 Activation (and inactivation) of cyclin-dependent kinases (CDKs). This schematic representation shows the five-step pathway (synthesis, assembly, activation, substrate phosphorylation, and cyclin degradation) that leads to CDK activation and proteolytic inactivation. The cyclin is oval, the CDK is round, the substrate is hexagonal, and activating phosphorylation is indicated by the inclusion of a white circle. Phosphorylation of the substrate may affect its structure, as shown here, or may only change the substrate's activity or make it a more attractive binding target for other proteins. Mechanisms that interfere with each step are listed above the step. Abbreviations: INK4, inhibitor of CDK4; KIP, CDK-inhibitory protein.

cycle. In the early *Xenopus* embryo, oscillations in cyclin B levels are mainly controlled by changes in the rate of cyclin degradation. Constant synthesis of cyclin B during the cell cycle provides a roughly linear increase in cyclin concentration until mitosis, at which point cyclin B degradation increases abruptly, resulting in a rapid decline in cyclin levels. Cyclin degradation seems to involve the ubiquitin-dependent proteolytic machinery, which requires the destruction box sequence motif near the N terminus of mitotic cyclins (Glotzer et al., 1991). The trigger for the initiation of cyclin B degradation is not entirely clear, but the proteolytic pathway is well defined.

Regulation of cyclin levels at the transcriptional level occurs in mammalian cells and yeast. In mammalian cells, sequential, periodic oscillations in the levels of the major cyclins largely reflect changes in messenger RNA levels (Pines and Hunter, 1989, 1990), but little is known about the mechanisms that generate these waves of gene expression. These mechanisms have been determined more comprehensively in the yeast *Saccharomyces cerevisiae*, where G_1 cyclins (CLNs) and M cyclins (CLBs) cooperate in overlapping feedback loops to ensure the properly timed execution of their transcription and degradation programs (Amon et al., 1993, 1994). Cyclins may act further to regulate CDK activity by targeting the bound CDK to specific substrates or subcellular locations.

Activation of CDK activity

Because cellular CDK levels tend to remain in constant excess throughout the normal cell cycle, most of the regulatory controls on catalytic activity are post-translational. In addition to cyclin binding, complete activation of the CDK requires phosphorylation at a conserved threonine residue (Thr-160/161). The enzyme responsible for this activating phosphorylation is itself a CDK and has been designated CAK for CDK-activating kinase. CAK is a multisubunit enzyme composed of a catalytic sub-

unit (CDK7 or MO15) and a regulatory subunit (cyclin H; Fisher and Morgan, 1994). CAK is capable of activating all of the major CDK-cyclin substrates involved in vertebrate cell cycle control. During a normal cell cycle, activation of phosphorylation at the threonine residue by CAK tends to oscillate in parallel with cyclin binding.

Changes in phosphorylation are probably not caused by changes in CAK activity, which remains relatively constant throughout the cell cycle. It is possible, therefore, that cyclin binding may stimulate CDK activation, perhaps by inducing conformational changes that permit CAK-mediated phosphorylation. CDK7, like its substrates, harbors a potential site of phosphorylation at a conserved threonine residue. Mutation of this residue greatly reduces kinase activity, suggesting that activation of CAK itself may require phosphorylation at this site (Fisher and Morgan, 1994). Therefore, although CAK activity is not rate limiting during normal cell proliferation, its regulation by other kinases or additional subunits may be critical under different environmental growth conditions.

Inactivation of CDK activity

Negative regulation of cell cycle progression by inactivation of CDK activity occurs during development, differentiation, senescence, and cell death. These negative controls most likely play a crucial role in preventing tumorigenesis, and an understanding of the balance between negatively and positively acting signals on CDK activities will be of utmost importance in future studies of the cell cycle.

A cyclin-CDK complex can be inactivated by a decrease in the synthesis or an increase in the degradation of cyclins, and through inhibitory phosphorylation at two sites near the N terminus (Thr-14 and Tyr-15 in human Cdc2 and CDK2). Phosphorylation of these two residues is particularly important in the control of Cdc2 activation during mitosis. Phosphorylation of Thr-14 and Tyr-15 parallels the rise in cyclin B levels that occurs as cells approach mitosis. The Cdc2–cyclin B complex is maintained in an inactive state until dephosphorylation of Thr-14 and Tyr-15 at the G_2/M border activates Cdc2 (Krek and Nigg, 1991).

The kinase responsible for phosphorylating Tyr-15 is Wee1, originally identified in *Schizosaccharomyces pombe* (Parker et al., 1991, 1992). Thr-14 and Tyr-15 are both dephosphorylated by the dual-specificity phosphatase Cdc25. Near the onset of mitosis, the Cdc25 protein undergoes a marked elevation in phosphatase activity that coincides with extensive phosphorylation of the protein in its N-terminal region. During mitosis, the kinase responsible for this phosphorylation is activated and the corresponding phosphatase is inhibited. The Cdc25-stimulatory kinase may be Cdc2 itself, creating an elegant positive-feedback loop to induce the mitotic dephosphorylation and activation of Cdc2.

Negative regulation of CDK activity occurs also through the actions of CKIs. Two families of these proteins exist that antagonize CDKs by binding to and inhibiting their kinase activity (reviewed in Sherr, 1994; Sherr and Roberts, 1995). The INK4 group (inhibitor of CDK4), whose members include p15, p16, p18, and p19, selectively compete with the D-type cyclins for binding to CDK4/6 to inhibit their kinase activity (Hannon and Beach, 1994; Serrano et al., 1993). The KIP family of inhibitors, which includes p21 (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993), p27 (Koff et al., 1993; Polyak et al., 1994a,b; Toyoshima and Hunter, 1994), and p57 (Lee et al., 1995; Matsuoka et al., 1995), is defined by a conserved domain that is sufficient for stable binding to cyclin-CDK complexes and inhibition of their kinase activity. The KIPs have been described as promiscuous inhibitors because they can bind to and inhibit each of the G_1 CDKs essential for G_1 progression and S-phase entry.

The CKIs seem to function as stoichiometric inhibitors by setting an adjustable threshold for cyclin-dependent activation of the CDKs. In the presence of growth inhibitory stimuli or antimitogenic signals the levels of CKIs in the cell increase. High levels of these proteins prevent proliferation by establishing an inhibitory threshold that cannot be suppressed by the steady-state levels of cyclin-CDK complexes present in the cell. Conversely, mitogenic signals lower the inhibitory threshold to below the level of cyclin-CDK complexes, permitting CDK activation and cell cycle progression. The decision to proliferate or not to proliferate relies, therefore, on a balance of growth-inhibitory and growth-stimulatory signals transduced from the extracellular environment and modulated by CDK activity and the antagonizing effects of the CKIs.

Proteolysis

CKIs have been shown to prevent cell proliferation by negatively regulating cyclin-CDK complexes. Recent findings suggest that the converse may be true as well, i.e., that cyclin-

CDK complexes may inhibit CKI function (Sheaff et al., 1997). Cyclin-CDK complexes in yeast and in mammalian cells seem to promote cell cycle progression through degradation of CKIs by the ubiquitin-proteasome pathway. This pathway also regulates the turnover of certain cyclins, thereby determining their periodic expression as well as their rapid proteolytic inactivation in response to growth factor deprivation.

The ubiquitin-proteasome pathway is involved in protein degradation in mammalian cells and is responsible for the tightly regulated selective turnover of intracellular proteins. Experimental evidence has implicated the ubiquitin system in the degradation of mitotic cyclins, oncoproteins, the tumor suppressor protein p53, several cell surface receptors, transcriptional regulators, and mutated and damaged proteins. Some of these proteolytic events occur throughout the cell cycle, whereas others occur following cell cycle–dependent posttranslational modifications (such as site-specific phosphorylation) of the target proteins. Although the signals that designate proteins for degradation are still being elucidated, the enzymes of the ubiquitin-proteasome proteolytic pathway have been well characterized.

Degradation of a protein by the ubiquitin pathway involves two distinct steps: targeting of the protein by covalent attachment of multiple ubiquitin molecules and degradation of the targeted protein. Conjugation of ubiquitin to proteins destined for degradation generally occurs in a three-step process involving three specific classes of enzymes called E1, E2, and E3. E1 is the ubiquitin-activating enzyme that catalyzes the ATP-dependent activation of ubiquitin to produce a high-energy thiol ester intermediate. Following activation, the E2 ubiquitin-carrier (or ubiquitin-conjugating) proteins transfer ubiquitin from E1 proteins to the E3 ubiquitin–protein ligase. Finally, the E3 ligase catalyzes the formation of an isopeptide bond between the ubiquitin polypeptide and the protein substrate. While E1 is a general factor that does not seem to be cell cycle dependent, many E2 and E3 proteins, whose expression might be regulated in a cell cycle–dependent manner, are yet to be identified.

Although the signals responsible for directing the ubiquitin-proteasome machinery to specific target proteins are still being elucidated, recent findings on the turnover of cyclins D1 and E have revealed exciting evidence for phosphorylation-dependent promotion of proteolysis. Unlike cyclins B1 (Brandeis and Hunt, 1996) and A, which are targeted for degradation by the ubiquitin-mediated pathway through a proteolysis targeting signal (known as the destruction box) located within their sequence, cyclins D1 and E are targeted for degradation by an alternative mechanism. Cyclin E degradation by the ubiquitin-proteasome system is regulated by both CDK2 binding and CDK2 catalytic activity (Clurman et al., 1996). The binding of cyclin E to CDK2 protects it from ubiquitination, while CDK2-activated pathways abrogate this protective effect.

Site-specific phosphorylation as a mechanism for substrate targeting in ubiquitin-mediated degradation is further supported by recent reports on the degradation of cyclin D1. It has recently been reported that cyclin D1 turnover is governed by ubiquitination and proteasomal degradation, which are positively regulated by cyclin D1 phosphorylation on Thr-286 (Diehl et al., 1997). Furthermore, this site-specific phosphorylation does not appear to be dependent on CDK4 catalytic activity, suggesting the existence of another kinase that can phosphorylate cyclin D1 to accelerate its destruction by the ubiquitin-proteasome machinery.

Significant findings on the ubiquitin-dependent degradation of CKIs in yeast as well as in mammalian cells (Pagano et al., 1995) have recently been reported. CDK-dependent phosphorylation seems to play a critical role in the targeting of cyclins and certain CKIs for degradation by the ubiquitin-proteasome degradation machinery. The two CKIs that have been identified in the budding yeast *S. cerevisiae* ($p40^{Sic1}$, which regulates entry into S phase by inhibiting the Cdc28p-Clnp kinases, and Far1p, which is specifically required to arrest the cell cycle in G_1 in response to pheromones) are degraded by a common ubiquitination system. The components of this G_1-S ubiquitination system in *S. cerevisiae* are Cdc34p, Cdc4p, Cdc53p, and Skp1p.

Henchoz et al. (1997) have shown that Far1p is regulated by this ubiquitination system and that an important determinant of the recognition signal for ubiquitination is specific phosphorylation of Far1p by the Cdc28p-Clnp kinase. A similar pathway has been proposed for controlling degradation of the CKI $p40^{Sic1}$ (Feldman et al., 1997). Analogously to Far1p, an essential requirement for ubiquitination and degradation of $p40^{Sic1}$ appears to be phosphorylation by the Cdc28p-Clnp kinase, thereby ensuring that S phase cannot be initiated before the G_1-specific Cdc28p-Clnp kinase has been activated.

The two components of the yeast ubiquitin machinery responsible for recruitment of phosphorylated substrates (in particular $p40^{Sic1}$) for degradation are Cdc4p and Skp1p. The interaction between Cdc4p and Skp1p is mediated by the F-box, a newly recognized structural motif that seems to be required for Skp1p association (Bai et al., 1996). Skp1p connects many cell cycle regulators to the ubiquitin proteolysis machinery through the F-box motif. It seems likely that Skp1p association may designate active CDKs as targets of the proteolysis machinery.

Ubiquitin- and phosphorylation-dependent degradation may serve as a general mechanism to regulate CKIs. Evidence for the operation of this mechanism in mammalian cells has been provided by reports on the phosphorylation-dependent degradation of the CKI p27 by the ubiquitin-proteasome machinery. Mutations that affect putative sites of phosphorylation by associated cyclin E–CDK2 kinase stabilize p27 in vivo (Clurman et al., 1996; Sheaff et al., 1997; Vlach et al., 1997), demonstrating that p27 is an inhibitor as well as a biologically relevant substrate of cyclin E–CDK2 kinase activity. Ubiquitin-dependent degradation triggered by phosphorylation is a conserved mechanism to regulate the activity of CKIs and cyclins throughout the cell cycle and in response to extracellular signals.

TARGETS OF THE CELL CYCLE MACHINERY

The molecular networking of the CDKs and CKIs seems to affect the fundamental cell cycle regulator Rb. The intricate nature of multiple regulatory systems designed to modulate the activation status of CDK proteins reflects the importance of the substrate protein Rb as a functional unit that controls G_1 phase and as an obligatory oncogenic target (Bartek et al., 1996). Rb is unique as a prototypic member of the "pocket-protein" family, whose members include p130 and p107. All three proteins share structural homology, bind to oncoproteins of small DNA tumor viruses, interact with the E2F transcription factors, and suppress proliferation when overexpressed in cell culture.

Rb is functionally different from p130 and p107 in several important ways, however. Rb-knockout mouse embryos die at embryonic day E14.5 (Jacks et al., 1992; Lee et al., 1992), while certain strains of mice lacking p130 or p107 are viable and fertile, and display no obvious developmental abnormalities (Cobrinik et al., 1996; Lee et al., 1996). Furthermore, heterozygous Rb mice all develop and eventually succumb to specific tumors, while neither heterozygous nor homozygous p130- or p107-mutant mice exhibit increased tumor incidence.

Rb is, therefore, a bona fide tumor suppressor protein whose upstream regulators and downstream effectors comprise a multicomponent biochemical pathway that serves as the molecular mechanism underlying cell cycle control. A better understanding of the functional relationship between Rb and its negative and positive regulators will provide greater insight into the control of cell proliferation and tumorigenesis.

Some targets for CDK activity reside in the replicative machinery, and a brief discussion of the role of CDKs in replication follows. This aspect of control is not yet clearly understood, however, and any discussion at this time will be incomplete.

Activation of a replication origin is controlled at two levels. First, a protein-DNA complex termed the prereplicative complex (pre-RC) forms at the origin; this complex forms only in G_1 phase. Second, the pre-RC is converted into a replicative complex; this occurs only during S phase (reviewed in Stillman, 1996). This control must ensure that origins fire only once per cell cycle; therefore, origin activation must be coordinated with cell cycle progression, presumably by CDKs. Proteins that participate in origin activation have been identified in yeast and *Xenopus*, and, more recently, mammalian homologs (pre-RCs) have been described. Briefly, pre-RCs form only in cells in G_1 phase and are activated at the G_1/S transition. An origin of replication complex (ORC) binds to the origin of replication and acts as a foundation upon which the pre-RC is assembled in G_1 cells. Assembly of the pre-RC occurs with production of Cdc6 and recruitment of MCM (minichromosome maintenance mutants) proteins. After it is assembled, the pre-RC is activated first at the G_1/S boundary, and later throughout S phase for late-firing origins. This event requires the activation of the Cdc7-dbf4 kinase complex. Phosphorylation by this complex stimulates the dissociation of Cdc6 and MCM from the ORC, and replication ensues.

Mitogens and their downstream effectors, the CDKs, can affect this process by regulating the expression of Cdc6 at both the transcriptional and post-transcriptional levels. Oscillations of CDK activities also prevent the formation of the pre-RC. For example, G_2-phase cells cannot support pre-RC formation even when

provided Cdc6 (Piatti et al., 1996). Thus, one active research area is to determine how a cell coordinates the activation of CDKs, the conversion of a pre-RC to a replicative complex, and ultimately does this once, per cell cycle.

A PARADIGM OF CELL CYCLE ANALYSIS

Researchers with little experience in cell cycle studies may benefit from use of the following analytical paradigm, distilled from the literature, which addresses the mechanism of cell cycle arrest following drug/cytokine treatment or arrest-inducing conditions (also see Table 8.1.1). This analysis should take approximately 3 months to complete. Subsequent units in this chapter will describe in detail some of the techniques that can be used.

First, it is necessary to establish that the cell is arrested in a particular stage of the cell cycle under arrest-inducing conditions. Second, the amount of CDK activity in treated and untreated cells is measured. This can be accomplished in a G_1 cell by determining the extent

Table 8.1.1 A Beginner's Guide to the Cell Cycle Engine[a]

Protein	Activity
Positive regulators of CDKs participating directly in the cell cycle	
Cyclin A	Required for G_1/S transition and progression through S-phase; a role in G_2/M transition
Cyclin B	The mitotic cyclin; essential for G_2/M transition
Cyclins D1, D2, D3	Not required in the absence of Rb; cell type–specific patterns of expression
Cyclin E	Required for G_1/S transition
Essential CDKs participating directly in the cell cycle	
Cdc2	Binds to cyclin B and cyclin A; essential for mitosis
CDK2	Binds to cyclin E and cyclin A; may associate with cyclin D1; essential for entry into S phase
CDK3	Unknown partner; essential for entry into S phase
CDK4	Binds to D-type cyclins; cell type–specific pattern of expression
CDK6	Binds to D-type cyclins; cell type–specific pattern of expression
Regulators	
CAK	Complex composed of cyclin H and CDK7 with or without a novel protein MAT1
Cdc25	Three isoforms with patterns of expression that suggest one works at G_1/S and the others in G_2 and M
CIP/KIP family (p21, p27, p57)	Inhibit activity of all G_1 CDK complexes by forming a ternary complex and either blocking activation by CAK or by inhibiting the active complex
INK4 family (p15, p16, p18, p19)	Inhibit activity of cyclin D–CDK4 and cyclin D–CDK6 complexes either by forming a ternary complex or by competing for the CDK subunit
Most frequently used targets of the engine	
Cdc6	Accumulation may be regulated by phosphorylation
E2F (1-5), DP (1-2)	Heterodimeric family of transcription factors that interact with Rb family members
Lamins	Phosphorylation by B-type cyclins correlates with dissolution of the lamin structure at mitosis
Rb, p107, and p130	Phosphorylation by CDKs generally correlates with inactivation
RFA, human SSB	One subunit of the trimeric single-stranded binding protein is phosphorylated by CDKs
Histone H1	One of the first-identified substrates of the CDKs and a convenient in vitro substrate

[a]Abbreviations: CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; CIP, CDK-inhibitory protein; KIP, CDK-inhibitory protein; INK4, inhibitor of CDK4; Rb, retinoblastoma; RFA, replication factor A; SSB, single-stranded DNA binding protein.

of phosphorylation of histone H1 by CDK2 and of glutathione-*S*-transferase-linked Rb by CDK4 and CDK6. These results can be correlated with the phosphorylation and expression of the various CDK substrates (i.e., Rb). Third, it is important to develop a steady-state expression profile of the cyclins, CDKs, and CKIs by immunoblotting as well as by northern blotting (if desired) to measure mRNA levels. Fourth, the formation of active CDK-cyclin complexes is investigated. If no kinase activity is detected, it may be interesting to determine whether this absence is due to CKI binding, post-translational modification of the ATP-binding cleft of the kinase, aberrant localization of one of the cyclins or CDKs, or other events. Finally, it may be relevant to determine whether cyclin-CDK complexes form at all. Are the components in the right place? Are the INK proteins sequestering CDK4 in the cytosol? Is cyclin D1 in the nucleus? These questions may provide insights into the mechanism of cell cycle arrest and may be a source of ideas for future research. If all the results indicate that the cell should not be, but is, in an arrested state, congratulations! You may be on your way to elucidating a novel mechanism. Good luck!

Literature Cited

Amon, A., Tyers, M., Futcher, B., and Nasmyth, K. 1993. Mechanisms that help the yeast cell cycle clock tick: G_2 cyclins transcriptionally activate G_2 cyclins and repress G_1 cyclins. *Cell* 74:993-1007.

Amon, A., Irniger, S., and Nasmyth, K. 1994. Closing the cell cycle circle in yeast: G_2 cyclin proteins initiated at mitosis persist until the activation of G_1 cyclins in the next cycle. *Cell* 77:1037-1050.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* 86:263-274.

Bartek, J., Bartkova, J., and Lukas, J. 1996. The retinoblastoma protein pathway and the restriction point. *Curr. Opin. Cell Biol.* 8:805-814.

Brandeis, M. and Hunt, T. 1996. The proteolysis of mitotic cyclins in mammalian cells persists from the end of mitosis until the onset of S phase. *EMBO J.* 15:5280-5289.

Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M., and Roberts, J.M. 1996. Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by CDK2 binding and cyclin phosphorylation. *Genes & Dev.* 10:1979-1990.

Cobrinik, D., Lee, M.-H., Hannon, G., Mulligan, G., Bronson, R.T., Dyson, N., Harlow, E., Beach, D., Weinberg, R.A., and Jacks, T. 1996. Shared role of the pRb-related p130 and p107 in limb development. *Genes & Dev.* 10:1633-1644.

Diehl, J.A., Zindy, F., and Sherr, C.J. 1997. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes & Dev.* 11:957-972.

Dulic, V., Lees, E., and Reed, S. 1992. Association of human cyclin E with a periodic G_1-S phase protein kinase. *Science* 257:1958-1961.

El-Deiry, W.S., Tokin, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. 1993. WAF1, a potent mediator of p53 tumor suppression. *Cell* 75:817-825.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. 1997. A complex of Cdc4p, Skp1p, and Cdc53p/Cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* 91:221-230.

Fisher, R.P. and Morgan, D.O. 1994. A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell* 78:713-724.

Glotzer, M., Murray, A.W., and Kirschner, M.W. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* 349:132-138.

Gu, Y., Turck, C.W., and Morgan, D.O. 1993. Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature* 366:707-710.

Hannon, G.J. and Beach, D. 1994. $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature* 371:257-261.

Harper, J., Adami, G., Wei, N., Keyomarsi, K., and Elledge, S. 1993. The p21 CDK-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. *Cell* 75:805-816.

Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R., and Peter, M. 1997. Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. *Genes & Dev.* 11:3046-3060.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., and Weinbert, R.A. 1992. Effects of an Rb mutation in the mouse. *Nature* 359:295-300.

Knoblich, J., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* 77:107-120.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, W., Elledge, S., Nishimoto, T., Morgan, D., Franza, R., and Roberts, J. 1992. Formation and activation of a cyclin E/CDK2 complex during the G_1 phase of the human cell cycle. *Science* 257:1689-1694.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J.M., and Massague, J. 1993. Negative regulation of G_1 in mammalian cells; inhibition of cyclin E–dependent kinase by TGF-β. *Science* 260:536-538.

Krek, W. and Nigg, E.A. 1991. Differential phosphorylation of vertebrate $p34^{Cdc2}$ kinase at the G_1/S and G_2/M transitions of the cell cycle: Identification of major phosphorylation sites. *EMBO J.* 10:305-316.

Kuerbitz, S.J., Plunkett, B.S., Walsh, W.W., and Kastan, M.B. 1992. Wild type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. U.S.A.* 89:7491-7495.

Lee, E., Chang, C.-Y., Hu, N., Wang, Y.-C., Lai, C.-C., Herrup, K., Lee, W.-H., and Bradley, A. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* 359:288-294.

Lee, M.-H., Reynisdottir, I., and Massague, J. 1995. Cloning of $p57^{Kip2}$, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes & Dev.* 9:639-649.

Lee, M.-H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., and Jacks, T. 1996. Targeted disruption of p107: Functional overlap between p107 and Rb. *Genes & Dev.* 10:1621-1632.

Lew, D.J., Dulic, V., and Reed, S.I. 1991. Isolation of three novel human cyclins by rescue of G_1 cyclin (CLN) function in yeast. *Cell* 66:1197-1206.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362:847-849.

Matsuoka, S., Edwards, M.C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper J.W., and Elledge, S.J. 1995. $p57^{Kip2}$, a structurally distinct member of the $p21^{CIP1}$ CDK-inhibitor family, is a candidate tumor suppressor gene. *Genes & Dev.* 9:650-662.

Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J., Hanks, S.K., Roussel, M.F., and Sherr, C.J. 1992. Identification and properties of an atypical catalytic subunit ($p34^{PSK-13}$)/CDK4) for mammaliam D-type cyclins. *Cell* 71:323-334.

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J., and Kato, J.-Y. 1994. D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* 14:2066-2076.

Meyerson, M. and Harlow, E. 1994. Identification of a G_1-kinase activity for CDK6, a novel cyclin D partner. *Mol. Cell. Biol.* 14:2077-2086.

Morgan, D. 1995. Principles of CDK regulation. *Nature* 374:131-133.

Murray, A. 1994. Cell cycle checkpoints. *Curr. Opin. Cell Biol.* 6:872-876.

Nevins, J.R. 1992. E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 258:424-429.

Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F., and Rolfe, M. 1995. Role of the ubiquitin-proteasome pathway in regulating amounts of the cyclin-dependent kinase inhibitor p27. *Science* 269:682-685.

Pardee, A.B. 1989. G_1 events and regulation of cell proliferation. *Science* 246:603-608.

Parker, L.L. and Piwnica-Worms, H. 1992. Inactivation of the $p34^{cdc-}$ cyclin B complex by the human Wee 1 tyrosine kinase. *Science* 257:1955-1977.

Parker, L.L., Atherton-Fessler, S., Lee, M.S., Ogg, S., Falk, J.L., Swenson, K.I., and Piwnica-Worms, H. 1991. Cyclin promotes tyrosine phosphorylation of $p34^{cdc2}$ in a $wee1^{+}$-dependent manner. *EMBO J.* 10:1255-1263.

Piatti, S., Bohm, T., Cocker, J.H., Diffley, J.F.X., and Nasmyth, K. 1996. Activation of S phase–promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. *Genes & Dev.* 10:1516-1531.

Pines, J. and Hunter, T. 1989. Isolation of human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. *Cell* 58:833-846.

Pines, J. and Hunter, T. 1990. Human cyclin A is adenovirus E1A–associated protein p60 and behaves differently from cyclin B. *Nature* 346:760-763.

Polyak, K., Kato, J.Y., Soloman, M.J., Sherr, C.J., Massague, J., Roberts, J.M., and Koff, A. 1994a. $p27^{Kip1}$, a cyclin-CDK inhibitor, links transforming growth factor–β and contact inhibition to cell cycle arrest. *Genes & Dev.* 8:9-22.

Polyak, K., Lee, M.-H., Erdjument-Bromage, H., Koff, A., Roberts, J.M., Tempst, P., and Massague, J. 1994b. Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 78:59-66.

Serrano, M., Hannon, G.J., and Beach, D. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366:704-707.

Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M., and Clurman, B.E. 1997. Cyclin E–CDK2 is a regulator of $p27_{KIP1}$. *Genes & Dev.* 11:1464-1478.

Sherr, C.J. 1994. G_1 phase progression: Cycling on cue. *Cell* 79:551-555.

Sherr, C.J. and Roberts, J.M. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes & Dev.* 9:1149-1163.

Stillman, B. 1996. Cell cycle control of DNA replication. *Science* 274:1659-1663.

Toyoshima, H. and Hunter, T. 1994. p27, a novel inhibitor of G_1 cyclin–CDK protein kinase activity, is related to p21. *Cell* 78:67-74.

Vlach, J., Hennecke, S., and Amati, B. 1997. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. *EMBO J.* 16:5334-5344.

Xiong, Y., Connolly, T., Futcher, B., and Beach, D. 1991. Human D-type cyclin. *Cell* 65:691-695.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* 366:701-704

Contributed by Michele S. Park
and Andrew Koff
Memorial Sloan-Kettering Cancer Center
New York, New York

Assays for CDK Activity and DNA Replication in the Cell Cycle

The somatic cell cycle is normally divided into four phases: S phase, during which DNA is replicated, M phase (mitosis), when chromosomes and cells divide, and the periods in between—G_1 phase (after M and before S) and G_2 phase (after S and before M). Progress through the cell cycle is regulated by a complex formed by a cyclin subunit and a cyclin-dependent kinase (CDK). The catalytic subunit (the CDK) is activated by binding to the cyclin subunit.

In mammalian cells, three different cyclins—denoted cyclins A, B, and E—are primarily involved in regulating the cell cycle. These bind to one of two different CDKs, CDK1 (often called cdc2) and CDK2, such that specific cyclin-CDK complexes are present and active at particular phases of the cell cycle (see below). The amount of protein kinase activity of these cyclin-CDK complexes indicates, therefore, where a cell is in the cell cycle. This is useful for analyzing effects on cell proliferation and on cell cycle arrest. Whether a cell is in S phase can be inferred by determining the activation status of specific CDK complexes, but it can also be assayed directly by the incorporation of nucleotide analogs into DNA, which can also show how many cells entered S phase in the course of the experiment. As well as marking particular cell cycle stages in normal cells, the behavior of some cyclin-CDK complexes is perturbed in some transformed cells; therefore, assays for cyclin-CDK activity are often informative in studies on cellular transformation. This unit outlines how to assay cyclin-CDK activity (see Basic Protocol 1) and how to detect DNA replication by incorporation of 5-bromodeoxyuridine (BrdU) into DNA (see Basic Protocol 2).

Particular stages in interphase can be identified according to which cyclin-CDK complexes are present and active as follows:

Cyclin E–CDK2 activity appears in late G_1 stage and disappears in early S phase.

Cyclin A–CDK2 activity appears in early S phase and disappears in early M. In some cell types, cyclin A–CDK1 activity appears in G_2 phase and also disappears in early M.

Cyclin B–CDK1 activity marks the end of G_2 phase and disappears at mid-M.

MEASURING CDK ACTIVITY

BASIC PROTOCOL 1

Cyclin-CDK activity is assayed by a protein kinase assay using either a peptide substrate or, more usually, histone H1. Histone H1 can also be phosphorylated by a number of other protein kinases, notably cAMP-dependent protein kinase (PKA). For this reason cyclin-CDK assays using histone H1 should be carried out in the presence of a PKA inhibitor. A sharp increase in the histone H1 kinase activity in a whole cell lysate can be measured during mitosis, which is a gross indication of cyclin B–CDK1 activity, the major mitotic kinase. To measure the activity of other cyclin-CDK complexes, however, or to assay specific B-type cyclin-CDK complexes, one must first isolate a specific cyclin-CDK complex by immunoprecipitation.

Alternatively, proteins of the cyclin-dependent kinase subunit (Cks) family can be used as an affinity matrix. These proteins, which include the $p13^{suc1}$ protein from the fission yeast *Schizosaccharomyces pombe* and the $p9^{Cks}$ proteins from *Xenopus* and from humans, bind a number of cyclin-CDK complexes in cell lysates—notably cyclin B–CDK1—with high affinity. Cks proteins covalently linked to Sepharose beads are commercially

Contributed by Jonathon Pines, Mark Jackman, and Karen Simpson

Current Protocols in Cell Biology (1998) 8.2.1-8.2.11

available from a number of companies and are often used as a quick and easy way to measure the increase in cyclin B–CDK1 activity at mitosis. They should not be considered specific for cyclin B–CDK1, however, because they bind other cyclin-CDK complexes as well as related kinases, such as MAP kinase.

In choosing an antibody before embarking on the immunoprecipitation and the protein kinase assay, there are two considerations that must be taken into account.

1. Because many CDKs are able to bind more than one cyclin, using an antibody directed against the cyclin will give a more precise indication of the stage of the cell cycle than using one that recognizes the CDK. Unfortunately, most anti-cyclin antibodies are fairly species specific, probably because the primary structure of the cyclins is much less well conserved than that of the CDKs.

2. Some domains of the cyclin and of the CDK are masked in the active complex (Jeffrey et al., 1995). Antibodies raised against the PSTAIRE motif for the conserved part of the CDK) of a CDK will recognize only the unbound, inactive kinase and, therefore, are useful only for immunoblotting, not for the kinase assay. Similarly, antibodies raised against the C terminus of the cyclin should not be used because these often only recognize the monomeric protein.

Basic Protocol 1 will give a good indication of cell cycle stage because it works well for cyclins A, B, and E, with their partner kinases CDK1 and CDK2. Complexes between cyclin D and CDK4 or CDK6 cannot be assayed in this manner, however, because they do not phosphorylate histone H1 and may be sensitive to some detergents. (See Background Information for references to more appropriate procedures.) Depending on the equipment available, the results can be assayed using a scintillation counter to measure the incorporation of radiolabeled phosphate into a substrate, or by SDS–polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*) followed by autoradiography (*UNIT 6.3*) or phosphorimaging.

Materials

Cells grown in 6-cm tissue culture dishes
1× PBS (*APPENDIX 2A*), ice cold
Lysis buffer (see recipe), ice cold
Protein A– or protein G–Sepharose conjugate (Amersham Pharmacia Biotech) or formaldehyde-treated *Staphylococcus aureus* cells (Pansorbin, Calbiochem)
Anti-cyclin antibody, anti-CDK antibody, or Cks–Sepharose bead conjugate (Upstate Biotechnology)
Kinase buffer (see recipe), ice cold
Histone H1 solution (see recipe) or consensus cdc2 peptide (New England Biolabs)
1 mM ATP (diluted from 100 mM ATP [see recipe] in distilled H_2O)
2000 Ci/mmol [γ-^{33}P]ATP or 3000 Ci/mmol [γ-^{32}P]ATP
100 mM EDTA (*APPENDIX 2A*)
2× SDS sample buffer (*UNIT 6.1*)
PKA-inhibitory peptide: 1 mM of peptide (Sigma) in 10 mM sodium phosphate, pH 7.2 (see *APPENDIX 2A* for buffer recipe; store peptide solution up to 1 year at –20°C)
50 mM roscovitine *or* 100 mM olomoucine (both from Calbiochem) in DMSO (store both solutions in aliquots up to 1 year at –20°C, and thaw only once; store olomoucine in the dark)
Negative control: immunoprecipitated preimmune serum or anti-IgG antibody
Positive control: purified cyclin B–CDK1
75 mM phosphoric acid (7.5 ml of 1 M phosphoric acid + 92.5 ml distilled water; store up to 2 years at room temperature)
96% ethanol

15% to 20% SDS-polyacrylamide gel (*UNIT 6.1*)
Coomassie blue G-250 staining solution (see *UNIT 6.1*)
Destaining solution: 10% (v/v) acetic acid

1-ml syringes and 21-G needles, prechilled to 4°C
1.5-ml microcentrifuge tubes, prechilled to 4°C
1-ml pipet tips, prechilled to 4°C
Phosphocellulose units (Pierce) *or* 1.5-cm squares of phosphocellulose P81 filter paper (Whatman)
Microcentrifuge, 4°C
End-over-end rotator or rotating wheel
Whatman 3MM filter paper
Phosphorimager (optional)

Additional reagents and equipment for SDS-PAGE and Coomassie brilliant blue staining (*UNIT 6.1*) and for autoradiography and densitometry (optional; *UNIT 6.3*)

Immunoprecipitate

1. Place 6-cm tissue culture dish containing cells on a glass plate on ice, preferably in a cold room. Remove culture medium with a Pasteur pipet, add 3 ml ice-cold PBS, and let stand for 1 min on ice. Tip the dish and remove PBS with a Pasteur pipet. Repeat wash with ice-cold PBS and carefully remove as much PBS as possible by draining the dish.

   Adjust the volumes appropriately for other size dishes. Ideally samples should be assayed in duplicate.

2. Add 1 ml lysis buffer and leave dish on ice for 20 min. Tip the dish and scrape the lysate to one edge with a rubber policeman or cell scraper.

   The lysis buffer, also known as RadioImmunoPrecipitation Assay (RIPA) buffer, is a fairly stringent buffer that removes some of the more weakly associated proteins from the cyclin-CDK complex. It is an appropriate buffer to measure the basic level of cyclin-CDK activity, although any modulation by weakly associated proteins will be lost. A variety of other lysis buffers have also been used to assay cyclin-CDK complexes, using the same basic procedure.

3. Take up the lysate through a prechilled 21-G needle attached to a prechilled 1-ml syringe, and transfer to a prechilled 1.5-ml microcentrifuge tube. Draw up the lysate through the needle three times to shear the DNA. Alternatively, sonicate the tube, keeping the lysate cold at all times.

4. Add 10 µl protein A– or protein G–Sepharose, or 100 µl formaldehyde-treated *S. aureus* cells, to the lysate. Cap the tube and microcentrifuge 20 min at 10,000 × *g*, 4°C, to clear lysate. Remove lysate (~900 µl) with a prechilled 1-ml pipet tip, being careful not to disturb the pellet.

   At this point, the lysate can be frozen in liquid nitrogen and stored at –80°C. Samples should be thawed only once and not refrozen.

5. Add lysate (~900 µl) to anti-cyclin antibody, anti-CDK antibody, or Cks-Sepharose beads in a prechilled 1.5-ml microcentrifuge tube on ice. Incubate 1 hr to overnight at 4°C, with continuous mixing on an end-over-end rotator or rotating wheel for Cks-Sepharose beads.

   The amount of antibody will vary depending on the titer. Most available anti-cyclin or anti-CDK polyclonal antibodies are used at 1:1000 or less. Most monoclonal antibodies are used at 0.1 to 1 µg/ml, which will be ~1 µl monoclonal or polyclonal antibody per 6-cm dish of cells.

   The tube does not need to be continuously mixed if antibodies are being used.

6. Microcentrifuge the tube 5 min at 10,000 × *g*, 4°C. Transfer lysate to a fresh prechilled 1.5-ml microcentrifuge tube containing 30 to 50 µl of a 50% mixture of protein A– or protein G–Sepharose in ice-cold lysis buffer. Mix on an end-over-end rotator or rotating wheel for 30 to 45 min at 4°C.

   When using protein A–Sepharose and a mouse monoclonal antibody, adding 1 µg of a rabbit anti–mouse antibody often increases the efficiency of immunoprecipitation, because some subtypes of mouse IgG do not bind well to protein A. In general, mouse monoclonal antibodies bind better to protein G–Sepharose than to protein A–Sepharose. See Harlow and Lane (1988) for a more detailed discussion and Table 7.2.1.

7. Microcentrifuge 5 sec at maximum speed, 4°C. Remove lysate with a 1-ml pipet tip or 21-G needle attached to an aspirator. Add 700 µl ice-cold lysis buffer to pellet and microcentrifuge 5 min at 10,000 × *g*, 4°C. Repeat microcentrifugation three times with 700 µl ice-cold lysis buffer each time.

Perform kinase reaction

8. Remove supernatant, add 1 ml ice-cold kinase buffer to pellet, and microcentrifuge 5 min at 10,000 × *g*, 4°C.

   For SDS-PAGE analysis, transfer the Sepharose beads in the last wash solution to a screw-cap microcentrifuge tube.

9. Prepare kinase assay cocktail:

   80 µl kinase buffer
   4 µl 1 mM ATP (40 µM final)
   4 µl 10 µCi/µl [γ-^{33}P]ATP (2000 Ci/mmol) *or* [γ-^{32}P]ATP (3000 Ci/mmol)
   0.5 µl 10 mg/ml histone H1 (50 µg/ml final)
   2 µl 1 mM PKA-inhibitory peptide (20 µM final)
   9.5 µl double-distilled water.

   Either ^{33}P- or ^{32}P-labeled ATP can be used. ^{33}P may be preferable, because it emits β particles of a lower energy, and is therefore less harmful to the researcher and requires less shielding to use.

10. Remove as much of the last wash from the pellet as possible without leaving it completely dry. Add 20 µl kinase assay cocktail to the immunoprecipitate or Cks-Sepharose beads on ice and incubate 30 min at 30°C. Add 20 µl kinase assay cocktail to negative control immunoprecipitate and positive control as well.

    The amount of protein kinase in an immunoprecipitate should always be compared with that of a negative control. Purified cyclin B–CDK1 to be used as a positive control can be purchased from a number of different suppliers.

    As a further test for the specificity of the assay, one can inhibit a parallel set of immunoprecipitates with chemical inhibitors of the cyclin-CDKS. The cyclin-CDK complexes can be inhibited by several different chemicals. Thus far the most specific inhibitors have proved to be roscovitine (use at a final concentration of 5 µM) and olomoucine (use at a final concentration of 10 µM). Either of these inhibitors can be added to a separate reaction to indicate whether the bulk of the protein-kinase activity is due to a cyclin-CDK complex.

Assay for kinase activity

Assay by scintillation counting

11a. Add 100 µl of 100 mM EDTA to the samples to stop the reaction. Set up phosphocellulose units in a rack and apply samples to phosphocellulose units using a pipet, being careful not to touch the membrane with the pipet tip. Alternatively, spot samples on 1.5-cm squares of phosphocellulose P81 paper in a glass dish.

Phosphocellulose units and P81 paper are negatively charged; therefore, the substrate must have some positively charged residues. This makes them appropriate to assay either histone H1 or a consensus cdc2 peptide.

12a. Microcentrifuge phosphocellulose units 30 sec at 10,000 × *g*, room temperature. Add 500 µl of 75 mM phosphoric acid and spin again. Transfer phosphocellulose units to fresh microcentrifuge tubes, add 500 µl 75 of mM phosphoric acid, and spin again. Alternatively, rinse P81 squares three times with 5 ml of 75 µM phosphoric acid, and one time with 5 ml 96% ethanol.

13a. Transfer phosphocellulose units or P81 filter papers to scintillation vials, add 2 to 10 ml scintillation fluid, and count on the ^{32}P channel if using ^{32}P, or on the ^{35}S channel if using ^{33}P.

Assay by SDS-PAGE

11b. Add 10 µl of 2× SDS-PAGE sample buffer to the samples to stop the reaction. Boil samples 3 min in a screw-cap microcentrifuge tube to reduce the risk of vaporizing radioisotope.

12b. Run the samples on a 15% to 20% SDS-polyacrylamide gel (*UNIT 6.1*).

13b. Place the gel in a flat container, add 500 ml staining solution, and let stand 15 min at room temperature. Remove staining solution and add 500 ml destaining solution for 20 min. Repeat destaining three times with 250 ml destaining solution for 15 min each time.

14b. Place the gel on Whatman 3MM filter paper, cover with plastic wrap, and dry the gel in a gel dryer for 1 hr at 80°C. Expose the gel in a phosphorimager or to X-ray film.

Phosphorylated histone H1 can usually be detected on the dried gel with a hand-held β counter. If using ^{32}P, place a lead screen with a hole punched in it over the gel to assess the amount of radioisotope in individual lanes. If using ^{33}P, use another piece of X-ray film to block the signal from the other lanes.

15b. Measure the amount of incorporated label using a phosphorimager or by densitometry of the exposed film.

The labeled histone H1 will appear as a doublet of bands running at ~30 kDa.

Given the limited linear response of X-ray film, densitometry is an inaccurate way to measure the incorporation of label. It will provide only a rough guide to the relative amount of kinase activity in each sample.

MEASURING DNA REPLICATION USING INCORPORATION OF BrdU

BASIC PROTOCOL 2

This protocol describes how to use the nucleotide analog BrdU to label and detect cells in the process of DNA replication. BrdU freely diffuses into cells and therefore can simply be added to the culture medium. Cells are grown on poly-L-lysine-coated coverslips, which allows them to be visualized using high-resolution optics on an epifluorescence microscope. The cells are then fixed on the coverslips and the DNA is denatured to expose the BrdU epitope to an anti-BrdU antibody. If BrdU is added for a short time, this method can be used to identify only those cells undergoing DNA replication. Alternatively, a continuous pulse of BrdU will identify all the cells whose DNA was replicated during the experiment. Anti-BrdU antibodies can be either unconjugated, in which case a secondary antibody labeled with a fluorophore must also be used, or conjugated to a fluorophore, in which case the antibodies can be visualized directly by fluorescence microscopy. Conjugated antibodies are generally less sensitive but are sufficient for most purposes, except for the very short pulses of BrdU that are used to visualize individual DNA replication foci.

Materials

1 mg/ml poly-L-lysine: 20 mg poly-L-lysine (average mol. wt. 400,000)/20 ml dH_2O; filter sterilize through 0.45 μm filter and store at –20°C for 3 months
Cell culture medium
Cell suspension: 10^5 cells/ml in PBS (*UNIT 1.1*; see *APPENDIX 2A* for PBS recipe)
10 mg/ml BrdU (see recipe)
1 mg/ml 5-fluorodeoxyuridine (FrdU; see recipe)
PBS (*APPENDIX 2A*)
Blocking solution: 3% (w/v) BSA in PBS
50% methanol/50% acetone (fixative; prepared fresh); *or* formaldehyde solution (cross-linking agent; see recipe) and Tris·Cl/$MgCl_2$/Triton X-100 (TSM; see recipe)
*Eco*R1 exonuclease and exonuclease III, *or* HCl/Triton X-100 (see recipe)
Anti-BrdU antibody, conjugated; *or* unconjugated anti-BrdU antibody and fluorophore-labeled secondary antibody (Amersham)
Slide-mounting solution containing antifade agent

Grade 1 (0.15-mm) glass coverslips, cleaned and sterilized with dry heat
Cytocentrifuge (optional)
Humidified chamber: e.g., inverted petri dish with dampened filter paper in lid

Prepare and seed cells onto coverslips

1. Incubate clean, dry heat–sterilized coverslips for 5 to 15 min at 37°C in poly-L-lysine solution in a 10-cm tissue culture dish. Wash the coverslips with water and air dry at room temperature.

   To handle the coverslips, use fine watchmakers forceps with one of the tips bent to ~20° angle.

2. Place coverslips in 6- or 10-cm tissue culture dishes and add enough cell culture medium to cover the surface of the dish (3 to 5 ml). Pipet in the cell suspension. Spin down suspension cells onto coverslips in a cytocentrifuge, or simply incubate them as a 10^5 cell/ml suspension in PBS with poly-L-lysine coverslips for 10 min at 37°C.

   The amount of cells added will depend on the experimental design, but cells should not be confluent after they have reattached to the dish. Note that cells often adhere better to coverslips than to plastic, so they will tend to be a little denser on the coverslip than in the rest of the dish.

   Seed cells at least 3 to 4 hr before staining to allow them to adhere properly. Some cell lines may require a different incubation temperature.

Label S-phase cells with BrdU

3. Add BrdU to the cell suspension at a final concentration of 25 to 100 μM. Incubate at 37°C for 15 min to several hours.

   Use higher concentrations for shorter times. For labelings >2 hr, FrdU can be added to a final concentration of 0.4 μM. This inhibits thymidylate synthetase, reducing endogenous pools of thymidine and increasing the effective concentration of BrdU in the cell.

4. Remove BrdU and wash coverslips three times with 5 ml PBS each time. Transfer coverslips to a glass petri dish containing 5 to 10 ml PBS. Proceed to either fixation or permeabilization of cells.

5a. *Fix/permeabilize cells with methanol/acetone:* Rinse coverslips once with 5 ml PBS and drain off the PBS. Carefully add enough fixative to cover the coverslips (3 ml) and incubate 2 min at room temperature. Remove fixative and rinse coverslips with 5 to 10 ml PBS.

   Other fixatives include 100% methanol or 100% acetone. Fixation can also be done at –20°C. If methanol or acetone is used, the permeabilization step is not necessary (go to step 6).

5b. *Fix cells with formaldehyde, then permeabilize with TSM:* Rinse the coverslips once with 5 ml PBS and drain off PBS. Carefully add enough formaldehyde solution to cover the coverslips (3 ml) and let stand for 2 to 5 min at room temperature. Carefully rinse the cells twice with 5 ml PBS. Drain off PBS, add enough TSM to submerge the coverslips (3 to 5 ml), and let stand for 5 to 10 min at room temperature. Drain off TSM and wash three times with 5 to 10 ml PBS for 2 min each.

An optional step is to wash once in 50 mM glycine in PBS after removing the TSM to quench the cross-linking reagent. This is not usually necessary unless there is a very high autofluorescent background at the end of the procedure.

6. Place a piece of Parafilm on top of the dampened filter paper in the humidified chamber and pipet ~25 µl blocking solution onto the paper. Drain the excess PBS from the coverslip by touching the edge to a Kimwipe. Lay the coverslip cell side down on top of the drop of blocking solution and leave for 15 to 30 min. Proceed to denaturation of DNA by either acid or nuclease treatment.

Nuclease treatment is easier to combine with other antibodies in double-immunofluorescence experiments; acid denaturation is sometimes more sensitive.

7a. *Denature DNA with acid:* Place coverslips in a glass petri dish, add 5 to 10 ml HCl/Triton X-100, and let stand 10 min at room temperature. Remove coverslips to a fresh petri dish and wash twice with 10 ml PBS for 5 min each to neutralize the acid.

In dual-labeling experiments, the sample should be incubated with the second primary and the secondary antibodies and then fixed again before acid treatment. The first secondary antibody should be labeled with Texas red or TRITC, not FITC, because FITC is sensitive to acid pH.

7b. *Denature DNA with nuclease:* Dilute *Eco*RI and exonuclease III in their recommended buffers according to the manufacturers' instructions. In the humidified chamber, place the coverslip cell side down on a drop of ~150 U/ml of *Eco*RI and incubate for 30 min at 37°C. Then place the coverslip on a drop of 300 U/ml exonuclease III and incubate 30 min at 37°C.

Some commercially available kits contain a cocktail of EcoR1, exonuclease III, and anti-BrdU antibody, or DNase I and anti-BrdU antibody, which will denature the DNA and stain with the antibody in a single step.

Incubate coverslips with antibodies

8. Dilute anti-BrdU antibody in blocking solution according to manufacturer's instructions. Place coverslips cell side down on a drop of anti-BrdU antibody. Incubate coverslips with anti-BrdU antibody for 30 to 60 min at room temperature.

Usually 2 to 5 µg/ml is a good dilution for monoclonal antibodies.

9. Lift the coverslip off the Parafilm with forceps and place cell side up in a petri dish with 5 ml PBS. Wash the coverslips four times in 5 ml PBS for 5 min each time.

Coverslips stained with anti-BrdU antibody conjugated to a fluorophore can now be mounted on slides (step 12).

10. Dilute fluorophore-labeled secondary antibody in blocking solution according to manufacturer's instructions. Remove coverslips from PBS with forceps and place cell side down on a drop of fluorophore-labeled secondary antibody. Wash the coverslips four times in 5 ml PBS for 5 min each time.

Antibodies and fluorophores vary in their affinity and brightness, so there is no universally appropriate dilution. Most suppliers give a recommended dilution; otherwise it will have to be determined empirically—1:200 to 1:5000 is usually appropriate. Higher dilutions will result in less background staining.

Mount coverslips on slides

11. Dip coverslips in a beaker of water to remove any salts and then drain the water off by holding the edge of the coverslip to piece of Kimwipe.

12. Place each coverslip cell side down onto a small drop of mounting solution containing antifade agent on a good-quality, clean microscope slide. Remove any excess mounting solution from around the edge of the coverslip with a Kimwipe, being careful not to dislodge the coverslip. Seal the coverslip around the edge with a small amount of nail polish.

    Commercial mounting solutions are readily available. All mounting solutions should be adjusted to pH 8.5 or greater, because FITC fluorescence is pH sensitive. Mounting solutions containing Gelvatol or Mowiol harden overnight, after which they do not need to be sealed.

    Slides can be stored in the dark up to 3 months at 4°C or −20°C.

13. View the slides under epifluorescence using the appropriate filter set for the fluorophore on the primary or secondary antibody.

    It is helpful to first locate the optical plane with the cells using transmitted light and then visualize the anti-BrdU staining by epifluorescence.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ATP, 100 mM

Dissolve ATP lithium salt (Boehringer Mannheim) in water. Adjust pH to 7.0 with 1 M NaOH. Store in aliquots at −20°C for 1 year.

Bromodeoxyuridine (BrdU), 10 mg/ml (27 mM)

Dissolve 100 mg BrdU in 10 ml water. Filter sterilize through 0.45-µm filter. Divide into aliquots and store up to 6 months at –20°C.

5-Fluorodeoxyuridine (FrdU), 1 mg/ml (4 mM)

Dissolve 10 mg FrdU in 10 ml water. Filter sterilize through 0.45-µm filter. Divide into aliquots and store up to 6 months at –20°C.

Formaldehyde solution

3.7% (v/v) formaldehyde
2% (w/v) sucrose in PBS (see *APPENDIX 2A* for PBS recipe)
Prepare fresh

HCl/Triton X-100

17.4 ml of 11.5 N HCl (2 N final)
0.5 g Triton X-100 (0.5% final)
82 ml double-distilled H_2O
Prepare fresh

Histone H1 solution

10 mg/ml histone H1 from calf thymus (Boehringer Mannheim)
10 mM $NaPO_4$, pH 7.2
Store in aliquots up to 1 year at −20°C

Kinase buffer

250 µl of 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*; 25 mM final)
300 µl of 5 M NaCl (*APPENDIX 2A*; 150 mM final)
100 µl of 1 M $MgCl_2$ (*APPENDIX 2A*; 10 mM final)
100 µl of 0.1 M dithiothreitol (DTT; *APPENDIX 2A*; 1 mM final)
Store in aliquots up to 6 months at −20°C

Lysis buffer

30 ml of 5 M NaCl (0.15 M final)
12 ml of 0.5 M Na_2HPO_4
8 ml of NaH_2PO_4
10 ml of 0.5 M EDTA (5.0 mM final)
10 ml Nonidet P-40 (1.0% v/v final)
10 g sodium deoxycholate (1.0 % w/v final)
1 g SDS (0.1% w/v final)
2.1 g NaF (50.0 mM final)
1.8 g $NaVO_4$ (1.0 mM final)
10 ml aprotinin (1.0% v/v final)
928 ml double-distilled H_2O
Filter through a 0.45-µm membrane
Store up to 1 month at 4°C

Final sodium phosphate concentration is 10.0 mM and final pH is 7.2. This buffer is also known as RIPA buffer.

Tris/sodium/magnesium detergent (TSM)

0.5 g Triton X-100 (0.5% v/v final)
2 ml of 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*; 20 mM final)
1 ml of 5 M NaCl (*APPENDIX 2A*; 50 mM final)
0.3 ml of 1 M $MgCl_2$ (*APPENDIX 2A*; 3 mM final)
96.2 ml double-distilled H_2O
Filter sterilize through 0.45-µm filter
Store up to 6 months at room temperature

COMMENTARY

Background Information

A number of markers can be used to assess the progress of a cell through the cell cycle. Cells in mitosis (M phase) are easily recognized under a phase contrast or differential interference contrast (DIC) microscope by their condensed chromosomes, and DNA replication (S phase) was first recognized many years ago by the incorporation of radiolabeled nucleotides into DNA. The long periods between mitosis and DNA replication were defined simply by the absence of any assayable event (except growth), as is signified by the name given to these periods, G_1 and G_2 (the gap phases). With the advent of flow cytometry, all four cell cycle phases can be easily recognized by the replication state of the DNA. G_1-phase cells have a diploid ($2n$) content of DNA, G_2- and M-phase cells have completed DNA replication and therefore have a tetraploid ($4n$) DNA content, and S-phase cells have an intermediate content.

Much more is understood now about the molecular events underlying progress through the cell cycle than when the phases were named. Progress is controlled by sequential waves of cyclin-dependent kinase (CDK) activity (Fig. 8.2.1). Precisely where a cell is in the cell cycle can be determined by which cyclin-CDK complex is present and active. Cyclin E–CDK2, cyclin A–CDK2, and cyclin B–CDK1 are particularly useful in this respect. The D-type cyclins, with their associated kinase subunits CDK4 and CDK6, are affected by a number of parameters in addition to the stage of the cell cycle. For example, D-type cyclins rapidly disappear when growth factors are removed (Matsushime et al., 1991). The D-type cyclin–CDK complexes must be assayed under different conditions from A-, B-, and E-type cyclin–CDKs because they recognize only a very limited set

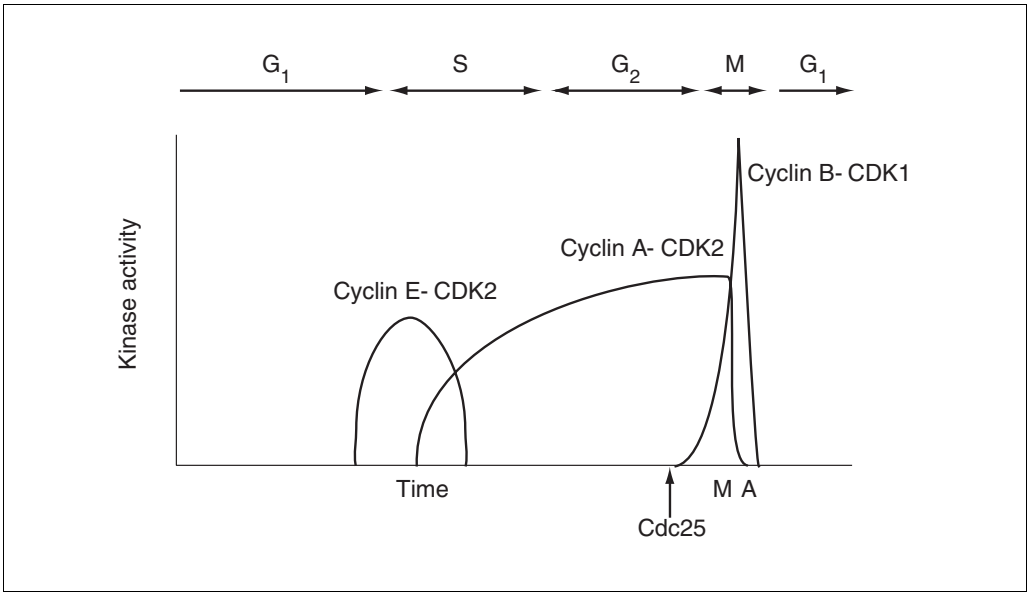


Figure 8.2.1 Appearance and disappearance of specific cyclin-cell-dependent kinase (CDK) activities through the cell cycle. The arrow marks the end of G_2 phase, at which point cyclin B–CDK1 is activated by the Cdc25 phosphatase. The relative kinase activities are not to scale. Abbreviations: M, metaphase; A, anaphase.

of substrates, which does not include histone H1. Of these, the retinoblastoma protein (Rb) is the best characterized (see Phelps and Xiong, 1997, for a more detailed discussion).

The amounts of CDK1 and CDK2 do not vary through the cell cycle, and therefore their activity is regulated by the levels of their cyclin partners. Cyclin levels are regulated both by their synthesis (effectively controlled at the level of transcription) and by their destruction. This is just the first level of regulation, however. Once the CDK has bound its partner cyclin, the complex must be phosphorylated on the T-loop threonine to generate a fully active and stable protein kinase complex. This step is performed by a specific enzyme, CDK-activating kinase (CAK), which in animal cells is itself another cyclin-CDK complex, cyclin H–CDK7, and is sometimes associated with a third protein, MAT1 (reviewed in Morgan, 1995). The phosphorylated cyclin-CDK complex can in turn be regulated by binding a member of a family of specific inhibitor proteins, and by phosphorylation of a threonine and/or tyrosine residue in the ATP-binding region of the kinase (reviewed in Lew and Kornbluth, 1996). These phosphorylation events alter the mobility of the CDK on SDS-PAGE. The final activation of a cyclin-CDK complex is performed by a specific family of phosphatases that dephosphorylate Tyr-14 and Thr-15. This event is responsible for the rapid activation of a preformed pool of cyclin B–CDK1 that drives cells into mitosis. The cyclin B–CDK1 complex is the most active histone H1 kinase in the cell, and its rapid activation is responsible for the peak of histone H1 kinase activity found in whole cell lysates when cells enter mitosis.

Critical Parameters and Troubleshooting

With the exception of the D-type cyclin–CDK4/6 kinases, the cyclin-CDKs are fairly robust kinases. Active cyclin-CDKs can be isolated from cell lysates that have been frozen immediately after isolation, although the kinases are not stable to repeated rounds of freeze/thaw. Cyclin-CDKs can be inactivated if the phosphate is removed from the T-loop threonine, however, and phosphatase inhibitors must be included in the lysis buffer. Okadaic acid is a poor choice as a phosphatase inhibitor, because it can prematurely activate cyclin B–CDK1 (Yamashita et al., 1991). Another phosphatase inhibitor, β-glycerophosphate, seems to have a stabilizing effect on cyclin-CDK complexes.

Negative results in CDK assays are more likely to be caused by failure to immunoprecipitate the kinase than by denaturation of the protein, which underlines the importance of choosing the correct antibody. If there is no detectable activity in samples that should contain a particular cyclin-CDK, the immunoprecipitates should be immunoblotted with anti-CDK and anti-cyclin antibodies. The anti-

PSTAIRE antibody, which recognizes a conserved alpha helix in CDKS with the sequence PSTAIRE in the center, is useful in this respect because it recognizes both CDK1 and CDK2 on immunoblots. Because the CDKs migrate at between 30 and 34 kDa, a normal reducing SDS-polyacrylamide gel can be used for immunoblots. Cyclins A and E migrate close to the heavy chain of the antibody in an immunoprecipitate, however, and can be masked if the secondary antibody binds to the heavy chain. This problem can be ameliorated by using antibodies from different species for the immunoprecipitation and the immunoblot; by cross-linking the antibody to the Sepharose beads; or by using *N*-ethyl maleimide or iodoacetamide in place of β-mercaptoethanol in the SDS-PAGE sample buffer.

A lack of signal in BrdU labeling is generally caused either by loss of cells from the coverslips during too-vigorous washing (especially before fixation) or by addition of the solutions to the wrong side of the coverslip. Check that there are cells on the slide at the end of the protocol by phase contrast or DIC microscopy. If the coverslips are dropped, it is very difficult to tell which side the cells are on. If the cells are dense enough, try scraping a small area with a needle and looking under a microscope to see if cells have been scraped away. Other problems are usually caused by defective antibodies (e.g., the fluorophore of the secondary antibody) or by defective enzymes in the nuclease method.

Anticipated Results

The cyclin-CDK complexes differ in their activity against histone H1 in the order: cyclin B–CDK1 > cyclin A–CDK1 = cyclin A–CDK2 >> cyclin E–CDK2. The amount of histone H1 kinase activity in any individual immunoprecipitate will vary, therefore, according to which cyclin-CDK is being measured and the stage of the cell cycle. Moreover, some transformed cells have altered levels of different cyclin-CDKs (e.g., cyclin E–CDK2 is overexpressed in a variety of cancer cell lines); therefore, results may differ between cell types.

The length of S phase will depend on the cell type being studied; S phase is ~6 to 8 hr long in many common human and mouse cell lines. When detecting DNA replication using BrdU, the number of positive cells will depend on the length of time for which BrdU is added to the medium and the proliferation state of the cells. With short pulses of BrdU, the sites of DNA replication will appear as distinct dots in the nucleus of the cell. They are easily seen with a ≥40× magnification lens. The number and size of the dots will depend on whether the cell is in early S phase (many small dots) or late S phase (fewer, bigger dots, more around the edge of the nucleus).

Time Considerations

Assuming a 1-hr incubation with the primary antibody, it should take ~3 hr to perform the steps from cell lysis through the kinase assay, and 90 min more to load the samples in the scintillation counter. Analysis of the samples by SDS-PAGE will take another 3 hr before the gel is exposed to X ray film.

For Basic Protocol 2, it should take ~3.5 hr from the time the coverslips are removed from the labeling medium until it is possible to view the slides under the microscope.

Literature Cited

Harlow, E. and Lane, D.P. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Jeffrey, P.D., Russo, A.A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N.P. 1995. Structure of a cyclin A–CDK2 complex. *Nature* 376:313-320.

Lew, D.J. and Kornbluth, S. 1996. Regulatory roles of cyclin-dependent kinase phosphorylation in cell cycle control. *Curr. Opin. Cell Biol.* 8:795-804.

Matsushime, H., Roussel, M.F., Ashmun, R.A., and Sherr, C.J. 1991. Colony-stimulating factor 1 regulates novel cyclins during the G_1 phase of the cell cycle. *Cell* 65:701-713.

Morgan, D.O. 1995. Principles of CDK regulation. *Nature* 374:131-134.

Phelps, D.E. and Xiong, Y. 1997. Assay for activity of mammalian cyclin D–dependent kinases CDK4 and CDK6. *Methods Enzymol.* 283:194-205.

Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T., and Nishimoto, T. 1991. Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO J.* 9:4331-4338.

Key Reference

Dunphy, W.G. (ed.) 1997. *Methods Enzymol.* Vol. 283: Cell Cycle Control. Academic Press, New York.

Presents a wide variety of protocols for analyzing the cell cycle.

Contributed by Jonathon Pines,
Mark Jackman, and Karen Simpson
Wellcome/CRC Institute and
Cambridge University
Cambridge, United Kingdom

Methods for Synchronizing Cells at Specific Stages of the Cell Cycle

The protocols presented here describe procedures used to synchronize cells in various stages of the cell cycle (Fig. 8.3.1). Synchronization is particularly useful for investigating a particular cell cycle–regulated event or preparing cells for extraction of transient factors whose expression is dependent on cell cycle stage. Exponentially growing cultures are generally asynchronous; i.e., each cell progresses through the cell cycle independently of the cell cycle stage of its neighboring cells. Cells that are synchronized are artificially induced to cycle in a homogeneous manner. The ability to continue cycling is an important distinction between a homogeneous population of cells created by synchronization and one created by blocking cells from cycling. Blocking cells from cycling may also result in a homogeneous population of cells at a particular stage of the cell cycle, but often results in death of the cell. In contrast, the purpose of synchonization is to create an enriched population of cells at a single stage of the cell cycle; these cells will then be able to continue through the cell cycle with as little disruption of normal events as possible. For a comprehensive review of events and explanation of the salient features of each of these stages see Pines (1995), Hartwell and Kastan (1994), *UNIT 8.1,* and the chapter introduction.

Techniques will be presented for synchronizing cells in the G_1, S, and M phases of the cell cycle. These techniques include a selection of methodologies that capitalize on the biology and biochemistry of eukaryotic cells, such as selective nutrient depletion (e.g., isoleucine deprivation, see Alternate Protocol 3; and serum withdrawal, see Basic Protocol 2), feedback control through addition of excess nutrients (e.g., thymidine, see Basic Protocol 4), morphological differences (e.g., mitotic shake-off, see Basic Protocol 1), or the use of chemical agents to reversibly arrest cells at a particular cell cycle stage (e.g., lovastatin, see Basic Protocol 3; mimosine, see Alternate Protocol 4; and nocodazole, see Alternate Protocol 2). The protocols can be modified to enhance for mitotic cells (see Alternate Protocol 1) or to provide sequential G_1/S blocks (see Alternate Protocol 5). Methods are provided for determining the mitotic index (see Support Protocol 1) and for measuring DNA synthesis by trichloroacetic acid (TCA) precipitation of [^{3}H]thymidine-labeled DNA (see Support Protocol 2).

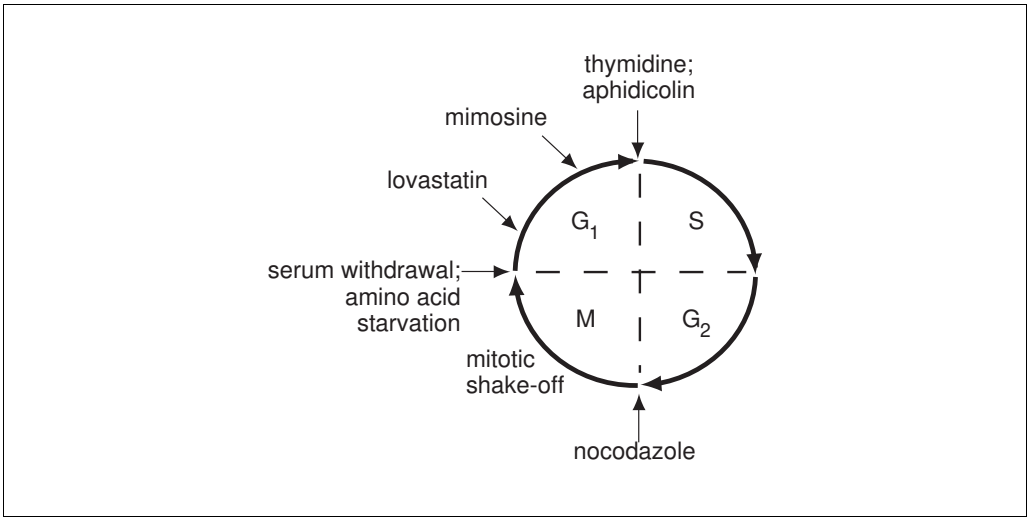


Figure 8.3.1 Relationship between synchronization methods and cell cycle. Arrows indicate the point in the cell cycle at which cells are enriched by each method.

Contributed by Joany Jackman and Patrick M. O'Connor
Current Protocols in Cell Biology (1998) 8.3.1-8.3.20

STRATEGIC PLANNING

Prior to selecting a method of synchrony there are several factors to consider.

Stage of Analysis Versus Stage of Synchrony

In general, it is best to synchronize cells as close to the start of the cell cycle phase to be studied (Fig. 8.3.2). In most cases, cells will not maintain a high degree of synchrony through several rounds of cell division. In order to follow the expression of factors as a function of progression through a cell cycle phase, it is usually best to synchronize cells in the phase prior to the actual phase to be investigated. For example, to investigate the S phase–specific expression of a G_1/S phase–cyclin protein (Gong et al., 1995), it may be necessary to synchronize cells in G_1 or at the G_1/S boundary using isoleucine deprivation or isoleucine deprivation coupled with aphidicolin treatment, respectively. The most difficult phase in which to investigate events related to both entry into and exit from is G_1, mainly because of the high degree of variability in G_1-phase progression.

Degree of Uniformity

Inherent in the strictness of this requirement is the specificity of the event to be analyzed. Ideally, cell cycle events that exhibit a broad window of action or expression would not require a high degree of synchrony, and measurements could be made over a longer period of time. In contrast, investigations of rare events with a short window of detection are often the most difficult, requiring a high degree of synchrony in the cell population in order to detect differences.

Number of Cells Required

This is determined in part by the sensitivity of the detection method selected. Cost and time are typically inversely related to the number of cells and degree of synchrony required. In cases where a large number of synchronized cells are required for biochemical

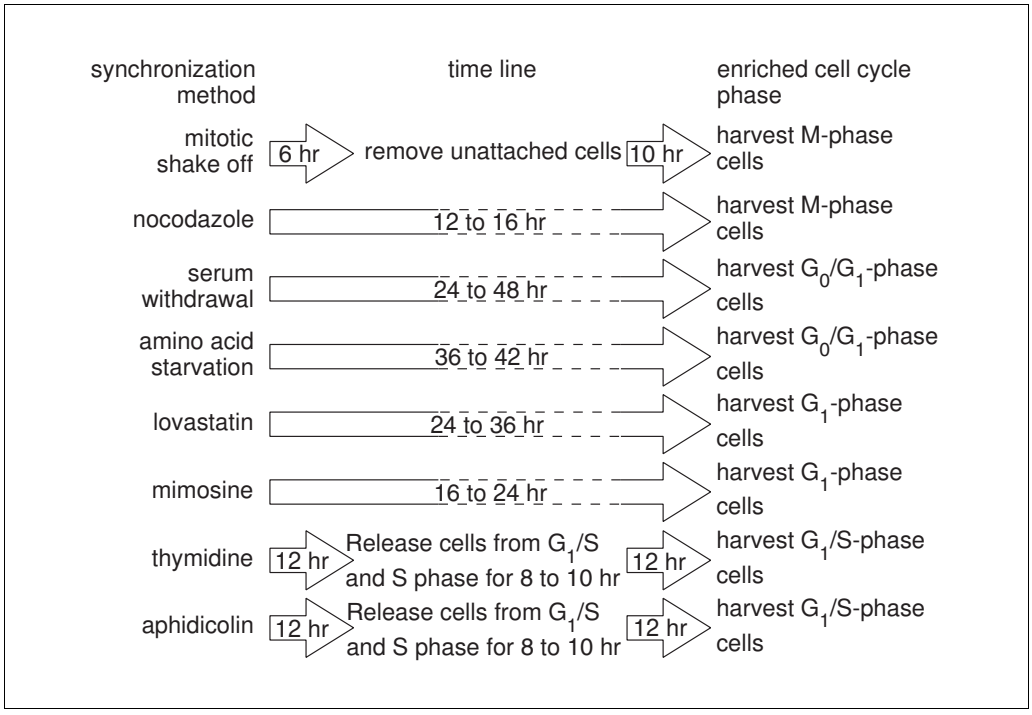


Figure 8.3.2 Quick reference guide to synchronization methods. Indicated times are for cultures with 24 hr doubling time.

characterization of a rare cell cycle event, some procedures, such as double aphidicolin exposure (see Alternate Protocol 5), can be expensive, while the same procedure may be an inexpensive and time-efficient means of obtaining a few hundred highly synchronized cells for analysis by immunofluorescent microscopy.

Doubling Time

All of the protocols discussed here are recommended for cells with doubling times <30 hr. Some may be used for cells with longer doubling times, but the efficacy of the protocol will depend on the specifics of the cells used. While all synchrony methods result in a population of cells enriched in a particular cell cycle phase, cell synchronization is relatively transient, producing synchronous populations for a single cycle. Synchrony generally diminishes each time cells pass through G_1 phase, since this is the most temporally variable stage of the cell cycle. Within a population of cells, G_1 phase averages between 8 and 16 hr for most rapidly dividing cell lines. Progression through S phase requires ~8 hr, through G_2 ~2 additional hours, and completion of M phase occurs 0.5 to 1 hr later.

Chemical Inhibitors

Chemicals that inhibit cell cycle progression are advantageous for synchronization because they are effective in a large variety of cell types, require no special equipment, and lend themselves to applications that require large numbers of cells. Indeed, chemical inhibitors are often the primary method of synchronizing cells at a particular stage of the cell cycle. There are two main disadvantages to chemical synchronization: (1) chemical treatment has been linked to the possible disruption of normal cell cycle regulatory processes (Schimke et al., 1991; Gong et al., 1995), and unbalanced cell growth induces an apoptotic phenotype in some cell types; and (2) chemicals may have more than one target in cells, some of which may not be fully known. With any experiment, however, careful design of controls is of greatest importance when using chemical synchronization.

Assessment of Synchronization

Assessment of the quality of synchronization is dependent on the equipment available. The best method for following overall changes in the distribution of cycling cells is flow cytometry. The most up-to-date benchtop versions of flow cytometers, such as the Becton Dickson FACScan/FACSCaliber and the Coulter Epics, are relatively simple to use, and the preparation of material for analysis is both rapid and inexpensive. If no flow cytometer is readily available, there are biochemical ways to determine cell synchrony. The first method measures the progression of cells through S phase. Short pulses of [^{3}H]thymidine in control and synchronized cultures are compared over time to monitor for peaks in [^{3}H]thymidine incorporation as an indicator of active replication and the maximum number of cells in S phase. This method can also be adapted to the fluorescent microscope using bromodeoxyuridine (BrdU)-labeled cells rather than [^{3}H]thymidine (*UNIT 8.2*). All cells that were in S phase during the pulse of BrdU can be followed through the cell cycle by labeling them with FITC. The second method measures the expression or sequential activation of cyclin-dependent kinases as an indicator of cell cycle stage. As demonstrated by Hunter, Pines and others (Minshull et al., 1989; Pines, 1995; Hunter and Pines, 1994; Brandeis and Hunt, 1996), cyclins are sequentially expressed and/or stabilized as cells move from G_1 into M phase (Table 8.3.1).

Immunoblotting (*UNIT 6.2*) for the various cyclins or in vitro kinase assays following cyclin-specific immunoprecipitation (*UNIT 8.2*) can be used to assess cyclin expression or activity. One caveat to this methodology is the fact that a large number of transformed cell lines inappropriately or constitutively express one or more cyclins (Buckley et al.,

Table 8.3.1 Characteristic Cyclin Expression Profiles and Activation as a Function of Cell Cycle Stage[a]

Cell cycle stage	Cyclin	Kinase activated
Early G_1	D-type	CDK4, CDK6
Mid-late G_1	E	CDK2
gc S phase	A	CDK2
G_2/M	A, B	cdc2

[a]Abbreviations: CDK, cell-dependent kinase; cdc, cyclin-dependent kinase.

1993; Keyomarsi and Pardee, 1993; Leach et al., 1993). This should be evaluated prior to using this method of assessment of cell cycle progression and synchronization. Finally, the mitotic index (a morphological evaluation of cell synchrony) can be used to compare asynchronous controls and synchronized cells for the appearance of rounded cells with condensed chromatin (O'Connor et al., 1993). This method has the advantage that no special equipment is required, although the evaluation of ≥500 events per sample is somewhat labor intensive and does not lend itself well to evaluating early G_1 synchronization.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

BASIC PROTOCOL 1

ENRICHMENT OF MITOTIC CELLS BY MITOTIC SHAKE-OFF

Mitotic shake-off is one of the oldest and perhaps easiest methods for obtaining mitotically enriched populations of cells (Morla et al., 1989; Pines and Hunter, 1989; Pagano et al., 1992). This method is based on the observation that as cells progress into metaphase during mitosis they become rounded and have fewer points of attachment with the culture vessel (Elvin and Evans, 1983; Zwanenburg, 1983).

This procedure is applicable to most monolayer cells. Collection of pure populations of mitotic cells allows the measurement of cell cycle–related events during the M/G_1/S phase transitions. Each cell type requires some characterization to determine the most suitable times at which cells should be harvested following release.

This procedure has several advantages. This technique is based on normal cellular processes rather than extrinsic control by experimental manipulation and does not require special equipment or reagents other than those commonly available in the average cell culture facility. However, this procedure is not applicable to suspension cultures or weakly adherent cultures, such as some neuronal cell types. Only a small proportion of the cells, 1% to 5% of the typical exponential asynchronous culture, may be in mitosis at any one time; therefore, a limitation of the procedure is the low yield of mitotic cells obtained from exponentially growing cultures. To overcome this drawback and preenrich for mitotic cells, one can carry out short incubations with mitotic inhibitors, and/or release cells from G_1/S-phase synchronization using the procedures described below, and capture cells by shake-off as they progress into M. Although this procedure is used for following the progression of cells into G_1, the same degree of synchrony is not maintained as cells progress through subsequent S and G_2 phases.

The following protocol describes mitotic shake-off using the adherent HT-29 colon cell line. It has also been used with CHO cells, HeLa cells, and a number of other colon and breast cell lines.

Materials

Human colon carcinoma cell line (HT-29 cells), grown in 162-cm^2 tissue culture flasks
RPMI-15: RPMI 1640 supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS; *APPENDIX 2A*), prewarmed to 37°C
RPMI 1640 medium (*APPENDIX 2B*), prewarmed to 37°C
0.5× trypsin solution (see recipe)

Additional reagents and equipment for trypsinizing and counting cells (*UNIT 1.1*)

1. Trypsinize (*UNIT 1.1*) subconfluent cultures of HT-29 cells and replate in prewarmed RPMI-15 at one-half to one-third maximum density. Incubate at 37°C until 70% to 80% confluent.

   This ensures that the maximum number of cells will be replicating in the culture.

2. Remove the growth medium and wash cells once with prewarmed RPMI 1640. Add 3 ml of 0.5× trypsin solution and incubate cells 5 min at 37°C.

3. Tap the flasks on the bench to shake off any loosely attached rounded cells and add 20 ml prewarmed RPMI-15. Transfer cell suspension to 15-ml centrifuge tubes and centrifuge 5 min at 500 × *g*, room temperature.

4. Discard supernatant, resuspend pellet in 20 ml prewarmed RPMI-15, and centrifuge 5 min at 500 × *g*, room temperature. Discard supernatant and resuspend pellet in 20 ml prewarmed RPMI-15.

5. Count cells (*UNIT 1.1*) and adjust concentration to 2.5 × 10^5 cells/ml in prewarmed RPMI-15.

6. Transfer 20 to 40 ml of cell suspension to 162-cm^2 flasks and incubate 6 hr at 37°C. Observe the cultures to see if cells have reattached.

7. Shake flasks, swirl medium over the flask surface, and pipet off the medium. Repeat wash twice with 20 ml prewarmed RPMI-15 each time. Add 20 ml fresh, prewarmed RPMI-15 and incubate 10 hr at 37°C.

   The washing step removes any unattached cells.

8. Observe the cultures for evidence of cell rounding. Gently shake or tap the flasks on the bench and swirl the medium around the flask. Remove the medium, which contains mitotic cells.

9. Pool the medium from several flasks and centrifuge 5 min at 500 × *g*, room temperature, to pellet the mitotic cells.

10. Resuspend cells in RPMI-15, count cells (*UNIT 1.1*), and adjust cell density to 5 × 10^5 cells/ml in RPMI-15. Analyze cells by flow cytometry, Giemsa staining for mitotic chromatin condensation, or phase-contrast microscopy for rounded cells.

    For Giemsa staining, submerge an air-dried slide in 0.1 to 0.2 mg/liter Giemsa (Sigma, Life Technologies) for 10 min. Gently rinse stain away by dipping in 1× PBS and air dry the slide.

ALTERNATE PROTOCOL 1

PRE-ENRICHMENT OF EXPONENTIAL CULTURES FOR MITOTIC CELLS

Short incubations with S phase–synchronizing agents, such as excess thymidine (see Basic Protocol 4), will increase the proportion of cells in M phase. Release cells from this block by thoroughly washing and collecting them ~10 hr later, when significant numbers of rounded cells are observable in the culture. Alternatively, add 0.4 µg/ml nocodazole 5 to 6 hr before shake-off to arrest cells as they progress through M phase.

ALTERNATE PROTOCOL 2

ENRICHMENT OF MITOTIC CELLS BY NOCODAZOLE ARREST

Nocodazole chemically interferes with the organization of microtubules in cells. Microtubule formation is an important structural feature of cells as they enter mitosis. Several drugs, including vincristine and colcemid, are similar to nocodazole in that they interfere with microtubules and cause arrest in G_2/M. Unlike nocodazole, however, the effects of these agents may not be completely or readily reversed.

Nocodazole arrests both suspension and adherent cells in a prophase to pseudo-metaphase state. Interpretation of results and discernment of mitotic figures may be more difficult when nocodazole is used since nocodazole disrupts microtubules. Microtubules are required for the condensation of chromatin and alignment on the metaphase plate. This procedure has been used to obtain large amounts of activated cyclin B-cdc2-dependent kinases.

Nocodazole is inexpensive and specific in acting on cells in the post-replicative period. It can be added to actively growing cultures without residual effects on other cell cycle stages. Nocodazole is useful for obtaining large numbers of highly synchronized cells. Some cell lines are defective in sensing disruption of the microtubular network and will not arrest in G_2/M, but will instead continue to cycle and undergo abnormal mitosis. Nocodazole can exhibit toxicity at concentrations close to its effective concentration for some cell lines, so the optimal concentration should be determined prior to preparing large amounts of material. The concentration used in this protocol works well with CA46 cells, but lower concentrations may be used with other cell lines. Effective concentrations range from 10 to 400 ng/ml. High concentrations of nocodazole notably diminish cell synchrony following reentry into the cell cycle.

The use of the suspension cell line, CA46, is described in this protocol. Other lymphoma cell lines, breast cell lines, colon cell lines, and HeLa cells have also been used.

Materials

CA46 cells, grown in 75-cm^2 tissue culture flasks
0.4 mg/ml nocodazole (diluted from 4 mg/ml nocodazole; see recipe)
RPMI-15: RPMI 1640 supplemented with 15% heat-inactivated FBS (*APPENDIX 2A*), prewarmed to 37°C

Additional reagents and equipment for counting cells (*UNIT 1.1*)

1. Grow CA46 cells to subconfluency (0.5 to 1.0 × 10^6 cells/ml). Count cells (*UNIT 1.1*) and adjust concentration to 5 × 10^5 cells/ml in 20 ml prewarmed RPMI-15.

   At this dilution, the maximal number of cells should be replicating within the culture.

2. Add 0.4 mg/ml nocodazole to cultures at a final concentration of 400 ng/ml.

3. Incubate cells 12 to 16 hr at 37°C.

4. Centrifuge cells 5 min at 500 × *g*, room temperature, discard supernatant, and resuspend pellet in 20 ml fresh, prewarmed RPMI-15. Repeat centrifugation and addition of 20 ml prewarmed RPMI-15.

5. Transfer cell suspension to fresh 75-cm^2 tissue culture flasks and incubate 4 hr at 37°C. Monitor cultures during this next 4-hr period for an increase in cell number.

An increase in cell number indicates progression into G_1 phase. Mitotic index, due to the effect of nocodazole on microtubules, may be more difficult to measure if nocodazole is used. The mitotic figures seen following release from nocodazole are sometimes aberrant and less distinct (these probably result from cells trapped in mitosis at the time of application). In addition it is easier and faster to merely count cells than to perform mitotic index measurements if progression through M phase is being measured.

ENRICHMENT OF CELLS AT G_0/G_1 BY SERUM STARVATION

BASIC PROTOCOL 2

This protocol describes enrichment of cells at G_0/G_1 by serum starvation (Campisi et al., 1984). Although some fibroblasts begin entering G_0 within 1 hr (Pardee, 1989), it may take 24 to 48 hr for an entire population to respond to serum withdrawal. Before using this technique, it is important to determine the confluent/maximum cell density the cells will achieve (cells/ml or cells/cm^2).

The method described below is for monolayer cultures, although the procedure can be adapted to suspension cultures. This procedure has been used in many cell cycle–related studies, including analysis of the cell cycle–dependent reversible tyrosine phosphorylation of cdc2 (Morla et al., 1989) and regulation of cyclin A (Girard et al., 1991; Carbonaro-Hall et al., 1993). This simple method for obtaining G_0 cells requires no special equipment or reagents and is effective on cell types that do not respond to confluence arrest. Serum withdrawal may require extensive optimization of both the amount of serum withdrawn and the length of withdrawal. Withdrawal of serum for extended periods will actually reduce the synchronicity of the restimulated population. For some cell types, serum starvation is not a viable method of synchronization, because cells permanently arrest in G_0, do not arrest, or undergo apoptosis.

The use of 3T3 cells, a fibroblastic cell line, is described in this protocol. This procedure has also been shown to be effective in producing a G_0-like state in myoblasts (Puri et al., 1997), CHO fibroblasts (Tesfaigzi and Carlson, 1996), and MOLT-4 leukemia cells (Dbaibo et al., 1995).

Materials

NIH-3T3 cells, grown in 100-mm tissue culture plates
1× trypsin (Life Technologies)
DMEM (*APPENDIX 2A*), prewarmed to 37°C
DMEM-0.5: DMEM supplemented with 0.5% (v/v) heat-inactivated FBS (*APPENDIX 2A*), prewarmed to 37°C
DMEM-10: DMEM supplemented with 10% (v/v) heat-inactivated FBS, prewarmed to 37°C

Additional reagents and equipment for trypsinizing and counting cells (*UNIT 1.1*)

1. Trypsinize (*UNIT 1.1*) exponentially growing NIH-3T3 cells grown in 100-mm tissue culture plates, using 1× trypsin.

2. Transfer detached cells to 15-ml conical centrifuge tubes and centrifuge cells 5 min at 500 × *g,* room temperature. Discard supernatant, resuspend pellet in 10 ml prewarmed DMEM, and repeat centrifugation. Repeat wash.

3. Plate cells at 30% to 40% of their confluent density in 15 ml DMEM-0.5 and incubate 24 to 48 hr at 37°C.

   A 24- to 48-hr period of serum starvation allows cells time to sequester into a G_0-like state. This time period gives cells that have passed the restriction point (Pardee, 1974, 1989) the opportunity to proceed through the remainder of the cell cycle and arrest upon reentry into G_1 of the next cell cycle.

4. Remove DMEM-0.5 and add 15 ml DMEM-10. Incubate at 37°C.

   The addition of medium containing 15% FBS stimulates the cells to reenter the cell cycle. NIH-3T3 cells progress into S phase ~12 hr after stimulation; this time will vary depending on the cell type.

ALTERNATE PROTOCOL 3

ENRICHMENT OF CELLS AT G_0/G_1 BY AMINO ACID STARVATION

A large number of synchronous G_0 cells can be obtained by amino acid starvation (Tobey and Crissman, 1972). Omission of isoleucine, an essential amino acid, from the medium causes cells to sequester in a G_0/G_1-like state. Methionine depletion has also been successfully used to synchronize cells (Sallot et al., 1996). In some tumor cell lines, however, this treatment may produce a late S/G_2-phase arrest (Hoshiya et al., 1996). In the author's experience, depleting both isoleucine and methionine does not result in greater synchronization, but rather increases the likelihood of irreversible G_1 arrest and cell death. Before using this technique, it is important to determine the doubling time of the exponentially growing culture.

This procedure can be used with suspension or adherent cell lines to follow the progression of early G_1 cells into the cell cycle. Cells cycle synchronously upon release (addition of complete medium) for an entire cell cycle through cytokinesis. This method does not require exogenous chemicals to cause cell cycle arrest but makes use of the intrinsic biology of the cell. Notably, not all cell types will become synchronous with this procedure. Some transformed cells lines are defective in nutrient checkpoint control and will continue through the cell cycle irregularly. In other cell types, nutrient deprivation induces cell death or partial differentiation rather than transient arrest.

Materials

CA46 cells, grown in 162-cm^2 tissue culture flasks
Isoleucine-free minimal essential medium (e.g., Select-Amine Minimal Medium, Life Technologies), prewarmed to 37°C
Dialyzed FBS (Life Technologies)
RPMI-15: RPMI 1640 supplemented with 15% (v/v) heat-inactivated FBS (*APPENDIX 2A*)

1. Centrifuge exponentially growing CA46 cells 5 min at 500 × *g*, room temperature. Discard supernatant, resuspend pellet in 50 ml prewarmed isoleucine-free minimal essential medium without serum, and centrifuge again. Repeat wash.

2. Resuspend cells at 35% to 40% of their confluent density in (4×10^6 cells/ml) isoleucine-free minimal essential medium/10% dialyzed FBS, transfer to appropriate size tissue culture flasks, and incubate 36 to 42 hr at 37°C.

   This time period is equivalent to one and one-half to two cell cycles for CA46 cells. This time should be appropriately adjusted for each cell type.

   Prolonged culture under these conditions will lead to increased toxicity, genetic aberrations, and, in some cases, irreversibility of the arrest.

3. Transfer cells to 50-ml centrifuge tubes and centrifuge 5 min at 500 × *g*, room temperature. Discard supernatant and resuspend pellet in RPMI-15 at a final cell density of 0.5×10^6 cells/ml.

   Avoid rough or unnecessary handling of the cells following the starvation procedure because the cells may be more fragile than exponentially growing cells.

   Adherent cells should be treated cautiously as they may not be as tightly attached to the plate and may be dislodged by the removal of the isoleucine-free medium or addition of the fresh complete medium.

ENRICHMENT OF G_1-PHASE CELLS USING LOVASTATIN

BASIC PROTOCOL 3

Lovastatin inhibits HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A reductase), resulting in the depletion of mevalonate, which is an essential precursor of cholesterol synthesis and a requirement for isoprenylation of substrate molecules, including p21 ras (Wejde et al., 1993).

Lovastatin has been demonstrated to block a variety of cell types, including adherent and suspension cells, in very early G_1 phase rather than in G_0, as defined by the presence of the proliferation-specific antigen Ki-67 (Keyomarsi et al., 1991). Lovastatin has been effectively used to synchronize breast cancer cells (Bonapace et al., 1996; Wilcken et al., 1997) and Jurkat cells (Martel et al., 1997). Lovastatin is a convenient and rapid inhibitor of early G_1 cells, requiring a single cell cycle to induce a G_1 block. Lovastatin is not effective with all cell types, and in some cell types it also induces a partial arrest of cells in G_2/M (Jakobisiak et al., 1991). Lovastatin is more expensive than some other methods described for generating G_1 populations, and it requires careful optimization of concentration and timing in order to obtain maximal synchronization.

This protocol describes a method by which lovastatin can be used to synchronize breast cancer cells in G_1 phase.

Materials

MCF-7 cells, grown in 100-mm tissue culture plates
1× trypsin (Life Technologies)
DMEM-10: DMEM supplemented with 10% (v/v) heat-inactivated FBS (*APPENDIX 2A*), prewarmed to 37°C
10 mM activated lovastatin (see recipe)
DMEM-10 containing 5 mM mevalonate (Sigma)

Additional reagents and equipment for trypsinizing and counting cells (*UNIT 1.1*)

1. Trypsinize (*UNIT 1.1*) exponentially growing MCF-7 cells using 1× trypsin and replate in 15 ml DMEM-10 at a cell density equivalent to 30% to 35% of maximum.

   This prevents growth arrest due to confluence.

2. Add 10 mM activated lovastatin to the culture medium at a final concentration of 40 µM and incubate 24 to 36 hr at 37°C.

   This incubation should last for at least one doubling time; times may vary according to the cell type.

   Effective concentrations of lovastatin vary according to the cell type and should be optimized; concentrations ranging between 10 and 60 µM have been reported (Keyomarsi et al., 1991).

3. Trypsinize cells in 3 to 5 ml 1× trypsin. Add 5 ml prewarmed DMEM-10 to cells and centrifuge 5 min at 500 × *g*, room temperature. Discard supernatant, resuspend pellet in 10 ml prewarmed DMEM-10 and repeat centrifugation. Repeat wash.

4. Resuspend cells at 35% to 40% of their confluent density in 15 ml DMEM-10 containing 5 mM mevalonate.

   The mevalonate concentration should be ~100-fold the concentration of lovastatin used. Key regulatory proteins that require isoprenylation for activity will be modified following release of the cells from the lovastatin block.

5. Monitor cells for progression through G_1 and S phases by flow cytometry, activation of cyclin E– or cyclin A–dependent kinases, or incorporation of labeled nucleotide precursors into DNA (see Support Protocol 2).

ALTERNATE PROTOCOL 4

ENRICHMENT OF G_1-PHASE CELLS BY MIMOSINE ARREST

Mimosine, a plant-derived amino acid whose cellular target is still unclear, blocks cells either in late G_1 phase (Lalande, 1990) or at the G_1/S boundary (Watson et al., 1991; Mosca et al., 1992), depending on the relevance of the recovery period to the timing of initial [^{3}H]thymidine incorporation. When used alone or in combination with prior isoleucine synchronization, mimosine arrests cells with a diploid DNA content (Mosca et al., 1992, Dijkwel and Hamlin, 1992). Mimosine is an effective inhibitor of S-phase entry in mammalian fibroblastic cell lines and human leukemic cell lines, and it inhibits DNA replication in *Xenopus* extracts (Gilbert et al., 1995). Mimosine is an inexpensive and stable inhibitor of cell cycle progression and is relatively non-toxic compared with other chemical inhibitors, such as aphidicolin. Unlike aphidicolin, however, mimosine requires several hours to inhibit cell cycle progression. Studies monitoring cell cycle progression indicate that replication is initiated 1 to 2 hr after release from mimosine-induced blockade.

There is some debate as to the target and position in the cell cycle at which mimosine arrests cells. It has been reported that mimosine inhibits enzymes involved with thymidine biosynthesis (Kalejta and Hamlin, 1997) and other enzymes involved with nucleotide biosynthesis (Gilbert et al., 1995). The lack of clarity as to the exact target and site of action of mimosine could complicate the use of mimosine to study defined events in nucleotide regulation or incorporation.

Materials

CA46 cells, grown in 75-cm^2 tissue culture flasks
RPMI-15/mimosine: RPMI supplemented with 15% (v/v) heat-inactivated FBS (*APPENDIX 2A*) and 400 μM mimosine (diluted from 100 mM mimosine; see recipe), prewarmed to 37°C
RPMI-15: RPMI supplemented with 15% (v/v) heat-inactivated FBS, prewarmed to 37°C

1. Centrifuge 5–25 × 10^6 exponentially growing CA46 cells or CA46 cells synchronized by prior isoleucine deprivation (see Alternate Protocol 3) 5 min at 500 × *g*, room temperature. Discard supernatant and resuspend pellet in prewarmed RPMI-15/mimosine to give a final cell concentration of 0.4 × 10^6 cells/ml.

   Effective concentrations of mimosine range from 100 to 400 μM.

2. Incubate cells 6 to 24 hr (exponentially growing cells) or 14 hr (isoleucine-deprived cells) at 37°C.

3. Centrifuge cells 5 min at 500 × *g*, room temperature. Discard supernatant, resuspend pellet in 10 ml prewarmed RPMI-15, and repeat centrifugation. Repeat wash and resuspend cells in prewarmed RMPI-15 at a cell density equivalent to 35% to 40% of their confluent density (0.4 × 10^6 cells/ml).

4. Monitor progression of cells into S phase by incorporation of labeled nucleotide precursors into DNA (see Support Protocol 2) or flow cytometry.

SYNCHRONIZING CELLS AT THE ONSET OF S PHASE BY DOUBLE-THYMIDINE BLOCK

Synchronization of cells at the G_1/S phase border is generally achieved by inhibition of DNA synthesis using chemical inhibitors, including aphidicolin, hydroxyurea, or excess thymidine (Tobey and Crissman, 1972; Thomas and Lingwood, 1975; Heintz et al., 1983).

Synchronization of cells with excess thymidine was the first reliable method to be widely used (Bootsma et al., 1964, Stein and Borun, 1972). The method involves two sequential exposures to high levels of thymidine, with each exposure separated by an interval determined from knowledge of the normal cell cycle distribution of the exponentially growing cells and the recovery time from the thymidine block. The method is most useful to measure S- and G_2/M-phase events. The procedure is good for one cell cycle passage; the cells lose synchrony rapidly thereafter.

Thymidine is an inexpensive and readily available inhibitor of the cell cycle. Unlike many inhibitors, the mechanism by which thymidine blocks the cell cycle is well understood and attributable to feedback inhibition of the nucleotide synthesis (Thomas and Lingwood, 1975). It may be difficult to accurately measure DNA synthesis by measuring [^{3}H]thymidine incorporation, because of the large pools of thymidine in cells, especially early in S phase. Prolonged disruption of the cell cycle with thymidine results in genetic aberrations presumably caused by misreplication events.

Before using this technique, it is important to determine the doubling time of the exponentially growing culture. The procedure described below is for the synchronization of HeLa cells based on a doubling time of 24 hr.

This protocol can be used for both adherent and suspension cells. The only requirement is that cells display a robust doubling time of less than 24 to 30 hr. Doubling times outside this range may require alterations in the timing of thymidine application and release (see Critical Parameters and Troubleshooting).

Materials

HeLa cells, grown in 162-cm^2 tissue culture flasks
DMEM-10/thymidine: DMEM supplemented with 10% (v/v) heat-inactivated FBS (*APPENDIX 2A*) and 2 mM thymidine (diluted from 100 mM thymidine; see recipe), prewarmed to 37°C
DMEM, prewarmed to 37°C
DMEM-10: DMEM supplemented with 10% (v/v) heat-inactivated FBS (*APPENDIX 2A*), prewarmed to 37°C
1× trypsin (Life Technologies)
1× PBS, pH 7.4 (*APPENDIX 2A*)

Additional reagents and equipment for trypsinizing and coating cells (*UNIT 1.1*)

1. Plate exponentially growing HeLa cells at 30% to 50% confluence in 15 ml DMEM-10/thymidine and incubate 12 hr at 37°C.

   This time period is equivalent to the progression through G_2/M and G_1 phases. During this period, the G_2/M cells will progress into G_1 and then with the original G_1 cells will achieve a biochemical state equivalent to G_1/S-phase border cells. Any cells in S phase when thymidine is added will become blocked in S phase.

2. Remove the the thymidine-containing medium with a pipet and rinse the dishes twice with 10 ml prewarmed DMEM each time.

   Thoroughly rinse all surfaces of the dish to prevent carryover of thymidine.

3. Trypsinize cells using 1× trypsin, resuspend in 10 ml DMEM-10, and count the number of cells in suspension (*UNIT 1.1*). Replate the cells at 30% to 35% of their confluent density in 15 ml prewarmed DMEM-10 and incubate 16 hr at 37°C.

   During this period, the cells will recover from the thymidine block (~1 to 2 hr), progress through the cell cycle, divide, and enter G_1 of the next cell cycle. If the cells are expected to progress through two more cycles, they should be plated at ~25% of their confluent density.

   Progression of cells through the first thymidine block can be monitored by determining cell number. Approximately 5 to 6 hr after removal of the thymidine, cells that were blocked at the end of S phase (the leading-edge cells) will enter G_1 of the next cell cycle and an increase in cell number will be detectable. Incubation in medium without added thymidine should occur ~8 to 10 hr later in order to give lagging-edge cells (those arrested at G_1/S in the first block) sufficient time to progress through S phase.

4. Remove medium with a pipet and replate with 15 ml prewarmed DMEM-10/thymidine and incubate 12 to 14 hr at 37°C.

   Cells in G_2/M or G_1 will progress and arrest at the G_1/S-phase border.

5. Remove the the thymidine-containing medium with a pipet and rinse the dishes twice with 15 ml DMEM-10 each time.

   Thoroughly rinse all surfaces of the dish to prevent carryover of thymidine.

6. Monitor the progression of cells into S phase by flow cytometry, into G_2/M by assembly and activation of cyclin B-cdc2-dependent kinase (*UNIT 8.2*), or through M phase by cell rounding, mitotic index (see Support Protocol 1), or increases in cell number.

ALTERNATE PROTOCOL 5

PERFORMING SEQUENTIAL G_1/S BLOCKS

Variations on the double thymidine–block strategy have evolved with time. A commonly used variant technique involves a first block with thymidine and a second block with aphidicolin (Heintz et al., 1983) or alternatively a double-aphidicolin block (O'Connor et al., 1993). Essentially the same basic principles as described in Basic Protocol 4 for the double-thymidine block apply to the use of aphidicolin. The use of thymidine and aphidicolin procedures for synchronizing cells at the G_1/S-phase border proved useful in determining the regulation of various cell cycle–related processes, including the regulation of histone gene expression (Heintz et al., 1983), regulation of cyclin B (Pines and Hunter, 1989), and effect of DNA damage on the formation and activation of cyclin A– and cyclin B–dependent kinases (O'Connor et al., 1993). Starvation methods paired with sequential aphidicolin treatment are particularly useful for obtaining highly synchronous populations of G_1/S cells (Heintz and Hamlin, 1982, Burhans et al., 1986).

Aphidicolin is a specific inhibitor of eukaryotic DNA polymerase (Heintz and Hamlin, 1982). Treatment of cells with aphidicolin prohibits S-phase cells from progressing through the remainder of S phase, while G_1 and G_2/M cells continue to cycle to the G_1/S boundary (Burhans et al., 1986). For this reason, a single aphidicolin block is not usually sufficient to obtain a highly synchronous culture. Aphidicolin can be substituted for thymidine during both blocks in Basic Protocol 4, by adding aphidicolin (see recipe) to exponentially growing cells at a final concentration of 0.1 μg/ml aphidicolin.

The point at which aphidicolin blocks the entry of cells into S phase is much better characterized and closer to the start of S phase than the point at which excess thymidine blocks. Incorporation of nucleotide precursors into DNA begins <15 min after release of cells from aphidicolin (Burhans et al., 1986); therefore, aphidicolin may be more appropriate for some studies examining S-phase progression. It is important to note that

aphidicolin blocks the replicative DNA polymerase but not other events related to replication such as unwinding of replication origins and assembly of replication complexes. For this reason, there is some controversy about the exact phase in which aphidicolin arrests cells from cycling. It is clear that aphidicolin blocks cells at a cell cycle phase before the incorporation of deoxynucleotides into long polymers of DNA at the very beginning of S phase.

Aphidicolin can be used in suspension cultures or adherent cell lines. While aphidicolin is rapid and dependable and produces a highly synchronous population of cells immediately poised to enter S phase, it is also more expensive to use than excess thymidine. In addition, prolonged treatment of cells with aphidicolin results in DNA fragmentation (Schimke et al., 1991).

DETERMINING MITOTIC INDEX

SUPPORT PROTOCOL 1

The mitotic index is used to monitor the progression of cells through M phase, when chromatin condensation occurs. This procedure is labor intensive, but it correlates well with the progression of cells through M phase and cytokinesis (O'Connor et al., 1993). This method is useful to monitor cell cycle progression into M phase in the presence of inhibitors of cell division, such as colcemid or vincristine, when observation of the progression of cells through a single cell cycle is desired.

This protocol is adaptable to attached cells that have been trypsinized or to suspension cells. When working with adherent cells it is important to guarantee that you are observing the entire population of cells and not a subset of easily detached cells. Adherent cells staged at certain phases in the cell cycle attach more tightly to their substratum (see previous protocol) and may detach more poorly than other cells in the same vessel. Therefore, to avoid an experimental artifact and overestimation of mitotic cells due to uneven detachment of cells, culture vessels should be carefully viewed after trypsinization and removal of cells to ensure that no or few cells are left behind. Mitotic index should be determined at several timepoints (see below). Elevation of the percentage of cells exhibiting mitotic figures relative to the asynchronous control is evidence that the cells are actively cycling and are synchronized.

Materials

Burkitt's lymphoma cell line (CA46 cells), grown in RPMI-15, synchronized and unsynchronized
RPMI-15: RPMI supplemented with 15% (v/v) heat-inactivated FBS
1× PBS, pH 7.4 (*APPENDIX 2A*), ice cold
0.5× PBS, pH 7.4 (*APPENDIX 2A*), *or* 75 mM KCl, ice cold
3:1 (v/v) ethanol/glacial acetic acid, ice cold
Giemsa stain (Sigma, Life Technologies; optional)

1. Stimulate $>1 \times 10^3$ CA46 cells for each timepoint to enter the cell cycle by the method indicated in the synchronization protocol or by changing to fresh RPMI-15.
2. At the appropriate timepoints, transfer cells to a 15-ml centrifuge tube and centrifuge 5 min at 500 × *g*, room temperature. Discard supernatant, resuspend cells in 2 vol ice-cold 1× PBS, and repeat centrifugation. Discard supernatant and resuspend cells in 0.5 ml ice-cold 0.5× PBS for 10 min or ice-cold 0.75 mM KCl for ≥20 min, on ice to permit swelling of the cells without rupture.

   This hypotonic swelling makes mitotic figures easier to distinguish. Alternatively, add 6 ml of 2% (v/v) 3:1 ethanol/glacial acetic acid and store overnight at 4°C.

   The number and frequency of timepoints depends on the experiment. For G_1- or S-synchronized cells, mitotic index can be monitored every 4 hr; for late S- or M-synchronized cells,

it should be monitored every 30 min. Unsynchronized cells should be analyzed at the same timepoints to assess the success of the synchronization protocol.

3. Centrifuge cells 5 min at 500 × *g*, room temperature. Discard supernatant and resuspend pellet in 0.5 ml of 3:1 ethanol/glacial acetic acid. Finger vortex gently while resuspending to avoid cell clumps.

   The final cell concentration should be 1-5× 10^3 cells/ml.

4. Drop ~100 µl cells onto glass slide, two to three slides per timepoint. Allow slides to air dry for 0.5 to 2 hr.

   Cells should not overlap, therefore, the optimal concentrations of cells to be placed on each slide can be determined empirically by dropping cells onto one or two slides and observing them for the distribution on the slide. To help cells spread evenly, the slide can be tilted at a slight angle to improve the spread of cells.

5. Stain cells with 0.1 to 0.2 mg/liter Giemsa stain for 10 min. Gently rinse Giemsa stain from slides by dipping in 1× PBS and air dry slides.

6. Observe slides under the microscope for the presence of cells with mitotic figures with condensed nuclear material and a lack of nuclear membrane. Represent the data as a percentage of mitotic cells in the total number of cells observed.

   Generally, 200 to 500 cells are scored for each timepoint.

SUPPORT PROTOCOL 2

MONITORING [^{3}H]THYMIDINE INCORPORATION INTO DNA BY TCA PRECIPITATION

Pulse-labeling of cells with [^{3}H]thymidine (Thomas and Lingwood, 1975) followed by TCA precipitation onto filters (Jackman et al., 1994) has been used to monitor synchronous cells for peak DNA synthesis (>3- to 4-fold increase over baseline incorporation of labeled nucleotides). Peak DNA synthesis reflects the point in time when the maximum number of cells are in S phase.

Materials

CA46 cells, released from synchronization
RPMI-15: RPMI supplemented with 15% (v/v) heat-inactivated FBS (*APPENDIX 2A*), prewarmed to 37°C
[^{3}H]thymidine (20 to 60 Ci/mmol)
1× PBS (*APPENDIX 2A*)
0.5× PBS (see recipe)
20% (v/v) TCA, ice cold
5% (v/v) TCA, ice cold
95% ethanol

Dry ice/methanol bath
Wet ice in bucket
Glass fiber filters
Vacuum manifold
Vacuum drying oven, 80°C

1. Following release of cells into the cell cycle by replacement of amino acids or growth factors (Basic Protocol 2, Alternate Protocol 3) or by removal of inhibitors (Basic Protocol 3, Basic Protocol 4, Alternate Protocol 4), transfer several aliquots of 1 × 10^5 CA46 cells to 15-ml plastic centrifuge tubes.

   The number of cells can be reduced 10-fold, if necessary

2. Centrifuge the cells 5 min at 500 × *g*, room temperature. Discard supernatant and resuspend pellet in an equivalent volume of prewarmed RPMI-15 supplemented with 5 µCi/ml [^{3}H]thymidine. Incubate 30 min at 37°C.

3. Centrifuge cells 5 min at 500 × *g*, room temperature, discard supernatant, and resuspend pellet in 5 ml ice-cold 1× PBS. Repeat PBS wash and resuspend pellet in 0.5 ml 0.5× PBS.

4. Place cells in tube rack in dry ice/methanol bath and let stand for 1 to 3 min. Allow cells to thaw for 3 to 5 min at 37°C. Repeat twice.

   The freeze-thaw process lyses the cells.

5. Place tubes containing cell lysates in bucket containing wet ice. Add an equal volume of ice-cold 20% TCA to the lysates and let stand 30 min on ice.

6. Moisten glass fiber filters with 5% TCA, then place in vacuum manifold and seal in place according to the manufacturer's directions.

   Label filters with pencil prior to wetting or, alternatively, label the caps of scintillation vials into which filters will be placed.

7. Transfer precipitates to glass fiber filters under vacuum. Wash each tube twice with 2 vol ice-cold 5% TCA and transfer this material also to the appropriate filter.

8. Wash filters three times under vacuum with 5 ml ice-cold 5% TCA and once with 5 ml ice-cold 95% ethanol.

9. Transfer filters to scintillation vials and set caps loosely in place. Place vials in 80°C vacuum oven for ~45 min.

   Include blank filters as a negative control for background.

10. Add 5 to 10 ml scintillation fluid to each vial. Place vials in scintillation counter and count on the ^{3}H channel for 5 min each. Average the cpms from multiple aliquots and plot against time from release.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Aphidicolin, 10 µM

10 µM aphidicolin (Sigma) in dimethylsulfoxide (DMSO)
Store <3 months at −80°C

Lovastatin, activated, 10 mM

Dissolve 52 mg lovastatin (Merck, Sharp, and Dohme Research Pharmaceuticals) in 1.04 ml of 95% ethanol. Add 813 µl 1 N NaOH. Adjust to pH 7.2 with 1 M HCl and dilute to 13 ml (4 mg/ml; 10 mM) with sterile-filtered, deionized H_2O or medium. Store <1 month at −20°C.

This preparation is "activated" lovastatin, which is ready for use.

Mimosine, 100 mM

100 mM L-mimosine (Sigma) in PBS, pH 7.4 (*APPENDIX 2A*)
Store <2 months at 4°C

Nocodazole, 4 mg/ml

DMSO in 4 mg/ml nocodazole (Aldrich)
Store <6 months at −20°C or <1 month at 4°C

Thymidine, 100 mM

100 mM thymidine (Sigma) in PBS, pH 7.4 (*APPENDIX 2A*)
Sterile filter or autoclave and store <3 months at −20°C or −80°C

Trypsin, 0.5×

Dilute 1× trypsin (Life Technologies) 1:1 with 50 ml 1× HBSS (*APPENDIX 2A*) supplemented with 1.25 ml of 0.5 M disodium EDTA and 1 ml of 0.5 M HEPES, pH 7.2. Store <3 months at −20°C or <1 week at 4°C.

COMMENTARY

Background Information

An essential task in the study of cell cycle–related processes is synchronization of cells into specific cell cycle phases. Synchronization improves the conditions by which an actual process under scrutiny can be studied and helps link the process to a particular cell cycle–phase transition. This unit provides methods that have proven effective in synchronizing mammalian cells into defined cell cycle phases. The advantages and disadvantages of each procedure are discussed and some key situations where such procedures have been employed successfully in the biochemical dissection of cell cycle–control processes are described. This unit focuses on the use of chemicals or growth-restriction conditions to synchronize exponentially growing cells.

An additional method for obtaining enriched populations of phase-specific cells, using centrifugal elutriation to obtain pure populations of cells, has been omitted from this unit. Centrifugal elutriation uses mechanical separation to isolate cells in different cell cycle phases. Centrifugal elutriation is often a superior method of synchronization: it allows one to obtain pure populations of cells without introducing potential artifacts of imbalanced growth, since no chemicals are employed and no transient arrests are induced. Its expense and specific requirements for optimization of the elutriator preclude the use of this methodology in most laboratories performing occasional synchronizations or just getting started in methods of cell cycle analysis. This method will, however, be described in a future unit.

Critical Parameters and Troubleshooting

The goal of cell synchronization is to create a completely synchronized population of cells that progress uniformly through the cell cycle. Achieving this task requires that (1) the cells be completely arrested at one defined point in the cell cycle, (2) the arrest procedure be completely reversible, and (3) the released cells recover and progress uniformly through the cell cycle. In the practical sense, the procedures currently available for cell synchronization aim towards, but do not entirely meet, these requirements. Problems arise in the degree of arrest obtainable and the uniformity with which the released population progresses through the cell cycle. For a cell line not yet characterized for synchronization, one may have to experiment with several different procedures to find the most suitable technique.

Mitotic shake-off: If cell lines are poorly adherent, synchrony obtained by this shake-off will be poor as well. Therefore, the time of trypsinization or the concentration of trypsin should be reduced. Care should be taken with more fragile cell lines not to damage their membranes during manipulation of cells, which might prevent their reattachment or reentry into the cell cycle.

Poorly growing cultures or cultures that are not in logarithmic growth will produce lower yields of cells than fresh, rapidly dividing cultures. If too few cells are obtained by this method despite optimal cell growth, removal of unattached cells to enhance synchrony should be omitted in favor of obtaining larger numbers of cells. Alternatively, this technique can be combined with nocodazole to enhance the number of cells and purity of the synchronized population.

Nocodazole: If an increase in cell number is not observed or a decrease in cell number is observed following release from nocodazole, the concentration of the drug is most likely too high, resulting in an irreversible block in G_2/M phase or causing cells to die. Reducing the concentration should alleviate this problem.

Increasing the concentration of nocodazole should prevent any leakiness of the G_2/M-phase block. In cell lines with doubling times >24 hr, 16 hr may not be sufficient time to accumulate the bulk of the cells in G_2/M. Caution should be used when extending the time of nocodazole

exposure, as longer exposure times may permit all cells to exit G_1 and S phases, but may be toxic to cells held for prolonged periods of time in G_2 and M phases. Finally, a number of tumor cell lines are defective in their ability to arrest at G_2/M. Nocodazole will not be an effective synchronizing agent for these checkpoint-defective cell lines.

Serum starvation: A range of reduced serum concentrations are effective (0.1% works well for most cell lines). Serum withdrawal can also be accomplished by complete withdrawal (0% serum) or performed with 1% serum, depending on the cell line. The optimal concentration must be determined for each cell line. In addition, plating cells too densely interferes with the ability of cells to fully arrest. Densely plated cells arrest as a result of confluence rather than nutritional depletion. If cells are overgrown following removal of serum, cells should be plated at a lower density prior to initiating serum withdrawal.

Amino acid depletion: Insufficient washing of the cells early in amino acid starvation will reduce the ability of the cells to become uniformly synchronized. It is very important to use dialyzed (not charcoal-treated) FBS rather than reduced serum concentrations of standard serum. Keeping cells below confluence is also important during amino acid depletion, as excess cell numbers can prevent maximum growth of synchronized cultures.

Low numbers of synchronized cells can result from toxicity of amino acid withdrawal. The unfortunate side effect of toxicity is that dying cells can provide a source of isoleucine, which will further reduce the quality of the synchrony. Toxicity can result if cells are exposed to amino acid–deficient medium for two or three cell cycles. Extended withdrawal times may also cause cells to remain in G_1 as statically arrested cells rather than transiently blocked cells.

Lovastatin: The results of synchrony obtained with lovastatin have shown some variation among cell types. Several fibroblastic cell lines have demonstrated strong G_1-arrest characteristics in the presence of lovastatin, while other tumor cell lines have demonstrated a persistent arrest in both G_1 and G_2. In those cases where cells show a significant G_2 rather than G_1 population of cells in the presence of lovastatin, it would be best to select another method of synchronization.

Mimosine: As mentioned previously, the site and mode of action of mimosine arrest has not been clearly defined. Therefore, it is not clear that mimosine inhibits cells from progressing into S phase or inhibits the progression of cells through S phase (Kalejta and Hamlin, 1997). If mimosine does, in fact, have two sites of action in the cell cycle, then a single mimosine treatment may produce populations contaminated with cells trapped in S phase. For this reason, it may be prudent to use mimosine as an adjunct to G_0/G_1-synchronizing protocols to obtain pure, late G_1/S–synchronized cultures.

Double-thymidine block: Timing is of the essence with this technique! With the procedure outlined in Basic Protocol 4, the leading-edge cells will be at the G_1/S phase border of the next cell cycle at the end of the sixteenth hour, while the lagging edge of cells will be in G_2/M at the end of the sixteenth hour. If thymidine is added too quickly, cells at the end of S phase will become trapped until released following the second thymidine block. If thymidine is added too late, cells already in S phase will become trapped in S phase during the block and may interfere with detection of early S-phase events.

Mitotic index: This is a relatively straightforward technique with few steps that require optimization. Gentle handling of the cells following resuspension and fixation with 3:1 ethanol/glacial acetic acid will improve cell yields and cell morphology.

[^{3}H]thymidine incorporation into DNA: Insufficient cell numbers, poorly growing cultures, and use of medium with a large pool of cold thymidine can all lead to poor labeling of DNA. Also insufficient washing of cultures synchronized by double-thymidine exposure can lead to a large pool of excess cold thymidine, which reduces the relative amount of [^{3}H]thymidine. If cell number is the problem, precipitating materials from increasing numbers of cells should demonstrate an optimum cell concentration for detection of labeled DNA. Alternatively, if cell numbers are limiting, the addition of 1 µg of cold carrier DNA or RNA to cell lysates may improve the recovery of labeled DNA. Poorly growing cultures are detectable by their reduced cell cycle time and failure to increase logarithmically in cell number with time. If the required medium for cell growth (e.g., some hematopoietic cell lines require the addition of hypoxanthine/thymidine to the medium), the medium used for labeling cells can be substituted for another medium with lower thymidine concentration (e.g., DMEM) during the 30-min labeling period with little or no effect on cell replication.

The lack of increase in DNA synthesis above background can be the result of insufficient washing of TCA precipitates, poor or ineffec-

tive synchronization of cells, or failure of cells to re-enter the cell cycle.

Failure of cells to re-enter the cell cycle can be determined by monitoring cell numbers. In cases where synchrony is poor, the cell number will increase uniformly rather than punctately, as occurs with synchronized cultures. Thorough and complete washing of cell precipitates is critical to the success and reproducibility of this protocol.

Anticipated Results

Mitotic shake-off: Cells obtained by mitotic shake-off should be enriched in G_2/M phase by at least 75%. Contamination of cells in M with cells in other stages is most often related to the stringency of trypsin treatment or rigor of shaking or tapping of the cultures. Pure populations of G_1 cells can be gained using the mitotic shake-off technique by placing the mitotic cells in a flask and incubating them for 2 hr at 37°C. The mitotic cells will re-attach to the flasks and flatten out as they enter G_1 phase. Mitotic laggards can be removed from the flask by gently swirling the medium over the attached cells and then decanting the medium. The remaining attached cells will consist of primarily G_1 cells, which can be confirmed by flow cytometry.

Nocodazole: It is possible to obtain nearly pure populations of G_2/M-phase cells (up to 95% with CA46 cells) by using nocodazole. It is necessary to confirm that the cell cycle block has been reversed by monitoring cell number. Nocodazole is generally used at 10 to 400 ng/ml (final concentration). Excessive concentrations or prolonged incubation times can be toxic. The authors have found that nocodazole-arrested lymphoma cells do not display the characteristic proportional increase in cell volume that is observed with other types of G_2-arrest agents, when evaluated using a Coulter counter and channelzyer. Therefore, methods that monitors changes in cell volume should be not be used to evaluate the effectiveness of nocodazole arrest.

Serum starvation: Over 90% of the population should be in G_0/G_1 following serum starvation. By 12 to 16 hr after addition of serum-containing medium, 40% to 60% of the population should enter S phase in a parasynchronous wave. Peak synthesis of DNA, as measured by [^{3}H]thymidine incorporation, should occur by 16 hr after restimulation.

Amino acid depletion: With this technique, >90% of CA46 cells will arrest in G_1 after two cell cycles. Following release into the cycle, >80% of these cells will re-enter the cell cycle and progress into S phase. The quality of the synchronization depends on obtaining maximal replication in cells prior to depletion of amino acids and thorough washing of cells prior to resuspension in isoleucine-free medium. Conditions that lead to extensive cell death during the procedure may interfere with this procedure, since lysed cells can act as a significant source of free isoleucine. Depending on the cell type, cells will enter S phase as a synchronous or parasynchronous wave within 8 to 12 hr. Starvation methods are useful when combined with other types of synchronizing agents, in particular, release from starvation in the presence of aphidicolin or mimosine to enrich for G_1/S cells (Heintz and Hamlin, 1982, Burhans et al., 1990, Dijkwel and Hamlin, 1992).

Lovastatin: Up to 80% reversible sequestration of cells in G_1 phase has been seen with the use of lovastatin (Wilcken et al., 1997). It is important to note that recovery from lovastatin does prolong the G_1 phase of many cell types (Keyomarsi et al., 1991).

Mimosine: Synchronization with mimosine produces late G_1/S populations of >70% purity. These cells initiate DNA synthesis <30 min after release from mimosine arrest.

Double-thymidine block: Cell populations treated in this manner can often be enriched to contain >65%, and sometimes as much as 80%, of the cells in S phase. The real trick to this procedure is to prevent the leading-edge cells from entering S phase of the next cell cycle while ensuring the lagging-edge cells have left S phase of the previous cell cycle. Most cells will progress into S phase within 1 to 2 hr following release from excess thymidine.

Mitotic index: As cells enter M phase, the number and percentage of mitotic figures observed in the cultures will increase.

[^{3}H]Thymidine incorporation: Peak DNA synthesis as reflected by an increase of at least 3-fold over background represents the point in time when the maximum number of cells are in S phase. The increase in peak DNA synthesis can be substantially >5-fold over background, depending on the culture conditions and the degree of synchrony obtained.

Time Considerations

Because these methods involve manipulations of cultures of cells, they required one or more days, although the hands-on time for any one day may be limited. For an estimate of the total time required for each synchrony protocol see Figure 8.3.2.

Literature Cited

Bonapace, I.M., Addeo, R., Altucci, L., Cicatiello, L., Bifulco, M., Laezza, C., Salzano, S., Sica, V., Bresciani, F., and Weisz, A. 1996. 17 beta-Estradiol overcomes a G_1 block induced by HMG-CoA reductase inhibitors and fosters cell cycle progression without inducing ERK-1 and -2 MAP kinase activation. *Oncogene* 12:753-763.

Bootsma, D., Budke, L., and Vos, O. 1964. Studies on synchronized division of tissue culture cells initiated by excess thymidine. *Exp. Cell Res.* 33:301-304.

Brandeis, M. and Hunt, T. 1996. The proteolysis of mitotic cyclins in mammalian cells persists from the end of mitosis until the onset of S phase. *EMBO J.* 15:5280-5289.

Buckley, M.F., Sweeney, K.J., Hamilton, J.A., Sini, R.L., Manning, D.L., Nicholson, R.I., deFazio, A., Watts, C.K., Musgrove, E.A., and Sutherland, R.L. 1993. Expression and amplification of cyclin genes in human breast cancer. *Oncogene* 8:2127-2133.

Burhans, W.C., Selegae, J.E., and Heinz, H.H. 1986. Isolation of the origin of replication associated with the amplified Chinese hamster dihydrofolate reductase domain. *Proc. Natl. Acad. Sci. U.S.A.* 83:7790-7794.

Burhans, W.C., Vasselev, L.T., Caddle, M.S., Heintz, N.H., and DePamphilis, M.D. 1990. Identification of an origin of bi-directional replication in mammalian chromosomes. *Cell* 62:955-965.

Campisi, J., Morreo, G., and Pardee, A.B. 1984. Kinetics of G_1 transit following brief starvation for serum factors. *Exp. Cell Res.* 152:459-462.

Carbonaro-Hall, D., Williams, R., Wu, L., Warburton, D., Zeichner-David, M., MacDougall, M., Tolo, V., and Hall, F. 1993. G_1 expression and multistage dynamics of cyclin A in human osteosarcoma cells. *Oncogene* 8:1649-1659.

Dbaibo, G.S., Pushkareva, M.Y., Jayadev, S., Schwarz, J.K., Horowitz, J.M., Obeid, L.M., and Hannun, Y.A. 1995. Retinoblastoma gene product as a downstream target for a ceramide-dependent pathway of growth arrest. *Proc. Natl. Acad. Sci. U.S.A.* 92:1347-1351.

Dijkwel, P.A. and Hamlin, J.L. 1992. Initiation of DNA replication in the DHFR locus is confined to the early S period in CHO cells synchronized with the plant amino acid mimosine. *Mol. Cell Biol.* 12:3715-3722.

Elvin, P. and Evans, C.W. 1983. Cell adhesiveness and the cell cycle: Correlation in synchronized Balb/c 3T3. *Biol. Cell* 48:1-9.

Gilbert, D.M., Neilson, A., Miyazawa, H., DePhamphilis, M.L., and Burhans, W.C. 1995. Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. *J. Biol. Chem.* 270:9597-9696.

Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N.J.C. 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* 67:1169-1179.

Gong, J., Traganos, F., and Darzynkiewicz, Z. 1995. Growth imbalance and altered expression of cyclins B1, A, E, D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. *Cell Growth Differ.* 6:1485-1493.

Hartwell, L.H. and Kastan, M.B. 1994. Cell cycle control and cancer. *Science* 266:1821-1828.

Heintz, N.H. and Hamlin, J.L. 1982. An amplified chromosomal sequence that includes the gene for DHFR initiates replication within specific restriction fragments. *Proc. Natl. Acad. Sci. U.S.A.* 79:4083-4087.

Heintz, N., Sive, H.L., and Roeder, R.G. 1983. Regulation of human histone gene expression: Kinetics of accumulation and changes in the rate of synthesis and the half-lives of individual histone mRNAs during the HeLa cell cycle. *Mol. Cell. Biol.* 3:539-550.

Hoshiya, Y., Kubota, T., Matsuzaki, S.W., Kitajima, M., and Hoffman, R.M. 1996. Methionine starvation modulates the efficacy of cisplatin on human breast cancer in nude mice. *Anticancer Res.* 16:3515-3517.

Hunter, T. and Pines, J. 1994. Cyclins and cancer. II. Cyclin D and CDK inhibitors come of age. *Cell* 79:573-582.

Jackman, J., Alamo, I., and Foranace, A.J. 1994. Genotoxic stress confers preferential and coordinate messenger RNA stability on the five gadd genes. *Cancer Res.* 54:5656-5662.

Jakobisiak, M., Bruno, S., Skierski, and Darzynkiewicz, Z. 1991. Cell cycle–specific effects of lovastatin. *Proc. Natl. Acad. Sci. U.S.A.* 88:3628-3632.

Kalejta, R.F. and Hamlin, J.L. 1997. The dual effects of mimosine on DNA replication. *Exp. Cell Res.* 231:173-183.

Keyomarsi, K. and Pardee, A.B. 1993. Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 90:1112-1116.

Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A.B. 1991. Synchronization of tumor and normal cells from G_1 to multiple cell cycles by lovastatin. *Cancer Res.* 51:3602-3609.

Lalande, M. 1990. A reversible arrest point in late G_1 phase of the mammalian cell cycle. *Exp. Cell Res.* 186:332-339.

Leach, F.S., Elledge, S.J., Sherr, C.J., Wilson, J.K., Markowitz, S., Kinzler, K.W., and Vogelstein, B. 1993. Amplification of cyclin genes in colorectal carcinomas. *Cancer Res.* 53:1986-1989.

Martel, J., Payet, M.D., and Dupris, G. 1997. The MDR1 (P-glycoprotein) and MRP (P-190) transporters do not play a major role in the intrinsic multiple drug resistance of Jurkat T lymphocytes. *Leuk. Res.* 21:743-752.

Minshull, J., Pines, J., Golsteyn, R., Standart, N., Mackie, S., Colman, A., Blow, J., Ruderman, J.V., Wu, M., and Hunt, T. 1989. The role of cyclin synthesis, modification and destruction in the control of cell division. *J. Cell Sci.* (Suppl.) 12:77-97.

Morla, A.O., Draetta, G., Beach, D., and Wang, J.Y.J. 1989. Reversible tyrosine phosphorylation of cdc2: Dephosphorylation accompanies activation during entry into mitosis. *Cell* 58:193-203.

Mosca, P.J., Dijkwel, P.A., and Hamlin, J.L. 1992. The plant amino acid mimosine may inhibit initiation of origins of replication in CHO cells. *Mol. Cell. Biol.* 12:4375-4383.

O'Connor, P.M., Ferris, D.K., Pagano, M., Draetta, G., Pines, J., Hunter, T., Longo, D.L., and Kohn, K.W. 1993. G_2 delay induced by nitrogen mustard in human cells affects cyclin A–CDK2 and cyclin B1–cdc2 complexes differently. *J. Biol. Chem.* 268:8298-8308.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. 1992. Cyclin A is required at two points in the human cell cycle. *EMBO J.* 11:961-971.

Pardee, A.B. 1974. A restriction point for control of normal animal cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.* 71:1286-1290.

Pardee, A.B. 1989. G_1 events and regulation of cell proliferation. *Science* 246:603-608.

Pines, J. 1995. Cyclins and cyclin-dependent kinases: Theme and variations. *Adv. Cancer Res.* 66:181-212.

Pines, J. and Hunter, T. 1989. Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* 58:833-846.

Puri, P.L., Balsano, C., Purgio, V.L., Chirillo, P., Natoli, G., Ricci, L., Mattei, E., Graessmann, A., and Levrero, M. 1997. MyoD prevents cyclin A-CDK2-containing E2F complexes formation in terminally differentiated myocytes. *Oncogene* 14:1171-1184.

Sallot, M., Ordener, C., Lascombe, I., Propper, A., Adessi, G.L., and Jouvenot, M. 1996. Differential EGF action on nuclear protooncogenes in human endometrial carcinoma RL95-2 cells. *Anticancer Res.* 16:401-406.

Schimke, R.T., Kung, A.L., Rush, D.F., and Sherwood, S.W. 1991. Differences in mitotic control among mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* 56:417-425.

Stein, G.S. and Borun, T.W. 1972. The synthesis of acidic chromosomal proteins during the cell cycle of HeLa cells. *J. Cell Biol.* 52:292-296.

Tesfaigzi, J. and Carlson, D.M. 1996. Cell cycle–specific expression of G(0)SPR1 in Chinese hamster ovary cells. *Exp. Cell Res.* 228:227-282.

Thomas, D.B. and Lingwood, C.A. 1975. A model of cell cycle control: Effects of thymidine on synchronous cell cultures. *Cell* 5:37-42.

Tobey, R.A. and Crissman, H.A. 1972. Preparation of large quantities of synchronized cells in late G_1 in the pre-DNA replicative phase of the cell cycle. *Exp. Cell Res.* 75:460-464.

Watson, P.A., Hanauke-Abel, H.H., Flint, A., and Lalande, M. 1991. Mimosine reversibly arrests cell cycle progression at the G_1/S-phase border. *Cytometry* 12:242-246.

Wejde, J., Carlberg, M., Hjertman, M., and Larrson, O. 1993. Isoprenoid regulation of cell growth: Identification of mevalonate-labelled compounds inducing DNA synthesis in human breast cancer cells depleted of serum and mevalonate. *J. Cell. Physiol.* 155:539-548.

Wilcken, N.R.C., Musgrove, E.A., and Sutherland, R.L. 1997. Different points of action of retinoids and anti-estrogens in G_1 phase identified in synchronized T-47D breast cancer cells. *Int. J. Cancer* 70:291-296.

Zwanenburg, T.S. 1983. Standardized shake-off to synchronize cultured CHO cells. *Mutat. Res.* 120:151-159.

Contributed by Joany Jackman
U.S.A.M.R.I.I.D.
Fort Dietrick, Maryland

Patrick M. O'Connor
Agouron Pharmaceuticals
San Diego, California

Determining Cell Cycle Stages by Flow Cytometry

The most common approach to determining the cell cycle stage is based on measurement of cellular DNA content. This allows one to discriminate between cells in the $G_{0/1}$ versus the S versus the G_2/M phases of the cell cycle (Fig. 8.4.1). DNA is generally stained with a fluorescent dye and cellular fluorescence is measured by flow, image, or laser scanning cytometry (Crissman and Steinkamp, 1990). A variety of fluorochromes can be used for DNA staining. The spectral properties of the dyes most frequently used for this purpose are presented in Figure 8.4.2. Following cell staining with one of these dyes, the intensity of fluorescence integrated over the analyzed cell is expected to be in stoichiometric relationship to DNA content, and thereby can be used to determine the cell cycle stage. Deconvolution of the DNA content frequency histograms, usually done using special computer software (Rabinovitch, 1994), reveals the percentage of cells residing in the respective phases of the cell cycle.

A plethora of techniques for DNA content measurement utilizing the fluorochromes depicted in Figure 8.4.2 have been published during the past two decades. Description of many methods, which are applicable to different cell systems including eukaryotes and prokaryotes, as well as to clinical samples, can be found in a separate monograph (Darzynkiewicz et al., 1994). The techniques differ primarily according to the mode of cell permeabilization (detergent versus fixation with alcohols) and composition of the stain solution. Four types of procedures are described in this unit. Relatively simple and rather universally applicable methods for staining ethanol-fixed cells are presented in Basic Protocol 1 and Alternate Protocol 1. Because cells may be stored in the fixative for extended periods of time and/or transported while in the fixative, these methods allow one to prepare and collect the cells without regard to the timing of their analysis. The methods presented utilize the two most commonly used DNA fluorochromes, propidium iodide (PI; see Basic Protocol 1) and 4′,6-diamidino-2-phenylindole (DAPI; see Alternate Protocol 1). The DAPI staining procedure, which does not require incubation with RNase A, is simpler and more rapid as compared to the one using PI. However, it requires a flow cytometer equipped with a UV excitation source.

The methods utilizing detergent to permeabilize cells are presented in Basic Protocol 2 and Alternate Protocol 2. These methods provide more accurate estimates of the DNA content, and therefore better discrimination of the cell-cycle phases as compared to measurement of fixed cells. This is due to the fact that exposure of live cells to detergents results in rupture of the plasma membrane and elimination of the cytoplasmic constituents, which contain components that are autofluorescent or that nonspecifically interact with DNA fluorochromes. Isolated nuclei are then stained rather than whole cells. It should be stressed that analysis of the detergent-treated cells may lead to an underestimation of M cells. Lacking a nuclear envelope, these cells may totally disintegrate into chromosomes or chromosome aggregates. In Basic Protocol 2 cells are stained with PI; in Alternate Protocol 2 they are stained with DAPI. As in the case of fixed cells, staining with of detergent-permeabilized cells with DAPI does not require incubations with RNase A. It should be stressed that fluorochromes other than PI or DAPI (Fig. 8.4.2) can be used in these protocols, provided that their fluorescence is excited and measured at appropriate wavelengths, as indicated in the figure.

A different approach is used for cell cycle analysis of live cells (see Basic Protocol 3). The main application of this method is for cell sorting, where cells that have been selected on the basis of their DNA content (cell cycle phase) can be cultured for analysis of their

Contributed by Zbigniew Darzynkiewicz, Gloria Juan, and Elzbieta Bedner
Current Protocols in Cell Biology (1999) 8.4.1-8.4.18

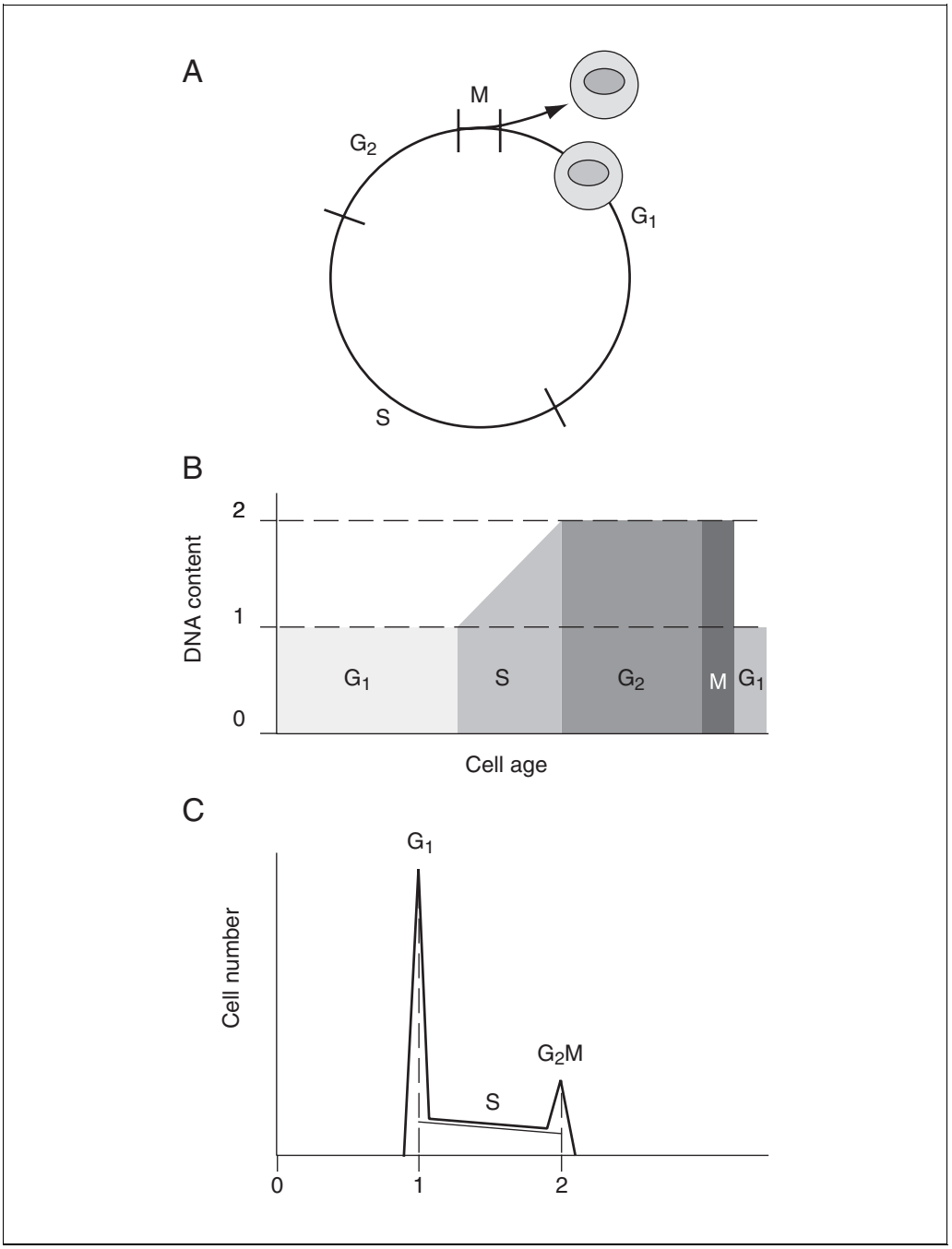


Figure 8.4.1 Relationship between DNA content and the cell cycle. (**A**) Stages of the cell cycle. (**B**) Estimation of cell position in the cell cycle based on DNA content measurement. Content of cellular DNA doubles during S, and therefore the cell age during S can be estimated based on the amount of replicated DNA (increase in DNA content). On the other hand, cells in G_1 and G_2/M are uniform with respect to DNA content, which is equivalent to the DNA ploidy index (DI) 1.0 (for G_1) and 2.0 (for G_2/M). (**C**) If DNA content could be measured with absolute accuracy, based on DNA-specific fluorescence, the G_1 and G_2/M cells would have uniform fluorescence values and be represented on the frequency histograms as the bars of a single channel width (dashed lines). Due to inaccuracy in DNA-content measurement, the actual data are in the form of G_1 and G_2/M peaks. Percentage of cells represented by these peaks, and in the S phase, are estimated by deconvolution of the histograms, using a variety of mathematical techniques (Bagwell, 1993; Rabinovitch, 1994). The interactive software based on these techniques deconvolutes the histograms and is available from several sources (see Background Information).

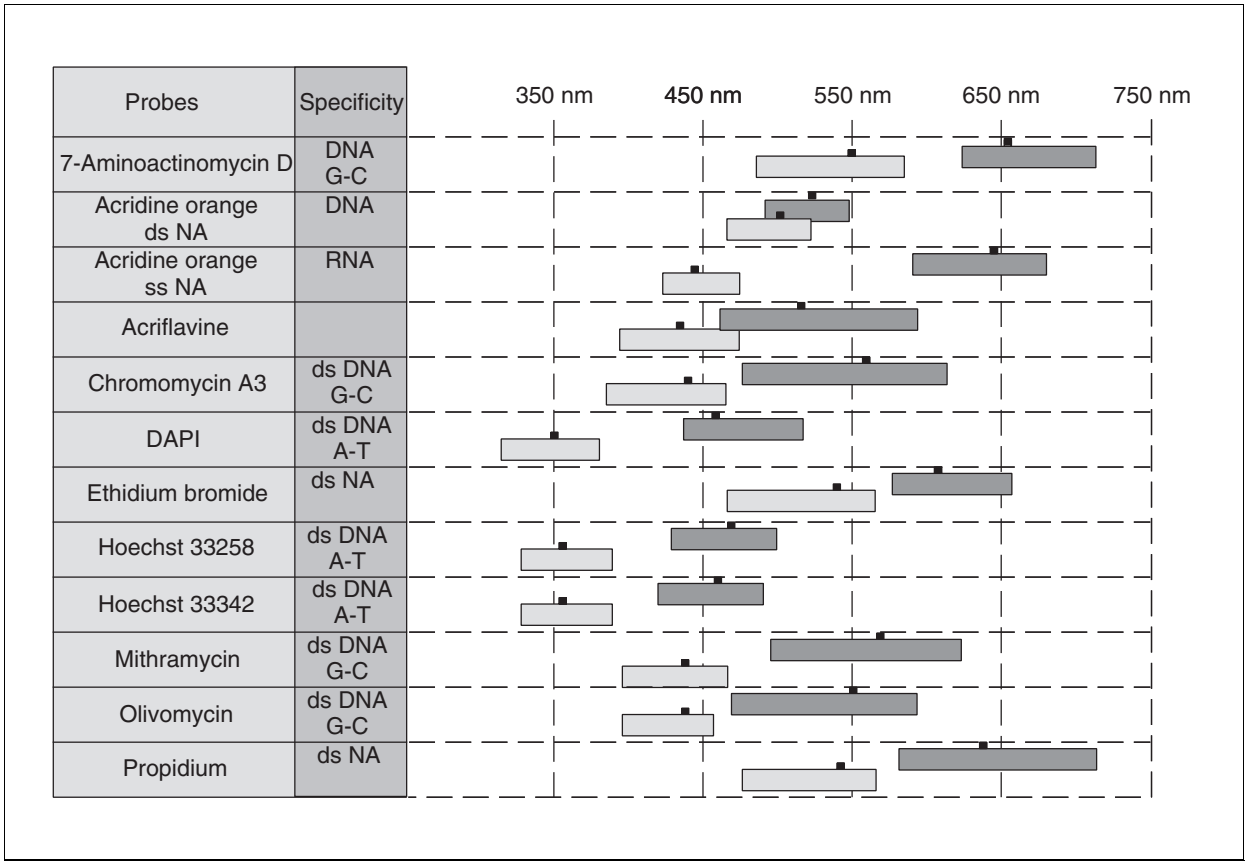


Figure 8.4.2 Fluorescence excitation (shaded bars) and emission (solid black bars) wavelengths of the most commonly used DNA fluorochromes. Abbreviations: ds, double-stranded; ss, single-stranded; NA, nucleic acid.

growth characteristics or sensitivity to drugs or for purposes of cloning or expansion. The method presented in Basic Protocol 3 is simple, based on cell staining with Hoechst 33242 fluorochrome, which, unlike PI or DAPI, is able to penetrate through the plasma membrane and stain DNA in live cells.

The final procedure combines analysis of cellular DNA content with expression of cyclins D, E, A, or B1. Cyclins are the key components of the cell cycle progression machinery and are expressed discontinuously during the cell cycle (see Background Information). Thus, the presence of a particular cyclin detected immunocytochemically within the cell, provides an additional marker of the cell cycle position. Simultaneous bivariate analysis of DNA content and expression of cyclins (Basic Protocol 4) makes it possible to distinguish additional stages of the cell cycle, which cannot be identified by analysis of DNA content alone (Darzynkiewicz et al*.,* 1996).

CELL CYCLE ANALYSIS OF FIXED CELLS STAINED WITH PROPIDIUM IODIDE

BASIC PROTOCOL 1

In this protocol, ethanol is used to fix and permeabilize cells to make them accessible to propidium iodide (PI). As mentioned above, the fixation step makes this protocol applicable in instances when samples have to be stored or transported before analysis. Following fixation, the cells are rinsed with PBS and stained with PI in a solution containing Triton X-100 and RNase A. Triton X-100 additionally permeabilizes the cells, and to some extent decreases cell loss resulting from electrostatic cell attachment to tubes. Because double-stranded sections of RNA also stain with PI, RNase A is included to digest these sections and thereby to increase specificity of DNA staining.

Materials

Cells to be stained
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fixative: 70% ethanol
Propidium iodide staining solution I (see recipe)

Low-speed centrifuge
12 × 75–mm centrifuge tubes, preferably polypropylene or silanized
Flow cytometer with 488-nm argon-ion laser fluorescence excitation source
Software to deconvolute cellular DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems)

Additional reagents and equipments for counting and trypsinizing cells (*UNIT 1.1*)

Prepare cell suspension for fixation

1a. *For cells growing in suspension or hematologic samples:* Rinse cells once by centrifuging 6 min at 200 × *g*, room temperature, with PBS. Count cells (*UNIT 1.1*) and thoroughly resuspend 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

1b. *For cells growing attached to tissue culture dishes:* Collect cells from flasks or petri dishes by trypsinization (*UNIT 1.1*) and pool the trypsinized cells with the cells floating in the medium (the latter consist of detached mitotic, apoptotic, and dead cells). Centrifuge the cell suspension 6 min at 200 × *g*, room temperature. Remove the supernatant and resuspend the pellet in medium containing serum (to inactivate trypsin), then centrifuge again and remove the supernatant. Count cells (*UNIT 1.1*) and thoroughly resuspend 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

Other means of trypsin inactivation, such as addition of protease inhibitors, may also be used.

1c. *For cells isolated from tissues (e.g., tumors):* Rinse free of any enzyme used for cell dissociation using the centrifugation technique described in step 1a, above, and thoroughly resuspend (well dispersed, not in aggregates) 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

Fix cells in ethanol

2. Prepare for fixation by adding 4.5 ml of 70% ethanol fixative to each of an appropriate number of 12 × 75-mm centrifuge tubes. Keep tubes on ice.

3. Using a Pasteur pipet, transfer 0.5-ml aliquots of cell suspensions prepared as in step 1a, 1b, or 1c into the appropriate tubes containing the cold 70% ethanol fixative and keep cells in fixative ≥2 hr on ice.

It is important to achieve a single-cell suspension. Fixation of cells that are in aggregates while suspended in PBS stabilizes the aggregates, which then become impossible to disperse. It is essential, therefore, to have a monodisperse cell suspension at the time that cells are mixed with ethanol.

Cells suspended in 70% ethanol can be stored at 0° to 4°C for several months if not years.

Stain cells with PI

4. Centrifuge the ethanol-suspended cells 5 min at 200 × *g*. Decant ethanol thoroughly.

5. Suspend the cell pellet in 5 ml PBS, wait 60 sec, then centrifuge again as in step 4. Decant supernatant

6. Resuspend cell pellet in 1 ml of propidium iodide staining solution I. Incubate either 15 min at 37°C or 30 min at room temperature.

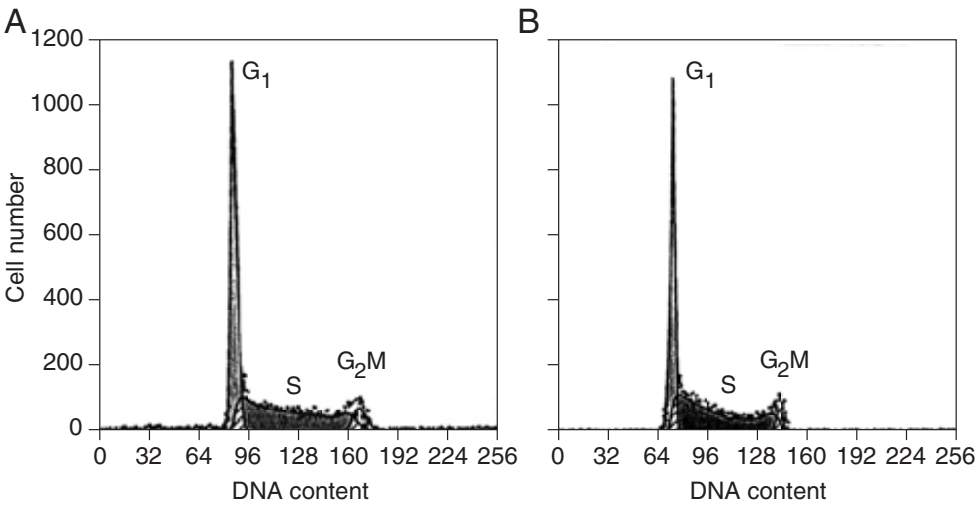


Figure 8.4.3 (**A**) DNA content frequency histograms of fixed HL-60 cells stained with DAPI (Alternate Protocol 1) and (**B**) detergent-permeabilized HL-60 cells stained with DAPI (Alternate Protocol 2). The histograms were deconvoluted using Multicycle software (Phoenix Flow Systems).

Measure cell fluorescence by flow cytometry

7. Set up and adjust flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

   For excitation, the 488-nm argon-ion laser line may be used. Alternatively use a BG 12 optical filter when the source of illumination is a mercury-arc or xenon lamp. A long-pass (>620-nm) emission filter is recommended.

8. Measure cell fluorescence by flow cytometry. Use pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using DNA content histogram deconvolution software (e.g., Multicycle from Phoenix Flow Systems).

CELL CYCLE ANALYSIS OF FIXED CELLS STAINED WITH DAPI

ALTERNATE PROTOCOL 1

This protocol is similar to Basic Protocol 1, except the cells are stained with DAPI rather than PI. Because DAPI does not stain RNA, there is no need to treat the cells with RNase A. Excitation of DAPI, however, requires UV light source, which is not universally available. Emission of DAPI is measured at blue wavelengths.

Additional Materials *(also see Basic Protocol 1)*

DAPI staining solution I (see recipe)
Flow cytometer with UV illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Collect cells, fix in 70% ethanol, and wash (see Basic Protocol 1, steps 1 to 5).
2. Resuspend cell pellet in 1 ml DAPI staining solution. Incubate 30 min in the dark at room temperature.
3. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI emission at blue wavelengths.

   For excitation, an UG-1 optical filter (short-pass, 390 nm) may be used when the source of excitation is a mercury-arc or xenon lamp. For detection of DAPI emission a band-pass filter at 470 ± 20 nm is recommended.

4. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

BASIC PROTOCOL 2

CELL CYCLE ANALYSIS OF UNFIXED, DETERGENT-PERMEABILIZED CELLS STAINED WITH PI

In this protocol the cells are lysed with detergent to aid in the staining of DNA for flow cytometric analysis. The cells in suspension are mixed with staining solution that contains Triton X-100, PI, and RNase A. DNA content of the stained nuclei is then measured by flow cytometry, using excitation with blue light. Because there is no fixation step, the procedure is simpler and more rapid, and because there are fewer centrifugations, there is less cell loss as compared to Basic Protocol 1

Materials

Cells to be stained: 1×10^6 to 5×10^6 cells/ml suspended in PBS (*APPENDIX 2A*) or culture medium
Propidium iodide staining solution II (see recipe)

Flow cytometer with 488-nm argon-ion laser fluorescence excitation source
Software to deconvolute cellular DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems)

1. Mix 0.2 ml of cell suspension with 2 ml of propidium iodide staining solution II. Incubate 20 min at room temperature.
2. Set up and adjust flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

   For excitation, the 488-nm argon-ion laser line may be used. Alternatively use a BG 12 optical filter when the source of illumination is a mercury-arc or xenon lamp. A long-pass (>620-nm) emission filter is recommended.

3. Measure cell fluorescence by flow cytometry. Use pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using DNA content histogram deconvolution software (e.g., Multicycle from Phoenix Flow Systems).

ALTERNATE PROTOCOL 2

CELL CYCLE ANALYSIS OF UNFIXED, DETERGENT-PERMEABILIZED CELLS STAINED WITH DAPI

This protocol is essentially identical to Basic Protocol 2 except that the cells are stained with DAPI. As mentioned previously, there is no need for incubation with RNase A, which simplifies the protocol, but the procedure requires a flow cytometer equipped with UV light excitation source.

Additional Materials (also see Basic Protocol 2)

DAPI staining solution II (see recipe)
Flow cytometer with UV-illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Mix 0.2 ml of cell suspension (1×10^5 to 1×10^6 cells suspended in PBS or culture medium) with 2 ml of DAPI staining solution II. Incubate 10 min at room temperature.
2. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI emission at blue wavelengths

For excitation, an UG-1 optical filter (short-pass, 390 nm) may be used when the source of excitation is a mercury-arc or xenon lamp. For detection of DAPI emission a band-pass filter at 470 ± 20 nm is recommended.

3. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

STAINING OF LIVE CELLS WITH HOECHST 33342

BASIC PROTOCOL 3

Supravital staining of DNA offers the possibility of sorting live cells at different phases of the cell cycle, based on differences in their DNA content. The protocol uses Hoechst 33342 fluorochrome, which stains cellular DNA without a need for cell fixation or permeabilization with detergent. The actual procedure for cell staining is simple. Cells are suspended in culture medium or PBS and incubated in the presence of 2.0 to 5.0 μg/ml of Hoechst 33342 for 20 to 90 min. Cell fluorescence is then measured directly, without any additional treatments or centrifugations. Because Hoechst 33342 is excited at UV wavelengths, the procedure requires a flow cytometer with a UV light illumination source. It should be stressed, however, that accuracy of DNA content measurement and therefore the ability to discriminate cells at different phases of the cycle, is much lower with supravital cell staining as compared to staining of ethanol-fixed or detergent-permeabilized cells.

Materials

1 mg/ml Hoechst 33342 in H_2O (store up to several weeks at 4°C in dark or foil-wrapped bottles)

Cells to be stained: 1×10^6 cells/ml suspended in PBS (*APPENDIX 2A*) or culture medium

Flow cytometer with UV light illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Add sufficient 1 mg/ml Hoechst 33342 to cells suspended in PBS or culture medium (1×10^6 cells/ml) to obtain a final fluorophore concentration of 2.0 μg/ml. Incubate 20 min at 37°C.
2. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm, and detection of Hoechst 33342 fluorescence at blue wavelengths.
3. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G_2/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

When intensity of cell fluorescence or resolution of cells in the cell-cycle phases is inadequate, prolong the staining time (up to 90 min) and/or increase Hoechst 33342 concentration in the medium (up to 5 μg/ml). The same sample may be reanalyzed after prolonged incubation and/addition of more staining solution.

This protocol is predominantly used for sorting live cells. However, because sensitivity of cells to Hoechst 33342 varies depending on the cell type (the dye also sensitizes cells to UV light), it is possible that viability and cell cycle progression of the sorted cells may be affected by the staining procedure.

BASIC PROTOCOL 4

BIVARIATE ANALYSIS OF DNA CONTENT AND EXPRESSION OF CYCLINS D, E, A, OR B1

A special category of methods for cell-cycle analysis combines measurement of DNA content and expression of the proliferation-associated proteins. The latter are detected immunocytochemically, using specific antibodies that are labeled with fluorochrome either directly, or indirectly via a secondary antibody. This protocol is devoted to cyclins, whose analysis provides an insight into the actual components of the cell-cycle progression machinery (see Background Information and *UNIT 8.2*). Because some cyclins are expressed transiently, at very specific time intervals in the cell cycle, their presence in the cell can be considered as a marker of this particular portion of the cycle. The protocol combines measurement of DNA content with expression either of one of the D-type cyclins, or cyclins E, A, or B1. Cells are fixed and labeled with anti-cyclin antibody followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody, and finally stained with PI for DNA measurement. If directly conjugated FITC-anti-cyclin antibody is available, secondary-antibody labeling (steps 8 and 9) can be omitted. This analysis is adapted to the most commonly used flow cytometers, such as those equipped with a single-laser (488-nm) illumination source.

Materials

Cells to be analyzed
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fixative: 80% ethanol or absolute methanol, −20°C.
0.25% (v/v) Triton X-100 in PBS, pH 7.4 (store at 4°C)
Rinsing buffer: 1% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4 (store at 4°C)
Anti-cyclin IgG1 antibodies: e.g., mouse monoclonal antibodies to cyclin B1 (clone GNS-1), cyclin A (clone BF-683), cyclin D1 (clone G124-326), cyclin D3 (clone G107-565), and cyclin E (clone HE12); all provided by PharMingen; antibodies to cyclin D1 may also be obtained from Immunotech/Coulter
Mouse IgG1 (isotypic control)
FITC-conjugated goat anti–mouse IgG
Propidium iodide staining solution III (see recipe)

15-ml conical tubes, polypropylene or silanized
Low-speed centrifuge
Flow cytometer equipped with 488-nm argon laser fluorescence excitation source

Additional reagents and equipment for collecting and preparing cells for fixation (see Basic Protocol 1, step 1a, b, or c)

Prepare and fix cell suspension

1. Collect cells and resuspend in PBS (see Basic Protocol 1, step 1a, b, or c).
2. Prepare for fixation by adding 10 ml of 80% ethanol or absolute methanol fixative to each of an appropriate number of 15-ml tubes. Keep tubes on ice (0° to 4°C).

   In addition to preparing a tube for each aliquot of cells to be tested with an anti-cyclin antibody, prepare an appropriate number of tubes for isotypic controls .

3. With a Pasteur pipet, transfer 1 ml of the each cell suspension into the appropriate tubes containing cold fixative. Incubate on ice.

   Time of fixation (storage) at 4°C may vary from 4 hr to several days.

   To minimize cell loss, all the subsequent steps should be done in the same tube.

Label cells with anti-cyclin primary antibody

4. Centrifuge fixed cells 5 min at 300 × *g*, room temperature. Remove alcohol, resuspend cells in 5 ml PBS, and centrifuge as before.

5. Remove supernatant and resuspend cell pellet (≤1 × 10^6 cells) in 1 ml of 0.25% Triton in PBS. Keep on ice for 5 min, then add 5 ml of PBS and centrifuge at 300 × *g* for 5 min at room temperature. Remove supernatant.

6. Dissolve each of the anti-cyclin antibodies of interest (primary antibodies) in rinsing buffer at 5 µg/ml. For each sample to be analyzed, take 100 µl of the 5 µg/ml anti-cyclin antibody solution. Also, prepare 100 µl of 5 µg/ml mouse IgG_1 (isotypic control) for each tube of control cells prepared.

7. Resuspend each cell pellet in 100 µl of rinsing buffer containing the appropriate primary antibody or isotypic control. Incubate 60 min at room temperature with gentle agitation or at 4°C overnight.

Label cells with FITC-conjugated secondary antibody

8. Add 5 ml of rinsing buffer to each tube and centrifuge 5 min at 300 × *g*, room temperature. Remove the supernatants.

9. Make a 1:30 dilution of the secondary antibody (FITC-conjugated goat anti–mouse IgG) in rinsing buffer. Resuspend each of the cell pellets in 100 µl of the diluted FITC-conjugated secondary antibody and incubate 30 min in the dark at room temperature with gentle agitation.

   If cyclin antibody that is directly conjugated to FITC is available, this protocol can be simplified by omitting steps 8 and 9.

Stain cells with PI

10. Add 5 ml of rinsing buffer to each tube and centrifuge 5 min at 300 × *g*, room temperature. Remove the supernatants

11. Resuspend each cell pellet in PI staining solution III. Incubate 20 min at room temperature in the dark before measurement.

Measure cell fluorescence by flow cytometry

12. Set up and adjust the flow cytometer for excitation with blue light (488-nm laser line). Use a 530 ± 20 nm band-pass filter for detection of FITC emission and 620-nm long-pass filter for PI emission.

13. Measure the cyclin-associated green fluorescence of FITC and DNA-associated red fluorescence of PI.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DAPI staining solution I

To 100 ml of 0.1% (v/v) Triton X-100 in PBS (*APPENDIX 2A*) add 0.1 mg 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Store up to 1 month at 4°C in the dark.

DAPI staining solution II

To 100 ml of PIPES buffer (see recipe) add 0.1 mg 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Store up to several weeks at 0° to 4°C in dark or foil-wrapped bottles.

PIPES buffer

3.02 g piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; Calbiochem; 10 mM final)
5.84 NaCl (0.1 M final)
406 mg $MgCl_2$ (2 mM final)
1 ml Triton X-100 (0.1% final)
H_2O to 1000 ml
Adjust to pH 6.8
Store up to 6 months at 0° to 4°C

Dissolve the dry ingredients and Triton X-100 in ~800 ml water, adjust pH with NaOH or HCl, and add water to 1000 ml.

Propidium iodide (PI) staining solution I

To 100 ml of 0.1% (v/v) Triton X-100 in PBS add 20 mg DNase-free RNase A and 2 mg of propidium iodide (PI; Molecular Probes). Store up to 2 weeks at 4°C in the dark.

If RNase is not DNase-free, boil a stock solution of RNase A (2 mg in 1 ml water) for 5 min, then use it to prepare the PI staining solution.

Propidium iodide (PI) staining solution II

Add 2 mg of propidium iodide (PI; Molecular Probes) to 100 ml of PIPES buffer (see recipe). Store up to several weeks in dark or foil-wrapped bottles at 0° to 4°C. Prior to use add DNase-free RNase A to 200 µg/ml final concentration.

Propidium iodide staining solution III

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing:
5 µg/ml propidium iodide (PI)
200 µg/ml DNase-free RNase A
Prepare fresh

COMMENTARY

Background Information

Univariate cellular DNA content analysis

Choice of protocol: Choosing a particular protocol from among those presented in this unit depends on the sample type (i.e., determining whether fixed or unfixed cells should be used), availability of UV light illumination in the flow cytometer (which will dictate preference for DAPI over PI), or the need to identify and quantify apoptotic cells in addition to discrimination of the cell cycle phases. In the discussion that follows, characteristics and applicability of each of the methods is presented. Because a variety of different fluorochromes can be used to stain cellular DNA, and occasionally some of them may be preferred over DAPI, PI, or Hoechst 33342, the spectral properties of the most commonly used dyes are presented in Figure 8.4.2.

In Basic Protocol 1 and Alternate Protocol 1, DNA content is measured in prefixed cell samples. The preference for analysis of fixed cells often is dictated by the need to store or transport samples. Extended storage of unfixed cells, unless done at low temperatures following suspension of the cells in cryopreservative medium, leads to cell deterioration and DNA degradation. Fixed cells, on the other hand, often can be stored for months if not years without much deterioration. The fixative essentially has two functions: (1) it preserves the cells by preventing lysis and autolytic degradation, and (2) it makes the cells permeable and hence makes DNA accessible to the fluorochrome. For DNA-content analysis, the precipitating fixatives (ethanol, methanol, and acetone) are preferred over the cross-linking agents (formaldehyde, glutaraldehyde). It should be stressed, however, that damaged DNA, especially DNA having a large number of double-strand breaks (e.g., as are present in apoptotic cells) leaks out from the ethanol-prefixed cells during hydration and subsequent staining. This allows one to identify apoptotic cells as those with fractional DNA content ("sub-G_1 cell population"). Methods for detecting apoptotic cells are reviewed in Darzynkiewicz et al. (1997).

As mentioned, a variety of DNA fluorochromes can be used to stain DNA in the prefixed cells. Staining with dyes that react with DNA and RNA, such as PI, requires incubation of cells with RNase A. The enzyme is included in the staining solution; a 30-min incubation at room temperature in that solution is adequate to remove RNA from the subsequently measured cells. Since most flow cytometers use blue light (488-nm line of the argon ion laser) as the fluorescence excitation source, PI is a useful label.

Alternate Protocols 1 and 2 employ DAPI instead of PI. One advantage of DAPI is its greater specificity towards DNA, which often is reflected in lower coefficient of variation (CV) values of the mean DNA content of G_1 cell populations. Another advantage is that it does not require incubation with RNase A. However, not all flow cytometers are equipped with a UV-light illumination source, which is needed for DAPI excitation.

The major advantage of detergent-based methods (see Basic Protocol 2 and Alternate Protocol 2) is greater accuracy in DNA-content estimates. However, because the cells are lysed by detergents, mitotic cells, which lack a nuclear envelope, may disintegrate to such an extent that individual chromosomes or chromosome clusters are measured. This usually happens when the samples are vigorously pipetted or vortexed. Therefore, one has to be cautious in interpreting the data, because mitotic cells may not be detected by methods utilizing detergents or hypotonic staining solutions. This is of special importance in instances when a large proportion of cells are in mitosis—e.g., during incubations with mitotic blockers. Furthermore, the presence of chromosomes or chromosome aggregates in the sample may contribute to an increased frequency of detection of objects with low fluorescence values, generally classified as debris or apoptotic cells ("sub-G_1" cell population, see Darzynkiewicz et al., 1997). Likewise, the lysis of apoptotic cells, which have fragmented nuclei, releases several fragments from a single cell. Each such fragment may erroneously be identified as an individual apoptotic cell.

DNA staining in live cells (see Basic Protocol 3) is generally performed in combination with cell sorting to obtain cells synchronized at particular cell cycle phases. The fluorochrome for supravital cell staining is expected to be nontoxic and not alter the cell metabolism. Such a probe has yet to be developed. Most DNA fluorochromes are charged molecules that do not adequately penetrate the plasma membrane. Some uncharged Hoechst dyes that can pass through the membrane, though at a limited rate, are exceptions. The most frequently used supravital DNA fluorochrome is Hoechst 33342. The procedure of staining with Hoechst 33342 followed by sorting appears not to induce immediate cytotoxicity (Loken, 1980). Delayed toxicity attributed to Hoechst 33342 has been observed, however, especially when the cells were treated with some antitumor drugs or radiation subsequent to staining. Hoechst dyes photosensitize cells that have BrdU incorporated into their DNA, in particular to UV light at ~300-nm wavelengths. Viability of the sorted BrdU-labeled cells, counterstained with Hoechst dyes and illuminated with UV light laser, is expected to be impaired.

The intensity of supravital cell staining with Hoechst 33342 and the resolution of DNA content (i.e., possibility of discrimination of cells in different cell-cycle phases) varies among different cell types. This variability, to a large degree, is due to a rapid efflux of the dye from the cell generated by the P glycoprotein transport pump. Cells characterized by rapid efflux mechanisms (e.g., multidrug-resistant tumor cells or stem cells) stain poorly with Hoechst 33342. It has been observed, however, that agents that may impair the efflux function (e.g., calcium channel–blocking drugs such as verapamil), at least in some cell types, improve stainability with Hoechst 33342 (Krishan, 1987).

Cell cycle and kinetic parameters: All cells in $G_{0/1}$ have a uniform DNA content, as do cells in G_2/M. Under ideal conditions of DNA staining and measurement, the fluorescence intensities of all $G_{0/1}$ and G_2/M cells are expected to be uniform, and, after digitization of the electronic signal from the photomultipler, to have uniform numerical values with G_2/M cells having twice the fluorescence of cells in $G_{0/1}$. This, however, is never the case, and on frequency histograms the $G_{0/1}$ and G_2/M populations are represented by peaks of various width because of inaccuracy of the measurement. The coefficient of variation (CV) of the mean value of DNA-associated fluorescence of the $G_{0/1}$ population is a measure of the width. The CV value, therefore, is a reflection of the inaccuracy of the DNA estimate. Because of the inaccuracy there is an overlap between early S and $G_{0/1}$ as well as between late S and G_2/M cells on the histograms. Several mathematical methods of deconvolution of the DNA content frequency histograms have been developed to estimate the

percentage of cells in the respective phases of the cell cycle (Bagwell, 1993; Rabinovitch, 1994). The goal of these techniques is to evaluate the extent of the overlap and thereby to correct for the inaccuracy of the measurement. These methods provided the basis for development of software that allows one to estimate percentage of cells in particular phases of the cycle from the histograms, through simple interactions with the computer. Such software is generally provided upon the purchase of instruments or can be obtained separately from other vendors. The most common software programs used to deconvolute DNA content frequency histograms are provided by Phoenix Flow and Verity Software House.

It is often assumed that the cells in S and G_2/M represent the "proliferative" cell fraction and that their frequency reflects the proliferative potential of the cell population. While this indeed may be the case in some cell systems, the DNA content frequency histograms alone do not provide any direct information on cell kinetics. For example, the histogram cannot reveal whether the cell progression through the cycle is slowed down (e.g., compared to control) if the slowdown affects all phases of the cycle proportionally. Likewise, the histogram cannot show whether the cells are "frozen" in the cycle, which may happen during treatment with high doses of certain drugs, hyperthermia, or radiation. However, when the cell-proliferation rate (e.g., the cell-doubling time in culture) is measured in parallel with DNA content, it is possible, with certain assumptions and approximations, to estimate cell kinetics, including the duration of individual phases of the cycle. One of the assumptions is that the cells are in exponential phase of growth. In such a situation there are always twice as many daughter cells as mother cells, and the cell age distribution across the cycle is represented by the diagram shown in Figure 8.4.4B. Duration of a particular phase of the cycle (e.g., G_1) can then be estimated from the equation:

$$\frac{T_{G1}}{T_C} = \frac{\ln(F_{G1} + 1)}{\ln 2}$$

where T_{G1} is duration of G_1 phase, T_C is duration of the cell cycle, and F_{G1} is a fraction of cells residing in G_1. T_C is estimated from the cell growth curves—i.e., when the number of live cells is plotted as a function of time (cell number on exponential scale versus time on linear scale of the x and y coordinates), the exponential growth is reflected by a straight slope. The slope allows one to calculate the cell doubling time in culture. The latter, with generally acceptable approximation, equals T_C. A similar formula, of course, applies for estimating duration of phases of the cell cycle other than G_1. Thus, for example, when the duration of the cell cycle is 24 hr and the fraction of cells in G_1 is 0.5 (50%), the duration of G_1 (T_{G1}) is $1n(1 + 0.5) \times 24/1n\ 2 = (0.405 \times 24)/0.693 =$ 14.0 hr. The graphical approach for measuring duration of the cell-cycle phases from the DNA content frequency histograms is illustrated in Figure 8.4.4.

Bivariate analysis of DNA content and expression of cyclins D, E, A, or B1

Cyclins are the key elements of the cell cycle progression machinery. They combine with particular cyclin-dependent protein kinases (CDKs) forming the holoenzymes that phosphorylate different sets of proteins at consecutive stages of the cell cycle, thereby driving the cell through the cycle (Pines and Hunter, 1991; Draetta, 1994; Hartwell and Kastan, 1994; Sherr, 1994; Cardon-Cardo, 1995; Morgan, 1995; Bartek et al., 1997). The function of cyclins in these holoenzymes is to activate their partner CDKs and to target them to specific protein substrates whose phosphorylation is essential for traversing a particular section of the cell cycle.

Several cyclins, notably D-type cyclins as well as cyclins E, A, and B, are expressed transiently during the cell cycle. Cyclins D and E belong to the family of G_1 cyclins, whereas cyclins A and B are G_2 cyclins (also see Fig. 8.2.1). During unperturbed growth of normal, nontumor cells, the scheduled timing of synthesis and degradation of cyclins takes place at very specific points of the cycle. The periods of expression of these cyclins by the cell, therefore, can be considered landmarks of the cell cycle. These landmarks complement the traditional milestones of the cycle detected by DNA content measurement, namely mitosis and DNA replication.

The development of antibodies to cyclins made it possible to detect them immunocytochemically and to investigate their expression in individual cells by cytometry (Gong et al., 1994; Lukas et al., 1995; Urbani et al., 1995; Juan et al., 1997). Bivariate analysis of DNA content and cyclin expression provides a framework for subdividing the cell cycle into several subcompartments and for defining, with a greater precision than before, the point at which the cell cycle is arrested by some antitumor

drugs (Darzynkiewicz et. al., 1996). Furthermore, the differences in cyclin expression make it possible to discriminate between cells having the same DNA content but residing at different phases, such as in G_2 versus M or G_2/M cells of a lower DNA ploidy versus G_1 cells of a higher DNA ploidy.

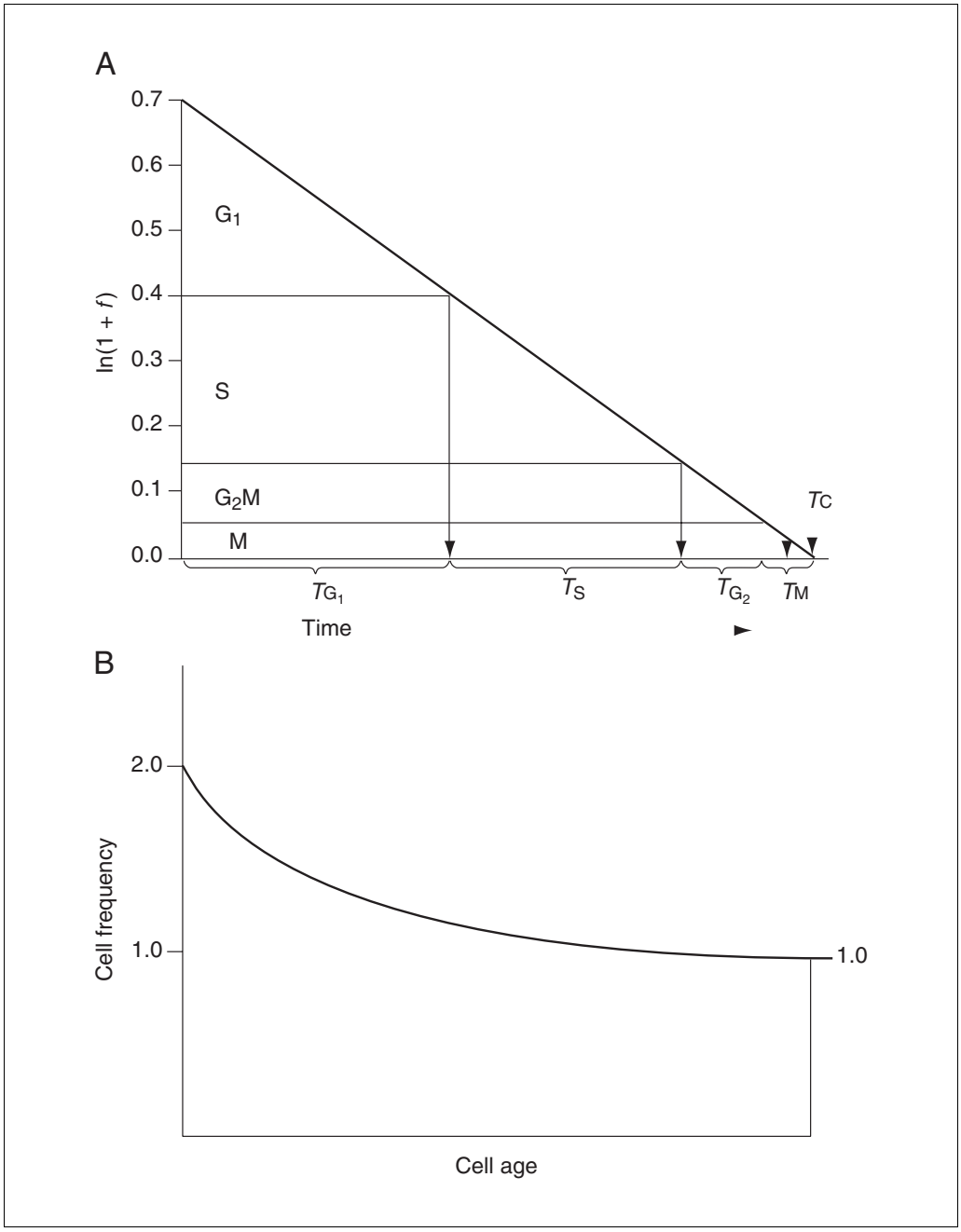


Figure 8.4.4 Graphical method for estimating duration of particular cell cycle phases (**A**) The method is based on the assumption that all cells are in the exponential phase of proliferation—i.e., that there are twice as many daughter as mother cells. (**B**) The proportions of cells in different phase of the cycle are obtained from DNA content frequency histograms (Fig. 8.4.3), and the value of T_C (cell generation, or cell cycle time), which is in turn calculated from the growth curves as the cell-doubling time in culture. T_C equals the cell-doubling time when all cells are in the proliferative cell pool—i.e., when the growth fraction equals 1.0. Fractions of cells in particular phases of the cycle (f) are plotted exponentially as ln(1 + f). T_C is then connected with ln 2 (0.693). The points of intersection of the line connecting ln 2 and T_C with the levels representing frequency of cells in particular phases of the cycle, projected on the time coordinate, represent the duration times of these phases, as shown.

The bivariate analysis of cyclin expression versus DNA content, unlike any other approach, detects the inappropriate ("unscheduled") expression of cyclins—i.e., the presentation of G_1 cyclins by cells in G_2/M and of G_2/M cyclins by G_1 cells—without the need for cell synchronization. Such unscheduled expression of cyclins B1 and A was seen when cell cycle progression was halted, e.g., after synchronization at the G_1/S boundary by inhibitors of DNA replication (Gong et al., 1995; Urbani et al., 1995). The unscheduled expression of cyclins B1 or E, representing a characteristic feature of a particular tumor phenotype, was also observed in some tumor cell lines when their growth was unperturbed (Gong et al., 1994). Likewise, while the expression of cyclins D1 or D3 in nontumor cells was restricted to an early section of G_1, the presentation of these proteins in many tumor cell lines was also seen during S and G_2/M (Juan et al., 1996). As specific markers of cell proliferation, cyclins are expected to reflect proliferative potential of tumors and therefore to be the key prognostic markers in neoplasia.

Critical Parameters

Univariate cellular DNA content analysis

The most critical issue in DNA content analysis is the accuracy of DNA content measurement. The accuracy, as mentioned (see Background Information), is reflected by the extent of variation in cellular fluorescence between the cells with identical DNA content, such as $G_{0/1}$ cells. The CV of the DNA-associated mean fluorescence of G_1 cells, therefore, is considered as an index of accuracy of DNA content measurement. High accuracy is required, in particular, in studies of DNA ploidy, to distinguish between DNA diploid and aneuploid cells, which may differ minimally in DNA content. High accuracy of DNA content measurement is also critical in analysis of the cell cycle distribution. Regardless of the type of the software used to deconvolute DNA frequency histograms, the accuracy in estimation of cell proportions in respective phases of the cell cycle directly correlates with accuracy of DNA content measurement. There is no formal consensus regarding the acceptable maximal CV value of the mean DNA content of $G_{0/1}$ cell population—i.e., maximal error in cellular DNA content estimate. Most researchers, however, would consider the accuracy to be poor and results unacceptable if CV values of $G_{0/1}$ populations of normal, nontumor cells exceed 6%.

A variety of factors can contribute to poor accuracy in DNA content analysis. The most common is inappropriate optical alignment (spatial position of laser beam with respect to sample flow and fluorescent light–collecting lenses) of the flow cytometer. When the instrument is optimally aligned, the measured cell passes precisely through the center of the laser beam, and at the same time, is in the focus of the fluorescence emission collection lenses. A minor adjustment of the sample flow or any of the optical parts leads to a loss in accuracy of DNA content measurement. Proper maintenance of the instrument, and careful adjustment with standard fluorescent beads of known uniformity prior to analysis of the experimental samples, is essential to achieve accurate DNA content measurements. Problems in sample preparation, either resulting in mechanical damage to the cells or involving incorrect composition of buffers and staining solutions, represent another common cause of poor resolution in DNA analysis.

It should be stressed that there may be situations when, in spite of good accuracy in DNA content measurement (in terms of proper instrument adjustments and sample staining), the CV of the mean DNA content of G_1 cell populations is relatively large. This may happen when significant numbers of dead or dying cells are present in the sample or when the cells were treated with drugs interacting with DNA. Many antitumor drugs are known to impair stainability of DNA with the fluorochromes used for flow cytometry. Furthermore, because of the nature of the tumor, which may either be multiclonal or have developed drug resistance by gene amplification mechanisms (e.g., as reflected by the presence of minute chromosomes), the tumor cell populations may have variable DNA content, and therefore intrinsically high CV values for the $G_{0/1}$ cell populations.

Bivariate analysis of DNA content and expression of cyclins D, E, A, or B1

The critical steps for immunocytochemical detection of intracellular proteins are cell fixation and permeabilization. These steps often have to be customized for particular antigens. The fixative is expected to stabilize the antigen in situ and preserve its epitope in a state where it continues to remain reactive with the available antibody. The cell has to be permeable to allow access of the antibody to the epitope.

Most studies on cyclins have employed precipitating fixatives such as 70% to 80% ethanol, absolute methanol, or a 1:1 mixture of methanol and acetone cooled to –20° to –40°C. Brief (15- to 30-min) treatment with 1% paraformaldehyde followed by 70% cold ethanol has been used for fixation of D-type cyclins although this cyclin can also be detected following fixation with cold methanol. The choice of fixative, thus, appears not to be a critical factor for cyclin detection and, although the absolute level of the immunofluorescence may vary, various fixation protocols yield essentially similar cyclin distributions with respect to the cell cycle position. Each fixative has some undesirable effects (e.g., increased cell clumping in the case of the ethanol/acetone mixture or cell autofluorescence and poor DNA stainability when formaldehyde is used), and one often has to compromise between these effects and the optimal detection of a particular cyclin. Fixation in 80% cold ethanol, as presented in this protocol, offers such a compromise.

Much more critical for the detection of cyclins is the choice of a proper antibody. Often an antibody that is applicable to immunoblotting fails in immunocytochemical applications, and vice versa. Likewise, the antibody may show nonspecific reaction with denatured proteins (i.e., reveal several bands on immunoblots), and yet be acceptable in immunocytochemical assays. These discrepancies may be due to differences in accessibility of the epitope or differences in the degree of denaturation of the antigen on the immunoblots as compared to the situation in its in situ location. Some epitopes may not be accessible in situ at all. This especially pertains to epitopes that are the cyclin segments involved in formation of complexes with the partner CDKs, CDK inhibitors, or other molecules such as proliferating cell nuclear antigen (PCNA). Since there is strong homology between different cyclin types, cross-reactivity may also be a problem. Because commercially available monoclonal antibodies may differ, e.g., in specificity and degree of cross-reactivity, it is important to use reagents that have been already tested and referenced in published papers. It is required that the authors provide information (the vendor and hybridoma clone number) on the reagent used in their study.

A very important control is examination of the stained cells by UV light microscopy, or preferably confocal microscopy. All cyclins, with the notable exception of cyclin B1, have nuclear localization. Cyclin B1 has cytoplasmic localization during late S and most of G_2, and is localized in the chromatin only at mitosis. Cyclin B2 is localized to Golgi (Jackman et al., 1995). Inappropriate localization of the fluorescence of the cells stained with the presumed anti-cyclin antibody may be an indication of its nonspecific reactivity with other cell constituents.

The relative cellular content of a particular cyclin plays a role in its detection. The signal-to-noise ratio (ratio of fluorescence intensity of the cyclin-positive cells to the control cells, stained with the isotype immunoglobin), for example, is higher in the case of cyclin B1 than in the case of cyclins E or A, most likely due to the fact that the absolute level of cyclin B1 is higher compared to cyclins E or A. The level of expression of D-type cyclins varies markedly depending on the cell type and the phase of cell growth. High sensitivity of the instrument and low level of cell autofluorescence, therefore, are of greater importance for the detection of cyclins E or A than of cyclin B1 or D-type cyclins.

While isotypic immunoglobin or irrelevant isotypic antibody are generally accepted as appropriate controls, they are not perfect for this purpose, as their fluorescence may also vary depending on the source (vendor) and may not be representative the actual background. Ideal controls represent the cells of the same type and of the same species, but with the gene that codes for the detected protein deleted. Such control cells should be subjected to identical immunocytochemical procedure as the studied cells. Unfortunately, few cell lines with deleted cyclin genes are currently available.

Anticipated Results

Univariate cellular DNA content analysis

Figure 8.4.3 presents DNA frequency histograms of HL-60 cells stained with DAPI according to Alternate Protocol 1 and Alternate Protocol 2, respectively. As is evident based on differences in DNA content, one can identify a population of $G_{0/1}$ cells with uniform low DNA content values, G_2/M cells with DNA content twice that of $G_{0/1}$ cells, and S phase cells with intermediate DNA content. To reveal the percentage of cells in $G_{0/1}$, S, and G_2/M, the DNA frequency histograms in the figure were deconvoluted using the Multicycle software (Phoenix Flow Systems). Similar results are expected when using Basic Protocols 1 and 2. Supravital cell staining with Hoechst 33342, however, yields less accurate DNA measurements, which

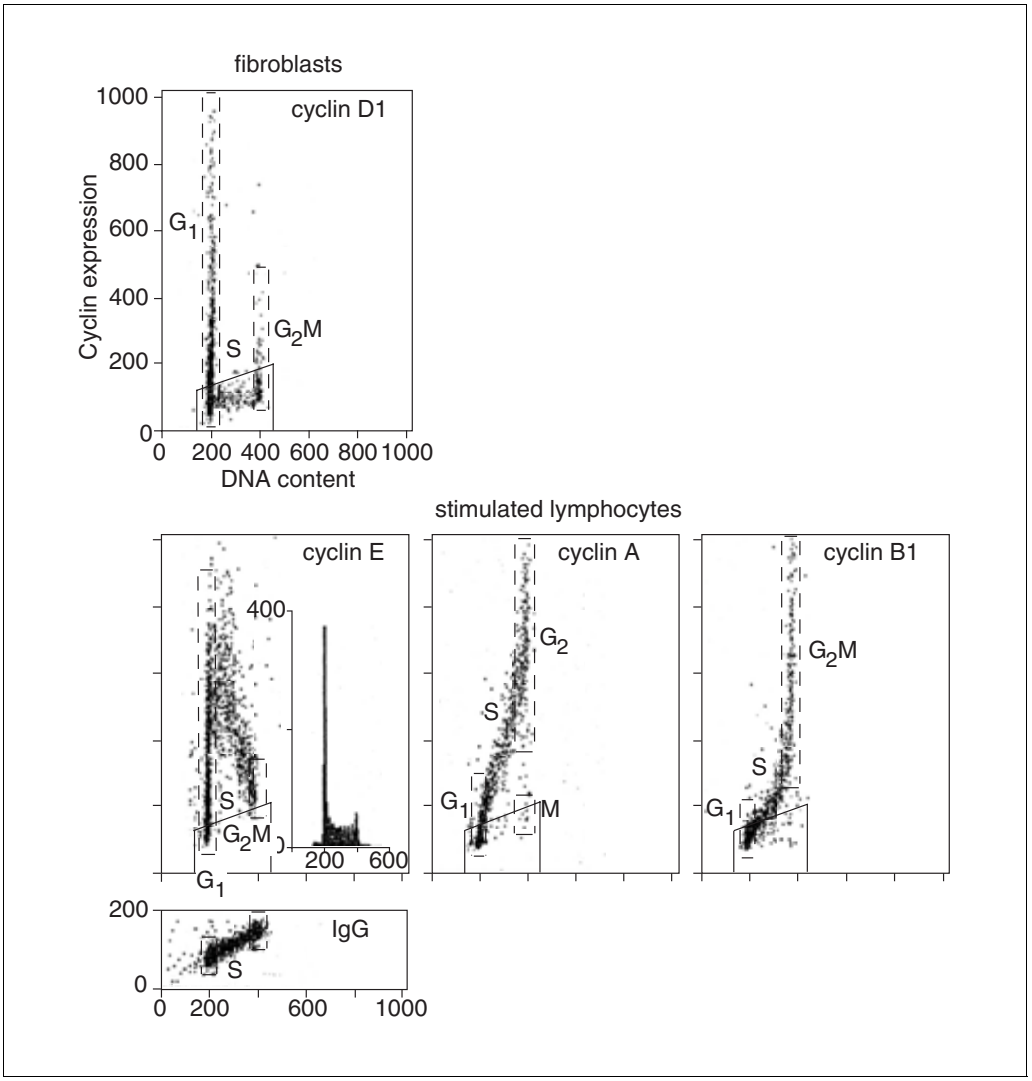


Figure 8.4.5 Bivariate cyclin versus DNA content distributions (scatter plots) showing expression of cyclin D1 in normal human fibroblasts and cyclins E, A, and B1 in mitogen-stimulated human lymphocytes. The trapezoidal windows represent level of fluorescence of the respective control cells stained with the isotype IgG, rather than the respective cyclin-anti-cyclin antibody. The $G_{0/1}$ and G_2/M populations gated based on differences in DNA content are marked by dashed lines.

are reflected by the increased width of the $G_{0/1}$ and G_2/M peaks (i.e., increased CV of the mean fluorescence of $G_{0/1}$ and G_2/M cell populations).

Bivariate analysis of DNA content and cyclin expression

The scheduled timing of expression of cyclins B1, A, E, and D1 in relation to the major phases of the cell cycle is reflected by a very characteristic pattern of the bivariate cyclin–versus–cellular DNA content distributions. These distributions are shown in Figure 8.4.5 for normal human proliferating lymphocytes (cyclins B1, A, and E) and fibroblasts (cyclin D1). As is evident from the cytograms, the expression of cyclin B1 is limited to late S phase cells and the cells with a G_2/M DNA content, while early- and mid–S phase cells show a very low level of this protein. Cells in G_1 phase are essentially cyclin B1–negative.

Similar to cyclin B1, expression of cyclin A is minimal in G_1 cells. It becomes pronounced, however, during S phase where its level progressively increases as the cells advance towards G_2. Maximal expression of cyclin A is seen in cells having a G_2/M DNA content (Fig. 8.4.5). It should be mentioned, however, that because cyclin A is abruptly degraded during prometaphase (Pines and Hunter, 1991), mitotic cells that have advanced past prometa-

phase are essentially cyclin A–negative (not shown).

Expression of cyclin E can be summarized as follows: (1) the maximal level of this protein is detected in the cells undergoing transition from G_1 to S; (2) its level continuously decreases during cell progression through S, with the result that most G_2/M cells are cyclin E–negative; and (3) a distinct threshold in cyclin E expression is apparent at the G_1/S transition. As it is evident from the continuity of the cell clusters on scatter plots (Fig. 8.4.5) the cells have to accumulate cyclin E above the threshold level to enter S phase.

The presence of cyclin D1 in exponentially growing normal fibroblasts is limited to cells in $G_{0/1}$ (Fig. 8.4.5). Most cells in S and G_2/M are cyclin D1–negative, with the exception of a few cells with a G_2/M DNA content. The latter may be G_1 cell doublets, since not all doublets can be identified by analysis of the shape (pulse width) of the electronic signal.

It should be stressed that, as mentioned before (see Background Information), the cyclin distributions as shown in Figure 8.4.5 characterize only those cells that are growing normally, exponentially, and asynchronously. The distributions are very different when the cell-cycle progression is perturbed, or in the case of some tumor cell lines that display unscheduled expression of these cyclins.

Time Considerations

For Basic Protocol 1 (PI staining), cell fixation takes ~10 min, but cells have to be kept in fixative 2 hr; the cell staining procedure takes ~45 min. Alternate Protocol 1 (DAPI staining) requires similar times for cell fixation. However, because there is no need to incubate cells with RNase, the time of staining is shorter. The detergent-based cell-staining procedure of Basic Protocol 2 takes ~60 min. For Alternate Protocol 2, which is simpler, cell staining takes ~15 min. Supravital staining of cells (Basic Protocol 3) requires ~20 min for staining, although extended staining times (up to 90 min) may be needed for some cell types.

For Basic Protocol 4 (DNA and cyclins) cell fixation takes ~10 min, but the cells have to be kept in fixative for ≥2 hr. The cell-staining procedure takes ~1 hr if the antibodies used are directly conjugated with a fluorochrome. The staining may take an additional 40 min if the primary antibody is not fluorochrome-tagged and therefore indirect labeling has to be used. It is sometimes convenient, however, to leave the cells for incubation with the primary antibody overnight, at 4°C.

Literature Cited

Bagwell, B.C. 1993. Theoretical aspects of flow cytometry data analysis. *In* Clinical Flow Cytometry. Principles and Application (K.D. Bauer, R.E. Duque, and T.V. Shankey, eds.) pp. 41-61. Williams & Wilkins, Baltimore.

Bartek, J., Bartkova, J., and Lukas, J. 1997. The retinoblastoma protein pathway in cell cycle control and cancer. *Exp. Cell Res.* 237:1-6.

Cardon-Cardo, C. 1995. Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. *Am. J. Pathol.* 147:545-560.

Crissman, H.A. and Steinkamp, J.A. 1990. Cytochemical techniques for multivariate analysis of DNA and other cell constituents. *In* Flow Cytometry and Sorting. 2nd ed. (M.R. Melamed, T. Lindmo, and M.L. Mendelsohn, eds.) pp. 227-247. Wiley-Liss, New York.

Darzynkiewicz, Z., Robinson J.P., and Crissman, H.A. 1994. Flow Cytometry. 2nd ed. Part A. Academic Press, San Diego (also published as vol. 41, *Methods Cell Biol.*, 1994).

Darzynkiewicz, Z., Gong, J., Juan, G., Ardelt, B., and Traganos, F. 1996. Cytometry of cyclin proteins. *Cytometry* 25:1-13.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., and Traganos, F. 1997. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 27:1-20.

Draetta, F.G. 1994. Mammalian G1 cyclins. *Curr. Opin. Cell Biol.* 6:842-846.

Gong, J., Ardelt, B., Traganos, F., and Darzynkiewicz, Z. 1994. Unscheduled expression of cyclin B1 and cyclin E in several leukemic and solid tumor cell lines. 1994. *Cancer Res.* 54:4285-4288.

Gong, J., Traganos, F., and Darzynkiewicz, Z. 1995. Growth imbalance and altered expression of cyclins B1, A, E, and D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. *Cell Growth Differ.* 6:1485-1493.

Hartwell, L.H. and Kastan, M.B. 1994. Cell cycle control and cancer. *Science* 266:1821-1823.

Jackman, M., Firth, M., and Pines, J. 1995. Human cyclins B1 and B2 are localized to strikingly different structures: B1 to microtubules, B2 primarily to the Golgi apparatus. *EMBO J.* 14:1646-1654.

Juan, G., Gong, J., Traganos, F., and Darzynkiewicz, Z. 1996. Unscheduled expression of cyclins D1 and D3 in human tumour cell lines. *Cell Prolif.* 29:259-266.

Juan, G., Li, X., and Darzynkiewicz, Z. 1997. Correlation between DNA replication and expression of cyclins A and B1 in individual MOLT-4 cells. *Cancer Res.* 57:803-807.

Krishan, A. 1987. Effect of drug efflux blockers on vital staining of cellular DNA with Hoechst 33342. *Cytometry* 8:642-645.

Loken, M.M. 1980. Simultaneous quantitation of Hoechst 33342 and immunofluorescence on viable cells using a fluorescence activated cell sorter. *Cytometry* 1:136-142.

Lukas, J., Bartkova, J., Welcker, M., Petersen, O.W., Peters, G., Strauss, M., and Bartek, J. 1995. Cyclin D2 is a moderately oscillating nucleoprotein required for G1 phase progression in specific cell types. *Oncogene* 10:2125-2134.

Morgan, D.O. 1995. Principles of CDK regulation. *Nature* 374:131-134.

Pines, J. and Hunter, T. 1991. Human cyclin A and cyclin B are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J. Cell Biol.* 115:1-17.

Rabinovitch, P.S. 1994. DNA content histogram and cell-cycle analysis. *Methods Cell Biol.* 41:263-296.

Sherr, C.J. 1994. G1 phase progression. Cycling on cue. *Cell* 79:551-555.

Urbani, L., Sherwood, S.W., and Schimke, R.T. 1995. Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. *Exp. Cell Res.* 219:159-168.

Contributed by Zbigniew Darzynkiewicz, Gloria Juan, and Elzbieta Bedner
New York Medical College
Valhalla, New York

Centrifugal Elutriation to Obtain Synchronous Populations of Cellsab

Counterflow centrifugal elutriation (CCE) is a generally noninvasive method for separating large numbers of mixed particles (bacteria, yeast, mammalian cells) into populations on the basis of their size and mass. For mammalian cells the method is particularly useful for separating mixed cell populations (e.g., T cells or monocytes from peripheral blood mononuclear cells or, as described here, for obtaining proliferating cell populations partitioned at progressive stages of the cell division cycle. CCE does not perturb cell metabolism, a fundamental problem encountered with metabolic synchronizing agents (see *UNIT 8.3*). Thus, the characteristics of populations separated by CCE more closely reflect normal cell cycle regulation. The standard protocol permits separation of ~2×10^8 cells into six to ten progressive populations using a Beckman JE-6B rotor (see Basic Protocol). For separation of a larger number of cells (~10-fold more), parameters are modified for use with a large JE-5.0 rotor (see Alternate Protocol). Because the task of harvesting cells from large volumes of culture medium by repetitive centrifugation is time consuming and can adversely effect cell viability, this protocol also presents a convenient method for concentrating cells from culture media by continuous flow using the large elutriation rotor. To accurately assess elutriated cell fractions for purity and to verify cell cycle position, short protocols are provided to quantify DNA content by flow cytometry with propidium iodide (see Support Protocol 1), to quantify nascent DNA synthesis by incorporation of [^{3}H]thymidine deoxyribose (see Support Protocol 2), and to quantify DNA synthesis and content by flow cytometry using PI and incorporated 5-bromo-2′-deoxyuridine (BrdU; see Support Protocol 3).

SEPARATION OF CELLS INTO PROGRESSIVE STAGES OF THE CELL DIVISION CYCLE BY COUNTERFLOW CENTRIFUGAL ELUTRIATION

BASIC PROTOCOL

This section describes the setup and equilibration of the Beckman J-6B elutriator system for sterile separation of mammalian cells into progressive populations through the cell division cycle based on cell size and mass.

NOTE: Methodical tissue culture technique (Chapter 1) is the single most important parameter to ensuring reproducible results and good yields at all cell cycle phases. Healthy cells are less prone to lysis and clumping from released DNA and eliminate the need for DNase treatment during the run. Large clumps in the elutriation chamber will adversely effect fluid flow and separation. Sustained log-phase growth is required for high viability, low debris, and sufficient levels of S- and G_2/M-phase cells.

Materials

70% (v/v) ethanol
Elutriation medium (see recipe) equilibrated ≥2 hr at ambient temperature
Nonadherent cells in log-phase growth: suspension cultures at 2–5 × 10^5 cells/ml with fresh medium added at each doubling

Beckman J-6B or J25I centrifuge with view port door and strobe assembly
Beckman JE-6B rotor with standard elutriation and bypass chambers
Elutriation liquid system assembly (Fig. 8.5.1), consisting of:
  Medium reservoir: 2-liter roller bottle and 2-ml pipet
  Loading syringe: 10-ml syringe barrel and three-way valve
  High-quality peristaltic pump with 10-turn potentiometer or digital control (e.g., Masterflex 7520 or 7550), capable of 100 rpm, 0 to 150 ml/min in 0.1 ml/min increments

continued

Contributed by Alan F. Wahl and Karen L. Donaldson
Current Protocols in Cell Biology (1999) 8.5.1-8.5.16

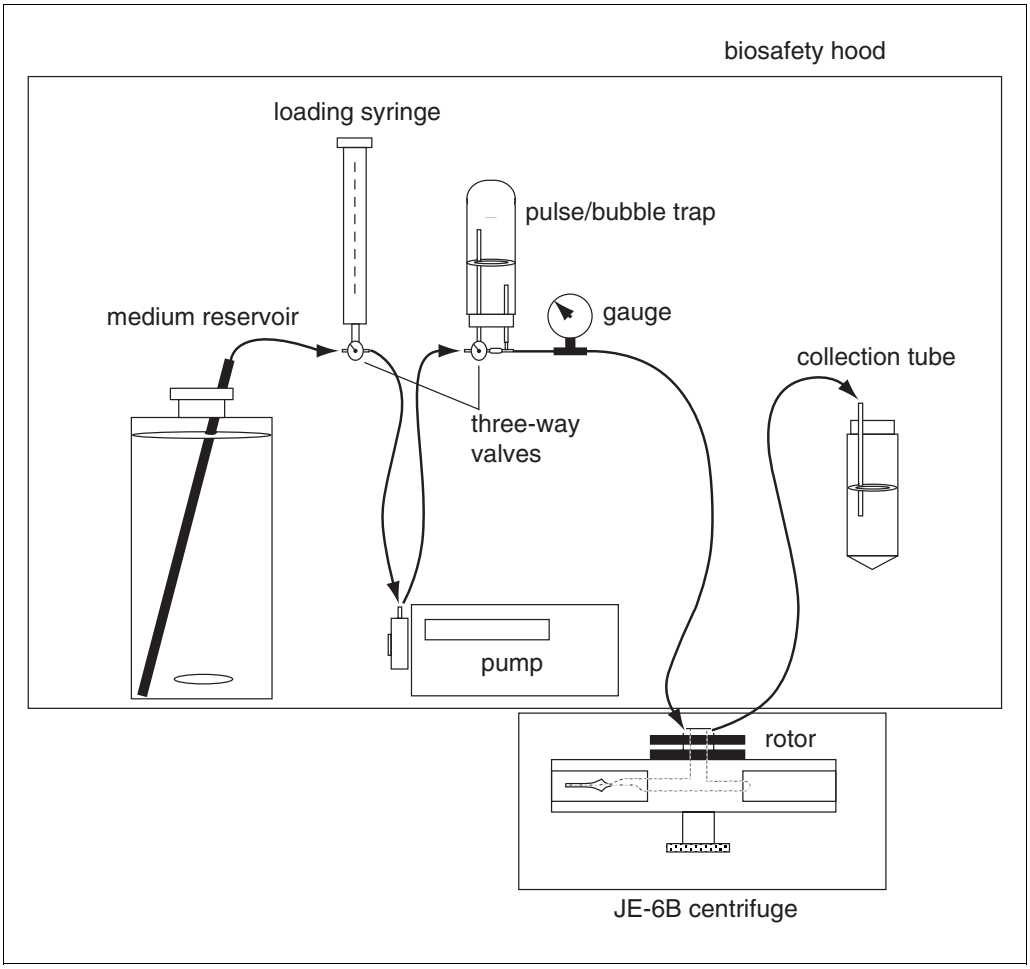


Figure 8.5.1 Schematic of the counterflow centrifugal elutriation system. Fluid is drawn from a reservoir past a valved loading syringe and through a peristaltic pump. It proceeds through a pulse/bubble trap compensator and pressure gauge to the rotor, and returns from the chamber to a 2-ml pipet into a collection vessel. The entire setup (with the exception of the centrifuge itself) is assembled in a hood.

Pulse/bubble trap and three-way valve (Beckman)
Pressure gauge (Beckman)
Collection tube: 250-ml conical centrifuge bottle (Corning)
¼-in.-o.d. (0.64-cm) Silastic tubing (Masterflex 6411-14)
Ring stand and clamps
18-G needle
10-ml syringe
250-ml conical centrifuge bottles
Beckman GPR centrifuge (or equivalent) with swinging bucket rotor
Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

NOTE: Elutriation is performed with the centrifuge, media, and cells at ambient temperature. Cell fractions are processed immediately after collection or stored on ice in multiple runs.

Set up elutriation system

1. Set up a JE-6B elutriation rotor, strobe assembly, and J-6B or J25I centrifuge according to manufacturer's specifications (Beckman Instruments, 1981, 1989).

Ensure that the internal rotating seal is free from wear and is correctly seated against the head assembly.

Failure of the rotating seal will either result in leakage into the centrifuge chamber or allow input and output fluid to mix and bypass the elutriation chamber.

2. Ensure that the elutriation and bypass chambers are in the correct positions and that the fluid flowing into the rotor enters the bottom (outermost) region of the elutriation chamber.
3. Set up the elutriation liquid system assembly as shown in Figure 8.5.1. Secure the loading syringe, pulse/bubble trap, and pressure gauge using clamps and a ring stand. Use ¼-in.-o.d. Silastic tubing throughout the system. Bevel the input and output pipet ends to prevent flow obstruction during the run.

   This design differs from that described by the manufacturer.

   The pulse/bubble trap is held inverted (as shown) with a three-way valve connected to the first input tee. This allows the cells to bypass this chamber. After loading, the chamber is switched in-line to compensate for any flow pulsation caused by the pump.
4. To ensure sterile sorting, place the entire assembly (step 3) in a laminar flow hood and place the centrifuge directly adjacent to the hood (Fig. 8.5.1). Minimize the length of tubing used in the system.

Sterilize elutriation system

5. With the centrifuge off, sterilize the entire system by pumping 70% ethanol through at ~30 to 40 ml/min, being careful to fill the loading reservoir, bypass chamber, pressure gauge, bypass tubing, and all valves. Allow to sit for 10 min.
6. Remove ethanol, being careful to drain all components by starting at the loading chamber and finally drain the rotor by lifting and tilting.
7. Rinse any residual ethanol by pumping sterile water through the system as described for ethanol.

   Residual ethanol will cause precipitate in the elutriation medium.
8. Allow to recirculate 5 to 10 min by putting both input and output lines into a sterile water vessel, then remove lines and drain the system.

Equilibrate elutriation system

9. Equilibrate the system with elutriation medium that is well equilibrated at the operating (ambient) temperature. Circulate the medium as described in step 5, placing both input and output lines into a 2-liter vessel containing elutriation medium.

   Cold medium placed in a warm system will degas and create bubbles in the system.
10. Begin removing trapped air from the system by pinching off tubing to create ~20 lb back pressure and quickly releasing. Start at the loading chamber and progress toward the elutriation chamber, switching valves as necessary to ensure that all bubbles are removed. Remove air in the elutriation chamber by lifting the rotor and tilting 90 degrees to allow medium to fill the chamber.
11. Turn on the centrifuge, bring the rotor to running speed, and repeat step 10 to remove trapped air.
12. Stop rotor and lift to check for leakage.

    This step also helps to dislodge additional trapped air.
13. Restart rotor and recheck the entire system for trapped air bubbles.

    Air that becomes trapped in the elutriation input will impede flow into the chamber and ruin the run.

An air cushion of ~1 in. (2.5 cm) should remain in the pulse/bubble chamber to dampen any pump pulsation during the run.

Calibrate pump

14. Set rotor speed to 1800 rpm and loading flow rate at 12 ml/min.

    As an example, this protocol loads cells at 12 ml/min at a rotor speed of 1800 rpm, with ten samples taken at 3 ml/min increments up to 42 ml/min. Elutriation of most mammalian cells is done at rotor speeds between 1800 and 2500 rpm, with corresponding loading flow rates ranging between 12 and 20 ml/min. See Table 8.5.1 for examples of flow rates used to elutriate Jurkat cells using the JE-6B rotor. See Beckman Instruments (1981, 1989) for a detailed discussion of the selection of rotor speed and flow rate based on particle size and mass.

    Although separation of populations is achieved by varying either speed or flow rate, the authors have found that varying flow rate with a digitally controlled pump provides the most reproducible results.

15. Calibrate the pump to the loading flow rate with the complete system in place and the rotor running at the desired speed. To calibrate, collect medium from output line into a graduated cylinder for 1 min and measure volume. If necessary, readjust pump calibration setting and collect again for 1 min. Repeat until pump speed is accurate. Also check maximum flow rate to ensure that this is obtainable with minimum back pressure (~5 psi).

    Obstruction of input and output tubes or a change in their respective heights will affect pressure, flow rates, and separation. A high-quality digital pump will retain linearity through the range of flow rates. For pumps utilizing a 10-turn potentiometer, settings should be determined to deliver each incremental flow rate.

Harvest cells

16. Transfer 2×10^8 nonadherent log-phase cells (~0.5 to 1 liter) to 250-ml conical centrifuge bottles and centrifuge for 10 min at $200 \times g$ (e.g., in a Beckman GPR centrifuge). Gently resuspend pellet in 10 ml elutriation medium and verify cell density (final 2×10^7/ml).

Table 8.5.1 Jurkat Elutriation Parameters[a] and Cell Cycle Phase Content of the Separated Populations

Fraction	Flow rate (ml/min)	Cell number	% of load	% G_1[b]	% S[b]	% G_2[b]
Load	13	2.0×10^8	100[c]	55.6	27.3	17.1
1	18	3.3×10^6	1.6	96.2[d]	3.6	0.2
2	21	2.1×10^7	10.5	91.0	5.2	3.8
3	24	3.7×10^7	18.5	86.8	11.0	2.2
4	27	3.2×10^7	16.0	78.0	19.4	2.6
5	30	2.3×10^7	11.5	59.7	35.4	4.9
6	33	1.9×10^7	10.6	29.9	55.0	15.1
7	36	1.5×10^7	7.5	13.0	50.7	36.3
8	39	1.2×10^7	6.0	4.8	40.3	54.9
9	42	7.7×10^6	3.8	4.1	32.3	63.6
10	45	5.7×10^6	2.8	2.9	19.8	77.3
11	48	4.3×10^6	2.2	2.7	10.4	86.9

[a] JE-6B rotor maintained at constant speed of 1800 RPM.

[b] Percentage distribution calculated from histograms of total DNA content (Fig. 8.5.3).

[c] The percentages from fractions 1 to 11 add up to <100% because small amounts are lost to the system during loading (load fraction) or aggregate in the chamber and are unrecoverable (dump fraction).

[d] Approximately 3% of these cells contain apoptotic DNA with sub-G_1 content.

For most efficient use of time and to maintain cells in optimal condition, cells can be centrifuged during equilibration of the elutriator.

17. Load concentrated cells into a 10-ml syringe and pass three times *slowly* through an 18-G needle just prior to loading. Check cells under a microscope to ensure that the culture is monomeric with high viability.

Load elutriator and collect fractions

18. Check that the pump and rotor are at speed (12 ml/min, 1800 rpm). Transfer the 10-ml cell suspension directly from the syringe used for dispersion (step 17) into the loading syringe. Place elutriator output line into a 250-ml conical centrifuge bottle (collection vessel). Set the valve at the pulse/bubble trap to bypass the trap.

    Loading can be done through the pulse/bubble trap, but this dilutes the cells and increases the time needed for loading and equilibration.

19. Open the valve to the loading syringe to allow cells to be drawn into the medium stream, being careful to prevent air from being drawn into the system. Do not allow the loading syringe to empty completely (shut the valve once all but ~250 µl of the cell suspension has been drawn into the system).

20. Add ~2 to 4 ml elutriation medium to the loading syringe and load the additional volume to ensure complete loading of the cells. Again, leave ~250 µl remaining in the loading syringe.

    If air has entered the elutriator line during loading, the rotor must be stopped and purged of air and the cells must be reloaded.

21. Once cells have bypassed the pulse/bubble trap, open this valve to trap bubbles and to compensate for pump pulsation. Carefully monitor cells through the view port of the centrifuge door.

    Cells appear as a broad translucent band that should fill the chamber to just below the elutriation boundary (Fig. 8.5.2), below the top of the chamber.

22. Watch the cells carefully. Do not allow the cells to pack (increase flow rate if necessary) or to flow over the top of the elutriation boundary (decrease flow rate). Allow cells to equilibrate while collecting 125 ml medium (load fraction).

    For pilot runs, examine the load fraction to ensure that it contains only debris. If a significant number of viable cells are present, the load should be repeated at a lower-flow rate.

    Elutriation is a balance of g force and counterflow. If cells do not appear to be filling the chamber, gradually increase pump speed. If cells are reaching the elutriation boundary, reduce the pump speed to prevent loss of cells and then gradually increase until they are at the appropriate level. Do not change rotor speed.

23. Once cells have equilibrated, transfer the output line to a fresh 250-ml collection vessel to collect fraction 1. Gradually increase pump speed by 0.1 ml/min increments from 12 ml/min to 15 ml/min and collect another 125 ml medium. Place cells on ice.

    Cold shock will cause sustained growth arrest of some cell types. Placing these populations on ice is not recommended when kinetic cell growth studies are to follow elutriation.

24. Collect remaining fractions in 125 ml medium each at 3 ml/min increments in pump speed. Collect ten samples up to 42 ml/min and place on ice.

25. Once the last fraction has been collected, remove any remaining cells from the rotor by stopping the rotor and continuing to pump 125 ml medium through the system.

    For multiple elutriation runs of the same cell line, fractions may be overlaid into the same 250-ml bottle; however, all run conditions must be reproduced. The same number of cells must be loaded and rotor/pump speeds and fractions must be kept the same in order to

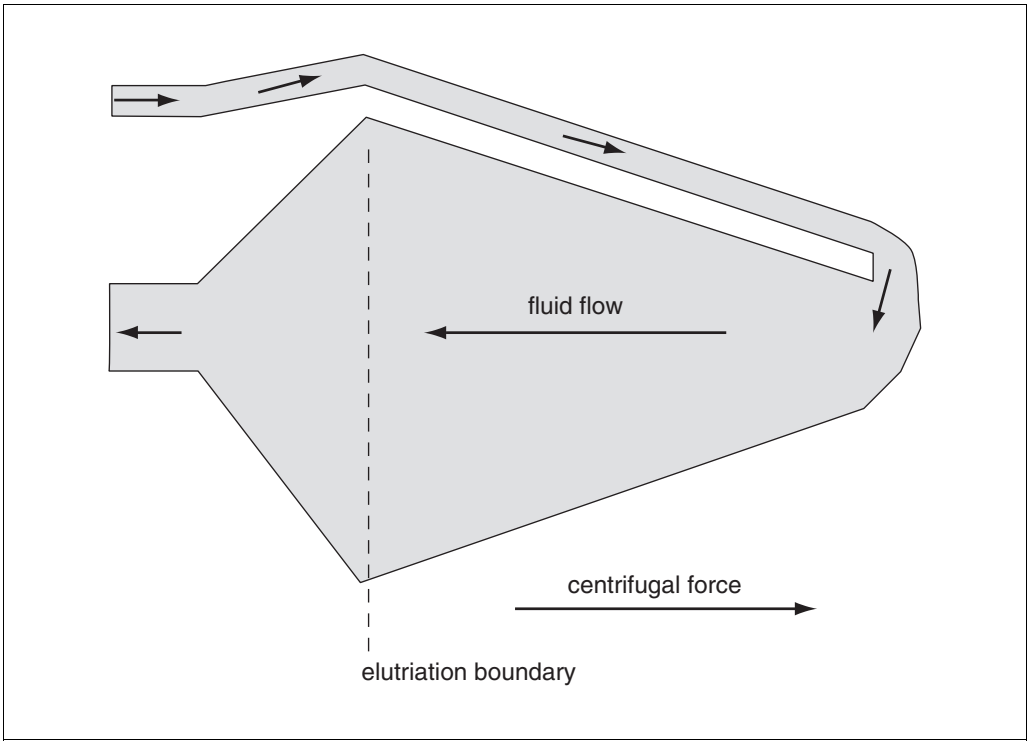


Figure 8.5.2 Schematic of the standard elutriation chamber.

duplicate each fraction. Store fractions on ice to slow cell cycle progression during subsequent runs.

26. Carefully and thoroughly resuspend cells in centrifuge bottles for each fraction. Count cells using a hemacytometer (*UNIT 1.1*) and concentrate the desired number of cells by centrifugation for use in experiments.

Very early and late fractions contain fewer cells. To obtain a more accurate cell count, cells from these fractions must first be concentrated by centrifugation.

ALTERNATE PROTOCOL

LARGE-SCALE ELUTRIATION WITH THE JE-5.0 ROTOR

Setup and equilibration of the larger JE-5.0 elutriator for concentration and large-scale elutriation is as described above (see Basic Protocol) with minor modifications (e.g., the size of the system tubing is increased to accommodate increased flow rate). If large and small rotors are used interchangeably, it is convenient to keep a separate, preassembled plumbing system (loading syringe, pulse/bubble trap chamber, gauges, and so on) for each rotor. The careful handling of healthy cells and the loading conditions are as described in the Basic Protocol.

To accommodate the volume of cells needed to fuel the large rotor, cells are concentrated from one large vessel (such as a 10-liter spinner flask) using the JE-5.0 rotor. With careful monitoring to prevent cells from pelleting or flowing through the chamber, a large volume of cells can be conveniently concentrated by gradually increasing rotor speed while maintaining a constant pump speed. The flowthrough medium is collected in a large waste flask and the concentrated cells are collected by transferring the output line to a 250-ml bottle and stopping the rotor. This concentration procedure is less time consuming and labor intensive than traditional centrifugation procedures and also preserves better viability. Thus, it is very useful on its own to concentrate cells for any purpose.

Additional Materials *(also see Basic Protocol)*

Beckman JE-5.0 rotor with 40-ml chamber
⅜-in.-o.d. (1.0-cm) Silastic tubing (Masterflex 6411-16)
30-ml syringes for loading chamber and for dispersing cells
4-liter vessel
Large sterile vessel
Large waste flask

Concentrate cells

1. Set up elutriator (see Basic Protocol, steps 1 to 4), but use a Beckman JE-5.0 rotor, ⅜-in.-o.d. Silastic tubing throughout the assembly, a 30-ml syringe for the loading chamber, and a 4-liter vessel for the medium reservoir.
2. Sterilize, equilibrate, and calibrate elutriator (see Basic Protocol, steps 5 to 15), calibrating at a pump speed of 70 ml/min and a rotor speed of 2000 rpm.
3. Pool nonadherent log-phase cultured cells into a large sterile vessel.

   At 2×10^5 cells per ml, this procedure will require ~10 liters of cells to load the chamber.
4. Place both input and output lines in the loading vessel and load cells into the chamber at a pump speed of 70 ml/min and rotor speed of 2000 rpm. Continue to recirculate for 8 to 10 min to ensure that cells are visible in the chamber.
5. Gradually increase rotor speed to 2400 rpm over a 5-min interval, and then transfer the output line into waste flask.
6. Continue to gradually increase the rotor speed to 2900 rpm, keeping cells well below the elutriation boundary near the top of the chamber (Fig. 8.5.2). Be careful not to allow cells to pack into a pellet (increase flow rate if necessary).
7. Once the desired number of cells are collected based on cells/ml medium, transfer the output line into a 250-ml centrifuge bottle. Stop the rotor and collect 250 ml medium in each of two centrifuge bottles (500 ml total).

Prepare cells and equipment for elutriation

8. Centrifuge cells for 10 min at $200 \times g$ (e.g., in a Beckman GPR centrifuge) and gently resuspend in 30 ml elutriation medium.
9. While the cells are centrifuging, reequilibrate the system with elutriation medium and recalibrate at a pump speed of 50 ml/min and a rotor speed of 1800 rpm.

   Preparing the system for elutriation while the cells are centrifuging provides for the most efficient use of time and helps maintain cells in optimal condition.

   See Table 8.5.2 for example of flow rates used for WEHI-231 cells elutriated using the JE-5.0 rotor.
10. Load concentrated cells into a 30-ml syringe and pass three times slowly through an 18-G needle just prior to loading. Check cells under a microscope to ensure mono-dispersion.

Load elutriator and collect fractions

11. Load elutriator (see Basic Protocol, steps 18 to 22), making sure pump and rotor speed are set at 50 ml/min and 1800 rpm. Follow cells with ~10 ml elutriation buffer to ensure complete cell loading (step 20), and leave 1 ml to prevent complete emptying of the syringe (steps 19 and 20). Collect 250 ml for the load fraction (step 22).

Table 8.5.2 WEHI-231 Elutriation Parameters[a] and Cell Cycle Phase Content of Separated Populations

Fraction	Flow rate (ml/min)	Cell number	% of load	% G_1[b]	% S[b]	% G_2[b]
Load	50	2.0×10^9	100[c]	24.3	58.8	16.9
1	60	7.8×10^6	0.4	98.7	1.2	0.1
2	70	2.6×10^8	13.0	67.7	32.2	0.1
3	80	3.5×10^8	17.5	39.0	60.4	0.6
4	90	3.3×10^8	16.5	18.4	79.2	2.4
5	100	2.0×10^8	10.0	7.5	82.2	10.3
6	110	2.1×10^8	10.5	4.0	69.4	26.6
7	120	2.1×10^8	10.5	3.8	50.5	45.7
8	130	1.5×10^8	7.5	5.2	36.3	58.5
9	140	4.2×10^7	2.1	6.2	29.5	64.3

[a]JE-5.0 rotor maintained at constant speed of 1800 RPM

[b]Percentage distribution calculated from dot plots of BrdU and total DNA (Fig. 8.5.5).

[c]The percentages from fractions 1 to 11 add up to <100% because small amounts are lost to the system during loading (load fraction) or aggregate in the chamber and are unrecoverable (dump fraction).

12. To collect first fraction, transfer output line to a fresh 250-ml collection vessel. Gradually increase pump speed by 1.0 ml/min increments to 60 ml/min and collect the next 250-ml fraction. Place cells on ice.

    Cold shock will cause sustained growth arrest of some cell types. Placing these populations on ice is not recommended when kinetic cell growth studies are to follow elutriation.

13. Collect remaining fractions in 250 ml medium each at 10 ml/min increments in pump speed. Collect ten samples up to 150 ml/min and place cells on ice.

14. Remove any remaining cells from the rotor using 250 ml medium, and count and concentrate cells as desired (see Basic Protocol, steps 25 and 26).

ANALYSIS OF CELL FRACTIONS TO DETERMINE CELL CYCLE POSITION

Three protocols are presented here for accurately assessing elutriated cell fractions for purity and for verifying cell cycle position. To determine DNA content, elutriated fractions may be examined by flow cytometry using the DNA-binding fluorophore propidium iodide (PI; see Support Protocol 1; also see *UNIT 8.4*). To determine cell cycle stage, nascent DNA synthesis can be assessed by [^{3}H]TdR incorporation into cells (see Support Protocol 2). This is one way of characterizing the proportion of cells that are in S phase. DNA content and active DNA synthesis can be assessed simultaneously by a dual staining method using both PI and 5-bromo-2′-deoxyuridine (BrdU) incorporation (see Support Protocol 3; also see *UNIT 8.2*). The PI staining procedures, both alone and in combination with BrdU incorporation, generally follow the protocols from Becton Dickinson Immunocytometry. Cell cycle stage can also be determined by characterizing the cyclin-dependent kinase (CDK) activity of the cells (see *UNIT 8.2*).

SUPPORT PROTOCOL 1

Standard PI Staining and Flow Cytometric Analysis

Materials

Elutriated cell populations (see Basic Protocol or Alternate Protocol)
PBS (*APPENDIX 2A*), ice cold
Methanol, ice cold

continued

Buffered PI solution (see recipe)
200 U/ml RNase in PBS

12×75–mm tubes
Nylon cloth, 41-μm mesh
Flow cytometer (Becton Dickinson Immunocytometry FACScan or equivalent)

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

1. Set up 12×75–mm tubes with 1×10^6 cells from each elutriated population in 1 ml ice-cold PBS.

   Maintain equal cell density and PI concentration for each sample for comparison of DNA in different populations (also see step 7).

2. While gently vortexing cells, gradually add 1 ml ice-cold methanol in a dropwise fashion. Incubate on ice for 30 min.

   The methanol must be ice cold. Be sure to begin vortexing cells before adding methanol to prevent clumping. Cells are now fixed and may be stored at 4°C for several days prior to analysis.

3. Centrifuge cells at $300 \times g$, room temperature, for 10 min. Carefully aspirate liquid from cell pellet and then loosen pellet by flicking tube or vortexing.

4. Combine equal volumes of buffered PI solution and 200 U/ml RNase solution, and add 500 μl to each tube.

5. Incubate for 30 min at room temperature, protected from light. Transfer tubes to ice and keep on ice until analysis.

6. Immediately before analysis, carefully vortex samples and then filter through a 41-μm nylon mesh to remove any cell clumps.

7. Analyze on a flow cytometer using an FL2 linear detector.

   A histogram of FL2 area versus number of events will show DNA content and cell cycle stage (see Fig. 8.5.3).

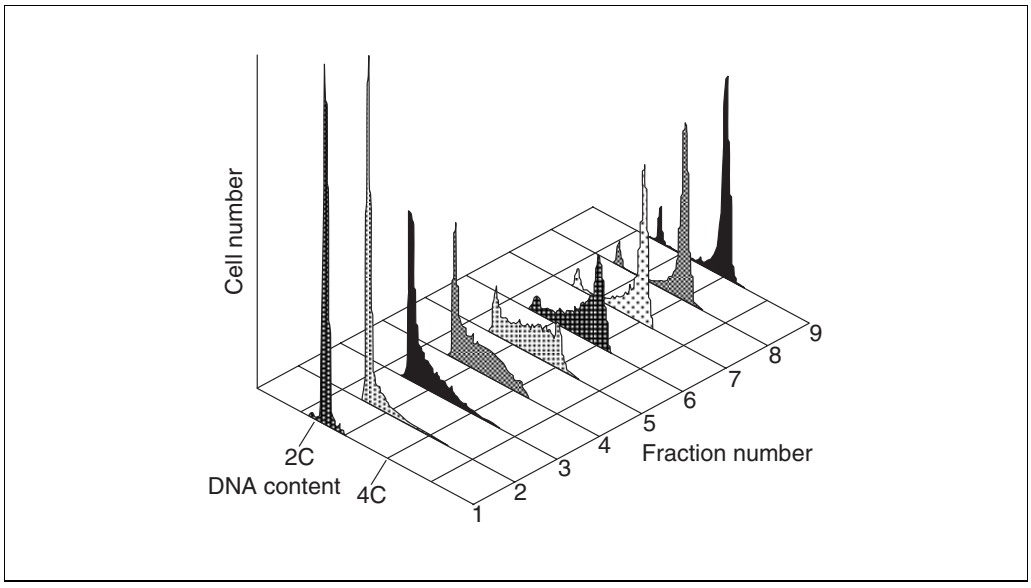


Figure 8.5.3 Three-dimensional histogram of DNA content by flow cytometry versus elutriation fractions of WEHI-231 cells, a murine lymphocytic B cell line. An aliquot of each fraction was fixed in methanol and stained with propidium iodide. The relative DNA content of each fraction was determined by flow cytometry, collecting 10,000 events (*x* axis, propidium iodide fluorescence intensity for DNA content; 2C, diploid; 4C, tetraploid; *y* axis, relative cell number).

Equal cell density between samples is important for equilibration of PI incorporation. Detection of fluorescence intensity will be decreased if greater than equal numbers are used (resulting in a leftward shift in the DNA histogram), or increased if using less than equal cell numbers (resulting in a rightward shift). If cell numbers between samples are fairly close, compensation for this shift may be made by setting a marker at 200 and adjusting FL2 detectors for each sample so that the G_1 peak appears at 200. This should only be done when the position of the G_1 peak is easily recognized.

SUPPORT PROTOCOL 2

[^{3}H]Thymidine Deoxyribose Incorporation

Materials

Elutriated cell populations (see Basic Protocol or Alternate Protocol)
Culture medium prewarmed to 37°C
[Methyl-^{3}H]thymidine deoxyribose ([^{3}H]TdR; 6.7 Ci/mmol)
Aqueous liquid scintillation fluid

96-well microtiter plate
Glass fiber filters
Cell harvester

1. Prepare an aliquot of each elutriated cell population at a density of 4×10^5 cells/ml in an appropriate culture medium. Plate 150 µl of each aliquot into triplicate wells of a 96-well microtiter plate and set up control wells with 150 µl culture medium. Warm the plate at 37°C for 10 min.

   Maintain equal cell numbers between samples for comparison of thymidine incorporation between cell populations.

2. Prepare an aliquot of 37°C culture medium containing [^{3}H]TdR at 10 µCi/ml. Add 50 µl to each well containing cells and each control well.

3. Incubate for 30 min at 37°C.

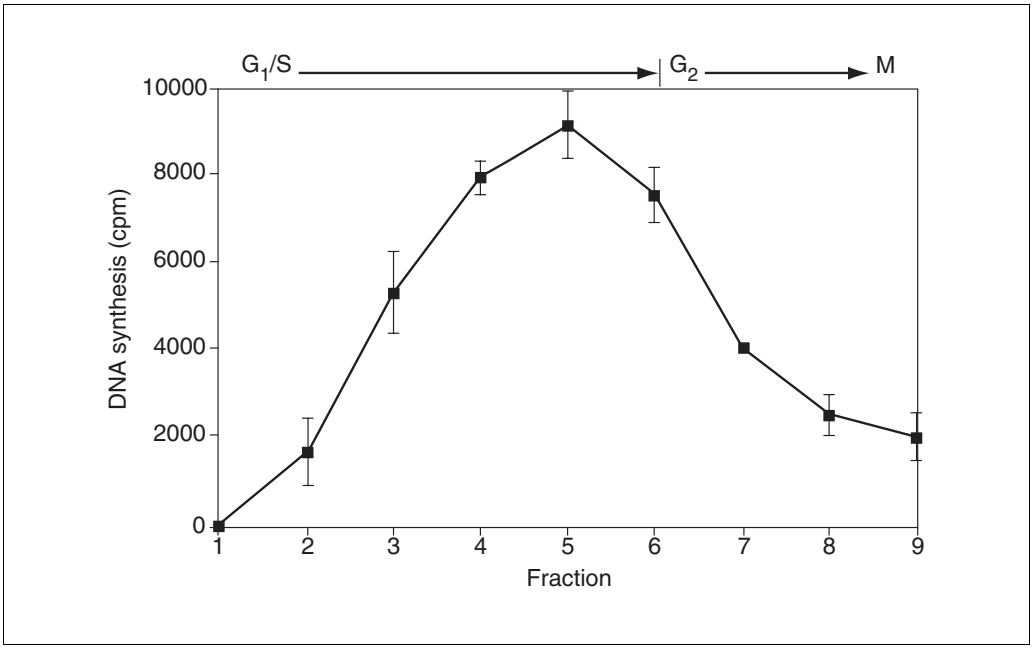


Figure 8.5.4 DNA synthesis profiles of elutriated WEHI-231 murine B cells by [^{3}H]TdR incorporation. The level of cellular DNA synthesis was determined by incubating equal numbers of cells from each elutriated fraction with [^{3}H]TdR for 30 min prior to cell harvest and scintillation counting (*x* axis, elutriation fraction number; *y* axis, DNA synthesis in cpm). On the basis of flow cytometry and DNA synthesis data, relative positions within the cell cycle are shown at the top of each plot. Each data point is the mean of triplicate determinations; bars are SD.

4. Transfer medium and cells onto glass fiber filters using a cell harvester and wash with water.
5. Air dry filters and seal each in a bag with aqueous liquid scintillation fluid.
6. Quantify radioactivity in a β scintillation counter.

A representative graph of DNA synthesis as a function of elutriation fraction number is shown in Figure 8.5.4.

Combined BrdU Incorporation and PI Staining

SUPPORT PROTOCOL 3

Materials

Elutriated cell populations (see Basic Protocol or Alternate Protocol)
Culture medium
1 mM BrdU solution (see recipe)
PTB buffer (see recipe)
PBS (*APPENDIX 2A*), ice cold
100% ethanol, ice cold
2 N HCl/0.5% (v/v) Triton X-100 (store at 4°C)
0.1 M sodium tetraborate ($Na_2B_4O_7$), pH 8.5 (store at 4°C)
Fluorescein isothiocyanate (FITC)–conjugated anti-BrdU antibody
5 µg/ml PI solution (see recipe)

12 × 75–mm tubes
Flow cytometer (Becton Dickinson Immunocytometry FACScan or equivalent)

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

1. Set up 12 × 75–mm tubes with 1×10^6 cells from each elutriated population in 1 ml culture medium.

Maintain equal cell density and PI and BrdU concentration for each sample for comparison of DNA in different populations (also see step 12).

2. Add 10 µl of 1 mM BrdU solution (final 10 µM) and incubate for 15 min at 37°C.

For cell lines with shorter cell division times, incubation time may be reduced to 2 min; slower cycling cells may require a 30-min incubation.

3. Wash cells twice in 3 ml PTB buffer and centrifuge at 300 × *g* for 10 min at room temperature. Aspirate medium, resuspend cell pellet in 500 µl ice-cold PBS, and place cells on ice.

Be certain that PBS is cold and cell pellets are placed on ice both before and after cold ethanol is added.

4. While gently vortexing cells, gradually add 1 ml ice-cold ethanol in a dropwise fashion. Incubate on ice for 30 min.

It is critical that ethanol be ice cold. Be sure to begin vortexing cells before adding ethanol and maintain vortexing to prevent clumping. Cells are now fixed and may be stored several days at 4°C.

5. Centrifuge cells at 300 × *g*, 10°C, for 10 min. Carefully aspirate liquid from cell pellet and then loosen pellet by flicking tube or vortexing.
6. Add 1 ml of 2 N HCl/0.5% Triton X-100 and incubate for 30 min at room temperature.

This step denatures the DNA, producing single-stranded molecules that allow the subsequent binding of anti-BrdU antibody.

7. Centrifuge at 300 × *g*, room temperature, for 10 min. Resuspend pellet in 1 ml of 0.1 M $Na_2B_4O_7$, pH 8.5, to neutralize. Incubate ~10 to 15 min at room temperature.
8. Centrifuge at 300 × *g*, room temperature, for 10 min. Resuspend pellet in 80 µl FITC-conjugated anti-BrdU antibody diluted 1:4 in PTB buffer. Incubate for 30 min at room temperature.
9. Add 3 ml PTB buffer to each tube, centrifuge cells, and resuspend pellet in 500 µl of 5 µg/ml PI solution.
10. Incubate for 15 min at room temperature, protected from light, and then place on ice.
11. Analyze on a flow cytometer using FL1 log (FITC) and FL2 linear (PI) detectors. Show dot plot as FL2 area versus FL1 height.

    A histogram of FL2 area will also show DNA content and cell cycle stage (see Fig. 8.5.5).

    Equal cell density between samples is important for equilibration of PI incorporation. Detection of fluorescence intensity will be decreased if greater than equal numbers are used (resulting in a leftward shift in the DNA histogram), or increased if using less than equal cell numbers (resulting in a rightward shift). If cell numbers are fairly close, compensation for this shift may be made by setting a marker at 200 and adjusting FL2 detectors for each sample so that the G_1 peak appears at 200. This should only be done when the position of the G_1 peak is easily recognized.
12. To calculate percentage of cells in each stage of the cell cycle, draw regions in dot plot and then calculate with Region Stats (standard with FACScan).

    Calculations can also be done using the PI histogram and CELLFIT (Becton Dickinson) formulas; however, the relatively pure cell cycle populations in transition between cell cycle phases obtained through elutriation cells cannot be analyzed, as the Becton Dickinson algorithm requires profiles with G_1, S, and G_2 content. Manually marking peak areas is also inaccurate as S phase overlaps into G_1 and G_2.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BrdU solution, 1 mM

30.7 mg 5-bromo-2′-deoxyuridine (BrdU)
100 ml PBS (*APPENDIX 2A*)
Divide into 1-ml aliquots
Store up to 6 months at −20°C (do not refreeze)

Buffered PI (propidium iodide) solution

10 mg propidium iodide
0.1 ml Triton X-100
3.7 mg EDTA
90 ml PBS (*APPENDIX 2A*)

Combine, stirring gently to dissolve completely (protect from light). Adjust volume to 100 ml with PBS. Store up to 1 month at 4°C protected from light.

CAUTION: *Use gloves. Propidium iodide is a mutagen and suspected carcinogen.*

Elutriation medium

1.9 liters RPMI culture medium
100 ml heat-inactivated FBS (*APPENDIX 2A*)
Make fresh and use at room temperature

The composition of elutriation medium can vary. Typically, it is either culture medium or PBS without Ca^{2+} or Mg^{2+}, and is supplemented with 5% (v/v) FBS or 5% (w/v) BSA.

PI (propidium iodide) solution, 5 μg/ml

Prepare a 100 μg/ml stock solution by mixing 10 ml propidium iodide in 100 ml PBS (*APPENDIX 2A*) and stirring gently to dissolve completely (protect from light). Store up to 6 months at 4°C protected from light. Immediately before use, dilute 1/20 in PBS (final 5 μg/ml).

CAUTION: *Use gloves. Propidium iodide is a mutagen and suspected carcinogen.*

PTB (PBS/Tween/BSA) buffer

2.5 ml Tween 20 (20% final)
5 g BSA (1%)
Adjust to 500 ml with PBS (*APPENDIX 2A*)
Mix slowly to dissolve
Pass through 0.2-μm filter
Store up to 6 months at 4°C

COMMENTARY

Background Information

A detailed characterization of the physical separation properties of the elutriation system can be found in Keng et al. (1981) and the respective JE-6B and JE-5.0 instruction manuals (Beckman Instruments, 1981, 1989). Additional information on cell cycle separation is found in Kaufman et al. (1990) and Donaldson et al. (1997). B cell separation is described in Thompson et al. (1983), separation of T cells in Marjanovic et al. (1991), and separation of mixed populations in Meistrich (1983). CCE is relatively simple to perform and has the advantage of providing large quantities of cells with minimal impact on cell metabolism.

The combined separation and analysis protocols presented here are generally applicable to a wide variety of human and mouse nonadherent cell lines. Specific parameters are given for Jurkat cells using the standard rotor chamber (Table 8.5.1) and for WEHI-231 cells with the large chamber (Table 8.5.2). Although published protocols and the rotor speed and flow rate Nomogram contained in the Beckman elutriator manual will provide approximate starting conditions, a pilot run is likely to be required for each cell line. Cycling cells will separate differently depending on a wide variety of parameters including culture and medium conditions and ploidy. Some primary cells such as stimulated human peripheral T cells are much smaller than their transformed (Jurkat) counterparts, allowing twice as many cells to be loaded (Donaldson et al., 1997). As the majority of stimulated proliferating cells are in the G_1 phase of the cell cycle, with few cells in G_2/M, it may be difficult even in the best logarithmic cultures to obtain highly enriched G_2 fractions. Although elutriation can be carried out with a refrigerated rotor and cold media, the authors have found little advantage to cell viability, and degassing becomes more problematic.

The JE-5.0 rotor with the 40-ml chamber can be used to elutriate ten times as many cells as the JE-6B rotor, and the JE-5.0 is useful for harvesting large volumes of cells from culture medium prior to elutriation. The general conversion from the low-volume rotor used for analysis and methods development to the high-volume rotor for preparative work requires an ~4-fold increase in flow rate. As concentrating cells from large volumes of culture media by repetitive centrifugation can be time consuming and diminish cell viability, harvesting by continuous flow using the JE-5.0 rotor is a convenient alternative. Although it is possible to proceed from harvest in the JE-5.0 directly to sorting the cells without emptying the chamber, in practice, most cells types must first be removed and passed through an 18-G needle to ensure monodispersion prior to sorting.

Cell cycle position and distribution of the elutriated cell populations can be confirmed by several methods, the simplest being pulse labeling of each population with [^{3}H]TdR (Fig. 8.5.4). DNA content can be determined by PI staining and flow cytometry. Fluorescence intensity is directly proportional to DNA content (Fig. 8.5.3). Three-dimensional histograms show total DNA content of each elutriated fraction of WEHI-231 cells determined by flow cytometry of PI-stained nuclear DNA. The method of combined labeling of cell populations with PI for total DNA content and pulse BrdU for nascent DNA synthesis allows for the simultaneous and accurate determination of total DNA and active synthesis (Fig. 8.5.5). Dot plots of DNA content detected by PI staining (*x*

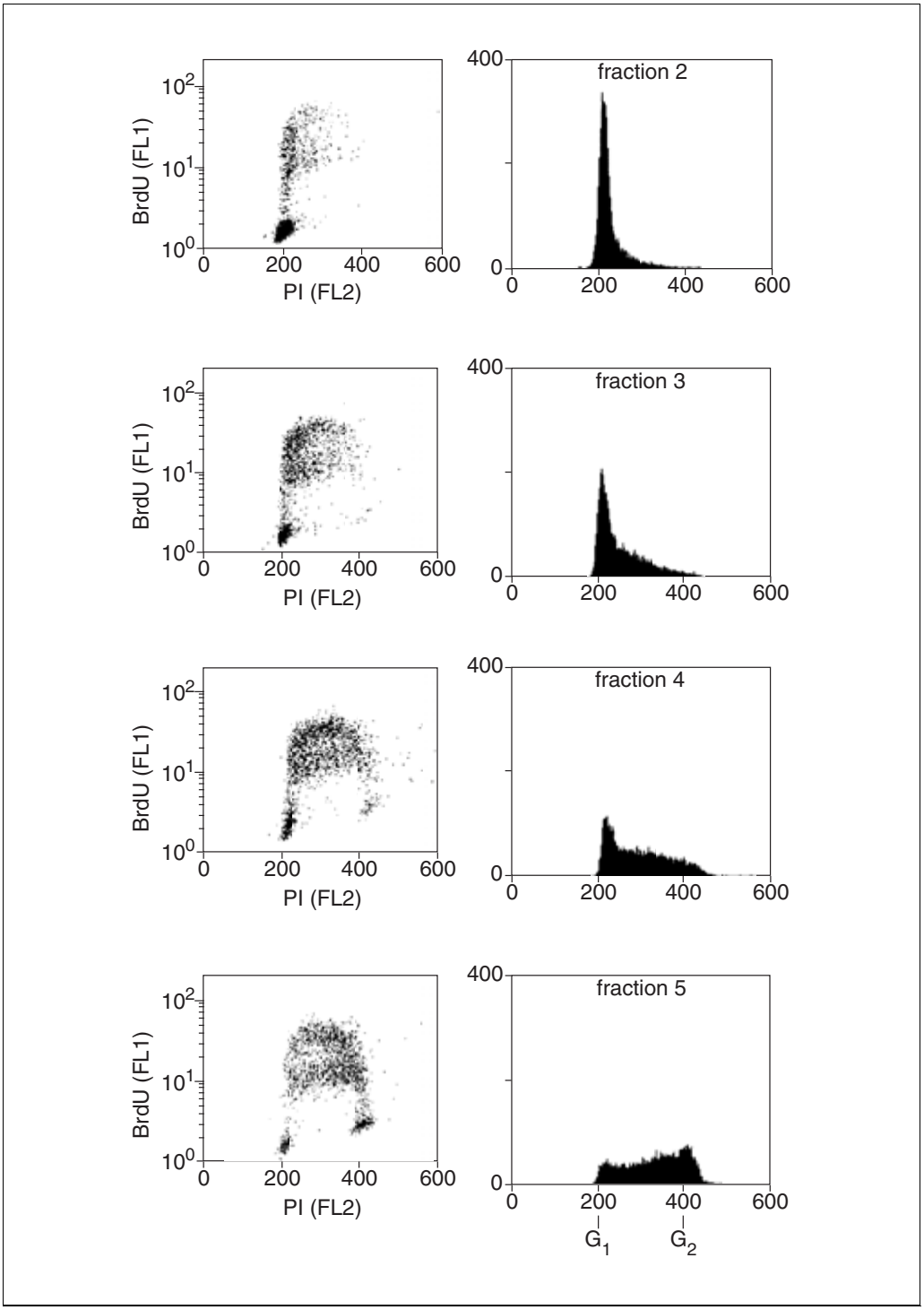
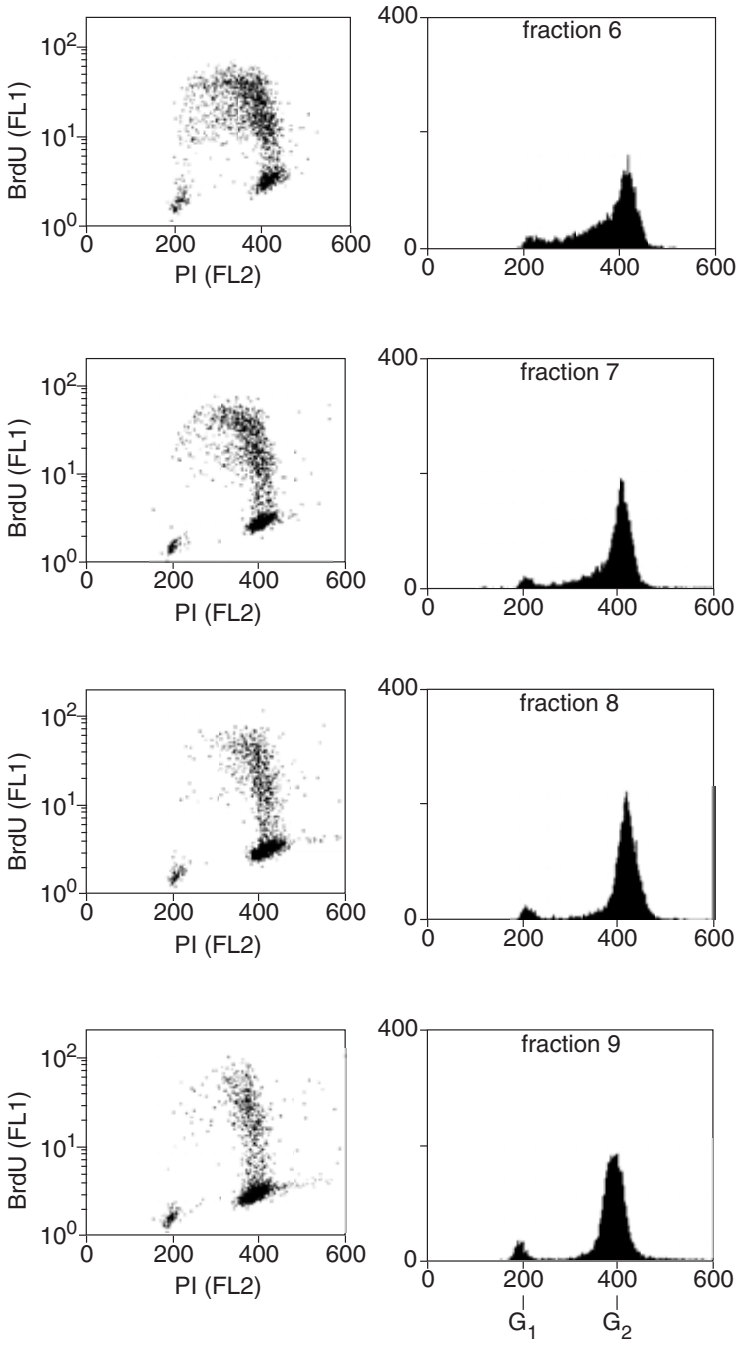


Figure 8.5.5 *(above and at right)* Simultaneous analysis of DNA synthesis and DNA content of elutriated WEHI-231 cells by BrdU incorporation, PI staining, and flow cytometry. Aliquots of cells from each elutriated fraction were pulsed with BrdU for 15 min, fixed in ethanol, and stained for DNA synthesis with FITC-conjugated anti-BrdU antibody and for DNA content with propidium iodide. Dot plots (left) were generated by analysis of 10,000 events and show DNA content by FL2 area (linear; *x* axis) and DNA synthesis by FL1 height (log; *y* axis). The corresponding histogram for DNA content is also shown immediately to the right (*x* axis, PI fluorescence intensity; *y* axis, relative cell number).



axis) with DNA synthesis by FITC-labeled anti-BrdU (*y* axis) are shown for each elutriated fraction. Cells synthesizing DNA shift upward on the *y* axis (FL1 height) and appear as an arc above G_1 and G_2 populations of cells. DNA content by PI staining on the *x* axis (FL2 area) is also shown as a histogram immediately to the right of each dot plot. Accurate percentages of cell cycle populations can be determined by gating separate regions around these G_1, S, and G_2 populations.

Critical Parameters

Experiments should be fully planned and all equipment, reagents, and tubes should be prepared and ready well in advance. Set up, sterilization, and equilibration are time consuming and it is helpful to coordinate these tasks with cell concentration. Even the best biophysical

predictions will not eliminate the need for a pilot elutriation run to verify speed and flow rates needed for optimal separation.

Cells in poor condition are not worth elutriating. Maintaining cells at $2–5 \times 10^5$ cells/ml with systematic 1:2 dilution with fresh medium for the week prior to elutriation will ensure healthy, log-phase cells. For cell cycle studies, this also ensures good yields of S- and G_2/M-phase populations. Cells must be monomeric. Doublets of G_1 cells can approximate the mass of a G_2 cell and contaminate later fractions. Similarly, highly aneuploid cells will not sort well due to overlaps in mass with near-diploid cells at different stages of their division cycle. Highly aneuploid cells can be eliminated from a mixed population by elutriating the mixed cells and harvesting the smallest population. Upon reculture, cells with high ploidy are greatly diminished.

Although the authors do not recommend it for these protocols, some protocols include EDTA or DNase treatment to reduce aggregation. The release of chromosomal DNA from damaged cells is the major cause of clumping within the chamber. Careful handling during concentration, resuspension, and syringe dispersion will greatly reduce lysis. Resuspend the cells in elutriation medium and load the same number of cells each time. Varying the medium or cell number can shift the buoyant density and eluting population.

Anticipated Results

A combination of CCE and analysis of elutriated fractions should provide ten fractions enriched for cells at specific stages of the cell cycle. The viability of the cells is maintained and the elutriated cells are suitable for culture and/or biochemical analysis.

Time Considerations

Effective CCE requires 90 min to set up, sterilize, equilibrate, and calibrate the equipment; 90 min to harvest the cells; and 30 min to elute the fractions. PI staining takes 2 hr, [^{3}H]TdR labeling takes 90 min, and PI staining plus detection of BrdU incorporation takes 4 hr.

Literature Cited

Beckman Instruments. 1981. The JE-6B Elutriation System and Rotor Instruction Manual (JE6B-IM-2). The Spinco Division of Beckman Instruments, Palo Alto, Calif.

Beckman Instruments. 1989. The JE-5.0 Elutriation System Instruction Manual (JE5-IM-1). The Spinco Division of Beckman Instruments, Palo Alto, Calif.

Donaldson, K.L., McShea, A., and Wahl, A.F. 1997. Separation by counterflow centrifugal elutriation and analysis of T- and B-lymphocytic cell lines in progressive stages of cell division cycle. *J. Immunol. Methods* 203:25-33.

Kauffman, M.G., Noga, S.J., Kelly, T.J., and Donnenberg, A.D. 1990. Isolation of cell cycle fractions by counterflow centrifugal elutriation. *Anal. Biochem*. 191:41-46.

Keng, P.C., Li, C.K.N., and Wheeler, K.T. 1981. Characterization of the separation properties of the Beckman elutriator system. *Cell Biophys.* 3:41-56.

Marjanovic, S., Skog, S., Heiden, T., Tribukait, B., and Nelson, B.D. 1991. Expression of glycolytic isoenzymes in activated human peripheral lymphocytes: Cell cycle analysis using flow cytometry. *Exp. Cell Res*. 193:425-431.

Meistrich, M. 1983. Cell separation: Methods and selected applications. *In* Experimental Factors Involved in Separation by Centrifugal Elutriation, Vol. 2 (T. Pretlow II and T. Pretlow, eds.) p. 33. Academic Press, New York.

Thompson, C.B., Ryan, J.J., Sieckmann, D.G., Finkelman, F.D., Mond, J.J., and Scher, I. 1983. A method for size separation of murine spleen cells using counterflow centrifugation. *J. Immunol. Methods* 63:299-307.

Contributed by Alan F. Wahl and Karen L. Donaldson
Seattle Genetics, Inc.
Bothell, Washington

Dynamic Proliferation Assessment in Flow Cytometry

Simone Diermeier-Daucher[1] and Gero Brockhoff[1]

[1]Department of Gynaecology and Obstetrics, University of Regensburg, Regensburg, Germany

ABSTRACT

Dynamic proliferation assessment via flow cytometry is legitimately supposed to be the most powerful tool for recording cell cycle kinetics in-vitro. The preeminent feature is a single cell–based multi-informative analysis by temporal high-resolution. Flow cytometric approaches are based on labeling of proliferating cells via thymidine substitution by a base analog (e.g., 5-bromo-2′-deoxyuridine, BrdU) that is added to cell cultures either for a short period of time (pulse labeling) or continuously until cell harvesting. This unit describes the alternative use of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) in place of BrdU for three different applications: (1) dynamic proliferation assessment by EdU pulse cell labeling; (2) the same approach as (1) but in combination with live/dead cell discrimination; and (3) dynamic cell cycle analysis based on continuous cell labeling with EdU and Hoechst fluorochrome quenching. In contrast to the detection of BrdU incorporation, EdU-positive cells can be identified by taking advantage of click chemistry, which facilitates a simplified and fast cell preparation. Further analysis options but also limitations of the utilization of EdU are discussed. *Curr. Protoc. Cell Biol.* 48:8.6.1-8.6.23. © 2010 by John Wiley & Sons, Inc.

Keywords: cell proliferation • EdU • flow cytometry • cell cycle kinetics

INTRODUCTION

Dynamic analysis of cell proliferation by flow cytometry enables high-resolution identification and quantification of effects and efficacies of growth factors and anti-cancer drugs (e.g., therapeutic antibodies, tyrosine kinase inhibitors) on tumor cells in-vitro (Brockhoff et al., 2001, 2007). In contrast to so-called static DNA analysis, dynamic techniques basically enable investigations into the regulation of cell proliferation under defined conditions: (1) calculation of the distribution of cells that actively contribute to cell multiplication according to their attribution to G1-, S-, and G2/M-phases of the cell cycle; (2) quantification of the percentage of resting cells, i.e., cells not actively contributing to cell multiplication (G0-phase cells); (3) determination of the absolute duration of the phases of cell cycle—up to three sequential cell cycles can be determined; (4) information about the probability with which resting cells are recruited into the "active cell cycle" and with which other cells cross from one phase into the next (transition of restriction points); and (5) retracing of the replication history of individual cells.

In the past, dynamic proliferation assays were usually based on the incorporation of the synthetic thymidine analog 5-bromo-2′-deoxyuridine (BrdU) into the DNA during its replication stage—cells are exposed to the base analog either continuously or during a brief pulse of ∼30 min. Currently, 5-ethynyl-2′-deoxyuridine (EdU, Fig. 8.6.1) can be used in place of BrdU, an alternative approach that features some advantages over the use of BrdU. The proof of thymidine substitution is determined either by immunological antibody staining (anti-BrdU) and/or "click chemistry" (EdU technique), or by using a fluorochrome whose fluorescence characteristics are dependent on the amount of BrdU/EdU incorporated.

Current Protocols in Cell Biology 8.6.1-8.6.23, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb0806s48

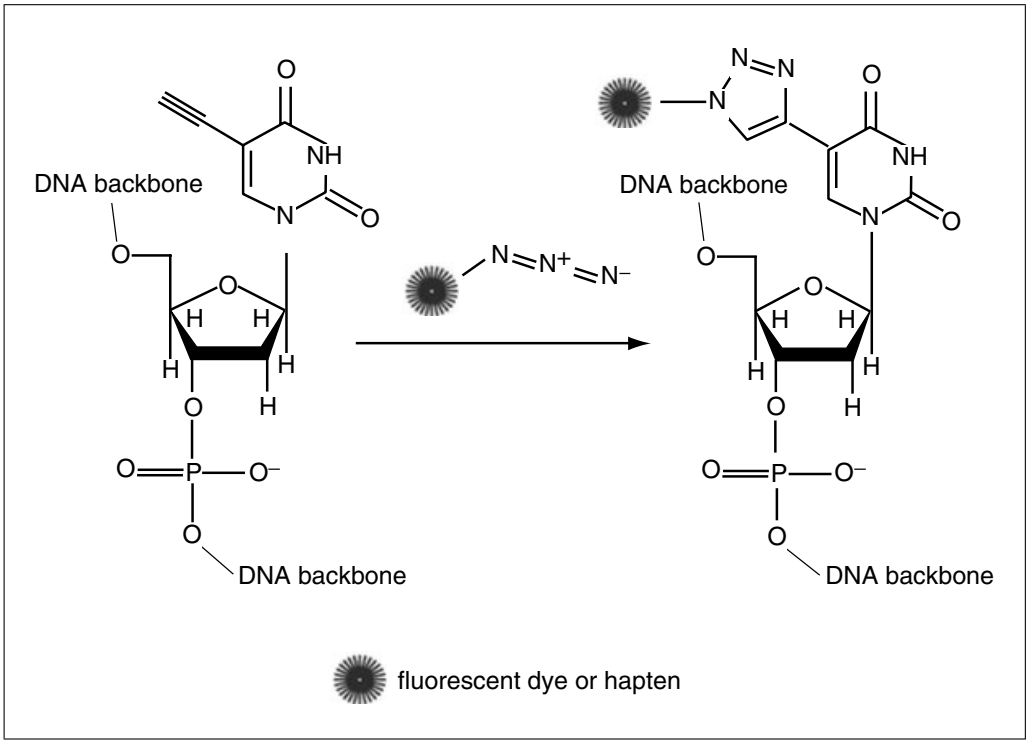


Figure 8.6.1 DNA labeling using the Click-iT EdU Cell Proliferation Assay (Molecular Probes/Invitrogen). The alkyne group of the EdU nucleoside analog reacts with an azide in a copper-catalyzed reaction (click chemistry) forming a very stable covalent bond. Prior denaturation of dsDNA with the antibody-based detection of BrdU-labeled DNA is not required.

S-phase labeling with thymidine analogs has expanded techniques and applications for detailed evaluation of cell cycle progression (Rabinovitch et al., 1988). However, cell line and cell type specific cytostatic effects of the widely used BrdU for cell cycle studies (Diermeier-Daucher et al., 2004) and a technically challenging antibody detection of BrdU demanded alternatives (Warren et al., 2009). 5-Ethynyl-2′-deoxyuridine (EdU, Fig. 8.6.1) is a nucleoside analog of thymidine that is incorporated into DNA during S-phase just like BrdU and is unreactive in biological systems (Buck et al., 2008). To prove substitution of thymidine by EdU, the protocols presented here use either click chemistry (see Basic Protocols 1 and 2; Salic and Mitchison, 2008) or the fluorochrome Hoechst 33258 whose fluorescence characteristics are dependent on EdU incorporation (EdU/Hoechst quenching technology, see Basic Protocol 3; Diermeier-Daucher et al., 2009).

The application of click chemistry to detect EdU incorporation into DNA is based on a copper-catalyzed covalent reaction between a dye-conjugated azide and the alkyne group of EdU (Fig. 8.6.1; Buck et al., 2008; Salic and Mitchison, 2008). Compared to antibody detection of BrdU-based techniques (Darzynkiewicz and Juan, 1997), click chemistry after EdU pulse-labeling allows a simple cell preparation and staining procedure based on aldehyde fixation and detergent permeabilization. This in turn enables penetration of the dye-conjugated azide into the cell, which facilitates coupling of the EdU-alkyne group. In combination with stoichiometric DNA counterstaining, EdU detection facilitates the detailed analysis of cell cycle kinetics and simple multiplexed cell analysis, as well as dead cell exclusion from subsequent analysis (Diermeier-Daucher et al., 2009). Dead cells can be labeled with a dye that permeates leaky cell membranes before DNA labeling takes place. Furthermore, the dead cell fraction can be quantified to evaluate drug-induced cell death in combination with cell cycle analysis of the surviving cell fraction (Diermeier-Daucher et al., 2009). A wide selection of available dyes for live/dead

discrimination (LIVE/DEAD Fixable Dead Cell Stain Kits, Molecular Probes/Invitrogen; see Internet Resources) and fluorescent EdU labeling with click chemistry (Click-iT EdU Cell Proliferation Assays, Molecular Probes/Invitrogen; see Internet Resources) offers an extensive variety of dye combinations that can be selected based on the available flow cytometer and excitation laser lines.

A high time resolution of flow cytometric proliferation assessment is a feature of the recently performed EdU/Hoechst quenching technique (Diermeier-Daucher et al., 2009), which requires a continuous incubation of cells with the nucleoside analog EdU. During the first cell cycle of DNA replication, complete thymidine substitution takes place in one DNA strand and during the second cell cycle in the second DNA strand. DNA of these cells is stained with two DNA dyes: Hoechst 33258 and a DNA intercalating dye, such as propidium iodide (PI). Hoechst 33258 fluorescence is quenched by the EdU/thymidine exchange while PI fluorescence is not affected by EdU incorporation (for excitation and emission wavelengths, see Table 8.6.3). Application of these dyes enables one to distinguish cells in different cell cycle phases (G0/G1, S, and G2/M) of successive cell cycles (Fig. 8.6.2; Diermeier-Daucher et al., 2009). Non-cycling cells (that are driven into the G0-phase by drug treatment) do not incorporate EdU into their DNA, which is a feature that distinguishes them from cycling cells and enables quantification.

This unit presents three protocols for dynamic analysis of cell proliferation. Basic Protocol 1 applies an EdU pulse to label cells in S-phase, click chemistry for EdU detection with Alexa Fluor 488 azide, and stoichiometric DNA labeling with Click-iT EdU cell cycle dye 633 (both Molecular Probes/Invitrogen). The Alternate Protocol extends the procedure described in Basic Protocol 1 to a three-parameter flow cytometric analysis for live/dead discrimination with LIVE/DEAD Fixable Violet dead cell stain (Molecular Probes/Invitrogen) and subsequent fixation prior to EdU and stoichiometric DNA labeling. Support Protocol 1 is suited for extended characterization and quantification of the dead cell fraction determined in Alternate Protocol by determination of the sub-G1 peak. Basic Protocol 2 outlines the procedure for the EdU/Hoechst quenching technique with continuous cell labeling with EdU, cell permeabilization, simultaneous nuclei

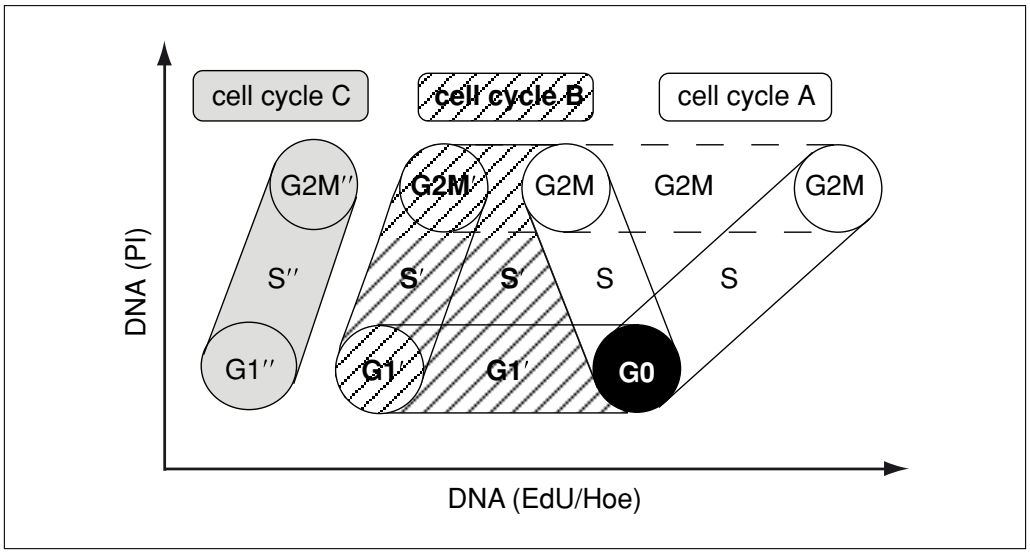


Figure 8.6.2 Schematic bivariate diagram of DNA/Hoechst 33258 versus PI fluorescence intensities. The figure shows the distribution of three successive cell cycles with cell cycle phases G0/G1, S, and G2/M of an asynchronous cell population continuously exposed to EdU. Superscript bars: first (none), second (′) and third (″) cell cycle. For quantitative calculation of the fraction of dormant cells, see instructions under Equation 8.6.1.

preparation, and subsequent staining with Hoechst 33258 and PI. Support Protocol 2 describes the calculation of the G0 cell fraction.

STRATEGIC PLANNING

Number of Samples for EdU Pulse–Labeling Experiments

Control samples

Two samples should be prepared for background staining control (see Table 8.6.1): (1) no EdU treatment, no staining (sample no. 1), (2) EdU treatment, no staining (sample no. 2). In addition, EdU negative cells are used for an unspecific Alexa Fluor 488 staining control (sample no. 3).

These protocols circumvent the need for compensation and compensation controls due to appropriate dye selection for the available flow cytometer. However, if the experimenter wants to check if the respective experiment requires compensation, prepare an unstained sample, single-stained control samples (see Table 8.6.1, samples no. 4 and 5; and Table 8.6.2, sample B), and one sample labeled with two (see Basic Protocols 1 and 2) or three (see Alternate Protocol) fluorescent dyes. Theoretical background on and the procedure of compensation in flow cytometry are described in detail in Roederer (2002).

Test samples

EdU cell proliferation assessment is highly suited for comparing the effects of different treatment modalities on cell cycle progression. Cells are labeled with EdU and treated with the agent of interest to slow down or accelerate proliferation of the respective cell type compared to untreated cells. Any treatment modality requires one additional (EdU-labeled) cell culture flask (Table 8.6.1: samples no. 7 to *x*) to be seeded. One drug-untreated (EdU-labeled) sample must be prepared to serve as a control (Table 8.6.1: sample no. 6).

The number of parallel cultures that is needed for dynamic cell proliferation assessment depends on the total period of time that the cell kinetic is to be analyzed. For pulse-labeling techniques, the observation period should span the time from the detection of initial EdU-positive, S-phase cells until they proceed into S-phase from the G1-phase of the second cell cycle. This procedure allows one to determine the duration of the entire cell cycle with single cell cycle phases in the order: G2/M-phase (1st cell cycle), S-phase (1st cell cycle), and G1-phase (2nd cell cycle). For most cell types, a period of 40 hr

Table 8.6.1 Untreated Control (1 to 6) and Drug-Treated Test Samples (7 to *x*) for Dynamic Cell Proliferation Assessment with EdU Cell Labeling and Click Chemistry for EdU Detection in Basic Protocol 1

No.	Drug treatment	EdU	Alexa Fluor 488	Click-iT EdU cell cycle dye 633	
1	–	–	–	–	Background staining control
2	–	+	–	–	Background staining control
3	–	–	+	–	Unspecific staining control
4	–	–	–	+	Positive single staining
5	–	+	+	–	Positive single staining
6	–	+	+	+	Double stained test sample
7 – *x*	+	+	+	+	Double stained test sample

Table 8.6.2 Samples Labeled Differently or Prepared in Addition to Basic Protocol 2 Compared to Samples Prepared for Basic Protocol 1

No.	Drug treatment	EdU	LIVE/DEAD Violet dead cell stain	Alexa Fluor 488	Click-iT EdU cell cycle dye 633	
6	–	+	+	+	+	Triple stained test sample
7 – *x*	+	+	+	+	+	Triple stained test sample
A	–	–	+	–	+	Unspecific staining control
B	NaN_3	–	+	–	+	Positive control for cell death/single staining

is sufficient for analysis (Brockhoff, 2009). For quenching analysis, the total period of continuous EdU labeling can span the total period of three successive cell cycles.

Additional control samples for live/dead discrimination

Live/dead discrimination as described in the Alternate Protocol requires additional control samples (Table 8.6.2) compared to Basic Protocol 1. While samples 1 through 5 remain the same, an additional sample A serves as a nonspecific staining control for the LIVE/DEAD Fixable dead cell stain. The NaN_3-treated (and LIVE/DEAD Fixable dead cell stained) sample B is used for BT474 cell death induction and consequently serves as a positive control. For analysis of other cell lines, select an appropriate treatment for the positive control sample. Samples no. 6 and 7 to *x* are triple stained (live/dead discrimination, EdU-labeled, and DNA content).

Time Schedule for Cell Culture

The protocols in this unit describe an equal growth period for all cell culture flasks of one experiment to ensure that the same growth density is obtained at time of harvesting. All cell culture flasks are seeded and harvested on the same day, but are incubated with EdU or drugs (e.g., growth factors, therapeutic antibodies, or cell cycle inhibitors) for different time intervals. The addition of these reagents must take place at different time points. For evaluation of cell cycle kinetics and of the time interval of interest (where differences in cell cycle progression become apparent, see Strategic Planning, Control samples), add EdU at 2-hr intervals (and wash out for pulse-labeling techniques) before harvesting to produce, e.g., 40, 38, 36, 34, 32, and 30 hr of cell culture before harvesting. Add drug of interest sufficiently before EdU addition takes place so that the effect of the drug will become apparent compared to untreated cells. Determination of cell number at harvest offers an additional parameter to check for an effect of the drug on cell multiplication.

Cell Culture for Optimal Growth

The total duration of the cell cycle depends on the specific cell line. To ensure subconfluent conditions at the time of harvest and to prevent cell-cell contact-induced inhibition of proliferation, the optimal plating density must be determined for each individual cell line.

Dye Combinations and Available Laser Lines

The choice of fluorescent dyes for EdU detection, DNA labeling, and live/dead discrimination depends on the number and wavelength of the available laser lines and must be adapted according to the available flow cytometer. Basic Protocol 1 applies Alexa Fluor 488 and Click-iT EdU cell cycle dye 633 and is suited for a two-laser flow cytometer

with two different laser lines (488 nm and 633 nm). The set of dyes used in the Alternate Protocol is expanded for the Live/Dead Fixable Violet dead cell stain, which represents an optimal dye combination for a three-laser flow cytometer (488 nm, 633 nm, 405 nm). For applying Basic Protocol 2, a 488-nm and UV (325 nm) excitation line is required for PI and Hoechst 33258 excitation, respectively. Dye characteristics of fluorochromes used in this unit are outlined in Table 8.6.3.

CAUTION: Dye solutions are potentially toxic and/or mutagenic. Handle with appropriate care. See manufacturer's safety datasheets.

For planning a three-color experiment, first assign one dye per available laser line before the two-fold assignment of two fluorochromes to the same laser line to minimize spectral overlap and to avoid the need for compensation (see Roederer, 2002, for background on compensation).

Alternative dyes for EdU detection (see Internet Resources) and live/dead labeling (see Internet Resources and Bradford and Buller, 2008) in Basic Protocol 1 and Alternate Protocol are available for custom-made multicolor experiments. For stoichiometric DNA labeling in EdU pulse-labeling experiments, alternative DNA dyes have been proven to be compatible with click chemistry for EdU detection (see Table 8.6.4; Bradford et al., 2008a,b).

PI is not recommended as a DNA dye in combination with EdU detection by click chemistry due to the increase of coefficients of variation (CV; for details about the importance of low CVs, see Brockhoff, 2009).

Table 8.6.3 Excitation/Emission Maxima, Excitation Wavelength, and Filter Characteristics for Flow Cytometric Detection of Dyes

Dye	Absorption maxima (nm)	Emission maxima (nm)	Excitation wavelength (nm)	Filter[a]
Hoechst 33258	345	478	325	450/50 BP
LIVE/DEAD Fixable Violet dead cell stain	416	451	405	450/50 BP
Alexa Fluor 488	495	519	488	530/30 BP
Propidium iodide (PI)	536	617	488	670 LP
Click-iT EdU cell cycle dye 633	640	658	633	660/20 BP

[a]BP, band-pass filter; LP, long-pass filter.

Table 8.6.4 Alternative DNA Dyes Compatible with Click Chemistry for EdU Detection Absorption and Emission Maxima, Excitation Wavelength, and Filter Characteristics for Flow Cytometric Detection[a]

Dye	Absorption maxima (nm)	Emission maxima (nm)	Excitation wavelength (nm)	Filter[b]
Hoechst 33258	343	483	325	450/50 BP
4′,6-Diamidino-2-phenylindol (DAPI)	358	461	325	450/50 BP
Vybrant DyeCycle Violet stain	369	437	325 or 405	450/50 BP
7-Aminoactinomycin (7-AAD)	546	647	488	695/40 BP
Sytox Red	640	658	633	660/20 BP

[a]Bradford et al., 2008a,b.
[b]band-pass filter.

Preparation of Nuclei

The detergent IGEPAL (see Basic Protocol 2) is used for nuclei preparation. Consequently, cytoplasmic or cell membrane–located antigens cannot be analyzed at the same time. However, the staining procedure may be simultaneously combined with an immunofluorescence staining to reveal detailed information on the expression of cell cycle–related nuclear antigen expression such as proliferation-associated antigens (Endl et al., 1997).

DYNAMIC CELL PROLIFERATION ASSESSMENT WITH EdU CELL LABELING AND DETECTION BY CLICK CHEMISTRY

BASIC PROTOCOL 1

In this protocol, parallel cultures of the cell line of interest are treated with the drug or growth factor of interest at different time points or are left untreated. After pulse-labeling of cell cultures with 5-ethynyl-2′-deoxyuridine (EdU) and removal of EdU by washing the cells thoroughly, the cells are cultured further until harvested in parallel. Detached cells are fixed and permeabilized for subsequent Alexa Fluor 488 azide coupling to EdU by click chemistry and DNA-labeling with Click-iT EdU cell cycle dye 633. This protocol is optimized for the human breast cancer cell line BT474.

Materials

Click-iT EdU Alexa Fluor 488 Cell Proliferation Assay Kit (store kit at 2°–6°C, do not freeze):
- 5-Ethynyl-2′-deoxyuridine (EdU)
- DMSO
- Click-iT fixative
- 10× saponin-based permeabilization and wash reagent
- Alexa Fluor 488 azide
- 10× Click-iT EdU reaction buffer
- 100 mM $CuSO_4$ stock solution
- Click-iT EdU buffer additive
- Click-iT EdU cell cycle dye 633
- 20 mg/ml RNase A

PBS/1% BSA: phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 1% bovine serum albumin (BSA); store up to 2 weeks at 4°C

BT474 breast cancer cells (ATCC #HTB-20; see *UNIT 1.1* for culture of mammalian cells)

Complete Dulbecco's modified Eagle medium (DMEM; *UNIT 1.1*)

Complete DMEM containing 5% fetal bovine serum (FBS; DMEM-5; *UNIT 1.1*)

Complete DMEM containing 2% FBS (DMEM-2)

Drug to be tested for its effect on cell proliferation (e.g., trastuzumab, also known as rhuMAb, 4D5, and herceptin, humanized monoclonal antibody acting on the ErbB2 (HER2/neu) receptor)

Trypsin/EDTA solution (*APPENDIX 2A*)

PBS/0.01% NaN_3/0.2% BSA: phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 0.01% NaN_3 and 0.2% BSA; store up to 2 weeks at 4°C

75-cm^2 cell culture flasks

15- and 50-ml centrifuge tubes (e.g., polypropylene tubes, Greiner Bio-one)

Refrigerated centrifuge, 4°C

Water jet vacuum pump (e.g., BRAND), optional

Hemacytometer (*APPENDIX 3B*)

5-ml sample tubes (e.g., 12 × 75–mm polystyrene round-bottomed tubes, BD)

Flow cytometer with 488-nm and 633-nm excitation lasers (see Table 8.6.3 for required/appropriate filters)

Acquisition software that stores data in list-mode flow cytometry standard (FCS) files (e.g., BD FACS Diva software v6.1.1, BD Biosciences)
Evaluation software (e.g., FlowJo 7.6.1, Tree star)

NOTE: Protect labeling solutions and labeled samples from light.

Prepare working solutions with components of Click-iT EdU Alexa Fluor 488 kit

1. Allow all solutions to warm to room temperature.
2. Dissolve 10 mg EdU in 4 ml DMSO (10 mM final EdU concentration), mix well.
3. Dilute 10× saponin-based permeabilization and wash reagent 1:10 with PBS/1%BSA.

   CAUTION: *This solution contains sodium azide. Acidic conditions liberate very toxic gas. Avoid contact with skin.*

4. Add 260 µl DMSO to Alexa Fluor 488 azide, mix well.
5. Dilute 10× Click-iT EdU reaction buffer 1:10 with distilled water to give a 1× Click-iT EdU reaction buffer working solution.
6. Reconstitute Click-iT EdU buffer additive with 4 ml of distilled water to give a 10× stock solution and mix well until powder has fully dissolved.
7. Add 270 µl DMSO to Click-iT EdU cell cycle dye 633 and mix well.

   CAUTION: *Click-iT EdU cell cycle dye is a nucleic acid–intercalating dye and is considered a potential mutagen that must be handled with care and stored according to the local regulations.*

   The 1× saponin-based permeabilization, wash solution, $CuSO_4$ stock solution, and 1× Click-iT EdU reaction buffer working solution can be stored for up to 6 months at 2° to 6°C. Reconstituted EdU solution, Alexa Fluor 488 azide working solution, 10× Click-iT EdU buffer additive solution, and Click-iT EdU cell cycle dye 633 can be stored for up to 1 year at ≤20°C.

   Saponin-based permeabilization and 1× wash solution supplied with the Click-iT EdU Alexa Fluor 488 Cell Proliferation Assay Kit may be substituted with PBS/1% BSA containing 0.05% (v/v) saponin.

   Commercially available formalin (e.g., SG Planung) or a freshly prepared 4% (w/v) paraformaldehyde (PFA; see recipe) may be used in place of Click-iT fixative.

Pulse label cells with EdU

8. Seed 1×10^6 exponentially growing cells in complete DMEM with 5% FBS (DMEM-5) in four 75-cm^2 cell culture flasks (day zero). Seed as many test samples that are to be treated both with EdU and the drug under investigation as required.

   Two flasks are labeled with EdU (cells from these flasks are used for samples no. 2, 5, and 6, Table 8.6.1). The remaining two flasks are used as EdU-negative control (cells from these flasks are used for samples no. 1, 3, and 4, Table 8.6.1).

   For running all control samples given in Table 8.6.1, 3×10^6 drug-untreated, EdU-positive cells and 3×10^6 drug-untreated, EdU-negative cells should be available after cell harvesting (steps 17 to 20). The cell culture protocol described here provides ~3×10^6 cells at time of harvesting per 75-cm^2 cell culture flask. Therefore, seeding four 75-cm^2 cell culture flasks (two for EdU treatment and two untreated) are sufficient to give this number of cells.

   As the total number of samples depends on the experimental setup (e.g., the number of control samples and drug-treated samples), the volumes for reagents in the protocols refer to 1×10^6 cells and therefore to one sample.

Larger cell culture flasks (e.g., 175-cm^2 cell culture flasks) and a higher number of cells (e.g., 2.3 × 10^6 cells per 175-cm^2 cell culture flasks) may be seeded to yield a sufficient number of control samples and to reduce work load and material consumption. However, for cell proliferation assessment and for comparing drug-untreated (Table 8.6.1, sample no. 6) versus drug-treated samples (Table 8.6.1, samples no. 7 to x), applying the same cell culture conditions (i.e., seeded cell number, size of cell culture flasks) is essential.

9. (Optional) Replace medium after 8 hr of growth with complete DMEM with 2% FBS (DMEM-2; day 0).

   For testing for the effect of different growth factors on cell cycle progression, reduction of FBS in the medium from 5% to 2% is recommended to reduce the amount of disturbing growth factors in the FBS.

10. Add drug to test samples (see Table 8.6.1, samples no. 7 to *x*), e.g., 10 μg/ml trastuzumab (day 1) and continue cell culturing.

11. Add 10 μM EdU for 3 hr to the two cell culture flasks for positive control samples (Table 8.6.1, samples no. 2, 5, and 6) and to all drug-treated test samples (Table 8.6.1, samples no. 7 to *x*) (day 2). Leave two cell culture flasks EdU-untreated as the negative control samples (Table 8.6.1, samples no. 1, 3, and 4). Provide standard cell culture conditions for cell culture flasks during the incubation time.

12. Add 10 μl DMSO (EdU solvent) to EdU-untreated cell culture flasks (Table 8.6.1, samples no. 1, 3, and 4).

    If preliminary experiments showed that addition of DMSO does not affect cell proliferation of the cell line under investigation, then this step can be omitted.

13. Remove the medium after 3 hr of EdU incubation.

14. Carefully wash cells with 10 ml PBS (37°C) to each culture flask and remove with vacuum. Repeat two times to completely remove the EdU from the cells.

15. Add fresh complete DMEM-5 (or DMEM-2, see above) and incubate under standard cell culture conditions until cells are harvested.

16. If cells have been treated with a drug in step 10, then replace the reagents in respective cell culture flasks and incubate under standard cell culture conditions until cells are harvested.

Harvest and fix cells

17. Harvest the cells using trypsin/EDTA solution (e.g., see *UNIT 1.1*) on day 4 for BT474. Collect cells into 50-ml centrifuge tubes, and transfer onto ice.

    If using trypsin/EDTA solution for cell harvesting, stopping the enzymatic reaction with medium containing FBS may stimulate the cells. However, each experimenter must decide, according to the applied drug treatment, if stopping the enzymatic reaction with FBS-containing medium is appropriate. Alternatively, trypsin enzyme activity may be stopped using ice-cold medium with FBS or accutase, or solely EDTA may be used for cell harvesting. Cells from the two EdU-labeled and EdU-unlabeled 75-cm^2 cell culture flasks may be merged.

18. Centrifuge cells 5 min at ~350 × *g*, 4°C.

19. Decant or remove the supernatant with a water jet vacuum pump.

20. Resuspend the cell pellet in 1 to 2 ml ice-cold PBS/0.01% NaN_3/0.2% BSA.

21. Repeat steps 18 to 20.

22. Determine cell number using a hemacytometer, as described in *UNIT 1.1*. Add 1×10^6 cells per sample (see Table 8.6.1) to 5-ml sample tubes.

    Samples no. 5 and 6 (Table 8.6.1) may be stained with Alexa Fluor 488 azide in one tube and divided before the DNA labeling steps.

23. Centrifuge cells 5 min at ~350 × *g*, 4°C.

24. Decant or remove the supernatant with a water jet vacuum pump.

25. Add 100 µl Click-iT fixative to each sample (containing 1×10^6 cells per sample) while vortexing to avoid cell clumping.

    CAUTION: *Click-iT fixative contains 4% PFA in PBS. PFA is harmful and should be handled with appropriate care.*

26. Incubate for 15 min at room temperature in the dark.

Wash and permeabilize cells

27. Add 3 ml of 1× saponin-based permeabilization buffer.

28. Centrifuge cells 5 min at ~350 × *g*, 4°C.

29. Decant or remove the supernatant with a water jet vacuum pump.

30. Repeat steps 27 to 29.

Couple EdU using click chemistry

31. Prepare 500 µl Click-iT reaction buffer per sample (1×10^6 cells) with the following components and use within 15 min:

    438 µl 1× Click-iT Edu reaction buffer (working solution)
    10 µl $CuSO_4$
    2.5 µl Alexa Fluor 488 azide
    50 µl reaction buffer additive

32. Add 500 µl Click-iT reaction buffer to samples no. 3, 5, 6, and to all test samples (Table 8.6.1, samples no. 7 to *x*), and mix well.

33. Add 500 µl Click-iT reaction buffer without adding the Alexa Fluor 488 dye to unstained samples (Table 8.6.1, samples no. 1, 2, and 4).

    Alternatively, 500 µl of 1× saponin-based permeabilization buffer is added to samples no. 1, 2, and 4. However, preliminary experiments to prove that incubation in 1× saponin-based permeabilization buffer does not give different results than the Click-iT reaction buffer without dye are required.

    Saponin temporarily permeabilizes the cell membrane to allow entry of reagents. Although saponin is not present in the Click-iT reaction buffer, it has been proved that permeability is sufficient for dye azide entry and the click reaction. However, for further wash steps and DNA labeling, saponin-containing buffer is used to allow dye entry into the cells.

34. Incubate 30 min at room temperature in the dark.

Label DNA content

35. Centrifuge cells 5 min at ~350 × *g*, 4°C.

36. Decant or remove the supernatant with a water jet vacuum pump.

37. Prepare 500 µl DNA labeling solution per sample (1×10^6 cells) with 493 µl of 1× saponin-based permeabilization buffer, 5 µl RNase A, and 2 µl Click-iT EdU cell cycle dye 633.

Usually, more than one sample is labeled with DNA dye in an experimental setup. To optimize the comparability of the measured fluorescence intensities and to reduce pipetting errors, all samples should be labeled with the same dye concentration. Therefore, preparing the necessary volume of the DNA labeling solution in one vial for all samples is recommended.

38. Add 500 µl of DNA labeling solution to each test sample (Table 8.6.1, samples no. 7 to *x*) and to control samples no. 4 and 6.

39. Add 500 µl of 1× saponin-based permeabilization buffer to the remaining control samples no 1, 2, 3, and 5.

40. Incubate 15 min at 37°C in the dark.

Measure fluorescence

41. Measure fluorescence without further washing using filter settings as described in Table 8.6.3.

42. If available, activate 633-nm excitation pulse processing option on Click-iT EdU cell cycle dye 633 detection channel (DNA content) for gating aggregates/doublets on the flow cytometer (for required filter settings, see Table 8.6.3)

43. Draw the following plots and histograms with the acquisition software:

    a. FC/SC dot blot,
    b. Histogram for Alexa Fluor 488 detection (optional),
    c. Histogram for Click-iT EdU cell cycle dye 633 detection (optional),
    d. Click-iT EdU cell cycle dye 633 area/Alexa Fluor 488 area dot plot.

    Use pulse-processing for gating of doublet cells (Brockhoff, 2009), if available, on the instrument. For example, using instruments from BD Biosciences draw a Click-iT EdU cell cycle dye 633 width/area dot plot.

44. Set the detector gains for Alexa Fluor 488 detection channel (EdU coupling) to logarithmic signal amplification and for Click-iT EdU cell cycle dye 633 detection channels (DNA content) to linear signal amplification in such a way as to enable discrimination of EdU-positive and EdU-negative cells in the Alexa Fluor 488 detection channel and to gain a cell cycle histogram in the Click-iT EdU cell cycle dye 633 detection channel.

    Alexa Fluor 488 and Click-iT EdU cell cycle dye 633 were selected for this assay as their excitation and emission maxima are largely separated from each other (see Table 8.6.3) and are suited for excitation with two different lasers. These dye characteristics allow avoiding the need for compensation and compensation controls. If the experimenter wants to check whether compensation is needed with his experimental system, see Strategic Planning, Dye Combinations and Available Laser Lines.

45. Acquire at least 1×10^4 cells per sample with appropriate software that stores data in list-mode flow cytometry standard (FCS) files (e.g., BD FACS Diva software v6.1.1).

Perform data analysis

46. Analyze data with appropriate software (e.g., FlowJo 7.6.1).

47. Gate live cells in FC/SC dot plot.

48. If the pulse-processing option is supported by the flow cytometer and was used during acquisition, gate single cells, e.g., in the Click-iT EdU cell cycle dye 633 width/area dot plot for BD instruments.

49. Visualize gated cells in the Click-iT EdU cell cycle dye 633 area/Alexa Fluor 488 area dot plot (see Anticipated Results; Fig. 8.6.3).

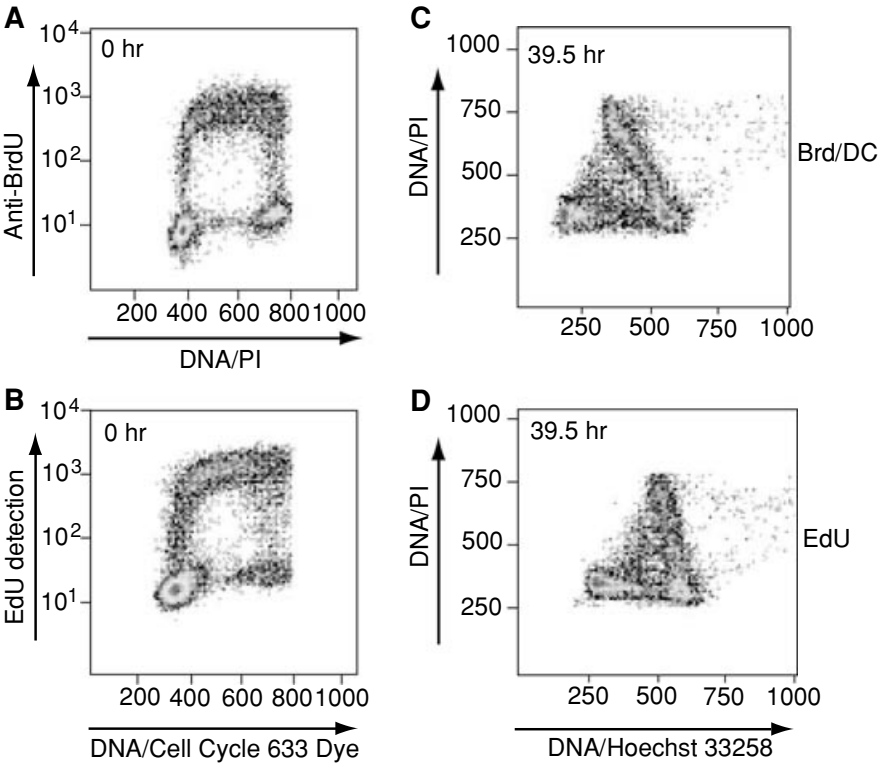


Figure 8.6.3 Comparison of BrdU/DC and EdU for dynamic cell proliferation assessment in BT474 human breast cancer cells. Anti-BrdU (**A**) versus click chemistry detection of EdU (**B**) measured on a FACSCanto II (BD Biosciences). Cells were pulse labeled with 20 μM BrdU/10 μM 2′-deoxycytidine (DC) for 30 min (A) or with 20 μM EdU for 3 hr (B). DNA labeling was performed with PI for BrdU-(A) and Click-iT EdU cell cycle dye 633 for EdU-labeled cells as described in Basic Protocol 1 (B). **C, D**: BrdU/Hoechst quenching versus EdU/Hoechst quenching measured on an LSRII (BD Biosciences). Cells were continuously labeled with 120 μM BrdU/60 μM DC (C) or 100 μM (D) EdU for 39.5 hr and stained with Hoechst 33258 and PI as described in Basic Protocol 3 (Diermeier-Daucher et al., 2009). The slope of BrdU/DC (C) and EdU (D) treated cells proliferating from G1- into S- and G2/M-phase in the first cell cycle depends on the ratio of the concentration of DNA dyes and the amount of nucleoside analog incorporated into the DNA and is therefore different for both nucleoside analogs (also see Critical Parameters and Troubleshooting: Hoechst/PI Concentrations).

ALTERNATE PROTOCOL

DYNAMIC CELL PROLIFERATION ASSESSMENT WITH EdU CELL LABELING AND DETECTION BY CLICK CHEMISTRY IN COMBINATION WITH LIVE/DEAD DISCRIMINATION

The LIVE/DEAD Fixable dead cell stains (Molecular Probes/Invitrogen) allow evaluation of the viability of mammalian cells by flow cytometry. This is accomplished by the reaction of a fluorescent reactive dye with cellular amines in combination with further antigen (which is beyond the scope of this unit) or DNA staining after cell fixation in a multicolor experiment. In necrotic cells, the reactive dye permeates the compromised membranes and reacts with free amines both in the interior of the cell and on the cell surface. Therefore, fluorescent staining is intense compared to staining of only cell-surface amines of viable cells showing a relatively dim staining (see Internet Resources; Diermeier-Daucher et al., 2009). Staining is completely preserved after fixation of the sample with formaldehyde.

The single-color LIVE/DEAD Fixable Dead Cell Stain Kits are available with different fluorescent colors of the included dye (blue, violet, aqua, yellow, green, red, far red,

or near IR; Internet Resources). This protocol describes the optimal dye selection for live/dead discrimination followed by cell cycle analysis with click chemistry with a three-laser flow cytometer: 405-nm (excitation of LIVE/DEAD Violet dead cell stain; live/dead discrimination), 488-nm (excitation of Alexa Fluor 488; EdU labeling), and 633-nm (excitation of Click-iT EdU cell cycle dye 633; DNA content) laser lines. For alternative dye selection, see Strategic Planning, Dye Combinations. This protocol is optimized for the human breast cancer cell line BT474.

***Additional Materials** (also see Basic Protocol 1)*

LIVE/DEAD Fixable Violet Dead Cell Stain Kit for 405-nm excitation (Molecular Probes/Invitrogen)
Drug for induction of cell death in the cell line of interest, e.g., sodium azide (NaN_3; Sigma) for BT474 (Diermeier-Daucher et al., 2004)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
4% (w/v) paraformaldehyde (PFA; see recipe)

NOTE: Protect labeling solutions and labeled samples from light.

Prepare working solution of LIVE/DEAD Fixable Violet dead cell stain

1. Allow one vial of LIVE/DEAD Fixable Violet dead cell stain and DMSO to warm to room temperature.
2. Dissolve LIVE/DEAD Fixable Violet dead cell stain in 50 µl DMSO and mix well until all dye has dissolved.
3. Use working solution as soon as possible (within a few hours after reconstitution).

   Once reconstituted, LIVE/DEAD Fixable Violet dead cell stain may be stored up to 2 weeks at ≤20°C.

Seed and pulse label cells with EdU

4. Seed cells as described in Basic Protocol 1. Provide for an additional 1×10^6 EdU-unlabeled and drug-untreated cells (unspecific staining control) and an additional 75-cm^2 cell culture flask to induce cell death (positive control, see Table 8.6.2).
5. Proceed with Basic Protocol 1, steps 9 through 16.
6. Add drug for induction of cell death to positive control cells (sample B) at an appropriate time point before harvesting.

   For the BT474 breast cancer cell line, incubation with 180 mM NaN_3 for 4 hr can be used as positive control (Diermeier-Daucher et al., 2004).

Harvest cells

7. Harvest the cells on day 4 for BT474 (the optimal time point for harvesting of other cell lines must be determined individually) using trypsin/EDTA solution (e.g., see *UNIT 1.1*), collect into 50-ml centrifuge tubes, and transfer onto ice.

   See annotation in Basic Protocol 1, step 17.

8. Centrifuge cells 5 min at ~350 × *g*, 4°C.
9. Remove the supernatant.

   If it is aimed to identify and to discriminate dead cells from the evaluation, the cell culture supernatant should be removed using a water jet vacuum pump. However, if the amount of cell death (e.g., in response to drug treatment) needs to be analyzed and quantified, collect the cell culture supernatant (including apoptotic and necrotic cells) and combine it with the cell suspension after trypsinization.

10. Resuspend the cell pellet in 1 to 2 ml ice-cold PBS.

    Do not use NaN_3- or BSA-containing buffers for cell washing and labeling with amino-reactive fluorescent dyes (e.g., LIVE/DEAD Fixable Violet dead cell stain).

11. Repeat steps 8 through 10.

12. Determine cell number using a hemacytometer, as described in *UNIT 1.1*. Add 1×10^6 cells per sample (see Table 8.6.1 and Table 8.6.2) to 5-ml sample tubes.

13. Centrifuge cells 5 min at ~350 × *g*, 4°C.

14. Decant or remove the supernatant with a water jet vacuum pump.

Label with LIVE/DEAD fixable dead cell stain

15. Prepare the same LIVE/DEAD Fixable Violet dead cell stain labeling solution for all samples by adding 1 µl LIVE/DEAD Fixable Violet dead cell stain to 1 ml PBS per sample.

16. Add 1 ml LIVE/DEAD Fixable Violet dead cell stain labeling solution to samples no. 6, 7 to *x*, A and B (see Table 8.6.2).

17. Incubate 30 min on ice in the dark.

Fix cells

18. Centrifuge cells 5 min at ~350 × *g*, 4°C.

19. Resuspend the cell pellet in 1 to 2 ml ice-cold PBS.

20. Centrifuge cells 5 min at ~350 × *g*, 4°C.

21. Decant or remove the supernatant with a water jet vacuum pump.

22. Dislodge cell pellet and add 1 ml of 4% PFA to each sample while vortexing.

    Commercially available formalin (e.g., SG Planung) may be used instead of PFA. If cell clumping is prevalent resuspend cells in PBS (e.g., 900 µl PBS), mix thoroughly by pipetting up and down and add concentrated PFA (100 µl of ~40% PFA) for fixation while vortexing.

23. Incubate for 15 min at room temperature in the dark.

Permeabilize cells and couple EdU with click chemistry

24. Add 2 ml of 1× saponin-based permeabilization buffer and proceed as described in Basic Protocol 1, steps 28 to 40.

Measure fluorescence

25. Measure fluorescence without further washing using filter settings as described in Table 8.6.3.

26. If available, activate the 633-nm excitation pulse processing option on Click-iT EdU cell cycle dye 633 detection channel (DNA content) for gating aggregates/doublets on the flow cytometer (for required filter settings see Table 8.6.3).

27. Draw the following plots and histograms with the acquisition software:

    a. FC/SC dot plot
    b. Histogram for LIVE/DEAD Fixable Violet dead cell stain detection
    c. Histogram for Alexa Fluor 488 detection (optional)
    d. Histogram for Click-iT EdU cell cycle dye 633 detection (optional)
    e. Click-iT EdU cell cycle dye 633 area/Alexa Fluor 488 area dot plot.

Use pulse-processing for gating of doublet cells (Brockhoff, 2009) if available for the instrument. For example, using instruments from BD Biosciences, draw a Click-iT EdU cell cycle dye 633 width/area dot plot.

28. Set the detector gains for LIVE/DEAD Fixable Violet dead cell stain and Alexa Fluor 488 detection channels (EdU coupling) to logarithmic signal amplification and for Click-iT EdU cell cycle dye 633 detection channels (DNA content) to linear signal amplification. Enable discrimination of live and dead cells in the LIVE/DEAD Fixable Violet dead cell stain detection channel and EdU-positive and EdU-negative cells in the Alexa Fluor 488 detection channel, respectively, and set detector gains to give a cell cycle histogram in the Click-iT EdU cell cycle dye 633 detection channel.

    LIVE/DEAD Fixable Violet dead cell stain, Alexa Fluor 488, and Click-iT EdU cell cycle dye 633 were selected for this assay as their excitation and emission maxima are largely separated from each other (see Table 8.6.3) and are suited for excitation with three different lasers. These dye characteristics allow one to avoid the need for compensation and compensation controls. If the experimenter wants to check whether compensation is needed with the specific experimental system, prepare unstained, single-stained, and one triple-stained sample and refer to Roederer (2002) for the procedure of compensation.

29. Acquire at least 1×10^4 cells per sample with appropriate software that stores data in list-mode flow cytometry standard (FCS) files (e.g., BD FACS Diva software v6.1.1).

Analyze data

30. Analyze data with appropriate software (e.g., FlowJo 7.6.1).

31. Gate live cells in FC/SC dot plot.

32. If the pulse-processing option is supported by the flow cytometer and was used during acquisition, gate single cells, e.g., in the Click-iT EdU cell cycle dye 633 width/area dot plot for BD instruments.

33. Gate LIVE/DEAD Fixable Violet dead cell stain–negative cells in the respective histogram (see step 27).

34. Visualize gated cells in the Click-iT EdU cell cycle dye 633 area/Alexa Fluor 488 area dot plot (see Anticipated Results, Fig. 8.6.3).

EVALUATION OF CELL DEATH BY DETERMINATION OF THE SUB-G1 FRACTION

SUPPORT PROTOCOL 1

Labeling with LIVE/DEAD Fixable Violet dead cell stain according to the Alternate Protocol allows one to exclude the dead cell fraction from evaluation of data in a multi-parametric assay. In addition, the dead cell fraction may also be quantified with LIVE/DEAD Fixable Violet dead cell stain by gating on the respective cell population. Furthermore, labeling according to the Alternate Protocol allows for a more detailed characterization of the dead cell fraction by visualization of the sub-G1 peak due to DNA labeling with Click-iT EdU cell cycle dye 633 and therefore provides the possibility to quantify the amount of cell death with a second marker for cell death in one assay. For a positive control, also label samples A and B (Table 8.6.2) with Click-iT EdU cell cycle dye 633 according to Basic Protocol 1, steps 37 to 40.

For flow cytometric measurements, reduce the threshold value so that data on cell fragments is also stored and not excluded from data storage. For visualization of the cells and cell fragments with lower than G1 DNA content, extend the gate in the FC/SC dot plot and the gate for single cells in the Click-iT EdU cell cycle dye 633 width/area dot plot to lower signal intensities.

BASIC PROTOCOL 2

DYNAMIC CELL PROLIFERATION ASSESSMENT BY EDU/HOECHST QUENCHING

Continuous exposure of proliferating cells to EdU results in substitution of thymidine in DNA during S-phase while quiescent/dormant cells remain EdU negative. Staining of cells with Hoechst 33258 and PI after detergent permeabilization leads to quenching of Hoechst 33258 fluorescence while PI fluorescence is stoichiometric to the DNA content. This allows distinguishing cells of different cell cycle phases (G0/G1, S, G2/M) of subsequent cell cycles. In addition, the EdU-negative G0 cell fraction is quantified. This technique is highly suited not only to determine drug-induced changes in cell cycle progression but also to quantify drug-caused cell cycle exit or re-entry. This protocol is optimized for the human breast cancer cell line BT474.

Materials

BT474 breast cancer cells (ATCC #HTB-20; see *UNIT 1.1* for culture of mammalian cells)
Complete DMEM containing 5% (v/v) fetal bovine serum (FBS; DMEM-5; *UNIT 1.1*)
Drug to be tested for its effect on cell proliferation (e.g., trastuzumab, also known as rhuMAb, 4D5, and herceptin, humanized monoclonal antibody acting on the ErbB2 (HER2/neu) receptor)
Complete DMEM containing 2% (v/v) FBS (DMEM-2), optional
10 mM 5-ethynyl-2′-deoxyuridine (EdU; e.g., Molecular Probes/Invitrogen) in DMSO
Trypsin/EDTA solution (*APPENDIX 2A*)
PBS/0.01% NaN_3/0.2% BSA: phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 0.01% (w/v) NaN_3 and 0.2% (w/v) BSA; store up to 2 weeks at 4°C
Freezing medium without phenol red (e.g., RPMI, 10% FBS, 10% DMSO; Brockhoff, 2009; see *UNIT 1.1*), optional
DNA washing buffer (see recipe)
DNA staining buffer (see recipe)
250 μg/ml propidium iodide (PI; e.g., Sigma) solution in PBS

75-cm^2 culture flasks
50-ml centrifuge tubes (e.g., Polypropylene tubes, Greiner Bio-one)
Refrigerated centrifuge, 4°C
Hemacytometer (*UNIT 1.1*)
Water jet vacuum pump (e.g., BRAND), optional
37°C water bath (optional)
5-ml sample tubes (e.g., 12 × 75–mm polystyrene round-bottomed tubes, BD)
70-μm nylon mesh, optional
Flow cytometer with a 488-nm and UV laser excitation (see Table 8.6.3 for required filters)
Acquisition software that stores data in list-mode flow cytometry standard (FCS) files (e.g., BD FACS Diva software V6.1.1, BD Biosciences)
Evaluation software (e.g., FlowJo 7.6.1, Tree star)

NOTE: Protect labeling solutions and labeled samples from light.

Seed cells and incubate cells with EdU

1. Seed 2×10^5 exponentially growing cells (day 0) in complete DMEM with 5% FBS (DMEM-5) in 75-cm^2 culture flasks.

   The number of necessary cell culture flasks depends on the number of time points or different treatment modalities with drugs (see below). For studying cell cycle kinetics, seed a sufficient number of cell culture flasks that allows iterative EdU addition at 2-hr intervals before harvesting simultaneously.

Provide EdU-negative cultures to serve as a negative control for whether the continuous EdU incubation has an inhibitory effect on cell proliferation. Examine these controls in a single-parameter histogram. The distributions of the cell cycle phases with and without EdU labeling must be similar.

Subject to the cell type–specific doubling time of the cells under investigation, seed a cell number to guarantee a sub-confluent cell culture after 7 days of growth.

2. (Optional) Add drug to test samples, e.g., 10 μg/ml trastuzumab (day 1); use untreated samples as controls. Continue cell culture.

   For testing for the effect of different growth factors on cell cycle progression, reduction of FBS in the medium from 5% to 2% is recommended.

3. Add 100 μg/ml EdU to both drug-treated and untreated cell samples on days 2, 3, and/or 4 to give different incubation times with EdU for comparison of cell cycle progression of the differentially treated test samples. Continue cell culture.

   The time point of EdU addition and the time interval of EdU continuous labeling of cells highly depends on the cell cycle time of the cell line under investigation and on the information required. Therefore, the optimal time points of EdU addition and the duration of continuous EdU exposure must be determined for each cell line separately. In addition, the EdU concentration that results in optimal quenching of Hoechst 33258 must be determined for each cell line.

   EdU has not yet been demonstrated to be a photosensitizer as it has been verified for BrdU. Hence there is no need to incubate and handle EdU-treated samples under dim conditions. However, since EdU has been found to potentially cause dsDNA breaks (Diermeier-Daucher et al., 2009), it should be applied at the lowest concentration possible.

4. Change medium on day 4 and replace EdU and drugs where appropriate. Continue cell culture.

Harvest cells

5. Harvest cells on day 7 for BT474 using trypsin/EDTA solution (e.g., see *UNIT 1.1*), collect into 50-ml centrifuge tubes, and transfer to ice.

   The optimal time point for harvesting cell lines must be determined individually according to the time point where differences in cell cycle progression become apparent. In addition, cells should be subconfluent at harvesting; see Strategic Planning, cell culture for optimal growth.

6. Centrifuge cells 5 min at ~350 × *g*, 4°C.

7. Decant or remove the supernatant with a water jet vacuum pump.

8. Resuspend the cell pellet in ice-cold PBS/0.01% NaN_3/0.2% BSA.

9. Repeat steps 6 to 8.

10. Determine cell number using a hemacytometer as described in *UNIT 1.1*. Store cells frozen at −20°C for later measurement, proceed to step 11, or label and measure on the same day, proceed to step 13.

Store cells for sample collection (Optional)

11. Freeze cells in freezing medium without phenol red (Brockhoff, 2009; see *UNIT 1.1*) at a final cell concentration of 1×10^6 cells/ml.

    Cells can be stored up to 6 months at −20°C without detectable degradation.

12. For labeling frozen cells, thaw cells in a 37°C water bath and immediately proceed to step 13.

Prepare and stain nuclei

13. Add 5×10^5 cells per sample to 5-ml sample tubes containing 1 to 2 ml DNA-washing buffer.
14. Centrifuge cells 5 min at ~350 × *g*, 4°C.
15. Decant or remove the supernatant with a water jet vacuum pump.
16. Resuspend the cell pellet in 1 to 2 ml ice-cold DNA-washing buffer.
17. Repeat steps 14 and 15 and proceed with step 18.
18. Add 994 µl DNA-staining buffer (containing 1.2 µg/ml Hoechst 33258).
19. Incubate 15 min at room temperature.
20. Add 6 µl PI (250 µg/ml, 1.5 µg/ml final concentration) and incubate for at least 15 min at 4°C.

Measure fluorescence

21. (Optional) Pass nuclei through a 70-µm nylon mesh to remove aggregates prior to flow cytometric analysis.
22. Measure fluorescence without further washing, using filter settings as described in Table 8.6.3.
23. If available, activate 488-nm excitation pulse processing option on PI detection channel (DNA content) for gating aggregates/doublets on the flow cytometer (for required filter settings, see Table 8.6.3).
24. Draw the following plots and histograms with the acquisition software:
    a. FC/SC dot plot
    b. Hoechst 33258/PI area dot plot.

    Use pulse-processing for gating of doublet cells (Brockhoff, 2009) if this option is available for the instrument. For example, using a BD Biosciences instrument draw a PI width/area dot plot.
25. Set the detector gains for PI and Hoechst 33248 detection channels to linear signal amplification.

    PI and Hoechst 33248 excitation and emission maxima are largely separated from each other (see Table 8.6.3) and are suited for excitation with two spatially separated lasers for time-resolved sample excitation. These dye characteristics allow neglecting compensation and compensation controls. Data shown in Anticipated Results were gained without compensation.
26. Acquire at least 1×10^4 events per sample with appropriate software that stores data in list-mode flow cytometry standard (FCS) files (e.g., BD FACS Diva software v6.1.1).

Analyze data

27. Analyze data with appropriate software (e.g., FlowJo 7.6.1).
28. Gate intact nuclei in FC/SC dot plot.
29. If the pulse-processing option is supported by the flow cytometer and was used during acquisition, gate single nuclei, e.g., in the PI width/area dot plot for BD instruments.
30. Visualize gated events in the Hoechst 33248 area/PI area dot plot.

QUANTIFICATION OF THE G0 CELL FRACTION WITH THE EDU/HOECHST QUENCHING TECHNIQUE

The fraction of quiescent cells (G0 cells) and their potential to re-enter the cell cycle via G0- to G1-transition can be quantified. Cells progress through successive cell cycles at different rates depending on the individual treatment. Therefore, calculation of the dormant cell fraction has to account for cells that have divided once, twice, or three times within the observation period (Brockhoff et al., 2007).

1. Draw regions with appropriate software (e.g., FlowJo 7.6.1) around the cells/nuclei that are attributed to G0 cells and to cells of the first, second, and third cell cycle, respectively, according to Figure 8.6.2 (Anticipated Results). Use the statistics displayed by the software to gain the cell number in the respective gates.
2. Calculate dormant cell/nuclei number according to Equation 8.6.1.

   Calculate the fraction of dormant cells ($G0_{korr.}$) analyzed with the EdU/Hoechst quenching technique by determining the number of G0 cells (G0) and cell numbers in the first (A), second (B), and third (C) cell cycles (also see Fig. 8.6.2).

$$\frac{G0}{\frac{A}{1}+\frac{B}{2}+\frac{C}{4}+G0} = G0_{korr.}$$

Equation 8.6.1

Divide the number of G0 cells by the sum of:

(1) the number of cells that have incorporated EdU, but have not divided (A, cells of the first cell cycle that are in S or G2/M-phase),
(2) the number of cells that have divided once (B, cells that are assigned to the second cell cycle) divided by 2 (two cells in the second cycle have resulted from one cell in the first cycle),
(3) the number of cells that have divided twice (C, cells that belong to the third cell cycle) divided by 4 (four cells in the third cycle have resulted from one cell in the first cycle), and
(4) the number of G0 cells.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DNA buffer

100 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
154 mM NaCl
1 mM $CaCl_2$
0.5 mM $MgCl_2$
Store up to 1 year at room temperature

DNA staining buffer

DNA buffer (see recipe)
0.2 % (w/v) BSA
0.1 % (v/v) IGEPAL CA-630 (nonylphenylpolyethylenglycol, e.g., Sigma)
40 μg/ml RNase A

continued

1.2 μg/ml Hoechst 33258
Prepare fresh

Cut off ~0.3 cm of the pipet tip for facilitating pipetting of the viscous IGEPAL.

Allow the necessary volume to warm to room temperature before use.

A stock solution consisting of DNA buffer, 0.2% (w/v) BSA, and 0.1% (v/v) IGEPAL may be prepared and stored for up to 2 weeks at 4°C. Add RNase A and Hoechst 33258 immediately before use of DNA staining buffer.

DNA washing buffer

DNA buffer (see recipe)
0.2 % (w/v) BSA
Store up to 2 weeks at 4°C

Paraformaldehyde (PFA), 4% (w/v)

Add 40 ml of PBS (*APPENDIX 2A*) to 2 mg of solid paraformaldehyde. Add 3 to 4 ml of 1 M NaOH and mix until the solid dissolves. Adjust the pH to 7.4. Bring the volume to 50 ml with water and check the pH again. Store up to 1 week at 4°C. Discard after 1 week.

CAUTION *Wear gloves and do not inhale PFA powder.*

COMMENTARY

Background Information

Nucleoside analog-based flow cytometric techniques constitute powerful tools to determine cell cycle kinetics and to disclose potential mitogenic, cytostatic, and cytotoxic effects upon specific cell treatment (Brockhoff et al., 2001, 2007; Diermeier-Daucher et al., 2005; Cappella et al., 2008a; Brockhoff, 2009). The high information content of flow cytometric studies of cell cycle kinetics has led to alternative procedures in addition to the classical BrdU pulse-labeling techniques (Darzynkiewicz and Juan, 1997) with the aim to circumvent the requirement of DNA denaturation and to allow for multicolor experiments and multiplexing with antigen staining.

Click chemistry has proven to fulfill these requirements and was recently introduced for labeling and detection of newly synthesized DNA by coupling the thymidine analog EdU with a fluorescent azide probe. Since its publication in 2002 (Rostovtsev et al., 2002; Tornoe et al., 2002) click chemistry has proven extremely useful for cell culture studies with flow cytometry (Buck et al., 2008; Cappella et al., 2008a; Brockhoff, 2009; Diermeier-Daucher et al., 2009), automated fluorescence microscopy (high content screening; Bradford et al., 2008c), and high-speed ImageStream image cytometry (George et al., 2008). Recent reports highlight the application of EdU for in vivo studies applying intraperitoneal injection of EdU into mice followed by click chemistry on paraffin tissue sections (Salic and Mitchison, 2008) and the combination of EdU incubation of whole-mount embryos with click chemistry and immunofluorescent antigen staining of tissue sections (Warren et al., 2009).

A strategy to avoid the need for denaturation of dsDNA in combination with an antibody-based detection protocol was recently published by Cappella et al. (2008b). EdU incorporated into DNA is covalently linked to a BrdU azide by click chemistry, which in turn is labeled with fluorophore-conjugated anti-BrdU antibodies.

The antibody detection method of DNA-labeled BrdU was augmented by Clarke et al. (2008) with the non-antibody approach of EdU detection by click chemistry, which enables double labeling of proliferating cells. As simultaneous addition of both nucleosides only results in the incorporation of BrdU into DNA of S-phase cells, the sequential addition of EdU followed by BrdU allows one to distinguish three nucleoside-labeled populations (EdU-positive, EdU/BrdU-double positive, and BrdU-positive) and was suggested for detection of rapid changes in cell proliferation upon drug treatment.

Furthermore, the highly informative Hoechst quenching technique with (continuous) EdU incubation was developed as an option rather than the BrdU/Hoechst quenching technique (Poot et al., 2001) for

detailed analysis of cell cycle kinetics of cell lines that show BrdU-induced effects on cell proliferation (Diermeier-Daucher et al., 2009). Continuous BrdU exposure has been shown to result in cell-type specific changes in cell cycle progression (Diermeier-Daucher et al., 2004). The EdU/Hoechst quenching technique offers an alternative. As an informative technique for high-resolution assessment of cell cycle kinetics, it represents a powerful tool for analysis of cell cycle distribution and kinetics with a broad field of applications, including studying effects of growth factors and drugs on a specific cell cycle phase.

Critical Parameters and Troubleshooting

EdU-induced effects on cell cycle progression

Continuous treatment with EdU may have cytostatic or cytotoxic effects (Diermeier-Daucher et al., 2009). However, different cell types of different origins differentially and individually respond to nucleoside treatment, which is valid for both BrdU (Diermeier-Daucher et al., 2004) and EdU (Diermeier-Daucher et al., 2009). Therefore, it is recommended to test the cell line under investigation for a potential impact of EdU on cell viability, DNA synthesis, and cell cycle progression before applying both pulse-labeling and continuous treatment with nucleoside analogs. Nevertheless, determination of the G0 cell fraction applying the EdU/Hoechst quenching technique with continuous EdU exposure of cells is even possible with cell lines that are affected by EdU in cell cycle progression. The comparison of EdU-labeled drug-untreated control cells with EdU-labeled drug-treated cells allows one to compensate for the EdU-caused effect and to solely analyze cell cycle exit (or re-entry) mediated by the drug of interest. However, incorporation of BrdU into DNA has been suggested to increase the sensitivity of cells towards certain drugs (Poot et al., 1991). EdU as an alternative nucleoside analog may have similar effects.

Hoechst/PI dye concentrations

The fluorescence of DNA-bound dye and the quenching effect may vary among different cell lines. Therefore, in the Hoechst 33258 versus PI dot plot, the slope of cells that were proliferating from G0/G1 into S and G2/M in their first cell cycle during EdU incubation may not be left-facing, (Fig. 8.6.3). To optimize the slope, change the concentration ratio of Hoechst 33258 versus PI dyes by variation of, e.g., the PI concentration while the Hoechst 33258 concentration remains constant.

Reduce artifacts

Detergent is present in cell suspensions of EdU/Hoechst quenching measurements. This may disturb the current streaming through the flow cytometer and artifacts may develop during signal detection. A reduction of the flow velocity <300 events/sec may improve the quality of the measurement.

Deficient discrimination of EdU-positive versus EdU-negative cells

To increase the separation of EdU-positive versus EdU-negative cells in pulse-labeling experiments, the EdU concentration used may be increased. However, this procedure may increase the probability for cytostatic or even cytotoxic effects of the nucleoside analog. Therefore, to minimize the risk for nucleoside-induced effects, the shortest incubation time in combination with the lowest EdU concentration is recommended for these experiments.

Weakly stained cells in addition to EdU-positive and EdU-negative cells

For EdU-pulse labeling, the nucleoside analog must be removed completely after the 3-hr pulse. Otherwise, cells can insert the EdU remnants into the DNA, which becomes visible after cell staining and results in a weak, positive reaction. These cells appear as dimly positively-stained cells in the two-dimensional dot-plot and distort the accurate temporal resolution of cell cycle phases. Therefore, to ensure a complete removal of non-incorporated EdU, wash the cultures thoroughly (at least three times) with warm PBS.

Anticipated Results

Interpretation of data gained with the quenching technique

Consecutive cell cycles of synchronized or asynchronously growing cell populations in vitro can be investigated. The measured fluorescence of PI is directly proportional to the DNA content of cells, which is twice as high in G2/M cells compared with G0/G1 cells. Fluorescence intensity of Hoechst 33258 depends on the amount of EdU incorporated into DNA over time and increases with cell cycle phases (from G0/G1- and S- to G2/M-phase) and in successive cell cycles. Quenching of Hoechst 33258 fluorescence occurs over the first cell cycle (Diermeier-Daucher et al., 2009) and is

due to the unifiliary thymidine substitution by EdU during the first cell cycle of EdU exposure and results in a decrease of Hoechst 33258 fluorescence intensity with increasing DNA content. During the second cell cycle, EdU is incorporated into the second DNA strand and the EdU substitution level increases from 50% to 75%. Since the percentage increase in thymidine substitution by EdU in the DNA is lower than in the first cell cycle (increase in nucleoside substitution after one cell cycle: 50%; after two cell cycles: 25%; Brockhoff, 2009), Hoechst 33258 fluorescence intensity is no more efficiently quenched but increases with DNA content to generate a mirror image between the first and second cell cycles. Cells of the third cell cycle run in parallel to cells of the second cycle.

Figure 8.6.2 shows that after EdU incubation non-proliferating cells remain at the starting point of the first cell cycle. After an extended incubation with the nucleoside analog (e.g., after a 96-hr EdU incubation), these dormant cells separate from the EdU-positive fraction of proliferating cells. Hence EdU-negative, non-proliferating cells can be quantified with appropriate gating and calculation procedures described in Support Protocol 2 and in Diermeier-Daucher et al. (2009). For further details on dynamic analysis of cell cycle kinetics, see Brockhoff (2009) or the original literature can be consulted (Latt et al., 1977; Kubbies et al., 1985; Poot et al., 1988, 1994; Rabinovitch et al., 1988). For calculation of the dormant cell fraction, see Support Protocol 2. Original data derived from an EdU/Hoechst 33258 quenching measurement are shown in Figure 8.6.3.

Examples for dynamic cell proliferation assessment with EdU

EdU can be used in place of BrdU for dynamic cell proliferation assessment of BT474 breast cancer cell line both for the pulse-labeling anti-BrdU technique (Fig. 8.6.3A,B) and for the continuous labeling BrdU/Hoechst quenching technique (Fig. 8.6.3C,D). Nucleoside analog–labeled S-phase and nucleoside analog–negative G1- and G2/M-phase cells separate to a similar extent for BrdU detection with anti-BrdU antibodies (49-fold difference) and for EdU labeling with click chemistry (55-fold difference) at the 0-hr time point (Fig. 8.6.3A,B). Compared to BrdU, EdU is capable of quenching Hoechst 33258 to a similar extent. Figure 8.6.3C,D shows one representative time point (39.5 hr; Diermeier-Daucher et al., 2009).

Time Considerations

Coupling of EdU with fluorescent dye by click chemistry and staining with DNA dye takes ~2 hr, including cell harvesting, cell counting, and all incubation and washing steps (see Basic Protocol 1). Staining for live/dead discrimination as a three-step staining protocol in addition to the two-step staining in Basic Protocol 1 requires an additional 1 hr (see Alternate Protocol). Flow cytometric measurement requires 30 min for about ten samples. Staining and measurement for the EdU/Hoechst quenching technique takes ~1 hr each (see Basic Protocol 2).

Literature Cited

Bradford, J.A. and Buller, G.M. 2008. Dead cell stains in flow cytometry: A comprehensive analysis. XXIV International Congress, Program and Abstracts, 417/P315:247.

Bradford, J.A., Clarke, S.T., Buck, S.B., Hill, D., Chen, Y.-T., Gee, K., Agnew, B., and Salic, A. 2008a. Detection of S-phase cell cycle progression using 5-ethynyl-2′-deoxyuridine incorporation with click chemistry. XXIV International Congress, Program and Abstracts, 279/P177:197-198.

Bradford, J.A., Clarke, S.T., Hill, D., and Chen, Y-T. 2008b. Characterization of DNA content, cyclin B1 and phosphorylated histone H3 with direct S-phase using EdU incorporation in multiparameter testing of cell lines with cell cycle blocking agents. XXIV International Congress, Program and Abstracts, 280/P178:198.

Bradford, J.A., Hill, D.M., Clarke, S.T., and Chen, Y.-T. 2008c. Detection of DNA synthesis by automated microscopy and image analysis: Comparison of BrdU method and a new click chemistry based EdU method. XXIV International Congress, Program and Abstracts, 283/P181:199.

Brockhoff, G. 2009. Flow cytometric DNA und proliferation analysis. *In* Cellular Diagnostics—Basic Principles, Methods and Clinical Applications in Flow Cytometry. (U. Sack and G. Rothe, eds.) pp. 390-425. Karger Publishing, Unionville, CT.

Brockhoff, G., Heiss, P., Schlegel, J., Hofstaedter, F., and Knuechel, R. 2001. Epidermal growth factor receptor, c-erbB2 and c-erbB3 receptor interaction, and related cell cycle kinetics of SK-BR-3 and BT474 breast carcinoma cells. *Cytometry* 44:338-348.

Brockhoff, G., Heckel, B., Schmidt-Bruecken, E., Plander, M., Hofstaedter, F., Vollmann, A., and Diermeier, S. 2007. Differential impact of cetuximab, pertuzumab and trastuzumab on BT474 and SK-BR-3 breast cancer cell proliferation. *Cell Prolif.* 40:488-507.

Buck, S.B., Bradford, J., Gee, K.R., Agnew, B.J., Clarke, S.T., and Salic, A. 2008. Detection of S-phase cell cycle progression using 5-ethynyl-2′-deoxyuridine incorporation

with click chemistry, an alternative to using 5-bromo-2′-deoxyuridine antibodies. *Biotechniques.* 44:927-929.

Cappella, P., Gasparri, F., Pulici, M., and Moll, J. 2008a. A novel method based on click chemistry, which overcomes limitations of cell cycle analysis by classical determination of BrdU incorporation, allowing multiplex antibody staining. *Cytometry A* 73:626-636.

Cappella, P., Gasparri, F., Pulici, M., and Moll, J. 2008b. Cell proliferation method: Click chemistry based on BrdU coupling for multiplex antibody staining. *Curr. Protoc. Cytom.* 45:7.34.1-7.34.13.

Clarke, S.T., Bradford, J.A., and Godfrey, B. 2008. Dual pulse labeling of S-phase populations using click chemistry. DGfZ-ESCCA joint abstracts from the 18th annual meeting of the German Society of Cytometry (DGfZ) and the 8th Euroconference of Clinical Cell Analysis by the European Society for Clinical Cell Analysis (ESCCA). *Cytometry B Clin. Cytom.* 74B:378-415.

Darzynkiewicz, Z. and Juan, G. 1997. Analysis of DNA content and BrdU incorporation. *Curr. Protoc. Cytom.* 2:7.7.1-7.7.9.

Diermeier-Daucher, S., Schmidt-Bruecken, E., Kubbies, M., Kunz-Schughart, L.A., and Brockhoff, G. 2004. Continuous bromodeoxyuridine (BrdU) exposition differentially affects cell cycle progression of human breast and bladder cancer cell lines. *Cell Prolif.* 37:195-206.

Diermeier-Daucher, S., Horvàth, G., Knuechel-Clarke, R., Hofstaedter, F., Szöllõsi, J., and Brockhoff, G. 2005. Epidermal growth factor receptor coexpression modulates susceptibility to herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation. *Exp. Cell Res.* 304:604-619.

Diermeier-Daucher, S., Clarke, S.T., Hill, D., Vollmann-Zwerenz, A., Bradford, J.A., and Brockhoff, G. 2009. Cell type specific applicability of 5-ethynyl-2′-deoxyuridine (EdU) for dynamic proliferation assessment in flow cytometry. *Cytometry A* 75:535-546.

Endl, E., Steinbach, P., Knüchel, R., and Hofstädter, F. 1997. Analysis of cell cycle-related Ki-67 and p120 expression by flow cytometric BrdUrd-Hoechst/7AAD and immunolabeling technique. *Cytometry* 29:233-241.

George, T.C., Bradford, J.A., Clarke, S.T., Godfrey, W.L., Kong, R., Hall, B.E., and Morrissey, P.M. 2008. Effect of p27KIP1 nuclear localization on cell cycle progression and apoptotic changes as measured using ImageStream imaging cytometry of cells labeled with Click-iT EdU and Vybrant DyeCycle stains. *XXIV International Congress, Program and Abstracts*. 20:106.

Kubbies, M., Schindler, D., Hoehn, J., and Rabinovitch, P.S. 1985. BrdU-Hoechst flow cytometry reveals regulation of human lymphocyte growth by donor age-related growth fraction and transition rate. *J. Cell. Physiol.* 125:229-234.

Latt, S.A., George, Y.S., and Gray, J.W. 1977. Flow cytometric analysis of bromodeoxyuridine-substituted cells stained with 33258 Hoechst. *J. Histochem. Cytochem.* 25:927-934.

Poot, M., Schindler, D., Kubbies, M., Hoehn, H., and Rabinovitch, P.S. 1988. Bromodeoxyuridine amplifies the inhibitory effect of oxygen on cell proliferation. *Cytometry.* 9:332-338.

Poot, M., Schuster, A., and Hoehn, H. 1991. Cytostatic synergism between bromodeoxyuridine, bleomycin, cisplatin and chlorambucil demonstrated by a sensitive cell kinetic assay. *Biochem. Pharmacol.* 41:1903-1909.

Poot, M., Hoehn, H., Kubbies, M., Grossmann, A., Chen, Y., and Rabinovitch, P.S. 1994. Cell-cycle analysis using continuous bromodeoxyuridine labeling and Hoechst 33358-ethidium bromide bivariate flow cytometry. *Methods Cell. Biol.* 41:327-340.

Poot, M., Rosato, M., and Rabinovitch, P.S. 2001. Analysis of cell proliferation and cell survival by continuous BrdU labeling and multivariate flow cytometry. *Curr. Protoc. Cytom.* 15:7.14.1-7.14.9.

Rabinovitch, P.S., Kubbies, M., Chen, Y.C., Schindler, D., and Hoehn, H. 1988. BrdU-Hoechst flow cytometry: A unique tool for quantitative cell cycle analysis. *Exp. Cell Res.* 174:309-318.

Roederer, M. 2002. Compensation in flow cytometry. *Curr. Protoc. Cytom*. 22:1.14.1-1.14.20.

Rostovtsev, V.V., Green, L.G., Fokin, V.V., and Sharpless, K.B. 2002. A stepwise huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed. Engl.* 41:2596-2599.

Salic, A. and Mitchison, T.J. 2008. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 105:2415-2420.

Tornoe, C.W., Christensen, C., and Meldal, M. 2002. Peptidotriazoles on solid phase: [1,2,3]-Triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* 67:3057-3064.

Warren, M., Puskarczyk, K., and Chapman, S.C. 2009. Chick embryo proliferation studies using EdU labeling. *Dev. Dyn.* 238:944-949.

Internet Resources

http://probes.invitrogen.com/media/pis/mp34955.pdf
Available dyes for live/dead discrimination with the LIVE/DEAD dead cell stain kit.

http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/Click-iT-Detection-Assays/click-iT-Edu.html#flow
Available dyes for EdU labeling with Click-iT EdU Cell Proliferation Assays.

http://www.isac-net.org/congress2008/documents/colorbars_isac_program.pdf
XXIV International Congress 2008, program and abstracts.

CHAPTER 9
Cell Adhesion

INTRODUCTION

Cell-adhesive interactions determine the organization of tissues and mediate and guide precise cell migrations during embryonic development, inflammation, the immune response, and wound repair. They also help to regulate gene expression, growth, differentiation, and apoptosis. Research into cell adhesion has undergone dramatic evolution over the past two to three decades, from phenomenological studies of the biophysical and morphological mechanisms used by cells to adhere to other cells and the extracellular matrix, to the discovery and in-depth mechanistic analyses of a variety of specific cell adhesion systems, and then to the recent paradigm shift whereby adhesion molecules are now viewed as activators or regulators of a remarkably wide range of signal transduction pathways.

FUNCTIONS OF ADHESION MOLECULES

Cell adhesion molecules function by forming specific protein-protein or protein-carbohydrate bonds at the cell surface to mediate cell interactions. In addition, cell adhesion molecules or cell adhesion receptors often form direct links to multimolecular protein complexes on the cytoplasmic face of the plasma membrane. These cytoplasmic adhesion and signaling complexes interact with the cytoskeleton and signal transduction pathways. As a result, cell adhesion not only links cells with other cells and the extracellular matrix, but also helps to integrate extracellular physical information with the major signal transduction pathways within cells.

Cell adhesion is often categorized into cell-to-cell adhesion and cell-to-substrate adhesion. In each case, the physical structures that mediate cell adhesion can be either specialized adhesive structures or broad expanses of plasma membrane. For example, cells can initially adhere to other cells along large areas of plasma membrane using general-purpose adhesive molecules such as cadherins. But they can also form attachments to each other by using specialized adhesive structures, such as desmosomes, adherens junctions, and tight junctions. Each type of junctional complex involves specific adhesion-molecule components, such as cadherins and associated cytoplasmic components involved in linkage to the actin-containing cytoskeleton. Fibroblasts can also form adhesions to other fibroblasts, but they most characteristically form adhesive interactions with extracellular matrix molecules. Such cell-to-matrix adhesion sites can also be either broad zones or specialized structures. For example, rapidly migrating cells typically interact with tissue culture substrates through broad areas of contact termed “close contacts,” and the entire basal surface of epithelial cells adheres via integrins to their underlying basement membranes. Specialized adhesive structures include the hemidesmosomes of certain epithelial cells and the focal adhesions of fibroblastic and endothelial cells. The protein composition of the complexes involved in cell-to-matrix adhesions can sometimes involve a few of the same proteins as in cell-to-cell adhesions, such as vinculin and actin, but they mainly consist of distinct types of adhesive, cytoskeletal, and signal transduction molecules.

Contributed by Kenneth M. Yamada
Current Protocols in Cell Biology (2003) 9.0.1-9.0.9

GENERAL CHARACTERISTICS OF CELL ADHESION PROTEINS

Cell adhesiveness generally involves specific binding of a cell adhesion protein or receptor to a target molecule. Adhesion mediated by the binding of an adhesion molecule to the same type of protein on a neighboring cell is termed a "homophilic" interaction. Members of the calcium-dependent cadherin family are major mediators of such interactions, and some calcium-independent adhesion proteins, such as N-CAM, can also provide homophilic interactions. Cadherin adhesion molecules characteristically form tightly packed dimeric and multimeric complexes that mediate adhesion with high avidity via cooperativity, even though the affinity of a single cadherin protein-protein interaction alone is not high.

A particularly common type of adhesive interaction involves the binding of a receptor to a specific ligand. In the process of cell-to-cell adhesion, the target protein of an adhesion receptor can be either a "counter-receptor" or a complex carbohydrate linked to a protein anchor in the plasma membrane. In cell-to-matrix interactions, a plasma membrane adhesion receptor such as an integrin binds to an adhesive extracellular matrix protein. For example, a number of types of integrin receptors can bind to fibronectin, laminin, or collagen. Integrin receptors directly mediate cell adhesion, migration, and anchorage to these structural components of the extracellular matrix.

Molecules that mediate cell-cell or cell-matrix adhesion fall into two broad structural classes. One type is anchored to the plasma membrane, often as a transmembrane protein. These membrane-anchored molecules are generally receptors, homophilic adhesion molecules, or counter-receptors. They often consist of an extracellular domain containing one or more specific cell interaction domains or sites, as well as a stalk region, a hydrophobic transmembrane domain, and a cytoplasmic domain or tail. This type of adhesion molecule is often likely to be involved in the transmembrane transmission of signals after binding to their target molecule.

The second broad class of adhesion molecules consists of cell surface or extracellular matrix proteins (see Chapter 10) that contain domains involved in cellular adhesion. Nearly all matrix proteins contain such sites. For example, this class includes fibronectin, laminins, vitronectin, collagens, and many other extracellular proteins. These proteins contain one or more cell-binding domains, which are comprised of a primary recognition motif consisting of a short peptide sequence (e.g., Arg-Gly-Asp or Leu-Asp-Val) and often a synergy site or other structural feature that substantially enhances receptor-binding specificity and affinity.

Adhesion molecules frequently have many of the following characteristics (for reviews, see Alberts et al., 1994; Chothia and Jones, 1997; Edelman and Thiery, 1985; Gumbiner, 1996; Hay, 1991; Richardson and Steiner, 1995).

1. The backbone structure of adhesion proteins is often based on multiple repeats of protein motifs, such as the immunoglobulin (Ig) motif, EGF repeat, or fibronectin motif. The basic immunoglobulin repeat is quite common, as is the structurally related fibronectin type III repeat.

2. They have specialized functional domains, including one or more domains for binding molecules on other cells or in extracellular matrix. Often a domain for forming dimers or higher polymers is also present, as well as another for binding to complex carbohydrates.

3. They often bind with only moderate affinity, e.g., in the range of $K_d = 10^{-6}$ to 10^{-7} M for fibronectin, and as low as 10^{-4} M for leukocyte adhesion molecules involved in rolling adhesion. This modest affinity appears to be important for allowing dynamic changes in cell adhesions and to permit cell migration involving cyclic attachment and detachment of cell adhesion sites to a substrate or other cells.

4. They often function by clustering to generate complexes of high avidity: adhesion molecules such as integrins and cadherins often organize into large local clusters or aggregates in the plane of the plasma membrane, which can produce strong total avidity as a result of the summation of the otherwise weak binding of many individual molecules.

5. Their binding functions can be regulated by activation, e.g., "inside-out" signaling that changes their ability to bind ligands effectively; examples are integrin activation during leukocyte adhesion and platelet activation, and the suppression of the cadherin system by tyrosine phosphorylation.

MAJOR FAMILIES OF CELL ADHESION MOLECULES

Adhesion molecules that are anchored in the plasma membrane include several large groups of proteins that share common structural motifs, such as the immunoglobulin repeat. **Cadherins** comprise a large family of proteins particularly centrally involved in cell-to-cell adhesive interactions (Takeichi, 1990; Yap et al., 1997; see *UNIT 9.3*). Cadherins on one cell bind in homophilic fashion to the same type of cadherin on other cells by means of specific cell interaction domains, which can include the short peptide recognition sequence His-Ala-Val. The cytoplasmic domain of cadherin molecules characteristically binds to catenins, which provide direct linkages to the actin cytoskeleton (e.g., via α- and β-catenins).

The "classical" cadherins such as E-cadherin and N-cadherin mediate adhesion over broad expanses of cell-cell contact, or they can become further organized into adherens-type junctions that are linked to cytoplasmic molecules such as vinculin and actin. A number of other types of cadherins have been discovered recently, whose functions are probably also adhesive, but which remain to be characterized. Finally, certain highly specialized cadherins found exclusively in desmosomes, termed desmocollins and desmogleins, link cells together at particularly strong attachment sites connected to intermediate filaments such as keratins or vimentin.

Cadherin activity is quite sensitive to calcium ion concentration, which accounts for the ability of calcium chelators such as EDTA to dissociate tissues into their individual component cells. Chelation of divalent cations appears to disrupt conformation, so that the cadherin molecules become quite sensitive to general proteolytic attack; this latter property has been used to classify cell-cell adhesion molecules (see *UNIT 9.3*). Although desmosomal and other cadherins can be found widely in epithelia of organisms at all ages, the cadherins are particularly important during embryonic development, when they appear to organize groups of cells and tissues by specific cell-cell adhesions. They help to define tissues by binding primarily to cadherins of the same type, rather than to other cadherins on unrelated cell types; this activity can lead to "sorting out" of different types of cells from others.

Integrins are nearly ubiquitous cell surface receptors for a wide variety of extracellular matrix proteins, as well as for "counter-receptor" ligands on other cells. There are more than 20 distinct integrin subunits, which are combined to form heterodimers that always consist of one α and one β subunit. Genetic loss of almost any integrin subunit leads to disease or death, often during embryonic development or near the time of birth. Integrin molecules have a head domain containing a ligand-binding site, two spindly legs, and usually rather short cytoplasmic domains. Integrin binding is often inhibited by depletion of divalent cations.

CAMs (cell adhesion molecules) and integrin counter-receptors are structurally related by their use of the immunoglobulin repeat, and they often have the term "-CAM" in their names, yet they differ functionally. Molecules such as NCAM are calcium-independent,

homophilic adhesive molecules that bind to the same type of molecule on an adjacent cell surface, often a cell of the same tissue type (Edelman and Crossin, 1991). In contrast, counter-receptors such as the ICAMs and VCAM have specialized peptide recognition sites that are bound specifically by integrins such as LFA-1 (CD11a/CD18 or $\alpha_L\beta_2$) or VLA-4 ($\alpha_4\beta_1$), which are often present on other types of cells (e.g., endothelial cells and lymphocytes). The functions and sites of expression of these molecules can consequently differ widely. For example, molecules such as NCAM are implicated in embryonic developmental events such as axonal guidance and bundling, whereas ICAM counter-receptors are present as targets for binding by cells circulating in blood. The levels of these counter-receptors on the cell surface can often also be regulated rapidly in response to cytokines, for example during inflammatory responses.

A family of transmembrane proteins termed **ADAMs** (membrane proteins with a disintegrin and metalloprotease domain; Wolfsberg and White, 1996) contain a protease-like domain plus an integrin recognition site in the disintegrin domain. They appear to play roles in cell adhesion; for example, one member of this family is present on sperm and has been implicated in sperm-egg adhesion. **Syndecans** are cell-surface heparan sulfate proteoglycans with a protein core that crosses the plasma membrane and terminates in a cytoplasmic tail. Syndecans appear to function as "co-receptors," mediating signaling in association with a primary adhesion molecule. For example, syndecans synergize with fibronectin during formation of focal adhesion sites and form linkages to protein kinase C (Woods and Couchman, 1998).

Adhesive extracellular matrix molecules are described in Chapter 10. One interesting aspect of their function involves the striking effects of adsorption or attachment to a substrate. Molecules such as fibronectin and vitronectin circulate in blood at relatively high concentrations (e.g., 100 to 300 μg/ml), yet binding to cell surface receptors is relatively limited. On the other hand, even very low concentrations of the same proteins (1 to 10 μg/ml) bind to substrates and then mediate strong cell adhesion. This functional enhancement of binding of cells to molecules attached to a substrate has been variously ascribed to activation of molecules such as fibronectin and vitronectin by conformational changes resulting from binding to a substrate, to enhanced ability to interact with cells due to multivalency, or to a physical chemical enhancement in free energy (overall avidity) due to immobilization of a ligand. Whatever the mechanisms, it is clear that the binding of extracellular adhesion molecules to substrates can enhance functional activity, which appears to be important biologically to generate strong adhesion only when a circulating ligand is immobilized. For example, in wounds, immobilized fibronectin forms a crucially important provisional matrix that permits adhesion and migration of cells to close the wound.

FUNCTIONS OF CELL ADHESION MOLECULES IN SIGNAL TRANSDUCTION AND CYTOSKELETON

The functions of cell adhesion molecules and their receptors can be understood intuitively as physical mechanisms by which cells attach to other cells, adhere to extracellular matrix molecules, and provide traction during cell migration. This concept can be extended to intracellular structural effects—i.e., the organizing of the cytoskeleton. For example, transmembrane adhesion proteins provide membrane anchors for a host of cytoskeletal proteins such as keratin or vimentin in desmosomes and hemidesmosomes, actin microfilaments and other proteins in epithelial adherens junctions, and a number of adhesion plaque proteins and actin in cell-substrate adhesion plaques (e.g., see Jockusch et al., 1995; Yamada and Geiger, 1997).

It is now clear, however, that adhesion molecules also play crucial roles in helping to trigger or modulate many major signal transduction pathways. In fact, their signaling

functions are probably at least as important in cell biology as their attachment and cytoskeletal activities. A notable characteristic of the intracellular complexes of proteins induced by binding of adhesion molecules is that their formation also induces accumulation of a variety of signal transduction molecules that can trigger downstream signaling pathways (Clark and Brugge, 1995; Schwartz et al., 1995; Lafrenie and Yamada, 1996). As a consequence, adhesion proteins are actually cell-interaction proteins that have multiple functions in the bidirectional transfer of information at the cell surface. They can mediate outside-in transfers of signaling information, such as cellular responses to binding of specific extracellular matrix proteins involving the activation of intracellular signaling, but they can also mediate inside-out information in which intracellular signals modulate the activity of integrins and assembly of extracellular matrix. Even classical adhesive proteins such as fibronectin can trigger a bewildering range of activities after binding by integrins; these can include activation or modulation of many well-known mammalian signal transduction pathways (such as tyrosine phosphorylation, MAP kinases, protein kinase C, Ca^{2+} and H^{+} fluxes, and phosphoinositide pathways) which can activate specific gene transcription, mediate anchorage-dependent growth stimulation, and prevent apoptosis.

Two structural regions in transmembrane adhesion molecules that allow them to function as signal transduction receptors are their extracellular ligand-binding domains and their cytoplasmic domains. The ligand-binding domains are obviously essential for binding to extracellular molecules, but in at least some cases, they have additional, intriguing roles. For integrin adhesion receptors, there appear to be separable functions for ligand occupancy (filling the binding site with a ligand), as opposed to receptor clustering (which can be induced by multivalent ligands such as fibrils of fibronectin or collagen). These two inputs can synergize to promote the accumulation of specific cytoskeletal proteins such as α-actinin and actin to form strong adhesions (Yamada and Miyamoto, 1995).

Even though they lack intrinsic enzymatic activities, integrins appear to function as signaling receptors and regulators of actin cytoskeletal organization by recruiting other molecules to bind to their cytoplasmic domains. Integrins appear to bind directly to certain cytoplasmic proteins, such as talin, α-actinin, and tensin, in a process that is sometimes regulated by ligand occupancy on the outside of the cell. Also important, however, are docking proteins such as focal adhesion kinase, which can bind to integrins as well as binding to at least eight cytoplasmic molecules to form molecular complexes. Other docking proteins such as $p130^{Cas}$ probably increase the repertoire of cross-linked and complexed proteins. Integrin clustering appears to play a central role in forming large complexes that can consist of over 30 different types of molecules. These integrin-induced multimolecular complexes can serve as signaling centers, e.g., for tyrosine phosphorylation and MAP kinase activation (reviewed by Yamada and Miyamoto, 1995; see *UNIT 14.2* and *UNIT 14.3* for protocols measuring these post-translational modifications).

Cell-to-cell adhesion molecules also appear to undergo a very similar process of local clustering, complex formation, binding of cytoskeletal proteins such as actin, and accumulation of signaling molecules (Kirkpatrick and Peifer, 1995; Yap et al., 1997; Yamada and Geiger, 1997). It will become increasingly important to understand how these processes are controlled. The cadherin system can be suppressed by tyrosine phosphorylation mediated by v-Src kinase, and certain integrin functions can be down-modulated by the phosphatase PTEN (Takeda et al., 1995; Tamura et al., 1998). There are likely to be large numbers of other regulatory pathways that affect the widespread intracellular effects of cell-cell and cell-substrate adhesion molecules on cytoplasmic processes. It is clear that adhesion molecules are crucial, integral components of the basic signaling and regulatory mechanisms of cells, providing dynamic links to the external environment.

CELL ADHESION ASSAYS

A crucial first step for effective analysis of a cell adhesive process is to characterize it in a quantitative in vitro assay. Cell-to-substrate adhesion assays are used to examine the ability of cells to attach to matrix molecules, such as fibronectin or laminin, and to determine which specific adhesion receptors are involved using antibodies or synthetic peptide inhibitors. Roles of cytoplasmic molecules such as cytoskeletal proteins and signaling molecules can also be evaluated with these assays using pharmacological inhibitors of intracellular processes—for instance inhibitors of kinases and other molecules.

There are several types of cell-substrate adhesion assays, which can quantitate (1) cell attachment, (2) cell spreading, or (3) detachment of previously adherent cells. Each assay measures different parameters of the adhesion process: cell attachment assays generally determine the numbers of cells that can attach to a substrate in a specific time period after washing off nonattached cells (*UNIT 9.1*); cell spreading assays determine the percentage of cells that show spread morphologies, or measure the surface area of spread cells, after certain times of incubation (*UNIT 9.1*); and cell detachment assays measure the ease with which cells can be detached after a particular period of time, for example by determining the numbers of cells detached from a dish using each of a series of increasing centrifugal force levels (*UNIT 9.2*). Although conceptually distinct, these three types of assays can in practice have features that overlap significantly.

In order to compare these assays, it is useful to consider the steps in cell-substrate adhesion and spreading. The first step in cellular adhesion to a substrate is the attachment of plasma membrane receptors or other cell interaction molecules to substrate molecules such as extracellular matrix (ECM) proteins (Chapter 10). For example, if cells are cultured on tissue culture dishes or glass in serum-containing medium, they often adhere to serum proteins such as vitronectin and fibronectin that are nonspecifically adsorbed to the substrate. Integrins frequently mediate such adhesions (e.g., attachment of cells to an extracellular matrix protein), although various other cell attachment proteins, such as the selectins, also exist.

The binding of integrins to ligands can occur even at 4°C, because it is a direct protein-protein interaction. However, this mechanism of cell adhesion is initially quite weak and can often require centrifugation of the cell against the substrate to produce close enough apposition of the plasma membrane to substrate proteins for effective binding. In contrast, normal adhesion appears to involve active cellular processes involving the plasma membrane and the cytoskeleton. The latter, poorly understood process of cytoskeletal strengthening of adhesions can produce enormous increases in attachment strength (see *UNIT 9.2* for a conceptual discussion). This strengthening phenomenon may be related to the formation of multimolecular complexes in adhesion structures containing a variety of cytoskeletal and signal transduction proteins (Burridge et al., 1997; Yamada and Geiger, 1997). Cell attachment assays are generally performed shortly after plating cells before much cell spreading can occur (e.g., within 10 to 20 min for fibroblasts).

Attachment is rapidly followed by spreading of the cell on the substrate in a dynamic adherence response involving cell movements; spreading can become maximal by 30 to 60 min. Even in simple attachment assays, it appears likely that there are some contributions from cell spreading, as the area of attachment of the cell to the substrate expands, accompanied by increasing organization of the cytoskeleton. As spreading and firmer adhesion ensue, cells often form strong focal adhesions to the substrate that are sites of termination of actin microfilament bundles (also known as stress fibers, since the bundles appear to follow lines of linear stress between focal adhesions). As cells attach and spread, they rapidly acquire resistance to detachment, for example by centrifugal force.

Although assays for cell attachment, spreading, or detachment each focus on one particu-

lar facet of cell-substrate adhesion, each assay has strengths, weaknesses, and partial overlaps with the others. Attachment assays are conceptually the simplest, but nonetheless usually unavoidably involve an element of measuring resistance to detachment of the cells by shear forces. In order to rinse unattached cells out of 96-well plates, medium must be poured in and then aspirated or flicked out, thereby generating substantial shear forces as the meniscus passes over attached cells. Cell spreading assays can provide rapid semiquantitative data and evaluate the cytoskeletal facet of cell-adhesive interactions. As a consequence, however, they do not measure only the simple interaction of an adhesion receptor with its ligand, but instead the more complete adhesion response. Spreading assays also involve an element of judgment in choosing to record whether a cell is spread or not spread, which can sometimes perturb a researcher unaccustomed to microscopy. The advantage of centrifugal detachment assays is that they can provide direct physical measurements of adhesive strength as measured in force units ($\times g$). However, this sort of assay is particularly sensitive to timing, and waiting slightly too long before an assay can permit rapid cytoskeletal organization and strengthening responses that anchor cells so tightly to the substrate that they cannot be detached without being destroyed. Nevertheless, centrifugal detachment assays arguably provide the most rigorous quantitative data.

Methods for quantification of cell-to-cell adhesion generally depend on determining the rates of reaggregation of dissociated cells (see *UNIT 9.3*). Although it is also possible to estimate strengths of adhesion by determining the force needed to separate two cells using microneedles, aggregation assays are the norm. These assays are conceptually related to the platelet aggregometer assays used routinely in clinical laboratories to measure platelet adhesion (aggregation). An added feature with nucleated cells, however, is that multiple types of adhesion molecules can often be involved in any particular cell-cell adhesive event. Three commonly observed types of adhesion molecule are the calcium-dependent cadherins, calcium-independent molecules such as N-CAM and other immunoglobulin-repeat molecules, and integrins, though other adhesion molecules may also be involved.

CHARACTERIZATION OF CELL ADHESION MECHANISMS

Once the adhesive characteristics of a cell toward a particular cellular or substrate adhesive system are established, a variety of inhibitors or modulators of adhesion can be tested in order to better define the adhesion system. Examples include function-blocking antibodies against the many dozens of known adhesion molecules to determine which are required for an adhesive event, competitive peptide inhibitors that target the active sites of adhesion receptors, and pharmacological and ionic activators and inhibitors. The latter categories include phorbol esters or Mn^{2+} to activate certain integrins, and chelators for depleting Ca^{2+} to inhibit cadherins and integrins. For routine analyses of integrins, the pertinent receptor(s) involved in a particular adhesive function can be identified by targeting a broad class of integrins first—for instance using an antibody that inhibits all β_1 or all α_v integrins—and then narrowing down the possible candidate receptors involved in a particular function by using more specific antibodies within that class (e.g., anti-α_5 within the β_1 integrin class). Monoclonal antibodies against integrins are widely available. For cadherins, specific antibodies against classical but not novel cadherins are also available, as are various cell biological approaches as described in this chapter. Reagents for analyzing other types of adhesion molecules should become increasingly available from commercial sources.

PROCEDURES DESCRIBED IN THIS CHAPTER

Two semiquantitative assays for measuring cell adhesion to a substrate are provided in *UNIT 9.1*. The first is a cell spreading assay in which adhesion is evaluated by determining

the percentage of cells that spread on a substrate, using microscopy to count spread cells. The second assay quantitates cell attachment after washing out nonattached cells, using a colorimetric protocol for quantification. The relative advantages of each of these widely used assays are compared.

The McClay centrifugation assay for directly quantitating the strength of adhesion of cells to a substrate is described in *UNIT 9.2*. This assay determines the proportion of cells detached by the graded application of centrifugal force. A new variation of the original method provides a simple method for quantitation by counting numbers of stained cells under a light microscope. This simplification of the assay should further enhance the applicability and popularity of this assay. *UNIT 9.2* also provides a helpful in-depth conceptual discussion of the issues involved in quantitating cell adhesiveness.

In *UNIT 9.3*, the original discoverer of the important cadherin cell-cell adhesion system provides an overview of cadherins, as well as detailed laboratory protocols for assaying cell-cell adhesion. Both quantitative and qualitative methods for identifying cadherin function are presented. Besides providing complete protocols for assaying cell-cell adhesion of a single cell type in short- and long-term cultures, this unit describes a method for assaying the sorting-out behavior of different types of cells in a mixed-cell aggregation culture. In addition, a variety of approaches for the detection and characterization of cadherin and associated catenin systems are discussed, along with dominant-negative inhibitor approaches to characterizing this centrally important cell-cell adhesion system.

UNIT 9.4 describes current experimental approaches used to analyze integrin functions in mediating adhesive and other interactions of cells with specific substrates. This unit also provides detailed procedures for in vitro analyses of binding interactions between purified integrins and their ligands.

UNIT 9.5 provides a variety of protocols for studying the major group of calcium-independent cell-cell adhesion proteins termed the immunoglobulin superfamily of cell adhesion molecules (IgSF-CAMs). This superfamily contains over 100 adhesion molecules. After describing how to purify IgSF-CAMs from tissues or culture supernatants, this unit describes a number of approaches to characterizing their biological functions in vitro and in living cells. Assays with fluorescent beads provide ways to mimic and to analyze IgSF-CAM adhesive functions in isolation or in interactions with cells. Complementary approaches using various transfection, adhesive-substrate, inter-molecular interaction, and functional disruption analyses provide a powerful collection or tools to understand the roles and mechanisms of this major class of adhesion proteins.

UNIT 9.6 turns to dynamic analyses of cell adhesion. Cell adhesion often consists of sequential steps in dynamic processes that can be difficult to visualize and experimentally analyze in terms of mechanisms using simple cell attachment protocols. For example, leukocytes in the blood stream do not simply settle by gravity onto surfaces such as endothelial cells or exposed matrix in vessel walls, but they instead interact dynamically in the presence of flow and shear stress. In addition, some adhesive events that occur rapidly are difficult to study in static assays but can be analyzed effectively using dynamic flow methods. *UNIT 9.6* describes methods for such analyses using commercially available flow chambers in which cells being carried in suspension attach in the presence of laminar flow. Flow assays can measure cell adhesion either to a monolayer of endothelial cells or to purified extracellular matrix proteins attached to a substrate. The resulting data obtained in the form of video microscopy recordings can then be analyzed using approaches described in the second basic protocol.

Further supplements will provide protocols for analyzing other types of cell adhesion systems.

LITERATURE CITED

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1994. Cell junctions, cell adhesions, and the extracellular matrix. *In* Molecular Biology of the Cell, 3rd ed, pp. 949-1009. Garland Publishing, New York.

Burridge, K., Chrzanowska-Wodnicka, M., and Zhong, C. 1997. Focal adhesion assembly. *Trends Cell Biol.* 7:342-347.

Chothia, C. and Jones, E.Y. 1997. The molecular structure of cell adhesion molecules. *Ann. Rev. Biochem.* 66:823-862.

Clark, E.A. and J.S. Brugge. 1995. Integrins and signal transduction pathways: The road taken. *Science* 268:233-239.

Edelman, G.M. and J.P. Thiery (eds.). 1985. The Cell in Contact: Adhesions and Junctions as Morphogenetic Determinants. John Wiley & Sons, New York.

Edelman, G.M. and Crossin, K.L. 1991. Cell adhesion molecules: Implications for a molecular histology. *Ann. Rev. Biochem.* 60:155-190.

Gumbiner, B.M. 1996. Cell adhesion: The molecular basis of tissue architecture and morphogenesis. *Cell* 84:345-357.

Hay, E.D. (ed.). 1991. Cell Biology of Extracellular Matrix. Plenum, New York.

Jockusch, B.M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. 1995. The molecular architecture of focal adhesions. *Ann. Rev. Cell Dev. Biol.* 11:379-416.

Kirkpatrick, C. and Peifer, M. 1995. Not just glue: Cell-cell junctions as cellular signaling centers. *Curr. Opin. Genet. Devel.* 5:56-65.

Lafrenie, R.M. and Yamada, K.M. 1996. Integrin-dependent signal transduction. *J. Cell Biochem.* 61:543-553.

Richardson, P.D. and Steiner, M. 1995. Principles of Cell Adhesion. CRC Press, Boca Raton, Fla.

Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. 1995. Integrins—Emerging paradigms of signal transduction. *Ann. Rev. Cell Dev. Biol.* 11:549-599.

Takeda, H., Nagafuchi, A., Yonemura, S., Tsukita, S., Behrens, J., and Birchmeier, W. 1995. V-src kinase shifts the cadherin-based cell adhesion from the strong to the weak state and β-catenin is not required for the shift. *J. Cell Biol.* 31:1839-1847.

Takeichi, M. 1990. Cadherins: A molecular family important in selective cell-cell adhesion. *Ann. Rev. Biochem.* 59:237-252.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K.M. 1998. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* 280:1614-1617.

Wolfsberg, T.G. and White, J.M. 1996. ADAMs in fertilization and development. *Dev. Biol.* 180:389-401.

Woods, A. and Couchman, J.R. 1998. Syndecans: Synergistic activators of cell adhesion. *Trends. Cell Biol.* 8:189-192.

Yamada, K.M. and Geiger, B. 1997. Molecular interactions in cell adhesion complexes. *Curr. Opin. Cell Biol.* 9:76-85.

Yamada, K.M. and Miyamoto, S. 1995. Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.* 7:681-689.

Yap, A.S., Brieher, W.M., and Gumbiner, B.M. 1997. Molecular and functional analysis of cadherin-based adherens junctions. *Ann. Rev. Cell Dev. Biol.* 13:119-146.

Kenneth M. Yamada

Cell-Substrate Adhesion Assays

This unit describes two standard assays for quantitating the adhesion of cells to an immobilized substrate. The first is an assay employing phase-contrast microscopy to measure the spreading of adherent cells (see Basic Protocol 1); the second is a colorimetric assay measuring attachment of cells (see Basic Protocol 2). The relative usefulness of spreading versus attachment assays is discussed later in this unit (see Background Information). Both assays employ microtiter plates and are performed in a humidified 5% to 10% (v/v) CO_2 atmosphere at 37°C. These protocols can be used to determine whether there is adhesion between a given cell type and a given adhesion molecule. Additionally, they can be used to test the regulatory effects of exogenous agents on cell-substrate adhesion.

When the adhesive substrate is a peptide, it should be conjugated to an inert protein to improve immobilization. An additional protocol is provided for preparation of such peptide-protein conjugates (see Support Protocol).

CAUTION: Glutaraldehyde and crystal violet are hazardous chemicals; follow appropriate precautions for handling, storage, and disposal.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be performed in a humidified 37°C, 5% to 10% (v/v) CO_2 incubator unless otherwise specified. CO_2 levels should be adjusted to maintain pH 7.4.

SPREADING ASSAY

BASIC PROTOCOL 1

In this protocol, cells are added to a microtiter plate coated with an adhesive molecule and are incubated to allow spreading. After the incubation period, all cells are fixed with glutaraldehyde, and the wells are examined for the percent spread cells using morphological criteria and phase-contrast microscopy.

Materials

Adhesion molecule of interest
Dulbecco's PBS (DPBS; Life Technologies; *APPENDIX 2A*)
10 mg/ml heat-denatured BSA solution (see recipe)
Cells of interest
Dulbecco's modified Eagle medium with 25 mM HEPES (DMEM/HEPES; Life Technologies; *APPENDIX 2B*), prewarmed to 37°C and gassed with 5% to 10% (v/v) CO_2
2× exogenous agent (e.g., antibody, peptides) in DPBS
5% (w/v) glutaraldehyde (dilute 50% stock solution with water)
CMF-DPBS/NaN_3: divalent cation–free Dulbecco's PBS (CMF-DPBS; Life Technologies; *APPENDIX 2A*) containing 0.05% (w/v) sodium azide (NaN_3)

96-well tissue culture microtiter plates (Costar)
Aspirator
Multichannel pipettor
15-ml polypropylene tubes
Glass coverslips

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

Contributed by Martin J. Humphries
Current Protocols in Cell Biology (1998) 9.1.1-9.1.11

Immobilize substrate

1. Dilute adhesion molecule with DPBS.

   For adhesion to extracellular matrices or purified extracellular matrix molecules (see Chapter 10), 1 to 20 μg/ml is usually adequate. Purified matrix proteins and other molecules can be purchased from a variety of commercial suppliers, including Sigma, Life Technologies, Becton Dickinson Labware, and Calbiochem. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration should be used (see Critical Parameters and Troubleshooting).

   The handling of adhesion molecules prior to dilution varies; some molecules (e.g., fibronectin) are best thawed quickly at 37°C, while others (e.g., laminin) are best thawed slowly on ice.

   Occasionally, it may be necessary to measure adhesion of cells to peptides rather than proteins. In this case, peptides should be conjugated to inert protein carriers as this permits efficient immobilization (see Support Protocol for details).

2. Add 100 μl diluted adhesion molecule to the appropriate wells of a 96-well, tissue culture microtiter plate. Leave a blank well or wells for measuring background spreading on blocked plastic.

   There is generally no need to carry out spreading assays with replicate wells, as quantification is performed by counting multiple fields from within the same well.

3. Incubate 60 min at room temperature or overnight at 4°C.

   Time-course studies have shown that there is substantial coating of proteins onto plastic within an hour at room temperature, which allows the assay to be performed quickly. However, if the adhesion molecule binds weakly to plastic, or if it is more convenient to carry out the experiment the next day, the wells can be coated overnight, usually without detrimental effects.

Block nonspecific adhesion

4. Aspirate fluid, add 200 μl of 10 mg/ml heat-denatured BSA solution to each well with a multichannel pipettor, and incubate 30 min at room temperature.

   Although simple BSA solutions, or other inert proteins, can be used for blocking plastic, heat-denatured BSA is the most effective agent in the author's hands. Nevertheless, its preparation is not straightforward and the procedure must be followed closely. To ensure blocking of the sides of the wells, 200 μl is used rather than 100 μl.

   The time can be varied at this step; 30 min is probably the minimum time for efficient blocking of plastic, but longer times are not detrimental. Even overnight blocking at 4°C produces good results.

Prepare cells

5. During blocking, prepare a suspension of the cells to be examined (e.g., *UNIT 1.1*) in a 15-ml polypropylene tube.

   Trypsin, EDTA, or trypsin/EDTA solutions are commonly used to detach adherent cells. The action of these reagents must be terminated—e.g., by resuspending the cells in DMEM with 10% (v/v) FBS—prior to using the cells in spreading assays. However, the use of these agents usually has no deleterious effect on adhesive activity provided that the cells are not overtrypsinized. It is important to guard against clumping or aggregation of cells; therefore, gentle conditions should be used when centrifuging and resuspending cells. All solutions used during the preparation of cell suspensions should be warmed to 37°C.

   Freshly isolated cells from tissue can also be used, but results are much more variable.

6. Count cells using a hemacytometer (*UNIT 1.1*), and resuspend the cells at 2×10^5/ml in warm DMEM/HEPES gassed with 5% to 10% (v/v) CO_2. Incubate 10 min at 37°C with the lid off in a CO_2 incubator.

   For most cells, this density prevents cells added to the microtiter plate from interfering with each other during the spreading process. If cells contact each other, substrate-dependent adhesive effects can be altered; on the other hand, if cells are too sparse, quantification becomes difficult.

   Cells are left in a polypropylene tube with the lid off to allow them to recover from the process of detachment. Alternatively, the tube can be capped and left on its side in an incubator to prevent the cells from settling and aggregating into a large clump at the bottom of the tube. However, they should not be left too long (to prevent nonspecific adhesion to the sides of the tube), and they should be pipetted gently prior to use (to ensure dispersion).

   Gassing the cell suspension with CO_2 can sometimes give enhanced spreading, although this is not always needed.

   Molecules that mediate spreading are not always resistant to trypsin treatment. However, this 10-minute recovery period is generally sufficient for reexpression.

Perform adhesion assay

7. Aspirate the BSA solution and wash the wells with 100 µl DPBS.

   The heat-denatured BSA is toxic for some cells.

8. To test spreading of specific cell types on given adhesion molecules, add 50 µl DPBS followed by 50 µl cells to the appropriate wells. To examine the effects of exogenous agents (e.g., antibodies, peptides) on spreading, add 50 µl of 2× exogenous agent followed by 50 µl cells. In this case, also add 50 µl DPBS followed by 50 µl cells to control wells.

   Spreading can sometimes be increased by incubating the plate containing exogenous agents several minutes at 37°C, allowing them to warm up prior to addition of cells.

   To ensure good spacing of cells, guard against swirling, tapping, or shaking the wells once cells have been added. In the author's experience, a single pipetting of cells down the side of the well and into the DPBS solution produces good dispersion.

   A number of adhesion studies have focused on divalent cation–dependence, particularly when integrins are involved. Calcium, magnesium, and manganese ions are the most frequently studied. It is worth noting that manganese ions will precipitate phosphate from PBS. Therefore, in the presence of manganese, HEPES-buffered saline should be used in place of DPBS.

9. Incubate 60 to 90 min at 37°C in a 5% to 10% (v/v) CO_2 incubator with the microtiter plate lid off.

   It is important that the DMEM/DPBS mixture has the opportunity to equilibrate as rapidly as possible with gaseous CO_2 in order to reestablish the buffer (see Critical Parameters and Troubleshooting).

   Ensure that the shelves holding the microtiter plates are level, as uneven shelves lead to uneven settling of cells.

Fix and analyze cells

10. Aspirate medium and fix cells by directly adding 100 µl of 5% (w/v) glutaraldehyde down the side of the well and incubating 30 min at room temperature.

11. Aspirate fixative and store cells in 200 µl CMF-DPBS/NaN_3.

    Fixed assay plates can be stored at 4°C for several weeks protected from evaporation.

12. Add sufficient CMF-DPBS/NaN_3 to form an inverted meniscus at the top of the well and carefully place an appropriately sized glass coverslip over the plate.

Understandably, the optical quality of the plastic that is used to make microtiter plates is not ideal for phase-contrast microscopy. However, adding a coverslip to an overly full well can greatly improve the observation of adherent cells in microtiter plates.

13. Using an inverted phase-contrast microscope, quantify percent spreading in each well by counting three separate, randomly selected fields of 100 cells each. Use an eyepiece graticle to aid the selection of cells and to minimize double counting. Use specific morphological criteria, such as a phase-dark cell body and cytoplasm that is visible around the entire circumference of the nucleus, to identify cells with spread morphology.

Alternatively, image analysis software can be used to measure average cell area (see Critical Parameters and Troubleshooting).

BASIC PROTOCOL 2

ATTACHMENT ASSAY

This protocol measures the attachment of cells to microtiter plates coated with an adhesion molecule. After incubation, nonattached cells are removed and the remaining attached cells are fixed with glutaraldehyde, washed, and stained with crystal violet. The plates are read by a microtiter plate reader, and the percent attached cells is calculated.

Materials

Adhesion molecule of interest
Dulbecco's PBS (DPBS; Life Technologies; *APPENDIX 2A*)
10 mg/ml heat-denatured BSA solution (see recipe)
Cells of interest
Dulbecco's modified Eagle medium with 25 mM HEPES (DMEM/HEPES; Life Technologies; *APPENDIX 2B*), prewarmed to 37°C and gassed with 5% to 10% (v/v) CO_2
2× exogenous agent (e.g., antibody, peptides) in DPBS
5% (w/v) glutaraldehyde (dilute 50% stock solution with water)
0.1% (w/v) crystal violet solution (see recipe)
10% (v/v) acetic acid

96-well tissue culture microtiter plates (Costar)
Aspirator
15-ml polypropylene tubes
Microtiter plate reader

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

Immobilize substrate

1. Dilute adhesion molecule with DPBS.

For adhesion to extracellular matrices or purified extracellular matrix molecules (see Chapter 10), 1 to 20 μg/ml is usually adequate. Purified matrix proteins and other molecules can be purchased from a variety of commerical suppliers, including Sigma, Life Technologies, Becton Dickinson Labware, and Calbiochem. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration should be used (see Critical Parameters and Troubleshooting).

The handling of adhesion molecules prior to dilution varies; some molecules (e.g., fibronectin) are best thawed quickly at 37°C, while others (e.g., laminin) are best thawed slowly on ice.

Occasionally, it may be necessary to measure adhesion of cells to peptides rather than proteins. In this case, peptides should be conjugated to inert protein carriers as this permits efficient immobilization (see Support Protocol for details).

2. Add 100 µl diluted adhesion molecule to the appropriate wells of a 96-well, tissue culture microtiter plate using a P200 pipettor. Prepare sufficient wells to perform assays in triplicate or quadruplicate. Leave sufficient blank wells to determine 100% attachment at three cell concentrations and to determine background binding of crystal violet to plastic.

   A standard pipettor should be used rather than a multichannel pipettor, as attachment assays rely much more heavily on the accuracy of pipetting than do spreading assays.

   The blank wells for 100% attachment are not blocked with BSA. The background wells are treated as shams, and therefore are blocked with BSA.

3. Incubate 60 min at room temperature or overnight at 4°C.

   Time-course studies have shown that there is substantial coating of proteins onto plastic within an hour at room temperature, which allows the assay to be performed quickly. However, if the adhesion molecule binds weakly to plastic, or if it is more convenient to carry out the experiment the next day, the wells can be coated overnight, usually without detrimental effects.

Block nonspecific adhesion

4. Aspirate fluid, add 200 µl of 10 mg/ml heat-denatured BSA solution to each sample well, and incubate 30 min at room temperature. Block wells for background crystal violet binding, but not those for 100% attachment.

   Although simple BSA solutions, or other inert proteins, can be used for blocking plastic, heat-denatured BSA is the most effective agent in the author's hands. Nevertheless, its preparation is not straightforward and the procedure must be followed closely. To ensure blocking of the sides of the wells, 200 µl is used rather than 100 µl.

   The time can be varied at this step; 30 min is probably the minimum time for efficient blocking of plastic, but longer times are not detrimental. Even overnight blocking at 4°C produces good results.

Prepare cells

5. During blocking, prepare a suspension of the cells to be examined in a 15-ml polypropylene tube. Prepare a sufficient number of cells to perform assays in triplicate or quadruplicate.

   Trypsin, EDTA, or trypsin/EDTA solutions are commonly used to detach adherent cells. The action of these reagents must be terminated—e.g., by resuspending the cells in DMEM with 10% (v/v) FBS—prior to using the cells in spreading assays. However, the use of these agents usually has no deleterious effect on adhesive activity provided that the cells are not overtrypsinized. It is important to guard against clumping or aggregation of cells; therefore, gentle conditions should be used when centrifuging and resuspending cells. All solutions used during the preparation of cell suspensions should be warmed to 37°C.

   Freshly isolated cells from tissue can also be used, but results are much more variable.

6. Count cell density on a hemacytometer (*UNIT 1.1*), and prepare working cell suspensions at 5×10^5/ml for fibroblasts and similarly sized cells, and at 1×10^7/ml for leukocytic cells in warm DMEM/HEPES gassed with 5% to 10% (v/v) CO_2. Incubate 10 min at 37°C with the lid off in a CO_2 incubator.

   It is advisable to use pipet tips that have their ends cut off for attachment assays. This is to prevent coated proteins and/or cells from being washed off directly by a fine stream of liquid.

These densities prevent cells from interfering with each other during the attachment process. If cells contact each other, substrate-dependent adhesive effects can be altered; on the other hand, if cells are too sparse, quantification becomes difficult.

Cells are left in a polypropylene tube with the lid off in a CO_2 incubator to allow them to recover from the process of detachment. Alternatively, the tube can be capped and left on its side in an incubator to prevent the cells from settling and aggregating into a large clump at the bottom of the tube. However, they should not be left too long (to prevent nonspecific adhesion to the sides of the tube), and they should be pipetted gently prior to use (to ensure dispersion).

It is more important to gas the medium for an attachment assay than it is for the spreading assay, particularly when the level of adhesion is predicted to be low. Gassing the cell suspension with CO_2 can sometimes give enhanced attachment, although this is not always needed.

Perform adhesion assay

7. Aspirate the BSA solution and wash the wells with 100 µl DPBS.

   The heat-denatured BSA is toxic for some cells.

8. To estimate 100% attachment, dilute cells to 20%, 50%, and 100% of the working cell suspension using warm DMEM/HEPES gassed with 5% to 10% (v/v) CO_2. Add 50 µl DPBS followed by 50 µl cells to uncoated wells.

   Cells can also be plated on poly-L-lysine-coated plastic (APPENDIX 2A) to determine 100% attachment.

   Using cell dilutions and extrapolating the resulting graph is the most accurate way of determining 100% attachment, as the absorbance value for 100% attachment with undiluted cells may be off the linear range of the plate reader.

   To ensure good spacing of cells, guard against swirling, tapping, or shaking the wells once cells have been added. In the author's experience, a single pipetting of cells down the side of the well and into the DPBS solution produces good dispersion.

9. To test attachment of specific cell types to given adhesion molecules, add 50 µl DPBS followed by 50 µl cells to the appropriate wells. To examine the effects of exogenous agents (e.g., antibodies, peptides) on attachment, add 50 µl of 2× exogenous agent followed by 50 µl cells. In this case, also add 50 µl DPBS followed by 50 µl cells to control wells.

   Attachment can sometimes be increased by incubating the plate containing exogenous agents several minutes at 37°C, allowing them to warm up prior to addition of cells.

   A number of adhesion studies have focused on divalent cation–dependence, particularly when integrins are involved. Calcium, magnesium, and manganese ions are the most frequently studied. It is worth noting that manganese ions will precipitate phosphate from PBS. Therefore, in the presence of manganese, HEPES-buffered saline should be used in place of DPBS.

10. Incubate 15 to 20 min at 37°C in a 5% to 10% (v/v) CO_2 incubator with the microtiter plate lid off.

    The incubation time for attachment assays may need to be adjusted depending on the cell type, as some cells adhere more quickly than others.

    It is important that the DMEM/DPBS mixture has the opportunity to equilibrate as rapidly as possible with gaseous CO_2 in order to reestablish the buffer (see Critical Parameters and Troubleshooting).

    Ensure that the shelves holding the microtiter plates are level, as uneven shelves lead to uneven settling of cells.

Remove loose cells and fix adherent cells

11. Fix cells only in the wells to be used for determining 100% attachment by adding 100 µl of 5% (w/v) glutaraldehyde.

12. Remove nonadherent and loosely attached cells from remaining wells by either tapping the plate or gently washing the wells 1 to 3 times with 100 µl DPBS and cut-off pipet tips.

    This is the most critical stage in an attachment assay, and needs to be optimized for each cell type used (see Critical Parameters and Troubleshooting).

13. Aspirate the final wash and fix attached cells by adding 100 µl of 5% (w/v) glutaraldehyde and incubating 20 min at room temperature (or at 4°C overnight, if necessary).

14. Wash wells three times with 100 µl water.

Stain and analyze adherent cells

15. Add 100 µl of 0.1% (w/v) crystal violet solution to each well and incubate 60 min at room temperature.

    Avoid getting crystal violet solution on the rims of the wells, as this dries during incubation and can be difficult to remove by washing.

    Staining can also be performed overnight without detriment to the final results.

16. Wash wells three times with 400 µl water.

    A larger wash volume is used to remove all traces of stain.

17. Solubilize dye in 100 µl of 10% (v/v) acetic acid and incubate 5 min on an orbital shaker at 150 rpm, room temperature.

18. Measure absorbance at 570 nm using a microtiter plate reader. Subtract background crystal violet staining from all experimental and 100% attachment results. Plot data from 20%, 50%, and 100% inocula (A_{570} versus cell density) and determine the value for 100% attachment by extrapolation. Use this value to express experimental data as percent attachment.

PREPARATION OF PEPTIDE-PROTEIN CONJUGATES

SUPPORT PROTOCOL

The discovery that many large adhesion proteins contain short peptide sequences that are recognized by cellular receptors has enabled these receptor sites to be probed with the use of synthetic peptide reagents. By definition, these peptides are small and usually bind poorly to plastic. In addition, immobilization of peptides may reduce their cellular adhesivity by masking key residues that endow adhesive activity. For these reasons, it is necessary to use an alternative method to simple coating from a peptide solution. The author prefers to prepare covalent conjugates of peptides to protein carriers that are inert in terms of adhesive activity. Many different cross-linkers and carriers are available for such syntheses, but consistent success can be obtained with *N*-succinimidyl 3-(2-pyridyl-dithio)propionate (SPDP), a heterobifunctional cysteine-lysine cross-linker, and rabbit IgG as carrier. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) also works well as a cross-linker, and BSA and keyhole limpet hemocyanin are good carriers. A detailed description of the use of these agents can be found in Humphries et al. (1994). The first step in the conjugation reaction is the conjugation of the cross-linker to the carrier. The second step is the conjugation of the cross-linker/carrier to the peptide, which requires disulfide bond formation between the cross-linker and peptide, and is slow. Thus, peptides are usually designed with a terminal cysteine residue.

Materials

6 mg/ml normal rabbit IgG (Sigma) in CMF-DPBS
3 mg/ml *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce) in ethanol
Divalent cation–free Dulbecco's PBS (CMF-DPBS; Life Technologies; *APPENDIX 2A*)
Cysteine-containing peptide, solid
Dulbecco's PBS (DPBS; Life Technologies; *APPENDIX 2A*)
10-ml Sephadex G25 columns (PD-10, Amersham Pharmacia Biotech)

Additional reagents and equipment for dialysis (*APPENDIX 3*)

1. Pipet 1 ml of 6 mg/ml normal rabbit IgG into a microcentrifuge tube. Add 0.2 ml of 3 mg/ml SPDP by rapid injection and mix thoroughly. Incubate 30 min at room temperature on a rotator.

   SPDP is not very soluble in aqueous solution. It should be added to and mixed with IgG rapidly to avoid precipitation.

2. Equilibrate a 10-ml Sephadex G25 column with 10 to 20 vol CMF-DPBS.
3. Add the rabbit IgG/SPDP mixture to the column followed by CMF-DPBS. Discard the first 2.5 ml eluate and collect the next 3.5 ml (containing cross-linker-derivitized IgG) in a single tube.
4. Aliquot the collected fraction as necessary (depending on the number of conjugates to be made) and add to 1 to 2 mg solid cysteine-containing peptide. Vortex briefly and incubate overnight at room temperature on a rotator.

   Radiolabeled peptide can be used to quantify coupling if necessary, although, in the author's experience, coupling is quite consistent from peptide to peptide.

5. Dialyze the mixture (*APPENDIX 3*) against several changes of DPBS (with divalent cations) to remove uncoupled peptide. Dialyze using standard dialysis tubing in ≥1000-fold DPBS at 4°C with gentle stirring. Dialyze >3 hr for each change of DPBS.
6. Divide into 100-µl aliquots and store indefinitely at −80°C.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Crystal violet solution, 0.1%

Dissolve 0.1% (w/v) crystal violet in 200 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6. Filter through a 0.22-µm filter. Store at room temperature.

CAUTION: *Crystal violet is hazardous; handle with caution and dispose of properly.*

It is imperative to filter this crystal violet solution. It can be difficult to determine whether it has dissolved properly due its intense color. If the solution is not filtered, specks of solid crystal violet can sometimes be added to the experimental wells, resulting in spuriously high absorbance readings.

Heat-denatured BSA solution, 10 mg/ml

Dissolve BSA at 10 mg/ml in divalent cation–free Dulbecco's PBS (CMF-DPBS; Life Technologies; *APPENDIX 2A*) in a 15-ml polystyrene tube. Filter through a 0.22-µm filter to remove undissolved protein, and incubate 10 to 12 min in an 85°C water bath. Cool to room temperature and use the same day.

The major aim is to prepare a solution that contains microparticulate BSA, as this will coat plastic very efficiently. The solution should go slightly hazy. A clear solution indicates insufficiently aggregated BSA, while a white solution indicates that aggregates are too large. After cooling, the solution is ready for use.

COMMENTARY

Background Information

As the name suggests, adhesion assays measure cell adhesion. However, because adhesion is a complex process involving receptor-ligand binding, modulation of intracellular signaling, and cytoskeletal modulation, these assays can also be a source of information about other cellular events and a means of probing the contribution of these processes to adhesion. In general, adhesion assays are used to test whether a certain cell type can adhere to a certain adhesive substrate, and to test the sensitivity of a particular cell-substrate interaction to inhibitors. This unit describes two standard assays for quantifying the adhesion of cells to an immobilized substrate. The spreading assay is based on an original publication by Yamada and Kennedy (1984), and the attachment assay is based on Kueng et al. (1989). As presented here, the attachment assay employs colorimetric detection of bound cells. However, this is actually one of many different readouts that can be applied to the basic assay. Common alternatives include the use of radiolabeled or fluorescently labeled cells (Dustin and Springer, 1989; Chan et al., 1992), or the measurement of endogenous gene activity (e.g., phosphatase; Prater et al., 1991). In general these are more time-consuming to perform.

A number of considerations will affect the decision whether to use a cell spreading assay or a cell attachment assay. Spreading assays take longer to perform, but are less prone to nonspecificity. For example, many molecules can mediate attachment of cells that is not physiologically relevant, but very few of these molecules can mediate morphological spreading. Importantly, by observing cells in a spreading assay, a lot of information can be gained about the biological response of the cells to the substrate. The morphology of cells can differ even if the percent spreading is the same. Additionally, now that signaling mechanisms that control cell morphology are better understood, spreading assays can give indirect indications of the intracellular events that are triggered by certain substrates. Spreading assays are more sensitive when used to measure the inhibitory activity of an exogenous agent, because the readout from the assay is more reliant on multiple adhesive interactions, and partial disruption by an inhibitor is sufficient to see a biological effect. A greater degree of receptor blockade is probably needed to observe an inhibitory effect in an attachment assay. Finally, as spreading assays do not need replicate wells, it is easier to construct detailed dose-response curves.

Nonetheless, use of attachment assays is sometimes obligatory, because not all cells are able to spread and because some cells only spread on certain substrates. It is misleading to think that attachment assays measure single adhesive contacts: multiple contacts are needed for a cell to withstand the washing steps in an attachment assay. Nevertheless, fewer contacts are needed for attachment than for a cell to spread.

Critical Parameters and Troubleshooting

General considerations for adhesion

The major problem likely to be encountered in both assays is that cells fail to adhere. Many factors can lead to this, including coating plates with insufficient amounts of adhesive substrate, using bad batches of adhesive substrate, using poor protein-binding microtiter plates or badly constructed plates with uneven wells, squirting liquids too vigorously onto the bottom of the wells, using cultures that are growing poorly or have a mycoplasma infection, having variations in pH during adhesion, and (for attachment assays) overwashing. Several of these factors are discussed in further detail below.

Substrate concentration. The concentration of adhesion molecule required for coating depends on a number of factors, including the size of the molecule, the efficiency with which it coats plastic, and the apparent affinity with which it is bound by cellular receptors. In most cases, spreading assays are used to measure the adhesion of cells to extracellular matrices or purified extracellular matrix molecules (also see Chapter 10). The key components of such matrices are usually large macromolecules that coat plastic relatively well; they are also bound with at least moderate affinity by cells. For these reasons, a concentration range between 1 and 20 μg/ml is usually adequate, although it is advisable to carry out a range-finding dose-response experiment before focusing on a narrow range. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration should be used.

Plates. Most tissue culture microtiter plates are adequate for spreading and attachment experiments, although the author finds Costar to be excellent. Immulon 4HB plates from Dynex

Technologies (formerly Dynatech Labs) have higher protein-binding capacity and are particularly good for assays involving small proteins.

Cultures. For cell spreading assays, an important parameter is the health of the cells. Cultures should be actively growing, but should have been passaged >24 hr previously. Relatively poor spreading responses have been observed in cells that were passaged the day before a spreading assay.

pH. When cells and DPBS (with or without exogenous agents) are combined in the coated well, it is important that the DMEM/DPBS mixture has the opportunity to equilibrate as rapidly as possible with gaseous CO_2 in order to reestablish the buffer. This process can be aided by leaving the lid off the microtiter plate in the incubator. It is also the author's experience that the adhesion of some cell types is improved by raising the concentration of gaseous CO_2 from 5% to 10% (v/v). This can be particularly effective at increasing binding to poorly adhesive substrates. It may also be advisable to use a particular incubator and/or time of day when the door to the incubator will not be opened, as this helps prevent alkalinization of the medium.

Washing. For attachment assays, the key parameter is the washing protocol, as this is the major determinant of the signal-to-noise ratio. When washing unattached cells (see Basic Protocol 2, step 12), different cells respond differently to tapping and washing, and it is recommended to vary the number of tapping or washing cycles to obtain the best signal-to-noise ratio (i.e., attachment to an adhesive substrate compared to attachment to BSA-blocked plastic). Sometimes this can even be judged by eye in a pilot experiment. Cut-off pipet tips are particularly important at this stage to avoid removal of attached cells.

More specific problems include cell death in the assay (which can be caused by exposure of sensitive cells to heat-denatured BSA) and clumping of cells either in the center of a well or around the perimeter (which is caused by swirling the plate). In addition, large errors in attachment assays can result from inaccurate pipetting, which can come from use of multichannel pipettors, or suboptimal washing of wells (note that the volume of BSA blocking solution is higher than the volume of adhesive substrate, and that the wash step after crystal violet staining is larger still).

Assessing cell spreading

Different methods can be used to determine whether a cell is spread or not. Perhaps the most quantitatively accurate method is to use image analysis software to measure average cell area; however, this tends to produce small differences that may be hard to interpret without the application of other criteria relating to cell shape. Instead, the author prefers to assign specific criteria to a definition of spreading and apply these to each cell individually. The usual criteria are that the cell body should be phase-dark and that cytoplasm should be visible around the entire circumference of the nucleus. Different cells adopt different morphologies and therefore these criteria might need to be slightly modified on a case-by-case basis.

Anticipated Results

Both the spreading and attachment assays measure percent adhesion, and the level of adhesion obtained depends upon the cell type and adhesive substrate under study. In spreading assays, a level of 80% should be anticipated, and often higher levels can be obtained. It is critical that the background level of spreading on BSA-coated plastic be as low as possible. Frequently, this is actually zero, but certainly this should not rise above 2% to 3%. The level of attachment observed by the crystal violet staining method is usually not as high as for spreading, but 60% to 70% should be attainable. The background level of attachment (i.e., on BSA-coated plastic) should also be as low as possible; however, for attachment assays, this level is usually higher (5% to 10%).

Time Considerations

Both spreading and attachment are relatively quick to perform. Coating and blocking the wells requires <2 hr. The actual assays can be carried out easily in half a day, and the subsequent quantification of spreading assays can take a similar length of time. Quantification of attachment assays is much quicker (i.e., <2 hr).

Literature Cited

Chan, B.M.C., Elices, M.J., Murphy, E., and Hemler, M.E. 1992. Adhesion to vascular cell adhesion molecule 1 and fibronectin. Comparison of α4β1 (VLA-4) and α4β7 on the human B cell line JY. *J. Biol. Chem.* 267:8366-8370.

Dustin, M.L. and Springer, T.A. 1989. T cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 341:619-624.

Humphries, M.J., Mould, A.P., and Weston, S.A. 1994. Conjugation of synthetic peptides to car-

rier proteins for cell adhesion studies. *J. Tissue Culture Methods* 16:239-242.

Kueng, W., Silber, E., and Eppenberger, U. 1989. Quantification of cells cultured on 96-well plates. *Anal. Biochem.* 182:16-19.

Prater, C.A., Plotkin, J., Jaye, D., and Frazier, W.A. 1991. The properdin-like type I repeats of human thrombospondin contain a cell attachment site. *J. Cell Biol.* 112:1031-1040.

Yamada, K.M. and Kennedy, D.W. 1984. Dualistic nature of adhesive protein function: Fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. *J. Cell Biol.* 99:29-36.

Contributed by Martin J. Humphries
University of Manchester
Manchester, United Kingdom

Quantitative Measurement of Cell Adhesion Using Centrifugal Force

The following protocol was developed in order to study the biophysical sequence of events in cell-substrate adhesion, and it can also be adapted to study cell-cell adhesion. The method allows quantification of the weak association between cells and their substratum at 4°C, thereby giving a measure of the receptor-ligand affinity only. The approach also allows measurement of the ATP-dependent events that strengthen adhesion and that involve the cytoskeleton following initial binding. This is done by incubating cells in contact with substrate at 37°C for increasing periods of time. Centrifugal force is the only shear force involved in the assay; thus the strength of any adhesion that resists this applied force can be accurately measured. The original centrifugal adhesion assay developed by McClay et al. (1981) used radioactively labeled cells in a rather cumbersome procedure to quantify adhesion. The protocol given here is much easier than any previously published version. At the same time, the assay remains highly quantitative and has simple innovations that can accommodate many kinds of adhesion studies.

The procedure is as follows: 96-well polyvinyl chloride (PVC) plates with flat bottoms are coated with the substrate target for the cells. The wells are then treated with BSA or another nonadherent protein that blocks nonspecific binding sites. Cells are added to the PVC plates on ice, the wells are filled to the brim, and then sealed with clear packing tape. The idea is to enclose each well as a sealed, fluid-filled compartment without any air bubbles. The cells are then gently centrifuged into contact with the substrate. To measure cell-substrate affinity alone, the wells are kept at 4°C, flipped upside down, and centrifuged to provide a defined dislodgement force for the cells (Fig. 9.2.1). Using replicates challenged with different centrifugal forces one can determine the relative avidity of cells for a substrate. If cells are incubated at 37°C after being centrifuged onto the substrate, one can follow the progress of adhesive strengthening due to cytoskeletal engagement. This process generally occurs rapidly, and progress can be monitored by this assay.

There are many ways to quantify cells adhering to the substrate. Currently, the simplest is to image the well bottom and count cells bound relative to controls and to the total

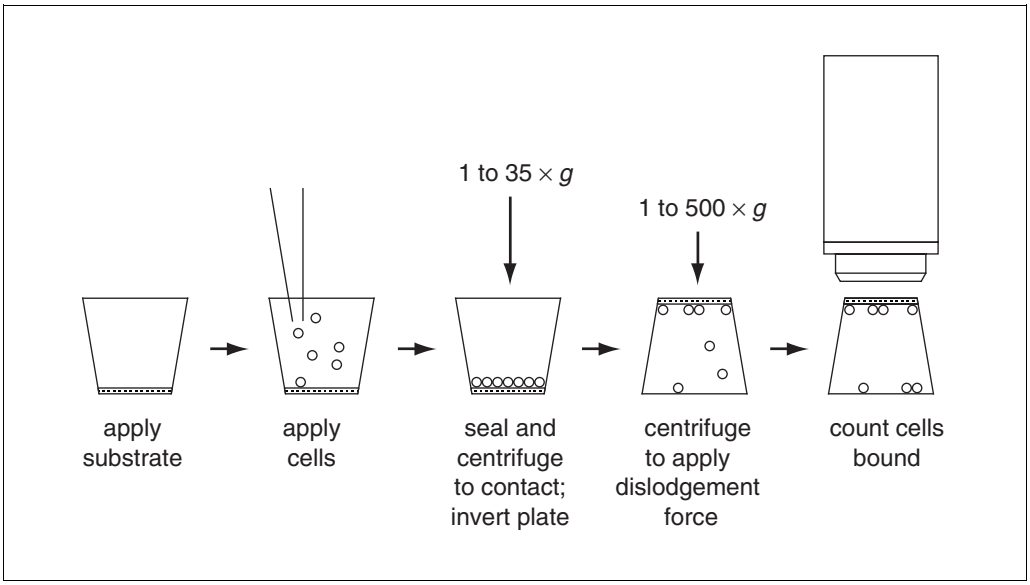


Figure 9.2.1 Centrifugal force adhesion assay with light microscopic readout.

Contributed by David R. McClay and Philip L. Hertzler
Current Protocols in Cell Biology (1998) 9.2.1-9.2.10

number of cells originally added. Other approaches can easily be adapted; however, this method provides a simple visual measure of adhesion that is quite easy to learn, is easily adapted by any laboratory at low cost, and allows for a number of variations to be applied, including morphometrics (see Background Information).

BASIC PROTOCOL

CENTRIFUGATION CELL ADHESION ASSAY

Materials

96-well flat-bottom polyvinyl chloride (PVC) plates (Falcon)
Substrate molecule of interest in PBS
40 mg/ml BSA (fraction V; Sigma) in PBS
PBS (*APPENDIX 2A*)
Tissue culture medium without FBS
Dissociated cells of interest, suspended at $5–10 \times 10^5$/ml in calcium-free physiological solution (see Critical Parameters, discussion of suitable media)
Clear packing tape (3M Scotch 375, 4.8 cm wide, although any clear packing tape will probably work)
Microtiter plate support template
Low-speed refrigerated cell centrifuge with microtiter plate carrier

1. Cut away the sides of a 96-well flat-bottom PVC plate with scissors, and then cut the plate into a 3 × 8–well rectangle.

   Only the six wells in the middle are used. Since the assay involves filling the wells over the brim to eliminate air bubbles, some of the medium will spill into the surrounding empty wells. Plates can be cut into other configurations depending upon the centrifuge carrier, the microscope, or other variables that are particular to the lab.

2. Add 50 µl substrate per well, leaving blank wells for 100% attachment (no substrate or blocking) and for background binding (no substrate). Use at least three replicates for each parameter tested. Incubate ~30 min at room temperature.

   Polyvinyl chloride has a natural avidity for many proteins.

   As a control, determine how much of the substrate protein is attached to the plate. In the first set of experiments with a substrate protein or peptide, determine the threshold concentration for binding (relative to the BSA control wells and at a low g force), and then operate just above that threshold in subsequent experiments (see Critical Parameters).

3. Wash three times with 100 µl PBS. Then block substrate-coated and background binding wells (but not 100% attachment control wells) ~30 min with 50 µl of 40 mg/ml BSA at room temperature. Wash three times with 100 µl PBS, flicking the contents of the wells into the sink to eliminate the fluid after each wash.

   It is important that the background control well does not bind cells. BSA treatment should block all spaces not covered by the substrate molecule. Typically 100% of cells bind to the untreated wells, and <1% bind to the background wells. Although this varies according to the cell type used and the dislodgement force applied, in most cases the conditions can be adjusted so that <1% of cells bind to the BSA-blocked wells. This leaves the test substrate with a wide range in which to examine adhesion avidity.

   If desired, poly-L-lysine can be added to the 100% attachment control well (no substrate, no blocking) to bind cells even more tightly.

4. Place the plate on ice. Add 100 µl tissue culture medium to all wells, add 100 µl cell suspension ($5–10 \times 10^4$ cells), and then fill all wells with another 100 µl tissue culture medium.

   Fewer cells can be added, especially if fluorescent cells are used for later quantification. For fluorescent cells, good results can be obtained with as few as 1000 cells per well. Also,

it is possible to employ two different fluorescent colors and unstained cells in the same well, so that two experimental cell types can be compared to a control cell population under exactly the same conditions.

Medium and cells should be added on ice to prevent cells from attaching and adhering prematurely. There should be a positive meniscus bulging above the top of the well, as excess fluid is necessary to seal the well without any air bubbles.

5. Gently lay a piece of clear packing tape over the wells, and place the plate on a microtiter plate support. Apply the tape, starting from one end and squeegeeing the excess medium to spill over into the empty wells next to the test wells. Press down on the tape to make sure it adheres to the PVC surrounding each well.

   The microtiter plate support is a rubber or metal template that holds the PVC plate while it is being sealed. A firm backing is necessary to support the pressure applied when sealing the tape. A metal plate can be made by a machine shop. Its thermal conductance keeps the wells cold during the manipulation and sealing procedures. If metal supports are unavailable, a 96-well plate can be used as a mold to make a plastic support device.

   With a little practice, this maneuver can be done without introducing any bubbles into the sealed wells. If sealing is done correctly, each well should now be a completely filled, enclosed chamber, and should survive centrifugal forces up to ~500 × g without leaking. Some cells will be lost in the process of sealing, but that will not add an error factor to the analysis because of the way cells are counted against controls in the assay.

6. Centrifuge cells into contact with the substrate for ~3 min at 35 × *g*, 4°C, in a low-speed refrigerated centrifuge with a microtiter plate carrier.

   Some larger cells can be allowed to settle onto the substrate at 1 × g. If cells are centrifuged onto the substrate, ~3 min of centrifugation will place 100% of the cells into contact with the substrate.

   If this is done at 4°C, receptor-ligand combinations are brought into contact. As long as the plate is left at 4°C, this receptor-ligand affinity can be measured independent of cytoskeletal contributions.

   One can substitute a big centrifuge bucket as a swinging bucket carrier if a microtiter plate carrier is not available.

7. *Optional:* To measure strengthening events in adhesion, simply move the plate, with cells in contact with the substrate, to a 37°C water bath and float the plate for the desired period of time.

   For many cell types, incubations <5 min are sufficient to convert the weak receptor-ligand binding into a highly strengthened adhesion that is strongly resistant to centrifugal shear forces.

8. Invert the plate and centrifuge the cells in the inverted position using a microtiter plate carrier, 5 min at the desired speed. Return the plate to ice, keeping it in the inverted position.

   This should dislodge cells if enough centrifugation force is applied and if the cells are not strongly adhering. Obviously there are a number of parameters that can be controlled in this step: e.g., duration of contact at 37°C, rate of dislodgement, force of centrifugation, and rate of strengthening.

9. Place the wells on the stage of a compound microscope, and count cells using a 10× objective. Subtract background binding from substrate binding, and determine the percent binding compared to the 100% attachment control.

   Imaging software can be used to capture frames and automatically count the cells in each frame. To increase accuracy, set up the microscope so it will image a well prior to centrifugation, then return to the same field after centrifugation to assess adhesion.

COMMENTARY

Background Information

Early cell adhesion assays used cell aggregation as a semiquantitative measure. These assays were useful for studying some adhesive phenomena, but suffered from an inability to quantify the sequence of adhesion. Attempts to quantify adhesion have used viscometers, cell particle counters, spectrophotometers, radioactivity, and other approaches. In most cases, adhesion assays were limited in that they required cells to associate rather tightly with one another in order to survive unknown, or poorly defined, shear forces intrinsic to the assay.

This centrifuge assay was designed to use a single shear force of defined magnitude. The force operating on a cell can be easily calculated, providing an impression of the strength of an adhesion. This approach, though quantitative, is still not perfect. For example, one would really like to measure the "on rate" as an adhesion is formed, but this is an exceedingly difficult parameter to measure. Also, the assay described here measures the proportion of adherent cells within a population, rather than the behavior of individual cells. Single cell assays may offer advantages not offered by the assay given here.

The present assay is based on previously published versions (McClay et al., 1981; Lotz et al., 1989; Burdsal et al., 1991, 1994) in which centrifugal force is used as a dislodgement force (F_D) for the cells. This assay is highly modified and simplified compared to the original assays, but enjoys a better and more versatile capacity for quantification.

The following relationship is important for understanding where force fits into the adhesion process. As measured in the centrifugal adhesion assay, $F_D = (\rho_{cell} - \rho_{medium}) \times V_{cell} \times$ RCF, where F_D is the dislodgement force (dynes/cell) tending to pull the cell from the substrate, $(\rho_{cell} - \rho_{medium})$ is the specific density difference between the cell and the medium (usually 1.07 g/cm^3), V_{cell} is the volume of the cell, and RCF is the relative centrifugal force (McClay et al., 1981). Because of the small difference in specific density between the fluid and the cell, cells in culture are little affected by alterations at $1 \times g$, since in reality these cells are already experiencing microgravity.

The assay as presented above allows for very precise comparisons between cells that are only slightly different from one another. For example, if one cell population is transfected with a construct and the other population is a non-transfected control, the two can be tested in the same well (with one of the populations fluorescently tagged) to accurately compare adhesive performance.

Most other cell-substrate assays have a certain unknown shear force that can confound quantification, especially when one wants simply to measure receptor-ligand interactions. The simple "stick-and-wash" assay, in which one allows cells to bind to the substrate for a period of time and then washes the wells, may be simpler to use than the assay presented above, but suffers from several problems. It has an undefined shear force (washing) that is difficult to control accurately. Stick-and-wash assays also cannot measure the initial receptor-ligand interactions, because cells must adhere at least somewhat tightly to the substrate in order to survive washing. Thus, the adhesion measured in a stick-and-wash assay is actually both adhesion and engagement of the cytoskeleton. Often such assays are completed hours after cells were added to wells, so that any number of postadhesion events could occur before measuring "adhesion." In contrast, the present assay allows measurement of the progression of several adhesion events separately, and therefore has the capacity to examine several parameters in the sequence of forming an adhesion. It should be mentioned that for more than two decades the simpler "stick-and-wash" assay was successfully employed by a number of laboratories to find and characterize most of the known adhesion molecules. The present assay is useful for detailed structure-function analyses of those molecules.

An additional parameter that can be measured with the centrifugal adhesion assay is the relationship between adhesiveness and cell phenotype. Since the cells are counted by microscopy, one can also score cell phenotypic properties (e.g., spreading or motility). This adds to the versatility of the assay. The assay can be used, with minimal adaptation, for a wide variety of cell types, including cells from several phyla, tumor cells, and cells of all stages of embryonic development.

Initial binding

In practice there are many events in the establishment of an initial adhesion. There is an on rate by which the first receptor-ligand interactions occur. Then there is a rate of recruitment of additional cellular adhesion molecules to the site of adhesion. This parameter has

been measured, and is related to the rate of diffusion of the protein in the phospholipid bilayer. As long as no additional molecules are added to the bilayer, the total recruitment of receptor-ligand combinations is limited to the total population in the membrane at the time of the assay. Finally, since the interaction is not covalent, there is an off rate. Most cell types bind to substrates with a force that resists 10^{-6} to 10^{-5} dynes/cell of applied centrifugal dislodgement force. In reality, cells come off the substrate when the off rate (increased by centrifugal force) exceeds the on rate. Red blood cells fail to adhere to most substrates at around 10^{-8} dynes/cell. Some cells, such as macrophages, naturally adhere with a receptor-ligand avidity that is slightly higher than the 10^{-5} dynes/cell level. When measuring initial binding one must measure the adhesion at very low substrate concentrations. Once the well becomes supersaturated with multiple layers of substrate, the initial binding loses specificity for the receptor-ligand combination under investigation. This presumably results from nonspecific effects of charge and substrate heterogeneity.

Cytoskeletal contribution to strengthening

After obtaining a measurement of initial binding (receptor-ligand affinity), a measurement can be made of the strengthening of that adhesive process. This cellular process is remarkably fast. Using this assay, most strengthening is completed within 5 min at 37°C. The degree of strengthening is usually more than 2 orders of magnitude, and may be as much as 5 to 6 orders of magnitude.

The limitations of this assay come into play during the strengthening process. Although we recommend a range of 1 to $500 \times g$ for the assay, the centrifuge plate carriers have a maximum safe speed that cannot be exceeded. Using large buckets as plate carriers can increase the centrifugation speed to ~4000 rpm. This means that the assay can be extended to forces between 1 and $\sim 2500 \times g$ in most centrifuges. In measurements using the ultracentrifuge to achieve very high forces, it was found that adherent fibroblast cells are not released by $15{,}000 \times g$, and only 30% are released at $\sim 59{,}000 \times g$ (Rich, 1978). Thus, for practical measurements of strengthening, one can quantify the early events to monitor the rate of strengthening, and one can study cells with modified cytoskeletons to experimentally address this mechanism.

Again it should be noted that the "stick-and-wash" assays that are employed in many laboratories simply measure strengthened adhesion. In contrast, this is an accurate assay as long as the rate-limiting step in the adhesion sequence is the adhesive step of interest. The stick-and-wash assays can be misleading in such tests. For example, consider a laboratory with an interest in cytoskeleton contributions. Hopefully, if the stick-and-wash assay is used with cytoskeletally impaired cells, the data will indicate that no adhesion has occurred. This must assume that the initial binding occurs as normal, but that the cells then fail to strengthen due to the missing critical cytoskeletal component. However, if the initial cell-substrate binding step fails to occur properly, the investigator might be misled into thinking the cytoskeleton is at fault. With the centrifugal assay, it is possible both to separate initial adhesion from strengthening, and to determine the extent of strengthening. This application has been very useful in a variety of circumstances involving embryonic cells of different stages, or transfected cells with different adhesion deficiencies.

Biophysical analogy of initial binding versus strengthened adhesion

There are many biophysical models of adhesion that attempt to explain how the cytoskeleton works to accomplish an increase in adhesive strength by orders of magnitude. Based largely on the knowledge that cells are malleable, and knowing that cells adhere by noncovalent receptor-ligand interactions, Dembo and Bell (1987) showed that initial binding was relatively weak and was similar for most adhesion molecules. Strengthened adhesion, on the other hand is highly resistant to shear forces. In the Dembo and Bell model, cells adhering by receptor-ligand interactions alone simply peel away from the substrate breaking bond by bond. When adhesion is strengthened by recruitment of the cytoskeleton into the adhesion complex, there is a rapid and very large increase in adhesive strength. As a simple analogy of the initial binding versus strengthened adhesion, consider a piece of double-stick Scotch tape. The following test is a simple means of demonstrating this principle.

Stick a piece of double-stick Scotch tape onto a desk. The Mylar backing of the tape is malleable so that the force needed to initially start peeling one end of the tape away from the substrate will be fairly small, and will remain fairly constant until the entire strip of tape is peeled away from the substrate. This is because the peeling force is the force necessary to break

the adhesive bonds only at the immediate point of contact as the tape is progressively peeled away (Fig. 9.2.2A). The rate of peeling depends upon the applied force, the number of bonds, the strength of an individual bond, and the on and off rates of the bonds. As the peeling force is increased, a point is reached when the off rate exceeds the on rate causing the adhesion to fail at that peeled interface. In the centrifugal assay, the centrifugal force just below the threshold point of cell removal is a measure of the initial binding affinity of the cell.

To demonstrate how adhesive strengthening appears to work, place a microscope slide on top of the same piece of double-stick tape on the desk. The tape is the same, the adherence to the substrate is the same, but now instead of a malleable backing the glass provides a very stiff backing for the tape. An exposed end of the tape peels away rather easily until the edge of the slide is encountered. Then a force that is orders of magnitude larger must be used to remove the tape from the substrate. The massive additional force needed to lift the tape from the substrate is a consequence of the hard backing provided by the glass slide. Because of that rigid backing, the entire piece of tape must be removed all at once. Thus, the entire adhesive surface of the tape under the glass slide simultaneously resists the removal force (Fig. 9.2.2B).

By analogy, this rigidity is a biophysical property provided by the cytoskeleton that enables the adhesion to be strengthened. By cooperatively linking multiple adhesive molecules into more of a unit, a vast increase in apparent adhesive strength can be accomplished without an increase in number of adhesion molecules. Mechanically, the cytoskeleton causes a local stiffening of the adhesion complex so that many more adhesion molecules are acting together to cooperatively strengthen the adhesion.

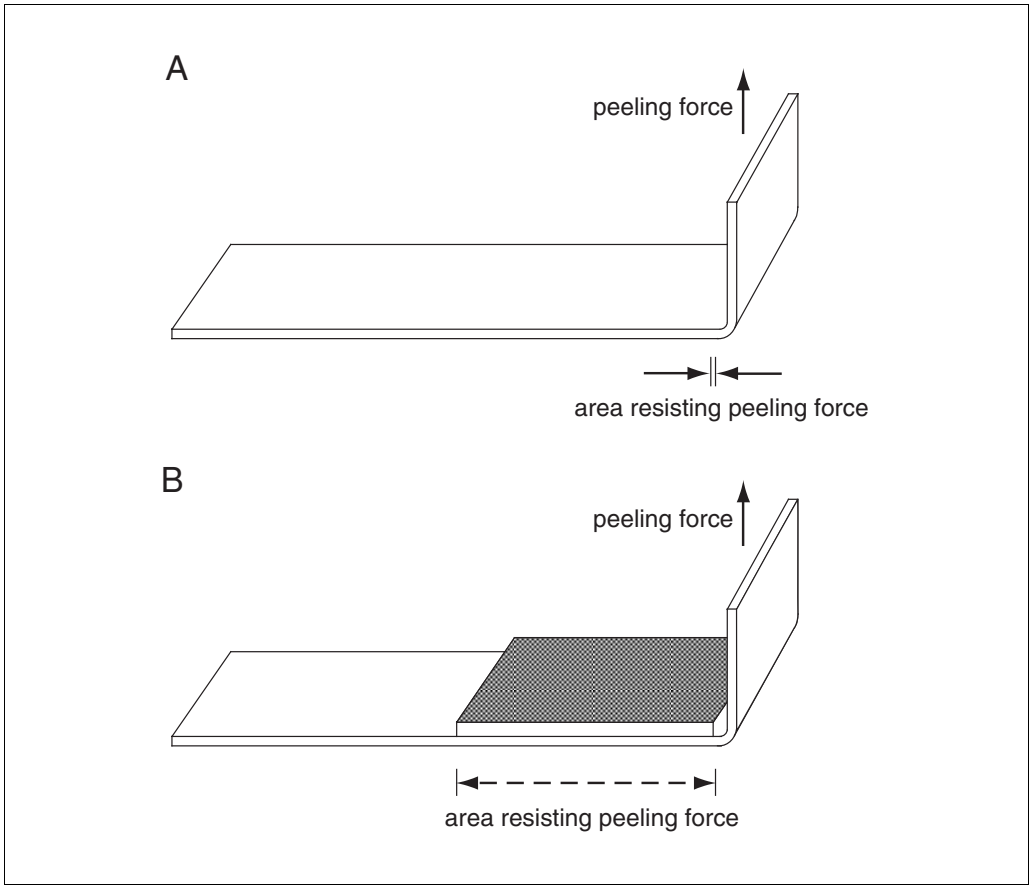


Figure 9.2.2 Tape analogy of two states of cell adhesion. (**A**) Double-stick tape applied to a surface is analogous to the adhesive behavior of cells that have only receptor-ligand association with the substrate. The adhesive area that resists the peeling force is small, limited to the area between the two arrows. (**B**) Adding a glass slide to the upper surface of the tape creates a situation analogous to the adhesive behavior of cells with cytoskeleton-supported cooperativity of cell-substrate association. The stiffened area of tape (membrane) increases the adhesive area that resists the peeling force.

Obviously, the cortex of the cell is not as rigid as the glass backing. Nevertheless, any coupling of the cytoskeleton to the group of adhesion molecules that allows these molecules to act as a unit rather than as freely diffusing molecules, provides an important cooperativity for strengthening. Conveniently, this allows the cell to control its own adhesiveness by controlling intracellular microcompartments (areas where adhesive molecules can couple with cytoskeleton and other areas where the cytoskeletal connections are released). An ability to measure many components of these dynamic associations is necessary to understand how adhesion molecules and cytoskeleton components work together to provide cells with a range of adhesive properties. The centrifugal adhesion assay can help with such analyses.

Critical Parameters

Concentration of substrate

As a rule, the substrate should be applied as a molecular monolayer on the plate. In the first set of experiments with a substrate protein or peptide, determine the threshold concentration for binding, and then operate just above that threshold in subsequent experiments. If one calculates the total surface area occupied by one substrate molecule, and calculates the total surface area of the well bottom, it is a simple calculation to figure out how much substrate would form a molecular monolayer. For example, ~1 μg of fibronectin per well is about the right amount. Some overlapping is assumed, since the protein is filamentous, but the point is that it takes very little protein to completely coat the plate. At a concentration of 10 μg/well, the assay loses specificity, presumably because many nonspecific factors can now contribute to the adhesion. Also, if the protein is layered on the plate, the effects of fibronectin peeling away from fibronectin may confound the assay.

Timing of adhesive strengthening

Significant strengthening is seen after even 1 min of incubation at 37°C. By 5 min at 37°C, the strengthening of most cells exceeds the ability of the cell centrifuge to provide enough force to dislodge them, because most bucket carriers or 96-well plates have a maximum RCF at which they can be centrifuged (usually on the order of 2500 × *g*). Note that this level of strengthening occurs well before any significant change can be seen in the cells, and before they establish visible focal contacts.

Increments in centrifugal force

For initial binding, cells can be centrifuged onto the substrate at ~35 × *g*. For dislodgement of cells kept at 4°C, the force can vary from 1 to ~2500 × *g*. Most cells will maintain adhesion to between 50 and 200 × *g*, but will be removed by higher *g* forces. For strengthened adhesions, a good guideline is to use a dislodgement force that is twice that necessary to remove the initially bound cells, and then ask how long it takes for the cells to strengthen at 37°C enough to remain adherent at that force. Usually that time is <2 min.

Suitable media

The selection of medium depends on the cell type used, and several kinds of media are used for each assay. Cells should be dissociated using established protocols and media for each cell type. The cells are then maintained in a calcium-free buffer for counting and handling during the short time prior to adding them to the wells (see discussion of cations below). Finally, tissue culture medium without any fetal bovine serum (or equivalent) should be used as the cell adhesion test medium. If necessary, fetal bovine serum can be included, provided that proper controls are done for the contribution of cell adhesive substrates in the serum.

Phosphate buffered saline (PBS) containing 40 mg/ml BSA is used as a blocking solution for all wells pretreated with the substrate in question, as well as for background binding wells. In the authors' experience, the BSA eliminates almost all background binding to the well bottom. PBS without BSA serves as the washing reagent and as the buffer into which the substrates are diluted when initially coating the wells.

Role of cations

Many cell-cell interactions require calcium for some aspect of association. The actual function of calcium in these interactions has a rich literature. For the present case, keeping cells in calcium-free solutions is a useful way to control the experiment and to prevent adhesion from occuring prematurely. Thus, cells are added in calcium-free saline to the medium in the wells. There is enough calcium in most tissue culture media to allow this dilution of calcium in the well. Note that the calcium-free medium does not contain EGTA or any other calcium chelator. If the dissociation protocol uses calcium chelators, resuspend the cells in calcium-free medium without the EGTA after they are dissociated.

Recovery period after dissociation

If cells are enzymatically dissociated, they will need a recovery period to regenerate surface adhesion molecules prior to using the centrifugation assay. It is preferable to use nonenzymatic methods for dissociation so that cells are ready to use without recovery. If a recovery period is necessary, cells can be resuspended in complete culture medium with serum, and incubated in flasks at a rotation speed >70 rpm. This provides a chronic fluid shear force that prevents cells from adhering, yet allows them to recover from enzymatic treatments. The length of recovery varies according to cell type, but 4 hr at 37°C is a good first approximation. The cells should then be washed into calcium-free, serum-free medium for the assay.

Fluorescent molecules for labeling

Many fluorescent tags are available, though some work better than others. Rhodamine isothiocyanate (RITC) is the authors' first choice simply because it works on all cells and is slow to fade. The method of labeling is simple. Take ~1 mg of RITC (a bit on the end of a spatula) and add it to 50 µl dimethylsulfoxide (DMSO). Add the RITC/DMSO to calcium-free medium (without phenol red as a pH indicator) until the color of the medium barely turns pink. If the medium turns red, too much RITC has been added and the medium is toxic. Allow the cells to accumulate the RITC from the slightly pink medium, and they will be healthy. Usually 30 min or less is all that is necessary for labeling.

Several markers can be used as a second label. Fluorescein isothiocyante (FITC) tends to be a bit difficult, as batches of FITC lose activity quickly. The fluorescein quenches easily as well. The easiest to use is Hoechst dye (33342 or 33258), which is easily loaded into cells, stains nuclei brightly, and is retained well. To label the cells, dissolve a small amount of Hoechst (or DAPI) dye in aqueous medium, add it to the cells at ~1:200, and incubate 30 min. Other dyes such as calcein AM or acridine orange can also be used to label other populations of cells. These dyes are easily loaded, and have good retention (i.e., do not bleed into unlabeled cells). To label cells, add 10 µl of a freshly prepared dye solution (up to 1 mg in 50 µl DMSO) to 100 ml cells in aqueous medium. Incubate 30 min, wash, and check labeling. Adjust as necessary, as labeling varies with cell type. High dye concentrations should be avoided because they may be toxic, and because most dyes are cumulative (i.e., they will gradually be accumulated from a weak solution).

Troubleshooting

There are three potential pitfalls to be avoided in the assay. All three can be managed with relative ease, but some tips are offered below if these problems confound the assay.

Bubbles from improper sealing

The biggest problem encountered by the novice is the introduction of bubbles of air into the chamber when the wells are sealed. The bubble will act like a bulldozer and sweep cells off the substrate when the wells are inverted. To avoid this, it may take some practice sealing the wells. The easiest way to put the tape on is to place the wells in a rubber support, which acts as a backstop when the tape is pressed down. The tape is then rolled onto the wells, and any remaining medium is squeegeed by rubbing the top either with fingers or a flat surface.

Bubbles generated during the warming incubation

If the sealed plate is incubated at 37°C for >5 min, air bubbles are often introduced. To avoid this, the plates can be sealed after the 37°C incubation. For long incubations, fill the wells with 100 µl of medium plus 100 µl of cell suspension (2/3 full). After the incubation, place the plates on ice and add 100 µl of medium to brim the wells. Add the tape and do the dislodgement step. In practice, this is really an easy step; however, it does introduce a potential error in that the late addition of medium adds a shear force other than centrifugal force to the system. One can easily control for this by examining the wells both prior to and after topping them off.

Quantifying cells

The cells will not be uniformly distributed on the bottom of the wells, which can potentially cause a sampling problem. This can be completely avoided by comparing an experimental population of cells, labeled with one dye, against a reference control population of cells, labeled with a different dye, in the same well. It is then possible to detect very small differences that might exist between the two cell populations. To count cells, it is simplest to capture images of the well bottoms, and either count the cells manually or use a computer-generated macro to automatically count the cells in the field.

Anticipated Results

With most cell types, two basic types of results will be obtained. Measuring adhesion as a function of *g* force will generate the two curves seen in Figure 9.2.3A. The cells adhering by a receptor-ligand interaction alone (4°C) will survive the dislodgement force until a certain force is reached and then there will be a rather sharp drop in their ability to resist the dislodgement force. On the other hand, if the cells are subjected to a brief incubation at 37°C with time held constant, they will tend to resist removal. Figure 9.2.3B shows an example of expected results when measuring adhesion as a function of time at 37°C, with the dislodgement force held constant.

Time Considerations

A typical assay requires 1 hr to complete. Extra time is required if the cells must recover afer dissociation or if strengthened adhesion is measured.

Literature Cited

Burdsal, C., Alliegro, M.C., and McClay, D.R. 1991. Echinonectin as a substrate for adhesion during development of the sea urchin embryo. *Dev. Biol.* 144:327-334.

Burdsal, C.A., Lotz, M.M., Miller, J., and McClay, D.R. 1994. A quantitative switch in integrin expression accompanies differentiation of F9 cells treated with retinoic acid. *Dev. Dynamics* 201:344-353.

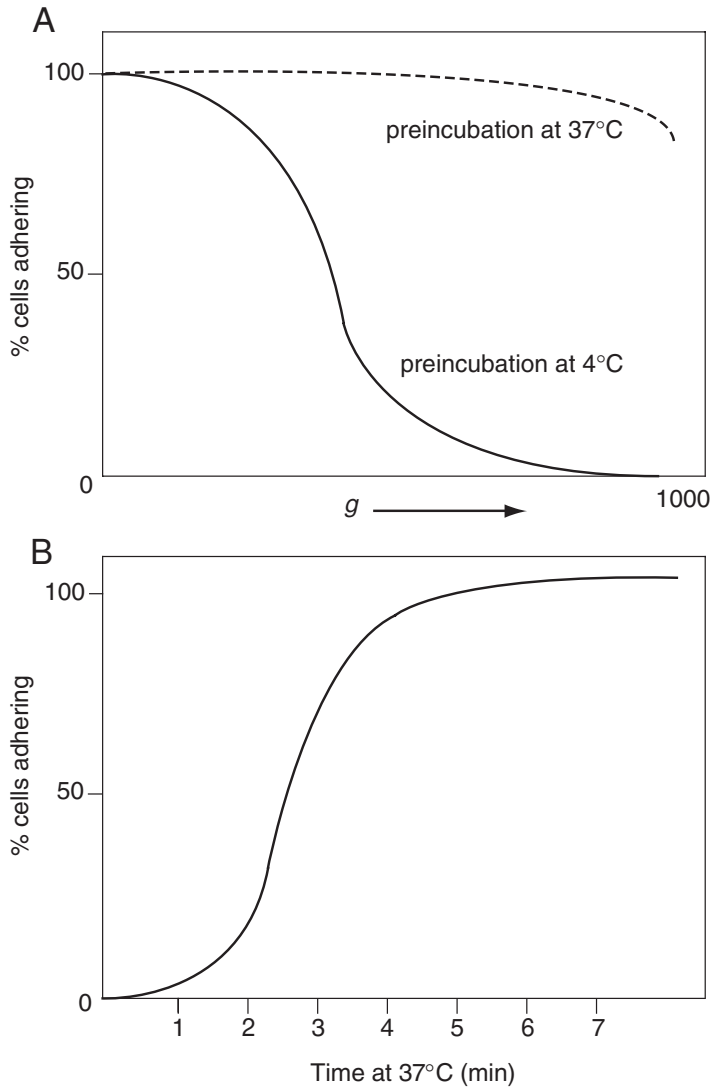


Figure 9.2.3 Theoretical data. (**A**) Force (in *g*) required to dislodge cells preincubated at 4°C and 37°C. (**B**) Time required to stabilize initial adhesion.

Dembo, M. and Bell, G.I. 1987. The thermodynamics of cell adhesion. *Curr. Top. Membr. Transp.* 29:71-89.

Lotz, M.M, Burdsal, C.A., Erickson, H.P., and McClay, D.R. 1989. Cell adhesion to fibronectin and tenascin: Quantitative measurements of initial binding and subsequent strengthening response. *J. Cell Biol.* 109:1795-1805.

McClay, D.R., Wessel, G.M., and Marchase, R.B. 1981. Intercellular recognition: Quantitation of initial binding events. *Proc. Natl. Acad. Sci. U.S.A.* 78:4975-4979.

Rich, A.M. 1978. Substratum amd solution parameters of cell adhesion. Ph.D. thesis. University of North Carolina, Chapel Hill.

Contributed by David R. McClay
and Philip L. Hertzler
Duke University
Durham, North Carolina

Cadherin-Dependent Cell-Cell Adhesion

Cell-cell adhesion mechanisms are subdivided into the Ca^{2+}-dependent system (CDS) and Ca^{2+}-independent system (CIDS), and cadherins are the major components of the former. In general, both systems are present in a single cell, and therefore these two adhesion systems must be discriminated for accurate assays of cell adhesion. This unit provides multiple protocols to assay cell-cell adhesion that is mediated by classic cadherins (see Background Information). Reaggregation of dissociated cells is described for measuring cadherin activity in a variety of cultures: short-term aggregation cultures (see Basic Protocol 1), long-term aggregation cultures (see Alternate Protocol), and mixed-cell aggregation cultures (see Basic Protocol 2). The methods for cell reaggregation should be modified depending on the purpose of the experiment. Two Support Protocols describe methods for dispersing cells while selectively preserving cadherins but removing CIDS. These protocols are optimized for using cultured fibroblastic cells (see Support Protocol 1) and embryonic tissue cells (see Support Protocol 2). Cells can also be dissociated by removing cadherins from the cell surface (see Support Protocol 3). The selection of a dissociation protocol is discussed in detail below (see Strategic Planning). Additional protocols describe how to detect and identify cadherins from cells (see Basic Protocol 3), how to inhibit cadherin-mediated cell adhesion (see Basic Protocol 4), and how to restore cadherin activity in cadherin- and catenin-deficient cell lines (see Basic Protocol 5).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

STRATEGIC PLANNING

Cells of multicellular solid tissues are connected to each other by various forms of cell-cell junctions—tight junctions, adherens junctions, desmosomes, and gap junctions—and by multiple classes of cell-surface molecules, each of which has distinctive functions. These structurally and molecularly complex adhesion systems can be subdivided operationally into CDS and CIDS (Takeichi, 1977, 1988). It is possible to selectively remove either of these two systems (or both) from cell surfaces by differential trypsin treatments (Takeichi et al., 1979; Urushihara et al., 1979). CDS is highly sensitive to trypsin even at low concentrations, but it can be protected from protease digestion by Ca^{2+}. On the other hand, CIDS can be removed only by treatment with relatively high concentrations of trypsin, and this proteolysis, in general, cannot be protected by Ca^{2+}. Accordingly, if cells are treated with a high concentration of trypsin in the presence of Ca^{2+} (TC treatment; see Support Protocols 1 and 2), CDS is left intact but CIDS is removed from the cell surface. If cells are treated with a low concentration of trypsin in the absence of Ca^{2+} (LTE treatment; see Support Protocol 3), CIDS is left intact, whereas CDS is digested and inactivated. Cells disaggregated by TC or LTE treatment can quickly reaggregate if placed in appropriate culture conditions, because adhesion molecules belonging to either CDS or CIDS are left intact on their surfaces.

As expected from the above properties of CDS and CIDS, treating cells with a high concentration of trypsin in the absence of Ca^{2+} (TE treatment; see Support Protocol 3) inactivates both adhesion systems. Thus, for most cell types, TE-treated cells temporarily lose all cell-cell adhesion activities, although they generally retain their cell-substrate adhesiveness. To have the TE-treated cells reaggregate, the adhesion molecules must be restored (see Basic Protocol 5).

Contributed by Masatoshi Takeichi and Shinichi Nakagawa

Current Protocols in Cell Biology (1998) 9.3.1-9.3.15

Most cell-cell adhesion molecules identified thus far can be grouped into either CDS or CIDS. Classic cadherins (Ca^{2+}-dependent cell-cell adhesion molecules) are the major components of CDS and, to the authors' knowledge, no other adhesion molecules are similar to the cadherins in their trypsin/Ca^{2+} sensitivity. Some of the other cell-cell adhesion molecules require Ca^{2+} for their activity, but are not preserved by Ca^{2+} during TC treatment. Therefore, CDS preserved from TC-treatment is represented virtually by a single family of adhesion molecules, the cadherins. CIDS includes multiple classes of adhesion molecules, especially members of the immunoglobulin superfamily; its entire biochemical profile remains to be clarified. Adhesion molecules left on LTE-treated cell surfaces may vary greatly according to cell type.

Cells retain a clustered state during and after TC treatment, because cadherins are active due to the presence of Ca^{2+}. To dissociate them into single cells, they must be washed with a Ca^{2+}-free medium. This strategy for cell disaggregation can be applied to many cell types, including fibroblasts (see Support Protocol 1), early embryonic cells (see Support Protocol 2), and neuroepithelial cells. However, it can be difficult to disaggregate certain cell types into single cells after TC treatment, especially differentiated epithelial cells. Some junctional structures seem to be resistant to the removal of Ca^{2+}. For these cells, it is not possible to obtain healthy, single cells after TC treatment, and only TE treatment (see Support Protocol 3) is an effective way to obtain single cells. In the latter case, cadherins must be sacrificed to allow cell dissociation. Their activity can be studied, however, by letting the cells recover by long-term culture of the cells (see Alternate Protocol).

Each cadherin type can confer specific adhesiveness on cells. This nature of cadherins can be assayed by mixing cells expressing different cadherin types (Nose et al., 1988; Murphy-Erdosh et al., 1995; Nakagawa and Takeichi, 1995; see Basic Protocol 2). Cadherins are associated with cytoplasmic proteins, collectively called catenins, and this association is essential for cadherin activity (Aberle et al., 1996; Barth et al., 1997). On this molecular basis, manipulation of either cadherin or catenins can alter cadherin activity (see Basic Protocols 4 and 5).

BASIC PROTOCOL 1

SHORT-TERM AGGREGATION CULTURE

This protocol describes a method for allowing dispersed cells to reaggregate within short incubation periods. Cells are cultured for up to two hours in a simple balanced salt solution. This method should be chosen when adhesion molecules are assumed to be left intact on cell surfaces after trypsin treatments, and their activity is to be assayed. Physiological recovery of the trypsinized molecules is not expected in this simple culture system.

Materials

1% (w/v) BSA (see recipe)
HCMF (see recipe)
Cells of interest (see Support Protocols 1 to 3), suspended in ice-cold HCMF at $0.5–1 \times 10^5$ cells/ml for cell lines, or at $2–5 \times 10^6$ cells/ml for early embryonic neurons
100 mM $CaCl_2$ (*APPENDIX 2A*)
8% (w/v) paraformaldehyde in HCMF

24-well tissue culture plate (Falcon)
Gyrating shaker (New Brunswick Scientific, Model G2)
BSA-coated Pasteur pipets (see recipe)

Additional reagents and equipment for counting cell aggregates with a hemacytometer (*UNIT 1.1*) or Coulter counter

1. Precoat the wells of a 24-well tissue culture plate with 0.5 ml of 1% (w/v) BSA overnight at 4°C. Rinse the wells a few times with HCMF just before use.
2. Place the plate on ice and add 0.5 ml cells of interest to each well. Add 56 µl of 100 mM $CaCl_2$ (1 mM final) to activate cadherins on TC-treated cells. Add an equal volume of water to controls wells to check the effect of Ca^{2+}.

   When desired, also add other substances, such as antibodies (Table 9.3.1) and inhibitors.

   Cell density varies between cell lines and embryonic cells, because the latter are often smaller than the former.

   Release of DNA from damaged cells tends to perturb cell aggregation processes (see Support Protocol 2). To avoid this, add DNase I (10 µg/ml final) and $MgCl_2$ (1 mM final)

Table 9.3.1 Commercially Available Monoclonal Antibodies to Cadherins and Catenins

Antigen	Clone	Inhibitory activity[a]	Supplier[b]
Cadherins			
Xenopus C-cadherin	6B6		DSHB
Canine E-cadherin	rr1		DSHB
Human E-cadherin	HECD-1, SHE78-7	+	Takara, Zymed
Human E-cadherin			Transduction Labs
Human E-cadherin			Chemicon
Human E-cadherin	6F9		ICN, BIB, PGN
Human E-cadherin	67A4		Cosmo Bio
Mouse E-cadherin	ECCD-1	+	Takara, Zymed
Mouse E-cadherin	ECCD-2	–	Takara, Zymed
Mouse uvomorulin (E-cadherin)	DECMA-1		Sigma
Xenopus E-cadherin	5D3, 8C2		DSHB
Rat K-cadherin (cadherin-6)			Transduction Labs
Chicken N-cadherin	NCD-2	+	Takara, Zymed
Chicken ACAM (N-cadherin)	FA-5, GC-4, ID-7.2.3		Sigma
Human P-cadherin	NCC-CAD-299	+	Takara, Zymed
Human P-cadherin			Transduction Labs
Mouse P-cadherin	PCD-1	+	Takara, Zymed
Rat R-cadherin			Transduction Labs
Human VE-cadherin	BV6		BLD, BIB
Human cadherin-5			Transduction Labs
Human cadherin-5	TEA1/31		Cosmo Bio
Pan cadherin	CH-19		Sigma
Chicken LCAM	7D6		DSHB
Catenins[c]			
α-Catenin			Transduction Labs
β-Catenin	CAT-5H10		Zymed
β-Catenin			Transduction Labs
β-Catenin	15B8, 6F9		Sigma
Plakoglobin (γ-catenin)	PG-11E4		Zymed
Plakoglobin (γ-catenin)			Transduction Labs
Plakoglobin (γ-catenin)	15F11		Sigma
Plakoglobin (γ-catenin)	PG5.1		PGN

[a]Inhibitory activity is indicated only for antibodies that have been tested directly by the authors. For other antibodies, refer to original descriptions.

[b]BIB, Boehringer Ingelheim Bioproducts; BLD, BioLine Diagnostics; ICN, ICN Biomedicals; DSHB, Developmental Studies Hybridoma Bank; PGN, Progen Biotechnik GmbH. See *SUPPLIERS APPENDIX* for addresses.

[c]Species name is omitted for catenins, as most antibodies can recognize a wide variety of species.

to the aggregation medium. The presence of DNase is often critical for accurate measurement of natural cell aggregation. Mg^{2+} might activate integrins, but this effect is generally negligible in cadherin assays. Cadherins themselves are insensitive to Mg^{2+}.

3. Place the plate on a gyrating shaker at ~80 rpm, and incubate 30 min to 2 hr at 37°C. Monitor the aggregation process every 30 min (or at shorter intervals during the initial 30-min period) with an inverted microscope, being careful not to disturb the central accumulation of cells.

   The cells soon become concentrated at the center of each well, and will aggregate at this position. Accumulation can be perturbed by contaminating fibrous materials (such as cotton fibers), affecting the pattern and rate of cell aggregation. Therefore, it is very important to avoid such contamination.

   TC-treated cells start to aggregate within 10 min in the presence of Ca^{2+}. Without Ca^{2+}, TC-treated cells should not aggregate. If they aggregate in the Ca^{2+}-free wells, it is possible that adhesion molecules other than cadherins are active in these cells. On the other hand, LTE-treated cells do aggregate in the absence of Ca^{2+}. TE-treated cells, in general, do not aggregate under any of these conditions. Sometimes, a low degree of aggregation may be observed for TE-treated embryonic cells, suggesting that some residual adhesion molecules are present.

   Maximum aggregation of the cells is generally attained within 2 hr under the above culture conditions, and 30 to 60 min incubation is often sufficient to assess their aggregation properties. Longer incubations may cause cell death, as well as nonspecific cell aggregation due to various factors.

4. Place the plate on ice.

   Cooling the cells stops cadherin-mediated aggregation. Ca^{2+}-independent aggregation may still proceed for LTE-treated cells.

5. Add an equal volume of 8% (w/v) paraformaldehyde to each well, mix, and incubate 15 min on ice.

   Light fixation with an aldehyde-based fixative helps prevent redissociation of the aggregates. Quick mixing of the fixative with the cell suspension is essential to ensure that there is no artificial (fixation-induced) cell aggregation.

6. Gently stir the wells, obtain an aliquot of the cell suspension (or the whole suspension) using a BSA-coated Pasteur pipet, and count the number of cell aggregates and single cells with a hemacytometer or automated cell counter. Determine N_t/N_0, where N_t is the total particle number (aggregates plus single cells) at incubation time t, and N_0 is the total cell number in the cell suspension (see Support Protocols 1 to 3, and the materials list above).

   Be certain that cells or aggregates are uniformly distributed in the well when an aliquot of the cell suspension is removed for counting. See Background Information for details on how to quantify cell aggregation.

ALTERNATE PROTOCOL

LONG-TERM AGGREGATION CULTURE

As mentioned above (see Strategic Planning), for some cell types including most epithelia, complete disaggregation of cells cannot be achieved by TC treatment, and TE treatment (see Support Protocol 3) must be used. The long-term aggregation culture involves incubating cells for several hours to days, by maintaining cells in nutritious culture medium in place of HCMF. Under these conditions, cells undergo de novo protein synthesis, and therefore TE-treated cells gradually recover adhesion molecules, allowing them to aggregate. Cadherin-mediated aggregation begins within a few hours of culture. This aggregation can be inhibited with specific blocking antibodies to cadherins, if they are available. Cadherin activities of TE-treated cells can be studied by this method. TC- and LTE-treated cells do not require long-term cultures.

Additional Materials *(also see Basic Protocol 1)*

1% (w/v) agar (e.g., Noble agar, Difco) in standard culture medium
Cells of interest (see Support Protocol 3), suspended at 0.5–1 × 10^5 cells/ml for cell lines, or at 2–5 × 10^6 cells/ml for early embryonic neurons, in standard cell culture medium (e.g., supplemented Dulbecco's modified Eagle medium, DMEM; *APPENDIX 2A*) with 10% FBS (*APPENDIX 2A*)

1. Pour a minimum amount of 1% agar into the wells of a 24-well tissue culture plate (or other dishes) to cover the entire bottom surface. Allow the gel to set.

   Agar is a powerful inhibitor of cell attachment to the substratum. For long-term cultures, BSA coating is not sufficient to prevent cells from attaching to the dishes.

2. Add cells of interest to agar-coated wells (see Basic Protocol 1, step 2). Incubate as in Basic Protocol 1 (step 3) for the desired period, placing the plate on a shaker in a 5% to 10% (v/v) CO_2 incubator if the medium is buffered with a $NaHCO_3/CO_2$ system.

   The cultures can be maintained for several days if the culture medium is properly refreshed (e.g., by changing half of the medium every day). Cell aggregation also can be induced without rotating the plates. CO_2 levels should be adjusted to maintain pH 7.4.

3. Fix the cells and determine the extent of aggregation (see Basic Protocol 1, steps 4 to 6).

MIXED-CELL AGGREGATION CULTURE

BASIC PROTOCOL 2

To evaluate whether two given populations of cells can adhere to each other, they are mixed and allowed to reaggregate in one of the above culture systems. The resultant cell aggregates are analyzed to determine whether or not the cells have segregated. To do this, the two cell populations must be distinguishable. This can be achieved by staining one population of cells with a fluorescent dye or by labeling two populations with optically distinct dyes. Suitable dyes include 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), Cell Tracker (Molecular Probes), PKH26 (ZYNAXIS), or Fluoro-gold (Fluorochrome).

Materials

Cell cultures
Standard cell culture medium (e.g., supplemented Dulbecco's modified Eagle medium, DMEM; *APPENDIX 2A*) with 10% FBS (*APPENDIX 2A*)
Fluorescent dye(s): e.g., 3 mg/ml 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) in dimethylsulfoxide
HEPES buffer (see recipe)
8% (w/v) paraformaldehyde in HCMF (see recipe for HCMF)
Glycerol-based mounting medium containing antifading reagents (e.g., 1 mg/ml *p*-phenylenediamine in 90% glycerol)
BSA-coated microcentrifuge tubes (see recipe)

Additional reagents and equipment for dispersing cultures (see Support Protocol 1 to 3), for counting cells (*UNIT 1.1*), and for short-term or long-term aggregation culture (see Basic Protocol 1 or Alternate Protocol)

1. Label one culture with a fluorescent dye, or label both cultures with optically distinct fluorescent dyes. For example, label a monolayer cell culture by incubating the cells overnight at 37°C with standard cell culture medium containing 15 µg/ml DiO, and wash 3 times with HEPES buffer. Disperse cultures into single cells (see Support Protocols 1 to 3) and resuspend.

   Cells can also be labeled after the dissociation step.

For other fluorescent dyes, follow the manufacturer's instructions. Make sure that all the cells have been equally labeled by examining with a fluorescence miscroscope.

2. Count the cells (*UNIT 1.1*) and adjust the density with medium to be used for the following aggregation culture. Mix the two disaggregated cell cultures in a 1:1 ratio and incubate for aggregation (see Basic Protocol 1, steps 1 to 3, or see Alternate Protocol, steps 1 and 2).
3. Add an equal volume of 8% paraformaldehyde to each well of the dish, and fix the cells 30 min at 4°C.
4. Transfer the contents of each well into a BSA-coated microcentrifuge tube, and centrifuge the sample 1 min at 700 × *g*, 4°C.

   The microcentrifuge tube should be coated with 1% BSA so that the cells will not stick to the wall of the tube.

5. Discard the supernatant, and gently wash the cells three times with HEPES buffer.
6. Mount the cells on a glass slide with a minimum amount of glycerol-based mounting medium containing antifading reagents.
7. Analyze the aggregation pattern of the aggregates using a fluorescence microscope.

   In mixed-cell aggregation assays, three kinds of pattern can be observed for the distribution of the two cell populations. (1) Cells of each population form separate aggregates, indicating that they can adhere only to like cells. (2) Two cell populations randomly intermix with each other, suggesting that they share common adhesion mechanisms. (3) Each cell aggregate contains the two cell types, but their distribution is not random, and they tend to segregate from each other within the aggregate, forming their own clusters. By analyzing these patterns, selectivity in the adhesiveness of two given cell populations can be evaluated.

SUPPORT PROTOCOL 1

DISSOCIATION OF FIBROBLASTS BY TC TREATMENT

This protocol describes cell dissociation employing a high concentration of trypsin in the presence of Ca^{2+} (TC treatment), which has been used successfully for cultured fibroblasts and similar cells. For other cell types, some modifications of the method may be necessary (see Support Protocol 2).

Materials

Semiconfluent monolayer of the cells of interest
HMF (see recipe)
TC solution (see recipe)
0.5% (w/v) soybean trypsin inhibitor solution (see recipe)
HCMF (see recipe), ice cold
BSA-coated 10-ml test tubes and Pasteur pipets (see recipe)

Additional reagents and equipment for disaggregation by TE treatment (see Support Protocol 3) and for counting (*UNIT 1.1*)

Prepare cultures

1. Prepare semiconfluent monolayer cultures of cells by seeding them 1 or 2 days before use. To confirm successful TC treatment, prepare parallel cultures treated with TE (see Support Protocol 3).

   Overcrowded or old cultures should not be used, as they are often difficult to dissociate into healthy single cells.

2. Rinse the cultures three times with HMF.

Perform TC treatment

3. Remove HMF and add TC solution to the rinsed cultures (e.g., ~4 ml for a 5-cm dish).

   The Ca^{2+} concentration can be modified, balancing protection against proteases and adequate cell disaggregation according to cell type (see Critical Parameters and Troubleshooting).

4. Incubate 15 to 30 min at 37°C on a rotary shaker, and then examine the cells with an inverted microscope.

   The suspended cells should exist as tight clumps if the TC treatment is successful. In sharp contrast, TE-treated cells should be virtually dispersed as single cells. If TC treatment results in a nearly complete dispersion of the cells, either the cells do not have any cadherins, or something was wrong with the treatment procedure, resulting in inactivation of cadherins. To test the latter possibility, the Ca^{2+} concentration in the TC solution can be increased to 5 mM.

Dissociate cells

5. Transfer cells into a BSA-coated 10-ml test tube. If necessary, collect attached cells by flushing the solution with a BSA-coated Pasteur pipet.

   In general, cells spontaneously detach from the dishes during trypsin treatment.

6. Centrifuge 3 to 4 min at 700 × *g*, 4°C, and remove the supernatant.

7. Add 100 µl of 0.5% soybean trypsin inhibitor solution to the cell pellet, followed by 5 ml ice-cold HCMF. Resuspend the cells by brief pipetting with a BSA-coated Pasteur pipet and centrifuge them again. Aspirate the supernatant.

8. Add 5 ml ice-cold HCMF to the cells and disperse the cell clumps by gentle pipetting. Centrifuge as in step 6 and aspirate the HCMF. Repeat.

   For complete dispersion of TC-treated cells, vigorous pipetting is generally required. After several repeats of pipetting, place a tiny aliquot of the cell suspension onto a glass slide and examine it under an inverted microscope. If cell clumps are still present, repeat the pipetting until essentially all cells become dissociated.

   Some cell types, such as epithelial cells, may resist being dissociated into single cells after TC treatment. For such samples, prolonged pipetting might eventually kill them. In this case, other methods for cell dispersion should be employed (e.g., TE treatment; see Support Protocol 3).

9. Resuspend the cells in 2 to 5 ml ice-cold HCMF, and pipet them using a BSA-coated Pasteur pipet in order to obtain completely dissociated cells.

10. Count cell density (*UNIT 1.1*), centrifuge the cells as in step 6, and finally suspend the cells at 0.5–1 × 10^5 cells/ml in ice-cold HCMF (see Basic Protocol 1). Store on ice, and use as soon as possible.

    Never leave the cell suspension at room temperature, as this causes cadherin degradation if Ca^{2+} is absent, and induces precocious cadherin-mediated aggregation if Ca^{2+} is present.

DISSOCIATION OF EMBRYONIC CELLS BY TC TREATMENT

SUPPORT PROTOCOL 2

This protocol describes how to modify TC treatment (see Support Protocol 1) for dissociating embryonic tissues. This protocol works nicely to dissociate early neural and fibroblastic tissues. For some tissues (e.g., early embryonic brains), Ca^{2+} concentration can be increased up to 10 mM to optimize preservation of cadherins without reducing the efficiency of cell disaggregation. On the other hand, it may be difficult to dissociate epithelial organs into single cells by the standard TC treatment (see Support Protocol 3 to resolve this problem).

Additional Materials (also see Support Protocol 1)

Embryo(s)
HEPES buffer (see recipe), ice cold
DNase I
1 M $MgCl_2$ (*APPENDIX 2A*)
BSA-coated 5-cm petri dish (see recipe)

1. Remove tissue from embryos and place in ice-cold HEPES buffer. Cut the tissue into small pieces in HEPES buffer.
2. Transfer the collected tissue with a minimum amount of HEPES buffer to the edge of a 5-cm BSA-coated petri dish, and mince with a pair of fine-tipped scissors.
3. Add 4 to 5 ml TC solution, and incubate 20 to 30 min at 37°C on a rotary shaker.

   Larger pieces of tissue may require longer incubation periods.
4. Lightly pipet the suspension with a BSA-coated Pasteur pipet to briefly disperse the tissue, and examine with an inverted microscope. If viscous DNA gels are present, digest them by adding an excess amount of soybean trypsin inhibitor (add 1/10th vol of a 0.5% solution) followed by 10 µg/ml DNase I and 10 mM $MgCl_2$ (final concentrations). Incubate without shaking at 37°C until the viscous material completely disappears (several minutes).

   DNA gels can cause nonspecific cell aggregation (see Critical Parameters and Troubleshooting).
5. Transfer the cell suspension to a BSA-coated 10-ml test tube, and wash (see Support Protocol 1, steps 6 to 9).

   If soybean trypsin inhibitor was added in step 4, it does not need to be added again here.

   The trypsinized cell suspensions may contain undigested, large cell clumps or tissue debris, mainly derived from extracellular matrices. These should be removed by low-speed centrifugation or with a filter (e.g., Cell Strainer, Falcon), at some step in the washing process.
6. Count and resuspend cells (see Support Protocol 1, step 10), bringing embryonic brain cells, for example, to $2–5 \times 10^6$ cells/ml.

SUPPORT PROTOCOL 3

DISSOCIATION OF CELLS BY LTE OR TE TREATMENT

Treatment of cells at a low concentration of trypsin (LTE treatment) or at a high concentration of trypsin (TE treatment), both in the presence of 1 mM EDTA, was designed to obtain cells lacking cadherins. In LTE-treated cells, CIDS remains active, whereas in TE-treated cells, both cadherins and CIDS are inactivated. Thus, TE treatment produces temporarily null-adhesive cells, and this treatment is useful for complete dissociation of most cell types. As mentioned above, cells of certain tissues (e.g., epithelial tissues) cannot be completely dissociated by TC treatment. In this case, TE treatment is the only choice for obtaining single-cell preparations. Many aspects of the LTE and TE treatments are identical to those for TC treatment; only those steps that must be modified are present here.

Additional Materials (also see Support Protocols 1 and 2)

TE solution (see recipe)
LTE solution (see recipe)

1a. *For cultured cells:* Wash cells three times with HCMF.

1b. *For embryonic tissue:* Rinse tissue with HCMF and transfer to a 5-cm BSA-coated petri dish for trypsin treatment.

2. Add 4 to 5 ml LTE or TE solution to the dish and incubate 15 to 30 min at 37°C on a rotary shaker.

   For LTE treatment, the best trypsin concentration should be determined for every tissue or cell line to ensure that a sufficient CIDS activity is preserved, that cadherins are fully inactivated, and that cells can be dissociated into single cells. For many cell lines, 0.0001% (w/v) trypsin gives satisfactory results; 0.0005% is suitable for embryonic brain.

3. Examine the cells with an inverted microscope. For cells from embryonic tissues, digest DNA gels as necessary (see Support Protocol 2, step 4).

   In contrast to TC-treated cells, which are tightly clumped, LTE-treated cells should be more loosely clustered, and TE-treated cells should be virtually dispersed as single cells.

4. Transfer cells to a BSA-coated 10-ml test tube and wash (see Support Protocol 1, steps 6 to 9).

5. Count and resuspend cells (see Support Protocol 1, step 10), bringing cell lines to $0.5–1 \times 10^5$ cells/ml, and embryonic cells to $2–5 \times 10^6$ cells/ml.

   For long-term culture, suspend cells in the appropriate culture medium (see Alternate Protocol).

DETECTION OF CADHERINS AND CATENINS

BASIC PROTOCOL 3

When working with cells for which no information is available concerning cadherin expression or activity, the following procedures should be helpful in determining their cadherin activity.

Examine morphology

Observe the morphology of cell colonies in relatively low-density cultures. If any cadherins are active, the cells should exhibit firm associations with each other at their margins. If the cells have no cadherin activity, individual cells should be independent (i.e., not forming any tight contacts with others). When these cells become confluent, they may temporarily form monolayer-like sheets, which are sometimes indistinguishable from cadherin-active cell sheets. However, upon further proliferation, overgrown cells tend to be excluded from the sheets if cadherins are inactive; many of the excluded cells may stay on the top of the sheets, exhibiting a round shape. This type of cell association pattern is generally not observed for cadherin-active confluent cell layers, as they maintain tight mutual associations even at high densities.

Examine catenin protein localization

Immunostain cell layers with fluorophore-conjugated antibodies to β-catenin, αE-catenin, or αN-catenin (*UNIT 4.3*; Table 9.3.1). If one of these molecules is concentrated at contact sites between the cells, it strongly suggests that a certain type of cadherin is present and colocalizes with the catenins.

Determine Ca^{2+}-dependent trypsin sensitivity

Treat the cells by the TC (see Support Protocols 1 or 2) and TE (see Support Protocol 3) methods. If cells are detached from the dish as tight clumps after TC treatment, but are dispersed into single cells by TE treatment, this is a strong indication that they have certain cadherins.

Examine cadherin expression

If anti-cadherin antibodies are available (Table 9.3.1), use them for immunoblotting (*UNIT 6.2*) or immunostaining (*UNIT 4.3*). If cadherins are active, immunostaining signals

should be concentrated at cell-cell contact sites. Note that most anti-cadherin antibodies can recognize only limited species.

If immunological strategies do not work, try reverse transcription PCR (RT-PCR) using primers that can amplify fragments of multiple cadherin types. Refer to Suzuki et al. (1991) and Sano et al. (1993) for appropriate PCR primers.

BASIC PROTOCOL 4

INHIBITION OF CADHERIN FUNCTION

There are at least three established ways for blocking cadherin activity: removal of Ca^{2+}, use of inhibitory antibodies, and expression of dominant negative cadherin constructs. Each is detailed below.

Inhibition by Ca^{2+} removal

Removal of Ca^{2+} is the most effective way for blocking cadherins. For most cell types, cadherins are inactivated at <0.1 mM Ca^{2+}. Incubation of cells with EGTA or EDTA (e.g., 1 mM) greatly facilitates Ca^{2+} removal and induces visible dissociation of the cells within several to 10 min at 37°C. Any cells treated with these chelators tend to round up, exhibiting formation of clear gaps between them. However, it should be stressed that many cells, especially epithelial cells, are not completely separated from each other by chelator treatment alone. They often maintain tight connections through fine processes. These chelator-resistant connections cannot be disrupted by a simple mechanical force. The molecular nature of these connections is not fully understood, and only TE treatment can disrupt them.

Inhibition with antibodies

Many antibodies raised against the extracellular domain of cadherins can inhibit their adhesion activities. In monolayer cultures, one can observe morphological changes in antibody-treated cells. These changes are not as drastic as those observed with Ca^{2+} removal, but careful investigators can detect clear differences between the antibody-treated and untreated cells. Most importantly, gaps often become visible between the antibody-treated cells. These changes can be recognized within a few hours after addition of the antibodies. High-density cultures should not be used for these observations, because it is difficult to detect morphological changes. When three-dimensional tissues are treated with anti-cadherin antibodies, their response is seen as a rounding-up of individual cells and a loss of the compact tissue appearance.

For anti-cadherin antibodies, the following points should be kept in mind. (1) Among available anti-cadherin antibodies, those with blocking activity are limited (Table 9.3.1). (2) Many antibodies show species-specific reactivity. (3) A single cell usually expresses multiple cadherin types. For complete inhibition of a cell's cadherin-mediated adhesion, all types must be blocked. Nevertheless, blocking the major cadherin expressed by the cell is often sufficient to induce a morphological response to the antibodies.

Inhibition with dominant negative cadherin constructs

The products of two groups of cadherin cDNA constructs are known to exhibit a dominant negative effect (Takeichi, 1995). One group (ΔN) encodes a molecule with a truncated extracellular domain and an intact cytoplasmic domain (Kintner, 1992; Fujimori and Takeichi, 1993). These molecules are not functional as adhesion molecules themselves, because they lack the extracellular domain. However, they can associate normally with the cytoskeletal system. Based on this nature, the ΔN molecules can compete with endogenous, intact cadherins for interactions with cytoplasmic components, thus interfering with the adhesion activity of the latter. ΔN can nonspecifically inhibit a wide variety of classic cadherins.

The other type of dominant negative cadherin construct (ΔC) was designed to encode a molecule consisting of the intact extracellular domain and a truncated cytoplasmic domain (Levine et al., 1994). Again, the products of these constructs are not functional as adhesion molecules, and they are assumed to interfere with the homophilic interactions at the extracellular domain of endogenous cadherins. In contrast with the use of ΔN, it is possible to specifically block the function of a particular cadherin type with these constructs, because each cadherin type has a unique sequence in its extracellular domain. ΔC has been used successfully when overexpressed in *Xenopus* embryos but not in cell lines. It may be that a large excess of the mutant molecule is required to fully compete with endogenous intact cadherins.

RESTORATION OF CADHERIN ACTIVITY IN CADHERIN- OR CATENIN-DEFICIENT CELL LINES

BASIC PROTOCOL 5

Cell lines with impaired cadherin function are useful materials for studying the mechanisms of cadherin-mediated adhesion. Cadherin dysfunction can be induced by various mechanisms, including loss or mutation of cadherin or catenin genes, or down-regulation of their expression. Cell lines that do not show any cadherin expression include L (Nagafuchi et al., 1987), Neuro 2a (Matsunaga et al., 1988), and S180 (Mege et al., 1988). α-Catenin-deficient lines include PC9 (Hirano et al., 1992) and HCT-8/R1 (van Hengel et al., 1997). HSC39 has a truncated β-catenin gene (Kawanishi et al., 1994; Oyama et al., 1995). In some lines, cadherin function is blocked despite the presence of all known components for the cadherin adhesion machinery. Such blocking is perhaps based on physiological mechanisms. Cadherin dysfunction is most frequently found in carcinoma cell lines. In many of them, not only cell-cell adhesion but also cell-substrate adhesion is often impaired, and they grow as suspended cells without forming tight clumps. If a cell line is found to grow as single cells, it is highly possible that the cells' cadherin activity is abnormal due to one of the above mechanisms.

In cadherin- or catenin-deficient cell lines, cadherin activity can be restored by transfecting with a cDNA encoding the affected protein products. Isolation of successfully transfected clones is relatively easy, because their morphology is dramatically changed from a dispersed to an adhesive type, allowing selection of positive colonies by microscopic observation. On the other hand, the physiologically blocked cadherin function cannot be rescued by a simple addition of cadherin or catenin cDNA.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BSA-coated glass- and plasticware

Place 1% (w/v) BSA solution (see recipe) into test tubes, dishes, and microcentrifuge tubes to be used for collection of cells. Submerge Pasteur pipets in the BSA solution. Let sit at room temperature for at least 1 hr before the experiment. Rinse with HCMF (see recipe) before use.

Cells suspended in protein-free solutions readily attach to plastic or glass surfaces, causing loss of and damage to the cells. To avoid this, all plastic or glass dishes, tubes, and pipets to be used for cell preparation must be precoated with BSA.

If necessary, BSA can be left in tubes/pipets overnight at 4°C.

BSA solution, 1% (w/v)

1 g bovine serum albumin (Fraction V; Sigma)
HCMF (see recipe) to 100 ml
Adjust pH to 7.4 with NaOH
Filter sterilize through a 0.45-µm filter
Store ≤1 week at 4°C

HEPES buffer

1 liter HCMF (see recipe)
1 ml 1 M $CaCl_2$ (*APPENDIX 2A*)
1 ml 1 M $MgCl_2$ (*APPENDIX 2A*)
Filter through a 0.45-µm pore-sized filter to remove any dusty materials
Store up to several months at 4°C

HEPES buffer, Ca^{2+}- and Mg^{2+}-free (HCMF), pH 7.4

8 g NaCl (0.137 M final)
0.4 g KCl (5.4 mM final)
0.12 g $Na_2HPO_4 \cdot 12H_2O$ (0.34 mM final)
1.0 g glucose
2.38 g *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 10 mM final)
H_2O to 1 liter
Adjust pH to 7.4 with 1 N NaOH
Filter through a 0.45-µm filter to remove any dusty materials
Store up to several months at 4°C

HEPES buffer, Mg^{2+}-free (HMF)

1 liter HCMF (see recipe)
1 ml 1 M $CaCl_2$ (*APPENDIX 2A*)
Filter through a 0.45-µm filter to remove any dusty materials
Store up to several months at 4°C

LTE solution

10 µl 0.1% (w/v) trypsin solution (see recipe; 0.0001% final)
100 µl 100 mM EDTA (*APPENDIX 2A*) or ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (1 mM final)
9.89 ml HCMF (see recipe)
Prepare fresh

EDTA or EGTA should give the same effect.

Soybean trypsin inhibitor solution, 0.5% (w/v)

50 mg soybean trypsin inhibitor (Sigma)
HCMF (see recipe) to 10 ml
Divide into 100-µl aliquots
Stored ≤1 month at −20°C

TC solution

1 ml 0.1% (w/v) trypsin solution (see recipe; 0.01% final)
100 µl 100 mM $CaCl_2$ (1 mM final)
8.9 ml HCMF (see recipe)
Prepare fresh

TE solution

1 ml 0.1% (w/v) trypsin solution (see recipe; 0.01% final)
100 µl 100 mM EDTA (*APPENDIX 2A*) or ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; 1 mM final)
8.9 ml HCMF (see recipe)
Prepare fresh

EDTA or EGTA should give the same effect.

Trypsin solution, 0.1% (w/v)

10 mg crystalline trypsin (Sigma)
HCMF (see recipe) to 10 ml
Divide into 1-ml aliquots
Store ≤1 month at −20°C

COMMENTARY

Background Information

The cadherins constitute a large superfamily of molecules with diverse primary structures (Takeichi, 1995). Among its members, those of the "classic cadherin" subfamily have most extensively been characterized as cell-cell adhesion molecules. More than 20 members of the classic cadherin subfamily are known, and they are subdivided into type I and II groups (Takeichi, 1995). All of them bind cells by homophilic interactions. Based upon specificity in their molecular interactions, cells tend to preferentially adhere to those expressing the same cadherin (Takeichi, 1995). Each cadherin is expressed in restricted tissues, and a single cell generally expresses multiple cadherin types. This expression profile should be kept in mind when identifying cadherins in a given cell. The protocols described in this unit should be applied only to the classic cadherins. Other cadherins such as desmosomal cadherins and protocadherins may show different biochemical and biological properties.

The cytoplasmic domain of classic cadherins is associated with catenins (Aberle et al., 1996; Barth et al., 1997). The catenins are subdivided into the β-catenin and α-catenin groups. The former includes β-catenin Armadillo in *Drosophila*), plakoglobin (γ-catenin), and $p120^{cas}$, and the latter consists of αE-catenin and αN-catenin. The β-catenin group directly associates with the carboxy terminal domain of cadherins. α-Catenins bind to β-catenin, and the cadherin/β-catenin/α-catenin complex formation is crucial for full activity of this adhesion machinery. Therefore, cadherin dysfunction can be brought about by loss of any of these three components. TE treatment cleaves only the cadherins.

The mechanism by which Ca^{2+} protects cadherins from proteolytic digestion is not fully understood, although the Ca^{2+}-binding sites have now been identified (Overduin et al., 1995; Shapiro et al., 1995). Although cadherins are generally resistant to TC treatment, this procedure can cleave the proximal portion of the cadherin extracellular domain if incubation is long (especially when isolated cell membranes are treated), resulting in the production of an extracellular fragment (Takeichi, 1988).

How the strength of cell-cell adhesion should be quantified is a classic controversial issue. The simplest way, which does not require sophisticated equipment, is to measure the decrease in the total particle number in cell suspensions. The degree of cell aggregation can be represented by the index N_t/N_0, where N_t is the total particle number (aggregates + single cells) at incubation time t, and N_0 is the total cell number in the cell suspension. Counting of particle number can be done using a hemacytometer (*UNIT 1.1*) but is greatly facilitated if a Coulter counter or its equivalent is available.

Critical Parameters and Troubleshooting

Calcium

When preparing cells for cadherin assays, the presence or absence of Ca^{2+} affects the preservation of cadherins on the cell surfaces, even when exogenous proteases are absent. With physiological concentrations of Ca^{2+}, cadherins are always protected from protease digestion. In contrast, in Ca^{2+}-free media, cadherins tend to be gradually degraded by proteases released from lysed cells in a temperature-dependent manner. Therefore, when the preservation of cadherins is desired, it is highly

recommended that 1 to 2 mM Ca^{2+} be added to all solutions used for cell preparation or tissue dissection. Higher concentrations of Ca^{2+} are more effective in preserving cadherins against tryptic digestion, but they can cause difficulty in cell disaggregation in subsequent steps. For cells that disaggregate with relative ease, the Ca^{2+} concentration should be increased to 5 to 10 mM. Conversely, the Ca^{2+} concentration can be reduced to 0.2 to 0.5 mM for other cell types; this enhances cell disaggregation but reduces cadherins left intact on the cell surface. Ca^{2+} at <0.1 mM fails to protect cadherins. It should also be remembered that more Ca^{2+} is required when higher concentrations of trypsin are used. Ca^{2+} can protect cadherins against many classes of proteases.

To dissociate cells, however, they must be exposed to Ca^{2+}-free conditions to inactivate cadherins. At this time, cadherins can be protected by low temperatures. Ca^{2+}-free cell suspensions should always be kept at an ice-cold temperature. If Ca^{2+} is added to a suspension of cells with cadherins, it induces immediate cell aggregation at physiological temperatures. Under ice-cold conditions, however, cadherin-mediated aggregation is inhibited. Thus, placing cells in an ice-cold, Ca^{2+}-containing medium is the best way to both preserve their cadherin activity and keep them dispersed.

Nonspecific aggregation or adhesion

In cell aggregation assays, the cell suspension should be as clean as possible, as any noncellular contaminant can disturb natural cell aggregation. For example, one piece of cotton fiber can act as a core to induce artificial clumping of the cells.

DNA gels most effectively clump cells. During the trypsin treatments, DNA may be released from lysed cells. The DNA forms viscous gels, which trap cells in loose aggregates. These artificial aggregates should be distinguished from those mediated by real cell-cell adhesion molecules. The presence of DNA gels can also block cell pelleting in the washes that follow trypsin treatment. Therefore, if a significant amount of viscous material is observed in the cell suspension, it should be removed by DNase treatment before the cell washes (see Support Protocol 2). This pretreatment with DNase is generally not required when cell lines are used, because DNA release is not as extensive.

BSA precoating of the dishes, tubes, and pipets to be used for the rotation culture or for cell preparation is an absolute requirement for the success of the experiments, as it prevents the attachment of cells to these surfaces. If cells attach to the dish during the cell aggregation assay, the aggregation process is severely affected, and such cultures should be discarded.

Anticipated Results

In suspension cultures, cadherin-mediated cell aggregation results in the formation of compacted cell masses, especially when the cells are cultured for a long period (e.g., overnight) in a nutritious medium. In the initial phase of the cadherin-mediated cell aggregation (e.g., during the first 2 hr after inoculation), cell aggregates often grow in a one-dimensional, branching pattern. Even in such aggregates, mutual cell-cell contacts are very tight. In principle, the cells in a pair, bound together by cadherins, tend to maximize their contact area by spreading onto one another. On the other hand, when only CIDS is active, cells are clustered maintaining their original round shape. This is the principal morphological difference between the cadherin-dependent and -independent cell-cell contacts.

Time Considerations

Short-term aggregation assays can be completed in <1 day; long-term assays require 1 to several days.

Literature Cited

Aberle, H., Schwartz, H., and Kemler, R. 1996. Cadherin-catenin complex: Protein interactions and their implications for cadherin function. *J. Cell. Biochem.* 61:514-523.

Barth, A.I.M., Näthke, I.S., and Nelson, W.J. 1997. Cadherins, catenins and APC protein: Interplay between cytoskeletal complexes and signaling pathways. *Curr. Opin. Cell Biol.* 9:683-690.

Fujimori, T. and Takeichi, M. 1993. Disruption of epithelial cell-cell adhesion by exogenous expression of a mutated non-functional N-cadherin. *Mol. Biol. Cell* 4:37-47.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S., and Takeichi, M. 1992. Identification of a neural α-catenin as a key regulator of cadherin function and multicellular organization. *Cell* 70:293-301.

Kawanishi, J., Kato, J., Sasaki, K., Fujii, S., Watanabe, N., and Niitsu, Y. 1994. Loss of E-cadherin-dependent cell-cell adhesion due to mutation of the β-catenin gene in a human cancer cell line, HSC-39. *Mol. Cell. Biol.* 15:1175-1181.

Kintner, C. 1992. Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. *Cell* 69:225-236.

Levine, E., Lee, C.H., Kintner, C., and Gumbiner, B.M. 1994. Selective disruption of E-cadherin

function in early *Xenopus* embryos by a dominant negative mutant. *Development* 120:901-909.

Matsunaga, M., Hatta, K., and Takeichi, M. 1988. Guidance of retinal nerve fibers by N-cadherin adhesion molecules. *Nature* 334:62-64.

Mege, R.M., Matsuzaki, F., Gallin, W.J., Goldberg, J.I., Cunningham, B.A., and Edelman, G.M. 1988. Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci. U.S.A.* 85:7274-7278.

Murphy-Erdosh, C., Yoshida, C.K., Paradies, N., and Reichardt, L.F. 1995. The cadherin-binding specificities of B-cadherin and LCAM. *J. Cell Biol.* 129:1379-1390.

Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K., and Takeichi, M. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* 329:341-343.

Nakagawa, S. and Takeichi, M. 1995. Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* 121:1321-1332.

Nose, A., Nagafuchi, A., and Takeichi, M. 1988. Expressed recombinant cadherins mediate cell sorting in model systems. *Cell* 54:993-1001.

Overduin, M., Harvey, T.S., Bagby, S., Tong, K.I., Yau, P., Takeichi, M., and Ikura, M. 1995. Solution structure of the epithelial cadherin domain responsible for selective cell adhesion. *Science* 267:386-389.

Oyama, T., Kanai, Y., Ochiai, A., Akimoto, S., Oda, T., Yanagihara, T., Nagafuchi, A., Tsukita, S., Shibamoto, S., and Ito, F. 1995. A truncated β-catenin disrupts the interaction between E-cadherin and α-catenin: A cause of loss of intercellular adhesiveness in human cancer cell lines. *Cancer Res.* 54:6282-6287.

Sano, K., Tanihara, H., Heimark, R.L., Obata, S., Davidson, M., St. John, T., Taketani, S., and Suzuki, S. 1993. Protocadherins: A large family of cadherin-related molecules in central nervous system. *EMBO J.* 12:2249-2256.

Shapiro, L., Fannon, A.M., Kwong, P.D., Thompson, A., Lehmann, M.S., Grubel, G., Legrand, J-F., Als-Nielsen, J., Colman, D.R., and Hendrickson, W.A. 1995. Structural basis of cell-cell adhesion by cadherins. *Nature* 374:327-337.

Suzuki, S., Sato, K., and Tanihara, H. 1991. Diversity of cadherin family: Evidence for eight new cadherins in nervous tissue. *Cell Regulation* 2:261-270.

Takeichi, M. 1977. Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* 75:464-474.

Takeichi, M. 1988. The cadherins: Cell-cell adhesion molecules controlling animal morphogenesis. *Development* 102:639-655.

Takeichi, M. 1995. Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* 7:619-627.

Takeichi, M., Ozaki, H.S., Tokunaga, K., and Okada, T.S. 1979. Experimental manipulation of cell surface to affect cellular recognition mechanisms. *Dev. Biol.* 70:195-205.

Urushihara, H., Ozaki, H.S., and Takeichi, M. 1979. Immunological detection of cell surface components related with aggregation of Chinese hamster and chick embryonic cells. *Dev. Biol.* 70:206-216.

van Hengel, J., Gohon, L., Bruyneel, E., Vermeulen, S., Cornelissen, M., Mareel, M., and van Roy, F. 1997. Protein kinase C activation upregulates intercellular adhesion of α-catenin-negative human colon cancer cell variants via induction of desmosomes. *J. Cell Biol.* 137:1-14.

Contributed by Masatoshi Takeichi
and Shinichi Nakagawa
Kyoto University
Kyoto, Japan

Analyzing Integrin-Dependent Adhesion

This unit describes methods for the analysis of integrin-ligand binding in both cell-based assays (see Basic Protocol 1) and solid-phase assays (see Basic Protocol 2). A major application of cell adhesion assays is in investigating whether a certain cell type can adhere to a specific adhesive substrate, and, if so, which receptors are involved. Particularly if the substrate is a matrix component (e.g., fibronectin), members of the integrin family are likely to play a dominant role in adhesion. Procedures are described here for assessing which integrins are involved in this process. A detailed analysis of ligand recognition by individual integrins can be performed using a solid-phase receptor-ligand binding assay. The unit also contains support protocols for integrin purification (see Support Protocol 1) and coupling of antibodies to Sepharose for use in this purification (see Support Protocol 2), as well as for biotinylation of integrin ligands to be used in the solid-phase assay (see Support Protocol 3).

ANALYZING INTEGRIN-DEPENDENT ADHESION IN CELL-BASED ASSAYS

BASIC PROTOCOL 1

Cell-based assays for integrin-dependent adhesion are conducted in the same manner as the spreading and attachment assays described in *UNIT 9.1*, except that antibodies to integrins (see Table 9.4.1), peptides, or other reagents are included to identify the integrin(s) and characterize the molecular events associated with adhesion. Function-blocking monoclonal antibodies are the most useful reagents for these analyses because of their high degree of specificity. If only a single integrin is involved in mediating adhesion, antibodies to either the α or β subunit should abrogate cell adhesion. For example, antibodies to either α5 or β1 should completely block HT-1080 fibrosarcoma cell attachment and spreading on fibronectin, showing that this interaction is mediated solely by α5β1 (Akiyama et al., 1989). If multiple integrins are involved, a combination of monoclonal antibodies will be required to block adhesion (Mould et al., 1990, 1994). Although peptides and other inhibitors have, in general, less specificity than monoclonal antibodies, they can be useful for determining what amino acid sequence in the ligand is recognized by the integrin. For example, CS1 peptide inhibits A375-SM melanoma cell attachment to fibronectin, demonstrating that the CS1 sequence is involved in adhesion to this substrate.

Materials

Adhesion molecule of interest
Dulbecco's phosphate-buffered saline (DPBS, Life Technologies; also see *APPENDIX 2A*)
10 mg/ml heat-denatured BSA solution (*UNIT 9.1*)
Cells of interest
DMEM/HEPES: Dulbecco's modified Eagle medium (Life Technologies; *APPENDIX 2B*) with 25 mM HEPES, gassed with 5% to 10% CO_2
Inhibitor: integrin-specific monoclonal antibody (see Table 9.4.1) or peptide, dissolved in DPBS at appropriate concentration
96-well tissue culture microtiter plate

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*) and spreading or attachment cell-substrate adhesion assays (*UNIT 9.1*)

1. Prepare the 96-well plate by coating the wells with the adhesion molecule of interest diluted to the appropriate concentration in DPBS and blocking with 10 mg/ml heat-denatured BSA solution (see *UNIT 9.1*, Basic Protocol 1, steps 1 to 4).

Contributed by A. Paul Mould
Current Protocols in Cell Biology (2002) 9.4.1-9.4.16

Unless the level of adhesion is low, use a concentration of adhesion molecule that promotes 50% to 70% maximal adhesion for coating the wells.

2. Prepare a suspension of the cells of interest at a density of 2×10^5 cells/ml in warm DMEM/HEPES gassed with 5% to 10% CO_2. Incubate cells in an open tube 10 min at 37°C in a CO_2 incubator.

3a. *For cell spreading assay:* Remove BSA solution from the 96-well plate, wash the wells with 100 µl DPBS, and add 50 µl DPBS containing the inhibitor at twice the desired final concentration to each well. Add 50 µl cell suspension to each well.

Table 9.4.1 Monoclonal Antibodies Suitable for Use in Cell Adhesion Assays

Integrin subunit[a]	Ligand[b]	mAb[c]	Function-blocking	Supplier
α1	COL/LN	5E8D9	Yes	Upstate Biotechnology
		TS2/7	No	Serotec
α2	COL/LN	Gi9	Yes	Beckman Coulter
		16B4	No	Serotec
α3	FN/COL/LN	P1B5	Yes	Chemicon
		M-Kid2	No	Beckman Coulter
α4	FN/VCAM-1	HP2/1	Yes	Serotec
		44H6	No	Serotec
α5	FN	JBS5	Yes	Serotec
		VC5	No	Pharmingen
		mAb 16	Yes	K.M. Yamada[d]
α6	LN	GoH3	Yes	Serotec
		4F10	No	Serotec
α9	TN	Y9A2	Yes	Chemicon
β1		4B4	Yes	Beckman Coulter
		K20	No	Beckman Coulter
		mAb 13	Yes	K.M. Yamada[d]
αL	ICAM-1, -2, -3	MHM 24	Yes	Dako
		BV17	No	Chemicon
αM	FG, ICAM-1	ICRF 44	Yes	Serotec
		LM11	No	Chemicon
β2		MHM 23	Yes	Dako
αV	VN/FN/FG/TSP	69-6-5	Yes	Beckman Coulter
		P3G8	No	Chemicon
β3		SZ21	Yes	Beckman Coulter
		PM6/13	No	Serotec
β4		ASC-3	Yes	Chemicon
		450-90	No	Serotec
β5		P1F6	Yes	Life Technologies

[a]Note that β1 associates with α1-α9, β2 associates with αL and αM, β1, β3, and β5 associate with αV, and β4 associates with α6; β2 integrins are expressed only on leukocytes, but most other integrins are widely expressed.

[b]Abbreviations: COL, collagen; FG, fibrinogen; FN, fibronectin; ICAM, intercellular adhesion molecule; LN, laminin; TN, tenascin, TSP, thrombospondin; VCAM, vascular cell adhesion molecule; VN, vitronectin.

[c]All mAbs, with the exception of GoH3, mAb 16, 69-6-5, and mAb 13, are mouse anti-human. GoH3, mAb 16, 69-6-5 and mAb 13 are rat anti-human. See above list of suppliers for antibodies to other species. Antibodies should be stored in small aliquots at −70°C.

[d]Available by Materials Transfer Agreement from K.M. Yamada, National Institutes of Health.

3b. *For cell attachment assay:* Mix an aliquot of the cell suspension with an equal volume of DPBS containing the inhibitor at twice the final concentration. Incubate 15 to 30 min at 37°C. Remove BSA solution from the 96-well plate, wash the wells with 100 µl DPBS, and add 100 µl of the cell/inhibitor mixture to each well.

In the authors' experience, a final mAb concentration of 10 µg/ml, or 1:500 dilution of ascites, gives a maximal inhibitory effect. However, the concentration of mAb to achieve a maximal level of inhibition should be tested using a range of mAb concentrations. An important consideration is that the mAbs should be azide-free. Many commercially available mAbs contain sodium azide as a preservative. This is toxic to all cells and will inhibit attachment and spreading. Therefore it is essential that the sodium azide be removed by dialysis into an appropriate buffer such as DPBS.

Peptides can be used in place of mAbs to analyze the contribution of integrins to cell adhesion. However, these are generally less useful than mAbs because only for a small number of integrins have specific inhibitor peptides been described. Examples are CS1 (DELPQLVTLPHPNLHGPEILDVPST) for α4β1 and α4β7, GACRRETAWACGA for α5β1, and KQGADV for αIIbβ3. RGD peptides (e.g., GRGDS) are of broad specificity and inhibit α5β1, αIIbβ3, and all αV integrins. However, RGD peptides may be useful as a "first screen" to determine if receptors of this class are important for adhesion, or alternatively to confirm their involvement if cell adhesion is inhibited by, for example, mAbs to α5 or αV. Concentrations of peptides required to inhibit cell adhesion are generally higher than for mAbs: typically 0.1 to 1 mg/ml. Higher concentrations are normally required for inhibiting cell attachment than for inhibiting cell spreading. GRGDS, KQGADV, and CS1 peptides are available from Bachem.

4. Incubate, fix, and analyze for spreading assay (see *UNIT 9.1*, Basic Protocol 1, step 8 to 13) or for attachment assay (see *UNIT 9.1*, Basic Protocol 2, steps 8 to 18).

It is essential that the effect of each mAb on cell attachment or spreading be compared with appropriate controls. The best controls to use are mAbs that are noninhibitory against the integrins under test—e.g., K20 for β1 (see Table 9.4.1). However, if these are not available, the following controls can be used: (1) wells to which no mAbs are added (DPBS only); (2) nonimmune mouse or rat IgG or ascites; (3) irrelevant isotype-matched mAb controls. Control peptides (e.g., GRGES) should be used in parallel with the authentic peptides to exclude any toxic or nonspecific effects.

If a partial inhibitory effect is observed with several different anti-integrin mAbs, the assay can be repeated using two or more mAbs in combination to analyze the relative contribution of each integrin to cell adhesion.

ANALYZING INTEGRIN-LIGAND INTERACTIONS IN SOLID-PHASE ASSAYS

BASIC PROTOCOL 2

This protocol describes a simple integrin-ligand binding assay in which the integrin is adsorbed to the wells of an ELISA plate. The plate is blocked using BSA (to reduce nonspecific binding of the ligand) and then biotin-labeled ligand is added. After washing to remove unbound ligand, bound ligand is detected by addition of an avidin-peroxidase conjugate followed by a colorimetric detection step. An example of an application of this procedure would be use of biotinylated fibronectin or fibronectin fragments as ligands for the integrin α5β1.

Materials

Purified integrin (see Support Protocol 1)
Dulbecco's phosphate-buffered saline (DPBS, Life Technologies; also see *APPENDIX 2A*)
Blocking solution (see recipe)
Biotin-labeled ligand (see Support Protocol 3)
Binding buffer (see recipe)

ExtrAvidin peroxidase reagent (Sigma)
ABTS reagent (see recipe)
2% (w/v) SDS in water (*APPENDIX 2A*)

ELISA plate (e.g., Immulon 1B or 4HBX; Dynex Technologies)
Plastic film (e.g., Nescofilm, Parafilm, Saran wrap)
Multichannel pipettor
21-G hypodermic needle
Side-arm flask
Microtiter plate reader

Coat ELISA plate with integrin

1. Dilute purified integrin to ~1 μg/ml with DPBS.

   At least a 50-fold dilution is required, otherwise the detergent present in the purified integrin interferes with adsorption to the plate.

2. Add the diluted integrin to the wells of an ELISA plate (100 μl/well). Leave a set of wells empty for measuring binding of the ligand to BSA.

   The authors normally perform the assay using 4 to 6 replicates for each sample.

   Immulon 1B and 4 HBX ELISA plates (Dynex) are suitable. More recently, the authors have found that one-half area EIA/RIA plates (Costar) also work well, and have the advantage that similar results can be obtained with half as much integrin (i.e., 50 μl/well)

3. Wrap the plate in plastic film and store overnight at room temperature.

   Alternatively, the plate can be stored for up to 1 week at 4°C.

Block the ELISA plate

4. Add 25 μl blocking solution to each integrin-containing well using a multichannel pipettor, then remove the solution by aspiration using a 21-G hypodermic needle attached by tubing to a side-arm flask connected to a vacuum source, or by inverting the plate over a sink and flicking out the liquid.

   A small amount of blocking solution is added to the wells before aspirating the integrin solution because the authors have found that this renders the wells hydrophilic and prevents them from drying out when they are aspirated. Drying out of the wells destroys the activity of some of the integrin.

5. Add 200 μl blocking solution to each well (including those used for testing binding to BSA alone) using a multichannel pipettor. Leave the plate at room temperature for 1 to 3 hr, then aspirate or flick out the blocking solution.

6. Add 200 μl of binding buffer to each well using a multichannel pipettor. Remove the buffer by aspirating or flicking it out. Repeat two times.

7. Remove residual liquid by inverting the plate and striking it hard several times onto adsorbent paper towels.

Add biotin-labeled ligand

8. Dilute the biotin-labeled ligand in binding buffer to the appropriate concentration. Add 100 μl of this solution to each experimental well.

   The appropriate concentration must be determined by pilot experiments. A concentration of 0.1 μg/ml works well for biotinylated 80-kD fragment of fibronectin (Mould et al., 1995a).

   Other reagents (e.g., mAbs, peptides, or synthetic compounds) can be added simultaneously with the ligand at this stage to test for their effects on ligand binding.

9. Cover the plate with plastic film and incubate 3 hr at 37°C.

   A cell culture incubator is suitable for this incubation.

10. Aspirate solutions from the wells to remove unbound ligand.

11. Wash wells three times with 200 µl binding buffer (see step 6). Remove the residual buffer (see step 7).

Detect bound biotin-labeled ligand

12. Dilute ExtrAvidin-peroxidase reagent 1:500 in binding buffer. Add 100 µl of the diluted ExtrAvidin-peroxidase reagent to each well using a repeating pipettor. Incubate the plate 10 to 15 min at room temperature. During this time prepare the ABTS reagent.

13. Aspirate solutions from wells to remove unbound ExtrAvidin peroxidase reagent.

14. Wash wells two times with 200 µl of binding buffer and then two times with 400 µl of binding buffer. Remove the residual buffer (see step 7).

15. Add 100 µl of ABTS reagent to each well using a repeating pipettor. Allow the reaction to proceed until a strong (but not dark) green color is obtained (typically 10 to 30 min).

16. Stop the reaction by adding 100 µl of 2% SDS solution to each well using a repeating pipettor.

17. Read the plate using an automatic microtiter plate reader at 405 nm.

18. Calculate the mean and standard deviation of the absorbance readings for ligand binding to integrin, and for ligand binding to BSA alone, using the following equations.

$$\text{net binding to integrin} = (\text{mean of absorbance readings for ligand binding to integrin}) - (\text{mean of absorbance readings for ligand binding to BSA alone})$$

$$\text{standard deviation of net binding} = \sqrt{(\text{standard deviation of absorbance readings for ligand binding to integrin})^2 + (\text{standard deviation of absorbance readings for ligand binding to BSA alone})^2}$$

INTEGRIN PURIFICATION

SUPPORT PROTOCOL 1

This protocol describes the affinity purification of the integrin α5β1 from human placenta using anti-β1 and anti-α5 mAbs. The procedure can be adapted to purify other integrins from different tissue sources or from pellets of cultured cells. If one is starting from cultured cells, typically enough cells should be used to give at least 10 ml of packed cells, and the volumes of reagents and resins should be scaled down 2- to 4-fold.

Materials

Human placenta (from maternity unit of local hospital; process within a few hours of delivery)
Homogenization buffer (see recipe)
1% (w/v) Virkon (Merck) in water

Extraction buffer (see recipe), 4°C
Sepharose 4B resin (Sigma)
Rat IgG–Sepharose resin (see Support Protocol 2)
mAb 13 (anti-β1)–Sepharose and mAb 16 (anti-α5)–Sepharose (see Support Protocol 2)
Wash buffer (see recipe), 4°C
Elution buffer (see recipe), 4°C
1 M Tris·Cl, pH 8.2 (*APPENDIX 2A*; store up to 6 months at 4°C), 4°C
0.1 M Tris·Cl, pH 8.3 (*APPENDIX 2A*)/0.1% (w/v) Triton X-100 (Ultra grade, Sigma), 4°C (store up to 3 months at 4°C)
Phosphate-buffered saline (PBS; prepare using 10× stock solution from Life Technologies) containing 0.05% (w/v) sodium azide (add from 20% w/v sodium azide stock in H_2O)
PBS (Life Technologies)
5× SDS-PAGE sample buffer (see recipe)
6% SDS-PAGE gel (*UNIT 6.1*)

Large scissors
Blender
Beckman J6-B centrifuge with JA-10 and JA-20 rotors (or equivalent refrigerated centrifuge)
500-ml polycarbonate centrifuge bottles (Nalgene)
50-ml polyallomer centrifuge tubes (Nalgene)
Econo-Pac 20-ml disposable polypropylene columns (Bio-Rad)
50-ml screw-top polypropylene tubes (Becton Dickinson Labware)
Rotating platform (Cole-Parmer)
1.6 × 20–cm C16 column (Amersham Pharmacia Biotech)
Peristaltic pump
Fraction collector
0.8-cm diameter Poly-Prep 2-ml disposable polypropylene column (Bio-Rad)

Additional reagents and equipment for SDS-PAGE and staining of gels (*UNIT 6.1*)

CAUTION: Human placenta should be treated as potentially biohazardous; take suitable precautions such as wearing latex gloves, eye protection, and a lab coat. The homogenization should be performed in a primary cell culture cabinet and any spillage of homogenate or extract should be treated with 1% Virkon. Centrifuge bottles and tubes should be soaked in 1% Virkon after use.

Homogenize placenta

1. Cut the placenta (minus umbilical cord and amniotic membranes) into small chunks using large scissors, and place the pieces in a blender with ~500 ml of cold homogenization buffer. Homogenize the placenta using a moderate speed for ~1 min. Pour the homogenate into 500-ml polycarbonate centrifuge bottles and store at −70°C until required (up to 2 years).

   One placenta yields ~1 liter of homogenate. Commercially available laboratory homogenizers may be used, but a robust domestic blender is adequate for this purpose.

Extract the homogenate

2. Thaw the homogenate in a cold room (preferable) or at room temperature overnight.

   Perform all subsequent operations at 4°C where possible.

3. Centrifuge the homogenate 10 min at 4400 × *g* (5000 rpm in a JA-10 rotor), 4°C. Discard the supernatant into a bucket containing 5 liters of 1% Virkon, and resuspend

the pellets in 600 ml of homogenization buffer. Centrifuge again under the same conditions and discard the supernatant as described.

Most of the soluble proteins have been removed at this stage. The next step uses detergent (Triton X-100) to solubilize proteins (including integrins) from cell membranes. The extraction buffer contains protease inhibitors and BSA to minimize proteolytic degradation of the integrins.

4. Extract pellet by adding 400 ml extraction buffer to the centrifuge bottle and keeping on ice for ~1 hr, shaking vigorously every few minutes to ensure that the pellets are fully resuspended.

   The extract obtained will be red in color because not all of the hemoglobin has been removed in step 3.

5. Centrifuge the extract 10 min at 6400 × *g* (6000 rpm in a JA-10 rotor), 4°C. Pipet the supernatant into 50-ml centrifuge tubes, then centrifuge 30 min at 48,000 × *g* (20,000 rpm in a JA-20 rotor), 4°C.

Purify the β1 integrins

6. Pack a 20-ml disposable column with 4 ml of Sepharose 4B by pouring 8 ml of a 50% suspension of Sepharose 4B into the column. Preclear and filter the supernatant from step 5 by passing it through the column and collecting the flowthrough.

   Several columns can be used simultaneously to speed up this step.

7. Mix the flowthrough with 8 ml of a 50% suspension of rat IgG–Sepharose in screw-top 50-ml polypropylene centrifuge tubes and agitate for 2 hr on a rotating platform. Remove the rat IgG–Sepharose by pouring through 20-ml disposable columns (filtration is accomplished by the fritted disc at the bottom of the column).

   This step removes proteins that bind to rat IgG. If a murine mAb is being used in the subsequent purification steps mouse IgG–Sepharose should be used in place of rat IgG–Sepharose. The authors normally discard the rat IgG–Sepharose at this stage.

8. Mix the flowthrough from step 7 with 8 ml of mAb 13–Sepharose in 50-ml polypropylene tubes and agitate for 2 hr on a rotating platform. Recover the mAb 13–Sepharose by pouring through a 20-ml disposable column. Retain the flowthrough and store at 4°C.

9. Resuspend the mAb 13-Sepharose in wash buffer, pack the suspension into a 1.6 × 20–cm C16 column, and wash the column overnight at ~10 ml per hr with wash buffer, delivered via a peristaltic pump.

10. Elute integrin by passing elution buffer through the column at 45 ml/hr for 30 min. During this time collect 2-min (1.5-ml) fractions (using a fraction collector) into tubes to which 0.5 ml of 1 M Tris·Cl, pH 8.2, has been added. Mix the fractions with this buffer as they elute from the column to ensure prompt neutralization. Store fractions at 4°C.

11. Neutralize the column immediately with 20 ml of 0.1 M Tris·Cl (pH 8.3)/0.1% Triton X-100 at a flow rate of 45 ml/hr. Reequilibrate the column with PBS/0.05% sodium azide and store the mAb 13–Sepharose (removed from the column) at 4°C.

    Because of the low pH used to elute the integrin, the mAb 13–Sepharose gradually deteriorates in its capacity for integrin purification. However, in our experience the column can be reused about ten times before replacement is necessary.

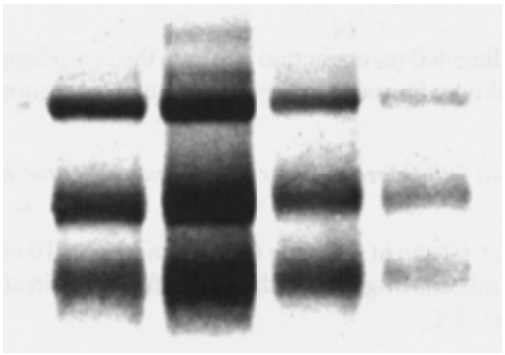


Figure 9.4.1 SDS-polyacrylamide gel of sequential fractions from low-pH elution of mAb 13 column. The major bands observed are (from top) α1 subunit, other α subunits, and β1 subunit. The migration positions of molecular weight standards are indicated (in kDa).

Analyze the purified β1 integrins

12. To 25-µl aliquots of the fractions, add 25 µl 1× PBS and 12.5 µl of 5× SDS-PAGE sample buffer to each aliquot. Heat the samples in a boiling water bath for 3 min and run on a 6% SDS-polyacrylamide gel (*UNIT 6.1*).

13. Stain the gel with Coomassie blue for 1 hr (*UNIT 6.1*). Destain the gel and check for elution of integrin. A typical elution profile is shown in Figure 9.4.1.

The flowthrough from step 8 can be reapplied to the mAb 13–Sepharose, after any precipitate has been removed by centrifuging for 10 min at 4400 × g (5000 rpm in a JA-10 rotor), 4°C (follow step 8 onwards; elute and neutralize the column before chromatographing the flowthrough a second time, then pool the β1 integrin fractions; repeat until the yield of β1 integrins is markedly lower than from the first purification). In the authors' experience, the mAb 13 purification step needs to be repeated several times before all the β1 integrins have been depleted from the extract.

Purify the integrin α5β1

14. Pool the fractions from step 10 that contain the purified β1 integrin. Centrifuge the pooled fractions 10 min at 48,000 × *g* (20,000 rpm in a JA-20 rotor), 4°C. Mix the supernatant with 2 ml of mAb 16–Sepharose for 2 hr on a rotating platform. Pack the suspension into a 0.8-cm diameter 0.8-cm diameter Poly-Prep 2-ml disposable column and wash with 12 ml of wash buffer.

15. Elute α5β1 with 5 ml of elution buffer, added in 0.4-ml aliquots. Collect 0.4-ml fractions in 1.5-ml microcentrifuge tubes containing 0.1 ml of 1 M Tris·Cl, pH 8.2. Mix the fractions with this buffer as they elute from column to ensure prompt neutralization. Store fractions up to 2 years at −70°C.

16. Neutralize the column with 5 ml of 0.1 M Tris·Cl (pH 8.3)/0.1% (w/v) Triton X-100. Re-equilibrate the column with PBS/0.05% sodium azide and store the mAb 16–Sepharose at 4°C.

Analyze the purified α5β1

17. Analyze 25-µl aliquots of the fractions by SDS-PAGE as described in steps 12 and 13.

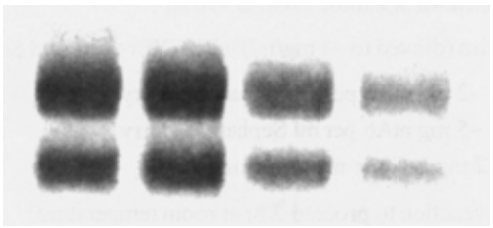


Figure 9.4.2 SDS-polyacrylamide gel of sequential fractions from low-pH elution of mAb 16 column. The major bands observed are (from top) α5 subunit and β1 subunit. The migration positions of molecular weight standards are indicated (in kDa).

The major bands detected by Coomassie blue staining should be those corresponding to expected positions of the α5 and β1 subunits (Fig. 9.4.2). The molecular weights are ~150 kDa for α5 and ~130 kDa for β1.

COUPLING OF ANTIBODIES TO SEPHAROSE

SUPPORT PROTOCOL 2

Suitable mAbs for purification may be available in-house or can be generated using published protocols (e.g., Akiyama et al., 1989). Some anti-integrin hybridomas are available from cell culture collections (e.g., ATCC). The authors have found that the anti-β1 mAb 13 and the anti-α5 mAb 16 are particularly suitable for affinity purification of α5β1. Both mAbs are available from K.M. Yamada, National Institutes of Health.

Materials

Antibodies/IgG to be coupled: anti-β1 and anti-α5 mAbs (e.g., mAb 13 and mAb 16) and rat IgG (Sigma)
Coupling buffer: 0.5 M NaCl/0.1 M $NaHCO_3$ (store up to 6 months at room temperature)
CNBr-activated Sepharose (Sigma)
1 mM HCl
1 M ethanolamine in H_2O (store up to 6 months at room temperature)
Acetate wash buffer: 0.1 M sodium acetate, pH 4 (adjust with glacial acetic acid)/0.5 M NaCl (store up to 6 months at room temperature)
Tris wash buffer: 0.1 M Tris·Cl, pH 8 (*APPENDIX 2A*)/0.5 M NaCl (store up to 6 months at room temperature)
Phosphate-buffered saline (PBS; prepare using 10× stock solution from Life Technologies) containing 0.05% (w/v) sodium azide (add from 20% sodium azide stock solution in H_2O)
PBS (Life Technologies)

Buchner funnel with medium-porosity fritted-glass disc
Conical flask with side arm
50-ml screw-top polypropylene centrifuge tubes
Rotating platform (Cole-Parmer)

Additional reagents and equipment for dialysis (*APPENDIX 3*)

1. Dialyze the antibodies (if necessary) into an amine-free buffer (e.g., PBS or coupling buffer). Measure the absorbance of an aliquot of the dialyzed mAb at 280 nm. Dilute or concentrate antibodies to ~1 mg/ml

   Dialysis is only necessary where the mAb is supplied in amine-containing buffer (e.g., Tris).

2. Pre-swell the CNBr-activated Sepharose in ~50 ml of 1 mM HCl. Wash with ~200 ml of 1 mM HCl per ml of resin in a Buchner funnel. Prepare a 50% resin slurry in PBS.

   3 g of CNBr-activated Sepharose swells to ~10 ml.

3. Mix the antibodies (diluted to ~1 mg/ml) with CNBr-activated Sepharose as follows.

   a. *For mAb 13:* ~2 mg mAb per ml Sepharose slurry
   b. *For mAb 16:* ~5 mg mAb per ml Sepharose slurry
   c. *For rat IgG:* 2 mg IgG per ml Sepharose slurry.

   Allow coupling reaction to proceed 2 hr at room temperature.

4. Following the coupling reaction, add the mixture to a Buchner funnel attached to a conical flask with side arm and remove the supernatant by vacuum filtration. Determine the absorbance at 280 nm for an aliquot of the recovered supernatant and compare to the starting value according to the following equation, where D is the dilution factor (total volume divided by volume of starting solution).

$$\text{coupling efficiency} = \left(1 - \frac{A_{280} \text{ of filtrate} \times D}{A_{280} \text{ of starting solution}}\right) \times 100\%$$

   Typical coupling efficiencies are 95% to 99%.

5. Wash the Sepharose in the Buchner funnel with 20 ml of coupling buffer and remove all buffer under suction. Remove the Sepharose from the funnel using a spatula, place it in a 50-ml conical polypropylene centrifuge tube with 20 ml of 1 M ethanolamine, and incubate 1 hr at room temperature on a rotating platform

   The buffer for washing may be added in several aliquots if the capacity of the funnel is low.

6. Return the Sepharose to the Buchner funnel and wash with three alternating applications, 50 ml each, of acetate wash buffer and Tris wash buffer.

7. Wash the Sepharose with 20 ml of PBS and store in PBS/0.05% sodium azide at 4°C.

   The buffer for washing may be added in several aliquots if the capacity of the funnel is low.

SUPPORT PROTOCOL 3

BIOTINYLATION OF INTEGRIN LIGANDS

This protocol describes labeling of ligands for in vitro integrin-binding assays (see Basic Protocol 2). Biotin is covalently conjugated to amino groups on the ligand to permit quantification of bound ligand.

Materials

Ligand of interest
Coupling buffer 0.5 M NaCl/0.1 M $NaHCO_3$ (store up to 6 months at room temperature)
Sulfo-NHS Biotin (Pierce)
Tris/saline: 25 mM Tris·Cl (pH 7.4)/150 mM NaCl

Tris/saline containing 0.05% sodium azide
Rotating platform (Cole-Parmer)

Additional reagents and equipment for dialysis (*APPENDIX 3*) and protein assay (*APPENDIX 3*)

1. Dialyze ligand into 1 liter of coupling buffer (*APPENDIX 3*) for at least 2 hr at room temperature.

   About 0.5 ml of ligand at a concentration of ~0.5 mg/ml gives sufficient material for a large number of assays.

2. Add an equal mass of sulfo-NHS-biotin (~0.25 mg) to the dialysate in a 1.5-ml microcentrifuge tube and mix on a rotating platform 30 min at room temperature.

   For some proteins, sulfo-NHS-LC-biotin (Pierce) gives higher signals than sulfo-NHS-biotin in the solid phase assay.

3. Dialyze the solution against two changes of 1 liter Tris/saline, and once against 1 liter of Tris/saline, 0.05% azide (at least 2 hr per dialysis) at room temperature.

4. Microcentrifuge the dialysate for 15 min in a 1.5-ml microcentrifuge tube at maximum speed, room temperature.

   This removes any large aggregates or precipitate from the solution.

5. Measure the concentration of biotinylated protein in the supernatant using, for example, the BCA assay (*APPENDIX 3*). Store up to 6 months at 4°C.

   Alternatively, many biotinylated proteins can be stored in aliquots at −70°C. This procedure will typically provide sufficient material for >100 α5β1 fibronectin assays. How much material will be required for any assay will depend upon the affinity of the integrin-ligand interaction.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ABTS reagent

ABTS buffer:
0.05 M Na_2HPO_4
0.1 M sodium acetate
Adjust pH to 5.0 using concentrated HCl
Store up to 3 months at room temperature

ABTS reagent: Immediately before assay, dissolve 11 mg of 2,2′-azinobis(3-ethylbenzthiazoline)sulfonic acid (ABTS; Sigma) in 0.5 ml water. Mix 67 µl of 30% H_2O_2 with 7 ml water. Add 0.5 ml of this ABTS solution to 10 ml ABTS buffer (see above) and 100 µl H_2O_2 solution. Mix thoroughly.

This amount of reagent is sufficient for one full 96-well plate assay.

Binding buffer

Mix the following components in the order indicated:
150 mM NaCl
25 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
1 mM $MnCl_2$
0.1% (w/v) BSA (fraction V; Sigma, 99% pure)
Prepare fresh

This is conveniently prepared from Tris/saline [25 mM Tris·Cl (pH 7.4)/150 mM NaCl] and a stock solution of 1 M $MnCl_2$. BSA is then added.

Blocking solution

Prepare a solution of 25 mM Tris·Cl containing 150 mM NaCl. Add sufficient 20% sodium azide stock for a final concentration of 0.05% (w/v). Add BSA (fraction V; Sigma, 98% pure) for a final concentration of 5% (w/v) and dissolve by vigorous stirring. Centrifuge the solution in 50-ml centrifuge tubes for 5 min at 2800 × *g* (4000 rpm in a JA-10 rotor), and filter the supernatant through a 20-ml disposable column. Store up to 3 months at 4°C.

This is conveniently prepared from Tris-saline [25 mM Tris·Cl (pH 7.4)/150 mM NaCl] to which sodium azide is added from a 20% stock solution. BSA is then added and dissolved by vigorous stirring. The solution is then centrifuged and filtered as above. Final concentrations are 150 mM NaCl; 25 mM Tris·Cl, pH 7.4, 5% (w/v) BSA, and 0.05% (w/v) sodium azide.

Elution buffer

10 mM sodium acetate, pH 3.25
1 mM $CaCl_2$
1 mM $MgCl_2$
0.1% (w/v) Triton X-100 (Sigma, Ultra grade)
Store up to 1 month at 4°C

Extraction buffer

150 mM NaCl
25 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
2% (w/v) Triton X-100 (Sigma, Ultra grade)
1 mM PMSF (100 mM stock solution prepared in isopropanol; also see *APPENDIX 1B*)
10 µg/ml leupeptin (1 mg/ml stock solution prepared in water; also see *APPENDIX 1B*)
2 mg/ml BSA (fraction V; Sigma, 98% pure)
Prepare fresh, then cool on ice

Homogenization buffer

150 mM NaCl
25 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.005% (w/v) digitonin
Store for up to 3 months at 4°C

SDS-PAGE sample buffer, 5×

25% (v/v) glycerol
125 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
10% (w/v) SDS
0.1% (w/v) bromophenol blue
Store indefinitely at 4°C. Warm in a hot water bath and mix well before use.

Wash buffer

150 mM NaCl
25 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
1 mM $CaCl_2$ (add from 1 M stock)
1 mM $MgCl_2$ (add from 1 M stock)
0.1% (w/v) Triton X-100 (Sigma, Ultra grade)
Store up to 1 month at 4°C

This is conveniently prepared from Tris-saline [25 mM Tris·Cl (pH 7.4)/150 mM NaCl] and stock solutions of 1 M $CaCl_2$ and 1 M $MgCl_2$.

COMMENTARY

Background Information

Monoclonal antibodies were of crucial importance in the initial identification of cell surface receptors that mediated adhesion of cells to extracellular matrix components (e.g., Wayner and Carter, 1987). A wide range of well characterized anti-integrin mAbs are now commercially available for use in cell attachment and spreading assays. The choice of which anti-integrin mAbs to use is determined in part by the substrate. For example, if collagen type I is the substrate, anti-α1 and anti-α2 mAbs should be tested, as these are the likely integrins involved. The complement of integrins expressed by the cell also helps to determine which mAbs should be tested. If the profile of integrin expression is unknown, it can be determined by flow cytometry or by immunoprecipitation of surface-labeled cells. Conversely, if a mAb blocks cell adhesion, it is important to demonstrate that the corresponding integrin subunit is expressed by the cells.

Historically, the first purification of an integrin receptor was a major advance in understanding the molecular basis of cell-matrix interactions (Pytela et al., 1985). This and later methods employed a ligand affinity column as the major purification procedure. In particular, fibronectin fragments have been used to purify α5β1, and RGD peptides have been used to purify αVβ3 and αIIbβ3 (Pytela et al., 1987; Smith and Cheresh, 1988; Yamada and Yamada, 1990). Ligand affinity columns remain the method of choice where mAbs are unavailable. However, the use of mAbs has the advantage that the purification is more specific (e.g., several different integrins bind to fibronectin affinity columns) and higher yields of integrins can be obtained. Multiple β1 integrins can also be obtained from the same source. For example, other β1 integrins from human placenta that are present in the flowthrough from the mAb 16 column can be purified using other specific anti-α mAbs. For some assays, and particularly where one β1 integrin predominates in the tissue or cell extract, it may be sufficient to purify the total β1 integrins and use this partially purified preparation in the solid-phase assay. For example, β1 integrins purified from MOLT-4 cells (Newham et al., 1998) contain ~75% α4β1, the remainder being α5β1. The protocol described here can be adapted to purify other integrins from other tissues or from pellets of cultured cells.

The major advantage of assays using purified integrins, as compared to cell-based assays, is that integrin-ligand binding can be studied in isolation. Integrin clustering, signaling, and cytoskeletal interactions are all known to affect the strength of adhesion in cell-based assays. Furthermore, adhesion may be modulated by indirect effects (e.g., by signaling from other cell-surface receptors). Although doubts are often expressed as to whether plastic-adsorbed integrin is representative of integrin in its native environment, a number of careful studies have shown no significant differences between the behavior of integrins in solid-phase assays and on cell surfaces. Hence, this approach has been broadly validated. The first solid-phase integrin-ligand binding assay was described by Charo et al. (1991) for studying fibrinogen binding to αIIbβ3. The α5β1-fibronectin assay developed by the author of this unit is both extremely sensitive and highly versatile. For example, the author has described how the assay can be used to investigate the effects on ligand binding of divalent cations, activating and inhibitory mAbs, peptide inhibitors, and mutations (Mould et al., 1995a,b, 1996, 1997). Another important area in which this type of assay is finding use is in the pharmacological screening of inhibitors of integrin-ligand interactions. This assay can give information about the inhibitory potency of a compound and whether it is a direct competitive or allosteric inhibitor of ligand binding. The attenuation of mAb epitopes can also provide data on the location of the binding site of an inhibitor on the integrin (Mould et al., 1997).

The author's preferred method for labeling of integrin ligands is biotinylation, because of its safety and simplicity. One potential drawback is that if one or more lysyl residues in the ligand are crucial for integrin binding, their modification may render the ligand inactive. In this case, a possible solution may be to reduce the amount of biotinylation reagent so that some of the lysyl residues remain unmodified. Other labeling methods such as radioiodination can also be used. Alternatively, if the ligand is a recombinant protein, a "tag" such as an epitope sequence or the Fc region of IgG can be incorporated for use in the detection of bound ligand.

Table 9.4.2 Troubleshooting Guide for Problems Encountered in Solid-Phase Assays

Problem	Possible cause	Solution
High background binding to BSA-coated wells	Insufficient blocking of wells Ligand concentration too high	Block for longer time (e.g., overnight) Test range of ligand concentrations for optimal signal/background
Spuriously high signal in some wells	Insufficient washing Tops of wells contaminated	Follow washing protocol carefully Add reagents to the center of wells
Wide variation in signal in experimental wells	Integrin added to plate insufficiently mixed Wells aspirated before adding blocking reagent Plate contaminated, e.g., by dust	Mix diluted integrin thoroughly before coating the plate Add blocking reagent before removing coating solution Use fresh, clean plates
Low signal above background binding to BSA	Insufficient integrin Ligand concentration too low Inactive ligand	Use lower dilution of integrin Use higher concentration of ligand Check activity of ligand in cell-based assay

Critical Parameters and Troubleshooting

For cell-based assays, as described in *UNIT 9.1*, the health of the cells and careful preparation are important for achieving optimal spreading or attachment. In order to optimize the sensitivity of cell attachment or spreading to inhibition, the author recommends that a concentration of substrate be chosen that gives 50% to 70% of maximal adhesion (unless this value is low, in which case a concentration of substrate that gives near maximal adhesion should be used). If the level of adhesion is near maximal, inhibitors are less effective at blocking adhesion.

Failure of a mAb to inhibit spreading or attachment can normally be taken to mean that the integrin it recognizes is not involved in adhesion. However, it is important to check that the antibody is effective in a system where inhibition should be observed (e.g., an anti-α5 mAb should block spreading of HT-1080 fibrosarcoma cells on fibronectin). Conversely, if an antibody does inhibit, it is important to test that it does not inhibit adhesion to an inappropriate ligand (e.g., an anti-α5 mAb should not perturb HT-1080 cell spreading on collagen). Most of the antibodies described in Table 9.4.1 are well characterized and should not show any non-specific effects. However, as described earlier, it is essential that the antibodies not contain sodium azide.

It is also important to bear in mind that inhibition of adhesion can be caused by "cross-talk"—i.e., where ligation of one integrin (e.g., by peptide) indirectly affects the activity of a second integrin via intracellular signaling (Diaz-Gonzalez et al., 1996). While it is often difficult to rule out such effects, they normally only cause a partial reduction in adhesion, whereas adhesion is frequently totally ablated by specific antibody or peptide inhibition. Finally, if anti-integrin mAbs or peptides do not completely block cell adhesion, it is possible that non-integrin receptors may play some role. This may be observed particularly in cell attachment assays. For example, cell-surface proteoglycans contribute to melanoma cell attachment to the heparin-binding domain of fibronectin (Mould et al., 1994).

For solid-phase assays, the specificity of the assay must be tested carefully. The most important test for specificity is the ability of unlabeled ligand to compete with labeled ligand for binding to the integrin. Hence, in the presence of a large excess of unlabeled ligand, very little binding of labeled ligand should be observed. Nearly all integrin-ligand interactions are divalent-cation dependent. Hence, replacing the Mn^{2+} in the binding buffer with EDTA should reduce binding to levels similar to that observed for BSA-coated wells. Further tests for specificity can be carried out. For example: (1) mAbs that are inhibitory in cell-based assays should also inhibit ligand binding in solid-phase assays, (2) mutations known to affect integrin binding sites should perturb ligand recognition, and (3) known ligand mimetics (e.g., RGD peptides for α5β1 or CS1 peptide for α4β1) should block ligand binding. All of these tests have been performed for the α5β1-fibronectin

Table 9.4.3 Troubleshooting Guide for Problems Encountered in Integrin Purification

Problem	Possible cause	Solution
Large number of proteins copurify with integrins	Inadequate preclearing or filtration of extract, or precipitate forms during purification procedures	Recentrifuge after preclearing and filtering extract on Sepharose 4B, or when any precipitate is visible
Small amounts of integrins purified	Insufficient mAb coupled to Sepharose Affinity of mAb too low Column has been used many times	Couple more mAb to Sepharose Use mAb with higher affinity Replace with fresh mAb-Sepharose
Integrin degraded	Insufficient levels of protease inhibitors in extraction buffer Extraction or other manipulations performed at too high a temperature	Increase levels of protease inhibitors and BSA in extraction buffer Perform all manipulations at 4°C or on ice

assay. Table 9.4.2 is a troubleshooting guide for solid-phase ligand-binding assays.

For integrin purification, it is essential to have sufficient tissue or cell pellets for the extraction and enough mAb-Sepharose to perform a successful purification. Table 9.4.3 is a troubleshooting guide for integrin purification.

Anticipated Results

For cell-based assays, in most cases, it is possible to obtain levels of spreading or attachment of >50%. It should be feasible using anti-integrin mAbs, either alone or in combination, to reduce the level of spreading or attachment to close to that seen on BSA.

For integrin purification, yields of α5β1 are ~2 mg per placenta (estimated by Coomassie blue staining). Each full 96-well-plate solid-phase assay uses <10 µg of integrin. Hence, this is enough to perform a large number (~200) of solid-phase assays. In these assays, a strong positive signal (OD_{405} ~1) is normally achieved in ≤30 min, whereas background binding to BSA is generally very low (OD_{405} ~0.1).

Time Considerations

Cell attachment and spreading assays are quick to perform. The actual assays can be carried out in about half a day or less; however, particularly if many different mAbs or peptides are being tested, quantification of spreading assays can take several hours.

Purification of α5β1 from placenta takes ~3 days in total. Allow an additional day for preparation of buffers and mAb-Sepharose. The solid-phase assay can be performed in 5 to 8 hr but only requires 1 to 2 hr of hands-on time. However, the assay needs to be planned in advance so that the appropriate number of wells can be coated with integrin overnight. Plates can be coated with integrin several days in advance.

Literature Cited

Akiyama, S.K., Yamada, S., Chen, W.-T., and Yamada, K. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: Roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell Biol.* 109:863-875.

Charo, I.F., Nannizzi, L., Phillips, D.R., Hsu, M.A., and Scarborough, R.M. 1991. Inhibition of fibrinogen binding to GP IIb-IIIa by a GP IIIa peptide. *J. Biol. Chem.* 266:1415-1421.

Diaz-Gonzalez, F., Forsyth, J., Steiner, B. and Ginsberg, M.H. 1996. Trans-dominant inhibition of integrin function. *Mol. Biol. Cell* 7:11939-11951.

Mould, A.P., Wheldon, LA., Komoriya, A., Wayner, E.A., Yamada, K., and Humphries, M.J. 1990. Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin α4β1. *J. Biol. Chem.* 265: 4020-4024.

Mould, A.P., Askari, J.A., Craig, S.E., Garratt, A.N., Clements, J., and Humphries, M.J. 1994. Integrin α4β1-mediated melanoma cell adhesion and migration on vascular cell adhesion molecule-1 (VCAM-1) and the alternatively spliced IIICS region of fibronectin. *J. Biol. Chem.* 269:27224-27230.

Mould, A.P., Akiyama, S.K., and Humphries, M. J. 1995a. Regulation of integrin α5β1-fibronectin interactions by divalent cations: Evidence for distinct classes of binding sites for Mn^{2+}, Mg^{2+}, and Ca^{2+}. *J. Biol Chem.* 270:26270-26277.

Mould, A.P., Garratt, A.N., Askari, J.A., Akiyama, S.K., and Humphries, M.J. 1995b. Identification of a novel monoclonal antibody that recognises a ligand-induced binding site epitope on the β1 subunit. *FEBS Lett.* 363:118-122.

Mould, A.P., Akiyama, S.K., and Humphries, M.J. 1996. The inhibitory anti-β1 integrin monoclonal antibody 13 recognises an epitope that is attenuated by ligand occupancy: evidence for

allosteric inhibition of integrin function. *J. Biol. Chem.* 271:20365-20374.

Mould, A.P., Askari, J.A., Aota, S., Yamada, K., Irie, A., Takada, Y., Mardon, H.J., and Humphries, M.J. 1997. Defining the topology of integrin α5β1-fibronectin interactions using inhibitory anti-α5 and anti-β1 monoclonal antibodies: Evidence that the synergy sequence of fibronectin is recognised by the amino-terminal repeats of the α5 subunit. *J. Biol. Chem.* 272:17283-17292.

Newham, P., Craig, S.E., Clark, K., Mould, A.P., and Humphries, M.J. 1998. Analysis of ligand-induced and ligand-attenuated epitopes on the leukocyte integrin α4β1: VCAM-1, MAdCAM-1 and fibronectin induce distinct conformational changes. *J. Immunol.* 160:4508-4517.

Pytela, R., Pierschbacher, M.D., and Ruoslahti, E. 1985. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* 40:191-198.

Pytela, R., Pierschbacher, M.D., Argraves, S., Suzuki, S., and Ruoslahti, E. 1987. Arginine-glycine-aspartic acid adhesion receptors. *Methods Enzymol.* 144:475-489.

Smith, J.W. and Cheresh, D.A. 1988. The Arg-Gly-Asp binding domain of the vitronectin receptor. *J. Biol. Chem.* 263:18726-18731.

Wayner, E.A. and Carter, W.G. 1987. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β subunits. *J. Cell Biol.* 105:1873-1884.

Yamada, K.M. and Yamada, S.S. 1990. Isolation of fibronectin receptors. In Receptor Purification (G. Litwack, ed.) pp 435-449. Humana Press. Clifton, NJ.

Contributed by A. Paul Mould
University of Manchester
Manchester, United Kingdom

Analysis of Cell-Cell Contact Mediated by Ig Superfamily Cell Adhesion Molecules

The calcium-independent cell adhesion molecules (CAMs) constitute a large family of cell surface molecules. A major group among these are the immunoglobulin superfamily (IgSF) molecules. IgSF-CAMs may be composed of immunoglobulin (Ig) folds only, Ig folds linked to fibronectin type III (FnIII) folds, or Ig folds linked to protein modules other than FnIII folds. The IgSF is a large protein superfamily comprising >100 proteins involved in cell-cell adhesion. Its members are found in vertebrates, invertebrates, and also in yeast. Most of the molecules of the IgSF are cell surface molecules that are membrane-anchored either by a single transmembrane segment or by a glycosylphosphatidylinositol (GPI) anchor that is posttranslationally attached to the C-terminus. Some of the IgSF-CAMs also occur in soluble form, e.g., in the cerebrospinal fluid or the vitreous fluid of the eye, due to a cleavage of the GPI-anchor or the membrane-proximal peptide segment. In some cases, such as NCAM, various forms may be generated by alternative splicing.

This unit provides protocols for the purification of IgSF-CAMs from tissue extracts and tissue culture supernatants and for the analysis of the adhesive functions of IgSF-CAMs with isolated molecules and in the cellular context. Following personal expertise, the authors have added a few frequently used functional assays demonstrating the role of IgSF-CAMs in neural development, such as neurite outgrowth from cultured neurons, and the use of antibodies for the inhibition of IgSF-CAM functions in vitro. The first group of protocols describe affinity purification of IgSF-CAMs (see Basic Protocol 1), preparation of the affinity column (see Support Protocol 1), solubilization of membrane proteins (see Support Protocol 2), transient transfection of HEK 293 cells to express IgSF-CAMs (see Support Protocol 3), and detection of IgSF-CAMs by dot blot analysis (see Support Protocol 4). Assays using fluorescent microspheres with coupled proteins are used for one type of functional analysis based either on interactions between microspheres (see Basic Protocol 2) or on interactions between microspheres and cultured cells (see Basic Protocol 3). There are two protocols for coupling proteins to microspheres: coupling proteins to fluorescent microspheres (see Support Protocol 5) and coupling proteins to glutaraldehyde-activated amino beads (see Support Protocol 6). A second group of protocols analyze the functions in cell-based assays. *Trans*-interactions are studied using IgSF-CAM-transfected myeloma cells (see Basic Protocol 4). This protocol requires stable transfection of myeloma cells (see Support Protocol 7). *Cis*-interactions are detected by chemical cross-linking (see Basic Protocol 5) and antibody co-capping (see Basic Protocol 6). IgSF-CAMs and other substrates have the ability to promote neurite outgrowth (see Basic Protocol 7), which requires coating of the growth surface with IgSF-CAM (see Support Protocol 8), nitrocellulose as a binder for the substrate of interest (see Support Protocol 9), poly-D-lysine (see Support Protocol 10), collagen (see Support Protocol 11), or laminin (see Support Protocol 12). Differential fixation protocols are used for fluorescent immunohistochemistry samples (see Support Protocol 13) or for morphological analyses (see Support Protocol 14). Finally, there is a protocol for assessing the effect of inhibiting CAM-CAM interactions in vitro (see Basic Protocol 8).

Contributed by Peter Sonderegger, Stefan Kunz, Christoph Rader, Daniel M. Suter, and Esther T. Stoeckli

Current Protocols in Cell Biology (2001) 9.5.1-9.5.52

BASIC PROTOCOL 1

Purification of IgSF-CAMs by Immunoaffinity Chromatography

The best method for the purification of native, functionally intact proteins is certainly the use of standard chromatography, such as ion exchange, hydrophobic interaction, and gel permeation columns. However, the establishment of such a standard purification protocol can be time-consuming and requires expensive equipment. As the specific characteristics exploited for chromatography differ from protein to protein, purification protocols cannot be generalized. A faster and less expensive way to purify IgSF-CAMs is to use affinity chromatography. Tissue homogenates, body fluids in the case of secreted proteins, or cell lines engineered to express a particular protein of interest, either transiently or stably, can be used as a source for IgSF-CAM purification. Affinity chromatography makes use of the specific binding properties of the proteins, e.g., receptors for their ligands, enzymes for their substrates, or antibodies for their antigens. For IgSF-CAMs, the purification by immunoaffinity is most commonly used. A disadvantage of affinity purification is the possibility of the loss of activity, as the protein is sometimes eluted from the column by rather harsh conditions. The protocol described below has been successfully used for the purification of functionally intact IgSF-CAMs (Stoeckli et al., 1991, 1996; Rader et al., 1993).

The general principle of immunoaffinity chromatography is the use of a resin-coupled monoclonal antibody directed against the protein to be purified. Generally, activated Sepharose resins are used. The resin is packed into a column connected to a peristaltic pump and to a UV-detector to monitor the elution profile of the column. The purity of the eluted protein is analyzed by SDS-PAGE (*UNIT 6.1*). Here, we describe a purification protocol for a membrane-bound IgSF from brain membranes (see Support Protocol 2 for membrane preparation and protein solubilization).

Materials

CNBr-activated Sepharose 4B column (see Support Protocol 1)
Loading buffer: 0.5% CHAPS in PBS with Ca^{2+}/Mg^{2+}
Elution buffer: 0.5% CHAPS in 50 mM diethylamine
Protein solution (see Support Protocol 2)
1 M Tris·Cl, pH 7.0 (*APPENDIX 2A*)
PBS with Ca^{2+}/Mg^{2+} (see recipe)
0.02% (v/v) merthiolate or equivalent bacteriostatic agent

Prepare column

1. Rinse the column extensively with loading buffer, especially for a column that was prepared earlier and has been stored for a while. Add 2 to 3 vol (i.e., 3 times the volume of the column) of elution buffer to the column to test the stability of the column under the elution conditions and to make sure that the column does not contain any contaminations, such as unspecifically bound proteins from previous use of the column.

2. Properly reequilibrate the column to loading conditions before loading the protein solution (see Support Protocol 2 for protein solution preparation).

   As the binding affinity of antibodies is usually not temperature-sensitive, the authors recommend running the affinity column at 4°C rather than at room temperature. Keeping the column and the protein solution to be loaded at 4°C helps to prevent contamination and slows down degradation of the proteins.

Load the column

3. During loading of the protein solution onto the column, adjust the flow rate to a sufficiently slow rate (usually 0.1 ml/min is chosen for good yields) to allow good interaction between antigen and antibodies coupled to the sepharose beads.

   The capacity of the column can be tested by collecting fractions of the flow-through and subsequent immunoblot analysis (see Support Protocol 4). For best yield, the capacity of the column should not be exhausted. Typically, column volumes of 1 ml are used.

Elute column

4. Incubate the column in 0.9 vol elution buffer for 10 min.

   This will increase the elution efficiency (a higher concentration of the eluted protein in eluate, rather than a broad elution peak with a long tail). Do not expose the column to elution conditions for longer times than necessary, because the high pH of the buffer could be detrimental for the affinity column.

5. Elute the column at a rate of 1 ml/min. Collect the eluate in vials containing enough 1 M Tris·Cl, pH 7.0, to buffer the eluate at a neutral pH value.

   For a 1-ml column, 1-ml fractions are collected in 1.5-ml microcentrifuge tubes.

   The volume of 1 M Tris·Cl, pH 7.0, required for restoring the pH should be determined at 4°C, as the pH value of Tris is extremely temperature sensitive.

   The flow rate for elution can be much higher than that for loading. However, check the maximal flow rate acceptable for a specific resin. For Sepharose resins a maximal flow rate of 30 ml $hr^{-1}cm^{-2}$ is recommended.

   The eluate can be stored a few days at 4°C; for longer storage below −20°C is recommended. However, keep in mind that repeated thawing and freezing is detrimental to the protein. Furthermore, freezing of dilute protein solutions is not recommended.

6. Re-equilibrate the column to loading conditions for a second run, or prepare the column for storage.

Regenerate and store the affinity column

7. Immediately after elution re-equilibrate the column to neutral pH values with PBS with Ca^{2+}/Mg^{2+}. For storage, add 0.02% merthiolate or an equivalent bacteriostatic agent to the PBS to prevent bacterial growth. Store the column at 4°C.

   Prevent drying of the column during storage. The column can be stored for several months at 4°C.

Preparation of the Affinity Column

SUPPORT PROTOCOL 1

This protocol only describes the preparation of an immunoaffinity column. Generally, because IgSF-CAMs have low binding affinities for their binding partners and have no enzymatic activity that could be used for substrate-based purification, the use of immunoaffinity columns is the method of choice.

However, a prerequisite is the availability of a monoclonal antibody against the protein to be purified. This antibody is covalently coupled to a Sepharose resin. Affinity columns are versatile, they can be used for tissue homogenates, solubilized membrane proteins, or culture supernatants from cell lines that are engineered to produce and release IgSF-CAMs. If stored appropriately, affinity columns can be reused many times over several months.

Materials

CNBr-activated Sepharose 4B gel
1 mM HCl
Buffer I: 0.5 M NaCl in 0.1 M $NaHCO_3$, pH 8.3
Monoclonal antibody against the protein to be purified
0.2 M glycine, pH 8.0
Buffer II: 0.5 M NaCl in 0.1 M sodium acetate, pH 4.0
Loading buffer (see Basic Protocol 1)

Sintered glass filter connected to a vacuum pump
Column (e.g., Poly-Prep column, Bio-Rad)
U-bottomed polypropylene vial that can be closed tightly

1. Soak 1:2 (w/v) CNBr-activated sepharose 4B in 1 mM HCl for 15 min at room temperature.

   For a 1-ml column, start with 350 to 400 mg Sepharose.

2. Transfer beads to a sintered glass filter connected to a vacuum pump, and wash with ≥25 vol of 1 mM HCl, followed by buffer I.

   It is very important to prevent the beads from drying between the additions of buffer (one gram dry resin yields ~3.5 ml swollen gel).

3. Transfer the slurry to a U-bottomed polypropylene vial containing the antibody solution in buffer I. Carry out the reaction for 2 hr at room temperature. Close the vial tightly.

   The final concentrations should be: 100 mg Sepharose (dry weight) and 5 mg antibody per ml coupling reaction mix.

   Ideally, rotating the vial end-over-head is used to maximize the coupling efficiency.

   Do not use a magnetic stirrer, which will damage the agarose beads.

4. Stop the reaction by gently centrifuging the Sepharose beads for 5 min at 2000 × *g*, room temperature.

5. Add 3 vol 0.2 M glycine, pH 8.0, to the pellet and continue to rotate the vial end-over-head for an additional 2 hr.

   Collect the supernatant of the coupling reaction to check the coupling efficiency.

6. Pack the slurry into a column.

   Typically, column volumes are ~1 ml with a column diameter of 0.5 cm.

7. Wash the column with 5 vol buffer I followed by 5 vol buffer II. Repeat wash procedure four times to remove excess uncoupled ligand.

8. Before loading the protein solution (see Support Protocol 2), wash the column thoroughly with 25 to 30 vol loading buffer.

   An affinity column can be used repeatedly, if stored appropriately with an antibacterial agent (e.g., 0.02% merthiolate) at 4°C. Before using the column after storage, rinse the column extensively with loading buffer. Use 2 to 3 vol elution buffer to clean the column, restore loading conditions by rinsing thoroughly with loading buffer (≥10 vol).

Solubilization of Membrane Proteins

Most IgSF-CAMs are either glycosyl-phosphatidylinositol-anchored or transmembrane proteins, therefore, they have to be solubilized from cell membranes with detergents. In the authors' experience, the use of CHAPS has given the best results with respect to yield and functional integrity of the purified proteins. Keep in mind that for many functional assays, detergent removal from the protein solution is necessary after purification; the presence of detergents can also interfere with binding assays. Especially sensitive assays are those that involve neurons, such as neurite outgrowth assays (see Basic Protocol 7). For removal of detergents from protein solutions, the authors have used SM-2 beads from BioRad or Calbiosorb from Calbiochem. The use of high concentrations of detergents can interfere with the purification by immunoaffinity columns, therefore, dilution of the protein solution after the solubilization step, i.e., before the solution is loaded onto the affinity column, is recommended. The protocol given below has been successfully used for the purification of functionally intact L1/NgCAM from E14 chicken brain membranes (e.g., Stoeckli et al., 1991, 1996). It is adapted from the purification protocol described by Grumet and Edelman (1984). However, the authors have used the same protocol for the solubilization and purification of other IgSF-CAMs (Rader et al., 1993; Fitzli et al., 2000). For storage, membranes and proteins can be frozen at the indicated steps. However, repeated freez-thaw cycles are detrimental for proteins and for high yields of intact proteins and should be minimized.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

14-day-old chicken embryo brains, freshly frozen in liquid nitrogen
Liquid nitrogen
Ca^{2+}/Mg^{2+}-free buffer (CMF buffer; see recipe)
0.8 M and 2.25 M sucrose in PBS
1 M and 2 M NaCl in PBS
50 mM triethylamine
0.5% and 1% CHAPS in PBS

Mortar and pestle
Dounce homogenizer
Centrifuge tubes for Sorval SS-34 or equivalent rotor
38-ml polycarbonate tubes for ultra high-speed centrifuge

Prepare membranes

1. Remove brains of 14-day-old chicken embryos and immediately freeze in liquid nitrogen.

   If necessary, brains can be stored at −70°C for extended periods of time.

2. Cool a mortar and pestle of sufficient size with liquid nitrogen and grind frozen brains in batches to a fine powder. Add small volumes of liquid nitrogen to keep brains/brain powder frozen during grinding.

   Carefully avoid thawing of the tissue at any time.

3. Add ~3 vol CMF buffer to 1 vol brain powder. Homogenize the brain powder in a Dounce homogenizer.

4. Centrifuge homogenate for 20 min at 45,000 × *g* (10,000 rpm in a SS-34 rotor), 4°C.

5. Resuspend the pellets in 1.5 to 2 vol of 2.25 M sucrose, use Dounce homogenizer to get homogenous suspension.
6. Transfer ~24 ml suspension to each polycarbonate ultracentrifuge tube, overlay with ¼ vol of 0.8 M sucrose.
7. Centrifuge for 60 min at 150,000 × *g*, 4°C.
8. Transfer the membranes that are accumulated in the interphase to the Dounce homogenizer, resuspend them in 25 vol PBS.
9. Centrifuge for 60 min at 150,000 × *g*, 4°C.
10. Decant supernatant and resuspend in PBS, centrifuge as in step 9, and resuspend pellets in smallest possible volume of PBS for storage at −20°C, or use directly for stripping.

Samples can be stored up to 1 month at −20°C, or at −70°C for longer storage.

Strip packed membranes

11. Add 1 vol of 2 M NaCl in PBS to packed membrane suspension (from step 10), homogenize suspension.
12. Add 1 M NaCl in PBS to a final volume of 4 to 5 times the volume of the packed membranes.
13. Stir suspension 1 hr on a magnetic stirrer at low speed, 4°C, to strip membranes from peripheral membrane proteins.
14. Centrifuge for 60 min at 150,000 × *g*, 4°C.
15. Resuspend pellets in 20 vol of 50 mM triethylamine, stir for 60 min at 4°C.
16. Repeat centrifugation step 14. Carefully remove the supernatant with a pipet, as the membranes do not form a stable pellet after the high pH extraction step.
17. Resuspend the pellets in PBS.
18. Centrifuge for 60 min at 150,000 × *g*, 4°C.
19. Repeat washing the membranes with PBS at least one time to restore a pH value between 7.2 and 7.6. Keep an aliquot for measuring the protein concentration.
20. Freeze the stripped membranes at −20°C or use directly for solubilization step.

For storage longer than 1 month, store at −70°C.

Solubilize integral membrane proteins

21. If frozen membranes are used, they should be washed once again with PBS (steps 17 and 18).
22. Transfer pellets into a Dounce homogenizer and resuspend in 1 vol of 1% CHAPS buffer. Add 4 vol of 0.5% CHAPS buffer.

For good solubilization the protein concentration should be adjusted to ~1 mg/ml.

23. Extract the membrane proteins by stirring the suspension for 60 min at 4°C.
24. Centrifuge for 60 min at 150,000 × *g* , 4°C.
25. Combine supernatants, measure the volume, and remove aliquot to determine the protein concentration.

Do not freeze the solubilized proteins, but use directly for affinity purification step.

Production of Recombinant CAM by Transient Transfection of HEK293 Cells with Calcium Phosphate

SUPPORT PROTOCOL 3

Transient transfection of HEK 293 cells with expression vectors containing cDNAs of IgSF-CAMs represents a fast and convenient method for the production of intermediate amounts (100 μg to 1 mg) of recombinant protein. The human embryonic kidney cell line 293 (HEK 293) is a well established, easily transfectable cell line that is widely used for the expression of recombinant proteins. The transfection method of choice with HEK 293 cells is always calcium phosphate transfection, an inexpensive, convenient technique that results in high efficiencies of transfection.

DNA can be introduced into a wide variety of cultured cell lines as a calcium phosphate complex (Graham and van der Eb, 1973; Wigler et al., 1977). The transfected DNA can either integrate into the genome of the recipient cell, resulting in stable transgene expression accompanied by a stably altered phenotype of the cell (stable transfection) or remain episomal resulting in only transient expression of the transgene (transient transfection). The following protocol can be used likewise for the generation of stable cell lines by subsequent selection for stable transfectants or for transient expression only.

Efficient transfection requires the formation of a fine precipitate of calcium phosphate in the presence of DNA. The formation of the DNA-containing calcium phosphate particles is initiated under defined chemical conditions, in the absence of cells or serum. The particle size is the most critical parameter regarding efficiency of transfection, that is uptake of DNA-containing calcium phosphate particles by the cells. The main determinants of particle size are calcium and phosphate concentrations, the concentration of DNA, size of DNA fragments involved, pH, temperature, and time of incubation. After initial formation of the DNA-containing calcium phosphate particles, the precipitate is added to the cells. During the incubation of the precipitate with the cells, the formation of DNA-containing calcium phosphate particles continues and preexisting particles grow in size. The particles adhere to the cells and are taken up by endocytosis. After a few hours of exposure, the medium is changed and the cells start to express the recombinant protein. The period of efficient transgene expression varies between different expression vectors but lasts generally for a few days.

Materials

HEK 293 cells
Cell culture medium for HEK 293 cells (see recipe)
Purified DNA of interest
$CaCl_2$ solution (see recipe)
HBS solution (see recipe)
175-cm^2 tissue culture flasks
Additional reagents and equipment for trypsinizing cells (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

1. For transient transfection, grow HEK 293 cells of low passage number (passage <40) and split cells every 2 to 3 days before 80% confluency is reached.

   By frequent splitting and growth in subconfluent density, the cells remain in their exponential growth phase and are highly susceptible for transfection up to about passage number 40.

2. One day prior to the experiment, trypsinize cells briefly (*UNIT 1.1*) and prepare a single cell suspension. Plate 5×10^6 cells in 30 ml of medium per 175-cm^2 flask and incubate for 16 to 20 hr at 37°C, 5% CO_2 incubator.

   For optimal transfection efficiency, 60% to 70% confluency of cells is required. This allows two additional cell divisions after transfection. Some 293 lines adhere only poor to tissue culture plastic, especially after transfection. To achieve more stable adherence, tissue culture flasks can be coated with poly-D-lysine (see Support Protocol 10).

   HEK 293 cells do not need a medium change prior to addition of the calcium phosphate precipitate.

3. Mix purified DNA (40 to 80 µg in a maximum volume of 100 µl) with 1.5 ml $CaCl_2$ solution and incubate for a few minutes at room temperature.

   For efficient formation of the DNA-containing calcium phosphate particles, temperature is critical. The solutions should be kept at room temperature for a couple of hours before use. The optimal amount of DNA depends on the particular expression construct used for transient transfection and has to be determined individually for each system.

4. Add 1.5 ml of HBS solution and mix well. Incubate for 1 to 15 min at room temperature and then add the reaction mixture to the cells (from step 2).

   The formation of the DNA-containing calcium phosphate particles starts immediately after mixing the two solutions and can be observed by a slightly opaque appearance of the solution. Longer incubation periods generally result in the formation of a precipitate with larger particles. The optimum time of incubation critically depends on the size of the plasmids used.

   The growth rate of the calcium phosphate particles frequently correlates inversely with the size of the plasmids. For very large plasmids(>30 kb), preincubation of no longer than 1 min is recommended.

5. Incubate the DNA-containing calcium phosphate particles with the cells for 4 to 6 hr at 37°C in an incubator.

   The formation of the calcium phosphate precipitate can be monitored under an inverted light microscope with a 63× objective. The precipitate should become visible as tiny particles (~300 nm in diameter) especially on the surface between the cells. However, long exposure to the atmosphere outside of the incubator should be avoided.

6. Change medium after the incubation period and continue incubation. Begin testing for expression of the recombinant protein 1 day after transfection.

   The cells should now express the recombinant protein for a few days.

   Uptake of the DNA-containing calcium phosphate particles by cells can be checked by examination of free surfaces between cells. The margins surrounding the cells should be cleared of particles, due to their uptake by the cells.

SUPPORT PROTOCOL 4

Detection of IgSF-CAMs by the Dot Immunoblot Method

A rapid and convenient method for the detection of IgSF-CAMs in eluates from chromatography or affinity columns are dot immunoblots. Similar to immunoblots, dot blots are a semi-quantitative method to detect proteins transferred onto a nitrocellulose membrane via a chromogenic reaction. For this purpose, secondary antibodies coupled to peroxidase or alkaline phosphatase are used to detect binding of the first antibodies directed against the protein of interest. However, in contrast to immunoblots, proteins are not separated on a polyacrylamide gel, therefore dot blots are a much faster way to demonstrate the presence of a specific protein in a given sample. Furthermore, as the proteins are directly applied to the nitrocellulose membrane for dot blots, there is no loss due to difficulties in

transfer from the gel to the membrane. The fact that only small volumes can be applied onto the membrane for dot blots may, however, limit its use for detection of proteins in dilute solutions.

The procedure described here can also be used to stain IgSF-CAMs after immunoblotting. Use manufacturer's manuals and protocols for sample preparation, SDS-PAGE, and blotting of proteins onto nitrocellulose (also see *UNITS 6.1* and *6.2*).

Materials

Protein solution of interest
TBS: 0.2 M NaCl in 50 mM Tris, pH 7.4
Blocking solution: 2% (w/v) milk powder in TBS with or without 0.1% (w/v) Tween 20
Antibody against the protein of interest diluted in blocking solution
Secondary antibody coupled with horseradish peroxidase (HRP) diluted in blocking solution
4-chloro-1-naphthalene solution (see recipe)

0.2-µm nitrocellulose membrane (e.g., Schleicher and Schuell)
96-well plates
Rotary shaker

1. Place matching round disks of 0.2-µm nitrocellulose membrane into wells of a 96-well plate.

   When large numbers of samples have to be analyzed, the use of a commercially available protein-dispersing device is advised (e.g., Dot Punch IM-96, Inotech AG). These devices allow the application of protein solutions to the membrane before they are put into the wells. This is more convenient and less time-consuming compared to the procedure described below.

2. Apply small volumes (<10 µl) of the protein solution onto the membrane.

   The solution should not cover the entire membrane but remain confined to a segment of the membrane (dot). This is important in order to ensure the attachment of sufficient amounts of proteins per area to reach detection limits. If the protein solution is very dilute, the dotting procedure can be repeated after drying the nitrocellulose after the first application. The repeated application of protein to the membrane increases the amount of the protein of interest.

3. Dry the membranes to optimize protein adherence to the nitrocellulose.

4. Wash membranes three times with 100 µl TBS per well.

5. Block protein adsorbance of the membrane by incubating ≥30 min with blocking solution, room temperature.

   Generally, 2% to 5% milk powder in TBS with or without 0.1% Tween 20 works well as a blocking solution. In some cases, addition of 2% to 10% (v/v) serum in TBS improves the signal-to-background ratio.

6. Apply 1 to 10 µg/ml first antibody diluted in blocking solution and shake for ≥1 to 2 hr, room temperature, on a rotary shaker.

   If necessary, incubate overnight.

   Shaking the 96-well plate improves antibody/antigen interaction.

7. Wash thoroughly with TBS (≥3 times).

8. Incubate with blocking solution for ≥30 min before applying the secondary antibody.

9. Apply 1 to 10 µg/ml of secondary antibody coupled with horseradish peroxidase diluted in blocking solution to the membrane and incubate 1 hr at room temperature.

   As a secondary antibody, the use of a peroxidase-coupled antibody is described, but good results can also be obtained with phosphatase-coupled antibodies. Generally, an antibody concentration of 1 to 10 µg/ml is used to get good signal-to-noise ratios but batches or products from different suppliers vary.

10. Remove antibody solution and wash membrane thoroughly with at least three changes of TBS.

11. Visualize the antibody by adding 0.1 ml of 4-chloro-1-naphthalene solution.

    Generally, the dark blue color develops immediately.

12. Stop the reaction by rinsing the membranes with water.

FUNCTIONAL ASSAYS WITH PURIFIED PROTEINS

BASIC PROTOCOL 2

Analysis of Protein Interactions with Fluorescent Microspheres

There are essentially two ways to analyze the binding properties of cell adhesion molecules, (1) binding assays based on purified proteins, and (2) binding assays based on heterologously expressed proteins in their physiological environment (cell aggregation assays). This protocol describes a method that utilizes biochemically purified proteins coupled to fluorescent microspheres, whereas Basic Protocol 4 is based on cell aggregation assays. If pure cell adhesion molecules are available, proteins can be coupled to polystyrene beads of different fluorescent colors. Coupling can be either by covalent linkage or by hydrophobic adsorption of the protein to the microsphere surface. After dispersion of the beads by ultrasonication, pairwise combinations of differently coupled beads aggregate during a particular incubation period. Binding activities are analyzed by the evaluation of aggregate formation both qualitatively, by inspection on a fluorescent microscope, and quantitatively, using a fluorescence-activated flow cytometer. Although this protocol is a reliable method to detect binding activities of IgSF-CAMs and has been used by several laboratories, one has to consider false negative results as well as discrepancies between results of bead and cell aggregation assays. Some discrepancies have been explained by differences in the orientation of proteins coupled to beads in comparison to proteins on the surface of cells.

Materials

Protein-conjugated fluorescent microspheres; stock solutions contain 10^{11} beads/ml in 0.5% (w/v) BSA (see Support Protocol 5; Duke Scientific; Bangs Laboratories; Polysciences)
0.5% (w/v) BSA solution (see recipe)
0.5 mg/ml Fab fragments of antibodies against proteins of interest in PBS (optional)
0.5% (w/v) trypsin (optional)
PBS (see recipe)

Water bath sonicator (Branson Ultrasonics)
Fluorescence microscope equipped with FITC and TRITC filters
0.5-ml microcentrifuge tube
Rotator
Glass microscope slides
Refrigerated microcentrifuge
Fluorescence-activated flow cytometer

Prepare mixtures of fluorescent microsphere

1. Sonicate test tubes containing stock solutions of protein-conjugated microspheres in a water bath sonicator for 2 min at room temperature. Use lowest sonicator output setting needed to achieve monodisperse bead solution to prevent protein structure damage.

   Make relatively small aliquots of protein-conjugated bead stocks because repetitive ultrasonication may damage protein structure.

   For the applications described here, fluorescent microspheres with a nominal diameter of 0.5 μm work best. Both covalent coupling of proteins via surface-exposed functional groups or hydrophobic adsorption may be used. The best mode of coupling may depend on the particular nature of the protein to be studied.

   The authors have consistently used Covaspheres fluorescent microspheres from Duke Scientific (Kuhn et al., 1991; Suter et al., 1995; Rader et al., 1996; Kunz et al., 1998; Fitzli et al., 2000). Fluorescent Covaspheres with a nominal diameter of 0.5 μm were easy to handle, allowed covalent coupling of the proteins, and were readily detectable in the light microscope and cell sorter. However, Duke Scientific has discontinued the distribution of Covaspheres, but a new line of beads with well characterized fluorescence and charge density is available. Different functional groups for covalent coupling of proteins will be offered, including carboxylate and aldehyde groups.

   Support Protocol 5 describes covalent coupling of proteins to Covaspheres as well as the hydrophobic adsorption of proteins to polystyrene beads. With some modifications, it may also be suited for other types of beads.

   The authors have added a support protocol that can be used for coupling proteins to microspheres exhibiting amino groups on their surfaces (see Support Protocol 6).

2. Check monodispersity of bead stock solution by quick inspection of a 1:100 dilution in 0.5% BSA under a fluorescence microscope.

3. Immediately after ultrasonication, prepare different test mixtures by combining protein-coupled beads of different fluorescent colors. Add 2 μl of green-fluorescent beads conjugated with protein 1 and 2 μl of red-fluorescent beads conjugated with protein 2 into a 0.5-ml microcentrifuge tube and bring to a final volume of 20 μl with 0.5% BSA solution (the concentration of each species of beads in the test mixture is 10^{10}/ml). Vortex beads.

   Use as many controls as possible. Typical control incubations are:

   a. Each type of beads alone.

   b. Beads conjugated with control proteins that should not bind to protein of interest (e.g., BSA, non-immune IgG).

   c. Protein-conjugated beads that were either incubated in boiling water for 10 min or treated with 0.5% trypsin overnight.

   These treatments should abolish specific protein interactions.

   d. Pretreat beads with Fab fragments that are specific to the conjugated protein of interest. For this purpose, incubate an appropriate volume of the bead stock solution with Fab from polyclonal IgG at a concentration of 0.5 mg/ml in PBS for 2 hr at room temperature. Remove unbound antibodies by three consecutive washes with PBS. For solution changes, centrifuge beads in a microcentrifuge tube for 10 min at 16,000 × *g*, 4°C. Dissolve possible aggregates of beads by ultrasonication as in step 1.

4. Incubate by slowly rotating test mixtures protected from light for 1 hr at room temperature on a rotator.

   If such a rotator is not available, invert tubes a few times every 15 min. Tubes can be light protected with aluminum foil.

Analyze by fluorescence microscopy

5. Analyze the test mixtures for aggregate formation by fluorescence microscopy. Prepare a 1:10 dilution of the different incubations in 0.5% BSA solution and place 20 µl of this dilution on a glass slide for inspection.

   Most fluorescent microspheres emit a relatively intense signal. Therefore, antifading agents are not necessary for inspection. After a while, aggregates will settle and pictures can be taken for documentation or quantification of aggregate sizes. Adhesive interactions between two different proteins coupled to beads labeled with FITC and TRITC, respectively, should result in aggregates consisting of up to 100 beads of both colors that are equally distributed throughout the clusters. Carefully check for bleed-through between the different fluorescence channels.

Analyze by fluorescence-activated flow cytometry

6. Use flow cytometric analysis for quantification of aggregate formation. Calibrate the flow cytometer correctly for nonaggregated beads of each color.

7. Dilute test mixtures 1:1000 in 0.5% BSA solution and inject into a fluorescence-activated flow cytometer equipped with appropriate FITC and TRITC filter sets (Kuhn et al., 1991).

   Always use fluorescence microscopy in parallel to flow cytometer analysis to obtain information on the distribution of the beads in aggregates (see Critical Parameters and Troubleshooting).

8. Use uncoated, nonaggregating beads to determine relative fluorescence intensity (RFI) of individual beads of each color. Compensate for the spectral overlap of the FITC and TRITC emission electronically.

9. Record the data output for number, size, and composition of mixed aggregates.

   Data can be presented as two-dimensional contour or dot plots with the RFIs in the FITC and TRITC channel as x- and y-coordinates indicating size and composition of aggregates (Kuhn et al., 1991; Suter et al., 1995). The signal intensity represents the number of aggregates of a certain size and composition. The RFI values of single beads will allow the investigator to set the boundaries between mixed aggregates and aggregates that consist only of one species of beads. Determination of these boundaries is necessary to calculate the percentage of beads found in mixed aggregates as an indicator for protein binding.

SUPPORT PROTOCOL 5

Coupling Proteins to Fluorescent Microspheres

This support protocol describes a method for the preparation of protein-conjugated fluorescent microspheres required for the protein interaction studies described in Basic Protocols 2 and 3. It is based on the procedure that the authors have used for the coupling of proteins to Covaspheres (Duke Scientific). However, the same procedure has successfully been used for the adsorption of proteins to polystyrene beads, which bind proteins by hydrophobic interactions.

Additional Materials *(also see Basic Protocol 2)*

10^{11} unconjugated beads/ml fluorescent polystyrene microspheres, 0.5-µm diameter (Duke Scientific; Bangs Laboratories; Polysciences)

Biochemically purified IgSF-CAMs (see Basic Protocol 1) in a phosphate-based buffer system (either PBS or 20 mM sodium phosphate, pH 7.0)

1. Sonicate tubes containing stock solutions of 10^{11} unconjugated beads/ml fluorescent polystyrene microspheres in a water bath sonicator for 2 min at room temperature.

2. If bead solution is monodisperse, transfer 100 µl of beads to a new tube and add 50 µg of biochemically purified IgSF-CAMs in PBS to a total volume of 1 ml (10^{10} beads/ml during coupling). Vortex tubes.

   Before coupling, test protein preparations carefully for purity with SDS-PAGE to avoid false positive, as well as false negative, results.

   Store proteins under sterile conditions in PBS or 20 mM sodium phosphate, pH 7.0, 4°C. For highly stable proteins, storage for up to 1 year at 4°C under sterile conditions is possible.

   Cell adhesion molecules with transmembrane and cytoplasmic domains (e.g., NgCAM and NrCAM) require the presence of detergents such as CHAPS and deoxycholate during purification. The authors found that biological activity, such as binding, is better preserved when these detergents are maintained during protein storage at 4°C and during coupling to beads.

3. Incubate the mixture for 1 hr in a 37°C water bath. Mix periodically by inverting tube.

4. Centrifuge for 10 min at 16,000 × *g*, 4°C. Remove supernatant containing unbound protein and save for coupling analysis. Resuspend beads in 1 ml of 0.5% BSA solution.

   This step is necessary to block residual binding sites that could result in unspecific bead-bead interactions.

5. Sonicate beads as in step 1 and incubate 30 min at room temperature.

6. Centrifuge beads as in step 4 and resuspend pellet in 100 µl of 0.5% BSA solution. Store beads at 4°C.

   Protein-conjugated microspheres should never be frozen and can be kept up to 1 year at 4°C (depending on protein stability).

7. (*Optional*) Determine coupling yield by SDS-PAGE of serial dilutions of protein samples taken before and after coupling.

   Densitometric analysis of bands after silver staining or immunoblot detection allows the determination of both coupling efficiency and the number of protein molecules bound per microsphere.

   Typically, a coupling yield of >50% is obtained under the conditions described. In the case of the IgSF-CAMs axonin-1 and NgCAM, ~16,000 molecules were found to bind per Covasphere bead (Kuhn et al., 1991).

Covalent Coupling of Proteins to Glutaraldehyde-Activated Amino Beads

SUPPORT PROTOCOL 6

As an alternative method for coupling proteins to beads, this protocol describes the glutaraldehyde-activated coupling of proteins to microspheres with amino-functional groups. Purified IgSF-CAMs can be coupled directly (not oriented) to amino beads. Alternatively, Fc-containing recombinant proteins can be bound to protein A–conjugated microspheres in an oriented manner.

Additional Materials *(also see Basic Protocol 2)*

Amino-functional microspheres (e.g., silica aminopropyl beads from Bangs Laboratories)
0.1 M NaOH or HCl (optional)
8% (v/v) EM-grade glutaraldehyde, newly opened bottle
400 µg/ml biochemically purified IgSF-CAMs (see Basic Protocol 1) in a phosphate-based buffer system (either PBS or 20 mM sodium phosphate, pH 7.0)
Blocking solution (see recipe)

1. Prepare an appropriate volume (e.g., 0.5 ml) of 1% amino-functional microsphere stock solution in ultra pure water. Wash beads two times with 1 ml water. Centrifuge for 4 min at 16,000 × *g*, room temperature, to separate microspheres from wash solution. Remove supernatant and resuspend microspheres in 0.5 ml water.

   The amino-functional microspheres are available as powder or 10% (w/v) solution. Keep microsphere stock solutions at 4°C and never freeze.

2. Before adding the glutaraldehyde solution, check under the microscope whether the bead solution is monodisperse. If the bead solution contains significant clumps, sonicate in the water bath sonicator for 5 min at room temperature. If clumps persist, use a Branson microtip sonicator at the lowest output power needed to disrupt the clumps. Check pH of bead solution with cut pH strips and, if necessary, adjust pH to 6.5 or 7.0 with 0.1 M NaOH or HCl, respectively.

   Glutaraldehyde activation works best at pH 6 to 7.

3. If the bead solution is monodisperse and has a pH of 6.5 to 7.0, add an equal volume of a newly opened bottle of 8% EM-grade glutaraldehyde and mix. Incubate beads on rotator for ≥6 hr or overnight at room temperature.

4. Centrifuge beads as in step 1, remove supernatant, and wash activated beads at least three times with 1 ml water, and once with the buffer in which the protein is dissolved (PBS or 20 mM sodium phosphate).

5. Add 0.5 ml of 400 µg/ml purified IgSF protein in PBS, pH 7.3, or in 20 mM sodium phosphate, pH 7.0, to bead pellet and resuspend beads in protein solution (1% beads during coupling reaction). Incubate on rotator for 4 hr at room temperature or overnight at 4°C.

   If less protein is available, scale down amounts of protein and beads proportionally.

6. Centrifuge beads 4 min at 16,000 × *g*, room temperature, save supernatant for coupling analysis on SDS-PAGE, and resuspend beads in 0.5 ml blocking solution. Incubate beads in blocking solution for 30 min at room temperature on rotator.

7. Centrifuge beads again. Resuspend beads in 0.5 ml blocking solution (1% bead stock) and store at 4°C for months.

BASIC PROTOCOL 3

Binding of Protein-Conjugated Microspheres to Cultured Cells

This protocol determines binding specificity of a cell adhesion molecule to a receptor on a particular cell type. Both primary cell cultures (Kuhn et al., 1991; Suter et al., 1995) and cell lines (Buchstaller et al., 1996; Rader et al., 1996) can be incubated with protein-conjugated microspheres under live conditions to determine interactions between specific proteins and cells. Antibody preincubations can be used as a control but also for receptor identification. Furthermore, cells transiently transfected with mutated receptor forms can be used for the identification of extracellular protein domains necessary for binding of the cell adhesion protein (Rader et al., 1996; Kunz et al., 1998; Fitzli et al., 2000).

Materials

Primary cultures of neuronal and/or glial cells or other cells
Complete medium used for cell cultures
Serum-free BSA-containing cell culture medium
10^{11} beads/ml protein-conjugated fluorescent polystyrene microspheres; stock solutions in 0.5% BSA (see Support Protocol 5)

0.5 mg/ml Fab against protein of interest in serum-free medium (optional)
Fixation solution (see recipe)
PBS (see recipe)
Mounting medium (see recipe)

Waterbath sonicator
37°C, 10% CO_2 humidified incubator
Glass microscope slides
Fluorescence microscope

Prepare and bind microspheres to cells

1. Cultivate primary neurons and/or glial cells in conditions that allow live cell incubations in relatively small volumes.

   For example, grow cells on substrate-coated coverslips using a removable donut-shaped Teflon ring to limit the incubation volume to 200 to 250 µl (Suter et al., 1995).

   If culture conditions include the use of serum, prepare also a corresponding serum-free, BSA-containing medium as in Stoeckli et al., 1991; see recipe.

2. Wash cultured cells one time with complete culture medium and one time with serum-free, BSA-containing cell culture medium.

   Medium exchanges are carried out carefully with a pipet.

3. Prepare 1:1000 dilutions of 10^{11} beads/ml protein-conjugated fluorescent polystyrene microspheres in serum-free cell culture medium, sonicate dilutions for 2 min, room temperature and immediately add to cultured cells.

4. (*Optional*) To test whether a specific CAM binds to a particular cell type via a characterized receptor (to which antibodies are available), preincubate cells with 0.5 mg/ml Fab fragments in serum-free medium, before adding the beads, for 2 hr in a 37°C, 10% CO_2 humidified incubator. Remove unbound antibodies by washing two times with serum-free medium before proceeding to step 5. To test for the specificity of bead binding to cells, use control protein–conjugated beads as well as beads that were preincubated with the corresponding Fab fragments.

5. Incubate the cells with the bead solution (step 3) for 1 hr in a 37°C, 10% CO_2 humidified cell culture incubator.

6. Use a Pasteur pipet to carefully aspirate medium with unbound beads. Immediately add serum-free medium. Repeat this step three times.

   Do not allow the cells to dry.

   Beads bound to live cells can now be inspected. However, if more time is needed for analysis, the authors recommend fixing the cells. Fixation also allows processing of the cells for immunostaining of marker proteins or CAMs and, therefore, correlative analysis of CAM binding and CAM expression.

Fix cells

7. Fix the cells by adding 80 µl of 4× fixation solution to the cultures, which are in 240 µl of serum free medium. Gently mix and incubate 1 hr at 37°C.

8. Wash fixed cells three times with PBS.

9. For immunofluorescence staining of the cells, proceed as described in *UNIT 4.3*. Otherwise mount cells on a glass microscope slide in mounting medium for inspection under a fluorescence microscope.

   Beads are generally intensely fluorescent. Therefore, relatively short exposure times are sufficient when taking pictures.

BASIC PROTOCOL 4

Trans-Interaction Assay with Myeloma Cells

Interactions between IgSF-CAMs that have been determined either by a fluorescent microsphere assay or by binding of fluorescent microspheres to cells do not provide any information on whether the interaction occurs between molecules located on different cells (*trans*-interaction) or between molecules residing on the membrane of the same cell (*cis*-interaction). In order to distinguish between a *trans*- and a *cis*-interaction, the IgSF-CAMs need to be studied in a natural environment, i.e., as membrane-bound molecules residing in their proper orientation in a biological membrane. Expression in nonadherent myeloma cells (see Support Protocol 7) provides a means of assessing IgSF-CAMs for *trans*-interactions. Two populations of stably transfected myeloma cell clones are stained with optically distinct intracellular fluorescent dyes. The cells are dissociated, incubated, and examined under a microscope in order to determine whether re-aggregation has occurred.

To evaluate whether two populations of myeloma cell clones expressing the IgSF-CAMs of interest on their surface can adhere to each other, they are labeled, mixed, dissociated, and allowed to re-aggregate. Labeling with optically distinct fluorescent dyes is necessary to distinguish the two populations.

Materials

Two populations of myeloma cell clones expressing the CAMs of interest
Selection medium, e.g., 5 mM L-histidinol in DMEM supplemented with 10% (v/v) FCS
PBS with Ca^{2+}/Mg^{2+} (see recipe)
Stock solution of green fluorogenic dye, e.g., 1 mM 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes) in DMSO
Stock solution of red fluorogenic dye, e.g., 7.5 mM 5-(and-6)-carboxynaphtofluorescein diacetate (Molecular Probes) in DMSO
1% (v/v) FCS in PBS with Ca^{2+}/Mg^{2+}
5 mg/ml *p*-phenylenediamine in 1% FCS in PBS with Ca^{2+}/Mg^{2+}

15-ml conical polypropylene centrifuge tubes
Hemacytometer
V-shaped 96-well microtiter plate (e.g., Costar, Corning)
Centrifuge and rotor for microtiter plates
22-G needle attached to 1-ml syringe
Glass microscope slide
Fluorescence microscope with appropriate filters for green and red fluorescence, e.g., FITC and Texas Red
Additional reagents and equipment for counting cells (*UNIT 1.1*)

Prepare cells

1. Grow transfected myeloma cell clones that express the CAM(s) of interest on their surface in selection medium to a cell density of ~5×10^5 cells/ml. Use a hemacytometer to monitor the cell density (the aggregation assay requires 5×10^5 cells per population per sample; see *UNIT 1.1*).

   Transfected myeloma cell clones that express high concentrations of a homophilically trans-interacting cell adhesion molecule can grow in very large cell aggregates consisting of thousands of cells that are easily visible by eye. The authors found that cultures of aggregating myeloma cell clones contain more dead cells than nonaggregating myeloma cell clones. This might, at least in part, be due to a limited oxygen and nutrition supply to

the center of the aggregate. In order to limit the number of dead cells, aggregating myeloma cell clones should be split more frequently avoiding large cell aggregates. The cell density can be determined with a hemacytometer after dissociating the cells by repeated pipetting through a 22-G needle.

2. Transfer 5 to 10 ml of cell culture into 15-ml conical polypropylene tubes and centrifuge for 3 min at 500 × *g*, room temperature. Aspirate the supernatant and resuspend the cells in 1 to 2 ml PBS with Ca^{2+}/Mg^{2+}. Determine the cell density with a hemacytometer (*UNIT 1.1*). Add PBS with Ca^{2+}/Mg^{2+} to give a cell density of 5×10^5 cells/150 µl. Pipet 150 µl/well to a V-shaped 96-well plate.

   The aggregation assay requires two populations in distinct wells per sample. Use wells A1 and A2 for the first sample, B1 and B2 for the next sample, etc.

   When desired, the cells can be pre-incubated with antibodies at this step by incubating the cells with Fab monoclonal or polyclonal antibodies in a concentration range of 10 to 500 µg/ml in 1% FCS in PBS with Ca^{2+}/Mg^{2+} for 1 hr at room temperature.

3. Centrifuge the microtiter plate for 2 min at 500 × *g*, room temperature, remove the supernatant by flicking plate into a sink (cells will remain in wells). Resuspend cells in 90 µl PBS with Ca^{2+}/Mg^{2+}.

Label cells

4. Prepare fresh working solutions of both, green and red, fluorogenic dyes by diluting 10 µl of the stock solution in 990 µl PBS with Ca^{2+}/Mg^{2+}. Add 10 µl of the appropriate working solution to the well with the appropriate 90-µl cell suspension. Use one column for one fluorogenic dye (e.g., stain A1, B1, etc. with green and A2, B2, etc. with red). Incubate 30 min at 37°C.

   The end concentration of the fluorogenic dyes is 1 µM of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester and 7.5 µM of 5-(and-6)-carboxynaphtofluorescein diacetate. The electrically neutral ester substrates freely diffuse through the cell membrane into the cell, where they are cleaved into fluorescent products by nonspecific intracellular esterases. The charged fluorescent products are retained by cells with intact plasma membranes. Serum of the cell culture medium contains esterases and has to be washed away prior to the incubation and avoided during the incubation.

5. Centrifuge microtiter plate and remove the supernatant as in step 3. Resuspend the cells in 150 µl PBS with Ca^{2+}/Mg^{2+}. Repeat step.

6. Centrifuge microtiter plate 2 min at 500 × *g*, room temperature and remove the supernatant by flicking plate into sink. Resuspend cells in 75 µl of 1% FCS in PBS with Ca^{2+}/Mg^{2+}.

Dissociate cells and allow re-aggregation

7. Combine complementarily stained cells of one sample in one well (e.g., add A2 to A1, B2 to B1, etc.). Dissociate cells by slowly pipetting up and down ten times through a 22-G needle attached to a 1-ml syringe. Avoid foaming. Incubate 45 min at 4°C.

   During re-association the plate should not be moved.

8. Centrifuge microtiter plate for 2 min at 500 × *g*, room temperature, and remove the supernatant by flicking plate into sink. Resuspend the cells in 150 µl of 1% FCS in PBS with Ca^{2+}/Mg^{2+}. Repeat step.

9. Centrifuge microtiter plate and remove the supernatant as in step 8. Resuspend the cells in 40 µl of 1% FCS in PBS with Ca^{2+}/Mg^{2+}.

Analyze cells

10. Immediately prior to microscopic analysis of an individual sample, add 10 µl of 5 mg/ml *p*-phenylenediamine in 1% FCS in PBS with Ca^{2+}/Mg^{2+}. Pipet the sample several times up and down using a 200-µl pipet tip. Mount 10 µl of the sample on a glass slide.

    The use of the antifading reagent p-phenylenediamine in an end concentration of 1 mg/ml markedly preserves the fluorescence intensity.

11. Analyze the aggregates with a fluorescence microscope using FITC and Texas Red filters.

    Filters that allow the simultaneous detection of green and red fluorescence facilitate the analysis.

SUPPORT PROTOCOL 7

Stable Transfection of Myeloma Cells by Protoplast Fusion

This protocol describes the stable transfection of myeloma cells with a vector that allows the surface expression of IgSF-CAMs. A vector particularly suited for myeloma cell expression was described by Traunecker et al. (1991). Expression by this vector is driven by an Ig κ promoter and enhancer. The 3′ end of the transcript of interest is spliced onto an exon encoding the Ig κ constant domain in order to mimic stable Ig transcripts. The vector contains a histidinol dehydrogenase gene that allows the selection of stable transfectants in the presence of L-histidinol. L-histidinol is a precursor of L-histamine and an inhibitor of protein synthesis. The vector has been stably transfected into the mouse myeloma cell line J558L for the production of soluble lymphocyte-derived cell-surface receptor proteins (Traunecker et al., 1991). The system has also been used for the surface expression of IgSF-CAMs (Rader et al., 1993; Buchstaller et al., 1996; Fitzli et al., 2000). Alternatively, other mammalian expression vectors and myeloma cells can be used.

Myeloma cell clones that stably express large amounts of IgSF-CAMs on their surface were generated by a transfection method known as protoplast fusion. Transfection by protoplast fusion is a highly efficient method for the direct transfer of mammalian expression vectors from bacteria to mammalian cells (Schaffner, 1980; Sandri-Goldin et al., 1981; Rassoulzadegan et al., 1982; Gillies et al., 1983). It involves digesting bacterial cell walls with lysozyme to produce protoplasts and then fusing the protoplasts to mammalian cells in the presence of polyethylene glycol. The following protocol is based on the myeloma expression system described by Traunecker et al. (1991) and can easily be adapted to other systems.

Materials

Glycerol stock of an *E. coli* strain 803 clone (ATCC #35581) transformed with a mammalian expression vector containing the cDNA of the IgSF-CAM of interest (store at −80°C)
LB agar/ampicillin plates (see recipe; store at 4°C)
DMEM supplemented with 10% (v/v) FCS
LB medium (see recipe), prewarmed to 37°C
50 mg/ml ampicillin (store at −20°C)
60 mg/ml chloramphenicol in ethanol (store at −20°C)
DMEM supplemented with 10% (w/v) sucrose and 10 mM $MgCl_2$, prewarm
20% (w/v) sucrose in 50 mM Tris·Cl, pH 8.0, ice cold
1 mg/ml lysozyme (Roche Molecular Systems), freshly dissolved 10 mg in 10 ml of 250 mM Tris·Cl, pH 8.0, and filtered through 0.22-µm filter
250 mM EDTA, pH 8.0, ice cold
50 mM Tris·Cl, pH 8.0, ice cold

10 mg/ml DNase I (Roche Molecular Systems; store at −20°C)
DMEM
PEG 1500 in DMEM supplemented with DMSO (see recipe)
Mouse BALB/c myeloma cell line J558L (ECACC #88032902) or another myeloma cell line
50 mg/ml kanamycin
50 mM L-histidinol (see recipe)
Polyclonal anti-IgSF-CAM antibody
Fluorescein-conjugated secondary antibody

25-ml cell culture flasks
12-ml and 50-ml polypropylene tubes
15- and 50-ml conical polypropylene centrifuge tubes
37°C bacterial shaker
500-ml Erlenmeyer flask
Refrigerated tabletop centrifuge
37°C water bath
Glass microscope slides
Microscope with 1000× magnification
Multipipet trays
24- and 96-well tissue culture plates
Multipipettor and tips
Plastic wrap (e.g., Saran)
96-well plates with V-shaped wells

Additional reagents and equipment for indirect immunofluorescence (*UNIT 4.3*) and freezing cells (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

NOTE: The following protocol is written for one sample. It is not recommended to handle more than four samples in parallel.

Day 1: grow transformed bacterial strain

1. Streak a glycerol stock of an *E. coli* strain 803 clone containing the mammalian expression vector onto an LB agar/ampicillin plate. Grow overnight at 37°C.

   E. coli strain 803 (also termed 1106) might be more efficient than other E. coli strains in producing stable protoplasts (Rassoulzadegan et al., 1982).

   The antibiotic has to be adapted to the prokaryotic selection marker of the mammalian expression vector.

2. Grow myeloma cells in DMEM supplemented with 10% (v/v) FCS in 25-ml cell culture flasks. Aim at a high cell density of ~1×10^6 cells/ml that is reached on day 3 (protoplast fusion requires 5×10^6 cells per sample).

Day 2: grow transformed bacterial cultures

3. Inoculate 2 ml LB medium prewarmed at 37°C in a 12-ml polypropylene tube with a single *E. coli* colony from the freshly streaked LB agar/ampicillin plate. Add 2 µl of 50 mg/ml ampicillin. Grow for 4 hr at 250 rpm in a 37°C bacterial shaker.

4. Dilute 100 µl of cell culture into a 500-ml Erlenmeyer flask with 100 ml of LB medium and 100 µl of 50 mg/ml ampicillin. Grow to an OD_{600} of ~0.6. Start checking the optical density after 3 hr.

5. After OD_{600} ~0.6 is reached, add 200 µl of 60 mg/ml chloramphenicol to a final concentration of 120 µg/ml. Grow overnight at 250 rpm in a 37°C bacterial shaker.

   Plasmids carrying the colE1 origin of replication can be amplified in the presence of chloramphenicol (Hershfield et al., 1974).

Day 3: harvest bacterial cells and form protoplasts

6. Transfer the overnight culture into two 50-ml conical polypropylene centrifuge tubes and centrifuge 10 min at 2500 × *g*, 4°C.

7. In the meantime, prewarm 20 ml DMEM supplemented with 10% sucrose and 10 mM $MgCl_2$ in a 50-ml polypropylene tube in a 37°C water bath.

8. Pour off the supernatants of the spun culture.

Prepare protoplasts

9. From here on proceed in a sterile laminar flow bench. Vortex and combine the two bacterial pellets in 2.5 ml ice-cold 20% sucrose in 50 mM Tris·Cl, pH 8.0.

10. Add 500 µl ice-cold 1 mg/ml lysozyme in 250 mM Tris·Cl, pH 8.0, mix gently (swirl), and incubate 5 min on ice.

11. Add 1 ml ice-cold 250 mM EDTA, pH 8.0, gently swirl, and store on ice 5 min.

12. Add 1 ml ice-cold 50 mM Tris·Cl, pH 8.0, gently swirl, and incubate 10 min at room temperature. During this incubation period, mount 10 µl of the sample on a glass microscope slide and analyze protoplast formation under a microscope.

    A microscope with 1000× magnification is required to distinguish between spherical protoplasts and rod-shaped bacteria. At the end of incubation, ~90% protoplasts should be formed.

13. Add 20 ml DMEM supplemented with 10% sucrose and 10 mM $MgCl_2$ very slowly to the protoplast preparation. To do this, swirl protoplasts gently, start adding drops of DMEM supplemented with 10% sucrose and 10 mM $MgCl_2$, and slowly increase the added volume.

    The prepared protoplasts are fragile and need to be handled with care. Protoplast lysis is indicated by an increasing viscosity of the preparation due to the release of genomic DNA. The preparation can be analyzed using a microscope as above.

14. Add 40 µl of 10 mg/ml DNase I and incubate 15 min at room temperature.

    Addition of DNase I reduces the viscosity of the protoplast preparation.

Prepare for fusions

15. In the meantime, prewarm the following in separate 50-ml propylene tubes in a 37°C water bath:

    15 ml DMEM
    10 ml DMEM supplemented with 10% (v/v) FCS
    50 ml DMEM supplemented with 10% (v/v) FCS.

    Thaw at room temperature:

    2 ml PEG 1500 in DMEM supplemented with DMSO.

16. Centrifuge protoplast preparation in the 50-ml polypropylene tube 30 min at 2500 × *g*, room temperature.
17. In the meantime, transfer 5×10^6 mouse BALB/c myeloma cells into 15-ml conical polypropylene centrifuge tubes and centrifuge 10 min at 500 × *g*, room temperature. Aspirate the supernatant and resuspend the cells in 5 ml prewarmed DMEM.

    The myeloma cell preparation should be serum-free.

    Other myeloma cell lines that have been transfected by protoplast fusion or electroporation include mouse P3-X63Ag8.653, mouse Sp2/0-Ag14, mouse NSO, and rat YB2/0 (Gillies et al., 1989; Nakatani et al., 1989; Bebbington et al., 1992; Shitara et al., 1994).

18. Pour off the supernatant of the centrifuged protoplast pellet.

    The protoplast pellet should have a smooth surface.

Carry out fusions

19. Slowly layer the myeloma cell preparation on top of the protoplast pellet in the 50-ml conical polypropylene centrifuge tube. Centrifuge 10 min at 500 × *g*, room temperature.
20. Aspirate the supernatant. Mix cell and protoplast pellet by hand-flicking the tube and tapping it on the benchtop.
21. Add 2 ml PEG 1500 in DMEM supplemented with DMSO. Resuspend the pellet by pipetting up and down several times.
22. After addition of the PEG solution (~1 to 2 min), very slowly add 10 ml prewarmed DMEM. To do this, swirl protoplasts gently, start adding drops of DMEM, and slowly increase the added volume.
23. Add 10 ml prewarmed DMEM supplemented with 10% FCS, swirl gently, and centrifuge 10 min at 500 × *g*, room temperature.
24. Aspirate the supernatant, resuspend the pellet in 50 ml prewarmed DMEM supplemented with 10% FCS, and add 100 µl of 50 mg/ml kanamycin.
25. Pour into a multipipet tray and distribute among five 96-well tissue culture plates by adding 100 µl/well using a multipipettor. Wrap tissue culture plates in plastic wrap and incubate for 48 hr in a 37°C, 10% CO_2 humidified incubator.

Day 5: select transfected cells

26. After 48 hr, prepare selection medium by adding 10 ml prewarmed 50 mM L-histidinol to 40 ml prewarmed DMEM supplemented with 10% FCS. Add 100 µl of 50 mg/ml kanamycin. Pour into a multipipet tray and add 100 µl/well using a multipipet. Rewrap tissue culture plates in plastic wrap and continue incubation in a 37°C, 10% CO_2 humidified incubator.

    The antibiotic, here L-histidinol, has to be adapted to the eukaryotic selection marker of the mammalian expression vector. Only transfected myeloma cells will survive the treatment with L-histidinol.

27. Examine plates visually for clones ~10 days after selection medium is added.

    No medium change or any other treatment is necessary during this time

Identify IgSF-CAM expressing clones

28. Once a clone becomes clearly visible by eye, analyze it for the expression of IgSF-CAM by indirect immunofluorescence staining (*UNIT 4.3*). Transfer ≤50% of the cloned cells to a well of a 96-well plate with V-shaped wells and perform indirect

immunofluorescence analysis with a polyclonal anti-IgSF-CAM and a fluorescein-conjugated secondary antibody. Identify clones that are expressing IgSF-CAM.

29. Expand positive clones into 24-well tissue culture plates.
30. Subclone positive clones by limiting dilution in 96-well tissue culture plates.
31. Maintain positive subclones in selection medium, e.g., 5 mM L-histidinol in DMEM supplemented with 10% FCS. Store backup cells frozen in liquid nitrogen using standard procedures (*UNIT 1.1*).

Cells from positive subclones are used in myeloma cell aggregation assays.

BASIC PROTOCOL 5

Detecting *Cis*-Interactions between IgSF-CAMs by Chemical Cross-Linking

The following basic protocols describe methods for the detection of *cis*-interactions, i.e., interactions between proteins that reside in the same membrane. In the chemical cross-linking methods described in this protocol, bifunctional reagents are used to establish covalent cross-bridges between associated proteins. The covalently linked protein complexes are then analyzed by SDS-PAGE (*UNIT 6.1*) and immunoblots (*UNIT 6.2*), using specific antibodies. In the antibody-induced co-capping methods (see Basic Protocol 6), a hypothesized *cis*-interaction between two cell surface proteins is evaluated by inducing a redistribution of one molecule and testing whether the putative binding protein follows. For both methods, the cells analyzed should be cultured at low density, in order to prevent contact between cells. Under these conditions, close associations of proteins are only possible between proteins residing in the membrane of the same cell.

Chemical cross-linking joins two molecules by means of a cross-linking reagent. The method critically depends on the cultivation of the cells of interest as single cells at low density to avoid the formation of cell-cell contacts. Moreover, the structural integrity of the cells should be maintained throughout the procedure. The use of hydrophilic, membrane-impermeable bifunctional cross-linking reagents restricts the cross-linking to extracellular domains of membrane proteins. Considering the close spatial association between proteins interacting within the same membrane, a high degree of specificity of chemical cross-linking is mandatory.

The specificity of chemical cross-linking is mainly determined by the chemical reactivity of the functional groups of the cross-linking reagent and the length of the spacer separating the reactive groups. The *N*-succinimidyl group combines efficient reactivity with a high selectivity for primary amino groups, thereby limiting the cross-linking to lysine side chains at the surface of proteins engaged in interactions. Since most IgSF-CAMs contain multiple lysine residues in their extracellular domains, the amino-group-specific homobifunctional *N*-succinimide-derivatives are suitable reagents for the detection of *cis*-complexes formed between such molecules. The following protocol will primarily focus on the application of hydrophilic homobifunctional di-*N*-succinimidyl derivatives with relatively short (0.6 to 1.2 nm) spacer sequences separating the reactive groups. In order to enhance the specificity of the chemical cross-linking reaction to stably associated proteins, lateral movement in the cell membrane is reduced by performing the cross-linking reaction on ice. Ideal spacer length of the cross-linking reagent, optimal concentrations of cross-linkers, and reaction times strongly depend on the specific system that is analyzed and must be evaluated empirically. The parameters described below turned out to be optimal in many experimental situations and represent a good starting point for further optimizations. After quenching the cross-linking reaction, cells are lysed and the cross-linked complexes ideally isolated by immunoprecipitation (*UNIT 7.2*) using specific antibodies against the molecule of interest. Immunoprecipitates are separated by SDS-PAGE and cross-linked complexes can be detected by immunoblot analysis. The presence of

known binding partners within the cross-linked complexes can be addressed by immunochemical techniques, which require only low amounts of proteins. However, immunochemical analysis clearly restricts the detection of cross-linked binding partners to known molecules against which antibodies are available. Scaling-up of the procedure may result in the isolation of sufficient amounts of cross-linked material for subsequent microsequencing allowing the detection of novel binding partners.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Materials

Cells of interest growing in tissue culture at low density
PBS with Ca^{2+}/Mg^{2+} (see recipe)
100 mM cross-linking reagent (see recipe):
Bis(sulfosuccinimidyl)suberate (BS^3) in water
Disuccinimidyl tartrate (DST) in water-free DMSO
Disulfo disuccinimidyl tartrate (Sulfo-DST) in water
3, 3′-Dithiobis(sulfosuccinimidyl propionate) (DTSSP) in water
5 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS
1 M glycine solution in water, pH 8.0
Lysis buffer (see recipe)
Primary antibody: serum, purified immunoglobulin, or purified immunoglobulin immobilized on agarose or Sepharose matrix
Protein A or protein G coupled to agarose or Sepharose matrix (optional)
Wash buffer (see recipe)
Sample buffer for SDS-PAGE (*APPENDIX 2A*)
10-cm tissue culture dishes precoated with poly-D-lysine combined with additional substrates, such as laminin (see Support Protocols 10 and 12)
Horizontal shaker
Cell scraper
2-ml microcentrifuge tubes
End-over-end rotator (model 750)
100- or 200-µl and 500-µl Hamilton syringe and 22-G needle
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblot analysis (*UNIT 6.2*), and immunoprecipitation (*UNIT 7.2*)

Prepare cells

1. Prior to the experiment, seed cells onto 10-cm tissue culture dishes at a low density. Plate slowly dividing (division rate of <1 division in 48 hr) or nondividing cells, e.g., primary neurons, 12 to 16 hr prior to the experiment at a density of 10^4 cells per cm^2 on cell culture dishes precoated with poly-D-lysine (Support Protocol 10) combined with additional substrates, such as laminin (see Support Protocol 12).

   Fast dividing cells, especially fast-growing cell lines, are problematic in this experiment as extensive contacts are formed between the progeny of cell divisions. With COS or HEK 293 cells, cultures of mainly single cells can be obtained by plating the cells 4 to 6 hr prior to the experiment on poly-D-lysine coated cell culture dishes in a density of 1-2 × 10^4 cells per cm^2. For the final passage of cells, do not trypsinize cells. Most cell lines can easily be detached when cultured on untreated cell culture plastic by incubation for a few minutes in 5 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS at room temperature. Care must be taken to generate single-cell suspensions without any cell aggregates by careful trituration using a large-bore

pipet tip. Glass pipets (Pasteur pipets) are not recommended, since the sharp glass edges and the strong shearing forces damage the cells resulting in the formation of clumps and aggregates.

2. Use one 10-cm tissue culture dish (containing ~10^6 cells) for each reaction. In the first set of experiments, include three different concentrations of cross-linking reagent and three to four different reaction times, resulting in a total of nine to twelve individual samples.

3. Prior to the experiment, check cells for homogenous distribution under an inverted microscope that allows the examination of living cells. Do not use cultures containing aggregates or clumps of cells.

4. Remove culture medium and wash the cells three times with PBS containing Ca^{2+}/Mg^{2+}. Add 10 ml PBS with Ca^{2+}/Mg^{2+} and incubate 5 to 10 min on ice.

   Incubation on ice not only reduces lateral movements of proteins within the cell membrane but also decreases endocytosis of membrane proteins.

   The cells are living and therefore, during this and subsequent steps, 5 through 8, are very fragile, especially primary neurons that have the tendency to detach spontaneously during the procedure. All steps must be carried out with the utmost care to protect the cultures from mechanical stress.

Cross-link cells

5. During the incubation period of step 4, freshly prepare 10 ml each of 0.1 mM, 1 mM, and 10 mM cross-linker solution per dish in PBS with Ca^{2+}/Mg^{2+}, using the 100 mM DST stock solution in water-free DMSO.

   The concentration of the cross-linking reagent is a very critical parameter. Most published protocols use concentrations of ~1 mM for efficient cross-linking. For a first set of experiments, the authors recommend 0.1 mM, 1 mM, and 10 mM for the reagents DST and DSSP.

   The cross-linker solution in PBS with Ca^{2+}/Mg^{2+} is unstable at room temperature and should be kept on ice for ≤5 to 10 min prior to adding to cells.

6. Remove PBS from cells and add the prechilled cross-linker solution carefully to the cells. Carry out the cross-linking reaction on ice with gentle shaking (20 to 30 rpm) on a horizontal shaker.

   Tilt cell culture dish slight and add cross-linker solution at the edge of the dish with a pipet. Distribute the cross-linker solution carefully over the entire surface of the dish.

Quench reaction and lyse cells

7. Quench the reaction after 0, 5, 15, and 45 min by adding 0.5 ml of 1 M glycine solution, pH 8.0 (final glycine concentration is 50 mM). Perform the quenching reaction for 15 min.

   The reaction time is a critical parameter regarding specificity and yields of chemical cross-linking. Quenching of the reaction in an initial experiment after 0, 5, 15, and 45 min results in a "kinetic profile" of the cross-linking reaction. This "kinetic profile" is often valuable, not only for the optimization of the reaction time as a parameter but also in the interpretation of the results.

8. Completely remove the reaction mixture from the cells without detaching them and add 2 ml of lysis buffer per dish. Remove cells from the culture dish with a cell-scraper and lyse them for 30 min on ice on a horizontal shaker.

9. Transfer cell lysate to 2-ml microcentrifuge tubes and microcentrifuge 15 min at 12,000 to 14,000 rpm, 4°C.

This centrifugation step clears the cell lysates from nuclei and cell debris. Still, the cleared lysates frequently have an opaque appearance. The lysates can be frozen and kept for several weeks at −20°C.

Immunoprecipitate cross-linked proteins

10. In 2-ml microcentrifuge tubes, prepare the primary antibody for immunoprecipitation.

    Primary antibodies for immunoprecipitation can be used in the form of whole serum, purified immunoglobulins, or coupled to an agarose or Sepharose matrix (for coupling of purified immunoglobulins to cyanogen bromide agarose or Sepharose see Support Protocol 1).

    Prior to the addition of the cell lysate, preincubate whole serum with Sepharose-coupled protein A (ideal for rabbit antisera) or protein G (for goat or sheep antisera) in a ratio serum/protein A/G Sepharose of 2:1 and purified immunoglobulins with protein A/G matrix in a ratio of 5 μg immunoglobulin/μl matrix for 30 min at room temperature. For an efficient immunoprecipitation ~10 to 20 μg of specific immunoglobulin are required. A total matrix volume between 10 and 20 μl is recommended.

11. Add the cleared cell lysate to the primary antibody and rotate for 4 to 16 hr end-over-head at 4°C.

12. Microcentrifuge samples for 1 min at 14,000 rpm, 4°C and carefully remove the supernatant; keep supernatant and store at −20°C.

13. Resuspend matrix in 1 ml wash buffer, vortex for 20 sec, and microcentrifuge 30 sec at 14,000 rpm, 4°C. Repeat the wash step three times and carefully remove the supernatant after final wash step. Remove the residual fluid from the matrix pellet with a 100- or 200-μl Hamilton syringe without aspirating matrix material.

14. Elute material bound to the matrix in 50 to 100 μl sample buffer for SDS-PAGE by boiling 5 min. Microcentrifuge samples for 1 min at 14,000 rpm, room temperature.

    After boiling, the protein sample in SDS-PAGE sample buffer can be stored several months at −20°C or years at −70°C.

15. Analyze protein samples by SDS-PAGE (*UNIT 6.1*) and detect the cross-linked complexes by immunoblot analysis (*UNIT 6.2*).

Detecting *Cis*-Interactions between IgSF-CAMs by Antibody-Induced Co-Capping

BASIC PROTOCOL 6

The lateral mobility of integral membrane proteins within the plane of the cell membrane allows the induction of large clusters of molecules by cross-linking their extracellular moieties with antibodies. This phenomenon is termed "antibody-induced capping". Interactions between two cell surface proteins in the plane of the same membrane (*cis*-interactions) can be addressed by examining the effects of antibody-induced capping of one molecule on the distribution of the other molecule. Reciprocal co-capping or co-distribution of two molecules after induction of capping with antibodies against one of them strongly indicates a (direct or indirect) *cis*-interaction. The following protocol describes a basic method for the induction of capping by antibodies and the detection of co-capping by fluorescence microscopy.

Cells of interest are cultivated at low density to avoid the formation of extensive cell-cell contacts. Incubation with the primary antibody against the first molecule (A) is performed on intact, live cells in the cold to reduce lateral movement of membrane proteins. Following the incubation with primary antibody against molecule A, unbound antibody is removed. Bound primary antibody is detected by a fluorochrome-conjugated secondary

antibody against the animal species in which the anti-A primary antibody was raised. Subsequently raising the temperature to 37°C restores the lateral mobility of proteins in the cell membrane and results in the formation of clusters of molecule A due to antibody-mediated cross-linking. The cells are washed, fixed, and counter-stained with a primary antibody against the putative binding partner of molecule A (molecule B). To this end, the primary antibody against molecule B must be raised in a different species than the anti-A antibody. The anti-B antibody is detected by a secondary antibody conjugated to a different chromophore than the secondary antibody against molecule A. Care should be taken that the two antibody combinations do not cross-react. Further, the emission wavelengths of the chromophores coupled to the secondary antibodies must be distinct to allow a complete separation of the signals by the use of narrow band-pass filters in the subsequent examination by immunofluorescence microscopy. Once the conditions for antibody-induced capping and for observing specific immunolocalization have been established, co-localization of the fluorescence signals elicited by the secondary antibodies allows the detection of co-clustering of molecules A and B on the cell surface. Quantitative analysis of the localization of the fluorescent signals requires a spatial resolution not normally achieved by standard light microscopes. To this end, due to its superior resolution power, confocal laser scanning microscopy represents a powerful technique for the quantitative assessment of co-distribution induced by antibody-induced capping.

Materials

Cells of interest growing in tissue culture (e.g., nondividing cells or primary neurons)
Cell culture medium (used for the cells of interest) without serum
PBS (see recipe)
Hank's Balanced Salt Solution (HBSS; *APPENDIX 2A*)
HBSS/1% FCS: HBSS containing 1% (v/v) fetal calf serum (FCS)
Primary antibodies (serum or purified immunoglobulin) for molecules A and B
Secondary antibodies of the appropriate sources coupled to different fluorescent dyes
4× Fixative solution (see recipe)
Vectashield mounting medium for fluorescence (H-1000, Vector Laboratories)
10-cm tissue culture dishes
12-mm no. 1 round glass coverslips, sterilized by autoclaving or soaking in 70% ethanol for 1 hr and precoated with poly-D-lysine (see Support Protocol 10)
Watchmaker's forceps
24-well tissue culture plates
Microscope slides
Nail polish
Fluorescence microscope with 63× and/or 100× oil immersion objectives

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Prepare cells

1. One day prior to the experiment, seed cells at low density onto 10-cm cell culture dishes, each containing 15 to 20 sterilized poly-D-lysine coated 12-mm round glass coverslips. Plate nondividing cells or primary neurons 12 to 16 hr prior to the

experiment at a density of 2×10^4 cells per cm^2 and fast-growing cell-lines 8 to 12 hr prior to the experiment at a density of 10^4 cells per cm^2.

These cell densities should result in ~10% confluency at the time of the experiment.

Coating of glass coverslips with poly-D-lysine is always recommended, in order to prevent cell detachment during the procedure.

2. Two to 4 hr prior to the experiment, transfer the glass coverslips with watchmaker's forceps from the 10-cm cell culture dish to wells 24-well tissue culture plates containing 1 ml cell culture medium per well. Incubate at 37°C

Aseptic technique is recommended, but not mandatory.

3. After 2 to 4 hr of incubation, remove the culture medium and wash the cells three times with 1 ml HBSS at room temperature, each time by adding 1 ml of HBSS, incubating 1 min, and aspirating wash solution. Add 200 µl HBSS/1% FCS per well and incubate the 24-well plate for 10 min on ice.

Do not let the cells dry throughout the procedure. The incubation with HBSS/1% FCS on ice will block potential unspecific sites for antibody adsorption. Incubation on ice not only reduces lateral movements of proteins within the cell membrane but also decreases endocytosis of membrane proteins.

The cells are living and therefore, during this and subsequent steps are very fragile, especially primary neurons that have the tendency to detach spontaneously during the procedure. Carry out all steps with the utmost care to protect the cultures from mechanical stress.

Prepare and bind primary antibody for molecule A

4. Prepare the appropriate dilutions of primary antibody and control antibody to molecule A in HBSS/1% FCS in microcentrifuge tubes and centrifuge for 1 min at maximum speed. Transfer supernatants to new tubes and put primary antibody solutions on ice ≥5 min prior to use.

For co-capping experiments the primary antibodies are generally applied at higher concentrations than for standard immunofluorescence protocols. Titrate optimal antibody concentrations for each experimental system individually. As a rule, dilute affinity-purified antibodies to a range of 5 to 20 µg/ml and antisera between 1:20 and 1:200. If using a commercially available antibody, multiply the concentration recommended for immunofluorescence staining by a factor of five. For a first set of experiments, try a range of dilutions and test for potential cross-reactivity of the primary antibodies in advance. Add negative controls to ensure specificity of antibody-induced capping. To this end, preimmune serum or purified antibodies from preimmune serum are required.

Antibody dilution is a critical parameter regarding cross-reactivity: this phenomenon may occur at the high concentrations that are used for co-capping experiments even if absent at concentrations based on own experience or recommended by manufacturers for standard immunofluorescence protocols.

5. Remove HBSS/1% FCS from cells and add 200 µl of cold anti-A antibody in HBSS/1% FCS to each well. Incubate with the primary anti-A antibody for 30 min on ice.

Prepare and bind secondary antibody for molecule A

6. Dilute fluorochrome-conjugated secondary antibody in HBSS/1% FCS in microcentrifuge tubes and centrifuge for 1 min at maximum speed. Transfer supernatants to new tubes and place secondary antibody solutions on ice ≥5 min prior to use.

Commercially available preparations of secondary antibodies must be tested for cross-reactivity with the primary and secondary antibodies against molecule B in advance. Even

if manufacturers assure that no such cross-reactivity occurs, consider that the concentrations of secondary antibodies used in co-capping experiments are well above those normally applied for immunofluorescence staining. Typical dilutions of commercial preparation are between 1:20 and 1:200 (~5 to 10 times higher than for standard immunofluorescence applications).

7. Aspirate primary antibody solution and wash cells two times with ice-cold HBSS. Add 250 µl/well of secondary antibody and incubate for 30 min on ice. Protect from light.

8. Remove the solution containing the secondary antibody and wash the cells two times with ice-cold HBSS. Add 200 µl of ice-cold cell culture medium without serum and put the 24-well cell culture plate for 30 min in a 37°C, 5% CO_2 humidified incubator.

   Never use HBSS or PBS for this incubation step. The phosphate buffer system present in HBSS and PBS is insufficient to buffer the pH efficiently in a 5% CO_2 atmosphere. The pH would drop too much and lead to false results. Always use the cell culture medium normally applied for the cell cultures of interest without serum. When stored on ice, keep the cell culture medium in air-tight tubes.

Fix cells

9. Add 66.7 µl of 4× fixative solution to each well and allow cells to fix for 15 µin at 37°C in the dark.

   A frequent source of experimental artifacts in co-capping experiments is fixation. It is necessary to block any further lateral movement of the capped proteins after fixation. The fixation in culture medium with 2% formaldehyde/0.1% glutaraldehyde results in total immobilization of membrane proteins without permeabilization of the cell membrane (for a detailed discussion, see Dubreuil et al., 1996).

10. Aspirate fixative and wash cells two times with PBS. Remove the PBS after the second wash step and add 500 µl PBS/1% FCS. Incubate for 15 min at room temperature.

Prepare and bind primary antibody for molecule B

11. Prepare dilutions of the primary antibody for molecule B in PBS/1% FCS as described in step 4.

    For the counter-staining (here of molecule B), the primary antibodies are generally applied in concentrations comparable to standard immunofluorescence protocols. Titrate optimal antibody concentrations for the counter-staining in a way that similar intensities of fluorescence signals result for both molecules. As a rule, dilute affinity-purified antibodies in a range of 1 to 10 µg/ml, purified Fab fragments 5 to 20 µg/ml, and antisera between 1:100 and 1:1000.

    If using a commercially available antibody, the concentration recommended for immunofluorescence staining is a good starting point for the optimization of the counter-staining. For a first set of experiments, try a range of dilutions and test again for potential cross-reactivity of the antibodies in advance. Add negative controls to ensure specificity of the counter-staining. In addition to preimmune serum or purified antibodies from preimmune serum, include further primary antibodies against molecules for which no co-capping with molecule A is expected in the experiment. The specificity of co-capping is the most critical issue in the whole experiment.

12. Remove the PBS/1% FCS and add 200 µl/well of diluted primary anti-B antibody in PBS/1% FCS. Incubate 1 hr at room temperature. Protect from light with aluminum foil or place 24-well plate in a drawer.

Prepare and bind secondary antibody for molecule B

13. Dilute the secondary antibody against anti-B antibody in PBS/1% FCS as described in step 6.

    If commercially available preparations of secondary antibodies are used for the counter-staining, dilutions between 1:100 and 1:500 are recommended in most cases. These concentrations are similar to those used for standard immunofluorescence applications. The issue of cross-reactivity with the primary and secondary antibodies against molecule B is much less critical than in case of the antibody used for capping but have to be tested anyway to use the system in reverse order to detect reciprocal co-capping (see below).

14. Remove primary anti-B antibody solution and wash cells two times with PBS/1% FCS. Add 200 µl/well secondary anti-B antibody in PBS/1% FCS and incubate 45 min at room temperature. Protect from light.

Fix cells

15. Wash cells two times with PBS/1% FCS and one time in PBS only. Add 250 µl of 1× fixative solution (2% formaldehyde/0.1% glutaraldehyde in PBS) and let cells fix for 5 min at room temperature in the dark.

16. Remove fixative and wash cells two times with PBS.

    The samples can be stored protected from light for up to 1 day at 4°C.

Mount and examine cells

17. Label microscope slides and place 1 drop of Vectashield mounting medium onto slide. Carefully remove each coverslip from the 24-well plate with watchmaker's forceps and blot excess fluid by touching edge with a paper towel. Invert coverslip, cell-side down onto mounting medium. Do not apply pressure. Blot excess mounting medium with a paper towel and allow slides to dry 5 min at room temperature in the dark. Seal around the rim of the coverslip with nail polish.

18. Examine specimen on a standard fluorescence microscope using a 63× or 100× oil immersion objective.

    Due to its superior resolution power, confocal laser scanning microscopy represents the preferred technique for the quantitative assessment of capping and co-capping.

Neurite Outgrowth Assay

BASIC PROTOCOL 7

This protocol describes a method to quantify the growth of neurites from cultured neurons. Neurite outgrowth assays are used to determine a potential role of an IgSF-CAM as a substratum for neurite outgrowth. The authors describe here the use of embryonic chicken sensory neurons (Stoeckli et al., 1991), however depending on the substratum to be tested and the responsiveness of the cells, other neurons such as tectal, cerebellar, and spinal cord neurons, may be used as well. It is important that for these assays the neurons are plated at a very low density in order to prevent contact between neurons and their axons, as well as contact between neurons and non-neuronal cells. Only then does the measured neurite length reflect the neurite outgrowth-promoting activity of the substratum used.

Many IgSF-CAMs were found to be potent neurite outgrowth-promoting substrates for various types of neurites. This protocol is an example of the one used for low-density cultures of dorsal root ganglia neurons on an IgSF-CAM substrate. Low-density cultures (Stoeckli et al., 1991, 1996), rather than explants or high-density cultures, are used to minimize effects derived from cell-cell contacts. To achieve reproducible results for growth assays use serum-free, chemically defined media wherever possible.

Many commercially available software packages (e.g., NIH Image, Metamorph) provide convenient methods for neurite length measurements. The goal of this protocol, therefore, is not the explication of a particular method of neurite length measurement, but rather the discussion of basic requirements and principles that need to be fulfilled to obtain reproducible values.

Materials

Chicken embryos (E8 to E10)
0.5% (v/v) glucose in PBS
0.25% (w/v) trypsin in PBS without Ca^{2+}/Mg^{2+} (Life Technologies)
Serum-free culture medium (see recipe)

Tissue culture dishes coated with substrate of choice (see Support Protocol 8)
Sterile dissecting tools
15-ml centrifuge tubes
Fire-polished Pasteur pipet, ~0.3-mm diameter bore
Neubauer chamber for cell counting (Fig. 1.1.1)
35-mm cell culture dishes
Image analysis software and required equipment
Additional reagents and equipment for counting cells (*UNIT 1.1*)

Dissect dorsal root ganglia and process cells

1. Coat 35-mm tissue culture dishes with appropriate substrate according to Support Protocol 8, 9, 10, 11, or 12.
2. Dissect dorsal root ganglia (DRG) from 10-day-old chicken embryos (Sonderegger et al., 1985) with sterile dissecting tools and collect DRGs in a noncoated 35-mm tissue culture dish on ice in 1 ml of 0.5% glucose in PBS.

   Younger embryos can also be used, but the dissection before E8 requires more skills and practice.
3. Transfer DRG to a 15-ml centrifuge tube and centrifuge 3 to 5 min at 300 to 500 × *g*, room temperature.
4. Carefully remove supernatant and add 2 ml of 0.25% trypsin solution to pellet. Resuspend ganglia and incubate 25 min in 37°C water bath.
5. Centrifuge DRG for 3 to 5 min at 300 to 500 × *g*, room temperature, decant trypsin solution supernatant, and add 1 ml serum-free culture medium.
6. Use a fire-polished Pasteur pipet with an opening of ~0.3-mm diameter to mechanically dissociate (triturate) the ganglia. Continue until no cell clumps are visible by eye.

   Less than ten passages should be sufficient to get a single cell suspension.

Count and plate cells

7. Count an aliquot of the cell suspension in a Neubauer chamber (*UNIT 1.1*).
8. Obtain a cell suspension dilution of 150,000 cells/ml. Plate 1 ml of cell suspension per 35-mm cell culture dish.
9. Incubate cultures for 24 to 30 hr in a 37°C, 5% CO_2 humidified incubator.

   Axons reach their maximal length after 24 to 30 hr. However, growth rate and onset of axon growth are substratum-dependent.

Measure and count neurites

10. At the appropriate time, measure neurite length for neurites extending from single cell bodies without contact to other neurites (see Fig.9.5.1 and legend).

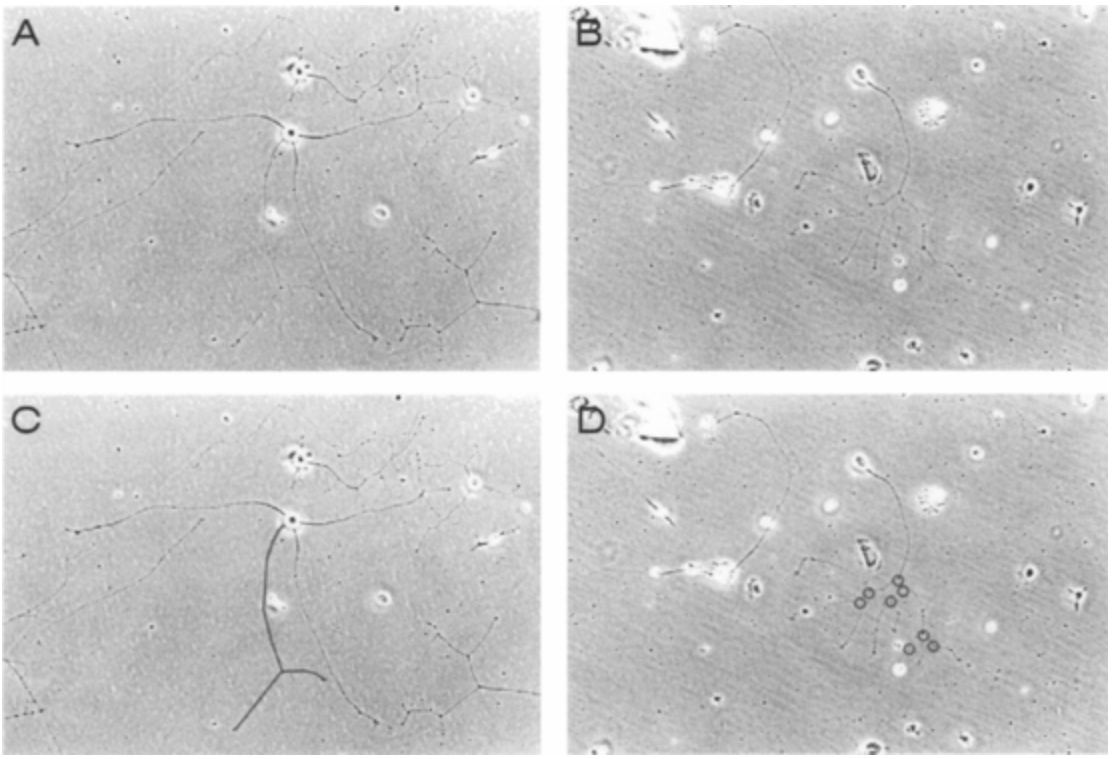


Figure 9.5.1 In order to be a valid assessment of the neurite outgrowth-promoting qualities of a test substrate, the evaluation of neurite outgrowth has to be carried out according to strict standards. Culture conditions have to be reproducible and identical. Therefore, chemically defined media should be used rather than serum containing media. Even minor changes in the composition of media can dramatically alter growth characteristics and the morphology of neurons (e.g., Savoca et al., 1995). The main criteria in the evaluation of neurite growth-promoting qualities of a test substratum is neurite length. However, keep in mind that only neurite lengths of single axons and not bundles of axons should be measured, as the latter would reflect a combination of neurite outgrowth promoted by the substratum and neurite growth along axons. Neurite length can be assessed either as length of the longest neurite (**C**) or as total neurite length of a neuron, in which case the lengths of all neurites of a neuron are added up and represented as one value. Different means can be used to measure the length of a neurite. The most convenient way is the use of a computerized system where one can trace the neurite with the joy stick and the computer will automatically determine its length. Make sure that the software allows the inclusion of the length of the side branches, as they have to be included into the measurements. The comparison between the neurons shown in (**A**) and (**B**) clearly demonstrates that the branching of a neurite is an important trait characteristic for a given substratum. The clear morphological differences between the neurons shown in (**A**) and (**B**) are reflected in the length plots (see Fig. 9.5.2). While the curves for the length of the longest neurite and the total length of all neurites of a neuron would greatly differ for neurons such as the one shown in (**A**), they would be superimposed for neurons like the one shown in (**B**). In other words, a strong deviation of the two curves indicates that neurons have, on average, multiple neurites, whereas a small deviation indicates that the majority of the neurons have only one neurite. Another way to quantify the morphological differences between neurons is the counting of branch points per neurite as shown in (**D**). While the neuron shown in (**A**) has 0 to 2 branch points per neurite, the neuron shown in (**B**) has 8 branch points (**D**).

A prerequisite for reliable neurite length measurements are low-density cultures of sufficient and reproducible quality. Care has to be taken to avoid the measurement of neurite fascicles rather than single neurites, as this confuses the results.

Fasciculation as well as cell-cell contact positively influences neurite length. Thus, for accurate determination of the neurite outgrowth-promoting capacity of a particular substrate or experimental condition, only single neurites extending from single cell bodies without contact to other neurites and especially without contact to non-neuronal cells should be included in the experiment. Cell numbers and culture conditions have to be chosen accordingly.

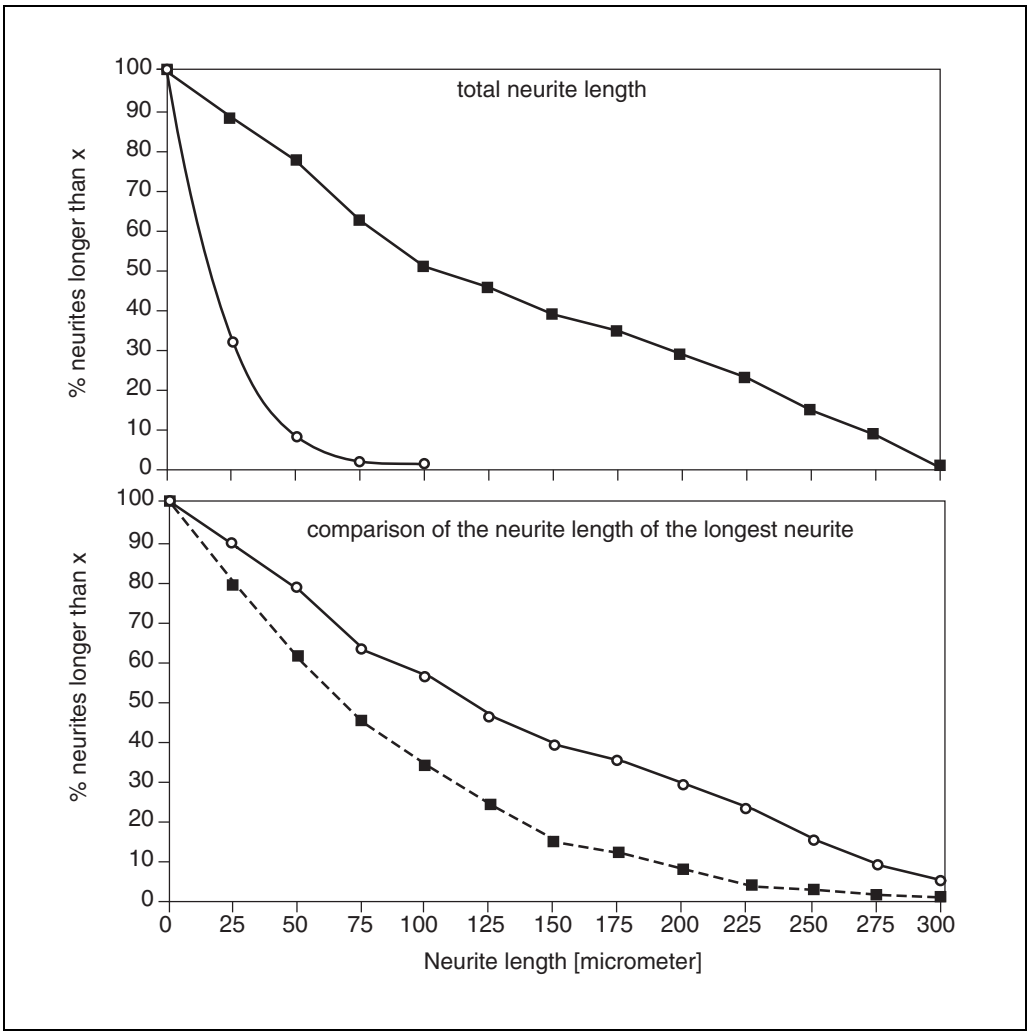


Figure 9.5.2 Graphic representation of neurite length. The neurite outgrowth-promoting activity of different IgSF-CAMs compared to each other or compared to a control protein can be represented in the type of plot that was introduced by S. Chang and colleagues (1987). In these plots the total length of all neurites extending from a neuron can be shown. The two curves in (**A**) represent a good (solid line) versus a poor substrate (dashed line). The same type of plot can also be used to represent a difference in neurite morphology (**B**). When the two curves representing the length of the longest neurite of a cell (dashed line with squares) and the total length of all neurites of a given cell (solid line with dots) differ a lot from each other, the neuron has most likely several long neurites, whereas cells with only one long neurite have plots where the two curves are almost the same.

11. Count the number of neurites per neuron, number of branches, and branching order.

    Very often the morphology of neurites is substratum-specific. Therefore, the total neurite length per neuron may only give you a partial account of the differences between distinct substrates. Therefore, additional information should be collected, such as number of neurites per neuron, number of branches per neurite, and branching order (i.e., appearance of secondary or even tertiary branches).

12. Analyze the growth cone. Measure the area, number of filopodia, and length of filopodia.

    Sometimes the analysis of the growth cone morphology is crucial. Characteristics, such as growth cone area, number of filopodia, or length of filopodia, can change dramatically depending on the substrate used. However, the culture conditions must be controlled carefully, because many of these characteristics can vary depending on the culture medium

as well as on the substratum used. Also, carefully control the origin of the neurons: only neurons from the same embryonic age can be compared. Neurites taken from adult animals can show completely different growth characteristics compared to embryonic neurons. This is especially true with respect to the dependence of neurons for trophic factors. Ideally, serum-free, chemically defined media are used in comparative studies of neurite length and morphology, as the content and concentration of serum components is subject to considerable batch-to-batch variability.

13. Represent the quantitative results.

A very common graphic representation of neurite length is the plot introduced by Chang et al. (1987). In these graphs the percentage of neurons with a total neurite length longer than x is easily visible (Fig. 9.5.2). Similarly, the length of the longest neurite per cell or the number of branches can be represented. The comparison between the curves for total neurite length per neuron and the length of the longest neurite can be used as a graphical representation of the difference between neurons with predominantly one neurite that is very long and neurons with many neurites per neuron that amount to the same total length when added together (Fig. 9.5.2B).

Inhibiting CAM-CAM Interactions In Vitro

BASIC PROTOCOL 8

One way to investigate the function of a particular protein is the analysis of changes resulting from blockage of function. This can be done with several methods both in vivo and in vitro. Whereas in vivo experiments are far more difficult and complex to perform, they more likely reflect the true function of the particular CAM because all parameters are the same, all binding partners are present, all interactions are possible, and even the most difficult parameter to test experimentally, the factor time, can be taken into account. However, there are also disadvantages; in vivo assays are far more complicated and time-consuming than in vitro studies. Most in vivo studies in vertebrates are done in "knock-out" mice, whereby homologous recombination in embryonic stem cells, a gene of interest is replaced by a construct that selects for the cells that have lost the expression of this gene. These "knock-out" mice can be analyzed for phenotypes resulting from the lack of expression of the gene of interest. While this is certainly a very potent method to analyze the function of CAMs, there are several major drawbacks; it is time-consuming, expensive, labor-intensive, and requires appropriate facilities with specially trained experimenters.

Furthermore, as the analysis of several knock-out lines has shown in the past, some mutations result in early embryonic lethality, in which case the organ of particular interest may not have developed by the time of death. This is especially true, when the function of a particular gene is to be analyzed in the nervous system. Alternatively, the lack of one gene may be compensated for, in which case, phenotypes are not detectable or only minor changes occur that are detectable only by very detailed analyses that often exceed the potential of one lab.

An alternative to these genetic loss-of-function analyses is an in vivo study where the blockage of function is done at the protein level (e.g., Stoeckli and Landmesser, 1995; Perrin and Stoeckli, 2000). Due to the relatively easy accessibility of the chicken embryo, it is the model of choice for this type of study. Obviously in contrast to the in vivo experiments described above, this is only possible for functional studies during embryonic development. Although less expensive and less dependent on an appropriate animal facility, in vivo studies using chicken embryos as a model system are time-consuming and require a considerable level of technical skills and training.

A description of in vivo loss-of-function studies would exceed the scope of this unit. For that reason, only in vitro methods for inhibition of IgSF-CAM function will be discussed.

The blockage of IgSF-CAM function in cultures of dissociated cells or tissue explants is much less complex than the inhibition of IgSF-CAM function in vivo; however, the results obtained in vitro may not reflect or only partially reflect the function of a particular IgSF-CAM in vivo. The advantages of in vitro experiments are: they are easier to perform, less time-consuming, and more suitable to study a particular question, due to lower degree of complexity. Assays as the one described in this protocol were used to study the role of NgCAM (Chang et al., 1987) and axonin-1 (Stoeckli et al., 1991) in the fasciculation of dorsal root ganglia axons.

Explants of intact dorsal root ganglia (DRG) are grown on a collagen substrate. Under these culture conditions axons extend from the explants in a radial fashion in the form of fascicles. The role of NgCAM as a candidate molecule mediating the axon/axon interaction under these conditions has been tested by the addition of Fab from anti-NgCAM antibodies to the culture medium.

Materials

10-day-old chicken embryos (E10)
0.5% (w/v) glucose in PBS
Chemically defined, serum-free cell culture medium (see recipe)
Control Fab
Fab against CAM of interest

15-ml centrifuge tubes
8-well-slide cell culture dishes (e.g., LabTek, Life Technologies) coated with IgSF-CAM (see Support Protocol 8)
Pasteur pipet or automatic pipettor with 200-µl tips

1. Dissect DRG from E10 embryos, collect in 0.5% glucose in PBS on ice.
2. Transfer DRG to a 15-ml centrifuge tube, and centrifuge for 3 to 5 min at 300-500 × *g*, 4°C or room temperature.
3. Decant supernatant and resuspend pellet in 1 ml serum-free cell culture medium by tapping the vial on the bench and swirling the tube.
4. Prepare 8-well-slide cell culture dishes coated with IgSF-CAM according to Support Protocol 8 by filling the wells with 300 µl medium containing no Fab, control Fab, or Fab against CAM of interest in different concentrations.

   Antibody concentrations ranging from 100 to 500 µg/ml for polyclonal and 10 to 500 µg/ml for monoclonal IgGs have been found useful for in vitro studies.

5. Plate DRG in the smallest possible volume either by using a Pasteur pipet or an automatic pipettor with a 200-µl tip.

   The DRG should be carefully placed on the surface of the dish rather than just put in the medium to facilitate attachment to the substratum. Take care not to scratch the coated surface of the cell culture dish.

   Carefully avoid shear forces that can destroy the ganglia. For inexperienced investigators, it is best to use an automatic pipettor set at 20 µl.

6. Grow DRG for 40 hr in a 37°C, 5% CO_2 humidified incubator.
7. Fix DRG as described in Support Protocol 13 by adding fixative directly to the culture medium to avoid detachment of the ganglia.

COATING OF CULTURE DISHES

To provide a suitable substratum for neurite growth, tissue culture dishes are coated with components of the extracellular matrix, such as laminin (see Support Protocol 12) or collagen (see Support Protocol 11). Alternatively, IgSF-CAMs can be used as substratum by either coating them directly onto tissue culture plastic (see Support Protocl 8) or on top of a nitrocellulose layer (see Support Protocol 9). The application of a nitrocellulose layer prior to coating of the protein substrate (Lagenaur and Lemmon, 1987; see Support Protocol 9) is useful to increase the coating efficiency. When glass has to be used instead of tissue culture plastic, precoating with nitrocellulose can be necessary. For coating with ECM components such as collagen (see Support Protocol 11) or laminin (see Support Protocol 12), glass is an acceptable surface even without precoating.

NOTE: All solutions and equipment must be sterile, and proper sterile technique should be used accordingly. Use filtration to sterilize solutions.

Coating with IgSF-CAM

SUPPORT PROTOCOL 8

Purified IgSF-CAMs can be used to coat culture surfaces for neurite growth assays. As purified IgSF-CAMs are usually available in limited amounts, draw a ~0.5-cm^2 circular area near the center of a 35-mm dish with a lab marker on the bottom outside surface of the dish. Then apply the protein to be tested to the marked area of the dish only. Coat the rest of the dish with bovine albumin (Albumax). This configuration provides better optical access to the cells than a 24 well plate. It also allows comparison of the growth-promoting effect of the protein of interest with that of bovine serum albumin in the same dish.

Materials

Protein to be coated
PBS (see recipe)
10 mg/ml bovine serum albumin (e.g., Albumax, Life Technologies) in PBS

1. Dissolve the proteins in PBS at concentrations of 10 to 100 µg/ml.

   Dilute protein solutions should not be stored for extended periods of time, no more than a few days at 4°C and never frozen. If freezing is required, add 100 µg/ml BSA as a carrier protein. Check for possible interference with the assay.

2. Pipet 20 µl protein solution per 0.5-cm^2 area of the culture dish and spread over marked area or use sufficient solution to cover entire surface.

   If glass is used, the volume per area should be doubled to reach sufficient dissipation of liquid.

3. Incubate for 2 hr in a 37°C humidified incubator.
4. Aspirate protein solution and rinse the entire surface of the dishes three times with PBS.
5. To saturate the protein adsorbance capacity of the tissue culture plastic or glass coverslip, incubate dishes for 30 min with a 10 mg/ml BSA solution. Cover the entire surface of the dish (1 ml per 35-mm culture dish).

   The use of BSA purified as fraction V from bovine serum is not advised, as most batches contain contaminations of endotoxins that are detrimental for cell survival, especially for neurons. The bovine serum albumin sold as Albumax from Life Technologies has given very satisfactory results.

6. Rinse dishes three times with PBS. Remove PBS immediately before plating cells. For reproducible results, do not allow the coated proteins to dry.

   Dishes should be coated immediately before use; storage is not recommended.

SUPPORT PROTOCOL 9

Pre-Coating Glass Surfaces with Nitrocellulose

IgSF-CAMs do not adhere well to glass surfaces unless those surfaces have previously been coated with nitrocellulose.

Materials

Methanol
Nitrocellulose (e.g., BA-83, Schleicher and Schuell), 0.2-µm pore size
Coverslips, 22-mm diameter (preclean glass with acetone before coating, dry, and autoclave)

1. Dissolve a 5-cm^2 piece of 0.2-µm pore size nitrocellulose membrane in 17 ml methanol.

   Make sure membrane is completely dissolved. The membrane becomes translucent and is difficult to see. The solution has to be prepared immediately before use. Do not store.

2. Dilute 300 µl of nitrocellulose membrane/methanol solution with 3.7 ml sterile water to obtain a coating solution.
3. Coat 22-mm glass coverslips by incubating with 200 µl coating solution for 2 hr in a laminar flow hood. Remove excess coating solution and let coverslips dry before use. Do not store. Proceed with coating with IgSF-CAM (Support Protocol 8).

   The same procedure has been used to precoat Thermanox coverslips for electron microscopic use.

   Tissue culture plastic can be coated with undiluted nitrocellulose solution, using 450 µl of the stock solution per 35-mm culture dish.

SUPPORT PROTOCOL 10

Pre-Coating with Poly-D-Lysine

The neurite outgrowth-promoting capacity of laminin is enhanced by precoating culture surfaces with poly-D-lysine. Alternatively, poly-D-lysine coating alone provides a good substrate for attachment of neurons and nonneuronal cells (also see Basic Protocol 5).

Materials

0.5 mg/ml poly-D-lysine (see recipe)

1. Make a stock solution of 0.5 mg/ml poly-D-lysine in sterile 150 mM sodium borate, pH 8.4. For coating tissue culture plastic, prepare a working solution immediately prior to use.

   Stock solution may be stored for a few weeks at 4°C. The sodium borate buffer can be autoclaved or filtered before poly-D-lysine is added.

   Depending on the type of culture, the coating concentrations range between 10 and 500 µg/ml. Dilute the appropriate volume of the poly-D-lysine stock solution with sodium borate.

2. Incubate dishes with 1 ml coating solution per 35-mm cell culture dish overnight in a 37°C humidified incubator.
3. Aspirate coating solution and rinse dishes at least three times with sterile water. Incubate the dishes overnight with sterile water in a 37°C humidified incubator.

   The dishes can be used immediately or dried and stored for several weeks.

Coating with Collagen

SUPPORT PROTOCOL 11

Collagen is a good substrate for many non-neuronal cells, but can also be used for axon growth assays e.g., for motor neurons. For most neuronal populations, laminin is the better neurite outgrowth promoting substrate than collagen.

Materials

2 mg/ml collagen in 0.1% (v/v) acetic acid, sterile
35-mm cell culture dishes

1. Dilute a 2 mg/ml stock solution of collagen in 0.1% acetic acid to get a final concentration of 0.25 to 0.5 mg/ml.
2. Coat 35-mm cell culture dishes with 750 µl of 0.25 to 0.5 mg/ml collagen solution. Evaporate the solution by incubating the dishes at 60°C overnight or until dry.

The dishes can be kept for a few weeks in a dry place at room temperature.

Coating with Laminin

SUPPORT PROTOCOL 12

Laminin is a very potent substrate for most neuronal and non-neuronal cells. It can be coated directly to tissue culture plastic and glass surfaces. However, laminin reveals best neurite outgrowth-promoting capacities when coated on dishes precoated with poly-D-lysine (see Support Protocol 10).

Laminin should not be stored in diluted solutions. For best results, keep small aliquots at −20°C, thaw slowly at 4°C, and prepare coating solution immediately before use. Use 750 to 1000 µl of 10 to 20 µg/ml solutions of stock per 35-mm cell culture dish. Coating is done as described in Support Protocol 8 for IgSF-CAMs. Dishes coated with laminin can be stored.

FIXATION OF CELLS FOR IMMUNOHISTOCHEMICAL STAINING PROCEDURES USING FLUORESCENT ANTIBODIES

SUPPORT PROTOCOL 13

In order to avoid the detachment of axons or the collapse of growth cones, the fixative should be added directly to the culture medium. For staining procedures involving fluorescent antibodies, glutaraldehyde can only be used in a very limited concentration, as it is autofluorescent. A concentration of 0.1% (v/v) is compatible with the use of fluorescent secondary antibodies. However, some antigens do not tolerate fixation by glutaraldehyde.

Materials

Paraformaldehyde solution (see recipe)
Cell culture medium

Add 350 µl of concentrated paraformaldehyde solution to 1 ml cell culture medium to get a final concentration of 2% paraformaldehyde and 0.1% glutaraldehyde. Fix cells for 30 min to 1 hr at 37°C or 2 hr to overnight at 4°C.

FIXATION FOR MORPHOLOGICAL ANALYSIS, NEURITE LENGTH MEASUREMENTS, AND FOR IMMUNOHISTOCHEMISTRY WITH NON-FLUORESCENT SECONDARY ANTIBODIES

If the autofluorescence of glutaraldehyde does not matter, higher concentrations than 0.1% (v/v) can be used. Glutaraldehyde has a higher crosslinking activity than formaldehyde and therefore, is the fixative of choice for good preservation of morphology. Final concentrations of up to 1% can be used. Add ⅓ vol 4× fixative solution (see recipe) to culture medium. Fix cells 30 min to 1 hr at 37°C or 2 hr to overnight at 4°C. For non-neuronal cells that are more strongly attached to the culture dish than neurons with axons, aspirate the culture medium and add 1× fixative solution.

NOTE: The preservation of antigens may restrict the use of high concentrations of glutaraldehyde, even if not used in combination with fluorescent secondary antibodies.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking solution (for amino bead coupling)

0.5 g bovine serum albumin (Fraction V; Sigma)
0.02% (w/v) NaN_3
50 mM Tris, pH 8.0, to 100 ml
Sterilize by filtering through a 0.45-µm filter
Store up to 1 month at 4°C

BSA solution, 0.5% (w/v)

0.5 g bovine serum albumin (Fraction V; Sigma)
0.02% (w/v) NaN_3
PBS (see recipe) to 100 ml
Sterilize by filtering through a 0.45-µm filter
Store up to 1 month at 4°C

Ca^{2+}/Mg^{2+}-free buffer (CMF buffer)

137 mM NaCl
4 mM KCl
0.4 mM $Na_2HPO_4 \cdot 2\ H_2O$
0.18 mM KH_2PO_4
12 mM $NaHCO_3$
11 mM glucose, pH 7.2
Filter sterilize through 0.22-µm filter
Store <2 weeks at 4°C

$CaCl_2$ solution

250 mM $CaCl_2$
Dissolve in ultrapure water
Sterilize by filtering through a 0.22-µm filter
Store at room temperature

Cell culture medium for HEK 293 cells

DMEM containing:
2 mM glutamine
2.4 g/liter sodium bicarbonate
10 mM HEPES, pH 7.5
10% (v/v) FCS
Filter sterilize through 0.45-µm filter
Store no more than a few days
Warm to 37°C before adding to cells

Chemically defined, serum-free medium

MEM containing
5 mg/ml lipid-rich bovine serum albumin with 0.1% IgG (e.g., AlbuMAX I, Life Technologies)
2 mM L-alynyl-L-glutamine or dipeptide glycyl-L-glutamine (e.g., GlutaMAX I, Life Technologies)
100 µg/ml transferrin
10 µg/ml insulin
20 ng/ml triiodothyronine
40 nM progesterone
200 ng/ml corticosterone
200 µM putrescine
60 nM sodium selenite
20 ng/ml nerve growth factor (NGF)
Prepare fresh

4-chloro-1-naphthalene solution

Stock solution: Dissolve 4-chloro-1-naphthalene in ethanol to give a 3 mg/ml solution. Store several weeks at −20°C.

Working solution: Immediately before use, dilute stock solution in TBS (*APPENDIX 2A*) and add 30% H_2O_2 to give final concentrations of 0.5 mg/ml chloronaphthalene and 0.01% H_2O_2 in TBS.

Cross-linking reagents

100 mM stock solution of cross-linker in water (BS^3, Sulfo-DST, and DTSSP) or water-free DMSO (DST). Stocks in DMSO can be stored at −20°C under dry nitrogen or argon for 1 to 2 months.
Bis(sulfosuccinimidyl)suberate (BS^3, Pierce)
Disuccinimidyl tartrate (DST, Pierce)
Disulfo disuccinimidyl tartrate (Sulfo-DST, Pierce)
3, 3′-Dithiobis(sulfosuccinimidyl propionate) (DTSSP, Pierce)

These reagents can be stored under water-free nitrogen or argon for up to 1 year at–20°C.

Working solution: Dilute stock solution of cross-linker in PBS with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} to a concentration of 1 mM. Prepare fresh and do not keep for >1 hr on ice.

Fixation solution, 4×

40 ml concentrated paraformaldehyde solution (see recipe)
5 ml 20× PBS
0.4 ml 50% (v/v) glutaraldehyde (reagent grade) in water
H_2O to 50 ml

For best results, prepare fresh. If necessary, the fixative can be stored 2 to 3 days at 4°C.

HBS solution

50 mM HEPES
1.5 mM Na_2HPO_4
140 mM NaCl
Dissolve in ultrapure water
Adjust pH to 7.05 with 1 M NaOH or 1 M HCl
Sterilize by filtering through a 0.22-µm filter
Store for months at 4°C

L-histidinol, 50 mM

Dissolve 535 mg L-histidinol dihydrochloride (Sigma) in 50 ml of 100 mM HEPES, pH 7.4, pass through a 0.22-µm filter, and store in aliquots for up to 1 year at −20°C.

LB medium

Dissolve 10 g NaCl, 10 g bacto-tryptone (Difco), and 5 g yeast extract (Difco) in 1 liter water, autoclave, and store at 4°C.

LB/ampicillin agar plates

Dissolve 15 g bacto-agar in 1 liter LB medium (see recipe) by autoclaving, cool down to 50°C, add 1 ml of 50 mg/ml ampicillin, mix, and pour into 10-cm polystyrene plates. Let sit at room temperature overnight and store protected from light at 4°C.

Lysis buffer

1% (w/v) CHAPS
0.1% (w/v) SDS
50 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
150 mM NaCl
5 mM EDTA
10 µg/ml leupeptin
10 µg/ml aprotinin
10 µg/ml pepstatin A
1 mM PMSF

Protease inhibitors except PMSF are stable in lysis buffer at −20°C for several months. However, PMSF has to be added fresh. Prepare a 100 mM PMSF stock solution in isopropanol and <5 min before use add to lysis buffer to a final concentration of 1 mM. PMSF stock solution can be stored several weeks at −20°C.

Mounting medium

10 ml 1M Tris·Cl, pH 9.0 (*APPENDIX 2A*)
70 ml glycerol (70% v/v final)
5.09 g *n*-propyl-gallate (0.24 M final)
H_2O to 100 ml
Adjust pH to 9.0
Make 1-ml aliquots and store at −20°C

Paraformaldehyde solution, 10% (w/v)

Add 5 g paraformaldehyde to 45 ml water, add 75 µl of 1 M NaOH and dissolve by heating to 60°C in a water bath. Let solution cool to room temperature, add water to a final volume of 50 ml, check that pH does not exceed 7.4. For best results prepare fixative immediately before use. Do not store >2 to 3 days at 4°C.

PBS

8.00 g NaCl (137 mM final)
0.20 g KCl (2.7 mM final)
2.16 g $Na_2HPO_4 \times 7\ H_2O$ (8 mM final)
0.20 g KH_2PO_4 (1.5mM final)
990 ml H_2O
Adjust pH to 7.4 with 1 N HCl or 1 N NaOH
Adjust volume to 1 liter with water
Filter sterilize through a 22-mm filter
Store at 4°C

PBS with Ca^{2+}/Mg^{2+}

8.00 g NaCl (137 mM)
0.20 g KCl (2.7 mM)
2.16 g $Na_2HPO_4 \times 7\ H_2O$ (8 mM)
0.20 g KH_2PO_4 (1.5 mM)
0.10 g $CaCl_2$ (0.9 mM)
0.10 g $MgCl_2 \times 6\ H_2O$ (0.5 mM)
990 ml H_2O
Adjust pH to 7.4 with 1 N HCl or 1 N NaOH
Adjust volume to 1 liter with water
Filter sterilize through a 0.22-mm filter
Store at 4°C

PEG 1500 in DMEM supplemented with DMSO

Melt 42.5 g polyethylene glycol 1500 (PEG 1500) in a microwave oven, add 50 ml DMEM and 10 ml DMSO, pass through a 0.22-μm filter, and store in aliquots up to 1 year at –20°C.

Poly-D-lysine solution

Dissolve 0.5 mg/ml poly-D-lysine (Sigma) in sterile 150 mM sodium borate, pH 8.4. Store a few weeks at 4°C.

Wash buffer

0.1% (w/v) CHAPS
0.1% (w/v) SDS
50 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
150 mM NaCl
5 mM EDTA
1 mM PMSF
Add <5 min before use

COMMENTARY

Background Information

IgSF-CAMs have been characterized as crucial molecules for both the development and the normal function of multicellular organisms. They exert their function by mediating the interactions between cells under various circumstances. Interactions mediated by IgSF-CAMs may determine whether a cell remains where it is or whether it moves somewhere else; they may be involved in determining the path of cell migration and the time and the location of the end of the migratory path. Other IgSF-CAMs have been implicated in processes such as the extravasation of white blood cells, inflammation, wound healing, as well as tumor invasion and metastasis. In the developing nervous system, IgSF-CAMs play important roles in cellular migration, in the regulation of axonal outgrowth and pathfinding, and in synaptogenesis. In the adult nervous system, IgSF-CAMs play a role in the regulation of neural plasticity and in nerve regeneration after injury. Defective

genes of IgSF-CAMs have been found to result in severe cerebral malformations and mental retardation.

Recent advances in the characterization of IgSF-CAMs have revealed that in several cases IgSF-CAMs not only mediate cell-cell adhesion by binding to each other, but that they are often capable of eliciting intracellular signals upon binding an extracellular ligand. Intracellular signals elicited by extracellular ligand contact of IgSF-CAMs comprise many signal pathways, including the production of second messengers and the activation of transmembrane or intracellular kinases and phosphatases.

The enormous complexity of binding partners is the most striking difference between the calcium-dependent cell adhesion molecules and the calcium-independent cell adhesion molecules of the IgSF. Many IgSF-CAMs have been found not only to bind to their own kind in so-called homophilic interactions, but also to engage in several heterophilic interactions. Therefore, the determination of ligands plays a very important role in the characterization of IgSF-CAMs. Only by identifying the macromolecular binding partners of IgSF-CAMs can their functional roles in living organisms be elucidated. Some of these very basic studies demonstrating interactions between IgSF-CAMs and other binding partners may be applicable to the study of cell surface molecules in general, including molecules specifically expressed in the immune system, virus receptors, tumor growth markers, or growth factor receptors. However, the analyses of the specific functional roles of these proteins is beyond the scope of this unit. For these, the reader is referred to more specialized applications.

Trans-interactions

IgSF-CAMs are composed of at least one Ig fold, which is also the molecular building block of antibodies (Rader and Sonderegger, 1998). It is therefore conceivable to express IgSF-CAMs in myeloma cells that are specialized in the production of antibodies. An expression system based on myeloma cells was established for the production of soluble lymphocyte-derived cell-surface receptor proteins by Traunecker et al. (1991). This system has also been used for the expression of IgSF-CAMs on the surface of myeloma cells (Rader et al., 1993). By studying the IgSF-CAMs in a natural environment, i.e., as membrane-bound molecules residing in their proper orientation in a biological membrane, *trans*-interactions between molecules residing in different membranes can be readily detected through cell aggregation. Nonadherent myeloma cells provide an established natural environment for these studies. The myeloma cell aggregation assay described here has contributed significantly to the elucidation of the complex interaction pattern of two IgSF-CAMs, axonin-1 and NgCAM, which were found to *trans*-interact homophilically and *cis*-interact heterophilically (Rader et al., 1993; Buchstaller et al., 1996; Rader and Sonderegger, 1998; Sonderegger et al., 1998). In addition, the myeloma cell aggregation assay can be used for the structure-to-function analysis of a *trans*-interaction. Monoclonal antibodies that have been mapped to particular epitopes on the IgSF-CAMs can be analyzed for interference with myeloma cell aggregation. Defined mutants of IgSF-CAMs, e.g., engineered domain deletion mutants or pathological point mutations, can be expressed in myeloma cells and assessed for their effect on *trans*-interactions (Freigang et al., 2000). Furthermore, the influence of soluble molecules, e.g., ligands or toxins, on *trans*-interactions of IgSF-CAMs can be studied using myeloma cell aggregation. The expression in myeloma cells has also facilitated the production of soluble variants of IgSF-CAMs and fragments thereof (Rader et al., 1993, 1996).

Chemical cross-linking

Chemical cross-linking is a special form of chemical modification that results in the covalent coupling of molecules by a cross-linking reagent. In contrast to the simple conjugation of two molecules, cross-linking refers to the covalent coupling of two molecules that undergo an interaction with each other. The interacting molecules involved can be proteins, peptides, nucleic acids, or carbohydrates. Cross-linkers for proteins are bifunctional molecules containing two reactive groups that undergo reactions with side chains of amino acids. Cross-linkers can be generally classified into two main families, the homobifunctional cross-linkers, which contain two identical functional groups, and the heterobifunctional cross-linkers, which contain different types of reactive moieties. Simple homobifunctional cross-linkers, such as dialkyl halides and bis-imidoesters, were introduced in the 1950's. Since then >300 different cross-linking reagents have been synthesized and a large number of these are commercially available (Ji, 1983; Staros and Anjaneyulu, 1989). Recent developments led to the design and synthesis of cleavable bifunctional compounds that allow the recovery of the

individual compounds present within a cross-linked complex after its isolation.

Using bifunctional reagents for cross-linking of macromolecules, such as proteins, two different reaction products are generated resulting from either intramolecular or intermolecular cross-linking. For the study of protein-protein interactions as described in the protocol, intermolecular cross-linking is of interest. In the past, intermolecular cross-linking was instrumental in the investigation of antigen-antibody complexes, membrane protein structures, and protein-protein interactions at the quarternary structural level, e.g., in receptor-ligand or multienzyme complexes. The application of chemical cross-linking to identify membrane receptors for macromolecular ligands has become an established technique that can be successfully applied to the analysis of interactions between IgSF-CAMs (Staros, 1988).

The choice of reagents for cross-linking membrane proteins must take into consideration the particular physicochemical properties of biological membranes. A major problem is represented by the unspecific, random-collision dependent cross-linking, due to the lateral mobility of membrane proteins. Chemical cross-linking of membrane proteins should, therefore, generally be carried out in the cold to reduce lateral movements of proteins. A second major factor to be considered is the hydrophobicity of the membrane core. Hydrophobic and hydrophilic reagents have the potential to probe different regions of integral membrane proteins for interactions with binding partners. To study interactions between extracellular domains of IgSF-CAMs, hydrophilic, membrane-impermeable cross-linkers that bear hydrophilic or charged groups can be used (Buchstaller et al., 1996; Kunz et al., 1998). The limitation of cross-linking to closely associated binding partners within complexes of membrane proteins requires reagents with short spacers between their reactive groups. However, cross-linkers with spacer lengths of <0.5 nm result in poor yields of intermolecular coupling, whereas extensive cross-linking with decreasing specificity is generally observed with reagents with spacer lengths of >1.1 nm (Middaugh et al., 1983). The use of a homologous series of cross-linking compounds is recommended for the evaluation of the system.

For efficient cross-linking between extracellular domains of membrane proteins, two functional groups that differ in reactivity and chemical selectivity are predominantly used, (1) the *N*-succinimidyl group and (2) the photoactivatable aryl azide group. The *N*-succinimidyl group combines efficient reactivity with a high selectivity for primary amino groups, thereby limiting the cross-linking to lysine side chains and free N-termini of proteins. Disuccinimidyl derivatives of variable spacer length and hydrophilicity are currently commercially available. Popular hydrophilic disuccinimidyl reagents are the disuccinimidyl suberate (DSS) derivative bis(sulfosuccinimidyl)suberate (BS^3) with a spacer length of 1.14 nm, disuccinimidyl tartrate (DST) and its disulfo-variant disulfo disuccinimidyl tartrate (Sulfo-DST) with a spacer length of 0.64 nm. A water-soluble, membrane-impermeable, thiol-cleavable disuccinimidyl reagent with a spacer length of 1.2 nm is represented by 3, 3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP).

The aryl azide group undergoes a UV light–induced chemical reaction resulting in the generation of a nitrene, a highly reactive, short-lived intermediate containing a nitrogen electron sextet (aza-analogon to a carbene). Analogous to carbenes, the nitrenes react immediately with their direct environment. The major reaction of interest is the insertion into C-H and N-H bonds resulting in the formation of a new covalent bond. This broad specificity allows the nitrenes to react with virtually all chemical groups present on a protein surface, making this type of cross-linking extremely efficient. Common nitrene precursors are arylazides with absorbance maxima in the long UV region. Nitro-substitution of the aromatic ring results in a further shift of absorbance towards longer wave lengths, away from the absorbance maxima of proteins. Photosensitive heterobifunctional cross-linkers containing one amine-reactive *N*-succinimidyl group with the photoactivatable aryl azide group are widely used reagents for the cross-linking of membrane proteins with their receptors (Hermanson, 1996). Commercially available products of this group are *N*-hydroxysulfosuccinimidyl-4-azidobenzoate (Sulfo-HSAB, spacer length of 0.9 nm) and the highly versatile thiol-cleavable reagent sulfosuccinimidyl 4-(*p*-azidophenyldithio) propionate (Sulfo-SADP, with a spacer length of 1.39 nm). For protocols regarding the use of these photoreactive cross-linkers see Jung and Moroi (1983) and Wood and O'Dorisio (1985).

Co-capping

The detection of *cis*-interactions by antibody-induced co-capping is based on the lateral mobility of integral membrane proteins within

the plane of the cell membrane. The freedom of lateral movement allows the induction of large clusters of these molecules at the cell surface by cross-linking their extracellular moieties with antibodies, so-called capping. Molecules that undergo (direct or indirect) interactions with the capped molecules in the plane of the cell membrane are co-clustered and exhibit co-capping when examined by immunofluorescence microscopy.

Antibody-induced co-capping has been an established technique, in particular in the field of immunology, for the last decades and was instrumental for the detection of many interactions of leukocyte surface molecules that are involved in antigen presentation and recognition (Rojo et al., 1989; Zhou et al., 1993). The co-capping approach allows detection of interactions between membrane proteins at the level of individual intact cells. The availability of novel fluorescent dyes allows the detection of three or four different fluorochromes simultaneously. This allows the examination of the cellular distribution of, for example, effector molecules of signal transduction or cytoskeleton components as a function of co-clustering of cell surface proteins by a combination of classical co-capping protocols with intracellular immunofluorescence staining.

Classical biochemical techniques, for example co-immunoprecipitation or chemical cross-linking (see Basic Protocol 5), require a homogeneous sample. In contrast, co-capping studies examine interactions between proteins at the level of individual cells and can, therefore, be performed on heterogeneous samples, like mixed primary cell cultures derived from animal tissue, if appropriate markers are available that allow one to distinguish between different cell types. A further major advantage is the requirement for only a limited number of cells for co-capping studies. This opens the possibility to study specific, rare cell types, which can be isolated only in small quantities. A powerful combination is represented by the prior isolation of a specific cell type, e.g., by fluorescence-activated cell sorting (FACS), using appropriate combinations of cell surface markers with subsequent co-capping studies.

The crucial prerequisite of the co-capping approach is the availability of suitable antibodies. This limits application of the technique to already described molecules against which antibodies are available. In addition, the antibodies used must meet the following criteria, (1) absence of any cross-reaction with other cell surface proteins on the cells of interest; and (2) no interference of antibody binding with the interaction that is studied (this point often represents an unknown factor that may influence the result). In addition, the two primary antibodies used must be raised in two different species.

Critical Parameters and Troubleshooting

Preparation of IgSF-CAMs

For the functional analysis of an IgSF-CAM, it is extremely important to have a highly purified protein sample. Due to the high complexity of their interaction pattern, a contamination of the protein sample to be analyzed by another IgSF-CAM could confound the results of binding assays (see Basic Protocols 2 and 3) or neurite outgrowth assays (see Basic Protocol 7). For this reason, protein samples purified with affinity columns should always be analyzed by SDS-PAGE followed by silver staining to confirm the absence of contaminating proteins. When possible contaminants are known, for instance based on their high expression level in the tissue used as a source for the purification, or when the absence of a particular IgSF-CAM is important for the experimental procedure, their absence can best be documented by immunoblot analysis of the purified fractions. As a quick alternative, dot blots described in Support Protocol 4 can be used. If contaminants are found, the following possibilities should be considered.

Affinity column was not rinsed properly after loading or has run dry. Make sure absorbance is back to baseline before starting elution. Take an aliquot from the wash fraction to confirm the absence of protein. It is extremely important that the column never runs dry.

Antibody used to make column is not specific or antibody is denatured because column is too old or was not stored properly. When the quality of the purified protein samples decreases, it is time to prepare a new affinity column. It is possible that the antibody is very sensitive to high pH conditions used for elution. Try an elution buffer with low pH (0.1 M glycine/HCl, pH 2.7) as an alternative. Change concentration and/or type of detergent used.

Because of the large number of IgSF-CAMs expressed in the nervous system and based on the enormous complexity of their interactions, the specificity of the antibodies used for function-blocking assays and the purity of IgSF-CAM preparations have to be tested carefully. It is important to include appropriate controls,

such as the use of preimmune IgG purified according to the same protocols as the specific IgGs to rule out toxicity of the antibody preparations. Assess the dose-dependence of the effect. A very convincing control for the specificity of the assay is the absence of an effect in the presence of a specific antibody against another IgSF-CAM that is expressed by the same cell.

Transfection

The most critical parameter determining the efficiency of the calcium phosphate protocol described is the 293 cells that are used. Cells of low passage numbers (not more than 40 passages) exhibit a much higher transfection efficiency than later passages.

The formation of the precipitate prior to the addition to cells is mainly influenced by the following parameters: concentrations of calcium, phosphate, and DNA, the nature of the DNA construct, pH, temperature, and time of incubation. The calcium and phosphate concentrations given in this protocol are optimized for this system. Optimal DNA concentration depends on the construct and must be determined for each system individually. A good starting point for optimizations when dealing with medium-sized plasmids of 5 to 10 kb is 40 to 80 μg DNA. Plasmids of different sizes vary considerably in behavior in calcium phosphate transfection. The protocol described works best for average sized expression plasmids (5 to 10 kb). Very large plasmids (>30 kb) frequently result in the formation of much larger precipitates, reducing the efficiency of transfection considerably. A reaction at pH 7.0, room temperature (20° to 25°C), and 1 min reaction time are good starting conditions for optimizations. The formation of precipitate after mixing $CaCl_2$ solution with HBS solution can be monitored by measuring the absorbance at 320 nm (e.g., for the testing of new batches of solutions).

After addition to the cells, the stability of the DNA-containing calcium phosphate particles is the most critical factor for the efficiency of transfection. One source of instability is a reduction in medium pH due to the metabolic activity of cells. It is therefore important to include 10 mM HEPES (final concentration) in the 293 cell culture medium. The reduction of the CO_2 partial pressure to 3% is another option to raise the pH value, if necessary. Increased time of exposure to the precipitate can further enhance the efficiency of transfection. An incubation time of 4 to 6 hr given in the protocol is a good starting point and can be extended up to 24 hr in cases where no cytotoxicity is observed. However, the combination of exposure to the precipitate with an osmotic shock, e.g., by adding 10% (w/v) glycerol to the cell culture medium, as suggested by some authors for the calcium phosphate transfection of Chinese hamster ovary (CHO) cells, is not recommended for 293 cells.

Immunoblotting

No positive dots. The concentration of the protein of interest may be below the detection limit. Use repeated applications of protein solution to the nitrocellulose membrane. Apply the purified protein onto the membrane as a positive control. If the total protein concentration of the solution applied to the membrane is high, even repeated applications of protein solution to the membrane may not give a satisfactory result.

High background. Make sure that the volumes of protein solution applied to the membrane are small. Let the dot dry before the next step. Wash membranes more thoroughly. Change the blocking solution and/or blocking time. For instance, try blocking with serum from the same species that was used to raise the secondary antibody.

Microsphere assays

False negative results in the bead aggregation assay. Coupling reaction results in protein orientation on the bead surface in a way that binding sites are not accessible. Alternatively, proteins could not be physiologically active after purification (e.g., elution after immunoaffinity column).

False positive results in the bead aggregation assay. Protein preparations that are not pure and contain another CAM could result in false positive binding results. Furthermore, if each of the two test proteins has homophilic binding properties, weak unspecific aggregation of preformed homophilic aggregates could result in false positive results with respect to heterophilic interactions. Therefore, check for unevenly distributed beads in mixed aggregates under the microscope (bead distribution in aggregates is not detectable in a flow cytometer).

Discrepancies between results of bead and cell aggregation assays. The orientation of proteins on the beads is likely to be random and not uniform as in a biological membrane. This has to be considered when interpreting and comparing the results with the ones of the cell aggregation assay. Furthermore, the orientation problem has to be taken into account when

analyzing *cis*- and *trans*-interactions (Kuhn et al., 1991; Buchstaller et al., 1996; Rader et al., 1996; Sonderegger et al., 1998).

Orientation of proteins on beads can be better controlled when using proteins with domains or tags that tightly bind to a linker protein that is coupled first to the microsphere. For example, recombinant Fc-fusion proteins can be coated on protein A-conjugated beads in an oriented manner. One has to consider that this linkage includes a noncovalent protein interaction.

Trans-interactions

The stably transfected myeloma cell clones may differ from the parental myeloma cell line in more than only the expression of the IgSF-CAM. The upregulation of endogenous cell adhesion molecules, such as integrins and IgSF-CAMs, can result in myeloma cell aggregation (Kawano et al., 1991). It is therefore essential to run a series of control experiments that address the question whether any cell aggregation is caused by a *trans*-interaction of the expressed IgSF-CAMs or by other factors. For this, follow these guidelines:

1. Confirm the functional surface expression of the IgSF-CAM using, for example, monoclonal antibodies directed against conformational epitopes.
2. Each aggregation assay should be performed in duplicate with cross-wise exchange of the fluorescent dyes.
3. Myeloma cell clones that express an IgSF-CAM should not form mixed aggregates with the parental myeloma cell line.
4. Independent myeloma cell clones that express the same IgSF-CAM should give the same aggregation pattern.
5. Independent myeloma cell clones that express different quantities of the same IgSF-CAM should be analyzed for a correlation between expression and aggregation.
6. Pre-incubation with polyclonal Fab directed to the IgSF-CAM should prevent aggregation.
7. Pre-incubation with an enzyme that removes the IgSF-CAM selectively from the surface should prevent aggregation. A very useful enzyme for this is phosphatidylinositol-specific phospholipase C, which selectively cleaves glycosyl-phosphatidylinositol-anchored proteins.

Chemical cross-linking

The application of cross-linking reagents on intact, live cells may result in possible artifacts due to the perturbation of the architecture of the cell membrane by the chemicals. Very often, the reagents are used at millimolar or higher concentrations (0.1 to 10 mM) and must be dissolved in organic solvents prior to use. It is therefore very important to assure that the reagents by themselves and especially organic solvents, if used, do not affect the structural integrity and viability of the cells tested. Water-soluble reagents like BS^3, Sulfo-DST, and DTSSP that carry sulfonyl groups can be used to circumvent the solvent problem.

Successful detection of *cis*-interactions between IgSF-CAMs requires a high specificity of chemical cross-linking on the one hand, and sufficient yields of cross-linked materials on the other hand, for subsequent biochemical characterization of cross-linked partners. As described above, the specificity and efficiency of the cross-linking reaction is determined by the following parameters: length of the spacer separating the reactive groups; chemical reactivity of the functional groups of the cross-linking reagent; concentration of cross-linking reagent; and reaction time.

Cross-linking reagents with short spacers between the reactive groups such as DST and Sulfo-DST restrict chemical coupling to closely associated molecules and are therefore preferable for the detection of *cis*-complexes between membrane proteins. However, due to the spatial proximity of their functional groups, these reagents exhibit an enhanced tendency for intramolecular cross-linking, i.e., coupling lysine side chains of the same molecule with each other, which may result in very low yields of cross-linked material. The application of reagents with longer spacers between the reactive groups such as BS^3 and DTSSP generally results in higher yields, but bears the risk of unspecific reactions. As previously discussed, the chemical reactivity of the functional groups present in commercially available cross-linking reagents range from highly selective, like the *N*-succinimidyl group, to rather unselective photoactivated groups, like aryl azides, which generate highly reactive, unstable intermediates that undergo reactions with a wide variety of chemical structures within a protein. Although the *N*-succinimidyl group is most frequently used for modifications and cross-linking of cell surface proteins, its selective reactivity with primary amino groups (mainly lysine side chains) excludes this class of cross-linkers from extended hydrophobic interfaces through which IgSF-CAMs may interact. Upon photoactivation, reagents containing aryl azide groups exhibit a high reactivity towards ali-

phatic hydrocarbon groups, like RCH_2R and R_2CHR, that allows cross-linking within a strongly hydrophobic environment. However, the high reactivity and low selectivity of photoactivated aryl azides frequently results in a high degree of unspecific cross-linking, not only among proteins, but also extensive protein-lipid cross-linking. For the detection of specific interactions among the closely associated proteins on the surface of intact cells, these reagents are, therefore, not recommended as first choice, but represent an option in cases where no cross-linking products are obtained using more specific reagents.

Optimal concentrations of cross-linking reagents must be evaluated empirically for every experimental system. A more detailed discussion of the chemical background can be found in Lomant and Fairbanks (1976), Lewis et al. (1977), and Smith et al. (1978). For homobifunctional *N*-succinimidyl derivatives, like DST and DSSP, optimal concentrations for cross-linking on intact cells are consistently in the range of 0.1 to 10 mM in published protocols. This range of concentrations represents a good starting point for optimizations. Cross-linking reagents with higher reactivity, like e.g., aryl azides, are generally applied in much lower concentrations, usually between 10 μM and 1 mM, for cross-linking on intact cells.

A further critical parameter is reaction time. Longer reaction times generally result in better yields of cross-linked products, but also in more unspecific reactions. Using homobifunctional *N*-succinimidyl reagents, quenching of the cross-linking reaction after 0, 5, 15, and 45 min results frequently in a "kinetic profile" of the process. Specifically, a different pattern of cross-linked products is observed during the time course of the cross-linking reaction. This reflects the tendency of many membrane molecules, such as IgSF-CAMs, to form oligomeric or even multimeric aggregates in the membranes of living cells. The appearance of initial cross-linked molecules, after a few minutes, is often followed by the appearance of further cross-linked complexes with higher molecular masses, generated from the coupling of the initial complexes with additional molecules. Initial cross-linked products that are generated within the first minutes of the reaction may correspond to the first assembly units from which larger oligomeric or multimeric complexes are formed. The "kinetic profile" of the cross-linking reaction may, therefore, give some information about the nature of the complexes or aggregates formed by molecules like IgSF-CAMs.

Co-capping

The demonstration of a *cis*-interaction by antibody-induced co-capping of cell surface molecules critically depends on many factors. To allow the induction of caps by antibody cross-linking and co-capping mediated by a *cis*-interaction, the molecules of interest must have a minimal lateral mobility within the plane of the cell membrane. In addition to mobility, the relative stoichiometry of the molecules of interest is critical for the detection of co-capping after antibody-induced capping. Ideally, comparable levels of expression at the cell surface allow reciprocal co-capping of two molecules that interact with each other in *cis* with sufficient affinity. However, large stoichiometric excess of one molecule due to different expression levels results in asymmetric results in reciprocal co-capping experiments; capping of the more abundant component is followed by clearly detectable co-capping of the less abundant molecule. In contrast, capping of the molecule expressed at lower level results in only partial co-capping of the more abundant component. For the detection of partial co-capping, confocal laser scanning microscopy is a powerful technique that allows the reliable detection of locally enhanced fluorescence signals (co-capping) versus a relatively high homogeneous background signal (uncapped monomers of the molecule present in stoichiometric excess).

In addition to the factors described above, the availability of specific primary antibodies against the molecules of interest, raised in different species, is an essential prerequisite for co-capping experiments. For antibody-induced capping, polyclonal or monoclonal antibodies can be used. Polyclonal antibodies are able to cross-link the cell surface antigen to some extent in the absence of secondary antibody, whereas in the case of monoclonal primary antibodies, cross-linking of bound primary antibody with the secondary antibody is required for the induction of caps. The most critical factors of every co-capping experiment is the specificity of the primary antibodies used. A major source of experimental artifacts is the potential of cross-reactivity of primary antibodies, either between the molecules of interest or with other, unidentified molecules expressed on the cell. It is therefore of pivotal importance to exclude any cross-reactivity under the conditions (antibody concentrations, temperature,

incubation times, etc.) used for co-capping and counter-staining. It is not recommended to test for cross-reactivity with other immunochemical techniques like immuno-blot or ELISA, because the antigens are presented in a different form on nitrocellulose and on plastic surfaces than on live cells. The absence of cross-reactivity of the partially or totally denatured proteins present in such immunochemical assays does, therefore, not necessarily exclude cross-reactivity of the native proteins present on the live cells under the conditions used for co-capping experiments. In situations where the proteins of interest are co-expressed in recombinant form in a cell type that does not normally express them, the test for cross-reactivity is straightforward. Specificity of detection can be checked by immunostaining of single transfectants and mock-transfected cells (as a negative control) with both antibodies (see, e.g., Buchstaller et al., 1996; Kunz et al., 1998). It should be noted that cross-reactivity of antibodies is a phenomenon that depends on the antibody concentrations used. Even in cases where no cross-reactivity is reported in the literature (e.g., based on experience with standard protocols for immunofluorescence), cross-reactivity may occur at the higher antibody concentrations that are normally applied in co-capping experiments. Cross-reactivity of the secondary antibodies represents only a minor problem, since highly specific preparations of fluorochrome-labeled secondary antibodies against a wide variety of species are commercially available. The use of phylogenetically more distant species is desirable since the potential of cross-reactivity between the secondary antibodies is lower. Combinations that are frequently documented in the literature are mouse/rabbit, mouse/goat (or sheep), rabbit/goat (or sheep). A considerable risk of cross-reactivity exists especially for the combination mouse/rat. In this case the choice of secondary antibodies has to be made with care.

Several controls must be included to ensure specificity of antibody-induced capping. The use of preimmune serum, or purified IgG from preimmune serum, is an essential negative control in cases where complete sera or total IgG fractions, respectively, are used as a source of primary antibodies. Additional controls should include the detection of unrelated molecules expressed by the cell of interest that are not expected to co-distribute with one of the molecules tested for *cis*-interaction.

Apart from false-positive results due to cross-reactivity of antibodies, false-negative results can be due to potential interference of antibody binding with the interaction between the molecules of interest. Polyclonal antibodies directed against a variety of epitopes can perturb molecular interactions. It is therefore worthwhile to test several different antibodies, polyclonal as well as monoclonal, if available. The potential interference of antibody binding with the interaction between the molecules of interest can be prevented by using heterologously expressed recombinant proteins. Specifically, different *N*-terminal peptide tags, like the myc-tag or the influenza hemagglutinin-tag can be introduced by molecular cloning into the polypeptide sequences of the studied proteins. Subsequent capping with antibodies specific for the N-terminally localized tag sequences will reduce the risk of interference with a binding site on the surface of the molecule.

It should always be kept in mind, that the detection of antibody-induced co-capping between two molecules is no proof of a direct molecular interaction between the two components. In order to demonstrate such a direct binding, additional experimental techniques, like chemical cross-linking (see Basic Protocol 5) or biochemical binding assays using isolated, purified proteins (see Basic Protocol 2) are required.

Neurite outgrowth

Many IgSF-CAMs have a neurite outgrowth-promoting activity, therefore, neurite growth assays are widely used for functional analysis. The preparation of neuronal cultures takes some practice and should best be learned in a laboratory where culture techniques are established. The reproducibility of the cultures is very important to get results. Keep in mind that the growth and the morphology of neurites depend on the substrate and the medium. As the quality and the components of serum differ considerably from batch to batch, the lot of serum used should not be changed during analysis. For best reproducibility, the use of a chemically defined, serum-free medium is recommended. All the solutions and the purified proteins to be tested for their neurite outgrowth-promoting activity have to be of high quality. Especially, the absence of endotoxins is extremely important for the survival of neurons. Make sure that detergents are removed carefully from protein solutions before using them in tissue culture.

The growth characteristics of neurites are age-dependent. In particular, the dependence on specific growth factors may change dramati-

cally. Therefore, it is essential not to mix different ages of tissue. Similarly, the response of neurons to different substrates may be species-specific. Remember that neurite length is not the only criterion that can be assessed in neurite growth assays. Closely monitor neurite and growth cone morphologies and branching patterns.

Anticipated Results

Transfection

For transient transfections with the protocol described, reporter constructs can be used to assess the efficiency of transfection and calculate the percentage of transfected cells. Using reporter constructs that express green fluorescent protein (GFP) as a reporter under the control of the human cytomegalovirus (CMV) immediate early promoter (plasmid size of 5 to 10 kb, DNA amounts for transfection between 40 and 80 μg), the authors repeatedly observed transfection efficiencies of 30% to 40% based on the detection of GFP expression 24 to 48 hr after transfection. Expression of recombinant protein under the control of strong viral promoters lasts, generally, for 48 to 96 hr after transfection.

Microsphere assays

With the appropriate controls, all the protocols described here that involve microsphere and cell aggregation techniques are powerful methods to study binding properties of IgSF-CAMs. Furthermore, these assays are not very time-consuming and enable processing of several tests in parallel.

Trans-interactions

Myeloma cell clones that were stably transfected to express IgSF-CAMs form aggregates when the IgSF-CAMs interact in *trans*. Homophilic *trans*-interactions are indicated by aggregates of cells that express the same IgSF-CAM. Heterophilic *trans*-interactions are indicated when two populations of cells that express distinct IgSF-CAMs form mixed aggregates.

Transfection-protoplast fusion

On average, 100 clones are obtained per 96-well tissue culture plate, i.e., 500 clones per 5×10^6 transfected myeloma cells. Thus, the transfection efficiency is in the range of 1×10^{-4}. Even with an optimized electroporation procedure, the transfection efficiency was ~20 times lower (Rader et al., 1993). While the majority of myeloma cell clones expresses moderate amounts of the IgSF-CAM, a small percentage typically reveals very high expression. Thus, the higher the transfection efficiency, the higher becomes the likelihood to obtain a myeloma cell clone with very high expression. This makes protoplast fusion the method of choice for the transfection of myeloma cells.

Chemical cross-linking

During optimization of the cross-linking protocol, the emphasis should be placed on the specificity of the reaction. As described, there is generally an inverse relationship between specificity of a cross-linking reaction and its efficiency. A protocol that ensures a high degree of specificity often has the drawback of low yields of cross-linked material. Based on published results and experience in the authors' laboratory, yields of cross-linking protocols, like the one described, range from 0.1% to 1%, corresponding to a few hundred nanograms of cross-linked material from a reaction performed on 10^6 cells. This amount of protein is normally sufficient for immunochemical characterization of the cross-linked molecules, e.g., by immunoblot analysis or re-immunoprecipitation (Buchstaller et al., 1996; Kunz et al., 1998).

The appearance of only one or a few complexes of a molecule of interest indicates some degree of specificity of the reaction. The cross-linked complexes isolated by immunoprecipitation can be separated by SDS-PAGE (ideally two-dimensional; *UNIT 6.4*). The detection of presumed binding partners in the cross-linked complexes by immunoblot analysis should always include controls, that is the detection of a membrane protein present in the cell used for cross-linking that is not expected to associate with the molecule of interest.

Co-capping

Antibody-induced co-capping can be observed between molecules that undergo direct or indirect *cis*-interactions. Very clear results can be obtained in cases where the two components are expressed at comparable levels and directly interact with each other with relative high affinity, as demonstrated in the example shown in Figure 9.5.3. Co-capping of molecule A (NgCAM) with molecule B (axonin-1) was studied on stably double-transfected CV-1 cells. Capping of molecule A (NgCAM), induced by the subsequent incubation with a mouse monoclonal primary and a rabbit-anti mouse secondary antibody (Fig. 9.5.3A), re-

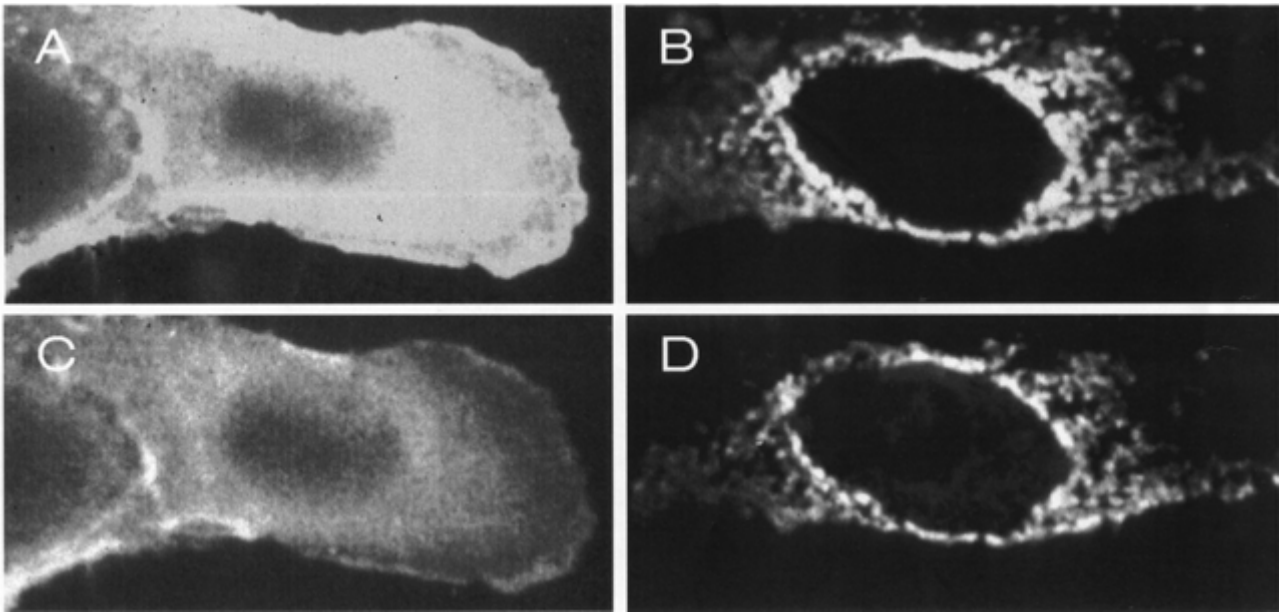


Figure 9.5.3 Antibody-induced co-capping of axonin-1 with NgCAM on stably double-transfected CV-1 cells. In double-transfected CV-1 cells, NgCAM (**A**) and axonin-1 (**C**) were randomly distributed. Capping of NgCAM was induced by a mouse monoclonal antibody and a rabbit anti-mouse IgG. NgCAM caps were detected by a Texas Red–labeled donkey-anti-rabbit IgG (**B**). The distribution of axonin-1 was detected by counter-staining with goat anti-axonin-1 Fab fragments and a FITC-labeled donkey anti-goat IgG (**D**). For examination of the cells, a confocal laser-scanning microscope equipped with an argon/krypton laser was used. Texas Red was detected using the 568-nm band-pass excitation filter (**A,B**) and FITC with the 488-nm band-pass excitation filter (**C,D**), minimizing cross-talk between the two fluorochromes.

sults in extensive co-capping of molecule B (axonin-1), as shown by counter-staining with goat anti-B (axonin-1) Fab fragments on the same cell (Fig. 9.5.3C). As expected, no capping or co-capping is detected in the absence of primary antibody against molecule A (NgCAM).

Time Considerations

Preparation of affinity column

The preparation of an affinity column takes 1 working day. Because affinity columns have to be loaded slowly, it is convenient to load large volumes of protein solution overnight to have the column ready for elution the following day. However, ensure that the column never runs dry.

Transfection

The entire transfection procedure can be performed in <1 hr for up to 12 samples.

Trans-interactions

Stable transfections of myeloma cells including selection, subcloning, and analysis require 4 to 6 weeks. Myeloma cell aggregation assays can be completed in <1 day.

Chemical cross-linking

The entire procedure can be performed within 2 days. The cross-linking reaction, including the making of the cell lysates, can be carried out within 3 to 4 hr. The lysates can be stored for several weeks at −20°C. Immunoprecipitation can be performed either with an incubation of 4 hr with the first antibody or with an incubation overnight.

Co-capping

The entire procedure can be performed in 4 to 5 hr. Incubation periods given here for antibody-induced capping are based on the authors' own optimized and published protocols. The time periods given for counter-staining can be extended for the primary antibody from 1 hr at room temperature to overnight at 4°C, if desired or necessary for higher sensitivity. The protocol can be interrupted after step 11 and 16 and the fixed coverslips stored at 4°C under light protection for up to 1 day before the subsequent steps are carried out.

Neurite outgrowth

The time required for neurite growth assays depends on the type of neurons used. Dissociated dorsal root ganglia neurons, for instance, will extend long neurites on many IgSF-CAMs

in <24 hr. DRG explants may take an additional day to reach their full length. Coating of IgSF-CAMs as a substrate is best done immediately before neurons or explants are plated, whereas dishes coated with poly-D-lysine or collagen can be stored. Dishes coated with laminin or IgSF-CAMs should not be stored and must not be dry.

Literature Cited

Bebbington, C.R., Renner, G., Thomson, S., King, D., Abrams, D., and Yarranton, G.T. 1992. High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Biotechnology* 10:169-175.

Buchstaller, A., Kunz, S., Berger, P., Kunz, B., Ziegler, U., Rader, C., and Sonderegger, P. 1996. Cell adhesion molecules NgCAM and axonin-1 form heterodimers in the neuronal membrane and cooperate in neurite outgrowth promotion. *J. Cell Biol.* 135:1593-1607.

Chang, S., Rathjen, F.G., and Raper, J.A. 1987. Extension of neurites on axons is impaired by antibodies against specific cell surface glycoproteins. *J. Cell Biol.* 104:355-362.

Dubreuil, R.R., MacVicar, G., Dissanayake, S., Liu, C., Homer, D., and Hortsch, M. 1996. Neuroglian-mediated cell adhesion induces assembly of the membrane cytoskeleton at cell contacts sites. *J. Cell Biol.* 133:647-655.

Fitzli, D., Stoeckli, E.T., Kunz, S., Siribour, K., Rader, C., Kunz, B., Kozlov, S.V., Buchstaller, A., Lane, R.P., Suter, D.M., Dreyer, W.J., and Sonderegger, P. 2000. A direct interaction of axonin-1 with NrCAM results in guidance, but not growth of commissural axons. *J. Cell Biol.* 143:351-358.

Freigang, J., Proba, K., Leder, L., Diederich, K., Sonderegger, P., and Welte, W. 2000. The crystal structure of the ligand-binding module of axonin-1/TAG-1 suggests a zipper mechanism for neural cell adhesion. *Cell* 101:425-433.

Gillies, S.D., Morrison, S.L., Oi, V.T., and Tonegawa, S. 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. *Cell* 33:717-728.

Gillies, S.D., Dorai, H., Wesolowski, J., Majeau, G., Young, D., Boyd, J., Gardner, J., and James, K. 1989. Expression of human anti-tetanus toxoid antibody in transfected murine myeloma cells. *Biotechnology* 7:799-804.

Graham, F.L. and van der Eb, A.J. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.

Grumet, M. and Edelman, G.M. 1984. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. *J. Cell Biol.* 98:1746-1756.

Hermanson, G.T. 1996. Bioconjugate Techniques. Academic Press, New York.

Hershfield, V., Boyer, H.W., Yanofsky, C., Lovett, M.A., and Helinski, D.R. 1974. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. *Proc. Natl. Acad. Sci. U.S.A.* 71:3455-3459.

Ji, T.H. 1983. Bifunctional reagents. *Methods Enzymol.* 91:580-609.

Jung, S.M. and Moroi, M. 1983. Crosslinking of platelet glycoprotein Ib by *N*-succinimidyl(4-azidophenyldithio) propionate and 3, 3'-dithiobis(sulfosuccinimidyl propionate). *Biochem. Biophys. Acta* 761:152-162.

Kawano, M.M., Huang, N., Tanaka, H., Ishikawa, H., Sakai, A., Tanabe, O., Nobuyoshi, M., and Kuramoto, A. 1991. Homotypic cell aggregations of human myeloma cells with ICAM-1 and LFA-1 molecules. *Br. J. Haematol.* 79:583-588.

Kuhn, T.B., Stoeckli, E.T., Condrau, M.A., Rathjen, F.G., and Sonderegger, P. 1991. Neurite outgrowth on immobilized axonin-1 is mediated by a heterophilic interaction with L1(G4). *J. Cell Biol.* 115:1113-1126.

Kunz, S., Spirig, M., Ginsburg, C., Buchstaller, A., Berger, P., Lanz, R., Rader, C., Vogt, L., Kunz, B., and Sonderegger, P. 1998. Neurite fasciculation mediated by complexes of axonin-1 and Ng cell adhesion molecule. *J. Cell Biol.* 143:1673-1690.

Lagenaur, C. and Lemmon, V. 1987. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. *Proc. Natl. Acad. Sci. U.S.A.* 84:7753-7757.

Lewis, R.V., Roberts, M.F., Dennis, E.A., and Allison, W.S. 1977. Photoactivated heterobifunctional cross-linking reagents which demonstrate the aggregation state of phospholipase A2. *Biochemistry* 16:5650-5654

Lomant, A.J. and Fairbanks, G. 1976. Chemical probes of extended biological structures: Synthesis and properties of the cleavable protein cross-linking reagent [^{35}S]dithiobis(succinimidyl propionate). *J. Mol. Biol.* 104:243-261.

Middaugh, C.R., Vanin, E.F., and Ji, T.H. 1983. Chemical crosslinking of cell membranes. *Mol. Cell. Biochem.* 50:115-141.

Nakatani, T., Nomura, N., Horigome, K., Ohtsuka, H., and Noguchi, H. 1989. Functional expression of human monoclonal antibody genes directed against pseudomonal exotoxin A in mouse myeloma cells. *Biotechnology* 7:805-810.

Perrin, F.E. and Stoeckli, E.T. 2000. The use of lipophilic dyes in studies of axonal pathfinding in vivo. *Microsc. Res. Tech.* 48:25-31.

Rader, C. and Sonderegger, P. 1998. Structural features of neural cell adhesion molecules belonging to the immunoglobulin superfamily. *In* Ig Superfamily Molecules in the Nervous System (P. Sonderegger, ed.) pp. 1-22. Harwood Academic Publishers, Chur, Switzerland.

Rader, C., Stoeckli, E.T., Ziegler, U., Osterwalder, T., Kunz, B., and Sonderegger, P. 1993. Cell-cell adhesion by homophilic interaction of the neuro-

nal recognition molecule axonin-1. *Eur. J. Biochem.* 215:133-141.

Rader, C., Kunz, B., Lierheimer, R., Giger, R.J., Berger, P., Tittmann, P., Gross, H., and Sonderegger, P. 1996. Implications for the domain arrangement of axonin-1 derived from the mapping of its NgCAM binding site. *EMBO J.* 15:2056-2068.

Rassoulzadegan, M., Binetruy, B., and Cuzin, F. 1982. High frequency of gene transfer after fusion between bacteria and eukaryotic cells. *Nature* 295:257-259.

Rojo, J.M., Saizawa, K., and Janeway, C.A. 1989. Physical association of CD4 and the T-cell receptor can be induced by anti-T-cell receptor antibodies. *Proc. Natl. Acad. Sci. U.S.A.* 86:3311-3315.

Sandri-Goldin, R.M., Goldin, A.L., Levine, M., and Glorioso, J.C. 1981. High-frequency transfer of cloned herpes simplex virus type 1 sequences to mammalian cells by protoplast fusion. *Mol. Cell. Biol.* 1:743-752.

Savoca, R., Ziegler, U., and Sonderegger, P. 1995. Effects of L-serine on neurons in vitro. *J. Neurosci. Methods* 61:159-167.

Schaffner, W. 1980. Direct transfer of cloned genes from bacteria to mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 77:2163-2167.

Shitara, K., Nakamura, K., Tokutake-Tanaka, Y., Fukushima, M., and Hanai, N. 1994. A new vector for the high level expression of chimeric antibodies in myeloma cells. *J. Immunol. Methods* 167:271-278.

Smith, R.J., Capaldi, R.A., Muchmore, D., and Dahlquist, F. 1978. Cross-linking of ubiquinone cytochrome c reductase (complex III) with periodate-cleavable bifunctional reagents. *Biochemistry* 17:3719-3723.

Sonderegger, P. Lemkin, P.F., Lipkis, L.E., and Nelson, P.G. 1985. Differential modulation of the expression of axonal proteins by non-neuronal cells of the peripheral and central nervous systems. *EMBO J.* 4:1395-1401.

Sonderegger, P., Kunz, S., Rader, C., Buchstaller, A., Berger, P., Vogt, L., Kozlov, S.V., Ziegler, U., Kunz, B., Fitzli, D., and Stoeckli, E.T. 1998. Discrete clusters of axonin-1 and NgCAM at neuronal contact sites: Facts and speculations on the regulation of axonal fasciculation. *Prog. Brain Res.* 117:93-104.

Staros, J.V. 1988. Membrane-impermeant cross-linking reagents: Probes of the structure and dynamics of membrane proteins. *Acc. Chem. Res.* 21:435-441.

Staros, J.V. and Anjaneyulu, P.S.R. 1989. Membrane-impermeant cross-linking reagents. *Methods Enzymol.* 172:609-628.

Stoeckli, E.T., Kuhn, T.B., Duc, C.O., Ruegg, M.A., and Sonderegger, P. 1991. The axonally secreted protein axonin-1 is a potent substratum for neurite growth. *J. Cell Biol.* 112:449-455.

Stoeckli, E.T. and Landmesser, L.T. 1995. Axonin-1, Nr-CAM, and Ng-CAM play different roles in the in vivo guidance of chick commissural neurons. *Neuron* 14:1165-1179.

Stoeckli, E.T., Ziegler, U., Bleiker, A.J., Groscurth, P., and Sonderegger, P. 1996. Clustering and functional cooperation of NgCAM and axonin-1 in the substratum-contact area of growth cones. *Dev. Biol.* 177:15-29.

Suter, D.M., Pollerberg, G.E., Buchstaller, A., Giger, R.J., Dreyer, W.J., and Sonderegger, P. 1995. Binding between the neural cell adhesion molecules axonin-1 and Nr-CAM/Bravo is involved in neuron-glia interaction. *J. Cell Biol.* 131:1067-1081.

Traunecker, A., Olivieri, F., and Karjalainen, K. 1991. Myeloma based expression system for production of large mammalian proteins. *Trends Biotechnol.* 9:109-113.

Wigler, M., Silverstein, S., Lee, L-S., Pellicer, A., Cheng, Y., and Axel, R. 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* 11:223-232.

Wood, C.L. and O'Dorisio, M.S. 1985. Covalent cross-linking of vasoactive intestinal polypeptide to its receptors on intact human lymphoblasts. *J. Biol. Chem.* 260:1243-1247.

Zhou, M.-J., Todd, R.F., van de Winkel, J.G.J., and Petty, H.R. 1993. Cocapping of the leukoadhesin molecules complement receptor type 3 and lymphocyte function-associated antigen-1 with Fc gamma receptor III on human neutrophils. Possible role of lectin-like interactions. *J. Immunol.* 150:3030-3041.

Contributed by Peter Sonderegger
University of Zurich
Zurich, Switzerland

Stefan Kunz and Christoph Rader
The Scripps Research Institute
La Jolla, California

Daniel M. Suter
Yale University
New Haven, Connecticut

Esther T. Stoeckli
University of Basel
Basel, Switzerland

Measurement of Adhesion Under Flow Conditions

This unit describes the analysis of dynamic cell adhesion using a flow chamber assay. The flow chamber enables the researcher to reconstruct cell systems in the presence of shear stress to assay adhesion under well-defined forces. These assays are most commonly used to study leukocyte adhesion, either to cultured endothelial cell monolayers or to purified substrates, simulating physiological interactions of leukocytes with endothelial cells. The assay described (Basic Protocol 1) utilizes commercially available parallel-plate flow chambers, in which cells are introduced under conditions of laminar flow between two flat surfaces, allowing for visualization of dynamic adhesion on a microscope (Lawrence and Springer, 1991). Flow cell assays are particularly useful to investigate adhesive events that occur very rapidly, on a time scale shorter than those assayed by static assays (Alon et al., 1995; Smith et al., 1999). Some adhesive events occur only in the presence of shear and, thus, cannot be characterized under static conditions (Finger et al., 1996; Lawrence et al., 1997). In addition, the flow cell assay allows visualization of the subprocesses of adhesion, including rolling, firm arrest, adhesion strengthening, spreading, and migration (Cinamon et al., 2001). Methods of data analysis are also discussed (Basic Protocol 2).

This assay can be also be used to characterize transient adhesive events or adhesion strengthening even for cells that do not normally experience shear stress, because contact time between cells and substrates and anti-adhesive forces can be closely regulated by stopping and starting the flow (Kassner et al., 1995). Flow chamber assays are also useful for measuring bacterial adhesion under flow (Mohamed et al., 2000; Poelstra et al., 2000).

FLOW ASSAY FOR CELL ADHESION

BASIC PROTOCOL 1

Commercial flow chambers incorporate the surface of either a culture dish or a coverslip as one of the two parallel plates between which laminar flow occurs. This surface should be coated with either endothelial cells or purified extracellular matrix proteins as an adhesive substrate.

Although several flow chambers are available, the same basic methods are used to conduct flow cell adhesion experiments regardless of the particular flow chamber apparatus used. Until recently, investigators often constructed their own laminar flow chambers, but with the advent of relatively inexpensive commercial models, this is not worthwhile unless special characteristics are required. The following method usually assumes that a GlycoTech flow chamber is used, but this protocol can easily be adapted for use with other chambers. Simply modify the assembly procedure according to the manufacturer's instructions and use cell- or ligand-coated glass coverslips instead of coated dishes if required.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Substrate coating solutions:
- Fibronectin, gelatin, or other extracellular matrix proteins, either alone or in combination, for cell monolayers only
- 10 μg/ml matrix protein, such as fibronectin or collagen, in PBS (*APPENDIX 2A*), pH 7.4, for dishes and coverslips coated with purified ligand only

Contributed by Dennis F. Kucik
Current Protocols in Cell Biology (2009) 9.6.1-9.6.10

Cultured endothelial cells (*UNIT 1.1*) growing as a confluent monolayer culture in tissue culture dish or on a coverslip of a size appropriate for the chamber used

1% (w/v) BSA in PBS (*APPENDIX 2A*), pH 7.4, for dishes and coverslips coated with purified ligand only

PBS (*APPENDIX 2A*), pH 7.4, for dishes and coverslips coated with purified ligand only

100 μg/ml poly-L-lysine (PLL; e.g., Sigma), for coverslips coated with purified ligand only

1% (v/v) glutaraldehyde, for coverslips coated with purified ligand only

Leukocytes or other cells of interest

HBSS (*APPENDIX 2A*), room temperature and 37°C

2 mM 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes) in DMSO

35-mm plastic tissue culture dishes for GlycoTech chamber or glass coverslips of a size appropriate for the flow chambers that require them, sterilized by autoclaving, UV light, or flaming with ethanol

35-mm tissue culture dishes or 6-well tissue culture plates, for glass coverslips only

Permanent marker or diamond stylus, for dishes coated with purified ligand only

25-ml syringes

Programmable syringe pump

Flow chamber (e.g., GlycoTech) and appropriate tubing

Inverted phase-contrast microscope with low-power (~10 to 20×) objective, fluorescence optics (recommended but not required), and stage incubator set to 37°C

Video camera (e.g., CCD-300T-RC; Dage-MTI) and other recording equipment (e.g., VCR, television monitor, video cables)

Prepare substrates

For cell monolayer preparation

1a. Incubate a 35-mm sterile plastic tissue culture dish or glass coverslip with 1 ml substrate coating solution of fibronectin, gelatin, or other extracellular matrix proteins, either alone or in combination (Table 9.6.1) for 30 min at 37°C.

There are a number of commercially available flow chambers that require glass coverslips of different sizes. In general, only a small portion of the surface area of the coverslip is used for the assay. The size of the coverslip required has more to do with the geometry of the chamber than the actual surface area used.

2a. Remove excess coating solution and, if using coverslip, place the coverslip in a 35-mm dish or in the well of a 6-well tissue culture plate.

Table 9.6.1 Endothelial Cells and Culture Conditions

Cell type[a]	Dish coating[b]	Plating density (cells/cm^2)	Days to confluence
HUVEC	0.05% (w/v) gelatin		
	10 μg/ml fibronectin	5000	3-4
HAEC	0.05% (w/v) gelatin		
	10 μg/ml fibronectin	5000	3-4
MAEC	1% (w/v) gelatin	6000	8-10

[a]Abbreviations: HAEC, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; MAEC, mouse aortic endothelial cells.

[b]The buffer used for the coating solution is HBSS (Hanks' balanced salt solution).

3a. Add 2 ml endothelial cell suspension (see Table 9.6.1 for cell density). Culture at 37°C.

4a. Feed cells every 2 to 3 days with an appropriate medium until confluent. Continue with step 9.

Coating density and the time to reach confluency depend on the cell type (Table 9.6.1). Optimal conditions for endothelial cell types other than those listed in Table 9.6.1 should be worked out empirically. Higher seeding densities result in less time to confluency, but lower densities and more time generally result in a more uniform monolayer. Depending on the cell type, cells are usually suitable for assays for 2 to 3 days after they reach confluency.

Endothelial cell adhesion to tissue culture plastic is generally superior to adhesion to glass coverslips, even if both are coated with extracellular matrix materials. Optical properties of plastic, however, are inferior to glass, because of a relatively high fluorescent background. Birefringence of plastic can also be a problem if Nomarski imaging is used. The optical properties of plastic are, however, adequate for the level of resolution needed for visualization and analysis of adhesion under flow, and they can provide better visualization of rolling cells than, for example, ex vivo preparations of blood vessels.

Leukocytes interact more with endothelial cells that have been stimulated. Interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), and lipopolysaccharide (LPS) have been considered good paradigms for pro-inflammatory mediators and are often used experimentally on cultured endothelial cells to simulate inflammation (Cines et al., 1998). Stimulation results in expression of a number of adhesion molecules, in addition to other effects. Whether and how the endothelial cells should be stimulated will depend on the physiologic conditions that are being mimicked.

For plastic dishes coated with purified ligand

1b. Use a permanent marker or diamond stylus to outline a 5-mm diameter area of a sterile plastic tissue culture dish for coating.

If a diamond stylus is used, the scratches should be shallow enough that they do not prevent the flow chamber gaskets from sealing.

The size of the area covered depends less on the size of the dish or coverslip than on the area of obseervation required. The authors find that a 5-mm diamerer circular area is sufficient to provide several fields of view for independent measurements of cell-substrate adhesion.

2b. Add 25 µl of 10 µg/ml matrix protein in PBS to the marked area and incubate 1 hr at room temperature.

3b. Remove coating solution and add 1 ml of 1% BSA in PBS. Incubate 1 hr at room temperature.

4b. Remove BSA solution. Wash twice with 5 ml PBS each. Continue with step 9.

The coated dish can be stored for 1 day at 4°C.

For glass coverslips coated with purified ligand

1c. Place sterile glass coverslip in a 35-mm tissue culture dish or in the well of a 6-well tissue culture plate.

2c. Add 0.2 ml of 100 µg/ml poly-L-lysine (PLL) to the dish. Incubate 10 min at room temperature.

The amount of PLL used should be scaled up as needed depending on the coverslip size (e.g., 0.5 ml should be used for a 22 × 22–mm coverslip). The authors routinely use PLL with a molecular weight of 70 to 150 kDa.

3c. Wash coverslips three times with 5 ml PBS each.

4c. Add 1% glutaraldehyde and incubate 30 min at room temperature.

5c. Wash coverslips three times with 5 ml PBS each.

6c. Add 25 μl of 10 μg/ml matrix protein and incubate 1 hr at room temperature.

For a 22 × 22–mm coverslip, 100 μl coating solution should be used.

7c. Add enough 1% BSA in PBS to completely cover the coverslip to block. Incubate 30 min at room temperature.

8c. Wash coverslip with 5 ml PBS. Add fresh PBS to keep moist until used for flow assay. Use within 1 day.

This protocol, in which matrix molecules are covalently linked to adsorbed PLL, results in tighter, more uniform binding of matrix materials to coverslips than direct adsorption of matrix molecules to the glass. If, however, PLL needs to be avoided, direct adsorption of matrix materials, as in the plastic dish protocol, may provide an adequate substrate.

Coverslips precoated with PLL are also commercially available, but the authors have not tried them.

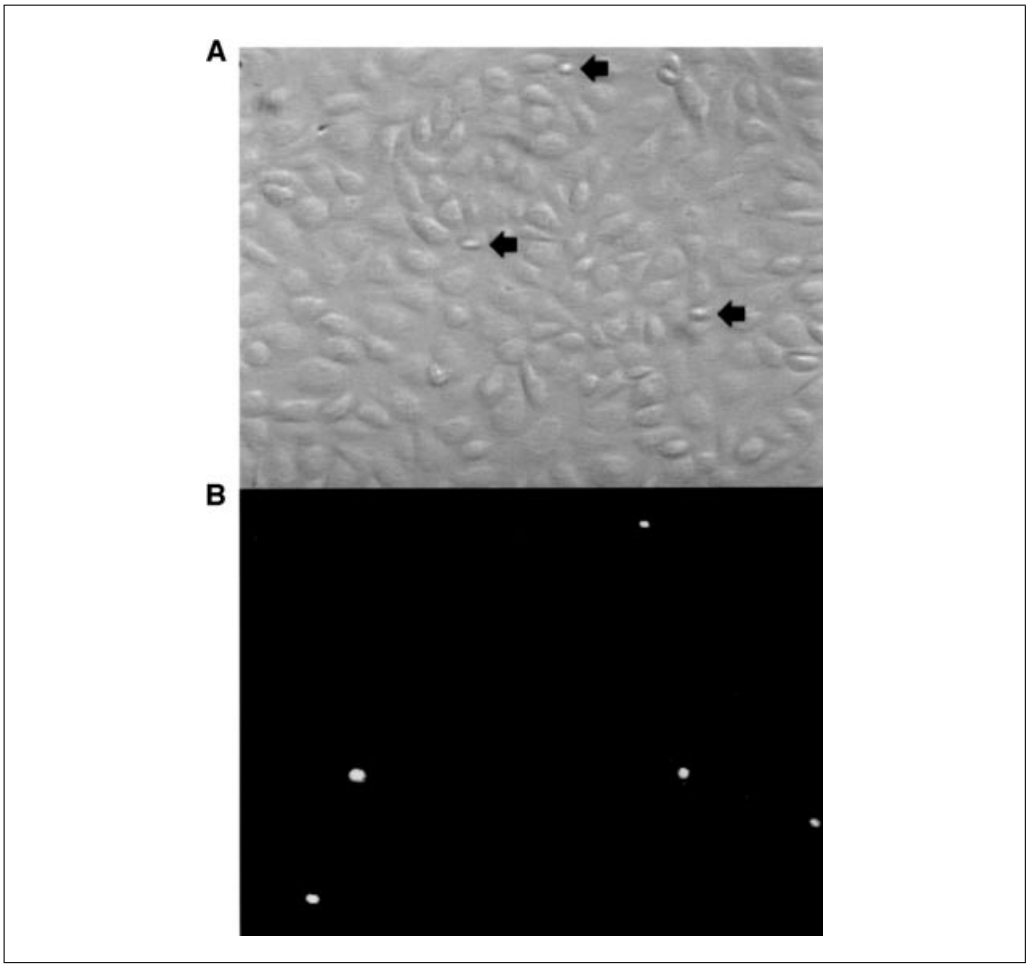


Figure 9.6.1 Visualization of endothelial cells and leukocytes using (**A**) phase contrast and (**B**) fluorescent optics. Arrows in (A) indicate leukocytes. Fluorescent optics provide high-contrast images that facilitate manual counting and are especially useful for computer tracking. When using endothelial cells as the substrate, however, it is important to observe the system with phase contrast or similar optics to assess the integrity of the monolayer before switching to fluorescence.

Load cells with flourescent dye(optional)

9. Wash leukocytes or other cells of interest in HBSS and then resuspend them at 1–2 $\times$ 10^6/ml in 25 ml HBSS.

   Although rolling and adherent cells can be visualized without fluorescence, the increase in contrast afforded by labeling the perfused cells greatly facilitates observation and counting (Figure 9.6.1) and is sometimes a requirement for object-tracking software.

10. Add 12.5 µl of 2 mM BCECF-AM and incubate 30 min at room temperature in the dark.

11. Wash cells three times with 35 ml HBSS each.

Carry out flow assay

12. Resuspend leukocytes in prewarmed HBSS to a concentration of 0.5 $\times$ 10^6 cells/ml

    Cell concentration should be high enough that sufficient events are observed in the few minutes of recording, but not so high that cell behavior is dominated by collisions with other rolling or adherent cells. The authors find that 0.5 $\times$ 10^6 cells/ml works well. The number of cells required will depend on the rate of flow and the length of observation needed to gather sufficient data to answer the particular experimental question. The authors usually use 3–5 $\times$ 10^7 cells/experiment (including controls).

    In general, it is a good idea to perform experiments at 37°C (this necessitates a stage warmer), although useful data can sometimes be obtained at room temperature, depending on the experimental question.

13. Load leukocytes into a 25-ml syringe, if using the pushing method, or into a centrifuge tube or other reservoir, if using the pulling method.

    There are two ways to use a syringe pump to perfuse cells through a flow chamber. The syringe can be filled with cells and the pump can be used to push them through the chamber. Alternatively, some investigators prefer to attach the syringe to the outlet side of the system to pull fluid and cells through the chamber.

14. Attach the tubing of a flow chamber to the syringe and remove any air bubbles from the system.

15. Assemble a flow chamber according to the manufacturer's instructions, using a tissue culture dish containing the monolayer of cultured endothelial cells (step 4a) or coated with purified ligand (step 4b or 8c). Again, work out any bubbles in the system.

    Flow chambers come in a wide variety of sizes. Generally, the smaller the chamber, the more economical the experiment is in terms of cells used per experiment. The dimensions of the GlycoTech flow chamber are determined by the size of the gasket used because the gasket forms the side walls of the chamber and also determines its height. GlycoTech supplies four gaskets with its basic kit; these range in width from 0.25 to 1.0 cm in width and in thicknesses ranging from 0.005 to 0.010 in. The length is fixed at 2 cm.

    Increasing the dimensions of the flow chamber will decrease the wall shear stress for a given flow rate. This has the disadvantage of requiring more volume (and thus more cells) to be perfused per unit time for a given shear. It can be advantageous, however, to use a larger chamber when low shears are desired because the syringe pump flow rates are less accurate and more pulsatile as flow is decreased. A wider chamber has the further advantage of providing more observation area if many fields of view are desired.

    Significant shear stresses can be created during assembly, which can sometimes tear endothelial cell layers loose from the dish. Therefore, care must be taken to assemble the system slowly and gently.

16. Program the syringe pump to give the desired flow according to the manufacturer's instructions.

    To determine the desired flow, flow rates must be converted to shear stresses. The manufacturer of the flow chamber will usually provide a handy conversion chart or table. Alternatively, shear stress can be calculated from the dimensions of the flow chamber and the rate of fluid flow (Lawrence et al., 1987). The shear stress desired will depend on the physiological condition to be mimicked. For example, simulation of arterial flow will require higher shear rates than simulation of venous or postcapillary venule conditions. The authors usually do a range of shears to determine the shear dependence of any phenomena observed. As a rule of thumb, physiologic shear stresses are typically thought to be in the range of 0.5 to 5.0 dynes/cm^2.

17. Mount the flow chamber on an inverted phase-contrast microscope with a prewarmed stage incubator. Focus the system and adjust the optics, video camera, and any other recording equipment as needed.

    If using endothelial cells, it is important to visualize the area of observation adequately to assess the integrity of the monolayer. Rips or other defects in the monolayer will confound interpretation of the results, because the data will reflect interactions of flowing cells not only with endothelial cells but also with bare plastic or glass.

    It is advisable to do a sample recording of a minute or so at this point and to play it back to be sure that all of the equipment is working properly.

18. Choose an area to observe, start the recording equipment, and begin the flow.

    The authors record on sVHS videotape for later digitization. Images can also be captured directly to computer. This may be necessary if the camera used does not have a video output.

19. Record a few minutes of data at each shear rate.

    The authors generally record ~3 to 6 min of data at each shear rate from each area of observation. For preliminary experiments they use at least three or four shear rates. Once the optimal shear and the shear dependence are determined, one or two shear rates may be sufficient. Considerations include the number of cells available (e.g., primary mouse cells are more precious than cell lines) and any time dependence of treatment regimens. It is important to record diligently the points on the videotape where shear rates are changed so that data can be interpreted when it is time to do the analysis.

20. Move to a new field of view and repeat steps 18 to 19. If using the pushing method, with the cells in the syringe, rotate the syringe 180° to compensate for any settling. If using the pulling method, with the cells drawn from a tube or other reservoir, make certain that the cells are still suspended.

    Most flow systems are large enough to allow a number of areas of observation for each preparation. It is important to observe multiple areas to control for any peculiarities in density of substrate coating or properties of endothelial cells. The number of fields to observe for each preparation will again depend on the number of cells available, any time dependence of treatment regimens, and the statistical power required.

21. Perform data analysis (see Basic Protocol 2).

BASIC PROTOCOL 2

DATA ANALYSIS

Quantifying Adherent Cells

The raw data from flow cell adhesion experiments will be videos of rolling and firmly adhering cells. The method of analysis will depend on the scientific questions to be answered. A simple count of adherent cells per minute can be obtained by visual review of videotapes. Just choose an appropriate time period (e.g., 1 min) and roll the tape to count the number of cells that become immobilized in the field of view during that time.

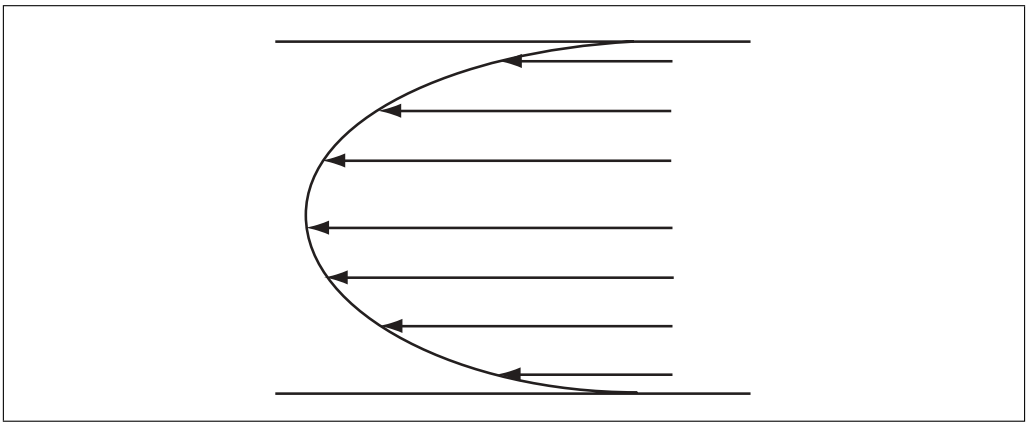


Figure 9.6.2 Velocity profile for fluid flow in a parallel-plate laminar flow apparatus. Longer arrows correspond to higher velocities. The velocity profile is parabolic, with a maximum velocity midway between the two plates and a slowest fluid flow near the plates.

It may be helpful to use two people to do this, one who counts and one who watches the time. Because the number of cells recorded will depend on the size of the field observed, it should be normalized for the area of observation. Area can be calculated using a stage micrometer, available from many scientific supply companies (e.g., Fisher), as a known length standard. A stage micrometer (also called a calibration plate) is a glass slide with accurate distance scales etched into the surface. By imaging this size standard, the size (in microns) of the video image or monitor screen can be determined.

An important consideration for meaningful comparisons of experimental conditions is that the number of cells perfused per minute must be the same under all experimental conditions. This requires that cells are loaded into the syringe or reservoir at a consistent density and that cells are not allowed to settle out of suspension.

Quantifying Rolling Cells

The number of rolling cells per minute can also be determined by visual review, but care must be taken in identification of rolling cells. By definition, under laminar flow there will be a parabolic hydrodynamic velocity profile, with the lowest velocity of the fluid occurring nearest the flow chamber wall (Figure 9.6.2). Thus, it may not be obvious which cells are moving slowly because of their position in the flow field and which are retarded by adhesive interactions (Goldman et al., 1967). Whereas under some optical conditions rolling cells can be distinguished by plane of focus, it is a good idea to calculate a critical velocity to verify that cells counted as rolling do indeed have adhesive interactions with ligand. The critical velocity is an objective measure that has been developed to identify rolling cells (Ley and Gaehtgens, 1991). A simple calculation for critical velocity is $V_c = \beta r \gamma$, where V_c is the critical velocity, β is a drag factor, r is the radius of the cell, and γ is the shear rate. A reasonable estimate of β that can be used for cells in a flow chamber is 0.5. Cells moving more slowly than the critical velocity can be confirmed as being rolling cells. Thus, even if the experimental question deals only with the number of rolling cells, and not their velocity, a few velocities should be measured to ensure that what appears to be rolling cells are moving more slowly than the critical velocity and, thus, are indeed rolling. With some practice, though, rolling cells can often be identified visually.

Rolling Velocities

The calculation of cell position in each digitized image can be done by comparison with a known size standard, again using a stage micrometer. A known length (in microns) can then be marked on the monitor screen with a laboratory marker. With a known distance

established, velocities can be determined by simply counting the number of video frames required for a cell to travel this known distance. Alternatively, more accurate velocity determinations can be made using more sophisticated image processing software that can accurately localize objects in each image and convert pixels to distances. A variety of commercial software packages are available. NIH Image, which is available online at no cost, will also track object motion (see Internet Resources).

COMMENTARY

Background Information

Leukocyte rolling and adhesion in vivo has been observed microscopically for more than 100 years (Cohnheim, 1889). More recently, investigators have used parallel-plate flow chambers to mimic the environment of the blood vessel under more controlled conditions. Much of what we know today about inflammation and leukocyte homing has been learned by examining leukocyte interactions with either endothelial cell monolayers or purified ligands in flow chambers (Butcher, 1991; Rossiter et al., 1997; Smith, 2000; Orsello et al., 2001). In addition, adhesion assays using flow chambers have provided important information about bacterial adhesion to surfaces. Today, adhesion under flow can readily be assayed economically using a variety of commercially available flow chambers. When mounted on a microscope and coupled to a video camera and a VCR, simple analysis of adhesion under shear is possible. More detailed analysis of rolling cell motion can be achieved by digitization of images coupled with motion analysis software.

Commercially available flow chambers, in general, work very well and make it easy to produce conditions that mimic physiological cell interactions with well-defined and tightly controlled shear stresses. Increased use of these assays will provide a fuller understanding of physiologic cell adhesion than could ever be achieved with static adhesion assays.

Critical Parameters and Troubleshooting

Flow experiments require some diligence to ensure that the system is working properly. The experimenter should watch the monitor carefully during the experiment to be sure that the flow is smooth and that perfused cell concentrations are uniform. Although a programmable syringe pump is capable of providing very steady perfusion, flow can become pulsatile when the syringe is almost empty or if the syringe is not mounted securely. Other sources of variability in flow are leaks or bubbles anywhere in the system. It is also a good idea to isolate the system from vibration.

If fluorescent dyes are used to label perfused leukocytes, it is a good idea to develop a protocol that labels a high percentage of the cells (>97%), because unlabeled cells will likely be missed during analysis, confounding quantification of the number of cells interacting with the endothelium or substrate. Care should be taken, however, not to overload the cells, because fluorescent dyes can be somewhat toxic. Too much label interferes with cell motility and may affect regulation of adhesion in general. The optimal dye-loading protocol can be determined by varying both dye concentration and incubation times and should be worked out for each new cell type.

When using object tracking software, the experimenter should observe the tracking closely. Although most available programs do a very good job and provide temporal resolution not obtainable by cruder methods, they also make occasional errors, briefly locking onto an object other than the cell of interest. The operator should confirm visually for each cell track that the software has faithfully tracked the object of interest and has not misidentified the cell at any point.

Uniformity and integrity of the substrate are also of vital importance. This is especially true of endothelial cell monolayers. High shear forces associated with assembly of the chamber or with manual flushing of bubbles can dislodge portions of the monolayer. Thus, to avoid mistaking leukocyte interactions with the chamber itself for interactions with the endothelium, the importance of a visual inspection of each recorded field of view cannot be overemphasized. Purified protein substrates present less of a problem, but care should be taken to ensure uniform distribution and density of adsorbed matrix materials through use of good experimental technique.

Cameras

The authors recommend cameras with a video (rather than digital) output, because storage of images is more economical on videotape than on digital media. This allows the experimenter to record continuously throughout the experiment. This can be an important

consideration if one is working with valuable perfused cells (e.g., leukocytes purified from gene-targeted mice). An economical recording system also encourages collection of the maximum amount of data from each experiment, avoiding the need to repeat experiments simply to increase statistical power. In addition, higher time resolution (30 frames/sec) is possible with video than with most digital systems. Images can be digitized later for computer analysis using a variety of commercially available systems.

If fluorescent cells are to be visualized, a highly light-sensitive camera must be used. This is because when rolling cells are visualized, the time to acquire the image is limited. Extended exposures cannot be used, as the cells will appear blurry from their movement. Several camera companies offer cameras that are sensitive enough for this application, but the authors know of few with both adequate sensitivity and video output. The authors have found the CCD-300T-RC (Dage-MTI) to work well. Equivalent cameras may be available from other manufacturers as well.

It is also possible to do flow cell experiments without fluorescently labeling the cells. Although this results in less contrast between rolling cells and underlying endothelial cells or substrate, the images can be perfectly acceptable (Fig. 9.6.1). Imaging without fluorescence also avoids possible deleterious effects of dye loading on the cells. In addition, the time required for each experiment is shortened by the amount of time needed to load the cells with fluorescent dye, which may be useful if short-acting treatments are used. A major determinant of whether fluorescence can be omitted is the quality of the microscope optics. Another factor to consider, however, is whether any tracking software to be used depends on cell intensity for tracking motion. Although some programs can track objects based on their size and shape, other packages require the tracked object to be brighter than its surroundings.

When imaging without fluorescence, it is especially important to avoid fingerprints and other smudges on the culture dish or coverslip and other optical elements of the flow chamber, to avoid degrading the image.

Video recording

It is recommended that video be recorded using an sVHS, rather than a VHS (the typical home-recording format), VCR. Not only will the sVHS spatial resolution be better, but the recordings are more resistant to degradation with repeated playback. Alternatively, images can be recorded directly to computer memory or hard drive. With some cameras, this may be the only option.

Image digitization and compression technologies are progressing so rapidly that any hardware recommendations are likely to be out of date within months. Therefore, it is recommended that the investigator research available products when setting up a system. An important factor to consider is the amount of compression necessary with each system, because increased compression can lead to decreased image quality and, consequently, a decrease in accuracy of cell position determination.

Anticipated Results

In general. leukocytes perfused over stimulated endothelial cells at physiological flow rates will be expected to interact with both rolling and firm adhesion. The amount and type of interaction will depend on the leukocyte type and its state of activation as well as the endothlial cell type (venous versus arteerial, mouse versus human). Endothelial cells can be stimulated by TNF-α (e.g., 10 to 100 ng/ml for 8 hr). This induces expression of a number of adhesion molecules and should dramatically increase adheive interactions with almost any leukocyte type. Human umbilical vein endothelial cells (HUVEC; *UNIT 2.3*) have been used in a number of flow chamber adhesion studies (Munn et al., 1994; Macconi et al., 1995; Thelilmeier et al., 1999; Patel, 1999; Kaur et al., 2001), are widely available, and make a good test system to validate experimental technique.

Time Considerations

The time required to prepare a culture monolayer is 1 to 2 hr with several days required for cells to reach confluency (Table 9.6.1). Preparing a pure ligand substrate requires 5 to 6 hr for either culture dishes or coverslips. The flow assay requires about 3 hr to do two cell types and three conditions, and at least 1 day, depending on the analysis performed is needed to analyze the data.

Literature Cited

Alon, R., Hammer, D.A., and Springer, T.A. 1995. Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. *Nature* 374:539-542.

Butcher, E.C. 1991. Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. *Cell* 67:1033-1036.

Cinamon, G., Grabovsky, V., Winter, E., Franitza, S., Feigelson, S., Shamri, R., Dwir, O., and Alon, R. 2001. Novel chemokine functions in lymphocyte migration through vascular endothelium under shear flow. *J. Leukocyte Biol.* 69:860-866.

Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., Barnathan, E.S., McCrae, K.R., Hug, B.A., Schmidt, A.M., and Stern, D.M. 1998. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 91:3527-3561.

Cohnheim, J. 1889. Lectures on General Pathology: A Handbook for Practitioners and Students. The New Sydenham Society, London.

Finger, E.B., Puri, K.D., Alon, R., Lawrence, M.B., Von Andrian, U.H., and Springer, T.A. 1996. Adhesion through L-selectin requires a threshold hydrodynamic shear. *Nature* 379:266-269.

Goldman, A.J., Cox, R.G., and Brenner, H. 1967. Slow viscous motion of a sphere parallel to a plane wall - II Couette flow. *Chem. Eng. Sci.* 22:653-660.

Kaur, J., Woodman, R.C., Ostrovsky, L., and Kubes, P. 2001. Selective recruitment of neutrophils and lymphocytes by thrombin: A role for NF-κB. *Amer. J. Physiol.-Heart and Circulatory Physiol.* 281:11784-11795.

Kassner, P.D., Alon, R., Springer, T.A., and Hemler, M.E. 1995. Specialized functional properties of the integrin alpha 4 cytoplasmic domain. *Mol. Biol. Cell* 6:661-674.

Lawrence, M.B. and Springer, T.A. 1991. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell* 65:859-873.

Lawrence, M.B., McIntire, L.V., and Eskin, S.G. 1987. Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. *Blood* 70:1284-1290.

Lawrence, M.B., Kansas, G.S., Kunkel, E.J., and Ley, K. 1997. Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E). *J. Cell Biol.* 136:717-727.

Ley, K. and Gaehtgens, P. 1991. Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric venules. *Circ. Res.* 69:1034-1041.

Macconi, D., Poppolo, M., Paris, S., Noris, M., Arelio, S., Remuzzi, G., and Remuzzi, A. 1995. PAF mediates neutrophil adhesion to thrombin or TNF-stimulated endothelial cells under shear stress. *Amer. J. Physiol.* 269:C42-C47.

Mohamed, N., Rainier, T.R., and Ross, J.M. 2000. Novel experimental study of receptor-mediated bacterial adhesion under the influence of fluid shear. *Biotechnol. Bioeng.* 68:628-636.

Munn, L.L., Melder, R.J., and Jain, R.K. 1994. Analysis of cell flow in the parallel plate flow chamber: Implications for cell capture studies. *Biophys. J* 67:889-895. [erratum appears in *Biophys. J.* 70:2033].

Orsello, C.E., Lauffenburger, D.A., and Hammer, D.A. 2001. Molecular properties in cell adhesion: A physical and engineering perspective. *Ophthalmic Genet* 19:310-316.

Patel, J.D. 1999. Mechanism of selective leukocyte recruitment from whole blood on cytokine-activated endothelial cells under flow conditions. *J. Immunol.* 162:6209-6216.

Poelstra, K.A., van der Mei, H.C., Gottenbos, B., Grainger, D.W., van Horn, J.R., and Busscher, H.J. 2000. Pooled human immunoglobulins reduce adhesion of *Pseudomonas aeruginosa* in a parallel plate flow chamber. *J. Biomed. Mater. Res* 51:224-232.

Rossiter, H., Alon, R., and Kupper, T.S. 1997. Selectins, T-cell rolling and inflammation. *Mol. Med.* 3:214-222.

Smith, C.W. 2000. Possible steps involved in the transition to stationary adhesion of rolling neutrophils: A brief review. *Microcirculation* 7:385-394.

Smith, M.J., Berg, E.L., and Lawrence, M.B. 1999. A direct comparison of selectin-mediated transient, adhesive events using high temporal resolution. *Biophys. J.* 77:3371-3383.

Theilmeier, G., Lenaerts, T., Remacle, C., Collen, D., Vermylen, J., and Hoylaerts, M. 1999. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interactiion under shear stress. *Blood* 94:2725-2734.

Internet Resource

rsb.info.nih.gov/nih-image/about.html
Web site for NIH Image.

Contributed by Dennis F. Kucik
University of Alabama at Birmingham
Birmingham, Alabama

CHAPTER 10
Extracellular Matrix

INTRODUCTION

Most cells of multicellular organisms interact routinely with extracellular matrix (ECM) molecules. The ECM provides structural support, as well as important regulatory signals governing cellular growth, metabolism, and differentiation. Epithelial cells of all types generally require various ECM components in the basement membranes to which they adhere for maintaining their characteristic polarized organization, differentiated state, and specific gene expression. Connective tissue cells nestle within matrices of collagens, proteoglycans, and other ECM components. Even circulating blood cells such as lymphocytes can interact extensively with ECM as they extravasate from blood vessels and localize in tissues during recirculation and inflammation. Two particularly dynamic tissue-remodeling processes in which ECM becomes critically important are cell movements during embryonic development and wound repair. In addition, tumor cells often must invade through basement membranes and connective tissue in order to metastasize (see *UNIT 19.1*).

Throughout these various cell-biological processes, ECM can act both as a structural scaffolding for cell adhesion and migration and as a trigger for signaling through ECM receptors. Binding of specific ECM molecules to their plasma-membrane receptors activates signal-transduction responses that can include activation of various tyrosine and serine-threonine kinase families, MAP kinase systems, ion fluxes, or phosphoinositide and arachidonic acid pathways. The study of these complex processes has blossomed recently due to the availability of individual purified ECM molecules, along with the realization that ECM modulates many crucial cell-biological functions.

UNIT 10.1 reviews key ECM functions, as well as the biochemistry of the major classes of ECM molecules. This comprehensive review provides a solid framework for understanding the initially somewhat bewildering complexity of ECMs, which range from basement membranes to loose connective tissue, cartilage, and ligaments.

Basement membranes are crucial for normal epithelial cell biology. *UNIT 10.2* describes the preparation of the basement-membrane extract termed Matrigel and the isolation of two important components, laminin and type IV collagen. Although these proteins are also available commercially, preparing them within the laboratory is much more economical for larger-scale studies. These purified proteins can be used in cell adhesion assays (*UNITS 9.1 & 9.2*) and for studying other cell-biological responses such as migration and differentiation.

Because extracellular matrices are generally three-dimensional, and their effects can be greater than the sum of their isolated components, cell biologists also use ECM gels. *UNIT 10.3* details the preparation and use of gels of purified collagen and of Matrigel basement-membrane extracts. Collagen gels and Matrigel can be used to study the three-dimensional behavior of epithelial, fibroblastic, and other cells. Matrigel can also be used in animals for angiogenesis assays (*UNIT 10.3*) and in tumor cell invasion assays (*UNIT 12.2*), as well as to promote the survival and growth of primary tumor cells that would not otherwise grow in vivo (*UNIT 10.3*).

Current Protocols in Cell Biology 10.0.1-10.0.4, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb1000s48

An alternative approach is to use intact extracellular matrices assembled by living cells in culture; the cell monolayers are extracted away to leave just the three-dimensional extracellular matrix. *UNIT 10.4* describes the preparation of two forms of such complex matrices, which can be used as substrates for cell biological studies in vitro.

A particularly widely used approach involves purified extracellular matrix molecules as substrates. Both fibronectin and vitronectin can be purified by affinity chromatography from human plasma, providing large quantities of an individual adhesive protein useful for cell culture studies using many types of cells. *UNIT 10.5* presents simple, reliable methods for purifying substantial quantities of fibronectin from plasma. Also included are two different protocols for purifying the cellular form of fibronectin, by extraction from cell surfaces or from fibronectin secreted by cells into serum-free medium; this form of fibronectin contains additional peptide sequences and has moderately enhanced adhesive activity. *UNIT 10.6* describes a simple protocol for the isolation of vitronectin, which is a cell adhesion protein used by many cells—e.g., by most adherent cells cultured in medium that contains 10% serum.

Even though proteoglycans are a major class of diverse extracellular and cell-surface molecules with important roles in a myriad of regulatory and structural functions, many researchers have hesitated to study them because they are technically difficult to analyze. These large, highly charged, readily aggregated molecules are now known to be present not only in extracellular matrices, but also linked to membranes by phospholipid, in transmembrane locations (e.g., the syndecans), or even in intracellular locations in some cells. *UNIT 10.7* provides numerous protocols and valuable tips for isolating and analyzing these widely distributed molecules implicated in signal transduction, adhesion, and ECM structure.

The glycoproteins and proteoglycans of the extracellular matrix are not static, but are instead in a state of dynamic balance between synthesis and degradation. The continuous turnover and active remodeling of the extracellular matrix in embryonic development, growth, and tissue repair depend critically on carefully regulated degradation by proteolytic enzymes, particularly by the matrix metalloproteinases (MMPs). The MMPs are regulated by activation and by their inhibitors (the TIMPs, as well as α2-macroglobulin). *UNIT 10.8* provides a series of methods for studying MMPs and their inhibitors, including a live-cell collagen degradation assay, analysis by either direct or reverse zymography, and enzyme-capture techniques.

The ECM of living organisms is often organized into a fibrillar, three-dimensional matrix. Although collagen gels and Matrigel provide valuable three-dimensional cell culture model systems, recent findings suggest that some cells require a matrix that more closely mimics their natural microenvironment in vivo in order to form normal cell adhesions; such matrices also enhance cell attachment, migration, and proliferation. *UNIT 10.9* provides methods for generating a fibroblast-derived three-dimensional ECM that closely resembles in vivo matrices. It also describes protocols for assessing cell adhesive signaling and morphological responses to such matrices, as well as methods for generating two-dimensional substrate controls.

ECM proteins in a group termed ‘matricellular’ proteins play complex and interesting roles in regulating the interaction of cells with other ECM molecules. These proteins include SPARC (also known as osteonectin; *UNIT 10.11*), thrombospondin (*UNIT 10.10*), tenascin, and other molecules that function to modulate a wide range of biological responses to the ECM. *UNIT 10.11* provides comprehensive protocols for isolating and purifying SPARC, a matricellular protein that can regulate cell adhesion, proliferation, and other processes. This unit presents methods for its purification from a cell line or

platelets, as well as for purifying recombinant SPARC from *E. coli* or insect cells. Finally, it also describes assays for its inhibitory effects on cell proliferation and adhesion.

Assembly of the extracellular matrix is currently thought to be a cell-directed process. For example, even though collagens can self-polymerize, their patterns of deposition are controlled by cells. The cell-mediated formation of a fibronectin fibrillar matrix is a particularly dramatic example, since cells have to convert soluble fibronectin dimers into an insoluble fibrillar matrix. *UNIT 10.12* provides detailed methods for analyzing this process of fibronectin matrix assembly based on measuring conversion of this protein to a detergent-insoluble form after polymerization. It describes the isolation and analysis of the resultant insoluble fibronectin matrix from cultured cells, as well as assays using exogenously added fibronectin or endogenous labeling. Because some laboratories may prefer to avoid using radioactivity, *UNIT 10.13* provides an alternative method of quantifying fibronectin matrix assembly using biotin-labeled fibronectin. This protocol includes an internal control to ensure equal recovery of detergent-insoluble material, as well as methods for parallel analyses of signal transduction processes that are involved in matrix assembly or result from this important process.

Cell interactions with three-dimensional matrices help to regulate cell growth, migration, and differentiation in vivo. Researchers now have increasing numbers of options for natural and synthetic extracellular matrices that can mimic various types of three-dimensional microenvironments. A new type of synthetic matrix termed Extracel is described in *UNIT 10.14* that consists of hyaluronan cross-linked to gelatin. It forms a hydrogel that is an effective culture system for both normal and malignant cells. This biodegradable scaffold can be designed to deliver growth factors to cells, and it provides an excellent environment for growth of tumor xenografts.

In order to study the roles of patterns and topology of matrix molecules, the newly developed process termed micro photopatterning provides a simple and highly flexible methodology to generate any protein pattern on a substrate. As described in detail in *UNIT 10.15*, this new technique uses a standard two-photon confocal microscope controlled by its built-in software to ablate any desired pattern in a polyvinyl alcohol thin film. Any protein can then be coated onto the exposed pattern, and the process can be repeated multiple times to generate complex patterns of multiple proteins positioned as close as a few micrometers from each other. Another advantage of this method compared to others is that it readily permits fluorescence and total internal reflection fluorescence (TIRF) microscopy.

Recent research on the mechanisms by which the extracellular matrix regulates processes as diverse as stem cell differentiation and tumor cell invasion has focused increasingly on physical properties of the local matrix microenvironment. Physical characteristics such as stiffness and surface topology are now known to provide important regulatory information that complements the microenvironmental effects of biochemical composition. *UNIT 10.16* provides a rigorously established protocol for experimentally controlling the stiffness or elasticity of extracellular matrices by using polyacrylamide hydrogels. This now-standard approach controls matrix stiffness by altering the concentrations of acrylamide and a molecular cross-linker to which a specific extracellular matrix molecule is attached to provide a substrate of known stiffness. Another physical property important for cell migration and invasion is the topology of matrix molecules, which is readily sensed by cells. *UNIT 10.17* provides two detailed protocols for generating aligned matrices comprised of collagen fibers. Cells align and migrate along these oriented fibers. This unit also provides methods for visualizing the aligned matrix containing cells by using second harmonics and for quantifying matrix alignment.

Studies of cells cultured in three-dimensional collagen gels have provided many valuable insights into cell behavior, but immunofluorescence staining of subcellular structures in these cells, such as cell-matrix adhesions, can be technically difficult—especially in thick collagen gels. *UNIT 10.18* provides detailed protocols and technical tips for obtaining high-quality confocal microscopy images of molecules in cells within three-dimensional collagen gels. It also provides methods for imaging the collagen fibrils in these gels by confocal reflection microscopy, as well as a method for sandwiching cells between very thin collagen gels to ensure that they are located in a three-dimensional microenvironment.

The units in this chapter span topics from individual purified ECM proteins to complex three-dimensional matrices consisting of many molecules interacting to form gels. They provide the opportunity to study the functions and mechanisms of the whole range of types of cell-ECM interactions, which are now recognized to play crucial roles in cell biology and pathology.

Kenneth M. Yamada

Overview of Extracellular Matrix

In all multicellular organisms, development is influenced by the interactions between cells and their extracellular matrix (ECM). Information contained in the ECM provides the cell with temporal and positional clues, such as where it is, where it should be going, how old it is (in terms of cellular differentiation), and in some instances, when it is time for it to die (through apoptosis). It is not surprising, then, that there has been a great deal of interest in defining the extracellular signals as well as the cell surface receptors that interact with these molecules and interpret the information. Now more than ever, understanding cell biology requires understanding the ECM.

Studying the ECM, however, is not for the faint of heart! In most instances, the functional form of matrix macromolecules is a large, sparingly soluble aggregate that cannot easily be solubilized or dissociated into component units. Even when dissociated matrix components are obtained, the biological properties of the constituent chains often differ from the intact form. To complicate matters, most ECM macromolecules participate in supramolecular assemblies where their biological properties are modified by the molecules with which they interact.

These unusual physical properties create serious problems for matrix characterization using a standard "wet chemistry" approach. They also create some, though not many, unique advantages. For example, the multimeric, cross-linked nature of ECM imparts an element of stability that is not found in other proteins. This is most obvious if one takes a historical look at techniques used for matrix purification (Partridge, 1962; Piez, 1997). In the early days of matrix biology, "connective tissue" was purified using extraction protocols that relied on the ability of the target matrix component to withstand relatively harsh conditions: acid solutions were used for purifying collagen, chaotropic agents for mucopolysaccharides (now called proteoglycans), and, the harshest of them all, boiling sodium hydroxide for purifying elastin. It is quite remarkable that so much of what we know about these three matrix classes resulted from experiments using products purified in this way. Although purification strategies are now a bit more sophisticated, modifications of these basic protocols are still used today. The use of molecular biology and mouse genetics has quickened the pace of matrix characterization and opened the door to functional studies of complex matrices that were unthinkable several years ago.

One of the most important properties of ECM is its functional diversity (Kleinman, 1993). Some components are designed to be rigid, others elastic; some wet, others sticky. All have modular designs that impart diverse roles, yet allow for highly specialized functions. The formation of a basement membrane, for example, requires the assembly of ECM molecules that have significant tensile strength (collagen), can act as charged molecular sieves (proteoglycans), and facilitate cell attachment (laminin). These molecules are woven together through processes that involve self-assembly and interactions with molecules that are specifically designed to serve as molecular bridges or linkers (nidogen/entactin; Yurchenco, 1994).

It is not possible to ascertain the functional properties of a complex matrix such as basement membrane without studying its individual components. At the same time, however, it is also clear that the functional complexity of the assembled basement membrane is greater than the sum of its component parts. To comprehend this greater sum requires shifting one's view away from a reductionist biochemical approach to one focused on cell and developmental biology. Here the cell becomes the reagent, interpreting informational signals contained in the ECM and adjusting its physiology accordingly. The researcher's task is to understand the readout.

The sections below contain an overview of the major classes of ECM. Molecules have been selected to illustrate specific functional or structural properties that are common to a matrix class or to ECM macromolecules generally. Where possible, recent reviews with references to more detailed literature are cited. Although somewhat dated, the text *Cell Biology of Extracellular Matrix* (Hay, 1991) provides an excellent overview of ECM biology. More detailed reviews can be found in various volumes of the *Biology of Extracellular Matrix* series, published by Academic Press.

COLLAGENS

Structure of Collagens

Collagen is the most ubiquitous ECM protein and is designed to provide structure and resiliency to tissues. It is defined by the pres-

Contributed by Robert P. Mecham
Current Protocols in Cell Biology (1998) 10.1.1-10.1.14

ence of a triple-helical domain containing peptide chains with repeating Gly-Xaa-Yaa triplets, and by the presence of hydroxyproline and hydroxylysine (Kühn, 1987; Prockop and Kivirikko, 1995). To date, nineteen distinct genetic collagen types have been identified. The characteristic molecular form of collagen is a triple helix made up of three polypeptides, called α chains, that coil into a right-handed triple helix. Collagens exist either as homotrimers composed of three identical α chains $(\alpha 1)_3$ or as heterotrimers consisting of two $([\alpha 1]_2\alpha 2)$ or three (α1α2α3) α chains.

The nomenclature for the collagen superfamily consists of an indication of their genetic type (a Roman numeral that generally denotes the chronological order in which the collagens were characterized) together with the α-chain composition. Type I collagen, for example, is a heterotrimer of two α1 chains and one α2 chain, and is indicated as $(\alpha 1[I])_2\alpha 2(I)$. Type II collagen is a homotrimer of three α1 chains and is written $(\alpha 1[II])_3$. Other collagens consist of three different α chains and (using type IX as an example) are written in the form α1(IX)α2(IX)α3(IX). It is important to note that each α chain within a collagen type is a distinct gene product; that is, an α1 chain in one collagen type is not the same protein as the α1 chain in any other collagen type. It is critical, therefore, to indicate the collagen type when referring to a particular α chain (e.g., the α1 chain of type I collagen).

Synthesis of Collagens

Collagen α chains are synthesized on membrane-bound ribosomes (ER) as large precursors, called pre-pro-α chains. In addition to the signal peptide (the "pre" part of the name) required for transport into the ER, each collagen precursor has extension peptides (the "pro" part) on both its N- and C-terminal ends (Fig. 10.1.1). Each pro-α chain combines with two others in the lumen of the ER to form the triple-helical molecule. The extension peptides are required for correct triple helix formation and remain with the triple-helical unit throughout the secretory pathway.

In the triple helix, the side chain of every third α-chain residue is directed towards the center of the helix, shifted by 30° from the preceding central residue of the same chain (Brodsky and Ramshaw, 1997). Steric constraints dictate that the center of the helix be occupied only by glycine residues; side chains of any other amino acid would perturb the triple-helical conformation.

Hydroxylation of proline residues in the Yaa position occurs as a post-translational modification in the lumen of the ER. The side-chain hydroxyl group of hydroxyproline stabilizes the helix through the formation of intermolecular hydrogen bonds. In fact, hydroxylation of ~100 prolyl residues is essential for the three pro-α chains of fibrillar collagens to form a triple helix that is stable at body temperatures. Hydroxylation of α chains is catalyzed by prolyl4-hydroxylase, a tetrameric enzyme consisting of two α and two β subunits (α2β2; Kivirikko and Myllyharju, 1998). Interestingly, the β subunit is protein disulfide isomerase, an ER protein that catalyzes thiol-disulfide interchange during protein folding (Koivu et al., 1987). The hydroxylation reaction catalyzed by

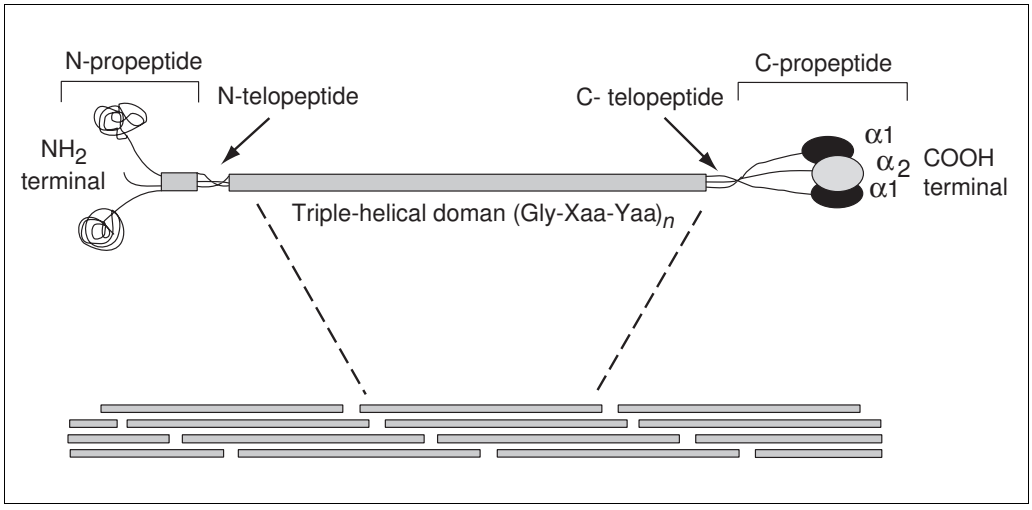


Figure 10.1.1 Functional domains of the type I procollagen molecule. Following cleavage of the propeptide domains in the extracellular space, collagen units assemble in a quarter-stagger arrangement to form a fibril.

prolyl 4-hydroxylase requires Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate. Conditions that prevent proline hydroxylation (such as nutritional deficiency of iron or of vitamin C) affect helix formation or stability. In scurvy, a human disease caused by a dietary deficiency of vitamin C, the nonhydroxylated pro-α chains are unstable and the skin and blood vessels become extremely fragile.

A second post-translational modification of procollagen that is crucial to its function is the hydroxylation of lysine residues. This reaction, which also occurs in the ER, is catalyzed by the enzyme lysyl hydroxylase. The active enzyme is a homodimer and, like prolyl hydroxylase, requires Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate. Hydroxylysine residues have two important functions: their hydroxy groups act as attachment sites for carbohydrate units, and they are essential for the stability of the intermolecular collagen cross-links that occur in the extracellular space after secretion. The glycosylation of hydroxylysine is unusual, consisting of a single galactose residue or a glucosylgalactosyl disaccharide attached to the hydroxyl group. The amount of carbohydrate added to procollagen varies greatly among different types of collagen, and its function is unknown.

Assembly of Collagens

After secretion into the extracellular space, the extension peptides of procollagen are removed by specific proteolytic enzymes. Both the N and C proteinases are members of the zinc metallopeptidase family and contain domains that suggest the ability to interact with cells and other matrix components (Kessler et al., 1996; Colige et al., 1997). Removal of the extension peptides converts the procollagen molecules to collagen (once called tropocollagen). Triple-helical collagen units then come together in the extracellular space to form the much larger collagen fibrils. The process of fibril formation is driven, in part, by the tendency of the collagen molecules to self-assemble. The fibrils form close to the cell surface, however, and it seems likely that the cell regulates the sites and rates of fibril assembly. The nonfibrillar collagens (see below) undergo only limited proteolytic processing prior to assembly. Here it is important to distinguish between collagen and gelatin. As stated above, collagen is the triple-helical form of the protein and can exist as single triple-helical units or triple-helical units polymerized into fibrils. Gelatin is denatured collagen. The individual α chains are no longer in a triple helix but can nevertheless polymerize into a random gel under appropriate conditions of temperature and ionic strength.

Collagen fibrils are greatly strengthened by covalent cross-links within and between the constituent collagen molecules. The types of covalent bonds involved are unique to connective tissue and are formed through deamination of certain lysine and hydroxylysine residues to yield highly reactive aldehyde groups. The aldehydes then undergo classical condensation reactions to form covalent bonds with each other or with other lysine or hydroxylysine residues. The extent and type of cross-linking varies from tissue to tissue, depending on tissue requirements. For example, collagen is highly cross-linked in tendons, where tensile strength is crucial. Lysyl oxidase, the enzyme that catalyzes cross-link formation, requires copper and molecular oxygen. If cross-linking is inhibited, collagenous tissues become fragile, and structures such as skin, tendons, and blood vessels tend to tear.

Collagen Classification

The polymeric structures formed by members of the collagen family vary depending on collagen type (Prockop and Kivirikko, 1995). The structures formed result, in large part, from the nontriple-helical "modules" found within many of the nonfibrillar collagens (Brown and Timpl, 1995). Based on structural similarities, the collagen superfamily can be divided into the following classes.

Fibril-forming collagens: types I, II, III, V, and XI. These collagens (Kühn, 1987; Kadler, 1994) all share a long triple-helical segment with a continuous Gly-Xaa-Yaa repeat over its entire length. They assemble into cross-striated fibers upon cleavage of N and C propeptides, with the individual units adopting a one-quarter stagger relative to their neighbors in the fibril. Types II and XI collagen undergo alternative splicing, and hybrid molecules containing both types V and XI collagen have been identified in some tissues.

Network-forming collagens: types IV, VIII, and X. α chains in the type IV collagen family (Hulmes, 1992; Kühn, 1994; Yurchenco, 1994) contain a large collagenous domain that is frequently interrupted by short noncollagenous sequences (i.e., something other than Gly-Xaa-Yaa). Noncollagenous domains are also found at the N and C termini of the chain, with the C-terminal domain being the larger of the two. Monomers associate at the C termini to form dimers and at the N termini to form tetramers.

The triple-helical domains intertwine to form supercoiled structures, resulting in a net-like structure. Type VIII collagen is found in Descemet's membrane in the eye and forms a stack of hexagonal lattices. A similar structure is formed by type X collagen synthesized by hypertrophic chondrocytes in the deep-calcifying zone of cartilage.

Fibril-associated collagens with interrupted triple helices (FACIT): types IX, XII, XIV, XVI, and XIX. These collagens (Mayne and Brewton, 1993; Olsen et al., 1995) are characterized by short triple-helical segments interrupted by short noncollagenous domains. They attach to the surface of fibril-forming collagens and do not form fibrils themselves. Type IX collagen is found on the surface of type II collagen, to which it is covalently bound. An unusual property of this collagen is the presence of a glycosaminoglycan (GAG) chain attached to a noncollagenous domain of the α2(IX) chain. Types XII and XIV collagen show structural similarities to type IX, including an attached GAG side chain. Types XVI and XIX have not been fully characterized but show similarities in structure to other members of the family.

Beaded filaments and anchoring fibrils: types VI and VII. Among the collagens of this family (Burgeson, 1993; Timpl and Chu, 1994), type VI collagen is characterized by α chains containing large N- and C-terminal globular domains separated by a small triple-helical segment. Alternative splicing produces variants of the α2(VI) and α3(VI) chains. Type VI collagen forms small beaded filaments in the ECM. Type VII collagen forms anchoring fibrils that link basement membranes to anchoring plaques of type IV collagen and laminin in the underlying ECM. Type VII collagen contains the longest triple helix of any known collagen, with only small interruptions throughout. The NC1 domain of type VII collagen binds to collagen types I and IV, fibronectin, and laminin 5.

Collagens with a transmembrane domain: types XIII and XVII. Types XIII and XVII collagen (Li et al., 1996) are unique in having a transmembrane domain with its N terminus predicted to be in the cytoplasm. Type XIII collagen undergoes extensive alternative splicing. Type XVII collagen is found primarily in the hemidesmosomes of the skin and is one of the antigens that produces the autoimmune disease bullous pemphigoid.

Other nonfibrillar collagens: types XV and XVIII. Types XV and XVIII collagen (Rehn and Pihlajaniemi, 1994) have large N- and C-terminal globular domains and a highly interrupted triple helix. Their large number of potential *N*- and *O*-linked glycosylation sites suggests that both types have the potential to be highly glycosylated.

ELASTIN AND MICROFIBRILLAR PROTEINS

Elastin

During evolution, with the advent of the closed circulatory system, came the requirement for blood vessels to accommodate the pulsatile blood flow of the heart. Vessels made mostly of collagen were too stiff, so in its place, we see the emergence of a matrix protein that has the properties of elastic recoil. This protein, elastin, is the predominant protein component of the elastic fiber that is of particular importance to the structural integrity and function of tissues in which reversible extensibility or deformability are crucial, such as the major arterial vessels, lungs, and skin.

In contrast to the genetic diversity evident in the collagen gene family, elastin is encoded by only one gene. Like collagen, elastin maturation in the ECM involves the assembly of a soluble precursor molecule (tropoelastin) into a highly cross-linked polymer. This assembly process is more complex than that for collagen, however, because the ability to self-assemble does not appear to be an intrinsic property of tropoelastin. Instead, elastin assembly requires helper proteins to align the multiple cross-linking sites on elastin monomers (Mecham and Davis, 1994).

Two functional domains repeat along the tropoelastin molecule (Fig. 10.1.2). One domain, related to cross-link formation, is an α helix containing alanine and lysine. The other, related to extensibility, is enriched in glycine, valine, and proline. The hydrophobic amino acids in this domain are arranged in repeating sequences that form a succession of β turns. The stacked β turns form a β spiral with a hydrophobic core. Stretching the elastin polymer exposes the hydrophobic core to water. Recoil occurs when the leaves of the β spiral contract to shield the hydrophobic amino acids from the aqueous microenvironment. Mature, cross-linked elastin is extremely hydrophobic and insoluble under most conditions (including when boiled in sodium hydroxide; Partridge, 1962). Its unusual physical properties make insoluble elastin one of the most stable proteins in the body—lasting the lifetime of the organism. Two polyfunctional cross-links, desmos-

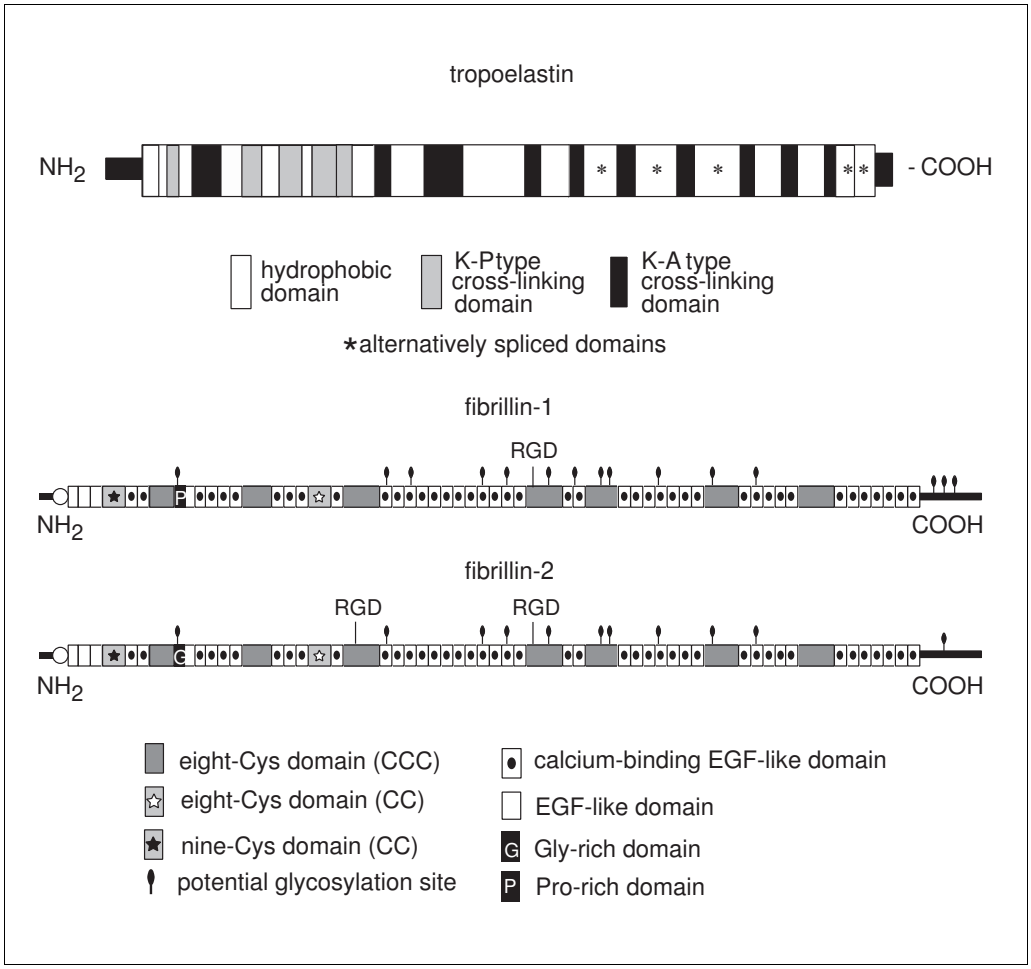


Figure 10.1.2 Domain map of tropoelastin and the fibrillins. Tropoelastin is secreted as a peptide of ~70 kDa and undergoes extensive covalent cross-linking during incorporation into the elastic fiber. Fibrillin-1 and fibrillin-2 each have a molecular weight of ~350 kDa and are the major structural elements of 10- to 12-nm-diameter microfibrils. Abbreviations: K-A, alanine-rich cross-linking domain; K-P, proline-rich cross-linking domain; RGD, Arg-Gly-Asp; CC, Cys-Cys sequences; CCC, Cys-Cys-Cys sequences; EGF, epidermal growth factor.

ine and isodesmosine, are unique to elastin and can be used as specific markers for this protein.

Fibrillin

Microfibrils were first identified as components of elastic fibers. They are found in greatest abundance in elastic tissues or in the ciliary zonules of the eye, although their distribution is widespread. Fibrillin-1 and -2 play key roles in microfibrillar architecture. These 350-kDa glycoproteins are highly homologous (Fig. 10.1.2), with modular structures consisting of repeating calcium-binding epidermal growth factor (EGF)–like domains interspersed between 8-cysteine domains similar to those found in the latent transforming growth factor-β (TGF-β)–binding protein family (Lee et al., 1991). Tandemly arranged EGF domains form a structural motif found frequently in ECM macromolecules (e.g., laminin, fibulin, latent TGF-β-binding protein, nidogen). When stacked together, these tightly folded, disulfide-bonded loop structures form a rigid, rod-like arrangement stabilized by interdomain calcium binding and hydrophobic interactions (Downing et al., 1996). The precise function of microfibrils is unclear, although their association with developing elastic fibers suggests a role in elastin assembly. Both fibrillin-1 and fibrillin-2 interact with the αvβ3 integrin through an Arg-Gly-Asp (RGD) sequence (see Adhesive Glycoproteins).

ADHESIVE GLYCOPROTEINS

Most, if not all, ECM macromolecules interact with binding proteins on the surface of cells. In many instances, this is through a unique sequence motif that is accessible as part

of the protein's folded functional structure, or cryptic and exposed only when the protein undergoes a conformational change induced by binding to another protein or as the result of degradation or denaturation. One such "recognition motif" is the well-known RGD sequence that is recognized by several members of the integrin family.

Fibronectin

A great deal of biochemical work has led to a model of the fibronectin molecule in which the protein's binding functions and its structure are clearly correlated (Hynes, 1990). The molecule is secreted as a dimer consisting of two similar subunits joined together at the C terminus by disulfide bonds (Fig. 10.1.3). Each chain has a molecular weight of ~220 to 250 kDa and is subdivided into a series of tightly folded domains. Each domain is responsible for one of fibronectin's binding functions. In plasma, fibronectin exists as a soluble dimer, but in the ECM it is found as an insoluble multimer.

Amino acid sequence analysis of fibronectin shows that the molecule is made up mostly of three repeating motifs, referred to as types I, II, and III repeats. These repeats are organized into functional domains that contain binding sites for ECM proteins and cell surface receptors (see Fig. 10.1.3). For example, there are two fibrin-binding domains consisting of multiple type I repeats on each subunit of the protein. Type I repeats are also found in the collagen-binding domain, and the first five type I repeats play an important role in matrix assembly. The N-terminal domain of fibronectin also mediates fibronectin's binding to gram-positive bacteria through type I modules. The type I module contains ~45 amino acids with four cysteines forming two disulfide bonds (Potts and Campbell, 1994). This module has also been found in a number of other proteins. In addition to type I repeats, the collagen-binding domain contains the only type II repeats found in fibronectin. Like type I repeats, these motifs contain two disulfide bonds, but they are larger than type I motifs.

The predominant structural feature of fibronectin consists of type III repeats, accounting for more than 60% of the sequence. No disulfide bonds are present in this structure, although two of the repeats contain a free cysteine. The cell-binding RGD sequence is located in the tenth type III repeat. This sequence is recognized by many members of the integrin family, including $\alpha5\beta1$, $\alpha v\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, $\alpha v\beta6$, $\alpha IIb\beta3$, and $\alpha8\beta1$. Other cell-binding regions include the C-terminal heparin-binding domain and the type III–connecting segment (IIICS), including the CS1 region. The type III consensus sequence is frequently found in other proteins.

Only one gene for fibronectin has been identified, but mRNAs for fibronectin have been shown to give rise to multiple versions of the protein through variable patterns of RNA splicing during gene transcription. Alternative splicing occurs predominately at three sites, termed extra type III domain A (EDA or EIIIA), extra type III domain B (EDB or EIIIB), and the

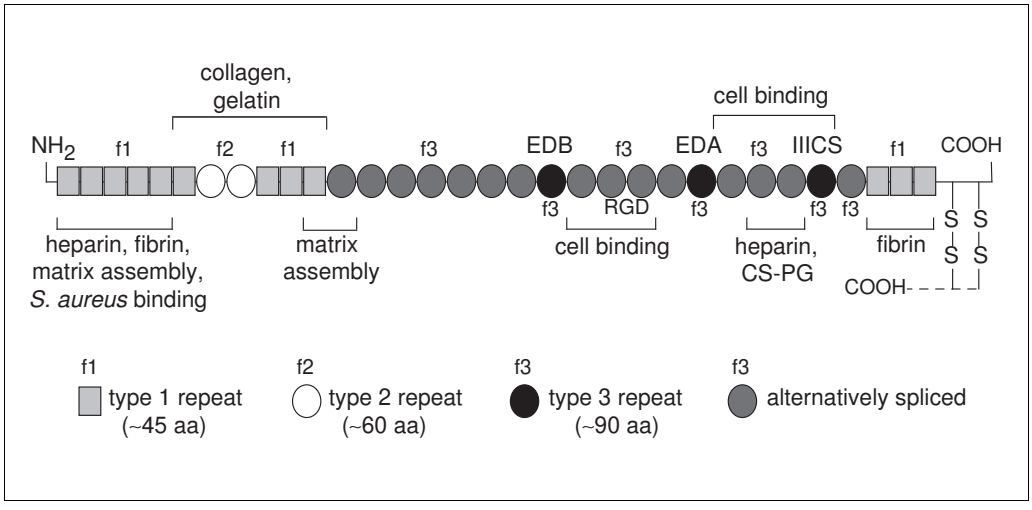


Figure 10.1.3 Domain map of fibronectin. The subunits of fibronectin vary in size between ~235 and 270 kDa. Alternative splicing occurs at three positions: EDA, EDB, and IIICS. Binding sites for other molecules and cells are indicated. Abbreviations: EDA, extra type III domain A; EDB, extra type III domain B; IIICS, connecting segment between the fourteenth and fifteenth type III repeats; RGD, Arg-Gly-Asp; CS-PG, chondroitin sulfate proteoglycan; aa, amino acids.

connecting segment between the fourteenth and fifteenth type III repeats (IIICS or V). Splicing within the IIICS segment produces five variants, such that twenty different fibronectin subunits can result from splicing within the three segments.

Subunits of plasma fibronectin produced by adult hepatocytes contain neither EDA nor EDB segments, and one subunit lacks the entire IIICS domain. Cultured fibroblasts, however, typically produce a form of fibronectin, referred to as cellular fibronectin, that contains the EDA and/or EDB segments. Fibronectins expressed in fetal and tumor tissues contain a greater percentage of EDA and EDB segments than those expressed in normal adult tissues. The biological functions of fibronectin isoforms are only poorly understood, despite having been studied extensively. Differences in solubility have been demonstrated, but it has been difficult to detect functional differences between plasma and cellular fibronectin in their ability to promote cell adhesion and spreading.

Vitronectin

Vitronectin (also called serum spreading factor, S-protein, and epibolin) is a multifunctional protein found in plasma and ECM. It is synthesized as a single chain that undergoes N glycosylation, tyrosine sulfation, and phosphorylation prior to secretion. In plasma, vitronectin circulates in two forms: a single chain of ~75 kDa and a proteolytically cleaved, two-chain form that dissociates into 65- and 10-kDa fragments upon reduction. It is present in fibrillar form in the ECM of a variety of tissues, where it sometimes colocalizes with fibronectin and elastic fibers. While little vitronectin immunoreactivity is detectable in most normal tissues, increased deposition has been observed in areas of tissue injury and necrosis. Tissue vitronectin was believed to be plasma derived, but recent studies indicate that extrahepatic cells have the biosynthetic potential to produce vitronectin and that its synthesis can be regulated under inflammatory conditions (Seiffert, 1997).

The cell attachment activity of vitronectin results from an RGD sequence that is recognized by a wide variety of integrins. Most of the cell adhesive activity of serum used for tissue culture can be attributed to vitronectin.

Laminin and Basement Membranes

Like fibronectin, the laminins are modular proteins with domains that interact with both cells and ECM (Ekblom and Timpl, 1996). They constitute a family of basement membrane glycoproteins that affect cell proliferation, migration, and differentiation. Eleven different laminins have been identified, each containing an α, β, and γ chain (Fig. 10.1.4). Electron microscopy has revealed that all laminins have a cross-like shape with three short arms and one rod-like long arm, a shape well suited for mediating interactions between sites on cells and components of the ECM (Beck et al., 1990; Maurer and Engel, 1996). The rod-like regions separating the globular units of the short arms are made up of repeating EGF-like domains. The long arm is formed by all three component chains folding into an α-helical coiled-coil structure, and is the only domain composed of multiple chains. It is terminated by a large globular domain composed of five homologous subdomains formed by the C-terminal region of the α chain.

Along with type IV collagen, laminins are a major structural element of the basal lamina (Timpl, 1996). The molecular architecture of these matrices results from specific binding interactions among the various components. The structural skeleton is formed by type IV collagen chains that assemble into a covalently stabilized polygonal network. Laminin self-assembles through terminal domain interactions to form a second polymer network. Nidogen (Mayer and Timpl, 1994) binds laminin near its center and interacts with type IV collagen, bridging the two. A large heparan sulfate proteoglycan (HS-PG), perlecan, binds laminin and type IV collagen through its GAG chains and forms dimers and oligomers through a core-protein interaction. Perlecan is important for charge-dependent molecular sieving, one of the critical functions of basement membrane. Other components that are sometimes found associated with basement membranes but may not be intrinsic components include fibronectin, type V collagen, fibulin, osteonectin (also known as BM-40 or SPARC), and chondroitin sulfate proteoglycans.

Cells attach to laminin through specific interaction sites created by its multidomain structure. For example, sites for receptor-mediated cell attachment and promotion of neurite outgrowth reside in the terminal region of the long arm. A second cell-attachment site and a cell-signaling site with mitogenic action are localized in the short arms. Cell binding to laminin occurs via a variety of receptors, including non-integrins (Mecham and Hinek, 1996) and integrins (Aumailley et al., 1996). The β1 family includes most of the laminin-binding integrins (α1β1, α2β1, α3β1, α7β1, α9β1). Other

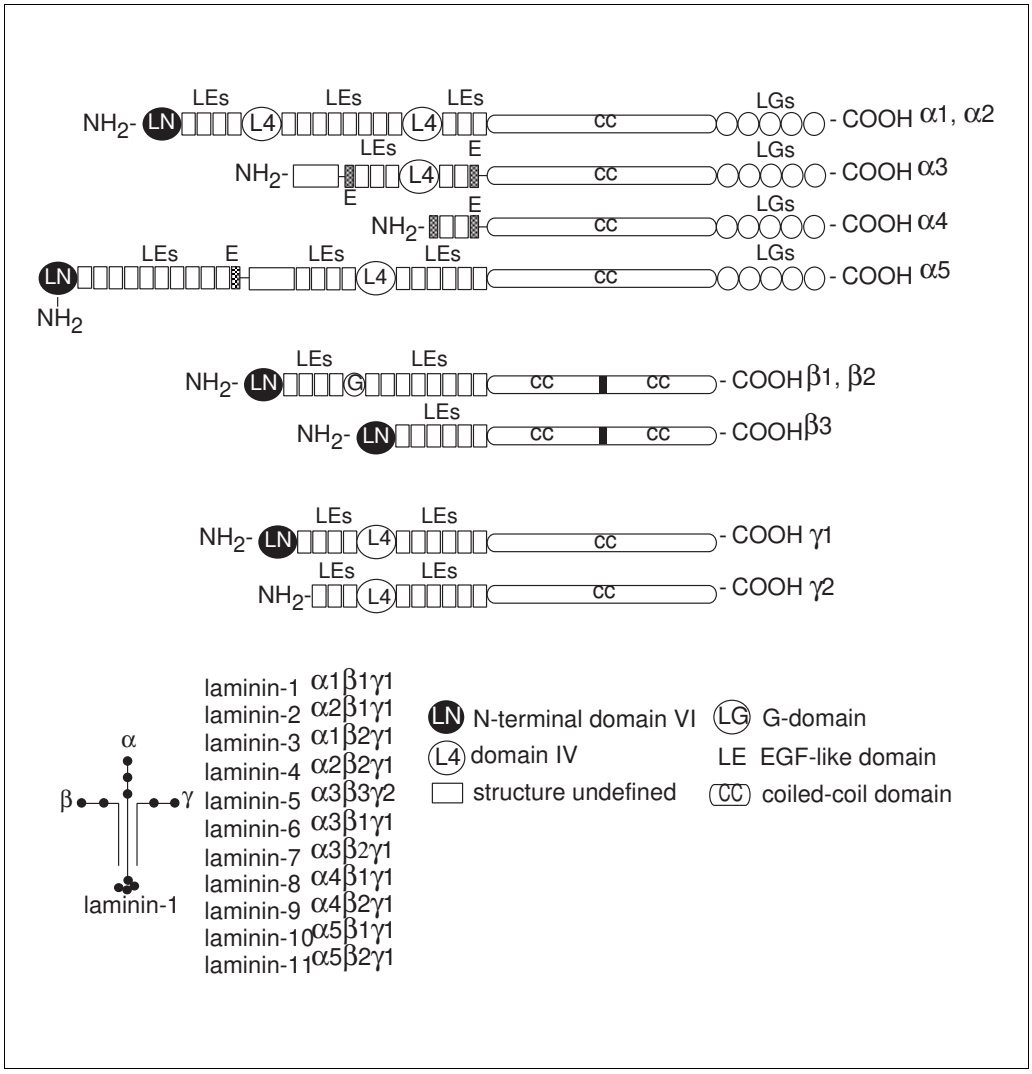


Figure 10.1.4 Domain map of laminin chains. Three polypeptide chains (α, β, and γ) form the laminin cross. The chain composition of known laminin types is shown in the insert. Abbreviation: EGF, epidermal growth factor.

integrins that bind laminin include αvβ3 and α6β4. Basement membrane can also have an indirect effect on cells by binding and sequestering growth and differentiation factors, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and TGF-β.

The importance of laminin to cell differentiation and migration has been demonstrated in developmental studies. Isoforms of laminin assembled from different chains are focally and transiently expressed and may serve distinct functions at early stages of development even before being deposited as components of basement membranes. Laminin is present at the two-cell stage in the mouse embryo, making it one of the first ECM proteins detected during embryogenesis.

MATRICELLULAR PROTEINS

The term "matricellular" has been applied to a group of extracellular proteins that function by binding to matrix proteins and to cell surface receptors, but do not contribute to the structural integrity of the ECM (Bornstein, 1995). Proposed members of this group include the thrombospondins, members of the tenascin protein family, SPARC/osteonectin (Lane and Sage, 1994), and osteopontin. These proteins are frequently called "antiadhesive proteins" because of their ability to induce rounding and partial detachment of some cells in vitro (Sage and Bornstein, 1991). Their ability to interact with many different matrix proteins and cell surface receptors may explain their complex range of biological functions.

Thrombospondin

The thrombospondin (TSP) family consists of five secreted glycoproteins (Adams et al., 1995). TSP-1 and TSP-2 have identical domain structures and are secreted as disulfide-bonded homotrimers (Fig. 10.1.5). TSP-3, TSP-4, and TSP-5/COMP (cartilage oligomeric matrix protein) are pentamers whose expression is more limited than that of TSP-1 and TSP-2. TSP-1 binds HS-PGs, various integrins, the integrin-associated protein, and CD36. It also binds plasminogen, fibrinogen, fibronectin, urokinase, and TGF-β (which it can also activate). TSP-1 exhibits variable effects on cell adhesion and cell proliferation (Bornstein, 1995). For example, TSP-1 promotes proliferation of vascular smooth muscle cells, yet inhibits proliferation of endothelial cells. It supports attachment and spreading of skeletal myoblasts but expresses antiadhesive activity toward endothelial cells. Thrombospondin is the most abundant protein of platelet alpha granules and is released when platelets are activated.

Tenascin

The tenascins constitute a gene family consisting of four members: tenascins-C, -R, -X, and -Y (Erickson, 1993; Chiquet-Ehrismann, 1995). Tenascin-C (early names include GMEM, cytotactin, J1, hexabrachion, and neuronectin) was the first form discovered and exists as a hexamer of disulfide-bonded subunits. Each subunit consists of a cysteine-rich N-terminal domain involved in oligomerization, EGF-like repeats, fibronectin type III–like repeats, and a fibrinogen-like globular domain (Fig. 10.1.5). The number of fibronectin type III–like repeats varies as a result of alternative splicing. Like TSP, tenascin-C has diverse biological effects when applied to cells. Both stimulation and inhibition of cellular proliferation have been observed in response to tenascin-C. In terms of cell adhesion, some cells do attach to tenascin, but weakly. In most instances, tenascin does not allow cell adhesion and can even inhibit cell attachment to other matrix proteins such as fibronectin and laminin. The finding that tenascin-C contains defined cell attach-

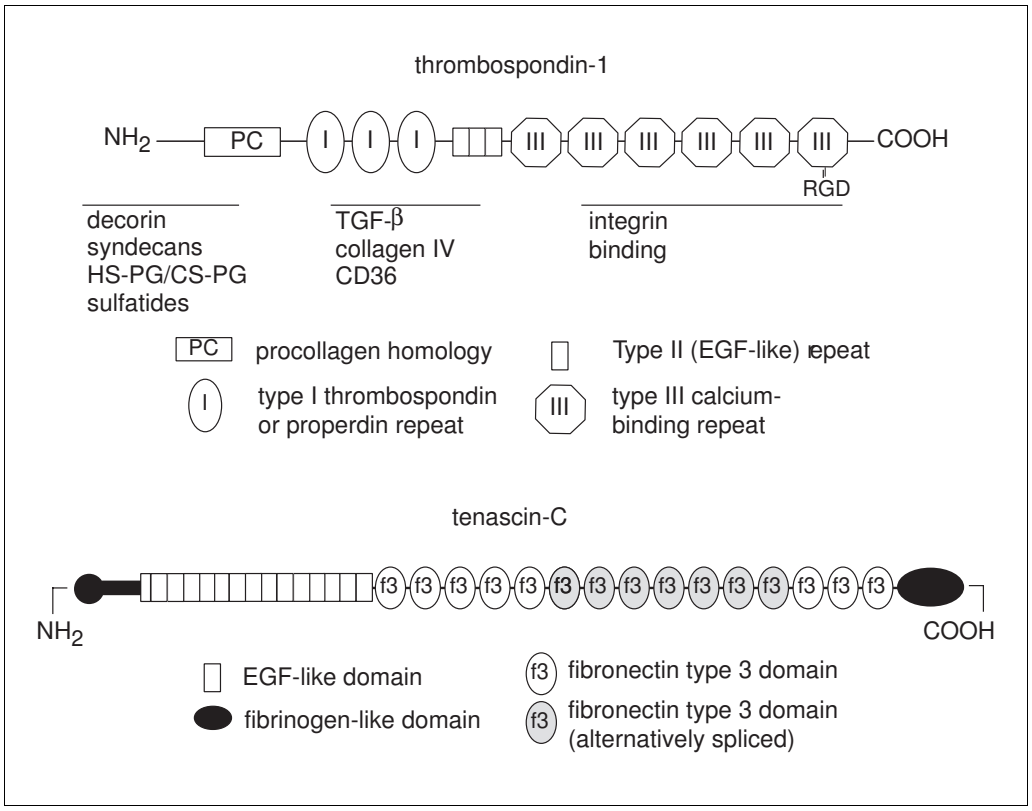


Figure 10.1.5 Domain map of thrombospondin-1 and tenascin-C. The functional form of thrombospondin is a homotrimer of ~420 kDa. It interacts with numerous matrix proteins and modulates cell attachment by interacting with various cell-surface receptors. Tenascin-C monomers form a hexameric structure joined at their N termini by disulfide bonds. Alternative splicing leads to subunits of differing molecular weights. Abbreviations: HS-PG, heparan sulfate proteoglycan; CS-PG, chondroitin sulfate proteoglycan; TGF-β, transforming growth factor-β; RGD, Arg-Gly-Asp; EGF, epidermal growth factor.

ment sites suggests that the overall antiadhesive properties of the glycoprotein are effected by separate domains that override the attachment domains.

PROTEOGLYCANS

The proteoglycans (once called acid mucopolysaccharides) constitute a number of genetically unrelated families of multidomain proteins that have covalently attached GAG chains. To date, more than 25 distinct gene products have been identified that carry at least one GAG chain (Iozzo and Murdoch, 1996). Like other matrix components discussed in this review, proteoglycans exist as structural variants, further increasing their functional and structural diversity.

For historical reasons, proteoglycans are named based on the type of attached GAG chain(s): (1) chondroitin sulfate and dermatan sulfate, consisting of a repeating disaccharide of galactosamine and either glucuronic acid or iduronic acid; (2) heparin and heparan sulfate, consisting of a repeating disaccharide of glucosamine and either glucuronic acid or iduronic acid; and (3) keratan sulfate, consisting of a repeating disaccharide of glucosamine and galactose. Hyaluronate is also a repeating disaccharide but is not sulfated and not bound to a core protein. GAG chains are usually attached through *O*-glycosidic linkages to serine residues in the proteoglycan core protein. A characteristic feature of GAG chains is that at physiological pH they contain one to three negative charges per disaccharide due to carboxylate and sulfate groups.

Knowledge of the structure and function of proteoglycans increased dramatically when molecular biology was used to study the core proteins (Hassell et al., 1993). The heterogeneity of this family of matrix proteins also became evident with the finding that there are no structural domains common to all proteoglycans. There are, however, distinguishing characteristics that allow them to be grouped into four broad categories.

Large Proteoglycans that Form Aggregates by Interaction with Hyaluronan

These proteoglycans interact with strands of hyaluronate to form a very-high-molecular-weight aggregate. A structural trait shared by these proteoglycans is the presence of three functional domains: a globular hyaluronan-binding domain at the N terminus, a central extended region that carries most of the GAG chains, and a modular C-terminal domain containing two EGF repeats, a C-type lectin domain, and a complement-regulatory-protein-like motif (Iozzo and Murdoch, 1996).

The largest member of this family is versican (Zimmermann and Ruoslahti, 1989), a major proteoglycan in blood vessels that is also expressed in nonvascular tissues. Aggrecan, the large aggregating proteoglycan of cartilage, has a smaller core protein than versican but contains nearly 3-fold more GAG chains (Fig. 10.1.6). The high charge density of aggrecan results in each monomer occupying a large hydrodynamic volume. Aggrecan's GAG chains result in a high density of fixed charge in cartilage, producing an osmotic swelling pressure that is balanced by tension in the collagenous network. The reversible redistribution of proteoglycan-bound water under loading gives cartilage the ability to absorb compressive loads (Wight et al., 1991). Two other members of this family include neurocan (Rauch et al., 1992) and brevican (Yamada et al., 1994), both found in brain tissues.

Basement Membrane Proteoglycans

HS-PGs appear to be ubiquitous components of all basement membranes. Perlecan is the largest basement membrane proteoglycan, with a modular core protein of 467 kDa (Fig. 10.1.6; Iozzo et al., 1994). It provides the basement membrane with a negative charge that is important to its sieving properties. The heparan sulfate chains of perlecan also bind growth factors and cytokines and sequester them into the basement membrane, where they may function as a reserve to be released during tissue repair. The interaction of heparan sulfate with the FGFs has been extensively studied (Aviezer et al., 1994). Perlecan interacts with other components of the basement membrane, particularly laminin and nidogen. The multidomain structure of perlecan core protein is reminiscent of other ECM proteins, and includes EGF repeats and repeats of structures found in the low-density lipoprotein receptor, laminin chains, and neural cell adhesion molecule.

Agrin was originally isolated from torpedo ray electric organ and was found to induce acetylcholine receptor aggregation. It is secreted by motor neurons and deposited in the synaptic cleft basement membrane. Agrin may also play a role in the sequestration of growth factors in the basement membrane. Like perlecan, agrin is a multidomain protein with regions of EGF and laminin G-domain homology. Agrin is found predominantly in the brain, but

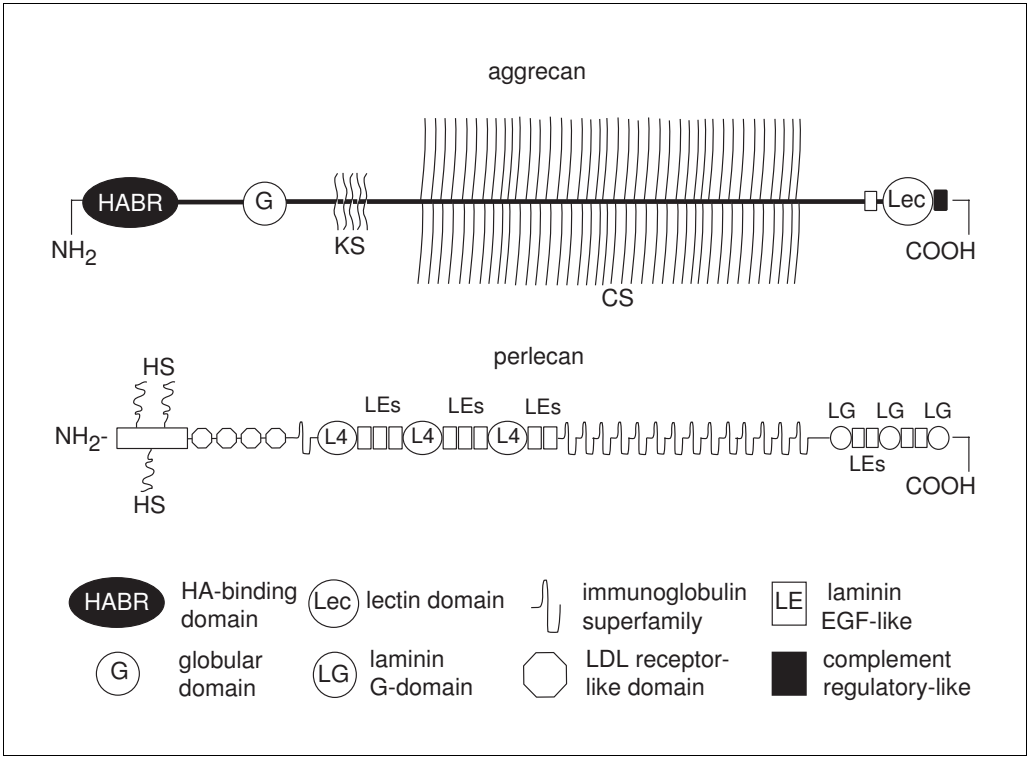


Figure 10.1.6 Domain map of two representative large proteoglycans. Aggrecan is the core protein of the aggregating proteoglycan found in cartilaginous tissues. The molecular weight of the aggrecan core protein is 210 to 250 kDa. There are 100 to 150 keratan sulfate chains and many more chondroitin sulfate chains that contribute to the 2500-kDa molecular weight of the mature proteoglycan. The glycosaminoglycans are attached to repetitive sequences in the middle two-thirds of the molecule, including several types of repeats containing Ser-Gly, the linkage site for chondroitin sulfate. Perlecan is the largest of the basement membrane proteoglycans and has two or three attached heparan sulfate side chains. Removal of heparan sulfate side chains by heparatinase produces a core protein of 400 to 450 kDa on SDS-PAGE. Abbreviations: HA, hyaluronate; KS, keratan sulfate; CS, chondroitin sulfate; HS, heparan sulfate; LDL, low-density lipoprotein; EGF, epidermal growth factor.

has also been localized to smooth and cardiac muscle.

Cell Surface Heparan Sulfate Proteoglycans

HS-PGs on the cell surface influence several important biological functions, including cell adhesion; the sequestration of heparin-binding ligands on the plasma membrane; and the promotion of dimerization/oligomerization of bound ligands, which enhances activation of primary signaling receptors.

Cell-associated HS-PGs have been divided into two major families, syndecan-like integral membrane HS-PGs (SLIPs) and glypican-related integral membrane HS-PGs (GRIPs; David, 1993; Carey, 1997). The SLIPs are transmembrane HS-PGs with a conserved intracellular domain that likely interacts with cytoskeletal and regulatory proteins. The GRIPs are linked to the cell surface by glycosyl phosphatidyl inositol in the outer leaflet of the lipid bilayer.

The syndecans, the principal form of cell-surface HS-PG, are synthesized by many cells. Syndecans bind a variety of extracellular ligands via their covalently attached heparan sulfate chains and are thought to play important roles in cell-matrix and cell-cell adhesion, migration, and proliferation. To date, four homologous syndecan core proteins have been cloned from vertebrate cells. All syndecans are type I transmembrane proteins, with an N-terminal signal peptide, an ectodomain that contains several consensus sequences for GAG attachment, a single hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain. The majority of GAG chains added to syndecan core proteins are of the heparan sulfate type, although syndecan-1 and syndecan-4 have chondroitin sulfate chains attached as well. Syndecans act as cell surface

receptors for a number of matrix molecules, thereby mediating cell attachment and tissue organization. They influence the interactions of basic FGF and other growth factors with their receptors on cells and are responsible for the maintenance of a nonthrombogenic surface on endothelial cells.

Small Leucine-Rich Proteoglycans

Small leucine-rich proteoglycans (SLRPs) comprise a class of secreted proteoglycans that include five structurally related members: decorin, biglycan, fibromodulin, lumican, and epiphycan (see Fig.10.1.7). Each has a leucine-rich core protein that assumes an arch-shaped structure with a concave surface capable of interacting with various other proteins. The N-terminal region contains one (decorin) or two (biglycan and epiphycan) GAG chains that can be either dermatan or chondroitin sulfate. Instead of GAG chains, fibromodulin and lumican have tyrosine sulfate in the N terminus, which provides an analogous negatively charged domain. These two SLRPs also contain N-linked keratan sulfate chains in their central domain.

SLRPs interact with numerous ECM proteins (e.g., fibronectin, TSP, fibrillin, microfibril-associated glycoprotein) and act to orient and order collagen fibers during development

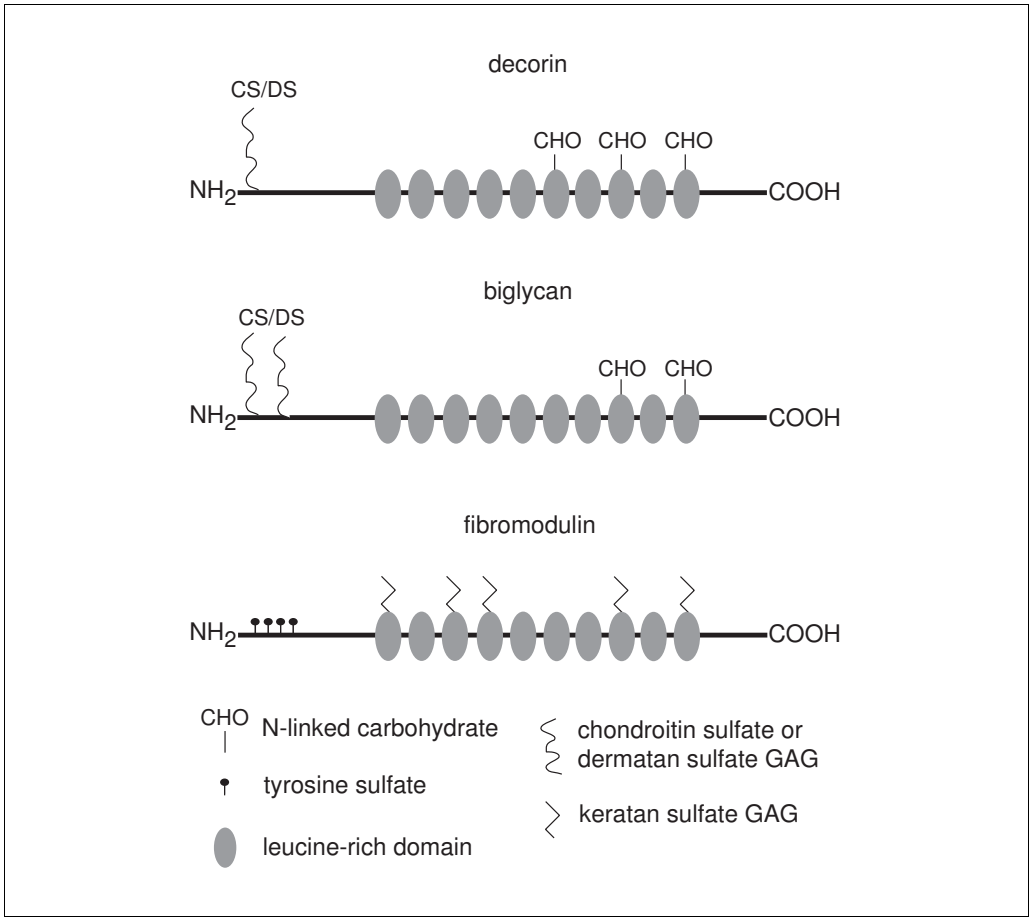


Figure 10.1.7 Domain map of representative members of the small leucine-rich proteoglycans. Decorin contains a single chondroitin or dermatan sulfate chain attached near the N terminus. The core protein is ~38 kDa. Decorin is heterogeneous with respect to glycosaminoglycan (GAG) chain size, such that the secreted proteoglycan shows a range of molecular weights centered between 100 and 250 kDa. The core protein of biglycan is similar in size to that of decorin, except biglycan contains two chondroitin or dermatan sulfate chains. The GAG chains are also heterogeneous in size, resulting in a broad band on SDS-PAGE centered anywhere from 200 to 350 kDa. Removal of GAG chains with chondroitin ABC-lyase results in a 45-kDa band. Fibromodulin has a core protein size of 42 kDa. Four of the five potential N-glycosylation sites in the leucine-rich region of the molecule are substituted with keratan sulfate chains. Five to seven closely spaced tyrosine sulfate residues are found in the N-terminal domain. Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate.

and tissue remodeling. Interactions with matrix proteins occur through the leucine-rich core which, in the case of type I collagen, influences collagen fibrillogenesis by binding to the surface of the collagen fibril at the d-band with the highly charged GAG chain extending out to regulate interfibrillar distances. Like other proteoglycans, SLRPs bind to growth factors (e.g., TGF-β) and thereby likely influence cellular differentiation and matrix synthesis. Decorin has recently been shown to directly regulate cell growth by activating the EGF receptor (Moscatello et al., 1998).

CONCLUSIONS

The furious pace of advances in the molecular biology of ECM has greatly expanded the knowledge of individual matrix components. The structure of many matrix macromolecules, for example, was determined from cloned cDNAs or genes long before complete protein information was available. With this increased knowledge as background, there is a growing realization that the information contained in the ECM is not a monosyllabic message encoded by individual molecules, but a complex and intricate arrangement dictated by the combinatorial organization of the supramolecular structure. As the focus of biological research changes from the letters to the message, understanding how cells read and interpret this information will undoubtedly reveal more about the letters in the code.

LITERATURE CITED

Adams, J.C., Rucker, R.P., and Lawler, J. 1995. The Thrombospondin Gene Family. R.G. Landis, Austin, Tex.

Aumailley, M., Gimond, C., and Rouselle, P. 1996. Integrin-mediated cellular interactions with laminins. *In* The Laminins (P. Ekblom and R. Timpl, eds.) pp. 127-158. Harwood Academic Publishers, New York.

Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. 1994. Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor receptor binding, mitogenesis, and angiogenesis. *Cell* 79:1005-1013.

Beck, K., Hunter, I., and Engel, J. 1990. Structure and function of laminin: Anatomy of a multidomain glycoprotein. *FASEB J.* 4:148-160.

Bornstein, P. 1995. Diversity of function is inherent in matricellular proteins: An appraisal of thrombospondin 1. *J. Cell Biol.* 130:503-506.

Brodsky, B. and Ramshaw, J.A. 1997. The collagen triple-helix structure. *Matrix Biol.* 15:545-554.

Brown, J.C. and Timpl, R. 1995. The collagen superfamily. *Int. Arch. Allergy Immunol.* 107:484-490.

Burgeson, R.E. 1993. Type VII collagen, anchoring fibrils, and epidermolysis bullosa. *J. Invest. Dermatol.* 101:252-255.

Carey, D.J. 1997. Syndecans: Multifunctional cell-surface co-receptors. *Biochem. J.* 327:1-6.

Chiquet-Ehrismann, R. 1995. Inhibition of cell adhesion by anti-adhesive molecules. *Curr. Opin. Cell Biol.* 7:715-719.

Colige, A., Li, S.W., Sieron, A.L., Nusgens, B.V., Prockop, D.J., and Lapiere, C.M. 1997. cDNA cloning and expression of bovine procollagen I N-proteinase: A new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. *Proc. Natl. Acad. Sci. U.S.A.* 92:2374-2379.

David, G. 1993. Integral membrane heparan sulfate proteoglycans. *FASEB J.* 7:1023-1030.

Downing, A.K., Knott, V., Werner, J.M., Cardy, C.M., Campbell, I.D., and Handford, P.A. 1996. Solution structure of a pair of calcium-binding epidermal growth factor-like domains: Implications for the Marfan syndrome and other genetic disorders. *Cell* 85:597-605.

Ekblom, P. and Timpl, R. 1996. The Laminins. Harwood Academic Publishers, New York.

Erickson, H.P. 1993. Tenascin-C, tenascin-R, tenascin X: A family of talented proteins in search of functions. *Curr. Opin. Cell Biol.* 5:869-876.

Hassell, J.R., Blochberger, T.C., Rada, J.A., Chakravarti, S., and Noonan, D. 1993. Proteoglycan gene families. *In* Extracellular Matrix. Advances in Molecular and Cellular Biology (H.K. Kleinman, ed.), Vol. 6, pp. 69-113. JAI Press, Greenwich, Conn.

Hay, E.D. (ed.) 1991. Cell Biology of Extracellular Matrix, 2nd ed. Plenum, New York.

Hulmes, D.J.S. 1992. The collagen superfamily: Diverse structures and assemblies. *Essays Biochem.* 24:49-76.

Hynes, R.O. 1990. Fibronectins. Springer-Verlag, New York.

Iozzo, R.V. and Murdoch, A.D. 1996. Proteoglycans of the extracellular environment: Clues from the gene and protein side offer novel perspectives in molecular diversity and function. *FASEB J.* 10:598-614.

Iozzo, R.V., Cohen, I.R., Grässel, S., and Murdoch, A.D. 1994. The biology of perlecan: The multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. *Biochem J.* 302:625-639.

Kadler, K. 1994. Extracellular matrix 1: Fibril-forming collagens. *Protein Profile* 1:519-638.

Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D.S. 1996. Bone morphogenetic protein-1: The type I procollagen C–proteinase. *Science* 271:360-362.

Kivirikko, K.I. and Myllyharju, J. 1998. Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. *Matrix Biol.* 16:357-368.

Kleinman, H.K. (ed.) 1993. Extracellular Matrix. Advances in Molecular and Cellular Biology, Vol. 6. JAI Press, Greenwich, Conn.

Koivu, J., Myllylä, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K., and Kivirikko, K.I. 1987. A single polypeptide acts both as the β subunit of prolyl 4-hydroxylase and as a protein disulfide-isomerase. *J. Biol. Chem.* 262:6447-6449.

Kühn, K. 1987. The classical collagens: Types I, II, and III. *In* Structure and Function of Collagen Types (R. Mayne and R.E. Burgeson, eds.) pp. 1-42. Academic Press, San Diego.

Kühn, K. 1994. Basement membrane (type IV) collagen. *Matrix Biol.* 14:439-445.

Lane, T.F. and Sage, E.H. 1994. The biology of SPARC, a protein that modulates cell-matrix interactions. *FASEB J.* 8:163-173.

Lee, B., Godfrey, M., Vitale, E., Hori, H., Mattei, M.-G., Sarfarazi, M., Tsipouras, P., Ramirez, F., and Hollister, D.W. 1991. Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. *Nature* 352:330-334.

Li, K., Tamai, K., Tan, E.M., and Uitto, J. 1996. Cloning of type XVII collagen. Complementary and genomic DNA sequences of mouse 180-kilodalton bullous pemphigoid antigen (BPAG2) predict an interrupted collagenous domain, a transmembrane segment, and unusual features in the 5′-end of the gene and the 3′-untranslated region of the mRNA. *J. Biol. Chem.* 268:8825-8834.

Maurer, P. and Engel, J. 1996. Structure of laminins and their chain assembly. *In* The Laminins (P. Ekblom and R. Timpl, eds.) pp. 27-49. Harwood Academic Publishers, New York.

Mayer, U. and Timpl, R. 1994. Nidogen: A versatile binding protein of basement membranes. *In* Extracellular Matrix Assembly and Structure (P.D. Yurchenco, D.E. Birk, and R.P. Mecham, eds.) pp. 318-416. Academic Press, San Diego.

Mayne, R. and Brewton, R.G. 1993. New members of the collagen superfamily. *Curr. Opin. Struct. Biol.* 5:883-890.

Mecham, R.P. and Davis, E.C. 1994. Elastic fiber structure and assembly. *In* Extracellular Matrix Assembly and Structure (P.D. Yurchenco, D.E. Birk, and R.P. Mecham, eds.) pp. 281-314. Academic Press, San Diego.

Mecham R.P. and Hinek, A. 1996. Non-integrin laminin receptors. *In* The Laminins (P. Ekblom and R. Timpl, eds.) pp. 159-183. Harwood Academic Publishers, New York.

Moscatello, D.K., Santra, M., Mann, D.M., McQuillan, D.J., Wong, A.J., and Iozzo, R.V. 1998. Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor. *J. Clin. Invest.* 101:4406-4412.

Olsen, B.R., Winterhalter, K.H., and Gordon, M.K. 1995. FACIT collagens and their biological roles. *Trends Glycos. Glycotech.* 7:115-127.

Partridge, S.M. 1962. Elastin. *Adv. Protein Chem.* 17:227-302.

Piez, K.A. 1997. History of extracellular matrix: A personal view. *Matrix Biol.* 16:85-92.

Potts, J.R. and Campbell, I.D. 1994. Fibronectin structure and assembly. *Curr. Opin. Cell Biol.* 6:648-655.

Prockop, D.J. and Kivirikko, K.I. 1995. Collagens: Molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* 64:403-434.

Rauch, U., Karthikeyan, L., Maurel, P., Margolis, R.U., and Margolis, R.K. 1992. Cloning and primary structure of neurocan, a developmentally regulated, aggregating chondroitin sulfate proteoglycan of brain. *J. Biol. Chem.* 267:19536-19547.

Rehn, M. and Pihlajaniemi, T. 1994. Alpha 1 (XVIII), a collagen chain with frequent interruptions in the collagenous sequence, a distinct tissue distribution, and homology with type XV collagen. *Proc. Natl. Acad. Sci. U.S.A.* 91:4234-4238.

Sage, E.H. and Bornstein, P. 1991. Extracellular proteins that modulate cell-matrix interactions. *J. Biol. Chem.* 266:14831-14834.

Seiffert, D. 1997. Constitutive and regulated expression of vitronectin. *Histol. Histopath.* 12:787-797.

Timpl, R. 1996. Macromolecular organization of basement membranes. *Curr. Opin. Cell Biol.* 8:618-624.

Timpl, R. and Chu, M.L. 1994. Microfibrillar collagen type VI. *In* Extracellular Matrix Assembly and Structure (P.D. Yurchenco, D.E. Birk, and R.P. Mecham, eds.) pp. 207-242. Academic Press, San Diego.

Wight, T.N., Heinegard, D.K., and Hascall, V.C. 1991. Proteoglycans: Structure and function. *In* Cell Biology of Extracellular Matrix, 2nd ed. (E.D. Hay, ed.) pp. 45-78. Plenum, New York.

Yamada, H., Watanabe, K., Shimonaka, M., and Yamaguchi, Y. 1994. Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family. *J. Biol. Chem.* 269:10119-10126.

Yurchenco, P.D. 1994. Assembly of laminin and type IV collagen into basement membrane networks. *In* Extracellular Matrix Assembly and Structure (P.D. Yurchenco, D.E. Birk, and R.P. Mecham, eds.) pp. 351-358. Academic Press, San Diego.

Zimmermann, D.R. and Ruoslahti, E. 1989. Multiple domains of the large fibroblast proteoglycan, versican. *EMBO J.* 8:2975-2981.

Contributed by Robert P. Mecham
Washington University School of Medicine
St. Louis, Missouri

Preparation of Basement Membrane Components from EHS Tumors

This unit describes methods for passaging and harvesting the basement membrane matrix–producing EHS tumor and for the subsequent isolation of a crude mixture of basement membrane components termed Matrigel (see Basic Protocol 1), which promotes the differentiation of a variety of epithelial, endothelial, and neuronal cells. Procedures for the isolation of the adhesive glycoprotein laminin-1 (see Basic Protocol 2) and of type IV collagen (see Basic Protocol 3) are also included.

All epithelial and endothelial cells produce a basement membrane matrix, but in most tissues they do so only in small amounts. The approach described here, using tumor cells, enables one to obtain basement membrane material of high purity in significant quantities. The material isolated from EHS tumors is structurally and antigenically identical to material from normal tissue basement membranes. The isolated material is native, intact, and homogeneous. It can be used in biological assays and for studying extracellular matrix assembly. The isolation methods for Matrigel and laminin-1 can also be applied to nontumor tissues, but yields are generally low and additional purification may be needed.

Type IV collagen is normally highly cross-linked; therefore it is difficult to extract it intact from tissues in quantity unless the animals are placed for several weeks on a lathrogenic diet, which makes collagens soluble by blocking cross-link formation. Support protocols cover the maintenance and harvesting of EHS tumors in mice (see Support Protocol 1) and maintenance of mice on a lathrogenic diet (see Support Protocol 2).

Procedures for preparing laminin-1 and type IV collagen are similar to that for Matrigel but require additional steps and different extraction buffers. Both laminin-1 and type IV collagen can be prepared from the same batch of tumor if the mice are lathyritic, but only laminin-1 can be prepared from nonlathyritic tissue. Once Matrigel is prepared from the tumor, the residue cannot be used for large-scale preparations of laminin-1 or type IV collagen because most of the material has been extracted.

PREPARATION OF MATRIGEL

BASIC PROTOCOL 1

Matrigel significantly promotes the differentiation of a variety of cell types, including endothelial, smooth muscle, fat, and neuronal cells. Untransformed cells generally do not proliferate on Matrigel, whereas transformed cells continue to proliferate. This preparative procedure requires 100 g of tumor tissue and the volumes used are important.

Materials

EHS tumor (see Support Protocol 1)
3.4 M NaCl buffer (see recipe), 4°C
2 M urea buffer (see recipe), 4°C
Tris-buffered saline (TBS; *APPENDIX 2A*), 4°C
Chloroform
Tissue culture medium (e.g., DMEM, RPMI)
70% ethanol

Electric homogenizer (e.g., Polytron)
Centrifuge and rotor, 4°C
Sterile hemostat

Additional materials and equipment for dialysis and protein assays (see *APPENDIX 3*)

Contributed by Hynda K. Kleinman
Current Protocols in Cell Biology (1998) 10.2.1-10.2.10

NOTE: All reagents and equipment should be prechilled to 4°C, and all procedures must be performed at 4°C.

1. If frozen, thaw the EHS tumor (100 g) at room temperature in 200 ml of 3.4 M NaCl buffer for ~1 hr. Homogenize the tumor until dispersed with an electric homogenizer.

   A Polytron homogenizer works well. Do not homogenize for too long, as it is important not to warm the tissue.

2. Centrifuge the homogenate 15 min at 8000 × *g*, 4°C, to pellet the tumor tissue. Discard the pink supernatant and repeat homogenization (step 1).

   The centrifuge and rotor must be cold before and during use for all manipulations.

3. Add 100 ml of 2 M urea buffer to the tumor pellets and homogenize to disperse the tumor. Stir overnight at 4°C.

4. Centrifuge the urea homogenate 20 min at 23,000 × *g*, 4°C, and decant the supernatant gently. Save the supernatant on ice.

   Pour off the thick supernatant gently, as the pellet may detach from the bottom of the tube.

5. Homogenize the pellets in 50 ml of 2 M urea buffer and centrifuge as in step 4. Combine the supernatants and discard the pellets.

6. Dialyze the supernatant against 2 liters TBS containing 10 ml chloroform/liter for ≥4 hr (see *APPENDIX 3*). Manually rotate the bags after 2 hr to assure dispersion of the contents.

   This step sterilizes the inside of the bag; chloroform will kill bacteria and spores. Subsequent steps remove the urea and chloroform. When discarding the chloroform-containing dialysis buffer, it is best to decant the buffer and discard the remaining chloroform, which settles to the bottom of the vessel, in a chemical waste container.

7. Dialyze twice more, for ≥2 hr each time, against 2 liters TBS and then once more against 2 liters tissue culture medium.

   Manually rotate the bags between changes to facilitate the exchange of solutions.

8. Working in a sterile hood and holding the dialysis bag with a sterile hemostat, spray it with 70% ethanol, cut it with sterile scissors, and empty the contents into a sterile container on ice.

9. Determine the protein concentration using a conventional protein assay (e.g., Lowry assay; see *APPENDIX 3*). Divide the preparation (Matrigel) into aliquots and store up to 12 months at −20°C.

   Matrigel concentration should be 10 to 15 mg/ml.

   It is not desirable to freeze and thaw Matrigel too many times, so generally 1- and 10-ml aliquots are most useful. The size of the aliquots should be based on need.

PREPARATION OF LAMININ-1

Laminin-1 promotes the adhesion, differentiation, migration, and growth of many cells in culture. In addition, most neuronal cells tested extend long neuritic processes when grown on laminin-1. Laminin-1 cannot be prepared from the same batch of EHS tissue as Matrigel, but it can be prepared from the same batch of tissue as type IV collagen (see Basic Protocol 3).

Materials

EHS tumor (see Support Protocol 1)
3.4 M NaCl buffer (see recipe), 4°C
0.5 M NaCl buffer (see recipe), 4°C
Ammonium sulfate
TBS (*APPENDIX 2A*), 4°C
NaCl
Chloroform

Electric homogenizer (e.g., Polytron)
Centrifuge and rotor, 4°C
Sterile hemostat

Additional materials and equipment for dialysis and protein assays (see *APPENDIX 3*)

NOTE: All reagents and equipment should be prechilled to 4°C, and all procedures must be performed at 4°C.

1. Thaw 100 g of EHS tumor in 200 ml of 3.4 M NaCl buffer for ~1 hr at room temperature. Homogenize tumor with an electric homogenizer until dispersed.

   The volumes given in all the protocols are based on 100 g of tissue. Larger or smaller amounts of tumor can be used with proportional volumes of buffers.

   A Polytron homogenizer works well. Do not homogenize for too long, as it is important not to warm the tissue.

2. Centrifuge the homogenate 15 min at 8000 × *g*, 4°C, to pellet the tumor tissue. Discard the pink supernatant and repeat homogenization (step 1).

3. Add 200 ml of 0.5 M NaCl buffer to the tumor pellets and homogenize. Stir overnight at 4°C.

4. Centrifuge the homogenate 15 min at 8000 × *g*, 4°C. Save the supernatant in a container on ice. Repeat step 3 and repeat centrifugation.

   The tumor homogenate pellet can be used to prepare type IV collagen (see Basic Protocol 3) if the tumor tissue was taken from lathyritic animals. Otherwise, the pellet should be discarded.

5. To the combined 0.5 M NaCl supernatants, slowly add ammonium sulfate to 30% saturation (16.4 g/100 ml) with vigorous stirring. Stir 1 hr after all of the salt has dissolved.

6. Centrifuge 15 min at 8000 × *g*, 4°C.

   The pellets should be large and white, with a reasonably clear supernatant. The supernatant should be saved unless it is certain that all the laminin-1 precipitated.

7. Dissolve the laminin-1 pellets in 300 ml TBS and dialyze for ≥2 hr against 2 liters TBS (see *APPENDIX 3*). Repeat dialysis twice against 2 liters TBS each time.

8. Empty the dialysis bags, measure the volume of the sample, and slowly add NaCl, with stirring, to a final concentration of 1.7 M. Centrifuge 15 min at 8000 × *g*, 4°C.

   The solution is already 0.15 M NaCl, so 1.55 M NaCl or 90.6 g should be added per liter.

   The pellet should be very small. This step removes the contaminating type IV collagen. If the pellet is large, repeat step 7 and omit step 8.

9. Dialyze the supernatant against TBS for three changes (as in step 7). Optionally, include 5 ml/liter chloroform to TBS in the first dialysis step to sterilize the laminin-1. If chloroform is added, then perform an additional dialysis with TBS.

10. Working in a sterile hood and holding the dialysis bag with a sterile hemostat, spray it with 70% ethanol, cut it with sterile scissors, and empty the contents into a sterile container on ice.

11. Determine the amount of protein with a standard protein assay (e.g., Lowry assay; see *APPENDIX 3*). Divide the preparation (laminin-1) into 1-ml aliquots and store at −70°C for 24 months.

    The protein concentration should be 3 to 6 mg/ml.

BASIC PROTOCOL 3

PREPARATION OF TYPE IV COLLAGEN

Type IV collagen is used for cell adhesion and migration studies. Type IV collagen can be prepared from the same tumor tissue as laminin as long as the animals have been on a lathrogenic diet for ≥2 weeks prior to tumor harvest (see Support Protocol 2).

Materials

Tumor homogenate (see Basic Protocol 2, step 4), prepared from animals fed a lathrogenic diet (see Support Protocol 2)
0.5 M NaCl buffer (see recipe), 4°C
2.0 M guanidine·HCl buffer (see recipe), 4°C
2.0 M guanidine·HCl buffer containing 32 mg/liter dithiothreitol (DTT), 4°C
0.5 M acetic acid (28.5 ml acetic acid/liter water), 4°C

Centrifuge and rotor, 4°C

Additional materials and equipment for dialysis and protein assays (see *APPENDIX 3*)

NOTE: All reagents and equipment should be prechilled to 4°C, and all procedures must be performed at 4°C.

1. Prepare tumor homogenate in 300 ml of 0.5 M NaCl buffer. Centrifuge 15 min at 8000 × *g*, 4°C. Discard the supernatant and repeat centrifugation.

   These steps and the subsequent ones remove contaminating laminin-1.

2. Homogenize pellets in 2.0 M guanidine·HCl buffer and centrifuge 15 min at 8000 × *g*, 4°C. Discard the supernatant and repeat the wash.

3. Homogenize pellets in 250 ml of 2.0 M guanidine·HCl buffer/DTT solution and stir overnight at 4°C.

4. Centrifuge tumor homogenate 15 min at 8000 × *g*, 4°C. Save the supernatant in a container on ice.

5. Homogenize pellets in 250 ml of 2.0 M guanidine·HCl buffer/DTT. Centrifuge immediately for 15 min at 8000 × *g*, 4°C. Discard pellet and combine supernatant with that from step 4.

6. Dialyze supernatants against four changes of 0.5 M acetic acid for ≥2 hr each (see *APPENDIX 3*).

   If the collagen concentration is >0.8 mg/ml, it will precipitate, and it is difficult to recover the precipitated material. To ensure that the entire batch does not precipitate, dialyze one-tenth of the sample through three changes before processing the remainder. If the collagen does precipitate, the remaining supernatant should be diluted by half with cold distilled water and then dialyzed. The material that has precipitated can be collected by centrifugation, redissolved in twice the original volume of 2.0 M guanidine·HCl buffer/DTT solution, and then dialyzed against 0.5 M acetic acid.

7. Determine the amount of protein by amino acid analysis or by lyophilizing 10 ml and weighing the sample. Divide the preparation (type IV collagen) into 1-ml aliquots and store at −20°C for 24 months.

   The solution should be clear. The presence of a precipitate indicates that collagen has come out of solution. The precipitate can be removed by centrifugation, leaving considerable type IV collagen in the supernatant.

MAINTENANCE AND HARVEST OF EHS TUMORS

SUPPORT PROTOCOL 1

This protocol describes a method for maintaining and harvesting the EHS tumor. In addition, information is provided on the lathrogenic diet that is necessary when the tissue is used for isolating type IV collagen (see Support Protocol 2). The tumor passage protocol requires live dispersed EHS tumor tissue or single cells, which can be obtained from American Type Culture Collection (see *SUPPLIERS APPENDIX*). A cell line can also be obtained from the developers of the line (R.V. Iozzo and J.R. Hassell). One 4-g tumor can be passaged into ten mice by subcutaneous or intramuscular injection. The tumor is ready for harvest in 3 to 4 weeks.

Materials

Fresh EHS tumor
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin
C57BL/6 mice
Anesthetic (e.g., 3.2% Avertin)
Dimethylsulfoxide (DMSO)
70% ethanol

50-ml plastic tubes
20- to 30-ml and 3-ml syringes
16-G needles
Centrifuge and rotor, 4°C

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

1. Mince fresh EHS tumor (~4 g) from each animal as much as possible with scissors. Add 7 ml PBS and transfer tissue chunks into a 20- to 30-ml syringe without a needle. Force tissue chunks through the syringe several times until the suspension goes through easily. Place a 16-G needle on the syringe and repeat the process.

   Have several ice buckets handy to keep the tumor cold. Handle each tumor separately, using clean tubes and syringes, in case there is too much bacterial contamination with blood or pus in one of the tumors. Number the tubes and respective syringes when working with large numbers of tumors. This step can be messy; gloves, lab coats, and masks are recommended.

2. Add 40 ml PBS and gently shake the tube to disperse the tissue. Allow tumor tissue to settle for a few minutes, or centrifuge for 5 to 10 sec to speed up the process. Discard supernatant and dislodge and disperse pellet by tapping the tube.
3. Repeat step 2. Add PBS containing penicillin and streptomycin to a final volume of 10 ml.

   At this stage the preparation should be a thick slurry.
4. Lightly anesthetize mice by intraperitoneal injection of 200 µl Avertin. Inject 1 ml cell slurry into each mouse, either subcutaneously or intramuscularly, using a 16-G needle with a 3-ml syringe.

   It is possible to use two injection sites per animal to increase the yield. Watch the animals carefully as the tumors grow to be sure that the animals can obtain food, etc.

   Female mice are more docile and easier to handle than males, and may be preferred as tumor recipients. The cages should be marked with the tumor number to allow tracking in case of contamination in the tumor.

   Dispersed tumor can also be frozen and stored for future use: add PBS containing 5% DMSO to dispersed tumor, transfer 1-ml aliquots to freezing vials, place in a −70°C freezer for 2 hr, and then store in liquid nitrogen. It generally takes >3 weeks for the initial tumor to become fully grown when frozen material is injected, probably because some cells die during the freezing and thawing process. Where possible, it is best to work with fresh tumor tissue.
5. Harvest the tumors after 3 weeks. Euthanize mice by CO_2 asphyxiation and dip each one in 70% ethanol. Carefully excise the tumor to remove any capsule-like material. Place each tumor in a separate 50-ml tube if the tumor is to be passaged, or in a freezer bag if it is to be stored for preparation of basement membrane components.

   Tumor size should not exceed 3 cm in length, height, or width as larger tumors will become necrotic. Tumors that are very bloody or full of yellow pus should be discarded. For convenience, freeze tumors at 100 g/bag and label the bags with tumor weight and date.

   If not used immediately, fresh tumor can be frozen in powdered dry ice or immersed directly in liquid nitrogen and then stored for ≤1 year at −70°C in freezer bags.

SUPPORT PROTOCOL 2

MAINTENANCE OF MICE ON A LATHROGENIC DIET

The lathrogenic diet prevents collagen from cross-linking; hence type IV collagen derived from lathyritic mice is more soluble and easily extractable, which increases the yield of material obtained. If mice are maintained too long on the diet (>3 weeks), they develop spontaneous bone fractures. It is important to begin monitoring the health of the mice after 2 weeks, and it may be necessary to euthanize mice at slightly different times to maximize the yield of tumor.

Materials

Lathrogenic chow (see recipe)

CAUTION: Lathrogenic chow contains toxic materials; gloves, mask, and lab coat should be worn when handling it.

1. Feed mice regular chow until 6 days after injection of tumor cells. Replace regular chow with lathrogenic chow (placed in containers inside the cage) after 6 days.

   There is no need to feed mice lathrogenic diet until the tumor starts to grow. Animals may spread the food around the cage, so the levels of food should be checked daily.
2. Keep mice on the diet for ≤3 weeks, at which time the tumor should be harvested.

   After 2 weeks on the diet, mice should be monitored for bone fractures, which are observed with this diet. Mice in distress should be sacrificed and the tumors harvested.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Guanidine·HCl buffer, 2.0 M

191.0 g guanidine·HCl (2 M final)
12.1 g Tris base (0.05 M final)
H_2O to 1800 ml
Adjust pH to 7.4 with 2 M HCl
Add H_2O to 2 liters
Prepare fresh as needed

Lathrogenic chow

100 g β-aminopropionitrile (Sigma)
2 g iproniazid (Sigma)
0.2 g pargyline (Sigma)
5 kg standard ground chow

Dissolve the first three ingredients in 300 ml distilled water and mix with the ground chow for 20 min in a commercial mixer. Store 6 months at 4°C.

When adding the solution, sprinkle it on different areas of the chow to help the mixing process.

CAUTION: *Gloves, mask, and lab coat should be worn when handling these toxic materials.*

NaCl buffer, 0.5 M

7.5 g NaCl (0.5 M final)
6.05 g Tris base (0.05 M final)
H_2O to 900 ml
Adjust pH to 7.4 with 2 N HCl
Add H_2O to 1 liter
Store 6 months at room temperature

NaCl buffer, 3.4 M

397 g NaCl (3.4 M final)
3 g EDTA (0.005 M final)
5 g *N*-ethylmaleimide (NEM; 0.02 M final)
12.1 g Tris base (0.05 M final)
H_2O to 1800 ml
Adjust pH to 7.4 with 2 N HCl
Add H_2O to 2 liters
Prepare fresh as needed

Urea buffer, 2 M

240 g urea (2 M final)
12.1 g Tris base (0.05 M final)
H_2O to 1800 ml
Adjust pH to 7.4 with 2 N HCl
Add H_2O to 2 liters
Store 6 months at 4°C

COMMENTARY

Background Information

The EHS tumor produces an abundant basement membrane matrix (Orkin et al., 1977). Basement membranes are thin extracellular matrices that underlie epithelial and endothelial cells and surround muscle, fat, and nerve cells (Martin and Timpl, 1987; Kleinman et al., 1993). Preparation of basement membrane and its major components, laminin-1 and type IV collagen, from tissues is difficult because they are present in normal tissues only in small amounts and are poorly soluble. These components have many uses in cell biology research and are generally needed in large quantities. Preparation of these components from EHS tumors is advantageous because the components are highly soluble and type IV collagen cross-linking can be prevented by passaging the tumor in mice fed a lathrogenic diet. In the past, type IV collagen could be isolated only in degraded forms.

The EHS tumor is a spontaneous tumor that was propagated from a wild mouse in the 1940s. Its tissue of origin is not known. It was initially characterized as a chondrosarcoma because of the large amount of extracellular material observed by histology. In 1977, the EHS tumor was defined as a basement membrane tumor when type IV collagen was discovered to be a major component (Orkin et al., 1977). The availability of basement membrane components from this tumor has greatly accelerated research on the structure and function of the basement membrane. All of the components isolated from this tumor are chemically, structurally, and antigenically identical to authentic tissue basement membrane components (Beck et al., 1990; Yurchenco and Schittny, 1990; Timpl and Brown, 1994).

The laminin isolated from the EHS tumor is composed of three chains, designated α1, β1, and γ1, and is termed laminin-1 (Timpl et al., 1979; Burgeson et al., 1994). Five α, three β, and two γ chains of laminin have been described to date in various tissues. Eleven variant forms of laminin, composed of different combinations of these chains, have been described. Good tissue or tumor sources of these latter laminins have not yet been described. A fragment of laminin-2 (merosin composed of α2β1γ1) is commercially available. Many of these laminins have tissue-specific locations and are expressed at specific times in these tissues.

Type IV collagen isolated from the EHS tumor is composed of two different chains, present in a 2:1 ratio, with the formula $\alpha1(IV)_2\alpha2(IV)$ (Orkin et al., 1977; Kleinman et al., 1982). Additional chains of type IV collagen have also been identified, including α3, α4, α5, and α6. Like laminin, these molecules are tissue specific in their localization, and good sources for their isolation have not yet been found.

The components of the EHS tumor are important because of their biological activities. Matrigel, a mixture of basement membrane components, promotes the differentiation of various cells and is also active with tissue explants (Carey et al., 1986; Kleinman et al., 1986). The response to Matrigel depends on the cell type. Endothelial cells form capillary-like structures when cultured on Matrigel, while mammary cells form cyst-like structures that show an 80-fold induction of casein (Li et al., 1986; Kubota et al., 1988). Explants of dorsal root ganglia extend long neuritic processes that are surrounded by Schwann cells producing myelin (Carey et al., 1986).

Matrigel can be used to coat culture dishes, with cells plated directly onto the gelled material. Cells can also be mixed with the Matrigel to form a suspension when warmed (Hadley et al., 1985; *UNIT 10.3*). In vivo, Matrigel has been used to assess angiogenesis (Passaniti et al., 1992) and to increase the incidence of tumor take and the rate of growth (Fridman et al., 1991). Matrigel has also been used to coat filters in migration assay chambers to assess tumor cell invasion (Albini et al., 1987; *UNIT 12.2*). Laminin-1 has been used mainly as a culture substratum. It promotes cell adhesion, growth, migration, and neurite outgrowth (Evercooren et al., 1982; Kleinman et al., 1993). It also reduces fibroblast growth. Type IV collagen promotes the adhesion and growth of many cells and is also used to coat filters in migration assays.

Critical Parameters and Troubleshooting

EHS tumor. A major problem with propagating EHS tumors is bacterial infection. Infection is easy to recognize as the tumors become watery, yellow in areas, and full of pus. Infected tumors should be discarded and not used for passage. Infection can be minimized by using antibiotics and by keeping all of the tumors separate when passaging. It is also important not to let the tumors become too large, as necrosis will reduce the yield of usable mate-

rial. Generally tumors >4 g are necrotic. For preparation of matrix components, tumors can be stored frozen at −70°C for ≤1 year and then thawed as described in the protocols.

Matrigel. Matrigel should be prepared as quickly as possible, as activity is lost if the preparation is left too long at any of the steps. The entire isolation and dialysis can be completed in ≤4 days. It is also critical that Matrigel be kept cold at all stages of the preparation. Once isolated, it is important to keep Matrigel at 4°C, because the material cannot be recovered in the liquid form after it has gelled. Using cold pipets and syringes helps to keep Matrigel liquid. The Matrigel protein concentration should not be <9 mg/ml; at lower protein concentrations, Matrigel will not form solid gels. Diluted Matrigel can be recovered by 40% ammonium sulfate precipitation, followed by suspension in a smaller volume and dialysis.

Laminin-1. It is safest not to throw anything away until the laminin-1 preparation is complete. It is also very important to keep all solutions cold during the preparation of laminin-1.

Type IV collagen. A major problem with the collagen preparation is having too much type IV collagen in the extraction buffer. The collagen will precipitate out of solution at concentrations >0.8 mg/ml when dialyzed against acetic acid in the final step, and the precipitated material is difficult to recover. The yield of collagen can vary, depending on how lathyritic the animals are; therefore the final dialysis step should be performed first on a small batch to be sure that the collagen will stay in solution. Type IV collagen is best stored in acetic acid. For culture, type IV collagen is used as a dried substratum. This generally requires overnight drying in a sterile hood.

Anticipated Results

In general, 1 g of tumor tissue yields ~1.5 ml of Matrigel or 10 mg of laminin-1 and 5 mg of type IV collagen.

Time Considerations

EHS tumors generally reach ~4 g and can be harvested 3 weeks after injection of the cells into mice. It is important to begin monitoring the health of the animals at 2 weeks. It may be necessary to sacrifice the animals at slightly different times to maximize the yield. The preparations of Matrigel, laminin-1, and type IV collagen should all be performed in ≤1 week. Arresting the preparation at intermediate stages can result in some degradation.

Literature Cited

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Kozlowski, J.M., and McEwan, R.N. 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res.* 47:3239-3245.

Beck, K., Hunter, I., and Engel, J. 1990. Structure and function of laminin: Anatomy of a multidomain protein. *FASEB J.* 4:148-160.

Burgeson, R.E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H.K., Martin, G.R., Meneguzzi, G., Paulsson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y., and Yurchenco, P.S. 1994. A new nomenclature for the laminins. *Matrix Biol.* 14:209-211.

Carey, D.J., Todd, M.S., and Rafferty, C.M. 1986. Schwann cell myelination: Induction by exogenous basement membrane–like extracellular matrix. *J. Cell Biol*. 102:2254-2263.

Evercooren, A.B., Kleinman, H.K., Ohno, S., Schwartz, J.P., and Dubois-Dalqc, M. 1982. Factors promoting neurite growth in human fetal sensory ganglia cultures. *J. Neurosci. Res.* 8:179-194.

Fridman, R., Kibbey, M.C., Royce, L.S., Zain, M., Sweeney, T.M., Jicha, D.L., Yannelli, J.R., Martin, G.R., and Kleinman, H.K. 1991. Basement membrane (Matrigel) enhances both the incidence and growth of subcutaneously injected human and murine cells. *J. Natl. Cancer Inst.* 83:769-774.

Hadley, M.A., Byers, S.W., Suarez-Quian, C.A., Kleinman, H.K., and Dym, M. 1985. Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ development. *J. Cell Biol.* 101:1511-1522.

Kleinman, H.K., McGarvey, M.L., Liotta, L.A., Gehron Robey, P., Tryggvason, K., and Martin, G.R. 1982. Isolation and characterization of native type IV collagen from the EHS sarcoma. *Biochemistry* 24:6188-6193.

Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W., and Martin, G.R. 1986. Basement membrane complexes with biological activity. *Biochemistry* 25:312-318.

Kleinman, H.K., Kibbey, M.C., Schnaper, W.H., Yamamura, K., Weeks, B.S., and Grant, D.S. 1993. The laminins: A family of basement membrane glycoproteins important in cell differentiation and tumor metastasis. *Vitamins Hormones* 47:161-186.

Kubota, Y., Kleinman, H.K., Martin, G.R., and Lawley, T.J. 1988. Role of laminin and basement membrane in the differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol*. 107:1589-1597.

Li, L., Aggeler, M.J., Farson, D.A., Hatier, C., Hassell, J.R., and Bissell, M.J. 1986. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 84:136-140.

Martin, G.R. and Timpl, R. 1987. Laminin and other basement membrane components. *Annu. Rev. Cell Biol.* 3:57-85.

Orkin, R.W., Gehron, P., McGoodwin, E.B., Martin, G.R., Valentine, T., and Swarm, R. 1977. A murine tumor producing a matrix of basement membrane. *J. Exp. Med.* 145:204-220.

Passaniti, A., Taylor, R.M., Pili, R., Guo, Y., Long, P.V., Haney, J.A., Pauly, R.R., Grant, D.S., and Martin, G.R. 1992. A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin and fibroblast growth factor. *Lab. Invest*. 67:519-528.

Timpl, R. and Brown, J.C. 1994. Supramolecular assembly of basement membranes. *BioEssays* 18:123-132.

Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidart, J.M., and Martin, G.R. 1979. Laminin—A glycoprotein from basement membrane. *J. Biol. Chem*. 254:9933-9939.

Yurchenco, P.D. and Schittny, J.C. 1990. Molecular architecture of basement membrane. *FASEB J*. 4:1577-1590.

Contributed by Hynda K. Kleinman
National Institute of Dental Research/NIH
Bethesda, Maryland

Preparation of Gelled Substrates

This unit describes the use of type I collagen as a gelled substrate for cell culture, as well as various ways to handle and manipulate the basement membrane matrix preparation Matrigel for use in cell culture and in vivo. Both matrix components form gels at high concentrations under physiological conditions. Type I collagen gels have been used mainly to promote cell growth and differentiation. Most cells in culture proliferate at a high rate and lose their differentiated phenotype, but do not proliferate when cultured on Matrigel. Matrigel is advantageous as a culture substratum because it promotes the differentiation of a variety of epithelial and endothelial cells; the response of the cells depends on the cell type (see Table 10.3.1). Differentiated cells in vitro have a variety of uses, including determining how genes are regulated and which factors control differentiation. Matrigel has been used in vivo to assess angiogenesis, increase tumor growth, and promote the survival of explanted cells and tissues.

The basic protocols outline the most common uses of type I collagen (see Basic Protocol 1) and Matrigel (see Basic Protocol 2) as gelled substrates. Gelled type I collagen can also be used as a floating substrate. Dried type I collagen substrates have been used in cell culture. Additional protocols describe how to grow cells inside Matrigel (see Alternate Protocol 1) and how Matrigel can be used in vivo to assess angiogenesis and to initiate and increase tumor growth (see Alternate Protocol 2).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

PREPARATION OF TYPE I COLLAGEN SUBSTRATES

BASIC PROTOCOL 1

Gelled collagen substrates should be prepared on the same day that they will be used for cell culture. Gelled type I collagen substrates are used for a variety of cell types, including hepatocytes and endothelial cells. Dried collagen substrates can be prepared the day before use and are often used to culture skeletal muscle cells. Type I collagen substrates allow for better growth, survival, and differentiation of various cell types, including skeletal muscle and hepatocytes, than does plastic or glass alone.

Materials

3 to 5 mg/ml type I collagen in dilute acid (e.g., Vitrogen 100, Collagen), ice cold
10× phosphate-buffered saline, pH 7.4 (10× PBS; *APPENDIX 2A*) or 10× medium salts (e.g., Medium 199, Life Technologies), ice cold
0.1 M NaOH, ice cold
0.1 M HCl, ice cold
Dilute acetic acid: 28.5 ml glacial acetic acid/liter
Sterile deionized H_2O
Cells
Tissue culture medium, 37°C

Wet ice in a bucket
Culture dishes

Preparing gelled type I collagen substrates

1a. Using prechilled solutions and keeping solutions on ice, mix 8 ml type I collagen with 1 ml of 10× PBS, pH 7.4, and 1 ml of 0.1 M NaOH (all solutions should be prechilled). Adjust pH to 7.4 with additional drops of 0.1 M NaOH or 0.1 M HCl as needed, using pH test paper or a pH meter to assess pH, and using aseptic technique.

Contributed by Hynda K. Kleinman
Current Protocols in Cell Biology (1998) 10.3.1-10.3.9

Type I collagen commercially obtained at 3 to 5 mg/ml in dilute acid should not be further diluted more than necessary or the final gel will be too weak. Below 3 mg/ml, the gel will be very weak and will not withstand the usual manipulations of plating and feeding the cells. Type I collagen obtained as a powder can be solubilized at 3 to 5 mg/ml by stirring overnight at 4°C in dilute acetic acid.

Alternatively, 10× medium salts can be used in place of the PBS.

When a dried film of fibrillar collagen is desired, coat the dish with a small amount of collagen and air dry the material, usually overnight, in a sterile culture hood. Skeletal muscle cells are cultured on dried collagen to encourage cell differentiation (i.e., fusion to myotubes).

2a. Pipet the desired amount of collagen onto the culture dish and warm the dish at least 1 hr in a 37°C incubator.

The collagen should completely cover the surface of the dish; 1 ml is generally sufficient for a 35-mm dish. The matrix usually turns white when it gels.

CO_2 is not important for gelling of the collagen, but subsequent incubations with cells should be performed in a humidified 37°C, 5% CO_2 incubator.

3a. Before use, rinse dried gels and collagen films with sterile distilled water to remove the salts. Add cells and medium as usual and culture.

Warm all solutions and gently pipet onto the culture dish.

In some cases, one may want to grow cells inside a type I collagen gel. This can be done by overlaying the collagen after the cells have attached, or by mixing the cells in the liquid type I collagen prior to gelation (see Alternate Protocol 1).

Floating collagen gels can be obtained by gently agitating around the edge of the collagen gel with a spatula after the cells have attached. When type I collagen gels are used as floating gels with fibroblasts attached, some contraction of the gel will occur. Hepatocytes have also been successfully cultured on floating collagen gels.

Preparing nongelled type I collagen substrates

1b. Dilute 3 to 5 mg/ml collagen solution to 0.1 mg/ml with either dilute acetic acid or 0.1 M HCl.

When directly drying collagen on tissue culture dishes without gelation, usually much less material is needed than for gelled substrates. Generally 10 to 20 µg collagen provides good activity on 16-mm-diameter-well (24-well) dishes.

The collagen can be diluted with 0.1 M HCl as an alternative to acetic acid.

2b. Plate the desired amount of 0.1 mg/ml collagen in acetic acid onto the culture dish. Allow to air dry overnight.

For example, 200 µl of a 0.1 mg/ml collagen solution can be used for each 16-mm-diameter well of a 24-well dish; the solution should completely cover the bottom of the well.

3b. Before use, rinse collagen films with sterile distilled water (to remove salts). Add cells and medium as usual and culture.

Warm all solutions and pipet gently onto the culture dish.

PREPARATION OF GELLED MATRIGEL SUBSTRATES

Matrigel is a crude basement membrane preparation that provides an excellent substrate for the differentiation of a variety of cells (see *UNIT 10.2*). Epithelial, endothelial, smooth muscle, fat, and Schwann cells are in contact with a basement membrane in vivo. These cells do not differentiate well on tissue culture plastic, but show high degrees of differentiation on Matrigel in vitro.

Materials

Matrigel (*UNIT 10.2*; Matrigel can also be obtained commercially from Sigma or Becton Dickinson Labware)
Medium salts, 4°C (optional, for making weak gels)
Cells
Tissue culture medium, 37°C

Wet ice in a bucket
Culture dishes

1. Thaw Matrigel at 4°C.

   Thawing can be accomplished by leaving the Matrigel in the refrigerator on ice overnight or by rolling the bottle in the hands until it is thawed. Do not allow the solution to become warm, as it will gel and cannot be recovered. It is not desirable to thaw and refreeze Matrigel too many times, so it should be stored in appropriate-sized aliquots based on the quantities generally needed.

   It is best to always have fresh wet ice in a bucket when working with Matrigel to keep it cold.

2. In a sterile hood, pipet desired amount of Matrigel onto culture dish.

   The Matrigel should be at ≥9 mg/ml for maximum gelling. Concentrations below that will form weak gels that may fall apart. Matrigel can be diluted to 4 mg/ml and will still gel, but the gel will be weak. In some cases, however, a weak gel is needed. Dilute the stock Matrigel with cold medium salts and mix well.

   If there are bubbles in the Matrigel, which can cause problems once it gels, let stand for a few minutes on ice to allow them to dissipate. Keeping the dish level, quickly pipet the Matrigel into the dishes; when performing multiple pipetting steps, do these quickly, and change the pipets if the Matrigel starts to gel inside them. For 16-mm-diameter wells, use 320 µl per well (this volume is necessary because a large meniscus forms at the edge of the well and distorts the surface). For larger dishes, a thinner coating works well, but one must be aware of the curve in the gel at the edges of the dish. For ≥100-mm-diameter dishes, pipet the Matrigel so that the bottom of the dish is covered, then pour off the excess and use it for another dish.

3. Cover the culture dish and incubate 30 to 60 min at 37°C to allow gelling of the Matrigel.

   Since Matrigel will gel at room temperature, place dishes in incubator immediately.

4. Gently add warm medium and cells to the culture dish as for normal culture. Return the plate to the incubator as usual and check the culture often for changes in cell behavior. Change the medium as usual, taking care not to disturb the gel layer.

   For example, for in vitro assays of angiogenesis (tube formation), incubate 40,000 endothelial cells in 100 µl complete medium in 16-mm-diameter wells containing 320 µl gelled Matrigel. Fix the culture and stain with Diff-Quik (Baxter) using only the fix and solution II (use of solution I will stain the Matrigel and make it difficult to distinguish the cells). The assay can be quantified with an image processor (e.g., NIH Image).

ALTERNATE PROTOCOL 1

GROWTH OF CELLS INSIDE MATRIGEL

Various cells may differentiate to different extents depending on whether the cells are plated on top of a Matrigel substrate or inside of Matrigel. For example, Sertoli cells are highly columnar on a Matrigel substrate but form cord-like structures when grown inside of Matrigel.

Materials

Matrigel (*UNIT 10.2*; Matrigel can also be obtained commercially from Sigma or Becton Dickinson Labware)
Cells
Tissue culture medium, 37°C

Wet ice in a bucket
Culture dishes

1. Thaw Matrigel at 4°C.

   Thawing can be accomplished by leaving the Matrigel in the refrigerator on ice overnight or by rolling the bottle in the hands until it is thawed. Do not allow the solution to become warm, as it will gel and cannot be recovered. It is not desirable to thaw and refreeze Matrigel too many times, so it should be stored in appropriate-sized aliquots based on the quantities generally needed.

   It is best to always have fresh wet ice in a bucket when working with Matrigel to keep it cold.

2. Prepare the cells as usual for cell culture. Centrifuge 5 min at 170 × *g*, room temperature, to collect cells. Decant supernatant, tap pellet to disperse cells, and put tube of cells in wet ice. Add desired amount of thawed Matrigel, mix well, and pipet onto culture dish.

   Take care to fully mix the cells into the Matrigel without causing bubbles. This can be accomplished by gently tipping the tube 180° several times. Approximately 320 µl of Matrigel in a 16-mm-diameter well works well with endothelial cells.

   The number of cells and volume of Matrigel needed for cell differentiation vary among cell types. It may be necessary to test various numbers of cells in a constant volume of Matrigel. A good range to test initially is 10,000 to 100,000 cells per 320 µl.

   The cells will be trapped inside the Matrigel when it gels. Previous studies have found that some cells will migrate towards each other and form differentiated structures. Histological sectioning and/or electron microscopy is necessary to fully observe these structures.

3. Cover the dish and incubate 30 to 60 min at 37°C to allow gelling of the Matrigel.

   Since Matrigel will gel at room temperature, place dishes in incubator immediately.

4. Gently add warm medium to the culture dish as for normal culture. Return the plate to the incubator as usual and check the culture often for changes in cell behavior. Change the medium as usual, taking care not to disturb the gel.

ALTERNATE PROTOCOL 2

USE OF MATRIGEL IN VIVO FOR ANGIOGENIC ASSAYS AND TUMOR GROWTH

Matrigel implanted subcutaneously in an animal remains as a plug with very few cells invading for several months. If certain angiogenic factors are mixed with the Matrigel before injection, however, considerable endothelial cell invasion into the plugs is observed within 1 week. This migration of endothelial cells and subsequent formation of vessels can be quantified and used as an assay for measuring the activity of angiogenic and antiangiogenic compounds.

If tumor cells or minced pieces of biopsy material are premixed in the cold with liquid Matrigel and injected, an increase in the incidence of tumor take and growth can be observed. This technique is highly useful for tumors that are hard to grow and for human biopsy specimens.

Materials

Matrigel (*UNIT 10.2*; Matrigel can also be obtained commercially from Sigma or Becton Dickinson Labware)
C57BL6 mice for angiogenesis assay *or* athymic nude mice for human tumor growth
Test compound for angiogenesis assay *or* tumor sample
Proteases (e.g., trypsin or collagenase) for dissociating tumor pieces, if necessary
Hemoglobin assay kit (e.g., Drabkin Reagent Kit, Sigma) or additional materials and equipment for histology, including image processor (e.g., NIH Image)

Wet ice in a bucket
Culture dishes
1- to 3-ml syringes
23- or 25-G needles

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Care and Use Committee (IACUL) and must follow officially approved procedures for the care and use of laboratory animals.

1. Thaw Matrigel at 4°C.

   Thawing can be accomplished by leaving the Matrigel on ice in the refrigerator overnight or by rolling the bottle in the hands until it is thawed. Do not allow the solution to become warm, as it will gel and cannot be recovered. It is not desirable to thaw and refreeze Matrigel too many times, so it should be stored in appropriate-sized aliquots based on the quantities generally needed.

   It is best to always have fresh wet ice in a bucket when working with Matrigel to keep it cold.

2. Mix Matrigel on ice with the test substance for an angiogenesis assay or with cells or dispersed tumor pieces for the subcutaneous growth of tumors.

   See Alternate Protocol 1, step 2, for details of mixing cells and Matrigel. The final injected volume should not exceed 1 ml. It is best not to reduce the concentration of Matrigel below 9 mg/ml.

   Angiogenic factors for testing and tumor cells/pieces should be suspended in as small a volume as possible. Basic fibroblast growth factor at 1 to 5 ng/ml should be included with Matrigel in some plugs as a positive control.

   If using tumor biopsy pieces, the material must be finely minced or dissociated with proteases, including trypsin or collagenase, so that it will pass through the syringe.

3. Cool a 1- to 3-ml syringe fitted with a 23- or 25-G needle by placing it in a beaker on ice. Load the syringe with 1 ml of the Matrigel/test compound or Matrigel/tumor cell mixture. For the angiogenesis assay, hold each mouse firmly in one hand and inject the material subcutaneously on the lower ventral side near one or both of the lower legs. For tumor-growth studies, restrain mice in a box and inject material subcutaneously in the upper dorsal area. After injecting, hold the needle in place for ~30 sec until the Matrigel starts to gel. Pull the needle out slowly and turn it to minimize leakage of Matrigel out of the injection site (some leakage may occur anyway).

   Female mice are easier to handle than male mice. Mice do not need to be anesthetized.

   If doing multiple animals, it may be necessary to change needles often, as the Matrigel will clog the needle.

4a. *Subcutaneous angiogenesis assay:* Sacrifice mice after 1 week. Gently cut the skin away and excise the Matrigel pellet, which will be clearly visible attached either to the undersurface of the skin or to the underlying muscle layer. Measure angiogenesis either using a hemoglobin assay kit (if there is an obvious abundance of vessels) or by histology.

For histology, fix the plugs, stain them with Masson trichrome, and quantify angiogenesis using an image processor by counting the number of vessels and/or measuring the area occupied.

4b. *Tumor growth:* Tumors are usually observable by 2 weeks but some cells, such as NIH 3T3, require >2 months.

COMMENTARY

Background Information

Most cells in tissues are in contact with an extracellular matrix. The use of collagen substrates in vitro enhances the growth and differentiation of many cell types over that observed with glass or plastic substrates (Elsdale and Bard, 1972; Emerman et al., 1979; Kleinman et al., 1981). Cells adhere directly to collagen through integrin receptors or via adhesion proteins such as fibronectin, which bind to both the cells (via integrin receptors) and the collagen. Collagen substrates are particularly useful for the differentiation of hepatocytes and breast epithelial cells. Dried collagen substrates have been used successfully for the culture of muscle cells (Hauschka and Konigsberg, 1966).

The important role of the basement membrane in cell differentiation was not realized until the crude basement membrane preparation known as Matrigel was developed and became widely used as a cell growth substrate (Kleinman et al., 1986, 1987). Most untransformed cells in culture form a monolayer and proliferate until confluence is reached. Cells cultured on Matrigel differentiate and their proliferation is arrested (Table 10.3.1). The cellular response depends on the cell type. For example, endothelial cells form capillary-like structures with a lumen, and melanoma cells produce melanin (Kleinman et al., 1986; Kubota et al., 1988). Salivary gland cells form acini (Hoffman et al., 1996) and bone cells form canaliculi (Vukicevic et al., 1990). Explanted neural tissue, such as dorsal root ganglion, shows extensive neurite outgrowth and myelin production (Carey et al., 1986). Another advantage of using Matrigel in culture is that fibroblasts (which can overgrow many primary cultures) do not grow well in its presence.

Attempts have been made to use whole natural basement membranes, such as the lens capsule or amnion, for cell culture and differentiation studies, with limited success. Lack of reproducibility and difficulty in handling the natural materials have restricted their use. Type I collagen (the most abundant collagen, comprising some 30% of the dry weight of vertebrates) at 3 to 5 mg/ml gels when it is brought to neutral pH and warmed. Type I collagen gels have been used as culture substrata, but most cells do not differentiate as well on collagen as they do on Matrigel.

Matrigel can be used with the cells either plated on top of the gel or entrapped inside it (Hadley et al., 1985). Although limited data are available, there is evidence that cells behave differently under the two culture conditions. For example, Sertoli cells remain as a monolayer on plastic, but become 15-fold more columnar with polarized nuclei when plated on Matrigel. When cultured inside Matrigel, Sertoli cells migrate into cord-like structures that resemble an immature testis with polarized nuclei. In some cases, the amount and type of growth factors in the Matrigel have been altered either by their removal, antibody binding, or exogenous addition (Taub et al., 1990; Vukicevic et al., 1992). Manipulation of growth factor levels greatly affects differentiation for both kidney cells and bone cells. Other cell types have not been fully evaluated for the effects of growth factors and Matrigel.

The differentiation of cells in culture has provided some useful assay models. For example, although tube formation by endothelial cells on Matrigel does not mimic all of the steps in angiogenesis, tube formation has been used as an assay to define angiogenic and antiangiogenic agents. For example, the sera of vasculitis patients contain angiogenic activity as determined by the tube assay (Cid et al., 1993). Purification of the causative factor, haptoglobin, was monitored using the tube assay, and

Table 10.3.1 Effect of Matrigel on Cell and Explant Differentiation

Cell/explant	Biological effect	Reference
Bone cells	Formation of canaliculi	Vukicevic et al., 1990
Dorsal root ganglion	Outgrowth and myelination	Carey et al., 1986
Endothelial cells	Formation of capillary-like structures with a lumen	Kubota et al., 1988
Hair follicle	Shaft formation	S. Yuspa and N. Martinet, pers. comm.
Hepatocytes	Maintenance of albumin synthesis and cytochrome P_{450}	Ben-Ze'ev et al., 1988
Mammary cells	Formation of ducts and lumina, increased casein production	Li et al., 1986
Melanoma cells	Rapid pigmentation	Kleinman et al., 1986
Notochord	Outgrowth	Bilozur and Hay, 1988
Olfactory neuroblast	Odorant responsiveness	Coon et al., 1989
Oviduct cells	Formation of tubes with a lumen with polarized secretion	Joshi, 1991
Pancreatic acinar cells	Acinar formation with secretory vesicles near lumen	Bendayan et al., 1986
Salivary gland cells	Acinar formation	Hoffman et al., 1996
Sertoli cells	15-fold columnar form, germ cell survival and differentiation	Hadley et al., 1985
Thyroid cells	Thyroglobulin production	Greenberg and Hay, 1986
Type II pneumocytes	Cuboidal cells, phosphatidylcholine synthesis	Blau et al., 1988

the activity was subsequently confirmed in several in vivo assays.

Critical Parameters and Troubleshooting

Matrigel and type I collagen must be maintained at 4°C when being manipulated. For long-term culture of >1 week, denser substrates may be needed, as the gels may begin to break apart over time in culture with certain types of cells. It is usually not desirable to significantly reduce the protein concentration of Matrigel. When testing new cell types, it may be necessary to try the cells on all the possible substrates (collagen gels, dried collagen films, on Matrigel, inside Matrigel) to determine the best configuration. Do not become discouraged if cells do not have a defined appearance before histological sectioning. Histology often reveals polarity and other indications of differentiation.

Anticipated Results

Histological analysis is recommended to assess the morphological differentiation of most cells, because the three-dimensional nature of the response is difficult to observe using a standard microscope. In some cases, however, the appearance of the cells in vitro may be obvious. Defined structures are observed for some cell type/substrate combinations, such as hepatocytes on type I collagen and endothelial cells on Matrigel; the latter form capillary-like structures with a lumen (Kubota et al., 1988). Normal cells will not continue to grow when cultured on Matrigel but rather will differentiate. The growth rate of tumors, on the other hand, generally increases 3- to 6-fold on Matrigel (Fridman et al., 1991). For tumor cells, the incidence of tumor take is increased several-fold: ~5% of most tumors grow in the absence of Matrigel and >50% grow in the presence of Matrigel.

Time Considerations

Type I collagen and Matrigel form gels within 30 min in the culture dish, but it is recommended that the dishes be kept at 37°C for a full hour before warm medium is added. The time required for differentiation to occur in vitro on Matrigel varies depending on the cell type. Endothelial cells require 18 hr to form capillary-like structures and should not be left more than 24 hr (Kubota et al., 1988). In contrast, salivary gland cells require several days to form acinar-like structures (Hoffman et al., 1996). The in vivo angiogenesis assay also shows some time variation depending on the factors being tested. Fibroblast growth factor is generally fully active by 5 days; other factors require more time (Passaniti et al., 1992). Generally the assay is quantified 7 days after injection.

Literature Cited

Bendayan, M., Duhr, M.A., and Gingros, D. 1986. Studies on pancreatic acinar cells in tissue cultures: Basal laminin (basement membrane) matrix promotes three-dimensional reorganization. *Eur. J. Cell Biol.* 42:60-67.

Ben-Ze'ev, A., Robinson, G.S., Bucher, N.R.L., and Farmer, S.R. 1988. Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* 856:2161-2165.

Bilozur, M.E. and Hay, E.D. 1988. Neural crest migration in 3D extracellular matrix utilizes laminin, fibronectin and collagen. *Dev. Biol.* 125:19-33.

Blau, H., Guzowski, D.E., Siddiqi, Z.A., Scarpelli, E.M., and Bienkowski, R.S. 1988. Fetal type 2 pneumocytes form alveolar-like structures and maintain long-term differentiation on extracellular matrix. *J. Cell. Physiol.* 136:203-214.

Carey, D.J., Todd, M.S., and Rafferty, C.M. 1986. Schwann cell myelination: Induction by exogenous basement membrane–like extracellular matrix. *J. Cell Biol.* 102:2254-2263.

Cid, M.C., Grant, D.S., Hoffman, G.S., Auerbach, R., Fauci, A.S., and Kleinman, H.K. 1993. Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. *J. Clin. Invest*. 91:977-985.

Coon, H.G., Curcio, R., Sakagushi, K., Brandi, M.L., and Swerdlow, R.D. 1989. Cell culture of neuroblasts from rat olfactory epithelium that show odorant responses. *Proc. Natl. Acad. Sci. U.S.A.* 85:1702-1717.

Elsdale, T. and Bard, J. 1972. Collagen substrate for studies on cell behavior. *J. Cell Biol*. 54:626-637.

Emerman, J.T., Burwen, S.J., and Pitleka, D.R. 1979. Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in tissue culture. *Cell* 11:109-119.

Fridman, R., Kibbey, M.C., Royce, L.S., Zain, M., Sweeney, T.M., Jicha, D.L., Yannelli, J.R., Martin, G.R., and Kleinman, H.K. 1991. Basement membrane (Matrigel) enhances both the incidence and the growth of subcutaneously injected human and murine cells. *J. Natl. Cancer Inst*. 83:769-774.

Greenberg, G. and Hay, E.D. 1986. Transformation of thyroid epithelium to mesenchyme-like cells in vitro: Changes in intermediate filament expression and cytodifferentiation. *J. Cell Biol*. 105:250a.

Hadley, M.A., Byers, S.W., Suarez-Quian, C.A., Kleinman, H.K., and Dym, M. 1985. Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation and germ development. *J. Cell Biol*. 101:1511-1522.

Hauschka, S.D. and Konigsberg, I.R. 1966. The influence of collagen on the development of muscle colonies. *Proc. Natl. Acad. Sci. U.S.A.* 55:119-126.

Hoffman, M.P., Kibbey, M.C., Letterio, J.J., and Kleinman, H.K. 1996. Role of laminin-1 and TGFβ3 in acinar differentiation of a human submandibular salivary gland cell line. *J. Cell Sci*. 109:2103-2021.

Joshi, M.S. 1991. Growth and differentiation of the cultured secretory cells of the cow oviduct on reconstituted basement membrane. *J. Exp. Zool*. 260:229-238.

Kleinman, H.K., Klebe, R.J., and Martin, G.R. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol*. 88:473-485.

Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W., and Martin, G.R. 1986. Basement membrane complexes with biological activity. *Biochemistry* 25:312-318.

Kleinman, H.K., Graf, J., Iwamoto, Y., Kitten, G.T., Ogle, R.C., Sasaki, M., Yamada, Y., Martin, G.R., and Luckenbill-Edds, L. 1987. Role of basement membranes in cell differentiation. *Ann. N.Y. Acad. Sci*. 513:134-145.

Kubota, Y., Kleinman, H.K., Martin, G.R., and Lawley, T.J. 1988. Role of laminin and basement membrane in the differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* 107:1589-1597.

Li, L., Aggeler, M.J., Farson, D.A., Hatier, C., Hassell, J.R., and Bissell, M.J. 1986. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 84:136-140.

Passaniti, A., Taylor, R.M., Pili, R., Guo, Y., Long, P.V., Haney, J.A., Pauly, R.R., Grant, D.S., and Martin, G.R. 1992. A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin and fibroblast growth factor. *Lab. Invest*. 67:519-528.

Taub, M., Wang, Y., Szczesny, T.M., and Kleinman, H.K. 1990. Epidermal growth factor or transforming growth factor alpha is required for kidney tubulogenesis in Matrigel cultures in serum-free medium. *Proc. Natl. Acad. Sci. U.S.A.* 87:4002-4006.

Vukicevic, S., Luyten, F.P., Kleinman, H.K., and Reddi, A.H. 1990. Differentiation of canaliculi-like bone network by basement membrane matrix components and defined domains of laminin. *Cell* 63:437-445.

Vukicevic, S., Kleinman, H.K., Luyten, F.P., Roberts, A.B., Roche, N.S., and Reddi, A.H. 1992. Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Exp. Cell Res*. 202:1-8.

Contributed by Hynda K. Kleinman
National Institute of Dental Research/NIH
Bethesda, Maryland

Preparation of Extracellular Matrices Produced by Cultured Corneal Endothelial and PF-HR9 Endodermal Cells

Cell behavior is regulated by the substratum upon which cells attach, migrate, and proliferate. Tissue culture surfaces are therefore coated with extracellular matrix (ECM) extracts or with single ECM components in order to better resemble the microenvironment of cells in vivo and promote cell adhesion, proliferation, and expression of differentiated functions. This unit outlines the coating of tissue culture surfaces with a natural ECM produced and deposited by cultured bovine corneal endothelial (BCE) cells or PF-HR9 mouse endodermal cells. BCE-ECM closely resembles the ECM of subendothelium in vivo, whereas ECM from PF-HR9 cells more closely resembles the composition of basement membranes of other epithelia in vivo (see Commentary).

The basic approach is to allow the cells to produce their own ECM, while avoiding extraction procedures and purification of single components that may alter the relative proportions of native ECM constituents, alter the ECM's supramolecular organization, and/or denature its active molecules. For this purpose, the ECM-producing cells are maintained in culture at a confluent cell density. Five to twelve days after seeding, cellular elements are removed by solubilization of the cell layer with Triton X-100 and NH_4OH, leaving the underlying ECM intact, free of cellular debris, and firmly attached to the entire area of the culture surface. The ECM is then washed and covered with PBS and stored at 4°C. Under these conditions there is no loss of biological activity (i.e., induction of cell attachment, proliferation, and differentiation) for up to 6 months.

The Basic Protocol describes the preparation of BCE-ECM-coated dishes. Alternate Protocols describe modifications for preparation of metabolically radiolabeled ECM (which can be used to study matrix degradation associated with cell invasion and metastasis) and ECM derived from PF-HR9 cells (HR9-ECM). To evaluate the quality of the ECM, Support Protocols present straightforward procedures for assessing the induction of cell attachment, cell proliferation, and cell differentiation by the ECM.

NOTE: All culture incubations should be performed in a humidified 37°C, 10% CO_2 incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

PREPARATION OF BOVINE CORNEAL ENDOTHELIAL CELL ECM (BCE-ECM)

BASIC PROTOCOL

This protocol describes the initiation, establishment, and maintenance of stock primary and early-passage bovine corneal endothelial cell (BCE) cultures, as well as the preparation and storage of tissue culture surfaces coated with ECM produced by these cells. This ECM closely resembles that of the subendothelium in vivo, and is used to initiate primary cultures; to study the effects of local environment on cell adhesion, proliferation, differentiation, and survival; and to study various aspects of cell motility. Major constituents include collagens, proteoglycans, laminin, fibronectin, entactin, and elastin (see Background Information).

Although this procedure can be modified to coat any type of tissue culture dish, the best ECM-coated dishes (i.e., those with a homogeneous matrix that coats the entire surface of the dish) are obtained by plating the cells at a high density in 35-mm dishes. Thus, the strategy is to have the main extent of cell growth in 10-cm dishes (the primary stock

Contributed by Israel Vlodavsky
Current Protocols in Cell Biology (1999) 10.4.1-10.4.14

cultures), followed by two to three population doublings in 35-mm dishes (the early-passage cultures, yielding ECM-coated dishes).

Materials

10 to 20 eyes of freshly slaughtered cows
95% (v/v) ethanol
PBS (*APPENDIX 2A*)
Supplemented low-glucose DMEM-10 (see recipe)
Recombinant human basic fibroblast growth factor (bFGF; Sigma)
Trypsin/EDTA solution (see recipe)
Dextran T40 (Sigma)
Triton/NH_4OH cell lysis solution (see recipe)
Gentamicin (Life Technologies)
Fungizone (amphotericin B; Life Technologies)

18-G needles
10-cm, 6-cm, and 35-mm tissue culture dishes
Groove director (curette or spoon-headed spatula), sterile
37°C, 10% (v/v) CO_2 incubator with 100% humidity
10-cm gelatin-coated tissue culture dishes (see recipe)

Establish and maintain stock BCE cell cultures

1. Obtain 10 to 20 eyes of freshly sacrificed cows from a local slaughter house. Transport at 4°C or room temperature and process within 24 hr.

   The eyes can be stored at 4°C.

2. Ensure that eyes have unscratched, thin, crystal-clear corneas. Wash eyes with 95% ethanol to sterilize the outside of the corneas and destroy the outer epithelial cell layer.

3. Puncture the edge of each cornea at the junction of the cornea and the sclera with an 18-G needle. Insert dissecting scissors into the hole, dissect out the cornea, and place it endothelial side up in a 10-cm tissue culture dish.

4. Wash one cornea gently with PBS to remove traces of iris, which appear as small black fragments and may have adhered to the endothelium during its dissection.

5. Gently scrape the endothelium with a sterile groove director, being careful not to apply pressure.

   Applying pressure while scraping the endothelium may cause one to penetrate the Descemet's membrane and pick up stromal cells.

6. Transfer tissue fragments by dipping the groove director into a 6-cm tissue culture dish containing 5 ml supplemented low-glucose DMEM-10. Repeat steps 5 and 6 two to three times.

   Visual examination of the plate should reveal small whitish tissue fragments floating in the medium. When examined under a phase-contrast microscope, the tissue fragments appear to be composed of closely apposed hexagonal cells organized into a honeycomb pattern.

7. Repeat steps 4 to 6 for all remaining corneas, preparing a separate primary culture for each one.

8. Incubate plates at 37°C in a 10% CO_2 incubator with 100% humidity. Leave them untouched for 5 days, except for occasional examination under a phase-contrast microscope.

   After 3 to 5 days, several tissue fragments attach to the plastic, and cells can be seen migrating out of the explants.

9. On day 5 to 6, replace medium with fresh medium and start adding recombinant bFGF every other day at a final concentration of 1 ng/ml.

10. On day 8 to 12, aspirate medium from 6-cm primary culture dish, add 2 ml trypsin/EDTA solution, and incubate at 37°C for 5 min or until the cells have rounded up but are not detached.

    By day 8 to 12, well-developed colonies are clearly visible and each 6-cm dish contains ~1 × 10^6 cells.

    One primary culture is selected for preparation of stock culture dishes. Other primary culture dishes can be processed in parallel or maintained in the incubator for up to 3 weeks.

11. Aspirate trypsin/EDTA solution and resuspend cells in 5 ml supplemented low-glucose DMEM-10.

12. Transfer the entire 5-ml cell suspension (~1 × 10^6 cells) into one regular or gelatin-coated 10-cm tissue culture dish containing 5 ml supplemented low-glucose DMEM-10. Add bFGF at 1 ng/ml every other day until the plate is nearly confluent (5–7 × 10^6 cells/dish).

    Gelatin-coated dishes are used, if necessary, to improve the plating efficiency of BCE cells.

13. On day 4 to 6 of the first subculture (at near confluency), aspirate medium, add 5 ml trypsin/EDTA solution, and incubate at 37°C for 5 min or until the cells have rounded up but are not detached.

    It is best to passage the cells when subconfluent, because after reaching confluence the cells produce a large amount of ECM and hence become less susceptible to trypsinization and prone to damage in the process. This will be reflected in poor plating efficiency and cell growth, as well as in aberrant cell morphology.

14. Aspirate trypsin/EDTA solution and resuspend cells in 11 ml supplemented low-glucose DMEM-10.

15. Add 1 ml cell suspension to each of eleven 10-cm regular or gelatin-coated culture dishes, each containing 10 ml supplemented low-glucose DMEM-10 with 1 ng/ml bFGF. Add bFGF at 1 ng/ml every other day until the plates are nearly confluent (5–7 × 10^6 cells/dish).

    The surface area of a 10-cm stock dish is approximately equal to that of eight 35-mm dishes. Therefore, to prepare a batch of two hundred 35-mm dishes for production of ECM (see step 19), it is advisable to use the cells from stock dishes. A group of eleven 10-cm stock dishes can be used so that one dish is kept as a stock dish, and cells of the remaining ten 10-cm dishes are used to produce two hundred 35-mm ECM-coated dishes.

    If ECM is produced on a routine basis, try to prepare primary cultures once a month. Maintain confluent BCE cultures for up to 3 weeks in the CO_2 incubator with no medium change. The cells remain viable and can be readily subcultured at any time. The cells can also be frozen in liquid nitrogen using 10% (v/v) dimethyl sulfoxide (DMSO) in supplemented low-glucose DMEM-10, but the results are often not as good as those obtained with freshly isolated cells.

Prepare ECM-coated 35-mm dishes

16. Prepare 500 ml supplemented low-glucose DMEM-10 with 4% (w/v) dextran T40 and 1 ng/ml bFGF.

    Dextran T40 is added to the growth medium to increase its viscosity and hence the phagocytosis/exocytosis activity of the BCE. As a result, the amount (thickness) of ECM deposited is at least two-fold higher with dextran T40.

17. Trypsinize cells from ten 10-cm stock culture dishes by aspirating the medium, adding 5 ml trypsin/EDTA solution to each dish, and incubating at 37°C for 5 min or until the cells have rounded up but are not detached.

    Best production of ECM in terms of thickness and homogeneity is obtained with secondary or early passage BCE. Do not use cells beyond their sixth passage in culture.

18. Aspirate trypsin/EDTA solution and resuspend the cells of each dish in 5 ml supplemented low-glucose DMEM-10. Pool the cell suspensions and add to 500 ml supplemented low-glucose DMEM-10 with 4% dextran and 1 ng/ml bFGF.

19. Distribute 2-ml aliquots (~2×10^5 cells) into each of two hundred 35-mm plastic tissue culture dishes. Add 1 ng/ml recombinant bFGF every other day until the cultures are confluent (ordinarily within 4 to 6 days).

20. Incubate cultures for an additional 5 to 8 days without medium change.

Remove cells

21. Warm Triton/NH_4OH cell lysis solution 10 to 20 min at 37°C. Aspirate the culture medium from each 35-mm culture dish and add 1 ml cell lysis solution.

    In certain experiments, Triton X-100 and NH_4OH should be avoided (see Critical Parameters). In this case, cells are removed with 2 M urea in supplemented low-glucose DMEM-10, for 10 to 20 min at 37°C (Gospodarowicz et al., 1983).

22. Incubate dishes for 3 to 5 min with gentle shaking at room temperature and examine one of the dishes under a phase-contrast microscope.

    Generally, complete cell lysis occurs within 2 to 3 min and the ECM remains firmly attached to the tissue culture plastic. If cell nuclei remain bound to the bottom of the dish, incubate for an additional 5 to 10 min at 37°C to obtain complete solubilization of the cell layer.

    ECM produced by BCE cells appears as a delicate uniform network of amorphous material closely associated with the plastic. The homogeneous nature and thickness of the ECM lattice can be appreciated by scratching the plate with a needle, which may detach and lift the ECM membrane along the edge of the scratch. A more fibrillar, nonhomogenous type of ECM is less active and is often produced by late-passage cells.

    Detached ECM can be used for purification or biochemical analysis; however, purification of a single component from bound or detached ECM is not straightforward.

23. Wash each plate four times with 2 ml PBS.

24. Cover ECM-coated plates with 2 ml PBS supplemented with 50 µg/ml gentamicin and 0.25 µg/ml Fungizone. Store up to 4 months at 4°C.

ALTERNATE PROTOCOL 1

PREPARATION OF METABOLICALLY LABELED ECM

The procedure described in the Basic Protocol can also be applied to prepare radioactive, metabolically labeled ECM. Metabolically labeled ECM can be prepared by adding labeled sulfate ($Na_2{}^{35}SO_4$), amino acids ([^{3}H]proline or [^{14}C]glycine), or sugars (i.e., [^{14}C]glucosamine) to the tissue culture medium. The labeled ECM synthesized in this manner can be utilized as a substrate to study degradation of specific ECM constituents by certain purified enzymes or invasive cells, such as metastatic tumor cells or activated cells of the immune system. For example, sulfate-labeled ECM-coated dishes are useful as a natural basement membrane-like substrate for measurement of heparanase activity expressed by highly metastatic tumor cells and activated cells of the immune system (Vlodavsky et al., 1983, 1992, 1995; Naparstek et al., 1984).

To prepare sulfate-labeled BCE-ECM, add 40 µCi $Na_2{}^{35}SO_4$ (540 to 590 mCi/mmol; Amersham) per 35-mm dish on days 2 and 5 after seeding the early-passage cultures (see

Basic Protocol, step 19), and incubate without medium change. Ten to twelve days after seeding, prepare ECM as described (see Basic Protocol, steps 21 to 24). To increase the specific activity of the labeled ECM, use Fischer's medium (which does not contain sulfate) rather than DMEM for the 35-mm ECM-producing cultures. Supplement Fischer's medium (Life Technologies) as for supplemented low-glucose DMEM-10 and add 4% (w/v) dextran and 1 ng/ml bFGF. Maintain stock cultures as described above (see Basic Protocol). About 80% of the ECM-associated sulfate radioactivity is incorporated into sulfated proteoglycans, primarily heparan sulfate proteoglycans.

PREPARATION OF HR9-ECM

ALTERNATE PROTOCOL 2

Initiation of BCE cultures requires certain skills and is time consuming. It may, therefore, be more convenient to work with a permanent cell line capable of producing ECM. Such a cell line (PF-HR9) was derived from a differentiated mouse endodermal carcinoma (Chung et al., 1977) and can be obtained from ATCC or any of the many laboratories that are working with it (Kramer and Vogel, 1984; Rogelj et al., 1989). Unlike BCE cells, PF-HR9 cells are epithelial in nature and, as such, they produce ECM that better resembles epithelial basement membranes (rather than the subendothelial ECM produced by BCE). While the BCE-ECM provides an excellent model for basement membranes of blood vessels, the HR9-ECM provides a model for basement membranes underlying epithelial cells in a variety of organs and tissues. Major constituents of this ECM are laminin, entactin, collagen type IV, and heparan sulfate proteoglycans. Unlike the BCE-ECM, HR9-ECM contains little or no fibronectin, collagen type III, or dermatan sulfate proteoglycans, and no detectable bFGF (Gospodarowicz et al., 1984; Rogelj et al., 1989). It induces cell attachment and flattening, and promotes growth and differentiation of kidney tubule epithelial cells (Gospodarowicz et al., 1984), but fails to induce endothelial cell proliferation and neurite outgrowth in PC12 cells (Rogelj et al., 1989).

This procedure is described for 35-mm tissue culture dishes, but can easily be modified to coat other tissue culture plates and flasks. In fact, all available culture dishes and multiwell plates have been successfully coated with BCE-ECM and HR9-ECM.

Additional Materials *(also see Basic Protocol)*

PF-HR9 cells (ATCC; Chung et al., 1977; Kramer and Vogel, 1984)
Supplemented high-glucose DMEM-10 (see recipe)
Ascorbic acid (Sigma)
Penicillin
Streptomycin
Fibronectin-coated tissue culture dishes (see recipe)

1. Maintain stock cultures of PF-HR9 cells in supplemented high-glucose DMEM-10 in 10-cm tissue culture dishes at 37°C in a humidified, 10% CO_2 incubator. Dissociate stock cultures with trypsin/EDTA solution (5 min at 37°C) and subculture weekly at a 1:10 split ratio.

   PF-HR9 cells can be frozen in liquid nitrogen in high-glucose DMEM containing 10% (v/v) FBS and 10% (v/v) DMSO.

2. Dissociate one 10-cm stock culture of PF-HR9 cells with trypsin/EDTA solution (5 min at 37°C), rinse cells, and resuspend in 100 ml supplemented high-glucose DMEM-10.

   One stock culture provides enough cells for fifty 35-mm dishes.

3. Seed 2×10^5 cells per 35-mm fibronectin-coated tissue culture dish in supplemented high-glucose DMEM-10 with 4% (w/v) dextran T40.

These dishes will be coated with HR9-ECM. Precoating with fibronectin enforces a firm adhesion of the HR9-ECM to the plastic substratum.

4. Incubate for 5 or 6 days, adding ascorbic acid at 50 µg/ml to the culture medium on days 2 and 4.

5. Warm Triton/NH_4OH cell lysis solution 10 to 20 min at 37°C. Aspirate the culture medium from each 35-mm culture dish and add 1 ml cell lysis solution.

   In certain experiments, denudation with Triton X-100 and NH_4OH should be avoided (see Critical Parameters).

6. Incubate the dishes for 3 to 5 min with gentle shaking at room temperature and examine one of the dishes under a phase-contrast microscope.

   Generally, complete cell lysis occurs within 2 to 3 min and the ECM remains firmly attached to the tissue culture plastic. If cell nuclei remain bound to the bottom of the dish, incubate for an additional 5 to 10 min at 37°C to obtain complete solubilization of the cell layer.

   The appearance of the HR9-ECM is similar to that of BCE-ECM (see Basic Protocol, step 22) except that it often contains cellular debris and is slightly more fibrillar.

7. Aspirate the solubilized material and wash each plate four times with 2 ml PBS.

   If needed, remove cellular debris by pipetting. If plates have not been precoated with fibronectin, the HR9-ECM may come off the plates during washing.

8. Add 1 ml PBS containing 50 µg/ml gentamicin and 0.25 µg/ml Fungizone to each 35-mm HR9-ECM plate. Store at 4°C for up to 4 months.

SUPPORT PROTOCOL 1

CELL ATTACHMENT ASSAY

A human colon carcinoma cell line, isolated from the liver and designated HS703T, is used to evaluate the cell adhesion–promoting activity of the ECM. The cells can be obtained from the Cell Culture Laboratory (Oakland, CA), under the auspices of the Office of Naval Research and the Regents of the University of California. HS703T cells have previously been shown to utilize laminin but not fibronectin in order to attach and spread (Vlodavsky and Gospodarowicz, 1981). On uncoated or fibronectin-coated tissue culture plastic, the cells grow as loosely attached aggregates of spheroid cells. In contrast, as early as 5 min after plating on ECM, the cells migrate out of the cell aggregates and become firmly attached and flattened (Vlodavsky et al., 1980; Vlodavsky and Gospodarowicz, 1981; Fig. 10.4.1). Attachment and spreading of HS703T human colon carcinoma cells therefore provide a rapid, convenient, and sensitive assay for the presence of a uniform ECM coating on plastic surfaces. This protocol can be used to assess BCE-ECM as well as HR9-ECM.

Materials

HS703T cells (Vlodavsky et al., 1980; Vlodavsky and Gospodarowicz, 1981)
Supplemented high-glucose DMEM-10 (see recipe)
Trypsin/EDTA solution (see recipe)
35-mm ECM-coated tissue culture dishes (see Basic Protocol; see Alternate Protocol 2)
PBS (*APPENDIX 2A*)

35-mm tissue culture dishes
Coulter counter

1. Maintain stock cultures of HS703T cells in supplemented high-glucose DMEM-10 in a humidified 37°C, 10% CO_2 incubator.

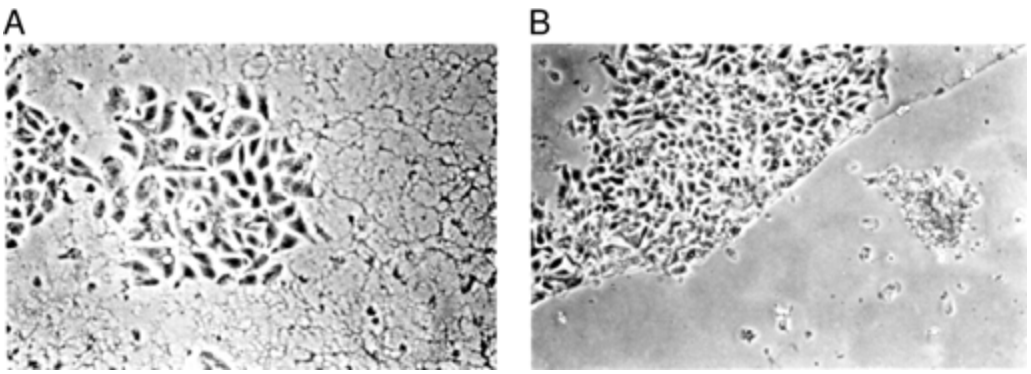


Figure 10.4.1 Adhesion and flattening of human colon carcinoma cells (HS703T) on BCE-ECM. (**A**) Intact ECM; (**B**) "wounded" ECM, where the ECM coating was scraped from part of the dish. Loosely attached aggregates of spheroid cells are seen on plastic (wounded ECM) as compared to firmly attached and flattened cells on ECM. Phase-contrast micrographs (180×) were taken 6 hr after seeding.

2. Dissociate cells with trypsin/EDTA solution (incubate 5 min at 37°C, then rinse with medium) or by repeated pipetting.
3. Seed 1×10^5 cells each onto duplicate 35-mm regular tissue culture dishes and duplicate ECM-coated tissue culture dishes.
4. At various times ranging from 5 to 60 min after seeding, remove unattached cells by gentle trituration and rinse the dishes twice with 2 ml PBS.
5. Dissociate remaining firmly attached cells with 1 ml trypsin/EDTA solution as above. Count the cells of duplicate dishes with a Coulter counter according to manufacturer's instructions.

   Alternatively, prelabel stock cultures with [^{3}H]thymidine (1 μCi/ml, 20 hr) and proceed as described above. Instead of counting cells, solubilize the firmly attached cells with 0.1 N NaOH (20 min, 37°C) and measure the radioactivity by liquid scintillation spectroscopy (Fridman et al., 1985).

CELL PROLIFERATION ASSAY

SUPPORT PROTOCOL 2

Bovine aortic vascular endothelial cells (BAEC; prepared according to Gospodarowicz, 1976) are applied to evaluate the growth-promoting activity of the BCE-ECM. When seeded in complete medium at a clonal cell density (300 cells/35-mm dish), the cells proliferate and form colonies on ECM (Fig. 10.4.2B), but not on regular tissue culture plastic (Fig. 10.4.2A; Gospodarowicz et al., 1980a,b; Rogelj et al., 1989). This protocol is not suitable for HR9-ECM.

Materials

Aortic tissue from freshly sacrificed cows
DMEM/BCS: supplemented low-glucose DMEM-10 (see recipe) in which FBS and newborn calf serum are replaced with 10% (v/v) bovine calf serum
Trypsin/EDTA solution (see recipe)
35-mm regular and BCE-ECM-coated tissue culture dishes (see Basic Protocol)
3.7% formaldehyde (prepared from commercial 37% formalin)
0.125% (w/v) crystal violet solution (see recipe)

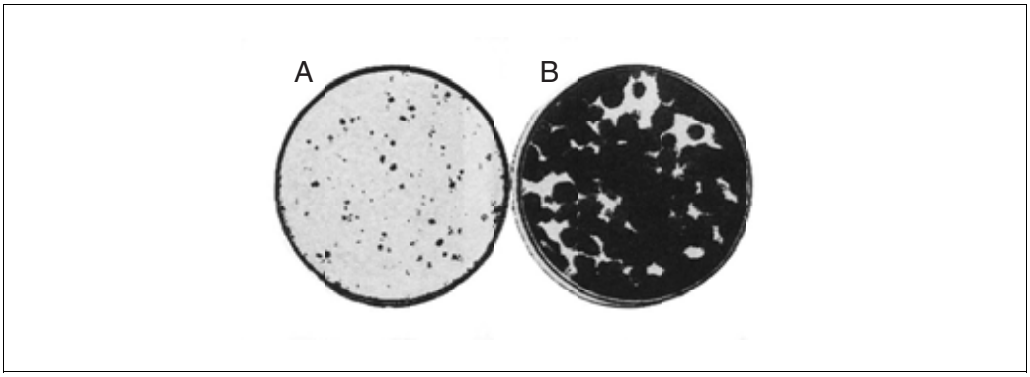


Figure 10.4.2 Clonal growth of bovine aortic endothelial cells on (**A**) plastic or (**B**) ECM. Cell colonies were fixed and stained with 0.125% crystal violet ten days after seeding (300 cells/35-mm dish).

1. Initiate clonal populations of bovine aortic endothelial cells (BAEC) from freshly obtained aortic tissue as described (Gospodarowicz et al., 1976), and maintain cells in DMEM/BCS in a humidified 37°C, 10% CO_2 incubator.
2. Dissociate a confluent stock culture into a single-cell suspension by incubating 5 min at 37°C with trypsin/EDTA solution. Rinse with medium.
3. Add 2 ml DMEM/BCS to duplicate 35-mm tissue culture dishes and duplicate BCE-ECM-coated tissue culture dishes, seed each with 300 cells, and incubate in CO_2 incubator.
4. At 10 to 12 days after seeding, aspirate medium and fix cells 1 hr at room temperature with 1 ml of 3.7% formaldehyde.
5. Stain cultures for 5 min with 1 ml of 0.125% crystal violet solution and rinse with tap water.

   At least 100 cell colonies, each containing >70 closely apposed, polygonal cells, should be seen on ECM. In contrast, uncoated dishes should have no cell colonies or very small and lightly stained colonies composed of senescent cells.

SUPPORT PROTOCOL 3

CELL DIFFERENTIATION ASSAY

The ability of PC12 cells, originally established from a transplantable rat pheochromocytoma, to extend neurites is used to evaluate the differentiation-promoting activity of BCE-ECM (Vlodavsky et al., 1982; Rogelj et al., 1989). This protocol is not suitable for HR9-ECM.

Materials

PC12 cells (ATCC)
Supplemented high-glucose DMEM-10 (see recipe)
Trypsin/EDTA solution (see recipe)
35-mm regular and BCE-ECM-coated tissue culture dishes (see Basic Protocol)

1. Maintain PC12 cells in supplemented high-glucose DMEM-10 in a humidified 37°C, 10% CO_2incubator.

   For PC12 cells, FBS in the medium should be heat inactivated (APPENDIX 2A).

2. Dissociate cells into a single-cell suspension by incubating 5 min at 37°C with trypsin/EDTA solution and rinse with medium.

3. Add 2 ml supplemented high-glucose DMEM-10 to each of the triplicate 35-mm tissue culture dishes triplicate ECM-coated tissue culture dishes, seed each with 1×10^5 cells, and incubate in a CO_2 incubator.

4. Examine cultures daily for neurite outgrowth.

   Extension of neurites to at least five-fold the length of the cell body is seen within 2 to 4 days on ECM. In contrast, there is almost no outgrowth on regular tissue culture plastic.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Crystal violet solution, 0.125% (w/v)

Dissolve 2.5 g crystal violet in 100 ml methanol. Stir overnight and filter through Whatman no. 3 paper. Store up to 4 months at room temperature. Dilute 1/20 in H_2O immediately before use.

Fibronectin-coated tissue culture dishes

Prepare a 25 µg/ml solution of human plasma fibronectin (Sigma) in PBS (*APPENDIX 2A*). Add 1 ml per dish to 35-mm tissue culture dishes and incubate 1 hr at room temperature. Remove fibronectin solution and rinse once with PBS. Store covered with PBS up to 1 month at 4°C. Rinse again after storage.

Gelatin-coated tissue culture dishes

Prepare a 0.2% (w/v) gelatin solution in PBS (*APPENDIX 2A*). Autoclave the solution, cool, and filter through a 0.2-µm filter. Add 5 ml solution per dish to 10-cm tissue culture dishes, and incubate ≥3 hr at 4°C. Remove the gelatin solution and wash the dishes once with 10 ml PBS. Store covered with PBS up to 1 month at 4°C. Rinse again after storage.

Supplemented low-glucose DMEM-10

Dulbecco's modified Eagle medium (DMEM) containing 1 g/liter glucose, supplemented with:
10% (v/v) FBS (*APPENDIX 2A*)
5% (v/v) newborn calf serum
50 µg/ml gentamicin
0.25 µg/ml Fungizone (amphotericin B; Life Technologies)
Store up to 1 month at 4°C

Supplemented high-glucose DMEM-10

Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/liter glucose, supplemented with:
10% (v/v) FBS (*APPENDIX 2A*)
50 U/ml penicillin
50 µg/ml streptomycin
Store up to 1 month at 4°C

Heat inactivation of FBS is only required for PC12 cells (see Support Protocol 3).

Triton/NH_4OH cell lysis solution

PBS (APPENDIX 2A) containing:
0.5% (v/v) Triton X-100
20 mM NH_4OH
Store up to 3 months at 4°C

Trypsin/EDTA solution

2.5 g trypsin
0.2 g EDTA
8 g NaCl
0.4 g KCl
1 g glucose
0.35 g $NaHCO_3$
0.01 g phenol red
H_2O to 1 liter
Store up to 3 months at –20°C

COMMENTARY

Background Information

The ECM is an organized complex of collagens, proteoglycans, and glycoproteins, all interacting to produce a highly stable structure upon which cells migrate, proliferate, and differentiate in vivo (Kleinman et al., 1981). Historically, ECM was regarded as a relatively inert scaffolding that stabilizes the physical structure of tissues. Subsequent studies indicated that the ability of cells to respond to various growth and differentiation factors is determined to a large extent by their shape and orientation, and that these are modulated by components of the ECM through interaction with specific transmembrane cell-surface integrin receptors (Gospodarowicz et al., 1978; Ingber, 1990; Hynes, 1992; Damsky and Werb, 1992). Studies with mammary gland epithelial cells led not only to the identification of genes that are dependent upon the ECM for their transcription, but also to the discovery of ECM response elements (Boudrean et al., 1995). It was also proposed that mechanical forces generated through cell-ECM interactions produce an altered cytoskeleton, altered nuclear morphology, and ultimately changes in the pattern of gene expression (Chen et al., 1997). Based on these and other observations, it is recognized that the ECM plays an active and complex role in regulating the morphogenesis of cells that contact it, influencing their development, migration, proliferation, survival, and metabolic functions (Meredith et al., 1993; Vlodavsky et al., 1993; Aharoni et al., 1997).

It appears that cellular responses to ECM are mediated by the combined action of basement membrane (BM) macromolecules (i.e., collagen IV, laminin, nidogen/entactin, proteoglycans) and active molecules (i.e., growth factors, enzymes) that are immobilized and stored in the ECM by means of binding to its macromolecular constituents, primarily to heparan sulfate proteoglycans (HSPG; Ruoslahti and Yamaguchi, 1991; Vlodavsky et al., 1993). Heparan sulfate also contributes to the assembly and integrity of the ECM through binding to various ECM molecules, including the fibrillar interstitial collagens (types I, III, and V), fibronectin, laminin, thrombospondin, and tenascin.

Elucidating specific components of the ECM involved in controlling cell proliferation and differentiation either in vivo or in vitro is a difficult task, mostly due to its intricate nature. Because the correct in vitro reconstruction of the ECM from its isolated native components into the highly ordered structure that it represents in vivo would be a formidable task, advantage is taken of the fact that cultured bovine corneal endothelial cells (BCE) and PF-HR9 endodermal cells have the ability to produce, underneath their basal surface, a thick ECM that adheres strongly to plastic (Vlodavsky et al., 1980; Gospodarowicz et al., 1984). The ECM produced by cultured BCE cells closely resembles the subendothelium in vivo in its morphology and molecular composition. It contains primarily collagens (mostly types III and IV, with smaller amounts of types I and V), proteoglycans (mostly heparan sulfate and dermatan sulfate proteoglycans, with smaller amounts of chondroitin sulfate proteoglycans), laminin, fibronectin, entactin, and elastin. Vascular endothelial cells and other cell types plated in contact with the subendothelial ECM no longer require the addition of soluble basic fibroblast growth factor (bFGF) and/or other growth-promoting factors in order to proliferate and express their differentiated functions (Gospodarowicz et al., 1980a,b; Vlodavsky et al., 1982). Unlike cells maintained on plastic (e.g., vascular smooth muscle cells), cells cultured in contact with BCE-ECM resemble their in vivo counterparts and proliferate in response to plasma factors (Gospodarowicz and Ill, 1980).

PF-HR9 cells are epithelial in nature. As such, they produce ECM composed primarily of characteristic epithelial basement membrane components such as collagen type IV, heparan sulfate proteoglycans, laminin, and entactin (Timpl et al., 1979; Leivo et al., 1982). This ECM resembles epithelial basement membranes better than the subendothelial ECM produced by BCE. While the BCE-ECM provides an excellent model for basement membranes of blood vessels, the HR9-ECM provides a model for basement membranes underlying epithelial cells in a variety of organs and tissues. Unlike the BCE-ECM, HR9-ECM contains little or no fibronectin, collagen type III, dermatan sulfate proteoglycans, and no detectable bFGF (Rogelj et al., 1989).

Although many cell types are capable of producing ECM in vitro, the ECM is most often removed together with the cells when the cell layer is solubilized with Triton/NH_4OH. This is due primarily to secretion of ECM components by the cultured cells (i.e., fibroblasts, smooth muscle cells) both in between the cells and toward the bottom and top of the cell layer. In contrast, the corneal endothelial cells and, to a lesser degree, the PF-HR9 cells deposit their ECM in a polar fashion, almost exclusively underneath the cell layer. This enables a preferential removal of the cell layer while leaving the ECM bound to the tissue culture plastic. In particular, this property and mode of polar deposition is characteristic of BCE cells whose ECM is more firmly bound to the tissue culture dish than that produced by PF-HR9 cells. Thus, BCE-ECM can be washed extensively to efficiently remove cellular debris.

The polar secretion of ECM by BCE and the firm interaction of this ECM with the tissue culture plastic provide an appropriate system to obtain a naturally produced substrate, free of cellular elements (Vlodavsky et al., 1980; Gospodarowicz et al., 1980a,b). The presence of nuclei or cytoskeletal elements could not be detected on the denuded ECM when coated plates were examined by phase-contrast microscopy, scanning electron microscopy, or indirect immunofluorescence, using anti-actin and anti-vimentin antibodies, or the benzimidole derivative Hoechst 33258 for nuclear staining (Gospodarowicz et al., 1983). Moreover, ECM prepared after a 24-hr exposure of subconfluent BCE cultures to [^{3}H]thymidine was devoid of labeled material. Likewise, no labeled cell surface components remained associated with the ECM after solubilization (Triton/NH_4OH) of lactoperoxidase-iodinated BCE that were plated on ECM for 24 hr. No serum proteins could be identified in the ECM (Gospodarowicz and Ill, 1980).

Unlike the BCE-ECM, cellular debris is always left adhering to the HR9-ECM. This is mostly due to the fact that part of the PF-HR9 cell population dies while proliferating. Cellular debris is therefore trapped in the ECM that is continuously produced by the living cells. Cellular debris adhering to the apical surface of the HR9-ECM are probably derived from dead cells that are present within the monolayer and that are not fully lysed when exposed to the Triton/NH_4OH solution.

The presence of heparan sulfate as a major glycosaminoglycan (GAG) in the ECM raised the possibility that ECM serves as a reservoir for heparin-binding growth factors that are tightly bound and stabilized by heparan sulfate in the ECM. Among these factors is bFGF, extracted from the subendothelial BCE-ECM produced in vitro (Vlodavsky et al., 1987) and from BM of the cornea in vivo (Folkman et al., 1988). bFGF was identified in a variety of endothelial and epithelial basement membranes in vivo (Cordon-Cardo et al., 1990; Vlodavsky et al., 1993). These and other results indicate that bFGF is an ECM component required for supporting cell proliferation, differentiation, and survival (Vlodavsky et al., 1991, 1993).

ECM-coated dishes are widely used for initiating primary cultures; for studying the control of cell adhesion, proliferation, differentiation, and survival by the local microenvironment (Vlodavsky et al., 1993); and for investigating cell migration, invasion, and metastatic behavior (Vlodavsky et al., 1983, 1992, 1995). ECM-coated dishes have been useful in restoring the normal phenotypic expression of a number of normal cell types that would otherwise have lost normal phenotypes when maintained on plastic (Gospodarowicz and Tauber, 1980; Gospodarowicz et al., 1980a,b). In the field of aging, cells maintained on ECM and exposed to serum-supplemented medium have a much longer life span in culture than cells maintained on plastic. This effect is attributed to cell survival signals that inhibit programmed cell death (Aharoni et al., 1997). ECM-coated dishes have also been useful in allowing the maintenance of a number of normal diploid cells and tumor cells under serum-free conditions, so that plasma factors involved in the control of their proliferation could be studied (Gospodarowicz and Ill, 1980; Gospodarowicz et al., 1980a,b, 1984). This property also facili-

tates the initiation of biopsy-derived epithelial cell cultures, while suppressing overgrowth by stromal fibroblasts (Biran et al., 1983). The BCE-ECM has been used in studies of nerve and glial cell differentiation (Vlodavsky et al., 1982; Lubetzki-Korn et al., 1983). The nerve cells can be either tumoral, such as the pheochromocytoma PC12 cell line, or normal sensory ganglion cells (Fujii et al., 1982; Vlodavsky et al., 1982). In either case, when cells are maintained on ECM, neurite outgrowth is initiated.

The HR9-ECM preferentially supports the proliferation and differentiation of epithelial cells. In the case of kidney tubule epithelial cells, it not only supports their growth but also promotes their differentiation into tubules that are indistinguishable from those seen in vivo (Gospodarowicz et al., 1984). HR9-ECM also allows the maintenance of kidney tubule epithelial cells under serum-free culture conditions; such cultures led to the identification of the two main plasma factors (transferrin and high-density lipoprotein) involved in supporting their growth (Gospodarowicz et al., 1984).

Critical Parameters

The goal of the described procedure is to obtain culture dishes entirely coated with a homogeneous, naturally produced, basement membrane–like matrix. The strategy is to have the main extent of cell growth in stock dishes and then seed the ECM-producing cells at a high density, allowing the ECM to be deposited over the course of two to three population doublings. The best production of ECM, in terms of thickness and homogeneity, is obtained with secondary or early-passage BCE. Cells beyond their sixth passage in culture should not be used. Also, 4% dextran T40 should be included in the growth medium in order to increase its viscosity and hence the phagocytotic/exocytotic activity of the BCE, which in turn increases the amount of ECM deposited. Unlike BCE-ECM, PF-HR9 cells produce little or no fibronectin. To enforce a firm adhesion of the HR9-ECM to the plastic substratum it is necessary to precoat the culture dishes with fibronectin. Even then, extensive washing of the HR9-ECM should be avoided, and cellular debris is often left adhering to this ECM. In contrast, no cellular debris remains associated with the BCE-ECM.

In certain experiments, denudation with Triton X-100 and NH_4OH should be avoided. For example, cell lysis should be avoided in experiments aimed to prove that bFGF is deposited into the ECM prior to the actual denudation of the ECM, and that it is not sequestered by heparan sulfate in the ECM when the cells are lysed with Triton and NH_4OH. For this purpose, the ECM-producing cells are removed by a procedure involving little or no cell lysis. This can be best achieved by exposing the cell monolayer to 2 M urea in DMEM for 10 to 20 min at 37°C (Gospodarowicz et al., 1983). Under these conditions, the cells detach from each other, round up, and can be removed by pipetting as intact and viable cells, leaving the underlying ECM firmly bound to the tissue culture plastic (Vlodavsky et al., 1987, Gospodarowicz et al., 1983).

Anticipated Results

The described procedures enable the coating of any available tissue culture surface with intact, naturally produced ECM, similar in its molecular organization to subendothelial (BCE-ECM) and subepithelial (HR9-ECM) basement membranes in vivo. The basic approach is to allow cells to deposit their own ECM and avoid using extraction/enzymatic procedures that may alter or denature the native ECM constituent proportion and supramolecular organization.

One primary BCE culture maintained in a 6-cm dish can be subcultured into eleven 10-cm stock dishes, yielding enough cells to coat two hundred 35-mm tissue culture dishes. For PF-HR9 cells, one 10-cm stock culture provides enough cells to coat fifty 35-mm dishes.

Time Considerations

For BCE-ECM, it takes 8 to 12 days to obtain primary cultures, 8 to 10 days to get the appropriate stock cultures, and ~10 additional days for the actual deposition of ECM. A shorter time is needed for preparation of ECM deposited by the PF-HR9 teratocarcinoma-derived cell line. The HR9-ECM is obtained 5 to 6 days after seeding the cells, but unlike the BCE-ECM, it contains some cellular debris and lacks bFGF.

Literature Cited

Aharoni, D., Meiri, I., Atzmon, R., Vlodavsky, I., and Amsterdam, A. 1997. Differential effect of components of the extracellular matrix on differentiation and apoptosis. *Curr. Biol.* 7:43-51.

Biran, S., Horowitz, A.T., Fuks, Z., and Vlodavsky, I. 1983. High-density lipoprotein and extracellular matrix promotes growth and plating efficiency of normal human mammary epithelial cells in serum-free medium. *Int. J. Cancer* 31:557-566.

Boudrean, N., Myers, C., and Bissell, M.J. 1995. From laminin to lamin: Regulation of tissue-specific gene expression by the ECM. *Trends Cell Biol.* 5:1-4.

Cordon-Cardo, C., Vlodavsky, I., Haimovitz-Friedman, A., Hicklin, D., and Fuks, Z. 1990. Expression of basic fibroblast growth factor in normal human tissues. *Lab. Invest.* 63:832-840.

Chen, C.S., Mrksich, M., Huang, Whitesides, G.M., and Ingber, D.E. 1997. Geometric control of cell life and death. *Science* 276:1425-1428.

Chung, A.E., Estes, L.E., Shinozuka, H., Braginski, J., Lorz, C., and Chung, C.A. 1977. Morphological and biochemical observations on cells derived from the in vitro differentiation of the embryonal carcinoma cell line PCC4-F. *Cancer Res.* 37:2072-2081.

Damsky, C.H. and Werb, Z. 1992. Signal transduction by integrin receptors for extracellular matrix. Cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772-781.

Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I. 1988. A heparin-binding angiogenic protein—basic fibroblast growth factor—is stored within basement membrane. *Am. J. Pathol.* 130:393-400.

Fridman, R., Alon, Y., Doljansky, R., Fuks, Z., and Vlodavsky, I. 1985. Cell interaction with the extracellular matrices produced by endothelial cells and fibroblasts. *Exp. Cell Res.* 158:461-476.

Fujii, D.K., Massoglia, S.L., Savin, N., and Gospodarowicz, D. 1982. Neurite outgrowth and protein synthesis by PC12 cells as a function of substratum and nerve growth factor. *J. Neurosci.* 2:1157-1175.

Gospodarowicz, D. and Ill, C.R. 1980. Do plasma and serum have different abilities to promote cell growth? *Proc. Natl. Acad. Sci. U.S.A.* 77:2726-2730.

Gospodarowicz, D. and Tauber, J.P. 1980. Growth factors and extracellular matrix. *Endocrine Rev.* 1:201-227.

Gospodarowicz, D., Moran, J., Braun, D., and Birdwell, C.R. 1976. Clonal growth of bovine vascular endothelial cells in tissue culture: Fibroblast growth factor as a survival agent. *Proc. Natl. Acad. Sci. U.S.A.* 73:4120-4124.

Gospodarowicz, D., Greenberg, G., and Birdwell, C.R. 1978. Determination of cellular shape by the extracellular matrix and its correlation with control of cellular growth. *Cancer Res.* 38:4155-4171.

Gospodarowicz, D., Delgado, D., and Vlodavsky, I. 1980a. Permissive effect of the extracellular matrix on cell proliferation in-vitro. *Proc. Natl. Acad. Sci. U.S.A.* 77:4094-4098.

Gospodarowicz, D., Vlodavsky, I. and Savion, N. 1980b. The extracellular matrix and the control of proliferation of vascular endothelial and vascular smooth muscle cells. *J. Supramol. Struct.* 13:339-372.

Gospodarowicz, D., Gonzalez, R., and Fujii, D.K. 1983. Are factors originating from serum, plasma or cultured cells involved in the growth promoting effect of the extracellular matrix produced by cultured bovine corneal endothelial cells? *J. Cell Physiol.* 114:191-202.

Gospodarowicz, D., Lepine, J., Massoglia, S., and Wood, I. 1984. Comparison of the ability of basement membranes produced by corneal endothelial and mouse-derived endodermal PF-HR-9 cells to support the proliferation and differentiation of bovine kidney tubule epithelial cells in vitro. *J. Cell Biol.* 99:947-961.

Hynes, R.O. 1992. Integrins: Versatility, modulation and signaling in cell adhesion. *Cell* 69:11-25.

Ingber, D.E. 1990. Fibronectin controls capillary endothelial cell growth by modulating cell shape. *Proc. Natl. Acad. Sci. U.S.A.* 87:3579-3583.

Kleinman, H.K., Klebe, R.J., and Martin, G.R. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473-485.

Kramer, H.R. and Vogel, K.G. 1984. Selective degradation of basement membrane macromolecules by metastatic cells. *J. Natl. Cancer Inst.* 72:889-899.

Leivo, I., Alitalo, K., Riteli, L., Vaheri, A., Timpl, R., and Wartiovaara, J. 1982. Basal lamina glycoproteins laminin and type IV collagen are assembled into a fine-fibered matrix in cultures of a teratocarcinoma-derived endoderm cell line. *Exp. Cell Res.* 137:15-23.

Lubetzki-Korn, I., Ovadia, H., Vlodavsky, I., Fuks, Z., and Abramsky, O. 1983. Enhanced growth and morphological differentiation of isolated adult rat oligodendrocytes in vitro: Use of a naturally produced extracellular matrix. *Brain Res.* 267:151-155.

Meredith, J.E. Jr., Fazeli, B., and Schwartz, M.A. l993. The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* 4:953-961.

Naparstek, Y., Cohen, I.R., Fuks, Z., and Vlodavsky, I. 1984. Activated T lymphocytes produce a matrix-degrading heparan sulfate endoglycosidase. *Nature* 310:241-243.

Rogelj, S., Klagsbrun, M., Atzmon, R., Kurokawa, M., Haimovitz, A., Fuks, Z., and Vlodavsky, I. 1989. Basic fibroblast growth factor is an extracellular matrix component required for supporting the proliferation of vascular endothelial cells and the differentiation of PC12 cells. *J. Cell Biol.* 109:823-831.

Ruoslahti, E. and Yamaguchi, Y. 1991. Proteoglycans as modulators of growth factor activities. *Cell* 64:867-869.

Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidart, J.M., and Martin, G.R. 1979. Laminin—A glycoprotein from basement membrane. *J. Biol. Chem.* 254:9933-9937.

Vlodavsky, I. and Gospodarowicz, D. 1981. Respective involvement of laminin and fibronectin in the adhesion of human carcinoma and sarcoma cells. *Nature* 289:304-306.

Vlodavsky, I., Liu, G.M., and Gospodarowicz, D. 1980. Morphological appearance, growth behavior and migratory activity of human tumor cells maintained on extracellular matrix vs plastic. *Cell* 19:607-616.

Vlodavsky, I., Levi, A., Lax, I., Schlessinger, J., and Fuks, Z. 1982. Induction of cell attachment and morphological differentiation in a pheochromocytoma cell line and embryonal sensory cells by the extracellular matrix. *Dev. Biol.* 93:285-300.

Vlodavsky, I., Fuks, Z., Bar-Ner, M., Ariav, Y., and Schirrmacher, V. 1983. Lymphoma cell mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: Relationship to tumor cell metastasis. *Cancer Res.* 43:2704-2711.

Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M. 1987. Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. *Proc. Natl. Acad. Sci. U.S.A.* 84:2292-2296.

Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R., Bashkin, P., and Fuks, Z. 1991. Extracellular sequestration and release of fibroblast growth factor: A regulatory mechanism? *Trends Biochem. Sci.* 16:268-271.

Vlodavsky, I., Eldor, A., Haimovitz-Friedman, A., Matzner, Y., Ishai-Michaeli, R., Levi, E., Bashkin, P., Lider, O., Naparstek, Y., Cohen, I.R., and Fuks, Z. 1992. Expression of heparanase by platelets and circulating cells of the immune system: Possible involvement in diapedesis and extravasation. *Invasion Metastasis* 12:112-127.

Vlodavsky, I., Bar-Shavit, R., Korner, G., and Fuks, Z. 1993. Extracellular matrix-bound growth factors, enzymes and plasma proteins. *In* Molecular and Cellular Aspects of Basement Membranes (D.H. Rohrbach and R. Timpl, eds.) pp. 327-343. Academic Press, San Diego.

Vlodavsky, I., Mohsen, M., Lider, O., Svahn, C.M., Ekre, H.-P., Vigoda, M., Ishai-Michaeli, R., and Peretz, T. 1995. Inhibition of tumor metastasis by heparanase inhibiting species of heparin. *Invasion Metastasis* 14:290-302.

Key References

Gospodarowicz et al., 1980a. See above.

See note for Vlodavsky et al. (1980).

Gospodarowicz et al., 1984. See above.

First article describing the effects of HR9-ECM on cell proliferation and differentiation.

Vlodavsky et al., 1980. See above.

This and Gospodarowicz et al. (1980a) are the first two articles describing the preparation, composition, and biological effects (induction of cell attachment, flattening, and proliferation) of BCE-ECM on normal and malignant cells.

Vlodavsky et al., 1993. See above.

Review article emphasizing the biological significance of ECM-resident bFGF and other active molecules.

Contributed by Israel Vlodavsky
Hadassah-Hebrew University Hospital
Jerusalem, Israel

Purification of Fibronectin

The adhesive glycoprotein fibronectin can be purified from body fluids, cultured cells, or tissues. Because of its relatively high fibronectin content, plasma is the most convenient source of this protein, which can be used to promote the adhesion, migration, and growth of virtually all cultured cells. The protocol presented in this unit for the purification of human plasma fibronectin (see Basic Protocol 1) uses immobilized gelatin (denatured collagen) to bind the protein. A pH 5.5 buffer is used to specifically elute the bound fibronectin without resorting to denaturing conditions. Because there are structural and functional differences between plasma and cell-derived fibronectins, purification protocols for the latter form are also given. Cellular fibronectin can be easily extracted from the surface of cultured cells (see Basic Protocol 2) and further purified if necessary (see Alternate Protocol 1) or harvested from cell-conditioned serum-free culture medium (see Alternate Protocol 2).

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed. Even screened human plasma should be treated as potentially contaminated with infectious agents such as HIV or hepatitis viruses. Two layers of gloves, safety glasses or goggles, and suitable protective clothing should be worn at all times. Plasma should be always be centrifuged in capped tubes or bottles. During thawing or warming, the plasma should be "double contained" (e.g., plastic bags of plasma should be contained in beakers before being placed in a water bath) in case of leakage. Any surfaces or skin on which plasma has been spilled or splashed should be disinfected with 10% sodium hypochlorite bleach or iodine-based disinfectant such as Wescodyne or Providone.

PURIFICATION OF PLASMA FIBRONECTIN

BASIC PROTOCOL 1

This protocol describes the purification of fibronectin from surplus or outdated human plasma. It can also been used to purify fibronectin from a variety of other species, including bovine, chicken, rat, and mouse from either plasma or sera. The yield of fibronectin will be much lower from sera because the process of precipitating the fibrin clot out of plasma to produce serum can also remove as much as 60% of the fibronectin. Surplus or outdated human plasma can be most easily obtained from local blood banks, or it may be purchased commercially. This protocol as described assumes 1 liter of human plasma as the starting material and can be easily scaled up proportionally.

Materials

1000 ml (3 to 4 units) surplus or outdated human plasma
Sepharose CL-4B (Amersham Pharmacia Biotech)
Gelatin-Sepharose (Amersham Pharmacia Biotech)
6 M urea
Tris-buffered saline (TBS): 0.9% (w/v) NaCl/10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.2 M EDTA, pH 7.0
0.2 M PMSF (see recipe)
ε-Amino-*n*-caproic acid
Column buffers A and B (see recipes)
Citrate elution buffer (see recipe)
0.5 M sodium phosphate, dibasic
Ammonium sulfate, ultrapure (Life Technologies or Sigma)
Dulbecco's PBS (*APPENDIX 2A*)
CAPS/saline (optional; see recipe)

5-cm-diameter column, ≥5 cm tall, siliconized with Aquasil (Pierce)
5-cm-diameter column, ≥10 cm tall, siliconized with Aquasil

continued

Contributed by Steven K. Akiyama
Current Protocols in Cell Biology (1999) 10.5.1-10.5.13

50-ml polycarbonate high-speed centrifuge tubes
250- or 500-ml centrifuge bottles
Dialysis tubing (12,000 to 14,000 MWCO; Spectrapor or equivalent)
High-speed refrigerated centrifuge with GS-3 and GSA rotors (Sorvall) or equivalent

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

Prepare columns

1. Pack a column (5 cm in diameter and ≥5 cm high) with 100 ml Sepharose CL-4B. Wash with at least 300 ml column buffer A as fast as possible by gravity flow.

   This will be used as a precolumn to remove plasma components that bind nonspecifically to gelatin or Sepharose. The flow rate can be increased by using tubing connected to the bottom of the column to siphon down the column eluate more rapidly.

2. Pack a column (5 cm in diameter, 10 cm high) with 200 ml gelatin-Sepharose. Wash the gelatin-Sepharose with at least 800 ml of 6 M urea in TBS, then at least 800 ml of column buffer A.

   Both of the preceding steps can be completed up to several days before the following steps.

Prepare plasma

3. Warm plasma to 37°C.

   All subsequent steps are performed at room temperature, except where noted.

4. Add protease inhibitors to plasma with stirring:

   25 ml 0.2 M EDTA (5 mM final)
   5 ml 0.2 M PMSF (1 mM final)
   6.6 g ε-amino-*n*-caproic acid (50 mM final).

5. Centrifuge 15 min at 10,000 × *g*, room temperature, and save the supernatant.

   Use 250- to 500-ml bottles appropriate for a Sorvall GSA or GS-3 rotor or equivalent. The rotor and the centrifuge should be at room temperature prior to and during centrifugation.

Affinity purify plasma fibronectin

6. Apply the plasma to the precolumn as fast as possible, then immediately apply the flowthrough to the gelatin-Sepharose column at a flow rate of no more than ~500 ml/hr.

   Treat the flowthrough fractions of the gelatin-Sepharose column as biohazardous waste and discard in a manner consistent with local and institutional regulations.

   The precolumn removes plasma components that bind nonspecifically to the gelatin or Sepharose beads.

7. Wash the column as rapidly as possible with 800 ml column buffer B, then with 400 ml column buffer A.

8. Elute the fibronectin from the column with citrate elution buffer and collect 40-ml fractions. Check fractions for the presence of eluted fibronectin by spectrophotometry (measuring A_{280}). Pool the fibronectin-containing fractions, and immediately add 4 ml of 0.5 M dibasic sodium phosphate per fraction to neutralize the solution.

   This is a possible stopping point. Neutralized fibronectin solutions can be stored up to several days at 4°C.

   The yield of fibronectin can be increased by following the citrate buffer elution by a second elution with 4 M urea in TBS. The urea-eluted fibronectin will be of lower purity and usually comprises less than half the yield of the citrate-eluted fibronectin; however, it is still potentially useful, especially as starting material for the preparation of fibronectin frag-

ments. The urea should be removed immediately by dialysis before the fibronectin is concentrated by ammonium sulfate precipitation as described in step 9.

To save the gelatin-Sepharose column for reuse, wash thoroughly with TBS containing 0.1% sodium azide immediately after the urea elution and store it at 4°C. The column can be used repeatedly for many years if it is kept hydrated. Reused gelatin-Sepharose can actually provide higher yields of fibronectin than "new" gelatin-Sepharose because of irreversible occupancy of nonspecific binding sites on the beads

Concentrate fibronectin by precipitation (optional)

9. If higher concentrations of fibronectin than those eluted from the column are necessary, precipitate the fibronectin by slowly adding 0.291 g ammonium sulfate per milliliter (50% saturation) while stirring on ice until the ammonium sulfate is completely dissolved. Let the ammonium sulfate-treated fibronectin incubate on ice without stirring at least 2 hr.
10. Centrifuge 15 min at 10,000 × *g*, 4°C. Resuspend the pellet and dialyze for several days at 4°C with stirring in 3.5 liters of a suitable buffer to remove any residual ammonium sulfate. Replace the buffer each day with 3.5 liters fresh buffer.

    A wide range of common buffers can be used to solubilize and dialyze the final plasma fibronectin product. It is generally easiest to use TBS or Dulbecco's PBS. It is possible to achieve fibronectin concentrations greater than ~10 mg/ml in most physiological buffers. If higher concentrations are needed, fibronectin is soluble at over 20 mg/ml in CAPS/saline.

    If necessary, fibronectin can be sterilized by filtration, although yields will be low due to binding of fibronectin to the filter.

11. Determine the concentration of fibronectin.

    This is most easily determined by measuring the A_{280} and using an extinction coefficient of 1.28 ml·mg^{-1}·cm^{-1}.

12. Analyze by SDS-PAGE (*UNIT 6.1*).

    In the presence of reducing agent, plasma fibronectin appears as a doublet of 230 and 220 kDa.

13. Freeze small aliquots on dry ice and store at −80°C.

    Stored at this temperature, fibronectin can remain active for 10 years or more.

    If storage at −80°C is not available, the second-best option is to store fibronectin as a liquid solution on wet ice. Storage of fibronectin at −20°C should be avoided—at this temperature, fibronectin tends to form a clot that cannot be redissolved. Thaw frozen fibronectin at 37°C with minimal shaking or agitation. Avoid repeated freezing and thawing.

PURIFICATION OF FIBRONECTIN FROM CULTURED CELLS

BASIC PROTOCOL 2

There are two major approaches for the purification of cellular fibronectin from monolayers of cultured fibroblastic cells: (1) extracting cell surface-bound fibronectin from the cell layer and (2) purifying fibronectin that is spontaneously released from the cell monolayer and secreted into the medium (see Alternate Protocol 2). The protocols have been used to produce cellular fibronectin from fibroblasts from several species including human, chicken, rat, and mouse. Although many cell types can be used, cultured fibroblasts are usually the cells of choice. These cells are easy to culture and produce relatively large amounts of fibronectin. Fibroblasts can either be prepared from embryonic or neonatal tissue from the appropriate animal species or be purchased from commercial sources, such as the American Type Culture Collection. Yields of fibronectin can vary widely depending on cell type and culture conditions, but can be surprisingly high. For example, fibronectin can comprise up to 3% of the total protein produced by chick embryo

fibroblasts. In general, earlier-passage cells provide higher yields of fibronectin than later-passage cells.

This protocol assumes that human fibroblasts have been cultured to confluency in ten 850-cm^2 tissue culture roller bottles. Alternatively, 150-cm^2 dishes or 175-cm^2 flasks can be used, with volumes adjusted accordingly. Since fibronectin yields depend on cell numbers, allow the cells to grow as dense as possible yet not so dense that they detach. Many fibroblastic cells (e.g., chick embryo fibroblasts) are not truly contact inhibited and can eventually form several cell layers in culture, although they will detach from the substrate if cultured too long without passaging.

Cellular fibronectin in substantially pure form can be extracted from fibroblast monolayers with 1 to 2 M urea. If 1 M urea is used, the yield will be lower but further purification is often not necessary. Extraction with 2 M urea is often necessary to get a good yield of fibronectin, especially when human fibroblasts are used as the starting material. Users are encouraged to do small pilot extraction of 35-mm or 60-mm dishes of cells to determine the yield and purity of extracted fibronectin at different urea concentrations. Although 2 M urea extracts cell surface fibronectin from fibroblasts better than 1 M urea, the higher concentration is also more efficient at extracting other cell surface proteins. Thus, further purification of cellular fibronectin extracted with 2 M urea is usually required.

Materials

Fibroblasts grown to dense confluence in roller bottles using serum-containing medium
Hank's balanced salt solution (HBBS; *APPENDIX 2A*)
Dulbecco's modified Eagle's medium (DMEM)
100 mM glutamine
0.2 M PMSF (see recipe)
Urea, ultrapure (Life Technologies or ICN)
Ammonium sulfate, ultrapure (Life Technologies or Sigma)
0.2 M CAPS buffer, pH 11.0 (see recipe)
CAPS/saline (see recipe)

Roller bottle apparatus
Refrigerated high-speed centrifuge
50-ml polycarbonate high-speed centrifuge tubes
Dialysis tubing (12,000 to 14,000 MWCO; Spectrapor or equivalent)
1.5-cm polypropylene column, 5 cm high
12 × 75–mm polypropylene tubes

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*).

Extract cells

1. Warm 2000 ml HBSS and 1500 ml DMEM supplemented with 60 ml of 100 mM glutamine (4 mM final) and 15 ml 0.2 M PMSF (2 mM final) to 37°C.

   Approximately 200 ml HBSS and 150 ml supplemented DMEM will be needed per bottle.

2. Gently rinse roller bottles four times with 50 ml warmed HBSS each time. Discard rinses.
3. Add 25 ml/bottle DMEM with 4 mM glutamine and 2 mM PMSF and incubate 1 hr at 37°C while rotating at 1 rpm to deplete the monolayer of adsorbed serum proteins.

4. Prepare 2 M urea in DMEM by dissolving 30 g ultrapure urea in DMEM supplemented with 4 mM glutamine in a total volume of 250 ml. Warm to 37°C. Just before use, add 2.5 ml of 0.2 M PMSF (2 mM final).
5. Discard the serum-free medium, then add 20 ml/bottle fresh 2 M urea in DMEM with 4 mM glutamine and 2 mM PMSF (prepared in step 4) and incubate 2 hr at 37°C while rotating at 1 rpm.

   This step extracts the fibronectin from the cell surface. Usually 2 M urea is needed to extract human fibronectin, but 1 M urea can be used with some cells, for example chick embryo fibroblasts. Alternatively, 1 M urea will extract human fibronectin, albeit at low yields. However, fibronectin extracted with 1 M urea is often sufficiently pure that affinity purification is not necessary.
6. Centrifuge the extracted fibronectin in eight 50-ml polycarbonate centrifuge tubes for 15 min at 15,000 × *g*, 4°C. Save the supernatant, which contains the fibronectin, and discard the pellets.

Precipitate fibronectin

7. Measure the volume and slowly add 0.472 g ultrapure ammonium sulfate per milliliter of supernatant (70% saturation) with stirring on ice. After all of the ammonium sulfate is dissolved, let the solution incubate ~1 hr on ice without stirring.
8. Centrifuge the precipitated fibronectin in 50-ml polycarbonate centrifuge tubes for 15 min at 15,000 × *g*, 4°C. Discard the supernatant and gently but rapidly resuspend all the pellets in a total of ~10 ml of 0.2 M CAPS buffer, pH 11.0. Readjust pH to 11.0 with NaOH as needed while resuspending.

Dialyze precipitate

9. Dialyze the resuspended cellular fibronectin against 3.5 liters CAPS/saline buffer at 4°C with stirring. Replace buffer twice.

   Care must be taken to seal the dialysis buffer from exposure to air as well as possible using Saran wrap or equivalent. To minimize the airspace in the beaker above the dialysis buffer, make sure the Saran wrap is touching the top of the buffer. Otherwise, carbon dioxide from the air can dissolve in the buffer, lowering its pH and precipitating the cellular fibronectin.
10. Centrifuge 20 min at 15,000 × *g*, 4°C, to clarify the purified fibronectin.
11. Determine the concentration of cellular fibronectin by measuring the A_{280} using an extinction coefficient of 1.28 ml·mg^{-1}·cm^{-1} and analyze by SDS-PAGE (*UNIT 6.1*).

    Cellular fibronectin should produce a band at ~250 kDa.
12. Rapidly freeze cellular fibronectin in small aliquots with liquid nitrogen.

    To avoid partial neutralization of the CAPS buffer, do not use dry ice. Cellular fibronectin should be stored in liquid nitrogen or at −80°C. In liquid nitrogen, cellular fibronectin will remain active for several years if it is not repeatedly frozen and thawed. Cellular fibronectin should be thawed at 37°C with a minimum of shaking or agitation.

AFFINITY PURIFICATION OF EXTRACTED CELLULAR FIBRONECTIN

ALTERNATE PROTOCOL 1

Cellular fibronectin extracted from cells can often be used without further treatment. However, this material can be contaminated with other cellular proteins, especially if 2 M urea is required to extract the fibronectin; in such cases further purification is necessary.

Additional Materials *(also see Basic Protocol 2)*

Tris-buffered saline (TBS; *APPENDIX 2A*)
Gelatin-Sepharose (Amersham Pharmacia Biotech)
5-ml siliconized glass or polypropylene column

1. Extract cellular fibronectin from monolayer cells (see Basic Protocol 2, steps 1 to 6).
2. Pack 5 ml of gelatin-Sepharose into a siliconized glass or polypropylene column.

   Do not use an excessively large affinity column. Recoveries of cellular fibronectin tend to be greater at higher ratios of fibronectin to gelatin-Sepharose, due a relative decrease in nonspecific binding to the beads. A 5-ml bed volume is sufficient.
3. Wash the gelatin-Sepharose with 2.5 ml of 8 M urea in TBS, then with 25 ml TBS.
4. Dilute the cellular fibronectin 1:3 with TBS to a final urea concentration of 0.5 M.
5. Apply the fibronectin to the affinity column and collect 3-ml fractions.
6. Wash the column-bound material extensively with TBS, checking the A_{280} of the effluent, until the A_{280} is <0.05.
7. Elute with 8 M urea in TBS and collect 3-ml fractions. Save and pool the fractions containing eluted fibronectin as determined by a peak in the A_{280}.
8. Proceed with dialysis of the fractions (see Basic Protocol 2, steps 9 to 12).

ALTERNATE PROTOCOL 2

PURIFICATION OF HUMAN CELLULAR FIBRONECTIN FROM CONDITIONED MEDIUM

Densely cultured fibroblastic cells can release substantial amounts of cellular fibronectin into culture medium. The approach described here is to culture roller bottles of human fibroblastic cells to dense confluence in serum-containing growth medium, and then culture the cells in serum-free medium. The use of serum-free medium is required in order to avoid contamination of the cellular fibronectin with plasma fibronectin. Fibroblasts usually cannot be expected to proliferate much under serum-free culture conditions, so they must be cultured in 850-cm^2 roller bottles in the presence of serum until they are heavily confluent. This procedure assumes that ten such roller bottles are used as the source of fibronectin.

This protocol was initially developed as described using a serum-free medium formulation called CRCM-30 that was commercially available from Life Technologies. Unfortunately, this medium is no longer available and neither is its formulation, so the protocol is described using an alternative serum-free medium consisting of a mixture of Ham's F-12 nutrient mixture and DMEM supplemented with antibiotics, pyruvate, amino acids, insulin, selenium, transferrin, and BSA. In trial experiments, this medium did not provide as high yields as CRCM-30, but it works adequately. A wide range of serum-free media is commercially available and any formulation without serum additives should be suitable.

Additional Materials *(also see Basic Protocol 2)*

Serum-free medium: e.g., 1:1 DMEM/Ham's F12; supplemented (see above)
Gelatin-Sepharose (Amersham Pharmacia Biotech)
BSA, crystalline (3× recrystallized; CalBiochem)
Tris-buffered saline (TBS; *APPENDIX 2A*)
Urea, ultrapure (Life Technologies or Sigma)
CAPS/saline (see recipe)
Ammonium sulfate

2.5-cm polypropylene or siliconized glass column, 5 cm high (siliconized with Aquasil, Pierce)
1-liter cylindrical polypropylene bottles
3-ml polypropylene tubes

Prepare cells and collect conditioned medium

1. Warm 2000 ml serum-free medium to 37°C.
2. Gently rinse roller-bottle fibroblast cultures four times each with 50 ml warmed serum-free medium.
3. Add 50 ml/bottle of serum-free medium and incubate at least 1 hr at 37°C while rotating at 1 rpm to deplete the monolayer of adsorbed serum proteins.

   The incubation period in this step can be as long as overnight with no deleterious effects.
4. Discard the serum-free medium. Add 100 ml of fresh serum-free medium to each roller bottle and incubate 24 to 48 hr at 37°C while rotating at 1 rpm.
5. Collect the serum-free, conditioned medium from the bottles and add 10 ml of 0.2 M PMSF per 1000 ml conditioned medium (2 mM final).
6. Centrifuge the extracted fibronectin in polypropylene centrifuge tubes for 15 min at 25,000 × *g*, 4°C. Save the supernatant, which contains the fibronectin, and discard the pellets.

   The conditioned serum-free medium from several bottles can be pooled and stored at –80°C, or used immediately in the next section.

   Secreted cellular fibronectin can often be repeatedly harvested from the same roller bottles by adding more serum-free medium. Usually the cells will eventually start to detach from the roller bottles. When this happens, they can be regrown to confluence by adding serum-containing medium before repeating steps 1 to 5. As the fibroblasts age, the amount of fibronectin they secrete will decrease, so this process cannot be repeated indefinitely. When the yield of fibronectin begins to decline, the cells should be discarded.

Affinity purify secreted cellular fibronectin

7. Prepare the gelatin-Sepharose affinity column. Pack 10 ml gelatin-Sepharose per liter of conditioned medium in a 2.5-cm polypropylene or siliconized glass column.
8. Wash the gelatin-Sepharose with 30 ml of 5 mg/ml BSA in TBS. Then wash the column with 50 ml of 8 M urea in TBS followed by 50 ml TBS.

   The column is prewashed with BSA in order to block nonspecific, irreversible protein binding to the gelatin-Sepharose.
9. Mix 1 ml of BSA-treated gelatin-Sepharose with 1 liter of conditioned medium and incubate for 24 hr at 4°C.

   This is most easily done by mixing the beads and medium together in 1-liter cylindrical polypropylene bottles placed on a roller bottle apparatus with rotation at 3 rpm at 4°C. During this time, the fibronectin in the conditioned medium should bind to the immobilized gelatin.

   Avoid mechanical stirring or excessive agitation of the gelatin-Sepharose, both of which can break up the Sepharose beads, decreasing subsequent column flow rates and the yield of fibronectin.

Recover fibronectin

10. Allow the beads to settle, decant off most of the medium, and pour the gelatin-Sepharose into a 2.5-cm diameter siliconized glass or polypropylene column.
11. Wash the column-bound material with 50 ml TBS at room temperature.
12. Elute with 8 M urea in TBS, collecting 3-ml fractions. Save and pool the fractions containing eluted fibronectin as determined by a peak in the A_{280}, until $A_{280} < 0.05$.

Concentrate fibronectin (optional)

13. If the concentration of cellular fibronectin obtained from the gelatin-Sepharose affinity column is >1 mg/ml, proceed to step 16. If a higher concentration of cellular fibronectin is needed, dialyze the eluted fibronectin against 3.5 liters CAPS/saline with stirring for at least 3 hr to remove most of the urea. Replace buffer at least twice.
14. Transfer the fibronectin to a polypropylene beaker, measure the volume, and slowly add 0.472 g ultrapure ammonium sulfate per ml (70% saturation) with stirring on ice. After all of the ammonium sulfate is dissolved, let the solution incubate at least 1 hr on ice without stirring.
15. Centrifuge the precipitated fibronectin in polypropylene centrifuge tubes for 15 min at 15,000 × *g*, 4°C. Discard the supernatant and gently but rapidly resuspend the cellular fibronectin pellets in 0.2 M CAPS buffer, pH 11.0. Readjust pH to 11.0 with NaOH if needed while resuspending.

Dialyze fibronectin

16. Dialyze the cellular fibronectin against 3.5 liters CAPS/saline buffer at 4°C with stirring. Replace buffer at least twice.

    Care must be taken to seal the dialysis buffer from exposure to air as well as possible using plastic (Saran) wrap. To minimize the airspace in the beaker above the dialysis buffer, make sure the Saran wrap is touching the top of the buffer. Otherwise, carbon dioxide from the air can dissolve in the buffer and lower its pH.

17. Clarify the purified fibronectin by centrifuging 20 min at 15,000 × *g*, 4°C.
18. Determine the concentration of cellular fibronectin by measuring the A_{280} using an extinction coefficient of 1.28 ml·mg^{-1}·cm^{-1} and analyze by SDS-PAGE (*UNIT 6.1*).
19. Freeze cellular fibronectin in small aliquots with liquid nitrogen.

    To avoid partial neutralization of the CAPS buffer, do not use dry ice. Cellular fibronectin should be stored in liquid nitrogen or at −80°C. In liquid nitrogen, cellular fibronectin will remain active for 10 years or more if it has not been repeatedly frozen and thawed. Cellular fibronectin should be thawed at 37°C with a minimum of shaking or agitation.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CAPS buffer, pH 11.0, 0.2 M

22.1 g CAPS (cyclohexylaminopropane sulfonic acid)
~450 ml H_2O
Adjust pH to 11.0 with 6 N NaOH
Add H_2O to 500 ml
Store protected from air up to several weeks at room temperature

After 1 to 2 months, check the pH and readjust with NaOH if necessary.

CAPS/saline

8.8 g sodium chloride
~900 ml H_2O
50 ml 0.2 M CAPS buffer, pH 11.0 (see recipe)
1 ml 1 M calcium chloride
H_2O to 1000 ml
Store protected from air up to 1 month at room temperature

After 1 month, check the pH and readjust with NaOH if necessary.

Citrate elution buffer

11.6 g NaCl
29.4 g sodium citrate (trisodium citrate, dihydrate)
~1800 ml H_2O
2 ml 20% (w/v) sodium azide stock (see recipe)
Adjust pH to 5.5 with 6 N HCl
Add H_2O to 2000 ml
Store up to 1 month at room temperature

Composition: 0.1 M NaCl and 50 mM sodium citrate, pH 5.5.

Column buffer A

12.1 g Tris·Cl
13.1 g ε-amino-*n*-caproic acid
11.8 g sodium citrate (trisodium citrate, dihydrate)
2 ml 20% (w/v) sodium azide stock (see recipe)
H_2O to ~1900 ml
Adjust pH to 7.6 with 6 N HCl
H_2O to 2000 ml
Prepare fresh on day of experiment and use within 2 days

Composition: 50 mM Tris·Cl, 50 mM ε-amino-n-caproic acid, and 20 mM sodium citrate, pH 7.6.

Column buffer B

58.4 g sodium chloride
6.0 g Tris·Cl
6.5 g ε-amino-*n*-caproic acid
5.9 g sodium citrate (trisodium citrate, dihydrate)
~800 ml H_2O
1 ml 20% (w/v) sodium azide stock (see recipe)
Adjust pH to 7.6 with 6 N HCl
Add H_2O to 1000 ml
Prepare fresh on day of experiment and use within 2 days

Composition: 1 M NaCl, 50 mM Tris·Cl, 50 mM 6-aminohexanoic acid, and 20 mM sodium citrate, pH 7.6.

PMSF stock solution, 0.2 M

Dissolve 69.6 mg PMSF (phenylmethylsulfonyl fluoride) in 2 ml isopropyl alcohol (Sigma). Store up to several days at room temperature, or divide into aliquots and store up to several years at −20°C.

Sodium azide, 20%

Dissolve 20 g sodium azide (Sigma) in water to 100 ml. Store for several months at room temperature.

COMMENTARY

Background Information

The fibronectins have been important as substrates to promote the adhesion and culture of cells in vitro and also as model systems for investigating mechanisms of cell adhesive interactions (Hynes, 1990; Mosher, 1989; Aota and Yamada, 1995; Potts and Campbell, 1994; Akiyama et al., 1995). Fibronectin contains two distinct regions that independently promote cell adhesion by binding integrin cell surface receptors. For most cells, adhesion is mediated by the central cell-binding domain of fibronectin through an Arg-Gly-Asp (RGD) sequence and a Pro-His-Ser-Arg-Asn (PHSRN) sequence that can interact synergistically with the RGD sequence to bind the α5β1 integrin (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984; Aota et al., 1994). A sec-

ond, independent cell-adhesive region of fibronectin that is located in the alternatively spliced IIICS (or V) module (Kornblihtt et al., 1985) binds the α4β1 integrin. At least two nonadjacent peptide sequences within the IIICS module contain cell adhesive activity—a Leu-Asp-Val (LDV) sequence and an Arg-Glu-Asp-Val (REDV) sequence (Humphries et al., 1987; Kormoriya et al., 1991). Unlike the central RGD and PHSRN sequences, the LDV and REDV sequences do not act in synergy but appear to have an additive effect on cell adhesion, although the REDV is much less active (~1% that of the LDV sequence).

Fibronectin is commonly found in most tissues and body fluids, but it is most easily purified from plasma and from cultured cells. The presence of cell adhesive factors in plasma or serum has been known for a long time and the existence of plasma fibronectin, in particular, has been known for about 50 years, although it has gone by a number of names, most commonly "cold insoluble globulin" or CIg (Morrison et al., 1948). Due to its relatively high content of fibronectin in soluble form, plasma is the most convenient source. Plasma-derived fibronectin is dimeric, consisting of two similar, but nonidentical, subunits, and is highly soluble under physiological conditions and over a wide range of pH values and salt concentrations.

Fibronectin has a number of ligands including heparin, collagen, and fibrin, as well as cell surface receptors. It actually binds denatured collagen (gelatin) better than it does native collagen; therefore, virtually all protocols for purifying fibronectin rely on its affinity for gelatin as described by Engvall and Ruoslahti (1977). The method in Basic Protocol 1 was first described by Miekka et al. (1982). It uses a pH 5.5 buffer to specifically elute the bound fibronectin without causing its denaturation and without eluting other gelatin-binding plasma proteins. The elution of fibronectin using a citric acid buffer provides a number of advantages over earlier procedures that use urea elution, including eliminating a second affinity purification column, preventing the denaturation the fibronectin, and avoiding exposure of the fibronectin to urea, which can chemically modify the fibronectin or irreversibly change its conformation.

Cellular and plasma fibronectins are derived from alternatively spliced variants of the same gene (Kornblihtt et al., 1985). Cellular fibronectin contains up to three inserted sequences not present in all chains of plasma fibronectin. The functional differences between plasma and cellular fibronectins are most striking in assays that measure morphological effects on cells, with the latter being more effective in certain adhesion and cell morphology assays (Yamada and Olden, 1978, Hashimoto-Uoshima et al., 1997; Manabe et al., 1997).

A number of cultured cell types synthesize fibronectin, including fibroblasts, endothelial cells, epithelial cells, myoblasts, and chondrocytes (Hynes, 1973; Jaffe and Mosher, 1978; Yamada and Weston, 1974; Chen et al., 1977; Dessau et al., 1978; Furcht et al., 1978; Ruoslahti et al., 1973). Cell-derived fibronectin is usually retained on the cell surface as insoluble fibers, but it can also be released into the culture medium. Virtually all cultured cells that synthesize fibronectin can be used a source of this protein, either by direct extraction with urea or by harvesting the fibronectin released into culture medium. The abundance of cellular fibronectin on fibroblasts and the ease with which these cells are isolated and cultured made these the most convenient choice.

Critical Parameters and Troubleshooting

The preparation procedures for plasma and cellular fibronectin are relatively simple and require a minimum of equipment, all readily available. The most common problem with these procedures is low yield, which is most often caused by improper handling of the purified fibronectin. Other potential causes are poor starting material and failure of fibronectin to either bind to or elute efficiently from the affinity column.

Except where noted, all of the steps in the purification protocols for fibronectin are performed at room temperature (~22°C); it is therefore necessary to include sodium azide in all column buffers to inhibit microbial growth. The protein content of all fractions is determined by the absorbance at 280 nm (A_{280}) and should be confirmed by SDS-PAGE (*UNIT 6.1*) with either Coomassie blue R-250 or silver staining.

In most cases, the final products of these protocols are obtained at relatively low concentrations—usually ≤1 mg/ml. Ammonium sulfate precipitation followed by resolubilization of fibronectin in a small volume and dialysis is usually the best method for increasing its concentration. Because all forms of fibronectin tend to adsorb readily onto surfaces, the use of membrane-type centrifugal concentrators

(such as Centricons or Centri-preps) is not recommended. Fibronectins of all forms are also quite susceptible to proteolytic cleavage. Therefore, these protocols usually require the addition of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF; see *APPENDIX 1B*). Solid PMSF is only slightly soluble and highly unstable when added to aqueous buffers, so it must be prepared as a 200 mM stock solution in isopropanol, which is then diluted into the aqueous buffer. PMSF dissolved in isopropanol is stable for several weeks at room temperature. However, PMSF is quite unstable in aqueous solution and, therefore, must be added to buffers immediately before use. All buffers containing PMSF that are not used within 20 min should be discarded.

Improper handling of fibronectin can arise from a number of sources, many of which are unintentional. Most low yields are the result of irreversible adsorption of the fibronectin onto the surfaces of tubes and beakers, and/or precipitation or clotting of the purified fibronectin. To insure maximal recovery of fibronectin, only polypropylene or siliconized glass beakers and tubes should be used. Agitation and other physical disturbances of the fibronectin should be avoided because such rough handling can cause denaturation of fibronectin at the air-liquid interface, aggregation, and precipitation. Fibronectin can also be lost due to improper handling during freezing and storage. In fact, small amounts of fibronectin are unavoidably lost during freezing and thawing. Fibronectin is best snap-frozen in powdered dry ice (for plasma-derived protein) or liquid nitrogen and stored at −80°C. Freezing at −20°C usually results in irreversible precipitation of the fibronectin, probably because slow freezing gives the protein a chance to aggregate in the liquid phase and form a clot during the time the solution is partially liquid as it cools. Fibronectin should be thawed rapidly at 37°C with no agitation. Storing fibronectin in liquid form at or near 0°C for a week or more is preferable to repeated freezing and thawing or freezing at −20°C.

Variations in starting material are almost unavoidable. Plasma and sera, whether from human or animal sources, will show individual variations in fibronectin content, even if collected in exactly the same manner. Cultured cells can vary in their fibronectin production and secretion, and yields usually decrease substantially as cells "age." Since the preparation of fibronectin requires only 1 to 2 days, it is usually not worth the time it takes to assay a fibronectin source prior to trying the purification protocol.

Fibronectin binds with high affinity to gelatin. Thus, low yields are rarely due to the fibronectin not binding to the affinity columns. When this does happen, it is usually because the affinity column is very old or has been stored improperly before use. The gelatin-Sepharose should always be stored near pH 7 in the presence of 0.05% sodium azide to retard microbial growth. Furthermore, although the gelatin is covalently bound to the beads, it can "bleed" off with time and repeated use, and it is susceptible to proteolysis by gelatinases in plasma. Thus, the urea prewash of the gelatin-Sepharose is a crucial step to remove gelatin fragments that can competitively inhibit fibronectin binding to the immobilized gelatin. It is also recommended that careful records be kept documenting the yield of a gelatin-Sepharose affinity column, and if a substantial drop in yields of consecutive preparations is noticed, the column should be discarded.

The most common cause of low yield is failure to elute the purified fibronectin efficiently off the gelatin-Sepharose affinity column. This is most likely due to small errors in the pH of the elution buffer. Citrate elution of fibronectin occurs because fibronectin binds poorly to gelatin at its isoelectric point. Thus, elution will not occur if the pH is either too high or too low. One way to avoid a total loss is always to elute the gelatin-Sepharose with 4 M urea following the citrate elution and collect these fractions as well. The urea-eluted fibronectin will be less pure than citrate-eluted material, but it will be useful for most cell culture purposes.

Cellular fibronectin is not very soluble at neutral pH and is best stored at pH 11. The solution can then be diluted into neutral buffers with no problems. Precautions should be taken to avoid exposing cellular fibronectin to air, and especially dry ice, for long periods of time, as carbon dioxide will eventually dissolve in the buffer, lowering its pH and precipitating the fibronectin.

Occasionally, the flow rate of the affinity column can become very slow. The most common cause is that the fibronectin has formed a "clot" at the top the affinity column, especially if the flow rate is too slow. If this happens, use a polypropylene rod to break up the top centimeter or so of the column bed, which should increase the flow rate.

The most commonly used alternative protocol for the purification of plasma fibronectin

involves the use of two affinity columns—one of gelatin and one of heparin—as described by Hayashi and Yamada (1983). In this protocol, plasma fibronectin is first bound to a gelatin-Sepharose column and eluted with 4 M urea. This material is immediately bound to a heparin-Sepharose column and washed with 4 M urea. The urea is then washed away and the purified fibronectin is eluted with 0.3 M sodium chloride. Although this protocol takes more than 50% longer, it yields fibronectin of higher purity at the expense of having exposed the fibronectin to denaturing conditions. The extra affinity column is worth the extra effort if extremely high-purity fibronectin is required. The specific cell adhesive activities of urea-eluted and citrate-eluted fibronectins are similar.

Anticipated Results

Basic Protocol 1 should yield 100 to 300 mg of fibronectin, which is usually a more-than-adequate amount for most uses. This material is usually of very high purity, and there should be no detectable impurities when it is analyzed by gel electrophoresis. This protocol has been optimized to promote efficient use of gelatin-Sepharose, and therefore specifies a relatively high plasma to affinity support ratio. If the fibronectin is to be purified from a particularly valuable plasma or serum sample, the column, wash and elution volumes can be doubled to increase (though not double) the yield.

It is much more difficult to predict yields of cellular fibronectin since these can depend on culture conditions, passage number of the cells, and cell type as well as the efficiency of the protocol. Low-passage-number human fibroblasts in ten 850-cm^2 tissue culture roller bottles should typically provide between 10 and 20 mg of cellular fibronectin.

The major impurity in preparations of cellular fibronectin is the extracellular protein tenascin-C. The level of contamination can be quite high, despite the use of great care. An early paper described the separation of tenascin from preparations of chicken cell surface fibronectin (Erickson and Iglesias, 1984). The gelatin-Sepharose affinity column chromotography step should separate the cellular fibronectin from tenascin because the latter protein does not bind to gelatin (Erickson, 1989; Lightner et al., 1988).

Time Considerations

All of these protocols take ≤1 day of actual hands-on time. Basic Protocol 1 will yield purified protein 2 to 3 days after starting. Usually the first ½ day is spent preparing buffers and packing and washing columns. The affinity purification steps are usually performed in one more day. Possible stopping points are noted in each protocol and, of course, the protocol can be stopped overnight or even over weekends at dialysis steps.

Literature Cited

Akiyama, S.K., Olden, K., and Yamada, K.M. 1995. Fibronectin and integrins in invasion and metastasis. *Cancer Metast. Rev.* 14:173-189.

Aota, S. and Yamada, K.M. 1995. Fibronectin and cell adhesion: Specificity of integrin-ligand interaction. *Adv. Enzymol. Relat. Areas Mol. Biol.* 70:1-21.

Aota, S., Nomizu, M., and Yamada, K.M. 1994. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. *J. Biol. Chem.* 269:24756-24761.

Chen, L.B., Maitland, N., Gallimore, P.H., and McDougal, J.K. 1977. Detection of the large, external, transformation-sensitive protein on some endothelial cells. *Exp. Cell Res.* 106:39-46.

Dessau, W., Sasse, J., Timpl, R., Jilek, F., and von der Mark, K. 1978. Synthesis and extracellular deposition of fibronectin in chondrocyte cultures. *J. Cell Biol.* 79:342-355.

Engvall, E. and Ruoslahti, E. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer* 20:1-5.

Erickson, H.P. 1989. Tenascin: An extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annu. Rev. Cell Biol.* 5:71-92.

Erickson, H.P. and Iglesias, J.L. 1984. A six-armed oligomer isolated from cell surface fibronectin preparations. *Nature* 311:267-269.

Furcht, L.T., Mosher, D.F., and Wendelschafer-Crabb, G. 1978. Immunocytochemical localization of fibronectin (LETS protein) on the surface of L6 myoblasts: Light and electron microscopic studies. *Cell* 13:263-271.

Hashimoto-Uoshima, M., Yan, Y.Z., Schneider, G., and Aukihil, I. 1997. The alternatively spliced domains EIIIB and EIIIA of human fibronectin affect cell adhesion and spreading. *J. Cell Sci.* 110:2271-2280.

Hayashi, M. and Yamada, K.M. 1983. Domain structure of the carboxy-terminal half of human plasma fibronectin *J. Biol. Chem.* 258:3332-3340.

Humphries, M.J., Kormoriya, A., Akiyama, S.K., Olden, K., and Yamada, K.M. 1987. Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type–specific adhesion. *J. Biol. Chem.* 262:6886-6892.

Hynes, R.O. 1973. Alterations of cell surface proteins and malignant transformation. *Biochim. Biophys. Acta* 458:73-107.

Hynes, R.O. 1990. Fibronectins. Springer-Verlag, New York.

Jaffe, E.A. and Mosher, D.F. 1978. Synthesis of fibronectin by cultured endothelial cells. *J. Exp. Med.* 147:1779-1791.

Kormoriya, A., Green, L.J., Mervic, M., Yamada, S.S., Yamada, K.M., and Humphries, M.J. 1991. The minimal essential sequence of a major cell type–specific adhesion site (CS1) within the alternatively-spliced type III connecting domain of fibronectin is leucine–aspartic acid–valine. *J. Biol. Chem.* 266:15075-15079.

Kornblihtt, A.R., Umezawa, K., Vibe-Pedersen, K., and Baralle, F.E. 1985. Primary structure of human fibronectin: Differential splicing may generate at least 10 polypeptides from a single gene. *EMBO J.* 4:1755-1759.

Lightner, V.A., Rajagopalan, P., and Erickson, H.P. 1988. Hexabrachion/tenascin does not bind to fibronectin, laminin, or collagen. *J. Cell Biol.* 107:600a.

Manabe, R., Oke, N., Maeda, T., Fukuda, T., and Sekiguchi, K. 1997. Modulation of cell-adhesive activity of fibronectin by the alternatively spliced EDA segment. *J. Cell Biol.* 139:295-307.

Miekka, S.I., Ingham, K.C., and Menache, D. 1982. Rapid methods for isolation of human plasma fibronectin. *Thromb. Res.* 27:1-14.

Morrison, P.R., Edsall, J.T., and Miller, S.G. 1948. Preparation and properties of serum and plasma proteins XVIII. The separation of purified fibrinogen from fraction I of human plasma. *J. Am. Chem. Soc.* 76:3103-3108.

Mosher, D.F., ed. 1989. Fibronectin. Academic Press, New York.

Pierschbacher, M.D. and Ruoslahti, E. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309:30-33.

Potts, J.R. and Campbell, I.D. 1994. Fibronectin structure and assembly. *Curr. Opin. Cell Biol.* 6:648-655.

Ruoslahti, E., Vaheri, A., Kuusela, P., and Linder, E. 1973. Fibroblast surface antigen: A new serum protein. *Biochim. Biophys. Acta* 322:352-358.

Yamada, K.M. and Kennedy, D.W. 1984. Dualistic nature of adhesive protein function and its biologically active peptide fragments can autoinhibit fibronectin function. *J. Cell Biol.* 99:29-36.

Yamada, K.M. and Olden, K. 1978. Fibronectins—adhesive glycoproteins of cell surface and blood. *Nature* 275:179-184.

Yamada, K.M. and Weston, J.A. 1974. Isolation of a major cell surface glycoprotein from fibroblasts. *Proc. Nat. Acad. Sci. U.S.A.* 71:3452-3496.

Contributed by Steven K. Akiyama
Chapel Hill, North Carolina

Purification of Vitronectin

BASIC PROTOCOL

The protocol described here has been successfully used to purify vitronectin from plasma or sera of a number of species. This method takes advantage of the observation that vitronectin must be activated or "opened" by denaturation before it can bind to heparin. Plasma or serum is first passed through a heparin-Sepharose column to remove fibronectin and other heparin-binding serum components. Then urea is added to the heparin-cleared plasma (or serum) to activate the heparin-binding activity of vitronectin. The activated vitronectin then binds to heparin-Sepharose and is eluted at very high purity.

As described here, this protocol assumes the starting material consists of 400 ml of citrate-anticoagulated human plasma, which should yield 10 to 20 mg of vitronectin of high (usually >98%) purity. A similar volume of human serum, or plasma or sera from other mammals, can also be used. This protocol can be easily scaled up by using correspondingly larger columns and buffer volumes.

NOTE: Only citrate-anticoagulated, not heparin-anticoagulated, plasma or serum should be used with this protocol. Because vitronectin binds to glass, any glassware to be used in this protocol should first be siliconized (see *APPENDIX 3*). Alternatively, use plastic—polypropylene, polycarbonate, or polyethylene—equipment (polypropylene is suggested throughout but the others may be used). Polystyrene is also acceptable and is better than glass.

CAUTION: When working with human blood cells or infection agents, appropriate biosafety practices must be followed. Even screened human plasma should be treated as if it is contaminated with HIV and hepatitis viruses. Always wear two layers of gloves, safety glasses or goggles, and suitable protective clothing. Keep a 10% bleach solution, Wescodyne, or Providone on hand at all times to use as an emergency disinfection agent in case of plasma spills or splashes.

Materials

Sepharose CL-4B (Amersham, Pharmacia Biotech)
Heparin-Sepharose (Amersham, Pharmacia Biotech)
Phosphate/saline/EDTA (see recipe)
2 M NaCl in phosphate/saline/EDTA (see recipe)
~400 ml (1 to 2 units) citrate-anticoagulated human plasma or serum
0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
0.2 M phenylmethanesulfonyl fluoride (PMSF; *UNIT 10.5*)
95% ethanol, ice cold
Ultrapure urea
8 M urea in phosphate/saline/EDTA (see recipe)
8 M urea/2 M NaCl in phosphate/saline/EDTA (see recipe)
2-Mercaptoethanol
8 M urea/0.3 M NaCl in phosphate/saline/EDTA (see recipe)
Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*)

20- and 50-ml (minimum) 2.5-cm-diameter polypropylene or siliconized glass columns
600- and 1000-ml polypropylene beakers
Polypropylene centrifuge tubes
Whatman no. 2 filter paper
10-ml polypropylene tubes
Quartz spectrophotomer cuvettes siliconized with Aquasil (Pierce)
Dialysis tubing (12,000 to 14,000 MWCO)

Contributed by Steven K. Akiyama
Current Protocols in Cell Biology (1999) 10.6.1-10.6.5

Prepare columns

1. Prepare a 2.5-cm-diameter, 8-ml Sepharose CL-4B precolumn. Wash with 24 ml phosphate/saline/EDTA.

   Follow manufacturer's instructions to prepare Sepharose CL-48 and heparin-Sepharose. All column chromatography steps in this protocol are at room temperature.

2. Prepare a 2.5-cm-diameter, 20-ml heparin-Sepharose column at room temperature. Wash with 80 ml of 2 M NaCl in phosphate/saline/EDTA, then with 80 ml phosphate/saline/EDTA.

   The columns can be washed up to several days in advance and kept at room temperature if 0.02% sodium azide is added to the phosphate/saline/EDTA.

3. To 400 ml citrate-anticoagulated plasma or serum, add 2.5 ml of 0.2 M EDTA and 0.5 ml of 0.2 M PMSF with rapid stirring at 4°C.

4. If serum is being used as the starting material, proceed to step 5. If plasma is used, place it in a 600-ml polypropylene beaker packed in ice and gradually add 48 ml ice-cold 95% ethanol with stirring. Continue to stir for 10 min.

   This step precipitates fibrinogen in plasma.

5. Centrifuge the plasma or serum 10 min at 12,000 × *g*, 4°C. Discard the pellet.

6. Save the supernatant and filter through Whatman no. 2 filter paper at 4°C.

   This is a possible overnight stopping point. The filtrate should be stored at 4°C.

Remove heparin-binding proteins

7. Apply the filtered plasma, preequilibrated at room temperature, to the Sepharose CL-4B precolumn to remove any plasma components that nonspecifically bind to the beads. After all the plasma has passed through the column, wash the column with 20 ml phosphate/saline/EDTA and add the wash to the plasma.

8. Apply the pooled flowthrough and wash from step 7 to the heparin-Sepharose column. After all the plasma has passed through the heparin-Sepharose column, wash the column with 50 ml phosphate/saline/EDTA and add the wash to the plasma.

   At this point, the procedure can be stopped until the next day or the vitronectin can be purified immediately. If the vitronectin is not to be purified immediately, wash the heparin-Sepharose with 80 ml of 2 M NaCl in phosphate/saline/EDTA to remove heparinases or glycosidases that could degrade the beads, and store the column-treated plasma containing the vitronectin at 4°C overnight. The column can be stored at room temperature if 0.02% sodium azide is added to the 2 M NaCl.

   This step removes plasma components (such as fibronectin) that bind to heparin in their native form.

Activate the vitronectin

9. Place the plasma in a 1000-ml polypropylene beaker at room temperature and add 384 g ultrapure urea to the plasma with gentle stirring. Then dilute the plasma to a final volume of 800 ml with phosphate/saline/EDTA. Let stand 1 to 2 hr at room temperature.

   This step activates the vitronectin so that it can bind to heparin. The final urea concentration is 8 M.

10. While the urea-treated plasma is left to stand, wash the heparin-Sepharose column used in step 8 above with 120 ml of 8 M urea/2 M NaCl in phosphate/saline/EDTA, then with 120 ml of 8 M urea in phosphate/saline/EDTA.

Urea is unstable in aqueous solution at room temperature. Therefore, all urea-containing solutions must be prepared fresh each day and subsequently discarded if not used the same day they are prepared.

Affinity purify vitronectin

11. Apply urea-treated plasma from step 11 to the heparin-Sepharose column at a flow rate of ≤100 ml/hr.

    This step can proceed overnight as long as precautions are taken to ensure that the column will not dry out.

    If step 11 proceeds overnight, prepare fresh solutions of 8 M urea in phosphate/saline/EDTA (see recipe) and 8 M urea/0.3 M NaCl in phosphate/saline/EDTA (see recipe).

12. Wash the column with 400 ml of 8 M urea in phosphate/saline/EDTA.

13. Add 140 µl 2-mercaptoethanol to 200 ml of 8 M urea in phosphate/saline/EDTA.

14. Wash the column with 100 ml of 8 M urea in phosphate/saline/EDTA supplemented with 2-mercaptoethanol.

    If this washing step takes ≤1 hr, stop the column so that the total exposure of the column to 2-mercaptoethanol is at least 1 hr to ensure removal of contaminants sensitive to reduction. Longer times, up to overnight, can also be used.

    If step 14 proceeds overnight, prepare fresh solutions of 8 M urea in phosphate/saline/EDTA (see recipe) and 8 M urea/0.3 M NaCl in phosphate/saline/EDTA (see recipe).

15. Wash the column with another 100 ml of 8 M urea in phosphate/saline/EDTA supplemented with 2-mercaptoethanol.

    The minimal exposure time of the column to 2-mercaptoethanol should be 1 hr.

16. Wash the column with 100 ml of 8 M urea in phosphate/saline/EDTA without 2-mercaptoethanol.

17. Elute the vitronectin with 8 M urea/0.3 M NaCl in phosphate/saline/EDTA. Collect 10-ml fractions. Pool and save the fractions containing eluted vitronectin as judged by A_{280} measured in siliconized quartz cuvettes. Save fractions with $A_{280} > 0.05$.

    It is recommended that the fractions containing higher concentrations ($A_{280} > 0.5$) be kept separate from those containing lower concentrations.

Dialyze fractions

18. Dialyze the fractions with higher concentrations ($A_{280} > 0.5$) at 4°C for 3 days against 3.5 liters DPBS, changing the buffer each day. Dialyze the lower-concentration fractions at 4°C for 3 days against 3.5 liters 10% DPBS (1 vol DPBS diluted with 9 vol H_2O), changing the buffer each day.

19. Centrifuge 20 min at 12,000 × *g*, 4°C, to clarify the purified vitronectin. Collect the supernatant.

20. Measure the concentration of vitronectin.

    The fastest way to estimate the protein concentration is to measure the A_{280} and calculate the concentration assuming an extinction coefficient of 1.36 ml/mgcm.

21. Aliquot 50 or 100 µl vitronectin into separate polypropylene tubes and lyophilize. Label the tubes to indicate which buffer was used, the original volume, and the amount of vitronectin in each tube.

    When the tubes are reconstituted with deionized water, the tubes that contained vitronectin in 10% DPBS should be reconstituted to one-tenth the original volume to yield higher concentrations of vitronectin in full-strength DPBS. Reconstituted vitronectin can be sterilized by autoclaving, if necessary.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

2 M NaCl in phosphate/saline/EDTA

29.4 g NaCl
5 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
6.3 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
0.25 ml 20% (w/v) sodium azide (*UNIT 10.5*)
Deionized H_2O to 250 ml
Store several weeks at room temperature

Phosphate/saline/EDTA

7.6 g sodium chloride (130 mM final)
20 ml 0.5 M sodium phosphate, pH 7.7 (see recipe; 10 mM final)
25 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*; 5 mM final)
1 ml 20% (w/v) sodium azide (0.02% final; *UNIT 10.5*)
Deionized H_2O to 1000 ml
Store several weeks at room temperature.

Sodium phosphate, pH 7.7, 0.5 M

79.7 g $Na_2HPO_4 \cdot 12H_2O$
4.29 g $NaH_2PO_4 \cdot 2H_2O$
Deionized H_2O to 1000 ml

8 M urea in phosphate/saline/EDTA

480 g ultrapure urea
7.6 g sodium chloride
20 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
25 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
Deionized H_2O to 1000 ml

The shelf life of this solution is <1 day.
Deionized H_2O to 500 ml

8 M urea/2 M NaCl in phosphate/saline/EDTA

60 g ultrapure urea
14.7 g NaCl
2.5 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
3.2 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
Deionized H_2O to 125 ml

The shelf life of this solution is <1 day.

8 M urea/0.3 M NaCl in phosphate/saline/EDTA

96 g ultrapure urea
3.5 g NaCl
4 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
5 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
Deionized H_2O to 200 ml

The shelf life of this solution is <1 day.

COMMENTARY

Background Information

Vitronectin, also known as serum spreading factor, S-protein, and epibolin, is found primarily in blood at concentrations ranging from 100 to 400 μg/ml and in other biological fluids as well as tissues (Preissner, 1991). Vitronectin can appear as two species of similar size. Human vitronectin appears as distinct bands of 65 and 75 kDa under reducing conditions. The ratio of the two species can vary. Vitronectin contains an Arg-Gly-Asp sequence which, as is the case for fibronectin, functions as a cell-adhesive recognition signal for integrins. However, cellular vitronectin and fibronectin receptors are usually distinct. Cells usually adhere to fibronectin primarily through β1 integrins while adhering to vitronectin primarily through β3 integrins.

The protocol described here was first developed by Yatohgo et al. (1988) and has been successfully used to purify vitronectin from plasma or sera of a number of species. This protocol can generally be used across species lines. It represents a major improvement over earlier methods, which relied on the propensity of vitronectin to bind to glass and used up to four different affinity columns and required several days (Barnes and Silnutzer, 1983). The protocol described here takes advantage of the observation that vitronectin, unlike the fibronectin present in plasma, must be activated or opened with 8 M urea before it can bind to heparin. Thus, plasma components that bind specifically or nonspecifically to heparin or the Sepharose beads can be removed prior to vitronectin activation, enabling this protocol to yield vitronectin of extremely high purity.

Critical Parameters and Troubleshooting

Low yields of vitronectin can be due to analogous causes as low yields in affinity-purification of plasma fibronectin. For example, heparin-Sepharose columns tend to bleed ligand. Therefore, extensive prewashing of the affinity columns is very important. Also, care must be taken to use only citrate-anticoagulated plasma. If heparin-anticoagulated plasma is used, the heparin can competitively inhibit vitronectin binding to the affinity column, greatly reducing the yields. A recurring problem in this protocol is the slowing of column flows to distressingly low rates. Thus, if it is necessary to scale up this protocol, it is recommended that column volumes be increased by changing the column diameters, rather than heights. Columns often run well initially but eventually slow down. A faster flow rate can sometimes be reestablished by breaking up the "crust" of aggregated proteins that can form on the top of the affinity column. It may be necessary to disturb the top of the gel to a depth of 1 to 2 mm. Vitronectin was so named from its propensity to bind to glass. Therefore, only polypropylene or siliconized glass columns, beakers, and tubes should be allowed to contact the vitronectin in all steps. Using untreated glass materials in this protocol can result in much lower yields. Finally, vitronectin can be stored at −80°C without lyophilization, but it slowly loses activity over the course of a year or two.

Anticipated Results

The protocol described here should yield 10 to 20 mg of vitronectin of relatively high purity (usually >98%). Purity can be checked by SDS-PAGE (*UNIT 6.1*). Human vitronectin usually appears as a doublet of bands at 65 and 75 kDa.

Time Considerations

This protocol requires a total of 2 to 3 days. Hands on time is between 1 and 2 days. Convenient stopping points are indicated in the protocol.

Literature Cited

Barnes, D.W. and Silnutzer, J. 1983. Isolation of human serum spreading factor. *J. Biol. Chem.* 258:12548-12552.

Preissner, K.T. 1991. Structure and biological role of vitronectin. *Annu. Rev. Cell Biol.* 7:275-310.

Yatohgo, T., Izumi, M., Kushiwagi, H., and Hayashi, M. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13:281-292.

Contributed by Steven K. Akiyama
Chapel Hill, North Carolina

Proteoglycan Isolation and Analysis

This unit describes specific methods for isolation and analysis of proteoglycans and glycosaminoglycans. These molecules can be difficult to work with due to their large size, high negative charge, and tendency to aggregate and form complexes with other molecules. This requires modification of standard protocols and protocols unique to proteoglycans. Proteoglycans vary in size from small (e.g., decorin ≈ 100 kDa) to very large (e.g., aggrecan ≈ 1×10^6 kDa). Proteoglycans are present in extracellular matrices (e.g., perlecan), linked to cell surfaces by phospholipid (e.g., glypicans), as transmembrane components (e.g., syndecans), and in intracellular granules in some cells (also see *UNIT 10.1*). As a class of molecules, they can be isolated and purified utilizing their glycosaminoglycan chains (e.g., by ion-exchange chromatography) or their core proteins (antibody-affinity chromatography). Their glycosaminoglycan content can be further analyzed by treatment with degradative enzymes followed by size-exclusion chromatography or SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety office (also see *APPENDIX 1D*).

NOTE: Even where chemical amounts of proteoglycan may be harvested, the addition of a trace of radiolabeled material aids in monitoring the purification process. In the absence of radiolabel, commercial assays based on the binding of 1,9-dimethylmethylene blue (a cationic dye) to sulfated glycosaminoglycans may be used to monitor isolation (e.g., Blyscan Glycosaminoglycan Assay, Biocolor; Accurate Chemical and Scientific).

ISOLATION AND PURIFICATION OF PROTEOGLYCANS

This group of protocols contains methods for isolating proteoglycans from cultured cells and for chromatographic purification.

Isolation of Proteoglycans from Cultured Cells

BASIC PROTOCOL 1

The following protocol is used to isolate proteoglycans from conditioned medium (soluble), as well as cell-associated proteoglycans (cell surface and intracellular) and proteoglycans in the extracellular matrix (insoluble). This is a general method applicable to all proteoglycans, as compared to Alternate Protocol 1, which is specifically designed to isolate different subcellular pools, especially those membrane-intercalated proteoglycans that act as links between the cytoskeleton and extracellular matrix. If only soluble proteoglycans are required, medium may be removed and new medium incubated with cells to increase yield.

Proteoglycans can be isolated from radiolabeled cultured cells. Since low yields are expected, particularly for some proteoglycans, the equivalent of five 10-cm-diameter culture dishes should be used for each experiment (8 ml medium/dish).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% to 10% CO_2 incubator (depending upon medium) unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Contributed by Anne Woods and John R. Couchman
Current Protocols in Cell Biology (2000) 10.7.1-10.7.19

Materials

Radiolabeled cultured cells in 10-cm tissue culture dishes (see Support Protocol)
PBS (*APPENDIX 2A*)
Urea, ultrapure
Solubilization buffer (see recipe) with and without Triton X-100

1. Collect supernatant from radiolabeled cultured cells by decanting into a 50-ml conical centrifuge tube. Rinse cell layer twice with 4 ml PBS each time, and add washes to the tube.
2. Centrifuge supernatant 10 min at 800 × *g*, room temperature, to remove any cellular material.
3. Add solid urea to the supernatant to a final concentration of 4 M. Store supernatant at 4°C; do not freeze. Use for analysis as soon as possible.

   All further stages with supernatant proteoglycans may be performed without detergent present in the buffers. The authors' experience, however, is that losses are decreased by the presence of 0.1% (v/v) Triton X-100.
4. Wash cell layers twice with 20 ml PBS to remove excess free radiolabel.
5. Add 2 ml solubilization buffer to the dish and scrape cells loose. Transfer suspension to a 15-ml conical tube.

   Do not substitute Tween 20 for Triton, as this detergent is not as effective in solubilizing syndecans.
6. Rinse cell layer and scraper sequentially twice with 2 ml solubilization buffer and add these washes to the tube. Incubate 30 min on ice with occasional vigorous shaking.
7. Centrifuge 10 min at 800 × *g*, room temperature.

   The supernatant should appear clear. If it is not, continue shaking and incubating until particulates are no longer seen. Proteoglycans (especially syndecans) aggregate readily, and may form large complexes that can be pelleted at higher g force if not solubilized.
8. Decant supernatant into a 100-ml graduated cylinder and dilute 1:5 with solubilization buffer without Triton to reduce detergent to 0.2%.
9. Store at 4°C; do not freeze. Use for analysis as soon as possible.

ALTERNATE PROTOCOL 1

Isolation of Proteoglycan Pools

Proteoglycans can be found soluble in culture supernatant, incorporated into insoluble matrix, attached to the cell surface, or as transmembrane components, some of which may link between the cytoskeleton and extracellular matrix. Methods presented here are adapted from Woods et al. (1985).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% to 10% CO_2 incubator (depending upon medium) unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Additional Materials *(also see Basic Protocol 1)*

Serum-free culture medium
Phosphatidylinositol-specific phospholipase C (PI-PLC; Boehringer Mannheim, Molecular Probes)
Lysis buffer (see recipe) with and without detergent

Isolate conditioned medium pool

1. Wash radiolabeled cultured cell monolayers twice with 20 ml PBS and incubate 1 hour at 37°C with serum-free culture medium containing 0.1 mU PI-PLC.

   This releases glycosylphosphotidylinositol (GPI)–linked proteoglycans (Schmidtchen et al., 1990).

2. Decant into a 15-ml conical centrifuge tube. Rinse cell layer twice with 2.5 ml serum-free medium without enzyme and add to tube.

3. Add solid urea to 4 M final, agitate to dissolve, and incubate on ice until used. Concentrate and purify (step 11) as soon as possible (within 24 hr).

Isolate lysate pool

4. Add 2 ml lysis buffer to the dish and incubate 15 min at 37°C.

5. Transfer lysate to a 15-ml conical tube, rinse cell layer twice with 1 ml lysis buffer, and add washes to the tube.

   If desired, lysates from up to three dishes can be pooled in a single 15-ml tube, and up to twelve can be combined in a 50-ml tube.

6. Add solid urea to 4 M and stir to dissolve.

7. Dilute 1:1 with lysis buffer without detergent to dilute detergent to 0.1%. Incubate on ice until used. Concentrate and purify (step 11) as soon as possible (within 24 hr).

   This is the "lysate" material, which will contain hydrophobic proteoglycans not retained due to linkage to cytoskeleton/matrix, and proteoglycans undergoing degradation or synthesis. Cell layers will lose phase density, but nuclei and stress fibers should remain.

Isolate cytoskeletal/matrix pool

8. Rinse dish three times with 20 ml PBS, 4°C.

9. Add 1 ml solubilization buffer and scrape cell remnants into the solution.

10. Dilute 1:10 with solubilization buffer without detergent to dilute detergent to 0.1%. Incubate on ice until used. Concentrate and purify (step 11) as soon as possible (within 24 hr).

    This is the cytoskeletal/matrix fraction, which will contain hydrophobic proteoglycans linked to the cytoskeleton/matrix, as well as matrix proteoglycans.

Concentrate and purify pools

11. Concentrate and purify all three samples by DEAE-Sephacel chromatography (see Basic Protocol 2).

    The steps above all contain protease inhibitors in the buffers because cell lysis is occurring. However, it is essential to perform anionic-exchange chromatography directly after isolation of the three pools, or proteolysis will still occur.

Radiolabeling of Proteoglycans with $^{35}SO_4$ or [^{3}H]Glucosamine

SUPPORT PROTOCOL

Because glycosaminoglycan chains (except hyaluronan) are usually highly sulfated, they can be metabolically labeled with $^{35}SO_4$. This is done by adding 40 µCi/ml $H_2{}^{35}SO_4$ (1050 to 1600 Ci/mmol; NEN Life Sciences) to the medium when seeding the cells, and allowing the cells to grow until just subconfluent (e.g., 95%). Sulfate-free medium should not be used, as it does not appear to increase incorporation of radiolabel, perhaps because sulfate becomes limiting. Streptomycin sulfate should not be used as an antibiotic in the medium, as this increases the level of unlabeled sulfate. If supernatant is not needed for analysis, it can be decanted at cell harvest and reused for labeling additional cells.

Glucosamine derivatives are readily incorporated into glycosaminoglycans, so [^{3}H]glucosamine (40 to 60 Ci/mmol; Amersham) can be used to radiolabel proteoglycans and hyaluronan. This is done by adding 10 μCi/ml [^{3}H]glucosamine at seeding and then growing cells to confluency.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% to 10% CO_2 (depending upon medium) incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

BASIC PROTOCOL 2

Anion-Exchange Chromatography Purification of Proteoglycans with DEAE-Sephacel

Anion-exchange chromatography is used to purify proteoglycans from conditioned medium or cells (Basic Protocol 1) or the isolated subcellular fractions (Alternate Protocol 1). Due to the high negative charge of the glycosaminoglycan chains, proteoglycans bind with higher affinity to DEAE than do most other cellular components.

Materials

Proteoglycan samples (see Basic Protocol 1 or Alternate Protocol 1)
Diethylaminoethyl (DEAE)–Sephacel: 50:50 (v/v) slurry in PBS
PBS (*APPENDIX 2A*)
DEAE-Sephacel equilibration buffer (see recipe)
Conditioned medium from cell line of interest, incubated with cells 24 to 48 hr
DEAE low-pH buffer (see recipe)
DEAE elution buffer (see recipe)
2 × 0.5–cm minicolumns

1. Prepare DEAE-Sephacel in 2 × 0.5–cm minicolumns with ~2 to 3 ml gel bed by adding a 50:50 slurry of DEAE-Sephacel in PBS.

   This is sufficient for isolation of proteoglycans from the equivalent of five 75-cm^2 tissue culture flasks. For larger amounts of material—e.g., from 0.5 to 10 liters of culture supernatants—use 5 to 10 ml gel volume per liter.

2. Attach inlet tubing to the top of the column. Place tubing in a PBS container and wash column with 10 column vol PBS using gravity or a pump at 16 ml/hr.

   An economic system for minicolumns is to place two syringe needles (18-G) through a rubber bung. Break one needle at the metal/plastic junction and attach tubing. Place a syringe on the other needle and use this to remove air and pull liquid down the tubing into the column. For smaller volumes, a reservoir can be inserted at the column mouth.

3. Equilibrate the column with 2 column vol DEAE-Sephacel equilibration buffer.

4. Condition the column by loading 20 ml conditioned medium from the cell line being used onto the column as in step 2, using a reservoir or tubing from a suitable container.

5. Wash the column twice with 5 ml each DEAE-Sephacel equilibration buffer.

6. Wash the column twice with 5 ml each DEAE low-pH buffer.

   Since this step removes most material other than proteoglycans, a less stringent protease inhibitor cocktail (i.e., lacking ε-aminocaproic acid and benzamidine·Cl) can be used in the low-pH and elution buffers.

7. Wash the column twice with 5 ml each DEAE elution buffer.

8. Reequilibrate as in step 3.
9. Load the proteoglycan sample onto the column. If a large volume is to be used, perform at 4°C.
10. Repeat washes in steps 5 and 6.
11. Elute the column with ten 1-ml aliquots of DEAE elution buffer.

    Typically the proteoglycans begin to elute after 0.5 ml and are fully eluted by 10 ml. A solution of 4 M guanididium·Cl can be used instead of high salt if the proteoglycan is to be concentrated by ethanol precipitation. If so, urea, salts, EDTA, NEM, and PMSF should be removed by washing with DEAE low-pH buffer without these ingredients.

12. Monitor elution by radiolabel if metabolically labeled, or by dye-binding assay (see unit introduction) if not. Combine fractions containing proteoglycans and store at 4°C. Do not freeze.

    DEAE-Sephacel columns can be reused indefinitely. The top 1 to 2 mm of gel can be discarded before reuse if it becomes heavily contaminated (grey in color). Columns should be stored upright at 4°C in equilibration buffer containing 0.02% (w/v) sodium azide. Rinse columns with two volumes equilibration buffer prior to use. If stored long-term (6 months), recondition as in steps 4 to 8 prior to use.

    Isolated proteoglycans can be dialyzed into new buffers or concentrated by precipitation and resuspended in the buffer of choice. For details, see Basic Protocol 4, steps 1a and 1b.

Isolation of Hydrophobic Proteoglycans

ALTERNATE PROTOCOL 2

This protocol is used to specifically isolate membrane-intercalated proteoglycans by virtue of their hydrophobic properties. It is used after Basic Protocol 2, which isolated all proteoglycans irrespective of hydrophobicity/hydrophilicity. Hydrophobic proteoglycans from Basic Protocol 2 have Triton associated with their core proteins. In the first part of this procedure, a cholate-containing buffer is used on the DEAE-Sephacel column (from Basic Protocol 2) to exchange Triton for cholate. This weaker detergent then allows binding of the hydrophobic proteoglycans to a purifying column of octyl-Sepharose.

Additional Materials *(also see Basic Protocol 2)*

DEAE-Sephacel eluate and column, *or* column loaded for elution (see Basic Protocol 2)
DEAE low-pH buffer (see recipe) without NaCl
Hydrophobic chromatography equilibration buffer (see recipe)
Hydrophobic chromatography exchange buffer (see recipe)
Hydrophobic chromatography washing buffer (see recipe)
Hydrophobic chromatography elution buffer (see recipe)
Octyl-Sepharose (Pharmacia): 50:50 (v/v) slurry in PBS containing 0.1% Triton X-100
0.1% (v/v) Triton X-100
Octyl-Sepharose equilibration buffer (see recipe) with and without NaCl
Octyl-Sepharose washing buffer (see recipe)
Octyl-Sepharose elution buffer I (see recipe)
Octyl-Sepharose elution buffer II (see recipe)
Minicolumn

Exchange detergent

1. Dilute DEAE-Sephacel eluate 1:10 (v/v) with DEAE low-pH buffer without NaCl to give a final NaCl concentration of 0.2 M.

2. Reequilibrate the DEAE-Sephacel column with 2 column vol hydrophobic chromatography equilibration buffer.

   The column is reequilibrated to increase the pH, and thus prevent precipitation of cholate.

3. Apply diluted eluate to the reequilibrated column.

   Alternately, start exchange while proteoglycans remain on DEAE-Sephacel (following step 10 of Basic Protocol 2). Rinse the column with hydrophobic chromatography equilibration buffer to increase pH, and then proceed with step 4 below.

4. Exchange proteoglycan-bound Triton with cholate by slowly passing 100 ml hydrophobic chromatography exchange buffer over the column.

   This is a slow process of exchange and is best achieved using gravity rather than a pump. The column will turn a brown color due to bound detergent.

5. Wash column with 10 ml hydrophobic chromatography washing buffer.

6. Elute with 20 ml hydrophobic chromatography elution buffer, collecting 1-ml fractions.

7. Monitor fractions by counts or dye binding (see unit introduction), and combine fractions that contain proteoglycans.

   The most concentrated fractions will be brown. These are typically fractions 1 to 5.

Purify hydrophobic proteoglycans

8. Prepare a minicolumn of octyl-Sepharose with a bed volume of 3 to 4 ml by adding 6 to 8 ml of 50:50 slurry in PBS containing 0.1% Triton X-100.

9. Wash with 20 ml of 0.1% Triton X-100 to remove ethanol, and then with 100 ml octyl-Sepharose equilibration buffer to remove Triton.

   This column is sufficient for analysis or isolation of 10,000 to 100,000 cpm hydrophobic proteoglycans from lysate or cytoskeletal/matrix pool (Alternate Protocol 1) purified by DEAE-Sephacel (Basic Protocol 2). Individual eluate fractions or pooled fractions may be used.

10. Dilute combined fractions (step 7) to 0.6 M NaCl with octyl-Sepharose equilibration buffer without NaCl, and add to octyl-Sepharose column. Incubate overnight at 4°C.

    The most efficient binding is achieved by allowing liquid above the column to drain, then adding 0.5 to 1.0 ml sample and allowing this to drain into the column before closing the column.

11. Wash column with twenty 1-ml aliquots of octyl-Sepharose washing buffer to remove cholate.

12. Wash column with twenty 1-ml aliquots of octyl-Sepharose elution buffer I to remove any material bound by ionic interactions.

13. Elute hydrophobic proteoglycans with twenty 1-ml aliquots of octyl-Sepharose elution buffer II, collecting 1-ml fractions.

    During this procedure the octyl-Sepharose will swell and shrink. This is normal.

    Following concentration by hydrophobic chromatography, proteoglycans may be highly aggregated. This is not reversed by boiling.

14. Use as soon as possible.

    If necessary, samples can be stored at 4°C, but not for more than 2 to 3 days. Samples should not be frozen.

ANALYSIS OF PROTEOGLYCANS

Analysis of proteoglycans can be very complex. This group of protocols contains methods for analyzing the size and composition of protoglycans isolated from cultured cells.

Analysis of Proteoglycans by Size-Exclusion Chromatography

BASIC PROTOCOL 3

Since proteoglycans are large and bear highly charged glycosaminoglycan chains, they often do not enter SDS-PAGE gels. One method of analysis is size-exclusion chromatography on Sepharose 4B (also see *UNIT 5.5*; Table 5.5.2). This procedure can be used for samples from Basic Protocol 2 or Alternate Protocol 2, or following immunoprecipitation (Basic Protocol 10). It is not suitable for unpurified samples (Basic Protocol 1 or Alternate Protocol 1), as high levels of unincorporated radiolabel may present a containment problem, and radiolabeled components other than proteoglycans will complicate the results obtained.

Materials

Sepharose 4B (Pharmacia)
SEC running buffer (see recipe)
Proteoglycan sample (see above)
Stock dye solution: 1 mg/ml each blue dextran (Sigma) and *N*-2,4-dinitrophenyl (DNP)–alanine (Sigma)

90 × 1–cm column
Column pump

1. Prepare a 90 × 1–cm column containing a 70-ml bed volume of Sepharose 4B according to the manufacturer's instructions. Pack the column at 9 ml/hr with SEC running buffer.
2. Decrease the rate to 6 ml/hr.

   This rate should be used for analysis to prevent collapse of the column.
3. Remove cap from the column and allow liquid to flow until the top of the gel is just dry. Add a maximum of 1 ml proteoglycan sample to be analyzed.

   For analytical purposes 8,000 to 10,000 cpm should be used.
4. Allow sample to enter gel, then rinse the top of the gel by adding 2 ml SEC running buffer and directly removing it.

   Failure to rinse will result in smearing of sample through the gel.
5. Replace cap and run column pump at 6 ml/hr. Collect 80 1-ml fractions and monitor content by radiolabel or dye-binding assay (see unit introduction).
6. Determine void volume (V_0; excluded volume) and total volume (V_t; total included volume) either by coelution (200 µl mixed dye in 800 µl sample) of a mixture of blue dextran (high molecular weight; totally excluded) and DNP-alanine (low molecular weight; totally included), or based on comparison with a previous run containing these dyes.

   V_0 is the volume at which blue dextran elutes (peak of blue color), and V_t is the volume at which DNP-alanine elutes (last fraction containing yellow color).

   See UNIT 5.5 for details on calibration with markers from a separate run.
7. Determine relative elution (K_{av}) using the equation $K_{av} = (V_s - V_0)/(V_t - V_0)$, where V_s is the elution volume of fractions where proteoglycans have peaks.

If HPLC equipment is available, a TSK 4000 column (TosoHaas) can be used to monitor proteoglycans. However, losses can be large if a prefilter is used, since many proteoglycans will tend to bind nonspecifically to the prefilter.

BASIC PROTOCOL 4

Analysis of Glycosaminoglycan Size Following Alkaline Elimination

Free glycosaminoglycan (GAG) chains can be obtained by alkaline elimination (here) or treatment with papain (see Basic Protocol 5) to destroy the core protein. Alkaline elimination removes all protein, leaving free intact glycosaminoglycans.

Materials

Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
Ethanol (optional)
1 M NaOH/2 M $NaBH_4$
10 M acetic acid
Dialysis tubing, MWCO 12-12,000 (optional)

1a. *For dialysis:* Transfer proteoglycan sample to distilled water by dialyzing against three changes of 100 vol water over 24 hr at 4°C. Use dialysis tubing with a MWCO of 12-12,000.

1b. *For precipitation:* Precipitate proteoglycan sample with 4 vol of 80% ethanol at −20°C, wash with 80% ethanol, and air dry. Resuspend in 0 to 5 ml distilled water.

2. Add an equal volume of 1 M NaOH/2 M $NaBH_4$. Incubate 16 hr at 4°C.

3. Neutralize with 10 M acetic acid, checking with pH paper until pH ≈ 7.

4. Reprecipitate with ethanol, if required to reconcentrate. Solubilize in water.

5. Analyze GAG size directly by size-exclusion chromatography (see Basic Protocol 3; *UNIT 5.5*) or HPLC.

NOTE: *If samples are not neutralized (step 3), analytical columns will be ruined.*

BASIC PROTOCOL 5

Analysis of Glycosaminoglycan Size Following Papain Digestion

Papain digestion degrades protein cores, leaving intact glycosaminoglycans. It requires no neutralization step.

Materials

Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
Papain digestion buffer (see recipe)
20 mg/ml papain (Sigma)
65°C water bath

Additional reagents and equipment for dialysis or ethanol precipitation (see Basic Protocol 4)

1. Transfer proteoglycan sample to papain digestion buffer (see Basic Protocol 4, step 1a or 1b).

2. Incubate with 2 mg/ml papain for 16 hr at 65°C.

3. Analyze GAG size directly by size-exclusion chromatography (see Basic Protocol 3; *UNIT 5.5*) or HPLC.

No enzyme inactivation is required.

Analysis of Glycosaminoglycan Content and Protein Core by GAG Degradation with Lyases

This analysis relies on the use of enzymes to selectively degrade glycosaminoglycans. Heparinase III will degrade heparan sulfate glycosaminoglycans. Chondroitinase ABC lyase will degrade all forms of chondroitin and dermatan sulfate glycosaminoglycans. Chondroitinase ACII lyase will degrade chondroitin but not dermatan sulfate glycosaminoglycans. Chondroitin B lyase will degrade dermatan sulfate. Each enzyme has different requirements and may not have maximum activity in the same buffer, but the heparinase III buffer listed below appears to work sufficiently well for most analyses if only core proteins are to be analyzed. Ovomucoid (Sigma) may be added at 100 µg/ml to prevent proteolysis of the protein core. These core treatments leave an intact case protein (unlike Basic Protocols 4 and 5), which can then be analyzed.

Materials

Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
Chondroitinase buffer (see recipe)
Heparinase III buffer (see recipe)
Chondroitin sulfate type C or B (chondroitin 6-sulfate or dermatan sulfate; Sigma)
Chondroitinase ABC (EC 4.2.2.4), ACII (EC 4.2.2.5), or B (no EC number) (Seikagaku America)
Heparinase III (a.k.a. heparitinase or heparitinase I; EC 4.2.2.8; Seikagaku America)
Additional reagents and equipment for dialysis or ethanol precipitation (see Basic Protocol 4)

For treatment with chondroitinases:

1a. Transfer proteoglycan sample to chondroitinase buffer (see Basic Protocol 4, step 1a or 1b).

Sample volumes depend on the proteoglycan concentration in the sample and the future method of analysis. For immunoblotting, a minimum volume is needed (i.e., resuspend precipitated sample from Basic Protocol 4, step 1b, in 50 to 200 µl of appropriate buffer). For SEC or HPLC, use 0.1 to 0.5 ml (≥2000 cpm if radiolabeled).

Since chemical amounts of isolated proteoglycans are usually small (µg levels), the amounts of carrier and enzyme do not need to be varied.

If performing dual digests with chondroitinases and heparinase, use heparinase III buffer. Chondroitinase B is inhibited by phosphate.

2a. Add ~2 µg chondroitin sulfate type C as carrier for chondroitinase ABC or ACII, or type B for chondroitinase B.

This increases the efficiency of digestion and recovery if low amounts of material (e.g., radiolabeled preparations) are used.

3a. Add 0.5 to 1 mU chondroitinase ABC, ACII, or B and incubate at 37°C.

Incubation times depend on the amount of proteoglycan present. For up to ~10 µg proteoglycan, an incubation of 2 to 3 hr should suffice; where amounts are higher, adding a second aliquot of enzyme for a further 2 hr may be needed.

4a. Monitor glycosaminoglycan digestion by size-exclusion chromatography (see Basic Protocol 3) or HPLC (degraded material will now elute in the V_t), or by immunoblotting (see Basic Protocol 8).

For treatment with heparinase III:

1b. Transfer proteoglycan sample to heparinase III buffer (see Basic Protocol 4, step 1a or 1b).

Sample volumes depend on the proteoglycan concentration in the sample and the future method of analysis. For immunoblotting, a minimum volume is needed (i.e., resuspend precipitated sample from Basic Protocol 4, step 1b, in 50 to 200 µl of appropriate buffer). For SEC or HPLC, use 0.1 to 0.5 ml (≥2000 cpm if radiolabeled).

Since chemical amounts of isolated proteoglycans are usually small (µg levels), the amounts of carrier and enzyme do not need to be varied.

2b. Add 2 to 5 µg heparan sulfate as carrier if low amounts of proteoglycan are present.

Do not add heparan sulfate if also digesting with chondroitinases. The products of heparan sulfate lyases are slightly inhibitory to chondroitinases. Dual digestions may, therefore, not be as efficient as separate incubations.

3b. Add 1 to 2 mU heparinase III and incubate at 37°C.

Time of incubation varies as indicated for chondroitinase treatment.

4b. Monitor glycosaminoglycan digestion by size-exclusion chromatography (see Basic Protocol 3) or HPLC (degraded material will now elute in the V_t), or by immunoblotting (see Basic Protocol 8).

BASIC PROTOCOL 7

Treatment with Nitrous Acid to Degrade Heparan Sulfate

Nitrous acid will degrade heparan sulfate glycosaminoglycans.

CAUTION: This procedure must be performed in a chemical fume hood, since nitrosamines are released.

Materials

1 N H_2SO_4
0.114 g/ml $Ba(NO_2)_2$
Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
10 N NaOH

1. Mix 1 ml of 1 N H_2SO_4 (0.5 mmol) with 1 ml of 0.114 g/ml $Ba(NO_2)_2$ on ice for 10 min, shaking occasionally.
2. Remove $BaSO_4$ precipitate by centrifuging 2 min at 500 × *g*, 4°C. Save the supernatant.

   The supernatant is nitrous acid at pH 1.5. This is stable for a few hours on ice.
3. Mix sample and nitrous acid at a 1:4 (v/v) ratio and leave 2 hr on ice.

   The proteoglycan sample should be unbuffered, preferably in water.
4. Neutralize with 10 N NaOH to a pH of ≈7, checking with pH paper.
5. Monitor heparan sulfate digestion by size-exclusion chromatography (see Basic Protocol 3) or HPLC (degraded material will now elute in the V_t), or by immunoblotting (see Basic Protocol 8).

   NOTE: *If samples are not neutralized (step 4), analytical columns will be ruined.*

Analysis of GAG Type and Core Protein

BASIC PROTOCOL 8

SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*) using standard procedures can be used to analyze GAG type and identify the core protein. These procedures can be used for analysis of whole cells or conditioned medium without proteoglycan isolation and purification, or they can be used on purified proteoglycans. Commercial antibodies are available for detection of carbohydrate "stubs" created on protein cores by lyase treatment. Monoclonal antibodies are available (Seikagaku America) against digested heparan sulfate (Δ heparan sulfate) and digested GAGs that had contained chondroitin sulfate that was nonsulfated (Δ di-0S) or was sulfated at the 4 or 6 position on galactosamine residues (Δ di-4S or Δ di-6S). These will detect all protein cores that had these specific saccharides. Specific proteoglycans can also be detected by core protein–specific antibodies. The appearance of a discrete polypeptide by SDS-PAGE analysis, representing the core protein, with a concomitant reduction in a high-molecular-weight smear characteristic of intact proteoglycans following treatment with specific lyases, determines which type of GAG was present on the protein core.

Nitrocellulose membranes may be used for immunoblotting, but intact proteoglycans will transfer poorly. Since this method relies on resolution of deglycanated core proteins following enzyme digestions, poor transfer of intact proteoglycan may not be important. Cationic membranes can, however, be used to capture intact proteoglycans more efficiently.

Size-exclusion chromatography of radiolabeled proteoglycans can also be used to analyze the composition of proteoglycans. Intact GAGs will elute in the V_0 of a Sephadex G-50 column, whereas disaccharides released by enzyme treatment will elute at or close to the V_t. Differential treatment susceptibility indicates the type (and relative amounts) of GAG in the sample. If HPLC is available, a TSK 4000 HPLC column (TosoHaas) column may be used.

Analysis of GAG Size

BASIC PROTOCOL 9

This is most easily performed using HPLC, but Sephadex G-200, Sepharose CL-6B, or equivalent resins may be used if HPLC is not available.

Materials

HPLC running buffer (see recipe)
Proteoglycan sample, enzymatically or chemically treated (see Basic Protocols 4 and 5)
DEAE-Sephacel column (see Basic Protocol 2)
PD-10 gel-filtration column (Amersham Pharmacia Biotech)
High-performance liquid chromatograph (HPLC) with TSK 4000 column (TosoHaas)

1. Isolate intact GAG chains from intact proteoglycans following treatments as above (see Basic Protocols 4 and 5), using anion-exchange chromatography on DEAE-Sephacel columns (see Basic Protocol 2).
2. Desalt samples on a PD-10 gel-filtration column according to manufacturer's instructions, using distilled water as buffer, and lyophilize.
3. Equilibrate a TSK 4000 HPLC column with HPLC running buffer.
4. Solubilize sample in HPLC running buffer. Load 100 to 500 µl sample and elute at a flow rate of 0.5 ml/min. Collect 0.5-ml fractions.

Isolated glycosaminoglycans do not bind the prefilter.

5. Assay fractions for radioactivity or dye binding (see unit introduction).

An alternate HPLC running buffer (see recipe) containing 4 M guanidium·Cl may be used to prevent self-association of GAGs.

GAG standards of known size should be used, since globular proteins are inappropriate for calibration.

BASIC PROTOCOL 10

Immunoprecipitation of Proteoglycans

Although some matrix proteoglycans can be immunoprecipitated by standard methods (*UNIT 7.2*), there are some specific problems. First, hydrophobic proteoglycans can be a problem, especially if analysis of associated components is required (Oh et al., 1997). The type of detergent used may result in differences in co-immunoprecipitation, as seen in studies with other membrane receptors (Serru et al., 1999). Second, GAG chains may limit accessibility of antibodies against core proteins. Use of a primary and secondary antibody, followed by immobilized protein A, may improve yield. Alternatively, GAG chains may be removed enzymatically prior to immunoprecipitation. Some proteoglycans may aggregate after GAG removal, resulting in precipitation.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Alternate HPLC running buffer

382.12 g guanidine·Cl (4 M final)
6.05 g Tris base (50 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 6.0 with 1 M HCl
Add 5 ml Triton X-100 (0.5% v/v final)
Add H_2O to 1 liter
Store up to 1 month at 4°C

Chondroitinase buffer

6.05 g Tris acetate (50 mM final)
4.08 g sodium acetate (30 mM final)
0.1 g bovine serum albumin (BSA; 0.1 mg/ml final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 8.0 with 1 M acetic acid
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (for hydrophobic proteoglycans; 0.1% v/v final)
Stir slowly until Triton is dissolved
Add H_2O to 1 liter
Store up to 1 week at 4°C

DEAE elution buffer

87.66 NaCl (1.5 M final)
4.102 g sodium acetate (50 mM final)
240 g urea (4 M final)

continued

1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 4.0 with 1 M acetic acid
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (0.1% v/v final)
Stir slowly until Triton is dissolved
Add H_2O to 1 liter
Store up to 1 week at 4°C

DEAE low-pH buffer

11.69 g NaCl (0.2 M final)
4.102 g sodium acetate (50 mM final)
240 g urea (4 M final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 4.0 with 1 M acetic acid
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (0.1% v/v final)
Stir slowly until Triton is dissolved
Add H_2O to 1 liter
Store up to 1 week at 4°C

DEAE-Sephacel equilibration buffer

11.69 g NaCl (0.2 M final)
50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
240 g urea (4 M final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
0.783 g benzamidine·Cl (5 mM final)
13.12 g ε-aminocaproic acid (0.1 M final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 8.0 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (0.1% v/v final)
Stir slowly until Triton is dissolved
Add H_2O to 1 liter
Store up to 1 week at 4°C

Heparinase III buffer

1.36 g sodium acetate (0.1 M final)
0.000158 g calcium acetate (0.01 mM final)
1 g ovalbumin (1% w/v final)
0.005 g leupeptin (50 µg/ml final)
0.0157 g benzamidine·Cl (1 mM final)
H_2O to 90 ml

continued

Adjust pH to 7.0 with 1 M acetic acid
Add 100 µl 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 100 µl Triton X-100 (for hydrophobic proteoglycans; 0.1% v/v final)
Stir slowly until Triton is dissolved
Add H_2O to 100 ml
Store up to 2 days at 4°C

HPLC running buffer

13.6 g KH_2PO_4 buffer, pH 6.0 (0.1 M final)
29.2 g NaCl (0.5 M final)
2 g Zwittergent (Aldrich; 0.2% w/v final)
H_2O to 1 liter
Store up to 1 month at 4°C

Hydrophobic chromatography elution buffer

4 g sodium cholate (0.4% w/v final)
175.2 g NaCl (3 M final)
2.422 g Tris base (20 mM final)
1.25 *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H_2O to 1 liter
Store up to 1 week at 4°C

Hydrophobic chromatography equilibration buffer

5.84 g NaCl (0.1 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H_2O to 1 liter
Store up to 1 week at 4°C

Hydrophobic chromatography exchange buffer

20 g sodium cholate (2% w/v final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H_2O to 1 liter
Store up to 1 week at 4°C

Hydrophobic chromatography washing buffer

4 g sodium cholate (0.4% w/v final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H_2O to 1 liter
Store up to 1 week at 4°C

Lysis buffer

3.03 g Tris base (25 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
0.783 g benzamidine·Cl (5 mM final)
13.12 g ε-aminocaproic acid (0.1 M final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.5 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 2 ml Triton X-100 (0.2% v/v final)
Stir slowly to dissolve Triton
Add H_2O to 1 liter
Prepare fresh just prior to use

Octyl-Sepharose elution buffer I

Prepare as for octyl-Sepharose washing buffer (see recipe), but adjust NaCl to 3 M final.

Octyl-Sepharose elution buffer II

175.2 g NaCl (3 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 10 ml Triton X-100 (1% v/v final)
Stir slowly to dissolve Triton
Add H_2O to 1 liter
Store up to 1 week at 4°C

Octyl-Sepharose equilibration buffer

4 g sodium cholate (0.4% w/v final)
35.04 NaCl (0.6 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (10 mM final)

continued

7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 20 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H_2O to 1 liter
Store up to 1 week at 4°C

Octyl-Sepharose washing buffer

5.84 g NaCl (0.1 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H_2O to 1 liter
Store up to 1 week at 4°C

Papain digestion buffer

11.69 g NaCl (2 M final)
0.744 g EDTA (10 mM final)
0.4102 g sodium acetate (50 mM final)
0.0175 g cysteine·Cl (0.01 M final)
H_2O to 90 ml
Adjust pH to 5.5 with 1 M acetic acid
H_2O to 100 ml
Prepare fresh just prior to use

Size-exclusion chromatography (SEC) running buffer

1 g SDS (0.1% w/v final)
20.47 g NaCl (0.35 M final)
6.06 g Tris base (50 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 8.0 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H_2O to 1 liter
Store up to 2 weeks at room temperature

Solubilization buffer

10 ml Triton X-100 (1% v/v final)
240 g urea (4 M final)
3.03 g Tris base (25 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
0.783 g benzamidine·Cl (5 mM final)

continued

13.12 g ε-aminocaproic acid (0.1 M final)
H_2O to 900 ml
Heat to dissolve
Adjust to pH 7.5 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
H_2O to 1 liter
Store up to 1 day at 4°C

COMMENTARY

Background Information

The analysis of proteoglycans has always been a specialized field due to the complexity of these molecules and their interactions with other molecules. Until relatively recently, it was only possible to isolate proteoglycans via the binding of their glycosaminoglycan chains to cationic matrices. Now, as core proteins are identified and characterized, antibody affinity chromatography can be used to purify individual proteoglycan species for analysis. However, analysis of glycosaminoglycans attached to the individual core proteins is still a tedious task, because the biological activity may depend on the fine structure of the glycosaminoglycan chains as well as the distinct core proteins. The glycosaminoglycan chain type can be readily identified by the use of selective enzyme degradation as detailed here. However, determining the fine structure of glycosaminoglycan chains—particularly the patterns of epimerization and sulfation of, for example, heparan sulfate chains—relies on techniques that are beyond the scope of this unit. For information on this, the reader should consult Vives et al. (1999).

Interest in proteoglycans, particularly cell-surface proteoglycans, has grown recently. This is due in part to the discoveries of the roles of cell-surface proteoglycans in signaling as co-receptors with integrins in adhesion, and with growth factors in cell cycle regulation (reviewed in Woods and Couchman, 1998; Bernfield et al., 1999; also see *UNIT 10.1*). It is often daunting for new researchers to enter the proteoglycan field, and it is hoped that these techniques will help.

Critical Parameters

The core proteins of proteoglycans are susceptible to proteolysis, but freezing often results in insoluble complexes. It is therefore important to design experiments so that isolation, purification, and analysis proceed as rapidly as possible. In addition, once the glycosaminoglycans are removed from the core protein, the core proteins aggregate, especially at high concentration or if hydrophobic. Again, speed is of the essence.

Troubleshooting

Core protein aggregation, particularly of the syndecans, results in SDS-resistant multimers, which are noncovalent. This results in several bands migrating more slowly on SDS-polyacrylamide gels than expected based on molecular weight. This has now been documented in several studies but often needs to be explained in manuscripts. It is not due to disulfide bonding; it appears to be an inherent capacity of the core proteins.

The enzymes used to degrade glycosaminoglycans are susceptible to inactivation and have a limited shelf life once they are hydrated. Where they are used infrequently, enzymes can be hydrated, divided into aliquots, and freeze dried. If a negative result is seen, the particular enzyme preparation should be checked for activity under the experimental conditions used. Manufacturer's data sheets explain this procedure.

Anticipated Results

Basic Protocol 1. Yield cannot be quantified since nonincorporated radiolabel is present. After Basic Protocol 2, the supernatant fraction will yield the highest amount of labeled materials (~4×10^6 cpm), with the cell-associated materials yielding 1×10^6 cpm. These results are typical of those obtained using rat embryonic fibroblasts (confluent monolayers from five 10-cm dishes).

Alternate Protocol 1. Yield cannot be quantified since nonincorporated radiolabel is present. After Basic Protocol 2, the supernatant fraction will yield the highest amount of labeled materials (~4×10^6 cpm), with the lysate yielding somewhat less (~950,000 cpm), and the cytoskeleton/matrix yielding the least (~150,000 cpm). These results are typical of

those obtained using rat embryonic fibroblasts (confluent monolayers from five 10-cm dishes).

Support Protocols 1 and 2. Each cell type synthesizes its own spectrum of proteoglycans in varying amounts. For fibroblasts, total incorporation into proteoglycans can be expected to be 5–6 × 10^6 cpm/5 dishes.

Basic Protocol 2. Anticipated yields from this procedure are listed under Basic Protocol 1 and Alternate Protocol 1 above.

Alternate Protocol 2. Little material should be lost on reapplication of isolated proteoglycans to DEAE-Sephacel and elution. If losses do occur, the column should be reconditioned with medium before use. Yields of the different fractions will differ with cell type. Typically, using rat embryonic fibroblasts, 40% to 60% of lysate proteoglycans and 20% to 40% of cytoskeleton/matrix proteoglycan pools, respectively, elute from octyl-Sepharose with detergent buffer.

Basic Protocol 3. It is possible to resolve a 1-ml sample of proteoglycan into three to six species by size-exclusion chromatography, depending upon the cell type.

Basic Protocol 4. Alkaline elimination quantitatively separates glycosaminoglycans from the core protein.

Basic Protocol 5. Treatment of proteoglycans with papain degrades the core protein and releases free glycosaminoglycans.

Basic Protocol 6. Treatment of proteoglycans with lyases followed by immunoblotting allows identification of three to six core proteins, depending on the species produced by the cell line.

Basic Protocol 7. Treatment of proteoglycans with nitrous acid degrades heparan sulfate to low-molecular-weight products.

Basic Protocol 8. Immunoblotting allows detection of which glycosaminoglycans are bound to specific proteoglycan protein cores.

Basic Protocol 9. HPLC analysis allows determination of GAG size.

Basic Protocol 10. Specific proteoglycans and protein cores can be immunoprecipitated from cell preparations, but yield is often low.

Time Considerations

Basic Protocol 1 and Alternate Protocol 1. The whole procedure should be completed in <30 min to keep proteolysis at a minimum. Once the samples have been applied to DEAE-Sephacel and the column is washed with low-pH buffer, proteolytic risk is reduced.

Support Protocol 1. Addition of radiolabel requires 15 min, but 3 to 4 days may be needed for growth to confluency.

Basic Protocol 2. The time needed to apply the extract to the DEAE column will depend on the volume of extract. Samples should not be loaded at speeds >16 ml/hr. Washing and elution each take ~30 min. A convenient stopping point, if needed, is after the second wash (step 6).

Alternate Protocol 2. The time taken to (re)load proteoglycans onto DEAE-Sephacel will depend on the volume to be loaded. Rates >16 ml/hr should not be used. Exchange to cholate detergent is most conveniently performed overnight. Loading onto octyl-Sepharose requires overnight incubation, and washing and elution of the column requires ~3 hr.

Basic Protocol 3. Each size-exclusion chromatography run takes ~16 hr. Several columns can be run simultaneously if multichannel pumps are used.

Basic Protocol 4. Alkaline elimination requires 16 hr, with 5 to 10 min for neutralization.

Basic Protocol 5. Papain digestion requires 16 hr.

Basic Protocol 6. Incubation times depend on the amount of proteoglycan present. For up to 10 µg proteoglycan, 2 to 3 hr should suffice. Where larger amounts are present, addition of enzymes for a further 2 hr may be needed.

Basic Protocol 7. Treatment with nitrous acid requires 2.5 hr.

Basic Protocol 8. Enzyme treatment requires 2.5 hr. SDS-PAGE, transfer, and immunoblotting require 24 hr.

Basic Protocol 9. HPLC analysis requires ~4 hr, while SEC requires 16 to 18 hr.

Basic Protocol 10. Immunoprecipitation requires ~4 hr.

Literature Cited

Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., and Zako, M. 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* 68:729-778.

Oh, E.S., Woods, A., and Couchman, J.R. 1997. Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C. *J. Biol. Chem.* 272:8133-8136.

Schmidtchen, A., Sundler, R., and Fransson, L.A. 1990. A fibroblast heparan sulphate proteoglycan with a 70 kDa core protein is linked to membrane phosphatidylinositol. *Glycoconjugate J.* 7:563-572.

Serru, V., LeNaour, F., Billard, M., Azorsa, D.O., Lanza, F., Boucheix, C., and Rubinstein, E. 1999.

Selective tetraspan-integrin complexes (CD81/α4β1, CD151/α3β1, CD151/α6β1) under conditions disrupting tetraspan interactions. *Biochem. J.* 340:103-111.

Vives, R.R., Pye, D.A., Salmavirta, M., Hopwood, J.J., Lindahl, U., and Gallagher, J.T. 1999. Sequence analysis of heparan sulphate and heparin oligosaccharides. *Biochem. J.* 339:767-773.

Woods, A. and Couchman, J.R. 1998. Syndecans: Synergistic activators of cell adhesion. *Trends Cell Biol.* 8:189-192.

Woods, A., Couchman, J.R., and Höök, M. 1985. Heparan sulfate proteoglycans of rat embryo fibroblasts. A hydrophobic form may link cytoskeleton and matrix components. *J. Biol. Chem.* 260:10872-10879.

Key References

Bernfield et al., 1999. See above.

An excellent review of cell surface glypican and syndecan proteoglycans.

Gallagher, 1997. Structure-activity relationships of heparan sulfate. *Biochem. Soc. Trans.* 25:1206-1209.

An expert review of heparan sulfate glycosaminoglycan structure and function.

Iozzo, R.V. 1998. Matrix proteoglycans: From molecular design to cellular function. *Annu. Rev. Biochem.* 67:609-652.

An in-depth, informative review of extracellular matrix proteoglycans.

Lindahl, U., Kusche-Gullberg, M., and Kjellén, L. 1998. Regulated diversity of heparan sulfate. *J. Biol. Chem.* 273:24979-24982.

A concise review of heparan sulfate glycosaminoglycan complexity and biosynthesis.

Internet Resources

www.glycoforum.gr.jp

A Web site supported by Seikagaku Corp. that contains articles on glycoscience, including proteoglycans and links to other sites.

Contributed by Anne Woods and
John R. Couchman
University of Alabama at Birmingham
Birmingham, Alabama

Matrix Metalloproteinases

Henning Birkedal-Hansen,[1] Susan Yamada,[1] Jack Windsor,[2] Anne Havernose Pollard,[3] Guy Lyons,[4] William Stetler-Stevenson,[5] and Bente Birkedal-Hansen[5]

[1]National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland
[2]University of Indiana School of Dentistry, Indianapolis, Indiana
[3]University of Copenhagen School of Dentistry, Copenhagen, Denmark
[4]Kanematsu Laboratories, Royal Prince Alfred Hospital, Sydney, Australia
[5]Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

ABSTRACT

Matrix metalloproteinases are a class of enzymes that play an important role in the remodeling of the extracellular matrix in development and cancer metastasis. This unit describes a set of methods—cell-mediated dissolution of type-1 collagen fibrils, direct and reverse zymography, enzyme capture based on α2-macroglobulin and TIMP-1 and -2, and demonstration of cryptic thiol groups in metalloproteinase precursors—that are used to characterize the functions of matrix metalloproteinases and their inhibitors. *Curr. Protoc. Cell Biol.* 40:10.8.1-10.8.23. © 2008 by John Wiley & Sons, Inc.

Keywords: matrix metalloproteinases • type-1 collagen • zymography • α2-macroglobulin • TIMP-1 and -2

This unit describes a set of methods that are relatively unique to studies of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs, α2M), including cell-mediated dissolution of type I collagen fibrils (see Basic Protocol 1), direct and reverse zymography (see Basic Protocols 2 and 3), enzyme capture techniques based on α2-macroglobulin (α2M) and TIMP-1, and -2 (see Basic Protocol 4 and Alternate Protocol), and detection and demonstration of cryptic thiol groups in MMP precursors (see Basic Protocol 5). Support Protocols are included for preparation (see Support Protocol 1) and labeling of collagen with a fluorophore (see Support Protocol 2).

DISSOLUTION AND DEGRADATION OF COLLAGEN FIBRILS BY LIVE CELLS

BASIC PROTOCOL 1

Comparatively few methods allow detailed analysis of how live cells orchestrate MMP and inhibitor functions in the degradation and remodeling of extracellular matrices. The methods described in this protocol were developed to study the function of matrix metalloproteinases (MMPs) in the degradation of type I collagen fibrils by live cells under controlled but readily variable conditions. In its simplest form, cells are seeded on a few-micron-thick film of reconstituted collagen fibrils, then incubated for a period of 1 to 7 days. The progressive dissolution of the film under the cell layer—in response, e.g., to changing environmental conditions, inducing agents, or inhibitors—may be monitored directly and related to the level of expression of key components of the requisite proteolytic machinery. The system is readily manipulated in a number of ways: by induction/repression of transcription of components of the signaling and effector systems; by transfection of new genes of potential importance to the process; or by selective or specific blocking strategies using antisense-, MMP-specific inhibitor–, or antibody-based approaches. The limited susceptibility of type I collagen fibrils to cleavage and dissolution by MMPs permits one to narrow the scope of the investigation to a small number

Current Protocols in Cell Biology 10.8.1-10.8.23, September 2008
Published online September 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1008s40

of ("collagenolytic") enzymes. This characteristic also makes it a realistic objective to dissect the entire sequence or set of reactions involved in cell-mediated dissolution of collagen fibrils, starting from the initial engagement of cell surface receptors by cytokines, growth factors, and other catabolic reagents, through the final enzymatic cleavage, dissolution, and disposal of the substrate. Important questions that may be addressed using this approach include the following:

a. What enzymes are actually involved in the cleavage reaction itself and in the precursor activation steps?
b. How do cells regulate the activity of the enzymes?
c. What role is played by TIMPs in modulating, containing, and blocking the response?
d. What is the ultimate fate of the collagen chains and peptides generated as a result of proteolysis?

Recent studies have shown that type I collagen (in solution or in reconstituted fibrillar form) may be cleaved by a larger number of enzymes than previously anticipated, including the three classical "collagenases," MMP-1, MMP-8, and MMP-13 (Birkedal-Hansen et al., 1993; Knäuper et al., 1996). In addition, reports suggest that MMP-14 (Ohuchi et al., 1997) and TIMP-free MMP-2 may also dissolve collagen fibrils at meaningful rates under physiologic conditions (Aimes and Quigley, 1995). It is of note that although the three classical collagenases (MMP-1, MMP-8, and MMP-13) were discovered because of their ability to dissolve reconstituted fibrils of type I collagen, no definitive proof has yet been rendered that cleavage of collagen fibrils is indeed the exclusive or even prevailing biologic function of any of these enzymes. Admittedly, the evidence seems compelling based on a large number of in vitro studies.

Earlier versions of this method have been published (Birkedal-Hansen, 1987; Birkedal-Hansen et al., 1989, 1993; Lin et al., 1987). The isolation and purification techniques of type I collagen and the methods for formation of reconstituted hydrated gels of type I collagen have been described elsewhere in detail (Birkedal-Hansen, 1987). The method relies on the ability of neutral solutions of type I collagen in an appropriate concentration range (0.1 to 5 mg/ml) to form hydrated gels of reconstituted fibrils by heating to 37°C. The method also takes advantage of the observation that such loose hydrated gels may be collapsed by gentle air-drying into a thin film of uniform, densely packed, randomly oriented fibrils which remain as highly resistant to proteolysis by enzymes such as trypsin, chymotrypsin, and plasmin as hydrated gels or natural fibrils (Fig. 10.8.1). Trypsin, which is often used as a standard for testing the resistance of collagen fibrils to "unspecific" proteolytic cleavage, is unable to dissolve the collagen fibril films prepared as described. The same is true for a large number of proteinases of all four classes, and it is this unique resistance to proteolysis which renders this assay system particularly valuable, as it greatly reduces the number of proteinases that are involved in the cleavage/dissolution reaction.

Several variants of the method may be used. While the authors often prefer (for ease of presentation and interpretation) to seed the cells in a small button in the middle of a much larger dish (35 mm; Fig. 10.8.1A, middle) in order to maintain medium excess, it is also possible to seed the cells over the entire collagen-coated surface, although a confluent monolayer rapidly exhausts the medium. The collagen may be used in its natural state or labeled either with radioactive or fluorescent tags to facilitate monitoring (see Support Protocol 2), retrieval, and quantification of dissolved collagen chains and fragments.

Depending on the casting conditions, collagen films may be generated with a thickness down to 1 to 2 μm, which is approximately the thickness of a single layer of well-spread cells. Most cell types seeded on this film spread within minutes to hours, although often

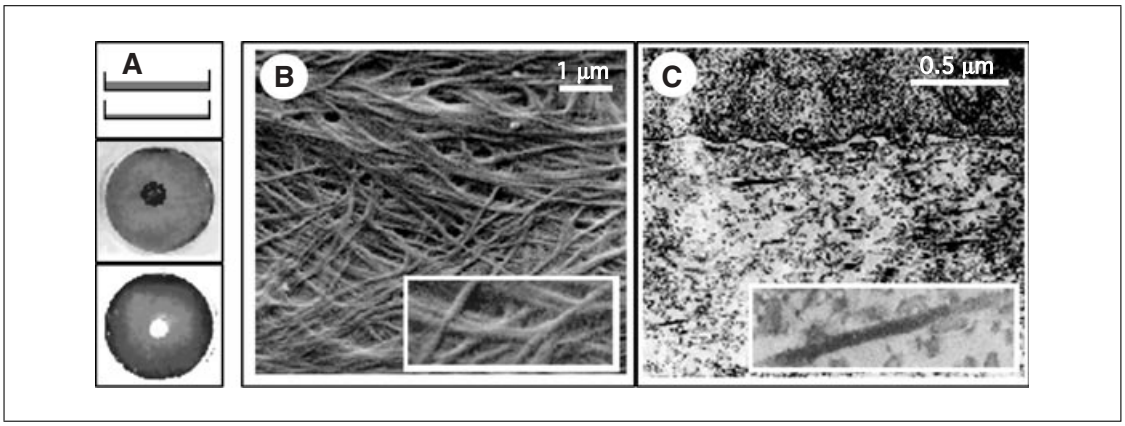


Figure 10.8.1 Reconstituted collagen fibril film. (**A**) Rat tail tendon type I collagen is polymerized by heat gelation. The gel is air dried and reduced in thickness to a few microns. Cells are seeded in the middle of the plate and incubated with culture medium. After incubation, the cells are removed and a clearing beneath the cell layer is exposed by staining with Coomassie blue. (**B**) The air-dried collagen fibril film consists of uniform, randomly oriented reconstituted fibrils. (**C**) Detail of cell attached to the collagen fibril film.

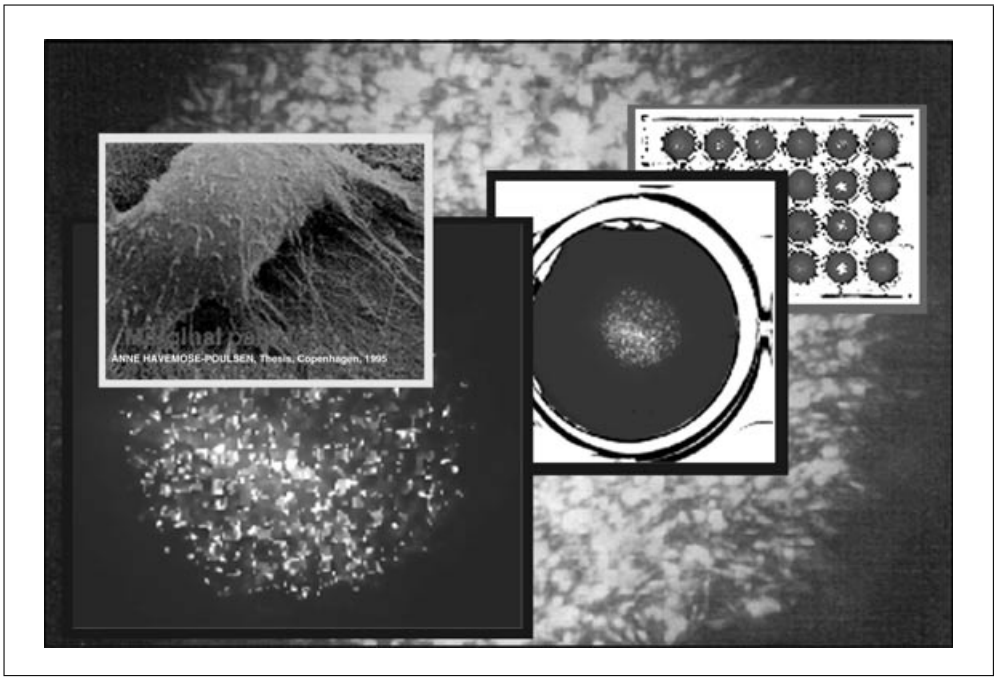


Figure 10.8.2 Dissolution of collagen fibrils by live adherent cells. Fibroblasts seeded in the center of the well dissolve the underlying collagen fibril film. Upper left panel shows scanning electron micrograph of fibroblast attached on collagen fibril film. Recreated from Havemose-Poulsen et al. (1998).

more slowly than on plastic. Cells that express an appropriate complement of MMPs either constitutively or after exposure to cytokines and growth factors (or phorbol ester) progressively dissolve the underlying fibril coating, and, within 24 to 96 hr, clear a path to the plastic surface (Fig. 10.8.1A, lower; Fig. 10.8.2). Coomassie blue staining of the residual collagen fibril film after removal of the cells is usually sufficient to visualize the dissolution of the underlying film (Fig. 10.8.2).

Materials

3 mg/ml rat tail tendon type I collagen in 13 mM HCl (see Support Protocol 1)
13 mM HCl, 4°C
Neutralizing buffer (see recipe), 4°C

Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (CMF-PBS; *APPENDIX 2A*) supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate
Cells of interest (e.g., fibroblasts, keratinocytes, or tumor cells)
DMEM (*APPENDIX 2A*) supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate with and without 10% (v/v) FBS (or other medium appropriate for cell type)
Growth factors/cytokines: e.g., IL-1β, TNF-α, TGF-α, or TPA; or phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA, or phorbol myristate acetate, PMA)
1% (v/v) Triton X-100
0.05% (w/v) trypsin/0.53 mM EDTA (Invitrogen)
Coomassie blue stain (see recipe)
6-well cell culture plates

Additional reagents and equipment for trypsinizing and counting cells (*UNIT 1.1*)

Prepare collagen-coated plates

1. To cast one 6-well plate, dilute 1 ml of 3 mg/ml type I collagen stock solution with 7 ml of 13 mM HCl at 4°C. Mix the collagen solution with 2 ml of cold neutralizing buffer in a precooled test tube either by gently pipetting up and down while avoiding formation of air bubbles (which will form defects in the gel) or by gently inverting the tube several times.

   The neutralizing buffer is designed to bring the pH of the solution to 7.4 (check with pH paper). The concentration of this buffer is 0.2 M inorganic phosphate (as Na_2HPO_4/NaH_2PO_4) and 0.47 M NaCl. The final collagen concentration is 300 μg/ml in 40 mM P_i/~0.10 M NaCl. Since pH dramatically influences the gelling properties of the collagen solution it is often advantageous to first test the efficacy of the neutralizing buffer by mixing 4 volumes of 13 mM HCl with one volume of neutralizing buffer and checking the final pH (7.4).

   The final thickness of the collagen film depends on the concentration of the collagen solution. A 300 μg/ml solution dispensed at a volume of 1.5 ml per (35-mm) dish yields a film of 1.5 to 2.0 μm in thickness after drying. Higher concentrations yield thicker films. The lower concentration limit for proper gelling is around 100 μg/ml using rat tail tendon collagen prepared as described (see Support Protocol 1) but somewhat higher (500 μg/ml) with commercial type I collagen preparations.

2. Immediately after mixing, add a 1.5-ml aliquot of neutralized collagen solution to each well of the 6-well culture plate. Rotate the plate to permit the collagen to cover the entire well bottom evenly. Incubate in humidified incubator for 2 hr at 37°C. Avoid movement of gel and plate during gelling.

3. Remove plate from incubator, remove lid, and place at room temperature in an air stream (laminar flow hood) overnight (during this process the gel dries down to a thin film). Wash three times with distilled water, each time for 30 min at room temperature or 37°C, to remove salt crystals formed during the drying (check efficacy of washing step using a phase-contrast microscope). Dry again overnight in laminar flow hood and check for absence of residual salt crystals.

   It is important that all salt crystals be removed by washing before the plates are used.

4. Add 2 ml CMF-PBS or DMEM supplemented with penicillin/streptomycin. Store in this solution in incubator at 37°C or in refrigerator at 4°C in closed plastic bag to prevent evaporation.

   The plate can be stored in this manner for up to 2 weeks as long as evaporation is avoided.

5. Immediately before seeding cells, remove medium from wells by aspiration and wash with 2 ml distilled water for 30 min. Remove water and leave plate to air dry in hood.

Plate cells

6. Trypsinize and count cells (see *UNIT 1.1*), then dilute cell suspension to the appropriate concentration in DMEM/10% FBS, or in medium appropriate for the cell type being used.

   Best results are obtained with 10,000 to 50,000 cells in a 25-μl aliquot, using a cell suspension of 4×10^5 to 2×10^6 cells/ml, somewhat depending on cell size. The intent is to form a coherent monolayer in a small central button (Fig. 10.8.1A, middle).

7. Deliver a 25-μl aliquot to the center of the well without touching the fragile collagen film. Fill plate volume between wells with distilled water to avoid evaporation during seeding and attachment. Place plate in plastic box on wet paper towels to avoid evaporation, and then place in incubator for 5 hr or overnight at 37°C to allow cells to attach.

8. Add to each well 2 ml DMEM/10% FBS or appropriate medium and incubate overnight at 37°C to allow cell spreading.

   Some cell types can be transferred immediately to serum-free medium while others require overnight incubation in serum-supplemented medium.

   Once the cells are spread, incubation may be performed either with or without serum. The result depends somewhat on cell type. Some cells tend to detach in the absence of serum while others can be maintained for 2 to 3 days in complete absence of serum while degrading the collagen fibril matrix.

9. If the experiment is to be performed in the absence of serum, thoroughly and repeatedly wash with CMF-PBS or serum-free DMEM for 10 min at 37°C, to remove remnants of serum.

   Some cells may require special media formulations, i.e., keratinocytes. Most fibroblast strains do well under serum-free conditions either in DMEM or DMEM/F12 (1:1).

*Induce expression of MMP*s

10. Induce cells for expression of MMPs at this stage by including in the medium cytokines such as IL-1β (10^{-9}M), TNF-α (10^{-8}M), TGF-α (10^{-8}M), or TPA (1 to 2×10^{-7} M).

    Alternatively, cytokine or TPA induction may be achieved during the last 24 hr before trypsinization and seeding. If incubated under serum-free conditions, plasminogen may be added to the medium. Some cells respond to exposure to plasminogen by greatly accelerating the rate of dissolution, while others do not. If desired, plasminogen is added from a stock solution in CMF-PBS to give a final concentration of 4 μg/ml. Human plasminogen is either purchased from one of several commercial sources (i.e., Pharmacia Hepar or Sigma-Aldrich) or prepared as described (Deutsch and Mertz, 1970) from outdated human plasma by lysine-Sepharose chromatography.

11. Incubate the plates at 37°C for 1 to 4 days (or up to 7 days) depending on the experimental design. Follow the progress of the process with a phase-contrast microscope.

    To avoid evaporation it may be advantageous to fill the volume between the wells with sterile distilled water.

Stain plate and quantitate results

12. In order to visualize the dissolution of the film beneath the cell layer, remove the cells either by dissolution in 1% (v/v) Triton X-100, by 0.5% trypsin/0.53 mM EDTA (10 min, 37°C), or by a combination thereof.

    Avoid use of SDS, which dissolves the collagen fibril film as well as the cells.

13. Rinse the wells with distilled water.

14. Stain with Coomassie blue stain for 5 to 15 min to visualize residual collagen film, then wash three times with distilled water.

15. Destain in distilled water for 30 min (or perform three quick washes with water) and finally allow plates to air dry.

    After drying the plates, they can be stored indefinitely (Fig. 10.8.2).

    In order to follow the progressive dissolution of the collagen fibril film it is advantageous to terminate sample wells on consecutive days and to contrast the dissolution after 1, 2, 3 ... days.

    If desired the plates can be scanned directly into Adobe Photoshop using a scanner capable of scanning transparent originals.

16. Determine the extent or rate of dissolution of the substrate

    The degree of dissolution at the conclusion of the experiment may be measured photometrically in Coomassie blue–stained plates by measuring the absorption of light in a conventional light microscope equipped with a exposure (photo)meter as described in Havemose-Poulsen et al. (1998). The relationship between amount of collagen present on the plate and exposure time is strictly linear at least up to three times the collagen layer thickness used in this protocol.

    Alternatively, if the cells are seeded evenly as a confluent monolayer over the entire collagen-coated well bottom (see below), progression may be monitored daily by removal of aliquots of medium and measuring the release of collagen chains and peptides. To this end the collagen may be labeled either with ^3H (Birkedal-Hansen, 1987; Birkedal-Hansen and Danø, 1981) or with fluorescent tags (Ghersi et al., 2002). This approach is less useful if the cells are seeded in a small 2- to 4-mm button at the center of the well, because the background release of radioactivity and fluorescent label from the entire film compromises the sensitivity of the analysis (typically only 10% to 20% of the collagen fibril film is covered by cells in this variation).

SUPPORT PROTOCOL 1

PREPARING RAT TAIL TENDON COLLAGEN TYPE I

Methods for isolation and preparation of rat tail tendon type I collagen have been described in detail elsewhere (Birkedal-Hansen, 1987; Birkedal-Hansen and Danø, 1981). Alternatively, rat, bovine or human type I collagen may be purchased from Becton Dickinson Biosciences Discovery Labware. Briefly, tendons teased from rat tails are washed with distilled water and with 0.5 M NaCl. The acid-soluble collagen fraction is then extracted in 0.5 M acetic acid, and type I collagen is purified by sequential salt precipitation at neutral to slightly alkaline pH, first with 5% NaCl, then (after redissolution in acetic acid) with 0.02 M Na_2HPO_4.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Tails of ~400 g rats (freshly removed or stored frozen at −80°C)
0.5 M NaCl in 50 mM Tris·Cl, pH 7.4 (see *APPENDIX 2A* for Tris·Cl)
5 mM, 50 mM, and 0.5 M acetic acid
NaCl (solid)
0.02 M Na_2HPO_4
13 mM HCl
Neutralizing buffer (0.2 M NaP_i)

Glass wool or cheesecloth
500-ml centrifuge bottles
High-speed centrifuge (Sorvall with SS-34 and GSA rotors, or equivalent centrifuge and rotors)

10,000 to 14,000 MWCO dialysis membrane
One large (25-liter) or several smaller (4-liter) dialysis tanks
Sterile scissors
125-ml glass Wheaton bottles

Additional reagents and equipment for dialysis (*APPENDIX 3C*)

Extract collagen

1. Skin 10 to 20 rat tails and place tails on ice. Break tails at joints and tease out individual collagen fibers. Wash in large volume distilled water (2 to 3 liter) for 1 hr with agitation. Change wash water three to four times.

   The yield is 200 to 400 mg collagen per rat.

2. Extract overnight at 4°C with agitation in 2 liters of 0.5 M NaCl/50 mM Tris·Cl, pH 7.4. Discard extract and repeat step.

3. Discard second salt extract and wash collagen fibers extensively (over a 3-hr period with change two to three times per hr) in distilled water to remove salt.

4. Extract overnight at 4°C with slow agitation in 2 liters of 0.5 M acetic acid.

5. Remove insoluble remnants by filtration through glass wool or cheesecloth, then centrifuge in 500-ml bottles for 30 min at 11,000 × *g*, 4°C. Add solid NaCl little by little to a final concentration of 5% w/v (50 g/liter) under constant vigorous stirring. When the salt is completely dissolved, turn off stirrer, cover beaker, leave in cold room overnight, and let precipitate gather at bottom of vessel.

   The collagen immediately starts to precipitate upon addition of the salt.

6. Collect precipitate by centrifugation for 30 min at 11,000 × *g*, 4°C. Discard supernatant.

7. Redissolve collagen by adding 450 ml of 0.5 M acetic acid to first centrifuge bottle, transfer liquid to the second bottle, and so on, until collagen is redissolved/redispersed into ~900 to 1000 ml in 0.5 M acetic acid.

8. Stir vigorously overnight at 4°C until collagen is completely dissolved.

   If not dissolved overnight, add more acetic acid and bring volume up to 1600 to 1800 ml.

Dialyze collagen solution

9. Place collagen solution, 300 to 400 ml at a time, in dialysis bags. Dialyze in tank against 25 liters of 0.5 M acetic acid, then for 3 to 4 days against 50 mM acetic acid. Change daily and mix content of bags.

   See APPENDIX 3C for additional details on dialysis.

10. Dialyze against several changes of 0.02 M Na_2HPO_4 in the 25-liter tank over the next 72 hr.

    Precipitation should happen as fast as possible, so change solution frequently in the beginning and massage bags frequently to facilitate even distribution of reagents. The collagen precipitates as a thick white gel.

11. Harvest precipitate by centrifugation in 500-ml bottles for 30 min at 11,000 × *g*, 4°C. Redissolve collagen in 0.5 M acetic acid by vigorous stirring overnight at 4°C.

12. Dialyze 3 to 4 hr against 0.5 M acetic acid, then overnight against 50 mM acetic acid, and, finally, overnight against several changes of 5 mM acetic acid.

13. Centrifuge 1 hr at 11,000 × *g*, 4°C. Lyophilize supernatant and store in dessicator at −80°C.

14. Redissolve as follows.
    a. Weigh out no more than 150 mg collagen.
    b. Cut into 1-cm pieces with sterile scissors.
    c. Place collagen pieces into a 125-ml glass Wheaton bottle that has been autoclaved with a stir bar inside.
    d. Add cold 13 mM HCl to make a 3 mg/ml solution and stir briskly at 4°C with occasional shaking for ~24 hr.

    The collagen solution should be slightly opalescent.

15. Centrifuge solution for 20 min at 50,000 × *g*, 4°C, to remove any insoluble material, if necessary.

    Note that the solution remains somewhat opalescent even after centrifugation. This solution may be stored for months at 4°C. Freezing should be avoided.

SUPPORT PROTOCOL 2

LABELING OF COLLAGEN

Rat tail tendon type I collagen may be labeled using [^{3}H]acetic anhydride as described in detail in Birkedal-Hansen and Danø (1981) and Birkedal-Hansen (1987), or with fluorescent reagents. The following fluorescent labeling method was adapted from a technique devised by the Chen laboratory (G. Ghersi and W.T. Chen, unpub. observ.).

Materials

3 mg/ml rat tail tendon type I collagen originally dissolved in or dialyzed into 13 mM hydrochloric acid (see Support Protocol 1)
Neutralizing buffer (see recipe)
Borate buffer: 0.05 M $NaB_4O_7 \cdot 10H_2O$, pH 9.3, containing 0.04 M NaCl, filter sterilized
20 to 30 mg tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) *or* fluorescein isothiocyanate (FITC) stock solutions, dissolved in DMSO
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
20 mM and 1 M hydrochloric acid, sterile

125-ml glass Wheaton bottle, autoclaved
Platform shaker

1. Mix 8 ml of 3 mg/ml rat tail tendon type 1 collagen with 2 ml neutralizing buffer in a sterile 125-ml bottle and incubate at 37°C overnight to form a gel.

   Rat tail tendon type I collagen may be prepared as described in Support Protocol 1, or purchased from BD Biosciences; bovine skin and human placental type I collagen are also available from the same supplier.

2. Wash for 1 hr with sterile borate buffer at room temperature by rotating at low speed on a platform shaker.

3. Remove buffer and replace with 10 ml borate buffer containing 2 to 3 mg/ml TRITC or FITC (prepared from 20 to 30 mg FITC or TRITC predissolved in a small volume of DMSO). Incubate at room temperature with gentle shaking for 20 to 30 min or until the dye diffuses through the gel. Protect from light from this point onward.

4. Wash with multiple changes of PBS at room temperature with rotation on a platform shaker for several days to remove free dye. Wash out salts with several changes of water.

   At some point, the collagen gel may become detached from the bottle. If so, pipet off solutions carefully in order to avoid breaking up the collagen.

   If unbound dye is not throughly washed from the collagen gel, subsequent experiments may be marred by high background fluorescence.

5. Add a sterile magnetic stir bar and stir to dissolve the gel in 8 ml pre-chilled 20 mM hydrochloric acid at 4°C. If the collagen is reluctant to dissolve, make sure the pH is around 2. If necessary adjust the pH by the addition of 1 M hydrochloric acid.

   Collagen can also be labeled with Alexa Fluor dyes using protein-labeling kits containing amine-reactive dyes from Molecular Probes/Invitrogen. Neutralize and gel 2.5 mg collagen as described in step 1, above, and equilibrate with several 10-min washes in PBS. Add 1 M bicarbonate buffer, pH 8.3 to 0.2 M. Resuspend a vial of Alexa Fluor dye (premeasured to label 1 mg protein) in a small amount of PBS and add immediately to the collagen. Incubate for 2 hr at room temperature and then wash extensively and redissolve as above.

   Collagen is labeled in the fibrillar state so that sites important for subsequent alignment and gelling are not being blocked by the labeling procedure. Consequently, collagen labeled in this fashion readily dissolves in dilute acid and gels again upon neutralization and mild heating. Depending on the need, the fluorescently labeled collagen may be diluted up to 10-fold with unlabeled rat tail tendon collagen and still yield a strong enough signal for quantification.

GELATIN/CASEIN ZYMOGRAPHY

BASIC PROTOCOL 2

Zymographic methods are designed to analyze the proteolytic capacity of latent and active MMPs (Heussen and Dowdle, 1980; Birkedal-Hansen and Taylor, 1982; Birkedal-Hansen, 1987). This set of techniques is based on a number of unique properties of MMPs: (1) MMPs retain (or refold to display) catalytic activity after electrophoresis in SDS-containing buffers as long as heating and reduction are avoided (Birkedal-Hansen and Taylor, 1982); (2) brief exposure to SDS opens the "cysteine switch" (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990) so that both precursor and proteolytically truncated ("activated") forms of the enzyme display catalytic activity; and (3) MMP catalytic activity is reversibly inhibited by SDS and readily restored when SDS is removed by washing with Triton X-100 (Birkedal-Hansen and Taylor, 1982). It is therefore possible to resolve a heterogenous group of MMPs and non-MMPs in SDS-containing gels copolymerized with a suitable substrate (gelatin, casein), remove the SDS, and develop (without distinction) the spontaneous or latent catalytic activity associated with each electrophoretic band. After appropriate incubation (to allow for proteolysis), the discrete bands of substrate lysis are made visible by Coomassie blue staining of the gel (Fig. 10.8.3). SDS opens the "cysteine switch" but instantly inhibits the switch-open enzyme and blocks autolytic truncation normally associated with activation. The proenzyme bands therefore migrate at their expected high-molecular weight, but display proteolytic activity because the switch is unable to again "close" after removal of the SDS with Triton X-100.

Zymography using gels containing 0.1 to 1.0 mg/ml gelatin are by far the most sensitive. Gels may either be purchased (Invitrogen) or prepared as described below. Gelatin works particularly well for MMP-2 and MMP-9, whereas MMP-1, MMP-3, MMP-7, MMP-8, and MMP-10 are better identified in casein-containing gels.

Materials

Gelatin (bovine skin, Sigma-Aldrich type B6-6269) or casein (Sigma-Aldrich, technical, C-0376)
2.0 M Tris·Cl, pH 8.8 (*APPENDIX 2A*)
30/0.8 acrylamide/bisacrylamide (*UNIT 6.1*)
Glycerol
10% (w/v) SDS (*APPENDIX 2A*)
TEMED
10% (w/v) ammonium persulfate

MMP preparation of interest (for standards, use 1 to 5 ng purified MMP)
5× electrophoretic sample buffer (see recipe)
Electrophoretic running buffer (see recipe)
Gel washing buffers 1 to 4 (see recipe)
Coomassie blue stain (see recipe)
Gel destaining solution (see recipe)

50-ml centrifuge tubes
57°C water bath
Whatman no. 1 filter paper *or* 0.5-μm syringe filter
Gel washing tray of appropriate size

Additional reagents and equipment for preparing SDS-PAGE gels according to Laemmli (*UNIT 6.1*)

NOTE: The following procedure is based on a standard 10% SDS-PAGE according to Laemmli (Laemmli, 1970; *UNIT 6.1*) using a 4% stacking gel and a pH 8.3 running buffer. It is important to avoid heating and/or reduction during sample preparation and running of the gel.

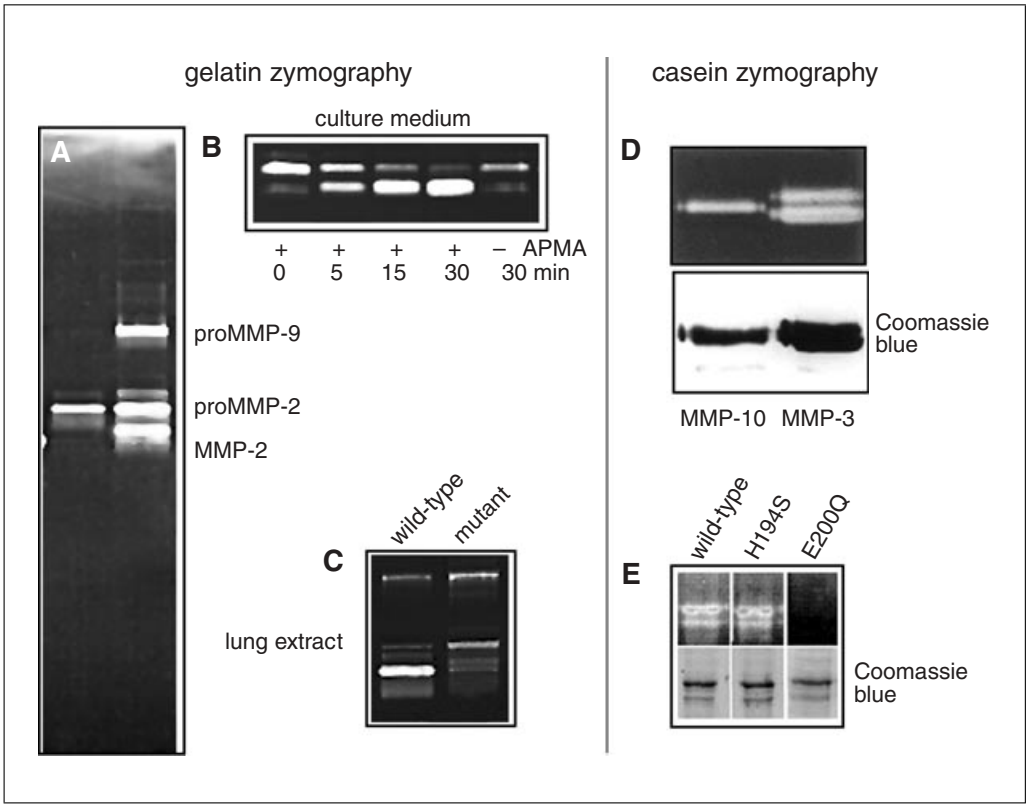


Figure 10.8.3 Zymography. (**A**) Zymography using gelatin-containing polyacrylamide gel. Culture medium containing proMMP-2 (left) or MMP-2/proMMP-2 and proMMP-9 (right). The proenzymes display catalytic activity because exposure to SDS during sample preparation opens the cysteine switch. (**B**) Detail showing conversion of proMMP-2 to MMP-2 by exposure to aminophenylmercuric acetate. From Caterina et al. (2000). (**C**) MMP-2 and MMP-9 activity in extracts of lungs of wild-type mice (left) or mice in which the TIMP-2 gene has been mutated to inactive form (modified from Caterina et al., 2000). (**D**) Zymography using casein-containing polyacrylamide gel. (pro)MMP-3 and MMP-10 cleave casein embedded in the gel (modified from Windsor et al., 1993). (**E**) Casein zymogram of mutant and wild-ype MMP-1. Inactivation of catalytic activity by mutation of catalytic site glutamic acid (E) to glutamine (Q) that abolishes casein cleavage. A histidine to serine replacement outside the active site does not. Modified from Windsor et al. (1994).

1. Weigh out appropriate amount of gelatin (for 0.1 to 1.0 mg/ml final concentration) or casein (for 1.0 mg/ml final concentration) and place in a 50-ml centrifuge tube.
2. For every 10 ml of solution to be prepared, add 4 ml of 2.0 M Tris·Cl, pH 8.8, and 6 ml water. Dissolve by heating in a 57°C water bath. Filter through Whatman no. 1 filter paper or syringe filter.
3. Prepare the 10% resolving gel (also see *UNIT 6.1*) by adding the following to 10 ml filtered gelatin or casein solution (0.2 to 13 mg/ml in 0.8 M Tris·Cl, pH 8.8; see step 2):

   6.6 ml 30/0.8 acrylamide/bisacrylamide
   2 g glycerol
   0.2 ml 10% (w/v) SDS
   13.3 μl TEMED
   67 μl 10% (w/v) ammonium persulfate

   Pour resolving gel as described in *UNIT 6.1*.
4. Prepare 4% stacking gel by combining the following (also see *UNIT 6.1*):

   1 ml 30/0.8 acrylamide/bis acrylamide
   0.36 ml 2 M Tris·Cl, pH 6.8
   75 μl 10% (w/v) SDS
   6 ml H_2O
   8 μl TEMED
   60 μl ammonium persulfate

   Pour stacking gel as described in *UNIT 6.1*.
5. Mix 1 part MMP solution (partially or fully purified MMP, culture medium, concentrated culture medium, or other preparation containing MMP) with 4 parts of 5× sample buffer (final concentration, 1% w/v SDS). Incubate at room temperature for 10 min, then load 20 to 30 μl into each well of the 15-ml gel prepared in steps 3 and 4.

   Alternatively, load 20 to 30 μl per well of an Invitrogen minigel.
6. Run gel at 200 V for 35 to 45 min or until dye front reaches bottom of gel using electrophoretic running buffer, pH 8.3.
7. Remove gel from electrophoretic apparatus and place in an appropriately sized container. Wash four times, 20 min each, successively, in washing buffers 1, 2, 3, and 4 at room temperature. Shake gently throughout.
8. Replace the last wash buffer with fresh washing buffer 4 and incubate 1 to 24 hr at 37°C.

   A few hours of incubation is usually sufficient to reveal MMP-2 and MMP-9 by gelatin zymography. Overnight incubation is required to visualize MMP-1, MMP-3, MMP-13, MMP-7, and MMP-10 by casein zymography.
9. Stain gel with Coomassie blue stain for 30 min and destain with gel destaining solution for several hours until bands are clear.

   Typical results are shown in Figure 10.8.3.

REVERSE ZYMOGRAPHY

BASIC PROTOCOL 3

Reverse zymography is specifically designed to identify electrophoretic bands which display MMP-inhibitory activity. The method is based on incorporation of both MMP activity and gelatin into the running gel. During the ensuing incubation, the SDS-activated MMP-2 (gelatinase A) cleaves the substrate everywhere in the gel except in and immediately around bands with inhibitory activity such as TIMPs. This method yields well

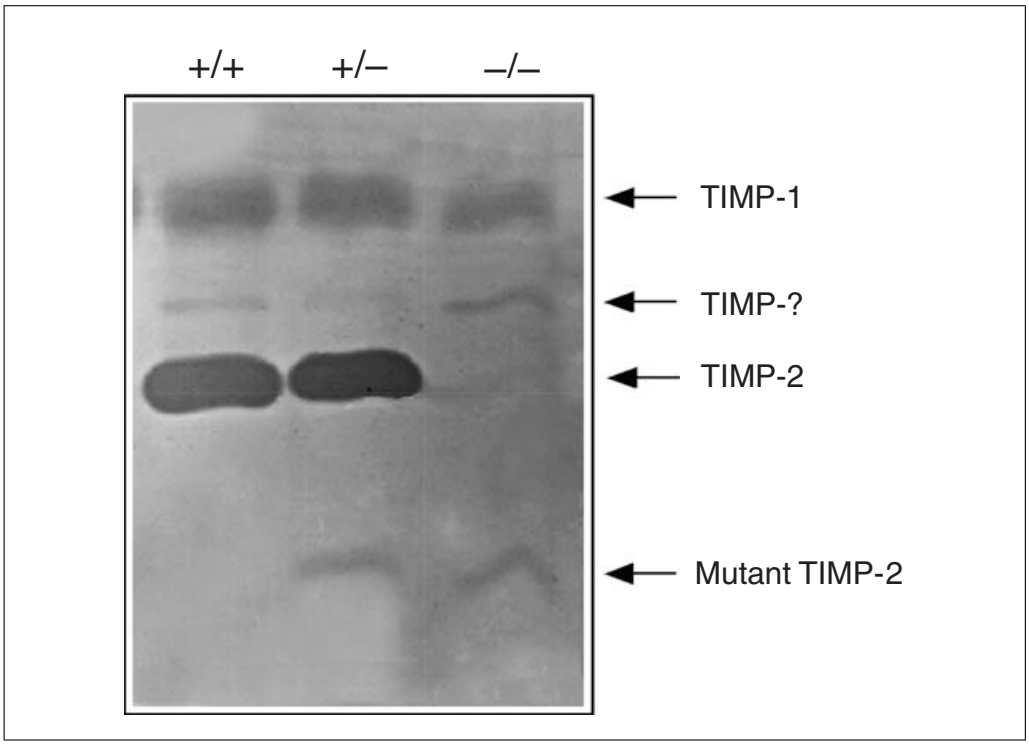


Figure 10.8.4 Reverse zymography. Inhibition of MMP-2 by TIMPs. Skin fibroblast culture medium obtained from wild-type, hemizygous, or homozygous TIMP-2-deficient mice was resolved by SDS-PAGE in a gel also containing MMP-2 and gelatin. During incubation, MMP-2 cleaves gelatin unless inhibited by electrophoretic bands of TIMPs. The TIMP-2-deficient cells still express TIMP-1 and unidentified component below TIMP-1, possibly TIMP-3 and a weakly inhibitory truncated mutant of TIMP-2. Modified from Caterina et al. (2000).

resolved bands of TIMP-1, TIMP-2, TIMP-3, and TIMP-4, as well as mutant forms of these inhibitors (Fig. 10.8.4). The following protocol is developed by the Stetler-Stevenson laboratory and used in the authors' laboratory as well. Quantities are for a 15-ml gel, but can be scaled down as necessary.

Materials

8.7 mg/ml gelatin solution (see recipe)
MMP-2 (Gelatinase A)
5× electrophoretic sample buffer (see recipe)
2.5% (w/v) Triton X-100
Incubation solution (see recipe)

Additional reagents and equipment for "forward" zymography (see Basic Protocol 2)

1. Prepare separating gel (17%), copolymerizing gel with gelatin (2.5 mg/ml) and purified gelatinase A (MMP-2), by mixing the following components (also see *UNIT 6.1*):

   4.2 ml 8.7 mg/ml gelatin solution
   0.16 μg/ml (final concentration) gelatinase A (MMP-2)
   8.25 ml 30/0.8 acrylamide/bisacrylamide
   2.1 ml H_2O
   0.29 ml 10% (w/v) SDS
   7.3 μl TEMED
   73 μl 10% (w/v) ammonium persulfate

Pour separating gel as described in *UNIT 6.1*.

Purified MMP-2 may be replaced with culture medium of cells that secrete this enzyme. The appropriate amount should be determined by trial and error.

2. Prepare 5% stacking gel by combining the following (also see *UNIT 6.1*):

   1.66 ml 30/0.8 acrylamide/bis acrylamide
   1.55 ml 2 M Tris·Cl, pH 6.8
   125 µl 10% (w/v) SDS
   8.2 ml H_2O
   10 µl TEMED
   200 µl ammonium persulfate

   Pour stacking gel as described in *UNIT 6.1*.

3. Mix samples with 5× sample buffer for reverse zymography. Incubate at room temperature for ≥10 min, then load 20 to 30 µl into each well of the gel.
4. Run gel at 150 V until buffer front reaches bottom of gel.
5. Remove gel and wash in three changes of 2.5% Triton X-100, each for 2 hr with gentle shaking.
6. Incubate overnight at 37°C in incubation solution.
7. Stain gel with Coomassie blue stain for 20 min and destain in gel destaining solution for several hours until background is clear.

   Typical results are shown in Figure 10.8.4.

α2-MACROGLOBULIN (α2M) CAPTURE

BASIC PROTOCOL 4

α2M capture is particularly valuable because it permits assessment of the proteolytic competence and activity of single bands of MMPs in a mixture of many partially or fully processed forms. The method was originally devised (Birkedal-Hansen et al., 1976) for separation of complexes from unreacted forms by molecular sieve chromatography (Fig. 10.8.5), but it is even more valuable when combined with electrophoretic analysis. The protocol is based on the observation that α2M forms complexes only with catalytically competent forms of MMPs. Unactivated MMP precursors or forms devoid of catalytic activity are not captured. The ensuing separation by SDS-PAGE permits easy identification of bands which have been captured and moved to the top of the gel because of the large molecular mass of the α2M (Fig. 10.8.5). Bands that escape capture continue to migrate at their usual position. Complexes formed with α2M are covalent and therefore not easily dissociated. The ability of α2M to discriminate between latent and overtly active forms of the enzyme is a result of the α2M inhibition mechanism. α2M is inert until the attacking proteinase cleaves a peptide bond in the bait region. This cleavage results in rapid conformational change and liberates a thiol ester which covalently bonds to and immobilizes the attacking proteinase.

Materials

MMP solution to be tested
2 to 3 mg/ml purified α2M in 50 mM Tris·Cl standard buffer (see recipe for buffer)
100 µg/ml TPCK-treated trypsin (e.g., Sigma) in 50 mM Tris·Cl standard buffer (see recipe), pH 7.4
1.0 mg/ml soybean trypsin inhibitor in 50 mM Tris·Cl standard buffer (see recipe), pH 7.4
5× electrophoretic sample buffer (see recipe)

Antibodies to MMPs of interest
Nitrocellulose paper

Additional reagents and equipment for SDS-PAGE according to Laemmli (*UNIT 6.1*) and for immunoblotting (*UNIT 6.2*)

1. Mix one half of the test solution with a sufficient volume of 1.5 mg/ml α2M to achieve a ≥10× molar ratio of inhibitor to MMP. Incubate 15 min at room temperature.

2. To compare "activated" and "unactivated" samples, preincubate the other half of the test sample with 10 μg/ml trypsin (added from 100 μg/ml stock) for 10 min at room

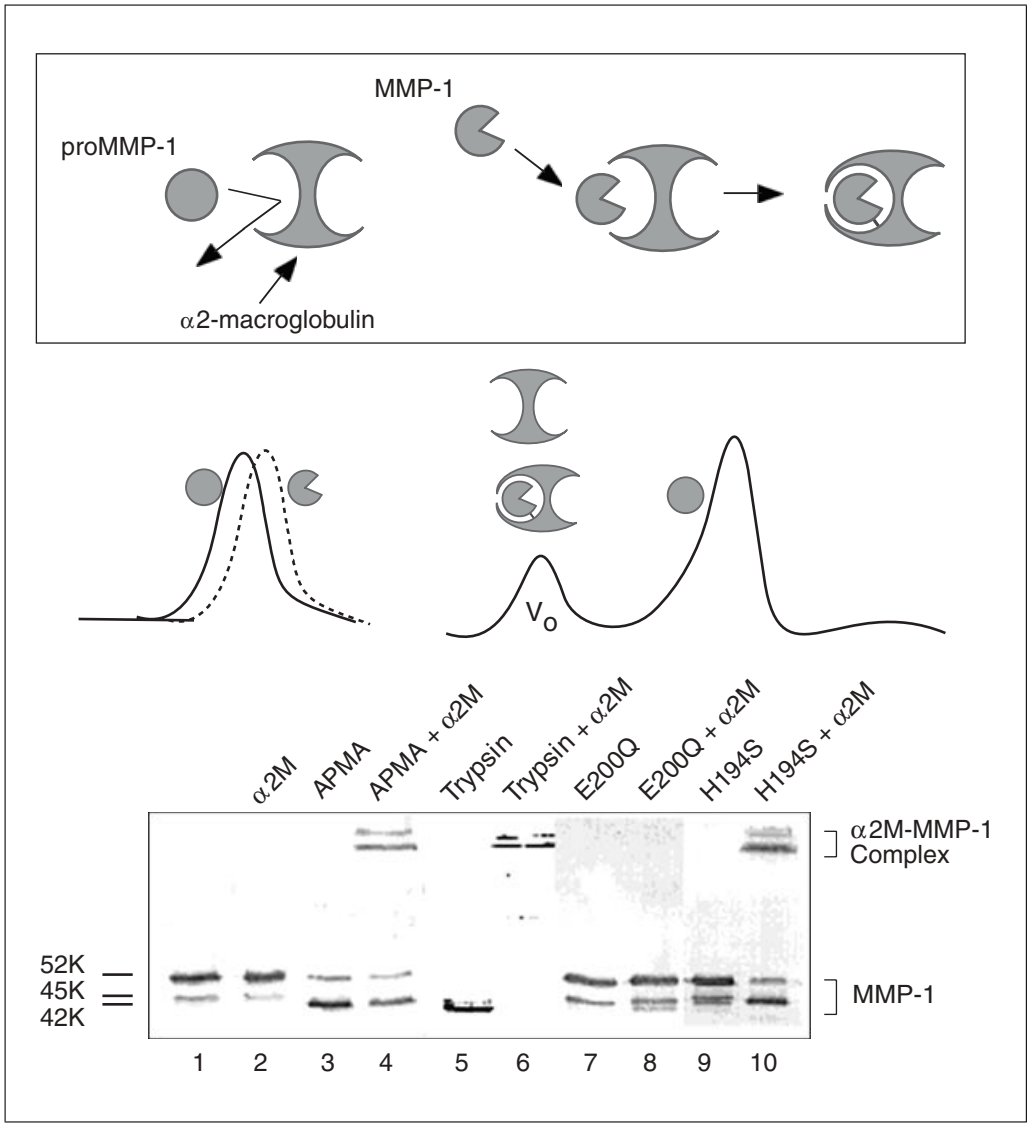


Figure 10.8.5 α2-macroglobulin (α2M) capture. The capture technique is based on the property that proteolytic cleavage of the α2M bait region results in conformational and eventually covalent capture of the attacking proteinase. Because of the large disparity in molecular weight, captured and free froms of the proteinase may be separated either by molecular sieve chromatography (upper panel; Birkedal-Hansen et al., 1976) or by SDS gel electrophoresis (lower panel; Windsor et al., 1994). Covalently bound proteinase is not released and is readily identified by appropriate antibody staining. Latent or inactive proteinases are not captured. The method therefore discriminates between enzyme forms with and without catalytic activity at the moment of testing. The panel shows wild-type and mutant forms of human MMP-1. Samples in lanes 3, 4, and 7 to 10 are pretreated with *p*-aminophenylmercury acetate (APMA). Samples in lanes 5, and 6 were preactivated by trypsin. Modified from Windsor et al. (1994).

temperature, then add 100 μg/ml soybean trypsin inhibitor (added from 1 mg/ml stock). Incubate separately with α2M as described in step 1.

Commercial sources of α2M are available but should always be checked for activity by titration with trypsin using a suitable substrate (Sottrup-Jensen and Birkedal-Hansen, 1989). Alternatively, the inhibitor may be prepared by standard techniques as described by Sottrup-Jensen and Birkedal-Hansen (1989) and Sottrup-Jensen et al. (1983). Activation with trypsin prior to addition of α2M often yields more complete capture than with organomercurials—e.g., NH_2PheHgAc (APMA)—which seem to gradually inactivate α2M. Samples preincubated with organomercurials, however, still show partial capture.

3. Mix with 5× electrophoretic sample buffer (final concentration, 1% w/v SDS, 2.5% v/v 2-ME) without heating, then resolve by by SDS-PAGE using a 10% gel according to Laemmli (Laemmli, 1970; *UNIT 6.1*).

4. Transfer to nitrocellulose paper and stain with appropriate MMP antibody using conventional immunoblotting techniques (*UNIT 6.2*).

Typical results are shown in Figure 10.8.5.

TIMP CAPTURE

ALTERNATE PROTOCOL

Complexes formed with TIMPs are not covalent, although several, but not all, withstand exposure to low concentrations of SDS, as originally observed by DeClerck et al. (1991), who first pioneered this technique. This method detects many but not all activated MMPs that bind TIMPs, including MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-10 (stromelysin-2), and MMP-13. Detection is most conveniently done by immunoblotting using specific antibodies to the two complex components (MMP and TIMP; Fig. 10.8.6). The method described below is the authors' adaptation of the method of DeClerck (DeClerck et al., 1991). It is based on capture with TIMP-1, but TIMP-2 capture works just as well.

Materials

0.1 to 1.0 mg/ml TIMP-1 (Oncogene Research Products, Chemicon International; also see Bodden et al., 1994) in 50 mM Tris·Cl standard buffer (see recipe), pH 7.4

10.0 mM NH_2PheHgAc (APMA; Sigma) in Tris·Cl standard buffer (see recipe), pH 7.4

5× electrophoretic sample buffer (see recipe, but use only 0.5% w/v SDS)

Antibodies to MMPs and TIMP-1 of interest (Calbiochem, Chemicon International)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Incubate control and activated samples with 40 to 100 μg/ml TIMP-1 (added from 0.1 to 1.0 mg/ml stock) with and without 1.0 mM NH_2PheHgAc (added from 10.0 mM stock) for 90 min at 37°C.

Molecules which are activated by NH_2PheHgAc are captured almost instantly by TIMP-1.

TIMP-1 may be prepared from cultures of fibroblasts or similar cell lines that express fairly high levels of TIMP-1 activity (Bodden et al., 1994). Concentrations of this compound in the range of 0.1 to 1.0 mg/liter may be recovered from the culture medium. The purification scheme is somewhat cumbersome but greatly facilitated by use of antibody-based affinity chromatography techniques.

2. Mix with 5× electrophoretic sample buffer containing 0.5% SDS. Resolve by SDS-PAGE using a 10% gel on ice at 100 V (*UNIT 6.1*).

Note that the SDS concentration of the sample buffer is reduced to 0.1% (final concentration) in order to avoid dissociation of these entirely noncovalent complexes. This change is crucial to the success of the technique.

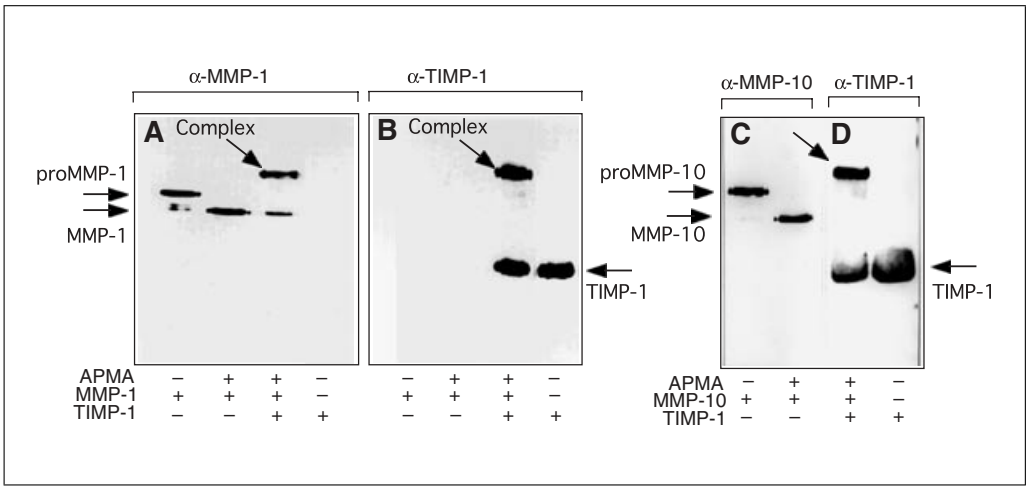


Figure 10.8.6 TIMP capture. (**A**, **B**) are identical panels stained with antibodies to either human MMP-1 or TIMP-1. Capture of activated human MMP-1 gives rise to a new band in the 70-kDa range containing both MMP-1 and TIMP-1 (arrow). (**C**) proMMP-10 and activated MMP-10 stained with antibody to human MMP-10. (**D**) Addition of TIMP-1 to activated MMP-10 results in capture of the enzyme now migrating in a complex with TIMP-1 in the M_r 70-kDa range. Modified from Windsor et al. (1993).

3. Transfer to nitrocellulose and stain adjacent lanes with antibodies to TIMP-1 and to MMP using standard immunoblotting techniques (*UNIT 6.2*).

   Typical results are shown in Figure 10.8.6.

BASIC PROTOCOL 5

FLUORESCENT LABELING OF CRYPTIC CYS-RESIDUE IN MMPs

Most MMP (and ADAM) precursors contain a cryptic thiol group derived from a single, unpaired cysteine residue in the propeptide. This group is coordinately bonded directly to the active site Zn ("cysteine switch") and in this manner plays a significant role in maintaining the catalytic latency of the proteinase precursors. The protocol below permits unmasking and detection of this cryptic thiol group (Fig. 10.8.7). The "switch" opens upon addition of SDS, which allows reaction of the liberated thiol group with a fluorescent maleimide compound (Yamamoto et al., 1977; Lyons et al., 1991).

Materials

MMP-containing samples
20 μM fluorescent maleimide N-(7-(di-methylamino-4-methyl-3-coumarinyl) maleimide (DACM) in Tris·Cl standard buffer (see recipe for buffer; prepare from 1 mM DACM stock in DMSO or ethanol)
2-mercaptoethanol stock in electrophoretic sample buffer (see recipe for buffer): concentration appropriate to obtain 5% final concentration in reaction mixture
Fluorescent lamp
Photographic equipment

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

1. Expose companion samples of 50 to 200 μg/ml MMP for 1 hr at room temperature to 20 μM DACM (final concentration) either in the presence or absence of 1% (w/v) SDS.

2. Stop reaction by adding 2-mercaptoethanol (as stock solution of appropriate concentration in electrophoretic sample buffer) to a final concentration of 5% (v/v).

3. Resolve proteins by SDS-PAGE (*UNIT 6.1*).

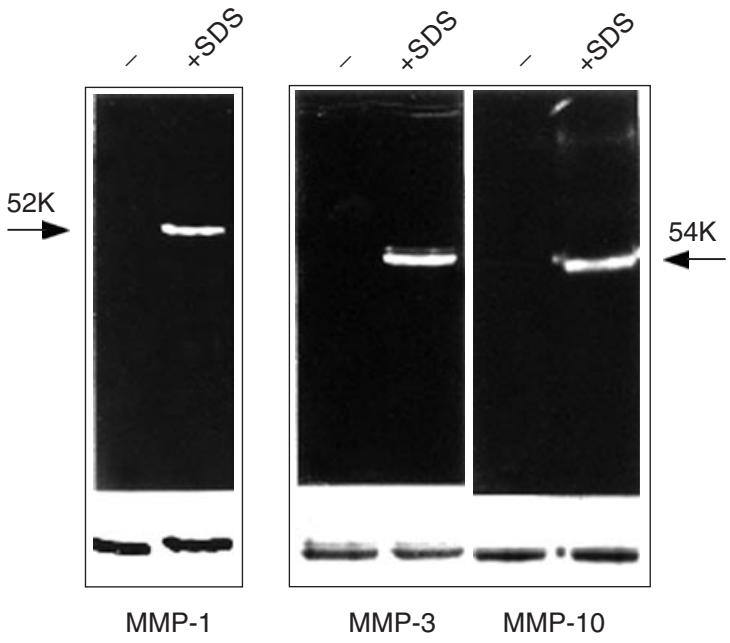


Figure 10.8.7 Fluorescent labeling of propeptide cryptic thiol residue by fluorescent maleimide. The cysteine switch is "closed" in the nascent proenzyme and therefore not reactive with a fluorescent maleimide compound (DACM). Exposure to SDS "opens" the switch and renders the cryptic thiol group reactive with the maleimide resulting in covalent modification of the proenzyme and generation of a readily detectable fluorescent band. Left panel: MMP-1. Right panel, MMP-3 and MMP-10. Lower edge of each panel shows Coomassie blue staining of the same bands. Modified from Windsor et al. (1993).

4. Photograph under long-wavelength UV illumination.

Typical results are shown in Figure 10.8.7.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX*** *2A; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Coomassie blue stain

0.5% (w/v) Coomassie blue R-250
30% (v/v) methanol
10% (v/v) acetic acid
Store up to 6 months at room temperature

Electrophoretic running buffer, pH 8.3

0.025 M Tris base
0.192 M glycine
0.1% (w/v) SDS
Store up to 1 year at room temperature

Electrophoretic sample buffer, 5×

0.2 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
5% (w/v) SDS
20% (w/v) glycerol

continued

0.1% (w/v) bromphenol blue
Store up to 1 year at room temperature

This is the sample buffer used in Basic Protocol 2.

Gelatin solution, 8.7 mg/ml

Add gelatin (bovine skin, Sigma-Aldrich type B6-6269) to 1 M Tris·Cl, pH 8.8 at 8.7 mg/ml. Dissolve by heating to 57°C, then filter through Whatman no. 1 filter paper.

Gel destaining solution

30% (v/v) methanol
10% (v/v) acetic acid
60% (v/v) H_2O
Store up to 1 year at room temperature

Gel washing buffers 1 to 4

Buffer 1:
2.5% (v/v) Triton X-100
3 mM NaN_3
Buffer 2:
2.5% (v/v) Triton X-100
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
3 mM NaN_3
Buffer 3:
2.5% (v/v) Triton X-100
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
3 mM NaN_3
5 mM $CaCl_2$
1 μM $ZnCl_2$
Buffer 4:
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
3 mM NaN_3
5 mM $CaCl_2$
1 μM $ZnCl_2$

Buffers may be stored up to 1 year at room temperature.

Incubation solution

50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.2 M NaCl
5 mM $CaCl_2$
0.02% (w/v) Brij-35
Store up to 1 year at 4°C

Neutralizing buffer (0.2 M naP_i)

Prepare the following stock solutions:
Solution A: 2.78 g NaH_2PO_4 in 100 ml H_2O
Solution B: 5.365 g $Na_2HPO_4 \cdot 7H_2O$ in 100 ml H_2O
Prepare working solutions as follows:
15.2 ml Solution A
64.8 ml Solution B
16.6 ml 5 M NaCl
Add 80 ml 0.1 N NaOH
Store up to 1 year at 4°C

Tris·Cl standard buffer, pH 7.4

50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.2 M NaCl
5 mM $CaCl_2$
Store up to 1 year at 4°C

COMMENTARY

Background Information

Dissolution of collagen type I

Substrate. Although collapsing the gel by air drying is advantageous for most purposes, and the resulting collagen film is more similar to the density of collagen in interstitial connective tissues (Fig. 10.8.1), it is possible to seed the cells on top of (or inside) fully hydrated gels and to monitor the process as the cells dissolve their way through the collagen gel. Electron microscopy confirms that hydrated gels are very loose, with the individual fibrils spaced far apart. The collagen content is quite low compared to the liquid phase and accounts for only 0.03% of the mass and for a similarly small volume fraction of the gel.

While use of reconstituted type I collagen fibrils as a substrate offers particular advantages because of its resistance to general proteolysis, it is possible to replace this substrate with other extracellular matrix components. Type II collagen does not form fibrils as readily as does type I but might prove useful after additional refinement of the system. Type III collagen appears to gel adequately for this purpose and may also be used as a substrate. Films and gels of type IV collagen may also be used, as may Matrigel (predominantly composed of laminin), fibrin, and fibronectin. An important variation using fluorescently labeled fibronectin was devised by Chen and coworkers (Chen et al., 1984; Chen and Chen, 1987).

Serum. Serum contains a number of factors expected to either promote or inhibit the proteolytic dissolution of the extracellular matrix including collagen fibrils. The high concentration of α2M (2 to 3 mg/ml or 3 to 4 × 10^{-6} M), which effectively blocks most MMPs in test tube experiments, however, does not inhibit cell-mediated dissolution of the collagen fibril film. Serum also contains plasminogen at a concentration of ~200 μg/ml (2 × 10^{-6} M). Addition of even low concentrations of plasminogen (4 μg/ml; 4 × 10^{-8} M) to serum-free cultures greatly accelerates the rate of dissolution of the collagen fibril film by human foreskin keratinocytes (or other cells) which express urokinase-type plasminogen activator (u-PA) or tissue-type plasminogen activator (t-PA). The mechanism is not quite well understood but may involve a role for plasminogen in the extracellular activation of certain proMMP precursors as an essential step in the dissolution of the substrate.

Cytokines, transcriptional activation. Addition of cytokines, growth factors, and agents such as TPA, which upregulate or induce expression of MMPs, generally accelerates dissolution of the fibril coating dramatically, but since these reagents upregulate a wide range of MMPs, it is not yet possible to determine whether a single MMP or group of MMPs is responsible for this effect.

Inhibition. That dissolution of the collagen fibril coating is mediated by metalloproteinase-dependent mechanisms is readily made evident by synthetic inhibitors. Inclusion of the Zn-chelating agent 1,10-phenanthroline completely blocks dissolution, as do synthetic MMP inhibitors such as BB94, BB2516 (British Biotech), and Galardin. A number of synthetic inhibitors currently exist; some of these may be obtained by directly contacting the pharmaceutical companies in question (British Biotech, Roche Diagnostics, Celltech). Serine proteinase inhibitors such as α1-antitrypsin (α1AT) and soybean trypsin inhibitor, as well as cysteine proteinase inhibitors such as E-64, have no effect on the rate of dissolution. These findings suggest that the process(es) that result in dissolution of the collagen fibrils are absolutely dependent on MMP activity.

Zymography

Gelatin zymography is a fairly straightforward yet very highly sensitive technique as long as heating and reduction are avoided during sample preparation. The method yields discrete, well-resolved, and distinct unstained bands on a blue background, which are clearly visible and easy to photograph and document with transillumination (Fig. 10.8.3). The activity may be quantified by comparison with standard curves of specific purified MMPs (Kleiner and Stetler-Stevenson, 1994), but the rate of lysis varies considerably from MMP to MMP, and the technique is primarily intended

to provide qualitative information. A variation described by Lyons et al. (1991) permits monitoring of real-time progress of the reaction under UV light by use of gelatin labeled by a fluorophore. Although gelatin zymography is highly sensitive, capable of detecting low picogram quantities of MMPs, the assay does not reflect the activity of these proteases present in the sample analyzed. This is because the addition of SDS to the sample prior to electrophoretic separation results in dissociation of many enzyme inhibitor complexes. Therefore, zymography represents an excellent technique for identification of MMP species present in a given sample, but overinterpretation of the results—e.g., assessment of specific activity—is a common pitfall. Casein zymograms develop more slowly, almost invariably require overnight incubation, and tend to produce less sharp bands. (Latent) proenzyme forms also show up because of the "switch"-opening effect of SDS, but these forms do not necessarily acquire full catalytic activity. "Activation" by organomercurials (0.5 to 1.0 aminophenylmercuric acetate in 50 mM Tris·Cl buffer, pH 7.5, for 20 min to 20 hr) before sample preparation often results in higher levels of proteolytic activity but also shifts the M_r of the individual bands because of autolytic cleavage and removal of the propeptide.

α2M capture

Capture techniques permit direct assessment of the ability of various forms of MMPs to bind to natural inhibitors in a manner that resists dissolution by exposure to low concentrations of SDS. Zymographic techniques are not capable of discriminating between latent and catalytically active forms of the enzymes. That, however, can readily be achieved by α2M capture. TIMP capture (see Alternate Protocol) on the other hand does not depend on proteolytic activity and merely requires a correctly folded, but not necessarily catalytically competent, active site (Windsor et al., 1994).

TIMP capture

The method is particularly useful for analysis of the binding capacity of mutants in TIMPs and in MMPs (Windsor et al., 1994; Caterina et al., 1997). It is important to recognize that TIMP binding is not necessarily synonymous with catalytic competence. Mutants of MMP which are correctly folded but devoid of catalytic activity, such as the E200Q mutant of MMP-1 in which the active site glutamate is replaced with glutamine (and therefore catalytically inactive), still form complexes with TIMP-1 fully, as well as the native enzyme (Windsor et al., 1994). TIMP-1 captures both truncated and full-length forms, as long as the "switch" is open (by APMA).

Fluorescent labeling of cryptic Cys residue

The nascent closure of the "cysteine switch" by bonding of the single unpaired propeptide Cys residue to the active-site Zn^{2+} converts a catalytic Zn-binding site to a structural Zn-binding site. In order to monitor the (re)opening of the switch as a preamble to zymogen activation, the authors of this unit reasoned that covalent linkage of a fluorophore to the free thiol group might render this process easily visible and potentially quantifiable. That is indeed the case. The method shows, for instance, that the phenomenon of "switch opening" can be readily visualized in the absence of propeptide cleavage by exposure either to SDS or to EDTA.

Critical Parameters and Troubleshooting

Dissolution of collagen type I

Even for the experienced operator, collagen is not an easy protein to work with. Its preparation and use require meticulous and stringent adherence to the rules and conditions that "work," often with very little leeway for shortcuts and modifications. The most important checkpoint comes after the initial gelling. Unless there is clear and unequivocal evidence of gelling after 2 hr, efforts should be made to identify and correct the problem. Since there is no simple way to measure collagen concentration, the authors have utilized initial dry powder weight from materials stored in refrigerated dessicator jars as a guide. The concentrations mentioned in this unit refer to powder weight under these conditions. It is absolutely necessary that the solution from which the collagen is lyophilized be completely salt-free following extensive dialysis against dilute acetic acid. This problem may be avoided by purchase of commercial preparations of rat or bovine type I collagen, but it is necessary to test the gelling properties of the particular brand in question at the desired concentration and under the desired conditions. After 2 hr of gelation, the gel should be reasonably firm, i.e., it should not disintegrate upon gentle flicking of the plate. If the gel disintegrates during this test, the problem must first be solved before proceeding.

The homogeneity of the gel is also very important. This is best checked following the first

air drying and washing step by staining a newly prepared film with Coomassie blue. This will instantly reveal whether the gel is uniform and homogenous and if it contains particulate matter (which can be removed by centrifugation) or air bubbles. Both must be avoided, and the technique must be improved until each gel is completely uniform and homogenous after staining. It is also important to ascertain after the first washing of the first-time dried gel that salt-crystal deposits (formed during the initial drying phase) have been completely removed by washing. This is most easily checked using the phase-contrast microscope. The gel should look granular but uniform; any trace of crystal patterns is a certain indication of inadequate washing.

Zymography

Gelatin zymography presents few, if any, technical challenges, hence the popularity and universal application of this technique. Because of the longer incubation time required and the lesser sensitivity, casein zymograms often give less distinct and more diffuse bands. Although it has not been widely explored, it is highly likely that a large number of other substrates could be substituted for either gelatin or casein. Reverse zymography, on the other hand, is technically challenging and requires great care and skill as well as considerable practice and experience. The latter method is, however, a uniquely powerful technique to identify discrete MMP-inhibitory bands.

Inhibitor capture

While commercial preparations of α2M are available, the method is critically dependent on the native configuration of the inhibitor. Consequently, the authors rely only on freshly isolated inhibitor. Occasionally, methods which are employed to activate MMPs, such as exposure to organomercurials, adversely affect the inhibitor and render the capture reaction partial rather than complete. In some cases trypsin activation (stopped by soybean trypsin inhibitor) is preferable, but many mutants are highly sensitive to trypsin and rapidly degrade during activation attempts.

Fluorescent labeling of cryptic Cys residue

The method is fairly straightforward, although care must be taken to exclude any chemicals from the solutions that interfere with the Cys-maleimide reaction (e.g., heavy metals, *N*-ethylmaleimide, or iodoacetate). Photographic documentation can be tricky, but usually works well when using reflected UV light.

Anticipated Results

Dissolution of collagen type I

Use of 1- to 2-μm films results in complete dissolution within 1 to 4 days. Initially the cells penetrate the collagen fibril coating in discrete spots, which eventually coalesce to form contiguous zones devoid of collagen fibrils (Fig. 10.8.2). Dissolution of the fibril coating is strictly limited to the area immediately beneath the cell layer and does not extend beyond the boundaries of the cell colony. A similar pattern is observed in the presence of serum or purified plasminogen.

Zymography

When performed correctly, the reverse zymography–stained gel shows discrete, well-resolved bands of TIMPs on a virtually unstained background, indicating that all of the gelatin has been degraded except in and around the TIMP bands (Fig. 10.8.4). While this method yields important information when used in qualitative or semiquantitative fashion, the read-out may be quantified as described by Kleiner and colleagues (Oliver et al., 1997).

As with direct zymography, reverse zymography is a highly sensitive technique that can detect as little as 50 to 100 pg of TIMPs in a given sample (Oliver et al., 1997). However, as with direct zymography, careful interpretation of results is essential. Again, use of SDS-containing sample buffers and electrophoretic separation of the sample results in dissociation of some protease-inhibitor complexes. Thus, the levels of TIMPs present may not accurately reflect the actual free TIMP levels present in the samples analyzed. Alternatively, as described for the TIMP capture assays, not all TIMP-MMP complexes may be dissociated by SDS, and TIMP-binding to an MMP active site does not necessarily reflect proteolytic competency of the enzyme.

α2M capture

Incubation of native α2M with activated proteinases that cleave the bait region result in full or partial capture of the attacking proteinase. Complete capture requires a significant molar excess of inhibitor (with the amount varying from proteinase to proteinase), which may be determined by titration in preliminary experiments. Because of the size difference, captured and uncaptured bands are readily resolved and identified on western blots by staining with anti-MMP antibodies. Latent or catalytically inactive forms are not captured and remain at their usual migration position in the gel.

TIMP capture

Remarkably, most TIMP-MMP complexes survive dilute SDS solutions at room temperature and permit electrophoretic separation of free and complexed forms. The M_r difference (20 to 30 kDa) is sufficient to fully resolve the bands. As with α2M capture, latent forms of MMPs ("switch closed") are not captured, and this method is therefore valuable in distinguishing "switch-open" and "switch-closed" forms before proteolytic excision of the propeptide during activation. The active site, however, does not have to possess catalytic activity, and inactive mutants (if correctly folded) readily form complexes with TIMPs.

Fluorescent labeling of cryptic Cys residue

Removal of Zn^{2+} with EDTA, as expected, also unmasks the cryptic thiol group. Fully converted ("activated") forms of the enzyme which have lost the entire propeptide no longer react. Note, however, that the free thiol group is only a few residues upstream of the ultimate proteolytic processing site. Partially processed forms of the proenzymes therefore may still react with DACM.

Time Considerations

Analysis of the degradation of collagen gels takes ~2 days to prepare the gels and 1 to 4 days for the assay itself. It requires ~2 weeks to prepare rat tail tendon collagen type I and 3 to 4 days to label the collagen with fluorophore.

Direct zymography takes 2 days to complete, while reverse zymography takes 2 days.

α2M and TIMP capture take 1 to 2 days depending on the duration of antibody incubation in immunoblotting.

Fluorescent labeling of the cryptic Cys residue can be completed in a single day.

Literature Cited

Aimes, R.T. and Quigley, J.P. 1995. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4-length and 1/4-length fragments. *J. Biol. Chem.* 270:5872-5876.

Birkedal-Hansen, H. 1987. Catabolism and turnover of collagens: Collagenases. *Methods Enzymol.* 144:140-171.

Birkedal-Hansen, H. and Danø, K. 1981. A sensitive collagenase assay using [^{3}H]collagen labeled by reaction with pyridoxal phosphate and [^{3}H]borohydride. *Anal. Biochem.* 115:18-26.

Birkedal-Hansen, H. and Taylor, R.E. 1982. Detergent-activation of latent collagenase and resolution of its component molecules. *Biochem. Biophys. Res. Commun.* 107:1173-1178.

Birkedal-Hansen, H., Cobb, C.M., Taylor, R.E., and Fullmer, H.M. 1976. Synthesis and release of procollagenase by cultured fibroblasts. *J. Biol. Chem.* 251:3162-3168.

Birkedal-Hansen, H., Birkedal-Hansen, B., Windsor, L.J., Lin, H.Y., Taylor, R.E., and Moore, W.G.I. 1989. Use of inhibitory (anticatalytic) antibodies to study extracellular proteolysis. *Immunol. Invest.* 18:211-224.

Birkedal-Hansen, H., Moore, W.G.I., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J.A. 1993. Matrix metalloproteinases: A review. *Crit. Rev. Oral Biol. Med.* 4:197-250.

Bodden, M.K., Harber, G.J., Birkedal-Hansen, B., Windsor, L.J., Caterina, N.C.M., Engler, J.A., and Birkedal-Hansen, H. 1994. Functional domains of human TIMP-1 (tissue inhibitor of metalloproteinases). *J. Biol. Chem.* 269:18943-18952.

Caterina, N.C.M., Windsor, L.J., Yermovsky, A.E., Bodden, M.K., Taylor, K.B., Birkedal-Hansen, H., and Engler, J.A. 1997. Replacement of conserved cysteines in human tissue inhibitor of metalloproteinases-1. *J. Biol. Chem.* 272:32141-32149.

Caterina, J.J., Yamada, S., Caterina, N.C.M., Longenecker, G., Holmback, K., Shi, J., Yermovsky, A.E., Engler, J.A., and Birkedal-Hansen, H. 2000. Inactivating mutation of the mouse tissue inhibitor of metalloproteinnases-2 (TIMP-2) gene alters proMMP-2 activation. *J. Biol. Chem.* 275:26416-26422.

Chen, J.M. and Chen, W.T. 1987. Fibronectin-degrading proteases from the membranes of transformed cells. *Cell* 48:193-203.

Chen, W.T., Olden, K., Bernard, B.A., and Chu, F.-F. 1984. Expression of transformation-associated protease(s) that degrade fibronectin at cell contact sites. *J. Cell Biol.* 98:1546-1555.

DeClerck, Y.A., Yean, T.D., Lu, H.S., Ting, J., and Langley, K.E. 1991. Inhibition of autoproteolytic activation of interstitial procollagenase by recombinant metalloproteinase inhibitor MI/TIMP-2. *J. Biol. Chem.* 266:3893-3899.

Deutsch, D.G. and Mertz, E.T. 1970. Plasminogen: Purification from human plasma by affinity chromatography. *Science* 170:1095-1096.

Ghersi, G., Goldstein, L.A., Wang, J.-Y., Yeh, Y., Hakkinen, L., Larjava, H., and Chen, W.-T. 2002. Regulation of fibroblast migration on collagenous matrix by novel cell surface protease complex. *J. Biol. Chem.* 277:29231-29241.

Havemose-Poulsen, A.P.H., Stolze, K., and Birkedal-Hansen, H. 1998. Dissolution of type I collagen fibrils by gingival fibroblasts isolated from patients of various periodontitis categories. *J. Periodontal Res.* 33:280-291.

Heussen, C. and Dowdle, E.B. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl

sulfate and copolymerized substrates. *Anal. Biochem.* 102:196-202.

Kleiner, D.E. and Stetler-Stevenson, W.G. 1994. Quantitative zymography: Detection of picogram quantities of gelatinases. *Anal. Biochem.* 218:325-329.

Knäuper, V., Lopez-Otin, C., Smith, B., Knight, G., and Murphy, G. 1996. Biochemical characterization of human collagenase-3. *J. Biol. Chem.* 271:1544-1550.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lin, H.Y., Wells, B.R., Taylor, R.E., and Birkedal-Hansen, H. 1987. Degradation of type I collagen by rat mucosal keratinocytes. *J. Biol. Chem.* 262:6823-6831.

Lyons, J.G., Birkedal-Hansen, B., Moore, W.G.I., O'Grady, R.L., and Birkedal-Hansen, H. 1991. Characteristics of a 95-kDa matrix metalloproteinase produced by mammary carcinoma cells. *Biochemistry* 30:1450-1456.

Ohuchi, E., Imai, K., Fuji, Y., Sato, H., Seiki, M., and Okada, Y. 1997. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.* 272:2446-2451.

Oliver, G.W., Leferson, J.D., Stetler-Stevenson, W.G. and Kleiner, D.E. 1997. Quantitative reverse zymography: Analysis of picogram amounts of metallopooteinase inhibitors using gelatinase A and B reverse zymograms. *Anal. Biochem.* 244:161-166.

Sottrup-Jensen, L. and Birkedal-Hansen, H. 1989. Human fibroblast collagenase-α-macroglobulin interactions. *J. Biol. Chem.* 264:393-401.

Sottrup-Jensen, L., Stepanik, T.M., Wierzbicki, D.M., Jones, C.M., Lonblad, P.B., Kristensen, T., Mortensen, S.B., Petersen, T.E., and Magnusson, S. 1983. The primary structure of α-macroglobulin and localization of a factor XIIIa cross-linking site. *Ann. N.Y. Acad. Sci.* 421:41-60.

Springman, E.B., Angleton, E.L., Birkedal-Hansen, H., and Van Wart, H.E. 1990. Multiple modes of activation of latent human fibroblast collagenase: Evidence for the role of a Cys^{73} active-site zinc complex in latency and a "cysteine switch" mechanism for activation. *Proc. Natl. Acad. Sci. U.S.A.* 87:364-368.

Van Wart, H.E. and Birkedal-Hansen, H. 1990. The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. U.S.A.* 87:5578-5582.

Windsor, L.J., Grenett, H., Birkedal-Hansen, B., Bodden, M.K., Engler, J.A., and Birkedal-Hansen, H. 1993. Cell-type-specific regulation of SL-1 and SL-2 genes. Induction of SL-2, but not SL-1, in human keratinocytes in response to cytokines and phorbolesters. *J. Biol. Chem.* 268:17341-17347.

Windsor, L.J., Bodden, M.K., Birkedal-Hansen, B., Engler, J.A., and Birkedal-Hansen, H. 1994. Mutational analysis of residues in and around the active site of human fibroblast-type collagenase. *J. Biol. Chem.* 269:26201-26207.

Yamamoto, K., Sekine, T., and Kanaoka, Y. 1977. Fluorescent thiol reagents. XII. Fluorescent tracer method for protein SH groups using *N*-(7-dimethylamino-4-methyl coumarinyl) maleimide. *Anal. Biochem.* 79:83-94.

Preparation of Extracellular Matrices Produced by Cultured and Primary Fibroblasts

Culturing fibroblasts on traditional two-dimensional (2-D) substrates induces an artificial polarity between lower and upper surfaces of these normally nonpolar cells. Not surprisingly, fibroblast morphology and migration differ once suspended in three-dimensional (3-D) collagen gels (Friedl and Brocker, 2000). However, the molecular composition of collagen gels does not mimic the natural fibroblast microenvironment. Fibroblasts secrete and organize the extracellular matrix (ECM), which provides structural support for their adhesion, migration, and tissue organization, in addition to regulating cellular functions such as growth and survival (Buck and Horwitz, 1987; Hay, 1991; Hynes, 1999; Geiger et al., 2001). Cell-to-matrix interactions are vital for vertebrate development. Disorders in these processes have been associated with fibrosis, developmental malformations, cancer, and other diseases.

This unit describes methods for generating tissue culture surfaces coated with a fibroblast-derived 3-D ECM produced and deposited by both established and primary fibroblasts. The matrices closely resemble in vivo mesenchymal matrices and are composed mainly of fibronectin fibrillar lattices. Utilizing in vivo–like 3-D matrices as substrates allows the acquisition of information that is physiologically relevant to cell-matrix interactions, structure, function, and signaling, which differ from data obtained by culturing cells on conventional 2-D substrates in vitro (Cukierman et al., 2001).

These protocols were initially derived from methods described in *UNIT 10.4*, which were modified to obtain fibroblast-derived 3-D matrices and to characterize cellular responses to them. The basic approach is to allow fibroblasts to produce their own 3-D matrix (see Basic Protocol). For this purpose, fibroblasts are plated and maintained in culture in a confluent state. After 5 to 9 days, matrices are denuded of cells, and cellular remnants are removed. Such extraction results in an intact fibroblast-derived 3-D matrix that is free of cellular debris and remains attached to the culture surface (see Figure 10.9.1). The fibroblast-derived 3-D matrices are then washed with PBS and can be stored 2 to 3 weeks at 4°C or up to 3 weeks frozen at −80°C. Moreover, to analyze the effect of matrix pliability on cellular behavior, prepared 3-D matrices can be rigidified by chemical cross-linking (see Support Protocol 1 and *UNIT 17.10*).

Additionally, to evaluate the quality of the fibroblast-derived 3-D matrices, support protocols present a variety of procedures for measuring cell responsiveness to the 3-D matrix microenvironment (see Support Protocols 2 and 3). The rapid cell attachment of fibroblasts plated within the matrix can be quantified. By plating isolated fibroblasts in the 3-D matrix, the acquisition of an in vivo–like spindle-shaped morphology can also be measured. To ascertain whether fibroblasts respond to the 3-D microenvironment when plated within specific (NIH-3T3) matrices, the phosphorylation level of nonreceptor focal adhesion kinase (FAK) pY^{397} can be quantified by immunoblotting (see Support Protocol 4).

This unit will also describe how to mechanically compress the fibroblast-derived 3-D matrices to obtain 2-D substrate controls (see Support Protocol 5). Moreover, a support protocol will illustrate how to solubilize the fibroblast-derived 3-D matrices to produce a matrix-derived protein mixture for additional 2-D coating controls and for subsequent

Contributed by Dorothy A. Beacham, Michael D. Amatangelo, and Edna Cukierman
Current Protocols in Cell Biology (2006) 10.9.1-10.9.21

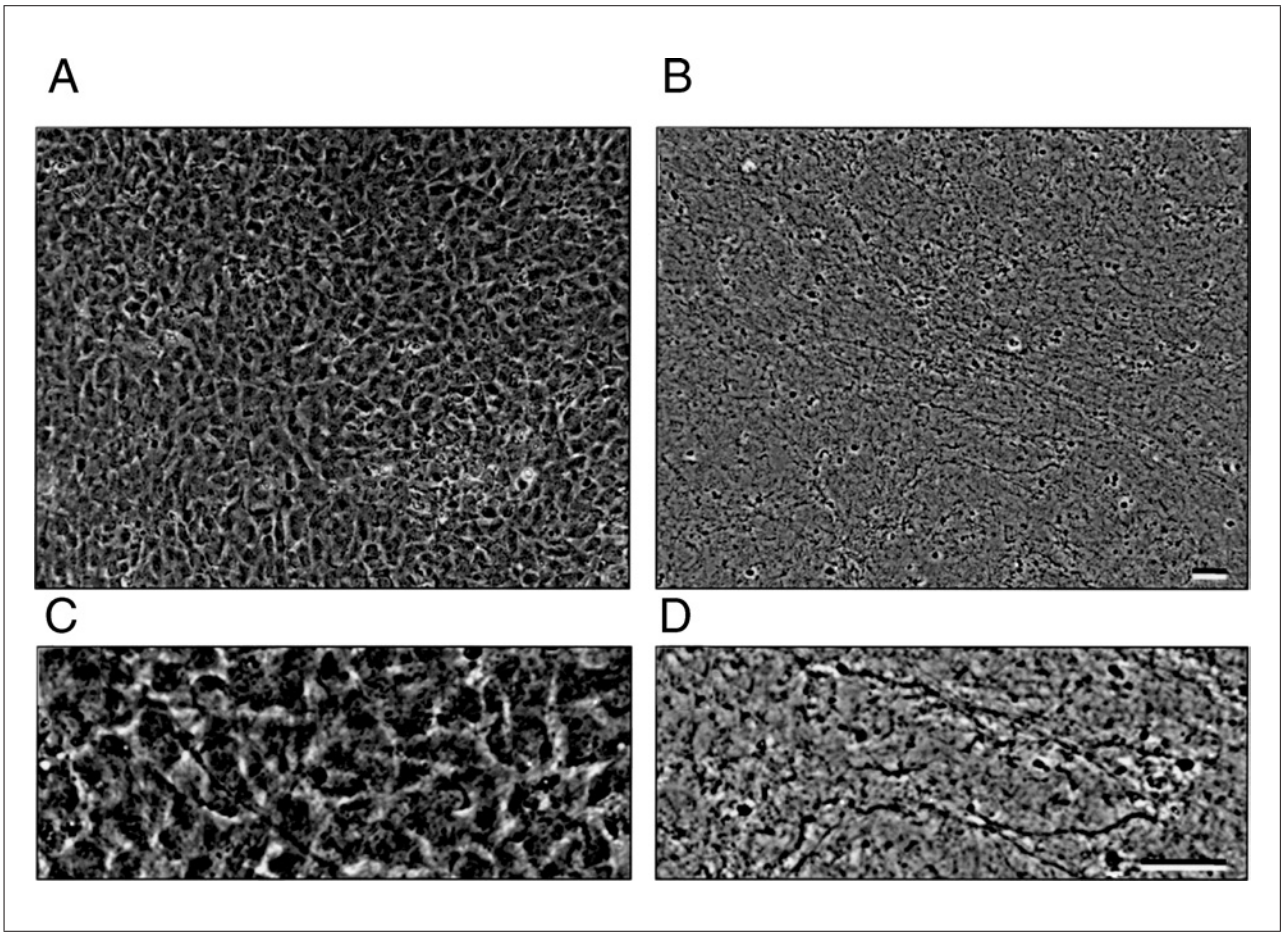


Figure 10.9.1 Fibroblast-derived 3-D matrices before and after extraction process. (**A**) Culture at day 5 prior to matrix extraction. (**B**) The resulting fibroblast-derived 3-D matrix. Panels **C** and **D** are magnified insets from **A** and **B**, respectively. Bars represent 50 μm.

biochemical analysis of the matrices (see Support Protocol 6 and Commentary). Lastly, there is a protocol for isolating primary fibroblasts from fresh tissue samples to produce additional types of fibroblast-derived 3-D matrices (see Support Protocol 7).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All cell-culture incubations should be performed in a 37°C, 10% CO_2 humidified incubator.

BASIC PROTOCOL

PREPARATION OF EXTRACELLULAR MATRICES PRODUCED BY CULTURED OR PRIMARY FIBROBLASTS

NIH-3T3 cells (for primary cell lines see Support Protocol 7) must be routinely cultured in high-glucose Dulbecco's modified Eagle medium supplemented with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin unless otherwise specified. Never allow cultured NIH-3T3 cells to become completely confluent while maintaining stock cultures. When cells reach 80% confluence (about once per week), subculture at a 1:20 dilution. However, prior to plating for matrix deposition, NIH-3T3 cells should be adapted to grow in 10% fetal bovine serum rather than calf serum for the cells to adopt an optimal phenotype for matrix production (see Critical Parameters).

Depending on the laboratory equipment available, and the anticipated uses of the fibroblast-derived 3-D matrices, a suitable surface on which the matrices will be produced (e.g., glass-bottom dishes, coverslips, or tissue culture dishes) must be selected as follows:

(1) Disposable glass bottom dishes (MatTek) can be utilized for real-time fluorescent experiments or for quality assessment assays (e.g., cell attachment and cell shape) using an inverted fluorescent microscope (see Support Protocols 1 and 2).

(2) Coverslips can be used for immunofluorescence experiments in which samples are fixed and mounted on microscope slides (see Support Protocol 3), or for mechanical compression of the fibroblast-derived 3-D matrices to be used as control 2-D surfaces (see Support Protocol 5).

(3) Regular tissue culture dishes (e.g., 35-mm diameter) can be used for in vivo observations using an inverted microscope, for matrix solubilization and further characterization, and/or for biochemical analyses (see Support Protocols 5 and 3, respectively). Tissue culture dishes are also used for real-time cell motility analyses (Cukierman, 2005).

Materials

NIH-3T3 cells (ATCC) or primary fibroblasts (see Support Protocol 7)
Confluent medium with fetal bovine serum (FBS; see recipe)
0.25% (w/v) trypsin/0.03% (w/v) EDTA solution (see recipe)
0.2% (w/v) gelatin solution (see recipe)
Ethanol (absolute)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1% (v/v) glutaraldehyde in PBS (see recipe)
1 M ethanolamine (see recipe)
Matrix medium with ascorbic acid (see recipe)
Extraction buffer (see recipe), 37°C
10U/ml DNase I (Roche) in PBS^+ (see recipe for PBS^+), optional
Penicillin/streptomycin (Invitrogen)
Fungizone (amphotericin B; Invitrogen)

37°C, 10% CO_2 humidified incubator
15-cm dishes plus the specific culture vessels for matrix production
Inverted phase-contrast microscope
6-well tissue culture plates or 35-mm dishes (optional)
22-mm circular high-quality coverslips (Carolina; optional)
Bacterial 6-multiwell petri plates for preparing matrices on coverslips
Parafilm strips
Small, sterile fine-pointed tweezers (e.g., Dumont no. 4), optional

Prepare cell cultures

1. Start with a semi-confluent (80% confluent) culture of NIH-3T3 cells cultured in 10 to 12 ml confluent medium containing fetal bovine serum (see Critical Parameters) or primary fibroblastic cells on a 15-cm culture plate (see Support Protocol 7); aspirate and discard the culture medium.

2. Rinse the cell layer briefly with 1.5 ml of 0.25% trypsin/0.03% EDTA (trypsin/EDTA) per 15-cm dish. Then gently aspirate off the solution.

   This rinse will remove traces of serum that contains trypsin inhibitors.

3. Add enough trypsin/EDTA solution to cover the cell layer, quickly aspirate excess liquid, and observe under an inverted microscope at room temperature until the cells have detached from the culture dish (1 to 3 min).

4. Collect the cells in 10 ml of confluent medium.
5. Add 2 ml of the suspended cells and 10 to 12 ml of confluent medium to a 15-cm plate and culture for 2 to 3 days (until semi-confluent, up to ~80% confluence).

   As many as five 15-cm culture dishes may be used.

Prepare surfaces for matrix deposition

Although not strictly required, both gelatin coating (steps 6 and 7) and cross-linking of gelatin (steps 8 through 11) with glutaraldehyde stabilizes the attachment of the matrices to the culture dish surface and greatly improves final yield. However, the resulting matrices may be thinner than those obtained without gelatin pre-coating and cross-linking. Therefore, matrix thickness should be determined for each fibroblastic cell type and a decision to follow the optional steps should be made for each cell type.

6a. *For tissue culture dishes:* Add 2 ml of 0.2% gelatin solution to a 35-mm tissue culture dish surface to be used for fibroblast-derived 3-D matrix deposition and incubate for 1 hr at 37°C.

   Choose 35-, 60-, or 100-mm dishes to be used in this protocol. For 60- or 100-mm dishes, scale up the volumes of all reagents added from 2 ml to 4 and 8 ml, respectively.

6b. *For coverslips:* Presterilize by flaming the coverslips after dipping in anhydrous ethanol (absolute). Then place coverslip in a tissue culture dish and rinse with PBS. Incubate coverslips in a 0.2% gelatin solution.

7. Aspirate gelatin and add 2 ml PBS.

   When using coverslips, after this rinse, transfer coverslips to individual wells of multiwell bacterial plates.

   When preparing coverslips for 3-D matrix deposition, multiwell bacterial petri dishes are preferred over tissue culture plastic dishes because the fibroblasts do not adhere well to bacterial petri plastic. Consequently, there is preferential fibroblastic growth on pretreated glass coverslips instead of on the surface of the petri plastic, conditions conducive to enhancing matrix production on the coverslip. Placing coverslips on bacterial petri plastic also facilitates lifting the coverslip off the dish surface with tweezers during matrix extraction (see step 19) because any cells growing underneath the glass coverslips are easily dislodged.

8. Aspirate PBS and add 2 ml of 1% glutaraldehyde (prediluted in PBS) to each dish or well and incubate 30 min at room temperature.
9. Wash coverslips or culture dishes three times for 5 min each with 2 ml of PBS.
10. Add 2 ml of 1 M ethanolamine to each dish and incubate 30 min at room temperature.
11. Repeat the PBS washes (step 9).

    This step is a good place to stop if time does not permit cell seeding. Dishes can be left for 1 to 7 days at room temperature under sterile conditions.

12. Aspirate PBS from dishes and replace with 2 ml matrix medium. If the medium appears purple, repeat steps 11 and 12 to remove any trace amounts of ethanolamine.

    At this point, the surfaces are ready to be seeded with matrix-producing fibroblasts.

Allow cells to deposit matrix

13. Repeat steps 1 to 3 to harvest cells from a semi-confluent dish.

    This protocol was developed for NIH-3T3 cells. Nevertheless, other fibroblast cell lines can be used. For example, the same protocol can be followed from this point on using human or other primary fibroblastic cells (see Suppport Protocol 7).

14. Collect cells from each dish in 10 ml of matrix medium, count cells (*UNIT 1.1*), and dilute to a final concentration of 2.5×10^5 cells/ml.

15. Aspirate medium (from step 12) and seed 5×10^5 cells in 2 ml of matrix medium per 35-mm dish and culture for 24 hr.

    Use as many dishes as needed; there should be enough cells for ~100 35-mm plates from each 15-cm semiconfluent dish. Remember to scale up volumes from 2 ml to 4 or 8 ml for 60- or 100-mm plates, respectively.

16. After 24 hr, carefully aspirate the medium from cells and replace with fresh matrix medium containing 50 μg/ml of ascorbic acid.

    For some primary cell lines, adding a ten-fold higher concentration of ascorbic acid on this first addition of ascorbic acid after cell plating can increase matrix thickness. Because the higher ascorbic acid concentration is detrimental for NIH-3T3 cells, whether or not to use this higher dose should be independently determined for each fibroblastic cell type.

17. Ascorbic acid degrades over time in culture, so change medium with freshly made matrix medium every 48 hr for a total of 5 to 9 days after step 16.

    At this time, the matrix should be sufficiently thick to achieve three-dimensionality (≥10 μm, see below). At this point it is ready to be extracted (see Fig. 10.9.1 A).

    Matrices should be extracted after they reach a thickness of at least 10 μm. The time required for each fibroblast cell type to produce a matrix of this thickness may vary. Therefore, this time period must be determined empirically for each cell type.

Remove cells from matrix

18. Carefully aspirate the medium and rinse gently with 2 ml PBS by touching the pipet against the dish wall rather than at the bottom of the dish where the cells are located.

19. Gently add 1 ml of prewarmed (37°C) extraction buffer.

    If coverslips are being used, gently lift the coverslips with the fine-pointed tweezers (or a syringe needle) so that extraction buffer reaches under the coverslip. This step will ensure that the matrix deposited on the coverslip will be separated successfully from the matrix deposited on the bottom of the culture dish. This will facilitate subsequent handling of the coverslips without tearing the delicate matrix.

20. Observe the process of cell lysis using an inverted microscope. Incubate at 37°C until no intact cells are visualized (~3 to 5 min; see Fig. 10.9.1B).

Remove cellular debris

21. Slowly add 2 to3 ml PBS to dilute the cellular debris. Gently pipet the PBS on the side of the dish to avoid disturbing the newly formed matrix. Store dishes overnight at 4°C to avoid disturbing the matrix.

    The above dilution process should be carried out gently to prevent turbulence that may cause the freshly denuded matrix-layer to detach from the surface.

22. As cautiously as possible (using a pipet), aspirate the diluted cellular debris, but without completely aspirating the liquid layer so that the matrix surface remains hydrated at all times.

    Do not attempt to aspirate the whole volume. This will prevent removing the matrix layer.

23. Gently add another 2 ml of PBS and gently aspirate the PBS as described in steps 21 and 22.

24. (*Optional*) If necessary, minimize DNA by treating the matrix with DNase I. Add 2 ml DNase I and incubate 30 min at 37°C.

25. At the end of the incubation, aspirate the enzyme and wash two times with 2 ml PBS.

26. Cover the matrix-coated plates (or coverslips) with at least 3 ml PBS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone. Seal with Parafilm and store for up to 2 or 3 weeks at 4°C.

    For signal transduction assays, store the matrix-coated plates in serum-free medium (see Commentary).

    Matrices can also be stored for at least 3 weeks at −80°C (longer times have not yet been tested) without compromising matrix integrity, when compared with matrices from the same batch stored at 4°C. To store matrices at −80°C, rinse matrix dishes two times with sterile, nanopure H_2O and carefully aspirate all the liquid. Then label dishes to indicate the date of freezing for future reference, seal with Parafilm, and place at −80°C. When needed, thaw matrix dishes at room temperature and rehydrate with PBS prior to use for cell attachment or replating (see Support Protocols 1 and 5).

27. Confirm the integrity of the matrices directly before use. Examine for matrix integrity using an inverted phase-contrast microscope.

    The matrices should be attached to the culture surface and appear similar to the example in Figure 10.9.1B.

SUPPORT PROTOCOL 1

FIXATION OF EXTRACTED MATRICES FOR LACK OF PLIABILITY ANALYSES

For certain experiments designed to analyze the effect of rigidity or pliability of the matrix on cell behavior, it is necessary to chemically rigidify the prepared matrices. To this end, matrices are fixed with 1% glutaraldehyde prior to cell plating and analysis.

Materials

1% (v/v) glutaraldehyde in PBS (see recipe)
Tissue culture dishes or coverslips with matrix
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1 M ethanolamine (see recipe)
Penicillin/streptomycin
Fungizone
Parafilm

1. Aspirate PBS, add 2 ml of 1% glutaraldehyde (prediluted in PBS) to each tissue culture dish or well, and incubate 30 min at room temperature.
2. Wash coverslips or culture dishes three times, for 5 min each, with 2 ml PBS at room temperature.
3. Add 2 ml of 1 M ethanolamine to each dish and incubate 30 min at room temperature.
4. Repeat the PBS washes (step 2).
5. Cover the matrix-coated plates (or coverslips) with at least 3 ml PBS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone. Seal with Parafilm. Store up to 2 or 3 weeks at 4°C or at −80°C as described for NIH-3T3 matrices (see Basic Protocol, step 26).

ASSESSING THE QUALITY OF FIBROBLAST-DERIVED THREE-DIMENSIONAL MATRICES

The quality of fibroblast-derived 3-D matrices can be tested by one of three assays presented here as support protocols. The first two assays, induction of rapid cell attachment (see Support Protocol 2) and rapid acquisition of spindle-shape morphology (see Support Protocol 3), are based on examination of fluorescently labeled cells plated on 3-D matrices. The prelabeling with a fluorescent dye is required to enhance the observation of cells

within fibroblast-derived 3-D matrices. NIH-3T3 matrix quality can also be assessed in a third assay to check for down regulation of activated FAKpY^{397} when normal fibroblasts are plated within prepared NIH-3T3 matrices (see Support Protocol 4). These are referred to as "re-plated" fibroblasts. To analyze the level of activated [FAKpY^{397}/total FAK] of normal fibroblasts re-plated within these matrices, the ratio of FAKpY^{397}/total FAK is compared to the level of FAKpY^{397}/total FAK of the same cells plated on a traditional fibronectin-coated tissue culture dish, (2-D surface; see Support Protocol 2, cell attachment assay). Typically, a 1.5- to 4-fold reduction in FAK pY^{397}/total FAK in normal fibroblasts replated into NIH-3T3 matrices is observed (Cukierman 2001; see Support Protocol 4).

Cell Attachment Assay

SUPPORT PROTOCOL 2

Human or mouse fibroblasts can be used to evaluate the cell adhesion–promoting activity of the fibroblast-derived 3-D matrices. It has been reported that these in vivo–like 3-D matrices (NIH-3T3) are about six-fold more effective than 2-D substrates in mediating cell adhesion as quantified by a 10-min cell attachment assay (Cukierman et al., 2001). Briefly, cell nuclei are prelabeled to avoid any background staining from DNA debris on the 3-D matrix. The live prelabeled cells are rinsed free of excess dye, trypsinized, and plated on the fibroblast-derived 3-D matrix to be assessed or onto control fibronectin-coated surfaces. After 10 min, nonattached cells are washed away, and attached cells are quantified by counting nuclei.

Materials

Semi-confluent fibroblasts (human or mouse) in a 15-cm dish
Confluent medium with fetal bovine serum (see recipe)
Hoechst 33342 stock solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), 4°C and room temperature
Trypsin/EDTA solution (see recipe)
Glass-bottom no. 1.5 dishes (MatTek Corporation): three containing fibroblast-derived 3-D matrix (see Basic Protocol) and three with pre-coated 2-D fibronectin (see recipe)
Fixing solution (see recipe)
15-ml polypropylene conical tubes
Tissue culture centrifuge equipped with rotor suitable for conical 15-ml tubes
Fluorescence inverted microscope equipped with an appropriate camera and set of filters to visualize Hoechst 33342 (see *APPENDIX 1E*)
Image analysis software capable of counting objects (optional)

Label cells

1. Start with a semi-confluent 15-cm culture dish containing fibroblasts (mouse or human); aspirate and discard the culture medium.
2. Add 20 ml of confluent medium containing 40 µl of Hoechst 33342 stock solution (1:500) to the cells. Incubate 15 min at 37°C.

Harvest cells

3. Rinse four times with 10 ml PBS at room temperature, 1 min each rinse.
4. Add enough trypsin/EDTA solution to cover the cell layer, aspirate excess liquid, and observe under an inverted microscope until cells are detached from the culture dish (1 to 3 min).

Prepare cell suspension

5. Collect the cells in 10 ml of confluent medium into a 15-ml polypropylene conical tube and take a sample for counting (*UNIT 1.1*).

6. Pellet the cells by centrifuging 5 min at 100 × *g*, room temperature.
7. Discard the supernatant and gently resuspend the cells with confluent medium to a final concentration of 3.5×10^5 cells/ml.
8. Rotate cells in suspension for 20 min at 37°C.

Allow cells to attach

9. Carefully place a 150-µl drop of cell suspension onto the glass-bottom part of the dishes coated with 3-D matrix or 2-D matrix controls. Incubate 10 min at 37°C.
10. Remove from the incubator and tilt the dishes slightly to dislodge the medium droplet containing unattached cells from the glass portion onto the plastic portion of the dish and then aspirate.
11. Rinse the dishes by slowly adding (to the plastic portion of the dishes) 3 ml of 4°C PBS.

Fix cells

12. Aspirate PBS carefully and add 2 ml of fixing solution. Incubate 20 min at room temperature.
13. Aspirate and add 2 ml PBS at room temperature.

Visualize and analyze attached cells

14. Using an inverted fluorescence microscope with appropriate excitation wavelength and excitation and emission filters (*APPENDIX 1E*), acquire five random images of the nuclei from each one of the six dishes utilizing a 10× or 20× objective and count the nuclei.

    Counting of the nuclei can be done automatically utilizing commercially available image analysis software capable of counting objects (e.g., MetaMorph from Universal Imaging Corporation). If the counting is done automatically, then images should be acquired with a 10× objective. However, if the nuclei are to be counted manually, then a 20× objective is recommended.

    The mean number of cells attached to the fibroblast-derived 3-D matrix should be up to six-fold higher than the number attached to the 2-D matrix control. This result will confirm the quality of the NIH-3T3-derived 3-D matrix (Cukierman et al., 2001).

SUPPORT PROTOCOL 3

Determination of Cell Shape

Human or mouse fibroblasts can be used to evaluate induction of spindle-shaped cell morphology promoted by a good-quality in vivo–like 3-D matrix. A recent report has established that fibroblasts will acquire an in vivo–like spindle-shaped morphology in cell-derived 3-D matrices 5 hr after plating (Cukierman et al., 2001). The protocol consists of prelabeling live fibroblast membranes with a fluorescent dye and incubating the cells on fibroblast-derived 3-D matrices or controls for a period of 5 hr. After this period of time, the fibroblast-derived 3-D matrix promotes a spindle-shaped morphology resembling in vivo fibroblast morphology, thereby confirming the quality of the 3-D matrices.

Materials

Fibroblast-derived 3-D matrix-covered coverslips (see Basic Protocol)
Fibronectin 2-D matrix-coated coverslips (see recipe)
2% (w/v) heat-denatured BSA (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Semi-confluent 15-cm dish of fibroblasts
Trypsin/EDTA solution (see recipe)
Confluent medium with fetal bovine serum (see recipe)

1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) stock solution (see recipe)
Fixing solution (see recipe)
Prolong Gold mounting medium (Invitrogen)

35-mm tissue culture dishes or 6-well plates
Inverted microscope
15-ml polypropylene conical tubes
Tissue culture centrifuge equipped with rotor suitable for 15-ml conical tubes
Fine-point forceps (e.g., Dumont 4)
Glass microscope slides
Fluorescent microscope equipped with digital camera
Image analysis software capable of measuring elliptical Fourier parameters

Block nonspecific cell binding with BSA

1. Cautiously place fibroblast-derived 3-D matrix and control-coated coverslips (matrix face up) into 35-mm tissue culture dishes (or 6-well plates).
2. Block nonspecific cell binding by adding 2 ml of 2% heat-denatured BSA and incubate for 1 hr at 37°C.
3. Rinse all blocked coverslips with 2 ml PBS.

   At this point, coverslips are ready to be seeded with the prelabeled cells.

Label cell membrane with DiI

4. Start with a semi-confluent 15-cm dish of fibroblasts; aspirate and discard the culture medium.
5. Rinse the cell layer briefly with 1.5 ml trypsin/EDTA.

   This rinse will remove traces of serum that contain trypsin inhibitors.

6. Add enough trypsin/EDTA solution to cover the cell layer, aspirate excess liquid, and observe under an inverted microscope until cells are detached from the culture dish (1 to 3 min).
7. Collect the cells in 10 ml of confluent medium containing 4 μg/ml DiI into a 15-ml polypropylene conical tube.
8. Incubate the cells with the dye in suspension by rotating gently for 30 min at 37°C.
9. Pellet the cells by centrifugation 5 min at 100 × *g*, room temperature.
10. Discard the supernatant by aspiration, and gently resuspend the cells in confluent medium to a final volume of 10 ml.
11. Repeat steps 9 and 10 four additional times to remove any remaining free dye.
12. Count cells (*UNIT 1.1*) and dilute (with confluent medium) to a final concentration of 1×10^4 cells/ml.

Plate labeled cells

13. Carefully aspirate PBS from the coverslips in step 3.
14. Add 2 ml of the diluted cell suspension to the dishes containing coverslips and incubate 5 hr at 37°C.

    For fast qualitative analysis, cells can be observed and photographed at the end of 5 hr with an inverted fluorescence microscope (see APPENDIX 1E for wavelength information).

15. Aspirate medium and rinse with 2 ml PBS.

Fix cells

16. Aspirate PBS and fix with 1 ml of fixing solution for 20 min at room temperature.
17. Aspirate fixing solution and rinse with 2 ml PBS.
18. Rinse with 2 ml water to eliminate residual salt.
19. Carefully lift coverslip with fine-point forceps and gently discard excess liquid by touching the edge of the coverslip onto a paper towel.
20. Mount coverslips (cells face down) on a droplet (~20 µl) of Prolong Gold mounting medium placed on a glass microscope slide.
21. Allow mounted samples to dry in the dark for ~1 hr at room temperature.

    At this point, samples are ready for morphometry analysis, or they can be stored overnight in the dark at room temperature before transferring to ≤4°C.

Perform morphometric analysis

22. Acquire fluorescent digital images, slightly over-exposing to visualize the contour of the cells (for wavelength, see *APPENDIX 1E*).

    Use a magnification that will allow visualization of an entire cell in each image. Randomly capture images of at least 12 cells per sample and a minimum of 36 cells per substrate.

23. Perform the measurements for both the length (span of the longest cord) and the breadth (caliper width) of each cell.
24. Calculate the inverse axial ratio by dividing length by breadth.

    The inverse axial ratio corresponds to the elliptical form factor (EFF) morphometric parameter found in the integrated morphometry analysis (IMA) function of MetaMorph software (Universal Imaging Corporation).

    The mean inverse axial ratio induced by a high-quality NIH-3T3-derived 3-D matrix should be about three-fold greater than that induced by the 2-D fibronectin control (Cukierman et al., 2001).

SUPPORT PROTOCOL 4

Lysis of Re-Plated Fibroblasts for Western Blot Analyses

To assure the quality of a batch of NIH-3T3-derived 3-D matrices, the levels of FAK activity (FAKpY^{397}) must be down-regulated at least 1.5 fold (Cukierman et al., 2001) when compared to classic 2-D cultures. This protocol describes how to lyse normal human or murine primary fibroblasts after re-plating within 3-D matrices for biochemical analysis by immunoblotting (see *UNIT 6.2*). In brief, re-plated normal fibroblasts are lysed and subjected to immunoblot analysis. Cell lysate extracts can also be stored for later analyses (see step 11).

Materials

Matrix-coated ≥35-mm dishes (see Support Protocol 2)
Fibronectin-coated ≥35-mm dishes
Cell suspension from confluent cultures of fibroblasts
Confluent medium with fetal bovine serum (see recipe)
Lysis buffer (modified RIPA) reagent (see recipe) supplemented with protease and phosphatase inhibitors (see recipe), ice cold
Normal human or murine fibroblasts re-plated in 3-D matrix dishes (see Basic Protocol, step 18)
Phosphate-buffered saline without Ca^{2+} or Mg^{2+} (CMF-PBS; *APPENDIX 2A*), ice cold
Dry ice/isopropanol bath

5× sample buffer supplemented with β-mercaptoethanol
Anti-FAKpY^{397} and anti-total FAK (see recipes)
Glutaraldehyde-3-phosphate dehydrogenase (GAPDH)

37°C, 10% CO_2 humidified incubator
Cell scraper (Costar, Fisher Scientific)
1.5-ml microcentrifuge tubes (Eppendorf)
Sonicator (e.g., Branson Sonifier 150)
Scion image software beta version 4.03

Additional reagents and equipment for calculating the amount of 5× sample buffer supplemented with β-mercaptoethanol (*UNIT 6.1*) and detecting proteins by immunoblotting (*UNIT 6.2*)

Re-plate fibroblasts within 3-D matrices

1. Block nonspecific binding in 3-D matrices with BSA in a 35-mm dish (or 6-well multiwell dish (see Support Protocol 2, steps 1 and 2).

   When using 2-D substrates, precoat 35-mm dishes with 1 ml of 5 μg/ml fibronectin or other matrix protein (see recipe for pre-coated 2-D fibronectin dishes) for 1 hr at 37°C. Alternatively, use uncoated dishes in PBS for 2-D control substrates.

2. Plate 2 ml of cell suspension at a final concentration of 1×10^5 cells/ml in confluent medium for each dish and incubate 20 hr in a 37°C, 10% CO_2 humidified incubator (see Basic Protocol, steps 1 to 4).

Lyse cells within matrices

3. Supplement 10 ml of ice-cold lysis buffer with protease and phosphatase inhibitors.

4. Carefully aspirate confluent medium from fibroblasts re-plated in 3-D matrix dishes (see Basic Protocol, step 18).

5. Gently add ice-cold PBS and repeat steps 4 and 5 for a total of two PBS washes.

6. Carefully aspirate again and tip the dishes for 1 min to accumulate the excess PBS on one side of the dish (~30° to bench top).

   It is important to remove all traces of PBS to prevent diluting lysates with PBS for the purpose of maintaining a uniform volume of lysate for different samples. This will yield a relatively consistent protein loaded onto SDS-PAGE gels for immunoblotting (see UNIT 6.2).

7. Carefully aspirate the excess PBS to avoid detaching the matrix layer.

8. Place the dishes (usually a 35-mm dish) on ice and add 200 to 300 μl of ice-cold lysis buffer.

   For larger dishes, add a proportionally larger volume of lysis buffer.

9. Incubate on ice for 5 min with gentle rocking.

Collect the lysate

10. Scrape the cells and matrix from the dish with the cell scraper. Then, tilt the dish toward one side and collect the lysate mixture into a 1.5-ml microcentrifuge tube.

11. Sonicate each tube of cell lysate using the remote setting of 3 (medium power) for 30 sec for each tube.

12. Centrifuge the lysates 15 min at 16,100 × *g*, 4°C.

13. Carefully remove the supernatant and transfer to a fresh, labeled tube. If not used immediately, quick freeze the lysates in a dry ice/isopropanol bath and store for 1 to 2 weeks at −80°C.

To quick-freeze cell lysates, prepare a dry ice/isopropanol bath by adding ~100 ml of isopropanol to a 400-ml beaker and placing in an ice bucket containing dry ice pellets in the fume hood. Allow the isopropanol to cool for 30 min. Add 1.5-ml microcentrifuge tubes containing freshly prepared lysates to a tube-rack and lower the rack into the isopropanol so that the lysate volume is completely immersed. The lysates should be frozen almost immediately (smaller aliquots are better). Finally, quickly transfer the tubes to a −80°C freezer. Samples are stable for 1 to 2 weeks. Each individual protein should be tested since there is some variability.

Analyze lysates

14. To analyze the matrix by immunoblotting, calculate the amount of 5× sample buffer supplemented with β-mercaptoethanol (*UNIT 6.1*) that is needed to make a 1× final concentration after addition to the sample; and add that amount to appropriate lysate samples.

15. Analyze signaling proteins by immunoblotting and immunodetection (*UNIT 6.2*) using antibodies to FAKpY^{397} and total FAK.

For analysis of phosphoproteins, incubate primary antibodies with TBST with a final concentration of 5% (w/v) BSA.

For total FAK and other non-phosphorylated protein epitopes, primary antibodies, all secondary antibodies, and for blocking buffer, use 5% (w/v) nonfat dried milk in TBST as the diluent.

16. Scan individual protein bands corresponding to FAKpY^{397} or total FAK and quantify their optical densities using the public Scion image software beta version 4.03 by means of the Gelplot2 macro. To adjust for sample loading, quantify glutaraldehyde-3-phosphate dehydrogenase (GAPDH, 40 kDa) as a total cellular protein control.

The software can be downloaded from the Scion image software Web site at: http://www.scioncorp.com/frames/fr_download_now.htm.

The average protein yield of the matrix and fibroblasitic proteins (lysate) is ~0.5 to 2 mg per 35-mm dish.

PREPARATION OF TWO-DIMENSIONAL CONTROLS

Any given cell response induced by in vivo–like fibroblast-derived 3-D matrices could be due to the three-dimensionality of the matrix, its molecular composition, or a combination of both. The following two support protocols provide methods for obtaining suitable 2-D control matrices with the same molecular composition as the 3-D matrices.

SUPPORT PROTOCOL 5

Mechanical Compression of the Fibroblast-Derived 3-D Matrix

This protocol describes how to apply pressure to the fibroblast-derived 3-D matrix to collapse the matrix to a flat substrate. Mechanical compression of the 3-D matrix ensures that all natural components of the 3-D matrix are present, lacking only the element of three-dimensionality. Briefly, the 3-D sample is compressed using a known weight applied to a given area. The surface that comes into contact with the matrix is covered with a Teflon film to prevent sticking and to avoid tearing the flattened matrix as the weight is retracted.

NOTE: Any other materials fulfilling the same purpose can be substituted.

Materials

Superglue
Absolute ethanol, optional
Fibroblast-derived 3-D matrix on 22-mm circular coverslips
Phosphate-buffered saline (PBS; see *APPENDIX 2A*)
Flat platform large enough to rest on the ring (see Fig. 10.9.2)
Suitable spacer smaller in width than the diameter of the ring but longer in height than the depth of the ring (see Fig. 10.9.2)
12-mm round coverslips (Carolina)
Teflon film: protective overlay composed of: 0.001-in. FEP film, on 0.008-in. vinyl film, with adhesive back (use to cover laboratory bench-tops, Cole-Parmer Instrument Company)
Cork borer (12-mm diameter)
Biological hood equipped with UV light
Stand equipped with a horizontal ring
Lifting laboratory jack
Parafilm
Weight (~158 g)
35-mm dishes
Inverted phase-contrast microscope

Construct weight holder for matrix compression

1. Glue the flat platform to the spacer in such a way that the spacer will protrude slightly beyond the bottom of the ring when the platform is placed on the ring (Fig. 10.9.2).

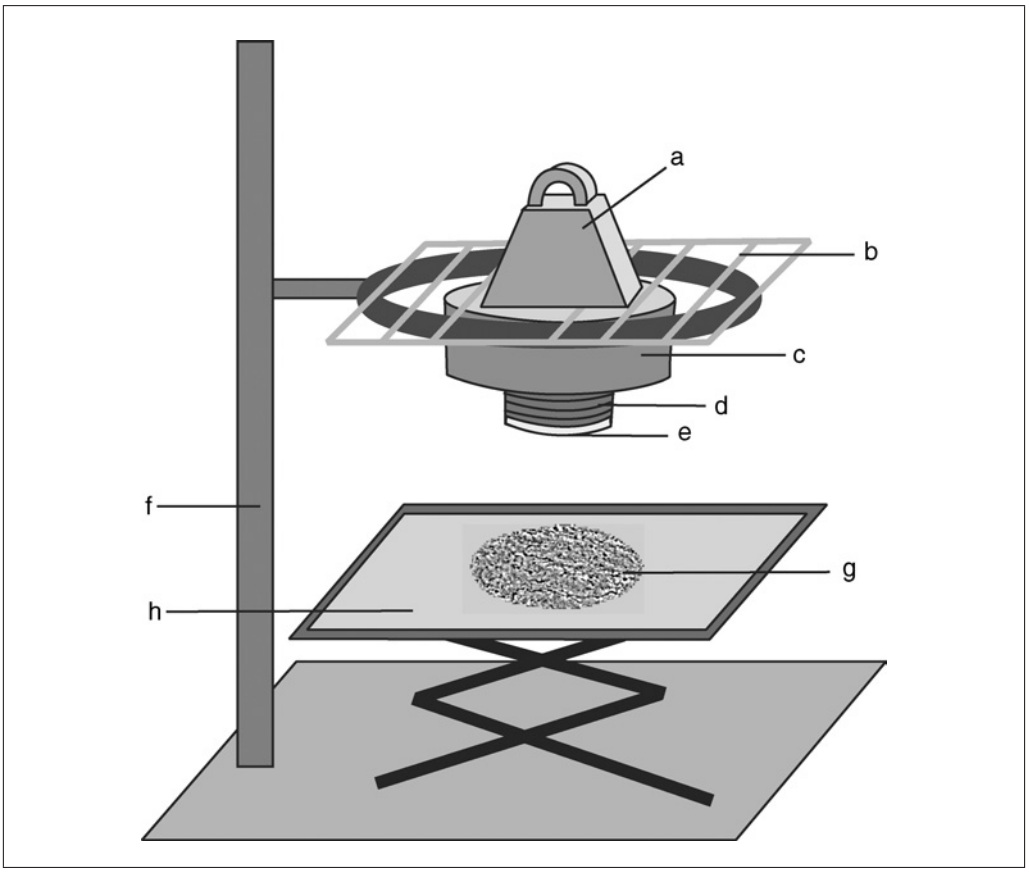


Figure 10.9.2 Diagram showing the components of the mechanical compression device. (a) Weight. (b) Flat platform. (c) Spacer. (d) 12-mm coverslips. (e) Teflon film. (f) Ring stand. (g) Fibroblast-derived 3-D matrix to be mechanically compressed. (h) Lifting laboratory jack.

2. Glue four 12-mm round coverslips to the end of the spacer (one on top of the other) as an extension of the spacer using superglue. Allow enough time for the superglue to completely dry.

   This will facilitate penetration of the coverslip portion into the matrix while avoiding contact between the matrix and the rest of the spacer, and it defines the area of compression.

3. Cut a Teflon circle (12-mm diameter) with the cork borer.

4. Cover the last coverslip with the Teflon film.

5. Sterilize materials by exposing them to a UV light in a biological hood for several hours with the Teflon film facing the light.

   If the compressed matrices are to be in contact with cells for only short periods of time (e.g., for the 10-min cell attachment assay), rinsing the Teflon film with ethanol and air-drying should be sufficient to prevent contamination.

6. Place the glued platform with spacer on the ring portion of the stand with the Teflon facing down.

7. Cover the flat upper surface of the laboratory jack with Parafilm and position the jack under the ring.

8. Set the weight on the platform and level the ring so that the Teflon film is situated parallel to the surface of the jack (see Fig. 10.9.2).

Mechanically compress the 3-D matrix

9. Position the fibroblast-derived 3-D matrix-coated coverslip (matrix face up) onto the jack directly underneath the Teflon film.

10. Slowly raise the laboratory jack until the matrix contacts the Teflon film and the platform rises above the ring. Wait for 2 min.

    At this point, the entire weight should be resting on the matrix, compressing it at a specific weight per unit area.

11. Slowly lower the jack until the platform rests once again on the ring, and the compressed matrix is separated from the Teflon film.

12. Place the coverslip with the compressed matrix into a 35-mm dish. Carefully add 2 ml PBS and examine by phase-contrast microscopy to confirm continued integrity of the compressed matrix.

SUPPORT PROTOCOL 6

Solubilization of Fibroblast-Derived 3-D Matrix

This protocol describes how to solubilize fibroblast-derived 3-D matrix to generate a protein mixture that can be used for subsequent coating of surfaces or biochemical analysis. Briefly, the matrices are treated with a guanidine solution to denature and solubilize the matrix components, thereby producing a liquid mixture that can be stored and used for coating surfaces.

Materials

Fibroblast-derived 3-D matrices on 35-mm dishes (see Basic Protocol)
Solubilization reagent (see recipe)

Cell scraper (e.g., rubber policeman, Costar brand, Fisher Scientific)
1.5-ml microcentrifuge tubes
Rotator at 4°C
Microcentrifuge

Prepare dishes

1. Aspirate PBS from fibroblast-derived 3-D matrix-covered dishes.
2. Tip the dishes for 1 min to accumulate the excess PBS on one side of the dish (~30° to bench top).
3. Aspirate the excess PBS carefully to avoid detaching the matrix layer.

Solubilize matrix

4. Place the dishes on ice and add 300 µl of solubilization reagent. Incubate 5 min on ice.
5. Scrape the dish with a cell scraper toward one side of the dish and pipet the mixture into a 1.5-ml microcentrifuge tube.
6. Add an additional 200 µl solubilization reagent. Rotate 1 hr at 4°C.

Collect solubilized matrix

7. Microcentrifuge 15 min at 12,000 × *g*, 4°C.
8. Transfer the supernatant into a fresh 1.5-ml microcentrifuge tube. Store the solubilized matrix in solubilization reagent indefinitely at 4°C.

The average protein concentration is 1 to 3 mg/ml.

ISOLATION OF PRIMARY FIBROBLASTS FROM FRESH TISSUE SAMPLES

SUPPORT PROTOCOL 7

NIH-3T3 cells are particularly well-suited for mesenchymal cell–derived matrix production because they are homogeneous and provide batch-to-batch consistency. When grown in FBS, their ability to grow at high densities and lack of contact inhibition allows the NIH-3T3 cells to produce a thicker matrix, usually ≥10 µm, which is optimal for cell studies of 3-D cultures (see Critical Parameters). However, other primary fibroblasts and fibroblast cell lines are also suitable for the production of cell-derived matrices. This protocol describes harvesting of primary fibroblasts from fresh tissue samples.

Materials

Fresh tissue samples (murine or human surgical)
Phosphate-buffered saline (PBS; *APPENDIX 2A*) supplemented with antibiotics (see recipe), 4°C
Confluent medium with fetal bovine serum (FBS; see recipe)
Ciprofloxicin (Invitrogen), optional
Trypsin/EDTA solution (see recipe)
60-mm tissue culture dishes
Dissecting scissors, tweezers, and scalpels (Fisher Scientific)
12-well or 6-well tissue culture plates
Sterile laminar flow hood
75-cm^2 tissue culture flasks
Inverted phase-contrast microscope

Prepare tissue samples

1. Rinse tissue samples obtained immediately after surgery (human or murine) three times in a 60-mm tissue culture dish with pre-cooled (to 4°C) PBS supplemented with antibiotics.
2. Aspirate supplemented PBS, finely chop the tissue sample into 1-mm^2 pieces using a sterile scalpel, assisting with sterile tweezers (see Amatangelo et al., 2005).
3. Using sterile dissecting scissors, make multiple scratches into the plastic surface of a 12-well or 6-well tissue culture dish in a star-like configuration.

4. Wash the dish two times with 1 ml (12-well plate) or 2 ml (6-well plate) PBS and gently press tissue pieces into the indentations created by the scratches.

Isolate primary fibroblasts

5. Allow plate to dry for 5 min under the sterile laminar hood.
6. Gently add 1 ml (12-well plate) or 2 ml (6-well plate) confluent medium with FBS to each of the wells, ensuring that the tissue samples remain attached to the scratched surfaces. Incubate 2 to 7 weeks. Replace the confluent medium every other day until primary fibroblasts emerge from the tissue pieces.

   This process normally takes 2 to 7 weeks depending on the tissue source.

7. (*Optional*) As an additional measure to prevent contamination of freshly isolated surgical samples, culture half of the tissue pieces in confluent medium supplemented with 250 ng/ml to 2.5 μg/ml Fungizone and 10 μg/ml ciprofloxacin.
8. After fibroblasts are grown to ~70% confluence in multiwell dishes remove the tissue pieces.
9. Trypsinize fibroblasts with trypsin/EDTA and passage into a 75-cm^2 tissue culture flask (see Basic Protocol, steps 1 to 4).
10. Once fibroblasts reach confluence in a 75-cm^2 flask, harvest and freeze them for future experimental analysis, and/or use them to produce fibroblast-derived matrices between passage 2 and 6 (see Basic Protocol).

    The authors start counting passages once the fibroblasts are initially transferred into a 15-cm dish. The fibroblasts are stable by morphological and biochemical criteria to at least passage 6. Morphological criteria include an elongated cell shape and the shape of the nuclei by Hoechst staining. For tumor-associated fibroblasts, elliptically shaped nuclei typical of myofibroblastic cells have been observed. They have not yet been genetically characterized.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Anti-FAKpY^{397}

Make a 1:1000 to 1:2500 dilution of anti-FAKpY^{397} (Biosource International or Covance) in 5% (w/v) BSA (Sigma)/TBST (see *UNIT 6.2*; see recipe for TBST). Store up to 12 months at −20°C.

Anti-total FAK

Use a 1:2500 dilution of anti-total FAK (Upstate Cell Signaling Solutions) in 5% (w/v) nonfat dried milk (Carnation, Fisher Scientific)/TBST (see *UNIT 6.2*; see recipe for TBST). Store up to 12 months at −20°C.

Confluent medium with fetal bovine serum

High-glucose Dulbecco's modified Eagle medium supplemented with:
10% (v/v) fetal bovine serum (*APPENDIX 2A*)
100 U/ml penicillin
100 μg/ml streptomycin
Store for 1 month at 4°C

For surgical or fine needle aspirates tissue samples, 250 ng/ml to 2.5 μg/ml Fungizone can be added to the cultures.

Culture medium with calf serum

High-glucose Dulbecco's modified Eagle medium supplemented with:
10% (v/v) calf serum
100 U/ml penicillin
100 μg/ml streptomycin
Store for 1 month at 4°C

DiI solution

Dilute 2.5 mg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) stock solution in ethanol to 4 μg/ml with confluent medium (see recipe) and sterilize by filtration using a 0.22-μm filter. Store up to 12 to 24 months at –20°C.

Ethanolamine, 1 M

Prepare a 1 M solution of ethanolamine (Sigma-Aldrich) sterile water by adding 0.062 ml of ethanolamine per milliliter of water. Filter sterilize through a 0.2-μm filter unit. Prepare fresh.

Extraction buffer

Phosphate-buffered saline (PBS; APPENDIX 2A) containing:
0.5% (v/v) Triton X-100
20 mM NH_4OH
Store up to 1 month at 4°C

Fixing solution

Into a 50-ml polypropylene conical tube, add:
2 g sucrose
10 ml 16% (w/v) solution paraformaldehyde (EM-grade from Electron Microscopy Sciences)
Phosphate-buffered saline (PBS; *APPENDIX 2A*) to a final volume of 40 ml
Prepare fresh

Gelatin solution, 0.2% (w/v)

Prepare a 0.2% (w/v) gelatin solution in PBS (*APPENDIX 2A*). Autoclave the solution, cool, and filter through a 0.2-μm filter. Prepare fresh.

Glutaraldehyde solution in PBS, 1% (v/v)

Dilute a 25% stock of glutaraldehyde (Sigma) to 1% glutaraldehyde in PBS (*APPENDIX 2A*). Thaw a 10-ml aliquot of the stock and dilute to a final volume of 250 ml in PBS. Filter sterilize through a 0.2-μm filter unit and store in 50-ml aliquots at −20°C.

Heat-denatured BSA, 2% (w/v)

Dissolve 2 g BSA fraction V (Sigma) in 100 ml water and filter sterilize using a low-protein-binding 0.22-μm filter. Store indefinitely at 4°C.

Just prior to use, heat the amount needed 5 min at 65°C or until the solution starts to appear slightly opaque (not milky). Cool to room temperature before using for blocking procedures. Do not store the heat-denatured BSA.

Hoechst 33342 stock solution

Prepare a 2 mM (MW 615.9 g) Hoechst 33342 (bisbenzimide H 33342 fluorochrome, trihydrochloride; Calbiochem) solution in water. Store at 4°C, protected from light.

Lysis buffer (modified RIPA) reagent

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
50 mM NaCl
1% (w/v) deoxycholic acid, sodium salt (Fisher)
48 mM NaF
1% (w/v) glycerol (Fluka)
1% (w/v) Triton X-100 (Sigma)
Adjust to 100 ml with MilliQ H_2O
Store 3 to 6 months at 4°C

Matrix medium with ascorbic acid

Confluent medium (see recipe) containing:
L-ascorbic acid sodium salt (Sigma) at a final concentration of 50 μg/ml
Filter sterilize with a 0.2-μm filter
Prepare fresh

Ascorbic acid should be freshly prepared just prior to use as a 1000× stock concentration of 50 mg/ml in PBS to yield a final concentration of 50 μg/ml. Remove a 5- to 10-ml aliquot of medium, add ascorbic acid, and after filtering, add the ascorbic acid–containing medium back to the total volume of medium. In cases where a 500 μg/ml final concentration of ascorbic acid is added on the first day after cell plating, the stock is diluted only 100-fold instead of 1000-fold (see Basic Protocol, steps 16 and 17).

PBS$^+$

Phosphate-buffered saline (PBS; APPENDIX 2A) containing:
1 mM $CaCl_2$
1 mM $MgSO_4$
Store 6 to 12 months at room temperature

PBS supplemented with antibiotics

Phosphate-buffered saline (PBS; APPENDIX 2A) containing:
100 U/ml penicillin
100 μg/ml streptomycin
2.5 μg/ml Fungizone (amphotericin B; Invitrogen)
10 μg/ml ciprofloxicin
Store 1 to 2 weeks at 4°C

Pre-coated 2-D fibronectin dishes

In phosphate-buffered saline (*APPENDIX 2A*), prepare a 5 μg/ml solution of human plasma fibronectin (see *UNIT 10.5* or Sigma). Immediately add 1 ml per 35-mm tissue culture dish and incubate for 1 hr at 37°C. Remove remaining fibronectin solution and rinse once with PBS. Prepare diluted fibronectin solution fresh for each experiment.

The above procedure can be used with any desired protein for coating dishes or coverslips. If solubilized matrix mixture is to be used (see Support Protocol 6), coat with a 30 μg/ml protein concentration.

Protease and phosphatase inhibitors

1 mM sodium pyrophosphate
1 mM nitrophenol phosphate
5 mM benzamidine
1 mM PMSF
1 mM sodium orthovanadate

continued

Serine/threonine phosphatase cocktail inhibitor 1 (100 μl/10 ml lysis buffer; Sigma)
Tyrosine phosphatase inhibitor cocktail 2 (100 μl/10 ml lysis buffer; Sigma)
Prepare fresh

Solubilization reagent

5 M guanidine containing:
10 mM dithiothreitol
Store indefinitely at 4°C

Tris-buffered saline with Tween (TBST)

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
0.5% (v/v) Tween-20 detergent (Sigma)
Adjust pH to 8.0 with 12 M HCl
Store up to 2 weeks at 4°C

Trypsin/EDTA solution

2.5 g trypsin
0.2 g EDTA
8 g NaCl
0.4 g KCl
1 g glucose
0.35 g $NaHCO_3$
0.01 g phenol red
Bring up to 1 liter with H_2O
Sterilize by filtration with a 0.2-μm filter and store up to 3 months at −20°C

COMMENTARY

Background Information

Extracellular matrix (ECM) was historically regarded as a passive scaffold that stabilizes the physical structure of tissues. With time, it became evident that the ECM is much more than a simple physical scaffold. The ECM is a dynamic structure capable of inducing (and responding to) a large variety of physiological cell responses regulating the growth, migration, differentiation, survival, and tissue organization of cells (Buck and Horwitz, 1987; Hay, 1991; Hynes, 1999). Integrins are receptors for matrix molecules and can mediate these cell responses by inducing the formation of membrane-associated multi-molecular complexes. These integrin-based structures (cell-matrix adhesions) mediate strong cell-substrate adhesion and transmit information in a bi-directional manner between ECM and the cytoplasm. There are three main cell-to-matrix adhesions. The "focal adhesion" mediates firm linkage to relatively rigid substrates (Burridge and Chrzanowska-Wodnicka, 1996). Focal adhesions cooperate with "fibrillar adhesions" that generate fibrils from pliable fibronectin (Katz et al., 2000; Pankov et al., 2000). Fibroblasts require culture for several days at high cell density to generate 3-D matrices and evolve "three-dimensional-matrix adhesions." The requirements for producing 3-D matrix adhesions include three-dimensionality of the ECM, integrin $\alpha_5\beta_1$, fibronectin, other matrix component(s), and pliability of the matrix (Cukierman et al., 2001). The fibroblast-derived matrix provides an in vivo–like 3-D environment for cultured fibroblasts, thereby restoring their normally nonpolar surroundings. The fibroblast-derived 3-D matrix can be used as a suitable in vitro system to investigate in vivo–like fibroblast-to-matrix interactions, such as 3-D matrix adhesion signaling.

Critical Parameters

The phenotype of cultured NIH-3T3 fibroblasts as monitored by cell morphology is extremely important for the successful preparation of 3-D matrix-coated dishes. The fibroblasts should be well-spread and flat under sparse culture conditions. If elongated cells are commonly observed in the cell population, recloning of the cell line may be necessary to achieve greater phenotypic homogeneity. The NIH-3T3 line obtained from ATCC (ATCC# CRL-1658) has this morphology and produces

an excellent matrix. The NIH-3T3 cells must be maintained routinely as sub-confluent cultures in a medium containing calf serum to retain the correct phenotype. However, if the matrix deposition at confluence is performed in the presence of calf serum, the resultant matrices are thicker but less stable and more likely to detach from the surface than matrices obtained after culture in fetal bovine serum. Therefore, NIH-3T3 cells should be changed to medium containing fetal bovine serum prior to matrix deposition. While the NIH-3T3 cells are being adapted for matrix production in FBS, they take on a more uniform polygonal morphology and are not as contact inhibited as those grown in calf serum. NIH-3T3 cells do not normally take on a very elongated morphology unless they are cultured within 3-D matrices. To pre-adapt the NIH-3T3 cells to fetal bovine serum–containing medium, it is recommended to culture the cells for 15 to 20 passages before plating for matrix deposition.

The Basic Protocol can be modified for other fibroblastic cell lines capable of secreting and assembling fibronectin-based matrices. As described in Support Protocol 7, the authors have adapted this protocol for the isolation of primary fibroblasts obtained from human and/or murine surgical tissue samples. Fibroblasts can be isolated from tissue samples after ~2 to 7 weeks in culture. In some cases, the resulting matrix may be too thick or dense to obtain efficient extraction. In such cases, more prolonged cell extraction may be needed with extensive DNase treatment until no cell debris is detected. The lack of contaminating cellular debris (in the case of NIH-3T3 cells) in the matrices has been confirmed by immunoblotting and immunofluorescence staining for cellular proteins like actin or GAPDH.

Pre-coating surfaces with gelatin promotes fibronectin binding and results in smooth layers of relatively homogenous matrices that will not detach from the surface.

The thickness of NIH-3T3-derived 3-D matrices is measured using a confocal microscope without dehydration of the matrix (no mounting or fixing). The resultant thickness observed varies between 8 and 20 μm. Basic molecular characterization of the matrices revealed the presence (among other molecules) of fibronectin organized in a fibrillar mesh, collagen I and III but not IV, and small traces of non-organized laminin and perlecan.

The integrity of these 3-D matrices must be confirmed prior to every use. This can be accomplished by using phase-contrast microscopy and discarding any matrices that are torn or detached (see Fig. 10.9.1 B). Moreover, if matrices are to be used for short-term signal transduction assays under serum-depleted conditions, then freshly made matrices must be utilized. Matrices that have been stored at 4°C or −80°C (up to 2 to 3 weeks) should be used only after assessment of their integrity by phase-contrast microscopy. Freshly prepared or stored matrices can be used to test the induction of cellular responses in the presence of serum such as attachment, morphology, motility, proliferation, and for immunofluorescence staining. Additionally, biochemical analysis of the 3-D matrix can be assessed by immunoblotting to test for cell responsiveness to three-dimensionality by phospho-FAK down regulation (see Support Protocol 3).

Anticipated Results

The Basic Protocol is based on the ability of densely cultured fibroblasts (start up at ~2.5×10^5 cells/ml) to coat any available tissue culture surface by deposition of their natural matrix, which gradually forms a 3-D matrix. This intact, naturally produced ECM is similar in its molecular organization to mesenchymal fibronectin-based extracellular matrices in vivo (Cukierman et al., 2001). The basic approach is to allow cells to deposit their own ECM followed by removal of cells, while avoiding procedures that may alter or denature the native ECM constituents and supramolecular organization.

One NIH-3T3 semi-confluent (80%) cultured 15-cm dish can yield enough cells to coat 100 35-mm tissue culture dishes.

Time Considerations

The adaptation step after switching NIH-3T3 cell medium to fetal bovine serum for future matrix deposition requires culturing the cells for 15 to 20 passages. This adaptation process could take between 5 and 22 weeks depending on the rate of NIH-3T3 cell proliferation and the dilution factor per passage. The rate of NIH-3T3 proliferation is dependent upon many factors, including the growth-promoting abilities of the fetal bovine serum, which unfortunately is largely batch-dependent. Therefore, the time required for adapting NIH-3T3 cells to growth in FBS should be determined empirically. At this point, the NIH-3T3 cells are between 20 and 25 passages, and can be cultured in FBS for at least 20 to 25 additional passages, resulting in a total of ≥50 passages. After that, their morphology starts to become more

spindle-shaped and, therefore, they can no longer form optimal matrices. When the NIH-3T3 cells become too spindle-shaped, they fail to form uniform monolayers that upon extraction can ultimately produce uneven matrix coverage. Matrix production will require between 5 and 9 days. About 2 to 7 weeks are required from the time of tissue isolation to harvesting primary fibroblasts.

Literature Cited

Amatangelo, M.D., Bassi, D.E., Klein-Szanto, A.J., and Cukierman, E. 2005. Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts. *Am. J. Pathol.* 167:475-488.

Buck, C.A., and Horwitz, A.F. 1987. Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* 3:179-205.

Burridge, K., and Chrzanowska-Wodnicka, M. 1996. Focal adhesions, contractility, and signaling. *Annu. Rev. Cell Dev. Biol.* 12:463-518.

Cukierman, E. 2005. Cell migration analyses within fibroblast-derived 3-D matrices. *Methods Mol. Biol.* 294:79-93.

Cukierman, E., Pankov, R., Stevens, D.R., and Yamada, K.M. 2001. Taking cell-matrix adhesions to the third dimension. *Science* 294:1708-1712.

Friedl, P., and Brocker, E.B. 2000. The biology of cell locomotion within three-dimensional extracellular matrix. *Cell Mol. Life Sci.* 57:41-64.

Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K.M. 2001. Transmembrane crosstalk between the extracellular matrix and the cytoskeleton. *Nat. Rev. Mol. Cell Biol.* 2:793-805.

Hay, E.D. 1991. Cell biology of extracellular matrix, 2nd ed. Plenum Press, New York.

Hynes, R.O. 1999. Cell adhesion: Old and new questions. *Trends Cell Biol.* 9:M33-M77.

Katz, B.Z., Zamir, E., Bershadsky, A., Kam, Z., Yamada, K.M., and Geiger, B. 2000. Physical state of the extracellular matrix regulates the structure and molecular composition of cell-matrix adhesions. *Mol. Biol. Cell* 11:1047-1060.

Pankov, R., Cukierman, E., Katz, B.Z., Matsumoto, K., Lin, D.C., Lin, S., Hahn, C., and Yamada, K.M. 2000. Integrin dynamics and matrix assembly: Tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. *J. Cell Biol.* 148:1075-1090.

Key References

Cukierman et al., (2001). See above.

The source for procedures and materials described in this unit.

Contributed by Dorothy A. Beacham, Michael D. Amatangelo, and Edna Cukierman

Fox Chase Cancer Center
Philadelphia, Pennsylvania

Purification and Analysis of Thrombospondin-1

Thrombosponding-1 (TSP-1) is a trimeric matricellular protein that is expressed by many cells. It contains several different domains that allow it to participate in cell adhesion, cell migration, and cell signaling. Recently, TSP-1 has been shown to activate transforming growth factor-β (TGF-β) and to inhibit both angiogenesis and tumor growth. This unit describes two protocols: the purification of TSP-1 from platelet-rich plasma (see Basic Protocol 1) and the purification of TSP-1 proteolytic fragments (see Basic Protocol 2).

ISOLATION OF THROMBOSPONDIN-1 FROM HUMAN PLATELETS

BASIC PROTOCOL 1

TSP-1 is released from platelet α-granules in response to thrombin and can therefore be readily purified from the supernatant of thrombin-treated platelets. Human platelets can be obtained from the Red Cross or from hospital blood banks. Outdated pheresis units of platelet-rich plasma are a good source of TSP-1. Platelets are separated from plasma and other blood components by a series of centrifugation steps. The isolated platelets are washed repeatedly to remove plasma proteins and the washed platelets are then activated by exposure to thrombin. Next the TSP-1-containing supernatant is passed over a heparin-Sepharose column. Lower-affinity heparin-binding proteins are washed away and the TSP-1 is eluted under conditions of high salt. The TSP-1-containing fractions are pooled, precipitated, and loaded onto a 10% to 20% continuous sucrose gradient and subjected to ultracentrifugation. The gradient is divided into fractions and the protein concentrations are determined by measuring optical density. The level of purity is normally >95% as determined by SDS-PAGE (*UNIT 6.1*).

Materials

Platelet-rich plasma
Baenziger A buffer (see recipe)
Baenziger B buffer (see recipe)
1 M $CaCl_2$ (*APPENDIX 2A*)
1 N NaOH (optional)
Thrombin
Diisopropyl fluorophosphate (DFP)
Heparin-Sepharose CL-6B (Amersham Pharmacia Biotech)
0.15, 0.25, 0.55, and 2.0 M heparin-Sepharose column buffers (see recipe)
Anti-vitronectin immunoaffinity column: prepare in advance according to manufacturer's instructions using an Affi-Gel Hz Immunoaffinity kit (Bio-Rad) and anti–human vitronectin antibody (e.g., GIBCO/BRL)
Ammonium sulfate
10% and 20% (w/v) sucrose gradient solutions (see recipe)

15- and 50-ml centrifuge tubes (conical bottom preferred)
Preparative centrifuge (Sorvall RC-B3 or equivalent) and rotor (H4000 or equivalent)
40-ml Oak Ridge centrifuge tubes
High-speed centrifuge (Beckman J2-MC or equivalent) and rotor (JA-20 or equivalent)
1 × 12–cm chromatography column
Fraction collector and appropriate tubes
Spectrophotometer set at 280 nm
Gradient maker
14-ml ultracentrifuge tubes
Ultracentrifuge (Beckman LM-80 or equivalent) and rotor (SW 41Ti or equivalent)

Contributed by Karen O Yee, Mark Duquette, Anna Ludlow, and Jack Lawler
Current Protocols in Cell Biology (2003) 10.10.1-10.10.13

NOTE: Platelets are temperature sensitive and activated by untreated glass surfaces; therefore, they should be handled at room temperature in plasticware, and centrifuges and buffers should be warmed to room temperature before use.

Prepare platelets

1. Transfer platelet-rich plasma to 50-ml centrifuge tubes (conical bottom preferred) and centrifuge in a Sorvall RC-B3 preparative centrifuge 20 min at 1400 × *g* (2800 rpm in an H4000 rotor), 20°C.

   Pheresis units are preferable, but random donor units of platelet-rich plasma also work well.

2. Carefully pour off the supernatant. Gently resuspend the cell pellet in Baenziger A buffer at a ratio of 15 ml buffer per 2 ml packed cells.

3. Transfer the platelet suspension to 15-ml centrifuge tubes and centrifuge 8 min at 120 × *g* (800 rpm in an H4000 rotor), room temperature.

   Most of the platelets will remain in suspension following this centrifugation, while erythrocytes and leukocytes will pellet.

4. Leaving behind the red cell pellet, carefully transfer the platelet suspension to 50-ml centrifuge tubes (~22 ml per tube).

5. Add Baenziger A buffer to a final volume of 50 ml. Mix by inverting the tube several times and centrifuge 20 min at 1400 × *g* (2800 rpm in an H4000 rotor), 20°C.

Wash platelet pellet

6. Carefully pour off the supernatant. Resuspend each cell pellet in 15 ml Baenziger A buffer and then add buffer to a final volume of 50 ml. Invert the tube to mix and centrifuge 20 min at 1400 × *g* (2800 rpm in an H4000), room temperature. Repeat once.

7. Remove the supernatant and resuspend the pellet in 15 ml Baenziger B buffer. Add sufficient Baenziger B to achieve a ratio of 50 ml buffer per 2 to 3 ml packed cells. Mix the tube by inversion.

8. Add 100 µl of 1 M $CaCl_2$ per 50 ml suspension.

   From this point on, 2 mM calcium must be present at all times to maintain the conformational integrity of the thrombospondin molecule.

9. Check the pH of the suspension using pH paper. Adjust to pH 7.6 by adding 1 N NaOH as necessary.

Activate platelets

10. *Optional:* If the platelets are from outdated units, enhance their response to thrombin by incubating 5 min in a 37°C water bath.

11. Add 50 U thrombin per 50 ml platelet suspension and immediately mix by gentle inversion. Continue mixing 2 to 3 min at room temperature, then place on ice.

    Platelet aggregation should be evident upon examination of the suspension. The platelets will form large clumps and settle to the bottom of the tube, causing the supernatant to appear somewhat clear after 2 to 3 min. Outdated platelets respond more slowly than fresh ones. Outdated units should therefore be mixed for an additional 2 to 3 min.

12. Remove the cellular debris by centrifuging the tubes 5 min at 1400 × *g* (2800 rpm in an H4000 rotor), 4°C. Transfer supernatant to a 40-ml Oak Ridge centrifuge tube.

From this point on the TSP-1-containing supernatant must be kept on ice and all subsequent steps must be performed at 4°C.

13. Add sufficient DFP to achieve a final concentration of 1 mM (i.e., 0.181 µl/ml).

    CAUTION: *DFP is a powerful serum protease inhibitor and is highly toxic. Great care should be taken in its use. DFP is volatile and should be used in a fume hood.*

Isolate TSP-1 supernatant

14. Centrifuge 20 min in a Beckman J2-MC high-speed centrifuge at 34,957 × *g* (17,000 rpm in a JA-20 rotor), 4°C.

15. Transfer the supernatant to a clean 50-ml tube. Place the sample on ice and leave overnight at 4°C.

    This incubation step is necessary to allow formation of fibrin fibrils, which are then removed by centrifugation (step 17). If the supernatants are applied to the heparin-Sepharose column without performing this procedure, the fibrin fibrils will form on the top of the column and the flow rate will be decreased significantly.

Isolate TSP-1

16. Prepare and pour enough heparin-Sepharose CL-6B, according to the manufacturer's instructions, to produce a 5-ml bed volume in a 1 × 12–cm chromatography column. Equilibrate the column with 50 ml of 0.15 M heparin-Sepharose column buffer.

17. Following the overnight incubation (step 15), centrifuge the supernatant 20 min at 1400 × *g* (2800 rpm in an H4000 rotor), 4°C. Transfer the supernatant to a new tube.

18. Load the supernatant onto the equilibrated heparin-Sepharose column at a flow rate of ~3 ml/min.

    The TSP-1 will be immobilized on the column following this step. If necessary, the protocol may be paused at this point; however, the column should be washed extensively with 0.15 M heparin-Sepharose column buffer before pausing. TSP-1 is stable on the column for 3 to 4 days.

19. Connect the column to a fraction collector with appropriate tubes and elute the column with 40 ml of 0.15 M heparin-Sepharose column buffer at a flow rate of ~3 ml/min, collecting twenty 2-ml fractions. Repeat with 0.25 M heparin-Sepharose column buffer.

    Little or no TSP-1 will be present in these first two elutions.

20. Elute TSP-1 by applying 40 ml of 0.55 M heparin-Sepharose column buffer and collect in 2-ml fractions. Determine which fractions contain protein by measuring their absorbance at 280 nm. Calculate the total amount of protein in milligrams using the following formula: total protein = $OD_{280} \times 1.08 \times$ volume.

    After elution, >80% of total protein is TSP-1.

21. Strip the heparin-Sepharose column by applying 100 ml of 2.0 M heparin-Sepharose column buffer. Equilibrate and store the column in 0.15 M heparin-Sepharose column buffer at 4°C.

    The column can be used repeatedly if treated in this manner.

22. Pool the protein-containing fractions and apply to an anti-vitronectin immunoaffinity column.

    Although vitronectin is present in only trace amounts in the TSP-1-containing fraction (<1%), cells adhere strongly to vitronectin, which can pose a problem in certain applications involving purified TSP-1. It is therefore advisable to remove it. The authors are unable to detect vitronectin in the immunoaffinity flowthrough by immunoblotting.

For storage and reuse of the immunoaffinity column, refer to the manufacturer's instructions.

23. Transfer the flowthrough to a 40-ml Oak Ridge tube. Precipitate the protein by adding ammonium sulfate to 40% (w/v). Mix by inverting the tube until the solid is dissolved.

24. Centrifuge the sample 20 min at 34,957 × *g* (17,000 rpm in a JA-20 rotor), 4°C.

    The precipitated protein should form a milky-white pellet following centrifugation. It may be useful to note the orientation of the tube in the rotor to aid in locating the pellet.

25. Carefully pour off the supernatant and briefly leave the tube inverted to drain away all remaining liquid.

26. Resuspend the pellet in sufficient 0.15 M heparin-Sepharose buffer so that the protein concentration is ~1 mg/ml.

    The concentration does not change significantly from the value determined in step 20.

27. Using a gradient maker, prepare 12 ml of a 10% to 20% continuous sucrose gradient in a 14-ml ultracentrifuge tube.

28. Carefully load the protein-containing sample onto the gradient, using no more than 2 mg protein on each gradient in order to achieve good resolution.

    Drawing up the solution into a pipet tip and slowly discharging it by turning the volume adjustment wheel on the pipettor works well. It is important not to disturb the gradient.

    See UNIT 5.3 for more information concerning sucrose gradients.

29. In a Beckman LM-80 ultracentrifuge, centrifuge the gradients 18 hr at 247,605 × *g* (38,000 rpm in an SW 41Ti rotor), 4°C.

30. Fractionate the sucrose gradients into 0.5-ml aliquots. Read the absorbance of each fraction at 280 nm.

    The concentration of TSP-1 in each fraction is determined using the equation $c = A_{280}/\varepsilon l$, *where* ε *is the molar extinction coefficient of TSP-1 (1.08 in* $M^{-1}cm^{-1}$*) and l is the pathlength of the cuvette.*

    TSP-1 is the major peak located in the middle third of the gradient. The peak usually appears in the eighth fraction from the bottom and continues over approximately eight fractions. There is also a minor peak located higher in the gradient that is composed mainly of β*-thromboglobulin. The purified TSP-1 can be frozen directly in the sucrose gradient solution and stored 3 to 5 years at −70°C. If necessary for specific applications, sucrose can be removed by dialysis (APPENDIX 3C).*

BASIC PROTOCOL 2

ISOLATION OF PROTEOLYTIC FRAGMENTS OF TSP-1

The identification of functional sites within larger proteins can be accomplished by producing individual domains for functional studies. The proteolytic digestion of native molecules or the expression of individual domains by recombinant approaches is typically used for this purpose. While the authors were developing the procedure for the purification of TSP-1, it was observed that molecules that lacked the 25,000-Da N-terminal domain were not retained by the heparin-Sepharose column. This observation led to the early identification of the N-terminal domain as a high-affinity heparin-binding site and to the development of a procedure to purify this domain. During the development of the isolation protocol, it was also found that TSP-1 purified in the presence of calcium was distinct from that purified in the presence of EDTA. The removal of calcium from the protein renders some regions much more labile to proteolysis (Lawler and Hynes, 1986). Subsequent sequencing studies revealed that the type 3 repeats are a contiguous set of calcium-binding sites. Removal of calcium causes the type 3 repeats and the adjacent

C-terminal domain to unfold and become more labile to proteolysis. Thus, it is difficult to design a protocol that utilizes proteolysis in order to isolate the type 3 repeats and the C terminus of TSP-1. These domains are either resistant to digestion in the presence of calcium or are readily digested in its absence. In the absence of calcium, the central 70,000-Da core of the protein can be produced by chymotryptic digestion. This structure contains the intrachain disulfide bonds and hence is trimeric with a molecular weight of 210,000 Da. A procedure for preparing the N-terminal heparin-binding domain and the central core region is provided below.

Materials

TSP-1 (see Basic Protocol 1)
TBS (see recipe) containing 2 mM $CaCl_2$
0.5 M EDTA (*APPENDIX 2A*)
Chymotrypsin
Diisopropyl fluorophosphate (DFP)
0.8 × 3–cm column of immobilized soybean trypsin inhibitor (Pierce)
0.8 × 3–cm column of heparin-Sepharose CL-6B (Amersham Pharmacia Biotech)
0.15, 0.25, and 0.55 M heparin-Sepharose column buffers (see recipe)

Centriplus centrifugal filter device (3000-Da cutoff; Millipore)
1.3 × 30–cm column of Sephadex G-200
Fraction collector
Spectrophotometer set at 280 nm

1. Begin with 5 mg purified TSP-1 in 3 ml TBS containing 2 mM $CaCl_2$ on ice. Add 0.5 M EDTA to a final concentration of 5 mM.
2. Dissolve chymotrypsin in TBS to a final concentration of 1 mg/ml and add 50 µl to the sample.

   This gives an enzyme-to-substrate ratio of 1:100 (w/w).
3. Digest 20 hr in a covered ice bucket in a 4°C cold room.

   This approach produces a very reproducible digestion pattern.
4. Terminate the digestion by adding DFP to 1 mM (0.181 µl/ml) and incubating an additional 2 hr on ice.
5. Pass the sample over a 0.8 × 3–cm column of immobilized soybean trypsin inhibitor to remove the chymotrypsin.
6. Collect the flowthrough from the column and apply to a 0.8 × 3–cm heparin-Sepharose column connected to a fraction collector with appropriate tubes.
7. Collect 1-ml fractions while the sample is flowing onto the column and throughout the elution. Elute the column in a stepwise fashion with 20 ml (each) of 0.15, 0.25, and then 0.55 M heparin-Sepharose buffer.

   The 25,000-Da fragment elutes in the 0.55 M heparin-Sepharose buffer. The protein is ~95% pure and can be used directly or concentrated further. Some applications may require dialysis to reduce the salt concentration.

   The 210,000-Da fragment is in the initial flowthrough fractions. It should be dialyzed and purified on a Sephadex G-200 column as described in the following steps.
8. Pool the flowthrough fractions and concentrate to 2 ml using a Centriplus centrifugal filter device.
9. Apply this sample to a 1.3 × 30–cm Sephadex G-200 column equilibrated with TBS at 4°C.

Sephadex G-200 is fragile and care should be exercised to follow the manufacturer's directions for removing fines and for pouring and eluting the column.

10. Apply TBS and collect fifty 1-ml fractions. Measure the OD_{280} of each fraction.

The 210,000-Da fragment is eluted in the molecular weight peak near the void volume of the column. This material can be concentrated using the Centriplus centrifugal filter device (step 8) and should be >95% pure.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Baenziger A buffer

0.102 M NaCl
0.0039 M K_2HPO_4
0.0039 M Na_2HPO_4
0.022 M NaH_2PO_4
0.0055 M glucose
Store up to 2 weeks at 4°C

Baenziger B buffer

0.015 M Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.14 M NaCl
0.005 M glucose
Store up to 2 weeks at 4°C

Heparin-Sepharose column buffers, 0.15, 0.25, 0.55, and 2.0 M

0.015 M Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.002 M $CaCl_2$
0.15, 0.25, 0.55, or 2.0 M NaCl
Store up to 3 weeks at 4°C

The molarity of the buffer refers to the concentration of the NaCl.

Sucrose gradient solutions, 10% and 20% (w/v)

0.015 M Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.14 M NaCl
0.002 M $CaCl_2$
10% or 20% (w/v) sucrose
Store up to 1 week at 4°C

TBS (Tris-buffered saline)

0.015 M Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.015 M NaCl
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

The thrombospondins are a family of extracellular matrix proteins currently consisting of five members, thrombospondins 1 to 4 and cartilage oligomeric matrix protein (COMP). For comprehensive reviews, see Adams (2001) and Chen et al. (2000). These proteins are synthesized by many tissues with patterns of expression that are temporally and spatially regulated. All thrombospondin family members are composed of a series of multidomain structures and have the ability to bind large numbers of calcium ions. Calcium binds to the thrombospondins through a cooperative mechanism that involves a significant conformational change in the protein. Through interactions with molecules on the cell surface and components of the extracellular matrix, the

thrombospondins play a role in cell adhesion, migration, differentiation, and proliferation.

Thrombospondin-1 (TSP-1) was the first member of the gene family to be identified and has been the most extensively characterized. TSP-1 is a large multifunctional glycoprotein with a molecular weight of 420,000 Da, and is a trimer composed of identical subunits each with a molecular weight of 142,000 Da. TSP-1 is expressed by both normal and tumor cells and has a number of domains that allow it to interact with cells and other proteins. These include (1) a heparin-binding domain that interacts with proteoglycans, integrin α3β1, and cell-surface glycosaminoglycans (Clezardin et al., 1997; Merle et al., 1997); (2) three type 1 repeats that interact with CD36, matrix metalloproteinases, fibronectin, and heparan sulfate proteoglycans, and also activate latent TGF-β (Bornstein, 1995; Schultz-Cherry et al., 1995; Crawford et al., 1998); (3) an RGDA sequence within the last type 3 repeat, which interacts with integrin αvβ3; and (4) a C-terminal cell-binding domain that contains a recognition sequence for the integrin-associated protein CD47 (Gao et al., 1996). In this unit, the authors focus on the activities of TSP-1 that involve the type 1 repeats and the interaction of TSP-1 with integrins (Fig. 10.10.1). The interaction of TSP-1 with proteoglycans is discussed in detail in a recent review by Chen et al. (2000).

TSP-1 and transforming growth factor-β

Recently, TSP-1 has been shown to activate transforming growth factor-β (TGF-β) by binding to the latency-associated protein and altering the conformation of TGF-β to make it accessible to its receptor (Schultz-Cherry et al., 1995; Crawford et al., 1998). The region of TSP-1 responsible for TGF-β activation is the amino acid sequence KRFK, which is found at the start of the second type I repeat (Schultz-Cherry et al., 1995; Crawford et al., 1998; Fig. 10.10.1). TGF-β is a 25-kDa homodimeric cytokine and a known tumor suppressor (Markowitz and Roberts, 1996). It is secreted in a latent complex consisting of mature TGF-β, the latency-associated protein, and sometimes an additional latent TGF-β-binding protein. The latent TGF-β-binding protein is thought to target latent TGF-β to sites in the extracellular matrix where it is sequestered until activated. Activation of TGF-β has been demonstrated in vitro by activators such as acids, plasmin, or cathepsin D (Munger et al., 1997). TSP-1 and the αvβ6 integrin have been shown to activate TGF-β in vivo (Crawford et al., 1998; Munger et al., 1999). Activation of TGF-β by TSP-1 was demonstrated in vivo when TSP-1-deficient mice were injected with a peptide containing the sequence KRFK. The lungs of the injected mice became morphologically more similar to wild-type mice and active TGF-β was detected in the bronchial epithelial cells (Crawford et al., 1998). In some contexts, however, TSP-1 does not appear to be a good activator of TGF-β

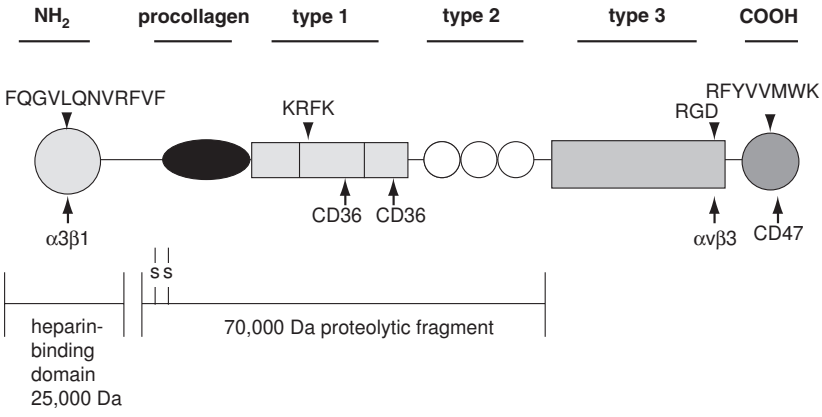


Figure 10.10.1 Representative model of TSP-1 identifying the different structural and functional domains. The binding sites for the various integrins, CD36, and CD47 are indicated below the model. Amino acid sequences that mediate receptor binding and activation of TGF-β are indicated above the model. The proteolytic fragments isolated in the protocol are shown at the bottom. The FQGVLQNVRFVF sequence is a GAG-independent cell binding site and the RFYVMWK sequence is an integrin-associated protein (CD47) binding site.

(Abdelouahed et al., 2000; Grainger and Frow, 2000). These data indicate that post-translational modification or other factors may regulate the ability of TSP-1 to activate TGF-β. Thus, co-expression of TSP-1 and TGF-β does not necessarily mean that TSP-1 will activate latent TGF-β in that tissue.

The role of TSP-1 in angiogenesis and cancer

TSP-1 has been shown to be an effective inhibitor of angiogenesis, tumor progression, and metastasis (Chen et al., 2000; Lawler, 2002). While TSP-1 levels are very low in many tumor cells, expression of TSP-1 is high in the tumor stroma (Brown et al., 1999). Overexpression of TSP-1 in MDA-MB-435 human breast carcinoma cells decreased tumorigenesis and metastasis in vivo (Weinstat-Saslow et al., 1994). Furthermore, the tumors derived from cells formed by a fusion of low-TSP-1-expressing human breast cancer cells and high-TSP-1-expressing normal breast epithelial cells were smaller in nude mice as compared to the tumors formed from the breast cancer cells alone (Zajchowski et al., 1990). Lastly, one group has shown that plasma TSP-1 secreted from primary HT1080 fibrosarcomas in nude mice inhibited growth of experimental metastases (Volpert et al., 1998). Moreover, if the implanted fibrosarcoma cells were transfected with an antisense TSP-1 construct prior to implantation, melanoma cell invasion of the lung was not inhibited.

Recently, the authors have shown that recombinant proteins comprising the second type 1 repeat of TSP-1 and containing the TGF-β activating sequence KRFK inhibited B16F10 tumor growth in mice (Miao et al., 2001). Furthermore, it was observed that treatment with a TGF-β antibody or soluble TGF-β receptor reversed this inhibition, suggesting that TSP-1 activation of TGF-β is part of the inhibitory pathway. By contrast, an effect of TGF-β was not observed with Lewis lung carcinoma because these cells have acquired mutations that have rendered them unresponsive. Vascular density was decreased in both B16F10 and Lewis lung carcinoma tumors treated with the recombinant proteins through a TGF-β-independent mechanism.

In another study, Streit et al. (1999) overexpressed full-length TSP-1 in A431 human carcinoma cells and implanted these cells in the flanks of nude mice. Decreased tumor growth and angiogenesis were observed in tumors expressing TSP-1. Recent work has demonstrated that the KRFK sequence in the second type 1 repeat of TSP-1 is partly responsible for this growth inhibition and the decrease in tumor angiogenesis (K. Yee, unpub. observ.). In another recent study, TSP-1 null mice were crossed with *c*-neu transgenic mice to create a mouse that develops breast tumors and does not express TSP-1. These mice developed tumors that were larger and more vascular than the tumors of mice overexpressing TSP-1 (Rodrídguez-Manzaneque et al., 2001). The authors also determined that the absence of TSP-1 in these tumors resulted in an increase in the amount of active matrix metalloproteinase 9 (MMP-9).

The effects of TSP-1 on endothelial cell migration and angiogenesis have been previously observed by several groups (Tolsma et al., 1993; Dawson et al., 1997; Qian et al., 1997; Iruela-Arispe et al., 1999; Jiménez et al., 2000; Nör et al., 2000). These studies demonstrate that TSP-1 is able to prevent tumor progression in several in vivo cancer models and that one of the ways TSP-1 inhibits tumor growth may be through decreasing tumor angiogenesis. In a different avenue of thinking, many groups have examined MMP-2 and MMP-9 with regards to breast cancer progression (Benaud et al., 1998; Martorana et al., 1998; Remacle et al., 1998; Rudolph-Owen et al., 1998; Lee et al., 2001). MMP-2 and -9 are gelatinases that degrade collagen types IV, V, VII, and X, as well as denatured collagen and gelatin (Dollery et al., 1995). Recently, TSP-1 has been shown to interact with MMP-2 and -9 and inhibit their activation (Bein and Simons, 2000; Rodrídguez-Manzaneque et al., 2001). This interaction is mediated by the type 1 repeats of TSP-1. Therefore, one of the mechanisms through which TSP-1 inhibits both tumor progression and tumor angiogenesis may be due to its ability to inhibit MMP activation and prevent growth factor and cell mobilization.

Angiogenesis is a complex process that involves multiple cell types. TSP-1 does have possible effects on the recruitment of immune cells and on the proliferation and migration of vascular smooth muscle cells. In some assays, these effects can predominate, leading to the conclusion that TSP-1 supports angiogenesis. The preponderance of in vivo data indicates that the anti-angiogenic effects predominate in tumors.

TSP-1 and CD36

CD36 is an integral membrane glycoprotein, a member of the class B scavenger receptor

family, and is located within the caveolae of the cell membrane. It is expressed in many cells including microvascular endothelium, adipocytes, skeletal muscle, dendritic cells, and hematopoietic cells including platelets and macrophages (Febbraio et al., 2001). CD36 is also a receptor for TSP-1 and binds to the specific sequence CSVTCG in the second and third type 1 repeats of TSP-1, while TSP-1 type 1 repeats bind the CD36 LIMP-II Emp sequence homology (CLESH) region of CD36 (Crombie and Silverstein, 1998). This binding initiates a signal that involves the nonreceptor tyrosine kinases *fyn*, *lyn*, and *yes* as well as p38MAPK (Huang et al., 1991). One of the endpoints of this cascade is activation of caspase 3 and endothelial cell apoptosis (Guo et al., 1997; Jiménez et al., 2000; Nör et al., 2000). CD36 signaling is one of the mechanisms by which TSP-1 inhibits angiogenesis and tumor progression (Dawson et al., 1997; Simantov et al., 2001).

The initial work on exploring the anti-angiogenic effect of TSP-1 through CD36 utilized peptides containing the CSVTCG sequence. These peptides inhibited endothelial cell migration and angiogenesis (Iruela-Arispe et al., 1991; Tolsma et al., 1993; Dawson et al., 1999). Antibodies to CD36 also inhibited endothelial cell migration (Dawson et al., 1997) and, in CD36-null mice, TSP-1 did not inhibit angiogenesis in a cornea pocket assay (Jiménez et al., 2000). Therefore, binding of TSP-1 to CD36 on endothelial cells inhibits angiogenesis and tumor progression.

TSP-1 and integrins

Integrins are a family of cell surface receptors composed of both an α and a β subunit (Hynes, 1992). TSP-1, in both soluble and matrix-bound forms, can interact with β1 and β3 integrins; however, the physiological consequences of binding are dependent upon the integrin engaged, the cell type, and in some cases the involvement of accessory proteins.

TSP-1 and β1 integrins

In breast carcinoma cells, α3β1 is essential for chemotaxis towards TSP-1 and cell spreading on an immobilized TSP-1 matrix (Chandrasekaran et al., 1999). This interaction is mediated through binding of the integrin to residues 190 to 201 of the N-terminal region of TSP-1 (Krutzsch et al., 1999). In the presence of a β1-activating antibody, the adhesive properties of the carcinoma cells on TSP-1 are enhanced. This is characterized by rearrangement of F actin filaments into filopodia that terminate at points that are rich in β1 and are in contact with TSP-1. Signaling through the insulin-like growth factor-I receptor (IGF-IR) can also potentiate this adhesion. Recent evidence suggests that IGF-IR signaling activates α3β1 by promoting association with the mitochondrial molecule heat shock protein 60 (Barazi et al., 2002).

Small-cell lung carcinoma cells also bind residues 190 to 201 of TSP-1 through α3β1 (Guo et al., 2000). This interaction stimulates the cells to extend neurite-like processes and differentiate along a neuronal pathway. When epidermal growth factor is added to these cultures, binding to TSP-1 through this receptor also suppresses cell proliferation. This mechanism may be important for the antitumorigenic effects of TSP-1.

In response to loss of cell-cell contact, endothelial cells engage immobilized TSP-1 through α3β1 and are stimulated to adhere to TSP-1 and proliferate (Chandrasekaran et al., 2000). This effect can be induced through disruption of cell contacts through wounding or by inhibiting vascular endothelial (VE) cadherin, indicating a role for TSP-1 in supporting repair of wounded endothelium. However, classically, TSP-1 is known for inhibiting endothelial cell proliferation and angiogenesis (Good et al., 1990). Indeed, endothelial cells exposed to a soluble TSP-1 peptide that recognizes α3β1 have decreased proliferation and motility (Chandrasekaran et al., 2000). These opposing effects on endothelial cells suggest that tight regulation of TSP-1/α3β1 interaction and signaling exists. Recent studies using melanoma cells demonstrated that the ability of TSP-1 to bind α3β1 is altered when TSP-1 is bound to fibronectin (Rodrigues et al., 2001). Conformational regulation of TSP-1 may represent one mechanism by which integrin-mediated cellular responses are controlled.

Activated T-lymphocytes can adhere to intact TSP-1 through α4β1 and α5β1 integrins (Yabkowitz et al., 1993). This may have implications for mediating T cell activation, as stimulation of the ERK pathway by TSP-1 in these cells can be inhibited using anti-β1 function-blocking antibodies (Wilson et al., 1999). A role for TSP-1 in modulating the inflammatory response would not be surprising since TSP-1-deficient mice suffer from inflammatory disease (Lawler et al., 1998).

TSP-1 and β3 integrins

In platelets, it was originally discovered that αvβ3 and, to a lesser extent, αIIbβ3 (GPIIbIIIa) function as adhesion receptors for TSP-1. The recognition site for these integrins is the RGD motif located in the type 3 repeats of TSP-1.

TSP-1 can influence integrin function directly and indirectly through its interaction with nonintegrin receptors. In platelets, binding of the C terminus of TSP-1 to the transmembrane receptor integrin-associated protein (IAP or CD47) leads to assembly of a TSP-1/IAPαIIbβ3 complex on the platelet surface. This complex can further activate αIIbβ3 and cause phosphorylation of focal adhesion kinase, resulting in both augmentation of platelet aggregation and attachment to fibrinogen (Chung et al., 1997). A necessity for G-protein signaling has since been added to this cascade of events (Frazier et al., 1999).

TSP-1/IAPαvβ3 complexes are also important in other cell types. On vitronectin substrates, C32 human melanoma cells are stimulated to spread in response to complex formation (Gao et al., 1996). More recently, an increase in latent TGF-β activation, induced by tamoxifen treatment of breast carcinoma cells, has been shown to be dependent on localization of TSP-1 to the cell surface by this mechanism (Harpel et al., 2001).

Another example of TSP-1 affecting integrin function through cooperation with other receptors occurs in the clearance of apoptotic neutrophils. Here, TSP-1 associates with CD36 on the macrophage surface and αvβ3 associates on the neutrophils where it forms a bridge, allowing the recognition of neutrophils for ingestion (Savill et al., 1992). This process can be modulated on a second exposure of macrophages to neutrophils by ligation of αvβ3, α6β1, and α1β2 (Erwig et al., 1999).

αvβ3 is also expressed on endothelial cells. In sickle cell anemia patients, both αvβ3 in the endothelium (Solovey et al., 1999) and TSP-1 plasma levels are elevated. These proteins have been implicated in recurring vaso-occlusion problems in sickle cell patients caused by exaggerated adhesion of the sickle cell red blood cells (SS-RBCs) to the endothelium. Indeed, it has been demonstrated that TSP-1 enhances adhesion of SS-RBCs to cultured endothelial cells and that antibodies to αvβ3 can block this event (Kaul et al., 2000). It is as yet unknown if this is a direct consequence of TSP-1/αvβ3 association.

Critical Parameters

The response of the platelets to thrombin is a critical factor contributing to the success of the purification procedure. Since platelets become less responsive during storage, the platelet-rich plasma should be processed as soon as possible after collection. Since platelets are temperature sensitive, buffers and centrifuges used in the purification procedure should be warmed to room temperature before beginning the procedure. The platelets should also be handled gently during the resuspension steps to prevent mechanical activation. Moreover, since platelets are activated by untreated glass surfaces, all transfer pipets and tubes should be plastic.

TSP-1 is susceptible to proteolysis following its secretion into the supernatant. It is important to work quickly following the activation step to minimize exposure to proteases secreted from the platelets and the thrombin used for the activation. The supernatant should be treated immediately with DFP following the debris-clearing centrifugation step in order to inactivate these proteases. The supernatant should be kept on ice at all times during the remaining purification steps.

The association of TSP-1 with calcium maintains the confirmation of the molecule. It is therefore essential that calcium be present in all solutions during and subsequent to thrombin treatment. A concentration of 2 mM is recommended.

Troubleshooting

The problem most likely to be encountered in the purification procedure is unresponsive platelets. To remedy this situation the procedure can be performed on a small scale using fresh platelets. This will provide a sense of how the aggregated platelets should appear following thrombin treatment. Another method for assaying platelet responsiveness is to perform electrophoresis on the supernatant from the thrombin-treated platelets. TSP-1 is a major component of the platelet α-granule and should appear as a prominent band running at an apparent molecular weight of 185,000 Da on discontinuous Laemmli SDS gels (*UNIT 6.1*). This anomalously high value for the molecular weight of the subunit is probably due to a decrease in the amount of SDS bound to the large number of negatively charged residues in the type 3 repeats.

Anticipated Results

The purification procedure should result in producing ~200 μg TSP-1 per 100 ml outdated platelet-rich plasma, which is most often >95% pure as determined by SDS-PAGE. There is evidence that some preparations of TSP-1 produced according to this method may contain trace amounts of active TGF-β bound to the TSP-1. It is possible to remove this contaminant by adjusting the pH of the sucrose gradient solutions to pH 11, as TGF-β will dissociate from TSP-1 under alkaline conditions (Murphy-Ullrich et al., 1992; Schultz-Cherry et al., 1994). The pH of the TSP-1-containing fractions should be returned to pH 7.6 immediately following centrifugation.

Whereas the protocol for purifying TSP-1 proteolytic fragments does not require many steps and is reasonably efficient, it is important to bear in mind that the N-terminal domain only represents ~18% of the total mass of the protein. Thus, if one starts with 5 mg total protein, a yield of 400 to 500 μg is appropriate. Since the 210,000-Da fragment represents about one-half of the protein, yields of 1 to 1.5 mg can be expected.

Time Considerations

The purification procedure is extended over a period of 3 days. The amount of time required to perform this procedure will depend in part on the amount of material to be processed. Approximately 3 to 4 hr should be allowed to isolate the TSP-1-containing supernatant (steps 1 to 15). Purification of TSP-1 (steps 17 to 28) will require another 3 to 4 hr. It is possible to leave the TSP-1 bound to the heparin-Sepharose column for a number of days prior to continuing the elution process.

The purification of proteolytic fragments also takes ~3 days. The limited tryptic digestion is done overnight. Elution of the heparin-Sepharose column can be done in ~1 day and the elution of the G-200 column requires another day.

LITERATURE CITED

Abdelouahed, M., Ludlow, A., Brunner, G. and Lawler, J. 2000. Activation of platelet-transforming growth factor β-1 in the absence of thrombospondin-1. *J. Biol. Chem.* 275:17933-17936.

Adams, J. 2001. Thrombospondins: Multifunctional regulators of cell interactions. *Annu. Rev. Cell Dev. Biol.* 17:25-51.

Barazi, H.O., Zhou, L., Smyth Templeton, N., Krutzsch, H.C., and Roberts, D.D. 2002. Identification of heat shock protein 60 as a molecular mediator of α3β1 integrin activation. *Cancer Res.* 62:1541-1548.

Bein, K. and Simons, M. 2000. Thrombospondin-1 type 1 repeats interact with matrix metalloproteinase 2: Regulation of metalloproteinase activity. *J. Biol. Chem.* 275:32167-73.

Benaud, C., Dickson, R.B. and Thompson, E.W. 1998. Roles of the matrix metalloproteinases in mammary gland development and cancer. *Breast Cancer Res. Treatment* 50:97-116.

Bornstein, P. 1995. Diversity of function is inherent in matricellular proteins: An appraisal of thrombospondin 1. *J. Cell Biol.* 130:503-506.

Brown, L.F., Guidi, A.J., Schnitt, S.J., Water, L.V.D., Iruela-Arispe, M.L., Yeo, T.-K., Tognazzi, K., and Dvorak, H.F. 1999. Vascular stroma formation in carcinoma *in situ*, invasive carcinoma and metastatic carcinoma of the breast. *Clin. Cancer Res.* 5:1041-1056.

Chandrasekaran, S., Guo, N.-H., Rodrigues, R.G., Kaiser, J., and Roberts, D.D. 1999. Pro-adhesive and chemotactic activities of thrombospondin-1 for breast carcinoma cells are mediated by a3b1 integrin and regulated by insulin-like growth factor 1 and CD98. *J. Biol. Chem.* 274:11408-11416.

Chandrasekaran, L., He, C.H., Al-Barazi, H., Krutzsch, H.C., Iruela-Arispe, M.L., and Roberts, D.D. 2000. Cell-contact-dependent activation of α3β1 integin modulates endothelial cell responses to thrombospondin-1. *Mol. Biol. Cell* 11:2885-2900.

Chen, H., Herndon, M.E., and Lawler, J. 2000. The cell biology of thrombospondin-1. *Matrix Biol.* 19:597-614.

Chung, J., Gao, A., and Frazier, W.A. 1997. Thrombospondin acts via integrin associated protein to activate the platelet integrin αIIbβ3. *J. Biol. Chem.* 272:14740-14746.

Clezardin, P., Lawler, J., Amiral, J., Quentin, G., and Delmas, P. 1997. Identification of cell adhesive active sites in the N-terminal domain of thrombospondin-1. *Biochem. J.* 321:819-827.

Crawford, S.E., Stellmach, V., Murphy-Ullrich, J.E., Ribeiro, S.M.F., Lawler, J., Hynes, R.O., Boivin, G.P. and Bouck, N. 1998. Thrombospondin-1 is a major activator of TGF-β1 in vivo. *Cell* 93:1159-1170.

Crombie, R. and Silverstein, R. 1998. Lysomsomal integral membrane protein II binds thrombospondin-1. *J. Biol. Chem.* 273:4855-4863.

Dawson, DW., Pearce, S.F.A., Zhong, R., Silverstein, R.L., Frazier, W.A., and Bouck, N.P. 1997. CD36 mediates the in vitro inhibitory effects of thrombospondin-1 on endothelial cells. *J. Cell Biol.* 138: 707-717.

Dawson, D.W., Volpert, O.V., Pearce, S.F.A., Schneider, A.J., Silverstein, R.L., Henkin, J., and Bouck, N. 1999. Three distinct *d*-amino acid substitutions confer potent antiangiogenic activity on an inactive peptide derived from a thrombospondin-1 type 1 repeat. *Molec. Pharmacol.* 55:332-338.

Dollery, C.M., McEwan, J.R., and Henney, A.M. 1995. Matrix metalloproteinases and cardiovascular disease. *Circ. Res.* 77:863-868.

Erwig, L.P., Gordon, S., Walsh, G.M., and Rees, A.J. 1999. Previous uptake of apoptotic neutrophils or ligation of integrin receptors downmodulates the ability of macrophages to ingest apoptotic neutrophils. *Blood* 93:1406-1412.

Febbraio, M., Hajjar, D.P., and Silverstein, R.L. 2001. CD36: A class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J. Clin. Invest.* 108:785-791.

Frazier, W.A., Gao, A., Dimitry, J., Chung, J., Brown, E.J., Lindberg, F.P., and Linder, M.E. 1999. The thrombospondin receptor integrin-associated protein (CD47) functionally couples to heterotrimeric G_i. *J. Biol. Chem.* 274:8554-8560.

Gao, A.-G., Lindberg, F.P., Dimitry, J.M., Brown, E.J., and Frazier, W.A. 1996. Thrombospondin modulates αvβ3 function through integrin-associated protein. *J. Cell Biol.* 135:533-544.

Good, D.J., Polverini, P.J., Rastinejad, F., Le Beau, M.M., Lemons, R.S., Frazier, W.A., and Bouck, N. 1990. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. U.S.A.* 87:6624-6628.

Grainger, D.J. and Frow, E.K. 2000. Thrombospondin-1 does not activate transforming growth factor β1 in a chemically defined system or in smooth muscle cell cultures. *Biochem J.* 350:291-298.

Guo, N.-H., Krutzsch, H.C., Inman, J.K., and Roberts, D.D. 1997. Thrombospondin-1 and type 1 repeat peptides of thrombospondin-1 specifically induce apoptosis of endothelial cells. *Cancer Res.* 57:1735-1742.

Guo, N.-H., Smyth Templeton, N., Al-Barazi, H., Cashel, J., Sipes, J.M., Krutzsch, H.C., and Roberts, D.D. 2000. Thrombospondin-1 promotes α3β1 integrin-mediated adhesion and neurite-like outgrowth and inhibits proliferation of small cell lung carcinoma cells. *Cancer Res.* 60:457-466.

Harpel, J.G., Shultz-Cherry, S., Murphy-Ullrich, J.E., and Rifkin, D.B. 2001. Tamoxifen and estrogen effects on TGF-βformation: Role of thrombospondin-1, αvβ3, and integrin-associated protein. *Biochem. Biophys. Res. Comm.* 284:11-14.

Huang, M.-M., Bolen, J.B., Barnwell, J.W., Shattil, S., and Brugge, J.S. 1991. Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn and Yes protein-tyrosine kinases in human platelets. *Proc. Natl. Acad. Sci. U.S.A.* 88:7844-7848.

Hynes, R.O. 1992. Integrins: Versatility, modulation and signaling in cell adhesion. *Cell* 69:11-25.

Iruela-Arispe, L., Bornstein, P., and Sage, H. 1991. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 88:5026-5030.

Iruela-Arispe, M.L., Lombardo, B., Krutzsch, H.C., Lawler, J., and Roberts, D.D. 1999. Inhibition of angiogenesis by thrombospondin-1 is mediated by 2 independent regions within the type 1 repeats. *Circulation* 100:1423-1431.

Jiménez, B., Volpert, O.V., Crawford, S.E., Febbraio, M., Silverstein, R.L., and Bouck, N. 2000. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nature Med.* 6:41-48.

Kaul, D.K., Tsai, H.M., Liu, X.D., Nakada, M.T., Nagel, R.L., and Coller, B.S. 2000. Monoclonal antibodies to αvβ3 (7E3 and LM609) inhibit sickle red blood cell-endothelium interactions induced by platelet-activating factor. *Blood* 95:368-374.

Krutzsch, H.C., Choe, B.J., Sipes, J.M., Guo, N.-H., and Roberts, D.D. 1999. Identification of an α3β1 integrin recognition sequence in thrombospondin-1. *J. Biol. Chem.* 274:24080-24086.

Lawler, J. 2002. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J. Cell. Mol. Med.* 6:1-12.

Lawler, J. and Hynes, R.O. 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* 103:1635-1648.

Lawler, J., Sunday, M., Thibert, V., Duquette, M., George, E.L., Rayburn, H., and Hynes, R.O. 1998. Thrombospondin-1 is required for normal pulmonary homeostasis and its absence causes pneumonia. *J. Clin. Invest.* 101:982-992.

Lee, J., Weber, M., Mejia, S., Bone, E., Watson, P., and Orr, W. 2001. A matrix metalloproteinase inhibitor, batimastat, retards the development of osteolytic bone metastases by MDA-MB-231 human breast cancer cells in BalbC nu/nu mice. *Eur. J. Cancer* 37:106-113.

Markowitz, S.D. and Roberts, A.B. 1996. Tumor suppressor activity of the TGF-βpathway in human cancers. *Cytokine Growth Factor Rev.* 7:93-102.

Martorana, A.M., Zheng, G., Crowe, T.C., O'Grady, R.L., and Lyons, J.G. 1998. Epithelial cells upregulate matrix metalloproteinases in cells within the same mammary carcinoma that have undergone an epithelial-mesenchymal transition. *Cancer Res.* 58:4970-4979.

Merle, B., Malaval, L., Lawler, J., Delmas, P., and Clezardin, P. 1997. Decorin inhibits cell attachment to thrombospondin-1 by binding to a KKTR-dependent cell adhesive site present within the N-terminal domain of thrombospondin-1. *J. Cell. Biochem.* 67:75-83.

Miao, W.-M., Seng, W.L., Duquette, M., Lawler, P., Laus, C., and Lawler, J. 2001. Thrombospondin-1 type 1 repeat recombinant proteins inhibit tumor growth through transforming growth factor β dependent and independent mechanisms. *Cancer Res.* 61:7830-7839.

Munger, J.S., Harpel, J.G., Gleizes, P.-E., Mazzieri, R., Nunes, I., and Rifkin, D.B. 1997. Latent transforming growth factor-β: Structural features and mechanisms of activation. *Kidney Int.* 51:1376-1382.

Munger, J.S., Huang, X., Kawakatsu, H., Griffiths, M.J.D., Dalton, S.L., Wu, J., Pittet, J.-F., Kaminski, N., Garat, C., Matthay, M.A. et al. 1999. The integrin αvβ6 binds and activates latent TGFβ-1: A mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 96:319-328.

Murphy-Ullrich, J.E., Schultz-Cherry, S., and Höök, M. 1992. Transforming growth factor-βcomplexes with thrombospondin. *Mol. Biol. Cell* 3:181-188.

Nielsen, B.S., Sehested, M., Kjeldsen, L., Borregaard, N., Rygaard, J., and Danø, K. 1997. Expression of matrix metalloprotease-9 in vascular pericytes in human breast cancer. *Lab. Invest.* 77:345-355.

Nör, J.E., Mitra, R.S., Sutorik, M.M., Mooney, D.J., Castle, V.P., and Polverini, P.J. 2000. Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway. *J. Vasc. Res.* 37:209-218.

Qian, X., Wang, T.N., Rothman, V. L., Nicosia, R.F., and Tuszynski, G.P. 1997. Thrombospondin-1 modulates angiogenesis in vitroby up-regulation of matrix metalloproteinase-9 in endothelial cells. *Exper. Cell Res.* 235:403-412.

Remacle, A.G., Noël, A., Duggan, C., McDermott, E., O'Higgins, N., Foidart, J.M., and Duffy, M.J. 1998. Assay of matrix metalloproteinases types 1, 2, 3 and 9 in breast cancer. *Br. J. Cancer* 77:926-931.

Rodrigues, R.G., Guo, N.-H., Zhou, L., Sipes, J.M., Williams, S.B., Smyth Templeton, N., Gralnick, H.R., and Roberts, D.D. 2001. Conformational regulation of the fibronectin binding and α3β1 integrin-mediated adhesive activities of thrombospondin-1. *J. Biol. Chem.* 276:27913-27922.

Rodrídguez-Manzaneque, J.C., Lane, T.F., Ortega, M.A., Hynes, R.O., Lawler, J., and Iruela-Arispe, M.L. 2001. Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and moblization of vascular endothelial growth factor. *Proc. Natl. Acad. Sci. U.S.A.* 98:12485-12490.

Rudolph-Owen, L.A., Chan, R., Muller, W.J., and Matrisian, L.M. 1998. The matrix metalloproteinase matrilysin influences early-stage mammary tumorigenesis. *Cancer Res.* 58:5500-5506.

Savill, J., Hogg, N., Ren, Y., and Haslett, C. 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J. Clin. Invest.* 90:1513-1522.

Schultz-Cherry, S., Ribeiro, S., Gentry, L., and Murphy-Ullrich, J.E. 1994. Thrombospondin binds and activates the small and large forms of latent transforming growth factor-βin a chemically defined system. *J. Biol. Chem.* 269:26775-26782.

Schultz-Cherry, S., Chen, H., Mosher, D.F., Misenheimer, T.M., Krutzsch, H.C., Roberts, D.D., and Murphy-Ullrich, J.E. 1995. Regulation of transforming growth factor-βactivation by discrete sequences of thrombospondin-1. *J. Biol. Chem.* 270:7304-7310.

Simantov, R., Febbraio, M., Crombie, R., Asch, A.S., Nachman, R.L., and Silverstein, R.L. 2001. Histidine-rich glycoprotein inhibits the antiangiogenic effect of thrombospondin-1. *J. Clin. Invest.* 107:45-52.

Solovey, A., Gui, L., Ramakrishnan, S., and Hebbel, R.P. 1999. Sickle cell anemia as a possible state of enhanced anti-apoptotic tone: Survival effect of vascular endothelial growth factor on circulation and unanchored endothelial cells. *Blood* 93:3824-3830.

Streit, M., Velasco, P., Brown, L.F., Skobe, M., Richard, L., Riccardi, L., Lawler, J., and Detmar, M. 1999. Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am. J. Pathol.* 155:441-452.

Tolsma, S.S., Volpert, O.V., Good, D.J., Frazier, W.A., Polverini, P.J., and Bouck, N. 1993. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have antiangiogenic activity. *J. Cell Biol.* 122:497-511.

Volpert, O.V., Lawler, J., and Bouck, N.P. 1998. A human fibrosarcoma inhibits systemic angiogenesis and the growth of experimental metastases via thrombospondin-1. *Proc. Natl. Acad. Sci. U.S.A.* 95:6343-6348.

Weinstat-Saslow, D.L., Zabrenetzky, V.S., VanHoutte, K., Frazier, W.A., Roberts, D.D., and Steeg, P.S. 1994. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res.* 54:6504-6511.

Wilson, K.E., Li, Z., Kara, M., Gardner, K.L., and Roberts, D.D. 1999. β1 integrin- and proteoglycan-mediated stimulation of T lymphoma cell adhesion and mitogen-activated protein kinase signaling by thrombospondin-1 and thrombospondin-1 peptides. *J. Immunol.* 163:3621-3628.

Yabkowitz, R., Dixit, V.M., Guo, N., Roberts, D.D., and Shimizu, Y. 1993. Activated T-cell adhesion to thrombospondin is mediated by the α4β1 (VLA-4) and α5β1 (VLA-5) integrins. *J. Immunol.* 151:149-158.

Zajchowski, D.A., Band, V., Trask, D.K., Kling, D., Connolly, J.L., and Sager, R. 1990. Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 87:2314-2318.

Contributed by Karen O Yee, Mark Duquette, Anna Ludlow, and Jack Lawler
Beth Israel Deaconess Medical Center
Boston, Massachusetts

Purification of SPARC/Osteonectin

SPARC (secreted protein acidic and rich in cysteine) is a founding member of the matricellular group of proteins that have been shown to mediate interactions between cells and the extracellular matrix (ECM; Bornstein and Sage, 2002). Other proteins within this family include thrombospondins 1 and 2, osteopontin, tenascins C and X, and Cyr61. Over the last several years, a wealth of data, largely from mice with targeted disruptions of the respective genes, has emerged identifying various targets of the matricellular proteins that influence cell behavior—e.g., growth factors, cell-cycle regulatory proteins, ECM components, adhesion proteins and/or their receptors, cell survival, collagen fibrillogenesis, and immune cell function. In vivo, these effects can be translated into abnormalities in blood vessel morphogenesis and connective tissues, wound healing, bone formation, and responses to various types of injury. Therefore, study of one or more of the matricellular proteins affords insight from a somewhat unusual and underexplored perspective: the interface between the cell surface and the extracellular milieu.

SPARC belongs to a family of several genes, only one other of which, *SC1/hevin*, has been characterized beyond a limited degree (Brekken and Sage, 2000). *SPARC*-null mice exhibit many phenotypic abnormalities that follow logically from the effects of SPARC on cultured cells (i.e., de-adhesion, antiproliferation, interaction with growth factors and ECM, and regulation of collagen production). These characteristics include (1) accelerated dermal wound healing and fibrovascular invasion of sponge implants, (2) reduced foreign body response, (3) thin skin with decreased collagen, which is deposited as small-diameter fibrils, (4) excessive accumulation of adipose tissue, (5) osteopenia, and (6) cataract formation (Bornstein and Sage, 2002). Providing a mechanistic explanation for any one of these phenotypes requires experiments, largely in vitro, with active purified protein in clearly defined assays with quantitative endpoints. This unit presents several protocols for the purification of SPARC (see Basic Protocol and Alternate Protocols 1, 2, and 3), and for the measurement of its biological activity and conformation (see Support Protocols 1 and 2). Since the end product—i.e., natural SPARC or recombinant (rSPARC)—differs according to the source, guidelines for the choice of each protocol, and its advantages and limitations, have been included with the Basic Protocol (purification of SPARC from cultured cells), Alternate Protocol 1 (rSPARC from *E. coli*), Alternate Protocol 2 (rSPARC from insect cells), and Alternate Protocol 3 (SPARC from blood platelets). A method for determining endotoxin levels is presented in Support Protocol 3.

NOTE: To prevent denaturation of SPARC due to adsorption to surfaces, only polypropylene or siliconized glass should be used.

NOTE: All solutions and equiptment coming into contact with live cells should be sterile and a septic technique should be used accordingly

PURIFICATION OF SPARC FROM PYS-2 CELLS

BASIC PROTOCOL

This protocol describes the purification of SPARC from cultured PYS-2 cells. This cell line, originally derived from a mouse parietal yolk sac carcinoma, has been a consistent reproducible source of biologically active SPARC for nearly two decades (Sage and Bornstein, 1995). The following procedure can be applied to most cell culture supernatants and involves essentially three steps: (1) precipitation of culture medium, (2) ion-exchange chromatography, and (3) molecular-sieve chromatography. Advantages of the PYS-2 cell line are its immortality, its high rate of growth, its copious production (secretion) of SPARC, and the presence of few other secreted products in the culture medium. It is also possible to radiolabel SPARC metabolically if desired. A commercial

Contributed by E. Helene Sage
Current Protocols in Cell Biology (2003) 10.11.1-10.11.23

source of SPARC, isolated according to this protocol and of ~80% purity, is available from Sigma-Aldrich.

Materials

50% to 70% confluent PYS-2 cells (see recipe)
DMEM (serum-free; *APPENDIX 2A*)
1100 Ci/mmol (12.5 Ci/ml) [*trans*-^{35}S]methionine/cysteine (ICN; optional)
DMEM minus methionine and cysteine (optional)
0.2 M PMSF stock solution (see recipe)
N-Ethylmaleimide (NEM)
Ammonium sulfate, ultrapure
DEAE buffer, 4°C (see recipe)
NaCl
~2 × 20–cm DEAE column (see recipe)
S-200 buffer (see recipe)
Scintillation fluid (optional)
Sephacryl molecular-sieve column (see recipe)
0.05 M acetic acid

Plastic pipets
50-ml polycarbonate high-speed centrifuge tubes
Low-speed GPKR (Beckman) centrifuge with swinging bucket rotor
High-speed refrigerated centrifuge with GSA (Sorvall) or JA-17 rotors (Beckman) or equivalent
12,000- to 14,000-MWCO dialysis tubing (Spectrapor) or equivalent, prewashed with DEAE buffer
Dialysis clips (optional)
Standard gradient maker (e.g., Amersham Biosciences)
Peristaltic pump
Fraction collector
Lyophilizer
50 or 250 ml centrifuge tubes

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) with autoradiography (*UNIT 6.3*), if appropriate, and determination of protein concentration by spectroscopy (*APPENDIX 3B*)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiments and dispose of wastes in appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Collect and precipitate tissue culture medium containing secreted SPARC

1. Replace medium in 20 to 30 dishes or flasks of PYS-2 cells (grown to 50% to 70% confluency) with 12 to 13 ml serum-free DMEM and preincubate 15 min at 37°C. Replace with fresh medium and then incubate 18 to 24 hr.

   If desired, purification can be monitored by adding 500 μCi of 1100 Ci/mmol [^{35}S]methionine to one dish and processing the medium in parallel with nonlabeled medium from the other dishes. Alternatively, if radiolabeled SPARC of high specific activity is required for experimental purposes, [^{35}S]methionine/cysteine can be added to all dishes. When using label, incubate cells in serum-free DMEM lacking methionine and cysteine.

2. Collect the medium from the cell layer by gentle aspiration via plastic pipet and transfer to centrifuge tubes. Remove cellular debris by centrifuging in a clinical (i.e.,

tissue-culture) centrifuge 5 min at 1,000 × *g*, room temperature, or in GPKR centrifuge at 1000 × *g*, 4°C.

3. Pool all supernatants in a siliconized flask. Add 0.2 M PMSF drop-wise with stirring to a final concentration of 0.2 mM, and NEM to a final concentration of 10 mM. Stir on ice until medium reaches 4°C.

   For 100 ml medium, add 0.1 ml PMSF stock solution and 62.5 mg NEM. Take care not to lyse cells in any of these procedures.

4. Add solid ultrapure ammonium sulfate to the medium in an amount equivalent to 50% (w/v) of the starting volume over a period of several hours. Stir 12 to 24 hr at 4°C.

   For 100 ml medium, add 50 g ammonium sulfate, in very small increments (e.g., 1 to 2 g) over several hours (e.g., 3 to 5). This detail is important for maintenance of neutral pH and for efficient precipitation of protein, which consists mainly of laminin 1, type IV collagen, bovine serum albumin (BSA), and SPARC.

   Do not allow the solution to foam by stirring too rapidly, as this indicates the proteins are denaturing.

5. Transfer medium to 50-ml polycarbonate high-speed centrifuge tubes and centrifuge in a high-speed refrigerated centrifuge with JA-17 rotor 30 min at 40,000 × *g*, 4°C. Discard the supernatant. Keep tubes containing pellets on ice or store up to 1 to 2 months at –70°C.

6. Thaw, if necessary, and dissolve each pellet by gentle vortexing in 2 to 5 ml DEAE buffer, 4°C. Pool these solutions and transfer to 12,000- to 14,000-MWCO dialysis tubing, prewashed with DEAE buffer and closed on one end. Rinse each centrifuge tube with 1 ml buffer and add this solution to the bag.

7. Close the open end of the dialysis bag with double knots or dialysis clips, leaving 1 to 2 in. (2.5 to 5 cm) extra space to allow for change in volume. Immerse the bag (containing ~40 ml) in a 500-ml graduated cylinder containing 500 ml DEAE buffer, 4°C. Dialyze with stirring overnight (or 4 to 6 hr), and change the dialysis buffer twice (2 to 3 hr each) for an additional 4 to 6 hr dialysis.

   Wear gloves when handling dialysis tubing to minimize exposure to radioactivity as well as to protect the sample from contamination. Mix bag contents several times by inversion.

8. Remove dialysis tubing, cut tip off carefully (if knotted) or remove clips, and empty contents into one or two 50-ml centrifuge tubes. Clarify the solution by centrifuging in a JA-17 rotor 20 min at 10,000 × *g*, 4°C. If appropriate, retain 10 to 25 µl for scintillation counting and for SDS-PAGE (*UNIT 6.1*) with autoradiography (*UNIT 6.3*), as assessment of starting material.

   The sample is now ready for ion-exchange chromatography.

Chromatograph on DEAE cellulose

9. Prepare gradient buffer B by adding 2.336 g NaCl to 200 ml DEAE buffer (200 mM NaCl final). Fill the front chamber of a standard gradient maker (containing a stir bar or paddle) with 200 ml DEAE buffer (gradient buffer A) and the second chamber with 200 ml gradient buffer B.

   Ensure that the narrow opening between the two chambers is filled with gradient buffer A before adding gradient buffer B. An air block will inhibit flow of B into A.

10. Use a peristaltic pump to add the entire sample onto an ~2 × 20–cm DEAE column, and follow with one to two column volumes DEAE buffer. Discard this eluate, which contains unbound protein.

11. If phenol red (from DMEM) is seen to bind to the resin, wash the column until it is no longer visible, or until the A_{280} of the flowthrough is at baseline.

Phenol red will interfere with the monitoring of the column effluent at 280 nm.

12. Connect the gradient maker to the peristaltic pump for delivery to the column bed. Connect a fraction collector to the column and set to collect 3-ml fractions of eluate in polypropylene or siliconized glass tubes. Elute bound proteins with a linear gradient of 0% to 100% buffer B over ~300 ml.

    All chromatographic procedures must be carried out at 4°C.

    A less complicated alternative to the continuous gradient is the use of two stepwise elutions, the first consisting of 100 ml of 75 mM NaCl in DEAE buffer, followed by 100 ml of 175 mM NaCl in DEAE buffer. SPARC will elute in the second buffer.

13. For radiolabeled SPARC (step 2), monitor the effluent by scintillation counting 20-µl aliquots from alternate fractions suspended in 3 ml scintillation fluid. For nonradiolabeled SPARC, monitor alternate fractions by absorbance at 280 nm.

    SPARC is eluted at 150 to 175 mM NaCl. See Sage et al. (1989) for an example of the elution profile. If the location of the peak containing SPARC is in doubt, individual fractions can be analyzed by SDS-PAGE (UNIT 6.1).

14. Pool fractions containing SPARC, and dialyze the pooled sample (~20 ml) against four changes of 4 liters (each) water over 24 to 48 hr, 4°C (see steps 6 to 8).

    After 24 to 48 hr, a precipitate containing SPARC, together with laminin and traces of BSA, should appear in the dialysis bag. Depending on the concentration of protein and/or the water used (pH 5.5 is optimal), precipitation may fail to occur. In this case, lyophilize the protein (step 16b), redissolve in DEAE buffer at 25% of the original volume, and repeat dialysis and precipitation (steps 14 and 15).

    If the column will be reused, it should be regenerated as described (see Reagents and Solutions).

15. Decant the entire contents of the bag into a centrifuge tube and centrifuge 30 min at 48,000 × *g*, 4°C. Discard the supernatant.

16a. *For immediate use:* Dissolve pellet in 2 ml S-200 buffer, clarify by microcentrifugation for 1 min at top speed or 10,000 × *g*, and proceed to molecular-sieve chromatography (step 18).

16b. *For storage before chromatography:* Resuspend pellet in 2 to 4 ml water, shell-freeze by twirling the tube in dry ice/ethanol to effect freezing of the solution on the sides of the vessel, and then lyophilize. Store up to 1 to 2 months at –70°C. Before use, resuspend in 1 to 2 ml S-200 buffer, stir 4 to 6 hr at 4°C, and clarify the solution by microcentrifugation at top speed for 1 min.

    Shell-freezing increases the efficiency of lyophilization and improves solubility of the protein after storage.

    Pellets from several preparations can be pooled prior to molecular-sieve chromatography.

Purify SPARC by molecular-sieve chromatography

17. Remove buffer from the top of a Sephacryl molecular-sieve column and apply the sample gently onto the resin. Allow the sample (optimally 1 to 2 ml) to flow into the bed. Add 2 to 4 ml S-200 buffer to the top of the column, reconnect the buffer reservoir, and allow effluent to flow by gravity at 8 to 10 ml/hr (0.17 ml/min) by adjustment of the pressure head (i.e., the reservoir containing S-200 buffer above the column).

    It is important not to disturb the column bed during sample loading, as the precision of elution can be affected.

In some cases it may be necessary to use a peristaltic pump, pulling buffer from the bottom of the column, at ~10 ml/hr. If the flow rate is too high, the column will pack too tightly and will cease to flow.

18. Collect 80 fractions of 1 to 1.5 ml each and monitor effluent by absorbance at 280 nm and/or by counting 10 to 25-µl aliquots in 3 ml scintillation fluid.

    The exact position of elution of SPARC will vary with chromatographic parameters (e.g., column size, sample size, flow rate). It is therefore advisable to monitor the column effluent and, if necessary, to check 10 to 25 µl of each fraction by SDS-PAGE (see below). The initial peak (at V_o) contains laminin, whereas the leading shoulder of the peak corresponding to the elution position of SPARC contains most of the BSA.

19. Pool peak fractions corresponding to SPARC (approximately ten fractions, corresponding to 55 to 65 ml total column effluent). Dialyze this pool against four changes of 4 liters of 0.05 M acetic acid each, 4°C, and lyophilize.

    Alternatively, the sample can be stored at –70°C in S-200 buffer without dialysis or lyophilization, or it can be dialyzed directly into another buffer as desired.

20. Determine the concentration of SPARC by absorbance at 280 nm, using the extinction coefficient (ε) 0.838 mg ml^{-1} cm^{-1} (*APPENDIX 3B*).

21. Analyze the purified protein by SDS-PAGE (*UNIT 6.1*) with autoradiography (*UNIT 6.3*). When heating samples at 95°C, use reducing (i.e., 50 mM DTT) and nonreducing conditions.

    For detection using Coomassie blue, from 1 to 5 µg SPARC is recommended; for detection by autoradiography, ~10^4 cpm is recommended. A single broad band, or occasionally a doublet, should be obtained with an apparent M_r of 39,000 (with DDT) or 43,000 (without DDT), the latter co-migrating with an ovalbumin molecular weight standard. The yield of purified SPARC is ~500 µg per 30 maxiplates (150-mm diameter) of PYS-2 cells (2 to 3 × 10^8 cells).

PURIFICATION OF rSPARC FROM *E. COLI*

ALTERNATE PROTOCOL 1

The preceding procedure (see Basic Protocol) allows for the purification of murine SPARC from cultured (tumor) cells. Limitations of a mammalian cell culture system as a protein source are its cost, potential contamination of the product by serum and cellular proteins/proteinases, and the low yield of product. To circumvent these problems, Bassuk et al. (1996a) expressed human rSPARC with a C-terminal histidine tag in *E. coli*. A soluble (monomeric) form and an insoluble (aggregated) form of SPARC were recovered, the latter sequestered in inclusion bodies within the host. Soluble (monomeric) SPARC from *E. coli* is biologically active and can be purified in relatively large quantities with minimal contamination by endotoxin or bacterial proteins. Isolation of the soluble form is accomplished by anion-exchange, nickel-chelate affinity, and gel-filtration chromatographies. Anion-exchange chromatography on DEAE-Sepharose is used as an initial isolation step. Metal-chelate affinity chromatography provides an efficient purification of rSPARC that has been expressed with a $(His)_6$ sequence. Gel-filtration chromatrography separates monomers of SPARC from dimers, trimers, and higher oligomers. This procedure is outlined below. It assumes that a competent strain of *E. coli*—e.g., BL21(DE3)—has been transformed with a SPARC expression plasmid—e.g., pSPARC wt (human)—with a hexahistidine $(His)_6$ sequence at the 3′ end (Bassuk et al., 1996a) and has been propagated and frozen as a glycerol stock.

Additionally, the aggregated form can be unfolded by urea treatment, purified by nickel-chelate affinity chromatography, and renatured by gradual removal of the denaturant. After disulfide bond isomerization, the disaggregated monomers are further purified by

high-resolution gel-filtration chromatography (Bassuk et al., 1996b). As the disaggregation/renaturation procedure is complicated and time consuming, the reader is referred to Bassuk et al. (1996b) for this additional protocol.

Additional Materials *(also see Basic Protocol)*

LB medium with appropriate selective reagents (*APPENDIX 2A*)
E. coli strain transfected with SPARC expression vector (Bassuk et al., 1996a)
Inducing agent (e.g., IPTG; *APPENDIX 3A*)
10 mM sodium phosphate, pH 7.0 (*APPENDIX 2A*)/10% (v/v) glycerol
90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF, 4°C (see recipe), with and without 0.5 M NaCl
DEAE-Sepharose Fast Flow anion-exchange resin (Amersham Biosciences): equilibrate in 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF and allow to settle
5 M NaCl (*APPENDIX 2A*)
0.2 M AEBSF stock solution (see recipe)
Nickel/nitrilotriacetic acid (Ni-NTA) metal-chelate affinity resin (Qiagen)
50 mM sodium phosphate (pH 5.3, 6.0, and 7.8)/0.5 M NaCl/10% (v/v) glycerol (see recipe)
1.6 × 60–cm Superdex 70 column (see recipe)
50 mM Tris·Cl (pH 8.0)/0.15 M NaCl (see recipe)
1× PBS (*APPENDIX 2A*) containing 1 to 4 mM Ca^{2+} (optional)

French press
2 × 20– and 1 × 10–cm chromatography columns
Flow cell coupled to a UV monitor set at 280 nm
Chart recorder
Conductivity meter (optional)
Disposable 10-ml gel-filtration column, sterile (optional)

Additional reagents and equipment for transfecting SPARC expression vector (*APPENDIX 3A*) and for SDS-PAGE on minigels (*UNIT 6.1*)

Extract E. coli

1. Inoculate 1.3 liters LB medium containing appropriate selective reagents with a suitable *E. coli* strain transfected with SPARC expression vector using standard techniques (*APPENDIX 3A*). Grow to midexponential phase (OD_{600} ~0.5) and induce with the appropriate agent.

   Induction of rSPARC in midexponential phase cells is necessary for high levels of expression. The procedure and chemical(s) used depend on the E. coli strain and the vector into which SPARC cDNA is cloned. For example, IPTG was used at a final concentration of 1 mM for SPARC cloned into pET22b vector and transfected into strain BL21(DE3) (Bassuk et al., 1996a).

2. After the cells have been induced, grow an additional 1 to 4 hr.

3. Recover the cells by centrifuging 20 min at 7000 × *g*, room temperature. Discard the supernatant and resuspend the pellet in 20 ml of 10 mM sodium phosphate, pH 7.0, containing 10% (v/v) glycerol. Disrupt by performing two cycles in a French press at 20,000 psi.

   Cells can alternatively be broken open by sonication on ice.

4. Separate soluble from insoluble material by centrifuging 30 min at 10,000 × *g*, 4°C. Decant soluble extract (supernatant) into a separate tube.

Soluble extracts and insoluble pellets at this stage can be stored up to 1 month at –80°C. Refer to Bassuk et al. (1996b) for details on processing pellets for aggregated SPARC.

Perform initial chromatography on DEAE-Sepharose

5. If necessary, thaw the soluble extracts on ice. Dilute to 100 ml with ice-cold 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF, 4°C.
6. Add 50 ml settled DEAE-Sepharose Fast Flow anion-exchange resin. Stir gently 12 to 18 hr at 4°C.
7. Pour slurry into a 2 × 20–cm chromatography column, allow to settle, and wash with ~250 ml of 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF until the absorbance at 280 nm is <0.01.
8. Assemble a linear gradient (see Basic Protocol 1, step 9) by adding 250 ml of 90 mM sodium phosphate buffer pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF to the front compartment of a gradient maker, and 250 ml of 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF with 0.5 M NaCl (7.3 g) to the other compartment.
9. At 4°C pump gradient onto the column at 3 ml/min. Collect 8-ml fractions and monitor the column eluate with a flow cell coupled to a UV monitor set at 280 nm and a chart recorder set at full scale equal to 1 OD unit.

   It is advisable also to monitor the eluate by conductivity; read every fourth fraction in a conductivity meter (clean probe after each reading). rSPARC elutes at a concentration of 0.10 to 0.25 M NaCl (conductivity of 14 to 20 mmho).

10. Analyze 50-µl aliquots of fractions by SDS-PAGE on minigels (see Basic Protocol and *UNIT 6.1*). Pool fractions containing rSPARC, and adjust solution to 0.5 M NaCl by adding 5 M NaCl.

    In the absence of post-translational modification, rSPARC migrates on an SDS-polyacrylamide gel with an apparent M_r of 34,000 to 38,000 Da after reduction. Adjustment of ionic strength can be monitored easily by conductivity measurement; the final conductivity of the pooled fractions containing SPARC should be equivalent to that of 50 mM sodium phosphate (pH 7.8)/0.5 M NaCl/10% (v/v) glycerol. At this point, fractions can be stored up to 1 month at –80°C.

Perform metal-chelate affinity chromatography

11. Add 0.2 M AEBSF stock solution to the pooled sample to a final concentration of 0.2 mM.
12. Mix sample with a slurry of Ni-NTA metal-chelate affinity resin, using 3 to 5 ml resin per liter original bacterial culture. Adjust pH to 7.8 with 1 N NaOH or 1 N HCl, and stir gently for 1 hr at 4°C.
13. Pour slurry into a chromatography column (e.g., 1 × 10 cm), allow to settle, and wash with ~60 ml of 50 mM sodium phosphate (pH 7.8)/0.5 M NaCl/10% (v/v) glycerol at a flow rate of 0.5 ml/min until the absorbance at 280 nm is <0.01.
14. Pass 15 column volumes of 50 mM sodium phosphate (pH 6.0)/0.5 M NaCl/10% (v/v) glycerol through the column to remove nonspecifically bound proteins (i.e., until A_{280} <0.01).
15. Elute rSPARC from the column with 20 ml of 50 mM sodium phosphate (pH 5.3)/0.5 M NaCl/10% (v/v) glycerol.
16. Store up to 1 month in ~1-ml aliquots at –80°C.

Perform gel-filtration chromatography

17. Apply ~1 ml rSPARC solution onto a 1.6 × 60–cm Superdex 70 column and elute by gravity using 50 mM Tris·Cl (pH 8.0)/0.15 M NaCl buffer at a flow rate of 0.1 ml/min. Collect fractions of 1.25 ml each, monitored at 280 nm.

    Over an elution range of 100 ml, rSPARC monomer elutes between 46 and 50 ml. This should be verified by SDS-PAGE, as should the removal of oligomers (compare migration with and without 50 mM DTT).

18. *Optional:* Perform buffer exchange as needed for experimental design using sterile disposable 10-ml gel-filtration columns. Elute in the buffer of choice (e.g., 1× PBS containing 1 to 4 mM Ca^{2+} for in vitro studies).

19. Store samples up to 3 months at –80°C.

ALTERNATE PROTOCOL 2

PURIFICATION OF rSPARC FROM INSECT (*Sf*9) CELLS

This protocol describes the purification of human rSPARC produced in a baculovirus expression system using insect (*Sf*9) cells (Bradshaw et al., 2000). Advantages of this system over those described above (see Basic Protocol and Alternate Protocol 1) are higher yield of rSPARC, production of protein in a nonbacterial system to minimize contamination by endotoxin (*E. coli*) or serum proteins (mammalian cells), and the potential for post-translational modifications of protein similar to those in mammalian cells.

This protocol assumes that the starting materials are human (or other species) SPARC cDNA (minus the signal sequence) subcloned into a baculovirus expression vector (Pharmingen), *Spodoptera frugiperda* 9 (*Sf*9) insect cells cotransfected with the SPARC expression vector and linearized baculovirus, and high-titer stocks of recombinant virus generated for subsequent infections of *Sf*9 cells grown in suspension in serum-free media (Invitrogen). Information on the latest versions of the *Sf*9/baculovirus expression system is readily available from the Pharmingen instruction manual, *Baculovirus Expression Vector System*, and the Invitrogen manual, *Growth and Maintenance of Insect Cell Lines for Expression of Recombinant Proteins using the Baculovirus Expression System*.

Materials

Sf9 cells (Invitrogen) infected with baculoviral SPARC expression vector, grown in serum-free *Sf*-900 II medium (Invitrogen)
200 mM MOPS, pH 6.5 (see recipe)
10 N NaOH (*APPENDIX 2A*)
Q-Sepharose Fast Flow column (see recipe)
200 and 400 mM LiCl/20 mM MOPS, pH 6.5 (see recipe)
0.1 N acetic acid, 4°C: 0.6 ml glacial acetic acid in 100 ml H_2O
Hanks' buffered saline solution (see recipe)

50-ml conical tube
0.22-µm filter bottle
AktaPrime automated liquid chromatography system (Amersham Biosciences) or equivalent conventional model
10,000-NMWL Ultrafree-15 (Millipore) or Centricon Plus-80 (Amicon) centrifugal filter device
0.22-µm sterile syringe-driven filter

Prepare Sf9 conditioned medium for chromatography

1. Transfer *Sf*9 cells infected at 2 to 4×10^5 cells/ml with baculoviral SPARC expression vector, grown 4 to 5 days in serum-free *Sf*-900 II medium, to 50-ml conical tubes. Centrifuge 45 min at $6000 \times g$, 4°C. Transfer the supernatant to a 0.22-µm filter bottle and discard the cell pellets.

   This system optimizes for the efficient secretion of recombinant protein. It is important to avoid lysis of the cells (and contamination of the medium) during this step.

2. Sterile-filter the supernatant and measure the volume. Add 1/10 vol 200 mM MOPS, pH 6.5, and adjust the pH to 6.5 with 6 N NaOH.

Purify rSPARC by anion-exchange chromatography

3. Pump the sample onto a Q-Sepharose Fast Flow column at a flow rate of 5 ml/min.

4. Using either an AktaPrime automated liquid chromatography system or equivalent conventional model, assemble a continuous linear salt gradient from 200 to 400 mM. Use 200 ml of 200 mM LiCl/20 mM MOPS, pH 6.5, supplied to the buffer valve (AktaPrime System) or front chamber (conventional gradient maker) and 200 ml of 400 mM LiCl/20 mM MOPS, pH 6.5, to the buffer switch valve or rear chamber.

5. Start the gradient pumping at a rate of 5 ml/min and collect 3.5-ml fractions over 300 ml, monitoring the column effluent at 280 nm.

   The fractions can also be checked by SDS-PAGE (see Basic Protocol and Alternate Protocol 1; UNIT 6.1). Human rSPARC produced by Sf9 cells migrates at ~38,000 to 40,000 Da after reduction, and the doublet shifts to a single band of ~36,000 Da in the absence of reducing agent. The doublet is the result of heterogeneous glycosylation (Bradshaw et al., 2000).

Dialyze sample

For dialysis using acetic acid

6a. Pool the fractions containing rSPARC in 12,000- to 14,000-MWCO dialysis tubing and dialyze against three 1- to 2-liter changes (each) of 0.1 N acetic acid, 4°C.

7a. Aliquot the samples according to use. Snap-freeze on dry ice or in liquid nitrogen, lyophilize, and store at –70°C.

   Acetic acid is used when the sample is to be lyophilized and concentrated.

   The above procedure results in rSPARC of ~80% purity by SDS-PAGE. An additional purification step (entailing molecular-sieve chromatography) can be performed after the sample has been lyophilized. Follow the procedure described above (see Basic Protocol, steps 17 to 21). Significant losses of rSPARC (Sf9) should be expected with this procedure, however.

For dialysis using saline solution

6b. Pool the fractions containing rSPARC in 12,000- to 14,000-MWCO dialysis tubing and dialyze against three 4-liter changes (each) of 1× Hanks buffered saline solution (HBSS) containing 1 µM $CaCl_2$. Concentrate in a 10,000-NMWL Ultrafree-15 or Centricon Plus-80 centrifugal filter device to 1 or 2 ml. Filter sterilize using 0.22-µm sterile syringe-driven filter.

   Saline solution is used when the sample is to be used for cell culture.

7b. Aliquot according to use. Snap freeze on dry ice or in liquid nitrogen and store at –70°C.

Analyze SPARC

8. Determine protein content by UV spectroscopy at 280 nm (*APPENDIX 3B*), using the extinction coefficient (ε) 0.838 mg ml^{-1} cm^{-1}. Estimate the purity of SPARC by SDS-PAGE (5 μg/sample lane; *UNIT 6.1*).

The yield is 2 to 4 mg SPARC (~80% purity) per 400 ml of initial Sf9 cell culture suspension (at 2 to 4 × 10^6 cells/ml).

An updated version of the Sf9/baculovirus expression system is now available from Invitrogen. Termed "InsectSelect," it is a virus-free system that relies on expression of protein from a single nonlytic, integrative plasmid transfected into Sf9 or other insect cells, and is claimed to be optimal for secreted proteins.

ALTERNATE PROTOCOL 3

PURIFICATION OF SPARC/OSTEONECTIN FROM TISSUES

SPARC was originally isolated from fetal bovine mineralized bone matrix, of which it is a major noncollagenous component, and was termed osteonectin (Termine et al., 1981). Two other significant sources of SPARC are platelets (osteonectin; Kelm and Mann, 1991) and the Engelbreth-Holm-Swarm (EHS) sarcoma, a murine basement membrane–producing tumor (termed BM-40; Sasaki et al., 1999, and references therein). SPARC, osteonectin, and BM-40 are now recognized as the same protein. Many of the functional properties of SPARC were deduced from biochemical/biophysical studies of the tissue-derived protein, which can be isolated in significantly greater quantities compared to yields typically described from in vitro sources. In this protocol, purification of SPARC from human platelets is described, based on an original report by Kelm and Mann (1990). There are several advantages to using platelets as a source of SPARC: (1) human blood is a readily available source for human SPARC; (2) bovine blood is an excellent source of SPARC and requires neither screening for pathogens nor the rigorous safety procedures associated with the use of human material; and (3) denaturing conditions are not involved (the extraction of osteonectin from bone matrix or EHS tumor includes the use of EDTA and, in some cases, guanidinium·HCl; Termine et al., 1981; Kelm and Mann, 1990; Sasaki et al., 1999). It is important to note, however, that differences have been reported between bone and platelet osteonectin from the same species, notably in the specificity of collagen binding that was attributed to differences in glycosylation (Kelm and Mann, 1991). Investigators interested in tissue-specific modifications of SPARC and their functional implications are encouraged to consult the references cited above. Bovine bone and human platelet osteonectin are available commercially from Calbiochem, although their method of purification is not specified. Haematologic Technologies sells human platelet osteonectin isolated by affinity chromatography on an anti-osteonectin monoclonal antibody column, as well as bovine bone osteonectin isolated from 0.5 M EDTA extracts of demineralized bone. All commercial preparations should be tested for activity in one or more of the assays described (see Support Protocols 1 to 3).

Materials

Platelet-rich plasma or platelet suspension, or informed, nonsmoking, aspirin-free, consenting adult blood donors
0.156 M citrate containing 0.1 M dextrose and 5.0 μM prostaglandin E_1 (Sigma; optional)
0.02 M Tris·Cl, pH 7.6/0.15 and 1.0 M NaCl (see recipe)
Thrombin
Sepharose 4B–AON IgG column (see recipe)
3.0 M NaSCN/0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl (see recipe)
0.05 M NH_4HCO_3

19-G butterfly needles
50 or 250-ml plastic centrifuge bottles with caps
12,000 to 14,000-MWCO dialysis tubing
Lyophilizer

Additional reagents and equipment for thrombin activation of platelets (Kelm and Mann, 1990) and SDS-PAGE (*UNIT 6.1*; also see Basic Protocol, step 22)

Prepare activated platelet supernatant

1a. *For predrawn plasma:* Purchase platelet-rich plasma or platelet suspensions from a local blood bank.

1b. *For in-house drawn plasma:* Draw 480 ml fresh blood from informed, nonsmoking, aspirin-free, consenting adults via 19-G butterfly needles into 0.156 M citrate containing 0.1 M dextrose and 5.0 μM prostaglandin E_1 (to prevent platelet activation). Remove red cells and leukocytes by centrifuging 30 min at 1000 × g, room temperature.

CAUTION: *Appropriate biosafety practices must be followed when working with human blood or blood products. Human blood must be screened for HIV and other infectious viruses. In addition, safety glasses, a double layer of gloves, and protective laboratory clothing should be worn at all times. Use double containment (e.g., place a tube or bag containing blood in a beaker prior to any manipulation) and ensure that all containers including centrifuge bottles are tightly capped.*

2. Centrifuge platelet-rich suspension 30 min at 27,000 × *g*, 4°C, to pellet the platelets. Decant supernatant.

3. Wash platelets with 200 ml of 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl, centrifuge as in step 2. Resuspend platelets in 50 ml of the same buffer. Count with a hemacytometer and suspend 4.5×10^{10} cells in 50 ml of 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl. Activate by adding 2.5 U/ml thrombin as described by Kelm and Mann (1990).

4. Transfer to plastic, capped centrifuge bottles and isolate activated platelets by centrifuging 30 min at 25,000 × *g*, room temperature. Discard the pellet using appropriate containment and retain the supernatant.

CAUTION: *Autoclave human products prior to disposal.*

Isolate SPARC/osteonectin by immunoaffinity chromatography

5. Apply platelet supernatant to a Sepharose 4B-AON IgG column (~2 × 20–cm). After the applied solution has permeated the resin, clamp off the column and allow the sample to remain within the column bed for 16 hr at 4°C.

6. Wash the column with 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl until the effluent shows an A_{280} of 0.01.

7. Wash the column with 0.02 M Tris·Cl (pH 7.6)/1.0 M NaCl until a baseline absorbance is achieved.

8. Elute SPARC/osteonectin with 3.0 M NaSCN/0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl and collect in a single tube or in fractions.

9. Transfer the effluent to 12,000 to 14,000-MWCO dialysis tubing, dialyze against two changes of 2 liters of 0.05 M NH_4HCO_3, and then lyophilize.

Peak fractions of the eluted SPARC can also be dialyzed against 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl, PBS, or DMEM (minus phenol red), as dictated by the intended use of the purified SPARC, and stored up to 1 month at –80°C. If the NH_4HCO_3 fails to lyophilize completely, redissolve the powder in water, dialyze against 0.01 N acetic acid, and repeat the lyophilization.

It is important to minimize the exposure of SPARC/osteonectin to NaSCN, and to keep all reagents at 4°C during affinity chromatography and dialysis.

10. Determine the concentration of SPARC/platelet osteonectin at A_{280} using the extinction coefficient (ε) 0.838 mg ml^{-1} cm^{-1}.

11. Monitor the purity of SPARC by SDS-PAGE (*UNIT 6.1*; also see Basic Protocol, step 22).

Platelet SPARC/osteonectin should be >80% pure by SDS-PAGE using a Coomassie blue stain. It exhibits an apparent M_r of ~3000 greater than that of bone osteonectin purified according to the same protocol (shown to be due to differences in glycosylation), but is comparable to that reported for SPARC isolated from PYS-2 cell culture media.

Immunoaffinity chromatography typically produces somewhat low recoveries of the protein antigen, albeit in a high state of purity given the minimal steps used in the isolation protocol. The total amount of SPARC/osteonectin in human platelets (prior to affinity purification) was reported by Kelm and Mann (1990) to range from 0.65 to 2.2 μg/10^8 platelets.

ASSAYS FOR THE EVALUATION OF SPARC ACTIVITY

All proteins need to be evaluated, not only for their extent of purity, but also for their activity and conformational integrity. The latter is especially critical in the case of recombinant proteins, which are produced either in biologically "inappropriate" hosts (e.g., SPARC in a prokaryotic system) or at levels that preclude proper processing, folding, and/or editing. Moreover, the importance of post-translational modification to the functions of many proteins is poorly understood. In the case of SPARC, N-linked glycosylation (one site) appears not to be critical for activity, at least in the assays that have been used; however, rSPARC (see Alternate Protocols 1 and 2) has consistently displayed less activity (up to 50%) than SPARC purified from PYS-2 cells (see Basic Protocol; Yost et al., 1994; Bassuk et al., 1996a; Bradshaw et al., 2000). These protocols describe biological assays that test two major effects of SPARC on cultured cells: de-adhesion and inhibition of proliferation. Other assays based on biochemical measurements (e.g., circular dichroism, binding assays) are standard procedures and are discussed elsewhere (see Commentary).

SUPPORT PROTOCOL 1

Proliferation Assay

Endotoxin will inhibit cell proliferation, and endothelial (especially BAE) cells are particularly sensitive. At 10 ng endotoxin/mg SPARC, there should be <10% inhibition of [^{3}H]thymidine incorporation in BAE cells exposed to 60 μg SPARC/ml. To determine the effect of endotoxin on other types of cells, treat the cells with a titration of CSE and measure proliferation.

Additional Materials *(also see Basic Protocol)*

Bovine aortic endothelial (BAE) cells
DMEM/0% and 10% (w/v) FBS (*APPENDIX 2A*)
Purified SPARC (see Basic Protocol or Alternate Protocol 1 to 3) and appropriate control buffer
6.71 Ci/mmol (1 mCi/ml) [methyl-^{3}H]thymidine (PerkinElmer)
10% (w/v) trichloroacetic acid (TCA), ice cold
95% (v/v) ethanol
0.4 N NaOH
Glacial acetic acid
Scintillation fluid

24-well tissue culture plate
15-ml conical tube
Radioactivity warning tape

Additional reagents and equipment for trypsinizing cells (*UNIT 1.1*)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiments and dispose of wastes in appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

1. Starve a 100-cm dish of confluent bovine aortic endothelial (BAE) cells in serum-free DMEM for 3 to 4 days.
2. Trypsinize cells (*UNIT 1.1*), resuspend in 10 ml DMEM/10% FBS, and centrifuge briefly (i.e., 5 min at 1000 × *g*, room temperature) to pellet.
3. Rinse cells twice with 5 ml serum-free DMEM.
4. In a 24-well tissue culture plate, plate triplicate wells containing 5×10^4 cells in 500 µl (final 1×10^5 cells/ml) of the following solutions, using the same volume for SPARC and buffer:

   Serum-free DMEM (control)
   DMEM/2%FBS containing 5 µg/ml SPARC dissolved in DMEM
   DMEM/2% FBS containing 20 µg/ml SPARC dissolved in DMEM
   DMEM/2% FBS containing buffer alone (control).

   Other buffers compatible with cell culture may be used but not acetic acid.
5. Incubate 16 to 18 hr at 37°C.

   By this time, cells will have begun to synthesize DNA (S phase).
6. Prepare label in a 15-ml conical tube by adding 20 µl (20 µCi) of 6.71 Ci/mmol [methyl-^{3}H]thymidine/ml DMEM. Place 55 µl (1.1 µCi) of this mixture into each well. Swirl plate gently to mix. Label plate with radioactivity warning tape, and incubate 4 hr at 37°C.

   CAUTION: *Perform this step in a laminar-flow hood with absorbent bench pad and radioactive waste receptacle.*
7. Wash each well twice with 500 µl ice-cold 10% TCA, and drain completely.

   CAUTION: *Collect radioactive media and washes for safe disposal.*
8. Wash with 500 µl of 95% ethanol. Remove ethanol and add 500 µl of 0.4 N NaOH per well. Incubate 30 min at room temperature with shaking.
9. Add 100 µl glacial acetic acid to neutralize the solution.

   Extremes of pH can result in precipitation of scintillation cocktail and/or quenching.
10. Place the contents of each well into a collection vial containing 3 ml scintillation fluid. Cap, mix by inversion, and measure cpm in a scintillation counter.

    There should be no precipitate in the vials; check pH if this occurs and adjust to neutrality.

    For rSPARC, expect >70% inhibition of [^{3}H]thymidine incorporation at 50 µg SPARC/ml. For SPARC purified from PYS-2 cells, the effective dose at which 50% inhibition of [^{3}H]thymidine incorporation occurs (ED_{50}) is 20 µg SPARC/ml.

De-adhesion Assay

SUPPORT PROTOCOL 2

This protocol is presented as a rapid, inexpensive, and diagnostic assay for the de-adhesive activity of SPARC on nontransformed cells in vitro. The activity is based on the diminishment of focal adhesions produced by cultured cells. These structures can be distinguished by immunofluorescence staining of vinculin in wedge-shaped structures at the periphery of the cell, which are diagnostic for focal adhesion complexes.

Additional Materials *(also see Basic Protocol)*

One 100-mm dish of nearly-confluent bovine aortic endothelial (BAE) cells, passaged not greater than ten times, grown in DMEM/10% FBS containing appropriate antibiotics
DMEM/2% and 10% FBS (*APPENDIX 2A*)
Purified SPARC (see Basic Protocol and Alternate Protocols 1 to 3) and appropriate control buffer

12-well tissue culture dishes
Phase-contrast microscope (*UNIT 4.1*)

Additional reagents and equipment for trypsinizing cells (*UNIT 1.1*)

1. Trypsinize (*UNIT 1.1*) a 100-mm dish containing a nearly confluent monolayer of bovine aortic endothelial (BAE) cells, passaged greater than ten times, and grown in DMEM/10% FBS containing appropriate antibiotics.

   The size of the dishes is optional and can be adjusted according to the availability of SPARC. Scale the volume of medium as appropriate for size of dish or well.

2. Transfer trypsinized cells to appropriate centrifuge tubes, pellet in a clinical centrifuge 5 min at $1000 \times g$, room temperature, and resuspend in an appropriate volume of DMEM/2% FBS. Plate 5 to 7.5×10^4 cells in triplicate wells of a 12-well tissue culture dish.

3. Add the following solutions to cells in triplicate, using the same volume for SPARC and buffer:

   No addition
   20 µg/ml SPARC
   40 µg/ml SPARC
   Appropriate control buffer.

4. Mix gently and incubate 1 hr at 37°C.

5. Check the plate carefully under a phase-contrast microscope. Examine several representative fields and count the number of cells in the following groups:

   Fully spread cells (group *a*)
   Partially spread cells (group *b*)
   Rounded cells (group *c*).

   Cells to which SPARC has not been added should be attached and beginning to spread. Cells to which SPARC has been added should be less spread (i.e., rounded). If control cells (i.e., no SPARC) have not spread, wait an additional 1 to 2 hr. There should be no toxicity or cell death.

6. Quantify the activity of SPARC according to the rounding index (RI):

   $RI = [(1 \times a) + (2 \times b) + (3 \times c)]/(a + b + c)$

   An RI = 1 represents a culture with only spread cells, whereas a culture with increasing numbers of round cells would approach the maximum, RI = 3.

   A titration curve can be generated using different concentrations of SPARC. Anticipate that different types of cells will show differential sensitivity to SPARC. Cell lines (e.g., 3T3, NRK) and transformed cells typically do not respond to SPARC.

Endotoxin Assay

Endotoxin is derived from gram-negative bacteria (e.g., *E. coli*) and is a commonly encountered contaminant of buffers, columns, and glassware. In addition, soluble (monomeric) SPARC purified from *E. coli* may contain endotoxin. Endotoxin interferes with bioassays for SPARC, so it is necessary to assess samples for the presence of endotoxin.

Materials

Purified SPARC (see Basic Protocol and Alternate Protocols 1 to 3) and appropriate buffer
Limulus Amoebocyte Lysate (LAL) Pyrochrome kit (Associates of Cape Cod) for the Detection and Quantification of Gram-Negative Bacterial Endotoxin:
Pyrochrome LAL reagent
Pyrochrome Reconstitution buffer
Control Standard Endotoxin (CSE)
50% (v/v) glacial acetic acid

Nonpyrogenic 96-well tissue culture plate
Microtiter plate reader

1. Prepare SPARC titrated at 0.2, 1, and 5 µg/ml in DMEM or HBSS. Pipet 50 µl of each into triplicate wells of a nonpyrogenic 96-well tissue culture plate, leaving 16 empty wells for standards and controls (step 5).
2. Tap vial containing Pyrochrome LAL reagent. Remove and discard stopper.
3. Add 3.2 ml Pyrochrome Reconstitution buffer to the LAL reagent. Mix gently but thoroughly. Cover with Parafilm and place for 3 to 5 min on ice. Store on ice up to 3 hr.
4. Prepare standards by adding 2.0 ml water to the vial containing the Control Standard Endotoxin (CSE) to yield 1.0 endotoxin units (EU)/ml endotoxin. Vortex and store on ice.
5. Place 200 µl water in each of five wells in a fresh 96-well plate. Make a five-step serial dilution using a ratio of 1:1 at each step, by adding 200 µl of 1.0 EU/ml endotoxin to the first well, mixing, transferring 200 µl to the next well, and repeating until the series is complete. Pipet 50 µl of each dilution into triplicate wells of the SPARC-containing plate (step 1), and include a 50-µl water-only control.

   The serial dilutions above will result in final concentrations of 0.5, 0.25, 0.125, 0.0625, and 0.0313 EU/ml endotoxin, respectively.
6. Pipet 50 µl of reconstituted Pyrochrome LAL reagent (step 3) into each well, shake on a microtiter plate shaker for 30 sec, and incubate at 37°C for 30 min.
7. Stop reaction by adding 25 µl of 50% glacial acetic acid per well.
8. Measure OD_{405} in a microtiter plate reader. Determine the concentration of endotoxin in the sample by comparison to the curve generated from the standards.

   The expected concentration of endotoxin is <10 ng/mg SPARC, at an estimated level of 10 EU/ng endotoxin. Endotoxin levels range from 5 to 15 EU/ng. The level of endotoxin that affects cells depends on the cell type.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AEBSF (aminoethylbenzenesulfonyl fluoride) stock solution, 0.2 M

Dissolve 4.794 g AEBSF (Calbiochem) in 100 ml water. Make fresh.

DEAE buffer

500 ml 8 M urea stock solution (see recipe)
50 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
448 ml H_2O
1 ml 0.2 M PMSF stock solution (see recipe)
625 mg *N*-ethylmaleimide (NEM)
Adjust pH to 8.0 with 10 N NaOH
Chill to 4°C
Make fresh buffer for each column run

Final concentrations are 50 mM Tris·Cl, pH 8.0, 0.2 mM PMSF, 10 mM NEM, and 4 M urea.

DEAE column

Pack an ~2 × 20–cm column (e.g., Amersham Biosciences) at 4°C with a 20% slurry of DE-52 cellulose (Whatman) equilibrated in DEAE buffer (see recipe). Equilibrate with several column volumes (~70 ml each) DEAE buffer, delivered via a peristaltic pump connected from a reservoir to the bottom of the column (pumping upward ensures more efficient utilization of theoretical plates for ion exchange). The column can be stored at 4°C and reused for several months. After storage, flush the column with several volumes of fresh DEAE buffer immediately before use. To regenerate a DEAE column, pump one column volume of DEAE buffer containing 500 mM NaCl (29.2 g/liter) followed by several column volumes of DEAE buffer until the absorption and conductivity of the elution buffer is restored to baseline (see Commentary).

Columns manufactured by Amersham Biosciences work well, as they are thick walled and are equipped with high-quality fittings that can withstand the pressures delivered by a peristaltic pump.

Hanks' buffered saline solution

0.14 g/l $CaCl_2$ (1.26 mM final)
40 g/l KCl (5.33 mM final)
0.6 g/l potassium phosphate, monobasic (0.44 mM final)
0.1 g/l magnesium chloride, hexahydrate (0.50 mM final)
0.1 g/l magnesium sulfate, heptahydrate (0.41 mM final)
0.35 g/l sodium bicarbonate (4.00 mM final)
0.048 g/l sodium phosphate, dibasic (0.30 mM final)
Store up to 3 months at 4°C

LiCl, 200 mM/20 mM MOPS, pH 6.5

200 ml of 200 mM MOPS, pH 6.5 (see recipe)
80 ml 5 M LiCl
Add H_2O to 1700 ml
Adjust pH to 6.5 with 6 N NaOH
Add H_2O to 2000 ml
Store up to 1 year at 4°C

LiCl, 400 mM/20 mM MOPS, pH 6.5

50 ml 200 mM MOPS, pH 6.5 (see recipe)
40 ml 5 M LiCl
Add H_2O to 480 ml
Adjust pH with 6 N NaOH
Add H_2O to 500 ml
Store up to 1 year at 4°C

LiCl, 2 M/20 mM MOPS, pH 6.5

50 ml 200 mM MOPS, pH 6.5 (see recipe)
200 ml 5 M LiCl
Add H_2O to 480 ml
Adjust pH with 6 N NaOH
Add H_2O to 500 ml
Store up to 1 year at 4 °C

Molecular-weight standards

Molecular-weight standards include a marker for the excluded (outer; V_o) and included (inner; V_i) volume of the column. For V_o, use 500 µl of 0.1% (w/v) blue dextran in S-200 buffer (see recipe). Clarify by centrifugation before applying to the column. For V_i, use 25,000 to 100,000 cpm [^{35}S]methionine or [^{3}H]proline (which can be detected by scintillation counting), or any small protein (M_r <10,000) or peptide that is minimally hydrophobic and nonglycosylated.

MOPS, 200 mM, pH 6.5

41.86 g 3-(*N*-morpholino)propanesulfonic acid (MOPS)
Add H_2O to ~800 ml
Adjust pH to 6.5 with 6 N NaOH
Add H_2O to 1000 ml
Store up to 1 year at 4°C

NaSCN, 3.0 M/0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl

243.24 g sodium isothiocyanate (NaSCN)
20 ml 1 M 0.22-µm-filter-sterilized Tris·Cl, pH 7.5 (*APPENDIX 2A*)
8.766 g NaCl
Add H_2O to ~900 ml
Adjust pH to 7.6 with 6 N NaOH
Add H_2O to 1000 ml
Store up to 1 year at 4 °C

PMSF (phenylmethylsulfonyl fluoride) stock solution, 0.2 M

Dissolve 3.48 g PMSF in 100 ml isopropanol. Store up to several years at 4°C or room temperature.

Add this reagent to aqueous solutions drop-wise while vortexing, or it will precipitate.

PYS-2 cells, 50% to 70% confluent

Using 150-mm tissue-culture dishes or equivalent plastic flasks, grow PYS-2 cells (ATCC CRL-2745) to between 50% and 70% confluence ($7–10 \times 10^6$ cells per plate) in DMEM/10% (v/v) FBS (*APPENDIX 2A*).

Cells undergo >1 population doubling in 24 hr under these conditions.

Q-Sepharose Fast Flow column

Pour a 1.7 × 20–cm column of Q-Sepharose Fast Flow resin (Amersham Biosciences) equilibrated in 200 mM LiCl/20 mM MOPS, pH 6.5 (see recipe). Equilibrate by running two to three column volumes of 200 mM LiCl/20 mM MOPS, pH 6.5 (see recipe), through the resin. After use, strip the column with 100 ml of 2 M LiCl/20 mM MOPS, pH 6.5 (see recipe), and equilibrate with 60 ml of 200 mM LiCl/20 mM MOPS, pH 6.5. Store up to 1 year at room temperature.

S-200 buffer

999 ml Hanks' balanced salt solution (HBSS; see recipe) with Ca^{2+} and Mg^{2+} (Life Technologies and *APPENDIX 2A*)
1 ml 0.2 M PMSF stock solution (see recipe)
Filter sterilize with a 0.22-μm filter
Prepare fresh for each run and keep at 4°C

Calcium is present in HBSS as 0.14 g/liter $CaCl_2$, and magnesium is present as 0.1 g/liter $MgCl_2 \cdot 6H_2O$ and 0.1 g/liter $MgSO_4 \cdot 7H_2O$.

Sephacryl molecular-sieve column

At 4°C, pour an ~1 × 100–cm column (e.g., Bio-Rad) of Sephacryl S-200 (Amersham Biosciences) in a slurry of cold, sterile S-200 buffer (see recipe). Allow column bed to pack slowly but steadily, with controlled elution from the bottom port of the column at ~10 ml/hr (0.17 ml/min), to a bed height of ~95 cm. Run several column volumes (~80 ml each) of S-200 buffer through the packed bed and then calibrate using molecular-weight standards (see recipe). Store in S-200 buffer at 4°C for up to several days prior to use. For longer storage and reuse (up to several months), store in S-200 buffer containing 0.1% (w/v) sodium azide at 4°C. Periodically clean (i.e., remove sample debris from the top of the column up) and flush with fresh S-200 buffer containing 0.1% sodium azide.

Azide must be flushed out completely (monitor at 280 nm) prior to chromatography of SPARC, as azide is toxic to cells and may also interfere with the properties of SPARC.

Sepharose 4B-AON IgG column

Following manufacturer's instructions, pour anti-osteonectin (AON-5031; 20 mg) monoclonal IgG_1 antibody (Haematologic Technologies) coupled to CNBr-activated Sepharose 4B (Amersham Biosciences) into a 5- to 10-ml column, at 4°C. Equilibrate in several column volumes of 50 mM Tris·Cl (pH 8.0)/0.15 M NaCl (see recipe), by gravity flow at 0.1 to 0.5 ml/min. After use reequilibrate column in 0.02 M Tris·Cl, pH 7.6/0.15 M NaCl. Store up to 1 month in that same buffer at 4°C.

Sodium phosphate, 50 mM (pH 5.3, 6.0, or 7.8)/0.5 M NaCl/10% (v/v) glycerol

29.2 g NaCl
5.75 g sodium phosphate dibasic
1.37 g sodium phosphate monobasic
100 ml glycerol
Add H_2O to ~800 ml
Adjust pH to 5.3 or 6.0 with 6 N HCl, or to 7.8 with 6 N NaOH
Add H_2O to 1000 ml
Store up to 1 to 2 days at 4°C

Sodium phosphate, 90 mM (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF

10.35 g sodium phosphate dibasic
2.466 g sodium phosphate monobasic
100 ml glycerol
1 ml 0.2 M AEBSF stock solution (see recipe)
Add H_2O to ~800 ml
Adjust pH to 7.8 with 6 N NaOH
Add H_2O to 1000 ml
Store up to 1 to 2 days at 4°C

Superdex 70 column

In the cold (i.e., 4°C), pour a 1.6 × 60–cm column of Superdex 70 gel-filtration resin (Amersham Biosciences) equilibrated in 50 mM Tris·Cl (pH 8.0)/0.15 M NaCl (see recipe). Calibrate using the molecular-weight standards (see recipe) blue dextran (V_o) and [^{3}H]proline (V_i) as described.

Tris·Cl, 0.02 M (pH 7.6)/0.15 and 1.0 M NaCl

20 ml 1 M 0.22-µm-filter-sterilized Tris·Cl, pH 7.5 (*APPENDIX 2A*)
8.77 or 58.44 g NaCl
Add H_2O to ~900 ml
Adjust pH to 7.6 with 1 N NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Tris·Cl, 50 mM (pH 8.0)/0.15 M NaCl

8.76 g NaCl
4.44 g Tris·Cl
2.65 g Tris base
Add H_2O to ~800 ml
Adjust pH to 8.0 with 6 N NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Urea stock solution, 8 M

Add 1920 g ultra pure urea (Life Technologies) in 2 liters water by dissolving ~200 g at a time. After all urea has dissolved, add water to 4 liters. Filter through Whatman no. 3 paper and store up to 1 month at 4°C.

COMMENTARY

Background Information

The abundance of SPARC in many tissues, and its high levels of secretion by most cells in vitro, belie the difficulty of its recovery as an intact, active protein after purification. SPARC (as osteonectin) was found to be a major non-collagenous component of fetal and adult bone (Termine et al., 1981). In situ hybridization of SPARC by numerous investigators has shown that the mRNA is abundant in most fetal tissues, presumably associated with morphogenesis, growth, and angiogenesis but is somewhat limited in the corresponding adult tissues (for reviews, see Lane and Sage, 1994; Brekken and Sage, 2000; Bradshaw and Sage, 2001). SPARC mRNA and protein are found in relatively high amounts in adult tissues that exhibit continuous turnover (gut epithelium) and remodeling (bone), and are produced in response to injury (wound healing) and certain types of pathologies (tumors, scleroderma). In more quiescent and/or established tissues, however, levels of SPARC are low. Since SPARC affects both the adhesion and proliferation of most normal cells, its association with angiogenesis and other processes requiring cell migration, differentiation, and synthesis of extracellular matrix (ECM) is not surprising.

There are several structural features of SPARC that should be considered in the context of a purification protocol.

1. SPARC is typically a secreted protein with two post-translational modifications that can be troublesome. There are fourteen cysteines, all of which are disulfide-bonded, and the folding and correct formation of disulfide bridges are not trivial in recombinant proteins produced at high levels, especially in yeast and bacteria. This has certainly been the case for SPARC (Yost et al., 1994; Bassuk et al., 1996b). Additionally, secreted SPARC contains a single complex-type carbohydrate chain (N-linked) which is not produced in nonmammalian systems. Interestingly, the carbohydrate has been shown to be variable in mammalian SPARCs—i.e., the carbohydrate from platelet SPARC is different from that from bone. In addition, cultured cells can assemble and process the oligosaccharide side-chain structures differently (Lane and Sage, 1994). It is important to remember that purification of SPARC from tissues such as bone will result in the recovery of nonsecreted SPARC that has unprocessed high-mannose-type oligosaccharide.
2. SPARC binds other proteins, including growth factors. The association of SPARC with albumin (probably through adventitious disulfide interchange) has been troublesome, but can be avoided by the use of serum-free culture (e.g., *Sf*9 cells, *E. coli*, or a serum-independent mammalian cell line). Anticipate that isolation of SPARC from tissues (including platelets) can result in contamination from plasma and tissue fluid components (e.g., albumin) as well as ECM proteins to which SPARC binds (collagen types I, III, IV, V, and thrombospondin 1). Moreover, SPARC also interacts with platelet-derived growth factor (PDGF) AB and BB and vascular endothelial growth factor (VEGF) with a $K_d \cong 10^{-9}$ M. If possible, it is best to avoid these proteins when choosing a source of SPARC, as additional purification steps to remove the contaminants will invariably result in lower yields and loss of activity.
3. SPARC binds to several cations (Cu^{2+}, Fe^{2+}) and has an absolute requirement for Ca^{2+}. The disulfide-bonded EF-hand, a Ca^{2+}-binding loop at the C terminus, is reasonably stable, with a K_d for $Ca^{2+} \cong 10^{-7}$ M, and is thought to serve a structural function. The N terminus, however, contains from five to eight low-affinity ($K_d \cong 10^{-3}$ to 10^{-5} M) Ca^{+2}-binding sites (glutamic acids). Association of Ca^{2+} with this region of SPARC serves to neutralize its excessive negative charge and confers α-helicity to this domain. It is therefore critical that SPARC is not exposed to EDTA or other chelating agents during purification, and that the protein is stored in the presence of 1 to 4 mM Ca^{2+}. One of the assays for native structure of SPARC, circular dichroism (see below), depends on α-helicity as a function of Ca^{2+} binding within this low-affinity site.

Three protocols have been discussed that maximize both the yield and the purity/native structure of either natural or rSPARC. Most cultured cells secrete reasonably high levels of SPARC into the culture medium, an environment in which SPARC is stable over several days at 37°C. Proteolytic degradation of SPARC has rarely been a problem, especially with the judicious use of protease inhibitors, as described in the protocols. Since both human and murine tumor cells can also secrete high levels of SPARC in vitro and in general are more tolerant of low serum (or, preferably, the absence of serum), they are a logical choice for the isolation of nonrecombinant SPARC, especially if they exhibit high rates of growth and secretion (see Basic Protocol).

Advantages of a recombinant protein expression system include the (theoretically) substantially higher yields of protein, as well as the potential of producing mutated versions of the protein. Both the *E. coli* and *Sf*9 cell systems can achieve these goals with respect to SPARC (see Alternate Protocol 1 and 2). Additionally, SPARC from any species for which the sequence is known can be engineered by the polymerase chain reaction (*APPENDIX 3F*) into a suitable expression vector. Disadvantages include potential problems with folding and post-translational modification of rSPARC; however, assessment of purity and activity of the SPARC produced in both *E. coli* and *Sf*9 cells has shown that these are both viable routes for the production of SPARC. Although the activity of rSPARC appears to be ~50% of that of the PYS-2-derived protein, the substantially greater yields may offset this limitation.

Any modification of the primary structure of SPARC must be considered as potentially deleterious to its conformation and/or activity. The $(His)_6$ sequence, tagged onto the C terminus of SPARC to facilitate its purification by metal-affinity chromatography, could affect one or more properties of SPARC (e.g., nuclear translocation, de-adhesion) and should be controlled for in subsequent experiments. As discussed in preceding paragraphs, post-translational differences need to be considered as well—i.e., the lack of carbohydrate in *E. coli* rSPARC (see Alternate Protocol 1), and a dif-

ferent or additional type of glycosylation conferred by *Sf*9 cells (see Alternate Protocol 2).

There may be situations in which the proper SPARC for study will be that isolated from a given tissue (e.g., bone). References have been included (see Alternate Protocol 3) for the extraction of SPARC from this tissue. The use of denaturants and EDTA could be problematic, although renaturation is always an option. Since both platelet and bone SPARC are available commercially (see Alternate Protocol 3), it is advisable to purchase a small amount and to test it according to the parameters required.

Critical Parameters and Troubleshooting

Many of the caveats at various stages of purification of SPARC have been detailed within each protocol. The principal problems are low recovery and poor bioactivity.

Recovery of SPARC depends on several factors, not the least important of which is the output of SPARC in vitro. Despite claims of immortality, transformed or tumor cells do not live forever in culture. Successive passages and cycling of cells on and off serum (or growth in the absence of serum) can affect their eventual viability. Therefore, it is important to monitor the secretion of SPARC over time (this also applies to the production of rSPARC). SPARC is produced optimally by subconfluent cells; at confluence or near-confluence, SPARC is secreted at a reduced rate, and will associate with the cell surface or ECM. Presented below is a list of other possible causes of recovery loss, as well as potential solutions; however, the reader should bear in mind that some losses are indeed unavoidable.

1. Failure of SPARC to redissolve completely in the various buffers used for purification or assay. Clarification of solutions is always recommended.
2. Precipitation of SPARC during freezing or thawing. Snap-freezing on dry ice, and quick-thawing at room temperature, are recommended.
3. Incomplete precipitation during dialysis against water, which can be checked by SDS-PAGE (*UNIT 6.1*) of a small aliquot of the supernatant.
4. Irreversible binding and/or denaturation of SPARC on membrane-type centrifugal concentrators (e.g., Centricons). Losses should be determined if the investigator chooses to concentrate purified SPARC in this manner. There are always new products on the market that claim to minimize this problem.
5. Degradation due to proteolysis by intrinsic proteinases or to bacterial contamination. Protease inhibitors should always be used during purification of SPARC, as described, and bacterial contamination should be minimal if sterile buffers or buffers containing sodium azide (NaN_3) are used.
6. Recovery can be compromised by the use of untreated glass vessels; only polypropylene or siliconized-glass containers should be used. Surface denaturation of SPARC occurs readily, either from adsorption to surfaces or from rapid stirring or overzealous mixing.

Denaturation of SPARC can be minimized with careful handling and attention to a few details.

1. The protein should be stored at –70° or –80°C, not at 4°C and especially not at –20°C.
2. 1 to 4 mM Ca^{2+} should be present in buffers containing SPARC.
3. Stirring of solutions should be steady but not rapid.
4. Purification of the protein should be conducted at 4°C whenever possible.
5. Only reagents (e.g., urea) of the highest purity should be used.
6. Reducing/oxidizing conditions, which can result in the scrambling of disulfide bonds, should be avoided.

Assays for SPARC bioactivity have been described elsewhere (see Support Protocols 1 to 3) and need not be repeated here. However, an important criterion for the correct folding of SPARC is the circular dichroism spectra obtained in the presence and absence of Ca^{2+}. These spectra are relatively easy to perform and interpret. Examples for SPARC purified from PYS-2 cells, *E. coli*, and *Sf*9 cells have been published (Sage et al., 1989; Bassuk et al., 1996a; Bradshaw et al., 2000). The method relies on a characteristic increase of the mean residue ellipticity (θ) at 220 nm as a function of increasing concentrations of Ca^{2+}, indicative of a shift toward α-helicity. SPARC preparations that do not exhibit this transition are likely to be contaminated by other components and/or denatured.

For the use of SPARC in proliferation (i.e., [^{3}H]thymidine incorporation) assays, it is important to measure levels (if any) of contaminating growth factors that could affect the results. Both PDGF and VEGF bind to SPARC (see Background Information) and are anticipated to stimulate the proliferation of smooth muscle cells, fibroblasts (PDGF), and endothelial cells (VEGF). Kits based on ELISA are now available for the detection of these factors;

alternatively, detection could be accomplished by immunoblot analysis after SDS-PAGE of SPARC under reducing conditions (*UNIT 6.2*), although the former method allows for greater sensitivity.

Anticipated Results

Isolation from PYS-2 cells (see Basic Protocol) should yield ~500 µg per 30 maxiplates (150-mm diameter) PYS-2 cells (~10^7 cells/plate). The protein is of high purity (>90% by SDS-PAGE) and retains maximal biological activity. For example, an ED_{50} of 20 µg/ml (0.6 µM) has been defined as an effective concentration for the induction of cell rounding by SPARC.

Yields of rSPARC from *E. coli* and *Sf*9 cells are greater than those from PYS-2 cells (see Alternate Protocols 1 and 2), but are in large part dependent on the efficiency of the expression system (i.e., the particular expression vector, the host and its growth properties, and whether the rSPARC is secreted or retained within the cell). Using a first-generation *Sf*9/baculovirus expression system, the authors' laboratory typically recovers 2 to 4 mg human rSPARC (of ~80% purity) from an initial suspension of ~10^9 cells. The InsectSelect system, which eliminates the need for viral infection, is likely to be an improvement over the earlier version. rSPARC can be purified to ≥80% and displays biological activity in cell rounding and proliferation assays.

The immunoaffinity-based chromatographic purification of SPARC from platelets will theoretically produce a highly purified protein, in reasonable yields, although the amount of SPARC in the starting material (α-granules of platelets) is low, from 0.7 to 2.2 µg/10^8 cells. One limiting factor is the availability of the monoclonal antibody used for the purification. This reagent must not only bind soluble SPARC with relatively and selectively high affinity, but must also release SPARC readily into the elution buffer without compromise of the SPARC or the antibody itself. Moreover, the antibody must function while coupled to an affinity resin. It is therefore important to ensure that a sufficient supply of the antibody is commercially available, as the column will have to be repacked periodically with new affinity-coupled resin. An alternative is to purchase a hybridoma cell line secreting a suitable anti-SPARC IgG that can be propagated in the laboratory.

Time Considerations

The Basic Protocol and Alternate Protocols 1 to 2 each require ~1 week from the time of medium (PYS-2 and *Sf*9 cells) or cell (*E. coli*) collection until the final lyophilization (or buffer exchange) step. Allow 1 to 2 days for the preparation of buffers and columns, and for the washing of columns. PYS-2 cells are usually ready for beginning the collection of medium 24 hr after plating, and medium is removed from the cells 18 to 24 hr later. Similar time frames apply to *E. coli* (grown overnight, diluted to an appropriate density in log phase, and induced) and to *Sf*9 cells (grown in flasks over 3 to 4 days to generate conditioned medium containing rSPARC).

In all the protocols, convenient stopping points have been noted. There is temporal flexibility in the purification process, especially during the dialysis steps.

Literature Cited

Bassuk, J.A., Baneyx, F., Vernon, R.B., Funk, S.E., and Sage, E.H. 1996a. Expression of biologically active human SPARC in *E. coli*. *Arch. Biochem. Biophys*. 325:8-19.

Bassuk, J.A., Braun, L.P., Motamed, K., Baneyx, F., and Sage, E.H. 1996b. Renaturation of secreted protein acidic and rich in cysteine (SPARC) expressed in *Escherichia coli* requires isomerization of disulfide bonds for recovery of biological activity. *Intl. J. Biochem. Cell Biol*. 28:1031-1043.

Bornstein, P. and Sage, E.H. 2002. Matricellular proteins: Extracellular modulators of cell function. *Curr. Opin. Cell Biol*. 64:608-616.

Bradshaw, A.D. and Sage, E.H. 2001. SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. *J. Clin. Invest*. 107:1049-1054.

Bradshaw, A.D., Bassuk, J.A., Francki, A., and Sage, E.H. 2000. Expression and purification of recombinant human SPARC produced by baculovirus. *Mol. Cell Biol. Res. Comm*. 3:345-351.

Brekken, R.A. and Sage, E.H. 2000. SPARC, a matricellular protein: At the crossroads of cell-matrix communication. *Matrix Biol*. 19:569-580.

Kelm, R.J. and Mann, K.G. 1990. Human platelet osteonectin: Release, surface expression, and partial characterization. *Blood* 75:1105-1113.

Kelm, R.J. and Mann, K.G. 1991. The collagen binding specificity of bone and platelet osteonectin is related to differences in glycosylation. *J. Biol. Chem*. 266:9632-9639.

Lane, T.F. and Sage, E.H. 1994. The biology of SPARC, a protein that modulates cell-matrix interactions. *FASEB J*. 8:163-173.

Sage, E.H., Vernon, R.B., Funk, S.E., Everitt, E.A., and Angello, J. 1989. SPARC, a secreted protein

associated with cellular proliferation, inhibits cell spreading in vitro and exhibits Ca^{+2} dependent binding to the extracellular matrix. *J. Cell. Biol.* 109:341-356.

Sage, E.H. and Bornstein, P. 1995. Matrix components produced by endothelial cells: Type VIII collagen, SPARC, and thrombospondin. *In* Extracellular Matrix: A Practical Approach. (M.A. Haralson and J. R. Hassell, eds.) pp. 131-160. Oxford University Press, Oxford.

Sasaki, T., Miosge, N., and Timpl, R. 1999. Immunochemical and tissue analysis of protease-generated neoepitopes of BM-40 (osteonectin, SPARC) which are correlated to a higher affinity binding to collagens. *Matrix Biology* 18:499-508.

Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L., and Martin, G.R. 1981. Osteonectin, a bone-specific protein linking mineral to collagen. *Cell* 26:99-105.

Yost, J.C., Bell, A., Seale, R., and Sage, E.H. 1994. Purification of biologically active SPARC expressed in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* 314:50-63.

Key References

Lane and Sage, 1994. See above.

This review of SPARC provides useful summaries of its location/abundance in tissues, sequence homologies, and physical characteristics.

Brekken and Sage, 2000. See above.

An up-to-date review of the structure and biology of SPARC.

Reed, M., Puolakkainen, P.A., Lane, T.F., Dickerson, D., Bornstein, P. and Sage, E.H. 1993. Differential expression of SPARC and thrombospondin-1 in wound repair: Immunolocalization and *in situ* hybridization. *J. Histochem. Cytochem.* 41:1467-1477.

A useful reference for immunostaining and in situ hybridization protocols for the detection of SPARC.

Acknowledgement

This manuscript has been prepared with the assistance of Sarah E. Funk and Gail Workman.

Contributed by E. Helene Sage
The Hope Heart Institute
Seattle, Washington

Analysis of Fibronectin Matrix Assembly

Fibronectin (FN) is one of the most ubiquitous components of the extracellular matrix (ECM). It plays a critical role in organizing ECM structure and influences cell behavior through interactions with cell surface receptors. Many types of cells secrete cellular FN and assemble it into a fibrillar network. Assembly proceeds via a step-wise process in which FN is initially organized into fine cell-associated fibrils and, through continued accumulation of FN, these fibrils are converted into a dense network of detergent-insoluble fibrils. Differential solubility in the detergent deoxycholate (DOC) is the principle for biochemical analysis of FN matrix (DOC-solubility assay).

In this unit, basic methods of detection, quantification, and visualization of the fibrillar FN matrix are described. The Basic Protocol for analysis of the matrix assembly process is based on the DOC-solubility assay and describes isolation and analysis of a FN matrix from cultured cells. Alternate protocols are also provided for analyzing matrix assembly using exogenous FN (see Alternate Protocols 1 and 2) or by metabolic labeling (see Alternate Protocol 3). In addition to biochemical analysis of matrix assembly, Alternate Protocol 4 and Alternate Protocol 5 describe visualization of matrix organization directly by incorporation of fluorescently labeled FN and by indirect immunofluorescence staining, respectively.

Protocols described in this section require cell culture (*UNIT 1.1*), purification of plasma FN (*UNIT 10.5*), metabolic labeling of cells (*UNIT 7.1*), immunoblotting (*UNIT 6.2*), immunoprecipitation (*UNIT 7.2*), and immunofluorescence staining (*UNIT 4.3*).

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO_2 incubator. Some media, e.g., DMEM, require increased levels of CO_2 to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

ANALYSIS OF MATRIX ASSEMBLY USING A DOC-SOLUBILITY ASSAY

BASIC PROTOCOL

Fibroblasts growing on tissue culture surfaces synthesize FN and assemble it into a fibrillar matrix. The assay is based on the insolubility of stable FN matrix in 2% DOC detergent (McKeown-Longo and Mosher, 1983). Cells are lysed in DOC lysis buffer and centrifuged to separate DOC-insoluble matrix from DOC-soluble material containing cell-associated and intracellular FN. The DOC-insoluble FN is solubilized in a buffer containing 1% SDS. The DOC-soluble and -insoluble fractions are resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting.

Materials

Sub-confluent (80% confluent) fibroblasts in a 10-cm tissue culture dish
PBS (*APPENDIX 2A*)
Trypsin/EDTA solution (GIBCO, Invitrogen)
Culture medium containing 10% FN-depleted serum (see *UNIT 10.5* for FN-depletion)
DOC lysis buffer (see recipe)
SDS-solubilization buffer (see recipe)
BCA protein assay kit (Pierce Chemical)
2× SDS sample buffer (see recipe)

Contributed by Iwona Wierzbicka-Patynowski, Yong Mao, and Jean E. Schwarzbauer
Current Protocols in Cell Biology (2004) 10.12.1-10.12.10

15-ml screw-cap tube
24-well tissue culture plate
Rubber policeman
1-ml syringe and 26-G, 3/8-in. needle
Additional reagents and equipment for cell culture (*UNIT 1.1*), gel electrophoresis (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

Prepare cell culture

1. Aspirate the medium from a sub-confluent culture of fibroblasts growing on a 10-cm tissue culture dish.
2. Rinse cells with 5 ml PBS to remove any residual serum.
3. Add 2 ml of trypsin/EDTA solution to cell layer and incubate for 1 to 5 min at room temperature.
4. When cells detach from the dish, add 2 ml of culture medium and transfer all cells to a 15-ml screw-cap tube. Count the cell number (*UNIT 1.1*) and centrifuge 5 min at 100 × *g*, room temperature.
5. Resuspend fibroblasts in culture medium containing FN-depleted serum at 2.5 × 10^5/ml (1 ml/well). Plate cells onto a 24-well tissue culture plate and incubate up to 8 hr in an incubator.

   The cell densities for matrix assembly vary for different cell lines and incubation times. The cells should be plated at subconfluency for overnight incubations. For incubations of several hours, cells should be plated almost confluent or touching. Usually, fewer cells are required for fibroblasts than other cell lines for a given surface area. If experiments are done in different-sized tissue culture dishes or wells, the amount of reagents and number of cells need to be scaled up or down accordingly. To avoid the introduction of exogenous FN from serum, FN-depleted serum should be used to supplement the medium instead.

Perform DOC-solubility assay

6. At desired time, aspirate medium from the wells and gently wash the cells with cold PBS.

   The desired time is determined by the purpose of the experiment and cell type used for FN matrix assembly. For cells expressing endogenous FN, it can take 4 to 6 hr to accumulate amounts of DOC-insoluble FN detectable by immunoblotting. DOC-insoluble matrix should be easily detectable in cells cultured overnight.

7. Add 200 µl of DOC lysis buffer to each well and scrape cells off of the dish using a rubber policeman. Collect cell lysate with a 1-ml syringe attached to a 26-G, 3/8-in. needle. To reduce viscosity, pass the cell lysate through the needle five times, transfer to a 1.5-ml microcentrifuge tube labeled "DOC-insoluble," and keep tube on ice.

   The amount of DOC lysis buffer should be adjusted accordingly for different-sized dishes (e.g., 2 ml for 10-cm dish, 1 ml for 6-cm dish, and 0.5 ml for 35-mm dish). Volumes can be adjusted to achieve the desired total protein concentration. Cell lysates should be thoroughly scraped off of the tissue culture surface. Lysates are passed (usually five passes) through a small-gauge needle to shear genomic DNA and reduce the viscosity. This procedure should be carried out without generating air bubbles. Alternatively, viscosity can be reduced by treating the samples with Triton X-100 followed by DNase I as described by Quade and McDonald (1988).

8. Microcentrifuge the lysates in 1.5-ml microcentrifuge tubes 15 min at 14,000 rpm, 4°C.

9. Carefully remove supernatant into a new 1.5-ml microcentrifuge tube labeled "DOC-soluble" and keep on ice.

   In some cases, the pellet of insoluble material is not very obvious, therefore, always mark the side where the pellet will reside after centrifugation. Remove supernatant as completely as possible and keep the pipet tip away from the pellet.

10. Add 25 μl of SDS-solubilization buffer to the insoluble pellet and mix thoroughly.

    It is important to thoroughly dissolve the pellet and to wash the walls of the tube. Do this by pipetting the SDS-solubilization buffer up and down and by vortexing. Scale up or down the volume of SDS-solubilization buffer for different sample sizes (e.g., 62.5 μl for 35-mm dishes). The amount of SDS-solubilization buffer or the concentration of SDS in the buffer can be increased if cells are plated on a protein-rich substrate (such as Matrigel, gelatin, or a 3-D matrix prepared from cultured fibroblasts).

Determine total protein concentrations

11. Estimate protein concentrations for DOC-soluble fractions using a BCA protein assay kit. Follow the manufacturer's instructions.

12. Normalize samples for the same amount of protein by adjusting volume with 2× SDS sample buffer and boil 2 min.

    Protein concentration in the DOC-soluble fraction is proportional to the number of cells in the culture and is used to adjust gel sample volumes on a per-cell basis. In a typical experiment, the total protein concentration ranges from 300 to 800 μg/ml from one well of a 24-well plate. The maximum amount of protein should be electrophoresed to ensure detection of FN (usually 3 to 10 μg/lane). DOC-insoluble sample volume for SDS-PAGE is based on protein concentration in the corresponding DOC-soluble fraction. To detect monomeric FN, samples should be reduced with 0.1 M DTT in the SDS sample buffer.

Analyze samples by immunoblotting

13. Resolve protein samples using a 5% polyacrylamide-SDS gel and transfer proteins to nitrocellulose. Perform electrophoresis and immunoblotting according to protocols described in *UNITS 6.1 & 6.2*, respectively.

    The amount of FN in both fractions can be detected using anti-FN antibodies, followed by secondary antibodies and ECL reagents.

QUANTIFICATION OF MATRIX ASSEMBLY USING ^{125}I-LABELED PROTEIN A

ALTERNATE PROTOCOL 1

Using ^{125}I-labeled protein A to detect FN in immunoblots allows the amount of assembled FN matrix to be quantified. After DOC-soluble and DOC-insoluble fractions are separated by SDS-PAGE and transferred to a nitrocellulose membrane, FN is detected with an anti-FN antibody and secondary antibody followed by radiolabeled protein A. The intensity of the protein band is then measured using a phosphorimager scanner as described below.

CAUTION: Experiments involving radioactive material handling have to be performed by trained personnel and in a designated area to avoid contamination. See *APPENDIX 1D* for safe use of radioisotopes.

Materials

Samples of DOC-soluble and -insoluble FN from cultures (see Basic Protocol, steps 1 to 10)
5% (w/v) BSA in TBS buffer (see *APPENDIX 2A* for TBS)
Primary anti-FN antibody (e.g., HFN7.1, ATCC)
Rabbit secondary antibody (e.g., unconjugated rabbit anti-mouse IgG, Pierce Chemical)

^{125}I-labeled protein A (10 μCi/μg,specific activity; MP Biomedicals)
Buffer A (see recipe)

Plastic wrap
Phosphorimager screen (cassette) and scanner
ImageQuant software

Additional reagents and equipment for gel electrophoresis (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Perform electrophoresis of samples of DOC-soluble and -incoluble FN from cultures and transfer to nitrocellulose according to protocols described in *UNITS 6.1 and 6.2*.
2. Block nitrocellulose with 5% BSA in TBS buffer overnight at 4°C.
3. Dilute primary antibody in 10 ml of 5% BSA in TBS and incubate with nitrocellulose filter for 1 hr at room temperature. Wash three times with 10 ml of 5% BSA in TBS buffer, 10 min each wash. Incubate with rabbit secondary antibody diluted to 1 μg/ml in 10 ml of 5% BSA in TBS 1 hr at room temperature. Wash three times with 10 ml TBS buffer, 10 min each wash.

   For optimal binding of protein A and to amplify the signal from the primary anti-FN antibody, rabbit secondary antibody should be used. The authors usually use unconjugated rabbit anti-mouse IgG (H+L) (Pierce Chemical) to detect monoclonal anti-FN antibodies.

4. Incubate with ~6 μCi of ^{125}I-protein A in 5% BSA in TBS buffer. Wash three times with 10 ml TBS, 10 min each wash.
5. Wrap the nitrocellulose in plastic wrap and place in phosphorimager cassette. Expose to phosphor screen for desired amount of time.

   The time of exposure is empirically determined. Bands can usually be detected after an overnight exposure but weaker signals may require exposure for ≥1 week.

6. Read the screen on phosphorimager scanner. Determine the number of counts associated with each band using ImageQuant software.

ALTERNATE PROTOCOL 2

ANALYSIS OF ASSEMBLY OF EXOGENOUS FN

Some cell lines (e.g., CHO and many tumor cell lines) do not produce significant levels of FN. To assess their matrix assembly capability and to study regulation of the assembly process, the addition of exogenous FN is required. For quantitation purposes, ^{125}I-labeled FN can be included in the exogenous FN.

Materials

Purified plasma FN (*UNIT 10.5*)
^{125}I-labeled FN (~1 μCi/μg; MP Biomedicals; optional)
Additional reagents and equipment for trypsinization and collection of cells, and isolation and analysis of DOC-insoluble and DOC-soluble FN (see Basic Protocol)

1. Prepare purified FN from blood plasma using the protocol described in *UNIT 10.5*.
2. Trypsinize and collect cells following Basic Protocol, steps 1 to 5.
3. Allow cells to attach and spread, usually 60 min at 37°C

4. Add 25 to 50 μg/ml of exogenous FN and incubate for desired amount of time.

   The amount of exogenous FN can be varied and the optimal amount for assembly should be determined empirically. To quantify the amount of FN in the matrix, ^{125}I-labeled FN can be added together with unlabeled FN. Alternatively, cells can be allowed to assemble exogenous FN for a period of time and then ^{125}I-labeled FN can be added for a shorter period. Additional reagents such as activators or inhibitors of matrix assembly can be added along with exogenous FN or at any time during the incubation.

5. Isolate and analyze DOC-insoluble and DOC-soluble FN (see Basic Protocol, steps 6 to 13).

   When ^{125}I-labeled FN is included, the SDS-polyacrylamide gel is dried and directly exposed to a phosphorimager screen (for gel drying, see Alternate Protocol 3).

ANALYSIS OF METABOLICALLY LABELED FN

ALTERNATE PROTOCOL 3

Using metabolically labeled cells for matrix assembly studies allows one to determine the incorporation of endogenous FN over specific time periods and also provides radiolabeled material for quantification. ^{35}S-labeled FN in DOC-soluble and -insoluble fractions are isolated by immunoprecipitation and analyzed using a phosphorimager after resolution by SDS-PAGE.

Materials

Cell cultures for labeling
Culture medium containing FN-depleted serum (see *UNIT 10.5* for FN-depletion)
Labeling medium (see recipe)
^{35}S-methionine (>1000 Ci/mmol)
IP buffer (see recipe)
Protein A–Sepharose beads
35-mm tissue culture dish or 6-well plate
Phosphorimager screen and scanner
ImageQuant software
Additional reagents and equipment for cell preparation (see Basic Protocol), IP protocol (*UNIT 7.2*)

1. Prepare cells according to Basic Protocol, steps 1 to 5.
2. Plate 1×10^6 cells in culture medium containing FN-depleted serum in 35-mm tissue culture dishes or 6-well plates. Let cells attach and spread.

   Alternatively, cells can be plated and allowed to grow until 80% to 90% confluent.

3. Aspirate medium, rinse cells with 2 ml labeling medium minus methionine, and replace with 1 ml labeling medium. Add 25 μCi of ^{35}S-methionine per milliliter of labeling medium and mix well. Incubate cells for desired amount of time.

   The optimal concentration of ^{35}S-methionine depends on cell type and length of labeling. A 24-hr labeling period with 25 μCi/ml of ^{35}S-methionine is typically used to determine assembly competence and FN expression by cells. Shorter labeling times with increased amounts of ^{35}S-methionine can be used (e.g., 50 μCi/ml for 6 to 8 hr or 100 μCi/ml for 2 hr).

4. Remove medium and save for detection of FN in the medium (see *UNIT 10.5*).
5. Wash cells with 2 ml ice-cold PBS.

   The waste PBS should be disposed of in a properly labeled radioactive waste container.

6. Prepare DOC-soluble and -insoluble fractions as described in Basic Protocol, steps 6 and 7, using 500 μl DOC-lysis buffer and 62.5 μl SDS-buffer.

7. Determine protein concentration. Normalize the samples to equal protein concentration in ~125 µl of DOC-soluble sample and 50 µl of DOC-insoluble sample.
8. Adjust volume to 500 µl using stock solutions to give composition of IP buffer.
9. Immunoprecipitate FN from the soluble and insoluble fractions using anti-FN antibody. Follow the IP protocol described in *UNIT 7.2*.
10. Run immunoprecipitated samples on 5% SDS-PAGE. Dry the gel and expose to phosphorimager screen. Scan the intensity of FN bands using a phosphorimager scanner and analyze using ImageQuant software.

Electrophoresis of one-third of the immunoprecipitate is usually sufficient to detect FN in fibroblast matrix. To dry gel, fix in 50% methanol/10% acetic acid solution 30 min at room temperature, rehydrate with several changes of water, place on a sheet of Whatman paper, place on gel dryer, cover with plastic wrap, and dry at 80°C under vacuum.

ALTERNATE PROTOCOL 4

DIRECT DETECTION OF MATRIX ASSEMBLY BY INCORPORATION OF FLUORESCENTLY LABELED FIBRONECTIN

In addition to the biochemical methods, FN matrix assembly can be monitored using fluorescence techniques. Fibrillar matrix can be detected by indirect immunofluorescence staining using antibodies with fluorescent tags (see Alternate Protocol 5) or matrix can be labeled directly by incorporation of fluorescently tagged FN.

Materials

Purified FN (*UNIT 10.5*)
50 mM sodium bicarbonate, pH 8.
Sulfo-NHS-rhodamine *or* fluorescein (Pierce Chemical)
CAPS-NaCl solution
Cell cultures (see Basic Protocol, steps 1 to 4)
Culture medium containing FN-depleted serum (see *UNIT 10.5* for FN-depletion)
PBS/Mg (PBS containing 0.5 mM $MgCl_2$)
3.7% (v/v) formaldehyde in PBS/Mg
0.5% NP-40 (v/v) in PBS/Mg
FluoroGuard (Bio-Rad)
Nail polish

Spectrophotometer
12-mm circular coverslips
24-well plate
Fine-tip forceps
Beakers
Paper towels
Kimwipes
Glass microscope slides

Prepare fluorescently labeled FN

1. Dialyze 1 mg/ml of purified FN in 50 mM sodium bicarbonate, pH 8.5, overnight at 4°C.
2. Immediately prior to use, make 1 mg/ml of sulfo-NHS-rhodamine in distilled water. Add 40 µg of sulfo-NHS-rhodamine per 1 mg of dialyzed FN.
3. Incubate 2 hr on ice in dark.
4. Dialyze the reaction mixture against CAPS-NaCl solution at 4° C. Use two changes of at least a 100-fold excess volume each.

5. Determine protein concentration by reading A_{280} using a spectrophotometer. Store 100-μl protein aliquots for 6 months at −80°C.

Prepare cultures

6. Place 12-mm circular coverslips in the wells of a 24-well plate.

   Sterilize coverslips prior to use by autoclaving.

7. Prepare cells in culture medium containing FN-depleted serum according to Basic Protocol, steps 1 to 4.

8. Plate cells on coverslips in wells of 24-well plates and let them attach and spread for 30 to 60 min.

   Make sure that coverslips are at the bottom of the wells rather than floating in the medium.

9. Add 25 μg/ml of rhodamine-labeled FN and incubate cells at 37°C for desired amount of time.

10. Aspirate medium and gently wash cells with 1 ml PBS/Mg. Fix with 1 ml of 3.7% formaldehyde in PBS/Mg 15 min at room temperature. Aspirate fixing solution and wash three times with 1 ml PBS/Mg.

11. Carefully remove coverslips from wells using fine-tip forceps. Set up three beakers containing 100 ml PBS/Mg. Wash coverslips by dipping several times in each beaker. Do a final wash in 100 ml water. Drain coverslips on a dry paper towel and dry the clean (non-cellular) face of coverslip with a Kimwipe.

    Alternatively, cells can be removed from the well prior to fixation. Place in a humidified chamber (e.g., a Petri dish lined with a moistened paper towel). Gently pipet 25 to 50 μl of fixing solution on top of cells and incubate 15 min at room temperature.

Visualize incorporated labeled FN

12. Place a drop (2 to 4 μl) of FluoroGuard on a glass microscope slide. Carefully place the coverslip with cells face down on top of the FluoroGuard.

13. Seal periphery of the coverslip with nail polish and let dry.

    Properly sealed slides can be stored several months at −20°C.

14. Examine slides with a fluorescence microscope equipped with rhodamine filters.

DETECTION OF MATRIX ASSEMBLY BY INDIRECT IMMUNOFLUORESCENCE STAINING

ALTERNATE PROTOCOL 5

Fibrillar matrix can be detected by indirect immunofluorescence staining using primary anti-FN antibody and secondary antibodies with fluorescent tags.

Materials

Cell cultures
Primary anti-FN antibody
2% (w/v) ovalbumin in PBS/Mg solution
Fluorescein-conjugated or rhodamine-conjugated goat anti-mouse IgG (or anti-rabbit IgG)

Petri dishes
Fluorescence microscope

Additional reagents and equipment for detection of FN matrix (see Alternate Protocol 4)

1. Follow Alternate Protocol 4, steps 6 to 10, except do not add fluorescently labeled FN.

   Exogenous FN can be added to cells if the cell type does not produce FN.

   For detection of intracellular proteins, fixed and washed cells can be permeabilized with 1 ml 0.5% NP40 in PBS/Mg, 15 min at room temperature followed by three washes with 1 ml PBS/Mg.

2. Carefully remove coverslips from wells using fine-tip forceps. Place in a humidified chamber (e.g., a Petri dish lined with a moistened paper towel).

3. Add 25 to 50 μl of diluted primary anti-FN antibodies in 2% ovalbumin/PBS/Mg solution and incubate for 30 min at 37°C.

   The optimal antibody dilution should be determined by the individual user. Typical dilutions are: 1:50 to 1:250 for hybridoma culture supernatant or polyclonal antisera and 1:500 to 1:2000 for ascites fluid or concentrated hybridoma supernatant. Antibody incubations can be done in a 37°C incubator (without CO_2).

4. Wash coverslips by dipping in 100 ml PBS/Mg three times.

5. Incubate cells with 25 to 50 μl of the secondary antibody (e.g., fluorescein- or rhodamine-conjugated goat anti-mouse or rabbit IgG) in 2% ovalbumin in PBS/Mg solution 30 min at 37°C.

6. Rinse and mount the coverslips according to Alternate Protocol 4, steps 12 to 13.

7. Examine coverslips using a fluorescence microscope with appropriate filters.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

25 mM Tris·Cl, pH 7.5
150 mM NaCl
0.1% (v/v) Tween-20
Store up to 6 months at 4°C

CAPS-NaCl solution

10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)
150 mM NaCl
Adjust to pH 11 with 5 N NaOH
Store up to 6 months at 4°C

Cell labeling medium

To cell culture medium without methionine, add non-radioactive methionine (tissue culture–grade) to a final concentration that is 0.1× the amount in normal medium.
Store up to 3 months at 4°C.

DOC lysis buffer

2% (w/v) sodium deoxycholate (from 10% DOC stock solution in dH_2O, kept for months at −20°C)
20 mM Tris·Cl, pH 8.8 (from 1 M Tris·Cl stock solution, pH 8.8, stored at room temperature)

continued

2 mM phenylmethysulfonyl fluoride (PMSF; 0.2 M PMSF in ethanol and stored at −20°C)
2 mM EDTA (from 0.5 M EDTA stock solution, stored at room temperature)
2 mM iodoacetic acid (freshly prepared 100 mM stock in H_2O)
2 mM *N*-ethylmaleimide (freshly prepared 100 mM stock in H_2O)
Prepare fresh

Dissolve any precipitated PMSF in the stock solution by vortexing.

IP buffer

50 mM Tris·Cl, pH 8.8
2.5 mM EDTA
2.5 mg/ml BSA
0.5% (v/v) NP-40
0.5% (w/v) DOC
0.1% (w/v) SDS
Prepare fresh

SDS sample buffer, 2×

200 mM Tris·Cl, pH 6.8
10% (v/v) glycerol
2 mM EDTA
4% (w/v) SDS
0.05% (w/v) bromphenol blue
Store up to 12 months at room temperature

SDS solubilization buffer

1% (w/v) SDS (from 20% SDS stock solution, kept at room temperature)
20 mM Tris·Cl, pH 8.8
2 mM PMSF
2 mM EDTA
2 mM iodoacetic acid (freshly prepared in H_2O)
2 mM *N*-ethylmaleimide (freshly prepared in H_2O)
Prepare fresh

COMMENTARY

Background Information

The extracellular matrix (ECM) is a protein network that acts as a framework for tissue architecture and dynamically regulates many cellular functions such as adhesion, migration, growth, and differentiation. A major component of most matrices, fibronectin (FN) is a multifunctional glycoprotein synthesized by many cell types including fibroblasts, endothelial cells, myoblasts, and astrocytes (Hynes, 1990; Pankov, 2002). In addition to cellular FN produced by cells in tissues, there is a considerable amount of FN in blood plasma. This plasma FN is made by hepatocytes and differs from cellular FN by alternative splicing (Schwarzbauer, 1991). FN is synthesized and secreted as a disulfide-bonded dimer. It is assembled by cells into a fibrillar matrix via a regulated, step-wise process (Schwarzbauer and Sechler, 1999; Wierzbicka-Patynowski and Schwarzbauer, 2003). Initiation of assembly depends on FN binding to cell surface integrin receptors. Once immobilized, dimeric FN is active to participate in FN-FN interactions leading to formation of fibrils. As the process proceeds, short fine fibrils become longer and denser. Initially the integrin-associated fibrils are soluble in deoxycholate (DOC) detergent. As additional FN dimers are assembled, the DOC-soluble pool is converted into DOC-insoluble matrix in which the FN dimers are quite tightly associated into detergent-stable high-molecular weight multimers. (McKeown-Longo and Mosher, 1983).

Thus, conversion from cell-associated fine fibrils to stable matrix can be monitored by analysis of the amounts of FN in these two pools. FN fibril organization can be examined by immunofluorescence staining.

Biochemical and microscopic analyses provide distinct types of information about FN matrix assembly. Immunoblotting of detergent lysates allows quantification of the amounts of FN in DOC-soluble and -insoluble fractions as well as determination of whether matrix FN is intact or has been proteolyzed. Immunofluorescence analyses allow one to follow the progression of FN assembly from fine fibrils to dense, stable matrix as well as to determine the overall distribution of FN in the cell layer.

Critical Parameters

At the time of analysis, cells should be sufficiently dense to ensure optimal conditions for fibril formation between adjacent cells but not so crowded that they are approaching quiescence. Critical steps in the DOC-solubility assay include reducing the viscosity of genomic DNA, gently removing DOC-soluble from the DOC-insoluble fraction after centrifugation, and completely dissolving the DOC-insoluble pellet.

Anticipated Results

Fibroblasts and other cells that synthesize significant levels of FN usually yield a visible amount of DOC-insoluble material within several hours after plating. Cell types that produce very little FN, such as many tumor cell lines, can require incubations as long as 24 hr for isolation of detectable DOC-insoluble FN. To increase the amount of FN available for assembly, cell cultures can be supplemented with exogenous FN allowing detection of FN matrix after much shorter incubation periods. The sensitivity of the DOC-solubility assay is in the nanogram range. Immunofluorescence staining usually shows a bright fibrillar pattern at times corresponding to detectable FN by immunoblotting.

Time Considerations

Time after plating cells is variable depending on the experiment. Performance time for the DOC-solubility assay depends on the number of samples but should be easily completed in 1 to 2 hr. SDS-PAGE and immunoblotting are described in *UNITS 6.1 & 6.2*. Similarly, antibody staining requires ~2 hr for fixation and incubations.

Literature Cited

Hynes, R.O. 1990. Fibronectins. Springer-Verlag, New York.

McKeown-Longo, P.J. and Mosher, D.F. 1983. Binding of plasma fibronectin to cell layers of human skin fibroblasts. *J. Cell Biol.* 97:466-472.

Pankov, R. and Yamada, K.M. 2002. Fibronectin at a glance. *J. Cell Sci.* 115:3861-3863.

Quade, B.J. and McDonald, J.A. 1988. Fibronectin's amino-terminal matrix assembly site is located within the 29-kDa amino-terminal domain containing five type I repeats. *J. Biol. Chem.* 263:19602-19609.

Schwarzbauer, J.E. 1991. Alternative splicing of fibronectin: Three variants, three functions. *BioEssays* 13:527-533.

Schwarzbauer, J.E. and Sechler, J.L. 1999. Fibronectin fibrillogenesis: A paradigm for extracellular matrix assembly. *Curr. Opin. Cell Biol.* 11:622-627.

Wierzbicka-Patynowski, I. and Schwarzbauer, J.E. 2003. The ins and outs of fibronectin matrix assembly. *J. Cell Sci.* 116:3269-3276.

Contributed by Iwona Wierzbicka-Patynowski, Yong Mao, and Jean E. Schwarzbauer

Princeton University

Princeton, New Jersey

Non-Radioactive Quantification of Fibronectin Matrix Assembly

Fibronectin (FN) matrix assembly is a cell-dependent process that converts soluble FN molecules into elaborate extracellular fibrillar matrices. This process relies on activated integrins, cellular contractility, and unmasking of cryptic fibronectin assembly sites for generation of insoluble fibrils (Geiger et al., 2001). The signaling pathways involved in matrix assembly have just begun to be elucidated (Wierzbicka-Patynowski and Schwarzbauer, 2002), and further studies will require simple and reliable assays for quantification of matrix assembly associated with parallel determinations of the activity of various signaling molecules.

This unit provides a protocol (see Basic Protocol) for non-radioactive determination of the rate of incorporation of biotinylated fibronectin into the insoluble matrix organized by cultured cells. This protocol provides a simple method for quantifying changes in matrix assembly that result from different experimental treatments or conditions with concomitant determinations of the activation state of various signaling molecules that may be involved in the process of matrix assembly. This unit also provides a protocol (see Support Protocol) for biotinylation of purified fibronectin.

QUANTIFICATION OF MATRIX ASSEMBLY USING BIOTINYLATED FIBRONECTIN

BASIC PROTOCOL

This protocol can be applied to nearly all cultured cell lines with little or no modifications. It describes labeling of the matrix assembled by cultured cells with biotinylated fibronectin, followed by isolation of detergent-insoluble fibronectin matrices (see *UNIT 10.12*). Quantification of the incorporated biotinylated FN is performed by electrophoresis (*UNIT 6.1*), electroblotting (*UNIT 6.2*), and detection with peroxidase-conjugated streptavidin. The quantities of intermediate filament proteins present in the detergent-insoluble fractions are determined by immunoblotting for use as the internal controls for isolation efficiency of the detergent-insoluble matrix in each fraction. The detergent-soluble fractions are used to monitor the ability of cells to bind fibronectin under the conditions being tested and to determine simultaneously the activation state of the signaling molecules of interest.

Materials

Fibroblasts (or any cell line of interest)
Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum (DMEM/10% FBS; *APPENDIX 2A*)
Biotinylated fibronectin (see Support Protocol)
PBS (*APPENDIX 2A*), ice cold
DOC extraction buffer (see recipe)
2× SDS sample buffer (*APPENDIX 2A*)
1 M NaF
0.1 M sodium orthovanadate solution (*APPENDIX 1B*)
10 mM leupeptin (*APPENDIX 1B*)
25 mM pepstatin A (*APPENDIX 1B*)
0.2 M phenylmethanesulfonyl fluoride (PMSF; *APPENDIX 1B*)
2× SDS sample buffer (*APPENDIX 2A*)

Contributed by Roumen Pankov and Kenneth M. Yamada
Current Protocols in Cell Biology (2004) 10.13.1-10.13.9

8% (w/v) polyacrylamide separating gels with 4% (w/v) stacking gels (*UNIT 6.1*) or commercially available pre-cast 4% to 12% gradient gels (e.g., Novex) for SDS gel electrophoresis

Prestained protein molecular size standards (e.g., Novex)

Transfer buffer (*UNIT 6.2*)

Ponceau S solution (*UNIT 6.2*)

Tris-buffered saline with 0.1% (v/v) Tween 20 (TTBS; *APPENDIX 2A*)

Blocking solution: TTBS containing 5% (w/v) dry nonfat milk (TTBS/milk)

Streptavidin, horseradish peroxidase (HRP)-conjugated (e.g., Jackson ImmunoResearch)

Enhanced chemiluminescence (ECL) detection reagent (*UNIT 14.2*)

Primary antibody: monoclonal anti-vimentin (e.g., Sigma)

Secondary antibody: horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (e.g., Amersham Bioscience)

35-mm tissue culture dishes

Plastic cell scraper (rubber policeman)

23-G needle and 1-ml syringe

1.5-ml microcentrifuge tubes

Micropipettors

Porous electrotransfer pads

Whatman 3MM filter papers cut to gel size

Two nitrocellulose membranes cut to gel size

SDS-PAGE/transfer apparatus (e.g., Bio-Rad, Novex)

Constant-voltage/current power supply (e.g., Bio-Rad)

Flat containers

Rocking shaker

Heat-sealable plastic bags and sealer

Sonicator/ultrasonic processor

Plastic wrap

X-ray film (e.g., Hyperfilm; Amersham Bioscience)

Tube heater (e.g., Thermomixer; Eppendorf) or boiling water bath

Film cassette for X-ray film

X-ray film developer

Additional reagents and equipment for dialysis (*APPENDIX 3C*), tissue culture (*UNIT 1.1*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

NOTE: All reagents and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a 37°C, 10% CO_2 humidified incubator. Use pre-warmed cell culture medium for all treatments.

Prepare and treat cells

1. Plate cells in 35-mm dishes so that after spreading they will be ~90% to 95% confluent, and culture overnight in DMEM/10% FBS.

   Depending on the size of fibroblasts used, the desired confluency can be obtained by plating between 0.25 × 10^6 cells (e.g., primary human fibroblasts) and 0.5 × 10^6 cells (e.g., NIH 3T3 cells).

2. After overnight incubation, wash cells with 1.0 ml DMEM/10% FBS and add 1 ml/plate of the same medium containing 20 μg/ml biotinylated FN.

   Treatment with reagents of interest can be incorporated in this step, which can include chemical compounds, peptides, antibodies, etc. Add an appropriate volume of stock

solution containing the reagent to one plate and the same volume of the solvent (e.g., DMSO) to another plate that will serve as a control. Label each plate.

If the presence of serum in the medium interferes with the action of the tested reagent (e.g., growth factors), replace serum with 1% (w/v) bovine serum albumin during the treatment period.

3. Incubate plates in a tissue culture incubator for 4 hr.

   Depending on the ability of the cells to form fibronectin matrix, this time period can be varied. For example, a 3-hr incubation is sufficient for primary human fibroblasts to incorporate readily detectable quantities of biotinylated fibronectin into the detergent-insoluble fraction, while 4 to 6 hr are necessary for β_1 null GD 25 cells to polymerize enough labeled FN for reliable detection.

4. Aspirate medium and wash cell monolayers three times with 2 ml ice-cold PBS each.

5. Lyse cells in 0.5 ml DOC extraction buffer, scrape plates with plastic scraper, pass the lysate five times through a 23-G needle attached to a 1-ml syringe, and transfer into labeled 1.5-ml microcentrifuge tubes. Keep lysates on ice.

   Shearing DNA by passing lysates through a thin needle is necessary to reduce viscosity and to allow sedimentation of small, insoluble matrix aggregates during centrifugation. Check that the needle is firmly attached to the syringe and gently aspirate and expel lysate from the syringe while avoiding the formation of bubbles.

6. Centrifuge lysates 20 min at 20,000 × *g*, 4°C.

Prepare detergent-insoluble matrix

7. Transfer a 100-µl aliquot of the supernatant into new 1.5-ml microcentrifuge tubes labeled DOC soluble, mix with 100 µl of 2× SDS sample buffer, and leave on ice.

8. Carefully remove the rest of the supernatant, leaving the DOC-insoluble pellet intact.

9. Wash the pellet by resuspending it in 100 µl DOC extraction buffer, pipetting up and down five times with a micropipettor.

10. Centrifuge 10 min at 20,000 × *g*, 4°C.

11. Carefully remove the supernatant and dissolve the pellet in 50 µl of 2× SDS sample buffer.

    Attention should be paid to avoid losing the pellet, which is usually very small and sometimes difficult to visualize.

Analyze samples by SDS-PAGE

12. Boil samples collected at steps 7 and 11 in a water bath for 3 min or heat 5 min on a 95°C heating block.

    The samples can be stored sealed and frozen at least 1 month at −20°C.

13. Cast an 8% polyacrylamide separating gel with a 4% stacking gel (*UNIT 6.1*) or use a commercially available pre-cast 4% to 12% gradient gel.

14. Load 25 µl of each sample/gel lane and a separate lane containing prestained protein standards on the gel.

    Load the set of DOC-insoluble samples first, followed by the set of DOC-soluble samples. Divide the two sets with marker proteins or an empty well, so that after the transfer the two portions of the membrane can be separated. Alternatively, load the two sets of samples on two separate gels.

15. Electrophorese the gel(s) at 150 V until the bromophenol blue dye reaches the bottom of the gel (see *UNIT 6.1*).

Transfer separated proteins from gel to membrane

16. When electrophoresis is complete, remove gel(s) from gel plates, cut off the stacking gel(s), and incubate the separating portion of gel(s) in 50 ml transfer buffer for 15 min.

    Use gloves to handle gels and membranes, since oil from hands can interfere with the transfer.

17. Assemble the transfer sandwich consisting of porous electrotransfer pad, Whatman 3MM filter paper, nitrocellulose membrane, equilibrated acrylamide gel, second Whatman 3MM filter paper, and second pad (Fig. 6.2.1).

    All pads, filter papers, and nitrocellulose membranes should be handled using gloves and pre-wetted with transfer buffer. The transfer cassette should be assembled submerged under the transfer buffer to avoid trapping air bubbles. Keep the orientation of the gel (judged by the position of the prestained protein standards) such that it will ensure the correct order of the samples after transfer onto the nitrocellulose membrane.

18. Place the transfer sandwich into the electroblotting apparatus filled with transfer buffer with the nitrocellulose membrane on the cathode side of the gel. Connect the apparatus to the power supply and transfer proteins for 1 hr at 100 V (constant voltage) with cooling (*UNIT 6.2*).

    Transfer time depends on the size of the proteins, acrylamide percentage, and thickness of gel. The completeness of transfer can be easily judged by the extent of transfer of the prestained protein standards.

19. After completing the electrotransfer, turn off the power supply and disassemble the apparatus and the transfer cassette. Remove the nitrocellulose membranes and stain with 50 ml Ponceau S solution in a flat container for 5 min. Destain membranes with several rinses of distilled water.

    If the two sets of samples were run on the same gel, cut membrane along the well that separates the two sets (see step 14).

    Two membranes can be incubated in the same container by orienting them back to back.

    Staining with Ponceau S does not interfere with subsequent reactions and provides a good estimate of protein loading, separation, and quality of transfer. The amounts of protein in the DOC-insoluble samples will be several-fold lower than the proteins in the DOC-soluble samples, because most cellular proteins are soluble in DOC, whereas only FN and a few other proteins are insoluble.

    The Ponceau S solution can be reused several times.

Probe membranes with streptavidin-HRP and antibodies

20. Rinse membranes one time with TTBS and incubate in 50 ml blocking solution for 30 min at room temperature with gentle shaking.

    Milk proteins in the blocking buffer are used to saturate free protein-binding sites and to prevent nonspecific binding. Do not incubate the membrane >1 hr in blocking buffer, since it has a slight stripping effect and may cause detachment of transferred proteins.

21. Dilute streptavidin-HRP as recommended by the manufacturer in a final volume of 10 ml TTBS containing 3% dry nonfat milk. Place membranes in a heat-sealable plastic bag, add diluted streptavidin-HRP, and seal the bag. Incubate membranes 1 hr at room temperature with gentle shaking.

    Two membranes can be incubated in the same bag by orienting them back-to-back. Remove all air bubbles from the bag before sealing.

22. Remove membranes from the bag and wash them three times, 15 min each, with 50 ml TTBS in a flat container with vigorous shaking.

23. Use the ECL immunodetection protocol (*UNIT 6.2*) to detect biotinylated fibronectin. Incubate membranes with ECL solution for 1 min, remove excess fluid by touching the edge of the membrane held vertically to a horizontal piece of filter paper, wrap membranes in plastic wrap, and expose to X-ray film.

    Do not allow membranes to dry after the exposure.

24. Rinse membranes with 10 ml TTBS.

Probe membranes for control proteins

25. Incubate the membrane containing the DOC-insoluble samples with anti-vimentin antibody, and the membrane containing DOC-soluble samples with anti-actin antibody. Dilute the antibodies according to the manufacturer's recommendations in a final volume of 10 ml TTBS containing 3% dry nonfat milk. Place membrane in a heat-sealable plastic bag, add diluted antibodies, and seal the bag. Incubate membranes for 1 hr at room temperature (or overnight at 4°C) with gentle shaking.

    This second reaction is used as an internal control for the efficiency of matrix isolation and loading of the gels. If primary cells are used in the experiment, they may not express detectable amounts of vimentin. In such cases, a different intermediate filament protein can be used as a marker. The choice of appropriate marker will depend on the origin of the primary cells (see Coulombe et al., 2001).

26. Repeat step 22.

27. Dilute the secondary antibody in a final volume of 10 ml TTBS containing 3% dry nonfat milk according to manufacturer's instructions. Place membranes in a new heat-sealable bag, add diluted antibody, and seal. Incubate 30 to 45 min at room temperature with gentle shaking.

    Either HRP- or alkaline phosphatase–conjugated secondary antibody can be used. HRP-conjugated secondary antibodies can be combined with the high-sensitivity ECL detection system.

    This system allows detection of signals from weak antibodies, although attention should be paid to linearity if accurate quantification of the signal is necessary (see Commentary).

28. Repeat step 22 and detect the secondary antibody using the ECL procedure described in step 23.

    The membrane containing resolved proteins from DOC-soluble fractions can be re-probed again with antibodies recognizing the phosphorylated (activated) forms of different signaling molecules of interest. This step is possible if the molecular masses of the signaling molecules are different from those of fibronectin (250 kD) and actin (45 kD) and if the new signals on the membrane will not interfere with any previous band detected. Alternatively, the remainder of DOC-soluble samples can be used for additional immunoblotting experiments with other antibodies.

Quantify gels

29. Measure the optical density (absorbance) of the signals from biotinylated FN and the antibodies for each sample using densitometry (*UNIT 6.3*) or image processing software (e.g., NIH Image).

30. Normalize the densitometry values from biotinylated FN to the readings for vimentin in the DOC-insoluble fraction and the densitometry readings from biotinylated FN to the readings for actin in the DOC-soluble fraction. Calculate fold or percent changes relative to the control.

SUPPORT PROTOCOL

BIOTINYLATION OF PLASMA FIBRONECTIN

Biotin is a vitamin that binds with high affinity to avidin and streptavidin. Because of its small size (244 Da), it can be used to label proteins without significant risk of affecting their function.

This protocol describes labeling of fibronectin with sulfo-NHS-biotin, followed by a dialysis step to remove unconjugated biotin.

Additional Materials *(also see Basic Protocol)*

Fibronectin (e.g., Sigma or purified as described in *UNIT 10.5*)
Bicarbonate buffer (see recipe)
Sulfo-NHS-biotin (e.g., Pierce)

1. Dialyze 0.5 mg fibronectin against 1 liter of bicarbonate buffer overnight at 4°C or 2 hr at room temperature, change bicarbonate buffer once, and dialyze for an additional 1 hr at room temperature.

   For a 25-sample preparation, 0.5 mg of fibronectin in 0.5 to 1.0 ml will be sufficient.

2. Immediately prior to use, dissolve 0.5 mg sulfo-NHS-biotin in 0.5 ml deionized water.

3. Immediately add 40 µl sulfo-NHS-biotin solution to 0.5 ml of fibronectin solution and incubate 30 min at room temperature on a gently rocking shaker.

   Avoid harsh mixing and foaming, because fibronectin tends to denature and precipitate at the liquid/air interface.

4. Dialyze the biotinylated fibronectin against 1 liter of TBS overnight at 4°C or 2 hr at room temperature, change TBS buffer once, and dialyze for an additional 1 hr at room temperature.

5. Centrifuge fibronectin solution in a microcentrifuge 15 min at maximum speed at room temperature and save the supernatant.

   This step will remove possible precipitates from the solution.

6. Determine protein concentration using BCA assay (*APPENDIX 3H*). Store 200-µl aliquots indefinitely at −70°C. Avoid repeated freeze-thawing.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DOC extraction buffer

1% (w/v) sodium deoxycholate
20 mM Tris·Cl, pH 8.5 (*APPENDIX 2A*)
2 mM *N*-ethylmaleimide, add fresh
2 mM iodacetic acid, add fresh
2 mM EDTA (*APPENDIX 2A*)
50 µM leupeptin (*APPENDIX 1B*), add fresh
50 µM pepstatin (*APPENDIX 1B*), add fresh
1 mM PMSF (*APPENDIX 1B*), add fresh
1 mM sodium vanadate (*APPENDIX 1B*), add fresh
50 mM NaF (*APPENDIX 1B*), add fresh
Prepare fresh

This DOC extraction buffer is based on the work of McKeown-Longo and Mosher (1983).

Bicarbonate buffer

50 mM $NaHCO_3$
100 mM NaCl
Adjust pH to 8.5 with 1 M NaOH if necessary
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

Fibronectin matrices not only provide substrates for cell attachment and tissue organization, but they also regulate migration, cell growth, and differentiation. These matrices are organized by cells from secreted cellular fibronectin and soluble plasma FN from blood, where this glycoprotein is present at high concentrations (300 μg/ml). In vitro, cells polymerize exogenous fibronectin from serum-supplemented culture medium together with the secreted FN (McKeown-Longo and Mosher, 1983). Even cells that do not produce fibronectin are able to form matrices when this molecule is provided exogenously (Sottile and Hocking 2002). This property permits the use of labeled exogenous FN as a tracer during the process of matrix assembly.

Experiments with iodinated fibronectin have shown that shortly (2 to 10 min) after addition to the culture medium, it binds to the cell culture and becomes resistant to simple rinses with buffers that preserve cell viability (McKeown-Longo and Mosher, 1983). Prolonged incubation leads to increased binding and formation of two different fibronectin pools that can be distinguished by their solubility in 1% deoxycholate (DOC). The DOC-soluble pool represents FN bound by cellular receptors and preexisting matrix fibrils, while the DOC-insoluble pool is believed to include bound fibronectin that is incorporated in the matrix through detergent-resistant interactions such as disulfide bonding. Based on the similarities between the incorporation of exogenous and endogenous FN, the quantities of labeled FN in different fractions appear to be proportional to the total amounts of fibronectin present in these fractions. Thus, the ability of cells to bind and organize fibronectin matrix under different conditions where a variety of signaling pathways are affected can be studied by following the relative distributions and quantifying the amounts of labeled (tracer) fibronectin between these two fractions.

Purification of the DOC-insoluble fraction from relatively small amounts of cultured cells very often poses technical difficulties in handling such small and often invisible pellets. Possible losses of part of the DOC fractions will lead to erroneous interpretation of the results. This serious problem can be avoided by a parallel determination of the amount of an intermediate filament protein present in this fraction. Due to their high insolubility, these proteins resist DOC extraction, and their quantities can be used as internal controls for the efficiency of isolation and recovery of the DOC-insoluble fraction.

Radioactive isotopes are widely used for quantification purposes, but this method demands special training, equipment, and disposal of reagents. Substitution of biotinylated fibronectin for radioiodinated fibronectin simplifies the technique while still preserving the necessary level of sensitivity. Moreover, covalently linking *N*-hydroxysulfosuccinimide (NHS)-coupled biotin to fibronectin is a routine and easy procedure. It permits detection of biotinylated FN with avidin through the strongest known noncovalent recognition reaction ($K_a = 10^{15}\ M^{-1}$).

Addition of phosphatase inhibitors to the DOC extraction buffer permits the use of the DOC-soluble fractions for determination of the activity of different phosphorylated signaling molecules by using simple immunoblotting techniques and phosphospecific antibodies, making this method more versatile than the classical DOC solubility assay.

Critical Parameters and Troubleshooting

Several parameters play critical roles for success in the quantification of fibronectin matrix assembly. Incorporation of readily detectable levels of biotinylated fibronectin into the DOC-insoluble fraction is necessary for accurate quantification. This step depends on the ability of the cells being studied to organize matrix, and it can be achieved by optimizing the duration of the labeling period.

Obtaining a high signal-to-noise ratio after immunoblotting is also essential for successful quantification of the amounts of biotinylated fibronectin and changes in signaling pathways. This goal can be achieved by: (1) loading

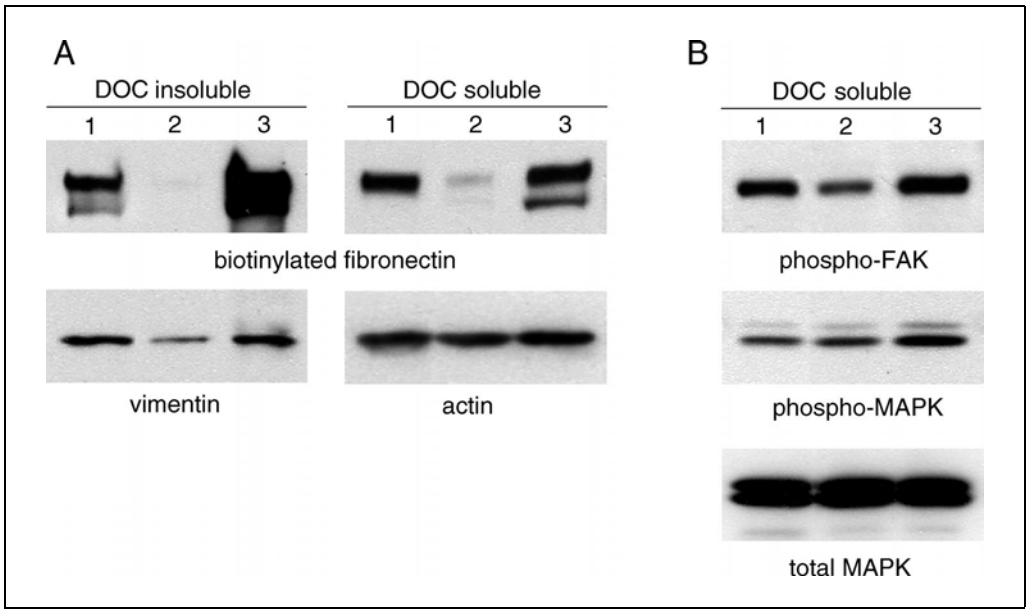


Figure 10.13.1 Determination of matrix assembly by the methods described in this unit. (**A**) Primary human fibroblasts were cultured overnight in normal medium, washed with medium without serum containing 1% BSA, and incubated in the same medium supplemented with 20 μg/ml biotinylated fibronectin without additional agents (lane 1), with 10 μM ROCK inhibitor Y27632 (lane 2), or with 2 μM lysophosphatidic acid (LPA; lane 3) for 4 hr. Deoxycholate (DOC)-insoluble and -soluble fractions were resolved on 4% to 12% gradient polyacrylamide gels, transferred to nitrocellulose membranes, and probed with HRP-conjugated streptavidin for determination of the amount of the incorporated FN (biotinylated fibronectin). The same membranes were re-probed with antibodies against vimentin and actin for determination of the efficiency of purification and gel loading. (**B**) Samples as in (**A**) from the DOC-soluble set were assayed by immunoblotting with antibodies against the activated form of focal adhesion kinase (phospho-FAK), activated form of mitogen-activated protein kinase (phospho-MAPK) and total MAPK for determination of the effect of Y27632 and LPA on the these signaling molecules.

sufficient amounts of proteins from both fractions to ensure trouble-free detection of the biotinylated FN; (2) use of freshly added phosphatase and protease inhibitors to the DOC extraction buffer; (3) use of sufficiently specific antibodies that recognize the phosphorylated but not the unphosphorylated forms of the signaling molecules of interest; and (4) following proper techniques for SDS-PAGE and immunoblotting (see *UNITS 6.1 & 6.2*).

Accurate comparison and quantification by densitometry of the amounts of biotinylated FN as well as the phosphorylation levels of the signaling molecules studied in different samples can be achieved if the detection system is kept in a linear range. That is, loading two times the amount of sample should be reflected in a doubling of signal. The enhanced chemiluminescence (ECL) system should be optimized to obtain linearity by adjustments of the amount of protein loaded on the gel, concentrations of primary and secondary antibody, and X-ray film exposure time. If the weakest signal is detectable and the strongest signal is still within the linear range of the film (e.g., not saturated), then the rest of the samples are also in the linear range and the results can be used for quantification.

Anticipated Results

Typical results expected after performing the Basic Protocol are presented in Figure 10.13.1. Easily detectable amounts of biotinylated FN should be present in the control lanes (Fig. 10.13.1 A, lane 1) in both DOC-insoluble and DOC-soluble sets of samples. Different treatments may have different effects on matrix assembly. In the example presented, blocking cellular contractility with Y27632 strongly decreased both binding of FN to the cells (Fig. 10.13.1 A, DOC soluble, lane 2) and its incorporation into the matrix (Fig. 10.13.1 A, DOC insoluble, lane 2). The opposite effect was observed after stimulation of cellular contractility with lysophosphatidic acid (LPA; Fig. 10.13.1 A, lane 3). Calculation of the differences observed (see Basic Protocol) revealed an 11.5-fold reduction in the incorporation of labeled FN into the DOC-insoluble matrix after treatment with Y27632 and a 4.9-fold

increase after stimulation with LPA. The reduction in the DOC-soluble fraction after treatment with Y27632 was 5-fold, suggesting that this agent may affect not only formation of the matrix, but also initial FN binding to the cell surface.

The decrease in matrix assembly after inhibition of cellular contractility was accompanied by a reduction in the activation of FAK, but not of MAPK (Fig. 10.13.1 B, lane 2), suggesting that in this particular experimental setting, the activity of FAK may be important for matrix assembly. While the effects of agents such as Y27632 and LPA on matrix formation are clear, drawing unambiguous conclusions about the relevant signaling events demands a number of additional experiments. Nevertheless, such initial correlative data between signaling and matrix assembly provide a good starting point.

Time Considerations

The procedure described in the Basic Protocol can be completed in 3 days.

The first day includes the time for cell attachment after plating (overnight); labeling with biotinylated FN (4 hr) and isolation of DOC-soluble and DOC-insoluble fractions (2 hr). The second day comprises SDS-PAGE and electrotransfer (3.5 hr for mini gels); probing with streptavidin-HRP and ECL reaction (2.5 hr); and overnight incubation with the primary antibody. The third day is for completion of the immunoreactions and ECL processing (4 hr).

There are a number of points where the procedure can be interrupted: (1) after preparation of the SDS-PAGE samples; (2) after the electrotransfer (membranes can be stored wet or dry in resealable plastic bags at 4°C); and (3) after completion of the first ECL development (membranes can be stored wet in resealable plastic bags at 4°C).

The procedure described in the Support Protocol can be completed in 1 day if the 2-hr dialysis period is employed, or 36 hr if the overnight dialysis is used.

Literature Cited

Coulombe, P.A., Ma, L., Yamada, S., and Wawersik, M. 2001. Intermediate filaments at a glance. *J. Cell Sci.* 114:4345-4347.

Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K.M. 2001. Transmembrane extracellular matrix–cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol.* 2:793-805.

McKeown-Longo, P.J. and Mosher, D.F. 1983. Binding of plasma fibronectin to cell layers of human skin fibroblasts. *J. Cell Biol.* 97:466-472.

Wierzbicka-Patynowski, I. and Schwarzbauer, J.E. 2002. Regulatory role for SRC and phosphatidylinositol 3-kinase in initiation of fibronectin matrix assembly. *J. Biol. Chem.* 277:19703-19708.

Contributed by Roumen Pankov and Kenneth M. Yamada

National Institute of Dental and Craniofacial Research, National Institutes of Health

Bethesda, Maryland

Use of Hyaluronan-Derived Hydrogels for Three-Dimensional Cell Culture and Tumor Xenografts

Monica A. Serban,[1] Anna Scott,[2] and Glenn D. Prestwich[1]

[1]Department of Medicinal Chemistry and Center for Therapeutic Biomaterials, The University of Utah, Salt Lake City, Utah
[2]Glycosan BioSystems, Salt Lake City, Utah

ABSTRACT

The practice of in vitro three-dimensional (3-D) cell culture has lagged behind the realization that classical two-dimensional (2-D) culture on plastic surfaces fails to mirror normal cell biology. Biologically, a complex network of proteins and proteoglycans that constitute the extracellular matrix (ECM) surrounds every cell. To recapitulate the normal cellular behavior, scaffolds (ECM analogs) that reconstitute the essential biological cues are required. This unit describes the 3-D cell culture and tumor engineering applications of Extracel, a novel semisynthetic ECM (sECM), based on cross-linked derivatives of hyaluronan and gelatin. A simplified cell encapsulation and pseudo-3-D culturing (on top of hydrogels) protocol is provided. In addition, the use of this sECM as a vehicle to obtain tumor xenografts with improved take rates and tumor growth is presented. These engineered tumors can be used to evaluate anticancer therapies under physiologically relevant conditions. *Curr. Protoc. Cell Biol.* 40:10.14.1-10.14.21. © 2008 by John Wiley & Sons, Inc.

Keywords: hyaluronan • semisynthetic extracellular matrix • hydrogel • biodegradable scaffold

INTRODUCTION

This unit describes the use of chemically modified, cross-linkable derivatives of hyaluronan (HA) hydrogels for more physiologically significant in vitro cell culturing and in vivo tumor and tissue engineering applications. Traditional two-dimensional (2-D) culturing conditions lead to aberrant cell behavior that may have limited relevance to in vivo conditions (Roskelley et al., 1994; Weaver et al., 1997; Wang et al., 1998; Cukierman et al., 2001). Mammalian cells do not grow in a physiologically realistic manner on plastic. In vivo, an extracellular matrix (ECM) surrounds the cells in all tissues. The ECM is a complex network of proteins and glycosaminoglycans (GAGs), which form a 3-D microenvironment that plays an integral part in signaling cells to proliferate, migrate, differentiate or invade (Galbraith et al., 1998; Geiger et al., 2001; Lutolf and Hubbell, 2005; Holmbeck and Szabova, 2006).

HA is a major constituent of the ECM and is the only nonsulfated GAG present (Knudson and Knudson, 2001). It is biocompatible and biodegradable, and it performs important biological functions such as stabilizing and organizing the ECM (Fraser et al., 1997; Dowthwaite et al., 1998), regulating cell adhesion and motility (Dowthwaite et al., 1998; Cheung et al., 1999), and mediating cell proliferation and differentiation (Entwistle et al., 1996).

The HA-derived hydrogel (Extracel) discussed in this unit is composed of chemically modified HA containing reactive thiol groups (known as CMHA-S, Glycosil, or Carbylan-S) and chemically modified gelatin containing reactive thiol groups (known as

Current Protocols in Cell Biology 10.14.1-10.14.21, September 2008
Published online September 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1014s40

Gtn-DTPH or Gelin-S), which are co-cross-linked with polyethylene glycol diacrylate (PEGDA or Extralink) to form a semisynthetic ECM (sECM; Shu et al., 2004, 2006; Prestwich, 2007, 2008). For clarity and consistency in this unit, we will use the names of the commercially available materials.

All three components of the hydrogel are available as lyophilized solids. Once reconstituted, the solutions can be easily pipetted and transferred into multiple formats (including any well size or tissue culture insert), or it can be injected into an animal model. The hydrogel is formed by mixing the chemical cross-linker, Extralink, with either Glycosil only or Glycosil mixed with Gelin-S. Once the cross-linker is added, the mixture will become more and more viscous until a solid hydrogel is formed. The gelation time can be controlled by the user depending upon requirements.

The HA component Glycosil can be cross-linked alone with Extralink, but most mature cell types do not adhere to HA-only hydrogels. Some cancer cells and stem cells will grow and proliferate in HA-only hydrogels, but usually some attachment factor (e.g., gelatin, an RGD peptide, collagen, laminin, or fibronectin) needs to be mixed with the Glycosil prior to cross-linking with PEGDA. The hydrogel retains proteins greater than 70 kDa in size, so even though the ECM-derived proteins are not covalently attached to the hydrogel, they are entrapped and will only be able to diffuse out as the sECM degrades. Growth factors are also retained within the hydrogel in a similar fashion (Cai et al., 2005; Pike et al., 2006; Riley et al., 2006).

Using three basic components to make an sECM simplifies the biological ECM to a consistent, fully defined, experimentally controllable material for research. Because ECM proteins and growth factors can be incorporated into these hydrogels, it is possible to make a fully defined mimic of specific ECMs found in mammalian tissues if the target tissue ECM composition is known. Additionally, since the basic hydrogel can be formed with only Glycosil and Extralink, animal-free hydrogels can also be made.

STRATEGIC PLANNING

For successful use of these protocols, the researcher must be familiar with how to culture the cells of interest in a 3-D environment or be prepared to conduct several experiments to determine the optimal conditions. Cells cultured in 3-D behave differently than those cultured 2-D on tissue culture–treated plastic. At a minimum, the cell morphology and gene expression patterns can change (Bissell et al., 2003). Because cells receive signals from the matrix on which they are grown (even if this matrix is plastic), the composition and stiffness (compliance) of this matrix help determine the growth and functional characteristics of the cells (Yeung et al., 2005; Engler et al., 2006). In the case of naïve mesenchymal stem cells, the matrix stiffness can cause lineage restriction. For fibroblasts, it changes the amount and arrangement of actin stress fibers (Ghosh et al., 2007). For many cell types, the differences when plating on stiff versus compliant surfaces is not yet characterized. Finally, cells respond differently when encapsulated within a hydrogel or when plated on the surface.

For in vitro cell growth, the culture medium and cell seeding density are very important. It is possible to use the optimal tissue culture–plastic culture conditions as a starting point for the hydrogel experiments. However, it is likely that some modification to these conditions will be required. For the in vivo tumor xenografts, the cell density, injection volume, and hydrogel dilution are critical for the experimental outcome (Liu et al., 2007a). The method of making the hydrogel affects its final properties. There are several variations to this general protocol which are discussed in subsequent sections. Prior to using the hydrogels, the following questions need to be addressed:

1. What gelation time is required?

2. Will cells be encapsulated in the hydrogel?

3. Will ECM proteins be incorporated into the hydrogel?

4. Will growth factors be incorporated into the hydrogel?

5. What hydrogel compliance is required?

Based on the answers to these questions, additional steps may be required in the hydrogel preparation. If it is your first time using these HA-derived hydrogels, performing a simple gelation test before starting the first experiment will greatly improve the chances of success. The test takes ~1 hour and will allow you to understand fundamentally how the materials work. A protocol for performing this test is given in Basic Protocol 1.

This unit contains six protocols which detail how to make the HA-derived hydrogels (Basic Protocol 1), vary their compliance (Basic Protocol 2) and composition (Basic Protocol 3), use them for cell growth in vitro (Basic Protocols 4 and 5), and implant them in mice for in vivo experimentation (Basic Protocol 6).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

STANDARD HA-DERIVED HYDROGEL PREPARATION

BASIC PROTOCOL 1

The basic hydrogel, Extracel, is the foundation tool for all the protocols discussed in this unit. Its preparation is required for 3-D cell and pseudo-3-D culture (encapsulation and surface growth) and tumor xenograft experiments. Extracel is composed of Glycosil (thiol-modified HA), Gelin-S (thiol-modified gelatin), Extralink (PEGDA), and degassed, deionized water (DG Water). Glycosil, Gelin-S, and Extralink are available as lyophilized solids. They must be reconstituted using DG Water prior to forming the hydrogel. When reconstituted, they form low-viscosity solutions in phosphate-buffered saline (PBS), pH ~7.4. The hydrogel is formed by mixing all three components together. The gelation time is highly dependent upon the pH of the Extracel solution: the higher the pH, the faster the gelation time. Additionally, depending upon the amount of Extralink used and the concentration of the Glycosil and Gelin-S solutions, gelation will occur in 10 min to >2 hr. Once the Extralink is added there is a time limit on using the hydrogel because it becomes impossible to pipet after the gelation point is reached.

Materials

7.5-ml Extracel Hydrogel Kit (Glycosan BioSystems) containing:
  Glycosil (three 1-ml vials)
  Gelin-S (three 1-ml vials)
  Extralink (three 0.5-ml vials)
  DG Water (one 10-ml vial)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Serum-free cell culture medium

37°C water bath
1-ml syringes with long-tip 20-G × 1½-in. needles, sterile
37°C shaking or rocking incubator
4-ml glass vials

Prepare the gel

1. Remove Glycosil, Gelin-S and Extralink vials from the −20°C freezer and heat them to 37°C (∼30 min).
2. Remove the DG Water from the −20°C freezer and thaw in a 37°C water bath (∼15 min).
3. Under aseptic conditions and using a syringe with the exact amount of liquid, add 1.0 ml DG Water to the Glycosil vial. Repeat for the Gelin-S vial.
4. Incubate both vials horizontally at 37°C, with shaking (for maximum mixing).

   NOTE: *Vigorous shaking will speed up dissolving time.*

   It will take <30 min for the solids to fully dissolve. Solutions will be clear and slightly viscous.
5. Under aseptic conditions and using a syringe with the exact amount of liquid, add 0.5 ml DG Water to the Extralink vial. Invert several times to dissolve.
6. As soon as possible and within 4 hr of making the solutions, mix equal volumes of Glycosil and Gelin-S in a sterile container. Mix by pipetting up and down gently or inverting the vial.
7. To form the hydrogel, add Extralink to the Glycosil + Gelin-S mix in a 1:4 volume ratio (0.25 ml of Extralink to 1.0 ml Glycosil + Gelin-S).

Perform gelation tests with Extracel

8. Follow steps 1 to 5 (above) for standard hydrogel reagent preparation.
9. Add 0.25 ml Glycosil and 0.25 ml Gelin-S to a small glass vial. Pipet up and down to mix.
10. Add 0.125 ml Extralink to the vial and pipet up and down to mix. Record the time.

    The initial solution of Glycosil + Gelin-S + Extralink will be low viscosity (similar to medium).
11. Every few minutes, invert the vial. Record the time at which the hydrogel no longer flows when the vial is inverted.

    As the hydrogel forms, the liquid will become more viscous.

    The gelation time is the difference between the two recorded times. This establishes the maximum length of time you will have to use Extracel after the Extralink is added.
12. Repeat steps 8 to 11, but in addition, add 0.5 ml PBS to the vial of Glycosil + Gelin-S from step 9.

    This gelation time will be substantially longer due to the dilution of the Glycosil and Gelin-S.
13. Repeat steps 8 to 11, but in addition, add 50 µl cell culture medium (no serum or additives) to the vial of Glycosil + Gelin-S in step 9. Pipet up and down to mix.

    This gelation time will be about the same as with the first trial (step 11). This simulates the addition of cells in medium into the hydrogel prior to cross-linking.

    These gelation tests will help the user determine the time constraints for working with Extracel, once the cross-linker Extralink is added to the Glycosil + Gelin-S. They will also help familiarize the user with how the hydrogel is formed, prior to working with it in an experiment.

HA-DERIVED HYDROGEL STIFFNESS VARIATION

As discussed above, hydrogel stiffness can have a dramatic effect on how cells behave in culture. Using the Extracel Hydrogel Kit as per the standard instructions results in a hydrogel compliance of ~100 Pa (J. Vanderhooft, unpub. observ.).

For the HA-derived hydrogels, compliance variation can be achieved in two different ways: (1) varying the concentration of the cross-linker used and (2) varying the concentrations of the Glycosil and Gelin-S solutions.

By increasing the concentration of Extralink, the compliance can be increased to ~500 Pa (Ghosh et al., 2007). Alternatively, diluting the Extracel solutions can decrease it to below the threshold of detection (~20 Pa). Glycosil-only hydrogels cross-linked with Extralink are ~300 Pa. Changing the concentration of Extralink significantly alters the gelation time, as does diluting the Glycosil and Gelin-S solutions. Doubling the Extralink concentration will decrease the gelation time by ~50%. A 2-fold volume dilution will more than double the time for the hydrogel to form.

Materials

7.5-ml Extracel Hydrogel Kit (Glycosan BioSystems) containing:
  Glycosil (three 1-ml vials)
  Gelin-S (three 1-ml vials)
  Extralink (three 0.5-ml vials; purchase additional vials separately, if required)
  DG Water (one 10-ml vial)
Serum-free cell culture medium
Phosphate-buffered saline (PBS; *APPENDIX 2A*), pH ~7.4 and ~7.6

37°C shaking or rocking incubator
37°C water bath
1-ml syringes with long-tip 20-G × $1^1/_2$-in. needles, sterile

Method 1: vary cross-linker concentration

1a. Prepare Glycosil and Gelin-S as in Basic Protocol 1, steps 1 to 4.

2a. Under aseptic conditions and using a syringe with the exact amount of liquid, add the appropriate amount of DG Water to the Extralink vial based on the desired hydrogel stiffness (see Table 10.14.1). Invert several times to mix.

3a. As soon as possible and within 4 hr of making the solutions, mix equal volumes of Glycosil and Gelin-S by pipetting up and down.

4a. To form the hydrogel, add Extralink to the Glycosil + Gelin-S mix in a 1:4 volume ratio (e.g., 0.5 ml Extralink to 2.0 ml Glycosil + Gelin-S).

The gelation time will decrease with the higher Extralink concentration.

For the stiffest hydrogel, there is insufficient Extralink in the standard Extracel 7.5-ml Hydrogel Kit to use all of the Glycosil and Gelin-S. Individual Extralink vials can be purchased, if required.

Table 10.14.1 Amounts of PBS Added to Extralink When Preparing Hydrogels of Different Stiffnesses by Cross-linker Concentration Variation

Condition	Volume PBS (ml)	Notes
A	0.25	Stiffest
B	0.5	Standard
C	1.0	Softest

Table 10.14.2 Amounts (ml) of PBS Added to Hydrogel Reagent Solutions When Preparing Hydrogels of Different Stiffnesses by Hydrogel Component Dilution

	Stiffest ⟶ Softest				
	Standard	A	B	C	D
Glycosil	0.00	0.25	0.50	0.75	1.00
Gelin-S	0.00	0.25	0.50	0.75	1.00
Extralink	0.00	0.13	0.25	0.38	0.50

Method 2: dilute hydrogel solutions

1b. Prepare the hydrogel kit reagents as in Basic Protocol 1, steps 1 to 5 (standard hydrogel reagent preparation).

2b. Based on the desired stiffness of hydrogel, aseptically add (using a syringe) varying volumes of sterile PBS to the prepared 1-ml Glycosil and Gelin-S vials (see Table 10.14.2). Invert to mix.

3b. Also add varying amounts of sterile PBS to the prepared 0.5-ml Extralink vial (see Table 10.14.2). Invert several times to mix.

4b. As soon as possible and within 4 hr of making the solutions, mix equal volumes of Glycosil and Gelin-S by pipetting up and down.

5b. To form the hydrogel, add Extralink to the Glycosil + Gelin-S mix in a 1:4 volume ratio (e.g., 0.5 ml Extralink to 2.0 ml Glycosil + Gelin-S).

The gelation time will increase with the solution dilution.

BASIC PROTOCOL 3

ECM COMPONENT INCORPORATION IN HYDROGELS

Gelin-S provides basic cell attachment sites for cell lines and some primary cell types (Shu et al., 2006; Prestwich et al., 2007). However, several cell types are dependent upon specific ECM components for growth and differentiation. For specific cell performance, matricellular and extracellular proteins (e.g., laminin, collagen, fibronectin, vitronectin, aggrecan, decorin) may be added to Glycosil-only hydrogels by the user (Mehra et al., 2006). These proteins are easily incorporated noncovalently into the hydrogel prior to gel formation and retained there after gel formation because of their size. The following protocol describes how to prepare Glycosil-only hydrogels mixed with a laminin isoform from a particular animal source.

Materials

1-ml vial of Glycosil (Glycosan BioSystems)
0.5-ml vial of Extralink (Glycosan BioSystems)
DG Water (Glycosan BioSystems)
500 μg/ml commercial (e.g., Sigma) or laboratory-prepared laminin stock solution (or other sterile, cellular matrix protein in aqueous solution): prepared according to the manufacturer's instructions, if commercially obtained

37°C water bath
1-ml syringes with long-tip 20-G × 1½-in. needles, sterile
37°C shaking or rocking incubator

1. Remove the Glycosil and Extralink vials from the −20°C freezer and heat them to 37°C (~30 min). Thaw the laminin solution (for commercial product, per the manufacturer's instructions).

For example, it is necessary to thaw Sigma laminin L6274 overnight.

2. Remove the DG Water from the −20°C freezer and thaw in a 37°C water bath (~15 min).
3. Under aseptic conditions and using a syringe with the exact amount of liquid, add 1.0 ml of DG Water to the Glycosil vial.
4. Place the vial horizontally at 37°C, with shaking (for maximum mixing).

   NOTE: *Vigorous shaking will speed up dissolving time.*

   It will take <30 min for the solids to fully dissolve. Solution will be clear and slightly viscous.
5. Under aseptic conditions and using a syringe with the exact amount of liquid, add 0.5 ml of DG Water to the Extralink vial. Invert several times to dissolve.
6. Add 125 µl of commercially obtained or laboratory-prepared laminin to the 1 ml of Glycosil solution. Mix thoroughly.
7. To form the hydrogel, add Extralink to the Glycosil + laminin mix in a 1:4 volume ratio (0.25 ml Extralink to 1.0 ml Glycosil + 0.125 ml laminin).
8. Vary the composition of the hydrogel, as desired, as follows:

   a. Increase or decrease the amount of laminin.
   b. Vary the source of the laminin.
   c. Use other ECM proteins (e.g., a specific type of collagen, fibronectin, vitronectin, decorin) in place of or in conjunction with laminin.

CELL GROWTH ON HA-DERIVED HYDROGEL SURFACE

BASIC PROTOCOL 4

This protocol describes how to make Extracel hydrogels in a 24-well plate format for cell growth on the surface. The protocol can easily be adapted for use with 6-, 12-, 48- and 96-well plates.

Materials

7.5-ml Extracel Hydrogel kit (Glycosan BioSystems)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile
1–2 × 10^4 cells/ml medium suspension of cultured cells of interest: prepared according to standard procedures (e.g., see *UNIT 1.1*)
Cell culture medium with serum
0.05% trypsin EDTA (VWR)
10× collagenase/hyaluronidase (StemCell Technologies)

37°C water bath
1-ml syringes with long-tip 20-G × 1½-in. needles, sterile
37°C shaking or rocking incubator
15-ml sterile, conical tubes
24-well tissue culture plates
Sterile plate-sealing film (e.g., Axy Seal, VWR) and roller
Light microscope (10× magnification)

Coat plates

1. Prepare the hydrogel kit reagents as in Basic Protocol 1, steps 1 to 5 (standard hydrogel reagent preparation).
2. Under aseptic conditions and using a syringe with the exact amount of liquid, add an additional 1.0 ml of sterile PBS to both the Glycosil and the Gelin-S vials. Shake to mix.

3. Under aseptic conditions and using a syringe with the exact amount of liquid, add an additional 0.5 ml of sterile PBS to the Extralink vial. Shake to mix.
4. Transfer Glycosil and Gelin-S solutions into a sterile 15-ml conical tube. Mix for at least 3 min with a 25-ml pipet by pipetting up and down.

   If the Extracel solutions are not well mixed, the hydrogel surface may not be uniform. This can cause variation in how the cells attach and grow on the hydrogel.
5. Remove a 24-well plate from the packaging.
6. Just before use, add the 1.0 ml Extralink to the tube containing Glycosil + Gelin-S. Mix at least 2 min by pipetting with a 25-ml pipet.
7. Pipet 500 µl into each of ten wells. Rock the plate by hand to ensure that the surface of the plate is equally coated.
8. Remove 300 µl from each well using a pipet, leaving 200 µl of hydrogel in each well. Repeat steps 7 and 8 until all wells are coated.
9. Cover each plate with a sterile film. Seal with a roller so that each well is isolated.

   Since these are thin coatings they will dehydrate very easily to form films if they are not completely sealed.
10. Allow gelation to occur on the bench top.

    It will take >2 hr for gelation to occur.
11. Store up to 4 months at 4°C until ready for use. Do not freeze.

Culture cells on the HA-derived hydrogel surface

12. Remove a precoated 24-well plate from storage at 4°C.
13. Allow it to warm to room temperature or place in the incubator to increase the temperature to 37°C prior to plating.
14. Add 500 µl of the cell suspension in medium to each well on top of the hydrogel.

    NOTE: *Cells should be cultured in the same medium as when they are grown on tissue culture–treated plastic. This medium may or may not contain serum, depending upon the cell type.*

    Cell seeding density depends upon the experiment and the cell type. As a rough guideline, follow the cell seeding density used for seeding a tissue culture–treated plastic 24-well plate.
15. Incubate at least 1 hr in a 37°C, 5% CO_2 incubator.
16. Verify cell attachment under the microscope. Once confirmed, add the appropriate amount of medium (0.5 to 1.5 ml) to each well and return the plate to the incubator.
17. Change the medium as required (based on changes in the medium's phenol red indicator) by carefully aspirating off the medium.

    The hydrogel can easily be removed by vacuum aspiration as well, so this must be done gently and carefully.
18. Pipet 1 to 2 ml medium into each well. Try to avoid disrupting the gel.
19. Return the plate to the incubator.

Recover cells from hydrogel surface

20. Aspirate the medium and wash the hydrogel surface with 1 to 2 ml PBS per well.

21. Add 0.5 ml trypsin solution to the hydrogel surface.

    Other products (e.g., Accutase, Detachin, TrypLE) that are gentler than trypsin and are better tolerated by cells can also be used. However, they may also degrade the hydrogel so that recovered cells carry some hydrogel particles with them. If this occurs, then use a 10× collagenase/hyaluronidase solution to digest the remaining hydrogel.

22. Incubate at 37°C until the cells begin to detach (~15 min).

23. Gently tap the plate to loosen the cells.

24. Add 2 ml medium with serum to the hydrogel surface and pipet up and down to get a uniform cell suspension.

25. Transfer the cells to a 15-ml culture tube. Add 8 ml medium with serum (10 ml final volume). Centrifuge the cells 5 min at 120 × *g*, room temperature.

26. Remove the supernatant and replace with 1 to 2 ml fresh medium.

    Cell viability will be similar to that of cells grown on plastic and detached with trypsin.

CELL ENCAPSULATION IN HA-DERIVED HYDROGELS

BASIC PROTOCOL 5

Encapsulating cells in HA-derived hydrogels and growing them in tissue culture inserts is the best way (in the absence of a bioreactor) to mimic in vivo conditions in vitro. This protocol describes how to make Extracel hydrogels in a 24-well plate format, using tissue culture inserts. Other insert formats also work, but the amount of hydrogel used per insert should be varied based on the insert volume.

It is not always necessary to recover cells from the hydrogels. Cells cultured by encapsulating them in tissue culture inserts can be treated like tissue. The hydrogel can be removed from the insert, embedded in paraffin, sectioned, and stained as per standard protocols. Note that small molecule dyes and stains that are less than 70 kDa in size will freely diffuse into the gel.

It is not possible to perform direct antibody staining of cells encapsulated in HA-derived hydrogels because the antibodies are too large to permeate the gel. If embedding, sectioning, and staining is not desirable, then the cells must be recovered from the hydrogel.

Materials

7.5-ml Extracel Hydrogel Kit (Glycosan BioSystems) containing:
10× collagenase/hyaluronidase (StemCell Technologies)
Sterile phosphate-buffered saline (PBS)
~0.4–2 × 10^4 cells/ml medium suspension of cultured cells of interest: prepared according to standard procedures (e.g., see *UNIT 1.1*)
Cell culture medium with and without serum

37°C water bath
1-ml syringes with long-tip 20-G × 1½-in. needles, sterile
37°C shaking or rocking incubator
24-well plate with tissue culture inserts (e.g., 6.5-mm Costar tissue culture–treated polycarbonate membrane polystyrene plates, Corning; 8.0-μm pore size Millicel, Millipore
35-mm sterile petri dishes
Surgical scalpel
15-ml conical centrifuge tube

Encapsulate cells

1. Prepare hydrogel kit reagents as in Basic Protocol 1, steps 1 to 5 (standard hydrogel reagent preparation).
2. Determine the volume of suspension required to obtain the desired seeding density in 2.5 ml of the hydrogel.

   Seeding density varies with cell type, but a typical range is 10,000 to 50,000 cells per insert.

3. Prepare two 24-well plates with tissue culture inserts by removing them from their sterile packaging.
4. Mix 1.0 ml of Glycosil and 1.0 ml of Gelin-S.
5. Centrifuge the volume determined in step 2 for 5 min at 120 × *g*, room temperature, and discard the supernatant. Resuspend the cell pellet in the 2 ml of Glycosil + Gelin-S.
6. Just before pouring the hydrogels, add 0.5 ml of Extralink to Glycosil + Gelin-S with cells. Mix completely by pipetting up and down.

   Once the Extralink is added you have <20 min before the hydrogel forms.

7. Quickly pipet 100 µl of Extracel mix into each insert.

   Do not add medium at this point because this will dilute the hydrogel and prevent it from gelling.

8. Incubate the plates for ~1 hr in a 37°C, 5% CO_2 incubator to allow the Extracel to gel.
9. Remove the plates from incubator and verify that the hydrogel is solid. If so, add 1.8 ml medium (with serum, if required) to each well. Incubate in a 37°C, 5% CO_2 incubator.
10. Change the medium as required:

    a. Move each tissue culture insert to an adjacent empty well.
    b. Aspirate the medium.
    c. Tap each insert carefully to remove the medium above the hydrogel in the insert.

       Aspiration can be used to remove the medium, but, the gel can also easily be removed by vacuum aspiration, so this must be done gently and carefully.

    d. Replace the insert into its original well.
    e. Slowly and carefully pipet 1.8 ml medium into each well.

       Try to avoid disrupting the gel.

    f. Return the plate to the 37°C, 5% CO_2 incubator.

       Cells behave differently when cultured in 3-D than when grown on the surface of a hydrogel or tissue culture–treated plastic. The cells will grow at different rates (typically slower) and have different morphologies (depending upon the hydrogel stiffness and composition). Additionally, the cells are not passaged in the traditional manner. Since the volume of the hydrogel provides a large volume for growth, the cultures can be maintained for many days, even weeks, before the cells become confluent.

Recover encapsulated cells

11. Dilute the 10× collagenase/hyaluronidase 10-fold in the cell culture medium (without serum) used to cultivate the cells.

    Do not use undiluted enzyme because this results in low cell viability.

If using medium that contains serum for culture, make sure to wash the hydrogels with serum-free medium or PBS before starting the digestion process because the serum will inactivate the enzymes. At a minimum, wash hydrogels twice for 1 hr to clear serum).

12. Remove the tissue culture insert from the 24-well culture plate. Place upside down in a petri dish.
13. Remove the membrane by using a surgical scalpel to cut it loose from the insert.

    The membrane will stay attached to the insert, but usually flips up out of the way.

14. Turn the insert right side up and using the back of a 10-µl pipet tip punch the hydrogel out of the insert into the petri dish.
15. Place the hydrogel in a 15-ml conical tube and add 5 ml diluted collagenase/hyaluronidase solution to the hydrogel for each 100 µl of hydrogel.
16. Incubate overnight at 37°C, with gentle shaking.

    At the end of the incubation there will still be some hydrogel left in the tube.

    If the 10-fold dilution of 10× collagenase/hyaluronidase is not satisfactory, try a 5-fold dilution with digestion overnight.

    Be cautious about mechanically breaking up the hydrogel prior to digestion because this can lower cell viability significantly.

17. Centrifuge in the conical tube 5 min at 120 × *g*, room temperature.
18. Aspirate and discard the supernatant.

Wash the cells

19. Add 5 ml PBS to wash the cell pellet.
20. Repeat steps 17 and 18.
21. Resuspend the cell pellet in 5 ml PBS.

    NOTE: *In the PBS you can see any remaining hydrogel.*

22. Centrifuge cell suspension 15 min at 120 × *g*, room temperature.
23. Aspirate and discard the supernatant.
24. Add 5 ml medium and repeat the centrifugation.
25. Aspirate off all medium but ~0.5 ml and resuspend pellet (in the remaining 0.5 ml) in the desired volume of cell culture medium.

HA-DERIVED HYDROGELS FOR TUMOR XENOGRAFTS

BASIC PROTOCOL 6

Clinically relevant cancer models are necessary to improve our ability to successfully treat the disease. Anticancer drug discovery efforts require models that can predictably translate preclinical results to efficacy in human patients. Most commonly used are the human tumor xenograft models, where human cancer cells are injected into immune compromised mice. Typically these cells are injected in serum-free medium or buffer or Matrigel (a tumor-derived basement membrane extract). Poor ‘‘take’’ is often a problem, and many cell lines or patient-derived cells will not form tumors by injection in buffer or medium.

HA-derived hydrogels can be used for the delivery and growth of cancer cells in vivo for the growth of orthotopic and subcutaneous tumors. Using a hydrogel to deliver cancer cells can offer several advantages (Liu et al., 2007a):

The incidence of cancer formation is increased and variability in tumor size is reduced.
The growth of organ-specific cancers is enhanced with improved tumor-tissue integration.
Vascularization is increased and necrosis is reduced in tumors.
Cancer seeding on adjacent tissues or organs is minimized.
The general animal health is improved, leading to better data with fewer animals.

Use the pilot study described below to determine the optimal:

Extracel dilution factor
Cell density
Hydrogel injection volume
Coordination of surgical or injection manipulations with hydrogel handling.

The protocol provided below is based on nine mice with two subcutaneous injections each (see Table 10.14.3), where each experimental condition (cell density and injection volume) has an "$n = 3$" (see Table 10.14.4). Please adjust this protocol (cell density and injection volume, especially) as required, based on experimental requirements and experience.

NOTE: These guidelines describe how to prepare Extracel hydrogels for encapsulation of cancer cells and injection of this suspension into experimental animals ***for research purposes only***.

NOTE: Researchers are responsible for obtaining a valid Institutional Animal Care and Use Committee (IACUC) protocol prior to initiation of any experiments (if applicable). The guidelines below only pertain to the operational use of the Extracel product in order to assist in preparing an IACUC protocol.

Table 10.14.3 Composition of Injections Mixes (μl)

	Glycosil	Gelin-S	Extralink	Cells + medium	Total volume
Six 100-μl injections					
Injection 1: 90% Extracel + 10% cell suspension	250	250	125	63	688
Injection 2: 50% Extracel + 50% cell suspension	130	130	65	325	650
Three 200-μl injections					
Injection 1: 90% Extracel + 10% cell suspension	120	120	60	30	330
Injection 2: 50% Extracel + 50% cell suspension	70	70	35	175	350

Table 10.14.4 Pilot Study Conditions

	Cells/ml	Injection volume (μl)
Mice 1-3	5×10^6	100
Mice 4-6	5×10^7	100
Mice 7-9	5×10^7	200

NOTE: We recommend conducting a benchtop study with Extracel to confirm the Extracel characteristics prior to initiating animal experiments and gain familiarity with handling and timing of use. The gelation time and final hydrogel properties are highly dependent upon the medium used, extent of hydrogel dilution, and final hydrogel pH (see Basic Protocol 1, steps 8 to 13).

NOTE: We ***strongly*** urge researchers to conduct pilot animal studies to optimize experimental conditions and familiarize the researcher with the handling of Extracel prior to doing large-scale animal testing. The pilot study will provide important information on the time course for tumor growth from a given cell line or primary tumor source, optimal injection size, cell concentration, and Extracel dilution.

Materials

Extracel Hydrogel kit (Glycosan BioSystems) containing:
  Glycosil
  Gelin-S
  Extralink
  DG Water
Tumor cells
Cell culture medium (without serum)
Research animals
Iodine and 70% (v/v) ethanol and sterile swabs

37°C water bath
1-ml syringes with long-tip 20-G × $1\frac{1}{2}$-in. needles, sterile
37°C shaking or rocking incubator

Prepare hydrogels

1. Remove Glycosil, Gelin-S, and Extralink vials from the −20°C freezer and heat them to 37°C (~30 min).
2. Remove the DG Water from the −20°C freezer and thaw in a 37°C water bath (~15 min).
3. Under aseptic conditions and using a syringe with the exact amount of liquid, add 1.0 ml of DG Water to the Glycosil vial. Repeat for the Gelin-S vial.
4. Place both vials horizontally at 37°C, with shaking (for maximum mixing).

   NOTE: *Vigorous shaking will speed up dissolving time.*

   It will take <30 min for the solids to fully dissolve. Solutions will be clear and slightly viscous.
5. Under aseptic conditions and using a syringe with the exact amount of liquid, add 0.5 ml DG Water to the Extralink vial. Invert several times to dissolve.

Prepare cells

6. Prepare cells for encapsulation by resuspending them in the relevant sterile cell culture medium (without serum) to the appropriate cell density and volume (5×10^7 cells/ml for 100-µl and 200-µl injections and 5×10^6 cells/ml for 100-µl injections).

   This protocol assumes that a suspension of 100 µl or 200 µl of Extracel + cells will be injected into nine research animals.

   The cell loading and amount of injected Extracel hydrogel used depends upon the application. The amounts discussed in these guidelines are based on published tumor xenograft experiments (Liu et al., 2007a; Prestwich et al., 2007; Prestwich 2008), where a cell concentration of 5×10^7 cells/ml was employed. Lower concentrations may also be effective; however, they will require longer tumor formation times.

Prepare animals and inject cell suspensions

7. Prepare the research animals for surgery as dictated by an approved IACUC protocol and sterilize the sites for surgery with iodine and alcohol swabs.

   For subcutaneous injections, the hydrogel with cells is injected under the skin. It is possible to perform two injections per animal, one on each side.

   For orthotopic (surgically implanted) injections, the animal is opened surgically and the hydrogel with cells injected into the desired location (e.g., onto the pancreas).

8. Add the appropriate volume of cell suspension to the appropriate amount of Glycosil + Gelin-S (see Tables 10.14.4). Mix the resulting suspension by gently vortexing or pipetting.

   The exact time for the hydrogel to become viscous and gel depends on the dilution factor of Extracel and the pH value of the hydrogel solution.

   The pH of medium used to dilute the Extracel and the dilution factor can profoundly affect the gelation time. As provided by the manufacturer, the gelation time is ∼20 min at ambient temperature. However, the greater the dilution factor, the longer the gelation time.

   The pH of Extracel as provided by the manufacturer is controlled to be approximately 7.4 prior to cell encapsulation and further dilution. However, the cell culture medium used can increase or decrease the pH and change the gelation time. For Extracel, a higher pH results in a faster gelation time. For multiple injections, many researchers desire a slower gelation time of 60 or more min. Lowering the pH by 0.1 or 0.2 units, to pH 7.3 or 7.2, combined with dilution with medium, allows researchers to identify an optimal pH/dilution condition for their specific operational needs.

   If stiffer hydrogels are required, increase the concentration of Extralink used or decrease the subsequent dilution factor (or resuspend the initial lyophilized Extracel components in half of the indicated water amounts).

   Extracel hydrogels form by the reaction of thiols in Glycosil and Gelin-S with the acrylate groups of the cross-linker Extralink. Both Glycosil and Gelin-S can form hydrogels in the absence of Extralink via disulfide bond formation; however, this reaction is normally very slow (hours instead of minutes).

9. When the animals are ready for injection of the hydrogel, add the appropriate amount of Extralink to the cells + Glycosil + Gelin-S. Mix the resulting suspension by gently vortexing or pipetting.

   Once the Extralink is added to the Glycosil + Gelin-S + cells, you have between 20 min and 2 hr before the hydrogel forms. Prepare accordingly. If you cannot inject all the animals within this amount of time, consider dispensing aliquots of the cells + Glycosil + Gelin-S into individual injection amounts and adding the Extralink just prior to injection into each animal.

10. Draw the Extracel + cells into a sterile 1-ml syringe with a 20-G needle.

11. Inject the required amount of hydrogel into the research animal at the desired location.

12. After injection, properly care for the research animals and monitor for tumor formation.

COMMENTARY

Background Information

Mammalian tissues are composed of a conglomerate of interconnected cells that perform similar functions within an organism. Cells can interact with each other directly or indirectly, and their activity is modulated by autocrine and paracrine regulatory mechanisms. In epithelial tissues, cells are in close contact with each other. The majority of other tissue types are comprised of cells that are surrounded by a complex network of macromolecules and proteins referred to as the ECM.

Cell culture is a vital tool for basic research in cell biology, drug discovery, drug evaluation processes, and protein biotechnology. Classical tissue culturing techniques were recently proven to be poor mimics of the physiological cellular environment (Bissel et al., 2003, 2005). Currently, two types of 3-D culturing methods are commonly used. One is referred to as 3-D "embedded" cell culture, while the other is known as 3-D "on-top" (Lee et al., 2007). Both methods require an extracellular matrix (ECM) equivalent as the 3-D culturing microenvironment.

At present, the leading ECM equivalent employed for 3-D culture is Matrigel. This is a natural, murine sarcoma–derived product. Its composition includes laminin, collagen, entactin, and growth factors. Matrigel was tested in numerous 3-D cell culturing applications, invasion assays, and tumor xenografts and yielded satisfactory results (Kleinman et al., 1986; *UNIT 12.2*). Nonetheless, Matrigel has drawbacks, the most serious of which pertain to difficulty of use, lack of experimental control of composition, batch-to-batch variability, and lack of utility in translational research for cell therapy (Prestwich, 2007).

A different natural ECM analog, PureCol (consisting of purified type I collagen; Nutracon, *http://www.purecol.nu*), is widely used in cell culture and tissue engineering and as a coating material for medical devices (Elsdale and Bard, 1972; Emerman and Pitelka, 1977; Bell et al., 1979; Schor et al., 1982; Weinberg and Bell, 1986). PureCol has long gelation times (45 to 60 min at 37°C) that make this material unsuitable as a vehicle for 3-D applications. For 3-D encapsulation, the gelation time of PureCol is such that the cells will settle by gravity prior to gelation, so they are not suspended throughout the hydrogel. However, this material is easy to use, has a very long history in cell culture, and is suitable for pseudo-3-D plate coating.

Although naturally derived ECM extracts provide biological recognition and meet key requirements such as presentation of receptor binding ligands and cell-induced proteolytic degradations, they are far from ideal. Issues of limited availability, batch-to-batch variability, pathogen transmission, immunogenicity, technical challenges in handling, and the inability to customize composition and compliance opened the door for a new generation of semisynthetic ECM equivalents.

One such commercially available material is PuraMatrix, a synthetic self-assembling peptide-based material that forms fibrous scaffolds which can be used for 3-D cell embedding or surface plating (Zhang et al., 1995; Holmes et al., 2000; Semino et al., 2003; Bokhari et al., 2005; Yamaoka et al., 2006). This nonanimal-derived material is nonimmunogenic and is suitable for in vivo studies. A major weakness of this material is its preparation protocol; the pH of the initial reagent is 3.0, which strictly limits the time of cell exposure to this environment. Furthermore, the gelation procedure for this material requires extensive handling. For example, the medium needs to be changed three times in 30 min. Increased handling increases the risk of cell culture contamination and thus limits use to small-scale experimental protocols.

In this unit, we introduced an sECM known commercially as Extracel, a hydrogel based on chemically modified hyaluronan (Glycosil) and gelatin (Gelin-S) that are co-cross-linked with polyethylene glycol diacrylate (Extralink). A generic synthetic scheme for this scaffold is presented in Figure 10.14.1. This biomaterial sustains cell growth and proliferation, while eliminating many of the issues posed by other biomaterials. Its preparation protocol is very user friendly and cell friendly and is suitable for large-scale experimental protocols. The gelation times can be adjusted by varying pH or temperature, and the compliance (stiffness) can be altered by adjusting the degree of cross-linking (Ghosh et al., 2007). In addition, its nature overcomes the issue of immunogenicity in in vivo applications (Liu et al., 2004, 2006a,b, 2007a,b; Duflo et al., 2006; Shu et al., 2006; Orlandi et al., 2007; Prestwich et al., 2007). The biological performance of the four aforementioned ECM equivalents both in pseudo-3-D and 3-D cell cultures were recently compared and contrasted (Serban et al., 2008).

Critical Parameters

The critical parameters required for experimental success were mentioned in each of the protocols. Below, we briefly summarize four key factors that can affect the experimental results:

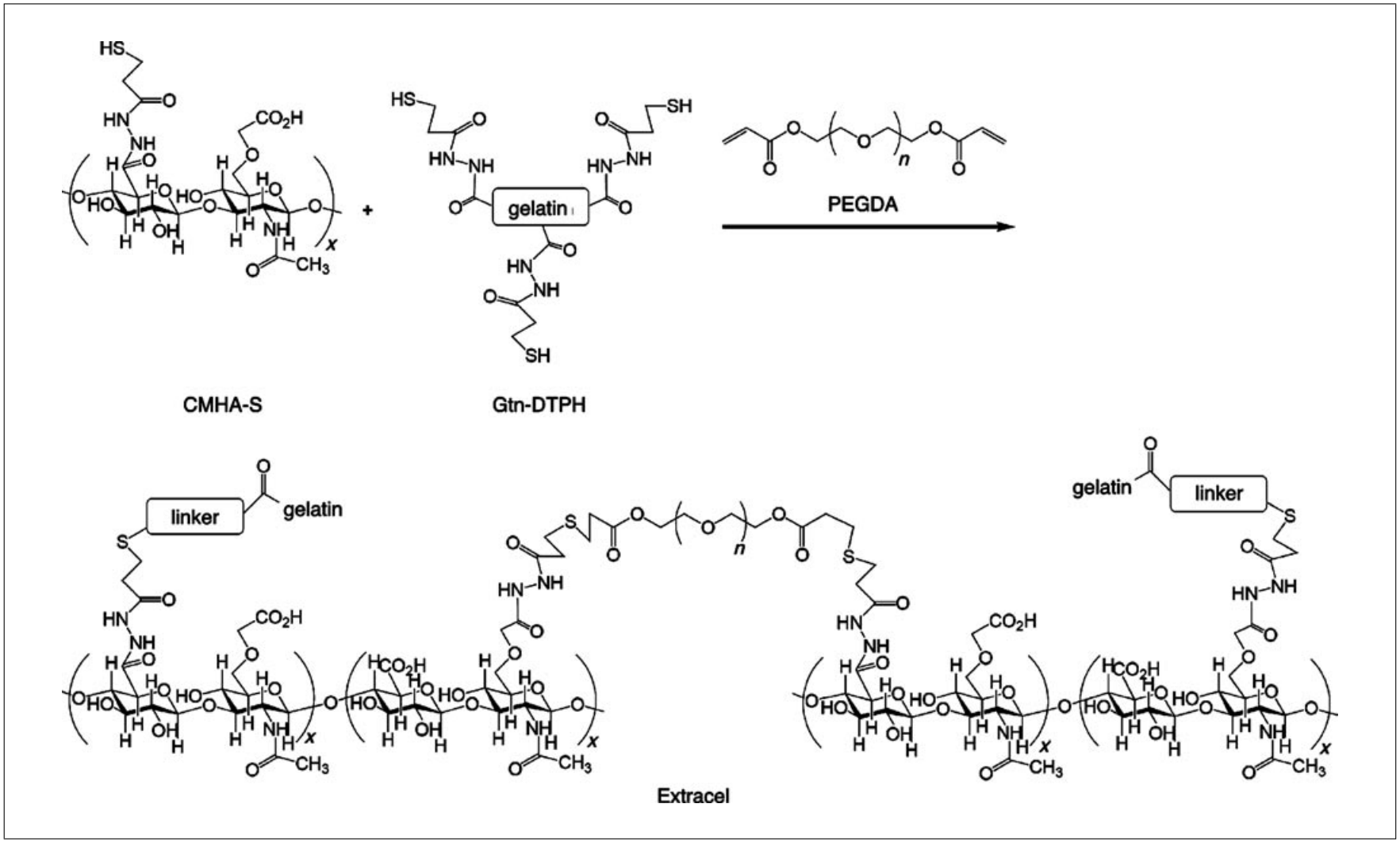


Figure 10.14.1 Generic synthetic scheme for Extracel. Extracel is composed of CMHA-S [thiol-modified hyaluronic acid (HA), trade name of Glycosil], Gtn-DTPH (thiol-modified gelatin, trade name of Gelin-S) and PEGDA (polyethylene glycol diacrylate, trade name of Extralink). In this schematic, "linker" refers to the PEGDA molecule. When mixed together, PEGDA chemically cross-links CMHA-S and Gtn-DTPH to form a hydrogel. *NOTE:* each CMHA-S and Gtn-DTPH molecule has multiple modification sites so that the covalent bonds are formed many times on each HA and gelatin molecule. Reprinted from *Methods*, Vol. 45, Serban, M.A. and Prestwich, G.D., Modular extracellular matrices: Solutions for the puzzle, Copyright 2008 with permission from Elsevier.

Table 10.14.5 Troubleshooting Guide to Working with Hydrogels

Problem	Possible cause	Solution
Hydrogel sets too quickly	High solution pH Extensive handling time High solution concentration	Adjust solution pH to ~7.4 Dilute solutions Aliquot gel components and cross-link near time of use
Hydrogel sets too slowly	Low solution pH Low solution concentration	Adjust solution pH to ~7.4 Reconstitute the lyophilized compounds with less water
Encapsulated cells settle to bottom	High solution dilution	Reconstitute the lyophilized compounds with less water
	Improper cross-linker-to-gel components ratio	Optimize cross-linker-to-gel components ratio
		Add cell suspension to the hydrogel only when mix is becoming viscous
Tumor formation not optimal	Improper solution pH Improper solution concentration	Adjust solution pH to ~7.4 Adjust solution concentrations
	Improper cross-linker-to-gel components ratio	Run a pilot, bench-top experiment to determine optimal hydrogel formulation based on experimental needs

Setup time
Solution pH
Solution dilution factor
Cell seeding density.

The last three factors mentioned can be customized to fit experimental requirements.

The duration of material handling is dictated by the chosen properties of the Extracel components (i.e., higher solution pH leads to faster gelation or lower dilution factor causes faster gelation). Although the protocols provided here are intended to serve as a general guide for experimental setup, it is important to recognize that individual cell types and lines might require optimization. For instance, human tracheal scar fibroblasts were found to prefer a gelatin-rich formulation of Extracel (Serban et al., 2008). Based on individual experimental needs, benchtop studies should be conducted to customize the protocols in order to fit the researcher's needs. These trials should only take a short time (a few hours) and can ensure experimental success.

The cell seeding density should be adjusted accordingly, especially when cell will be 3-D encapsulated. It is important to differentiate between surface (2-D) versus embedded (3-D) culturing. To extrapolate an initial 3-D cell seeding density if the 2-D seeding number is known, simply tripling the cell number is a good starting point. Then, work from this cell density to optimize the cell density for a particular experiment. Using classical analytical methods, cell proliferation or viability for both pseudo-3-D or 3-D culturing conditions can easily be determined. Colorimetric (MTS) assays and staining procedures such as fluorescein diacetate/propidium iodide (FDA/PI) or hematoxylin and eosin (H&E), are perfectly compatible with Extracel.

Troubleshooting

See Table 10.14.5 for troubleshooting hints for these protocols.

Anticipated Results

For cells grown on the surface of Extracel hydrogels (Basic Protocol 4), you should notice cell attachment in ~2 hr. Cells will elicit a morphology consistent with the hydrogel on which they are grown (Fig. 10.14.2). Cell viability should be similar to the classical 2-D culturing conditions.

Cells that are encapsulated in Extracel hydrogels should be homogeneously distributed in the hydrogel in a 3-D environment (you can

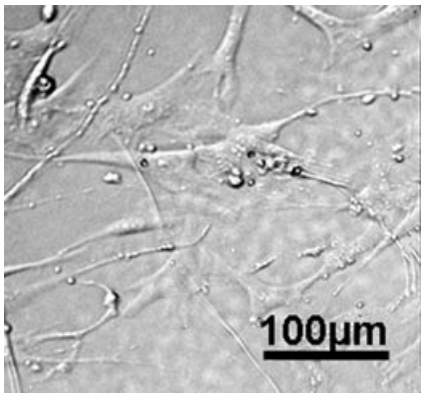


Figure 10.14.2 T31 human tracheal scar AM/Ethidium fibroblasts grown on Extracel. Reprinted from *Acta Biomater.*, Vol. 4, Serban, M.A., Liu, Y. and Prestwich, G.D., Effects of extracellular matrix analogues on primary human fibroblast behavior, *pp.* 67-75, Copyright 2008 with permission from Elsevier.

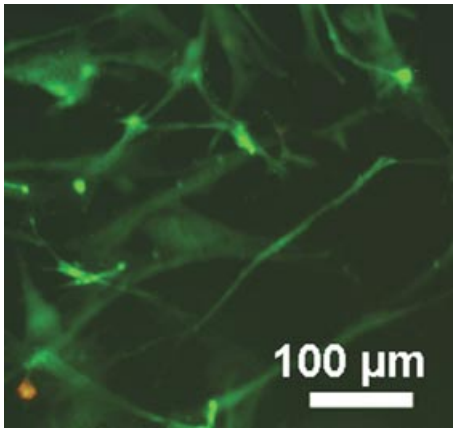


Figure 10.14.3 Calcein-homodimer-1 staining of Extracel-embedded T31 fibroblasts (M.A. Serban, Y. Lue, and G.D. Prestwich unpub. observ.) For color version of this figure see *http://www.currentprotocols.com.*

monitor this microscopically by changing the focal planes; see Fig. 10.14.3). Cell viability should be similar to the classical 2-D culturing conditions.

For tumor xenografts (Basic Protocol 6), both subcutaneous and orthotopic (surgically implanted) injections should result in well localized, vascularized, and differentiated tumors (Fig. 10.14.4 and Fig. 10.14.5). The use of Extracel as a delivery vehicle for tumor engineering leads to increased incidence of cancer formation, reduced variability in tumor size, enhanced growth of organ-specific cancers, improved vascularization, and lower occurrence of core necrosis and adjacent cancer seeding (Liu et al., 2007a).

Time Considerations

The time considerations for hydrogel handling for each of the six protocols were discussed during the process description. Gelling and incubation times are specific to the applications.

Conflict of Interest Statements

Glenn, D. Prestwich is Chief Scientific Officer and equity holder as cofounder for Glycosan BioSystems, Inc., and Senior Scientific Advisor and equity holder as cofounder for Carbylan BioSurgery, Inc., and Sentrx Animal Care, Inc.

Anna Scott is the Director of Operations and equity holder as cofounder for Glycosan BioSystems, Inc.

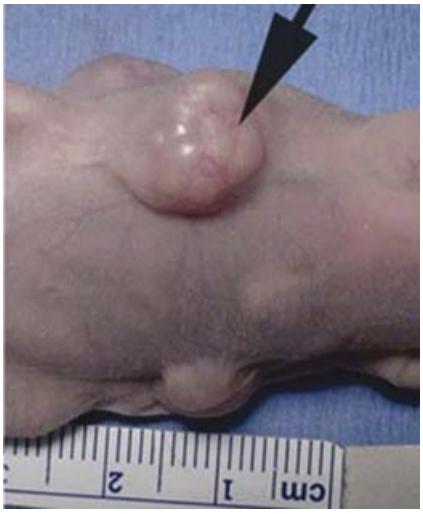


Figure 10.14.4 Gross view of breast tumors 4 weeks after subcutaneous injection of breast cancer cells in Extracel (reprinted from Liu et al., 2007a).

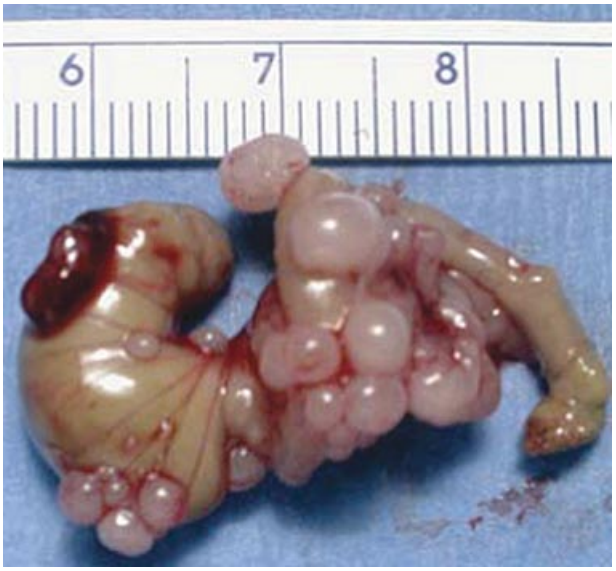


Figure 10.14.5 Gross view of colon tumors 4 weeks after subserous injection of colon cancer cells in Extracel (reprinted from Liu et al., 2007a).

Literature Cited

Bell, E., Ivarsson, B., and Merrill, C. 1979. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 76:1274-1278.

Bissell, M.J, Rizki, A., and Mian, I.S. 2003. Tissue architecture: The ultimate regulator of breast epithelial function. *Curr. Opin. Cell Biol.* 15:753-762.

Bissell, M.J, Kenny, P.A., and Radisky, D.C. 2005. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: The role of the extracellular matrix and its degrading enzymes. *Cold Spring Harb. Symp. Quant. Biol.* 70:343-356.

Bokhari, M.A., Akay, G., Zhang, S., and Birch, M.A. 2005. The enhancement of osteoblast growth and differentiation in vitro on a peptide hydrogel-polyHIPE polymer hybrid material. *Biomaterials* 26:5198-208.

Cai, S., Liu, Y., Shu, X.Z., and Prestwich, G.D. 2005. Injectable glycosaminoglycan hydrogels for controlled release of human basic fibroblast growth factor. *Biomaterials* 26;6054-6067.

Cheung, W.F., Crue, T.F., and Turley, E.A. 1999. Receptor for hyaluronan mediated motility

(RHAMM), a hyaladherin that regulates cell responses to growth factors. *Biochem. Soc. Trans*. 27:135-142.

Cukierman, E., Pankov, R., Stevens, D.R., and Yamada, K.M. 2001. Taking cell-matrix adhesions to the third dimension. *Science* 294:1708-1712.

Dowthwaite, G.P., Edwards, J.C., and Pitsillides, A.A. 1998. An essential role for the interaction between hyaluronan and hyaluronan-binding proteins during joint development. *J. Histochem. Cytochem*. 46:641-651.

Duflo, S., Thibeault, S.L., Li, W., Shu, X.Z., and Prestwich, G.D. 2006. Vocal fold tissue repair in vivo using a synthetic extracellular matrix. *Tissue Eng*. 12:2171-2180.

Elsdale, T. and Bard, J. 1972. Collagen substrata for studies on cell behavior. *J. Cell Biol.* 54:626-637.

Emerman, J.T. and Pitelka, D.R. 1977. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro* 13:316-328.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E.,2006. Matrix elasticity directs stem cell lineage specification. *Cell* 126:677-689.

Entwistle, J., Hall, C.L., and Turley, E.A. 1996. Hyaluronan receptors: Regulators of signalling to the cytoskeleton. *J. Cell. Biochem*. 61:569-577.

Fraser, J.R., Laurent, T.C., and Laurent, U.B. 1997. Hyaluronan: Its nature, distribution, functions and turnover.. *J. Intern. Med*. 242:27-33.

Galbraith, C.G. and Sheetz, M.P. 1998. Forces on adhesive contacts affect cell function. *Curr. Opin. Cell Biol*. 10:566-571.

Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K.M. 2001. Transmembrane crosstalk between the extracellular matrix-cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol*. 2:793-805.

Ghosh, K., Pan, Z., Guan, E., Ge, S., Liu, Y., Nakamura, T., Ren, X., Rafailovich, M., and Clark, R. 2007. Cell adaptation to a physiologically relevant ECM mimic with different viscoelastic properties. *Biomaterials* 28:671-679.

Holmbeck, K. and Szabova, L. 2006. Aspects of extracellular matrix remodeling in development and disease. *Birth Defects Res. C Embryo Today* 78:11-23.

Holmes, T.C., de Lacalle, S., Su, X., Liu, G., Rich, A., and Zhang, S. 2000. Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. *Proc. Natl. Acad. Sci. U.S.A*. 97:6728-6733.

Kleinman, H.G., McGarvey, M.L., Hassel, J.R., Star, V.L., Cannon, F.B., Laurie, G.W. and Martin, G.R. 1986. Basement membrane complexes with biological activity. *Biochemistry* 25:312-318.

Knudson, C.B. and Knudson, W. 2001. Cartilage proteoglycans. *Semin. Cell Dev. Biol*. 12:69-78.

Lee, G.Y., Kenny, P.A., Lee, E.H., and Bissell, M.J. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat. Methods*. 4:359-365.

Liu, Y., Shu, X. Z., Gray, S. D., and Prestwich, G. D. 2004. Disulfide-cross-linked hyaluronan-gelatin sponge: Growth of fibrous tissue in vivo. *J. Biomed. Mater. Res. A* 68:142-149.

Liu, Y., Ahmad, S., Shu, X.Z., Sanders, R.K., Kopesec, S.A., and Prestwich, G.D. 2006a. Accelerated repair of cortical bone defects using a synthetic extracellular matrix to deliver human demineralized bone matrix. *J. Orthop. Res*. 24:1454-1462.

Liu, Y., Shu, X.Z., and Prestwich, G.D. 2006b. Osteochondral defect repair with autologous bone marrow-derived mesenchymal stem cells in an injectable, in situ, cross-linked synthetic extracellular matrix. *Tissue Eng*. 12;3405-3416.

Liu, Y., Shu, X.Z., and Prestwich, G.D. 2007a. Tumor engineering: Orthotopic cancer models in mice using cell-loaded, injectable, cross-linked hyaluronan-derived hydrogels. *Tissue Engineering*. 13;1091-1110.

Liu, Y., Shu, X.Z., and Prestwich, G.D. 2007b. Reduced post-operative intra-abdominal adhesions using Carbylan-SX, a semisynthetic glycosaminoglycan hydrogel. *Fertil. Steril.* 87:940-948.

Lutolf, M.P. and Hubbell, J.A. 2005. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotechnol.* 23:47-55.

Mehra, T., Ghosh, K., Shu, X.A., Prestwich, G.D. and Clark, R.F. 2006. Molecular stenting with a cross-linked hyaluronan derivative inhibits collagen gel contraction. *J. Invest. Dermatol*. 126:2202-2209.

Orlandi, R. R., Shu, X. Z., McGill, L., Petersen, E., and Prestwich, G. D. 2007. Structural variations in a single hyaluronan derivative significantly alter wound-healing effects in the rabbit maxillary sinus. *Laryngoscope* 17:1288-1295.

Pike, D.B., Cai, S., Pomraning, K.R., Firpo, M., Fisher, R.J., Shu, X.Z., Prestwich, G.D., and Peattie, R. 2006. Heparin-regulated release of growth factors in vitro and angiogenic response in vivo to implanted hyaluronan hydrogels containing VEGF and bFGF. *Biomaterials*. 27:5242-5251.

Prestwich, G.D. 2007. Simplifying the extracellular matrix for 3-D cell culture and tissue engineering: A pragmatic approach. *J. Cell. Biochem*. 101:1370-1383.

Prestwich, G.D. 2008. Evaluating drug efficacy and toxicology in three dimensions: Using synthetic extracellular matrices in drug discovery. *Acc. Chem. Res*. 41:139-48

Prestwich, G.D., Liu, Y., Yu, B., Shu, X.Z., and Scott, A. 2007. 3-D culture in synthetic extracellular matrices: New tissue models for drug toxicology and cancer drug discovery. *Adv. Enzyme Regul.* 47:196-207.

Riley, C.M., Fuegy, P.W., Firpo, M.A., Shu, X.Z., Prestwich, G.D., and Peattie, R.A. 2006. Stimulation of in vivo angiogenesis using dual growth factor-loaded cross-linking glycosaminoglycan hydrogels. *Biomaterials*. 27:5935-5943.

Roskelley, C.D., Desprez, P.Y., and Bissell, M.J. 1994. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* 91:12378-12382.

Schor, S.L., Schor, A.M., Winn, B., and Rushton, G. 1982. The use of three-dimensional collagen gels for the study of tumour cell invasion in vitro: Experimental parameters influencing cell migration into the gel matrix. *Int. J. Cancer* 29:57-62.

Semino, C.E., Merok, J.R., Crane, G.G., Panagiotakos, G., and Zhang, S. 2003. Functional differentiation of hepatocyte-like spheroid structures from putative liver progenitor cells in three-dimensional peptide scaffolds. *Differentiation* 71:262-270.

Serban, M.A., Liu, Y., and Prestwich, G.D. 2008. Effects of extracellular matrix analogues on primary human fibroblast behavior. *Acta Biomater*. 4:67-75.

Shu,X.Z., Liu, Y., Palumbo, F., Luo, Y., and Prestwich, G.D. 2004. In situ cross-linkable hyaluronan hydrogels for tissue engineering. *Biomaterials* 25:1339-1348.

Shu, X.Z., Ahmad, S., Liu, Y., and Prestwich, G.D. 2006. Synthesis and evaluation of injectable, in situ cross-linkable synthetic extracellular matrices for tissue engineering. *J. Biomed. Mater. Res. A* 79:902-912.

Wang, F., Weaver, V.M., Petersen, O.W., Larabell, C.A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M.J. 1998. Reciprocal interactions between beta 1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: A different perspective in epithelial biology. *Proc. Natl. Acad. Sci. U.S.A*. 95:14821-14826.

Weaver, V.M., Petersen, O.W., Wang, F., Larabell, C.A., Briand, P., Damsky, C., and Bissell, M.J. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J. Cell. Biol*. 137:231-245.

Weinberg, C.B. and Bell, E. 1986. A blood vessel model constructed from collagen and cultured vascular cells. *Science* 231:397-400.

Yamaoka, H., Asato, H., Ogasawara, T., Nishizawa, S., Takahashi, T., Nakatsuka, T., Koshima, I., Nakamura, K., Kawaguchi, H., Chung, U.I., Takato, T., and Hoshi, K. 2006. Cartilage tissue engineering using human auricular chondrocytes embedded in different hydrogel materials. *J. Biomed. Mater. Res. A* 78:1-11.

Yeung, T., Georges, P.C., Flanagan, L.A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P.A. 2005. Effects of substrate stiffness on cell morphology, cytoskeletal structure and adhesion. *Cell Motil. Cytoskel.* 60:24-34.

Zhang, S., Holmes, T.C., DiPersio, C.M., Hynes, R.O., Su, X., and Rich, A. 1995. Self-complementary oligopeptide matrices support mammalian cell attachment. *Biomaterials* 16:1385-1393.

Generation of Micropatterned Substrates Using Micro Photopatterning

Andrew D. Doyle[1]

[1]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland

ABSTRACT

Micro photopatterning (μPP) has been developed to rapidly test and generate different patterns for extracellular matrix adsorption without being hindered by the process of making physical stamps through nanolithography techniques. It uses two-photon excitation guided through a point-scanning confocal microscope to locally photoablate poly(vinyl) alcohol (PVA) thin films in user-defined computer-controlled patterns. PVA thin films are ideal for surface blocking, being hydrophilic substrates that deter protein adsorption and cell attachment. Because gold substrates are not used during μPP, all live-cell fluorescent-imaging techniques including total internal reflection fluorescence microscopy of GFP–linked proteins can be performed with minimal loss of fluorescence signal. Furthermore, because μPP does not require physical stamps for pattern generation, multiple ECMs or other proteins can be localized within microns of each other. This unit details the setup of μPP. It also provides troubleshooting techniques. *Curr. Protoc. Cell Biol.* 45:10.15.1-10.15.35. © 2009 by John Wiley & Sons, Inc.

Keywords: micro photopatterning • micropatterning • extracellular matrix • two-photon confocal microscopy • photoablation • polyvinyl alcohol • thin film

INTRODUCTION

This unit describes the generation of micropatterned substrates using a direct-writing method known as micro photopatterning or μPP. Micropatterning of extracellular matrix (ECM) components, where ECM proteins are applied to a two-dimensional surface in a specified pattern, has been an important technique for understanding how cells respond and react to their physical surroundings. The most common manner in which to generate micropatterns is through the process known as microcontact printing or μCP, where a physical stamp is used to "ink" ECM patterns onto a gold-coated coverslip (Lehnert et al., 2004). More details about this process can be found in the Background Information section of the Commentary.

While μCP has been refined and can now generate sub micron-sized patterns (Lehnert et al., 2004), it has several limitations. One major limitation is that the patterns of a master cannot be readily changed. This requires a new master for each new pattern or stamp. A second limitation is the use of gold for alkanethiol attachment. Being an electron-dense metal, gold strongly quenches green fluorescent protein (GFP) fluorescence, leaving live-cell fluorescence imaging nearly impossible.

Here, we describe in detail how to generate ECM micropatterned glass-bottomed dishes or coverslips using μPP that bypasses many of the issues associated with other micropatterning techniques (Doyle et al., 2009). This technique utilizes high-powered two-photon (TP) laser excitation channeled through a point-scanning confocal microscope in order to physically ablate or remove a thin film of poly(vinyl) alcohol (PVA). This exposes the underlying glass surface to which ECM proteins can directly attach. PVA's high hydrophilicity and relative inertness make it an optimal candidate for deterring protein

Current Protocols in Cell Biology 10.15.1-10.15.35, December 2009
Published online December 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1015s45

adsorption to non-ablated regions of the thin film. Furthermore, PVA's high refractive index (~1.5) allows for all fluorescence techniques, including total internal reflection fluorescence (*UNIT 4.12*), even through nonablated regions of the film. In addition, by performing several rounds of μPP in series, multiple proteins can be deposited locally within microns of each other.

STRATEGIC PLANNING

While μPP is a fairly straightforward methodology, it does call for experience and knowledge using a confocal microscope. While the day-to-day processing requires little expense, two important pieces of equipment are necessary: (1) A spincoater to evenly and thinly distribute PVA over the glass surface, and (2) a two-photon laser point-scanning confocal microscope to locally ablate patterns in the PVA thin film. While each piece of equipment is an added expense (spincoater: ~$6,000 to $12,000; Zeiss 510 LSM NLO system: >$500,000) both can be found on most university campuses. Check with Material Science or Bioengineering departments for spincoating devices, and Biology or Physics departments for availability of two-photon confocal systems. Throughout this unit, we refer to the Zeiss 510 LSM NLO system (NLO stands for NonLinear Optics) as the confocal microscope and the AIM software version 4.2, which runs the microscope. It is assumed that the user has basic knowledge using the software in expert mode. Although not described here, it is our view that with slight alterations to these protocols, other two-photon confocal microscope types (i.e., Olympus) could be used to attain local ablation of the PVA thin film.

The process of μPP can be broken down into five stages: (1) glass surface activation, (2) PVA thin film deposition, (3) photoablation, (4) surface quenching, and (5) ECM adsorption. Each part of the process can be a stopping point, with the steps that follow occurring up to several days or even weeks apart. For example, following glass surface activation, dishes/coverslips can be kept desiccated for over month at 4°C, which allows you to generate 30 or more dishes at a time, but other steps can be performed in smaller batches more frequently. Table 10.15.1 shows the longevity of samples after their given stage of processing. The following sections will describe in detail each of the protocols associated with the stages, as well as provide background information and troubleshooting tips.

Table 10.15.1 Longevity of Samples After the Given Stage of Processing

Processing stage	Stability time (days)
Glass surface activation	30+
PVA thin film deposition	7-14
Photoablation	30+
Quenching	30+
ECM adsorption	1-3

BASIC PROTOCOL 1

ACTIVATION OF THE GLASS SURFACE

This protocol describes how to prepare the glass surface for direct conjugation to the PVA thin film. The first order of business is to clean the glass surface of any organic residues through acid washing. This is crucial so the silanes are uniformly distributed over the glass to provide covalent attachment of the PVA thin film. The term "activated glass" or "activation" here refers to adding a reactive aldehyde group conjugated directly to the glass surface, through silanes. The aldehydes can react directly with hydroxyl groups found on the PVA polymer, and hence the thin film generated in later steps is covalently attached

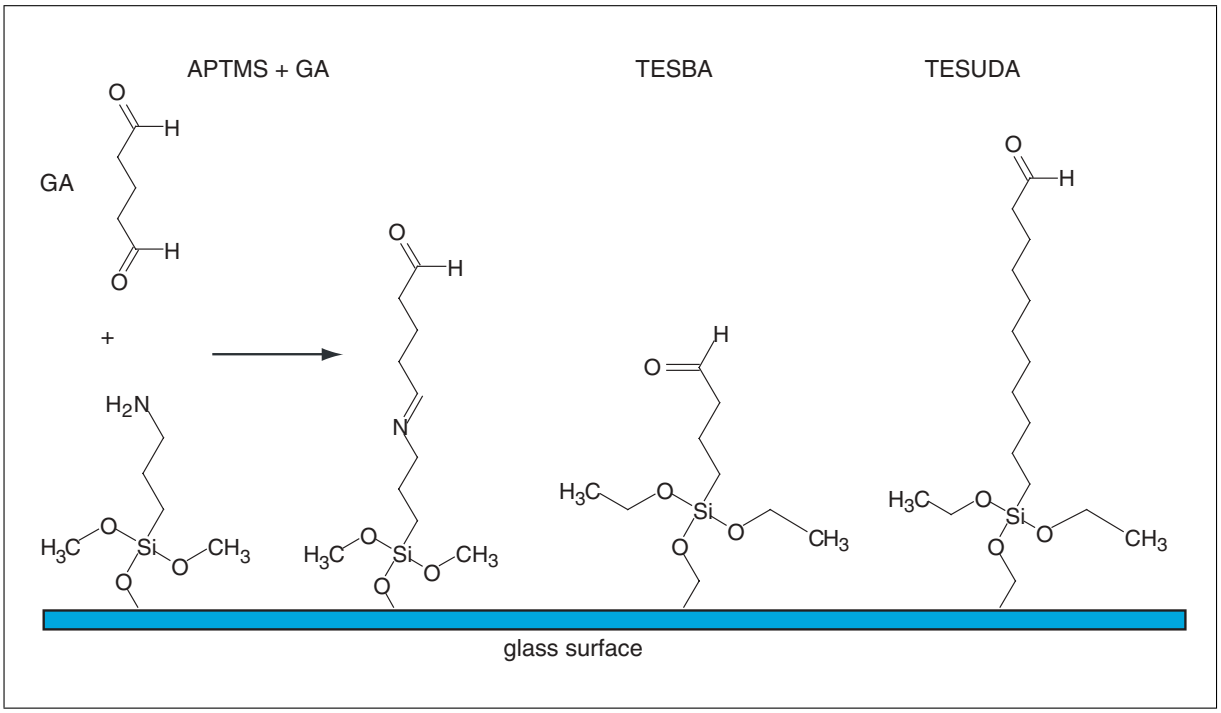


Figure 10.15.1 Schematics of APTMS, TESBA, and TESUDA conjugated to a glass surface. The addition of glutaraldehyde (GA) to APTMS-coated glass results in "activation" by attachment to the free amino group, leaving a free aldehyde to react with PVA during spin coating.

to the glass. The silanes in question are (3-aminopropl)trimethoxysilane (APTMS), triethoxysilylbutraldehyde (TESBA), and 11-(triethoxysilyl)undecanal (TESUDA). The first is an amino-terminated silane, which requires glutaraldehyde for "activation," while the latter two are both aldehyde terminated (Fig. 10.15.1). Protocols for using both types are detailed below. Caution should be used when handling silanes; they can cause severe burns and damage many surfaces.

NOTE: After the completion of each process (i.e., acid washing, silanization, etc.) it is important to visually inspect all dishes under a microscope with a 10× objective to observe if there are any imperfections in the surface. If there are, it is best to discard the affected dishes before any further steps.

Materials

50% (v/v) nitric acid
Deionized or distilled water
200 mM NaOH solution (see recipe)
(3-aminopropyl)trimethoxysilane (APTMS: 97% or higher; Gelest)
50% (v/v) glutaraldehyde (Electron Microscopy Sciences)
Aldex (Waste and Compliance Management)
Drierite (W.A. Hammond Drierite Company)

R-3603 Tygon tubing (Norton Performance Plastics)
1000-µl barrier filter pipet tips
Low-pressure air jet
Fume hood
Thirty MatTek glass-bottomed dishes (P35G-1.5-10-CMatTek)
Carrying tray
Pasteur pipets
Automatic pipettor

2000-ml beaker
Scale
Scintillation vials or other small glass containers
Drying oven
500-ml screw-top container (e.g., Nalgene) or other similar desiccated containers for storage

Prepare the glass surface for silanization

1. Before starting the acid washing, create a compressed air blower using 3 ft of Tygon tubing and a 1000-µl barrier filter pipet tip. Connect one end of the tubing to a low-pressure air jet (commonly found on most laboratory benches). Insert the pipet tip into the other end, tip out.

   Alternatively, this can be attached to a compressed air or nitrogen tank. This will used throughout the process to dry the dishes. Use low pressure for drying, $^1/_4$ to $^1/_3$ of the way open or under 10 psi.

2. In a fume hood, arrange thirty MatTek dishes on a carrying tray.

3. Using a Pasteur pipet fit into an automatic pipettor, add a small amount of 50% (v/v) nitric acid to each dish, just enough to cover the glass area, usually between 300 and 500 µl. Incubate for 25 min at room temperature.

4. After the incubation period has elapsed, place dishes in a large 2000-ml beaker and rinse with deionized or distilled water, under continuous flow for a minimum of 4 hr or overnight.

   Be sure that dishes are not floating.

5. Remove dishes from water and aspirate remaining water from dishes.

6. Arrange dishes on a carrying tray again, add 300 to 500 µl of 200 mM NaOH to each dish, and incubate for 15 min at room temperature.

   This step helps to exchange H^+ residues associating with the glass from the acid washing and neutralizing or replacing them with OH^-, which is more conducive for the binding of the methoxy or ethoxy portion of the silane.

7. Rinse dishes two times, each time with 3 ml deionized or distilled water, then dry under compressed air (device created in step 1).

Silanize glass surfaces

8. Place a scintillation vial on a scale, tare, and weigh out 1% (w/v) APTMS.

   CAUTION: *Silanes corrode metals, plastics, and all organics. Only use glass for transferring and wear chemical-resistant gloves when handling.*

9. Insert a glass Pasteur pipet into the APTMS and tilt container to 45°. Allow capillary action to bring the APTMS into the pipet.

10. Place your gloved finger on the top open-end of the pipet, transfer, and release a total of 100 mg into the tared vial. Add 9900 µl distilled water to the scintillation vial. Replace vial cap, mix gently, and incubate for 1 min at room temperature.

11. Add ~300 to 500 µl of the 1% APTMS solution to each dish, only covering the glass portion. Incubate 5 min at room temperature.

12. Aspirate APTMS and dispose of properly (contact your chemical safety officer for the proper disposal).

13. Rinse two times, each time with 3 ml distilled water over 10 min.

14. Aspirate the water and dry the dishes with compressed air.

15. Replace the dish lids and incubate at 65°C in a drying oven for a minimum of 3 hr.

    This step cures the silanes. They can also be left desiccated at room temperature overnight and achieve the same level of curing.

    Incubation above 65°C leads to warping of the dishes.

Activate amino-terminated silanes with glutaraldehyde

16. Mix 100 µl of 50% glutaraldehyde with 9900 µl distilled water to make a 0.5% glutaraldehyde solution. Add ~300 to 500 µl of the solution to only the glass surface of the silanized dishes. Incubate for 30 min at room temperature.

    To convert this amino-terminated silane (APTMS) into an active aldehyde, glutaraldehyde (a bi-functional aldehyde) is added to react directly with the amino group.

17. Remove the glutaraldehyde solution by aspiration and discard properly in Aldex.

    Aldex is used to inactivate the reactive aldehydes allowing for proper disposal.

18. Rinse dishes three times, each time in 3 ml distilled water over 20 min.

19. Aspirate the water and blow-dry the surface again.

20. Store up to 1 month at 4°C in a desiccated storage container (add Drierite to the container bottom or to a small vial placed inside it).

USING ALDEHYDE-TERMINATED SILANES FOR SURFACE ACTIVATION

ALTERNATE PROTOCOL 1

In order to bypass the glutaraldehyde-activation steps of the Basic Protocol, TESBA or TESUDA, which are already terminated in a reactive aldehyde group, can be used for silanization. Both are triethoxysilanes, which renders them water insoluble and requires ethanol as the solvent.

***Additional Materials** (also see Basic Protocol 1)*

Absolute ethanol (200 proof)
Triethoxysilylbutraldehyde (TESBA) *or* 11-(Triethoxysilyl)undecanal (TESUDA; both from Gelest)

Prepare the dishes

1. Follow steps 1 through 7 of Basic Protocol 1 for acid washing of glass-bottomed dishes.

Silanize the glass surface

2. Place a scintillation vial on a scale, tare, and weigh out 1% (w/v) of either silane.

3. Insert a glass Pasteur pipet into the silanes and tilt container to 45°. Use capillary action to bring the silanes into the pipet.

4. Place your gloved finger on the top open-end of the pipet, transfer and release a total of 100 mg into the tared vial.

5. Add 9900 µl of absolute ethanol to the scintillation vial. Mix gently with the cap on and incubate for 5 min at room temperature.

6. Add ~300 to 500 µl of the 1% silane solution to each dish, only covering the glass portion. Incubate for 5 min at room temperature.

7. Dispose of the silanes properly (contact your chemical safety officer for the proper disposal).

Rinse and dry the silanized dish

8. Flooding the dish, rinse twice, each time with 3 ml absolute ethanol followed by a single rinse with 3 ml distilled water for 2 min.
9. Aspirate the distilled water and dry dishes with compressed air.
10. Replace the dish lids and incubate at 65°C in a drying oven for a minimum of 3 hr.

    This step cures the silanes. They can also be left desiccated at room temperature overnight to achieve the same level of curing.

    Curing at temperatures above 65°C leads to warping of the dishes.
11. Store up to 1 month at 4°C in a desiccated storage container (add Drierite to the container bottom or to a small vial placed inside it).

BASIC PROTOCOL 2

GENERATING POLYVINYLALCOHOL (PVA) THIN FILMS

Here, we describe the mixing of the PVA solution and spincoating it into a thin film on the activated glass dishes. Spincoating is a process by which a solution (in this case PVA) is thinned evenly across a surface using centripetal force. This is often used in the semiconductor industry to apply agents to silicon wafers. A flat glass disc or dish is attached via vacuum suction to a central "chuck" (Fig. 10.15.2). Chucks come in many sizes to fit the size of the disc/dish. The vacuum secures the disc from moving during spinning. Most Spincoaters require a vacuum source (in our case a gel pump) and a source of high-pressure air, (a normal compressed air cylinder). The latter is to keep liquids and other materials from coating the rotor.

Materials

Distilled water
Poly(vinyl) alcohol powder (mol. wt. between 13,000 and 100,000; 98% hydrolyzed minimum; Sigma)
2 N HCl
5 M NaCl

400-ml glass beaker
Stirrer/hot plate
50-ml conical tubes
Scale
Flea Micro magnetic stir bar (VWR)
50-ml Steriflip (0.2-μm pore size)
Gel vacuum pump
High-pressure compressed air source (e.g., compressed air cylinder)
Vortex
5 to 10 MatTek dishes with activated glass surface (Basic Protocol 1 or Alternate Protocol 1)
Pipettor
Spincoater with a chuck capable of accepting 50-mm or smaller items (A WS-400B-6NPP/LITE spincoater from Laurell Technologies shown in Fig. 4.15.2 is used here)
Scintillation vial or 35-mm tissue culture dish
Nalgene 500-ml screw top or other similar containers for storage

Make the PVA solution

The PVA solution can be used repeatedly over a period of 1 week. We recommend making it fresh weekly.

Figure 10.15.2 Laurell Technologies Spincoater with an "activated" MatTek dish in position before spincoating PVA/HCl solution. The chuck (arrow) can be changed according to the size required for the dish/disc being spun.

1. Add ~270 ml of water to a 400-ml glass beaker and begin heating on a stirring hot plate at medium-high heat.
2. Weigh out 2.835 g of PVA into a 50-ml conical tube.
3. With the tube still on the scale, bring the weight/volume up to 50 g with distilled water. Add a stir bar to the conical tube and cap.
4. Loosen the cap of the tube slightly and place the PVA-containing tube into the beaker of hot, near boiling water. Be sure the water line of the beaker is between the 35 to 40 ml lines on the conical tube.

   PVA will not go into solution until it reaches ~90°C.
5. Turn on the stirring mechanism to medium and continue heating until PVA goes into solution, ~10 to 15 min. Have the 50-ml Steriflip ready.
6. Inspect the PVA solution and be sure no PVA crystals/powder remains. When sure, immediately filter the PVA solution into the Steriflip (0.2 μm) using vacuum.

   The PVA must be hot (above 90°C) to filter. Once cooled, the viscosity of the solution increases and makes filtering nearly impossible.
7. Allow the PVA solution to cool to room temperature.
8. Once cooled, transfer 8876 μl to a new 50-ml conical tube. To this, add 1124 μl of 2 N HCl and mix by vortexing.

   This acidified PVA solution can now be used for spincoating.

Spincoat thin films of PVA

9. Retrieve 5 or 6 of the "activated" MatTek dishes that were stored desiccated at 4°C (Basic Protocol 1 or Alternate Protocol 1).

10. Add ~300 to 500 µl of the PVA/HCl solution with a pipettor to the glass surfaces of the dishes and incubate for 5 min at room temperature. Be sure to completely cover the glass area.

11. Center a single dish on the chuck of the spincoater. Follow the manufacturer's instructions for operation. Pull a vacuum and turn on your compressed air. Initiate your spin. When finished, repeat for each dish.

    For spincoating, we have found that a relatively short spin of 40 sec at a high velocity (7000 rpm) works best for generating a thin coating of PVA, with few imperfections. Acceleration of the spincoater is also important: 550 rpm gets the dish up to speed within 18 sec. However, you may need to adjust all variables (time, velocity, and acceleration) to determine which best suits your needs.

12. Add 2 to 3 ml of 5 M NaCl to either a scintillation (no cap) vial or a 35-mm tissue culture dish and place in the storage container.

13. Place dishes within the storage container and incubate a minimum of 2 hr before ablating. Store dishes within the container at 4°C for up to two weeks prior to photoablation.

    High molarity or super-saturated salt solutions are effective in regulating humidity within a closed environment with each salt type maintaining a different relative humidity. For NaCl, it is ~50% to 60%, which is optimal for reducing issues with PVA crystallization, and loss of surface hydrophilicity.

    At this point in the processing, the activated glass surface is now coated with a submicron (between ~100 to 200 nm) thin film of PVA, which will deter protein adsorption and cell attachment (Fig. 10.15. 3).

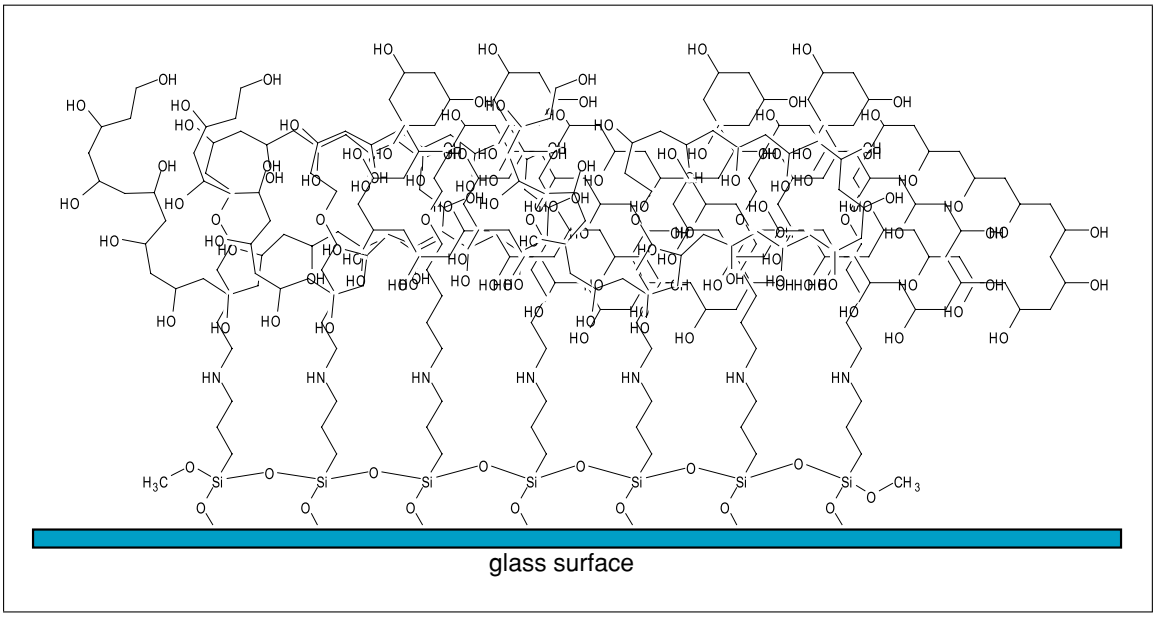


Figure 10.15.3 Representation of the PVA thin film generated on dishes following processing through Basic Protocol 2 using APTMS together with glutaraldehyde as the cross-linker.

PHOTOABLATION WITH TWO-PHOTON CONFOCAL MICROSCOPY

In this section, the actual process of photoablation is described. It is divided into several sections for clarity. First, the configuration settings for the confocal microscope are addressed, followed by the pre-ablation setup and how to generate pattern templates. We then detail how to photoablate single fields of view (FOV), and finally how to automate the process using the Multi Time macro and tiling functions. Throughout this section and the ones that follow, we will often be referring to dialog boxes and buttons within the AIM software. The title of each dialog/button will be **bolded** for easier referencing. For all intents and purposes, the actual photoablation process merely utilizes the intrinsic capabilities of a point-scanning confocal microscope. However, the major difficulty is in setting the proper configurations to elicit localized ablation efficiently. Once configuring is complete, the process can be performed with relative ease.

Materials

Glass cleaner
Immersion oil

Zeiss 510 LSM NLO confocal microscope or later model with 1.5-W minimum tunable two-photon titanium:Sapphire laser, and a 633-nm HeNe2 laser (5 mW power output)
AIM software (Zeiss MicroImaging)
63× oil immersion objective with numerical aperture of 1.3 or higher capable of NLO transmission
PVA thin film-coated MatTek dishes (Basic Protocol 2)

Set up confocal microscope configuration

The following steps are meant to guide you through configuration setup and scan settings that are required for the ablation process. Throughout this section are screen shots of the AIM software (version 4.2) to help in understanding how and where to change settings (indicated by bolded numbers in figures). It is important to note that the direct light path from the TP laser to the confocal scan head should be aligned at least once a month. Ablation efficiency is greatly reduced when mirrors are misaligned. Have a qualified individual align the mirrors (microscope facility director, Zeiss service representative, etc.) at 755 nm before beginning the ablation process.

1. Turn on the confocal microscope and boot the computer. Be sure to turn the two-photon laser from the standby position to the on position.

   There is no need to ignite the mercury arc lamp since it is not used to find the thin film.

2. Open the AIM software in expert mode.

3. Go into the **Acquire** menu and open the Laser window (Fig. 10.15.4, 1; i.e., the inserted numeral 1 in Fig 10.15.4); turn on the 633-nm laser and tune the two-photon to 755 nm.

4. In the same Acquire menu, select **Configuration** (Fig. 10.15.4, 2) to open up the configuration window (Fig. 10.15.5).

5. In Channel Mode, select **Singletrack** or **Multitrack**.

6. For the primary dichroic (Fig. 10.15.5A), select the HFT KP 700/488 (1).

   This allows the near-infrared (NIR) light from the two-photon to be reflected to your sample. While the 633-nm light is not properly matched for the dichroic, it provides back reflection of the glass/thin film interface, similar to backscatter, helping you find the appropriate z-plane for ablation.

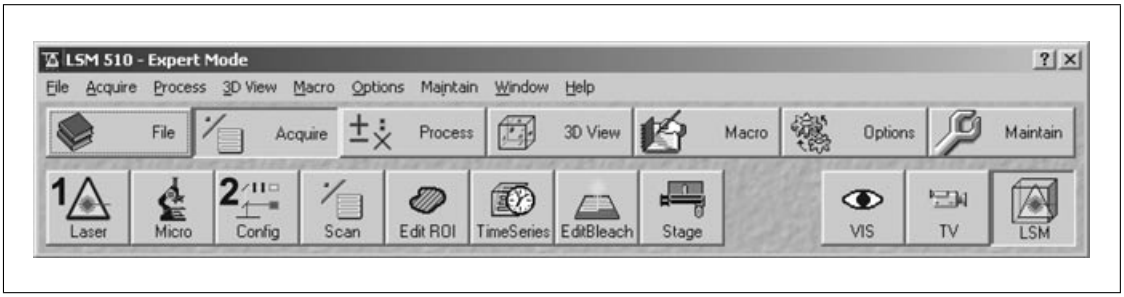


Figure 10.15.4 The LSM 510 AIM software Expert Mode window with the Acquire menu open. All screen shots of the AIM software are courtesy of Carl Zeiss MicroImaging.

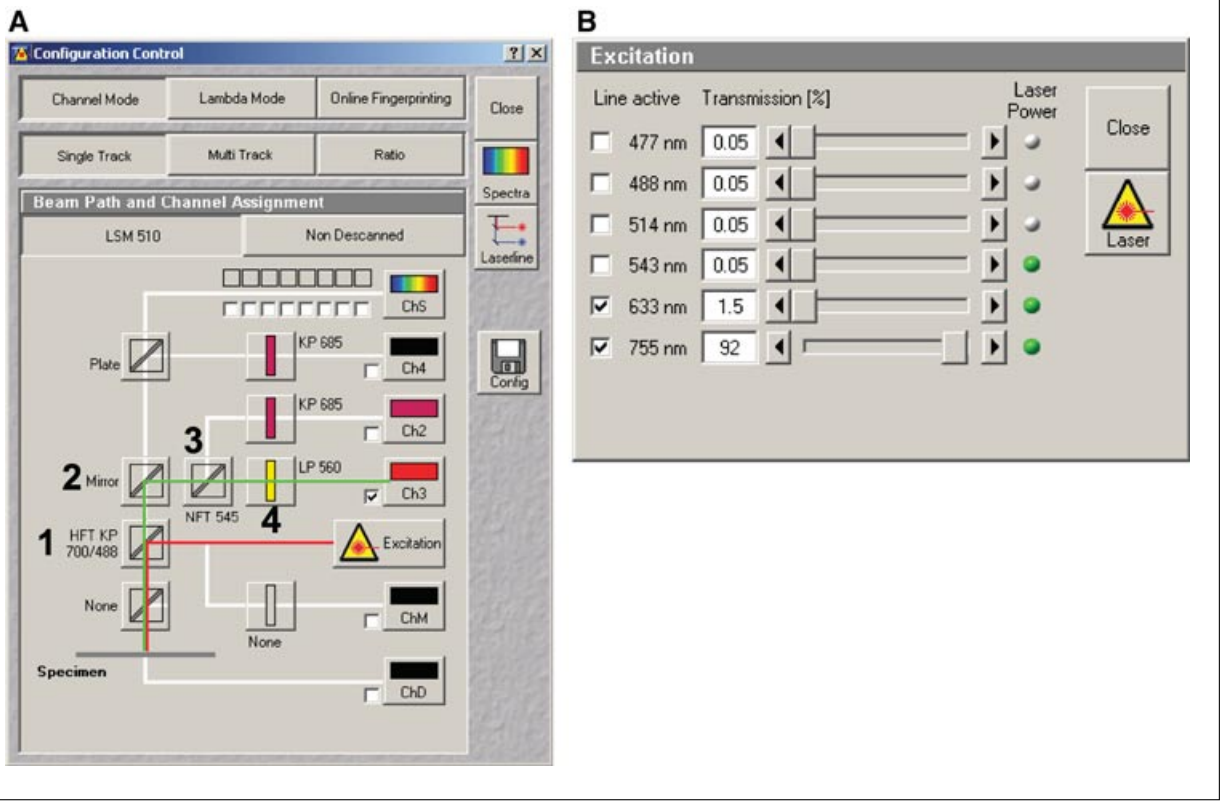


Figure 10.15.5 Configuration Control window screen shot for Zeiss' AIM software (version 4.2). (**A**) Red line depicts the light path from the lasers to the PVA-coated dish, while the green line illustrates the reflected light path from the dish to the channel 3 photomultiplier tube (Ch3). Numbers show the primary dichroic, (1) mirror (2), and filters (3 and 4) required for obtaining a reflected light image of the thin film surface. (**B**) The laser excitation panel with 633-nm and 755-nm TP settings. Courtesy of Carl Zeiss MicroImaging. For color version of this figure go to *http://www.currentprotocols.com/protocol/cb1015*.

7. Select photomultiplier tube or channel 3 (**Ch3**) and set the following filters along the light path illustrated below: **Mirror** (2), **NFT 545** (3), and **LP 560** (4).

8. Set the 633-nm laser to 1.5% power and the 755-nm TP to ~90% by selecting **Excitation**, this opens the Excitation window (Fig. 10.15.5B).

   The percent power is a reflection of the total amount allowed through the AOTF or AOM for the 633-nm HeNe2 and the 755-nm TP, respectively. Using the TP at 90% does not affect the lifetime of the laser.

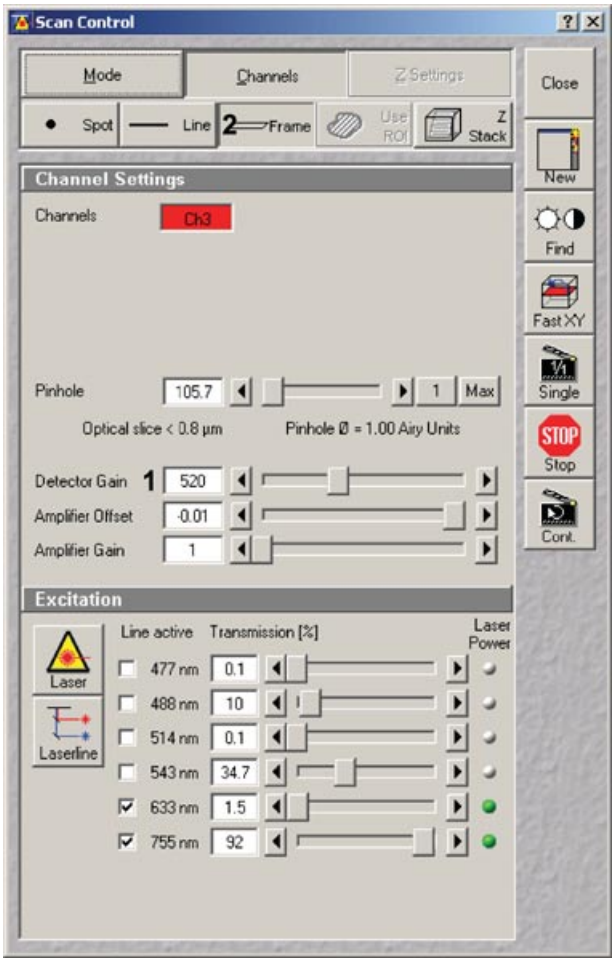


Figure 10.15.6 Scan Control window illustrating the Channels dialog panel. Courtesy of Carl Zeiss MicroImaging.

9. Click on the **Config** button on the middle right-hand side of the window. Save the configuration at this point.

   You may want the title to be basic at this point. Later on, this basic version can be changed and saved to reflect your zoom and scan settings (discussed in the next few steps).

10. Now that you have created and saved the proper basic configuration, open the **Scan** window and select the **Channels** menu (Fig. 10.15.6). Set the detector **gain** to approximately halfway up, in the low 500s (Fig. 10.15.6, 1).

11. Be sure that frame scan is selected (Fig. 10.15.6, 2) and the pinhole is set to 1 Airy for a 63× objective.

12. Select the **Mode** window (Fig. 10.15.7).

    The Mode window is divided vertically into four separate control boxes: (1) objective, line stepping, and frame control, (2) scan speed, (3) pixel depth, scan direction, and averaging, and (4) zoom, rotation, and offset. Because of the complexity of this window, each control box will be addressed in separate steps below.

13. **Objective, line stepping, and frame control** (Fig. 10.15.8): For µPP, this box in the Mode window is important for controlling the pixel size, which will impact the

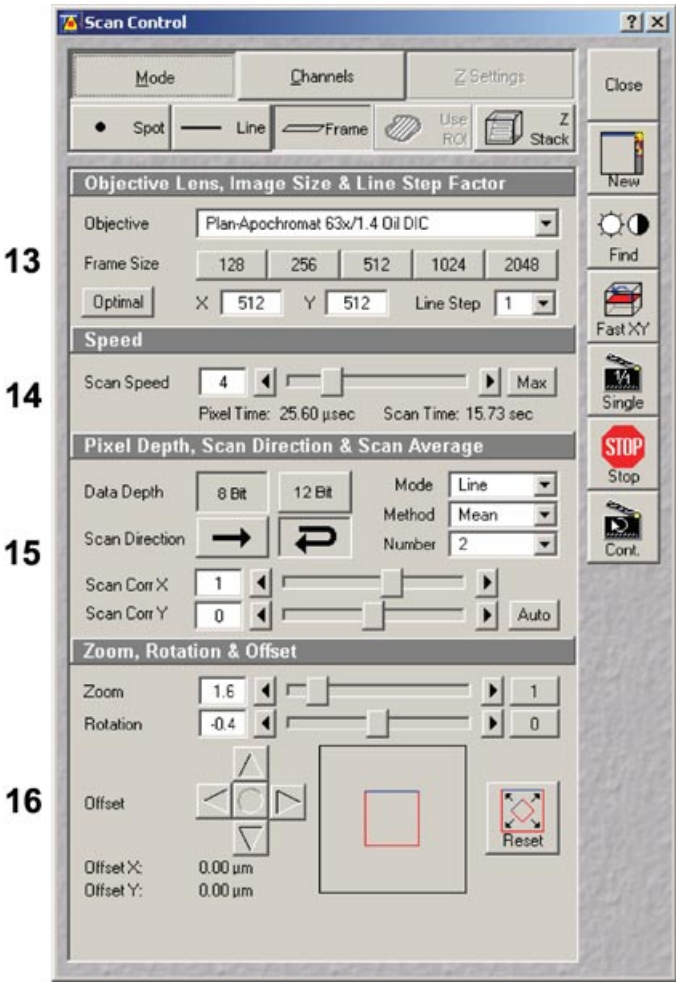


Figure 10.15.7 Scan Control window illustrating the Mode dialog panel. The four boxes within the panel are discussed in detail in the step numbered on the left side of the panel with enlarged images. Courtesy of Carl Zeiss MicroImaging.

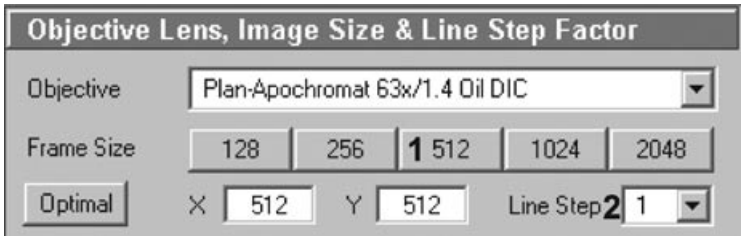


Figure 10.15.8 Objective, line stepping, and frame control box in the Scan control window of the AIM software. Courtesy of Carl Zeiss MicroImaging.

region of interest (ROI) template, how detailed or defined the ablation patterns are, as well as the total scan time (the larger the pixel array the longer the scan time).

We have found that using a 512 × 512 array is a good intermediate (Fig. 10.15.8, 1): fast scanning with a reasonably high level of detail. ***Line step*** *should be 1 (Fig. 10.15.8, 2). Objective can be varied but should have a high numerical aperture (N.A), minimum of 1.0.*

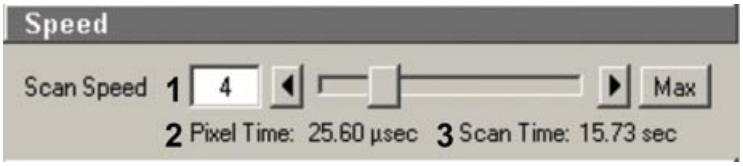


Figure 10.15.9 Scan speed box in the Scan control window of the AIM software. Courtesy of Carl Zeiss MicroImaging.

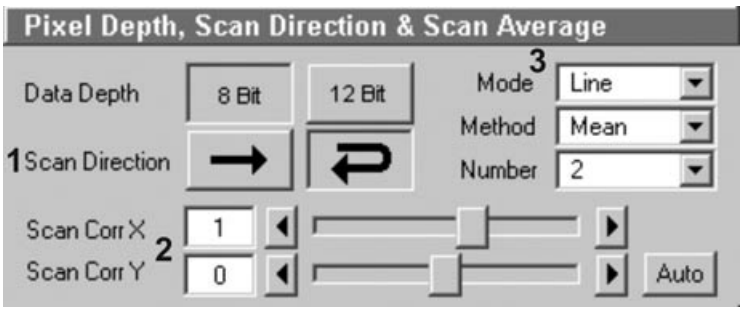


Figure 10.15.10 Pixel depth, scan direction, and averaging box in the Scan control window of the AIM software. Courtesy of Carl Zeiss MicroImaging.

14. **Scan speed** (Fig. 10.15. 9): This sets the relative rate of speed the galvanometric mirrors move and scan the FOV or the ablation area. As scan speed increases (Fig. 10.15. 9, 1), both pixel time and Scan time (Fig. 10.15.9, 2 and 3, respectively) decrease. The Pixel Time (or dwell time) is the amount of time the laser dwells on any given pixel in the FOV. Since efficiency of the ablation process is directly dependent on the total amount of light energy (in µjoules/µm^2), the dwell time is dependent on the total power output of the TP laser (in our case ~1200 mW at 755 nm). A scan speed of 4 using a 63× provides efficient ablation with our setup.

    Scan speed will need to be decreased when the pixel size changes, for instance, when a digital zoom is used to generate smaller patterns with the same objective lens. This will be discussed in later sections.

15. **Pixel depth, scan direction, and averaging** (Fig. 10.15.10): **Scan direction** and **scan average** are equally important here. Choose to reverse scan direction (Figure 10.15.10, 1; reverse arrow). This decreases the scan rate by half when compared to the single direction. When choosing a scan, the correction dialog box is opened. This helps to align the scanning properly (Fig. 10.15.10, 2; see your Zeiss representative or manual for more on how to do this). Scan average (Fig. 10.15.10, 3) provides the same basic function it normally does when imaging: repeated scanning of the same line or frame in the FOV. However, instead of decreasing background noise it adds a second (or more) pass over the area being ablated. For example, increasing this number from 1 to 2 overall doubles the laser dwell time by performing a second pass. It will also double the scan time (from step 14). Leave the scanning in line mode. The method does not matter since you are not saving the image files.

16. **Zoom, rotation, and offset** (Fig. 10.15.11): In this box, you can set the appropriate zoom (Fig. 10.15.11, 1). This is helpful if you want to decrease the size of an entire ROI template. For example, you can generate a circle pattern with a diameter of 10 µm simply by zooming in by 2× with a ROI template containing a circle with a 20-µm diameter. Here it is set to 1.6, which, when using a 63× objective, is equivalent to 100×. This is not an optical or a true digital zoom: the same pixel array is scanned but

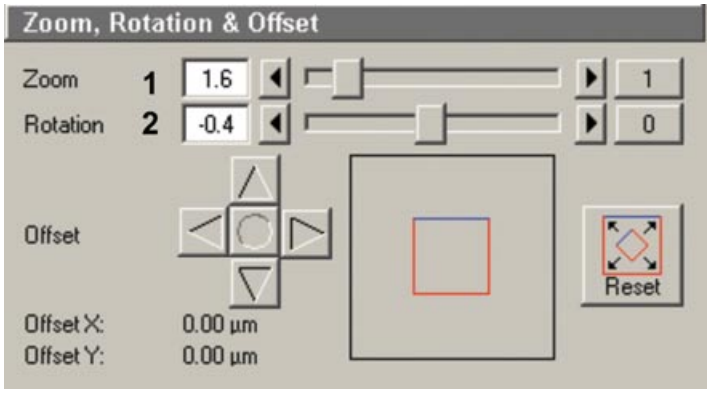


Figure 10.15.11 Zoom, Rotation, & Offset box in the Scan control window of the AIM software. Courtesy of Carl Zeiss MicroImaging.

simply in a smaller region on the galvanometric mirrors. Rotation (Fig. 10.15.11, 2) is important only when generating multiple patterns (same or different) next to each other, and comes into play in later sections when using the automated tiling function. In many confocal microscopes, the galvometric mirrors are slightly offset from the true horizontal or vertical plane of the stage. By correcting the rotation for this offset, larger patterns (i.e., long lanes or lines) can be flawlessly connected and generated. Setting the proper rotation offset is discussed later in Support Protocol 1. FOV offset is normally not changed.

The Zoom function can also go below 1 to 0.7 giving you a larger FOV. However, the rotation dialog will be reset to 0 and cannot be changed.

17. At this point, the confocal configurations are properly set. Save the Configuration again in the Configuration control panel, as you did in step 3 above.

Pre-ablation set up

While the previous section led you through the proper confocal configurations required for efficient ablation of the PVA thin film, this section will guide you through how to find the proper *z*-plane to ablate the thin film, how to align the *z*-plane, and how to generate ROI templates for use in ablation. After these steps, ablation can be performed.

18. Bring your PVA thin film dishes in their container to room temperature. Place the dishes you will be patterning near or on the microscope to allow them to acclimate to the temperature of the confocal microscope since differences in oil, objective, and dish temperature will cause focus drift.

19. Boot the system as before in step 1, turning on the appropriate lasers.

20. Clean the bottom of the dish with glass cleaner thoroughly and dry. Add a small drop of oil directly to the bottom of the dish and place it in the single dish holder.

21. Before proceeding, make sure the stage insert is clean and properly fit into the stage with all adjustment screws up (not contacting the stage plate).

22. After loading the software, load the configuration for ablation saved in previous steps.

23. Bring the 63× objective up to your sample using the course focus knobs until the oil hits the glass.

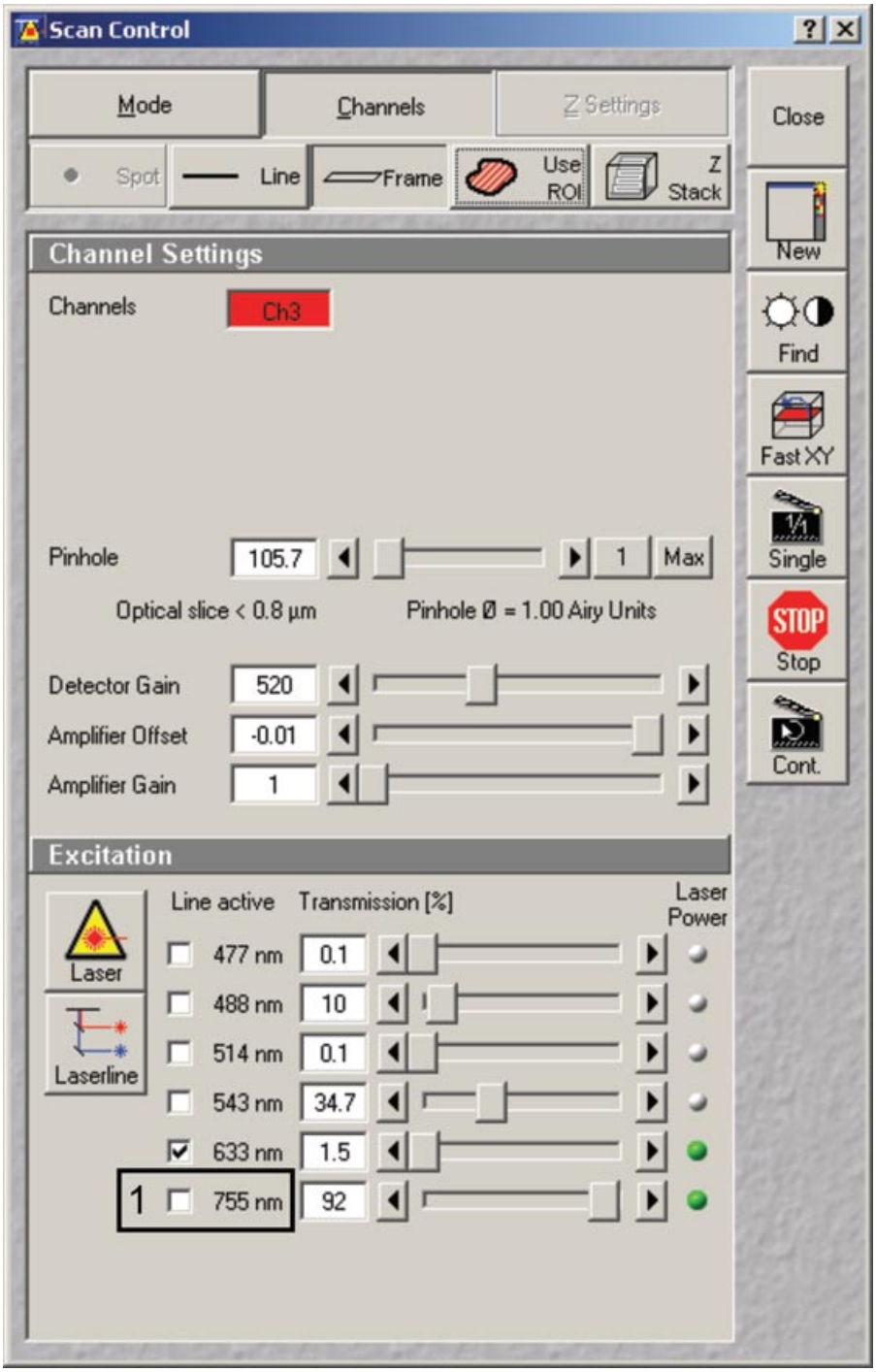


Figure 10.15.12 Scan Control window with Channels panel displayed. Box indicates an unchecked 755-nm laser line. Courtesy of Carl Zeiss MicroImaging.

24. Before using Fast XY to find your sample, be sure to inactivate the 755-nm laser line in the **Scan control** window under the **Channels** panel (Fig. 10.15.12, 1 black box).

    This is imperative since this amount of light will ablate the surface as soon as you reach the focal plane.

25. Using the **Fast XY** function, begin scanning for the PVA thin film surface by rotating the fine focusing knob (up: clockwise on the right-hand side of the microscope). Do

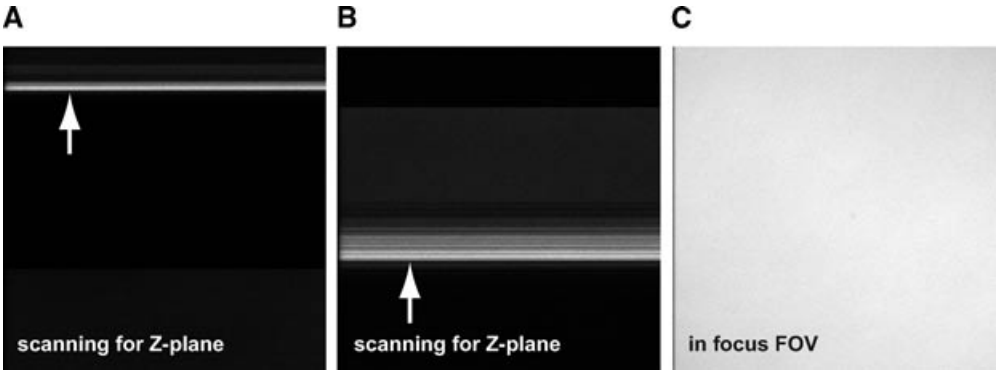


Figure 10.15.13 Reflected light images while trying to find the PVA thin film. (**A,B**) Images were taken while scanning in the *z*-plane. The bright linear areas indicate passing by the glass/PVA thin film (arrows). (**C**) Once found, the field of view (FOV) should appear equally (uniformly) bright if the surface is level.

this at a moderate to fast pace moving 300 to 400 μm in ~10 sec time. While doing this, keep your eye on the monitor. When you reach or pass the focal plane it will appear as a bright line or set of lines on the screen in Fast XY mode (Fig. 10.15.13A,B).

26. Adjust the objective *Z* position until nearly the entire FOV is in focus (Fig. 10.15.13C).

    Once in focus, you may need to adjust the PMT gain up or down depending on the brightness. The brightest z-plane, which is the glass interface, should be near pixel saturation (~255 for an 8-bit image).

27. More often than not, the FOV's brightness is unevenly distributed, such as in Figure 10.15.14A, and requires adjustment since the TP light is maximally absorbed only at the focal plane. To adjust properly you first need to know which corner or edge is low. Adjust the focus so the focal plane is below the glass. Slowly raise the objective until you start to see brightness in the FOV: whichever area appears bright first is low and needs to be raised using the adjustment screws in the stage insert plate (Fig. 10.15.14B). In the example in Figure 10.15.10A, both the upper-right and lower-right adjustment screws need to be turned clockwise, or screwed in to raise the stage up. Perform several half turns. The image should go black, indicating the stage has been adjusted. Refocus with the fine focusing knobs. Repeat this process until the FOV demonstrates even illumination, as in Figure 10.15.13C. This adjustment process should be performed before ablation of any dish. When finished with each dish return adjustments screws to their neutral positions.

28. Wait 5 min for the focus to adjust due to temperature variations, and then refocus to the brightest FOV. Open the **Stage and Focus control** window (Fig. 10.15.15). In the stage control window, set the **Z Focus step** (Fig. 10.15.15, 1) to 0.25 μm.

29. Using the focusing arrows (Fig. 10.15.15, 2), raise the focal plane to 0.75-μm above the brightest FOV. This is the proper height to perform ablations. The FOV should be slightly dimmer. Set this point to zero (**Z**: Fig. 10.15.15, 3).

30. In the stage position box, select **zero** (Fig. 10.15.15, 4). Now you will know the position of your first ablation site.

31. Now that the dish has been leveled, ablation can commence once you have generated a ROI template. In essence, this is the same way you would generate ROIs for FRAPing (Fluorescence Recovery After Photobleaching, *UNIT 21.1*) a sample.

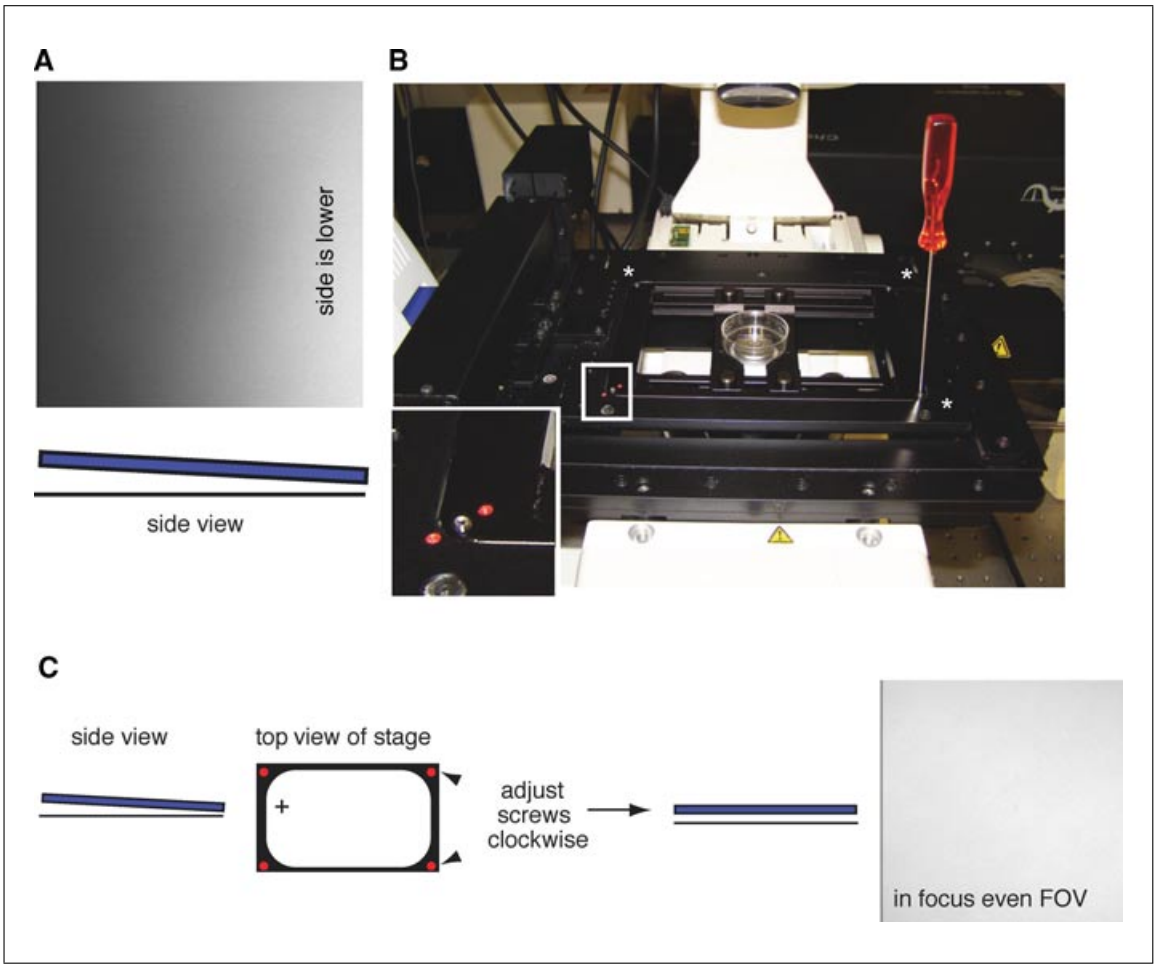


Figure 10.15.14 Dish leveling. (**A**) Reflected light image of a dish that is slightly tilted down on the right, as shown by the increase in brightness as you focus up to the glass surface. Below the image is a cartoon side view representing the glass (gray) with respect to the horizon (black line). (**B**) Image of the confocal stage. Inset shows a magnified view (white box) of one of four adjustment screws in the stage plate. The other adjustment screws are indicated by asterisks. (**C**) Simplified schematic of the tilted dish in (A) and how to correct the tilt by screwing in both right-side adjustment screws, until an in-focus evenly illuminated FOV is attained (right-side of arrow).

32. To generate ROIs, select the **Edit ROI** window (Fig. 10.15.16A,C). The window allows you to generate and save hundreds of different ROIs in a single FOV as templates.

33. To begin, in the Scan Control window select **New** to generate a new image window.

34. From the bottom dialog boxes in the Edit ROI window (Fig. 10.15.16A, 1 and black box), select a shape (i.e., circles, polygons rectangles, etc.). Then draw the shape onto the new image window (Fig. 10.15.16B). Once drawn, the shapes size and location information will appear in the Edit ROI window, checked (Fig. 10.15.16C, 4). From here, you can resize or reposition the shape or uncheck it and remove it.

    Several macros are available free from Zeiss that allow you to repeat a single ROI multiple times on the same window, which is helpful for generating a dot-based matrix, etc. It is also helpful to know the size of a single pixel with the objective and zoom you are using. This can be found by selecting the Info button in the image window (Fig. 10.15.16B, 2).

35. The **X and Y Scaling** (Fig. 10.15.16B, 3) can then be used for pattern spacing and sizing, as well as knowing the size of the FOV, important for moving the stage

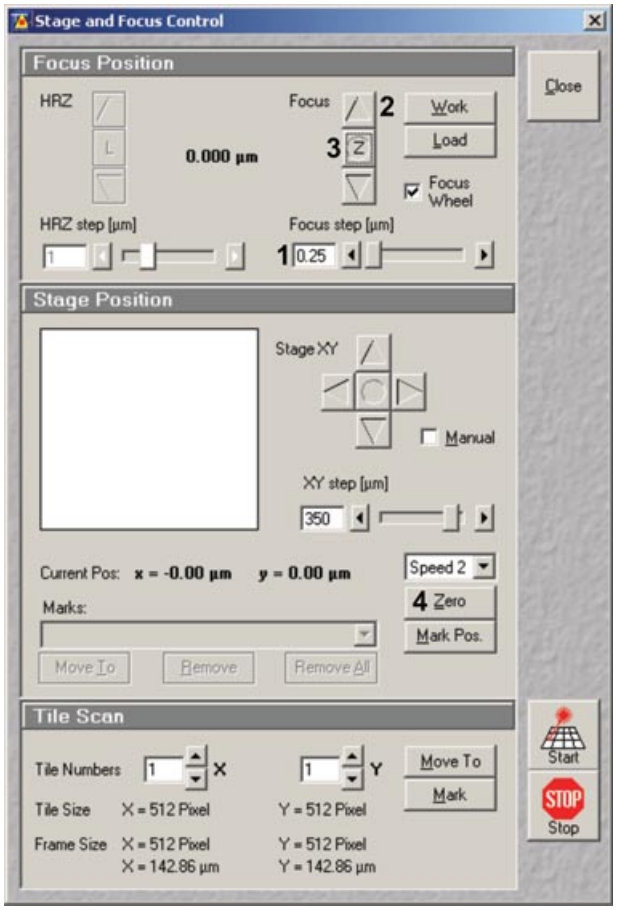


Figure 10.15.15 Stage and Focus Control window in the Zeiss AIM software. 1 indicates the focus step set at 0.25 µm. 2 indicates toggle arrows for focus position. 3 indicates the focus zeroing button. 4 indicates the Stage position zeroing button. Courtesy of Carl Zeiss MicroImaging.

horizontally or vertically when generating larger repeated patterns. Save and name the template when completed.

Photoablate the thin film

36. With the above steps completed, you can now proceed with photoablation. First, check that you are still focused ~0.75-µm above the brightest focal plane and readjust if necessary.

37. In the **Scan control** window under the **Channels** panel, check the 755-nm TP to on.

38. Select the ROI template of your choice from the **Edit ROI** window. In the Scan control window, select the **ROI** button (2nd row from top 2nd from the right, see Fig. 10.15.12).

39. Select **Single** scan button. The ROIs in the template should be slowly scanned from top to bottom, taking ~15 sec with the parameters set and discussed earlier.

    Because of the intense level of light hitting the sample, the ROIs will appear saturated with light (255 on an 8-bit scale).

40. Once the Scan has completed, uncheck the TP 755-nm line in the scan window, deselect the ROI tab, and **Fast XY** scan the FOV to observe the post ablation result as shown in Figure 10.15.17.

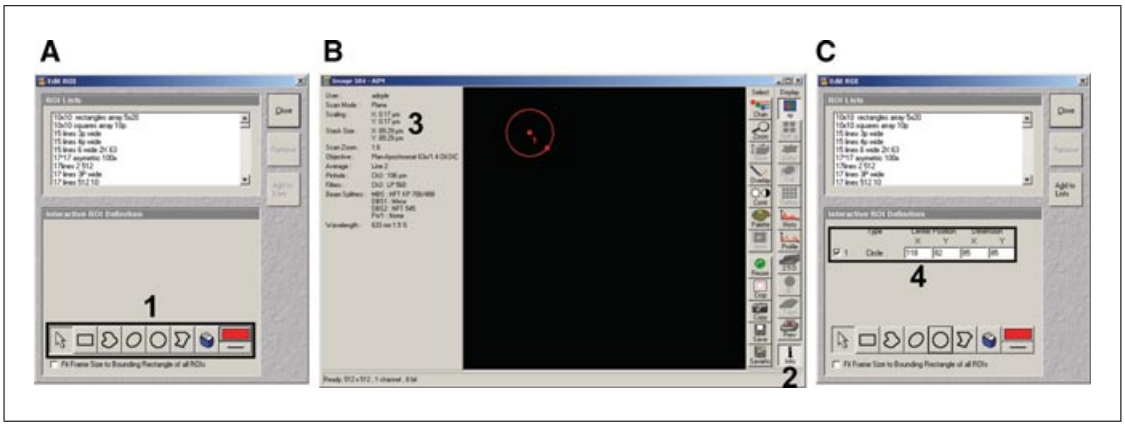


Figure 10.15.16 Edit ROI window and pattern generation. (**A**) Edit ROI window with the shape dialog boxes (1 and black box). (**B**) Example of generating a single circle once the circle dialog button is selected in the Edit ROI window in (A). 2 indicates the information button, which shows on the on the left-hand side of the image window. 3 (upper left) indicates where pixel scaling information can be found. (**C**) Edit ROI with the ROI definitions (4 and black box) for the circle shown in panel (B). Courtesy of Carl Zeiss MicroImaging.

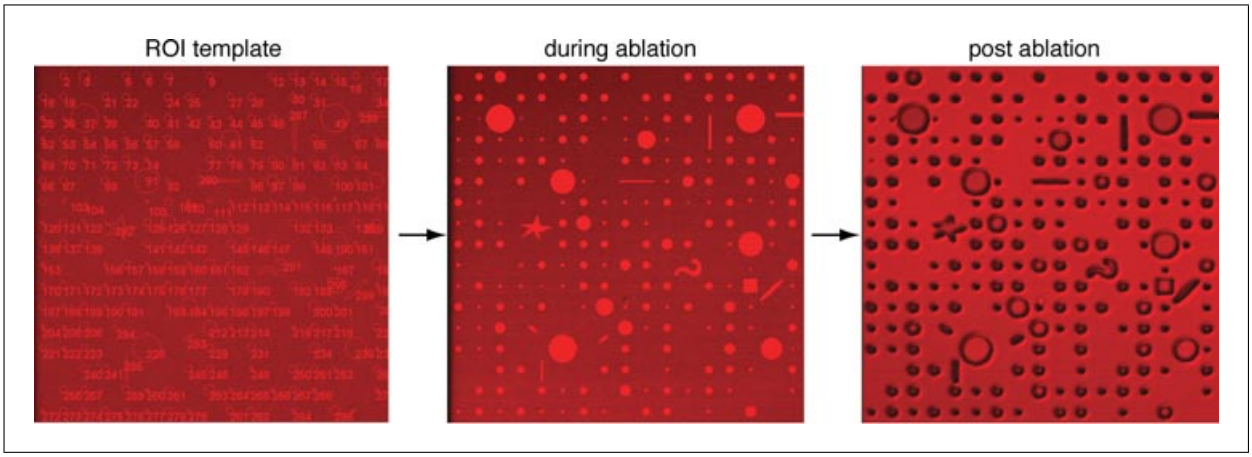


Figure 10.15.17 From template to ablated pattern. The three images representing the ROI template (left), what is observed during the ablation (middle), and the post-ablation pattern (right).

41. To generate multiple FOVs of the same pattern use the **Stage and Focus control** window to move the stage over by setting the *xy* step to exactly one FOV (use the calibrated *xy* information multiplied by your scanning window size, assuming *x* and *y* are equal).

    This works best for dot or separated patterns such as the example illustrated in Figure 10.15.17. However, if using a linear pattern, which needs to be seamlessly continued, we recommend reducing the xy step by 1 or 2 μm to provide an appropriate overlap.

Automate μPP with macro functions

To this point, you should be able to efficiently photoablate PVA thin films for a single FOV. Once the configurations have been set correctly and the ROI templates are generated, there is no true need to sit at the scope moving the stage from place to place if the process can be automated. This can be achieved through the **Multitime macro** in the AIM software. Originally intended for time-lapse imaging, Multitime allows the user to choose multiple locations within the dish. Instead, here we use Multitime to automate the μPP process. An additional feature is the ability to "tile" around a specified point; that is to image an array of images around a single point in a tile or grid-like fashion. The

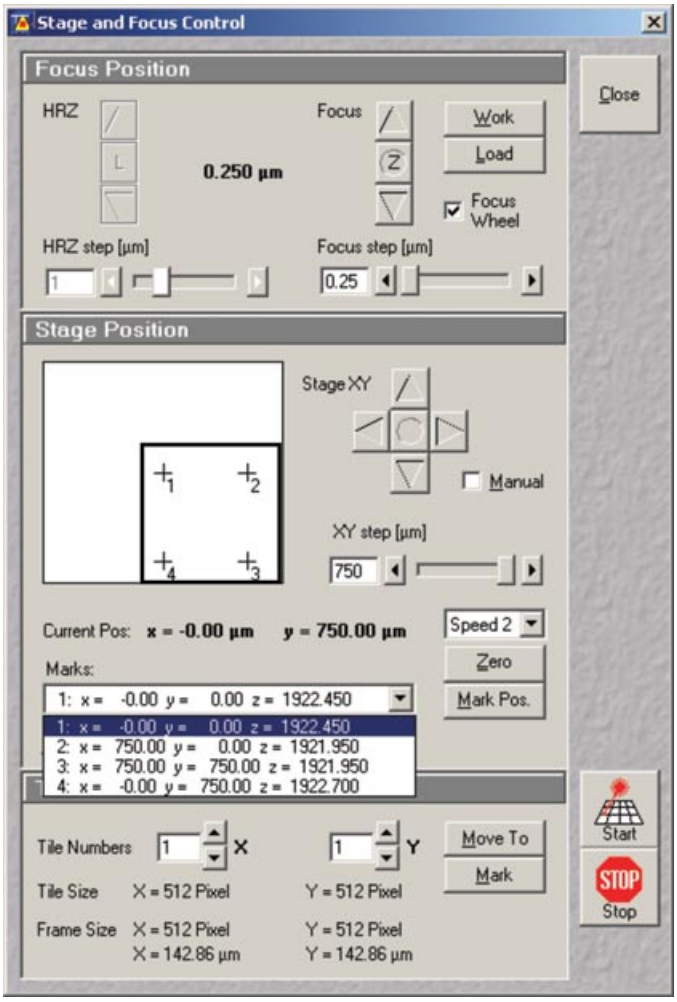


Figure 10.15.18 Using the Stage and Focus Control window to mark multiple tiling positions. The black box indicates four points that were selected for tiling. Courtesy of Carl Zeiss MicroImaging.

next several steps will help you to achieve this automation process. While the Multitime macro has many functions, we are only going through those that directly pertain to automating µPP.

42. After you have found the proper *z*-plane and zeroed your position as in steps 28 to 30, go to the **Stage and Focus control** window (Fig. 10.15.18). In the Stage position box (center), select **Mark Pos.** (Fig. 10.15.18, 1). Keep this window open since you will be referring to it later.

    The example in Figure 10.15.18 shows how this was repeated four times 750-µm apart. Each mark is listed in the dropdown menu below. These marked positions will be used for tiling in the Multitime macro.

    It is helpful to move the stage while in Fast XY mode. Once to the correct xy position, readjust the z-plane to be ~0.75-µm above the brightest FOV then mark the position, but do not zero.

43. Prior to starting, create a new database (**File>New File**) and save it in an appropriate place.

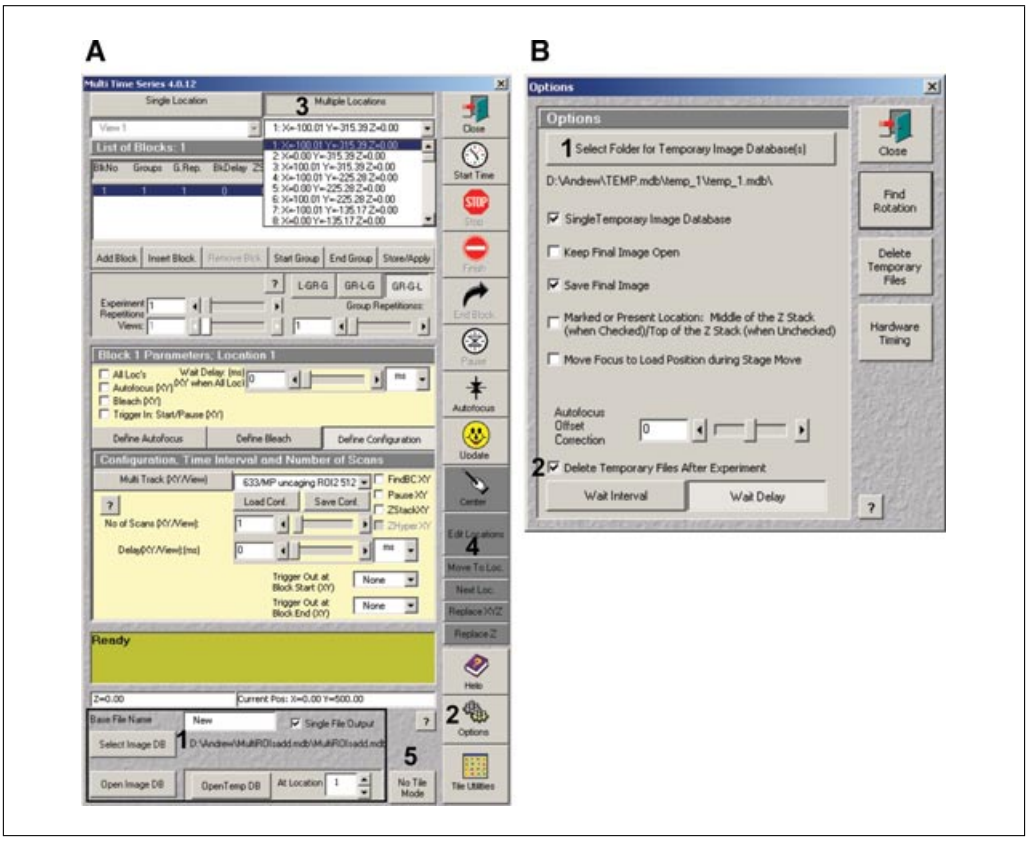


Figure 10.15.19 Multitime macro window (**A**) and the Options window (**B**). The bold numbers indicate the order each step is to be performed. Courtesy of Carl Zeiss MicroImaging.

44. In the main AIM window, select the **Macros** menu. If the Multitime macro does not appear in the window, load it (if unfamiliar with Macros ask your Microscope facility director or your Zeiss representative for help with installation).

45. Open the Multitime window. At the bottom of the window (Fig. 10.15.19A, bottom, 1), select the **Image DB** dialog button to choose the database you generated earlier.

46. From the window buttons on the right-hand side, select **Options** (Fig. 10.15.19A, 2 and 10.15.19B). In the Option window, first create a temporary image database (Fig. 10.15.19B, 1).

    This is where the software saves your ablation images.

47. Next, check the dialog box that says "Delete Temporary files after final experiment" (Fig. 10.15.19B, 2). Close window.

48. From the top of the window, select **Multiple locations** (Fig. 10.15.19A, 3), which will allow you to choose more than one point to scan.

    Steps 44 to 48 in Multitime only need to be performed once. After performing these actions once, Multitime will remember the settings.

49. Next, choose **Edit location** (Fig. 10.15.19A, 4). The edit location window (Fig. 10.15.20A) will allow you to tile around the marked positions from step 42.

50. Under the **Grid** tab, change the *x* and *y* Grid numbers to the appropriate number of FOVs to tile in each plane (Fig. 10.15.20A, 1), and then select **Create Grid Locations** (Fig. 10.15.20A, 2).

    This generates a list of points in the Multiple locations dropdown menu.

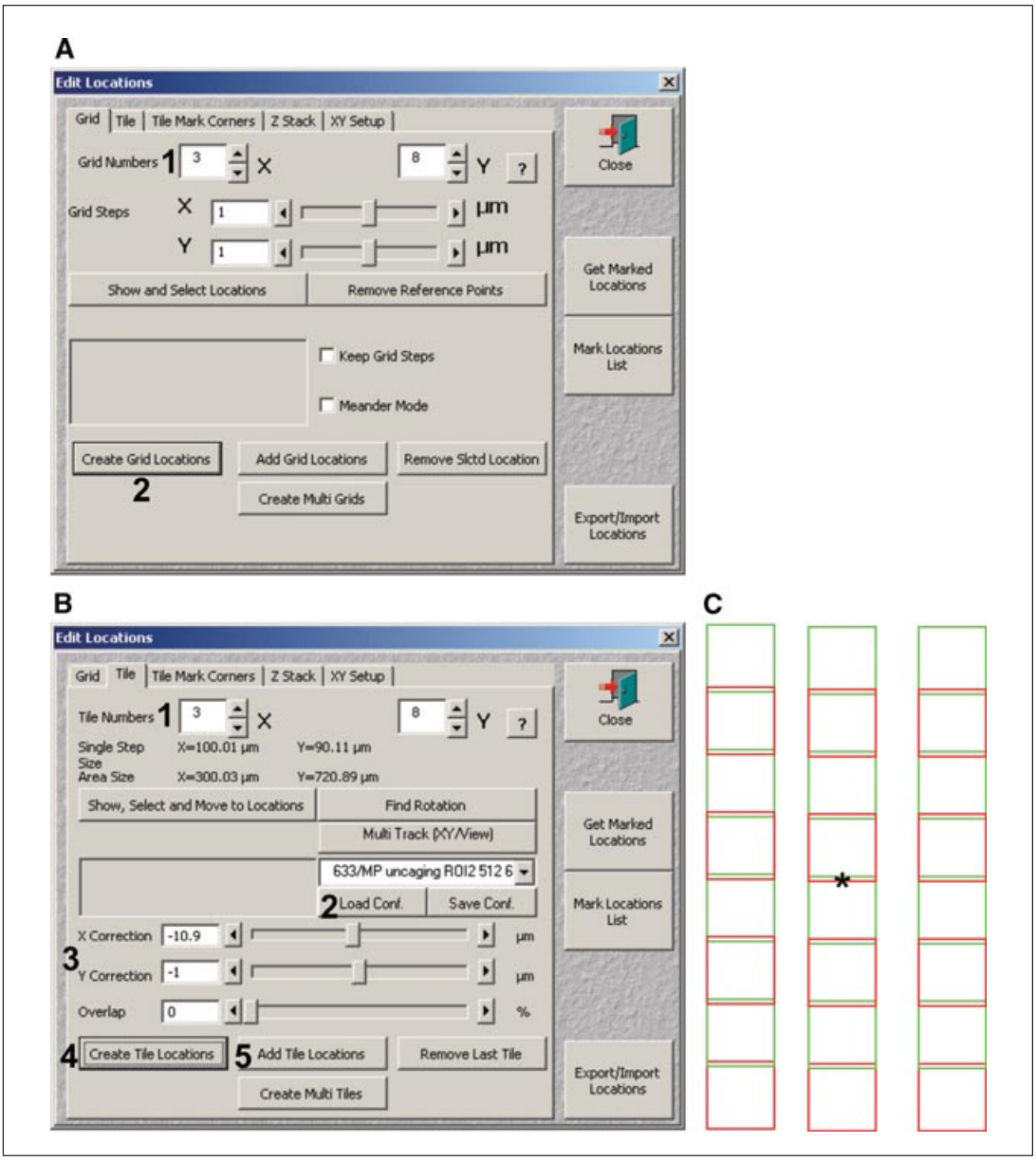


Figure 10.15.20 Tiling with Edit Locations window. (**A**) The Edit Locations window grid tab showing the Grid Numbers dialog (1) and the Create Grid Locations button (2) for grid generation. (**B**) The Edit locations window tile tab with Tile Numbers (1), configuration (2), XY correction (3), and Create Tile Locations (4) highlighted. (**C**) Schematic representation of the tiled grid generated based on the parameters shown in (B). The asterisk represents the marked positions chosen in the Stage and Focus control window. Courtesy of Carl Zeiss MicroImaging.

51. Next, select the **tile** tab (Fig. 10.15.20B). From top to bottom (1), change the **tile numbers** *x* and *y* (Fig. 10.15.20B, 1), (2) select the configuration to be used from the dropdown menu, then load it by hitting **Load Conf.** (Fig. 10.15.20B, 2), and (3) set your **X and Y correction** (Fig. 10.15.20B, 3; used for overlapping or spacing FOVs apart).

52. In the **Stage and Focus control** window, select a position from the dropdown menu, and select **Move To**.

53. Then, back in the **Edit Location** window select **Create Tile Locations** (Fig. 10.15.20B, 4). If more than one location is to be tiled, move to its position then select **Add Tile Location** (Fig. 10.15.20B, 5; after your initial locations all other locations are added in this manner).

54. Go to the **Scan Control** window and select **ROI**.

    IMPORTANT NOTE: *This is very important. If not selected, the Multitime Macro will photoablate the entire FOV.*

55. Select the **No Tile Mode** (Fig. 10.15.19, 5).

    This stops the tiling of the images collected into a larger single image.

56. Select **Start** in the Multitime window.

    You should start seeing Multitime running. At the bottom of the Multitime window, information should begin appearing telling you the position, scan number, and other information. It is helpful to time the process for a single FOV plus the stage movement to estimate your finishing time.

57. Once this Multitime has finished, check several FOVs to be sure the ROI template has been repeated. Close the **Multitime Macro** window.

58. If more patterning is required in the same dish (the same or a different pattern), simply move to a new nonablated region being sure to know your current position with respect to all previously ablated areas.

59. Repeat finding the *z*-plane, reset zero for *x*, *y*, and *z*.

    *The original zero point (0, 0, 0 for x, y, and z, respectively) should now be listed in the **Stage and Focus control** window as something different. For example, if you moved over 2000 μm in x, 0 μm in y, and 1.0 μm in z, the original position should read: $x = -2000.00$ $y = 0.00$ $z = -1.00$.*

DISH QUENCHING

BASIC PROTOCOL 4

Following the photoablation of the PVA thin films, the next step is quenching any unreacted aldehydes. Quenching the thin film is important for three reasons: (1) it reduces any reacted aldehydes leading to a stronger covalent attachment of the thin film to the glass surface; (2) the reduction of the aldehydes, especially if glutaraldehyde is used, decreases autofluoresence in the blue to green wavelengths (490 to 540 nm); and (3) it acts to block any free radicals that may be produced in the thin film during photoablation. Because sodium borohydride is very hygroscopic, we suggest desiccating it at room temperature in small aliquots in microcentrifuge tubes. Use a single tube 4 to 5 times and discard the remainder. The reaction that occurs when $NaBH_4$ comes in contact with water is temperature dependent, being more vigorous as temperature increases.

Materials

μPP-patterned dishes (Basic Protocols 1 through 3)
200 mM ethanolamine buffer (see recipe)
Sodium borohydride solution ($NaBH_4$; see recipe)
1 M NaOH solution (see recipe)
Phosphate-buffered saline (PBS; Hyclone, cat. no. SH30264.02)
Phosphate-buffered saline (PBS) with penicillin/streptomycin and fungizone (see recipe)

Storage containers
Scale
1.5-ml microcentrifuge tubes

1. To each photoablated dish, add 2 ml of ethanolamine buffer after photoablation. Store dishes containing ethanolamine buffer up to 1 month at 4°C in an airtight container.

   The ethanolamine buffer should be added on the same day (preferably within an hour) of photoablation.

2. If photopatterned dishes have been stored at 4°C, allow them to warm at room temperature for 5 min.
3. Weigh out 40 mg of $NaBH_4$ into a single 1.5-ml microcentrifuge tube. Add 1 ml of 1 M NaOH. Triturate several times.
4. To each dish, add 20 µl of the $NaBH_4$ solution mixed in step 3. Replace the dish lid, swirl several times to mix, and incubate up to 8 min at room temperature.

   You should start to see bubbling after 1 to 2 min, which shows the reaction is occurring.
5. Aspirate $NaBH_4$ solution and rinse two times, each time with 3 ml PBS. Add 1 to 2 ml of PBS with penicillin/streptomycin and fungizone. Store up to 1 month at 4°C.

BASIC PROTOCOL 5

ADSORBING EXTRACELLULAR MATRIX AND PLATING CELLS

The final step of µPP is adsorption of an extracellular matrix (ECM) molecule to the photoablated patterns. Here, we describe the attachment of fibronectin to photoablated dishes; however, any other ECM molecule or even growth factors can be absorbed in this fashion. Once the ECM is adsorbed to the surface, it is important to block attachment of other molecules (other ECMs, growth factors, etc.) found in serum using heat-denatured bovine serum albumin (BSA). For fluorescence microscopy techniques where the patterns are invisible, prior direct conjugation of a fluorescent dye to the ECM molecule of choice is helpful for pattern visualization. A general fluorescent dye labeling protocol for N-hydroxy succimidyl ester-based dyes can be found in Support Protocol 2. Pluronic F-127 is a nonionic detergent/surfactant, which is used here to help with blocking nonspecific protein adsorption to nonablated surfaces.

Materials

Fibronectin at 2 mg/ml concentration in PBS or other suitable buffer
Phosphate-buffered saline (PBS) with 0.1% (v/v) pluronic F-127 (see recipe)
µPP patterned dishes (Basic Protocols 1 through 4)
Lyophilized bovine serum albumin (BSA)
Phosphate-buffered saline (PBS; Hyclone, cat. no. SH30264.02)
2 M NaCl solution
Phosphate-buffered saline (PBS) with penicillin/streptomycin and fungizone (see recipe)
NIH/3T3 cells (ATCC) grown to 60% to 70% confluency in a 100-mm diameter dish in 10% CO_2 incubator
Hanks balanced salt solution (HBSS; Invitrogen)
0.5% (w/v) trypsin/EDTA solution (Invitrogen)
NIH/3T3 fibroblast culture medium (see recipe)

Tissue culture hood
37°C waterbath
400-ml beaker
Stirrer/hot plate
Scale
50-ml conical tubes
Glass test tube capable of holding 30 to 50 ml
Flea Micro magnetic stir bar (VWR)
Digital thermometer
Ice in an ice bucket
Vacuum aspirator
Airtight storage container

Benchtop swinging-bucket rotor centrifuge with adapters for 50-ml conical tubes
Inverted microscope equipped with a 10× phase contrast objective

Prepare coating solution

1. Calculate the total amount of fibronectin solution needed for all dishes.

   Because the glass surface only needs to be covered, we use ~100 μl per dish.

2. Next, calculate the volume of fibronectin (at 2 mg/ml) needed to attain the proper concentration (10 μg/ml).

   For example, for four dishes, add 2 μl of 2 mg/ml fibronectin to a microcentrifuge tube followed by 398 μl of PBS with 0.1% pluronic F-127 buffer for a 10 μg/ml solution.

3. Prewarm the concentrated fibronectin solution to ~37°C prior to mixing the solution. Fibronectin is an active molecule, which over time will lose activity when left at 4°C for extended periods. We suggest keeping small aliquots (~20 μl) frozen at −80°C and defrosting and using an aliquot for only 1 week stored at 4°C. Once diluted, the fibronectin solution must be used promptly and cannot be stored.

Coat the dish in the pattern

4. In a tissue culture hood, add 100 μl of the 10 μg/ml fibronectin to each dish, cover, and incubate for 1 hr at 37°C.

5. While waiting, heat 250 ml of water in a 400-ml beaker on the stirrer/hot plate to ~85°C.

Heat-denature BSA

6. Weigh out 0.3 g of BSA in a 50-ml conical tube. Bring volume/weight up to 30 g with PBS.

7. Incubate in a 37°C waterbath until BSA goes into solution, ~15 min.

   This BSA solution should be made fresh and can be used for a maximum of only 1 day.

8. Once the BSA has gone into solution, transfer the 1% BSA solution to a glass test tube and add the stir bar. Next place the test tube in the ~85°C water. Turn on the stirplate to medium.

9. Directly measure the temperature of the 1% BSA solution with the digital thermometer. Set a timer for 3 min. Wait until the solutions temperature reads 83°C and then start the timer. Monitor the temperature over the next 3 min.

   If the solutions temperature goes above 85°C, remove the test tube from the water bath and cool briefly, keeping the temperature a minimum of 83°C.

10. After 3 min, remove 1% BSA solution from the beaker of water and cool in an ice bath until the solution's temperature is below 37°C.

11. After the 1-hr incubation of the fibronectin on the μPP dishes, bring the dishes to the tissue culture hood, aspirate fibronectin, and rinse three times, each time with 3 ml PBS.

Rinse and block

12. Add 2 ml of 2 M NaCl solution to each dish and incubate 5 min at room temperature to reduce nonspecific protein binding.

13. Aspirate NaCl solution, and rinse three times, each time with 3 ml PBS.

14. Add 2 ml of heat-denatured 1% BSA solution (from step 10) to each μPP dish and again incubate for 1 hr at 37°C.

15. Rinse dishes three times, each time with 3 ml PBS, and store in an airtight container in 2 ml of PBS plus penicillin/streptomycin and fungizone until use. Use within 3 days of this last step.

Attach NIH/3T3 fibroblasts to μPP patterns

16. Detach 60% to 70% confluent NIH/3T3 fibroblasts from 100-mm tissue culture dish by rinsing twice, each time with 6 ml of 37°C HBSS.
17. Add 5 ml of 37°C trypsin/EDTA solution and wait 30 to 60 sec.
18. Aspirate excess solution and incubate for 2 to 3 min at 37°C.
19. Add 10 ml of 37°C NIH/3T3 fibroblast culture medium to the dish, triturate, and transfer to a 50-ml conical tube.
20. Centrifuge the cells in the swinging-bucket centrifuge 4 min at 1000 × *g*, room temperature.
21. After centrifugation, aspirate excess medium leaving the cell pellet undisturbed.
22. Tap the tube firmly on the tissue culture hood working area. Add 10 ml of NIH/3T3 fibroblast culture medium to tube, and triturate several times to loosen cell clumps.
23. Add 1 to 2 ml of cells to each μPP dish. Incubate for 10 to 15 min at 37°C, and then check cell attachment to patterns using a 10× phase contrast objective on an inverted microscope.

    When cells begin to attach they should appear phase dense when compared to non-adherent cells.

24. If the proper number of cells is not attached, check dishes every 5 min.
25. When the proper number of cells has attached, gently aspirate the excess and add 1.5 to 2 ml of fresh NIH/3T3 fibroblast culture medium. Incubate for a further 30 min at 37°C before imaging.

SUPPORT PROTOCOL 1

SETTING THE CONFOCAL SCAN HEADS' ROTATION OFFSET

As mentioned earlier in Basic Protocol 3, the *x* and *y* galvanometric scan head mirrors can be slightly misaligned or offset from a true horizontal or vertical plane. This becomes an issue when using the Multitime Macro for tiling: improper scanner alignment will result in the pattern being askew from FOV to FOV. For example, if generating a lined pattern that is meant to be continuous, lines may be offset. The following protocol alleviates this rotation alignment issue. This can be done simply in one of two ways: first, is to use a grid slide supplied by Zeiss for the alignment, and second is to generate a grid of your own using μPP. Both require the same steps. Any differences between the methods are discussed.

Materials

Zeiss 510 LSM NLO confocal microscope or later model with 1.5 W minimum tunable two-photon titanium:sapphire laser, and a 633-nm HeNe2 laser (5-mW power output), and a 543-nm HeNe1 laser (1-mW power output)
AIM software (Zeiss MicroImaging)
PVA thin film–coated MatTek dishes (Basic Protocol 2; for option 1)
Arc lamp
Grid slide, Objektträger (for option 2; Zeiss, cat. no. 474028)
Fluorescent highlighter, any color (for option 2)
Kimwipes

For option 1

1a. Turn on the microscope, the AIM software, and the appropriate lasers for performing μPP.

2a. Load your μPP configuration.

3a. Place a PVA thin film–coated dish on the stage and follow the pre-ablation setup in Basic Protocol 2, steps 18 through 30 in order to find the proper *z*-plane for μPP.

4a. Create a new ROI template in a pattern similar to Figure 10.15.21.

> *The grid and circle pattern help to determine the rotational offset. The spacing of the grid is not important, just that horizontal and vertical lines, as well as curved lines are incorporated into the pattern.*

5a. Being sure to have your zoom set for 1×, photoablate the pattern in the PVA thin film. Save the configuration with the TP 755-nm line unchecked.

6a. In the Focus and Stage control window, mark the position.

For option 2

1b. Turn on the arc lamp followed by the microscope, the AIM software, and the 543-nm HeNe1 laser.

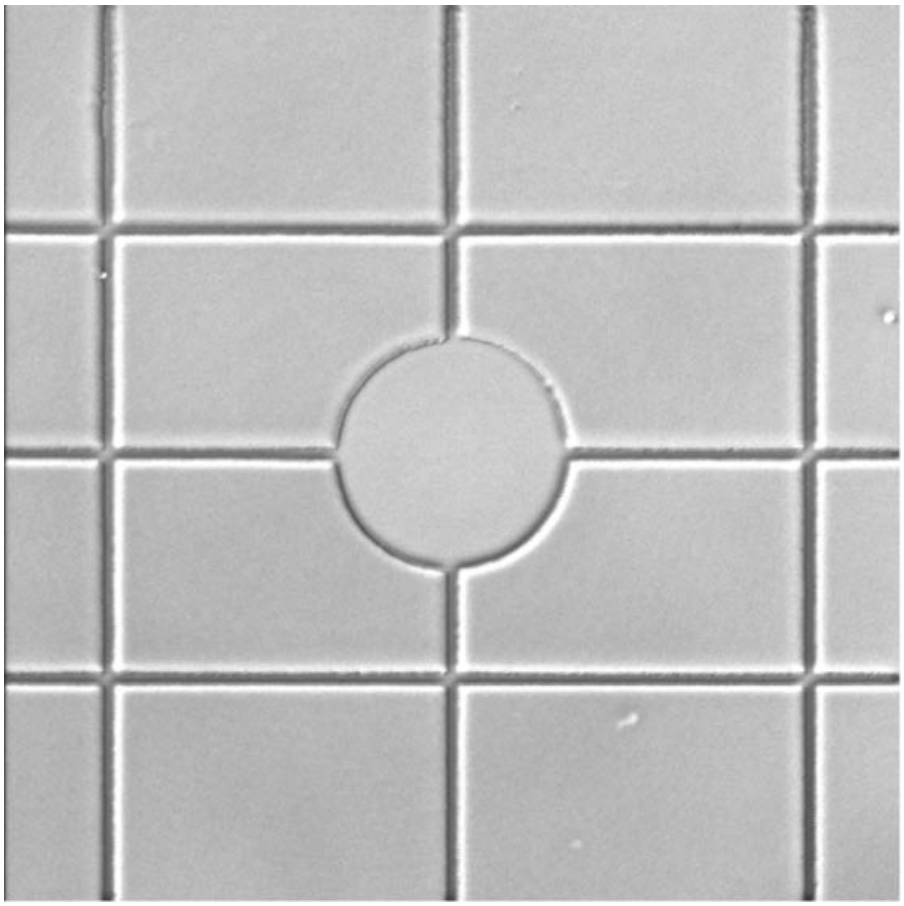


Figure 10.15.21 Microphotopatterned grid used for rotational alignment of the XY galvanometric scanning mirrors in the confocal head with the stage.

Figure 10.15.22 Tile Scan Rotation dialog window in the AIM software. Courtesy of Carl Zeiss MicroImaging.

2b. Select a configuration to image Cy3, Alexa Fluor 543, or Rhodamine dyes.

If needed, ask your microscope facility manager for help.

3b. On the grid slide, locate the grid in the center. Mark the grid with the fluorescent highlighter. Gently wipe off excess with a Kimwipe.

4b. Add a drop of oil over the grid and position the slide properly in the stage holder. Use epifluorescence to find the grid using a 63× 1.4 NA objective. Once the grid is found, switch to LSM mode, and adjust the *z*-plane while in Fast XY. Adjust the settings (PMT gain, laser output, etc.) and save as a new configuration.

5b. Scan the grid pattern until you find a region that contains both the vertical and horizontal lines, as well as part of a curved arc.

The grid pattern usually has a small and large circle pattern.

6b. In the Focus and Stage control window, mark the position.

For options 1 and 2

7. Open the Macros menu and select the Multime Macro. Open the Edit Locations window and select the tile tab (**Macros>Multime>Edit Locations> Tile tab**).

8. Select the configuration you saved in step 5a or 4b for either option and select **load Config.**

9. Select the **Find Rotation** button.

A new window should appear similar to the one shown in Figure 10.15.22.

10. Choose **Calibrate** (Fig. 10.15.22, 1). This should take ~20 sec before a number will appear in the **Rotation [°]** dialog on the left, which represents the rotational offset (Fig. 10.15.22, 2).

11. Go to the **Zoom, Rotation and Offset** box in the **Mode** panel of the **Scan control** window. Use the number found in the **Rotation [°]** dialog for your rotational offset in your μPP configurations. Save the configurations once complete.

SUPPORT PROTOCOL 2

DIRECT FLUORESCENT LABELING OF FIBRONECTIN

For any type of micropatterning including μPP, it is important to know whether the ECM is being adsorbed to the patterns and not nonspecifically. While antibodies can help with this determination, the direct approach is often best, especially when conducting live-cell fluorescence imaging. Below, we detail one method of directly labeling fibronectin with N-hydroxy succimidyl (NHS) ester-based fluorescent dyes. Before proceeding, several items should be noted: (1) NHS ester reactions are pH and temperature dependent, (2) the reactions are hygroscopic and once in contact with aqueous solutions begin reacting immediately, and (3) the ratio of protein to dye is important, with the reaction time being based on this and the parameter (1) above. For every 1 mg of protein, 5 to 10 μg of dye should be used (200:1 or 100:1 ratio). This is slightly below a 10-molar excess

normally suggested for dye-to-protein conjugations. Over-labeling ECM proteins can negatively affect cell attachment and/or migration. If using different starting protein amounts, recalculate the amount of dye to match these ratios. More information about these other types of fluorescent conjugations can be found in "Bioconjugate Techniques" written by Greg Hermanson (Hermanson, 1996).

Materials

NHS-ester-based fluorescent dye of choice (several are available from Invitrogen and Pierce)
Dimethyl sulfoxide (DMSO)
500 to 1000 μl of fibronectin at 2 mg/ml concentration or 2 mg of lyophilized fibronectin
100 mM borate buffer, pH 9.0 (see recipe)

Slide-A-Lyzer (Pierce)
1.5-ml microcentrifuge tubes
Aluminum foil
End-over-end rotating mixer, e.g., Labquake rotating mixer (sometimes termed a rotisserie shaker)
Desalting spin column *or* dye-removal columns capable of ~1 ml volumes (Pierce)
Centrifuge capable of 10,000 × *g* with 15-ml conical tube holders

1. Keep NHS-ester-based fluorescent dyes, which are hygroscopic, in DMSO until use. Dilute the lyophilized dye with DMSO to a concentration of 1 mg/ml. Split into aliquots of ~25 μl. Store at −20°C until use.
2. If starting from lyophilized fibronectin, add 1 ml of 100 mM borate buffer (pH 9.0) to make a 2 mg/ml concentration. If starting from fibronectin in PBS or other buffers (~pH 7.4), dialyze in borate buffer (see *APPENDIX 3C*).
3. Warm 2 mg/ml fibronectin to room temperature prior to reacting with NHS ester dyes.
4. Defrost NHS-ester dye (1 mg/ml concentration) prior to opening the tube since it will absorb moisture from the air.
5. Add 1 ml of fibronectin (2 mg/ml, in borate buffer) to a 1.5-ml microcentrifuge tube.
6. Add 10 to 20 μl of the concentrated dye (1 mg/ml) to the fibronectin solution. Close the tube and wrap with aluminum foil.
7. Incubate 1 hr at room temperature on an end-over-end mixer at ~8 rpm.

   For other ECM molecules, it is recommended that the reaction be incubated at 4°C for 2 hr.

8. After 1 hr, remove unreacted dye using either a desalt spin column or dye removal column and follow the manufacturer's protocol.

   Alternatively, gel filtration or dialysis of the unreacted dye can be performed.

USING MULTIPLE ECM PROTEINS WITH μPP

SUPPORT PROTOCOL 3

One advantage of μPP over other patterning techniques is the ability to repeat the process after an initial photoablation, quenching, ECM adsorption, and blocking. This allows placement of different ECMs within microns of each other at the subcellular levels (Fig. 10.15.23). It is crucial here to use 0.1% pluronic F-127 for protein dilution, as well as for rinsing steps to deter nonspecific protein adsorption. The following protocol details the process.

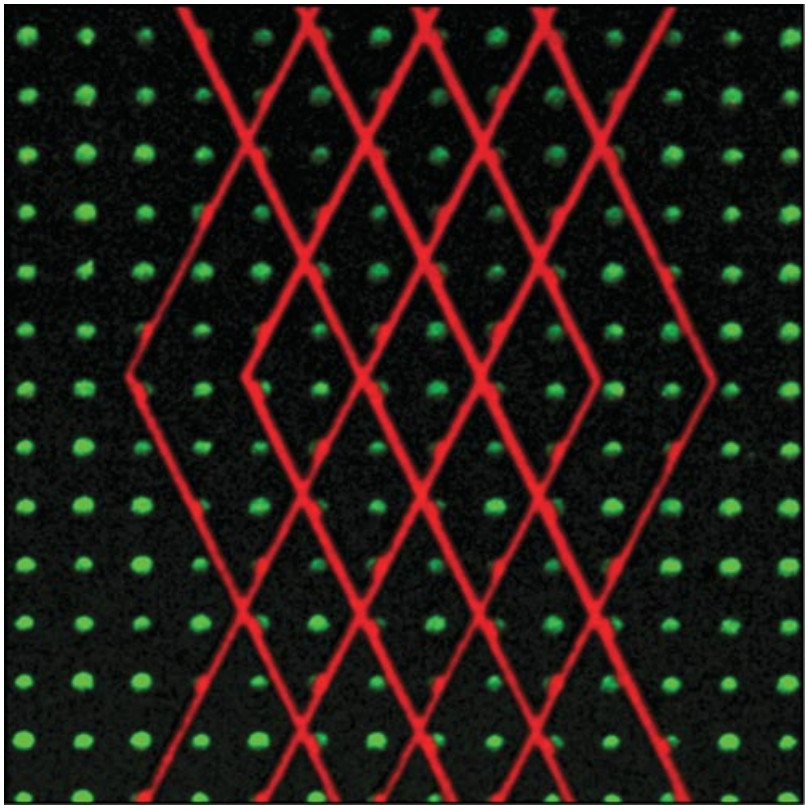


Figure 10.15.23 Dual ECM patterns by performing μPP twice in series. A first ablation was performed followed by quenching, ECM adsorption, and blocking. A second round of ablation was done is the presence of the second ECM. Green dots are fibrinogen and red lines are vitronectin. Dots are spaced 5-μm apart. For color version of this figure go to *http://www.currentprotocols.com/protocol.cb1015*.

Additional Materials *(also see Basic Protocol 3)*

Two different, fluorescently labeled ECM molecules/growth factors at the proper final concentration (user defined)
1% (w/v) heat-denatured BSA solution (prepare fresh and keep <1 day)
Phosphate-buffered saline (PBS) with 0.1% pluronic F-127 (see recipe)

PVA thin film–coated MatTek dishes (Basic Protocol 2)
Permanent marker
AIM software (Zeiss MicroImaging)

1. Prior to photoablation (Basic Protocol 3), mark a single side of a PVA thin film–coated dish along the edge of the attached coverslip.
2. Align the marked edge with the front of the microscope stage.
3. Proceed with rest of the photoablation procedure.

   This part can be automated.

4. Continue with the full coating, blocking, and cell plating process through Basic Protocol 5, substituting a fluorescently labeled protein during the adsorption step (step 4 of Basic Protocol 5).

   We suggest that this first labeled protein have a fluorophore in the visible range of emission, between 510 and 610 nm. Far red dyes such as Cy5, Alexa Fluor 633 and 647, or Dylight 649 are not recommended for this first stage.

5. After blocking the fluorescently labeled protein with 1% heat denatured BSA (step 14, Basic Protocol 5), perform a second photoablation, if desired.

   Here there are several options that may depend on the first adsorbed protein: (1) the surface of the film can be dried using compressed air immediately before photoablation, (2) the surface can be left in PBS, or (3) the second fluorescently labeled protein can be added to the surface. The photoablation process can still occur in solution (options 2 and 3); however, due to the presence of water, its efficiency can be reduced.

6. Align the marked side of the photoablated dish with the front of the stage. Use epifluorescence to scan the area(s) of the dish for the first photoablation site.

7. Once found, realign the dish to the best of your ability by hand. Fast XY scan the FOV using the most suitable configuration for the fluorophore used.

8. While scanning, make the fine alignment adjustments using the rotation dialog box in the Mode panel of the Scan control window. To help with this, open the Edit ROI window and choose the ROI template that was used to generate the first pattern. Leave the Edit ROI window open without selecting the ROI tab in the Scan control window, and continue scanning.

   This keeps the ROI template visible while scanning a full FOV.

9. Manually or using the Stage and Focus control window, align the ROI template over the fluorescent patterns. If using the same pattern, offset the template from the original position in either the *x* or *y* planes, or both.

   The amount of offset will depend on the original ROI.

10. Load the μPP photoablation configuration into the AIM software. Photoablate single FOVs at a time.

    We recommend this step not be automated.

11. Once finished, follow steps 6 through 8 in Basic Protocol 5, substituting PBS with 0.1% pluronic F-127 for PBS.

12. Following each protein added to the photoablated dish, block the surface with 1% heat-denatured BSA to prevent nonspecific protein attachment.

    Quenching with sodium borohydride is only required after the initial photoablation. Requenching will reduce fluorophore fluorescence and is not recommended.

13. Repeat the process (steps 3 through 5), if needed.

14. Plate cells on the surface as in steps 16 through 25 of Basic Protocol 5.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Borate buffer, 100 mM, pH 9.0

3.092 g boric acid (powder 99.5%; Sigma)
Add distilled water to 400 ml
Add several solid NaOH pellets at a time while mixing until the pH is ~9.0
Add distilled water to 500 ml
Filter sterilize using a 0.2-μm filter
Store up to 6 months at room temperature

Ethanolamine buffer, 200 mM

4.88 g ethanolamine hydrochloride ($EtOHNH_3$, crystalline form, 99%; Sigma)
Add sodium phosphate buffer (see recipe) to 250 ml
Filter sterilize using a 0.2-μm filter
Store up to 6 months at room temperature

NIH/3T3 fibroblast culture medium

440 ml Dulbecco's modified Eagle's medium (DMEM, high-glucose modified; Hyclone)
5 ml penicillin/streptomycin (10,000 U/μg per ml each, respectively; Invitrogen)
50 ml bovine calf serum (BCS; Hyclone)
Sterile filter using a 0.2-μm filter
Store up to 1 month at 4°C

Phosphate-buffered saline (PBS) with 0.1% pluronic F-127

199 ml phosphate-buffered saline (PBS; Hylcone)
1 ml of 20% pluronic F-127 in DMSO (Invitrogen)
Mix well on a stirplate
Sterile filter
Store up to 2 months at room temperature

Warming to ~37°C during mixing will help mix the solutions.

Phosphate-buffered saline (PBS) with penicillin/streptomycin and fungizone

490 ml DPBS/modified containing calcium and magnesium (Hyclone)
5 ml Amphotericin B (250 μg/ml; Invitrogen)
5 ml penicillin/streptomycin (10,000 U/μg per ml each, respectively; Invitrogen)
Mix and store up to 6 months at 4°C

Sodium borohydride solution

40 mg sodium borohydride ($NaBH_4$, hygroscopic powder; Sigma)
1 ml of 1 M NaOH (see recipe)
Mix well
Prepare fresh each time.

Sodium hydroxide, 1 M

20 g NaOH pellets
Distilled water to 500 ml
Mix well
Filter sterilize and store up to 6 months at room temperature

Sodium hydroxide solution, 200 mM

50 ml of 1 M NaOH solution (see recipe)
200 ml of distilled water
Mix well
Filter sterilize
Store up to 6 months at room temperature

Sodium phosphate buffer, 100 mM (pH 8.0)

6.90 g sodium phosphate monobasic (NaH_2PO_4)
Add distilled water to 400 ml
Add several solid NaOH pellets at a time while mixing until pH is ~8.0
Add distilled water to 500 ml
Filter sterilize using a0.2-μm pore-size filter
Store up to 6 months at room temperature

COMMENTARY

Background Information

Micropatterning using self-assembled monolayers

Micropatterning of ECM molecules originated by using self-assembled monolayers (SAMs) of alkanethiolates attached to gold-coated surfaces (Singhvi et al., 1994; Mrksich et al., 1996). The alkanethiol molecules consist of a sulfhydryl end terminal, a middle spacer, which is normally an ethylene-glycol backbone, and a head group that differs from the end terminal. Sulfhydryls or thiols have a high affinity for electron-dense gold and they bind, leaving the head groups pointing upward away from the gold surface. By changing the head group of the alkanethiol to a hydrophobic methyl (CH_3) or a hydrophilic hydroxyl (OH) group, the surface chemistry is altered; this will promote or deter ECM protein adsorption, respectively. Traditionally, in order to physically isolate hydrophobic from hydrophilic regions on a two-dimensional surface, a "rubber stamp" is generated that can physically ink the hydrophobic alkanethiol onto a gold surface. The remaining regions are backfilled with a hydrophilic alkanethiol, and finally an ECM protein can be added, which will only attach to the patterned hydrophobic regions of the surface. This process, known as microcontact printing (μCP), relies mostly on nanolithography techniques to generate a silicon "master" mold from which the polydemetylsiloxyane (PDMS) stamp is created (Singhvi et al., 1994).

Poly(vinyl) alcohol properties

As alluded to earlier, PVA is a highly hydrophilic polymer. It consists of a carbon backbone and hydroxyl groups located on every other carbon. PVA comes in varying molecular weights (mol. wt.), from as low as 6000 to >100,000 Da. PVA is generated from the hydrolysis of poly(vinyl) acetate. The percent hydrolysis that is listed with most PVAs defines the total amount of poly(vinyl) acetate hydrolyzed to PVA. The percent hydrolyzed should be as high as possible and is related to its hydrophilicity, with 98% to 99% being ideal for this application. With regards to the mol. wt., the larger the PVA monomer, the thicker the thin film becomes. We have found that using any of the molecular weights between 13,000 and 100,000 in a 5% solution can be used for μPP. Interestingly, after ablation of a high mol. wt. PVA film, the patterns remain visible via phase contrast and DIC imaging after submersion in buffer. This is not the case with 13,000 mol. wt. PVA, although labeling with fluorescent ECM proteins confirms proper local ablation (A.D.D., unpub. observ.). Because of this, low-molecular-weight PVA thin films are generally better for higher resolution fluorescence microscopy, and high mol. wt. PVA is helpful for visualizing the ECM patterns at lower magnifications.

Photoablation with two-photon microscopy

The process of photoablation is based on the ability of the PVA polymer to absorb light in the ultraviolet (UV) range (100 to 380 nm; Matsumoto et al., 1958). Other large polymers that have the ability to form a hydrogel such as polyacrylamide and polyethylene glycol can undergo photolytic degradation (Chen et al., 2003; Yamato et al., 2003). Two-photon femto-second pulse lasers mimic UV wavelengths using 720- to 760-nm light, and can excite UV-based fluorophores such as DAPI, coumarin, and Hoechst. For more information on properties and the process of two-photon excitation and confocal microscopy, we suggest reviewing *UNITS 4.5 & 4.11* on confocal and two-photon excitation microscopy, respectively. Absorption of UV light can initially result in polymerization of many polymer solutions (Du, 2007). However, continued exposure can disrupt primary bonds; in PVA's case the –OH bond to the carbon backbone. Further exposure results in a breakdown of the carbon backbone itself. With μPP, we use this property of PVA to locally breakdown or ablate the thin film, exposing the underlying glass to which ECM proteins can later be adsorbed.

Critical Parameters and Troubleshooting

The key elements during the multi-step μPP process requiring consideration are: (1) the PVA conjugation to the glass surface, (2) how the PVA thin film is stored, and (3) several parameters associated with the two-photon laser for proper photoablation. Many other troubleshooting tips are found throughout the text, where they are directly pertinent to the protocols.

Improper cleaning and/or activation of the glass surface can result in thin film detachment. Checking dishes for debris during each phase of the processing is important and should not be overlooked.

The PVA thin films need to be hydrated. PVA hydrogels can undergo crystallization

when dehydrated (Peppas and Merrill, 1977). While this process greatly increases their tensile strength, it greatly reduces their hydrophilicity from removal of hydroxyl groups and, hence, PVA's ability to deter protein adsorption and cell attachment is compromised. This will have no effect on the photoablation process, however. Critical times where dehydration can greatly affect the thin film are initially after spincoating, when the macromolecular monolayer may still be reacting with the activated glass surface. Short-term exposure to dry conditions such as during the photoablation on the microscope is tolerated.

Many issues stem from proper maintenance of the two-photon laser. We found that the amount of fluorescently labeled fibronectin attached to a given area is highly dependent on the total amount of light energy reaching the PVA thin film (Doyle et al., 2009). Small alignment issues of the two-photon source with the confocal scan head will greatly reduce the light throughput to your sample, and can result in improper photoablation within the whole field of view or just a part of it. If the two-photon system is heavily used by multiple users, more frequent mirror alignments should be performed. Other issues with uneven photoablation can arise from focus drift, bubbles in the immersion oil, and an uneven FOV. Another issue to factor into proper photoablation is the tuning of the TP laser. Technically speaking, the two-photon absorption of a given wavelength should be the same between different two-photon sources. However, there can be variation in the best or most suitable wavelength to maximize wavelength absorption. As it is recommended by most experts, you should test several wavelengths until the best one is found for your particular two-photon source to illuminate, or in this case photoablate, your sample. Starting with 755 nm, tune the Ti:Sapphire laser up or down ten nanometers at a time. Photoablate a simple pattern such as a field of same-sized dots at the particular wavelength, and then document the time taken and whether the ablation was efficient (complete removal of the PVA thin film) or not (only partial removal). Partial photoablation of patterns, where the PVA thin film is not completely removed from the glass surface, can lead to an inability of ECM protein adsorption and, hence, can affect cell attachment and/or migration.

Anticipated Results

It is expected that after generating the PVA thin film dishes and creation of the configurations and ROI templates, you should be able to generate micropatterns to which ECM or other proteins of interest can readily adsorb. Once patterns have been produced, you should find that cells should readily attach to patterns, especially linear structures (lines and lanes). There should be limited autofluorescence from the dish surface and all fluorescence microscopy techniques, from TIRF to spinning-disk confocal and two-photon confocal microscopy, should be effortlessly performed.

Time Considerations

As described in the Strategic Planning section, the many different parts of μPP can be performed not only on separate days, but weeks, if not months, apart between glass activation (Basic Protocol 1) and ECM adsorption/cell attachment (Basic Protocol 5). It is best to plan accordingly. For example, activating 30 dishes or more depending on your usage should be enough for 4 weeks. However, it is not prudent to continue all 30 dishes through the thin film deposition stage (Basic Protocol 2), unless you can process 30 dishes in a single week. Furthermore, how many of the 30 dishes can be used in a 1 to 3 day period after ECM adsorption needs also to be considered. Preplanning for dish need and usage will decrease your issues at key steps. One important time consideration is between formation of the PVA thin film through spincoating and the addition of the ethanolamine buffer after photoablation. Although as rare as this would occur, a short time (<2 hr) between these two steps could cause release of the thin film from the glass surface due to a lack of covalent attachment. Hence, the ethanolamine buffer should be added as soon as practical after spincoating.

Literature Cited

Chen, S., Kancharla, V.V., and Lu, Y. 2003. Laser-based microscale patterning of biodegradable polymers for biomedical applications. *Int. J. of Material & Product Technol.* 18:457-468.

Doyle, A.D., Wang, F.W., Matsumoto, K., and Yamada, K.M. 2009. One-dimensional topography underlies three-dimensional fibrillar cell migration. *J. Cell. Biol.* 184:481-490.

Du, J.Z., Sun, T.M., Weng, S.Q., Chen, X.S., and Wang, J. 2007. Synthesis and characterization of photo-cross-linked hydrogels based on biodegradable polyphosphoesters and poly(ethylene glycol) copolymers. *Biomacromolecules* 8:3375-3381.

Hermanson, G.T. 1996. Bioconjugate Techniques. 1st ed. Academic Press, San Diego, Calif.

Lehnert, D., Wehrle-Haller, B., David, C., Weiland, U., Ballestrem, C., Imhof, B., and Bastmeyer, M. 2004. Cell behavior on micropatterned substrata: Limits of extracellular matrix geometry for spreading and adhesion. *J. Cell Sci*. 117:41-52.

Matsumoto, M., Imai, K., and Kazusa, Y. 1958. Ultraviolet spectra of polyvinyl alcohol. *J. Polymer Sci*. 117:426-428.

Mrksich, M., Chen, C.S., Xia, Y., Dike, L.E., Ingber, D.E., and Whitesides, G.M. 1996. Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold. *Proc. Natl. Acad. Sci. U.S.A.* 93:10775-10778.

Peppas, N.A. and Merrill, E.W. 1977. Development of semicrystalline poly(vinyl alcohol) hydrogels for biomedical applications. *J. Biomed. Mater. Res*. 11:423-434.

Singhvi, R., Kumar, A., Lopez, G.P., Stephanopoulos, G.N., Wang, D.I., Whitesides, G.M., and Ingber, D.E. 1994. Engineering cell shape and function. *Science* 264:696-698.

Yamato, M., Konno, C., Koike, S., Isoi, Y., Shimizu, T., Kikuchi, A., Makino, K., and Okano, T. 2003. Nanofabrication for micropatterned cell arrays by combining electron beam-irradiated polymer grafting and localized laser ablation. *J. Biomed. Mater. Res. A* 67:1065-1075.

Preparation of Hydrogel Substrates with Tunable Mechanical Properties

Justin R. Tse[1] and Adam J. Engler[1]

[1]Department of Bioengineering, University of California, San Diego, La Jolla, California

ABSTRACT

The modulus of elasticity of the extracellular matrix (ECM), often referred to in a biological context as "stiffness," naturally varies within the body, e.g., hard bones and soft tissue. Moreover, it has been found to have a profound effect on the behavior of anchorage-dependent cells. The fabrication of matrix substrates with a defined modulus of elasticity can be a useful technique to study the interactions of cells with their biophysical microenvironment. Matrix substrates composed of polyacrylamide hydrogels have an easily quantifiable elasticity that can be changed by adjusting the relative concentrations of its monomer, acrylamide, and cross-linker, bis-acrylamide. In this unit, we detail a protocol for the fabrication of statically compliant and radial-gradient polyacrylamide hydrogels, as well as the functionalization of these hydrogels with ECM proteins for cell culture. Included as well are suggestions to optimize this protocol to the choice of cell type or stiffness with a table of relative bis-acrylamide and acrylamide concentrations and expected elasticity after polymerization. *Curr. Protoc. Cell Biol.* 47:10.16.1-10.16.16. © 2010 by John Wiley & Sons, Inc.

Keywords: extracellular matrix • elasticity • hydrogel • gradient • polyacrylamide

INTRODUCTION

The extracellular matrix (ECM) provides both mechanical support for surrounding cells and a variety of biochemical and biophysical signals that influence cellular behavior. These are largely the result of the ECM composition that includes adhesive glycoproteins, fibrous matrix proteins, proteoglycans, and glycosaminoglycans (Badylak, 2005). These signals are coupled in the body and together they create a 3-dimensional microenvironment for cell growth (Cukierman et al., 2001). In particular, the modulus of elasticity E, or "stiffness," is a characteristic of the ECM that certain anchorage-dependent cells can sense and respond to with a variety of cellular processes (Discher et al., 2005). E of a material represents the intrinsic resistance of organs and tissues to stress, and in its simplest mathematical form can be expressed as the tensile stress, σ, or force applied per unit area, divided by the resultant strain, ε, or relative change in length (Discher et al., 2005). Though highly nonlinear (Fung, 1993), at the physiologically appropriate strains, the degree of stiffness varies dramatically between tissues: brain (E_{brain} ~0.1-1 kPa) is clearly softer than striated skeletal muscle (E_{muscle} ~8-17 kPa), which is less stiff than precalcified bone ($E_{precalcified\ bone}$ = 25-40 kPa; Engler et al., 2006). At the cellular level, such changes in substrate elasticity have been observed to influence several cellular behaviors, including cell proliferation, locomotion, adhesion, spreading, morphology, striation, and even differentiation of stem cells (Pelham and Wang, 1997; Wang et al., 2000; Flanagan et al., 2002; Engler et al., 2004b, 2006; Khatiwala et al., 2006; Reinhart-King et al., 2005; Discher et al., 2009). That said, these properties are not static within the body; they are often displayed in highly complex gradients, such as those of stiffness at tissue interfaces. In vitro, the variation of E across a substrate has been shown to elicit directly cell migration or "durotaxis" in fibroblasts and vascular smooth muscle cells (Lo et al., 2000; Wong et al., 2003), indicating again that intrinsic matrix properties can influence cell behavior. Moreover, they are coupled, meaning that both mechanics and biochemical signals can

Current Protocols in Cell Biology 10.16.1-10.16.16, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1016s47

Table 10.16.1 Expected Modulus of Elasticity after Polymerization of Relative Acrylamide and Bis-Acrylamide Concentrations[a]

Acrylamide %	Bis-acrylamide%	Acrylamide from 40% stock solution (ml)	Bis-acrylamide from 2% stock solution (ml)	Water (ml)	$E \pm$ St. Dev. (kPa)
3	0.03	0.75	0.15	9.1	0.20 ± 0.03
3	0.06	0.75	0.3	8.95	0.48 ± 0.16
3	0.1	0.75	0.5	8.75	1.10 ± 0.34
3	0.15	0.75	0.75	8.5	1.37 ± 0.22
3	0.225	0.75	1.125	8.125	1.67 ± 0.14
3	0.3	0.75	1.5	7.75	1.78 ± 0.19
4	0.03	1	0.15	8.85	0.71 ± 0.24
4	0.06	1	0.3	8.7	1.16 ± 0.54
4	0.1	1	0.5	8.5	2.01 ± 0.75
4	0.15	1	0.75	8.25	2.55 ± 0.17
4	0.225	1	1.125	7.875	3.13 ± 0.42
4	0.3	1	1.5	7.5	3.24 ± 0.58
5	0.03	1.25	0.15	8.6	1.00 ± 0.31
5	0.06	1.25	0.3	8.45	1.80 ± 0.44
5	0.1	1.25	0.5	8.25	3.15 ± 0.85
5	0.15	1.25	0.75	8	4.47 ± 1.19
5	0.225	1.25	1.125	7.625	8.44 ± 0.82
5	0.3	1.25	1.5	7.25	8.73 ± 0.79
8	0.048	2	0.24	7.76	2.61 ± 0.82
8	0.264	2	1.32	6.68	19.66 ± 1.19
8	0.48	2	2.4	5.6	40.40 ± 2.39
10	0.03	2.5	0.15	7.35	2.83
10	0.06	2.5	0.3	7.2	7.43
10	0.1	2.5	0.5	7	10.61
10	0.15	2.5	0.75	6.75	16.70
10	0.225	2.5	1.125	6.375	23.43
10	0.3	2.5	1.5	6	34.88

[a]This table shows the relative concentrations of acrylamide and bis-acrylamide and their expected modulus of elasticity after polymerization in water. Acrylamide and bis-acrylamide can be kept premixed in solution for weeks to months at 4°C. At least 3 hydrogels per sample and 50 indentations per hydrogel were assessed for these measurements.

jointly influence cells, e.g., stem cells differentiate in response to an appropriately compliant material, but only when coupled to the right matrix ligand (Rowlands et al., 2008).

To study these affects in isolation and together, this unit describes the fabrication of hydrogel substrates of a defined *E* for the culture of cells. Basic Protocol 1 describes procedures for fabricating hydrogels of a constant *E*, termed statically compliant hydrogels, and of a gradient *E*, termed radial-gradient hydrogels, onto glass coverslips from non-biodegradable polyacrylamide. Basic Protocol 2 describes a technique to covalently coat the matrix substrate with an ECM protein, while the Support Protocol describes how to create an apparatus to make radial-gradient hydrogels. Table 10.16.1 provides expected *E* values after polymerization of various ratios of acrylamide and bis-acrylamide solutions.

FABRICATION OF STATICALLY COMPLIANT MATRIX SUBSTRATES OF VARYING STIFFNESS

BASIC PROTOCOL 1

Several different types of polymers, including both nonbiodegradable and biodegradable types, can be used to fabricate matrix substrates of varying stiffness. Here, we introduce a simple method to create matrix substrates from polyacrylamide (PA) with a tunable elasticity. Besides producing statically compliant hydrogels, this technique can be adapted to produce hydrogels with a gradient of elasticity (Alternate Protocol). PA offers several important advantages for its application as a biomaterial for cell culture. (1) The modulus of elasticity of the substrate can be varied by changing relative concentrations of acrylamide and bis-acrylamide (Pelham and Wang, 1997). (2) The surface chemistry of PA can be kept constant while changing its mechanical properties (Pelham and Wang, 1997; Khatiwala et al., 2006), a characteristic whose importance is highlighted by a study demonstrating the dependence of cell spreading on ligand density (Engler et al., 2004a). (3) PA is generally nonfouling, meaning that the adsorption of serum proteins or the nonspecific binding of cell surface receptors is typically negligible. As a result, only adhesive molecules chosen to be covalently attached to the surface of the gel can serve as ligands for cell attachment (Georges and Janmey, 2005). (4) The pore sizes of the gel are on the order of 100 nm, preventing cells (~15 μm in diameter) and their extensions, such as growth cones (~5 μm in diameter) from entering the substrate (Flanagan et al., 2002). (5) Immunofluorescence is made possible at high magnifications because of the thin, translucent quality of PA gels.

PA gels are produced in this protocol by mixing various acrylamide and bis-acrylamide concentrations and inducing free radical polymerization. A table of expected modulus of elasticity values given the input concentrations of acrylamide and bis-acrylamide is provided (Table 10.16.1), while we also include a plot in Figure 10.16.3 that presents the spatial elasticity gradient possible using the gradient gel method detailed here. PA gel modulus of elasticity was quantified using atomic force microscopy (AFM), which is a nano-indentation method of calculating elasticity. This technique has been extensively detailed elsewhere (Rotsch et al., 1999; Rotsch and Radmacher, 2000).

Materials

0.1 M NaOH
Distilled H_2O
3-Aminopropyltriethoxysilane (APES)
0.5% (v/v) glutaraldehyde in phosphate-buffered saline (PBS; Cellgro, cat. no. 46-013-CM)
Dichlorodimethylsilane (DCDMS)
40% (w/v) acrylamide stock solution (Sigma-Aldrich, cat. no. A4058)
2% (w/v) bis-acrylamide stock solution (Sigma-Aldrich, cat. no. M1533)
Phosphate-buffered saline (PBS), optional
Tetramethylethylenediamine (TEMED)
10% (wv) ammonium persulfate (APS)

25-mm circular coverslips (for 6-well plate)
Hot plate
35-mm petri dish(es)
Kimwipes
25 × 75–mm glass slides
Vacuum desiccator
Vortex mixer
6-well plate, optional

Prepare amino-silanated coverslip(s)

1. Place 25-mm coverslip(s) on a hot plate and add 500 µl of 0.1 M NaOH to the coverslip so that the solution covers the entire glass surface.

2. Heat the coverslip with solution at ~80°C until the liquid is evaporated.

   The solution should not boil, and there should be a thin semi-transparent film of NaOH remaining on the coverslip(s) after evaporation.

3. Repeat step 1 by diluting the NaOH by adding 500 µl of distilled H_2O to the coverslip and heating the solution at 80°C until the film of NaOH is uniform.

   This step should be performed if and only if steps 1 and 2 resulted in a non-uniform film.

   A uniform film of NaOH is important for uniform gel attachment.

4. Place coverslip(s) in a nitrogen-filled tent. Add 200 µl of APES to the surface of the coverslip(s). Allow 5 min for the APES to react.

   If a nitrogen tent is unavailable, this step can be done in the fume hood. Since APES will react with the oxygen in the air, use 250 µl of APES to compensate for the loss of reactivity. A thin film will likely result on the surface of the APES solution and additional washing cycles in step 6 may be necessary to remove it.

5. Rinse the coverslip(s) with distilled H_2O under the distilled H_2O tap to ensure both the top and bottom of the coverslip(s) is rinsed.

   It is important to completely rinse off the unreacted APES, for it will create an orange-brown precipitate with glutaraldehyde (see step 8) that fluoresces under UV light and can thus interfere with immunostaining techniques.

6. Place the coverslip(s) in distilled H_2O into a petri dish and rinse the coverslips twice, each time in ~10 ml (or enough to immerse the coverslip) distilled water for 5 min each.

7. Aspirate the second distilled H_2O wash solution and add ~10 ml (or enough to immerse the coverslip) of 0.5% glutaraldehyde in PBS. Let the solution stand for 30 min.

8. Aspirate the solution and dry the coverslips with a Kimwipe, by allowing the coverslips to dry naturally in air, or by blowing nitrogen on them.

   The amino-silanated coverslips remain viable for 48 hr. However, to prepare radial-gradient hydrogels, it is best to use the amino-silanated coverslips immediately after they are created to ensure uniform gel attachment.

Prepare chloro-silanated glass slide(s)

9. Using separate glass slides, spread about 100 µl of DCDMS onto each slide in the fume hood. Ensure that the solution coats the entire surface of the slides. Allow to react for up to 5 min before removing the excess DCDMS with a Kimwipe and rinse 1 min under distilled H_2O.

Prepare statically compliant hydrogel(s)

10. Mix acrylamide and bis-acrylamide to their desired concentrations in either distilled H_2O or PBS.

    See Table 10.16.1 for concentrations and corresponding elastic modulus.

    The elastic moduli will be slightly lower if the solutions are made in water, due to gel swelling when placed in cell culture media. This effect can be directly measured by AFM or other mechanical techniques.

    Acrylamide and bis-acrylamide can be kept together in solution for weeks to months, though the sterility of the stock solution should be closely maintained by filter sterilization.

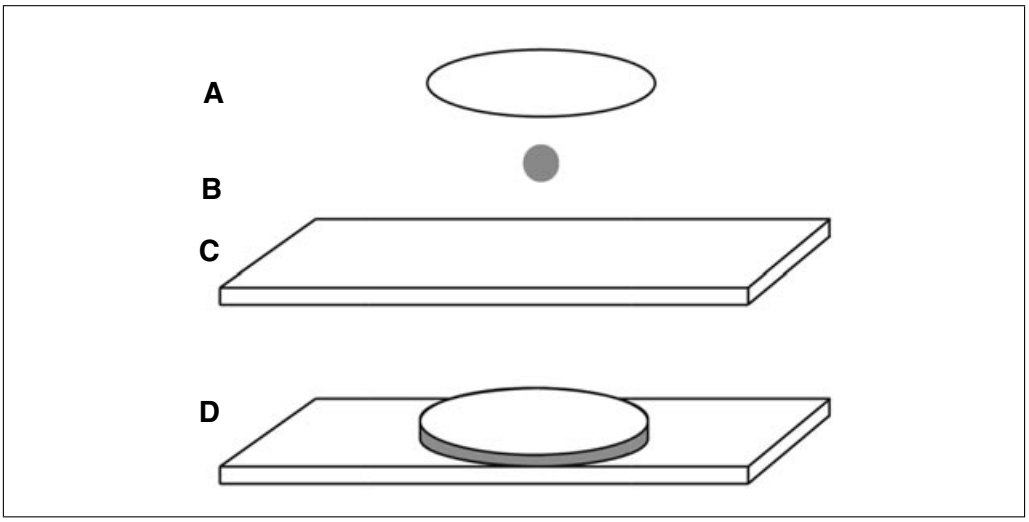


Figure 10.16.1 Exploded schematic of the setup for statically compliant PA hydrogels. The gel-glass composite includes (**A**) the amino-silanated coverslip, (**B**) polymerizing solution, and (**C**) chloro-silanated glass slide (C). (**D**) The completed setup is shown.

If not sterile, contaminants from the gel could adversely affect cell culture and induce infections.

11. Degas the mixture under strong vacuum for 15 min to exhaust the solution of dissolved oxygen.

    Dissolved oxygen in the solution will act as a sink for the subsequent free radical polymerization. Degassing the solution not only speeds up polymerization but ensures more uniform polymerization as well.

12. Add 1/100 total volume of APS and 1/1000 total volume of TEMED to gel solutions.

13. Vortex the polymerizing solution.

14. Quickly pipet 25 µl of the gel solution (see Fig. 10.16.1B) onto the treated side of the chloro-silanated glass slide(s) (see Fig. 10.16.1C) and add the amino-silanated coverslip(s) with the treated side down (see Fig. 10.16.1A).

    See Figure 10.16.1D for how the completed glass-gel-coverslip composite should appear.

    Essentially, the setup resembles a "sandwich" in which the polymerizing solution sits in between the chloro-silanated glass slide and the amino-silanated coverslip.

15. Allow the gel to polymerize for 5 to 30 min. Monitor the unused solution to determine when the solution is fully polymerized.

    Shorter polymerization times may result in insufficient polymerization of all available monomers and may cause the mechanical properties of the hydrogels to vary from the values noted here.

16. Remove the bottom glass slide and discard. Place the top coverslip-gel composite in a 35-mm petri dish or 6-well plate in PBS or dH_2O depending on what was used to dilute the acrylamide. Make sure that the gel-coated side faces up.

17. To remove unpolymerized acrylamide rinse twice, each time for 5 min in PBS or distilled H_2O depending on what was used to dilute the acrylamide.

    These hydrogels can be stored for long periods of time without losing any of their mechanical properties. To store them, immerse the hydrogels in water or PBS to keep them hydrated and store them at 4°C.

ALTERNATE PROTOCOL

FABRICATION OF RADIAL-GRADIENT MATRIX SUBSTRATES OF VARYING STIFFNESS

Radial-gradient hydrogels can be fabricated by photoinitiated polymerization as this technique allows for spatial control over the mechanical properties of the material. Among the commercially available photoinitiators, 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone, also known as Irgacure 2959, is commonly used for gel encapsulation and other photopolymerization reactions (Fedorovich et al., 2009). However, there are several limitations to using Irgacure 2959 that affect the maximum attainable polymerization rate, including its low solubility in water and its relatively narrow excitation spectrum. Nevertheless, we found that Irgacure 2959 as a photoinitiator is sufficient in producing spatial gradients of stiffness for our hydrogels. A detailed study on the kinetic properties of Irgacure 2959 and a novel photoinitiator that can be applied for this technique can be found in Fairbanks et al. (2009).

In this polymerization system, UV light of the appropriate wavelength catalyzes the free radical polymerization of acrylamide and bis-acrylamide by cleaving the Irgacure 2959 molecules into free radicals. While the mechanism for polymerization is similar to that of the fabrication of statically compliant hydrogels, UV light and Irgacure 2959 serve as the catalyst and the free-radical donor for the reaction, respectively, instead of TEMED and APS. The photomask filters the exposure of the UV light and creates a gradient of UV light intensity, which will result in a spatial gradient of polymerization rate. Because the polymerization reaction is stopped before completion by removing the UV light source, a polymer with a gradient of cross-linking density, or elasticity, is created through this process.

While the fabrication of statically compliant PA hydrogels is relatively straightforward, there are many parameters that need to be adjusted before the gradient gel can be created. These parameters include UV intensity, wavelength, and exposure time as well as distance from the UV light source, photoinitiator concentration, gel thickness, photomask grayscale, and acrylamide and bis-acrylamide concentration. Because of variations in the UV light source and photomask, several trials by the user are necessary optimize the protocol. It is recommended to only vary the UV exposure time and the acrylamide and bis-acrylamide concentrations while keeping the other parameters constant.

Materials

40% (w/v) acrylamide stock solution (Sigma-Aldrich, cat. no. A4058)
2% (w/v) bis-acrylamide stock solution (Sigma-Aldrich, cat. no. M1533)
Distilled water *or* phosphate-buffered saline (PBS; Cellgro, cat. no. 46-013-CM)
Irgacure 2959 (Ciba)

Vacuum desiccator
Gradient gel apparatus
Photomask
254-nm UV light source
Chloro-silane-treated 25 × 75–mm glass slides (Basic Protocol 1)
APES-coated glass coverslips (Basic Protocol 1)
35-mm petri dish(es) *or* 6-well plates

1. Mix acrylamide and bis-acrylamide to their desired concentrations in distilled H_2O or PBS.

   See Table 10.16.1 for concentrations and corresponding elastic modulus. The corresponding elastic modulus represents the maximum elastic modulus for the gradient gel. In our setup, we used a concentration of 10% acrylamide and 0.3% bis-acrylamide to obtain a gradient with a range of ~1 kPa to 14 kPa. Often, the range of the gradient is limited by the diffusion of acrylamide monomers during polymerization. These concentrations can be adjusted to obtain a desired range. However, some optimization will be necessary.

2. Add Irgacure 2959 at a concentration of 0.5% (w/v).

   It is best to use this solution immediately after mixing and to remake a new solution for each batch of gels because the initiator, Irgacure 2959, reacts slowly with the acrylamide and bis-acrylamide due to incidental light exposure.

   Irgacure 2959 is relatively insoluble in water; the solution can be placed in a warm water bath to speed up dissolving.

   As with the statically compliant gels, sterility is important for longer-term culture. Filter sterilization of all solutions is recommended.

3. Degas the mixture under strong vacuum for 60 min to speed up polymerization.

   Because the polymerization time is a function of both the amount of UV exposure and the concentration of dissolved gas in solution, it is important to remove all dissolved gases that may limit the free radical polymerization.

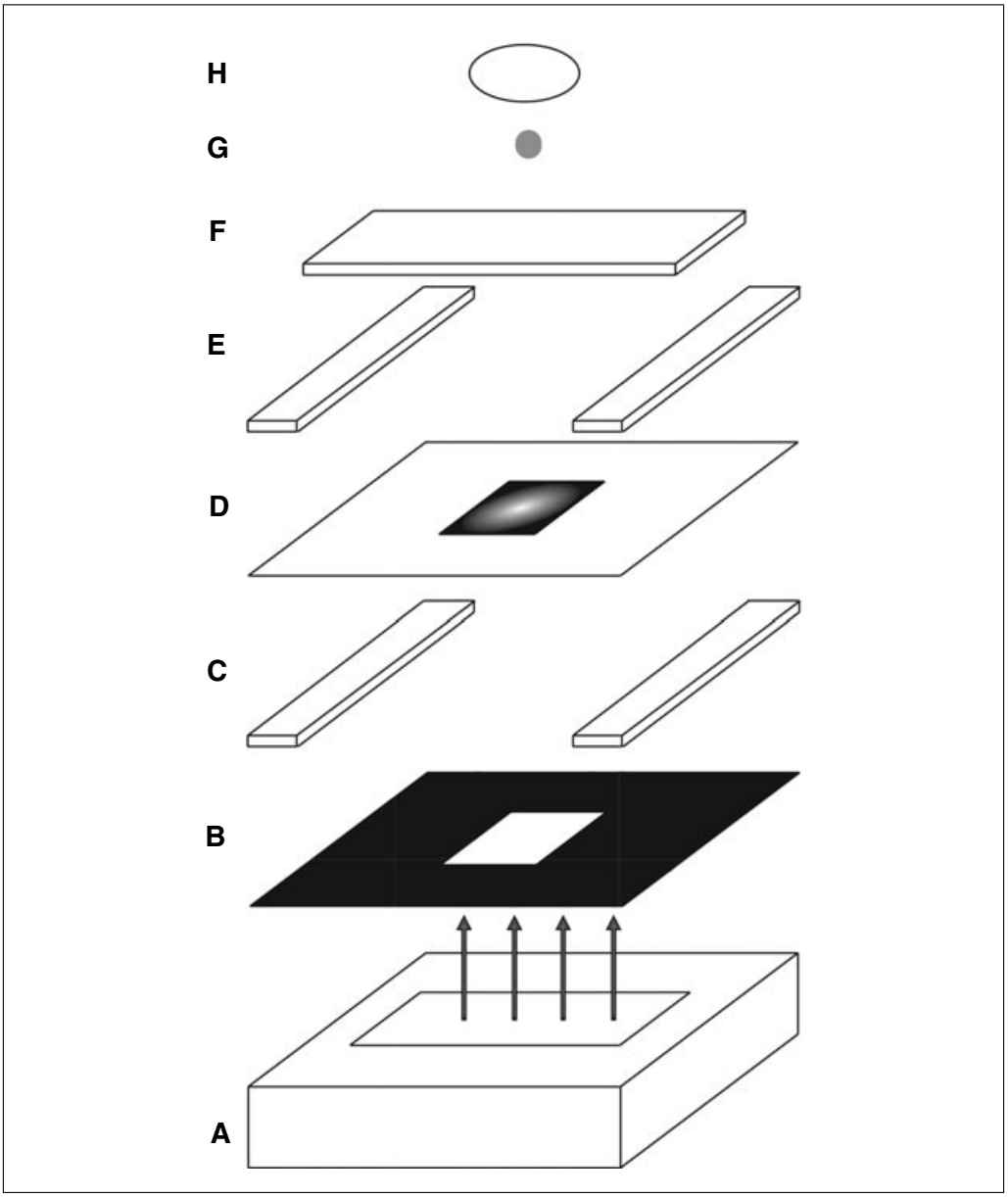


Figure 10.16.2 Exploded schematic of the setup for radial-gradient hydrogels. The setup includes (**A**) the UV light box, (**B**) negative photomask, (**C** & **E**) spacers, (**D**) photomask, (**F**) chloro-silanated glass slide, (**G**) polymerizing solution, and (**H**) amino-silanated coverslip.

4. Set up in the gradient-gel apparatus (Support Protocol 1) by placing the negative photomask on the UV light box (see Fig. 10.16.2A and B), the photomask on top of the UV light box (see Fig. 10.16.2D), and the chloro-silanated glass slide on top of the photomask (see Fig. 10.16.2D). Use spacers as necessary to separate each component (see Fig. 10.16.2C and E).

   The distance between the photomask and the chloro-silanated glass slide should be minimized to ensure the greatest resolution. For our setup, the distance was kept within 1 mm.

5. Pipet 30 µl of the gel solution (see Fig. 10.16.2G) onto the treated side of the chloro-silanated glass slide (see Fig. 10.16.2F) as set up in the gradient-gel apparatus (Support Protocol 1) and add the amino-silanated coverslip (see Fig. 10.16.2H) with the treated side down.

   To maintain the same approximate gel thickness as that of the statically compliant hydrogels, slightly more gel solution is added to compensate for the incomplete polymerization that takes place at the edge of the gels.

   It is possible to make thicker gels by increasing the volume of gel solution added to the chloro-silanated glass slide. However, the thickness of the gel introduces another parameter to the gradient elasticity as thicker gels allow greater diffusion of monomers in the x-y plane during polymerization, effectively decreasing the range of stiffness.

6. Turn on the UV light for the desired exposure time.

   The amount of UV light exposure can be varied to produce a desired gradient range. Since UV intensity, distance, photomask quality, and other parameters differ for each setup, it is important to optimize this parameter to produce the desired range. For our setup and gradient range, we used a polymerization time of 3 min 30 sec.

   Fig. 10.16.3 shows the spatially elastic gradient that can be obtained by using this technique.

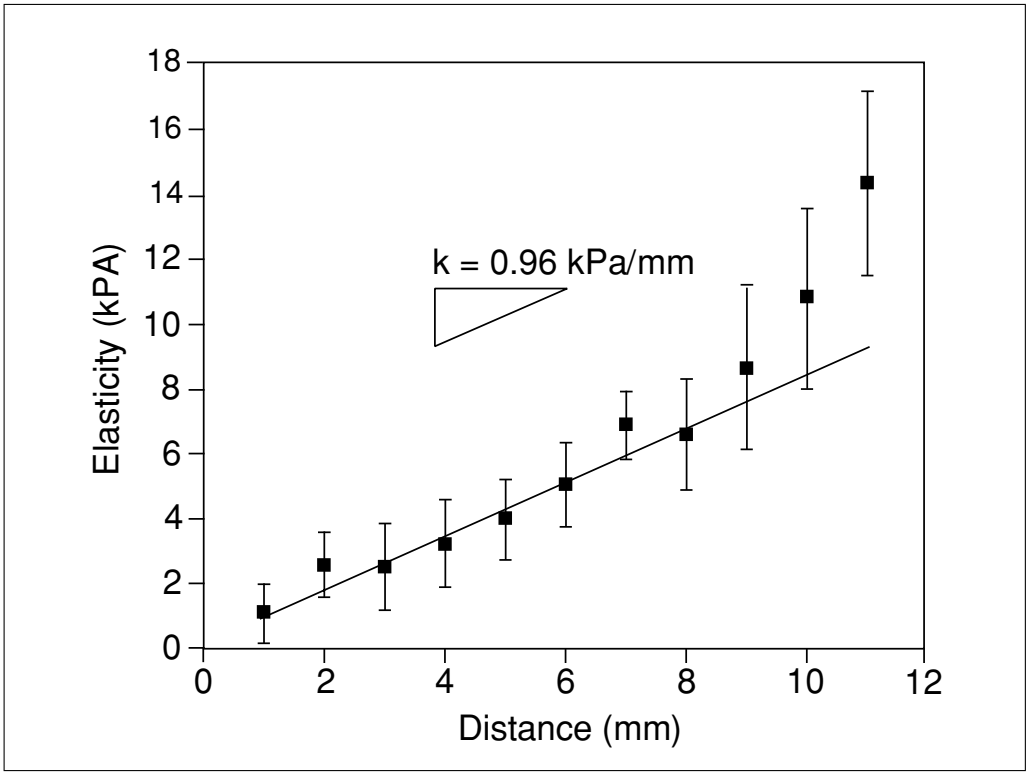


Figure 10.16.3 Spatial map of the elasticity (kPa) versus distance from the center of the gel (mm) characterized by AFM that can be obtained by fabricating the radial-gradient hydrogel detailed in the Alternate Protocol. The PA hydrogels represented in the figure were cast on coverslips of 25-mm diameter.

7. Remove the bottom glass slide and discard. Place the top coverslip-gel composite in a 35-mm petri dish or 6-wll plate in PBS or distilled H_2O depending on what was used to dilute the acrylamide. Make sure that the gel-coated side faces up.

8. Rinse twice to remove unpolymerized acrylamide with 2 ml PBS or distilled H_2O depending on what was used to dilute the acrylamide.

These hydrogels can be stored for long periods of time without losing any of their mechanical properties. To store them, immerse the hydrogels in water or PBS to keep them hydrated and store them at 4°C.

PREPARATION OF MATRIX PROTEIN SUBSTRATES OF VARYING STIFFNESS FOR CELL CULTURE

BASIC PROTOCOL 2

For many synthetic materials, before cell attachment can take place, an ECM protein coating is needed to promote adhesion. However, polyacrylamide hydrogels do not readily adsorb proteins, and thus it is essential to covalently bind proteins to the gel to ensure efficient cell attachment (Pelham and Wang, 1997). In this protocol, sulfo-SANPAH, a heterobifunctional protein cross-linker, is used to covalently bind proteins to the polyacrylamide substrates. Sulfo-SANPAH contains a nitrophenylazide group that is most photoreactive at wavelengths of 320 to 350 nm toward polyacrylamide. Exposure of the gel in a solution of Sulfo-SANPAH with a UV light source at 365 or 320 nm covalently links the sulfo-SANPAH to the polyacrylamide hydrogel. The *N*-hydroxysuccinimide ester in sulfo-SANPAH can then react with the primary amines of proteins to complete the attachment of proteins to the surface of the gel. Figure 10.16.4 shows a schematic of the steps in the functionalization of the PA hydrogel. Although not detailed here, an alternative conjugation method would be to incorporate 6-((acryloyl)amino) hexanoic acid into the gel solution of Basic Protocol 1 as it polymerizes. Reinhart-King and coworkers have successfully employed such a technique where the hexanoic acid copolymerizes into the gel and displays an *N*-hydroxysuccinimide ester for amine-containing protein binding (Reinhart-King et al., 2008). Carbodiimide has also been employed, though details may be found elsewhere (Grabarek and Gergely, 1990).

One of the concerns with ECM protein coatings is the variability of the ligand density among the different substrates and homogeneity of the ligand coating. With regards to this protocol, data suggests that the ligand is the limiting reagent in this reaction, and thus, the surface density of the ligand can be varied by changing the concentration of the ligand (Gaudet et al., 2003). This directly affects key cellular properties, e.g. cell spreading (Engler et al, 2004a). On the other hand, the surface chemistry of the matrix stays constant while the mechanical properties of the substrate are varied. Pelham and Wang (1997) report that the relative surface concentration of collagen varied $<3\%$ among the substrates, regardless of the modulus of elasticity, and several other studies confirm this observation (Wang et al., 2000; Khatiwala et al., 2006; Rowlands et al., 2008). Importantly, two gels of differing acrylamide/bis-acrylamide concentration but similar mechanical properties can bind similar amounts of matrix protein. To optimize the hydrogel coating protocol to the end user's specific proteins and conditions, such a check is necessary. Verification schemes used by Pelham and Wang (1997), as well as other groups, to confirm that relatively uniform protein attachment occurred, are also detailed here.

Finally, there are several choices of ECM proteins for PA hydrogel conjugation. Common ECM proteins include collagen I, collagen IV, laminin, and fibronectin. While these proteins can effectively promote cell attachment, the choice of the ECM protein is important as both integrin signaling and substrate elasticity are cues that affect cell

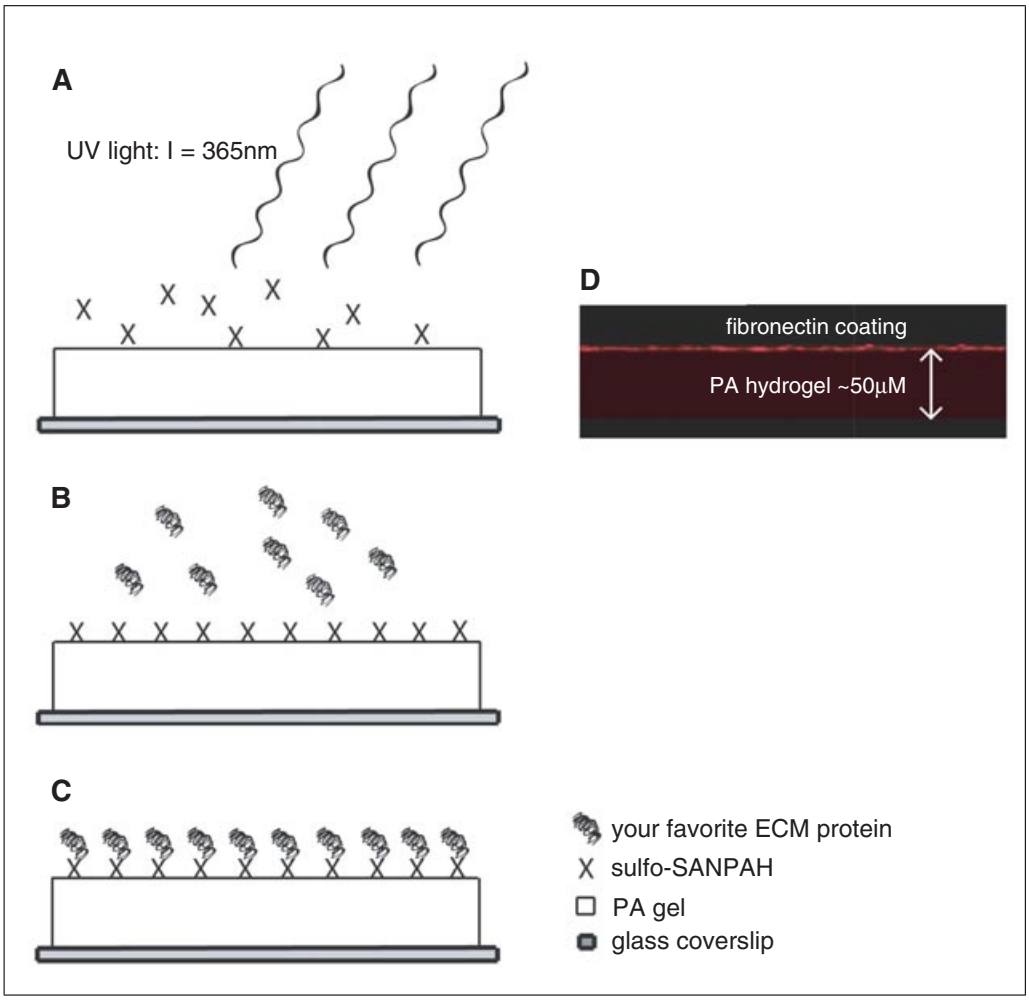


Figure 10.16.4 Schematic of the functionalization procedure for PA hydrogels. (**A**) The surface becomes activated upon addition of sulfo-SANPAH to the PA hydrogel, a reaction catalyzed with 365-nm UV light. (**B**) Overnight attachment of your favorite ECM protein in a 50 mM HEPES solution, pH 8.5. (**C**) Completed functionalization of your favorite ECM protein to the PA hydrogel. (**D**) Confocal cross-sectional fluorescence image of a 34-kPa PA hydrogel functionalized with rat fibronectin, demonstrating that the ECM protein represents a relatively uniform, thin layer of the PA hydrogel. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb1016*.

behavior (Rehfeldt et al., 2007). For example, while culturing mesenchymal stem cells (MSCs) on relatively stiff substrates promotes osteogenic differentiation of mesenchymal stem cells when collagen I (Engler et al., 2006) or fibronectin is bound (Rowlands et al., 2008), the use of collagen IV and laminin, proteins found in low concentrations in bone, on these same substrates does not result in any osteogenic marker expression (Rowlands et al., 2008). Nevertheless, because collagen I is the most abundant type of collagen found in the body, it is often used as a default surface coating for a variety of cell types (Badylak, 2005). The optimal choice of ECM protein or combinations of proteins thus depends on what will best mimic the in vivo microenvironment of the specific cell type.

Materials

PA hydrogels (Basic Protocol 1 or Alternate Protocol)
0.2 mg/ml sufosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)-hexanoate (sulfo-SANPAH; Pierce Biotechnology)

50 mM HEPES buffer, pH 8.5, filter sterilized
ECM protein(s) of choice
Distilled H_2O *or* phosphate-buffered saline (PBS; Cellgro, cat. no. 46-013-CM), sterile

320-nm or 365-nm UV light source
37°C tissue culture incubator

1. Remove dH_2O or PBS from the petri dish with coverslip-gel composite.
2. Add ~500 µl of sulfo-SANPAH solution to the gel surface or enough to cover the entire gel.

   Complete coverage is necessary to ensure even protein coating.

   Sulfo-SANPAH is light-sensitive and should be shielded from light until use.

3. Place the gel in the 365-nm UV light source at a distance of ~3 in. and expose for 10 min.

   Repeat as necessary should insufficient protein bind, or if the coating does not appear uniform using the methods detailed below.

   See Figure 10.16.4A for schematic of the attachment of sulfo-SANPAH to the PA hydrogel, catalyzed by 365-nm UV light.

4. Rinse with 2 ml 50 mM HEPES at least two to three times to eliminate excess sulfo-SANPAH.

   The HEPES solution should be filter sterilized to ensure that the hydrogels remain as sterile as possible.

5. Add an appropriate amount of ECM protein to 50 mM HEPES, and incubate this solution with the gel overnight at 37°C.

   Due to the aqueous instability of the sulfosuccinimidyl ester, conjugation should start immediately upon activation.

   Ensure that the protein used in the conjugation has a primary amine group in it. Most extracellular matrix proteins contain such a reactive group. The end user should optimize the density of ligand coating for the specific cell type used.

   If making larger gels, placing the gel on a rocker plate can ensure that the solution remains well mixed and evenly coats the gel.

   For our setup, we employ concentrations of 0.10 mg/ml for collagen and 10 µg/ml of fibronectin or laminin, which have previously been shown to promote optimal cell behavior in smooth muscle cells (Engler et al., 2004a), fibroblasts (Rajagopalan et al., 2004), and epithelial cells (Williams et al., 2008), respectively. Increasing the collagen concentration within the solution to enhance binding may cause the collagen to precipitate or form a gel once heated to a physiological temperature. When the hydrogels are used for cell culture, media proteins will not continue to attach to unlinked sulfo-SANPAH sites because the reaction will only take place when the pH is 8.5, and media pH is usually 7 to 7.4.

   It is critically important that, for successful conjugation of the protein to the PA gel, the protein solution not precipitate. Given the relatively high pH of 8.5 for the reaction, collagen will have a tendency to precipitate. To avoid this, we suggest adding HEPES buffer to the concentrated collagen stock solution slowly, with vigorous vortexing.

   See Figure 10.16.4B,C for schematic of the overnight attachment of an ECM protein to the sulfo-SANPAH at the surface of the PA hydrogel.

6. Rinse with 2 ml distilled H_2O or PBS, depending on what was used to dilute the acrylamide.

7. To verify binding amounts, we suggest either using fluorescently labeled (Engler et al., 2004c; Rowlands et al., 2008) or radioactively labeled (Rajagopalan et al., 2004) protein to relate the measured signal to the amount of protein from reference standards. Alternatively, antibody-coated bead binding (Lo et al., 2000) or enzyme-linked immunosorbent assays (ELISA) may be used to confirm protein binding.

   Using fluorescently bound proteins may be most effective as they will also enable detection of uniform protein coating. Fluorescently labeled proteins can be added at up to a 1:9 ratio to unlabeled protein in step 5 of this protocol. When observing the gel with a confocal microscope, it will be possible to confirm that functionalization was confined to the top surface of the gel and that it does not vary in intensity by more than 10% (see Fig. 10.16.4D, Rowlands et al., 2008, or Winer et al., 2009).

   If there is an intensity variation of more than 10%, additional functionalization steps using sulfo-SANPAH should performed, i.e., repeat steps 1 to 4 of this protocol prior to adding protein in step 5. Alternatively, increasing sulfo-SANPAH concentration is not advisable, as it will reach its solubility limit.

8. After confirmation of protein binding, or in parallel cultures, add 1 ml of sterile distilled H_2O or PBS to the petri dish or well and place in the tissue culture hood for 30 min under UV for sterilization.

9. Plate cells using standard tissue culture techniques. For isolated cells with only cell-ECM contacts, plate cells at $<10^4$ cells/cm^2 in their standard medium.

SUPPORT PROTOCOL

CONSTRUCTION OF A GRADIENT-GEL APPARATUS

The construction and setup of the gradient-gel apparatus is presented here. The three main components of the apparatus are the gradient and negative photomasks and the UV light source. Photomask design can simply be made using the gradient tool in Adobe Photoshop and by adjusting the percent gray-scale or using silvered glass etching techniques in a microfabrication facility. Given that the former is a much more accessible technology, we will focus our discussion here to desktop versions for photomask generation. For our particular setup, we use a radial-gradient of 0% to 70% gray-scale, with the center at 0% and the edge at 70% gray-scale. While the gray-scale range can be modulated to any value, 70% was chosen as the maximum gray-scale because a higher value blocked efficient polymerization. The 0% to 70% range allows us to achieve a maximal range of elasticity of the gel associated with a maximal range of gray-scale. Because the edges of the photomask would transmit the least amount of light, the PA hydrogel would be expected to show the least polymerization and thus the lowest modulus of elasticity at the edge of the gels. This radial-gradient design is beneficial because it is easy to find the lowest attainable modulus of elasticity by monitoring the amount of time required for the edge of the hydrogel to just complete polymerization. Our setup uses a small UV light box, which typically emits UV light at 254-nm and 365-nm. It is acceptable to use a UV wavelength outside the optimal Irgacure 2959 absorbance peak of 280 nm with the precaution that longer UV exposure time is needed to deliver the same results. The negative photomask is essentially a black screen that blocks out all excess light by transmitting only an area of light the size of the actual photomask.

While the setup of the gradient-gel apparatus can be altered so that the light box is on top, it is important for the relative order of each component to stay the same. Figure 10.16.2 shows an exploded diagram of our apparatus that illustrates how the UV light should transmit through the chloro-silanated glass slide first before reaching the polymer solution. This order would initiate polymerization at the interface between the polymer solution and the chloro-silanated glass slide allowing the gel surface to receive the most UV light.

Materials

Laser printer
Nitrocellulose film
Photomask design
4 mil transparency film
Negative photomask design
254-nm UV light box
Spacers

1. Create photomask by printing out the desired pattern at 1200 dpi using a laser printer onto nitrocellulose film (see Fig. 10.16.2D as an example).

   For our photomask design, the center of the photomask had a gray-scale intensity of 0%, while the edge photomask had a gray-scale intensity of 70%. The photomask design can be created using the gradient tool in Adobe Photoshop.

   The photomask should be the same dimensions as the gel itself.

   Nitrocellulose film was chosen for the photomask material because it transmits UV light at a wavelength of 254 nm. Typical commercially purchased transparency films do not transmit UV light below ~300 nm due to the presence of benzene rings of the poly(ethylene terephthalate) of the material that absorb at these wavelengths.

2. Print out the negative photomask design at 1200 dpi onto 4 mil transparency film using a laser printer (see Fig. 10.16.2B).

   The negative photomask design should be a black print that would transmit UV light in the shape of the photomask.

3. Place the negative photomask on top of the 254-nm UV light box (see Fig. 10.16.2A and B), separated by spacers (see Fig. 10.16.2B).

4. Place the nitrocellulose paper with printed photomask on top of the negative photomask (see Fig. 10.16.2D).

5. Because nitrocellulose paper is sticky, use spacers to avoid contact between the negative photomask and the chloro-silanated glass slide (see Fig. 10.16.2E).

6. Align the negative photomask so that the area of light that is transmitted is directly below the photomask.

7. Proceed to the Alternate Protocol to continue the preparation of radial-gradient hydrogels.

COMMENTARY

Background Information

PA gels were originally used for the separation of proteins in gel electrophoresis as PA is both nearly inert and has tunable cross-linking density. For the same reasons, PA gels can be useful for cell culture techniques, as varying these concentrations results in a quantifiable stiffness known as the modulus of elasticity that cells can sense and respond to. The application of polyacrylamide gels to cell culture was first developed in 1997 to study the responses of 3T3 fibroblasts and rat kidney epithelial cells to mechanical properties of the substrate (Pelham and Wang, 1997). While this protocol uses PA as the polymer of choice, other materials, such as polydimethylsiloxane, (PDMS), polyethylene glycol (PEG), and hyaluronic acid (HA) are some of the other synthetic and natural substrates with a tunable elasticity that may be used for cell culture (Rehfeldt et al., 2007). The application of this protocol allows one to study how matrix elasticity can affect cell locomotion, focal adhesion structure, cytoskeleton, differentiation, and other potential mechanosensitive cellular processes. Recently, the interest in the effects of matrix elasticity has prompted other researchers to develop

methods to fabricate hydrogels with mechanical properties that oscillate or change with time, temperature, and/or pH (Kaehr and Shear, 2008).

Critical Parameters and Troubleshooting

PA gradient-gel

The greatest challenge to fabricating a PA matrix with a gradient of elasticity is finding the correct set of parameters that can reliably reproduce the gradient. There are many parameters that can affect the modulus of elasticity of the gradient gel. The parameters include the UV bulb intensity, UV light wavelength, distance from the UV light box, Irgacure 2959 concentration, gel thickness, photomask gray-scale range, acrylamide and bis-acrylamide concentration, and UV exposure time. Because even minute changes in the setup can affect the final values of the gradient gel, it is necessary for the researcher to optimize these parameters to create the desired results. It is recommended to vary the UV exposure time and the acrylamide and bis-acrylamide concentrations, keeping all other parameters constant. To increase the range of the modulus of elasticity, increase the relative acrylamide and bis-acrylamide concentrations. To increase the modulus of elasticity of the hydrogels, increase the UV exposure time. There are several references to measure the modulus of elasticity of the gels by AFM (Domke and Radmacher, 1998; Rotsch et al., 1999; Rotsch and Radmacher, 2000).

Gel attachment

When separating the chloro-silanated glass slide from the gel, the gel may not be completely attached to the amino-silanated coverslip, resulting in uneven gel attachment. This is especially common when creating the gradient-gels, in which the polymerization starts on the side of the gel facing the chloro-silanated glass slide. To ensure uniform gel attachment, make sure that the glass slide is freshly coated with DCDMS and that the amino-silanated coverslips are used as quickly as possible after preparation.

Gel thickness

Another obstacle in the fabrication of the hydrogels is uneven gel thickness. Uneven gel thickness usually results from completing the protocol on a tilted surface, causing the polymer solution to aggregate to one side. To ensure uniform gel thickness, make sure that a flat surface is chosen to complete the protocol.

Anticipated Results

Support Protocol. A basic gradient-gel apparatus that can be assembled in a few minutes and used repeatedly for the fabrication of gradient-gels.

Basic Protocol 1. The researcher should observe that the amino-silanated glass coverslips are hydrophilic while the chloro-silanated glass slides are hydrophobic. After gel attachment, a translucent PA gel of ~50 μl should be uniformly attached to the amino-silanated glass coverslip. The modulus of elasticity of the statically compliant hydrogels should match the values found in Table 10.16.1 and can be confirmed by using AFM analysis.

Basic Protocol 2. After overnight incubation with the ECM protein, the surface of the hydrogel should have covalent linkages to the protein. The researcher can confirm protein attachment by using immunofluorescent staining such as that employed by Rowlands and coworkers (Rowlands et al., 2008) or with bead assays as with Lo and coworkers (Lo et al., 2000). If one uses the conditions described in this unit, the surface concentration of the ligand should be ~340 to 450 molecules per μm^2 (Gaudet et al., 2003).

Time Considerations

Support Protocol. The gradient-gel apparatus can be completed within 1 hr.

Basic Protocol 1. It takes ~15 min to prepare NaOH-coated glass coverslips, which can be stored indefinitely. The rest of the preparation of the chloro-silanated glass slides and amino-silanated glass coverslips takes ~50 min. The steps that complete the attachment of the statically compliant PA hydrogel takes ~40 min. For the radial-gradient hydrogel, it generally takes ~15 min for the Irgacure 2959 with acrylamide and bis-acrylamide to completely dissolve and ~60 min to degas. Each gel then takes a few minutes to polymerize, depending on the acrylamide and bis-acrylamide concentration and the desired gradient.

Basic Protocol 2. The set up and UV exposure to cross-link the sulfo-SANPAH to the PA hydrogel takes ~20 min. The attachment of the ECM protein to the PA hydrogel can be completed overnight. Finally, before plating cells, the PA hydrogels should be sterilized for 30 min by placing them in the tissue culture hood under UV.

Literature Cited

Badylak, S.F. 2005. Regenerative medicine and developmental biology: the role of the extracellular matrix. *Anat. Rec. B New Anat.* 287:36-41.

Cukierman, E., Pankov, R., Stevens, D.R., and Yamada, K.M. 2001. Taking cell-matrix adhesions to the third dimension. *Science* 294:1708-1712.

Discher, D.E., Janmey, P., and Wang, Y.L. 2005. Tissue cells feel and respond to the stiffness of their substrate. *Science* 310:1139-1143.

Discher, D.E., Mooney, D.J., and Zandstra, P.W. 2009. Growth factors, matrices, and forces combine and control stem cells. *Science* 324:1673-1677.

Domke, J. and Radmacher, M. 1998. Measuring the elastic properties of thin polymer films with the atomic force microscope. *Langmuir* 14:3320-3325.

Engler, A., Bacakova, L., Newman, C., Hategan, A., Griffin, M., and Discher, D. 2004a. Substrate compliance versus ligand density in cell on gel responses. *Biophys. J.* 86:388a-388a.

Engler, A.J., Griffin, M.A., Sen, S., Bonnemann, C.G., Sweeney, H.L., and Discher, D.E. 2004b. Myotubes differentiate optimally on substrates with tissue-like stiffness: Pathological implications for soft or stiff microenvironments. *J. Cell Biol.* 166:877-887.

Engler, A.J., Richert, L., Wong, J.Y., Picart, C., and Discher, D.E. 2004c. Surface probe measurements of the elasticity of sectioned tissue, thin gels and polyelectrolyte multilayer films: Correlations between substrate stiffness and cell adhesion. *Surf. Sci.* 570:142-154.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. 2006. Matrix elasticity directs stem cell lineage specification. *Cell* 126:677-689.

Fairbanks, B.D., Schwartz, M.P., Bowman, C.N., and Anseth, K.S. 2009. Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2,4,6-trimethylbenzoylphosphinate: Polymerization rate and cytocompatibility. *Biomaterials* 30:6702-6707.

Fedorovich, N.E., Oudshoorn, M. H., van Geemen, D., Hennink, W. E., Alblas, J., and Dhert, W.J.A. 2009. The effect of photopolymerization on stem cells embedded in hydrogels. *Biomaterials* 30:344-353.

Flanagan, L.A., Ju, Y.E., Marg, B., Osterfield, M., and Janmey, P.A. 2002. Neurite branching on deformable substrates. *Neuroreport* 13:2411-2415.

Fung, Y.C. 1993. Biomechanics: Mechanical Properties of Living Tissues, 2nd ed. Springer-Verlag, New York.

Gaudet, C., Marganski, W.A., Kim, S., Brown, C.T., Gunderia, V., Dembo, M., and Wong, J.Y. 2003. Influence of type I collagen surface density on fibroblast spreading, motility, and contractility. *Biophys. J.* 85:3329-3335.

Georges, P.C. and Janmey, P.A. 2005. Cell type-specific response to growth on soft materials. *J. Appl. Physiol.* 98:1547-1553.

Grabarek, Z. and Gergely, J. 1990. Zero-length crosslinking procedure with the use of active esters. *Anal. Biochem.* 185:131-135.

Kaehr, B. and Shear, J.B. 2008. Multiphoton fabrication of chemically responsive protein hydrogels for microactuation. *Proc. Natl. Acad. Sci. U.S.A.* 105:8850-;8854.

Khatiwala, C.B., Peyton, S.R., and Putnam, A.J. 2006. Intrinsic mechanical properties of the extracellular matrix affect the behavior of pre-osteoblastic MC3T3-E1 cells. *Am. J. Physiol. Cell Physiol.* 290:C1640-C1650.

Lo, C.M., Wang, H.B., Dembo, M., and Wang, Y.L. 2000. Cell movement is guided by the rigidity of the substrate. *Biophys. J.* 79:144-152.

Pelham, R.J. Jr. and Wang, Y. 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc. Natl. Acad. Sci. U.S.A.* 94:13661-13665.

Rajagopalan, P., Marganski, W.A., Brown, X.Q., and Wong, J.Y. 2004. Direct comparison of the spread area, contractility, and migration of balb/c 3T3 fibroblasts adhered to fibronectin- and RGD-modified substrata. *Biophys. J.* 87:2818-2827.

Rehfeldt, F., Engler, A.J., Eckhardt, A., Ahmed, F., and Discher, D.E. 2007. Cell responses to the mechanochemical microenvironment–implications for regenerative medicine and drug delivery. *Adv. Drug Deliv. Rev.* 59:1329-1339.

Reinhart-King, C.A., Dembo, M., and Hammer, D.A. 2005. The dynamics and mechanics of endothelial cell spreading. *Biophys. J.* 89:676-689.

Reinhart-King, C.A., Dembo, M., and Hammer, D.A. 2008. Cell-cell mechanical communication through compliant substrates. *Biophys. J.* 95:6044-6051.

Rotsch, C. and Radmacher, M. 2000. Drug-induced changes of cytoskeletal structure and mechanics in fibroblasts: An atomic force microscopy study. *Biophys. J.* 78:520-535.

Rotsch, C., Jacobson, K., and Radmacher, M. 1999. Dimensional and mechanical dynamics of active and stable edges in motile fibroblasts investigated by using atomic force microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 96:921-926.

Rowlands, A.S., George, P.A., and Cooper-White, J.J. 2008. Directing osteogenic and myogenic differentiation of MSCs: Interplay of stiffness and adhesive ligand presentation. *Am. J. Physiol. Cell Physiol.* 295:C1037-C1044.

Wang, H.B., Dembo, M., and Wang, Y.L. 2000. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. *Am. J. Physiol. Cell Physiol.* 279:C1345-C1350.

Williams, C.M., Engler, A.J., Slone, R.D., Galante, L.L., and Schwarzbauer, J.E. 2008. Fibronectin expression modulates mammary epithelial cell

proliferation during acinar differentiation. *Cancer Res.* 68:3185-3192.

Winer, J.P., Janmey, P.A., McCormick, M.E., and Funaki, M. 2009. Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Eng. Pt A* 15:147-154.

Wong, J.Y., Velasco, A., Rajagopalan, P., and Pham, Q. 2003. Directed movement of vascular smooth muscle cells on gradient-compliant hydrogels. *Langmuir* 19:1908-1913.

Engineering Three-Dimensional Collagen Matrices to Provide Contact Guidance during 3D Cell Migration

Paolo P. Provenzano,[1,2] Kevin W. Eliceiri,[2] David R. Inman,[2] and Patricia J. Keely[2]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington
[2]University of Wisconsin, Madison, Wisconsin

ABSTRACT

Cell invasion requires that cells navigate complex three-dimensional matrices in vivo. Topological cues are provided and three-dimensional cell migration and invasion facilitated by the alignment of collagen fibers proximal to tumors. In order to better understand the molecular mechanisms by which cells recognize and migrate along aligned matrices, in vitro assays are needed that recapitulate topological features of the in vivo matrix. Here, we describe two approaches for creating aligned three-dimensional collagen matrices, both dependent and independent of cell-mediated alignment. Approaches to quantify alignment and visualize the collagen matrix relative to the cells by second-harmonic generation are included. These assays are readily adaptable to a variety of cells and biological questions related to three-dimensional cell migration. *Curr. Protoc. Cell Biol.* 47:10.17.1-10.17.11. © 2010 by John Wiley & Sons, Inc.

Keywords: collagen alignment • three-dimensional cell migration • invasion

INTRODUCTION

Cell migration is an essential process in which physical and chemical cues are provided by the extracellular matrix (ECM; Lauffenburger and Horwitz, 1996; Petrie et al., 2009). Contact guidance from topographical features (Teixeira et al., 2003; Doyle et al., 2009) or three-dimensional (3D) microenvironments (Guido and Tranquillo, 1993; Dickinson et al., 1994; Dallon et al., 1999; Provenzano et al., 2008a) is the phenomenon by which the ECM provides directional cues that influence cell orientation and migration direction. In 3D collagen matrices, contact guidance from collagen fibers regulates cell migration via anisotropy in the local microenvironment (Dickinson et al., 1994; Provenzano et al., 2008a), with cells migrating preferentially along aligned collagen fibers.

In this unit, basic methods for engineering 3D collagen matrices to generate contact guidance cues are presented. The matrices closely resemble architectures observed in vivo (Provenzano et al., 2006). Utilizing in vivo–mimicking ECM conditions facilitates the acquisition of physiologically relevant information, while permitting manipulation of the system within a manageable experimental window. Therefore, to achieve this, two basic methods for generating 3D migration/invasion assays with defined architecture are described. The first system, described in Basic Protocol 1 makes use of placing cell-seeded collagen gels (CSCGs) or tissue explants into collagen matrices in a defined manner to create collagen architectures via cell contraction–mediated collagen reorganization. The second protocol (Basic Protocol 2) describes methods to align collagen matrices during matrix polymerization using a magnetic field. The aligned matrices may then be oriented and combined to develop 3D migration assays in which the cells face different contact guidance cues.

Current Protocols in Cell Biology 10.17.1-10.17.11, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1017s47

NOTE: All solutions and equipment used during the protocols must be sterile for use with cell culture. Likewise, all procedures should be performed in a sterile tissue culture hood with proper aseptic technique used throughout the protocols.

NOTE: Collagen matrix polymerization times should be optimized for each individual's laboratory conditions. Moreover, parameters may need to be adjusted to accommodate different cell types, experimental designs, and collagen matrix concentrations.

BASIC PROTOCOL 1

PREPARATION OF A 3D MIGRATION ASSAY USING NESTED CELL-SEEDED COLLAGEN GELS

Cell-seeded collagen gels (CSCGs) placed into an encasing matrix will contact and reorganize the matrix to generate regions of collagen alignment and regions of non-alignment that provide contact guidance cues for motile cells. In this assay, cells are seeded into collagen matrices to make plugs that are then inserted into an encasing collagen matrix. Over time, cells reorganize the matrix and invade into the newly organized encasing matrix.

Materials

Sub-confluent epithelial or fibroblast cells on a standard tissue culture dish/flask
Culture medium of choice for the chosen cell type
Phosphate-buffered saline (PBS) or serum-free culture medium
Trypsin/EDTA solution (Cellgro)
Rat tail collagen (BD Biosciences)
0.1 M HEPES buffer in 2× PBS

15-ml screw-cap conical tubes
96-well ultra-low adhesion culture plate (Corning)
37°C cell culture incubator
28-G syringe needle
1000-μl pipets with the tips cut off
6-well culture dishes (Corning ultra-low attachment plates work very well, but other more standard plates may be substituted as needed)

Additional reagents and equipment for counting the cells (*UNIT 1.1*) and optical imaging (Support Protocol)

Detach and count cells

1. Aspirate the cell culture medium from the culture dish containing sub-confluent cells. Rinse the cells with PBS or serum-free culture medium to remove residual serum. Aspirate buffer/medium from the dish.

   The volume of PBS or serum-free medium will depend on the size of the culture plate, as discussed in step 2.

2. Add 2 to 3 ml of trypsin/EDTA solution to the cell dish and incubate for ∼3 to 5 min (or until sufficient cell detachment is achieved) at room temperature. Use gentle agitation of the dish, if necessary, to detach cells.

   The concentration of the trypsin/EDTA solution may depend on the chosen cell line and the volume of trypsin/EDTA that is necessary will depend on the size of the culture dish. In general, the lowest concentration and volume of trypsin/EDTA solution that results in sufficient cell detachment within 10 min should be used. Furthermore, any cell culture reagent that adequately detaches the cells of interest may be substituted for trypsin/EDTA solution.

3. After the cells detach from the dish, transfer the cell-containing trypsin/EDTA solution to a 15-ml screw-cap conical tube and add 2 vol of culture medium for every one volume of trypsin/EDTA solution to the tube. Centrifuge the tube 3 to 5 min at 300× *g*, 4°C, to pellet the cells.

4. Discard the supernatant and resuspend the cells in 1 to 2 ml of culture medium. Count the cells (*UNIT 1.1*).

Prepare cell-seeded collagen gels (CSCGs)

5. Determine the volume of cell-containing medium that contains at least 150,000, but no more than 500,000 cells.

   The number of cells per CSCG may be adjusted for cell type and experimental design, but in general <150,000 cells do not adequately fill the CSCG with cells, while cell numbers >500,000 cells/CSCG are commonly too cell dense.

6. Calculate the volume of collagen mixture needed for the experiment.

   The amount of collagen added will depend on the desired final concentration of the collagen matrix and the initial concentration (in mg/ml) of the collagen solution. The volume of neutralizing HEPES buffer solution will be equal to the volume of collagen solution. Add regular culture medium to achieve the final volume. In our hands, 1 to 2 mg/ml collagen matrices have worked very well for MDA-MB-231 and T47D breast carcinoma cells. Therefore, as an example, to make a 2 mg/ml collagen matrix from a 10 mg/ml initial stock of collagen, add 200 μl of collagen solution, 200 μl of HEPES buffer solution, and 600 μl of culture medium for every ml of matrix solution needed. The 600 μl of culture medium includes the volume of culture medium needed to add the correct number of cells. For each CSCG, 150 to 250 μl (150-μl CSCGs work very well but larger volumes may used to accommodate more cells, i.e., 250 μl for 500,00 cells) of the collagen/HEPES/medium mixture is added to a well within the 96-well plate.

   Since the collagen solution is very viscous, pipet very carefully, allowing adequate time for the collagen solution to enter and exit the pipet. After releasing the collagen solution from the pipet allow the remaining volume to pool in at the bottom of the pipet tip and then clear the pipet again. In addition, make extra solution to ensure that enough CSCGs can be poured for the experiment.

7. In a new conical tube, add the calculated volume of collagen solution to an equal volume of 100 mM HEPES buffer (in 2× PBS). Add culture medium and culture medium containing cells to achieve the correct final volume and cell number per CSCG.

   All solutions should be kept on ice until the mixture is pipetted to the culture plate.

8. Pipet 150 to 250 μl of the collagen mixture (containing cells as described in step 6) to an individual well in the 96-well culture plate.

9. Allow the collagen matrix to polymerize for 20 min at room temperature, then at least 2 hr but not more than 16 hr in a cell culture incubator at 37°C (Fig. 10.17.1A).

Implant CSCGs into the encasing matrix

10. Gently detach the CSCG from the sides of the culture plate well using a fine needle, such as 28-G syringe needle. Insert the needle at the side of the CSCG, touching the well wall, and while maintaining contact with the wall, move the needle along the perimeter of the CSCG (Fig. 10.17.1B). Gently shake and tap the plate to help detach the CSCGs from the culture well surfaces.

11. Using a 1000-μl pipet with the tip cut off to increase the opening diameter (Fig. 10.17.1C), gently pull the CSCG up out of the culture well.

    The CSCG should never enter the pipet. The CSCG should remain suspended below the entrance to the pipet tip (Fig. 10.17.1D).

12. Suspend the CSCGs in culture medium in a tissue culture plate for 20 min to allow the matrix to contract.

    Allow ample room in the culture plate for the CSCGs to float without coming into contact with one another (Fig. 10.17.1E,F).

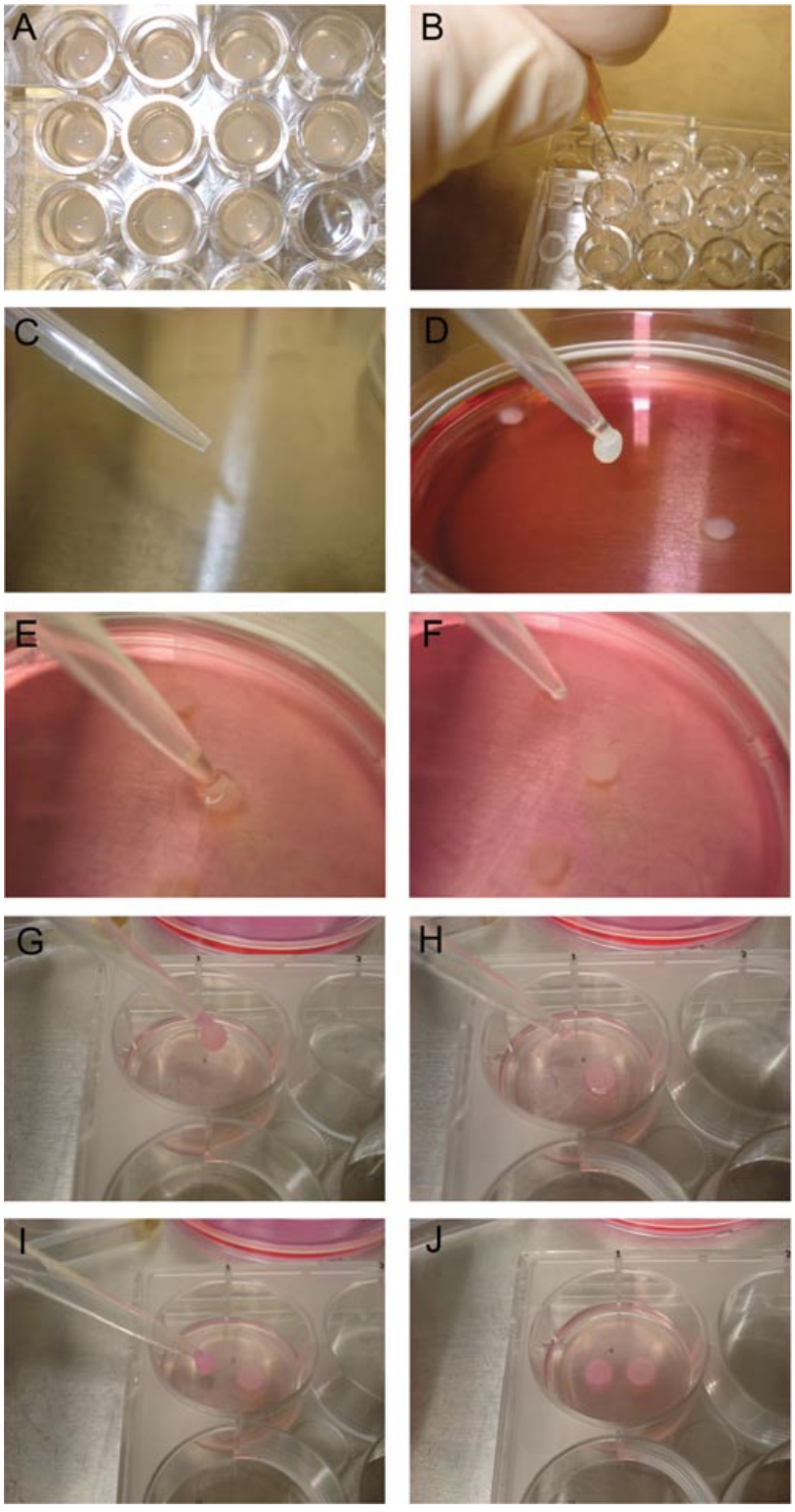


Figure 10.17.1 Key steps in Basic Protocol 1. (**A**) Polymerized CSCGs in a 96-well plate. (**B**) Detaching CSCGs from the culture dish wall. (**C**) A 1000-μl pipet with the tip cut off. (**D**) Transferring the CSCG from the culture well with care taken not to pull the CSCG into the pipet. (**E**) Placing the CSCG into a culture plate. (**F**) Floating the CSCG in culture medium with care not to allow multiple CSCGs to contact one another. (**G-J**) Placing CSCGs into the encasing matrix.

13. During step 12, make an additional collagen mixture for the encasing matrix.

    For each well of a 6-well tissue culture plate, 1.5 ml of 2 mg/ml collagen mixture is needed.

14. Add 1.5 ml of the collagen mixture to a well of the 6-well plate. Allow the collagen matrix to polymerize for ~10 to 15 min at room temperature.

    This step helps ensure that the CSCG will not directly rest on the bottom of the culture dish and attach to the bottom surface. By placing the CSCG into a matrix that is just starting to polymerize, and is therefore less fluid, the CSCG becomes suspended in the encasing matrix.

15. Place the CSCGs into the encasing matrix, making sure that the CSCGs does not sink to the bottom of the culture dish. Place the CSCGs 7 mm apart (from edge to edge) along the maximal diameter midline of the well (Fig. 10.17.1G-J).

    A custom reference grid may be placed under the well to help facilitate placement.

16. Allow the collagen matrix to polymerize undisturbed at room temperature for at least 20 min. After the matrix has sufficiently polymerized to allow movement of the plate without disturbing the CSCG placement, move the plate to the cell culture incubator at 37°C.

17. Sixteen to 24 hr after matrix construction, detach the encasing matrix and float the matrix in 2 ml cell culture medium.

    Detach the matrix from the culture dish by gently inserting a small pipet tip on the side of the matrix (while touching the well wall with the pipet tip). Gently move the tip around the perimeter of the matrix while maintaining contact with the well wall. Place the pipet tip at the side of the well and gently pipet 2 ml of medium into the well, under the matrix. Gently rotate the plate to detach the matrices and float them in the medium. Extreme care must be taken during this step so that the CSCGs do not tear away from the encasing matrix.

18. Incubate the nested CSCGs for one to several days, changing the medium every 3 days or for more even stimulation change 25% of the medium daily.

    We find that collagen alignment can be observed within the first 24 hr, but that collagen alignment is maintained and enhanced over 6 to 10 days.

19. Analyze three-dimensional cell migration/invasion with optical imaging (see Support Protocol)

    A single-plug nested assay can be employed as well (Grinnell et al., 2006; Provenzano et al., 2006; Miron-Mendoza et al., 2008), but does not provide the consistent contact guidance cues as the alignment is more distributed in random locations around the plug. However, this assay still provides a very good 3D migration assay, particularly for cancer biology studies where the CSCG can simulate an epithelial mass where carcinoma cells invade into the collagenous stroma. Likewise, more than two CSCGs can be employed to create more complex architectures.

PREPARATION OF ALIGNED COLLAGEN MATRICES TO CONSTRUCT A 3D MIGRATION ASSAY WITH DEFINED CONTACT GUIDANCE CUES

BASIC PROTOCOL 2

One limitation of the nested-plug assay, above, is that it does not readily allow separation of signaling events that lead to an aligned matrix from those necessary to migrate along a matrix that is already aligned. Here, we describe an approach to create a pre-aligned collagen matrix [as presented in Provenzano et al. (2008b) and adapted from Guo and Kaufman (2007)], which provides cells with contact guidance cues from perpendicular, parallel, and/or unaligned matrices. This approach allows one to address the question of which molecular events are specifically necessary for recognizing the topography of the matrix and mediating contact-guided 3D cell migration.

Materials

Sub-confluent epithelial or fibroblast cells on a standard tissue culture dish/flask
Culture medium of choice for the chosen cell type
Trypsin/EDTA solution (Cellgro)
Rat tail collagen (BD Biosciences)
0.1 M HEPES buffer in 2× PBS

Rectangular mold (~1- × 3-in.) *or* Nunc multidish, 4-well rectangular
1.5-μm diameter streptavidin-coated iron oxide beads (Bangs Labs, cat. no. BM551)
Cylindrical magnet (~2G; McMaster, cat. no. 5862K32)
37°C culture incubator
Small spatula *or* cell scraper

Additional reagents and equipment for detaching and counting cells and preparing collage solution (Basic Protocol 1) and optical imaging (Support Protocol)

Detach and count cells

1. Detach and count cells as described in steps 1 to 4 of Basic Protocol 1.

Prepare rectangular CSCGs

2. In a new conical tube, add the appropriate volume of collagen solution (as described in step 6 of Basic Protocol 1) needed to make 2.5 ml (for every two samples) of a mixture that will generate matrices with a final collagen matrix concentration of 2.0 mg/ml. Add an equal volume of 100 mM HEPES buffer (in 2× PBS). Add culture medium and culture medium containing cells to achieve the correct final volume and cell number per CSCG.

   At least 2 million cells per 2.5 ml matrix should be included. This number may vary by cell type and experimental question, but in general a smaller number of cells will not sufficiently fill the CSCG and the cell distribution at the CSCG-aligned matrix interface will be uneven.

3. Pipet the collagen mixture into a rectangular mold (~1 × 3 in.) or culture dish (Nunc 4-well rectangular multidishes work very well), and allow the matrix to polymerize for at least 2 hr at 37°C. During this time, move forward with step 4.

   While herein rectangular CSCGs are described and they have worked well, in general, any CSCG geometry that is needed to satisfy the final assay architecture may be substituted. Moreover, the size of the assay can be scaled as needed. However, the matrix volume should be optimized for each condition.

Generate aligned collagen matrices

4. In a new conical tube, add the appropriate volume of collagen solution (as described in step 6 of Basic Protocol 1) needed to make 2.5 ml of a mixture that will generate matrices with a final collagen matrix concentration of 2.0 mg/ml. Add an equal volume of 100 mM HEPES buffer (in 2× PBS). Add culture medium and then streptavidin-coated iron oxide beads (1.5-μm diameter) to achieve the correct final volume with a bead concentration of 0.1 mg/ml.

5. Pipet the collagen mixture (containing magnetic beads) into a rectangular dish with the same geometry as the dish used in step 3.

6. Place the cylindrical magnet underneath the culture well with the poles of the magnet along the long axis of the gel mold, in the direction of alignment, to align the collagen matrix during collagen fibrillogenesis at room temperature for 20 min.

   The culture plate well should be slowly translated over the magnet continuously, along the direction of alignment, during the first 10 min with each pass taking ~15 sec of the polymerization period in a single direction. If multiple matrices are being generated, an

array of magnets can be employed. In this case, four magnets can be separated by plastic so that a magnet is below each well (in the case of a Nunc 4-well plate). Note that care must be taken when handling the magnets as they will strongly attract or repel (depending on their orientation to one another) and can cause injury.

7. Place the culture dish, with the magnets remaining underneath the wells, in a cell culture incubator at 37°C for an additional 60 min.

Implant aligned matrices CSCGs into the encasing matrix

8. Remove the CSCG(s) and aligned matrices from the incubator. Using a small spatula or cell scraper, carefully lift each CSCG from the culture plate and float the matrix in culture medium for 20 min.

9. During step 8 make an additional collagen mixture for the encasing matrix at 2 mg/ml.

   The volume of the encasing matrix will depend on the choice of final geometry.

10. Cut a CSCG and an aligned matrix into two equal pieces and then pipet a thin, evenly distributed, volume of encasing matrix onto the culture plate. Immediately place the CSCG at the center of the dish and place the aligned matrices parallel and perpendicular to the CSCG. Gently pipet the encasing matrix over CSCG and aligned matrix pieces to achieve the configuration shown in Figure 10.17.2.

    The aligned matrices should be pressed directly against the CSCG with no space between the matrices at the CSCG/aligned matrix interface. Care should be taken so that the thin volume of collagen mixture at the bottom of the plate is not pushed between the CSCG and the aligned matrices. Likewise, when pipetting the encasing matrix, care should be taken not to flow an excess amount of collagen into the interface. Note that the CSCGs

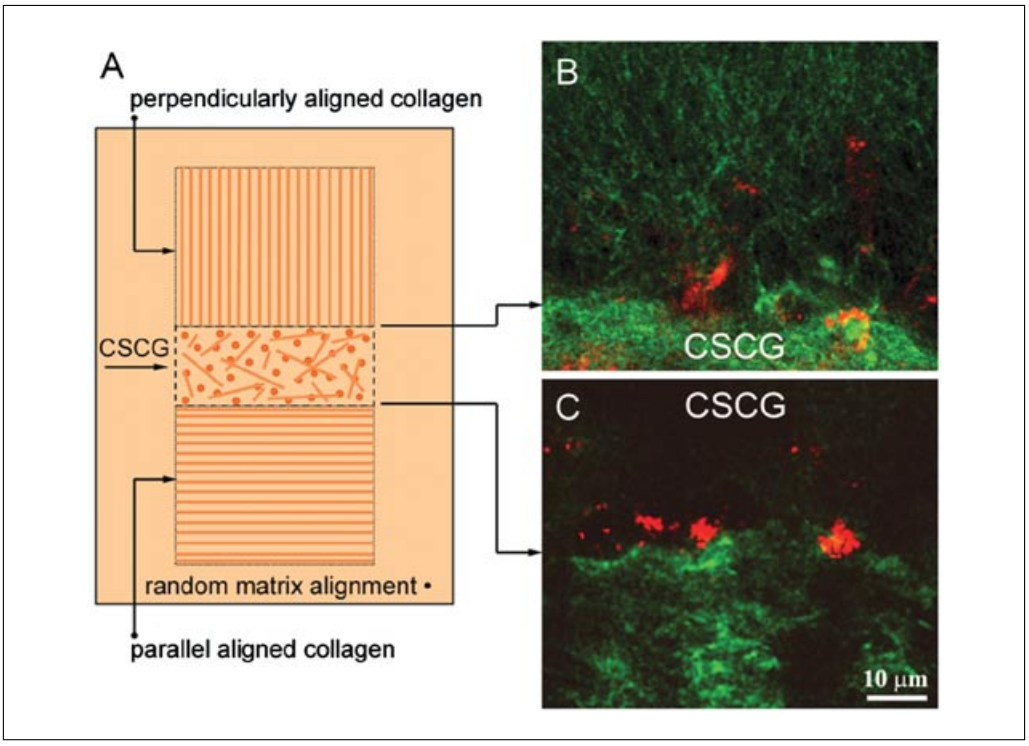


Figure 10.17.2 (**A**) Diagram illustrating 3D migration/invasion assay described in Basic Protocol 2. In the assay, collagen is magnetically aligned and aligned collagen matrices are connected to the CSCG so that the collagen alignment is either perpendicular (**B**) or parallel (**C**) to the CSCG boundary. Collagen is shown in green and cells in red. Reproduced from Provenzano et al., 2008a with permission. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb1017*.

and aligned collagen matrix can be carefully cut and shaped to achieve the desired architecture as shown in Figure 10.17.2. However, care should be taken to prevent an uneven surface from being placed against the CSCG.

11. Culture for one to several days, changing the medium every 3 days or for even more stimulation change 25% of the medium daily.

12. Analyze 3D cell migration/invasion with optical imaging (see Support Protocol)

SUPPORT PROTOCOL

IMAGING COLLAGEN MATRIX ALIGNMENT AND CELL MIGRATION WITH MULTIPHOTON EXCITATION AND SECOND-HARMONIC GENERATION MICROSCOPY

A number of multiphoton laser-scanning microscopy (MPLSM) systems are now commercially available that can be used to image collagen and the cells in the assays described above. Custom systems are also commonplace and the reader is encouraged to examine the references included in this unit for additional details (e.g., Denk 1990; Diaspro 2002; Condeelis 2003; Mohler 2003; Zipfel 2003; Provenzano 2006, 2008a, 2008b; Lee 2009).

The magnetic beads used in Basic Protocol 2 scatter light and can overload the microscope detector if care is not taken. Begin imaging with a low power or detector gain until it is determined how the beads will influence the microscope setup.

Multiphoton laser-scanning microscopy, MPLSM (Denk et al., 1990), is an optical sectioning technique where excitation is restricted to the plane of focus because the probability of two or more photons simultaneously exciting a fluorophore outside the focal volume is extremely low, significantly reducing phototobleaching by not exciting the out-of-focus-plane tissue volume [for detailed reviews of MPLSM and second-harmonic generation (SHG) see Diaspro and Sheppard, 2002; Mohler et al., 2003; Zipfel et al., 2003; Helmchen and Denk, 2005; Provenzano et al., 2009a. Detailed protocols for live-cell imaging and SHG, including equipment considerations, are provided in *UNITS 19.7 & 4.15*, respectively]. With MPLSM, the excitation wavelengths are long, typically 650- to 1050-nm excitation for 2- and 3-photon excitation, which has practical advantages for being able to excite a wide range of fluorophores (both endogenous and engineered) while facilitating deeper penetration into tissues, since the longer wavelengths are less prone to scatter. Additionally, MPLSM is advantageous due to its ability to produce second-harmonic generation signals useful for imaging fibrillar collagen. As such, MPLSM has emerged as a popular and powerful tool for analyzing cells and the extracellular matrix under live culture conditions.

To image the collagen matrix of the three-dimensional migration assays described in Basic Protocols 1 and 2, we image the live cell constructs with MPLSM (as described in Provenzano et al., 2006, 2008a,b) at an 890-nm excitation wavelength to simultaneously generate SHG signals from fibrillar collagen and endogenous fluorescence from the metabolite FAD (Provenzano et al., 2008a). Since SHG signal is found at exactly half of the excitation wavelength, SHG signals and endogenous fluorescence are separated with a 445-nm narrow-band-pass filter and a 464-nm long-band-pass filter (TFI Technologies), respectively. Collagen fiber orientation is examined at regions of the CSCG-encasing or -aligned matrix interface and at the level of the single cell using a Nikon CFI Plan Apo 60× water-immersion lens (NA = 1.2). The number of cells invading into the collagen gel is collected within three focal plains (center ± 30 μm) in regions of interest. Alternatively, full *z*-stacks can be created in regions of interest.

Alternative approaches for imaging 3D cell migration in collagen matrices exist. For instance, confocal reflectance microscopy has been used to successfully image the collagen matrix (Hegerfeldt et al., 2002). In combination with confocal reflectance or MPLSM, live cells can be imaged while expressing fluorescent proteins (Hegerfeldt et al., 2002;

Provenzano et al., 2009b). Furthermore, the cells in 3D constructs can be imaged with transmitted or phase-contrast light microscopy. In samples fixed with paraformaldehyde the distribution and number of cells that have migrated out of the CSCG can be analyzed following staining for cellular components, such as the nucleus (Grinnell et al., 2006; Miron-Mendoza et al., 2008), while MPSLM can be used to image cells and collagen in fixed samples as well (Gehler et al., 2009). Note that when studying cell migration in the context of 3D collagen matrix architecture, as is the case in both Basic Protocols, a method to image the collagen matrix in regions of interest for cell migration must be included, as changes in the organization of collagen strongly influence cell behavior.

COMMENTARY

Background Information

In three-dimensional microenvironments in vivo, cell migration plays a fundamental role in numerous physiologic and pathological processes, including tissue morphogenesis and cancer metastasis (Condeelis and Segall, 2003; Friedl and Gilmour, 2009). In vivo, alignment of collagen fibers has been shown to facilitate gland development (Ingman et al., 2006) and carcinoma cell invasion (Provenzano et al., 2006), while endothelial tube formation in three-dimensional constructs in vitro also shows a dependence on collagen matrix alignment (Lee et al., 2009). Therefore, in order to recapitulate matrix architecture associated with invasion into the collagenous stroma in intact tumors (Provenzano et al., 2006), we engineered 3D collagen matrix assays for studying contact guidance in vitro (Provenzano et al., 2008a). The first assay makes use of the cells themselves to generate contact guidance cues through cell contraction-mediated matrix reorganization, while the second assay pre-aligns the collagen matrix to facilitate separation of signals associated with 3D migration and motility events from those needed to reorganize the collagenous ECM.

Live-cell imaging with MPLSM has emerged as a powerful tool for studying cell migration in 3D microenvironments not only due to it ability to image endogenous fluorescence in live unlabeled cells, but also due to its ability to simultaneously image the collagen matrix. The use of SHG to image collagen fibers in 3D volumes provides novel insight into how the stromal ECM influences cell behavior. Furthermore, it also provides a means to understand how the cell influences matrix architecture under both normal and pathological conditions. While other techniques are valuable and can often be used in conjunction with MPLSM (i.e., imaging live cells with transmitted light, fixing samples after imaging to stain for specific features, etc.), MPLSM is unique in its ability to noninvasively image deep into 3D collagen environments and concurrently image cells and collagen with high resolution and good viability.

Critical Parameters and Troubleshooting

The ability of cells to contract a collagen matrix and cause the realignment (Basic Protocol 1) is dependent in part on the stiffness of the matrix, and in part on the ability of the cells to generate a contractile force. We have found that a 2 mg/ml collagen gel works well for many cell types including MDA-MB-231 cells, NMuMG cells, mouse embryonic fibroblasts, and mammary tumor explants. Dramatically increasing the collagen concentration of the encasing matrix may diminish the extent of the collagen rearrangement, and consequently may alter 3D cell migration. However, stiffer matrices may be useful for highly contractile cells, or when one wants to determine if changes in signaling events have altered cellular contractility (Gehler et al., 2009). In addition, the density of cells in the CSCG can influence the time course of collagen reorganization and cell migration. Our interests have revolved around simulating carcinoma cells invading into the collagenous stroma. As such, we have utilized a high cell density within the CSCGs; however, lower cell densities may be used if desired. The optimal cell density for a specific experimental condition should be optimized by each investigator. Finally, if the CSCG is detaching from the encasing matrix, lengthening the time of CSCG contraction before implantation may reduce pulling away from the encasing matrix. Alternatively, using low-adhesion culture plates can help facilitate detachment of the encasing matrix with the CSCG from the culture plate reducing the need to agitate the plate. Lastly, increasing the collagen density of the CSCG relative to the encasing matrix can help reduce detachment.

Anticipated Results

Cells migrate preferentially through collagen aligned perpendicular to the CSCG. In addition, cells migrate efficiently through aligned collagen matrices in the direction of alignment. With the assay described in Basic Protocol 1, the cells exert contractile forces on the matrix between the CSCGs, aligning the collagen matrix (collagen fibers oriented ~90° relative to the CSCG boundary). In regions 90° from the CSCG-CSCG axis (i.e., the "top" and "bottom" of the CSCG) collagen is oriented parallel to the CSCG boundary, likely due to collagen being pushed during growth expansion of the CSCG. Moreover, due to force balance resistance to contraction, in regions in the "back" (i.e., 180° from the interface nearest to the other CSCG) collagen is also reorganized perpendicular to the CSCG. This provides a valuable control to discount the possibility that cell migration from one explant to another in regions of alignment is being driven by contact guidance and not being driven exclusively by chemoattraction between the two CSCGs. Likewise, in pre-aligned matrices (Basic Protocol 2), cells are expected to migrate preferentially into the collagen matrix with alignment perpendicular to the CSCG.

Time Considerations

After cells are detached from the culture plate for seeding into CSCGs, the steps should be performed decisively so that the cells do not remain in suspension for prolonged periods of time. In addition, care must be taken to ensure that the collagen matrices are adequately polymerized before transferring out of the culture, so that matrix integrity is maintained. Likewise, in step 14 of Basic Protocol 1, the time should be optimized so that the matrix is neither under or over polymerized, so that the CSCG can easily flow into the matrix, but does not immediately drop to the bottom of the culture dish. Overall, once optimized, the entire assembly procedure for Basic Protocol 1 can be performed in 1 to 2 hr, depending on the number of samples being constructed. Basic Protocol 2 takes ~2.5 hr to assemble, with additional time required as the number of samples increases.

Migration into the aligned collagen regions in both protocols begins within 24 hr and continues for days. The exact time will depend on the cell lines used as well as the dimensions of the assay and the matrix conditions. However, following migration over 14 days is not uncommon. It is worth noting, however, that in prealigned matrices, cells that migrate into the matrix can remodel the matrix and in some regions reduce alignment while promoting alignment in other regions. As such, time points for these experiments are typically shorter (~12 to 72 hr) to limit the influence of cell-mediated matrix remodeling. Longer times may be used, but changes in matrix architecture should be noted as they may influence the migration pattern.

Literature Cited

Condeelis, J. and Segall, J.E. 2003. Intravital imaging of cell movement in tumours. *Nat. Rev. Cancer.* 3:921-930.

Dallon, J.C., Sherratt, J.E., and Maini, P.K. 1999. Mathematical modelling of extracellular matrix dynamics using discrete cells: Fiber orientation and tissue regeneration. *J. Theor. Biol.* 199:449-471.

Denk, W., Strickler, J.H., and Webb, W.W. 1990. Two-photon laser scanning fluorescence microscopy. *Science* 248:73-76.

Diaspro, A. and Sheppard, C.J.R. 2002. Two-photon excitation fluorescence microscopy. *In* Confocal and Two-Photon Microscopy: Foundations, Applications, and Advances. (A. Diaspro, ed.) pp. 39-73. Wiley-Liss, Inc., New York.

Dickinson, R.B., Guido, S., and Tranquillo, R.T. 1994. Biased cell migration of fibroblasts exhibiting contact guidance in oriented collagen gels. *Ann. Biomed. Eng.* 22:342-356.

Doyle, A.D., Wang, F.W., Matsumoto, K., and Yamada, K.M. 2009. One-dimensional topography underlies three-dimensional fibrillar cell migration. *J. Cell Biol.* 184:481-490.

Friedl, P. and Gilmour, D. 2009. Collective cell migration in morphogenesis, regeneration and cancer. *Nat. Rev. Mol. Cell Biol.* 10:445-457.

Gehler, S., Baldassarre, M., Lad, Y., Leight, J.L., Wozniak, M.A., Riching, K.M., Eliceiri, K.W., Weaver, V.M., Calderwood, D.A., and Keely, P.J. 2009. Filamin A-beta1 integrin complex tunes epithelial cell response to matrix tension. *Mol. Biol. Cell* 20:3224-3238.

Grinnell, F., Rocha, L.B., Iucu, C., Rhee, S., and Jiang, H. 2006. Nested collagen matrices: A new model to study migration of human fibroblast populations in three dimensions. *Exp. Cell Res.* 312:86-94.

Guido, S. and Tranquillo, R.T. 1993. A methodology for the systematic and quantitative study of cell contact guidance in oriented collagen gels. Correlation of fibroblast orientation and gel birefringence. *J. Cell Sci.* 105:317-331.

Guo, C. and Kaufman, L.J. 2007. Flow and magnetic field induced collagen alignment. *Biomaterials.* 28:1105-1114.

Hegerfeldt, Y., Tusch, M., Brocker, E.B., and Friedl, P. 2002. Collective cell movement in primary melanoma explants: Plasticity of cell-cell

interaction, beta1-integrin function, and migration strategies. *Cancer Res*. 62:2125-2130.

Helmchen, F. and Denk, W. 2005. Deep tissue two-photon microscopy. *Nat. Methods* 2:932-940.

Ingman, W.V., Wyckoff, J., Gouon-Evans, V., Condeelis, J., and Pollard, J.W. 2006. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev. Dyn*. 235:3222-3229.

Lauffenburger, D.A. and Horwitz, A.F. 1996. Cell migration: A physically integrated molecular process. *Cell* 84:359-369.

Lee, P.F., Yeh, A.T., and Bayless, K.J. 2009. Nonlinear optical microscopy reveals invading endothelial cells anisotropically alter three-dimensional collagen matrices. *Exp. Cell Res*. 315:396-410.

Miron-Mendoza, M., Seemann, J., and Grinnell, F. 2008. Collagen fibril flow and tissue translocation coupled to fibroblast migration in 3D collagen matrices. *Mol. Biol. Cell* 19:2051-2058.

Mohler, W., Millard, A.C., and Campagnola, P.J. 2003. Second harmonic generation imaging of endogenous structural proteins. *Methods* 29:97-109.

Petrie, R.J., Doyle, A.D., and Yamada, K.M. 2009. Random versus directionally persistent cell migration. *Nat. Rev. Mol. Cell Biol*. 10:538-549.

Provenzano, P.P., Eliceiri, K.W., Campbell, J.M., Inman, D.R., White, J.G., and Keely, P.J. 2006. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med*. 4:38.

Provenzano, P.P., Inman, D.R., Eliceiri, K.W., Trier, S.M., and Keely, P.J. 2008a. Contact guidance mediated three-dimensional cell migration is regulated by Rho/ROCK-dependent matrix reorganization. *Biophys. J*. 95:5374-5384.

Provenzano, P.P., Rueden, C.T., Trier, S.M., Yan, L., Ponik, S.M., Inman, D.R., Keely, P.J., and Eliceir, K.Wi. 2008b. Nonlinear optical imaging and spectral-lifetime computational analysis of endogenous and exogenous fluorophores in breast cancer. *J. Biomed. Opt*. 13:031220.

Provenzano, P.P., Eliceiri, K.W., and Keely, P.J. 2009a. Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin. Exp. Metastasis*. 26:357-370.

Provenzano, P.P., Inman, D.R., Eliceiri, K.W., and Keely, P.J. 2009b. Matrix density-induced mechanoregulation of breast cell phenotype, signaling, and gene expression through a FAK-ERK linkage. *Oncogene*. 10:4326-4343.

Teixeira, A.I., Abrams, G.A., Bertics, P.J., Murphy, C.J., and Nealey, P.F. 2003. Epithelial contact guidance on well-defined micro- and nanostructured substrates. *J. Cell Sci*. 116:1881-1892.

Zipfel, W.R., Williams, R.M., and Webb, W.W. 2003. Nonlinear magic: Multiphoton microscopy in the biosciences. *Nat. Biotechnol*. 21:1369-1377.

Key References

Provenzano, P.P. et al. 2008a. See above
The source of procedures described in this unit.

Guo, C. and L.J. Kaufman. 2007. See above.
The original work from which the protocol for aligning collagen with magnetic beads was adapted.

Miron-Mendoza, M. et al. 2008. See above.
This study describes the use of nested matrices to study fibroblast migration in collagen matrices.

Imaging Cells in Three-Dimensional Collagen Matrix

Vira V. Artym[1,2] and Kazue Matsumoto[1]

[1]Laboratory of Cell and Developmental Biology, NIDCR, NIH, Bethesda, Maryland
[2]Lombardi Comprehensive Cancer Center, Georgetown University, Washington, D.C.

ABSTRACT

The use of in vitro three-dimensional (3-D) collagen matrices to mimic an in vivo cellular environment has become increasingly popular and is broadening our understanding of cellular processes and cell-ECM interactions. To study cells in in vitro 3-D collagen matrices, both cellular proteins and the collagen matrix must be visualized. In this unit, the authors describe the protocol and provide troubleshooting for immunolabeling of cells in 3-D collagen gels to localize and visualize cellular proteins with high-resolution fluorescence confocal microscopy. The authors then describe confocal reflection microscopy as a technique for direct imaging of 3-D fibrillar collagen matrices by discussing the advantages and disadvantages of the technique. They also provide instrument settings required for simultaneous imaging of cellular proteins with fluorescence confocal imaging and 3-D collagen fibrils with confocal reflection microscopy. Additionally, the authors provide protocols for a "cell sandwiching" technique to prepare cell cultures in 3-D collagen matrices required for high-resolution confocal imaging. *Curr. Protoc. Cell Biol.* 48:10.18.1-10.18.20. © 2010 by John Wiley & Sons, Inc.

Keywords: three-dimensional collagen matrix • collagen type I • Nutragen • confocal reflection microscopy • immunostaining of three-dimensional cell samples • invasion

INTRODUCTION

The research of the last decade shows that cells can sense chemical composition of the extracellular matrix (ECM) and its physical properties such as dimensionality, stiffness, and architecture. The use of in vitro 3-D collagen matrices to mimic in vivo cellular environments has become increasingly popular, and broadens our understanding of cell growth, survival, migration, and cell-ECM interactions that occur in vivo under physiologically normal and diseased conditions. To study cellular processes and cell-ECM interactions in an in vitro 3-D collagen matrix, both cellular proteins and the 3-D collagen matrix need to be visualized. Cellular protein localization and dynamics can be visualized using fluorescence microscopy of living cells expressing proteins of interest as a chimera tagged with green fluorescent protein (GFP) or a GFP variant, or of fixed cells immunostained with fluorescently labeled antibodies. The collagen matrix can be visualized through second harmonic generation using multiphoton microscopy, by directly incorporating fluorescently labeled collagen molecules/monomers into polymerized collagen fibrils and using fluorescence microscopy or confocal reflection microscopy.

In this unit, the authors describe the protocol for immunolabeling of cells in 3-D collagen gels to localize and visualize cellular proteins with high-resolution fluorescence confocal microscopy (see Basic Protocol 1). The protocol for fluorescence confocal microscopy of cellular proteins is designed for simultaneous direct imaging of 3-D collagen matrices with confocal reflection microscopy, in order to visualize cell-ECM interactions (see Basic Protocol 2). The authors also provide protocols for preparing cell cultures in 3-D

Current Protocols in Cell Biology 10.18.1-10.18.20, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb1018s48

collagen matrices, which are required for high resolution confocal imaging (see Support Protocols 1 and 2).

STRATEGIC PLANNING

In Vitro Three-Dimensional Collagen Type I Matrices

The in vitro 3-D collagen type I matrix is a convenient system that allows for the mimicking of the in vivo 3-D ECM of connective tissue, where collagen type I is a predominant ECM molecule. However, the modeling of an in vivo environment means taking into consideration (1) the preparation method of collagen type I used, (2) collagen concentration, and (3) conditions for 3-D collagen matrix polymerization. These factors determine the characteristics of 3-D collagen matrix produced, which affect how cells interact with the collagen matrix, and thus result in distinct cell morphology and cellular protein localization.

First, an in vitro 3-D fibrillar collagen matrix is a meshwork of collagen fibrils polymerized from collagen molecules or monomers. Collagen fibril formation occurs when soluble collagen is brought to physiological conditions of neutral pH and warmed to 20° to 37°C (Williams et al., 1978; Gelman et al., 1979). Conversely, collagen solution is prepared by solubilizing native fibrillar collagen at pH 2 and at a temperature reduced to 4°C. The native collagen type I molecule contains nonhelical amino acid sequences on the C- and N- ends that are called telopeptides. These C- and N- telopeptides aid in collagen fibril alignment and provide sites for the cross-linking of collagen fibrils in 3-D ECM (Eyre et al., 1984). Depending on the conditions of collagen monomer preparation from native fibrillar collagen, telopeptides can be preserved or removed from the collagen molecule. Thus, acid extraction of native fibrillar collagen with hydrochloric or acetic acid preserves telopeptides, whereas extraction of collagen with pepsin cleaves off telopeptides. It has been shown that cells cultured in reconstituted collagen matrices that lack telopeptides do not require proteases for matrix transmigration (Packard et al., 2009; Sabeh et al., 2009). Therefore, it is important to remember that the choice of the matrix could determine cell morphology and behavior as well as cellular protein localization and dynamics.

Second, the concentration of collagen monomers used to polymerize fibrillar collagen networks determines the density of the matrix and pore size. It has been proposed that the cells can undergo mesenchymal-ameboid transitions that allow for protease-independent cell transmigration through matrices of low density and high pore size, where cells squeeze through pores in the matrix (Even-Ram and Yamada, 2005). However, for the cell to navigate through a dense fibrillar collagen network, it needs to employ proteases to cleave collagen fibrils, which facilitate the squeezing of the cell body through the narrow pores (Sabeh et al., 2009).

Third, morphology of collagen fibrils and alignment of collagen microfibrils in reconstituted collagen matrices depends on ionic strength, pH, temperature, and amount of phosphate (Williams et al., 1978). The conditions selected for production of 3-D collagen matrices are usually experimentally convenient and produce reconstituted fibrils that mimic native fibrils but do not exactly recreate the morphology and alignment of native collagen fibrils. To avoid possible heterogeneity in results due to changes in collagen fibril morphology, one needs to follow the same conditions for 3-D collagen matrix assembly in each experiment.

"Cell Sandwiching" in A Three-Dimensional Collagen Matrix

High-resolution fluorescence microscopy requires the use of 63× and 100× high numerical aperture oil-immersion objectives that have a working distance in the range of 100 to 200 μm. The working distance of the objective is defined as the distance from

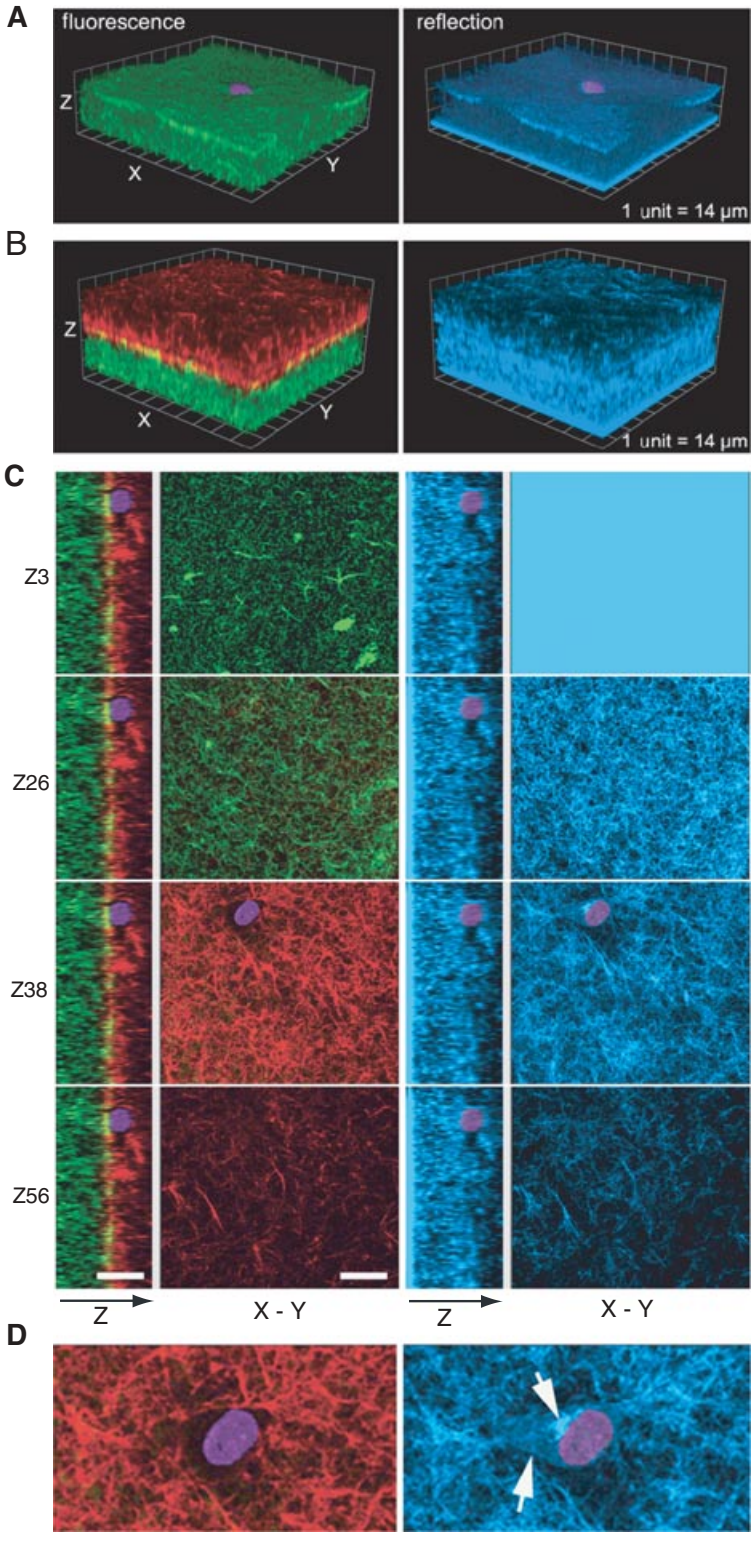


Figure 10.18.1 *(legend appears on next page)*

the front lens element of the objective to the closest surface of the coverslip when the specimen is in sharp focus. The working distance of the objective defines how deep into a 3-D collagen matrix one can image, i.e., it sets the limit on the distance of the cell to be examined with microscopy from the coverslip. The coverslip has its own thickness that is defined by the coverslip number (0.085 to 0.130 mm for number 0; 0.15 mm for number 1.0; 0.17 mm for number 1.5). Therefore, when subtracting the thickness of the glass coverslip from the working distance of the objective, one can estimate how far from the coverslip the cells can be located in the 3-D collagen matrix in order to be imaged with an inverted microscope. With the number 1 coverslip, which has a thickness of ~150 μm and a working distance of the objective of ~200 μm, one can image cells located in the range of 0 to ~50 μm away from the coverslip and deep into the 3-D collagen matrix. To position cells at the required distance from the coverslip and suspended in the 3-D collagen matrix, the authors of this unit developed conditions for "sandwiching" cells between two layers of collagen as described in Support Protocol 2. The authors used collagen conjugated to either fluorescent dye AlexaFluor 568 or AlexaFluor 647 to demonstrate the assembly of a 3-D collagen sandwich (Fig. 10.18.1). Using conditions provided in Support Protocol 2, the first collagen layer polymerized is between 30 and 35 μm in thickness. Cells plated on top of the first layer are consequently initially located at a distance from the glass coverslip that is suitable for high-resolution imaging. The second layer is polymerized on top of the first collagen layer and locks the cells in a 3-D collagen environment. Using collagen labeled with two different colors for labeling the two collagen layers demonstrates that collagen molecules from the second layer also penetrate into the first layer and bind to collagen fibrils in the first layer. This initiates fibril formation for the second collagen layer, ensuring that two collagen layers are "stitched" together with no free spaces in between (Fig. 10.18.2). The cells transmigrating within the 3-D collagen matrix move with different directions and angles relative to the glass surface of the coverslip and form large free spaces or tunnels. The alternative to the cell-sandwiching technique is mixing cells in collagen reconstituted to pH 7 before polymerizing collagen into a 3-D fibrillar matrix. The authors have found, however, that when using this latter procedure, many cells end up sinking through the matrix to the glass and then attaching to the glass coverslip. The cells that remain in the 3-D collagen matrix are usually situated at different distances from the glass coverslip, and are often too deep in the 3-D matrix to be imaged with high-numerical-aperture objectives.

Figure 10.18.1 *(appears on previous page)* Comparing fluorescence and reflection confocal microscopy for imaging of three-dimensional collagen sandwiches. (**A**) Assembly of a 3-D collagen sandwich involves polymerization of first collagen layer on glass surface, plating of the cells on this collagen layer, and (**B**) polymerization of the second collagen layer to encapsulate cells in a 3-D environment. The authors used collagen of two colors: one labeled with AlexaFluor 568 (in green) and another labeled with AlexaFluor 647 (in red) to demonstrate cell sandwiching between two collagen layers. Fluorescence microscopy was employed to visualize both collagen layers labeled with fluorescent AlexaFluor dyes (green and red). The collagen from the second layer penetrates into the first layer and stitches both layers together. The overlay of both layers is seen as yellow when green and red fluorescent dyes colocalize. Reflection confocal microscopy was used to visualize the collagen matrix of the sandwich (in blue). Cell nuclei were labeled with Hoechst (in magenta). (**C**) A series of X-Y slices through 3-D collagen sandwich taken at different Z-distances from the glass surface. Light reflected and scattered by the glass coverslip obscures images of collagen 3 μm in proximity to the glass surface in reflection confocal microscopy (Z3). Scale bar = 28 μm. (**D**) Light reflected from the cellular membranes contributes the cell outline to the image of collagen fibers (arrows). For color figure go to *http://www.currentprotocols.com/protocol/cb1018*.

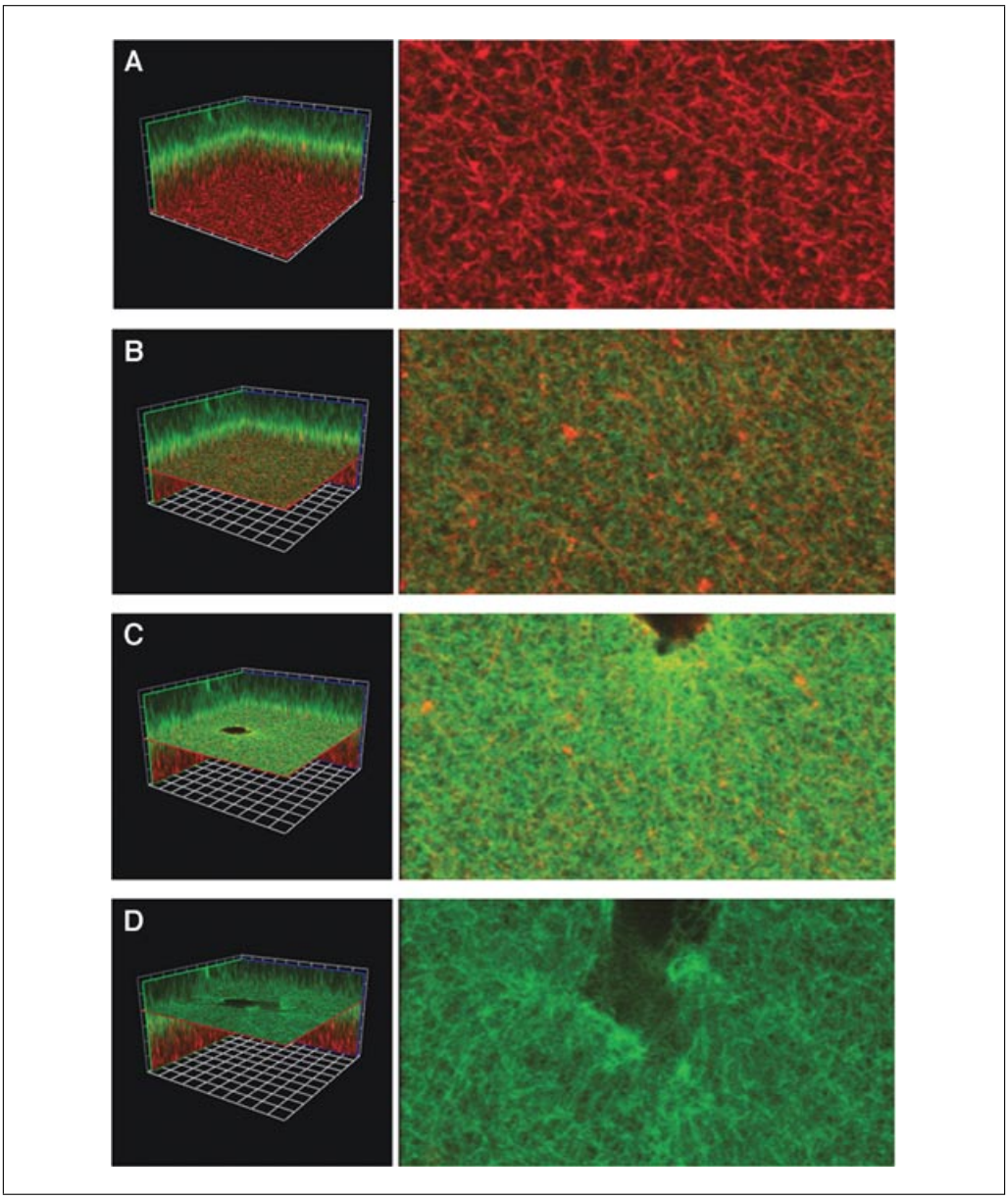


Figure 10.18.2 Morphology of collagen fibers in three-dimensional collagen sandwich. The authors used collagen labeled with AlexaFluor 647 (in red) to polymerize the first collagen layer, and collagen labeled with AlexaFluor 568 (in green) to polymerize the second collagen layer of a 3-D sandwich. X-Y slices taken at different Z-distances from the glass demonstrate that collagen from the second layer penetrates into the collagen of the first layer (**A**) and initiates collagen fibrils for the second layer of the three-dimensional collagen sandwich (**B**). The collagen fibers close to the glass surface appear straight (A and B), possibly from experiencing the tension from the stiffness of the glass. The collagen fibers of the second layer at a distance of about 30 μm and greater from the glass surface appear more wavy and relaxed (**C** and **D**). Unit size for the XYZ projection is 10 μm. For color figure go to *http://www.currentprotocols.com/protocol/cb1018*.

When culturing cells in an in vitro 3-D collagen system, one should test several conditions to establish what incubation time is appropriate for the cell line being studied, and for the collagen concentration to polymerize into a 3-D collagen matrix. Different cell types have different requirements regarding the time required to establish cell polarity and begin migrating within 3-D collagen matrices. The speed of cell migration within a 3-D collagen matrix depends on the density of the collagen matrix.

Microscope Instrumentation

Fluorescence imaging of cells in 3-D collagen matrices with simultaneous direct imaging of fibrillar collagen using reflection microscopy requires a laser scanning confocal microscopy system. Commercial confocal microscopes are equipped with multiple lasers and several photomultiplier tube detectors, which allow for simultaneous multilaser excitation, imaging of several different fluorophores, and confocal reflection imaging. Commercial laser scanning confocal systems include the Zeiss LSM510 and 710 META and NLO versions, Olympus FluoView, Leica TCS SPE, and Nikon Eclipse.

Confocal Reflection Microscopy

ECM fibers that differ in refractive index from their surroundings can reflect light. Laser-scanning confocal microscopy in reflection mode allows for the collection of reflected or back-scattered light from collagen fibers for each confocal plane, generating 3-D structural details of ECM that is not labeled with a fluorochrome (Fig. 10.18.1). Imaging of an unlabeled collagen 3-D matrix provides many advantages, by saving time and resources required for collagen labeling with fluorescent dye, by eliminating the issue of photobleaching that exists for fluorescently labeled collagen matrices, and by allowing for the use of unmodified collagen matrix for cell culture. Confocal reflection microscopy is easily combined with simultaneous fluorescence confocal imaging and does not require any additional modifications of the confocal microscope hardware. However, confocal reflection microscopy also has some limitations that need to be taken into account when preparing a sample for imaging. First, images of Z-planes in the proximity of 3 μm from the glass surface of the coverslip are obscured by bright uniform or nonuniform sources of background, which are caused by light reflection from the coverglass and interference from the optical pathway (Fig. 10.18.1C; Z3). Second, the intensity and contrast of the fibers diminish with the depth of the focus, due to laser light scattering and refraction (Fig. 10.18.1C; compare intensities of Z26, Z38, and Z56). The authors have found that the optimal range for confocal reflection imaging of collagen matrices is between 10 and 60 μm from the surface of the coverslip, and 10 to 60 μm deep into the 3-D collagen matrix. Third, cellular membranes, including the plasma membrane, Golgi, and endocytic vesicles, can also reflect light, contributing to the intensity of light reflected from the cell to the image of collagen fibers (Fig. 10.18.1D). The authors have found that the longer cells are incubated in the dense three-dimensional collagen matrices, the more the cells degrade and internalize collagen, which results in an increase in the number of endocytic vesicles that reflect laser light and contribute to the confocal reflection image.

BASIC PROTOCOL 1

IMMUNOLABELING CELLS IN THREE-DIMENSIONAL COLLAGEN MATRICES FOR IMMUNOFLUORESCENCE MICROSCOPY

This method describes how to fix and label samples of cells cultured in in vitro three-dimensional collagen type I matrices with fluorescently tagged antibody. Authors successfully applied this method of fixation and immunolabeling to different cell lines cultured in 3-D collagen type I matrices: fibroblasts, carcinoma, melanoma, and fibrosarcoma cell lines. The authors have used this protocol for immunolabeling of cells embedded in collagen matrices of different collagen type I concentrations that varied from 1.3 mg/ml to 6.5 mg/ml.

This method produced comparable immunostaining when different sources of collagen type I were used to prepare 3-D collagen matrices for cell culture. The authors used this protocol for the labeling of cells cultured in pepsin-extracted bovine collagen type

I that was devoid of telopeptide sequences, such as VITROGEN 100 (a former product of Cohesion; this product is now available as PureCol from INAMED Biomaterials) and Nutragen (a former product of INAMED Biomaterials), as well as collagen with intact telopeptide sequences, such as acid-extracted rat tail collagen type I (BD Biosciences), and mouse tail collagen type I (prepared by authors).

This protocol was developed for immunolabeling of 3-D collagen samples polymerized in the LabTek 8-well chambered coverglass systems that call for small volumes (400 μl) and allow for conserving antibodies. However, authors also used this protocol for immunolabeling of 3-D collagen samples polymerized in MatTek dishes (MatTek Co.) by scaling up all the reagents for volumes in the range of 1 to 2 ml.

Materials

4% (w/v) paraformaldehyde/5% (w/v) sucrose in PBS (see recipe)
Sample of cells in 3-D collagen gel polymerized in LabTek 8-well chambered coverglass (Support Protocol 2)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), filtered through a 0.22-μm filter
0.5% (v/v) Triton X-100 in PBS (see recipe)
Blocking solution (see recipe)
PBS/Tween (see recipe)
Primary antibody solution (see recipe)
Secondary antibody solution (see recipe)

Additional reagents and equipment for confocal microscopy (*UNIT 4.5*)

Fix the sample

1. Place the solution of 4% paraformaldehyde/5% sucrose in PBS in a water bath and preheat to 37°C.

2. Retrieve a sample of cells in 3-D collagen gel polymerized in LabTek 8-well chambered coverglass from the 37°C tissue culture incubator.

   Do not keep cell samples sitting at room temperature. Start fixing cell samples as described in the next step as soon as possible. Many cell lines are sensitive to temperature shifts. Keeping cell samples at temperatures that are lower than 37°C may cause cells to retract cellular protrusions. This will result in changes in cell morphology and, as a result, in altered immunostaining patterns for cellular proteins.

3. Gently aspirate cell culture medium from the well without damaging the 3-D gel, and slowly add 400 μl of the 4% paraformaldehyde/5% sucrose in PBS to the side of the well to fix the 3-D cell sample.

   Authors recommend using short glass Pasteur pipets attached to vacuum aspiration systems. Fit a 1000-μl plastic pipet tip without a filter on the Pasteur pipet and adjust the vacuum to a low setting to provide gentle aspiration. Always tilt the LabTek chamber to one side while aspirating to accumulate most of the liquid at one corner of the well where one inserts the pipet tip. Keep the pipet tip close to the well wall to avoid sucking up the matrix. Always leave about 25 μl of liquid above the matrix to ensure that you do not touch the matrix with the pipet tip, which can damage the matrix.

4. Incubate 20 min at room temperature.

5. Gently aspirate 4% paraformaldehyde/5% sucrose in PBS and wash the cell sample with 400 μl of PBS three times, each time adding and aspirating the liquid as described in step 3.

   Always add liquids slowly and to the side of the well to prevent damaging the 3-D matrix.

6. Aspirate PBS and add 400 μl of the 0.5% Triton X-100 in PBS to permeabilize the cells for immunostaining of the intracellular proteins. Incubate 10 min at room temperature.

7. Gently aspirate Triton X-100 solution from the well and wash sample with 400 μl of PBS in two short washing steps and one long washing step. For short washing, simply aspirate the liquid from the well and add fresh PBS; for long washing, add fresh PBS and incubate the sample for 30 min at room temperature.

   It is crucial to get rid of all the Triton X-100 in order to stop the permeabilization process and to ensure that it will not interfere with staining.

   At this point the sample can be stored at 4°C for ~1 week. Seal the LabTek chambered dish with Parafilm to prevent liquid evaporation and cell sample drying.

Stain the cells

8. Gently aspirate PBS and add 400 μl of blocking solution to the well to block the sample and prevent nonspecific binding of the antibodies. Incubate 30 min at room temperature.

9. Wash the sample with 400 μl of PBS/Tween one time.

10. Aspirate PBS/Tween and add 300 μl of primary antibody solution and incubate for 90 min at room temperature.

    Authors decrease the volume of the antibody solutions to 300 μl in order to spare the antibody used for the immunolabeling.

    One can use monoclonal or polyclonal antibody against the cellular proteins of interest. Refer to Critical Parameters and Troubleshooting on how to choose the right concentration of the antibody for immunostaining of 3-D samples. The primary antibody directly labeled with fluorochrome can also be used instead of the combination of primary antibody followed by fluorescently-conjugated secondary antibody.

    For the purposes of this unit, authors used phalloidin-AlexaFluor 488 to immunostain actin cytoskeleton, and mouse monoclonal anti-phosphotyrosine 4G10 primary antibody (Upstate Biotechnology) followed by donkey anti-mouse Cy5-conjugated secondary antibody (Jackson ImmunoResearch) to identify sites of tyrosine phosphorylation in carcinoma cells invading 3-D collagen matrix (Figure 10.18.6).

11. Wash the sample with 400 μl of PBS/Tween three times, each time incubating the sample for 10 min at room temperature.

12. Apply 300 μl of secondary antibody solution to the sample and incubate 60 min at room temperature.

13. Wash the sample with 400 μl of PBS/Tween three times, each time incubating the sample for 10 min at room temperature.

14. Wash with PBS three times for 10 min each.

15. Add 400 μl PBS to give the final sample.

Visualize the sample

16. Examine the sample with confocal microscopy (*UNIT 4.5*).

    At this stage, storage of the sample depends on the affinity of the antibody used for immunostaining. The sample is in the liquid and if stored, it will lose fluorescence labeling by many antibodies due to molecular diffusion, resulting in an increase of the background signal. Therefore, it is recommended that one proceed promptly to confocal microscopy after immunolabeling is complete. For some high-affinity antibodies, the sample can be stored overnight at 4°C and examined in the morning the next day. The possibility of sample storage needs to be determined by testing each antibody preparation.

COLLAGEN PREPARATION FOR THREE-DIMENSIONAL COLLAGEN MATRICES

Aliquot and neutralize collagen in the tissue-culture hood to prevent collagen contamination.

Materials

10× DMEM with phenol red (see recipe)
10× reconstitution buffer (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), filtered through a 0.22-μm filter
2 N NaOH
2 N HCl
9.44 mg/ml solution of collagen type I in 0.02 N hydrochloric acid (from BD Biosciences)
ColorpHast pH-indicator strips with pH range 6.5–10.0 (EMD Biosciences, *http://www.emdchemicals.com/*)

Prepare reagents

1. Defrost aliquots of 10× DMEM and 10× reconstitution buffer on ice.
2. Pre-chill microcentrifuge tubes, PBS, 2 N NaOH, and 2 N HCl that will be used for collagen preparation, on ice.
3. Calculate the amount of collagen that is needed for the experiment.

   Always calculate 20% more to account for pipetting difficulties due to the high viscosity of collagen.

   If a positive-displacement pipet is available to aliquot the viscous collagen solution, then the calculated volume of collagen solution should be used without additional 20% of collagen volume.

   To prepare a sample of cells sandwiched in three-dimensional collagen type I in one well of the LabTek 8-well chambered coverglass, as described in Support Protocol 2, one will need 150 μl of collagen at the final concentration required for 3-D matrix preparation. For the example, the authors used collagen at 2.5 mg/ml final concentration to prepare 3-D collagen sandwiches. If multiple wells of the LabTek chambered coverglass to be used, then the amount of the collagen needs to be adjusted accordingly.

Prepare collagen

4. Pipet the required amount of stock collagen solution into a prechilled microcentrifuge tube. Keep the tube with collagen on ice during the subsequent steps.
5. First, add 10× DMEM at one-eighth of the volume of collagen solution added, and mix by slowly and gently pipetting up and down. Observe the change in color of phenol red of DMEM.

   The phenol red will change from red to yellow, indicating that the pH of collagen solution is acidic.

6. Second, add 10× reconstitution buffer at one-eighth of the volume of collagen solution added, and mix by slowly and gently pipetting up and down.

   The phenol red will change from yellow to light pink, indicating that the pH of the collagen solution is shifting to basic.

   As an example, to neutralize 400 μl of rat tail collagen stock, add 50 μl of 10× DMEM and 50 μl of 10× reconstitution buffer.

   Always pipet the collagen solution up and down slowly and gently without introducing air bubbles. Harsh pipetting can cause collagen to precipitate and form clumps. Always keep the tube with collagen solution on ice, since a change in the pH of collagen solution to neutral, and an increase in temperature will induce polymerization of collagen fibrils.

7. Use a pH paper strip to measure the pH of the neutralized collagen: pipet a small drop of neutralized collagen on the strip of pH paper, and, using the color scale, determine the pH of the neutralized collagen solution.

   The pH must be in the range of 7.1 to 7.4. Use the ice-cold solution of 2 N NaOH or 2 N HCl to adjust the pH if needed. Add the solution of NaOH or HCl in small portions, mix gently by pipet, and re-measure pH with pH paper.

8. Incubate the neutralized solution of collagen on ice for 3 to 5 min to allow the pH of the solution to equilibrate.

9. Microcentrifuge collagen solution 3 min at 10,000 rpm, 4°C, to get rid of air bubbles.

10. Re-measure the pH of the neutralized collagen solution to ensure that it falls in the range between 7.1 and 7.4. If needed, adjust pH again, as described in step 7, using the ice-cold solution of 2 N NaOH or 2 N HCl, and then repeat steps 8 to 9.

Dilute collagen solution

11. Depending on the final concentration of the collagen that one needs to use, calculate a dilution ratio of neutralized collagen solution and PBS. Use ice-cold PBS to dilute neutralized collagen.

    Mix collagen and PBS solution slowly and gently by pipetting up and down—collagen can clump if this procedure is performed with haste.

    As an example, the following calculations can be used for preparing 2.5 mg/ml collagen solution from 400 μl of 9.44 mg/ml collagen stock. First, add 100 μl of 10× DMEM and 10× reconstitution buffer mixture to neutralize the collagen solution. This brings the collagen concentration to 7.56 mg/ml. Next, dilute the neutralized collagen with PBS in 1:2 dilution by mixing 500 μl of neutralized collagen with 1000 μl of ice-cold PBS in order to obtain a collagen solution with a final concentration of 2.5 mg/ml.

12. Microcentrifuge collagen solution 3 min at 10,000 rpm, 4°C, to get rid of air bubbles.

    Keep neutralized collagen solution on ice and use within 1 hr. Do not store it.

SUPPORT PROTOCOL 2

POLYMERIZATION OF COLLAGEN THREE-DIMENSIONAL GELS AND PREPARATION OF THREE-DIMENSIONAL CELL CULTURE

Perform all steps of the protocol under the tissue-culture hood to prevent bacterial contamination of the sample.

Materials

Neutralized solution of rat tail collagen diluted to a final concentration of 2.5 mg/ml (Support Protocol 1)
Cell culture medium
Cell suspension (e.g., MDA-MB-231 breast cell carcinoma line; ATCC no. HTB-26) at 1.25×10^4 cells/ml in cell culture medium
LabTek 8-well chambered coverglass (borosilicate no. 1.0 coverglass; Nunc, cat. no. 155411)
37°C, 10% CO_2 cell culture incubator
Tissue culture microscope with 10× objective and phase-contrast optics

Prepare collagen gel on coverglass

1. Place LabTek 8-well chambered coverglass on ice and allow it to chill. Keep neutralized solution of rat tail collagen on ice.

2. Warm cell culture medium in a 37°C water bath.

3. Drop 15 μl of ice-cold neutralized collagen solution on the glass surface of the well of the LabTek chambered coverglass, and, with gentle strokes, spread the collagen solution on the glass surface using the pipet tip. Keep LabTek chambered coverglass on ice while spreading collagen.

   There are eight chambers in each LabTek 8-well chambered coverglass. All eight of them can be used to prepare separate 3-D collagen cultures by following this protocol and assembling individual "collagen cell sandwiches" in each well.

4. Place LabTek coverglass in a 37°C, 10% CO_2 cell culture incubator for 25 min to allow collagen to polymerize and form fibrillar meshwork.

5. Use a tissue culture microscope equipped with 10× objective and phase-contrast optics to confirm formation of fibrillar collagen matrix.

   Neutralized collagen is a transparent viscous solution. Warming neutralized collagen to 37°C triggers polymerization of collagen fibrils, which result in formation of fibrillar matrix. Collagen fibrils are detectable with phase microscopy as a network of the fine filaments.

   Slight changes in pH or temperature affect the rate of collagen polymerization as well as the size of collagen fibrils. The concentration of collagen used for matrix polymerization also affects the size of collagen fibrils. It is required that one monitor fibril formation with phase-contrast microscopy to confirm polymerization of fibrillar collagen and to ensure consistency in the size of collagen fibrils. Plating cells on a collagen matrix that did not polymerize well will result in cell adhesion to the glass bottom of the well, detachment of the collagen matrices from the glass, and floating of the matrices.

   While polymerizing collagen matrix, do not allow matrix to dry. Dry matrix becomes opaque in appearance to the naked eye. Drying of collagen matrices changes the physical properties of the matrix, which affects its cellular response.

Prepare cells

6. During the time of collagen polymerization, detach cells to be examined, count them, and prepare cell suspension of 1.25×10^4 cells/ml in cell culture medium.

   Cell density should be adjusted for each cell line and for the requirements of the experimental design. It will vary depending on the size of the cells, on the cells' ability to grow and divide in 3-D collagen matrix, and on the duration of cell incubation in 3-D collagen matrices.

7. Retrieve LabTek coverglass system from the incubator and place it on a Kimwipe under the hood.

   Place LabTek coverglass system on a Kimwipe to avoid direct contact with a cold surface, since decrease in temperature can destabilize the collagen matrix at the glass surface, resulting in the matrix detaching and floating.

8. Add 400 μl of cell suspension on top of the polymerized collagen layer.

   Add cell suspension slowly to the well with polymerized collagen matrix, and always pipet to the side of the well to prevent damaging the matrix.

9. Incubate LabTek coverglass in a 37°C, 10% CO_2 for 30 min, allowing cells to attach to the collagen matrix.

10. Use tissue culture microscope equipped with 10× objective to check if cells are attached to the matrix.

    Place LabTek coverglass on microscope stage, focus on the cells, and, while examining cells under the microscope, gently shake the LabTek coverglass system. If cells are attached, they will move together with the matrix and will not float in the culture medium.

11. Retrieve LabTek coverglass system from the incubator and place it on the Kimwipe under the hood. Gently aspirate cell culture medium from the well.

Use Pasteur pipet equipped with 1000-μl pipet tip for liquid aspiration from the well. Always place pipet tip on the wall of the well above the matrix and tilt the LabTek coverglass system to gather all the liquid in the corner where the pipet tip is inserted.

Prepare the sandwich

12. Coat adherent cells with a layer of collagen by applying 80 μl of collagen dropwise on top of the cells adherent to the first collagen layer.

13. Place LabTek coverglass system in the 37°C, 10% CO_2 incubator and incubate for 30 min to polymerize the second layer of fibrillar collagen.

14. Confirm collagen layer polymerization with phase-contrast microscopy.

15. Retrieve LabTek coverglass system from the incubator and place it on the kimwipe under the hood. Gently add 400 μl of cell culture medium (preheated to 37°C) to the well.

16. Place LabTek coverglass system into the 37°C, 10% CO_2 incubator and incubate for 24 hr to allow cells to establish polarity and start migrating in the 3-D collagen matrix.

Incubation time for cells in 3-D matrices should be established experimentally and will depend on cell type, density of the matrix, and experimental design.

BASIC PROTOCOL 2

SIMULTANEOUS FLUORESCENCE CONFOCAL IMAGING OF CELLS AND CONFOCAL REFLECTION IMAGING OF COLLAGEN IN IN VITRO THREE-DIMENSIONAL GELS

Here the authors provide general procedures for configuring the confocal microscope hardware for simultaneous fluorescence and reflection imaging of three-dimensional collagen samples. They also provide practical suggestions on image acquisition and analysis.

Materials

Immersion oil

Sample of cells in three-dimensional collagen gel polymerized in LabTek 8-well chambered coverglass and immunostained for proteins of interest (Basic Protocol 1)

Inverted confocal microscope equipped with high NA 63× and/or 100× oil immersion objectives (also see *UNIT 4.5*)

Emission filters for specific emission wavelength of chromophores conjugated to anti-bodies used for immunostaining (Chroma, Omega, or available from confocal microscope manufacturer)

Image analysis software

Additional reagents and equipment for confocal microscopy (*UNIT 4.5*)

1. Clean the microscope objective with cleaning solution provided by the manufacturer using lens paper.

A 63× 1.4 NA or 100× 1.4 NA oil immersion objective is recommended for high-resolution imaging. Presence of oil or a dirty objective can result in the formation of interference patterns that deteriorate image quality.

2. Apply one small drop of the immersion oil on the objective lens.

3. Place the LabTek chamber with the specimen on the microscope stage and focus on the sample.

4. Configure confocal microscope hardware for simultaneous imaging in fluorescence and reflection modes.

   Conventional confocal systems with three photomultiplier (PMT) detectors allow for the use of two channels for simultaneous fluorescence imaging of two different fluorophores, and one channel for reflection imaging. To set a configuration for confocal reflection imaging, simply use a separate laser line to illuminate the sample and collect all the light reflected from the sample to the separate PMT detector. Do so by putting no emission filter in front of the detector, or by using a band-pass emission filter that collects the light of the wavelength used to illuminate the sample. Any laser line or PMT can be used for confocal reflection imaging; however the variations in reflection signal intensity and contrast may occur depending on the laser light intensity and PMT sensitivity. In Figures 10.18.3 to 10.18.5, authors present configuration settings that they have used for simultaneous fluorescence imaging with 488-nm and 633-nm laser lines, and reflection imaging with a

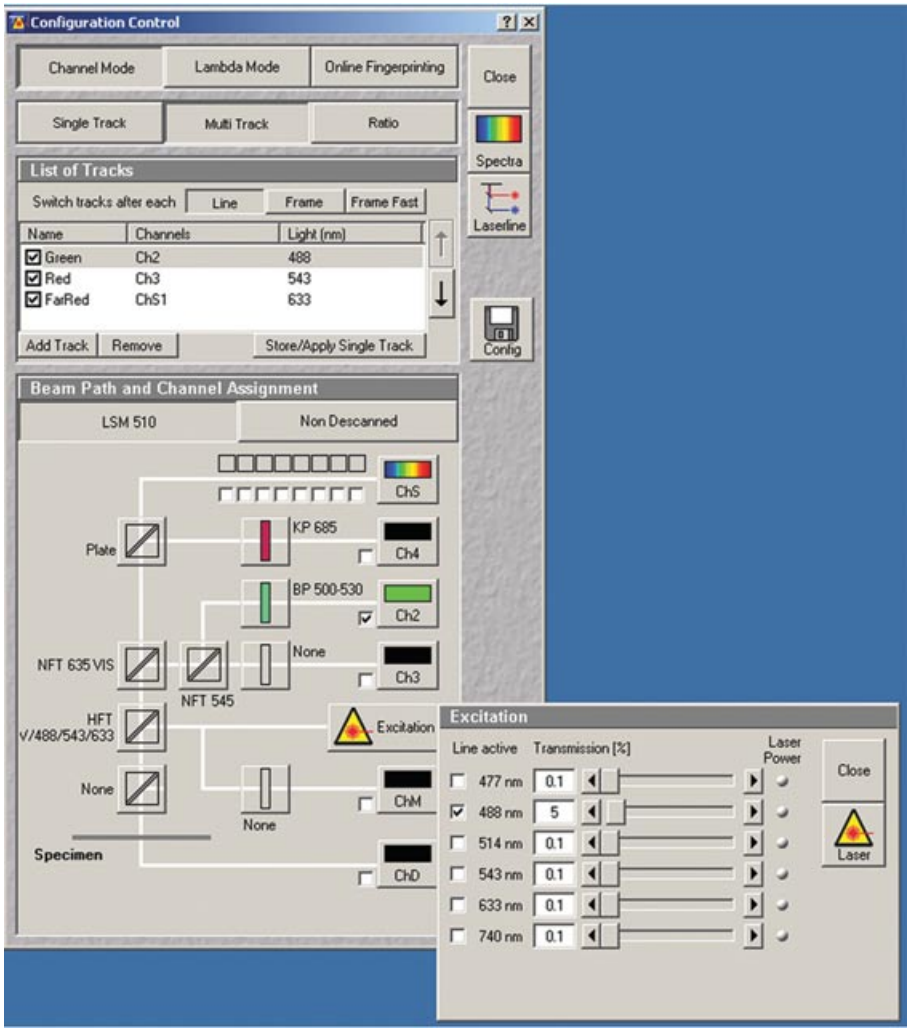


Figure 10.18.3 Configuration for fluorescence imaging with 488-nm excitation for three-channel simultaneous fluorescence, and reflection confocal microscopy with the Zeiss510 NLO imaging system.

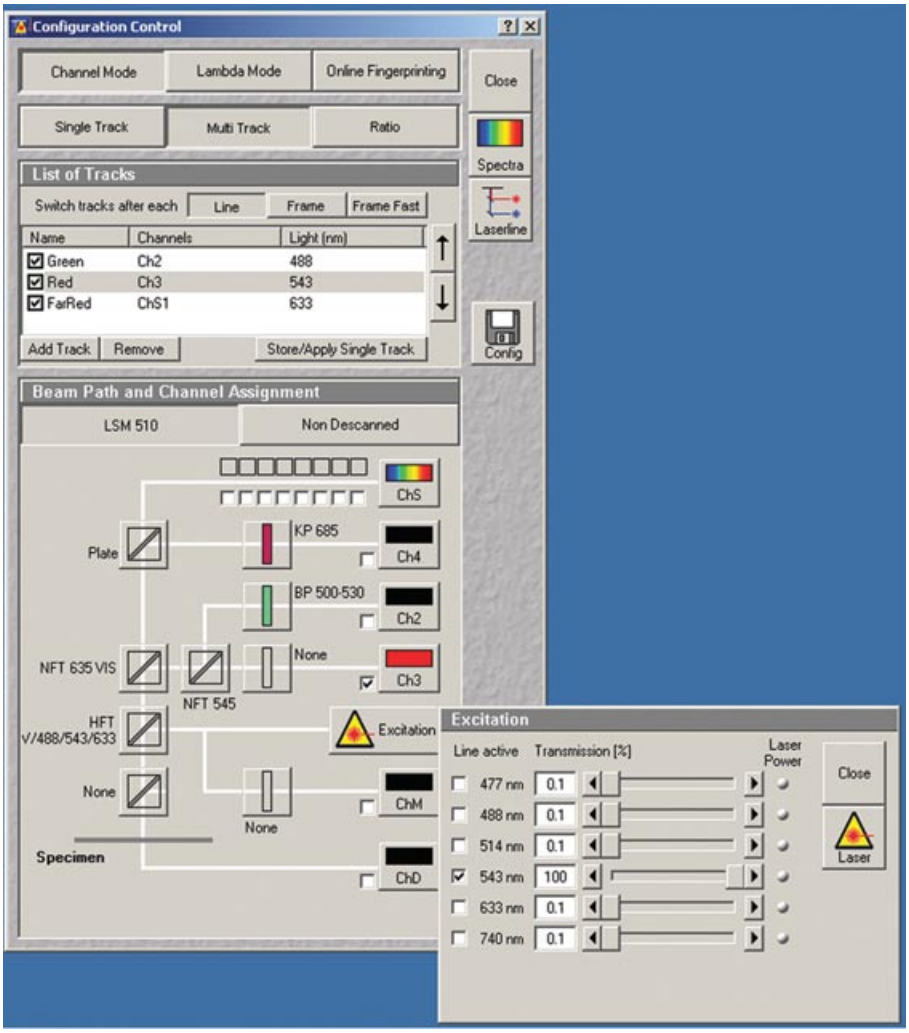


Figure 10.18.4 Configuration for reflection imaging with 543-nm excitation for three-channel simultaneous fluorescence, and reflection confocal microscopy with the Zeiss510 NLO imaging system.

543-nm laser line with a Zeiss510NLO system equipped with META detector. For detailed discussion on the principles and practice of confocal microscopy, see UNIT 4.5.

5. Initiate image acquisition.

Image acquisition is specific to the particular question being addressed, and should be developed by the researcher accordingly. A single confocal plane or Z-stacks of multiple confocal planes can be acquired to demonstrate the localization of the protein/proteins being studied in the cell, colocalization of the proteins, and/or cell interaction with the collagen three-dimensional matrix. Acquiring Z-stacks of whole 512×512 images takes a matter of minutes, and timing depends on the image-acquisition settings such as the number of confocal planes, the speed of acquisition, and the number of scans being averaged.

When acquiring Z-stacks, consider the following: (1) the reflection signal from the collagen matrix attenuates with the increase in three-dimensional volume being imaged, and therefore the differences in collagen matrix intensities will be greater between the first and last Z-slices, with the increase in distance between them; (2) increasing the size of the Z-stack and using low scan speed and a high number of scans for averaging, which is required for a contrast image, can result in photobleaching of some of the fluorophores used for cell immunostaining. To obtain the best image, one can consider selecting the

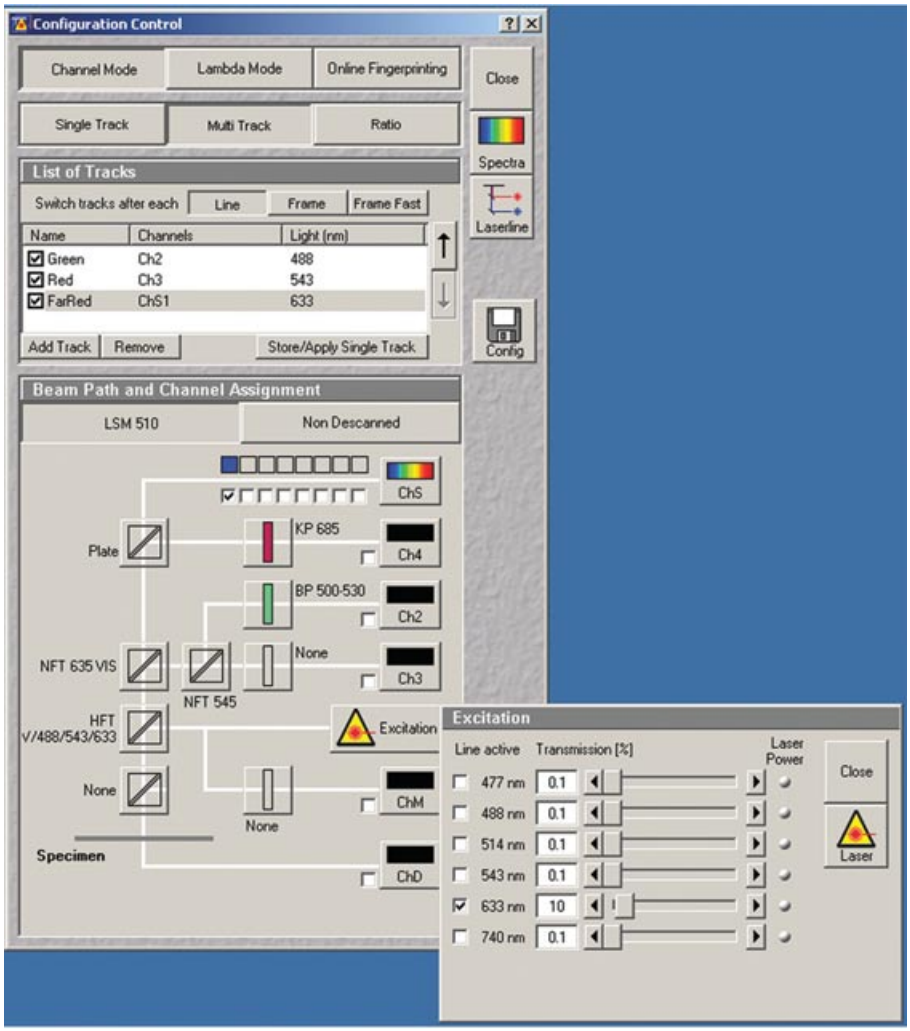


Figure 10.18.5 Configuration for fluorescence imaging with 633-nm excitation for three-channel simultaneous fluorescence, and reflection confocal microscopy with the Zeiss510 NLO imaging system.

region of interest (ROI) for imaging smaller three-dimensional volume with oversampling of Z-slices for the best three-dimensional image reconstruction.

6. Record and store an image.

7. Proceed with image visualization and analysis.

   Image analysis usually depends on the question being addressed by the researcher and the way the data needs to be presented to visualize the experimental result (Fig. 10.18.6). There are many commercial and free software packages available for image visualization and analysis. Among them the most popular are: MetaMorph (Molecular Devices), Volocity (PerkinElmer), AutoQuant (MediaCybernetics), and ImageJ (a free, stand-alone software available through NIH, http://rsbweb.nih.gov/ij/). The MetaMorph package offers great presentation, manipulation, and image analysis of single Z-planes; however its three-dimensional image reconstruction function is inferior to that of the Volocity and AutoQuant software, or ImageJ plug-ins. Commercial laser-scanning confocal imaging systems usually come with software that incorporates three-dimensional image reconstruction of different degrees of complexity depending on the manufacturer.

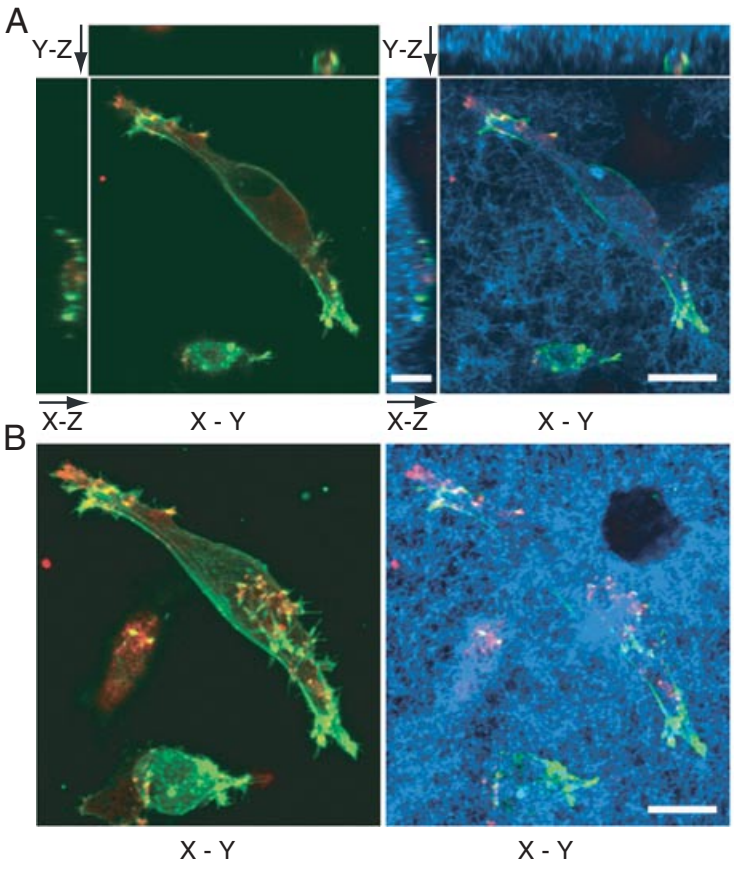


Figure 10.18.6 Breast carcinoma cells transmigrating through the three-dimensional collagen matrix. The actin cytoskeleton was labeled with phalloidin-AlexaFluor 488 (green) and the sites of tyrosine phosphorylation were labeled with anti-phospho-tyrosine primary antibody, followed by secondary Cy5-conjugated antibody (red). Collagen matrix was imaged with reflection confocal microscopy (blue). Images were acquired with a 63× 1.4 NA oil objective. Volocity software was used for image presentation as XYZ projections (**A**) and extended focus (**B**). Scale bars = 19 μm. For color figure go to *http://www.currentprotocols.com/protocol/cb1018*.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking solution

Prepare a fresh solution of 20% (v/v) donkey serum in 1× M.O.M. blocking reagent from the VECTOR M.O.M. Immunodetection kit (Vector Laboratories). Always make this solution fresh and do not store.

Collagen type I, 9.44 mg/ml, in 0.02 N hydrochloric acid

Obtain solution from BD Biosciences. Always keep the solution of collagen on ice. Refrigerate collagen solution up to 6 months at 4°C and never freeze.

There are several different commercially available sources of collagen type I. The protocol for 3-D cell culture polymerization can be used with any mouse tail collagen, commercially-available rat tail collagen type I (BD Biosciences; cat. no. 354249) or PureCol bovine type I collagen (INAMED).

DMEM, 10×, with phenol red

Dissolve DMEM powder (Invitrogen) into 0.1 volume (i.e., one-tenth that recommended by the manufacturer) of distilled water to make a 10× solution. Centrifuge 5 min at 13,000 × *g* to remove all the precipitate. Collect supernatant and filter it through a 0.22-μm vacuum filter to sterilize. Aliquot collected supernatant (500 μl) and store at up to 1 year at −20°C.

Always defrost fresh aliquot on ice before preparing 3-D collagen gels.

A precipitate will form; however this is normal because DMEM does not dissolve completely in this reduced amount of water. The loss of the DMEM components during precipitate formation does not effect the preparation of the neutralized collagen solution.

Paraformaldehyde, 4% (w/v)/sucrose, 5% (w/v)

Prepare a fresh solution of 4% (w/v) paraformaldehyde (electron microscopy grade) and 5% (w/v) sucrose in PBS (see *APPENDIX 2A* for PBS). Filter the solution through a 0.22-μm vacuum filter. Store up to 2 weeks at room temperature. Preheat this solution to 37°C on water bath before fixing cells.

PBS/Tween

Prepare a fresh solution of 0.05% (v/v) Tween 20 in PBS (*APPENDIX 2A*). Put stir bar into a bottle with the solution and stir on magnetic stirring plate until all the Tween 20 is dissolved. Filter the solution through a 0.22-μm vacuum filter. Prepare fresh.

Primary antibody solution

Prepare a fresh solution of primary antibody at 20 μg/ml final concentration in M.O.M. diluent of VECTOR M.O.M. Immunodetection kit (Vector Laboratories). Always make this solution fresh and do not store. To prepare M.O.M. diluent, mix 80 μl of M.O.M. (from the kit) in 1 ml of PBS (*APPENDIX 2A*).

Reconstitution buffer, 10×

Dissolve 2.2 g of sodium bicarbonate and 4.8 g of HEPES in 100 ml of distilled water. Filter through a 0.22-μm vacuum filter to sterilize, then aliquot (500 μl) and store up to 1 year at −20°C. Always defrost fresh aliquot on ice before preparing 3-D collagen gels.

Secondary antibody solution

Prepare a fresh solution of fluorescently-conjugated secondary antibody (Jackson ImmunoResearch), appropriate for the primary antibody used, in PBS/Tween (see recipe) containing 5% donkey serum. Use twice the concentration of secondary antibody that is recommended by the manufacturer. Prepare secondary antibody as recommended by the manufacturer and then dilute the amount required for immunostaining of the antibody in PBS/Tween (see recipe) containing 5% donkey serum. Always make this solution fresh and do not store.

Triton X-100, 0.5% (v/v) in PBS

Prepare 0.5% solution of Triton X-100 in PBS (see *APPENDIX 2A* for PBS) by diluting 10% stock solution of Triton X-100 in PBS, with stirring. Filter the solution through a 0.22-μm vacuum filter. Store up to 2 weeks at room temperature.

COMMENTARY

Background Information

Cellular processes and their underlying signaling events have been studied extensively in two-dimensional (2-D) cell models where cells are plated on glass, plastic, or 2-D non-fibrillar matrices representing different ECM ligands absorbed on glass/plastic surfaces. However, the flat and rigid 2-D surface neither represents the intricate topography of fibrillar matrices nor conveys physico-elastic properties of the in vivo ECM. Recent advances in comparing cell growth and function on different culture surfaces, as well as the efforts made to design model systems that mimic physiological in vivo conditions, have provided strong evidence that matrix topography, stiffness/rigidity, dimensionality, and composition are essential for reproducing physiological patterns of cell adherence, migration, cytoskeleton organization, signal transduction, differentiation, and cellular response to extracellular cues (Even-Ram and Yamada, 2005; Pedersen and Swartz, 2005; Schindler et al., 2006; Petrie et al., 2009). Therefore, 3-D in vitro models have become widely used for the studies of cellular biology.

Collagen type I is the most abundant fibrous protein of interstitial ECM. For that reason, it is commonly used for in vitro 3-D assays to study various cell types and different cellular processes, such as cell migration, invasion, angiogenesis, differentiation, and many others. Although reconstituted three-dimensional collagen type I cannot completely recreate in vivo ECM, it provides the structural integrity of a 3-D environment and basic cell adhesion functions (Pedersen and Swartz, 2005).

Microscopy is the most informative way to study cells embedded in 3-D collagen matrices. Confocal microscopy allows for optical sectioning of 3-D samples, enabling identification of cellular protein localization in differential cellular compartments as well as its relationship to the fibrillar ECM. Time-lapse confocal imaging provides valuable information on dynamics of cellular processes; however, this method requires the expression of cellular proteins tagged with green fluorescent protein (GFP) or GFP variants. Confocal microscopy of fixed and immunolabeled cellular proteins provides valuable information on endogenous proteins that supplements and verifies the understanding of cellular processes identified with the expression of exogenous GFP–tagged proteins. The biggest advantage of applying laser-scanning confocal microscopy to imaging of 3-D collagen cell samples is that it facilitates the simultaneous fluorescence imaging of cellular proteins and reflection imaging of the 3-D fibrillar ECM. Reflection imaging allows for studies and visualization of intact unmodified fibrillar matrix, eliminating any effect of matrix perturbation on the cell. Applying high-resolution confocal imaging for cellular imaging in 3-D collagen matrices sets limitations on 3-D sample preparation with regard to working distance of an objective. The cells need to be distanced from the glass coverslip to be accessible for imaging with a high-numerical-aperture objective. Plating cells in 3-D collagen matrix at the depth required for high-resolution imaging can be achieved with "cell sandwiching" between two collagen layers (Support Protocol 2).

Critical Parameters and Troubleshooting

Immunostaining of cells in three-dimensional collagen matrix

The choice of which antibody is used for immunostaining of cells embedded in 3-D collagen matrix is extremely important and may require some modification of the immunostaining protocol to accommodate differences in antibody affinity. To achieve successful immunostaining of 3-D samples, it is highly recommended to first test the antibody for immunostaining using 2-D cell samples. Simply plate cells on glass coverslips or, if required, coverslips precoated with the ECM molecule that facilitates cell adhesion, and then incubate cells to allow them to adhere. Next, fix the cells with 4% glutaraldehyde/5% sucrose in PBS for 20 min. If the antibody is to be used for immunostaining of intracellular proteins, permeabilize cells with 0.5% Triton X-100 in PBS for 10 min. Then, proceed with immunolabeling of the cell sample using the primary and secondary antibodies. If immunostaining of the cells in the 2-D cell sample gives a weak signal, using that antibody combination will result in poor staining of the cells in 3-D collagen gels. The described protocol for immunostaining of cells in 3-D collagen gels requires double the amount of the primary and secondary antibodies used for immunostaining of the cells in the 2-D cell system. This is necessary to achieve a sufficient concentration of the antibody required for immunolabeling, since in 3-D collagen gels, the solid fraction of

the fibers is about 1% by mass while the other 99% consists of water (Pedersen and Swartz, 2005). If a low-affinity antibody is to be used for immunolabeling of three-dimensional collagen samples, the authors recommend tripling the amount of the primary antibody or increasing incubation time for the labeling. It is crucial to remember that increasing the thickness of the 3-D collagen gels will require increasing incubation times for the primary and secondary antibodies to diffuse through the 3-D matrix.

Controls

It is recommended that the binding specificity of the primary and secondary antibodies be checked by performing the following controls. (1) Perform immunostaining of the 3-D collagen samples with the secondary antibody only to check for specificity of the secondary antibody. (2) Use GFP chimeras of the protein being studied to compare and confirm the pattern of endogenous protein localization in cells cultured in 3-D collagen gels. (3) If available, use specific protein knock-out cells to test for the specificity of the primary antibody.

Sample fixation and permeabilization

Immunostaining of different intracellular proteins may require different conditions for sample fixation and permeabilization to obtain the best immunostaining and, as a result, the best confocal image. This rule applies to immunostaining of samples in two- as well as three-dimensional in vitro systems. As alternatives to the provided protocol, one might consider testing several conditions for fixation and permeabilization by varying the duration of permeabilization with Triton X-100, or by combining fixation and permeabilization in one step. For immunostaining of some of the focal adhesion proteins, the authors have used the following fix/permeabilize protocol: permeabilize cells with 4% paraformaldehyde/5% sucrose/0.5% Triton X-100 in PBS for 5 min at room temperature, and then fix with 4% paraformaldehyde/5% sucrose in PBS for 20 min at room temperature. One should keep in mind that the variations in fixation/permeabilization procedures followed by the same immunolabeling protocol could yield differences in protein immunostaining patterns. This could be due to the effect of the fixation and permeabilization on the retention and accessibility of the protein being labeled, or the sample-penetration capacity for the antibody.

"Cell sandwiching" in three-dimensional collagen

Proper polymerization of the cells between two collagen layers is important in positioning cells at the distance from the glass that allows for confocal imaging with high-numerical-aperture objectives. If it is difficult to focus on the cells with 63× or 100× objectives, then the working distance of the objective does not permit viewing the cells. In this case, consider switching to a low-magnification objective that has a long working distance to assess the distance of the cells from the glass, by acquiring a Z-stack using reflection microscopy. Then, measure the distance between the first Z-slice with the image of the matrix at the glass surface, which will have a high background of light scattered from the glass, and the Z-slice with the image of the reflection from the cell body. This measurement, performed for several cells in the different parts of the 3-D sample, will give an approximation of the thickness and uniformity of the first collagen layer polymerized on the glass surface. To correct for collagen layer thickness, determine if spreading of the collagen solution on the glass surface of the coverglass system pre-chilled on ice is sufficient. If it is required to make the collagen layer thinner to match the working distance of the objective, then the amount of collagen used for polymerization of the collagen layer can be slightly decreased. Alternatively, select a high-numerical-aperture objective with a longer working distance. Also, in some cases, it becomes impossible to focus on the cells because the 3-D collagen matrix is detached from the glass surface and floats in the medium. Such samples are not suitable for imaging. Usually, 3-D collagen matrices detach from the glass for the following reasons. (1) The first layer was not well polymerized before plating cells on it. Always use phase microscopy to check for collagen fibril formation when preparing the sample as described in the protocol. (2) The sample underwent harsh treatment resulting from applying excessive suction forces when aspirating solutions from the well, or from dropping solution on top of the matrix instead of letting it drip and flow on the side wall of the well.

Anticipated Results

Support Protocols 1 and 2

Bringing collagen solution to a physiological pH of 7.1 to 7.4 will allow for polymerizing fibrillar collagen for 3-D collagen matrix using the "cell sandwiching"

technique. Polymerizing cells between two collagen layers should result in a cell sample in the 3-D collagen matrix suitable for high-resolution confocal microscopy.

Basic Protocol 1

Immunolabeling of the cells in a 3-D collagen matrix should result in a sample that is ready for fluorescence confocal imaging of the cellular proteins being studied.

Basic Protocol 2

Simultaneous fluorescence and reflection confocal imaging of the 3-D cell sample will result in collecting images of fluorescently labeled cellular proteins with fluorescence microscopy and unperturbed collagen matrix with reflection confocal microscopy. Z-stacks collected with confocal microscopy will allow for 3-D image reconstruction. The typical confocal immunofluorescence image is presented in Figure 10.18.6.

Time Considerations

Support Protocol 1

Preparation of the solution of collagen required for polymerization of 3-D collagen matrices should take about 60 min. It is important to remember that neutralized collagen can be stored on ice for only 1 hr; therefore it is recommended that the cell suspension be prepared right away, and that the Support Protocol 2 follow immediately.

Support Protocol 2

It takes about 2 hr to prepare the sample of cells in 3-D collagen matrix using the "cell sandwiching" technique described in Support Protocol 2. In addition, it will take at least 16 hr of incubation at 37°C to allow cells to polarize and start migrating in the 3-D collagen matrix. The total incubation time for this for the cell line being studied must be determined by the researcher.

Basic Protocol 1

All steps of this protocol, starting with the 3-D collagen cell sample fixation and finishing with immunolabeling, takes ~6.5 hr. It is required that confocal imaging commence after immunolabeling of the sample is complete. To avoid a lengthy day, authors recommend splitting the protocol into two groups of steps that can be accomplished over a 2-day period. First, perform fixation and permeabilization of the sample (steps 1 to 7) on the first day. After step 7 is complete and the sample has been washed with PBS, it can be stored overnight at 4°C. On the second day, proceed with blocking and immunostaining of the sample with primary and secondary antibodies (steps 8 to 15), followed by confocal microscopy of the sample.

Basic Protocol 2

Acquisition of Z-stacks of whole 512×512 images takes a matter of minutes; therefore 2 to 3 hr are required for image acquisition. Another 1 to 2 hr are required for data analysis and image presentation.

Acknowledgements

The project described was supported by Pathway to Independence Award K99CA129205 from NCI (V. Artym) and NIH/ NIDCR Intramural DE 000719.

Literature Cited

Even-Ram, S. and Yamada, K.M. 2005. Cell migration in three-dimensional matrix. *Curr. Opin. Cell Biol.* 17:524-532.

Eyre, D.R., Paz, M.A., and Gallop, P.M. 1984. Cross-linking in collagen and elastin. *Annu. Rev. Biochem.* 53:717-748.

Gelman, R.A., Williams, B.R., and Piez, K.A. 1979. Collagen fibril formation: Evidence for a multistep process. *J. Biol. Chem.* 254:180-186.

Packard, B.Z., Artym, V.V. Komoriya, A., and Yamada, K.M. 2009. Direct visualization of protease activity on cells migrating in three-dimensions. *Matrix Biol.* 28:3-10.

Pedersen, J.A. and Swartz, M.A. 2005. Mechanobiology in the third dimension. *Ann. Biomed. Eng.* 33:1469-1490.

Petrie, R.J., Doyle, A.D., and Yamada, K.M. 2009. Random versus directionally persistent cell migration. *Nat. Rev. Mol. Cell Biol.* 10:538-549.

Sabeh, F., Shimizu-Hirota, R., and Weiss, S.J. 2009. Protease-dependent versus -independent cancer cell invasion programs: Three-dimensional amoeboid movement revisited. *J. Cell Biol.* 185:11-19.

Schindler, M., Nur, E.K.A., Ahmed, I., Kamal, J., Liu, H.Y., Amor, N., Ponery, A.S., Crockett, D.P., Grafe, T.H., Chung, H.Y., Weik, T., Jones, E., and Meiners, S. 2006. Living in three dimensions: Three-dimensional nanostructured environments for cell culture and regenerative medicine. *Cell Biochem. Biophys.* 45:215-227.

Williams, B.R., Gelman, R.A., Poppke, D.C., and Piez, K.A. 1978. Collagen fibril formation: Optimal in vitro conditions and preliminary kinetic results. *J. Biol. Chem.* 253:6578-6585.

Overview of Eukaryotic In Vitro Translation and Expression Systems

EARLY HISTORY

Today, the protocols and commercial systems for expressing gene products in vitro are well known to molecular biologists and researchers in many other fields. The present-day systems have been steadily improved in efficiency and convenience over their early predecessors. The earliest in vitro translation extracts, developed in the 1960s, were central to the breaking of the genetic code. The late 1960s and early 1970s saw the emergence of a variety of translation extracts prepared from different sources, such as Krebs II ascites cells, CHO cells, rat and mouse liver, and HeLa cells. Most of these systems faithfully translated viral and eukaryotic mRNA pools but were limited in their efficiency and by the competitive translation of endogenous mRNAs. One system that overcame these limitations was the efficient wheat germ–based expression system (Roberts and Paterson, 1973). Another system based on lysates of rabbit reticulocytes that was being developed during this period would eventually become the most widely used option. In 1968, this reticulocyte system was improved through the addition of hemin (Adamson et al., 1968; Zucker and Schulman, 1968), which allowed a steady rate of translation for 60 min. However, these early systems contained high levels of globin mRNA, which made detection and quantification of added mRNA products difficult at best. One approach to this problem was to create a reconstituted system from fractions of reticulocyte lysate from which reticulocyte mRNA had been removed (Schreier and Staehelin, 1973). Unfortunately, the reconstituted fractionated system primed with added mRNAs did not retain high activity.

IMPROVED SYSTEMS

In 1976, Pelham and Jackson described a breakthrough protocol consisting of a micrococcal nuclease treatment to destroy endogenous mRNA followed by addition of EGTA to remove the Ca^{2+} required for nuclease activity (Pelham and Jackson, 1976). This system produced the low background of fractionated systems and yet, in contrast to those systems, exhibited remarkably high activity and an exceptional response to added mRNA. In addition, the nuclease-treated reticulocyte translation system exhibited much lower nuclease activity than the wheat germ system and could therefore generate much larger full-length gene products—up to 200 kDa. The system also retained the translational regulatory responses of the parental lysate. The system became more widely used and was continuously improved through the 1980s. Refinements included addition of exogenous calf liver tRNA to complement the limiting endogenous tRNA population strictly suited for globin expression, optimization of Mg^{2+} and K^{+} ion concentrations, addition of ribonuclease inhibitors (such as RNasin) and protease inhibitors, and inclusion of sugars to maintain reducing potential (Jackson and Hunt, 1983; Jagus, 1987). Concurrently, the wheat germ system was also improved through the use of high spermidine concentrations and the addition of human placental RNase inhibitor, facilitating efficient translation of high-molecular-weight proteins (Morch et al., 1986). Many translational mechanisms were investigated and defined using these systems.

With the growing availability of cloned genes, one of the most important advancements of in vitro expression during this period was the development of a system capable of synthesizing large quantities of gene-specific mRNA (Melton et al., 1984). Genes cloned behind a phage polymerase promoter, such as the T7 or SP6 promoter, could produce mRNA transcripts through incubation of linearized DNA templates with the appropriate polymerase and reaction conditions. Capped (or uncapped) transcript mRNAs were then added to the in vitro expression systems, resulting in expression of a single gene product, rather than expression from viral genomes or mRNA pools (Krieg and Melton, 1984). In vitro translation of transcripts from cDNAs allowed the identities of many cloned genes to be verified. An added bonus of this method is that any desired mutant protein can be generated simply by altering the DNA template (Struhl, 1989).

The efficiency of this in vitro translation method was increased by maximizing the amount of RNA produced for a given amount of DNA template (Milligan et al., 1987; Gurevich et al., 1991), allowing products of the transcription reaction to be added to a cell-free translation system without prior purification of the RNA (Andrews, 1989). The greatest im-

Contributed by Rosemary Jagus and Gregory S. Beckler

Current Protocols in Cell Biology (1998) 11.1.1-11.1.13

provement in RNA yields involved uncapped transcripts, as new vectors were introduced that could create transcripts that did not need to be capped before they could be translated. The vectors pTM1 (Moss et al., 1990) and pCITE (Novagen) place the 5′ untranslated leader region (UTR) of encephalomyocarditis virus (EMCV), which contains an internal ribosome entry site (IRES), upstream of the initiation codon to facilitate cap-independent translation in the reticulocyte system.

NEWER SYSTEMS AND APPROACHES

Continuous-Flow Systems

The late 1980s and the 1990s witnessed an evolution in the use of translation expression systems, from studies of translation mechanisms and simple verification of expected gene products toward the rapid and efficient production of larger quantities of functional proteins. Continuous-flow cell-free (CFCF) systems designed to work for tens of hours and produce hundreds of micrograms of synthesized protein were introduced in the late 1980s (Spirin et al., 1988). Use of the CFCF systems described by Spirin remains technically challenging, requires expensive flow cells, and has yet to demonstrate consistent results. For these reasons such systems have not been widely utilized, although they hold great potential for the production of gene products that are too toxic for production in vivo. Successes have been reported with CFCF systems involving simpler devices such as dialysis tubes, which have been used for prokaryotic systems (Davis et al., 1996; Kim and Choi, 1996) and a wheat germ system (K.J. Rothschild, pers. comm.). These dialysis tube–based systems are able to produce from hundreds of micrograms to >1 mg of expressed protein per milliliter of reaction. Another group has reported the use of affinity ligands to continuously remove the protein products, thereby improving translation efficiency (Marszal and Scouten, 1996).

Coupled Eukaryotic Systems

In 1992, the first eukaryotic coupled transcription/translation batch systems were introduced; these allowed direct expression from DNA containing cloned genes positioned behind T7, T3, or SP6 promoters in rabbit reticulocyte lysate or wheat germ extracts (Craig et al., 1992; Thompson et al., 1992). Transcription and translation reactions exhibit very different requirements for monovalent and divalent cations. The conditions that give optimal activity of the coupled system are closer to those needed for translation; although transcription is relatively inefficient in the coupled reaction, sufficient RNA is produced to saturate the protein synthetic capacity of the system. The fidelity and efficiency of expression in the coupled system is high. Unexpectedly, the coupling of transcription with translation often results in greater protein production (Jagus, 1993). Moreover, the ability to generate and utilize cloned DNA and the products of PCR reactions, rather than purified mRNA, has generated a multitude of new applications (Beckler, 1998—see Internet Resources).

Incorporation of Non-Natural Amino Acids

One primary advantage of in vitro translation is the ability to manipulate expression easily by adding exogenous compounds directly to the reaction. Incorporation of non-natural amino acids into synthesized proteins is a powerful tool for the creation of novel or mutant proteins. A technique for incorporating epsilon-amino-modified lysines into proteins synthesized in rabbit reticulocyte lysate has been described by Johnson et al. (1976). Charged tRNA complexes containing epsilon-modified lysines, such as biotinylated lysines, can be added to the translation reactions and the modified lysines will be incorporated into the synthesized protein. Biotinylated proteins can be detected nonisotopically using streptavidin–alkaline phosphatase or horseradish peroxidase conjugates (Kurzchalia et al., 1988; Hoeltke et al., 1995); these detection systems became commercially available in 1993 (Beckler and Hurst, 1993). Other groups developed alternative site-directed non-native amino-acid replacement (SNAAR) using nonsense codon suppression methods to introduce non-natural amino acids at specific sites (Noren et al., 1989). These approaches use RNA ligase to ligate in vitro–transcribed amber tRNA lacking the 3′ terminal pCpA from the acceptor stem to a chemically acylated pCpA-X, where X can be almost anything, such as a fluorescent, spin-label, or isotopic group (Noren et al., 1990).

NEW APPLICATIONS

The PCR revolution, the genome sequencing projects, and the plethora of techniques available to look at differentially expressed genes have led many researchers to ask what a particular gene of interest codes for, what function it has, and with what other gene products

it interacts. Many basic questions centered on understanding this "genetic output" are being answered using in vitro expression approaches. The following applications are made possible or desirable due to the current ease with which a particular gene or gene segment can be amplified through PCR, with concurrent incorporation of a phage polymerase promoter, such as the T7 promoter (Kain et al., 1991). The PCR-generated DNA can be expressed in a eukaryotic coupled transcription/translation reaction and the activity of the translated product(s) assessed using an in vitro assay.

Molecular Interactions

Protein-protein interactions

One of the most popular current applications of coupled eukaryotic systems is the detection of protein-protein interactions. Often, researchers will use an in vitro approach to verify or confirm results obtained in vivo, for example using the yeast two-hybrid approach (Boyd et al., 1995; see *APPENDIX 3*). To define the region of protein-protein interaction, a series of constructed deletion mutants or occasionally specific point mutants are synthesized in vitro and compared to full-length wild-type proteins.

Fusion-tag approach. Additions to the native gene product in the form of a fusion "tag" can be extremely useful if specific antibodies are not available. [^{35}S]Methionine-labeled proteins can be synthesized in coupled reactions in vitro from either full-length cDNAs or deletion mutants. These radioactive proteins can be used to detect interactions with suspected protein partners that have been expressed as GST, His-tag, or epitope-tagged (*UNIT 5.2*) fusion proteins and purified after expression in *Escherichia coli* (Chinnaiyan et al., 1995). Fusion proteins can be bound to an affinity matrix along with the radioactive protein with which they interact (Cowell and Hurst, 1996; Sharp et al., 1997). The bound radioactive proteins are then eluted and analyzed by SDS-PAGE (*UNIT 6.1*) or immunoblot (western) analysis (*UNIT 6.2*).

Immunoprecipitation. Another approach involves detection of a radioactive fusion partner by immunoprecipitation (*UNIT 7.2*) using antibodies against a particular antigenic domain. For example, an influenza hemagglutinin (HA) epitope incorporated in the carboxyl terminus of an in vitro–expressed protein can be immunoprecipitated using anti-HA antibodies (Benedict and Clawson, 1996). Alternatively, if an antibody against one of the partners is available, detection can be accomplished by coimmunoprecipitation (Leng et al., 1995; Sharp et al., 1997). Again, the bound radioactive proteins are eluted and analyzed by SDS-PAGE or immunoblot analysis. A variation on this analysis method utilizes in vitro–coupled expression of several proteins simultaneously. The relative protein expression levels can be controlled by varying the concentrations of each DNA construct. For instance, using cDNAs for chicken NF-κB p105, NF-κB p100, c-Rel, and v-Rel, cotranslated in vitro, and protein complex detection by immunoprecipitation with specific antiserum, it has been shown that one of the complexes in v-Rel-transformed spleen cells can be reconstituted in vitro (Sif and Gilmore, 1993).

Far western analysis. A direct detection method for identifying protein-protein interaction is the so-called "far western" analysis. In this approach, radioactive proteins are synthesized in vitro and then used as "probes" to detect binding to membrane-bound renatured proteins directly (Johnston et al., 1996).

Isolation of protein complexes by capture of biotinylated lysine residues. One drawback of fusion-protein techniques is the need to make the fusion protein construct. Another approach that obviates the need to develop novel constructs is to to incorporate non-natural amino acids, such as biotinylated lysines, into in vitro–translated proteins. This approach can be taken with any gene containing lysine codons. Incorporation of non-natural amino acids does not significantly alter the efficiency of polypeptide synthesis, and usually incorporation of biotinylated lysines does not affect the function of the protein. This approach has been used to develop a novel method for capturing protein complexes that associate with biotinylated Rab5, a member of the Rab family of GTP-binding proteins (Sanford et al., 1995). The ability to bind biotinylated lysine tightly to streptavidin-linked agarose can be utilized to capture in vitro–synthesized biotinylated Rab5. A promising and potentially powerful modification of the biotinylated-lysine capture technique for detection and gentle purification of in vitro–generated polypeptides, using photocleavable (PC) biotin, has recently been described (Rothschild et al., 1997). In a recent example, the nascent PC-biotin-labeled polypeptides were captured using streptavidin-coated magnetic beads, and after a short exposure to UV, the native polypeptide was released (with 70% to 95% efficiency) with no remaining "tags" (Olejnik et al., 1995, 1998; Rothschild et al., 1997).

Protein folding, chaperonins, and luciferase. Another emerging area utilizing in vitro systems to define protein-protein interactions is the field of protein folding and chaperonin interactions. Researchers have combined the advantages of in vitro expression with the power of instantaneous reporter gene product assays. The folding of polypeptides emerging from ribosomes has been analyzed using firefly luciferase as a model protein (Frydman et al., 1994; Frydman and Hartl, 1996). The growing polypeptide interacts with a specific set of molecular chaperones, including Hsp70, the DnaJ homologue Hsp40, and the chaperonin TRiC. The ordered assembly of these components on the nascent chain forms a high-molecular-mass complex that allows the cotranslational formation of protein domains and the completion of folding once the chain is released from the ribosome.

Real-time translation/folding assays

A novel approach has been developed using a wheat germ system in which the components for the luciferase enzymatic assay have been added directly to the translation reaction and monitored continuously in real time (Kolb et al., 1994). To demonstrate that luciferase exhibits cotranslational folding, the activity of translation products produced from wild-type mRNAs was compared with that of products produced from mutant mRNAs lacking stop codons to prevent release of the polypeptide from the ribosome. Luciferase was shown to be completely folded and enzymatically fully active immediately upon release from the ribosome (Kolb et al., 1994). However, no luciferase activity was observed while full-length luciferase remained attached to the ribosome as a peptidyl-tRNA, probably because the C-terminal portion of the enzyme is masked by the ribosome and/or ribosome-associated proteins. The investigators demonstrated that the ribosome-bound enzyme acquires enzymatic activity when its C-terminus is extended by at least 26 additional amino acid residues (Makeyev et al., 1996). The results demonstrate that the acquisition of the final native conformation by a nascent protein can occur as the protein is being synthesized and that folding does not require release of the protein from the ribosome.

Macromolecular assembly and frameshifting

Many in vivo translational control mechanisms are faithfully replicated in vitro. In addition, a variety of macromolecular complexes can be expressed and properly assembled in vitro. For example, an in vitro synthesis and assembly system for the protypical type D retrovirus, Mason-Pfizer monkey virus (M-PMV), has been developed utilizing rabbit reticulocyte reactions expressing M-PMV–Gag precursor polyprotein precursors as the result of two ribosomal frameshift events (Sakalian et al., 1996). The frameshift efficiency in vitro is identical to that observed in vivo. These polyproteins assemble to form immature retrovirus capsids indistinguishable from those formed in the host cell cytoplasm. More importantly, this system can be utilized in combination with anti-Gag antibodies to search for potential inhibitors of retrovirus assembly.

Protein-DNA interactions

The electrophoretic mobility shift assay (EMSA) is a widely used procedure to detect DNA binding sites for proteins or protein complexes, such as transcription factors. In this technique in vitro–generated proteins with putative DNA-binding activity are combined with an oligonucleotide containing a target consensus sequence site, and changes in DNA migration due to the bound protein are detected by PAGE. Either partner can be radiolabeled (usually 5′-end-labeled [^{32}P]DNA is used) and with proper controls, the translation extract containing the synthesized candidate binding factor can be used directly (Lee and Chang, 1995). Many researchers investigating transcription-factor binding use coupled wheat germ rather than rabbit reticulocyte translation systems, as wheat germ extract does not contain endogenous transcription factors such as NFκB (Sif and Gilmore, 1993). If the reticulocyte system must be used, it is possible to remove endogenous DNA-binding proteins from the reticulocyte system by a rapid method using biotinylated DNA and streptavidin-conjugated magnetic beads (Ebel and Sippel, 1995).

Protein-RNA interactions

Protein-RNA interactions can also be studied with in vitro translated proteins. To demonstrate the direct interaction of the U1 snRNP-A protein with SV40 late mRNAs, [^{35}S]Met-labeled U1 snRNP-A fusion protein containing a g10 epitope tag was synthesized in vitro, mixed with ^{32}P-labeled in vitro transcribed RNA, and immunoprecipitated. Coprecipitating RNAs were extracted and analyzed by PAGE (Lutz and Alwine, 1994).

Characterization of molecular environments

In vitro expression systems have also been utilized extensively to understand the molecular environment of nascent polypeptides as they are translated and translocated, including the very nature of the ribosome "tunnel" the polypeptide passes through. These approaches utilize the incorporation of non-natural amino acids containing photoactivatable cross-linking groups or fluorescent groups.

Photo-cross-linking. Studies of a secretory protein utilizing the incorporation of modified Lys-tRNAs containing a photoreactive cross-linker group, with subsequent photo-cross-linking, revealed a sequential passage of the transmembrane domain through three different proteinaceous environments, and helped define the multistep process of cotranslational integration of a nascent protein into the endoplasmic reticulum membrane (Do et al., 1996).

Fluorescent quenching. Fluorescent probes incorporated into nascent secretory proteins using modified Lys-tRNAs have been used to report on the ability of various-sized quenching agents to enter the endoplasmic reticulum pore and quench fluorescence. (Hamman et al., 1997).

Membrane association. The features within the N-terminal membrane-targeting domain of the cyclic AMP phosphodiesterase RD1 that are required for membrane association have been analyzed using in vitro expression of deletion mutants of RD1-CAT chimeras. Radioactive products were generated in vitro, incubated with membranes, and then identified in either the membrane pellets or soluble supernatant fractions (Smith et al., 1996).

Characterization of transmembrane domains. The G protein–coupled receptors involved in signal transduction across the plasma membrane have been viewed as having seven transmembrane helices and a three-dimensional structure similar to that of bacteriorhodopsin. These receptors appear to insert into membranes using signal-anchor sequences followed by stop-transfer sequences. Hydropathy plots and various experimental techniques have been used to predict the seven membrane domains, yet no one technique has allowed a direct demonstration. In vitro translation in the presence or absence of microsomal membranes was used not only to determine the presence of independent signal-anchor and stop-transfer sequences, but also to identify the amino acid sequences accounting for transmembrane folding (Bayle et al., 1997a,b). This method used constructs containing the N-terminal region of the gastric H^+/K^+ ATPase or the N-terminal region of the cholecystokinin-A (CCK-A) receptor, coupled via a linker region to the last 177 amino acids of the beta subunit of the gastric H^+/K^+ ATPase. The latter contains five potential N-linked glycosylation sites. Translation of the mRNA encoding one, two, or more putative transmembrane domains in the absence or presence of microsomes allowed determination of signal-anchor or stop-transfer properties of the putative transmembrane domains from the molecular weight shift after SDS-PAGE. Translation of segments of the gastric H^+/K^+ ATPase provided evidence for only seven transmembrane segments, but coupled with other data established a ten-membrane-segment model. The CCK-A receptor showed the presence of six of the seven transmembrane segments postulated for this protein. This technique provides a useful addition to methods of determining membrane domains of integral membrane proteins, but must in general be combined with other methods to establish the number of transmembrane alpha helices.

MOLECULAR FUNCTION

Functional Genomics

Ribosomal display for cell-free protein evolution

Ribosomal display is an emerging technology for performing cell-free protein evolution in vitro as an alternative to phage display. This approach utilizes the cell-free system to transcribe a DNA library, translate the mRNA pools, and, using a variety of techniques, retain the proteins and their encoding mRNAs attached to the ribosomes. The protein-mRNA-ribosome complexes are screened for binding to a target and the retained mRNA is amplified using RT-PCR, with the resulting DNA used for another round of selection. Initially the technique utilized the prokaryotic *E. coli* coupled transcription/translation system to generate large libraries of peptides used for receptor-ligand screening (Mattheakis et al., 1994). This system was improved to allow folding of whole proteins into their native structure while on the ribosome (Hanes and Pluckthun, 1997). The first eukaryotic-based system, centered around a rabbit reticulocyte coupled transcription/translation system, used antibody-ribosome-mRNA (ARM) complexes for rapid selection to monitor the evolution of antibody combining sites (He and Taussig, 1997). ARMs carrying single-chain (VH/K) binding frag-

ments specific for progesterone have been selected using antigen-coupled magnetic beads. Selection simultaneously captured the genetic information as mRNA, making it possible to generate and amplify cDNA by single-step RT-PCR on the ribosome-bound mRNA for further manipulation. Using mutant libraries, antigen-binding ARMs were enriched by a factor of 10^4- to 10^5-fold in a single cycle, with further enrichment in repeated cycles. Such an approach has obvious potential for the selection of receptors or peptides from libraries.

An interesting cell-free system for performing evolution studies in vitro has been developed to find reaction conditions in which isothermal RNA amplification would occur simultaneously with coupled transcription and translation (Joyce, 1993). After unsuccessfully testing wheat germ extracts and coupled *E. coli* systems, investigators were able to operate the "two" reactions simultaneously using the rabbit reticulocyte coupled transcription/translation system. This system can be used to perform laboratory "evolution" by putting selective pressure on functional protein products necessary for RNA amplification.

In vitro expression cloning (IVEC)

To a considerable extent, as the era of functional genomics dawns, the current scientific focus is shifting from genomic bioinformatic analyses as such to the development of approaches to understand gene-product function. A new alternative approach to functional gene identification utilizes the manipulative ability, speed, and convenience of eukaryotic coupled transcription/translation. In vitro expression cloning (IVEC) consists of adding small cDNA plasmid pools (of 50 to 100 clones per pool) directly into a rabbit reticulocyte coupled transcription/translation system. The resulting protein pools can be used to identify cDNAs rapidly on the basis of almost any biochemical property for which an assay can be developed (King et al., 1997; Lustig et al., 1997). The first report of this technology detailed the screening of a total of ~500 pools of cDNAs encoding ~12,000 proteins and identification of 105 pools with potential positives. Twenty mitotically phosphorylated proteins were identified by incubating in vitro protein pools with interphase *Xenopus laevis* egg extracts and identifying phosphorylated protein targets from their altered SDS-PAGE migration (Stukenberg et al., 1997). Interestingly, while fifteen of the identified phosphorylated proteins share sequence similarity with proteins previously characterized, the other five are novel. This approach has also been used to screen 100,000 cDNA clones to identify gelsolin as a substrate of caspase-3 protease, demonstrating that gelsolin is an effector of morphological changes in apoptosis (Kothakota et al., 1997). This technology appears to be an effective bridge connecting genomics and proteomics.

Molecular Characterization Studies

Enzymatic activity and mutation analysis

In vitro expression can be used to analyze synthesized proteins, whether wild-type or mutant, for enzymatic activity. While deletion mutation analysis is useful for defining protein- or DNA-binding regions, site-specific mutation analysis can be useful for understanding and modifying enzymatic activity. In one study, the hypothesis that histidine 21 of diphtheria toxin A subunit is important in ADP-ribosyltransferase activity was tested by mutagenizing codon 21 to encode all possible amino acids. The products were translated in vitro in a reticulocyte coupled transcription/translation system and analyzed for ADP-ribosylating activity, resulting in the determination that histidine 21 does not play a major role in diphtheria toxin catalysis (Johnson and Nicholls, 1994). In vitro systems offer substantial advantages over in vivo systems in terms of safety and expression efficiency for toxin expression.

Another group expressed a cDNA encoding a large (110-kDa) six-transmembrane protein, type IV adenylyl cyclase (ACIV), in a coupled transcription/translation system with the same specific enzymatic activity as baculoviral-produced ACIV (Warner et al., 1995). Interestingly, the in vitro–generated ACIV was very stable and demonstrated linear activity for 90 min at 30°C, which is much longer than impure samples (<15 min) and somewhat longer even than pure adenylate cyclase (1 hr). Use of such a system may provide a good first screen for identifying interesting mutants or producing active chimeric constructs of topologically complex proteins like ACIV.

Similarly, generation of in vitro–translated aromatase in the presence of canine pancreatic microsomes gives rise to an active enzyme when supplemented with recombinant P_{450} reductase (Pancharatnam et al., 1996). The in vitro–made enzyme has properties equivalent to those of aromatase in tissue extracts. This use of the coupled transcription/translation systems may provide a general procedure for examining structure/function relationships

among the cytochrome P_{450} superfamily of enzymes without the need for transient or stable transfection studies.

Post-translational modifications

A number of post-translational activities have been extensively analyzed using in vitro expression systems. For example, rabbit reticulocyte lysate contains proteolytic, phosphorylation, myristylation, farnsylation, isoprenylation, and adenylation activities (Glass and Pollard, 1991; Hancock, 1995). With the addition of canine microsomal membranes, methylation, glycosylation, and signal-sequence cleavage activities can be studied (Walter and Blobel, 1983; also see *UNIT 11.4*). A reticulocyte lysate assay for studying the post-translational modifications of Ras proteins has described the analysis of a number of different modifications (Roy et al., 1997).

Proteolytic cleavage analysis

For many years, in vitro expression systems have been used to study viral and cellular proteolytic activity. Examples include characterization of the autoprocessing cascade of HSV-1 (Godefroy and Guenet, 1995) and of the expression of a 250-kDa polyprotein that results in detectable self-cleavage products when expressed in a coupled transcription translation system, but is incompletely processed in a standard mRNA-based translation system (Hemmer et al., 1995). Recent work has described an in vitro assay to activate the hepatitis C virus N2-3 protease post-translationally and methods to study the effects of several common inhibitors on the enzymatic activity (Pieroni et al., 1997).

Antisense regulation

Antisense oligonucleotides have been shown to inhibit gene expression at either the transcriptional or translational level (Curcio et al., 1997). The mechanism for translational inhibition is thought to involve activation of RNase and is currently a target of intense investigation. Attempts to rationally design effective antisense RNAs have yet to yield consistent results, giving rise to the use of an empirical approach using randomized oligonucleotide arrays to identify ideal candidate antisense oligonucleotides (Lima et al., 1997; Milner et al., 1997). An in vitro reticulocyte lysate coupled transcription/translation system has been established for rapid screening of antisense oligodeoxyribonucleotides to determine which are the most effective in arresting mRNA translation (Chen et al., 1997). The potential for heteroduplex formation of an oligonucleotide has been shown to correlate closely with inhibition of translation in vitro.

Development of antiviral agents

The rapid reticulocyte coupled transcription/translation screening system has been applied to the identification of antisense oligodeoxynucleotides capable of inhibiting hepatic D viral replication (Chen et al., 1997). Other groups developing specific viral inhibitors have utilized in vitro expression to test different target regions with modified oligonucleotides (peptide nucleic acid and phosphorothioate oligonucleotides; Alt et al., 1997; Koppelhus et al., 1997). Additional studies have shown the circular 2′-deoxyribo-oligonucleotides to be potent inhibitors of luciferase expression in an in vitro coupled transcription/translation system (Azhayeva et al., 1997).

Ligand binding for identification of novel orphan receptors

The binding of ligands to in vitro–synthesized receptors can be an important aspect of identifying new receptors. For example, a search for novel "orphan" nuclear receptors and ligands cloned and characterized a novel estrogen receptor (Kuiper et al., 1996). Saturation ligand-binding and ligand-competition assays of the in vitro–expressed clone allowed this novel receptor to be distinguished from one previously cloned.

Characterization of antiserum

In studies utilizing antiserum, immunoprecipitation of in vitro–synthesized proteins can be used to demonstrate the specificity of the antiserum (Murrell et al., 1995).

Studies of Molecular Structure

Understanding of integral membrane protein function is currently limited by the difficulty of producing three-dimensional crystals. A method has been developed for probing conformational changes in proteins in which Fourier-transform infrared-difference spectroscopy is combined with amber suppression to insert a single isotopic label into a specific site within in vitro–synthesized polypeptides that fold into their native structure (Sonar, 1994). Unlike earlier methods using site-directed mutagenesis, this does not disrupt the protein structure. It should be applicable to a wide range of proteins, including those involved in enzyme catalysis, ion transport, and signal transduction.

Diagnostic Detection

Protein truncation test

Perhaps the fastest-growing application of coupled transcription/translation systems has been for the diagnostic detection of genetic diseases, a predominantly DNA-dominated arena. The protein truncation test (PTT), sometimes referred to as the in vitro–synthesized protein truncation (IVSP) assay, was first reported in 1993 as a rapid method for detecting translation-terminating mutations in the very large gene responsible for Duchenne muscular dystrophy (Roest et al., 1993) and in the familial adenomatous polyposis (APC) gene responsible for a type of hereditary colon cancer (Powell et al., 1993). In these and other diseases, such as hereditary breast cancer (Hogervorst et al., 1995), 70% to 95% of the mutations that cause disease result in a truncated gene product. In the PTT, genomic DNA or mRNA is first purified from the patient's blood or tissue. This is subjected to either RT-PCR or PCR with concurrent incorporation of a T7 promoter and an optimal translation initiation sequence surrounding the desired start codon. Often, when the source mRNA is limiting, a second nested PCR reaction is required. Large exons are amplified from genomic DNA while smaller exons are amplified together from mRNA, and the gene is segmented into overlapping amplified fragments. The amplified DNA is added directly into a coupled transcription/translation reaction, and translation-terminating mutations are detected as faster-migrating bands after SDS-PAGE analysis. The main advantages of the PTT include the ability to scan large (2- to 3-kb) DNA or RNA segments quickly and the fact that the method detects only disease-causing mutations, avoiding the fruitless evaluation of silent polymorphisms. A disadvantage has been that the interpretation of real results can be complicated by the presence of internally translated protein products and other nonspecific bands. The PTT has been improved through addition of reporter-tag sequences in the 5′ primer, and by the design of overlapping segments, gel parameters, and methods to prevent nonsense-mediated mRNA decay (Rowan and Bodmer, 1997; Garvin, 1998). A recent review comparing PTT to other detection methods describes it as the first choice for mutation screening (Frayling and Rowan, 1997). Perhaps the most unusual example of the power of this method for detecting previously unknown mutations was provided by a new discovery by the Kinzler and Vogelstein groups that uncovered a novel mechanism for producing germline mutations with resulting predisposition to colon cancer (Laken et al., 1997). IVSP screening analysis of a patient with a family history of colon cancer was found to produce truncated gene products, yet sequence analysis of the DNA revealed only an T-to-A transversion resulting in a lysine-for-isoleucine substitution. The truncation thus appeared to be an in vitro phenomenon caused by the transversion. However, further work revealed that this mutation creates a small hypermutable region of the gene, resulting in secondary, truncating mutations. It appears this region contains unusual secondary structure that is incorrectly processed by both the polymerases present in human cells and the phage polymerases used in the IVSP reaction. This finding has led to the development of a specific genetic screen which has demonstrated that this mutation is present in 6% of Ashkenazi Jews.

An approach similar to PTT has been applied to screening of the expression of the simian immunodeficiency virus (SIV) *nef* gene in the course of disease progression in macaque monkeys (Switzer and Heneine, 1995). SIV undergoes constant evolution, and an intact *nef* gene is important to maintain the high viral loads required for disease progression to AIDS in SIV-infected macaques. The coupled transcription/translation system has been used as a screening tool to examine the reading frames of SIV *nef* genes for the presence of premature stop codons. The screen has been further simplified by using a colony PCR step to simultaneously screen for correct recombinants and generate the T7-tagged PCR products required for analysis. Demonstrating the reliability of this technique, a 100% correlation was found between the results from coupled transcription/translation and the sequence analysis of 47 *nef* clones. This approach is much faster, cheaper, and less labor-intensive than conventional gene sequencing techniques.

Drug Screening and Pharmacology

The use of in vitro systems for high-throughput drug screening (HTS) is an emerging area in which there are few publications to date. However, this is not reflective of the intense activity in this area. A number of pharmaceutical companies are currently evaluating and developing in vitro screening protocols; material from company press releases was used for some of the descriptions below.

Screening for pharmacological agents

The in vitro luciferase folding/chaperonin assay described earlier has been extended to understanding the role of heat-shock factors, such as Hsp90. It is now understood that disruption of folding pathways can result in proteolytic degradation. Several groups are currently using this information to ascertain the pharmacological activities of benzoquinone ansamycins, such as geldanamycin (Schneider et al., 1996; Thulasiraman and Matts, 1996). These potentially medically important compounds were first identified as interesting because of their ability to inhibit tyrosine kinase activity. This ability appears to be due to their interaction with Hsp90, which prevents correct folding of tyrosine kinases and is followed by their proteolytic degradation. Other potentially important drugs affecting protein folding through inhibition of chaperonin function could be identified using this approach.

High-throughput screening for translation-inhibiting compounds

Viruses contain a number of different genetic elements used for promoting viral expression at the expense of host mRNA translation. Several groups are currently developing screens using in vitro expression of gene constructs containing a viral element—such as the 5′ UTR that can harbor an internal ribosome entry site—followed by a firefly or *Renilla* luciferase gene. Chemical or antibiotic libraries can be screened for specific translation-inhibiting effects. The viral element can be placed between the firefly and *Renilla* luciferase genes, with translation of the first gene relying on normal cap-dependent initiation. Use of the two luciferase genes allows normalization of the second reporter behind the viral element. Compound efficacy can be assessed rapidly (<30 sec) by assaying light output for both the reporter and the luciferase control. As a variation on this theme, RiboGene has reported that they are developing a high-throughput screen capable of screening several hundred thousand compounds for the ability to diminish or block the required ribosomal frameshifting that occurs during translation of the HIV *gag-pol* mRNA. This screen utilizes a reporter gene in which luciferase (and light) is produced only when the frameshift occurs.

Other Applications

A highly sensitive immunoassay, known as the ultrasensitive expression immunoassay (USEI), was developed based on a "solid-phase" coupled transcription/translation system described by Christopoulos and Chiu (1995). A DNA fragment encoding firefly luciferase is biotinylated and complexed with streptavidin. Biotinylated specific antibodies are used to quantify antigen immobilized on microtiter wells. After completion of the immunoreaction, streptavidin-DNA complex is bound to the immunocomplex. Subsequent expression of the solid phase–bound DNA by a coupled transcription/translation reaction produces luciferase. As few as 3000 molecules of DNA label can detect a minimum of 50,000 antigen molecules. The luminescence is a linear function of the number of antigen molecules in a range extending over 3 orders of magnitude. The high sensitivity achieved results from the combined amplification due to transcription/translation and the substrate turnover. The same investigators developed a similar approach for DNA detection (Chiu and Christopoulos, 1996). A denatured target DNA is hybridized to two probes, one biotinylated and one labeled with digoxigenin. The DNA label contains the firefly luciferase coding sequence downstream from a T7 RNA polymerase promoter. The target DNA (200 bp) is denatured and hybridized simultaneously with two oligonucleotide probes. One probe is immobilized in microtiter wells, via the digoxigenin/anti-digoxigenin interaction, and the other is biotinylated. After hybridization is complete, the hybrids are reacted with a streptavidin-luciferase-DNA complex. Subsequently, the solid phase–bound DNA is expressed by coupled transcription/translation. This system too is very sensitive due to the coupled transcription/translation system, and the resulting luminescence is linearly related to target DNA levels for between 5 and 5000 amol. Because the assay is performed in microtiter wells and avoids membrane hybridization and blotting, it can be adapted to automatable HTS detection.

A sensitive nonradioactive method for detecting eukaryotic nuclear extract transcription products has been developed using in vitro synthesis of mRNAs encoding the firefly luciferase gene. Transcriptional activity can be assessed by using vectors containing the luciferase gene (downstream of a range of different promoters) upstream of the nuclear extract transcription reactions and using the resulting transcripts to program a rabbit reticulocyte translation system. When the translation reaction products are tested using the sensitive luciferase assay, there is a linear relationship between transcriptional activity and light gener-

ated. This approach can be used either to test the transcriptional activity of the nuclear extract with regard to specific promoters or to compare promoter strengths in different extracts (Ayoubi and Van de Ven, 1995).

THE FUTURE OF IN VITRO TRANSLATION SYSTEMS

Although the process of translation—or coupled transcription/translation—is complex, the use of convenient, rapid in vitro expression systems for many different applications is simple. The emergence of the eukaryotic coupled systems, combined with the standard molecular biology techniques of PCR and RT-PCR, has produced an explosion of new applications in such diverse fields as diagnostic detection, HTS for anti-HIV drugs, screening applications for functional genomics, and ribosomal display methods for evolving novel binding partners. For rapid generation of high levels of in vitro–translated protein, continuous-flow systems currently hold more promise than concrete utility, although their practicality appears to be improving. Development of coupled transcription/translation systems from the hyperthermophilic *Archaea* may improve CFCF methodology, since these coupled systems should be more stable and longer lasting than systems derived from mesophilic organisms. The ability to manipulate both the reaction and the end product of in vitro translation systems can be expected to generate even more useful and novel tools and applications in the future.

Literature Cited

Adamson, S.D., Herbert, E., and Godchaux, W. 1968. Factors affecting the rate of protein synthesis in lysate systems from reticulocytes. *Arch. Biochem. Biophys.* 67:247-256.

Alt, M., Renz, R., Hofschneider, P.H., and Caselmann, W.H. 1997. Core specific antisense phosphorothioate oligodeoxynucleotides as potent and specific inhibitors of hepatitis C viral translation. *Arch. Virol.* 142:589-599.

Andrews, D. 1989. Examining protein translocation in cell-free systems and microinjected *Xenopus* oocytes. *BioTechniques* 7:960-962.

Ayoubi, T.A. and Van de Ven, W.J. 1995. A nonradioactive method to determine levels of in vitro transcription in nuclear extracts. *Anal. Biochem.* 225:190-192.

Azhayeva, E., Azhayev, A., Auriola, S., Tengvall, U., Urtti, A., and Lonnberg, H. 1997. Inhibitory properties of double-helix-forming circular oligonucleotides. *Nucl. Acids Res.* 25:4954-4961.

Bayle, D., Weeks, D., and Sachs, G. 1997a. Identification of membrane insertion sequences of the rabbit gastric cholecystokinin-A receptor by in vitro translation. *J. Biol. Chem.* 272:19697-19707.

Bayle, D., Weeks, D., Hallen, S., Melchers, K., Bamberg, K., and Sachs, G. 1997b. In vitro translation analysis of integral membrane proteins. *J. Recept. Signal Transduct. Res.* 17:29-56.

Beckler, G.S. and Hurst, R. 1993. Non-radioactive detection of in vitro translation products labeled using biotinylated lysine tRNA. *Promega Notes* 43:24-31.

Benedict, C.M. and Clawson, G.A. 1996. Nuclear multicatalytic proteinase subunit RRC3 is important for growth regulation in hepatocytes. *Biochemistry* 35:11612-11621.

Boyd, J.M., Gallo, G.J., Elangovan, B., Houghton, A.B., Malstrom, S., Avery, B.J., Ebb, R.G., Subramanian, T., Chittenden, T., Lutz, R.J., and Chinnadurai, G. 1995. Bik, a novel death-inducing protein, shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* 11:1921-1928.

Chen, T.Z., Wu, J.C., Au, L.C., and Choo, K.B. 1997. Specific inhibition of delta antigen by in vitro system by antisense oligodeoxynucleotide: Implications for translation mechanism and treatment. *J. Virol. Methods* 65:183-189.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 81:505-512.

Chiu, N.H. and Christopoulos, T.K. 1996. Hybridization assays using an expressible DNA fragment encoding firefly luciferase as a label. *Anal. Chem.* 68:2304-2308.

Christopoulos, T.K. and Chiu, N.H. 1995. Expression immunoassay. Antigen quantitation using antibodies labeled with enzyme-coding DNA fragments. *Anal. Chem.* 67:4290-4294.

Cowell, I. and Hurst, H. 1996. Protein-protein interaction between the transcriptional repressor E4BP4 and the TBP-binding protein Dr1. *Nucl. Acid Res.* 24:3607-3613.

Craig, D., Howell, M.T., Gibbs, C.L., Hunt, T., and Jackson, R.J. 1992. Plasmid cDNA-directed protein synthesis in a coupled eukaryotic in vitro transcription/translation system. *Nucl. Acids Res.* 20:4987-4995.

Curcio, L.D., Bouffard, D.Y., and Scanlon, K.J. 1997. Oligonucleotides as modulators of cancer gene expression. *Pharmacol. Ther.* 74:317-332.

Davis, J., Thompson, D., and Beckler, G.S. 1996. Large scale dialysis reactions using *E. coli* S30 extract system. *Promega Notes* 56:14-19.

Do, H., Falcone, D., Lin, J., Andrews, D.W., and Johnson, A.E. 1996. The cotranslational integration of membrane proteins into the phospholipid bilayer is a multistep process. *Cell* 85:369-378.

Ebel, T. and Sippel, A. 1995. A rapid method to deplete endogenous DNA-binding proteins from reticulocyte lysate translation systems. *Nucl. Acids Res.* 23:2076-2077.

Frayling, I.M. and Rowan, A.J. 1997. Searching for mutations. Familial adenomatous polyposis as a case study. *In* Molecular Diagnosis of Genetic Diseases (R. Elles, ed.) pp. 63-98. Humana Press, Totowa, N.J.

Frydman, J. and Hartl, F.U. 1996. Principles of chaperone-assisted protein folding: Differences between in vitro and in vivo mechanisms. *Science* 272:1497-1502.

Frydman, J., Nimmesgern, E., Ohtsuka, K., and Hartl, F.U. 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* 370:111-117.

Garvin, A.M. 1998. A complete protein truncation test for BRCA1 and BRCA2. *Eur. J. Hum. Genet*. In press.

Glass, C.A. and Pollard, K.M. 1990 Post-translational isoprenylation of human lamin B in the reticulocyte lysate in vitro translation system. *Promega Notes* 26:6-9.

Godefroy, S. and Guenet, C. 1995. Autoprocessing of HSV-1 protease: Effect of deletions on autoproteolysis. *FEBS Lett.* 357:168-172.

Gurevich, V.V., Pokrovskaya, I.D., Obukhova, T.A., and Zozulya, S.A. 1991. Preparative in vitro mRNA synthesis using SP6 and T7 RNA polymerases. *Anal. Biochem.* 195:207-213.

Hamman, B.D., Chen, J.C., Johnson, E.E., and Johnson, A.E. 1997. The aqueous pore through the translocon has a diameter of 40-60 Å during cotranslational protein translocation at the ER membrane. *Cell* 89:535-544.

Hancock, J.F. 1995. Reticulocyte lysate assay for in vitro translation and posttranslational modification of Ras proteins. *Methods Enzymol.* 255:60-65.

Hanes, J. and Pluckthun, A. 1997. In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. U.S.A.* 94:4937-4942.

He, M. and Taussig, M.J. 1997. Antibody-ribosome-mRMA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. *Nucl. Acids Res.* 25:5132-5134.

Hemmer, O., Greif, C., Dufourcq, P., Reinbolt, J., and Fritsch, C. 1995. Functional characterization of the proteolytic activity of the tomato black ring nepovirus RNA-1-encoded polyprotein. *Virology* 206:362-371.

Hoeltke, H.J., Ettl, I., Strobel, E., Leying, H., Zimmermann, M., and Zimmermann, R. 1995. Biotin in vitro translation, nonradioactive detection of cell-free synthesized proteins. *BioTechniques* 18:900-904.

Hogervorst, F.B., Cornelis, R.S., Bout, M., van Vliet, M., Oosterwijk, J.C., Olmer, R., Bakker, B., Klijn, J.G., Vasen, H.F., and Meijers-Heijboer, H. 1995. Rapid detection of BRCA1 mutations by the protein truncation test. *Nature Genet.* 10:208-212.

Jackson, R.J. and Hunt, T. 1983. Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Methods Enzymol.* 96:50-74.

Jagus, R. 1987. Translation in cell-free systems. *Methods Enzymol.* 152:267-275.

Jagus, R. 1993. TNT system–based production of [^{35}S]eIF-4E for functional studies. *Promega Notes* 42:17.

Johnson, V.G. and Nicholls, P.J. 1994. Histidine 21 does not play a major role in diphtheria toxin catalysis. *J. Biol. Chem.* 269:4349-4354.

Johnson, A.E., Woodward, W.R., Herbert, E., and Menninger, J.R. 1976. Nε-acetyllysine transfer ribonucleic acid: A biologically active analogue of aminoacyl transfer ribonucleic acids. *Biochemistry* 15:569-575.

Johnston, S., Yu, X.M., and Mertz, J.E. 1996. The major transcriptional transactivation domain of Simian virus 40 large T antigen associates nonconcurrently with multiple components of the transcriptional preinitiation complex. *J. Virol.* 70:1191-1202.

Joyce, G.F. 1993. Evolution of catalytic function. *Pure & Appl. Chem.* 65:1205-1212.

Kain, K.C., Orlandi, P.A., and Lanar, D.E. 1991. Universal promoter for gene expression without cloning: Expression PCR. *BioTechniques* 10:366-370.

Kim, D.M. and Choi, C.Y. 1996. A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane. *Biotechnol. Prog.* 12:645-649.

King, R.W., Lustig, K.D., Stukenberg, P.T., McGarry, T.J., and Kirschner, M.W. 1997. Expression cloning in the test tube. *Science* 277:973-974.

Kolb, V.A., Makeyev, E.V., and Spirin, A.S. 1994. Folding of firefly luciferase during translation in a cell-free system. *EMBO J.* 13:3631-3637.

Koppelhus, U., Zachar, V., Nielsen, P.E., Liu, X., Eugen-Olsen, J., and Ebbesen, P. 1997. Efficient in vitro inhibition of HIV-1 *gag* reverse transcription by peptide nucleic acid (PNA) at minimal ratios of PNA/RNA. *Nucl. Acids Res.* 25:2167-2173.

Kothakota, S., Azuma, T., Reinhard, C., Klippe, l.A., Tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Koths, K., Kwiatkowski, D.J., and Williams, L.T. 1997. Caspase-3-generated fragment of gelsolin: Effector of morphological change in apoptosis. *Science* 278:294-298.

Krieg, P.A. and Melton, D.A. 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNA. *Nucl. Acids Res.* 12:7057-7070.

Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.A. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. U.S.A.* 93:5925-5930.

Kurzchalia, T.V., Wiedmann, M., Breter, H., Zimmermann, W., Bauschke, E., and Rapoport, T.A. 1988. tRNA-mediated labelling of proteins with biotin. A nonradioactive method for the detection of cell-free translation products. *Eur. J. Biochem.* 172:663-668.

Laken, S.J., Petersen, G.M., Gruber, S.B., Oddoux, C., Ostrer, H., Giardiello, F.M., Hamilton, S.R., Hampel, H., Markowitz, A., Klimstra, D., Jhanwar, S., Winawer, S., Offit, K., Luce, M.C., Kinzler, K.W., and Vogelstein, B. 1997. Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC. *Nature Genet.* 17:79-83.

Lee, H.J. and Chang, C. 1995. Identification of human TR2 orphan receptor response element in the transcriptional initiation site of the simian virus 40 major late promoter. *J. Biol. Chem.* 270:5434-5440.

Leng, P., Brown, D.R., and Deb, S. 1995. Human oncoprotein MDM2 interacts with the TATA-binding protein in vitro and in vivo. *Int. J. Oncol.* 6:251-259.

Lima, W.F., Brown-Driver, V., Fox, M., Hanecak, R., and Bruice, T.W. 1997. Combinatorial screening and rational optimization for hybridization to folded hepatitis C virus RNA of oligonucleotides with biological antisense activity. *J. Biol. Chem.* 272:626-638.

Lustig, K.D., Stukenberg, P.T., McGarry, T.J., King, R.W., Cryns, V.L., Mead, P.E., Zon, L.I., Yuan, J., and Kirschner, M.W. 1997. Small pool expression screening: Identification of genes involved in cell cycle control, apoptosis, and early development. *Methods Enzymol.* 283:83-99.

Lutz, C.S. and Alwine, J.C. 1994. Direct interaction of the U1 snRNP-A protein with the upstream efficiency element of the SV40 late polyadenylation signal. *Genes Dev.* 8:576-586.

Makeyev, E.V., Kolb, V.A., and Spirin, A.S. 1996. Enzymatic activity of the ribosome-bound nascent polypeptide. *FEBS Lett.* 378:166-170.

Marszal, E. and Scouten, W.H. 1996. Dihydrofolate reductase synthesis in the presence of immobilized methotrexate. An approach to a continuous cell-free protein synthesis system, *J. Mol. Recognit.* 9:543-548.

Mattheakis, L.C., Bhatt, R.R., and Dower, W.J. 1994. An in vitro polysome display system for identifying ligands from very large peptide libraries. *Proc. Natl. Acad. Sci. U.S.A.* 91:9022-9026.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* 12:7035-7056.

Milligan, J.F., Groebe, D.R., Witherell, G.W., and Uhlenbeck, O.C. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucl. Acids Res.* 15:8783-8798.

Milner, N., Mir, K.U., and Southern, E.M. 1997. Selecting effective antisense reagents on combinatorial oligonucleotide arrays. *Nature Biotechnol.* 15:537-541.

Morch, M.D., Drugeon, G., Zagorski, W., and Haenni, A.L. 1986. The synthesis of high-molecular-weight proteins in the wheat germ translation system. *Methods Enzymol.* 118:154-164.

Moss, B., Elroy-Stein, O., Mizukami, W.A., and Fuerst, T.R. 1990. New mammalian expression vectors. *Nature* 348:91-92.

Murrell, A.M., Bockamp, E.O., Gottgens, B., Chan, Y.S., Cross, M.A., Heyworth, C.M., and Green, A.R. 1995. Discordant regulation of SCL/TAL-1 mRNA and protein during erythroid differentiation. *Oncogene* 11:131-139.

Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C., and Schultz, P.G. 1989. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* 244:182-188.

Noren, C.J., Anthony-Cahill, S.J., Suich, D.J., Noren, K.A., Griffith, M.C., and Schultz, P.G. 1990. In vitro suppression of an amber mutation by a chemically aminoacylated transfer RNA prepared by runoff transcription. *Nucl. Acids Res.* 18:83-88.

Olejnik, J., Sonar, S, Krzymanska-Olejnik, E., and Rothschild, K.J. 1995. Photocleavable biotin derivatives: A versatile approach for the isolation of biomolecules. *Proc. Natl. Acad. Sci. USA* 92:7590-7594.

Olejnik, J., Krzymanska-Olejnik, E., and Rothschild, K.J. 1998. Photocleavable affinity tags for isolation and detection of biomolecules. *Methods Enzymol.* 291:135-154.

Pancharatnam, J., Jagus, R., Lance, V., and Place, A.R. 1996. The role of $P450_{arom}$ in sex determination of prototheria and nonmammalian vertebrates. *In* Molecular Zoology (J.D. Ferraris and S.R. Palumbi, eds.) pp. 369-400. Wiley-Liss, New York.

Pelham, H.R.B. and Jackson, R.J. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.

Pieroni, L., Santolini, E., Fipaldini, C., Pacini, L., Migliaccio, G., and La Monica, N. 1997. In vitro study of the NS2-3 protease of hepatitis C virus. *J. Virol.* 71:6373-6380.

Powell, S.M., Petersen, G.M., Krush, A.J., Booker, S., Jen, J., Giardiello, F.M., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. 1993. Molecular diagnosis of familial adenomatous polyposis. *New Engl. J. Med.* 329:1982-1987.

Roberts, B.E. and Paterson, B.M. 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. U.S.A.* 70:2330-2334.

Roest, P.A., Roberts, R.G., Sugino, S., van Ommen, G.J., and den Dunnen J.T. 1993. Protein truncation test (PTT) for rapid detection of translation-terminating mutations. *Hum. Mol. Genet.* 2:1719-1721.

Rothschild, K.J., Sonar, S.M., and Olejnik, J. 1997. Methods for the Detection and Isolation of Proteins: U.S. Patent no. 5:643,722.

Rowan, A.J. and Bodmer, W.F. 1997. Introduction of a *myc* reporter tag to improve the quality of mutation detection using the protein truncation test. *Hum. Mutat.* 9:172-176.

Roy, S., Lane, A., Yan, J., McPherson, R., and Hancock, J.F. 1997. Activity of plasma membrane-recruited Raf-1 is regulated by Ras via the Raf zinc finger. *J. Biol. Chem.* 272:20139-20145.

Sakalian, M., Parker, S.D., Weldon, R.A., and Hunter, E. 1996. Synthesis and assembly of retrovirus Gag precursors into immature capsids in vitro. *J. Virol.* 70:3706-3715.

Sanford, J.C., Yu, J., Pan, J.Y., and Wessling-Resnick, M. 1995. GDP dissociation inhibitor serves as a cytosolic acceptor for newly synthesized and prenylated Rab5. *J. Biol. Chem.* 270:26904-26909.

Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouerfelli, O., Danishefsky, S., Rosen, N., and Hartl, F.U. 1996. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc. Natl. Acad. Sci. U.S.A.* 93:14536-14541.

Schreier, M.H. and Staehelin, T. 1973. Translation of duck-globin messenger RNA in a partially purified mammalian cell-free system. *Eur. J. Biochem.* 34:213-218.

Sharp, T.V., Witzel, J.E., and Jagus, R. 1997. A homologous region of eIF2α and vaccinia virus K3L gene product interact with the same domain in PKR. *Eur. J. Biochem.* 250:85-92.

Sif, S. and Gilmore, T.D. 1993. NF-κB p100 is one of the high–molecular weight proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. *J. Virol.* 67:7612-7617.

Smith, K.J., Scotland, G., Beattie, J., Trayer, I.P., and Houslay, M.D. 1996. Determination of the structure of the N-terminal splice region of the cyclic AMP–specific phosphodiesterase RD1 (RNPDE4A1) by ^{1}H NMR and identification of the membrane association domain using chimeric constructs. *J. Biol. Chem.* 271:16703-16711.

Sonar, S. 1994. Site-directed isotope labelling and FTIR spectroscopy of bacteriorhodopsin. *Struct. Biol.* 1:512-516.

Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y., and Alakhov, Y.B. 1988. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* 242:1162-1164.

Struhl, K. 1989. Synthesizing proteins in vitro by transcription and translation of cloned genes. *In* Current Protocols in Molecular Biology (F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 10.17.1-10.17.5. John Wiley & Sons, New York.

Stukenberg, P.T., Lustig, K.D., McGarry, T.J., King, R.W., Kuang, J., and Kirschner, M.W. 1997. Systematic identification of mitotic phosphoproteins. *Curr. Biol.* 7:338-348.

Switzer, W.M. and Heneine, W. 1995. Rapid screening of open reading frames by protein synthesis with an in vitro transcription and translation assay. *BioTechniques* 18:244-248.

Thompson, D., Van Oosbree, T., Beckler, G., and Van Herwynen, J. 1992. The TNT lysate systems: One step transcription/translation in rabbit reticulocyte lysate. *Promega Notes* 35:1-4.

Thulasiraman, V. and Matts, R.L. 1996. Effect of geldanamycin on the kinetics of chaperone-mediated renaturation of firefly luciferase in rabbit reticulocyte lysate. *Biochemistry* 35:13443-13450.

Walter, P. and Blobel, G. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84-93.

Warner, D.R., Basi, N.S., and Rebois, R.V. 1995. Cell-free synthesis of functional type IV adenylyl cyclase. *Anal. Biochem.* 232:31-36.

Zucker, W.V. and Schulman, H.M. 1968. Stimulation of globin-chain initiation by hemin in the reticulocyte cell-free system. *Proc. Natl. Acad. Sci. U.S.A.* 59:582-589.

Internet Resources

http://www.promega.com/promtech/tntbib.html

Bibliography of references using the TNT coupled trancription/translation system, compiled by G.S. Beckler in 1998. Promega Corp., Madison, Wis.

Contributed by Rosemary Jagus
Center of Marine Biotechnology and
Greenebaum Cancer Center
Baltimore, Maryland

Gregory S. Beckler
Promega Corporation
Madison, Wisconsin

In Vitro Translation

This unit describes the production and use of the two most widely employed eukaryotic in vitro translation systems—those derived from rabbit reticulocytes (see Basic Protocol 1) and from wheat germ (see Basic Protocol 2). These systems are useful for the production of proteins from mRNA isolated from eukaryotic cells or tissues or from mRNAs transcribed in vitro from cloned cDNAs that contain promoters for bacteriophage RNA polymerases. Also described is a time-saving variation that combines transcription and translation in a one-tube reaction (see Basic Protocol 3).

Although in vitro translation systems were developed primarily for the identification of mRNAs and the characterization of their products, the systems have been widely used for the identification of viral genes and the verification of cloned cDNAs. More recently, the systems have found a plethora of applications, including generation of protein variants that can be assayed for function in vitro (mutagenesis combined with expression-PCR), analysis of molecular interactions, characterization of molecular function, screening for mutants in disease states (protein truncation test), and high-throughput drug screening (see *UNIT 11.1*). The systems can be used to translate mRNAs attached to polysomes (see Alternate Protocol 1), purified from tissues or cell lines (see *APPENDIX 3*), or transcribed in vitro (see Support Protocols 1 and 2). Depending on the application, the systems can be used as small or large static reactions, in continuous-flow mode, and with either nonradioactive, radioactive, or modified amino acids (see Alternate Protocol 2 and Support Protocols 3 and 4).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

PRODUCTION AND USE OF mRNA-DEPENDENT CELL-FREE TRANSLATION SYSTEM FROM RABBIT RETICULOCYTES

BASIC PROTOCOL 1

Experimentally induced anemia results in increased accumulation of circulating reticulocytes. Treatment of rabbits with phenylhydrazine, which oxidizes red blood cell membranes, leads to premature lysis of these cells and induction of reticulocytosis. A number of variations on the phenylhydrazine protocol given here have been used successfully. However, since the translation machinery is lost as reticulocytes mature into red blood cells, it is extremely important that the treatment program be standardized to give high reticulocyte counts. It is good practice to keep rabbits for 1 week before starting to inject them to ensure a healthy starting condition. Ideally, the rabbits should weigh 3 kg. Reticulocyte count should be monitored by drawing blood from the marginal vein of the ear and examining cells stained with Brilliant Cresyl Blue. The interval between the last injection and bleeding allows the rabbits to clear the toxic products of phenylhydrazine from the circulation and increases the hematocrit and, hence, the yield of lysate.

Processing should be conducted as quickly as possible to produce a lysate of high protein-synthetic activity. If the need for lysate is high, it is possible to process five to ten rabbits at a time, since this should not increase processing time significantly. However, it is probably prudent to become familiar with the procedure with one or two rabbits before tackling ten. If processing more than one rabbit, the blood should be pooled prior to processing. If the need for lysate is low or occasional, it is probably better to purchase whichever commercially available system is most suitable. The activity of the translation systems should be tested after preparation and compared to that of previous batches or of

Contributed by Rosemary Jagus, Bhavesh Joshi, Suzanne Miyamoto, and Gregory S. Beckler
Current Protocols in Cell Biology (1998) 11.2.1-11.2.25

commercially available systems such as those provided by Amersham, Boehringer, Novagen, or Promega. The system described below can be used to translate mRNA purified from tissues or cells (see *APPENDIX 3*) or polysomes (see Alternate Protocol 1), or from in vitro–transcribed mRNAs, which can be uncapped (see Support Protocol 1) or capped (see Support Protocol 2) depending on the application and the choice of expression vector.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Female New Zealand white rabbits, 2 to 3 kg
2.5% (w/v) phenylhydrazine (see recipe)
100 mg/ml ketamine hydrochloride
20 mg/ml xylazine hydrochloride
500 U/ml heparin in sterile water (store at −20°C)
70% ethanol
0.4 mg/ml sodium pentobarbital
Reticulocyte wash buffer I (see recipe), with and without 1 U/ml heparin (from 500 U/ml stock)
Reticulocyte wash buffer II (see recipe)
Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*), sterile
100 mM $CaCl_2$ (*APPENDIX 2A*)
1 mM hemin hydrochloride (see recipe)
200 U/ml creatine phosphokinase (see recipe)
15,000 U/ml *Staphylococcus aureus* nuclease S7 (see recipe; prepare fresh)
200 mM EGTA (see recipe)
5 mg/ml calf liver tRNA (see recipe)
2 M KCl/10 mM magnesium chloride (K/Mg; see recipe)
1 mM amino acids minus methionine (see recipe)
[^{35}S]Methionine, translation grade (e.g., NEN Life Sciences or Amersham)
20 to 40 U/µl RNasin ribonuclease inhibitor (Promega) or equivalent
1 M phosphocreatine (see recipe)
Translation substrate: mRNA purified from cells or tissues (see *APPENDIX 3*) *or* uncapped or capped in vitro transcript (see Support Protocols 1 and 2)
5% and 10% trichloroacetic acid (TCA; see recipe)

60-ml disposable syringes
1-ml syringes
60-ml spring for 60-ml syringes
No. 16 and 1.5-in.-long no. 20 Huberpoint needles (Fisher)
Liquid nitrogen
Glass-fiber filters (e.g., Whatman GFC or equivalent)
Liquid scintillation counter and scintillation fluid

Prepare reticulocytes

1. Each day for 5 days, at the same time in the late afternoon, inject immature female New Zealand white rabbits (2 to 3 kg) subcutaneously in the interscapular region with 0.25 ml/kg of 2.5% phenylhydrazine hydrochloride using no. 20 Huberpoint needles.

   The weight of the rabbit is only a good guide for dosage with younger rabbits, since the drug does not equilibrate with adipose tissue.

2. Allow rabbits to recover for 2 days. Monitor reticulocyte count.

3. Early in the morning of day 8, inject rabbits intramuscularly in the thigh with 15 to 20 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride using a no. 20 Huberpoint needle.

4. Coat the inside of a spring-loaded 60-ml syringe with 0.1 ml of 500 U/ml heparin and attach to a no. 16 Huberpoint needle. When the rabbit is completely unconscious, lay it on its back and spray the thorax with 70% ethanol. With the spring attached to the syringe, push the plunger in completely and insert the needle under the sternum, pointing towards the heart.

   Use of the spring-loaded syringe takes most of the skill requirement out of blood collection by heart puncture. Once inside the thoracic cavity, the spring will prevent the syringe plunger from withdrawing. When the needle punctures the heart, the pressure of blood will allow the syringe to fill unassisted, a clear indication that the heart has been reached.

   The combination of ketamine hydrochloride and xylazine hydrochloride anesthetizes the rabbit and relaxes the muscles while maintaining heart rate, blood pressure, and ventricular function. It is preferable to sodium pentobarbital, which decreases blood oxygen and can result in lower translation activity in the lysates prepared from isolated reticulocytes as well as death of the rabbit before bleeding is complete. Note that ketamine/xylazine anesthesia abolishes the righting reflex, but does not affect the toe-web pinch reflex.

5. Recoat inside of syringe with 0.1 ml heparin and repeat bleeding procedure in same rabbit until no more blood can be extracted.

   It should be possible to withdraw between 60 and 100 ml blood from each rabbit.

6. After bleeding, euthanize each rabbit by injecting intraperitoneally with 200 mg/kg sodium pentobarbital.

Wash reticulocytes

7. Discharge blood into ~5 vol reticulocyte wash buffer I containing 1 U/ml heparin. Collect the cells by centrifugation for 10 min at 1400 × *g*, 4°C. Remove the supernatant by gentle suction and discard.

   When discharging blood from the syringe into the wash buffer, remove needle first to avoid rupturing the reticulocytes.

   After centrifugation the supernatant should be clear and pale straw-colored. Any redness in the supernatant suggests that the reticulocytes have not pelleted or lysis has occurred.

8. Wash twice by resuspending in ~5 to 10 vol reticulocyte wash buffer I without added heparin and centrifuging 10 min at 1400 × *g*, 4°C, discarding the supernatant each time.

   The washes are to remove serum (including clotting factors) and heparin, which is inhibitory to protein synthesis. Use maximum possible rinse volumes or at least 5 times the volume of blood collected. Reticulocyte wash buffer I contains 7.5 mM $MgCl_2$ to stabilize the reticulocyte cell membrane, along with 1 mM glucose to prevent the reticulocytes from becoming depleted of ATP and reducing equivalents. Some published procedures call for removal of the "buffy coat," the fluffy layer of white cells that sediment on top of the heavier reticulocytes, to prevent contamination by ribonucleases contained in the white cells. In practice, however, the white cells will not lyse under the conditions used to lyse reticulocytes, so their removal is unnecessary, and it reduces the yield of reticulocytes obtained.

9. Wash once with reticulocyte wash buffer II. Centrifuge as in step 7. Remove as much supernatant as possible to avoid carryover of salts into the lysate.

   Reticulocyte wash buffer II used in the final rinse is lacking the Mg^{2+} contained in reticulocyte wash buffer I. This is to prevent carryover into the reticulocyte lysate, since protein synthesis can be inhibited by Mg^{2+} at higher concentrations.

Prepare lysate

10. Transfer the cell pellet to a sterile graduated cylinder and record the volume of packed cells. Add 1.5 vol sterile DEPCW. Stir on ice for 5 min to maximize lysis.

11. Centrifuge 20 min at 20,000 × *g*, 4°C, to remove cell membranes and mitochondria. Decant the supernatant, and snap-freeze a few aliquots as "parent lysate" to compare with the mRNA-dependent lysate (MDL). Subject the rest to endonuclease treatment.

    The parent lysate is useful to provide an indication of how much incorporation to expect in a cell-free translation system working at maximum efficiency/capacity. It is also useful if the MDL is not very active and it is necessary to determine whether the problem came from the reticulocyte lysate prep itself or arose during the endonuclease treatment. In addition, the parent lysate can be used to optimize each batch for K^+, Mg^{2+}, and hemin concentrations.

12. To each 25 ml of lysate, add:

    250 µl 100 mM $CaCl_2$
    500 µl 1 mM hemin hydrochloride
    250 µl 200 U/ml creatine phosphokinase
    250 µl 15,000 U/ml nuclease S7.

    Incubate 15 min at 20°C.

13. Add 250 µl of 200 mM EGTA to chelate the Ca^{2+} and inactivate the nuclease. Add 250 µl calf liver tRNA (5 mg/ml). Snap-freeze 500-µl aliquots and store in liquid nitrogen.

    After nuclease treatment, the lysate is referred to as mRNA-dependent lysate or MDL. This preparation should produce between 30 and 40 ml MDL per rabbit. This is sufficient for 600 to 800 50-µl assays.

    Calf liver tRNA is added not because endogenous tRNA is hydrolyzed by endonuclease, but because the composition of reticulocyte tRNA is related to the amino acid composition of globin and may not always support the production of full-length proteins.

    Translation systems from reticulocytes retain activity for years when stored in liquid nitrogen. At −70°C, loss of activity is discernible and the lysate can only be used for 3 to 4 months. Storage at −20°C is not recommended.

14. Test each batch of MDL produced for translational activity by performing steps 15 to 20 using a suitable test RNA (mRNA purified from cells or tissues or transcribed in vitro).

    A good test transcript is that produced from Promega's pGEM-luc DNA, since a functional assay measuring luciferase activity can be used. This has the advantage of not requiring a radioactive amino acid and ensures that only full-length polypeptides are scored.

Translate mRNA

15. Remove reagents from storage and place on ice. Rapidly thaw MDL by hand warming and place on ice.

16. Assemble the following reaction components in a 0.5- to 1.5-ml sterile microcentrifuge tube (for 50 µl):

    2.5 µl K/Mg
    2.5 µl amino acid mixture minus methionine
    2.5 µl [^{35}S]methionine
    0.35 µl 20 to 40 U/µl RNasin
    0.5 µl 1 M phosphocreatine
    0.5 µl 200 U/ml creatine phosphokinase

continued

0.5 µl 1 mM hemin hydrochloride
35 µl MDL
0.5 to 2 µl (~0.5 µg) mRNA substrate
Nuclease-free water to 50 µl final.

Mix by gentle vortexing and, if necessary, microcentrifuge 5 sec to return reaction mix to bottom of tube.

The reaction volume can be modified from 10 µl to 1 ml depending on application. Capped microcentrifuge tubes should be used to avoid changes in reaction volume.

Include a control reaction without added template to assess if any translation products are arising from undegraded endogenous mRNA. Note that the composition of the reaction described above is optimal for MDL prepared as described in the preceding steps. The preparation of commercially available MDL may vary, and the manufacturer's recommendations for use should be followed.

Increased expression levels may be obtained by optimizing Mg^{2+} and K^{+} concentrations for a particular mRNA species. The mRNA used may be purified from cells or tissue or transcribed in vitro from cDNAs downstream of a phage polymerase promoter. Unfractionated cytoplasmic RNA preparations contain 90% to 95% rRNA and translate very poorly.

If synthesizing a recombinant protein such as an enzyme for functional analysis, higher protein levels can be achieved by substituting 1 mM nonradioactive methionine (2.5 µl/50-µl reaction) for [^{35}S]methionine.

17. Incubate 30 min to 1 hr at 30°C.
18. Determine amount of radioactivity incorporated into TCA-precipitable material by diluting 1 to 2.5 µl of the reaction in 1 ml ice-cold water and adding 1 ml of 10% TCA. Heat the mixture 10 min at 95°C to discharge aminoacylated tRNAs, then cool on ice.
19. Collect precipitated protein by vacuum filtration onto glass-fiber filters. Rinse with 5% TCA, and dry filters under an infrared lamp. Measure radioactivity by liquid scintillation spectroscopy.
20. Use remaining incubation mix for assay of function or analysis by SDS-PAGE (*UNIT 6.1*).

PRODUCTION AND USE OF mRNA-DEPENDENT CELL-FREE TRANSLATION SYSTEM FROM WHEAT GERM

BASIC PROTOCOL 2

In this procedure, wheat germ extract is prepared by grinding wheat germ in an extraction buffer, then centrifuging the resulting paste to remove cell debris. Endogenous amino acids and plant pigments that are inhibitory to protein synthesis are removed by gel filtration. Although the level of endogenous mRNA is low compared to that in untreated reticulocyte lysate, the response to added mRNAs is improved by treatment with calcium-dependent micrococcal nuclease with subsequent addition of sufficient EGTA to chelate the free calcium. The most important consideration in preparing wheat germ extract of high translational activity is to find a good batch of fresh commercial wheat germ low in endosperm-rich fragments. There can be considerable variability in the quality of wheat germ from one manufacturer and the activity of the resulting cell-free translation system, depending on growth conditions and conditions at harvest.

The wheat germ system can be used with all the alternative methods described for use of the reticulocyte system (see Support Protocols 1 and 2; see Alternate Protocols 1 and 2). It can also be used as a coupled transcription/translation system (see Basic Protocol 3).

Materials

Wheat germ
Wheat germ extraction buffer (see recipe; prepare fresh)
100-ml Sephadex G-25 column (Amersham Pharmacia Biotech) preequilibrated in extraction buffer
100 mM $CaCl_2$ (*APPENDIX 2A*)
1 mM hemin hydrochloride (see recipe)
200 U/ml creatine phosphokinase (see recipe)
15,000 U/ml nuclease S7 (see recipe; prepare fresh)
200 mM EGTA (see recipe)
5 mg/ml calf liver tRNA (see recipe)
50 mM ATP (see recipe for 4NTP mixture)
50 mM GTP (see recipe for 4NTP mixture)
50 mM and 1 M magnesium acetate
20 to 40 U/µl RNasin ribonuclease inhibitor (Promega) or equivalent
1 mM amino acids minus methionine (see recipe)
1 M potassium acetate
1 M phosphocreatine (see recipe)
10 mCi/ml [^{35}S]methionine (1000 Ci/mmol)
Translation substrate: mRNA purified from cells or tissues (see *APPENDIX 3*) *or* uncapped or capped in vitro transcript (see Support Protocols 1 and 2)
Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*)
Sterile, RNase-free glass powder (see recipe)

Prepare wheat germ cell-free translation system

1. Working at 4°C with all components and apparatus prechilled, grind 5 g wheat germ with 5 g sterile glass powder and 22.5 ml wheat germ extraction buffer to give a smooth paste. Centrifuge for 10 min at 30,000 × *g*, 4°C.

2. Remove top 20 ml of supernatant and load onto a 100-ml Sephadex G-25 column preequilibrated in extraction buffer. Use ~100 ml of the same buffer for elution.

   A low-molecular-weight inhibitor of protein synthesis and a deep yellow pigment are removed by the column.

3. As soon as all the extract has penetrated into the column, follow with 100 ml of extraction buffer. Collect 2.5-ml fractions and measure A_{260} by spectrophotometry (see *APPENDIX 3*). Pool the highest-absorbance fractions (which are also the most opaque fractions), usually fractions 20 to 30.

4. Let pooled fractions stand 5 min on ice, then centrifuge 10 min at 23,000 × *g*, 0°C. Carefully remove upper 20 ml.

   A high-molecular-weight inhibitor of protein synthesis aggregates as the column fractions incubate on ice. This will disperse if temperature rises above 4° or 5°C.

5. Add the following per 20 ml of lysate:

   200 µl 100 mM $CaCl_2$
   400 µl 1 mM hemin hydrochloride
   200 µl 200 U/ml creatine phosphokinase
   200 µl 15,000 U/ml nuclease S7.

   Incubate 15 min at 20°C.

6. Add 200 µl of 200 mM EGTA per 20 ml (to chelate Ca^{2+} and inactivate the nuclease).

7. Add the following per 20 ml of lysate:

   200 µl 5 mg/ml calf liver tRNA
   625 µl 50 mM ATP
   125 µl 50 mM GTP
   37.5 µl 1 M magnesium acetate.

   Mix, then snap-freeze in 200- to 500-µl aliquots and store in liquid nitrogen.

8. Test each batch of wheat germ extract produced for translational activity prior to use (see Basic Protocol 1, step 14).

Translate mRNA

9. Remove reagents from storage and place on ice. Rapidly thaw wheat germ extract by hand warming and place on ice.

10. Assemble the following reaction components in a 0.5- to 1.5-ml sterile microcentrifuge tube (50 µl):

    25 µl wheat germ extract
    1 µl 20 to 40 U/µl RNasin
    4 µl amino acid mix minus methionine (or other amino acid, as appropriate)
    3.75 µl 1 M potassium acetate
    1 µl 50 mM magnesium acetate
    2.5 µl 10 mCi/ml [^{35}S]methionine (1000 Ci/mmol)
    0.5 to 2 µl mRNA substrate (to give ~0.5 to 1 µg)
    DEPCW to 50 µl.

    Mix by gentle vortexing and, if necessary, microcentrifuge 5 sec to collect the reaction mix in the bottom of the tube.

    Include a reaction without added template to measure any background incorporation from endogenous mRNA. Note that the composition of the reaction described above is optimal for wheat germ extract prepared as described above. Preparation of commercially available wheat germ extract may vary, and the manufacturer's recommendations for use should be followed.

    Increased expression levels may be obtained by optimizing Mg^{2+} and K^{+} concentrations for a particular mRNA species. As with MDL, the mRNA used may be purified from cells or tissue (see APPENDIX 3) or transcribed in vitro from cDNAs downstream of a phage polymerase promoter (see Support Protocols 1 or 2).

11. Incubate 1 to 2 hr at 25°C.

12. Use 1.0 to 2.5 µl of reaction mixture for determining radioactivity incorporated into TCA-precipitable material (see Basic Protocol 1, steps 18 and 19). Use remainder for assay of function or analyze by SDS-PAGE (*UNIT 6.1*).

IN VITRO PROTEIN SYNTHESIS IN COUPLED TRANSCRIPTION/TRANSLATION SYSTEMS

BASIC PROTOCOL 3

Transcription of cDNA clones by bacteriophage RNA polymerases may be carried out simultaneously with translation in a single reaction by adding DNA directly to the translation system along with the appropriate RNA polymerase (T3, T7, or SP6). This adaptation obviates the need for three prior reactions: (1) linearization of DNA, (2) in vitro transcription, and (3) 5′ capping and methylation of mRNA. Optimization of the conditions for the combined reactions are closer to those required for in vitro translation than transcription. Under the conditions outlined, transcription is relatively inefficient, but sufficient levels of trancripts are produced to saturate the protein synthetic capacity

of the system. The reaction buffer is modified to provide the NTPs and additional Mg^{2+}, with the Mg^{2+} concentration being the critical parameter.

Materials

2 M KCl/10 mM $MgCl_2$ (K/Mg; see recipe)
Amino acid mixture minus methionine (see recipe)
25× transcription/translation buffer (TX/TL buffer; see recipe)
10 mCi/ml [^{35}S]methionine (1000 Ci/mmol)
20 to 40 U/µl RNasin ribonuclease inhibitor (Promega) or equivalent
1 M phosphocreatine (see recipe)
200 U/ml creatine phosphokinase (see recipe)
1 mM hemin hydrochloride (see recipe)
mRNA-dependent lysate (MDL; see Basic Protocol 1, step 14, or see Basic Protocol 2, step 8)
Plasmid DNA, at ≥0.1 mg/ml (*APPENDIX 3*)
Appropriate bacteriophage RNA polymerase (e.g., T3, T7, or SP6)
Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*)

1. Remove reagents from storage and place on ice. Rapidly thaw MDL by hand warming and place on ice.

2. Assemble the following reaction components in a 0.5- to 1.5-ml sterile microcentrifuge tube (50 µl):

   2.5 µl K/Mg
   2.5 µl amino acid mixture minus methionine
   2.0 µl 25× TX/TL buffer
   2.5 µl 10 mCi/ml (1000 Ci/mmol) [^{35}S]methionine
   0.35 µl 20 to 40 U/µl RNasin
   0.5 µl 1 M phosphocreatine
   0.5 µl 200 U/ml creatine phosphokinase
   1 µl 1 mM hemin hydrochloride
   25 µl MDL
   0.5 to 2 µl plasmid DNA (to give ~0.5 µg)
   RNA polymerase to give 600 to 1000 U/ml
   DEPCW to 50 µl.

   Mix by gentle vortexing, and if necessary microcentrifuge 5 sec to return reaction mixture to bottom of tube.

   Note that the amino acid mix minus methionine and [^{35}S]methionine can be replaced by complete amino acid mix and biotinylated lysine-tRNA. Alternatively, if synthesizing a recombinant protein such as an enzyme for functional analysis, higher protein levels can be achieved by substituting 1 mM nonradioactive methionine for [^{35}S]methionine (2.5 µl/50-µl reaction).

   Ensure that all ethanol is removed from DNA before adding to the reaction. The smaller the volume of DNA added to the reaction, the less likely it will be that a nonspecific inhibitor of protein synthesis will be introduced. Include a control incubation to which no DNA has been added. The reaction volume can be modified from 10 µl to 1 ml depending on application. Capped microcentrifuge tubes should be used to avoid changes in reaction volume.

3. Incubate 1 hr at 30°C.

4. Analyze as for the noncoupled system (see Basic Protocol 1, steps 18 to 20).

Best results are obtained with DNA from which all contaminating RNAs are removed by a method that does not involve RNase. However, miniprep DNA prepared by a variety of methods (see APPENDIX 3) has proved to be quite adequate. Optimal production of proteins appears to be obtained with ~1 μg plasmid DNA/100 μl reaction volume, although this varies somewhat with the purification method. However, adequate levels of product may be obtained with 0.1 μg plasmid DNA/100 μl reaction. Circular plasmid DNA gives the best results although linearized versions of most plasmids can be translated. Best results will be obtained from constructs containing the EMCV 5′ UTR and a 3′ poly(A)$^+$ sequence.

PRODUCTION OF UNCAPPED IN VITRO TRANSCRIPTS

SUPPORT PROTOCOL 1

Transcripts produced in vitro can be used in place of purified mRNA in either the reticulocyte or wheat germ translation systems. To produce high levels of uncapped mRNA, transcription reactions containing 4 mM of each nucleotide triphosphate (NTP) are used since the production of mRNA is proportional to the amount of NTP in the reaction up to this concentration (Milligan et al., 1987). Yields are also increased by the inclusion of 0.01% Triton X-100 and BSA (Milligan et al., 1987). The transcript yield is measured by the inclusion of trace amounts of radiolabeled NTP (usually [α-^{32}P]CTP). The percentage of [α-^{32}P]CTP incorporated is measured, and the total amount of CTP is calculated based on the ratio of labeled to unlabeled nucleotide. From that the amount of RNA synthesized is determined. For translation of uncapped transcripts, the efficiency of translation will be increased by using the cDNA of interest in a vector that places an internal ribosome entry site (IRES) upstream of the initiation codon to generate a mRNA that is processed via cap-independent translation. Suitable vectors for achieving this include pCITE from Novagen and the commercially unavailable pTM1 (Moss et al., 1990).

The transcripts produced by in vitro transcription can be used in place of purified mRNA in any of the basic protocols. The integrity of the RNA should be verified by agarose gel electrophoresis prior to use. Because high concentrations of RNA are produced, sufficient RNA to saturate the translation system can be added in such a small volume that prior cleanup is not necessary, as long as the free Mg^{2+} is titrated out after completion of the transcription reaction.

Materials

Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*), sterile
5× modified transcription buffer (MTB; see recipe)
12.5 mM 4NTP mix (see recipe)
1 M $MgCl_2$ (*APPENDIX 2A*)
250 mM DTT (see recipe)
[α-^{32}P]CTP (3000 Ci/mmol) in tricine, diluted in DEPCW to 10,000 to 50,000 cpm/μl
20 to 40 U/μl RNasin ribonuclease inhibitor (Promega) or equivalent
Plasmid DNA or PCR product containing appropriate phage polymerase promoter (see *APPENDIX 3*), linearized using a restriction enzyme that generates 5′ protruding ends, at 0.2 to 0.5 mg/ml concentration
20 U/μl RNA polymerase (SP6, T3, or T7; Promega)
0.5 mg/ml yeast tRNA (carrier for TCA precipitation)
5% trichloroacetic acid (TCA; see recipe)
0.5 M EDTA (*APPENDIX 2A*)
RQ1 RNase-free DNase (Promega)
TE buffer, pH 8 (*APPENDIX 2A*)
1:1 (v/v) TE-saturated phenol/chloroform
24:1 (v/v) chloroform/isoamyl alcohol

7.5 M ammonium acetate
100% and 70% ethanol

Dry ice/ethanol bath
Glass-fiber filters (e.g., Whatman GFC or equivalent)
Liquid scintillation counter

Produce uncapped transcripts

1. Remove reagents from storage and place on ice.
2. Assemble the following reaction components, in the order listed, in a 1.5-ml sterile screw-cap microcentrifuge tube at room temperature (50 µl total):

   14.7 µl DEPCW
   10 µl 5× MTB
   16 µl 12.5 mM 4NTP mix
   0.8 µl 1 M $MgCl_2$
   2 µl 250 mM DTT
   2.5 µl 20 to 40 U/µl RNasin
   1 µl 10,000 to 50,000 cpm/µl [α-^{32}P]CTP (200 to 1000 cpm/µl final)
   1 µl 0.2 to 0.5 mg/ml linearized DNA
   2 µl 20 U/µl appropriate RNA polymerase (800 U/ml final).

   Mix by gentle vortexing and microcentrifuge 5 sec to collect the reaction mix in the bottom of tube.

   Care should be taken in the choice of restriction enzyme for linearizing the DNA, since templates containing 3′ protruding ends allow the transcription of RNA from these sites. Enzymes such as BamHI and NcoI, which produce 5′ protruding ends, are good choices, whereas those such as Apa I, Sac I, and PstI that generate 3′ protruding ends should be avoided.

3. Place 1-µl spots of the mixture onto two glass-fiber filters and allow filters to dry; meanwhile, incubate remainder of reaction mixture 30 min to 1 hr at 37°C.
4. After the reaction incubation is over, pipet duplicate 1-µl samples of the transcription mixture into 100 µl of 0.5 mg/ml yeast carrier tRNA and add 2 ml of 5% TCA on ice. Collect precipitate on glass-fiber filters by vacuum filtration and allow to dry.
5. Determine total radioactivity (total cpm/µl) of pre- and postincubation samples on filters by liquid scintillation spectroscopy.

   Dividing the value for the second sample by that for the first gives the percentage of [α-^{32}P]CTP incorporated into the transcript. These reactions give between 25% and 30% incorporation of [α-^{32}P]CTP, which means that 50 to 60 nmol of CTP and ~200 to 240 nmol of RNA (depending on coding sequence) are made. Based on 0.33 µg/nmol, between 70 and 80 µg RNA is produced in a 50-µl reaction, giving enough RNA for ~50 50-µl translation assays (assuming some losses during cleanup). Once this has been determined for a particular DNA preparation, scale up the reaction 10-fold to give a greater supply of transcript for use in cell-free translation reactions.

6. Add EDTA to 10 mM final (from 0.5 M stock) to chelate free Mg^{2+}, and store at −20°C (good for several months).

   The reaction mix can be used directly in in vitro translation reactions without further processing. When using frozen reaction mix, warm briefly to 37°C prior to direct use in translation reactions (at 0.5 to 1 µl per 50-µl reaction). Alternatively, the transcripts can be purified from the reaction mix as follows.

   Because high concentrations of RNA are produced, sufficient RNA to saturate the translation system can be added in such a small volume that prior cleanup is not necessary, as long as the free Mg^{2+} is titrated out after completion of the transcription reaction.

Optional: Purify transcripts by phenol/chloroform extraction and ethanol precipitation

7. Add 1 U RQ1 DNase/µg template DNA and incubate 15 min at 37°C.
8. Add 200 µl TE buffer to the reaction mixture, then extract with 250 µl of 1:1 (v/v) TE-saturated phenol/chloroform. Vortex 1 min, then microcentrifuge 5 min at maximum speed, 4°C.
9. Transfer the upper (aqueous) phase to a fresh tube and repeat extraction with 250 µl of 24:1 (v/v) chloroform/isoamyl alcohol.
10. To the final aqueous phase, add 0.5 vol of 7.5 M ammonium acetate and 2.5 vol of 100% ethanol. Place mixture in dry ice/ethanol bath for 10 min. Microcentrifuge 5 min at maximum speed, 4°C.
11. Aspirate supernatant and wash pellet with 1 ml of 70% ethanol. Dry under vacuum and resuspend in a volume of DEPCW that will give ~1 mg/ml transcript. Store at −85°C.

Dilution of the transcription reaction is only necessary to avoid large losses when dealing with small volumes. For scaled-up reactions, this dilution is not necessary.

Equivalent or higher levels of uncapped transcripts can be produced using kits from Ambion, Novagen, or Promega.

It is possible that some double-stranded RNA (dsRNA) may be produced during in vitro transcription reactions from promiscuous binding of the polymerase. This will not be removed by the usual cleanup procedures, but it can be kept to a minimum provided the DNA is not linearized with restriction enzymes that generate 3′ protruding ends. If significant dsRNA is produced in the reaction, this will lead to activation of endogenous PKR, the dsRNA-activated kinase that phosphorylates and downregulates the translation factor eIF2. Higher levels of translation products may be generated by including additives that prevent PKR activation or eIF2α phosphorylation. These include 2 to 10 mM 2-aminopurine, high levels (10 µg/ml) of dsRNA such as poly(I)·poly(C), or 100 to 200 pmol/ml of the vaccinia virus K3L gene product, the pseudosubstrate of eIF2α.

PRODUCTION OF CAPPED IN VITRO TRANSCRIPTS

SUPPORT PROTOCOL 2

The production of capped transcripts is more expensive and more challenging than the production of uncapped transcripts and may not be necessary for the translation of many RNAs. Because of the presence of a cap analog as well as the lower concentration of transcripts generated, the reaction mix cannot be used directly in cell-free translation systems but must be purified prior to use.

Additional Materials *(also see Support Protocol 1)*

5× transcription buffer (TB; see recipe)
10 mM each ATP, CTP, UTP, and GTP (see recipe for 4NTP mixture)
10 mM ^{7}m(5′)Gppp(5′)G (cap analog; Amersham, Pharmacia Biotech)

1. Remove reagents from storage and place on ice.
2. Assemble the following reaction components, in the order listed, in a 1.5-ml sterile screw-cap microcentrifuge tube at room temperature (50 µl total):

13.375 µl DEPCW
10 µl 5× TB
5 µl *each* 10 mM ATP, CTP, and UTP
0.625 µl 10 mM GTP
2.5 µl 10 mM ^{7}m(5′)Gppp(5′)G

continued

1 µl 10,000 to 50,000 cpm/µl [α-^{32}P]CTP (200 to 1000 cpm/µl final)
2 µl 250 mM DTT
2.5 µl 20 to 40 U/µl RNasin
1 µl 0.2 to 0.5 mg/ml linearized plasmid DNA
1 µl 20 U/µl appropriate RNA polymerase (400 U/ml final).

Mix by gentle vortexing and microcentrifuge 5 sec to collect reaction mixture in bottom of tube.

The cap analog is present at a higher concentration than GTP, which is rate limiting for transcription. This ensures that the majority of transcripts are initiated with the cap analog. The cap analog is not used internally to any significant extent.

3. Place 1-µl spots of the mixture onto two Whatman GFC filters and allow filters to dry; meanwhile, incubate reaction mixture 45 min at 37°C.
4. After the 45-min incubation, pipet duplicate 1-µl samples of the reaction mixture into 100 µl of 0.5 mg/ml yeast carrier tRNA and add 2 ml of 5% TCA on ice. Collect precipitate on glass-fiber filters by vacuum filtration and allow to dry.
5. Determine total radioactivity (cpm/µl) of the pre- and postincubation samples on filters by liquid scintillation spectroscopy.

   Dividing the value for the second sample by that for the first gives the percent of [α-^{32}P]CTP incorporated into the transcript.

6. Add another 0.625 µl of 10 mM GTP and 1 µl of 20 U/µl RNA polymerase. Continue incubation for 45 min, then determine percentage of [α-^{32}P]CTP incorporated as in step 5.

   As the reaction proceeds, the GTP concentration falls, although the concentration of m^7(5′)Gppp(5′)G does not decrease significantly. The late addition of more GTP allows more synthesis to occur without affecting the use of the cap analog for initiation. These conditions allow 18% to 22% incorporation of CTP, which is equivalent to 9 to 11 nmol CTP, 36 to 44 nmol RNA, and 12 to 15 µg RNA. Between 75% and 90% of these trancripts are capped. This gives enough RNA for ~10 50-µl translation assays (assuming some losses during cleanup).

7. Stop reaction with RQ1 DNase and purify by phenol extraction and ethanol precipitation (see Support Protocol 1, steps 7 to 11).

ALTERNATE PROTOCOL 1

TRANSLATION OF POLYSOMAL mRNAs FROM CELLS AND TISSUES

Translation of polysome-bound mRNAs in vitro provides a means to assess in vivo recruitment of mRNAs. Polysomes or subfractions of polysomes either in a free or membrane-associated state have long been a source of mRNAs that can be translated in vitro with the aid of rabbit reticulocyte lysate or other cell-free fractions. The translation of polysomal mRNAs allows a finer assessment of changes in gene expression than can be determined by studying the changes in mRNA steady-state levels or transcriptional activity.

The method described here involves gentle lysis of the cells with the nonionic detergent Nonidet P-40 (NP-40) and isolation of polysomes by ultracentrifugation. The polysome fraction contains ribosomes, mRNA, and associated proteins, but translation is suspended in the elongation process. Translation can resume in vitro in a translation system that provides all other requirements. In vitro translation of the polysome-bound mRNA fractions in the presence of [^{35}S]methionine allows quick assessment of what mRNAs are being utilized in the cell by a variety of methods using a radioactive amino acid (e.g.,

methionine) or biotinylated lysine-tRNA. The translation products arise not just from translation runoff of polysomes, but also by reinitiation. Sufficient product can be produced to allow identification of the translation products by immunological techniques or even sequencing.

Materials

10× cell lysis buffer (see recipe; prepare working solution fresh before use)
1 M 4-(2-aminoethyl)benzenesulfonyl fluoride hydrocholoride (AEBSF)
8 mg/ml aprotinin
2 mg/ml leupeptin
250 mM DTT (see recipe)
20 to 40 U/µl RNasin ribonuclease inhibitor (Promega) or equivalent
0.5 M EDTA (*APPENDIX 2A*)
10% Nonidet P-40 (NP-40)
Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*), sterile
10 mg/ml cycloheximide
Cells from which the polyribosomes are to be isolated
PBS (*APPENDIX 2A*), ice-cold liquid *and* frozen to a slurry
Sucrose buffer (see recipe; prepare fresh)
HEPES/KCl buffer (see recipe)

Beckman polyallomer thick-walled tubes
Beckman TL100 centrifuge and TLA100.3 rotor

Additional reagents and equipment for in vitro translation (see Basic Protocols 1, 2, and 3)

Prepare lysis buffer and cells

1. Prepare two batches of complete lysis buffer by adding the following to 100 µl of 10× cell lysis buffer on ice:

   1 µl 1 M AEBSF
   1 µl 8 mg/ml aprotinin
   1 µl 2 mg/ml leupeptin
   4 µl 250 mM DTT
   10 µl 20 to 40 U/µl RNasin
   2 µl 0.5 M EDTA.

   Add 40 µl of 10% NP-40 to one tube. Adjust final volume in each tube to 1 ml with DEPCW. Keep tubes on ice.

2. Add 1 µl of 10 mg/ml cycloheximide per milliliter of cell medium to be used. Incubate 5 min at 37°C.

   Incubation with cycloheximide prior to cell harvest piles up mRNAs in polysomes, increasing the yield of polysome-bound mRNA and increasing the translational activity. The cycloheximide is washed away during sedimentation of the polyribosomes and so does not prevent translation of the polysome-bound mRNAs in a cell-free translation system.

Lyse cells

For cells in suspension:

3a. To ~1 × 10^8 cells and add an equal volume of a frozen slurry of PBS to reduce temperature rapidly. Centrifuge 7 min at 1200 × *g*, 4°C.

4a. Aspirate supernatant carefully. Resuspend cells in 10 ml ice-cold PBS and transfer to a sterile 15-ml conical centrifuge tube. Centrifuge as in step 3a, and discard supernatant.

5a. Resuspend cells in 1 ml PBS and transfer to 1.5-ml microcentrifuge tube. Microcentrifuge 10 sec at 4°C. Aspirate supernatant and discard.

6a. Resuspend pellet in 0.5 ml complete lysis buffer without NP-40 (from step 1); vortex very gently. Add 0.5 ml complete lysis buffer with 0.4% NP-40 to cells and mix by pipetting up and down. Incubate on ice for a maximum of 10 min to allow cells to lyse.

For cells grown in monolayers:

3b. Remove medium from 10 × 100–mm plates (~1×10^8 cells) and place on metal blocks sitting in an ice bath.

4b. Rinse twice with ice-cold PBS, tilting plates after final rinse to ensure complete removal of PBS.

5b. Add 500 µl complete lysis buffer with NP-40. Incubate 5 min on ice to allow cells to lyse. Incubate on ice for a further 5 min.

6b. Scrape lysate into appropriate-sized microcentrifuge tubes.

The number of cells needed and exact lysis conditions may need to be determined for each cell type or cell line used. Since mRNAs remain in a cytoplasmic fraction, they may be contaminated with RNase, although association with polysomes seems to afford protection from degradation.

Isolate polysomes

7. Microcentrifuge 10 min at maximum speed, 4°C.

8. Layer supernatant over a 400-µl cushion of sucrose buffer in a Beckman polyallomer thick-wall tube. Centrifuge 50 min at 125,000 × *g* (80,000 rpm in Beckman TL100 centrifuge with TLA100.3 rotor), 4°C.

Use of a sucrose pellet traps any contaminating cell membranes that may carry ribonuclease or protease activity.

9. Aspirate supernatant and discard. Invert pellet on a laboratory wipe to remove last traces of supernatant.

The remaining polysomal pellet should be clear and slightly straw-colored.

10. Snap-freeze immediately on dry ice. Store in liquid nitrogen (good for use 1 to 2 years).

Translate polysomal bound mRNA

11. Resuspend polyribosomal pellet in 50 µl HEPES/KCl buffer.

The volume in which the polysomes are resuspended may need to be modified depending on polysome yield.

12. For a 50-µl translation reaction, combine components as described in Basic Protocol 1, step 16, or Basic Protocol 2, step 10, substituting the resuspended polysomes (1 to 4 µl) for mRNA. Adjust the final volume appropriately and incubate 1 hr at 30°C.

Alternatively, to allow capture of the newly synthesized proteins, include 1 to 2 µl biotinylated lysine-tRNA in the reaction mix. Similarly, if biotinylated lysine-tRNA is used, 1 mM methionine may be substituted for [^{35}S]methionine to give nonradioactive products. This decision should reflect anticipated use and detection method. This reaction can be scaled up (e.g., to 1 ml) to allow the isolation of sufficient biotinylated translation product for sequencing. For scaleup, optimization of the nonradioactive lysine concentration is important so that lysine is not limiting but the majority of polypeptide chains still contain biotinylated amino acids.

13. Analyze by SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*) or by immunoblotting (*UNIT 6.2*), as appropriate.

IN VITRO TRANSLATION WITH BIOTINYLATED AMINO ACIDS

ALTERNATE PROTOCOL 2

Biotinylated lysine residues can be used for detection of proteins synthesized in cell-free translation systems, obviating the need for [^{35}S]methionine or other radioactive amino acids. These residues are provided as precharged biotinylated lysine-tRNA complexes in which the biotin moiety is linked to lysine by a spacer arm at the ε-amino group. Biotinylated lysine-tRNA is available commercially as Transcend tRNA (Promega) and the Biotin-Lysine-tRNA Set (Boehringer Mannheim).

The modified amino acid is added to the in vitro translation reactions along with the amino acid mixture, which may or may not contain unmodified lysine depending upon the amount of incorporation of biotinylated lysine desired. The protein products are subjected to SDS-PAGE (*UNIT 6.1*) and electroblotted for immunoblot analysis (*UNIT 6.2*). The proteins are detected by binding either streptavidin–alkaline phosphatase (streptavidin-AP) or streptavidin–horseradish peroxidase (streptavidin-HRP) conjugate, followed by colorimetric or chemiluminescent detection, respectively. The sensitivity is equivalent to that achieved with [^{35}S]methionine incorporation and autoradiographic detection, with the advantage that no radioisotope handling, storage, or disposal is required. Furthermore, lysine is more highly represented in proteins than methionine (average lysine content is 6.6% compared to 1.7% for methionine).

The biotin tag is stable for at least 12 months as lysine-tRNA and within synthesized proteins. In addition, the use of biotinylated lysine incorporation can open up some new applications, since the protein product can be captured with streptavidin affinity chromatography, using either immobilized streptavidin-agarose columns (see Support Protocol 3) or streptavidin-linked magnetic beads (see Support Protocol 4).

Additional Materials *(also see Basic Protocols 1 and 2)*

mRNA-dependent lysate (see Basic Protocol 1, step 14, or see Basic Protocol 2, step 8)
Biotinylated lysine-tRNA complex (Promega's Transcend tRNA, or Boehringer Mannheim's Biotin-Lysine-tRNA Set) at 0.5 µg/µl (12 pmol/µl)
Amino acid mix, complete (see recipe)
Streptavidin-alkaline phosphatase (streptavidin-AP) or streptavidin-horseradish peroxidase (streptavidin-HRP)
Optional: Streptavidin-linked magnetic beads (Dynal) *or* capture resin such as Streptavidin MagneSphere Paramagnetic Particles or Softlink Soft Release Avidin Resin (Promega), Dynabeads M-280 Streptavidin (Dynal), or Streptavidin Magnetic Particles, Streptavidin, immobilized (gel suspension; Boehringer Mannheim)
Additional reagents and equipment for production and use of reticulocyte lysate (see Basic Protocol 1) or wheat germ lysate (see Basic Protocol 2) for in vitro translation, SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

Translate biotinylated proteins

1. Remove reagents from storage and place on ice. Rapidly thaw MDL by hand warming and place on ice.

2. Combine components for a 50-µl translation reaction (see Basic Protocol 1, step 16, or see Basic Protocol 2, step 10), substituting 2 µl of 0.5 µg/µl biotinylated lysine tRNA (1 µg; 24 pmol) for [^{35}S]methionine and complete amino acid mix for amino acids minus methionine. Adjust final volume appropriately.

   Complete amino acid mix is used rather than amino acids minus lysine because lysine represents a significant percentage of amino acids; thus, since the reticulocyte contains only ~7 to 10 µM lysine, without lysine supplementation the amount of protein produced will be reduced. Alternatively, it is possible to increase the incorporation of biotinylated lysine by using amino acid mixtures minus lysine, but the total yield of protein may be less.

Biotin labeling of proteins containing few lysines can be increased by doubling the amount of biotinylated lysine-tRNA added, increasing the specific activity of the biotinylated form.

3. Incubate reaction mixture 1 hr at 30°C.

   The translation reaction mix can be stored for several months at −20° to −85°C prior to analysis. Optionally, detection can be enhanced by capturing the biotinylated protein from a larger volume of translation reaction (see Support Protocols 3 and 4) prior to performing the analysis outlined in steps 4 and 5.

   Reticulocyte lysate contains one minor streptavidin-binding protein that migrates at 100 kDa; some batches also contain an additional streptavidin-binding protein of ~45 kDa. Comparison with the no-template control will distinguish the endogenous streptavidin-binding protein(s) from the expressed product.

4. Analyze reaction mixture by SDS-PAGE (*UNIT 6.1*) and transfer to PVDF membrane (*UNIT 6.2*).
5. Detect with streptavidin-AP or streptavidin-HRP using either chemiluminescent or colorimetric substrates (*UNIT 6.2*).

SUPPORT PROTOCOL 3

CAPTURE OF BIOTINYLATED PROTEINS WITH STREPTAVIDIN-AGAROSE

After in vitro translation, the biotinylated proteins may be captured from the translation reaction using streptavidin immobilized on agarose. This technique can be used to increase the sensitivity of detection methods, and provides a quick and easy way to isolate translation products for a number of downstream applications.

Materials

Translation reaction mixture (see Alternate Protocol 2, step 3)
Softlink Soft Release Avidin Resin (Promega), washed with 5 vol TBS at 40°C
TBS (*APPENDIX 2A*)
2× SDS-PAGE sample buffer (see recipe)

1. Add 10 µl of translation reaction mixture to 20 µl TBS. Incubate 5 to 10 min at 30°C.
2. Add 40 µl of Softlink Soft Release Avidin Resin, prewashed with TBS, to the reaction mix and incubate for 1 hr at 4°C with periodic stirring.
3. Briefly microcentrifuge, or let stand to allow resin to settle.
4. Wash the pellet three to five times with TBS.
5. Elute the bound proteins from the resin by adding 20 to 50 µl of 2× SDS-PAGE sample buffer and heating 5 min at 95°C to release biotinylated protein from beads.

   The protein is now ready for analysis (see Alternate Protocol 2, steps 4 and 5).

SUPPORT PROTOCOL 4

CAPTURE OF BIOTINYLATED PROTEINS WITH MAGNETIC BEADS

Another, rapid method of isolating biotinylated proteins from the other reticulocyte proteins in the translation mix is the use of streptavidin-linked magnetic beads. These beads consist of small metallic particles coated with streptavidin. The biotinylated protein binds the streptavidin, and the beads are removed and recovered by exposing them to a magnetic field. After elution, the protein of interest is relatively pure and may be analyzed by immunodetection, SDS-PAGE, or other methods.

Materials

Translation reaction mixture (see Alternate Protocol 2, step 3)
2× biotin/streptavidin binding buffer (2× BSBB; see recipe)
Streptavidin-linked magnetic beads (e.g., Dynabeads M-280 Streptavidin, Dynal; or MagneSphere Paramagnetic Particles, Promega)

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
2× SDS-PAGE sample buffer (see recipe)
Magnetic tube holder

1. Add an equal volume of 2× BSBB to translation reaction mixture in a microcentrifuge tube. Bring the total volume up to 250 µl with 1× BSBB. Add 10 to 50 µl of streptavidin-linked magnetic beads equilibrated in BSBB.
2. Incubate with rotation 30 min at 4°C.
3. Place microcentrifuge tube in magnetic holder for 2 min to capture the magnetic beads.
4. Carefully aspirate supernatant away from magnetic beads bound to side of tube.
5. Wash magnetic beads twice with 500 µl of 1× BSBB and once with 10 mM Tris·Cl, pH 8.0, using magnetic holder to separate magnetic beads from wash buffer. Remove supernatants. Resuspend magnetic beads between washes by gentle vortexing.
6. After removal of final supernatant, add 50 µl of 2× SDS-PAGE sample buffer.
7. Incubate 5 min at 95°C to release biotinylated proteins from beads.

The protein is now ready for analysis (see Alternate Protocol 2, steps 4 and 5).

Other methods of capturing biotinylated proteins using streptavidin-coated magnetic beads are also possible; consult the section on Biomagnetic Techniques in Molecular Biology in the Dynal Technical Handbook, 2nd ed. (Dynal, 1995)

REAGENTS AND SOLUTIONS

Use deionized, Milli-Q-purified water in all recipes and solutions except for transcription buffers and reagents for transcription reactions, which should be made with diethylpyrocarbonate-treated Milli-Q-purified water (DEPCW). Storage time for solutions kept at 4°C apply only if sterility is maintained. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Amino acid mixes

Amino acid:	*Quantity:*
Alanine	13.35 mg
Arginine	31.5 mg
Asparagine	22.5 mg
Aspartic acid	20 mg
Cysteine	26.25 mg
Glutamine	22 mg
Glutamic acid	22 mg
Glycine	11.25 mg
Histidine	31.35 mg
Isoleucine	19.5 mg
Leucine	39 mg
Lysine	27.3 mg
Methionine	15 mg
Phenylalanine	24.75 mg
Proline	17.25 mg
Serine	15.75 mg
Threonine	17.85 mg
Tryptophan	30.6 mg
Tyrosine	27 mg
Valine	33 mg

Add water to 80 ml. Adjust pH to 7.2 with 1 N KOH, then add water to 100 ml. Filter sterilize and store in 1-ml aliquots up to 2 years at –20°C.

continued

This yields 100 ml of a stock solution containing 3 mM leucine and valine, 1 mM methionine, and 1.5 mM each of the other 18 amino acids.

For amino acids minus valine, leucine, lysine, or methionine, prepare as described except omit the appropriate amino acid.

***Biotin/streptavidin binding buffer (BSBB), 2×** (10 ml)*

Components:	*Concentration of 2× stock:*
100 µl 1 M Tris·Cl, pH 8.0	10 mM
5 ml 4 M NaCl	2 M
20 µl 500 mM EDTA	1 mM
4.88 ml DEPCW	

Prepare stock solutions with DEPCW and autoclave. Store 2× BSBB up to 6 months at 4°C.

Calf liver tRNA, 5 mg/ml

250 mg calf liver tRNA
DEPCW to 50 ml
Filter sterilize and store in 1-ml aliquots up to 2 years at −20°C

***Cell lysis buffer, 10×** (10 ml)*

Components:	*Final concentration of 10× stock:*
1 ml 1 M Tris·Cl, pH 7.4	0.1 M
1 ml 1 M $MgCl_2$	0.1 M
5 ml 2 M KCl	1 M
3 ml DEPCW	

Prepare stock solutions of reagents for this recipe with DEPCW and autoclave. Divide 10× buffer into 1-ml aliquots and store up to 2 years at −20°C.

Prepare complete lysis buffer from 10× cell lysis buffer stock immediately before use in the preparation of polysome-bound mRNA (see Alternate Protocol 1).

Creatine phosphokinase, 200 U/ml

500 U creatine phosphokinase
1.25 ml 100 mM KCl
20 mM HEPES-KOH (see recipe), pH 7.2
1.25 ml glycerol
Store in 500-µl aliquots up to 1 year at −20°C

Glycerol prevents the enzyme from freezing at −20°C.

DTT, 250 mM

Add 154 mg DTT to 4 ml ice-cold deaerated water. Filter sterilize and snap-freeze in 25- to 250-µl aliquots. Store up to 1 year at −20°C.

Once an aliquot is thawed for use, discard residual solution.

EGTA, 200 mM

7.6 g EGTA
H_2O to 100 ml
Adjust pH to 7.0 with 1 N NaOH
Autoclave
Store up to 6 months at 4°C

***Glass powder** (for production of wheat germ extract)*

Suspend glass powder (Sigma) in at least 20 vol water. Add 1 ml DEPC per liter. Stir at room temperature overnight in a loosely covered container. Autoclave for 45 min. Allow glass powder to settle, remove supernatant, and dry in oven at 185°C.

Hemin hydrochloride, 1 mM

Mix:
65 mg hemin hydrochloride (Sigma)
2 ml 100 mM KOH
Sufficient 100 mM Tris·Cl, pH 7.8, to adjust pH to 7.8
Then add:
20 ml sterile H_2O
80 ml ethylene glycol
Store in light-proof bottle up to 2 years at −20°C

HEPES-KOH, pH 7.2, 1 M

238.3 g HEPES, free acid
H_2O to 800 ml
Adjust pH to 7.2 with 10 N KOH
Add water to 1 liter
Filter sterilize
Store in 1-ml aliquots up to 2 years at −20°C

HEPES/KCl buffer *(10 ml)*

Components:	*Final concentration:*
100 µl 1 M HEPES-KOH, pH 7.2	10 mM
1 ml 1 M KCl	100 mM
20 µl 1 M $MgCl_2$	2 mM
2 µl 500 mM EDTA	0.1 mM
40 µl 250 mM DTT (see recipe)	1 mM
8.84 ml DEPCW	

Prepare all stock solutions of reagents for this recipe with DEPCW and filter sterilize. Prepare solution as described above, then store 1-ml aliquots up to 1 year at −20°C. Add 7 µl/ml RNasin RNase inhibitor (Promega) just prior to use.

Used for resuspension of polysomes.

KCl/10 mM $MgCl_2$ (K/Mg), 2 M

14.9 g KCl
0. 214 g $MgCl_2$
H_2O to 100 ml
Autoclave
Store in 1-ml aliquots up to 1 year at −20°C

Modified transcription buffer (MTB), 5× *(10 ml)*

Components:	*Concentration of 5× stock:*
2 ml 1 M Tris·Cl, pH 8.1	200 mM
300 µl 1 M $MgCl_2$	30 mM
500 µl 1 M NaCl	50 mM
200 µl 250 mM spermidine	5 mM
250 µl 10 mg/ml BSA	250 µg/ml
200 µl 2.5% Triton X-100	0.05%
6.55 ml DEPCW	

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Store 1-ml aliquots of 5× MTB up to 1 year at −20°C.

See Milligan et al. (1987) for discussion of this solution.

4NTP mixture, 12.5 mM

50 mM single-nucleotide stocks (ATP, CTP, GTP, and UTP): Transfer 500 mg of each to a separate sterile 50-ml tube. Add DEPCW to appropriate volume. Check pH of each stock by pipetting 1 µl onto pH paper, and adjust by adding 1 N NaOH drop by drop until pH reaches 7.5. Filter sterilize and store in 1-ml aliquots up to 1 year at −20°C.

10 mM single-nucleotide stocks: Dilute 1/5 in DEPCW. Store in 500-µl aliquots up to 1 year at −20°C.

12.5 mM 4NTP mix: Mix 2.5 ml each of 50 mM ATP, CTP, GTP, and UTP stocks (10 ml total). Store in 500-µl aliquots up to 1 year at −20°C.

Nuclease S7, 15,000 U/ml

Add 1 ml water to lyophilized *Staphylococcus aureus* nuclease S7 enzyme to obtain a concentration of 15,000 U/ml. Make fresh for each use, and discard residual solution.

Used at 1/100 dilution.

Phenylhydrazine, 2.5% (w/v)

Dissolve 5 g phenylhydrazine (Sigma) in 200 ml deaerated sterile 0.85% NaCl and adjust to pH 7.0 with 1 N NaOH (the solution should be a pale straw color, not brown). Store single-use aliquots in air-tight, light-proof containers up to 3 months at −20°C.

Once an aliquot is thawed for use, discard residual solution.

Phosphocreatine, 1 M

Dissolve 0.255 g phosphocreatine in ice-cold water to 1 ml. Snap-freeze in 25- to 250-µl aliquots and store up to 2 weeks at −20°C.

Once an aliquot is thawed for use, discard residual solution.

Reticulocyte wash buffer I

100 ml 10× reticulocyte wash buffer II (see recipe)
7.5 ml 1 M $MgCl_2$
10 ml 100 mM glucose
H_2O to 1 liter
Store up to 1 year at 4°C

Reticulocyte wash buffer II, 10×

38.5 g NaCl
1.875 g KCl
H_2O to 500 ml
Autoclave and store up to 1 year at 4°C

Before use, add 100 ml of 10× stock and 5 ml of 1 M HEPES-KOH, pH 7.2, to 900 ml sterile H_2O.

SDS-PAGE sample buffer, 2× (50 ml)

Components:	*Concentration of 2× stock:*
12.5 ml 1 M Tris·Cl, pH 6.8	250 mM
3 g SDS	6%
2.3 g DTT	300 mM
5 ml 2-mercaptoethanol	1.4 M
500 µl 2% bromphenol blue	0.02%
20 ml glycerol	20%
H_2O to 50 ml	

Filter and store in 1-ml aliquots up to 1 year at −20°C. Once an aliquot has been thawed for use, discard residual solution.

Sucrose buffer *(for cushion; 10 ml)*

Components:	*Concentration:*
3.42 g sucrose	1 M
1 ml 1 M KCl	100 mM
100 µl 1 M $MgCl_2$	10 mM
2 µl 0.5 M EDTA	0.1 mM
100 µl 1 M Tris·Cl, pH 7.4	10 mM
DEPCW to 10 ml	
Filter sterilize	
Use fresh	

Transcription buffer (TB), 5× *(10 ml)*

Components:	*Concentration of 5× stock:*
2 ml 1 M Tris·Cl, pH 7.5	200 mM
300 µl 1 M $MgCl_2$	30 mM
500 µl 1 M NaCl	50 mM
250 µl 10 mg/ml BSA	250 µg/ml
400 µl 250 mM spermidine	10 mM
6.55 ml DEPCW	

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Store 1-ml aliquots up to 1 year at −20°C.

Transcription/translation (TX/TL) buffer, 25× *(10 ml)*

Components:	*Concentration of 25× stock:*
1.4 ml 1 M HEPES-KOH (see recipe), pH 7.2	140 mM
400 µl 1 M $MgCl_2$	40 mM
200 µl 250 mM spermidine	5 mM
8 ml 12.5 mM 4NTP mix (see recipe)	10 mM each

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Store 1-ml aliquots at −20°C.

Trichloroacetic acid (TCA), 5% and 10%

Weigh reagent bottle containing TCA and record weight full. Wearing rubber gloves, goggles, and plastic apron, add water and dissolve by shaking closed container. Pour solution into measuring cylinder, record weight of bottle empty, calculate amount of TCA used, and adjust volume to obtain a 10% solution. Dilute part of solution to 5% with an equal volume of water. Store both solutions in brown plastic bottles.

This method prevents burns from TCA crystals.

Wheat germ extraction buffer *(50 ml)*

Components:	*Final concentration:*
5 ml 1 M potassium acetate	0.1 M
100 µl 1 M magnesium acetate	2 mM
1 ml 1 M HEPES-KOH, pH 7.2 (see recipe)	20 mM
8 µl 250 mM spermidine	40 µM
35.9 ml DEPCW	

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Make buffer fresh, and immediately prior to use, add 200 µl of 250 mM DTT stock (1 mM final).

COMMENTARY

Background Information

Choice of system

The two most widely used eukaryotic in vitro translation systems are the mRNA-dependent reticulocyte lysate (Pelham and Jackson, 1976), an adaptation of an earlier mRNA-independent reticulocyte system (Adamson et al.,1968; Zucker and Schulman, 1968), and the wheat germ system developed by Roberts and Paterson (1973) with later refinements (Morch et al., 1986; Van Herwynen, 1990).

Perhaps the first question a researcher will have is "Which system should I use?" Both systems are very active and express a large range of polypeptide sizes. The most obvious advantages of the wheat germ system are its ease of preparation and its low cost. In addition, no animal use is required. With the inclusion of spermidine and RNase inhibitor in the reaction and supplementation with calf liver tRNA, an earlier drawback of the wheat germ system, its lower ability to translate high-molecular-weight proteins, has been overcome (Morch et al., 1986; Van Herwynen, 1990; Van Herwynen and Beckler, 1995). Furthermore, the wheat germ extract readily translates mRNA preparations containing contaminating double-stranded RNA or oxidized thiols, which are inhibitory in the reticulocyte system. It is also the system of choice for expressing eukaryotic transcription factors, since it does not contain endogenous counterparts (Mercurio et al., 1993).

However, the wheat germ system exhibits rather narrow ionic requirements that are very sensitive to the nature of the mRNA to be translated and can require optimization for each template used. Overall, the reticulocyte system remains the most highly used and the most widely available commercially.

The choice of system should be determined by the nature of the mRNA to be translated and the chosen assay or required function for the expressed protein. Items to consider include whether the protein will fold better in one system versus the other, whether there are endogenous enzymes available for post-translational modifications required for activity of the translation product, whether there are endogenous activities or compounds that interfere with a functional assay, and whether there are endogenous streptavidin-binding proteins that could interfere with detection of biotinylated products. Based on the above considerations, use of the reticulocyte translation system may be advantageous for studies of post-translational modification, including the use of microsomal membranes (*UNITS 11.3 & 11.4*). The reticulocyte system is also better for high-level expression using constructs containing the encephalomyocarditis 5′ untranslated leader region (EMCV 5′ UTR) and for nonisotopic detection using biotinylated lysine-tRNA. Conversely, the wheat germ system is preferable for studies of eukaryotic transcription factors, real-time assays monitoring low levels of luciferase, and cross-linking and fluorescent detection assays involving incorporation of non-natural amino acids. However, there are exceptions to all these generalizations.

To couple or not

The development of coupled eukaryotic transcription/translation systems had allowed direct expression from DNA containing cloned genes positioned behind T7, T3, or SP6 promoters in rabbit reticulocyte lysate or wheat germ extracts (Craig et al, 1992; Thompson et al., 1992). The availability of the coupled systems necessitates another choice—the production of mRNA prior to the translation reaction versus the use of DNA directly using the coupled system. In most cases the coupled system is more convenient, faster, and produces greater protein yields. Unless there is a problem with termination of the mRNA sequence, circular DNA can be used in the coupled system, obviating the need for linearization and subsequent clean-up of DNA. Interestingly, even though the coupled system does not use capped mRNA, several groups have reported the coupled system still produces greater protein yields than optimized expression from capped mRNA (Jagus, 1993). The eukaryotic coupled systems are much more forgiving of DNA purity than their sister prokaryotic (*E. coli*) coupled systems. Most common commercially available DNA purification systems will provide adequate DNA purity. In many cases where PCR DNA is used, the PCR reaction mix can be added directly to the coupled transcription/translation system without purification, although care should be taken not to add too much DNA (Renshaw-Gregg and Guiltinan, 1996).

On a cautionary note, when coexpressing more than one gene in a coupled transcription/translation system, care should be taken that no inhibitory antisense mRNA is generated from the use of dual opposable promoter vectors (i.e., T3/T7 or SP6/T7). If vectors using dual opposable promoters are used in such an

instance, linearization of the DNA 3′ to the gene of interest will prevent the problem.

Critical Parameters

One of the most common questions of researchers expressing gene products in vitro is "How much protein will the system generate?" Unfortunately, there is no simple answer. A multitude of factors will affect the final protein yield, some of them outside the researcher's control. Almost all researchers will be most interested in the translation of transcripts synthesized in vitro from cloned cDNAs using the bacteriophage promoters such as those for T3, T7, and SP6 RNA polymerases (Melton et al., 1984; Milligan et al., 1987). There are now a number of improved commercial systems for generating high levels of mRNA from linearized DNA (e.g., Ambion, EpiCenter, Novagen, and Promega).

The highest yields of transcripts are obtained for uncapped mRNA. Production of uncapped transcripts is also cheaper, since the cap analog needed to produce capped trancripts is expensive. This makes it advantageous to use a vector that places a 5′ UTR, allowing cap-independent translation, upstream of the initiation codon. Such vectors include pCITE (Novagen) and pTM1 (Moss et al., 1990), which use the internal ribosome entry site (IRES) of EMCV to promote cap-independent translation. The addition of the EMCV 5′ viral IRES has been shown to increase the expression of luciferase 16-fold over a control luciferase construct without the sequence (Beckler, 1992). Interestingly, the EMCV IRES sequence does not enhance translation in the wheat germ translation system and is actually detrimental. Alternatively, high expression levels can be achieved with uncapped mRNA by optimizing salt concentrations.

To generate well-translated in vitro transcripts, care must be taken not only in the choice of vector but also in the design of the construct. For instance, the sequence surrounding the AUG start should be optimized to match the so-called "Kozak" consensus sequence of A/GCCAUGG to provide optimal translation initiation (Kozak, 1989). Similarly, the 5′ UTR of the mRNA should not contain any significant secondary structure that may retard migration of the ribosome from the 5′ cap to the initiation codon (Kozak, 1988). In general, it is safer not to include any 5′ UTR sequences from the cDNA of interest since they may be inhibitory to translation. It is preferable to choose a vector that provides a 5′ UTR that will ensure good translation (such as pCITE and pTM1) and to clone in at an *Nco*I or *Nde*I restriction site that becomes the initiation codon.

However, when transcripts requiring cap-dependent translation are used, decreasing the distance of the initiation codon from the 5′ cap can reduce translational efficiency (Kozak, 1991a). Conversely, inclusion of long 5′ sequence that cannot form secondary structures has been shown to increase translational efficiency through the buildup of small ribosomal subunits in the 5′ UTR (Kozak, 1991b). Addition of an artificial 30-nucleotide poly(A)$^+$ sequence to the 3′ end of an mRNA has also been shown to boost expression 7-fold (Beckler, 1992). Note that in mRNA templates containing an added 3′ poly(A)$^+$ tail, increasing mRNA concentration will decrease translation due to titration of poly(A)$^+$ binding protein, which is involved in translation initiation.

Enhancement additives

For the rabbit reticulocyte system, a variety of factors lead to the activation of protein kinases that phosphorylate the α-subunit of initiation factor eIF2. This leads to a cessation of translation initiation. Hemin is always added to reticulocyte systems to prevent activation of the heme-sensitive eIF2α kinase. The addition of 5′-cAMP (1 to 10 mM) may also stimulate translation by preventing eIF2α kinase activation (Gross et al., 1988). Glucose-6-phosphate or 2-aminopurine (0.5 mM) may also stimulate translation by a variety of mechanisms (Jagus and Safer, 1981; Jackson et al., 1983; Michelson et al., 1984; Gross et al., 1988). The addition of eIF2α homologues, such as the K3L gene product from vaccinia virus or the vaccinia virus double-stranded RNA binding protein (the E3L gene product), to the reticulocyte system can prolong the linear rate of translation, resulting in increased protein yields (reviewed by Jagus and Gray, 1994; Clemens and Elia, 1997). If the codon usage of the open reading frame contains rare codons, or is from an organism using a different genetic code (such as trypanosomes), it may be desirable to add exogenous tRNA purified from the source organism.

Optimization of ionic conditions

Optimization of Mg^{2+} and K^+ can enhance the translation of a particular transcript (Beckler, 1992; Beckler et al., 1995). Mg^{2+} is the most critical component of the translation reaction to optimize because the Mg^{2+} optimum is very narrow: small changes in Mg^{2+} concentration can dramatically affect the efficiency and fidel-

ity of translation (Beckler, 1992). In addition, each RNA transcript exhibits a unique Mg^{2+} optimum. Capped mRNAs generally require a higher K^+ concentration than uncapped mRNAs (Kemper and Stolarsky, 1977). mRNAs containing the 5′ UTR of EMCV also require higher K^+ concentrations (Jackson, 1991). The form and concentration of K^+ salts is also important for initiation at the correct site; more efficient and accurate translation is achieved using potassium chloride rather than potassium acetate (Jackson, 1991). Uncapped mRNAs produced in in vitro transcription reactions are also translated with greater fidelity using the chloride rather than the acetate (Jackson, 1991).

Anticipated Results

As stated above, there is no simple expectation of protein yield in the cell-free translation systems. The reticulocyte lysate should contain between 25 and 30 A_{260} units of ribosomes, or 250 to 300 pmol per ml. Using the MDL with added mRNA in a reaction in which the MDL constitutes 70% of the reaction volume (i.e., 175 to 200 pmol ribosomes/ml), under optimal conditions and saturating levels of globin mRNA, the yield will be up to 1 mol globin/mol ribosome/min at 37°C (Jagus, 1987). This works out to be 262.5 to 300 pmol/50-µl reaction/30-min incubation, or 3.5 to 4.25 µg globin. Globin mRNA is translated very efficiently. The expression level of other mRNAs will vary, depending on the integrity and purity of the mRNA and on all the characteristics of mRNA structure known to affect translatability (outlined above; see Critical Parameters).

In the coupled system, the MDL is usually used at 50% of the final reaction mix, but the yield per ribosome has the same or higher potential. However, the purity of the DNA, type of vector, and design of the construct will all have an impact. Similarly, use of enhancement additives can increase the yield by allowing translation to continue for longer.

Estimating expected yields when using biotinylated amino acids is more difficult, since in this case precharged biotinylated lysine-tRNA competes with endogenous lysine-tRNA, which is continuously recharged, so that specific activity of the biotinylated lysine-tRNA decreases throughout the incubation. With biotinylated lysine-tRNA it is difficult to determine the percent incorporation of biotinylated lysines into a translated protein. However, using 15 pmol biotinylated NBD-[^{14}C]lysine-tRNA per 25-µl reaction, supplemented with amino acid mix minus lysine, ~50% of the biotinylated NBD-[^{14}C]lysine was incorporated into protein (Crowley et al., 1993). This is much less yield than is seen using conventional radioisotope labeling, but in this circumstance, lysine was limiting and use of a complete amino acid mix increases both protein yield and detection (Beckler and Hurst, 1993).

Time Considerations

The time taken for in vitro translation reactions will vary depending on which system is used, the source of mRNA, the detection method, and whether a coupled system is used. Assuming that the source of mRNA consists of trancripts generated in vitro from a DNA template, then ~4 hr will be needed for synthesis and purification of the mRNA, 1 to 1.5 hr for the translation reaction, and ~7 to 12 hr for analysis by SDS-polyacrylamide minigels and fluorography, assuming a 4- to 8-hr exposure of the autoradiogram. This gives a total of ~12 to 16 hr. In comparison, use of the coupled system requires only 8 to 12 hr (Thompson et al., 1992).

If standard fluorographic detection is replaced by analysis using phosphoimaging, the time can be reduced by 2 to 6 hr. If fluorographic detection is replaced by the use of biotinylated lysine-tRNA immunodetection, the time can be cut down by 3 to 8 hr. If speed of production of translation product is a critical factor, the system of choice would be a coupled reticulocyte system purchased as a kit. If detection of the protein product is the only experimental need, the use of biotinylated lysine-tRNA with detection using avidin or streptavidin conjugated to alkaline phosphatase coupled with colorimetric detection is the fastest choice (Beckler and Hurst, 1993).

Literature Cited

Adamson, S.D., Herbert, E., and Godchaux, W. 1968. Factors affecting the rate of protein synthesis in lysate systems from reticulocytes. *Arch. Biochem. Biophys.* 67:247-256.

Beckler, G.S. 1992. Optimization of in vitro translation reactions using the Salt Select Lysate system. *Promega Notes* 35:5-10.

Beckler, G.S. and Hurst, R. 1993. Non-radioactive detection of in vitro translation products labeled using biotinylated lysine tRNA. *Promega Notes* 43:24-31.

Beckler, G.S., Thompson, D., and Van Oosbree, T. 1995. In vitro translation using rabbit reticulocyte lysate. *Methods Mol. Biol.* 37:215-232.

Clemens, M.J. and Elia, A. 1997. The double-stranded RNA-dependent protein kinase PKR: Structure and function. *J. Interferon Cytokine Res.* 17:503-524.

Craig, D., Howell, M.T., Gibbs, C.L., Hunt, T., and Jackson, R.J. 1992. Plasmid cDNA-directed protein synthesis in a coupled eukaryotic in vitro transcription/translation system. *Nucl. Acids Res.* 20:4987-4995.

Crowley, K.S., Reinhart, G.D., and Johnson, A.E. 1993. The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. *Cell* 73:1101-1115.

Dynal. 1995. Biomagnetic techniques in molecular biology. *In* Dynal Technical Handbook, 2nd ed. Dynal A.S., Oslo.

Gross, M., Rubino, M.S., and Starn, T.K. 1988. Regulation of protein synthesis in rabbit reticulocyte lysate. Glucose 6-phosphate is required to maintain the activity of eukaryotic initiation factor (eIF)-2B by a mechanism that is independent of the phosphorylation of eIF-2α. *J. Biol. Chem.* 263:12486-12492.

Jackson, R.J. 1991. Potassium salts influence the fidelity of mRNA translation initiation in rabbit reticulocyte lysates: Unique features of encephalomyocarditis virus RNA translation. *Biochim. Biophys. Acta* 1088:345-358.

Jackson, R.J., Campbell, E.A., Herbert, P., and Hunt, T. 1983. The preparation and properties of gel-filtered rabbit-reticulocyte lysate protein-synthesis systems. *Eur. J. Biochem.* 131:289-301.

Jagus, R. 1987. Translation in cell-free systems. *Methods Enzymol.* 152:267-275.

Jagus, R. 1993. TNT system-based production of [^{35}S] eIF-4α for functional studies. *Promega Notes* 42:17.

Jagus, R. and Gray, M.E. 1994. Proteins that interact with PKR. *Biochimie* 76:779-791.

Jagus, R. and Safer, B. 1981. Activity of eukaryotic initiation factor 2 is modified by processes distinct from phosphorylation. II. Activity of eukaryotic initiation factor 2 in lysate is modified by oxidation-reduction state of its sulfhydryl groups. *J. Biol. Chem.* 256:1324-1329.

Kemper, B. and Stolarsky, L. 1977. Dependence on potassium concentration of the inhibition of the translation of messenger ribonucleic acid by 7-methylguanosine 5′-phosphate. *Biochemistry* 16:5676-5680.

Kozak, M. 1988. Leader length and secondary structure modulate mRNA function under conditions of stress. *Mol. Cell Biol.* 8:2737-2744.

Kozak, M. 1989. The scanning model for translation: An update. *J. Cell Biol.* 108:229-241.

Kozak, M. 1991a. A short leader sequence impairs the fidelity of initiation by eukaryotic ribosomes. *Gene Expr.* 1:111-115.

Kozak, M. 1991b. Effects of long 5′ leader sequences on initiation by eukaryotic ribosomes in vitro. *Gene Expr.* 1:117-125.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* 12:7035-7056.

Mercurio, F., DiDonato, J., Rosette, C., and Karin, M. 1993. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. *Genes Dev.* 7:705-708.

Michelson, A.M., Ernst, V., Levin, D.H., and London, I.M. 1984. Effects of glucose 6-phosphate and hemin on activation of heme-regulated eIF-2α kinase in gel-filtered reticulocyte lysates. *J. Biol. Chem.* 259:8529-8533.

Milligan, J.F., Groebe, D.R., Witherell, G.W., and Uhlenbeck, O.C. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucl. Acids Res.* 15:8783-8798.

Morch, M.D., Drugeon, G., Zagorski, W., and Haenni, A.L. 1986. The synthesis of high-molecular-weight proteins in the wheat germ translation system. *Methods Enzymol.* 118:154-164.

Moss, B., Elroy-Stein, O., Mizukami, W.A., and Fuerst, T.R. 1990. New mammalian expression vectors. *Nature* 348:91-92.

Pelham, H.R.B. and Jackson, R.J. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.

Renshaw-Gegg, L. and Guiltinan, M. 1996. Optimization of PCR template concentrations for in vitro transcription-translation reactions. *Biotechnol. Lett.* 18:670-682.

Roberts, B.E. and Paterson, B.M. 1973. Efficient translation of Tobacco Mosaic Virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. U.S.A.* 70:2330-2334.

Thompson, D., Van Oosbree, T., Beckler, G., and Van Herwynen, J. 1992. The TNT lysate systems: One step transcription/translation in rabbit reticulocyte lysate. *Promega Notes* 35:1-4.

Van Herwynen, J. 1990. Improved incorporation with wheat-germ extract. *Promega Notes* 23:7.

Van Herwynen, J.F. and Beckler, G.S. 1995. Translation using a wheat-germ extract. *Methods Mol. Biol.* 37:245-251.

Zucker, W.V. and Schulman, H.M. 1968. Stimulation of globin-chain initiation by hemin in the reticulocyte cell-free system. *Proc. Natl. Acad. Sci. U.S.A.* 59:582-589.

Contributed by Rosemary Jagus
Center of Marine Biotechnology and
Greenebaum Cancer Center
Baltimore, Maryland

Bhavesh Joshi
Center of Marine Biotechnology
Baltimore, Maryland

Suzanne Miyamoto
Molecular Hematology Branch, NHLBI
Bethesda, Maryland

Gregory S. Beckler
Promega Corporation
Madison, Wisconsin

In Vitro Analysis of Endoplasmic-Reticulum-to-Golgi Transport in Mammalian Cells

This unit describes in vitro reconstitution of vesicular trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus in mammalian cells. The assays outlined here allow for the examination of factors that are required for and/or regulate the budding of vesicles from the ER and the subsequent fusion of these vesicles with the Golgi. The assays are based on the ability to follow the movement of a temperature-sensitive mutant of vesicular stomatitis virus–encoded glycoprotein (VSV-G ts045) as it moves out of the ER and through the Golgi. VSV-G ts045 is retained in the ER at the restrictive temperature (39.5°C), which allows for synchronized movement of this protein out of the ER upon switching to the permissive temperature (32°C). Transport is measured by following the maturation of two asparagine-linked oligosaccharide chains on VSV-G as the protein moves from the ER to the *cis*-Golgi and subsequently to the medial/*trans*-Golgi.

The first transport assay (see Basic Protocol 1) measures the movement of VSV-G in semi-intact cells (SICs). SICs are a population of cells that have lost their cytosolic contents due to mechanical perforation of the plasma membrane, but that retain their subcellular organelles. SICs prepared from normal rat kidney cells infected with VSV ts045 faithfully reconstitute vesicular transport upon addition of rat liver cytosol and ATP. The organelles in SICs are accessible to exogenous factors ranging in size from small molecules to large proteins, allowing the effects of different factors on the extent and rate of transport from the ER to the Golgi to be determined. Alternatively, the protocol can be modified to isolate and measure ER-to-*cis*-Golgi transport alone, using clone 15B CHO cells infected with the temperature-sensitive virus VSV ts045 (see Alternate Protocol).

The second transport assay uses microsomes isolated from VSV-infected mammalian cells (see Basic Protocol 2), and is similar to the SIC assay in that it involves a single incubation that reconstitutes ER-to-Golgi transport. A more detailed two-stage protocol allows for ER-derived vesicles to be formed in vitro (see Basic Protocol 3) and used in a separate second-stage reaction that measures fusion with purified Golgi membranes (see Basic Protocol 4). This microsome-based two-stage assay allows vesicle formation and the budding reaction to be studied as separate biochemical events from the targeting and fusion reactions. Furthermore the ER-derived vesicles can be purified and examined as separate entities (see Basic Protocol 3).

The unit also presents Support Protocols for preparing the necessary reagents for these assays. Preparation of microsomal membranes and Golgi membranes is described (see Support Protocols 1 and 4, respectively).VSV ts045 is propagated to generate a high-titer stock (see Support Protocol 2), which is then used as the source of virus for preparation of both SICs and microsomes. Cytosol from rat liver (see Support Protocol 3) is used in each transport assay, and is gel-filtered for use in fusion reactions of ER-derived vesicles with Golgi membranes.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

NOTE: All culture incubations should be performed in a humidified 5% CO_2 incubation unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

NOTE: VSV stocks and samples should be treated with bleach prior to disposal.

Contributed by Bernard B. Allan and William E. Balch
Current Protocols in Cell Biology (1998) 11.3.1-11.3.22

BASIC PROTOCOL 1

RECONSTITUTION OF ER-TO-GOLGI TRANSPORT IN SEMI-INTACT CELLS

This first protocol describes the infection of normal rat kidney (NRK) cells with VSV ts045, the labeling of infected cells with ^{35}S, and the mechanical perforation of these cells by hypotonic swelling to generate labeled SICs. The labeled SICs are then used in a reaction that reconstitutes transport of VSV-G ts045 from the ER to and through the Golgi in a cytosol-, ATP-, and temperature-dependent manner. The progress of VSV-G is measured by following the acquisition of endoglycosidase H (endo H) resistance by the N-linked oligosaccharide side chains of VSV-G, which increases with time as the protein moves through the Golgi. The endo H–sensitive and endo H–resistant forms of VSV-G are separated by SDS-PAGE and quantified, and transport is expressed as the percentage of VSV-G in the endo H–resistant form.

Materials

Normal rat kidney (NRK) cells
Alpha minimal essential medium (α-MEM), serum-free and with 5% (v/v) FBS (*APPENDIX 2A*)
VSV ts045 stock (~2×10^9 pfu/ml; see Support Protocol 2)
1 mg/ml actinomycin D in ethanol
Methionine-deficient labeling medium (see recipe)
[^{35}S]Methionine (~11 mCi/ml, 1175 Ci/mmol; Trans^{35}S-label, ICN Biomedicals)
20 mM unlabeled methionine (tissue culture grade; Sigma)
Perforation buffer (see recipe), ice cold
Swelling buffer (see recipe), ice cold
1% (w/v) trypan blue
Rat liver cytosol (see Support Protocol 3)
1 M HEPES acid, pH 7.4
0.1 M magnesium acetate
1 M potassium acetate
10× Ca^{2+} buffer (see recipe)
20× ATP-regenerating system (see recipe)
40 mM UDP-*N*-acetylglucosamine
Endo H buffer (see recipe)
75 mU/ml endoglycosidase H (endo H; Boehringer Mannheim) in 0.1 M sodium acetate, pH 5.6
4× SDS sample buffer (*APPENDIX 2A*)
Fluorographic enhancement solution: 125 mM salicylic acid (sodium salt), pH 7.0, in 30% (v/v) methanol

Culture incubator at 32°C
Water baths at 32°, 37°, and 39.5°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and for autoradiography and densitometry (*UNIT 6.3*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Infect and label NRK cells

1. Grow NRK cells to confluency on 100-mm tissue culture dishes.
2. Prepare an infection cocktail containing (per dish) 0.9 ml serum-free α-MEM, 0.1 ml of VSV ts045 stock (final ~2×10^8 pfu/ml), and 5 µl of 1 mg/ml actinomycin D (final 5 µg/ml).

Table 11.3.1 Reaction Mix for ER-to-Golgi Transport Assay in Semi-Intact Cells

Solution	Volume in mix (µl)	Final concentration
SICs[a]	5	
Rat liver cytosol	5	3 mg/ml
1 M HEPES acid, pH 7.4	1	25 mM
0.1 M magnesium acetate	1	2.5 mM
1 M potassium acetate	2	50 mM
10× Ca^{2+} buffer	4	1×
20× ATP-regenerating system	2	1×
40 mM UDP-*N*-acetylglucosamine	0.5	0.5 mM
Water	To 40 µl (final)	

[a]Abbreviation: SICs, semi-intact cells.

3. Add 1 ml infection cocktail per dish and rock continuously (or by hand at 5-min intervals) for 45 min in a 32°C culture incubator, ensuring even distribution of the infection cocktail.
4. Add 5 ml α-MEM with 5% FBS to each dish and incubate at 32°C for 3 hr and 40 min.
5. Move the cells to a 39.5°C water bath and wash three times with 3 ml methionine-deficient labeling medium. Incubate the cells in 3 ml methionine-deficient labeling medium 15 min at 39.5°C.
6. Remove labeling medium, replace with 1.5 ml methionine-deficient labeling medium supplemented with 100 µCi [^{35}S]methionine, and incubate 10 min at 39.5°C with occasional rocking.
7. Add 30 µl of 20 mM unlabeled methionine and incubate 2 min at 39.5°C.

   It is essential that the temperature does not drop below 39.5°C at any point during this procedure to ensure that all of the ^{35}S-labeled VSV-G remains in the ER.

Perforate labeled cells

8. Transfer cells to ice and wash three times with 5 ml ice-cold perforation buffer.
9. Cover cells with 5 ml ice-cold swelling buffer and allow cells to sit on ice for 10 min.
10. Aspirate swelling buffer, add 3 ml ice-cold perforation buffer, and immediately scrape the cells with a rubber policeman.
11. Transfer cells to a 15-ml polypropylene tube and pellet by centrifugation at 800 × *g*, 3 min at 4°C.
12. Wash the pellet in 3 ml ice-cold perforation buffer and recentrifuge.
13. Resuspend in 200 to 300 µl ice-cold perforation buffer.
14. Determine the extent of perforation by mixing 10 µl SICs with 1 µl of 1% trypan blue on a cover slip and examining by light microscopy.

    The trypan blue–stained nuclei identify the perforated cells. There should be 90% to 95% perforated cells.

    SICs should be used immediately (i.e., stored no more than 1 hr on ice).

Reconstitute ER-to-Golgi transport

15. Prepare each reaction mix in a microcentrifuge tube as shown in Table 11.3.1. Perform reactions in duplicate along with two negative controls: complete reaction mix incubated on ice and reaction mix without cytosol, incubated at 32°C.

16. Vortex gently 3 sec and incubate 90 min in a 32°C water bath.
17. Pellet SICs by microcentrifuging 20 sec at 16,000 × *g*, 4°C.
18. Resuspend pellet in 20 µl endo H buffer.
19. Boil 5 min, microcentrifuge briefly, and cool. Add 40 µl of 75 mU/ml endo H (3 mU) in 0.1 M sodium acetate, pH 5.6.

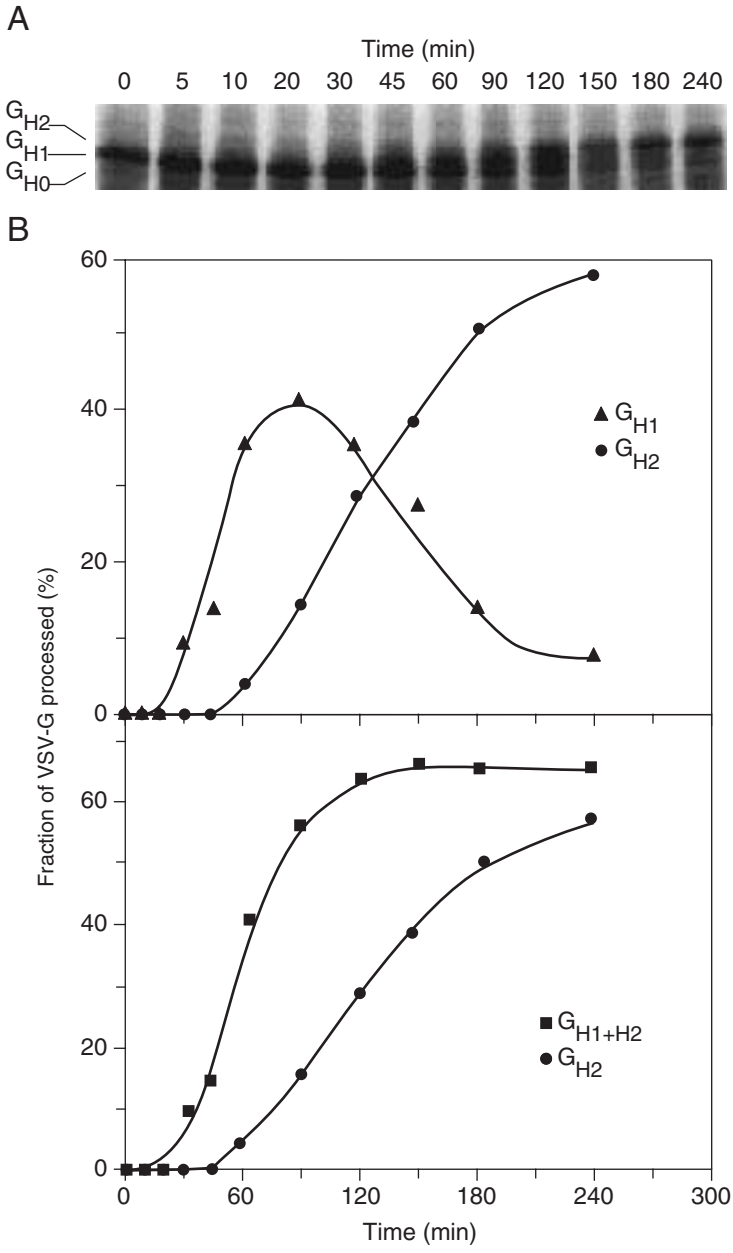


Figure 11.3.1 (**A**) Time course of ^{35}S-labeled VSV-G transport in semi-intact NRK cells, analyzed by endo H digestion. At the early time points, only G_{H0} is detected. G_{H1} appears as a transient intermediate and is replaced by G_{H2} as transport proceeds. These bands reflect discrete intermediates in VSV-G processing (see Background Information and Fig. 11.3.3). (**B**) Kinetics of VSV-G transport in semi-intact NRK cells as measured by the appearance of the G_{H1} and G_{H2} forms of VSV-G. Reprinted from Schwaninger et al. (1991) with permission from the American Society for Biochemistry and Molecular Biology.

20. Incubate overnight in a 37°C water bath.

21. Stop reaction by adding 20 µl of 4× SDS sample buffer, and separate the endo H–sensitive and endo H–resistant forms of VSV-G on a large 6.75% (w/v) polyacrylamide gel (*UNIT 6.1*).

22. Treat the polyacrylamide gel with fluorographic enhancement solution 20 min at room temperature. Dry the gel and autoradiograph (*UNIT 6.3*) at −80°C.

    Alternatively, expose the gel to a Phosphoimager screen.

23. Quantitate the intensities of the different forms of VSV-G by densitometry (Fig. 11.3.1; see *UNIT 6.3* for densitometry).

RECONSTITUTION OF ER-TO-*cis*-GOLGI TRANSPORT IN SEMI-INTACT CELLS

ALTERNATE PROTOCOL

It is sometimes desirable to separate ER-to-*cis*-Golgi transport from ER-to-*cis*-Golgi/medial-Golgi transport. This can be accomplished in an assay using the CHO cell line Clone 15B. These cells cannot process VSV-G oligosaccharide side chains beyond the $Man_5GlcNAc_2$ (Man_5; endo D_s in Fig. 11.3.3) form, due to a lack of the enzyme *N*-acetylglucosaminetransferase I (GlcNAc T I). The Man_5 form is uniquely sensitive to endoglycosidase D (endo D), and therefore the acquisition of endo D sensitivity is a measure of ER-to-*cis*-Golgi transport exclusively.

Additional Materials *(also see Basic Protocol 1)*

Clone 15B chinese hamster ovary (CHO) cells (ATCC)
Endo D buffer (see recipe)
0.5 mU/µl endoglycosidase D (endo D; Boehringer Mannheim)

1. Grow Clone 15B CHO cells to confluency on 100-mm tissue culture dishes and prepare VSV-infected, ^{35}S-labeled SICs (see Basic Protocol 1, steps 2 to 14).

2. Prepare each reaction mix in a microcentrifuge tube as shown in Table 11.3.1, except omit the UDP-*N*-acetylglucosamine. Perform reactions in duplicate along with two negative controls: complete reaction mix incubated on ice and reaction mix without cytosol, incubated at 32°C.

3. Vortex gently 3 sec and incubate 90 min in a 32°C water bath.

4. Pellet SICs by microcentrifuging 20 sec at 15,000 × *g*, 4°C.

5. Resuspend pellet in 60 µl endo D buffer containing 0.5 mU endo D.

6. Incubate the reaction overnight in a 37°C water bath.

7. Analyze results (see Basic Protocol 1, steps 21 to 23; see Fig 11.3.2).

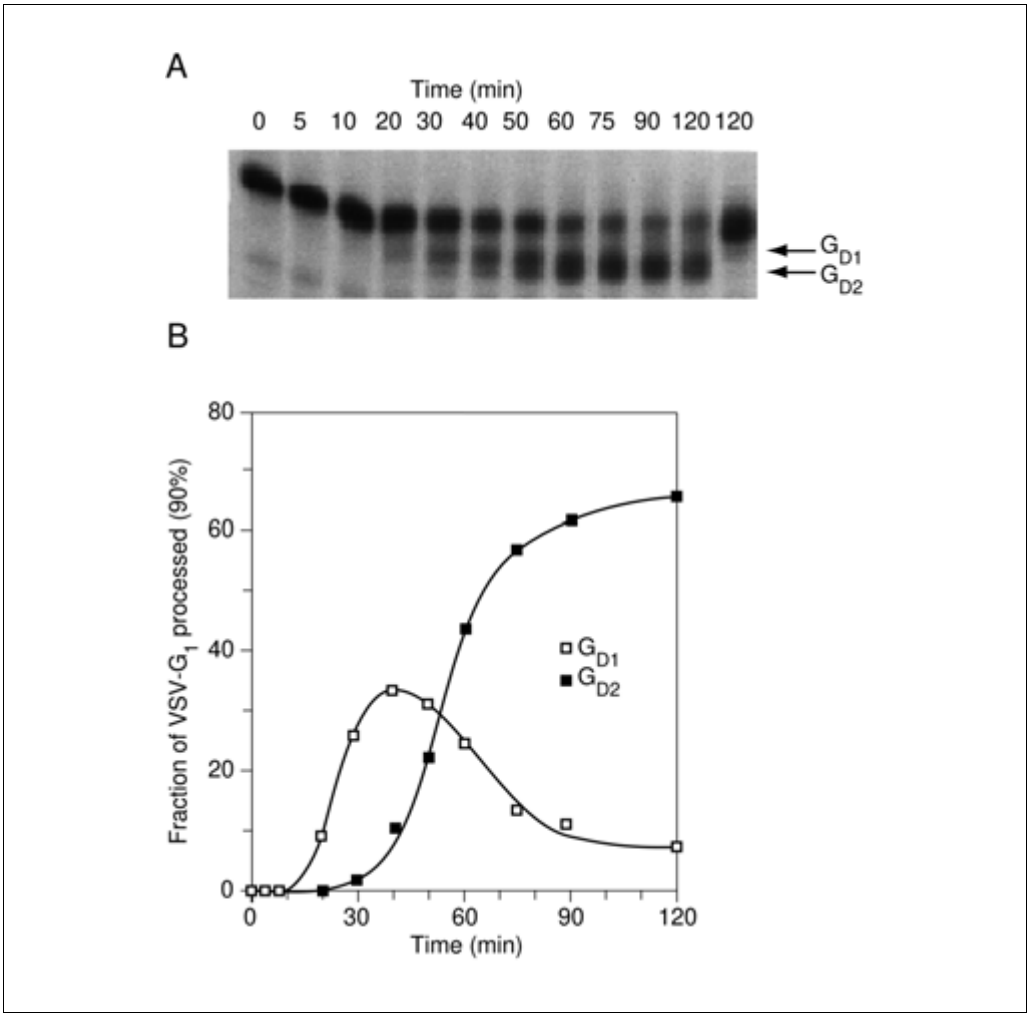


Figure 11.3.2 (**A**) Time course of ^{35}S-labeled VSV-G transport in semi-intact Clone 15B CHO cells, analyzed by endo D digestion. Clone 15B CHO cells are missing the enzyme GlcNAc T I, which is required for development of endo H resistance. At the early time points only G_{H0} is detected. Only G_{D2} is detected under standard SDS-PAGE conditions, although G_{D1}, which is produced when the first of the N-linked oligosaccharides is processed to the endo D–sensitive form, can sometimes be seen as an intermediate by running the dye front off the end of the gel to improve resolution. Reprinted from Schwaninger et al. (1991) with permission from the American Society for Biochemistry and Molecular Biology. (**B**) Kinetics of VSV-G transport as measured by the appearance of the G_{D1} and G_{D2} forms of VSV-G.

BASIC PROTOCOL 2

IN VITRO RECONSTITUTION OF ER-TO-GOLGI TRANSPORT IN MAMMALIAN MICROSOMES

This protocol describes a simple reaction that reconstitutes ER-to-Golgi transport in a microsomal fraction isolated from VSV-infected NRK cells. This assay is similar to the SIC assay in that it is cytosol-, ATP-, and temperature-dependent, and because transport is also measured by assessing the endo H resistance of the VSV-G oligosaccharide side chains.

Materials

Microsomes (see Support Protocol 1)
Rat liver cytosol (see Support Protocol 3)
1 M HEPES acid, pH 7.4
0.1 M magnesium acetate
1 M potassium acetate

Table 11.3.2 Reaction Mix for In Vitro ER-to-Golgi Transport Assay and for In Vitro Formation of ER-Derived Vesicles

Solution	Volume in mix (µl)	Final concentration
Microsomes	7.5	0.5-1 mg/ml
Rat liver cytosol	7.5	4.5 mg/ml
1 M HEPES acid, pH 7.4	1	25 mM
0.1 M magnesium acetate	1	2.5 mM
1 M potassium acetate	1.4	35 mM
10× Ca^{2+} buffer	4	1×
20× ATP-regenerating system	2	1×
2.5 M sorbitol	3	187.5 mM
40 mM UDP *N*-acetyl glucosamine	1	1 mM
Water	To 40 µl (final)	

10× Ca^{2+} buffer (see recipe)
20× ATP-regenerating system (see recipe)
2.5 M sorbitol
40 mM UDP-*N*-acetylglucosamine
Endo H buffer (see recipe)
75 mU/ml endoglycosidase H (endo H; Boehringer Mannheim) in 0.1 M sodium acetate, pH 5.6.
4× SDS sample buffer (*APPENDIX 2A*)
Anti-VSV-G monoclonal antibody p5D4 (Kreis, 1986)
Horseradish peroxidase (HRP)–conjugated secondary antibody
Water baths at 32° and 37°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and densitometry (*UNIT 6.3*)

1. Prepare each reaction mix as described in Table 11.3.2 and vortex gently. Perform reactions in duplicate, using an additional complete reaction incubated on ice as a negative control.

   The final concentration of potassium acetate in the assay should be 70 to 80 mM, with ~45 mM supplied by the microsomes and cytosol. The addition of other components to the assay may require altering the amount of potassium acetate added, so that the final concentration in the assay remains at 70 to 80 mM.

2. Transfer the reaction to a 32°C water bath and incubate 60 to 90 min.
3. Stop reaction by placing the tube on ice. Collect membranes by centrifuging 10 min at 20,000 × *g*, 4°C (e.g., in a Beckman TLA 100.3 rotor). Remove the supernatant by aspiration.
4. Resuspend pellet in 20 µl endo H buffer.
5. Boil 5 min, microcentrifuge briefly, and cool. Add 40 µl of 75 mU/ml endo H (3 mU) in 0.1 M sodium acetate, pH 5.6.
6. Incubate in a 37°C water bath overnight.
7. Stop reaction by adding 20 µl of 4× SDS sample buffer, and separate the endo H–sensitive and endo H–resistant forms of VSV-G on a large 6.75% (w/v) polyacrylamide gel (*UNIT 6.1*).

8. Transfer the proteins to nitrocellulose at 40 V for 6.5 to 10 hr in Tris/glycine/methanol transfer buffer, and immunoblot (*UNIT 6.2*) using the anti-VSV-G monoclonal antibody p5D4 and an HRP-conjugated secondary, each at 1:10,000 in BLOTTO. Quantitate the intensities of the different forms of VSV-G by densitometry (Fig. 11.3.1; see *UNIT 6.3* for densitometry).

   In this protocol only the G_{H0} and G_{H1} forms are seen.

BASIC PROTOCOL 3

IN VITRO FORMATION AND ISOLATION OF ER-DERIVED VESICLES

This protocol describes an in vitro reaction that generates VSV-G-containing ER-derived vesicles from VSV-G-containing microsomes. After incubation in the presence of cytosol and ATP at 32°C, the ER-derived vesicles are separated from the microsomal membranes by differential centrifugation. The ER-derived vesicles are harvested by high-speed centrifugation, and the amount of VSV-G in the high-speed pellet is determined by SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*). This gives a direct measure of the vesicle budding reaction. Timed reactions are used to examine the rate and/or extent of budding.

This protocol also describes a method for purifying the ER-derived vesicles by immunoprecipitation using an antibody against the C terminus of VSV-G. The protein constituents of the purified vesicles can then be examined by SDS-PAGE and immunoblotting. Alternatively, the ER-derived vesicles can be isolated and retained for use in a second-stage fusion reaction (see Basic Protocol 4).

Materials

Microsomes (see Support Protocol 1)
Rat liver cytosol (see Support Protocol 3)
1 M HEPES acid, pH 7.4
0.1 M magnesium acetate
1 M potassium acetate
10× Ca^{2+} buffer (see recipe)
20× ATP-regenerating system (see recipe)
2.5 M sorbitol
Resuspension buffer (see recipe), ice cold
1× SDS sample buffer (*APPENDIX 2A*)
Anti-VSV-G monocolonal antibody p5D4 (Kreis, 1986)
Horseradish peroxidase (HRP)–conjugated secondary antibody
p5D4-Dynabeads: p5D4 coupled to M-500 Dynabeads (Dynal; see manufacturer's instructions)
Immunoprecipitation buffer (see recipe)
FBS (*APPENDIX 2A*)
100 mM EDTA (adjust to pH 8.0 with KOH)
Transport buffer (see recipe)

Water bath at 32°C
Magnetic microcentrifuge tube holder

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and densitometry (*UNIT 6.3*)

1. Set up a 40- to 100-µl reaction for each desired time point as described in Table 11.3.2, except omit the UDP-*N*-acetylglucosamine. For a negative control, perform one complete reaction on ice.

   To determine the rate of budding, use 40-µl reactions with time points ranging from 0 to 60 min at 10-min intervals. To immunoprecipitate vesicles, perform three 10-min, 100-µl

reactions. For a two-stage transport assay (see Basic Protocol 4), use one 10-min, 100-µl reaction.

2. Incubate the reactions in a 32°C water bath, stopping each reaction at the appropriate time point by placing the tube on ice for 2 min.
3. Collect membranes by centrifugation at 135,000 × *g* for 10 min, 4°C (e.g., in a Beckman TLA 100.3 rotor).
4. To resuspend pellets, add 40 to 90 µl ice-cold resuspension buffer, resuspend by repeated pipetting, leave on ice for 10 min, and continue pipetting.

   The membranes are very difficult to resuspend, but can be completely resuspended by this method.

5. Add 0.1 to 0.2 vol of a salt mix so that the final concentration of potassium acetate is 150 mM and that of magnesium acetate is 2.5 mM. Mix by pipetting and microcentrifuge immediately at 16,000 × *g* for 5 min at 4°C.
6. Retain 80% of the medium-speed supernatant (MSS) and carefully aspirate the remainder away from the medium-speed pellet (MSP).

To determine the rate/extent of budding

7a. Centrifuge the MSS at 135,000 × *g* for 10 min, 4°C (e.g., in a Beckman TLA 100.3 rotor). Aspirate the supernatant very carefully or remove it using a yellow pipet tip. Keep the high-speed pellet (HSP).

8a. Resuspend the MSP in 50 µl of 1× SDS sample buffer and the HSP in 40 µl of 1× SDS sample buffer.

9a. Separate the proteins by loading 10 to 20 µl of each resuspended pellet on a 7.5% (w/v) polyacrylamide minigel (*UNIT 6.1*). Transfer the proteins to nitrocellulose and immunoblot for VSV-G (*UNIT 6.2*) using the anti-VSV-G monocolonal antibody p5D4 and an HRP-conjugated secondary, both at 1:10,000 in BLOTTO. Quantify signals by densitometry (*UNIT 6.3*) and determine the ratio of VSV-G in the HSP compared to total VSV-G in the HSP plus the MSP.

To purify vesicles by immunoprecipitation

7b. Mix 150 to 350 µl MSS from a 10-min time point with 1×10^7 p5D4-Dynabeads in a final volume of 372 µl immunoprecipitation buffer. Add 20 µl FBS (final 5%) and 8 µl of 100 mM EDTA (final 2 mM), and mix by continuous rotation at 4°C for 2 hr.

   Coupling of antibodies to the beads is performed exactly as described in the manufacturer's instructions.

8b. Wash beads four times with 500 µl immunoprecipitation buffer, collecting the beads each time with a magnetic tube holder.

9b. Solubilize vesicles on the beads into 1× SDS sample buffer by boiling 5 min and analyze the proteins by SDS-PAGE (*UNIT 6.1*).

To use vesicles in a second-stage fusion reaction

7c. Prepare an HSP from a 100-µl, 10-min reaction as described in step 7a.

8c. Resuspend the HSP in 25 µl transport buffer and keep on ice for stage 2 (see Basic Protocol 4).

   Each 100-µl budding reaction is sufficient for duplicate 50-µl fusion reactions.

SUPPORT PROTOCOL 1

PREPARATION OF MICROSOMAL MEMBRANES FROM NRK CELLS

This protocol describes the preparation of microsomes from VSV-infected NRK cells. These microsomes are used in the microsome-based transport assay (see Basic Protocol 2) and as the source of ER-derived vesicles for a more detailed two-stage assay (see Basic Protocols 3 and 4).

Additional Materials *(also see Basic Protocol 1)*

PBS (*APPENDIX 2A*), ice cold
Homogenization buffer I (see recipe)
100× PIC (see recipe)
Potassium acetate buffer (see recipe)
Transport buffer (see recipe)
1-ml ball-bearing homogenizer (Balch and Rothman, 1985)
Culture incubator at 39.5°C

NOTE: The method described below is for a twelve-dish microsome preparation.

Infect and collect cells

1. Grow NRK cells to confluency on twelve 150-mm tissue culture dishes.
2. Prepare an infection cocktail containing (per dish) 4.5 ml serum-free α-MEM, 0.5 ml VSV ts045 stock (final ~2×10^8 pfu/ml), and 25 µl of 1 mg/ml actinomycin D (final 5 µg/ml).
3. Add 5 ml per dish and rock continuously (or by hand at 5-min intervals) for 45 min in a 32°C culture incubator, ensuring even distribution of the infection cocktail.
4. Add 20 ml α-MEM with 5% FBS to each dish and incubate in a 39.5°C culture incubator (restrictive temperature) for 3 hr and 40 min.
5. Place each dish on ice. Immediately aspirate the medium and wash the cells with 10 ml ice-cold PBS.
6. Add 5 ml homogenization buffer I and scrape the cells using a rubber policeman. Transfer the cells to three 50-ml polypropylene tubes on ice, pooling cells from four dishes into each tube. Repeat the scraping procedure once.

Prepare membranes

7. Centrifuge at 720 × *g* for 3 min at 4°C and remove the supernatant by aspiration.
8. Resuspend each cell pellet in 0.9 ml homogenization buffer I with 1× PIC. Homogenize each by three complete passes through a 1-ml ball-bearing homogenizer.
9. Pool all cell homogenates and add an equal volume of homogenization buffer I with 1× PIC. Divide the diluted homogenate into six 1-ml aliquots in 1.5-ml microcentrifuge tubes.
10. Centrifuge at 720 × *g* for 5 min at 4°C.
11. Transfer each postnuclear supernatant to a 15-ml polypropylene tube. Add 0.5 vol potassium acetate buffer and mix.
12. Divide into 0.8- to 1-ml aliquots in 1.5-ml microcentrifuge tubes, and microcentrifuge at 12,000 × *g* for 2 min, 4°C.
13. Remove the supernatant by aspiration and resuspend all pellets in a total volume of 1 ml transport buffer with 1× PIC. Transfer 0.5 ml into each of two microcentrifuge tubes.

14. Microcentrifuge at 12,000 × *g* for 2 min, 4°C.

15. Resuspend the microsomal pellets in 6 to 8 vol transport buffer with 1× PIC. Pool the microsomes, divide into 50- to 100-µl aliquots, freeze in liquid nitrogen, and store at −80°C.

    In a typical twelve-dish preparation, 1 to 1.5 ml of resuspended microsomes (at 3 to 5 mg protein/ml) is obtained.

    These microsomes can be stored for several months without the loss of transport activity.

PROPAGATION OF VSV ts045

SUPPORT PROTOCOL 2

Stocks of VSV ts045 are prepared from infected baby hamster kidney cells to provide material for these protocols.

Materials

Baby hamster kidney (BHK) cells (ATCC)
Glasgow minimal essential medium (G-MEM; Life Technologies)
Tryptose phosphate broth (TPB; Sigma)
FBS (*APPENDIX 2A*)
TD buffer (see recipe)
Vesicular stomatitis virus (VSV) ts045 stock (Indiana serotype; multiplicity of infection = 0.1; ATCC)
Culture incubator at 32°C

1. Grow BHK cells to a confluent monolayer on 100-mm tissue culture dishes in G-MEM containing 10% (v/v) TPB and 5% (v/v) FBS.
2. Wash cells with TD buffer.
3. Add 10 ml G-MEM/10% TPB containing ~10^6 plaque forming units (pfu) of VSV ts045 stock.
4. Incubate 36 to 48 hr (until cells begin to round) in a 32°C culture incubator.
5. Remove medium, which now contains the amplified virus, and centrifuge at 720 × *g* for 10 min at 4°C.
6. Divide the supernatant into 100-µl aliquots, freeze in liquid nitrogen, and store indefinitely at −80°C.

    Generally the yield of virus is 1-2 × 10^9 pfu/ml. The viral stock can be refrozen twice without significant loss of titer.

FUSION OF ER-DERIVED VESICLES WITH GOLGI MEMBRANES

BASIC PROTOCOL 4

This protocol describes an assay that measures the fusion of ER-derived vesicles with purified Golgi membranes. The vesicles are made in vitro, isolated, and incubated at 32°C with rat liver Golgi membranes in the presence of cytosol and ATP. The acquisition of endo H resistance by the VSV-G oligosaccharide side chains is measured to follow fusion and expressed as the percentage of VSV-G in the endo H–resistant form.

Materials

ER-derived vesicles (HSP; see Basic Protocol 3, step 8c)
Desalted rat liver cytosol (see Support Protocol 3)
Enriched rat liver Golgi membranes (see Support Protocol 4)
1 M HEPES acid, pH 7.4
0.1 M magnesium acetate
10× Ca^{2+} buffer (see recipe)

Table 11.3.3 Reaction Mix for Fusion of ER-Derived Vesicles With Golgi Membranes

Solution	Volume in mix (µl)	Final concentration
HSP[a]	10	
Desalted rat liver cytosol	8	4 mg/ml
Golgi membranes	4	1.5 mg/ml
1 M HEPES acid, pH 7.4	1.25	25 mM
0.1 M magnesium acetate	1.25	2.5 mM
10× Ca^{2+} buffer	5	1×
20× ATP-regenerating system	2.5	1×
40 mM UDP *N*-acetylglucosamine	1.25	1 mM
2.5 M sorbitol	3.6	180 mM
Water	To 50 µl (final)	

[a]Abbreviation: HSP, high-speed pellet.

20× ATP-regenerating system (see recipe)
40 mM UDP-*N*-acetylglucosamine
2.5 M sorbitol
Endo H buffer (see recipe)
75 mU/ml endoglycosidase H (endo H; Boehringer Mannheim) in 0.1 M sodium acetate, pH 5.6
4× SDS sample buffer (*APPENDIX 2A*)
Anti-VSV-G monoclonal antibody p5D4 (Kreis, 1986)
Horseradish peroxidase (HRP)–conjugated secondary antibody
Water bath at 37°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and densitometry (*UNIT 6.3*)

1. Prepare each reaction mix as described in Table 11.3.3. Perform reactions in duplicate and include two negative controls: a complete reaction incubated on ice and a reaction without Golgi membranes, incubated at 32°C.

   Note that desalted rat liver cytosol is used in this assay.

2. Incubate in a 32°C water bath for 75 min.
3. Stop reaction by transferring the tubes to ice.
4. Collect membranes by centrifuging at 135,000 × *g* for 10 min, 4°C (e.g., in a Beckman TLA 100.3 rotor).
5. Resuspend pellet in 20 µl endo H buffer.
6. Boil 5 min, microcentrifuge briefly, and cool. Add 40 µl of 75 mU/ml endo H (3 mU) in 0.1 M sodium acetate, pH 5.6.
7. Incubate overnight in a 37°C water bath.
8. Stop reaction by adding 20 µl of 4× SDS sample buffer, and separate the endo H–sensitive and endo H–resistant forms of VSV-G on a large 6.75% (w/v) polyacrylamide gel (*UNIT 6.1*).
9. Transfer the proteins to nitrocellulose at 40 V for 6.5 to 10 hr in Tris/glycine/methanol transfer buffer and immunoblot (*UNIT 6.2*) using the anti-VSV-G monocolonal antibody p5D4 and an HRP-conjugated secondary, both at 1:10,000 in BLOTTO. Quantify the

intensities of the different forms of VSV-G by densitometry (Fig. 11.3.1; see *UNIT 6.3* for densitometry).

In this protocol only the G_{H0} and G_{H1} forms are seen.

PREPARATION OF RAT LIVER CYTOSOL

SUPPORT PROTOCOL 3

Rat liver cytosol is required as an additive for the transport assays. The first part of this protocol describes steps used for cytosol preparation and is performed in the same manner for each type of assay. For use in the ER-vesicle-to-Golgi fusion assay (see Basic Protocol 4), it must also be desalted by gel filtration, as described in the second part of the protocol.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male Sprague-Dawley rats
PBS (*APPENDIX 2A*), ice cold
Cytosol buffer (see recipe), ice cold
100 mM ATP
100× PIC (see recipe)

40-ml Dounce homogenizer with type A (tight-fitting) and type B (loose-fitting) glass pestles
Cheesecloth
Sephadex G-25M/PD-10 column (Pharmacia Biotech)

Prepare rat liver cytosol

1. Sacrifice two male Sprague-Dawley rats. Remove the livers and weigh them.

   Two livers should have a wet weight of 15 to 20 g. The age of the rats does not seem to affect yield or performance of the preparation.

2. Wash tissue three times with 50 ml ice-cold PBS and twice with 50 ml ice-cold cytosol buffer.
3. Add 3 vol (vol/gram wet weight of liver) ice-cold cytosol buffer supplemented with 1 mM ATP and 1× PIC, and cut the tissue into small pieces on ice using scissors.
4. Transfer the diced tissue to a homogenizer in a cold room and homogenize by hand using 10 strokes with a type B pestle (loose) followed by 10 strokes with a type A pestle (tight).
5. Filter the homogenate through cheesecloth into a beaker on ice.
6. Centrifuge the filtered homogenate at 12,000 × *g* (e.g., in a Beckman JA-20 rotor) for 10 min, 4°C.
7. Transfer the supernatant to appropriate centrifuge tubes (e.g., for a Beckman SW41 rotor) and centrifuge at 150,000 × *g* for 90 min, 4°C.
8. Remove the overlying lipid layer by aspiration and retain the remaining supernatant (cytosol).
9. Divide the cytosol into 250-µl aliquots, freeze in liquid nitrogen, and store up to 6 months at −80°C.

   This cytosol is ready for use in Basic Protocols 1 and 2 and in the Alternate Protocol.

   The normal protein concentration of the cytosol is 25 to 30 mg/ml. Two livers should provide ~150 aliquots (30 to 40 ml).

Prepare gel-filtered (desalted) cytosol

10. In a cold room, remove the caps from a Sephadex G-25M/PD-10 column and equilibrate the column with 25 ml ice-cold cytosol buffer supplemented with 1× PIC.
11. Add exactly 2.5 ml cytosol to the top of the column.
12. Allow the eluent to flow through the column until it stops, and discard the flow-through.
13. Elute the proteins with exactly 3.5 ml ice-cold cytosol buffer supplemented with 1× PIC.
14. Divide the filtered cytosol into 100-µl aliquots, freeze in liquid nitrogen, and store up to 6 months at −80°C.

Gel-filtered cytosol is used for Basic Protocol 4.

SUPPORT PROTOCOL 4

PREPARATION OF GOLGI MEMBRANES FROM RAT LIVER

Rat livers are used as a source for Golgi membranes, which are purified by sucrose-density fractionation of liver homogenate.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male Sprague-Dawley rats
Homogenization buffer II (see recipe), ice cold
100× PIC (see recipe)
Sucrose solutions in 10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*): 0.5 M (refractive index 1.3575), 1.0 M (1.3815), 1.1 M (1.3865), 1.25 M (1.3939), and 2.35 M (1.4464)
Dilution buffer (see recipe)
Transport buffer (see recipe)
Homogenizer (Potter-Elvehjem tissue grinder, size C) and Teflon pestles with 0.026-in. (66-mm) and 0.012-in. (30-mm) clearance
Cheesecloth
Refractometer
18-G needle

Prepare postnuclear supernatant

1. Remove the livers from two sacrificed male Sprague-Dawley rats, place on adsorbent paper, and weigh them.

   The two livers should have a wet weight of 15 to 20 g. The age of the rats does not seem to affect yield or performance of the preparation.

2. Place tissue in a beaker, add 5 vol (w/v) ice-cold homogenization buffer II with 1× PIC, and cut the livers into small pieces using scissors.
3. Transfer the diced tissue to a homogenizer and homogenize using a mechanical drive at a speed of 1000 rpm. Use 3 passes with a pestle of 0.026-in. clearance and 3 passes with a pestle of 0.012-in. clearance.
4. Filter the homogenate through cheesecloth into a beaker on ice. Wash the residue on the cheesecloth with 10 ml ice-cold homogenization buffer II.

5. Centrifuge the filtered homogenate at 1450 × *g* (e.g., in a Beckman JA-20 rotor) for 10 min, 4°C.
6. Collect the supernatant.

Separate membrane fractions

7. Layer 30-ml portions of supernatant on top of 8 ml of 1.25 M sucrose solution (e.g., in Beckman SW28 tubes).
8. Centrifuge at 112,000 × *g* (25,000 rpm in a Beckman SW28 rotor) for 90 min, 4°C.
9. Remove the top layer by aspiration and collect the membranes from the 0.5 M/1.25 M interface using a Pasteur pipet.
10. Use a refractometer to determine the sucrose concentration and adjust to 1.2 M (refractive index = 1.3915) by adding 2.35 M sucrose.
11. Place 13-ml portions of this membrane fraction at the bottom of SW28 tubes and overlay with 10 ml of 1.1 M sucrose, 10 ml of 1.0 M sucrose, and 5 ml of 0.5 M sucrose.
12. Centrifuge at 112,000 × *g* (25,000 rpm in a Beckman SW28 rotor) for 2.5 hr, 4°C.
13. Recover the band at the 0.5 M/1.0 M interface using an 18-G needle.
14. Divide into ~300-µl aliquots in 1.5-ml microcentrifuge tubes and add 4 vol dilution buffer.
15. Microcentrifuge at 16,000 × *g*, 4°C, for 10 min.

    After this centrifugation the membranes smear along the side of the tube rather than forming a compact pellet.

16. Aspirate the supernatant and resuspend the membranes gently with 0.5 ml transport buffer.
17. Pool the membranes from the two tubes and repeat microcentrifugation (as in step 15).
18. Resuspend membranes in a total of 4 ml transport buffer. Divide into 75- to 100-µl aliquots, freeze with liquid nitrogen, and store up to 3 months at −80°C.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

NOTE: The pH of all buffers is adjusted with KOH, where appropriate.

ATP-regenerating system, 20×

12 ml 46 mM ATP, pH 7.0 (20 mM final)
12 ml 233 mM creatine phosphate (100 mM final)
4 ml 700 U/ml creatine phosphate kinase (100 U/ml final)
Divide into 100- to 200-µl aliquots
Store up to 3 or 4 months at −80°C

Ca^{2+} buffer, 10×

20 mM HEPES acid, pH 7.4
50 mM EGTA
18 mM $CaCl_2$
Store up to 6 months at 4°C

Cytosol buffer

25 mM HEPES acid, pH 7.4
125 mM potassium acetate
Store up to 6 months at 4°C

Dilution buffer

25 mM HEPES acid, pH 7.2
87.5 mM potassium acetate
1.25 mM magnesium acetate
Store up to 6 months at –20°C

Endo D buffer

50 mM sodium phosphate, pH 6.2
5 mM EDTA
0.2% (v/v) Triton X-100
Store up to 6 months at –20°C

Endo H buffer

0.1 M sodium acetate, pH 5.6
0.3% (w/v) SDS
Store up to 1 month at 4°C
Add 20% (v/v) 2-mercaptoethanol just before use

Homogenization buffer I

20 mM HEPES acid, pH 7.4
375 mM sorbitol
Store up to 6 months at –20°C

Homogenization buffer II

10 mM Tris·Cl, pH 7.4
0.5 M sucrose
5 mM EDTA
5 mM EGTA
1× PIC (see recipe)
Prepare fresh

Immunoprecipitation buffer

20 mM HEPES acid, pH 7.4
150 mM potassium acetate
0.25 M sucrose
0.1% (w/v) BSA
Prepare fresh from stock solutions stored up to 1 week at 4°C

Methionine-deficient labeling medium

Methionine-deficient labeling medium (Sigma)
1× leucine (200× solution, 79 mM; tissue grade culture; Sigma)
1× lysine (200× solution, 80 mM; tissue grade culture; Sigma)
20 mM HEPES acid, pH 7.2
Store up to 1 year at 4°C

Methionine-deficient labeling medium is minimal essential medium (MEM) with Earle's salts and L-glutamine (but without L-leucine, L-lysine, L-methiodine, or sodium bicarbonate).

Perforation buffer

50 mM HEPES acid, pH 7.2
90 mM potassium acetate
Store up to 6 months at 4°C

Potassium acetate buffer

20 mM HEPES acid, pH 7.4
210 mM potassium acetate
3 mM magnesium acetate
Store up to 6 months at –20°C

Protease inhibitor cocktail (PIC), 100×

1 mg/ml leupeptin
1 mg/ml chymostatin
0.05 mg/ml pepstatin A
0.05 M phenylmethylsulfonyl fluoride (PMSF; *APPENDIX 2A*)
Prepare in water fresh before use from stock solutions stored at –20°C

Resuspension buffer

20 mM HEPES acid, pH 7.4
0.25 M sucrose
Store up to 6 months at –20°C

Swelling buffer

10 mM HEPES acid, pH 7.2
18 mM potassium acetate
Store up to 6 months at 4°C

TD buffer

138 mM NaCl
5 mM KCl
25 mM Tris base
0.4 mM Na_2HPO_4
Prepare fresh

Transport buffer

20 mM HEPES acid, pH 7.4
250 mM sorbitol
70 mM potassium acetate
1 mM magnesium acetate
Store up to 6 months at –20°C

COMMENTARY

Background Information

The transport of proteins along the early secretory pathway is mediated by small vesicles that bud from the ER and subsequently fuse with the Golgi apparatus (Palade, 1975). A number of biochemical assays have been developed to study ER-to-Golgi transport in vitro in mammalian cells (Beckers et al., 1987; Rowe et al., 1996) and yeast (Rexach and Schekman, 1991). The assays described in this unit allow the biochemical events of ER-to-Golgi trafficking to be studied in mammalian cells and for the role of distinct components involved in these events to be elucidated.

The assays are based on a viral glycoprotein that is transported to the cell surface in infected cells along the same pathway as endogenous plasma membrane proteins. The assays take advantage of a temperature-sensitive mutant of vesicular stomatitis virus (VSV ts045; Lafay, 1974), which encodes a glycoprotein (VSV-G ts045) that suffers from a thermoreversible folding defect. At the restrictive temperature (39.5°C), VSV-G ts045 is retained in the ER because it is misfolded. Switching to the per-

missive temperature (32°C) corrects the misfolding and triggers transport from the ER. The ability to turn on transport selectively allows the synchronized movement of VSV-G ts045 to be followed through the early secretory pathway.

The ability to retain VSV-G ts045 in the ER and control its release makes this protein an ideal reporter molecule. In theory, however, any cargo protein could be used and the assays described here could be modified to study any protein of interest. The limiting factor is the ability to distinguish cargo protein (coming from the ER during the assay) from protein that is already present in transport vesicles and the Golgi at the beginning of the assay.

The transport of VSV-G is monitored by assessing the maturation of two N-linked oligosaccharide side chains on VSV-G as the protein moves from the ER to and through the Golgi. The processing of the oligosaccharide side chains of VSV-G is a complex process and the formation of different forms of oligosaccharides can be manipulated in vitro (Davidson and Balch, 1993). In the protocols described here, four different forms of VSV-G can be distinguished (Fig. 11.3.1 and Fig. 11.3.2). In the ER, VSV-G exists in a high-mannose form ($Man_9GlcNAc_2$; G_0; Fig. 11.3.3). In the *cis*-Golgi, α-1,2-mannosidase I (Mann I) trims G_0 to the $Man_5GlcNAc_2$ (G_D) form. The oligosaccharide side chains of G_0 are resistant to cleavage by the enzyme endoglycosidase D (endo D), while G_D is uniquely sensitive to endo D. Under standard SDS-PAGE conditions, only a single G_D form is detected (G_{D2}). However a second G_D form (G_{D1}) can be seen by running the dye front off the end of the gel to improve resolution (Fig. 11.3.2). G_{D1} is a transient intermediate formed when only one of the VSV-G N-linked oligosaccharide side chains is processed.

G_D is further processed by *N*-acetylglucosaminetransferase I (GlcNAc T I) and α-1,2-mannosidase II (Mann II) in the *cis* and medial compartments of the Golgi to the G_{H1} and G_{H2}

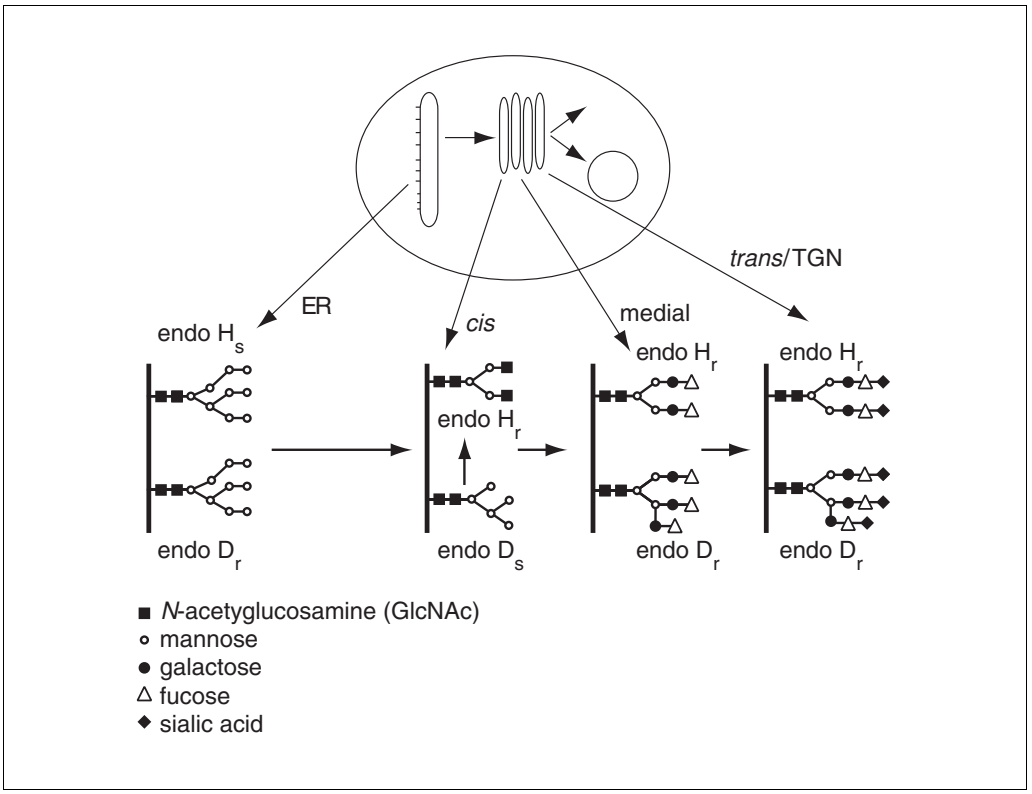


Figure 11.3.3 A schematic representation of the different oligosaccharide side chain processing forms of VSV-G. G_{H0} is the ER Man9 form, which is endo H sensitive (endo H_s) and endo D resistant (endo D_r). G_D is formed in the *cis*-Golgi by trimming G_{H0} to the Man_5 form, which is endo D sensitive (endo D_s). This is the only intermediate in the oligosaccharide processing of VSV-G that is sensitive to endo D and cannot normally be detected in wild-type cells due to its transient formation. Further processing of G_D in the *cis*-Golgi generates G_{H1}, which is endo H resistant (endo H_r). In the medial Golgi, G_{H1} is processed to the G_{H2} form, which is also endo H resistant (endo H_r), but which migrates more slowly than G_{H1} in SDS/polyacrylamide gels. TGN, *trans*-Golgi network.

forms. G_{H1} and G_{H2} are both resistant to endoglycosidase H (endo H), while G_0 is sensitive to endo H. Under the conditions described here, G_{H1} is a transient intermediate in the pathway, and after 90 min almost all VSV-G is in the G_{H2} form. Each oligosaccharide processing form of VSV-G has a unique mobility during SDS-PAGE, allowing for a direct quantitative assay for transport (Beckers et al., 1987).

Because Clone 15B CHO cells lack the enzyme GlcNAc T I (Gottlieb et al., 1975), there is no further processing of G_D as the protein moves through the Golgi in these cells. Therefore the appearance of the G_D form is a measure of transport from the ER to the *cis*-Golgi, and the alternative semi-intact cell assay using Clone 15B CHO cells should be used when one wants to study ER-to-*cis*-Golgi trafficking exclusively.

Further processing of VSV-G that involves the addition of galactose, sialic acid, and additional GlcNAc residues (to generate a complex form of VSV-G, $G_{complex}$) occurs in the medial and *trans*-Golgi network (TGN), and cannot be measured using the protocols described here. However, the protocols can be modified to measure delivery of VSV-G to the TGN (Davidson and Balch, 1993).

The first protocol describes the semi-intact cell (SIC) assay, which is a relatively quick and simple way to determine the effect of any particular factor on the extent and/or rate of transport of protein from the ER to the Golgi. This assay has been used to show that GTP and ATP hydrolysis, Ca^{2+}, members of the Rab protein family, as well as general trafficking proteins such as *N*-ethylmaleimide-sensitive factor (NSF) and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are required for ER-to-Golgi transport. The use of immunodepleted cytosols, the addition of transdominant mutants, and the use of neutralizing antibodies to inhibit transport are the main approaches used to show a role for a particular protein in transport.

The SIC assay can be separated into three distinct kinetic stages (Fig. 11.3.4). The first stage, in which there is no processing of VSV-G oligosaccharide side chains, represents vesicle formation and subsequent budding and targeting of the vesicles. The second stage, in which there is a progressive increase in side chain processing, represents fusion of ER-derived vesicles with the Golgi. A plateau of processing defines the third stage, which presumably represents the maximum capacity of the SIC assay

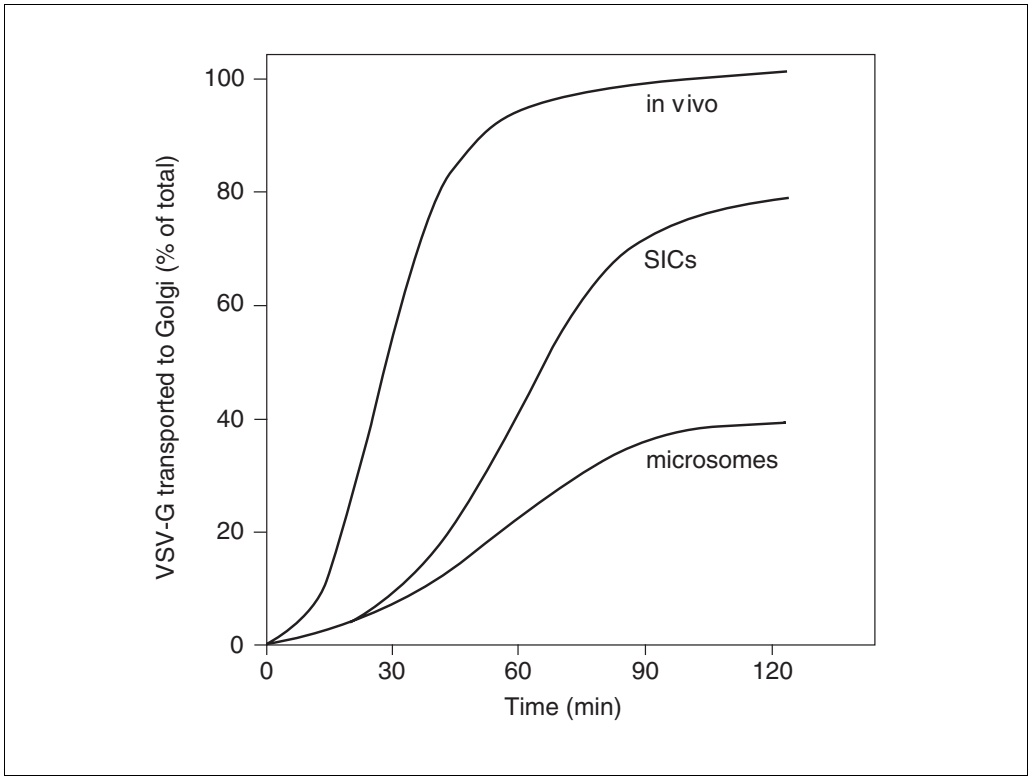


Figure 11.3.4 Comparison of the rates and extent of VSV-G transport from the ER to the Golgi apparatus measured either in vivo, in SIC preparations, or reconstituted using the microsome-based protocol.

to support ER-to-Golgi transport. Modifying this protocol to involve the timed addition of inhibitors during the incubation period can allow the investigator to assign a role of a particular factor to the early or late stages of transport. Furthermore, by accumulating transport intermediates with a reversible inhibitor, the requirements for further transport can be examined upon removal of the inhibitor.

The SIC assay cannot, however, accurately define a role for any factor in the separate biochemical events of ER-to-Golgi transport. The microsome-based assay described here was developed to allow for this. The protocol is a one-stage reaction in which ER-derived vesicles are formed, targeted, and allowed to fuse with Golgi membranes already present in the microsomal fraction. This assay is essentially the same as the SIC assay, except that its overall efficiency is lower (Fig. 11.3.4). It is used mainly to confirm that the microsome and cytosol preparations are competent to reconstitute ER-to-Golgi transport.

The more detailed two-stage assay allows for vesicle formation and budding, targeting of ER-derived vesicles, and fusion with the Golgi to be studied independently. This allows the investigator to accurately assign a role for any particular factor to a distinct biochemical event. Similar assays have been developed in the yeast system for studying both ER-to-Golgi transport (Rexach and Schekman, 1991) and vacuolar inheritance (Conradt et al., 1994). These assays have proved to be powerful tools in determining the order of the distinct biochemical events that are involved and the role of general trafficking proteins in these events.

The first stage of the two-stage assay involves vesicle formation in vitro. When used on its own, this assay allows for the factors involved in vesicle budding from the ER to be studied, as the appearance of VSV-G in the high-speed pellet (HSP) is a direct measurement of vesicle formation. The ER-derived vesicles can also be studied as separate entities by immunopurification using anti-VSV-G antibodies. This allows the protein constituents of the vesicles to be determined. The combination of the first-stage vesicle formation reaction with a separate second-stage fusion reaction allows the targeting and fusion events involved in ER-to-Golgi transport to be studied independently of vesicle formation and budding and independently of each other. The relative contributions of vesicular components, cytosolic factors, and Golgi components to the separate biochemical events can be determined using this assay.

Critical Parameters

All assays described in this unit are based on the synchronized movement of the ts045 mutant of VSV-G from the ER. To keep VSV-G ts045 in the ER, it is essential that the temperature does not drop below 39.5°C during the preparation of SICs and microsomes.

The preparation of SICs by mechanical perforation of hypotonically swollen cells produces a yield of 90% to 95% SICs for the cell lines used routinely in the authors' laboratory. The swelling procedure can be adjusted to achieve maximum perforation of other cell lines by changing the swelling time, the composition of the swelling buffer, or the degree of adherence to the tissue culture plate. In general, cells that adhere weakly require longer swelling times, while strongly adherent cells may not even require swelling before scraping. As an alternative to scraping, perforation can be performed using nitrocellulose stripping, which tears pieces of the plasma membrane away from the surface of the cell. Transport can then be assayed in the cells that remain attached to the nitrocellulose, or the cells that remain on the plate can be harvested by gentle scraping for an assay in suspension. In all cases, the degree of perforation can be determined by assessing trypan blue staining of the nuclei of perforated cells.

The preparation of microsomes with high budding activity is dependent on the homogenization conditions. The ball-bearing homogenizer breaks the cells efficiently, giving a high yield of membranes with high budding activity. The use of other types of homogenizers is not recommended.

In the microsome-based assay, the separation of budded vesicles from the microsomes is achieved by differential centrifugation. The largest reaction volume that does not require alterations in the centrifugation conditions is 100 µl. Larger reaction volumes may change the sedimentation properties of the membranes; therefore, the centrifugation conditions for larger reaction volumes should be characterized empirically.

Sodium ions inhibit both the SIC assay and the microsome-based assay. The pH of all buffers used in these assays is therefore adjusted with KOH, rather than NaOH. Any protein added to the assays should first be dialysed against 25 mM HEPES acid, pH 7.2, containing 125 mM potassium acetate. If it is necessary to add a component in any buffer not described

Table 11.3.4 Troubleshooting Guide

Problem	Possible cause	Solution
High percent transport in cytosol-free control in SIC assay	Poor perforation of cells	Determine percent perforation; optimize protocol for the specific cell line
	Cytosol not completely washed out of cells	Incubate cells on ice 10 min after perforation[a]
Cannot resuspend membranes completely in preparing ER-derived vesicles[b]	Concentration and/or pH of resuspension buffer are incorrect	Check concentration of sucrose using a refractometer; check pH
	Salts added before resuspension	Use salt-free resuspension buffer and add salts only after complete resuspension
No transport in SIC assay; no budding in microsome assay	Sodium ions in reaction	Adjust pH of all solutions with KOH; dialyze additives against 125 mM potassium acetate, 25 mM HEPES, pH 7.2
No fusion in second-stage fusion reaction	Sodium ions and/or sucrose in reaction	Make sure that sucrose has not been used instead of sorbitol

[a]See Basic Protocol 1, step 12.
[b]See Basic Protocol 3, step 4.

here, the effects of this buffer on the assay should be determined. See Table 11.3.4 for a brief troubleshooting guide.

Anticipated Results

The preparation of SICs from NRK, BHK, and CHO cells by scraping results in the perforation of 90% to 95% of cells. The percentage of perforation should be determined using trypan blue staining, particularly if other cell lines are to be used (see Critical Parameters).

The typical result obtained for the SIC assay is shown in Figure 11.3.1. The amount of VSV-G transported is determined by comparing the amount of the endo H–resistant form with the total amount of VSV-G in the reaction. The appearance of the different forms of VSV-G is the same after immunoblotting to measure transport in the two-stage assay, as is shown in Figure 11.3.1 for ^{35}S-labeling in the SIC assay.

In the SIC assay, 65% to 70% of VSV-G is transported after 60 to 90 min. Transport efficiency is lower in the microsome-based assay, where 35% to 40% of VSV-G is transported after 60 to 90 min (Fig. 11.3.2).

Stage 1 of the two-stage microsome assay allows for the rate of budding to be measured. Under standard conditions, VSV-G appears in the HSP after ~5 min, peaks at between 10 and 20 min, and then decreases over time. The amount of VSV-G in the HSP depends on the budding activity of the microsomes as well as on the fusion competence of the membranes in the reaction. In general, 15% to 20% of the VSV-G is found in the HSP at the peak time point. In stage 2 of the two-stage assay, 35% to 40% of the VSV-G in the HSP appears as the endo H–resistant form after 75 to 90 min (Fig. 11.3.2).

The purification of ER-derived vesicles using immunoprecipitation allows 25% to 30% of the VSV-G in the medium-speed supernatant (MSS) to be isolated with 2.5 to 5 µg p5D4 antibody/10^7 beads. This represents only ~5% of the total starting VSV-G. In general, therefore, the MSS from three 100-µl budding reactions (stage 1) are pooled for every immunoprecipitation reaction.

Time Considerations

The SIC assay and the microsome-based assay take 3 days from beginning the experiment to analyzing the data. The actual hands-on time for the SIC assay is 4.5 hr for infection of the cells and 3 to 3.5 hr from the preparation of SICs to the addition of endo H. The hands-on time for the microsome-based assay is considerably shorter, requiring only 2 to 2.5 hr on the first day. Digestion is done overnight (minimum 16 hr). The polyacrylamide gel (200 × 150 × 1.5 mm) takes 4 to 5 hr to run. For ^{35}S analysis, the gel is incubated in fluorographic enhancement solution for 20 min, dried for ~1 hr, and exposed to film overnight. For the microsome-based assay, the endo H–treated proteins are transferred to nitrocellulose overnight (6.5 to 10 hr total transfer time), and immunoblot analysis is done the next day.

The budding reaction (stage 1) and the immunoprecipitation of vesicles can be done in one day. Preparation of the MSS, MSP, and HSP requires ~1.5 to 2 hr. Immunoprecipitation requires 2 hr, with an additional 30 to 45 min for washing the beads. A minigel takes ~1 hr to run, and the proteins are transferred to nitrocellulose for 1 hr. Immunoblot analysis takes 3 to 4 hr.

The two-stage assay also takes a total of three days, with 1.5 to 2 hr for stage 1 and ~2 hr for stage 2, both on the first day. After the addition of endo H, the time required is the same as for the microsome assay.

Literature Cited

Balch, W.E. and Rothman, J.E. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: Asymmetric properties of donor and acceptor activities in a cell-free system. *Arch. Biochem. Biophys.* 240:413-425.

Beckers, C.J.M., Keller, D.S., and Balch, W.E. 1987. Semi-intact cells permeable to macromolecules: Use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell* 50:523-534.

Conradt, B., Haas, A., and Wickner, W. 1994. Determination of four biochemically distinct, sequential stages during vacuole inheritance in vitro. *J. Cell Biol.* 126:99-110.

Davidson, H.W. and Balch, W.E. 1993. Differential inhibition of multiple vesicular transport steps between the endoplasmic reticulum and trans Golgi network. *J. Biol. Chem.* 268:4216-4226.

Gottlieb, C., Baenziger, J., and Kornfeld, S. 1975. Deficient uridine diphosphate-*N*-acetylglucosamine: Glycoprotein *N*-acetylglucosaminyltransferase activity in a clone of Chinese hamster ovary cells with altered surface glycoproteins. *J. Biol. Chem.* 250:3303-3309.

Kreis, T.E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO J.* 5:931-941.

Lafay, F. 1974. Envelope viruses of vesicular stomatitis virus: Effect of temperature-sensitive mutations in complementation groups III and V. *J. Virol.* 14:1220-1228.

Palade, G.E. 1975. Intracellular aspects of the process of protein transport. *Science* 189:347-354.

Rexach, M.F. and Schekman, R.W. 1991. Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114:219-229.

Rowe, T., Aridor, M., McCaffery, J.M., Plutner, H., and Balch, W.E. 1996. COPII vesicles derived from mammalian endoplasmic reticulum (ER) microsomes recruit COPI. *J. Cell Biol.* 135:895-911.

Schwaninger, R., Beckers, C.M.J., and Balch, W.E. 1991. Sequential transport of protein between the endoplasmic reticulum and successive Golgi compartments in semi-intact cells. *J. Biol. Chem.* 266:13055-13063.

Contributed by Bernard B. Allan
and William E. Balch
The Scripps Research Institute
La Jolla, California

Cotranslational Translocation of Proteins into Canine Rough Microsomes

This unit describes the isolation of small vesicular fragments of rough endoplasmic reticulum (ER), known as "rough microsomes" (RMs), and their use in cell-free protein translocation assays. By faithfully reproducing the events associated with the transport of proteins into the ER, a cell-free assay provides a tool for analyzing ER targeting signals, signal-sequence cleavage, N-linked glycosylation, and membrane-protein biogenesis. Because translocation has been shown to be a strictly cotranslational process, purified RMs are added directly to wheat germ or rabbit reticulocyte translation reactions that are programmed with appropriate mRNAs (see *UNITS 11.1 & 11.2*). A variety of post-translational treatments are then performed to facilitate interpretation of the results of the translocation reaction. Finally, the proteins in the reaction mix are resolved by SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*). Translocation or membrane insertion of the translation product is indicated by mobility shifts due to signal-sequence cleavage and glycosylation, or by cofractionation with membrane components.

This unit describes the use of RMs isolated from canine pancreas (see Support Protocol 1) in cotranslational translocation assays and optional post-translational treatments (see Basic Protocol). Also included are methods of processing and purifying RMs that optimize their compatibility with in vitro translation reactions (see Support Protocols 2 and 3).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

TRANSLOCATION INTO CANINE ROUGH MICROSOMES

BASIC PROTOCOL

In this protocol secreted, lumenal, and integral membrane proteins are translocated cotranslationally into RMs that are added to rabbit reticulocyte or wheat germ translation reactions. Because many proteins contain an N-terminal, ~20- to 30-amino-acid "signal sequence" that is removed by signal peptidase as the protein is transported into the RMs, translocation can often be assessed simply by looking for a slight increase in mobility on polyacrylamide gels in the presence of RMs (see Fig. 11.4.1). Proteins that are translocated into RMs are also resistant to protease digestion. The extent of N-linked glycosylation can be assessed by looking for a retardation of mobility on polyacrylamide gels that can be reversed by the addition of glycosidases. Finally, the integration of membrane proteins can be analyzed by testing for resistance to alkaline extraction.

Although a standard cotranslational translocation reaction is performed to analyze the transport of all proteins, a variety of different post-translational treatments can be used to generate additional information about protein topology and protein modifications. Because these treatments are applicable only to specific classes of proteins and only in certain experimental situations, the investigator should assess in advance which, if any, of these optional treatments (steps 5, 6, and 7) will be appropriate. Studies by Hansen et al. (1986) and Gafvelin et al. (1997) illustrate the utility of these post-translational treatments.

Contributed by Harris D. Bernstein

Current Protocols in Cell Biology (1998) 11.4.1-11.4.11

Materials

Wheat germ *or* reticulocyte translation mix (see *UNIT 11.2*)
1 eq/µl RM solution (see Support Protocol 1)
mRNA encoding a protein of interest
mRNA encoding a control cytoplasmic protein
Buffer C (see recipe)
30% trichloroacetic acid (TCA; store at 4°C), ice cold
For optional steps:
  20 mg/ml proteinase K (store at −20°C)
  Nonionic detergent: e.g., 20% Triton X-100 or Nonidet P-40 (NP-40; store at −20°C)
  25% sodium dodecyl sulfate (SDS; store at room temperature)
  1 M dithiothreitol (DTT; *APPENDIX 2A*)
  Buffer D (see recipe)
  Endoglycosidase H (purchased as solid or stock solution)
  100 mM sodium carbonate, pH 11.5, ice cold
  Glacial acetic acid
1× SDS sample buffer (*APPENDIX 2A*)

Water bath or heating block, 26° or 30°C and (for optional step) 100°C
Tabletop ultracentrifuge and rotor (e.g., Beckman TLA 100.2 rotor; for optional step)

Additional reagents and solutions for in vitro translation (*UNIT 11.2*) and one-dimensional SDS-PAGE (*UNIT 6.1*)

1. Determine the number and total volume of in vitro translation reactions that are required for a given experiment (*UNIT 11.2*). Keeping all reagents on ice, make a master translation mix of the components that are going to be needed in all reactions (e.g., wheat germ extract, radioactive amino acids). Pipet the appropriate amount of master mix into each reaction tube, including tubes to be used for control reactions.

   Making a master mix considerably diminishes pipetting errors and reduces the number of pipetting steps needed to set up the reactions. It is a good idea to include separate control reactions lacking mRNA and lacking RMs. Because translocation of proteins into RMs is often highly efficient, small reaction volumes (e.g., 10 µl) are generally sufficient. Small-scale reactions should be performed in 0.5-ml microcentrifuge tubes. Larger reaction volumes can be used in cases where reactions are to be divided for post-translational treatments (e.g., digestion with endoglycosidase H).

2. Add 0.1 to 1 eq of RMs (0.1 to 1 µl of solution) per 10 µl of translation mix to each reaction; for RM-free control reactions, add an equivalent volume of buffer C instead. Then add appropriate amounts of mRNA (or, for RNA-free controls, buffer C). Incubate the reactions 40 to 60 min at 26°C for wheat germ reactions, or 30°C for rabbit reticulocyte translations.

   Generally 10 to 50 ng of an efficiently translated mRNA per 10 µl of translation mix will give a good signal, but higher amounts can also be added.

   The concentration of RMs that is added to the reaction is generally not critical, but in some cases it may be necessary to determine an optimal concentration empirically. Use one that promotes efficient translocation of a given protein and has a minimal inhibitory effect on the translation reaction. Addition of an equimolar amount of an mRNA that encodes a cytoplasmic protein (e.g., globin) to each reaction serves as a good control.

3. Stop the reactions by transferring the tubes to an ice bath.

4. If post-translational treatment(s) of the samples (e.g., protease digestion) are desired, proceed to steps 5, 6, and/or 7. Otherwise, precipitate the proteins by adding 1 vol of 30% TCA and incubating 10 min on ice; then proceed to step 8.
5. *To show that a protein or domain has been transported into the RM lumen (optional):*
   a. Add proteinase K to 100 µg/ml (final concentration). Incubate separate aliquots in the presence and in the absence of 0.5% to 1% nonionic detergent for 30 to 60 min at 0°C.
   b. Stop the protease digestion by adding 2 mM PMSF (final). Incubate an additional 15 min at 0°C.
   c. Precipitate proteins with 30% TCA (see step 4).

      As an additional control, addition of detergent will lyse the RMs and expose a translocated protein to protease digestion. Optionally, addition of 1 mM $CaCl_2$ to the samples prior to protease digestion can help to stabilize the membranes and may increase the yield of protease-protected protein.
6. *To remove N-linked carbohydrates from translocated proteins (optional):*
   a. Add 2% SDS and 75 mM DTT (final concentrations). Boil samples for 3 min.
   b. Dilute samples 20-fold in buffer D and add 1 µg/ml endoglycosidase H (final). Incubate 12 to 16 hr at 37°C.
   c. Precipitate proteins using 30% TCA (see step 4).

      Although endoglycosidase H is most commonly used, other glycosidases that remove N-linked carbohydrates (e.g., endoglycosidase F) can be substituted.
7. *To test for membrane integration (optional):*
   a. Dilute translation reactions 100-fold with ice-cold 100 mM sodium carbonate, pH 11.5. Incubate 30 min on ice.
   b. Centrifuge 30 min at 360,000 × g_{av} (100,000 rpm in a Beckman TLA 100.2 tabletop ultracentrifuge rotor), 4°C.
   c. Carefully remove the supernatant and neutralize with glacial acetic acid. Precipitate proteins with 30% TCA (see step 4).
   d. Meanwhile, dissolve the pellet, containing ribosomes and membrane fragments, directly in 1× SDS sample buffer (this can be run on an SDS-polyacrylamide gel without further manipulation).

   After the centrifugation, integral membrane proteins will be found in the pellet but soluble proteins will be found in the supernatant.

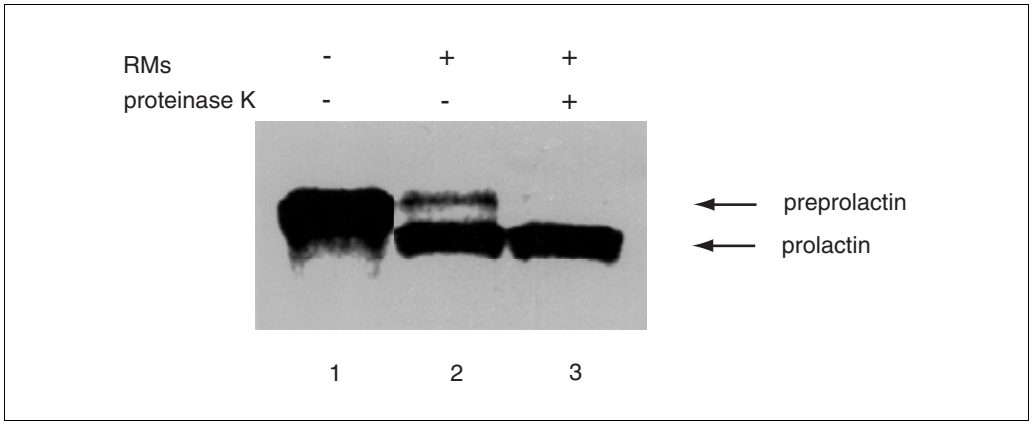


Figure 11.4.1 Translocation of preprolactin into canine pancreatic RMs. An mRNA encoding bovine preprolactin was translated in a wheat germ extract in the absence of microsomes (lane 1) or in the presence of RMs (lanes 2 and 3). After the translation was stopped, proteinase K was added to the sample shown in lane 3.

8. Pellet precipitated proteins by microcentrifuging 5 min at full speed, 4°C. Remove supernatant carefully. Boil in 1× SDS sample buffer for 3 to 5 min or until the pellet is dissolved.

   If the TCA is not removed carefully, the samples may remain acidic (the bromphenol blue in the sample buffer will turn yellow). This problem can be remedied by adding small amounts of 1 M Tris base.

9. Assess protein translocation and modification by SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*).

   The gel shown in Figure 11.4.1 illustrates how protein translocation can be assessed by this method. In the absence of RMs, an mRNA encoding bovine preprolactin directs the synthesis of a 26-kDa protein (lane 1). In the presence of microsomes, efficient translocation of the preprotein results in the removal of the signal sequence and the production of the faster-migrating prolactin form (lane 2). In the presence of proteinase K, essentially all of the prolactin is protected from digestion, but residual untranslocated preprolactin is degraded (lane 3).

SUPPORT PROTOCOL 1

PREPARATION OF RMs FROM CANINE PANCREAS

This protocol describes the isolation of RMs from canine pancreas. The extremely high yield, purity, and translocation competence of RMs obtained from canine pancreas makes it an ideal source for these vesicles. In the method described here, the cells of the pancreas are first disrupted by homogenization. Then nuclei, secretory granules, plasma membranes, and mitochondria are removed to produce a "post-mitochondrial supernatant" containing Golgi and vesicular fragments of ER produced by shearing. The RMs are pelleted through a discontinuous sucrose gradient and resuspended in a buffer that is compatible with their use in protein translocation assays.

NOTE: All steps in this protocol (except dissection and centrifugation) should be performed in a cold room at 4°C. Because it is important to work as quickly as possible after dissecting the pancreas and to keep the tissue cold, all solutions should be prechilled and all equipment and glassware should be placed in the cold room in advance.

Materials

Buffer A (see recipe), ice cold
Dog (e.g., beagle weighing 10 to 12 kg)
Buffer B (see recipe), ice cold
Buffer C (see recipe), ice cold
1% (w/v) SDS (*APPENDIX 2A*), ice cold
Liquid nitrogen

Dissecting tools: small scissors, regular and fine forceps, scalpel (with new blade)
Small plastic cutting board
Single-edge razor blades (at least 5)
Potter-Elvejhem-style tissue homogenizer (e.g., B. Braun Biotech) with 60-ml cylinder and matched Teflon plunger
50-ml polycarbonate or polypropylene centrifuge tubes (~28.5-mm diameter)
Medium-speed centrifuge and rotor (Sorvall SS-34 or Beckman JA20, or equivalent)
25 × 89–mm (26-ml) polycarbonate ultracentrifuge bottles with tops
10-ml glass syringe fitted with a round-tipped pipetting needle
Ultracentrifuge and rotor (Beckman Ti 50.2 or equivalent)
60-ml Dounce homogenizer fitted with type A pestle

Homogenize the pancreas

1. Cover work area with absorbent plastic-coated bench covering. Place ~100 ml ice-cold buffer A (enough to keep the tissue covered) in a 250-ml glass beaker and immerse in ice until fully chilled.

2. Sacrifice the dog, then make a large ventral incision into the abdominal cavity and isolate the pancreas. Dissect the pancreas as quickly as possible and immediately transfer it to the beaker of ice-cold buffer A.

   The pancreas is an elongated, lobulated, pinkish-gray organ that runs along the small intestine and stomach. To locate it, displace the liver and find the stomach. The pancreas will reside at the junction of the stomach and descending duodenum. Grasp the pancreas and gradually detach it from other tissues. The portion of the pancreas that is attached to the duodenum ("right lobe") is most accessible and should be detached first. Generally it is helpful to have a second person keep the pancreas exposed by holding or pushing away other organs.

   If available, tissue can be obtained from dogs that are used as research subjects in other projects. Most common anesthetics, methods of sacrifice, and experimental manipulations that do not affect pancreatic function are compatible with the isolation of translocation-competent RMs. It is absolutely essential, however, to remove the pancreas as quickly as possible after sacrifice to avoid tissue necrosis and degradation of ER components that are required for protein translocation. Provided that the animal is in good health, age and sex do not appear to have a significant effect on the isolation of RMs. Because the weight of the pancreas correlates with the size of the animal, the largest quantities of RMs are obtained from large dogs.

3. Rinse the pancreas in fresh ice-cold buffer A to remove blood and other debris. Place the pancreas on a piece of Parafilm. Using a scissors and fine forceps, remove major blood vessels and adipose and connective tissues from the pancreas.

   The Parafilm will help to visualize fat and connective tissue that is connected to the pancreas. It is critical to clean the pancreas carefully because extraneous tissues can interfere with effective homogenization. It is also important, however, to work rapidly to minimize necrosis of the pancreas.

4. Weigh the pancreas (keeping it in a chilled beaker) and mince finely using fresh razor blades. Change razor blades often as they become dull.

   The pancreas of a 10- to 12-kg dog typically weighs 20 to 30 g.

5. Place the tissue pieces in a clean beaker and add a volume of ice-cold buffer A equal to four times the weight of the pancreas.

6. Stir the tissue pieces and buffer together to create a uniform mixture. Homogenize 60-ml aliquots of the mixture using a motor-driven Potter-Elvejhem-style instrument.

   Five complete up-and-down strokes is generally sufficient to break the cells effectively. It is essential to avoid significant heating of the homogenate during this step; for this reason instruments in which the homogenizer cylinder fits into a container that can be filled with ice (e.g., the unit made by B. Braun Biotech) are recommended.

Isolate RMs

7. Pour the homogenate into a clean beaker and mix well, then transfer to 50-ml centrifuge tubes. Centrifuge 10 min at $700 \times g_{av}$ (3000 rpm in a Sorvall SS-34 or Beckman JA-20 rotor), 4°C.

8. Carefully remove the fat layer at the top of each centrifuge tube by aspiration, then pour the supernatants into a clean beaker. Mix the pooled supernatants well and transfer to clean 50-ml centrifuge tubes. Centrifuge 10 min at $8000 \times g_{av}$ (10,000 rpm in a Sorvall SS-34 or Beckman JA-20 rotor), 4°C.

9. Pour the "post-mitochondrial" supernatants into a clean beaker.

The pellets that are formed after the second centrifugation step are very soft, so pour off the supernatants carefully.

10. Pipet 16 ml of the pooled supernatants (cell extract) into 25 × 89–mm (26-ml) polycarbonate ultracentrifuge bottles.

11. Fill a 10-ml glass syringe fitted with a round-tipped pipetting needle with 8 ml ice-cold buffer B. Underlay the cell extract with this buffer. Close the bottles and centrifuge 2.5 hr at 145,000 × g_{av} (40,000 rpm in a Beckmann 50.2 Ti rotor), 4°C.

In this centrifugation step the RMs should form a tight pellet. If substantial degradation of microsomal proteins and attached ribosomes occurs during the preparation, a tight pellet will not be formed; such samples should be discarded.

12. Remove the top layer carefully by aspiration and discard. Next, remove the broad cloudy band at the interface, which is enriched with Golgi. Finally, remove the buffer B cushion.

13. Add a few milliliters of ice-cold buffer C to each tube and resuspend the pelleted RMs with a rubber policeman. Transfer the resuspended pellets to a 60-ml Dounce homogenizer. Collect residual RMs by washing the tubes with a few more milliliters of buffer C.

The total volume of buffer C used to resuspend the RMs should not exceed 80% of the initial weight of the pancreas. The large off-white pellets will be resuspended as large clumps that require further dispersal.

14. Disperse the RMs in the Dounce homogenizer using five complete up-and-down strokes. Transfer the RMs to a clean 100-ml graduated cylinder.

15. Remove two 10-µl aliquots and add each to 1 ml ice-cold 1% SDS; wipe off the the pipet tip before adding its contents to the SDS solution, because a considerable volume will stick to the outside. Pipet back and forth several times to transfer all of the RMs from inside the tip.

16. Read the absorbance of each sample at 280 nm. Using the average of the two readings, adjust the concentration of the microsomes to 50 A_{280} U/ml.

By definition this solution contains one equivalent (eq) of RMs per microliter.

17. Split the RMs into aliquots of an appropriate size and freeze in liquid nitrogen.

After adjusting the concentration of RMs as described here, the final yield should be ~1 to 1.5 ml per gram of pancreas. The RMs can be frozen and thawed at least several times without loss of activity, so a single aliquot can be used for multiple experiments.

SUPPORT PROTOCOL 2

PREPARATION OF EDTA-STRIPPED ROUGH MICROSOMES

When RMs are added to reticulocyte lysate translations, a high background of polypeptide synthesis can sometimes result from the readout of mRNAs that are attached to membrane-bound polysomes. To solve this problem, membrane-bound ribosomes can be removed by a simple EDTA treatment.

NOTE: All steps in this protocol should be performed in a cold room at 4°C. All solutions should be prechilled and all equipment and glassware should be placed in the cold room in advance.

Additional Materials *(see Support Protocol 1)*

Crude RM fraction (see Support Protocol 1)
Buffer C (see recipe)/50 mM EDTA, ice cold
Buffer C/0.5 M sucrose, ice cold

1. Add 1 vol buffer C/50 mM EDTA to a crude RM fraction. Incubate 15 min at 0°C.
2. Place sucrose cushion, consisting of buffer C/0.5 M sucrose, that is approximately half the volume of the RM mixture, into an ultracentrifugal tube. Carefully load the RMs over the sucrose cushion using a pipet, holding the pipet against the side of the tube to avoid disturbing the cushion. Centrifuge 1 hr at 145,000 × g_{av} (40,000 rpm in a Beckmann 50.2 Ti rotor), 4°C.
3. Resuspend the pelleted membranes to half their original volume in buffer C (without EDTA or sucrose). Disperse the vesicles in a Dounce homogenizer (see Support Protocol 1, steps 13 and 14) and transfer to a graduated cylinder.
4. Adjust to the original volume with buffer C. The stripped RMs are now ready to be used or stored.

PREPARATION OF COLUMN-WASHED ROUGH MICROSOMES

SUPPORT PROTOCOL 3

Occasionally batches of RMs can inhibit in vitro translation reactions. This problem can be solved by separating the RMs from ribosomes and other contaminants using a gel filtration column.

NOTE: All steps in this protocol should be performed in a cold room at 4°C. All solutions should be prechilled and all equipment and glassware should be placed in the cold room in advance.

Additional Materials *(also see Support Protocol 1)*

Sepharose CL-2B resin (Pharmacia Biotech)
Buffer E (see recipe), ice cold
Crude RM fraction (see Support Protocol 1)
Chromatography column of volume equivalent to ten times the volume of RMs to be treated
Ultracentrifuge and rotor (Beckman 45 Ti rotor or equivalent)

1. Prepare a Sepharose CL-2B column with a column volume equivalent to ten times the volume of RMs to be treated. Equilibrate with several column volumes buffer E.
2. Load RMs onto Sepharose CL-2B column.
3. Collect fractions that represent the void volume.
4. Pool the turbid fractions and centrifuge 30 min at 85,000 × g_{av} (35,000 rpm in a Beckman 45 Ti rotor), 4°C.
5. Resuspend the pelleted membranes to half their original volume in buffer C.
6. Disperse the vesicles in a Dounce homogenizer (see Support Protocol 1, steps 13 and 14) and transfer to a graduated cylinder.
7. Adjust to the original volume with buffer C. The washed RMs are now ready to be used or stored.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****. Adjust the pH of all stock solutions with acetic acid.*

Buffer A

50 mM triethanolamine, pH 7.5
50 mM potassium acetate, pH 7.5
6 mM magnesium acetate
1 mM EDTA
250 mM sucrose
1 mM DTT (added immediately before use; *APPENDIX 2A*)
0.5 mM PMSF (added immediately before use; *APPENDIX 2A*)

Store solution without DTT and PMSF indefinitely at 4°C.

Preparation of one canine pancreas requires 500 ml of buffer A.

Buffer B

50 mM triethanolamine, pH 7.5
6 mM magnesium acetate
1 mM EDTA
1.3 M sucrose
1 mM DTT (added immediately before use; *APPENDIX 2A*)
0.5 mM PMSF (added immediately before use; *APPENDIX 2A*)

Store solution without DTT and PMSF indefinitely at 4°C.

Preparation of one canine pancreas requires 100 ml of buffer B.

Buffer C

50 mM triethanolamine, pH 7.5
250 mM sucrose
1 mM DTT (added immediately before use; *APPENDIX 2A*)

Store solution without DTT indefinitely at 4°C.

Preparation of one canine pancreas requires 100 ml of buffer C.

Buffer D

50 mM triethanolamine, pH 7.5
1.5 mm magnesium acetate
1 mm EDTA
1 mM DTT (added immediately before use; *APPENDIX 2A*)

Store solution without DTT indefinitely at room temperature.

Buffer E

100 mM sodium citrate, pH 5.5
0.5 mM PMSF (added immediately before use; *APPENDIX 2A*)

Store solution without PMSF indefinitely at room temperature.

COMMENTARY

Background Information

Pioneering studies conducted by Palade and co-workers in the 1960s demonstrated that proteins that travel through the secretory pathway in mammalian cells are synthesized on endoplasmic reticulum (ER)–bound ribosomes and are translocated across the ER membrane (Palade, 1975). The subsequent discovery that the transport of proteins across the ER membrane, and the associated modifications, can be reproduced in vitro by adding rough microsomes (RMs) to heterologous cell-free translation reactions (Blobel and Dobberstein, 1975; Katz et al., 1977) was a remarkable breakthrough. These studies led to the development of methods for assessing protein translo-

cation (signal-sequence cleavage, glycosylation), formulation of the "signal hypothesis" (the idea that proteins contain sequence information that earmarks them for transport into the ER), and molecular dissection of the transport process itself.

Current understanding of the molecular mechanism whereby proteins are targeted to and translocated across the ER membrane has been derived largely from experiments in which canine pancreatic RMs have been added to wheat germ translation reactions. In early studies it was shown that a soluble ribonucleoprotein complex called the signal recognition particle (SRP) is required for protein transport into RMs. SRP recognizes the signal sequences of secreted proteins as they emerge from translating ribosomes (Walter and Blobel, 1981) and then targets ribosome-nascent chain complexes to the ER via an interaction with the ER-bound SRP receptor. Because SRP binds only to signal sequences that are part of a nascent polypeptide chain, protein translocation does not occur if microsomes are added post-translationally. The interaction between SRP and the SRP receptor facilitates release of the nascent chain (Gilmore et al., 1982; Meyer et al., 1982) and its insertion into a proteinaceous translocation channel comprised of the heterotrimeric Sec61p complex and an auxiliary protein called TRAM (Görlich et al., 1992; Görlich and Rapoport, 1993). Available evidence suggests that the translocation channel has properties associated with gated ion channels (Simon and Blobel, 1991; Crowley et al., 1994). During translocation, signal sequences are cleaved by the hexameric signal peptidase complex (Evans et al., 1986) and N-linked sugars are attached by the heterotrimeric oligosaccharyl transferase (Kelleher et al., 1992).

In addition to providing information about protein localization in vivo and insight into the mechanism of protein transport, the translocation assay described here has many other applications. Because signal-sequence processing occurs faithfully in vitro, N-terminal sequencing of the mature portion of a protein can be used to locate the signal-sequence cleavage site. For the large class of proteins that do not have an N-terminal signal sequence, deletion analysis can be performed to identify targeting sequences, as demonstrated, for example, by the work of Spiess and Lodish (1986). In addition, protease protection analysis following the insertion of membrane proteins can be used to study the topology of membrane proteins, which often cannot be deduced from the primary amino acid sequence (see, for example, Lipp and Dobberstein, 1986; Gafvelin et al., 1997).

Although canine pancreas is the most common source of RMs used in protein translocation studies, translocation-competent RMs have also been derived from a wide variety of other tissues including sheep pancreas (Kaderbhai et al., 1995), rat liver (Bielinska et al., 1979), ascites tumor cells (Bielinska et al., 1979), hen oviduct (Das et al., 1980), and *Drosophila* embryos (Brennan et al., 1980). Translocation-competent microsomes have also been obtained from *Saccharomyces cerevisiae* (Hansen et al., 1986), but these vesicles promote a post-translational, SRP-independent translocation reaction. Attempts to isolate translocation-competent microsomes from pig, rat, fish, and cow pancreas have not been successful (Kaderbhai et al., 1995; P. Walter, pers. comm.). At least in some of these cases it appears that the presence of high concentrations of endogenous ribonucleases destroys the integrity of the RMs. The ability to isolate translocation-competent RMs from different sources may facilitate the analysis of tissue-specific protein translocation or protein modification phenomena.

Most methods for isolating RMs are based on the observation that the attachment of ribosomes to rough ER imparts an especially high density to vesicles derived from that organelle. This high density facilitates purification of RMs on a discontinuous density gradient. In the method described here, RMs are pelleted through a 1.3 M sucrose layer whereas "smooth" microsomes, comprised mostly of Golgi and smooth ER, float at the interface between the low- and high-sucrose layers. This method works well for the isolation of RMs from specialized secretory organs such as the pancreas in which a very large percentage of the total membrane is rough ER. In a successful preparation, >90% of the high-density pellet is comprised of RMs; contamination with free ribosomes and other cellular components is minimal. Alternatively, RMs can also be purified by flotation on a very high-density (2 M) sucrose layer (Mechler and Vassalli, 1975; Behrens et al., 1996). This also yields highly purified RMs, but has the disadvantage that the vesicles may be recovered in a larger volume than desired and may require concentration by centrifugation. This method may be particularly useful in cases where pelleting the RMs would lead to unwanted contamination with free ribosomes or other cellular components.

Critical Parameters and Troubleshooting

It is essential to homogenize the pancreas effectively to obtain a good yield of RMs. Although it is neither necessary nor practical to remove all of the connective tissue and fat associated with the pancreas, excess extraneous tissue can interfere with homogenization. A good way to minimize this problem is to mince the pancreas as finely as possible before homogenization. Pieces of pancreas that prove difficult to disrupt can be removed from the homogenizer and minced again. In pelleting RMs through a sucrose cushion, the rotor and the size of the centrifuge bottles are not critical. It is important, however, to have a 2:1 to 3:1 ratio of cell extract to sucrose cushion to ensure a good separation of the large RM pellet from the smooth microsome interface.

It is very likely that pancreatic RMs obtained at the expected yield (see Support Protocol 1) will work well in translocation assays. Because the efficiency of translocation and glycosylation of different proteins can vary (see Anticipated Results), a new batch of RMs should be tested for activity using a protein that is known to be a good translocation/glycosylation substrate (e.g., preprolactin or influenza hemagglutinin). Sometimes RMs can inhibit translation or give rise to a high background of unwanted protein synthesis, but these problems can generally be eliminated by pretreating the RMs (see Support Protocols 2 and 3). Translocation assays can also be difficult to interpret if the translation reaction itself is inefficient. The optimization of translation conditions is discussed in *UNIT 11.2*.

Anticipated Results

Many but not all proteins are translocated across or inserted into canine RMs with high efficiency (50% to 100%). A few secreted proteins, in particular those that contain unusual signal sequences, are translocated very poorly under the conditions described here (Belin et al., 1996). It is possible that proteins in this class require as yet unidentified tissue-specific factors to promote their translocation.

Whereas signal-sequence cleavage is extremely effective in vitro, the efficiency of core glycosylation is much more variable. In some cases only a small fraction of translocated protein is glycosylated. Because glycosylation is often incomplete, several different partially modified forms of a protein that has multiple glycosylation sites may be observed on polyacrylamide gels. Post-translational treatment of translocation reactions with protease (see Basic Protocol, step 5) will reduce the translational background and may facilitate the visualization of weakly glycosylated forms.

A single pancreas from a 10- to 12-kg dog should weigh 20 to 30 g and yield a high-density pellet that is >90% rough microsomes with minimal contamination from free ribosomes and other cellular components. This preparation yields sufficient rough microsomes for many thousands of small-scale assays (using 1 µl of microsomes per reaction).

Time Considerations

The preparation of canine RMs (Support Protocol 1) can be completed in ~5 to 6 hr. The time required depends in part on the size of the pancreas; it takes longer to process a larger amount of tissue. Dissection of the pancreas, homogenization, and preparation of the post-mitochondrial supernatant requires ~1.5 to 2 hr of continuous work. Glassware and buffer C should be chilled during the subsequent 2.5-hr centrifugation step, but otherwise no work is required. Resuspension of the RMs and adjustment of the RM concentration should require <1 hr of hands-on time.

The time required to perform cotranslational translocation assays and post-translational treatments varies widely. A simple experiment involving a small number of reactions may be completed in 1.5 to 2 hr. The reactions can be assembled in <10 min. After a 40- to 60-min incubation period, an additional 20 min is required to precipitate and collect the proteins. A complex experiment with a large number of samples and different post-translational treatments may require a couple of hours of hands-on work plus incubation times (endoglycosidase H digestion usually requires an overnight incubation). The individual components of the translation and translocation reaction are very stable at 0°C, so the length of time required to assemble the reactions is not a concern. In addition, the incubation time for the translation-translocation reaction is not critical. These reactions are limited by the duration of the protein synthesis reaction, which is generally ~60 min. Shorter incubations will produce a lower signal but otherwise will give essentially the same results. After proteins are precipitated, samples may be left on ice for several hours. After the proteins are pelleted and the TCA is removed, the pellets are stable at −80°C for at least 1 week.

Both EDTA stripping of RMs (Support Protocol 2) and column washing of RMs (Support

Protocol 3) should take ~2 hr, although the time required for the latter procedure will depend to some extent on the flow rate of the column.

Literature Cited

Behrens, T.W., Kearns, G.M., Rivard, J.J., Bernstein, H.D., Yewdell, J.W., and Staudt, L.M. 1996. Carboxyl-terminal targeting and novel post-translational processing of JAW1, a lymphoid protein of the endoplasmic reticulum. *J. Biol. Chem.* 271:23528-23534.

Belin, D., Bost, S., Vassalli, J.-D., and Strub, K. 1996. A two-step recognition of signal sequences determines the translocation efficiency of proteins. *EMBO J.* 15:468-478.

Bielinska, M., Rogers, G., Rucinsky, T., and Boime, I. 1979. Processing in vitro of placental peptide hormones by smooth microsomes. *Proc. Natl. Acad. Sci. U.S.A.* 76:6152-6156.

Blobel, G. and Dobberstein, B. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* 67:852-862.

Brennan, M.D., Warren, T.G., and Mahowald, A.P. 1980. Signal peptides and signal peptidase in *Drosophila melanogaster*. *J. Cell Biol.* 87:516-520.

Crowley, K.S., Liao, S., Worrell, V.E., Reinhart, G.D., and Johnson, A.E. 1994. Secretory proteins move through the endoplasmic reticulum via an aqueous, gated pore. *Cell* 78:461-471.

Das, R.C., Brinkley, S.A., and Heath, E.C. 1980. Factors affecting the efficiency of co-translational processing of a de novo synthesized glycosylated immunoglobulin light chain. *J. Biol. Chem.* 255:7933-7940.

Evans, E.A., Gilmore, R., and Blobel, G. 1986. Purification of microsomal signal peptidase as a complex. *Proc. Natl. Acad. Sci. U.S.A.* 83:581-585.

Gafvelin, G., Sakaguchi, M., Andersson, H., and von Heijne, G. 1997. Topological rules for membrane protein assembly in eukaryotic cells. *J. Biol. Chem.* 272:6119-6127.

Gilmore, R., Walter, P., and Blobel, G. 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* 95:470-477.

Görlich, D. and Rapoport, T.A. 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell* 75:615-630.

Görlich, D., Hartmann, E., Prehn, S. and Rapoport, T.A. 1992. A protein of the endoplasmic reticulum involved early in polypeptide translocation. *Nature* 357:47-52.

Hansen, W., Garcia, P.D., and Walter, P. 1986. In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent post-translational translocation of the prepro-α-factor. *Cell* 45:397-406.

Kaderbhai, M.A., Harding, V.J., Karim, A., Austen, B.M., and Kaderbhai, N.N. 1995. Sheep pancreatic microsomes as an alternative to the dog source for studying protein translocation. *Biochem. J.* 306:57-61.

Katz, F.N., Rothman, J.E., Lingappa, V.R., Blobel, G., and Lodish, H.F. 1977. Membrane assembly in vitro: Synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. *Proc. Natl. Acad. Sci. U.S.A.* 74:3278-3282.

Kelleher, D.J., Kreibich, G., and Gilmore, R. 1992. Oligosaccharyltransferase activity is associated with a protein complex composed of ribophorins I and II and a 48 kd protein. *Cell* 69:55-65.

Lipp, J. and Dobberstein, B. 1986. SRP dependent membrane insertion of mouse invariant chain: A membrane spanning protein with a cytoplasmically exposed amino-terminus. *J. Cell Biol.* 102:2169-2175.

Mechler, B. and Vassalli, P. 1975. Membrane-bound ribosomes of myeloma cells. I. Preparation of free and membrane-bound ribosomal fractions. Assessment of the methods and properties of the ribosomes. *J. Cell Biol.* 67:1-15.

Meyer, D.I., Krause, E., and Dobberstein, B. 1982. Secretory protein translocation across membranes—The role of the "docking protein." *Nature* 297:647-650.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. *Science* 189:347-358.

Simon, S.M. and Blobel, G. 1991. A protein-conducting channel in the endoplasmic reticulum. *Cell* 65:371-380.

Spiess, M. and Lodish, H.F. 1986. An internal signal sequence: The asialoglycoprotein receptor membrane anchor. *Cell* 44:177-185.

Walter, P. and Blobel, G. 1981. Translocation of proteins across the endoplasmic reticulum. II. Signal recognition particle (SRP) mediates the selective binding to microsomal membranes of in-vitro-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* 91:551-556.

Key Reference

Blobel and Dobberstein, 1975. See above.

Landmark paper containing the first clear demonstration that a presecretory protein synthesized in a cell-free translation system is transported vectorially into ER vesicles.

Contributed by Harris D. Bernstein
National Institute of Diabetes and Digestive and Kidney Disesases/NIH
Bethesda, Maryland

In Vitro Analysis of SV40 DNA Replication

The early studies of bacterial DNA replication provided a platform for constructing the theory of eukaryotic DNA replication. In the absence of well-defined origins of DNA replication, however, study of the eukaryotic DNA replication was almost impossible until the efficient cell-free system that supported replication from the SV40 origin of replication was developed. The system was designed to bypass the de novo replication initiation by providing SV40 origin of replication and viral initiator protein T antigen in the presence of replication-competent extract. Replication is carried out completely by cellular proteins in the presence of the viral helicase T antigen and viral origin–containing plasmid. Replication initiates at the replication origin, which is recognized by T antigen, and proceeds bilaterally in a semiconservative manner. Many cellular replication proteins have been identified through study of the SV40 replication system.

This unit outlines general methods for in vitro SV40 DNA replication and product analysis, including incorporation of nucleotides by binding to DE81 paper (see Basic Protocol) and visualization of products after alkaline denaturing gel electrophoresis (see Alternate Protocol); methods for growing a large suspension culture for the extract (see Support Protocol 1) and for preparing a replication-competent S100 cell extract from 293 cells (see Support Protocol 2); support information for the expression of T antigen in insect cells (see Support Protocol 3); and procedures for purifying T antigen from baculovirus-infected insect cells (see Support Protocol 4) and preparing the Pab419 immunoaffinity column used for the purification (see Support Protocol 5).

IN VITRO REPLICATION OF PLASMIDS BEARING SV40 ORIGIN SEQUENCES

BASIC PROTOCOL

This protocol is used to study in vitro SV40 replication. The reaction consists of a mixture of T antigen and replication-competent S100 cell extract in the presence of SV40 origin–containing plasmid and [α^{32}P]ATP. Radiolabeled reaction products are analyzed by liquid scintillation counting. The reaction products can also be analyzed by agarose gel electrophoresis and autoradiography (see Alternate Protocol).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

Materials

5× replication buffer (see recipe)
Supercoiled SV40 origin–containing DNA template
2 U/µl creatine phosphokinase in 10 mM Tris·Cl, pH 8.0/50% (w/v) glycerol
Recombinant T antigen (see Support Protocol 4)
[α^{32}P]dATP (800 or 6000 Ci/mmol; final volume 0.0125 ml)
Replication-competent S100 cell extract (see Support Protocol 2)
DE81 paper (Whatman)
0.5 M Na_2HPO_4
100% ethanol
Scintillation cocktail

Contributed by Yi-Ling Lin and Anindya Dutta
Current Protocols in Cell Biology (1999) 11.5.1-11.5.12

1. Set up a reaction in a microcentrifuge tube containing:

   10 µl 5× replication buffer
   300 ng supercoiled SV40 origin–containing DNA template
   0.1 µl 2 U/µl creatine phosphokinase
   0.7 µg T antigen
   0.05 µl [α^{32}P]dATP (~200 to 1000 cpm/pmol)
   5 to 20 µl replication-competent S100 cell extract
   H_2O to 50 µl.

   Incubate 1 hr at 37°C.

   A negative control without T antigen should be included in the reaction, and the result should be treated as a baseline. Titration of the S100 is recommended to obtain optimal conditions for the reaction. Trichloroacetic acid precipitation can also be used for quantification of incorporated radioactivity.

   Because the volume of reagents in a single replication reaction is often minute, it can be helpful to make a master mix before setting up the reactions. The specific activity is determined as follows (where 125 pmol is the amount of dATP present in one-tenth input):

   cpm of one-tenth input from master mix for each reaction ÷ 125 pmol

   The replication assay can also be done with purified replication proteins, protein fractions, or combinations instead of the S100 cell extract. The reaction buffer and conditions for SV40 replication remain the same.

2. Stop the reaction by placing tubes on ice.

   The sample can then be analyzed for incorporation of radioactivity and by agarose gel electrophoresis and autoradiography (see Alternate Protocol).

3. Draw a grid of 1 × 1-cm squares on a sheet of DE81 paper and label the squares accordingly.

4. Spot 5 µl (one-tenth of output) of each reaction on the corresponding 1 × 1–cm square of DE81 paper.

   Do not allow the spots to dry.

5. Wash DE81 paper successively three times with 0.5 M Na_2HPO_4, once with double-distilled water, and once with 100% ethanol, 5 min per wash.

6. Dry paper with hair dryer or table lamp. Cut and put the squares in scintillation vials.

7. Add liquid scintillation cocktail. Count the radioactivity on squares. To determine specific activity of dATP, spot one-tenth of master mix on a square and count without washing; then calculate specific activity as described in step 1.

8. Determine the amount of dAMP incorporated in each reaction (in picomoles) using the following formula:

   [(cpm of the DE81 square) × 10] ÷ specific activity.

ALTERNATE PROTOCOL

ANALYSIS OF DNA REPLICATION REACTION PRODUCTS BY ALKALINE DENATURING AGAROSE GEL

Reaction products can also be analyzed by alkaline denaturing agarose gel electrophoresis to visualize the replicated DNA. Alternatively, one can analyze the replication products using a regular TAE or TBE agarose gel; however, the size range of nascent DNA products cannot be appreciated when using a nondenaturing gel.

Additional Materials *(also see Basic Protocol)*

3× stop solution (see recipe)
20 mg/ml high-quality glycogen (Life Technologies)
5 M NaCl (*APPENDIX 2A*)
TE buffer, pH 8.0 (*APPENDIX 2A*)
Agarose
1 N NaOH
0.5 M EDTA (*APPENDIX 2A*)
2× alkaline loading buffer (see recipe)
Alkaline running buffer, made fresh (see recipe)
7% trichloracetic acid (TCA)

Additional reagents and equipment for phenol/chloroform extraction and ethanol precipitation of DNA (*APPENDIX 3*), agarose gel electrophoresis (*APPENDIX 3*), and autoradiography (*UNIT 6.3*)

1. Perform in vitro replication reaction (see Basic Protocol, step 1).
2. Add ⅓ vol of 3× stop solution to the replication reaction. Incubate the reaction 30 min at 37°C for protein digestion.
3. Dilute the reaction volume to 100 µl with water. Remove the proteins by phenol/chloroform extraction.
4. Precipitate the DNA by adding 1 µl of 20 mg/ml high-quality glycogen (as carrier) and 250 µl of 100% ethanol.
5. Microcentrifuge 10 min at room temperature or 4°C, discard supernatant, and dissolve the DNA pellet in TE buffer.
6. Make a 1% (w/v) alkaline agarose gel: dissolve agarose in water by microwaving, cool the gel to 50°C, and add 1 N NaOH and 0.5 M EDTA to final concentrations of 30 mM and 1 mM, respectively.

   Do not microwave agarose with NaOH, as this will prevent the gel from solidifying.
7. Add 1 vol of 2× alkaline loading buffer to tube and add the DNA samples. Run gel at 2 to 7 V/cm in freshly made alkaline running buffer.
8. Stop electrophoresis when xylene cyanol has migrated two-thirds of the length of the gel (12 to 14 hr). Neutralize gel in 7% TCA 30 min at room temperature.

   Do not shake, as this may allow the solution to splash, contaminating the shaker with isotope.
9. Vacuum dry gel at low temperature (60°C) and autoradiograph (see *UNIT 6.3*).

PREPARING 293 CELL SUSPENSION CULTURE

SUPPORT PROTOCOL 1

This protocol provides guidance for expanding cells from a small-scale monolayer to a large-scale spinner culture for S100 cell extract. The cells are split to 1×10^5 cells/ml whenever the concentration reaches $0.5–1 \times 10^6$ cells/ml and are periodically checked for viability and density.

Materials

Ten 10-cm plates of confluent 293 cell culture
DMEM with 5% (v/v) calf serum
Joklik's medium (JRH Bioscience) with 5% (v/v) calf serum

Additional reagents and equipment for trypsinizing monolayer cells (*UNIT 1.1*), counting cells, and assessing viability (*UNIT 1.1*).

1. Trypsinize 10-cm plates of confluent 293 cells (*UNIT 1.1*). Resuspend the trypsinized cells from ten 10-cm plates in variable amount of medium.
2. Pool cells together and determine cell count by hemacytometer.
3. Inoculate cells at a density of 1×10^5 cells/ml in suitably sized spinner flask, and maintain as suspension culture in Joklik's medium.

   A good way to check if the cells are maintained as a suspension culture is to examine a drop of the culture under a light microscope: The cells should be individualized and round.

4. Monitor cells regularly (every 3 days) for viability (by Trypan blue; see *UNIT 1.1*) and density.
5. When cell density reaches 1×10^6 cells/ml, split cells by adding Joklik's medium directly to the growing cells to adjust their density to 1×10^5 cells/ml. Change to a bigger container when culture exceeds the recommended volume of the flask. Repeat this step several times, until there are enough cells for a 10-liter inoculation.
6. Split each 10-liter culture into one 8-liter and one 2-liter spinner culture. Check cell viability and density regularly (every 3 days).
7. When the cell density reaches 1×10^6 cells/ml, harvest the 8-liter culture for S100 cell extract, and split the 2-liter culture for another round of 10-liter inoculation (2 liters + 8 liters).

   Maintain a 2-liter suspension culture of 293 cells to significantly shorten the time required for preparation of lab stock S100.

SUPPORT PROTOCOL 2

PREPARATION OF A REPLICATION-COMPETENT S100 CELL EXTRACT

This protocol describes how to make replication-competent cytosolic cell extracts from an exponentially growing 293 suspension culture. The replication-competent S100 cell extract is used to provide replication factors in the reaction mix.

Materials

8 liters mid-log-phase 293 cells (see Support Protocol 1)
PBS (*APPENDIX 2A*), 4°C
Hypotonic lysis buffer (see recipe), 4°C
5 M NaCl
Protein assay dye reagents (Bio-Rad)

IEC PR7000 and rotor or equivalent
IEC Clinical centrifuge or equivalent
40-ml Dounce homogenizer (type B pestle)
RC5B Sorvall centrifuge with SS34 rotor
Beckman ultracentrifuge with SW 55.1 rotor

Harvest cells

1. Harvest cells from 8 liters of mid-log-phase 293 cells at 0.5–1×10^6 cells/ml by centrifuging cells in six 1-liter centrifuge bottles 20 min at $1000 \times g$, 4°C, in an IEC PR7000 or equivalent.
2. Carefully decant supernatant, and add more 293 cell culture to the centrifuge bottles until all culture has been pelleted. Carefully decant supernatant until a small amount of medium is left.

3. Resuspend pellets with residual medium, and transfer suspension into several 50-ml centrifuge tubes.
4. Centrifuge in an IEC Clinical centrifuge or equivalent 10 min at 1000 × *g*, 4°C, and resuspend each pellet with 15 ml cold PBS. Combine resuspended pellets and repeat centrifugation until all cells fit in one tube.

   This normally yields about a 10-ml cell pellet.

Lyse the cells

5. Resuspend the pellet with an equal volume (~10 ml) of freshly prepared hypotonic buffer, 4°C. Pellet the cells in the Clinical centrifuge for 10 min at 1000 × *g*, 4°C, and remove the supernatant.
6. Resuspend pellets in an equal volume of hypotonic buffer, 4°C, and incubate 10 min on ice to swell the cells.
7. Lyse cells by 30 strokes of Dounce homogenizer (with a type B pestle) on ice. Incubate the lysate 30 min on ice.

   The cell lysis may be checked under a microscope.

8. Transfer the lysate into a 30-ml Corex tube, and centrifuge in an SS34 rotor of a Sorvall RC5B centrifuge 10 min at 17,000 × *g*, 4°C. Save both supernatant and pellet. Store the pellet (which contains the nuclei) at −70°C.

   This pellet contains nuclear proteins, which are useful for many other purposes.

9. Measure the volume of the supernatant, and adjust the concentration to 0.1 M NaCl with 5 M NaCl. Divide the supernatant into several SW55.1 Beckman ultracentrifuge tubes, and centrifuge 1 hr at 100,000 × *g*, 4°C.

   Replication factor C (RF-C) is unstable in low salt.

10. Measure protein concentration of the extracts by Bio-Rad protein assay dye reagent, and divide into desired (~500-µl) aliquots. Snap freeze the extract and store at −70°C.

    This protocol usually yields 5 to 10 ml extract, with a protein concentration of 20 to 30 mg/ml. For the assay to work, the protein concentration of the extract must be ≥10 mg/ml.

EXPRESSION OF RECOMBINANT T ANTIGEN

SUPPORT PROTOCOL 3

Insect cells infected with recombinant T antigen baculovirus are used to express T antigen, which is then purified by immunoaffinity chromatography (see Support Protocol 4). The recombinant virus is not commercially available; however, most researchers will share cells.

Materials

Insect cells (Hi-five cells, Invitrogen)
Grace's medium with 10% fetal bovine serum (FBS)
Recombinant T antigen baculovirus stock, high titer
Serum-free medium

1. Seed ten 150-mm tissue culture plates with 2×10^7 Hi-five cells per plate.

   Alternatively, if starting with fewer cells, split them into ten 150-mm plates and grow until 80% confluent.

2. Add fresh Grace's medium to make up a final volume of 25 ml per plate before infection. Infect cells with high-titer recombinant T antigen baculovirus stock to achieve a multiplicity of infection (MOI) of 5 for optimal protein production.

   The amount of medium is not important, as long as the cells are resuspended evenly.

3. Incubate cells 2 days at 27°C. Check for signs of infection (cytopathologic effect) 24 to 48 hr later.

   Cytopathologic effects are usually visible by 24 hr after infection.

4. Two days after infection, harvest cells by scraping them off plates with a rubber policeman. Pellet and wash cells once with serum-free medium and store at −70°C.

   The pellet can be stored 6 months to 1 year.

SUPPORT PROTOCOL 4

IMMUNOAFFINITY PURIFICATION OF T ANTIGEN

In this protocol, recombinant T antigen is purified by immunoaffinity chromatography from insect cells (Hi-five cell, Invitrogen) infected with a recombinant baculovirus containing the T antigen gene.

NOTE: All the steps in this protocol must be carried out at 4°C. Use cold buffers with proteinase inhibitor added fresh throughout.

Materials

1 ml Sepharose CL-4B (Amersham Pharmacia Biotech) column packed in a 3-ml syringe plugged with siliconized glass wool
Pab419–protein A–Sepharose (Pab419-PAS) immunoaffinity column (see Support Protocol 5)
T antigen lysis buffer (see recipe), 4°C
Insect cell pellet containing recombinant T antigen (see Support Protocol 3)
Wash buffer 1 (see recipe), 4°C
Wash buffer 2 (see recipe), 4°C
Elution buffer (see recipe), 4°C
Protein assay dye reagent (Bio-Rad)
Dialysis buffer (see recipe), 4°C
0.1 M sodium borate, pH 9.0
0.5 M piperazine-*N,N′*-bis(2-hydroxypropanesulfonic acid) (PIPES), pH 7.0, 4°C
Dialysis tubing

1. Link a 1-ml Sepharose CL-4B column (for nonspecific absorption) serially to a Pab419-PAS column so the eluate from the former loads onto the latter. Equilibrate both columns with 10 ml T antigen lysis buffer.

   When using the Pab419-PAS column for the first time, pre-elute it with 20 ml elution buffer to remove any noncovalently coupled antibody.

2. Resuspend cell pellet containing recombinant T antigen in 5 to 10 vol (based on pellet volume) of ice-cold T antigen lysis buffer. Let cells stand 30 min on ice to lyse, mixing occasionally.

3. Clear lysate from cellular debris by centrifuging 30 min at 8000 × *g*, 4°C.

4. Load the cleared lysate onto the Sepharose CL-4B column, and collect flowthrough from the Pab419-PAS column. Repeat the loading once, and collect and save the flowthrough.

   Repeat the loading after disconnecting from the Sepharose CL-4B column. The second flowthrough is collected and saved in case the column condition is bad and most of the protein is still in the flowthrough.

5. Uncouple the columns, and wash the Pab419-PAS column with 100 ml wash buffer 1 followed by 50 ml wash buffer 2.
6. Elute T antigen from the Pab419-PAS column with 10 ml freshly prepared elution buffer. Collect 0.25-ml fractions in tubes containing 25 µl of 0.5 M PIPES, pH 7.0, to neutralize each fraction immediately. Determine T antigen concentration using Bio-Rad protein assay dye reagent.

   It is important to prepare the elution buffer fresh for immediate use. It is also important to add PIPES immediately to the fractions to neutralize the pH. This prevents T antigen from being inactivated owing to the high pH elution used in this protocol.
7. Pool the peak fractions, and dialyze the protein overnight at 4°C against 1 liter dialysis buffer. Divide protein into 100-µl aliquots, and snap freeze at −70°C.
8. Regenerate the Pab419-PAS column with 10 ml elution buffer, and wash with 10 ml of 0.1 M sodium borate, pH 9.0.

   The 100-µl aliquots can be further divided into 10-µl aliquots for the first time they are thawed to prevent repeated freeze/thaw cycles.

   The Pab419-PAS column can be reused four to five times and has a shelf life of ≥1 year at 4°C. Make a fresh Sepharose CL-4B column each time.

PREPARATION OF Pab419 IMMUNOAFFINITY COLUMN

SUPPORT PROTOCOL 5

Purification of T antigen requires an immunoaffinity column in which mouse ascites Pab419 antibody is coupled to protein A–Sepharose (PAS). The immunoabsorbent is stabilized by cross-linking with dimethylpimelimidate. Pab419 ascites is not commercially available; however, most researchers will share the Pab419 hybridoma. A commercial laboratory can produce the ascites.

NOTE: Most of the steps in preparing an immunoaffinity column can be performed at room temperature, because both the antibody and protein A–Sepharose are fairly stable. The column is stable for up to 1 year when stored at 4°C.

Materials

Pab419 ascites
Protein A–Sepharose (PAS; 1 ml packed volume) equilibrated in coupling buffer
Coupling buffer (see recipe)
Coupling buffer with 0.5% (v/v) NP-40 (4.2 ml of 10% NP-40 in 100 ml buffer)
0.1 M sodium borate, pH 9.0
400 mM dimethylpimelimidate (freshly prepared)
0.2 M ethanolamine, pH 8.0
0.2% sodium azide
5-ml syringe plugged with silanized glass wool

1. Mix 1.6 ml Pab419 ascites with 1 ml PAS in a 15-ml tube. Bring volume to 10 ml with coupling buffer, and incubate in a rotating wheel 1 hr at room temperature.

   Spinning down the particulate matter of ascites before mixing with the antibody prevents the column from clogging in later steps. Antibody sources other than ascites can be used but should be concentrated to 1 to 10 mg immunoglobulin/ml for best results.

   The reaction mix can be incubated at 4°C, 4 to 5 hr or overnight, if necessary.
2. Wash off the unbound antibody with three 10-ml washes of coupling buffer followed by five 10-ml washes of coupling buffer with 0.5% NP-40.
3. Equilibrate beads with five 10-ml washes of 0.1 M sodium borate, pH 9.0. Bring up the volume to 9 ml with 0.1 M sodium borate, pH 9.0.

Cross-linking with dimethylpimelimidate must be performed above pH 8.3. Check the pH of the sodium borate solution before use.

4. Remove 100 µl resuspended beads (~10 µl packed beads) and retain separately.

   This sample serves as a check for antibody conjugation before the cross-linking step, as described in step 5.

5. Add 1 ml freshly made 400 mM dimethylpimelimidate to the larger tube of beads (final concentration = 40 mM) and incubate 1 to 2 hr, room temperature, on a rotating wheel or rocker. Remove 100 µl resuspended beads after cross-linking, but before adding ethanolamine.

   Cross-linking efficiency can be determined by comparing the antibody released from the PAS beads before and after cross-linking by boiling the beads in Laemmli's buffer and conducting SDS-PAGE (UNIT 6.1). If the cross-linking took place efficiently, little antibody will be released from the beads after cross-linking compared to before cross-linking.

6. To stop the reaction, wash beads with two 15-ml washes of 0.2 M ethanolamine, pH 8.0, and then incubate 2 hr in 10 ml of 0.2 M ethanolamine, room temperature, on a rocker.

7. Transfer resuspended beads to a 5-ml syringe plugged with silanized glass wool, and wash with 20 ml of 0.1 M sodium borate, pH 9.0.

   The beads can be stored stably at 4°C in 0.1 M sodium borate, pH 9.0/0.2% sodium azide for more than 1 year.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Alkaline loading buffer, 2×

60 mM NaOH
2 mM EDTA
6% (w/v) Ficoll (type 400; Amersham Pharmacia Biotech)
0.05% (w/v) bromocresol green
0.08% (w/v) xylene cyanol FF
Store at 4°C; stable ≥1 year

Alkaline running buffer

30 mM NaOH
1 mM EDTA, pH 8.0
Prepare fresh before use

Coupling buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
5 mM EDTA
0.1% (v/v) NP-40
Store at room temperature; stable ≥1 year
Add 1 mM DTT and 1 mM PMSF before use

Dialysis buffer

10 mM PIPES, pH 7.0
0.1 mM EDTA
5 mM NaCl
10% glycerol
Store at room temperature; stable ≥1 year
Add 1 mM DTT and 1 mM PMSF before use

Elution buffer

56.5 µl triethylamine
2 ml glycerol
H_2O to 20 ml
Prepare fresh before use

Hypotonic lysis buffer

20 mM HEPES, pH 8.0
5 mM KCl
1.5 mM $MgCl_2$
Store at room temperature; stable ≥1 year
Add 1 mM DTT and 1 mM PMSF before use

Replication buffer, 5×

150 mM HEPES, pH 7.8
2.5 mM DTT
35 mM $MgCl_2$
20 mM ATP
1 mM each CTP, GTP, and UTP
0.125 mM dATP
0.5 mM each dCTP and dGTP
0.6 mM TTP
200 mM creatine phosphate
Adjust pH to 7.4 to 7.8 with 10 N NaOH
Store in −20°C freezer; stable ≥1 year

Stop solution, 3×

20 mM EDTA
0.3% (w/v) SDS
20 µg pronase
Store in −20°C freezer; stable ≥1 year

T antigen lysis buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
1 mM EDTA
10% (v/v) glycerol
0.5% NP-40
Store at room temperature; stable ≥1 year
Add 1 mM DTT and 1 mM PMSF before use

Wash buffer 1

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
0.5 M LiCl
1 mM EDTA
10% (v/v) glycerol
Store at room temperature; stable ≥1 year
Add 1 mM DTT and 1 mM PMSF before use

Wash buffer 2

10 mM PIPES, pH 7.4
5 mM NaCl
1 mM EDTA
10% glycerol
Store at room temperature; stable ≥1 year
Add 1 mM DTT and 1 mM PMSF before use

COMMENTARY

Background Information

The SV40 replication system was established in 1984 by Li and Kelly (1984). It provides the first biochemical tool for studying eukaryotic DNA replication. By providing an SV40 replication origin in plasmid form and the initiator protein (and helicase) T antigen in the presence of a replication-competent extract, the system can replicate the plasmid. This approach bypasses difficulties posed by the absence of known cellular initiation proteins and origins of replication.

The mechanisms of SV40 replication have been extensively studied and are summarized here. T antigen initiates the replication of the SV40 origin–containing plasmid by recognizing the replication origin, which is a single 64-bp core origin DNA sequence from the SV40 virus. The DNA is unwound with the assistance of cellular replication protein A (RPA). Synthesis of a primer RNA and the first Okazaki fragment is carried out by the DNA polymerase α/primase complex after the unwinding. Both RPA and replication factor C (RF-C) interact with polymerase α/primase and stimulate lagging-strand DNA synthesis (Tsurimoto and Stillman, 1991a,b). RPA functions as a stabilizer of single-stranded DNA during the replication process. Saturating amounts of RPA, however, block polymerase α and arrest the DNA synthesis after laying down the first Okazaki fragment. Proliferating cellular nuclear antigen (PCNA), RF-C, and ATP then interact with the polymerase α/primase and form a primer recognition complex. At this stage, polymerase δ recognizes the 3′ end of the first Okazaki fragment. RF-C loads PCNA onto the DNA; the latter forms a sliding clamp (Mossi et al., 1997), which interacts with polymerase δ (Lee et al., 1991) and results in the formation of a highly processive holoenzyme that efficiently replicates the leading-strand DNA. DNA polymerase α/primase, together with RPA, RF-C, and PCNA, continue the cycles of priming and DNA polymerization on the lagging strand, producing primer fragments that are extended into Okazaki fragments by DNA polymerase δ. The completion of lagging-strand DNA synthesis requires the processing of Okazaki fragments. RNase HI removes most of the RNA at the 5′ ends of the Okazaki fragments, but flap endonuclease 1 (FEN-1) is required to remove the last monoribonucleotide. After dissociation of cleaved RNA, DNA ligase comes in and seals the nick (Turchi et al., 1994). Thus the DNA replicates bidirectionally from the origin; polymerase δ continuously synthesizes the leading strand from the 3′ end of the first Okazaki fragment, and the DNA polymerase α/primase complex synthesizes the Okazaki fragments along the lagging-strand template by translocating away from the replication fork in a discontinuous manner. It is believed that similar replication mechanisms exist in cellular chromosomes, except that unknown initiation proteins and origins of replication are involved.

Assays representing various stages of SV40 DNA replication are useful in studying the replication mechanism, including origin binding by T antigen (Borowiec and Hurwitz, 1988), origin DNA untwisting (Dean and Hurwitz, 1991), DNA unwinding (Dean et al., 1987; Wold et al., 1987; Dutta and Stillman, 1992; Goetz et al., 1998), RNA primer formation (Murakami et al., 1992), primer DNA formation (Bullock et al., 1991; Denis and Bullock, 1993), and interactions between various replication factors (Dornreiter et al., 1993). The details of these protocols can be found in the cited literature.

The dependence of viral initiation proteins in the SV40 system makes it difficult to study issues related to chromosomal replication initiation. The *Xenopus* in vitro replication system has some advantages over the SV40 system in this regard, because it relies totally on cellular proteins and replicates only once in the cell cycle. Yet this model is not as easily manipulated as the SV40 system. Furthermore, because both are in vitro systems that depend on cellular extracts, possible participation of insoluble cellular components such as nuclear membrane, nuclear matrix, and chromatin cannot be properly determined. Recent introduction of an in vivo mammalian replication system (Krude et al., 1997) has provided a third approach to the study of DNA replication at the biochemical level. The synthesis of knowledge acquired from all three types of in vitro reactions will probably best illuminate the biochemistry of DNA replication.

Critical Parameters

It is important to have an active cell lysate and an active T antigen for the SV40 replication assay. The cells for the replication extracts must be monitored regularly for their viability. Antifungal reagents such as amphotericin B may be needed to avoid fungal contamination in the

long-term culture. The extracts have to be made and frozen on the day of harvesting for optimal reactions. An active lysate must have a protein concentration ≥10 mg/ml. Repeated freezing and thawing of T antigen and S100 extract inactivates the reaction; therefore, store the proteins in small aliquots. The pH of the replication buffer is a key issue for a successful SV40 replication reaction. It is necessary to measure the pH of the replication buffer after adding nucleotides (which are usually very acidic) unless prebuffered nucleotides are used.

Troubleshooting

When incorporation of the nucleotides is poor, four possible causes must be suspected: low protein concentration of the extract, repeated freezing and thawing of the extract, inactive T antigen, and acidic replication buffer. Low yield of T antigen or inactive T antigen is usually the result of improper immunoaffinity column preparation. Check the antibody conjugation and cross-linking procedures by running an SDS-PAGE, as suggested in Support Protocol 5. Also pre-elute the column before using it the first time.

Anticipated Results

The yield of T antigen from ten 150-mm plates with a 1-ml Pab419 column is ~2 to 3 mg. The 8-liter 293 suspension culture usually yields 5 to 8 ml replication-competent S100 cell extract, with a concentration of 20 to 30 mg/ml. A total of 10 to 15 µl of this extract usually gives maximal incorporation for a 50-µl SV40 replication reaction. A total of 60 to 100 pmol nucleotide is usually incorporated in a 50-µl reaction replication. Nucleotide incorporation decreases substantially when the conditions are not optimal.

Time Considerations

When it is started from one monolayer plate, the protocol takes ~3 to 4 weeks to produce enough cells for the replication competent S100 cell extract. The harvesting of cells and preparation of the extract usually takes 1 day. Insect cells infected with T antigen baculovirus are ready to be harvested 2 days after infection, and the resulting cell pellet can be stored at −70°C before purification. Preparation of the Pab419 immunoaffinity column with mouse ascites takes ~4 to 5 hr; most of the steps can be performed at 4°C when a longer incubation time is preferred. T antigen purification procedures take ~2 to 3 hr. The SV40 replication reaction takes ~2 hr to set up and finish. The products can be stored at −20°C for a few days before quantification and gel analysis. Between 1 and 2 hr are required for quantification of incorporated nucleotides for 10 to 20 reactions by the DE81 paper procedure. Alkaline gel analysis of the replication products usually takes 12 to 14 hr plus time for autoradiography.

Literature Cited

Borowiec, J.A. and Hurwitz, J. 1988. ATP stimulates the binding of simian virus 40 (SV40) large tumor antigen to the SV40 origin of replication. *Proc. Natl. Acad. Sci. U.S.A.* 85:64-68.

Bullock, P.A., Seo, Y.S., and Hurwitz, J. 1991. Initiation of simian virus 40 DNA synthesis in vitro. *Mol. Cell. Biol.* 11:2350-2356.

Dean, F.B., Bullock, P., Murakami, Y., Wobbe, C.R., Weissbach, L., and Hurwitz, J. 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. *Proc. Natl. Acad. Sci. U.S.A.* 84:16-20.

Dean, F.B. and Hurwitz, J. 1991. Simian virus 40 large T antigen untwists DNA at the origin of DNA replication. *J. Biol. Chem.* 266:5062-5071.

Denis, D. and Bullock, P.A. 1993. Primer-DNA formation during simian virus 40 DNA replication in vitro. *Mol. Cell. Biol.* 13:2882-2890.

Dornreiter, I., Copeland, W.C., and Wang, T.S. 1993. Initiation of simian virus 40 DNA replication requires the interaction of a specific domain of human DNA polymerase alpha with large T antigen. *Mol. Cell. Biol.* 13:809-820.

Dutta, A. and Stillman, B. 1992. cdc2 family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. *EMBO J.* 11:189-199.

Goetz, G.S., Dean, F.B., Hurwitz, J., and Matson, S.W. 1998. The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen–associated DNA helicase activity. *J. Biol. Chem.* 263:383-392.

Krude, T., Jackman, M., Pines, J., and Laskey, R.A. 1997. Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell* 88:109-119.

Lee, S.H., Kwong, A.D., Pan, Z.Q., and Hurwitz, J. 1991. Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen–dependent DNA polymerase delta. *J. Biol. Chem.* 266:594-602.

Li, J.J. and Kelly, T.J. 1984. Simian virus 40 DNA replication in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 81:6973-6977.

Mossi, R., Jonsson, Z.O., Allen, B.L., Hardin, S.H., and Hubscher, U. 1997. Replication factor C interacts with the C-terminal side of proliferating cell nuclear antigen. *J. Biol. Chem.* 272:1769-1776.

Murakami, Y., Eki, T., and Hurwitz, J. 1992. Studies on the initiation of simian virus 40 replication in

vitro: RNA primer synthesis and its elongation. *Proc. Natl. Acad. Sci. U.S.A.* 89:952-956.

Tsurimoto, T. and Stillman, B. 1991a. Replication factors required for SV40 DNA replication in vitro. I. DNA structure–specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins. *J. Biol. Chem.* 266:1950-1960.

Tsurimoto, T. and Stillman, B. 1991b. Replication factors required for SV40 DNA replication in vitro. II. Switching of DNA polymerase alpha and delta during initiation of leading and lagging strand synthesis. *J. Biol. Chem.* 266:1961-1968.

Turchi, J.J., Huang, L., Murante, R.S., Kim, Y., and Bambara, R.A. 1994. Enzymatic completion of mammalian lagging-strand DNA replication. *Proc. Natl. Acad. Sci. U.S.A.* 91:9803-9807.

Wold, M.S., Li, J.J., and Kelly, T.J. 1987. Initiation of simian virus 40 DNA replication in vitro: large-tumor-antigen- and origin-dependent unwinding of the template. *Proc. Natl. Acad. Sci. U.S.A.* 84:3643-3647.

Contributed by Yi-Ling Lin
Genetics Institute
Cambridge, Massachusetts

Anindya Dutta
Brigham and Women's Hospital
Boston, Massachusetts

In Vitro Transcription

Growth, differentiation, and development in eukaryotic organisms are largely regulated at the level of transcription. To gain an understanding of the molecular mechanisms of transcriptional regulation, it is necessary to utilize an in vitro transcription system by which the identity and function of various factors can be elucidated. The initial development of cell-free extracts from HeLa cells, capable of accurate initiation of transcription by RNA polymerase II, was an important advance in the analysis of gene regulation (Dignam et al., 1983; Manley et al., 1980). Since then, several extracts have been prepared, using diverse sources ranging from cultured mammalian (HeLa) cells (Dignam et al., 1983; Manley et al., 1980) to *Drosophila* embryos (Heiermann and Pongs, 1985; Soeller et al., 1988; Kamakaka et al., 1991), and yeast (Lue and Kornberg, 1987; Woontner and Jaehning, 1990), which mediate accurate initiation of basal and activated transcription. These extracts have been utilized to isolate and characterize the various general, as well as sequence-specific, transcription factors. The extracts have also been used to elucidate the detailed molecular mechanisms of transcription initiation in eukaryotes.

This unit describes an in vitro transcription reaction (see Basic Protocol) using either a mammalian (HeLa) cell extract (see Support Protocol 1) or a *Drosophila* embryo extract (see Support Protocol 2 or Alternate Protocol). This reaction is capable of transcribing from naked DNA or chromatin templates. Upon completion of transcription, the products are analyzed by primer extension analysis (see Support Protocol 3).

NOTE: All reagents used for these protocols should be high-quality molecular biology grade and should be RNase- and DNase-free. Likewise, all plasticware should be RNase-free, and great care should be taken to minimize contamination by RNases (*APPENDIX 2A*).

IN VITRO TRANSCRIPTION REACTIONS WITH NUCLEAR EXTRACTS

BASIC PROTOCOL

In this protocol, the nuclear extract is preincubated with a plasmid template containing an RNA polymerase II promoter to form preinitiation complexes at the promoter. Transcription is initiated by the addition of a mixture of ribonucleotide-5′-triphosphates (rNTPs) and is allowed to proceed for 30 min. After completion of the reaction, a stop solution is added. The RNA transcripts are deproteinized and precipitated. The transcripts can then be analyzed by primer extension analysis (see Support Protocol 3), S1 nuclease protection analysis of RNA, or runoff transcription assays.

NOTE: This protocol describes the conditions for 25-µl reactions. However, this reaction can be scaled up to accommodate various experimental parameters as described below.

Materials

Nuclear extract (see Support Protocol 1, Support Protocol 2, or Alternate Protocol)
10% (w/v) polyvinyl alcohol (PvOH; mol. wt. 10,000; Sigma)
10% (w/v) polyethylene glycol (PEG; mol. wt. 15,000 to 20,000)
1 M HEPES (potassium salt), pH 7.6 and 8.0, adjusted with KOH
200 ng/µl plasmid DNA template in TE buffer, pH 8.0 (*APPENDIX 2A*)
5 mM ribonucleotide-5′-triphosphates (rNTPs; see recipe)
Transcription stop solution (see recipe)
0.3 M sodium acetate
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (PCIAA), equilibrated with 10 mM Tris·Cl, pH 7.5
75% and 100% ethanol
30° and 37°C water baths

Contributed by Rohinton T. Kamakaka and W. Lee Kraus
Current Protocols in Cell Biology (1999) 11.6.1-11.6.17

Prepare transcription reaction mix

1. Thaw a sufficient number of aliquots of nuclear extract for the experiment quickly in an ice-water bath. Also thaw aliquots of 10% PvOH, 10% PEG, and 5 mM rNTPs.

   Keep all reagents on ice once thawed.

2. Prepare the following mix (multiplying the quantities by the number of tubes to be used in the assay):

   2.5 µl 10% PvOH
   2.5 µl 10% PEG
   0.5 µl 1 M HEPES (potassium salt), pH 8.0.

3. For each sample, combine the following reagents on ice, in the order listed, in a 1.5-ml microcentrifuge tube, mixing after each addition:

   6.0 µl H_2O
   5.5 µl PvOH/PEG/HEPES mix (from step 2)
   10.0 µl nuclear extract (or optimal amount determined empirically)
   1.0 µl 200 ng/µl DNA template (or optimal amount determined empirically).

   The water can be added to the PvOH/PEG/HEPES mix in step 2, to decrease the amount of pipetting. Purified transcription factors or column fractions from chromatographic purifications can be added at this step in addition to the extract in place of some or all of the water, if desired.

   The optimal amount of nuclear extract for each application will have to be determined empirically, but it typically ranges from 5 to 15 µl. If <10 µl of nuclear extract is used, the volume should be brought up with TM buffer containing 0.1 M KCl; if >10 µl of nuclear extract is used, the volume of the water should be reduced.

   The DNA template should be double-banded on CsCl gradients (see APPENDIX 3). The optimal amount of DNA template per reaction will also have to be determined empirically. It may range from 30 to 300 ng, depending on the strength of the promoter. A good starting point is 200 ng. The DNA is present in TE buffer, pH 8.0.

   The reaction for each set of experimental conditions should be performed in duplicate to compensate for any pipetting errors or loss of sample. Additionally, an internal reference plasmid can be added, if desired.

4. Incubate 15 min at room temperature (~21°C) to allow the formation of preinitiation complexes.

5. Prepare the rNTP mix just before use by combining:

   0.5 µl 1 M HEPES (potassium salt), pH 7.6
   2.5 µl 5 mM rNTPs
   4.5 µl H_2O.

Perform reaction

6. Initiate transcription by adding 2.5 µl of the rNTP mix prepared in step 5 to the incubated reaction from step 4. Incubate 30 min at 30°C.

   To confirm that the RNA was synthesized by RNA polymerase II, and not some other polymerase, inhibition with α-amanitin should be performed as a control. To do this, add 1 µl of 100 µg/ml α-amanitin (in water; also see APPENDIX 1B) to the reaction mix to give a final concentration of 4 µg/ml α-amanitin. RNA polymerase II is inhibited by 0.5 µg/ml α-amanitin; RNA polymerase III is inhibited by 200 µg/ml α-amanitin; and RNA polymerase I is resistant to α-amanitin.

7. Stop the reactions by adding 100 µl transcription stop solution. Mix well and incubate 10 min at 37°C.

Isolate transcripts

8. Add 250 µl of 0.3 M sodium acetate to each sample.
9. Extract each sample with 400 µl of 25:24:1 PCIAA. Transfer the aqueous phase from each tube to a new 1.5-ml microcentrifuge tube.
10. Add 1 ml of 100% ethanol and mix well. Microcentrifuge each sample 15 min at maximum speed, room temperature, to collect the RNA in the pellet.
11. Carefully aspirate the liquid from the small white pellets and add 400 µl of 75% ethanol to each tube. Microcentrifuge 5 min at maximum speed, room temperature.
12. Carefully aspirate all the liquid from the pellets. Dry the pellets in a rotary evaporator (e.g., a SpeedVac). Store the samples at −20°C, or proceed immediately with the primer-extension analyses (see Support Protocol 3).

PREPARATION OF A NUCLEAR EXTRACT FROM HELA CELLS

SUPPORT PROTOCOL 1

This protocol describes the preparation of a nuclear extract from HeLa cells and is based largely on the protocol described by Dignam et al. (1983). HeLa cells are incubated in a hypotonic buffer to swell them by osmotic action. The swollen cells are disrupted by Dounce homogenization, and the nuclei are collected in a pellet by centrifugation. The nuclei are then incubated in a high-salt buffer to extract the soluble proteins, including the transcription factors and RNA polymerase II, without lysing the nuclei. The nuclei are removed from the extract by centrifugation, and the extract is subjected to ammonium sulfate precipitation to concentrate the transcription factors and to remove histone H1 (a repressor of transcription). Finally, the precipitated proteins are collected by centrifugation, dissolved in buffer, dialyzed into a low-salt buffer, and used for transcription.

NOTE: This protocol describes the preparation of a HeLa nuclear extract from 12 liters of HeLa cell suspension culture (i.e., all volumes listed in the protocol below are based on an original culture volume of 12 liters). However, the preparation can be scaled up or down.

Materials

~5–8 × 10^5 cell/ml suspension culture of HeLa cells grown in spinner flasks
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Freezing buffer (see recipe)
Glycerol
PBS (*APPENDIX 2A*) containing 1 g/liter $MgCl_2$
Hypotonic buffer (see recipe)
High-salt buffer (see recipe)
Powdered ammonium sulfate
TM buffer (see recipe) with and without 0.1 M KCl

Beckman JS-4.2 and SW-28 and Sorvall GSA and SS-34 rotors (or equivalents) and appropriate centrifuge bottles and tubes
40-ml Wheaton Dounce homogenizer with loose (B) pestle (or equivalent)
Insulated magnetic stir plate
Coffee bean grinder or mortar and pestle
Dialysis tubing (12,000 to 14,000 MWCO)
Conductivity meter (optional)

Additional reagents and equipment for dialysis (*APPENDIX 3*)

NOTE: Perform all procedures at 4°C (i.e., on ice and in a cold room) using precooled solutions, glassware, and equipment. Perform all centrifugations at 4°C with precooled rotors. All low-speed centrifugations are done in a low-speed centrifuge (e.g., Sorvall RC-5) while high-speed centrifugations are done in an ultracentrifuge.

Isolate HeLa cell nuclei

1. Centrifuge 12 liters of HeLa cells grown under standard conditions (~5–8 × 10^5 cell/ml) in spinner cultures for 15 min at 1800 × *g*, 4°C, in a large, low-speed preparative rotor (e.g., Beckman JS-4.2).

2. Resuspend the cell pellets in PBS (25 to 50 ml for each liter of original culture volume), combine, and centrifuge again as in step 1 in one or two bottles.

   If cells are not to be used immediately, add glycerol and freeze as in steps 3 and 4. Alternatively, the cells can be used immediately without freezing, in which case collect the cells from the spinner culture by centrifugation as in the previous steps and proceed directly to step 5.

3. Resuspend the cell pellets in 2 ml of freezing buffer for each liter of original culture volume and add glycerol to 20% (v/v), taking into account the original glycerol concentration of the freezing buffer (30% v/v), the volume contributed by the cells, and the final volume of the cell suspension.

4. Freeze the cells in aliquots in 50-ml plastic, conical tubes in liquid N_2 and store at −80° to −100°C.

5. Thaw HeLa cells (if frozen) from 12 liters of culture in an ice water bath as quickly as possible. Centrifuge 10 min at 1400 × *g* (3000 rpm in a Sorvall GSA rotor), 4°C, add 200 ml of PBS containing 1 g/liter $MgCl_2$, then centrifuge again at 1400 × *g*. Aspirate the supernatant from the loose cell pellet and wash again in the same manner.

   This protocol can be readily scaled up or down by a factor of two (i.e., for preparations from 6 to 24 liters of cells).

6. Aspirate the supernatant from the washed cell pellet and resuspend the cells in 60 ml hypotonic buffer. Incubate the cells on ice 15 min to allow them to swell.

7. Lyse the cells by 12 strokes in a Dounce homogenizer (with loose "B" pestle). Centrifuge the cell lysate 8 min at 3000 × *g* (5000 rpm in a Sorvall SS-34 rotor), 4°C, to collect the nuclei in the pellet.

   Cell lysis can be monitored under a microscope by staining an aliquot of cells with trypan blue (UNIT 1.1), which stains the nuclei of lysed cells. Aim for 90% cell lysis.

   Work quickly after lysis of the cells to minimize the leakage of transcription factors from the nuclei.

8. Aspirate the supernatant and resuspend the isolated nuclei in a total final volume of 20 ml hypotonic buffer. Pool into one tared SS-34 tube and centrifuge 8 min at 3000 × *g*, 4°C. Aspirate the supernatant and weigh the nuclei.

   A typical yield of nuclei from a 12-liter culture of HeLa cells is ~12 g.

Prepare a high-salt nuclear extract

9. Resuspend the nuclei in 70 ml high-salt buffer and transfer to a 200-ml glass beaker. Stir the suspension slowly in an ice-water bath on an insulated magnetic stir plate for 30 min.

10. Transfer the suspension to thick-walled tubes for a Beckman SW-28 rotor. Balance the tubes and centrifuge 60 min at 100,000 × *g* (25,000 rpm in an SW-28 rotor), 4°C.

    A swinging bucket rotor is preferable to a fixed-angle rotor for this centrifugation step. With a fixed angle-rotor, undesirable shearing of the nuclei can occur.

11. Collect the supernatants into a graduated cylinder and measure the volume (expect 70 to 75 ml).

Precipitate the extract

12. Transfer the pooled supernatants to a 200-ml glass beaker. Start stirring the suspension slowly in an ice-water bath on an insulated magnetic stir plate. Slowly, over a 15 to 20 min period, add 0.33 g powdered ammonium sulfate per ml of supernatant (final saturation 55% at 0°C). After all the ammonium sulfate has been added and has dissolved, let the mixture stir for an additional 25 to 30 min.

    Pulverize the ammonium sulfate by using a coffee bean grinder or a mortar and pestle no more than 5 to 10 min before use. Powdered ammonium sulfate is hygroscopic and cannot be weighed accurately when it is wet.

13. Centrifuge the mixture 20 min 35,000 × *g* (17,000 rpm in an SS-34 rotor), 4°C. Remove the supernatants by aspiration, trying to remove as much of the liquid as possible.

14. Dissolve the pellets in a total final volume of 5 to 6 ml TM buffer and pool into one tube.

    The pellets from the ammonium sulfate precipitation are difficult to dissolve. For best results, add ~1.5 ml of TM buffer to each pellet. Then, pipet up and down gently to dislodge and resuspend the pellets. After pooling, use ~1 ml of buffer to rinse the tubes and the pipet, and add the rinse to the sample. The solution will be milky white after the pellets are dissolved.

Dialyze the extract

15. Desalt the extract by dialysis (*APPENDIX 3*) in 12,000 to 14,000 MWCO dialysis tubing as follows:

    1 hr versus 2 liters TM buffer
    1 hr versus 2 liters TM buffer containing 0.1 M KCl
    2 hr versus fresh change of TM buffer (2 to 4 liters) containing 0.1 M KCl.

    The dialysis is complete when the conductivity of the sample is the same as the conductivity of the dialysis buffer. If a conductivity meter is not available, it should be possible to assume complete or near-complete dialysis with this scheme.

16. Transfer the extract from the dialysis bag and centrifuge 10 min at 12,000 × *g* (10,000 rpm in an SS-34 rotor), 4°C, to remove any insoluble material. Divide the supernatant into aliquots, freeze in liquid N_2, and store at −80° to −100°C.

    If desired, save an aliquot of the extract for determination of the protein concentration by the Coomassie blue binding assay. A convenient aliquot size for in vitro transcription experiments is 150 to 200 µl. Avoid multiple freeze-thaw cycles.

    The yield is ~7 ml of HeLa extract from 12 liters of cells.

SUPPORT PROTOCOL 2

PREPARATION OF HIGH-SALT *DROSOPHILA* EXTRACTS

Two transcription extracts that are highly active for transcription in vitro can be prepared from *Drosophila* embryos (also see Alternate Protocol). Transcription extracts from *Drosophila* are particularly attractive for studies on transcriptional regulation because: (1) they are highly active and (2) many mechanisms of activation have been conserved between mammals and flies with various transcription factors being easily interchangeable in vitro. Furthermore, *Drosophila* embryos are inexpensive compared to cultured cells. Finally, *Drosophila* transcription extracts allow the use of in vitro–reconstituted chromatin as the template for transcription studies (Kamakaka et al., 1991).

This protocol is based on the method of Soeller et al. (1988) and Parker and Topol (1984). It involves the purification of nuclei from 0- to 12-hr *Drosophila* embryos, followed by extraction of nuclear proteins such as transcription factors and histones using a high-salt buffer. After removal of the cellular debris, the transcription factors are precipitated with ammonium sulfate (which, incidentally, removes nonspecific inhibitors of transcription such as histone H1). The precipitated proteins, after solubilization, are desalted by gel filtration, resulting in an active transcription extract. This extract is a rich source of both general and sequence-specific transcription factors which can be purified by further chromatography steps.

NOTE: This protocol is for use with ~100 g of embryos. The preparation, however, can be scaled up or down.

Materials

Drosophila cultures
Molasses/agar plates (see recipe)
Bleach (5.25% sodium hypochlorite) diluted 1:1 in distilled H_2O (store at room temperature)
Embryo wash solution: 0.7% (w/v) NaCl/0.04% (v/v) Triton X-100 (store at room temperature)
Disruption buffer (see recipe)
Resuspension buffer (see recipe)
4 M ammonium sulfate, pH 7.0
Solid ammonium sulfate
HEMG containing 0.1 M KCl (see recipe)

Embryo collection apparatus (construct according to Fig. 11.6.1) and paint brush
Nylon mesh (Tetko, #3-70/43 and 3-500/49) and interlocking rings for constructing embryo collection apparatus
Yamato LH-21 homogenizer (Thomas)
4 pieces of Miracloth (9 in. × 9 in.; Calbiochem)
GSA, 45-Ti, and SS-34 rotors (or equivalents) and appropriate centrifuge bottles and tubes
40-ml Wheaton Dounce homogenizer with a B pestle and 15-ml homogenizer with A pestle
Rotating mixer
Coffee grinder
5 × 22–cm G-25 SF desalting column (430 ml; Pharmacia Biotech)
Conductivity meter

Additional reagents and equipment for growing *Drosophila* (Goldstein and Fyrberg, 1994)

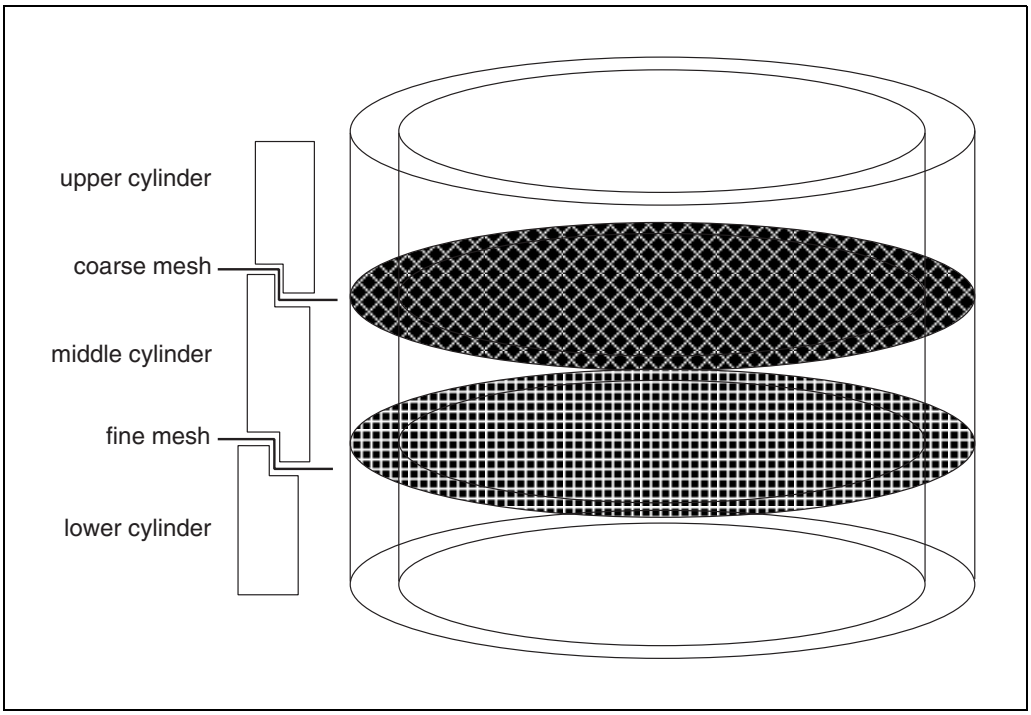


Figure 11.6.1 The embryo collection apparatus consists of three interlocking rings, which hold two nylon meshes of different porosity. The coarse mesh (Tetko 3-500/49) is placed between the upper and middle cylinders to collect any dead flies and large particulate material. The fine mesh (Tetko 3-70/43) is placed between the middle and lower cylinders to collect the embryos but allow the yeast to flow through. The dimensions of this apparatus can vary; 3-in. high, 10-in. diameter segments work well.

NOTE: Perform all procedures at 4°C (i.e., on ice and in a cold room) using precooled solutions, glassware, and equipment. Perform all centrifugations at 4°C with precooled rotors.

NOTE: The use of frozen embryos or embryos stored >3 days results in transcription extracts of a very poor quality.

Collect embryos

1. Grow wild-type flies at 25°C and 70% humidity in population cages (Goldstein and Fyrberg, 1994). Collect the embryos onto molasses/agar plates.

   Embryos collected between 0 and 12 hr after fertilization will be used. The plates can be stored for up to 3 days at 4°C (Biggin and Tjian, 1988).

2. Harvest 0- to 12-hr embryos in embryo collection apparatus (Fig. 11.6.1). Wash the embryos off the molasses/agar plates onto the coarse mesh of the collection apparatus in a sink, using a paint brush and running distilled water. Once all the embryos are collected, remove the upper cylinder and the coarse mesh containing the dead flies and particulate matter.

3. Remove large foreign particles, if present, in the embryos, and wash well in the collection apparatus consisting of the middle and lower cylanders to remove the yeast.

4. Move the apparatus containing the embryos into a Nalgene tub. Pour 3 liters of 1:1 bleach/distilled water into embryo collection apparatus in the Nalgene wash tub and swirl to mix. Dechorionate the embryos by soaking 90 sec in the bleach. Lift the embryos in the bleach solution out of the tub and allow the bleach solution to drain away.

   Do not soak embryos for >90 sec.

5. Quickly, rinse with 1 liter embryo wash solution by pouring embryo wash solution directly into embryo collection apparatus and swirling to mix.
6. Rinse with 1 to 3 liters water to remove excess embryo wash solution and bleach.
7. Dry the embryos to a moist cake by placing paper towels under the fine nylon mesh of the embryo collection apparatus to absorb excess water.
8. Weigh the embryos in a tared beaker.

Homogenize embryos

9. Add 3 ml disruption buffer per gram of embryos and stir with a rod to disperse the embryos evenly. Disrupt embryos with a single passage through the Yamato LH-21 homogenizer at 1000 rpm.
10. Pour the homogenate into a funnel lined with 1 layer Miracloth over a GSA bottle. After the liquid in each funnel has flowed into the GSA bottle, wash the debris remaining in the Miracloth with additional disruption buffer (2 ml per g of embryos).

    The final volume of disruption buffer should be ~5 ml/g embryos.

    Depending on the volume of the homogenate, between one and four funnels are used.

    Once the embryos are homogenized, work quickly—proteins can leak out of the nuclei into the buffer solution.

11. Centrifuge 15 min at 10,000 × *g* (8000 rpm in a GSA rotor), 4°C, to collect the nuclei in a pellet. Carefully decant the supernatant (the pellet will be very loose). Wipe lipids from the walls of the centrifuge bottles with Kimwipes.

    Try to remove as much lipid as possible.

12. Resuspend the nuclei in 3 ml disruption buffer per gram of embryos by swirling carefully. Do not resuspend the yellow yolk, which sticks very tightly to the bottle. Transfer nuclear suspension to a Dounce homogenizer.
13. Use a 40-ml Dounce homogenizer with a B pestle to disperse the nuclei. Transfer to clean GSA bottles.
14. Centrifuge again as in step 11. Carefully decant the supernatant, and wipe the lipids from the walls of the centrifuge bottles.
15. Resuspend the nuclei in 1 ml resuspension buffer per g embryos. Use a 40-ml Dounce homogenizer with a B pestle to break up clumps of nuclei (three strokes of the Dounce are sufficient).
16. Measure the volume accurately with a 250-ml graduated cylinder and transfer to 45-Ti bottles. Add a quantity of 4 M ammonium sulfate equivalent to 1/10 vol of the liquid in 45-Ti bottles to each bottle. Mix by inversion, then rotate 20 min on a rotating mixer.

    The final ammonium sulfate concentration is 0.364 M = 9% saturation at 20°C.

17. Centrifuge the lysed nuclei 1 hr at 142,400 × *g* (35,000 rpm in 45-Ti rotor), 4°C.
18. During the centrifugation, make the following preparations:
    a. Pulverize solid ammonium sulfate crystals in a coffee grinder.
    b. Prepare ice bath over a magnetic stirrer for ammonium sulfate precipitation.
    c. Begin to equilibrate the G-25 SF desalting column with 2 column volumes of HEMG containing 0.1 M KCl at 400 ml/hr.

Precipitate proteins

19. Remove supernatant from the lysed nuclei with a pipet. Avoid lipid globules on the top of the tube.

    It is normal for the solution to become cloudy upon standing.

20. Accurately measure the volume of the combined supernatants. Transfer the liquid to a 400-ml beaker containing a magnetic stirrer, placed in the previously prepared ice bath. Slowly (over a period of ~5 min) add 0.3 g pulverized ammonium sulfate/ml liquid. Stir for an additional 15 min.

    The final ammonium sulfate concentration is 2.26 M = 56% saturation at 20°C.

21. Centrifuge ammonium sulfate precipitate 20 min at 27,000 × *g* (15,000 rpm in an SS-34 rotor), 4°C. Decant supernatant, and drain pellets well.

    The supernatant is a good source for histone H1.

22. Resuspend pellets in 0.1 ml HEMG containing 0.1 M KCl per g embryos. Use a 15-ml Dounce homogenizer with an A pestle to disperse protein clumps.

23. Centrifuge the resulting mixture 10 min at 12,000 × *g* (10,000 rpm in an SS-34 rotor), 4°C, to remove insoluble material.

Desalt extract

24. Apply the protein extract to an equilibrated G-25 SF desalting column. Run the column at 400 ml/hr, and monitor the absorbance at 280 nm. Collect 1-min fractions (~7 ml).

    In ~20 min, the protein peak will elute from the column, the protein peak will be followed by a salt peak.

25. Check the protein concentrations of the peak fractions with the Coomassie blue binding assay (*APPENDIX 3*), then measure the conductivity of the last protein fraction that is to be pooled.

    If the conductivity of this fraction is identical to that of HEMG containing 0.1 M KCl (i.e., 20 mS/cm), then the desalting is complete. Dilute 20 ml of sample in 5 ml distilled water, and measure the conductivity of the resulting solution. 80 mS/cm corresponds to an extract of 20 mS/cm. If the conductivity does not match then use a larger column or dialyze the sample.

26. Pool the peak protein fractions, and centrifuge the solution 10 min as in step 23 to remove insoluble debris. Avoid the lipids at the top of the tubes.

27. Quick-freeze the extract in a 50-ml polypropylene screw-cap tube in liquid N_2 and store at −100°C.

    Typically 100 g of embryos will yield ~40 to 50 ml of high-salt transcription extract.

PREPARATION OF THE SOLUBLE NUCLEAR FRACTION FROM ISOLATED DROSOPHILA EMBRYO NUCLEI

ALTERNATE PROTOCOL

An alternative protocol for the preparation of *Drosophila* transcription extracts, based on the method of Kamakaka et al. (1991), has the advantages of speed, removal of nonspecific inhibitors of transcription, and allowing the preparation of extracts from very few embryos (extracts can be made from between 10 and 150 g of embryos, and quantities are simply scaled proportionately). This method involves the isolation of nuclei in a relatively low-salt buffer followed by a high-speed centrifugation of the nuclei in a small volume

to maximize the concentration of transcription factors while avoiding the extraction of nonspecific inhibitors of transcription.

Additional Materials *(also see Support Protocol 2)*

HEMG20 containing 0.1 M KCl (see recipe)
Ultracentrifuge with SW-28 rotor

1. Prepare nuclei (see Support Protocol 2, steps 1 to 14).
2. Resuspend the nuclei in 1 ml resuspension buffer per gram of embryos. Disperse nuclei in a 40-ml Dounce homogenizer with a B pestle (three strokes of the Dounce should be sufficient).
3. Transfer the entire suspension of nuclei into one tared GSA bottle.
4. Centrifuge 10 min at 10,000 × *g* (8000 rpm in GSA rotor), 4°C.
5. Decant the supernatant and weigh the bottle to determine the mass of the nuclei.
6. Add 0.5 ml per gram of nuclei of HEMG20 containing 0.1 M KCl.

   Example: if there are 10 g nuclei, then add 5 ml HEMG20 containing 0.1 M KCl.

7. Resuspend the pellet by shaking and swirling the nuclei (do not use a homogenizer). Place the suspension on ice for 15 to 60 min.
8. Centrifuge mixture 1 hr at 100,000 × *g* (24,000 rpm in SW-28 rotor), 4°C.

   It is important to always use a swinging-bucket rotor for the preparation of the soluble nuclear fraction to reduce shearing of chromatin.

9. After centrifugation, observe the four distinct layers, which will appear from top to bottom as follows:
   a. A thin gray-white lipid layer. Remove this with a spatula and discard.
   b. A yellow liquid layer, which comprises ~50% of the total volume of the tube and is the soluble nuclear fraction. Remove this extract with a pipet and transfer into a 50-ml screw-cap polypropylene tube.
   c. A gray liquid layer. Avoid the gray layer below the extract as this is enriched in transcription inhibitors and nucleases and phosphatases.
   d. At the bottom, a solid off-white layer of nuclear material which includes DNA, scaffold etc.
10. Quick-freeze the extract (step 9, substep b, above) in 50-ml polypropylene screw cap tube in liquid N_2 and store at −100°C.

    Usually, 100 g of embryos will yield 20 g of wet nuclei, which will then yield ~15 ml of the soluble nuclear fraction.

SUPPORT PROTOCOL 3

PRIMER EXTENSION ANALYSES OF IN VITRO TRANSCRIPTION PRODUCTS

This protocol describes primer extension analyses of in vitro transcribed RNA products. First, a short (~25 base) DNA oligonucleotide is end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Next, the radiolabeled oligomer is annealed to the RNA products from the in vitro transcription reactions, and the primers are extended with reverse transcriptase. Finally, the primer extension products are analyzed by denaturing-polyacrylamide gel electrophoresis.

Materials

~25-base DNA oligonucleotide complimentary to the RNA products from the in vitro transcription reactions
T4 polynucleotide kinase
150 µCi/µl [γ-^{32}P]ATP (7000 Ci/mmol; ICN Biomedicals)
2.5 M ammonium acetate
10 mg/ml glycogen
100% ethanol
1× TE buffer, pH 8.0 (*APPENDIX 2A*)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (PCIAA), equilibrated with Tris·Cl, pH 7.5
3 M sodium acetate
5× annealing buffer (see recipe)
RNA product (e.g., from Basic Protocol)
Primer extension mix (see recipe)
50 U/µl Moloney murine leukemia virus (MMLV) reverse transcriptase
Formamide loading buffer (FLB; see recipe)
8% urea-polyacrylamide sequencing gel
1× TBE buffer (*APPENDIX 2A*)

37°, 58°, 70°C, and boiling water baths
Whatman 3MM filter paper

Additional reagents and equipment for primer synthesis, agarose gel electrophoresis, DNA elution, ethanol precipitation, DNA quantification, primer labeling (*APPENDIX 3*), and phosphorimaging or autoradiography (*UNIT 6.3*)

NOTE: All reagents used for this protocol should be high-quality molecular biology reagents. All procedures should be performed at room temperature unless otherwise noted. All microcentrifugations are done at maximum speed.

CAUTION. When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out experiment and dispose of waste in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Radiolabel DNA oligonucleotide

1. Design, synthesize, and purify a DNA oligonucleotide primer ~25 bases in length (*APPENDIX 3*).

   The oligo should produce a primer-extension product 100 to 150 bases in length (from the 5′ end of the annealed oligo to the transcription start site). The oligo should be gel purified on high-percentage (~10%) polyacrylamide gels run in 1× TBE, eluted, ethanol precipitated, dissolved in water, and quantified. Oligonucleotides with an annealing temperature of ~58°C should be designed.

2. Make a working dilution of the primer at 2.5 pmol/µl.

3. In a 1.5-ml microcentrifuge tube, radiolabel 5 pmol (2 µl) of the primer (*APPENDIX 3*). Use T4 polynucleotide kinase according to the manufacturer's specifications (in a 20-µl reaction) in the presence of 2 µl of 150 µCi/µl [γ^{32}P]ATP. Incubate the reaction at 37°C for 1 hr.

   CAUTION: *Great care should be taken, and appropriate shielding used, when labeling with [γ-^{32}P]ATP. All bench surfaces, pipettors, tube racks, hands, and forearms should be carefully monitored with a Geiger counter (see APPENDIX 1D).*

Precipitate DNA

4. Add 100 µl of 2.5 M ammonium acetate. Heat the reaction to 70°C for 15 min to inactivate the T4 polynucleotide kinase.
5. Allow the tube to cool to room temperature, then add 2 µl of 10 mg/ml glycogen and 375 µl of 100% ethanol to precipitate the radiolabeled primer. Mix by vortexing.

   When vortexing to mix the radioactive solutions or to dissolve precipitated primer in this protocol, use careful, gentle vortexing, avoiding contact of the liquids with the inside of the tube lid. This will minimize leakage of radioactive material from the tube and reduce contamination of the microcentrifuge and the investigator's hands.
6. Microcentrifuge the ethanol precipitates 15 min. Carefully remove the ethanol supernatant from the pellet with a pipet and discard in an appropriate waste container. Add 100 µl TE buffer, pH 8.0, and dissolve the pellet by vortexing.
7. Add 100 µl PCIAA and mix by vortexing. Microcentrifuge the tube 5 min to separate the organic and aqueous phases. Remove the upper (aqueous) phase and transfer to a new 1.5-ml microcentrifuge tube.
8. Add 10 µl of 3 M sodium acetate and 300 µl of 100% ethanol to precipitate the radiolabeled primer. Mix by vortexing, and microcentrifuge 15 min.
9. Carefully remove and discard the ethanol supernatant. Dry the pellet 10 min at room temperature.

   Do not dry the pellets under vacuum or they will be very difficult to dissolve.
10. Dissolve the pellet in 150 µl TE buffer, pH 8.0 (to a final concentration of 0.033 pmol/µl), by vortexing. Store the radiolabeled primer at −20°C.

    Primers labeled in this manner may be stored under these conditions for 2 to 3 weeks pending primer-extension analyses.

Perform primer extension

11. Thaw an aliquot of radiolabeled primer complementary to the in vitro transcription RNA products. Make a mix containing 10 µl of 1× annealing buffer (2 µl of 5× annealing buffer and 8 µl TE, pH 8.0) and 0.25 µl of labeled primer (~8 fmol) for each sample to be analyzed in the assay.
12. Add 10 µl of this primer extension annealing mix to each tube of RNA product. Dissolve the RNA by vortexing.
13. Heat the samples to 70°C for 2 min, then incubate 40 min at 58°C to anneal the primer to the RNA product/template.

    The optimal annealing conditions will vary depending on the length and G+C content of the primer.
14. Microcentrifuge the samples quickly to collect condensation from the insides of the lids to the bottoms of the tubes, and allow the samples to cool to room temperature.
15. Add 40 µl primer extension mix and 0.5 µl MMLV reverse transcriptase combined just before use and mix by gentle vortexing. Incubate 40 min at 37°C.
16. Add 225 µl of 100% ethanol. Mix the samples by gentle vortexing and microcentrifuge 15 min.
17. Remove the ethanol supernatant and dry the pellets in a rotary evaporator (e.g., SpeedVac). Dissolve the pellets in 6 µl FLB. Store samples at −20°C or proceed immediately with denaturing polyacrylamide gel electrophoresis.

    Avoid storage of the primer extension products for more than 2 or 3 days before gel analysis.

Analyze the products

18. Pour a standard 8% urea-polyacrylamide sequencing gel (*APPENDIX 3*). Boil the primer extension samples 4 min in a water bath. Run the samples on the gel in 1× TBE until the bromphenol blue tracking dye is 3 to 5 cm from the bottom of the gel.

    A 25-base primer will run in the vicinity of the bromphenol blue tracking dye on an 8% sequencing gel.

19. Dry the gel on a piece of Whatman 3MM paper. Subject the dried gel to phosphorimaging analysis or autoradiography (*UNIT 6.3*).

    If the aim of the experiment is to quantify the amount of primer-extension product, fixing the gel in 10% acetic acid for 15 min is recommended. Prior to fixation of the gel, cut the gel to remove the free primer. Fixation of a gel with the free primer band results in the diffusion of the primer during fixation which increases the background.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Annealing buffer, 5×

10 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
1 mM EDTA
1.25 M KCl
Store up to 6 months at –20°C

Disruption buffer

15 mM HEPES (potassium salt), pH 7.6
10 mM KCl
5 mM $MgCl_2$
0.1 mM EDTA
0.5 mM EGTA
350 mM sucrose
Store up to 1 month at 4°C
Just before use add:
1 mM DTT
1 mM sodium metabisulfite
0.2 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

Formamide loading buffer (FLB)

80% formamide
10 mM EDTA
1 mg/ml xylene cyanol
Store up to 6 months at –20°C

Freezing buffer

50 mM Tris·Cl, pH 7.9
30% glycerol
1 mM EDTA
0.5 mM DTT
Prepare fresh at 4°C

HEMG containing 0.1 M KCl

25 mM HEPES (potassium salt), pH 7.6
100 mM KCl

continued

12.5 mM $MgCl_2$
0.1 mM EDTA
10% (v/v) glycerol
Store up to 1 month at 4°C
Just before use add:
1.5 mM DTT
1 mM sodium metabisulfite
0.1 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

HEMG20 containing 0.1 M KCl

25 mM HEPES (potassium salt), pH 7.6
100 mM KCl
12.5 mM $MgCl_2$
0.1 mM EDTA
20% (v/v) glycerol
Store up to 1 month at 4°C
Just before use add:
1.5 mM DTT
1 mM sodium metabisulfite
0.1 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

It is also possible to use 0.4 M potassium glutamate monohydrate (Sigma), instead of 0.1 M KCl, for the preparation of "nuclei juice."

High-salt buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10% (w/v) sucrose
0.42 M KCl
5 mM $MgCl_2$
0.1 mM EDTA
20% (v/v) glycerol
Store up to 1 month at 4°C
Just before use add:
2 mM DTT
0.2 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

The 0.42 M KCl in the high-salt buffer can be substituted with 0.42 M NaCl, if desired.

Hypotonic buffer

10 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
10 mM KCl
0.1 mM EDTA
0.1 mM EGTA
Store up to 1 month at 4°C
Just before use add:
2 mM DTT
0.2 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine
2 µg/ml aprotinin
2 µg/ml leupeptin
0.75 mM spermidine
0.15 mM spermine
2 µg/ml pepstatin

Molasses/agar plates

100 ml water
14.6 ml molasses
3.6 g fine-ground agar

Autoclave, pour into styrofoam meat trays, and let set. Just before use, spread the top with thick yeast paste (prepared by mixing Red Star yeast with water to a consistency that can be easily spread but is not so thin that the flies get stuck, approximately that of canned tomato paste). Store up to 1 week at 4°C.

Primer extension mix (1 ml)

Evaporate 62.5 µl of 2 mg/ml actinomycin D in ethanol to dryness. Add to this 886 µl water followed by 62.5 µl of 1 M Tris·Cl, pH 8.3. Vortex to dissolve actinomycin D. Add 12.5 µl of 100 mM $MnCl_2$ (do not use a 1 M stock). Add 3.5 µl of each 10 mM dNTP (14 µl total). Vortex again. Finally, add 25 µl of 0.5 M DTT.

Resuspension buffer

15 mM HEPES (potassium salt), pH 7.6
110 mM KCl
5 mM $MgCl_2$
0.1 mM EDTA
Store up to 1 month at 4°C
Just before use add:
2 mM DTT
1 mM sodium metabisulfite
0.2 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

Ribonucleotide-5′-triphosphates (rNTPs), 5 mM

Dilute 100 mM stocks of each rNTP (Amersham Pharmacia Biotech) in buffer (the authors use 20 mM sodium phosphate buffer, pH 7.5, but others—including potassium phosphate, Tris, and HEPES—would work) such that each nucleotide is at a final concentration of 5 mM. Store up to 1 year at −70°C

TM buffer

50 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
0.1 M KCl (omit where specified)
12.5 mM $MgCl_2$
1 mM EDTA
10% (v/v) glycerol
Store up to 1 month at 4°C
Just before use add:
1 mM DTT
0.1 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine
1 mM sodium metabisulfite

Transcription stop solution

20 mM EDTA, pH 8.0
0.2 M NaCl
1% (w/v) SDS
0.25 µg/µl glycogen
Store up to 1 month at room temperature

COMMENTARY

Background Information

In general, transcription extracts are prepared from whole cells or nuclei by the extraction of factors using high salt concentrations—e.g., 0.42 M NaCl or 0.36 M ammonium sulfate (Dignam et al., 1983; Manley et al., 1980). These extracts are a rich source of general transcription factors, as well as some sequence-specific factors, and they are capable of accurate initiation of transcription from eukaryotic promoters mediated by RNA polymerase II. Results from several laboratories have demonstrated that addition of sequence-specific factors during the transcription reaction results in activated transcription in a binding site–dependent manner. Typically, most of these high-salt extracts utilize between 2% and 5% of the DNA template in a single round of transcription, and most systems allow two to three rounds of transcription. Unfortunately, these high-salt extracts are also a rich source of transcription repressors such as the histones (Croston et al., 1991) and other nonspecific DNA-binding proteins which inhibit elevated amounts of transcription in vitro. Removal of these inhibitors is important in order to dissect the exact mechanisms of transcription activation by sequence-specific factors (see, e.g., Croston et al., 1991).

Use of low-salt nuclear extracts avoids the extraction of certain transcription repressors such as the histones. The low-salt extract is therefore very efficient in template usage, and, depending on the promoter, it is possible to use up to 20% of the template per round of transcription with the possibility of multiple rounds of transcription (Kamakaka et al., 1991). The absence of histones in the extract makes it particularly useful in the transcriptional analysis of reconstituted chromatin templates (Kamakaka et al., 1993). Since the soluble nuclear fraction is prepared with low-salt buffers, it can be used directly in in vitro reactions without desalting.

Critical Parameters

The protocol for preparation of transcription extracts involves the isolation of nuclei followed by extraction of the factors using either high-salt buffers or high-speed centrifugation.

The most critical consideration after nuclear isolation speed is that all subsequent steps be performed at 4°C as rapidly as possible.

During the high-speed centrifugation, the authors have noticed that use of a fixed-angle rotor generates extracts of very poor quality; hence it is recommended that only a swinging-bucket rotor be used.

In the authors' experience, dilute extracts are inefficient for in vitro transcription reactions.

The use of sterile, high-grade reagents and clean glassware is critical.

High-salt extracts, in general, are more efficient in extracting both general and sequence-specific factors from nuclei. However the method is more tedious (taking 7 to 10 hr) and it is impractical to generate extracts from very small quantities of material. The soluble nuclear fraction does not suffer from these problems.

Anticipated Results

Extracts with a protein concentration of between 10 and 30 mg/ml are expected, and these extracts are usually competent for transcription.

All of these extracts should be initially tested for transcription competence using well characterized eukaryotic promoters and sequence-specific transcription activators. The adenovirus major late promoter should be used to test the HeLa extract while the Kruppel promoter is recommended for the *Drosophila* extract. Gal4-VP16 is a potent transcription activator that can be used with promoter templates containing binding sites for Gal4 with the HeLa and *Drosophila* extracts. In studies carried out in the authors' laboratory RNA synthesis has been monitored by primer extension analysis of the RNA (see Kamakaka et al., 1991). Generation of a primer extension product of a specific size is an indication of a functional extract.

Extraction of 12 liters of HeLa cells should provide 7 ml of active extract. Extraction of 100 g of *Drosophila* embryos should provide 40 to 50 ml high-salt extract or 15 ml soluble nuclear extract.

Time Considerations

The in vitro transcription reaction takes 1 to 2 hr and analysis by primer extension another 2 to 3 hr. Preparation of the HeLa extract requires 11 hr (~6 hr to make the extract from frozen cells with an additional 4 to 5 hr for dialysis and freezing). Preparation of the *Drosophila* embryo high salt extract requires 8 to 9 hr and the soluble nuclear extract requires 4 to 5 hr.

Literature Cited

Biggin, M.D. and Tjian, R. 1988. Transcription factors that activate the Ultrabithorax promoter in developmentally staged extracts. *Cell* 53:699-711.

Croston, G.E., Kerrigan, L.A., Lira, L., Marshak, D.R., and Kadonaga, J.T. 1991. Sequence-specific antirepression of histone H1-mediated inhibition of basal RNA polymerase II transcription. *Science* 251:643-649.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl. Acids Res.* 11:1475-1489.

Goldstein, L.S.B. and Fyrberg, E.A. 1994. *Drosophila melanogaster*: Practical uses in cell and molecular biology. *Methods Cell Biol.* Vol. 44.

Heiermann, R. and Pongs, O. 1985. In vitro transcription with extracts of nuclei of *Drosophila* embryos. *Nucl. Acids Res.*13:2709-2730.

Kamakaka, R.T., Tyree, C.M., and Kadonaga, J.T. 1991. Accurate and efficient RNA polymerase II transcription with a soluble nuclear fraction derived from *Drosophila* embryos. *Proc. Natl. Acad. Sci. U.S.A.* 88:1024-1028.

Kamakaka, R.T., Bulger, M., and Kadonaga, J.T. 1993. Potentiation of RNA polymerase II transcription by Gal4-VP16 during but not after DNA replication and chromatin assembly. *Genes Dev.* 7:1779-1795.

Lue, N.F. and Kornberg, R.D. 1987. Accurate initiation at RNA polymerase II promoters in extracts from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* 84:8839-8843.

Manley, J.L., Fire, A., Cano, A., Sharp, P.A., and Gefter, M.L. 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. *Proc. Natl. Acad. Sci. U.S.A.* 77:3855-3859.

Parker, C.S. and Topol, J. 1984. A *Drosophila* RNA polymerase II transcription factor contains a promoter-region-specific DNA-binding activity. *Cell* 36:357-369.

Soeller, W.C., Poole, S.J., and Kornberg, T. 1988. In vitro transcription of the *Drosophila* engrailed gene. *Genes Dev.* 2:68-81.

Woontner, M. and Jaehning, J.A. 1990. Accurate initiation by RNA polymerase II in a whole cell extract from *Saccharomyces cerevisiae. J. Biol. Chem.* 265:8979-8982.

Contributed by Rohinton T. Kamakaka
NICHD
Bethesda, Maryland

W. Lee Kraus
Cornell University
Ithaca, New York

Nuclear Import in Digitonin-Permeabilized Cells

The development of the nuclear import assay in permeabilized cells in 1990 was a windfall for the examination of the mechanism of nuclear import. This assay (see Basic Protocol) has been used to detect and examine the factors required for import of nuclear substrates and has lately been adapted for the study of nuclear export as well. This unit will primarily deal with how to analyze the nuclear import of a substrate in an in vitro system. These experiments generally have two goals: first, to demonstrate that a given protein is imported into the nucleus and second, to define the particular sequence (termed the nuclear localization sequence or NLS) in the protein which is responsible for that localization. The nuclear import assay as described here uses *Xenopus* ovarian cytosol as a source of import factors to support import of fluorescent substrate into the nuclei of cells in which the plasma membrane has been permeabilized with digitonin (see Basic Protocol). The presence or absence of import substrate within the nuclei is subsequently detected by fluorescence microscopy.

More challenging than the nuclear import assay itself is generating the reagents necessary to perform the assay. This includes generation of the fluorescent nuclear import substrate and production of cytosol to support nuclear import of that substrate. Production of *Xenopus* ovarian cytosol is described here (see Support Protocol 1). It is a simple, dependable, and inexpensive procedure which does not require a relatively large tissue culture capability. Many laboratories instead use HeLa cytosol or reticulocyte lysate (*UNIT 11.2*) as a source of import factors.

Two protocols are given for production of fluorescent nuclear import substrate controls. The first protocol (see Support Protocol 2) describes how to make TRITC-BSA-NLS, which consists of bovine serum albumin (BSA) that is made fluorescent by coupling to rhodamine (for ease of detection) and then chemically coupled to NLS peptides to confer nuclear localization. A more recent version of import substrate (developed by Dr. Manfred Lohka; see Support Protocol 3) is a fusion protein composed of green fluorescent protein (GFP), glutathione *S*-transferase (GST), and an NLS. The recombinant import substrates have the advantage of containing a specific number of NLSs (one) and fluorophores (one) per molecule. This is not true of chemically conjugated import substrates which, due to the method of their production, are a heterogeneous population. However, the recombinant substrate has not been used as extensively and is therefore not as thoroughly characterized as the chemical conjugates.

Finally, the method of detection of imported substrate must be adapted to the protein in question. There are three common alternatives. First, if the protein has been cloned, it can be fused to GFP and its import assayed directly, assuming the total molecular weight of the substrate is greater than ~50 kDa. Second, the protein can be made fluorescent by direct conjugation to a fluorophore. Third, the protein can be detected by indirect immunofluorescence microscopy (see *UNIT 4.3*).

NUCLEAR IMPORT ASSAY IN ATTACHED HELA CELLS

BASIC PROTOCOL

The following protocol describes a nuclear import assay using a known NLS-containing substrate. The HeLa cell plasma membrane is permeabilized with digitonin, and soluble cellular components are washed away. These soluble components include proteins and nucleotides required for nuclear import to occur. The components are therefore added back to the permeabilized cells in the form of *Xenopus* ovarian cytosol, ATP, and GTP.

Contributed by Mary Shannon Moore and Eric D. Schwoebel

Current Protocols in Cell Biology (2000) 11.7.1-11.7.17

There are two choices of positive control substrate (see Support Protocols 2 and 3), both of which have the same basic properties: (1) the substrate is fluorescent to aid in detection and, (2) the substrate contains an NLS directing import into the nuclei. This substrate is mixed with the cytosol and nucleotide, and added to the cells. The import reaction is allowed to continue for 15 min, and is stopped by addition of cold buffer and fixation.

Materials

70% ethanol
30,000 cell/ml suspension of HeLa cells (ATCC CCL-2) in complete DMEM/10% FBS
DMEM/10% FBS (see recipe)
1× transport buffer (TB; see recipe), ice cold
Digitonin working solution (see recipe), ice cold
Import reaction mixture (see recipe)
Paraformaldehyde working solution (see recipe), ice cold
p-phenylendiamine mounting medium (see recipe)
Clear nail polish

12-mm circular glass coverslips (Fisher) sterilized by autoclaving
24-well tissue culture plates
Whatman 3 mm filter paper
Fine-tipped forceps with large radius curved shanks (e.g., Dumont #7, Fine Science Tools)
Fluorescence microscope

NOTE: All solutions and equipment coming into contact with tissue culture cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Prepare HeLa cultures

1. Place 12-mm coverslips in wells of 24-well plate using forceps that have been sterilized by immersion in 70% ethanol and air dried. Add 1 ml of HeLa cells at 30,000 cells/ml in complete DMEM/10% FBS. Return plate to incubator and incubate 16 hr.

   The number of cells plated may have to be optimized to give ~50% confluence after 16 hr.

Permeabilize cells

2. Place plate on ice for 5 min. Aspirate medium and gently add 1 ml ice-cold TB.

   If using a repeating pipettor, designate one syringe for each solution (TB, digitonin, and fixative). Be gentle when adding solutions to the coverslips. Pipet slowly and do not spray directly onto the cells, but rather down the side of the well. From this point on, sterility is not required.

3. Aspirate TB and add 1 ml ice-cold digitonin solution. Incubate 5 min on ice.

   Digitonin preferentially solubilized cholesterol from membranes, which leaves large holes through which soluble material passes. Because the plasma membrane contains higher concentrations of cholesterol than the nuclear envelope, it is selectively permeabilized while the nuclear envelope is not.

4. Aspirate the digitonin solution and add 1 ml of ice-cold TB. Continue to hold plates on ice up to 30 min.

Perform import reaction

5. Tape Parafilm onto the benchtop, ensuring that it is flat on the surface, and mark with numbers in the same pattern as the plate (i.e., in rows of six). Place 40-µl drops of the import reaction mixture on the Parafilm so as to correspond to each well. Using forceps, remove coverslips from wells and wick off excess TB by touching the edge of the coverslip to filter paper. Gently invert each coverslip (cell-side-down) on its designated drop of import mixture.

   The coverslip will float on top of the drop of import mix.

   Controls for nuclear import include reaction mixes prepared with probes containing positive and negative NLS sequences (see Critical Parameters).

6. Cover the reactions with an opaque tray to prevent fading, and incubate 15 min at room temperature.

7. Gently pipet 250 µl of ice-cold TB underneath the coverslip to stop the import reaction and to float the coverslip up from the Parafilm. Gently remove coverslip and place back in the TB (cell-side-up) in the 24-well plate on ice.

Fix cells

8. Aspirate TB and add 1 ml of fresh ice-cold TB to each coverslip. Aspirate TB and add 1 ml of ice-cold 3% paraformaldehyde. Cover to protect from light, and incubate on ice for 15 min.

9. Remove coverslip, wick off excess fixative on filter paper, and gently invert on a small drop of mounting medium (cell-side-down). Allow top of coverslip to dry for 5 min before sealing the edges of the coverslips with clear nail polish.

Assess cells for nuclear import

10. Observe by fluorescence microscopy using 63× or 100× oil objective.

    The simplest way to record the results is photographically. To ensure that different panels of a given figure are comparable, the different samples should be photographed with the same exposure time and printed identically. The relative differences in fluorescence intensity between different samples is generally very reproducible. However, the overall fluorescence intensity of the assay does show some inter-assay variation from day to day. Therefore all the relevant samples for a given figure should be done in a single experiment. If desired, the prints from these photographs can be scanned and quantitated. However, if quantitation is to be used on a regular basis, it is strongly recommended that the investigator access or purchase a quantitation program and a system (such as a CCD camera or confocal microscope; UNIT 4.5) specifically designed for that purpose.

PREPARATION OF *XENOPUS* OVARIAN CYTOSOL

SUPPORT PROTOCOL 1

Cytosol from *Xenopus* ovaries is used to provide the cytosolic factors necessary for protein import.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Eight female *Xenopus* frogs
PBS (see recipe), ice-cold
Protease inhibitor tablets (Boehringer Mannheim Complete, EDTA-free)
1× homogenization buffer (see recipe), ice cold
1× transport buffer (TB; see recipe), ice cold

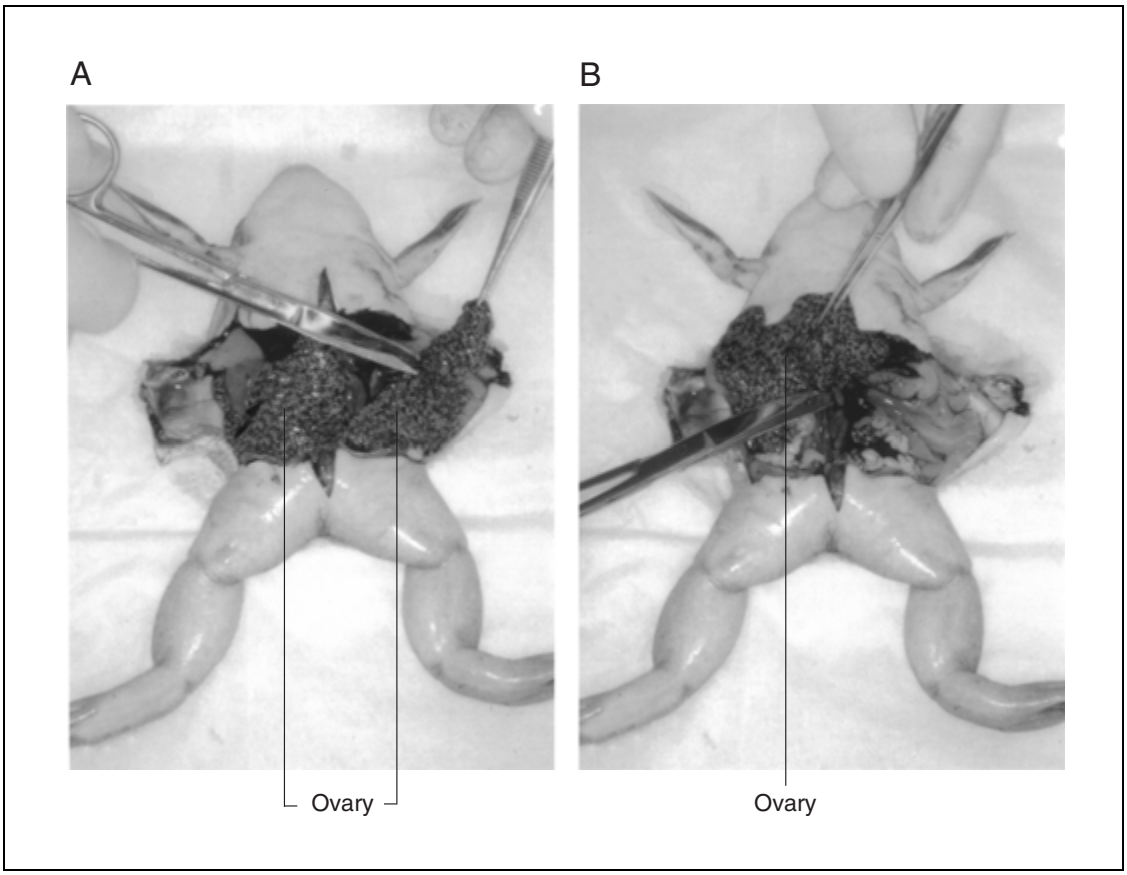


Figure 11.7.1 Frog ovary dissection. These panels show the exposed abdominal cavity of the female *Xenopus*. (**A**) Upon opening the abdominal cavity, the ovary is by far the most conspicuous organ. (**B**) To remove, gently lift the ovary with forceps and clip attachment points as near to the ovarian tissue as possible.

Carbon dioxide tank with attached tubing
Polytron homogenizer, PRO300D (PRO Scientific)
250-ml plastic centrifuge bottles
Sorvall centrifuge with GSA centrifuge rotor (or equivalent)
Cheesecloth
Beckman ultracentrifuge with Ti 70.1 rotor (or equivalent), and appropriate ultracentrifuge tubes
Plastic tubing, thin
20-ml syringe
Dialysis tubing (4000 to 6000 MWCO)
Centriplus-10 concentrators (Amicon)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

Dissect ovaries

1. Place frogs and water into a covered bucket with two pipet-sized holes drilled in the top for gas exchange. Sacrifice frogs by bubbling carbon dioxide through the frog water for 20 min under a fume hood. Transfer dead frogs into ice to keep cold.

   Approximately 350 mg of cytosol can be prepared from eight frogs.

2. Remove ovaries (Fig. 11.7.1) and place in a beaker (kept on ice) containing 1 liter of cold PBS.

   Try to avoid harvesting connective tissue.

3. After all of the ovaries are collected, wash them several times with a total of 4 liters of ice-cold PBS. Let the ovaries settle between washes and decant the supernatant.
4. Place the ovaries in a large graduated cylinder containing cold PBS to measure the wet volume of the ovaries.
5. Using a large pair of forceps, remove ovaries from beaker and briefly blot on paper towel to remove excess liquid. Transfer to a plastic beaker.

Prepare homogenate

6. Add an equal volume of cold homogenization buffer containing dissolved protease tablets (1 tablet per 50 ml buffer).
7. Homogenize with a Polytron homogenizer using several short bursts (5 to 10 sec each).

   This tissue is very soft and homogenizes easily. Use a relatively low setting on the homogenizer. Do not operate at high enough speeds to foam the slurry.

8. Transfer homogenate to 250-ml plastic centrifuge bottles and centrifuge 20 min in a GSA rotor at 16,000 × *g*, 4°C.
9. Decant the supernatant, avoiding the floating fat layer as much as possible. Filter supernatant through several layers of cheesecloth. Discard pellet and fat layer.
10. Transfer the supernatant to ultracentrifuge tubes using homogenization buffer to top off the last tube. Centrifuge 2 hr in a Ti 70.1 rotor at 150,000 × *g*, 4°C.

Collect clarified cytosol

11. Attach thin tubing to a 20-ml syringe. Insert tubing approximately one-half of the distance into the tube, between the floating fat layer and the loose yellow precipitate near the bottom. Draw off this clear middle layer.

    It is better to leave a little cytosol behind than to draw up contaminants at this step.

12. Transfer this cytosol to dialysis tubing (4000 to 6000 MWCO). Dialyze into a total of 6 liters of TB at 4°C. Change buffer three times with the third change followed by overnight dialysis.

    Additional insoluble material will form during dialysis, making the cytosol appear cloudy.

13. Transfer dialysate to ultracentrifuge tubes and centrifuge 1 hr in a Ti 70.1 rotor at 100,000 × *g*, 4°C.
14. Perform a Bio-Rad protein assay on the resulting cytosol (*APPENDIX 3B*). Concentrate the cytosol to ~20 mg protein/ml using Centriplus-10 concentrators.

    The protein concentration at the end of the cytosol preparation is usually 4 to 6 mg/ml. Typically, the optimal final concentration required to support nuclear import is ~10 mg/ml. Because additional constituents will be added during the import assay, it is convenient to concentrate the cytosol to ~20 mg/ml. A concentration curve must be performed on each batch to determine the optimal concentration required to support nuclear import.

15. Divide the concentrated cytosol into aliquots, snap freeze, and store at −80°C up to 6 to 8 months.

    A 550-µl 10 mg/ml aliquot would be enough cytosol for 24 cover slips.

PRODUCTION OF FLUORESCENT IMPORT SUBSTRATE: TRITC-BSA-NLS

A nuclear localization substrate is prepared by coupling BSA to an NLS and conjugating the resulting protein to TRITC.

Materials

Sephadex G-25 and G-50 (Pharmacia Biotech)
0.1 M sodium phosphate, pH 7.0 and 8.0 (*APPENDIX 2A*), ice cold
Bovine serum albumin, fatty-acid free (Boehringer Mannheim)
0.1 M sodium carbonate, pH 9.0/50 mM NaCl (store indefinitely at room temperature)
Tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC; Molecular Probes)
DMSO
1.5 M hydroxylamine·HCl, pH 8.5 (see recipe)
Sulfo-SMCC (Pierce)
NLS or mutant NLS peptide (~1 mg)
20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate (prepare fresh)
1× transport buffer (TB; see recipe)

Disposable 20-ml columns (Bio-Rad Laboratories, Cat # 732-1010)
Rocking platform
Centricon-30 concentrators (Amicon)

Additional reagents and equipment for dialysis (*APPENDIX 3C*), determining protein concentration (*APPENDIX 3B*), and SDS-PAGE (*UNIT 6.1*)

Prepare columns

1. Using disposable 20-ml columns, prepare three 15-ml bed volume desalting columns containing the following media.

   Sephadex G-25 equilibrated with 0.1 M sodium phosphate, pH 7.0
   Sephadex G-25 equilibrated with 0.1 M sodium phosphate, pH 8.0
   Sephadex G-50 equilibrated with 0.1 M sodium phosphate, pH 7.0

   Equilibrate each column at 4°C with at least 75 ml of the appropriate cold phosphate buffer.

Label BSA with TRITC

2. Dissolve 10 mg BSA in 1 ml of 0.1 M sodium carbonate, pH 9.0/50 mM NaCl. Save a 20-µl aliquot of this solution at 4°C as standard for SDS-PAGE analysis of conjugated products.

   This protocol will yield ~8 mg of TRITC-BSA-NLS import substrate.

   If more (or less) import substrate is required, scale entire procedure including BSA, TRITC, sulfo-SMCC, and peptide proportionally.

3. Dissolve 0.4 mg TRITC in 40 µl DMSO.

4. Add 12.5 µl of the TRITC/DMSO solution to dissolved BSA, 2.5 µl at a time, mixing after each addition. After all of the TRITC is added, wrap the tube in foil and incubate on a rocking platform 1 hr at room temperature.

   TRITC and its subsequent conjugates are sensitive to fading by ambient light, and prolonged exposure will result in noticeably dimmer import substrate. Protect from light whenever possible from this point onward.

5. Add 100 µl of 1.5 M hydroxylamine·HCl, pH 8.5, to the TRITC-BSA, wrap in foil, and mix for an additional 1 hr on the rocking platform at room temperature.

Hydroxylamine terminates the conjugation reaction and removes unstable conjugates from the BSA.

6. Microcentrifuge 10 min at 15,000 × *g*, 4°C, and save the supernatant.

Some insoluble complexes may form during steps 9 and 10.

Purify TRITC-BSA

7. Load the TRITC-BSA onto the 15-ml Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 8.0 (see step 1). Elute with 0.1 M sodium phosphate, pH 8.0, collecting 1-ml fractions. Pool the fractions containing the first red peak.

The separation of the TRITC-BSA (faster) and unconjugated TRITC (slower) should be obvious.

8. Concentrate eluate to ~0.5 ml in a Centricon-30. Remove a 10-µl aliquot and save at 4°C for SDS-PAGE analysis.

Note that if the separation of TRITC from TRITC-BSA on the Sephadex G-25 was effective, the filtrate from the Centricon should be colorless.

Activate TRITC-BSA

9. Dissolve 2 mg of sulfo-SMCC in 200 µl water (23 mM final). Add 166 µl of sulfo-SMCC to the TRITC-BSA. Wrap in foil and incubate 30 min at 37°C. Vortex lightly every ~5 min.

10. Microcentrifuge 10 min at 15,000 × *g*, 4°C, and collect the supernatant.

11. Load sample onto the 15-ml Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 7.0 (see step 1). Elute with 0.1 M sodium phosphate, pH 7.0, collecting 1-ml fractions. Pool the fractions containing the red eluate, which is the sulfo-SMCC activated TRITC-BSA. Remove 20 µl and save for SDS-PAGE analysis.

This column will separate unbound sulfo-SMCC (slower and colorless) from that bound to the TRITC-BSA (faster and red).

Conjugate NHS peptide to TRITC-BSA

12. Add a 5- to 20-fold molar excess of NLS peptide to TRITC-BSA-sulfo-SMCC. Cover with foil and incubate on a rocking platform overnight at 4°C.

13. Microcentrifuge 10 min at 15,000 × *g*, 4°C, to remove insoluble material.

14. Concentrate to 0.75 ml in Centricon-30.

15. Load the concentrated TRITC-BSA-NLS onto the 15-ml Sephadex G-50 column equilibrated with 0.1 M sodium phosphate, pH 7.0. Elute with 0.1 M sodium phosphate, pH 7.0, collecting 1-ml fractions Pool fractions containing the red eluate, which is the TRITC-BSA-NLS.

This column separates the final product, TRITC-BSA-NLS (faster and red) from the unconjugated NLS peptide (slower and colorless).

16. Dialyze (*APPENDIX 3C*) against a total of 3 liters of 20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate at 4°C. Change the buffer three times, with the last change followed by overnight dialysis.

17. Centrifuge dialysate 10 min at 10,000 × *g*, 4°C. Save the supernatant. Remove 20 µl and save for SDS-PAGE analysis.

18. Determine protein concentration using the Bio-Rad assay (*APPENDIX 3B*).

19. Concentrate in Centricon-30 if necessary.

The concentration of the stock import substrate should be at least 40× the final concentration used in the import assay. The authors' TRITC-BSA-NLS conjugates typically exhibit maximal import at 5 to 10 μg/ml.

Analyze conjugates

20. Analyze aliquots collected at steps 2, 8, 11, and 17 by SDS-PAGE (*UNIT 6.1*).

Conjugation of sulfo-SMCC to BSA (without peptide) will result in an apparent increase in molecular weight on SDS-PAGE. Successful coupling of peptide to the sulfo-SMCC-BSA will cause a further decrease in mobility. The number of peptides conjugated can be roughly estimated by the change in molecular weight.

21. Divide the conjugate solutions into aliquots and snap freeze in liquid nitrogen. Store at −80°C (for up to a year).

22. When required, briefly thaw aliquot in 37°C water bath, dilute to 40× with TB, and microcentrifuge 20 min at 15,000 × *g*, 4°C. Transfer supernatant to a fresh tube. Store in a dark container at 4°C for up to 1 month.

SUPPORT PROTOCOL 3

PRODUCTION OF FLUORESCENT RECOMBINANT IMPORT SUBSTRATE: GFP-GST-NLS

As an alternative to coupling of BSA to the NLS and TRITC (see Support Protocol 2), the nuclear localization substrate can be a recombinant GFP-GST-NLS produced in *E.coli*.

Materials

LB medium (*APPENDIX 2A*) containing 100 μg/ml ampicillin
DH5α strain of *E. coli*
GST-GFP-NLS and GST-GFP-mutNLS constructs in *E. coli* (Dr. Manfred Lohka, Biological Sciences, University of Calgary, Calgary, Canada)
1 M IPTG in H_2O, sterile filtered
Phosphate-buffered saline (PBS; see recipe)
Protease inhibitors tablets (Boehringer Mannheim Complete, EDTA-free)
PBS/EDTA (see recipe)
10% (v/v) Triton X-100
Glutathione–Sepharose 4B (Pharmacia Biotech)
Glutathione/Tris (see recipe)
20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate (prepare fresh)
Transport buffer (TB; see recipe)

Shaking incubator
Sorvall centrifuge with GSA and SS-34 rotors (or equivalents)
50-ml conical centrifuge tubes
French press or sonicator
Rocking platform
Disposable 2-ml column (Bio-Rad)
Centricon-30 (Amicon)

Additional reagents and equipment for dialysis (*APPENDIX 3B*), determining protein concentration (*APPENDIX 3B*), and SDS-PAGE (*UNIT 6.1*)

1. Inoculate a 250-ml culture of LB medium containing 100 μg/ml ampicillin with a single colony of *E. coli* DH5α containing GST-GFP-NLS. Grow overnight in a shaking incubator at 300 rpm, 37°C.

This 1-liter culture yields ~9 mg of GFP-GST-NLS.

2. Add the overnight culture to 750 ml of the LB medium. Mix and split into two flasks of 500 ml each. Add IPTG (from 1 M stock) to 0.4 mM, and continue shaking at 28°C for 24 hr.

3. Harvest cells by centrifuging 10 min in a GSA rotor at 4000 × *g*, 4°C. Discard the supernatant.

   Pellets can be frozen at –80°C at this time if desired.

4. Resuspend pellets in a total of 15 ml of 1× PBS and transfer suspension to a 50-ml conical tube. Rinse centrifuge bottles with an additional 3 ml PBS and pool the wash with the suspension. Vortex thoroughly to completely resuspend bacterial pellet.

5. Dissolve one protease inhibitor tablet in 1 ml of 1× PBS/EDTA and add to suspension. Add 3 ml of 10% Triton X-100 (1% final), and bring volume to to 30 ml with 1× PBS/EDTA. Vortex to mix.

   Dissolve tablet in room temperature PBS/EDTA. Crush tablet with a spatula to aid in dissolution and vortex until dissolved.

6. Disrupt cells by passage through a chilled French press at 16,000 psi.

   Chill the cylinder prior to use to minimize warming the sample. The authors find that lysis is most efficiently achieved with the French press. Alternately, cells can by lysed by sonication using three pulses of 1 min with a 1-min interval on ice after each pulse for cooling.

7. Clarify lysate by centrifuging 20 min at 16,000 × *g*, 4°C, in an SS-34 rotor or equivalent. Discard the pellet.

8. Gently resuspend glutathione-Sepharose 4B slurry in the stock bottle. Remove 2 ml (corresponding to ~1.5 ml of packed resin) and transfer to a 50-ml conical centrifuge tube. Centrifuge 2 min at 500 × *g* to sediment resin and remove supernatant. Resuspend the resin in 30 ml of 1× PBS/EDTA. Sediment resin by centrifuging 5 min at 500 × *g*. Discard supernatant.

9. Transfer clarified lysate from step 7 to the 50-ml conical centrifuge tube containing the glutathione–Sepharose 4B. Invert to mix and incubate with gentle rocking at 4°C for 2 hr.

   Brief exposure of GFP to ambient light does not noticeably affect its brightness, but protect the GFP from light during any prolonged step.

10. In the cold room, pour resin/homogenate from step 9 into a 2-ml disposable column and collect flowthrough for future analysis. Wash the column with 50 ml of cold PBS. Minimize disturbance of the column bed during addition of PBS. After the last wash, allow the meniscus of the buffer to approach the top of the column bed, then stopper the column.

    The presence of GFP should be particularly evident as the resin packs at the bottom of the column. The bedding will appear bright green.

11. Elute GFP-GST-NLS at room temperature with glutathione/Tris by adding 10 ml of glutathione/Tris to the top of the column bed. Unstopper column and collect 1-ml fractions of the eluate in 1.5-ml microcentrifuge tubes. Continue until the eluate is almost colorless and column bed appears nearly white. Place fractions on ice as they are collected and protect from light. Pool green fractions.

12. Dialyze (*APPENDIX 3C*) against a total of 3 liters of 20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate at 4°C. Change buffer three times with the last change followed by overnight dialysis.

13. Centrifuge dialysate 20 min in an SS-34 at 10,000 × *g*. Save the supernatant.

    Some protein may precipitate during dialysis.

14. Determine protein concentration using the Bio-Rad assay (*APPENDIX 3B*). Concentrate using Centricon-30 if necessary. Determine purity by SDS-PAGE (*UNIT 6.1*).

    The authors typically find that the activity of this substrate in the nuclear import assay is maximal at 25 to 50 µg/ml. It is convenient to concentrate the substrate to at least 40× its estimated final concentration, corresponding to ~2 mg/ml.

15. Aliquot, snap freeze in liquid nitrogen, and store at −80°C.

    The GST-GFP-NLS can be stored at −80°C for long periods. The size of the aliquots depends on the size of the experiment.

16. Perform a preliminary import assay (see Basic Protocol) to determine the optimal concentration of import substrate.

    Test several concentrations between 0 and 100 µg/ml. Too little substrate gives a faint signal, while too much substrate increases background and also decreases signal.

17. When required, thaw aliquot briefly in 37°C water bath, then dilute with TB to 40× the optimal concentration determined in step 16. Microcentrifuge 10 min at 15,000 × *g*, 4°C, to remove any precipitated protein, and transfer the supernatant to a fresh tube. Store at 4°C in a dark container (for up to a few weeks).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Digitonin solution

Stock solution (20 mg/ml):
40 mg digitonin (Calbiochem-Novabiochem)
Bring to 2 ml with DMSO
Store at room temperature for up to 1 month

Working solution: Dilute to 70 µg/ml in transport buffer (TB; see recipe) on day solution is to be used.

DMEM/10% FBS

Dulbecco's Modified Eagle Medium (e.g., Life Technologies, 1× liquid, high glucose) containing:
10% fetal bovine serum (*APPENDIX 2A*)
10 mM HEPES (pH 7.55)
100 U/ml penicillin G sodium
100 µg/ml streptomycin sulfate
Store up to 1 month at 4°C

Glutathione/Tris

2.5 ml 1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*; 50 mM final)
H_2O to 45 ml
76.8 mg reduced glutathione (Sigma; 5 mM final)
Adjust pH to 8.0 at room temperature with NaOH
Bring to 50 ml with H_2O
Divide into aliquots
Store up to several months frozen at −20°C

Glutathione noticeably alters the pH of the Tris buffer, which strongly affects the elution of some proteins.

Homogenization buffer

Prepare 10 × stock by combining the following:
200 ml 1 M HEPES, pH 7.3 (use KOH to adjust the pH)
20 ml 1 M magnesium acetate, pH 7.3
Bring volume to 1 liter with H_2O
Filter sterilize using a 0.2-µm filter
Store up to several months at 4°C
Prepare 1× working solution on day of use:
Dilute 10× stock to to 1× with H_2O
Add 1 M dithiothreitol (DTT) stock (store frozen in aliquots at −20°C) to 2 mM final

Hydroxylamine·HCl, 1.5 M, pH 8.5

Dissolve 2.1 g hydroxylamine hydrochloride in 10 ml water. Adjust pH to 8.5 with 5 M NaOH and adjust volume to 20 ml with H_2O. Prepare fresh.

Import reaction mixture

~10 mg/ml *Xenopus* ovarian cytosol (see Support Protocol 1)
0.5 mM GTP: add from 20 mM GTP stock in transport buffer (TB; see recipe)
1 mM ATP: add from 20 mM ATP stock in transport buffer (TB; see recipe)
Import substrate (for approximate concentrations, see Support Protocols 2 and 3)
Transport buffer (TB; see recipe) to 40 µl
Mix and store on ice until immediately before use.

The 20 mM ATP and GTP stock solutions are stored frozen in aliquots at −80°C.

Paraformaldehyde solution

Stock solution (6%): Under a fume hood, heat 150 ml of water to 60°C. Remove from heat and add 9 g paraformaldehyde. While stirring, add 2 drops 5 N NaOH from a Pasteur pipet. Continue stirring until dissolved. Store at 4°C for up to 1 month.

Working solution (3%): Dilute to 3% with 10× transport buffer (TB; see recipe) and water on the day of use.

PBS/EDTA, 4×

46 ml 0.2 M sodium phosphate, monobasic (NaH_2PO_4)
154 ml 0.2 M sodium phosphate, dibasic (Na_2HPO_4)
35.1 g NaCl
Bring to 900 ml with H_2O
Add 8 ml of 0.5 M EDTA
Adjust pH to 7.4 with NaOH
Bring to 1 liter with H_2O
Store at 4°C

Phenylendiamine mounting medium

Dissolve 100 mg *p*-phenylenediamine (an antifading agent) in 10 ml of 1× PBS (see recipe). Add 90 ml of glycerol and stir until homogenous. Divide into aliquots and store at −80°C. Warm to room temperature before use.

Prolonged exposure to light causes the mounting medium to turn brown.

Phosphate-buffered saline (PBS), 10×

2.3 mM NaH_2PO_4
7.7 mM Na_2HPO_4
150 mM NaCl
Store up to several months at 4°C

Transport buffer (TB)

Prepare 10× stock by combining the following:

200 ml 1 M HEPES, pH 7.3 (use KOH to adjust the pH)
275 ml 4 M potassium acetate, pH 7.3
20 ml 0.1 M magnesium acetate
3.8 g EGTA
Bring to 900 ml with H_2O and adjust pH to 7.3 with KOH or acetic acid
Bring volume to to 1 liter with H_2O
Filter sterilize using a 0.2-µm filter
Store up to several months at 4°C (months)

Prepare 1× working solution on day of use:

Dilute 10× stock to 1× with H_2O (working concentration)
Add 1 M dithiothreitol (DTT) stock (store frozen in aliquots at −20°C) to 2 mM final

The 1× TB contains 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, and 1 mM EDTA.

COMMENTARY

Background Information

Development of the permeabilized cell assay permitted a biochemical dissection of nuclear import mechanisms (Adam et al., 1990). This assay depends on the specificity of the detergent digitonin for cholesterol, which is present at higher concentrations in the plasma membrane than in the nuclear envelope (Colbeau et al., 1971). During the permeabilization process, the soluble contents of the cytoplasm leak out into the surrounding solution and are washed away. Included in these soluble contents are proteins and nucleotides (ATP and/or GTP) required for nuclear import. These must be re-added to permeabilized cells, in this case in the form of crude cytosol, for nuclear import to occur (Fig. 11.7.2A). Energy, in the form of nucleoside triphosphate (NTP), must also be added for most types of nuclear import to occur. While GTP alone appears to be required, the authors have included both ATP and GTP in this protocol. This is a common practice within the field when energy requirements are not a specific focus of the experiment. NTP can be removed from the import assay by addition of apyrase in place of ATP and GTP. The absence of NTP in the reaction mixture results in docking of the substrate at the NPC, yielding a characteristic rim pattern (Fig. 11.7.2B). Many kinds of nuclear import can also be inhibited by the addition of wheat germ agglutinin (WGA; Finlay et al., 1987; Fig. 11.7.2C), which binds to certain glycosylated NPC proteins (Hanover et al., 1987). This demonstrates that transport occurs through the NPC and is not the result of nuclear envelope damage.

There are a number of variations on the basic nuclear import assay. Several cell types have been used, but to date no significant differences have been found in import mechanics (see for instance Adam et al., 1990; Moore and Blobel, 1992; Adam and Adam, 1994). Cells in suspension can also be permeabilized, divided into aliquots, and frozen for future use (Leno et al., 1992; Görlich et al., 1994). The optimal digitonin concentration required for permeabilizing can, however, differ between cell types. At the ideal concentration, treatment of cells with digitonin results in permeabilization of the plasma membranes in the vast majority of cells (>95%), and nuclear envelope damage in very few cells (<5%). The integrity of the plasma membrane can be tested with DAPI, which is membrane impermeable and strongly stains DNA only in cells in which the plasma membrane has been permeabilized. The DAPI molecule is below the diffusion size limit of the NPC, and can therefore diffuse through the NPC into nuclei with intact nuclear envelopes. The integrity of the nuclear envelope can be tested with antibodies against nuclear components—i.e., lamina proteins or DNA itself (Adam et al., 1990). These antibodies cannot diffuse through the NPC, and will therefore only yield nuclear signal when the nuclear envelope has been damaged. Both the plasma membrane and the nuclear envelope are permeabilized with 0.1% Triton X-100.

There are also several variations on the duration of the nuclear import reaction and the temperature at which the reaction is incubated, extending up to 1 hr at 37°C. These longer

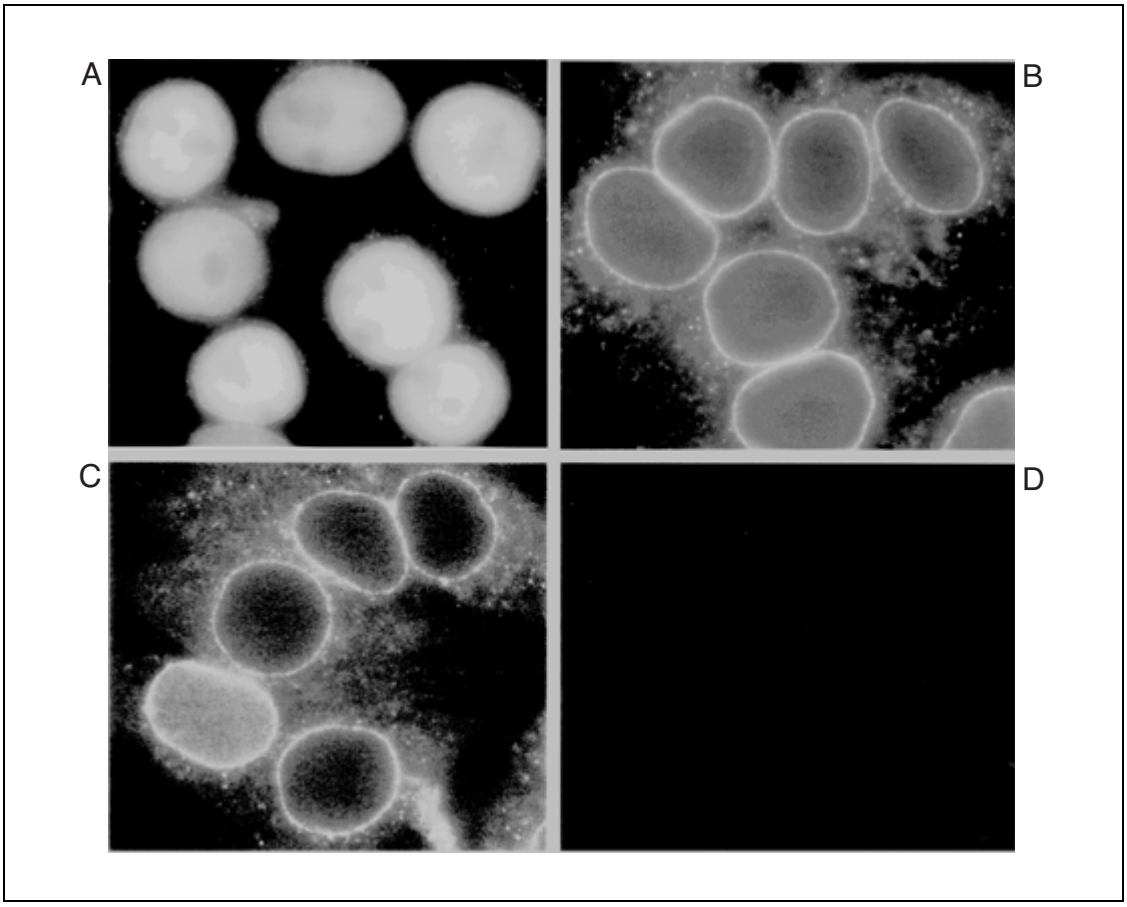


Figure 11.7.2 Fluorescence micrographs of the nuclear import assay performed under various conditions. All panels show representative nuclei from HeLa cells photographed and printed identically. (**A**) This positive control import reaction contained 10 mg/ml *Xenopus* cytosol, 0.5 mM GTP (0.5 mM), 1.6 mM ATP, and 50 μg/ml GFP-GST-NLS import substrate in TB, and was incubated for 15 min at room temperature prior to washing and fixation. (**B**) Same as (A) but GTP and ATP were replaced with 50 U/ml apyrase (50 U/ml), which at this concentration hydrolyzes any NTP. Note strong "rim" staining, reflecting the presence of substrate docked at the nuclear envelope. The punctate cytoplasmic signal may represent annulate lamellae (Davis and Blobel, 1986). (**C**) Same as (A), but in the presence of WGA (500 μg/ml), which binds to a subset of glycosylated NPC proteins and prevents import of NLS substrates. Note that the rim staining is still present but diminished. (**D**) Same as (A), but GFP-GST-NLS was replaced with GFP-GST-mutNLS. The mutant is neither imported into the nucleus nor bound to the nuclear envelope.

incubations should be performed in a humidified chamber to prevent the reaction mixture from evaporating. Long incubations may also require addition of an energy-regenerating system to maintain high NTP levels for the duration of the reaction. This regenerating system generally consists of 5 mM phosphocreatine and 20 U/ml creatine phosphokinase (final concentrations). The protocol given here is of relatively short duration by comparison, and addition of regenerating systems is not required.

Included is a support protocol describing the production of *Xenopus* ovarian cytosol (Moore and Blobel, 1992). While not as widely used as some other cytosols, it is simple and relatively inexpensive to produce. Alternatively, HeLa cytosol or reticulocyte lysate can either be produced (Adam, 1998) or be purchased from the NIH. Many people also use *Xenopus* egg extract produced from ovulated eggs (Görlich et al., 1994). Note that in ovulated eggs the nuclear envelope is broken down and the NPC is dissembled, so the constituents of this extract differ from those in ovarian cytosol.

Four proteins have been purified from cytosol on the basis of their ability to restore import in permeabilized cells. Karyopherin α/importin α (Adam and Gerace, 1991) binds to the NLS on nuclear proteins, and karyopherin β/importin β (Adam and Adam, 1994) in turn binds to the karyopherin α-NLS protein heterodimer and docks the complex at the NPC,

probably through karyopherin β interactions with a subclass of NPC proteins (Radu et al., 1995). Ran, a small GTPase (Moore and Blobel, 1993), and p10/NTF2 (Moore and Blobel, 1994), another protein known to interact with NPC proteins, are required for translocation of the NLS protein through the NPC and into the nuclear interior. Karyopherins α and β are sensitive to oxidation (Adam and Gerace, 1991; Adam and Adam, 1994), and the continuous presence of DTT in cytosol purification and during the import assay helps assure that these components are active (glutathione performs this role in vivo). Ran is in the GDP-bound form in the cytosol, but GTP is required for import, and it appears that Ran must be converted to the GTP-bound state to release substrate into the nuclear interior. Formation of stable nucleotide-Ran complexes requires Mg^{2+} (Bischoff and Ponstingl, 1991) which is present throughout the procedure. Potassium acetate is the preferred salt, as the buffer is designed to mimic the cellular interior, which is high in potassium. The total salt concentration is ~150 mM, similar to the situation in vivo.

The second common objective in nuclear import studies is to identify the NLS(s) within a given protein responsible for its localization. There are three cautions with this analysis (reviewed by Jans and Hübner, 1996). First, a protein may contain more than one NLS. Second, NLS activity may require a modification of the protein (e.g., phosphorylation/dephosphorylation or ligand binding) and that modification may not occur in the in vitro system. Along the same line, some proteins have both NLSs and NESs (nuclear export signals), and the steady-state localization of that protein can be a dynamic balance between these activities. The dynamics of these NLS and NES activities can also be modulated by secondary modifications. Third, some proteins are "piggy-backed" into the nucleus by binding to proteins that contain an NLS, so a protein above the diffusion limit may be exclusively nuclear and yet have no intrinsic NLS at all.

An active NLS is most accurately identified by a two-pronged approach (e.g., Kalderon et al., 1984a,b; Lanford and Butel, 1984). First, the protein is scanned for regions containing NLS activity. Cleavage products of the cDNA are joined to a reporter (e.g., GFP-GST), and the resultant fusion proteins are tested for import activity in nuclear import assays. Regions of the protein that confer nuclear localization on the reporter can be examined in more detail to specify the exact peptide sequence required for nuclear localization. There are no exact consensus sequences for NLSs, but there are some common themes that may be useful in their identification (Jans and Hübner, 1996). As mentioned above, this analysis may reveal more than one NLS. However, this analysis can also reveal NLSs that are not actually active in the native protein. There are portions of some proteins that act as NLSs in isolation, but which are not topologically exposed in the full-length protein and are, therefore, not active in vivo. For this reason, the putative NLS should also be mutated/deleted in the full-length protein to verify that inactivation of that putative NLS actually affects the localization of the protein.

This protocol is specifically designed to demonstrate nuclear import of test proteins and identify NLS(s) present in those proteins. Assays examining the intricacies of the nuclear import process itself require more specialized reagents, but are theoretically similar. In these studies, it is necessary to use more defined import reaction mixtures. This is most commonly achieved by depleting cytosol of certain import factors in order to determine their function, or, as is becoming more common, reconstituting nuclear import de novo using a complement of bacterially produced recombinant proteins.

Critical Parameters

The control import substrates are necessary to determine that the import assay is active. Typically, positive control substrates contain either an NLS like that of the SV40 T antigen (**PKKKRKV;** Kalderon et al., 1984a), or a bipartite NLS like that of nucleoplasmin (**KR**PAATKKAGQA**KKKK**L; Dingwall et al., 1988) in which two separate regions (shown here in bold) are required for activity. A longer peptide encoding the SV40 T antigen NLS is often used for coupling purposes (CYTP**PKKKRKV**). In addition to the NLS, the cysteine is required for the reaction with sulfo-SMCC cross-linker. The tyrosine residue allows for monitoring the peptide by A_{280} during chromatography to ensure that excess peptide is separated from the import substrate after cross-linking. It also allows the peptide to be iodinated if so desired. There are two common mutant NLS sequences used in negative controls. One contains a critical lysine mutation that abolishes NLS activity (PKTKRKV; Lanford and Butel, 1984). The second is simply the reverse amino acid sequence of the wild-type NLS, which also has no NLS activity (Adam et al., 1989). This reverse NLS may be a better

negative control because the charge of the peptide is conserved; both the wild-type and the mutant are highly basic. This high degree of positive charge confers high electrostatic binding properties, in particular a tendency to nonspecifically bind to DNA and proteins. Thus, the reverse sequence retains these nonspecific properties, unrelated to import activity, which may skew experimental results. These negative controls show neither rim staining (because they do not bind karyopherin α) nor nuclear import (Fig. 11.7.2D).

It is easiest to produce recombinant import substrates, such as GFP-GST-NLS, that encode both the fluorescent and targeting characteristics. The inclusion of GFP obviates the need to chemically attach fluorophores to the protein in order to detect nuclear import. The NLS directs the recombinant protein to the nucleus. The GST moiety serves a dual role. First, commercially available affinity resins greatly simplify the purification of this protein directly from bacterial lysates. Second, GST forms dimers under physiological conditions (reviewed in Mannervik and Danielson, 1988), raising the molecular weight of the import substrate to 106 kDa (2 × 53 kDa, the monomeric molecular weight of GST-GFP). This is well above the diffusion limit of the nuclear pore complex.

Alternatively, NLS peptides and fluorophores can be attached to BSA import substrate by chemical conjugation (Goldfarb et al., 1986). This method is a little more difficult. Both the sulfo-SMCC (which subsequently attaches to the peptide) and the TRITC react with lysines on the BSA, so it is important not to saturate the lysines with TRITC during the first reaction. Doing so will prevent subsequent binding of the sulfo-SMCC, and therefore prevent conjugation of the NLS (or mutant NLS) peptides. The terminal cysteine in the synthetic peptide is required for conjugation, because this residue reacts with the sulfo-SMCC on the BSA.

Many laboratories simplify this conjugation procedure by directly labeling nucleoplasmin (Newmeyer et al., 1986), which already encodes an NLS. In this manner a second reaction to attach NLS peptide is not required. The nucleoplasmin NLS is very protease sensitive, so negative control import substrate (lacking an NLS) can be produced by protease cleavage of the NLS from the nucleoplasmin, which is then referred to as nucleoplasmin core (Dingwall et al., 1982). In the authors' experience the nucleoplasmin-based import substrates have displayed higher background in the import assay, but this can be overcome by the addition of high concentrations of nucleoplasmin core as a blocking agent (Görlich et al., 1994).

The experimental protein must also, of course, be detectable to measure import, either by using an antibody against the protein or by fluorescently labeling the protein directly. Both of these approaches have potential drawbacks. TRITC reacts with lysines, which are often critical amino acids within the NLS. Thus, it is important not to utilize a high molar excess of TRITC, lest an endogenous NLS be inactivated. Indirect immunofluorescence microscopy avoids this detection problem, but the additional steps required to perform immunofluorescence microscopy after the import assay can have detrimental effects on cell morphology. Care must be taken to treat the cells gently, particularly when adding solutions to the coverslips.

If the protein has been cloned, it can be produced as a recombinant attached to GFP-GST and detected directly. Alternatively, a recombinant protein can be produced with an antigen site attached (such as FLAG, 6×His, or HA) and detected by indirect immunofluorescence microscopy. The nuclear pore complex can allow diffusion of substrates <50,000 Da (Bonner, 1975; Paine et al., 1975), so the molecular weight of the experimental substrate must be greater than that limit. If not, the possible nuclear accumulation demonstrated in the import assay could be the result of diffusion of the substrate through the nuclear pores and subsequent binding to sites within the nuclear compartment. This would not be considered active nuclear import. Note that GST forms a dimer under physiological salt conditions, so the GST-GFP-NLS positive control, with a monomeric molecular weight of 53 kDa, is actually a dimer of 106 kDa, well above the diffusion limit of the NPC.

Troubleshooting

The import assay itself is quite forgiving. It is important to titer the import substrate and cytosol relative to one another for every new batch. Incorrect concentrations (either too high or too low) will decrease the resulting signal. Poor morphology is generally due to rough handling of the cells. Be careful with all manipulations involving the coverslips, especially when adding solutions. Always pipet down the side of the well and not directly onto the cells. If no cells are present, check that the fixative was made according to the directions. Addition

of excess NaOH to the paraformaldehyde causes cells to detach from the coverslip.

Likewise, it is unusual for a batch of *Xenopus* cytosol to lack activity. Low activity generally correlates to long preparation time or extended storage. The entire prep should take ~1.5 days, including dialysis, concentration, and freezing. Be sure that DTT is added fresh to solutions where indicated to maintain the karyopherins α and β in their active forms, and likewise that Mg^{2+} is present to maintain Ran activity.

TRITC-NLS-BSA is a little more difficult to make. The resulting import substrate stock, at a few milligrams per milliliter, should appear bright red. At the recommended ratio of fluorophore to BSA (~1.5:1) most of the TRITC should conjugate to the BSA. This should be obvious during the subsequent chromatography. If the first peak (BSA-TRITC) is darker than the second peak (TRITC), there is enough label on the BSA. Monitor each step by SDS-PAGE to verify that peptide is actually conjugated. There should be a noticeable molecular weight increase with the addition of the sulfo-SMCC, and a further increase with the addition of peptide. TRITC-BSA or TRITC-BSA-NLS can also be visualized in the SDS-PAGE gel, prior to fixation and staining of the gel, by exposing the gel to UV light. Too many NLS peptides conjugated to the BSA may result in relatively bright rim staining and relatively low levels of import showing strong nucleolar staining. Too few NLS peptides may decrease the rate of import.

GFP-GST-NLS production and purification is relatively straightforward. The NLS may be susceptible to proteolysis, so maintain the protein at 4°C throughout the procedure (except for the elution).

Anticipated Results

The anticipated yield for each of the support protocols is ~350 mg *Xenopus* ovarian cytosol (see Support Protocol 1), 8 mg TRITC-BSA-NLS (see Support Protocol 2), and 9 mg GST-GFP-NLS (see Support Protocol 3). After optimization of these components, assays using 36 coverslips can be performed, viewed and photographed in one day. It is recommended that all experimental samples be performed in duplicate, with negative controls (WGA, apyrase, and/or mutant NLS) and NLS positive control performed on each experiment. This leaves room for testing ~14 experimental samples (in duplicate).

Time Considerations

A typical import assay requires 2.5 hr to perform, with an additional 3 hr to record the results photographically. It should take ~1.5 days to prepare the *Xenopus* ovarian extract. Production of the TRITC-BSA-NLS requires 2.5 days and GFP-GST-NLS 3 days.

Literature Cited

Adam, S.A. 1998. Permeabilized cell assay for nuclear protein import. *In* Cells, A Laboratory Manual, Vol. 1: Culture and Biochemical Analysis of Cells (D.L. Spector, R.D. Goldman, and L.A. Leinwand, eds.) pp. 45.1-45.8. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Adam, E.J.H. and Adam, S.A. 1994. Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.* 125:547-555.

Adam, S.A. and Gerace, L. 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell* 66:837-847.

Adam, S.A., Lobl, T.J., Mitchell, M.A., and Gerace, L. 1989. Identification of specific binding proteins for a nuclear location sequence. *Nature* 337:276-279.

Adam, S.A., Sterne-Marr, R., and Gerace, L. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807-816.

Bischoff, F.R. and Ponstingl, H. 1991. Mitotic regulator protein RCC1 is complexed with a nuclear *ras*-related polypeptide. *Proc. Natl. Acad. Sci. U.S.A.* 88:10830-10834.

Bonner, W.M. 1975. Protein migration into nuclei. I. Frog nuclei in vivo accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. *J. Cell Biol.* 64:421-430.

Colbeau, A., Nachbaur, J., and Vignais, P.M. 1971. Enzymatic characterization and lipid composition of rat liver subcellular membranes. *Biochim. Biophys. Acta*. 249:462-492.

Davis, L.I. and Blobel, G. 1986. Identification and characterization of a nuclear pore complex protein. *Cell* 45:699-709.

Dingwall, C., Sharnick, S.V., and Laskey, R.A. 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* 30:449-458.

Dingwall, C., Robbins, J., Dilworth, S.M., Roberts, B., and Richardson, W.D. 1988. The nucleoplasmin nuclear location sequence is larger and more complex than that of SV-40 large T antigen. *J. Cell Biol.* 107:841-849.

Finlay, D.R., Newmeyer, D.D., Price, T.N., and Forbes, D.J. 1987. Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.* 104:189-200.

Goldfarb, D.S., Gariépy, J., Schoolnik, G., and Kornberg, R.D. 1986. Synthetic peptides as nuclear localization signals. *Nature* 332:641-644.

Görlich, D., Prehn, S., Laskey, R.A., and Hartmann, E. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* 79:767-776.

Hanover, J.A., Cohen, C.K., Willingham, M.C., and Park, M.K. 1987. *O*-linked *N*-acetylglucosamine is attached to proteins of the nuclear pore. *J. Biol. Chem.* 262:9887-9894.

Jans, D.A. and Hübner, S. 1996. Regulation of protein transport to the nucleus: Central role of phosphorylation. *Physiol. Rev.* 76:651-685.

Kalderon, D., Richardson, W.D., Markham, A.F., and Smith, A.E. 1984a. Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* 311:33-38.

Kalderon, D., Roberts, B.L., Richardson, W.D., and Smith, A.E. 1984b. A short amino acid sequence able to specify nuclear location. *Cell* 39:499-509.

Lanford, R.E. and Butel, J.S. 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* 37:801-813.

Leno, G.H., Downes, C.S., and Laskey, R.A. 1992. The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in *Xenopus* egg extract. *Cell* 69:151-158.

Mannervik, B. and Danielson, U.H. 1988. Glutathione transferases—structure and catalytic activity. *CRC Crit. Rev. Biochem.* 23:283-337.

Moore, M.S. and Blobel, G. 1992. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell* 69:939-950.

Moore, M.S. and Blobel, G. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* 365:661-663.

Moore, M. S. and Blobel, G. 1994. Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 91:10212-10216.

Newmeyer, D.D., Finlay, D.R., and Forbes, D.J. 1986. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* 103:2091-2102.

Paine, P.L., Moore, L.C., and Horowitz, S.B. 1975. Nuclear envelope permeability. *Nature* 254:109-114.

Radu, A., Blobel, G., and Moore, M.S. 1995. Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. U.S.A.* 92:1769-1773.

Contributed by Mary Shannon Moore and Eric D. Schwoebel
Baylor College of Medicine
Houston, Texas

In Vitro Translation Using HeLa Extract

This unit describes the production and use of HeLa extract as a cell-free translation system. HeLa extract provides a useful alternative to rabbit reticulocyte lysate (RRL), the most widely used eukaryotic translation system (*UNIT 11.2*). HeLa extract has advantages over RRL in that it can translate mRNAs that cannot be translated in RRL, is more cap-dependent, has a higher capacity for faithful initiation at the correct start codon, and is likely more representative of translation in intact human cells. HeLa extract has been used for routine translation of mRNAs to characterize viral infections, for de novo synthesis of viruses, and for high-throughput drug screening. The HeLa cell-free translation system described here has applications in protein production, biotechnology, and for answering fundamental questions in biochemistry.

The following protocol describes the preparation of HeLa extract and its use in in vitro translation reactions (see Basic Protocol). The Basic Protocol is based on methods published over the past 25 years, but contains modifications that simplify the procedure, save time, reduce cost, and most importantly, provide highly reproducible translation signals for independent replicates. There is also a protocol for producing and purifying mRNA (see Support Protocol).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

PRODUCTION AND USE OF mRNA-DEPENDENT CELL-FREE TRANSLATION SYSTEM FROM HeLa EXTRACT

BASIC PROTOCOL

HeLa extract is produced by collecting HeLa cells grown in suspension, lysing the cells, removing cell debris, nuclei, and mitochondria, removing an inhibitory lipid layer, and exchanging the buffer by dialysis. Translation reactions are performed by adding an energy regeneration system, tRNAs, amino acids, and salts to the HeLa extract, Dounce homogenizing the mixture, removing the inhibitory lipid layer, adding purified mRNA, and incubating at 30°C for 3 hr. Translation signals are improved by removal of the lipid layer and treatment of the extract with calcium-dependent micrococcal nuclease to remove endogenous mRNAs. Dounce homogenizing the translation reaction prior to addition of mRNA provides a more homogenous mixture and greatly improves the reproducibility of the translation signal.

Materials

HeLa S3 cells (ATCC #CCL 2.2)
Joklik's modified Eagle medium for suspension cultures (SMEM)
Heat-inactivated iron-supplemented calf serum (Sigma)
200 mM L-glutamine
10,000 U/ml penicillin
10,000 µg/ml streptomycin
Hypotonic lysis buffer (see recipe)
Dialysis buffer (see recipe)
Diethylpyrocarbonate-treated H_2O (DEPCW; *APPENDIX 2A*)
1× phosphate-buffered saline (PBS; *APPENDIX 2A*)
0.4% (w/v) trypan blue stain
Bradford Reagent (Bio-Rad)

Contributed by Gary Witherell
Current Protocols in Cell Biology (2000) 11.8.1-11.8.10

1 mg/ml bovine serum albumin (BSA)
10× translation mix (see recipe)
0.1 M $CaCl_2$
1 mg/ml micrococcal nuclease (Pharmacia or Fluka)
0.2 M ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA), pH 7.5
1 M and 2 M stocks of potassium acetate (KOAc), pH 7.5
10 mM magnesium acetate [$Mg(OAc)_2$]
1 mM amino acid mix minus methionine (Promega)
1 mm methionine or ^{35}S-labeled methionine (15 mCi/ml; >1000 Ci/mmole; e.g., NEN Life Sciences or Amersham)
150 nM purified mRNA (capped or uncapped; see Support Protocol)

0.5, 1, and 6-liter glass spinner flasks (autoclaved)
40-ml Wheaton Dounce homogenizer type A
8-ml collodion dialysis bags, MWCO 12 kDa (Sartorius)
50-ml plastic conical tubes
30-ml glass centrifuge tubes, one of known weight
500-ml or 1-liter centrifuge bottles
Preparative centrifuge and rotor (e.g., Beckman J6-HC centrifuge and JS-4.2 rotor or equivalent)
High-speed centrifuge and rotor (e.g., Beckman J2-21 centrifuge and JA-20 rotor or equivalent)
2-ml screw-cap cryogenic vials with O-ring seal
1-ml Wheaton Dounce homogenizer
96-well microtiter plates

Additional reagents and equipment for assessing cell disruption by trypan blue dye exclusion (*UNIT 1.1*) and measurement of [^{35}S]methionine incorporation (*UNIT 11.2*)

NOTE: All solutions and equipment coming in contact with the cells or extract during the preparation must be sterile and at 4°C and aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Grow HeLa cells

1. Grow 6 liters of HeLa S3 cells in suspension culture at 37°C with 5% CO_2 in SMEM supplemented with 10% (v/v) iron-supplemented calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Strictly maintain cell density between 2×10^5 and 8×10^5 cells/ml by passaging every other day. Transfer cells into larger spinner flasks as needed. Start initial 0.4-liter cultures with 2×10^7 cells from a frozen stock.

2. The day before preparation of the extract, prepare hypotonic lysis buffer and dialysis buffer. Place buffers and any apparatus that will come into contact with cells or extract at 4°C. Autoclave 40-ml Dounce homogenizer and place at 4°C.

3. Place two collodion bags in separate 50-ml conical tubes and wash with DEPCW twice to remove residual ethanol. Fill tubes to the top with DEPCW and store at 4°C until ready for use.

4. Weigh one 30-ml glass centrifuge tube and place at 4°C.

   The weight of the tube will be used to calculate the weight of the cell pellet.

5. Determine the cell density of the culture, and if necessary, dilute with fresh medium to give the desired density (4 to 5×10^5 cells/ml) the next day.

Collect cells for HeLa extract

6. Obtain 6 liters of HeLa S3 cells in log-phase (4 to 5×10^5 cells/ml). Centrifuge cells 10 min at $2800 \times g$.
7. Wash cells three times with ice-cold 1× PBS: 50 ml for first wash and 25 ml for second and third washes. After each wash, pellet the cells by centrifuging 8 min at $640 \times g$. Use a pre-weighed 30-ml glass centrifuge tube for the third centrifugation. Remove buffer and weigh tube and pellet. Calculate cell volume assuming a density of 1 mg/ml.

   The volume of the cell pellet should be ~10 ml.
8. Resuspend the cell pellet in 1.5 times its volume of hypotonic lysis buffer (~15 ml) and swell on ice for 10 min.
9. Homogenize cells with ~110 strokes of a 40-ml Wheaton Dounce homogenizer type A (tight). Check cell disruption visually or by dye exclusion using trypan blue (*UNIT 1.1*) after 40, 60, 70, 80, and 100 strokes.

   The goal is to lyse >95% of the cells.
10. Transfer extract to 30-ml glass centrifuge tube. Remove cell debris and nuclei by centrifuging 5 min at $640 \times g$ for 5 min and then increasing the speed to $10{,}400 \times g$ for an additional 20 min to remove mitochondria.

    The supernatant should be cloudy.
11. Carefully remove the upper white lipid layer if it is present.

    This lipid material inhibits translation.

Prepare extract

12. Dialyze the extract for 2 to 3 hr in two collodion dialysis bags (8 ml, MWCO 12 kDa) against 1 liter of dialysis buffer to clean and replace buffer.
13. Transfer extract into a 30-ml glass centrifuge tube and centrifuge 10 min at $12{,}100 \times g$. Carefully remove the upper white layer.
14. Remove 2 µl of extract and determine the protein concentration using the Bradford reagent (*APPENDIX 3B*). Use BSA to create a standard curve.

    Typical extracts have protein concentrations of 20 to 30 mg/ml.
15. Transfer 380-µl aliquots of extract to 2-ml screw-cap cryogenic tubes with O-rings and freeze and store in liquid nitrogen.

Prepare for translation reactions

16. Prepare translation mix, aliquot, and store at −80°C.
17. Autoclave 1-ml Dounce homogenizer and place at 4°C overnight.

Prepare extract for translation

18. Partially thaw vial containing 380 µl of HeLa extract in hand and place on ice.
19. (*Optional*) Treat extract with calcium-dependent micrococcal nuclease. Add 10 µl 0.1 M $CaCl_2$ and 2 µl of 1 mg/ml micrococcal nuclease to 380 µl of extract. Mix by pipeting gently up and down. Incubate 15 min at 25°C.

    Optimal micrococcal nuclease concentration may vary with different suppliers and different lots (see Critical Parameters).
20. Place extract on ice and add 15 µl of 0.2 M EGTA to chelate Ca^{2+} and inactivate the nuclease.

21. If micrococcal nuclease treatment is not performed, add 10 µl of 0.1 M $CaCl_2$, 15 µl of 0.2 M EGTA, and 2 µl of DEPCW in place of nuclease.

Perform in vitro translation

22. Add 70 µl of translation mix. For internal ribosome entry site (IRES)-dependent translation add 30 µl of 1 M KOAc (pH 7.5) and 60 µl of 10 mM $Mg(OAc)_2$. For non-IRES-dependent translation add 47 µl of 2 M KOAc (pH 7.5), 30 µl of 10 mM $Mg(OAc)_2$, and 13 µl of DEPCW.

    Optimal KOAc and $Mg(OAc)_2$ concentrations may vary for different mRNAs.

23. For non-radioactive assays add 7 µl of 1 mM methionine and 7 µl of DEPCW. For radioactive assays add 14 µl (15 mCi/µl) of [^{35}S]methionine.

24. Transfer extract to a 1-ml Wheaton Dounce homogenizer and homogenize with 15 strokes at 4°C.

25. Transfer the translation extract to a 1.5-ml microcentrifuge tube and microcentrifuge 10 min at 10,000 × *g* (e.g., 11,000 rpm in an Eppendorf model 5415C centrifuge), 4°C. Remove the upper white lipid layer.

26. Transfer extract to a clean 1.5-ml microcentrifuge tube and microcentrifuge again 10 min at 10,000 × *g* and remove the upper white lipid layer.

27. Transfer 22 µl of extract to a new 1.5-ml microcentrifuge tube or microtiter plate containing 3 µl of 150 nM mRNA and 3 µl of DEPCW. Incubate at 30°C for 3 hr.

    Optimal mRNA concentrations may vary for different mRNAs. DEPCW may be replaced with test compound.

    Microtiter plates should be covered to avoid evaporation.

28. Assay translation efficiency by reporter activity or [^{35}S]methionine incorporation (*UNIT 11.2*).

SUPPORT PROTOCOL

PRODUCTION AND PURIFICATION OF mRNA

mRNAs can be produced or purified from cells by a variety of methods. Purification of the mRNA results in higher translation signals and increased reproducibility for independent replicates. mRNA is purified by phenol/choloroform extraction, isopropanol precipitation, and desalting using a Microspin column. The integrity of the mRNA should be verified by agarose gel-electrophoresis prior to use.

Materials

Template: plasmid, synthetic, or PCR-amplified DNA
RNase-free DNase (optional)
0.5 M ammonium acetate
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (IAA)
24:1 (v/v) chloroform/IAA
Isopropanol
Diethylpyrocarbonate-treated H_2O (DEPCW; *APPENDIX 2A*)
10 mg/ml ethidium bromide or Stains-All (Kodak)
Microspin S-400 columns (Pharmacia)

Additional reagents and equipment for production of capped and uncapped mRNAs (*UNIT 11.2*) agarose gel-electrophoresis and spectrophotometric determination of RNA and DNA concentrations (*APPENDIX 3*)

1. Produce preparative amounts of both capped and uncapped mRNAs by transcription from plasmids, synthetic DNA templates, or PCR templates using bacteriophage RNA polymerases (*UNIT 11.2*).

   Large-scale RNA production systems using bacteriophage RNA polymerases are available from Promega and Ambion. mRNAs may also be isolated from cells (UNIT 11.2) or chemically synthesized to a limited degree (Stiege and Erdmann, 1995).

2. (*Optional*) Remove DNA template by treatment with RNase-free DNase (*UNIT 11.2*).

   The presence of template DNA does not affect the translation efficiency or mRNA quantitation.

3. Transfer mRNA to 1.5-ml microcentrifuge tube. Add ammonium acetate to the mRNA to a final concentration of 0.5 M. Remove protein by extraction with 1 vol 25:24:1 phenol/chloroform/IAA followed by extraction with 1 vol 24:1 chloroform/IAA.

4. Precipitate mRNA by adding an equal volume of isopropanol, incubating 15 min at –20°C, and microcentrifuging 15 min at 16,000 × *g* (e.g., 14,000 rpm in an Eppendorf model 5415C centrifuge), 4°C.

5. Remove as much supernatant from the pellet as possible but do not dry the pellet. Resuspend the pellet in 100 µl of DEPCW.

6. Remove salts and nucleotides with a Microspin S-400 column. Resuspend resin by vortexing and pre-spinning column 1 min at 735 × *g* (e.g., 3,000 rpm in an Eppendorf model 5415C centrifuge) to remove storage buffer. Place column in a clean 1.5-ml microcentrifuge tube and load 100 µl of mRNA sample to the top of the resin. Centrifuge 2 min at 735 × *g* to recover mRNA.

7. Quantitate mRNA concentration by absorbance at 260 nm (*APPENDIX 3*) using an extinction coefficient of 25 ml/mg-cm.

   The absorbance of template DNA is typically insignificant compared to the absorbance of mRNA.

8. Resuspend to 150 nM in DEPCW. Confirm integrity of mRNA by agarose gel-electrophoresis (*APPENDIX 3*) and staining with ethidium bromide or Stains-All. Store mRNA up to 1 month at –20°C.

REAGENTS AND SOLUTIONS

*Use DEPC-treated deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Dialysis buffer

10 mM HEPES, pH 7.6, from a 1 M stock (see recipe)
90 mM potassium acetate (KOAc)
1.5 mM magnesium acetate [$Mg(OAc)_2$]
Adjust to pH 7.2 with KOH
Filter sterilize
Store overnight at 4°C
Add 1 mM DTT just before use

1 M HEPES, pH 7.6

Add 11.2 g HEPES to 38 ml DEPCW. Adjust solution to pH 7.6 with KOH and final volume to 50 ml with DEPCW.

Hypotonic lysis buffer

10 mM HEPES buffer, pH 7.6, from a 1 M stock (see recipe)
10 mM potassium acetate (KOAc)
1.5 mM magnesium acetate [$Mg(OAc)_2$]
Adjust to pH 7.6 with KOH
Filter sterilize
Store overnight at 4°C
Add 2 mM DTT just before use

Translation mix, 10×

152 mM HEPES, pH 7.6, from a 1 M stock (see recipe)
80 mM creatine phosphate (Boehringer Mannheim), from a 1 M stock
200 µg/ml creatine phosphokinase (Boehringer Mannheim), from a 10 mg/ml stock
8 mM ATP dipotassium salt
480 µM GTP disodium salt
200 µg/ml calf liver tRNA, from a 10 mg/ml stock
100 µM amino acid mix minus methionine, from a 1 mM stock (Promega)
2 mM spermidine
16 mM DTT
diethylpyrocarbonate-treated H_2O (DEPCW; *APPENDIX 2A*)
Aliquot and store for up to 1 year at −80°C

Creatine phosphate and creatine phosphokinase stocks are prepared in DEPCW and stored at −20°C for several months.

Calf liver tRNA is purified by 25:24:1 phenol/chloroform/IAA extraction, 24:1 chloroform/IAA extraction, ethanol precipitation using 0.3 M sodium acetate (NaOAc). Resuspend the pellet to 10 mg/ml in DEPCW and store for up to 1 year at −20°C.

COMMENTARY

Background Information

A variety of mammalian, yeast, plant, and bacterial cell-free extracts have been used to assay translation in vitro (*UNIT 11.1*; Villa-Komaroff et al., 1974; Iizuka and Sarnow, 1997; Jermutus et al., 1998). Cell-free extracts contain ribosomes, translation factors, aminoacyl tRNA synthetases, and other macromolecular components required for translation. Extracts are generally supplemented with amino acids, tRNAs, energy sources, salts, and energy regeneration systems. RRL and HeLa extract are the most commonly used mammalian translation systems. RRL has advantages in that it is simple to use, will translate both capped and uncapped mRNAs, and is commercially available in large amounts. The HeLa cell-free translation system has advantages in that it is more cap-dependent, will translate mRNAs that do not translate well in RRL, and is more likely to represent translation in intact human cells.

HeLa extract has been prepared and used for in vitro translation reactions for over 25 years, using a large variety of protocols (Villa-Komaroff et al., 1974; Celma and Ehrenfeld, 1975; Rose et al., 1978; Lee and Sonenberg, 1982; Molla et al., 1991; Carroll and Lucas-Lenard, 1993). The protocols used to prepare HeLa extract are similar in that they involve growing, collecting, and lysing cells, removing the cell debris, nuclei, and mitochondria, and storing the extract. The protocols differ in the type of serum used to grow the cells (horse, fetal bovine, calf), the cell lysis method (Dounce homogenization or detergent), lysis buffer composition and amount used, and the method used to exchange the buffer (dialysis, column chromatography, or none). In some protocols additional steps (freeze/thaw), treatments (micrococcal nuclease), or reagents (translation mix, buffer, or glycerol) are added before storage. In some cases these protocols fail to consistently produce useable extract, and the translation signals from in vitro translation reactions may vary 2- to 10-fold for independent replicates (Carroll and Lucas-Lenard, 1993; G. Witherell,

unpub. observ.). The method described here is a simplified protocol that saves time, lowers cost, and produces large amounts of HeLa extract. The quality of the HeLa extract produced is consistent from one preparation to another and the translation signals are highly reproducible.

The availability of a highly reproducible HeLa cell-free translation system has many applications in basic research, protein production, and biotechnology. Many of the applications performed with RRL and wheat germ extract (*UNIT 11.1*) have been or could be performed with HeLa extract. These applications include analysis and characterization of molecular interactions, functions, and structure of synthesized proteins. When studying biological processes, HeLa extract provides a translation system that is more cap-dependent, has a higher capacity for faithful initiation at the correct start codon, and is likely to be more predictive of results in intact human cells. HeLa extract has been used to translate mRNAs on a routine basis and is particularly useful for translation of mRNAs that do not translate well in RRL.

Human rhinovirus and enterovirus mRNAs are examples of mRNAs that translate poorly in RRL, producing low yields and a high degree of aberrant translation initiation (Belsham and Sonenberg, 1996; Hunt et al., 1999). Translation initiation of human rhinovirus and enterovirus mRNAs does not occur by a conventional 5′ cap-dependent translation mechanism. Cap-dependent translation, used by the vast majority of cellular mRNAs in eukaryotes, involves the interaction between components of the cell's translation machinery and the m^7G capped 5′ end of the mRNA (Dever, 1999). The m^7G cap, found on all nonorganellar mammalian mRNAs, is added post-transcriptionally. Translation initiation begins with the association of the 43S initiation complex (consisting of the 40S ribosomal subunit, charged initiator tRNA, and several initiation factors) with the m^7G cap structure. The "scanning model" describes the two-dimensional movement of the 43S initiation complex along the RNA, from the 5′ cap to the start codon of the protein coding region (Dever, 1999). To facilitate ribosome scanning, cellular 5′ untranslated regions (5′ UTRs) are typically <100 nucleotides in length, lack extensive secondary structure, and lack non-initiating AUG start codons.

In contrast, human rhinovirus and enterovirus 5′ UTRs lack a 5′ terminal m^7G cap structure, are several hundred nucleotides long, contain a high degree of secondary structure, and contain multiple noninitiating AUG codons preceding the authentic start codon of the polyprotein (Borman and Jackson, 1992). These features preclude ribosomes from binding the 5′ end of the viral RNA and scanning to the polyprotein start codon. Rather than by a scanning mechanism, translation initiation of human rhinovirus and enterovirus mRNAs occurs by a mechanism in which the 43S initiation complex locates the AUG start codon of the protein coding region by binding directly to a region of the viral 5′ UTR termed the internal ribosomal entry site, or IRES (Borman and Jackson, 1992). Human rhinovirus and enterovirus mRNAs can be translated under conditions in which cellular translation is inhibited. In fact, human rhinoviruses and enteroviruses encode a proteinase that is involved in the irreversible inactivation of 5′ end-dependent translation, resulting in shut-off of host cell translation. IRES-dependent translation in these viruses has been shown to require HeLa specific factors that stimulate translation and correct aberrant initiation (Svitkin et al., 1996; Hunt et al., 1999). HeLa extract supports all steps of enterovirus replication including translation, protein processing, RNA replication, and viral assembly and can therefore be used for de novo synthesis of infectious virus (Molla et al., 1991).

The high reproducibility of the HeLa cell-free translation system makes it suitable for high-throughput drug screening. RiboGene has used HeLa extract to identify defined chemical entities, natural product extracts, antisense oligonucleotides, antisense oligoribonucleotides, and RNA decoys that inhibit human rhinovirus and coxsackievirus IRES-dependent translation (G. Witherell, unpub. observ.). RiboGene has also used mRNA translation in HeLa extract as a selectivity assay to identify compounds that specifically inhibit viral, fungal, and bacterial translation systems (G. Witherell, unpub. observ.). Application of the HeLa translation system to screen for inhibitors or stimulators of other viral or cellular idiosyncratic translation mechanisms may lead to the discovery of new drugs for human diseases.

Critical Parameters

There are several critical parameters for the successful preparation of HeLa extract. The growth of the HeLa cells must be strictly maintained between 2×10^5 and 8×10^5 cells/ml. Allowing the cells to become more concentrated or more dilute typically results in less predictable growth cycles and a less efficient extract. Healthy cells should double every 24 to 36 hr.

Cells are grown in iron-supplemented calf serum, rather than fetal bovine serum, to reduce cost. Substitution of fetal bovine serum with iron-supplemented calf serum does not affect cell health or growth of the cells. The night before the preparation is to take place the cells are counted and a calculated amount of fresh medium is added to feed the cells and ensure that they will be at the correct concentration and volume the following morning. In addition, fresh medium is added to ensure that the cells are growing at optimal rates when harvested. Monitor the cell growth carefully and harvest between 4×10^5 and 5×10^5 cells/ml. Failure to adequately lyse the cells by Dounce homogenization, or resuspending the cell pellet in more than 1.5 times the packed-cell volume with hypotonic lysis buffer, results in a low protein concentration and a less active or inactive extract. Dialyzing the extract in collodion bags is preferred over dialysis tubing because they are firm bags that can be suspended in the dialysis beaker, saving significant time and greatly increasing the recovery of extract. Removing the inhibitory white lipid layer before and after dialysis is essential. The extract appears to maintain activity longer with storage in liquid nitrogen than at −80°C. Once the extract is thawed it is best not to reuse it. Each freeze/thaw cycle reduces the activity to approximately one-half the original activity. Extract may be stored as a cocktail with translation mix and salts at −80°C, for a few days or weeks, with little loss of activity.

Obtaining reproducible signals in the in vitro translation reaction requires Dounce homogenizing the translation cocktail and removing the upper white lipid layer in two subsequent centrifuge steps before addition of mRNA. Micrococcal nuclease treatment is not necessary for IRES-dependent translation but may stimulate scanning-dependent translation, depending on the mRNA. The pH of potassium acetate must be adjusted to 7.6 to maintain the pH of the reaction. Incubation temperature is optimal at 30°C, decreasing dramatically if the temperature is increased or decreased only a few degrees. Translation signals tend to be very low during the first hour of incubation, increase linearly over the next 2 hr, plateau at 3 hr, and then decrease after 4 hr. Addition of nuclease inhibitors has little effect on the translation signal, suggesting that the extract is largely nuclease free.

The purity and quality of reagents in the HeLa translation reaction is important. Micrococcal nuclease quality varies greatly between suppliers and even between lots, with some lots unusable due to contamination with other ribonucleases. Therefore, the optimal concentration of micrococcal nuclease must be determined for each supplier and each lot of micrococcal nuclease. Dipotassium ATP is used rather than disodium ATP to avoid high concentrations of sodium that could potentially reduce the translation efficiency of the HeLa extract. However, disodium ATP may be substituted for dipotassium ATP under most translation conditions. Purification of the mRNA and removal of salts with a Microspin column significantly improves the translation signal. Calf liver tRNA must be purified before use.

Suggested translation conditions for viral IRES-dependent translation are 50% (v/v) HeLa extract, 16 nM RNA, 40 mM potassium acetate, and 0.8 mM magnesium acetate for 3 hr at 30°C. Translation conditions for scanning-dependent translation are similar except that 126 mM potassium acetate and 0.4 mM magnesium acetate are suggested. Salts are added in addition to endogenous salts present in the extract. For radioactive assays, ^{35}S-labeled methionine may be substituted with a variety of commercially available labeled amino acids (e.g., Amersham).

During extract preparation, cells, extract, and all solutions and apparatus coming in contact with cells or extract, should be kept at 4°C unless otherwise specified. Latex or polyvinyl chloride gloves should be worn when handling extract, and only DEPCW used in buffers and reactions, to avoid contamination with ribonucleases.

Troubleshooting

A poor translation signal can typically be overcome by optimizing the translation conditions. Different mRNAs may require dramatically different translation conditions. The most critical factors for optimizing translation are Mg^{2+}, K^+, and mRNA concentrations.

The absence of a translation signal is typically due to degradation of the mRNA, an impaired energy regeneration system, or low protein concentration in the extract. Sources for ribonucleases include water, micrococcal nuclease stocks, and hands. An impaired energy regeneration system can typically be overcome with a new stock of creatine phosphokinase.

Anticipated Results

The yield of final extract should be ~10 to 15 ml per 6 liters of cells. Successful extracts contain between 20 and 30 mg/ml of protein. There is a positive correlation between protein concentration and translation efficiency.

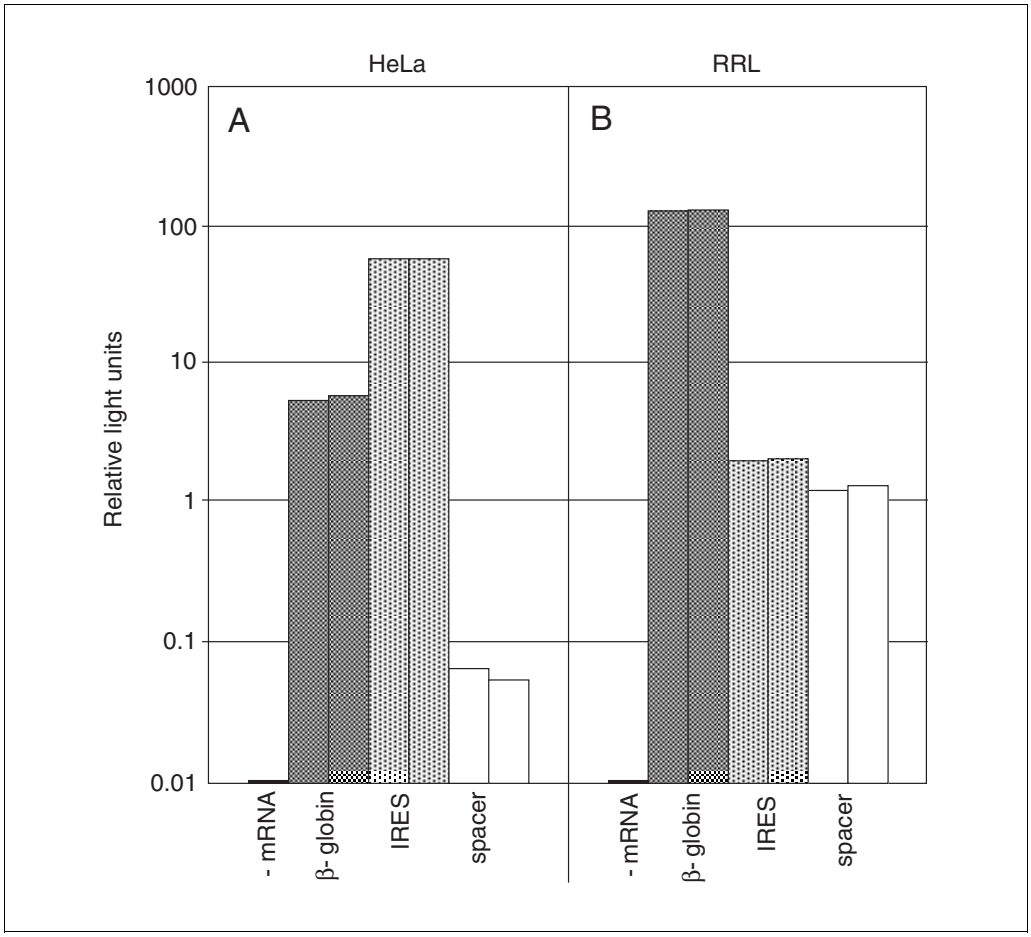


Figure 11.8.1 HeLa extract supports both viral IRES-dependent and human scanning-dependent translation. (**A**) HeLa in vitro translation reactions were performed as described in the Basic Protocol. (**B**) RRL translation reactions were performed as described previously (Witherell et al., 1995). Luciferase activity in relative light units (RLUs) was quantitated as a measure of translation. Translation reactions of luciferase driven by the β-globin 5′ UTR (β-globin), rhinovirus IRES (IRES), and a nonfunctional spacer sequence (spacer) are shown. A control reaction was performed in the absence of mRNA (–mRNA). RRL, rabbit reticulocyte lysate.

HeLa extract translates both scanning-dependent and IRES-dependent mRNAs (see Fig. 11.8.1). Translation of a monocistronic mRNA containing the human β-globin 5′ untranslated region (5′ UTR) driving translation of the luciferase reporter gene was used to represent human scanning-dependent translation. Translation of a dicistronic mRNA containing the human rhinovirus IRES (Borman and Jackson, 1992) driving translation of the luciferase reporter gene was used to represent viral IRES-dependent translation. A control dicistronic mRNA containing a non-functional spacer sequence in place of the rhinovirus IRES was used as a control mRNA. Uncapped mRNAs were prepared by in vitro transcription with T7 RNA polymerase using the MEGAscript kit (Promega) and purified as described in the Support Protocol. HeLa extract was prepared and in vitro translation reactions performed as described in the Basic Protocol. After incubation, luciferin reagent (Analytical Bioluminescence) was added and the relative light units (RLUs) quantitated using a Dynatech ML3000 luminometer. Luciferase activity was used as a direct measure of translation, and results were confirmed with a [^{35}S]-methionine incorporation assay (data not shown).

The HeLa cell-free translation system provides efficient and highly reproducible signals for both IRES- and scanning-dependent translation. Luciferase signals obtained from in vitro translation reactions were highly reproducible, with <5% variation for independent replicates (Fig. 11.8.1A). This degree of reproducibility is comparable to that of RRL (Fig. 11.8.1B). In vitro translation reactions using HeLa extract produced high luciferase activity from mRNAs containing the rhinovirus IRES and β-globin 5′ UTR (Fig. 11.8.1A). Translation driven by the

rhinovirus IRES was greater than 10,000-fold above background (Fig. 11.8.1A; compare IRES to −mRNA) and 1000-fold above translation driven by the nonfunctional spacer sequence (Fig. 11.8.1A; compare IRES to spacer) The low translation signal produced with the spacer sequence is likely due to a low level of aberrant initiation in the HeLa extract. These results demonstrate that the rhinovirus IRES is functional in HeLa extract. As expected, the rhinovirus IRES was not functional in RRL, producing a translation signal similar to that of the spacer sequence (Fig. 11.8.1B; compare IRES to spacer). RRL is known to lack certain factors required for rhinovirus IRES-dependent translation (Hunt et al., 1999). Translation driven by the spacer sequence was 20-fold higher in RRL than in HeLa extract (compare spacer in Fig. 11.8.1A to Fig. 11.8.1B), demonstrating that RRL initiates translation at incorrect start codons far more often in RRL than HeLa extract. Translation driven by the β-globin 5′ UTR in HeLa extract was greater than 1000-fold above background (Fig. 11.8.1A; compare β-globin to −mRNA), but 10-fold lower than rhinovirus IRES-dependent translation (Fig. 11.8.1A; compare β-globin to IRES). This was not surprising since the translation conditions were optimized for IRES-dependent translation rather than scanning-dependent translation. In addition, HeLa extract contains factors that limit translation of uncapped (non IRES-dependent) mRNAs (Svitkin et al., 1996). The translation signal produced by the β-globin 5′ UTR is far higher when capped mRNA and optimized conditions are used (data not shown). Translation of luciferase driven by the β-globin 5′ UTR was stimulated 26-fold in RRL compared to HeLa extract (compare β-globin in Fig. 11.8.1A to Fig. 11.8.1B). This result is consistent with the increased ability of uncapped mRNAs to translate in RRL (Svitkin et al., 1996).

Time Considerations

It takes ~4 days to grow the HeLa cells to 6 liters from a 400-ml starter culture. Preparation of the extract takes ~6 hr. Translation reactions take ~1 hr to set up followed by a 3-hr incubation.

Literature Cited

Belsham, G.J. and Sonenberg, N. 1996. RNA-protein interactions in regulation of picornavirus RNA translation. *Microbiol. Rev.* 60:499-511.

Borman, A. and Jackson, R.J. 1992. Initiation of translation of human rhinovirus RNA: Mapping the internal ribosome entry site. *Virology* 188:685-696.

Carroll, R. and Lucas-Lenard, J. 1993. Preparation of a cell-free translation system with minimal loss of initiation factor eIF-2/eIF-2B activity. *Anal. Biochem.* 212:17-23.

Celma, M.L. and Ehrenfeld, E. 1975. Translation of poliovirus RNA in vitro: Detection of two different initiation sites. *J. Mol. Biol.* 98:761-780.

Dever, T.E. 1999. Translation initiation: Adept at adapting. *TIBS* 24:398-403.

Hunt, S.L., Hsuan, J.J., Totty, N., and Jackson, R.J. 1999. unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. *Genes Dev.* 13:437-448.

Iizuka, N. and Sarnow, P. 1997. Translation-competent extracts from *Saccharomyces cerevisiae*: Effects of L-A RNA, 5′ cap, and 3′ poly(A) tail on translational efficiency of mRNAs. *Methods* 11:353-360.

Jermutus, L., Ryabova, L.A., and Pluckthun, A. 1998. Recent advances in producing and selecting functional proteins by using cell-free translation. *Curr. Opin. Biotechnol.* 9:534-548.

Lee, K.A. and Sonenberg, N. 1982. Inactivation of cap-binding proteins accompanies the shut-off of host protein synthesis by poliovirus. *Proc. Natl. Acad. Sci. U.S.A.* 79:3447-3451.

Molla, A., Paul, A.V., and Wimmer, E. 1991. Cell-free, de novo synthesis of poliovirus. *Science* 254:1647-1651.

Rose, J.K., Trachsel, H., Leong, K., and Baltimore, D. 1978. Inhibition of translation by poliovirus: Inactivation of a specific initiation factor. *Proc. Natl. Acad. Sci. U.S.A.* 75:2732-2736.

Stiege, W. and Erdmann, V.A. 1995. The potentials of the in vitro protein biosynthesis system. *J. Biotechnol.* 41:81-90.

Svitkin, Y.V., Ovchinnikov, L.P., Dreyfuss, G., and Sonenberg, N. 1996. General RNA binding proteins render translation cap dependent. *EMBO J.* 15:7147-7155.

Villa-Komaroff, L., McDowell, M., Baltimore, D., and Lodish, H.F. 1974. Translation of reovirus mRNA, poliovirus RNA, and bacteriophage Qβ RNA in cell-free extracts of mammalian cells. *Methods Enzymol.* 30:709-723.

Witherell, G.W., Schultz-Witherell, C.S., and Wimmer, E. 1995. *Cis*-acting elements of the encephalomyocarditis virus internal ribosomal entry site. *Virology* 214:660-663.

Contributed by Gary Witherell
RiboGene, Inc.
Hayward, California

Analysis of Eukaryotic Translation in Purified and Semipurified Systems

This unit describes analysis of eukaryotic translation in purified and semipurified systems. Sucrose gradients are used to separate ribosomal complexes to analyze eukaryotic translation by reconstituting the steps in initiation (see Basic Protocol 1 and Alternate Protocols 1 and 2). While gel-filtration chromatography is excellent for resolution of low-molecular-weight components (M_r <50,000 Da), sucrose gradients are most useful with high-molecular-weight complexes, often in excess of 1,000,000 Da. The large size of ribosomes has made sucrose gradients the method of choice for purification of ribosomes and ribosomal subunits. More importantly, this method has also proven highly useful for analyzing the formation of intermediates in translation initiation and identifying the protein translation factors associated with initiating ribosomes. Much of the current understanding of the sequential steps involved in translation initiation has been obtained by utilizing sucrose gradients in conjunction with purified translation factors to analyze translation in vitro.

Sucrose gradients are useful for other purposes as well. Because they provide an entirely liquid system, losses due to the sticking of material to a solid matrix are nonexistent. Thus, the gradients may be used in a preparative manner (see Support Protocol 1). Determining relative sedimentation coefficients can be accomplished by using analytical sucrose gradients (see Support Protocol 2).

Sucrose gradients have also been widely used to study translational regulation in intact cells. Interpretation rests on the assumption that the distribution of an mRNA in different complexes (e.g., polysomes versus free mRNP) is a reflection of the rate-limiting step for translation. For most situations, initiation is the affected step and the association of mRNA with polyribosomes is affected (Hershey, 1991; see Basic Protocol 2 and Alternate Protocol 3). Ribosomal complexes fron cultured cells can also be purified for biochemical studies (see Basic Protocol 2 and Alternate Protocol 4). Yeast cells can also be used as source material (see Support Protocol 3).

A term that is often associated with sucrose gradients is density (as in sucrose density gradients), which is misleading. For the experiments described in this unit, density is not a consideration. The materials to be separated are either proteins, RNA, or both, and as such will all pellet if the centrifugation is carried out long enough. Thus, an early consideration is the type of gradient (i.e., 5% to 20% versus 10% to 40% sucrose) and the length of time that centrifugation will be necessary (3 to 24 hr). The advantage of gradients that have a linear increase in sucrose concentration is that, due to the retarding effects of both the density and the viscosity of the solution, the distance a particle sediments is linear with time. Thus, if an 80S particle has traveled a third of the way down the gradient in time t, then in time $2t$ the particle will have traveled twice as far. This facilitates fine tuning of the time required for optimal resolution of components. However, the optimal time must be determined empirically for each rotor and bucket, even when identical g forces are considered, due to variability in length of centrifuge tubes.

The protocols in this unit are primarily focused on the sedimentation of large particles (>40S), although they could be adapted for smaller ones (4S to 25S; see Support Protocol 1). The two Basic Protocols focus on in vitro experiments with particles of 40S to 80S (see Basic Protocol 1) and on the analysis of particles from cultured cells, which range in size from 40S to 300S (see Basic Protocol 2). Basic Protocol 1 has been the primary method for analysis of intermediate steps in the formation of eukaryotic 80S initiation complexes, which contain 40S and 60S subunits, mRNA (or AUG codon), and Met-tRNA$_i$. Basic Protocol 2 has been used to study cell culture systems and ascertain under specific

Contributed by William C. Merrick and Jack O. Hensold
Current Protocols in Cell Biology (2000) 11.9.1-11.9.26

conditions whether there has been a change in the initiation or elongation rate of protein synthesis. At the same time, this protocol also allows one to determine if there has been a selective change in the distribution of various mRNAs in polysomes.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

NOTE: Diethylpyrocarbonate (DEPC)-treated water (*APPENDIX 2A*) should be used for all protocols in this unit.

BASIC PROTOCOL 1

IDENTIFYING INTERMEDIATES IN THE FORMATION OF PROTEIN SYNTHESIS INITIATION COMPLEXES

Sucrose gradients have been a key methodology to analyze the binding of Met-tRNA$_i$, mRNA, and factors to 40S and 80S ribosomes because of the resolving power achieved. Free components are resolved from either of several 40S complexes (40S, 43S, and 48S; see Alternate Protocol 1), 60S, or 80S complexes (see Alternate Protocol 2). No other technique accomplishes this resolution. The procedure below describes an example of the methodology for isolation of a 43S complex (40S subunits bound to eIF2, eIF3, AUG, and Met-tRNA$_i$, but not mRNA); with some simple modifications, 48S or 80S complexes can be identified in a similar manner. For the preparation of [^{3}H]Met-tRNA$_i$, eukaryotic initiation factor 2 (eIF2), and eIF3, see Merrick (1979a,b) and Grifo et al. (1983) and references therein.

Materials

10% and 40% (w/v) sucrose gradient solutions (see recipe)
1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 M KCl
100 mM $MgCl_2$ (see recipe)
100 mM dithiothreitol
100 A_{260} units/ml AUG
100 mM GTP (see recipe)
150 mM phosphoenolpyruvate
3000 IU/ml pyruvate kinase
10 µM [^{3}H]Met-tRNA$_i$ (15 Ci/mmol)
16 µM 40S subunits (see Support Protocol 1)
8 µM eucaryotic initiation factor 2 (eIF2)
15 µM eIF3
Aqueous scintillation solution (e.g., Ecoscint, National Diagnostics)
10% (w/v) trichloroacetic acid (TCA) solution, ice cold
Acetone
SDS sample buffer (see recipe)

Gradient maker
Refrigerated high-speed centrifuge (e.g., Beckman L7-55)
Swinging bucket rotor and appropriate tubes (e.g., Beckman SW56 rotor with 5-ml tubes)
UV absorbance detector and chart recorder
Syringe or peristaltic pump
Fraction collector (e.g., ISCO model 640 gradient fractionator)
90°C oven or heating block

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

1. Use a gradient maker to prepare linear 10% to 40% sucrose gradients in 5-ml SW56 centrifuge tubes. Use 2.5 ml each of 10% and 40% sucrose gradient solutions to prepare the gradient. Fill the tubes to within ¼ inch (6.4 mm) from the top (see Fig. 5.3.1). Keep gradients at 4°C.

   It is important to have the tube filled nearly to the top, as tubes that are only 80% filled tend to collapse. To determine the correct volumes for other tubes, fill a tube with water to within ¼ inch and measure the volume contained.

   The gradients may be stored overnight at 4°C prior to use. It is important that the gradients be cold at the beginning of the experiment.

2. Combine the following components in a reaction mixture (100 µl total):

   2 µl 1 M Tris·Cl, pH 7.5 (final 20 mM)
   10 µl 1 M KCl (final 100 mM)
   3 µl 100 mM $MgCl_2$ (final 3 mM)
   1 µl 100 mM dithiothreitol (final 1 mM)
   2 µl 100 A_{260} units/ml AUG (final 0.2 units)
   0.8 µl 100 mM GTP (final 0.8 mM)
   2.7 µl 150 mM phosphoenolpyruvate (final 4 mM)
   0.15 µl 3000 IU/ml pyruvate kinase (final 0.5 IU)
   3 µl 10 µM [^{3}H]Met-tRNA$_i$ (30 pmol)
   2 µl 16 µM 40S subunits (30 pmol)
   6 µl 8 µM eIF2 (50 pmol)
   7 µl 15 µM eIF3 (100 pmol).

   Incubate 15 min at 30°C and then chill on ice.

   While the inclusion of most of the above ingredients is standard, the inclusion of phosphoenolpyruvate and the enzyme pyruvate kinase are not. These two components make up an energy regenerating system that converts any GDP to GTP (i.e., phosphoenolpyruvate + GDP → pyruvate + GTP). This is important for translation initiation experiments, as eIF2 has a 100-fold higher affinity for GDP than GTP. Therefore, any GDP present in the original GTP stock solution or generated during the incubation is quite inhibitory.

   It was experimentally determined that the inclusion of 0.1 mM GDPNP in the gradients improved the yield of 43S complexes. The rationale is that GTP is lost from the ternary complex at a slow rate. This loss labilizes the Met-tRNA$_i$, and it dissociates from the 40S subunit. By having guanylyl-5′-imidodiphosphate (GDPNP; Calbiochem) or GTP in the gradient, the complex of eIF2·Met-tRNA$_i$ is able to bind GDPNP (or GTP) and reform the ternary complex, which is more stably bound to the 40S subunit.

3. Carefully layer the chilled reaction mixture onto the top of the 10% to 40% sucrose gradient and carefully place the tube in the rotor. Make sure the tubes are balanced in the rotor. Centrifuge gradients 150 min at 270,000 × *g* (55,000 rpm in SW56 rotor), 4°C.

   To avoid disturbing the gradients, great care should be taken when buckets are placed in and removed from the rotor, and when the rotor is placed in and removed from the centrifuge.

   If the number of samples is less than the number of positions in the rotor, all of the other positions must be occupied by similar gradients in buckets, but lacking a sample for analysis.

   Slow acceleration should be used if the centrifuge has that capability. Too fast of an acceleration causes mixing of the sample with the gradient or loss out of the tube. At the end of the run, normal braking is slow enough to not disturb the gradients (i.e., it is not necessary to coast to a stop).

If an SW56 swinging bucket rotor is not available, it is possible to calculate conditions for other rotors. For rotors with the same size bucket (4.4 to 5 ml; e.g., Beckman SW65L, SW50L, SW39L, SW60Ti, SW50.1), the relative k factor can be used to correct for time and speed (see Beckman Instruments, http://beckmancoulter.com; McEwen, 1967). The general formula is: (time × rpm of SW56)/SW56 k factor = (time × rpm of other rotor)/k factor of other rotor, where the k factor of the SW56 rotor is 55. Thus, to find the equivalent centrifugation using an SW39L rotor (k = 112), the calculation would be: (150 min × 55,000 rpm)/55 = (x min × 39,000 rpm)/112, giving x = 431 min. For centrifugation using alternate sucrose gradients, see McEwen (1967).

4. Carefully take the rotor out of the centrifuge, remove tubes, and place the tubes on ice.

   Warming causes thermal mixing and adversely affects the resolution of the gradients.

5. Unload samples from the tube by one of two alternative methods shown in Figure 11.9.1. In either case, use an in-line UV absorbance detector and chart recorder to monitor absorbance, and collect ~20 to 30 fractions (e.g., twenty-five 200-µl fractions) with a fraction collector.

   The tRNA and ribosomal RNAs will show up well by monitoring absorbance at 254 or 260 nm.

   At this point, the radioactivity in each fraction can be determined (step 6a). Alternatively, the location of eIF2 and eIF3 proteins can be determined by SDS-PAGE (steps 6b to 9b).

To measure radioactivity

6a. Mix 100 µl of each fraction with 5 ml of an aqueous scintillation solution and measure radioactivity by scintillation spectrometry (see Fig. 11.9.2).

   For the above example, two peaks of radioactivity should be observed, one of low molecular weight representing free [^{3}H]Met-tRNA$_i$ and a second, more rapidly sedimenting peak representing the labeled 43S complex (40S subunits, eIF2, GTP, Met-tRNA$_i$, eIF3, AUG).

To detect proteins

6b. Mix a 50- to 100-µl aliquot from each fraction with 1 ml ice-cold 10% TCA solution in a microcentrifuge tube and incubate 30 min on ice to precipitate the protein.

7b. Pellet protein by microcentrifuging 20 min at 10,000 × *g*, 4°C.

8b. Discard supernatant, wash pellet twice with 1 ml acetone to remove residual TCA, and allow to dry.

9b. Add 20 µl SDS sample buffer to the pellet, mix, heat 10 min at 90°C, and then analyze by SDS-PAGE (*UNIT 6.1*; Laemmli, 1970).

   If the sample is yellow in color, not all of the trichloroacetic acid was removed. Add 1 to 3 µl of 1 M Tris·Cl, pH 8, until the sample is blue.

   The peptide bands diagnostic for each factor can be visualized after the gel has been stained with Coomassie blue (UNIT 6.5). In general, only those peptide bands that are >40 kDa can be visualized in the 43S region of the gradient, as the 40S subunit has numerous subunits with molecular weights generally <40 kDa.

   An alternate approach to identify the proteins of interest is possible if antibodies are available. The proteins are transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane (immunoblotting; UNIT 6.2). The fractions containing the protein of interest are identified using standard methods for antibody binding and detection.

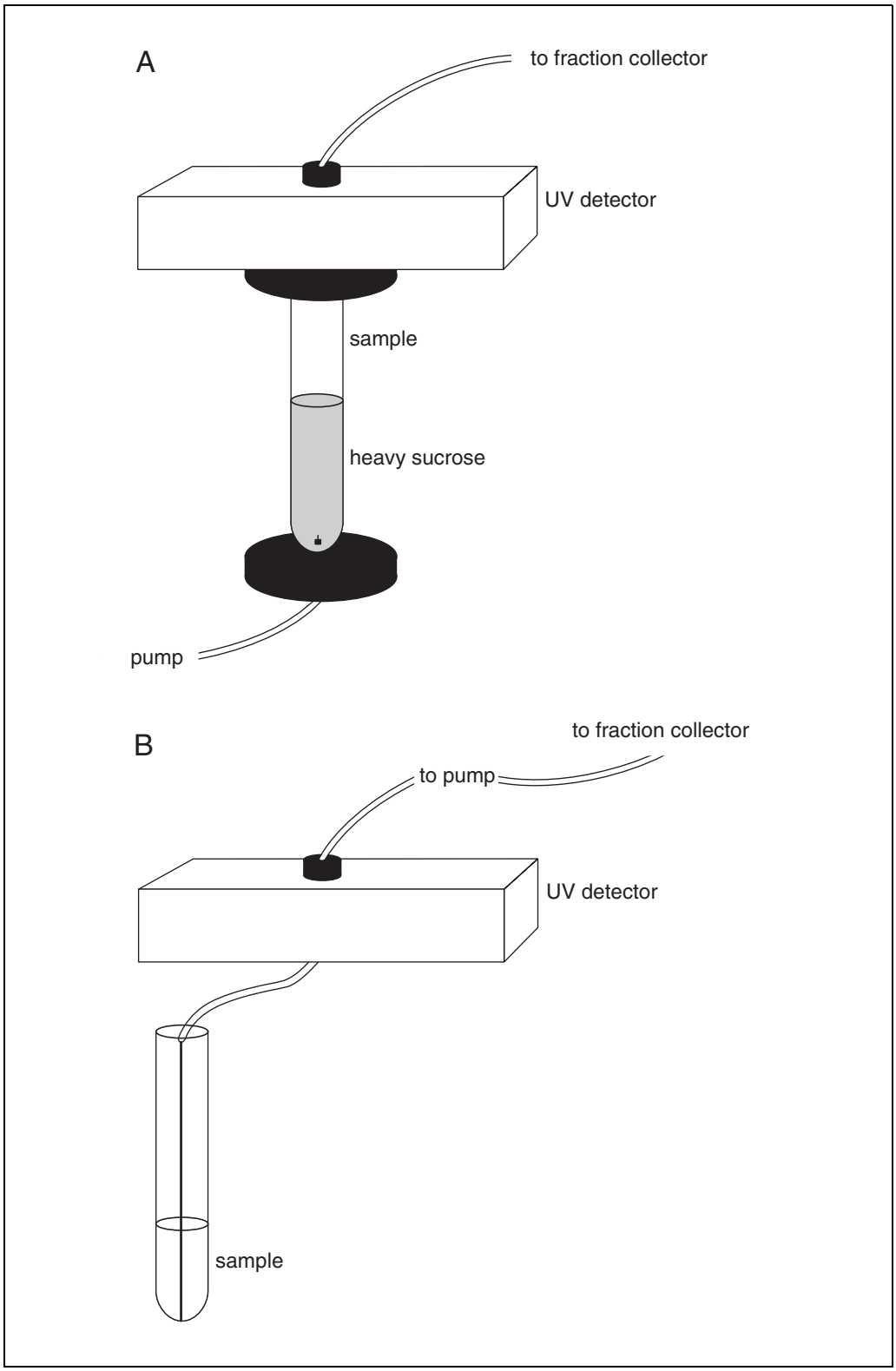


Figure 11.9.1 Methods for collecting sucrose gradients. In the example shown in (**A**), the top of the centrifuge tube is fitted into an adaptor that fits into the UV detector. A dense sucrose solution (i.e., >50% w/v) is introduced into the bottom of the centrifuge tube with the aid of a tube-piercing apparatus that seals the bottom of the tube. As the heavy sucrose is introduced, the gradient is displaced upwards through the UV detector and then to a fraction collector. In the example shown in (**B**), a thin metal tube is carefully inserted to the bottom of the centrifuge tube. The sample is pumped out of the bottom of the tube, through the UV detector, and then to the fraction collector.

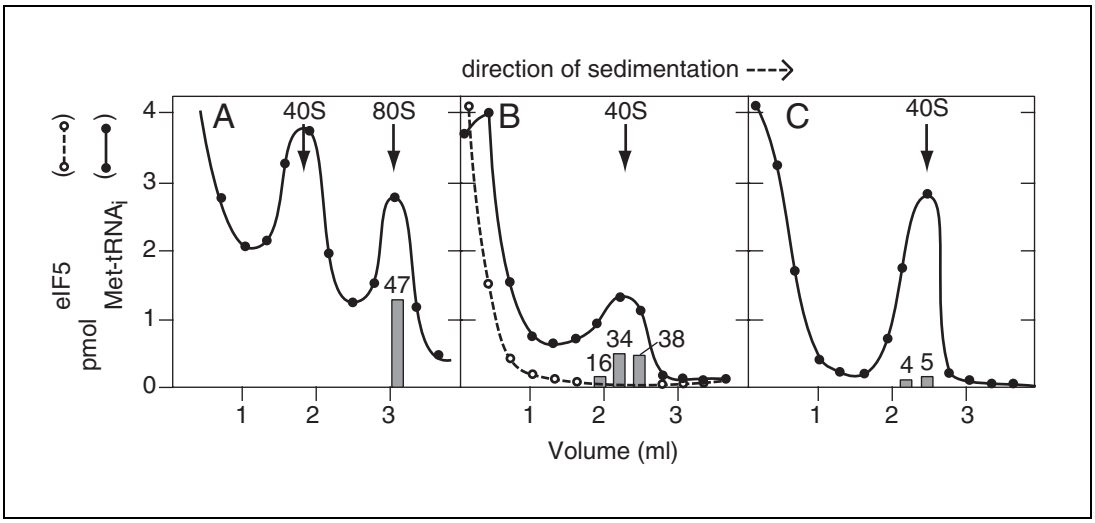


Figure 11.9.2 Reactivity of Met-tRNA$_i$ bound to 40S and 80S initiation complexes. In this experiment, eIF2, eIF3, AUG, GTP, [^{3}H]Met-tRNA$_i$, and 40S subunits were incubated 15 min at 30°C in a 100-µl volume. After incubation, the reaction was layered onto a 10% to 35% sucrose gradient (buffered in 20 mM Tris·Cl, pH 7.5, 100 mM KCl, and 5 mM $MgCl_2$). After centrifugation at 55,000 rpm at 4°C for 140 minutes, the gradients were fractionated (250-µl fractions) by displacement with 40% sucrose (Fig. 11.9.1A). Aliquots were sampled for radioactivity (closed circles) and for reactivity of Met-tRNA$_i$ with puromycin (expressed as a percentage of cpm bound Met-tRNA$_i$, shaded bars). (**A**) Also included in the incubation were eIF5 and 60S subunits. (**B**) [^{14}C]eIF5 (open circles) was present in the incubation; 60S subunits were added to test for reactivity with puromycin. (**C**) GDPNP replaced GTP in the incubation; eIF5 and 60S subunits were added to test for reactivity with puromycin. (Adapted from Peterson et al., 1979b.) This figure represents an experiment where different complexes are resolved by sucrose gradients. Met-tRNA$_i$, present in all experiments, was monitored by liquid scintillation spectrometry. The ability of bound Met-tRNA$_i$ to form methionyl-puromycin was determined separately. Previous studies had shown that formation of methionyl-puromycin required 40S and 60S subunits, AUG, eIF2, eIF3, eIF5, and Met-tRNA$_i$. eIF1A and eIF5A stimulate this process as well.

ALTERNATE PROTOCOL 1

FORMATION OF 48S PREINITIATION COMPLEXES

Using the same sucrose gradients, a variety of other components can be added to determine their influence on the binding of tRNA and mRNA to 40S subunits to form 48S preinitiation complexes (Benne and Hershey, 1978; Merrick, 1992). These include the substitution of globin mRNA (or an mRNA transcript of choice made with T7 polymerase) for AUG, the addition of ATP (required to obtain binding of mRNA to 40S subunits), or the addition of other translation initiation factors (most importantly eIF4A, eIF4B, eIF4F, and eIF4H to obtain maximal binding of mRNA to 40S subunits). As long as 60S subunits are not added, all of the products should be either free components or components bound to 40S subunits. Depending on additions, the 40S complexes could have the following characteristics: (1) 40S subunits: no factors, Met-tRNA$_i$, or mRNA are bound; (2) 43S complexes: eIF2, eIF3, AUG, and Met-tRNA$_i$ are bound, but not mRNA; (3) 48S complexes: eIF2, eIF3, mRNA, and Met-tRNA$_i$ are bound. The procedure is identical to that in Basic Protocol 1, except that the reaction in step 2 is replaced with the one in step 2a below.

Additional Materials *(also see Basic Protocol 1)*

1 M KCl
1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 mM dithiothreitol

2a. Prepare a reaction for formation of 48S complexes (total 100 µl):

2 µl 16 µM 40S subunits (40 pmol)
3 µl 10 µM [^{3}H]Met-tRNA$_i$ (30 pmol)
7 µl 15 µM eIF3 (100 pmol)
6 µl 8 µM eIF2 (50 pmol)
3 µl 100 A_{260} units/ml of AUG (0.3 units)
10 µl 1 M KCl
2 µl 1 M Tris·Cl, pH 7.5
1 µl 100 mM dithiothreitol
4 µl 100 mM $MgCl_2$
1 µl 100 mM GTP
61 µl H_2O.

Incubate 15 min at 30°C and then chill on ice.

It has been the authors' experience that, when using purified translation factors and 40S subunits, factors other than eIF2 and eIF3 are not found associated with the above complexes. However, since their initial purification is based on their association with polysomes, it is likely that either the ribosomal subunits are damaged or that an unidentified factor that stabilizes the association of these other factors with the 40S subunit is lost during purification.

If the presence of mRNA in the 48S complexes is to be studied, 10 µl of a 100 µg/ml solution of mRNA and 1 µl of 100 mM ATP should be substituted for the AUG codon. In addition, translation factors eIF4A, eIF4B, and eIF4F should be added (Merrick, 1979a; Grifo et al., 1983).

FORMATION OF 80S INITIATION COMPLEXES

ALTERNATE PROTOCOL 2

When 60S subunits are also added, it is possible to obtain complexes that contain 40S subunits, 60S subunits, or 80S subunits. The gradient conditions described above should allow the separation of all of these complexes. Polysomes, if formed, will be found pelleted at the bottom of the tube. If the original reaction mixture contains GDPNP, then no 80S complexes will be seen, and usually more 40S complexes are seen than with GTP. In the presence of GTP, 43S (with AUG) or 48S (with mRNA) complexes may be converted to 80S complexes (40S and 60S subunits, mRNA, Met-tRNA$_i$) if eIF1A, eIF5, and eIF5A (Merrick, 1979a; Grifo et al., 1983) are present in the original reaction mixture. The isolated 80S complexes should be reactive with puromycin to form methionyl puromycin (see Fig. 11.9.2).

Additional Materials *(also see Basic Protocol 1)*

16 µM 60S subunits (see Support Protocol 1)
70 µM eucaryotic initiation factor 1A (eIF1A)
70 µM eIF5A
20 µM eIF5
1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 M KCl
100 mM dithiothreitol
100 mM KPO_4, pH 8.0
Ethyl acetate
Scintillation solution (e.g., Econofluor)

13 × 100–mm test tubes
Scintillation vials

Separate and analyze complexes

1. Prepare 10% to 40% sucrose gradients in 5-ml SW56 tubes (see Basic Protocol 1, step 1).

2. Prepare a reaction for the formation of 80S complexes (total 100 µl):

   2 µl 16 µM 40S subunits (final 40 pmol)
   2 µl 16 µM 60S subunits (final 40 pmol)
   3 µl 10 µM [^{3}H]Met-tRNA$_i$ (final 30 pmol)
   7 µl 15 µM eIF3 (final 100 pmol)
   6 µl 8 µM eIF2 (final 50 pmol)
   0.7 µl 70 µM eIF1A (final 50 pmol)
   0.7 µl 70 µM eIF5A (final 50 pmol)
   0.5 µl 20 µM eIF5 (final 10 pmol)
   3 µl 100 A_{260} units/ml AUG (final 0.3 units)
   2 µl 1 M Tris·Cl, pH 7.5
   10 µl 1 M KCl
   1 µl 100 mM dithiothreitol
   4 µl 100 mM $MgCl_2$
   1 µl 100 mM GTP
   57 µl H_2O.

   Incubate 15 min at 30°C and then chill on ice.

3. Separate subunits and analyze radioactivity as described (see Basic Protocol 1, steps 3 to 6a).

   If desired, protein content may be determined for factions at this point.

Determine puromycin reactivity of peaks

4. Mix 100 µl of each fraction in a 13 × 100–mm test tube with 100 µl reaction buffer II (200 µl total). Incubate 15 min at 37°C.

5. Terminate reaction by sequential addition of 0.9 ml of 100 mM KPO_4, pH 8.0, and 3 ml of ethyl acetate. Vortex vigorously for 30 sec.

6. Separate aqueous and organic phases by centrifuging 10 min at 1000 × *g* (~2000 rpm), room temperature, in a tabletop centrifuge.

7. Carefully remove 2 ml of the organic phase (upper phase, containing the product methionylpuromycin) to a scintillation vial. Add 10 ml scintillation solution and measure radioactivity by scintillation spectrometry.

   The reactivity of the [^{3}H]Met-tRNA$_i$ in the 80S peak can be determined from the ratio of the amount of radioactivity obtained as methionylpuromycin relative to the radioactivity added to the reaction mixture in step 4 (equal to the radioactivity of the 100-µl aliquot measured in step 3).

SUPPORT PROTOCOL 1

ISOLATION OF RIBOSOMAL SUBUNITS USING PREPARATIVE SUCROSE GRADIENTS

The standard preparative use of sucrose gradients is for the isolation of 40S and 60S ribosomal subunits (Merrick, 1979b), although any macromolecular component with an S value of 10S or larger may be a candidate for purification by this method.

Materials

Rabbit reticulocyte lysate (Green Hectares)
Standard sucrose solution (see recipe)
KCl
Subunit buffer (see recipe)
5% and 20% (w/v) sucrose gradient solutions (see recipe)
0.25 M sucrose solution (see recipe)

Refrigerated high-speed centrifuge (e.g., Beckman L7-55)
Type 35 rotor and 70-ml polycarbonate centrifuge tubes (Beckman)
Ti60 or Ti70 rotor and 26-ml polycarbonate centrifuge tubes (Beckman)
Gradient maker
SW27 rotor and 32-ml polyallomer or cellulose nitrate centrifuge tubes (Beckman)

Prepare salt-washed ribosomes

1. In 70-ml polycarbonate centrifuge tubes for a Beckman type 35 rotor, centrifuge 420 ml rabbit reticulocyte lysate 4 hr at ~100,000 × *g* (35,000 rpm), 4°C.
2. Decant and discard supernatant. Rinse the surface of the polysomal pellets once with 3 ml standard sucrose solution.
3. Add 5 ml standard sucrose solution to each tube and tease the pellet using a glass stirring rod to give a solution of dissolved and partially dissolved polysomes.
4. Transfer contents of all tubes to a beaker in ice with a stir bar. Stir the polysome solution until all of the particles have dissolved (no more than 30 min).
5. Measure the volume of the polysome solution and add solid KCl to a final concentration of 0.5 M. Stir 30 min until KCl has dissolved completely.
6. Pour into 26-ml polycarbonate centrifuge tubes for a Ti60 or Ti70 rotor. Centrifuge 2 hr at 180,000 × *g* (50,000 rpm in Ti60 or Ti70 rotor), 4°C.
7. Decant supernatant and save as an enriched source of protein synthesis initiation factors (Merrick, 1979a). Store frozen at −120°C until needed (stable up to several years).
8. Dissolve pellet in a minimal amount of standard sucrose solution.

   This dissolved pellet is referred to as "salt-washed ribosomes." They may be stored frozen at −120°C.

Prepare ribosomal subunits

9. Dissolve salt-washed ribosomes in sufficient subunit buffer to achieve a concentration of 50 A_{260} units/ml.
10. Incubate 10 min on ice and then 10 min at 37°C.

    Puromycin causes release of the nascent peptide chains that stabilize ribosomal subunit interactions in translating polysomes during the initial high-salt wash. Release of the nascent chains followed by exposure to high salt causes the 80S ribosomes to dissociate into the 40S and 60S subunits.

11. Prepare 5% to 20% sucrose gradients in 32-ml SW27 polyallomer centrifuge tubes as described above (see Basic Protocol 1, step 1).
12. Layer 2- to 3-ml samples onto sucrose gradients and centrifuge 8 hr at 95,000 × *g* (27,000 rpm in SW27 rotor), 4°C.

13. Collect the gradients in sixty 0.5-ml fractions, monitoring A_{260} with a UV detector.

    The region used to obtain 40S subunits represents the front two-thirds of the 40S peak, while the region used for 60S subunits represents the middle half of the 60S peak. In this manner, 40S subunits are not contaminated with 60S subunits and 60S subunits are not contaminated with either 40S subunits or 80S subunits.

14. Concentrate individual subunits by centrifuging 18 hr at 170,000 × *g* (50,000 rpm in a Ti60 rotor), 4°C.

15. Dissolve pelleted subunits in a minimal amount of 0.25 M sucrose solution. Once the pellets are dissolved, determine the concentration of the subunits and adjust to 16 µM (135 A_{260} units/ml for 40S subunits and 270 A_{260} units/ml for 60S subunits). Store in 100-µl aliquots at the vapor temperature of liquid nitrogen for up to 2 years.

ALTERNATE PROTOCOL 3

MONITORING THE POSITION OF THE RIBOSOME ON GLOBIN mRNA

This protocol describes a method called "toeprinting" that is used for locating the position of the mRNA on the 40S subunit. For this assay (Anthony and Merrick, 1992), the mRNA of interest is hybridized to a radiolabeled oligonucleotide (≥20 nucleotides) that is complimentary to a region of the mRNA located ~50 nucleotides 3′ of the initiating AUG. In this case, the reaction is described for globin mRNA as an example.

Additional Materials *(also see Basic Protocol 1)*

mRNA for globin or other gene of interest
^{32}P-end-labeled oligonucleotide primer: e.g.,
5′-TCACCACCAACTTCTTCCAC-3′ for globin (5000 Ci/mmol), or primer appropriate to gene of interest
Micrococcal nuclease–treated rabbit reticulocyte lysate (Promega)
1 M HEPES·KOH, pH 7.5
100 and 500 mM $Mg(CH_3COOH)_2$
100 mM dithiothreitol
10 mM anisomycin
10% and 35% (w/v) sucrose gradient solutions (see recipe)
100 mM each dATP, dGTP, dCTP, dTTP
AMV reverse transcriptase
1:1 (v/v) phenol/chloroform (*APPENDIX 3*)
Ethanol
3 M potassium acetate, pH 5.0
SW56 rotor and 5-ml polyallomer centrifuge tubes (Beckman)

Additional reagents and equipment for DNA sequencing gels (*APPENDIX 3*)

Label message

1. For ten reactions, place 15 µCi of ^{32}P-labeled oligonucleotide primer and 10 µg globin mRNA in a microcentrifuge tube, place the tube in a small beaker of boiling water, and incubate 1 min.

   The probe should be a 20- to 30-oligonucleotide sequence complimentary to a portion of the mRNA of interest and located ~50 nucleotides 3′ of the initiating AUG.

2. Place the beaker on the bench top and allow to cool to room temperature (~30 min).

3. Prepare reaction mixtures (150 µl each):

50 µl micrococcal nuclease–treated rabbit reticulocyte lysate
2.25 µl 1 M HEPES·KOH, pH 7.5 (final 15 mM)
15 µl 1 M KCl (final 100 mM)
6.0 µl 100 mM $Mg(CH_3COOH)_2$ (final 24 mM)
1.5 µl 100 mM dithiothreitol (final 1 mM)
1.5 µl 10 mM anisomycin (final 0.1 mM)
$^1/_{10}$th volume of mRNA-oligonucleotide duplex (1 µg mRNA, 1.5 µCi oligonucleotide)
1 µl 100 mM dithiothreitol
2 µl 100 mM ATP
1 µl 100 mM GTP
55 µl H_20.

Incubate 15 min at 30°C and then chill 2 min on ice.

4. Prepare 10% to 35% sucrose gradients in 5-ml SW56 centrifuge tubes as described above (see Basic Protocol 1, step 1).

5. Layer the reaction on the sucrose gradients and centrifuge 150 min at 270,000 × *g*, 4°C.

6. Collect 0.2-ml fractions.

Reverse transcribe message

7. Combine the following:

200-µl aliquots of gradient fractions
0.2 mM each dATP, dGTP, dCTP, dTTP
4 U AMV reverse transcriptase.

Incubate 30 min at 37°C.

The concentrations of components in the sucrose gradient solution are designed to be compatible with the ionic and divalent ion requirements of the reverse transcriptase.

8. Extract reactions with an equal volume of 1:1 (v/v) phenol/chloroform. Separate phases by centrifuging 10 min at 1000 × *g*, 4°C. Remove the aqueous (upper) phase and add 0.1 vol 3 M potassium acetate, pH 5.0 and 2.5 vol ethanol. Let stand overnight at −20°C. Collect the precipitated nucleic acid by centrifuging 20 min at 10,000 × *g*, 4°C. Decant the aqueous/ethanol and allow tube to dry.

9. Resolve products on an 8% (w/v) acrylamide DNA sequencing gel (*APPENDIX 3*). Compare with a sequence ladder obtained by performing an extension reaction in the presence of dideoxynucleotides.

For the bottom sequencing gel, mix 10 ml of 8% acrylamide/urea bottom solution (see recipe), 40 µl of 10% ammonium persulfate, and 15 µl TEMED. For the top sequencing gel, mix 35 ml of 8% acrylamide/urea top solution (see recipe), 120 µl of 10% ammonium persulfate, and 50 µl TEMED.

10. Quantify the amount of mRNA in each complex (48S or 80S) based upon the radioactivity from the [^{32}P]primer.

DETERMINATION OF RELATIVE SEDIMENTATION COEFFICIENTS USING ANALYTICAL SUCROSE GRADIENTS

SUPPORT PROTOCOL 2

Sucrose gradients are a standard means for determining the relative sedimentation coefficient of proteins (for example see Merrick and Anderson, 1975) and to get a rough estimate of molecular mass. The advantage of this method over use of the analytical ultracentrifuge (which is much more accurate; see *UNIT 5.3*) is that either crude or pure

samples can be analyzed. The location of the desired protein can be determined by biological assay, by band pattern on SDS gels (*UNIT 6.1*), by antibody detection (immunoblotting; *UNIT 6.2*), or, in some instances, by ligand binding (far western blot; *UNIT 17.2*). For these determinations, 5% to 20% sucrose gradients are used. These lower concentrations of sucrose reflect the much smaller *S* values of proteins relative to ribosomal subunits. Buffer composition and ionic strength should be optimized for the protein in question, either for preservation of activity or for suitability in the subsequent assay. A variety of proteins can be used as markers, although a convenient set of proteins includes ovalbumin (3.55S), aldolase (7.8S), catalase (11.2S), and β-galactosidase (16.1S). As the shape of the protein will influence the *S* value (greater asymmetry will decrease the *S* value relative to a symmetric protein of the same molecular weight), this value is often used in conjunction with the Stokes radius, which can be determined by gel filtration (*UNIT 5.5*). The formula $M_r = 6\pi\eta Nsa/(1 - \bar{\nu}\rho)$ allows for the determination of the molecular weight, where η is viscosity, N is Avogadro's number, s is the sedimentation coefficient, a is the Stokes radius, $\bar{\nu}$ is the partial specific volume of the protein, and ρ is the solvent density. In this formula, the apparent increase in molecular weight that asymmetry causes in the determination of the Stokes radius is balanced by the decrease in *S* value that asymmetry induces.

Additional Materials *(also see Basic Protocol 1)*

5% and 20% (w/v) sucrose gradient solutions in suitable buffer (e.g., 20 mM Tris·Cl, pH 7.5/100 mM KCl/1 mM dithiothreitol)
Protein standards in same buffer:
10 mg/ml ovalbumin (3.55S)
10 mg/ml alddase (7.8S)
10 mg/ml catalase (11.2S)
10 mg/ml β-galactosidase (16.1S)
Unknown protein

1. Prepare 5% to 20% sucrose gradients in 5-ml SW56 centrifuge tubes as described above (see Basic Protocol 1, step 1).
2. Mix 50 to 100 µl of each protein standard together to yield 200 to 400 µl of standard mixture containing 10 mg/ml of each marker.

   Prepare 200 µl for SW rotors with small volumes (~5 ml/tube) or 400 µl for SW rotors with large volumes (~12 ml/tube).
3. Layer standard mixture on top of a 5% to 20% sucrose gradient.
4. Layer 100 to 300 µl unknown protein on top of a separate 5% to 20% sucrose gradient or mix with one or more of the standard proteins if an internal standard is desired.

   If an internal standard is used, it is best to choose a standard that is anticipated to have a significantly higher or lower S value, if possible.
5. Centrifuge 4.5 hr at 300,000 × *g* (65,000 rpm in SW56 rotor), 4°C.
6. Place the tubes on ice.
7. Collect twenty-five 0.2-ml fractions.
8. Analyze fractions by precipitating and electrophoresing the proteins (see Basic Protocol 1, steps 6b to 9b) or by assaying fractions directly for biological activity to determine the distance the protein has sedimented.
9. Determine the distance sedimented for each of the standard proteins by measuring UV absorbance at 280 nm as the gradient is removed from the tube or by analyzing aliquots of each fraction by SDS-PAGE (see Basic Protocol 1, steps 6b to 9b).

10. Construct a plot of $S_{20,w}$ of the standards versus the distance migrated (e.g., as milliliters or fraction tube number). Determine the S value for the unknown protein based upon its distance sedimented.

The plot should be a straight line.

The $S_{20,w}$ is the rate of sedimentation for a particle analyzed in distilled water at 20°C. Normally, one would have to correct for the usual centrifugation conditions, which would include both salt and temperature differences. However, as the protein standards are measured under the same conditions as the unknown, this correction is not necessary. For proteins that contain extensive sugar or lipid modifications, there will be a slight error as their partial specific volumes will not be 0.73 g/ml.

ANALYSIS OF TRANSLATION IN CULTURED CELLS

BASIC PROTOCOL 2

Initiation of translation is rate-limiting for translation of most mRNAs and is subject to regulation at multiple steps (Hershey, 1991). Thus, most changes in the translational efficiency of a given mRNA can be assessed by determining the percentage of that mRNA associated with actively translating polysomes. An approach to assess the relative amount of an individual mRNA in polysomes is outlined below.

The procedures described in this protocol have been broadly applied to analyze translation in adherent cells (BALB/c 3T3, Chinese hamster ovary, normal rat kidney, and C6 glioma cells), suspension cells (Friend erythroleukemia, Da3, and 32D hematopoietic cell lines), and in yeast (*Saccharomyces cerevisiae*). However, while broadly applicable, the necessity of lysing cells under conditions that do not denature ribosomes may preclude analysis of cells that have high concentrations of endogenous nucleases and that require strong denaturants (such as guanidinium or TriZol reagent) for preparation of intact RNA.

Materials

10% and 50% (w/v) sucrose gradient solutions (see recipe)
Cultured cells or yeast lysate (see Support Protocol 3)
PBS (*APPENDIX 2A*) or other neutral, buffered isotonic solution
TMK_{100} lysis buffer (see recipe), ice cold
Tris-buffered, water-saturated phenol: for buffering, use 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Chloroform
Ethanol
RNA-loading buffer (see recipe)

Gradient maker
Refrigerated high-speed centrifuge (e.g., Beckman L7-55)
Swinging bucket rotor and appropriate tubes (e.g., Beckman SW28.1 rotor and 17-ml tubes)
15-ml polyethylene tubes with caps
UV absorbance detector and chart recorder
Syringe or peristaltic pump
Fraction collector (e.g., ISCO model 640 gradient fractionator)
Water bath or heating block at 65°C

Additional reagents and equipment for analyzing RNA on agarose or acrylamide gels and for northern blotting (*APPENDIX 3*)

NOTE: Experiments involving RNA require careful precautions to prevent contamination and RNA degradation (see *APPENDIX 2A*).

NOTE: All procedures are carried out on ice with solutions that have been precooled to 4°C.

Prepare gradients

1. Prepare a linear sucrose gradient in a 17-ml SW28.1 centrifuge tube using a gradient maker and 8.15 ml each of 10% (light) and 50% (heavy) sucrose gradient solutions. Store gradient on ice, or at 4°C, until lysate is ready for loading.

   The addition of nuclease inhibitors to the sucrose gradients is not necessary. Heparin has occasionally been added to gradients as a nonspecific inhibitor of nucleases. However, this practice is discouraged because heparin competes with RNA for nucleic acid–binding proteins, and 80S ribosomal/mRNA complexes are not stable in heparin (W.C. Merrick and J.O. Hensold, unpub. obser.).

   For analysis of interactions of mRNAs (or proteins) with individual ribosomal subunits, insufficient separation is obtained using 10% to 50% sucrose gradients. For analysis of these smaller complexes, 10% to 25% sucrose gradients should be used.

Prepare lysate

2a. *For suspension cultures:* Collect cells in a 15-ml sterile centrifuge tube. Rinse three times in 10 ml ice-cold PBS, centrifuging 10 min at 1500 × *g*, 4°C, each time. Proceed to step 3.

   For optimal results, it is best to empirically determine the number of cells necessary for analysis. While increasing the load can increase detection of proteins or RNAs of low abundance, there is a resultant loss of resolution. For practical purposes, 2 to 4 × 10^7 cells provides optimal resolution on 10% to 50% gradients, while still allowing for detection of most proteins or RNAs of interest by immunoblotting or northern blotting, respectively. For analysis of more slowly sedimenting complexes on 10% to 25% gradients, the number of cells is increased by 1.5 to 2 fold.

2b. *For adherent cultures:* Rinse adherent cells three times with 10 ml ice-cold PBS, detach cells with a rubber policeman, and transfer to a microcentrifuge tube. Pellet cells by microcentrifuging 10 min at 1500 × *g*, 4°C. Proceed to step 3.

   Trypsin should be avoided because it may affect cell signaling and its use is not practical at the low temperatures employed.

2c. *For yeast:* Grow and lyse yeast cells as described below (see Support Protocol 3). Proceed to step 5.

3. Discard supernatant and lyse cell pellet by repeated pipetting in 400 µl ice-cold TMK_{100} lysis buffer.

   RNase inhibitors may be added to the lysis buffer (see Critical Parameters).

4. Transfer lysed pellet to a microcentrifuge tube and pellet nuclei by microcentrifuging 5 min at 10,000 × *g*, 4°C.

Fractionate lysate

5. Remove supernatant and layer onto the 10% to 50% sucrose gradient. For yeast, load 10 A_{254} units lysate per gradient.

6. Centrifuge 4 hr at 100,000 × *g* (27,000 rpm in SW28.1 rotor), 4°C.

   The methods described have been optimized for use with the SW28 rotor and SW28.1 buckets. This rotor accommodates a centrifuge tube that is 15% longer than that accommodated by the SW41 rotor, and thus provides better resolution of ribosomal complexes. However, for most purposes the separations achieved with the SW41 rotor are adequate. Centrifugation conditions for the SW41 and other rotors can be found in a variety of references (Steel and Jacobson, 1987; Kaspar and Gehrke, 1994; Proweller and Butler, 1994).

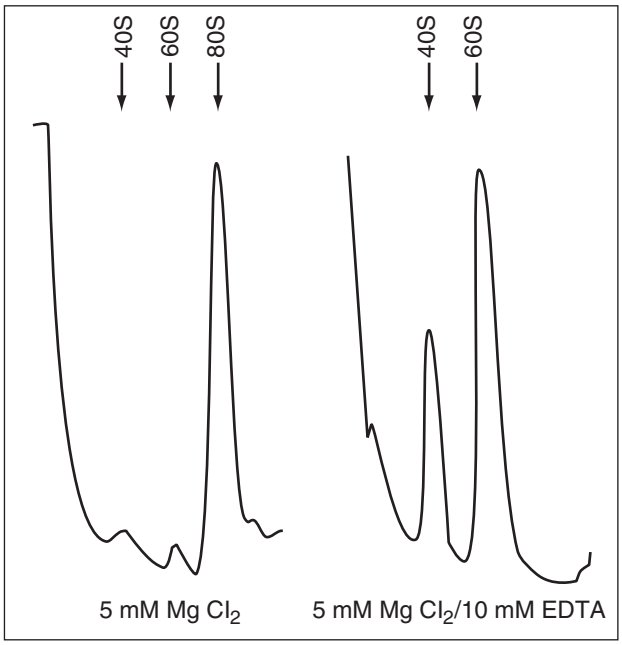


Figure 11.9.3 Sedimentation of ribosomes from erythroleukemic cells in 10% to 50% and 10% to 25% sucrose gradients. Erythroleukemic cells in log phase of growth were lysed as described in the text, and post-nuclear supernatants were loaded onto 10% to 25% sucrose gradients. The gradients were collected with an ISCO model 640 gradient fractionator by displacement from the bottom with 60% sucrose into a flow cell (Fig. 11.9.1A). UV absorbance was continuously monitored during the collection. The gradients contained 20 mM HEPES·KOH, pH 7.4, 5 mM $MgCl_2$, 100 mM KCl, and 2 mM dithiothreitol. In the experiment shown in the far right panel, 10 mM EDTA was added to the post-nuclear supernatants and to the gradients. The positions of sedimentation of the 40S, 60S, and 80S ribosomes are indicated by arrows above the figures. These assignments were confirmed by analysis of the rRNA content of the inclusive fractions. The direction of sedimentation is from right to left.

For analysis of 40S to 80S complexes in 10% to 25% sucrose gradients, centrifugation is 16 hr at 55,000 × g (20,000 rpm in SW28.1 rotor), 4°C. This completely pellets the polysomes while separating the 40S, 60S, and 80S complexes into distinct fractions (see Fig. 11.9.3). By increasing the sedimentation speed of these gradients to 23,000 rpm, 80S complexes are also pelleted, and separation of 43S from 48S (preinitiation) complexes is possible.

7. Collect gradients in 5-ml polyethylene tubes containing 1 ml Tris-buffered, water-saturated phenol using a fraction collector with continuous monitoring of absorbance at 254 nm (see Fig. 11.9.1).

   For most purposes, fractionation of the gradients into fourteen 1.2-ml fractions provides sufficient detail to determine the position of sedimentation of mRNAs in the gradient.

   To ensure that the amount of RNA in each fraction is representative of its relative distribution in the gradient, it is imperative that equal volumes of phenol and chloroform are added to the fractions. The amount of aqueous phase recovered following phase separation must also be constant.

Extract RNA

8. Immediately following collection, tightly cap the tubes, vortex to mix the phases, and place on ice until all fractions are collected.

9. Add 1 ml chloroform to each tube, mix phases by vortexing, and separate phases by centrifuging 15 min at 3000 × *g*, 4°C, in a tabletop centrifuge.

   The stepwise addition of phenol and then chloroform, rather than addition of a phenol/chloroform solution, facilitates mixing of the phenol with the viscous sucrose solution.

   Alternatively, the distribution of proteins in gradient fractions can be analyzed. In this case, the fractions are collected directly into microcentrifuge tubes containing 1/9 volume of 100% trichloroacetic acid (e.g., 133 µl for a 1.2-ml fraction). The pelleted proteins are recovered and analyzed by SDS-PAGE (UNIT 6.1).

10. To ensure equal recovery of extracted RNA from the fractions, transfer a constant volume of the aqueous phase (for example, separate the aqueous phase into two 500-µl fractions) to 1.5-ml microcentrifuge tubes and precipitate with 2 vol ethanol.

    The salt concentration in the buffer is sufficient for precipitation of RNA.

    The precipitated RNA can be stored for extended periods of time (3 to 6 months) in ethanol at −20°C prior to analysis.

11. Pellet RNA by microcentrifuging 20 min at 10,000 × *g*, 4°C.

Analyze RNA content

12. Carefully aspirate the ethanol-buffered sucrose solution, taking care not to disturb the RNA pellet. Resuspend pellet in 20 µl RNA-loading buffer.

    Excess sucrose remaining with the pellet can inhibit migration of the RNA out of the wells, particularly in the fractions that contain the highest concentration of sucrose. Complete removal of the sucrose can be facilitated by a second, brief (i.e., 30-sec) centrifugation in a microcentrifuge and aspiration of the residual supernatant.

13. Denature RNA by heating 15 min at 65°C.

14. Load the entire sample onto either an agarose or acrylamide gel prepared for electrophoresis (*APPENDIX 3*).

    Care must be taken to ensure that the entire RNA pellet is loaded onto the gel if the results are to be reflective of the actual distribution of RNA across the gradient fractions.

15. Blot the gel for northern blot hybridization and hybridize (*APPENDIX 3*).

ALTERNATE PROTOCOL 4

PURIFICATION OF RIBOSOMAL COMPLEXES FROM CULTURED CELLS FOR BIOCHEMICAL ASSAYS

When it is necessary to analyze ribosomal complexes in their native state, collection into either phenol or TCA is not possible. Collection of pooled polysomes is simplified by sedimentation in 10% to 25% sucrose gradients, since these conditions will pellet complexes that are larger than 80S.

Additional Materials *(also see Basic Protocol 2)*

Cultured cell lysate (Basic Protocol 2) or yeast lysate (Support Protocol 3)
25% sucrose gradient solution (buffered as for 10% and 50% solutions in Basic Protocol 2)
TMK_{100} lysis buffer (see recipe) without detergent
22% (w/v) sucrose solution (see recipe)
Swinging bucket rotor and tubes (e.g., Beckman SW50.1 rotor and 5-ml tubes)

1. Prepare and fractionate lysates as described (see Basic Protocol 2, steps 1 to 6), but use 10% and 25% sucrose gradient solutions and centrifuge 16 hr at 55,000 × g (20,000 rpm in SW28.1 rotor), 4°C.

2. Solubilize polysomal pellet in 0.4 ml TMK_{100} buffer without detergent.

   To obtain other complexes (such as individual subunits or monosomes), the relevant fractions from the 10% to 25% gradient are removed from the fractionator and placed on ice as soon as they are collected. The pooled fractions are then diluted in TMK_{100} lysis buffer.

3. Wash ribosomes and concentrate by centrifuging through 22% (w/v) sucrose solution in 5-ml tubes for 16 hr at 110,000 × *g* (34,000 rpm in an SW50.1 rotor), 4°C.

   This pellet contains all complexes larger than 40S.

4. Dissolve pelleted ribosomes and subunits in 0.25 M sucrose solution and store up to 2 years in liquid nitrogen.

PREPARATION OF YEAST LYSATES

SUPPORT PROTOCOL 3

Preparation of lysates from yeast follows general, previously described methods (Baim et al., 1985; Moritz et al., 1991).

Materials

Yeast culture
Breaking buffer (see recipe)
Dry acid-washed glass beads (425- to 600-µm; Sigma)
1 mg/ml cyclohexamide

Spectrophotometer

Additional reagents and equipment for growing yeast (*UNIT 1.6*)

1. Grow 100-ml yeast cultures to an OD_{600} of ~1 (log phase; *UNIT 1.6*).
2. Pellet cells by centrifuging 5 min at 1500 × *g*, 4°C.
3. Resuspend pellet in 5 ml sterile 4°C water containing 100 µg/ml cyclohexamide, incubate 1 min on ice, and pellet again.
4. Resuspend pellet in 500 µl breaking buffer and 400 µl acid-washed glass beads. Vortex eight times for 20 to 30 sec each, with 30-sec incubations on ice between vortexing steps.
5. Pellet beads and cellular debris in a refrigerated microcentrifuge 8 min at 10,000 × *g*, 4°C.
6. Transfer supernatant to a fresh tube and repeat centrifugation.
7. Measure the absorbance of an aliquot of the sample at 254 nm (nucleic acid).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acrylamide solution, 40% (w/v)

380 g acrylamide
20 g *N,N*-methlyenebisacrylamide
H_2O to 600 ml
Store up to 2 months at 4°C

Acrylamide/urea bottom solution, 8% (w/v)

40 ml 40% (w/v) acrylamide solution (see recipe)
90 ml 5× TBE electrophoresis buffer (*APPENDIX 2A*)
92 g urea
20 g sucrose
10 mg bromphenol blue
H_2O to 200 ml
Store up to 2 days at room temperature

Acrylamide/urea top solution, 8% (w/v)

100 ml 40% (w/v) acrylamide solution (see recipe)
50 ml 5× TBE electrophoresis buffer (*APPENDIX 2A*)
230 g urea
H_2O to 500 ml
Store up to 2 days at room temperature

ATP, 100 mM

Dissolve ATP to 100 mM in 20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*). Check the concentration by measuring A_{259}. Store 100-µl frozen aliquots, preferably at −80°C, for up to 6 months.

A 1:100 dilution of this solution (1 mM) should have an absorbance of 15.4 at 259 nm.

Breaking buffer

20 mM HEPES·KOH, pH 7.4
2 mM $MgCl_2$
100 mM KCl
14.4 mM 2-mercaptoethanol
100 µg/ml cyclohexamide
Store up to 2 weeks at −70°C

GTP, 100 mM

Dissolve GTP to 100 mM in 20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*). Check the concentration by measuring A_{252}. Store 100-µl aliquots frozen, preferably at −80°C, for up to 6 months.

A 1:100 dilution (1 mM) should have an absorbance of 13.7 at 252 nm.

$MgCl_2$, 100 mM

Since most bottles of $MgCl_2$ tend to contain visible H_2O (i.e., the crystals appear wet), assume that the original manufacturer's weight is correct and dissolve the entire contents of the bottle in H_2O to the desired concentration.

Recently, the authors have substituted magnesium acetate [$Mg(CH_3COO)_2$] for $MgCl_2$, as $Mg(CH_3COO)_2$ comes as a dry powder. However, since acetate is a great carbon source for bacteria or fungi, the solution should be autoclaved and stored cold.

RNA-loading buffer

20 mM MOPS, pH 7.0
8 mM sodium acetate
1 mM EDTA
50% (v/v) formamide
6.5% (v/v) formaldehyde
5% (v/v) glycerol
0.025% (w/v) bromphenol blue
0.025 (w/v) xylene cyanol
Store up to 2 months at 4°C

SDS sample buffer

1 mM dithiothreitol
80 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
0.002% (w/v) bromphenol blue
2% (w/v) sodium dodecyl sulfate (SDS)
10% (v/v) glycerol
Store up to 6 months at −20°C

Standard sucrose solution

0.25 M sucrose
20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.1 mM EDTA
1 mM dithiothreitol
Store up to 6 months at −20°C

Subunit buffer

50 mM HEPES·KOH, pH 7.5
2 mM $Mg(CH_3COO)_2$
1 mM puromycin
500 mM KCl
Prepare fresh

If necessary, may be stored 1 day at 4°C

Sucrose gradient solutions, 5% and 20% (w/v)

5% or 20% (w/v) sucrose
50 mM HEPES·KOH, pH 7.5
5 mM $Mg(CH_3COO)_2$
2 mM dithiothreitol
500 mM KCl
0.1 mM EDTA
Prepare fresh immediately before use

These solutions are used to prepare gradients for isolating 40S and 60S ribosomal subunits (see Support Protocol 1).

Sucrose gradient solutions, 10% and 35% (w/v)

10% or 35% (w/v) sucrose
60 mM NaCl
6 mM $Mg(CH_3COOH)_2$
50 mM Tris·Cl, pH 8.4 (*APPENDIX 2A*)
10 mM dithiotreitol
Prepare fresh immediately before use

The solutions are used to prepare gradients for toeprinting (see Alternate Protocol 3).

Sucrose gradient solutions, 10% and 40% (w/v)

10% or 40% (w/v) sucrose
20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 mM KCl
5 mM $MgCl_2$
100 μM GDPNP
Prepare fresh immediately before use

These solutions are used to prepare gradients for isolating ribosomal complexes (see Basic Protocol 1 and Alternate Protocols 1 and 2). They may be stored overnight at 4°C, but GDPNP should be added just before use.

Sucrose gradient solutions, 10% and 50% (w/v)

10% or 50% (w/v) sucrose
100 mM KCl
5 mM $MgCl_2$
2 mM dithiothreitol
20 mM HEPES·KOH, pH 7.4
Prepare in diethylpyrocarbonate (DEPC)–treated water (*APPENDIX 2A*)
Store up to 3 months at 4°C

These solutions are used for analyzing translation in cultured cells (see Basic Protocol 2). General procedures to limit contamination with exogenous nucleases (use of gloves, buffer preparation with ultrapure reagents and DEPC-treated water; see APPENDIX 2A) should be employed at all times.

Sucrose solution, 22% (w/v)

22% (w/v) sucrose
100 mM KCl
5 mM $MgCl_2$
2 mM dithiothreitol
20 mM HEPES/KOH, pH 7.4
Prepare in diethylpyrocarbonate (DEPC)-treated water (*APPENDIX 2A*)
Prepare immediately before use

This solution is used for preparing ribosomal complexes for biochemical assays (see Alternate Protocol 4).

Sucrose solution, 0.25 M

0.25 M sucrose
1 mM dithiothreitol
0.2 mM EDTA
10 mM KCl
1 mM $Mg(CH_3COO)_2$
Store up to 6 months at −20°C

TMK_{100} ($Tris/Mg^{2+}/K^+$) lysis buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
5 mM $MgCl_2$
100 mM KCl
1% (v/v) Triton X-100
0.5% (w/v) deoxycholate
Prepare in diethylpyrocarbonate (DEPC)–treated water (*APPENDIX 2A*)
1 U/ml placental ribonuclease inhibitor, added fresh just before use
2 mM dithiothreitol, added fresh just before use
Store up to 3 to 6 months at 4°C

General procedures to limit contamination with exogenous nucleases (use of gloves, buffer preparation with ultrapure reagents and DEPC-treated water; see APPENDIX 2A) should be employed at all times.

COMMENTARY

Background Information

Translation of an mRNA can be conveniently separated into several discrete steps. Translation initiation is the sequential association of an mRNA with a 40S ribosomal subunit to form a 48S preinitiation complex, which is followed by joining of the 60S subunit to form an 80S ribosome. This complex can then initiate the repetitive cycle of tRNA binding, peptide bond formation, and translocation that defines elongation. Repeated episodes of initiation and elongation on an individual mRNA result in the formation of polysomes, which can include over fifteen translating ribosomes per

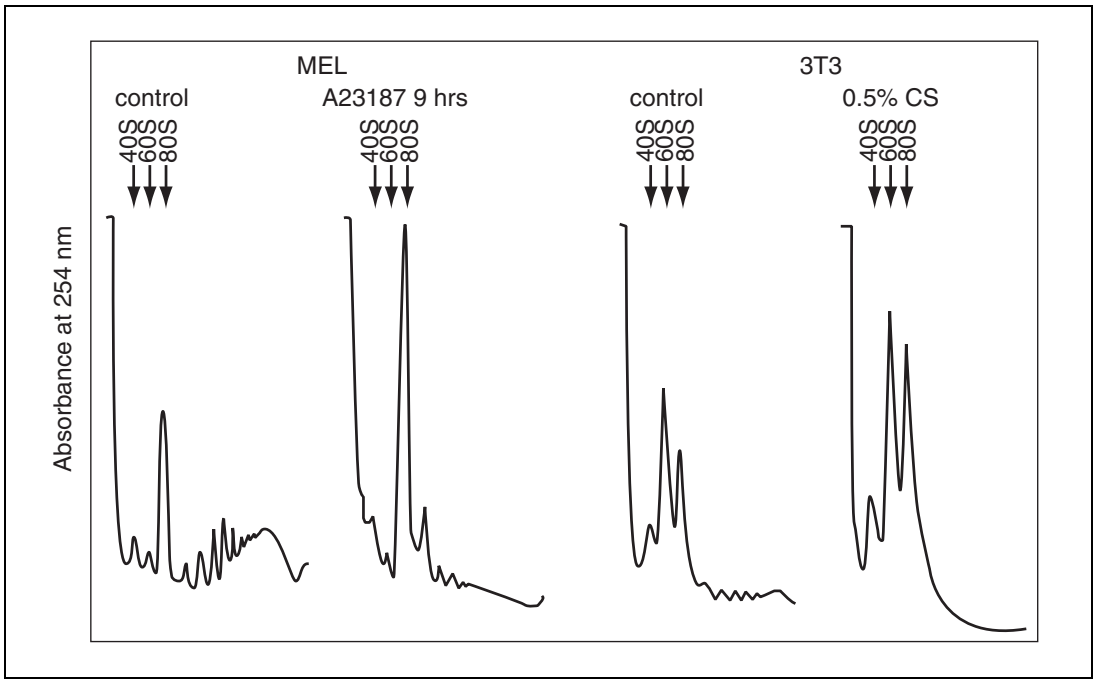


Figure 11.9.4 Serum-starved 3T3 cells and erythroleukemic cells exposed to inducers of differentiation demonstrate evidence of a decrease in the rate of translation initiation. Murine erythroleukemic (MEL) cells were grown for 9 hr in the presence or absence of the inducer of differentiation A23187 (0.75 μg/ml). BALB/c 3T3 cells were grown to confluence, and then either (1) replated in fresh medium containing 10% calf serum (control), or (2) washed with PBS, refed with medium containing 0.5% calf serum (0.5% CS), and incubated for an additional 18 hr. Cell lysates were prepared for analysis on 10% to 50% sucrose gradients as described in the text. Gradients were collected with an ISCO 640 gradient fractionator with continuous monitoring of the UV absorbance at 254 nm (Fig. 11.9.1A). Positions of 40S, 60S, and 80S ribosomal peaks are indicated by arrows above each tracing. The direction of sedimentation is from right to left.

mRNA. Due to the quantal increase in complex size as 40S and 60S subunits are added to an individual mRNA, it is possible to identify a number of different translational complexes by sucrose gradients. Using the techniques described here, it is possible to resolve a number of different complexes including uninitiated mRNAs (such as 7S globin mRNAs), 40S subunits (43S complexes), 48S preinitiation complexes, 60S subunits, 80S ribosomes, and polysomal complexes containing up to eight to ten ribosomes/mRNA.

Interpreting results of sucrose gradient separation of cell lysates

The distribution of ribosomes or mRNAs across a gradient ultimately reflects the combined rates of initiation, elongation, and termination of translation. Therefore, it is possible for different combinations of effects on these processes to produce similar changes in distribution. For example, a decrease in initiation rate would be expected to decrease the size of the average polysome as well as to decrease the total amount of polysomes (and polysomal mRNAs; e.g., Fig. 11.9.4). However, a similar result would be expected if initiation rate remained constant while elongation and termination rates were increased. Therefore, the results are best interpreted in the context of an independent determinant of translation, for example, as determined by the incorporation of radioactive amino acids into protein.

Suggested approaches for demonstrating physical association of mRNAs or proteins with ribosomes

To demonstrate that the proteins or RNAs found in individual fractions are in fact components of ribosomal complexes, several different approaches are commonly used. Digestion of extracts with RNase prior to their sedimentation can be used to confirm that the identified proteins are components of a ribonucleoprotein (RNP) complex. However, this approach does not distinguish between different types of RNPs and is not useful if the component of interest is itself an RNA. A more generally

useful approach takes advantage of the magnesium requirement for formation of higher-order ribosomal structures (Spirin, 1986). Addition of EDTA (5 mM in excess of $MgCl_2$) to the gradients results in complete dissociation of polyribosomes, 80S ribosomal complexes, and 40S ribosomal/mRNA preinitiation complexes (and also dissociates the 5S and 5.8S RNAs from the 60S ribosomal subunit). However, since most RNA/protein interactions are not dependent on magnesium, the presence of EDTA does not disrupt sedimentation of less complex RNPs. EDTA (10 mM) may be added to the postnuclear supernatant prior to loading on the gradient. (Its presence before separation of the nuclei results in nuclear dissolution.) An example of the effect of EDTA on ribosome sedimentation in 10% to 25% gradients is shown in Fig. 11.9.3.

A functional approach to demonstrate translation of an mRNA that cosediments with polysomes is to determine the sensitivity of the complex to puromycin. This inhibitor occupies the "A" site of the elongating ribosome, terminates translation, and causes release of the prematurely terminated peptide. Puromycin treatment therefore results in the "collapse" of polysomes, with an associated increase in monosomes and disomes, as determined by UV absorbance of the fractionated RNA. A similar effect should be evident for individual mRNAs in the fractions. For the purposes of these experiments, a 10-min incubation at 37°C in normal growth medium containing 100 µM puromycin is sufficient to observe the effects of this agent on translation.

As an alternative approach to determining translational activity, the stability of the ribosomal complexes in high concentrations of monovalent cations has occasionally been employed. This technique derived from the early observations that: (1) 80S fractions frequently contain a high content of ribosomal subunits, (2) radioactive amino acid incorporation does not occur in 80S fractions (Warner et al., 1963), and (3) 80S ribosomes can be dissociated into 40S and 60S complexes by sedimentation in 500 mM KCl (Martin and Hartwell, 1970; Zylber and Penman, 1970; Martin, 1973). On the basis of these observations, salt-labile subunits were considered to be "vacant couples" that did not contain bound mRNA. The addition of KCl at a concentration of 500 mM to the standard gradient conditions described in these protocols is sufficient to dissociate 80S ribosomal complexes in both mammalian cells and yeast, while polyribosomes remain intact (Martin and Hartwell, 1970; Zylber and Penman, 1970; Martin, 1973). However, while published data from this laboratory is consistent with the observations that salt-labile 80S complexes are translationally inactive, the authors have demonstrated that these complexes contain mRNA stably bound to the 40S ribosomal subunit (Hensold et al., 1996). Thus, while stability in 500 mM KCl can be used as a surrogate marker for translational activity, it cannot be assumed that these complexes do not contain mRNA, although the significance of these 80S complexes remains to be established.

When assessing the presence of a protein in a given ribosomal complex, it is important to keep in mind that the absence of a protein from a fraction does not exclude its existence in that complex. For example, elongation factors are not routinely found in polysomal fractions. Thus, loosely adherent proteins may be lost during sedimentation through sucrose or under the conditions used for lysis.

Critical Parameters

Cell translation is rapidly responsive to a variety of signals that are generated from both external and internal signaling pathways (Hershey, 1991; Merrick, 1992). Thus, care must be taken to ensure that extract preparation does not superimpose independent effects upon the cells to be assayed. For this reason, as a first step in the preparation of extracts, cells should be rapidly equilibrated in ice-cold PBS to cause a general arrest of cellular processes. This also serves to inhibit endogenous nucleases that may be released during extract preparation. Thus, it is critical that all steps in extract preparation are performed on ice or in refrigerated centrifuges. While some protocols for polysome analysis include cyclohexamide (50 to 100 µg/ml) to prevent "run-off translation" from polysomes, this is unnecessary if the extracts are maintained at 4°C during their preparation. Further, transient elevations in temperature that occur while the cells are exposed to cyclohexamide results in polysome profiles with characteristics of inhibited elongation. Thus, if cyclohexamide is included in buffers, precautions must be taken to avoid exposure to temperatures above 4°C.

The presence of ribonuclease inhibitors in the lysis buffer is also not absolutely necessary, since it is possible to fractionate intact RNA from MEL and 3T3 cells without added nuclease inhibitors as long as the extracts are prepared and fractionated at 4°C. For cell lines that have higher levels of endogenous RNases, lysis

buffer should be supplemented with placental ribonuclease inhibitor (1 U/μl) or RNase Block (Stratagene; Kaspar and Gehrke, 1994). The inclusion of deoxycholate in the lysis buffer increases the yield of polysomes, presumably due to the dissolution of the cytoskeleton that occurs in the ionic detergent. However, if ribosomal complexes are to be isolated for use in biochemical assays, it may be advisable to avoid this detergent, since it is more denaturing than nonionic detergents.

When assessing the distribution of mRNAs (or proteins) in gradient fractions, it is important that the amount of material recovered from each fraction be representative of the actual amount of material present in that fraction. As described here, this is ensured when care is taken to maintain equal volumes throughout all steps of the extractions. Further, it is equally important that the pellets be resuspended in a constant volume, and that either all of the material or a constant fraction of the volume be loaded onto gels, if the distribution of material is to be representative of its actual distribution across the gradient. Alternatively, a radiolabeled standard may be added to each fraction (such as an in vitro–transcribed RNA) prior to recovery of the RNA. Any variability in recovery of extracted RNA can then be corrected by loading equal amounts of the recovered radioactive RNA, as determined by scintillation counting of an aliquot of each fraction.

Troubleshooting

Translation in lysates

Translation in a system reconstituted from fractionated components is very inefficient and the molar yield of product is often on the same order of magnitude as the input level of ribosomes and factors. Reaction rates are also considerably reduced, especially for elongation. To circumvent this problem, unfractionated reticulocyte lysate can be used (see *UNIT 11.2*). However, in this case any added component must compete with the endogenous equivalent. Suggested approaches for the use of these lysates are discussed below.

The individual steps in initiation can be monitored by determining the binding of specific translation factors to either 40S, 60S, or 80S ribosomes. Purified factors can be radiolabeled by reductive methylation (Peterson et al., 1979a), or the factor may be translated in vitro in the presence of radioactive amino acids (Joshi et al., 1994). Alternatively, association of factors with specific translational complexes could be monitored by immunoblotting (*UNIT 6.2*), as previously described. Met-tRNA$_i$ may be monitored by addition of exogenously labeled [^{35}S]Met-tRNA$_i$. However, the aminoacyl linkage in Met-tRNA$_i$ is one of the least stable and generally has a half-life of ~8 to 10 min. Thus, under normal protein synthesis conditions, the percentage of radioactive Met-tRNA$_i$ falls quite rapidly. The stability of the initiator tRNA can be increased considerably by the addition of a formyl group (using *E. coli* transformylase) to yield fMet-tRNA$_i$, but this initiator tRNA is utilized about a third as well as the normal Met-tRNA$_i$.

For the analysis of mRNAs, radioactive mRNAs can be conveniently transcribed in vitro using bacterial polymerases (SP6, T7, or T4) in the presence of an mRNA cap analog, m^{7}GpppG. By setting the ratio of cap analog to GTP at 5:1 to 10:1, most of the transcripts emerge with an m^{7}G cap. The use of lysates that have been treated with micrococcal nuclease (Pelham and Jackson, 1976) reduces the level of endogenous mRNAs to the point where there is little competition for translation as long as the level of added transcript is <1 μg. The use of in vitro–transcribed RNAs allows one to systematically alter the RNA sequence/structure to determine the influence of either specific structural elements (stem-loops of specific sequences) or context (i.e., the nucleotides surrounding the AUG start codon) on the formation of 40S or 80S initiation complexes. If desired, the binding site of the ribosome/subunit to the mRNA can be mapped by toeprinting, as described previously (Anthony and Merrick, 1992). Both the 40S and 80S regions can be toeprinted. While it might appear ideal to perform toeprinting in the original translation mixture without gradient fractionation, it has been observed that it is not possible to reverse transcribe in the crude lysate. This may be due to the presence of phosphatases or other undefined inhibitors in the lysate.

Given that lysates contain all the ingredients necessary for complete polypeptide chain synthesis, normally most of the components end up in polysomes, and thus it is difficult to identify useful intermediate complexes. In this case, the use of inhibitors may facilitate their identification (Peterson et al., 1979a,b; Safer et al., 1979). To block the joining of the 40S and 60S subunits, the lysates can be supplemented with the drug edeine (which is increasingly difficult to obtain) or by the inclusion of GDPNP (as subunit joining requires the hydrolysis of the GTP in the ternary complex

eIF2-GTP-Met-tRNA$_i$). The presence of GDPNP also ensures that no elongation will take place. In the presence of these inhibitors, these experiments yield complexes that are either 40S, 43S, or 48S. Similarly, to monitor the flow of components (mRNA or Met-tRNA$_i$) into 80S complexes, anisomycin (an inhibitor of elongation) is used. It should be mentioned that in these studies with a specific inhibitor there will be a relative buildup of complexes at or before the point of the block. Inhibitors are therefore useful in magnifying the signal at individual steps.

Translation in intact cells

The most common problem in using gradients to analyze translation in cells is degradation of the RNA. If this occurs during extract preparation, the result will be a decrease in the amount of polysomal material, due to cleavage of the mRNA between translating ribosomes. If this is severe, it may be apparent on the UV absorbance tracing during collection of the gradient. Lesser degrees of degradation may only become apparent during subsequent gel electrophoresis of the RNA and be detected as widespread "nicking" of the rRNA by ethidium bromide staining of the gel. More subtle nicking may only be detected following northern blot hybridization and visualization of individual mRNAs. Suggestions to decrease nuclease activity during extract preparation are discussed. It is worth reemphasizing that keeping the cells and extracts on ice and precooling the gradients and centrifuge to 4°C before centrifugation provides an important safeguard against nuclease activity, even in the absence of exogenously added RNAse inhibitors.

If nicking is detectable in only one or two fractions, this is usually due to a nuclease that was inadequately removed following phenol and chloroform extraction of the gradient fractions. Ribonuclease can be recovered from polysome preparations (Caruccio and Ross, 1994), so the most likely cause of this problem is recovery of an incompletely (or reversibly) denatured nuclease from the interface during the phenol and chloroform extractions. In this case, greater care should be taken to avoid disturbing the interface, including removing a smaller volume of the aqueous fraction. In either case, if evidence of even partial RNA degradation is detected, the gradients should be repeated, since any conclusions obtained from even partially degraded complexes would be invalid.

When recovering proteins from TCA precipitates of gradient fractions, difficulty is sometimes encountered in solubilizing the proteins in the first one or two fractions. This is generally due to the high concentration of protein in these fractions. Complete solubilization may not be possible in the small volume of sample buffer necessary for loading the entire sample in a single well of a gel. In this case, it is best to increase the volume to ensure complete solubilization of the proteins and load a predetermined percentage of this volume in the well. This should then be taken into account when quantifying the results determined by immunoblotting.

During preparation of cell extracts, nuclear dissolution may occur due to the presence of the ionic detergent deoxycholate. This will be manifest as an increase in viscosity of the extract and the lack of a detectable nuclear pellet following centrifugation of the initial lysate. The concentration of deoxycholate used in Basic Protocol 2 was empirically determined for MEL cells, and cells differ in their sensitivity to this detergent. Thus, while 1% Triton X-100/0.5% deoxycholate is a useful starting point, the optimal concentration for other cells may need to be empirically determined. In addition, since mixed micelles of Triton X-100 and deoxycholate are formed in the extraction buffer, it is important that these detergents be well mixed in the lysis buffer before addition to the cells.

Anticipated Results

In analyzing the distribution of ribosomes or mRNA in cells, it is important to avoid preconceived notions of how the profile should appear. Most knowledge of translational mechanisms derives from insights gained from studies in reticulocyte lysates, and although these observations have been generally applicable to cells, it is likely that additional mechanisms of regulation have yet to be identified. For example, while phosphorylation of ribosomal protein S6 is one of the most highly conserved sequelae of mitogenic stimulation, the biochemical effect of this modification has yet to be conclusively established. Recent evidence suggests that this may influence translation of mRNAs that have a 5′-terminal oligopyrimidine tract (5′-TOP; Jefferies et al., 1994).

Changes in translation rate may also be due to changes in total ribosomal content (Rudland and de Asua, 1979). Recent studies in yeast suggest that ribosomal abundance may differentially affect mRNA translation (Proweller and Butler, 1997). However, unless care is taken

to standardize the amount of lysate loaded for total cell number, changes in cellular ribosomal abundance would not be detected by assessing the distribution of ribosomes across a gradient. In this case, it may be best to use alternative means to quantitate changes in ribosome content, such as blot hybridization with radiolabeled probes to rRNAs. In this case, RNA extractions and gel loading should be standardized for cell number.

Cell type differences in translational regulation are also likely to occur. The expression of the two isoforms of eIF4A demonstrate tissue-specific differences in their expression (Nielsen and Trachsel, 1988), and the mRNA levels of eIF4B and eIF4H are quite variable in different tissues (Richter et al., 1999). In addition, translational regulation may be abnormal in transformed cells (Rinker-Schaefer et al., 1992; Sonenberg, 1993) and thus in immortal cell lines. To emphasize these cell type differences in the distribution of ribosomes in sucrose gradients, Figure 11.9.4 illustrates differences between two murine cell lines. These differences are independent of cell growth conditions and cannot be attributed to differences in commonly identified means of translational regulation: phosphorylation of the initiation factors eIF2, eIF4E, or the 4E-binding protein PHAS I. Analysis of other cell types in this laboratory has identified at least two other distinct patterns of ribosomal distribution in mammalian cell lines. It is likely that the continued analysis of cellular growth and differentiation will provide insights into novel mechanisms of translational regulation.

Time Considerations

The time required for completion of these experiments will be largely dependent on the centrifugation time, since the 3 to 16 hr required for this step will be longer than any of the preceding or ensuing steps. For analyzing translation in cells, the preparation of the extracts should take 15 to 20 min, and a similar period of time should be allotted for pouring the gradients. Gradients can be poured the preceding day and stored at 4°C overnight without any ill effects. The time required for extraction of RNA or protein from the gradient fractions will depend on the number of gradients and the number of fractions collected from each gradient. In general, this should take 1 to 3 hr for most experiments.

Literature Cited

Anthony, D.D. and Merrick, W.C. 1992. Analysis of 40S and 80S complexes with mRNA as measured by sucrose density gradients and primer extension inhibition. *J. Biol. Chem.* 267:1623-1632.

Baim, S., Pietras, D., Eustice, D., and Sherman, F. 1985. A mutation allowing an mRNA secondary structure diminishes translation of *Saccharomyces cerevisiae* iso-1-cytochrome C. *Mol. Cell. Biol.* 5:839-1846.

Beckman Instruments. Rotors and Tubes for Preparative Ultracentrifuges. Beckman Instruments, Palo Alto, Calif.

Benne, R. and Hershey, J.W.B. 1978. The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes. *J. Biol. Chem.* 253:3078-3087.

Caruccio, N. and Ross, J. 1994. Purification of a human polyribosome-associated 3′ to 5′ exoribonuclease. *J. Biol. Chem.* 269:31814-31821.

Grifo, J.A., Tahara, S.M., Morgan, M.A., Shatkin, A.J., and Merrick, W.C. 1983. New initiation factor activity required for globin mRNA translation. *J. Biol. Chem.* 258:5804-5810.

Hensold, J., Barth-Baus, D., and Stratton, C. 1996. Inducers of erythroleukemic differentiation cause mRNAs that lack poly(A)-binding protein to accumulate in translationally inactive, salt-labile 80S ribosomal complexes. *J. Biol. Chem.* 271:23246-23254.

Hershey, J. 1991. Translational control in mammalian cells. *Annu. Rev. Biochem.* 60:717-755.

Jefferies, H., Reinhard, C., Kozma, S., and Thomas, G. 1994. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proc. Natl. Acad. Sci. U.S.A.* 91:4441-4445.

Joshi, B., Yan, R., and Rhoads, R. 1994. In vitro synthesis of human protein synthesis initiation factor 4g and its localization on 43 and 48S initiation complexes. *J. Biol. Chem.* 269:2048-2055.

Kaspar, R. and Gehrke, L. 1994. Peripheral blood mononuclear cells stimulated with C5a or lipopolysaccharide to synthesize equivalent levels of IL-1β mRNA show unequal IL-1β protein accumulation but similar polyribosome profiles. *J. Immunol.* 153:277-286.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Martin, T. 1973. A simple general method to determine the proportion of active ribosomes in eukaryotic cells. *Exp. Cell. Res.* 80:496-498.

Martin, T. and Hartwell, L. 1970. Resistance of active yeast ribosomes to dissociation by KCl. *J. Biol. Chem.* 245:1504-1508.

McEwen, C.R. 1967. Tables for estimating sedimentation through linear concentration gradients of sucrose solution. *Anal. Biochem.* 20:114-149.

Merrick, W.C. 1979a. Purification of protein synthesis initiation factors from rabbit reticulocytes. *Methods Enzymol*. 60:101-108.

Merrick, W.C. 1979b. Assays for eukaryotic protein synthesis. *Methods Enzymol.* 60:108-123.

Merrick, W. 1992. Mechanism and regulation of eukaryotic protein synthesis. *Microbiol. Rev*. 56: 291-315.

Merrick, W.C. and Anderson, W.F. 1975. Purification and characterization of homogeneous protein synthesis initiation factor M1 from rabbit reticulocytes. *J. Biol. Chem.* 250:1197-1206.

Moritz, M., Pulaski, B., and Woolford, J. 1991. Assembly of 60S ribosomal subunits is perturbed in temperature-sensitive yeast mutants defective in ribosomal protein L16. *Mol. Cell Biol*. 11:5681-5692.

Nielsen, P. and Trachsel, H. 1988. The mouse protein synthesis initiation factor 4A gene family includes two related functional genes which are differentially expressed. *EMBO J.* 7:2097-2105.

Pelham, H.R.B. and Jackson, R.J. 1976. An efficient mRNA-dependent translation system from rabbit reticulocyte lysates. *Eur. J. Biochem*. 67:247-256.

Peterson, D.T., Merrick, W.C., and Safer, B. 1979a. Binding and release of radiolabelled eukaryotic initiation factors 2 and 3 during 80S initiation complex formation. *J. Biol. Chem*. 254:2509-2516.

Peterson, D.T., Safer, B. and Merrick, W.C. 1979b. Role of eukaryotic initiation factor 5 in the formation of 80S initiation complexes. *J. Biol. Chem*. 254:7730-7735.

Proweller, A. and Butler, S. 1994. Efficient translation of poly(A)-deficient mRNAs in *Saccharomyces cerevisiae*. *Genes & Devel*. 8:2629-2640.

Proweller, A. and Butler, S. 1997. Ribosome concentration contributes to discrimination against poly(A)-mRNA during translation initiation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 272:6004-6010.

Richter, N.J., Rogers, G., Hensold, J.O., and Merrick, W.C. 1999. Further biochemical and kinetic characterization of human eukaryotic initiation factor 4H. *J. Biol. Chem*. 274:35415-35424.

Rinker-Schaeffer, C., Austin, V., Simmer, S., and Rhoads, R. 1992. Ras transformation of cloned rat embryo fibroblasts results in increased rates of protein synthesis and phosphorylation of eukaryotic initiation factor 4E. *J. Biol. Chem.* 267:2593-2598.

Rudland, P. and de Asua, L.J. 1979. Action of growth factors in the cell cycle. *Biochim. Biophys. Acta* 560:91-133.

Safer, B., Jagus, R., and Kemper, W.M. 1979. Analysis of initiation factor function in highly fractionated and unfractionated reticulocyte lysate systems. *Methods Enzymol*. 60:61-87.

Sonenberg, N. 1993. Translation factors as effectors of cell growth and tumorigenesis. *Curr. Opin. Cell Biol*. 5:955-960.

Spirin, A. 1986. Ribosome Structure and Protein Biosynthesis. Benjamin/Cummings, Menlo Park, Calif.

Steel, L. and Jacobson, A. 1987. Translational control of ribosomal protein synthesis during early *Dictylostelium* development. *Mol. Cell. Biol.* 7:965-972.

Warner, J., Knopf, P., and Rich, A. 1963. A multiple ribosome structure in protein synthesis. *Proc. Natl. Acad. Sci. U.S.A*. 49:122-129.

Zylber, E. and Penman, S. 1970. The effect of high ionic strength on monomers, polyribosomes and puromycin-treated polyribosomes. *Biochim. Biophys. Acta* 204:221-229.

Contributed by William C. Merrick and Jack O. Hensold
Case Western Reserve University School of Medicine
Cleveland, Ohio

Preparation and Use of Interphase *Xenopus* Egg Extracts

Xenopus interphase egg extracts (see Basic Protocol 1 and Alternate Protocol 1) are useful as sources of cytoplasmic and organelle material for in vitro reconstitution of cellular events such as nuclear assembly (see Basic Protocol 2) from demembranated sperm chromatin (see Support Protocol 2), nuclear protein import (see Basic Protocol 3), and nuclear DNA replication (see Basic Protocol 4 and Alternate Protocol 2). To characterize the molecular bases of these and other processes, the extracts can be modified by immunodepletion (see Support Protocol 3) or protein addition (see Support Protocol 4). Eggs are obtained from primed female frogs (see Support Protocol 1). These extracts can also be modified to support cycling (see *UNIT 11.11*) and apoptosis (see *UNIT 11.12*). To study meiotic maturation, extracts can be prepared from oocytes (see Basic Protocol 5).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

PREPARATION OF INTERPHASE EGG EXTRACTS

BASIC PROTOCOL 1

Prior to preparation of an interphase extract, the frog is injected and eggs are laid overnight in 100 mM NaCl (see Support Protocol 1). The mature *Xenopus* eggs are arrested in metaphase of meiosis II and therefore have high mitotic kinase or cyclin B/cdc2 activity. Egg lysis by centrifugation causes a transient release of Ca^{2+} from intracellular stores, resulting in proteolysis of cyclin B and subsequent loss of cdc2 kinase activity. After inactivation of cyclin B/cdc2, the extract moves from M phase to S phase. Addition of cycloheximide to the extract prevents synthesis of any new proteins, most notably cyclin B. Therefore, the extract remains in interphase of the cell cycle. Upon addition of sperm chromatin and an ATP regenerating system, membrane vesicles present in the extract fuse to the chromatin to form interphase nuclei (see Basic Protocol 2) which are capable of replicating their DNA (see Basic Protocol 4) and have intact nuclear pore complexes.

Materials

Eggs obtained from female frogs, in 100 mM NaCl (see Support Protocol 1)
2% (w/v) L-cysteine free base (Sigma) in H_2O
0.25× modified Ringer's solution (MMR), pH 7.7 (see recipe for 10×)
Egg lysis buffer, pH 7.7 (see recipe)
5 mg/ml cytochalasin B (Calbiochem) in DMSO
5 mg/ml aprotinin (Roche Diagnostics) in H_2O
5 mg/ml leupeptin (Roche Diagnostics) in H_2O
10 mg/ml cycloheximide in H_2O

200-ml beaker
100-mm glass petri dish
Pasteur pipets
Dissecting microscope
15-ml conical polypropylene centrifuge tubes
IEC Clinical centrifuge
Sorvall HB-4 swinging bucket rotor
18-G needle attached to 5-ml syringe

Contributed by Maureen Powers, Erica K. Evans, Jing Yang, and Sally Kornbluth
Current Protocols in Cell Biology (2001) 11.10.1-11.1.24

Wash and dejelly eggs

1. Collect eggs in a 200-ml glass beaker and pour off any excess solution.
2. Rinse eggs once in 2% L-cysteine, pH 8.0, then continue to incubate in 100 ml of 2% L-cysteine solution per frog for ~5 min at room temperature, gently swirling the eggs periodically.

   Before incubation with cysteine solution, a transparent jelly coat, which prevents the eggs from directly contacting each other, will surround each individual egg. Once eggs are dejellied, they will pack closely together

3. Pour the cysteine solution off of the dejellied eggs and wash eggs in 0.25× MMR by decantation to remove debris. Repeat 2 times. Pour eggs into a 100-mm glass petri dish.
4. Preferably working under a dissecting microscope, remove bad eggs from the petri dish with a Pasteur pipet.

   Mature Xenopus eggs have a darkly pigmented half on top and a light half on the bottom. The dark half of the egg should have a small white spot present in the middle created by breakdown of the oocyte nucleus (germinal vesicle breakdown) during the process of oocyte maturation. This spot can be seen by eye but is more easily seen using a dissecting microscope. Anything without this white spot is an oocyte and should be removed. Any eggs that are white, enlarged, or not uniformly pigmented should also be removed.

5. Wash eggs 2 to 3 times, each time by pouring egg lysis buffer on them and carefully pouring the buffer off, minimizing the number of eggs that are spilled out.

Lyse eggs

6. Transfer eggs with an inverted Pasteur pipet (with the bulb on the thin, pointed end) into a 15-ml polypropylene tube.

   The Pasteur pipets must be inverted because a wide-mouthed pipet is required to avoid lysing the eggs.

7. Centrifuge eggs 15 sec in a clinical centrifuge at 400 × *g*. Remove extra buffer from the top of the packed eggs by decantation.

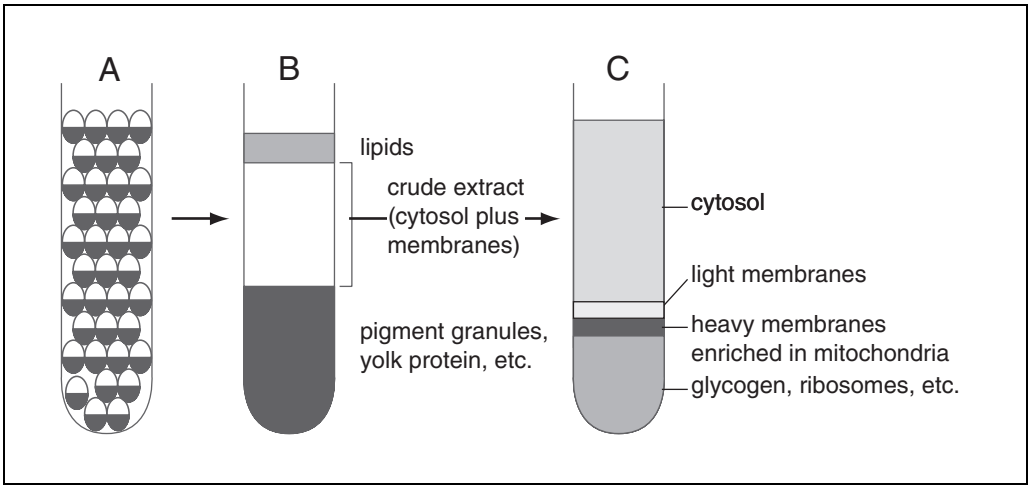


Figure 11.10.1 Preparation of the interphase egg extract. Eggs are packed in tubes by low-speed centrifugation (**A**) and then are centrifuged further at 12,000 × *g* to produce layers containing lipids, crude extract, and pigment granules (**B**). The crude extract can be further fractionated into cytosol, light membranes, and a heavy membrane fraction enriched in mitochondria (**C**).

8. On the basis of the volume of the packed eggs, add the following directly to the top of the packed eggs:

   5 µg/ml aprotinin (1:1000 dilution of 5 mg/ml stock)
   5 µg/ml leupeptin (1:1000 dilution of 5 mg/ml stock)
   5 µg/ml cytochalasin B (1:1000 dilution of 5 mg/ml stock)
   5 µl/ml of 10 mg/ml cycloheximide (50 µg/µl final)

   Aprotinin and leupeptin inhibit proteases, cytochalasin B inhibits actin polymerization, and cycloheximide inhibits protein synthesis.

9. Lyse eggs by centrifuging 15 min at 12,000 × *g*, 4°C, in Sorvall HB-4 swinging bucket rotor.

   The lysed eggs will be separated into three layers, a yellow lipid layer on top, the crude interphase extract in the middle, and a dark pellet (see Fig. 11.10.1B).

Collect extract

10. Remove the crude interphase extract by piercing the side of the polypropylene tube with an 18-G needle attached to a 5-ml syringe.

    The crude extract contains cytoplasm, light membrane from the ER and nuclear envelope, ribosomes, and heavy membrane organelles including mitochondria.

    When incubated at room temperature in the presence of sperm chromatin and an ATP-regenerating cocktail, the crude extract supports nuclear reconstitution (see Basic Protocol 2). However, this extract will not support nuclear assembly after freeze/thawing.

    The extract can be stored ~1 hr on ice.

PREPARING FRACTIONATED INTERPHASE EXTRACTS

ALTERNATE PROTOCOL 1

Using the following protocol, the crude interphase egg extract may be separated by high-speed centrifugation into discrete cytoplasmic, light-membrane, and heavy-membrane (mitochondrially-enriched) fractions. The cytosolic and light-membrane fractions can be snap-frozen in liquid nitrogen, stored at −80°C, and later reconstituted with sperm chromatin for nuclear assembly.

Additional Materials *(also see Basic Protocol 1)*

Crude interphase extract (see Basic Protocol 1)
2.5-ml Ultraclear centrifuge tubes
TLS centrifuge and TLS-55 rotor (Beckman) or equivalent

1. If this has not been done already (see Basic Protocol 1, step 8), supplement the isolated crude interphase extract with aprotinin and leupeptin (1:1000 dilution of 5 mg/ml stock) and cytochalasin B (1:1000 dilution of 5 mg/ml stock).

2. Transfer extract to a 2.5-ml Ultraclear centrifuge tube. Centrifuge the crude extract 70 min in a Beckman TLS-100 centrifuge at 250,000 × *g* (55,000 rpm in TLS-55 rotor), 4°C.

   The extract will separate into a clear cytoplasmic portion with a pale yellow membrane fraction below. A dark membrane fraction consisting of mitochondria and other organelles will lay below the light membranes (Fig. 11.10.1C).

3. Keeping extract on ice, remove clear cytoplasmic fraction with 200-µl pipet tip, taking care not to disrupt the light membrane layer. Retain both the cytoplasmic fraction and membrane layer and keep on ice.

4. Centrifuge cytoplasmic fraction again, in a new 2.5-ml Ultraclear centrifuge tube, 25 min at 250,000 × *g*, 4°C. Collect the top cytoplasmic fraction as in step 3, divide into aliquots (50 or 100 µl/microcentrifuge tube), freeze in liquid nitrogen, and store at −80°C until ready for use.

   Steps 5 and 6 should be performed while the above centrifugation is in progress.

5. Using a micropipettor with a cut-off or wide-bore pipet tip, carefully remove the light membrane fraction from the membrane layer (step 3), leaving dark membranes undisturbed. Dilute all of the light membrane in 1.5 ml egg lysis buffer and store on ice until ready to perform step 7.

6. Add 0.42 g sucrose to 5 ml egg lysis buffer to create 2× sucrose/egg lysis buffer. Chill to 4°C

7. When ready to centrifuge the membranes, place the diluted membranes (step 5) into 2.5-ml Ultraclear centrifuge tube. Underlay the membranes with 2× sucrose/egg lysis buffer by releasing it from a Pasteur pipet at the bottom of the membrane-containing tube. Add enough buffer to fill the tube.

   A clear layer will easily be seen below the membranes.

   The 2× sucrose/egg lysis buffer acts as a cushion to remove any proteins peripherally or nonspecifically associated with the membranes.

8. Centrifuge 20 min at 26,000 × *g* in the TLS-55 rotor, 4°C.

   After centrifugation, the membrane will be in a pellet at the bottom of the tube.

9. Pipet off the clear buffer until only the membrane fraction remains in the tube. With a wide-bore pipet tip, divide membranes into aliquots (5 to 10 µl per 0.5-ml microcentrifuge tube), flash freeze, and store at −80°C until ready for use.

   If there are any contaminating mitochondria/heavy membranes in the light membrane fraction, they will appear as a dark spot in the center of the light membrane pellet. By gently flicking the tube, the light membrane will dislodge, leaving an adherent mitochondrial pellet. It is important to avoid contaminating the light membrane with this fraction; therefore, should any dark spot be apparent, carefully use a wide-bore pipet tip to remove the light membranes to a separate tube before aliquoting.

SUPPORT PROTOCOL 1

INJECTION OF FROGS TO OBTAIN EGGS

Xenopus laevis mature females can be obtained from a number of commercial sources, including Nasco, Xenopus I, and Xenopus Express (see *SUPPLIERS APPENDIX*). Frogs should be kept in clean, dechlorinated tap water, at a maximal density of one frog per liter (though lower densities are desirable). Although dechlorination can be achieved by storing water in an open tank for 24 hr with aeration, it is faster to pass the water through a carbon filter. Optimal egg production is maintained by housing frogs at 18°C. Frogs can be fed a variety of foods, but the easiest is frog brittle from Nasco or Purina trout chow (1 g/frog, 3 to 4 days/week). Water should be changed on the days following feeding.

To produce eggs, frogs are primed with pregnant mare serum gonadotropin and are then induced to lay eggs at least 2 days later by injection of human chorionic gonadotropin.

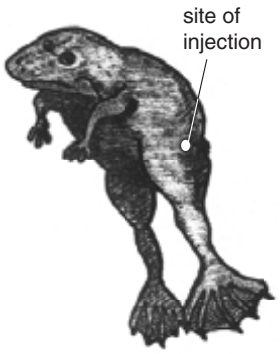


Figure 11.10.2 *Xenopus* injection site. The dorsal lymph sac, where the frog is injected, lies at the juncture of the torso and the upper portion of the leg. There is a loose fold of skin at this location under which the needle is slipped. Do not push the needle down into the muscle.

Materials

Female frogs, mature (e.g., Nasco, Xenopus I, or Xenopus Express)
100 mM NaCl (25 g NaCl/5 liters H_2O)
200 U/ml pregnant mare serum gonadotropin (PMSG; Calbiochem): reconstitute powder in sterile water to allow final injection volume of 0.5 ml (i.e., 200 U/ml final concentration); store in aliquots indefinitely at −20°C
1000 U/ml human chorionic gonadotropin (HCG; Sigma or Amersham/USB): reconstitute powder in sterile water to allow final injection volume of 0.5 ml (i.e., 1000 U/ml final concentration); store up to 1 month at 4°C
1× modified Ringer's solution (MMR; optional; see recipe)

Water tanks
1-ml syringes
25-G needles

1. For priming frogs, prepare a tank containing 100 mM NaCl to house frogs after injection.

   Primed frogs should be kept at a density of ~1 frog/liter.

2. Using a 25-G needle attached to a 1-ml syringe, inject frogs subcutaneously into the dorsal lymph sac (see Fig. 11.10.2) with 0.5 ml of 200 U/ml PMSG (total of 100 U). Return primed frogs to the tank prepared in step 1.
3. After a 2- to 3-day interval, induce egg laying by injecting frogs with 0.5 ml of 1000 U/ml HCG (total of 500 U), by the same route as PMSG injection (step 2). After injection, place each frog in 5 liters of 100 mM NaCl (or place the frog in 1× MMR if the eggs are to be used for preparation of cycling or CSF extracts; see *UNIT 11.11*).

   If more than one frog is injected, it is best to put each frog in its own container as the quality of eggs varies from frog to frog.

4. Approximately 16 to 22 hr after HCG injection (if frogs are kept at 18°C), remove the frog to a clean water tank and pour the eggs into a beaker.

   It is advisable to observe the frogs for 1 to 2 days for signs of infection at the site of injection.

5. Sort through the eggs and remove any bad eggs and oocytes.

   A "good egg" is one with even, unmottled pigmentation. A white pinpoint-sized spot, indicative of oocyte maturation, should be visible in the center of the darkly pigmented hemisphere of the mature egg. A particularly "bad" egg will appear as a white puffy ball, with the boundary between the hemispheres entirely obscured.

BASIC PROTOCOL 2

NUCLEAR ASSEMBLY IN THE INTERPHASE EGG EXTRACT

When sperm chromatin or other DNA templates (e.g., λ DNA) are added to interphase *Xenopus* egg extracts, membrane vesicles present in the extract bind to the surface of the chromatin, fuse, and incorporate nuclear pore components to form fully functional synthetic nuclei. These nuclei are competent to replicate their DNA (see Basic Protocol 4), transport macromolecules (see Basic Protocol 3), and undergo apoptotic nuclear fragmentation (see *UNIT 11.12*). This protocol describes the steps in setting up a nuclear assembly reaction using sperm chromatin as a template. This can be done using either crude interphase extract (Basic Protocol 1) or previously stored fractionated cytosol and light membranes (Alternate Protocol 1).

Materials

Interphase extract: crude (see Basic Protocol 1) or fractionated (see Alternate Protocol 1)
0.2 M phosphocreatine (store in small aliquots at −20°C)
5 mg/ml creatine kinase (store in small aliquots at −20°C)
0.2 M ATP (store in small aliquots at −20°C)
Sperm chromatin (see Support Protocol 2)
Fixative for visualizing nuclear assembly (see recipe)

Microscope slides
18-mm^2 glass coverslips
Fluorescence microscope

Prepare mix for nuclear assembly

1. To either crude extract or fractionated cytosol add phosphocreatine (from 0.2 M stock) to a final concentration of 20 mM, creatine kinase (from 5 mg/ml stock) to 50 µg/ml, and ATP (from 0.2 M stock) to 20 µM.

   Alternatively, It is also acceptable to use the 20× energy regenerating mix described in UNIT 11.11.

2. If fractionated extract is being used, add light membranes to the cytosol at a 1:10 dilution.

   This approximates the amount of membrane present in the original crude extract.

3. After vigorous mixing of the sperm chromatin preparation by repeated pipetting, add sperm chromatin to the crude or fractionated extract at the desired concentration and mix extract/sperm by up-and-down pipetting.

   Generally, these reactions are set up in 500-µl microcentrifuge tubes with a total volume <100 µl. Egg extracts will support the formation of up to 8000 to 9000 nuclei/µl. As more and more chromatin is used, the nuclei will become smaller in size due to depletion of nuclear-forming membranes. Below 50 nuclei/µl, it becomes increasingly difficult to locate nuclei by microscopy.

4. Incubate 60 min to 2 hr at room temperature.

   Nuclear formation in crude extracts is considerably faster than in the fractionated extract. In a robust extract, large nuclei can be seen in the crude extract within 60 min. Equivalently sized nuclei will form in the fractionated extract in ~2 hr.

Assess formation of nuclei

5. Mix the assembly reaction by gently pipetting up and down several times using a wide-bore or cut-off 200 µl pipet tip.

   This evenly suspends the nuclei which, especially as they grow larger, tend to settle out.

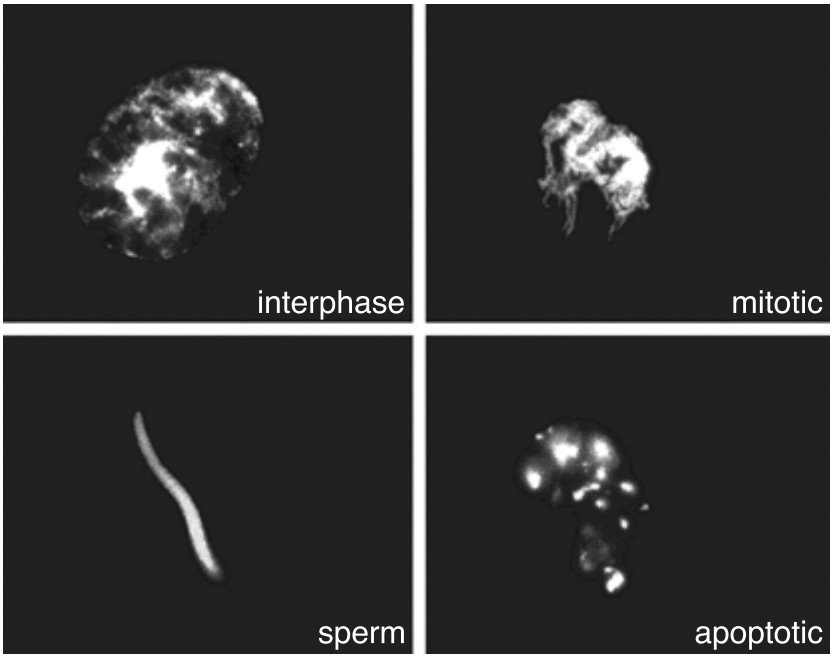


Figure 11.10.3 Nuclei in *Xenopus* egg extracts. Typical nuclei formed in *Xenopus* interphase extracts are shown as well as mitotic extracts (note condensed chromosomes) and apoptotic extracts (note fragmented nucleus). Also shown is a *Xenopus* sperm head prior to nuclear formation.

6. Remove a 4-µl aliquot of the assembly reaction mixture using a cut-off tip and spot it onto a glass microscope slide. Add 1 µl of fixative and gently swirl it into the sample by circling the pipet tip. Slowly and very gently lay a glass coverslip (18-mm^2) over the sample and examine the nuclei under the fluorescence microscope.

   The nuclei undergo a series of morphological changes as nuclear formation progresses. In a crude extract, after ~30 min, the newly formed nuclei will be small and oval in shape. The DNA is still quite condensed, and the nucleus should appear to be a solid light blue (from Hoechst binding) when observed in the UV channel. As the DNA decondenses further, the blue staining will be less homogeneous, with black "gaps" eventually appearing (e.g., Fig. 11.10.3). The formation of an intact nuclear envelope is evaluated by the ability of intact nuclei to exclude the high-molecular-weight FITC-dextran (which can be added to the fixative). Since the dextran does not contain a nuclear localization signal, once a fully closed nuclear membrane has formed, this large molecule will not enter the nucleus.

   As an alternative, the membrane can be visualized with 3,3′-dihexyloxacarboxyanine (DHCC; Kodak; 1 µg/ml included in the fix in place of FITC dextran). This is a fluorescent, lipophilic dye that is visualized in the fluorescein channel. In early stages of assembly, membrane vesicles are bound on the chromatin surface and begin to fuse in patches. This results in an uneven and discontinuous appearance of the forming membrane. Once the membrane is complete, it "smooths out" over the nuclear surface and will appear as an even, unbroken rim around the DNA (note that this can also be observed as a dark phase-dense line around the nucleus using phase-contrast microscopy). There will be a fair amount of background fluorescence in the surrounding cytosol due to membrane vesicles which have not been (or cannot be) incorporated into nuclei (see Fig. 11.10.3).

SUPPORT PROTOCOL 2

PREPARATION OF DEMEMBRANATED SPERM CHROMATIN TO USE AS TEMPLATES FOR NUCLEAR ASSEMBLY

Male *Xenopus* testes provide an abundant source of easily prepared chromatin to use as templates for nuclear assembly in the extract. Male frogs, which can be purchased from the same suppliers as the females (see Support Protocol 1), are most visibly distinguished from females by their relatively small size and by the presence of darkly pigmented patches on their forearms. Because frogs are external fertilizers, they produce large amounts of sperm. Consequently it is reasonable to expect to obtain 1 to 2×10^7 sperm heads per frog. These are generally used for nuclear formation in egg extracts at anywhere from 100 to 4000 per µl, depending upon the application. This protocol has been adapted from one developed by Drs. C. Macaulay and D. Forbes (University of California, San Diego).

Materials

4 to 5 male *Xenopus* (e.g., Nasco, Xenopus I, or Xenopus Express)
0.1% (w/v) Tricaine (see recipe)
Extraction buffer (see recipe), 4°C
Extraction buffer (see recipe) containing 200 mM, 2 M, 2.3 M, and 2.5 M sucrose
5 mg/ml aprotinin (Roche Diagnostics) in H_2O
5 mg/ml leupeptin (Roche Diagnostics) in H_2O
Dithiothreitol (DTT)
Triton X-100
Sucrose, ultrapure
Bovine serum albumin (BSA; fraction V)

Dissecting tools including scissors and 2 pairs of sharp forceps
60-mm glass petri dish
15-ml conical polypropylene tubes
Tabletop centrifuge
Refrigerated centrifuge with Sorvall HB-4 swinging-bucket rotor or equivalent
2.5 ml Beckman Ultraclear centrifuge tubes
TL-100 tabletop centrifuge and TLS-55 rotor (Beckman) or equivalent

Additional reagents and equipment for counting with a hemacytometer (*UNIT 1.1*)

Dissect testes

1. Anesthetize male frogs by immersion in 0.1% Tricaine (or immobilize in ice water) and sacrifice either by cervical dislocation or by pithing, followed by cutting through the spinal cord. Using dissection scissors, cut through the skin into the peritoneum along the midline of the frog.

   As indicated in the diagram (Fig. 11.10.4), the testes are located on either side of the midline in the center of the abdominal cavity. They are beige-colored 0.5-cm organs.

2. After pushing the liver aside, grasp the fatty material at mid-abdomen and pull gently until the testes emerge on either side of the midline. Gently cut them free of adherent tissue with either a forceps or scissors, and place in a 60-mm glass petri dish containing 4°C extraction buffer.

   To prepare a sizeable stock of sperm, testes should be isolated from 4 to 5 frogs and then placed in the petri dish containing extraction buffer.

3. Using 2 pairs of sharp forceps, mince the testes into small pieces and transfer them to a 15-ml conical polypropylene tube.

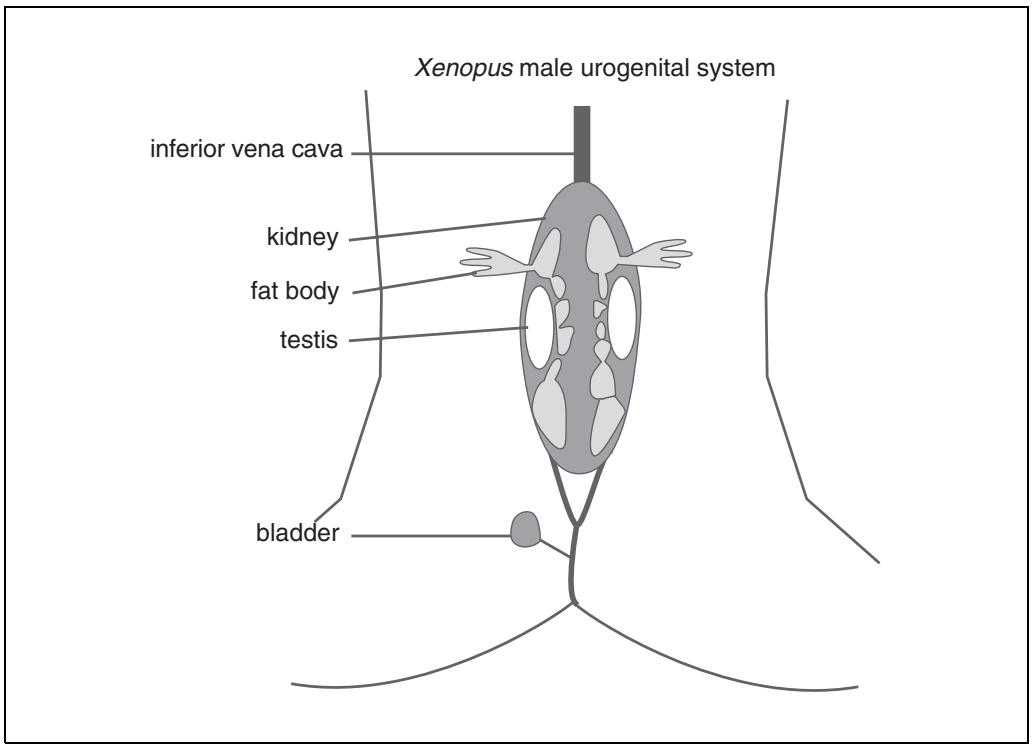


Figure 11.10.4 Schematic of the male *Xenopus* urogenital system. Testes are two white organs flanking the midline.

4. Vortex the minced testes vigorously and pellet the larger pieces by gentle centrifugation (10 sec at ~200 × *g*, room temperature in a clinical tabletop centrifuge).

Isolate sperm heads

5. Remove the supernatant to a new tube and add 3 ml extraction buffer supplemented with 200 mM sucrose to the pellet. Vortex 1 min, and briefly recentrifuge for 10 sec at 200 × *g*, room temperature. Combine the supernatants and repeat the extraction of the pellets 2 to 3 times until the supernatant is not very cloudy.
6. Centrifuge the combined supernatants 50 sec at 450 × *g*, room temperature, to pellet any remaining large pieces of tissue. Transfer the supernatants to a 15-ml tube and centrifuge 10 min at 2600 × *g* (4000 rpm in a Sorvall HB4 rotor), room temperature.
7. Prepare gradients by adding 0.2 ml extraction buffer containing 2.5 M sucrose to each of four 2.5-ml Beckman Ultraclear tubes and overlaying with 1.7 ml of extraction buffer containing 2.3 M sucrose.
8. Resuspend sperm in 0.8 ml extraction buffer containing 2 M sucrose and overlay gently onto the top of the sucrose gradients (0.2 ml per tube). After stirring the interface between the sperm and the 2.3 M sucrose with a pipet tip, centrifuge the sucrose gradients in a TL-100 tabletop ultracentrifuge, 25 min at 93,000 × *g* (33,000 rpm a TLS-55 rotor), 4°C.
9. Aspirate the top half of the gradient, which contains contaminating red blood cells. Transfer the lower half of the gradient to a new tube.

   Although the majority of the sperm bands on top of the 2.3 M sucrose cushion, the authors generally keep the entire lower half of the gradient.

10. Dilute sperm to 12 ml with extraction buffer containing 200 mM sucrose and pellet the sperm by centrifuging 10 min in a swinging-bucket rotor at 4100 × *g* (5000 rpm in a Sorvall HB4 or HB6 rotor or equivalent), 4°C.

Demembranate sperm

11. Supplement extraction buffer with additives at the following concentrations:

    200 mM sucrose
    5 µg/ml aprotinin (add from 5 mg/ml stock)
    5 µg/ml leupeptin (add from 5 mg/ml stock)
    1 mM DTT
    0.4% (v/v) Triton X-100.

    Resuspend the pellet in 1 ml of this supplemented extraction buffer/200 mM sucrose. Incubate 30 min on ice.

    This serves to demembranate the sperm.

12. Prepare sucrose cushions in two 1.5-ml microcentrifuge tubes by adding:

    0.5 ml extraction buffer
    0.5 M sucrose
    5 µg/ml leupeptin
    5 µg/ml aprotinin
    1 mM DTT
    3% (w/v) bovine serum albumin (BSA; fraction V).

    Overlay each sucrose cushion with one-half of the sperm preparation from step 11 and centrifuge 10 min in a clinical tabletop centrifuge at 870 × g, room temperature, to pellet the sperm.

13. Supplement extraction buffer with additives at the following concentrations:

    200 mM sucrose
    3% (w/v) bovine serum albumin (BSA; fraction V)
    5 µg/ml aprotinin (add from 5 mg/ml stock)
    5 µg/ml leupeptin (add from 5 mg/ml stock)
    1 mM DTT.

    Remove the supernatant from the pellets obtained in step 12 and resuspend the sperm pellet in 0.1 ml of the supplemented extraction buffer prepared in this step. Transfer the resuspended sperm to a clean microcentrifuge tube.

    At this point, it is important to avoid contaminating the preparation with residual Triton X-100 from the sides of the original tube.

Prepare final sperm suspension

14. Resuspend the sperm chromatin pellet in a total of ~0.25 ml of the supplemented extraction buffer/200 mM sucrose prepared in step 13.

15. Count the sperm using a hemocytometer and dilute the suspension to 50,000 to 100,000 sperm/µl. Snap freeze the sperm in small aliquots in liquid nitrogen and store at −70°C.

    The typical yield is 1–2 × 10^7 sperm per frog.

    At this point, the sperm are discrete masses of tightly condensed chromatin, unbounded by membranes.

NUCLEAR PROTEIN IMPORT IN VITRO

Once nuclei have been formed from interphase extracts, they can be assayed for the ability to import nuclear proteins by the addition to the extract of a fluorescently labeled import substrate (see *UNIT 11.7*). This provides a relatively rapid, visual assay that can be used to confirm that nuclei in the extract are intact and functional (see Basic Protocol 2). Nuclear transport can be monitored over time by quantitation of the amount of substrate that accumulates in the nucleus at various time points.

This following protocol can be used to quantitate nuclear import for comparison between different conditions or extracts, or simply to assess functional competence of nuclei in which another parameter is being altered.

Materials

Interphase egg extract: crude (see Basic Protocol 1) or fractionated (see Alternate Protocol 1)
ATP-regenerating system (see Basic Protocol 2)
Xenopus sperm chromatin (see Support Protocol 2)
Nuclear import substrate (*UNIT 11.7*)
Fix 1 (see recipe for fixatives for visualizing nuclear protein import)
16% (w/v) paraformaldehyde
Fix 2 (see recipe for fixatives for visualizing nuclear protein import)

Microscope slides
Coverslips
Fluorescence microscope equipped with:
64× objective (e.g., Zeiss 64× planapochromat, large aperture)
CCD camera
Frame grabber
Computer running image analysis software (e.g., NIH Image)

Additional reagents and equipment for assembling nuclei (see Basic Protocol 2)

1. Assemble nuclei using sperm chromatin and the interphase extract, along with the ATP-regenerating system (phosphocreatine, creatine kinase, and ATP; see Basic Protocol 2, step 1 and materials list), or add ~1000 nuclei per µl of extract from another source (e.g., rat liver nuclei) into the extract. Incubate 30 min, at room temperature.

2. After ~30 min incubation, check that nuclei have been formed (see Basic Protocol 2, step 5).

Where time course is not to be performed

3a. Once nuclei are formed, add the nuclear import substrate (*UNIT 11.7*) to the assembly reaction and mix by gently pipetting. Incubate 30 min, or longer, at room temperature.

> *The exact amount of substrate to add must be determined empirically, depending upon the particular batch of substrate and the purpose of the assay. For a simple determination of function, RITC import substrate should be added at a final concentration of ~5 µg/ml (1/200 dilution). For quantitation of a time course of import, the concentration should be somewhat lower in order to keep the signals within a linear response range over the time of the assay.*

4a. After the nuclei have been incubated with import substrate for 30 min or longer, remove and fix aliquots, using Fix 1, to detect uptake of the substrate. Store fixed samples in the dark at 4°C, if necessary.

> *Fixation is essentially immediate upon exposure to Fix 1.*

For quantitation of the time course of import

3b. Set up an assembly reaction (see above) of sufficient volume to allow for removal of at least 5-µl aliquots at each timepoint.

Quantitation of a import assay requires at least 15 to 20 min. For this reason, when performing a time course, it is best to fix each sample with 16% paraformaldehyde and then quantitate import after all samples have been collected.

4b. Immediately after the addition of import substrate, remove an aliquot from the reaction (5 µl) and transfer to a microcentrifuge tube on ice. Fix this sample (T_0) by adding 1.5 µl of 16% paraformaldehyde to bring the final concentration to 3.7% paraformaldehyde. Mix gently with a cut-off pipet tip. Store fixed samples in the dark at 4°C. At desired time points, continue to remove aliquots and fix as above.

5. Prepare samples for microscopy immediately before use. Place 4 µl of the fixed aliquot on a slide, and add 1 µl of Fix 2. Gently place a coverslip over the sample.

As the nuclei grow larger, they become increasingly susceptible to breakage when prepared for microscopy so the coverslip must be placed on the sample very gently and gradually. Breakage of a nucleus during processing can allow leakage of imported substrate. For this reason, the exclusion of FITC-dextran is used to identify intact nuclei.

6. Quantitate transport immediately after adding the coverslip and score only nuclei that exclude the FITC-dextran.

Because of the possibility of nuclear breakage, finding sufficient scorable nuclei can be a significant problem and thus, if possible, it is best to have large enough aliquots that more than one sample can be prepared if needed.

7. For quantitation, observe nuclei using a 64× objective on a microscope equipped with CCD camera, frame grabber, and computer running image analysis software.

The authors use a Zeiss 64× planapochromat large aperture objective which is very light sensitive.

NIH Image is available at no cost and is sufficient for this application, although more sophisticated and expensive software packages are available.

8. Once an appropriate nucleus is identified, capture the image and encircle the outline of the nucleus on the computer using the mouse. Determine the average fluorescence intensity within the nucleus, as well as the nuclear area using the software. Multiply average fluorescence by the area to yield the total nuclear fluorescence.

BASIC PROTOCOL 4

DNA REPLICATION WITH CONTINUOUS LABELING

DNA replication assays can be done using unfractionated interphase extracts (Basic Protocol 1), fractionated, reconstituted interphase extracts (Alternate Protocol 1), or cycling extracts (*UNIT 11.11*). While it takes ~1 hr to replicate 1000 synthetic nuclei/µl of crude extract, it can take 2 to 4 hr to fully replicate nuclei in a reconstituted extract. Although the protocol detailed below is useful for measuring replication of sperm chromatin nuclei in the extract, it can also be used to measure plasmid replication rates in the extract, and to examine replication of intact nuclei from other sources (e.g., nuclei prepared from tissue culture cells) added to extracts. It is worth bearing in mind the fact that these templates may replicate more slowly than sperm chromatin nuclei and that plasmid replication, in particular, will be slowed by inefficient nuclear formation (a prerequisite for replication in this system).

Two distinct replication assay protocols, continuous and pulse labeling (see Alternate Protocol 2) which depend upon the incorporation of radiolabeled nucleotides (e.g.,

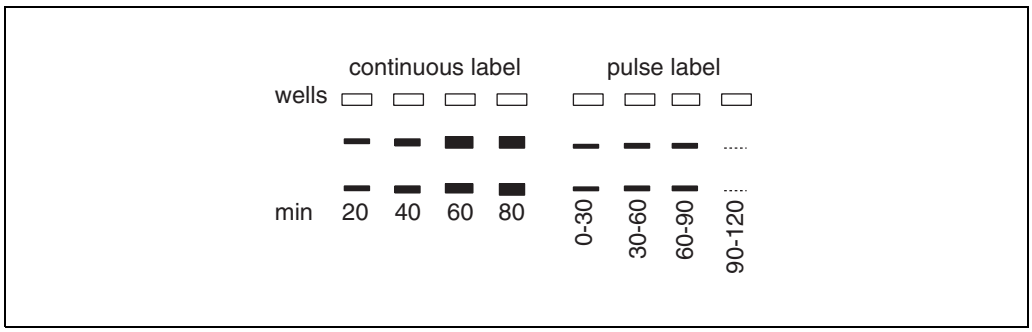


Figure 11.10.5 The banding pattern of labeled DNA following replication of DNA in egg extracts. When the products of a DNA replication reaction in *Xenopus* egg extract are resolved by agarose gel electrophoresis, two distinct bands are apparent (shown here schematically). In the case of continuous labeling, the intensity of these bands increases with time as increasingly more label is incorporated into DNA. In the case of pulse labeling, incorporation generally peaks and then diminishes as replication is completed (hence, as shown schematically here, no incorporation of label is seen at the later time point).

[α–^{32}P]dCTP) into replicating DNA, are routinely used. Direct inclusion of these nucleotides in extracts, withdrawal of aliquots, and resolution by agarose gel electrophoresis will give an estimate of total DNA replication, which will increase over time. Conversely, replication can be measured by inclusion of radiolabeled nucleotide in aliquots of extract over specific time intervals (e.g., 0 to 20 min, 20 to 40 min, 40 to 60 min, etc., after the start of nuclear formation) to compare, for instance, the replication that has occurred in one time interval with that which occurs in another. This "pulse replication" methodology is a more sensitive method than continuous labeling for comparing replication efficiency after different experimental treatments of the extract (e.g., depletion of particular components, addition of inhibitors; see Support Protocol 3 and Support Protocol 4, respectively).

When using sperm chromatin as the replication template, there should be two radiolabeled bands of replicated DNA. The pattern of incorporation differs when the two different replication protocols are used, as would be expected for continuous or pulsed incorporation of label (see schematic, Figure 11.10.5).

Materials

Interphase extract (see Basic Protocol 1)
0.2 M phosphocreatine (store in small aliquots at −20°C)
5 mg/ml creatine kinase (store in small aliquots at −20°C)
0.2 M ATP (store in small aliquots at −20°C)
Demembranated sperm chromatin (see Support Protocol 2)
10 μCi/μl [α–^{32}P]dCTP (3000 Ci/mmol)
Replication stop buffer (see recipe)
10 mg/ml proteinase K (Worthington)

Additional reagents and equipment for agarose gel electrophoresis (*APPENDIX 3A*) and autoradiography or phosphorimaging (*UNIT 6.3*)

1. To interphase extract add phosphocreatine (from 0.2 M stock) to a final concentration of 20 mM, creatine kinase (from 5 mg/ml stock) to 50 μg/ml, and ATP (from 0.2 M stock) to 2 mM.

2. For replication assays using sperm chromatin, pipet the demembranated sperm chromatin vigorously to eliminate clumping and add directly to extract at a final concentration of 100 to 4000 sperm heads/μl of extract.

3. Add [α–^{32}P]dCTP (or one of the other three deoxynucleotides in radiolabeled form) directly to the extract at 0.1 µCi/µl of extract and incubate at room temperature.
4. Withdraw 10-µl aliquots at 20-min intervals into an equal volume of replication stop buffer.
5. Add 3 µl of 10 mg/ml proteinase K stock to each aliquot and incubate 2 hr at 37°C.
6. Resolve samples on a 1% agarose gel.

   Any of the standard agarose gel running buffers will work fine.

   Loading the gel may be difficult if proteinase K treatment is not complete as the DNA will tend to adhere to the pipet tip.
7. Dry the gel and expose to film for autoradiography or develop by phosphorimager (*UNIT 6.3*).

ALTERNATE PROTOCOL 2

PULSE-LABELING DNA TO ASSESS REPLICATION

For this protocol, the replication reaction is initiated in the absence of labeled precursor. At defined time points (e.g., at 15- or 30-min intervals), samples of the reaction mixture are taken. Radiolabeled precursor is added to the sample for a defined period to measure replication during that time period.

Additional Materials *(also see Basic Protocol 4)*

XB buffer (see recipe)

1. Dilute 1 part [α–^{32}P]dCTP in 4 parts XB buffer and add 0.5 µl (1 µCi) of the resulting mix to an empty microcentrifuge tube for each time point to be taken. Do this for each condition to be tested (e.g., control extract, extract with added protein of interest, extract with depleted protein of interest, etc.). Let stand at room temperature.

   These are the assay tubes. To use fractionated extracts which have been reconstituted (the ultra S extract with added membrane), it should be suitable to set up assay tubes for the following intervals: 0 to 30 min, 30 to 60 min, 60 to 90 min. 90 to 120 min, and 120 to 150 min. For crude interphase extracts, it is better to have shorter time points; for example: 0 to 15 min. 15 to 30 min, 30 to 45 min, 45 to 60 min, 60 to 75 min, 75 to 80 min. Leave at room temperature.
2. Set up the reactions (Basic Protocol 4, steps 1 and 2). Place on ice.

   These are the master sample tubes.
3. To start the assay, move the master sample tubes to room temperature and remove 5 µl from each master sample tube to the first of each assay tube containing the [α–^{32}P]dCTP (e.g., the 0 to 30 min assay tube for fractionated extract or 0 to 15 min assay tube for unfractionated extract tube). Continue incubating the master sample and assay tubes at room temperature.
4. At the end of the specified time interval (e.g. 30 min or 15 min), add 5 µl of replication stop buffer and remove sample to ice. Withdraw 5 µl more from each of the master sample tubes and transfer to the next assay tube in the series (e.g., 30 to 60 min, 15 to 30 min). Again, stop the reaction by addition of replication stop buffer at the end of the specified time interval. Continue this until all time intervals have been done.

   This will give you "snapshots" of replication that occurred during each time interval.

   It is worthwhile to examine the morphology of nuclei in the samples at several time points during the experiment (see Basic Protocol 2) to make sure experimental treatments have not compromised nuclear architecture, which might in itself impair replication.
5. Digest samples with proteinase K and resolve by agarose gel electrophoresis as in Basic Protocol 4.

PREPARATION OF OOCYTE EXTRACT

Mature *Xenopus* Stage VI oocytes are physiologically arrested in prophase of the first meiotic division and therefore contain inactive cdc2/cyclin B complexes. Progesterone treatment of prophase I–arrested oocytes triggers meitotic maturation, concomitant with activation of the cdc2/cyclin B kinase. An additional Ser/Thr kinase, mos, is also required for meiotic maturation of oocytes. The oocyte extracts described below mimic several features of in vivo prophase I–arrested oocytes, including the ability of mos to evoke activation of MAP kinases.

Materials

Female frogs, mature (e.g., Nasco, Xenopus I, or Xenopus Express)
0.1% (w/v) Tricaine (see recipe)
Modified Barth's (MB) with and without calcium (see recipe)
1 mg/ml collagenase A (Roche Diagnostics) in MB (see recipe) without calcium
EB buffer (see recipe)
1000× CLAP protease inhibitor cocktail (see recipe)
1000× (5 mg/ml) cytochalasin B in DMSO

Dissecting microscope
Dissecting scalpel
Sharp forceps
50-ml plastic tubes
Tube rotator
Clinical centrifuge
Tabletop ultracentrifuge and swinging-bucket rotor
1-ml syringe
18-G needle

Dissect out ovary

1. Place female mature frog in 1 liter of 0.1% (w/v) tricaine until she is immobile, usually 30 to 60 min.

   Oocytes must first be dissected out of the anesthetized female frog

2. Place the frog on her back atop a bed of ice. Swab the abdomen first with deionized water and then with ethanol. Clean and sterilize all instruments with ethanol.

3. Make a small incision (~1 cm) in the abdomen, vertically and slightly off center. Cut through the skin and muscle layers.

   The oocytes, present in great abundance, should be readily apparent at this stage.

4. Gently tease oocytes out of the abdominal cavity and excise with a scalpel or scissors. Place oocytes in a petri dish containing MB without calcium.

   Precoating the dish with 6% (w/v) poly-HEMA in ethanol (dilute 12% poly-HEMA from Polysciences to 6% in ethanol) will prevent oocytes from sticking to the plastic dish. Simply pour the poly-HEMA solution onto the surface of the dish to coat, then pour off and allow to air dry in a hood. The dishes can be stored at room temperature (with lids).

5. Tear ovary (the mass of oocytes) into chunks of ~50 oocytes. Store oocytes that are not going to be used immediately in MB plus calcium at 18°C.

Free oocytes

6. Place ~10 ml oocytes in 50-ml tubes containing 30 ml collagenase A solution. Place on a tube rotator. Periodically check oocytes under the dissecting microscope until the majority have fallen away from the follicular cells.

Since prolonged treatment with collagenase can damage the oocytes, it is worthwhile to observe them carefully and remove them as soon as defolliculation is complete.

7. Remove and save the collagenase at −20°C.

   Collagenase A solution can be reused 3 to 4 times.

Wash and sort oocytes

8. Wash the oocytes extensively with MB minus calcium. Fill the 50-ml tube containing the oocytes with buffer, wait a few seconds for the larger, full-grown oocytes to settle and then, using a wide-bore pipet, aspirate the upper layer of buffer which contains the smaller, immature oocytes. Repeat this process five times to remove collagenase and to select for mature oocytes.

9. After washing, put oocytes in a petri dish for scanning with a dissecting microscope. Remove any oocytes that appear to be unhealthy (lysed or mottled), immature, or which have undergone germinal vesicle breakdown (GVBD).

   If oocytes have undergone GVBD, a while spot will appear at the top, center of the dark hemisphere of the oocyte.

   If oocytes are not to be used immediately to make an extract, store in MB plus calcium in a petri dish at 18°C for up to 2 days.

10. Carefully transfer oocytes to 50-ml conical tubes with the wide-end of a glass Pasteur pipet (place the bulb on the sharp end of the pipet, after snapping off most of the end). Wash twice with EB buffer using the method described in step 8.

11. Prepare 10 ml EB containing protease inhibitor cocktail CLAP (1:1000 dilution of stock solution). Wash oocytes with EB/CLAP using the method described in step 8.

12. Transfer oocytes to a thin-walled 2.5-ml polyallomer centrifuge tube for use in a tabletop ultracentrifuge rotor (e.g., for the Beckman TLS-55 rotor for the TL-100). Drop the filled small tube into a 15-ml centrifuge tube and pack the oocytes by gently spinning in a clinical centrifuge at 400 × *g* for 30 sec. Remove as much buffer as possible from the surface of the oocytes to prevent eventual dilution of the extracts.

Prepare oocyte extract

13. Lyse oocytes by centrifugation in a TL-100 ultracentrifuge (or equivalent), 15 min at 15,000 × *g* in a Beckman TLS-55 SW Ti rotor, 4°C.

    After centrifugation, three layers will be apparent: a black/green bottom layer (yolk platelets, mitochondria, etc.), a milky middle layer (oocyte extract), and a yellow top layer (lipids). The oocyte extract is a mixture of cytosolic and membranous components.

14. Using a 1-ml syringe with an 18-G needle, puncture the side of the tube at the base of the oocyte extract. Remove this layer and transfer to a second thin-wall centrifuge tube.

15. Add 1000× cytochalasin B to a final concentration in extract of 50 µg/ml (i.e., 1:1000 dilution of stock). Centrifuge as in step 13.

16. Transfer the large middle extract layer as in step 14 to a microcentrifuge tube and discard the pellet.

17. Divide the extract into 100-µl aliquots in mictocentrifuge tubes and snap freeze in liquid nitrogen. Store aliquots at −80°C.

IMMUNODEPLETION OF EXTRACTS

The ease with which components can be added to or depleted from in vitro reconstitution reactions is one of the chief advantages of using these systems to study biological processes. This protocol provides general guidelines for immunodepleting proteins from *Xenopus* egg extracts.

Complete immunodepletion of proteins from the egg extract often requires multiple rounds of depletion using a good, high-affinity antibody. One of the primary concerns is that depletion itself may cause artifactual perturbations in the process being studied. Therefore, it is critical to have a preimmune or IgG-depleted extract as a control. Moreover, it is important to avoid excessive dilution of the extract during the depletion procedure, since this may impair the proper functioning of the extract.

Materials

Antibody/antiserum
Preimmune serum or control IgG
Protein A–Sepharose
Phosphate-buffered saline (PBS; see recipe)
Bovine serum albumin (BSA), fraction V
XB buffer (see recipe)

1. Prepare antibody beads by incubating the affinity-purified antibody with protein A Sepharose in PBS containing 2 mg/ml BSA fraction V (also see *UNIT 7.2*). Prepare preimmune serum (control) beads in the same manner.

   If crude serum, rather than affinity-purified serum is being used, the protein A–Sepharose can be incubated directly with the serum without addition of BSA.

   Control protein A–Sepharose beads should be incubated with preimmune sera. If affinity-purified antibody is being used, the control can be pure IgG linked to protein A–Sepharose. While approximate volumes depend upon the antisera being used, ~15 µl of packed protein A–Sepharose can be incubated with ~10 µg of IgG in 200 µl of PBS containing 2 mg/ml BSA.

2. Pellet the beads in a microcentrifuge and remove all liquid.

3. *For crude or cycling extracts only:* Wash beads very rapidly with 100 µl or less of fresh extract that will be used for depletion experiments. Again, remove all liquid.

   Add extract to the beads, shake, centrifuge, and remove the extract; do this all in a cold room at 4°C. This allows the interstitial buffer of the beads to be replaced with extract and helps to avoid dilution of the extract to be depleted.

4. Add 100 µl of extract to be depleted to the 15 µl of packed antibody beads and rotate at 4°C for 40 min.

5. Pellet the beads by microcentrifuging and remove the supernatant to a new tube of antibody beads, washed as above.

6. Rotate at 4°C for 40 min and microcentrifuge. Remove the extract to a fresh tube.

   At this stage the supernatant contains a twice-depleted extract. A third (or even fourth) round of depletion may be necessary to completely deplete the protein of interest. The extent of depletion should be assessed by immunoblotting an aliquot of the depleted extract with the antibody used for the depletion or with an alternative antibody directed against the same protein. Occasionally, it is impossible to deplete a protein of interest, but addition of antibody directly to the extract has a "blocking" effect on the process being examined. If this effect can be reversed by adding an excess of the antigen, this can be an indication that the protein participates in the process under study.

ADDITION OF PROTEIN TO EXTRACTS

Addition of wild-type or mutant recombinant proteins to egg extracts often provides a relatively rapid assay of protein function. As with antibody addition, one of the most important considerations is adding a sufficient amount of protein to achieve an effect without excessively diluting the extract.

Materials

Recombinant protein of interest
XB buffer (see recipe)

Additional reagents and equipment for dialysis and microconcentration (*APPENDIX 3C*)

1. Dialyze or microconcentrate recombinant protein of interest into XB buffer.
2. Add proteins at more than a 1:5 dilution into the extract; if possible, a 1:10 or greater dilution is preferable.

   When using in vitro-translated proteins, the reticulocyte lysate will not grossly disrupt extract function if added directly to the extract at 1:10 or greater dilutions.

   This extract can be used for studying protein-protein interactions or for looking at biochemical reactions occurring in oocytes specifically. For example, MAPK can be activated in oocytes by addition of exogenous MOS protein.

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CLAP protease inhibitor cocktail, 1000×

Prepare in DMSO:
10 mg/ml chymostatin
10 mg/ml leupeptin
10 mg/ml antipain
10 mg/ml pepstatin
Store in aliquots indefinitely at −70°C

EB buffer (for preparation of oocyte extract; Basic Protocol 5)

42.68 g sucrose (0.25 M final)
10 ml of 5 M NaCl (0.1 M final)
1.25 ml of 1 M $MgCl_2$ (2.5 mM final)
2.4 g HEPES (20 mM final)
Adjust pH to 7.2 with NaOH and bring the volume to 500 ml with water.
Store until cloudy at 4°C

Egg lysis buffer, pH 7.7

250 mM sucrose
2.5 mM $MgCl_2$
1 mM DTT
50 mM KCl
10 mM HEPES
Prepare fresh

Extraction buffer (for preparation of demembranated sperm chromatin; Support Protocol 2)

10 mM HEPES, pH 7.4
80 mM KCl
15 mM NaCl
5 mM $MgCl_2$
1 mM EDTA
Store until cloudy at 4°C

Fixative for visualizing nuclear assembly (Basic Protocol 2)

10 mM HEPES, pH 7.5
200 mM sucrose
1 µg/ml Hoechst 33258
1 mg/ml FITC-dextran
12% (w/v) paraformaldehyde
Store at −20°C wrapped in foil

Fixatives for visualizing nuclear protein import (Basic Protocol 3)

Fix 1:

1 µl of 1 M HEPES, pH 7.5 (10 mM final)
10 µl of 2 M sucrose (200 mM final)
1 µl of 1 mg/ml Hoechst 33258 (bisbenzimide; Calbiochem; 1 µg/ml final)
10 µl of 10 mg/ml fluorescein isothiocyanate–dextran (FITC-dextran, 150 kDa, Sigma; 1 mg/ml final)
77 µl of 16% paraformaldehyde (methanol-free, Polysciences; 12% final)

After opening 16% paraformaldehyde vial, divide solution into aliquots and store at –20°C.

Fix 2: (for use on previously fixed samples)

10 mM HEPES, pH 7.5
200 mM sucrose
1 µg/ml Hoechst 33258
1 mg/ml FITC-dextran
3.7% (w/v) paraformaldehyde
Store at −20°C wrapped in foil

Modified Barth's (MB) solutions

Prepare the following stock solutions:

Stock solution A:

64 g NaCl
1 g KCl
2.5 g $NaHCO_3$
44.5 g HEPES

Adjust pH to 7.6 with NaOH and bring the volume to 500 ml with water. Store in 20-ml aliquots at −20°C.

Stock solution B:

0.95 g $Ca(NO_3)_2 \cdot 4H_2O$
0.75 g $CaCl_2 \cdot 2H_2O$
1.23 g $MgSO_4$ anhydrous
Bring volume to 500 ml with water.
Store in 20-ml aliquots at −20°C

continued

Stock solution B without Ca:

Weigh out 1.23 g anhydrous $MgSO_4$ and bring volume to 500 ml with water. Store 20-ml aliquots at −20°C.

For Modified Barth's with calcium:

460 ml H_2O
20 ml stock solution A (see above)
20 ml stock solution B (see above)
2.5 ml 10 mg/ml gentamycin solution (Sigma)
Store at 4°C

For Modified Barth's without Ca:

460 ml H_2O
20 ml stock solution A (see above)
20 ml stock solution B without Ca (see above)
2.5 ml 10 mg/ml gentamycin solution (Sigma)
Store up to several months at 4°C

Modified Ringer's solution (MMR), pH 7.7, 10×

1 M NaCl
20 mM KCl
10 mM $MgSO_4$
25 mM $CaCl_2$
5 mM HEPES, pH 7.8
0.8 mM EDTA
Store indefinitely at room temperature
Dilute 10× MMR to 0.25× MMR in water (1:40) before extract is made

~200 ml of 0.25× MMR are required to make the interphase extract.

Phosphate buffered saline (PBS)

8 g NaCl
0.2 g KCl
0.1 g $CaCl_2$
0.1 g $MgCl_2 \cdot 6H_2O$
1.15 g $NaH_2PO_4 \cdot 2H_20$
0.2 g KH_2PO_4
Add water to 1 liter
Store indefinitely at room temperature

Replication stop buffer

80 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
8 mM EDTA
0.13% phosphoric acid
10% Ficoll
5% (w/v) SDS
0.2% (w/v) bromphenol blue
Store at −20°C

Tricaine, 0.1% (w/v)

Dissolve 1 g 3-aminobenzoic acid ethyl ester (Tricaine; Sigma) in 1 liter water. Prepare fresh.

XB buffer

50 mM sucrose
100 mM KCl
0.1 mM $CaCl_2$
1 mM $MgCl_2$
10 mM potassium HEPES, pH 7.7
Prepare fresh

COMMENTARY

Background Information

The *Xenopus* egg is a very large cell (>0.5 mm in diameter) which, upon fertilization, gives rise to a 4000-cell embryo without any input from gene transcription and without any cell growth. In practical terms, this means that the egg contains a stockpile of cellular components poised for the assembly of cellular structures and for the execution of diverse cellular functions. While the normal somatic cell may contain the same range of cellular components as the egg, the vast excess of "uncommitted" components makes the egg an ideal source of material for in vitro reconstitutions.

There are multiple advantages to using the *Xenopus* egg cell-free system to study complex cellular processes such as cell cycle progression, DNA replication, apoptosis, nuclear envelope assembly and disassembly, and nuclear trafficking. In particular:

1. Because *Xenopus* are external fertilizers, each animal lays a large number of eggs, providing large amounts of homogeneous, cell cycle–synchronous material.

2. Pharmacological agents, recombinant proteins, and antibodies can be directly added to the in vitro extract, allowing direct manipulation and analysis of the biological process under examination.

3 Egg extracts are easily accessible to biochemical fractionation, facilitating immediate identification of factors involved in the process of interest.

4. Complex processes can be broken down into "partial reactions" that can be dissected individually (e.g., how does the first step in chromatin condensation occur?). Taken together, these features offer the opportunity to dissect even the most complex of cellular functions.

Extracts in interphase of the cell cycle

The key regulator of entry into mitosis in all eukaryotes studied to date is the Ser/Thr kinase cdc2/cyclin B (reviewed in Nurse 1990; Lew and Kornbluth 1996). This kinase complex is believed to catalyze mitotic cellular rearrangements either directly or indirectly through the phosphorylation of critical cellular substrates. Although cdc2 itself is inactive in its monomeric form, binding of the cyclin subunit activates cdc2 to promote mitosis. *Xenopus* eggs are physiologically arrested in metaphase of meiosis II with high cdc2/cyclin B kinase activity. Crushing these eggs by centrifugation releases Ca^{+2} from internal stores, leading to proteolysis of the cyclin subunit and loss of cdc2-associated kinase activity. Consequently, at the time of preparation, egg extracts transit from M phase to interphase of the cell cycle (S phase). Since cyclin B protein must be newly synthesized for each cell cycle, cycloheximide addition to extracts prevents reaccumulation of cyclin protein, and hence the extracts remain in interphase (Basic Protocol 1).

S phase extracts prepared from *Xenopus* eggs can spontaneously form nuclei upon addition of chromatin (Basic Protocol 2). While the most commonly used source of chromatin for these reconstitutions is demembranated sperm chromatin prepared from the testes of male *Xenopus* (see Support Protocol 2), even lambda phage DNA will suffice to provide a template for nuclear assembly, since it, too, will be packaged into chromatin by the extract (Forbes et al., 1983; Hartl et al., 1994). Although the precise molecular details of nuclear assembly have not yet been described in full, nuclear formation in the extract generally involves (1) swelling of the chromatin mediated, at least in part, by the nucleoplasmin protein, (2) binding of membrane vesicles to the surface of the chromatin, (3) fusion of bound membranes to form enclosed nuclear structures, and (4) further decondensation of the enclosed chromatin (Sheehan et al., 1988; Wilson and Newport, 1988; Philpott et al., 1991). At some point during this progression, functional nuclear pores are incorporated into the synthetic nuclei, allowing bidirectional nuclear transport of macromolecules. Indeed, it has been shown that nuclear pores can be incorporated de novo

into fully enclosed nuclear structures (Macaulay and Forbes, 1996).

Double-stranded DNA templates added to extracts will undergo a single round of semiconservative replication (see *UNIT 11.11*), provided they are assembled into nuclear structures (Blow and Laskey, 1986; 1988). In contrast, single-stranded templates will replicate even in the absence of nuclear structure, concurrently assembling into chromatin (Mechali and Harland, 1982).

Although primases in the extract will prime DNA synthesis from single-stranded templates, primers preannealed to the single-stranded DNA will be incorporated during replication. As is the case for nuclear assembly, the DNA sequence of templates to be replicated in the extract is not important, as highlighted by the ability of lambda DNA to replicate efficiently. This suggests that initiation at specific sequences may not be a feature of early embryonic DNA replication in *Xenopus*, though proteins critical for replication in all eukaryotes studied are of demonstrated importance in *Xenopus* (e.g., Orcs; Carpenter et al., 1996).

Nuclear transport and Xenopus egg extracts

Nuclei formed in egg extracts or added exogenously from another source are capable of supporting nuclear import of proteins. Nuclei can be assayed for the ability to import nuclear proteins by the addition to the extract of a fluorescently labeled nuclear import substrate (see Basic Protocol 3; Newmeyer et al., 1986; Finlay et al., 1989). This assay is often used as a relatively rapid and visual confirmation that nuclei are intact and functional (see Basic Protocol 2), and that they can be used to assess the rate of nuclear transport by quantitating the increase in nuclear fluorescence over time. Like nuclear transport in tissue culture cells, import into reconstituted nuclei is dependent upon temperature, energy in the form of ATP or GTP (which are readily interconverted in the extract), and a number of cytoplasmic protein factors. The specific functions of individual cytoplasmic factors have been most extensively studied using tissue culture cells which have been digitonin-treated to permeabilize their plasma membranes. This leaves the nuclear envelope intact, removes the cytoplasmic contents, and allows direct addition of proteins or cytosol to the environment outside the nucleus (see *UNIT 11.7*). Indeed, *Xenopus* extracts from eggs or oocytes have proven to be a convenient source of cytoplasmic material in such assays (e.g., Moore and Blobel, 1993; Gorlich et al., 1994). However, the unique advantage of the *Xenopus* extract in analysis of nuclear transport is the ability to manipulate the composition of the nuclear pore complex. Because the proteins that will make up the nuclear pore complex are found in the extract as soluble, individual components or small complexes, they are accessible for immunodepletion with specific antibodies (see Support Protocol 3; Finlay and Forbes, 1990; Powers et al., 1997). These depleted extracts are then used to form nuclei, and the effect of such "biochemical mutants" on nuclear transport can be monitored. In this manner, it is possible to address the function of individual proteins of the nuclear pore. Such depletion experiments can be performed on proteins involved in any process that is recapitulated in the extract and have proven invaluable in the study of cell cycle regulation and DNA replication.

Critical Parameters

The goal of extract preparation is to make a concentrated extract as close to the original composition of the cell as possible. Therefore, it is critical to remove as much of the buffer as possible after packing the eggs, even if it means loss of some of the eggs. During dejellying, it is important to wait just until the eggs settle against one another without a separation imposed by the jelly coat; however, dejellying the eggs for too long will result in damage to the egg, which can compromise extract function. It is also critical to handle the eggs gently until the time of centrifugation. Therefore, eggs should be transferred between containers either by direct pouring or by pipetting with a wide-bore cut-off and fire-polished (or upside-down) Pasteur pipet. If the eggs break prior to centrifugation, the extract will be sub-optimal.

Avoiding excessive dilution of the extract is also critical to the successful functioning of immunodepletion or protein addition experiments. For immunodepletions, this is accomplished by adding extract directly to packed beads (after washing and removal of all buffer) which have already been linked to antibody. It may require multiple immunodepletions to remove a protein of interest; therefore, it is important to examine the depleted extract by immunoblotting to confirm that the protein in question has actually been removed. It is also imperative that antibodies containing sodium azide not be used for the immunodepletions. This will disrupt extract function.

During preparation of light membranes, it is important to avoid contamination with the dark,

mitochondrial fraction. Since the light membranes are usually used after some time in frozen storage, contaminating mitochondria will lyse upon thawing, releasing cytochrome *c*. Any contaminating cytochrome *c* will induce activation of apoptotic proteases, or caspases (see *UNIT 11.12*), which will destroy nuclear function in the extract.

Many laboratories that prepare extracts also refrain from feeding any of the frogs once they have been primed. Any regurgitated food or stomach contents from the injected frogs may damage the eggs, compromising the extract.

To avoid damage to the sperm chromatin that will be used in the in vitro reconstitutions, it is important that they not be demembranated for longer than the allotted 30 min. In addition, it is best to avoid contamination with the red blood cells present in the gradient. These are much smaller than the sperm nuclei, and can produce confusion when examining samples under the microscope (e.g., it can be difficult to distinguish a nucleus with tightly condensed chromatin from a small red cell nucleus).

In examining nuclei, particularly in nuclear import experiments, it is important not to disrupt nuclear integrity. Therefore, when preparing slides, it is best to use a cut-off pipet tip and very gently lower the coverslip to the slide with a forceps.

Anticipated Results

These protocols can be used to produce extracts arrested in interphase. Upon addition of sperm chromatin, nuclei competent to transport macromolecules should be formed; these nuclei should replicate their DNA and undergo mitotic DNA condensation in response to addition of exogenous cyclin B.

Time Considerations

Interphase extracts require ~30 min to prepare and must be used within 2 hr of preparation if they are to preserve the ability to perform complex functions such as DNA replication and nuclear import. The fractionated egg extract requires an additional 2 hr of preparation time, but can be frozen indefinitely at −80°C.

Nuclear assembly in these extracts takes a variable amount of time. However, expect to see nice round nuclei in crude interphase extracts ~1 hr after the beginning of incubation at room temperature; similar nuclei can take up to 2 hr to form in a fractionated interphase extract. The kinetics of nuclear import in these two extracts are also different, with nuclear accumulation of a fluorescent import substrate to detectable levels requiring approximately twice as much time in a fractionated as in a crude extract.

Although preparation of the oocyte extract appears superficially as simple as the crude egg extract, sorting through the oocytes to remove mottled oocytes or oocytes that have undergone GVBD can be quite time-consuming. It is best to budget a full day to prepare an oocyte extract and perform a basic experiment using the extract.

Literature Cited

Blow, J.J. and Laskey, R.A. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell* 47:577-587.

Blow, J.J. and Laskey, R.A. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature* 332:546-548.

Carpenter, P.B., Mueller, P.R., and Dunphy, W.G. 1996. Role for a *Xenopus* Orc2–related protein in controlling DNA replication. *Nature* 379:357-360.

Finlay, D.R. and Forbes, D.J. 1990. Reconstitution of biochemically altered nuclear pores: Transport can be eliminated and restored. *Cell* 60:17-29.

Finlay, D.R., Newmeyer, D.D., Hartl, P.M., Horecka, J., and Forbes, D.J. 1989. Nuclear transport in vitro. *J. Cell Sci.* 11:225-242.

Forbes, D.J., Kirschner, M.W., and Newport, J.W. 1983. Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into *Xenopus* eggs. *Cell* 34:13-23.

Gorlich, D., Prehn, S., Laskey, R.A., and Hartmann, E. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* 79:767-778.

Hartl, P., Olson, E., Dang, T., and Forbes, D.J. 1994. Nuclear assembly with lambda DNA in fractionated *Xenopus* egg extracts: An unexpected role for glycogen in formation of a higher order chromatin intermediate. *J. Cell Biol*. 124:235-248.

Lew, D.J. and Kornbluth, S. 1996. Regulatory roles of cyclin-dependent kinase phosphorylation in cell cycle control. *Curr. Opin. Cell Biol*. 8:795-804.

Macaulay, C. and Forbes, D.J. 1996. Assembly of the nuclear pore: Biochemically distinct steps revealed with NEM, GTP gamma S, and BAPTA. *J. Cell Biol*. 132:5-20.

Mechali, M. and Harland, R.M. 1982. DNA synthesis in a cell-free system from *Xenopus* eggs: Priming and elongation on single-stranded DNA in vitro. *Cell* 30:93-101.

Moore, M.S. and Blobel, G. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* 365:661-663.

Newmeyer, D.D., Finlay, D.R., and Forbes, D.J. 1986. In vitro transport of a fluorescent nuclear protein and exclusion of nonnuclear proteins. *J. Cell Biol*. 103:2091-2102.

Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature* 344:503-508.

Philpott, A., Leno, G.H., and Laskey, R.A. 1991. Sperm decondensation in *Xenopus* egg cytoplasm is mediated by nucleoplasmin. *Cell* 65:569-578.

Powers, M.A., Forbes, D.J., Dahlberg, J.E., and Lund, E. 1997. The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways. *J. Cell Biol.* 136:241-250.

Sheehan, M.A., Mills, A.D., Sleeman, A.M., Laskey, R.A., and Blow, J.J. 1988. Steps in the assembly of replication-competent nuclei in a cell-free system from *Xenopus* eggs. *J. Cell Biol*. 106:1-12.

Wilson, K.L. and Newport, J. 1988. A trypsin-sensitive receptor on membrane vesicles is required for nuclear envelope formation in vitro. *J. Cell Biol*. 107:57-68.

Contributed by Maureen Powers
Emory University School of Medicine
Atlanta, Georgia

Erica K. Evans, Jing Yang, and Sally Kornbluth
Duke University Medical Center
Durham, North Carolina

Analysis of the Cell Cycle Using *Xenopus* Egg Extracts

This unit describes the preparation of cycling extracts that recapitulate, in vitro, the cell cycle of the intact fertilized egg. These extracts oscillate between interphase and mitosis, reproducing many of the in vivo events of the cell cycle including DNA replication, cyclin B synthesis and degradation, cdc2/cyclin B activation and inactivation, nuclear envelope breakdown and reformation, and chromosome condensation.

Preparation of a cycling extract requires activation of mature *Xenopus* eggs (see Basic Protocol 1). Extracts prepared in the presence of a calcium chelator are arrested in mitosis (see Basic Protocol 2), but these extracts can resume cycling upon addition of calcium (see Support Protocol 3). Extract stably arrested in mitosis requires not only a calcium chelator, but also a general phosphatase inhibitor (see Basic Protocol 3). It is also possible to drive interphase extracts into mitosis with the addition of recombinant cyclin. Changes in nuclear morphology (see Support Protocol 1) and in the extent of histone H1 phosphorylation (see Support Protocol 2) are used to follow cell cycle progression in these various extracts.

PREPARATION OF THE CYCLING EXTRACT

BASIC PROTOCOL 1

Mature *Xenopus* eggs are activated to provide an extract that cycles between interphase (S phase) and mitosis. Freshly collected eggs are activated in a specially constructed activation chamber and lysed for a crude cycling extract.

Materials

1× and 0.2× MMR (see recipe)
Unfertilized eggs laid in 1× MMR (see recipe)
Versilube F-50/silicone oil (see note in Basic Protocol 2)
XB buffer (see recipe)
CL protease inhibitor cocktail (see recipe)
2% (w/v) cysteine (see recipe)
5-ml polyallomer centrifuge tubes for Beckman SW 55 Ti rotor
Activation chamber (see Fig. 11.11.1)
12 V (AC) power supply with a toggle switch to rapidly control the delivery of current
Wide-bore Pasteur pipet
15-ml centrifuge tubes
Clinical centrifuge
Ice-water bath
Beckman floor model ultracentrifuge (with a SW 55 Ti rotor or equivalent)
18-G needle attached to a 5-ml syringe
1.5-ml microcentrifuge tubes and microcentrifuge at 4°C
Additional reagents and equipment for frog injection and egg collection (*UNIT 11.10*)

NOTE: Versilube F-50 silicon oil is the oil traditionally used in preparing cycling extracts. However, the company has recently replaced this with an M-20 oil, which purportedly has the same properties; this has not yet been tested extensively.

Contributed by Sally Kornbluth, Jing Yang, and Maureen Powers
Current Protocols in Cell Biology (2005) 11.11.1-11.11.14

Collect and wash eggs

1. Inject frogs (*UNIT 11.10*, Support Protocol 1), but place the frogs in 1× MMR after injection, so that the eggs will be laid in this solution.
2. The next morning, pour off the eggs into a beaker, rinse them once in room temperature distilled water, and then let them stand in water for 10 min.

   This facilitates the subsequent activation step.
3. Place 1 ml of Versilube F-50 oil into each of two 5-ml polyallomer centrifuge tubes for the Beckman SW 55 Ti rotor.

   It may be necessary to prepare more than two tubes to accommodate the eggs of one frog.
4. In a separate tube, mix 10 ml XB buffer with CL protease inhibitor cocktail to a final concentration of 10 μg/ml. Add 3 ml of this to each of the oil-containing tubes.

   This solution should sit on top of the oil layer.
5. Incubate the eggs in 2% cysteine for ~5 min until the jelly coat is removed and rinse three times with 0.2× MMR.

Activate eggs

6. Fill the activation chamber with 0.2× MMR so that the upper electrode will contact the solution. Pour in the eggs. For activation, deliver a 3-sec pulse (12 V AC), followed by a 5-sec pause and a subsequent 3-sec pulse. At the time of activation, start a timer (in the "counting up" mode) to keep the total time elapsed from activation until step 10 to <15 min.

   The activation chamber, made of Plexiglas, should be ~11 cm^2 on a side and 5 cm high (Fig. 11.11.1). The bottom of the chamber and the underside of the chamber lid

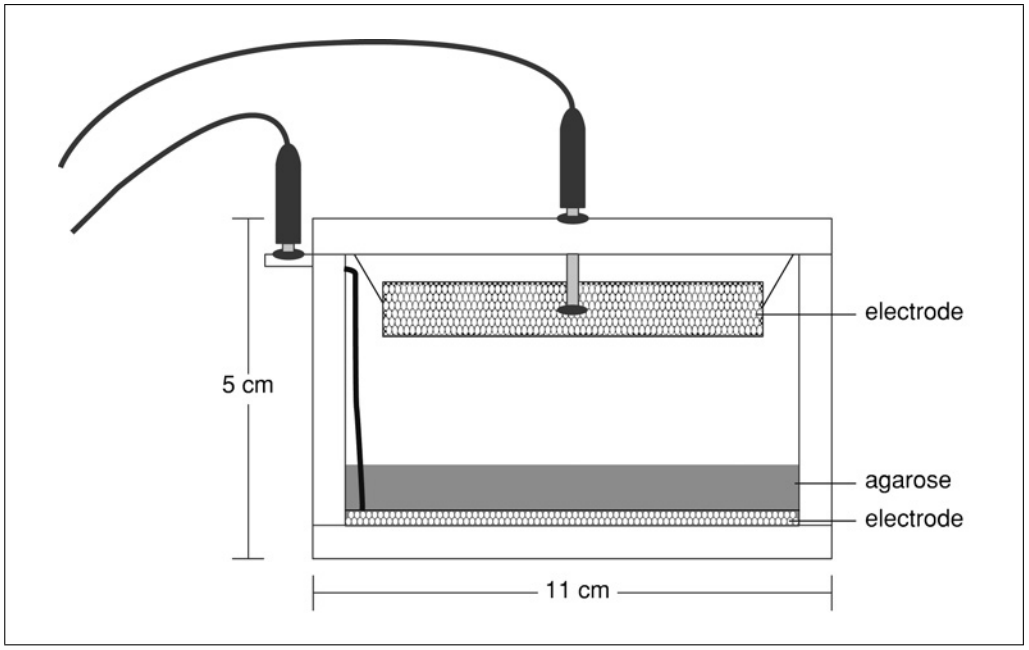


Figure 11.11.1 The activation chamber. The chamber is 11 cm on a side and 5 cm high and made of Plexiglas. The bottom of the chamber and the underside of the lid are covered with stainless steel plate electrodes. There is a pad of 0.2% (w/v) agarose in 0.2× MMR buffer covering the bottom of the chamber. Eggs and 0.2× MMR buffer are poured onto the agarose pad at the base of the chamber. The electrodes are attached via leads to a 12-V AC power supply. It is critical that the buffer make contact with the upper plate electrode in order for current to pass through the chamber, thereby activating the eggs.

are covered with stainless steel plate electrodes. A pad of 0.2% agarose prepared in 0.2× MMR is poured onto the bottom of the chamber.

Successful activation should induce the contraction of the pigment in the animal hemisphere. If the eggs are examined from above with the dark side up, a white rim (from the vegetal hemisphere) can be seen around the circumference of the egg after activation.

7. After activation, pour the eggs into a petri dish and rinse three times with XB buffer.
8. Using a wide-bore Pasteur pipet, drop the eggs gently into the centrifuge tubes containing oil and buffer, taking care to avoid dilution of the buffer in the tube with the buffer surrounding the eggs.
9. Place the tubes containing the eggs into 15-ml centrifuge tubes and centrifuge in a clinical centrifuge for 30 sec at 400 × *g*, followed by 20 sec at 1350 × *g*, room temperature.

   The eggs will have spun through the F-50 oil, resulting in elimination of excess buffer.

10. Remove all excess buffer and oil from above the eggs with a Pasteur pipet.

Lyse eggs

11. Continue incubation at room temperature until 15 min post-activation, then incubate a further 15 min in an ice-water bath.
12. Lyse the eggs by centrifuging them 10 min at 12,000 × *g*, 4°C (in a SW 55 Ti swinging-bucket rotor or equivalent).

    The extracts will now be fractionated into three layers: lipid, extract (cytoplasm plus membrane), and yolk, from top to bottom.

13. Collect the extract layer (cytosol plus membrane) by piercing through the side of the tube using a 18-G needle attached to a 5-ml syringe.
14. Place the extract into a 1.5-ml microcentrifuge tube and microcentrifuge 10 min at 12,000 × *g*, 4°C, to remove pigment granules and other particulate material.

    This extract should be used immediately for experiments requiring cycling extract; it should not be stored.

PREPARATION OF CSF-ARRESTED EXTRACTS

BASIC PROTOCOL 2

Xenopus eggs contain a cytoplasmic factor, termed cytostatic factor (CSF), that helps to stabilize the mitotic state, at least in part, through stabilization of the mitotic regulator cyclin B. Extracts prepared in the presence of a calcium chelator will maintain high levels of both CSF activity and cyclin B proteins. Extracts prepared in this manner are referred to as "CSF-arrested" extracts and are often used to study biochemical/morphological events of mitosis (but keep in mind that these are actually meiotically arrested extracts). While these extracts are in M phase at the time of preparation, they can be released into interphase by addition of calcium, leading to loss of CSF activity and destruction of cyclin B. Although these extracts will then behave like oscillating, or cycling extracts (see Basic Protocol 1), progressing to a subsequent mitosis as cyclins are synthesized, they tend to arrest at the next mitosis, rather than continuing to cycle, presumably because of residual, incompletely destroyed, CSF activity.

Materials

Xenopus eggs in 1× MMR (see recipe)
2% (w/v) cysteine (see recipe)
0.2× MMR (see recipe)

XB buffer (see recipe)
Versilube F-50/silicon oil (Andpak-EMA)
0.5 M EGTA stock solution, pH 8.0
1 M $MgCl_2$ stock solution
CL protease inhibitor cocktail (see recipe)
2% (w/v) cytochalasin B (Calbiochem)

5-ml polyallomer tubes for Beckman SW 55 Ti rotor (or equivalent)
Sawed-off and fire-polished Pasteur pipet
15-ml centrifuge tube
Clinical centrifuge
Floor model high-speed centrifuge (e.g., Sorvall, Beckman)
Beckman SW 55 Ti swinging-bucket rotor, or equivalent
5-ml syringe with an 18-G needle attached
Polycarbonate ultraclear microcentrifuge tubes (Beckman)
Microcentrifuge, 4°C

NOTE: Versilube F-50 silicon oil is the oil traditionally used in cycling extracts. However, the company has recently replaced this with an M-20 oil, which purportedly has the same properties; this has not yet been tested extensively.

Collect and wash eggs

1. Dejelly unfertilized eggs previously laid into 1× MMR in 2% cysteine and rinse with 0.2× MMR (see Basic Protocol 1).

   The amount of material to use depends on the amount of extract needed; 1 ml eggs yields ~0.5 ml packed eggs which yields ~0.25 ml extract.

2. Wash eggs three times in XB buffer.

3. Wash eggs three times in XB buffer containing 5 mM EGTA and an additional 1 mM $MgCl_2$.

4. Add 1 ml Versilube F-50 oil to each of two 5-ml polyallomer centrifuge tubes for an SW 55 Ti centrifuge (or equivalent).

5. In a clean tube, supplement a 10-ml aliquot of XB with EGTA to 5 mM, $MgCl_2$ to 1 mM, and add the CL protease inhibitor cocktail and cytochalasin B to a final concentration of 10 μg/ml each. Pipet 3 ml of this solution onto the top of the F-50 oil.

Isolate eggs

6. Using a sawed-off and fire-polished Pasteur pipet, drop the eggs into the centrifuge tubes containing oil and buffer until the tube is full.

   In lieu of a sawed-off pipet, use a short Pasteur pipet "backwards," with the sharp, long end inside the pipet bulb and the wide end to pick up the eggs. It is important that the eggs not be lysed at this step, so it is not advisable to use a narrow-bore pipet.

7. Place these egg-containing tubes inside 15-ml centrifuge tubes and centrifuge 40 sec in a clinical centrifuge at 400 × *g*, room temperature.

   This packs the eggs on the bottom of the tubes and the F-50 oil separates them from excess buffer.

8. Remove excess buffer and all of the oil with a Pasteur pipet.

Lyse eggs

9. Lyse the eggs by centrifuging 10 min at 12,000 × *g*, 4°C, in an SW 55 Ti (or equivalent) rotor.

   The extracts will now be fractionated into three layers, from top to bottom: a bright yellow lipid-containing layer, a brown extract layer (cytoplasm and membrane), and a dark greenish layer containing yolk and pigment granule.

10. Collect the extract layer (middle, brown layer) using an 18-G needle and a 5-ml syringe.

11. Place the extract into a microcentrifuge tube and microcentrifuge 10 min at 12,000 × *g*, 4°C. Using a needle, again withdraw the extract layer and place on ice.

    This extract can be used for mitotic reconstitutions or released into interphase by addition of $CaCl_2$ to a range of final concentrations between 0.1 and 0.4 mM (see Support Protocol 3).

PREPARING A MITOTIC EXTRACT

BASIC PROTOCOL 3

To make an extract more stably arrested in M phase than the CSF-arrested extract, it is necessary to include not only calcium chelators, but also a general phosphatase inhibitor. Together, these buffer components not only prevent the proteolytic destruction of cyclin B, but also maintain mitotic protein phosphorylation in the extract.

Materials

Xenopus eggs laid in 100 mM NaCl
2% (w/v) cysteine (see recipe)
Mitotic buffer (see recipe)
AL protease inhibitor cocktail (see recipe)
5 mg/ml cytochalasin B stock (see recipe)
Mitotic buffer (see recipe) supplemented with 250 mM sucrose (optional)

15-ml polycarbonate Ultraclear centrifuge tubes
Clinical centrifuge
High-speed floor model centrifuge (e.g. Sorvall or Beckman)
Large-capacity swinging-bucket rotor (e.g. Sorvall HB-4 or HB-6)
18-G needle attached to a 5-ml syringe

1. Collect eggs laid in 100 mM NaCl. Dejelly them in 2% cysteine, wash, and sort them (see *UNIT 11.10*).

2. After dejellying, rinse the eggs three times with mitotic buffer. Pour the eggs into a 15-ml polycarbonate Ultraclear centrifuge tube.

3. Pack the eggs by centrifuging 20 sec at 400 × *g*, room temperature, in a clinical centrifuge and remove all excess buffer with a Pasteur pipet.

   As in the interphase extract, it is better to lose a few eggs than to leave buffer behind.

4. Add the AL protease inhibitor cocktail to 5 μg/ml and the 5 mg/ml cytochalasin B stock to 5 μg/ml.

   Both reagents are diluted 1:1000 in the total egg volume.

5. Centrifuge the packed eggs 10 min at 12,000 × *g* (10,000 rpm in Sorvall HB-4 rotor), 4°C.

   As for the interphase extract, the mitotic extract will appear as a brownish layer sandwiched between a bright yellow lipid layer and a dark green layer.

6. Collect the brown extract layer, containing both cytoplasm and membrane, by inserting an 18-G needle attached to a 5-ml syringe through the wall of the centrifuge tube at the base of the extract layer.

7. *Optional:* Further separate the crude mitotic extract described above into cytoplasmic and membrane fractions using the method described for preparing fractionated interphase extracts (see *UNIT 11.10*, Alternate Protocol 1). In this case, use mitotic buffer supplemented with 250 mM sucrose to make a sucrose cushion for membrane fractionation.

   Note that this extract is an excellent source of mitotically vesiculated membranes.

BASIC PROTOCOL 4

DRIVING INTERPHASE EXTRACTS INTO MITOSIS

An interphase egg extract will not spontaneously enter mitosis because the added cycloheximide prevents synthesis of the cyclin proteins required for mitotic entry. To drive these extracts into mitosis, one can add recombinant forms of mitotic cyclins (e.g., cyclin B) that will associate with and activate the mitotic kinase, cdc2.

Since mitotic cyclins are degraded after cdc2 activation, mutant variants of cyclin lacking the signal for ubiquitination (the "destruction box") must be used if the extract is to be permanently arrested in mitosis (e.g., Murray et al., 1989). An alternative method to induce mitosis after nuclear assembly is to dilute the extract 1:1 with mitotic extract (see Basic Protocol 3). This provides active cdc2/cyclin complexes that will catalyze disassembly of the preformed nuclei.

Materials

Interphase extract (crude or fractionated) containing newly formed nuclei (see *UNIT 11.10*)

Recombinant cyclin protein (Desai et al., 1995)

Additional reagents and equipment for nuclear disassembly (*UNIT 11.10*)

1. After nuclear formation in either a crude or fractionated interphase extract, add recombinant cyclin protein (produced in either bacteria or baculovirus, e.g., Desai et al., 1995) to the extract and incubate at room temperature.

   It should take 30 to 60 min to observe nuclear envelope breakdown.

   It is best to titrate the added proteins over a range of concentrations since preparations vary in their potency.

   Typically, 15 to 20 nM recombinant cyclin B will drive extracts into mitosis.

2. To monitor nuclear disassembly, observe nuclei using the same methodologies described for nuclear assembly (see *UNIT 11.10*).

   In this case, the nuclear envelope will break down, as observed by loss of a dark ring by phase-contrast microscopy, loss of nuclear exclusion of the FITC-dextran, and loss of binding of the DHCC. More strikingly, the chromatin condenses into discrete packets (Fig. 11.10.3). Although prolonged incubation allows visualization of discrete chromosomes, in general, the mitotic DNA stained with Hoechst 33258 appears as a tangled, spaghetti-like mass.

   Although nuclear morphology is a good indicator of conversion into mitosis, the activity of added cyclin can be confirmed by assaying the kinase activity of the bound cdc2, using histone H1 as an exogenous substrate (see Support Protocol 2).

GENERATING A REPLICATION CHECKPOINT IN VITRO

ALTERNATE PROTOCOL

It is imperative that cells not enter mitosis prior to the completion of replication. To prevent this from occurring, cell-cycle checkpoints monitor the status of DNA replication and prevent onset of mitosis until DNA replication is finished. If DNA replication is artificially blocked using polymerase inhibitors, cells will arrest in G2, prior to mitotic entry. This form of cell cycle regulation can be recapitulated in vitro using cycling extracts and replicating sperm chromatin. While a permanent arrest prior to entry into mitosis can be achieved if extracts are supplemented with DNA replication inhibitors, the cell cycle can also be delayed by simple addition of large numbers of nuclei to the extract. Indeed, as increasing amounts of sperm chromatin are added, S phase and the time prior to entry into M phase lengthen concomitantly. Extracts will remain arrested prior to entry into mitosis until the DNA has been completely replicated.

Materials

Sperm chromatin (*UNIT 11.10*)
20× energy-regenerating mix (see recipe)
Cycling extract (see Basic Protocol 1)
5 mg/ml aphidicolin stock solution (Sigma) in high-quality DMSO (e.g., Pierce) (see recipe)

1. Supplement cycling extract with 20× energy-regenerating mix to a final concentration of 1×. Add sperm chromatin to a final concentration of 500 nuclei/μl extract. Supplement the reaction with 50 μg/ml aphidicolin and mix thoroughly. Incubate at room temperature and monitor the cell cycle state (see Support Protocol 1).

   Approximately 50 μl cycling extract is used per sample.

   Aphidicolin is a DNA polymerase inhibitor that will prevent replication of the sperm chromatin, thereby generating a cell cycle arrest prior to entry into mitosis. Note that the extract may not function well if the final DMSO concentration (the solvent base for the aphidicolin) in the extract exceeds 1% (v/v). To confirm that the failure to enter mitosis is due to the DNA replication checkpoint, extracts can be supplemented with 5 mM caffeine, which will override the checkpoint and drive the extracts into mitosis. Add the caffeine from a 0.5 M stock solution in water. It is necessary to boil the mixture to prepare the stock solution, since caffeine will only enter solution at this concentration if boiled.

MONITORING THE CELL CYCLE STATE OF EXTRACTS

SUPPORT PROTOCOL 1

As the cycling extract progresses through the cell cycle, nuclei form, DNA replication proceeds, and, after replication is complete, the extract enters mitosis. This series of events can be monitored by observation of nuclear morphology, by biochemical assessment of cdc2/cyclin kinase activity or phosphorylation state, and by direct measurements of DNA replication (see *UNIT 11.10*).

Materials

Cycling extract
Sperm chromatin (*UNIT 11.10*)
20× energy regenerating mix (see recipe)

1. To observe nuclear morphology through the cell cycle, supplement extracts with sperm chromatin (~500 nuclei/μl), and add a 1:20 dilution of the 20× energy regenerating mix. Mix the extract thoroughly by pipetting and incubate at room temperature. At 10-min intervals, withdraw samples for observation by fluorescence microscopy (see *UNIT 11.10*, Basic Protocol 2).

Since the extract is fairly viscous, it is best to gently invert the sample periodically to ensure uniform distribution of the nuclei. Initially, samples should appear identical to those described in UNIT 11.10 for interphase nuclei. These should then progress abruptly into mitosis, as described for nuclei in UNIT 11.10, Basic Protocol 4, and Figure 11.10.3. The transition from interphase into mitosis should occur between 60 and 120 min after the start of the room temperature incubation.

2. Use histone H1 as an exogenous substrate for cdc2/cyclin B (see Support Protocol 2). In general, withdraw aliquots of extract at 10-min intervals and snap freeze in liquid nitrogen for later histone H1 kinase assays.

The peak in histone H1-directed kinase activity typically appears ~10 min before observable chromatin condensation or nuclear envelope breakdown. Oscillations in histone H1 kinase activity occur even in extracts without sperm chromatin (or any other source of DNA).

As an alternative means of assessing the cell cycle status of the extract, one can measure the tyrosine phosphorylation state of cdc2. Phosphorylation of cdc2 at its two negative regulatory sites, Thr 14 and Tyr 15, is high in interphase and then drops precipitously as cdc25 dephosphorylates cdc2 at the time of mitotic entry. Therefore, aliquots of extract withdrawn at 10-min intervals can be resolved by SDS-PAGE (UNIT 6.1) and immunoblotted (UNIT 6.2) with anti-phosphotyrosine antisera.

SUPPORT PROTOCOL 2

ASSAYING HISTONE H1 KINASE ACTIVITY

Since entry into mitosis is regulated by the cyclin-dependent kinase, cdc2, progression into mitosis in the egg extract is often assayed by measuring cdc2 activity using histone H1 as an exogenous substrate. Aliquots of extracts are withdrawn at closely spaced time intervals (10 to 15 min) from either cycling extracts (see Basic Protocol 1) or interphase extracts converted to mitosis using recombinant cyclin protein (see Basic Protocol 4).

Materials

Extract: cycling (see Basic Protocol 1) or interphase driven into mitosis (see Basic Protocol 4)
EB buffer (see recipe)
Liquid nitrogen
10× kinase buffer (see recipe)
1 mg/ml histone H1 (Roche Diagnostics)
500 μM protein kinase inhibitor peptide (PKI; Sigma)
0.2 M ATP
5 mCi/ml [γ-^{32}P]-ATP (3000 Ci/mmol)
2× SDS sample loading buffer (*APPENDIX 2A*)

1. Dilute aliquots of extract to be assayed 1:1 with EB buffer (e.g., 2 μl of extract in 2 μl EB buffer will suffice) and place immediately into liquid nitrogen. Then transfer the frozen aliquots to a −80°C freezer without thawing.

2. Prepare the following kinase cocktail so there is sufficient amount for 10 μl per kinase reaction (i.e., for each aliquot of extract to be assayed). Prepare a master mix, multiplying the following amounts by the number of reactions plus 1 for pipetting error.

1 μl 10× kinase buffer
1 μl 1 mg/ml histone H1
0.4 μl 500 μM PKI
0.1 μl 0.2 M ATP
4-8 μCi γ-^{32}P]ATP
Water to 10 μl.

3. For each sample to be assayed, add 10 μl of kinase cocktail to a fresh microcentrifuge tube.

4. Transfer samples to be assayed from the −80°C freezer directly into a container containing finely crushed dry ice.

   The samples should be arrayed in the order in which they will be assayed.

5. To the first sample to be assayed (containing 2 μl extract and 2 μl EB buffer) add 240 μl EB buffer and pipet up and down rapidly. As soon as the sample is thawed, transfer 10 μl of the mixture to the corresponding tube containing 10 μl of kinase cocktail and start a timer counting upwards.

6. Stop the reaction after 10 min by adding 20 μl of standard 2× SDS sample loading buffer.

   Since reactions proceed for 10 min, for multiple aliquots start and stop reactions at staggered 1-min intervals.

7. Store samples by freezing or electrophorese directly on an 11% SDS-polyacrylamide gel (*UNIT 6.1*).

   Since most of the radioactive ATP runs below the dye front, it is best not to run the gel until the dye front reaches the bottom to avoid contamination of the SDS-running buffer. Indeed, it is best to cut the gel off at the dye front before drying and exposing the gel. After autoradiography, histone will be identifiable as a very closely spaced radiolabeled doublet.

RELEASE OF CSF-ARRESTED EXTRACTS AND THEIR PROGRESSION INTO INTERPHASE

SUPPORT PROTOCOL 3

To determine if removal or addition of particular components from an extract has a specific effect on exit from mitosis, it is easiest to use CSF extracts that can transit from mitosis to interphase in a controlled manner. Supplementation of CSF extracts with calcium drives them into interphase. If these extracts are supplemented with sperm chromatin, they will form nuclei and initiate DNA replication in the same manner as in cycling extracts described in Basic Protocol 1. However, the released CSF extracts have a tendency to arrest on re-entry into mitosis, unlike the cycling extracts that will continue through multiple cycles.

Materials

CSF-arrested extract (see Basic Protocol 2)
20× energy-regenerating mix (see recipe)
Sperm chromatin (*UNIT 11.10*)
1 M calcium chloride
Fixative for visualizing nuclear assembly (see recipe)
Cycloheximide (optional)

1. Supplement CSF extract with 20× energy-regenerating mix to a final concentration of 1×.

2. Add sperm chromatin to a final concentration of 100 to 500 nuclei/μl.

3. Determine the minimal concentration of calcium required to drive the extract into mitosis. Supplement small aliquots of extract containing energy-regenerating mix and sperm chromatin with 1 M $CaCl_2$ to 0.1 mM, 0.2 mM, or 0.4 mM and incubate at room temperature.

   While adding calcium to this extract will release it into interphase, the precise concentration of calcium required varies somewhat from extract to extract. Hence, to perform a large experiment it is probably worthwhile to first determine the minimal concentration of calcium required to drive a particular extract into interphase.

4. At 10-min intervals, remove a 4-µl sample and add 1 µl Hoechst 33258 to stain the DNA. Observe using fluorescence microscopy. Identify the lowest concentration of $CaCl_2$ that allows good release of CSF-arrested extract into interphase.

   Stain nuclei as described in UNIT 11.10, except FITC-dextran may be omitted here.

   In the absence of calcium addition, condensed mitotic chromosomes should be easily visible by fluorescence microscopy after 20 to 30 min of room-temperature incubation. For the calcium-released samples, find the lowest concentration allowing good release from CSF arrest into interphase. At the appropriate concentration, the sperm chromatin should decondense to form membrane-enclosed nuclei (see UNIT 11.10).

5. *Optional:* To arrest these extracts in interphase after calcium addition, add cycloheximide to a final concentration of 50 µg/ml at the time of calcium addition.

   The crude CSF-arrested extract can be further separated into cytoplasmic and membrane fractions (see UNIT 11.10, Alternate Protocol 1) and stored at −80°C. The freeze/thawed cytoplasmic fraction of CSF extracts can fully support chromosome condensation and spindle formation.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX** 2A; for suppliers, see **SUPPLIERS APPENDIX**.*

AL protease inhibitor cocktail

Dissolve aprotinin to 10 mg/ml in water and leupeptin to 10 mg/ml in water. Mix these two solutions 1:1 to give a final 5 mg/ml solution. Store at −70°C.

Aphidicolin stock solution, 5 mg/ml

Dissolve aphidicolin to 5 mg/ml in high-quality DMSO (e.g., Pierce). Store several months at −70°C.

CL protease inhibitor cocktail

Dissolve leupeptin to 10 mg/ml in distilled water and chymostatin to 10 mg/ml in DMSO and mix them at a ratio of 1:1 to give a final concentration of 5 mg/ml. Store at −70°C.

Cysteine, 2% (w/v)

Prepare 2% (w/v) cysteine in water. Adjust pH to 8.0 with NaOH. Prepare fresh for each use.

Cytochalasin B stock solution, 5 mg/ml

Prepare a 5 mg/ml stock of cytochalasin B (Calbiochem) in DMSO. Store up to 1 year at −20°C.

EB buffer

42.68 g sucrose (0.25 M final)
10 ml of 5 M NaCl (0.1 M final)
1.25 ml of 1 M $MgCl_2$ (2.5 mM final)
2.4 g HEPES (20 mM final)
Adjust pH to 7.2 with NaOH and bring the volume to 500 ml with water.
Store until cloudy at 4°C

Energy-regenerating mix, 20×

150 mM creatine phosphate
20 mM ATP
2 mM EGTA, pH 7.7
20 mM $MgCl_2$
Store up to several months at −20°C, but avoid repeated freeze/thaw

Fixative for visualizing nuclear assembly

10 mM HEPES, pH 7.5
200 mM sucrose
1 μg/ml Hoechst 33258
1 mg/ml FITC-dextran
12% (w/v) paraformaldehyde
Store at −20°C wrapped in foil

Kinase buffer, 10×

200 mM HEPES, pH 7.3
5 mM EGTA
10 mM $MgCl_2$
Store indefinitely at 4°C, unless it becomes cloudy

Mitotic buffer

24 ml 1 M β-glycerophosphate, pH 7.3
12 ml 0.5 M EGTA, pH 8.0
4.5 ml 1 M $MgCl_2$
100 μl 1 M DTT
Add water to 100 ml
Adjust pH to 7.3 with NaOH
Store indefinitely at 4°C, unless a precipitate forms

Modified Ringer's solution (MMR), pH 7.7, 10×

1 M NaCl
20 mM KCl
10 mM $MgSO_4$
25 mM $CaCl_2$
5 mM HEPES, pH 7.8
0.8 mM EDTA
Store indefinitely at room temperature
Dilute 10× MMR to 0.25× MMR in water (1:40) before extract is made

~ 200 ml of 0.25× MMR are required to make the interphase extract.

XB buffer

50 mM sucrose
100 mM KCl
0.1 mM $CaCl_2$
1 mM $MgCl_2$
10 mM potassium HEPES, pH 7.7
Prepare fresh

COMMENTARY

Background Information

Eggs arrested in metaphase have not only high levels of cdc2/cyclin B activity but also a cytoplasmic component, CSF (cytostatic factor), which stabilizes this activity. During normal fertilization, sperm entry promotes the destruction of CSF, initiating the oscillation between S and M phases seen in early embryonic development. Destruction of CSF and cyclin B can be achieved synchronously in a large population of eggs by electrical stimulation of the eggs (see Basic Protocol 1). If cycloheximide is not added to these extracts (so that protein synthesis can occur), extracts made from these electrically activated eggs faithfully recapitulate the embryonic cell cycle oscillations with respect to nuclear envelope formation and breakdown, DNA replication, cyclin accumulation and destruction, and cdc2 kinase oscillations (Murray and Kirschner, 1989; Murray et al., 1989). Indeed, in these extracts, DNA replication occurs only once per cell cycle and multiple rounds of replication occur only because they are separated by intervening mitosis.

Although cycling extracts are useful for studying the basic cell cycle machinery, they can also be used to reconstitute more complex phenomena, such as the operation of cell cycle checkpoints. When cellular events regulated by the cell cycle machinery malfunction or do not occur on time, subsequent cell cycle events are delayed. For example, if DNA replication is inhibited or DNA is damaged, mitosis will not occur, preventing chromosome segregation and almost certain breakage of partially replicated or damaged chromosomes. In cycling extracts, if nuclei are formed in the presence of the DNA polymerase α inhibitor, aphidicolin, mitosis will not occur since replication cannot be completed. This cell cycle arrest can be overridden by caffeine, an agent known to override this cell cycle checkpoint in intact cells, reinforcing the idea that the extract uses the same biochemical pathways to implement the cell cycle arrest as are used in vivo (Dasso and Newport, 1990). Hence, this system can be used to unravel the biochemical pathways that underlie the operation of the DNA replication checkpoint.

Extracts in Mitosis

Although synthesis of cyclin will drive a cycling extract into mitosis, the extract will not remain in mitosis since cyclin is destroyed by regulated ubiquitination and proteasome-mediated degradation. To study mitotic functions in isolation (e.g., nuclear disassembly, chromosome condensation), inclusion of EGTA in the buffer used during egg lysis results in chelation of the Ca^{+2} normally released upon egg lysis, thereby preventing the loss of CSF and cdc2/cyclin B activity, and locking these extracts in M phase (Glotzer et al., 1991; Murray et al., 1989). If Ca^{+2} is then added to these "CSF-arrested extracts," they can be released into interphase. Because these extracts are competent to synthesize protein, cyclin will accumulate, driving this extract into a subsequent mitosis (see Support Protocol 3). These extracts are excellent for the study of mitotic enzyme activities and for microscopic and biochemical analysis of mitotic chromosome condensation and spindle assembly. In addition, they have recently been used to great effect in the purification of proteins interacting with mitotic chromosomes (Hirano and Mitchison, 1994). Ca^{+2} addition allows controlled release of CSF extracts into interphase, making this extract the preferred choice for examining the M to S phase transition. This feature has been exploited to study the biochemistry of the "spindle checkpoint" that prevents anaphase progression if chromosomes are not properly attached to the mitotic spindle (Minshull et al., 1994). The CSF extract is also particularly useful if removal and addition of cellular components is desired prior to commencement of DNA replication.

If β-glycerophosphate (as well as EGTA) is included in the egg lysis buffer, the mitotic state is stabilized indefinitely, not only by making CSF and cyclin impervious to degradation, but also by stabilizing mitotic phosphorylations. These "mitotic" extracts (see Basic Protocol 3) can be fractionated into cytosolic and membranous components, which can be stored indefinitely and retain the mitotic character of the original extract.

An alternative method to prepare extracts arrested in mitosis is to convert S phase extracts to the mitotic state by addition of a recombinant cyclin B whose "destruction box" (the region of the protein that renders it susceptible to proteolytic degradation) has been removed (see Basic Protocol 4; Solomon et al., 1990). This leads to constitutively high levels of cdc2/cyclin B activity with a consequent mitotic arrest.

Critical Parameters

Excessive dilution of the cycling extract will interfere with its ability to cycle. This is probably because protein synthesis in these extracts is sensitive to dilution, and cyclin synthesis is required to complete the various cell cycle transitions. When preparing the cycling extract, it is imperative that all of the eggs in the preparation have been properly activated. If a significant proportion of the eggs have not undergone activation after the electrical pulse, the extract probably will not cycle unless the unsuccessfully activated eggs are removed. Rarely, a batch of eggs will be contaminated with small numbers of immature oocytes; these lack the white spot characteristic of eggs (resulting from germinal vesicle breakdown and displacement of pigment granules) and should be removed before extract preparation.

When triggering a checkpoint in the extract, the aphidicolin should be prepared so as not to exceed 1% final DMSO concentration in the extracts. Higher amounts of DMSO will impair extract function. If caffeine is being used to override the replication checkpoint, make sure it is fully in solution and do not place on ice (to avoid precipitation of the caffeine).

To monitor progression of the cell cycle in the in vitro extract, it is convenient to assay kinase activity directed against histone H1 (catalyzed by cdc2/cyclin B complexes). Each time point should be assayed immediately after thawing. If the whole time course is thawed at once and then assayed, the peaks of H1-directed kinase activity will be lost.

After preparing the CSF extract, it is worthwhile to test that it can be successfully released from metaphase into interphase with the desired concentration of calcium. Occasionally, release does not occur, indicating a requirement for higher calcium levels or indicating that the extract is nonfunctional. The easiest way to determine if release can occur is to supplement the extract with sperm chromatin and examine samples by Hoechst staining and fluorescence microscopy.

When driving an extract into mitosis using exogenous cyclin, bear in mind that cyclin preparations vary in their potency to induce mitosis. All batches of cyclin should be titered with regard to mitosis-inducing activity. If a nondestructible cyclin B is used, the extract will remain arrested in mitosis. With native cyclin B, the extract will complete mitosis and return to interphase concomitant with cyclin destruction.

Anticipated Results

Cycling extracts should complete interphase, go into mitosis, and come out again two or three times. Histone H1 kinase activity should peak ~10 min before visual breakdown of added nuclei. In the CSF extract, added sperm chromatin should look like tangled threads. Upon addition of calcium, these should now form round nuclei similar to those formed either in interphase extracts or at the start of a cycling extract incubation.

Time Considerations

Once beginning a cycling extract, it is not possible to stop at any point. It is particularly important that the time from egg activation to incubation on ice not exceed 15 min. The cycling extract can remain on ice for 1 to 2 hr before use, but it cannot be frozen for later use.

For assaying progression of the cycling extracts using histone H1 kinase assays, it is critical that all of the time points be incubated for precisely the same amount of time; hence, the authors have recommended starting the sample incubations at 1-min intervals so that they can be subsequently stopped at 1-min intervals.

Entry into mitosis in interphase extracts supplemented with recombinant cyclin can take a variable amount of time, depending on the particular extract and preparation of cyclin. However, it is advisable to aim for an amount of cyclin that takes 30 to 45 min to promote mitotic entry. If, after 60 min, entry into mitosis is not observed, it is likely that the extract is not responsive, that suboptimal levels of cyclin have been used, or that the preparation is inactive.

Literature Cited

Dasso, M. and Newport, J.W. 1990. Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: Studies in *Xenopus*. *Cell* 61:811-823.

Desai, D., Wessling, H.C., Fisher, R.P., and Morgan, D.O. 1995. Effects of phosphorylation by CAK on cyclin binding by CDC2 and CDK2. *Mol. Cell. Biol.* 15:345-350.

Glotzer, M., Murray, A., and Kirschner, M. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* 349: 132-138.

Hirano, T. and Mitchison, T. 1994. A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro. *Cell* 79:449-458.

Minshull, J., Sun, H., Tonks, N.K., and Murray, A.W. 1994. A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. *Cell* 79:475-486.

Murray, A. and Kirschner, M. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature* 339:275-280.

Murray, A., Solomon, M.J., and Kirschner, M.W. 1989. The role of cyclin synthesis and degradation in the control of MPF activity. *Nature* 339: 280-286.

Solomon, M., Glotzer, M., Lee, T., Philippe, M., and Kirschner, M. 1990. Cyclin activation of $p34^{cdc2}$. *Cell* 63: 1013-1024.

Contributed by Sally Kornbluth and Jing Yang
Duke University Medical Center
Durham, North Carolina

Maureen Powers
Emory University School of Medicine
Atlanta, Georgia

Analysis of Apoptosis Using *Xenopus* Egg Extracts

Recapitulation of the apoptotic phenotype in the *Xenopus* egg extract system was first described by Newmeyer et al. (1994) where it was noted that prolonged incubation of the crude interphase extract resulted in extracts that were capable of undergoing apoptosis characterized by chromosome condensation and nuclear fragmentation. The presence of a subcellular fraction enriched in mitochondria is essential for the induction of apoptosis in the extracts. These extracts share many of the biochemical characteristics described in apoptotic cells from other systems, such as caspase activation and DNA fragmentation. In addition, apoptosis in these extracts can be inhibited by addition of caspase inhibitors as well as the anti-apoptotic protein Bcl-2.

PREPARATION OF APOPTOTIC EXTRACTS AND ASSESSING APOPTOSIS

BASIC PROTOCOL

This unit describes preparation of an apoptotic extract from a crude interphase extract (see Basic Protocol) and an extract fractionated into latent and execution phases (see Alternate Protocol). An apoptotic extract can also be reconstituted from a fractionated interphase extract (*UNIT 11.10*) and purified mitochondria (see Support Protocol 2). Protocols are also included for monitoring apoptotic progression in the extract either by following activation of apoptotic proteases (caspases; see Support Protocol 1) or by assessing translocation of cytochrome *c* from the mitochondria to the cytosol (see Support Protocols 3 and 4).

In essence, the apoptotic extract does not differ from the crude interphase extract described in *UNIT 11.10*. Prolonged incubation of the extract at room temperature will result in release of mitochondrial cytochrome *c*, activation of caspases, and if nuclei are present, nuclear fragmentation. Therefore most other applications using crude extract require that incubation times not exceed 3 to 4 hr or the extract may enter apoptosis confounding analysis of the process under study (e.g., nuclear transport, nuclear envelope assembly). However, extracts lacking mitochondria (e.g., the fractionated interphase extract; *UNIT 11.10*) will not enter apoptosis even after prolonged incubation.

Materials

Crude interphase extract (*UNIT 11.10*)
0.2 M phosphocreatine
0.5 mg/ml creatine kinase
0.2 M ATP
Hoechst 33258 (Bisbenzimide H 33258; Calbiochem)
Fluorescence microscope

Additional reagents and equipment for preparation of *Xenopus* crude interphase extract (*UNIT 11.10*)

1. To a crude interphase extract, add 0.2 M phosphocreatine to a concentration of 20 mM (1:10), 0.5 mg/ml creatine kinase to a concentration of 5 µg/ml (1:100 dilution), and 0.2 M ATP to a concentration of 2 mM (1:100) to create an ATP-regenerating system. Mix and incubate this extract 4 to 8 hr at room temperature.

   Since extracts undergo apoptotic nuclear fragmentation at slightly different times, it is a good idea to start observing the nuclei at 0.5-hr intervals starting at ~3 hr after beginning the room temperature incubation.

Contributed by Sally Kornbluth and Erica K. Evans
Current Protocols in Cell Biology (2001) 11.12.1-11.12.8

2. Determine the onset of apoptosis by observing synthetic nuclei stained with Hoechst 33258 and visualized using fluorescence microscopy.

Nuclei assembled around sperm chromatin in the extract can be used as morphological indicators of apoptosis. For assembly of nuclei in vitro and slide preparation, see UNIT 11.10.

At the time of apoptosis, condensation of the chromatin and nuclear fragmentation are clearly visible (see Figure 11.10.3).

For a more quantitative assessment of apoptosis, it is easiest to measure activation of the apoptotic proteases, or caspases in the extract. It is generally possible to detect caspase activity from 2 to 3 μl of apoptotic extract (see Support Protocol 1). It is not necessary to include nuclei in an extract when measuring apoptosis by caspase activation or cytochrome c release.

It is also possible to measure mitochondrial cytochrome c release in these extracts; this is tightly correlated with the onset of apoptosis (see Support Protocol 3).

ALTERNATE PROTOCOL

SEPARATING APOPTOSIS INTO LATENT AND EXECUTION PHASES

This protocol separates the apoptotic reaction into two steps. The first step is the latent phase where a reaction or series of reactions that prepare the extract for apoptosis takes place. The second step of the apoptotic reaction is the execution phase where the apoptotic phenotype characterized by caspase activation, DNA degradation, and nuclear fragmentation is manifested.

The latent phase requires only the cytosolic and light membrane fractions of an interphase extract. Because this extract lacks the mitochondrial fraction, it cannot support caspase activation or full-blown apoptosis. However, when the cytoplasmic and membrane components are combined and allowed to incubate at room temperature for 2.5 hr, a "latent" apoptotic activity develops that is able to accelerate apoptosis in an extract that contains mitochondria. It is likely that activities which trigger mitochondrial cytochrome *c* release develop during the latent incubation. After transfer to the execution extract containing mitochondria, the latent extract triggers release of mitochondrial cytochrome *c* to the cytosol, which then serves as a cofactor in caspase activation.

The execution phase of the apoptotic reaction requires the presence of mitochondria, which are provided by the crude extract. Once a latent extract has been incubated for the appropriate time, 1/10 vol is transferred into a crude extract (execution extract) containing nuclei and the apoptotic morphology of the nuclei is observed after 60 to 120 min. The advantage of setting up the apoptotic reaction in this manner is that signaling molecules operating during the latent phase of apoptosis (prior to detectable caspase activation) can be studied separately from those involved in the apoptotic dismantling of cellular components. The fractionated extract is also easier to immunodeplete (see *UNIT 11.10*), since the soluble extract can be depleted in the absence of membrane components and then later reconstituted with membrane.

Materials

Freshly obtained *Xenopus* eggs, washed with egg lysis buffer (*UNIT 11.10*)
0.2 M phosphocreatine
0.5 mg/ml creatine kinase
0.2 M ATP
20× energy-regenerating mix (*UNIT 11.11*)
Demembranated sperm chromatin (*UNIT 11.10*)
Hoechst 33258 (Bisbenzimide H 33258; Calbiochem)

15-ml polypropylene tubes
5-ml syringe and 18-G needle

Additional reagents and equipment for preparing crude and fractionated interphase extracts (*UNIT 11.10*)

Prepare a fractionated interphase extract

To create a latent extract, a fractionated interphase extract is made exactly as described in *UNIT 11.10*, Basic Protocol 1, and Alternate Protocol 1, with the following variations.

1. Once the eggs are washed with egg lysis buffer and transferred to 15-ml polypropylene tubes (see step 6 of Basic Protocol 1 in *UNIT 11.10*), keep them on ice until crushed (lysed) by centrifugation (step 9 in that protocol), and keep them on ice again at the completion of the lysis centrifugation, until high-speed centrifugation (performed in step 2 of Alternate Protocol 1, *UNIT 11.10*).
2. After removing the crude extract from the 15-ml polypropylene tube with a 5-ml syringe and 18-G needle, do not supplement the crude extract with additional protease inhibitors or cytochalasin B (i.e., skip step 1 of Alternate Protocol 1 in *UNIT 11.10*).
3. Proceed with the steps required for making a fractionated interphase extract (see *UNIT 11.10*, Alternate Protocol 1).

Prepare the latent extract

4. Thaw an aliquot of fractionated cytosol and membrane (*UNIT 11.10*). Recombine 10 µl of membrane with 100 µl of cytosol.
5. Add 0.2 M phosphocreatine to 20 mM (1:10), 0.5 mg/ml creatine kinase to 5 µg/ml (1:100), and 0.2 M ATP to 2 mM (1:100). Mix and incubate 2.5 hr at room temperature.

Prepare execution extract

6. Make fresh crude interphase extract (see *UNIT 11.10*, Basic Protocol 1).
7. Dilute 20× energy regenerating mix to 1× in fresh crude extract.

   This energy mix is used in the execution extract because it does not dilute the extract as much as separate addition of phosphocreatine, creatine kinase, and ATP.

8. Dilute demembranated sperm chromatin to 1000 nuclei/µl in the execution extract.

Mix extracts and assess for apoptosis

9. Transfer 1/10 vol latent extract into execution extract (e.g., 5 µl latent extract into 50 µl execution extract). Mix by flicking tube.
10. Incubate at room temperature. Withdraw samples at 10-min intervals and stain with Hoechst 33258 (as for visualizing nuclear protein import; see *UNIT 11.10*, Basic Protocol 3). Examine for apoptotic nuclei.

    Nuclei should form by 30 min. Nuclei usually undergo apoptosis between 60 and 120 min after transfer.

SUPPORT PROTOCOL 1

MEASURING CASPASE 3–LIKE ACTIVITY

When apoptosis is activated in the egg extracts, it is possible to detect a caspase activity with a substrate specificity very similar to that seen for mammalian caspase 3, one of the "effector" caspases. Measurement of this activity provides an easy way to quantitate apoptosis in the extract, particularly to compare apoptosis in extracts subjected to different experimental treatments.

In vitro caspase assays are based on the cleavage of synthetic peptide substrates of the caspases linked to either chromophores or fluorophores. Upon cleavage of the substrate by relevant caspases from the apoptotic extract, release of the chromophore or fluorophore can be detected by fluorimeter or spectrophotometer. In the case of caspase 3, the synthetic substrate, Ac-DEVD-pNA is used.

Materials

Assay buffer (see recipe), room temperature
Apoptotic egg extract (see Basic Protocol or Alternate Protocol)
Cleavage substrate: 2 mM Ac-DEVD-pNA (see recipe)
Cleavage inhibitor: 20× Ac-DEVD-CHO (see recipe)
96-well microtiter plate

1. For each sample/time point to be assayed, place 85 µl of room temperature assay buffer in a well of a 96-well microtiter plate. Keep at room temperature.

   Assays are done in a 96-well plate to facilitate reading of multiple samples.

2. Add 1 to 5 µl of the apoptotic egg extract to be assayed to the well.

3. Add 10 µl of caspase substrate, 2 mM Ac-DEVD-pNA. Mix by tapping the sides of the plate. Incubate 1 hr in a 37°C incubator.

4. Stop the assay by addition of 5 µl of caspase inhibitor, 20× Ac-DEVD-CHO. Read all samples at 405 nm at the very end of the experiment.

   Alternatively, read the samples immediately using a microtiter plate reader at 405 nm.

   Addition of the caspase inhibitor stops further development of the caspase assay, allowing samples to be read later. If the peptide inhibitor is not added, the caspase assay will continue to develop, making it impossible to compare samples from different time points.

SUPPORT PROTOCOL 2

PREPARATION OF MITOCHONDRIA FROM *XENOPUS* EGG EXTRACTS

As noted in the Basic Protocol, the mitochondrial fraction of *Xenopus* egg extracts is absolutely required for in vitro apoptosis. When the cytosol and light membrane fractions of the extract (*UNIT 11.10*) are combined, the reconstituted extract will not enter apoptosis. However, if a purified heavy membrane fraction enriched in mitochondria is added, the reconstituted extract becomes competent to support apoptosis. Indeed, it has been reported that adding twice the concentration of mitochondria found endogenously in the crude extract results in the production of a robust and reproducible apoptotic extract (Faure et al., 1997). This is almost certainly due to the fact that mitochondrial cytochrome *c* release, which triggers activation of apoptotic proteases, is the downstream target of many apoptotic signaling pathways. Therefore, increasing the mitochondrial concentration results in increased cytochrome *c* release and more rapid apoptosis. The following protocol describes a procedure, adapted from the laboratory of Dr. Don Newmeyer of La Jolla Institute of Allergy and Immunology, to prepare a heavy membrane fraction enriched in mitochondria.

Materials

Fractionated interphase extract (Alternate Protocol 1 in *UNIT 11.10*)
Percoll solutions (see recipe)
MIB buffer (see recipe)

2.5-ml ultracentrifuge tubes (e.g., Beckman)
Beckman TL-100 table-top ultracentrifuge and TLS-55 swinging-bucket rotor

Additional reagents and equipment for preparing fractionated *Xenopus* interphase extract (*UNIT 11.10*)

1. Harvest the cytosol and light membrane fractions of a fractionated interphase extract. After removing the light membranes, gently remove the heavy membrane fraction using a cut-off pipet tip and a P-200 micropipettor. Place the material on ice.

   The mitochondrial/heavy membrane fraction is the brown opaque layer that lies directly below the light membrane layer. The translucent layer directly below the heavy membranes consists largely of pigment granules and glycogen.

2. For each sample, prepare a Percoll step gradient by slowly adding successive 0.45 ml layers of each of the following Percoll solutions to a 2.5-ml tube for the TLS-55 swinging-bucket rotor: 42%, 37%, 30%, and 25%.

   These solutions must be layered on top of each other slowly to prevent mixing.

3. Add the entire mitochondrial layer from the extracts of eggs of one to two frogs on top of the gradient. Centrifuge 25 min at 53,500 × *g* (25,000 rpm in TLS-55 rotor), 4°C, without the brake.

   After centrifugation, any contaminating light membranes will be at the top of the Percoll gradient. Heavy membranes/mitochondria will form a discrete dark band between Percoll concentrations.

4. Withdraw the heavy membranes/mitochondria using a Pasteur pipet. Resuspend them in 50 vol MIB buffer. Mix by gently inverting the tube.

5. Pellet the mitochondria by centrifuging 10 min at 1700 × *g*, 4°C.

6. Discard the supernatant and resuspend the mitochondrial pellet in an equal volume of MIB buffer to give a 10× stock for addition to reconstituted extract.

   Although some laboratories use frozen stocks of mitochondria, freeze/thaw can result in puncturing of mitochondria. Should this occur, direct cytochrome c leakage from mitochondria may produce rapid, unregulated apoptosis upon addition to extracts. This is obviously not useful for studying regulation of the apoptotoic process. Consequently, the authors prefer to use the mitochondrial fraction on the day of preparation. The entire procedure takes ~2.5 hr.

CYTOCHROME *c* RELEASE ASSAYS

SUPPORT PROTOCOL 3

In many systems, execution of the apoptotic program depends upon release of cytochrome *c* from the mitochondria. Once released to the cytoplasm, cytochrome *c* serves as a cofactor in activation of the apoptotic proteases, or caspases. Indeed, addition of purified cytochrome *c* to *Xenopus* egg extracts results in rapid caspase activation and fragmentation of subcellular structures (e.g., nuclei, endoplasmic reticulum). Therefore, one way to assess apoptotic progression in the *Xenopus* egg extract is through measurement of mitochondrial cytochrome *c* release. This can be done in crude egg extracts or, alternatively, purified mitochondria (see Support Protocol 2) can be mixed with cytosol from the fractionated interphase extract (*UNIT 11.10*), which will also support mitochondrial cytochrome *c* release.

Materials

Crude extract for apoptotic reaction (see Basic Protocol)
0.1-µm ultrafree-MC filters (Millipore)
Anti-cytochrome *c* antibody (Pharmingen)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. At various time intervals (e.g., 0.5-hr to 1-hr intervals are generally sufficient), withdraw 25-µl aliquots of crude extract for apoptotic reaction and pass through a 0.1-µm ultrafree-MC filter.

   This removes both mitochondria and other particulate matter. The supernatant above the filter should contain any cytochrome c that has been released into the cytosol.

2. Separate 7 µl of the supernatant retained above the filter by SDS-PAGE (*UNIT 6.1*) and immunoblot (*UNIT 6.2*) with anti-cytochrome *c* antibody.

SUPPORT PROTOCOL 4

CYTOCHROME *c* RELEASE ASSAY USING PURIFIED MITOCHONDRIA AND CYTOSOL

Under some circumstances it is desirable to prepare cytosol and mitochondria separately for reconstitution of cytochrome *c* release. For example, separate preparations are used when immunodepleting the cytosol of a particular protein to determine whether the protein in question affects cytochrome *c* release. Moreover, using purified mitochondria it is possible to determine if a given recombinant protein can induce mitochondrial cytochrome *c* release in the absence of accessory cytosolic components. Under these circumstances, egg lysis buffer containing the protein of interest would be substituted for cytosol in this protocol.

Materials

Heavy membrane fraction containing mitochondria (see Support Protocol 2)
Cytosolic fraction containing energy regenerating mix (*UNIT 11.10*)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Combine 14 µl of the heavy membrane fraction containing mitochondria with 170 µl cytosol containing an energy-regenerating mix. Incubate at room temperature.
2. At 0.5-hr intervals, withdraw 25-µl aliquots. Filter, electrophorese (*UNIT 6.1*), and immunoblot (*UNIT 6.2*) the supernatant for cytochrome *c*.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Assay buffer

50 mM HEPES, pH 7.4
100 mM NaCl
0.1% (w/v) CHAPS
1 mM EDTA
10% (v/v) glycerol
10 mM dithiothreitol, added fresh before each use
Store indefinitely at 4°C

Table 11.12.1 Preparation of Percoll Solutions for Gradients

Ingredient	% Percoll			
	42%	37%	30%	25%
MIB buffer concentrate[a]	0.612 ml	0.612 ml	0.612 ml	0.612 ml
Percoll	1.05 ml	0.92 ml	0.75 ml	0.25 ml
Water	0.838 ml	0.968 ml	1.14 ml	1.263 ml

[a]See recipe for MIB buffer.

Cleavage inhibitor: Ac-DEVD-CHO, 20×

Prepare 0.1 mM or 0.05 mg/ml stock of Ac-DEVD-CHO (Biomol) in DMSO. Dilute stock 1:50 in assay buffer (see recipe). Store up to several months at −20°C.

Cleavage substrate: Ac-DEVD-pNA, 2 mM

Dissolve 1.3 mg/ml Ac-DEVD-pNA (Biomol) in assay buffer (see recipe) for a 2 mM final concentration. Store in light-proof tube at −20°C.

MIB buffer

Prepare MIB buffer concentrate:
50 mM sucrose
10 mM KCl
10 mM sodium succinate
10 mM HEPES adjusted to pH 7.5 with KOH
5 mM EGTA
210 mM mannitol
0.5 mM dithiothreitol, added fresh before each use
Store indefinitely at 4°C

To prepare MIB buffer: Dilute 2.45 ml MIB buffer concentrate to 10 ml final volume in water.

Discard MIB buffer concentrate if microbial growth is detected.

Percoll solutions

Prepare Percoll solutions according to Table 11.12.1.

COMMENTARY

Background Information

Recently, a cell-free extract of *Xenopus* eggs that can support apoptosis was described (Newmeyer et al., 1994). The preparation of these extracts and assays of apoptosis are described in this unit (see Basic Protocol and Alternate Protocol). The ability to use eggs as a source of material to reconstitute apoptotic processes in vitro most likely reflects the in vivo process of oocyte atresia, wherein oocytes that have matured but have not been laid due to a lack of appropriate hormonal stimuli, are systematically resorbed by apoptosis.

As is the case in vivo, the apoptotic nuclear fragmentation seen in the in vitro extract of *Xenopus* eggs is characterized by a series of stereotyped phenotypic changes in subcellular structures. The extract offers the opportunity to examine nuclear events of apoptosis and also to study biochemically the regulation and activation of proteases critical for apoptotic execution. In extracts, as in vivo, the onset of apoptosis is marked by the condensation of DNA, after which the condensed DNA is distributed into many small membrane-enclosed structures (see Figure 11.10.3). Gel electrophoresis reveals the characteristic "laddering" of DNA, indicative of internucleosomal cleavage. Nuclear fragmentation in these extracts is accompanied by the activation of apoptotic proteases, the caspases (Newmeyer et al., 1994; Cosulich et al., 1996), and this is completely inhibited by addition of the broad range apoptotic inhibitor, bcl-2 protein (Bellamy et al., 1995; Villa et

al., 1997; Chao and Korsmeyer, 1998). By these crititeria, the extract faithfully recapitulates a number of the biochemical events of in vivo apoptosis. Additionally, other known positive regulators of apoptosis (e.g., Drosophila reaper protein, activated caspases, and cytochrome *c*) and negative regulators of apoptosis (e.g., tetrapeptide caspase inhibitors) behave in the *Xenopus* extract as in other systems, again supporting the notion that the extract accurately reconstitutes the apoptotic process (Evans et al., 1997; Kuwana et al., 1998). Coupled with the fact that elements of the apoptotic machinery have proven to be well conserved from worms to man, these observations make it highly likely that the *Xenopus* system will provide an excellent model for understanding key events in the regulation of apoptosis, as has been the case for the study of cell cycle progression, DNA replication, nuclear transport, and nuclear architecture.

Critical Parameters

Although most extracts will eventually enter apoptosis, the time required varies a great deal from extract to extract. A CSF-arrested extract or an extract otherwise in mitosis is extremely refractory to apoptosis.

When handling mitochondria to be used for apoptotic or cytochrome *c* release assays, they must be treated gently to prevent breakage. Freeze-thawing the mitochondria can cause them to leak cytochrome *c*; therefore, fresh mitochondria are optimal for apoptosis experiments.

In extracts containing in excess of 1000 sperm nuclei/µl, the nuclei may fragment very slowly, despite relatively high levels of caspase activity. Therefore, to use nuclear fragmentation as an indicator of apoptosis, nuclei should be kept at concentrations <1000 sperm nuclei/µl.

When resolving samples by SDS-PAGE for anti-cytochrome *c* immunoblots, it is worth noting that many of the commercially available molecular weight markers include cytochrome *c* as one of the molecular weight standards. This band will also "light up" with the cytochrome *c* antibodies, providing a convenient size marker for the cytochrome *c* on immunoblots.

Anticipated Results

Extracts entering apoptosis should fragment added nuclei and activate DNases that can cleave the added DNA. Caspase activity should be detectable prior to visible chromatin fragmentation, and this activity should be preceded by observation of cytochrome *c* release from the mitochondria to the cytoplasm. Expect to obtain a volume of mitochondria ~1/20 the volume of the starting crude extract.

Time Considerations

The time for an extract to enter apoptosis varies enormously depending upon the extract. Although the typical extract takes 4 to 6 hr to enter apoptosis, extracts can enter apoptosis in as little as 1 hr or after as long as 12 hr. When doing an experiment dependent upon an extract having entered apoptosis, it is advisable to monitor apoptotic progression, either through examination of nuclei in the extract or through measurement of caspase activity. The authors have also observed that extract containing large numbers of nuclei may be slower to enter apoptosis than extracts lacking nuclei. Cytochrome *c* release from mitochondria will precede caspase activation slightly.

Literature Cited

Bellamy, C.O.C., Malcomson, R.D.G., Harrison, D.J., and Wyllie, A.H. 1995. Cell death in health and disease: The biology and regulation of apoptosis. *Semin. Cancer Biol.* 6:3-16.

Chao, D.T. and Korsmeyer, S.J. 1998. BCL-2 family: Regulators of cell death. *Annu. Rev. Immunol.* 16:395-419.

Cosulich, S.C., Green, S., and Clarke, P.R. 1996. Bcl-2 regulates activation of apoptotic proteases in a cell-free system. *Curr. Biol.* 6:997-1005.

Evans, E.K., Kuwana, T., Strum, S.L., Smith, J.J., Newmeyer, D.D., and Kornbluth, S. 1997. Reaper-induced apoptosis in a vertebrate system. *Eur. Mol. Biol. Organ. J.* 16:7372-7381.

Faure, S., Vignerson, S., Doree, M., and Morin, N. 1997. A member of the Ste/PAK family of protein kinases is involved in both arrest of *Xenopus* oocytes at G2/prophase of the first meiotic cell cycle and in prevention of apoptosis. *Eur. Mol. Biol. Organ. J.* 16:5560-5561.

Kuwana, T., Smith, J.J., Muzio, M., Dixit, V., Newmeyer, D.D., and Kornbluth, S. 1998. Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome *c*. *J. Biol. Chem*. 273:16589-16594.

Newmeyer, D.D., Farschon, D.M., and Reed, J.C. 1994. Cell-free apoptosis in *Xenopus* egg extracts: Inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 79:353-364.

Villa, P., Kaufmann, S.H., and Earnshaw, W.C. 1997. Caspases and caspase inhibitors. *Trends Biochem. Sci.* 22:388-393.

Contributed by Sally Kornbluth and Erica K. Evans
Duke University Medical Center
Durham, North Carolina

Mitotic Spindle Assembly In Vitro

This unit presents in vitro assays for studying the process of mitotic spindle assembly using *Xenopus laevis* egg extracts. These protocols all make use of cytostatic factor (CSF)–arrested extracts. CSF extracts arrested in metaphase are open to biochemical study and manipulation and allow for extensive cytological analysis of experiments. A detailed protocol for the preparation of CSF extracts can be found in *UNIT 11.11*, and the authors' modifications are given in Support Protocol 1.

The basic protocols in this unit can be used to assay the roles of different cellular factors in the many processes and events that must take place to properly assemble a mitotic spindle. All of these assays are performed in a test tube environment. The researcher combines CSF extract with factors that generate mitotic microtubule structures, such as centrosomes or dimethyl sulfoxide (DMSO), which give rise to mitotic asters (see Basic Protocol 1), sperm nuclei (see Basic Protocols 2 and 3), or DNA beads (see Basic Protocol 4), which lead to the formation of spindles. Rhodamine-labeled tubulin (see Support Protocol 2) is added to allow quick and easy visualization by fluorescence microscopy of microtubule growth and organization in samples taken at various time points. Experimental conditions are created by pretreating the extract to specifically inactivate or remove proteins of interest (see *UNIT 11.11* and Support Protocol 3). Finally, the results of individual experiments are preserved by transferring the extract reactions to coverslips for fixation and storage (see Support Protocol 4). This same fixation method also allows for immunofluorescence analysis of individual experiments (*UNIT 4.3*).

The first basic protocol (see Basic Protocol 1) describes a method for assembling microtubule asters in extracts using either DMSO or purified centrosomes. Although spindles are not formed in these reactions, they are useful, because of their simplicity of method and mechanism, for studying microtubule nucleation, dynamics, and organization. Subsequent protocols build on the same basic reaction, but substitute sperm nuclei (see Basic Protocols 2 and 3) or DNA beads (see Basic Protocol 4) to promote spindle formation. Purified *Xenopus* sperm nuclei added to the CSF extract support assembly of monopolar spindles ("half-spindles"), which eventually fuse to form bipolar spindles. This protocol is useful as a fast and simple assay of spindle assembly in the extract. A more physiological method for assembling spindles involves cycling the extract containing sperm nuclei through interphase and back into mitosis, allowing the chromosomes and centrosome to duplicate (see Basic Protocol 3). After reentry into a mitotic state, the extract assembles true bipolar spindles. This protocol allows the researcher to study the importance of interphase events in spindle assembly. The last basic protocol (see Basic Protocol 4) describes a method for assembling spindles in extract around DNA-coated beads (for preparation see Support Protocol 5). These beads are sufficient to support spindle assembly in the extract and yet lack centromeric DNA sequences and centrosomes. This protocol, therefore, allows study of the spindle assembly process in a more simplified system that depends on the microtubule-stabilizing and -organizing capacity of mitotic chromatin.

NOTE: Schematic diagrams illustrating the anticipated time course of extract reactions are shown in Figure 11.13.1, while actual micrographs of asters and spindles are shown in Figure 11.13.2.

Contributed by John Merlie, Jr. and Rebecca Heald

Current Protocols in Cell Biology (2001) 11.13.1-11.13.23

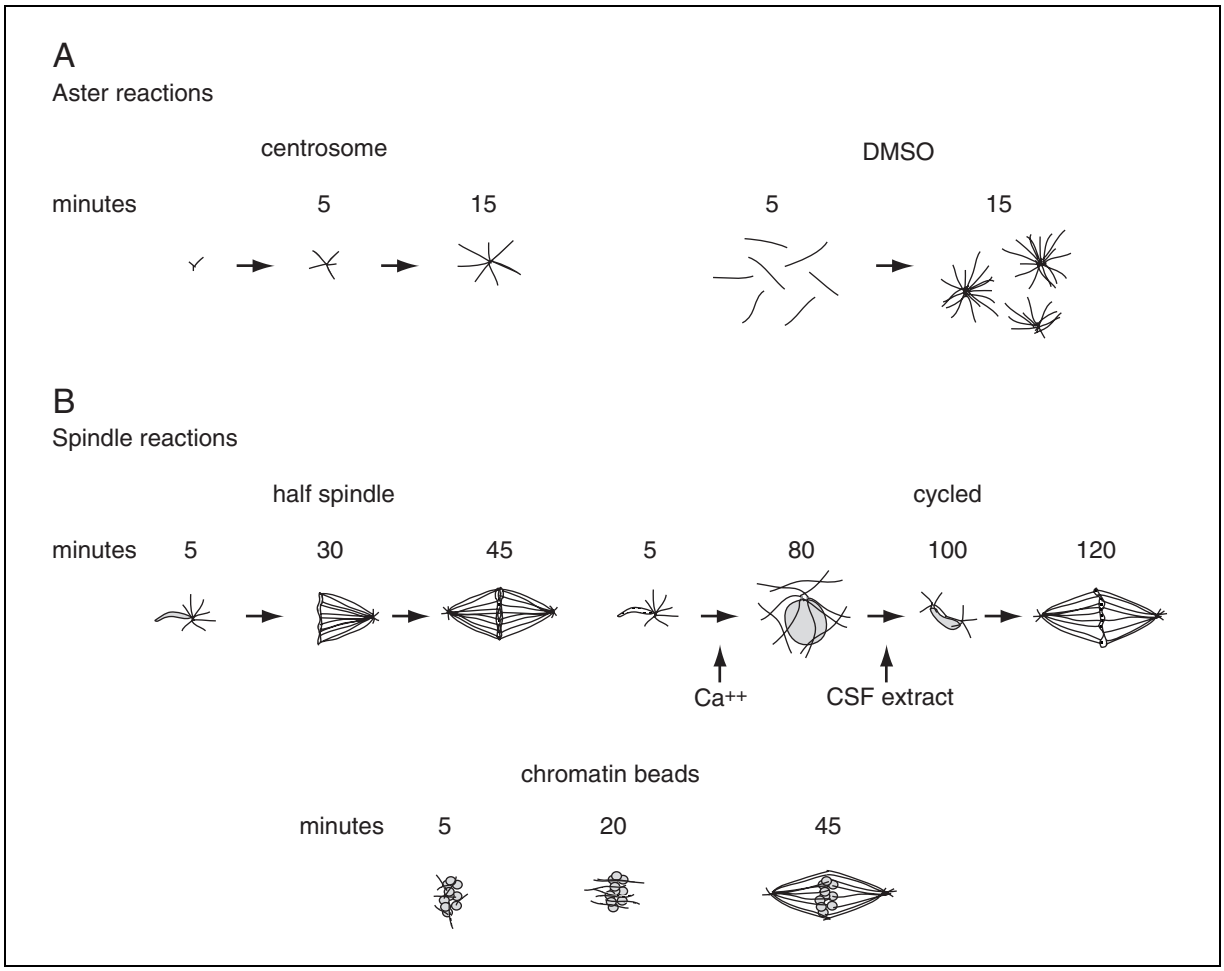


Figure 11.13.1 Schematic drawings illustrating the different extract reactions. (**A**) Aster assembly reactions showing centrosome-nucleated microtubules and DMSO-stabilized microtubules that are organized by microtubule-based motor proteins. (**B**) Spindle assembly reactions with sperm nuclei and chromatin beads.

BASIC PROTOCOL 1

ANALYZING DMSO AND CENTROSOME ASTER REACTIONS

Aster reactions provide a fast and simple method for determining the ability of an extract to support microtubule nucleation, polymerization, and organization. This protocol makes use of either purified centrosomes or DMSO to induce aster assembly. Centrosomes provide focal sites for microtubule nucleation and organization. A protocol for purifying centrosomes from human lymphocytes is given in Blomberg-Wirschell and Doxsey (1998). In contrast, DMSO acts as a microtubule-stabilizing agent that leads to the spontaneous nucleation of microtubules, which are then organized into focused asters by microtubule-based motor proteins. CSF extract is combined with either of the above and rhodamine-labeled tubulin (see Support Protocol 2). Asters begin to form within minutes after incubation at 20°C. Samples taken at various time points are squashed on slides with fixative. Reactions can be "spun down" onto coverslips for fixation, immunofluorescence analysis, and long-term storage (see Support Protocol 4).

Materials

20 to 30 mg/ml rhodamine-labeled tubulin (see Support Protocol 2)
CSF extract (*UNIT 11.11*; also see Support Protocol 1)
Dimethyl sulfoxide (DMSO), anhydrous *or* ~5×10^8 purified centrosomes/ml
Spindle fix (see recipe)
Nail polish

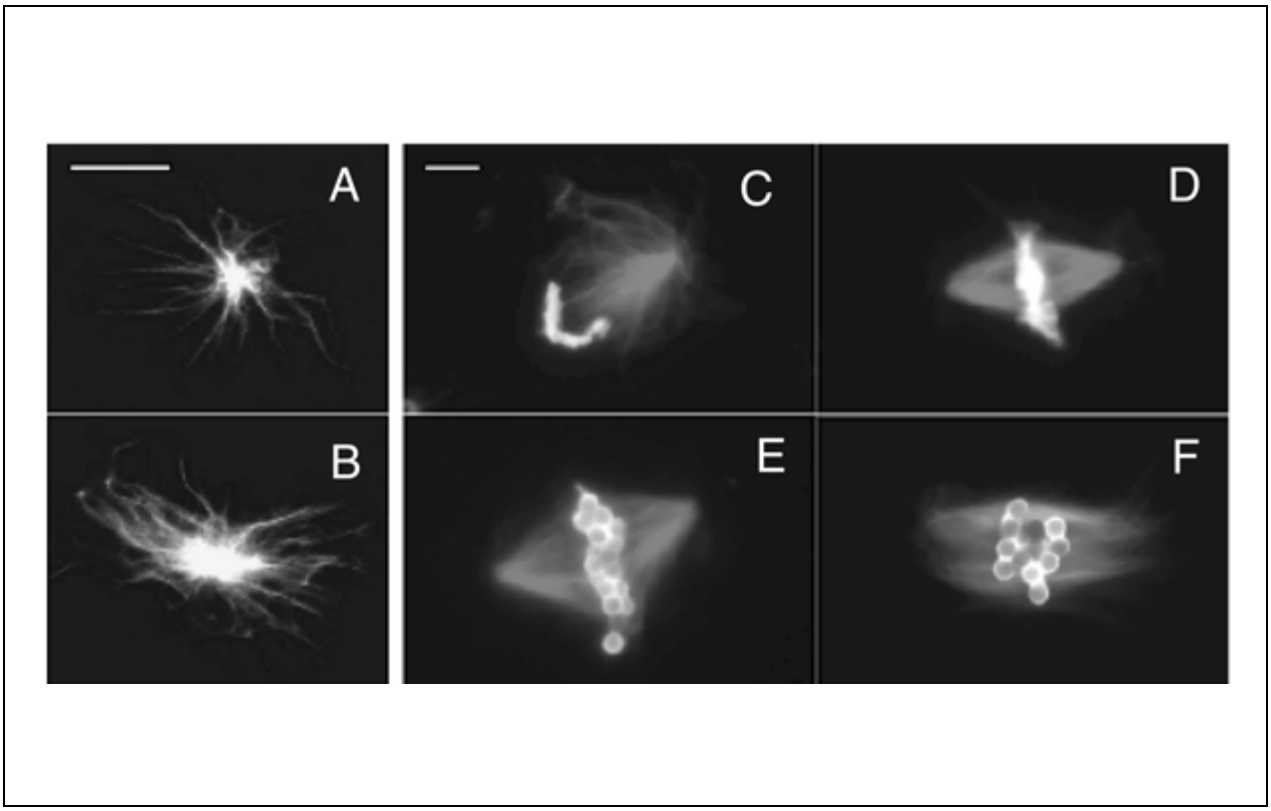


Figure 11.13.2 Examples of asters and spindles visualized by fluorescence microscopy. (**A**) Centrosome-nucleated aster. (**B**) DMSO aster. (**C**) Sperm half-spindle. (**D**) Sperm bipolar spindle. (**E**) DNA bead spindle. (**F**) DNA bead spindle assembled in the presence of antibody 70.1 that recognizes dynein intermediate chain. Microtubules are visualized by incorporation of rhodamine-labeled tubulin and appear gray. DNA is seen in the DAPI channel and appears white. Bar is equal to 5 μm.

1.5-ml microcentrifuge tubes
Wide-orifice 1- to 200-μl pipet tips
Water bath
Microscope slides and 18 × 18–mm square coverslips
Fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets

1. On ice, prepare a 1:10 dilution of rhodamine tubulin stock by adding 1 μl stock to 10 μl CSF extract. Prepare the reaction tube on ice by adding 1.25 μl of the 1:10 dilution of rhodamine tubulin to 25 μl CSF extract (1:20) in a 1.5-ml microcentrifuge tube to give a final reaction mixture with a tubulin dilution of 1:200.

   IMPORTANT NOTE: *Always mix and transfer extract and extract reactions with wide-orifice (or cut-off) pipet tips, and avoid generating bubbles. It is important to use 1.5-ml tubes and not let reaction volumes exceed 100 μl, to permit better gas exchange.*

   For multiple reactions, dilute labeled tubulin stock, to a concentration of 0.10 to 0.15 mg/ml (1:200), into the total volume of extract needed, then aliquot into 25-μl reactions.

2. Add 1.25 μl of anhydrous DMSO or purified centrosomes.

   Centrosome stock should be ~5 × 10^8 centrosomes/ml.

3. Prepare a 20°C bath by adding sufficient ice to bring a room temperature bath to the appropriate temperature. Add additional ice, as needed, to maintain temperature.

   IMPORTANT NOTE: *Reactions are initiated by placing samples at 20°C. Do not place samples on ice before fixation, as this will cause the microtubules to depolymerize.*

4a. *For centrosome reactions:* Incubate for 5 to 15 min then take a "squash sample" by transferring 1 µl of the reaction to a microscope slide. Carefully place 5 µl of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 × 18–mm coverslip.

Always mix reactions by pipetting gently before removing "squash samples" for mounting on slides.

For best results, lower the coverslip at an angle and let it touch the extract/fixative drop before releasing. Because preservation of microtubule structures is somewhat variable, take duplicate samples.

Two or three coverslips can be fit side-by-side on each slide.

Analysis using the rhodamine channel should reveal red asters of microtubules emanating from single focal points. Aster size will increase with increasing incubation time.

4b. *For DMSO reactions:* Incubate for 5 to 30 min then take a 1-µl sample of the reaction to fix and squash as described above.

At early time points extensive microtubule polymerization is apparent. By 20 min, asters have organized, and these contain many more microtubules than centrosome asters.

Analysis using the rhodamine channel should reveal red asters of microtubules emanating from single focal points. Aster size will increase with increasing incubation time.

5. Seal the edges of the coverslips with nail polish and store squash samples in the dark at 4°C.

Alternatively, reaction mixtures can be spun onto coverslips, fixed, and mounted (see Support Protocol 4).

Spin-downs and squash samples, if sealed, can be stored at 4°C for months to years after the experiment is performed.

6. Analyze on a fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets.

Analysis using the rhodamine channel should reveal red asters of microtubules emanating from single focal points. Aster size will increase with increasing incubation time.

BASIC PROTOCOL 2

ANALYZING SPERM DNA "HALF-SPINDLE" REACTIONS

"Half-spindle" reactions provide a fast and easy assay for spindle assembly in an extract. This method of spindle assembly proceeds in a nonphysiological manner. The centrosome of a sperm nucleus nucleates a single microtubule aster that interacts with sperm chromosomes, forming a monopolar (half-) spindle. Half-spindles then fuse pairwise to form bipolar spindles; however, these reactions are still quite sensitive to perturbations in the extract, making them quite useful for studying the roles of specific proteins and factors in spindle assembly. CSF extract is combined with demembranated *Xenopus* sperm nuclei (for preparation, see *UNIT 11.10*) and rhodamine-labeled tubulin. Incubation at 20°C allows microtubule polymerization and spindle assembly. Fluorescence microscopy analysis of squash samples will reveal a few fully-formed bipolar spindles and many half-spindles as early as 15 to 30 min after the start of incubation; however, a reaction is not considered "complete" until at least 45 or 60 min. At this point, an unperturbed extract of good quality will have produced bipolar spindles around 75% to 95% of the sperm nuclei that it contains. Reactions can then be spun down onto coverslips for fixation, immunofluorescence analysis, and long-term storage (see Support Protocol 4).

Materials

20 to 30 mg/ml rhodamine tubulin stock (Murray, 1991)
CSF extract (*UNIT 11.11*; also see Support Protocol 1)
20× demembranated sperm nuclei (~100 sperm/µl extract; *UNIT 11.10*)

Spindle fix (see recipe)
Nail polish

1.5-ml microcentrifuge tubes
Water bath
Microscope slides and 18 × 18–mm square coverslips
Fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets

1. On ice, prepare a 1:10 dilution of rhodamine tubulin stock by adding 1 µl stock to 10 µl CSF extract. Prepare the reaction tube on ice by adding 1.25 µl of the 1:10 dilution of rhodamine tubulin to 25 µl CSF extract (1:20) in a 1.5-ml microcentrifuge tube to give a final reaction mixture with a tubulin dilution of 1:200.

   IMPORTANT NOTE: *Always mix and transfer extract and extract reactions with wide-orifice (or cut-off) pipet tips, and avoid generating bubbles. It is important to use 1.5-ml tubes and not let reaction volumes exceed 100 µl, to permit better gas exchange.*

   For multiple reactions, dilute labeled tubulin stock, to a concentration of 0.10 to 0.15 mg/ml (1:200), into the total volume of extract needed, then aliquot into 25-µl reactions.

2. Add 2.5 µl of 20× demembranated sperm nuclei (~100 sperm/µl extract).

   More concentrated sperm stocks can be diluted to 20× in 100 mM KCl/150 mM sucrose/1 mM $MgCl_2$, flash frozen, and stored in aliquots at −80°C.

3. Incubate the reaction for 15 min in a 20°C water bath.

   The bath is prepared by adding sufficient ice to bring a room temperature bath to 20°C (see Basic Protocol 1, step 3) and adding additional ice as necessary to maintain temperature.

4. Prepare a squash sample by transferring 1 µl of the reaction to a microscope slide. Carefully place 5 µl of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 × 18–mm coverslip. Analyze samples on a fluorescence microscope with 40× or 63× objective and rhodamine/Hoechst filter sets. Seal with nail polish.

   Always mix reactions by pipetting gently before removing "squash samples" for mounting on slides.

   For best results, lower the coverslip at an angle and let it touch the extract/fixative drop before releasing. Because preservation of microtubule structures is somewhat variable, take duplicate samples.

   Two or three coverslips can be fit side-by-side on each slide.

   Analysis of early time points on a fluorescence microscope should reveal condensed chromatin structures in the channel associated with microtubules in early stages of organization visible in the rhodamine channel.

5. Take additional squash samples at 30, 45, and 60 min to assess spindle assembly as described above. Analyze samples, seal with nail polish and store in the dark at 4°C.

   By 30 min, samples should contain condensed chromatin structures associated with astral arrays of microtubules focused into a single pole.

   At 45 to 60 min samples should also contain half-spindles that have fused to produce bipolar spindles that resemble those produced by the more physiological "cycling" reactions described in the next protocol (see Basic Protocol 3). At this point the reaction is complete. Continued incubation will lead to the formation of large aggregates of spindles.

   Alternatively, reaction mixtures can be spun onto coverslips, fixed, and mounted as described (see Support Protocol 4).

   Spin-downs and squash samples, if sealed, can be stored at 4°C for months to years after the experiment is performed.

BASIC PROTOCOL 3

ANALYZING SPERM DNA "CYCLING" REACTIONS

This protocol provides an assay for the importance of interphase events (i.e., DNA replication, centrosome duplication, and chromatin packaging) in the process of spindle assembly. Unlike half-spindle reactions, this protocol cycles the extract through interphase and back into mitosis. Any sperm nuclei present during the reaction undergo chromatin decondensation, DNA replication, and centrosome duplication. Returning the extract to mitosis promotes spindle assembly through the more physiological pathway of microtubule nucleation and growth from duplicated centrosomes around a replicated set of chromosomes containing duplicated kinetochores.

In this protocol, as in half-spindle reactions (see Basic Protocol 2), CSF extract is combined with rhodamine-labeled tubulin and demembranated *Xenopus* sperm nuclei. After a 10-min incubation at 20°C, calcium is added to the reaction to promote entry into interphase. After 80 min of further incubation at 20°C, nuclei will appear large and round, and microtubules should be long and abundant. Addition of fresh CSF extract to the reaction drives the reaction back into mitosis and spindle assembly begins. Spindles are formed 45 to 60 min after addition of fresh CSF extract, and, as with previous reactions, can be spun down onto coverslips (see Support Protocol 4).

Materials

20 to 30 mg/ml rhodamine tubulin stock (see Support Protocol 2)
CSF extract (*UNIT 11.11*; also see Support Protocol 1)
20× demembranated sperm nuclei (~100 sperm/µl extract; *UNIT 11.10*)
10× calcium solution (see recipe)
Spindle fix (see recipe)

20°C water bath
Microscope slides and 18 × 18–mm square coverslips
Fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets

Prepare reaction

1. On ice, prepare a 1:10 dilution of rhodamine tubulin stock by adding 1 µl stock to 10 µl CSF extract. Prepare the reaction tube on ice by adding 1.25 µl of the 1:10 dilution of rhodamine tubulin to 25 µl CSF extract (1:20) in a 1.5-ml microcentrifuge tube to give a final reaction mixture with a tubulin dilution of 1:200.

   IMPORTANT NOTE: *Always mix and transfer extract and extract reactions with wide-orifice (or cut-off) pipet tips, and avoid generating bubbles. It is important to use 1.5-ml tubes and not let reaction volumes exceed 100 µl, to permit better gas exchange.*

   For multiple reactions, dilute labeled tubulin stock, to a concentration of 0.10 to 0.15 mg/ml (1:200), into the total volume of extract needed, then aliquot into 25-µl reactions.

2. Add 2.5 µl of 20× sperm nuclei (~100 sperm/µl extract).

3. Incubate the reaction 10 min in a 20°C water bath.

   The bath is prepared by adding sufficient ice to bring a room temperature bath to 20°C (see Basic Protocol 1, step 3) and adding additional ice as necessary to maintain temperature.

Release extract into interphase

4. Add 5 µl 10× calcium solution and mix well to release extract into interphase.

   Calcium leads to the degradation of the cell cycle regulatory proteins cyclin B and CSF, causing inactivation of the mitotic cdc2 kinase and exit from mitosis.

5. Incubate the reaction 80 min in a 20°C water bath.

6. Transfer 1 µl of the reaction to a microscope slide. Carefully place 5 µl of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 × 18–mm coverslip. At 30 and 80 min after calcium addition, check that the extract is entering interphase. Analyze on a fluorescence microscope with 40× or 63× objective and rhodamine/Hoechst filter sets.

   If sperm nuclei do not appear to be decondensing at 30 min after calcium addition, add an additional 2.5 µl (1/20 dilution) of 10× calcium.

   By 80 min, nuclei are visible in the DAPI channel and should appear large, round, and uniform, while microtubules should be long and abundant.

Drive the reaction into mitosis

7. At 90 min post calcium addition, add 0.5 vol (25 µl) of fresh CSF extract to the reaction. Continue incubation at 20°C.

   Addition of fresh extract will send the reaction back into mitosis.

8. Take squash samples at 15, 30, 45, 60, and 90 min after addition of fresh CSF extract to assess the spindle assembly reaction. Examine the samples using a fluorescence microscope with 40× or 63× objective and rhodamine/Hoechst filter sets.

   Bipolar spindles should be visible by 45 min after addition of fresh CSF extract.

9. Seal the edges of the coverslips with nail polish and store squash samples in the dark at 4°C.

   Alternatively, entire reaction mixtures can be spun onto coverslips, fixed, and mounted (see Support Protocol 4).

ANALYZING DNA-BEAD REACTIONS

BASIC PROTOCOL 4

This protocol assays the ability of an extract to support spindle assembly around chromatin in the absence of centrosomes or special chromosomal sequences. This reduces the system even more towards a minimal set of necessary components and allows for detection of otherwise subtle phenotypes (such as those resulting from the disruption of motor proteins) in the absence of the strong structural cues normally provided by the centrosomes.

DNA-coupled Dynabeads are retrieved on a magnet, washed with CSF extract to remove the storage buffer, and resuspended in fresh CSF extract. After 10 min of incubation at 20°C, the reaction is sent into interphase by the addition of calcium. Incubation for 2 hr at 20°C allows for proper assembly of chromatin on the beads. Addition of fresh CSF extract and incubation at 20°C for 30 min brings the reaction back into mitosis. The beads are retrieved using a magnet, or flash frozen for later use. After removal of the extract, the beads are resuspended in fresh CSF extract. The reaction is again incubated at 20°C. Bipolar spindles should form after 30 to 90 min of incubation, and, as with previous protocols, reactions can then be spun down onto coverslips for fixation, immunofluorescence analysis, and long-term storage.

Materials

DNA beads (see Support Protocol 5)
CSF extract (*UNIT 11.11*; also see Support Protocol 1) with and without 1:200 rhodamine-labeled tubulin (Support Protocol 2)
10× calcium solution (see recipe)
Spindle fix (see recipe)

0.5-ml microcentrifuge tubes

Magnetic particle concentrator (MPC; Dynal)
20°C water bath
Microscope slides and 18 × 18–mm square coverslips
Fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets

1. Transfer 3 µl of DNA beads (about 0.5 µg DNA) to a 0.5-ml microcentrifuge tube and place on a magnetic particle concentrator (MPC) on ice. Remove supernatant using a pipet. Be careful not to disturb the pellet. Wash beads by resuspending them in 20 µl of CSF extract.

2. Retrieve beads using the magnet, pipet off supernatant, and resuspend in 100 µl CSF extract. Transfer to a 1.5-ml microcentrifuge tube and incubate 10 min in a 20°C water bath.

   The bath is prepared by adding sufficient ice to bring a room temperature bath to 20°C (see Basic Protocol 1, step 3) and adding additional ice as necessary to maintain temperature.

3. Send CSF extract into interphase by adding 10 µl of 10× calcium solution. Incubate for 2 hr in a 20°C water bath, mixing periodically.

   Mixing the reactions periodically (every 20 to 30 min) is important to prevent aggregation of beads into large clumps.

4. Return the extract containing the beads to mitosis by adding 50 µl of fresh CSF extract. Incubate for 30 min at 20°C.

   At this point the entire reaction can be flash frozen in liquid nitrogen and stored at –80°C. This provides a stock of preformed chromatin beads thereby avoiding the lengthy chromatin-assembly steps.

5. Incubate the bead mixture on ice for several minutes. Retrieve the beads by incubating in the MPC (magnet) on ice for 10 to 15 min.

   Due to the high viscosity of the extract, bead retrieval is slow. To accelerate the process, mix by pipetting the mixture every few minutes, keeping the tube on the MPC.

6. Remove the supernatant with a pipet. Resuspend the beads in 100 µl of fresh CSF extract containing rhodamine-labeled tubulin at a 1:200 dilution. Incubate at 20°C.

7. Take squash samples to monitor the spindle assembly process by transferring 1 µl of the reaction to a microscope slide. Carefully place 5 µl of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 × 18–mm coverslip. Analyze on a fluorescence microscope with 40× or 63× objective and rhodamine/Hoechst filter sets.

   Spindle assembly requires between 30 and 90 min, depending on the extract.

8. Seal the edges of the coverslips with nail polish and store squash samples in the dark at 4°C.

   Alternatively, entire reaction mixtures can be spun onto coverslips, fixed, and mounted (see Support Protocol 4).

PREPARATION OF CSF EXTRACT

The authors' method for extract preparation, presented here, is very similar to that presented in *UNIT 11.11*, but is somewhat more simple; i.e., oil is not used, eggs are crushed by centrifugation in a Sorvall HB-4 or HB-6 rotor, a second clarifying centrifugation is not performed, cytochalasin D is used instead of cytochalasin B, and a different mix of protease inhibitors is used. If the extract is to be cycled, EGTA should be omitted from the 20× energy mix.

Materials

Xenopus eggs, dejellied (*UNIT 11.10*)
Extract buffer (XB; see recipe)
CSF-XB (see recipe)
10 mg/ml LPC (see recipe)
10 mg/ml cytochalasin D in DMSO: store in aliquots at −20°C
20× energy mix (*UNIT 11.11*)

400-ml beaker
Polished, cut-off glass pipet
SW-50 tubes (Beckman)
13-ml adapter tubes (Sarstedt)
Clinical centrifuge
Sorvall centrifuge and HB-4 or HB-6 rotor with rubber adapters for 15-ml tubes
18-G needle and 1-ml syringe

Additional reagents and equipment for injecting frogs, and collecting and dejellying eggs (*UNIT 11.10*)

1. Inject frogs, collect eggs in 400-ml beaker, and dejelly (*UNIT 11.10*).
2. Wash dejellied eggs several times with 50 to 100 ml extract buffer (XB) and then twice with 50 to 100 ml CSF-XB (XB containing 5 mM EGTA and 1 mM $MgCl_2$). Swirl beaker gently and decant as much buffer as possible between washes.

   Preparation of a good extract is critical for the success of in vitro aster and spindle assembly reactions. Pay close attention to the protocols describing how to collect, handle, and dejelly eggs (UNIT 11.10).

3. Perform one final wash with 50 ml CSF-XB to which LPC has been added to a final concentration of 10 µg/ml.
4. Using a polished, cut-off glass pipet, rinsed with CSF-XB, load eggs into SW-50 tubes containing 1 ml CSF-XB with 10 µg/ml LPC, to which 10 µl of 10 mg/ml cytochalasin D has been added (100 µg/ml final).

   Immerse glass pipet into solution before expelling eggs, so that they do not contact air.

5. Transfer SW-50 tubes containing eggs to 13-ml Sarstedt adapter tubes containing 0.5 ml water to prevent tubes from collapsing.
6. Centrifuge in a room temperature clinical centrifuge at 150 × *g* for 1 min, then at 700 × *g* for 30 sec. Remove all buffer from the top of the packed eggs with a cut-off glass pipet.

   Removal of all the buffer is essential to obtain concentrated cytoplasm.

7. Place the tubes in an HB-4 or HB-6 rotor containing rubber adapters. Centrifuge 15 min at 16,460 × *g* (10,000 rpm), 16°C, to crush eggs.

8. Place tubes on ice. Pierce tube at the bottom of the yellow cytoplasmic layer with an 18-G needle attached to a 1-ml syringe, with the opening directed upwards. Collect cytoplasmic layer.

   Some of the grayish layer just below the cytoplasm should also be collected, but avoid the gray layer above the cytoplasm.

9. Dilute 10 mg/ml LPC to 1:1000, 10 mg/ml cytochalasin D to 1:500, and 20× energy mix to 1× in the cytoplasmic layer.

   If extract is to be cycled through interphase, omit EGTA from the 20× energy mix. The extract can be kept on ice for up to 6 hr before use.

SUPPORT PROTOCOL 2

PREPARATION OF RHODAMINE-LABELED TUBULIN

In the assays described in this unit, microtubules are visualized by incorporation of fluorescently-labeled tubulin subunits. This allows for easy analysis of the reaction without the need for immunostaining techniques. Combining a succinimidyl ester fluorochrome with pure bovine brain microtubules produces fluorescent tubulin derivatives (Hyman et al., 1991). The activated esters are thought to label random surface lysines and give good labeling stoichiometry and yield of functional tubulin. A variety of different fluorochromes can be used, including tetramethylrhodamine, X-rhodamine, Texas Red, and fluorescein. The following protocol requires 100 mg of purified tubulin for the eventual recovery of around 20 mg of labeled product.

Phosphocellulose column–purified tubulin is generally recovered and stored frozen in column buffer. After thawing the tubulin, 20× conversion buffer is added to convert this buffer to BRB80, a buffer that better promotes microtubule polymerization. Addition of GTP and glycerol, and incubation at 37°C, induces polymerization of the tubulin into microtubules. The microtubules are then pelleted, gently resuspended in labeling buffer, and incubated with the fluorochrome while still polymerized. After the addition of quenching buffer to end the reaction, the microtubules are again pelleted to remove unincorporated fluorochrome. The pellets are depolymerized and the resulting soluble tubulin subunits are separated from denatured protein by centrifugation. The recovered supernatants are incubated to again polymerize microtubules, which are then pelleted and depolymerized. After a final centrifugation, the tubulin-containing supernatants are frozen in small aliquots in liquid nitrogen and stored at −80°C until needed for use in extract reactions.

Materials

100 to 200 mg phosphocellulose column–purified tubulin, frozen (Ashford et al., 1998)
20× conversion buffer, ice-cold (see recipe)
200 mM GTP
Glycerol, 37°C
Labeling cushion (see recipe)
Labeling buffer, 37°C (see recipe)
Tetramethylrhodamine succinimidyl ester (Molecular Probes)
Dimethyl sulfoxide (DMSO), anhydrous (Sigma)
BRB80 cushion, 37°C (see recipe)
Quenching buffer, 37°C (see recipe)
IB, ice-cold (see recipe)
2× and 1× BRB80, ice-cold (see recipe for 5×)

37°C water bath
Ultracentrifuge with Ti50 rotor and polycarbonate screw-cap centrifuge tubes, prewarmed to 35°C (Beckman)
3-ml wide-orifice plastic transfer pipets
Wash bottle with H_2O
TLA100 table-top ultracentrifuge with TLA100.3 rotor, TL100 rotor, and polycarbonate centrifuge tubes, prewarmed to 35°C (Beckman)
Sonicator

Additional equipment and reagents for determining protein concentration by Bradford assay (*APPENDIX 3B*)

Prepare reaction mixture

1. Thaw phosphocellulose column–purified tubulin aliquots quickly in a 37°C water bath until mostly liquid. Transfer tubes to ice and measure the volume in a graduated cylinder.

   The starting tubulin amount should be at least 100 mg.

2. Add 1/20 vol ice-cold 20× conversion buffer to convert the column buffer to BRB80.

3. Add 200 mM GTP to 1 mM, and 0.5 vol glycerol, 37°C.

4. Mix well and cover with Parafilm. Immediately transfer to a 37°C water bath and incubate for 45 min to polymerize tubulin.

   Mix the tubulin periodically during this incubation. Bubbles should form and remain suspended in the viscous microtubule solution.

5. During the incubation, load polycarbonate screw-cap centrifuge tubes appropriate for a Ti50 rotor with 5 ml of labeling cushion. Prewarm along with Ti50 rotor to 37°C.

Perform first warm spin

6. Load polymerized tubulin on top of cushions with a 3-ml wide-orifice plastic transfer pipet. Ultracentrifuge 1 hr at 242,000 × *g* (50,000 rpm), 35°C.

   Keep polymerized tubulin warm at all times, for best results perform this step in a 37°C warm room. Fill each tube with 5 ml sample.

   Prewarm labeling buffer to 37°C at this step.

7. After centrifuging, keep tubes at 37°C. Aspirate down to the cushion. Wash the interface with water by filling each tube gently with water from wash bottle, then aspirate the wash and the cushion.

   This step removes inactive tubulin subunits.

8. Resuspend pellets in a minimal volume (50 µl each) of labeling buffer, 37°C, by drawing up and down into a cut-off pipet tip, and keep at 37°C. Wash tubes with 100 µl of labeling buffer, 37°C, to recover the remaining tubulin. Combine all resuspensions and the wash. Note the total volume, which should be around 1.5 ml.

   During this step, be sure to keep the microtubules at 37°C to maintain polymerization. The high pH of the labeling buffer is best for high-stoichiometry labeling, but it is not favorable for tubulin polymerization.

9. Prepare 100 mM tetramethylrhodamine succinimidyl ester in anhydrous DMSO. Add 1/10 volume 100 mM tetramethylrhodamine succinimidyl ester to the resuspended microtubules and vortex gently.

   As an alternative to 100 mM tetramethylrhodamine succinimidyl ester, a different succinimidyl ester fluorochrome can be used instead.

   Tetramethylrhodamine succinimidyl ester reacts with water and is not stable for storage.

10. Incubate 45 min at 37°C. Gently vortex the mixture every few minutes.

    Vortexing is especially important during the first few minutes of incubation. Do not vortex vigorously or the microtubules will depolymerize.

11. Add 1.5 ml BRB80 cushion to polycarbonate tubes appropriate for a TLA100.3 rotor.

    BRB80 cushion, rotor, and tubes should all be prewarmed to 37°C. During this step the quenching buffer can be prewarmed to 37°C as well.

12. Stop the labeling reaction (step 10) by adding 2 vol of quenching buffer, 37°C. Mix the reaction well and keep at 37°C.

Perform second warm spin

13. Load the mixture onto the prewarmed BRB80 cushions with a 3-ml wide-orifice plastic transfer pipet. Ultracentrifuge in a TLA 100.3 rotor for 30 min at 265,070 × *g* (70,000 rpm), 35°C. After the centrifugation, begin cooling the centrifuge to 4°C.

14. Aspirate the supernatants, washing the interface as before (step 8). Place both the rotor and the tubes on ice.

15. Resuspend each pellet in 100 µl ice-cold IB to depolymerize the microtubules. Pool the resuspensions. Draw up and down in a pipet to break up tubulin clumps. Incubate the mixture on ice for 15 min.

    Depolymerization can be accelerated by gentle sonication on ice for 1 to 2 sec pulses.

16. Add 1 vol of ice-cold 2× BRB80, and sonicate briefly. Check the tubulin concentration by Bradford analysis (*APPENDIX 3B*). If it exceeds 30 mg/ml, dilute tubulin with 1× BRB80 to 25 mg/ml before proceeding to the next step.

Perform first cold spin

17. Load depolymerized tubulin into a tube appropriate for the TLA100.3 rotor on ice. Centrifuge 15 min at 265,070 × *g* (70,000 rpm), 4°C. After the centrifugation, begin warming the centrifuge to 35°C.

18. Recover the supernatant and transfer to a 15-ml conical tube. Add 0.5 vol 37°C glycerol and GTP to 1 mM. After recovering the supernatants begin warming the rotor to 37°C.

19. Polymerize the microtubules by incubating 45 min at 37°C.

    Mix the reaction periodically during this step.

20. During the incubation add 1.5 ml of BRB80 cushion to tubes appropriate for a TLA100.3 rotor, and begin warming to 37°C.

Perform third warm spin

21. Load the polymerized microtubules onto the prewarmed cushions with a wide orifice (i.e., a cut 200- to 1000-µl pipet tip). Centrifuge 20 min at 265,070 × *g* (70,000 rpm), 35°C. After the centrifugation, begin cooling the centrifuge to 4°C. Also begin cooling a TLA100 rotor and polycarbonate tubes to 4°C.

22. Aspirate the supernatants and resuspend each pellet in 100 µl of ice-cold IB.

23. Add 1 vol cold 2× BRB80 and sonicate briefly. Check the concentration by Bradford assay (*APPENDIX 3B*). If necessary, dilute labeled tubulin to 30 mg/ml with cold 1× BRB80.

Perform final cold spin

24. Load mixture into TLA100 tubes on ice. Centrifuge 15 min at 265,070 × *g* (70,000 rpm), 4°C.

25. Recover the supernatant on ice and determine tubulin concentration by Bradford assay (*APPENDIX 3B*), and record.

26. Freeze in 1-µl and 50-µl aliquots in liquid nitrogen and store at −80°C.

    The 1-µl aliquots are for use with extract reactions. The 50-µl aliquots may be thawed when needed, split into 1-µl aliquots, flash frozen, and stored at −80°C.

MOTOR DISRUPTION

SUPPORT PROTOCOL 3

The following protocol illustrates a technique that can be used to assess the function of an individual protein in spindle assembly using the *Xenopus* egg extract system. The protocol describes how to disrupt the microtubule-based motor cytoplasmic dynein, which is required for organization of spindle poles. Disruption is achieved by adding inhibitory antibodies, which have been shown to inhibit association of dynein to microtubules in spindles (Heald et al., 1997). Antibodies dialyzed against extract buffer (XB) can be added to the CSF extract either before initiating the reaction, or after microtubule structures have formed. The effects of dynein disruption are monitored by squash or spin-down analysis.

Materials

Dynein intermediate chain antibody 70.1 (Sigma), as ascites
Unreactive antibody (e.g., mouse IgG or ascites; Sigma)
Extract buffer (XB; see recipe)

12,000 to 14,000 mol.-wt. cut-off dialysis tubing
4°C microcentrifuge
Low-volume concentrators with mol. wt. 50,000 cut-off (Microcon)

1. Dialyze 0.5 ml of 70.1 ascites against two changes of 1 liter XB, 4 hr each, using 12 to 14 kDa cut-off dialysis tubing at 4°C. Concentrate to 15 to 20 mg/ml using microconcentrator in a microcentrifuge at 4°C.

   Dynein inhibition requires a relatively high concentration of this antibody.

   Dialysis into XB removes any sodium azide, buffer, or salts that could disrupt the extract.

2. Add 1.25 µl diluted rhodamine tubulin and 2.5 µl dynein intermediate chain antibody 70.1 to 25 µl CSF extract (a dilution of 1:10). Mix well and incubate on ice for 10 to 20 min. Prepare a control sample containing a similar amount of control antibody such as mouse IgG in parallel.

3. Proceed with the aster or spindle assembly protocol (see Basic Protocols 1 to 4).

   Antibody can also be added to extract after reactions are complete. For dynein inhibition the same effects are observed.

4. Analyze squash or spin-down samples taken at different time points, and compare the phenotypes observed in the antibody-treated samples with the control samples.

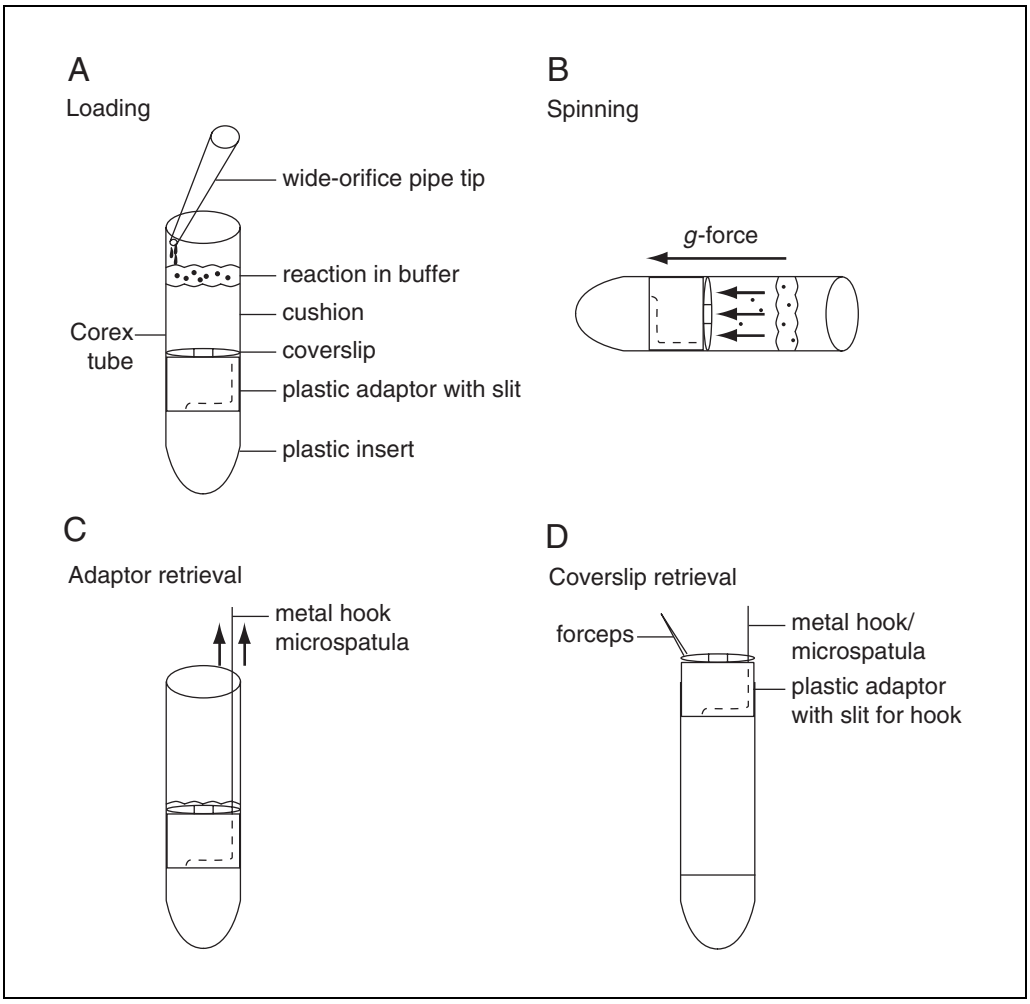


Figure 11.13.3 Schematic showing how spin-down tubes work to transfer spindles or asters from extract to coverslips for immunofluorescence and long-term storage. (**A**) Using a wide-orifice pipet tip, the extract reaction is layered on top of a glycerol cushion in a modified Corex tube containing a plastic insert and adapter with a coverslip on top. (**B**) Centrifugation in a swinging bucket rotor pellets asters and spindles through the cushion onto the coverslip. (**C**) After the cushion is aspirated, the adaptor is retrieved using a metal hook or spatula that fits into groove on one side of the adaptor. (**D**) The coverslip is retrieved from the top of the adaptor with forceps and transferred to –20°C methanol to fix.

SUPPORT PROTOCOL 4

REACTION SPIN-DOWNS

Spin-downs allow an entire reaction mixture to be transferred onto glass coverslips, fixed, and, if desired, subjected to immunostaining techniques. Because reactions are diluted into detergent and spun through a cushion, much of the soluble material is removed, including unincorporated fluorochrome-labeled tubulin subunits. This procedure dramatically reduces background fluorescence compared to squash samples.

Reactions to be processed are first diluted with the appropriate spin-down dilution buffer. The mixture is then gently layered over the appropriate spin-down cushion in a modified Corex centrifuge tube containing a 12-mm coverslip (Mitchison and Kirschner, 1984; Fig. 11.13.3). Centrifugation in a swinging-bucket rotor pellets spindles and other insoluble structures like asters, free nuclei, and DNA beads onto the coverslips. The coverslips are then fixed in methanol, washed in PBS/0.1% NP-40, immunostained if necessary, stained with Hoechst DNA dye, mounted onto slides, and sealed with nail polish. Slides are then observed on a fluorescence microscope and, over the long-term, stored in the dark at 4°C.

Additional Materials *(also see Basic Protocol 1)*

Extract reaction
30% or 15% spin-down dilution buffer (see recipe)
40% or 25% spin-down cushion (see recipe)
Methanol, −20°C
Wash buffer: PBS, sterile/0.1% (v/v) NP-40
PBS, sterile/3% (w/v) BSA
Primary and secondary antibodies in PBS, sterile/3% BSA (e.g., GTU88 anti γ-tubulin and FITC-conjugated goat anti-mouse; Sigma)
10 mg/ml Hoechst dye, in water
Mounting medium (see recipe)

1.5-ml microcentrifuge tube
Modified 15-ml Corex tube containing 12-mm round coverslip (Aladin Enterprises; see Fig. 11.13.3)
Wide-orifice 200- to 1000-µl pipet tips
Sorvall centrifuge, HB-4, HB-6, or HS-4 rotor and appropriate rubber adapters
Metal hook or microspatula
Watchmaker's forceps
Ceramic staining rack
Glass staining dish
Incubation chamber: parafilm cut to fit in bottom of 150-cm round plastic tissue culture dish with lid

NOTE: When using this protocol for either type of aster reaction (see Basic Protocol 1), substitute 15% spin-down dilution buffer and 25% spin-down cushion in place of those called for below.

1. Transfer up to 50 µl extract reaction to a 1.5-ml microcentrifuge tube and add 1 ml of 30% spin-down dilution buffer.

   Add the dilution buffer quickly and evenly to avoid clumping.

2. Layer the mixture over 5 ml of 40% spin-down cushion in a 15-ml modified Corex tube containing a 12-mm round coverslip using a wide-orifice 200- to 1000-µl pipet tip.

   Use care when layering. The mixture will form a distinct layer over the cushion.

3. Centrifuge tubes 15 min at 17,000 × *g* (10,000 rpm) in an HB-4 or HB-6 rotor, 16°C.

   If large numbers of samples are generated, an HS-4 rotor with 16-tube capacity can be used. Run the HS-4 centrifuge at 5930 × g (6,000 rpm) for 25 min, 16°C.

4. Aspirate supernatant and gently pull out plastic support with a metal hook or microspatula.

5. Pick up each coverslip off of support using watchmaker's forceps and place in ceramic staining rack in methanol, −20°C, in glass staining dish for 5 min.

   Take care to be aware which side of the coverslip is facing up. Once the proper orientation has been lost it can be very difficult to reestablish.

6. Transfer each coverslip right side up to Parafilm in an incubation chamber, placing it in a drop of wash buffer. Gently push each coverslip through the drop so that it adheres to the Parafilm.

7. Block coverslips to reduce background by aspirating wash buffer and adding 200 µl sterile PBS/3% BSA to each coverslip. Incubate 10 min at room temperature.

8. *Optional:* If desired, stain coverslips with primary and secondary antibodies in PBS/3% BSA for 15 min each. After each antibody incubation wash coverslips four times with copious amounts of wash buffer.

9. Stain DNA with 10 mg/ml Hoechst dye diluted 1:2000 in wash buffer (5 µg/ml final) for 1 min. After four washes in wash buffer, place coverslips upside down on a 3-µl drop of mounting medium on microscope slide. Aspirate excess mounting medium and seal coverslip edges with nail polish. Store slides in the dark at 4°C for up to one year.

SUPPORT PROTOCOL 5

DNA-COATED BEADS

The use of bead-bound plasmid DNA allows for assays of spindle assembly in a more minimal system. DNA beads are produced using the high-affinity interaction between biotin and streptavidin molecules to couple plasmid DNA to paramagnetic Dynabeads. Large batches of DNA beads can be produced and stored for long periods at 4°C until needed for use in extracts.

Purified plasmid DNA is cut with restriction enzymes to produce a linear DNA fragment containing two sticky ends. Klenow is used to fill in one end with biotin-conjugated nucleotides, while the other is filled in with thionucleotides to help prevent exonuclease digestion. After removing enzyme and unincorporated nucleotides, the biotin-labeled DNA is combined with streptavidin-coated Dynabeads in a specialized binding solution. Overnight incubation at 16°C on a rotator provides sufficient time for binding. After incubation, the beads are washed, resuspended, and stored in bead buffer. The amount of DNA bound is quantified by spectrophotometric analysis of the binding reaction supernatant before and after incubation with the beads.

Materials

50 µg plasmid DNA (<5 kb) purified by column (Qiagen) chromatography
Appropriate restriction enzymes (e.g., *Not*I, *Bam*HI)
TE buffer, pH 8, sterile (*APPENDIX 2A*)
Klenow DNA polymerase (exo$^-$) and buffer (New England Biolabs)
Nucleotides: biotin-dATP (Life Technologies), biotin-dUTP (Clontech), thio-dCTP, and thio-dGTP (Pharmacia)
G-50 Nick columns (Pharmacia)
Washing and binding solutions from Kilobase BINDER kit (Dynal)
Streptavidin Dynabeads from Kilobase BINDER kit (Dynal)
Bead buffer (see recipe)

Magnetic particle concentrator (MPC; Dynal)
Rotator at 16°C

Additional reagents and equipment for ethanol precipitation and restriction endonuclease digestion of plasmid DNA (see *APPENDIX 3A*), and quantification by absorption spectroscopy (*APPENDIX 3D*)

Prepare plasmid DNA

1. Prepare plasmid DNA by Qiagen-style column purification.

   While the sequence of the DNA is not important, the plasmid should be more than 5 kb to effectively induce chromatin assembly in extracts.

2. Cut 50 µg of the DNA with two restriction enzymes (see *APPENDIX 3A*) that have unique sites in the polylinker to produce one short and one long DNA fragment.

The choice of the enzyme should be such that one end of the long fragment terminates in an overhang containing guanines and cytosines, while the other contains only adenines and thymidines (e.g., NotI, BamHI).

3. Ethanol precipitate the linearized DNA (see *APPENDIX 3A*), and resuspend in 25 µl TE buffer, pH 8. Quantify recovery by A_{260} measurement (*APPENDIX 3D*).
4. Prepare fill-in reaction containing 1× Klenow buffer, 30 µg linearized DNA, 50 µM nucleotides (biotin-dATP, biotin-dUTP, thio-dCTP, and thio-dGTP), 20 U Klenow polymerase, and sufficient H_2O to make the final volume 70 µl. Incubate for 2 hr at 37°C.
5. Remove unincorporated nucleotides and small DNA fragments, using a Pharmacia G-50 Nick column, following manufacturer's instructions. Quantify recovery by A_{260} measurement (*APPENDIX 3D*).

   The DNA is eluted in a large volume (400 µl), but the recovery is better than with spin columns.

Couple DNA to beads

6. Prepare coupling mix by combining 400 µl biotinylated DNA (step 4) and 400 µl binding solution. Set aside 25 µl of the coupling mix for later evaluation of coupling efficiency.
7. Prepare 4 µl of streptavidin Dynabeads for each microgram of DNA to be coupled according to manufacturer's instructions.
8. Retrieve beads using the magnetic particle concentrator (MPC). Wash once with 5 vol binding solution (600 µl for 120 µl beads). Retrieve the beads and resuspend them in coupling mix containing DNA.
9. Incubate bead/coupling mixture for several hours (or overnight) on a rotator at 16°C.

Retrieve and wash beads

10. Retrieve the beads using the MPC. Save the supernatant. Compare the A_{260} of the supernatant to that of the sample taken before coupling (step 3) to determine the amount of DNA immobilized.

    Typically two-thirds of the DNA is coupled.

11. Wash beads twice with 500 µl washing solution, and then twice with 500 µl bead buffer. After the last wash, resuspend the beads in sufficient bead buffer such that the final concentration of immobilized DNA is 1 µg/5 µl of beads. Store at 4°C for up to one year.

    If beads are "clumpy," pass through a 12-G needle.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Bead buffer

29.2 g NaCl (2 M final)
0.30 g Tris base (10 mM final)
73 mg EDTA, free acid (1 mM final))
200 ml H_2O
Adjust pH to 7.6 with 2 M HCl
H_2O to 250 ml
Store up to 1 year at room temperature

BRB80, 5×

30.24 g PIPES free acid (0.4 M final)
0.26 ml 4.9 M $MgCl_2$ (5 mM final; Sigma)
0.48 g EGTA (5 mM final)
200 ml H_2O

While stirring, add KOH pellets until the PIPES dissolves. Adjust to pH 6.8 with 10 M KOH. Add H_2O to 250 ml. Sterilize by filtration. Store up to 1 year at 4°C.

BRB80 cushion

60 ml glycerol (60% v/v final)
20 ml 5× BRB80 (1× final; see recipe)
20 ml H_2O
Sterilize by filtration
Store up to 1 year at 4°C

Calcium solution, 10×

1 ml 400 mM $CaCl_2$ (4 mM final)
20.4 µl 4.9 M $MgCl_2$ (1 mM final; Sigma)
0.75 g KCl (100 mM final)
H_2O to 100 ml
Store in aliquots indefinitely at −20°C

Conversion buffer, 20×

47.6 g PIPES free acid (630 mM final)
857 µl 4.9 M $MgCl_2$ (16.8 mM final; Sigma)
0.5 ml 0.5 M EGTA (1 mM final)
200 ml H_2O

While stirring, add KOH pellets until the PIPES dissolves. Adjust the pH to 6.8 with 10 M KOH, and bring the volume to 250 ml with water. Sterilize by filtration. Store up to 1 year at 4°C.

CSF-XB

198 ml extract buffer (XB; see recipe)
2 ml 0.5 M K·EGTA (5 mM final): store indefinitely in 2-ml aliquots at −20°C
40.8 µl 4.9 M $MgCl_2$ (1 mM final; Sigma)
Prepare fresh on day of use

The 0.5 M K·EGTA is made by dissolving 9.51 g EGTA in 40 ml water, pH is adjusted to 7.7 with 10 M KOH, and the volume is adjusted to 50 ml.

Extract buffer (XB)

25 ml 20× XB salts (see recipe)
12.5 ml 2 M sucrose (50 mM final); store in aliquots at −20°C
5 ml 1 M HEPES (0.1 M final), adjusted to pH 7.7 with 10 M KOH
H_2O to 500 ml
Make up fresh on day of use

IB

1.852 g monopotassium glutamate (50 mM final)
15 mg free glutamic acid (0.5 mM final)
20.4 µl 4.9 M $MgCl_2$ (Sigma; 0.5 mM final)
H_2O to 200 ml
Sterilize by filtration
Store in 50-ml aliquots indefinitely at −20°C

Labeling buffer

40 ml glycerol (40% v/v final)
2.38 g HEPES, free acid (0.1 M final)
20 µl 4.9 M $MgCl_2$ (1 mM final)
200 µl 0.5 M EGTA (1 mM final)
20 ml H_2O

Adjust the pH to 8.6 with 10 M NaOH. Add water to 100 ml. Sterilize by filtration. Store up to 6 months at 4°C.

Labeling cushion

60 ml glycerol (60% v/v final)
20 ml 0.5 M HEPES (0.1 M final)
20 µl 4.9 M $MgCl_2$ (1 mM final)
200 µl 0.5 M EGTA (1 mM final)
Adjust pH to 8.6 with 10 M NaOH
H_2O to 100 ml
Sterilize by filtration
Store up to 6 months at 4°C

LPC, 10 mg/ml

Combine leupeptin, pepstatin, and chymostatin to a concentration of 10 mg/ml each in DMSO. Store in aliquots at −20°C.

Mounting medium

9 ml glycerol (90% v/v final)
1 ml 0.2 M Tris·Cl, pH 8 (*APPENDIX 2A*; 10% v/v or 0.02 M final)
Store up to 1 year at room temperature

Quenching buffer

20 ml 5× BRB80 (2× final; see recipe)
20 ml glycerol (40% v/v final)
5 ml 1 M potassium glutamate (100 mM final)
H_2O to 50 ml
Make up fresh on day of use

Spin-down dilution buffer, 30%, 15%

30 ml glycerol (30% v/v final)
1 ml Triton X-100 (1% v/v final)
20 ml 5× BRB80 (1× final)
H_2O to 100 ml
Store up to 1 year at room temperature

For 15% spin-down dilution buffer, only add 15 ml glycerol.

Spin-down cushion, 40%, 25%

200 ml glycerol (40% v/v final)
100 ml 5× BRB80 (1× final)
H_2O to 500 ml
Sterilize by filtration
Store up to 1 year at 4°C

For 25% spin-down cushion, only add 125 ml glycerol.

Spindle fix

600 µl 80% glycerol (48% v/v final)
300 µl 37% formaldehyde (11% final)
100 µl 10× MMR (1× final; *UNIT 11.10*)
0.5 µl 10 mg/ml Hoechst dye (5 µg/ml final; Sigma)
Store up to 1 week at room temperature

Best prepared fresh on day of use.

XB salts, 20×

74.6 g KCl (2 M final)
2.04 ml 4.9 M $MgCl_2$ (Sigma; 20 mM final)
147 mg $CaCl_2$ (2 mM final)
H_2O to 500 ml
Sterilize by filtration
Store up to 1 year at 4°C

COMMENTARY

Background Information

The function of the mitotic spindle is essential for proper segregation of chromosomes during cell division. At the onset of mitosis, the radial interphase array of microtubules breaks down, and then forms a bipolar spindle around condensed chromosomes. Microtubules from each pole attach to sister chromatids and align them in the center of the spindle before segregating them to opposite poles during anaphase. Spindle assembly is a complex process, requiring intricate regulation of microtubule dynamics (Andersen, 1999; Walczak, 2000) and of microtubule and chromosome movements mediated by multiple different microtubule-based motor proteins (Heald and Walczak, 1999; Mountain and Compton, 2000).

Spindle assembly and function has been studied in a variety of model organisms, including genetic systems such as yeast, *C. elegans,* and *Drosophila*, and in cultured cells; however, *Xenopus* egg extracts provide the only system in which spindle assembly has been reconstituted in vitro. This is possible because eggs are synchronized in the cell cycle, and concentrated cytoplasm can be obtained without harsh disruption techniques. Pioneered by Lohka, Masui, and Maller (Lohka and Masui, 1983; Lohka and Maller, 1985), *Xenopus* egg extracts have been optimized to study cell cycle transitions (Murray and Kirschner, 1989), spindle assembly (Sawin and Mitchison, 1991), and anaphase (Shamu and Murray, 1992; Murray et al., 1996), as well as many other cellular processes (see *UNITS 11.10* to *11.12*).

Xenopus eggs are arrested in metaphase of meiosis II, a "mitotic-like" division, by cytostatic factor (CSF), which is thought to be the product of the *c-mos* protooncogene (Sagata et al., 1989). This calcium-sensitive activity is preserved if extracts are prepared in the presence of EGTA, a calcium chelator. CSF-arrested extracts allow experimentation in mitotic cytoplasm, without transiting through the cell cycle. CSF extracts can be induced to cycle by the addition of calcium, which causes the degradation of CSF and cyclin B, thereby inactivating the mitotic cdc2 kinase and promoting entry into interphase.

The power of egg extracts derives from the extensive structural and functional analyses, facilitated by the "open" nature of the system, which nevertheless maintains many properties of intact cytoplasm. Individual components can be manipulated in order to compromise their function by techniques such as immunodepletion (*UNIT 11.10*) or antibody addition (see Support Protocol 3). The excellent cytology of the system permits detailed analysis of the effects of the disruption on spindle assembly. In addition, the system has been ideal for dissecting subsets of processes required for spindle assembly, including microtubule nucleation, dynamics, chromosome condensation, and kinetochore assembly (Belmont et al., 1990; Verde et al., 1990; Hirano and Mitchison, 1991; Zheng et al., 1995; Desai et al., 1997).

The protocols in this unit highlight several of the reactions possible in *Xenopus* egg extracts, and serve to illustrate some of the mechanisms underlying spindle assembly. Microtubule nucleation and organization can be assayed using aster reactions (see Basic Protocol 1); however, there is an important distinction between the two agents used to induce aster formation. Centrosomes are found in most so-

matic cells and serve as focal microtubule nucleation sites that organize spindle poles. In contrast, DMSO, like the microtubule-stabilizing drug taxol, leads to random polymerization of microtubules, which are subsequently organized into focused asters in mitotic cytoplasm (Verde et al., 1991). This process is dependent on microtubule-based motors, including cytoplasmic dynein (Heald et al., 1997) and is thought to mimic the formation and organization of spindle poles (Gaglio et al., 1996, 1997).

Reactions containing *Xenopus* sperm nuclei (Basic Protocols 2 and 3) introduce another level of complexity to the reaction. The centrosome associated with each sperm nucleus nucleates microtubules that interact with sperm chromosomes. In a CSF extract, this leads directly to the formation of mitotic structures. If the reaction is cycled through interphase, the chromosomes and centrosomes duplicate and subsequent spindle assembly is more physiological. DNA bead spindle assembly reactions (see Basic Protocol 4) are similar to female meiosis in that both pathways occur in the absence of centrosomes and illustrate the microtubule-stabilizing force of mitotic chromatin (Heald et al., 1996). Such a mechanism of spindle assembly has been termed "self-organization," because obvious bipolar cues are absent. This system has been particularly useful in dissecting the roles of different microtubule-based motors in setting up the bipolar spindle structure (Walczak et al., 1998).

Therefore, CSF extracts provide a system to examine both centrosome-directed assembly and the self-organization mechanisms of spindle assembly. This system is also amenable to immunofluorescence techniques. Spindle assembly reactions can be easily transferred from reaction tubes onto coverslips, fixed, and stained for desired components.

Critical Parameters and Troubleshooting

The single most important factor in successful in vitro spindle assembly reactions is the quality of the extract; therefore, special attention should be paid to protocols outlining frog handling and extract preparation (*UNIT 11.10*). Once prepared, extract should always be transferred with wide-orifice pipet tips to minimize disruption. Vortexing or any violent agitation of extract or reactions should always be avoided. Flash-frozen extracts can be used in these assays, but generally give less reproducible results. Freeze/thawing can disrupt proteins and rupture vesicles, both of which lower the quality of the extract, and can result in poorly organized microtubule structures or loss of CSF arrest.

The authors find that the most common problem with these assays relates to the inconsistency of the extracts. Although the quality of eggs is by far the most important parameter for a good extract, it is nevertheless problematic to predict when an extract will be of sufficiently low quality to preclude use in these assays. When setting up an experiment involving manipulation of the extract, it is advisable to first run a standard half-spindle reaction to assess the quality of the extract. Running such a reaction will reveal the level of "mitotic" arrest, and the speed and quality of spindle assembly, before extensive time or scarce resources are invested. If assays that require cycling the extract through interphase are to be run, it is advisable to run a test "cycling" reaction with sperm nuclei. After the normal addition of calcium, squash samples can be taken to monitor the extract's ability to properly enter interphase, which is not always reproducible. In this case, a second addition of calcium may be made 10 to 30 min after the first addition. Non-extract-related problems frequently involve observations such as high background in spin-downs or squashes, absence of structures in squash samples, and the "stickiness" of DNA beads. High background in squash samples can be caused by a concentration of rhodamine-labeled tubulin in the reaction that is too high, the reagent should then be used at a higher dilution. High background in spin-down samples normally results from improper immunofluorescence techniques and can usually be remedied in subsequent reactions by titration of the antibodies used and more extensive washes or blocking incubations. A lack of observable structures in squash samples usually results from inadequate mixing of reactions before taking samples, as spindles tend to settle to the bottom of the tube. Problems with "stickiness" and aggregation of DNA beads can be avoided by occasional mixing of bead reactions during both the chromatin assembly and spindle assembly steps. Additionally, if a particular batch of DNA beads has proven to be quite "sticky," they should be passed through a 27-G needle immediately before being used for "bead spindle" reactions.

Anticipated Results

The consistency of results from these assays depends upon the quality of the CSF extracts, which is variable even under normal circum-

stances, as *Xenopus* have seasonal variations in egg yields. Extracts may exhibit differences in the amount of time necessary for spindle assembly, and microtubule and chromosome morphology can also vary from experiment to experiment; therefore, reactions should always be repeated several times to ensure interpretable results. With experience, a user of the system can run assays that show clear and reproducible differences between control and experimental reactions. With good extracts and effective disruption of target proteins, these assays can provide up to hundreds of scorable structures (spindle or aster) per coverslip.

Time Considerations

The assays described in this unit work best with freshly prepared extracts, which require ~2 hr of preparation. Once extracts are prepared, the assays generally take from 15 min for aster reactions, to 4 hr for bead-spindle reactions, to run to completion. Chromatin beads can also be prepared ahead of time and stored frozen in extract, reducing the time for this assay to that of a sperm spindle reaction. If reactions are spun down, an additional 30 to 60 min must be added depending upon whether immunofluorescence is also performed. Spindowns and squash samples, if sealed, can be stored in the dark at 4°C for months to years after the experiment is performed.

Preparation of DNA beads is generally a 2-day project only because of the need for long incubations for restriction digests and coupling. Once prepared, the beads can be kept for months to years at 4°C without significant loss of activity in extracts.

The rhodamine-labeled tubulin preparation requires considerable attention and a full day to complete, and, for best results, it should not be performed simultaneously with other experiments.

Literature Cited

Andersen, S.S.L. 1999. Balanced regulation of microtubule dynamics during the cell cycle: A contemporary view. *BioEssays* 21:53-60.

Ashford, A.J., Anderson, S.S.L., and Hyman, A.A. 1998. Preparation of tubulin from bovine brain. *In* Cell Biology: A Laboratory Handbook, 2nd ed. (J.E. Celis, ed.) Vol. 2, pp. 205-212. Academic Press, San Diego.

Belmont, L.D., Hyman, A.A., Sawin, K.E., and Mitchison, T.J. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell* 62:579-589.

Blomberg-Wirschell, M. and Doxsey, S.J. 1998. Rapid isolation of centrosomes. *Methods Enzymol.* 298:228-238.

Desai, A., Deacon, H.W., Walczak, C.E., and Mitchison, T.J. 1997. A method that allows the assembly of kinetochore components onto chromosomes condensed in clarified *Xenopus* egg extracts. *Proc. Natl. Acad. Sci. U.S.A.* 94:12378-12383.

Gaglio, T., Saredi, A., Bingham, J.B., Hasbani, M.J., Gill, S.R., Schroer, T.A., and Compton, D.A. 1996. Opposing motor activities are required for the organization of the mammalian mitotic spindle pole. *J. Cell Biol.* 135:399-414.

Gaglio, T., Dionne, M.A., and Compton, D.A. 1997. Mitotic spindle poles are organized by structural and motor proteins in addition to centrosomes. *J. Cell Biol.* 138:1055-1066.

Heald, R. and Walczak, C.E. 1999. Microtubule-based motor function in mitosis. *Curr. Opin. Struct. Biol.* 9:268-274.

Heald, R., Tournebize, R., Blank, T., Sandaltzopoulos, R., Becker, P., Hyman, A., and Karsenti, E. 1996. Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature* 382:420-425.

Heald, R., Tournebize, R., Habermann, A., Karsenti, E., and Hyman, A. 1997. Spindle assembly in *Xenopus* egg extracts: Respective roles of centrosomes and microtubule self-organization. *J. Cell Biol.* 138:615-628.

Hirano, T. and Mitchison, T.J. 1991. Cell cycle control of higher-order chromatin assembly around naked DNA in vitro. *J. Cell Biol.* 115:1479-1489.

Hyman, A.A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L., and Mitchison, T.J. 1991. Preparation of modified tubulins. *Methods Enzymol.* 196:478-485.

Lohka, M.J. and Maller, J.L. 1985. Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. *J. Cell Biol.* 101:518-523.

Lohka, M.J. and Masui, Y. 1983. Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science* 220:719-721.

Mitchison, T.J. and Kirschner, M.W. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature* 312:232-236.

Mountain, V. and Compton, D.A. 2000. Dissecting the role of molecular motors in the mitotic spindle. *Anat. Rec.* 261:14-24.

Murray, A.W. 1991. Cell cycle extracts. *Methods Cell Biol.* 36:581-605.

Murray, A.W. and Kirschner, M.W. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature* 339:275-280.

Murray, A.W., Desai, A.B., and Salmon, E.D. 1996. Real-time observation of anaphase in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 93:12327-12332.

Sagata, N., Watanabe, N., Vande, W.G., and Ikawa, Y. 1989. The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* 342:512-518.

Sawin, K.E. and Mitchison, T.J. 1991. Mitotic spindle assembly by two different pathways in vitro. *J. Cell Biol.* 112:925-940.

Shamu, C.E. and Murray, A.W. 1992. Sister chromatid separation in frog egg extracts requires DNA topoisomerase II activity during anaphase. *J. Cell Biol.* 117:921-934.

Verde, F., Labbe, J.C., Doree, M., and Karsenti, E. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of *Xenopus* eggs. *Nature* 343:233-238.

Verde, F., Berrez, J.M., Antony, C., and Karsenti, E. 1991. Taxol-induced microtubule asters in mitotic extracts of *Xenopus* eggs: Requirement for phosphorylated factors and cytoplasmic dynein. *J. Cell Biol.* 112:1177-1187.

Walczak, C.E. 2000. Microtubule dynamics and tubulin interacting proteins. *Curr. Opin. Cell Biol.* 12:52-56.

Walczak, C.E., Vernos, I., Mitchison, T.J., Karsenti, E., and Heald, R. 1998. A model for the proposed roles of different microtubule-based motor proteins in establishing spindle bipolarity. *Curr. Biol.* 8:903-913.

Zheng, Y., Wong, M.L., Alberts, B., and Mitchison, T. 1995. Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. *Nature* 378:578-583.

Key References

Murray, 1991. See above.

This article documents the Xenopus system and is the basis for many of the protocols presented here.

Contributed by John Merlie, Jr. and Rebecca Heald
University of California, Berkeley
Berkeley, California

Analysis of RNA Export Using *Xenopus* Oocytes

Microinjection of *Xenopus* oocytes has long been a tremendously useful tool in the study of nucleocytoplasmic transport, as well as many other aspects of intracellular function. A mature *Xenopus* oocyte (stage V, VI) is an extremely large cell (~1 mm). Indeed, following maturation, the oocyte (now egg) will respond to fertilization by dividing into thousands of embryonic cells with no significant increase in total cellular volume. The large size of the *Xenopus* oocyte makes it possible to quantitatively address questions in cell biology on a single-cell basis. For instance, in the case of nucleocytoplasmic transport, it is uniquely possible to definitively monitor the nucleocytoplasmic distribution of a wide range of transport substrates simultaneously in individual cells. When studying RNA export, this panel typically includes transcripts that represent different classes of RNA, such as tRNA, snRNA, and mRNA. The experimental strategy is straightforward: transport cargos of choice are injected into either the nucleus or cytoplasm; then, at different points over time, each oocyte nucleus is dissected away from its respective cytoplasm. These physically divided pools of nuclear and cytoplasmic components are rapidly processed and analyzed for specific proteins or RNAs.

This unit will focus on the analysis of RNA export using oocyte microinjection. The procedure for the injection itself is described along with a method for recovering RNA from oocyte nuclear and cytoplasmic fractions (Basic Protocol). Obtaining the oocytes is a simple surgical procedure and is also presented (Support Protocol 1). Additional information is included to describe how RNA cargo can be synthesized and labeled in vitro prior to injection (Support Protocol 2).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer; also see *APPENDIX 1D*.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Experiments involving RNA require careful technique to prevent contamination (see *APPENDIX 2A*). All equipment should be RNase free and all solutions should be RNase free or made with DEPC-treated water.

ANALYSIS OF RNA EXPORT IN MICROINJECTED *XENOPUS* OOCYTES

BASIC PROTOCOL

Although the nucleus is not visible in an intact oocyte, material can be delivered specifically to this intracellular compartment because the position of the nucleus is relatively fixed within the cell (Fig. 11.14.1). Thus, by orienting the oocyte with respect to the needle and then pushing the needle a fixed distance into the oocyte, there is a high probability of hitting the nucleus. One procedure that can be used to ensure that the nucleus is in an obvious place is to centrifuge the oocytes. This causes the nucleus to rise to the top of the oocyte (just under the animal pole). It also can cause a slight clearing in the pigment granules at this central point, creating a bull's eye for the injection needle. This makes injections initially easier to master; however, with practice, this step is not necessary (it simply takes a little more effort to learn how far the needle must penetrate the oocyte when the nucleus has not been spun to the surface). In either case, it is important

Contributed by Deborah Duricka and Katharine S. Ullman
Current Protocols in Cell Biology (2001) 11.14.1-11.14.13

to include markers that will confirm a nuclear hit. One marker is a visual tag, namely a high-molecular-weight dye. This allows the accuracy of the hit to be assessed at the time of dissection. Depending on the experimental design, more than one dye can be used in successive injections to create a color mix indicative of two nuclear hits. In the case of RNA export, the other useful marker is an RNA that is not an export (or import) cargo. Selective recovery of this marker with the nuclear fraction confirms that RNA was delivered to the nucleus and that the dissection was performed accurately.

Not only does the large size of the *Xenopus* oocyte facilitate the process of nuclear microinjection, but this feature is also key to the analysis of RNA export following microinjection. To analyze the localization of RNA, each oocyte is physically dissected into nuclear and cytoplasmic fractions, and RNA is prepared from these fractions. To begin this process, individual oocytes are retrieved, blotted dry, and then placed under oil prior to dissection. The nucleus is removed by puncturing a small hole in the middle of the animal hemisphere and then applying slight pressure to the oocyte with forceps. Due to the relatively light density of the nucleus compared to other contents of the cell, it will emerge first from this hole. A needle can then be used to guide the nucleus away from the cytoplasm. Performing this dissection under oil ensures that there will be no loss of material from the nucleus during the process (Lund and Paine, 1990). The nucleus and cytoplasm can then be pipetted out of the oil and into a microcentrifuge tube containing the appropriate buffer. Proteins are removed by a combination of proteinase K digestion and phenol extraction. The RNA is then precipitated and electrophoresed on a denaturing acrylamide gel.

Materials

RNA sample (see Support Protocol 2)
Xenopus oocytes (see Support Protocol 1)
Light mineral oil (e.g., Fisher Scientific)
RNA harvest buffer (see recipe)
4 mg/ml proteinase K solution: 1:4 (v/v) 20 mg/ml proteinase K (e.g., Ambion, Worthington) in RNA harvest buffer, prepared fresh
5:1 (v/v) acid phenol/chloroform, pH 4.5 (Ambion)
20 mg/ml glycogen or seeDNA (Amersham Pharmacia Biotech)
RNA sample buffer (see recipe)

Glass capillary tubes, 1-mm o.d., 0.75-mm i.d., 10-cm length (e.g., Sutter Instruments)
Needle-pulling apparatus (e.g., Sutter Instruments micropipet puller, model P-87)
Pico-injector, basic model (e.g., Model PL1-100, Medical Systems Corp.)
Nitrogen

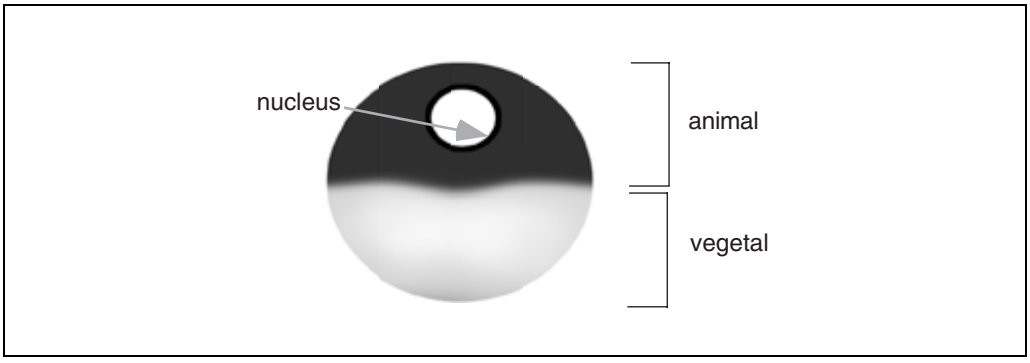


Figure 11.14.1 Stage VI oocyte with approximate location of nucleus and animal and vegetal hemispheres indicated.

Dumont no. 3 and Dumont SS forceps (Fine Science Tools), including one pair of no. 3 forceps set aside for use with oil

Disecting microscope (e.g., MZ7.5, Leica) with optional cold stage (e.g., Labworks # Equipment Service, Inc.; also requires circulating cold bath), 4°C

18°C incubator

Disposable transfer pipets (e.g., Fisher Scientific)

Whatman 3MM filter paper, cut into ~0.5-in × 3-in strips

Mid-sized polystyrene weighing dishes

1-ml syringes

27.5-G needles

100-µl glass capillary tubes with 25-µl microcaps (e.g., Drummond Scientific)

Additional reagents and equipment for phenol/chloroform extraction, ethanol precipitation (*APPENDIX 3A*), and polyacrylamide gel electrophoresis of RNA (*UNIT 6.1*)

Make injection needle

1. Wearing gloves to protect against RNase contamination, clamp a glass capillary tube into a needle-pulling apparatus. Adjust the apparatus and make needles with a 20-µm-diameter tip.

   This type of instrument automatically heats the capillary tube and pulls it in two. Each half is then considered a needle and has an extremely fine tapered point.

   There may be some variability in settings, depending on the instrument and the heating filament. With the Sutter Flaming/Brown micropipet puller, model P87, the authors have used the following parameters with success: P100, heat 340, velocity 120, time 150, and pressure 225.

   It is best to do this step ahead of time. Several needles can be pulled and then stored indefinitely in a large plastic petri dish. The needles should be anchored by embedding the center of the needle in a small piece of clay affixed to the dish. Depending on design (i.e.,whether successive injections, different cargo, etc), each experiment may take 4 to 10 needles. It is possible to reuse needles after extensive rinsing with DEPC-treated water. The needles do break easily, but with experience this becomes less of a problem.

2. Mount a needle into the injection arm of a pico-injector according to manufacturer's instructions. Place the threaded metal cuff over the back of the needle, followed by a freshly cut silicon gasket. Push the needle into the arm until gasket seals. Then slide the metal collar onto the injection-arm threads and screw firmly in place.

   These directions will vary with the specific type of injection apparatus.

3. Gently break the sealed tip of the needle by grasping the tip with a pair of Dumont no. 3 forceps and gently turning them perpendicular to the needle.

   The opening at this point should be <20 µm. The seal must be broken before the sample can be loaded.

4. Test the seal by pulling up some sterile water into the needle and then ejecting the water completely.

5. Spot ~3 µl RNA sample onto a piece of Parafilm and draw sample up into the needle using the load feature of the pico-injector. Avoid air bubbles.

   Alternatively, the needle can be backfilled using a gel-loading tip before it is mounted on the injection arm. If the needle is immobilized in an upright position for several minutes, the sample will slide into place at the tip through capillary action.

6. Adjust the size of the needle tip such that the ejected volume is 5 to 20 nl by successively breaking the tip with forceps and checking the ejected drop size under a microscope. Use the eye-piece reticule to compare the drop to a previously calculated conversion between drop diameter and volume.

The desired ejected volume depends on the particular injection. Although the cytoplasm can be injected with up to 50 nl, it is best to divide this total volume into more than one drop or injection.

Drop size is dependent on three main factors: the pressure of nitrogen (or air) in the output, the duration of the output, and the needle tip size. Generally, for an ~7-nl drop, the authors keep the pressure and time at ~15 psi and 100 msec, respectively, although these parameters can be used to make fine adjustments in volume.

Some pico-injectors also have a back-pressure feature that allows a constant low-level pressure to be continuously applied. This prevents dilution of the sample that can result from small amounts of buffer influx through the needle tip due to capillary action. Depending on the viscosity of the sample, back pressure is generally ~1 psi but should be adjusted empirically such that a minimal amount of sample flows out of the needle to counteract capillary flow into the needle.

There are alternative methods of perfecting the needle. One important technique is beveling the needle. This procedure can be found in Terns and Goldfarb (1998).

Inject oocytes

7. Chill *Xenopus* oocytes ~10 min on ice.

   Chilling the oocytes serves two purposes: (1) it stops active nucleocytoplasmic transport, allowing the transport experiment to be synchronized by the shift back to 18°C, and (2) it causes microtubules to depolymerize, allowing the nucleus to move towards the surface of the oocyte, which creates a more reproducible target.

   If not using a cold-stage, it is usually sufficient to work with ice-cold buffer and inject oocytes in small enough groups that they are returned to 4°C or 18°C without significant warming. Alternatively, an outer petri dish with ice-water can be used to keep the oocytes in chilled buffer if this is critical (for many experiments, the kinetics are slow enough that this is not a critical point).

   Oocytes can also be centrifuged (15 min, 1000 × g) to ensure that the nucleus is directly under the outer surface of the oocyte (Terns and Goldfarb, 1998). However, this will also cause the nucleus to flatten out, restricting the range of depth for an accurate nuclear injection.

8. Bring a petri dish with oocytes into the field of view of the microscope and push the oocytes into a group at one side of the dish using a pair of Dumont SS forceps.

   Alternatively, a mesh grid can be used to organize and orient the oocytes.

9. Hold one oocyte with forceps and orient the animal hemisphere (dark pigmented half) toward the needle such that the central axis of the oocyte is aligned with the path of the needle. For cytoplasmic injection, aim to hit the vegetal hemisphere, to assure avoiding the nucleus.

   The yield of RNA recovery per oocyte is quite efficient, so knowing the detectability of the input material should allow one to almost directly extrapolate to the number of oocytes needed in the harvest. One to two oocytes worth of material is typically sufficient for one lane of a gel. Since it is good to have duplicate samples and since not every injection will lead to a clean hit and a clean dissection, it is recommended to inject at least 10 to 12 oocytes per sample, more if multiple time points are being analyzed.

10. Lower the needle to the oocyte and advance the needle into the oocyte.

    This works best when a bit of pressure is applied to the oocyte with the forceps, creating a taut surface.

11. Deliver material by depressing the foot pedal of the pico-injector.

12. Retract needle and push oocyte to opposite side of the petri dish.

13. Repeat process on remaining oocytes. Return oocytes to an 18°C incubator until the desired time point (typically between 30 min and 18 hr).

    Some experiments require harvesting oocytes injected with the same material at different time points, in order to analyze kinetics.

    Intermittently between injections, a drop of sample should be ejected directly into the buffer to confirm that the needle has not become blocked. With experience, observing this drop also gives a rough idea of the ejection volume. If there is any concern that the volume has decreased, the drop size should be measured at high magnification.

Dissect oocytes

14. Using a disposable transfer pipet, place a few oocytes onto a strip of Whatman 3MM filter paper and allow buffer to absorb.

15. Gently nudge an oocyte with a second strip of filter paper until the oocyte sticks to the second strip. Using Dumont no. 3 forceps set aside for working with oil, immediately place oocyte into a mid-sized polystyrene weighing dish containing a layer of light mineral oil. Repeat until six or seven oocytes are in the oil.

    It is important to remove the right amount of liquid at this point, as any delay in transfer of the oocyte will result in dehydration, but premature transfer of the oocyte will result in a residual aqueous layer that is problematic during dissection. It may help to have a drop of oil on the forceps used to transfer oocytes from the filter paper to the oil.

16. To dissect the nucleus away from the cytoplasm, hold the oocyte with the same forceps and puncture the center of the animal hemisphere using a 27.5-G needle attached to a 1-ml syringe.

17. Gently squeeze the oocyte with the forceps until the nucleus emerges. Use the back side of the needle to brush the nucleus away from the cytoplasm.

    Some practice is required to know how much pressure to apply during dissection. The size of the hole punctured in the oocyte is important. It should be large enough to allow the nucleus to pass through intact and yet small enough to exclude cytoplasm while the nucleus is emerging.

18. Using a 100-µl glass capillary tube and 25-µl microcap, transfer nucleus and remaining cytoplasm (enucleated oocyte) to separate microcentrifuge tubes containing 165 µl RNA harvest buffer. Keep samples on ice (if processing within the hour or so) or frozen until ready for further processing.

    The samples are relatively stable once frozen, but for longer-term storage, proceed through RNA harvest step. Even then, analysis within a few days is optimal due to the radioactive nature of the sample.

    The cytoplasm can also be transferred with forceps.

Process RNA

19. Add 55 µl of 4 mg/ml proteinase K solution to each tube. Vortex and place at 37°C for 90 min. During the first 60 min, vortex periodically.

    Repeated vortexing is especially important for the cytoplasmic samples.

20. Extract samples with 5:1 acid phenol/chloroform, pH 4.5.

    To ensure that the interface is completely avoided, it is advisable to carry forward only 90% of the sample after extraction.

21. Ethanol precipitate RNA (*APPENDIX 3A*). Include a carrier such as 20 to 50 µg glycogen or seeDNA, especially in the nuclear fractions.

22. Air dry pellets and resuspend in RNA sample buffer (generally, 10 µl per oocyte).

    RNA in sample buffer can be stored for several weeks, but again since it is radioactive, it is advised to analyze it within a few days if possible or up to 2 weeks.

23. After heating the sample 5 min at 68°C and vortexing, analyze RNAs by electrophoresis on a denaturing polyacrylamide gel (*UNIT 6.1*).

    It is highly recommended to quantitate these results using a phosphorimager.

SUPPORT PROTOCOL 1

PREPARATION OF *XENOPUS* OOCYTES FOR MICROINJECTION

The ovary lies spread beneath the ventral surface of the female frog. The ovary consists of a mixed population of oocytes, representing various stages of maturation, that are held in loose proximity--much like a cluster of grapes. A small section, or lobe, of the ovary can easily be retrieved through a small incision towards the side of the abdomen. The desired amount of ovarian tissue is then clipped off the ovary and the incision sutured. The clump of oocytes is next teased apart and the immature oocytes are sorted away from the larger, mature oocytes.

Materials

Female *Xenopus laevis* (e.g., Nasco, Xenopus I, Xenopus Express)
0.05% (w/v) benzocaine (Sigma) in H_2O from 10% (w/v) stock in ethanol, stored at 4°C.
MBS (see recipe), 4°C

Toothed and Dumont no. 3 forceps (Fine Science Tools)
Surgical scissors
Sutures, Vicryl 4.0 (Ethicon) with attached needle
Hemostat
Recovery tank with lid
1-ml syringes
18-G needles bent into triangular loops
Disposable transfer pipets (e.g., Fisher)
18°C incubator

Make surgical incisions

1. Wearing gloves, place a female *Xenopus laevis* in a 4-liter beaker with 1 liter of 0.05% benzocaine for 10 min to anesthetize. Check efficacy of anesthetic by pinching the frog's toes.

   The frog should remain flaccid.

2. Place frog on its back on a paper towel soaked in 0.05% benzocaine and spread on top of ice chips.

3. Make a small (~1 cm) incision with the corner of a razor blade in the skin towards the lower side of the abdomen. To facilitate the incision, grip the skin with a pair of toothed forceps and then start the incision between the prongs of the forceps.

   A small amount of bleeding sometimes occurs and can simply be blotted with a tissue. At this point, it is usually possible to see the oocytes underlying the next layer of abdominal wall.

4. Using a pair of Dumont no. 3 forceps, grab the inner abdominal muscle wall, lift it up, and cut it with a pair of surgical scissors (so that this incision aligns with the initial one through the skin).

   Again, this can be a small incision (slightly <1 cm).

Harvest ovarian lobe

5. Reach through the incision with the forceps. Retrieve a lobe of the ovary by closing the forceps and gently pulling them out through the incision.

   Retrieving a lobe should be possible even if the view of the oocytes is somewhat obscured.

6. Clip off the desired amount of ovary with scissors and place in ice-cold MBS. Gently ease the remainder of the lobe back in through the incision.

   At this point, the benzocaine-soaked towel can be removed.

Suture incisions

7. To suture the inner incision, draw a needle and Vicryl 4.0 suture through the layers of the wall on each side of the incision using a hemostat. Pull until there is only a short (~2 cm) piece of thread remaining on the opposite side.

8. Using the hemostat, loosely wrap the long piece of thread twice around the pair of forceps. Grab the short end of the thread with the forceps. Pull on thread wrapped around the forceps and slip it off the forceps to make a knot. Cut the ends of the thread close to this knot.

9. Repeat steps 7 and 8 one to two more times to suture the inner incision. Then repeat the same steps to suture the incision in the skin.

   Alternatively, it is possible to suture both layers with the same stitches.

   Because the skin can be more difficult to push the needle through, it helps to brace against the force with forceps that are spread such that the needle pushes between the two arms of the forceps.

10. Rinse frog in frog tank water and move it to a recovery tank with a shallow layer of water. Initially prop its head up out of the water.

    Usually, the anesthetic wears off after 20 to 60 min and the frog can then be returned to a normal tank. It is advisable to monitor the frog an additional 12 hr or so before returning it to the colony.

    A lid with air holes should be kept on the recovery tank as some frogs will try to jump out as soon as they recover.

Collect and sort oocytes

11. Using two pairs of Dumont no. 3 forceps, grab two parts of the outer layer of tissue that encloses the ovary. Pull forceps apart to partially invert the ovary and expose the oocytes.

12. Using the forceps, pull the ovary apart into smaller pieces (~50 oocytes each). Rinse chunks of ovary by swishing through ice-cold MBS and place in a clean petri dish with ice-cold MBS.

    These clumps can be stored for a couple of days. In some cases they can be used for up to a week following the surgery; in this case, the buffer should be changed daily.

13. Using a 1-ml syringe attached to an 18-G needle bent into a triangular loop, press down on a section of stalk that attaches one oocyte to the rest of the ovary. Grab an adjacent piece of this connective tissue with no. 3 forceps and pull gently, detaching the oocyte from the ovary. Repeat until the desired number of oocytes are freed from the ovary.

14. Sort the oocytes, keeping only those that are large (stage V and VI) and in perfect condition. Use a disposable transfer pipet to place these in a new petri dish with fresh, ice-cold MBS and store overnight in an 18°C incubator.

Smith et al. (1991) provides illustrations of oocyte stages. Oocytes at earlier stages of maturation are also used in some types of experiments (Feldherr et al., 1998).

Oocytes with mottled pigmentation or any scarring where the stalk was detached should be discarded. Sometimes a small tear in the follicle layer will cause a slight bulge in the oocyte at this point. There is nothing wrong with these oocytes per se, but these bulges make the oocytes prone to breaking when they are blotted dry, so they are best discarded. Also, oocytes that appear to be softening (sometimes this is not obvious until dissection) should be discarded.

Some groups remove the layer of follicle cells that encase each oocyte. This can either be done manually or by collagenase treatment (Terns and Goldfarb, 1998). Removal of the follicle layer decreases resistance to the needle during injection. It is also useful for some procedures, such as when endogenous material is being examined. However, defolliculated oocytes tend to be more difficult to handle during the dissection.

SUPPORT PROTOCOL 2

PREPARATION OF RNA FOR INJECTION

RNA export cargo is most easily monitored by the injection of radioactively labeled RNA. Such RNA can be generated in vitro following standard procedures for in vitro transcription driven by bacteriophage polymerases (*UNIT 11.6*). In some cases, it is important to include a nucleotide cap analog in order to mimic the bona fide 5′ end of a typical polymerase II transcript. During the analysis, this structural feature will contribute to RNA stability as well as to transport. When choosing which RNAs to monitor for export, an important point to keep in mind is that some steps in RNA biogenesis--such as splicing and export--are coupled (Luo and Reed, 1999). This may impact the decision of how an RNA should be produced for a particular experiment (i.e., with or without an intron). It should also be noted that as an alternative to injecting RNA cargo directly, oocytes can be injected with plasmids in conjunction with radioactive nucleotide. In this case, the plasmid template should contain a gene under the control of a eukaryotic RNA polymerase promoter. It is also possible to monitor the export of endogenous RNAs labeled in the presence of radioactive nucleotide. This procedure has been done to monitor the export of ribosomal RNAs (Powers et al., 1997). Although there are specific circumstances when these alternatives would be desirable, they do involve the injection of more radioactive material than in the case of the labeled RNAs.

Table 11.14.1 Reaction Mixture for RNA Labeling[a]

Ingredient	Amount in one reaction	Stock concentration
ATP, CTP, UTP	0.5 µl	5 mM each
DEPC-treated H_2O	to 10 µl total	—
DNA template, linearized[b]	1-2 µl	250-500 ng/µl
GTP	0.5 µl	1 mM
m7GpppG[c]	1 µl	5 mM
[^{32}P]GTP	3 µl	3000 Ci/mmol
RNA polymerase buffer	1 µl	10×
RNase inhibitor	0.25 µl	40 U/µl
SP6 or T7 RNA polymerase[d]	0.5 µl	20 U/µl

[a]To transcribe multiple RNAs, make a mix of common components and distribute this pool to tubes containing reaction-specific ingredients, such as DNA template.

[b]Template DNA can be either a linearized plasmid or a PCR product (where the 5′ oligo used to generate the PCR product contains the appropriate promoter sequence).

[c]Include only for capped RNA.

[d]The choice of polymerase is dependent on the type of promoter in the particular construct being used.

Materials

5 mM each ATP, CTP, UTP
20 U/µl SP6 or T7 RNA polymerase and 10× buffer
DEPC-treated H_2O (*APPENDIX 2A*)
250 to 500 ng/µl DNA template, linearized (*APPENDIX 3A*)
1 mM GTP
5 mM m^7GpppG (for capped analogs only)
3000 Ci/mmol [^{32}P]GTP
40 U/µl RNase inhibitor (e.g., RNasin, Promega)
5 M ammonium acetate
20 mg/ml glycogen or seeDNA (Amersham Pharmacia Biotech)
70% and 100% (v/v) ethanol
Blue dextran (avg. mol. wt. 2,000 kDa; e.g., Sigma-Aldrich) or rhodamine B dextran (mol. wt. 70 kDa; e.g., Molecular Probes)

1. Set up reaction mixture in a microcentrifuge tube to include components listed in Table 11.14.1. Mix well and incubate 60 min at 37°C.

2. Add the following and mix well:

   16 µl 5 M ammonium acetate
   60 µl DEPC-treated H_2O
   1 µl 20 mg/ml glycogen or seeDNA
   240 µl 100% ethanol.

   Again, when processing multiple samples it is easiest to make a master mix of these reagents.

   Some protocols include DNase treatment and phenol/chloroform extraction (APPENDIX 3A) before precipitation.

3. Chill on ice ≥15 min. Pellet in a microcentrifuge ~30 min at maximum speed, 4°C.

4. Wash twice with 70% ethanol at room temperature.

5. Resuspend in 15 µl DEPC-treated water.

6. To analyze RNA, make a 1:15 (v/v) dilution in DEPC-treated water and quantitate the radioactivity in a 2-µl aliquot using a scintillation counter.

   It is also useful to electrophorese 2 µl of the dilution on a 6% denaturing gel (UNIT 6.1). After a quick exposure to film (~30 min), the RNA can be assessed for size and uniformity.

7. Prepare the mixture of RNAs and dyes to be injected. For nuclear injections, add blue dextran or rhodamine B dextran to a final concentration of ~6 mg/ml or ~4 mg/ml, respectively.

   Generally, 0.5 to 2 × 10^6 cpm/µl of an RNA is used for an injection. A quick phosphorimager scan of a gel with the newly-synthesized RNAs can be used to generate the desired ratio of RNAs when injecting a mixed population. This avoids the problem of counting residual free nucleotide, which can be variable. In terms of absolute levels of RNA, typically total levels of RNAs should be in the low femtomole range to prevent saturation of an export pathway.

   A high-molecular-weight dye can be mixed into the sample to monitor the injection accuracy (see Basic Protocol introduction). There are alternative markers for a nuclear hit, such as colloidal gold (Bataille et al., 1990).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Modified Barth's solution (MBS; Sive et al., 2000)

88 mM NaCl
1 mM KCl
2.4 mM $NaHCO_3$
15 mM HEPES
0.74 mM $CaCl_2$
0.82 mM $MgSO_4$
Autoclave and store up to 6 months at room temperature or 4°C
Add penicillin/streptomycin (Life Technologies) to 100 U/ml before use

HEPES is not included in all MBS recipes. The original recipe had a mix of $CaCl_2$ and $Ca(NO_3)_2$.

RNA harvest buffer

100 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
10 mM EDTA, pH 8.0
300 mM NaCl
2% (w/v) SDS
Store up to 6 months at room temperature

RNA sample buffer

1× TBE buffer (*APPENDIX 2A*) containing:
10 M urea
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol
Store at room temperature

COMMENTARY

Background Information

Oocyte microinjection studies have laid much of the groundwork for our understanding of the nuclear pore complex. Among the many important functional parameters that have been elucidated by this approach are the size limits of both diffusion and active transport through the pore (Bonner, 1975; Feldherr et al., 1984) and the identification of steps in the transport of different cargo that are either unique or shared with other pathways through the pore (Jarmolowski et al., 1994; Pokrywka and Goldfarb, 1995). Injection experiments have also been extremely useful in delineating both the *cis* signals within RNA and the *trans*-acting factors that are required for efficient export (e.g., Guddat et al., 1990; Fischer et al., 1995). More recently, microinjection studies have been used to address the specific roles of particular pore proteins (Stutz et al., 1996; Powers et al., 1997; Ullman et al., 1999).

The experimental strategies made possible by oocyte microinjection take advantage of the ability to survey a wide variety of transport cargo simultaneously in an intact cell. The function of individual components of the transport machinery can be probed with reagents ranging from antibodies to dominant-negative recombinant proteins.

As both the nuclear pore and the soluble accessory factors involved in transport become understood at a molecular level, the oocyte will continue to be an indispensable tool in the field of nucleocytoplasmic transport. Of course, this method is most powerful when complemented with other approaches. For instance, examining a specific interaction biochemically in vitro provides critical substantiating evidence for a functional relationship that is identified through microinjection analysis. Similarly, structural and biochemical studies of the pore and the soluble factors involved in transport provide important clues to function that can then be tested in an in vivo setting. Thus, in combination with other techniques, further analysis of transport in the oocyte will be used to gain insight into how the transport machinery

coordinates the multitude of trafficking events that occur each minute through each pore.

Critical Parameters

One critical parameter for oocyte microinjection is simply the quality of the oocytes. There is a certain amount of variability from frog to frog and, however frustrating this may be, it is well worth demanding a high standard at the very first step of oocyte isolation. In other words, if the oocytes cannot be recovered from the ovary without damage or if they have any abnormalities in their pigment distribution in the first place, try a new frog! Once you find a frog that has good oocytes, this is usually a reproducible attribute, and the frog can be operated on several times (with a week or so of rest in between).

Some problems with the oocytes are not immediately evident. This is one reason why it is beneficial to store the oocytes overnight before use and cull those that do not remain stable during this time. Finally, it pays to inject more oocytes per experimental point than you actually want to harvest. Then, at harvest time, you can once again select only those oocytes that have remained stable through the treatment.

Another important parameter to consider is the amount of RNA that you inject. Export pathways are saturable. Thus, too much of any one RNA will result in a much slower apparent export rate. It is important to note that this problem can actually be used to an advantage. Saturation of a particular pathway with unlabeled RNA has been used to identify steps in transport that are shared by more than one substrate (Jarmolowski et al., 1994; Pokrywka and Goldfarb, 1995). This strategy has also been employed to purposefully create a situation where a particular factor is limiting, allowing an add-back experiment to be performed where a candidate factor is tested for its ability to overcome the saturation (Gruter et al., 1998; Kutay et al., 1998).

Finally, it is important to strictly control the drop size and needle tip size. Given that the volume of an oocyte nucleus is approximately equivalent to 40 nl, it is best if the volume of the injected material does not exceed 10 to 12 nl. This is a somewhat arbitrary cutoff, and some experiments have been done successfully with larger volumes; however, erring on the side of caution in terms of maintaining nuclear integrity is advisable. The same can be said for needle size. The authors have found that a 10-nl volume generated by a burst of ~100 msec with ~15 PSI pressure translates into a needle that is fine enough for these experiments. The question of whether both drop size and needle size are within acceptable parameters can be assessed empirically by including appropriate controls for injection and dissection.

Troubleshooting

The needle becomes clogged during the injection procedure. There could be particulate matter in the injection material. Clear the solution with a high-speed spin prior to loading the needle. Sometimes the needle clogs simply because the sample at the tip has dried. To circumvent this, minimize the amount of time that the tip of the needle is out of the buffer solution. When the needle has clogged due to a dry tip, it can often be cleared by advancing and retracting the needle in and out of the buffer system.

The needle won't penetrate into the oocyte. The layer of follicle cells can sometimes make the oocyte somewhat resistant to injection. In most cases, this is easily overcome simply by slightly squeezing the oocyte with forceps to counter the pressure of the needle. If this does not work, make a slightly larger needle tip. If this is still a general problem, either bevel the needle or defolliculate the oocytes (Terns and Goldfarb, 1998). The latter procedure may lead to more difficulty in handling the oocyte at later steps.

U6 snRNA (or U3 snRNA) is entirely or partially in the cytoplasmic fraction. These two RNAs do not get exported and therefore can serve to confirm that the nucleus was both selectively injected and cleanly dissected. If these RNAs do not appear exclusively in the nuclear fraction, either the injection or dissection was not clean. Looking at the distribution of pre-mRNA and the splice products can help narrow the problem. Whether due to injection or dissection, the solution is more practice!

Pre-mRNA is in the cytoplasmic fraction. These two RNAs do not get exported and therefore can serve to confirm that the nucleus was both selectively injected and cleanly dissected. If these RNAs do not appear exclusively in the nuclear fraction, RNA was injected into the cytoplasm. Practice making nuclear injections with blue dextran dye. It is possible to get some export of pre-mRNA when there is a large excess of RNA injected. Injecting less pre-mRNA (that has a greater specific activity) may help. Also note that pre-mRNA containing a specific *cis* element (such as the constitutive transport element from type D retroviruses) can in fact be actively exported. Make sure that

there has not been an error in the templates used for generating injection RNAs.

The intron is in the cytoplasmic fraction. This indicates that there was a problem in the dissection, because the intron is generated only in the nucleus and is not exported. Again, more practice is necessary. Watch carefully for the entire nucleus to extrude followed by a small amount of cytoplasm. Also see comment about *cis* elements above.

After the injections, the oocytes seem mushy. These oocytes may not have been of good enough quality from the beginning. Sorting the oocytes very carefully is important. Switching frogs might also overcome the problem. Alternatively, the needle tip used in the injection may have been too large. Try breaking needles closer to the tip to create a finer opening and adjusting the pressure and time of the pico-injector to compensate. It also might help to divide the injection volume into two drops (or more for cytoplasmic injections).

RNA harvested from oocytes has a smeary appearance on gels. If the injected RNA looks good (eliminating an early problem with degradation), then this indicates that the RNA is being degraded at some point in the procedure. If this seems specific to mRNA and U1 RNA, the cap analog may not have been incorporated. Double-check concentrations used; try fresh stock if nothing else looks incorrect. Otherwise, there may be a general problem with degradation during the procedure. Try making clean, fresh solutions.

An oocyte has protruding bulges. This indicates that there is a rupture in the follicle layer. Be extremely gentle when pulling each oocyte off of the stalk that holds it in the ovary. In some cases, the oocytes are particularly difficult to isolate without damage. If this is the case, the simplest solution is to try another frog.

Anticipated Results

Once this procedure is up and running, it should yield a very definitive answer to the question of where different RNA export cargos localize under conditions of interest. By harvesting the oocytes at multiple time points, it is also possible to assess the kinetics of export, which may prove to be an important parameter. By altering the sequence of the RNA itself, the *cis* signals that contribute to the export of an RNA can be readily assessed. Just as importantly, mutational analysis of the RNA can be used to delineate which features contribute to the retention of certain RNAs.

Time Considerations

The entire procedure typically takes a minimum of 3 days: (1) a day to synthesize and double-check the RNA cargo (surgery can be performed the same day); (2) a day to perform the microinjections and harvest the oocytes (time points will range from minutes to a few hours); (3) a day to prepare and analyze the RNA distribution. It often makes sense to do >1 day of injections in between day 1 and day 3, because ultimately this is more time efficient than having to do a second operation to get more oocytes for a second injection.

It is also important to consider the amount of time it takes to learn this technique. It will take a few to several weeks to develop the eye-hand coordination and the experience needed to reproducibly inject the nucleus (and note that when using oocytes that have not been spun, the hit rate can vary from 70% to 90% even with experience). It also takes some practice to dissect the oocytes--mostly this is the experience it takes to know how much to squeeze the oocytes to get the nucleus to extrude as well as how big a hole to puncture in the first place. It is advisable to start by injecting blue dextran as a way to practice both the injection and the dissection.

Literature Cited

Bataille, N., Helser, T., and Fried, H.M. 1990. Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus* oocyte nucleus: A generalized, facilitated process. *J. Cell Biol.* 111:1571-1582.

Bonner, W.M. 1975. Protein migration into nuclei. I. Frog oocyte nuclei in vivo accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. *J. Cell Biol.* 64:421-430.

Feldherr, C.M., Kallenbacj, E., and Schultz, N. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* 99:2216-2222.

Feldherr, C., Akin, D., and Moore, M.S. 1998. The nuclear import factor p10 regulates the functional size of the nuclear pore complex during oogenesis. *J. Cell Sci.* 111:1889-1896.

Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W., and Luhrmann, R. 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* 82:475-483.

Gruter, P., Tabernero, C., von Kobbe, C., Schmitt, C., Saavedra, C., Bachi, A., Wilm, M., Felber, B.K., and Izaurralde, E. 1998. TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol. Cell* 1:649-659.

Guddat, U., Bakken, A.H., and Pieler, T. 1990. Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell* 60:619-628.

Jarmolowski, A., Boelens, W.C., Izaurralde, E., and Mattaj, I.W. 1994. Nuclear export of different classes of RNA is mediated by a specific factor. *J. Cell Biol.* 124:627-635.

Kutay, U., Lipowsky, G., Izaurralde, E., Bischoff, F.R., Schwarzmaier, P., Hartmann, E., and Gorlich, D. 1998. Identification of a tRNA-specific nuclear export receptor. *Mol. Cell* 1:359-369.

Lund, E. and Paine, P.L. 1990. Nonaqueous isolation of transcriptionally active nuclei from *Xenopus* oocytes. *Methods Enzymol.* 181:36-43.

Luo, M.J. and Reed, R. 1999. Splicing is required for rapid and efficient mRNA export in metazoans. *Proc. Natl. Acad. Sci. U.S.A.* 96:14937-14942.

Pokrywka, N.J. and Goldfarb, D.S. 1995. Nuclear export pathways of tRNA and 40S ribosomes include both common and specific intermediates. *J. Biol. Chem.* 270:3619-3624.

Powers, M.A., Forbes, D.J., Dahlberg, J.E., and Lund, E. 1997. The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways. *J. Cell Biol.* 136:241-250.

Sive, H.L., Grainger, R.M., and Harland, R.M, eds. 2000. Early Development of *Xenopus Laevis*: A Laboratory Manual, pp. 283-284. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Smith, L.D., Xu, W., and Varnold, R.L. 1991. Oogenesis and oocyte maturation. *Methods Cell Biol.* 36:45-60.

Stutz, F., Izaurralde, E., Mattaj, I.W., and Rosbash, M. 1996. A role for nucleoporin FG repeat domains in export of human immunodeficiency virus type 1 Rev protein and RNA from the nucleus. *Mol. Cell Biol.* 16:7144-7150.

Terns, M.P. and Goldfarb, D.S. 1998. Nuclear transport of RNAs in microinjected *Xenopus* oocytes. *Methods Cell Biol.* 53:559-589.

Ullman, K.S., Shah, S., Powers, M.A., and Forbes, D.J. 1999. The nucleoporin nup153 plays a critical role in multiple types of nuclear export. *Mol. Biol. Cell* 10:649-664.

Key References

Lund and Paine, 1990. See above.

Describes the specific steps and advantages of dissecting oocytes under oil.

Terns and Goldfarb, 1998. See above.

Covers many technical aspects of oocyte microinjections, including a detailed description and illustration of suggested equipment.

Internet Resources

http://oacu.od.nih.gov/ARAC/oocyte.htm

This site contains Animal Research Advisory Committee guidelines for oocyte harvest from Xenopus laevis.

http://froglab.biology.utah.edu/Oogenesis/oogenesis.html

This site contains a summary of the different stages of oocyte maturation.

Contributed by Deborah Duricka and Katharine S. Ullman
University of Utah
Salt Lake City, Utah

In Vitro Analysis of Peroxisomal Protein Import

This unit describes a quantitative in vitro assay for peroxisomal protein import. The assay (outlined in Fig. 11.15.1) is ELISA-based and employs semi-permeabilized human cells and a biotinylated import substrate (see Basic Protocol). In the Basic Protocol, import is assessed directly in cells; in the Alternate Protocol, import is quantitated after isolation of cellular organelles/peroxisomes. A method for preparing biotinylated luciferase is also described (see Support Protocol).

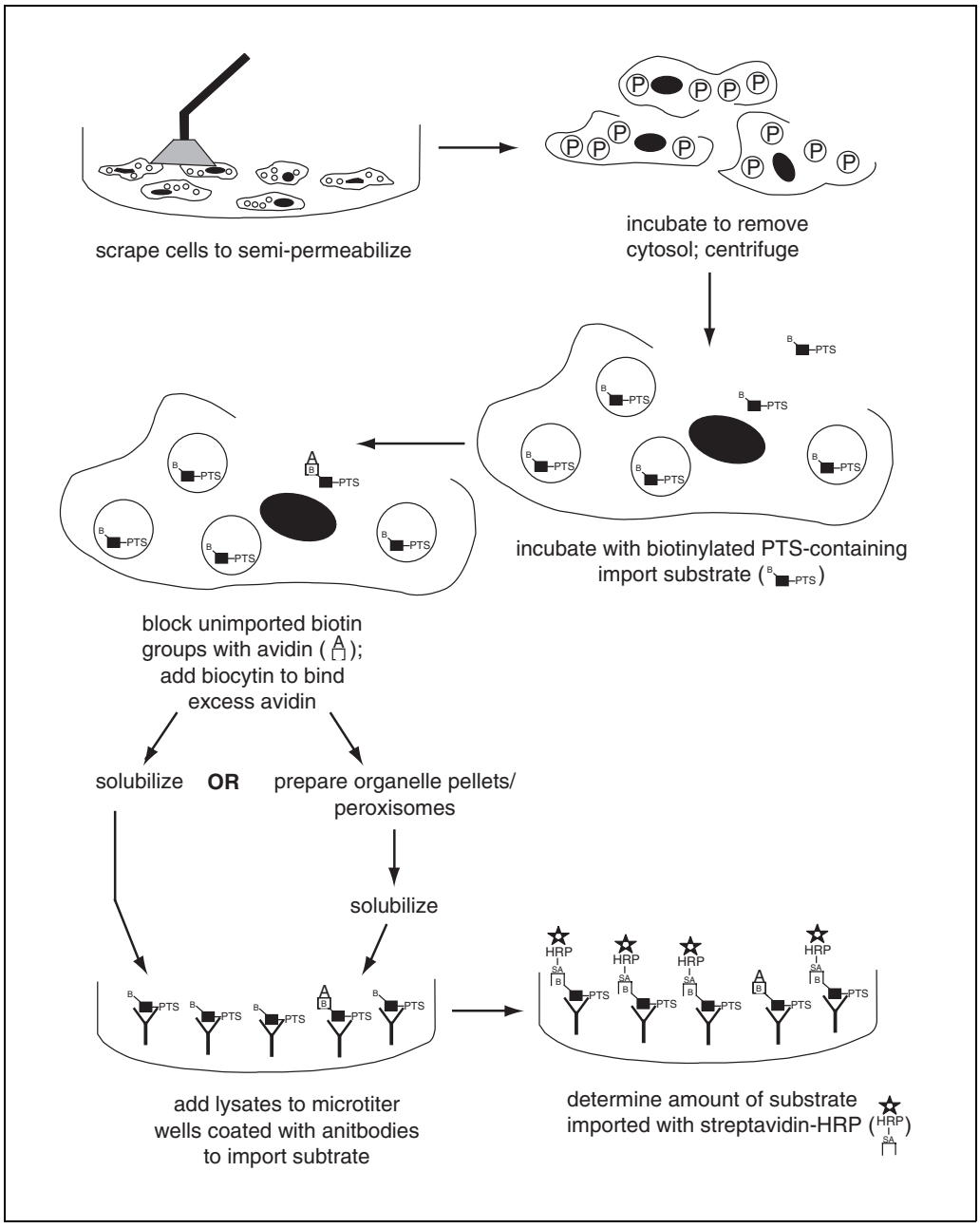


Figure 11.15.1 Quantitative in vitro assay for peroxisomal protein import. A, avidin; B, biotin; HRP, horseradish peroxidase: P, peroxisome; PTS, peroxisomal targeting signal; SA, streptavidin.

Contributed by Stanley R. Terlecky
Current Protocols in Cell Biology (2002) 11.15.1-11.15.10

BASIC PROTOCOL

PEROXISOMAL PROTEIN IMPORT IN VITRO

Semi-permeabilized cells are incubated with import substrate, biotinylated luciferase. After the import reaction, cells are isolated and biotin groups on unimported substrate blocked. Cells are then lysed and import quantified by ELISA.

Semi-permeabilized cells are incubated with import substrate, biotinylated luciferase, and then unimported substrate is blocked. The extent of import is quantified by ELISA.

Materials

Rabbit polyclonal *APPENDIX 2A* anti-luciferase antibodies (*UNIT 16.2*)
ELISA coating solution: 50 mM sodium carbonate, pH 9.0
ELISA blocking solution (EBS; see recipe), pH 7.4
Human epidermoid carcinoma (A431; ATCC CRL-1555) cells or diploid lung (IMR90; ATCC CCL-186) fibroblasts growing in tissue culture
Hanks balanced salt solution (HBSS, Life Technologies; *APPENDIX 2A*)
Dulbecco's minimum essential medium containing 10% (defined) fetal bovine serum (FBS) (DMEM-10)
Import reaction buffer (IRB, see recipe), pH 7.4
ATP and regenerating system (ATP, see recipe)
800 µM ZnCl in IRB
Biotinylated luciferase (B-Luc, see Support Protocol)
IRB containing 1.25% or 0.2% (w/v) bovine serum albumin (IRB/1.25% BSA or IRB/0.2% BSA, respectively)
Avidin (Calbiochem)
Biocytin (Calbiochem-Novabiochem)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4
Streptavidin horseradish peroxidase (Roche Diagnostics)
30% (v/v) hydrogen peroxide (H_2O_2)
o-Phenylenediamine
ELISA development buffer: 27 mM citric acid/51 mM sodium phosphate, pH 5.0
4 N sulfuric acid (H_2SO_4)

ELISA microtiter well strips (Maxisorp-Immunomodule, Nunc)
15-cm cell culture dishes (Corning)
Rubber policeman
50-ml conical centrifuge tubes (Corning)
Platform rocker
1.5-ml microcentrifuge tubes (Eppendorf)
37°C water bath
Microtiter plate washer (optional)
Microtiter plate reader

Prepare semi-permeabilized cells

1. Coat microtiter wells with antibody by adding 200 µl of a 1:500 dilution of rabbit polyclonal anti-luciferase antibodies in ELISA coating solution to ELISA microtiter well strips and incubating overnight at 4°C.

   Determine optimal concentration of coating antibody (and biotinylated ligand) added by serial dilution titration analysis. It is critically important that the amount of biotinylated ligand added to a given microtiter well not exceed the binding capacity of the coated antibody. Ideally, the amount of biotinylated ligand added should approach the upper range of where capture is still linear. This analysis must be performed for each ligand/antibody combination.

2. Wash ELISA microtiter well strips two times with 200 µl PBS and block residual binding capacity with 200 µl EBS. Incubate overnight at 4°C.

   Coated ELISA microtiter well strips may be stored in EBS until use—typically within 2 days of preparation.

3. Wash cells near, but not at, confluence, two times with 25 ml HBSS and add 25 ml fresh Dulbecco's minimum essential medium containing 10% FBS for 30 min prior to permeabilizing.

   Growing cells on 15-cm tissue culture plates works well for these procedures. These plates will contain ~5-6 × 10^6cells (sufficient for ~24 import reactions) when nearly confluent. The following steps describe procedures for treatment of each 15-cm plate.

   Pool cells from different plates at the end of the permeabilization procedure.

4. Wash cells three times with 25 ml IRB and harvest in a small residual volume (~5 ml) by scraping firmly with a stiff rubber policeman.

   Scraping in this manner removes portions of the plasma membrane and thus semi-permeabilizes cells.

5. Repeat scraping with an additional 5 to 10 ml IRB to harvest remaining cells.

6. Bring volume to 50 ml with IRB in a 50-ml conical centrifuge tube and rock 15 min at 4°C to remove cytosol.

7. After 15 min, centrifuge cells 5 min at 800 × *g*, 4°C. Aspirate supernatant.

   Properly semi-permeabilized cells will not exclude trypan blue or contain more than ~15% of their cytosolic contents. Lactate dehydrogenase and Hsc70 are convenient cytosolic marker proteins that may be examined by enzyme assay or immunoblotting, respectively.

8. Carefully resuspend cells in ~275 µl IRB and keep on ice until use.

   Once prepared, the cells should be used as quickly as possible. Therefore, step 7 should be initiated prior to completion of cell preparation.

Prepare and incubate import reactions

9. Add the following import reaction components, excluding cells, to labeled 1.5-ml microcentrifuge tubes on ice (final reaction volume is 40 µl, which includes 10 µl of cells):

   2 µl ATP
   5 µl zinc chloride
   5 µl 80 µg/ml B-Luc diluted in IRB/1.25% BSA
   18 µl IRB

   The 18 µl IRB addition may be reduced to accommodate such components as cytosol, (inhibitory) antibodies, chelators, and combinations or fractions thereof.

   Consolidating pipetting steps by pooling components improves reproducibility. Minimally, each condition should be performed in duplicate.

10. Initiate import reaction by adding 10 µl semi-permeabilized cells to microcentrifuge tubes containing import reaction mixture. Gently mix the contents and incubate 45 min at 37°C. Tap the microcentrifuge tubes gently every 5 min to ensure an even distribution.

    For zero-time incubations, add cells to import reaction components at the end of the incubation, i.e., at 45 min.

11. Terminate import reaction by microcentrifuging tubes for 1 min at 13,000 rpm, 4°C. Aspirate supernatant.

    The vast majority of unimported B-Luc is removed in this step. The small amount remaining in the pellet is "inactivated" in the following steps.

Block unimported B-Luc

12. Add 100 µl of 75 µg/ml avidin diluted in IRB/0.2% BSA and resuspend cell pellet. Incubate for 1 hr at 4°C on a platform rocker.

    Avidin blocks biotin groups on unimported B-Luc.

13. Add 10 µl of 750 µg/ml biocytin diluted in IRB/0.2% BSA and incubate for an additional 15 min at 4°C.

    Biocytin binds excess avidin.

14. Solubilize by adding 100 µl EBS and vortexing. Incubate 30 min at 4°C.

    Unmodified B-Luc, the hallmark of import, is now available for capture and detection in microtiter wells coated with anti-luciferase antibodies.

Capture B-Luc

15. Just prior to plating lysates, wash ELISA microtiter well strips two times with 200 µl PBS. Add 200 µl of cell (or organelle) lysates to appropriate microtiter wells and incubate overnight at 4°C.

Develop plates

16. Wash ELISA microtiter well strips three times with 200 µl PBS and add 200 µl EBS for 5 min. During this time, dilute the streptavidin horseradish peroxidase 1:5000 in EBS.

17. After 5 min, wash ELISA microtiter well strips three times with 200 µl PBS, and add 200 µl of the streptavidin horseradish peroxidase/EBS mixture. Incubate for 1 hr at room temperature.

18. After 1 hr, wash ELISA microtiter well strips three times with 200 µl PBS and add 200 µl EBS for 5 min. During this 5-min period, prepare the development solution by adding 10 µl of 30% H_2O_2 and 10 mg *o*-phenylenediamine to 25 ml ELISA development buffer.

    The development solution should not be prepared ahead of time.

    CAUTION: *o-Phenylenediamine is a carcinogen and contact should be avoided. All o-phenylenediamine-containing solutions should be discarded appropriately.*

19. After 5 min, wash ELISA microtiter well strips three times with 200 µl PBS, and add 200 µl of H_2O_2/*o*-phenylenediamine/ELISA development buffer mixture. Allow development to proceed for 1 to 2 min.

20. Stop development with 50 µl of 4 N H_2SO_4 and, in a microtiter plate reader, measure absorbance at 490 nm.

IMPORT INTO ORGANELLE PELLETS/PEROXISOMES

ALTERNATE PROTOCOL

In this protocol, import is carried out in semi-permeabilized cells. Then the cells are homogenized and fractionated, and import assessed in the resultant organelles.

Additional Materials *(also see Basic Protocol)*

Antibody-coated microtiter well strips (Basic Protocol, steps 1 and 2)
Homogenization buffer (see recipe), pH 7.8

15-ml conical centrifuge tubes (Corning)
27-G needle
1-ml syringe
2-ml glass Dounce tissue grinder, with loose- and tight-fitting pestles (Kontes)

Prepare semi-permeabilized cells

1. Wash cells near, but not at confluence, two times with 25 ml HBSS and add 25 ml fresh medium for 30 min prior to permeabilizing.

   For this protocol, two nearly confluent 15-cm tissue culture plates will provide sufficient cells for ~6 import reactions. The following steps describe procedures for treatment of two 15-cm plates.

2. Wash cells three times with 25 ml IRB and harvest in a small residual volume (~5 ml) by scraping firmly with a stiff rubber policeman.
3. Repeat scraping with an additional 5 to 10 ml IRB added to harvest remaining cells. Pool cells from two plates at this step.
4. Bring volume to 50 ml with IRB in a 50-ml conical centrifuge tube and centrifuge cells 5 min at 800 × *g*, 4°C. Carefully aspirate supernatant.

   Avoid touching cell pellet or it too may be aspirated. Leave a small amount of supernatant if necessary.

5. Carefully resuspend cells in ~12 ml IRB and dispense ~2-ml aliquots into each of six 15-ml conical centrifuge tubes on ice.

   Repeated gentle pipetting with a 5-ml pipet helps reduce clumping of the cells. It is critically important that each 15-ml conical centrifuge tube receive an equal volume/number of cells.

6. Centrifuge the cells 5 min at 800 × *g*, 4°C. Carefully aspirate supernatant.

   Once prepared, the cells should be used as quickly as possible. Therefore, the import reaction components should be prepared and pooled such that they may be added to cells in a one-step addition immediately after the supernatant is aspirated.

Prepare import reactions/incubations

7. Prepare a 187.5-µl import reaction mix with the following components:

   12.5 µl ATP
   31.3 µl zinc chloride
   31.3 µl 80 µg/ml B-Luc diluted in IRB/1.25% BSA
   112.4 µl IRB

   The 112.4 µl IRB addition may be reduced to accommodate such components as cytosol, (inhibitory) antibodies, chelators, and combinations or fractions thereof.

8. Gently resuspend the cells in the import reaction mix and immediately transfer the contents to a 1.5-ml microcentrifuge tube. Incubate for 45 min at 37°C. Tap the microcentrifuge tubes every 5 min to ensure an even distribution.

   The cell volume is ~60 µl, therefore the final reaction volume is ~250 µl. For zero-time incubations, add import reaction components to cells at the end of the incubation, i.e., at 45 min.

9. Terminate import reaction by microcentrifuging tubes 1 min at 13,000 rpm, 4°C. Aspirate supernatant.

Block unimported B-Luc

10. Add 250 µl of 187.5 µg/ml avidin diluted in homogenization buffer and resuspend cell pellet. Incubate for 1 hr at 4°C with rocking.

    Avidin blocks biotin groups on unimported B-Luc.

11. Add 25 µl of 1.875 mg/ml biocytin diluted in homogenization buffer and incubate for an additional 15 min at 4°C.

    Biocytin binds excess avidin.

Prepare organelle pellets/peroxisomes

12. Homogenize cells on ice by passing through 27-G needle attached to 1-ml syringe five times, followed by ten strokes with a loose-fitting pestle and ten strokes with a tight-fitting pestle in a 2-ml Dounce homogenizer.

13. Centrifuge 10 min at 1000 × *g* at 4°C to remove nuclei and unbroken cells.

14. Transfer supernatant to a fresh 1.5-ml microcentrifuge tube and microcentrifuge 20 min at 17,000 × *g*, 4°C, to prepare organelle pellet/peroxisomes. Aspirate supernatant.

15. Solubilize pellet by adding 200 µl EBS and vortexing. Incubate for 30 min at 4°C.

    Unmodified B-Luc, the hallmark of import, is now available for capture and detection in microtiter wells coated with anti-luciferase antibodies in the ELISA assay (see Basic Protocol, steps 1, 2 and 15 to 20). Alternatively, proteins in the organelle pellet are separated by SDS-PAGE (UNIT 6.1), transferred to nitrocellulose, and probed with streptavidin alkaline-phosphatase (see Terlecky et al., 2001; also see UNIT 6.2).

SUPPORT PROTOCOL

LUCIFERASE BIOTINLYATION

Biotinylated luciferase is used as the import substrate for the assays described in this unit. The protein contains a peroxisomal targeting signal and is readily available comercially

Materials

Firefly (*Photinus pyralis*) luciferase (Sigma-Aldrich)
Phosphate-buffered saline (PBS; *APPENDIX 2A*) pH 7.4
8.25 mM 6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, succinimidylester (Molecular Probes) in dimethylsulfoxide (B-XX-SE)
PBS containing 0.2% (w/v) BSA (PBS/0.2% BSA)
Streptavidin alkaline-phosphatase (KPL)
Rabbit polyclonal anti-luciferase antibodies (*UNIT 16.2*)
Micro Bio-Spin P-6 chromatography column (spin column, Bio-Rad)

1. Dissolve 2 mg of firefly luciferase in 200 µl PBS.

2. Add 20 µl of 8.25 mM B-XX-SE (about five times molar excess over luciferase). Incubate 1 hr at room temperature.

3. During 1-hr incubation, wash four spin chromatography columns first three times with 500 µl PBS, then one time with 500 µl PBS/0.2% BSA, followed by a 500-µl PBS wash. Centrifuge each wash for 1 min at 1000 × *g*, room temperature.

4. After 1 hr, add 55 µl of biotinylation reaction (step 2) to each column and centrifuge for 4 min at 1000 × *g*. Collect and pool filtrates.

   This step effectively separates the modified protein from unincorporated labeling reagents.

5. Adjust volume of biotinylated luciferase (B-Luc) with PBS such that final protein concentration is ~2 mg/ml. Dispense into 10-µl aliquots and store at −80°C.
6. Confirm biotinylation by blotting with streptavidin alkaline-phosphatase and rabbit polyclonal anti-luciferase antibodies.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ATP and regenerating system (ATP)

20 mM adenosine 5′-triphosphate (disodium salt, Amersham Biosciences)
100 mM creatine phosphate (disodium salt, Calbiochem-Novabiochem)
8.0 IU creatine phosphokinase (Sigma-Aldrich)
Store up to 6 months at −80°C

ELISA blocking solution (EBS)

0.2% (w/v) BSA
1 mM EDTA (disodium salt)
50 mM sodium chloride
0.1% (w/v) sodium dodecylsulfate salt (SDS)
10 mM Tris·Cl
1.0% (v/v) Triton X-100
Adjust pH to 7.4 with HCl
Store up to 1 month at 4°C

Homogenization buffer

10 mM acetic acid
0.2% (w/v) BSA
1 mM EDTA (disodium salt)
0.1% (v/v) ethanol
0.25 M sucrose
10 mM triethanolamine
Adjust pH to 7.8 with NaOH
Store up to 1 month at 4°C

Import reaction buffer (IRB)

40 mM *N*-2-hydroxyethyl-piperazine-*N*′-2-ethane-sulfonic acid (HEPES)
2 mM magnesium acetate
100 mM potassium acetate
85 mM sucrose
Adjust pH to 7.4 with KOH
Store up to 1 month at 4°C

COMMENTARY

Background Information

Peroxisomes

Peroxisomes are critically important, ubiquitous subcellular organelles whose biology impacts human health, disease, and aging. In recent years, considerable efforts have been expended to try to understand how these single-membrane bounded multifunctional organelles arise. Powerful genetic approaches in a number of diverse organisms have identified 23 proteins, called peroxins, which participate in various aspects of peroxisome biogenesis. Biochemical approaches have also contributed significantly to an understanding of how peroxisomes are formed from a mechanistic standpoint.

Peroxisome biogenesis appears to involve three steps: (1) synthesis and assembly of membrane proteins and lipids, (2) signal-mediated

post-translational import of proteins from the cytosol, and (3) growth and division of the organelle (reviewed in Terlecky and Fransen, 2000). It is the processes associated with protein import into the organelle, i.e., step 2, that are reconstituted in the assay described here. An important question at this point is what sets this in vitro system apart from others currently available?

In vitro assays

Three other in vitro assays for peroxisomal protein import are in use. The first. involves isolating organelles from rat liver or yeast cells and incubating them with import substrates (Lazarow et al., 1991). Protease protection is used as the hallmark of import. The second involves microinjecting intact cells with import substrates and examining their redistribution to peroxisomes over time (Walton et al., 1992). Import is detected by immunofluorescence. The third involves bacterial cytolysin semi-permeabilizing cells and incubating them with import substrates (Wendland, 1994). Once again, import is detected by immunofluorescence. All three have contributed and will continue to contribute to the field. The assay described here is the most recently developed for peroxisomal protein import. Based on a cell-free system first designed to quantitate receptor-mediated endocytosis (Smythe et al., 1992), it employs mechanically disrupted cells and biotinylated import substrates. The hallmark of import is the accumulation of avidin-inaccessible biotin groups inside the peroxisome. The assay is a reliable and robust multisample system designed to permit examination of a number of parameters within a single experiment. It faithfully recapitulates the import process, is easy to use and reproducible, and it should greatly facilitate the search for novel biochemical factors and mechanisms fundamental to peroxisomal protein import.

Quantitative assay specifics/advantages

Nearly all peroxisomal proteins are directed to the organelle by virtue of a peroxisomal targeting signal (PTS); specifically, PTS1 or PTS2. Although the protocols described here employ the PTS1-containing protein luciferase, the assay may, in principle, be applied to other PTS1- and PTS2-containing substrates. Interestingly, PTSs exist as compositional motifs, and not as absolutely conserved sequences. The assay could be used to test the relative strength of signal variants; a powerful advantage over nonquantitative immunofluorescence-based assays that cannot discriminate between potentially subtle differences in import efficiencies.

The assay does not detect significant import in cells from patients who suffer from the fatal peroxisomal disorder Zellweger syndrome (Terlecky et al., 2001). In these particular cells, the basis of the import deficiency is known—they lack a functional PTS1 import receptor, called Pex5p. Perhaps in other patient cell lines, the system could be used as a "biochemical complementation assay" to identify missing or defective factors.

The assay is presented in two forms; as a Basic Protocol and as an Alternate Protocol. The two may be used interchangeably. The advantage of the ("cell") Basic Protocol is that it is easier to use and is less time-consuming. It is the approach of choice in most circumstances. The ("organelle pellet/peroxisome") Alternate Protocol is especially effective when trying to avoid problems associated with cell clumping in small volumes. Human fibroblasts, especially if over-confluent, can clump after being semi-permeabilized and concentrated. This can make dispensing equal (10 µl) volume aliquots problematic. In the Alternate Protocol, cells are (equally) divided while in a larger and more easily divided (12 ml) volume.

Critical Parameters and Troubleshooting

Biotinylation

A procedure is detailed for the biotinylation of luciferase (see Support Protocol). Clearly, there are other methods for the biotinylation of this enzyme—some involve isolation of the biotinylated-species on (monomeric) avidin columns. The latter is an effective approach but can be problematic if elution involves more stringent conditions (e.g., acid). Under such circumstances, care should be taken to ensure that the renaturation/refolding steps result in a protein that is still recognized by antibodies and captured in antibody-coated microtiter wells, and remains a substrate for the peroxisomal protein import pathway.

Import assays

Peroxisomal protein import is blocked at low temperatures (e.g., 4°C). Therefore, in addition to a zero-time incubation, (complete) import reactions should also be carried out at 4°C. Typically, import will be reduced to <10% of the 37°C value.

Factors that modulate import should be determined empirically for each ligand. For the PTS1-containing protein, B-Luc, import is stimulated some six-fold by zinc ions (100 µM) and an additional two- to three-fold by cytosol (10 µg/ml; Terlecky et al., 2001). For other substrates, the concentrations of these and other factors should be titrated to maximize the import signal. It should be noted that the cytosol effect on B-Luc import is variable, depending on the source, the concentration, the amount of endogenous cytosol removed from cells, and other factors (Terlecky et al., 2001). As such, it is recommended that each cytosol preparation be individually evaluated for activity with a given substrate.

Lastly, the assay described in the ("cell") Basic Protocol is performed in a final volume of 40 µl. It is, therefore, exquisitely sensitive to pipetting errors. Where possible, reduce or eliminate the potential for variance by pooling components.

ELISA quantitation

It is extremely important that the signal-to-noise ratio of the assay be high. Contributing to background noise (i.e., the absorbance detected in an import reaction at time-zero or at time 45 min at 4°C) is (1) impure or poor quality development reagents, (2) ineffective blocking of microtiter wells, and (3) incomplete washing of microtiter wells between steps. Contributing to reduced signals (i.e., the absorbance detected in an import reaction at 45 min at 37°C) is (1) using poorly permeabilized cells, (2) exceeding the binding capacity of the coated antibody, and (3) inactivation of the import substrate.

Suggested remedies for these problems are as follows. For high background noise: (1) use the reagents and sources suggested in the protocols, (2) modify the blocking buffer to include higher concentrations of BSA or switch to gelatin-, casein-, or non-fat milk-based blocking buffers (however, be sure not to interfere with antibody-capture of the biotinylated-import substrate), and (3) wash thoroughly (a plate washer helps increase reproducibility and simplifies the process). For low signals: (1) scrape the cells more vigorously, or consider growing the cells on fibronectin or other attachment factor, (2) reduce the amount of cell or organelle lysates loaded (from the Basic Protocol or Alternate Protocol, respectively), or, alternatively, reduce the amount of substrate added to the import reaction, and (3) biotinylate the import substrate in another way. With respect to the latter point, it is possible that biotinylation of the import substrate results in a

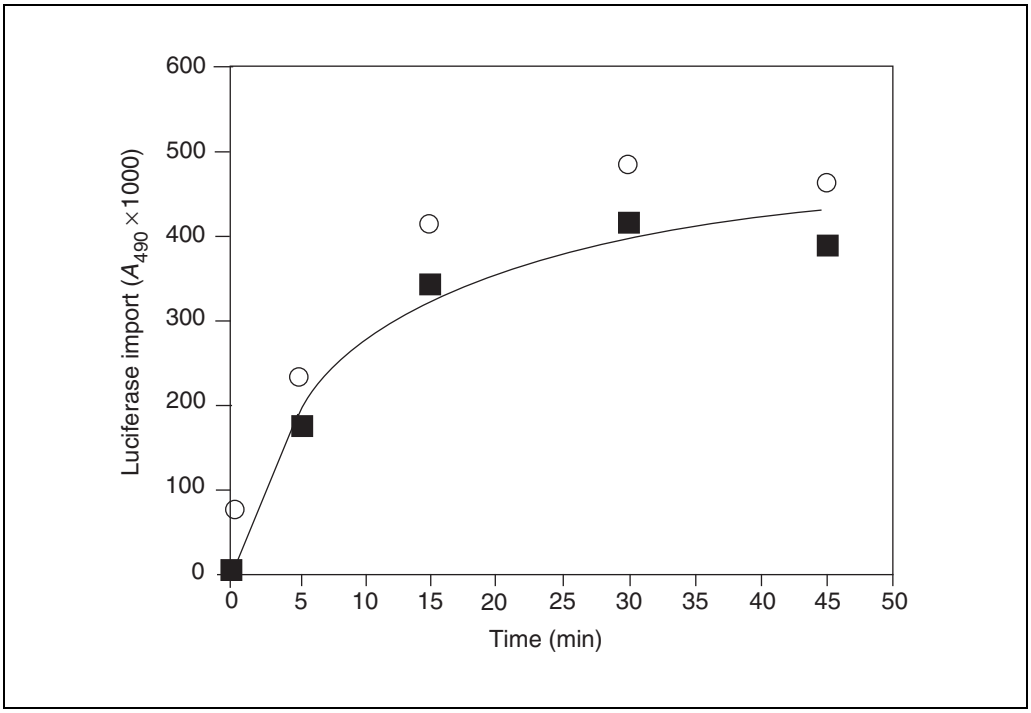


Figure 11.15.2 Peroxisomal import kinetics. Semi-permeabilized human A431 cells were incubated with biotinylated luciferase in the in vitro peroxisomal import reaction described in the Alternate Protocol. At the times indicated, the extent of import was quantified by ELISA. The raw absorbance units (×10^2) obtained at each time point (open circle); adjusted absorbance values reflecting subtraction of the time 0 value (filled square and solid line).

protein that is still recognized by antibodies and therefore captured in antibody-coated microtiter wells, but has had its PTS inactivated or shielded. Under such circumstances, consider using a different biotinylation method, or reducing the number of biotin groups coupled per protein molecule. If necessary, employ the immunofluorescence-based semi-permeabilized cell import assay (Wendland, 1994; Terlecky et al., 2001) to confirm import competence of the biotinylated substrate.

Anticipated Results

The protocols outlined here will result in a measurable absorbance at 490 nm. For example, Figure 11.15.2 illustrates results of an import time course. The challenge is to ensure that the signal is obtained under appropriate conditions (e.g., import conducted for 45 min at 37°C) and not inappropriate ones (e.g., import conducted at 4°C or for zero-time). Furthermore, to permit valid comparisons between experiments, every effort should be made to keep assay parameters as consistent as possible. This includes the number of cells and their treatment, the concentrations and preparations of assay components, and the conditions employed. Clearly, if the positive and negative controls are similar in experiments conducted on different days, then the results obtained for "experimental" conditions may be usefully and accurately compared.

Time Considerations

The biotinylation reaction described in the Support Protocol will require ~2 hr to complete. This excludes the time required for blotting the resultant biotinylated-species. Clearly, the Support Protocol must be completed before initiating import assays.

A typical import assay will require 30 min and an overnight incubation 2 days before the assay for coating microtiter wells with antibodies, and 30 min and an overnight incubation 1 day prior to the assay for blocking of wells. On the day of the import experiment, ~4.5 hr will be required for the "cell" Basic Protocol, and 6 hr for the "organelle pellet/peroxisome" Alternate Protocol. An overnight incubation is once again required, and on day 2 of the experiment, the assay is developed. This step requires ~2 hr to complete. Every effort should be made to design the experiment, prepare reagents, label tubes, and check on the availability of required equipment well in advance of beginning the assay. The only other time consideration is that of optimizing the ELISA assay by serial dilution titration analysis. Performed after biotinylation of the ligand, it will require the 2-day preparation of antibody-coated and blocked microtiter wells, and ~2 hr to add appropriate amounts of the biotinylated-ligand. After an overnight incubation, once again ~2 hr is required to develop the assay.

Literature Cited

Lazarow, P., Thieringer, R., Cohen, G., Imanaka, T., and Small, G. 1991. Protein import into peroxisomes in vitro. *Methods Cell Biol*. 34:303-326.

Smythe, E., Redelmeir, T.E., and Schmid, S.L. 1992. Receptor-mediated endocytosis in semiintact cells. *Methods Enzymol.* 219:223-234.

Terlecky, S.R. and Fransen, M. 2000. How peroxisomes arise. *Traffic* 1:465-473.

Terlecky, S.R., Legakis, J.E., Hueni, S.E., and Subramani, S. 2001. Quantitative analysis of peroxisomal protein import in vitro. *Exp. Cell Res*. 263:98-106.

Walton, P.A., Gould, S.J., Feramisco, J.R., and Subramani, S. 1992. Transport of microinjected proteins into peroxisomes of mammalian cells: Inability of Zellweger cell lines to import proteins with the SKL tripeptide peroxisomal targeting signal. *Mol. Cell. Biol*. 12:531-541.

Wendland, M. 1994. A permeabilized cell system to study peroxisomal protein import. *In* Cell Biology: A Laboratory Handbook (J. Celis, ed.) pp. 140-147. Academic Press. San Diego.

Key References

Smythe et al., 1992. See above.

Highly recommended first description of an ELISA-based in vitro internalization assay using biotinylated substrates and semi-intact cells. This method is the basis for the assay described in this unit.

Terlecky et al., 2001. See above.

First description of the use of the system outlined in the previous reference for assaying peroxisomal protein import.

Contributed by Stanley R. Terlecky
Wayne State University School of Medicine
Detroit, Michigan

In Vitro Analysis of Chloroplast Protein Import

This unit describes methods for isolating intact chloroplasts from pea (*Pisum sativum;* see Support Protocol 1) and *Arabidopsis thaliana* (see Support Protocol 2) for the study of the import of nuclear-encoded plastid precursor proteins. Chloroplasts from both preparations are competent for the in vitro import of recombinant preproteins synthesized using in vitro translation systems derived from reticulocyte lysates or wheat germ lysates (see *UNIT 11.2* and Support Protocol 3). Preproteins expressed in *E. coli* also have been used for import studies using isolated pea chloroplasts (Pilon et al., 1990; Schnell and Blobel, 1993). These assays can be used to test whether a particular protein is targeted to chloroplasts, for analyzing the suborganellar location of newly imported preproteins, or to study the mechanism of import itself. The in vitro chloroplast protein import assay is described in the Basic Protocol, and methods for fractionating the chloroplasts and analyzing the results of import are provided in the Alternate Protocol.

IN VITRO CHLOROPLAST PROTEIN IMPORT ASSAY

BASIC PROTOCOL

Intact chloroplasts isolated from either pea or *Arabidopsis* using the methods described in Support Protocols 1 and 2 can be used for in vitro chloroplast protein import assays. These assays are often used to confirm the ability of proteins to be imported into chloroplasts, as chloroplast targeting prediction programs (Emanuelsson et al., 1999, 2000) are not 100% accurate, or to study the mechanism of import itself.

Generally, the intact chloroplasts are incubated with in vitro—translated, radiolabeled chloroplast preproteins in the presence of energy in the form of ATP (Pain and Blobel, 1987; Theg et al., 1989). The chloroplasts are re-isolated to remove any precursor not imported during the assay and analyzed using a PhosphorImager or by fluorography (*UNIT 6.3*) following SDS-PAGE (*UNIT 6.1*) to detect imported protein. The majority of chloroplast proteins are synthesized with a cleavable *N*-terminal transit peptide that is removed following import to the stroma (Bauer et al., 2001). This cleavage results in a shift in molecular weight, which provides a convenient indication that import has occurred. Chloroplasts are treated with exogenous protease (e.g., thermolysin), following the import assay, to digest any excluded preprotein and confirm that the processed preprotein has been imported, and thereby, protected from protease digestion (Cline et al., 1984). For proteins targeted to the outer envelope membrane, many of which do not have cleavable transit peptides, resistance to alkali extraction or the generation of a characteristic pattern of protease resistance can confirm integration into the membrane (Muckel and Soll, 1996; Froehlich et al., 2001).

Materials

Freshly isolated, intact chloroplasts (see Support Protocols 1 and 2)
HEPES-sorbitol buffer, pH 7.5 (if using pea chloroplasts, see recipe) or pH 8.0 (if using *Arabidopsis* chloroplasts, see recipe)
Chloroplast import master mix (see recipe)
0.1 M dithiothreitol (DTT)
0.1 M ATP
In vitro—translated [^{35}S]methionine-labeled chloroplast protein (see Support Protocol 3 or *UNIT 11.2*)
40% (v/v) Percoll (see recipe)
2 mg/ml thermolysin (see recipe)
0.5 M EDTA
SDS-PAGE sample buffer (see recipe)
Destain solution (see recipe)

Contributed by Matthew D. Smith, Lynda Fitzpatrick, Kenneth Keegstra, and Danny J. Schnell
Current Protocols in Cell Biology (2002) 11.16.1-11.16.21

26°C water bath
1.5-ml microcentrifuge tubes
Refrigerated microcentrifuge
Enlightening fluorography enhancer (NEN)
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and fluorography or phosphorimagery (*UNIT 6.3*)

Perform in vitro import assay

1. Adjust the chlorophyll concentration of freshly prepared chloroplasts (see Support Protocols 1 and 2) to 1 mg chlorophyll/ml using the appropriate HEPES-sorbitol buffer.

   Use the equivalent of 50 μg chlorophyll in a standard 100-μl assay.

   The amount of chlorophyll can be increased to 100 μg chlorophyll/100 μl assay if the sensitivity of import (i.e., the import efficiency of a particular substrate) is low.

2. Mix the following components on ice:

   50 μl 1 mg/ml chloroplasts (50 μg chlorophyll)
   10 μl chloroplast import master mix
   1 μl 0.1 M DTT
   2 μl 0.1 M ATP
   62 μl appropriate HEPES-sorbitol buffer

   Pre-incubate for 5 min at 26°C in the dark.

3. To begin import, add 1 to 5 μl [^{35}S]methionine-labeled in vitro translation product (equivalent to at least 10,000 cpm). Incubate 20 to 30 min at 26°C in the dark.

   The amount of in vitro–translated import substrate added to each reaction can vary considerably depending on the efficiency of import of the substrate. A model import substrate, such as the precursor to the small subunit of rubisco (preSSU), that is synthesized in a reticulocyte lysate system and radiolabeled with [^{35}S]methionine is imported with efficiencies of up to 65% of added substrate. However, the authors recommend that investigators assume an import efficiency of ~20% for previously untested preproteins. With this in mind, the amount (cpms) of substrate should be increased proportionately to ensure sensitivity of detection with film or a PhosphorImager. Also, be aware that transit peptide processing might remove [^{35}S]methionine residues, thereby, decreasing sensitivity.

4. Stop import by diluting the reaction into 0.5 ml of ice-cold HEPES-sorbitol buffer and hold on ice.

Re-isolate the chloroplasts

5. Layer the diluted chloroplasts over a 0.5- to 1-ml cushion of ice-cold 40% Percoll in a 1.5-ml microcentrifuge tube and microcentrifuge for 5 min at 2000 × *g*, 4°C.

6. Carefully aspirate the supernatant and 40% Percoll, containing any broken chloroplasts and nonimported translation product.

   It is important to remove all of the supernatant without disturbing the chloroplast pellet in order to minimize contamination of the chloroplasts with excess precursor.

Treat chloroplasts with thermolysin

7. Resuspend the chloroplasts in 100 μl of ice-cold HEPES-sorbitol buffer and transfer to a fresh 1.5-ml microcentrifuge tube. Add 5 μl of 2 mg/ml thermolysin, mix gently, and incubate for 30 min on ice.

   A concentration of 100 μg thermolysin/ml during the protease treatment is standard for the analysis of proteins imported to the inner envelope, stroma, or thylakoid. For integral outer

envelope proteins that will be susceptible to proteolysis, it may be desirable to titrate the level of thermolysin to generate a digestion pattern that is characteristic of the integrated protein (Muckel and Soll, 1996; Tu and Li, 2000; Froehlich et al., 2001). The final thermolysin concentration can be varied from 10 to 200 μg/ml depending on the substrate being studied and the susceptibility to degradation. The optimal concentration to be used for each substrate should be determined empirically.

8. Stop the reaction by adding 0.5 M EDTA to a final concentration of 10 mM. Re-isolate the chloroplasts by centrifugation through a 40% Percoll cushion, containing 5 mM EDTA, as described in step 5.

Analyze imported proteins

9. Resuspend the chloroplast pellet (step 6 or step 8) directly in 25 μl SDS-PAGE sample buffer and analyze by SDS-PAGE (*UNIT 6.1*) and fluorography or phosphorimagery (*UNIT 6.3*).

   As an alternative to analyzing the chloroplast pellet directly by SDS-PAGE, the chloroplasts may first be fractionated to determine the precise suborganellar location of the imported protein (see Alternate Protocol).

10. Analyze the results of import by SDS-PAGE using a 10% or 12% polyacrylamide gel (*UNIT 6.1*).

    Samples from each step of the import assay and subsequent treatments should be analyzed. For example, chloroplasts reisolated directly following the import assay (step 6) and following protease treatment (step 8) should be analyzed along with samples from alkaline extraction (see Alternate Protocol, steps 9 and 12) and each fraction from chloroplast subfractionation (Alternate Protocol, steps 18 and 23). A small aliquot of the in vitro translation product used in the import assay, usually equivalent to 1/10 of the amount added to the reaction, should be included on the gel. This will serve as a molecular weight reference, to confirm the transit peptide has been removed, and as a reference for the amount of radioactive substrate added to the reaction (keeping in mind that the processed protein might have fewer labeled methionine residues than the precursor protein with transit peptide). This will provide a complete and quantitative analysis of the import experiment.

11. Following electrophoresis, fix the gel in destain solution for 30 to 60 min. For exposure using a PhosphorImager, dry the gel under vacuum, wrap in plastic and expose to the phosphor storage screen overnight. For fluorography using film, soak the gel in Enlightening fluorography enhancer for 30 to 60 min, dry the gel under vacuum at 65°C for 1.5 to 2 hr, and expose to X-ray film overnight at −80°C.

FRACTIONATION OF RE-ISOLATED CHLOROPLASTS FOLLOWING IMPORT

ALTERNATE PROTOCOL

It may be desirable to subfractionate chloroplasts following an import reaction to determine the suborganellar localization of the newly imported preprotein. To assess stromal versus membrane localization, chloroplasts can simply be lysed and separated into stromal versus membrane fractions by differential centrifugation. To confirm that a suspected membrane protein is integrated, the membrane fraction can be subjected to alkaline extraction. Alternatively, the chloroplasts can be fractionated into outer and inner envelope membranes, thylakoids, and stroma if the precise suborganellar compartment must be determined (Keegstra and Yousif, 1986). Subfractionation into the three membrane components should not be attempted with chloroplast samples corresponding to <0.5 mg chlorophyll because of the low yield of envelope membrane. The inner and outer envelope membranes constitute ≤1% of the total chloroplast membrane fraction.

Materials

Chloroplast pellet from the import reaction (see Basic Protocol)
HEPES-sorbitol buffer (see recipe), ice cold
2 mM EDTA, ice cold
4 M NaCl
0.5% and 100% (w/v) trichloroacetic acid (TCA; see recipe)
SDS-PAGE sample buffer (see recipe)
0.1 M $NaCO_3$, pH 11.5, ice cold
TE/DTT buffer (see recipe) containing 0.6 M sucrose and buffer containing 0.2 M sucrose
TE/DTT buffer (see recipe)
1 M, 0.8 M, and 0.46 M sucrose

3-ml Beckman polyallomer thick-walled ultracentrifuge tubes
Ultracentrifuge and rotor (Beckman TL 100 and TLA 100.3 rotor, or equivalent)
Dounce homogenizer or Potter homogenizer and pestle
Swinging-bucket rotor (e.g., Beckman SW 50.1)
5-ml polyallomer tubes (e.g., for Beckman SW 50.1)
1-ml micropipettor
Gradient former (e.g., Buchler DensiFlow gradient former)
Gradient fractionater (e.g., ISCO gradient fractionater)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and fluorography or phosphorimagery (*UNIT 6.3*)

Lyse chloroplasts for stromal versus membrane association

1. Resuspend the chloroplast pellet from the import reaction (see Basic Protocol, step 6 or 8) in 150 µl of ice-cold HEPES-sorbitol buffer.
2. Dilute the suspension with 800 µl of ice-cold 2 mM EDTA. Vortex vigorously and let stand for 10 min on ice to lyse the chloroplasts. Add 50 µl of 4 M NaCl, mix, and microcentrifuge the lysed chloroplasts for 30 min at 15,000 × *g*, 4°C, to pellet both the thylakoid and envelope membranes.
3. Carefully transfer the supernatant (stroma) to a fresh 3-ml polyallomer thick-walled tube and precipitate the protein by adding 100% TCA to a final concentration of 12%. Incubate for 1 hr on ice. Resuspend the membrane pellet directly in 25 µl SDS-PAGE sample buffer.
4. Collect the TCA precipitate by centrifugation for 30 min at 15,000 × *g*, 4°C. Remove and discard the supernatant.
5. Wash the pellet with 500 µl 0.5% TCA and resuspend the pellet of precipitated stromal proteins directly in 25 µl SDS-PAGE sample buffer.

Prepare chloroplasts for alkaline extraction

6. Resuspend and lyse the chloroplast pellet from the in vitro import assay (see Basic Protocol, step 6 or 8) in 100 µl of ice-cold 0.1 M $NaCO_3$, pH 11.5, by pipetting the pellet into solution. Transfer to a fresh microcentrifuge tube.
7. Add another 900 µl of ice-cold 0.1 M $NaCO_3$, pH 11.5, and transfer the solution to a 3-ml polyallomer thick-walled ultracentrifuge tube. Incubate for 10 min on ice.
8. Ultracentrifuge the lysed chloroplasts for 30 min at 40,000 × *g*, 4°C, to pellet the membranes.

9. Carefully collect the supernatant containing the stroma and transfer to a fresh 3-ml ultracentrifuge tube. Resuspend the membrane pellet in 25 µl SDS-PAGE sample buffer.

Precipitate the alkaline extract and chloroplast stroma

10. Precipitate the alkaline supernatant by adding 100% TCA to a final concentration of 12%. Incubate for 1 hr on ice.

11. Collect the TCA precipitate by centrifuging for 30 min at 15,000 × *g*, 4°C.

12. Wash the pellet with 500 µl of 0.5% TCA and resuspend the pellet directly in 25 µl SDS-PAGE sample buffer.

13. Analyze all fractions by SDS-PAGE (*UNIT 6.1*) and fluorography or phosphorimagery (*UNIT 6.3*).

Fractionate chloroplasts

14. Resuspend the chloroplast pellet from the in vitro import assay (see Basic Protocol, step 6 or 8) in TE/DTT buffer containing 0.6 M sucrose to a concentration of 1 to 2 mg/ml chlorophyll. Let stand for 10 min on ice.

    If the protein import assay was performed with <0.5 mg chlorophyll, the chloroplasts from the import assay should be mixed with a nonradioactive chloroplast fraction to bring the chlorophyll content >0.5 mg. Fractionation of samples containing 1 to 2 mg chlorophyll by this procedure is ideal.

15. Freeze the chloroplast suspension for 1 to 2 hr at −20°C.

    It is essential to lyse the chloroplasts under hypertonic conditions to ensure maximum separation of outer and inner envelope membranes in the subsequent sucrose gradient fractionation (Keegstra and Yousif, 1986). The sample may be transferred to −80°C at this stage and stored indefinitely.

16. Thaw the suspension and dilute with 3 vol of TE/DTT buffer. Homogenize with 20 strokes in a Dounce or Potter homogenizer with a tight pestle.

17. Ultracentrifuge the lysed chloroplasts in a swinging-bucket rotor for 1 hr at 40,000 × *g*, 4°C.

18. Remove the brownish supernatant containing the stromal contents using a pipet and store at −20°C.

    The supernatant also might appear yellowish. This color indicates the presence of high levels of plastoglobules in the stroma. These low-density stromal lipid bodies are high in carotenoids.

Fractionate the chloroplast membranes

19. Resuspend the membrane pellet in TE/DTT buffer containing 0.2 M sucrose to a concentration of 1 to 2 mg chlorophyll/ml with 20 strokes in a Dounce or Potter homogenizer.

    The total chloroplast membranes may be stored at −80°C for at least 6 months or separated into thylakoid and envelope fractions, as described in subsequent steps.

20. Set up a sucrose step gradient consisting of 2 ml of 1 M sucrose, 1 ml of 0.8 M sucrose, and 1 ml of 0.46 M sucrose in 5-ml polyallomer tubes. Layer the membrane suspension onto the top.

    The gradients can be made during the centrifugation at step 17.

21. Apply 1 ml of membrane suspension (not >2 mg chlorophyll content) on each gradient. Ultracentrifuge the samples for 1.5 hr at 270,000 × *g*, 4°C, in a Beckman SW 50.1 rotor using low acceleration and deceleration rates.

    Alternatively, the membranes can be separated using a TLS-55 rotor in a Beckman TL 100 or equivalent centrifuge. The volumes of the steps for this rotor are 0.75 ml of 1 M sucrose, 0.5 ml of 0.8 M sucrose, and 0.5 ml of 0.46 M sucrose. The membrane suspension should not exceed 0.5 ml in volume and 1 mg chlorophyll content. Centrifuge the samples for 1.5 hr at 55,000 rpm at low acceleration and deceleration rates.

22. Collect the interface from each step of the gradient (~0.6 ml from each interface) with a 1-ml micropipettor.

    The upper (0.2:0.46 M sucrose) interface contains residual stroma and plastoglobules. The middle (0.46:0.8 M sucrose) interface is highly enriched in outer envelope membranes. The lower (0.8:1 M sucrose) interface is enriched in inner envelope membranes. The thylakoid membranes form a tight pellet at the bottom of the tube.

23. To collect the membranes, dilute each fraction with 3 to 5 vol TE/DTT buffer and centrifuge in a swinging-bucket rotor (e.g., Beckman SW-40) for 1.5 hr at 270,000 × *g*, 4°C. Remove the supernatant with a pipet and discard. Resuspend the pellets in a small volume of SDS-PAGE sample buffer for analysis.

SUPPORT PROTOCOL 1

ISOLATION OF INTACT CHLOROPLASTS FROM PEA

Intact chloroplasts capable of importing nuclear-encoded plastid proteins can be isolated from pea seedlings grown on soil (Chua and Schmidt, 1978; Cline, 1986; Pain and Blobel, 1987; Bruce et al., 1994). Peas have been used as the model system for chloroplast protein import because large amounts of material can be grown quickly and easily. Plants are typically grown in standard potting trays, in either greenhouse conditions or in growth chambers under long-day (12 to 16 hr) conditions. However, peas are easily propagated without growth chambers or greenhouses and can be grown on a well-lit windowsill. Plants should be watered regularly, and 10- to 14-day-old seedlings will yield ample import-competent chloroplasts.

Prior to beginning the isolation procedure, all buffers should be prepared and chilled on ice. All procedures should be performed on ice or in a cold room, and all centrifuges and rotors should be precooled to 4°C. Maintaining the temperature of the extract at or below 4°C throughout the procedure is essential for preventing degradation of chloroplast proteins by proteases. Proceeding as quickly as possible will also limit proteolysis. To limit degradation of chloroplast components that are particularly susceptible to proteolysis, a protease inhibitor cocktail can be included in all buffers throughout the procedure.

This procedure can also be used for the isolation of intact chloroplasts from other species, most commonly spinach (Joyard et al., 1982). Fresh young spinach leaves can be obtained from a local grocer for use with the procedure. However, the yield and import capacity of chloroplasts obtained using this source is unpredictable.

Materials

Pea seeds (*Pisum sativum* Green Arrow, Jung Seed)
Potting soil (e.g., Premier Brand Pro-Mix BX or Scotts Brand Metro Mix)
85% and 40% (v/v) PBF-Percoll (see recipe)
Grinding buffer containing 0.25% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) ascorbic acid (see recipe), ice cold
Protease inhibitor cocktail (optional, e.g., Sigma-Aldrich, *UNIT 3.4*)
HEPES-sorbitol, pH 7.5 buffer (see recipe)
80% acetone
40% (v/v) DMSO (see recipe), ice cold
Liquid nitrogen

Standard potting trays (21.5 × 11 × 2.5–in.)
30-ml glass centrifuge tubes (Corex)
Scissors
2-liter beakers
Rotary homogenizer (Polytron or comparable with a 30-mm saw-tooth generator)
50-cm Miracloth squares
Large funnel (180-mm diameter)
250-ml centrifuge bottles with screw-cap lids
Superspeed centrifuge (e.g., Sorvall RC-5B)
Large rotor capable of holding 250-ml bottles (e.g., Sorvall GSA)
Swinging-bucket rotor capable of holding 50-ml tubes (e.g., Sorvall HB-4)
Large-bore pipet
Cryotubes

Prepare seeds and grow plants

1. Weigh 120 to 150 g of dry pea seeds and imbibe by submerging the seeds in water for ≥5 hr or overnight at room temperature.

2. Fill two standard potting trays 2/3-full with potting soil, and spread the seeds evenly over the surface of the soil. Cover seeds with a thin layer of soil, and water thoroughly, until soil is saturated.

3. Transfer the tray to a greenhouse or growth chamber, and grow plants under 12- to 16-hr days for 10 to 14 days at 20° to 25°C.

   The light intensity is not critical. Plants can be grown on a sunlit windowsill if growth facilities are not available. Peas prefer a moderate growth temperature not exceeding 30°C.

Prepare pea tissue

4. Prepare three PBF-Percoll step gradients by gently overlaying 7 ml of 40% PBF-Percoll onto a 5 ml layer of 85% PBF Percoll in 30-ml glass centrifuge tubes. Hold tubes on ice until needed (see step 12).

5. Harvest the aerial portions of 10- to 14 day-old pea seedlings from two flats (~100 to 140 g of tissue) by clipping the seedlings at the base of the first leaves with sharp scissors. Place the tissue in a 2-liter beaker placed on ice and chill for 10 min. Avoid transferring soil with the seedlings.

   The amount of tissue harvested may be scaled up or down depending on requirements; however, the ratio of tissue to buffer (see step 6) should be kept constant.

6. Add 1 liter of ice-cold grinding buffer containing 0.25% BSA and 0.1% ascorbic acid to the harvested tissue.

   (Optional) Add a protease inhibitor cocktail to the grinding buffer to a final concentration of 0.05% (v/v) and mix well immediately prior to adding to the tissue (Chen et al., 2000).

Homogenize pea tissue

7. Grind the pea leaves for 30 sec to 1 min using a rotary homogenizer at 80 rpm.

   Take care not to over aerate the solution with the generator. Grind until all seedlings have been homogenized and continue for 15 sec to adequately disrupt the tissue.

8. Pass the homogenate through two layers of 50-cm Miracloth squares held in place by a 180-mm diameter funnel, into another 2-liter beaker that has been pre-chilled on ice. Use two filtration systems to facilitate rapid filtration of the homogenate, passing equal volumes of the homogenate through each filter.

9. Transfer the filtrate immediately into four pre-cooled 250-ml centrifuge bottles and centrifuge in a pre-cooled rotor for 2 min at 1500 × *g*, 4°C, with the brake on and stop immediately.

Isolate chloroplasts

10. Carefully decant and discard the majority of the supernatant, leaving ~5 ml in each bottle.

    The chloroplasts will be in the pellet, which is very loose. Therefore, use caution when decanting the supernatant, so as not to disturb and lose the pellet. Do not be alarmed if the supernatant remains dark green after the centrifugation step. These are broken chloroplasts. Do not leave >5 ml of supernatant on the chloroplast pellet. If too much supernatant is decanted, simply add back several ml of grinding buffer to aid in resuspending the chloroplast pellet.

11. Carefully resuspend the chloroplast pellets in the remaining 5 ml of grinding buffer by gently swirling the bottles and then combine all the pellets into a single bottle.

    Resuspend the pellet with a gentle rotary motion. This step might require 5 min. Do not shake vigorously or resuspend by pipetting.

12. Transfer the chloroplast suspension equally onto the top of three previously prepared PBF-Percoll step gradients (see step 4). Centrifuge in a pre-cooled swinging-bucket rotor for 10 min at 1500 × *g*, 4°C, with the brake off.

    The PBF-Percoll step gradients should be prepared prior to beginning the preparation (step 4) in order to proceed as quickly as possible and limit proteolysis. The protease inhibitor cocktail may also be added to the gradient to prevent degradation of particularly susceptible chloroplast components. Transfer the chloroplasts using a large-bore pipet and avoid rapid pipetting.

13. Following centrifugation, observe two green bands. The upper band at the 40% PBF-Percoll/grinding buffer interface contains broken chloroplasts and cellular debris. The lower band located at the interface between the 40% and 85% PBF-Percoll layers contains the intact chloroplasts. Aspirate the grinding buffer and most of the 40% PBF-Percoll layers, leaving the 40%:85% PBF Percoll interface and the band of intact chloroplasts.

    It might be difficult to distinguish the two bands at the interfaces of the PBF Percoll gradients if the yield of chloroplasts is high. Aspirate the upper layers within 1 cm of the 40% and 85% PBF-Percoll interface.

14. Carefully remove the bands of intact chloroplasts from the three gradients with a large-bore pipet and transfer equal volumes into two 30-ml glass centrifuge tubes that each contain 20 ml of HEPES-sorbitol buffer, pH 7.5. Centrifuge for 2 min at 4000 × *g*, 4°C, with the brake on.

    The band of intact chloroplasts forms a tight, viscous layer in the PBF-Percoll gradient. Remove the layer and the chloroplasts that adhere to the side of the tube, being careful to transfer as little PBF-Percoll as possible.

15. Carefully decant and discard the supernatant as the pellet is very loose. Gently resuspend the pellet of intact chloroplasts in a small volume (1 to 2 ml) of HEPES-sorbitol buffer, pH 7.5, by swirling.

Quantitate chloroplast yield

16. Measure chlorophyll content (Arnon, 1949). Dilute 5 µl of the chloroplast resuspension into 1 ml of 80% acetone. Mix vigorously and microcentrifuge the extract for 2 min at maximum speed to remove the protein precipitate. Remove the supernatant to a clean tube.

The supernatant should be bright green and the pellet completely white. If the pellet retains some pigment, the sample should be mixed and microcentrifuged again before proceeding.

17. Measure the A_{652} of the sample against an 80% acetone blank. Calculate the chlorophyll concentration (mg/ml) by:

    $(A_{652}/36) \times 200$

    to compensate for the dilution factor.

    The typical yield of chloroplasts from two trays of 10- to 14-day-old seedlings (100 to 140 g tissue) is 25 to 40 mg chlorophyll.

    The chloroplasts also can be inspected visually with a bright-field microscope to assess intactness. Intact chloroplasts appear as smooth spherical bodies with enclosed green thylakoids. Chloroplasts from this procedure should be ~90% intact. Counting the chloroplasts using a hemacytometer will allow the investigator to establish the conversion of chlorophyll content to chloroplast number (i.e., chloroplasts/mg chlorophyll). This unit can be used for absolute quantitation of molecules of substrate imported per chloroplast in the in vitro import assays.

    The yield of chloroplasts from two trays of peas is greater than typically needed for a protein import assay with controls. However, the larger-scale preparation is provided because the chloroplasts from pea can be cryopreserved (see below) and used for subsequent import assays. Chloroplasts corresponding to 2 mg chlorophyll will provide sufficient material for a standard import assay with controls. As a result, the pea chloroplast isolation procedure can be scaled down if desired.

Cryopreserve chloroplasts

Intact chloroplasts isolated from pea seedlings can be stored in liquid N_2 and can be subsequently used for in vitro import assays (Yuan et al, 1991). Cryopreservation will lead to the loss of some intact chloroplasts (typically 40% to 50% loss). It is preferable to use freshly prepared chloroplasts whenever possible.

18. Gently resuspend the chloroplast pellet (step 15) in HEPES-sorbitol buffer, pH 7.5, to a concentration of 5 mg chlorophyll/ml by swirling on ice.

19. Measure the volume with a pipet. Add an equal volume of ice-cold 40% DMSO buffer drop-wise to the chloroplast suspension while gently swirling to give a final concentration of 20% DMSO. Allow the chloroplast mixture to equilibrate for 5 min on ice. Immediately pipet 0.5-ml aliquots into cryotubes on ice and snap freeze in liquid nitrogen.

20. Store the tubes under liquid nitrogen.

    Chloroplasts can be stored under these conditions for up to 3 months.

21. When ready to use, thaw the chloroplast suspension on ice and quickly dilute with an equal volume of HEPES-sorbitol buffer, pH 7.5. Layer over a cushion of 40% Percoll and centrifuge for 10 min at $3000 \times g$, 4°C.

22. Remove the buffer and 40% Percoll layers by aspiration.

    The yield of chloroplasts from the 40% Percoll step following cryopreservation is ~50% to 60%.

23. Gently resuspend the chloroplast pellet in the appropriate volume of ice-cold HEPES-sorbitol buffer, pH 7.5.

    The chloroplasts are ready to be used for an import assay (see Basic Protocol).

SUPPORT PROTOCOL 2

ISOLATION OF INTACT CHLOROPLASTS FROM *ARABIDOPSIS THALIANA*

Intact chloroplasts can be produced from *Arabidopsis* plants grown either on soil or on agar plates (Fitzpatrick and Keegstra, 2001). The growth conditions required to produce intact chloroplasts from soil-grown plants are not critical. A 12- to 16-hr day, at 20° to 25°C with a light intensity of 80 to 200 $\mu Em^{-2}sec^{-1}$ will suffice. Plants can be grown on trays or in pots. However, for a high yield, it is important to prevent the plants from experiencing stress, particularly from watering too much or too little. Yields may be improved slightly by destarching these plants by incubating for 12 to 24 hr in the dark before harvesting. Soil-grown plants that are between 3- and 5-weeks-old typically give the best yield of intact chloroplasts. However, chloroplasts isolated from plants grown on soil do not import precursor proteins efficiently in vitro. Therefore, to obtain import-competent chloroplasts from *Arabidopsis*, plants should be grown on agar plates.

The following method is geared to produce chloroplasts from petri plate–grown plants that are capable of importing precursor proteins. However, if a capacity for importing proteins is not required, the method also works for soil-grown plants, observing the modifications noted in the text.

The aerial portions of the plants are incubated with cell-wall-degrading enzymes to release protoplasts. These are collected, washed, and then ruptured. The chloroplasts that are released are purified on a Percoll gradient. Harvesting of protoplasts and chloroplasts, should be done as quickly as possible to ensure a good yield of high-quality chloroplasts. In *Arabidopsis*, this method has the advantage over the traditional method described in Support Protocol 1, as it produces a higher yield of chloroplasts (up to ten times greater), which import precursor proteins well. This method uses two petri plates containing 4-week-old plants (~10 g total of tissue), and yields 0.5 to 1 mg chlorophyll of intact chloroplasts. This method can be scaled up if more chloroplasts are desired, but caution is advised as yields can suffer because of the longer time needed to harvest protoplasts and chloroplasts from large amounts of tissue.

Materials

Murashige and Skoog growth medium (see recipe)
Seed sterilizing solution (see recipe)
Arabidopsis thaliana seeds, 30 to 40 mg per plate
Sterile, autoclaved water
0.1% (w/v) agarose (autoclaved, sterile)
Digestion buffer (see recipe)
Digestion enzyme solution (see recipe)
40% and 85% (v/v) AT Percoll (see recipe)
Protoplast resuspension buffer (see recipe)
Protoplast breakage buffer (see recipe)
HEPES-sorbitol buffer, pH 8.0 (see recipe)

Plastic petri plates, 20-25 mm × 150–mm diameter, sterile
Sterile laminar-flow hood
1.5-ml microcentrifuge tubes or 15-ml tubes
Platform shaker
Parafilm
Growth room or chamber (16-hr day, 70 to 120 $\mu Em^{-2}sec^{-1}$, 20° to 25°C)
Single-edge razor blades
100-mm petri dishes
500-ml beakers (optional)

Plastic wrap (optional)
60 W light source (optional)
30-ml glass centrifuge tubes (e.g., Corex)
200-µm nylon mesh (e.g., Sefar America), 100- to 120-mm squares, fashioned into a cone and stapled to hold its shape
Small funnel
50-ml centrifuge tubes
Tabletop centrifuge with swinging-bucket rotor, capable of slow acceleration and deceleration
Protoplast rupturing device (see recipe)
Superspeed centrifuge capable of 39,000 × *g*, with a 50-ml tube swing-out rotor
23-cm Pasteur pipet

Prepare plates

1. After autoclaving, allow the Murashige and Skoog growth medium to cool before pouring into the 150-mm diameter plates. Pour 50 ml of growth medium per plate in a sterile laminar-flow hood and allow to set.

   Plates may be used immediately, or stored in a sealed, sterile environment for up to 6 weeks at 4°C.

   Plants from about two plates, yielding 0.5 to 1 mg chlorophyll intact chloroplasts, are used for each chloroplast isolation procedure.

Prepare seeds and grow plants

2. Add 1 or 12 ml of seed sterilizing solution to seeds in a microcentrifuge tube (if sowing two plates) or a sterile 15-ml tube (for sowing 3 to 12 plates), respectively. Rock for 20 min at room temperature on a platform shaker.

3. Wash seeds four times with sterile water.

   Seeds may be centrifuged for 15 sec at 100 × g to facilitate washing. The supernatant can be decanted in a laminar-flow hood to maintain sterility.

4. Resuspend seeds in 0.1% (w/v) sterile agarose and dispense ~1 ml per plate in the laminar-flow hood. Add 2 to 3 ml sterile water to each plate. Tip and shake the plates to evenly distribute the seeds. Allow the plates to dry with lids slightly askew in a laminar-flow hood.

   Excess water can be removed from the plates by pipetting to speed the drying process.

5. When the plates are dry, seal with Parafilm and vernalize the seeds by incubating the plates at 4°C for 24 to 48 hr.

6. Transfer to a growth room (16-hr day, 70 to 120 $\mu Em^{-2}sec^{-1}$, 20° to 25°C).

   After several days, condensation may accumulate on the lids. Plants remain small in these conditions, growing no larger than 15 mm in diameter. Import-competent chloroplasts can be obtained from plants between 2 and 6 weeks of age (assuming obvious senescence or death has not occurred by 6 weeks). Plants that are 4-weeks-old work well as they provide ample tissue without evidence of senescence. Destarching of plate-grown plants, by incubating them in the dark, is not necessary.

Harvest the tissue

7. Harvest the entire aerial portions of the plants with a razor blade, and place immediately in a 100-mm petri dish containing 15 ml digestion buffer on ice. When all the tissue has been harvested, place the petri dish on a firm surface and chop the tissue rapidly for up to 1 min using a fresh razor blade.

The tissue should separate from the agar easily, and be chopped such that it is no longer matted. If soil-grown tissue is used, leaves or the entire green portion of the plants may be harvested. The larger soil-grown leaves can be gently sliced such that each leaf is cut at least once.

8. Drain the tissue and wash with 10 to 20 ml digestion buffer. Allow the dish to sit on ice for several minutes and swirl to release cell contents released during the chopping. Pour off the digestion buffer and repeat the washing process until the discarded buffer is no longer, or only faintly, green.

   The petri dish, with the lid held firmly on, may be held vertically over a beaker and allowed to drain, to facilitate washing. Typically, two to three washes are required.

Digest the tissue

9. Add 10 ml digestion enzyme solution to the drained tissue, swirl to mix, and distribute the tissue uniformly with fingertips.

   The ratio of tissue to enzyme solution is high, but there should be sufficient liquid such that it is visible when the petri dish is tilted. A small amount of additional digestion buffer may be added if necessary, to achieve this.

   The amount of digestion enzymes should be halved for soil-grown plants, such that the final concentrations are 2% cellulase, 0.4% macerozyme.

10. Place the petri dish by a window that receives indirect sunlight at ~50 to 100 $\mu Em^{-2}sec^{-1}$ and a temperature of 21° to 25°C for 4 to 5 hr.

    Alternatively, the digestion may be carried out in a 500-ml beaker, covered with plastic wrap, placed in a water bath at 23°C, and illuminated with a 60 W bulb.

    The digestion time required for soil-grown tissue is ~3 to 3.5 hr.

11. When digestion nears completion, prepare a 40% (10 ml): 85% (7 ml) AT Percoll step gradient in a 30-ml glass centrifuge tube and hold on ice.

    Alternatively, a 50% continuous AT Percoll gradient may be used. Prepare by mixing well 15 ml Percoll and 15 ml gradient buffer in a 50-ml centrifuge tube. Centrifuge for 30 min at 39,000 × g, 4°C, with the brake off. A swinging-bucket rotor is not essential at this step. Hold on ice.

12. When the tissue is sufficiently digested, it becomes a darker green and the bathing medium becomes green when swirled gently. Transfer the petri dish to ice.

    Refrain from swirling the tissue until it is reasonably certain that digestion is complete. Protoplasts may be less stable once released into the medium and yields can suffer. In soil-grown tissue, tiny holes appear in the leaves when digestion nears completion.

Harvest protoplasts

13. Harvest the protoplasts by gently swirling the petri dish for 30 to 60 sec and filtering the medium through a 200-µm mesh cone, fitted inside a small funnel, into a 50-ml centrifuge tube on ice.

    Using a 5-ml Gilson pipetman, with ~15 mm cut from the end of the tip, facilitates harvesting. Avoid splashing and dripping of the protoplast suspension by allowing it to run down the inside of the tube.

14. Transfer any tissue from the nylon mesh back into the petri dish and wash the tissue with 10 to 15 ml of fresh digestion buffer, filtering it into the same 50-ml centrifuge tube. Wash the tissue one or two times more until little green is released from the tissue.

    Usually this coincides with the centrifuge tube becoming full.

15. Centrifuge the protoplasts for 5 min at 100 × *g*, 4°C, in a swinging-bucket rotor using a slow-accelerating tabletop centrifuge.

16. Remove and discard the green supernatant carefully with a pipet. Be careful not to disturb the protoplast pellet as it is very loose. Resuspend the pellet in 5 ml protoplast resuspension buffer by gently swirling the pellet while the buffer is dispensed down the side of the tube.

    A few drops of the protoplast suspension may be removed at this stage for inspection under a light microscope.

17. Centrifuge the protoplasts for 2 min at 100 × *g*, 4°C, in a tabletop centrifuge.

Break the protoplasts

18. Remove the supernatant and resuspend the pellet in 5 ml protoplast breakage buffer using the technique described in step 16.

    Before resuspending the pellet, ensure that the tools needed are at hand and the centrifuge is prepared. Once the protoplasts are broken, the goal is to load the released chloroplasts on the Percoll gradient as quickly as possible to minimize their exposure to proteases or other inhibitory compounds from either the digestion enzymes or from the ruptured protoplasts.

19. Immediately transfer the resuspended protoplast pellet into the barrel of the protoplast rupturing device (see recipe). Holding the end of the device over a 50-ml centrifuge tube on ice, carefully replace the plunger and gently and firmly force the suspension through the layers of mesh. Repeat this procedure.

    A few drops of the suspension may be removed at this stage for inspection under a light microscope to ensure that all of the protoplasts have been ruptured. If this cannot be done quickly, it is prudent to assume that this has occurred so as not to delay loading the gradient.

Isolate chloroplasts

20. Quickly and carefully layer the broken protoplasts onto the AT Percoll step gradient (step 11). Centrifuge in a swing-out rotor for 10 min at 2500 × *g*, 4°C, with the brake off.

    If using a continuous gradient, centrifuge in a swing-out rotor for 10 min at 8000 × g, 4°C, with the brake off.

21. Following centrifugation, visualize two green bands in the gradient: an upper band of broken chloroplasts at the protoplast breakage buffer/40% AT Percoll interface, and a lower band of intact chloroplasts at the 40%/85% AT Percoll interface. Remove the load zone and the upper band of broken chloroplasts by aspiration. Harvest the lower band using a 23-cm Pasteur pipet and transfer to a 50-ml centrifuge tube.

    About 5 ml is typically harvested from the gradient.

22. Dilute with 40 to 45 ml HEPES-sorbitol buffer, pH 8.0, and centrifuge for 5 min at 700 × *g*, 4°C.

    The authors routinely use an AT Percoll step gradient because of the ease of reproducibility of the gradient and the sharp resolution of chloroplasts at the step interfaces. The linear gradient also will yield two major bands: one containing broken chloroplasts near the top of the gradient, and a second band of intact chloroplasts near the bottom of the tube. Occasionally, a third band may be visible between the upper and lower bands towards the top of the Percoll gradient. It is composed of mostly broken chloroplasts and should be ignored.

23. Carefully decant the supernatant and, without righting the tube, blot away all excess buffer. Resuspend the pellet in a small volume (200 to 300 µl) of HEPES-sorbitol buffer, pH 8.0 and quantify the chlorophyll (see Support Protocol 1).

Occasionally, Arabidopsis chloroplasts have a tendency to clump together when being resuspended. To minimize this, swirl the pellet gently to loosen it, and continue to swirl while adding the HEPES-sorbitol buffer, pH 8.0, drop-wise. It may be necessary to gently pipet the suspension up and down to completely resuspend the pellet.

SUPPORT PROTOCOL 3

PRODUCTION OF [^{35}S]METHIONINE-LABELED IMPORT SUBSTRATE BY IN VITRO TRANSLATION

Typically, chloroplast-targeted proteins to be used as import substrates are synthesized as [^{35}S]methionine-labeled proteins using an in vitro translation system. These proteins can be made using the methods outlined in *UNIT 11.2* or using commercially available coupled transcription-translation systems (e.g., Promega). The coupled systems are relatively easy to use and generally produce enough substrate to be used in multiple import reactions. There has been some suggestion that wheat-germ extracts are preferable for producing import substrates to be used in chloroplast import assays, since they are plant-based. However, in practice, import substrates produced using rabbit reticulocyte lysates are efficiently imported into chloroplasts as well. Therefore, the method used to produce the import substrate will depend on which system is best suited for the individual protein being tested.

Materials

Expression vector harboring the gene that encodes protein to be used as import substrate
Plasmid isolation kit (e.g., Qiagen Plasmid Midi kit)
In vitro translation kit (e.g., TNT Coupled Reticulocyte Lysate system, Promega)
Destain solution (see recipe)
Image acquisition software (e.g., Molecular Dynamics)
Additional reagents and equipment for SDS-PAGE gel (*UNIT 6.1*)

In vitro translate [^{35}S]methionine-labeled import susbstrate using commercial reticulocyte lysate coupled transcription-translation system

1. Isolate the expression vector harboring the gene that encodes the protein to be used as an import substrate from an overnight culture of *E. coli*.

   Many commercial plasmid-isolation kits can be used for this purification. It is recommended that the isolated plasmid be extracted with phenol/chloroform prior to being used for in vitro transcription/translation. Novagen's pET series of vectors that use the T7 promoter work well for in vitro transcription/translation of proteins.

2. Follow the manufacturer's instructions for synthesizing [^{35}S]methionine-labeled protein.

   Promega's TNT Coupled Reticulocyte Lysate system produces ample, high-quality translation product that can be used for numerous import assays.

3. Run a small aliquot (0.5- to 1-µl) of the translation product on an SDS-PAGE gel (*UNIT 6.1*). Fix the gel in destain solution for 30 min and dry under a vacuum. Expose to a storage phosphor screen overnight (*UNIT 6.3*).

4. View an image of the gel and quantify the radioactivity for each of the translation products using commercial software.

   Adjust the amount of translation product used in the in vitro import assay to be at least 10,000 cpm (see Basic Protocol).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AT Percoll, 40% (v/v)

40% (v/v) Percoll (Sigma-Aldrich)
50% (v/v) gradient buffer (see recipe)
Store up to 1 month at −20°C and thaw before use

Optional, for isolating Arabidopsis chloroplasts using a Percoll step gradient.

AT Percoll, 85% (v/v)

For 50 ml:
42.5 ml Percoll (Sigma-Aldrich; final 85%)
2.5 ml 1 M HEPES-KOH, pH 7.3 (final 50 mM)
3.01 g sorbitol (final 330 mM)
H_2O to 50 ml
Store up to 1 month at −20°C and thaw before use

Optional, for isolating Arabidopsis chloroplasts using a Percoll step gradient.

Chloroplast import master mix, 50 ml

For 50 ml:
2.5 ml 1 M HEPES-KOH, pH 7.5 (final 50 mM)
3.01 g sorbitol (final 330 mM)
2.5 ml 1 M magnesium acetate (final 50 mM)
2.5 ml 5 M potassium acetate (final 250 mM)
H_2O to 50 ml
Store up to 4 weeks at 4°C

DMSO, 40% (v/v)

For 10 ml:
250 µl 1 M HEPES-KOH, pH 7.5 (final 25 mM)
0.0613 g sorbitol (final 330 mM)
4 ml DMSO (final 40% v/v)
H_2O to 10 ml
Store up to 2 weeks at 4°C

Destain solution

45% (v/v) methanol
10% (v/v) acetic acid
Store indefinitely at rom temperature

Digestion buffer

For 400 ml:
8 ml 1 M MES-KOH, pH 5.2 (final 20 mM)
29.13 g sorbitol (final 400 mM)
200 µl 1 M $CaCl_2$ (final 0.5 mM)
H_2O to 400 ml
Store up to 2 weeks at 4°C

Digestion enzyme solution

Dissolve 0.4 g cellulase "onozuka" R 10 (e.g., Yakult, Kanematsu) and 0.08 g macerozyme R 10 (e.g., Yakult, Kanematsu) in 10 ml digestion buffer (see recipe). Centrifuge for 5 min at 2000 × g to pellet insoluble materials. Use supernatant immediately.

The volume, 10 ml, is enough for two plates of Arabidopsis.

continued

Gradient buffer

For 100 ml:

10 ml 1 M HEPES-KOH, pH 7.3 (final 100 mM)
12.03 g sorbitol (final 660 mM)
100 µl 2 M $MgCl_2$ (final 2 mM)
200 µl 1 M $MnCl_2$ (final 2 mM)
800 µl 0.5 M EDTA (final 4 mM)
0.2 g BSA (final 0.2%)
H_2O to 100 ml
Store up to 2 weeks at 4°C

Grinding buffer

For 2 liters:

50 ml 1 M HEPES-KOH, pH 7.7 (final 25 mM)
120.25 g sorbitol (final 330 mM)
8 ml 0.5 M EDTA (final 2 mM)
1 ml 2 M $MgCl_2$ (final 1 mM)
2 ml 1 M $MnCl_2$ (final 1 mM)
H_2O to 2 liters
Store up to 2 weeks at 4°C

Just prior to use, add BSA to a final concentration of 0.25% (w/v) and sodium ascorbate to a final concentration of 5 mM (0.1% w/v).

HEPES-sorbitol buffer, pH 7.5

For 500 ml:

12.5 ml 1 M HEPES-KOH, pH 7.5 (final 25 mM)
30.06 g sorbitol (final 330 mM)
H_2O to 500 ml
Store up to 2 weeks at 4°C

HEPES-sorbitol buffer, pH 8.0

For 100 ml:

5 ml 1 M HEPES-KOH, pH 8.0 (final 50 mM)
6.01 g sorbitol (final 330 mM)
H_2O to 100 ml
Store up to 2 weeks at 4°C

Murashige and Skoog growth medium

1 liter for 20 plates:

4.4 g Murashige and Skoog salt and vitamin mix with buffer (e.g., Life Technologies)
10 g sucrose
H_2O to 800 ml
Adjust pH to 5.7 with 1 M KOH
H_2O to 1 liter
Add 8 g Phytagar (e.g., Life Technologies)
Autoclave
Allow to cool to touch
Pour into large, sterile petri dishes
Allow plates to set and dry
Store unused plates, sealed in a bag, up to 6 weeks at 4°C

If a Murashige and Skoog salt and vitamin mix without buffer is used, supplement with 50 mM MES-KOH, pH 5.7.

PBF-Percoll

For 170 ml:
5.1 g polyethylene glycol 4000
1.7 g BSA
1.7 g Ficoll
Percoll (Sigma-Aldrich) to 170 ml
Store up to 1 month at −20°C

PBF-Percoll, 40% (v/v)

For 50 ml:
20 ml PBF-Percoll (final 40%)
1.25 ml 1 M HEPES-KOH, pH 7.5 (final 25 mM)
3.01 g sorbitol (final 330 mM)
200 µl 0.5 M EDTA (final 2 mM)
25 µl 2 M $MgCl_2$ (final 1 mM)
0.0077 g glutathione (reduced; final 500 µM)
0.05 g sodium ascorbate (final 0.1%)
H_2O to 50 ml
Store up to 1 month at −20°C, thaw immediately before use

PBF-Percoll, 85% (v/v)

For 50 ml:
42.5 ml PBF-Percoll (final 85%)
1.25 ml 1 M HEPES-KOH, pH 7.5 (final 25 mM)
3.01 g sorbitol (final 330 mM)
200 µl 0.5 M EDTA (final 2 mM)
25 µl 2 M $MgCl_2$ (final 1 mM)
0.0077 g glutathione (reduced; final 500 µM)
0.05 g sodium ascorbate (final 0.1%)
H_2O up to 50 ml
Store up to 1 month at −20°C, thaw immediately before use

Percoll, 40% (v/v)

For 50 ml:
20 ml Percoll (Sigma-Aldrich; final 40%)
2.5 ml 1 M HEPES-KOH, pH 7.5 (final 50 mM)
3.01 g sorbitol (final 330 mM)
H_2O to 50 ml
Store up to 1 month at −20°C, and thaw before using

Protoplast breakage buffer

For 50 ml:
1 ml 1 M tricine-KOH, pH 8.4 (final 20 mM)
2.733 g sorbitol (final 300 mM)
0.5 ml 0.5 M EDTA (final 5 mM)
0.5 ml 0.5 M EGTA (final 5 mM)
0.042 g $NaHCO_3$ (final 10 mM)
H_2O to 50 ml
Store up to 2 weeks at 4°C
Immediately before use add 0.05 g BSA (final 0.1% w/v)

continued

Protoplast resuspension buffer

For 50 ml:
8 ml 1 M MES-KOH, pH 6.0 (final 20 mM)
29.13 g sorbitol (final 400 mM)
200 µl 1 M $CaCl_2$ (final 0.5 mM)
H_2O to 50 ml
Store up to 2 weeks at 4°C

Protoplast rupturing device

10-ml disposable syringe
10-µm nylon mesh, 30- to 35-mm square
20-µm nylon mesh, 30- to 35-mm square
Electrical tape, 15- to 20-mm wide

Cut off the end of the syringe barrel so that it resembles a hollow tube. Put the 10-µm mesh on top of the 20-µm mesh and place both over the cut end of the syringe such that the 10-µm mesh faces the outside and the 20-µm mesh is against the syringe barrel. Fix the mesh in place using the electrical tape to hold the two layers of mesh to the sides of the syringe leaving the mesh exposed at the end of the barrel.

This procedure is tricky. The meshes must be very firmly taped to the barrel or leaks will occur during use. Use a lot of electrical tape, wrapping it multiple times, overlapping the tape as far as half way down the syringe barrel and back up to the top. Constantly, and very firmly pull and stretch the tape to ensure a good seal. Both 20- and 10-µm meshes are used to more gently rupture large protoplasts (with 20-µm mesh), before more rigorously rupturing smaller protoplasts (with 10-µm mesh).

Seed sterilizing solution

1.5% (w/v) sodium hypochlorite (dilute commercially available bleach accordingly)
0.02% (v/v) Triton-X 100
Store at room temperature until used

SDS-PAGE sample buffer

For 1 ml:
350 µl 1 M Tris base (final 350 mM)
250 µl 20% (w/v) SDS (final 5% (w/v)
80 µl 1 M dithiothreitol (DTT; final 80 mM)
150 µl 50% (v/v) glycerol (final 7.5%)
10 µl saturated bromophenol blue
160 µl H_2O
Store at −20°C until used

TE/DTT buffer

For 50 ml:
2.5 ml 1 M tricine-KOH, pH 7.5 (final 50 mM)
200 µl 0.5 M EDTA (final 2 mM)
50 µl 1 M dithiothreitol (DTT; final 1 mM)
47.25 ml H_2O
Store up to 2 weeks at 4°C

Thermolysin, 2 mg/ml

Dissolve 2 mg thermolysin in 1 ml HEPES-sorbitol buffer (see recipe). Divide into 50-µl aliquots and store up to 6 months at −80°C. Do not freeze/thaw more than one time.

Trichloroacetic acid (TCA), 100% and 0.5%

To make 100% (w/v) TCA, add 227 ml of water directly to a bottle containing 500 g of solid TCA. Dissolve by shaking the closed container. Dilute part of the 100% solution to 0.5% (w/v) with an appropriate volume of water. Store indefinitely at room temperature.

COMMENTARY

Background Information

Traditionally, pea chloroplasts have been used as a model system for measuring chloroplast precursor protein import. The pea offers the advantages of the ability to obtain large amounts of material that can be grown quickly and easily, and rapid and simple isolation of intact import-competent chloroplasts. The basic mechanism of protein import into chloroplasts appears to be conserved across diverse plant species. In fact, isolated pea chloroplasts are capable of importing preproteins from species as far removed as the algae (Mishkind et al., 1985; Yu et al., 1988; Su and Boschetti, 1994). This makes the pea an ideal system for investigating protein targeting to chloroplasts, and in most cases, the demonstration of preprotein import and processing in pea chloroplasts is sufficient to establish chloroplast localization. However, the growing importance of *Arabidopsis thaliana* as a model system for the investigation of genetic, molecular, and cellular processes in plants makes it desirable to use a homologous biochemical system to analyze chloroplast targeting. In particular, the identification of gene families encoding components of the import apparatus in the complete *Arabidopsis* genome has focused considerable attention on *Arabidopsis* as a model for investigating the molecular mechanism of the import reaction (Jackson-Constan and Keegstra, 2001). The methods for isolating functional chloroplasts from *Arabidopsis* are less developed than those for pea, but experimental amounts of import-competent chloroplasts can now be isolated from *Arabidopsis* (Fitzpatrick and Keegstra, 2001).

Critical Parameters

The yield and import-competence of chloroplasts is directly related to the age and condition of the plant material. The plant material should be obtained from young seedlings. The protein import capacity of chloroplasts is developmentally regulated (Dahlin and Cline, 1991). The highest import capacities are observed in chloroplasts from young, rapidly growing tissues. Therefore, allowing plants to grow larger to obtain more material or isolating chloroplasts from mature plants will result in low import yields.

Temperature is one of the most important parameters that will determine the quality of chloroplasts isolated by these protocols. As stated above, it is essential that all reagents for the isolation of chloroplasts from both species be kept ice-cold and that all procedures be performed on ice or in a cold room to ensure that the extracts never warm up during isolation. The degradation of components of the import machinery during chloroplast isolation has been documented, and although protease inhibitor mixtures can aid in preventing degradation, they are only moderately effective (Bolter et al., 1998; Chen et al., 2000). The best approach to minimize degradation is to work quickly and in the cold. In addition, it is preferable to work in moderate to low light conditions during the isolation procedure. This will prevent photooxidation-induced damage to the chloroplasts.

Freshly isolated chloroplasts can be stored on ice for up to 30 min before initiating the import assay. However, as stated for the isolation procedure, it is best to have all reagents for the import assay assembled and ready to proceed directly to the import assay following the isolation procedure.

Troubleshooting

The potency of the cell wall–degrading enzymes used to generate the *Arabidopsis* protoplasts can vary. Therefore, if yields of protoplasts or chloroplasts from *Arabidopsis* (see Support Protocol 2) seem low, it may be necessary to adjust the digestion time accordingly. Since the enzyme preparations are rather crude and may contain many other degradative enzymes that could decrease yield, use caution when extending digestion times. In addition, it is important not to agitate the tissue during the digestion but to swirl the tissue thoroughly to release the protoplasts only when digestion is complete.

Import efficiency of precursor proteins (see Basic Protocol) varies widely. Should effi-

ciency be <20% of the added import substrate or if it is difficult to detect the imported product, it may be necessary to increase the amount of translation product or chloroplasts used in the assay.

Some proteins, particularly those targeted to the chloroplast envelope, may be susceptible to proteolysis by proteases that co-purify with chloroplasts. It may be possible to limit this proteolysis by performing the import reaction at 20°C, rather than 26°C (see Basic Protocol).

Anticipated Results

The expected results from an import assay will vary depending on the preprotein being studied. All proteins targeted to the internal compartments of the chloroplast contain a cleavable *N*-terminal transit peptide that is removed in the stroma after translocation across the envelope. Therefore, a protein imported into the stroma will have a lower molecular weight than its precursor form prior to import. While the length of transit peptides varies greatly (Chen and Schnell, 1999; Keegstra and Cline, 1999), in most cases the reduction in molecular weight (3000 to 10,000 Da) should be large enough to be easily detected by SDS-PAGE analysis. In order to confirm that the lower molecular weight protein has been imported, chloroplasts are treated with the protease thermolysin following the import reaction. At least 70% of the lower-molecular-weight protein should be protected from proteolysis to confirm that import to the stroma has taken place. Often, both processed and unprocessed forms of the import substrate will be detected in chloroplasts before treatment with exogenous protease. The unprocessed form of the precursor protein represents protein that is nonspecifically associated with the outer chloroplast envelope or bound to components of the import apparatus at the early, binding stages of import. The unprocessed form should therefore be removed by treatment with thermolysin. Thermolysin treatment can also be used to confirm that outer envelope membrane proteins have been integrated. In this case, the thermolysin will degrade only those portions of the protein exposed to the exterior of the chloroplast, leaving a predictable portion of the protein protected from proteolysis. In addition, resistance to alkaline extraction can be used to confirm the integration of membrane proteins.

Time Considerations

Chloroplast isolation from pea should not take >1 hr from the time of tissue homogenization to isolation of the intact chloroplasts. The entire time for the procedure, including gradient preparation and chlorophyll determination, may take ~2 hr. Isolation of chloroplasts from *Arabidopsis* takes considerably longer, as the tissue is digested with protoplast-forming enzymes for 4 to 5 hr. Isolation of intact chloroplasts from protoplasts takes an additional 45 to 60 min. The in vitro import assay requires 20 to 30 min of incubation time and additional time to re-isolate and process the chloroplasts following import. This time can vary from 2 to 8 hr depending on the method used to treat the chloroplasts and the degree of chloroplast subfractionation. A polyacrylamide gel takes 2 to 3 hr to run, 30 min to fix, and 30 to 60 min to dry. The gel is then exposed to a storage phosphorimaging screen or X-ray film overnight (16 hr) or longer, depending on the strength of signal and efficiency of import.

Literature Cited

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenooxidase in Beta vulgaris. *Plant Physiol*. 24:1-15.

Bauer, J., Hiltbrunner, A., and Kessler, F. 2001. Molecular biology of chloroplast biogenesis: Gene expression, protein import and intraorganellar sorting. *Cell Mol. Life Sci.* 58:420-433.

Bolter, B., May, T., and Soll, J. 1998. A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. *FEBS Lett*. 441:59-62.

Bruce, B.D., Perry, S., Froehlich, J., and Keegstra K. 1994. In vitro import of proteins into chloroplasts. *In* Plant Molecular Biology Manual (B.S. Gelvin and R.A. Schilperoort, eds.) pp. 1-15. Kluwer Academic Publishers Group, Dordrecht, The Netherlands.

Chen, X. and Schnell, D.J. 1999. Protein import into chloroplasts. *Trends Cell Biol*. 9:222-227.

Chen, K., Chen, X., and Schnell, D.J. 2000. Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. *Plant Physiol.* 122:813-822.

Chua, N.H. and Schmidt, G.W.. 1978. Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. U.S.A.* 75:6110-6114.

Cline, K. 1986. Import of proteins into chloroplasts: Membrane integration of a thylakoid precursor protein reconstituted in chloroplast lysates. *J. Biol. Chem.* 261:14804-14810.

Cline, K., Werner-Washburne, M., Andrews, J., and Keegstra, K. 1984. Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. *Plant Physiol* 74:675-678.

Dahlin, C. and Cline, K. 1991. Developmental regulation of the plastid protein import apparatus. *Plant Cell* 3:1131-1140.

Emanuelsson, O., Nielsen, H., and von Heijne, G. 1999. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci.* 8:978-984.

Emmanuelsson, O., Nielson, H., Brunak, S., and von Heijne, G. 2000. Prediciting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol*. 300:1005-1016.

Fitzpatrick, L.M. and Keegstra, K. 2001. A method for isolating a high yield of *Arabidopsis* chloroplasts capable of efficient import of precursor proteins. *Plant J.* 27:59-65.

Froehlich, J.E., Benning, C., and Dormann, P. 2001. The digalactosyldiacylglycerol (DGDG) synthase DGD1 is inserted into the outer envelope membrane of chloroplasts in a manner independent of the general import pathway and does not depend on direct interaction with monogalactosyldiacylglycerol synthase for DGDG biosynthesis. *J. Biol. Chem.* 276:31806-31812.

Jackson-Constan, D. and Keegstra, K. 2001. *Arabidopsis* genes encoding components of the chloroplastic protein import apparatus. *Plant Physiol* 125:1567-1576.

Joyard, J., Grossman, A., Bartlett, S.G., Douce, R., and Chua, N.-H. 1982. Characterization of envelope membrane polypeptides from spinach chloroplasts. *J. Biol. Chem.* 257:1095-1101.

Keegstra, K. and Cline, K. 1999. Protein import and routing systems of chloroplasts. *Plant Cell* 11:557-570.

Keegstra, K. and Yousif, A.E. 1986. Isolation and characterization of chloroplast envelope membranes. *Methods Enzymol.* 118:316-325.

Mishkind, M.L., Wessler, S.R., and Schmidt, G.W. 1985. Functional determinants in transit sequences: Import and partial maturation by vascular plant chloroplasts of the ribulose-1,5-bisphosphate carboxylase small subunit of *Chlamydomonas*. *J. Cell Biol.* 100:226-234.

Muckel, E. and Soll, J. 1996. A protein import receptor of chloroplasts is inserted into the outer envelope membrane by a novel pathway. *J. Biol. Chem.* 271:23846-23852.

Pain, D. and Blobel, G. 1987. Protein import in chloroplasts requires a chloroplast ATPase. *Proc. Natl. Acad. Sci. U.S.A.* 84:3288-3292.

Pilon, M., De Boer, A.D., Knols, S.L., Koppelman, M.H.G.M., Van der Graaf, R.M., de Kruijff, B., and Weisbeek, P.J. 1990. Expression in *Escherichia coli* and purification of a translocation-competent precursor of the chloroplast protein ferredoxin. *J. Biol. Chem.* 265:3358-3361.

Schnell, D.J. and Blobel, G. 1993. Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. *J. Cell Biol.* 120:103-115.

Su, Q. and Boschetti, A. 1994. Substrate- and species-specific processing enzymes for chloroplast precursor proteins. *Biochem. J.* 300:787-792.

Theg, S.M., Bauerle, C., Olsen, L.J., Selman, B.R., and Keegstra, K. 1989. Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. *J. Biol. Chem.* 264:6730-6736.

Tu, S.L. and Li, H.M. 2000. Insertion of OEP14 into the outer envelope membrane is mediated by proteinaceous components of chloroplasts. *Plant Cell* 12:1951-1960.

Yu, L.M., Merchant, S., Theg, S.M., and Selman, B.R. 1988. Isolation of a cDNA clone for the gamma subunit of the chloroplast ATP synthase of *Chlamydomonas reinhardtii*: Import and cleavage of the precursor protein. *Proc. Natl. Acad. Sci. U.S.A.* 85:1369-1373.

Yuan, J., Cline, K., and Theg, S.M. 1991. Cryopreservation of chloroplasts and thylakoids for studies of protein import and integration. *Plant Physiol.* 95:1259-1264.

Contributed by Matthew D. Smith and Danny J. Schnell
University of Massachusetts
Amherst, Massachusetts

Lynda Fitzpatrick and Kenneth Keegstra
Michigan State University
East Lansing, Michigan

In Vitro RNA Splicing in Mammalian Cell Extracts

Almost every eukaryotic pre-mRNA generated by RNA polymerase II transcription requires the removal of introns to create mRNA. The correct splicing of constitutive exons is thus critical for normal protein expression and function. Moreover, the removal of many introns by the spliceosome is controlled in a tissue-specific or developmentally specific manner. The differential skipping or inclusion of exons in the final mRNA is called alternative splicing, and often leads to changes in the final protein product. By this mechanism, a finite number of genes in the genome can code for a more complex proteome. Aberrant splicing can lead to a disease state by altering the protein products of a gene. Thus, understanding the regulation of many genes requires a study of the splicing of their RNA products.

In order to study RNA splicing at a biochemical level, it is necessary to employ an in vitro, or cell-free, system. Cell-free splicing systems require two main components: (1) an extract made from mammalian cell nuclei and (2) the introns and exons of the eukaryotic gene of interest, cloned downstream from a prokaryotic phage promoter, such as T7 or SP6. This minigene construct allows the synthesis of sufficient quantities of pre-mRNA substrates in vitro, which are then incubated in the nuclear extract and analyzed for splicing. Nuclear extracts, first developed for studying transcription in vitro (*UNIT 11.6*), were subsequently modified for splicing (Support Protocol 1). Extracts are commonly generated from the HeLa cell line, but other cell lines and tissues can be used (Support Protocol 2). This is important when studying the regulation of an exon that is alternatively spliced in different cell types. These extracts can be used for functional studies of splicing and for isolation and characterization of splicing factors. This unit describes how to set up an in vitro splicing reaction (see Basic Protocol 1) using a mammalian nuclear extract derived from either cell line or tissue, and how to analyze the splicing reaction products (see Basic Protocol 2).

CREATING A MINIGENE CONSTRUCT FOR TESTING A NOVEL SUBSTRATE

To study the splicing of a gene of interest, its exons and introns must be cloned downstream of a bacteriophage promoter to create a minigene construct. Minigenes are subcloned from genomic DNA. It is difficult to analyze the splicing of introns larger than 1000 nucleotides in vitro. This is because lariat intermediates containing long introns cannot be resolved in a single denaturing acrylamide gel with smaller splicing intermediates and products (see Basic Protocols 1 and 2). Since mammalian introns can be very long (much longer than 1000 nucleotides), it is often not possible to test a full-length intron in an in vitro splicing reaction. This problem is overcome by deleting stretches of intron sequence that are not critical for splicing regulation.

Before testing the splicing of the original genomic clone and deletion mutants in vitro, it is necessary to test them in vivo. This will confirm that any shortened minigene version of the gene shows proper splicing and regulation. To test the splicing substrates in vivo, the equivalent minigene can be constructed in a mammalian expression vector and transfected into the appropriate cell line(s). After transfection and the appropriate incubation period, RNA is harvested from the cells and the percentage of inclusion or skipping of the exon in the mRNA is assayed by primer extension, RT-PCR, and ribonuclease protection assays. Further deletions can be made based on the initial in vivo results. One

Contributed by Nanette Rooke and Jason Underwood
Current Protocols in Cell Biology (2003) 11.17.1-11.17.20

way to guess which sequences are important in splicing regulation is through comparing the same intronic sequences of different species. Phylogenetically conserved intronic sequences are likely to be more important in splicing regulation than those sequences that are divergent. The goal is to create a minigene with short introns while maintaining the regulation of splicing of the exon. Once this has been achieved, the minigene can then be subcloned downstream of a bacteriophage promoter and used in the in vitro system.

NOTE: All reagents for these protocols should be high-quality, molecular-biology grade, and RNase- and DNase-free. All glass and plasticware should be RNase-free. Care should be taken to avoid RNase contamination (also see *APPENDIX 2A* for considerations when working with RNA).

BASIC PROTOCOL 1

IN VITRO SPLICING OF PRE-MESSENGER RNA

Methods for in vitro splicing of pre-mRNA in nuclear extract derived from animal cell lines or tissues have been described for many years (Hernandez and Keller, 1983; Padgett et al., 1983; Hardy et al., 1984; Krainer et al., 1984; Rio, 1988; Kramer and Keller, 1990; Black, 1992; and Eperon and Krainer, 1994). In these protocols, a uniformly radiolabeled pre-mRNA is incubated for several hours in nuclear extract supplemented with salts and other required cofactors. The RNA is then extracted from the nuclear extract, subjected to denaturing polyacrylamide gel electrophoresis, and visualized by autoradiography of the dried gel on film or a phosphor imager screen. The pre-mRNA and the reaction products and intermediates are identified as bands on the autoradiogram (see Basic Protocol 2).

Materials

Buffer DG (see recipe)
10 mM ATP
0.5 M creatine phosphate
55 mM $MgCl_2$
20% (w/v) polyethylene glycol 3350 (PEG; mol.wt. 3350; Sigma)
Nuclear extract (Support Protocols 1 and 2)
20 U/µl RNase inhibitor (RNA guard; Amersham Pharmacia Biotech; Ribonuclease Inhibitor, porcine)
5 to 20 fmol/µl uniformly radiolabeled pre-mRNA splicing substrate (Support Protocol 3)
20 mg/ml proteinase K (Amersham Pharmacia Biotech)
10% (w/v) sodium dodecyl sulfate (SDS; *APPENDIX 2A*)
1.1× PCA buffer (see recipe)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (PCA)
20 mg/ml glycogen (Roche)
100% ethanol, ice-cold
RNA loading buffer (see recipe)

30°C and 80°C water baths or heating blocks
Whatman 3MM filter paper
Kodak XAR X-ray film (standard 14 × 17–in.) with intensifying screen, or phosphor imager screen and imager (e.g., PhosphorImager from Molecular Dynamics or equivalent; see *UNIT 6.3*)

Additional reagents and equipment for denaturing PAGE of nucleic acids (*APPENDIX 3A*) and autoradiography (*UNIT 6.3*)

Prepare splicing reaction

1. Place microcentrifuge tube(s) on ice and add in the order indicated:

≤15 µl buffer DG (for total of 15 µl buffer DG and nuclear extract)

1 µl 10 mM ATP

1 µl 0.5 M creatine phosphate

1 µl 55 mM $MgCl_2$ (2.2 mM final)

2.5 µl 20% PEG 3350

≤15 µl nuclear extract (for total of 15 µl buffer DG and nuclear extract)

H_2O to bring total volume of reaction to 24 µl

0.5 µl 20 U/µl RNase inhibitor.

The sum of the volumes of buffer DG and the nuclear extract should equal 15 µl, or 60% of the total reaction volume. The splicing activity of the nuclear extract will vary with each nuclear extract preparation. Also, different pre-mRNAs will splice with different efficiencies in each nuclear extract preparation; thus the amount of extract used in a splicing reaction should be titrated with respect to each pre-mRNA being tested. In addition, the splicing efficiencies of different pre-mRNAs can be affected by the concentration of $MgCl_2$ (see Critical Parameters). Therefore, the amount of $MgCl_2$ should also be titrated to optimize the splicing reaction. The use of PEG 3350 and RNase inhibitors is not always necessary. For notes on RNase contamination, see Critical Parameters in the Commentary section.

The total reaction volume can be scaled up if needed. Other protocols have been used in which the total reaction volume can vary from 10 to 50 µl.

2. Remove the reaction mixture(s) from the ice, mix by gently flicking the tube, and incubate in a 30°C water bath or heating block for 8 to 10 min.

3. Add 1 µl of a solution containing 5 to 20 fmol radiolabeled pre-mRNA to bring the total reaction volume to 25 µl. Mix the reagents by gently flicking the microcentrifuge tube. Centrifuge briefly to collect any droplets on the side of the tube and continue incubating at 30°C for 2 to 4 hr.

   The duration of the incubation is dependent on the rate of splicing for a particular pre-mRNA substrate, i.e., the percent conversion to intermediates and products as a function of time. The rate and overall splicing efficiency of a particular substrate is determined by doing a time-course experiment. Doing a time course can also help in identifying the splicing reaction products (see Basic Protocol 2). For some pre-mRNAs, splicing is readily detectable within 2 hr or less, but weaker substrates may take up to 4 hr to accumulate sufficient intermediates and products for analysis.

4. Terminate the splicing reaction by adding 3 µl of 20 mg/ml proteinase K and 2 µl of 10% SDS to the reaction. Continue to incubate at 30°C for 20 to 30 min.

Extract and separate RNA products

5. Add 370 µl 1.1× PCA buffer and 200 µl of 25:24:1 PCA. Vortex well. Microcentrifuge 1 min at maximum speed, room temperature.

   Adding 370 ml of 1.1× PCA buffer to the splicing reaction (25 µl) will bring the final concentration of PCA buffer to ~1×.

6. Transfer the upper, aqueous layer to a new microcentrifuge tube.

7. Add 1 µl of 20 mg/ml glycogen (to act as a carrier for the RNA) and 1.0 ml of ice-cold 100% ethanol. Mix well. Incubate on dry ice for 10 min or overnight at −20°C. Microcentrifuge 10 min at maximum speed, 4°C, to collect the pellet of RNA at the bottom. Carefully remove as much supernatant as possible, being careful not to disturb the pellet.

A drawn-out Pasteur pipet can aid in removing the last droplets.

8. Dry the pellet in a Speedvac evaporator for 1 min.

   If a Speedvac evaporator is not available, the pellet may be dried on the benchtop for 10 min.

9. Pour the appropriate percentage denaturing polyacrylamide gel (*APPENDIX 3A*).

   The gel should be poured using a large gel-running apparatus (30 × 40-cm gel or approximate equivalent), and it should contain 8 M urea, 0.5× or 1× TBE, 19:1 acrylamide:bisacrylamide, and 4% to 12% total acrylamide. The lariat intermediates formed during splicing will have anomalous electrophoretic mobility when run in a denaturing gel. This, and the sizes of the pre-mRNA and free exon, should be taken into account when deciding what percentage polyacrylamide gel to use and how far to run it. For pre-mRNAs between 350 nucleotides to 550 nucleotides, 6% to 8% polyacrylamide gels are standard. See Basic Protocol 2 for additional considerations regarding this denaturing PAGE step.

10. Resuspend the pellet in 8 to 10 µl RNA loading buffer by vortexing, heat to 80°C for 5 min, load on the denaturing polyacrylamide gel, and separate the RNA by denaturing PAGE (*APPENDIX 3A*).

11. Dry the gel on Whatman 3MM filter paper on a gel dryer. Visualize the RNA by autoradiography on 14 × 17–in. Kodak film or a large phosphor imager screen.

    Both of these visualization techniques are described in UNIT 6.3

BASIC PROTOCOL 2

ANALYSIS OF SPLICING REACTION RNA PRODUCTS

During the first step of splicing, the RNA is cleaved at the 5′ splice site between the first exon and the intron, and this intron 5′ end forms a 2′-5′ phosphodiester bond at the branch-site adenosine (Fig. 11.17.1). This generates a free 5′ exon and lariat-3′ exon intermediate. During the second step of splicing, the 3′ splice site is cleaved and the two

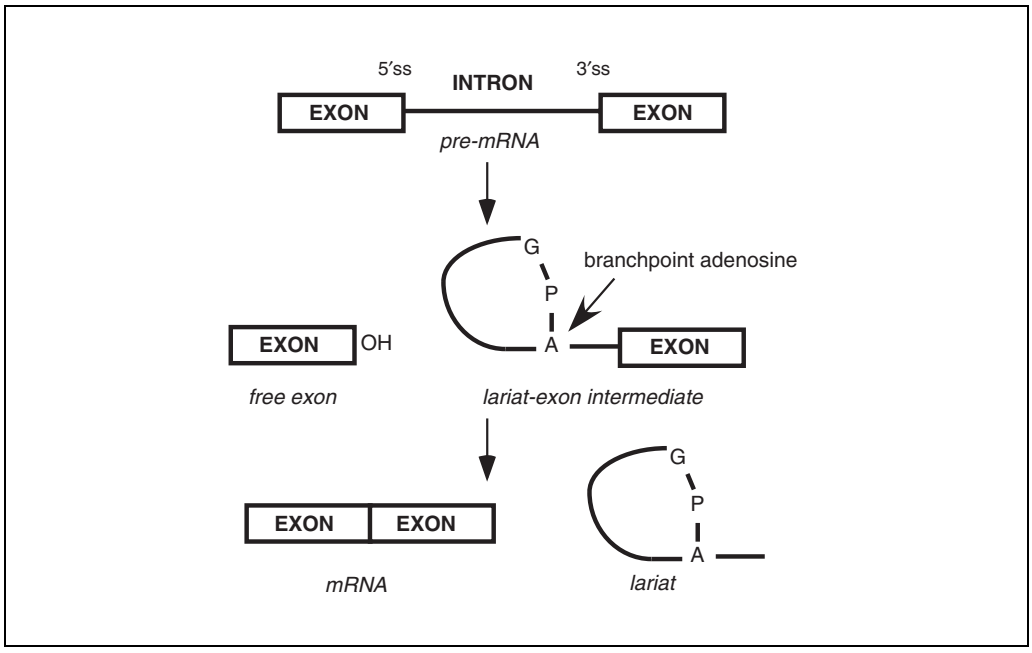


Figure 11.17.1 The splicing pathway of a two-exon, one-intron pre-mRNA. In the first step of splicing, the 2′ hydroxyl of the branchpoint adenosine attacks the 5′ end of the intron, creating a 2′-5′ phosphodiester bond. Two intermediates are generated, a free exon and a lariat-exon intermediate. In the second step, the 3′ hydroxyl of the free exon attacks the phosphate at the 3′ splice site. The lariat is released, and the two exons are ligated together, generating the final mRNA.

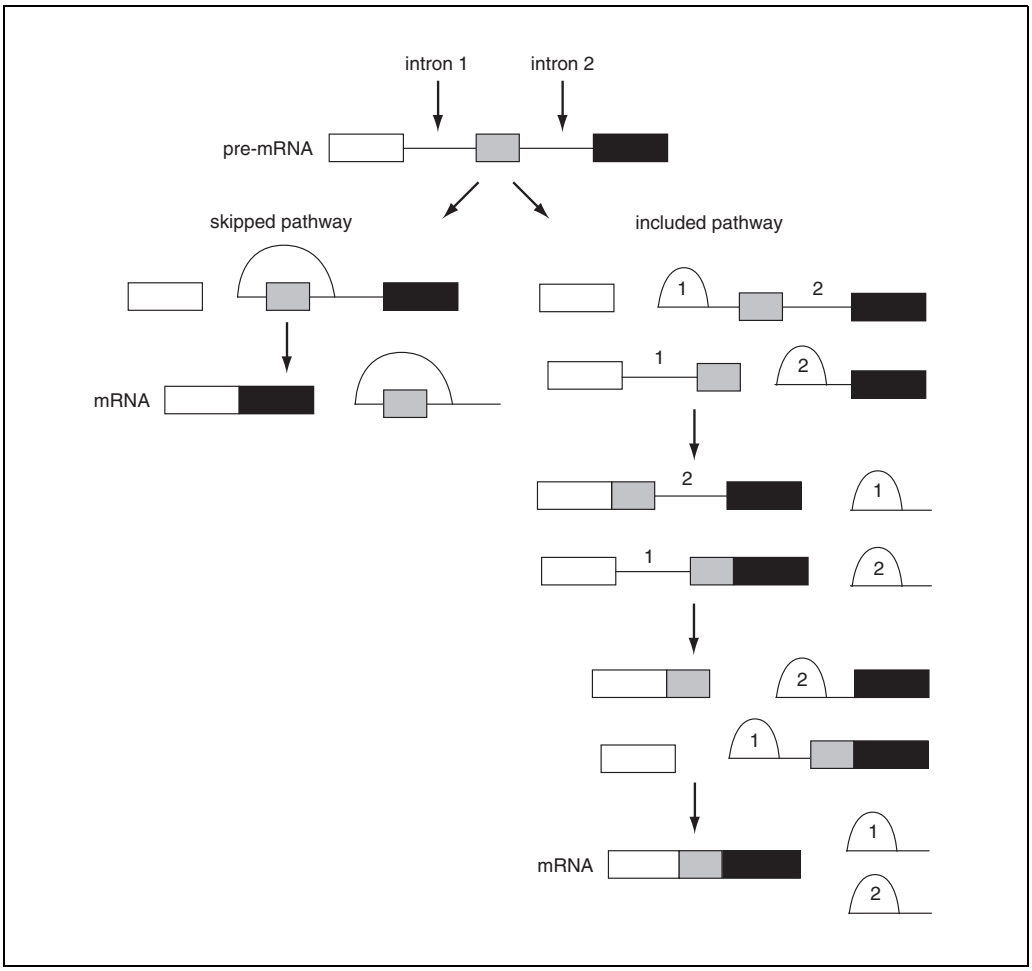


Figure 11.17.2 The splicing pathway of a three-exon, two-intron pre-mRNA. A pre-mRNA with three exons and two introns can proceed through two pathways, where the middle exon is either skipped or is included. In the included pathway, either the upstream or downstream intron can be spliced out first. Illustrated are the intermediates and products in both the skipped and included pathways.

exons are joined together, generating the final linear mRNA and the excised lariat intron. The pre-mRNA, lariat intermediate, free exon intermediate, lariat product, and final mRNA product will have distinct mobilities on a denaturing polyacrylamide gel, creating a pattern of bands. These bands will appear differentially over time. For example, the bands for the lariat intermediate and the 5′ exon intermediate will appear earlier than the final mRNA and lariat intron bands.

The splicing pattern of a two-exon, single-intron pre-mRNA is relatively simple. However, when studying regulated exons it may be useful to utilize a three-exon, two-intron pre-mRNA. The complexity of products resulting from pre-mRNAs that undergo multiple splicing patterns makes the identification of bands and the interpretation of the results more difficult. Figure 11.17.2 illustrates the intermediates and products expected in the splicing pathway of a three-exon, two-intron pre-mRNA. Figure 11.17.3 is a gel image for such a pre-mRNA, which was spliced in vitro in HeLa nuclear extract. The latter figure demonstrates the type of splicing pattern expected with a more complex substrate. Note that, in addition to the more prominent darker bands corresponding to splicing intermediates and products, a number of other bands may appear. These are most often caused by RNA degradation in the extract, as well as by radiolysis, and are often unavoidable.

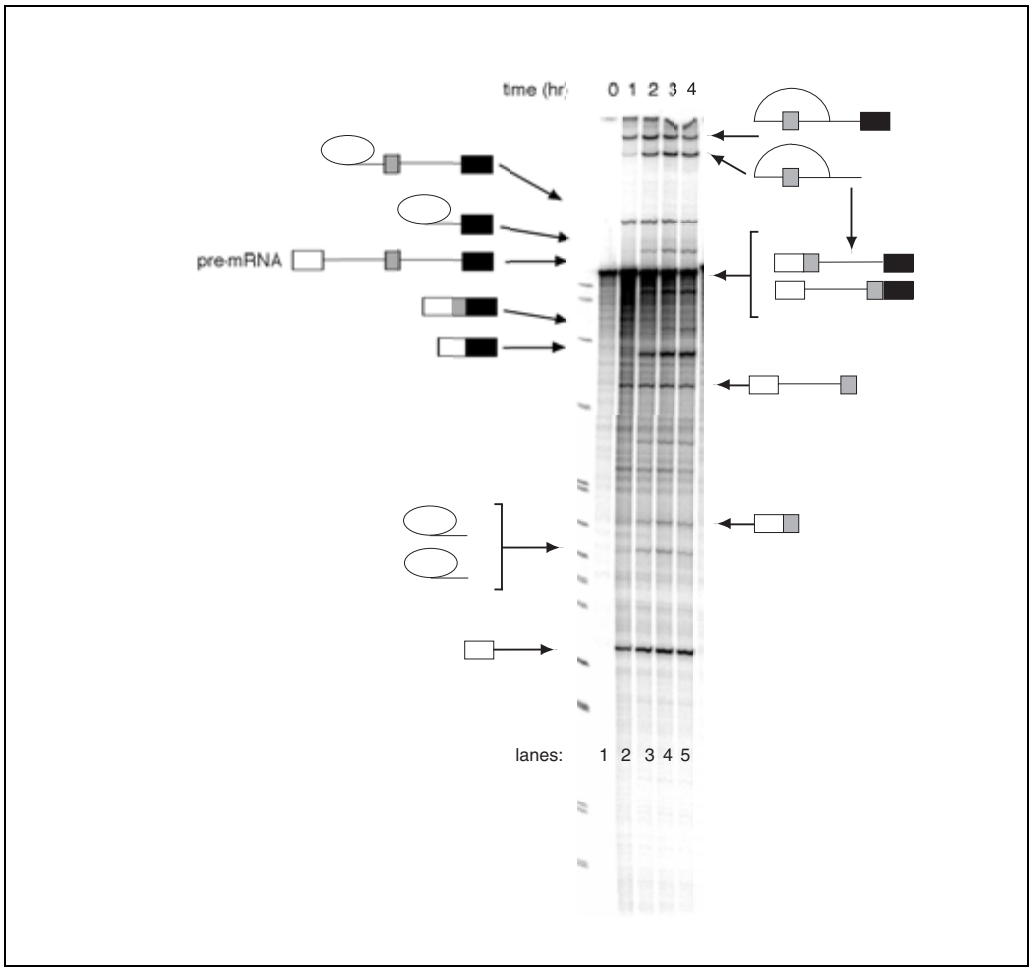


Figure 11.17.3 Autoradiogram of a splicing reaction with a three-exon, two-intron pre-mRNA. Splicing reactions containing the pre-mRNA were incubated in HeLa nuclear extract under the conditions described in Basic Protocol 1. A time course was conducted by terminating the reactions at 1-hr intervals after the initial start of incubation. The pre-mRNA used in this experiment has equal-sized introns, somewhat simplifying the pattern of splicing. It is possible to see the appearance of intermediates during the first hour of splicing, but the final products do not appear until the second hr or later (compare lanes 2 and 3).

Determining if an RNA Contains a Lariat

Determining which bands on the gel correspond to the different RNAs generated during the splicing reaction can be laborious. The linear pre-mRNA product and 5′ exon intermediate can be tentatively identified by their gel mobility. Their time of appearance in a time-course experiment is also diagnostic, in that the 5′ exon should appear earlier. Conclusive demonstration of the mRNA comes from RT/PCR amplification of the suspected band and sequencing of the product. This also confirms the position of the splice sites. Lariat molecules will also show diagnostic appearance times in the time course. The lariat intermediate should appear with the 5′ exon and the lariat product with the mRNA. The anomolous gel mobility of the looped RNA lariat molecules is also used to determine which bands correspond to the lariats. As the percentage of polyacrylamide increases, the mobility of lariat molecules decreases dramatically relative to linear molecules. A splicing reaction can be analyzed in two denaturing gels, one of low-percentage acrylamide (4% to 5%) and one of high-percentage acrylamide (8% to 10%), and the mobility of the different bands compared. RNA lariats will migrate with a marked difference between the two gels relative to linear markers. Often, in a high-percentage gel, the lariats will

shift above the precursor RNA and be the only bands in this region of the gel. This method is further described in Grabowski (1994).

Another approach to identifying a lariat is by debranching using the lariat debranching enzyme. RNA extracted from a band of interest is incubated with cytoplasmic S100 extract (see Support Protocol 1, step 8) or recombinant debranching enzyme (Ruskin and Green, 1990; Ooi et al., 2001). The S100 extract contains the lariat debranching enzyme, which specifically recognizes and cleaves the 2′-5′ phosphodiester bond. Samples of the RNA before and after debranching are separated on a denaturing acrylamide gel and their mobilities compared. The debranched lariat will migrate faster on a high-percentage gel than the intact lariat, at a position diagnostic of the linearized molecule.

Finally, it is possible to identify splicing intermediates and products by comparing related substrates. For example, increasing the length of the 3′ exon in a particular substrate will cause the intermediates containing this exon to migrate more slowly in a denaturing gel. Thus, comparison of the mobilities of the intermediates and products of the initial substrate versus the substrate with a longer 3′ exon can aid in the identification of bands.

Mapping the Branchpoint

Once an RNA is demonstrated to contain a lariat, it is possible to determine the site of the branchpoint. Primer extensions are used to map the site of the branchpoint. During the primer extension reaction, the 2′-5′ phosphodiester bond formed at the branchpoint partially blocks extension by reverse transcriptase at the base prior to the branch. The branchpoint location is determined by finding the primer extension stop. Primer extension on a debranched or linear RNA is used as a control to determine the positions of natural stops in the RNA. These reactions are run next to a sequencing ladder to identify the position of the stops. The most widely used protocol for debranching and primer extension to determine lariat intermediates and branchpoint location is that of Ruskin and Green (1990).

PREPARATION OF NUCLEAR EXTRACT FROM TISSUE CULTURE CELLS

SUPPORT PROTOCOL 1

HeLa cells are the standard source for a nuclear extract that is active in pre-mRNA splicing. This method was originally developed to prepare nuclear extracts competent for RNA polymerase II transcription, and was later adapted to study pre-mRNA splicing (Dignam et al., 1983 and *UNIT 11.6*). Subsequent revisions of this protocol have improved the splicing activity on some pre-mRNA substrates (Krainer et al., 1984; Reichert and Moore, 2000). Nuclear extracts from other tissue-culture cells have also been successfully prepared that reflect alternative splicing phenotypes present in vivo (Noble et al., 1987; Rio, 1988; Black, 1992; Guo and Kawamoto, 2000). Briefly, the cells are swelled in a hypotonic buffer and lysed by Dounce homogenization. After centrifugation of the lysed cells, the pelleted nuclei are recovered and resuspended in a high-salt buffer. During gentle mixing, this buffer extracts RNA processing factors, but does not lyse the nuclei. After extraction, the nuclei are pelleted by centifugation and the supernatant is the nuclear extract. After dialysis into a low-salt buffer, the extracts are ready for RNA processing reactions.

This extract protocol may be scaled down for smaller numbers of cells. Monolayer cell cultures may also be used, but should be grown on a scale that will produce at least 1 ml of packed cell volume and thus make the use of a small Dounce homogenizer feasible. Other extraction protocols have been described for preparing extracts from smaller cell numbers (Lee et al., 1988).

NOTE: This protocol uses HeLa cells, but the general technique can be used for other cell types.

Materials

~5–8 × 10^9 HeLa cells (~12 liters of suspension culture grown in spinner flasks; roller bottles are utilized for some cell types) *or* other mammalian cell of interest, in suspension or monolayer culture
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*) with 1 mM EDTA
Buffer A (see recipe)
Buffer C420 (see recipe)
Buffer DG (see recipe)

Beckman centrifuge with JS-4.2 and JA-20 rotors (or equivalents) and appropriate centrifuge tubes/bottles
50-ml (or other appropriate size) conical tubes, graduated
Rubber policeman or cell scraper
40-ml Dounce homogenizer with tight (type A) and loose (optional) pestle (Wheaton)
Dialysis tubing (10,000 to 14,000 MWCO), prepared as described in *APPENDIX 3C*
Conductivity meter (optional)
Liquid nitrogen

Additional reagents and equipment for trypan blue staining (*UNIT 1.1*), dialysis (*APPENDIX 3C*), and Bradford protein assays (*APPENDIX 3H*)

NOTE: Perform all steps of this procedure at 0° to 4°C. All buffers, vessels, and centrifuge rotors should be precooled to this temperature.

Isolate cell nuclei

For suspension cultures

1a. Grow cells to log phase. Transfer a quantity of suspension containing a total of ~5–8 × 10^9 log-phase cells to 1-liter centrifuge bottles and centrifuge for 20 min at 1800 × *g* in a Beckman JS-4.2 rotor, 4°C. Decant and discard the supernatants.

Overgrowth of cells can result in low splicing activity in the final extract.

2a. Resuspend the cells in 25 ml of PBS for each liter of suspension culture volume and transfer to graduated 50-ml graduated conical tubes. Consolidate the cells into one tube by recentrifuging, resuspending in a smaller volume of PBS, and transferring all pellets to on 50-ml tube.

For monolayer cultures

1b. Grow cells on ten 100- to 150-mm dishes to ~80% to 90% confluency. Remove the medium from the cells and wash the monolayer once with PBS. After washing, add fresh PBS containing 1 mM EDTA to cover the cells, then scrape the cells from the dish with a rubber policeman or cell scraper. Pool cells into conical tubes of the appropriate size for the volume of cells.

Overgrowth of cells can result in low splicing activity in the final extract.

2b. Centrifuge cells 10 min at 1800 × *g* in a JS-4.2 rotor, 4°C. Decant the supernatant and discard. If necessary, resuspend cells in a small volume of PBS, combine, and centrifuge again at 1800 × *g* to consolidate cells into a single conical tube.

3. Using the graduations on the conical tube, estimate the packed cell volume (pcv).

This starting pcv is utilized to estimate the volume of each buffer that is used in the preparation of the extract.

4. Gently resuspend the cells in a volume of buffer A that is ~5 times the pcv. Centrifuge cells 5 min at 1800 × *g* in a JS-4.2 rotor, 4°C,and discard the supernatant.

   This step washes residual PBS from the cells so that efficient osmostic swelling can occur in the subsequent step. Some swelling may occur during this first buffer A wash, so perform this step quickly.

5. Resuspend the cell pellet in a volume of buffer A that is ~3 times the starting pcv (from step 3). Incubate on ice for 10 min.

6. Transfer the cells to a precooled Dounce homogenizer. Homogenize using 5 to 15 slow strokes with a tight pestle.

   Homogenize slowly and use special care during the down stroke to avoid loss of the sample. Lysis can be monitored by staining a small aliquot of the cells with trypan blue (UNIT 1.1). Lysis should be ~90% before proceeding. Different cell types may require different amounts of homogenization to achieve cell lysis.

7. Transfer the lysed cells to appropriate centrifuge tubes and pellet the nuclei by centrifuging 15 min at 3300 × *g* in a JA-20 rotor, 4°C.

8. Discard supernatant or save it to prepare a cytoplasmic S100 extract. Estimate the packed nuclear volume (pnv) using the tube graduations.

   A cytoplasmic extract can be prepared by slow addition of 0.11 volumes of 10× cytoplasmic extract buffer (see recipe) to the above supernatant. After centrifugation for 1 hr at 100,000 × g, the supernatant is the S100 extract. This should be dialysed and subsequently cleared of precipitates in the same manner as a nuclear extract (see steps 11 to 15, below).

Preparation of nuclear extract

9. Resuspend the nuclear pellet in 1 pnv of buffer C420. If clumps of nuclei are difficult to resuspend, homogenize the sample in a Dounce with a loose pestle. Transfer to a small precooled glass beaker containing a small magnetic stir bar.

   The salt concentration in buffer C is one parameter that can be optimized during extract preparation. Active splicing extracts from HeLa cell nuclei are usually obtained by extraction in 0.4 to 0.6 M KCl. Lysis of the nuclei can sometimes be reduced by resuspending the cells in 0.5 pcv of buffer C100 and then adding 0.5 pcv of buffer C (high salt) dropwise to yield the appropriate final concentration.

10. Extract the nuclei by stirring very slowly for 30 min.

11. Transfer the extract to a centrifuge tube and centrifuge 30 min at 25,000 × *g* in a JA-20 rotor, 4°C.

    The supernatant after this spin is the nuclear extract.

12. If conductivity will be used to monitor the dialysis, save a small sample before transferring the extract into dialysis tubing.

13. Dialyze the nuclear extract against 2 liters of buffer DG for 2.5 hr using 10,000 to 14,000 MWCO dialysis tubing (*APPENDIX 3C*). Change the dialysis buffer to new buffer DG and dialyze for another 2 hr. If desired, check the conductivity to monitor the dialysis.

    Conductivity is checked by diluting 10 µl of the extract into 1 ml of distilled water. An equivalent dilution of the dialysis buffer (buffer DG) is used for comparison. Diluted buffer DG should read ~16 to 18 µS/cm. Dialysis is complete when the conductivity of the extract matches that of buffer DG. These dialysis times and volumes are based upon a large extract preparation. Smaller volumes can be dialyzed in less time. Overnight dialysis can result in a decrease in RNA splicing activity.

14. Remove the extract from the dialysis tubing and transfer to a centrifuge tube. Centrifuge 20 min at 12,000 × *g* in a JA-20 rotor, 4°C. Keep the supernatant, which is the completed nuclear extract, and discard the pellet.

    This step removes protein and nucleic acid precipitates that form during dialysis.The precipitates are normal and result from the drop in salt concentration.

15. Remove a small aliquot of the supernatant and use it to determine the protein concentration by the Bradford method (*APPENDIX 3H*). Divide the remaining nuclear extract into aliquots in separate tubes and quick-freeze in liquid nitrogen. Store the extracts at −80°C.

    Aliquots of 200 or 500 μl are convenient for in vitro splicing experiments.

    Nuclear extracts vary widely in final protein concentration (2 to 20 mg/ml), and the concentration does not always correlate with the splicing activity. All extracts should be tested for splicing on constitutive splicing substrates as a positive control, as well as any regulated splicing substrate that will be studied. The most common constitutive substrates are derived from the adenovirus major late first intron (Grabowski et al. 1984) and the β-globin first intron (Krainer et al. 1984).

SUPPORT PROTOCOL 2

PREPARATION OF NUCLEAR EXTRACT FROM PRIMARY TISSUE

In this protocol, the nuclear extract is prepared from a tissue that is isolated from an animal immediately after euthanasia. Like the extract protocol for cultured cells (Support Protocol 1; Dignam et al., 1983), this protocol was initially used to isolate extracts that display tissue-specific RNA polymerase II promoter activity (Sierra at al., 1993). The Grabowski laboratory adapted this protocol to study neuron-specific splicing and demonstrated that splicing-competent nuclear extracts could be generated from whole rat brain (Ashiya and Grabowski, 1997). They have further developed this procedure to produce rat cerebellum extracts that have higher activity for certain neuron-specific exons (Zhang et al., 1999). The protocol that follows is for rat brain and will require optimization for other tissues. Specifically, the salt concentration used for nuclear extraction is a critical parameter and may be changed to maximize the extraction of the nuclei and to minimize nuclear lysis. Note that some tissues (e.g., pancreas) have very high RNase content and may not generate usable extracts.

Briefly, the protocol involves homogenization of primary tissue to break open the cells. The nuclei are subsequently fractionated away from cellular debris by centrifugation through a sucrose cushion. The nuclei are then extracted and the resulting nuclear extract is processed in a manner similar to the standard Dignam extract (Support Protocol 1).

Materials

Sucrose cushion buffer (see recipe)
Dissected whole brains from six 28-day Sprague-Dawley rats
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Homogenization buffer (see recipe)
Buffer C230 (see recipe)
Buffer DG (see recipe)

Dissection tools
Small Petri dish or tissue-culture plate
Beckman SW 28 rotor (or equivalent) and polyallomer ultracentrifuge tubes
Motor-driven Teflon-glass homogenizer (e.g., Wheaton Overhead Stirrer; Wheaton Science Products)
Small dialysis cassette (0.1 to 0.5 ml Pierce "Slide-a-lyzer" or equivalent), 10,000 MWCO (also see *APPENDIX 3C*)

Additional reagents and equipment for dialysis (*APPENDIX 3C*), trypan blue staining (*UNIT 1.1*), and Bradford protein assays (*APPENDIX 3H*)

NOTE: Chill homogenization buffer and glass-Teflon homogenization apparatus overnight at −20°C before the procedure and perform all steps at 0° to 4°C. All other buffers, vessels and centrifuge rotors should be precooled. The volumes in this procedure assume a starting material of six whole brains from 28-day rats, ~15 grams of tissue. For other tissues, this protocol should be scaled according to the mass of starting material.

Isolate nuclei

1. Transfer 10 ml of sucrose cushion buffer into each of two ultracentrifuge tubes. Chill at −20°C.
2. Dissect the tissue and transfer the material of interest to a Petri dish on ice. Rinse tissue in cold PBS. Remove as much blood as possible by gently dabbing with a Kimwipe. Mince the washed tissue with a razor blade or sterile scissors.
3. Add 5 ml of homogenization buffer (containing DTT and protease inhibitors added immediately before use) to the homogenizer. Transfer the minced tissue to the homogenizer vessel and add another 25 ml of homogenization buffer with DTT and protease inhibitors.

   As a rule of thumb, the tissue should not constitute more than 10% to 15% of the total volume at the homogenization stage. However, the final homogenate must be less dense than the sucrose cushion on which it is layered.

4. Turn the homogenizer motor on at ~200 rpm. Begin homogenization by slowly raising the glass homogenizer over the rotating Teflon "pestle" to bring the pestle into contact with the homogenate. Set the motor to ~400 to 500 rpm. Raise the tube until the pestle reaches the bottom of the glass vessel. Slowly move it back out of the homogenate. Repeat this process one or two times until nuclei and not intact cells are observed when monitoring the homogenization by light microscopy and trypan blue staining (*UNIT 1.1*).

   It requires significant pressure to raise and lower the tube over the pestle. Glass homogenizers can break if the pestle does not seat properly, so safety glasses should be worn during this step. Plastic-coated homogenizer tubes reduce the shattering danger and are recommended. Keep the homogenizer dipped in an ice water bath to maintain maximum cooling during the procedure. Upon monitoring of the homogenate by light microscopy, the majority of the cells should be broken, but the nuclei should be intact.

5. Dilute the homogenate to 50 ml with homogenization buffer. Carefully layer 25 ml of the homogenate onto each 10-ml sucrose cushion.
6. Centrifuge 1 hr at 75,000 × *g* in SW 28 rotor, 0° to 4°C.
7. Remove any floating material and carefully aspirate and drain the solution without disturbing the nuclear pellet. Cut off the tube above the pellet with a clean razor blade or scissors.
8. Fill a 10-ml syringe with cold Milli-Q water and attach an 18-G needle. Holding the centrifuge tube upside down, rinse the tube walls with the syringe. Rinse the pellet twice with water and then twice with buffer C230 using a pipet. Dry the tube with a Kimwipe without touching the pellet.

Prepare nuclear extract

9. Resuspend the two pellets in a total volume of 600 µl of Buffer C230 using a micropipettor with a 1-ml tip that has had the end cut off to generate a 3-mm aperture. Transfer to a microcentrifuge tube and incubate on ice for 30 min.

   Brain nuclei are very sensitive to lysis and thus require the lower-salt (C230) buffer rather than the C420 buffer used for the tissue culture cells (Support Protocol 1). The salt concentration in buffer C is one parameter that can be optimized during extract preparation. Lysis of the nuclei can sometimes be reduced by resuspending the cells in 0.5 pcv of buffer C100 and then adding 0.5 pcv of buffer C (higher-salt) dropwise to yield the appropriate final concentration. Nuclei from different tissue types may require altered conditions. The buffer C100 suspension can be divided into several aliquots and various buffer Cs, differing in salt concentration, can be added to the aliquots in parallel to optimize the procedure.

10. Microcentrifuge 10 min at maximum speed, 0° to 4°C. Keep the resulting supernatant, which is the nuclear extract.

11. Load the supernatant into a small dialysis cassette ("Slide-a-lyzer") with a 1-ml syringe and 18-G needle (*APPENDIX 3C*).

12. Dialyze for 1 hr against 1 liter of buffer DG. Change to fresh buffer DG and dialyze for an additional 1 hr.

    The small volume of the extract and the high relative surface area of the Slide-a-lyzer assures that the dialysis is rapid. Monitoring the dialysis by conductivity is recommended for larger preparations (see Support Protocol 1).

13. Remove the extract from the dialysis cassette with a 1-ml syringe (18-G needle). Transfer to a microcentrifuge tube and microcentrifuge 15 min at 16,000 × *g*, to clear any precipitates. Retain the supernatant.

14. Determine the protein concentration by the Bradford assay (*APPENDIX 3H*). Divide the the extract into aliquots in microcentrifuge tubes and quick-freeze in liquid nitrogen. Store the nuclear extracts at −80°C.

    See the comments in Support Protocol 1 on testing nuclear extracts. Some tissue-derived nuclear extracts benefit from concentrating the extract (e.g., using Amicon centrifugal concentrators). This is a possible option if initial splicing assays are unsuccessful with a particular batch of extract.

SUPPORT PROTOCOL 3

IN VITRO TRANSCRIPTION OF CAPPED, RADIOLABELED RNA

The substrate for in vitro splicing reactions is usually produced by in vitro transcription of a DNA template by a bacteriophage RNA polymerase (e.g., T3, T7, SP6) in the presence of an [α-^{32}P]NTP. The resulting radiolabeled RNAs are of high specific activity and possess a 5′ mRNA cap structure (diguanosine 5′-5′ triphosphate). The cap structure stabilizes the RNA against exonuclease activity and may bind proteins that are important for RNA processing efficiency. The relative importance of the cap-binding complex to RNA splicing varies by substrate, but the cap is usually necessary to reduce nuclease digestion and thus produce cleaner results.

The DNA template can be derived by two methods. Commonly, the pre-mRNA sequence is cloned into a plasmid downstream of a bacteriophage promoter, and the resulting plasmid can be linearized after the pre-mRNA sequence with a restriction endonuclease to generate the template. Alternatively, the corresponding pre-mRNA sequence can be amplified by PCR with a special "tailed" sense primer. The primer (5′ to 3′) consists of the top strand sequence of a bacteriophage promoter followed by at least one guanosine

followed by the region that complements the pre-mRNA 5′ end. The antisense primer is designed at the 3′ end of the pre-mRNA sequence to specify the point where the polymerase "runs off." The resulting PCR product can be purified by agarose gel electrophoresis, cleaned up by standard methods (Beulsey et al., 1991; e.g., "freeze and squeeze" or kit), and used as transcription template. Any DNA used for transcription should be free of nuclease activity and thoroughly desalted.

NOTE: All water and microcentrifuge tubes used in this protocol should be RNase-free (see *APPENDIX 2A*).

CAUTION: All proper guidelines for radioisotopes should be followed. Use care when handling transcription reactions to avoid radioactive contamination. Monitor all work spaces, pipets, and the microcentrifuge for counts. Dispose of waste as specified by the local radiation safety officer (also see *APPENDIX 1D*).

Materials

RNase-free water (*APPENDIX 2A*)
10× transcription buffer (provided with polymerase, or see recipe)
0.1 M DTT
NTP mix (see recipe)
Cap analog solution (see recipe)
12.5 µM [α-^{32}P]UTP (800 Ci/mmol)
0.1 µg/µl DNA template (linearized plasmid or PCR product) in 10 mM Tris·Cl, pH 8.1 (see *APPENDIX 2A* for Tris buffer)
10 U/µl bacteriophage RNA polymerase (T3, T7, or SP6)
RNase-free DNase I (if PCR product is used as template)
Formamide loading buffer (see recipe)
RNA elution buffer (see recipe)
70% and 100% ethanol, –20°C
TE-acetate buffer (see recipe)

85°C heating block
Scintillation counter and vials appropriate for Cerenkov counting

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*APPENDIX 3A*) and autoradiography (*UNIT 6.3*)

1. Bring all solutions to room temperature. Assemble the 25-µl reaction in the following order:

   6.5 µl RNase-free water
   2.5 µl 10× transcription buffer
   2 µl 0.1 M DTT
   2 µl NTP mix
   3 µl cap analog solution
   5 µl 12.5 µM [α-^{32}P]UTP (800 Ci/mmol)
   2 µl DNA template
   2 µl 10 U/µl bacteriophage RNA polymerase.

   The final concentrations of the components are: 40 mM Tris·Cl, pH 7.9; 6 mM $MgCl_2$; 2 mM spermidine; 8 mM DTT; 1.2 mM cap analog; 0.2 mM each of GTP, ATP, and CTP; 0.01 mM cold UTP; 2.5 µM [α-^{32}P]UTP; ~20 µM DNA template; 0.8 U/µl bacteriophage RNA polymerase.

   The order of addition and the assembly at room temperature is critical. This order prevents precipitation of the DNA from contact with high concentrations of spermidine.

2. Incubate at 37°C for 1 to 2 hr. While the mix is incubating, prepare a 0.8-mm-thick analytical-size 4% 1× TBE urea-acylamide gel with wells capable of holding 50 µl. Pre-run the gel.

   APPENDIX 3A provides references for preparing and running denaturing PAGE gels.

3. If a plasmid serves as the template, proceed to step 4. If a PCR product is being used, add 2.5 U of RNase-free DNase I and incubate at 37°C for 10 min.

   PCR templates and the transcript RNA are so similar in size that they run in nearly the same position on a denaturing gel. Degrading the DNA after transcription resolves this problem.

4. Stop the reactions by addition of 25 µl of formamide loading buffer.

5. Incubate the stopped reactions at 85°C for 5 min.

6. Load 50 µl per well of each reaction directly onto the pre-run 4% 1× TBE urea-acylamide gel. Electrophorese at 25 V/cm for 10 min and then 40 V/cm until the bromphenol blue reaches the bottom of the gel.

7. Disassemble the gel so as to leave the radioactive gel slab on one of the glass plates. Cover in plastic wrap.

   CAUTION: *The bottom buffer chamber will be radioactive due to the unincorporated α-^{32}P-UTP. Dispose of the radioactive buffer appropriately (also see APPENDIX 1D).*

8. Visualize the transcript by brief autoradiography (*UNIT 6.3*).

   Exposure of the gel for 15 sec is usually adequate.

9. Align the film with the gel to locate the position of the radiolabeled RNA. Cut out the band using a razor blade and transfer to a microcentrifuge tube.

   Shredding the gel does not usually improve the RNA yield significantly and makes a clean transfer (step 11) more difficult.

10. Incubate overnight in 400 µl of RNA elution buffer.

    For large RNAs (> 600 nt), incubation at 37°C can generate more complete elution. For small RNAs (<300 nt), SDS can be omitted from the elution buffer and thus the second precipitation is not necessary.

11. Transfer the elution buffer to a new microcentrifuge tube.

    Elution efficiency can be monitored with a Geiger counter by comparing the elution buffer and gel slice counts.

12. Add 1 ml of 100% ethanol. Mix well and incubate on dry ice for 10 min.

13. Microcentrifuge 10 min at maximum speed, 0° to 4°C. Carefully dispose of supernatant as radioactive waste.

14. Rinse the pellet with 1 ml of cold 70% ethanol.

15. Resuspend pellet in 400 µl TE/acetate buffer and repeat the ethanol precipitation (steps 12 to 14).

    This step removes any residual SDS contamination from the first precipitation. Gel filtration over a disposable G-50 spin column (Amersham Pharmacia Biotech) can also be utilized.

16. Resuspend the pellet in 50 µl of RNase-free water and assay 1 µl by Cerenkov counting.

    Radiolysis occurs relatively rapidly with transcripts of high specific activity. The RNA should be used within 48 hr of completing this procedure, to assure the highest quality RNA.

REAGENTS AND SOLUTIONS

*Use RNase-free (**APPENDIX 2A**) high-purity deionized (Milli-Q or Nanopure) water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Buffer A

10 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)
1.5 mM $MgCl_2$
10 mM KCl
Store buffer with above components up to 3 months at 4°C
Add the following immediately before use, with rapid mixing:
0.2 mM PMSF (from 100 mM stock in ethanol)
1 mM DTT
1 µg/ml pepstatin A (from 1 mg/ml stock in ethanol)
1 µg/ml leupeptin (from 10 mg/ml stock in H_2O)
1 µg/ml aprotinin (from 10 mg/ml stock in 10 mM HEPES, pH 8.0)
Prepare fresh buffer with DTT and protease inhibitors for each extract preparation

Buffer C20

20 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)
25% (v/v) glycerol
1.5 mM $MgCl_2$
0.2 mM EDTA
20 mM KCl
Store buffer with above components up to 3 months at 4°C
Add the following immediately before use, with rapid mixing:
0.2 mM PMSF (from 100 mM stock in ethanol)
1 mM DTT
1 µg/ml pepstatin A (from 1 mg/ml stock in ethanol)
1 µg/ml leupeptin (from 10 mg/ml stock in H_2O)
1 µg/ml aprotinin (from 10 mg/ml stock in 10 mM HEPES, pH 8.0)
Prepare fresh buffer with DTT and protease inhibitors for each extract preparation

Buffers C230 and C420

Prepare as for Buffer C20 (see recipe) but use 230 mM and 420 mM KCl, respectively.

Buffer DG

20 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)
20% (v/v) glycerol
80 mM monopotassium glutamate
0.2 mM EDTA
Store buffer with above components up to 3 months at 4°C
Add the following immediately before use, with rapid mixing:
0.2 mM PMSF (from 100 mM stock in ethanol)
1 mM DTT
1 µg/ml pepstatin A (from 1 mg/ml stock in ethanol)
1 µg/ml leupeptin (from 10 mg/ml stock in H_2O)
1 µg/ml aprotinin (from 10 mg/ml stock in 10 mM HEPES, pH 8.0)
Prepare fresh buffer with DTT and protease inhibitors for each extract preparation.

Many in vitro splicing protocols use buffer D, which contains 100 mM KCl instead of potassium glutamate. These can sometimes work as well as the buffer DG used here. However, the use of glutamate as a potassium counterion instead of chloride improves in vitro splicing efficiency of some substrates and lowers RNA degradation (Black, 1992; Reichert and Moore, 2000; also see Commentary).

Cap analog solution

Dissolve 25 A_{260} units of dry diguanosine 5′-5′ triphosphate dinucleotide (New England Biolabs or Amersham Pharmacia-Biotech) in 130 µl of water to obtain a final concentration of 10 mM diguanosine 5′-5′ triphosphate. Store up to one year at −20°C.

Cytoplasmic extract buffer, 10×

0.3 M HEPES, pH adjusted to 7.9 with KOH (at 25°C)
1.4 M KCl
30 mM $MgCl_2$
Prepare fresh for each extract preparation

Formamide loading buffer

95% (v/v) formamide
15 mM EDTA pH 8.0 (*APPENDIX 2A*)
0.05% (w/v) bromphenol blue
Store in aliquots for up to 3 months at −20°C

Homogenization buffer

10 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)
15 mM KCl
1 mM EDTA
2.2 M sucrose
5% (v/v) glycerol
0.15 mM spermine
0.5 mM spermidine
Store buffer with above components up to 3 months at 4°C
Add the following immediately before use, with rapid mixing:
0.2 mM PMSF
1 mM DTT

Prepare fresh buffer with DTT and protease inhibitor for each extract preparation

NTP mix

2.5 mM GTP
2.5 mM ATP
2.5 mM CTP
0.125 mM UTP
Store up to one year at −20°C

Ribonucleotide triphosphates can be purchased as 100 mM stock solutions at neutral pH. Alternatively, the dry NTP salts can be dissolved in water and titrated to pH 8.0 with Tris base.

PCA Buffer, 1.1×

0.33 M sodium acetate
0.05 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
0.005 M EDTA, pH 8.0 (*APPENDIX 2A*)
0.2% (w/v) SDS
Store up to 1 year at room temperature

RNA elution buffer

20 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
2 mM EDTA
0.5 M ammonium acetate
0.4% (w/v) SDS
Store for up to 1 year at room temperature

RNA gel loading buffer

0.1× TBE buffer (*APPENDIX 2A*)
8 M urea
0.025% (w/v) xylene cyanol
0.025% (w/v) bromphenol blue
Store up to one year at room temperature

Sucrose cushion buffer

10 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)
15 mM KCl
1 mM EDTA
2.0 M sucrose
10% (v/v) glycerol
0.15 mM spermine
0.5 mM spermidine
Store buffer with above components up to 3 months at 4°C
Add the following immediately before use, with rapid mixing:
0.2 mM PMSF
1 mM DTT
1 µg/ml pepstatin A
1 µg/ml leupeptin
1 µg/ml aprotinin
Prepare fresh solution with DTT and protease inhibitors for each extract preparation and filter sterilize

Transcription buffer, 10×

400 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
60 mM $MgCl_2$
20 mM spermidine
Store for up to 1 year at–20°C

TE-acetate buffer

10 mM Tris·Cl pH 8.0 (*APPENDIX 2A*)
1 mM EDTA
0.5 M ammonium acetate
Store for up to 1 year at room temperature

COMMENTARY

Background information

Nuclear extracts from cultured mammalian cells were developed for analysis of transcription and were later adapted to the study of pre-mRNA splicing (Dignam et al., 1983; Krainer et al., 1984). Nuclear extracts that splice pre-mRNAs into mRNAs in vitro have become an indispensable tool for the analysis of splice site choice and splicing factors. In particular, the preparation of nuclear extract from various cell lines or tissues has enabled the study of regulated exons. Nuclear extracts produced by these methods are also used to study other RNA processing events such as 3′ cleavage and polyadenylation (Moore and Sharp, 1985; Takagaki et al., 1988; Christofori and Keller, 1988). Recently, nuclear extracts were shown to possess factors involved in mRNA export (Le Hir et al., 2000 and Zhou, et al. 2001). Laboratories are also now using nuclear extracts as a tool to investigate the coupling of the processes of transcription, splicing, 3′ end processing, and export.

Critical Parameters

The original splicing extract procedure and many subsequent adaptations utilized a final dialysis step into a buffer containing 100 mM KCl. Most laboratories still utilize these conditions. Several laboratories have reported an increase in splicing efficiency for some substrates by replacing potassium chloride (KCl)

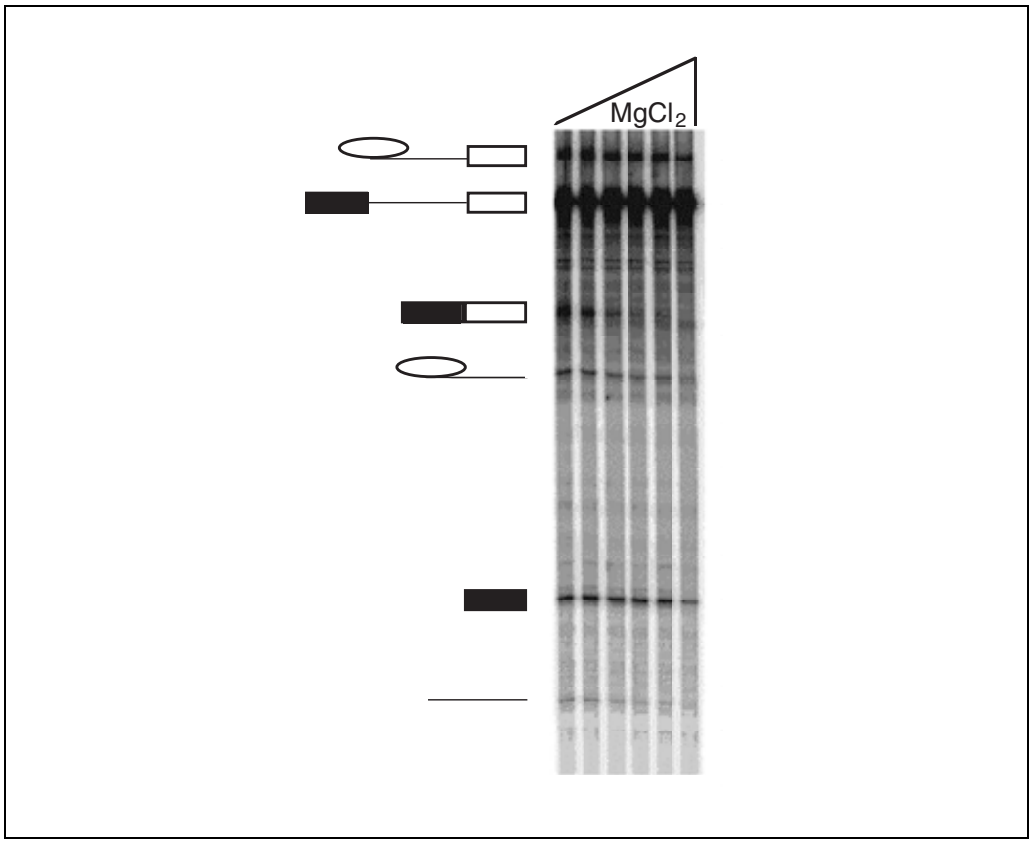


Figure 11.17.4 Magnesium titration on the β-globin first intron splicing substrate, HBΔ6 (Krainer et al., 1984). The final $MgCl_2$ concentration was titrated in 0.5 mM steps from 2.5 mM to 5 mM. Both the stability of the mRNA product and the efficiency of the second catalytic step of splicing are reduced as Mg is increased above 3 mM, while the efficiency of the first catalytic step is relatively constant over a wider Mg range.

with potassium acetate or potassium glutamate in the preparation of the nuclear extract (Black, 1992; Reichert and Moore, 2000). Moreover, some introns that are inactive in KCl are spliced in potassium glutamate. It is found that most constitutive and regulated substrates splice as well, if not better, in glutamate. This anion effect is seen in other processes involving nucleic acid binding proteins (Ha et al., 1992). The protocols presented in this unit all use glutamate.

Consistent preparation of the nuclear extract is also important to allow the technique to be optimized to yield the highest splicing activity. The condition of the starting material, whether cells or tissue, should be carefully monitored. Overgrowth of tissue culture cells will result in a loss of splicing activity in the prepared extract. The time that it takes to perform each step of the extract protocol, as well as any errors, should be noted so as to improve future preparations. For extracts made from tissues, it is important to minimize the time between isolation of the tissue from the animal and the homogenization stage.

Several parameters can be changed during optimization of the nuclear extraction. Extraction of the nuclear contents takes place in buffer C, and the KCl concentration of this extraction can affect the activity. Too low a salt concentration will result in a poor protein yield, while too high a salt concentration will result in lysis of the nuclei. This can be optimized by dividing the nuclei into several batches to be extracted under different salt conditions. Nuclei from various tissues or cell types are likely to require different conditions.

Extracts vary widely in concentration, and there is not always a direct corrolation between extract concentration and splicing activity. All new extracts should be tested on constitutive and regulated substrates. It is recommended that a simple two-exon/single-intron constitutive pre-mRNA be used when optimizing the reactions, because the splicing efficiency of these substrates is usually greater than a multiple intron substrate. As mentioned earlier, new extracts may require reoptimization of the splicing reaction (e.g., magnesium concentration). An extract that is dilute may show activity

on an efficiently spliced substrate (e.g., adenovirus first intron), but have no activity on a weaker splicing substrate.

Splicing conditions may need to be reoptimized for different pre-mRNA substrates. Pre-mRNA substrates have different optimal magnesium and monovalent salt concentrations, different requirements for macromolecular crowding reagents (e.g. PEG), and different optimal extract concentrations. For an example of a magnesium titration, see Figure 11.17.4.

As is standard when working with RNA, it is important to avoid contamination with RNases. Should contamination occur, the problem is usually most easily solved by discarding all solutions and preparing new ones. The bench area in which the experiment is being performed should be cleaned as well. This is true not only for the splicing reaction but also for the nuclear extract preparation. It is extremely important that all of the reagents used in the preparation be RNase-free and of the highest available purity. Filter sterilize all solutions and use only highly pure deionized (Milli-Q or Nanopure) water.

Anticipated Results

Using this protocol, it is possible to produce extracts between 4 to 20 mg protein/ml that will splice a uniformly labeled pre-mRNA generated by in vitro transcription

For most pre-mRNAs, the splicing reaction is more robust in vivo than in vitro. A standard in vitro splicing reaction does not usually convert all of the pre-mRNA to products during a standard reaction time of 1 to 4 hr. Much of the pre-mRNA input is still intact at the end of the incubation. With weak splicing substrates, the pre-mRNA is usually the most intense band on the splicing gel. Usually the linear molecules of the splicing reaction are identified by their migration relative to a radiolabelled DNA marker set (e.g., end labelled pBR322 *Msp1* digest). Linear RNA polynucleotides will migrate slightly slower through the urea-PAGE than the same length DNA. Branched products are identified by their retarded mobility in high percentage (8% to 12%) gels and by their susceptibility to debranching. Time course experiments run on the same splicing gel can often elucidate which products are from Steps 1 and 2 of the reaction. Normally, a splicing gel shows a number of radiolabeled RNA bands that are splicing-independent and splicing-dependent. Splicing-independent bands are often sites in the RNA with a high propensity for hydrolysis or radiolysis. Splicing-dependent bands will require the energy source ARP and creatine phosphate for their appearance, so this can be used as criteria for assigning band identities.

Time Considerations

Nuclear extract preparation takes 9 to 12 hr, including the final dialysis step. The extract preparation prior to dialysis requires 3 to 5 hr. Additional time for dissection is necessary for extracts from tissues and will depend upon the complexity of the tissue isolation. The entire procedure should be completed in 1 day. In vitro transcription and gel purification can be performed in ~4 hr, and the RNA is eluted overnight and used for splicing the next day. The in vitro splicing reaction requires ~3 to 5 hr. The preparation of the polyacrylamide gel and electrophoresis of the splicing reaction requires 4 to 5 hr.

Literature Cited

Ashiya, M. and Grabowski, P.J. 1997. A neuron-specific splicing switch mediated by an array of pre-mRNA repressor sites: Evidence of a regulatory role for the polypyrimidine tract binding protein and a brain-specific PTB counterpart. *RNA* 3:996-1015.

Bewsey, K.E., Huff, J.P., and Johnson, M.E. 1991. Rapid isolation and purification from agarose gels: The phenol freeze-fracture method. *Biotechniques.* 10:724-725.

Black, D.L. 1992. Activation of c-src neuron-specific splicing by an unusual RNA element in vivo and in vitro. *Cell* 69:795-807.

Christofori, G. and Keller, W. 1988. 3′ cleavage and polyadenylation of mRNA precursors in vitro requires a poly(A) polymerase, a cleavage factor, and a snRNP. *Cell* 54:875-889.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl. Acids Res.* 11:1475-1489.

Eperon, I.C. and Krainer, A.R. 1994. Splicing of mRNA precursors in mammalian cells. *In* RNA Processing: A Practical Approach (D. Rickwood and B.D. Hames, eds.), pp. 57-98. IRL Press, Oxford, U.K.

Grabowski, P.J. 1994. Characterization of RNA. *In* RNA Processing: A Practical Approach (D. Rickwood and B.D. Hames, eds), pp. 1:31-55. IRL Press, Oxford, U.K.

Grabowski, P.J., Padgett, R.A., and Sharp, P.A. 1984. Messenger RNA splicing in vitro: An excised intervening sequence and a potential intermediate. *Cell* 37:415-427.

Guo, N. and Kawamoto, S. 2000. An intronic downstream enhancer promotes 3′ splice site usage of a neural cell-specific exon *J. Biol. Chem.* 275:33641-33649.

Ha, J.H., Capp, M.W., Hohenwalter, M.D., Baskerville, M., and Record, M.T. Jr. 1992. Thermodynamic stoichiometries of participation of water, cations and anions in specific and nonspecific binding of lac repressor to DNA. Possible thermodynamic origins of the "glutamate effect" on protein-DNA interactions. *J. Mol. Biol.* 228:252-264.

Hardy, S.F., Grabowski, P.J., Padgett, R.A., and Sharp, P.A. 1984. Cofactor requirements of splicing of purified messenger RNA precursors. *Nature* 308:375-377.

Hernandez, N. and Keller, W. 1983. Splicing of in vitro synthesized messenger RNA precursors in HeLa cell extracts *Cell* 35:89-99.

Krainer, A.R., Maniatis, T., Ruskin, B. and Green, M.R. 1984. Normal and mutant human β-globin pre-mRNAs are faithfully and efficiently spliced in vitro. *Cell* 36:993-1005.

Kramer, A. and Keller, W. 1990. Preparation and fractionation of mammalian extracts active in pre-mRNA splicing. *Methods Enzymol.* 181:3-20.

Le Hir, H., Izaurralde, E., Maquat, L.E., and Moore M.J. 2000. The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. *EMBO J.* 19:6860-6869.

Lee, K.A., Bindereif, A., and Green, M.R. 1988. A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Anal. Tech.* 5:22-31.

Moore, C.L. and Sharp, P.A. 1985. Accurate cleavage and polyadenylation of exogenous RNA substrate. *Cell* 41:845-855.

Noble, J.C., Pan, Z.Q., Prives, C., and Manley, J.L. 1987. Splicing of SV40 early pre-mRNA to large T and small t mRNAs utilizes different patterns of lariat branch sites. *Cell* 50:227-236

Ooi, S.L., Dann, C. 3rd, Nam, K., Leahy, D.J., Damha, M.J., and Boeke, J.D. 2001. RNA lariat debranching enzyme. *Methods Enzymol.* 342:233-248.

Padgett, R.A., Hardy, S.F., and Sharp, P.A. 1983. Splicing of adenovirus RNA in a cell-free transcription system. *Proc. Natl. Acad. Sci. U.S.A.* 80:5230-5234.

Reichert, V. and Moore, M.J. 2000. Better conditions for mammalian in vitro splicing provided by acetate and glutamate as potassium counterions. *Nucl. Acids Res.* 28:416-423.

Rio, D.C. 1988. Accurate and efficient pre-mRNA splicing in *Drosophila* cell-free extracts. *Proc. Natl. Acad. Sci. U.S.A.* 85:2904-2908.

Ruskin, B. and Green, M.R. 1990. RNA lariat debranching enzyme as a tool for analyzing RNA structure. *Methods Enzymol.* 181:180-188.

Sierra, F., Tian, J.M., and Schibler, U. 1993. In vitro transcription with nuclear extracts from differentiated tissues. *In* Gene transcription: A practical approach (B.D. Hames and S.J. Higgins, eds.), pp. 125-152. Oxford University Press, New York.

Takagaki, Y., Ryner, L.C., and Manley, J.L. 1988. Separation and characterization of a poly(A) polymerase and a cleavage/specificity factor required for pre-mRNA polyadenylation. *Cell* 52:731-742.

Zhang, L., Liu, W., and Grabowski, P.J. 1999. Coordinate repression of a trio of neuron-specific splicing events by the splicing regulator PTB. *RNA* 5:117-30.

Zhou, Z., Luo, M.J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. 2001. The protein Aly links pre-messenger RNA splicing to nuclear export in metazoans. *Nature* 407:401-405.

Contributed by Nanette Rooke and Jason Underwood
University of California, Los Angeles
Los Angeles, California

Endocytosis Assays in Intact and Permeabilized Cells

Clathrin-coated pits and vesicles represent the major ports of entry into most eukaryotic cells. As well as performing housekeeping functions (e.g., allowing cells to take up essential nutrients), the endocytic pathway participates in a number of tissue-specific events such as synaptic-vesicle recycling, control of morphogen gradients during development, downregulation of receptor tyrosine kinases, and immune surveillance (Conner and Schmid, 2003). To understand the role played by clathrin-mediated uptake, it is therefore essential to have robust endocytosis assays in intact cells (Basic Protocol 1). The clathrin-coated vesicle cycle requires a complicated interplay of proteins and lipids that is regulated in space and time. Reconstitution assays in permeabilized cells (Basic Protocol 2 and Alternate Protocols 1 and 2) provide a powerful approach to understanding how this complex process is regulated. Support Protocols 1 to 9 describe the preparation of cells and reagents for the assays.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator unless otherwise indicated.

MEASUREMENT OF RECEPTOR-MEDIATED ENDOCYTOSIS IN INTACT CELLS

BASIC PROTOCOL 1

A number of methods have been described to measure the internalization of ligands such as transferrin (Hopkins and Trowbridge, 1983). In general, they rely on the ability to distinguish cell surface from intracellular pools of ligand. Routinely, the authors use transferrin that has been biotinylated via a cleavable disulfide linkage (B-SS-Tfn) as a reporter molecule to distinguish between intracellular and cell surface ligand by accessibility either to avidin or to the small membrane-impermeant thiol-reducing reagent, sodium 2-mercaptoethanesulfonate (MesNa). Transferrin is a ligand of choice to study the core machinery because the transferrin receptor is abundant on most cells, the route followed by transferrin when bound to its receptor is well characterized (Hopkins and Trowbridge, 1983), and transferrin is inexpensive and easy to modify by biotinylation or iodination. It is possible to use other ligands, however (see Background Information). Essential prerequisites are a reasonable number of receptor molecules on the cell surface and effective methods to label the receptor with either ligand or antibody or by cell surface labeling.

This protocol describes a method whereby avidin and the small membrane-impermeant reducing agent MesNa quench B-SS-Tfn at the cell surface so that the internalized B-SS-Tfn may be measured. Following addition of avidin or MesNa, these reagents are themselves functionally inactivated. Biocytin (a conjugate of biotin and lysine) is added to bind to remaining binding sites on avidin, which is tetrameric. Excess MesNa is removed by reaction with iodoacetamide (IAA). In each case, the cells are solubilized and all of the transferrin is captured on an ELISA plate coated with antibodies to transferrin. In the case of the avidin assay, cell surface B-SS-Tfn will be bound to avidin, whereas in the case of the MesNa assay, cell surface transferrin will no longer be biotinylated. In this case, internalized B-SS-Tfn may be detected using streptavidin conjugated with the enzyme horseradish peroxidase (HRP). The principles of the avidin and MesNa assays are outlined for permeabilized cells in Figure 11.18.1. For many experiments in intact cells, either the avidin or MesNa assays may be used, as under most conditions they give identical results

Contributed by Andrew Osborne, Alexander Flett, and Elizabeth Smythe
Current Protocols in Cell Biology (2005) 11.18.1-11.18.24

(Schmid and Smythe, 1991). There may be occasions, however, when a comparison of the results of these assays may provide valuable information. For example, under conditions where scission is inhibited in intact cells but invagination is not, the avidin assay will measure a larger amount of apparently internalized ligand as compared with the MesNa assay (Damke et al., 1994; Hill et al., 2001). The avidin and MesNa assays may be used with either adherent cells or cells growing in suspension.

Materials

35-mm tissue culture dishes of A431 cells (or other adherent cells; ~80% confluent) *or* cell suspension (~1 × 10^6 cells/200 μl)
Serum-free medium: DMEM containing 1 mg/ml BSA
PBS/BSA: CMF-DPBS (*APPENDIX 2A*) containing 0.2% (w/v) BSA, ice cold and 31°C
1 mg/ml biotinylated transferrin (B-SS-Tfn; see Support Protocol 1)
50 μg/ml avidin (Sigma) in PBS/BSA, for avidin assay only
1 mg/ml biocytin (Sigma), for avidin assay only
10 and 100 mM sodium 2-mercaptoethanesulfonate (MesNa; Sigma) in MesNa buffer (see recipe), prepare fresh (for MesNa assay only)
500 mM iodoacetamide (Sigma), prepare fresh (for MesNa assay only)
Blocking buffer (see recipe)
ELISA plates coated with anti-transferrin antibodies (see Support Protocol 2)
0.1 U/ml streptavidin–horseradish peroxidase (HRP; Pierce) in blocking buffer
Dulbecco's PBS (*APPENDIX 2A*)
o-Phenylenediamine tablets (Sigma)
H_2O_2 (Sigma)
HRP assay buffer: 51 mM phosphate/27 mM citrate, pH 5.0
2 M H_2SO_4 (see recipe)
31°C incubator or water bath with metal tray to support tissue culture dishes
Microscope suitable for examining cells in culture, for adherent cells only
ELISA plate reader with 492-nm filter

Incubate cells with B-SS-Tfn

1. Incubate a 35-mm dish of A431 cells in 2 ml serum-free medium for 30 min at 37°C. For cells in suspension, centrifuge 200 μl (~1 × 10^6 cells) cell suspension 5 min at 1000 × *g*, at room temperature. Remove supernatant and replace with 200 μl serum-free medium. Agitate gently for 30 min at 37°C.

   The purpose of this incubation is to remove bovine transferrin present in serum.

   A 35-mm dish of adherent cells or ~1 × 10^6 cells in suspension is used for each time point.

Figure 11.18.1 *(at right)* Principles of the avidin and sodium 2-mercaptoethanesulfonate (MesNa) assays. Permeabilized cells are incubated with transferrin (Tfn) that has been biotinylated via a cleavable disulfide linkage (B-SS-Tfn) under conditions where endocytosis can occur (1). Some of the B-SS-Tfn is sequestered into deeply invaginated coated pits or internalized into coated vesicles (2). The permeabilized cell mixes are then chilled and treated with avidin (3). Cell surface B-SS-Tfn is accessible to avidin, whereas sequestered B-SS-Tfn (either in deeply invaginated coated pits or internalized in coated vesicles) is inaccessible. Excess binding sites on avidin are quenched by the addition of biocytin (4). The cells are then lysed and applied to ELISA plates coated with anti-transferrin antibodies (anti-Tfn Ab). Sequestered and internalized B-SS-Tfn may be detected by binding of streptavidin–horseradish peroxidase (HRP; 5). Alternatively, following the endocytosis reaction, cell surface B-SS-Tfn is reduced using the small membrane-impermeant reducing agent MesNa. MesNa can access B-SS-Tfn sequestered in deeply invaginated coated pits, whereas B-SS-Tfn internalized in coated vesicles is resistant to MesNa reduction (6). Iodoacetamide (IAA) is used to quench excess MesNa (7). The cells are solubilized, and internalized B-SS-Tfn is detected using streptavidin-HRP (8).

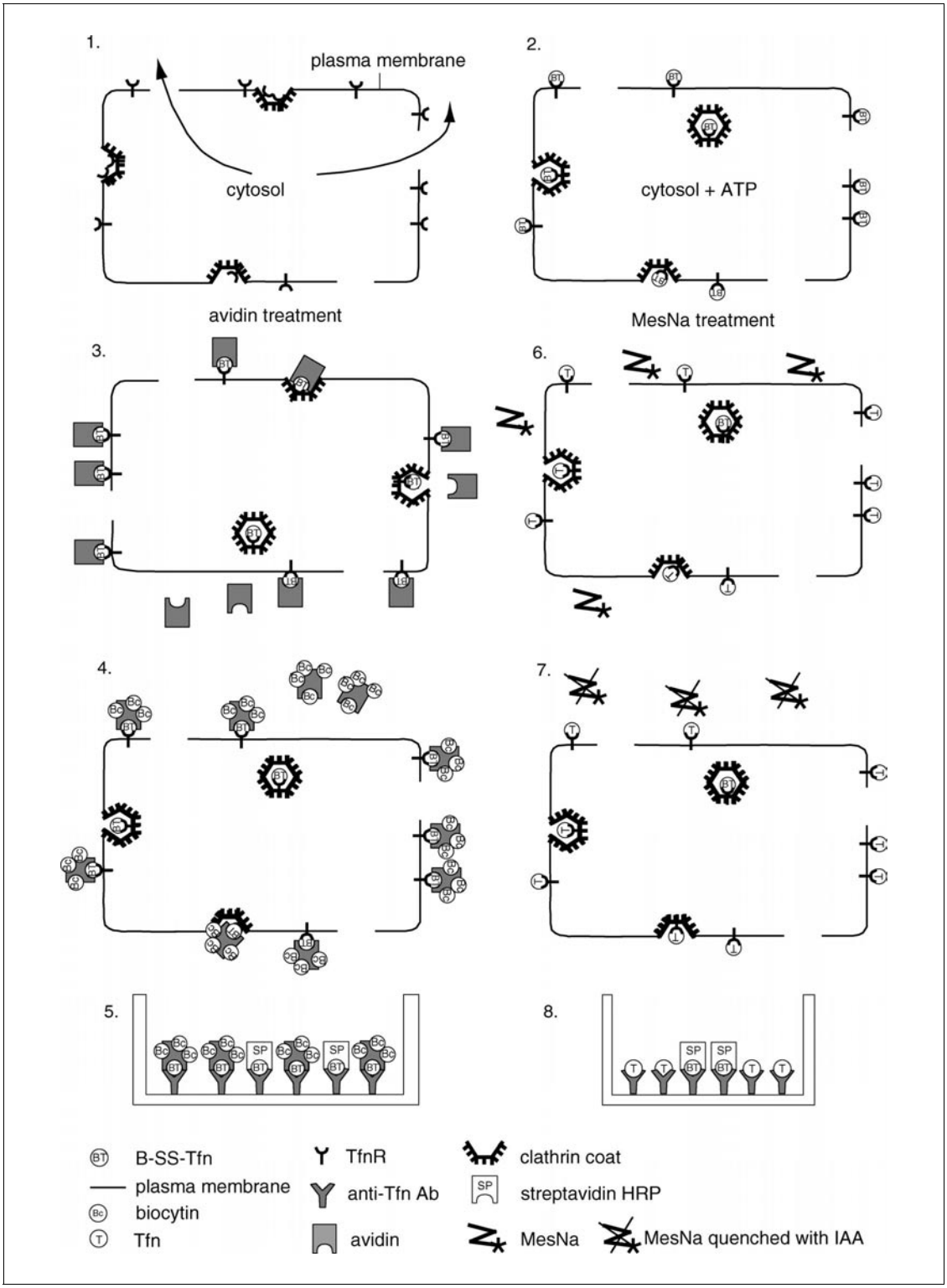


Figure 11.18.1 Legend at left.

2. Wash cells three times with ice-cold PBS/BSA. Use 2 ml for adherent cells and 1 ml for cells in suspension followed by centrifugation conditions given in step 1. Add 1 μg/ml B-SS-Tfn in PBS/BSA to sample (1 ml for adherent cells and 200 μl for cells in suspension) and incubate 30 to 60 min at 4°C.

   To minimize the amount of B-SS-Tfn to be used, dishes may be incubated with ligand in a volume of 0.6 ml with gentle rocking.

3. Wash cells three times with ice-cold PBS/BSA as in step 2. Warm cells at 31°C in 1 ml warm PBS/BSA for adherent cells or in 200 μl warm PBS/BSA for cells in suspension for various times.

   In general, to measure the internalization of a cell surface receptor, the incubation times that are chosen would range from 0 to 60 min, with a greater emphasis on early time points. Typical time points would include 0, 2, 5, 10, 15, 20, 30, and 60 min.

   The incubation is carried out at 31°C to slow down the rate of endocytosis. Ligands such as transferrin are endocytosed at a very fast rate at 37°C (∼15% of the surface pool per minute in many cell types; Watts and Marsh, 1991), making estimation of internalization difficult. Hence, the lower temperature facilitates data collection at early time points.

4. Return cells to 4°C and continue with either avidin (step 5a) or MesNa (step 5b) assay. For either assay, hold a set of duplicate cells (total B-SS-Tfn samples) at 4°C (i.e., do not treat with avidin or MesNa) and process with the other samples (step 8).

   The duplicate samples that are held at 4°C are processed with the other samples (step 8) to determine the total amount of B-SS-Tfn available to be internalized.

For avidin assay

5a. Wash cells two times with ice-cold PBS/BSA (2 ml for adherent cells and 1 ml for cells in suspension followed by 5 min centrifugation at 1000 × *g*). Incubate B-SS-Tfn-labeled cells with 50 μg/ml avidin in PBS/BSA (600 μl for adherent cells and 200 μl for cells in suspension) for 1 hr at 4°C with gentle agitation.

6a. Quench avidin with 30 μl of 1 mg/ml biocytin for adherent cells or 10 μl of 1 mg/ml biocytin for cell suspension at 4°C for 10 min. Continue with step 8.

For MesNa assay

5b. Incubate cells at 4°C with freshly prepared 10 mM MesNa in MesNa buffer (600 μl for adherent cells or 200 μl for cells in suspension) for 20 min with gentle agitation.

6b. Make two further additions of freshly prepared 100 mM MesNa (72 and 96 μl for adherent cells or 24 and 32 μl for cells in suspension) and incubate 20 min at 4°C after each addition.

   For effective reduction of B-SS-Tfn, it is critical that the MesNa solution is freshly prepared just prior to addition to the cells.

7b. Quench MesNa by addition of freshly prepared 500 mM IAA (150 μl for adherent cells or 50 μl for cells in suspension).

Solubilize cells

8. Remove avidin or MesNa mixture (use centrifugation for cells in suspension) and solubilize cells by adding blocking buffer. Add 600 μl to dish of adherent cells and pipet up and down; check under a microscope to ensure that all cells are removed. For cells in suspension, resuspend cell pellet in 200 μl blocking buffer. Treat total B-SS-Tfn samples (step 4) in parallel.

9. Add 100 μl blocking buffer to each well of an ELISA plate coated with anti-transferrin antibodies. Add 100 μl solubilized cells to duplicate wells of the ELISA plate. Plate a titration of different amounts of total B-SS-Tfn samples diluted in blocking buffer to maintain a final volume of 100 μl. Incubate plates overnight at 4°C or for 3 hr at 37°C.

   To ensure that color development falls within a linear range, different amounts of the total B-SS-Tfn samples should be plated.

   Incubation of the ELISA plate at 37°C does not require a humidified, 5% CO_2 incubator.

Develop assay

10. Wash plate three times with ~300 μl PBS per well.

    A plate washer can be used for the washes. In the absence of a plate washer, the washes may simply be removed by flicking into a sink or waste container, but all wells must be emptied on each wash.

11. Add 200 μl of 0.1 U/ml streptavidin-HRP in blocking buffer and incubate 1 hr at room temperature.

12. Wash three times with 300 μl PBS per well.

13. Prepare the following reaction mix:

    10 mg *o*-phenylenediamine
    10 μl H_2O_2
    25 ml HRP assay buffer.

    CAUTION: *During the development of the HRP reaction product, gloves should be worn at all times. o-Phenylenediamine is carcinogenic, but preweighed tablets are commercially available that can be added directly to assay buffer.*

14. Add 200 μl reaction mix to each well of the ELISA plate to develop the reaction.

15. Stop the reaction by adding 50 μl of 2 M H_2SO_4, which also enhances color development.

    CAUTION: *2 M H_2SO_4 is a strong and corrosive acid and should be treated with appropriate care. The ELISA plates containing the reaction product should be disposed of in accordance with local practices for the disposal of hazardous material.*

    The time taken for color to develop will be variable and will depend on the number of cells in a particular endocytosis assay. In general, when the darkest wells are a strong yellow color, it is time to add the acid. With experience, it becomes obvious when the reaction needs to be stopped so that the absorbance at 492 nm falls within the linear range.

16. Read absorbance on an ELISA plate reader at 492 nm. Check that the values obtained with the total B-SS-Tfn samples are within the linear range of absorbance.

    In general, there is a linear relationship up to an absorbance of ~0.8. Background values should have an absorbance <0.08.

Quantify results

17. Use total B-SS-Tfn samples (step 4) to estimate the total amount of B-SS-Tfn available for internalization.

18. Express the amount of avidin-inaccessible or MesNa-resistant B-SS-Tfn at each time point as a percentage of total B-SS-Tfn present in the samples held at 4°C.

BASIC PROTOCOL 2

MEASUREMENT OF RECEPTOR-MEDIATED ENDOCYTOSIS IN PERMEABILIZED CELLS

The avidin and sodium 2-mercaptoethanesulfonate (MesNa) assays are used extensively with permeabilized cells to reconstitute different stages in the clathrin coated–vesicle cycle (Schmid and Smythe, 1991; Smythe et al., 1992; Carter et al., 1993). The size difference between the two probes allows the difference between cargo sequestration into deeply invaginated coated pits and its internalization into coated vesicles to be distinguished (Fig. 11.18.1). Avidin is a tetramer composed of four subunits of 64 kDa and so is relatively bulky and is unable to access B-SS-Tfn that is sequestered in deeply invaginated coated pits or internalized in coated vesicles. MesNa is considerably smaller (165 Da) and so can access sequestered cargo but, because it is membrane impermeant, it fails to reduce B-SS-Tfn within coated vesicles. Reconstitution of the clathrin coated–vesicle cycle in permeabilized cells generates intermediates such as deeply invaginated coated pits that would be very transient and thus seldomly detected in vivo. This has the advantage of allowing the components required to generate these intermediates to be identified and characterized (Benmerah et al., 1998; Jost et al., 1998; McLauchlan, 1998; Hill et al., 2001; Olusanya et al., 2001; Jackson et al., 2003).

Materials

15-cm tissue culture dishes of A431 cells (~80% confluent)
Serum-free medium: DMEM containing 1 mg/ml BSA
KSHM buffer (see recipe), ice cold
4 μg/ml biotinylated transferrin (B-SS-Tfn; see Support Protocol 1) in KSHM containing 0.2% (w/v) BSA (KSHM/BSA)
ATP-regenerating system (see Support Protocol 6)
ATP-depleting system (see Support Protocol 7)
Cytosol (see Support Protocols 4 and 5)
50 μg/ml avidin (Sigma) in KSHM/BSA, for avidin assay only
1 mg/ml biocytin (Sigma), for avidin assay only
10 mM and 50 mM sodium 2-mercaptoethanesulfonate (MesNa; Sigma) in MesNa buffer (see recipe), prepare fresh (for MesNa assay only)
500 mM iodoacetamide (IAA; Sigma), prepare fresh (for MesNa assay only)
Blocking buffer (see recipe)
ELISA plates coated with anti-transferrin antibodies (see Support Protocol 2)
0.1 U/ml streptavidin–horseradish peroxidase (HRP; Pierce) in blocking buffer
Dulbecco's PBS (*APPENDIX 2A*)
o-Phenylenediamine tablets (Sigma)
H_2O_2 (Sigma)
HRP assay buffer: 51 mM phosphate/27 mM citrate, pH 5.0
2 M H_2SO_4 (see recipe)
Microcentrifuge, 4°C
ELISA plate reader with 492-nm filter
Additional reagents and equipment for permeabilizing cells (see Support Protocol 3)

Permeabilize cells

1. Incubate a 15-cm dish of A431 cells in 20 ml serum-free medium for 30 min at 37°C.
2. Wash cells four times with 20 ml each ice-cold KSHM.
3. Permeabilize cells (see Support Protocol 3).
4. Remove cytosol by dilution of cells into 50 ml KSHM and then collect permeabilized cell membranes by centrifugation at 1000 × *g* for 5 min at 4°C.

Set up reconstitution assay

5. Discard supernatant and resuspend permeabilized cell membranes (i.e., the pellet) in 500 μl of 4 μg/ml B-SS-Tfn in KSHM/BSA.

   For the number of A431 cells present in a confluent layer, 2×10^5 cells/cm^2 is a reasonable assumption. Therefore, a 15-cm dish at 80% confluency will yield 2.4×10^7 cells. Thus, each 15-cm plate should provide sufficient cells for ~50 to 70 assays. Each sample will therefore contain ~1×10^6 cells.

6. Add 10 μl membrane-B-SS-Tfn mixture to a microcentrifuge tube containing 4 μl of either an ATP-regenerating or an ATP-depleting mix, and/or cytosol, and/or purified components, and KSHM to give a final volume of 40 μl. Set up two sets of duplicates without cytosol and with an ATP-depleting system. Also set up two other sets of duplicates without cytosol and with an ATP-regenerating system. Incubate samples 30 min at 31°C except for one set of duplicates without cytosol and with an ATP-depleting system, which is held at 4°C.

   Information about purified components that might be added to the permeabilized cells is described elsewhere in more detail (see Alternate Protocols 1 and 2).

   The two sets of duplicate tubes without cytosol will be used as controls.

7. Incubate assay mixes 20 to 30 min at 37°C with gentle agitation every 5 min.

8. Pellet permeabilized cells by microcentrifugation for 20 sec at 22,000 × *g*, 4°C. Aspirate supernatant and continue with either avidin (step 9a) or MesNa (step 9b) assay. For either assay, hold a set of duplicate samples without cytosol but with an ATP-regenerating system at 4°C (i.e., do not treat with avidin or MesNa) and process with the other samples (step 13).

   The samples that are held at 4°C will be used to measure the total amount of B-SS-Tfn available for endocytosis.

For avidin assay

9a. Resuspend pelleted membranes in 100 μl KSHM containing 0.2% BSA and 50 μg/ml avidin. Incubate 60 min on ice.

10a. Quench remaining binding sites on avidin by the addition of 10 μl of 1 mg/ml biocytin. Leave for 10 min at 4°C. Continue with step 13.

For MesNa assay

9b. Resuspend pelleted membranes in 50 μl freshly prepared 10 mM MesNa in MesNa buffer and agitate gently for 30 min at 4°C.

10b. Add 12.5 μl freshly prepared 50 mM MesNa in MesNa buffer and incubate for a further 15 min at 4°C.

   For effective reduction of B-SS-Tfn, it is critical that the MesNa solution is freshly prepared just prior to addition to the cells.

11b. Add 16 μl freshly prepared 50 mM MesNa in MesNa buffer and incubate 15 min at 4°C.

12b. Quench unreacted MesNa by adding 30 μl of 500 mM freshly prepared IAA. Incubate 10 min at 4°C.

Solubilize cells

13. Add 100 µl blocking buffer to lyse the cells. Process total B-SS-Tfn samples (step 8) in parallel.

14. Add 100 µl blocking buffer to each well of an ELISA plate coated with anti-transferrin antibodies. Add 100 µl lysate to duplicate wells of the ELISA plate. Plate a titration of different amounts of the total B-SS-Tfn samples diluted in blocking buffer to maintain a final volume of 100 µl. Incubate 3 hr at 37°C or overnight at 4°C.

    To ensure that color development falls within a linear range, different amounts of the total B-SS-Tfn samples should be plated.

    Incubation of the ELISA plate at 37°C does not require a humidified, 5% CO_2 incubator.

Develop assay

15. Wash plate three times with 300 µl PBS per well.

16. Add 200 µl of 0.1 U/ml streptavidin-HRP in blocking buffer to each well and incubate 1 hr at room temperature.

17. Wash plate three times with 300 µl PBS per well.

18. Prepare the following reaction mix:

    10 mg *o*-phenylenediamine
    10 µl H_2O_2
    25 ml HRP assay buffer.

    CAUTION: *During the development of the HRP reaction product, gloves should be worn at all times. o-Phenylenediamine is carcinogenic, but preweighed tablets are commercially available that can be added directly to assay buffer.*

19. Add 200 µl reaction mix to each well of the ELISA plate to develop the reaction.

20. Stop the reaction by adding 50 µl of 2 M H_2SO_4, which also enhances color development.

    CAUTION: *2 M H_2SO_4 is a strong and corrosive acid and should be treated with appropriate care. The ELISA plates containing the reaction product should be disposed of in accordance with local practices for the disposal of hazardous material.*

21. Read absorbance on an ELISA plate reader at 492 nm. Check that the values obtained from titration of total samples (step 14) are within the linear range of absorbance.

    In general, there is a linear relationship up to an absorbance of ~0.8. Background values should have an absorbance <0.08.

Quantify results

22. Use the samples that were not treated with avidin or MesNa to estimate the total amount of B-SS-Tfn available for internalization.

23. Express the amount of avidin-inaccessible or MesNa-resistant B-SS-Tfn as a percentage of total B-SS-Tfn present in the samples held at 4°C.

    The signal (A_{492}) obtained from samples incubated on ice without cytosol and in the presence of ATP-regenerating system (step 6) indicates the background signal and is typically identical to that of an empty well. The signal from samples incubated without cytosol and in the presence of ATP-depleting system corresponds to that from any remaining intact cells in the preparation. This value should be subtracted from samples where B-SS-Tfn has been incubated with permeabilized cells under conditions where endocytosis occurs.

STAGE-SPECIFIC ASSAYS USING PERMEABILIZED CELLS

The clathrin coated–vesicle cycle can be divided into a series of steps with different biochemical requirements. These include the assembly of new coated pits onto the cell surface, cargo recruitment into these pits, invagination, and scission to form a coated vesicle.The permeabilized cell assay as described above distinguishes between the sequestration of cargo into deeply invaginated coated pits versus its internalization into coated vesicles (Schmid and Smythe, 1991). The assay system can be further refined, however, to select for particular intermediates. For example, although there are already coated pits present that are capable of cargo sequestration when the cells are permeabilized, addition of purified coat components can stimulate cargo sequestration into newly formed coated pits.

Coat Protein–Dependent Cargo Sequestration

ALTERNATE PROTOCOL 1

Addition of purified AP2 to assay mixes containing cytosol as described in Basic Protocol 2 results in enhanced B-SS-Tfn sequestration into newly formed clathrin-coated pits (Smythe et al., 1992; McLauchlan et al., 1998). The clathrin that participates in forming these new pits is recruited from the permeabilized cell membranes (McLauchlan et al., 1998). The permeabilized cell system can also be manipulated so that it is dependent on the addition of exogenously added clathrin (Jackson et al., 2003).

For AP2-dependent sequestration, perform the assay as described for the avidin inaccessibility assay in permeabilized cells (see Basic Protocol 2, steps 1 to 23), with the following changes. In step 6, add AP mix or purified AP2 (see Support Protocol 8) to the reaction mix, keeping the final volume at 40 μl. Also set up control incubations containing permeabilized cell membranes, ATP-regenerating system, and AP mix or AP2 only. The AP2 concentration that gives maximal AP2 dependence will vary with different cytosol preparations and needs to be determined empirically. The concentration of each batch of AP2 should also be titrated to optimize the signal. In general, 0.1 to 0.25 mg/ml AP2 gives maximum stimulation. AP2 is most stable in 10 mM Tris·Cl, pH 7.5, and no more than 10 μl of this solution should be added to the assay mixes to prevent buffer effects. The control reactions containing ATP-regenerating system and AP2 only give a signal that is generally equivalent to incubations in the presence of permeabilized cell membranes and ATP alone. If the signal is higher, then the difference should be subtracted from the AP2-dependent signal.

For clathrin- and AP2-dependent sequestration, perform the assay as described for the avidin inaccessibility assay in permeabilized cells (see Basic Protocol 2, steps 1 to 23), with the following changes. To generate a requirement for exogenous clathrin, add 0.4 μl of 100 μM stock (prepared in methanol) of the protein phosphatase inhibitor microcystin (1 μM final) to the reaction mixes in step 5 and incubate 10 min on ice and then 30 min at 37°C. Set up controls that include assay mixes containing coat components and ATP-regenerating system but omit cytosol. Coat proteins are added as described (see Basic Protocol 2, step 6). Typically, AP2 is added as described above. Microcystin is carcinogenic, so gloves should always be worn while carrying out these assays, and material must be disposed of in accordance with local practices. AP2-dependent cargo sequestration in permeabilized cells relies on recruitment of clathrin from the permeabilized cell membranes to participate in new coated-pit formation. There may, however, be occasions when it is necessary to add soluble clathrin. The amount of clathrin required (prepared as described, see Support Protocol 8, and dialyzed into KSHM) needs to be determined empirically but is generally in the range of 0.1 to 0.25 mg/ml. Microcystin is effective presumably because a dephosphorylation event(s) is required for the mobilization of clathrin from membranes (Jackson et al., 2003).

ALTERNATE PROTOCOL 2

Further Manipulation of Endocytosis in Permeabilized Cells

The list of components involved in coated-vesicle formation is expanding, and hence there is a need to further dissect the temporal and spatial requirements for coated-vesicle formation.

Manipulation of cytosolic components

Soluble components may be manipulated by addition of purified components directly to assay mixes or by supplementing cytosol with purified components. Alternatively, to test for a specific requirement for a particular protein, cytosol can be immunodepleted using antibodies specific for the protein before being added to the assay mix (McLauchlan et al., 1998). Controls should include cytosol that has been mock depleted with an irrelevant antibody.

Manipulation of membrane components

Permeabilized cell membranes may also be manipulated. For example, truncated proteins or particular domains of endocytic proteins can act as dominant-negative inhibitors of endocytosis by targeting interacting proteins and locking them into a dead-end nonfunctional complex. In particular, the SH3 domains of proteins that interact with the proline-rich domain of dynamin (e.g., amphiphysin and endophilin) have been utilized to inhibit ligand sequestration and internalization in permeabilized cells (Simpson et al., 1999; Hill et al., 2001). These protein modules bind to targets on the membrane and render them incapable of functioning in the coated-vesicle cycle.

Treatment of permeabilized cells with SH3 domains

The use of SH3 domains is one example of targeting a membrane-associated factor. Other possibilities are to use inhibitory antibodies or other protein modules. In each case, it is essential to include a negative control that mimics as closely as possible the inhibitory component. Care should be taken in the interpretation of experiments where membrane components are targeted with, for example, a specific antibody, in case there are issues of steric hindrance. The most meaningful results will be obtained if it is possible to rescue the inhibited membranes by addition of the target protein in a soluble form (e.g., Hill et al., 2001). The authors have found that the best working range for inhibition by the SH3 domains of amphiphysin and endophilin is 1 to 5 μM.

Many peripheral membrane proteins that cycle from the cytoplasm to the membrane during the coated-vesicle cycle remain associated with permeabilized cell membranes. If no specific inhibitor is available, it is sometimes possible to remove peripheral membrane proteins by stripping the membranes with high concentrations of NaCl and KCl or urea. The extent of removal of components may be assessed by immunoblotting (*UNIT 6.2*). The concentrations of chaotropic reagents required to remove peripheral proteins may, however, have detrimental effects on the ability of the membranes to support the reconstitution of clathrin-coated pit formation, and so it is important to titrate the concentration of stripping agent and test whether cargo sequestration and internalization may be reconstituted with a reasonable efficiency following the treatment.

Additional Materials *(also see Basic Protocol 2 and see Alternate Protocol 1)*

Purified GST fusion protein containing an SH3 domain of interest

1. Prepare permeabilized cell membranes and incubate with B-SS-Tfn as described (see Basic Protocol 2, steps 1 to 4).

2. Preincubate permeabilized cell membranes with GST fusion protein containing SH3 domain of choice for 5 min at 30°C.

   The minimum amount of GST fusion protein required to inhibit the membrane target needs to be determined empirically by titration of different amounts. A useful working range with which to start is 1 to 5 μM.

3. Pellet membranes by centrifugation for 5 sec at 22,000 × *g*, 4°C. Aspirate supernatant containing excess GST fusion protein.

4. Resuspend membranes and continue with assay as described (see Basic Protocol 2, steps 5 to 23).

   Controls should include membranes treated either with GST alone or with another similar SH3 domain that has no apparent role in endocytosis.

PREPARATION OF BIOTINYLATED TRANSFERRIN

SUPPORT PROTOCOL 1

Biotinylated transferrin (B-SS-Tfn) is used as a marker to follow endocytosis in intact and permeabilized cells. It binds to the transferrin receptor, which is internalized by receptor-mediated endocytosis.

Materials

Transferrin (Sigma)
Dulbecco's PBS (*APPENDIX 2A*)
NHS-SS-biotin: sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (EZ-Link Sulfo-NHS-SS-Biotin; Pierce)
Dimethyl sulfoxide (DMSO; Sigma)
500 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
PBS/BSA: PBS containing 1 mg/ml BSA
Liquid nitrogen
Dialysis tubing (10,000 MWCO) or buffer exchangers (e.g., Vivaspins; Vivascience)
Quartz cuvettes
Spectrophotometer with UV source (280 nm)

1. Prepare a 10 mg/ml solution of transferrin in distilled water.

   Water is used rather than buffer because transferrin is usually sold as a desiccated solution and so already contains salt.

2. Dialyze or buffer-exchange the transferrin solution into PBS to ensure that any amines present in the buffer salts are removed.

   Amines could interfere with the biotinylation reaction.

3. Using a quartz cuvette, measure the absorbance at 280 nm to determine the exact concentration of transferrin.

   A solution of 1 mg/ml transferrin has an absorbance of 1.2 at 280 nm.

4. Use 1 mg transferrin for the biotinylation reaction. Incubate 30 min at 30°C with an ~20-fold molar excess of NHS-SS-biotin freshly prepared as a 100× stock in DMSO.

   This ratio of biotin to transferrin results in one or two molecules of biotin per transferrin molecule as determined with the HABA reagent (Green, 1970). This is optimal because too many biotin moieties per transferrin molecule could potentially interfere with binding to the transferrin receptor.

5. Stop the biotinylation reaction by adding 10 μl of 500 mM Tris·Cl, pH 7.5.

6. Exchange buffer by dialysis against PBS/BSA to remove free NHS-SS-biotin.

7. Prepare a 1 mg/ml solution of B-SS-Tfn. Divide into aliquots (usually 10 µl) in microcentrifuge tubes and snap freeze in liquid nitrogen before storing at −80°C.

   The B-SS-Tfn is stable for at least 1 year at −80°C. A fresh aliquot is used for each in vitro assay, and repeated freeze-thawing is avoided.

   CAUTION: *When dispensing and snap-freezing in liquid nitrogen, a face mask and protective gloves should always be worn.*

SUPPORT PROTOCOL 2

PREPARATION OF ELISA PLATES

This protocol describes the preparation of enzyme-linked immunosorbent assay (ELISA) plates for analysis of the samples.

Materials

Anti-transferrin antibodies (available as sheep serum from Alba Bioscience)
100 mM sodium bicarbonate, pH 8.3
Dulbecco's PBS (*APPENDIX 2A*)
Blocking buffer (see recipe)
Flat-bottomed ELISA microtiter plates

1. Coat flat-bottomed ELISA microtiter plates with 200 µl anti-transferrin antibody diluted to 1:5000 in 100 mM sodium bicarbonate, pH 8.3.
2. Incubate plates either at 37°C for 3 hr or overnight at 4°C.
3. Wash three times with 300 µl PBS followed by incubation with 250 µl blocking buffer for a minimum of 1 hr at room temperature.
4. Store plates in blocking buffer for up to 1 week at 4°C. Remove blocking buffer before using plates.

SUPPORT PROTOCOL 3

METHODS FOR PERMEABILIZATION OF CELLS

Freeze-Thaw Permeabilization

Freeze-thaw permeabilization is carried out by freezing the cells in liquid nitrogen and thawing them rapidly at 37°C. Further cycles of freeze-thawing may be required to efficiently permeabilize >90% of the cells, but it is important to assess the degree of cell breakage after each cycle to ensure that the cells have not been grossly damaged. The degree of cell breakage can be assessed either by measurement of release of lactate dehydrogenase (see Support Protocol 9) or by assessing accessibility to trypan blue using light microscopy (*UNIT 1.1*).

Streptolysin O Permeabilization

Bacterial toxin streptolysin O (SLO) has been used extensively to permeabilize various cell types (Bhakdi et al., 1985; Ullrich et al., 1995). As SLO makes relatively small holes in the plasma membrane, it is less likely to be physically disruptive to the cell as compared with, for example, scraping of the cells. SLO is sold in amounts measured in International Units, but these units do not necessarily correspond to the ability of the toxin to permeabilize cells (Ahnert-Hilger et al., 1985), and so each batch needs to be titrated and optimized accordingly for each particular cell type. A useful working range is 0.05 to 0.1 IU/ml diluted in PBS. SLO is perhaps more useful for cells grown in suspension rather than adherent cells as there may be problems with cell loss of adherent cells. A general procedure is to incubate the cells in the appropriate amount of SLO at 37°C followed by incubation at 4°C, although the lengths of these incubations need to be optimized.

Permeabilization by Mechanical Shear

Human carcinoma A431 cells can be effectively permeabilized (>90% of the cytosolic components are released) by simply scraping from the tissue culture dish.

PREPARATION OF CYTOSOL FROM TISSUE

SUPPORT PROTOCOL 4

Cytosol from bovine, porcine, and murine brain, tissue culture cells (e.g., A431 and K562), and even yeast cells supports endocytosis in permeabilized cells, although with varying efficiency. Brain cytosol is most often used because of the availability of relatively large amounts of tissue and the abundance of endocytic proteins in this tissue (Morris and Schmid, 1995). Cytosol prepared from liver may not be used to measure the sequestration and internalization of transferrin because of the high levels of blood found in liver. This may contain sufficient transferrin to compete for binding with biotinylated transferrin (B-SS-Tfn). See Support Protocol 5 for preparation of cytosol from tissue culture cells.

Materials

KSHM buffer (see recipe), ice cold
Tissue (brain or liver), freshly harvested or stored at −80°C
Liquid nitrogen

Motor-driven 50-ml Dounce homogenizer with Teflon restle
Medium-speed centrifuge and rotor (e.g., Beckman J2-21 with JA 25.5 rotor), 4°C, and centrifuge tubes
Ultracentrifuge (e.g., Beckman LE-80K with 45Ti rotor), 4°C, and ultracentrifuge tubes

1. Add an equal volume of KSHM buffer to brain or liver tissue that has been chopped into small pieces.

   Tissue can be used either fresh or frozen. If frozen tissue is used, it must be frozen as quickly as possible following slaughter, preferably in liquid nitrogen, and must be stored at −80°C.

   If mouse or rat brains are used, it is possible to pool several brains to make a larger batch of cytosol.

2. Homogenize in a motor-driven 50-ml Dounce homogenizer at 4°C. Homogenize until most material is in a smooth paste, but avoid foaming, which will lead to protein denaturation.

   CAUTION: *When using a motor-driven Dounce homogenizer, eye protection and protective gloves should always be worn.*

3. Centrifuge homogenate 20 min at 10,000 × *g*, 4°C, to remove large aggregates.

4. Transfer supernatant to an ultracentrifuge tube and centrifuge 1 hr at 100,000 × *g*, 4°C, to yield a cytosol supernatant and a microsomal pellet.

5. Transfer supernatant to microcentrifuge tubes in small aliquots (100 to 500 µl). Snap-freeze cytosol in liquid nitrogen before storage at −80°C for up to at least 1 year.

   For each experiment, a fresh aliquot is thawed rapidly at 37°C before being placed on ice for subsequent addition to the assay tubes.

   CAUTION: *When snap-freezing in liquid nitrogen, a face mask and protective gloves should always be worn.*

SUPPORT PROTOCOL 5

PREPARATION OF CYTOSOL FROM TISSUE CULTURE CELLS

Although adherent cells may be used for the preparation of cytosol, cells grown in suspension, (e.g., K562 or sHeLa cells) are generally used, as they can be conveniently grown in reasonable quantities.

Additional Materials *(also see Support Protocol 4)*

Cells grown in suspension
KSHM buffer (see recipe) containing protease inhibitors (e.g., Complete EDTA-Free Protease Inhibitor Cocktail; Roche), ice cold
10-ml Dounce homogenizer with Teflon pestle, optional

1. Maintain cells in suspension at a density between 0.2×10^5 and 1×10^6 cells per ml.

   The authors generally prepare cytosol from 1 to 2 liters of cells in suspension (or twenty 15-cm plates if adherent cells are used).

2. Collect cells by centrifugation at 1,000 × *g* for 5 min.

   Adherent cells should be collected by trypsinization (UNIT 1.1) before pelleting.

3. Resuspend cell pellet in 5 vol ice-cold KSHM buffer containing protease inhibitors.

4. Lyse cells either by homogenization using a 10-ml Dounce homogenizer (10 to 20 passes) or by one or two cycles of freeze-thawing. Maintain homogenate at 4°C at all times.

   Freeze-thawing is carried out by freezing the cell pellet in liquid nitrogen followed by thawing rapidly at 37°C before placing on ice. The degree of cell breakage can be assessed either by measurement of release of lactate dehydrogenase (see Support Protocol 9) or by assessing accessibility to trypan blue using light microscopy (UNIT 1.1). More than one cycle of freeze-thawing may be required to permeabilize the majority of the cells. In the case of both homogenization and freeze-thawing, however, care should be taken to avoid extensive damage to a limited number of cells, as this could result in the release of lysosomal enzymes with potential proteolytic activity.

5. Prepare cytosol by ultracentrifugation of the cell lysate for 1 hr at 100,000 × *g*, 4°C.

6. Transfer supernatant to cryovials in small aliquots (100 to 500 µl). Snap-freeze cytosol in liquid nitrogen before storage at −80°C for up to 1 year.

   For each experiment, a fresh aliquot is thawed rapidly at 37°C before being placed on ice for subsequent addition to the assay tubes.

   CAUTION: *When snap-freezing in liquid nitrogen, a face mask and protective gloves should always be worn.*

SUPPORT PROTOCOL 6

PREPARATION OF AN ATP-REGENERATING SYSTEM

ATP is required for endocytosis. To allow endocytosis to occur in permeabilized cells, it is thus necessary to provide ATP. A regenerating system ensures that the concentration of ATP is maintained at a constant level and hence never becomes limiting. The ATP-regenerating system is prepared as a 10× stock and consists of a mixture of ATP, creatine phosphokinase, and phosphocreatine. When ATP is hydrolyzed to ADP, creatine phosphokinase transfers phosphate from phosphocreatine to regenerate ATP, thus maintaining ATP at a constant level.

Materials

ATP (Sigma)
Phosphocreatine (Sigma)
Creatine phosphokinase (Sigma)
50% (v/v) glycerol in KSHM buffer (see recipe)

1. Prepare the following stock solutions:

   200 mM ATP
   200 mM phosphocreatine
   0.5 mg/ml creatine phosphokinase in 50% (v/v) glycerol in KSHM buffer.

   Stocks can be stored in aliquots at −20°C for up to 6 months.

   It is critical that stocks of ATP are maintained at neutral pH. Addition of ATP to many buffers at pH 7 will cause a substantial reduction in pH, and so the solution needs to be titrated to pH 7 or above before dividing into aliquots and freezing. The concentration of ATP should be determined by spectrophotometry using a molar extinction coefficient of 15.4×10^3 *at pH 7.0 and 260 nm.*

2. Prepare ATP-regenerating system just prior to use by making a solution of the following:

   10 μl 200 mM ATP
   200 μl 200 mM phosphocreatine
   10 μl 0.5 mg/ml creatine phosphokinase.

PREPARATION OF AN ATP-DEPLETING SYSTEM

SUPPORT PROTOCOL 7

Because endocytosis is dependent on ATP, certain control experiments in permeabilized cells require the use of an ATP-depleting system, which effectively removes all of the available ATP. Inclusion of an ATP-depleting system allows an estimate of the contribution made by any remaining intact cells to the endocytosis signal measured, because the ATP-depleting system is not cell permeable and so will only prevent endocytosis in permeabilized cells. The ATP-depleting system is prepared as a 10× stock and consists of a mixture of hexokinase and glucose. Hexokinase catalyzes the formation of glucose-6-phosphate from glucose, using ATP as a source of phosphate. Hexokinase is often supplied as an ammonium sulfate precipitate, which is stable for long periods at 4°C.

NOTE: Prepare ATP-depleting system just prior to use.

Materials

Ammonium sulfate suspension of hexokinase (Sigma)
500 mM glucose
KSHM buffer (see recipe)
Microcentrifuge, 4°C

1. Centrifuge 10 μl of an ammonium sulfate suspension of 10,000 U/ml hexokinase at 22,000 × *g* for 1 min at 4°C.
2. Discard supernatant and resuspend pellet in 10 μl of 500 mM glucose and 40 μl KSHM.

SUPPORT PROTOCOL 8

PREPARATION OF COAT PROTEINS

Coat proteins are prepared from either bovine or porcine brains according to modifications of the method of Campbell et al. (1984).

Materials

Bovine or porcine brains
Buffer A (see recipe)
12.5% (w/v) Ficoll/12.5% (w/v) sucrose in buffer A
Buffer B: 1 vol buffer A and 3 vol of 1 M Tris·Cl, pH 8 (*APPENDIX 2A*)
Ammonium sulfate
1 M DTT (*APPENDIX 2A*)
100 mM PMSF in 100% ethanol or isopropanol, store at −20°C
Staining solution: 40% (v/v) methanol/7% (v/v) acetic acid/25 g/liter Coomassie brilliant blue R250
Destaining solution: 15% (v/v) methanol/7% (v/v) acetic acid in water
10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*), for AP mix purification only
KSHM buffer (see recipe), for clathrin purification only
Liquid nitrogen
Kitchen blender
Medium-speed centrifuge and rotor (e.g., Beckman J2-21 with JLA 10.5 and JA 25.5 rotors), 4°C, and 500- and 50-ml centrifuge tubes
Ultracentrifuge (e.g., Beckman LE-80K with 45Ti rotor), 4°C, and 65-ml ultracentrifuge tubes
50-ml Dounce homogenizer with Teflon pestle
2-liter graduated cylinder
Sephacryl S-400 column (26 mm × 1 m)
Fraction collector and tubes
50-ml Vivaspin concentrators (Vivascience)
Dialysis membrane (10,000 MWCO)
Cryovials
Additional reagents and equipment for SDS–polyacrylamide gel electrophoresis (*UNIT 6.1*)

NOTE: All procedures are carried out at 4°C and all solutions should be ice cold.

Homogenize brains

1. Collect 1 kg bovine or porcine brains as soon as possible after animal sacrifice.

   Brains should be used as soon as possible after slaughter. If this is not possible, the tissue should be transported on dry ice from the slaughterhouse and stored frozen at −80°C. If frozen, the brains should be thawed overnight at 4°C or for 2 hr at room temperature.

2. Homogenize brains in an equal volume of buffer A with two 30-sec pulses in a kitchen blender.

Isolate coated vesicles

3. Centrifuge homogenate 30 min in a 500-ml tube at 12,000 × *g* in a JLA 10.5 rotor. Transfer supernatant to a clean 500-ml centrifuge tube.
4. Re-extract pellet in 600 ml buffer A and centrifuge at 12,000 × *g* for 30 min.
5. Combine supernatants, divide between 65-ml ultracentrifuge tubes, and ultra centrifuge 1 hr in a 45Ti rotor at 49,000 × *g*.

6. Discard supernatant and resuspend resulting microsomal pellet in 110 ml buffer A using a 50-ml Dounce homogenizer (10 to 20 strokes). Use a smaller volume of buffer A for initial resuspension and then mix thoroughly with the remaining volume. Add 110 ml of 12.5% Ficoll/12.5% sucrose in buffer A.
7. Divide mixture between 50-ml centrifuge tubes and centrifuge 40 min at 41,400 × *g* in a JA 25.5 rotor.
8. Transfer supernatant to a 2-liter graduated cylinder and dilute to 1 liter in buffer A. Divide between 65-ml ultracentrifuge tubes and ultracentrifuge 1 hr at 85,000 × *g* to pellet the coated vesicles.

Isolate coat proteins

9. To strip the coat proteins from the vesicles, resuspend pellet in ∼120 ml buffer B using the Dounce homogenizer (five to ten strokes) and incubate ≥15 min on ice.
10. To remove membranes, ultracentrifuge 1 hr at 158,000 × *g*. Re-extract membrane pellets by resuspension in buffer B and centrifugation as before.
11. Precipitate coat proteins from the combined supernatants by addition of solid ammonium sulfate to 50% saturation (e.g., 29.5 g per 100 ml supernatant).
12. To recover the precipitate, centrifuge 15 min at 12,000 × *g* and resuspend in ∼8 ml buffer B.

Purify clathrin and adaptor proteins

13. Apply this material to a 26-mm × 1-m Sephacryl S-400 column equilibrated in 800 ml (∼2 column vol) buffer B containing 0.1 mM DTT and 0.1 mM PMSF, added just before use. Elute with 600 ml buffer B. Collect 5-ml fractions with a fraction collector.
14. Identify fractions containing clathrin and the adaptor proteins by SDS-PAGE (*UNIT 6.1*) on an 8% SDS–polyacrylamide gel. Use staining and destaining solutions to visualize the proteins with Coomassie blue.

    Following gel filtration in Sephacryl S-400, the adaptor pool is a mixture of the plasma membrane adaptor AP2 and the trans-Golgi network adaptor AP1. Previous studies have compared the activity of this AP mix with AP2 that had been separated from AP1 and found that they exhibit the same activity, indicating that AP1 has no apparent competitive effect on AP2 for clathrin-coated pit and vesicle formation at the plasma membrane (Smythe et al., 1992). This means that for many experiments in the permeabilized cells, the mix of APs does not require further purification. In some cases, however, it may be important to prepare purified AP2. AP2 can be purified from AP1 according to the method of Matsui and Kirchhausen (1990) or alternatively according to the method of Pauloin and Thurieau (1993). Both methods rely on the differential binding of AP1 and AP2 to hydroxyapatite. The difference in the methods is that one yields AP2 that still has the μ2 kinase associated with it (Matsui and Kirchhausen, 1990) whereas the other yields AP2 free of μ2 kinase (Pauloin and Thurieau, 1993).

    For more information about staining protein gels, see UNIT 6.6.

15. Pool fractions containing clathrin (630 kDa) and adaptor proteins (∼320 kDa) separately.

    Clathrin can easily be identified by the presence of an ∼180-kDa heavy chain and two light chains of ∼30 kDa. The APs may be identified by several bands at ∼100 kDa and by the 50-kDa μ2 subunit.

16. Concentrate to ∼1 mg/ml using 50-ml vivaspin concentrators according to manufacture's instructions.

17. Dialyze against two changes of 1 liter of 10 mM Tris·Cl, pH 7.5 (for APs) or KSHM (for clathrin) for 2 to 3 hr or overnight at 4°C.

18. Using liquid nitrogen, snap-freeze in 100-µl aliquots in microcentrifuge tubes before storage at −80°C for up to at least 1 year.

    CAUTION: *When dispensing and snap-freezing in liquid nitrogen, a face mask and protective gloves should always be worn.*

    A fresh aliquot is thawed rapidly at 37°C for each assay.

SUPPORT PROTOCOL 9

LACTATE DEHYDROGENASE ASSAY

Lactate dehydrogenase (LDH) is a cytosolic enzyme, the release of which provides a good estimate of how well cells are permeabilized. LDH catalyzes the conversion of pyruvate to lactate coupled to the oxidation of NADH (Kornberg, 1955). The oxidation of nicotinamide adenine dinucleotide (NADH) is followed spectrophotometrically at 340 nm. A small aliquot of the total permeabilized cells is used to estimate the extent of cell breakage.

Materials

Cell suspension samples, unpermeabilized and permeabilized with method of choice (see Support Protocol 3)
KSHM buffer (see recipe)
0.5% (v/v) Triton X-100 in PBS (*APPENDIX 2A*)
LDH reaction buffer (see recipe)

Microcentrifuge, 4°C
Spectrophotometer with UV detector (340 nm) and quartz cuvettes

1. After initial permeabilization, dilute cells to 2×10^5 cells/ml with KSHM to promote the release of cytosol and then pellet by centrifugation at $1000 \times g$ for 5 min at 4°C. Analyze an equal number of unpermeabilized (intact) cells in parallel.

2. Resuspend cell pellet in 0.5% Triton X-100 in PBS. Leave on ice for 10 min.

3. Add a small volume of the cell suspension (1 to 5 µl) to a quartz cuvette and start the reaction by the addition of 1 ml LDH reaction buffer.

4. Follow the rate of oxidation of NADH by inserting the cuvette into a spectrophotometer and taking readings at 340 nm for 1 to 10 min, depending on the activity of the sample.

   The initial rate of enzyme activity is used to determine the amount of LDH remaining in the cells and is compared with that of an equal number of intact cells to assess the efficiency of permeabilization. Cell permeabilization should be >90% efficient (i.e., the LDH signal from permeabilized cells should be <10% of that from the intact cells).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.2% (w/v) BSA
1% (w/v) Triton X-100
0.1% (w/v) SDS (*APPENDIX 2A*)
1 mM EDTA

continued

50 mM NaCl (*APPENDIX 2A*)
Store up to 1 week at room temperature

Blocking buffer can also be stored as a 5× stock without BSA for up to 2 months at room temperature.

Buffer A

100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5
0.5 mM $MgCl_2$ (*APPENDIX 2A*)
1 mM EDTA
1 mM DTT (*APPENDIX 2A*)
Store up to 1 week at 4°C (without PMSF)
Add 0.1 mM PMSF immediately before use

PMSF is a serine protease inhibitor that has a very short half-life in aqueous solution. A stock solution (100 mM) can be prepared in 100% ethanol or isopropanol. Immediately prior to use, this solution can be diluted into the relevant buffer, which should be vigorously stirred to enhance the solubility of PMSF in aqueous solution.

H_2SO_4, 2 M

Dilute 53.30 ml of 98% pure H_2SO_4 liquid (specific gravity, 1.84 g/ml; mol. wt., 98.07) into water to make 500 ml of a 2 M solution. Store up to 1 year at room temperature.

CAUTION: *Dilution of sulfuric acid is an exothermic reaction, and thus this solution should be prepared in a fume hood by the slow addition of the acid to distilled water with gentle stirring. Adequate eye protection should be worn.*

KSHM buffer

20 mM HEPES, pH 7.4
100 mM potassium acetate
85 mM sucrose
1 mM $MgCl_2$ (*APPENDIX 2A*)
Store up to 1 week at 4°C

The composition of this buffer has been chosen to mimic the intracellular environment as closely as possible.

Protease inhibitors should be included when preparing cytosol samples. Tablets containing a mixture of protease inhibitors with a broad substrate specificity are commercially available (e.g., Complete EDTA-Free Prolease Inhibitor Cocktail; Roche)

LDH reaction buffer

0.33 mM pyruvate (Sigma)
0.132 mM reduced NADH (Sigma; diluted from a 1.32 mM stock made fresh in 1 mM $NaHCO_3$)
250 mM sucrose (Sigma)
6.6 mM HEPES, pH 7.2 (Merck)
Prepare fresh

MesNa buffer

50 mM Tris·Cl, pH 8.6 (*APPENDIX 2A*)
100 mM NaCl (*APPENDIX 2A*)
1 mM EDTA
0.2% (v/v) BSA
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

Clathrin-mediated endocytosis is responsible for the cellular uptake of a variety of biologically important macromolecules, including nutrients, growth factors, and hormones (Mellman, 1996). Material destined for internalization is packaged into specialized regions of the membrane called coated pits. The coated pits invaginate and pinch off forming coated vesicles. Following uncoating and fusion with the early endosomal compartment, internalized material is sorted to a variety of destinations within the cell. It may be recycled to the cell surface, delivered to late endosomes and ultimately lysosomes, or transported across the cell in the process of transcytosis. Material can be internalized either bound to specific transmembrane receptors (receptor-mediated endocytosis) or by being present in the soluble material captured by coated pits as they become coated vesicles (fluid-phase endocytosis). Bulk membrane is similarly internalized if included in clathrin-coated vesicles (absorptive endocytosis).

As well as being a fundamental process in almost all eukaryotic cells, coated-vesicle formation plays a critical role in a number of tissue-specific events such as synaptic-vesicle recycling, the control of morphogen gradients during development, immune surveillance, and downregulation of receptor tyrosine kinases that have mitogenic effects (Conner and Schmid, 2003). Furthermore, the endocytic pathway is often hijacked by pathogens in order to infect cells (Marsh and Pelchen-Matthews, 2000). For these reasons, it is important to have robust assays that allow the measurement of clathrin-mediated endocytosis in intact cells.

Coated pits form from the assembly of clathrin, the AP2 adaptor complex, and other accessory proteins from the cytosol onto the membrane. Clathrin polymerizes onto the membrane to form the characteristic lattice diagnostic of coated pits. The heterotetrameric AP2 complex is the other major protein component of isolated coated vesicles. Within the clathrin lattice, it links clathrin to transmembrane cargo such as the transferrin receptor (Ohno et al., 1995). AP2 is composed of two 100-kDa subunits (α- and β2-adaptin), a 50-kDa subunit (μ2), and a 17- to 19-kDa subunit (σ2). AP2 interacts with clathrin via the hinge of β2-adaptin (Shih et al., 1995) and with cargo via the μ2 subunit (Ohno et al., 1995; Owen and Evans, 1998). The large-molecular-weight GTPase dynamin functions in the invagination and scission of clathrin-coated pits, although its precise mechanism of action remains controversial (Song and Schmid, 2003). In addition to clathrin, AP2, and dynamin, a host of other proteins (including eps15, epsin, β-arrestin, Dab2, numb, ARH, AP180, amphiphysin, endophilin, and rab5) are involved in coated-vesicle formation, and their functions are beginning to be defined (Brodsky et al., 2001; Traub, 2003). Some of these proteins, such as epsin, Dab2, ARH, and β-arrestin, may act as adaptors for specific cargo (Bonifacino and Traub, 2003). Many of these proteins are involved in a complex series of interactions with each other and with AP2 and clathrin. Furthermore, there is increasing evidence for links between coated-vesicle formation and the actin cytoskeleton with such proteins as intersection, syndapin, and mAbp1 being important in mediating these interactions (Schafer, 2002; Smythe and Ayscough, 2003). This implies that coated-vesicle formation results from a complex series of interactions between different components that is regulated in space and time. The real challenge for the future lies in deciphering how these interactions are regulated.

One approach to understanding the complexity of these interactions is through the use of cell-free assays that reconstitute individual stages in the coated-vesicle cycle. These assays allow the manipulation of endocytic components so that their temporal and spatial interactions can be determined. These assay systems are not only applicable to understanding the regulation of the core endocytic machinery but might also be used to investigate how specific cargo (e.g., receptor tyrosine kinases) modulate the core machinery to facilitate their internalization (Lamaze and Schmid, 1995; Lanzetti et al., 2000; Haglund et al., 2002).

Other ligands

The uptake of transferrin bound to its receptor is used extensively to study receptor-mediated endocytosis because the pathway followed by transferrin has been extensively characterized (*UNIT 15.3*). It is known that the kinetics of the internalization of the receptor alone are identical to those of the ligand-bound receptor (Watts, 1985). Although transferrin is a valuable reporter molecule that allows examination of the core endocytic machinery, the uptake of other ligands and

receptors may also be measured in permeabilized cells. The assay systems may, for example, be modified to measure the uptake of other transmembrane proteins. A ligand may be biotinylated as described for transferrin. If no ligand is readily available, specific antibodies that can be biotinylated may also be used as ligands. Care needs to be taken using this approach because antibodies are divalent and can potentially cross-link receptors and induce their internalization via other endocytic routes (e.g., phagocytosis; *UNIT 15.7*). Therefore, it is advisable either to confirm that antibody cross-linking is not occurring using intact cells (e.g., by potassium depletion of cells, which is a reasonably specific inhibitor of clathrin-mediated endocytosis; Larkin et al., 1983) or, perhaps more cleanly, to use antibody Fab fragments (Harlow and Lane, 1988).

If no suitable ligands are available, it is still possible to measure internalization of particular transmembrane proteins by biotinylation of the entire cell surface using NHS-SS-biotin. Biotinylated cargo protected from avidin or MesNa may then be collected following immunoprecipitation (*UNIT 7.2*) or by immunoblotting (*UNIT 6.2*) of material captured on avidin agarose (Schmidt et al., 1997; Palacios et al., 2002).

When examining the stimulated uptake of cell surface receptors such as growth factor receptors in vitro, it should be borne in mind that there is a significant uptake of membrane simply by inclusion into clathrin-coated pits as they invaginate and pinch off. This must be taken into account when designing a permeabilized cell assay for this type of receptor, and the amount of sequestration or internalization by bulk flow must be subtracted from the total signal. This has been demonstrated for epidermal growth factor (EGF) receptor uptake (Lamaze et al., 1993; Lamaze and Schmid, 1995). In this case, an antagonistic antibody to the receptor was used to assess bulk internalization in the absence of EGF under conditions where this antibody had been shown not to induce internalization in intact cells. Analysis showed that bulk uptake of material accounted for ~50% of the signal seen, demonstrating the importance of this measurement in accurately assessing the role of individual components in stimulated uptake.

Critical Parameters

Cell maintenance and permeabilization

The permeabilized assays may be performed in a variety of different cell types. The major criteria are that the cells are in good condition and that permeabilization of the cells occurs efficiently, as the success of the assay depends on this.

Cell maintenance. For optimal efficiency of reconstitution of the coated-vesicle cycle in permeabilized cells, care should be taken to maintain cells in logarithmic phase. It is worth screening a variety of conditions of serum and growth and degrees of confluency to establish the optimum conditions of permeabilization for particular cell types. In the authors' experience, for example, they reconstitute different stages of the coated-vesicle cycle in A431 cells most efficiently when cells are grown in defined fetal bovine serum (HyClone) and seeded <24 hr prior to permeabilization, with a final confluency of 70% to 90%.

Cell permeabilization. The optimal method used to permeabilize the cells also needs to be determined empirically. The biochemical assays described in this unit for cargo sequestration and internalization are reliant on efficiencies of permeabilization of >90% (measured by LDH release; see Support Protocol 9). Cell preparations where ≤90% of the cells are permeabilized are unsuitable because endocytosis by the remaining intact cells in the preparation will make a significant contribution to the background signal measured in incubations containing an ATP-depleting system only. Different cell lines show different degrees of permeabilization, however, and it is beneficial to screen several different conditions of permeabilization to ensure maximum efficiency of reconstitution of the clathrin coated–vesicle cycle. Several possible methods are listed in Support Protocol 3. One criterion by which to judge the effectiveness of the permeabilization procedure is visualization of the cells by light microscopy following staining with trypan blue (*UNIT 1.1*). Analysis of the permeabilized cell preparation by electron microscopy will provide further confidence that the overall morphology of the cells is maintained. Ultimately, however, the best measure of a good permeabilization protocol is efficient ligand sequestration and internalization.

Ligand detection

Transferrin receptors are abundant on most cell types, but for other transmembrane proteins, it is important to ensure that the method of detection will be sufficiently sensitive to allow detection of small amounts of endocytosed protein. For example, if surface biotinylation is used to label a transmembrane protein, it is essential that enough biotin moieties are

attached so that between 1% and 5% of the total biotinylated transmembrane protein can still be detected, as this will be the amount internalized at early time points. Another important parameter that should be determined is the linearity of the ELISA plate signal by optimizing the amount of cell lysate that is plated.

Anticipated Results

In intact cells (Basic Protocol 1), the avidin and MesNa assays will measure the same amount of internalized B-SS-Tfn in control cells. This will peak to a maximum of 40% to 60% of the total cell-associated B-SS-Tfn after 5 to 10 min. There is then a drop in the amount of B-SS-Tfn that is apparently cell associated as a result of recycling of B-SS-Tfn back to the cell surface and discharge into the medium (Hopkins and Trowbridge, 1983). Differences in the extent of internalization measured by these assays will be detected under conditions where, for example, scission is inhibited but invagination is not. This could result from transfection of a dominant-negative mutant form of a protein required specifically for scission. Under these conditions, the avidin assay will measure increased apparent internalization as compared with the MesNa assay, although the difference in signals will actually be due to increased sequestration in deeply invaginated coated pits.

In Basic Protocol 2, where endocytosis is being measured in permeabilized cells, the avidin assay measures B-SS-Tfn sequestration into deeply invaginated coated pits and internalization into coated vesicles, whereas the MesNa assay will measure internalization into coated vesicles only. This difference is reflected in the signal obtained with the two assays. Routinely, 25% to 50% of total B-SS-Tfn becomes avidin inaccessible, whereas 10% to 30% becomes MesNa resistant. Coat protein–dependent sequestration results in 10% to 20% of total B-SS-Tfn becoming avidin inaccessible. If results lower than these values are obtained, care should be taken in the interpretation of results, as this will indicate that the reconstitution of a particular step in the coated-vesicle cycle is suboptimal.

Time Considerations

Following preincubation with serum-free medium (0.5 hr), endocytic assays in intact cells (see Basic Protocol 1) require binding of B-SS-Tfn (0.5 to 1 hr); washing and internalization (0.5 to 1 hr); and incubation with avidin and biocytin, solubilization in blocking buffer, and plating on ELISA plates (2.5 to 3 hr). The ELISA plates may be incubated at 37°C for 3 hr or overnight at 4°C. Because the ELISA plates need to be incubated with streptavidin coupled to horseradish peroxidase (HRP; 1 hr) and the HRP reaction product is then developed and detected at 492 nm (0.5 to 0.75 hr), the overnight incubation at 4°C is usually the preferred option. It is also possible to interrupt the procedure following solubilization of the samples by freezing them, storing them at –20°C for up to 1 month, and applying them to ELISA plates at a later stage.

For endocytic assays in permeabilized cells (see Basic Protocol 2), following preincubation in serum-free medium, preparation of the permeabilized cells (which may include specific treatments of the membranes), incubation at 37°C to allow endocytosis to proceed, treatment with avidin and biocytin, solubilization, and plating onto ELISA plates requires 4 to 5 hr. Thus, incubation of the ELISA plates overnight at 4°C is usually most convenient, with addition of streptavidin-HRP and development of the HRP reaction product taking place the next day (1 to 2 hr).

Preparation of B-SS-Tfn (Support Protocol 1) will take between 1.5 and 2.5 hr. Preparation of ELISA plates (Support Protocol 2) involves ~30 min to plate the antibodies and ~15 min (either 3 hr later or on the next day) to wash the plates and prepare for storage. If streptolysin O permeabilization is the method of choice (Support Protocol 3), this could increase the time taken to permeabilize the cells to 40 min depending on the optimal incubation times at 37° and 4°C. Preparation of cytosol from brain (Support Protocol 4) will take ~15 min for the homogenization followed by 1.5 to 2 hr for centrifugation and freezing. Preparation of cytosol from cells in culture (Support Protocol 5) will take ~45 min for washes and homogenization followed by 1.5 to 2 hr for centrifugation and freezing. The preparation of coat proteins (Support Protocol 8) takes 2 to 3 days in total. In general, steps 1 to 12 are carried out on day 1 and steps 13 to 18 take a further 1 to 2 days. The lactate dehydrogenase assay (Support Protocol 9) requires 1 to 2 hr.

Literature Cited

Ahnert-Hilger, G., Bhakdi, S., and Gratzl, M. 1985. Minimal requirements for exocytosis. A study using PC 12 cells permeabilized with staphylococcal alpha-toxin. *J. Biol. Chem.* 260:12730-12734.

Benmerah, A., Lamaze, C., Begue, B., Schmid, S.L., Dautry, V.A., and Cerf, B.N. 1998. AP-2/Eps15

interaction is required for receptor-mediated endocytosis. *J. Cell Biol.* 140:1055-1062.

Bhakdi, S., Tranum-Jensen, J., and Sziegoleit, A. 1985. Mechanism of membrane damage by streptolysin-O. *Infect. Immun.* 47:52-60.

Bonifacino, J.S. and Traub, L.M. 2003. Signals for sorting transmembrane proteins from endosomes to lysosomes. *Annu. Rev. Biochem.* 72:395-447.

Brodsky, F.M., Chen, C.Y., Knuehl, C., Towler, M.C., and Wakeham, D.E. 2001. Biological basket weaving: Formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Dev. Biol.* 17:517-568.

Campbell, C., Squicciarini, J., Shia, M., Pilch, P.F., and Fine, R.E. 1984. Identification of a protein kinase as an intrinsic component of rat liver coated vesicles. *Biochemistry* 23:4420-4426.

Carter, L.L., Redelmeier, T.E., Woollenweber, L.A., and Schmid, S.L. 1993. Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. *J. Cell Biol.* 120:37-45.

Conner, S.D. and Schmid, S.L. 2003. Regulated portals of entry into the cell. *Nature* 422:37-44.

Damke, H., Baba, T., Warnock, D.E., and Schmid, S.L. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* 127:915-934.

Green, N.M. 1970. Spectrophotometric determination of avidin and biotin. *Methods Enzymol.* 18:51-67.

Haglund, K., Shimokawa, N., Szymkiewicz, I., and Dikic, I. 2002. Cbl-directed monoubiquitination of CIN85 is involved in regulation of ligand-induced degradation of EGF receptors. *Proc. Natl. Acad. Sci. U.S.A.* 99: 12191-12196.

Harlow, E. and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Hill, E., van der Kaay, J., Downes, C.P., and Smythe, E. 2001. The role of dynamin and its binding partners in coated pit invagination and scission. *J. Cell Biol.* 152:1-16.

Hopkins, C.R. and Trowbridge, I.S. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97:508-521.

Jackson, A.P., Flett, A., Smythe, C., Hufton, L., Wettey, F.R., and Smythe, E. 2003. Clathrin regulates cargo recruitment via activation of the AP2 associated μ2 kinase. *J. Cell Biol.* 163:231-236.

Jost, M., Simpson, F., Kavran, J.M., Lemmon, M.A., and Schmid, S.L. 1998. Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. *Curr. Biol.* 8:1399-1402.

Kornberg, A. 1955. Lactic dehydrogenase of muscle. *Methods Enzymol.* 1:441-454.

Lamaze, C. and Schmid, S.L. 1995. Recruitment of epidermal growth-factor receptors into coated pits requires their activated tyrosine kinase. *J. Cell Biol.* 129:47-54.

Lamaze, C., Baba, T., Redelmeier, T.E., and Schmid, S.L. 1993. Recruitment of epidermal growth-factor and transferrin receptors into coated pits in-vitro—differing biochemical requirements. *Mol. Biol. Cell* 4:715-727.

Lanzetti, L., Rybin, V., Malabarba, M.G., Christoforidis, S., Scita, G., Zerial, M., and Di Fiore, P.P. 2000. The Eps8 protein coordinates EGF receptor signaling through Rac and trafficking through Rab5. *Nature* 408:374-377.

Larkin, J.M., Brown, M.S., Goldstein, J.L., and Anderson, R.G. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell* 33:273-285.

Marsh, M. and Pelchen-Matthews, A. 2000. Endocytosis in viral replication. *Traffic* 2000. Aug. I:525-532.

Matsui, W. and Kirchhausen, T. 1990. Stabilization of clathrin coats by the core of the clathrin-associated protein complex AP-2. *Biochemistry* 29:10791-10798.

McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M., and Smythe, E. 1998. A novel role for rab5-GDI in ligand sequestration into clathrin-coated pits. *Curr. Biol.* 8:34-45.

Mellman, I. 1996. Membranes and sorting. *Curr. Opin. Cell Biol.* 8:497-498.

Morris, S.A. and Schmid, S.L. 1995. Synaptic vesicle recycling: The Ferrari of endocytosis? *Curr. Biol.* 5:113-115.

Ohno, H., Stewart, J., Fournier, M.C., Bosshart, H., Rhee, I., Miyatake, S., Saito, S., Gallusser, A., Kirchhausen, T., and Bonifacino, J.S. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* 269:1872-1875.

Olusanya, O., Andrews, P.D., Swedlow, J.R., and Smythe, E. 2001. Phosphorylation of threonine156 of the μ2 subunit of the AP2 complex is essential for endocytosis in vitro and in vivo. *Curr. Biol.* 11:896-900

Owen, D.J. and Evans, P.R. 1998. A structural explanation for the recognition of tyrosine-based endocytotic signals. *Science* 282:1327-1332.

Palacios, F., Schweitzer, J.K., Boshans, R.L., and D'Souza-Schorey, C. 2002. ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. *Nat. Cell Biol.* 4:929-936.

Pauloin, A. and Thurieau, C. 1993. The 50 kDa protein subunit of assembly polypeptide (AP) AP-2 adapter from clathrin-coated vesicles is phosphorylated on threonine-156 by AP-1 and a soluble AP50 kinase which co-purifies with the assembly polypeptides. *Biochem. J.* 296:409-415.

Schafer, D.A. 2002. Coupling actin dynamics and membrane dynamics during endocytosis. *Curr. Opin. Cell Biol.* 14:76-81.

Schmid, S.L. and Smythe, E. 1991. Stage-specific assays for coated pit formation and coated vesicle budding in vitro. *J. Cell Biol.* 114:869-880.

Schmidt, A., Hannah, M.J., and Huttner, W.B. 1997. Synaptic-like microvesicles of neuroendocrine cells originate from a novel compartment that is continuous with the plasma membrane and devoid of transferrin receptor. *J. Cell Biol.* 137:445-458.

Shih, W., Gallusser, A., and Kirchhausen, T. 1995. A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes. *J. Biol. Chem.* 270:31083-31090.

Simpson, F., Hussain, N.K., Qualmann, B., Kelly, R.B., Kay, B.K., McPherson, P.S., and Schmid, S.L. 1999. SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat. Cell Biol.* 1:119-124.

Smythe, E. and Ayscough, K. 2003. The Ark/Prk kinase family: Regulators of endocytosis and the actin cytoskeleton. *EMBO Rep.* 4:246-251.

Smythe, E., Carter, L.L., and Schmid, S.L. 1992. Cytosol-dependent and clathrin-dependent stimulation of endocytosis in vitro by purified adapters. *J. Cell Biol.* 119:1163-1171.

Song, B.D. and Schmid, S.L. 2003. A molecular motor or a regulator? Dynamin's in a class of its own. *Biochemistry* 42:1369-1376.

Traub, L.M. 2003. Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. *J. Cell Biol.* 163:203-208.

Ullrich, O., Horiuchi, H., Alexandrov, K., and Zerial, M. 1995. Use of Rab-GDP dissociation inhibitor for solubilization and delivery of Rab proteins to biological membranes in streptolysin O–permeabilized cells. *Methods Enzymol.* 257:243-253.

Watts, C. 1985. Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. *J. Cell Biol.* 100:633-637.

Watts, C. and Marsh, M. 1992. Endocytosis: What goes in and how? *J. Cell Sci.* 103:1-8

Contributed by Andrew Osborne
Harvard Medical School
Boston, Massachusetts

Alexander Flett and Elizabeth Smythe
University of Sheffield
Sheffield, England

In Vitro Analysis of Yeast Mitochondrial Protein Import

Mitochondrial targeting prediction programs (e.g., Mitoprot, *http://ihg.gsf.de/mitoprot.html*) are often used to predict whether a protein contains a mitochondrial targeting sequence, (usually at the N-terminus) or if the protein resides in the mitochondrion. However, these programs can give inaccurate results. For example, they can predict that known mitochondrial resident proteins localize to other organelles, or they can fail to recognize a mitochondrial protein due to lack of an N-terminal targeting sequence (Guda et al., 2004). Intact mitochondria isolated from *Saccharomyces cerevisiae* using the methods described in *UNIT 3.8* (Glick and Pon, 1995) can be used for in vitro mitochondrial protein import assays to confirm or reject the predicted ability of proteins to be imported into mitochondria. In addition, because protein import is highly conserved among metazoans, yeast mitochondria can be used to model mitochondrial protein import in higher eukaryotes such as plants and animals, which are not always amenable to genetic and biochemical manipulation. Alternatively, these protocols can be adapted to assays for import of proteins into plant and mammalian mitochondria. Finally, the import assay, when used in combination with genetic and biochemical strategies, can be used to study the mechanism of mitochondrial biogenesis.

Isolated *S. cerevisiae* mitochondria can also be used for in organello translation assays for studying export of mitochondrial proteins translated in the yeast matrix into the inner mitochondrial membrane. These assays are performed by incubating isolated mitochondria in a buffer containing high concentrations of ATP, GTP, and amino acids. One of the amino acids added, usually methionine, is radiolabeled, and new synthesis of proteins in the isolated mitochondria is monitored by the incorporation of the radiolabeled amino acid into the newly synthesized polypeptide chains. Following translation, the mitochondria are re-isolated by centrifugation and lysed in an SDS-containing buffer. The mitochondrial proteins are resolved by SDS-PAGE and the newly synthesized proteins detected through fluorography or by using a PhosphorImager.

This unit describes methods for importing in vitro–translated (see *UNIT 11.2*) or recombinant (e.g., see Ausubel et al., 2007 or Coligan et al., 2007) proteins into isolated yeast mitochondria (*UNIT 3.8*) and exporting mitochondrial proteins translated in the yeast mitochondrial matrix into the inner mitochondrial membrane. Yeast cells are generally grown in a nonfermentable carbon source (e.g., lactate, ethanol, or glycerol) to promote respiration and hence increase mitochondrial proliferation and improve bioenergetic fitness and yield. Mitochondria isolated from yeast (*UNIT 3.8*) can be quickly frozen in liquid nitrogen and stored at −80°C for extended periods of time before use in import assays.

Mitochondrial precursor proteins (proteins not yet imported into mitochondria) can be derived from an in vitro transcription/translation reaction in reticulocyte lysate or purified from an *E. coli* recombinant protein expression system. The translocation (import and export) assays described in this unit can be used to test whether a protein is targeted to mitochondria, determine its location within the mitochondrion, and study its import mechanism. In addition, translation in the mitochondrial matrix and export of the translated proteins can also be investigated. The in vitro mitochondrial import and export methods are described in the Basic Protocols 1 and 2. Methods for fractionating mitochondria and analyzing the results of import and export assays are provided in Alternate Protocols 1 and 2.

Contributed by Rosemary A. Stuart and Carla M. Koehler

Current Protocols in Cell Biology (2007) 11.19.1-11.19.20

BASIC PROTOCOL 1

IN VITRO MITOCHONDRIAL PROTEIN IMPORT ASSAY: IMPORT OF LABELED PROTEINS

Generally, intact mitochondria are incubated with in vitro–translated, radiolabeled mitochondrial precursor protein in the presence of an energy form (ATP) and metabolites (e.g., NADH) to generate a membrane potential. The mitochondria, often treated with protease (e.g., trypsin or proteinase K) to remove any nonimported precursor, are re-isolated, and their proteins are analyzed to detect the imported protein by SDS-PAGE (*UNIT 6.1*) followed by fluorography (*UNIT 6.3*) or use of a PhosphorImager. The mitochondrion contains four compartments—the outer and inner membranes, intermembrane space, and matrix—to which the precursor can be imported (Koehler, 2004). Many proteins are synthesized with a cleavable N-terminal targeting sequence that is removed following import. As an example, this protocol describes the import of a typical precursor, the targeting sequence from *Neurospora crassa* ATPase subunit 9 fused to the reporter dihydrofolate reductase (Su9-DHFR; Pfanner et al., 1987), which is cleaved when imported into the matrix. This cleavage results in a shift in molecular weight and provides a convenient indication that import has occurred. However, many proteins (e.g., those of the inner and outer membrane and intermembrane space) may not be proteolytically processed during import. To confirm import of this category of proteins, other techniques are used: (1) In the case of an inner membrane–targeted protein, the membrane potential can be dissipated with uncouplers that generally prevent the import of a precursor. (2) For membrane proteins, resistance to alkali extraction or generation of a characteristic pattern of protease-resistant fragments can confirm integration into the membrane. Basic Protocol 1 should serve as a starting point for performing basic import assays.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

Materials

Freshly isolated or frozen, intact mitochondria (*UNIT 3.8*)
Buffer C (see recipe)
2× import buffer (see recipe)
0.1 M ATP (see recipe)
0.5 M NADH (see recipe)
1 mg/ml valinomycin in ethanol: store in 50-µl aliquots up to 6 months at −20°C
In vitro–translated [^{35}S]methionine-labeled mitochondrial precursor protein (Support Protocol 1) (e.g., *Neurospora crassa* Su9-DHFR)
10 mg/ml trypsin in 20 mM potassium HEPES, pH 7.4 (see recipe): prepare 0.5 ml fresh
20 mM potassium HEPES, pH 7.4 (see recipe)
20 mg/ml trypsin inhibitor (see recipe), freshly prepared
SDS sample buffer (*APPENDIX 2A*)

15-ml centrifuge tubes
25°C water bath
Refrigerated microcentrifuge

Prepare mitochondria

1. Thaw the mitochondrial aliquot quickly by hand and then place on ice. Dilute the mitochondria to 10 mg protein/ml with buffer C, if necessary.

2. To remove BSA (if used in the storage medium), wash the mitochondria by adding 0.5 ml buffer C, centrifuging 5 min at 14,000 × *g*, 4°C, and resuspending the pellet in buffer C to 10 mg protein/ml.

   The mitochondria are typically frozen in 25 mg/ml aliquots supplemented with 10 mg/ml BSA (see UNIT 3.8). If not maintained in an osmotically stabilized buffer, mitochondria can rupture or shrink, becoming import incompetent.

   Removal of excess BSA facilitates the protease treatment to remove nonimported precursors in subsequent steps.

Perform import assay

3a. *To perform the import reaction in the presence of a membrane potential* ($\Delta\Psi$): Add the reagents in the order presented to a 15-ml centrifuge tube (500 µl total reaction volume). Mix well after each addition by gentle vortexing.

   250 µl 2× import buffer
   10 µl 0.1 M ATP
   2 µl 0.5 M NADH
   207 µl H_2O
   10 µl mitochondria (10 mg/ml)
   1 µl ethanol

3b. *To perform the import reaction in the absence of a membrane potential (control):* Add the reagents in the order presented to a 15-ml centrifuge tube (500 µl total reaction volume). Mix well after each addition by gentle vortexing.

   250 µl 2× import buffer
   10 µl 0.1 M ATP
   2 µl 0.5 M NADH
   207 µl H_2O
   10 µl mitochondria (10 mg/ml)
   1 µl 1 mg/ml valinomycin

   This is a general import reaction; volume, mitochondria amount, and temperature can be varied. Because protein import generally requires a membrane potential, it is important to include a control import reaction in which mitochondria are dissipated of $\Delta\Psi$ by uncoupling, i.e., dissipating aspects of the proton motive force across the energized inner mitochondrial membrane. The uncoupler valinomycin has limited solubility in water and therefore should be added to the tube after the mitochondria. An alternative uncoupler is FCCP.

4. Incubate the reaction 1 to 2 min at 25°C.

5. Add 20 µl in vitro–translated radioactive precursor protein (e.g., Su9-DHFR), either directly from the reticulocyte lysate (Support Protocol 1, step 2) or denatured with urea (Support Protocol 1 step 6) while gently vortexing the import reaction to ensure adequate mixing.

   Intact mitochondria should be handled gently.

   Some proteins such as those that reside in the inner membrane and lack an N-terminal cleavable targeting sequence (e.g., Tim22 or the adenine nucleotide translocator) must be imported directly from the reticulocyte lysate. The chaperone proteins in the reticulocyte lysate are bound to the precursors and maintain them in an import competent state. However, most precursors that contain an N-terminal targeting sequence can be imported either directly from the reticulocyte lysate or in a urea denatured form.

The amount of in vitro–translated precursor protein added to each reaction can vary considerably depending on the efficiency of labeling and import. A model import precursor Su9-DHFR (Neurospora crassa subunit 9 fused to dihydrofolate reductase) is imported with efficiencies of up to 50% of the added precursor. However, the recommended import efficiency is estimated at 5% to 20% for previously untested precursors. With this in mind, the amount of a precursor should be increased proportionately to ensure sensitivity in detection with film or a PhosphorImager. In addition, processing of the targeting sequence may result in the removal of [^{35}S]methionine residues, thereby, decreasing sensitivity. For urea-denatured precursors, the dilution into the import assay should be at least 1:13.3 because the final urea concentration cannot exceed 0.6 M to ensure that mitochondria integrity is unaffected.

6. Incubate 10 min at 25°C. Agitate gently every other minute to facilitate gas exchange.

   The import time can be varied depending upon the robustness of import. In addition, the reaction can be expanded into an import time course by removing aliquots at particular time points.

7. Stop the import reaction by adding 1 µl of 1 mg/ml valinomycin to each tube.

Characterize import results

8. Remove two aliquots of 200 µl each from each reaction and transfer to separate 1.5-ml microcentrifuge tubes.

9. Treat mitochondria in one tube with protease to remove nonimported precursor. Treat mitochondria in the other tube (the control) in the same manner, but add buffer instead of trypsin solution.

   a. Add 2.5 µl 10 mg/ml trypsin or 20 mM potassium HEPES, pH 7.4 (control) to the aliquot from the import assay and incubate on ice for 30 min.

   b. Add 2.5 µl 20 mg/ml trypsin inhibitor to inhibit the trypsin and incubate on ice for 15 min.

   A concentration of 50 µg protease/ml during the protease treatment is standard for the analysis of imported proteins across the outer membrane. As an alternative protease, 10 µg/ml proteinase K can be used followed by 200 mM PMSF to inactivate the protease, but trypsin is generally used. A precursor that is imported into the outer membrane facing the cytosol will be protease sensitive; carbonate extraction (Alternate Protocol 2) can be used to evaluate import insertion into the outer membrane.

10. Microcentrifuge 5 min at 20,000 × *g*, 4°C.

11a. *To fractionate mitochondria and analyze the reaction for localization:* Discard the supernatant and resuspend the pellet as described in Alternate Protocol 1.

11b. *To prepare samples for SDS-PAGE:* Discard the supernatant and resuspend the protein pellet in 20 µl SDS sample buffer and proceed to Support Protocol 2 or store up to 3 days at −20°C before proceeding with SDS-PAGE.

   Steps 11a and 11b may be used as alternate or parallel steps, depending on the information desired. Step 11a is used when proceeding to Alternate Protocol 1 to determine where in the mitochondria (matrix, membranes, or intermembrane space) the imported proteins were incorporated. Step 11b can be used alone when characterizing the overall import and calculating the efficiency of import for a given precursor.

IN VITRO MITOCHONDRIAL IMPORT ASSAY: MITOCHONDRIAL FRACTIONATION AND PROTEIN LOCALIZATION

It may be desirable to fractionate mitochondria following an import reaction to determine the suborganellar localization of the newly imported protein. To assess matrix versus intermembrane space localization of the imported protein, mitochondria can be treated by osmotic shock to rupture the outer membrane, followed by centrifugation to separate the matrix, along with the inner and outer membranes, from the soluble intermembrane space components. Protection from added protease during osmotic shock confirms localization of the protein in the matrix, whereas protease sensitivity confirms localization in the intermembrane space or association with the mitochondrial membranes facing the intermembrane space. To confirm that a protein is integrated into the membrane, mitochondria can be subjected to alkali extraction (Fujiki et al., 1982); integral membrane proteins remain in the pellet and peripheral membrane proteins are released to the supernatant. Protease treatment that results in a protected fragment supports the idea of membrane integration for a protein that contains both membrane and soluble domains and can be used to confirm localization within the mitochondrion. For membrane proteins, the authors suggest using the procedures for both mitochondrial fractionation and alkali extraction. If the protein of interest is soluble, the alkali extraction can be eliminated. It is important to use controls when performing mitochondrial fractionation, and immunoblotting with antibodies for mitochondrial markers for the various compartments is suggested. Increasing the reaction volume or running replicate reactions of the import assays will provide the additional material required for immunoblot analysis (see Support Protocol 2).

Materials

Mitochondrial pellet from the import reaction (Basic Protocol 1, step 10)
1× import buffer + ATP + NADH (see recipe)
20 mM potassium HEPES, pH 7.4 (see recipe), cold
100% trichloroacetic acid (TCA): add 227 ml H_2O directly to bottle containing 500 g solid TCA and shake to dissolve; store up to 2 months at 25°C.
SDS sample buffer (*APPENDIX 2A*)
10 mg/ml proteinase K/20 mM potassium HEPES, pH 7.4 (see recipe): prepare 0.5 ml fresh using 5 mg proteinase K (Sigma)
0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol: prepare 1 ml fresh using 34.8 mg PMSF in 1 ml ethanol
10% (v/v) Triton X-100
Acetone
0.1 M Na_2CO_3: store up to several weeks at 4°C
Refrigerated microcentrifuge

Fractionate mitochondria

1a. Resuspend the mitochondrial pellet from the import reaction (Basic Protocol 1, step 11a) in 50 µl of 1× import buffer + ATP + NADH with a pipet, gently pipetting up and down to displace the pellet. Do not introduce air bubbles.

The samples should be kept in ice during subsequent manipulations.

2a. Dilute the mitochondria with 450 µl of 20 mM potassium HEPES, pH 7.4. Vortex briefly and incubate on ice for 15 to 30 min to rupture the outer membrane.

Dilution with 20 mM potassium HEPES, pH 7.4 causes hypotonicity of the solution which results in the rupture of the mitochondrial outer membrane while the inner membrane should remain intact, securing the matrix from protease. Mitochondria hypotonically swollen in this manner are referred to as mitoplasts.

3a. Microcentrifuge the lysed mitochondria 10 min at 20,000 × *g*, 4°C. Save the pellets of mitoplasts (intact inner membrane and matrix) and lysed outer membrane fragments for processing in step 8a, and proceed to step 4a with the supernatant.

4a. Carefully transfer the supernatant (soluble intermembrane space fraction) to a 1.5-ml microcentrifuge tube and precipitate the protein by adding 100% TCA to a final concentration of 10%.

5a. Keep the supernatant samples on ice until they have all been acid denatured, and then heat the TCA-precipitated samples 5 min at 65°C to inactivate proteases.

6a. Incubate the samples 10 min on ice.

7a. Microcentrifuge 10 min at 20,000 × *g*, 4°C.

8a. Resuspend the protein pellet from step 7a and the mitoplast membrane pellet from step 3a in 20 µl SDS sample buffer. Use immediately or hold up to 3 days at −20°C until ready to analyze by SDS-PAGE (Support Protocol 2).

The radioactive signal may be reduced upon prolonged storage.

If the protein of interest is recovered in the pellet after centrifugation, it may be localized in the matrix, inner or outer membrane, or intermembrane space tightly associated with a membrane. If the protein is released to the supernatant, it is a soluble intermembrane space protein.

Confirm suborganellar localization with protease treatment

1b. Resuspend the mitochondrial pellet from the import reaction (Basic Protocol 1, step 10) in 50 µl of 1× import buffer + ATP + NADH by gentle pipetting. In a parallel control reaction resuspend a second mitochondrial pellet from the import reaction in 50 µl of 1× import buffer + ATP + NADH containing 1% Triton X-100 (5 µl of 10% Triton X-100).

The inclusion of Triton X-100 in the fractionation protocol will solubilize the mitochondrial membranes thus giving the endogenously added proteases full access to the imported radiolabeled protein. This control is often added because it is important to confirm that the imported protein can, in principle, be degraded by the protease when the barrier of the membranes is removed. Once this has been controlled, the recovery of radiolabeled imported proteins following protease treatment of mitochondria/mitoplasts can be safely interpreted as an "inaccessibility" (i.e. import across the membrane) of the imported protein, rather than merely due to an inherent stability of the protein towards the exogenously added protease.

2b. Dilute the mitochondria in both reactions with 450 µl of cold 20 mM potassium HEPES, pH 7.4. Vortex briefly.

3b. Add 2.5 µl of 10 mg/ml proteinase K and incubate 15 to 30 min on ice. Add 2.5 µl of 0.2 M PMSF, incubate for an additional 5 min.

4b. Microcentrifuge the lysed mitochondria 10 min at 20,000 × *g*, 4°C, to separate the mitoplast pellet from the intermembrane space. In the case of the Triton X-100–containing control samples, do not centrifuge, but proceed directly to the TCA precipitation in step 5b. Save the pellets of the mitoplasts following centrifugation for processing in step 9b and proceed to step 5b with the supernatant.

Centrifuging the Triton X-100–treated controls is not necessary because there will be no mitoplast pellet.

5b. Carefully transfer the supernatants (soluble intermembrane space fractions) to 1.5-ml microcentrifuge tubes and precipitate the protein by adding 100% TCA to a final

concentration of 10%. Also add 125 µl acetone to the parallel reaction tube that originally included Triton X-100.

Because Triton X-100 precipitates in TCA, the addition of acetone until the mixture is hazy resolubilizes the Triton X-100.

6b. Keep the samples on ice until they have all been acid denatured, and then heat the TCA-precipitated samples at 5 min 65°C to inactivate proteases.

7b. Incubate the samples 10 min on ice.

8b. Microcentrifuge 10 min at 20,000 × *g*, 4°C.

9b. Resuspend the protein pellet and the mitoplast pellet from step 4b in 20 µl SDS sample buffer. Use immediately or hold up to 3 days at −20°C until ready to analyze by SDS-PAGE (Support Protocol 2).

The radioactive signal may be reduced upon prolonged storage.

Precipitate integral membrane proteins with alkaline extraction

1c. Resuspend the mitochondrial pellet from the import reaction (Basic Protocol 1, step 10) in 100 µl of 0.1 M Na_2CO_3, pH 11.0 by gentle pipetting.

2c. Incubate 10 min on ice.

Treatment of mitochondria with carbonate releases the soluble proteins from the mitochondria, whereas integral membrane proteins are retained and are pelleted with the membranes following centrifugation.

3c. Microcentrifuge the mitochondrial extract 30 min at 20,000 × *g*, 4°C.

4c. Carefully transfer the supernatant containing the soluble proteins to a new microcentrifuge tube and precipitate the soluble protein by adding 100% TCA to a final concentration of 10% and proceed with steps 5c to 8c. Save the pellet from step 3c and process further as in step 8c.

5c. Keep the samples on ice until they have all been acid denatured, and then heat the TCA-precipitated samples 5 min at 65°C to inactivate proteases.

6c. Incubate the samples 10 min on ice.

7c. Microcentrifuge 10 min at 20,000 × *g*, 4°C.

8c. Resuspend the membrane pellet from step 4c and the precipitated proteins from step 7c in 20 µl SDS sample buffer. Use immediately or hold up to 3 days at −20°C until ready to analyze by SDS-PAGE (Support Protocol 2).

The radioactive signal may be reduced upon prolonged storage.

IN ORGANELLO TRANSLATION OF MITOCHONDRIALLY ENCODED PROTEINS

The vast majority of mitochondrial proteins are encoded by the cell's nucleus, synthesized in the cytosol, and imported into mitochondria. Understanding of the process of protein import into mitochondria over the years has been greatly advanced through the use of in vitro protein import assays, such as those described in Basic Protocol 1. A small percentage of mitochondrial proteins are encoded by the mitochondrial DNA and are synthesized on ribosomes located in the mitochondrial matrix (Stuart, 2002). In the yeast *S. cerevisiae*, eight proteins are translated within the mitochondria: cytochrome oxidase subunits 1, 2 and 3 (Cox1, Cox2, and Cox3); cytochrome *b* (cyt *b*) of the cytochrome bc_1 complex; subunits 6, 8 and 9 of the F_1F_o-ATP synthase complex (Atp6, Atp8, and Atp9); and the Var1 protein of the small ribosomal subunit.

One can monitor the synthesis and membrane association of these proteins in vitro, using isolated yeast mitochondria; this process is termed in organello translation of mitochondrially encoded proteins. With the exception of Var1, which is soluble in the mitochondrial matrix, the other seven proteins synthesized within the mitochondria are integral inner membrane proteins. These proteins become inserted into the inner membrane during their synthesis on matrix-localized ribosomes, and post-translational insertion can be monitored in the isolated mitochondria largely through proteolytic accessibility assays.

In organello assays can address the bioenergetics of the translation and membrane insertion steps by performing them in the presence of uncouplers, which dissipate aspects of the proton motive force across the energized inner mitochondrial membrane. In addition, the in organello translation assay, followed by protease (e.g., proteinase K) accessibility studies, can be used to study the roles of certain proteins (e.g., Oxa1) in the membrane insertion steps (Stuart, 2002), using mitochondria isolated from null mutants or from conditional mutants proposed to be defective in the membrane insertion process. The translation pattern and protease accessibility profile of the radiolabeled translation products can be compared between wild-type and mutant mitochondria. For example, Cox2 is synthesized as a precursor form (pCox2), containing a cleavable N-terminal targeting sequence (Rojo et al., 1999). The presequence is removed following insertion of the protein into the inner membrane by a peptidase (Imp1), located on the intermembrane space side of the inner membrane. pCox2 is approximately 1 kDa larger than the processed mature form; thus these two forms of Cox2 can be resolved by SDS-PAGE. It should be noted that in wild-type mitochondria, and in the presence of a fully energized membrane, the processing of pCox2 occurs very efficiently, and under these optimal insertion conditions the pCox2 species is not normally observed. Failure to undergo efficient insertion (i.e., in absence of energized inner membrane, or following translation in mitochondria with a defective insertion machinery, e.g., in *oxa1* mutants) may result in the accumulation of pCox2 species.

The in organello translation assay is described in Basic Protocol 2, and methods for fractionating mitochondria and analyzing membrane insertion of the newly synthesized proteins are provided in Alternate Protocol 2.

BASIC PROTOCOL 2

IN ORGANELLO MITOCHONDRIAL PROTEIN ASSAY: TRANSLATION AND TRANSLOCATION OF MITOCHONDRIALLY ENCODED PROTEINS

Mitochondria isolated from *S. cerevisiae* according to methods described in *UNIT 3.8* and Glick and Pon, (1995) can be used for the in organello translation assays. Generally, the intact mitochondria are incubated with [^{35}S]methionine, translation buffer, which contains the other nonlabeled amino acids, in the presence of energy in the form of ATP, GTP, and metabolites (e.g., NADH) to generate a membrane potential. After an incubation period, further incorporation of radiolabeled methionine into the newly synthesized proteins is prevented by the addition of excess cold (unlabeled) methionine, followed by the addition of puromycin, a drug that results in the release of nascent chains from the ribosomes. The mitochondria are then re-isolated and, following SDS-PAGE (*UNIT 6.1*), analyzed using fluorography or a PhosphorImager E (*UNIT 6.3*) to detect the newly synthesized, radiolabeled proteins.

For protease accessibility studies (Alternate Protocol 2), the authors have found that it is better to pre-swell the mitochondria, i.e., convert them to mitoplasts, prior to the in organello translation procedure, rather than afterwards. The efficiency of both the mitoplast formation and the protease accessibility of exported segments of the newly synthesized radiolabeled proteins are greater if the hypotonic swelling procedure precedes the translation step, rather than vice versa. For other types of investigations (e.g., translation pattern studies) unswollen mitochondria may be used.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

Materials

Frozen, intact mitochondria (see *UNIT 3.8*)
Buffer C (see recipe)
20 mM potassium HEPES, pH 7.4 (see recipe)
0.2 M ATP (see recipe)
1.5× translation buffer (see recipe), freshly prepared
0.5 mg/ml pyruvate kinase: prepare fresh using 2.5 mg pyruvate kinase (Roche) and 5 ml sterile H_2O
0.2 M NADH: prepare fresh using 14.2 mg NADH and 100 µl sterile H_2O
[^{35}S]methionine (15 mCi/ml >1000 Ci/mmol in vitro–translation grade; GE Healthcare)
0.2 M methionine: prepare using 30 mg methionine and 1 ml sterile H_2O; store up to 1 year at −20°C
1 mg/ml puromycin: prepare 1 ml fresh using sterile H_2O
SDS sample buffer (*APPENDIX 2A*)

Heating block set at 25°C
Refrigerated microcentrifuge
Rotary shaker

Additional reagents and equipment for hypotonic swelling of mitochondria (Alternate Protocol 2; optional), SDS-PAGE (*UNIT 6.1*)

Prepare mitochondria or mitoplasts

1. Preheat the microcentrifuge tube heating block to 25°C.
2. Thaw the mitochondria quickly by warming in the hand immediately before beginning the experiment. If necessary, dilute the mitochondrial concentration to 10 mg protein/ml with buffer C. Store on ice until needed.

3a. *For studies using mitochondria:* Proceed with step 4.

3b. *For studies using mitoplasts*: Perform the hypotonic swelling procedure as follows.

i. Add the following to a 1.5-ml microcentrifuge tube:
440 µl cold 20 mM potassium HEPES, pH 7.4
10 µl 0.2 M ATP.

The hypotonic swelling reaction should contain ATP to ensure that ATP levels are not depleted from the mitochondrial matrix. Incubation of mitochondria in an ATP-free buffer would result in the release of ATP via the ADP/ATP carrier protein from the mitochondrial matrix to the external medium.

ii. Mix well and add 50 µl of isolated mitochondria (10 mg protein/ml) in buffer C.
iii. Mix gently and leave on ice for 30 min.
iv. Collect the swollen mitochondria (mitoplasts) by microcentrifuging 10 min at 20,000 × *g*, 4°C.
v. Remove the supernatant and resuspend the mitoplasts in 50 µl cold buffer C.

The mitoplasts should be handled gently. If the preparation does not perform well in the in organello translation, the inner membranes may have been ruptured. If necessary, the presence of matrix proteins can be investigated by immunoblot analysis (see Support Protocol 2 and UNIT 6.2) with the supernatant. Should a significant proportion of the matrix-localized proteins be present in the supernatant, the membrane may have lysed during osmotic shock.

Perform in organello translation

4. Mix the following in a 1.5-ml microcentrifuge tube:

   40 µl 1.5× translation buffer, freshly prepared
   1.5 µl 0.5 mg/ml pyruvate kinase
   6.5 µl H_2O.

5. Premix the tube by vortexing lightly and then add:

   5 µl 10 mg/ml mitochondria (from step 2) or mitoplasts (from step 3b)
   6 µl of 0.2 M NADH.

   Mix well by vortexing gently. Incubate the sample for 5 min at 25°C.

   It is important to maintain the mitochondria in an osmotically buffered solution so that they remain intact. The translation buffer should be made fresh.

6. Add 1 µl [^{35}S]methionine, mix well and incubate at 25°C, typically for 20 min.

   The incubation time may need to be varied with mitochondria from different strains or mutants.

Perform chase

7. Add 30 µl 0.2 M methionine and incubate for an additional 5 min at 25°C.

8. Add 15 µl 1 mg/ml puromycin, mix, and incubate 10 min at 25°C.

   The addition of cold methionine serves as a chase to ensure that translation is completed, whereas the addition of puromycin induces release of translating polypeptides from the ribosomes. Failure to add methionine and puromycin can result in the observation of a high level of partially synthesized radiolabeled proteins.

Analyze mitoplasts or mitochondria

9a. *To localize in organello–translated proteins in mitoplasts:* Proceed with protease accessibility analysis as in Alternate Protocol 2.

9b. *To re-isolate mitoplasts and mitochondria:* Microcentrifuge 12 min at 20,000 × *g*, 4°C. Carefully remove the supernatant with a pipet tip and add 500 µl cold buffer C. Vortex lightly, to wash tube walls and microcentrifuge 12 min at 20,000 × *g*, 4°C.

   If not proceeding with Alternate Protocol 2 (step 9a), step 9b would be used to isolate mitochondria and mitoplast samples directly for SDS-PAGE in step 10 (e.g., for translation pattern studies).

10. *To prepare mitoplasts and mitochondria directly for SDS-PAGE:* Remove the supernatant from the mitoplast or mitochondrial pellet and add 35 µl SDS sample buffer to the pellet. Shake 30 min at 4°C to achieve full solubilization of the mitochondrial proteins. Use immediately or hold up to 3 days at −20°C until ready to analyze by SDS-PAGE (Support Protocol 2).

    *The mitochondrial and mitoplast samples should **not** be heated to 95°C prior to SDS-PAGE, as is customary for sample preparation. Exposure of the samples to these elevated temperatures can lead to aggregation of the mitochondrially encoded proteins because of their high hydrophobicity.*

IN ORGANELLO MITOCHONDRIAL PROTEIN ASSAY: PROTEIN LOCALIZATION BY PROTEASE TREATMENT

The insertion of translation products into the mitochondrial inner membrane results in the translocation of hydrophilic segments of these proteins into the intermembrane space. Insertion of these integral membrane proteins can be assessed by analyzing the protease accessibility of their intermembrane space–exposed segments to exogenously added proteases (e.g., proteinase K). Because the mitochondria are double-membrane organelles, exogenously added proteases only gain access to the outer surface of the inner membrane when the integrity of the outer membrane is compromised. The outer membrane can be disrupted by hypotonic swelling, which when carefully performed results in rupture of the outer membrane while leaving the inner membrane intact. The resulting hypotonically swollen mitochondria are termed mitoplasts. The soluble intermembrane space components will be released from mitoplasts and exogenously added protease can access the outer surface of the inner membrane, but not the matrix space, because the integrity of the inner membrane should not have been compromised using this procedure. It is important to include controls for the integrity of the inner membrane to ensure that the hypotonic swelling procedure does not also result in rupture of the inner membrane.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

Materials

Labeled mitoplasts (Basic Protocol 2 step 8)
20 mM potassium HEPES, pH 7.4 (see recipe)
0.2 M ATP (see recipe)
Buffer C (see recipe)
0.5 mg/ml pyruvate kinase: prepare fresh using 2.5 mg pyruvate kinase (Roche) and 5 ml sterile H_2O
1 mg/ml proteinase K in 20 mM potassium HEPES, pH 7.4 (see recipe), cold: prepare 0.5 ml fresh using 0.5 mg proteinase K (Sigma)
0.2 M phenylmethylsulfonyl fluoride (PMSF): prepare fresh using 34.8 mg PMSF and 1 ml alcohol
SDS sample buffer (*APPENDIX 2A*)

Refrigerated microcentrifuge
Rotary shaker

Treat mitoplasts with protease

1. Divide the labeled mitoplasts into two equal aliquots of 50 µl and keep on ice.

   One sample will be treated with protease and the second sample will be included as a control.

2. Add 2.5 µl 1 mg/ml proteinase K to one sample and add 2.5 µl H_2O to the second sample, which will serve as the mock-treated control.

3. Incubate the samples for 20 min on ice and then add 0.5 µl of 0.2 M PMSF to each sample and vortex gently.

4. Re-isolate the mitoplasts by microcentrifuging 15 min at 20,000 × *g*, 4°C.

5. Remove the supernatant and add 500 µl buffer C containing freshly added PMSF (2 mM final concentration).

   The addition of PMSF to buffer C ensures that the proteinase K remains inactive.

6. Vortex gently and centrifuge again 15 min at 20,000 × *g*, 4°C.

Prepare samples for SDS-PAGE

7. Remove all supernatant and add 35 µl SDS sample buffer to the pellet. Resuspend the pellet by shaking 30 min at 4°C, to achieve full solubilization of the mitochondrial proteins.

   The samples should not be heated to 95°C prior to SDS-PAGE, as is customary for sample preparation. Exposure of the samples to these elevated temperatures can lead to aggregation of the mitochondrially encoded proteins because of their high hydrophobicity.

8. Electrophorese the samples as in Support Protocol 2.

SUPPORT PROTOCOL 1

PREPARATION OF PRECURSOR PROTEINS

Typically, mitochondrial-targeted proteins used as import precursors (e.g., *Neurospora crassa* Su9-DHFR) are synthesized as [^{35}S]methionine-labeled proteins using an in vitro translation system. These proteins can be made using the methods outlined in *UNIT 11.2* or using commercially available coupled transcription/translation systems (e.g., Promega). The coupled systems are relatively easy to use and generally produce enough precursor to be used in multiple import reactions. In general, imported substrates are produced using a rabbit reticulocyte lysate as opposed to a wheat germ lysate because rabbit reticulocyte lysate contains necessary chaperone proteins and allows the high mitochondrial import efficiency of precursors proteins required for import experiments. Alternatively, precursors can be purified as recombinant proteins from *E. coli*. These proteins can be rendered detectable by radiolabeling during *E. coli* expression or by immunoblotting after import. Precursors can be presented to mitochondria from the reticulocyte lysate directly or from a urea solution that is diluted to a final concentration of 0.6 M urea or less upon addition to the import reaction.

Materials

Expression vector (e.g., pET vector using the SP6 or T7 promoter; Novagen) carrying the gene encoding the protein of interest
Plasmid isolation kit (e.g., Qiagen or Promega)
In vitro translation kit (e.g., TNT coupled reticulocyte lysate system; Promega)
[^{35}S]methionine (10 mCi/ml; >1000 Ci/mmol in vitro translation grade; GE Healthcare)
Ammonium sulfate solution, saturated (see recipe)
8 M urea solution (see recipe)
Refrigerated microcentrifuge

Addition reagents and equipment for performing SDS-PAGE (*UNIT 6.1*)

1. Isolate the expression vector containing the gene encoding the protein of interest using a commercial plasmid isolation kit.

   Many commercial plasmid isolation kits can be used for this purification. It is recommended that the isolated plasmid be extracted with phenol/chloroform prior to being used for in vitro transcription/translation.

2. Using an in vitro translation kit, follow the manufacturer's instructions for synthesizing [^{35}S]methionine–labeled protein.

   The TNT coupled reticulocyte lysate system from Promega produces an abundant, high-quality translation product that can be used for numerous import assays.

3. Electrophorese a small aliquot (0.5 to 1 μl) of the translation product in 20 μl SDS sample buffer using SDS-PAGE (see *UNIT 6.1*) to confirm successful translation.

   Adjust the amount of translation product used in the in vitro import assay to be at least 10,000 cpm or to an amount that can be detected after 1 to 2 days on film. The ability of the reticulocyte lysate to translate the precursor should be tested before assaying import. Translation should generate a major radiolabeled band of the predicted size upon SDS-PAGE. Prestained molecular weight markers can be used on the gel for size comparison.

4. Add two volumes of saturated ammonium sulfate solution to the translation reaction. Mix well and incubate 30 min on ice to allow precipitation.

5. Microcentrifuge 10 min at 20,000 × *g*, 4°C.

6. Remove the supernatant and resuspend the pellet (precursor protein) in the same volume of 8 M urea as the original volume before precipitation. Keep the precursor at room temperature.

FINAL PROCESSING OF SAMPLES AND ANALYSIS BY SDS-PAGE

SUPPORT PROTOCOL 2

Samples should be loaded in the gel in a logical order, including a standard for the translation reaction. For example, mitochondria re-isolated directly following the import assay (Basic Protocol 1, step 8) and the protease treatment (Basic Protocol 1, step 10) can be analyzed with the samples from mitochondrial subfractionation and alkaline extraction. A sample of the translation product used in the in vitro import assay, usually equivalent to 1/10 to 1/20 of the amount added to the reaction, should be included on the gel. This will serve as a molecular weight reference, to confirm that the presequence has been removed, and as a reference for the amount of radioactive precursor imported into the mitochondrion. Note that the processed protein may contain fewer radioactive methionine residues than the precursor with the presequence. This will provide a complete and quantitative analysis of the import experiment. If additional manipulations, such as an import time course have been included, those samples should be loaded in a logical order.

Materials

Labeled protein samples (from Basic Protocol 1, Alternate Protocol 1, Basic Protocol 2, or Alternate Protocol 2)
25% (w/v) ammonium hydroxide solution
Labeled precursor proteins (Support Protocol 1)
10 mg/ml heat-denatured mitochondria: heat mitochondria (see *UNIT 3.8* and Basic Protocol 1) 5 min at 95°C
SDS sample buffer (*APPENDIX 2A*)
Antibodies against endogenous mitochondrial markers (e.g., Hsp60, ADP/ATP carrier, and cytochrome b_2), optional (not commercially available; contact authors at *rosemary.stuart@marquette.edu* or *koehler@chem.ucla.edu* for more information)
5% (w/v) trichloroacetic acid (TCA) solution
1 M Tris base (store up to 2 months at 4°C)
1 M sodium salicylate (store up to 6 months at 25°C)

65°C water bath
Rotary shaker
Nitrocellulose membrane, optional
Gel vacuum, optional
Storage phosphor screen and image acquisition software and instrumentation (e.g., PhosphorImager; GE Healthcare) *or*
X-ray film and cassette and densitometer

Additional reagents and equipment for performing SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and detecting and quantitating radiolabeled proteins (*UNIT 6.3*)

Prepare samples

1. If the labeled protein samples sample turns bright yellow, use a disposable Pasteur pipet to overlay the sample with NH_3 gas obtained from the vapors of a 25% (w/v) ammonium hydroxide solution (**do not** aspirate liquid). Agitate to mix the gaseous NH_3 into the sample buffer until the mixture turns blue.

   A yellow color indicates that the sample pH is acidic and that the proteins may still be precipitated. A blue color indicates that the sample pH is basic and the proteins are in solution.

2. Incubate the samples 5 min at 65°C. If the sample turns yellow, treat with NH_3 vapors again.

Obtain a 10% translation standard

To estimate the efficiency of the import of radiolabeled precursor proteins in the import assay, the amount of precursor added to the import reaction has to be determined.

3. To obtain a 10% translation standard based on the basic import assay (20 µl of precursor from the translation reaction; see Basic Protocol 1, step 5), mix 2 µl of the translation reaction (Support Protocol 1) with 10 µl of heat-denatured mitochondria and add an equal volume of 2× SDS sample buffer.

   Mitochondria are first denatured in sample buffer at 95°C for 5 min to prevent any reaction with the precursor. Including denatured mitochondria in the translation standard is important because a precursor may migrate differently when mixed with mitochondrial proteins in comparison to the translation reaction.

Analyze proteins by SDS-PAGE

4. Electrophorese the labeled protein samples and the import assay 10% standard (if appropriate) as described in *UNIT 6.1*.

   The authors use a gel system with 0.2% bisacrylamide and 16% acrylamide to separate 10- to 80-kDa proteins.

5. *Optional, but recommended:* Transfer proteins following SDS-PAGE to a nitrocellulose membrane for immunoblotting (*UNIT 6.2*) with antibodies against endogenous mitochondrial markers (e.g., Hsp60 for matrix, ADP/ATP carrier for inner membrane, and cytochrome b_2 for intermembrane space) to confirm successful mitochondrial swelling, extraction, or lysis.

6. To reduce radioactive background in the gel after electrophoresis, incubate the gel 30 min in 5% TCA in a beaker in a fume hood. Alternatively, heat the 5% TCA to 65°C to hasten the process.

   The proteins in the gel will precipitate and turn an opaque white color.

7. Remove the gel to a tray and wash briefly with water. Neutralize the gel by incubating it 5 min in 1 M Tris base at room temperature, with gentle shaking.

8. Wash the gel briefly with water. Add 1 M sodium salicylate and incubate 20 min at room temperature, with gentle shaking.

9. Dry the gel under vacuum and expose to a storage phosphor screen or X-ray film at −70°C overnight (see *UNIT 6.3*).

   Alternatively, the gel can be prepared as described in UNIT 6.3 with commercial enhancers.

Estimate import efficiency

10. Estimate the efficiency of the import reaction by quantifying the intensity of the imported precursor's radioactive signal and the intensity of the 10% translation standard's signal using a PhosphorImager or densitometer (see *UNIT 6.3*). Compare the intensity of the imported precursor signal to the intensity of the 10% translation standard.

    If the intensity is similar, the import efficiency is 10%. The import efficiency is typically between 5% and 50%.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Amino acid stock solution

For 10 ml:

20 mg each of the amino acids: alanine, arginine, aspartic acid, asparagine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, and valine
Dispense in 100-µl aliquots and store at −20°C

Ammonium sulfate solution, saturated

For 25 ml:

25 g $(NH_4)_2SO_4$ (ammonium sulfate)
H_2O to 25 ml
Stir for 30 min at room temperature. Remove the supernatant and keep at room temperature.

The $(NH_4)_2SO_4$ will not dissolve entirely.

ATP, 0.1 M

For 10 ml:

551 mg ATP
20 mM potassium HEPES, pH 7.4 (see recipe)
H_2O to 10 ml
Adjust pH to 7.4 using 10 M KOH and pH indicator paper
Store in 1-ml aliquots at −20°C

ATP, 0.2 M

For 1 ml:

130 mg ATP
Sterile H_2O to 1 ml
Adjust pH to 7.4 using 10 M KOH and pH indicator paper.
Store in 100-µl aliquots at −20°C

Buffer C, pH 7.4

For 100 ml:

25 ml 2.4 M sorbitol stock (0.6 M final), sterile
2 ml 1 M potassium HEPES, pH 7.4 (see recipe; 20 mM final), sterile
Sterile H_2O to 100 ml
Store up to several weeks at 4°C

Import buffer, 2×

For 100 ml:

2.38 g HEPES
150 mg L-methionine
200 mg BSA, fatty acid free
50 ml 2.4 M sorbitol
0.4 ml 1 M KH_2PO_4, pH 7.4
4 ml 2.5 M KCl
2 ml 1 M Mg_2Cl
1 ml 0.5 M EDTA, pH 7.4
Sterile H_2O to 100 ml
Adjust pH to 7.4 with 10 M KOH, if necessary
Store in 15-ml aliquots at −20°C

Import buffer, 1× + ATP + NADH

For 1 ml:

0.5 ml 2× import buffer (see recipe)
20 µl 0.1 M ATP (see recipe)
4 µl 0.5 M NADH (see recipe)
Sterile H_2O to 1 ml
Prepare fresh.

NADH, 0.5 M

For 1 ml:

355 mg NADH
20 mM potassium HEPES, pH 7.4 (see recipe) to 1 ml
Store in 20-µl aliquots at −80°C

Potassium HEPES, pH 7.4

For 100 ml:
2 ml 1 M potassium HEPES, pH 7.4 (20 mM final)
H_2O to 100 ml
Adjust pH to 7.4 with 10 M KOH
Autoclave and store up to several weeks at 4°C

Translation buffer, 1.5×

For 1 ml:

1.7 mg α-ketoglutarate
3.5 mg phosphoenolpyruvate
375 µl 2.4 M sorbitol
225 µl 1 M KCl
22.5 µl 1 M potassium phosphate buffer, pH 7.4
30 µl 1 M Tris·Cl, pH 7.4
19 µl 1 M $MgSO_4$
45 µl 100 mg/ml BSA (fatty acid free; Sigma)
30 µl 0.2 M ATP (see recipe)
15 µl 50 mM GTP
9 µl amino acid stock solution (see recipe)
10 µl 10 mM cysteine
18.5 µl 1 mg/ml tyrosine (see recipe)
Sterile H_2O to 1 ml

continued

Store on ice until ready to use
Prepare fresh

The individual components of this buffer can be made in advance, sterilized by passing through a 0.22-μm filter, and stored up to 1 year at −20°C.

Trypsin inhibitor, 20 mg/ml

For 0.5 ml:

10 mg soybean trypsin inhibitor
0.5 ml 20 mM potassium HEPES, pH 7.4 (see recipe)
Prepare fresh

Tyrosine, 1 mg/ml

For 1 ml:

1 mg tyrosine
Sterile H_2O to 900 μl
Adjust pH to 7.2 with 5 N KOH
Sterile H_2O to 1 ml
Store in 100-μl aliquots at −20°C

Urea, 8 M

For 10 ml:

4.85 g urea
0.039 g dithiothreitol
0.25 ml 1 M Tris·Cl, pH 7.5
H_2O to 10 ml
Store up to 4 weeks at −20°C

COMMENTARY

Background Information

Yeast mitochondria have been typically used as a model system for studying import of mitochondrial precursor proteins because it is easy to purify large quantities and store them frozen at −80°C for extended periods of time without affecting import performance. In addition, because yeast can be manipulated genetically, the system is amenable to the development of mutant mitochondria. The basic mechanism of protein import into mitochondria is conserved across metazoan species. Mammalian mitochondria for import assays can be purified from mouse and rat organs such as liver, heart, and brain as well as from cultured cells. Mitochondria for import studies can also be purified from plants and lower eukaryotes (e.g., *C. elegans* and *Xenopus*; Curran et al., 2004). Most researchers use fresh rather than frozen mitochondria from mouse and rat organs, purifying them immediately before use.

Precursors from a wide array of organisms, including mammals and plants, can be imported into mitochondria using the import assay in Basic Protocol 1. For example, the ADP/ATP carrier that resides in the *Trichomonas vaginalis* hydrogenosome can be imported into yeast mitochondria (Dyall et al., 2000), indicating that protein translocation pathways are highly conserved. Because of the possibility of inappropriate import into yeast mitochondria, import studies of heterologous precursors into yeast mitochondria should be confirmed by localization studies in the organism from which the precursor originates.

In organello methods (Basic Protocol 2 and Alternate Protocol 2) can easily be adapted to mitochondria from organisms other than yeast, particularly plants and mammals. This assay can be particularly useful for studying cell lines derived from patients with mitochondrial diseases, which are often characterized by defects in mitochondrial translation (Boulet et al., 1992). Isolation of mitochondria is not required for in organello translation, the process can be studied in intact cells treated with cycloheximide to inhibit translation from cytosolic ribosomes (Barrientos et al., 2004).

Critical Parameters

In vitro import assay

The quality of the mitochondria preparation influences the success of the import assay. The yield of mitochondria from a yeast culture and their ability to import proteins are directly related to the cell density at harvest. If the cells have reached stationary phase, purification of mitochondria may be difficult because the cell wall is resistant to zymolyase treatment, reducing the yield.

The carbon source influences the quality of the mitochondrial preparation. Cells grown in a nonfermentable carbon source, such as lactate, ethanol, and glycerol, have more respiratory-active mitochondria than cells grown in glucose, making them bioenergetically more robust for import.

Mitochondria should be kept cold during isolation to minimize proteolysis of the proteinaceous surface receptors, which are required for efficient precursor import. Purification of mitochondria also should be performed on ice once the cells are being homogenized. Buffers should be kept ice cold and rotor and homogenizers should be prechilled. Mitochondria should be prepared quickly. After isolation, mitochondria should be frozen quickly in liquid nitrogen in small aliquots and then transferred to a −80°C freezer.

Several parameters affect the robustness of the import reaction itself. Precursors vary in their ability to be imported and each one must be evaluated empirically. For example, membrane proteins such as those of the mitochondrial carrier family, Tim22 and Tim23, lose import competence when treated with urea, which most likely reduces the ability of chaperone proteins in the reticulocyte lysate to help maintain the mitochondrial protein import competency.

In contrast, precursors with a typical N-terminal targeting sequence usually import equally well whether used directly from the reticulocyte lysate or treated with urea. Urea denaturation is the typical method for preparing recombinant precursors from *E. coli* for import assays.

In steps where proteases have been added, subsequent inactivation of the proteases is important to maintain the stability of the products. PMSF addition to the sample buffer ensures protease inactivation before separation by electrophoresis.

TCA precipitation is necessary when the volume of the sample is too large to load on a gel for SDS-PAGE, e.g., supernatants from osmotic shock or carbonate extraction.

In organello assays

For in organello assays, the quality of the mitochondrial preparation is also important for the same reasons as in the in vitro assays.

During the translation reaction, inclusion of the methionine chase and subsequent puromycin treatment are important for release of the mature polypeptide from the ribosome. Failure to do so can result in a high level of partially synthesized radiolabeled proteins.

Because the mitochondrial proteins are especially hydrophobic, samples should not be heated to 95°C prior to SDS-PAGE, as is customary for sample preparation. Exposure of the samples to these elevated temperatures can result in aggregation of the mitochondrially encoded proteins due to their high hydrophobicity.

The inclusion of high ATP levels is important for solutions used in hypotonic swelling of mitochondria. Hypotonic treatment of mitochondria may result in leaking of the inner membrane, causing depletion of ATP from the mitochondrial matrix. Limiting the ATP levels in the mitoplasts will severely hinder the efficiency of the translation reaction and also possibly inhibit protein insertion into the inner membrane. Including ATP during osmotic lysis ensures that the mitochondria are not energetically compromised during the translation reaction.

Troubleshooting

Import efficiency of precursors varies widely. If efficiency is less than 5% of the added import substrate, or if it is difficult to detect the imported product, it may be necessary to increase the amount of translation product or mitochondria used in the assay. The in vitro transcription/translation reaction can be altered to increase the yield of the radiolabeled precurson. Alternatively, the protein may lack numerous methionine residues. In this case a template can be generated to add methionine residues at the C-terminus or in another position that does not alter targeting.

Controls to verify that manipulations to the mitochondria (e.g., osmotic swelling and carbonate extraction) are successful should be included. For example, when a membrane protein is extracted using carbonate, additional reactions should be included so that the samples can be immunoblotted with antibodies against endogenous marker proteins, (e.g., Hsp60 for matrix, ADP/ATP carrier for inner membrane, and cytochrome b_2 for intermembrane space). These proteins serve as excellent controls for verifying that hypotonic lysis is successful.

For the in organello translation assays, it is especially important to control the efficiency of the hypotonic swelling procedure and ensure integrity of the inner membrane. Failure to efficiently form mitoplasts will prevent protease accessibility to the intermembrane space. The translation products will not be degraded by the protease, despite being correctly inserted into the membrane. Immunoblot analysis should be included with antisera against inner membrane control proteins, known to have protease-accessible domains in the intermembrane space (e.g., ADP/ATP carrier protein, Tim23 of the Tim23 translocase, and D-lactate dehydrogenase protein).

On the other hand, the Var1 protein is a soluble protein in the mitochondrial matrix and hence should not be accessible to proteases exogenously added to the mitoplasts. Degradation of Var1 by the added proteases would indicate that the integrity of the inner membrane has been compromised during the mitoplast preparation procedures or in subsequent steps. Therefore, it is important to include the mock-protease treated sample in parallel, so the Var1 levels can be directly compared with the protease-treated sample. Immunoblot analysis of the samples with known matrix-localized proteins (e.g., Mge1, Hsp70, Hsp60) is also advised for this reason.

Anticipated Results

The results from an import assay will vary depending on the precursor being studied. All proteins targeted to mitochondria that at least partially cross the mitochondrial inner membrane require a membrane potential for import. Most precursors with an N-terminal targeting sequence are processed by mitochondrial proteases in the matrix and intermembrane space to remove the targeting sequence; these precursors will have a lower molecular weight than the precursor prior to import. The length of the targeting sequence can vary greatly, but in most cases the reduction of molecular weight (2000 to 10,000 Da) should be large enough to be easily detected by SDS-PAGE analysis. To confirm that the lower-molecular-weight protein has been imported, mitochondria are treated with an uncoupler such as valinomycin that renders the mitochondria defective in import because the membrane potential across the inner membrane has been dissipated.

Often the unprocessed and processed forms of the import substrate will be detected by comparing mitochondria before treatment with exogenous protease (trypsin or proteinase K). The unprocessed form of the precursor represents protein that is nonspecifically associated with the outer membrane or potentially bound to components of the import apparatus at the early, binding stages of import. The unprocessed form should be removed by treatment with exogenous protease; the processed form that has completely crossed the outer membrane should be protected. In addition, resistance to alkaline extraction in combination with protease treatment can be used to confirm integration into the membrane.

For SDS-PAGE analysis of the in organello import reaction, a gel with final concentrations of acrylamide and bisacrylamide of 16% and 0.2% (w/v), respectively, is recommended. The relative mobilities of the translation products using this gel system correspond to proteins with molecular masses as follows: Var1 (~45 to 47 kDa, depending on the genetic background of the strain used for mitochondrial isolation), Cox1 (36 kDa), Cox2 (34 kDa), cyt *b* (30 kDa), Cox3 (23 kDa), Atp6 (21 kDa), Atp8 (9 kDa), and Atp9 (8 kDa).

In analysis of the insertion of exported proteins into the mitochondrial inner membrane, correctly inserted Cox1, Cox2, Cox3, cyt *b*, and Atp6 proteins are accessible to exogenously added proteases from the intermembrane space side of the inner membrane. Thus, proteinase K treatment of mitoplasts, following the translation reaction, should result in the degradation of these proteins, if they became correctly inserted into the membrane during the translation reaction. The protease treatment results in the generation of proteolytic fragments of these proteins with SDS-PAGE mobilities that are ~10 kDa and smaller. These fragments are membrane associated and, therefore, are re-isolated with the mitoplast fraction. Due to their abundance and also the fact that the sizes of these proteolytic fragments are similar to those of the full length Atp8 and Atp9 proteins, it is not possible to distinguish these fragments from the Atp8 and Atp9 proteins. For this reason it is not possible to ascertain whether the Atp8 and Atp9 proteins are accessible to the protease, i.e., have been correctly inserted into the membrane, using this assay.

Time Considerations

Isolation of mitochondria from yeast takes ~4 to 8 hr after the cells have reached the correct density. Mitochondria are then frozen in small aliquots of ~1 mg at a concentration of 10 to 25 mg/ml in an osmotically stabilized buffer (see *UNIT* 3.8). The transcription-translation assay to generate radiolabeled

precursor proteins requires ~1 to 2 hr (Support Protocol 1).

The import assay takes ~1 hr (Basic Protocol 1), whereas additional manipulation requires another 1 to 2 hr (Alternate Protocol 1), depending upon the assays that will be done. A polyacrylamide gel takes 2 to 3 hr to run, 30 min to fix, and 30 to 60 min to dry. The gel is then exposed to a storage phosphorimaging screen or X-ray film overnight (16 hr or longer), depending upon the strength of the signal and efficiency of import (see Support Protocol 2 and *UNIT 6.3*). If gels are used for immunoblotting (see *UNIT 6.2*), an additional 1 to 2 hr is required.

The in organello assay (including buffer preparation time) takes ~2 hr (Basic Protocol 2), and additional manipulations (e.g., mitoplast formation and protease accessibility studies) require an additional 1 to 2 hr (Alternate Protocol 1). A polyacrylamide gel takes 2 to 3 hr to run (Support Protocol 2), with an additional 2 hr to transfer the proteins (using a semidry blotting chamber) to a nitrocellulose membrane if the gels are used for immunoblotting (see *UNIT 6.2*). Drying the gels under a heating lamp takes 10 to 15 min and exposing them to a storage phosphorimaging screen or X-ray film takes 5 hr or longer, depending upon the strength of the efficiency of the translation reaction and the age of the [^{35}S]methionine used (see *UNIT 6.3*).

Literature Cited

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D, Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 2007. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Barrientos, A., Zambrano, A., and Tzagoloff, A. 2004. Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in *Saccharomyces cerevisiae*. *EMBO J*. 23:3472-3482.

Boulet, L., Karpati, G., and Shoubridge, E.A. 1992. Distribution and threshold expression of the tRNA(Lys) mutation in skeletal muscle of patients with myoclonic epilepsy and ragged-red fibers (MERRF). *Am. J. Hum. Genet.* 51:1187-1200.

Coligan, J.E., Dunn, B.M., Speicher, D.W., and Wingfield, P.T. (eds.) 2007. Current Protocols in Protein Science. John Wiley & Sons, Hoboken, N.J.

Curran, S.P., Leverich, E.P., Koehler, C.M., and Larsen, P.L. 2004. Defective mitochondrial protein translocation precludes normal *Caenorhabditis elegans* development. *J. Biol. Chem.* 279:54655-54662.

Dyall, S.D., Koehler, C.M., Delgadillo, M.G., Bradley, P.J., Plümper, E., Leuenberger, D., Turck, C.W., and Johnson, P.J. 2000. The presence of a member of a mitochondrial carrier in the hydrogenosomal membranes: Conservation of membrane targeting pathways indicates a progenitor organelle gave rise to hydrogenosomes and mitochondria. *EMBO J*. 20:2488-2497.

Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. *J. Cell Biol*. 93:97-102.

Glick, B.S. and Pon, L. 1995. Isolation of highly purified mitochondria from *S. cerevisiae*. *Meth. Enzymol*. 260:213-233.

Guda, C., Fahy, E., and Subramaniam, S. 2004. MITOPRED: A genome-scale method for prediction of nucleus-encoded mitochondrial proteins. *Bioinformatics*. 20:1785-1794.

Koehler, C.M. 2004. New developments in mitochondrial assembly. *Ann. Rev. Cell Dev. Biol.* 20:309-335.

Pfanner, N., Muller, H.K., Harmey, M.A., and Neupert, W. 1987. Mitochondrial protein import: Involvement of the mature part of a cleavable precursor protein in the binding to receptor sites. *EMBO J*. 6:3449-3454.

Rojo, E.E., Guiard, B., Neupert, W., and Stuart, R.A. 1999. N-terminal tail export from the mitochondrial matrix. Adherence to the prokaryotic "positive-inside" rule of membrane protein topology. *J. Biol. Chem.* 274:19617-19622.

Stuart, R. 2002. Insertion of proteins into the inner membrane of mitochondria: The role of the Oxa1 complex. *Biochim. Biophys. Acta*. 1592:79-87.

Contributed by Rosemary A. Stuart
Marquette University
Milwaukee, Wisconsin

Carla M. Koehler
UCLA
Los Angeles, California

CHAPTER 12
Cell Motility

INTRODUCTION

The ability of eukaryotic cells to move and respond to their environment is critical for morphogenesis, and underlies the invasiveness of tumor cells. The majority of cells have the capacity to actively migrate toward a chemoattractant. For a cell to move in this manner, it must be able to detect the direction of a chemical gradient, orient toward the gradient, and move directionally toward it. The cellular processes that mediate this response are numerous, including extension of a pseudopod, attachment of the cell to the substratum, and breakage of existing attachments. Chapter 12 describes methods for analyzing these processes.

UNIT 12.1 describes basic assays for studying directed cell movement along a chemical gradient. These are useful for identifying new chemoattractants and studying the cellular processes required for such movement. Among the assays described are those that monitor a population of cells after exposure to a chemoattractant in a steep gradient across a thin porous filter or over short distances through a gel of extracellular matrix or agarose. In these assays, the gradient is applied gradually or suddenly depending on the purpose of the experiment. The latter approach is useful for biochemical analysis of chemoattractant signal transduction, where a rapid change in chemoattractant concentration is required to generate a signal. Cell migration in these assays is detected using radioactive, fluorescent, or enzymatic cell markers, or cytochemistry. Direct observation of an individual cell's behavior in response to a chemoattractant is an alternative visual approach described in several assays in this unit. These assays can be used to dissect the complex processes involved in the chemotatic response; the questions of how a cell detects a chemical gradient and determines its direction, orients its movement toward the signal, and moves along the gradient are emphasized in this unit. Selection of an appropriate assay is important because cells move at vastly different rates in response to a chemotactic signal (e.g., neutrophils and *Dictyostelium* amoebae move about ten times faster than fibroblasts and neurons) and require different time scales and conditions for monitoring their movements.

UNIT 12.2 describes a protocol for assessing the invasiveness of cells through a basement-membrane matrix. Cells are placed in the upper well of a Boyden migration chamber that is coated with basement-membrane matrix, and a chemoattractant is applied in the lower well. After a short incubation period, cells that have migrated through the matrix-coated filter are counted and can be recovered for further analysis. This type of assay can be used to identify compounds that either promote or inhibit invasion of cells across the matrix-coated filter, and is easily adapted for large-scale screening of compounds. In addition, because cells that have moved to the lower chamber can be recovered, subpopulations that have increased invasiveness can be isolated and studied. A useful application of the assay is for analysis of metastatic tumor cells that invade somatic tissues through blood vessel basement membrane and underlying connective tissue.

UNIT 12.3 describes protocols for monitoring the traction forces exerted by migrating cells as they move along a substratum. These assays provide important information relevant to understanding subcellular events occurring during migration, including breakage of adhesive contacts with the extracellular matrix, cytoskeletal rearrangements involved

Contributed by Jennifer Lippincott-Schwartz
Current Protocols in Cell Biology (2005) 12.0.1-12.0.3

in forward motion, and the formation of new contacts. The least expensive and easiest method described for studying cell traction uses a silicone sheet to measure traction forces under the entire cell. The orientation of wrinkles generated in the substrate by locomoting cells is used to describe the global orientation of the forces under the cell. A newer, more expensive technique that is also included measures subcellular traction forces by employing a system of deformable levers with variable stiffnesses to measure forces directly under regions of the cell as small as 4 μm^2.

Many cells reside in environments where they frequently experience disruptions in membrane integrity. When such tearing or wounding of the cell surface occurs, an active and complex resealing mechanism rapidly repairs the membrane disruptions. *UNIT 12.4* describes methods for studying the processes of cell wounding and resealing. The methods are based on the finding that wounded cells will take up tracer macromolecules to which they are normally impermeable; upon resealing, the tracers are trapped in the cell. Because dead cells do not reseal, they do not trap the tracers and are thereby distinguishable from live wounded cells. Protocols in this unit describe how to detect wounded cells using fluorescent dextrans or immunostaining for rat serum albumin added to the medium. Also covered are electron microscopic analysis of wounded cells and quantification of wounded cells by image analysis and flow cytofluorometry. Because plasma membrane disruptions provide a route of entry for otherwise impermeable macromolecules that an experimentalist might want to introduce into the cytosol of cells (including enzymes, antibodies, and expression vectors), understanding how plasma membrane disruptions arise and reseal is of considerable practical utility.

A powerful system for studying cell dynamics is *Dictyostelium discoideum* amoebae, which grow as separate independent cells, but upon starvation actively cluster together to form multicellular structures. During this process the amoebae undergo a range of different behaviors, including chemotaxis, signal transduction, plasma membrane ruffling, phagocytosis, and cytokinesis. *UNIT 12.5* describes how to grow and image *D. discoideum* amoebae in order to study motile and chemotactic behavior in wild-type and genetically modified strains. Protocols are described for growing and maintaining the cells and how to transform them with GFP fusion plasmids. Methods are also provided for characterizing the functionality of GFP fusion proteins expressed in the amoebae. The unit also includes methods for imaging GFP fusion proteins in chemotaxing cells, aggregates, mounds, and slugs.

UNIT 12.6 describes a microscopy-based assay for analyzing the migration of human neutrophils. Neutrophils play an important role in initiating the inflammatory response in higher organisms. They accomplish this by migrating into an area of infection in response to a chemotactic signal. The protocol in this unit describes how to perform time-lapse confocal imaging of neutrophils migrating in two-dimensions and techniques for analyzing the speed and directional persistence of such movement. This type of analysis, in which migration parameters are quantified, is important for understanding potential causes of cell migration defects. A protocol for the isolation of human neutrophils from blood is also detailed in this unit, as is a method for preparing microscope chambers for imaging.

UNIT 12.7 presents assays relevant for studying actin-based motility within cells. The assays are based on the propulsion of the *Listeria* and *Shigella* or functionalized microspheres reconstituted from five proteins, including profilin, actin-depolymerizing factor, gelsolin, neural Wiskott-Aldrich syndrome protein (N-WASP) and the actin-related protein, Arp2/3 complex (all of whose purification protocols are described). By quantifying the dependence of motility parameters on the concentration and/or combination of these

components, these assays provide an approach for characterizing the molecular processes underlying actin-based motile behavior of cells.

UNIT 12.8 presents a novel technique—photoactivation—for marking cells deep within an embryo so that these cells can be followed using confocal microscopy techniques over time. In this approach, standard electroporation techniques are used to deliver photoactivatable GFP attached to a protein of interest into cells in an embryo. The expressing cells are then excited with ~400 nm light. This causes the chromophore population to undergo photoconversion to a form whose baseline fluorescence at 488 nm excitation increases 100-fold, resulting in the cells becoming optically highlighted. By using photoactivation to selectively highlight cells in this manner, it becomes possible to follow individual cells over time in an embryo. This method provides a way to trace cell lineages and to study cell migration patterns, noninvasively.

Jennifer Lippincott-Schwartz

Chemotaxis Assays for Eukaryotic Cells

Chemotaxis is defined as a bias in the direction of cell movement along a chemical gradient. Chemotaxis contributes to morphogenetic movements and to cell accumulation at sites of inflammation. In recent years, interest in the field of chemotaxis has focused on identifying new chemoattractants, especially the chemokines, which attract various leukocytes (Baggiolini et al., 1997; Baggiolini, 1998), and the netrins and semiphorins/collapsins, which attract or repel neuronal growth cones (McKenna and Raper, 1988; Tessier-Lavigne and Goodman, 1996; Song et al., 1997). Much attention is also focused on the cellular and molecular basis of chemotaxis (Devreotes, 1994; Schleicher et al., 1995; Van Haastert, 1995). Assays appropriate for identifying new chemoattractants often differ from those appropriate for investigating the mechanisms of chemotaxis. This unit describes both types of assays in general terms, provides detailed instructions for performing various assays, and considers issues specific to neutrophils, lymphocytes, fibroblasts, and *Dictyostelium discoideum* amoebae.

Chemotaxis is most clearly documented by observing individual cells moving up a gradient, e.g., toward a pipet or a well containing a chemoattractant. However, to assay a number of test materials in this manner can be time-consuming. Thus, assays have been developed that, although less definitively detecting chemotaxis, allow an efficient search for potential chemoattractants. Most of these assays monitor the redistribution of a population of cells after exposure to a test material established in a steep gradient (change in concentration ≥2% per 10 μm) across a thin porous filter (filter assay; see Basic Protocol 1), or over short distances (several mm) through a gel of extracellular matrix or agarose (under-agarose and small population assays; see Basic Protocols 2 and 3, respectively). The rigid matrix allows a gradient to form by diffusion, while protected from disruption due to fluid flow. Cell migration toward the test material, detected microscopically or through radioactive, fluorescent, or enzymatic cell markers, is quantified after a set incubation time. Such assays allow a large number of materials and concentrations to be tested in parallel. Increased migration toward a test material in these assays suggests that the material might be a chemoattractant. However, increased migration might also be seen if the material merely enhances the number of cells migrating and/or their rate of migration (chemokinesis; see Background Information). A protocol is also included for distinguishing enhanced migration from chemotaxis (see Support Protocol 1).

For a cell to exhibit chemotaxis, it must detect the direction of a chemical gradient, orient its translocation in that direction, and then move along the gradient. The signaling pathways that mediate this complex response are being investigated. Often it is helpful to consider different aspects of the response. For example, a particular cellular component (i.e., a mutated gene or a pharmacologically altered enzyme) might be required for a cell to extend a pseudopod, direct the pseudopod up the gradient, limit pseudopods from extending in other directions, stimulate an attachment to the substratum, or break attachments. To investigate the responsible mechanism, assays are used that give detailed information about an individual cell's behavior through direct observation of the cell during the response. Such assays include observation of cells in established gradients (under-agarose, bridge assays; see Basic Protocols 2 and 4, respectively), video recording of cells presented with a pipet containing attractant (pipet assay; see Basic Protocol 5), and observation of cells exposed to a sudden increase or decrease in concentration of chemoattractant (upshift assay; see Basic Protocol 6). These methods can be modified to allow monitoring by fluorescence microscopy to follow the time course of redistribution of GFP-tagged proteins (Maniak et al., 1995; Moores et al., 1996; Westphal et al., 1997). Image analysis systems may also be used to analyze results (see Support Protocol 2).

Contributed by Sally H. Zigmond, Ellen F. Foxman, and Jeffrey E. Segall

Current Protocols in Cell Biology (1998) 12.1.1-12.1.29

STRATEGIC PLANNING

In all assays, the accuracy of cell orientation in a gradient of chemoattractant (Fig. 12.1.1) depends on both the steepness of the gradient and the mean concentration of the chemoattractant. To exhibit chemotaxis, a cell must detect a change in the concentration of chemoattractant over time and/or distance. A cell detects the change in concentration by a change in the occupancy of its chemoattractant receptors. The change in occupancy is greatest when the gradient is steepest and when the concentration of the attractant is well below that which saturates the receptors. In linear gradients of a constant steepness (e.g., a 10-fold increase in concentration over 1 mm), a concentration of chemoattractant somewhat less than the K_d of the receptor (i.e., the concentration that results in occupancy of half of the receptors) gives optimal cell orientation. Because the geometry of the gradient varies between assays, the concentration needed to achieve a steep gradient in the appropriate concentration range also varies. The gradient needs to have an appropriate concentration and steepness throughout the time the cells are migrating.

Selection of an appropriate assay is influenced by four principal factors. (1) The choice of assay depends on the rate of locomotion of the cell under study. Neutrophils and *Dictyostelium* amoebae move about ten times faster than fibroblasts and neurons (~10 μm/min versus ~1 μm/min). Since the gradient in most assays is both established and then dissipated by diffusion, assays of slower moving cells may require a special means of renewing or maintaining a gradient (Fisher et al., 1989). Filter assays are useful for most cell types because the filter is so thin, a steep gradient is established, and even slowly moving cells can move across a thin filter before the gradient is lost. For specific information about different cell types and media considerations, see Critical Parameters.

The choice of assay also depends upon (2) the purpose of the assay (i.e., whether to determine if a factor is chemotactic or to assess the mechanism of chemotaxis) and (3)

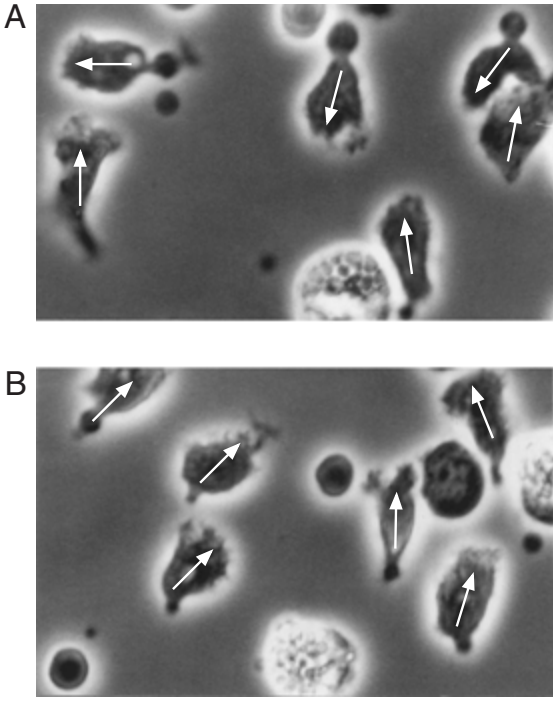


Figure 12.1.1 Cell orientation in (**A**) random migration (chemokinesis) versus (**B**) chemotaxis. Arrows indicate direction of individual cell movement; direction of chemotaxis in (B) is to the top.

Table 12.1.1 Appropriate Chemotaxis Assays for Desired Information

Cell type	Assay to detect a response	Assay to determine mechanism of response
Leukocyte	Filter, under-agarose	Under-agarose, bridge, pipet, upshift
Dictyostelium	Small population, pipet, aggregation[a]	Bridge, pipet, upshift
MTLn3/fibroblast	Filter	Filter, pipet, upshift

[a]Normal aggregation of *Dictyostelium* is mediated in part by chemotaxis. For assay details see Soll (1987).

Table 12.1.2 Experimental Requirements for Chemotaxis Assays

Assay type	Cells per assay	Chemoattractant		Replicates
		Concentration ($\times K_d$)	Minimum volume (µl)	No. of simultaneous assays
Multiwell filter	10^4-10^5	0.1-10	25[a]	12-48 per chamber
Culture insert	$0.5–5 \times 10^6$	0.1-10	10	24 per chamber
Under-agarose	10^5	10-1000	10	10-20 per chamber
Small population	10^3	0.1-10	0.3[b]	~8 per chamber
Bridge	10^3	0.1-10	50[a]	5
Pipet	1-50	10^3-10^4	5	1

[a]Volumes indicated for 48-well multiwell chambers and for glass bridge chambers. Other multiwell chambers and Plexiglas bridge chambers require more chemoattractant.
[b]From three sequential 0.1-µl applications.

the time available to perform the assay. Assays vary in their time commitment and in the type of information that they can provide (Table 12.1.1 and Table 12.1.2). "Survey assays" are those that can be performed on many samples simultaneously and scored at a later time, either automatically or at least at one's leisure. These are useful for screening for potential chemoattractants. These assays would need to be modified to examine directional neurite extension from neurons. Information about how a particular agent affects cell behavior is often best obtained from "visual assays," which allow direct monitoring of cell movement and behavior. However, direct visualization (and video recording) must usually be performed by altering one parameter at a time. Finally, the choice of assay is dependent upon (4) the availability of cells and chemoattractants, because assays vary in the number of cells and the total amount of chemoattractant required (Table 12.1.2).

FILTER ASSAY FOR CHEMOTAXIS

BASIC PROTOCOL 1

In this protocol, cells suspended in buffer are placed on top of a porous filter while chemoattractant is placed below the filter. After incubation, cell migration into or through the filter is measured (Boyden, 1962). Prior to performing the assay, it is necessary to select the appropriate (1) chamber (e.g., blind-well or multiwell chambers from Neuro Probe, Fig. 12.1.2, or 24-well tissue culture plates with filter inserts), (2) filter type (thick cellulose nitrate or thin polycarbonate), (3) filter pore size, and (4) filter coating for the cell type being assayed (see Critical Parameters). Migration is quantified by measuring (1) the distance cells have migrated into the filter, (2) the number of cells attached to the bottom of the filter, and/or (3) the number of cells that have migrated through the filter and fallen into the lower chamber. It is surprising that both of the latter measures have been used, because they would be expected to shift in opposite directions if the test factor

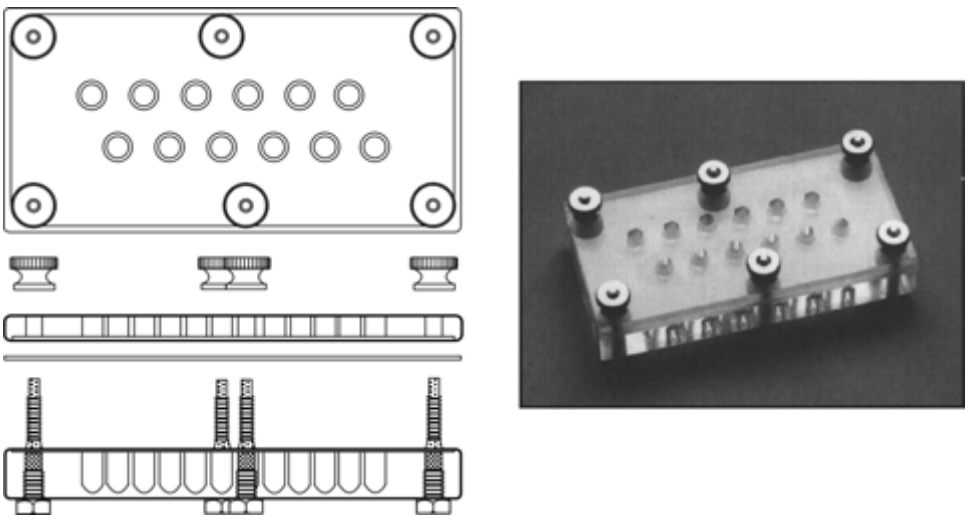


Figure 12.1.2 A standard 12-well multiwell chemotaxis chamber from Neuro Probe is made of acrylic and has 4.75-mm-diameter wells that expose 18 mm^2 of filter. Volumes of upper and lower wells are 100 and 150 μl, respectively. A 48-well multiwell chamber is also available (upper and lower well volumes 50 and 25 μl, respectively). A blind-well chamber, which is a nonstandard special-order chamber, is also available with upper and lower well volumes of 200 and 75 μl, respectively. Figure redrawn with permission from Neuro Probe.

alters all adhesivity. However, each measure has been used successfully with particular cell types. In general, fibroblasts have been quantified on the lower surface while lymphocytes, which are less adhesive, have been quantified in the lower chamber. Neutrophils are frequently in both places (Jungi, 1975); thus, it is advisable to initially monitor the number of cells both on the filter bottom and in the lower chamber.

If a factor induces an increase in cell migration, additional tests are required to determine whether this is due to an increase in the rate of random migration (chemokinesis) or in a directional movement (chemotaxis) toward the test material, or both (Zigmond and Hirsch, 1973). One can examine the effects on chemokinesis by measuring migration in a series of homogeneous (isotropic) concentrations of test material placed both above and below the filter. The results from these assays are compared to those from cells migrating in a gradient, to determine whether migration in a gradient exceeds that expected from random locomotion alone. Alternatively one can use a different assay in which directional migration is assayed directly (e.g., small population assay, see Basic Protocol 3; bridge assay, see Basic Protocol 4; pipet assay, see Basic Protocol 5). For thick filter assays, the migration distance expected in the absence of chemotaxis (see Support Protocol 1) can be used to determine if chemotaxis is present.

This assay is described for neutrophils; adaptations for other cell types are indicated in annotations.

Materials

Buffer for upper and lower wells (see Critical Parameters discussion on media)
Chemoattractant in buffer
Cells of interest
3.7% (w/v) formaldehyde in PBS (*APPENDIX 2A*)
Giemsa stain (see recipe) or Diff-Quick (EM Science) for neutrophils; hematoxylin for lymphocytes

70% and 100% isopropanol
1:1 (v/v) isopropanol/Americlear
Americlear (Baxter Biotech)
Permount (EM Science)
15-µm polystyrene beads (Polysciences)

Chemotaxis chamber: blind-well or multiwell chamber (Fig. 12.1.2; Neuro Probe) with appropriate filters, or 24-well tissue culture plate with filter inserts (see Critical Parameters)
Filter scraper (Neuro Probe; optional)
Microscope with 40× objective and micrometer (16× objective optional)

Additional reagents and equipment for counting cells using a hemacytometer (*UNIT 1.1*) or cell sorter

Perform assay

1. Fill the lower wells of a chemotaxis chamber with buffer or with chemoattractant in buffer. Use sufficient volume to slightly overfill the well, to prevent air bubbles from being trapped when the filter is lowered (see Table 12.1.3).

   The cells in the middle of the filter will be exposed to the mean of the concentrations of chemoattractant present above and below the filter. Thus, when the upper well contains no chemoattractant, the optimal chemoattractant concentration in the lower well is approximately that of the dissociation constant, K_d, of the receptor. Initially, if the K_d is known, one might test concentrations from one tenth to ten times K_d (Table 12.1.2). If K_d is not known, one could test 10-fold dilutions between 0.1 nM and 10 µM. Most leukocyte chemokines are active at nM concentrations.

2. Lower a filter smoothly onto the chamber, checking that the liquid in each well makes full contact with the filter (i.e., that there are no air bubbles). Assemble rest of chamber and tighten screws. Before adding cells, aspirate off any fluid from the lower well that came through the filter.

   When using multiwell chambers, prewetting the filter with buffer helps prevent wicking of fluid out of wells. Avoid moving the filter after it is lowered so that chemoattractant concentrations in different wells are not mixed.

   When using a 24-well tissue culture plate, place a tissue culture insert containing the appropriate pore size into each well, just prior to adding the cells.

3. Dilute cells into the upper-well buffer such that the correct number of cells is added when the chamber is filled (Table 12.1.3).

   The number of cells is based on the exposed filter area. The cells will rapidly settle by gravity onto the upper surface of the filter.

4. Incubate chamber in a humid environment at 37°C.

   The duration of incubation depends of the cell type, filter, and parameter assayed (see Table 12.1.4).

Table 12.1.3 Characteristics of Assay Chambers Used for Filter Assay

Assay chamber	Lower well volume (µl)[b]	Filter surface area	Upper well volume (µl)	Number of cells
Blind-well[a]	200	18 mm^2	200	50,000
12-well multiwell[a]	150[b]	18 mm^2	100	48,000
48-well multiwell[a]	25	3.2 mm^2	50	15,000
24-well tissue culture plate	600	0.3 cm^2	100	1,000,000

[a]Available from Neuro Probe.
[b]It is best to load a small excess to prevent air bubbles.

Fix and stain cells

5. Drain off top wells with a pipet. Disassemble chamber and remove filter carefully. Immediately put empty chambers in water to soak. If a thin filter is used, scrape the upper side with a filter scraper.

   It is easier to clean chambers if they are not allowed to dry.

   It is difficult to separate the focal planes at the top and bottom of thin filters. Most investigators, therefore, wipe the cells off the upper surface before attempting to count those on the lower surface (Harvath et al., 1980). As an alternative to a filter scraper, a clip can be attached to either end of the filter, and a moist Q tip can be used to scrape the cells from the top side of the filter while the filter hangs from one clip. It is important to work quickly before the filter dries.

6. Place the filter in 10 ml of 3.7% formaldehyde in a small Coplin jar and incubate 30 min to overnight at room temperature.

   Alternatively, a thin filter can be fixed in methanol and air dried for staining with Diff-Quick (Harvath et al., 1980).

7. Stain the cells on the filter ≥1 hr at room temperature (see Table 12.1.4). Make sure multiple filters are separated from one another so that stain readily penetrates each filter.

   For Diff-Quick and hematoxylin, follow manufacturer's instructions.

8. Destain and clear the filter by incubating 5 min each as follows: twice in water, once in 70% isopropanol, twice in 100% isopropanol, once in 1:1 isopropanol/Americlear, and three times in Americlear. Incubate the filter 30 min in Permount and mount on a microscope slide.

   Large slides (e.g., 75 × 50–mm) are convenient for mounting and viewing the larger filters (e.g., 48-well filter).

Score cell migration

9a. *To score distance moved in a thick filter:* Under a 40× objective, use the micrometer on the fine focus knob of the microscope to measure the distance from the top of the filter to the furthest two cells in a single focal plane (or some other convenient measure). Repeat in 5 separate fields across the filter.

   In some instances, investigators have measured the population distribution in the filter (Zigmond and Hirsch, 1973; Buettner et al., 1989; Table 12.1.5).

Table 12.1.4 Incubation Times and Staining Procedures for Filter Assays

Cell type	Filter type	Pore size (μm)	Duration of incubation	Stain
Neutrophils	Thick	3.0	0.5-2.0 hr for migration into filter; 3-4 hr for migration through filter	Formaldehyde fixed: freshly prepared Giemsa, 1 hr to overnight
Neutrophils	Thin	3.0	20 min	Methanol-fixed, air dried: Diff-Quick
Lymphocytes	Thin	3.0-5.0	4 hr	Hematoxylin, overnight
Fibroblasts	Thin	8.0	3-4 hr	Hematoxylin, 1 hr to overnight

Table 12.1.5 Response of Neutrophils to *f*-Met-Met-Met: Mean Distance From the Top of the Filter to the Front PMNs[a]

Concentration above the filter (M)	Distance to front PMNs (μm) at concentration below the filter (M)			
	10^{-9}	10^{-8}	10^{-7}	10^{-6}
10^{-9}	27	30 [27]	57 [30]	69 [35]
10^{-8}	27 [30]	30	47 [30]	62 [35]
10^{-7}	34 [33]	36 [34]	34	60 [38]
10^{-6}	38 [52]	34 [52]	48 [52]	56

[a]Numbers in brackets are the distances to the front two PMNs calculated for a hypothetical population undergoing random migration (see Support Protocol 1), based on PMN migration observed in a uniform concentration gradient (numbers along the diagonal of the table). Data from Showell et al. (1976); reproduced with permission from Rockefeller University Press. *f*-Met-Met-Met, *N*-formyl-methionyl-methionyl-methionine; PMN, polymorphonuclear leukocyte.

9b. *To score number of cells on lower surface of a thin filter:* Using a 16× or 40× objective, count the number of cells per field that are on the under side of the filter for each well. Repeat for at least 5 fields.

When using a new cell type, it is important to check that cells are not falling off the filter into the bottom well.

9c. *To count cells recovered from lower chamber:* Collect fluid from the lower well, pipetting fluid up and down to remove cells stuck to bottom and sides of the well. Use a hemacytometer or cell sorter to count the number of cells in the cell suspension. To quantify by cell sorting, mix half of the cell suspension with a known number of 15-μm polystyrene beads. Run the suspension through the sorter and acquire a plot of forward scatter versus side scatter. Determine the ratio of beads to cells, and thereby calculate the number of cells in the cell suspension.

Since they have different light-scattering properties, beads and cells will appear as two discrete populations, allowing quantitation of cells from the known number of beads (Campbell et al., 1997b).

Differentiate chemotaxis from chemokinesis

10. If enhanced migration is observed at a particular concentration in the lower well, test the effects of that concentration on migration (both rate and number of migrating cells) when it is present both above and below the filter. Also test ½, ¼, and ⅛ of that concentration.

This approach allows a rough evaluation of whether the results observed under gradient conditions are due to chemotaxis or to effects on the rate of migration or on the number of migrating cells (Wilkinson, 1982). It makes the reasonable assumptions that the gradient through the filter is linear, and that cells moving from one concentration to another rapidly adjust their rate of locomotion to that observed when cells are incubated exclusively at that concentration. Simple mathematical analysis of chemokinesis versus chemotaxis can also be applied (see Support Protocol 1).

CALCULATING THE DISTANCE CELLS ARE EXPECTED TO MOVE IN THICK FILTERS IN THE ABSENCE OF CHEMOTAXIS

This calculation, which comes from Zigmond and Hirsch (1973), can be used to roughly estimate the contribution of unstimulated migration and chemokinesis to the extent of migration into a thick filter (see Basic Protocol 1). More sophisticated calculations are found in Buettner et al. (1989). Migration beyond this value can be considered a chemotactic response. Calculation of migration in the absence of chemotaxis is based on a number of assumptions: (1) a linear gradient exists across the filter, (2) acceleration of the cells between two known velocities is constant, and (3) movement of the leading two cells of the population is equal to that of a single particle moving with a velocity that is a function of the concentration of stimulatory material.

For this sample calculation, migration of cells was initially examined in a gradient of 10% to 50% serum across a 130-µm-thick filter. To test migration in the absence of chemotaxis, the velocity of migration was determined at three homogeneous concentrations (i.e., same above and below the filter): 10%, 30%, and 50%. The duration of the assay (30 min, or 1 time unit) is the same in both gradient and homogeneous assays. The following assay parameters/results are used. (1) The velocity (V) in a given concentration (both above and below the filter) is the distance from the top of the filter to the front two cells after an incubation of 1 time unit. In this example, velocities were determined to be: V_o = 84 µm/unit time (10% serum), V_1 = 73 µm/unit time (30% serum), and V_2 = 60 µm/unit time (50% serum). (2) The distance from the top of the filter to the first concentration for which a velocity is determined is d_1, the distance from the first concentration to the second is d_2, and so on. Since this example calculated velocities for three evenly distributed concentrations over a linear gradient, d_1 is the distance from the top of the filter (10%) to the middle of the filter (30%), and d_2 is the distance from the middle to the bottom of the filter (50%). Thus, $d_1 = d_2$ = 65 µm.

Since acceleration is constant, the mean velocity over distance d_1 is $\overline{V}_1 = (V_0 + V_1)/2$ = 78.5 µm/unit time. Thus, the time required to move from the top of the filter over distance d_1 is $T_1 = (d_1)/(\overline{V}_1)$ = 0.828 unit time. Similarly, the mean velocity over distance d_2 is $\overline{V}_2 = (V_1 + V_2)/2$ = 66.5 µm/unit time, and the time to move over d_2 is $T_2 = (d_2)/(\overline{V}_2)$ = 0.977 unit time.

Since $T_1 + T_2 > 1$ unit time, the front two cells will not get all the way across $d_1 + d_2$ in 1 unit time. To calculate the distance the cells will move into d_2 in one unit time, acceleration over d_2 is used, where $a_2 = (V_2 - V_1)/T_2 = -13.3$ µm/(unit time)^2. The time remaining after the cells have crossed d_1 (i.e., the time they migrate through d_2) is $T_f = 1 - T_1$ = 0.172 unit time. The distance migrated into d_2 is

$$V_1(T_f) + \frac{a_2(T_f)^2}{2}$$

Thus, the total distance the front cells would be expected to move is

$$d_t = d_1 + V_1(T_f) + \frac{a_2(T_f)^2}{2}$$

$$d_t = 65\,\mu\text{m} + (73\,\mu\text{m / unit time} \times 0.172\text{ unit time}) +$$

$$\frac{[-13.3\,\mu\text{m / (unit time)}^2] \times (0.172\text{ unit time})^2}{2} = 77\,\mu\text{m}$$

Table 12.1.6 Sample Data for Cell Response to Serum[a]

Concentration above the filter (%)	Distance migrated (μm) at concentration below the filter (%)		
	10	30	50
10	84		[77]
30		73	
50			60

[a]Unbracketed numbers are measured values at homogenous concentrations. Bracketed number is the calculated value for the 10% to 50% gradient.

The measured distances at homogeneous concentrations and the calculated distance for this gradient are presented as for a checkerboard assay in Table 12.1.6. To determine whether chemotaxis occurs with these cells in a 10% to 50% serum gradient, the measured distance for migration in the gradient is then compared to this calculated value. Movement greater than 77 μm would suggest that chemotaxis occurs.

UNDER-AGAROSE CHEMOTAXIS ASSAY

BASIC PROTOCOL 2

In this assay (Nelson et al., 1975), a tissue culture dish is filled with a mixture of agarose and cell culture medium. Wells are cut in the hardened agarose, and chemoattractant added to one well forms an approximately exponential gradient as it diffuses into the agarose (Foxman et al., 1997). Cells are placed in a nearby well, from which they migrate in the small space between the agarose layer and the culture dish. The cell distribution, in the presence and absence of chemoattractant, is determined after an incubation period (Fig. 12.1.3). The assay is useful for monitoring migration of neutrophils, monocytes, and possibly lymphocytes (Nelson et al., 1975; Laroche et al., 1983), as well as more adherent cells (Orredson et al., 1983; Stokes et al., 1990).

This protocol describes the analysis of cells fixed after a set migration time. Alternatively, the behavior of migrating cells can be monitored under the agarose by video microscopy using an inverted microscope fitted with a charge-coupled device (CCD) camera. It is important to determine the best parameters (e.g., magnification, contrast) to observe the behavior of interest. To maintain the proper environment, a stage warmer should be used to keep the cells at 37°C, a humidified chamber should be used to prevent drying, and HEPES-buffered agarose and migration medium should be used to maintain pH.

Materials

Agarose plate mixture (see recipe)
Migration medium (see recipe)
Cell suspension (10^7/ml) in migration medium
Chemoattractant solution in migration medium
Absolute methanol
37% (w/v) formaldehyde
0.5% (w/v) Fields stain B (Gallard-Schlesinger)
2.5% (w/v) Fields stain A (Gallard-Schlesinger)

35-mm tissue culture dishes
3-mm hole cutter (e.g., steel punch or plastic pipet tip), sterile, with a vacuum line and a catch flask
Template for cutting 3-mm wells separated by 2 mm in a single line
Counting grid (optional)

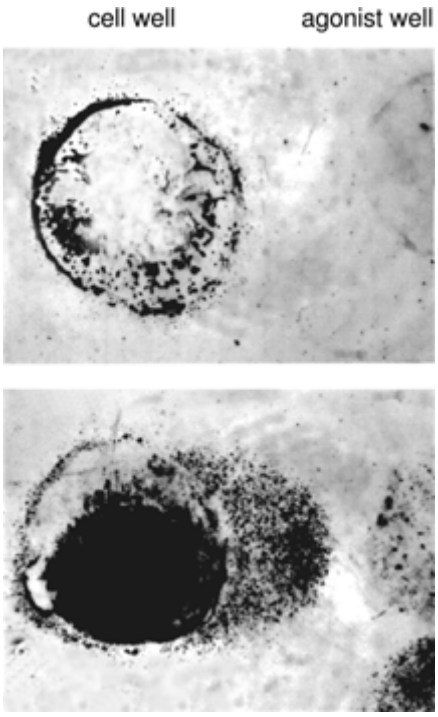


Figure 12.1.3 Under-agarose assay: response of neutrophils to interleukin 8 (IL-8). (**A**) Neutrophils exposed to a well containing no IL-8. (**B**) Neutrophils exposed to a well containing 1 pmole IL-8. Reproduced from Foxman et al. (1997) with permission from Rockefeller University Press.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Prepare plates

1. Add 3 ml agarose/plate mixture to each 35-mm tissue culture dish and allow to cool and solidify at room temperature. Transfer plates to a humidified 37°C, 5% (v/v) CO_2 incubator, and incubate several hours to overnight.

2. Attach a sterile 3-mm hole cutter to a vacuum line with a catch flask. Use a template to make five 3-mm wells in a straight line, separated by 2 mm. Push the implement through the agarose layer until it reaches the plastic plate, using the vacuum to remove the agarose plug.

   If using a steel punch, avoid scratching the plastic; scratches become barriers to cell migration.

   The agarose plugs collect in the catch flask. To prevent the tubing from clogging, flush with water after cutting holes.

   Cut wells no more than several hours before the assay. If a well is cut too long before it is filled with fluid, the agarose around the well dries and sticks to the plastic, preventing the cells from crawling underneath.

   A good template is a piece of metal containing precisely positioned holes (Nelson et al., 1975) and constructed to fit over a 35-mm dish. Alternatively, an actual-size diagram of the desired well pattern can be placed underneath the dish and used as a guide for cutting wells.

   The pattern of wells can be varied. Linear and parallel cell and attractant wells can be cut, yielding a steeper gradient of attractant, with a linear rather than radial distribution (Tranquillo et al., 1988). A triangular placement of wells allows analysis of migration in the presence of two chemoattractant sources (Foxman et al., 1997).

Prepare cells and chemoattractants

3. Suspend chemoattractants at the desired upper concentrations in migration medium. Suspend cells at 10^7 cells/ml in migration medium.

   To form an effective gradient, the chemoattractant concentration should be ~10 to 1000 times the chemoattractant receptor K_d (Table 12.1.2).

Perform assay

4. At the start of the assay, fill the two most peripheral wells with 10 µl migration medium. Next, place 10 µl cell suspension (10^5 cells) in each of the two intermediate wells. Finally, place 10 µl chemoattractant in the central well.

5. Return the plate to the 37°C, 5% CO_2 incubator for 2 hr (neutrophils) or longer (other cell types).

Fix and stain cells

6. Flood each plate with 1 ml absolute methanol. Allow cells to fix 30 min at room temperature, or overnight at 4°C.

7. Pour off methanol and flood each plate with 1 ml of 37% formaldehyde. Allow cells to fix 30 min at room temperature (longer if cold).

8. Remove agarose, using a Pasteur pipet to pry the edge loose, if necessary.

   The cells should now be fixed to the plastic and thus remain on the dish.

9. Add 1 ml of 0.5% Fields stain B followed by 1 ml of 2.5% Fields stain A, and allow to stain for 1 to 2 min. Rinse plates with water and allow to dry.

Measure cell migration

10a. *Measure leading edge distance:* Along a line running through the center of the five wells, measure the distance from the edge of the cell well to the edge of the migrating cell front. Compare the distance cells have migrated toward the chemoattractant well to the distance they have migrated toward a well containing only medium (i.e., the peripheral wells).

   A magnifier with reticles (Fisher Scientific) is a useful tool for performing these measurements.

10b. *Determine number of migrating cells:* Use a counting grid to count the number of cells that have migrated into squares at different distances from the cell well.

   This method allows comparison between the number of cells migrating in one assay versus another. A plot of cell density at different distances from the starting well provides more information about cell migratory behavior than an estimate of the leading front distance alone (Lauffenburger et al., 1983).

11. Evaluate chemokinesis and chemotaxis coefficients.

   Because most chemoattractants stimulate cell motility in a dose-dependent manner, a careful analysis is required to distinguish chemoattractant-stimulated motility (chemokinesis) from directional migration (chemotaxis). One approach is to measure chemoattractant-stimulated motility by observing the effect of a uniform field of chemoattractant (i.e., incorporated into the agarose) on cell migration, at a variety of different concentrations. Using these measurements, a chemokinesis coefficient can be determined, which can be used to calculate the relative contribution of chemokinesis and chemotaxis to the cell migration pattern in a chemoattractant gradient (Tranquillo et al., 1988).

   The orientation and directionality of migration of individual cells can be observed by viewing migrating cells using video microscopy. In fixed cells, some investigators have used nuclear orientation to determine if cells are oriented toward the chemoattractant source (Palmblad et al., 1982; Krauss et al., 1994).

BASIC PROTOCOL 3

SMALL POPULATION CHEMOTAXIS ASSAY

This assay utilizes a hydrophobic agar surface to allow the placement of two small drops of liquid extremely close to each other (Konijn, 1970; Konijn and Van Haastert, 1987; Fig. 12.1.4). Diffusion through the agar generates a transient gradient, which allows a qualitative (yes or no) judgment as to whether there is a chemotactic response. This assay has not been used for any cell types other than *Dictyostelium*.

Materials

Purified agar (e.g., Noble agar, Difco)
Bonner's salts (see recipe)
Dictyostelium cells at $\leq 5 \times 10^6$ cells/ml
17 mM Sorensen's phosphate buffer, pH 6.2 (see recipe)
Chemoattractant solution in Sorensen's phosphate buffer
10-cm petri dishes

1. Prepare 0.5% to 1% (w/v) purified agar in Bonner's salts.

   Purified agar can be replaced by less-expensive bacterial agar (e.g., Bacto agar, Difco) if it is extensively washed. A 20-g aliquot should be washed five times with 1 liter distilled water, followed by ten to fifteen times with an equal volume of deionized water, using no. 1 Whatman filter paper and a funnel to remove each wash (Konijn and Raper, 1961).

   The correct hydrophobicity and agar density is reached when 0.1-µl drops of cell suspension form half spheres and do not spread out from their original deposition site, and when the cells are unable to move out of the area covered by the original drop.

2. Pour 10 ml agar/salt solution in 10-cm petri dishes and allow to solidify.

   Plates can be stored 1 day at room temperature followed by up to one week at 4°C.

3. Wash *Dictyostelium* cells twice in ice-cold 17 mM Sorensen's phosphate buffer, pH 6.2, and resuspend at 10^7 cells/ml. Shake suspension 1 hr (for measuring responses to folate) or 6 to 8 hr (for cAMP) at 150 rpm, 21° to 23°C.

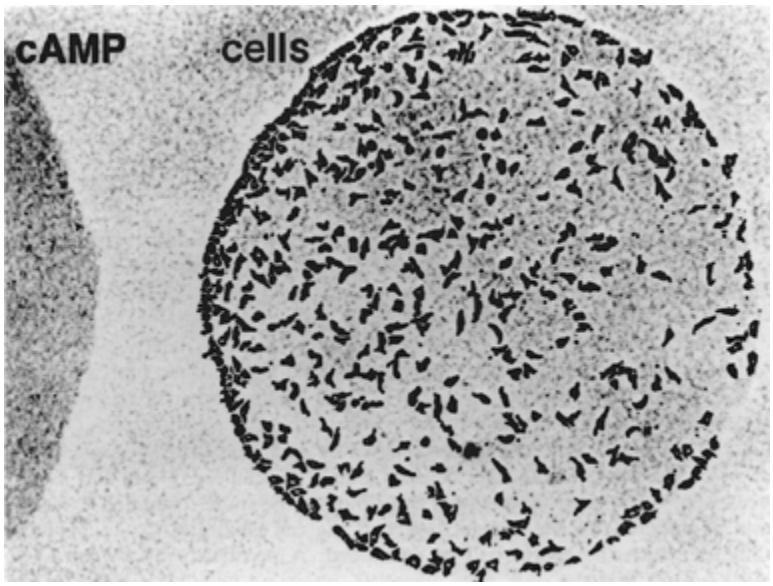


Figure 12.1.4 Small population assay. A single drop containing 500 to 1000 cells was exposed to cAMP (applied on the left in three 0.1-µl drops containing 10 nM cAMP) and was photographed 5 min after stimulus. Note the higher denisty of cells on the side nearest the stimulus. Reproduced from Konijn (1970) with permission from Birkhauser Verlag AG.

It is important that starting cells be grown at or below 5×10^6 cells/ml so that they are in log growth phase. For information on preparing Dictyostelium, consult Devreotes et al. (1987). Grow axenic strains in axenic media such as HL5 (Watts and Ashworth, 1970). Grow nonaxenic strains in association with bacteria (Sussman, 1987).

4. Wash cells once more and resuspend at 10^7/ml in 17 mM Sorensen's phosphate buffer. Place 0.1-µl drops (10^3 cells) at regular intervals on the agar surface.

   The small drops can be generated by using a Pasteur pipet with the tip drawn out by hand over a flame, and broken off (once cooled) to provide a small bore (40- to 70-µm inside diameter). Fine pressure control can be achieved by using rubber tubing and either mouth pipetting or using a pipet bulb. Uniform speed of deposition of the drops is important to minimize variation in cell density. The pipet should be refilled after every ten to twenty drops to prevent cells from settling in the pipet and ensure uniformity of cell density. It is useful to set up rows of 10 replicate drops, with each row stimulated by a different concentration of chemoattractant.

5. Add 0.1-µl drops of chemoattractant ~100 µm away from the cell drops. Repeat at 5-min intervals for a total of three applications.

   The concentration of chemoattractant should span a range between one tenth and ten times K_d (Table 12.1.2).

6. Incubate ~30 min at 21° to 23°C.

   The plates should be checked every 10 min, since the precise details of when the gradient forms and cells respond will vary slightly from assay to assay, and the gradient may be relatively transient.

7. Examine the distribution of cells within each drop to determine whether more cells are pressing against the side of the drop that is next to the stimulus (Fig. 12.1.4). Quantify the response by calculating the percent of responding drops, with 100% showing a strong response.

   See Konijn and Van Haastert (1987) for images of responding populations.

BRIDGE CHEMOTAXIS ASSAY

BASIC PROTOCOL 4

Cells are prepared on coverslips and are then inverted and observed on a 1-mm-wide bridge that connects two wells via a thin layer of fluid (Fig. 12.1.5). When different concentrations of chemoattractant are placed in the two wells, an approximately linear gradient develops across the bridge. The orientation or movement of cells toward one or the other well is monitored.

Materials

Cells: whole blood, neutrophils in suspension, or *Dictyostelium discoideum*
0.9% (w/v) NaCl
HEPES-buffered HBSS (prepare as for HBSS in *APPENDIX 2A*, but replace bicarbonate with 10 mM HEPES acid) containing 0.2% (w/v) BSA (for neutrophils in suspension)
17 mM Sorensen's phosphate buffer, pH 6.2 (for *Dictyostelium*; see recipe)
Chemoattractant in HEPES-buffered HBSS/1% (w/v) gelatin
HEPES-buffered HBSS/1% (w/v) gelatin

20 × 40–mm coverslips, unwashed (neutrophils) or acid-washed (*Dictyostelium*; see recipe)
Glass (Neuro Probe) or Plexiglas bridge chamber (Fig. 12.1.5; see Critical Parameters)
Microscope with 40× phase objective

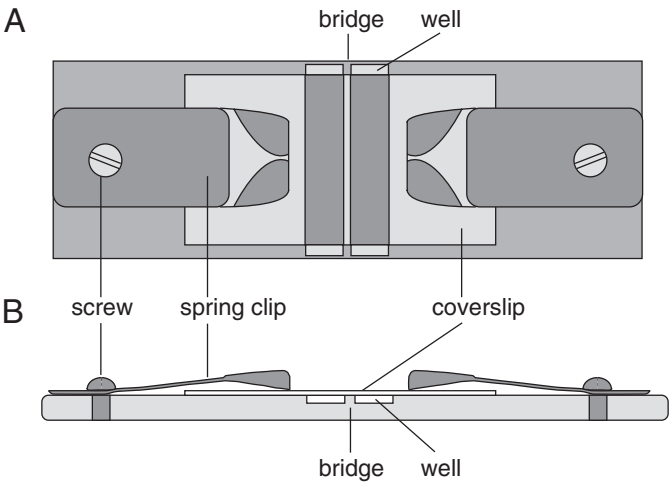


Figure 12.1.5 Apparatus for bridge assay. (**A**) Top view; (**B**) side view. A Plexiglas (or glass) base (3 mm high × 2.5 cm wide) contains two wells (4 to 5 mm wide × 1 mm deep) separated by a 1-mm-wide bridge. A 20 × 40–mm coverslip is placed across the wells and secured by two spring clips, so that a thin gap (e.g., 5 μm) is made between the coverslip and the bridge. Cells on the underside of the coverslip are flattened between the coverslip and bridge, and the direction of migration (to one or the other wells) is monitored.

Prepare cells on coverslips

For neutrophils from whole blood:

1a. Place ~0.1 ml human blood (e.g., from finger prick) across the center of a 20 × 40–mm coverslip.

Enough blood must be placed on each coverslip to allow it to clot and partially retract without drying.

2a. Place the coverslip in a moist chamber (e.g., a petri dish containing a wet piece of filter paper) at 37°C (with or without 5% to 10% CO_2, depending on whether the medium is buffered with bicarbonate). Incubate ~45 min, until the blood has clotted and is beginning to retract so that fluid is visible around the edges.

3a. Gently rinse the clot and red blood cells off with 0.9% NaCl.

A monolayer of cells, mostly neutrophils, remains on the coverslip. Care must be taken not to let the cells dry.

For neutrophils in suspension:

1b. Prepare a suspension of human or rabbit cells at 3×10^5 cells/ml in HEPES-buffered HBSS containing 0.2% BSA.

Neutrophils do not stick well in gelatin, which is used during the assay.

2b. Apply 0.1 ml cell suspension in a ~200-mm^2 strip across the center of a 20 × 40–mm coverslip. Allow to settle for ~5 min at room temperature.

3b. Rinse the cell layer with a few drops of incubation medium immediately before placing coverslip on bridge chamber (step 4).

For Dictyostelium:

1c. Prepare a single-cell suspension of *Dictyostelium* at 5×10^4 cells/ml in 17 mM Sorensen's phosphate buffer, pH 6.2.

2c. Place 50 µl cell suspension in a thin strip across the center of an acid-washed 20 × 40–mm coverslip.

Dictyostelium adhere best to acid-washed coverslips (Soll, 1988; Segall, 1992). The coverslips can also be coated with 2 mg/ml BSA.

3c. Allow cells to settle and attach for 5 to 10 min at room temperature.

Assemble bridge chamber

4. Quickly remove most of the fluid over the cells by tipping the coverslip and allowing fluid to flow onto a Kimwipe.

5. Invert the coverslip onto a clean chamber so that cells lie over the bridge. Lower the coverslip slowly, allowing one edge to touch first.

A clean chamber is essential to successful chemoattractant-induced orientation of cells (see Critical Parameters). Touching one side of the coverslip to the bridge and then lowering the other side helps eliminate air bubbles.

6. Secure the clips on each side without moving the coverslip.

Any movement of the coverslip will cause enough sheer force to lyse the cells on the bridge.

Chamber assembly requires practice, as short distances between the coverslip and the bridge are required for optimal cell orientation (see Critical Parameters). The chamber thickness can be measured by the difference in focal plane between the top of the bridge and the bottom of the coverslip, using the micrometer on the fine focus knob of the microscope.

Perform assay

7. Fill the two wells by capillary action with ~0.1 ml HEPES-buffered HBSS or chemoattractant both containing 1% gelatin.

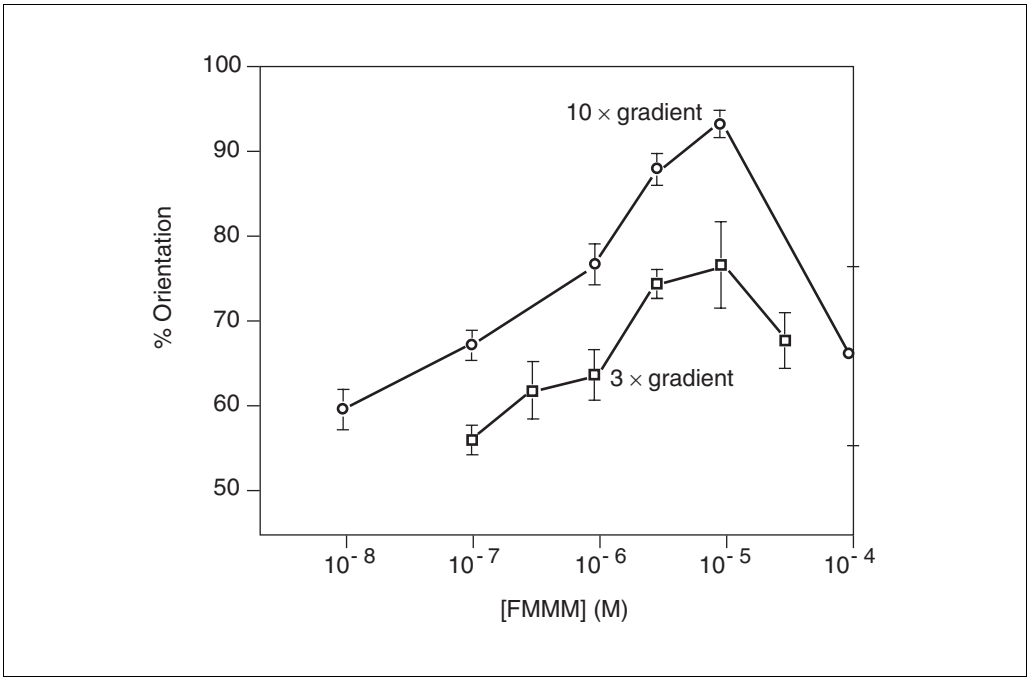


Figure 12.1.6 Results from bridge assay expressed as percent oriented cells versus chemoattractant concentration. The two curves represent different gradient steepnesses. The *x* axis corresponds to the high-concentration well. The other well contained one-tenth (circles) or one-third (squares) this value. *f*MMM, *N*-formyl-methionyl-methionyl-methionine. Reproduced from Zigmond (1977) with permission from Rockefeller University Press.

The chemoattractant concentration in the middle of the bridge will be approximately the mean of the two concentrations in the wells. Thus, the concentration of chemoattractant should range from one tenth to ten times the K_d of the receptor (Table 12.1.2).

A gradient of formyl peptide is ~90% established within 10 min and is roughly stable for 1 hr (Lauffenburger and Zigmond, 1981).

8. Incubate chambers 10 to 20 min at 37°C (or room temperature for *Dictyostelium*) to allow the cells to respond.

9. View cells at the middle of the bridge with a 40× phase objective. Determine the direction of cell migration for 100 cells based on morphological criteria, and plot percent cells oriented toward chemoattractant versus chemoattractant concentration (Fig. 12.1.6).

The front of a locomoting cell has a broad lamellipodium, while the tail is thinner and can be knob-like or drawn out into retraction fibers. The polarized morphology is clearest when the cells are moving well. When cooled, even to room temperature, neutrophils can round up and are difficult to score. The direction of movement can also be evaluated by video recording.

BASIC PROTOCOL 5

PIPET CHEMOTAXIS ASSAY

A pipet containing chemoattractant is brought up to a cell or group of cells, and the behavior of the cells is observed directly or by video microscopy.

Materials

Chemoattractant solution in assay buffer

Assay buffer: 17 mM Sorensen's phosphate buffer, pH 6.2 (see recipe), with or without $CaCl_2$ and $MgCl_2$ (for *Dictyostelium*), or DPBS (*APPENDIX 2*); JRH Biosciences; for MTLn3 cells)

Cells of interest

Omega dot tubing (4-in. glass capillary tubing, 1-mm o.d. × 0.58-mm i.d.; A-M Stevens)

Pipet puller for microinjection or neurobiology, capable of producing ~0.1-μm tip diameters (e.g., David Kopf Instruments, Narishige, Sutter Instruments)

Syringe with fine-bore needle (e.g., 3-in., 30-G)

Micromanipulator that can be attached to a microscope stage (e.g., Leitz, Narishige)

1. Prepare pipets (~0.1-μm tip diameter) using omega dot tubing and a pipet puller.

The thin tip allows a steep concentration gradient to be set up outside the pipet by diffusion, rather than by bulk flow. Omega dot tubing is used because it provides an internal filament to allow backfilling via surface tension (capillary action).

Alternatively, microloader micropipet tips are available from Eppendorf. To evaluate the suitability of the pipet, trial pipets can be filled with a high concentration (0.1 to 1 mM) of fluorescein or other fluorescent dye, and then viewed with a fluorescence microscope. If the tip is too large, a polarized stream of fluorescent material will be seen coming out of the tip. With the correct size tip, a spherically symmetric ball of fluorescence will be seen, brightest near the pipet tip, and getting dimmer with distance from the pipet, reflecting the spatial gradient generated by diffusion. Appropriate tips will be too small to resolve well with a 40× long–working distance objective.

2. Backfill the pipet by inverting and placing a drop of chemoattractant solution on the base. When the tip is full, fill the shank using a syringe with a fine-bore needle.

For expensive protein chemoattractants, load 5 μl directly to the tip with a microloader (Eppendorf).

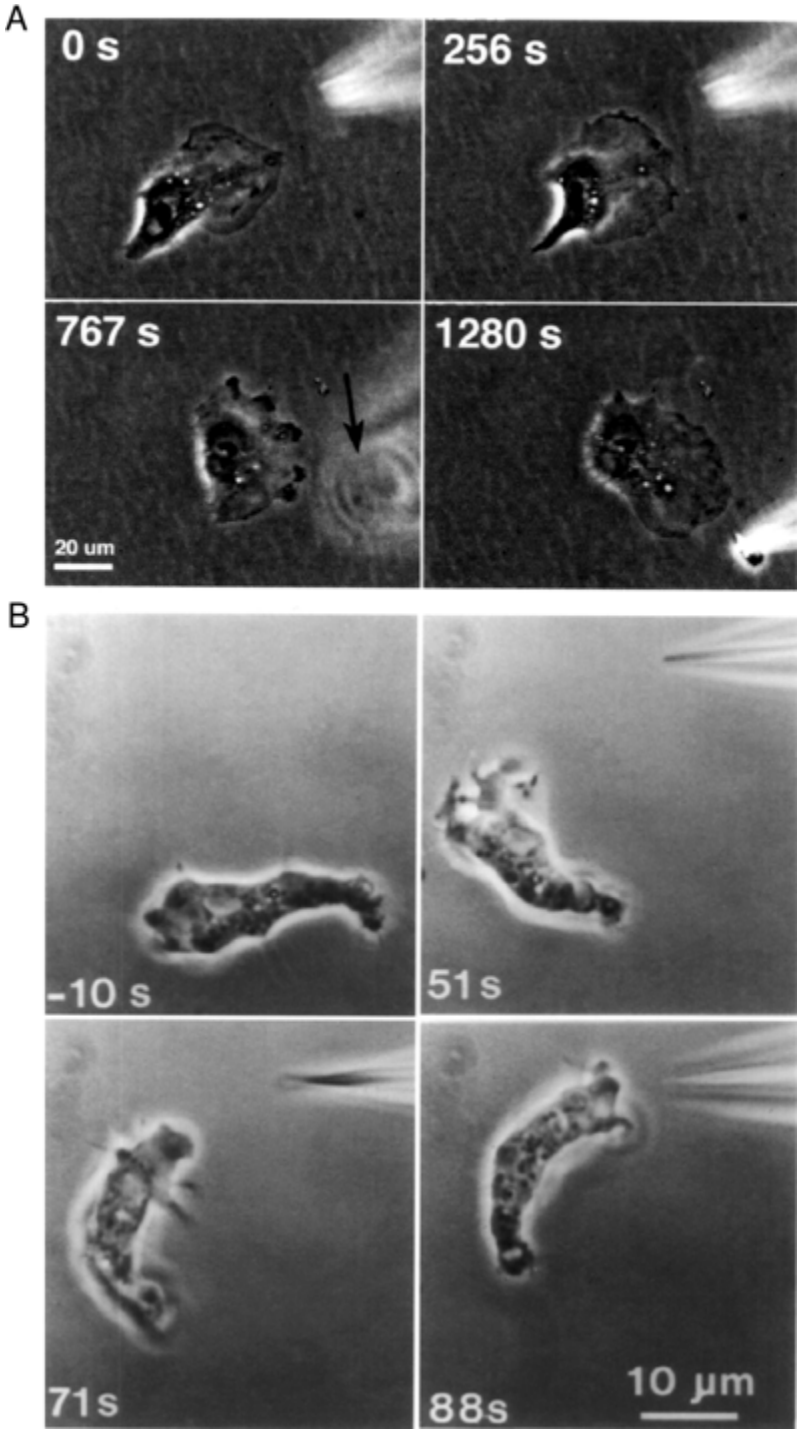


Figure 12.1.7 Pipet assay. (**A**) Response of mammary adenocarcinoma cell to a micropipet filled with 50 µM epidermal growth factor. The cell moved toward the micropipet (0 and 256 sec) and then reoriented when the pipet was moved (767 and 1280 sec). Arrow indicates movement of pipet. Reproduced from Bailly et al. (1998) with permission from Academic Press. (**B**) Response of *Dictyostelium discoideum* cell to a micropipet containing 0.1 mM cAMP. At −10 sec, the cell is moving to the left. At 0 sec, the pipet was placed in the upper right corner of the image field and the cell reoriented rapidly, moving toward the pipet. Reproduced from Segall and Gerisch (1989) with permission from Current Biology Limited.

A high concentration of chemoattractant (10^3 to 10^4 times K_d of the receptor; Table 12.1.2) is required inside the pipet. For example, in Dictyostelium, with a receptor K_d of 20 to 100 nM, 0.1 to 1 mM cAMP in the pipet provides a strong stimulus (>1000 times K_d). For MTLn3 tumor cells, with receptor K_d of 1 to 10 nM, 50 μM EGF in the pipet is necessary.

3. Place cells in a petri dish and cover with a thin layer of assay buffer.

   If temperature control is important, the buffer can be covered by mineral oil to minimize evaporation.

4. Place the micropipet in the micromanipulator and carefully bring the tip of the pipet to within 2 to 5 μm of the surface of the dish. If necessary, use positive pressure to increase the ejection rate.

   Note, however, that fluid flow generated by positive pressure may perturb a gradient that is generated purely by diffusion.

   Depending on the concentration of chemoattractant in the pipet and the chemotactic abilities of the cells, the tip may need to be within 10 μm of the cell, or it may be better at 50 μm away (for pipets containing high concentrations of chemoattractant). However, it is important to note that the gradient becomes shallower as the pipet is moved away from the cell, so that the steepest gradients are produced by pipets near the cell but perhaps utilizing a lower chemoattractant concentration so as to avoid saturating the receptors.

5. Incubate for the appropriate time.

   For Dictyostelium, responses can occur within seconds to minutes, while for slower-moving cells (e.g., MTLn3 cells), reorientation and movement may take 10 to 20 min.

6. Score the response of the cells (Fig.12.1.7) by measuring (1) the percent of cells that move toward the pipet, (2) the accuracy of redirection (cosine of the angle between the direction of cell movement and the direction of the pipet), or (3) the speed of cell movement toward the pipet.

   Typically, a 40× long–working distance objective provides adequate resolution. Lower magnification objectives may be useful in order to visualize the responses of multiple cells.

BASIC PROTOCOL 6

UPSHIFT CHEMOTAXIS ASSAY

In this assay, cell behavior is observed after a sudden homogeneous increase (or decrease) in concentration of chemoattractant. This assay is useful for biochemical analysis of chemotactic signal transduction, which almost always relies upon bath application of chemoattractant to cells followed by lysis and biochemical assays. In such studies, there is little or no significant spatial gradient. Rather, a sudden temporal change in chemoattractant concentration is the stimulus.

Although this is different from the stimulus experienced by cells in a spatial gradient, it provides useful information about the kinetics of various biochemical responses. A similar evaluation of behavioral responses to such a stimulus is also possible, allowing the correlation of biochemical changes with specific behaviors. In its simplest form, the upshift assay can be performed by microscopic evaluation of cells moving in a petri dish. By adding an equal volume of chemoattractant solution, a relatively efficient concentration increase is effected and cell behavior can be observed. An example of this approach is shown in Figure 12.1.8, and a detailed protocol is provided in Cammer et al. (1997). Alternatively, a number of specialized chambers or procedures can be utilized to allow a more precise control of the concentration (Varnum-Finney et al., 1988; Segall, 1992) or of the rate of increase in concentration (Omann and Sklar, 1988).

USING IMAGE ANALYSIS PROGRAMS TO ASSESS CHEMOTAXIS

In many chemotaxis assays (e.g., under-agarose, bridge, micropipet, and upshift assays), the area, shape, speed, and direction of movement of individual cells must be measured. Although it is possible to trace video or still images on the monitor screen, a number of image processing programs that aid in subsequent quantification are available. DIAS (Dynamic Image Analysis System) is specifically designed for such analysis (Soll, 1995). Depending on the image itself, the program can directly determine cell borders (for slightly defocused bright-field images). Alternatively, it allows a user to trace high-resolution differential-interference-contrast (DIC) or phase-contrast images. The program then calculates the center of mass, area, roundness, speed, and direction of movement of each cell in the field. A number of other parameters can be calculated as well. Other programs, such as NIH Image (Cammer et al., 1997) and Metamorph (Universal Imaging), can also be adapted to provide a limited subset of similar measurements.

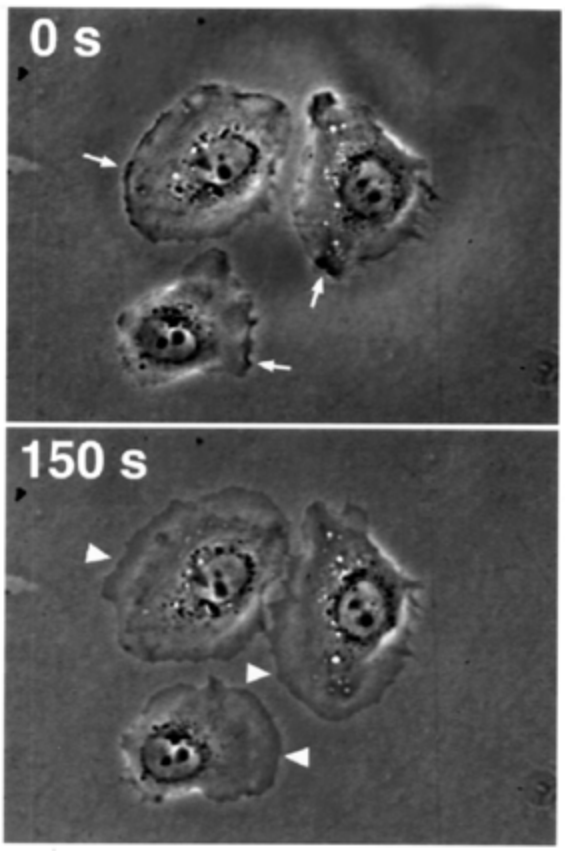


Figure 12.1.8 Upshift assay. MTLn3 cells were grown 24 hr on tissue culture plastic in growth medium in a 37°C CO_2 incubator. They were transferred to a microscope with a 37°C incubator box. The lid of the dish was removed and the medium was covered with a layer of prewarmed mineral oil (Sigma). After tracking for 5 to 10 min, an equal volume of 10 nM epidermal grown factor (EGF) in 37°C growth medium was added under oil using a sterile pipet (final 5 nM stimulus). The top panel shows cell morphology immediately after addition of EGF. The phase-dark areas at the edges of the cells are vertical ruffles (arrows). The bottom panel shows cell morphology 150 sec after addition of EGF. The ruffles have disappeared and flat hyaline lamellipodia have extended (arrowheads), increasing the total area of the cells. Reproduced from Segall et al. (1996) with permission from Kluwer Academic Publishers.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acid-washed coverslips

Soak coverslips 10 min in 2.7% HCl. Rinse extensively in water and dry in a clean area or a laminar-flow hood. Store several days to a week at room temperature in a clean, sealed container to reduce the rate at which the surfaces become hydrophobic.

Agarose plate mixture

For bicarbonate-buffered under-agarose plates:

Boil agarose in HBSS (*APPENDIX 2A*) at a final concentration of 2.4% (w/v). Make a separate solution of 20% (v/v) heat-inactivated bovine calf serum (BCS) in bicarbonate-buffered RPMI 1640. When agarose solution is cooled to 50°C, mix solutions 1:1 (final 1.2% agarose, 10% BCS, 0.5× RPMI, 0.5× HBSS) and use immediately.

For HEPES-buffered under-agarose plates:

Boil 2.4% (w/v) agarose in sterile water. In a separate container, dilute 1 M HEPES (Life Technologies) to 20 mM in 2× RPMI 1640 without bicarbonate (from low-endotoxin 10× RPMI 1640 stock; Sigma). Add 20% (v/v) heat-inactivated BCS to RPMI/HEPES and adjust to pH 7.2. When agarose solution is cooled to 50°C, mix solutions 1:1 (final 1.2% agarose, 10% BCS, 1× RPMI) and use immediately.

For both types of plates, the agarose must be boiled and cooled before addition of protein solution, to avoid denaturing the protein. For serum-free assays, replace 20% BCS with 1% (w/v) BSA or 0.25% gelatin (see Critical Parameters).

Media with serum/protein (but no agarose) can be filter sterilized and stored up to 6 months at 4°C.

Bonner's salts

10 mM NaCl
10 mM KCl
2.7 mM $CaCl_2$
Store at 4°C (stable at least 1 month)

Giemsa stain

4 ml 95% (v/v) ethanol
3 ml Giemsa stock (Ricca Chemical)
43 ml 15 mM sodium or potassium phosphate buffer, pH 6.8
Prepare fresh before use

Migration medium

For bicarbonate-buffered under-agarose plates (see agarose plate mixture above), use 1× bicarbonate-buffered RPMI 1640 with 10% (v/v) heat-inactivated BCS. For HEPES-buffered under-agarose plates, use 1× RPMI without bicarbonate (from low-endotoxin 10× RPMI 1640 stock; Sigma) containing 10 mM HEPES and 10% (v/v) heat-inactivated BCS. Filter sterilize and store up to 6 months at 4°C.

For either medium, 10% BCS can be replaced with 0.5% (w/v) BSA (see Critical Parameters).

Sorensen's phosphate buffer, 17 mM, pH 6.2

For 10× stock solution:
20.6 g/liter KH_2PO_4
5.05 g/liter $Na_2HPO_4 \cdot 7H_2O$
Store at room temperature or 4°C (stable at least 1 month)
Before use, dilute to 1× and supplement with 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$

COMMENTARY

Background Information

Distinguishing chemotaxis from chemokinesis

Chemotaxis assays are designed to determine if a biological compound has the ability to induce directed migration of cells. To demonstrate that a factor elicits chemotaxis, it is important to show that a gradient of the factor biases the direction of locomotion rather than simply stimulating locomotion in random directions (chemokinesis). Unfortunately, merely demonstrating that cell migration is not stimulated when the gradient is reversed does not prove that the test substance is a chemoattractant. This is because many factors, including many chemoattractants, stimulate the rate of migration in a concentration-dependent manner, and because very high concentrations of some substances can be less stimulatory than lower concentrations (Harvath et al., 1980; Tranquillo et al., 1988).

To determine if chemotaxis is present in a spatial gradient, one must show that the enhanced migration cannot be accounted for quantitatively by enhanced rates of random migration. To do this one needs to first define the effects on random migration, by quantifying migration in a series of homogeneous concentrations of the test material (a checkerboard assay as in Tables 12.1.5 and 12.1.6). Once the effects of different concentrations on random migration are known, it is possible to determine if they account fully for the migration observed in a gradient. Migration in a positive gradient that exceeds that expected from random migration is evidence for chemotaxis. This analysis does not correct for the possibility that migration is stimulated as cells are exposed to higher concentrations of attractant and inhibited as the concentrations decrease (Rhodes, 1982). Such effects on migration rates have been observed, but their magnitude is probably too small to appreciably alter the results. It is also possible to determine if a factor testing positive in an initial assay is truly chemotactic by testing it in an assay that directly monitors the movement of individual cells.

When and why should one determine if a factor is really a chemoattractant?

In some cases the investigator may not care if the response is due to enhanced migration or chemotaxis. If documentation of chemotaxis is not shown, a valid way to report the results is that the factor "stimulated (or enhanced) cell migration." However, understanding the cellular basis for the increased migration is important for predicting how the factor will function in a given situation. Consider a test factor that merely enhances migration in a concentration-dependent manner. A purely chemokinetic factor can appear to attract cells in one assay but repel them in a different assay. If, in one assay, cells are placed in a well and exposed to a gradient of this factor emanating from an adjacent well, cells exposed to the higher concentration will move faster and spread further toward the well than cells exposed to the lower concentration. However if, in a second assay, the cells are distributed evenly throughout a gradient, again emanating from an adjacent well, the cells exposed to the higher concentration will again move the fastest, but now the movement results in net dispersion of cells away from the well containing the factor. Thus, the factor could appear to be a chemoattractant in one assay and a repellent in a second. In reality, it simply stimulates migration in a concentration-dependent manner.

Chemotaxis when the gradient is attached to the substratum

There are many situations in which the chemoattractant is not in solution but rather is attached to the substratum. Cells can respond to a chemoattractant gradient even when the chemoattractant is attached in a fixed gradient (Wilkinson and Allan, 1978). In vivo, it is likely that binding of chemoattractant to the extracellular matrix allows a gradient to be maintained in the midst of water flux through the extracellular space (McCormick et al., 1993, 1995; Webb et al., 1995). In other cases, the chemoattractant acts directly through adhesive receptors that signal to the cell interior to stimulate spreading and process outgrowth (Aznavoorian et al., 1996; Condic and Letourneau, 1997). Finally, adhesivity itself can result in cell accumulation in regions of high adhesivity, a phenomenon initially termed haptotaxis (Carter, 1965). This cell accumulation can result merely from the trapping of cells in regions of very high adhesivity, not from directed movement (Harris, 1973).

Variations in the basic assays

The assays presented in this unit represent basic starting procedures. They can be modified

for a variety of specific purposes. Some examples of such modifications are described here.

Filter assays. Cell migration assays performed with filters can be automated through the use of radiolabeled or fluorescently tagged cells. In addition, instead of filters, cell migration can be assayed across epithelial cell monolayers (Nash et al., 1987), endothelial cell monolayers (Huang et al., 1988), or gels of extracellular matrix components (Loike et al., 1995).

Under-agarose assays. An interesting variation has been developed by Tranquillo and collegues (Moghe et al., 1995). These investigators initiate the assay with neutrophils evenly distributed throughout a fibrin gel rather than concentrated in a well. This geometry allows analysis of cell movements that are uncomplicated by the effects of high cell concentration and gradients of cell concentration.

Bridge assays. The chemoattractant can be added after the cells have had time to equilibrate in the chamber. With *Dictyostelium* amoebae, where the thickness of the fluid layer does not appear to be so critical, it is possible to first fill the wells with buffer and incubate the bridge chamber in a humidified chamber for 20 min at room temperature to allow the cells to equilibrate. Unstimulated cell behavior can be measured under these conditions. One well can then be flushed with 10 nM to 2 μM cAMP, while the other is flushed with just buffer. After allowing 10 min for gradient formation, cell behavior over the next 20 to 30 min will demonstrate the chemotactic response. Cells move at ~10 μm/min with a strong bias toward the well filled with cAMP (cosine of direction of movement relative to the direction of the gradient is typically 0.3 to 0.6). With neutrophils, also, it is possible to remove the fluid from a well and replace it with a different solution. However, care must be taken not to move the coverslip, which will shear the cells on the bridge.

For slower moving cells such as fibroblasts or epithelial cells, a chamber that may have a more stable, longer-lasting gradient has been designed (Zicha et al., 1997) and is available from Weber Scientific. An alternate approach to generating a stable spatial gradient utilizes hollow fibers embedded in an agarose gel that is continuously supplied with new medium. This method provides the longest-lasting gradients, but is quite delicate and is not commercially available. Details on construction of the chamber are described in Fisher et al. (1989).

Pipet assay. A two-pipet assay has been used to analyze the dynamics of leukocyte pseudopod extension (Zhelev et al., 1996).

Critical Parameters

Equipment options for filter assays

For filter assays (see Basic Protocol 1), there are a number of chamber and filter options to select from (see Tables 12.1.3. and 12.1.4).

Chamber design. Filter assays can be performed in individual, blind-well chambers (Fig. 12.2.1), or in multiwell chambers (Fig. 12.1.2) containing 12 or 48 wells. These are available from Neuro Probe. Alternatively, cells can be induced to migrate through porous tissue culture inserts (Collaborative Biomedical Products, Corning Costar) that fit in standard 24-well tissue culture dishes.

Filters. The original Boyden filter assay used thick cellulose acetate or nitrate filters (Boyden, 1962). They are ~100 μm thick, and have torturous pores (Millipore or Neuro Probe). Because these filters are thick, measurable parameters include: the distance of migration into the filter (Zigmond and Hirsch, 1973; see Support Protocol 1), the number of cells that have moved a given distance into the filter, and the number of cells that have migrated all the way through the filter. These filters may be used with blind-well or multiwell Neuro Probe chambers.

Thin polycarbonate filters (Nucleopore) are only ~10 μm thick and have pores that are straight channels through the filter. These filters can be used to count the number (or fraction) of cells that have migrated through the filter to the lower surface (Harvath et al., 1980) and to count the number of cells that have fallen off into the lower chamber. Since the filters are only one cell diameter thick, it is impossible to further define the distance migrated. Polycarbonate membranes are available for blind-well and multiwell Neuro Probe chambers. In addition, two types of thin filters are available as tissue culture inserts for standard 24-well tissue culture plates, including polyethylene terephthalate (PET) BIOCOAT control inserts (Collaborative Biomedical Products) and polycarbonate Transwell inserts (Corning Costar). It appears that chemokinetic effects are less pronounced in thin polycarbonate filters than in thick cellulose nitrate filters (Bignold, 1988).

Pore size. Both filter types come in different mean pore sizes. The appropriate pore size depends on the cell type being studied (see

Table 12.1.4). Monocytes can be assayed using filters with 5.0- to 8.0-μm pores, and mesenchymal cells using filters with 8.0-μm pores. Neutrophils are usually assayed with 3.0-μm pores. They can migrate into very small pores (1 μm) when induced by a gradient of chemoattractant, but not when exposed to a homogeneous concentration of attractant (Campbell et al., 1997a). Thus, a small pore size can be chosen to minimize the contribution of chemokinesis.

Filter coating. Usually the clean filters are toxic and some protein coat must be included. Albumin can be added to the assay medium, or the filter can be precoated with an extracellular matrix protein. For example, for MTLn3 cells or fibroblasts it is helpful to coat the filter 2 hr with type I collagen (27 μg/ml rat tail collagen) in Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (JRH Biosciences; also see DPBS and CMF-DPBS in *APPENDIX 2A*). The filter is then rinsed in DPBS before use. Depending on the cell type, better responses may be obtained by drying the matrix molecules onto the filter.

Equipment considerations for bridge assays

Glass chambers are available from Neuro Probe. The glass chamber is thin and provides excellent optics. Plexiglas chambers can usually be cut locally. The shop should be advised that the Plexiglas must be completely flat (i.e., not warped). After cutting the wells, the burrs on the edge of the wells must be removed, but the top of the chamber should not be polished, as this lowers the height of the bridge.

The distance between the bridge and coverslip must be precise for proper cell orientation. For instance, optimal neutrophil orientation occurs when the distance between the coverslip and bridge is ~5 μm. Improved orientation when the fluid layer is thin probably reflects the fact that the gradient across the bridge is formed by diffusion. The thin space creates resistance to fluid flow between compartments, which disturbs the gradient. The thin space, and resultant high cell concentration, probably allows the cells to steepen the gradient by removing/degrading the chemoattractant.

The chamber thickness can be measured by the difference in focal plane between the top of the bridge and the bottom of the coverslip, using the micrometer on the fine focus knob of the microscope. When the distance is 5 μm, the cells are somewhat flattened. To obtain a fluid layer in the 5- to 10-μm range requires a clean chamber and sufficient force applied by the clips. Chambers exposed to room air rapidly absorb "junk." Therefore, chambers should be washed just before use in warm water with tissue culture detergent, rinsed well with distilled water, and wiped dry. The bridge can also be wiped with 70% or 95% ethanol. To obtain the proper fluid layer, it also helps to use a minimal amount of fluid when making the cell preparation. It is usually necessary to prepare extra chambers and then incubate and score only those of appropriate thickness.

Cell-specific issues

Neutrophils like to be squeezed and move rapidly through filters, under agarose, and when compressed between two surfaces as in the bridge assay. When merely placed on a surface, neutrophils show a variable ability to translocate, and are frequently described as being "stuck by their tails". These "stuck" cells extend processes into the medium but appear unable to form effective new attachments. This behavior is remedied by altering the extracellular matrix protein present (Marks et al., 1991; Maxfield, 1993), and is rarely seen when cells are in contact with (squeezed between) two or more surfaces, such as in a filter, bridge, or under-agarose assay.

Neutrophils respond to various chemoattractants, including bacterial-derived peptides (*N*-formyl peptides), a cleavage product of complement factor 5 (C5a), lipids such as leukotriene B_4 (LTB_4), proteolytic fragments of the extracellular matrix (Senior et al., 1980), and many of the C-X-C family of chemokines (Baggiolini et al., 1997).

With neutrophils, the method of cell isolation and the time between drawing the blood and starting the assay can have important effects. Different labs use different protocols. Endotoxin-free neutrophils can be stored at room temperature for several hours without activity. Note that some investigators have demonstrated that neutrophils change phenotype when assayed >2 hr after cell preparation (>5 hr after blood drawing; Seligmann et al., 1981). Rabbit peritoneal neutrophils can be stored overnight at 4°C, as long as they are kept in the peritoneal fluid.

Monocytes move more slowly than neutrophils but are readily assayed in filter assays. They respond to many of the CC family of chemokines as well as to *N*-formyl peptides.

Lymphocytes (T and B cells) respond chemotactically to a variety of chemokines. Lymphocytes migrate well through thin poly-

carbonate or polyethylene terephthalate membranes.

Dictyostelium amoebae respond well in filter, under-agarose, small population, bridge, and micropipet assays. *Dictyostelium* amoebae do not typically show tight attachment to surfaces and do not require surfaces coated with specific adhesion molecules. The sensitivity of *Dictyostelium* cells to specific chemoattractants can vary with cell state. Cells taken directly from growth medium are chemotactic to folate. As cells are starved, they undergo a developmental program that involves formation of aggregates of cells by chemotaxis to extracellular cAMP. During morphogenesis in aggregates to form pseudoplasmodia or fruiting bodies, responsiveness to cAMP may be reduced and responses to monapterin derivatives develop. Thus far, the best characterized chemotactic responses have been those to extracellular cAMP by cells starved for 6 to 8 hr.

Mammalian fibroblasts and epithelial cells (including many tumor cell types) typically move slowly (0.2 to 2 μm/min). This may reflect the slow detachment of focal contacts to surfaces coated with typical extracellular matrix molecules such as collagen, laminin, vitronectin, or fibronectin. Chemotactic responses of such cells have been characterized using filter assays. Identified chemoattractants for these cells include ligands for G protein–coupled receptors—such as autocrine motility factor (AMF)—and growth factors that bind to receptor tyrosine kinases—such as fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, platelet-derived growth factor (PDGF), and hepatocyte growth factor/scatter factor (HGF/SF). The methods described in this unit have been developed for a chemotactic tumor cell line (termed MTLn3 cells), but similar conditions should work for most fibroblast and epithelial cells. Extracellular matrix molecules can be used instead of chemoattractants to evaluate haptotactic responses.

Neurons have shown a variety of motile responses. One distinctive feature of neurons is the formation of a growth cone, which shows polarized movement while the main cell body may remain stationary. Growth factors, neurotransmitters, and adhesion molecules have been shown to attract or repel neuronal growth cones. The assays typically utilized for growth cone responses are micropipet or tissue fragment assays. Thus far, the focus has mainly been on identifying chemoattractants for various neuronal types. Future studies will begin to focus on mechanistic questions, given the recent advances in characterizing chemoattractants for neurons.

Media

Since most neutrophil assays are short, the cells can be assayed in Hanks' balanced salt solution (HBSS). Alternatively, a full medium such as RPMI can be used. Neutrophils move best in slightly acidic (pH 7.1 to 7.2) and slightly hypotonic conditions. Bicarbonate-buffered tissue culture medium can be used when assays are incubated in 5% to 10% CO_2. For incubations in room air, both RPMI and HBSS can be purchased without bicarbonate, and then supplemented with 10 to 20 mM HEPES. All solutions should be kept sterile and as endotoxin-free as possible, since endotoxin activates neutrophils.

All assays require some protein to allow migration and prevent toxicity from glass, filters, and agarose. The protein can be albumin, gelatin, or serum proteins. Most commonly, bovine serum albumin is added to a final concentration of 0.1% to 1% (w/v). Although albumin itself can stimulate migration, this effect is diminished through use of fatty acid–free albumin. Gelatin is dissolved fully in boiling water as a 10% (w/v) stock, which is then warmed to remelt the gelatin and diluted tenfold (final 1%) into medium. Gelatin is nonstimulatory (Chenoweth et al., 1979) and minimizes neutrophil adhesion to various surfaces. Gelatin enhances migration in the bridge assay but does not provide enough traction to allow cells to enter pores of the Millipore filter. However, once cells have entered the pores, substitution of gelatin for BSA stimulates subsequent migration (Zigmond, unpub. observ.). Heat-inactivated serum (≤10%) may also be used as a protein source. Serum is heat-inactivated by warming to 56°C for 25 min. However, even after heat inactivation, serum contains chemoattractants.

The optimal medium for studies with *Dictyostelium* is 17 mM Sorensen's phosphate buffer, pH 6.2, with 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$.

For MTLn3 cells/fibroblasts, α-MEM with 12 mM HEPES, pH 7.4, can be used. BSA can be included at 0.35% (w/v) to provide an osmotic strength similar to 5% BCS. Optimal responses to EGF are obtained by always growing and passing cells at <80% confluency, and by serum starving the cells in HEPES-buffered MEM with 0.35% BSA for 3 to 24 hr just before stimulation.

Decreased orientation in the bridge assay

The level of orientation in the bridge assay is reduced when competing chemoattractants are present. Neutrophils adjacent to air bubbles or cell clumps produce chemoattractants, and thus become foci for attracting adjacent cells. These foci reduce the level of orientation induced by the chemoattractant gradient across the bridge. When chemoattractant is present in both wells, cells near the edge of the well containing the lower concentration are sometimes observed to orient toward this edge. This "edge effect" appears to be due to a high concentration of cells on the bridge degrading chemoattractant until there is a lower concentration at the center of the bridge than in the well containing the lower concentration.

Controls

Under all conditions, the most important control is using medium without chemoattractant. Cell responses under these conditions provide a baseline for comparison with medium containing chemoattractant. Distinguishing between chemokinesis and chemotaxis can then be done, if desired, by evaluating cell responses when the same concentration of chemoattractant is present in all solutions.

Anticipated Results

Filter assay

With a thick cellulose nitrate filter, unstimulated cells typically move 10 to 15 μm into the filter and stimulated cells move 80 to 90 μm into the filter. When measuring the distance to the cell front, it is important to stop the incubation before cells have moved all the way through the filter (~100 μm after staining and clearing; Zigmond and Hirsch, 1973). When measuring the number of cells on the bottom of the filter, between 20% and 50% of the cells move to the bottom following stimulation (Harvath et al., 1980).

For thin-filter assays, with neutrophils migrating through tissue culture inserts with 1-μm pores, typically 5% to 20% of the cells added to the top well traverse the filter and can be counted in the bottom well in response to an optimal gradient. Without stimulation, <1% of cells appear in the bottom well (Campbell et al., 1997a).

The percentage of lymphocytes/lymphoid cells that migrate through 3- or 5-μm pores varies widely (from 1% to 50%) with cell type and chemoattractant used. However, when the optimal pore size is used, the background migration without chemoattractant is typically very low (<1% of cells added).

Under-agarose assay

Neutrophils migrating in response to an optimal chemoattractant source can migrate up to 1.6 mm toward the attractant during the 2-hr migration period. Neutrophils in this assay tend to migrate farther toward some chemoattractants than others, e.g., migrating more toward interleukin 8 or *N*-formyl-methionyl-leucyl-phenylalanine (*f*MLP) than C5a or LTB_4 (Foxman et al., 1997). The differences in migration may reflect differences in gradient formation, difference in cellular responsiveness to chemoattractants, or both. Neutrophils in the migrating cell population are densely packed. In the absence of stimulation, individual neutrophils can be observed to migrate as far as 0.5 mm away from the starting well, but the cells are much more sparsely distributed (Fig. 12.1.3).

Small population assay

This assay is designed to score rapid movement of *Dictyostelium* cells toward a chemoattractant. As the chemoattractant concentration is increased, the fraction of responding cells will go from 0% to a maximum of near 100%, followed by a decrease when the concentration becomes too high. The optimal concentration range may be very narrow.

Bridge assay

Random migration results in ~50% of the cells moving toward each well. In an optimal gradient of a potent chemoattractant, it is possible to observe 100% of the cells with polarized morphology, all oriented toward the attractant. Often, only between 50% and 80% have clear enough polarity to be scored, and of these, between 80% and 90% are oriented toward the chemoattractant (Zigmond, 1977). In video studies, up to 100% of the cells are observed to move toward the chemoattractant, although the accuracy of the orientation toward the attractant varies.

Pipet assay

For *Dictyostelium* or neutrophils, changes in cell shape and extensions are seen within seconds after placement of the pipet, with net movement toward the pipet observed within 1 min. For epithelial or mesenchymal cells, such as MTLn3 cells or fibroblasts, a slow response involves extension of projections followed by net cell movement, which may take up to 20 to

30 min. In the most extreme cases, such as orientation of *Sacchyromyces cerevisiae* cells in a gradient of mating factor, responses may take hours (Segall, 1993). In all cases, too high of a concentration of chemoattractant in the pipet will result in responses similar to those seen in the upshift assay (see below), with no oriented movement toward the pipet.

Upshift assay

Within 20 min a series of shape changes should occur. The specific changes and their kinetics vary with cell type. For *Dictyostelium,* cells stop and round up within 30 sec, and extend lamellipodia at 1 to 2 min, followed by at least a partial restoration of motility (Varnum-Finney et al., 1988; Segall, 1992). Restoration can be observed within 10 min, especially at low chemoattractant concentrations. For neutrophils, dramatic lamellipodial extension occurs 1 min after stimulation, followed by motility at 3 to 5 min (Zigmond and Sullivan, 1979). For MTLn3 cells, maximal lamellipodial extension is seen at 3 to 5 min (Segall et al., 1996). For mammalian cells, restoration may take 30 min, or a permanent change (either increase or decrease) in rate of movement may be seen.

Time Considerations

Filter assay

Isolating cells from blood or a peritoneal exudate takes 3 to 6 hr, respectively. It takes 30 min to prepare medium and 10 min to prepare a 12-well multiwell chamber. The assay itself requires 30 min to 3 hr for incubation (depending on cell and filter type), 2 to 3 hr for staining and clearing the filter (staining can be performed overnight), and 5 to 15 min per filter for mounting and scoring.

When the filter assay is performed using a 24-well plate with inserts, ~30 min are required to prepare the medium, but it may be prepared the night before and equilibrated overnight in a tissue culture incubator. It takes ~30 min to set up the chamber with filter and medium. The amount of time required to prepare the cells for the assay depends on the cells, on average from a few minutes to 2 to 3 hr or more. Incubation time for the assay is 1.5 hr for neutrophils and 3 to 4 hr for lymphocytes or lymphoid cell lines. Scoring the assay requires 5 min per well.

Under-agarose assay

It takes ~1 hr to prepare the medium and pour the plates, and this can be done the night before the assay. On the day of the assay, it takes ~1 min per plate to cut the wells. The time required to prepare cells for the assay ranges from a few minutes for cultured cells to 2 to 3 hr for cells isolated from blood. It takes ~1 min to set up each plate, 2 hr to incubate neutrophils (longer for other cells), 1.5 hr to fix and stain cells, and ~2 min per plate to score migration distance.

Small population assay

It takes 1 to 2 days to prepare the agar dishes for this assay. Practice is required to prepare Pasteur pipets of the appropriate tip diameter. Cell preparation time will vary depending on the cell type. For example, for optimal chemotactic response to cAMP, *Dictyostelium* requires starvation for 4 to 8 hr. Performance of the assay itself requires 15 to 20 min to place the various drops, and 30 min to record the responses.

Bridge assay

The time to perform an individual assay includes 3 to 6 hr to obtain the cells from blood or peritoneal exudate, or 45 min from a finger prick sample; ~30 min to prepare the medium, which can be done during cell preparation; 1 to 3 min to prepare each chamber; 20 min to incubate; and 5 to 10 min to score orientation or migration on a video recording.

Pipet assay

For rapidly moving cells such as *Dictyostelium* or neutrophils, the responses of individual cells can be determined within 2 to 4 min. For slowly moving cells such as fibroblasts or epithelial cells, responses occur after 10 to 20 min. The time required to quantitate the results depends upon the number of cells and images analyzed. On average, using NIH Image or DIAS, 10 to 30 min per cell are required to quantitate area, speed, and direction of motion (Cammer et al., 1997; Bailly et al., 1998; Fig. 12.1.7).

Upshift assay

Preparing and warming solutions requires 20 to 30 min. Recording of cell responses in a microscope requires 20 min per upshift compartment. Quantitation of results depends on the method used. For NIH Image or DIAS, roughly 10 min are required to trace out the perimeter and calculate the area for one cell if one image per min is recorded.

Literature Cited

Aznavoorian, S., Stracke, M.L., Parsons, J., McClanahan, J., and Liotta, L.A. 1996. Integrin αvβ3 mediates chemotactic and haptotactic motility in human melanoma cells through different signaling pathways. *J. Biol. Chem.* 271:3247-3254.

Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature* 392:565-569.

Baggiolini, M., Dewald, B., and Moser, B. 1997. Human chemokines: An update. *Annu. Rev. Immunol.* 15:675-705.

Bailly, M., Yan, L., Whitesides, G.M., Condeelis, J.S., and Segall, J.E. 1998. Regulation of protrusion shape and adhesion to the substratum during chemotactic responses of mammalian carcinoma cells. *Exp. Cell Res.* 241:285-299.

Bignold, L.P. 1988. Kinetics of chemo-attraction of polymorphonuclear leukocytes towards N-formyl peptide studied with a novel polycarbonate (Nucleopore) membrane in the Boyden chamber. *Experientia* 44:518-521.

Boyden, S.V. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J. Exp. Med.* 115:453-466.

Buettner, H.M., Lauffenburger, D.A., and Zigmond, S.H. 1989. Measurement of leukocyte motility and chemotaxis parameters with Millipore filter assay. *J. Immunol. Methods* 123:25-37.

Cammer, M., Wyckoff, J., and Segall, J.E. 1997. Computer-assisted analysis of single-cell behavior. *Methods Mol. Biol.* 75:459-481.

Campbell, J.J., Foxman, E.F., and Butcher, E.C. 1997a. Chemoattractant receptor crosstalk as a regulatory mechanism in leukocyte adhesion and migration. *Eur. J. Immunol.* 27:2571-2578.

Campbell, J.J., Qin, S., Bacon, K.B., Mackay, C.R., and Butcher, E.C. 1997b. Biology of chemokine and classical chemoattractant receptors: Differential requirements for adhesion-triggering versus chemotactic responses in lymphoid cells. *J. Cell Biol.* 134:255-266.

Carter, S.B. 1965. Principles of cell motility: The direction of cell movement and cancer invasion. *Nature* 208:1183-1187.

Chenoweth, D.E., Rowe, J.G., and Hugli, T.E. 1979. A modified method for chemotaxis under agarose. *J. Immunol. Methods* 25:337-353.

Condic, M.C. and Letourneau, P.C. 1997. Ligand-induced changes in integrin espression regulate neuronal adhesion and neurite outgrowth. *Nature* 389:853-855.

Devreotes, P.N. 1994. G protein-linked signaling pathways control the developmental program of *Dictyostelium*. *Neuron* 12:235-241.

Devreotes, P., Fontana, D., Klein, P., Sherring, J., and Theibert, A. 1987. Transmembrane signaling *Dictyostelium. Methods Cell Biol.* 28:299-331.

Fisher, P.R., Merkl, R., and Gerisch, G. 1989. Quantitative analysis of cell motility and chemotaxis in *Dictyostelium discoideum* by using an image processing system and a novel chemotaxis chamber providing stationary chemical gradients. *J. Cell Biol.* 108:973-984.

Foxman, E.F., Campbell, J.J., and Butcher, E.C. 1997. Multi-step navigation and the combinatorial control of leukocyte chemotaxis. *J. Cell Biol.* 139:1349-1360.

Harris, A.K. 1973. Behavior of cells on substrata of variable adhesiveness. *Exp. Cell Res.* 77:285-297.

Harvath, L., Falk, W., and Leonard, E.J. 1980. Rapid quantitation of neutrophil chemotaxis: Use of polyvinylpyrrolindone-free polycarbonate membrane in a multiwell assembly. *J. Immunol. Methods* 37:39-45.

Huang, A.J., Furie, M.B., Nicholson, S.C., Fischbarg, J., Liebovitch, L.S., and Silverstein, S.C. 1988. Effects of human neutrophil chemotaxis across human endothelial cell monolayers to ions and macromolecules. *J. Cell Physiol.* 135:355-366.

Jungi, T.W. 1975. Assay of chemotaxis by a reversible Boyden chamber eliminating cell detachment. *Int. Arch. Allergy Appl. Immunol.* 48:341-352.

Konijn, T.M. 1970. Microbiological assay of cyclic 3′,5′-AMP. *Experientia* 26:367-369.

Konijn, T.M. and Raper, K.B. 1961. Cell aggregation in *Dictyostelium discoideum. Dev. Biol.* 3:725-756.

Konijn, T.M. and Van Haastert, P.J.M. 1987. Measurement of chemotaxis in *Dictyostelium*. *Methods Cell Biol.* 28:283-298.

Krauss, A.H., Nieves, A.L., Spada, C.S., and Woodward, D.F. 1994. Determination of leukotriene effects on human neutrophil chemotaxis in vitro by differential assessment of cell motility and polarity. *J. Leukocyte Biol.* 55:201-208.

Laroche, L., Papiernik, M., and Bach, J.F. 1983. Sezary syndrome: Specific skin-directed migration of peripheral blood lymphocytes. *J. Immunol.* 130:2467-2470.

Lauffenburger, D.A. and Zigmond, S.H. 1981. Chemotactic factor concentration gradients in chemotaxis assay system. *J. Immunol. Methods* 40:45-60.

Lauffenburger, D.A., Rothman, C., and Zigmond, S.H. 1983. Measurement of leukocyte motility and chemotaxis parameters with a linear under-agarose assay. *J. Immunol.* 131:940-947.

Loike, J.D., el Khoury, J., Cao, L., Richards, C.P., Rascoff, H., Mandeville, J.T., Maxfield, F.R., and Silverstein, S.C. 1995. Fibrin regulates neutrophil migration in response to interleukin 8, leukotriene B4, tumor necrosis factor, and formyl-methionyl-leucyl-phenylalanine. *J. Exp. Med.* 181:1763-1772.

Maniak, M., Rauchenberger, R., Albrecht, R., Murphy, J., and Gerisch, G. 1995. Coronin involved in phagocytosis: Dynamics of particle-induced relocalization visualized by a green fluorescent protein Tag. *Cell* 83:915-924.

Marks, P.W., Hendrey, B., and Maxfield, F.R. 1991. Attachment to fibronectin or vitronectin makes human neutrophil migration sensitive to alterations in cytosolic free calcium concentration. *J. Cell Biol.* 112:149-158.

Maxfield, F.R. 1993. Regulation of leukocyte locomotion by Ca^{2+}. *Trends Cell Biol.* 3:386-391.

McCormick, B.A., Colgan, S.P., Delp-Archer, C., Miller, S.I., and Madara, J.L. 1993. *Salmonella typhimurium* attachment to human intestinal epithelial monolayers: Tanscellular signalling to subepithelial neutrophils. *J. Cell Biol.* 123:895-907.

McCormick, B.A., Hofman, P.M., Kim, J., Carnes, D.K., Miller, S.I., and Madara, J.L. 1995. Surface attachment of *Salmonella typhimurium* to intestinal epithelia imprints the subepithelial matrix with gradients chemotactic for neutrophils. *J. Cell Biol.* 131:1599-1608.

McKenna, M.P. and Raper, J.A. 1988. Growth cone behavior on gradients of substratum bound laminin. *Dev. Biol.* 130:232-236.

Moghe, P.V., Nelson, R.D., and Tranquillo, R.T. 1995. Cytokine-stimulated chemotaxis of human neutrophils in a 3-D conjoined fibrin gel assay. *J. Immunol. Methods* 180:193-211.

Moores, S.L., Sabry, J.H., and Spudich, J.A. 1996. Myosin dynamics in live *Dictyostelium* cells. *Proc. Natl. Acad. Sci. U.S.A.* 93:443-446.

Nash, S., Stafford, J., and Madara, J.L. 1987. Effects of polymorphonuclear leukotye transmigration on the barrier function of cultured intestinal epithelial monolayers. *J. Clin. Invest.* 80:1104-1113.

Nelson, R.D., Quie, P.G., and Simmons, R.L. 1975. Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.* 115:1650-1656.

Omann, G.M. and Sklar, L.A. 1988. Response of neutrophils to stimulus infusion: Differential sensitivity of cytoskeletal activation and oxidant production. *J. Cell Biol.* 107:951-958.

Orredson, S.U., Knighton, D.R., Scheuenstuhl, H., and Hunt, T.K. 1983. A quantitative in vitro study of fibroblast and endothelial cell migration in response to serum and wound fluid. *J. Surg. Res.* 35:249-258.

Palmblad, J., Uden, A., and Venizelos, N. 1982. The quantification of neutrophil orientation and migration under agarose—A new method for detecting directed and random movements. *J. Immunol. Methods* 44:37-53.

Rhodes, J.M. 1982. Measurement of chemotaxis in Boyden chamber filter assays. Is the checkerboard correction valid? *J. Immunol. Methods* 49:235-236.

Schleicher, M., Andre, B., Andreoli, C., Eichinger, L., Haugwitz, M., Hofmann, A., Karakesisoglou, J., Stockelhuber, M., and Noegel, A.A. 1995. Structure/function studies on cytoskeletal proteins in *Dictyostelium* amoebae as a paradigm. *FEBS Lett.* 369:38-42.

Segall, J.E. 1992. Behavioral responses of streamer F mutants of *Dictyostelium discoideum*: Effects of cyclic GMP on cell motility. *J. Cell Sci.* 101:589-597.

Segall, J.E. 1993. Polarization of yeast cells in spatial gradients of a mating factor. *Proc. Natl. Acad. Sci. U.S.A.* 90:8332-8336.

Segall, J.E. and Gerisch, G. 1989. Genetic approaches to cytoskeleton function and the control of cell motility. *Curr. Opin. Cell Biol.* 1:44-50.

Segall, J.E., Tyerech, S., Boselli, L., Masseling, S., Helft, J., Chan, A., Jones, J., and Condeelis, J. 1996. EGF stimulates lamellipod extension in metastatic mammary adenocarcinoma cells by an actin-dependent mechanism. *Clin. Exp. Met.* 14:61-72.

Seligmann, B., Chused, T.M., and Gallin, J.I. 1981. Human neutrophil heterogeneity identified using flow microfluorimetry to monitor membrane potential. *J. Clin. Invest.* 68:1125-1131.

Senior, R.M., Griffin, G.L., and Mecham, R.P. 1980. Chemotactic activity of elastin-derived peptides. *J. Clin. Invest.* 66:859-862.

Showell, H.J., Freer, R.J., Zigmond, S.H., Schiffmann, E., Aswanikumar, S., Corcoran, B., and Becker, E.L. 1976. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal secretion for neutrophils. *J. Exp. Med.* 143:1154-1169.

Soll, D.R., 1987. Methods for manipulating and investigating developmental timing in *Dictyostelium discoideum. Methods Cell Biol.* 28:413-431.

Soll, D.R. 1988. "DMS," a computer-assisted system for quantitating motility, the dynamics of cytoplasmic flow, and pseudopod formation: Its application to *Dictyostelium* chemotaxis. *Cell Motil. Cytoskeleton* 10:91-106.

Soll, D.R. 1995. The use of computers in understanding how animal cells crawl. *Int. Rev. Cytol.* 163:43-104.

Song, H.-J., Ming, G.-L., and Poo, M.M. 1997. cAMP-induced switching in turning direction of nerve growth cones. *Nature* 388:275-279.

Stokes, C.L., Rupnick, M.A., Williams, S.K., and Lauffenburger, D.A. 1990. Chemotaxis of human microvessel endothelial cells in response to acidic fibroblast growth factor. *Lab. Invest.* 63:657-668.

Sussman, M. 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* 28:9-29.

Tessier-Lavigne, M. and Goodman, C.S. 1996. The molecular biology of axon guidance. *Science* 274:1123-1133.

Tranquillo, R.T., Zigmond, S.H., and Lauffenburger, D.A. 1988. Measurement of the chemotaxis coefficient for human neutrophils in the under-

agarose migration assay. *Cell Motil. Cytoskeleton* 11:1-15.

Van Haastert, P.J.M. 1995. Transduction of the chemotactic cAMP signal across the plasma membrane of *Dictyostelium* cells. *Experientia* 51:1144-1154.

Varnum-Finney, B., Schroeder, N.A., and Soll, D.R. 1988. Adaptation in the motility response to cAMP in *Dictyostelium discoideum. Cell Motil. Cytoskeleton* 9:9-16.

Watts, D.J. and Ashworth, J.M. 1970. Growth of myxameobae of the cellular slime mold *Dictyostelium discoideum* in axenic culture. *Biochem. J.* 119: 171-174.

Webb, L.M.C., Ehrengruber, M.U., Clark-Lewis, I., Baggiolini, M., and Rot, A. 1995. Binding to heparin sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc. Natl. Acad. Sci. U.S.A.* 90:7158-7162.

Westphal, M., Jungbluth, A., Heidecker, M., Muhlbauer, B., Heizer, C., Schwartz, J.M., Marriott, G., and Gerisch, G. 1997. Microfilament dynamics during cell movement and chemotaxis monitored using a GFP-actin fusion protein. *Curr. Biol.* 7:176-183.

Wilkinson, P.C. 1982. The measurement of leukocyte chemotaxis. *J. Immunol. Methods* 51:133-148.

Wilkinson, P.C. and Allan, R.B. 1978. Chemotaxis of neutrophil leukotyes towards substratum-bound protein attractants. *Exp. Cell Res.* 117:403-412.

Zhelev, D.V., Alteraifi, A.M., and Hochmuth, R.M. 1996. F-actin network formation in tethers and in pseudopods stimulated by chemoattractant. *Cell. Motil. Cytoskel.* 35:331-344.

Zicha, D., Dunn, G.A., and Jones, G. 1997. Analyzing chemotaxis using the Dunn direct-viewing chamber. *In* Basic Cell Culture Protocols, 2nd ed. (J.W. Pollard and J.M. Walker, eds.) pp. 449-458. Humana Press, Totowa, N.J.

Zigmond, S.H. 1977. The ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol.* 75:606-616.

Zigmond, S.H. and Hirsch, J.G. 1973. Leukocyte locomotion and chemotaxis: New methods for evaluation, and demonstration of a cell-derived chemotactic factor. *J. Exp. Med.* 137:387-410.

Zigmond, S.H. and Sullivan, S.J. 1979. Adaptation of leukocytes to peptide chemotactic factors. *J. Cell Biol.* 82:517-527.

Contributed by Sally H. Zigmond
University of Pennsylvania
Philadelphia, Pennsylvania

Ellen F. Foxman
Stanford University Medical School
Stanford, California

Jeffrey E. Segall
Albert Einstein College of Medicine
Bronx, New York

Invasion Assays

The basement membrane is a thin extracellular matrix that underlies epithelial and endothelial cells and separates these tissues from stroma. Tumor cells must cross the vessel basement membrane and penetrate the underlying stroma in order to invade tissue and form distant metastases. They do this by producing proteases that degrade the extracellular matrix. Several in vitro invasion assays have been developed using various extracellular matrix barriers including amnion, type I collagen gels, and a reconstituted basement membrane termed Matrigel (see *UNITS 10.2 & 10.3*). Matrigel-based invasion assays (see Basic Protocol) are the most reliable, reproducible, and representative of in vivo events, and they are the assays most frequently used. Porous filters are coated with a thin layer of Matrigel and placed in a Boyden migration chamber with a chemoattractant in the lower well and tumor cells in the upper well (Fig. 12.2.1). The entire chamber is then incubated for ~3 to 10 hr, depending on the tumor cells used. After incubation, the filter is removed, fixed, and stained, and the cells on the lower surface of the filter are quantitated. This assay is advantageous because it is quick, reliable, quantitative, and in most cases does not require sterility. Compounds that either promote or inhibit invasion can be assayed. In addition, at the end of the assay, the invasive cells can be recovered and used for further study. The invasion assay can be used to screen for a large variety of compounds in 48-well chambers, with the advantages that smaller amounts of test material and fewer cells are needed. Commercial kits are also available. This protocol is provided for those investigators who seek to test a large number of samples or to modify the basic protocol (for example, by increasing or decreasing the amount of Matrigel coated on to accommodate cells with high or low invasive activity, respectively).

MEASURING INVASION THROUGH A MATRIX

BASIC PROTOCOL

This protocol describes a method for assessing tumor cell invasion through a basement membrane matrix in vitro. Tumor cells are placed in the upper well of a Boyden migration chamber, which is separated from the lower well by a porous filter coated with basement membrane matrix (Matrigel). A chemoattractant is placed in the lower well to facilitate cell migration. After a short incubation, invasive cells are counted or recovered from the lower surface of the filter.

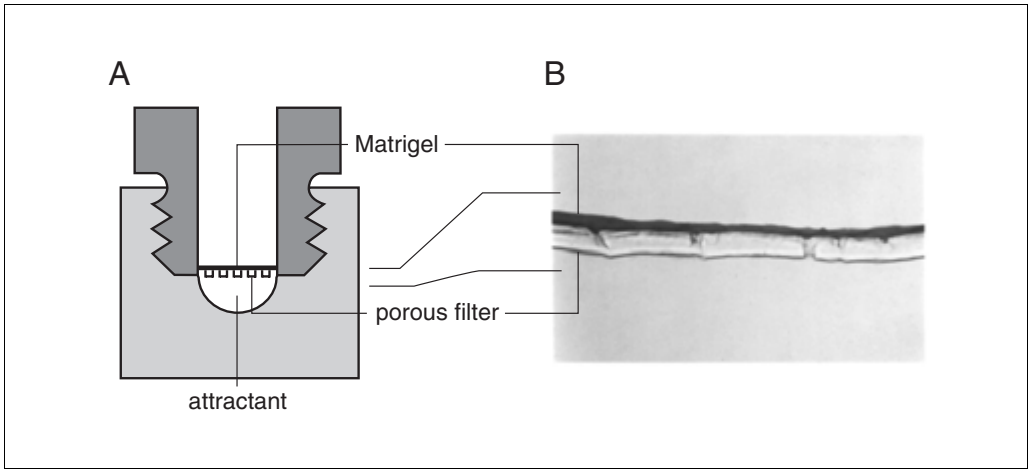


Figure 12.2.1 Configuration of chamber used for invasion assay. (**A**) Schematic representation of the Boyden chamber assembly. (**B**) Photomicrograph of matrix barrier cross-section, consisting of a porous polycarbonate filter coated with Matrigel. Reproduced with permission from the American Association for Cancer Research (Albini et al., 1987).

Contributed by Hynda K. Kleinman and Karin Jacob
Current Protocols in Cell Biology (1998) 12.2.1-12.2.5

Materials

3T3-conditioned medium (see recipe) or selected chemoattractant or inhibitor
Tumor cells in culture
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
Culture medium containing 0.1% (w/v) BSA (varies by cell type)
Diff-Quik fixative and stains (Baxter)

Boyden chambers (Neuro Probe; available as single or 48-well chambers)
Polyvinylpyrrolidone (PVP)-free polycarbonate membranes, 8- or 12-μm pore size (e.g., Nucleopore filters, Neuro Probe), sized to fit Boyden chambers (13-mm for single chambers and 25 × 80–mm for 48-well chambers) coated with Matrigel (see Support Protocol)

1. Add 3T3-conditioned medium or other chemoattractant to be tested to lower wells of Boyden chambers until a small meniscus appears.

   Each assay data point is generally based on triplicate filters/wells. Always include three negative control wells that lack the migration factor in the lower well. If using 48-well chambers, each filter should include three positive- and three negative-control wells (i.e., medium alone). Generally nano- or microgram amounts of chemoattractants are used. It is best to test several different concentrations.

2. Place a Matrigel-coated filter over the lower well of each Boyden chamber and secure the filter with a gasket.

   For 48-well chambers, start putting the filter down from the middle of the chamber to the edges to avoid bubbles. Never move the filter after it has been placed or there will be a possibility of cross-contamination of the wells. Do not screw the gasket too tight.

3. Release tumor cells from their culture vessel by first removing the culture medium and then adding enough 0.5 M EDTA to fully cover the cell layer in the culture dish. Centrifuge the cell suspension 5 min at 170 × *g* in a tabletop centrifuge, room temperature. Decant the supernatant and resuspend the pellet in serum-free medium at 5×10^5 cells/ml (1×10^6 cells/ml for 48-well chambers).

   The cells should not be confluent. Best results are obtained when the cells are split 24 hr before the assay and always seeded at the same density. It is very important to use EDTA rather than trypsin to release the cells from the plate, as trypsin will degrade the Matrigel.

4. Add cells to the upper wells of the chambers.

   Generally 100,000 to 200,000 cells per 0.3-ml well are placed in the upper chamber. When using the smaller 48-well chambers, 50,000 cells per 0.05-ml well are placed in each upper chamber well.

5. Incubate 3 to 6 hr in a humidified 5% CO_2, 37°C incubator.

   The time of incubation is dependent on the tumor cells used; 3 to 6 hr is the most frequently used range of incubation times. The optimal time can be determined in an initial test assay with and without attractant in the lower well, looking for the greatest difference between the two conditions. Usually <2% of the added cells migrate in the presence of the attractant.

   NOTE: *All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.*

6. Stop the incubation by removing and fixing the filters using the Diff-Quik system. Remove the filters, dip in fixative for 10 sec, and place in solutions I and II for 2 min each. Wash in water for a few seconds.

   Small weigh boats or Coplin jars may be used for these steps and the solutions can be reused. The cells will appear dark blue with a pale blue to white background.

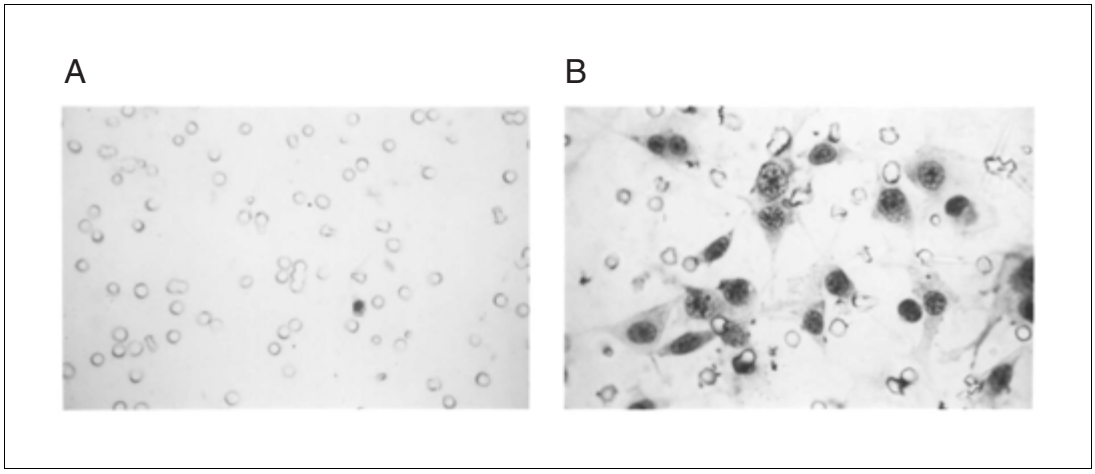


Figure 12.2.2 Photomicrographs showing results of an invasion assay: filter underside (attractant side) after incubation with (**A**) noninvasive and (**B**) invasive cells. Reproduced with permission from the American Association for Cancer Research (Albini et al., 1987).

7. Wipe cells from the upper surface with a cotton-tipped swab and mount filter on a slide with the lower side down.

   This step removes the cells that have attached to the upper surface of the filter and makes counting easier. The migrated cells will be on the underside of the filter facing the glass slide.

8. Count the cells.

   This can be done with a computer system or by eye (see Fig. 12.2.2). Be sure to survey the entire filter, as the distribution of migrated cells may be uneven. Count the most representative areas. Counting with a 10× magnification using a grid has proven to be reliable and reproducible.

PREPARATION OF MATRIGEL-COATED FILTERS

SUPPORT PROTOCOL

Coating the filter with Matrigel provides a surface similar to that encountered by cells migrating in vivo.

Materials

PVP-free polycarbonate membranes, 8- or 12-µm pore size (e.g., Nucleopore filters, Neuro Probe), sized to fit Boyden chambers (13-mm for single chambers; 25 × 80–mm for 48-well chambers)

Matrigel (*UNIT 10.2*; Matrigel can also be obtained commercially from Becton Dickinson Labware or Sigma)

1. Number the filters on the dull side with a permanent ink pen.

2a. *If using single chambers:* Dilute Matrigel to 1 µg/µl and pipet 25 µl as a center spot on the dull side of the 13-mm-diameter filter. Air dry overnight.

2b. *If using 48-well chambers:* Dilute Matrigel to 0.5 µg/µl in cold water. Place the filter shiny side down and cover the top with 1 ml of the diluted Matrigel. Incubate 15 to 20 min, then hang the filter to air dry for 5 to 10 min (a pin or needle stuck in one corner through the filter and into a board is convenient for drying). Cut the corner so that the orientation of the filter can be determined at the end of the assay.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

3T3-conditioned medium

Grow 3T3 cells to confluence and replace medium with serum-free medium containing freshly prepared 50 μg/ml ascorbic acid. DMEM or RPMI 1640 can be used, but it is generally best to use the medium in which the invasive cells to be tested are normally cultured. Collect the conditioned medium after 24 hr and store in 1-ml aliquots at −20°C for up to 2 years.

COMMENTARY

Background Information

To metastasize, tumor cells invade through vessel basement membrane and the underlying connective tissue by attaching to, degrading, and migrating across the basement membrane matrix (Liotta, 1989). The in vitro assay described in this unit is performed to determine the invasive character of tumor cells, to test for factors that promote or inhibit invasion, and to select for malignant subpopulations, which are invasive (Kramer et al., 1986; Albini et al., 1987). The assay is rapid, quantitative, and proven to be highly reliable in a number of research laboratories.

Matrigel is a better barrier than amnion or other natural substrates, because it shows high reproducibility in its activity, and the assay time is much shorter (Hendrix et al., 1989). Many known inhibitors of tumor metastases, such as protease inhibitors, block invasion in this assay (Reich et al., 1988; Stahl and Meuller, 1994; Kobayashi et al., 1995). Likewise certain growth factors, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor α (TGFα), hepatocyte growth factor (HGF), and thyroid-stimulating hormone (TSH), have all been found to promote invasion (Nicolson et al., 1993; Hoelting et al., 1994; Rong et al., 1994; Sunitha et al., 1994; Chambers et al., 1995). Having an assay that is quick, reliable, and accurate can facilitate diagnosis and aid in the development of therapeutics. The assay can be used as a prescreen for a large number of test compounds.

Critical Parameters and Troubleshooting

The exact time of incubation varies depending on the cells used and the amount of coated Matrigel. Trials using different incubation time in the presence and absence of a chemoattractant such as 3T3-conditioned medium should be performed initially to determine the optimum time of incubation. The optimum is the time at which there is the largest difference in the number of infiltrating cells between the control and positive attractant. In some cases, it may also be desirable to vary the number of cells added. For example, if the cells are highly invasive and too numerous to count, fewer cells can be used. In addition, because of the assay's sensitivity, if possible stimulators or inhibitors are being assessed, several different concentrations of the test substance should be tested.

Choosing an appropriate attractant is important. Many studies employ conditioned medium from 3T3 cells, which is collected for 24 hr with serum-free medium containing 50 μg/ml ascorbic acid. The conditioned medium is generally used directly without preconcentration. With cells that have not been previously tested, it may be desirable to test a range of concentrations. Several other chemoattractants have also been identified and include various growth factors, such as EGF (Hoelting et al., 1995), bFGF (Hasegawa et al., 1994), and HGF (Rong et al., 1994). Tissue extracts have been found to act as chemoattractants with some cell types (Hujanen and Terranova, 1985), and laminin as well as other matrix molecules can also promote invasion when placed in the lower well of the chamber (Koochepour et al., 1995). To determine the best chemoattractant, it is advisable to test 3T3-conditioned medium first and then various growth factors and matrix molecules if needed. Cells generally respond by migrating toward growth factors for which they have receptors. The attractant needs to be in the lower well of the chamber and should be present during the entire assay. It may be necessary to test various concentrations as cells usually migrate to factors at concentrations approximately one-tenth of that needed for growth, and many chemoattractants are inhibitory for migration when used at high concen-

trations. Preincubation with the chemoattractant is not required.

It is important to include controls in each assay. Generally the negative control is medium alone (lacking attractant), whereas the positive control is either 3T3-conditioned medium or a medium containing a substance already identified as a chemoattractant for the cells being tested. The assay should be performed in triplicate for the best results.

Anticipated Results

Results are assessed quantitatively by comparing the cell counts for experimental and control wells or plates: for example, when testing an attractant, very few cells invade in its absence, whereas more cells (usually at least twice background) should be observed in its presence.

Time Considerations

The assay should be completed in 1 day. Filters can be coated the night before the assay. Once fixed, the filters are stable for months and can be easily stored in slide boxes.

Literature Cited

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M., and McEwan, R.N. 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res.* 47:3239-3245.

Chambers, S.K., Wang, Y., Gertz, R.E., and Kacinski, B.M. 1995. Macrophage colony stimulating factor mediates invasion of ovarian cancer cells through urokinase. *Cancer Res.* 55:1578-1585.

Hasegawa, Y., Takada, M., Yamamoto, M., and Saitoh, Y. 1994. The gradient of basic fibroblast growth factor concentration in human pancreatic cancer cell invasion. *Biochem. Biophys. Res. Commun.* 200:1435-1439.

Hendrix, M.J.C., Seftor, E.A., Seftor, R.E.B., Minorowski, R.L., Saba, P.Z., Sundareshan, P., and Welch, D.R. 1989. Comparison of tumor cell invasion assays: Human amnion versus reconstituted basement membrane barriers. *Invasion Metastasis* 9:278-297.

Hoelting, T., Siperstein, A.E., Clark, O.H., and Duh, Q.Y. 1994. Epidermal growth factor enhances proliferation, migration, and invasion of follicular and papillary thyroid cancer in vitro and in vivo. *J. Clin. Endocrinol. Metab.* 79:401-408.

Hoelting, T., Siperstein, A.E., Clark, O.H., and Duh, Q.Y. 1995. Epidermal growth factor (EGF)– and transforming growth factor α–stimulated invasion and growth of follicular thyroid cancer cells can be blocked by antagonism to the EGF receptor and tyrosine kinase in vitro. *J. Endocrinol.* 132:229-235.

Hujanen, E.S. and Terranova, V.P.T. 1985. Migration of tumor cells to organ-derived chemoattractants. *Cancer Res.* 45:3517-3521.

Kobayashi, H., Gotoh, J., Hirashima, Y., Fujie, M., Sugino, D., and Terao, T. 1995. Inhibitory effects of a conjugate between human urokinase and urinary trypsin inhibitor on tumor invasion in vitro. *J. Biol. Chem.* 270:8361-8368.

Koochepour, S., Merzak, A., and Pilkington, G.J. 1995. Extracellular matrix proteins inhibit proliferation, upregulate migration and induce morphological changes in human glioma cell lines. *Eur. J. Cancer* 31A:375-380.

Kramer, R.H., Bensch, K.G., and Wong, J. 1986. Invasion of reconstituted basement membrane by metastatic human tumor cells. *Cancer Res.* 46:1980-1989.

Liotta, L.A. 1989. Tumor invasion and metastasis. Role of the extracellular matrix. *Cancer Res.* 46:1-7.

Nicolson, G.L., Nakajima, M., Herrmann, J.L., Menter, D.G., Cavanaugh, P.G., Park, J.S., and Marchetti, D. 1993. Malignant melanoma metastasizes to brain: Role of degradative enzymes and responses to paracrine growth factors. *J. Neurooncol.* 18:139-149.

Reich, R., Thompson, E.W., Iwamoto, Y., Martin, G.R., Deason, J.R., Fuller, G.C., and Miskin, R. 1988. Effects of inhibitor of plasminogen activator, serine proteases and collagenase IV on invasion of basement membrane by metastatic cells. *Cancer Res.* 48:3307-3312.

Rong, S., Segal, S., Avner, M., Ressau, J.H., and Van der Woude, G.F. 1994. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. *Proc. Natl. Acad. Sci. U.S.A.* 91:4731-4735.

Stahl, A. and Meuller, B.M. 1994. Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells in vitro. *Cancer Res.* 54:3066-3071.

Sunitha, I., Meighen, D.L., Hartman, D.P., Thompson, E.W., Byers, S.W., and Avigan, M.I. 1994. Hepatocyte growth factor stimulates invasion across reconstituted basement membrane by a new human small intestinal cell line. *Clin. Exp. Metastasis* 12:1143-1154.

Contributed by Hynda K. Kleinman
and Karin Jacob
National Institute of Dental Research/NIH
Bethesda, Maryland

Cell Traction

Traction forces are exerted by cells against their substrates during migration and can be measured under the entire cell or under subcellular regions. This unit describes several protocols for making silicone sheets to measure traction forces under the entire cell, as well as a protocol for developing a micromachined device to measure forces under subcellular regions.

The original technique used to measure tractions, which examined the orientation of wrinkles generated in the substrate by locomoting cells, is described first (see Basic Protocol 1). The technique is useful for providing a global orientation of the forces under cells such as fibroblasts, which typically exert large traction forces (10 nN/μm of leading cell edge; Harris et al., 1980). Calibrated microneedles (see Support Protocol 1) are used to calibrate all of the substratum (see Basic Protocols 1 and 2 and Support Protocols 1 and 2).

Also described (see Alternate Protocol 1) is a modification of the original protocol that provides increased optical resolution as well as a simple method for quantitatively decreasing the stiffness of the substrate. This second substratum can be made with stiffnesses ranging from 0.26 to 50 nN/μm (Burton and Taylor, 1997).

Another modification is described in Alternate Protocol 2. This method measures forces with enhanced spatial resolution by determining the displacement of fiduciary markers embedded within the substrate. This protocol can also produce substrates of varying stiffness, ranging from 5 to 20 nN/μm (Lee et al., 1994; Oliver et al., 1995); however, the time constant for the beads to recover to their original position increases from ~1 to 15 sec (Lee et al., 1994; Oliver et al., 1995) between these two levels of stiffness. A modified airbrush apparatus (see Support Protocol 2) is used to coat the substrate with the fiduciary markers (Oliver et al., 1995).

Finally, a new technique is described for measuring subcellular traction forces using a micromachined substrate (see Basic Protocol 2). This substrate is based on a system of deformable levers that have stiffnesses ranging from 0.09 to 89 nN/μm. It can measure forces under regions of the cell as small as 4 μm^2 in real time, but cannot measure forces under multiple regions of the cell simultaneously (Galbraith and Sheetz, 1997). A procedure for creating silanized coverslips is presented in Support Protocol 3. The assay requires polarized reflection microscopy for visualization (see Support Protocol 4).

The protocols are presented in order of increasing cost and complexity. All three of the silicone-substrate protocols can be implemented in a reasonable amount of time, are not particularly expensive, and can measure forces under the entire cell. The protocol using micromachined substrates is more costly, but it has the advantage of measuring forces under subcellular regions. The appropriate protocol should be selected according to the magnitude of the forces that a particular cell exerts, the time constant of the events to be measured, and the resources available for development of the substrate.

MEASURING CELL TRACTION ON WRINKLING SUBSTRATES

BASIC PROTOCOL 1

This protocol outlines the construction and calibration of wrinkling silicone sheets and the use of these sheets in measuring cell traction. The fabrication of the sheets follows the procedure developed by Harris et al. (1980), and the calibration follows the procedure developed by Burton and Taylor (1997). Sheets that monitor forces by observing the displacement of fiduciary markers are discussed in Alternate Protocol 2. For a given

Contributed by Catherine G. Galbraith and Michael P. Sheetz
Current Protocols in Cell Biology (1998) 12.3.1-12.3.16

experiment, sheets should be chosen so that their stiffness matches the forces expected to be be generated by the cell type of interest (see Table 12.3.1).

Materials

Cells of interest
Culture medium specific for cells being used, phenol red–free and supplemented with 20 to 50 mM HEPES
Dimethylpolysiloxane (60,000 or 30,000 centistokes; Sigma)
Phenol red–free culture medium degassed using house vacuum

Temperature-control system for microscope stage (or hair dryer and controller)
Glass coverslips, acid washed
Petri dishes slightly larger than the coverslips used
Inverted microscope with long-working distance-objective *or* upright microscope with a multi-immersion-medium objective, equipped with video system
Micromanipulator (adjustable in three dimensions; e.g., Nanishige) with needle holder (World Precision Instruments)
Calibrated microneedles (see Support Protocol 1)
Computer with a video-frame capture card and image-analysis software (optional; e.g., NIH Image, available by anonymous FTP from *zippy.nimh.nih.gov*)
Macintosh computer with Scion frame grabber running

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

Construct the wrinkling substrate

1. Spread a thin layer, 20 to 50 μm deep, of dimethylpolysiloxane fluid over the surface of a clean glass coverslip.

Table 12.3.1 Traction Forces Exerted by Various Cell Types

Traction assay	Cell type and method	Force	Reference
Microneedle (Basic Protocol 1)	Chick heart fibroblast, force exerted against particle attached to dorsal surface	50 pN/μm^2	Felder and Elson, 1990
	Chick sensory neuron growth cone, force pulling neurite during growth cone advance	2 nN	Lamoureux et al., 1989
	Chick heart fibroblast, force required to stall cell per micron of cell leading edge	10 nN/μm	Harris et al., 1980
	Fish keratocyte, force required to stall cell	60 nN	Oliver et al., 1995
Silicone substrates with fiduciary markers (Alternate Protocol 2)	Fish keratocyte, forces from local markers under cell pincer region	~45 nN	Dembo et al., 1996; Lee et al., 1994; Oliver et al., 1995
Wrinkling silicone substrates (Alternate Protocol 1)	3T3 fibroblast, forces at cleavage furrow during division	~75 nN	Burton and Taylor, 1997
	3T3 fibroblast, force of respreading after division	300-1200 nN	Burton and Taylor, 1997
Micromachined substrates (Basic Protocol 2)	Chick embryo fibroblast, forces under subcellular regions	0.9-4.0 nN/μm^2	Galbraith and Sheetz, 1997

2. Set the gas on a Bunsen burner to its minimal level. Invert the coverslip and use forceps to pass it over the upper part of the flame for ~1.5 sec.

This method typically cross-links only the topmost layer (1 μm) of fluid. The silicone sheet will spontaneously wrinkle into many folds, making it opaque, but the sheets become transparent again within 5 to 20 sec as they cool. If the sheets are exposed to the flame for too long, permanently roughened surfaces which are less flexible will be made. These sheets contain an appreciable number of "prestress" wrinkles that will not diminish with time.

Prepare cells for traction assays

3. Set up a temperature-controlled environment for the cells on the stage of the microscope that will be used for making the measurements (if they are normally not cultured at room temperature).

This usually takes the form of a 37°C incubator that surrounds the microscope stage. Incubators can be purchased from most microscope manufacturers; alternatively, inexpensive incubators can be constructed by building a plastic enclosure around the microscope and then heating it with a hair dryer. The temperature can be regulated manually by adjusting a Variac rheostat connected to the hair dryer or by purchasing an independent controller. Controllers are available from a variety of sources (e.g., Fisher). It is important to pick a controller that is capable of supplying the current needed to run a hair dryer (a 1500-W hair dryer requires ~13 A).

4. Grow cells or isolate primary cultures in the phenol red–free formulation of the regular medium supplemented with 20 to 50 mM HEPES.

5. If cells can be passaged, remove cells from the culture vessel using a mild trypsin/EDTA treatment (*UNIT 1.1*), then inactivate the trypsin by adding an excess of degassed phenol red–free culture medium supplemented with HEPES. Resuspend cells in the same medium.

Cells to be used in traction assays should be removed from the culture vessel using as gentle a procedure as possible. The normal method of passaging any cell line or isolating any primary cell culture can be used.

The medium used to inactivate trypsin should contain serum or BSA.

Plate cells on substrate and measure wrinkling

6. Place the coverslip, fluid side up, into a petri dish just slightly larger than the coverslip. Flood the petri dish with degassed tissue culture medium to a depth of ≥0.5 cm. Incubate for 1 to 3 hr at normal culture temperature or until the cells resume their normal shape and migration speed.

Plate the cells onto the traction-assay substratum at densities that do not allow cells to contact each other but provide a sufficient number of cells on the surface for measurement. This number must be determined empirically for all of the assays. The density of cells will depend upon the stiffness of the substrate, since the wrinkles (Basic Protocol 1 and Alternate Protocol 1) and bead displacements (Alternate Protocol 2) generated by cells on very compliant substratum can interfere with each other. The density of cells will also depend upon the density of the measuring devices (see Basic Protocol 2).

There are a number of difficulties wetting the membrane that are due to the hydrophobic nature of the silicone surface. If there is insufficient medium in the petri dish, then the coverslip will float to the surface and the silicone sheet will "unwet," killing the cells and possibly rupturing the surface. A rapid wetting from one end of the dish to the other usually works well, but expect to rupture a number of membranes while practicing.

Normal migration speed typically refers to the migration speed on glass coverslips that are coated with the same extracellular matrix (ECM) as the traction assay substrate.

7. Place cells on the temperature-controlled microscope stage and record the length of the wrinkles produced by the cells. Record a calibration scale for the video field and use this field to measure the length of the wrinkles either directly or with a computer equipped with a frame grabber and image-analysis software.

Calibrate the substrate

8. Mount the substrate in a petri dish containing cell culture medium on the stage of a microscope equipped with a video system. Position a micromanipulator equipped with a needle holder so that the tip of the needle can reach the center of the optical field.

   Do not coat the membrane with cells if you are going to calibrate the membrane. Note that it is difficult to do the calibration under sterile conditions, so several duplicate membranes that will never be plated with cells should be calibrated instead of the actual "experimental" membrane.

   The calibration technique described in Alternate Protocol 2 for nonwrinkling substrates is more accurate, but it requires that the substrate be larger (>10-fold) than the diameter of the needle and that the substrate be approximately parallel to the coverslip. However, the substrate is often concave with respect to the coverslip in this assay.

9. Mount a calibrated microneedle in the micromanipulator and use it to push laterally on the surface of the sheet until it produces wrinkles that are shorter and longer than those produced by cells.

   An alternative method (Burton and Taylor, 1997) is to plate cells on the sheet and then fix the cells and sheets in 1% glutaraldehyde for 10 min. The fixed cells now act as "handles" that can be pushed by the microneedles.

10. Determine the displacement of the tip of the needle that is needed to produce each wrinkle length from a video recording of the calibration experiment. Record a calibration scale for the video field, and then use this field to calculate the displacement of the needle tip from its position before and after producing the wrinkle as described in step 7.

11. For each wrinkle length, calculate the force needed to generate the wrinkle as the product of the needle stiffness (see Support Protocol 1) and the needle deflection. This produces a curve of force versus wrinkle length.

Calculate traction force

12. Use the force versus wrinkle length curve generated in step 11 to calculate the force needed to produce the wrinkles measured in step 7.

SUPPORT PROTOCOL 1

CALIBRATING MICRONEEDLES

Calibrated microneedles can be used to measure the force of a locomoting cell by determining the amount of force required to stall the forward movement. The microneedles are also a necessary tool for measuring the stiffness of any of the devices whose construction is described in this unit.

Materials

25-µm-diameter Chromel thermocouple wire (Omega Engineering)
Pipet puller (David Kopf Instruments)
Pyrex capillary glass of various diameters and wall thicknesses (Drummond Scientific)

Small microscope mounted with its optical axis parallel to the bench
Eyepiece reticule (i.e., a calibration scale that fits into the eyepiece of the microscope; Edmund Scientific)

1. Cut a long (~30-cm) piece of 25-μm-diameter Chromel wire, weigh it, and carefully determine the length. Use the mass/length ratio to determine the mass of smaller pieces of Chromel wire that will be used in step 3.

2. Pull glass needles using a pipet puller.

   As a guide, 0.86-mm-o.d., 0.51-mm-i.d. Pyrex glass pulled on a vertical puller (David Kopf Instruments) at heat 27 and pull 10 should produce a microneedle in a useful starting range. Make sure that the diameter of the needle is less than the width of the wrinkles and that it can fit within the micromachined substrate (Basic Protocol 2).

3. Calibrate the needle by mounting it on the stage of the horizontal microscope (microscope turned onto its back so that its optical axis is parallel to the bench) and hanging a series of different-mass pieces of Chromel wire off the end of the needles. Measure the deflection of the tip by each mass using the eyepiece reticule. Pieces of wire ranging from 0.02 to 1.0 mg should provide a useful starting range.

   It is important to place the weights consistently on the very tip of the needle so that the deflection is consistent throughout the calibration.

   If needles that are too flexible to be calibrated by hanging weights are desired, then calibrate the needles by measuring the relative stiffness of the more flexible needles against stiffer needles that are calibrated as described above (Nicklas, 1983).

   As an alternative to the stiffer calibrated reference needles, 8- and 16-mm lengths of the Chromel wire can be used to make the "reference needles" (Dennerll et al., 1988). These lengths of wire can be glued into the center of capillary glass and tested with the same techniques as the needles. This should yield a stiffness (see Basic Protocol 2 for mathematical definition) of 2.43×10^{-2} N/m for the 8-mm length of wire. Note that the moment of inertia (I) for a circular beam is $1/4\pi r^4$.

4. Calculate the slope of the curve for force (mass of wire in g × 980 cm/sec^2) versus tip deflection to obtain the stiffness of the needle.

MEASURING CELL TRACTION ON WRINKLING SUBSTRATES WITH AN ALTERNATIVE POLYMER

ALTERNATE PROTOCOL 1

Deformable substrates can be formed with an alternative silicone polymer that has been described by Burton and Taylor (1997). Substrates formed with this polymer (phenylmethylpolysiloxane) have several advantages over those formed with the polymer described in Basic Protocol 1 (dimethylpolysiloxane)—i.e., they are suitable for differential interference microscopy and their stiffnesses can be decreased by exposure to ultraviolet light. This polymer can be cross-linked with the Bunsen burner technique described in Basic Protocol 1, the sputter-coater technique described in Alternate Protocol 2, or the tungsten-wire technique described by Burton and Taylor (1997). An example showing fibroblasts wrinkling a sheet that was made with this polymer cross-linked in a sputter coater is shown in Figure 12.3.1.

Additional Materials *(also see Basic Protocol 1 or Alternate Protocol 2)*

Phenylmethylpolysiloxane, trimethyl terminated (Dow Corning 710 fluid)
Ultraviolet lamp (UVGL-58 from UVP)

1. Prepare coated coverslips, replacing the dimethylpolysiloxane with trimethyl-terminated phenylmethylpolysiloxane. Perform the measurements in Basic Protocol 1 or Alternate Protocol 2.

   The advantage of this silicone is that it has a high index of refraction (1.536), which is suitable for polarized (differential interference) and interference reflection microscopy. Additionally, the silicone absorbs ultraviolet light (peak absorbance λ = 254 nm),which

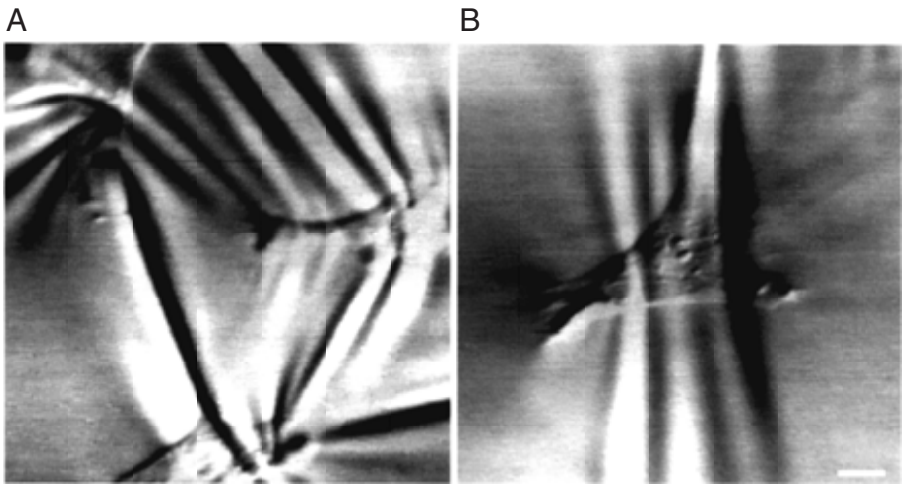


Figure 12.3.1 Chick embryo fibroblasts plated on a membrane made from phenylmethylpolysiloxane (J.A. Galbraith, unpub. observ.). The membrane was cross-linked in a sputter coater according to Alternate Protocol 2. Cells were plated in phenol red–free DMEM that was supplemented with 10% fetal bovine serum and antibiotics. Substrates were visualized with differential interference microscopy on a Zeiss inverted microscope using a 40 × 0.75–NA long-working-distance objective. (**A**) A group of three fibroblasts generates wrinkles that are perpendicular to their long axis. (**B**) A single fibroblast that is moving from left to right also generates wrinkles perpendicular to its long axis and the direction of migration. Bar is 10 μm.

weakens the sheet, making it more distensible. The disadvantage of this polymer is that it is considerably more expensive than dimethylpolysiloxane.

2. If calibration experiments indicate that the sheets are too stiff, make the sheets more distensible by exposing them to ultraviolet irradiation (6 W at 15 to 20 mm above the sheet, λ = 254 nm, 300 μWcm^2 at 15 cm for the surface) using a UVGL-58 lamp. Repeat the measurements.

   The curve for stiffness versus time of irradiation needs to be determined for each method of curing the silicone fluid. The curve given by Burton and Taylor (1997) for creating a sheet of any stiffness should be used as a starting point, but the actual time of exposure may depend upon the method of cross-linking.

ALTERNATE PROTOCOL 2

MEASURING CELL TRACTION ON NONWRINKLING SUBSTRATES

This protocol describes the use of nonwrinkling silicone sheets containing 1-μm diameter beads that act as fiduciary markers for measuring displacements and calculating force (Lee et al., 1994; Oliver et al., 1995).

Additional Materials *(also see Basic Protocol 1)*

Vacuum grease or Valap (*UNIT 13.1*)
Dimethylpolysiloxane (12,500 centipoise; Sigma)
1-μm liquid bead suspension (Polysciences)
Phenol red–free formulation of culture medium
Culture medium degassed using house vacuum

22 × 8–mm Pyrex cylinders (Bellco Glass)
Glass coverslips, acid washed
Airbrush apparatus (see Support Protocol 2)
Vacuum source
Sputter coater (e.g., Electron Microscopy Sciences)

Construct nonwrinkling substrate

1. Make Rappaport chambers by sealing the Pyrex cylinders to acid-washed coverslips with vacuum grease or Valap.
2. Pour 60 mg dimethylpolysiloxane into each chamber and allow it to spread evenly.
3. Disperse 1-μm beads on the surface of the dimethylpolysiloxane at a density of ~3×10^4 beads/mm^2, using a modified airbrush apparatus as described in Support Protocol 2.

   Although this can be done with a bristle brush using freeze-dried beads, a modified airbrush apparatus (see Support Protocol 2) using a liquid suspension of beads produces fewer aggregates.

4. Allow air bubbles from the dimethylpolysiloxane to escape or outgas under vacuum.
5. Cross-link the top layer of gel using a sputter coater at a vacuum of 0.15 torr and a target height of 60 mm. Apply the voltage to maintain a constant current of ~2 mA for 2 sec.

   The time, vacuum, and target height may need to be adjusted between different sputter coater models.

6. Add degassed culture medium to each cross-linked chamber (see Basic Protocol 1, step 6).

   Degassing the medium prevents the nucleation of small air bubbles on the silicone surface which can pull and distort the membrane. The presence of serum seems to stabilize the surface (Oliver et al., 1995).

Calibrate the substrate

7. Place the chamber on the microscope stage. Mount two calibrated microneedles of the same stiffness in the micromanipulators and position them toward the center of the sheet.

   A more accurate method of calibrating silicone sheets was introduced by Dembo et al. (1996). This method is similar to the one described in Basic Protocol 1, except that two identical calibrated needles and two manipulators are needed. The revised technique has the advantages of eliminating boundary effects due to the size of the Rappaport chamber and of satisfying global force balance during the calibration. See Oliver et al. (1998) for a more complete discussion.

8. Simultaneously move the needles toward each other, pinching the sheet between them.
9. Determine the stiffness of the sheet (force versus bead displacement curve) from the bending of the needles (see Oliver et al., 1998).

Measure cell traction

10. Add a suspension of cells to another cross-linked chamber, incubate for 1 to 3 hr, and measure the displacement of the beads (see Basic Protocol 1, steps 6 and 7).
11. Use the stiffness of the sheet determined in step 9 and the displacement of the beads by the cells determined in step 10 to calculate the force exerted by the cells.

SUPPORT PROTOCOL 2

PREPARING A MODIFIED AIRBRUSH APPARATUS

The airbrush is used to evenly disperse beads on the surface of polymer-coated coverslips (see Alternate Protocol 2). This device is illustrated in Oliver et al. (1995) and Figure 12.3.2. The details of this protocol were kindly supplied by Tim Oliver.

Materials

Nebulizer (DeVilbiss model 646)
Drying reservoir (16-liter container; Nalgene)
T-connector
Connecting tubing
Glass transfer pipet with tip drawn to 1-mm-i.d. opening
Impactors (37-mm Air Sampling Cassettes; model 4339, Gelman)

Assemble the apparatus

1. Connect one end of the nebulizer to a supply of filtered air.
2. Connect the other end of the nebulizer to the drying reservoir (16-liter Nalgene container).

   This is the input side of the drying reservoir.

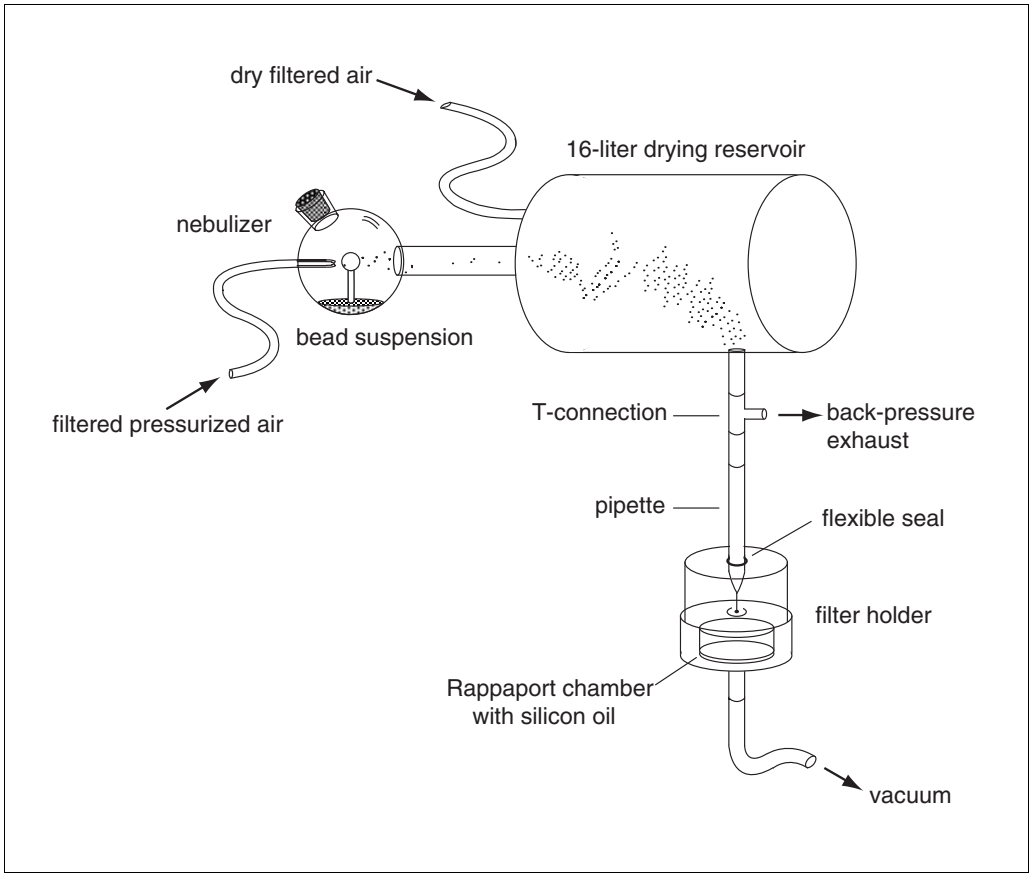


Figure 12.3.2 Illustration of a modified airbrush apparatus based on that of Oliver et al. (1995). This device separates a solution of beads into single dried particles. One end of the nebulizer is connected to a supply of filtered air. The other end of the nebulizer is connected to the input side of the drying reservoir. Another, independent filtered air supply is also connected to the input side of the reservoir. The output side of the reservoir is connected to a pipet with a 1-mm-i.d. tip. The pipet tip enters a modified impactor that contains the Rappaport chamber filled with silicone oil. The tip can be moved around to disperse the beads.

3. Supply the input side of the reservoir with dry filtered air, independent of the supply of air to the nebulizer.

   A pressure of 5 psi is recommended, but the air flow should be adjusted so that beads remain in the reservoir for several seconds.

4. Attach a T-connector to the output side of the reservoir.

   The perpendicular end of the T will be used as an exhaust.

5. Use a piece of tubing to connect the other end of the T to the drawn glass pipet.

6. Create an impactor thick enough to accommodate the Rappaport chambers by stacking the middle inserts of several cassettes between the top and bottom of one of them. Place a Rappaport chamber containing degassed silicone inside this thicker impactor.

7. Place the drawn pipet into the top of the impactor, and connect the two with a piece of flexible tubing.

8. Connect the bottom of the impactor to a vacuum.

Use airbrush apparatus to disperse beads (also see Alternate Protocol 2, step 3)

9. Add a suspension of the beads in ethanol to the nebulizer, then turn on the air and vacuum.

10. Move the pipet to disperse the beads over the top of the silicone.

    A recommended concentration is 3×10^4 beads/mm^2.

MEASURING CELL TRACTION ON MICROMACHINED SUBSTRATES

BASIC PROTOCOL 2

This protocol describes a method for developing micromachined substrates to measure traction forces under subcellular regions. An example of this is shown in Figure 12.3.3 (Galbraith and Sheetz, 1997). This device is based on a system of 5904 cantilevers of different lengths and therefore different stiffnesses. The levers are buried beneath the surface of the device, but on the free end of the lever there is a small pad that is planar with the surface of the device. The pads range in area from 4 to 25 μm^2 and are surrounded by a square hole that provides a 2-μm space on each side of the pad to allow the lever to move when a cell pulls on the pad. The forces that the cells exert on the pads can be determined by measuring the displacement of the pads and calculating the product of the pad displacement and the stiffness of the cantilevered lever. This approach is conceptually similar to the use of calibrated microneedles to measure forces.

Materials

50% (v/v) hydrofluoric acid
Appropriate extracellular matrix (ECM; e.g., 40 μg/ml laminin)
25% (w/v) $MgSO_4$
Phenol red–free culture medium

Nanofabrication facility
Computer-aided drawing (CAD) software package
Polarized reflection cube (see Support Protocol 3)
Fluorescence illumination for microscope
Red filter (645 nm long-pass)
Cloning cylinders
Silanized coverslips (see Support Protocol 3)
Sylgard 184 (Dow Corning)

1. Contact a nanofabrication facility that can perform the actual fabrication of the device.

   MCNC (Web site, http://www.mcnc.org; email, pr@mcnc.org) is one facility that can design and fabricate devices.

Design cantilever-beam force-measuring device

2. Estimate the traction forces that will be generated by the cells (see Oliver et al., 1994, and Table 12.3.1). Be certain to provide over- and underestimates of force in addition to the best guess.

3. Determine the maximum deflection allowable for beam displacement.

   A general guideline is several microns, but the actual number will depend upon the precision of the micromachining.

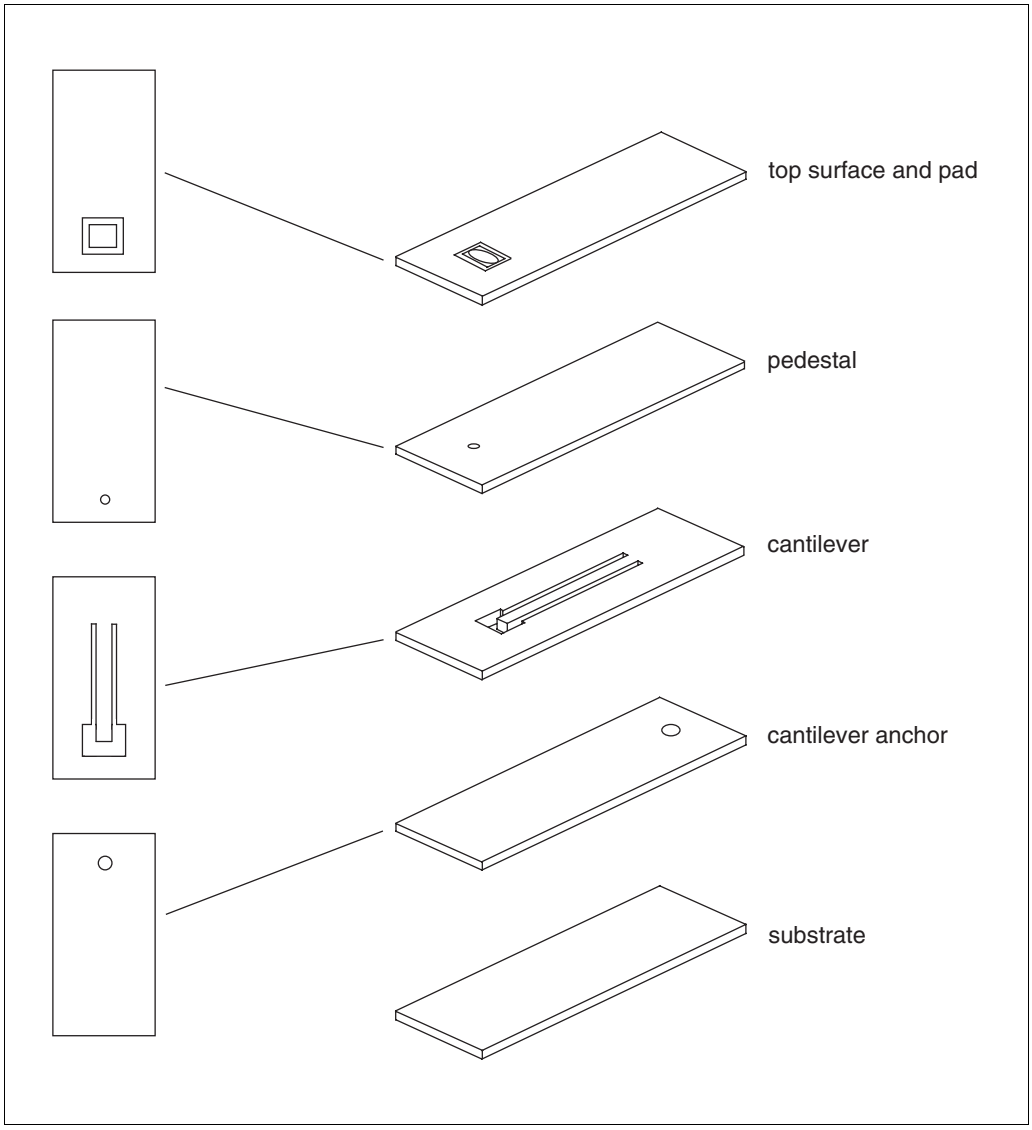


Figure 12.3.3 Masks and layers that are involved in making a single cantilever. A silicon wafer is used as substrate. Phosphosilicate glass (PSG) is placed on the silicon wafer, and a mask with holes is used to lithographically pattern the PSG to form anchors for the beams. Polycrystalline silicon is deposited over the PSG, and the mask with the beams and surrounding wells is used to pattern these structures. A layer of spin-on glass is added, and another mask is used to place a small hole at what will be the free end of the beam. The surface is then coated with a plasma-deposited amorphous silicon that fills the holes and forms a pedestal that anchors the pad to the beam. A final mask is used to etch the pad and square hole in the top layer.

4. Determine the appropriate beam stiffness by using the following equation:

$$\text{stiffness} = \frac{\text{traction force}}{\text{beam displacement}}$$

5. Calculate the appropriate geometric factors that will provide beams of this stiffness, using the following formulas, which give the relationship between the pertinent parameters for a square beam.

$$\text{stiffness} = \frac{3EI}{l}$$

$$I = \frac{h^4}{12}$$

In these equations, E is the Young's modulus of the silicone substrate, l is the length of the beam, I is the moment of inertia, and h is the height of the beam, which is equal to the width. Note that the height and width of the beam may be limited by constraints imposed by the fabrication facility. In the case of the authors' device, E is between 145 and 160 GPa, the width and height of the beams are 2 μm, and the lengths vary from 0.86 to 0.086 mm. These geometric parameters yield beams with stiffnesses ranging from 89 to 0.09 nN/μm. Additionally, very long beams may have "stiction" problems that will cause them to adhere to the walls of the channels in which they reside. The authors believe that they have encountered this with the longest beams used, which often appear immovable.

6. Determine the spacing and orientation of the measurement sensors (beams).

This will determine the layout for each unit, which will be repeated to make up the device or chip. Numerous chips can then be fabricated from one wafer of silicon. A typical layout has 9 repeating units, and ~40 chips are fabricated on one wafer.

Fabricate the device

7. Using a CAD package, generate mechanical drawings of each mask that is needed to create the layers of the repeating unit.

This step can be done by the fabrication facility. Micromachining is similar to a multiple-step photolithography: silicon layers are grown, exposed, and etched, and the process is repeated. For each layer a separate mask must be generated to create the appropriate pattern for etching. Figure 12.3.4 illustrates the types of masks and layers that are involved in making a single cantilever.

8. Use the CAD drawings to generate the actual masks that will be used for machining each layer.

9. Have the nanofabrication facility manufacture wafers containing numerous chips, then cut the wafer into individual pieces and perform the final etch (using 50% hydrofluoric acid, HF) that frees the assembly.

Initial releases that are performed by the facility can cause many of the pads to be dissociated from the ends of the beams. Titrate the time of exposure to HF to obtain free beams that still have pads on their ends.

CAUTION: *HF is an aggressive reagent requiring appropriate protective clothing and appropriate tools such as Teflon containers and forceps.*

Different reagents are needed to release other substrates.

Prepare the device for cells

10. Remove any residual HF from the chip to ensure the biocompatibility of the chip. After release, soak the chips in deionized water for 1 hr and then 25% $MgSO_4$ for 2 hr.

    This procedure seems to effectively sequester any free fluoride left trapped beneath the surface of the chip.

11. Prepare chambers for chips by attaching cloning cylinders to silanized coverslips with Sylgard gel.

    Follow manufacturer's instructions for curing. Note that large batches of the 2-part gel can be mixed and frozen in aliquots at −80°C until use.

12. Place the chips in chambers and coat the chip with an appropriate extracellular matrix (ECM) in the same way that you would coat a glass coverslip. However, take care

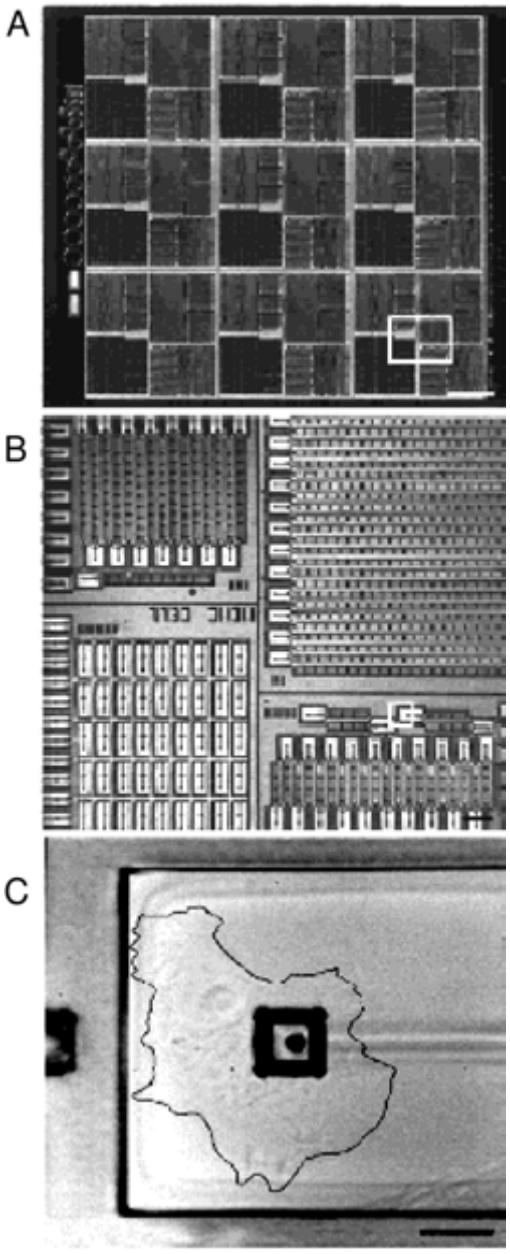


Figure 12.3.4 Micrographs of micromachined substrate. (**A**) Entire surface of a device that has nine repeats of a basic pattern containing four different lever lengths. Box indicates area shown in the next panel. Bar is 1 mm. (**B**) The four different lengths of beams. Box indicates area shown in the next panel. Bar is 0.1 mm. (**C**) A chick embryo fibroblast crossing a 25-μm^2 pad. Bar is 10 μm.

never to let the chip completely dry out, since changes in surface tension can remove the pads from the ends of the chips.

13. Plate cells on the chips by placing a suspension of cells in the cloning cylander chambers. Follow step 6 of Basic Protocol 1 to determine concentration and incubation time. View the chips by polarized reflection microscopy, using a polarized reflection cube (see Support Protocol 3) and a microscope with fluorescence illumination, a 645-nm long-pass filter, and temperature control if needed.

    Reflection microscopy must be used since the chips are opaque and do not transmit light. This viewing method produces high-contrast images of the chip, but low-contrast images of the cell.

14. Calibrate the movable pieces of the device with calibrated microneedles (see Basic Protocol 1, steps 8 to 11).

    For this procedure, a long working distance water-immersion objective on an upright microscope is convenient.

15. Compare the calibrated stiffness with the theoretical calculations.

SILANIZING COVERSLIPS

SUPPORT PROTOCOL 3

Silanized coverslips are used as a base for assays of cell traction using micromachined substrates (see Basic Protocol 2). This protocol describes a simple procedure for coating glass coverslips.

Materials

20% HNO_3
Acetone, reagent grade
1,1,1,3,3,3-Hexamethyldisilazane (HMDS; Aldrich)
70% ethanol

Glass coverslips and coverslip racks (Thomas Scientific)
Pyrex dish large enough to hold coverslip racks
Drying oven
Nitrogen gas cylinder with regulator
3-cc syringe with needle

1. Place coverslips on coverslip racks and soak in 20% HNO_3 for 1 hr.
2. Rinse coverslips in Milli-Q-purified water for 1 hr.
3. Soak in reagent-grade acetone for a few minutes. Pour off acetone and allow excess fluid to air dry.
4. Place coverslip racks in Pyrex dish and cover with aluminum foil. Bake 1 hr in 140°C oven.
5. Collect a plastic bag full of nitrogen gas. Puncture the bag with a needle attached to a 3-cc syringe. Withdraw 2.5 cc of nitrogen gas, insert the needle into the top of the HMDS container, and withdraw 2.5 cc HMDS.

    The gas is used to equalize the pressure in the container without oxidizing the HMDS.

6. Dispense HMDS into the Pyrex dish through the aluminum foil, coating the coverslips.
7. Bake 20 min at 140°C.

8. Evacuate the oven for 5 min, then lift foil cover off the Pyrex dish and continue to evacuate for a further 15 min.
9. Continue baking for 1 hr at 140°C.
10. Cool coverslips and store in 70% ethanol.
11. Drain excess ethanol and allow coverslips to air dry before use.

SUPPORT PROTOCOL 4

PREPARING A POLARIZED REFLECTION CUBE

Reflection microscopy must be used with micromachined substrates (see Basic Protocol 2), because the chips are opaque. While commercial polarization microscopes may be used, this method provides a convenient way to modify existing laboratory microscopes for use in this technique.

Materials

Fluorescence slider or cube (available from microscope manufacturers)
50-50 mirror (Chroma)
Polarizers (Chroma)

1. Place a 50-50 mirror in the position of the fluorescent slider or cube that normally holds the dichroic mirror.
2. Place polarizers where the excitation and emission filters normally reside, and orient the polarizers to be perpendicular each other.

Chroma will provide polarizers backed with glass in the appropriate sizes and mounts.

COMMENTARY

Background Information

Migrating cells exert traction forces against their substrates. These tractions result from the interactions between the force-generating cytoskeleton, the focal adhesion complex, and the extracellular matrix (ECM). The interaction of the intracellular forces results in a net forward movement of the cell. However, the majority of the force acts to break the adhesive contacts with the ECM, since traction forces are several orders of magnitude larger than the force required to move a cell swimming in solution. Traction forces measured on the ventral surface of the cell are also much larger (Galbraith and Sheetz, 1997) than forces generated on the dorsal surface (Felder and Elson, 1990), and this may be due to differences in the cytoskeletal organization between the ventral and dorsal surfaces (Cramer et al., 1997). Given this difference between tractions on the two surfaces, it has been important to develop techniques and technologies that can measure many of the subcellular events of cell migration on the dorsal surface of the cell.

Most of the techniques for measuring traction forces have been developed around sheets of cross-linked silicone polymer (see Basic Protocol 1). The original technique used to measure tractions examined the orientation of wrinkles generated in the sheets by locomoting cells. This technique has been useful for providing a global orientation of the forces under cells (Harris et al., 1980) and for comparing the relative forces of different cell types (Harris et al., 1981). These original experiments did not determine the magnitude of the forces generated underneath a cell, but they did establish the force required to stop a locomoting chicken heart fibroblast (~200 nN if the cell has a 20-μm-long leading edge). These sheets also demonstrated that cells that migrated at the highest velocities generated the smallest traction forces, since fast-moving cells such as leukocytes and nerve growth cones were unable to wrinkle the sheets (Harris et al., 1981).

This assay was modified to produce nonwrinkling substrates that measure traction forces by examining the displacement of beads embedded within the sheet (Lee et al., 1994; see Alternate Protocol 2). These sheets were able to determine the small traction forces generated under different regions of rapidly moving keratocytes. The forces measured by these sheets were limited in temporal resolution,

since the half time for recovery was ~0.5 sec for the stiffest membranes. However, the forces were easier to quantitate than those measured by wrinkles, and the bead displacements have been used to provide detailed information about the orientation and location of traction forces (Oliver et al., 1995; Dembo et al., 1996).

A more recent modification of the original traction assay used a different polymer to generate another wrinkling sheet whose stiffness can be easily modified by exposure to ultraviolet light (Burton and Taylor, 1997; see Alternate Protocol 1). These less stiff sheets can be wrinkled by cells undergoing cytokinesis (Burton and Taylor, 1997) and can measure forces as small as 10 to 20 nN. All three of these silicone substrates are useful for measuring forces under the entire cell.

Another technique for measuring traction forces is based on micromachining technology (Galbraith and Sheetz, 1997; see Basic Protocol 2). The device is constructed with a system of movable levers that have stiffnesses ranging from 0.09 to 89 nN/μm. This device can measure forces under regions of the cell as small as 4 μm^2 in real time, but it cannot measure forces under multiple regions of the cell simultaneously. Therefore, the protocols presented in this unit either provide detailed real-time subcellular information from a small number of adhesive contacts or extensive spatial information of slower events under the entire cell.

Critical Parameters

The time of cross-linking for the silicone sheets will critically affect their stiffness. It may be necessary to manufacture a number of sheets using an array of different conditions. This will provide a number of specimens, some of which will be suitable.

The other critical parameter in using the membranes is how they are wetted with the cell-containing medium. Rapid coating, starting from one side and moving to the other, produces the fewest membrane ruptures, but any coating method requires practice.

Troubleshooting

The traction forces generated by several different cell types have been reported in the literature. It is therefore important to compare the measurements obtained with values presented in the literature (Table 12.3.1; Oliver et al., 1994). If the measurements obtained are vastly different, recheck the calibration. Begin by recalibrating the 8-mm length of Chromel wire and comparing its stiffness with the value obtained by Dennerll et al. (1988). If this is accurate, then check the calibration of the needles. Finally, recalibrate the film or micromachined device.

Anticipated Results

Typical traction forces generated by various cell types are listed in Table 12.3.1.

Time Considerations

The time required to manufacture any of the sheets is not extensive, however finding the conditions under which the sheets are produced with suitable stiffness may require some experimentation. In contrast, the time required to manufacture the micromachined devices may be considerable. Depending upon the schedule of the microfabrication facility and the number of process iterations required, the time from concept inception to product can easily consume 6 months to 1 year.

Additionally, the time required to collect data will vary greatly, depending upon the migration speed of the cells of interest. It should also be noted that the time required to analyze the video data is severalfold greater than the amount of time required to collect the data.

Literature Cited

Burton, K. and Taylor, D. 1997. Traction forces of cytokinesis measured with optically modified elastic substrata. *Nature* 385:450-454.

Cramer, L., Siebert, M., and Mitchison, T. 1997. Identification of novel polarity actin filament bundles in locomoting heart fibroblasts; Implications of the generation of motile force. *J. Cell Biol.* 136:1287-1305.

Dembo, M., Oliver, T., Ishihara, A., and Jacobson, K. 1996. Imaging the traction stresses exerted by locomoting cells with the elastic substratum method. *Biophys. J.* 70:2008-2022.

Dennerll, T., Joshi, H., Steel, V., Buxbaum, R., and Heidemann, S. 1988. Tension and compression in the cytoskeleton of PC-12 neurites II: Quantitative measurements. *J. Cell Biol.* 107:665-674.

Felder, S. and Elson, E. 1990. Mechanics of fibroblast locomotion: Quantitative analysis of forces and motions at the leading lamellas of fibroblasts. *J. Cell Biol.* 111:2513-2526.

Galbraith, C. and Sheetz, M. 1997. A new bend on measuring local traction forces: Micromachined substrate. *Proc. Natl. Acad. Sci. U.S.A.* 94:9114-9118.

Harris, A., Wild, P., and Stopak, D. 1980. Silicone rubber substrata: A new wrinkle in the study of cell locomotion. *Science* 208:117-118.

Harris, A., Stopak, D., and Wild, P. 1981. Fibroblast traction as a mechanism for collagen morphogenesis. *Nature* 290:249-251.

Lamoureux, P., Buxbaum, R., and Heidemann, S. 1989. Direct evidence that growth cones pull. *Nature* 340:159-162.

Lee, J., Leonard, M., Oliver, T., Ishihara, A., and Jacobson, K. 1994. Traction forces generated by locomoting keratocytes. *J. Cell Biol.* 127:1957-1964.

Nicklas, R. 1983. Measurements of the force produced by the mitotic spindle in anaphase. *J. Cell Biol.* 97:542-548.

Oliver, T., Lee, J., and Jacobson, K. 1994. Forces exerted by locomoting cells. *Semin. Cell Biol.* 5:139-147.

Oliver, T., Dembo, M., and Jacobson, K. 1995. Traction forces in locomoting cells. *Cell Motil. Cytoskeleton* 31:225-240.

Oliver, T., Jacobson, K., and Dembo, M. 1998. Design and use of substrata to measure traction forces exerted by cultured cells. *Methods Enzymol.* 298:497-521.

Contributed by Catherine G. Galbraith and Michael P. Sheetz
Duke University Medical Center
Durham, North Carolina

Cell Wound Assays

It is often assumed that plasma membrane continuity is a constant feature of the life of the normal, healthy cell, and that when there is evidence of a disruption in continuity, one can conclude that cell death has occurred. Neither of these assumptions is correct. The plasma membrane, like other moving parts, is vulnerable to mechanically induced wear and tear. Many animal cells that reside in mechanically active tissue environments normally and frequently experience disruptions in plasma membrane integrity (McNeil, 1993; McNeil and Steinhardt, 1997; McNeil et al., 1981). Cells locomoting in culture periodically tear off small pieces of cytoplasm as their trailing end is drawn out into long retraction fibers that eventually break (Chen, 1981). Pathological levels of mechanical stress can, of course, exacerbate these constitutive levels of cell "wounding." An active and complex Ca^{2+}-regulated resealing mechanism rapidly repairs disruptions, preventing influx of potential toxins and loss of vital cytosolic constituents (Steinhardt et al., 1994). Indeed, many cells survive surprisingly large membrane disruptions. Skeletal muscle cells and certain free-living amoebae, for example, recover after being cut in half, and sea urchin eggs can be fertilized and undergo cleavage after a 20×40–μm^2 patch of plasma membrane and underlying cortex is ripped from their surface (Terasaki et al., 1997).

The study of cell wounding, whether it has biological or practical aims (see Commentary), requires methods for identifying wounded cells, often present as a small minority in a culture or tissue. This unit describes such methods, which can also yield quantitative data concerning the frequency and magnitude of wounding. Wounding can be detected microscopically in cultures of adherent cells and in tissues using fluoresceinated dextrans (see Basic Protocol and Alternate Protocol 1, respectively) or using an animal's endogenous serum albumin and enzyme immunohistochemistry (see Alternate Protocol 2). Electron microscopy of wounded cells or tissues (see Alternate Protocol 3) can be used to verify the cytoplasmic location of the tracer molecule. Wounding can be quantified by image analysis (see Support Protocol 1) or flow cytofluorometry (see Support Protocol 2).

STRATEGIC PLANNING

The wounded cell is identified by virtue of cytosolic labeling with a normally impermeant tracer macromolecule (Fig. 12.4.1). An open plasma membrane disruption allows the tracer to enter the cytosol, and resealing traps it there. Dead cells, which do not reseal, are not labeled as long as all exogenous tracer is washed away after the disruption. There are several wound tracers to choose from. Fluoresceinated dextrans are relatively inexpensive and are available in a range of sizes (mol. wt. 5,000 to 2,000,000). If aldehyde-fixed specimens will be employed, dextrans containing conjugated lysine residues must be used. The advantage of an exogenously added tracer, such as fluoresceinated dextran, is that its time of addition and removal are known, and thus it can be used in pulse-chase labeling protocols to determine the timing of wound events. Albumin is an excellent endogenous wound tracer. For whole-animal studies it has the prominent advantage of normally bathing the cells of many tissues at high concentration.

Detection of a wound tracer by microscopy provides useful structural detail. For example, one can identify wounded cells on a cell-by-cell basis in a background of nonwounded cells, and determine which cell types in a tissue are vulnerable and at what location. Microscopy also allows one to determine whether the labeling pattern is cytosolic, the pattern expected of a wound tracer. Endocytosed tracer is recognizable as a punctate staining of the cytoplasm only. In contrast, tracer entering through a disruption results in

Contributed by Paul L. McNeil, Mark F.S. Clarke, and Katsuya Miyake
Current Protocols in Cell Biology (1999) 12.4.1-12.4.15

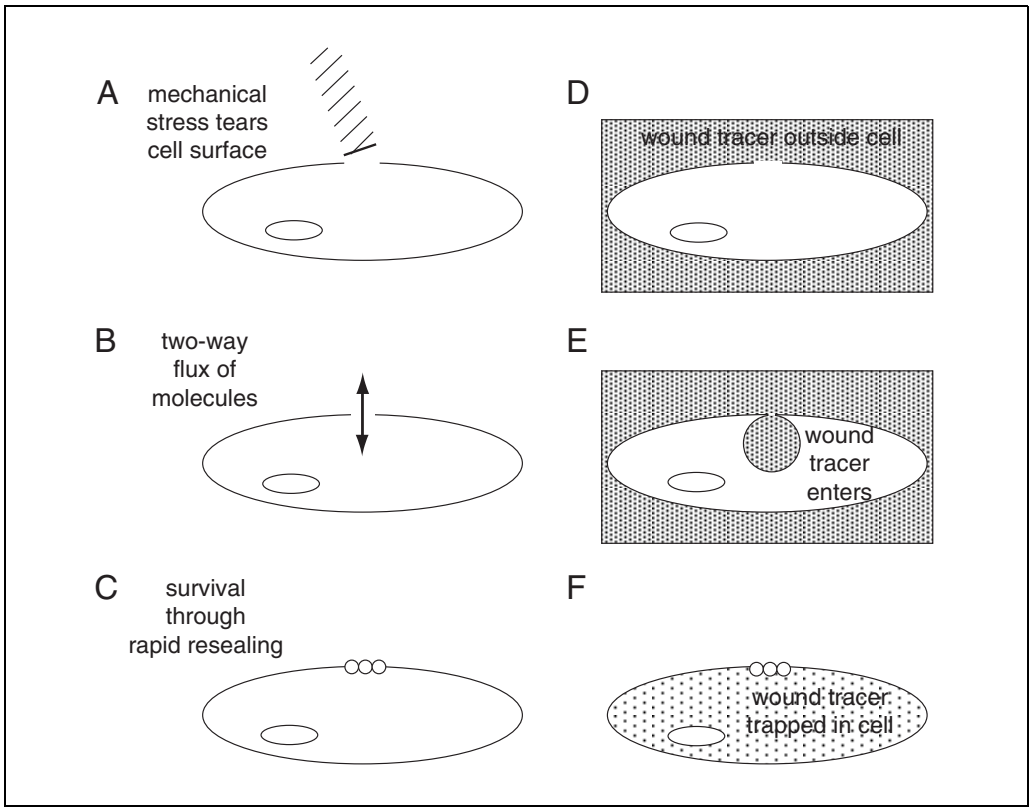


Figure 12.4.1 Cell wounding defined and identified. Mechanical stress creates a plasma membrane disruption (**A**) through which normally impermeant molecules may enter and leave the cell (**B**). Rapid resealing (<5 sec, mediated by Ca^{2+}-induced membrane mobilization and fusion events) allows the cell to survive the wound (**C**). When the disruption occurs in the presence of a normally impermeant wound tracer molecule (**D**), it can enter the cell (**E**), and become trapped there upon successful resealing (**F**). If the wound tracer is visible microscopically or fluorometrically, the cell can be identified as having been wounded.

a diffuse, even labeling of cytoplasm and nucleoplasm (if a probe <40,000 mol. wt. is employed). Often such patterns are discernible by fluorescence microscopy, but electron microscopic localization provides a more definitive indication of tracer location, especially in tissues.

BASIC PROTOCOL

WOUND DETECTION IN CULTURED MONOLAYERS USING FLUORESCEIN DEXTRAN

A procedure for microscopical detection of wounded cells growing in adherent culture is described in this protocol, along with a useful positive control (mechanical injury of the monolayer; Swanson and McNeil, 1987). This procedure is written for human umbilical vein endothelial cells, but is applicable to many and perhaps most cell types commonly grown in the laboratory. The authors have applied it to HeLa, COS, fibroblast (NIH and Swiss 3T3), various endothelial, and smooth muscle cells.

Materials

Adherent cells of interest (e.g., human umbilical vein endothelial cells; HUVEC; *UNIT 2.3*)

Cell culture medium (e.g., DMEM/10% FBS for HUVEC; Life Technologies)

Dulbecco's phosphate-buffered saline (DPBS), sterile (Life Technologies; *APPENDIX 2A*)

continued

5 mg/ml FDxLys solution: lysine-fixable fluorescein dextran, 10,000 mol. wt. (Molecular Probes) in DPBS
3.7% (w/v) formaldehyde (Fisher) in DPBS
Antibleaching microscopic mounting medium (e.g., ProLong, Molecular Probes)
22 × 22 × 1–mm glass coverslips (Fisher), sterilized by flaming with ethanol (*UNIT 1.3*)
Sterile petri dishes (Falcon; e.g., 100 × 15–mm with four compartments)

1. Plate cells of interest onto flame-sterilized coverslips and culture in an appropriate cell culture medium until the desired degree of confluency is reached.

   The wound protocol will work at any cell density, although higher densities induce a greater number of wounding events per coverslip.

2. Remove coverslip from its culture dish or well with tweezers and wash two times by dipping in two 50-ml beakers containing sterile DPBS. Touch the coverslip edge to a paper towel to wick off excess DPBS after each wash, but do not allow cells to dry out.

   In this and all following manipulations, be sure to keep track of which side of the coverslip the cells are plated on.

3. Place the coverslip cell-side up in the bottom of a plastic petri dish that is large enough to comfortably accommodate the coverslip. Pipet 100 µl of 5 mg/ml FDxLys solution onto and off of the coverslip several times.

   This ensures that the cells, which reside in an "unstirred" layer, are thoroughly and uniformly exposed to the tracer solution.

4. Initiate experimental conditions suspected of causing wounding. Leave negative controls undisturbed.

   Examples of mechanical stress that might be suspected of causing wounding are shear stress due to medium flow over or around cells and tensile stresses due to compression or stretching of the cell or its substratum. Negative controls receive FDxLys but are not mechanically disturbed.

   Time in the dye should be minimized after wounding has been completed.

5. As a positive control, scratch the coverslip approximately ten times using a single-edge razor blade. View under phase-contrast optics to ensure that zones of cells (2 to 4 cells wide) have been denuded from the substratum by this maneuver.

6. Wash all coverslips as in step 2 and return to normal culturing conditions (add culture medium, return to incubator) for the desired interval.

7. Wash each coverslip three times in DPBS and then immerse in 3.7% formaldehyde for 10 to 30 min.

   The investigator may need to determine the appropriate fixative for other fluorescent wound tracers.

8. Wash the coverslip twice in distilled water and then mount in an antibleaching microscopic mounting medium according to manufacturer's instructions. View microscopically using a standard fluorescein filter set.

   An example of FDxLys-labeled cells is shown in Figure 12.4.2.

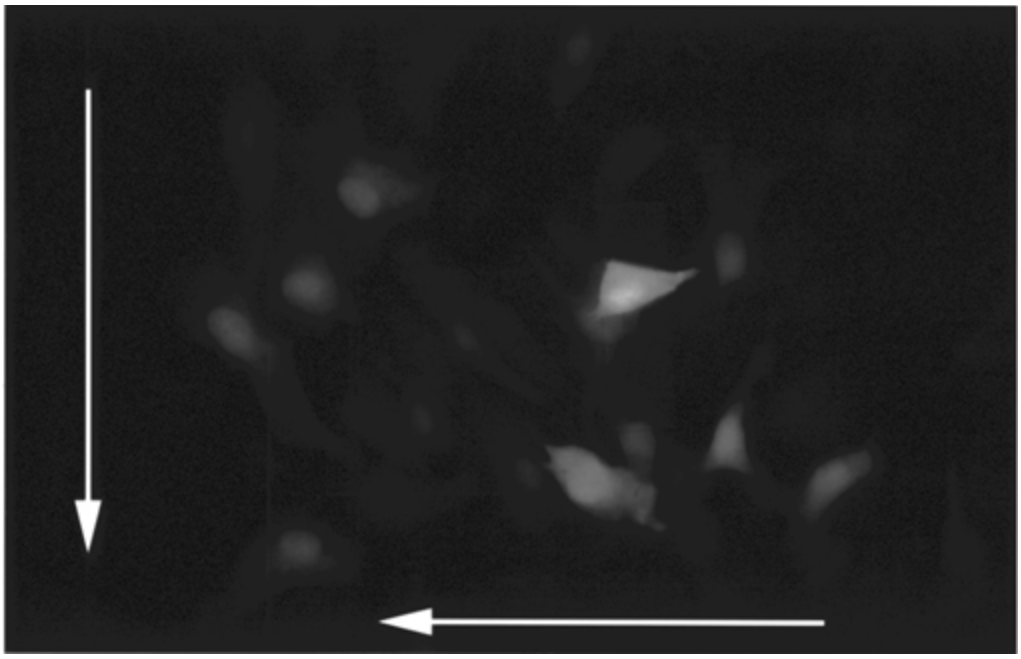


Figure 12.4.2 Example of detection of mechanically induced cell wounding in vitro. A monolayer of bovine aortic endothelial cells growing on a glass coverslip was scratched with a syringe needle in two directions (arrows indicate scratch tracks) in the presence of the wound tracer FDxLys (1 mg/ml). The culture was fixed in 4% formaldehyde 3 hr later. Cells that incurred plasma membrane disruptions that were successfully resealed are labeled with FDxLys (green). Cells that were not labeled are present in the upper right-hand corner of the micrograph. In the absence of this wound tracer, it would have been impossible to tell which of the cells in this field had been wounded, since morphologically they are indistinguishable from undisturbed neighbors. **See color figure.**

ALTERNATE PROTOCOL 1

WOUND DETECTION IN MAMMALIAN TISSUES USING FLUORESCEIN DEXTRAN

This procedure describes detection of wounded cells when the wound is induced in an intact animal by physiological or pathological mechanical stress. As with the in vitro procedure (Basic Protocol), wounded cells are detected by virtue of labeling with an impermeant marker. However, experimental access to the extracellular environment of cells in tissues is severely restricted compared to that available in the monolayer tissue culture situation. In vivo, tracer addition and removal and cell fixation are most efficiently accomplished with minimal tissue perturbation by delivering reagents (tracer, wash solution, fixative) through the blood vascular system (Clarke et al., 1995; McNeil and Khakee, 1992). Hence, the technique of vascular perfusion is described in detail in this protocol. The technique presented here focuses on the triceps skeletal muscle of the rat front leg. It should provide adequate perfusion for detecting cell wounding in most other mammalian tissues/organs, but may not be adequate for all. For example, it is not suitable for the heart, because the heart is purposefully injured in gaining access to the blood vascular system and its circulation is then bypassed during the perfusion.

In this procedure, as in the Basic Protocol, wounded cells are labeled by virtue of uptake of fluorescein dextran. Alternatively, tissue sections can be immunostained for rat serum albumin (see Alternate Protocol 2).

CAUTION: Due to the highly reactive and volatile nature of freshly prepared formaldehyde solution, the perfusion apparatus and the animal being perfused should be contained in a fume hood.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male Sprague-Dawley rats (250 g)
Lysine-fixable fluorescein dextran (FDxLys; Molecular Probes; 10,000 mol. wt.)
Dulbecco's phosphate-buffered saline (DPBS), sterile (Life Technologies; *APPENDIX 2A*)
1% (w/v) procaine solution in DPBS, 37°C
4% and 8% (w/v) freshly prepared formaldehyde solution in DPBS
10%, 20%, and 30% (w/v) sucrose solutions in DPBS
Tissue-Tek OCT compound (Fisher)
OCT/sucrose solution: DPBS containing 50% (v/v) OCT compound and 30% (w/v) sucrose
Isopentane
Liquid nitrogen
100 mM Tris·Cl (*APPENDIX 2A*; but adjust to pH 7.0)
Antibleaching microscope mounting medium (e.g., ProLong; Molecular Probes)

500-ml reservoirs
0.3-meter lengths of 0.2-mm-i.d. plastic tubing
1.5-meter length of 0.1-mm-i.d. plastic tubing
Three-way valve
5-cm-long, 1- to 2-mm-o.d. blunt cannula
18-G needles
1-ml syringes
Cryostat microtome
Superfrost plus microscope slides (Fisher) coated with the appropriate adhesion factor (*UNITS 2.3 & 4.3*)

Set up perfusion apparatus

1. In a fume hood, set up two 500-ml reservoirs connected by two separate 0.3-meter lengths of 0.2-mm-i.d. plastic tubing to a three-way valve. At the third end of the valve, connect a single 1.5-meter length of 0.1-mm-i.d plastic tubing to a 5-cm-long, 1- to 2-mm-o.d. blunt cannula. Elevate the solution reservoirs ≥1 meter above the animal to produce an adequate perfusion pressure.

   This setup, illustrated in Figure 12.4.3, allows perfusion of the animal first with procaine solution and then with formaldehyde solution.

Introduce tracer into blood vascular system

2. Grasp a male Sprague-Dawley rat firmly by the skin over its neck/shoulder region. With the free hand,administer 0.25 ml sterile DPBS containing ~400 mg FDxLys/kg body weight by i.p. injection with an 18-G needle and 1-ml syringe.

   FDxLys is rapidly distributed throughout the vascular system and extracellular spaces within 2 min of successful injection into the peritoneal cavity. This can be confirmed by illuminating the animal's ear with a hand-held UV lamp; it should rapidly become fluorescent.

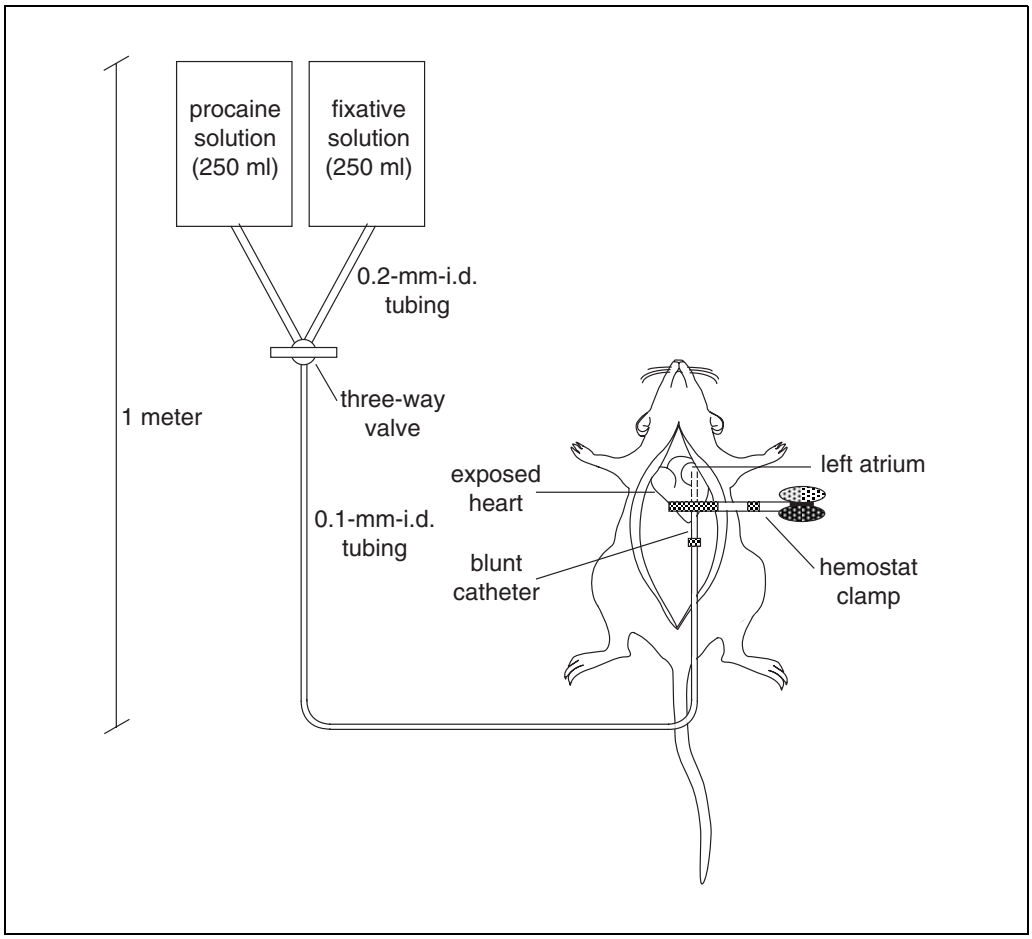


Figure 12.4.3 Perfusion setup for use after wounding in situ. Procaine and fixative solutions are elevated ≥1 meter above the subject and are administered sequentially to the left atrium via a three-way valve and a 5-cm-long cannula.

Initiate experimental conditions suspected of causing wounding

3. Expose the rat to the experimental condition of interest (e.g., exercise or administration of positively inotropic agents).

   Negative controls are not exposed to these conditions; positive controls are exposed to pathological levels of mechanical stress (e.g., a needle-puncture injury; Fig. 12.4.4).

Perfuse blood vascular system to remove exogenous tracer

4. Deeply anesthetize rat by administering an i.p.. injection of 200 mg/kg pentobarbital.
5. Place the animal on a dissection board and immobilize the front limbs with surgical tape so that the triceps muscles are in an unstretched, uncontracted state (Fig. 12.4.3).
6. With the three-way valve in the closed position, fill one reservoir of the perfusion apparatus with 250 ml warm (37°C) 1% procaine solution and the other with 250 ml of 8% formaldehyde solution. Displace any air bubbles trapped in the plastic tubing.
7. Make a small horizontal incision through the abdominal wall of the rat. Using this incision to gain access to the abdominal cavity, expose the abdominal organs by making a longitudinal incision in the abdominal wall along the full length of the animal. Expose the ribcage, puncture the diaphragm using a small pair of surgical scissors, and, working quickly, dissect out the diaphragm to expose the heart.

   Be careful not to damage any of the major blood vessels, heart, or lungs within the chest cavity.

8. Make a small incision (0.2 mm) in the apex of the left ventricle. Turn the three-way valve of the perfusion apparatus so that procaine solution is flowing, and insert the blunt cannula through the small incision, positioning the end of the cannula so that it rests in the left atrium close to the aortic arch. Clamp the cannula in place with a hemostat so that the perfusion solutions cannot exit the heart except through the aorta.
9. Begin perfusing the animal with procaine solution.
10. A few seconds after initiating perfusion with procaine, make an incision in the right atrium to allow the perfusion solution to exit the vasculature. Perfuse all 250 ml procaine.

    Adequate whole-body perfusion (with the exception of the heart) has been achieved if the liver is clear of blood (i.e., has changed from a deep red to a light brown color).

11. Without allowing air to enter the three-way valve, switch to the formaldehyde perfusion solution and perfuse the animal with 250 ml formaldehyde.

    Fixation is rapid and can be checked by gently pressing on the liver, which should become hard to the touch.

12. Fill the body cavity with absorbent pads to contain the fixative solution and leave the animal for 1 hr in the fume hood.

Prepare tissue for microscopy

13. Carefully dissect out the tissue or organ of interest and place it in fresh 8% formaldehyde solution overnight.
14. Wash tissue once quickly with DPBS and cut into pieces of a suitable size for mounting for frozen sectioning.
15. Infiltrate tissue sequentially with 10%, 20%, and 30% sucrose solutions and with OCT/sucrose solution for a minimum of 24 hr per solution.
16. Immerse infiltrated tissue in OCT compound for 3 hr and slowly freeze the tissue onto a sectioning stub using liquid nitrogen–cooled isopentane.
17. Cut 10-µm-thick frozen sections on a cryostat microtome and collect onto adhesion factor–coated Superfrost plus microscope slides.
18. Postfix sections in 4% formaldehyde solution for 10 min at room temperature.
19. Wash sections in 100 mM Tris·Cl, pH 7.0, for 5 min. Mount in antibleaching microscope mounting medium and coverslip.
20. View sections directly using a fluorescence microscope equipped with a standard fluorescein filter set.

    Cells that have suffered a membrane wound during the exercise period will contain the green FDxLys fluorescence signal (Fig. 12.4.4A).

WOUND DETECTION USING ALBUMIN AS A WOUND TRACER

ALTERNATE PROTOCOL 2

Albumin, a prominent protein constituent of blood (~10 mg/ml; ~60,000 mol. wt.), is present in most mammalian extracellular fluids. Hence, it can be used as an endogenous wound tracer in whole animal experiments (Clarke et al., 1995; McNeil and Khakee, 1992). Cultured cells can be labeled with albumin as described in the Basic Protocol. Wounded cells are labeled with an anti–rat serum albumin antibody directly conjugated with horseradish peroxidase, and are then detected enzymatically. The protocol can be

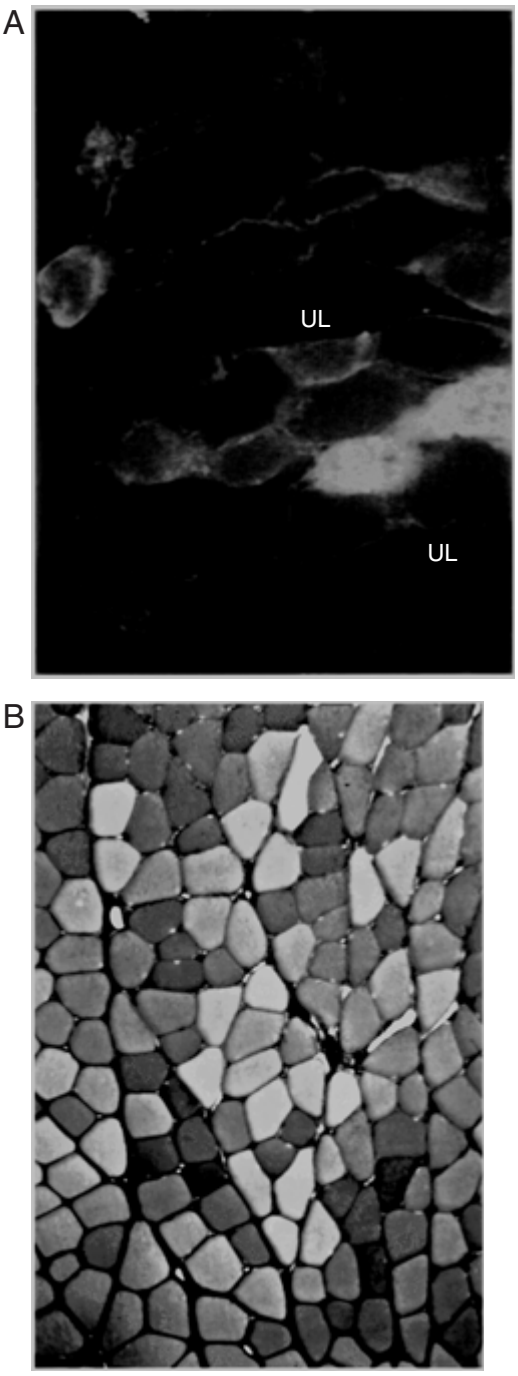


Figure 12.4.4 Example of wounded cells in skeletal muscle detected using FDxLys (**A**) or albumin (**B**) as the wound tracer. Mouse quadriceps muscles were injured by penetration with a narrow-gauge needle. Wounded cells (myofibers) are labeled with FDxLys as described in Alternate Protocol 1 (UL, unlabeled cells) or with rat serum albumin as described in Alternate Protocol 2. **See color figure.**

modified for use with alkaline phosphatase–conjugated antibodies or for secondary antibody amplification of the albumin signal.

CAUTION: Diaminobenzidine (DAB) is carcinogenic. Wear gloves and work in a fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

10 mM ammonium chloride solution in DPBS
0.1% (v/v) Triton X-100 solution in DPBS
Wash buffer: 0.05% (v/v) Triton X-100 in DPBS
H_2O_2/NaN_3/DPBS: 0.3% (v/v) hydrogen peroxide and 2% (w/v) sodium azide in DPBS (add stock H_2O_2 to azide buffer immediately before use)
Heat-inactivated sheep serum (Life Technologies)
Horseradish peroxidase (HRP)–conjugated sheep anti–rat serum albumin (RSA) polyclonal antibody (Organon Teknika Cappel)
100 mM Tris·Cl, pH 7.0 (*APPENDIX 2A*; but adjust pH to 7.0)
HRP substrate kit (Vector Laboratories) with 3,3′-diaminobenzidine (DAB)
50%, 75%, 90%, 95%, 99%, and 100% (v/v) EM-grade ethanol
EM-grade xylene
Permanent mounting medium (e.g., Cytoseal 60, Stephen's Scientific)

NOTE: All staining procedures are carried out at room temperature unless otherwise specified.

1a. *For cultured monolayers:* Prepare experimental and control cultured cells as described (see Basic Protocol, steps 1 to 7), but replace 5 mg/ml FDxLys with 5 mg/ml rat serum albumin (step 3).

1b. *For tissues:* Prepare experimental and control tissue sections as described (see Alternate Protocol 1, steps 1 to 18), but omit the FDxLys injection.

2. Wash the specimen twice with DPBS and incubate in 10 mM ammonium chloride solution for 30 min to inactivate reactive aldehyde groups.

3. To permeabilize tissue sections, incubate 5 min in 0.1% Triton X-100 solution, followed by 5 min in wash buffer.

   Use gentle agitation for these and all subsequent incubations and washes.

4. To block endogenous peroxidase activity, incubate 30 min in H_2O_2/NaN_3/DPBS followed by 5 min in wash buffer.

5. To block nonspecific protein binding, incubate tissue section 1 hr in wash buffer containing 4% (v/v) heat-inactivated sheep serum, followed by 5 min in wash buffer alone.

6. Incubate overnight at 4°C with HRP-conjugated sheep anti-RSA polyclonal antibody made up in wash buffer/1% sheep serum.

   Due to batch-to-batch variations, titration of primary antibody concentration will be required to achieve optimal staining intensity of wounded cells; a range of 1:100 to 1:1000 is recommended as a starting point.

7. Wash five times with wash buffer and two times with 100 mM Tris buffer.
8. Prepare fresh HRP substrate buffer per manufacturer's instructions and incubate tissue section in substrate buffer for 30 min in the dark.
9. Wash section in distilled water for 5 min and dehydrate in a graded ethanol series: 10 min each in 50%, 75%, 90%, 95%, 99%, and 100% ethanol.
10. Incubate in fresh xylene with two to three changes over a total of 10 min. Mount in permanent mounting medium and view by light microscopy.

Cells that have suffered a membrane wound within the 24 to 48 hr prior to animal sacrifice will be positively stained for serum albumin in their cytoplasm (Fig. 12.4.4B).

ALTERNATE PROTOCOL 3

ELECTRON MICROSCOPE VISUALIZATION OF WOUND TRACERS

It is often desirable to use electron microscopical (EM) localization techniques to demonstrate that the wound tracer is present in cytosol and not in a membrane-bounded cytoplasmic compartment. This resolves the issue of whether the tracer has entered directly into the cytosol after crossing the plasma membrane boundary (the localization predicted if entry occurred through a disruption) or gained access to the cell interior by an endocytotic process. In this procedure, cells that have wounded in tissues in the presence of albumin are prepared for EM as well as preliminary light microscopy. The procedure requires expertise in preparing and handling ultrathin sections.

CAUTION: Osmium tetroxide and uranyl acetate are hazardous. Wear gloves when handling. Perform steps in a fume hood to avoid vapors. Dispose of used osmium and uranyl acetate according to institutional guidelines for hazardous chemicals.

Additional Materials *(also see Alternate Protocols 1 and 2)*

70% (v/v) EM-grade ethanol
LR-White acrylic resin, hard (EM Science)
Goat anti–rat serum albumin (RSA) primary antibody (Oregon Teknika Cappel)
Biotinylated rabbit anti–goat IgG secondary antibody (Oregon Teknika Cappel)
Streptavidin-gold (10-µm particles; Auroprobe EM kit; Amersham)
Intense Silver Enhancement kit (Amersham) containing silver salt (solution A), initiator (solution B), and sodium thiosulfate solution
Immersion oil
100 mM sodium cacodylate, pH 7.4/1% (w/v) osmium tetroxide

60°C oven
Formvar-coated nickel grids (Monsanto)

Additional reagents and equipment for preparing and handling ultrathin sections and for uranyl acetate/lead citrate staining (e.g., Glauert, 1975)

Prepare sample

1. Wound tissues in the presence of albumin and perfuse/dissect tissues as described (see Alternate Protocol 1, steps 1 to 13).
2. Wash tissue in DPBS and cut into 5-mm^3 or smaller pieces.
3. Dehydrate in 70% ethanol with constant agitation for 2 hr.

Prepare thin sections

4. Transfer samples from ethanol directly into LR-White acrylic resin and incubate overnight with constant specimen agitation.

5. Polymerize at 60°C for 2 hr according to manufacturer's instructions.
6. Prepare 1-µm-thick sections according to standard ultramicrotomy methods.

Stain sections for preliminary light microscopy

7. Perform immunostaining for RSA as described (see Alternate Protocol 2, steps 5 to 7) but use an unlabeled goat anti–RSA primary antibody.

   These and all subsequent incubations and washes should be done with gentle agitation.

8. Wash thoroughly (5 min) with wash buffer and incubate 2 hr in biotinylated rabbit anti–goat IgG secondary antibody at 1:200 in wash buffer/1% sheep serum.
9. Wash thoroughly (5 min) with wash buffer and incubate 2 hr in streptavidin-gold according to manufacturer's instructions.
10. Mix silver salt (solution A) and initiator (solution B) 1:1 (v/v) immediately before use and float a gold-labeled section on a drop of this solution. Monitor the intensity of staining to determine the optimal staining time (typically 5 to 10 min). Rinse the stained section with distilled water.
11. Incubate section for 2 to 3 min in sodium thiosulfate solution to stop the reaction, and rinse again with water.
12. Dry section thoroughly, mount in immersion oil, and view by transmitted-light microscopy (see example in Fig. 12.4.5A).

Prepare and stain ultrathin sections

13. Prepare 70-nm ultrathin sections from the same block according to standard ultramicrotomy techniques and collect on a formvar-coated nickel grid.
14. Perform immunostaining as described above (steps 7 to 9). Do not use silver enhancement (step 10).
15. Incubate overnight in 100 mM sodium cacodylate, pH 7.4/1% osmium tetroxide at room temperature.
16. Stain with uranyl acetate and lead citrate and view by standard transmission EM (for example see Fig. 12.4.5B).

QUANTIFICATION OF WOUNDING FREQUENCY AND INTENSITY: IMAGE ANALYSIS

SUPPORT PROTOCOL 1

Because the details of the following procedure (Clarke et al., 1995; McNeil and Khakee, 1992; McNeil et al., 1981) are computer and software dependent, the following protocol is written in generic form and refers to common image analysis software capabilities. Universal Imaging (*http://www.image1.com*) sells appropriate software for Windows-based PCs. For Macintosh, NIH Image software can be downloaded free of charge at *http://www.rsb.info.nih.gov*.

1. Acquire a digitized 256–grey scale image of the specimen in a blinded fashion or using a predetermined pattern of image acquisition from the microscopic specimen. Acquire all images that are to be compared with one another under identical conditions with regard to magnification, specimen illumination, camera sensitivity and gain settings, and image acquisition time.
2. Import the digitized images into an image analysis program.

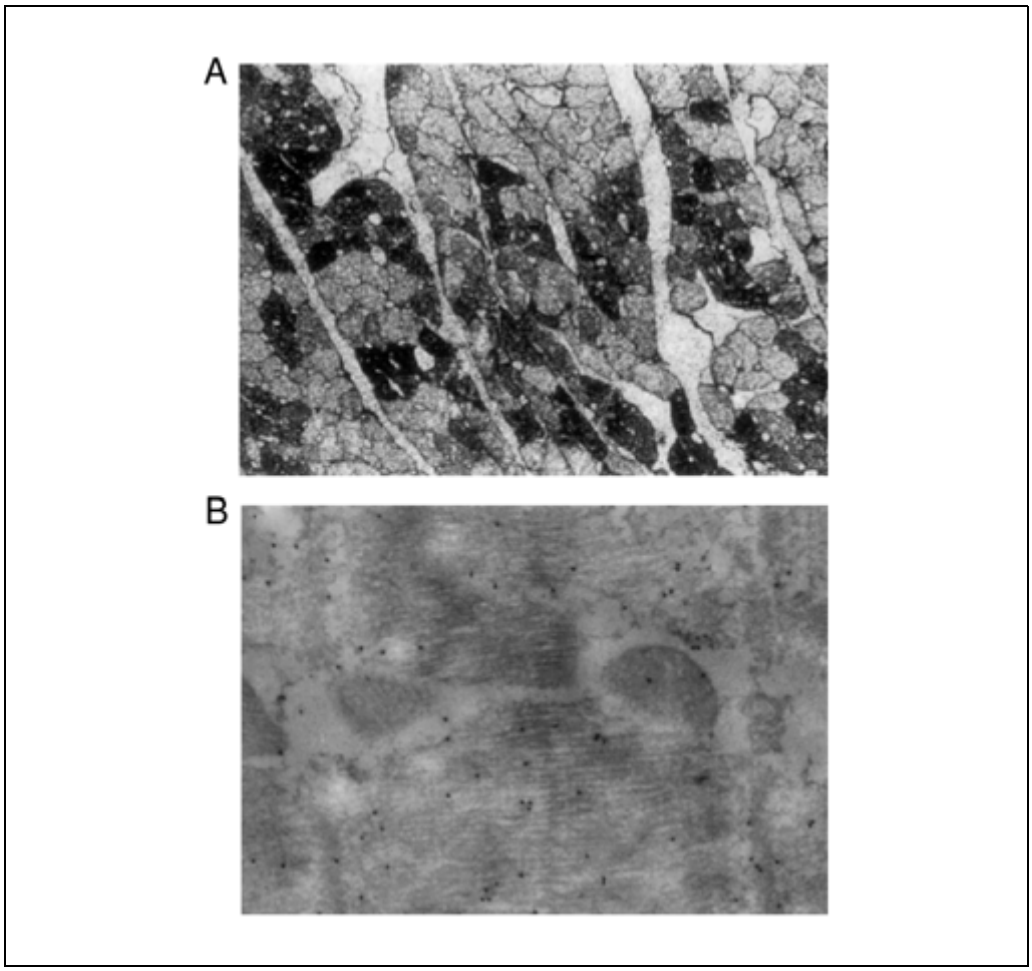


Figure 12.4.5 An example of EM localization of a wound tracer (albumin). (**A**) Light microscope image of an ~1-µm-thick plastic section of rat cardiac muscle stained with an antibody to rat serum albumin, a biotinylated secondary antibody, and streptavidin-gold according to Alternate Protocol 3. The gold is visualized by intensification with silver, resulting in dark labeling of the albumin-positive (wounded) cells. (**B**) Electron microscope image of the same specimen showing that the gold label, and therefore the wound tracer, is present in the cytosol, not in an organellar compartment. This rules out uptake of the rat serum albumin by endocytosis or through this cell's T-tubule system.

3. Utilizing the densitometry option of the software (or functional equivalent), create a measurement "window" that comfortably fits within the on-screen image of the cell or portion of a cell from which a measurement will be made.

   This can be repeated for as many cells/areas as are present on the screen. By varying the size of the measurement window to suit the cell type being studied, membrane wounding in a variety of different tissues/in vitro specimens can be analyzed using this protocol.

4. Instruct the image analysis software to measure the average pixel intensity in each measurement window, and to record the data in a file for later analysis.

5. Export these values to a spreadsheet software package.

6. Determine a control, nonwounded level of labeling by measuring the average pixel intensities of cells that clearly contain no wound tracer.

7. Calculate an average threshold value for designating wounded cells by calculating the average pixel value of the control data pool obtained in step 6.

8. Using this threshold value, calculate the percentage of wounded cells with the spread sheet.
9. To compare two different experimental conditions, plot data as a population histogram or calculate mean population intensities.

QUANTIFICATION OF WOUNDING FREQUENCY AND INTENSITY: FLOW CYTOFLUOROMETRY

SUPPORT PROTOCOL 2

This protocol is written with the assumption that the flow cytofluorometer will be operated by a skilled technician, and that the goal is to assess the degree of wounding in a cell population (Clarke and McNeil, 1994; McNeil et al., 1984). The compatibility of potential wound tracers with the available flow apparatus should be determined in advance by consultation with its operator or other local expert. This procedure requires additional reagents and equipment for trypsinization (*UNIT 1.1*) and flow cytometry (Shapiro, 1995; Robinson et al., 1999).

Prepare single-cell suspensions

1. Wound and label adherent cells (see Basic Protocol, steps 1 to 6). For controls, include cells that were exposed to the wound tracer but not to wound-inducing conditions (pinocytosis control) and wounded cells that were not exposed to the tracer (autofluorescence control).
2. Wash twice with DPBS.
3. Trypsinize adherent cells from substratum (*UNIT 1.1*) or otherwise manipulate cells or tissues to obtain a single-cell suspension.
4. Resuspend in 0.25 to 1 ml DPBS or similar saline suitable for flow equipment.

   If the cells tend to form clumps, filter through a nylon mesh (20-mm mesh Nitex, Tetko) before use.

   For most efficient flow analysis (important when paying per unit time of instrument usage), the density of the suspension should be 10^6 cells/ml or higher. Discuss this, and the exact method employed locally for filtering out cell clumps, in advance with the flow operator.

Perform flow analysis

5. Acquire 20,000 events from the autofluorescence control, measuring both forward scatter and fluorescence.

   Excitation and emission settings, filters, and so on will depend on the instrument and wound tracer used.

6. Use this data to set a low threshold for the fluorescence channel acquisition.

   Living cell and dead cell populations, as well as cell doublets, should be discernible from the forward scatter data. If not, adjust instrument accordingly.

7. Acquire 20,000 events from the experimental population. Use this data to adjust the high end of parameters such as gain and laser intensities so that the entire range of fluorescence intensities in the population is captured.

   Fluorescence intensities may range over several log values.

8. Acquire 20,000 events from the pinocytosis control population. Use the fluorescence profile of this population to set a threshold allowing discrimination between this population and that subset of the experimental population whose fluorescence intensities fall above this threshold.

9. Calculate the percentage of cells in the experimental population that are above the level derived from the pinocytosis control and display the distribution of fluorescent cells within this population.

This displays wound-induced uptake of the tracer.

COMMENTARY

Background Information

Why is survivable plasma membrane disruption, referred to here as cell wounding, of interest to the cell and molecular biologist? There are number of reasons. First, such disruptions provide a route out of cytosol for numerous impermeant molecules that are not capable of being exported by the classical secretory pathway but nevertheless are present outside cells and/or have an extracellular function that can only be understood in terms of a release mechanism (Smallheiser, 1996). An example is basic fibroblast growth factor (bFGF). This polypeptide lacks a classical signal sequence for import into the endoplasmic reticulum, but is a potent extracellular cell growth–promoting signal. One route of release of bFGF is through disruptions. Characterization of cell wounding using the methods described here has demonstrated how this mode of release may have both physiological and pathological relevance in vivo (McNeil, 1993; McNeil and Steinhardt, 1997).

Second, disruptions provide a route into the cytosol for potentially potent second messengers, such as Ca^{2+}. When a cell suffers a plasma membrane disruption, Ca^{2+} can enter down a 10,000-fold concentration gradient until resealing intervenes. This entry of a potent, broadly acting intracellular second messenger might have profound consequences on subsequent cell functioning, both in the short term through the activation of Ca^{2+}-sensitive proteins and in the long-term through changes in gene expression. For example, Ca^{2+} entry is now known to evoke massive intracellular vesicle-vesicle fusion (Terasaki et al., 1997) and vesicle transport to and docking and fusion with the plasma membrane (Bi et al., 1995; Miyake and McNeil, 1995; Steinhardt et al., 1994). Ca^{2+} entry also may evoke *cfos* and other gene expression events (Grembowicz et al., 1999).

Third, plasma membrane disruptions can be of considerable practical utility to the cell and molecular biologist. They provide a route of entry for otherwise impermeant macromolecules that an experimenter might wish to introduce into cytosol. Enzymes, antibodies, oligonucleotides, and expression vectors are all examples of macromolecules that can be loaded into living cells through disruptions (McNeil, 1989; McNeil et al., 1981). Skeletal muscle cells can be transfected with naked DNA simply by injecting it through a syringe needle into a muscle (Wolff et al., 1990). It is clear from studies using the methods described in this unit that the injected DNA enters muscle cells through plasma membrane disruptions, which are prominently induced by the needle puncture.

Critical Parameters

Certain artifacts must be avoided. First, artifactual membrane wounding induced by animal handling must be kept to a minimum prior to removal of wound tracers and fixation. For example, early studies indicated that relatively low levels of mechanical force applied to skin caused significant membrane wounding of the epithelial cells (McNeil and Ito, 1990). Second, as membrane wound quantification relies on densitometry, section thickness is extremely important. The authors routinely mount two or more specimens for quantitative comparisons on the same stub to ensure equivalency of section thickness. However, any such potential problems associated with differing section thickness can be avoided if the experimenter has access to a confocal microscope, which allows the volumetric quantification of fluorescent wound tracer within the specimen in optical sections of identical thickness.

For additional critical parameters related to immunofluorescence and immunoperoxidase staining procedures, see *UNITS 4.3 & 4.6*, respectively.

Troubleshooting

Poor staining of wounded cells in positive control experiments could be due to insufficient wound tracer. A higher concentration of the wound tracer should be used, and its access to the extracellular environment of interest verified. Artificially high labeling in negative control experiments not subject to mechanical stress prior to fixation is most likely due to probe entry during and/or after cell fixation. The cells/tissue must be more thoroughly

washed to remove all exogenous tracer prior to fixation.

Anticipated Results

In all mechanically active tissues/culture environments tested by the authors, evidence of plasma membrane disruption events has been obtained. However, the percentage of cells affected and the range of labeling intensities varies tremendously (<1% up to 80%). It is therefore impossible to generalize as to expected results.

Time Considerations

Most of these protocols can be completed in a day or less. Exceptions are the EM immunostaining and image analysis quantitation protocols, which may take several days to complete.

Literature Cited

Bi, G-.Q., Alderton, J.M., and Steinhardt, R.A. 1995. Calcium-regulated exocytosis is required for cell membrane resealing. *J. Cell Biol.* 131:1747-1758.

Chen, W.T. 1981. Mechanism of retraction of the trailing edge during fibroblast movement. *J. Cell Biol.* 90:187-200.

Clarke, M.S.F. and McNeil, P.L. 1994. Syringe loading: A method for inserting macromolecules into cells in suspension. *In* Cell Biology: A Laboratory Handbook, Vol. 3. (J.E. Celis, ed.) pp. 30-36. Academic Press, San Diego.

Clarke, M.S.F., Caldwell, R.W., Miyake, K., and McNeil, P.L. 1995. Contraction-induced cell wounding and release of fibroblast growth factor in heart. *Circ. Res.* 76:927-934.

Glauert, A.M. 1975. Fixation, dehydration and embedding of biological specimens. *In* Practical Methods in Electron Microscopy, Vol. 3 (A.M. Glauert, ed.). Elsevier Scientific Publishing, New York.

Grembowicz, K.P., Sprague, D., and McNeil, P.L. 1999. Temporary disruption of the plasma membrane is required for c-fos expression in response to mechanical stress. *Mol. Biol. Cell.* In press.

McNeil, P.L. 1989. Incorporation of macromolecules into living cells. *Methods Cell Biol.* 29:153-173.

McNeil, P.L. 1993. Cellular and molecular adaptations to injurious mechanical force. *Trends Cell Biol.* 3:302-307.

McNeil, P.L. and Ito, S. 1990. Molecular traffic through plasma membrane disruptiuons of cells in vivo. *J. Cell Sci.* 96:549-556.

McNeil, P.L. and Khakee, R. 1992. Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. *Am. J. Pathol.* 140:1097-1109.

McNeil, P.L. and Steinhardt, R.A. 1997. Loss, restoration and maintenance of plasma membrane integrity. *J. Cell. Biol.* 137:1-4.

McNeil, P.L., Hohman, T.C., and Muscatine, L. 1981. Mechanisms of nutritive endocytosis. II. The effect of charged agents on phagocytic recognition by digestive cells. *J. Cell Sci.* 52:243-269.

McNeil, P.L., Murphy, R.F., Lanni, F., and Taylor, D.L. 1984. A method for incorporating macromolecules into adherent cells. *J. Cell Biol.* 98:1556-1564.

Miyake, K. and McNeil, P.L. 1995. Vesicle accumulation and exocytosis at sites of plasma membrane disruption. *J. Cell Biol.* 131:1737-1745.

Robinson, J.P., Darzynkiewicz, Z., Dean, P.N., Orfao, A., Rabinovitch, P.S., Stewart, C.C., Tanke, H.J., and Wheeless, L.L. 1999. Current Protocols in Cytometry. John Wiley & Sons, New York.

Shapiro, H.M. 1995. *Practical Flow Cytometry*, 3rd ed. Wiley-Liss, New York.

Smallheiser, N.R. 1996. Proteins in unexpected locations. *Mol. Biol. Cell* 7:1003-1014.

Steinhardt, R.A., Bi, G., and Alderton, J.M. 1994. Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. *Science* 263:390-393.

Swanson, J.A. and McNeil, P.L. 1987. Nuclear reassembly excludes large macromolecules. *Science* 238:548-550.

Terasaki, M., Miyake, K., and McNeil, P.L. 1997. Large plasma membrane disruptions are rapidly resealed by Ca^{2+}-dependent vesicle-vesicle fusion events. *J. Cell Biol.* 139:63-74.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P.L. 1990. Direct gene transfer into mouse muscle in vivo. *Science* 247:1465-1468.

Contributed by Paul L. McNeil
Medical College of Georgia
Augusta, Georgia

Mark F.S. Clarke
Universities Space Research Associates
Houston, Texas

Katsuya Miyake
Fukushima Medical University
Fukushima, Japan

Dictyostelium Cell Dynamics

Dictyostelium discoideum is a powerful, genetically accessible system that can be used to study fundamental cellular processes, including cytokinesis, motility, phagocytosis, chemotaxis, signal transduction, and aspects of development such as cell sorting, pattern formation, and cell-type determination (Maeda et al., 1997; Daunderer et al., 1999; Kay and Williams, 1999). *D. discoideum* amoebae grow as separate, independent cells but, upon starvation, interact to form multicellular structures (see Commentary). Among the great strengths of this model system is the capacity to track the dynamic behaviors of individual cells. Most importantly, phenotypic complementation of null mutants with green fluorescent protein (GFP) fusion proteins is providing an extremely useful tool for cell biology.

This unit describes how to grow and image *D. discoideum* amoebae and focuses primarily on cell motility and chemotaxis processes. Specific step-by-step procedures are described for imaging GFP-labeled proteins in live amoebae (see Basic Protocol 1), aggregation stream and mound preparations (see Basic Protocol 2), and slug preparations (see Basic Protocol 3). Alternate Protocols 1 and 2 describe the imaging of GFP-labeled proteins in the presence of a chemoattractant. Support Protocols 1 and 2 give an in-depth description of how the cells are cultured and maintained. Support Protocol 3 describes the transformation of *D. discoideum* with a GFP fusion plasmid. Support Protocols 4 and 5 describe two different ways to characterize the functionality of the fusion protein generated by the complementation of null mutants or by overexpression in wild-type backgrounds.

IMAGING GFP-LABELED PROTEINS IN LIVE SINGLE CELLS

BASIC PROTOCOL 1

Once the functionality of a GFP fusion protein expressed in *Dictyostelium* amoebae is established (see Support Protocols 4 and 5), studying its cellular localization can be performed by examining single cells (as described here) or by analyzing the behavior of cells as they come together to form aggregates or mounds (see Basic Protocol 2) and slugs (see Basic Protocol 3). The methods described in this protocol are divided in two sections. First, a brief overview of the imaging arrangement is presented. Second, a detailed step-by-step description of how samples are prepared for live microscopy is provided.

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) expressing desired green fluorescent protein (GFP) fusion protein
Phosphate buffer (PB; see recipe)
Developmental buffer (DB; see recipe)
Stock solution of adenosine 3′,5′-cyclic monophosphate (cAMP, sodium salt) to give appropriate final concentration, for developed cells only

Inverted microscope equipped with a charge-coupled device (CCD) camera, mercury light source, and appropriate filter sets for GFP imaging
Computer with either IPLab-Spectrum (Scanalytics) or Openlab (Improvision) acquisition software
Chambered coverglass (e.g., Lab-Tek II, Nalge Nunc International)
Brass holder designed to fit in a standard mechanical stage holder
Peristaltic pump connected to timer (for developed cells only)

Additional materials and reagents for growing *D. discoideum* cells (see Support Protocols 1 and 2) and for counting cells (*UNIT 1.1*)

Contributed by Carole A. Parent
Current Protocols in Cell Biology (2001) 12.5.1-12.5.19

Set up imaging hardware

1. Set up an inverted microscope and its associated hardware.

   The CCD camera must be capable of acquiring images rapidly and with high sensitivity. Good examples of this type are the Photometrics PXL (Roper Scientific) and the Hamamatsu Orca (Hamamatsu Photonic Systems) cameras.

   For double-labeling experiments, a filter wheel is required to ensure rapid changes in the filters while the cells are moving.

2. Set up a computer with IPLab-Spectrum or Openlab acquisition software to control image acquisition.

   These software programs handle image acquisition, processing, measurement, and analysis for a variety of applications. Since the amoebae are light sensitive, the exposure time and the intensity of the fluorescence excitation light should be kept at a minimum using neutral-density and UV filters and shutters. Of course, the exposure time will be highly dependent on the level of expression of the GFP fusion protein, and will therefore have to be empirically determined.

3. Mount the chambered coverglass on a standard stage holder.

   Alternatively, a 35-mm coverslip can be positioned on an Attofluor cell chamber (Molecular Probes) and directly mounted in a standard 35-mm-diameter stage holder.

Visualize cells

For growing cells:

4a. Grow transformed *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) or on bacterial lawns (see Support Protocol 2).

5a. Count cells (*UNIT 1.1*), dilute in PB to ~1×10^6 cells/ml, and deposit 4 µl (4000 cells) onto a mounted chambered coverglass.

6a. Let cells adhere 5 min.

7a. Gently add PB to cover the surface of the coverglass, and examine for GFP.

For developed cells:

4b. Grow transformed *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) and dispense 50-ml aliquots into conical tubes.

5b. Centrifuge 4 min at 1500 × *g*, 22°C, and wash cells in DB.

6b. Centrifuge again as in step 5b. Pool and count cells (*UNIT 1.1*), and resuspend in a flask at 2×10^7 cells/ml in DB.

7b. Shake 1 hr at 100 rpm at 22°C.

8b. To optimize development, while shaking at 100 rpm for 4 to 5 hr add cAMP stock solution at a final concentration of 75 nM to the cells every 6 min using a peristaltic pump connected to a timer (see Commentary).

9b. Remove 1 ml cells, centrifuge 4 min at 1500 × *g*, 22°C, and resuspend in 1 ml PB.

10b. Vortex to break up cell aggregates.

11b. Dilute 20 fold in PB and deposit 4 µl onto a mounted chambered coverglass.

12b. Let cells adhere 5 min.

13b. Gently add PB to cover the surface of the coverglass, and examine for GFP.

IMAGING GFP-LABELED PROTEINS FOLLOWING A UNIFORM INCREASE IN CHEMOATTRACTANT

ALTERNATE PROTOCOL 1

The distribution of the GFP fusion protein can be studied under basal, nonstimulated conditions where processes such as cytokinesis and phagocytosis (for growth stage cells) as well as random motility can be measured. In addition, the effect of receptor stimulation on the localization of the labeled protein can be easily assessed on developed cells. This can be performed in two ways: in the presence of a uniform concentration of chemoattractant (Figure 12.5.1A) or a chemoattractant gradient (Figure 12.5.1B,C; see Alternate Protocol 2).

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) expressing desired green fluorescent protein (GFP) fusion protein
Developmental buffer (DB; see recipe)
Adenosine 3′,5′-cyclic monophosphate (cAMP, sodium salt) to give appropriate final concentration
Phosphate buffer (PB; see recipe)

Inverted microscope equipped with a charge-coupled device (CCD) camera, mercury light source, and appropriate filter sets for GFP imaging
Computer with either IPLab-Spectrum (Scanalytics) or Openlab (Improvision) acquisition software
Eight-chambered coverglass (Lab-Tek II, Nalge Nunc International)
Peristaltic pump connected to timer

Additional materials and reagents for growing *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) and for counting cells (*UNIT 1.1*)

Set up imaging hardware

1. Set up an inverted microscope and its associated hardware.

   The CCD camera must be capable of acquiring images rapidly and with high sensitivity. Good examples of this type are the Photometrics PXL (Roper Scientific) and the Hamamatsu Orca (Hamamatsu Photonic Systems) cameras.

   For double-labeling experiments, a filter wheel is required to ensure rapid changes in the filters while the cells are moving.

2. Set up a computer with IPLab-Spectrum or Openlab acquisition software to control image acquisition.

   These software programs handle image acquisition, processing, measurement, and analysis for a variety of applications. Since the amoebae are light sensitive, the exposure time and the intensity of the fluorescence excitation light should be kept at a minimum using neutral-density and UV filters and shutters. Of course, the exposure time will be highly dependent on the level of expression of the GFP fusion protein, and will therefore have to be empiricallly determined.

3. Mount an eight-chambered coverglass on a standard stage holder.

Prepare developed D. discoideum cells

4. Grow transformed *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) and dispense 50-ml aliquots into conical tubes.

5. Centrifuge 4 min at 1500 × *g*, 22°C, and wash cells in DB.

6. Centrifuge again, pool and count cells (*UNIT 1.1*), and resuspend in a flask at 2×10^7 cells/ml in DB.

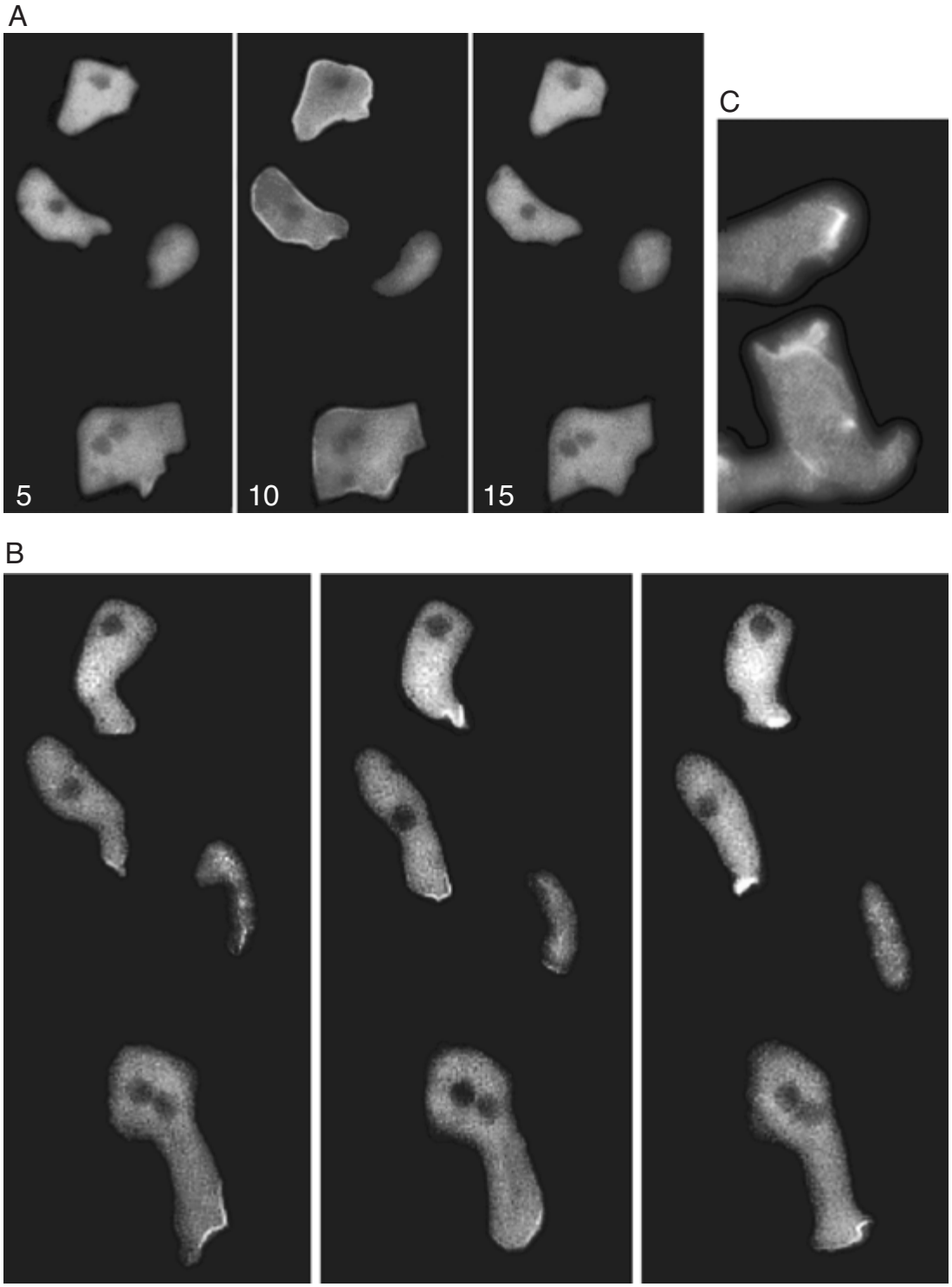


Figure 12.5.1 Translocation of pleckstrin homology (PH) domain–containing proteins to the plasma membrane at the leading edge of chemotaxing *D. discoideum* cells. Endogenous cytosolic regulator of adenylyl cyclase (CRAC), a PH domain–containing protein of *D. discoideum* amoebae, was replaced with a CRAC-GFP fusion protein. Transformants were grown, starved in the presence of pulses of cAMP, and processed for microscopy analysis. (**A**) Chemoattractant was applied uniformly by increasing the pressure on the microinjector (this can also be done by adding a bolus of attractant to the chamber). The cells respond with uniform translocation of CRAC to the plasma membrane. The colors were obtained by merging the 5-sec frame (in blue) with the 10- and 15-sec frames (in yellow). The association of CRAC-GFP appears in yellow. (*Legend continues on next page.*)

7. Shake 1 hr at 100 rpm.
8. To optimize development, while shaking at 100 rpm for 4 to 5 hr, add cAMP to a final concentration of 75 nM to the cells every 6 min using a peristaltic pump connected to a timer (see Commentary).
9. Remove 1 ml cells, centrifuge 4 min at 1500 × *g*, 22°C, and resuspend in 1 ml PB.
10. Vortex to break up cell aggregates.
11. Dilute 20 fold in PB and deposit 4 µl into each chamber of the eight-chambered coverglass.
12. Let cells adhere 5 min and gently add 300 µl PB to each well.

Acquire GFP images

13. Mount coverglass on the inverted microscope and set the acquisition software to time-lapse mode.

    D. discoideum amoebae have a diameter of ~10 to 20 µm. Using 63× or 100× oil-immersion objectives will give the best results, increasing both spatial resolution and brightness. The exact time frame to be chosen will depend on the protein of interest.

14. Start the acquisition process and record baseline images from a chamber for about 4 to 5 frames.
15. Between two frames, delicately add 30 µl of cAMP stock solution to the chamber.

    This will give a 10× dilution factor. Prepare stock solutions of cAMP to give final concentrations ranging from 10^{-9} to 10^{-5} M.

16. Finish data acquisition.

    The length of the acquisition process will depend on the protein studied.

17. Move to a different chamber and repeat steps 14 to 16, using a different concentration of cAMP.

    A dose-response curve with cAMP concentrations ranging from 10^{-9} to 10^{-5} M should be performed.

IMAGING GFP-LABELED PROTEINS IN A CHEMOATTRACTANT GRADIENT

ALTERNATE PROTOCOL 2

The spatial localization of the GFP fusion protein in chemotaxing cells can be assessed by observing cells exposed to a chemoattractant gradient (Parent and Devreotes, 1999). This is achieved using a micropipet to deliver a constant amount of attractant on the coverglass (Figure 12.5.1B,C).

Figure 12.5.1 *(continued from previous page)* (**B**) These three images are from the same series as in (A). The gradient of cAMP was restored, and a polarized CRAC-GFP signal was observed as the cells moved up the gradient (from low concentrations at the top of the frame to high concentrations at the bottom). The micropipet was located just outside the bottom of the frames. In both (A) and (B), the numbers in the bottom left corner are seconds after the start of the experiment. (**C**) Wild-type amoebae expressing the PH domain of PKB (protein kinase B or Akt) linked to GFP were placed in a gradient of chemoattractant. As the cells were moving up the gradient, the fluorescent signal was observed at the front of the cells. The micropipet was located at the top right corner of the frame. Reprinted from Parent and Devreotes (1999) with permission from the American Association for the Advancement of Science. **See color plate.**

Additional Materials *(see Basic Protocol 1 and Alternate Protocol 1)*

10^{-5} to 10^{-6} M adenosine 3′,5′-cyclic monophosphate (cAMP, sodium salt)
One-chambered coverglass (Lab-Tek II, Nalge Nunc International) or Attofluor cell chamber (Molecular Probes) fitted with a 35-mm coverslip
Micropipet consisting of pulled glass capillary with an opening of ~0.5 µm (e.g., Femtotips, Eppendorf Scientific)
Microinjector (e.g., Eppendorf Scientific)
Additional reagents and equipment for imaging GFP-labeled proteins following a uniform increase in chemoattractant (see Alternate Protocol 1)

NOTE: A Microloader (Eppendorf Scientific) can be used to load the micropipet.

1. Set up an inverted microscope and prepare developed transformed *D. discoideum* cells as described (see Alternate Protocol 1, steps 1 to 10).
2. Dilute cells 20 fold in PB and deposit 4 µl onto a one-chambered coverglass or on an Attofluor cell chamber fitted with a 35-mm coverslip.
3. Let cells adhere 5 min and gently add ~2 ml PB.
4. Mount coverglass or cell chamber on the inverted microscope.
5. Load a micropipet with 10^{-5} to 10^{-6} M cAMP and secure it in a microinjector.

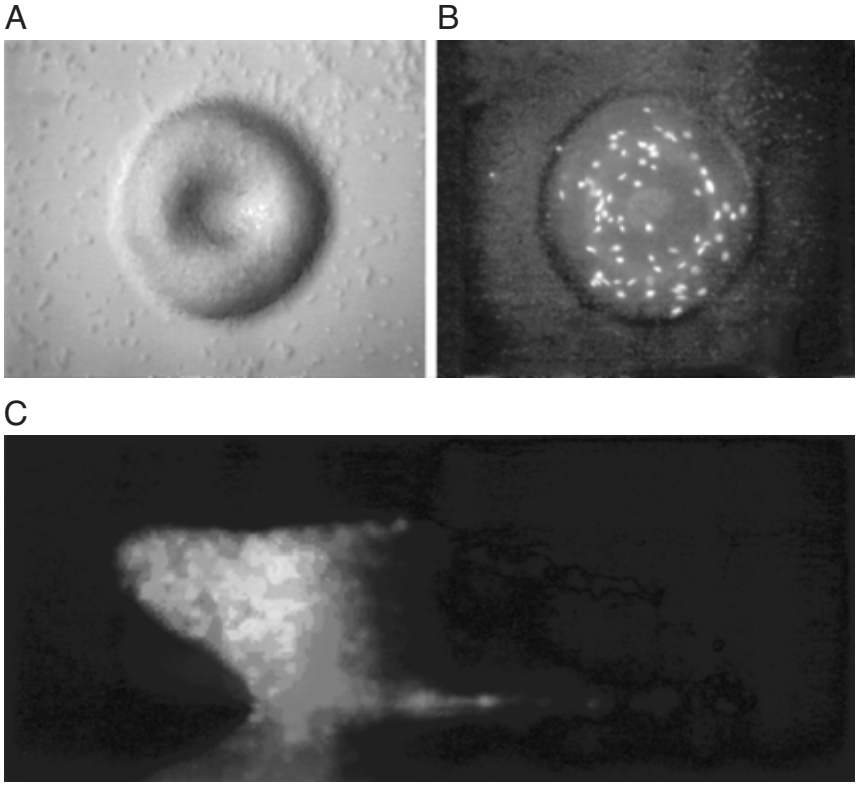


Figure 12.5.2 Visualizing GFP-labeled cells in *D. discoideum* mounds and slugs. (**A**) Bright field and (**B**) fluorescence images of a mound containing 0.1% cells expressing GFP under the actin 15 promoter, viewed at 10× magnification. (**C**) Merged image of a slug co-expressing wild-type GFP under the ecmA promoter and the red-shifted S65T GFP mutant under the ecmO promoter. The slug is viewed from the side at 10× magnification as described (see Alternate Protocol 2). Separate images were taken <0.5 sec apart using excitation at 400 and 485 nm, respectively, to show the wild-type (in green) and red-shifted (in red) GFP. These images were then merged to give the image shown. Regions that express both forms of GFP appear in yellow. Figure and methods generously provided by D. Dormann and C.J. Weijer (Dormann et al., 1996). **See color plate.**

6. Using phase optics and a 40× oil-immersion objective, carefully position the micropipet in the vicinity of a group of cells.

   A 40× objective is used to view larger field of cells.

   Because the cells move rapidly towards cAMP (~15 ìm/min), the micropipet should be positioned far enough from them so the cells do not aggregate on the micropipet and clog it.

7. Adjust the microinjector to manual mode with a compensation pressure of ~20 hPa.

   The gradient generated can be assessed by placing a diluted solution of rhodamine (which has a molecular weight similar to cAMP) in the micropipet and observing the fluorescence signal after the gradient reaches steady state (Parent et al., 1998).

8. Set the acquisition software to time-lapse mode at one frame every 15 sec and begin data acquisition.

   Acquisition can be performed for as long as desired.

   The distribution of the fusion protein can be observed while the gradient is re-established if the micropipet is repositioned while the cells are chemotaxing.

IMAGING GFP-LABELED PROTEINS IN AGGREGATION STREAM AND MOUND PREPARATIONS

BASIC PROTOCOL 2

GFP-labeled cells can be used to study the patterns of cell movement during multicellular development in *D. discoideum*. This is achieved by mixing nonexpressing cells with cells expressing GFP under a constitutive promoter. Alternatively, GFP (and/or a color variant of GFP) can be fused to cell-specific promoters, thereby allowing the visualization of different cell types in a live organism. This technique not only allows the observation of wild-type organisms, but it can also be used to precisely study the developmental defects of mutants.

This protocol describes the procedures used to visualize aggregation streams and mounds. The aggregation streams are formed in early development as the cells are chemotaxing toward centers. Visualization of these streams is best observed at higher magnification. For the visualization of mounds and later structures, lower magnification is preferred (Fig. 12.5.2, panels A and B; also see Rietdorf et al., 1996).

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) grown on non-nutrient agar (see Support Protocol 5)
Silicone oil (e.g., Dow Corning 200/20cs, BDH Chemicals)

Incubator, 22°C
Attofluor cell chamber (Molecular Probes) fitted with a 35-mm coverslip (optional)
Additional reagents and equipment for imaging GFP-labeled proteins (see Basic Protocol 1)

1. Set up an inverted microscope and its associated hardware as described (see Basic Protocol 1, steps 1 to 2).

2. Place transformed *D. discoideum* cells grown on non-nutrient agar in an incubator at 22°C until aggregation streams or mounds have formed (~6 to 9 hr).

3. Directly mount the 35-mm petri dish containing the *D. discoideum* cells on the inverted microscope and observe with a 10× objective.

   The agar layer is thin enough to allow imaging through the agar with a 10× objective.

4. *For observation at higher magnifications (20×, 40×, and 63×):* Excise a small piece of agar containing aggregation streams or mounds with a scalpel and deposit the agar piece on its side onto a one-chambered coverglass or an Attofluor cell chamber fitted with a 35-mm coverslip.
5. Carefully pick up the agar piece, invert it, and lower it with the cells facing down onto the surface of the coverglass such that the mounds will be squashed against the glass.
6. Fill the chamber with silicone oil to prevent the agar from drying.
7. Mount the chamber on the microscope and start data acquisition.

BASIC PROTOCOL 3

GFP-LABELED PROTEINS IN SLUG PREPARATIONS

This protocol includes methods used to visualize structures appearing in later development such as slugs. Here again, optimal visualization will be obtained under lower magnification conditions.

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) grown to log phase in liquid medium (see Support Protocol 1)
35-mm petri dish containing 0.5 ml of 1% (w/v) aqueous Bacto Agar (Difco)
Silicone oil (e.g., Dow Corning 200/20cs, BDH Chemicals)

Incubator, 22°C
Small rubber ring (1 mm high and 5 mm in diameter)

Additional reagents and equipment for imaging GFP-labeled proteins (see Basic Protocol 1)

Prepare D. discoideum slugs

1. Set up an inverted microscope and its associated hardware as described (see Basic Protocol 1, steps 1 to 2).
2. Dispense transformed *D. discoideum* cells, grown to log phase in liquid medium, in 50-ml aliquots into sterile conical tubes.
3. Centrifuge 4 min at 1500 × *g*, 22°C, and wash cells in DB.
4. Centrifuge as in step 3 and wash cells in water.
5. Centrifuge again, count cells (*UNIT 1.1*), and resuspend at 10^8 cells/ml in water.
6. Spot 10 µl cells on a 35-mm petri dish containing 0.5 ml of 1% aqueous Bacto Agar.
7. After 30 min, tilt the plate and blot excess fluid.

   The 30-min period will allow cells to adhere.
8. Place plate in the dark in an incubator at 22°C until slugs are formed (~16 to 36 hr).
9. Place a small rubber ring around a slug and carefully push the ring against the agar surface.

   This eliminates light scattering at the slug's surface.
10. Fill ring with a few drops of silicone oil to cover the slug.

View D. discoideum slugs

11. Directly mount the 35-mm petri dish on the inverted microscope and observe with a 10× objective.

    The slug can be viewed through the agar.

12a. *To view the slug from its side:* Carefully cut an agar piece with a slug on it, turn agar 90° around its long axis (relative to the long axis of the slug), and place it on a one-chambered coverglass. Immediately fill chamber with silicone oil and mount on the microscope.

12b. *To view at higher magnification:* Carefully cut an agar piece with a slug on it, invert agar 180°, and deposit it on a one-chambered coverglass, slug-side down. Fill chamber partly with silicone oil and mount on the microscope.

The agar should be cut right next to the slug tip. If the tip protrudes between the edge of the agar and coverglass, the slug usually continues to migrate.

13. Start data acquisition.

GROWING *D. DISCOIDEUM* AXENICALLY

SUPPORT PROTOCOL 1

The availability of axenic strains (i.e., strains that can grow in liquid cultures) renders growth of *D. discoideum* amoebae in the laboratory very simple (Sussman, 1987). The most common wild-type strains used are Ax2 and Ax3. The standard growth medium, HL5, is easily made in the laboratory. Over 10^{11} clonal amoebae can be grown and harvested in a few days without sophisticated equipment. The optimal growth temperature for the amoebae is 22°C; the cells will die at temperatures above 29°C. Amoebae are handled aseptically and grown in temperature-controlled incubators. Cells can be harvested at growth stage or at any developmental stage. In the early stages of development, the genetically identical cells differentiate synchronously and the population remains homogenous (Sussman, 1987). This allows biochemical analyses to be performed using a variety of physiologically relevant conditions. Amoebae can also easily and rapidly be grown on a lawn of bacteria (see Support Protocol 2). *D. discoideum* amoebae can readily be stored under liquid nitrogen as amoebae in the presence of glycerol or dimethyl sulfoxide. Alternatively, spores can be stored virtually indefinitely with silica gel at 4°C (Sussman, 1987).

Materials

D. discoideum cells (ATCC)
HL5 medium (see recipe)
Phosphate buffer (PB, see recipe)

100-mm tissue culture plate
250-ml tissue culture flask
Incubator, 22°C, with shaker

1. Aseptically grow *D. discoideum* cells to confluence in 12 ml HL5 medium in a 100-mm tissue culture plate in an incubator at 22°C.

The procedure can be scaled up as needed by increasing the number of plates used.

2. Using a 10-ml sterile pipet, transfer cells from the plate to a 250 ml tissue culture flask containing 100 ml HL5 medium.

This procedure can be scaled up or down depending on the need. A flask/medium volume ratio of ~2.5 is preferred for good aeration.

3. Shake at 200 rpm in the incubator at 22°C until log phase is reached.

Under these conditions, cells double every 10 to 12 hr and reach log-phase densities of 5 × 10^6 cells/ml in ~2 days.

4. Collect log-phase cells as needed by centrifuging 4 min at 1500 × *g*, 22°C.

5. Passage the mass culture plates every 3 to 5 days (split ratio of ~1:5) in HL5 medium (*UNIT 1.1*).

   These cultures can usually be kept for ~2 months.

SUPPORT PROTOCOL 2

GROWING *D. DISCOIDEUM* ON BACTERIAL LAWNS

Alternatively, *D. discoideum* can be grown on bacterial lawns (Sussman, 1987). This type of growth is used when studying phagocytosis.

Materials

Klebsiella aerogenes (ATCC)
HL5 medium (see recipe) without dihydrostreptomycin
D. discoideum amoebae (ATCC)
100-mm SM plates (see recipe)
Phosphate buffer (PB; see recipe)
Incubator, 22°C

1. Grow a dense culture of *Klebsiella aerogenes* in HL5 medium without dihydrostreptomycin overnight at room temperature with shaking at 250 rpm.

   Start cells from a single colony.

2. Mix ~10^6 *D. discoideum* amoebae with 200 µl grown bacterial culture and plate on a 100-mm SM plate.

   Use log-phase-grown amoebae.

   The procedure can be scaled up as needed by increasing the number of plates used.

3. Store plate in a humidified environment in an incubator at 22°C.

   Placing the plates in a closed plastic box in the incubator will provide an appropriately humid environment. Within 36 to 48 hr, the bacterial lawn will be cleared by the growing cells and ~10^9 amoebae can be recovered per 100-mm plate. It is important to recover the amoebae before they eat up the bacteria and enter the development program.

4. Add ~6 ml PB to the plate, scrape the layer of cells from the plate, and transfer to a 50-ml conical tube.

5. Repeat step 4 three times.

6. Centrifuge 1 min at 1000 × *g*, 22°C. Discard supernatant.

   This supernatant should remain turbid from the remaining bacteria.

7. Resuspend pellet in 50 ml PB.

8. Repeat steps 6 to 7 until supernatant is clear (usually three times).

9. Resuspend pellet as required for subsequent analysis.

SUPPORT PROTOCOL 3

PLASMID CONSTRUCTION AND TRANSFORMATION

Transformed lines of *D. discoideum* amoebae can be established using either integrating or episomal vectors (Nellen and Firtel, 1985; Manstein et al., 1995). Several selectable markers and promoters active during the developmental program have been well characterized (Egelhoff et al., 1991; Kay and Williams, 1999; Pang et al., 1999). Episomal vectors carrying Ddp1 sequence, which can amplify up to 100 copies per cell, and the actin 15 promoter/2H3 terminator expression cassette are used to establish cell lines expressing GFP fusion proteins (Hughes et al., 1994; Parent and Devreotes, 1996a,b).

This cassette gives rise to high constitutive levels of protein expression, and the extrachromosomal vector leads to little or no variation in protein expression between clones. Since the *D. discoideum* genome is ~70% A+T, a *recA* bacterial stain should be used to propagate the plasmid. In order for the gene to be translated, a ribosome binding site (RBS) must precede the ATG of the gene to be expressed. The 9-nucleotide RBS sequence, TTATAAAAA, has been routinely used for the constitutive expression of a variety of genes. The position of the GFP label within the protein will vary depending on the protein of interest. For information on plasmid construction, see *APPENDIX 3A*.

The enhanced version of GFP (eGFP; Clontech Laboratories), which harbors the F64L and S65T mutations and fluoresces about 35 times more intensely than wild-type GFP, has been used successfully in *D. discoideum*. This is the so-called red-shifted GFP because its maximal excitation peak is 490 nm compared to 395 nm for the wild-type GFP. Moreover, with the availability of eGFP variants in different colors (blue, cyan, green, and yellow; Clontech Laboratories), it is easy to perform double-and triple-labeling experiments.

When studying the distribution of a *D. discoideum* protein whose deletion by homologous recombination gives rise to a phenotype, gene replacement becomes a powerful tool to assess the functionality of the GFP fusion protein (see Commentary). Transformation of *D. discoideum* amoebae is accomplished by electroporation using supercoiled DNA preparations (Howard et al., 1988). The plasmid is introduced in both the wild-type and the appropriate null cells. Typically, transformants will appear 2 weeks after addition of G418. The cells should be kept under selection at all times during growth when episomal vectors are being used.

Materials

D. discoideum cells grown to log phase in liquid medium (see Support Protocol 1)
Electroporation buffer (EB; see recipe), ice cold
Supercoiled DNA vector encoding a GFP fusion protein
100-mm SM plate (see recipe)
Healing solution (see recipe)
HL5 medium (see recipe)
20 mg/ml (w/v) G418 solution (see recipe)
Klebsiella aerogenes prepared for bacterial lawns (see Support Protocol 2)

Electroporation cuvettes (0.2-cm electrode gap; e.g., Bio-Rad), ice cold
Electroporator (e.g., Gene Pulser II, Bio-Rad)
Incubator, 22°C, with shaker
24-well plates

Additional reagents and equipment for counting cells (*UNIT 1.1*), immunoblotting (see Support Protocol 4 and *UNIT 6.2*), and microscopic visualization (see Basic Protocols 1, 2, and 3; see Alternate Protocols 1 and 2)

NOTE: Unless otherwise mentioned, all manipulations are performed aseptically on ice.

Prepare cells

1. Dispense *D. discoideum* cells, grown to log phase in liquid medium, in 50-ml aliquots into sterile 50-ml conical tubes.
2. Centrifuge 4 min at 1500 × *g*, 22°C, count cells (*UNIT 1.1*), and resuspend at 4×10^7 cells/ml in ice-cold EB.
3. Remove 400 µl cells and mix in a sterile microcentrifuge tube with 5 to 10 µg supercoiled DNA vector encoding a GFP fusion protein.

   A vector containing GFP alone should be used as a control.

4. Incubate tube 4 min on ice.

Transform cells

5. Transfer cells to ice-cold electroporation cuvettes.
6. Place cuvettes in an electroporator and electroporate at 1.1 to 1.2 kV and 3 µF using a 5-Ω resistor in series with the chamber.

   A τ value of 0.5 to 0.7 msec should be obtained.

7. Move cuvette to ice and incubate 10 min.
8. Transfer electroporated cells to a 100-mm tissue culture plate using a sterile Pasteur pipet.
9. Add 2 µl healing solution and gently mix.
10. Incubate 15 min at room temperature.
11. Add 12 ml HL5 medium and incubate overnight in an incubator at 22°C.
12. Add G418 to 20 µg/ml.

Clone cells

13. When the plate is confluent, isolate clones by clonally plating cells on *Klebsiella aerogenes* bacterial lawns at ~100 amoebae per plate (see Support Protocol 2, steps 1 to 3).
14. Grow cells 4 to 5 days at 22°C (Fig. 12.5.3).
15. Select individual clones by scraping the feeding edge of the plaques and transferring into 24-well plates containing 20 µg/ml G418 in HL5 medium.
16. Move plate to 22°C incubator and allow growth.

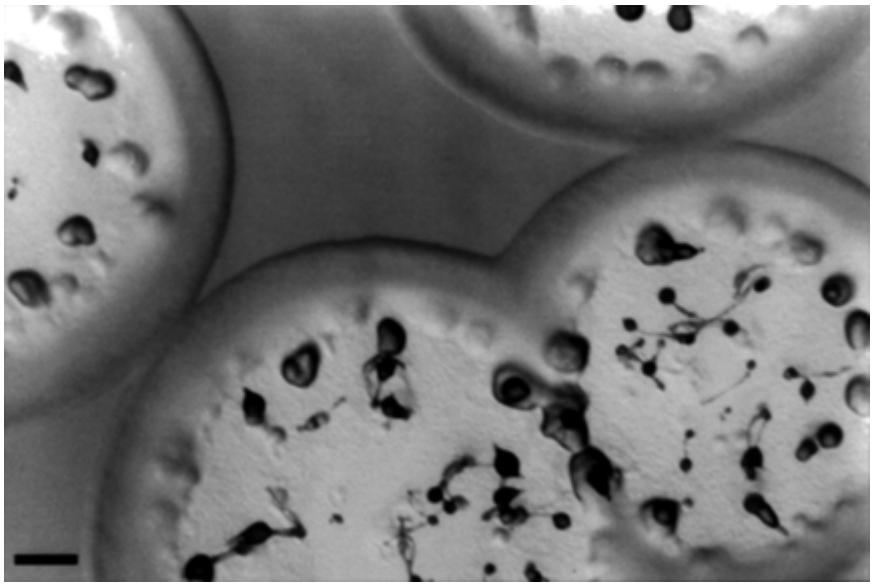


Figure 12.5.3 Cloning *D. discoideum* transformants on bacterial lawns. *D. discoideum* cells were mixed with *Klebsiella aerogenes* bacteria, plated on SM plates, and incubated at 22°C. This picture was taken 5 days after plating. Four cleared plaque regions are partially visible. Each plaque arose from a unique clone. Bar is 1.5 mm.

17. Analyze individual clones for GFP expression by immunoblotting (see Support Protocol 4 and *UNIT 6.2*) or microscopic visualization (see Basic Protocols 1, 2 and 3 and Alternate Protocols 1 and 2).

PHENOTYPIC SCREENING BY IMMUNOBLOTTING

SUPPORT PROTOCOL 4

The level of expression of the fusion protein is assessed by performing immunoblot analysis using commercially available antibodies directed against GFP (also see *UNIT 6.2*). This is especially important because it will confirm that the full-length GFP fusion is expressed and will provide information on the level of expression. In addition, probing with an antibody directed against the protein of interest allows one to compare the level of expression of the endogenous protein to that of the fusion protein. This is sometimes difficult as the epitope recognized by the antibody can be masked by the presence of the GFP.

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) grown to log phase in liquid medium (see Support Protocol 1)
Developmental buffer (DB; see recipe)
4× SDS sample buffer (*APPENDIX 2A*)
Anti-GFP antibody (e.g., Clontech Laboratories)
Antibody directed against protein of interest (optional)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Dispense transformed *D. discoideum* cells, grown to log phase in liquid medium, into 50-ml tubes.

   A cell line that does not express GFP should be used as a control.

2. Centrifuge 4 min at 1500 × *g*, 22°C, and wash cells in DB.
3. Centrifuge as in step 2 and resuspend at 2×10^7 cells/ml in DB.
4. Transfer cells to a flask and shake 2 hr at 100 rpm.

   This starvation period will downregulate the expression of proteases and therefore limit protein degradation.

5. Mix 200 µl cells with 67 µl of 4× SDS sample buffer in a microcentrifuge tube.
6. Boil 2 min.
7. Carry out SDS-PAGE (*UNIT 6.1*), loading ~53 µl sample (8×10^5 cellS) per lane.
8. Transfer gel to nitrocellulose membrane.
9. Perform immunoblot analysis (*UNIT 6.2*) using an anti-GFP antibody and an antibody directed against the protein of interest (optional).

PHENOTYPIC SCREENING BY DEVELOPMENT ON NON-NUTRIENT AGAR

SUPPORT PROTOCOL 5

The functionality of the GFP fusion protein is assessed by determining if it behaves as its wild-type counterpart when expressed in the null background. In order to assess the effect (or noneffect) of the GFP fusion protein on normal development, this characterization is also performed when the fusion protein is expressed in wild-type cells. The procedure is simple and fast—wild-type cells will differentiate and form fruiting bodies 24 hr after the

initiation of the starvation period (Fig. 12.5.4). Moreover, the whole developmental program can be readily recorded using time-lapse video. Alternatively, development can be assessed by clonally plating amoebae on bacterial lawns (see Support Protocol 2).

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) grown to log phase in liquid medium (see Support Protocol 1)
Developmental buffer (DB; see recipe)
Developmental buffer agar (DB agar; see recipe)
35-mm petri dish
Incubator, 22°C

1. Dispense transformed *D. discoideum* cells, grown to log phase in liquid medium, in 50-ml aliquots into sterile conical tubes.

   Wild-type cells should be used as controls.

2. Centrifuge 4 min at 1500 × *g*, 2°C, and wash cells in DB.
3. Centrifuge again, count cells (*UNIT 1.1*), and resuspend at 1×10^7 cells/ml in DB.
4. Transfer 1 ml cells to a 35-mm petri dish containing 0.5 ml DB agar.
5. Let cells adhere 5 min.
6. Tilt plate, wait 5 min, and aspirate excess buffer.
7. Let air dry 2 min.
8. Replace lid and store plate in a humidified environment in an incubator at 22°C.

   Placing the plates in a closed plastic box in the incubator will provide an appropriately humid environment.

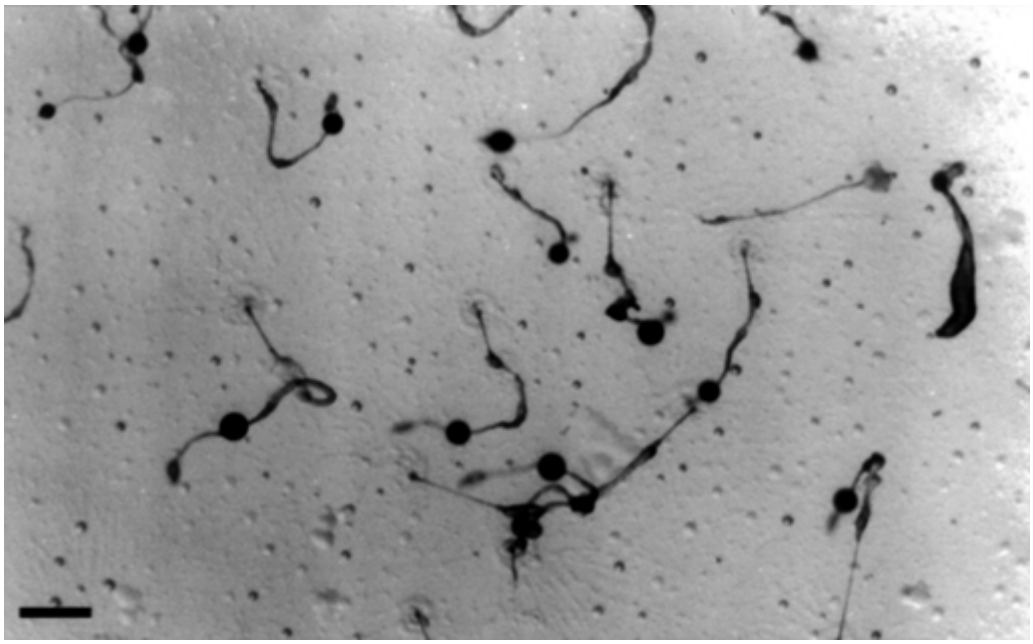


Figure 12.5.4 Development of *D. discoideum* on non-nutrient agar. Wild-type *D. discoideum* cells were grown axenically (see Support Protocol 1), plated on non-nutrient agar (see Support Protocol 5), and incubated at 22°C. This picture was taken 24 hr after plating. Bar is 1.5 mm. Note the characteristic spore heads on top of the stalks that form the fruiting bodies. Mutants can give rise to a wide variety of developmental phenotypes.

9. Assess development as appropriate.

The developmental program of the cells can be recorded so that the 24-hr period is compressed into a few minutes. This is easily accomplished by connecting a video camera to the microscope and using a time-lapse video recorder to capture images. For best results, a low-magnification objective should be used (2.5× to 5×). Since the cells are sensitive to high temperature, care should be taken to use a very dim light source. The functionality of the GFP fusion protein is then easily assessed by comparing the phenotype of the transformed cell line with that of wild-type cells. A comprehensive analysis of the developmental program of wild-type cells can be found in Kessin (2000) and references within.

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Developmental buffer (DB)

5 mM $Na_2HPO_4 \cdot 7H_2O$
5 mM KH_2PO_4
2 mM $MgSO_4$
200 µM $CaCl_2$
Final pH 6.2
Store up to 6 months at 4°C

Developmental buffer agar (DB agar)

Add 1% (w/v) Difco Bacto Agar to 50 ml DB (see recipe) in 100-ml bottles and autoclave on liquid cycle for 20 min. Store up to 6 months at room temperature. When ready for use, unscrew the cap and microwave until the agar is melted.

Electroporation buffer (EB)

10 mM sodium phosphate buffer, pH 6.1 (*APPENDIX 2A*)
50 mM sucrose
Final pH 6.1
Filter sterilize using 0.22-µm filters
Store up to 6 months at −20°C

G418 solution, 20 mg/ml

Prepare a 20 mg/ml solution in 10 mM HEPES, pH 7.2. Filter sterilize using 0.22-µm filters, dispense into 0.5-ml aliquots, and store at −20°C.

Healing solution

100 mM $CaCl_2$
100 mM $MgCl_2$
Filter sterilize using 0.22-µm filters
Store up to 6 month at 4°C

HL5 medium

10 g glucose (55 mM final)
10 g Difco Peptone (1% w/v final)
5 g yeast extract (0.5% w/v final)
0.95 g $Na_2HPO_4 \cdot 7H_2O$ (3.5 mM final)
0.5 g KH_2PO_4 (3.7 mM final)
0.03 g dihydrostreptomycin (41 µM final)
H_2O to 1 liter
Final pH 6.5

continued

Mix all components and divide into aliquots in bottles and flasks. Cover flasks with a stopper made of cotton cheesecloth. Autoclave 20 min on liquid cycle. Store up to 1 month at 22°C.

Autoclaving for longer periods will cause the glucose to caramelize (making the medium appear darker) and serious growth problems to occur.

Phosphate buffer (PB)

5 mM $Na_2HPO_4 \cdot 7H_2O$
5 mM KH_2PO_4
Final pH 6.2
Store up to 6 months at 4°C

SM plates

10 g dextrose (55 mM final)
10 g Difco Bacto Peptone (1% w/v final)
1 g yeast extract (0.1% w/v final)
1.9 g KH_2PO_4 (13.9 mM final)
0.9 g K_2HPO_4 (5.2 mM final)
20 g Difco Bacto Agar (2% w/v final)
H_2O to 1 liter
Final pH 6.4
Autoclave 20 min on liquid cycle
Pour 25 ml per 100-mm petri dish
Let solidify at room temperature
Store up to 6 months at 4°C

COMMENTARY

Background Information

D. discoideum amoebae live in two distinct phases (Bonner, 1982). Vegetative or growth-stage cells function independently and use phagocytosis or pinocytosis to ingest bacteria or liquid medium. Similar to mammalian cells, amoebae grown in liquid medium divide using binary fission. Upon starvation, the amoebae acquire the capacity to communicate with one another and enter a developmental program that leads to the formation of a multicellular organism. In the early stages of development, ~10^5 amoebae use chemotaxis to come together and form a tight aggregate. This aggregate then differentiates into stalk and spore cells and, through a series of morphological changes, forms a spore head atop a stalk of vacuolated cells—the so-called fruiting body. Once environmental conditions are favorable again, the spores germinate and repeat the life cycle. The unique feature of *D. discoideum*'s life cycle allows one to study in great detail the dynamic behavior of individual cells. Moreover, the advent of the GFP technology now allows one to readily assess the dynamic behavior of individual proteins within living, migrating cells.

The growth and developmental programs of *D. discoideum* are mediated via the evolutionarily conserved G protein–coupled signaling cascade (Parent and Devreotes, 1996a; Aubry and Firtel, 1999). Four hours after the initiation of starvation, cells maximally synthesize and secrete cAMP. Through the action of an extracellular phosphodiesterase, cAMP is produced at 6-min intervals. These oscillations in the levels of cAMP produce propagating waves that generate gradients directing the cells toward the aggregating centers. At the same time, G protein–coupled receptors that specifically bind cAMP are expressed on the cell surface, and binding of the nucleotide to its receptor leads to a variety of biochemical responses. As is observed when leukocytes are stimulated with chemoattractants, rapid and transient increases in polymerized actin, Ca^{2+} influx, intracellular messengers such as IP_3, cAMP, and guanosine 3′,5′-cyclic monophosphate (cGMP), and in the phosphorylation of myosins I and II have all been measured following cAMP addition to amoebae.

In the early aggregation period, these biochemical changes give rise to chemotactic movement and changes in gene expression, both of which are essential components of normal development. Genetic and biochemical analyses have established that, throughout

growth and development, four cAMP receptors (cAR1 to 4) coupled to eleven G proteins (composed of eleven distinct α subunits associated with a unique βγ-complex) are expressed. Similarly, various conserved signaling effectors are expressed at specific times in the course of the *D. discoideum* life cycle. A number of these effectors have been identified by cloning and through information emerging from the genome and cDNA sequencing projects. These effectors include: adenylyl and guanylyl cyclases, phosphodiesterases, phospholipase C, protein kinase A, glycogen synthase 3, small guanosine trisphosphatases and their regulators, actin and a plethora of actin binding proteins, conventional and unconventional myosins, MAP kinases, two-component histidine kinases, and STATs, which are not present in yeast (Harwood et al., 1995; Parent and Devreotes, 1996a; Kawata et al., 1997; Maeda et al., 1997; Brown and Firtel, 1998; Loomis, 1998; Loomis et al., 1998; Aubry and Firtel, 1999; Eichinger et al., 1999; Thomason et al., 1999; Williams, 1999).

D. discoideum's highly accessible genetics renders it a unique model system to study fundamental cellular processes including cell motility and directed migration (Eichinger et al., 1999). Most of the molecular genetic techniques typically associated with *S. cerevisiae* are available in *D. discoideum*. Random mutagenesis can be performed using high-efficiency extrachromosomal vectors (Parent and Devreotes, 1996a). Because the genome is haploid, novel genes can be identified using insertional mutagenesis or restriction-enzyme-mediated integration (REMI; Kuspa and Loomis, 1992). Nonessential genes can be easily disrupted by homologous recombination (De Lozanne and Spudich, 1987). So far, over 400 genes involved in cell motility, signal transduction, and cell differentiation have been targeted, and important generalizations for eukaryotic cells have been derived using these cell lines. Strains with multiple gene deletions can be constructed by consecutive transformations using different selectable markers. Since the cells are free living, gene deletions that might be lethal in other organisms can often be productively studied in *D. discoideum* (Kay and Williams, 1999).

Using phenotypic complementation of null mutants with GFP fusion proteins is extremely useful for cell biology. Because heterologous expression of yeast and mammalian genes is possible in *D. discoideum*, this technique can be used to assess the functionality of endogenous as well as heterologous genes (Manstein et al., 1995; Slade et al., 1997; Pang et al., 1999; Parent and Devreotes, unpub. observ.). Moreover, studying the phenotypic behavior of wild-type cells overexpressing wild-type or mutated genes is very useful in assessing the function of a given protein. Consequently, the high degree of conservation between amoebae and higher eukaryotes at the cellular and molecular levels coupled to the accessible genetics of this model system renders the study of fundamental biological processes observed in *D. discoideum* indispensable for deciphering complex biological responses.

Critical Parameters and Troubleshooting

Using *D. discoideum* to study cell motility has many advantages. Compared to mammalian cells, amoebae are easy to grow and manipulate. The medium is readily made in the laboratory and is inexpensive. As amoebae eat bacteria, the risk of bacterial contamination is small, and the optimal growth temperature of 22°C is very convenient. The cells adhere well to plastic and glass but do not require trypsinization procedures. Stable transformants are easily obtained and maintained, and phenotypic characterization is rapid. Nonetheless, there are some aspects of *D. discoideum* research that require special attention.

1. The quality of the peptone used to make the HL5 medium is critical for proper growth. It has been observed that the Difco brand gives by far the best results. Suboptimal medium will result in slow or no growth.

2. When growing cells on bacterial lawns, the author has found that using overgrown cultures of *Klebsiella aerogenes* results in serious growth problems. Growing the bacterial cultures overnight at room temperature gives rise to the best results.

3. A transformation efficiency of ~1/1000 is expected when using extrachromosomal vectors. Good quality DNA preparations free of contaminants and salt will give the best results. Also, because the heat generated by the electrical shock can kill the cells, prechilling the cuvette and using ice-cold buffer is critical. Looking at the cells 24 hr after electroporation, before the selection antibiotic is added, is a good way to see if the cells were harmed by the electrical burst. At that time, most of the cells should be adhering to the bottom of the plate.

4. Performing a dose-response curve analysis for the effectiveness of G418 on the survival of wild-type cells is preferable. This is

achieved by simply adding various concentrations of G418 to wild-type cells and monitoring cell survival. The optimal concentration should be close to 20 μg/ml.

5. Development on non-nutrient agar is influenced by environmental conditions, and most of the abnormal developmental problems are caused by an inadequate level of humidity on the plate. This can be fixed by not air drying the plate after aspirating the excess buffer and simply leaving it slightly tilted with the lid on.

6. When assessing the effect of receptor stimulation on the cellular distribution of the GFP fusion protein, it is essential to make sure that the cells are expressing cAMP receptors (a key point when studying cells that display a developmental phenotype). This is accomplished by performing immunoblot analysis using a specific polyclonal antibody directed against cAR1 (a receptor expressed in early development) on samples taken from developed cells.

Time Considerations

The limiting step in these procedures is obtaining the transformants, which under normal conditions will require ~2 to 3 weeks. Once the phenotypic characterization is completed (~1 week), the live-cell imaging is fast and rewarding.

Literature Cited

Aubry, L. and Firtel, R.A. 1999. Integration of signaling networks that regulate *Dictyostelium* differentiation. *Annu. Rev. Cell Dev. Biol.* 15:469-517.

Bonner, J.T. 1982. Comparative biology of cellular slime molds. *In* The Development of *Dictyostelium discoideum* (W.F. Loomis, ed.) pp. 1-33. Academic Press, New York.

Brown, J.M. and Firtel, R.A. 1998. Phosphorelay signalling: New tricks for an ancient pathway. *Curr. Biol.* 8:R662-R665.

Daunderer, C., Schliwa, M., and Graf, R. 1999. *Dictyostelium discoideum*: A promising centrosome model system. *Biol. Cell* 91:313-320.

De Lozanne, A. and Spudich, J.A. 1987. Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. *Science* 236:1086-1091.

Dormann, D., Siegert, F., and Weijer, C.J. 1996. Analysis of cell movement during the culmination phase of *Dictyostelium* development. *Development* 122:761-769.

Egelhoff, T.T., Titus, M.A., Manstein, D.J., Ruppel, K.M., and Spudich, J.A. 1991. Molecular genetic tools for study of the cytoskeleton in *Dictyostelium. Methods Enzymol.* 196:319-334.

Eichinger, L., Lee, S.S., and Schleicher, M. 1999. *Dictyostelium* as a model system for studies of the actin cytoskeleton by molecular genetics. *Micros. Res. Tech.* 47:124-134.

Harwood, A.J., Plyte, S.E., Woodgett, J., Strutt, H., and Kay, R.R. 1995. Glycogen synthase kinase 3 regulates cell fate in *Dictyostelium*. *Cell* 80:139-148.

Howard, P.K., Ahern, K.G., and Firtel, R.A. 1988. Establishment of a transient expression system for *Dictyostelium discoideum*. *Nucl. Acids Res.* 16:2613-2623.

Hughes, J.E., Kiyosawa, H., and Welker, D.L. 1994. Plasmid maintenance functions encoded on *Dictyostelium discoideum* nuclear plasmid Ddp1. *Mol. Cell. Biol.* 14:6117-6124.

Kawata, T., Shevchenko, A., Fukuzawa, M., Jermyn, K.A., Totty, N.F., Zhukovskaya, N.V., Sterling, A.E., Mann, M., and Williams, J.G. 1997. SH2 signaling in a lower eukaryote: A STAT protein that regulates stalk cell differentiation in *Dictyostelium*. *Cell* 89:909-916.

Kay, R.R. and Williams, J.G. 1999. The *Dictyostelium* genome project: An invitation to species hopping. *Trends Genet.* 15:294-297.

Kessin, R.H. 2000. *Dictyostelium*: Evolution, Cell Biology, and the Development of Multicellularity. Developmental and Cell Biology Series. Cambridge University Press, Cambridge.

Kuspa, A. and Loomis, W.F. 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. U.S.A.* 89:8803-8807.

Loomis, W.F. 1998. Role of PKA in the timing of developmental events in *Dictyostelium* cells. *Microbiol. Mol. Biol. Rev.* 6:684-694.

Loomis, W.F., Kuspa, A., and Shaulsky, G. 1998. Two-component signal transduction systems in eukaryotic microorganisms. *Curr. Opin. Microbiol.* 1:643-648.

Maeda, Y., Inouye, K., and Takeuchi, I. 1997. *Dictyostelium*, A Model System for Cell and Developmental Biology. Universal Academy Press, Tokyo.

Manstein, D.J., Schuster, H.P., Morandini, P., and Hunt, D.M. 1995. Cloning vectors for the production of proteins in *Dictyostelium discoideum*. *Gene* 162:129-134.

Nellen, W. and Firtel, R.A. 1985. High-copy-number transformants and co-transformants in *Dictyostelium*. *Gene* 39:155-163.

Pang, K.M., Lynes, M.A., and Knecht, D.A. 1999. Variables controlling the expression level of exogenous genes in *Dictyostelium*. *Plasmid* 41:187-197.

Parent, C.A. and Devreotes, P.N. 1996a. Molecular dissection of G protein–mediated signal transduction using random mutagenesis in *Dictyostelium*. *Microb. Genome Methods* 1:1-13.

Parent, C.A. and Devreotes, P.N. 1996b. Molecular genetics of signal transduction in *Dictyostelium*. *Annu. Rev. Biochem.* 65:411-440.

Parent, C.A. and Devreotes, P.N. 1999. A cell's sense of direction. *Science* 284:765-770.

Rietdorf, J., Siegert, F., and Weijer, C.J. 1996. Analysis of optical density wave propagation and cell movement during mound formation in *Dictyostelium discoideum. Dev. Biol.* 177:427-438.

Slade, M.B., Emslie, K.R., and Williams, K.L. 1997. Expression of recombinant glycoproteins in the simple eukaryote *Dictyostelium discoideum*. *Biotechnol. Genet. Eng. Rev.* 14:1-35.

Sussman, M. 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* 28:9-29.

Thomason, P.A., Traynor, D., and Kay, R.R. 1999. Taking the plunge: Terminal differentiation in *Dictyostelium*. *Trends Genet.* 15:15-19.

Williams, J.G. 1999. Serpentine receptors and STAT activation: More than one way to twin a STAT. *Trends Biochem. Sci.* 24:333-334.

Key References

Kessin, 2000. See above.

This is the most recent book on Dictyostelium cell biology to date. It covers a wide variety of valuable information on the development of Dictyostelium.

Ludin, B. and Matus, A. in association with *Trends Cell Biol.* 1999. "GFP in motion".

This CD contains beautiful movies highlighting the use of GFP in live cells. Several sections describe results obtained using D. discoideum. Contact Trends in Cell Biology (www.elsevier.com) for more details.

Spudich, J.A. (ed.) 1987. *Dictyostelium discoideum*: Molecular Approaches to Cell Biology. *Methods Cell Biol.* Vol. 28.

This volume contains details of a variety of molecular and cell biological tools used to study D. discoideum.

Experientia Vol. 51. 1995.

This volume contains a series of reviews pertaining to genomics, cell biology, and biochemistry of D. discoideum.

Internet Resources

http://dicty.cmb.nwu.edu/dicty/dicty.html

This site serves as a single entry point for the online resources for the D. discoideum community. It provides access to an online database of investigators, current and past editions of the newsletter "Dicty News", links to cDNA and genome sequencing projects, and a wide variety of useful resources.

Contributed by Carole A. Parent
National Cancer Institute, NIH
Bethesda, Maryland

Optical Microscopy–Based Migration Assay for Human Neutrophils

This unit describes an in vitro microscopy assay for examining the migration of human neutrophils in two dimensions. The method is particularly useful for identifying the underlying cause of a migration defect, and it can be used to evaluate a variety of migration parameters (e.g., speed and directional persistence) that cannot be easily discerned from assays based on migration through a porous barrier (e.g., Boyden chamber assays; *UNIT 12.4*). For this assay, freshly isolated human neutrophils are added to microscopy chambers and then stimulated to migrate with a chemotactic agent. Images of fields of cells are acquired at multiple time points after stimulus addition. The images are used to create time-lapse movies that can be analyzed to determine the effects of specific pharmacological treatments on migration of individual cells.

The Basic Protocol describes the method for acquiring time-lapse images of migrating neutrophils, while support protocols describe isolation of human neutrophils from blood (see Support Protocol 1) and preparation of microscopy chambers (see Support Protocol 2).

NOTE: There are special ethical and safety considerations for working with human cells.

NOTE: All solutions and materials used with living neutrophils should be sterile and free of lipopolysaccharide (LPS, endotoxin) contamination, which will activate the neutrophils (see Critical Parameters).

ACQUIRING TIME-LAPSE IMAGES OF MIGRATING NEUTROPHILS

BASIC PROTOCOL

A crucial initial step in an inflammatory response is the migration of neutrophils and other immune cells toward chemotactic stimuli. Cell migration requires a highly orchestrated series of attachment and detachment events, which occur in concert with protrusive and contractile forces. Importantly, each step in the process must be regulated in both space and time, and so investigations of migration mechanisms often warrant assays that provide both types of information. The assay described here measures the extent to which neutrophils translocate (i.e., migrate in two dimensions) in response to an isotropic bath of chemoattractant. This assay is based on the observation of cells by optical microscopy, which provides intracellular and extracellular spatial information. Temporal information is available because the cells are imaged at various times after stimulation, rather than at a single fixed timepoint.

Materials

HBS, pH 7.4 (see recipe)
50× (0.5 M) glucose stock solution (filter sterilize and store at 4°C)
Human neutrophils, freshly isolated (see Support Protocol 1)
Formyl-Met-Leu-Phe (fMLF)

1.5-ml polypropylene microcentrifuge tubes
End-over-end rotator or platform rocker
Air-curtain heater (Arenberg Sage)
35-mm fibronectin-coated coverslip dishes (see Support Protocol 2)
Humidified slide warmer (LabLine Instruments)
Inverted microscope equipped for digital imaging with 20×, 40×, or 63× objective

Contributed by Lynda M. Pierini and Frederick R. Maxfield
Current Protocols in Cell Biology (2003) 12.6.1-12.6.15

NOTE: Neutrophils are readily activated by shear forces or by contact with certain surfaces. Cells should be handled gently, transferred using wide-bore pipet tips, and kept in polypropylene (as opposed to glass or polystyrene) vessels.

Prepare cells, coverslip dishes, and microscope

1. On the day of the experiment, prepare ~25 ml of HBS, pH 7.4, with 10 mM glucose (added from 50× glucose stock) for every ten coverslip dishes to be used. Prewarm the HBS/glucose to 37°C and maintain at that temperature.

2. For every coverslip dish to be used, prepare 50 µl of neutrophil suspension at 1–2 × 10^6 cells/ml in HBS/glucose. Prepare in bulk (up to 1.5 ml per 1.5-ml conical tube) and place on a slow-moving rotator or rocker at room temperature.

   If testing the effects of a particular pretreatment (e.g., pharmacological agent) on the migration of neutrophils, split the cells into the appropriate number of aliquots and treat the test samples at this time.

3. Prewarm the microscope stage to 32° to 35°C with an air-curtain heater for 30 to 60 min before the start of the experiment.

   Neutrophil migration is completely abrogated at temperatures above ~39°C, so it is essential that the temperature of the microscope stage and objective be carefully monitored throughout the experiment.

   Typically, the air-curtain heater should be placed 4 to 6 in. (10 to 15 cm) away from the microscope stage and aimed so that warm air is blown across both the stage and the objective. As an alternative, a microscope objective heater may be used (e.g., Bioptechs; http://www.bioptechs.com).

   If using an oil-immersion objective, prewarm the immersion oil to the same temperature as the stage and objective. This will minimize focus drift caused by temperature-dependent changes in oil viscosity.

4. When ready to begin, remove excess PBS from a 35-mm fibronectin-coated coverslip dish and add 50 µl of cell suspension to the well in the center of the dish. Place the coverslip dish onto a humidified slide warmer maintained at 37°C, and let cells settle onto the coverslip for 5 min.

5. Gently add 1 ml of warm HBS/glucose to the coverslip dish, adding the buffer off to the side of the dish so that cells do not become dislodged.

   Neutrophils will not become tightly adherent until after they are stimulated. For this reason, it is easy to accidentally wash them away.

6. Transfer the coverslip dish to the microscope stage and look at the cells using transmitted light illumination and any contrast method available (e.g., phase contrast or differential interference contrast) to confirm by morphology that the cells are healthy and resting.

   Neutrophils are relatively thick cells that are best imaged with differential interference contrast optics, which allows detailed observation of morphological changes. Phase-contrast imaging is usually less informative because the cells become extremely refractile at some points during migration.

   This is a good time to evaluate the activation state of the neutrophils. As noted above, resting neutrophils should not be tightly adherent and they should not spread on the coverslip. Usually <10% of the cells will attach, spread, and become spontaneously polarized; the remainder of the cells will be rounded with smooth plasma membranes (see Fig. 12.6.1). If much greater than 10% of the neutrophils appear to be activated in the absence of exogenously added chemoattractant, subtle effects of experimental treatments may be difficult to detect. In this case, the investigator may wish to discard the samples and

begin again with a fresh blood draw from a different donor. If >40% of the neutrophils appear to be activated, neutrophils should be re-isolated from a new donor. If the basal level of activation of the neutrophils from various donors is consistently high, all solutions used in the isolation procedure should be discarded and prepared anew. LPS contamination should be considered (see Critical Parameters). Mechanical stimulation (e.g., from an unbalanced centrifuge) can also cause activation.

Add chemoattractant and begin image acquisition

7. Prepare a 20 nM solution of fMLF in HBS/10 mM glucose (see step 1) and maintain at 37°C in a water bath. Gently add 1 ml of the warm 20 nM fMLF solution to the periphery of the coverslip dish on the microscope stage and let stand for 2 min.

   Because the cells are in an isotropic bath of chemoattractant (i.e., there is no gradient), they will become oriented in random directions.

8. Begin image acquisition. Acquire a transmitted-light image of the same field of cells every 10 to 20 sec for 4 min. Save this series of images, then acquire a second and third series of images of different fields from the same dish.

   Alternatively, a single, longer time series can be acquired if detailed analyses of cell paths and behaviors are to be performed.

   After data acquisition is complete, the sample can be discarded or used for immunofluorescence staining (see UNIT 4.3).

9. Repeat steps 4 to 8 with fresh cells and a new coverslip dish.

Analyze cell behavior

10a. *To determine the migration capacity of neutrophils:* Draw a circle of a fixed diameter around each cell within the first image of the time series. For each cell in the field, step through the images in the sequence and note whether the cell has moved out of its circle (i.e., for each cell, determine if the cell's centroid has translocated beyond the perimeter of the circle).

   It is not sufficient just to compare the first and last images in a time series because cells may have moved out and then back into their circles and would thus be scored as nonmigratory. Alternatively, cells may become detached during the course of the assay and so may no longer reside within their starting circles; these cells would mistakenly be scored as migratory.

   Some cells become very elongated. Depending on the intent of the experiment, a cell whose front, but not rear, moves outside the circle can be counted or not, as long as the criterion is specified and consistent throughout the analysis.

   There are sophisticated image-analysis programs that can perform this sort of analysis in an automated fashion. A description of these programs and their use in analyzing time-lapse movies is beyond the scope of this unit. It should be noted that automated analysis of migration is most easily accomplished using fluorescence images of labeled cells rather than transmitted-light images in which object identification is more difficult.

   Choosing the diameter of the circle to draw around the cells in the initial images is somewhat arbitrary. For historical reasons, the authors ordinarily use a 14-μm-diameter circle, so a migrating neutrophil is defined as one that has translocated >7 μm in 4 min. With this definition, 60% to 90% of control cells are migratory. All samples on a given day should be evaluated using circles with the same diameter.

   The neutrophil preparation used for these studies will contain a small percentage (1% to 5%, depending on the donor) of eosinophils. Eosinophils are relatively easy to identify because their cytoplasm is full of distinct spherical granules that are generally larger and more refractile than the granules in neutrophils (see Fig. 12.6.3). Cells that are clearly identifiable as eosinophils should not be considered in the data analysis.

   To compare data from experiments performed on different days, results should be normalized relative to the migratory level of the control cells.

10b. *To determine displacement, persistence, and turning behavior of individual cells:* Track and analyze each cell manually or by computer. To track cell paths manually, mark and record the positions of every cell's center in each field, then enter these positions into a graphing program for further analysis (Mandeville et al., 1995).

SUPPORT PROTOCOL 1

ISOLATING HUMAN NEUTROPHILS

Primary human neutrophils cannot be maintained in culture, making it necessary to isolate neutrophils from volunteers each day of an experiment. The first step of the isolation procedure is based on a centrifugal technique that separates neutrophils from red blood cells (RBCs) and most other leukocytes. The neutrophil-containing layer of cells isolated in this way will contain a small percentage of contaminating RBCs and eosinophils. RBCs are nearly completely removed from the preparation by using hypoosmotic shock to preferentially lyse the RBCs, leaving the neutrophils and eosinophils unharmed. Contaminating eosinophils typically comprise only 1% to 5% of the remaining cells and can be easily identified and disregarded during image analysis.

This separation technique is based on the density of human neutrophils and the osmolality of the separation medium. It is likely that the density of neutrophils from other animals will differ slightly from that of human neutrophils, so the density of the separation medium (i.e., Polymorphprep) will have to be adjusted if the protocol is to be used for isolation of neutrophils from animals other than humans. Suggestions for adjustments can be obtained from the manufacturer.

CAUTION: Only qualified and experienced personnel, following governmental and institutional guidelines for working with human subjects, should draw blood from donors.

NOTE: It is important to verify that the donor is healthy and not taking any medications because neutrophil function may be altered under these circumstances.

Materials

Sodium heparin
Healthy volunteer blood donor
Polymorphprep density gradient medium (Accurate Chemical & Scientific Corp.)
5× PBS (see recipe)
HBS/glucose: HBS, pH 7.4 (see recipe), containing 10 mM glucose added from 0.5 M stock (see Basic Protocol)

15- and 50-ml conical polypropylene centrifuge tubes
Tourniquet
Alcohol wipes
21-G needles
10-ml disposable syringes
Tabletop centrifuge with swinging-bucket rotor

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

NOTE: Use PBS without calcium or magnesium throughout the isolation procedure until the final resuspension step. Divalent cations in HBS will cause cells to aggregate.

Obtain donor blood sample

1. Place 200 U sodium heparin in a 15-ml conical tube.

   The authors have avoided using Vacutainers because they tend to stimulate the neutrophils. Newer plastic Vacutainers have not been evaluated for this assay.

2. Place tourniquet on a healthy donor's arm, swab arm with alcohol wipe, and then draw 10 ml blood using a 21-G needle and a 10-ml syringe.

   The health of the blood donor is essential to the success of the assay. For further details, see Critical Parameters.

3. Immediately transfer blood into the heparin-containing conical tube. Mix the heparin and blood by gently inverting the capped tube several times, and then let stand 10 min to allow blood to cool to room temperature.

   The blood should be used within 2 hr of being drawn from the donor.

Isolate neutrophils

4. Place 5 ml Polymorphprep into each of two 15-ml conical tubes. Carefully layer 5 ml of the heparinized blood onto the top of the Polymorphprep in each tube using a sterile polypropylene transfer pipet. Cap the tubes and centrifuge 30 min at 450 × *g*, room temperature (18° to 22°C), in a swinging-bucket rotor.

   At the end of the centrifugation step, several layers can be discerned within each tube, including (from bottom to top) a dark red layer consisting largely of RBCs, a clear layer containing Polymorphprep with some RBCs and some neutrophils, a fuzzy white band containing neutrophils and eosinophils, a tight white band of mononuclear cells (T cells, B cells, and monocytes) at the sample/medium interface, and a yellow layer of plasma. The two white bands of leukocytes are typically separated by 0.5 to 1.0 cm. If RBCs are clumped at the top of the clear Polymorphprep layer such that the band of neutrophils is not clearly defined, centrifuge the samples for an additional 5 to 10 min.

5. From each tube, carefully aspirate the yellow plasma layer and top tight white band (mononuclear cells), leaving the fuzzy white band (containing neutrophils and eosinophils). Remove the neutrophil-containing band from each tube with a sterile polypropylene transfer pipet and combine in a single 50-ml conical tube. Collect the clear Polymorphprep layer from each tube and add to the neutrophils.

   When removing the tight white band of mononuclear cells (i.e., the upper band), it is best to err on the side of losing some neutrophils so that purity is optimized over yield. The number of neutrophils obtained from this procedure is far greater than needed for the migration assay.

6. Fill the 50-ml conical tube with 1× PBS and sediment cells by centrifuging 10 min at 400 × *g*, room temperature (18° to 22°C).

7. Aspirate the PBS. Resuspend neutrophil pellet in 1 ml of 1× PBS and transfer to a 1.5-ml microcentrifuge tube.

   The cell suspension appears reddish because it contains contaminating RBCs in addition to neutrophils and eosinophils.

Remove contaminating RBCs

8. Microcentrifuge 6 to 8 sec at 10,000 rpm (7,500 × *g*). Aspirate supernatant, leaving reddish pellet.

9. Using a wide-bore pipet tip, carefully resuspend pellet in 800 µl water.

10. Allow RBCs to lyse for 30 sec and then add 200 µl of 5× PBS. Rapidly mix the contents of the tube by capping the tube and inverting several times. Immediately sediment cells in microcentrifuge as in step 8, then aspirate the supernatant.

    Keeping the cells in water for <30 sec will result in incomplete lysis of RBCs, while waiting significantly longer than 30 sec may damage the neutrophils.

11. Resuspend the pellet from step 10 in 1 ml of 1× PBS. Microcentrifuge as in step 8 and aspirate the supernatant. Repeat wash and resuspend final pellet in 1 ml HBS/glucose.

12. Determine concentration of cells using a hemocytometer (see *UNIT 1.1*).

Neutrophils can be kept on ice or at room temperature for several hours before use. In general, neutrophils should be used within 4 to 6 hr of isolation.

SUPPORT PROTOCOL 2

PREPARING COVERSLIPS AND MICROSCOPY CHAMBERS

Long-term observation of live cells often requires specialized chambers to maintain cell viability. Neutrophil migration assays are typically completed within 30 min, so environmentally controlled perfusion chambers are unnecessary. Instead, the open-air chambers described below are adequate, and they are less expensive and easier to use than most perfusion setups. Prefabricated chambers with poly-D-lysine-coated coverslips can be purchased from commercial sources (e.g., MatTek) for approximately twice the cost of making them.

NOTE: These chambers are open to the air, so bicarbonate-buffered media cannot be used.

NOTE: Chemotaxis chambers can be constructed from these sample chambers by covalently attaching opsonized erythrocytes to the coverslip surface (Pytowski et al., 1990).

Materials

Nochromix glass cleaning reagent (Godax Laboratories) mixed with H_2SO_4 per manufacturer's instructions
Paraffin
Petroleum jelly
1× PBS (see recipe)
0.1 mg/ml fibronectin (see recipe)

Glass coverslips (thickness should be matched with recommendation of microscope manufacturer)
35-mm tissue culture dishes
Sheet metal hole punch
Cotton-tipped applicator or fine-tipped paint brush

Prepare coverslips

1. Clean glass coverslips by immersing in Nochromix/H_2SO_4 solution for 1 hr. Make sure that coverslips are well separated so that the surfaces of all of the coverslips are exposed to the cleaning solution.

   CAUTION: *Nochromix cleaning solution is extremely caustic. Use appropriate safety gear and procedures.*

2. Remove the Nochromix/H_2SO_4 solution, rinse the coverslips with water three times, then soak for 1 hr in water. Repeat this water rinse and soaking process two more times.
3. Remove clean coverslips from the last wash one at a time using a pair of tweezers. Place coverslips in a single layer onto filter paper and let dry. Store clean, dry coverslips in a covered receptacle.

Prepare coverslip dishes

4. Prepare a mixture of paraffin/petroleum jelly at a ratio of 3:1 (v/v) by first melting enough paraffin over low-medium heat to yield 75 ml. Bring the volume of the liquefied paraffin up to 100 ml by adding petroleum jelly. Stir the mixture well.

   This mixture can be stored for up to 6 months and remelted over low-medium heat when needed. The mixture should be stirred before each use, and the last ~25 ml of the mixture should be discarded because the composition changes with repeated heatings.

5. Punch a 1.2-cm-diameter hole in the bottom center of a 35-mm tissue culture dish using a sheet metal punch.
6. Keeping the dish in an inverted orientation, use a cotton-tipped applicator or a fine-tipped paint brush to spread a thin layer of melted paraffin/petroleum jelly around the perimeter of the hole on the outside surface of the dish. Allow paraffin/petroleum jelly to resolidify on the dish.
7. Place a single clean, dry coverslip atop the solid ring of paraffin/petroleum jelly on the underside of the dish.
8. To affix the coverslip to the dish, warm the inverted dish (with coverslip on top) briefly to liquify the paraffin/petroleum jelly, and then let cool to room temperature.

   To warm the inverted dish, an inverted hot plate or an infrared lamp can be positioned such that heat from the hot plate or infrared lamp will warm items underneath. The heating rate should be set so that the paraffin/petroleum jelly mixture melts in ~5 sec when the inverted dish (with its coverslip on top) is warmed. Immediately after the paraffin/petroleum jelly melts, remove the dish from the heat and let it cool to room temperature.

   The result will be a well in the center of the right-side-up dish. The coverslip forms the bottom of the well, and the sides of the hole in the dish form the sides of the well. The thin solidified ring of paraffin/petroleum jelly, which seals the coverslip to the dish, does not contribute significantly to the depth of the well.
9. Store coverslip dishes for up to 6 months at room temperature. Dishes should be stored in an inverted position to prevent scratching of the coverslips and to keep debris from collecting in the wells.

Coat coverslip dishes with fibronectin (perform on day of assay)

10. Rinse the well of each coverslip dish three times with 1× PBS. Apply 50 µl of 0.1 mg/ml fibronectin to the well of each coverslip dish and allow to stand at room temperature for 30 to 60 min. Carefully wash the coverslip dishes three times with 1× PBS, and then leave covered with PBS until use.

REAGENTS AND SOLUTIONS

Use enodotoxin-free deionized water in all recipes and protocol steps. For common solutions see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Fibronectin, 0.1 mg/ml

Purchase lyophilized human fibronection (1.0 mg per vial) from BD Biosciences. To rehydrate lyophilized fibronectin, add 1 ml of distilled water and let stand at room temperature for 1 hr, gently swirling the solution periodically to completely dissolve the fibronectin (do not vortex). Store fibronectin at 4°C and use within one month of rehydration. Dilute fibronectin 1:10 (v/v) with 1× PBS (see recipe) on day of use.

HEPES-buffered saline (HBS), pH 7.4

200 µl 5 M $MgCl_2$ (1 mM final)
0.147 g $CaCl_2$ (1 mM final)
0.37 g KCl (5 mM final)
8.8 g NaCl (150 mM final)
4.8 g HEPES (20 mM final)
H_2O to 1 liter
Adjust to pH 7.4 with 1 M NaOH or 1 M HCl
Filter sterilize and store up to 4 months at 4°C

The authors prefer to use a commercial $MgCl_2$ stock solution (e.g., 49 M $MgCl_2$ from Sigma) because $MgCl_2$ is very hygroscopic and difficult to weigh accurately.

Phosphate-buffered saline (PBS), 5×

1.0 g KH_2PO_4 (7.5 mM final)
40.0 g NaCl (685 mM)
1.0 g KCl (13.5 mM)
5.8 g Na_2HPO_4 (40.5 mM)
H_2O to 1 liter
Adjust to pH 7.4 with 1 M NaOH or 1 M HCl
Filter sterilize and store up to 4 months at 4°C

COMMENTARY

Background Information

Neutrophils, with their capacity to migrate in response to a variety of stimuli, are essential for maintaining human health. In the absence of functional neutrophils people would rapidly succumb to infection. It has been over a century since Metchnikoff suggested that neutrophils serve as active defenders against invaders (Metchnikoff, 1893), yet there are still many questions about neutrophil locomotion that remain to be answered. How do neutrophils polarize in an isotropic bath of stimuli? How can they migrate in persistent paths when there is no gradient of stimulus? What are the molecular components of the migration machinery? How are these components regulated? To address these sorts of questions, visual assays have been used for over fifty years and have provided detailed descriptions and quantification of cell locomotion (Dixon and McCutcheon, 1936; McCutcheon, 1946). Microscopy-based assays are still one of the most powerful means of gaining insight into the mechanics of cell migration.

Precise terminology has been developed to describe migration phenomena (Keller et al., 1977a,b,c), and brief definitions of the relevant terms (shown in italics) are given here. Cells may possess or lack an *intrinsic capacity* to migrate. For example, promyelocytic cells are nonmotile, and it is only after they differentiate into monocytes or neutrophils that they acquire the capacity for migration. Once migration has been induced, the cells' behavior can be described as *random* or *directed*. Cells that are migrating randomly move along a path with no particular orientation relative to the environment. Neutrophils and other cells have been shown to exhibit a "persistent random walk" as opposed to "Brownian" random movement (Dunn, 1981). The direction of persistence is completely random with no correlation between individual cells or between cells and environmental factors. In contrast, cells that migrate directionally move with a preference for (or avoidance of) a particular direction, usually dictated by an environmental cue such as a *chemoattractant*, a soluble factor that activates cells to migrate (e.g., bacterially generated formyl-peptides and inflammatory chemokines). Cells within a gradient of a chemotactic chemoattractant will be oriented nonrandomly with their front-to-rear axis aligned parallel to the gradient (Zigmond, 1977). Chemoattractants can affect the speed or frequency of migration (*chemokinesis*) and/or the direction of migration (*chemotaxis*). Chemokinesis and chemotaxis are *not* synonymous with random and directed migration, respectively. Directed migration can result from either chemotaxis or chemokinesis coupled with *contact guidance*, where the physical properties of the environment dictate the direction of migration—e.g., aligned fibrils within tissue matrices (Mandeville et al., 1997; Wilkinson and Lackie, 1983). Refer to Keller et al. (1977a,b,c) for more in-depth definitions of the abovementioned italicized terms.

The assay described in this unit can only determine if an agent is chemokinetic; it cannot establish the chemotactic potential of a chemoattractant. To directly show that a chemoattractant is chemotactic, an orientation chamber, such as the one introduced by Zigmond (1977) and modified by others (Zicha et al., 1991, 1997) should be used. Alternatively, the assay chamber described here can be converted into a chemotaxis chamber by attaching bacterial spores (Allan and Wilkinson, 1978) or opsonized erythrocytes (Pytowski et al., 1990) to the surface of the coverslip. Addition of fresh serum to the chambers activates complement and generates radial gradients around the spores or erythrocytes when these particles are widely separated on the coverslip (~20 to 100 μm apart). Time-lapse imaging of neutrophils migrating towards these point sources provides a direct measure of chemotaxis. To address chemotactic potential, researchers have also used assays based on migration through micropore filters—e.g., Boyden chamber as-

says (Boyden, 1962). These are indirect assays that determine the end-point distribution of a cell population after exposure to chemoattractant. Although much larger numbers of cells are analyzed, filter assays can only provide information on cell populations as opposed to individual cells and/or subsets of cells. With a visual assay, the direction and speed of migration can be measured to directly demonstrate chemotaxis or chemokinesis, and displacement, persistence, and turning behavior of individual cells can be extracted from detailed analyses of cell paths (Haston and Wilkinson, 1987, 1988; Mandeville et al., 1995). Excellent discussions on the benefits and limitations of various filter assays can be found elsewhere (Wilkinson, 1996, 1998).

It should also be noted that the assay described here is a two-dimensional one. Thus, the analogous in vivo situation is likely to be cells migrating along the luminal surface of vascular endothelium rather than through the three-dimensional environment of connective tissues. Cells use somewhat different mechanisms for migration through three-dimensional matrices as compared to migration across two-dimensional surfaces, but there are enough similarities that a simple two-dimensional migration assay is an excellent starting point for dissecting the complexities of locomotion.

Clearly, no single type of migration assay is suitable for answering every question, so a variety of complementary assays should be used. See *UNITS 12.1, 12.2 & 12.4* for descriptions of other types of migration assays, and reviews by Wilkinson for an overview and critique of popular methods for studying cell migration (Wilkinson, 1996, 1998).

Critical Parameters

The success of neutrophil migration assays hinges on just two main parameters, the neutrophils and their environment. In vivo, the role of the neutrophil is to sense and respond to environmental signals that may indicate the presence of pathogens. The exquisite sensitivity of neutrophils to the presence of microbes is one of the main obstacles to performing an in vitro migration assay. Cells that are slightly prestimulated (or "primed") often respond differently to chemoattractants than naïve cells, so it is important to start with truly unstimulated neutrophils. The neutrophils from a donor who is coming down with or just getting over a cold may be slightly activated and should not be used. Similarly, many medications are known to affect cell migration, and for that reason

Table 12.6.1 Troubleshooting Guide for Optical Microscopy–Based Neutrophil Migration Assays

Problem	Possible cause	Solution
Cells appear jagged prior to stimulation and migrate poorly or not at all in response to chemoattractant	Endotoxin contamination	Test H_2O supply for endotoxin contamination; decontaminate H_2O and remake all solutions
	Cells were activated during isolation	Isolate neutrophils from a different donor taking extra care not to activate cells by rough handling
Cells appear round and nonadherent	Chemoattractant not added	Remake solution of chemoattractant
	No divalent cations in assay buffer	Remake assay buffers; verify that HBS (not PBS) has been used as the assay buffer
Cells appear very spread and tightly adherent after stimulation, and they do not migrate	Temperature too high	Adjust temperature
	Substrate too adhesive	Prepare coverslip dishes with varying amounts of substrate to optimize conditions
	Chemoattractant concentration too high	Perform dose-response analysis with chemoattractant to optimize concentration
Cells are slow to polarize and migrate only a short distance after stimulation	Temperature too low	Adjust temperature

neutrophils should not be isolated from donors taking medication. Donor health needs to be carefully assessed prior to drawing blood, and all results ought to be confirmed with neutrophils from various donors.

Neutrophils are fragile cells that are readily activated by rough handling. Extreme care is required during the isolation procedure to minimize shear stress and to avoid cell contact with activating surfaces, such as glass. An unbalanced centrifuge, overzealous resuspension technique, or excessive handling of cells can all contribute to cell activation. Isolated neutrophils are still adept at sensing microorganisms in the environment, and they will become activated in the presence of even very low levels of bacterial lipopolysaccharides (LPS, endotoxin). It is crucial that the water supply be tested for the presence of LPSs prior to making isolation and assay buffers, and that all stock buffers be prepared and maintained under sterile conditions. Recirculating reverse osmosis (RO) water systems can become contaminated with algae or other microorganisms. It is important to check for LPSs because contamination anywhere in a closed-loop system can release LPSs, which then spread throughout the loop. Filtration or UV light at the tap will not remove LPSs. The water supply can be tested for LPS contamination using a commercially available kit (e.g., Chromogenic Limulus Amebocyte Lysate Assay Kit from BioWhittaker).

After cells with a low basal level of activation have been isolated, they will be added to the assay chamber and stimulated to migrate. At this point, the ability of the cells to migrate and the extent of migration will depend upon the agent used to stimulate the cells, the matrix on which they will migrate, and the temperature during the assay. The absolute concentration of chemoattractant, even in the absence of a gradient, is an important determinant of migration. Neutrophils exhibit dose-dependent migration that varies between chemoattractants, making it necessary to generate dose-response curves to determine the optimal concentration of a

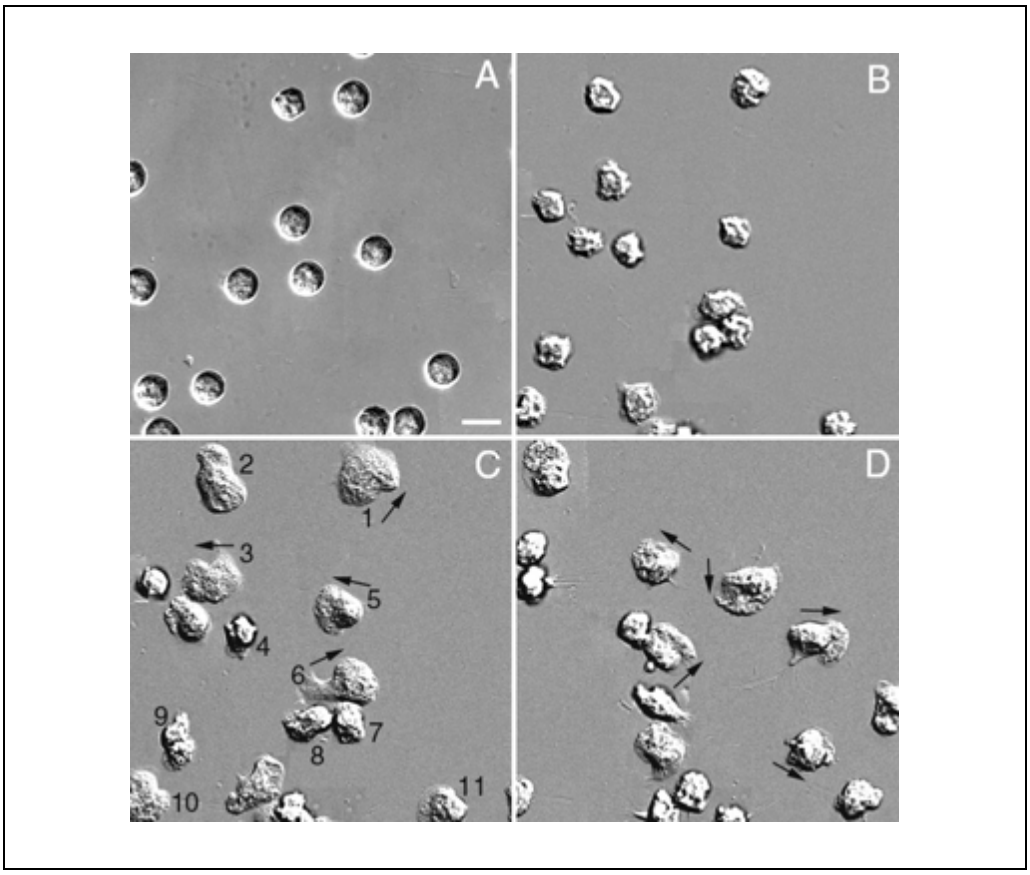


Figure 12.6.1 Morphological changes induced in human neutrophils by stimulation with the chemoattractant fMLF. Neutrophils were plated onto a fibronectin-coated coverslip dish for 5 min at 37°C and then left unstimulated (**A**) or stimulated with fMLF for 15 sec (**B**), 1 min (**C**), or 6 min (**D**). The images shown in panels A, B, and C are each from different fields of cells, whereas the image shown in panel D is the same field of cells shown in C, but imaged 5 min later. Numbers in C correspond to cell paths traced in Figure 12.6.2. Arrows indicate direction of neutrophil migration. Bar is equal to

particular chemoattractant. Because migration is dependent upon the precise regulation of cell attachments in space and time, the adhesive nature of the substratum is another critical parameter that can be varied. Substrata of differing adhesiveness can yield very different migration data in response to a given chemoattractant at a fixed concentration. If the substratum is too adhesive, cells will be unable to detach from the surface and therefore they will be unable to translocate. On the other hand, if the substratum is not adhesive enough, cells will not be able to form tight enough attachments to generate the tractional forces necessary for locomotion. Thus, the ability of cells to migrate, and their speed of migration, depend upon the tightness of the attachments that the cell forms with the substratum. This in turn depends upon the concentration of the ligand (e.g., the density of the matrix element deposited on the coverslip), the concentration of the ligand receptor (e.g., integrin expression levels), and the ligand receptor binding affinity (Palecek et al., 1997). Optimal migration conditions for substrates can be determined empirically by measuring migration of cells plated onto coverslips coated with varying concentrations of substrate. When using a range of substrate concentrations, one should add an appropriate amount of albumin to keep the concentration of total protein constant, otherwise there may be areas of exposed glass at the low end of the substrate concentration range. Because contact guidance or exposed glass surfaces can bias cell migration, it is wise to verify that substrates have been deposited evenly. This can be accomplished by fluorescently labeling the substrate directly or indirectly via immunofluorescence (Marks et al., 1991) and evaluating the fluorescence intensity profile across the coverslips.

Finally, neutrophil migration is sensitive to temperature, with the complete abrogation of migration occurring at temperatures not much above 37°C. Neutrophils migrate well, though more slowly, even at room temperature, so it is sensible to perform migration assays slightly below 37°C (~35°C) to avoid deleterious effects of higher temperatures.

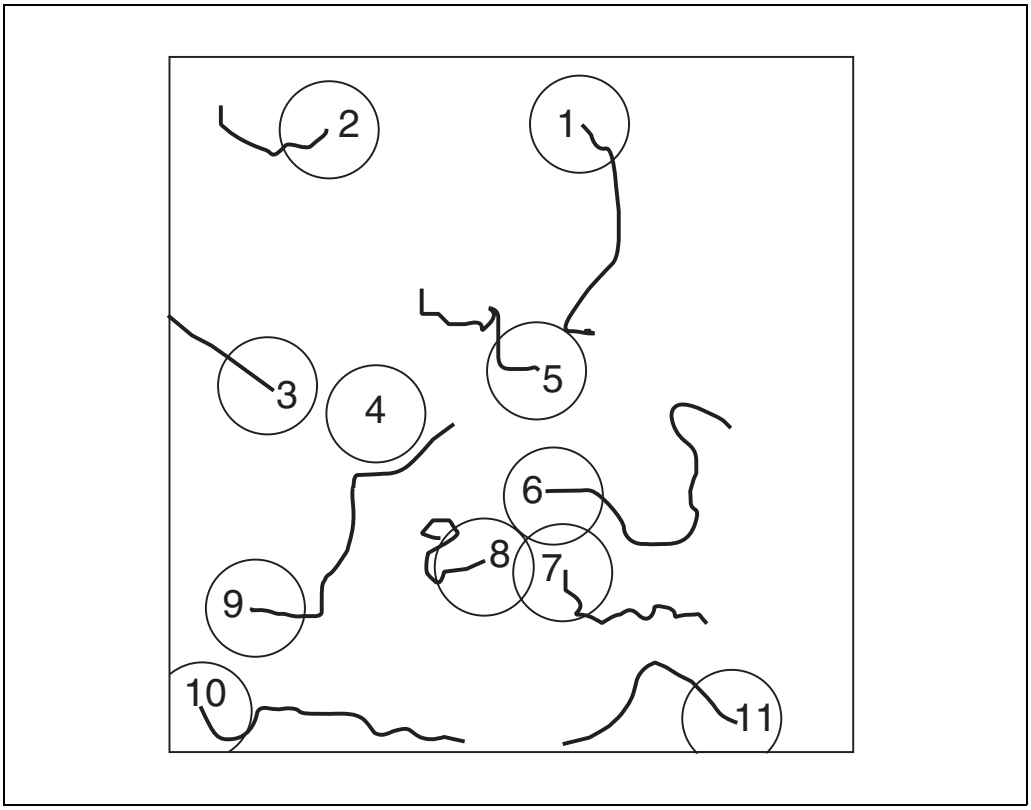


Figure 12.6.2 Tracings of cell paths. An image of a field of fMLF-stimulated neutrophils was acquired every 12 sec for 5 min (see Fig. 12.6.1C and D for initial and final images, respectively). 14-μm-diameter circles were drawn around the cells in the first image (Fig. 12.6.1C), and the positions of cell centroids were marked for each image in the sequence. Centroid positions were then connected to indicate the paths traveled by each cell.

Table 12.6.2 Possible Phenotypes of Neutrophils Observed in Optical Microscopy–Based Migration Assays

Phenotype	Potential cause	Reference
Prominent, elongated uropod (Fig. 12.6.4B)	Inability to detach from substratum. Cells with this defect will make attempts to move forward, but will usually "snap back" to their starting positions because their rears are tightly attached to the substratum. Inability to retract tail. Cells with this defect will move forward slowly, dragging along their elongated tails.	Hendey and Maxfield (1993); Marks et al. (1991); Pierini et al. (2000)
Few or no cells on dish, but remaining cells are still polarized	Adhesion inhibited. Neutrophils do not require adhesive interactions to polarize. Separate polarization and adhesion assays can be performed to verify conclusions.	Graham and Brown (1991); Wilkinson (1998)
Cells remain symmetrical in shape, without a discernible front or rear (Fig. 12.6.4D,F)	Inhibition of cell contraction. Cells with this defect can send out membrane protrusions, but cannot undergo the contractions required to form a polarized morphology. Inhibition of membrane extension. Cells with this defect may look as if they are unstimulated.	Eddy et al. (2000)

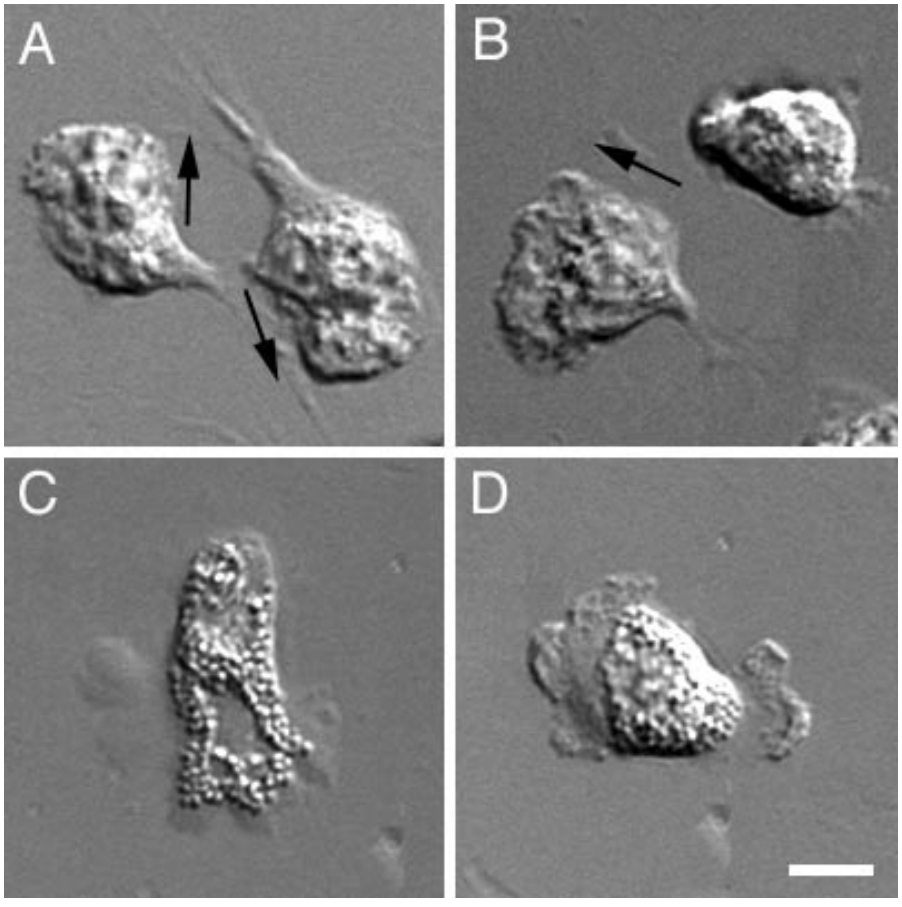


Figure 12.6.3 Comparison of eosinophil and neutrophil morphology. Differential interference contrast images of neutrophils (**A**, **B**) and eosinophils (**C**, **D**) are shown. Note the large refractile granules in the eosinophils. Arrows indicate direction of neutrophil migration. Bar is equal to 10 μm.

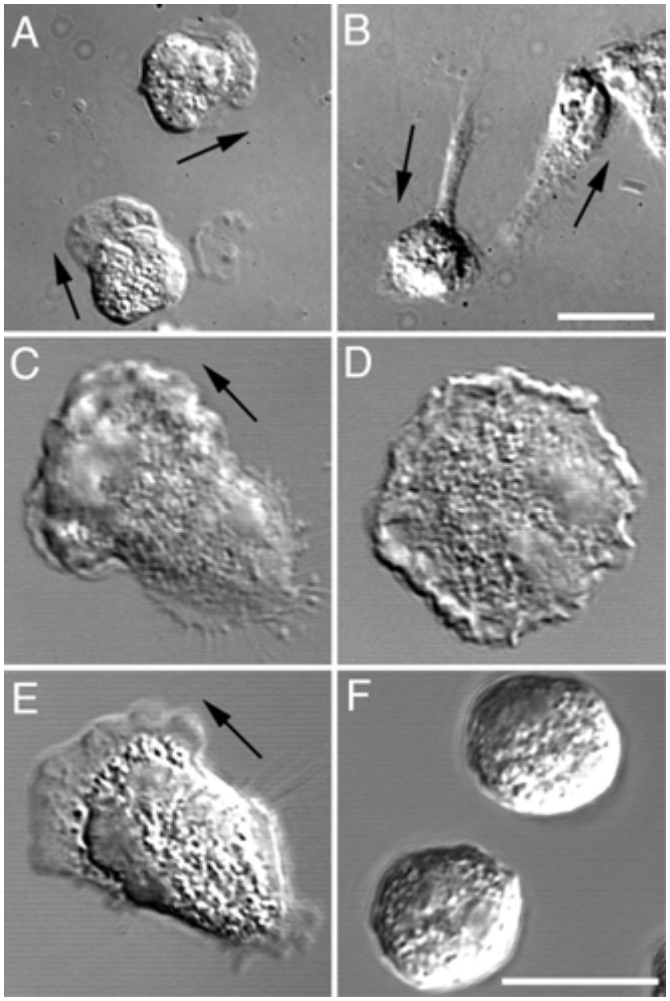


Figure 12.6.4 Examples of phenotypes resulting from various treatments of neutrophils. Images of control (**A**, **C**, **E**) and treated (**B**, **D**, **F**) neutrophils are shown after 2 min stimulation with fMLF. When intracellular calcium is buffered, neutrophils can polarize, but they cannot migrate because they are unable to detach from the substratum (Hendey and Maxfield, 1993; Marks et al., 1991; Pierini et al., 2000). Note the prominent elongated tails in panel B. In contrast, when either myosin light chain kinase is inhibited (Eddy et al., 2000; panel D) or cholesterol is depleted (Pierini and Maxfield, unpub. observ.; panel F), neutrophils can neither polarize nor migrate. These images make it evident that the underlying cause for each of these migration defects is different (see Table 12.6.2). Arrows indicate direction of neutrophil migration. Bars are equal to 10 μm. Images in panels C and D courtesy of Dr. Robert J. Eddy.

Troubleshooting

Table 12.6.1 provides a list of problems that can arise in the neutrophil migration assay, along with their possible causes and solutions.

Anticipated Results

Neutrophils go through a sequence of morphological changes following activation (Seveau et al., 2001; Zigmond and Sullivan, 1979; Fig. 12.6.1). When neutrophils are unstimulated, they are very nearly spherical in shape (Fig. 12.6.1A) and do not adhere tightly to the coverslip. Within 15 to 30 sec after the addition of chemoattractant, the cell periphery becomes irregular, with small membrane protrusions and retractions occurring in every direction (Fig. 12.6.1B). By 2 min, the cells take on a polarized morphology with a distinct front (lamellipod) and rear (uropod) that will orient randomly in the field when chemoattractant is present isotropically (Fig. 12.6.1C).

Less than 10% of the neutrophils should polarize and migrate spontaneously (that is, in the absence of chemoattractant), compared to 60% to 90% of the cells polarizing and migrating after addition of chemoattractant (compare

panels A and C in Fig. 12.6.1). The numbers for spontaneous and stimulated migration will vary somewhat from day to day and will depend on the donor, the basal level of activation of the isolated neutrophils, the temperature of the microscope and buffers during the assay, and other parameters such as the migration matrix and chemoattractant used.

Representative of a typical data set, panels C and D in Figure 12.6.1 show images of a field of neutrophils at 1 min and 6 min after stimulation with fMLF, respectively. Figure 12.6.2 shows the paths traveled by the cells as they moved from their starting positions in panel C to their final positions in panel D. Note that most of the cells have moved several cell lengths during the 5-min course of the assay. Though not readily apparent from this relatively short migration assay, neutrophils normally exhibit a "persistent random walk," with long stretches of approximately straight-line runs between infrequent turns (Fig. 12.6.2; see cells 1, 3, 9, and 10).

Up to 5% of the cells in the field may be eosinophils, as opposed to neutrophils, and it is important to be able to distinguish these cells from neutrophils so that they can be removed from the analysis. Eosinophils (Fig. 12.6.3C,D) tend to have more prominent granules than neutrophils (Fig. 12.6.3A,B).

Often it is informative to evaluate the effects of a pharmacological agent or other treatment on cell migration, using visual analysis to discern the likely basis for any effects on migration. Some phenotypes that can result, along with their possible causes, are listed in Table 12.6.2 and illustrated in Figure 12.6.4. This table is not meant to be comprehensive, only illustrative of how observation of cell morphology and/or behavior can lead to a mechanistic understanding. A particular treatment may have significantly subtler effects on cell migration that may only become apparent after careful analysis of cell paths and behavior.

Time Considerations

The entire procedure, from isolation of cells (~1.25 hr) and preparation of coverslip dishes (~1 hr) to acquisition of three 4-min image series from each of ten dishes can be accomplished in ~4 hr. Coverslips and coverslip dishes can be prepared and assembled ahead of time, with the exception of coating the coverslips with extracellular matrix component, which should be done on the day of the experiment. The time required for image analysis will depend on the exact nature of the analysis and whether automation is employed.

Literature Cited

Allan, R., and Wilkinson, P. 1978. A visual analysis of chemotactic and chemokinetic locomotion of human neutrophil leucocytes. Use of a new chemotaxis assay with *Candida albicans* as gradient source. *Exp. Cell Res*. 111:191-203.

Boyden, S.V. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. Exp. Med*. 115:453-466.

Dixon, H.M., and McCutcheon, M. 1936. Chemotropism of leukocytes in relation to their rate of locomotion. *Proc. Soc. Exp. Biol. Med*. 34:173-176.

Dunn, G.A. 1981. Chemotaxis as a form of directed cell behaviour: Some theoretical considerations. *In* Biology of the Chemotactic Response (J.M. Lackie and P.C. Wilkinson, eds.) p. 1-26. Cambridge University Press, Cambridge, U.K.

Eddy, R., Pierini, L., Matsumura, F., and Maxfield, F. 2000. Ca^{2+}-dependent myosin II activation is required for uropod retraction during neutrophil migration. *J. Cell Sci*. 113:1287-1298.

Graham, I.L., and Brown, E.J. 1991. Extracellular calcium results in a conformational change in Mac-1 (CD11b/CD18) on neutrophils. Differentiation of adhesion and phagocytosis functions of Mac-1. *J. Immunol.* 146:685-691.

Haston, W. and Wilkinson, P. 1987. Gradient perception by neutrophil leucocytes. *J. Cell Sci.* 87:373-374.

Haston, W. and Wilkinson, P. 1988. Locomotion and chemotaxis of leukocytes: Gradient perception and locomotor capacity. *Curr. Opin. Immunol.* 1:5-9.

Hendey, B. and Maxfield, F.R. 1993. Regulation of neutrophil motility and adhesion by intracellular calcium transients. *Blood Cells* 19:143-164.

Keller, H., Wilkinson, P., Abercrombie, M., Becker, E., Hirsch, J., Miller, M., Ramsey, W., and Zigmond, S. 1977a. A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Cell Biol. Int. Rep*. 1:391-397.

Keller, H., Wilkinson, P., Abercrombie, M., Becker, E., Hirsch, J., Miller, M., Ramsey, W., and Zigmond, S. 1977b. A proposal for the definition of terms related to locomotion of leukocytes and other cells. *J. Immunol*. 118:1912-1914.

Keller, H., Wilkinson, P., Abercrombie, M., Becker, E., Hirsch, J., Miller, M., Scottramsey, W., and Zigmond, S. 1977c. A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Clin. Exp. Immunol*. 27:377-380.

Mandeville, J., Ghosh, R., and Maxfield, F. 1995. Intracellular calcium levels correlate with speed and persistent forward motion in migrating neutrophils. *Biophys. J*. 68:1207-1217.

Mandeville, J., Lawson, M., and Maxfield, F. 1997. Dynamic imaging of neutrophil migration in three dimensions: Mechanical interactions between cells and matrix. *J. Leukocyte Biol*. 61:188-200.

Marks, P.W., Hendey, B., and Maxfield, F.R. 1991. Attachment to fibronectin or vitronectin makes human neutrophil migration sensitive to alterations in cytosolic free calcium concentration. *J. Cell Bio*. 112:149-158.

McCutcheon, M. 1946. Chemotaxis in leukocytes. *Physiol. Rev*. 26:319-336.

Metchnikoff, E. 1893. Lectures on the comparative pathology of inflammation. Kegan Paul, London.

Palecek, S., Loftus, J., Ginsberg, M., Lauffenburger, D., and Horwitz, A. 1997. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* 385:537-540.

Pierini, L., Lawson, M., Eddy, R., Hendey, B., and Maxfield, F. 2000. Oriented endocytic recycling of alpha5beta1 in motile neutrophils. *Blood* 95:2471-2480.

Pytowski, B., Maxfield, F., and Michl, J. 1990. Fc and C3bi receptors and the differentiation antigen BH2-Ag are randomly distributed in the plasma membrane of locomoting neutrophils. *J. Cell Biol*. 110:661-668.

Seveau, S., Eddy, R., Maxfield, F., and Pierini, L. 2001. Cytoskeleton-dependent membrane domain segregation during neutrophil polarization. *Mol. Biol. Cell* 12:3550-3562.

Wilkinson, P. 1996. Cell locomotion and chemotaxis: Basic concepts and methodological approaches. *Methods* 10:74-81.

Wilkinson, P. 1998. Assays of leukocyte locomotion and chemotaxis. *J Immunol. Methods* 216:139-153.

Wilkinson, P. and Lackie, J. 1983. The influence of contact guidance on chemotaxis of human neutrophil leukocytes. *Exp. Cell Res*. 145:255-264.

Zicha, D., Dunn, G., and Brown, A. 1991. A new direct-viewing chemotaxis chamber. *J. Cell Sci*. 99:769-775.

Zicha, D., Dunn, G., and Jones, G. 1997. Analyzing chemotaxis using the Dunn direct-viewing chamber. *Methods Mol. Biol*. 75:449-457.

Zigmond, S. 1977. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol*. 75:606-616.

Zigmond, S. and Sullivan, S. 1979. Sensory adaptation of leukocytes to chemotactic peptides. *J. Cell Biol*. 82:517-527.

Contributed by Lynda M. Pierini and
Frederick R. Maxfield
Weill Medical College of Cornell University
New York, New York

Actin-Based Motility Assay

This unit describes an in vitro assay to study actin-based motility in which the propulsion of the bacteria *Listeria* and *Shigella* or functionalized microspheres is reconstituted from five pure proteins (see Basic Protocol). The pathogens *Listeria* and *Shigella* are acknowledged models of the leading edge of motile cells. By quantitating the dependence of motility parameters on the concentrations of components of the assay, the molecular processes that support the motile behavior of living cells can be understood, predictions of different theoretical models can be evaluated, and insight into the role of newly discovered proteins in motility can be obtained. Data analysis for the assay is described in Support Protocol 1.

This unit includes support protocols for biochemical purification of profilin (see Support Protocol 2), actin-depolymerizing factor (ADF; see Support Protocol 3), gelsolin (see Support Protocol 4), neural Wiskott-Aldrich syndrome protein (N-WASP; see Support Protocol 5), and Arp2/3 complex (actin-related protein; see Support Protocol 6), and for the preparation of N-WASP-coated microspheres (see Support Protocol 7).

STRATEGIC PLANNING

Performing a successful motility assay requires 1 month of preparatory biochemistry. Preparation of the main biochemical components for the motility assay is detailed in Support Protocols 2 to 6. These components must be prepared in advance, dispensed into aliquots of appropriate size, and stored at −80°C. Two different types of animal tissue must be obtained for the various preparations: bovine brain and bovine spleen. The tissues must be obtained from a freshly slaughtered cow in a slaughterhouse and used immediately or flash-frozen. Actin purification from rabbit muscle is also required for the motility assay (see *UNIT 13.2*).

The biochemical purifications represent a challenging task. The reader should also note that several proteins used in the assay (actin, rhodamine-labeled actin, and Arp2/3) are now commercially available (Cytoskeleton, Inc).

Motility assays are performed under optimal conditions using functionalized microspheres. Such particles can indeed be coated with N-WASP (see Support Protocol 7) or ActA in a chemically controlled and reproducible fashion. Use of bacteria (*Listeria* or *Shigella*) is recommended in restricted cases where *Listeria* strains mutated in ActA or *Shigella* strains mutated in IcsA (the bacterial activator of N-WASP) are to be examined.

ACTIN-BASED MOTILITY ASSAY

BASIC PROTOCOL

This protocol describes the execution of an assay that reconstitutes the propulsion of *Listeria*, *E. coli* expressing the *Shigella* protein IcsA, or N-WASP-functionalized microspheres in a minimum motility medium containing Arp2/3 complex, ADF, gelsolin, profilin, and actin (Loisel et al., 1999).

Materials

Profilin (see Support Protocol 2)
ADF (see Support Protocol 3)
Gelsolin (see Support Protocol 4) or other capping protein
VASP (for use with *Listeria* or ActA-functionalized microspheres only; prepare as for N-WASP, see Support Protocol 5)

Contributed by Christophe Le Clainche and Marie-France Carlier
Current Protocols in Cell Biology (2004) 12.7.1-12.7.20

Arp2/3 complex *or* Alexa488-Arp2/3 complex (see Support Protocol 6)
Assay buffer (see recipe) containing 1% (w/v) bovine serum albumin (BSA)
10% (w/v) bovine serum albumin (BSA) in distilled water
5 μM α-actinin (Sigma) in distilled H_2O
48 μM Mg-F-actin (see recipe)
1% (w/v) methylcellulose (see recipe)
ATP/DABCO/DTT mix (see recipe)
40 μM rhodamine-G-actin (Cytoskeleton, Inc.) in assay buffer/1% BSA (store up to 1 month at −80°C)
4×10^9 microspheres/ml suspension of N-WASP-functionalized microspheres (see Support Protocol 7) *or* 4×10^9 bacteria/ml suspension of *Listeria* or *E. coli* IcsA in assay buffer/1% BSA
Valap (*UNIT 13.1*)

Glass slides (Superfrost Plus, Fisher, or Menzel-Gläser; 25 × 75 × /mm)
Glass coverslips (22 × 22 mm)
Microscope, phase-contrast or fluorescence (with appropriate filters; *UNIT 4.2*)

1. Rapidly thaw ADF, profilin, Arp2/3 complex, gelsolin, and VASP, and dilute to the appropriate stock concentrations (see Table 12.7.1) with assay buffer/1% BSA.
2. Combine the components of the actin-based motility assay in a 0.5 ml tube in the (top-to-bottom) order indicated in Table 12.7.1.

Table 12.7.1 Components of Actin-Based Motility Assay

Ingredient[a]	Stock concentration	Volume added	Final concentration
BSA	10% (w/v)	1.2 μl	0.5%
ADF	55 μM	1.6 μl	3.7 μM
Profilin	50 μM	1.2 μl	2.5 μM
Gelsolin[b]	0.5 μM	2.4 μl	50 nM
α-Actinin	5 μM	1.2 μl	0.25 μM
Arp2/3[c]	1 μM	1.8 μl	75 nM
VASP[d]	8 μM	1.4 μl	0.47 μM
Mg-F-actin	48 μM	3.8 μl	7.6 μM
Methylcellulose	1% (w/v)	5.4 μl	0.23%
ATP/DABCO/DTT mix	see Reagents and Solutions	3.0 μl	—
Rhodamin-G-actin	40 μM	0.6 μl	1 μM
N-WASP functionalized microspheres or *Listeria* or *E. coli* IcsA	4×10^9 microspheres or bacteria per ml	0.6 μl	2×10^8 microspheres or bacteria/ per ml
Final volume		24 μl	

[a]Abbreviations: Arp2/3, actin-related protein; ADF, actin-depolymerizing factor; ATP, adenosine triphosphate; BSA, bovine serum albumin; DABCO, 1.4.-diazobicyclo[2,2,2]octane; DTT, dithiothreitol; N-WASP, neural Wiskott-Aldrich syndrome protein; VASP, vasodilator-stimulated phosphoprotein.
[b]Other capping proteins may be used.
[c]Alexa488-Arp2/3 (also prepared as in Support Protocol 6) may be used.
[d]Add only for assays using *Listeria* or ActA-functionalized microspheres in place of the N-WASP functionalized microspheres (see Strategic Planning).

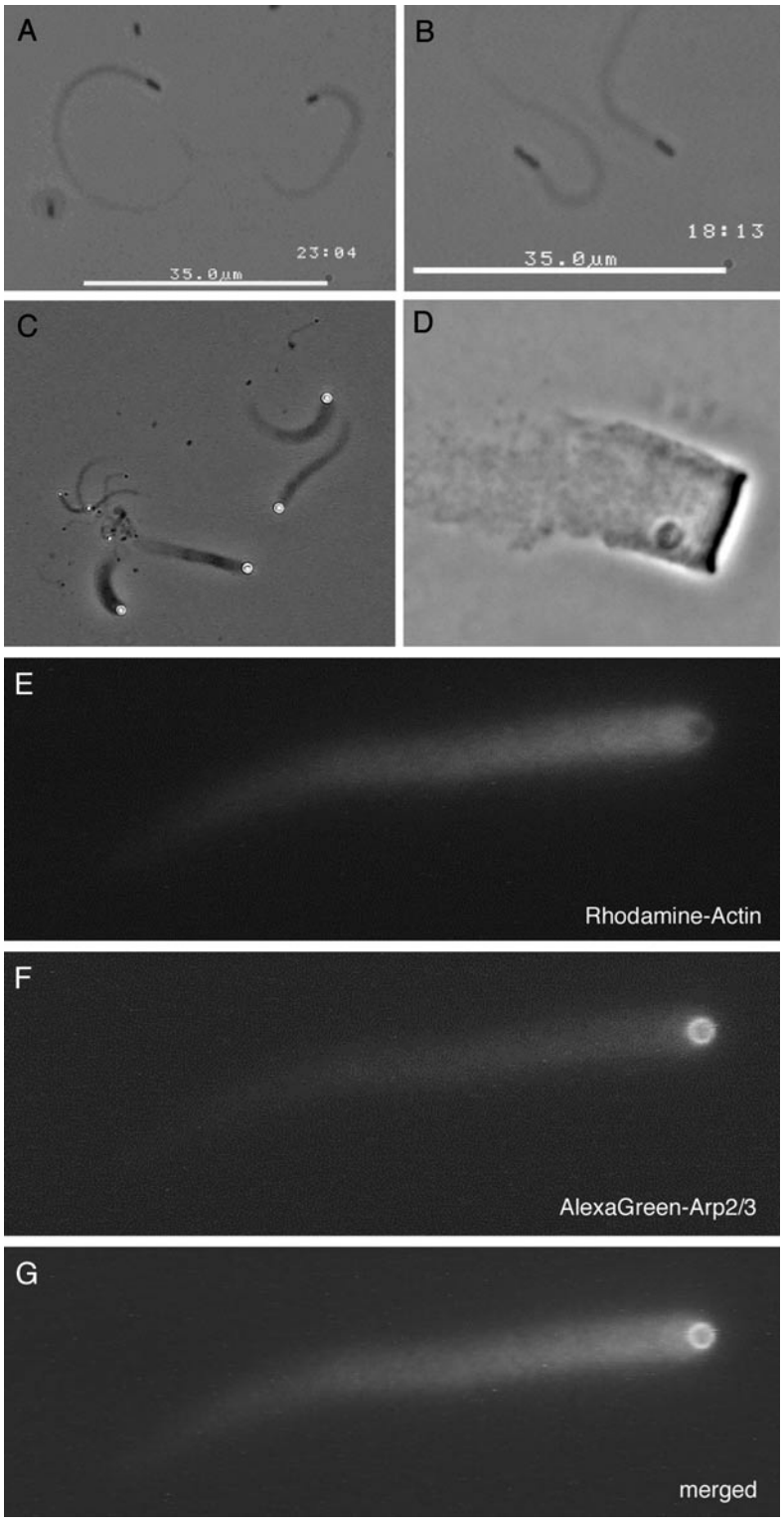


Figure 12.7.1 Examples of actin-based motility of bacteria or functionalized particles in the reconstituted motility assay. Synchronous films of up to four selected fields per chamber were recorded with a CCD camera on an Olympus AX70 microscope equipped with a motorized stage (Märzhäuser) using MetaMorph 4.6 for microscope control and image acquisition. (**A**) *Listeria monocytogenes*; (**B**) *E. coli* IcsA (substitute for *Shigella flexneri*); (**C**) N-WASP-coated polystyrene beads of different diameters; (**D**) N-WASP-coated glass rod (1 µm in diameter); (**E, F, G**) double-label experiment using N-WASP-coated beads in motility assay containing rhodamine-labeled actin and Alexa488-labeled Arp2/3 (from Wiesner et al., 2003). Both the surface of the bead and the tail are labeled by Arp2/3.

Because high concentrations of methylcellulose can cause excessive filament bundling, all components excluding methyl cellulose and F-actin must be mixed first.

Although α-actinin cross-links actin filaments and increases the density of the actin tail, this protein is not essential and can be omitted.

If the pipetting scheme given in Table 12.7.1 is modified (e.g., to vary the concentration of some component), the resulting change in volume of the assay should be such that all concentrations of other protein, BSA, and nonprotein components remain unchanged. To make up the final volume use assay buffer/1% BSA.

3. Pipet 3 μl of the mixture on a glass slide and cover with a coverslip. Seal the preparation with Valap.

   Be careful to avoid introducing bubbles between the slide and the coverslip.

   For microspheres of diameter >5 μm, do not compress the coverslip onto the slide; rather, use a larger sample volume and chamber slide to avoid sticking of the bead to the glass surfaces of the slide and coverslip.

4. Observe under a phase-contrast or fluorescence microscope at room temperature (see Anticipated Results and Figure 12.7.1). Record up to four selected fields per chamber with a CCD camera, using MetaMorph 4.6 for microscope control and image acquisitiion. Analyze data (Support Protocol 1).

   When optimal conditions are used, the actin tails can be observed in phase contrast using a 20× phase-contrast objective (NA 0.5). Fluorescence is useful to colocalize a fluorescent protein like Alexa488-labeled Arp2/3 with the fluorescent rhodamine-actin tail.

   If the rhodamine-labeled actin and/or Alexa488-labeled Arp2/3 photobleach rapidly, the concentrations of DABCO, an oxygen-scavenging component, and DTT, a reducing agent, should be checked (see Reagents and Solutions, recipe for ATP/DABCO/DTT mix).

SUPPORT PROTOCOL 1

DATA ANALYSIS

The reconstituted motility assay can be used as an analytical tool. Parameters that have been exploited are speed, percentage of motile particles, length of the actin tail, and density of actin and Arp2/3 in the actin tail.

Average rates of movement are determined by selecting freely moving microspheres from several different fields in the motility assay recordings. For microspheres of 0.5-μm diameter and larger, the template recognition–based tracking tool of MetaMorph (Universal Imaging) is used to measure mean velocities. In this assay, 100% of the beads move. Generally, when bacteria are used (see Strategic Planning), less than 100% of the bacteria move. The fraction of motile bacteria, in this case, is an additional parameter that should be considered in characterizing the motile behavior.

To determine F-actin and Arp2/3 densities in the actin tails, first, rhodamine-labeled actin and Alexa488-labeled Arp2/3 are used in the motility assays, and their respective fluorescence intensities are recorded with a LHESA 72LL CCD camera using appropriate filters and a 100 objective (NA 1.35). The extent of Arp2/3 labeling (100%) and of actin labeling (10%) is chosen to yield intensities in the linear response range of the camera for both fluorophores. Control experiments are performed to make sure that no emission overlap exists between the two fluorophores. Images of comets are recorded at steady state, and average emission intensities of rhodamine (IR) and Alexa488 (IA) are measured as mean gray values in a square section of the actin tail at least one bead diameter behind the bead. An average IR/IA ratio is determined from sets of at least five microspheres. To estimate the absolute stoichiometry of actin and Arp2/3 from these values, a calibration

is performed using solutions of the two fluorescent proteins at different concentrations (Wiesner et al., 2003).

The percentage of motile microspheres is determined manually by counting the ratio of motile particles to total particles. Only central regions are used because the edge of the preparation displays significant differences in the percentage of motile microspheres.

PROFILIN PURIFICATION

SUPPORT PROTOCOL 2

Recombinant profilin from different sources or profilin purified from bovine spleen can be used. Here, the purification of profilin from bovine spleen (Perelroizen et al., 1996) is described.

Materials

Bovine spleen (see Strategic Planning)
Profilin buffer A (see recipe) containing 0.5% (v/v) Triton X-100
200 mM PMSF stock in absolute ethanol
4 M and 7 M urea in profilin buffer A (see recipe for buffer)
Profilin buffer B (see recipe)
Liquid nitrogen
Centrifuge with Sorvall SLA 1500 rotor (or equivalent)
Cheescloth
Ultracentrifuge with Sorvall A641 rotor (or equivalent)
15-cm length × 2.5-cm inner diameter poly-L-proline-Sepharose column (see recipe)
10,000-kDa MWCO dialysis membrane
Centriprep 10 centrifugal concentrator (Millipore; also see *APPENDIX 3C*)
Ultracentrifuge with Beckman TLA 100.3 rotor (or equivalent)
Additional reagents and equipment for dialysis and concentration of protein solutions (*APPENDIX 3C*) and determination of protein concentration (*APPENDIX 3B*)

Prepare tissue homogenate

1. Homogenize 240 g of frozen spleen in 350 ml of profilin buffer A/0.5 % Triton X-100. Add PMSF (from 200 mM stock) to 1 mM final and homogenize 45 sec.
2. Centrifuge 45 min at 22,000 × *g* (12,000 rpm in a Sorvall SLA 1500 rotor), 4°C. Filter the supernatant through eight layers of cheesecloth.
3. Centrifuge the filtered supernatant for 1 hr and 45 min at 200,000 × *g* (39,000 rpm in a Sorvall A641 rotor), 4°C. Filter again through eight layers of cheesecloth.

Purify by affinity chromatography

4. Add the sample to a 15-cm length × 2.5-cm inner diameter poly-L-proline-Sepharose column equilibrated with profilin buffer A. Wash the column with 400 ml profilin buffer A.
5. Elute actin with 250 ml 4 M urea in profilin buffer A, monitoring absorbance at 280 nm.
6. Elute profilin with 250 ml 7 M urea in profilin buffer A, monitoring absorbance at 280 nm.
7. Wash the column with 500 ml water.

Purify by dialysis

8. Combine fractions containing protein eluted by 7 M urea and dialyze 18 hr against two changes of 1000 ml profilin buffer B, using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).

9. Concentrate dialyzed protein on Centripep 10 centrifugal concentrator according to the manufacturer's instructions.

10. Dialyze again once for 18 hr against 1000 ml profilin buffer B, using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).

11. Ultracentrifuge 15 min at 334,000 × *g* (90,000 rpm in a Beckman TLA 100.3 rotor), 4°C. Retain supernatant.

12. Measure the protein concentration of the supernatant using UV absorbance (*APPENDIX 3B*). Freeze in liquid nitrogen in 0.1-ml aliquots and store at −80°C.

Profilin can be stored for months at −80°C. Thawed aliquots can be stored on ice for a few weeks without loss of activity.

SUPPORT PROTOCOL 3

ADF PURIFICATION

Bacterial recombinants of ADFs from different sources (plant, amoeba, and vertebrate) have been used successfully in the motility medium. Here the purification of recombinant human ADF expressed in *E. coli* (Carlier et al., 1997) is described.

Materials

E. coli BL21 DE3 strain transformed with pET16-ADF (see *APPENDIX 3A* for transformation of *E. coli* with plasmid)
LB medium (*APPENDIX 2A*) containing 0.2 mg/ml ampicillin
ADF buffers A, B, C, D, and E (see recipes)
10 mg/ml lysozyme
250 mM EDTA
200 mM PMSF in absolute ethanol
1 M $MgCl_2$
2 mg/ml DNase I
Liquid nitrogen

Centrifuge with Sorvall GSA rotor (or equivalent)
Probe sonicator (Fisher)
Centrifuge with Sorvall SS-34 rotor (or equivalent)
10,000-kDa MWCO dialysis membrane
Ultracentrifuge with Beckman TL-100 rotor (or equivalent)
DEAE (DE-52; Whatman) column (20 × 2.5 cm; *UNIT 10.11*)
Stirred cell with Diaflo PM 10 ultrafiltration membrane (Amicon)
SP trisacryl column (15 × 1.5 cm; BioSepra)

Additional reagents and equipment for transforming and growing bacteria and induction of protein expression with IPTG (*APPENDIX 3A*), dialysis (*APPENDIX 3C*), and determination of protein concentration (*APPENDIX 3B*)

Express the protein and lyse the cells

1. Grow *E. coli* BL21 DE3 strain transformed with pET16-ADF in LB medium containing 0.2 mg/ml ampicillin at 37°C. At OD = 1, add 0.5 mM IPTG to induce ADF expression. Incubate 2 hr and 30 min at 37°C.

APPENDIX 3A provides cross-references for the molecular biology techniques used in this step.

2. Centrifuge 10 min at 4000 × *g* (5000 rpm in a Sorvall GSA rotor), 4°C. Resuspend pellet in 100 ml ADF buffer A. Rapidly freeze-thaw the homogenate.
3. Add 1 ml of 10 mg/ml lysozyme. Incubate 5 min, then add 1 ml of 250 mM EDTA and incubate an additional 5 min.
4. Add 250 µl of 200 mM PMSF. Sonicate three times, each time for 1 min at 150 W power in a probe sonicator on ice.
5. Add 500 µl of 1 M $MgCl_2$ and 1 ml of 2 mg/ml DNase I. Incubate 5 min at room temperature.

Purify the protein

6. Centrifuge 30 min at 17,000 × *g* (12,000 rpm in a Sorvall SS-34 rotor), 4°C.
7. Dialyze supernatant overnight at 4°C against 1000 ml of ADF buffer B using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
8. Ultracentrifuge 45 min at 100,000 × *g*, 4°C.
9. Load the sample onto a 20 × 2.5–cm size DEAE column. Elute with ADF buffer B Collect fractions at 2 ml/min and 2 min/fraction.
10. Concentrate the flowthrough to 10 ml by ultrafiltration in a stirred cell with a Diaflo PM 10 membrane.
11. Dialyze overnight at 4°C against 1000 ml ADF buffer C using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
12. Load the sample onto a 1.5 × 15 size SP trisacryl column. Elute with 100 ml ADF buffer D. Collect fractions at 1 ml/min and 2 min/fraction.
13. Concentrate to 3 ml and dialyze overnight at 4°C against 1000 ml ADF buffer E using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
14. Ultracentrifuge the dialysate 15 min at 313,000 × *g* (90,000 rpm in a Beckman TL-100 rotor), 4°C.
15. Measure the concentration using UV absorbance (*APPENDIX 3B*). Freeze 0.1-ml aliquots in liquid nitrogen and store at −80°C.

ADF can be store for months at −80°C. Thawed aliquots can be stored on ice for a few weeks without loss of activity.

GELSOLIN PURIFICATION

SUPPORT PROTOCOL 4

Although in the original protocol published by Loisel et al. (1999) Capping Protein was used, further assays showed that any other capping protein can work as well as Capping Protein, i.e., the general barbed-end capping function is required for motility. In particular, gelsolin, which can easily be obtained as a bacterially expressed recombinant protein, supports motility.

Materials

E. coli BL21 DE3 strain expressing gelsolin (see *APPENDIX 3A* for transformation of *E. coli*)
Gelsolin buffers A, B, C, and D (see recipes)
1 M $MgCl_2$
2 mg/ml DNase I
500 mM NaCl in gelsolin buffer B (see recipe for buffer)
Anti-gelsolin antibody (Sigma)

500 mM NaCl in gelsolin buffer C (see recipe for buffer)
Liquid nitrogen

Probe sonicator (Fisher)
Refrigerated centrifuge
Centriprep 30 centrifugal concentrator
30,000-kDa MWCO dialysis membrane
2.5 × 20–cm DEAE-cellulose (DE-52; Whatman) column (*UNIT 10.11*)
Gradient maker
11 × 1.25–cm SP trisacryl column (BioSepra)

Additional reagents and equipment for transforming and growing bacteria and induction of protein expression with IPTG (*APPENDIX 3A*), dialysis (*APPENDIX 3C*), and determination of protein concentration (*APPENDIX 3B*), and immunodetection (*UNIT 6.2*)

1. Grow *E. coli* BL21 DE3 expressing gelsolin (see *APPENDIX 3A* for molecular biology techniques).
2. Add 100 ml gelsolin buffer A per liter of bacteria grown. Incubate 10 min at 4°C, then sonicate five times, each time for 1 min at 150 W power.
3. Add $MgCl_2$ from 1 M stock to a final concentration of 5 mM and 2 mg/ml DNase I to a final concentration of 0.02 mg/ml. Incubate 10 min at 4°C.
4. Centrifuge 30 min at 18,000 × *g*, 4°C.
5. Dialyze overnight at 4°C against 1000 ml gelsolin buffer B using a 30,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
6. Add the sample to a DEAE column equlibrated in gelsolin buffer B. Prepare a 1-liter, 0 to 500 mM linear NaCl gradient by placing 500 ml gelsolin buffer B in one compartment of a gradient maker and 500 ml of 500 mM NaCl (in gelsolin buffer B) in the other compartment. Apply the gradient to elute.
7. Identify the gelsolin-containing fractions by immunodetection (*UNIT 6.2*) using anti-gelsolin antibody. Combine desired fractions, then concentrate using a Centriprep 30 centrifugal concentrator and dialyze overnight at 4°C against 1000 ml gelsolin buffer C using a 30,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
8. Add the sample to an 11 × 1.25–cm SP trisacryl column equilibrated in gelsolin buffer C. Prepare a 300-ml, 0 to 500 mM linear NaCl gradient by placing 150 ml gelsolin buffer C in one compartment of the gradient maker and 150 ml of 500 mM NaCl (in gelsolin buffer C) in the other compartment. Apply the gradient to elute.
9. Identify the gelsolin-containing fractions by immunodetection (*UNIT 6.2*) using anti-gelsolin antibody. Combine desired fractions and dialyze overnight at 4°C against 1000 ml gelsolin buffer D. Freeze in liquid nitrogen and store at −80°C.

SUPPORT PROTOCOL 5

N-WASP PURIFICATION

His-tagged human N-WASP is expressed in *Sf*9 cells using the baculovirus system (Egile et al., 1999). His-tagged human VASP (see Basic Protocol and Table 12.7.1 for applications) is purified using the same protocol (Laurent et al., 1999).

Materials

Insect (*Sf*9) cells expressing N-WASP (see *APPENDIX 3A* for transformation of insect cells)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

PBS (*APPENDIX 2A*) containing 0.5 mM PMSF (add from 200 mM PMSF stock in absolute ethanol)
N-WASP buffers A, B, C, D, E, and F (see recipes)
NaCl
Imidazole
50% (w/v) suspension of Ni-NTA resin (Qiagen)
Liquid nitrogen

Cell scraper
Clinical (tabletop) centrifuge
Dounce homogenizer
Probe sonicator (Fisher)
Ultracentrifuge with Beckman TL-100 rotor (or equivalent)
2.5 × 20–cm DEAE-cellulose (DE-52; Whatman) column (*UNIT 10.11*)
1 × 5 cm, 10-ml chromatography column
30,000-kDa MWCO SpectraPor 2 dialysis membrane (Spectrum)

Additional reagents and equipment for protein expression in insect cells (*APPENDIX 3A*) and dialysis (*APPENDIX 3C*)

1. Collect 5×10^8 insect cells with a cell scraper in PBS. Centrifuge 5 min at 500 × *g* (2000 rpm in a clinical centrifuge), room temperature.
2. Wash by adding 100 ml PBS containing 0.5 mM PMSF and centrifuging again as described above. Remove supernatant.
3. Resuspend cells in 10 ml N-WASP buffer A. Homogenize in a Dounce homogenizer, then sonicate three times, each time for 1 min at lowest power.
4. Ultracentrifuge 40 min at 334,000 × *g* (90,000 rpm in a Beckman TL-100 rotor), 4°C.
5. Dilute the supernatant to obtain 0.1 M NaCl. Add the sample to a DEAE column equilibrated in N-WASP buffer B.
6. Save the flowthrough, adjust the NaCl concentration up to 0.3 M, and add imidazole to a final concentration of 10 mM.
7. Equilibrate 4 ml of a 50% suspension of agarose Ni-NTA resin with 30 ml N-WASP buffer C.
8. Mix the DEAE flowthrough and the Ni-NTA resin for 1 hr at 4°C on an end-over-end rotator. Load the sample/resin onto a 10-ml column and wash twice, each time with 4 ml N-WASP buffer D. Elute by adding ten 0.4-ml aliquots of N-WASP buffer E.
9. Dialyze eluate twice, each time for 30 min at 4°C in 500 ml N-WASP buffer F using a 30,000-kDa MWCO SpectraPor2 dialysis membrane (*APPENDIX 3C*). Freeze in liquid nitrogen and store at −80°C.

N-WASP can be stored for months at −80°C. Thawed aliquots kept on ice can be used for motility for a week.

ARP2/3 PURIFICATION

SUPPORT PROTOCOL 6

Arp2/3 can be purified from a variety of sources, including amoeba (Mullins et al., 1998) and vertebrate tissues like thymus (Higgs et al., 1999) or brain (Egile et al., 1999). It is also possible to purify recombinant Arp2/3 expressed in insect cells. Here, the purification of Arp2/3 from bovine brain (Egile et al., 1999) is described.

Materials

Bovine brain (see Strategic Planning)
Arp2/3 buffers A, B, C, D, E, F, G, and H (see recipes)
2 M sucrose
100 μM Alexa488-C5-maleimide (Molecular Probes) in Arp2/3 buffer H (see recipe for buffer)
DTT

Hammer
Waring blender
Refrigerated centrifuge
30,000-kDa MWCO dialysis membrane
SP trisacryl column (4 × 30 cm; BioSepra)
Sepharose GSH-GST-VCA as described by Egile et al. (1999) 2-ml column containing
Disposable NAP-10 column (Amersham Biosciences)

Additional reagents and equipment for dialysis (*APPENDIX 3C*) and determination of protein concentration (*APPENDIX 3B*)

Purify Arp2/3 complex

1. Break 300 g frozen bovine brain in small pieces using a hammer and mix with 600 ml Arp2/3 buffer A at 4°C. Homogenize in a Waring blender three times at high speed, each time for 20 sec.
2. Centrifuge the homogenate 1 hr at 100,000 × *g*, 4°C.
3. Dialyze the supernatant overnight at 4°C in 4 liters Arp2/3 buffer B using a 30,000-kDa MWCO dialysis membrane.
4. Centrifuge the supernatant 30 min at 100,000 × *g*, 4°C.
5. Load the supernatant onto a 4 × 30–cm SP trisacryl column equilibrated with Arp2/3 buffer B. Wash the resin with 5 volumes of Arp2/3 buffer B, wash again with 150 ml Arp2/3 buffer C, then elute with 150 ml Arp2/3 buffer D.
6. Dialyze the peak fractions 18 hr against 500 ml of Arp2/3 buffer E using a 30,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
7. Load the dialysate on a 2-ml column containing 1 ml of Sepharose-GSH-GST-VCA equilibrated in Arp2/3 buffer E.
8. Wash with 10 ml Arp2/3 buffer F, then elute with 10 ml Arp2/3 buffer G.
9. Combine the fractions showing absorbance at 290 nm and dialyze 18 hr against 1000 ml of Arp2/3 buffer F using a 30,000-kDa dialysis membrane.
10. Adjust the concentration of the solution to 200 mM sucrose using a 2 M sucrose stock solution. Measure protein concentration. Freeze in liquid nitrogen and store at −80°C.

Arp2/3 can be stored for months at −80°C. Thawed aliquots kept on ice can be used for 2 days.

The activity of Arp2/3 can be monitored during the purification by measuring the stimulation of actin assembly using pyrene fluorescence as a probe. In such an assay, the cuvette contains 2.5 μM G-actin (10% pyrenyl-labeled), 0.5 μM VCA, and an aliquot of the fraction to be tested, in polymerization buffer (see recipe).

Fluorescently label Arp2/3 complex (optional)

11. Filter Arp2/3 complex through a NAP-10 column equilibrated with Arp2/3 buffer H, to remove DTT.
12. Incubate Arp2/3 complex with 100 μM Alexa488-C5-maleimide in Arp2/3 buffer H for 1 hr on ice, then add DTT to 1 mM to stop the labeling.
13. Add the sample to a NAP-10 column to separate the labeled protein from the free unreacted fluorophore. Elute with Arp2/3 buffer F. Keep Alexa488-Arp2/3 on ice and use within 3 days.

    Determine the labeling stoichiometry from the UV-visible spectrum of the labeled protein.

PREPARATION OF N-WASP-FUNCTIONALIZED MICROSPHERES

SUPPORT PROTOCOL 7

The successful reconstitution of actin-based motility of *Listeria* and *Shigella* opened the way for the design of a biomimetic system using carboxylated polystyrene microspheres functionalized with an activator of the Arp2/3 complex (N-WASP or ActA) and placed in the minimum motility medium. This protocol describes the procedure to functionalize the beads with N-WASP (Wiesner et al., 2003). The same procedure can be used to make ActA-coated beads (Samarin et al., 2003).

Materials

Polystyrene carboxylated microspheres of diameters from 0.2 to 3 μm (Polysciences)
N-WASP buffers A and C (see recipes)
4 μM N-WASP (see Support Protocol 5)
10 mg/ml BSA
Refrigerated centrifuge

1. Incubate polystyrene carboxylated microspheres of diameters from 0.2 to 3 μm in N-WASP buffer A containing 400 nM N-WASP (added from 4 μM stock) for 1 hr on ice.

   To prepare ActA-functionalized microspheres, use the same protocol but incubate the microspheres with 0.7 μM full-length ActA.

   The quantity of microspheres in a 50-μl suspension is adjusted so that the total surface of solid per unit volume of solution was 7.5 × 10^9 μm^2/ml, independent of the diameter of the microspheres.

2. Add 5 μl of 10 mg/ml BSA to block free adsorption sites and incubate 15 min on ice.
3. Centrifuge the microspheres 5 min at 10,000 × *g*, 4°C, then wash twice, each time by adding 250 μl N-WASP buffer A and centrifuging 5 min at 10,000 × *g*, 4°C. Resuspend final pellet in 50 μl N-WASP buffer C containing 1 mg/ml BSA.

   Functionalized microspheres have been stored on ice and used for up to 1 week without any change in their motile properties.

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ADF buffer A

10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EDTA
Store up to 1 week at 4°C

ADF buffer B

10 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
50 mM NaCl
5 mM DTT
0.2 mM EGTA
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

ADF buffer C

10 mM PIPES, pH 6.5
25 mM NaCl
5 mM DTT
0.2 mM EGTA
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

ADF buffer D

10 mM PIPES, pH 6.5
50 mM NaCl
5 mM DTT
0.2 mM EGTA
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

ADF buffer E

5 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM DTT
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

Arp2/3 buffer A

10 mM PIPES, pH 6.8
5 mM EGTA
1 mM DTT
0.1 mM PMSF added just before use
Store up to 1 week at 4°C

Arp2/3 buffer B

20 mM PIPES pH 6.8
1 mM DTT
Store up to 1 week at 4°C

Arp2/3 buffer C

20 mM PIPES, pH 6.8
30 mM KCl
1 mM DTT
Store up to 1 week at 4°C

Arp2/3 buffer D

20 mM PIPES, pH 6.8
100 mM KCl
1 mM DTT
Store up to 1 week at 4°C

Arp2/3 buffer E

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
25 mM KCl
0.25 mM DTT
0.1 mM ATP
1 mM $MgCl_2$
0.5 mM EDTA
Store up to 1 week at 4°C

Arp2/3 buffer F

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
25 mM KCl
0.25 mM DTT
10 μM ATP
1 mM $MgCl_2$
0.5 mM EDTA
Store up to 1 week at 4°C

Arp2/3 buffer G

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
25 mM KCl
0.25 mM DTT
10 μM ATP
200 mM $MgCl_2$
0.5 mM EDTA
Store up to 1 week at 4°C

Arp2/3 buffer H

20 mM HEPES, pH 7.2
0.2 mM $MgCl_2$
0.2 mM ATP
Store up to 1 week at 4°C

Assay buffer

10 mM HEPES pH 7.5
0.1 M KCl
1 mM $MgCl_2$
0.1 mM $CaCl_2$
1 mM ATP
Store up to 1 week at 4°C

ATP/DABCO/DTT mix

Add 15 μl of 0.2 M ATP, pH 7.0, and 6 μl 1 M $MgCl_2$ to 79 μl of distilled water to prepare a 30 mM ATP/60 mM $MgCl_2$ stock solution. Next, prepare the

continued

ATP/DABCO/DTT mix by mixing 40 µl of the 30 mM ATP/60 mM $MgCl_2$ stock solution with 20 µl of 2.2 mM 1,4-diazabicyclo[2.2.2]octane (DABCO; Molecular Probes) and 10 µl 0.2 M DTT. Store on ice and use within 3 days.

Gelsolin buffer A

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM EDTA
100 mM NaCl
Store up to 1 week at 4°C
Just before use, add:
0.1 mg/ml lysozyme
0.5 mM PMSF (added just before use from 200 mM stock in absolute ethanol)
10 µg/ml benzamidine

Gelsolin buffer B

20 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
1 mM EGTA
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

Gelsolin buffer C

10 mM MES pH 6.5
1 mM EGTA
Store up to 1 week at 4°C

Gelsolin buffer D

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EGTA
0.15 M NaCl
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

Methylcellulose, 1%

Prepare 1% (w/v) methylcellulose in distilled water, heat to 60°C, and stir until the solution clears. Store up to 1 week at 4°C.

Mg-F-actin

Prepare KME:
2 M KCl
20 mM $MgCl_2$
4 mM EGTA

Prepare low-ionic-strength buffer:
5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.1 mM $CaCl_2$
0.2 mM ATP
1 mM DTT
0.01% (w/v) NaN_3

Prepare 100 µl of Mg-F-actin by adding 5 µl of KME (see above) to 95 µl Ca-G-actin (prepare as in *UNIT 13.2*) in low-ionic-strength buffer (see above). Store up to 1 week at 4°C.

N-WASP buffer A

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
0.1% (v/v) Tween 20
Store up to 1 week at 4°C
Just before use, add:
1 mM PMSF
5 μg/ml leupeptin
5 μg/ml chemostatin
5 μg/ml pepstatin
10 μg/ml benzamidine

N-WASP buffer B

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.1 M NaCl
Store up to 1 week at 4°C

N-WASP buffer C

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
10 mM imidazole
Store up to 1 week at 4°C

N-WASP buffer D

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
30 mM imidazole
Store up to 1 week at 4°C

N-WASP buffer E

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
100 mM imidazole
Store up to 1 week at 4°C

N-WASP buffer F

10 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.1 M NaCl
1 mM DTT
Store up to 1 week at 4°C

Poly-L-proline-Sepharose column

Conjugate poly-L-proline (Sigma) to Sepharose-CNBr (Amersham Biosciences) according to the coupling instructions supplied by the manufacturer of the Sepharose-CNBr. Pack the poly-L-proline-Sepharose in a 15-cm length × 2.5-cm inner diameter chromatography column.

Polymerization buffer

5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.2 mM ATP
1 mM DTT
0.1 mM $CaCl_2$
1 mM $MgCl_2$
0.2 mM EGTA
0.1 M KCl
Store up to 1 week at 4°C

Profilin buffer A

10 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.1 M KCl
0.1 M glycine
1mM DTT
Store up to 1 week at 4°C

Profilin buffer B

5 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1mM DTT
Store up to 1 week at 4°C

COMMENTARY

Background Information

Early studies of the movement of motile cells and associated dynamics of actin filaments revealed that the polarized array of actin filaments turns over rapidly in the lamellipodium. Filament barbed ends (dynamic ends) facing the plasma membrane grow, while filament pointed ends depolymerize at the rear of the lamellipodium in a treadmilling process. In the lamellipodium, actin filaments turn over 100 times faster than pure actin. To account for the rapid movement of eukaryotic cells, actin treadmilling is enhanced by several factors. ADF enhances the rate of pointed-end depolymerization and increases the concentration of actin monomers at steady state. Profilin specifically binds monomeric actin in a complex that has the property of associating exclusively at the barbed ends. The flux of actin monomer provided by ADF-mediated depolymerization is converted into the profilin-actin complex, which polymerizes at the barbed ends only. Hence, the processivity of treadmilling is enhanced by profilin in the presence of ADF. In addition to ADF and profilin, a capping protein is required for efficient motility in the reconstituted medium. By blocking a large fraction of the barbed ends, capping proteins funnel the flux of pointed-end depolymerization to feed the growth of a few noncapped filaments, which individually grow faster than if the other filaments were not capped. In conclusion, ADF, profilin and capping proteins act synergistically in enhancing pointed-end depolymerization and barbed-end polymerization, resulting in an increase in treadmilling (Pantaloni et al., 2001).

In the treadmilling process, capping proteins are recycled after depolymerization of capped filaments and eventually cap the growing barbed ends. Maintenance of a steady number of transiently growing barbed ends requires the constant generation of barbed ends. The cellular factor that generates new filaments in a site-directed, signaling-controlled fashion is the Arp2/3 complex. The Arp2/3 complex, a conserved ubiquitous complex of seven subunits including the two actin-related proteins Arp2 and Arp3, stimulates the formation of actin filaments by branching pre-existing filaments. In higher eukaryotic cells, Arp2/3 alone is inactive and needs to be activated by a variety of activators like WASP family proteins (Rohatgi et al., 1999; Egile et al., 1999) and cortactin. (Weaver et al., 2001; Uruno et al., 2001). The multimodular proteins WASP interact with the Arp2/3 complex and G-actin via its C-terminal WA domain to stimulate branching of actin filaments. The activity of N-WASP is enhanced by the binding of effectors like Cdc42 (Egile et al., 1999; Rohatgi et al.,

1999), phosphatidylinositol bisphosphate (Rohatgi et al., 1999), Grb2 (Carlier et al., 2000), and Nck (Rohatgi et al., 2001).

The propulsive movement of the pathogens *Listeria* and *Shigella* involves the same molecular mechanism as the protrusive behavior of the leading edge of motile cells. The *Listeria* protein ActA directly activates Arp2/3 complex (Welch et al., 1998). The protein VASP interacts with the *Listeria* protein ActA and increases actin-based movement of *Listeria* 10-fold, but has no effect on *Shigella* (Laurent et al., 1999). The *Shigella* protein IcsA does not activate Arp2/3 complex but recruits and activates the host protein N-WASP, which in turn activates Arp2/3 complex (Egile et al., 1999).

In view of the abovementioned information, a number of biochemical assays have been developed to study actin-based propulsion of the bacteria *Listeria* and *Shigella*. First, the reconstitution of *Listeria* movement in cell extracts has converted the complex cell biology problem of motility into a simpler biochemistry problem (Theriot et al., 1994). This assay has been instrumental in identifying the Arp2/3 complex as the cellular factor responsible for stimulating actin assembly at a bacterial surface (Welch et al., 1997). This assay was also useful in understanding the function of the key players of actin-based motility, like VASP (Laurent et al., 1999) and ADF (Carlier et al., 1997). The design of a minimal motility medium using pure proteins (Loisel et al., 1999), presented in this unit, comes as a logical conclusion from discoveries of the factors involved in the reorganization of actin cytoskeleton at the surface of *Listeria* and *Shigella* and at the leading edge of motile cells.

The assay is based on two essential biochemical functions: (1) local nucleation of actin filaments by the Arp2/3 complex at the surface of a solid particle (bacteria or microsphere) coated with an activator of Arp2/3 and (2) constant renewal of the Arp2/3-induced actin array by treadmilling regulating proteins (ADF, gelsolin, profilin; Loisel et al., 1999).

This chemically controlled medium offers advantages in analyzing the mechanism at work, because the dependence of velocity on the concentration of different factors and their interplay with the surface density of Arp2/3 activator can be addressed.

The minimum motility medium has the potential to test the function of proteins involved in signaling pathways or actin dynamics. For instance, the function of Grb2, a protein that links N-WASP to receptor tyrosine kinase, has been tested in the motility medium. Grb2 shortens the delay preceding actin comet, supporting the biochemical demonstration that Grb2 is an activator of N-WASP (Carlier et al., 2000). Similarly, potential inhibitors or drugs can be tested as well. Another application is the study of the effect on motility of actin-binding proteins like thymosin β4, actobindin, and ciboulot. Ciboulot and actobindin enhance motility and substitute for profilin in the minimum motility medium. In contrast, thymosin β4 has no effect on motility and cannot replace profilin. These results and in-depth biochemical studies support the view that ciboulot-actin and actobindin-actin complexes assemble exclusively at the barbed end. Table 12.7.2 recapitulates examples of applications of the motility assay.

The physical mechanism of force protrusion can be addressed using the minimum motility medium. This biomimetic motility assay has been used to address the force-velocity relationship, by varying the size of the bead and the viscous drag (see Fig. 12.7.1C). Movement was not slowed down by increasing the diameter of the microspheres (0.2 to 3 μm) or by increasing the viscosity of the medium by several thousand-fold. This result shows that forces due to actin polymerization are balanced by internal forces resulting from transient attachment of filament ends at the surface. These forces are greater than the viscous drag (Wiesner et al., 2003).

Critical Parameters and Troubleshooting

The actin-based motility assays described in this unit involve the purification of seven different proteins. The major source of problem is the loss of activity of one or more components of the motility medium. Several experiments that are commonly used to verify the activity of the proteins are listed below, but a detailed description of the protocols is outside the scope of this unit.

To verify actin assembly, measure the pyrenyl-labeled actin polymerization by the increase of pyrenyl fluorescence.

To verify the activity of Arp2/3 complex and N-WASP, measure the increase of pyrenyl-actin assembly in the presence of Arp2/3 activated by N-WASP.

The activity of gelsolin can be tested by its ability to bind NBD-labeled actin in a 1:2 complex. Add increasing concentrations of NBD-labeled actin in the presence of a fixed

Table 12.7.2 Applications of Actin-Based Motility Assay

Protein	Experiment	Result	Interpretation
Ciboulot	Substitution of profilin by ciboulot	Ciboulot functionally replaces profilin	The complex ciboulot-G-actin participates in barbed-end assembly in a profilin-like fashion (Boquet et al., 2000).
Actobindin	Substitution of profilin by actobindin	Actobindin functionally replaces profilin	The complex actobindin-G-actin participates in barbed-end assembly in a profilin-like fashion (Boquet et al., 2000).
Thymosin β4	Substitution of profilin by thymosin β4	Thymosin β4 does not replace profilin	The complex actin-thymosin β4 does not participate in barbed end (Boquet et al., 2000).
Grb2	Addition of Grb2	Grb2 shortens the delay preceding actin comet tail formation	The protein Grb2 is an activator of N-WASP (Carlier et al., 2000).
VASP	Addition of VASP in the motility medium containing *Listeria* or ActA- or WASP-coated microspheres	VASP enhances actin-based propulsion of *Listeria* (Laurent et al., 1998), of ActA-coated beads (Boujemaa-Paterski et al., 2001), and WASP-coated beads (Castellano et al., 2001)	VASP enhances the dissociation of the newly formed branch junction from the immobilized branching enzyme ActA (Samarin et al., 2003).

concentration of gelsolin. Monitor the formation of this complex by the increase of NBD-labeled actin fluorescence. At saturation, the ratio of gelsolin to actin is 1:2. Capping activity of gelsolin is highly dependent on the presence of Ca^{2+} at concentrations of the order of 10^{-4} M, at least. If one protein is added to the medium, be sure that the buffer in which it is equilibrated does not contain EDTA in amounts sufficient to chelate all Mg^{2+} and/or Ca^{2+} ions in the medium. ATP-Mg is the ligand of actin; the affinity of ATP decreases by six orders of magnitude upon removal of Mg^{2+} ions. Subsequent dissociation of ATP leads to irreversible denaturation of actin. EDTA can be removed by dialysis or gel filtration (on a G-25 column) in the appropriate buffer.

ADF binds to filaments and destabilizes them, causing partial depolymerization. The effect of ADF on the treadmilling is a suitable probe of its active state. In the presence of etheno-ATP-F-actin and a large excess of ATP, the decrease of etheno-ATP fluorescence reflects the rate of pointed-end depolymerization. ADF increases the rate of depolymerization. ADF activity is highly dependent on the pH, which should be >7.8.

To verify the labeling of Alexa488-labeled Arp2/3, first resolve the different subunits of Arp2/3 complex on SDS-PAGE and identify subunits by UV illumination of the gel. Typically 80% of the label is bound to the ARCp41 subunit, which contains five cysteines and 20% is bound to the Arp3 subunit (Wiesner et al., 2003).

To verify the branching activity of Alexa488-labeled Arp2/3 complex, polymerize actin (2.5 μM, 10% pyrenyl-labeled) in the presence of 0.5 μM VCA, in the absence or presence of 30 nM Arp2/3 before addition and after a 1-hr incubation with Alexa488-C5-maleimide. The authors find that the activity of Arp2/3 complex is not affected by Alexa488 labeling (Wiesner et al., 2003).

The reader will also find helpful information regarding the dependence of bacterial velocity on the concentration of essential components (Arp2/3, ADF, and gelsolin) and nonessential components (α-actinin, profilin, and VASP) in Figure 12.7.2.

Anticipated Results

A successfully completed assay is defined by the following criteria. Typically, the actin tails appear 5 to 15 min following addition to the motility medium. Typical examples of actin tails are shown in Figure 12.7.1. The percentage of motile bacteria/microspheres

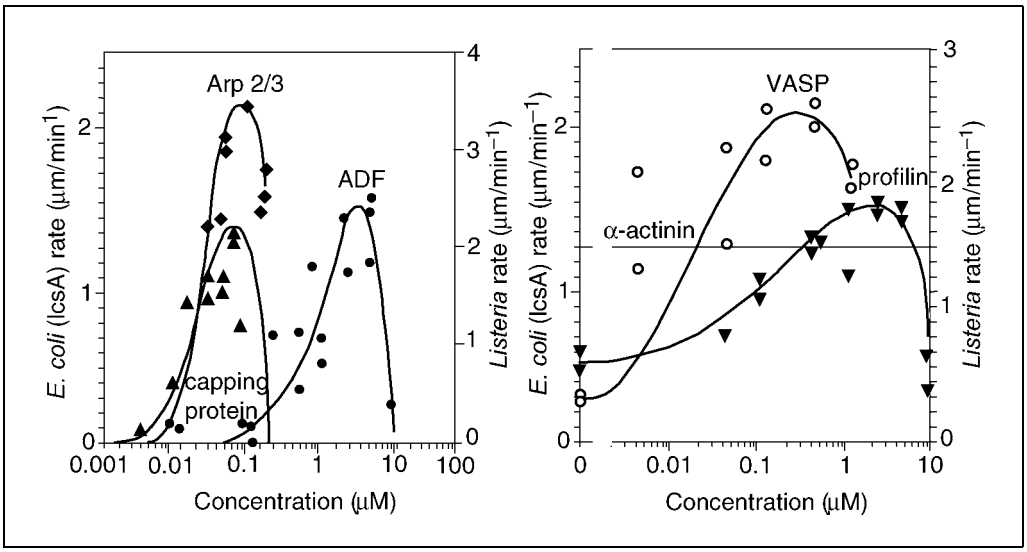


Figure 12.7.2 Dependence of bacterial rate on the concentration of essential components (left) and nonessential components (right). Filled symbols, *E. coli* (IcsA); open symbols, *Listeria*. Note the logarithmic abscissa scale. Duplicate data points represent independent measurements made by two experimentalists. Each data point is the average of 10 measurements. The standard deviation was generally 20%. From Loisel et al. (1999).

under optimal conditions falls between 70% and 80% for *Listeria* or *E. coli* IcsA and near 100% for N-WASP-coated polystyrene microspheres. At all surface densities of N-WASP, the steady-state velocity of microspheres was reached after 10 to 15 min incubation in the medium and remained constant for 1 hr. Average rates of movement at steady state are 1 to 2 μm/min for N-WASP or ActA-coated microspheres, 2 μm/min for *E. coli* IcsA and *Listeria*.

Time Considerations

Actin preparation requires 1 day to prepare an acetone powder from rabbit muscle and 6 days to purify actin extracted from acetone powder. Purifications of the proteins takes 3 days for Arp2/3 complex, 2 days for N-WASP, 2 days for gelsolin, 2 days for ADF, and 2 days for profilin. The motility assay only takes 1 hr.

Literature Cited

Boquet, I., Boujemaa, R., Carlier, M.F., and Preat, T. 2000. Ciboulot regulates actin assembly during *Drosophila* brain metamorphosis. *Cell* 102:797-808.

Boujemaa-Paterski, R., Gouin, E., Hansen, G., Samarin, S., Le Clainche, C., Didry, D., Dehoux, P., Cossart, P., Kocks, C., Carlier, M.F., and Pantaloni, D. 2001 *Listeria* protein ActA mimics WASp family proteins: It activates filament barbed end branching by Arp2/3 complex. *Biochemistry* 40:11390-11404.

Carlier, M.F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G.X., Hong, Y., Chua, N.H., and Pantaloni, D. 1997. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: Implication in actin-based motility. *J. Cell Biol.* 136:1307-1322.

Carlier, M.F., Nioche, P., Broutin-L'Hermite, I., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P., and Pantaloni, D. 2000. GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASp) with actin-related protein (ARP2/3) complex. *J. Biol. Chem.* 275:21946-21952.

Egile, C., Loisel, T.P., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P.J., and Carlier, M.F. 1999. Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J. Cell Biol.* 146:1319-1332.

Higgs, H.N., Blanchoin, L., and Pollard, T.D. 1999. Influence of the C terminus of Wiskott-Aldrich syndrome protein (WASp) and the Arp2/3 complex on actin polymerization. *Biochemistry* 38:15212-15222.

Laurent, V., Loisel, T.P., Harbeck, B., Wehman, A., Grobe, L., Jockusch, B.M., Wehland, J., Gertler, F.B., and Carlier, M.F. 1999. Role of proteins of the Ena/VASP family in actin-based motility of *Listeria monocytogenes*. *J. Cell Biol.* 144:1245-1258.

Loisel, T.P., Boujemaa, R., Pantaloni, D., and Carlier, M.F. 1999. Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature* 401:613-616.

Mullins, R.D., Heuser, J.A., and Pollard, T.D. 2000. The interaction of Arp2/3 complex with actin: Nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. U.S.A.* 95:6181-6186.

Pantaloni, D., Le Clainche, C., and Carlier, M.F. 2001. Mechanism of actin-based motility. *Science* 292:1502-1506.

Perelroizen, I., Didry, D., Christensen, H., Chua, N.H., and Carlier, M.F. 1996. Role of nucleotide exchange and hydrolysis in the function of profilin in actin assembly. *J. Biol. Chem.* 271:12302-12309.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M.W. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 97:221-231.

Rohatgi, R., Nollau, P., Ho, H.Y., Kirschner, M.W., and Mayer, B.J. 2001. Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J. Biol. Chem.* 276:26448-26452.

Samarin, S., Romero, S., Kocks, C., Didry, D., Pantaloni, D., and Carlier, M.F. 2003. How VASP enhances actin-based motility. *J. Cell Biol.* 163:131-142.

Theriot, J.A., Rosenblatt, J., Portnoy, D.A., Goldschmidt-Clermont, P.J., and Mitchison, T.J. 1994. Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell-free extracts. *Cell* 76:505-517.

Uruno, T., Liu, J., Zhang, P., Fan, Y.X., Egile, C., Li, R., Mueller, S.C., and Zhan, X. 2001. Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat. Cell Biol.* 3:259-266.

Weaver, A.M., Karginov, A.V., Kinley, A.W., Weed, S.A., Li, Y., Parsons, J.T., and Cooper, J.A. 2001. Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. *Curr. Biol.* 11:370-374.

Welch, M.D., Iwamatsu, A., and Mitchison, T.J. 1997. Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. *Nature* 385:265-269.

Welch, M.D., Rosenblatt, J., Skoble, J., Portnoy, D.A., and Mitchison, T.J. 1998. Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation. *Science* 281:105-108.

Wiesner, S., Helfer, E., Didry, D., Ducouret, G., Lafuma, F., Carlier, M.F., and Pantaloni, D. 2003. A biomimetic motility assay provides insight into the mechanism of actin-based motility. *J. Cell Biol.* 160:387-398.

Contributed by Christophe Le Clainche and Marie-France Carlier
Laboratoire d'Enzymologie et Biochimie Structurales (LEBS)
Centre National de la Recherche Scientifique (CNRS)
Gif-sur-Yvette, France

In Vivo Marking of Single Cells in Chick Embryos Using Photoactivation of GFP

This unit describes a technique that uses a photoactivatable variant of GFP (PAGFP) and confocal laser scanning microscopy to selectively mark single cells within living embryos (see Basic Protocol). This method can be used to trace short-term cell lineage and study cell migratory behaviors. Initially, a region of interest within an embryo is microinjected with a glass needle containing the PAGFP construct. Electroporation opens pores in the cells through which the PAGFP construct enters. Individual cells that incorporate PAGFP are selected, focused in on, and made visually distinct from the host population by photoactivation with 405-nm laser light. Migratory behaviors of cells with photoactivated GFP are captured and analyzed using previously developed chick embryo culture and confocal time-lapse imaging techniques. Alternatively, embryos can be reincubated until a desired stage to survey the lineage of photoactivated cells. In addition, this unit includes a protocol for in ovo and whole chick embryo explant culture and time-lapse imaging (see Alternate Protocol).

PHOTOACTIVATION OF GFP IN SINGLE CELLS IN CHICK EMBRYOS

BASIC PROTOCOL

Early stage chick embryos are microinjected with photoactivatable GFP (PAGFP) and control constructs that are electroporated into the cells. Cells are imaged and the GFP is photoactivated to label individual or small groups of cells. These cells can be followed to analyze migratory behavior or cell lineage (see Fig. 12.8.1).

Materials

Fertilized white Leghorn chick eggs incubated at 37°C
India ink (drawing ink, Pelikan)
Howard Ringer's solution (J.A. Webster), sterile
10 mg/ml Fast Green FCF (Fisher) in Howard Ringer's solution, optional
PAGFP construct (available from J. Lippincott-Schwartz; *jlippin@helix.nih.gov*)
H2B-mRFP construct (optional; available from P. Kulesa; *pmk@stowers-institute.org*)
70% ethanol
High-vacuum silicone grease (Dow Corning)
B27 supplement (Invitrogen)
Neural basal medium (Invitrogen)
L-glutamine (Sigma-Aldrich)
H_2O, sterile
Human fibronectin (Invitrogen), diluted to 20 μg/ml in phosphate butter

Egg incubator (G.Q.F. Manufacturing Co., Model 1550)
15-ml tubes
Glass capillary tubes; borosilicate with filament (Sutter Instrument)
Glass needle puller (Sutter Instrument, Model P-87)
Micropipet loader
20-μl pipettor
Picospritzer (Picospritzer III, Parker Hannifin Corporation)
Dumont no. 5 forceps and iris scissors (Fine Science Tools)
Electroporator (BTX)
Platinum electrodes (A.M. Systems)

Contributed by D. A. Stark and P. M. Kulesa
Current Protocols in Cell Biology (2005) 12.8.1-12.8.11

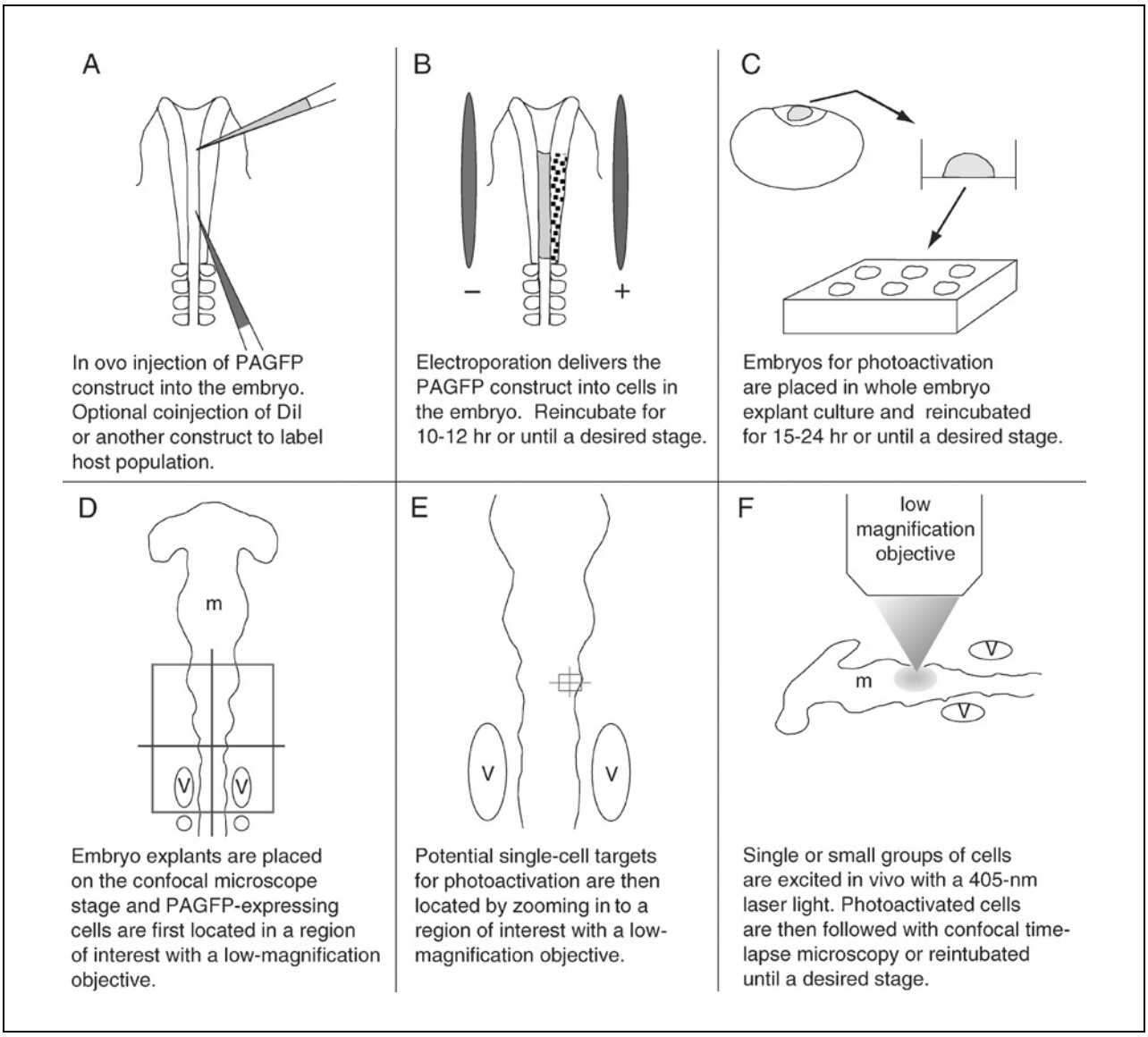


Figure 12.8.1 A diagram of the photoactivation process in a chick embryo. A summary outline of the photoactivation technique starting with the injection of PAGFP and H2B-mRFP constructs and ending with the photoactivation in the whole chick embryo. From left to right this figure shows a flowchart of the photoactivation process in a chick embryo, including: (**A**) injection, (**B**) electroporation, (**C**) mounting, (**D**) region of interest targeting, (**E**) selection of an individual cell for photoactivation, and (**F**) the photoactivation process.

18- and 25-G needles (Becton Dickinson)
1- and 5-ml syringes (VWR)
Tape (e.g., Scotch)
Plastic transfer pipet, sterile
Tungsten needle (A.M. Systems)
Micromanipulator (World Precision Instruments)
6-well culture plates
Soldering tool (Weller)
Sandpaper, medium grade (3M)
Glass cover slips, 22-mm round (VWR)
Culture insert (Millipore)
Petri dishes (Falcon)
Fluorescence stereo microscope with halogen light source and appropriate filters (LP-DAPI, TRITC; Leica)

Whatman no.1 filter paper
Dissecting scissors
Stereo dissecting microscope
Confocal inverted laser scanning microscope with 488- and 405-nm laser lines (Zeiss LSM5 PASCAL) and cell tracking software (optional)
Parafilm
Cardboard (1/4-in. thick)
Velcro (sticky back tape)
Insulation for cardboard box (5/16-in. thick; Reflectix)
Clear plastic packaging tape
Chick incubator heater (Model 115-20, Lyon)

Prepare reagents and equipment for injection

1. Incubate fertilized chick eggs at 37°C until the desired stage in an egg incubator.

   Incubate eggs for 40 to 43 hr for HH (Hamburger and Hamilton, 1951) stage 8 to 9 embryos.

2. Mix 1 ml of India ink in 9 ml of Howard Ringer's solution in a 15-ml tube.

3. Prepare a glass needle from a glass capillary tube using a needle puller with the following settings: heat, 400; pull, 50; velocity, 50; and time, 100.

4. (Optional) Prepare a very small amount of 10 mg/ml Fast Green to aid in visualizing the amount of injected dye.

5. Load the glass needle with a 1:1 mixture of the PAGFP construct (concentration of 1 to 2 μM/μl) and H2B-mRFP using a micropipet loader and a 20-μl pipettor.

   Both constructs should incorporate into the cells in a stochastic manner. Some cells will have one or the other of the constructs, and some will have both. Figure 12.8.2 (see Commentary) illustrates only the photoactivated cells (green) and not all of the cells that took up the PAGFP construct.

   H2B-mRFP is another fusion protein construct (H2B-mRFP) with a different emission spectrum such that the host population is fluorescently labeled. DiI (Molecular Probes) may also be used.

6. Trim the tip of the glass needle such that the construct is able to pass through the tip of the glass needle at small quantities with the application of the picospritzer. To do this, bring the tip of the glass needle to make gentle contact with the tip of a pair of Dumont no. 5 forceps. Tap the picospritzer foot pedal to visualize the amount of dye exuding from the tip of the needle.

   The picospritzer settings need to be adjusted with the size of the capillary needle; however, the recommended settings are: duration, 10 msec; and pressure, 10 psi.

7. Set the electroporator to a desired value.

   For an HH stage 10 embryo and platinum electrodes, the recommended settings are: 20 V, 55 pulse-length, and 5 pulses.

8. Attach an 18-G needle to a 5-ml syringe and a 25-G needle to a 1-ml syringe.

9. Bend the 25-G needle near the tip at a 90° angle in the direction of the pivot end and fill the syringe with the India ink solution from step 2.

Prepare embryo

10. Remove eggs from the incubator and lightly rinse the tops of the eggs with 70% ethanol.

11. With an 18-G needle, remove 3 ml of albumin from the caudal part of each egg.

12. Place a strip of tape over the top portion of the egg and use iris scissors to carefully cut a window in the egg without penetrating the yolk.

13. Locate the embryo within the window and angle the 1-ml syringe with the attached 25-G needle to inject India ink solution directly under the embryo. Try to use only enough ink to make the embryo visible (e.g., ~100 μl).

14. Place a few drops of Howard Ringer's solution around the embryo with a transfer pipet to prevent drying.

15. With a sharpened tungsten needle, carefully penetrate the vitelline membrane and pull it away from the desired injection site.

Inject and deliver constructs to embryos

16. Position the micromanipulator next to the egg and direct the injection needle to the injection site. Inject the PAGFP and control constructs into a selected region (Fig. 12.8.1).

    For example, PAGFP is injected into the lumen of the neural tube of HH stage 9 to 10 embryos to label cells in the neural tube and premigratory neural crest cells. The timing of the injection and the electroporation depends on the cells or tissue to be labeled.

17. After injection of the PAGFP construct, quickly retract the injection needle and place electroporator electrodes at the edge of the blastoderm (Fig. 12.8.1). Place the positive electrode on the right side of the embryo (as viewed from above the egg) and the negative electrode on the left side of the embryo.

18. Start the electroporation process using the parameters suggested in step 7.

    As the embryo develops, it will roll on its left side and this will expose the right side as the construct is attracted to the positive electrode.

19. Add a few more drops of Howard Ringer's solution around the embryo with the transfer pipet.

Incubate egg

20. Tape over the eggshell window and place the egg back into the 37°C incubator for 10 to 12 hr.

Prepare for embryo culture

21. Prepare a 6-well culture plate. In one well, cut a hole in the plastic bottom by melting a coin through the plastic with a soldering tool. Use sandpaper to smooth over the hole.

22. Secure a 22-mm glass coverslip to the bottom of the hole with a thin ring of silicone grease.

23. Mix 0.5 ml of B27 supplement with 25 ml of neural basal medium and add 0.0025 g of L-glutamine. Place 1 ml of culture medium into the well with the coverslip and fill the other wells half full with sterile water.

24. Pipet 200 μl of 20 μg/ml fibronectin onto a culture insert and swirl the insert such that the fibronectin covers the entire surface. Place the culture insert into a covered petri dish for 20 min at room temperature.

Dissect embryo

25. Remove the tape from the eggshell window and observe the health of the embryo and fluorescence labeling (construct expression) using a fluorescence stereo microscope.

    If the PAGFP is not highly expressed, then the construct will not be seen under a GFP filter cube; however, the construct can be seen under a long-pass DAPI filter cube at the

risk of photoactivation and UV exposure to the embryo. If under low expression, it would be most suitable to observe the control construct (H2B-mRFP) with a TRITC filter cube and assume the PAGFP cells have the equivalent (approximately) level of expression in the cells.

26. Cut a piece of Whatman no. 1 filter paper into a donut-shaped ring with an inner diameter large enough to encompass the whole embryo and blastoderm. Dip filter paper ring into Howard Ringer's solution, and place over the embryo.

    The embryo should be taken out of the egg and cultured prior to the photoactivation process.

27. Use dissecting scissors to cut around the outside edge of the filter paper.

28. Take the Dumont no. 5 forceps and pull out the paper ring with the embryo attached. Place the embryo into a petri dish containing Howard Ringer's solution.

29. Place the dish under a stereo microscope and use the forceps to tease off the vitelline membrane and filter paper. Rinse away any yolk or ink that may have accumulated around the embryo.

Establish embryo culture

30. Remove all excess fibronectin from the culture insert.

31. Cut the end off of a plastic transfer pipet so that the embryo can be easily picked up. Place the embryo onto the culture insert. Orient the embryo such that it is dorsal-side down (for photoactivation of PAGFP with an inverted confocal microscope).

    If the embryo is difficult to manipulate, place a few extra drops of Howard Ringer's solution onto the culture insert. After the embryo is positioned, gently pipet excess Howard Ringer's solution from the culture insert surface and let the embryo continue to spread out onto the culture insert surface.

32. Position the embryo in the center of the culture insert such that the edges of the embryo do not come in contact with the culture insert edges.

    If contact occurs, the embryo will be drawn to the point of contact with the culture insert.

33. Carefully place the culture insert inside the well of a 6-well dish containing medium. Position the culture insert directly over the center of the coverslip bottom. Cover the 6-well plate with the lid.

    The culture is now prepared for imaging. If not imaging immediately, place the dish into the 37°C incubator. Incubate 15 to 24 hr or until the desired stage.

Visualize embryo

34. Place the culture dish onto the microscope stage and bring the sample into focus using a low-magnification objective, e.g., using a 10× or 20× objective lens. Use a mercury lamp source and the filter cube needed to locate the population of cells expressing the control construct.

    A long-pass DAPI filter can be used to view PAGFP; however, avoid prolonged exposure of the embryo to UV light. The expression may be very dim depending on how many hours it is post injection.

35. If there is an option for multi-track excitation scanning available with the confocal imaging software, choose a multi-track configuration to include GFP and any excitation that is required for highlighting the host cell population, if necessary.

    If multi-tracking is not an option, select the single-track configuration. The multi-track configuration will create an image without any bleed through from the two constructs.

36. Create an image of the PAGFP-expressing cells, as if it were very low-expressing GFP, by turning up the detector gain, pinhole diameter, and applying four to eight averages before smoothing out the noise. If the expression is still too low to visualize, then increase the laser power past 10% on an argon laser.

37. Locate a region where the cells are expressing PAGFP.

Photoactivate GFP

38. To target an individual cell for photoactivation of PAGFP, continue to zoom in on a particular region. To focus on an individual cell, crop the region of excitation and scan around the area of the cell.

    The PAGFP construct is located in the cytoplasm and will diffuse rapidly during and post photoactivation. The selected cell does not have to be completely located inside the cropped box before photoactivation can occur.

39. To photoactivate PAGFP within a cell, use a 405-nm laser with a power setting between 2% and 5%. Then, scan a single image to photoactivate the PAGFP within the cell.

    A single scan may not be enough to optimize the photoactivation.

40. Evaluate the increase in mean GFP fluorescence in the cell by following the 405-nm excitation scan with a 488-nm excitation scan.

    A second or third scan at 405 nm may be necessary. Repeat as needed for other cells in the embryo.

    As the image is zoomed in, the power from the laser increases by:

    $$\textit{laser dosage} = (\textit{laser intensity} \times \textit{scan time})/\textit{scan area}.$$

    The higher the zoom, the less percent laser power is required. It is recommended to have 2% to 3% laser power if $\geq$35$\times$ zoom and 3% to 5% laser power if <34$\times$ zoom.

    If immediate photobleaching occurs, the 405-nm power is too high and must be lowered accordingly for the subsequent cell(s). The current cell is no longer suitable for photoactivation.

    Often, photoactivated cells become so bright that they are over exposed. At this point, the detector gain and pinhole can be reduced to create a more acceptable confocal image. If laser power levels were increased, then begin by reducing the power level.

41. Once photoactivation is complete in a single or number of cells in an embryo, begin collecting time-lapsed images or reincubate the embryo.

    Cell-lineage and cell-migratory behavioral studies use time-lapse imaging or reincubation of embryos.

42. To reincubate the embryo, add 0.5 ml of culture medium to the well.

    If the culture insert begins to float, remove some medium so that the culture insert sits on the bottom of the dish.

43. Wrap the dish in Parafilm and place in a 37°C incubator for the designated amount of time for post-photoactivation.

Construct a heater box for the microscope

44. Construct a 1/4-in. thick cardboard box around the microscope stage. Use Velcro strips to attach the box sides together.

    The box should fit around the stage but not enclose any lamp sources.

45. Cut holes in the box to allow for access to the microscope.

46. Wrap insulation around the outside of the box and attach it with clear packaging tape.

47. Near the back of the box, place the chick incubator heater within the box.

Perform time-lapse visualization

48. Configure the confocal imaging software to collect images at a desired time-interval.

    A minimum of a 2-min interval setting between time points is recommended.

IN OVO PHOTOACTIVATION

ALTERNATE PROTOCOL

In ovo photoactivation is an easier protocol that requires less time and allows the chick embryo to develop more naturally. Unfortunately, the technique requires more specialized equipment. An upright Zeiss LSM 5 Pascal was specifically ordered to provide the working distance needed to place a chicken egg under the objective for imaging. Another limitation to this protocol is that cells targeted for photoactivation must be accessible to confocal laser excitation. Once the embryo starts to turn, it becomes more difficult to target a cell for photoactivation. An advantage of this technique is that it allows a longer incubation period post photoactivation versus that for the cultured embryos.

Additional Materials *(also see Basic Protocol)*

Beeswax (Eastman Kodak)
Microinjected and electroporated eggs (see Basic Protocol)
3.8-cm × 7.5-cm × 15-μm Teflon membrane, high-sensitivity, oxygen permeable (Fisher)
~2.2-cm i.d. × 2.6-cm o.d. × 0.5-cm high acrylic ring (constructed in machine shop)
2.4-cm i.d. × 2.1-cm o.d. rubber O ring (constructed in machine shop)
Upright, laser scanning confocal microscope with 488-nm and 405-nm laser excitation, equipped with a long working distance 10× Plan Neofluar objective (NA = 0.3)

Prepare equipment

1. Prepare the Teflon membrane to mount over the embryo in ovo to allow oxygen transfer to the embryo without drying the contents of the egg.

   a. Warm beeswax enough to melt it (~37°C). Dip one edge of the acrylic ring in the heated beeswax. Quickly place the ring in the center of the Teflon membrane and use the rubber O ring to pull the membrane taught around the acrylic ring. Hold the O ring in place until the beeswax has completely dried.

   b. Cut away the extra Teflon and roll the edges of the ring over a hot plate to adhere the membrane to the ring.

2. Prepare the upright LSM 5 Pascal microscope by turning it on, removing any unnecessary objectives, dropping the removable scanning stage and focus drive to the lowest position, and placing a protective cover of plastic wrap over the stage to prevent any egg accidents.

3. Ten to 12 hr post injection, remove microinjected and electroporated eggs from the incubator and observe for fluorescence.

4. Cut out a window of an egg so it is large enough to place the membrane directly over the embryo.

5. Place a 1 ml of Howard Ringer's solution over the embryo to prevent any drying that may occur and drop the membrane so that the center is directly over the embryo.

Photoactivate GFP

6. Carefully place the egg under the long-working distance objective and settle it in a low platform holder.
7. Photoactivate as described in Basic Protocol, steps 38 to 40.
8. Remove the egg from the microscope and gently pull the membrane and acrylic ring off of the embryo. Retape the egg and place it back into the 37°C egg incubator.
9. Rinse the membrane in Howard Ringer's solution before preparing it for the next embryo.

SUPPORT PROTOCOL

IMAGING USING HIGH-MAGNIFICATION ACQUISITION

To image post photoactivation, use a high-magnification acquisition protocol because once the embryo grows older, the tissue becomes too thick and deep to collect quality images. To acquire images at a higher magnification, the embryo must be removed from the culture disk and placed on a slide and coverslipped.

Additional Materials *(also see Basic Protocol)*

Embryos on culture inserts (see Basic Protocol)
Glass-bottomed petri dishes (Mat Tek)
Glass microscope slides (VWR)
Coverslips, 22-mm

1. Remove the embryo on a culture insert from a 6-well plate and place into a larger glass-bottomed petri dish filled with Howard Ringer's solution. Gently rinse the disk until the embryo detaches and floats off of the membrane.

   If the embryo remains attached, then forceps may be used to pull away the edges of the blastoderm from the membrane.

2. On a glass microscope slide, use silicone grease to create a circle large enough to place the whole embryo inside.
3. Cut the tip off of a plastic transfer pipet so that the embryo may be freely picked up from the petri dish. Place the embryo inside of the ring of grease on the glass microscope slide.
4. Position the embryo so that the dorsal side will contact the coverslip. Using a small-tip transfer pipet, remove excess Howard Ringer's solution from the grease ring.
5. Place a 22-mm round coverslip over the embryo and gently seal the glass against the grease. Continue to image as described above.

COMMENTARY

Background Information

The photoactivatable GFP (PAGFP) was developed at the National Institutes of Health (Patterson and Lippincott-Schwartz, 2002). The technique takes advantage of the inherent brightness of the green fluorescent protein (Chalfie et al., 1994) and previously developed photoactivation methods (Yokoe and Meyer, 1996; Sawin and Nurse, 1997). The mechanism of PAGFP is not completely understood; however, when it is excited with ultraviolet or an ~400-nm light, the chromophore population undergoes photoconversion and shifts predominantly to the anionic form. This results in an increase in minor peak absorbance. PAGFP is designed with a lower initial minor peak absorbance that greatly increases after photoconversion, giving a more noticeable optical contrast with 488-nm excitation. Upon intense illumination with ultraviolet or an ~400-nm light, the baseline fluorescence in a PAGFP-labeled cell increases ~100-fold under 488-nm excitation (Patterson and Lippincott-Schwartz, 2002).

Other groups have taken advantage of these types of protein characteristics to create fluorescent reporters such as Kaede (Ando et al., 2002) and photoswitchable CFP (PS-CFP; Chudakov et al., 2004). Kaede takes advantage of a photoconversion technique that changes the green emission, excited at 488 nm, to red when exposed to 405-nm light. A 2000-fold increase can be seen as the green expression decreases and the red expression increases. Both the green and the converted red forms have the same excitation wavelength of 488 nm. Kaede serves as an excellent marker to selectively distiguish a single cell while maintaining the ability to observe other neighboring cells. PS-CFP is similar to of Kaede with a UV-induced ability to convert from cyan-to-green emission. However, both excitation and emission spectra are affected during the switch when exposed to 405-nm laser light creating a 1500-fold increase in fluorescence. The PS-CFP construct has been used to study protein trafficking and fluorescence resonance energy transfer (FRET) analysis.

The PAGFP technique has distinct advantages over existing techniques, including the ability to mark a cell deep within an embryo and sustain individual cell health with minimal invasiveness. PAGFP can be re-excited with 405-nm light to extend the duration of the increased fluorescent signal in a cell versus non-photoactivated cells. Previous cell labeling techniques, for the most part, rely on the ability to inject a fluorescent dye into a cell(s) using a glass needle. A brief history of cell tracing methods is found in Stern and Fraser (2001). Iontophoretic labeling is described in Fraser (1996). A comparison of optical techniques is found in Patterson and Lippincott-Schwartz (2003). Standard electroporation techniques are used to deliver the PAGFP construct into cells in an embryo (Itasaki et al., 1999).

Critical Parameters and Troubleshooting

During the photoactivation process, photobleaching may be observed during high magnification or zoom using the 405-nm laser. Once cells start to photobleach, they are no longer suitable for photoactivation. This can be prevented by starting the experiment with a low-power laser or using an objective with a low magnification such as the 10× 0.3 NA Plan Neofluar objective.

An increase of intensity from non-photoactivated cells can be seen over time. Eventually, the photoactivated cells may be lost among the host population, usually after 24 hr. This is an effect that appears to depend on the embryo reincubation time and the efficiency of the photoactivation. The longer an embryo has to reincubate post injection, the more effective is the photoactivation of the cells.

Photoactivated cells can be reactivated. Follow the same procedure as photoactivation, zooming in on cells and irradiate with a 405-nm light. There may not be another 100-fold intensity increase; however, a strong discrimination will be seen in comparison to the non-photoactivated cells. There are several reasons why a cell may not be visually distinguishable when it is followed over time. These include: cell death, migration deep into the embryo, or simply poor photoactivation. When final images are taken and some or all of the photoactivated cells are missing, then the image histogram must be used to determine which cells have the highest fluorescence intensity. PAGFP proves to be stable in 488-nm light, which makes it a good candidate for z-stack and time-lapse usage; however, under 405-nm light, the cell can start to rapidly bleach if scanning persists after photoactivation.

Anticipated Results

A cell with good expression of PAGFP can be photoactivated such that there is a 100-fold increase in mean fluorescence intensity (Fig. 12.8.2, panel B). The photoactivated cell will be visually distinguishable when compared to other cells that have not been irradiated with 405-nm light (Fig. 12.8.2, panels A, C, and D). By using confocal microscopy, three-dimensional renderings of the photoactivated cell(s) can be created to visualize the morphology during active migration or post migration. This data is best collected under the conditions of a healthy embryo, strong PAGFP photoactivation in the target cell, and active cellular activity.

Time Considerations

The total protocol as written will take up to 3 days to complete with a 12-hr post-photoactivation incubation period. The majority of this time is taken up with culture and egg incubation times. For example, to photoactivate cells in the neural tube and premigratory neural crest cells, eggs were incubated for 36 to 40 hr to bring them to an HH 9 to 10 somite stage before injecting PAGFP.

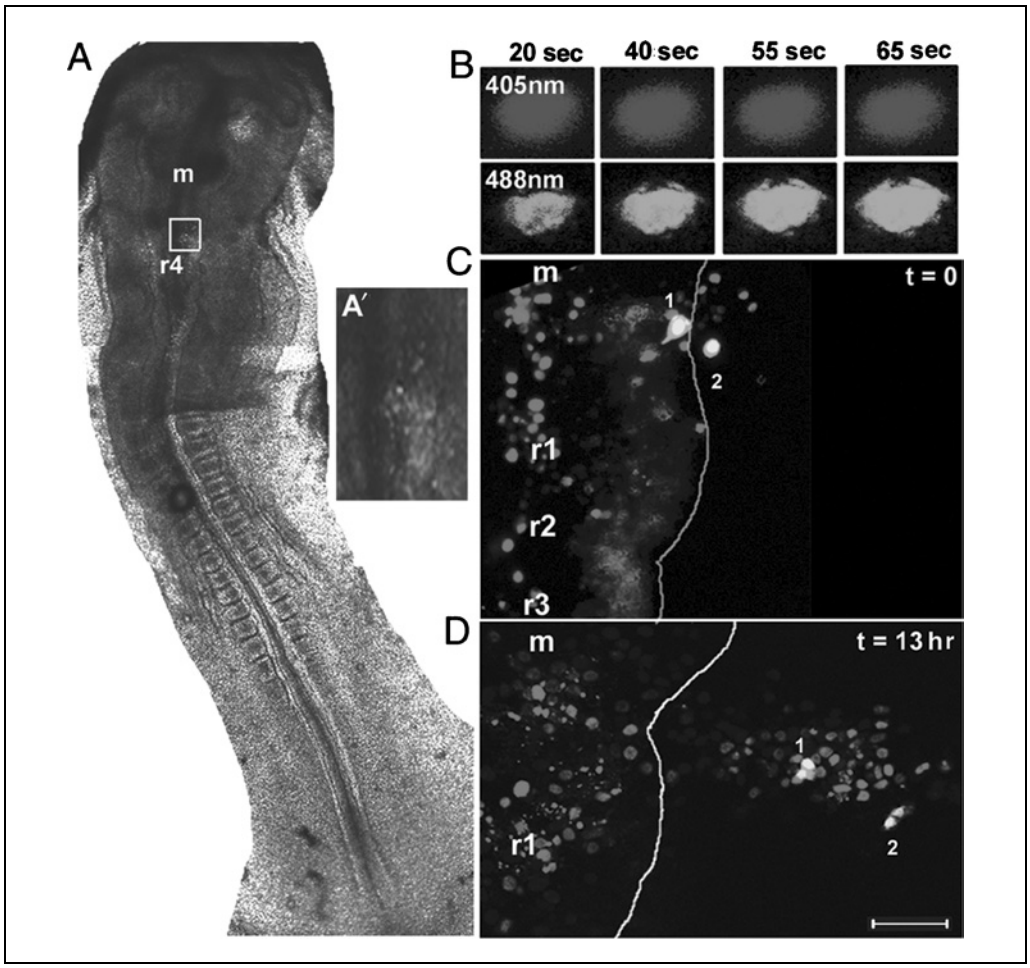


Figure 12.8.2 Photoactivation of PAGFP in a single cell in a living chick embryo. (**A**) A global view of a 16-somite chick embryo (5× magnification) showing post-photoactivation of PAGFP in a single cell (green-colored cell, within the box region) surrounded by a subpopulation of host cells (red). (**A′**) A magnified view of the box region in **A** shows the photoactivated cell (green-colored). (**B**) The photoactivation process in a single cell within the chick embryo is shown as a sequential series of confocal excitation scans. The top row represents the 405-nm excitation scans of the cell (blue) at various time points of a typical sequence. The second row shows the resulting increase in GFP-fluorescence after each 405-nm excitation scan is followed by a 488-nm excitation scan. (**C,D**) As an example of the ability to observe individual cells within the chick embryo, two individual migrating cells (labeled as 1 and 2) were photoactivated separately at a time when the cells were just starting to emerge and migrate into the surrounding unlabeled tissue. The subpopulation of cells (red) within the chick neural tube provide a background view of other adjacent migrating and non-migrating cells. After a 13-hr reincubation of the whole embryo explant in culture, the final image (**D**) shows the location of the original cells that have migrated further laterally away from the neural tube. The cells (1 and 2) appear to have divided. The scale bar is 50 μm. The embryo in (**A**) is ∼2 mm in length. The individual cell in **B** has a diameter of ∼15 μm. The labels refer to the midbrain (m) and rhombomeres 1 to 4 (r1, r2, r3, r4). This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.interscience.wiley.com/c_p/colorfigures.htm*.

Culture and photoactivation can be done within 2 to 3 hr after a minimum 10- to 12-hr incubation period. The length of the time-lapse imaging session or reincubation can vary depending on the desired stage and morphological event that is to be probed.

Literature Cited

Ando, H., Furuta, T., Tsien, R.Y., and Okamoto, H. 2001. Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos. *Nat. Genet.* 28:317-325.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. 1994. Green fluorescent

protein as a marker for gene expression. *Science* 263:802-805.

Chudakov, D.M., Verkhusha, V.V., Staroverov, D.B., Souslova, E.A., Lukyanov, S., and Lukyanov, K.A. 2004. Photoswitchable cyan fluorescent protein for protein tracking. *Nat. Biotechnol.* 22:1435-1440.

Fraser, S.E. 1996 Iontophoretic dye labeling of embryonic cells. *Methods Cell Biol.* 51:147-160.

Hamburger, V. and Hamilton, H.L. 1951. A series of normal stages in the development of the chick embryo. *J. Embryol. Exper. Morph.* 88:49-92.

Itasaki, N., Bel-Vialar, S., and Krumlauf, R. 1999. Shocking developments in chick embryology: Electroporation and in ovo gene expression. *Nat. Cell Biol.* 1:E203-E207.

Patterson, G.H. and Lippincott-Schwartz, J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297:1873-1877.

Patterson, G.H. and Lippincott-Schwartz, J. 2003. Development and use of fluorescent protein markers in living cells. *Science* 300:87-91.

Sawin, K.E. and Nurse, P. 1997. Photoactivation of green fluorescent protein. *Curr. Biol.* 7:R606-R607.

Stern, C.D. and Fraser, S.E. 2001. Tracing the lineage of tracing cell lineage. *Nat. Cell Biol.* 3:E216-E218.

Yokoe, H. and Meyer, T. 1996. Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nat. Biotechnol.* 14:1252-1256.

Contributed by D. A. Stark and P. M. Kulesa
Stowers Institute for Medical Research
Kansas City, Missouri

CHAPTER 13
Organelle Motility

INTRODUCTION

The cytoplasm of eukaryotic cells is comprised of a complex network of filaments, including microtubules and actin, that extend throughout the cytoplasm. These filaments are highly dynamic and continuously remodel as the cell changes shape, divides, and interacts with the environment. They play a key role in organizing the cytoplasm by connecting protein compartments and organelles in different regions of the cell. Microtubules, for example, are known to provide communication paths between organelles, controlling the spatial location of these structures. Actin filaments, on the other hand, form contractile bundles with myosin and mediate muscle contraction and contractile-ring formation during cytokinesis. In this chapter, assays are described for examining the characteristics and functions of the microtubule and actinomyosin filament systems. These assays are useful for identifying accessory proteins that attach microtubules to organelles and/or modulate sliding of myosin molecules across actin fibers.

UNIT 13.1 describes an in vitro assay for examining interactions between microtubules and isolated organelles. Microtubules are polar structures with their plus (i.e., rapidly growing) ends extending out to the cell periphery, and their minus ends associated with the centrosome adjacent to the nucleus. They emanate as a star-like network out from the centrosome, with new microtubules constantly extending out to replace old ones that have depolymerized. The endoplasmic reticulum (ER) uses microtubules to move out to the cell periphery, while membranes of the Golgi complex cluster inwards toward the centrosome along microtubules. These organelle movements are mediated by microtubule-associated motor proteins, including kinesin and cytoplasmic dynein. The in vitro motility assay described in this unit provides a useful system for studying organelle transport along microtubules. It can be used for dissecting the molecular machinery involved in such movement, including proteins that regulate microtubule motors or that cross-link motors to membranes. Effects of pharmacological or biochemical perturbations on microtubule structure and dynamics can also be investigated. In the assay described in *UNIT 13.1*, microtubules are nucleated from a stationary point (i.e., the flagellar axoneme), allowing their plus ends to grow and shorten in a way analogous to what happens in normal cells. Both motor-driven motility of organelles and microtubule polymerization–driven movement of organelles can be studied. The unit also provides detailed methods for preparation of purified tubulin, cytosol enriched in motor proteins, and axonemes from sea urchin sperm, as well as for isolation of membrane-bound organelles. The assay is performed in a simple perfusion chamber and the results are visualized using video-enhanced DIC microscopy.

UNIT 13.2 provides a motility assay for transport of actin by myosin. F-actin filaments labeled with rhodamine-phalloidin are imaged in an in vitro system that contains purified myosin, an ATP-activated motor protein that hydrolyzes ATP to ADP and P_i when stimulated by binding to actin filaments. This assay uses two purified proteins, actin and myosin, to study the contractile forces produced by the sliding of actin filaments along the myosin filaments. During muscle contraction, the head regions of myosin molecules engage in an ATP-driven cycle in which they attach to adjacent actin filaments, undergo a conformational change that pulls the myosin filament, and then detach. This results in

Contributed by Jennifer Lippincott-Schwartz
Current Protocols in Cell Biology (2005) 13.0.1-13.0.2

the sliding of actin filaments against the myosin, which can be directly visualized and quantified in this assay.

The use of GFP fusion proteins has revolutionized the study of organelle motility by providing a method to visualize diverse organelles directly under the microscope. Virtually any protein can be tagged with GFP, expressed in cells, then visualized by applying blue light. *UNIT 13.3* describes how to express and image GFP fusion proteins in plants in order to study organelle dynamics in these cell types. Both transient and viral-mediated expression methods are described for expressing GFP fusion proteins. Tips are given for imaging GFP proteins targeted to the endoplasmic reticulum and Golgi complex, two organelles that function in secretory transport. Solutions to common problems with expression and imaging GFP fusion proteins–e.g., misfolding and autofluorescence–are also discussed.

Nuclear migration (*UNIT 13.4*) occurs in a wide variety of cell types, including newly fertilized eggs, muscle, nerves, and dividing cells. It is vital for the cell to properly locate its nucleus and surrounding endomembrane system. This unit describes an in vitro assay for monitoring nuclear motility of the pronucleus from frog eggs. This motility is, as are other examples of nuclear motility, driven by microtubule-dependent motility. Purified nuclei are added to a motility extract that includes *Xenopus* cytosol, centrosomes, purified microtubules, dynein, and regulatory factors. The system is then visualized using video enhanced-differential interference contrast microscopy. Once they associate with microtubules, the nuclei normally move toward the centrosomes in microtubule minus end-directed (due the dynein in the extract) motility. There they accumulate over time. Regulators or inhibitors of this movement can be assayed, and the effects on motility can be quantified using this system.

UNIT 13.5 describes dynamic live cell imaging approaches employing photobleaching to measure the kinetics of nuclear proteins. Three major methods of photobleaching microscopy are described fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), and inverse fluorescence recovery after photobleaching (iFRAP). The unit highlights how each technique differs in permitting the determination of distinct particular parameters of protein behavior in vivo. In addition to describing the photobleaching techniques, this unit details transfection methods for introducing GFP-expression vectors into mammalian cells by electroporation or by lipofection. Moreover, it provides a method for the determination of the number of GFP molecules in a single, living cell, which is critical for the quantification of GFP-fusion proteins.

UNIT 13.6 describes a number of in vitro assays for characterizing the function of putative actin-binding proteins. One assay described uses a change in fluorescence of pyrenyl-labeled actin to assess a protein's actin capping activity. The read-out of this assay includes kinetic measurements of filament growth or depolymerization at one or the other end of the actin filament. Another assay describes assays of filament growth from actin seeds. This allows proteins that interact with G-actin to be analyzed, and permits the determination of whether the actin association rate constant for filament assembly is the same or different from that of free G-actin. The unit also provides protocols for measuring the turnover of a population of filaments in vitro using a fluorescent analog of ATP whose fluorescence changes when bound to actin. It further describes mathematical modeling approaches for understanding the mechanism by which the basic features of actin spontaneous assembly (nucleation-growth process) are modified by the severing or branching agents. Finally, the unit highlights light microscopy approaches that can be used to observe the dynamics of individual actin filaments, including total internal reflection fluorescence microscopy (TIRF).

Jennifer Lippincott-Schwartz

Microtubule/Organelle Motility Assays

This unit describes an in vitro assay that uses video-enhanced differential interference contrast (VE-DIC) microscopy to examine the motile interactions between isolated organelle fractions and microtubules (MTs; see Basic Protocol). The method can be used to dissect the molecular requirements for organelle movement and membrane trafficking. A field of axoneme-nucleated MTs, growing and shortening as they would in a living cell (dynamic MTs), is generated in a simple microscope perfusion chamber. Various combinations of isolated endoplasmic reticulum (ER) and Golgi apparatus organelles, cytosol containing motor proteins and other soluble factors, nucleotides, and specific pharmacological reagents are then added to the dynamic MT, and the motile interactions between the organelles and MTs are observed by VE-DIC microscopy.

In addition, this unit includes protocols for biochemical preparation of phosphocellulose-purified tubulin from porcine brain (see Support Protocol 3), axonemes from sea urchin sperm (see Support Protocol 2), rat liver cytosol (see Support Protocol 4), and rat liver organelle fractions (see Support Protocol 5). To ensure more reproducible results, a protocol for preparing thoroughly cleaned ("squeaky clean") coverslips and simple microscope perfusion chambers is also included (see Support Protocol 1).

STRATEGIC PLANNING

Performing a successful motility assay requires ~2 weeks of preparatory biochemistry and considerable skill in obtaining VE-DIC images. Detailed description of how to set up the sophisticated optical system required for imaging single MTs by VE-DIC is outside the scope of this unit and is not included here. Instead, the reader should consult the unit on microscopy by E.D. Salmon (*UNIT 4.1*) and other more comprehensive descriptions of the techniques required for achieving such images (Walker et al., 1988; Salmon and Tran, 1998).

Preparation of the principal biochemical components for the motility assay is detailed in Support Protocols 2 to 5. These components must be prepared in bulk in advance, dispensed into appropriately sized aliquots, and stored at −70°C. Unopened samples can be stored for >2 years. Three different types of animal tissue must be obtained for the various preparations. The animals that are most difficult to acquire are the sea urchins, *Strongylocentrotus purpuratus*, used for the preparation of axonemes. Sea urchins can be obtained from early winter through mid spring from Marinus, Inc., but their availability depends on the seasonal catch. Porcine brains for the tubulin preparation must be obtained from freshly slaughtered pigs, and the tubulin preparation should begin within 3 to 4 hr after the tissue is harvested. A local butcher can supply information regarding the location of the closest slaughterhouse. Fresh rat livers are fairly easy to obtain; alternatively, flash-frozen livers can be purchased from Pel-Freez.

In contrast to the biochemistry and microscopy, setting up the motility assay in the Basic Protocol is relatively simple. Note, however, that specific brands of microscope coverslips and slides are required for the preparation of the microscope perfusion chambers (see Support Protocol 1), and the coverslips should be cleaned according to the steps outlined. Rigorous attention to the detailed instructions presented in Support Protocol 1 is crucial to the success of the assay. Inexpensive microscopy supplies are often coated with oils and dirt that can lead to spurious and inconsistent results.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

Contributed by Clare M. Waterman-Storer

Current Protocols in Cell Biology (1998) 13.1.1-13.1.21

MT/ORGANELLE MOTILITY ASSAYS

This protocol describes the set-up and execution of an assay that combines dynamic MTs with cellular organelle fractions and cytosolic proteins to reconstitute organelle motility in vitro (see Fig. 13.1.1).

Materials

Axoneme fragments (see Support Protocol 2)
Golgi or ER membranes (see Support Protocol 5)
45 μM purified brain tubulin (see Support Protocol 3)
Rat liver cytosol (see Support Protocol 4)
PM buffer (see recipe)
PM buffer containing 1 mM GTP
20× energy regeneration system (see recipe)
15 mM MgGTP, prepared by diluting 100 mM MgGTP stock (see recipe) in PM buffer
Valap (see recipe)

Simple perfusion chambers (see Support Protocol 1)
Filter paper cut into 2-cm squares

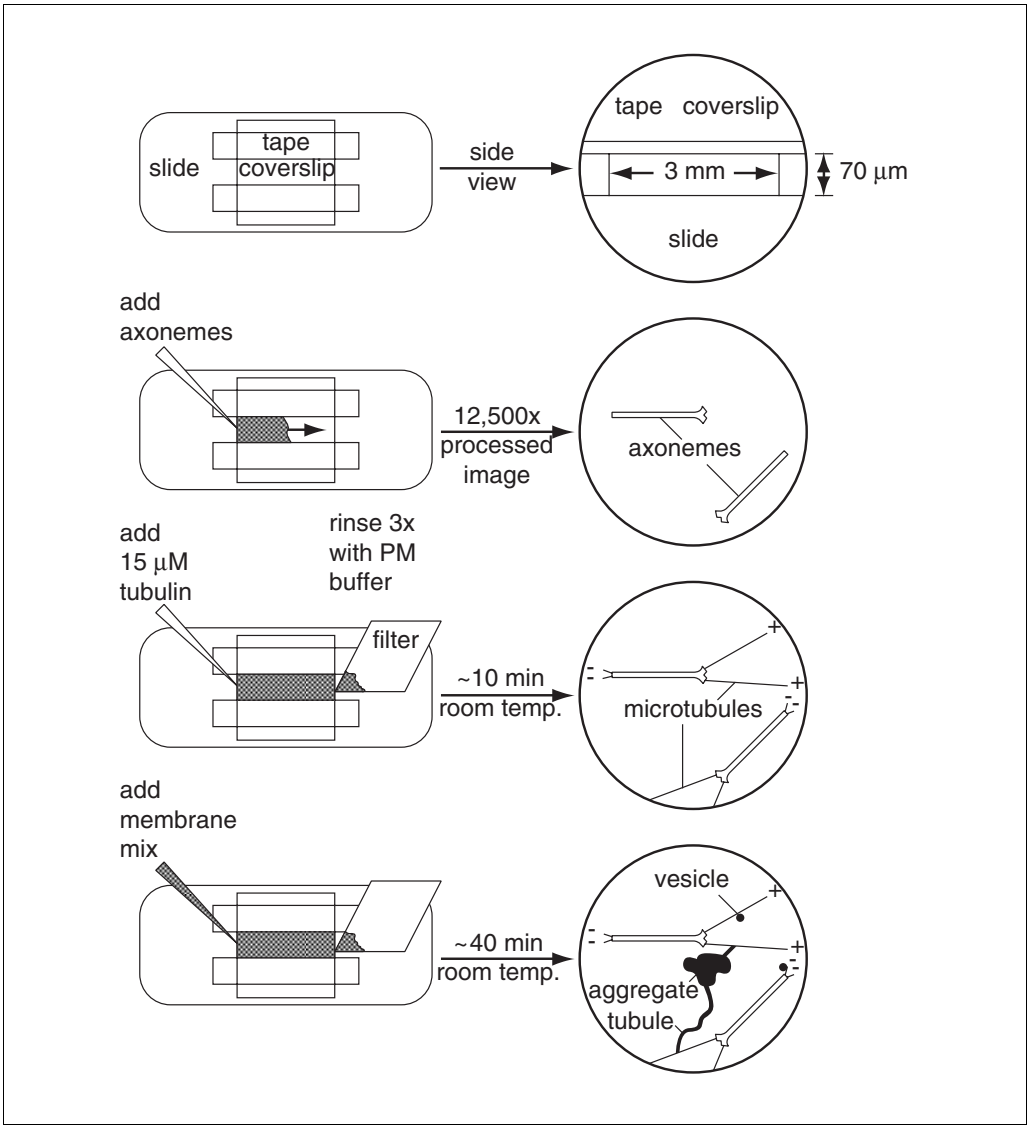


Figure 13.1.1 Flow chart for performing MT/organelle motility assay.

Humid chamber made of a 90-mm glass petri dish containing moist paper towels
High-resolution VE-DIC microscope system (as described in Salmon and Tran, 1998 or equivalent)

1. Rapidly thaw and immediately place on ice one aliquot each of axonemes, Golgi or ER membranes, 45 μM purified brain tubulin, rat liver cytosol, and 20× energy regeneration system.
2. Dilute axonemes with PM buffer to the proper concentration as determined in Support Protocol 2. Prepare 6× Golgi or ER membranes by diluting organelles with PM buffer/1 mM GTP (see Support Protocol 5, step 10).
3. Prepare and place on ice a 30-μl membrane mix:

   5 μl 6× Golgi or ER membranes
   1.5 μl 20× energy regeneration system
   10 μl 45 μM tubulin
   1 μl 15 mM MgGTP
   12.5 μl cytosol.

4. Add axonemes to a simple perfusion chamber by slowly pipetting ~10 μl of diluted axonemes against one open end of the chamber and allowing the chamber to fill.

   Be careful to avoid introducing large bubbles into the chamber.

5. Place the perfusion chamber into the humid chamber and incubate 10 min at room temperature to allow the axonemes to adhere to the glass.
6. Wash out unadhered axonemes by slowly pipetting 10 μl PM buffer against one end of the perfusion chamber while simultaneously wicking excess buffer from the opposite side of the chamber with the tip of a square of filter paper. Repeat wash two more times.
7. Dilute 5 μl of 45 μM tubulin with 10 μl PM buffer/1 mM MgGTP. Perfuse the diluted tubulin into the chamber containing the washed axonemes. Place a drop of immersion oil on the top and bottom of the slide, and transfer it to the VE-DIC microscope stage.

   Briefly, the microscope system consists of illumination from an HBO100-W mercury arc lamp introduced into an upright microscope stand (equipped with optical components for DIC image formation) via a fiber-optic scrambler. Illumination is passed through IR reflecting and 546-nm narrow band-pass filters before being focused for Köhler illumination onto the specimen via a 1.4-NA oil-immersion condenser. The light is collected by a 100 × 1.3- or 1.4-NA objective and magnified 12.5× before being collected by a scientific-grade Newvicon tube type video camera (equivalent of Hamammatsu C2400). The video signal is processed by frame averaging, background subtraction, and contrast enhancement by a real-time image processor (equivalent to the Hamammatsu Argus 10), and then recorded in real time onto high-resolution S-VHS video tape.

8. Focus on the axonemes with the 100× objective lens. Align the slide on the stage so that one edge of the double-stick tape that forms the perfusion chamber perfectly bisects the area illuminated by the microscope condenser lens. Immerse the 100× objective lens in oil and focus on the edge of the tape. With the edge of the tape in view, back off fine focus until the very edge of the tape begins to go out of focus. Move the slide so the lens is within the area coated with axonemes, which should now be quite close to focus.

   It can be difficult to focus on axonemes on the surface of the coverslip because of their very small size and the very bright illumination needed for VE-DIC. This procedure should make focusing on the axonemes easier.

Table 13.1.1 Pharmacologic Agents for Addition to Membrane Mix[a]

Pharmacologic agent	Final concentration	Function	Stock solution	Amount added to 30-µl membrane mix
Brefeldin A	60 µM	Removes Golgi coat proteins	Dilute 1.5 µl of a 3.6 mM Brefeldin A stock in ethanol[b] 1:1 into PM buffer[b] prior to use	3 µl
Aluminum fluoride		Activates hetero-trimeric G proteins		Add 1 µl of 30× NaF[b] and 1 µl of 30× $AlCl_3$[b]
MgGTP-γ-S	1 mM	Activates hetero-trimeric G proteins	30 mM MgGTP-γ-S[b]	1 µl
MgAMP-PNP	1 mM	Inhibits kinesin-like proteins[c]	150 mM MgAMP-PNP[b]	1 µl
Sodium ortho-vanadate	25 µM	Inhibits cytoplasmic dynein[d]	Dilute 1 µl of 100 mM stock into PM buffer[b]	1 µl

[a]Also see *APPENDIX 1B*.

[b]See recipes for instructions on solution preparation. Abbreviations: MgAMP-PNP, 5′ adenylylimidodiphosphate magnesium salt.

[c]Vale et al., 1985.

[d]Shpetner et al., 1988.

9. Optimize the image for visualization of individual MTs by aligning the microscope for Köhler illumination. Use the real-time image processor to perform background subtraction, contrast enhancement, and frame averaging. Observe and record onto S-VHS video tape images of polymerization dynamics of individual MTs as they are nucleated off the axonemes.

   Note the difference between the plus (longer, faster-growing MTs) and minus (shorter, slower-growing MTs) ends of the axonemes. For details on microscopy techniques, refer to UNIT 4.1 or Salmon and Tran (1998).

10. During the observation of MT dynamics, allow the membrane mix to warm to room temperature.

11. Perfuse 12 µl of membrane mix into the simple perfusion chamber on the microscope stage. Seal the chamber edges on both sides with a drop of melted valap. Observe and record the dynamic interactions between the organelles and MTs.

    Note that often it takes up to 45 min for motility to develop. This time period is proportional to room temperature.

    Pharmacological agents (see Table 13.1.1) may be added to the membrane mix prepared in step 3 to test the involvement of Golgi coat proteins and MT motor proteins in organelle movement in vitro. For review of the effects of these pharmacological agents on membrane trafficking, see Klausner et al. (1992). These agents should be added to the mix, correcting all components for concentration, and incubated 15 min at 37°C prior to being introduced into the flow chamber.

    Organelles may also be pretreated to strip them of specific subsets of peripheral proteins prior to addition to the mix. This will allow examination of the involvement of these proteins in organelle movement (see Support Protocol 5, steps 12 to 14).

PREPARATION OF SIMPLE PERFUSION CHAMBERS AND COVERSLIPS

SUPPORT PROTOCOL 1

For the MT/organelle motility assay to be reproducible, the perfusion chambers and coverslips must be "squeaky clean."

Materials

Versa Clean dish detergent (Fisher)
1 mM EDTA
70% and 100% ethanol

22 × 22–mm no. 1.5 coverslips (Corning)
Water-bath sonicator
Double-stick tape
Clay-Adams precleaned Goldseal 3 × 1–in. microscope slides (Becton Dickinson)
Dumont no. 5 forceps

Prepare "squeaky clean" coverslips

1. Place coverslips, one at a time, into a 500-ml glass beaker filled halfway with hot tap water containing ~5 ml Versa Clean detergent. Take care to separate coverslips that are stuck together. Sonicate 45 min in a water-bath sonicator.

   Prepare three packages of coverslips at one time.

2. Rinse coverslips ten times by swirling with hot tap water. Sonicate 30 min in hot tap water.
3. Rinse coverslips ten times by swirling with double distilled water. Sonicate 30 min in double distilled water.
4. Rinse coverslips three times by swirling with 1 mM EDTA. Sonicate 30 min in 1 mM EDTA.
5. Rinse coverslips three times by swirling with 70% ethanol. Sonicate 30 min in 70% ethanol.
6. Rinse coverslips three times by swirling with 100% ethanol. Sonicate 30 min in 100% ethanol.
7. Rinse coverslips once with 100% ethanol. Transfer clean coverslips to a 500-ml screw cap jar, cover with 100% ethanol, and store at room temperature until use.

Prepare simple perfusion chambers

8. Cut pieces of double-stick tape into 25 × 5–mm strips. Apply two strips of tape to the center of a slide, side by side, 3 mm apart, and parallel to the long axis of the slide.
9. Using Dumont no. 5 forceps, retrieve a single squeaky clean coverslip from the storage jar. Dab the edge of the coverslip with a Kimwipe to remove any large drops of ethanol and quickly pass it through the flame of a Bunsen burner. Allow ethanol to burn off.
10. Center the coverslip over the tape strips on the slide and apply the coverslip to the strips of tape, pressing firmly with the back of the forceps to make a good seal.
11. Repeat until ~20 perfusion chambers have been prepared and store them in a slide storage box until use.

SUPPORT PROTOCOL 2

PREPARATION OF SEA URCHIN SPERM AXONEMES

This protocol (as adapted from Bell et al., 1982) should yield enough axoneme fragments to perform thousands of motility assays.

Materials

4 male *S. purpuratus* sea urchins (Marinus)
0.55 M KCl
Artificial sea water (mixes available from aquarium supply stores; prepare per manufacturer's instructions)
20% (w/v) sucrose in distilled water
Isolation buffer (see recipe)
High-salt buffer (see recipe)
Isolation buffer (see recipe) containing 50% glycerol

60-ml syringe with 18-G needle
Tabletop clinical centrifuge
Refrigerated superspeed centrifuge (Sorvall RC-5B) with Sorvall SS-34 rotor (or equivalent)
50-ml polycarbonate centrifuge tubes (e.g., Sorvall)
50-ml Dounce glass homogenizer with type A and B pestles

NOTE: Because it is impossible to tell what sex a sea urchin is until it sheds gametes from the pores located on its dorsal surface, order at least twice as many animals as needed.

Collect sperm

1. Sacrifice the sea urchins. Fill a 60-ml syringe with 0.55 M KCl and attach an 18-G needle. Pierce the urchin from the bottom center of the animal, insert the needle ~1 in., and inject body cavity with fluid until resistance is felt. Place the animal upright on paper toweling. Allow several minutes for gametes to begin to exude from the five pores located on top of the urchin.

   The urchins must be injected and sacrificed to induce their gametes to shed. Upon arrival from shipment, some urchins may have shed a small amount of gamete. In this case, identifying the sex of the animal should be simple: sperm is white and eggs are peach or orange colored.

2. Using a glass Pasteur pipet with a rubber bulb, collect white sperm dropwise as it is exuded. Pool sperm from all four animals into a single test tube on ice.

   Expect to get 3 to 4 ml of sperm per animal over a ~20-min collection period. Do not collect orange- or peach-colored eggs.

3. Dilute the sperm with 3 vol artificial sea water. Let stand 20 min on ice.

4. Centrifuge 5 min at 500 rpm in a tabletop clinical centrifuge to pellet debris.

5. Transfer supernatant to 50-ml polycarbonate centrifuge tubes. Pellet sperm by centrifuging 5 min at 3000 × *g* (5000 rpm in SS-34 rotor), 4°C.

Isolate sperm tails

6. Remove and discard supernatant. Resuspend pellet by trituration in 5 vol of 20% sucrose to osmotically remove the plasma membranes.

7. Transfer the demembranated sperm to a Dounce glass homogenizer partially immersed in slushy ice. Homogenize with fifteen rapid passes of a type B pestle to break the sperm heads from the tails.

   The pestle should be moved from the top of the liquid to the very bottom of the homogenizer, and upon return to the top, the pestle should not break the surface of the liquid.

8. Transfer the homogenate to 50-ml centrifuge tubes, pellet sperm heads by centrifuging 10 min at 12,000 × *g* (10,000 rpm in SS-34 rotor), 4°C.

9. Collect the supernatant containing the demembranated tails into new 50-ml tubes and pellet the tails by centrifuging 15 min at 20,000 × *g* (13,000 rpm in SS-34 rotor), 4°C.

10. Discard the supernatant. Using a metal weighing spatula and a Pasteur pipet, collect only the top white layer that contains the demembranated tails. Resuspend pelleted tails by trituration in 4 vol isolation buffer.

    The bottom yellow layer of the pellet contains sperm heads and debris.

Disrupt tails and extract axonemes

11. Transfer resuspended tails to Dounce glass homogenizer on ice. Homogenize with five rapid passes of a type A pestle to break the tails into fragments. Transfer fragment suspension into 50-ml centrifuge tubes.

12. Pellet the tail fragments in 50-ml tubes by centrifuging 10 min at 12,000 × *g,* 4°C.

    This step will also result in a two-layer pellet.

13. Discard the supernantant and collect only the top white layer. Resuspend this white pellet by trituration in 4 vol isolation buffer. Transfer to new tubes and centrifuge as in step 12. Repeat this cycle of resuspension and centrifugation one or two more times to completely separate the tail fragments from heads and debris. Continue until the pellet is a single layer of pure white.

14. Discard the supernatant and resuspend the white pellet in 4 vol high-salt buffer.

15. Transfer to Dounce glass homogenizer on ice. Homogenize with five passes of a type A pestle. Incubate 45 min on ice to extract dyneins and central pair MTs from the tail fragments.

Isolate axonemes

16. Transfer extraction suspension to 50-ml centrifuge tubes and separate the extracted axonemes from soluble proteins by centrifuging 15 min at 20,000 × *g,* 4°C.

17. Discard the supernatant and resuspend the axoneme pellet by trituration in 4 vol high-salt buffer. Transfer to a new 50-ml centrifuge tube and reextract the axoneme fragments by incubating 15 min on ice.

18. Pellet the extracted axonemes by centrifuging 15 min at 20,000 × *g,* 4°C.

19. Discard supernatant and resuspend extracted axoneme pellet by trituration in ⅓ the original volume of sperm (in step 2) in isolation buffer/50% glycerol.

20. Distribute the axonemes into 20-µl aliquots in 0.5-ml microcentrifuge tubes and freeze by immersing tubes in liquid nitrogen.

21. Store frozen axonemes at −70°C until use.

22. Determine the proper axoneme dilution for use in the MT/organelle motility assay. Thaw an aliquot of axonemes and observe various dilutions by VE-DIC (at the same magnification as will be used in the assay) in a simple perfusion chamber (see Support Protocol 1). Note the dilution required such that two to three axonemes are visible per ~30-μm^2 microscopic field.

SUPPORT PROTOCOL 3

PREPARATION OF PORCINE BRAIN TUBULIN

Although many protocols for tubulin preparation are available, this procedure (as adapted from Walker et al., 1988) is the simplest and, in the author's experience, provides the highest yields of tubulin. Three pig brains should yield ~60 mg of purified tubulin.

Materials

100-g P-11 cellulose phosphate fibrous cation exchanger (Whatman)
0.1 M HCl
0.1 M and 10 M NaOH
0.1 M $MgSO_4$
10× and 1× column buffer (see recipe)
3 fresh pig brains (use <3 hr after slaughter)
Homogenization buffer (see recipe; freshly prepared)
PM buffer (see recipe)
100 mM MgATP (see recipe)
PMG buffer (see recipe)
100 mM MgGTP (see recipe)
1 M dithiothreitol (DTT; *APPENDIX 2A*)
Glutamic acid, sodium salt

2-liter sintered-glass filter funnel and 2-liter sidearm Erlenmeyer flask
Waring blender
Temperature-controlled ultracentrifuge (Beckman L7-55) with Beckman 50.2Ti rotor (or equivalent)
31.5-ml thick-walled polycarbonate ultracentrifuge tubes (e.g., Beckman) with screw caps
30-ml Dounce type A glass homogenizer
44 × 250–mm adjustable volume column for low-pressure liquid chromatography (e.g., Amicon model #95240 or equivalent)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3*)

DAY 1: Prepare phosphocellulose column

1. Add 90 g of Whatman P-11 phosphocellulose to 2 liters of 0.1 M NaOH in a 4-liter beaker while mixing very gently with a glass rod. Mix suspension gently 5 min, then allow the solids to settle 20 min. Aspirate and discard excess solution. Transfer remaining phosphocellulose slurry to a 2-liter sintered-glass funnel on a 2-liter sidearm Erlenmeyer flask connected to a vacuum line. Carefully vacuum filter the remaining 0.1 M NaOH from the phosphocellulose, but do not allow the resin to run dry.

2. Gently scrape the phosphocellulose from the funnel and return it to the 4-liter beaker. Add 2 liters of 0.1 M NaOH, mix gently 5 min, and check the pH with pH paper. If the pH of the slurry is not >12, repeat the settling, aspiration, and filtering treatment and resuspend phosphocellulose in 2 liters of 0.1 M NaOH. Repeat until pH >12.

3. Rinse phosphocellulose with 4 liters of distilled water by vacuum filtration, again being careful never to let the resin run dry.

4. Transfer phosphocellulose from the funnel to the 4-liter beaker and add 2 liters 0.1 M HCl. Mix gently, allow the resin to settle, aspirate excess solution, and vacuum filter the resin (as in step 1). Repeat the 0.1 M HCl treatment cycle until the pH of the phosphocellulose slurry is <3.

5. Rinse the phosphocellulose with 4 liters of distilled water by vacuum filtration.

6. Transfer the phosphocellulose resin to the 4-liter beaker and add 2 liters of 0.1 M $MgSO_4$. Mix, settle, aspirate, and vacuum filter.

7. Transfer the phosphocellulose resin to the 4-liter beaker and add 2 liters of 10× column buffer. Mix gently 10 to 15 min, allow the resin to settle, aspirate excess column buffer, and vacuum filter.

8. Transfer resin to the 4-liter beaker, add 2 liters of 1× column buffer, and mix gently for 5 min. Check the pH of the slurry with a pH meter and adjust to 6.6 with 10 M NaOH. Allow resin to settle, aspirate excess buffer, and vacuum filter. Repeat the 1× column buffer treatment cycle, using 10 M NaOH to adjust the pH of the slurry to 6.6, until the pH of the slurry is 6.6 without adjustment after resuspension and mixing. Allow resin to settle 20 min and aspirate buffer until the settled resin:buffer ratio is 3:1 (v/v).

Pour column

9. Mix the resin gently until evenly suspended in the buffer, then rapidly pour the slurry into an empty 44 × 250–mm liquid chromatography column (clamped to a support in the cold room). Fill column to the top. Cover with Parafilm and allow the resin to settle for several hours.

10. Fill the column adjuster plunger with column buffer, and very slowly insert it into the column, being very careful not to disturb the phosphocellulose resin. Insert the adjuster plunger until all air bubbles are expelled from column through the column inlet tubing which is immersed in a 4-liter reservoir of column buffer. Tighten and seal the adjuster plunger fittings, leaving the inlet tube in the buffer reservoir.

11. Attach the outlet tubing to a peristaltic pump and set the pump to run at 0.25 ml/min to allow the column to pack for ~48 hr.

    A well-packed phosphocellulose column should be perfectly even in color with no evidence of cracks in the resin. The better the phosphocellulose column is packed, the more concentrated the peak of elution of tubulin protein.

DAY 2: Prepare MT protein

12. Transport brains from slaughterhouse to laboratory in an evacuated plastic ziplock bag buried in ice.

    Brains must be kept on ice from the time the animals are slaughtered.

13. Working in a 4°C cold room, carefully and thoroughly remove the meninges and any blood-red tissue from the surface, stem, and within the folds of each brain. Pick tissue away by hand and use Kimwipes to peel membranes from surface of brain.

    The dry wipe will stick to the meninges; as it is drawn gently across the surface of the brain, it will peel away the deep red membrane from the pinkish-gray nervous tissue underneath.

14. Cut cleaned brains into 2- to 3-cm^2 cubes and weigh the tissue. Transfer the tissue to a Waring blender and add 0.5 ml/g freshly prepared homogenization buffer containing 1 mM MgATP.

Homogenize brain tissue

15. Homogenize the tissue by blending 5 sec on high speed and then 45 sec on low speed.

    This should result in a suspension with the color and consistency of a strawberry milkshake.

16. Using a 50-ml serological pipet with one-third of the tip cut off and a pipet bulb with strong suction, transfer the brain homogenate to several 31.5-ml polycarbonate

ultracentrifuge tubes. Note the homogenate volume and pair the tubes by weight (to 0.01 g). Discard any extra homogenate that does not fit into a full rotor-full of centrifuge tubes.

Tubulin is sensitive to proteases and easily denatured; keeping extra homogenate that must be left for 1 hr unprocessed while the first set of tubes is centrifuged does not significantly increase the final yield.

17. To remove undisrupted tissue from cell cytosol, centrifuge homogenate 60 min at 100,000 × *g* (29,000 rpm in 50.2Ti rotor), 4°C.

18. Working at room temperature, carefully collect the cytosolic supernatants from the tubes with a pipet. Pool supernatants in a graduated cylinder and add an equal volume of PMG. To promote MT polymerization, add MgGTP to 0.2 mM. Distribute into 31.5-ml ultracentrifuge tubes and pair tubes by weight.

Isolate MT protein

19. Immerse the portion of the tubes containing the cytosol in a 37°C water bath and incubate 45 min to allow MT proteins to polymerize into MTs. During this incubation, warm the ultracentrifuge and 50.2Ti rotor to 25°C.

20. Pellet MTs from the cytosol by centrifuging 45 min at 100,000 × *g,* 25°C.

21. In the cold room, discard the supernatant and resuspend the MT pellet in a volume of PM buffer containing 0.2 mM GTP equal to one-fifth the volume of homogenate.

To resuspend the pellet, add a few milliliters of resuspension buffer to each centrifuge tube and to a small glass Dounce homogenizer (on ice).

22. Using a round-ended weighing spatula, scrape out the sticky pellets and transfer them to the Dounce homogenizer. Resuspend any pellet remaining in the ultracentrifuge tubes by trituration and transfer to the homogenizer.

23. Homogenize the pellets with five to ten passes of a type A pestle. Dispense the resuspended MTs into 31.5 ml-ultracentrifuge tubes and pair tubes by weight.

24. Incubate the resuspended MTs on ice for 30 min with gentle mixing every 5 min to allow for MT depolymerization. During this incubation, chill the ultracentrifuge and rotor to 4°C.

25. Clarify the MT protein by centrifuging 45 min at 100,000 × *g,* 4°C.

26. At room temperature, collect the supernatant containing the MT protein, add an equal volume of PMG, and add GTP to a concentration of 0.2 mM. Dispense into 31.5-ml centrifuge tubes and pair tubes by weight.

27. Immerse the portion of the tubes containing the MT protein solution in a 37°C water bath and incubate 45 min to allow MTs to polymerize. During the incubation, warm the ultracentrifuge and rotor to 25°C.

28. Pellet the MTs from polymerization-incompetent tubulin by centrifuging 45 min at 100,000 × *g,* 25°C.

29. In the cold room, discard the supernatant. Add 1 ml of column buffer containing 0.5 mM GTP to each tube. Making sure that the buffer is covering the pellet, immerse tube in liquid nitrogen to freeze pellet.

Store the tubes at −70°C until the next day or whenever phosphocellulose column purification of tubulin is to be carried out.

DAY 3: Purify tubulin from MT protein on phosphocellulose column

30. Chill ultracentrifuge and 50.2Ti rotor to 4°C.

31. Prepare and equilibrate phosphocellulose column with column buffer containing 0.5 mM GTP and 1 mM DTT. Turn off peristaltic pump, and if resin bed has settled, carefully loosen the seals on adjuster plunger. Slowly insert adjuster plunger further into column until it barely touches the top of the resin and retighten the seals. Switch the inlet tube from the reservoir of 1× column buffer to a 1-liter reservoir of 1× column buffer containing 0.5 mM MgGTP and 1 mM DTT. Be careful not to introduce bubbles into the inlet tube. Turn the peristaltic pump on and adjust speed to 1.8 ml/min. Allow ≥300 ml of buffer to be drawn through the column before loading the MT protein.

32. While column equilibrates, thaw MT pellets (from step 29) by immersing ultracentrifuge tubes in a 37°C water bath until pellets turn from chalky white to completely translucent white. When pellets are thawed, place tubes immediately on ice and put them in the cold room.

33. Add a volume of 1× column buffer containing 0.2 mM GTP equal to ~3× vol of pellets. Resuspend pellets on ice with a Dounce glass homogenizer and type A pestle (as in step 21). Transfer resuspended MTs to a 31.5-ml ultracentrifuge tube. Allow MTs to depolymerize by incubating on ice for 30 min with gentle mixing every 5 min.

34. Clarify the MT protein by centrifuging 45 min at 100,000 × *g,* 4°C.

35. In the cold room, collect the clarified MT protein supernatant and add MgGTP to a final concentration of 0.5 mM (an additional 0.3 mM) and DTT to 1 mM.

Load MT protein

36. When column has equilibrated, switch the inlet tube from the buffer reservoir to the clarified MT protein. Load the MT protein onto the column at 1.8 ml/min. When all of the protein is loaded, switch the inlet back to the buffer reservoir and begin to collect 10-ml fractions. As each aliquot comes off, add MgGTP to a final concentration of 1 mM (an additional 0.5 mM).

    Tubulin will pass through the column and come off after ~100 ml. MT binding proteins will remain bound to the phosphocellulose resin.

37. Monitor the elution of tubulin by using a UV monitor to measure absorption at 280 nm. Pool fractions containing tubulin (usually ~100 ml).

    Alternatively, the protein concentration of a 100-µl aliquot of each fraction can be determined by colorimetric assay (APPENDIX 3).

Separate MTs from denatured tubulin

38. Working at room temperature, add 0.186 g/ml glutamic acid (sodium salt) to the tubulin solution and stir slowly until dissolved. Dispense the solution into 31.5-ml ultracentrifuge tubes and pair tubes by weight.

39. Immerse the centrifuge tubes to the level of the liquid in a 37°C water bath and incubate 30 min to allow MTs to polymerize. During this time, warm the ultracentrifuge and rotor to 25°C.

40. Separate MTs from polymerization-incompetent tubulin by centrifuging 30 min at 100,000 × *g,* 25°C.

41. In the cold room, resuspend MT pellets on ice in 3 vol of PM buffer containing 0.5 mM MgGTP using a glass Dounce homogenizer. Incubate resuspended MTs on ice 30 min to allow MT depolymerization.

42. During this time, determine the tubulin concentration by measuring the absorbance at 280 nm (using PM buffer containing 0.5 mM MgGTP as a blank). Calculate the tubulin concentration using the formula: [tubulin] = (A_{280} × dilution factor)/extinction coefficient), where the extinction coefficient of tubulin = 115,000 mol/cm. Add PM buffer containing 0.5 mM MgGTP to adjust the final protein concentration to 45 µM.

43. Dispense into several 1-ml aliquots (stock aliquots) and several 50-µl aliquots (to be used directly in the MT/organelle motility assays). Freeze by immersing in liquid nitrogen and store at −70°C until needed.

SUPPORT PROTOCOL 4

PREPARATION OF RAT LIVER CELL CYTOSOL

This protocol (modified from Donaldson et al., 1991) should yield enough cytosol for up to 200 MT/organelle motility assays.

Materials

Fresh or flash-frozen (Pel-Freez) rat livers
PBS (*APPENDIX 2A*)
Homogenization buffer (see recipe)
Homogenization buffer containing 0.5 mM MgGTP (from 100 mM MgGTP stock; see recipe)
PM buffer (see recipe) containing 0.25 M sucrose

Superspeed centrifuge (Sorvall RC-5B) with Sorvall SS-34 rotor (or equivalent)
Ultracentrifuge (Beckman L7-55) with Beckman 50.2Ti and SW-28 rotors (or equivalents)
20-ml glass homogenizer with Teflon pestle
Homogenizer or drill press
50-ml polycarbonate centrifuge tubes (e.g., Sorvall)
31.5-ml thick-walled polycarbonate ultracentrifuge tubes (e.g., Beckman) with screw caps

Additional reagents and equipment for determination of protein concentration (*APPENDIX 3*)

1. Chill the superspeed centrifuge, ultracentrifuge, and rotors to 4°C.
2. In the cold room, rinse liver well in PBS, then rinse in freshly prepared homogenization buffer.
3. Weigh liver, return to the cold room, and mince tissue finely with scissors. Transfer minced tissue to 20-ml glass homogenizer and add 1 vol homogenization buffer containing 0.5 mM MgGTP. Keeping the homogenizer immersed in slushy ice, homogenize tissue with a Teflon pestle attached to a homogenizer or drill press using six slow passes at 3000 rpm.
4. Transfer homogenate to 50-ml centrifuge tubes, pair tubes by weight, and remove cellular debris by centrifuging 10 min at 10,000 × *g* (9000 rpm in SS-34 rotor), 4°C.
5. In the cold room, collect supernatant, transfer to 31.5-ml ultracentrifuge tubes, and pair tubes by weight. Clarify cytosol by centrifuging 60 min at 100,000 × *g* (29,000 rpm in 50.2Ti rotor), 4°C.
6. Collect clarified cytosolic supernatant, dispense into 50-µl aliquots, and freeze by immersion in liquid nitrogen. Store at −70°C until use.

PREPARATION OF RAT LIVER ORGANELLE FRACTIONS

This protocol should yield enough organelles for up to 200 MT/organelle motility assays.

Additional Materials *(also see Support Protocol 4)*

Homogenization buffer (see recipe) containing 0.5 mM MgGTP (from 100 mM stock; see recipe) and 0.25 M sucrose
Homogenization buffer containing 0.5 mM MgGTP
2.3 M sucrose
PM buffer (see recipe) containing 0.25 M sucrose and 0.5 mM GTP
3 M KI stock (optional)
0.5 M EDTA stock (optional; *APPENDIX 2A*)
0.5 M Na_2CO_3 stock, pH 11.5 (optional)
PM buffer containing 0.25 M sucrose (optional)

25-ml Ultraclear ultracentrifuge tubes (e.g., Beckman)
Beckman SW-28 and 50.2Ti rotors or equivalents
31.5-ml thick-walled polycarbonate ultracentrifuge tubes (e.g., Beckman) with screw caps
TLA tabletop ultracentrifuge (Beckman) with TLS-55 swinging bucket rotor or equivalent and mini-ultracentrifuge tubes (e.g., Beckman)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3*)

Clarify rat liver homogenate

1. Rinse liver, mince, and perform initial centrifugation as in the preparation of rat liver cytosol (see Support Protocol 4, steps 1 to 4), except in step 2, homogenize minced liver in homogenization buffer containing 0.5 mM MgGTP and 0.25 M sucrose.

2. In the cold room, collect the supernatant from the clarified homogenate and add 2.3 M sucrose to a final concentration of 1.25 M sucrose.

Isolate Golgi and ER

3. Set up a sucrose density step gradient in a 25-ml Ultraclear ultracentrifuge tube as follows, using 2.0, 1.25, 1.1, and 0.25 M sucrose solutions prepared by diluting the 2.3 M sucrose stock solution with homogenization buffer containing 0.5 mM MgGTP, chilled to 4°C. Add a 2-ml 2.0 M sucrose cushion to the bottom of the tube. Being careful to avoid disturbing the sucrose cushion, layer 12 ml of clarified homogenate containing 1.25 M sucrose on the 2.0 M sucrose by using a pipet and allowing the solution to slowly run in a steady stream down the side of the centrifuge tube. Add a 12-ml layer of 1.1 M sucrose and then an 8-ml layer of 0.25 M sucrose. Discard any excess homogenate. Pair the gradient with a balance tube of equal weight.

4. Separate the organelles on the basis of density by centrifuging the gradient 3 hr at 100,000 × *g* (28,000 rpm in SW-28 rotor), 4°C.

5. Working in the cold room, use a Pasteur pipet to carefully harvest the off-white band of Golgi membrane at the interface between the 0.25 M and 1.1 M sucrose layers and the off-white band of ER membrane at the interface between the 1.1 M and 1.25 M sucrose layers. Place each in separate 31.5-ml ultracentrifuge tubes on ice.

6. Determine protein concentration of the Golgi and ER membrane fractions (*APPENDIX 3*).

7. Dilute both membrane fractions with 3 vol of 0.25 M sucrose in homogenization buffer containing 0.5 mM GTP. Pellet membranes by centrifuging 60 min at 100,000 × *g* (29,000 rpm in 50.2Ti rotor), 4°C.
8. In the cold room, discard the supernatants and resuspend the dense, sticky ER and Golgi membrane pellets separately by extensive trituration in PM buffer containing 0.25 M sucrose and 0.5 mM GTP to achieve a protein concentration of 5 mg/ml.
9. Dispense into 20-µl aliquots and freeze by immersion in liquid nitrogen. Store at −70°C until use.
10. To determine the proper dilution of ER of Golgi membranes for use in the motility assay, view various dilutions of membranes in PM buffer in simple perfusion chambers by VE-DIC microscopy. Note the dilution required such that ~20% of the area of the microscopic field is covered with organelles.
11. When preparing membranes prior to the MT motility assay, thaw one of the aliquots frozen in step 9 and make a stock membrane preparation 6× the dilution determined in step 10.

Prepare stripped membranes (optional)

12. To strip the membranes, incubate a small aliquot (~50 to 100 µl) of isolated organelles with one of the following solutions for 30 min on ice:

    Salt-washed organelles: 0.6 M KI (from 3 M KI stock);

    EDTA-stripped organelles: 10 mM EDTA (from 0.5 M EDTA stock);

    Carbonate-washed organelles: 150 mM Na_2CO_3 (from 0.5 M Na_2CO_3 stock, pH 11.5).

13. Transfer to a mini-ultracentrifuge tube and pellet membranes by centrifuging 1 hr at 110,000 × *g* (50,000 rpm in TLS-55 rotor) at 4°C in a Beckman TLA tabletop ultracentrifuge.
14. Carefully remove supernatant and resuspend membrane pellet by gentle trituration in the original volume of PM buffer containing 0.25 M sucrose.

Treat membranes with pharmacological agents

15. Incubate membranes, cytosol, and energy mix with brefeldin A, aluminum fluoride, or GTP-γ-S (30 min at 37°C) prior to adding them to the mix (see Table 13.1.1). Isolate treated membranes by centrifugation as in step 13. Resuspend and use as described for stripped membranes.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

$AlCl_3$, 30× (1.5 mM)

20 mg $AlCl_3$
Distilled water to 100 ml
Store <1 year at room temperature

Brefeldin A, 3.6 mM

10 mg brefeldin A (Epicenter Technologies)
100% ethanol to 12 µl
Store <1 year at −20°C

Column buffer, 10×

500 ml 1 M PIPES (see recipe; 250 mM final)
40 ml 0.5 M EGTA (see recipe; 10 mM final)
40 ml 1 M $MgSO_4$ (5 mM final)
Distilled H_2O to 1800 ml
Adjust pH to 6.7
Add distilled H_2O to 2 liters
Store <1 month at 4°C

EGTA, 0.5 M

19.02 g EGTA sodium salt
Distilled H_2O to 90 ml
Adjust pH to 7.0
Distilled H_2O to 100 ml
Store <1 year at room temperature

Energy regeneration system, 20× (see Murray, 1991)

150 mM creatine phosphate (Boehringer Mannheim)
2 ml 100 mM MgATP (see recipe; 20 mM ATP/20 mM $MgSO_4$ final)
40 ml 0.5 M EGTA (see recipe; 2 mM final)
Distilled H_2O to 10 ml
Dispense into 100-µl aliquots and store indefinitely at −20°C

HEPES, 1 M, pH 7.0

119.15 g *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (free acid)
Distilled H_2O to 400 ml
Add solid NaOH, a few pellets at a time while mixing, until the pH is ~6.8
Add concentrated NaOH dropwise to pH 7.0
Add distilled H_2O to 500 ml
Filter sterilize and store <6 months at 4°C

High-salt buffer

120 ml 5 M NaCl (0.6 M final)
4 ml 1 M $MgSO_4$ (4 mM final)
2 ml 0.5 M EDTA (*APPENDIX 2A*; 1 mM final)
10 ml 1 M HEPES (see recipe; 10 mM final)
Distilled water to 900 ml
Adjust pH to 7.0
Add H_2O to 1 liter
Store <1 year at 4°C
Add 2-mercaptoethanol (2-ME) to 7 mM final and DTT (*APPENDIX 2A*) to 1 mM final just before use

Homogenization buffer

300 ml PM buffer (see recipe)
52.3 mg PMSF (1 mM final)
3 g leupeptin (10 mg/ml final)
300 µg pepstatin A (1 µg/ml final)
3 mg *N*-α-*p*-tosyl-L-arginine methyl ester (TAME, 10 µg/ml final)
Mix well to dissolve; use immediately

Isolation buffer

20 ml 5 M NaCl (0.1 M final)
4 ml 1 M $MgSO_4$ (4 mM final)
2 ml 0.5 M EDTA (*APPENDIX 2A*; 1 mM final)
10 ml 1 M HEPES (see recipe; 10 mM final)
Distilled H_2O to 900 ml
Adjust pH to 7.0
Add distilled H_2O to 1 liter
Store <1 year at 4°C
Add 2-mercaptoethanol (2-ME) to 7 mM final just before use

MgAMP-PNP, 150 mM

25 mg AMP-PNP (Boehringer Mannheim)
90 µl 1 M $MgSO_4$ (150 mM final)
Add 225 µl distilled H_2O (to 315 µl)
Store <2 years at −20°C

MgATP, 100 mM

Check formula weight of the lot of ATP and determine the amount required for 10 ml of 100 mM solution. Add 8.5 ml distilled H_2O to the determined amount of ATP. Add 1 ml 1 M $MgSO_4$ stock and adjust pH to 7.0 using NaOH. Add H_2O to 10 ml. Dispense into 200-µl aliquots and store <2 years at −20°C.

MgGTP, 100 mM

Check formula weight of the lot of GTP and determine the amount required for 10 ml of 100 mM solution. Add 8.5 ml distilled H_2O to the determined amount of GTP. Add 1 ml 1M $MgSO_4$ stock and adjust pH to 7.0. Add H_2O to 10 ml. Dispense into 200-µl aliquots and store <2 years at −20°C.

MgGTP-γ-S, 30 mM

10 mg GTP-γ-S tetralithium salt (Boehringer Mannheim)
18 µl 1 M $MgSO_4$ stock (30 mM final)
Add 572 µl distilled H_2O
Store <2 years at −20°C

NaF, 30×

378 mg NaF (0.9 M final)
Distilled H_2O to 10 ml
Store indefinitely at −20°C

Na_2CO_3, 0.5 M

531 mg Na_2CO_3
Distilled H_2O to 10 ml
Store <1 year at room temperature

PIPES, 1 M, pH 6.9

151.2 g piperazine-*N,N′*-bis(2-hydroxypropanesulfonic acid), free acid
Distilled H_2O to 400 ml
Add solid NaOH a few pellets at a time while mixing until the pH is ~6.7
Add concentrated NaOH dropwise to pH 6.9
Add distilled H_2O to 500 ml
Filter sterilize and store <1 year at 4°C

PM buffer, pH 6.9

100 ml 1 M PIPES (see recipe; 100 mM final)
2 ml 1 M $MgSO_4$, (2 mM final)
2 ml 0.5 M EGTA (see recipe; 1 mM final)
Add distilled H_2O to 900 ml
Adjust pH to 6.9
Add distilled H_2O to 1 liter
Store <2 months at 4°C

PMG buffer

80 ml 1 M PIPES (see recipe; 80 mM final)
2 ml 1 M $MgSO_4$, (2 mM final)
2 ml 0.5 M EGTA (see recipe; 1 mM final)
600 ml glycerol
Distilled H_2O to 900 ml
Mix well
Adjust pH to 6.9
Add distilled H_2O to 1 liter
Store <1 month at 4°C

Sodium orthovanadate, 100 mM

1.839 g sodium orthovanadate
Distilled H_2O to 8 ml in a screw-cap tube
Adjust pH to 10 with NaOH or HCl
If solution is yellow, place in boiling water until clear, then recheck pH (repeat as necessary)
Adjust to final concentration by checking A_{265} and adding distilled water as needed
Store indefinitely at −20°C

The extinction coefficient for sodium orthovanadate is 2925 $M^{-1}cm^{-1}$.

Valap

50 g Vaseline (Fisher)
50 g lanolin (Fisher)
50 g paraffin (Fisher)

Place vaseline, lanolin, and paraffin in a 1-liter Pyrex beaker. Heat on low setting on a hot plate, stirring occasionally, until all components are melted and well mixed. Pour into several small screw-cap jars (~50 ml capacity). Store indefinitely at room temperature.

COMMENTARY

Background Information

As proteins mature and are processed and secreted, they move continuously between the membranous network compartments of the endoplasmic reticulum (ER) and Golgi apparatus, and from the Golgi apparatus to the cell surface. In living cells, this dynamic membranous organelle traffic occurs through a process in which membrane tubules or vesicles bud from one membrane compartment and fuse with another to deliver their contents. Membrane tubulation, budding, and fusion is believed to be controlled by G proteins and specific "coat" proteins that reversibly associate with the organelle surface (Klausner et al., 1992; Pfeffer, 1994; Bennett, 1995; Schmid and Damke, 1995). In addition, in living cells, ER and Golgi membrane tubules extend and branch in a microtubule (MT)-dependent fashion (Terasaki et al., 1986; Dailey and Bridgman, 1989; Lee et al., 1989; Cooper et al., 1990; Terasaki and Reese, 1994). MT-dependent membrane trafficking is believed to involve the MT-based motor proteins kinesin and cytoplasmic dynein (Lippincott-Schwartz et al., 1995; Presley et al., 1997; reviewed in Cole and Lippincott-

Schwartz, 1995; Sheetz, 1996). The MT/organelle motility assay provides an in vitro system in which MT-based membrane tubule movement and fusion can be observed and the effects of pharmacological, immunochemical, or biochemical treatments can be tested. As mentioned in the Basic Protocol, manipulations of the system may include: the addition of known effectors of coat proteins, G proteins, or MT-based motors to the membrane mix; the removal of subsets of peripheral proteins from the isolated organelles; and fractionation or immunoprecipitation of specific proteins from the cytosol prior to addition to the membrane mix.

ER-like membrane tubules have been shown to form in vitro in a MT-dependent fashion by two mechanisms. The first involves motor proteins and occurs when crude organelle fractions are mixed with cytosolic extracts and taxol-stabilized MTs in the presence of ATP. Here, membranes make gliding attachments to MTs or stable attachments to gliding MTs (Dabora and Sheetz, 1988; Vale and Hotani, 1988; Allan and Vale, 1991, 1994; Gill et al., 1991; Schroer and Sheetz, 1991; Allan, 1995). The second mechanism is independent of motor ATPase activity and occurs in extracts of *Xenopus laevis* eggs. In this case, membranes attach to the growing ends of MTs, and membrane tubules extend by a MT polymerization-dependent mechanism (Waterman-Storer et al., 1995). Similar MT polymerization-dependent movements of chromosomes have also been demonstrated in vitro (Coue et al., 1991; Lombillo et al., 1995a,b). The assay described in the Basic Protocol improves on previously published membrane motility assays in that the MTs are nucleated from a stationary point (a fragment of a flagellar axoneme), allowing their free ends to grow and shorten similar to the way they would behave in a living cell. This assay permits observation of both motor-driven motility of organelles and MT polymerization–driven mechanisms of organelle movement. Nucleation of MTs from stationary axonemes also provides a good marker for determining the polarity of organelle movement, as MTs grow more robustly from the plus ends of axonemes than from their minus ends. Thus axoneme-nucleated MTs with free plus ends are much longer than those with free minus ends (Walker et al., 1988).

Critical Parameters and Troubleshooting

The success of the membrane MT motility assay depends on two basic parameters: the cleanliness of the slides, coverslips, and perfusion chamber, and the quality of the biochemical components used in the assay. Adherence to the recommended choice of brand names and proper cleaning of coverslips is tantamount to making a good perfusion chamber for the motility assay.

Motile interactions between membranes and MTs take 30 to 45 min to develop. If no motility occurs after this period of time, try raising the room temperature. Be aware that many of the manipulations of membrane mixes suggested in the Basic Protocol have not yet been described in published work. Thus they may not yield any results, much less interesting results.

For all biochemical preparations, pay attention to the cleanliness of the glassware. Use double glass-distilled water for the preparation of solutions. Plan manipulations carefully in advance and follow the protocols with expedience.

The most important step for getting pure axonemes (Support Protocol 2) with very little debris is the careful collection of only the top, white layer of the pellet (containing sperm tails) without collecting any of the yellowish bottom layer of the pellet (containing heads and debris). The pellet must be washed until it is pure white and free of debris before completing the protocol. When determining the proper axoneme dilution, it is possible to check the purity of the preparation. If excess non-axoneme material appears to be present, dilute the axonemes 1:4 with isolation buffer (final glycerol concentration is 12.5%), centrifuge, and resuspend pellet in isolation buffer plus 50% glycerol. Check the purity of the axonemes by microscopy. If the preparation still appears "dirty," repeat the wash step until the axonemes appear "clean."

Several steps are critical for the preparation of tubulin (Support Protocol 3). First, proper packing of the phosphocellulose column is necessary to obtain a good concentrated peak of tubulin. If the resin has a cracked appearance after packing, it should be emptied, resuspended in column buffer, and repoured. If this procedure requires an extra day, keep the pellets of MT protein frozen until the column is packed properly. Never use phosphocellulose resin that is >1 week old. Tubulin is susceptible to proteolysis and easily denatured; therefore time is critical throughout the preparation of MT protein. Thorough removal of brain meninges, which contain high levels of blood proteases, and addition of protease inhibitors to the homogenization buffer just prior to use is very

important. Improper resuspension of microtubule pellets in a Dounce homogenizer can greatly reduce tubulin yields; be very careful not to cause bubbling in the solution and always keep the homogenizer on ice when moving the pestle. Be sure that rotor temperatures are equilibrated prior to use and that enough centrifuge tubes are clean. Fortunately, even if timing is drastically off or slightly careless technique is used, the preparation should yield some usable protein.

When preparing rat liver organelles (Support Protocol 4), pour the step gradients so that there is as little mixing between steps as possible. This is the most critical step to clean separation of the different fractions of organelles. To avoid diluting organelles at the interface between the gradient steps, avoid collecting solution above and below the interface; it is better to leave some membrane behind than to collect too much from adjacent fractions that may contain soluble protein complexes. Finally, some labs insist that interactions between motor proteins and organelles are better preserved when organelle fractions are isolated on Nycodenz gradients. If active motor proteins are present in the MT/organelle motility assay (i.e., microtubules or whole axonemes glide on the coverslip surface), but organelles do not form motile interactions with microtubules, consider isolating organelles on Nycodenz gradients (McIlvain et al., 1993; Kumar et al., 1995).

Anticipated Results

The membrane MT motility assay should generate the formation of moving membrane tubules and vesicles similar to those seen in Figure 13.1.2. Key observations may include the following: (1) What form do the membranes take on: tubular, vesicular, or both? (2) Are both static and dynamic interactions between membranes and MTs observed in the same preparation? (3) How are the membranes attached to the MT: along the shaft or at the end? (4) What direction do the membranes move on the MT? (5) Do axonemes or free MTs glide on the coverslip surface, and in what direction do they glide? (6) Does the motility change direction? Comparison of different assays, using differently treated membrane mixes, may yield interesting differences in these behaviors.

Sperm axonemes prepared from four sea urchins (Support Protocol 2) should yield enough fragments for thousands of motility assays. Isolation of tubulin from three pig brains (Support Protocol 3) should yield ~60 mg of purified tubulin. One rat liver should yield enough cytosol (Support Protocol 4) or organelles (Support Protocol 5) for up to 200 assays.

Time Considerations

Once all biochemical components are prepared, their proper dilutions determined, and a supply of perfusion chambers is made, the MT/organelle motility assay should take 2 hr to perform, allowing 30 to 45 min to record the motility on video tape. Thus, several different membrane mixes or treatment conditions can be tested in a single day. New aliquots of membrane and cytosol should be thawed for each new assay done in a single day.

Axonemes, rat liver organelles, and rat liver cytosol are all relatively easy to prepare; each requires two 3- to 6-hr days. Reagents and

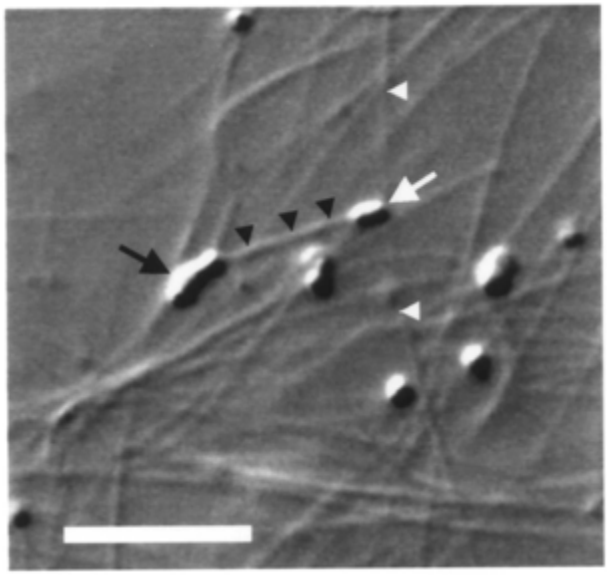


Figure 13.1.2 VE-DIC micrograph of a membrane/microtubule motility assay. Membrane associated with an axoneme fragment (black arrow) has extended a thin membrane tubule (black arrowheads) via a motile attachment (white arrow) to a single microtubule (white arrowheads). Many single microtubules and membrane vesicles can be seen in this field. Bar = 5 μm.

buffers are prepared on the first day and the components are purified the second day. Tubulin purification is much more difficult, and requires 4 long (8- to 12-hr) days of work. Buffers and reagents are prepared on the first day. The phosphocellulose column is prepared during the second day. The pig brains should be acquired fresh on the third day for the preparation of MT protein, and on the fourth day tubulin is purified from the MT protein on the phosphocellulose column. The first tubulin preparation attempted will probably not give the best results. The organization, timing, and motor skills required for the preparation must be practiced and developed over time, and the preparation must often be adapted to the constraints of the particular laboratory in which it is performed (types of rotors available, cold room space available) before this purification results in high protein yields.

Literature Cited

Allan, V. 1995. Protein phosphatase 1 regulates the cytoplasmic dynein-driven formation of endoplasmic reticulum networks in vitro. *J. Cell Biol.* 128:879-891.

Allan, V.J. and Vale, R.D. 1991. Cell cycle control of microtubule-based transport and tubule formation in vitro. *J. Cell Biol.* 113:347-359.

Allan, V.J. and Vale, R.D. 1994. Movement of membrane tubules along microtubules in vitro: Evidence for specialized sites of motor attachment. *J. Cell Sci.* 107:1885-1897.

Bell, C.W., Fraser, C., Sale, W.S., Tang, W.-J.Y., and Gibbons, I.R. 1982. Preparation and purification of dynein. *J. Methods Cell Biol.* 24:373-397.

Bennett, M.K. 1995. SNAREs and the specificity of transport vesicle targeting. *Curr. Opin. Cell Biol.* 7:581-586.

Cole, N.B. and Lippincott-Schwartz, J. 1995. Organization of organelles and membrane traffic by microtubules. *Curr. Opin. Cell Biol.* 7:55-64.

Cooper, M.S., Cornell-Bell, A., Chernjavsky, A., Dani, J.W., and Smith, S.J. 1990. Tubulovesicular processes emerge from trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans Golgi elements into a reticulum. *Cell* 61:135-145.

Coue, M., Lombillo, V.A., and McIntosh, J.R. 1991. Microtubule depolymerization promotes particle and chromosome movement in vitro. *J. Cell Biol.* 112:1165-1175.

Dabora, S.L. and Sheetz, M.P. 1988. Microtubule dependent formation of a tubular vesicular network with characteristics of the endoplasmic reticulum from cultured cell extracts. *Cell* 54:27-35.

Dailey, M.E. and Bridgman, P.C. 1989. Dynamics of the endoplasmic reticulum and other membranous organelles in growth cones of cultured neurons. *J. Neurosci.* 9:1897-1909.

Donaldson, J.G., Lippincott-Schwartz, J., and Klausner, R.D. 1991. Guanine nucleotides modulate the effects of brefeldin A in semipermeable cells: Regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* 112:579-588.

Gill, S.R., Schroer, T.A., Szilak, I., Steuer, E.R., Sheetz, M.P., and Cleveland, D.W. 1991. Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *J. Cell Biol.* 115:1639-1650.

Klausner, R.D., Donaldson, J.G., and Lippincott-Schwartz, J. 1992. Brefeldin A: Insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071-1080.

Kumar, J., Yu, H., and Sheetz, M.P. 1995. Kinectin, an essential anchor for kinesin-driven vesicle motility. *Science* 267:1834-1837.

Lee, C., Ferguson, M., and Chen, L.B. 1989. Construction of the endoplasmic reticulum. *J. Cell Biol.* 109:2045-2055.

Lippincott-Schwartz, J., Cole, N.B., Marotta, A., Conrad, P.A., and Bloom, G.S. 1995. Kinesin is the motor for microtubule-mediated Golgi-to-ER membrane traffic. *J. Cell Biol.* 128:293-306.

Lombillo, V.A., Stewart, R.J., and McIntosh, J.R. 1995a. Minus-end-directed motion of kinesin-coated microspheres driven by microtubule depolymerization. *Nature* 373:161-164.

Lombillo, V.A., Nislow, C., Yen, T.J., Gelfand, V.I., and McIntosh, J.R. 1995b. Antibodies to the kinesin motor domain and CENP-E inhibit microtubule depolymerization-dependent motion of chromosomes in vitro. *J. Cell Biol.* 128:107-115.

McIvain, J.M., Jr., Lamb, C., Dabora, S., and Sheetz, M.P. 1993. Microtubule motor-dependent formation of tubulovesicular networks from endoplasmic reticulum and Golgi membranes. *Methods Cell Biol.* 39:227-236.

Murray, A.W. 1991. Cell cycle extracts. *Methods Cell Biol.* 36:581-605.

Pfeffer, S.R. 1994. Rab GTPases: Master regulators of membrane trafficking. *Curr. Opin. Cell Biol.* 6:522-526.

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J., and Lippincott-Schwartz, J. 1997. ER-to-Golgi transport visualized in living cells. *Nature* 389:81-85.

Salmon, E.D. and Tran, P. 1998. High resolution video enhanced differential interference contrast microscopy. *Methods Cell Biol.* 56:153-184.

Schmid, S.L. and Damke, H. 1995. Coated vesicles: A diversity of form and function. *FASEB J.* 9:1445-1453.

Schroer, T.A. and Sheetz, M.P. 1991. Two activators of microtubule-based vesicle transport. *J. Cell Biol.* 115:1309-1318.

Sheetz, M.P. 1996. Microtubule motor complexes moving membranous organelles. *Cell Struc. Funct.* 21:369-373.

Shpetner, H.S., Paschal, B.M., and Vallee, R.B. 1988. Characterization of the microtubule-activated ATPase of brain cytoplasmic dynein (MAP 1C). *J. Cell Biol.* 107:1001-1009.

Terasaki, M. and Reese, T.S. 1994. Interactions among endoplasmic reticulum, microtubules, and retrograde movements on the cell surface. *Cell Motil. Cytoskel.* 29:291-300.

Terasaki, M., Chen, L.B., and Fujiwara, K. 1986. Microtubules and the endoplasmic reticulum are highly interdependent structures. *J. Cell Biol.* 103:1557-1568.

Vale, R.D. and Hotani, H. 1988. Formation of membrane networks in vitro by kinesin-driven microtubule movement. *J. Cell Biol.* 107:2233-2241.

Vale, R.D., Reese, T.S., and Sheetz, M.P. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* 42:39-50.

Walker, R., O'Brien, E., Pryer, N., Soboeiro, M., Voter, W., Erikson, H., and Salmon, E.D. 1988. Dynamic instability of individual microtubules analyzed by video light microscopy: Rate constants and transition frequencies. *J. Cell Biol.* 107:1437-1448.

Waterman-Storer, C., Gregory, J., Parsons, S., and Salmon, E.D. 1995. Membrane/microtubule tip attachment complexes (TACs) allow the assembly dynamics of plus ends to push and pull membranes into tubulovesicular networks in interphase *Xenopus* egg extracts *J. Cell Biol.* 130:1161-1169.

Key References

Coue et al., 1991 and Waterman-Storer et al., 1995. See above.

In these papers, in vitro assays show that organelles can be moved by microtubule polymerization and depolymerization in the absence of motor ATPase activity.

Dabora and Sheetz, 1988 and Vale and Hotani, 1988. See above.

These papers were the first to use in vitro assays to reconstitute microtubule motor-based organelle motility.

Klausner et al., 1992. See above.

This review clearly describes the roles and regulation of coat proteins in the morphology and movement of organelles through the secretory pathway.

Salmon and Tran, 1998. See above.

This paper provides an in-depth description of the theory and practice of setting up the microscope system required to perform the MT/organelle motility assay.

Contributed by Clare M. Waterman-Storer
University of North Carolina
Chapel Hill, North Carolina

In Vitro Motility Assay to Study Translocation of Actin by Myosin

A most basic property of myosin is its ability to interact with and translocate actin. This unit describes an in vitro motility assay that can be used to study the translocation, or sliding, of actin filaments by myosin bound to a coverslip. The assay makes use of the ability to image single F-actin filaments labeled with rhodamine phalloidin, a high-affinity fluorescent ligand using fluorescence microscopy. The system is fast, easy to set up and maintain, uses only small amounts of protein, and yields quantitative results.

Several preliminary steps are required before the in vitro motility assay can be conducted. Special flow cells that are used in the assay must be prepared (see Support Protocol 1). The assay also requires purified myosin and actin. Other proteins, such as the regulatory proteins tropomyosin and troponin must be prepared if needed for assay variations. The method for preparing myosin varies greatly depending on the type of myosin being studied (see Table 13.2.1). Actin can be purified by the method in Support Protocol 2, and then it must be labeled with rhodamine phalloidin (see Support Protocol 3). When the in vitro motility assay is conducted (see Basic Protocol), the flow cell is placed under the objective of a fluorescent microscope, and the image is recorded on videotape. Afterward, the data from the videotape can be quantified.

ANALYZING ACTIN TRANSLOCATION BY MYOSIN

BASIC PROTOCOL

Translocation of actin by myosin is followed using rhodamine phalloidin–labeled actin exposed to immobilized myosin in a flow cell. The assay can be modified for specific purposes. For example, myosin can be applied to the coverslip surface either as filaments or as monomers. The enzymatically active fragments of myosin, heavy meromyosin (HMM) or subfragment-1 (S1), can also be used. The ionic strength can be varied over a range extending up to physiological levels and the temperature can be varied over a wide range. The movement of pure actin, or of actin complexed with regulatory proteins, can be measured.

The assay is best conducted with freshly prepared myosin, although it is possible to use myosin that has been flash frozen and stored in liquid nitrogen. The presence of damaged myosin heads (dead heads) that bind actin, but do not hydrolyze ATP, contributes to poor quality of movement. Two procedures can minimize this problem. The myosin-coated coverslip can be washed with a solution containing unlabeled actin in the presence of ATP before the rhodamine phalloidin–labeled actin is introduced, as is done in this protocol. This has the effect of complexing the dead heads with the unlabeled actin. If this simple treatment does not prove sufficient, a stoichiometric amount of F actin can be added to myosin in the presence of ATP at high ionic strength and sedimented at 480,000 × *g* for 15 min in a Beckman TL100 ultracentrifuge. This removes dead heads before the myosin sample is applied to the coverslip.

Quantification of the images can be done in several ways. The most thorough method makes use of a dedicated automatic tracking system such as the Cell Trak system from Motion Analysis (Homsher et al., 1992). Another alternative involves the use of frame grabbers to digitize images coupled with the use of tracking software run on a desktop computer (Work and Warshaw, 1992; Marston et al., 1996).

Contributed by James R. Sellers

Current Protocols in Cell Biology (1998) 13.2.1-13.2.10

Table 13.2.1 Purification Protocols for Myosins

Myosin type	Purification protocol
Rabbit skeletal muscle myosin	Margossian and Lowey, 1982
Invertebrate striated muscle myosin	Sellers, 1981
Chicken smooth muscle	Sellers et al., 1981
Nonmuscle myosin II	Daniel and Sellers, 1992
Native thick filaments	Sellers and Kachar, 1990
Myosin I	Collins et al., 1990
Myosin V	Nascimento et al., 1996
Baculovirus/*Sf* 9-expressed recombinant myosins	Trybus, 1994[a]

[a]If the recombinant myosin is HIS or FLAG tagged, specific affinity columns can be used to aid in purification.

Materials

0.2 mg/ml myosin monomers (see Table 13.2.1)
1 mg/ml BSA in G-actin buffer (see recipe)
Wash buffer (see recipe)
Wash buffer containing 1 mM ATP and 5 μM actin (see Support Protocol 2)
Wash buffer containing 20 nM rhodamine phalloidin–labeled actin (see Support Protocol 3)
Assay buffer (see recipe)

Flow cell (see Support Protocol 1)
Fluorescent microscope, with high-numerical-aperture objective (60× to 100×, 1.3 to 1.4 NA) and 100-W mercury lamp
SIT camera or an intensified CCD
VHS or sVHS video recorder
Image processor
Cell Trak image analysis system (Motion Analysis)

1. Fill the flow cell with myosin monomers (or the soluble fragments HMM and S1) at a concentration of 0.2 mg/ml. Allow the solution to remain in the cell for 1 to 2 min to allow myosin to bind.

   Myosin monomers are applied in a high-ionic-strength buffer (e.g., 0.5 M NaCl/10 mM MOPS [pH 7.0]/0.1 mM EGTA/1 mM DTT), whereas HMM or S1 can be applied at a low ionic strength (e.g., 10 mM MOPS [pH 7.0]/0.1 mM EGTA/1 mM DTT). Myosin filaments must be applied at low ionic strength in a buffer where myosin filaments are stable such as 50 mM KCl/10 mM MOPS (pH 7.0)/1 mM $MgCl_2$/0.1 mM EGTA/1 mM DTT.

2. Wash the flow cell with 2 to 3 volumes of 1 mg/ml BSA in actin buffer. Allow the last volume of buffer to remain in the cell for 1 to 2 min to block non-specific binding.

   This serves to wash unbound myosin from the flow cell and block the surface. Note that if myosin filaments are being applied to the surface, it is necessary to wash the flow cell with BSA in a low-ionic-strength buffer that will not depolymerize myosin filaments.

3. Wash the flow cell with 2 to 3 vol wash buffer containing 1 mM ATP and 5 μM actin. Allow the last volume of solution to remain in the flow cell for 1 to 2 min.

   Note that prior to use this actin/ATP–containing solution is vortexed intensely or passed through a narrow-gauge syringe in order to sheer the unlabeled actin. The actin added in this step binds to damaged, noncycling myosins and dramatically improves the quality of measurements. The actin is sheared to increase the accessibility to the surface.

4. Wash the flow cell with 2 to 3 vol wash buffer to remove ATP, followed by 2 vol wash buffer containing 20 nM of rhodamine phalloidin–labeled actin. Allow to bind for 0.5 to 1 min.

5. Begin the motility assay by adding assay buffer.

   Note that this buffer is viscous due to the methylcellulose and it may be necessary to use a piece of filter paper placed at the outflow of the flow cell as a wick to pull the solution into the cell.

6. Place the flow cell on the microscope stage and observe the motion of the rhodamine phalloidin–labeled actin using a filter cube for detecting rhodamine fluorescence and a high-NA 60× to 100× objective.

   Movement of actin by myosin is dependent on temperature, and thus it is necessary to regulate the temperature of the slide. Most investigators use 25° or 30°C as standard. This can be accomplished in several ways. The least expensive way is to place a blow dryer at an appropriate distance to yield the desired temperature. It is also possible to regulate the temperature of the assay by jacketing the objective using either a water jacket or a Peltier-type device. Simply regulating the temperature of the stage is usually not sufficient to control the temperature because the objective constitutes a large heat source. The temperature of the sample in the flow cell can be estimated using a fine wire thermister mounted between a coverslip and a slide. This "dummy" flow cell is mounted on the stage, and the objective is brought to the same position as the one used when imaging a flow cell.

   It is useful to have several neutral density filaments that can be placed in the light path to attenuate the light intensity to avoid bleaching of rhodamine fluoresence.

7. Process the results with either an intensified CCD (charge-coupled device), a neuvicon camera, or a SIT (silicon-intensified target) camera to detect the image and an sVHS video recorder to record the image movements, which can be displayed on a standard black-and-white video monitor.

   It is useful to have an image processor to conduct frame averaging and background subtraction. This is especially helpful if the myosin being studied moves actin filaments slowly. This can be accomplished using either a dedicated image processor such as the Argus-20 (Hamamatsu) or image-processing software running on a desktop computer. If an image processor is used, it is useful to have two monitors, one for the processed image and one for the raw image.

8. Quantify results by measuring the position of an actin filament over time, measuring five to ten time points for each actin filament. Calculate and record the mean velocity for the measurements.

   Quantification of the rate of movement is most easily accomplished using an automated tracking system such as the Cell Track System by Motion Analysis. This system digitizes the image of all actin filaments in a video sequence, determines the centroid position, determines the path, and calculates the main velocity of each actin filament. In the absence of such an automated system, some investigators have written their own software using commercially available frame grabbers (Work and Warshaw, 1992; Winkleman et al., 1995).

SUPPORT PROTOCOL 1

PREPARATION OF FLOW CELLS

The actin translocation assay requires nitrocellulose-coated coverslips applied to a simple flow cell.

Materials

1% (w/v) nitrocellulose (Ernest F. Fullham) in amyl acetate
Apiezon M grease (Thomas; optional)

Double-sided tape
18-mm^2 no. 1 coverslips
Glass microscope slides
24 × 60–mm no. 0 coverslips (optional)

1. Place a 2-µl drop of 1% nitrocellulose in amyl acetate onto a no. 1 coverslip and spread the drop over the surface using the long axis of the pipet as a swab. Allow the surface to dry (5 min).

   Alternatively, to create a thin film of nitrocellulose, apply 1 drop of 1% nitrocellulose from a Pasteur pipet to the surface of deionized water in a 10-cm round dish and allow the amyl acetate to evaporate. Place no. 1 coverslips onto the dried film. Tear excess film away from the area surrounding the coverslips using forceps. Remove the coated coverslips by submersing and inverting them while lifting them from the water. Air dry the coated coverslip with the film side up. Coverslips and microscope slides are typically used as they come from the box.

2. Apply two parallel strips of double-sided tape ~10 mm apart on a glass microscope slide. Place a nitrocellulose-coated coverslip with the coated side down on top of the tape strips to create a quick and simple flow cell.

   Alternatively, cut spacers (24 × 3 to 4 mm) from a no. 0 coverslip using a diamond scribe. Place parallel tracks of Apiezon M grease ~10 mm apart and 25 mm long on the long axis of a glass microscope slide. Place the thin coverslip spacers on the outside of the grease tracks and cover them with a nitrocellulose-coated coverslip with the coated side down. It is also possible to glue the spacers and coverslip to the slide with fingernail polish.

SUPPORT PROTOCOL 2

PURIFICATION OF ACTIN

Rabbit muscle actin used for the translocation assay is most often purified by this method (Spudich and Watt, 1971). After it is prepared, a portion is labeled with rhodamine phalloidin (see Support Protocol 3).

Materials

Back and leg muscles of freshly sacrificed 350-g rabbit
0.1 M KCl/0.15 M potassium phosphate, pH 6.5
0.05 M $NaHCO_3$
1 mM EDTA, pH 7.0
Acetone
G-actin buffer (see recipe)
2 M KCl (stock solution)
1 M $MgCl_2$ (stock solution)

Meat grinder, prechilled
Cheesecloth
Filter paper
Sorvall centrifuge and SS-34 rotor (or equivalent)
Beckman ultracentrifuge and 55 Ti rotor (or equivalent)
Potter-Elvehjem tissue grinder

Additional reagents and equipment for dialysis (*APPENDIX 3*)

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All procedures are performed at 4°C unless otherwise specified and all buffers should be prechilled to this temperature.

Prepare actin powder

1. Excise the back and leg muscles of a freshly sacrificed rabbit, wash them free of blood with distilled water, and chill on ice.

   Steps 2 to 6 should be carried out in a cold room at 4°C.

2. Grind the muscle in a meat grinder that has been prechilled.

3. Extract the mince with 1 liter of 0.1 M KCl/0.15 M potassium phosphate, pH 6.5, for 10 min with stirring. Filter the mince by squeezing it through several layers of cheesecloth.

   Stirring in steps 3 to 7 is done by hand using a glass rod or spatula or by using an overhead motorized stirrer. The suspension is too viscous to use a magnetic stirrer.

   Filtration is most easily accomplished by spreading several layers of cheesecloth over a large funnel which is supported by a ring stand. This method of filtering is also used in steps 4 to 7.

4. Extract the retained mince with 2 liters of 0.05 M $NaHCO_3$ for exactly 10 min with stirring, and filter as in step 3.

5. Extract the filtered mince with 1 liter of 1 mM EDTA, pH 7.0, for 10 min with stirring.

6. Extract the mince twice with 2 liters distilled water for 5 min each time.

7. Extract the mince five times at room temperature with chilled acetone for 10 min each time. Stir to break up residue clumps and filter the extract.

8. Spread out the acetone-washed, filtered residue on a large piece of filter paper and dry overnight in a hood.

   The resulting acetone powder can be stored for months at −20°C.

Extract the acetone powder

9. Extract the acetone powder (typically 5 g) in 20 ml of G-actin buffer per gram of powder on ice, 30 min with stirring.

10. Filter the extract through cheesecloth (see step 3 annotation) and retain the filtrate. Reextract the residue using 20 ml of the same buffer per gram of original dried acetone powder, 10 min with stirring.

11. Filter through cheesecloth again, retaining the filtrate, and combine the filtrates.

12. Centrifuge 1 hr at 40,000 × *g*, 4°C. Carefully decant the supernatant and discard the pellet.

Polymerize the actin

13. Add KCl to 50 mM and $MgCl_2$ to 2 mM to polymerize actin. Allow polymerization to continue for 2 hr on ice.

14. Slowly add solid KCl to a final concentration of 0.8 M and stir gently 30 min.

    This step helps to remove tropomyosin and α-actinin contaminants.

15. Centrifuge 1.5 hr at 150,000 × *g*, 4°C, to sediment the polymerized actin.
16. Resuspend the F-actin pellets by homogenization in a Teflon-lined homogenizer using 3 ml G-actin buffer per gram of acetone powder. Dialyze for 2 days in G-actin buffer with repeated changes to depolymerize the actin.
17. Centrifuge the depolymerized actin 1.5 hr at 150,000 × *g*, 4°C. Retain the supernatant.
18. Add KCl and $MgCl_2$ to concentrations of 50 mM and 2 mM, respectively, to polymerize the actin. Dialyze in the buffer of choice containing 1 to 2 mM $MgCl_2$ to maintain the polymerized state. Store on ice at a concentration of 100 to 200 µM for ~1 month.

If the actin is stored longer than 1 month, it should be put through a new depolymerization/polymerization cycle by repeating steps 16 to 18.

SUPPORT PROTOCOL 3

PREPARATION OF RHODAMINE PHALLOIDIN–LABELED ACTIN

Actin is labeled with rhodamine phalloidin so that its translocation can be followed in the assay described in the Basic Protocol. Rhodamine phalloidin serves two purposes: the phalloidin moiety binds to actin and stabilizes the filaments against depolymerization; and the rhodamine moiety is a common flurophore that is excitable by green light.

Materials

Rhodamine phalloidin (Molecular Probes)
Methanol
Labeling buffer (see recipe)
Actin (see Support Protocol 2, step 18)
Wash buffer (see recipe)
Speed-Vac evaporator (Savant) or equivalent

1. Place 60 µl of rhodamine phalloidin in a microcentrifuge tube and dry in an evaporator.
2. Redissolve the dried powder in 5 µl methanol.
3. Add 85 µl labeling buffer.
4. Dilute actin to 20 µM in labeling buffer. Add 10 µl of 20 µM actin and incubate overnight. Store on ice up to 1 month.
5. Dilute an aliquot to 20 nM actin with wash buffer the day of use.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Assay buffer

Wash buffer (see recipe) containing:
0.7% (w/v) methylcellulose (see recipe)
1 mM ATP
50 mM DTT
2.5 mg/ml glucose
0.1 mg/ml glucose oxidase
0.02 mg/ml catalase
Prepare fresh daily

continued

Assay buffer should be prepared fresh daily from stocks of ATP (0.1 M ATP, pH 7.0, stored at −20°C), DTT (1 M, prepared fresh daily), glucose (2.5 mg/ml, stored at −20°C), glucose oxidase (Sigma), and catalase (Sigma).

The ionic conditions of this buffer (KCl, $MgCl_2$, $CaCl_2$ concentrations) and the pH and temperature can be varied according to the experimental protocol.

G-actin buffer

2 mM Tris base
0.2 mM Na_2ATP
0.5 mM DTT
0.2 mM $CaCl_2$
3 mM NaN_3
Adjust pH to 8.5 with HCl
Store at 4°C

Labeling buffer

10 mM MOPS [3-(*N*-morpholino) propane sulfonic acid], pH 7.0
0.1 mM EGTA
3 mM NaN_3
Store at 4°C

Methylcellulose, 14% (w/v)

Prepare a 1.4% (w/v) solution of methylcellulose (Sigma). Dialyze the solution against deionized water for 24 hr. Store at −20°C until used.

Methylcellulose should have a viscosity of 4000 centipoise for a 2% solution at 20°C.

Wash buffer

80 mM KCl
5 mM $MgCl_2$
20 mM MOPS, pH 7.4
0.1 mM EGTA
Store at 4°C

COMMENTARY

Background Information

Myosin is an ATP-dependent molecular motor that interacts with actin (Sellers and Goodson, 1995). In muscle fibers actin and myosin are arranged in interdigitating filaments that slide past each other during contraction. There is a superfamily of myosins that also perform mechanical functions such as cytokinesis and vesicle transport in nonmuscle cells. The in vitro motility assay to study actomyosin interactions fills a void between biochemical measurements of the actin-activated Mg-ATPase activity of myosin and mechanical measurements of skinned muscle fibers. In this assay, myosin molecules bound to a nitrocellulose-coated surface interact with actin filaments in solution and translocate them in an ATP-dependent process. This is a simple assay that reconstitutes the essence of a muscle fiber using only two purified proteins, actin and myosin. It requires only small quantities of these proteins (e.g., 1 to 2 μg myosin per assay) and is quick to perform. Thin filament regulatory proteins can be added to examine actin-linked regulatory systems (Shirinsky et al., 1992; Fraser and Marston, 1995; Lin et al., 1996). The ionic conditions can be easily varied as needed. The assay is much simpler than a previous in vitro motility assay that followed the movement of myosin-coated microbeads over actin cables exposed by dissection of the long cells of *Nitella axillaris*, a green alga (Sheetz and Spudich, 1983).

Many novel techniques for the study of actomyosin interaction have evolved from the in vitro motility assay, including assays that allow for the imaging of both the myosin and actin filaments (Sellers and Kachar, 1990), assays in which the force generation of myosin is measured, and assays in which single molecules of myosin can be imaged as they interact with actin. Force measurement is accomplished by two methods. In the first case, actin filaments

attached to flexible microneedles are lowered onto a myosin-coated surface (Kishino and Yanagida, 1988; VanBuren et al., 1995). Force per unit length of actin is monitored by imaging the bending of the needle. In the other mechanical assay, beads are attached to the opposite ends of an actin filament, and optical trapping techniques are used in which the position of each bead is controlled by dual optical traps. The filament is lowered to a surface coated with low-density myosin, and force-producing events are measured by monitoring the position of the beads at the end of the actin filament (Finer et al., 1994; Molloy et al., 1995). The use of single-molecule fluorescence methods allows one to image individual myosin molecules or observe their interactions with fluorescently labeled nucleotides (Funatsu et al., 1995; Vale et al., 1996).

Critical Parameters

Myosin can be attached to the coverslip surface by a variety of methods. It can be bound as filaments or as monomers (Toyoshima et al., 1987; Umemoto and Sellers, 1990). Fragments of myosin such as HMM or S1 can also be used (Toyoshima et al., 1987). A more specific attachment of myosin to the surface is accomplished using site-specific antibodies, usually directed against the carboxyl terminus of the myosin or subfragment (Winkelmann et al., 1995). Isoform-specific antibodies can also be used to select a specific myosin isoform from a mixture of myosins or from a crude cell or tissue extract (Cuda et al., 1993; Kelley et al., 1996). In these cases, the affinity-purified antibody (0.2 mg/ml) is first bound to the nitrocellulose surface; this is followed by washing and blocking with BSA. Myosin (or an extract) is then added and allowed to complex with the antibody for 10 to 30 min. Subsequent steps are performed as described in the Basic Protocol.

The rate of actin-filament sliding generated by myosin varies greatly among myosin isoforms (Sellers and Goodson, 1995). In addition, assay conditions such as ionic strength, temperature, and pH can modulate the rate of sliding. Various parameters of the assay must therefore be varied. With slowly moving myosins (<0.2 μm/sec), longer imaging intervals are required, necessitating lower illumination levels, and as a consequence, it is very useful to average 16 to 64 frames to improve the signal-to-noise ratio. With more rapidly moving myosins (4 to 10 μm/sec), short imaging intervals can be used along with higher illumination levels. Under these conditions, it is preferable to have an intensified CCD camera as opposed to a SIT camera, which has a slower temporal response that may result in the appearance of comet-tail-like images of the actin filaments.

There are many high-quality manufacturers of the required equipment. It is advisable to have representatives demonstrate the various pieces of equipment before purchase. A detailed treatise on video microscopy is available (Inoue and Spring, 1997).

In general, the assay does not work well at pH 6.5 or below, and the rate of actin filament sliding by myosin slows dramatically as the temperature falls below 20°C.

Troubleshooting

There are several common problems in the assay that are usually easily remedied.

1. *The actin filaments are too dim.* Increase the light intensity or the number of frames that are averaged. Remove any unnecessary glass surfaces between the actin filaments and the camera. If necessary, prepare fresh actin. It is important that the dried rhodamine phalloidin powder be predissolved in methanol to ensure complete solubilization.

2. *Rhodamine label attached to actin filaments photobleaches rapidly.* The light intensity should be reduced by means of neutral density filters, and frame averaging should be used to increase signal-to-noise ratio. The oxygen-scavenging components of the in vitro motility assay (glucose oxidase and catalase) should be checked to ensure they are active. Use 50 mM DTT, if not already doing so.

3. *The actin filaments move intermittently or rapidly shear into small pieces.* This is typical in myosin preparations containing rigorlike, noncycling "dead heads" (see Basic Protocol). Reducing the surface density of myosin also leads to less shearing. If necessary, prepare fresh myosin.

4. *Actin filaments detach from the surface or exhibit wobbly motions.* Increase the density of myosin attached to the surface or decrease the ionic strength. Use of methylcellulose generally allows for movement over lower myosin surface densities by decreasing the lateral Brownian movement of actin filaments. In the presence of methylcellulose, Brownian movement occurs only in the long dimension of the actin filament.

Anticipated Results

A successfully completed assay should allow measurement of translocation of actin by myosin and dissection of the biochemistry of

the process. Typically, translocation rates range from <0.2 μm/sec to 10 μm/sec depending upon the source of myosin and the assay conditions.

Time Considerations

Actin preparation requires ~1 day to prepare an acetone powder and 3 to 4 days to prepare polymerized filaments. Myosin preparation takes from 1 day to 5 days, depending on the myosin isoform. Performing the translocation assay requires 30 min to set up. Data acquisition is in real time. Data analysis can be very slow and laborious unless an automated tracking system is used. The time required for data analysis is a function of how many filaments are being followed and the system being used for analysis.

Literature Cited

Collins, K., Sellers, J.R., and Matsudaira, P. 1990. Calmodulin dissociation regulates brush border myosin I (110-kD calmodulin) mechanochemical activity in vitro. *J. Cell Biol.* 110:1137-1147.

Cuda, G., Fananapazir, L., Zhu, W.-S., Sellers, J.R., and Epstein, N.D. 1993. Skeletal muscle expression and abnormal function of β-myosin in hypertrophic cardiomyopathy. *J. Clin. Invest.* 91:2861-2865.

Daniel, J.L. and Sellers, J.R. 1992. Purification and characterization of platelet myosin. *Methods Enzymol.* 215:78-88.

Finer, J.T., Simmons, R.M., and Spudich, J.A. 1994. Single myosin molecule mechanics: Piconewton forces and nanometre steps. *Nature* 368:113-119.

Fraser, I.D.C. and Marston, S.B. 1995. In vitro motility analysis of smooth muscle caldesmon control of actin-tropomyosin filament movement. *J. Biol. Chem.* 270:19688-19693.

Funatsu, T., Harada, Y., Tokunaga, M., Saito, K., and Yanagida, T. 1995. Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature* 374:555-559.

Homsher, E., Wang, F., and Sellers, J.R. 1992. Factors affecting movement of F-actin filaments propelled by skeletal muscle heavy meromyosin. *Am. J. Physiol. Cell Physiol.* 262:C714-C723.

Inoue, S. and Spring, K.R. 1997. Video Microscopy: The Fundamentals. Plenum, New York.

Kelley, C.A., Sellers, J.R., Gard, D.L., Bui, D., Adelstein, R.S., and Baines, I.C. 1996. *Xenopus* nonmuscle myosin heavy chain isoforms have different subcellular localizations and enzymatic activities. *J. Cell Biol.* 134:675-687.

Kishino, A. and Yanagida, T. 1988. Force measurements by micromanipulation of a single actin filament by glass needles. *Nature* 334:74-76.

Lin, D., Bobkova, A., Homsher, E., and Tobacman, L.S. 1996. Altered cardiac troponin T in vitro function in the presence of a mutation implicated in familiar hypertrophic cardiomyopathy. *J. Clin. Invest.* 97:2842-2848.

Margossian, S.S. and Lowey, S. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85B:55-71.

Marston, S.B., Fraser, I.D.C., Bing, W., and Roper, G. 1996. A simple method for automatic tracking of actin filaments in the motility assay. *J. Muscle Res. Cell Motil.* 17:497-506.

Molloy, J.E., Burns, J.E., Kendrick-Jones, J., Tregear, R.T., and White, D.C.S. 1995. Movement and force produced by a single myosin head. *Nature* 378:209-212.

Nascimento, A.A.C., Cheney, R.E., Tauhata, S.B.F., Larson, R.E., and Mooseker, M.S. 1996. Enzymatic characterization and functional domain mapping of brain myosin-V. *J. Biol. Chem.* 271:17561-17569.

Sellers, J.R. 1981. Phosphorylation-dependent regulation of *Limulus* myosin. *J. Biol. Chem.* 256:9274-9278.

Sellers, J.R. and Goodson, H.V. 1995. Motor proteins 2: Myosin. *Protein Profile* 2:1323-1423.

Sellers, J.R. and Kachar, B. 1990. Polarity and velocity of sliding filaments: Control of direction by actin and of speed by myosin. *Science* 249:406-408.

Sellers, J.R., Pato, M.D., and Adelstein, R.S. 1981. Reversible phosphorylation of smooth muscle myosin, heavy meromyosin, and platelet myosin. *J. Biol. Chem.* 256:13137-13142.

Sheetz, M.P. and Spudich, J.A. 1983. Movement of myosin-coated fluorescent beads on actin cables in vitro. *Nature* 303:31-35.

Shirinsky, V.P., Biryukov, K.G., Hettasch, J.M., and Sellers, J.R. 1992. Inhibition of the relative movement of actin and myosin by caldesmon and calponin. *J. Biol. Chem.* 267:15886-15892.

Spudich, J.A. and Watt, S. 1971. The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* 246:4866-4871.

Toyoshima, Y.Y., Kron, S.J., McNally, E.M., Niebling, K.R., Toyoshima, C., and Spudich, J.A. 1987. Myosin subfragment-1 is sufficient to move actin filaments in vitro. *Nature* 328:536-539.

Trybus, K.M. 1994. Regulation of expressed truncated smooth muscle myosins. Role of the essential light chain and tail length. *J. Biol. Chem.* 269:20819-20822.

Umemoto, S. and Sellers, J.R. 1990. Characterization of in vitro motility assays using smooth muscle and cytoplasmic myosins. *J. Biol. Chem.* 265:14864-14869.

Vale, R.D., Funatsu, T., Pierce, D.W., Romberg, L., Harada, Y., and Yanagida, T. 1996. Direct observation of single kinesin molecules moving along microtubules. *Nature* 380:451-453.

VanBuren, P., Harris, D.E., Alpert, N.R., and Warshaw, D.M. 1995. Cardiac V_1 and V_3 myosins differ in their hydrolytic and mechanical activities in vitro. *Circ. Res.* 77:439-444.

Winkelmann, D.A., Bourdieu, L., Ott, A., Kinose, F., and Libchaber, A. 1995. Flexibility of myosin attachment to surfaces influences F-actin motion. *Biophys. J.* 68:2444-2453.

Work, S.S. and Warshaw, D.M. 1992. Computer-assisted tracking of actin filament motility. *Anal. Biochem.* 202:275-285.

Contributed by James R. Sellers
National Heart, Lung, and Blood Institute
Bethesda, Maryland

Organelle Motility in Plant Cells: Imaging Golgi and ER Dynamics with GFP

This unit describes the use of green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* as a vital marker for endoplasmic reticulum (ER) and Golgi apparatus (GA) in higher plant cells. GFP is expressed as a chimeric protein either with selected peptide targeting sequences or with complete proteins that are resident in the ER or GA. Expression of GFP constructs can be observed in living material using conventional, or preferably, confocal laser–scanning epifluorescence microscopes. GFP has a number of intrinsic properties that make it attractive as a marker for cell biological studies. For instance, it is highly fluorescent, yet requires no cofactors or substrates. In addition, it is relatively stable, is pH insensitive, shows no detectable level of cytotoxicity (when expressed in organelles), and in many instances, does not interfere with the functioning of native proteins when expressed as chimeric constructs. Moreover, there are genetically modified variants of GFP with altered spectral and other properties, such as folding rate (Cubitt et al., 1995).

This unit describes two protocols for rapid transient expression of GFP in the plant endomembrane system using *Agrobacterium* and virus vectors. These protocols can be used for the expression of GFP targeted to most organelles. Besides *Agrobacterium*-mediated transformation (see Basic Protocol), virus transformation (see Alternate Protocol), has been optimized for GFP expression in some *Nicotiana* species.

TRANSIENT EXPRESSION FOR VISUALIZATION OF ER AND GOLGI PROBES IN LEAVES

BASIC PROTOCOL

This protocol describes the production of GFP-transcripts in *Nicotiana* using an *Agrobacterium tumefaciens*–mediated transient expression system. There are a number of techniques for transient expression of reporter constructs in plant cells, such as electroporation and PEG-mediated transformation of protoplasts, and microprojectile bombardment and virus-mediated expression in tissues; however, transient expression mediated by *Agrobacterium* containing a reporter construct in a suitable binary vector (e.g., pVKH18En6) is one of the easiest, quickest, and more reliable methods. It requires minimum laboratory equipment, and it allows the study of intact cells and tissues from leaves still attached to the plant with minimal tissue disruption, although it is not clear to what extent the bacterial infection alters cell physiology. Moreover, transformed leaf tissue may be used to generate stable transformants.

The basic principle of the transformation is infiltration of the intracellular spaces of *Nicotiana* leaves with a suspension of *A. tumefaciens*, which is injected into the leaf tissue by pressure though the stomata of the abaxial leaf epidermis. The infectious *Agrobacterium* cells then transfer the T-DNA carrying the genes to be transferred to the host cell resulting in a transient build-up of reporter transcripts in the cell. With GFP-based reporters, fluorescence can be detected in the infected area from abaxial and adaxial epidermis, guard cells, and palisade and spongy mesophylls, but rarely trichomes and vascular tissue. The technique gives high expression levels of GFP constructs, which may fade in a week to ten days after inoculation of the leaves due to progressive depletion of the foreign DNA.

This method offers a number of advantages including speed and relatively little variability between cells within the infected area in which most, if not all cells, appear to express the construct. Moreover, Rossi et al. (1993) have shown that expression from T-DNA

Contributed by Chris Hawes, Federica Brandizzil, Henri Batoko, and Ian Moore
Current Protocols in Cell Biology (2001) 13.3.1-13.3.10

varies linearly with bacterial concentration over three orders of magnitude offering the opportunity to control GFP expression levels. Finally, the geometry of epidermal cells, which have large vacuoles and a thin layer of cytoplasm immediately below the cuticular wall, greatly facilitates observation by both conventional epifluorescence microscopy (*UNIT 4.2*) and confocal imaging (*UNIT 4.5*). For example, the authors have been able to distinguish the distribution of ER- and Golgi-targeted GFP in these cells using a conventional epifluorescence microscope.

Materials

Suitable *Agrobacterium* vector (e.g., pVKH18En6) with multiple cloning site and appropriate selectable marker
Agrobacterium tumefaciens (e.g., GV3101::pMP90)
YEB medium containing appropriate selective antibiotic (see recipe)
5% (v/v) sodium hypochlorite or 1% (w/v) Virkon (Amtec Int. Ltd.)
Infiltration medium (INM; see recipe)
Four-week-old greenhouse plants of *Nicotiana tabacum*, *N. clevelandii*, and *N. benthamiana*

Shaking incubator, 28°C
1.5-ml microcentrifuge tubes, sterile
Spectrophotometer
1-ml disposable plastic syringe, without needle
22° to 25°C greenhouse for growing plants
Permanent marker pen
Fine scissors
Slides and coverslips (use thickness 0 for confocal microscopes)
Electrical or waterproof tape
Conventional epifluorescence microscope, laser-scanning confocal microscope (preferred), or equivalent, with appropriate filters (e.g., standard FITC filter block)
Confocal-imaging time-lapse software (Zeiss, Leica, Biorad)

Additional reagents and equipment for vector construction, transformation, and cell culture (see *APPENDIX 3*) adapted for plants

CAUTION: All solutions used to culture and wash bacteria should be treated with a suitable disinfectant before discarding in an autoclavable waste container. Resulting plant material should be treated as biological hazard and handled accordingly.

NOTE: Sterile conditions are required for culture and handling of *Agrobacterium*, but not during tobacco leaf infiltration and subsequent incubation of the plant. All equipment coming into contact with bacteria should be autoclaved.

Clone GFP into bacteria and grow Agrobacterium

1. Using standard molecular biology techniques (see *APPENDIX 3*), clone GFP chimera into a suitable vector with an appropriate selectable marker (e.g., kan^r, amp^r) and transform into a suitable *Agrobacterium* strain.

   A number of binary Agrobacterium vectors can be used for the cloning of GFP (Bevan, 1984). In the authors' laboratories a vector with an enhanced 35S promoter, pVKH18En6, is used to drive high levels of GFP expression (Hawes et al., 2000).

2. Pick a single colony of *Agrobacterium* from a selection plate and inoculate 2 to 5 ml YEB medium containing appropriate selectable antibiotic.

3. Culture *Agrobacterium* at 28°C in a shaking incubator to stationary phase (24 to 48 hr).
4. Transfer 1.0 ml of culture to a 1.5-ml sterile microcentrifuge tube and pellet bacteria by microcentrifuging for 5 min at 4000 rpm.
5. Discard the supernatant in a disinfecting solution of either 5% (v/v) sodium hypochlorite ($NaOCl_3$) or 1% (w/v) Virkon.
6. Wash the bacterial pellet twice with 1.0 ml INM each.
7. Microcentrifuge for 5 min at 4000 rpm.
8. Resuspend in INM to OD_{600} 0.5 to 0.6 or higher to increase the level of expression.

Inoculate plants

9. Inject bacterial suspension into the abaxial epidermis of plant leaves from a 1-ml disposable plastic syringe by simply pressing the nozzle against the leaf surface (do not use a needle), holding the leaf on the other side with a gloved finger at the point of contact to support the pressure.

   The infiltration is easier when the leaf stomata are open. Illuminating the plants from underneath with a bright light prior to infiltration is recommended.

   The spread of liquid entering the leaf via stomata is visualized by a darkening of the leaf tissue. The boundaries of the infiltrated area should be outlined with a permanent marker pen.

   Expression can also be achieved in whole Arabidopsis plants by immersing plantlets in bacterial suspension under vacuum (Rakousky et al., 1998).

10. Incubate plants for 2 to 3 days under normal growing conditions in a greenhouse, except at 20° to 22°C to optimize infection.

11a. *For observation with a UV lamp:* Check for GFP fluorescence in leaves with a hand-held long-wavelength UV lamp after 2 days (assuming a GFP variant with a UV-excitation peak).

   The time required for the fluorescence to appear may depend on the construct. It is wise to check a piece of leaf tissue 2 days after the inoculation of Agrobacterium cells into the leaf, and every 4 to 6 hr thereafter, to establish the optimal time for expression of each construct used.

11b. *For observation using a microscope:* After 2 days, cut out a segment of leaf tissue with fine scissors, mount in a drop of water on a microscope slide, and cover with a long coverslip held in position with strips of electrical or waterproof tape at either end.

   It is important to remember that GFP fluorochrome cyclization will not occur in an anoxic environment so it is preferable that microscope preparations are not sealed.

Visualize expression

12. Observe the specimen with a conventional epifluorescence microscope or laser-scanning confocal microscope (preferred) and appropriate filters.

   Most forms of GFP can be observed with a standard FITC filter block; however, the exact filter configuration will depend upon the excitation and emission wavelengths of the GFP (or its spectral variant) used.

   Depending on the levels of expression, for ER- or GA-targeted constructs, image capture by confocal microscopy may require high laser power. This is possible with GFP as it is relatively resistant to photobleaching. Moreover, a wide pinhole aperture may be required

to obtain a satisfactory signal-to-noise ratio. Although, this strategy reduces confocality, and the ultimate resolution of the image, it can help overcome problems of image blurring due to organelle movement during the image capture process.

13. (Optional) To make a confocal video of endomembrane dynamics, select a region of interest, increase the laser power to the maximum available, select a wide pinhole aperture, and collect images as fast as possible using confocal-imaging time-lapse software.

    If cytoplasmic movement is not dramatic then line (not sequential) image averaging can be used to improve the signal-to-noise ratio.

ALTERNATE PROTOCOL

VIRUS MEDIATED EXPRESSION OF GFP CONSTRUCTS

Viruses are often used as vectors for transient expression of DNA in foreign organisms, due to the high level of replication of the virus and the high level of protein synthesis (results may be obtained 4 to 10 days after inoculation of leaves of the host plants). In general the level of protein expression is high because of the activity of the strong viral promoter. The limitation associated with the virus expression technique resides in the fact that not all cells in plants expressing the viral genome become infected. This may in turn limit the reproducibility of biochemical assays. Moreover, the high levels of expression of the desired protein may be a reason for their mistargeting in the cell. Some inserts may be unstable in the vector, and the likelihood of instability increases with the size of the insert, such that 3 kb may be close to the practical limit. Finally, the pathogenic effects of the viral infection must be taken into account.

For plants, potato virus X (PVX) has been used successfully to express the marker proteins GFP and β-glucuronidase (GUS; Chapman et al., 1992; Baulcombe et al., 1995), and numerous other foreign proteins, often as fusions with GFP (Blackman et al., 1998; Boevink et al., 1998, 1999). The foreign genes inserted into the multiple cloning site are under the transcriptional control of the subgenomic promoter. A site at the 3′ end of the PVX sequence is used to linearize the plasmid before infectious run-off transcripts are made with T7 RNA polymerase (the T7 promoter is present immediately upstream of the PVX sequence).

Additional Materials *(also see Basic Protocol)*

GFP chimera construct
PVX vector (pTXS.P3C2) with multiple cloning site and T7 promoter (available from various laboratories)
Midiprep kit, without RNase (Qiagen)
T7 RNA polymerase transcription kit (e.g., Ambion T7 message in machine kit)
*Spe*I or *Sph*I restriction enzymes (see *APPENDIX 3*)

Aluminum oxide
Aluminum oxide dispenser: small glass flask containing abrasive, sealed with miracloth

Additional equipment and reagents for restriction digestion, phenol/chloroform extraction, and ethanol precipitation (see *APPENDIX 3*), and quantification of DNA concentration by spectroscopy (see *APPENDIX 3D*)

IMPORTANT NOTE: In some countries in order to handle plant viruses as vectors, a license must be obtained from the appropriate authorities.

IMPORTANT NOTE: All solutions used for virus work must be RNase-free.

1. Insert the GFP chimera in the multiple cloning site of the PVX vector in the correct orientation with respect to the T7 promoter (see *APPENDIX 3*).

2 Prepare a high-quality, medium-scale, RNase-free DNA preparation using a midiprep kit. Avoid using RNase in preparation of the DNA (i.e., do not add RNases to solution P1 and increase the volume of the wash buffer as recommended by the manufacturer).

3. Linearize the vector with *Spe*I or *Sph*I restriction enzymes to make infectious RNA.

4. (Optional) Clean the DNA by phenol/chloroform extraction and ethanol precipitation (see *APPENDIX 3*).

5. Synthesize capped transcripts using 0.2 to 1.0 µg linearized DNA for a single infection, as template for the T7 RNA polymerase transcription kit, following the manufacturer's instructions.

   The transcripts are generally not phenol/chloroform extracted or precipitated before inoculating.

6. Dust host plants *lightly* with abrasive aluminum oxide using an aluminum oxide dispenser.

7. Drop 5 µl of transcripts (1 to 5 µg/µl) onto four leaves and spread lightly with a glove-protected fingertip.

8. After inoculation wash the abrasive off the leaves. Keep plants at ~23°C in a culture room or sealed greenhouse.

9. Four to seven days post inoculation with PVX-GFP virus, screen plants for small fluorescent lesions on inoculated leaves with a hand-held long-wavelength UV-light or an epifluorescence microscope.

10. Analyze as described elsewhere (see Basic Protocol, steps 11 to 13).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Infiltration medium (INM)

In a 10-ml measuring cylinder, add 50 mg glucose, 1 ml 0.5 M MES, pH 5.6 (see recipe), 1 ml 20 mM Na_3PO_4 (see recipe), and 5 µl 200 mM acetosyringone (Aldrich) in dimethyl sulfoxide (DMSO; final concentration 100 µM). Bring volume to 10 ml with water, seal the cylinder with 1 cm^2 Parafilm, and mix thoroughly by inversion. Make fresh prior to use.

200 mM acetosyringone in DMSO and 20 mM Na_3PO_4 can be stored at 4°C up to two weeks, or aliquoted and kept at –20°C for longer (i.e., 6 months) storage.

NOTE: *No antibiotic must be added to this medium.*

MES, 0.5 M, pH 5.6

In a 20-ml beaker add 1.95 g MES and 18 ml water. Adjust the pH to 5.6 with KOH and bring the volume to 20 ml with water.

Store up to 2 weeks at 4°C, or for longer storage (i.e., 6 months), aliquot and store at –20°C.

Na_3PO_4, 20 mM

Dissolve 0.152 g $Na_3PO_4 \cdot 12H_2O$ in 20 ml water. Store up to 2 weeks at 4°C, or for longer storage (i.e., 6 months) aliquot and store at –20°C.

YEB medium

In a 1-liter beaker, dissolve 5 g beef extract (Difco), 1 g yeast extract (Merck), 5 g peptone (Difco), 5 g sucrose (BDH Laboratory), and 0.5 g $MgSO_4 \cdot 7H_2O$, in 0.8 liters of water. Add water to 1 liter, stirring continuously. Autoclave and store up to 6 months at room temperature.

It may take some time to dissolve completely.

COMMENTARY

Background Information

While many experimental programs may ultimately require the production of stable transformants, much useful information can be obtained through the use of transient expression systems. The production of transgenic plants and culture cell lines expressing GFP can be achieved using standard transformation and gene expression technologies. One of the major advantages of GFP and its wavelength-shifted derivatives, when used to tag proteins of interest, is that cellular events can be studied in vivo in planta and monitored by conventional epifluorescence microscopy. However, with tissue samples it may be necessary to use either confocal microscopy or conventional fluorescence microscopy combined with low-light-level cameras and deconvolution software.

The introduction of jellyfish fluorescent protein has provided cell biologists with a powerful new tool with which to study cell structure and function in vivo.

GFP chimeras were first used in plant cell biology to investigate the movement of viruses by the construction of both fluorescent viruses and fluorescent viral proteins, such as the movement proteins (Baulcombe et al., 1995; Oparka et al., 1995; Itaya et al., 1997). Subsequently, most major organelles have been successfully labeled with GFP chimeras, including the nucleus (Grebenok et al., 1997), vacuole (Di Sansebastiano et al., 1998), mitochondria (Köhler et al., 1997), plastids (Köhler and Hanson, 2000), cell plate (Gu and Verma, 1997), GA and ER (Boevink et al.,1998, 1999), and cytoskeleton (Köst et al., 1998; Marc et al., 1998).

In plant cells, GFP can be expressed on its own, fused to targeting peptides, or as a chimera with a complete protein of interest. Targeting the protein into the ER can be achieved by the addition of an appropriate N-terminal signal peptide (such as sporamin, patatin, or chitinase signal; Boevink et al., 1999; Haseloff et al., 1997) to translocate the protein into the ER lumen, and a C-terminal His/Lys, Asp, Glu, Leu (H/KDEL) retrieval sequence to maintain the protein in the ER (see *APPENDIX 1C*). For GA visualization, targeting (signal-anchor) sequences from plant or mammalian transferases can be spliced onto the C terminus of GFP to locate the Golgi (Boevink et al., 1998), as can complete Golgi enzyme–coding sequences (Nebenführ et al., 1999). For instance the signal-anchor sequence of a rat sialyl transferase, incorporating the transmembrane and cytoplasmic amino acid domains, targets GFP to the *trans*-Golgi, while a soybean mannosidase I-GFP fusion locates towards the *cis*-face (Nebenführ et al., 1999). Both ER and Golgi can be targeted with the *Arabidopsis* homolog of the yeast H/KDEL receptor, *aERD2* (Boevink et al., 1998).

The transient expression protocol described in this unit can be used for species other than *Nicotiana*. The authors have been able to transiently express ER-targeted GFP in *Arabidopsis*, *Petunia*, and cucumber.

Critical Parameters

There are now a number of variants of GFP, obtained by genetic modification of the wild-type gene, which have been optimized to change various characteristics of the protein. This reengineering has been aimed at changing the spectral properties of the protein and improving the speed of maturation of the chromophore, including photoisomerisation and reduction in photobleaching of the protein (Cubitt et al., 1995, 1999); therefore, it is advisable to make a careful choice of the GFP to be used before embarking on extensive transformation procedures. For instance, mutations with enhanced blue excitation peaks may have reduced UV excitation, making rapid screening of plants with UV lamps impossible.

In order to avoid possible artifacts, it is important to ensure that the detected GFP fluorescence in transiently expressing plant cells is not derived from *Agrobacteria* synthesizing the protein. With other reporter systems this problem has been overcome by insertion of a plant intron into the reporter gene. Unfortunately such intron-containing GFPs have yet to be

constructed. Furthermore, as high expression levels may result in mistargeting, it is advisable that serial dilutions of bacteria be used to optimize expression levels.

Due to the availability of the spectral variants of GFP, including yellow (YFP), cyan (CFP) and the newly released red fluorescent proteins (dsRed), co-expression of constructs is possible. Care must then be taken with the choice of filter sets to prevent bleed through of signal when excitation and emission spectra are close. This of course is not the case for specific techniques, such as fluorescence resonance energy transfer (FRET; *UNIT 17.1*), in which the overlapping spectra are essential parameters (Gadella et al., 1999).

GFP will remain fluorescent after both paraformaldehyde and light glutaraldehyde fixation, thus permitting the use of other fluorescent probes, such as rhodamine conjugated phalloidin (Boevink et al., 1998), or standard immunofluorescence protocols, with red or far-red emitting fluorochromes. GFP will also retain antigenicity after fixation and preparation for immunogold labeling using the progressive-lowering-of-temperature technique and embedding in acrylic resins (Vanden-Bosch, 1991; Boevink et al., 1998; *UNIT 4.7*). Thus, it is possible to confirm the location of GFP fusion proteins at the ultrastructural level. This is important when proteins are targeted to some of the smaller organelles, such as mitochondria, plastids, and Golgi. One may encounter difficulties in obtaining GFP antibodies that are suitable for immunogold labeling of plant tissue. Consequently, it may be advisable to incorporate one of the widely used epitope tags into the GFP fusion if at some stage in the future immunogold labeling is envisaged.

Finally, with the observation of living plant material, specific problems, such as autofluorescence of chlorophyll and rapid cytoplasmic streaming, or organelle movements, are often encountered; these problems can be overcome by selective use of filter sets and optimization of microscope settings.

Troubleshooting

Sometimes a GFP chimera does not fluoresce, which can be due to several reasons. For example, frame shifts when GFP-fusions are used. If the resulting cDNAs have been checked and confirmed by sequencing, it is advisable to check that the binary plasmid carrying the construct is stable in *Agrobacterium*. Another possible explanation for lack of fluorescence may be that the GFP chimera is misfolded. Levels of expression can be checked by conventional immunoblot assay of protein extract (*UNIT 6.2*). This is also useful when with a particular construct, one encounters a relatively low-fluorescence level, which may result from protein instability or reduced folding kinetics.

On occasion a mislocalization of the fusion protein can be observed. If the quality of the targeting signals is trusted, then the phenome-

Table 13.3.1 Optimization of Images of GFP Targeted to GA and ER at the Confocal Microscope

Problem	Solution	Result
Autofluorescence of chlorophylls	Use a narrow-band emission filter set (e.g., 515–525 nm) in combination with optimum brightness and contrast settings	Reduction of fluorescence from chloroplasts
Blurred image due to the dynamics of the ER and GA	Increase laser intensity and/or increase pinhole aperture in combination with single-line scan or collect data from a defined region of interest (ROI)	Reduction in resolution and confocality may be experienced. A collection of time-resolved data sets for presentation as movies can be obtained
Low signal from GFP that is not compensated by change of brightness/contrast settings	Increase the laser intensity and/or increase the pinhole aperture	Increase of signal from GFP and reduction of confocality and resolution
Photobleaching of GFP fluorescence	Reduce the laser intensity, the number of image scans for averaging, and the number of sections per Z-series	Reduction of resolution and photobleaching damage

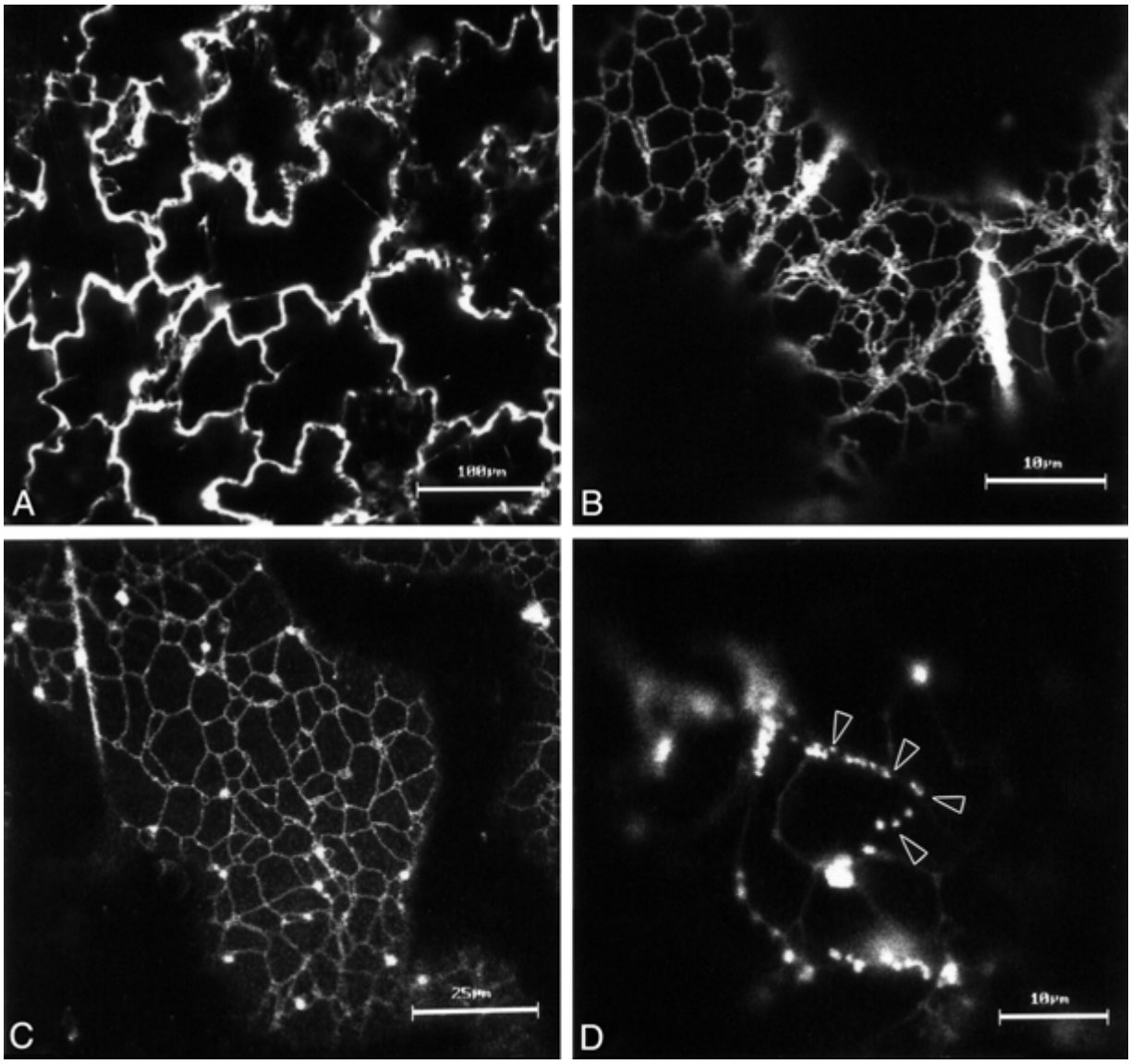


Figure 13.3.1 (**A**) *Agrobacterium*-mediated expression of a signal peptide-GFP-HDEL construct in leaf epidermal cells of *Nicotiana clevelandii*. The low magnification micrograph shows high levels of expression in the endoplasmic reticulum at the cortex of the cells. (**B**) Potato virus X–mediated expression of a signal peptide-GFP-calreticulin construct in a leaf epidermal cell of *N. clevelandii*. (**C**) Virus-mediated expression of an ER/Golgi–targeted construct (aERD2-GFP) in a leaf epidermal cell. Note the bright Golgi bodies are associated with the cortical ER tubules. (**D**) Golgi dynamics. Sequential frame capture of Golgi (arrows) moving over static cortical ER tubules. All micrographs taken with a Zeiss LSM 410 laser scanning confocal microscope.

non may be due to overexpression of the construct. Optimization of the bacterial concentration (as measured by optical density) is then suggested. Mislocalization can be also due to instability of the targeting signal within the fusion protein.

During observation of leaf tissue, the autofluorescence of the chlorophylls can mask low levels of GFP fluorescence. The use of a narrow-band emission filter set (e.g., 515 to 525 nm) in combination with optimum brightness and contrast settings will reduce the autofluorescence from chloroplasts. Increasing the laser power and/or altering brightness and contrast settings can usually compensate for the overall reduction in signal that may result. Movement of labeled organelles in plant cells may result in a blurred image. Modifying laser and/or pinhole settings in combination with single-line scanning can help limit this problem. If necessary, the authors recommend collecting data from a defined region of interest (ROI). A reduction in resolution and confocality will occur, but quick collection of time-resolved

data sets for presentation as movies can be obtained. GFP is an ideal marker for time-lapse studies because of its relative resistance to photobleaching; however, if a reduction of signal from GFP and its variants due to photobleaching is observed, the laser intensity, the number of scans for image averaging, and the number of sections per Z-series should be reduced. Recommendations for the optimization of the ER and GA image at the confocal microscope are summarized in Table 13.3.1.

Anticipated Results

Typical results can be seen in Figure 13.3.1. The results illustrated in the figure are typical for those organelles to which GFP has been deliberately targeted. The percentage of transformed cells can vary with the time and with the concentration of bacteria. With the experimental conditions suggested in the Basic Protocol usually, 3 to 4 days after inoculation of plants, 20% to 40% of the cells are transformed within the area of initial bacterial penetration. Similarly, the virus transformation may produce approximately the same yield of transformed cells (after 4 to 5 days), and this number of cells may increase with time due to the virus spread which is not a feature of *Agrobacterium* transformation.

Time Consideration

As mentioned above, fluorescence may be detected 2 days after plant transformation. In the authors' experience, this depends on the particular construct, but in general, a detectable expression is obtained from 2 to 4 days and may last about 7 to 10 days.

Literature Cited

Baulcombe, D.C., Chapman, S.N., and Santa Cruz, S. 1995. Jellyfish green fluorescent protein as a reporter for virus infection. *Plant J.* 7:1045-1053.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* 12:8711-8721.

Blackman, L.M., Boevink, P., Santa Cruz, S., Palukaitis, P., and Oparka, K.J. 1998. The movement of cucumber mosaic virus traffics into sieve elements in minor veins of *Nicotiana clevelandii*. *Plant Cell* 10:525-537.

Boevink, P., Oparka, K., Santa Cruz, S., Martin, B., Betteridge, A., and Hawes, C. 1998. Stacks on tracks: The plant Golgi apparatus traffics on an actin/ER network. *Plant J.* 15:441-447.

Boevink, P., Martin, B., Oparka, K., Santa Cruz, S., and Hawes, C. 1999. Transport of virally expressed green fluorescent protein through the secretory pathway in tobacco leaves is inhibited by cold shock and brefeldin A. *Planta* 208:392-400.

Chapman, S.N., Kavanagh, T., and Baulcombe, D.C. 1992. Potato-Virus X as a vector for gene expression in plants. *Plant J.* 2:549-557.

Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A., and Tsien, R.Y. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:448-455.

Cubitt, A.B., Woolenweber, L.A., and Heim, R. 1999. Understanding structure-function relationships in the *Aequoria victoria* green fluorescent protein. *In* Green Fluorescent Proteins (K.F. Sullivan and S.A. Kay eds.) pp. 19-30. Academic Press, San Diego.

Di Sansebastiano, G.-P., Paris, N., Marc-Martin, S., and Neuhaus, J.-M. 1998. Specific accumulation of GFP in a non acidic vacuolar compartment via a C-terminal propeptide-mediated sorting pathway. *Plant J.* 15:449-457.

Gadella, T.W.J., van der Krogt, G.N.M., and Bisseling T. 1999. GFP-based FRET microscopy in living plant cells. *Trends Plant Sci.* 4:287-291.

Grebenok, R.J., Pierson, E., Lambert, G.M., Gong, F.-C., Afonso, C.L., Haldeman-Cahill, R., Carrington, J.C., and Galbraith, D.W. 1997. Green-fluorescent protein fusions for efficient characterisation of nuclear targeting. *Plant J.* 11:573-586.

Gu, X.J. and Verma, D.P.S. 1997. Dynamics of phragmoplastin in living cells during cell plate formation and uncoupling of cell elongation from the plane of cell division. *Plant Cell* 9:157-169.

Haseloff, J., Siemering, K.R., Prasher, D.C., and Hodge, S. 1997. Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. U.S.A.* 94:2122-2127.

Hawes, C., Boevink, P., and Moore, I. 2000. Green fluorescent protein in plants. *In* Protein Localization by Fluorescence Microscopy: A Practical Approach (V.J. Allen, ed.) pp. 163-177. Oxford University Press, Oxford.

Itaya, A., Hickman, H., Bao, Y.M., Nelson, R., and Ding, B. 1997. Cell-to-cell trafficking of cucumber mosaic virus movement protein: Green fluorescent protein fusion produced by biolistic bombardment in tobacco. *Plant J.* 12:1223-1230.

Köhler, R.H. and Hanson, M.R. 2000. Plastid tubules of higher plants are tissue-specific and developmentally regulated. *J. Cell Sci.* 113:81-89.

Köhler, R.H., Zipfel, W.R., Webb, W.W., and Hanson, M.R. 1997. The green fluorescent protein as a marker to visualise plant mitochondria in vivo. *Plant J.* 11:613-621.

Köst, B., Spielhofer, P., and Chua, N.-H. 1998. A GFP mouse talin fusion protein labels plant actin filaments *in vivo* and visualises the actin cy-

toskeleton in growing pollen tubes. *Plant J.* 16:393-401.

Marc, J., Granger, C.L., Brincat, J., Fisher, D.D., Kao, T.-H., McCubbin, A.G., and Cyr, R.J. 1998. A GFP-MAP4 reporter gene for visualising cortical microtubule rearrangements in living epidermal cells. *Plant Cell* 10:1927-1939.

Nebenführ, A., Gallagher, L.A., Dunahay, T.G., Frohlick, J.A., Mazurkiewicz, A.M., Meehl, J.B., and Staehelin, L.A. 1999. Stop-and-go movements of plant Golgi stacks are mediated by the acto-myosin system. *Plant Physiol.* 121:1127-1141.

Oparka, K.J., Roberts, A.G., Prior, D.A.M., Baulcombe, D., and Santa Cruz, S. 1995. Imaging the green fluorescent protein in plants–Viruses carry the torch. *Protoplasma* 189:133-141.

Rakousky, S., Kocabek, T., Vincenciova, R., and Ondrej, M. 1998. Transient beta-glucuronidase activity after infiltration of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*. *Biologia Plantarum* 40:33-41.

Rossi, L., Escudero, J., Hohn, B., and Tinland, B., 1993. Efficient and sensitive assay for T-DNA-dependent transient gene expression. *Plant Mol. Biol. Reporter* 11:220-229.

VandenBosch, K.A. 1991. Immunogold labelling. *In* Electron Microscopy of Plant Cells (J.L. Hall and C. Hawes, eds.) pp. 181-218. Academic Press, London.

Key References

Boevink, P., Santa Cruz, S., Hawes, C., Harris, N., and Oparka, K.J. 1996. Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells. *Plant J.* 10:935-941.

This paper illustrates the production of GFP targeted to the ER of living plant cells using a PVX-based expression system.

Boevink et al., 1998. See above.

The authors describe the fusion of the wild-type GFP to the transmembrane domain of a rat sialyl transferase in Nicotiana cells and its localization at the GA. Moreover, they describe the splicing of the wild-type GFP to the C-terminus of the Arabidopsis homologue of the yeast HDEL receptor, aERD2 and the localization of the protein chimera at the ER and GA, using the potato virus X expression system.

Haseloff et al., 1997. See above.

In this paper the authors describe how the wild-type GFP was engineered to be expressed in plants in a non-virus mediated system and to be targeted to the ER.

Nebenführ et al., 1999. See above.

The authors describe the fusion of a soybean Gm-Man1, encoding the resident Golgi protein α-1,2 mannosidase-1, to the green fluorescent protein, and its targeting to the Golgi of Bright Yellow 2 suspension-cultured cells.

Internet Resources

http://www.brookes.uk/schools/bms/research/molcell/hawes/gfp/gfpold.html

Web site for movies obtained at the confocal laser microscope in Nicotiana clevelandii epidermal cells expressing GFP fused to the C-terminus of the transmembrane domain of a rat sialyl transferase (localized at the GA) and the GFP spliced to the C-terminus of the Arabidopsis homologue of the yeast HDEL receptor, aERD2 (localized at the ER and GA).

http://www.plantsci.cam.ac.uk/Haseloff/Home.html

Web site for movies of GFP expressed in Arabidopsis root tips showing different patterns of GFP expression generated by enhancer detection.

http://www.mbg.cornell.edu/kohler/kohler_Trends.html

Web site with movies showing GFP targeting to plastids and mitochondria.

Contributed by Chris Hawes and Federica Brandizzil
Oxford Brookes University
Oxford, United Kingdom

Henri Batoko and Ian Moore
University of Oxford
Oxford, United Kingdom

Movement of Nuclei

This unit presents an in vitro assay for nuclear motility along microtubules (see Basic Protocol). This assay system mimics the movement of the female pronucleus of the fertilized frog egg. Dynamic microtubule asters are assembled from purified centrosomes in *Xenopus* cytosol. Purified nuclei are then added and the motility is observed using video-enhanced differential interference contrast (VE-DIC) microscopy. This assay has been used to demonstrate that cytoplasmic dynein drives the motility of nuclei along microtubules in this system. Dynein activity causes nuclei to move to microtubule minus-ends. Therefore, the nuclei move to and accumulate at centrosomes. Other components of the nuclear motility apparatus can be defined by this assay including regulatory factors present in the cytosol, or binding partners for dynein on the nuclear membrane. Pharmacological and biological inhibitors of nuclear motility can be assayed and the effects on motility can be quantitated.

The assay system requires the following components: purified nuclei assembled in *Xeno*pus egg extracts (see Support Protocol 5); purified centrosomes (see Support Protocols 1 and 2); and *Xenopus* cytosol that has been clarified by high-speed centrifugation (see Support Protocol 4) from fractionated interphase extracts (see Support Protocol 3). The clarified extract provides the subunits for microtubule assembly, the dynein motor complex that drives motility, and regulatory factors that have not yet been defined.

STRATEGIC PLANNING

These experiments require familiarity with handling *Xenopus laevis* for obtaining eggs used in this protocol. Protocols for handling *Xenopus* for egg production and preparation of active cytoplasmic extracts are described elsewhere and should be reviewed (Murray, 1991; *UNIT 11.10*). Included here are those procedures specifically required for nuclear motility experiments. These experiments require significant microscopy expertise, especially using VE-DIC. VE-DIC theory and techniques are not presented here. Refer to *UNIT 4.1* for basic microscopy theory and techniques. Specific techniques for imaging microtubules and organelles using VE-DIC are presented elsewhere (Walker et al., 1988; Salmon and Tran, 1998; *UNIT 13.1*). The support protocols describe the procedures to prepare the different components used in the Basic Protocol.

NUCLEAR MOTILITY ASSAY

BASIC PROTOCOL

This protocol describes the set-up and execution of an assay that combines dynamic microtubules with synthetic nuclei assembled in *Xenopus* egg extracts (see Fig. 13.4.1 for a schematic of the assay). All assay components are stored separately in small aliquots at –70°C and recombined during the assay. (Assay components have been stored as long as 6 months without any loss of activity. Longer storage is conceivable, but has not been tested.) The use of aliquotted, frozen components allows reproducibility between assays as well as the possibility to inactivate or modify individual components to study the function of individual proteins in microtubule-mediated nuclear motility.

Contributed by Sigrid Reinsch

Current Protocols in Cell Biology (2001) 13.4.1-13.4.28

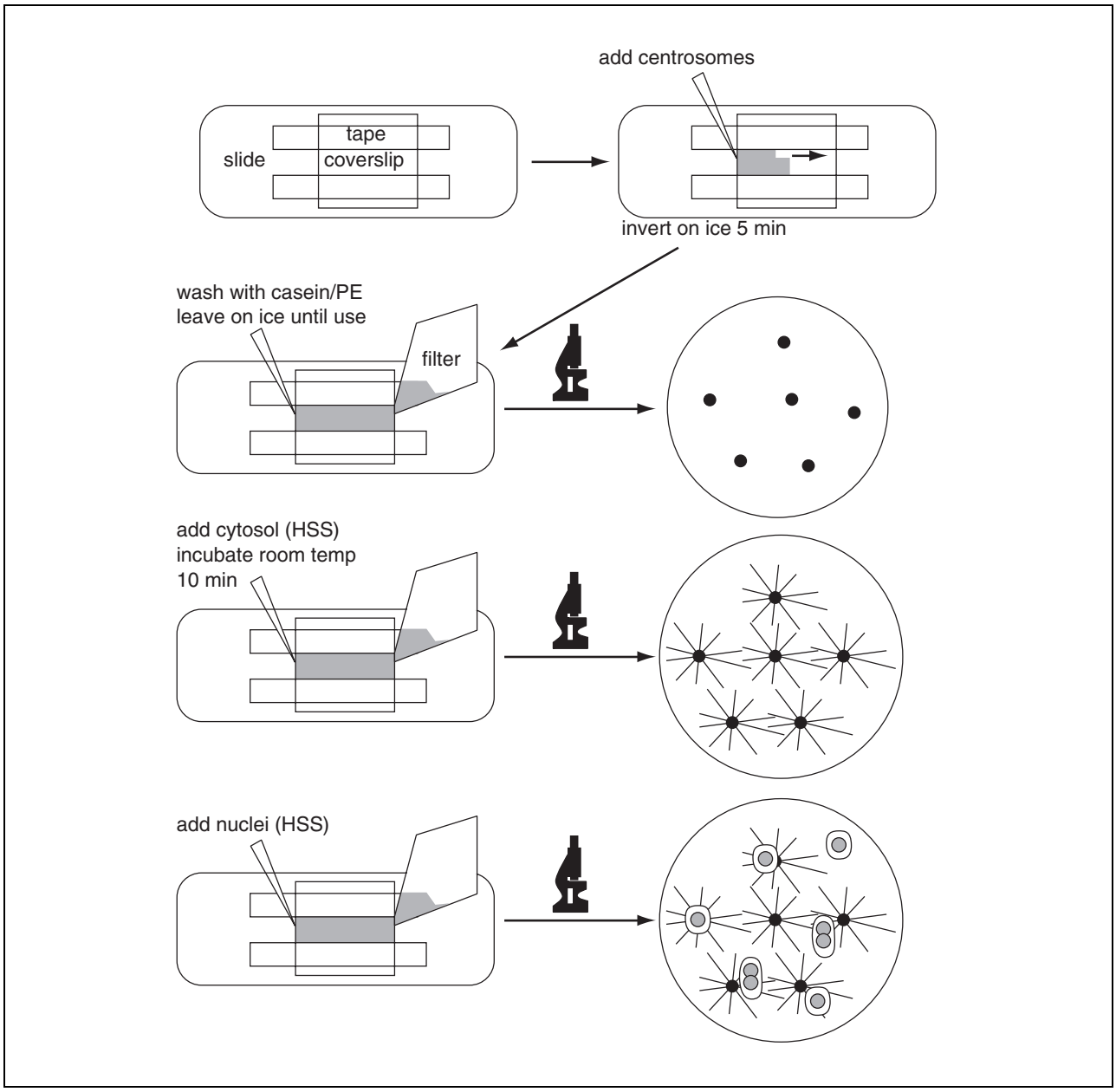


Figure 13.4.1 Flow chart of nuclear motility assay. Schematic showing the slide/coverslip chamber assembly used in this assay, and the flow-through technique. The individual steps are shown on the left panels and a representative microscope field is shown on the right panels. Centrosomes appear as round dots scattered on the field, microtubules are the lines emanating from the centrosomes in the second step, and nuclei moving on the asters are shown in the third step.

Materials

Centrosomes (see Support Protocol 1)
PE buffer (see recipe)
5 mg/ml casein in PE buffer (see recipe)
ABC buffer (see recipe)
HSS cytosol (see Support Protocol 4)
DNA-bead nuclei (see Support Protocol 5)
Valap (*UNIT 13.1*) at 37°C
Immersion oil
Assay reagent (e.g., drug, antibody, expressed protein)
Antibodies bound to beads (for immunodepletion experiments)

Inverted microscope equipped for high-resolution DIC optics
Simple perfusion chambers made from: clean slide, double-stick tape, and clean 18 × 18–mm coverslips (for preparation see *UNIT 13.1*, Support Protocol 1)
Humid chamber: a covered 10- or 15-cm glass petri dish with strip of moist filter paper around perimeter
Magnetic particle concentrator (Dynal)
Inverted microscope
Camera (e.g., Hamamatsu CCD C307, Hamamatsu)
Image processor (e.g., Argus 10, Hamamatsu or equivalent)

Assay nuclear motility

1. Prior to the experiment, optimize inverted microscope for DIC imaging of microtubules.

2. Assemble perfusion chambers according to the instructions in *UNIT 13.1*, Support Protocol 1. Assemble by placing two pieces of double-sided tape 2 mm apart on a microscope slide and attach an 18 × 18–mm coverslip.

   The chambers should have an approximate volume of 7 µl.

3. Thaw an aliquot of centrosomes. Dilute with PE buffer to the optimal concentration determined in Support Protocol 2. Flow 7 µl of diluted centrosomes into each chamber to be used in the day's set of experiments. Be careful not to introduce bubbles into the chamber. Invert perfusion chambers onto ice and allow the centrosomes to settle onto the coverslip and attach for 5 min.

   For all perfusion steps, samples are pipetted at one opening of the perfusion chamber and wicked on the opposite side with a small piece of filter paper.

4. Flow 30 µl of 5 mg/ml casein in PE through perfusion chamber to block binding sites on the glass and rinse out unattached centrosomes. Incubate on ice until use (up to 4 hr maximum).

   From this point, only one chamber will be used at a time because each must be viewed individually on the microscope.

5. Ten minutes before the start of the assay, rinse chamber with 30 µl of ABC buffer. Flow 10 µl of HSS cytosol into chamber and incubate for 10 min at room temperature in the humid chamber.

   This allows recruitment of pericentriolar material from the cytosol, nucleation of microtubules, and aster formation.

6. During the 10-min incubation, quickly thaw an aliquot of DNA-bead nuclei (see Support Protocol 5) and immediately place on ice. Resuspend in 150 µl ABC buffer. Retrieve on a magnetic particle concentrator, carefully remove the ABC buffer, and resuspend in 10 ml HSS cytosol.

7. Flow the nuclear suspension into the chamber to start the assay. Seal the chamber with Valap, oil top and bottom of chamber with immersion oil, and observe at 20° to 22°C. Use DIC optics, a 100× objective, and a high-resolution condenser.

   If appropriate, use additional magnification to obtain an optimal on-screen field size ~50 µm. Use image background subtraction to enhance the microtubules (Walker et al., 1988; Salmon and Tran, 1998).

   Simultaneous imaging of nuclei and microtubules is a bit trickier than imaging only microtubules or other organelles due to the high refringence of the nuclei (especially magnetic-bead nuclei). Nuclei move rapidly on the microtubules. By 20 min, most nuclei have stopped moving and reached the centrosomes.

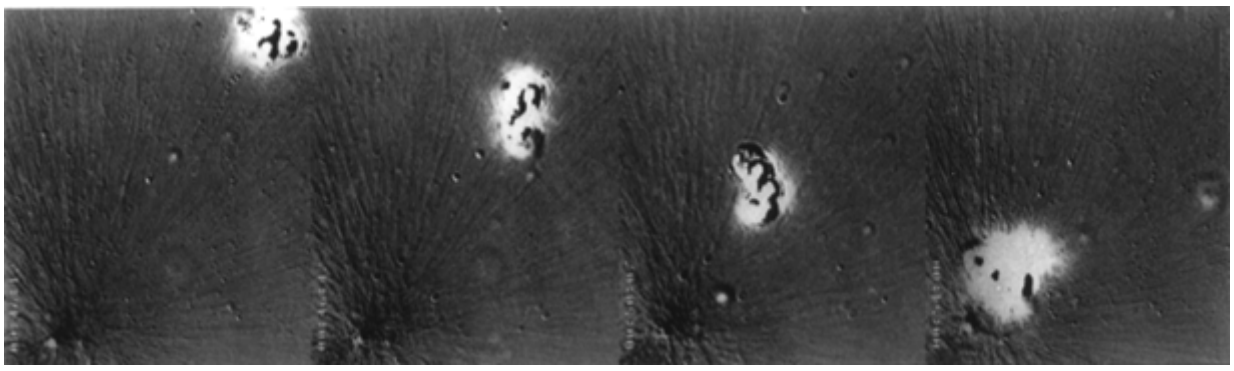


Figure 13.4.2 DIC images of a nucleus moving on a microtubule aster. A sequence of four stills are shown from a motility assay over a period of 10 min. The centrosome is in the lower left corner of each panel, and a robust microtubule aster emanates from this focus. A synthetic nucleus containing several paramagnetic beads starts in the upper right corner and moves to the centrosome during the course of the experiment. The nucleus appears refractory under AVEC-DIC imaging conditions. Image processing, including background subtraction and frame averaging, enhances the microtubules for clearer visualization. The time points are not indicated, but would occur during the first 20 min following assembly of the assay. By the 20-min time-point where quantitation is performed, 90% of nuclei will have reached the centrosome.

Collect images

8. To film nuclear movements, collect shuttered images with a camera. Perform rolling image averaging and background subtraction with an Argus 10 image processor, or equivalent image processing program.

   An appropriate interval between shuttered images is 5 sec. Figure 13.4.2 shows several sequential frames of a movie of a nucleus migrating on a microtubule aster.

Quantitate nuclear motility

9. To perform a quantitative assay for accumulation at centrosomes, repeat steps 1 through 8. Starting at 20 min after adding DNA-bead nuclei, for an additional 10 min, count as many fields as possible to determine percentage of nuclei present at centrosomes versus those at a distance (>5 μm) from centrosomes.

   One should be able to count 50 to 100 nuclei in the 10-min interval. Typical values are 90% to 95% of the nuclei present at centrosomes. Because many nuclei contain multiple DNA beads, each centrosome containing beads/nuclei should be counted as "1". Therefore numbers for accumulation are minimal values. Values <90% to 95% indicate difficulties with the assay that must be addressed before continuing (see Troubleshooting). If the assay looks optimized, then proceed to the next step.

Perform addition experiments

To assess pharmacological or biological reagents for effect

10a. Perform steps 1 through 4.

11a. Add 2 μl of drug, antibody, or expressed protein to 18 μl of HSS cytosol.

12a. Resuspend an aliquot of DNA-bead nuclei in 150 μl of ABC buffer. Retrieve on a magnet particle concentrator and discard supernatant.

13a. Resuspend DNA-bead nuclei in 10 μl of HSS/test compound mixture (step 11a). Use the remaining 10 μl to flow through perfusion chamber after the nucleation step (step 4) to replace the normal HSS cytosol with that containing the test compound.

14a. Flow in the nuclei/HSS/test compound mixture, incubate 20 min at 20° to 22°C and count as in step 9.

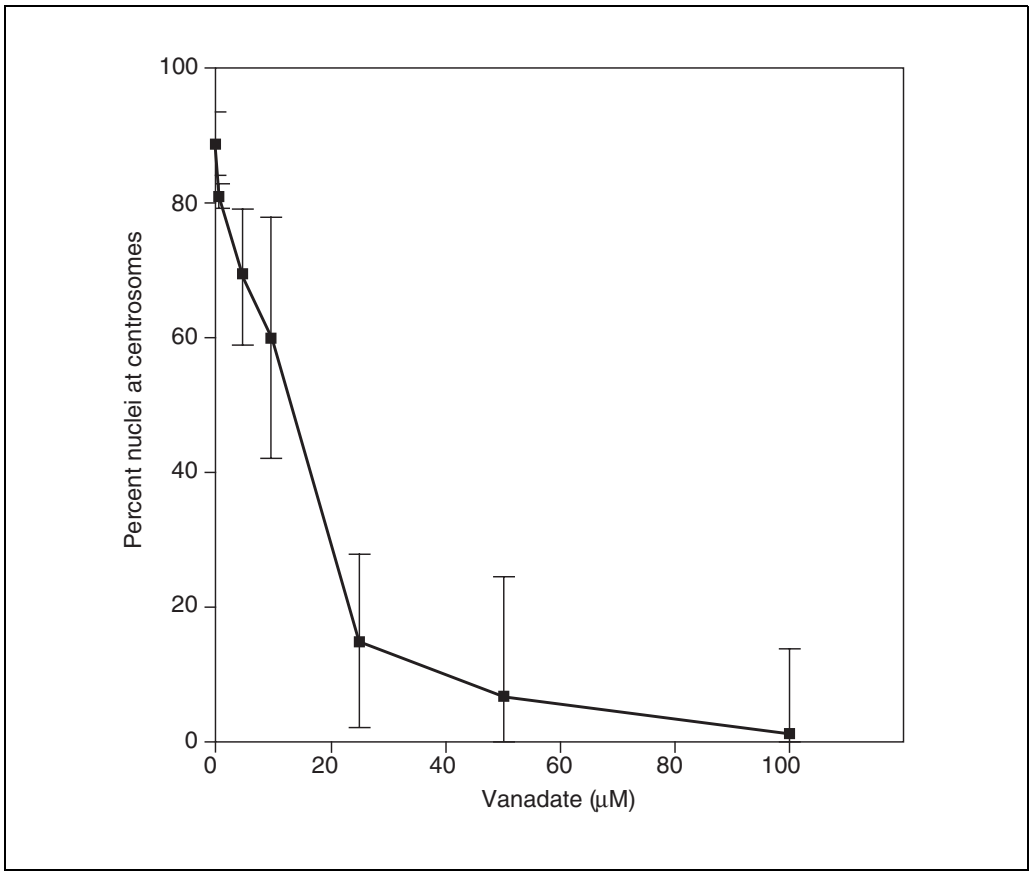


Figure 13.4.3 Effect of vanadate on accumulation of nuclei at centrosomes is an example of how this assay is used to derive quantitative data. Accumulation of nuclei at centrosomes was compared in HSS containing varying amounts of sodium orthovanadate, a general ATPase inhibitor. Assays were performed in triplicate for each vanadate concentration to generate a dose-response curve. The experiment indicates that vanadate significantly inhibits nuclear accumulation at centrosomes at a concentration of 20 mM. This concentration has been shown to be effective in inhibiting the motility of cytoplasmic dynein, while inhibition of kinesins requires concentrations of vanadate in the 100 mM range.

Perform each assay three to four times to obtain statistically significant data. The control mixture should contain 2 μl of appropriate buffer. Figure 13.4.3 shows a titration curve for the effects of different concentrations of vanadate on the accumulation of nuclei at centrosomes.

For some reagents, it is important to incubate the cytosol with the reagent for 30 to 60 min before the assay. This is especially true for proteins expressed in bacteria, which may need to undergo refolding, or other post-translational modification in the extract to be effective (S. Reinsch, unpub. observ.).

To perform depletion experiments

10b. Immunodeplete specific components from HSS cytosol using antibodies bound to beads. Use depleted HSS cytosol for motility cytosol in the assay.

For examples of successful depletion experiments using Xenopus extracts see Merdes et al. (1996); Walczak et al. (1996); or Tournebize et al. (2000).

These experiments are technically more challenging.

SUPPORT PROTOCOL 1

CENTROSOME ISOLATION FROM LYMPHOCYTES

Centrosomes in fibroblastic and lymphoid cells are anchored in the cytoplasm by microtubules and interact with the nucleus in an unknown way. The centrosome-nucleus association is dynamic in vivo but becomes irreversible in vitro if cells are lysed at physiological ionic strength. Most procedures for making nuclei lead to the preparation of nucleus-centrosome complexes that are almost impossible to dissociate. Therefore, in this protocol, the nucleus-centrosome interaction in vivo is first disrupted, then cells are lysed under conditions where centrosomes can be purified away from the nucleus.

Human lymphoid cells in culture are used here because they grow in suspension and have a low cytoplasm/nucleus ratio. Evidence indicates that both the microtubule cytoskeleton and the actin network are involved in centrosome anchoring in the cytoplasm (Euteneuer and Schliwa, 1985; Buendia et al., 1990; Mack and Rattner, 1993). To purify centrosomes, cells are first preincubated with cytochalasin and nocodazole to disrupt connections between centrosomes and nucleus (Bornens et al., 1987). Extremely fast lysis of cells at very low ionic strength in the presence of Triton X-100 leads to chromatin dispersion and release of centrosomes in suspension. The addition of a low concentration of magnesium ions to the lysis buffer leads to partial chromatin stabilization and still allows centrosome release. This reduces contamination of centrosomes by chromatin components. Centrosomes are rapidly sedimenting particles. Their purification is therefore achieved by separation on sucrose gradients.

It takes ~1 week to grow enough cells for the preparation. The preparation itself takes 1 day. One or two people can do the preparation, and a minimum of two people are needed to perform steps 6 to 7, which need to be done very rapidly. It is important to have highly concentrated centrosomes (~2×10^8/ml) for this assay. The yield is typically 30% to 50%, with several fractions containing centrosomes at the optimal concentration.

Materials

KE37 human lymphoblastic cells (ACC46; DSMZ German Collection of Microorganisms and Cell Cultures)
RPMI-10: RPMI 1640 medium with 10% (w/v) FBS
10 mM nocodozole stock solution in DMSO (see recipe)
10 mg/ml cytochalasin D stock solution in DMSO (see recipe)
PBS (see recipe), ice cold
PBS/10 with 8% sucrose (see recipe)
Lysis buffer (see recipe)
4 ml 0.5 M K-PIPES (pH 7.2)/1 mM EDTA (see recipe)
1 mg/ml DNase I (see recipe)
40%, 50%, and 70% sucrose in gradient buffer (see recipe)
Liquid nitrogen
PE buffer (see recipe)
Methanol, −20°C
PBS/0.1% Triton X-100
Monoclonal anti-tubulin antibody (Amersham)
Secondary antibodies
Hoechst
Polyclonal anti-pericentriolar antibody (e.g., γ-tubulin, pericentrin; optional)

250-ml plastic flask
2-liter spinner culture flask
500-ml centrifuge bottles
Clinical centrifuge or equivalent with swinging bucket rotor for 50-ml tubes

Super-speed centrifuge (e.g., Sorvall RC-26 or equivalent)
Large volume rotor (GSA or equivalent)
50-ml capped conical tubes
10-ml plastic pipet
125-µm nylon mesh (Millipore)
Beckman SW28 and SW28.1 centrifuge tubes
Ultracentrifuge
Ultracentrifuge SW28 rotor with SW28 and SW28.1 buckets
18-G needles
Refractometer
Modified Corex tubes (Evans et al., 1985; *UNIT 11.13*)
11- to 12-mm diameter coverslips (acid-washed)
HB-4 or HB-6 rotor
Forceps
Fixing jar with coverslip holder

Additional reagents and equipment for immunofluorescence staining (*UNIT 4.3*) and analyzing centrosomes by spinning onto coverslips (*UNIT 11.13*)

Grow KE37 cells

1. Grow 1 liter of KE37 cells in four 250-ml plastic flasks in RPMI 1640-10.
2. One day before the centrosome preparation, transfer 1-liter cells to a 2-liter spinner culture bottle and add 1 liter RPMI 1640-10.
3. On the day of purification have 2 liters of cells at a density close to 1.5×10^6 cells/ml (3×10^9 cells total).

   It is important that the cells are maintained in exponential phase at densities between 1 and 4×10^6 cells/ml.

4. Add 60 µl of 10 mM nocodazole to a final concentration of 33 µM and 200 µl of 10 mg/ml cytochalasin D to 1 µg/ml final. Incubate 1 hr at 37°C with continuous stirring.
5. Transfer to 500-ml centrifuge bottles and centrifuge for 15 min at 650 × g (2000 rpm in GSA rotor), 4°C.

   During all subsequent centrifugations, do not decant supernatant, but rather aspirate supernatant.

6. Resuspend cells in ice-cold PBS to a maximum total volume of 160 ml.
7. Transfer to four 50-ml capped conical tubes. Centrifuge for 5 min at 500 × *g* (in a clinical centrifuge), 4°C. Repeat PBS wash and centrifugation once.
8. Resuspend cells in ≥25 ml/tube PBS/10 with 8% sucrose. Centrifuge for 5 min at 250 × g (1000 rpm in a clinical centrifuge), 4°C.

   Do this step and step 9 as quickly and gently as possible.

9. Lyse cells in each tube by adding 10 ml of lysis buffer to pellet. Disperse pellet by pipetting up and down with a 10-ml plastic pipet without generating bubbles. Bring volume in each tube up to 20 ml with lysis buffer. Invert 2 to 3 times slowly. Incubate 5 min on ice.

   Cells should not form an aggregate. They should lyse immediately. The maximum total volume for the preparation should not exceed 90 ml at this point.

10. Centrifuge 10 min at 2000 × *g* (3000 rpm in a clinical centrifuge), 4°C.

11. Filter supernatant through 125-µm nylon mesh. Remove a 300-µl sample for counting.

12. To the pooled 90 ml supernatants add 1.8 ml of 0.5 M K-PIPES (pH 7.2)/1 mM EDTA and 90 µl DNase I stock solution.

Purify centrosomes on sucrose gradients

13. Load 30 ml of lysate into three Beckman SW28 ultracentrifuge tubes and using a 10-ml plastic pipet underlay with 5 ml of 50% sucrose in gradient buffer.

14. Ultracentrifuge 20 min at 20,000 × *g* (11,100 rpm in SW28 rotor), 4°C.

15. Aspirate supernatant leaving ~2 ml of lysate above the cushion in each tube.

    To estimate the 2-ml volume above the cushion, prepare a blank tube containing the cushion volume and 2 ml above this volume. Mark tube at the appropriate height to compare with the tubes containing the centrosome samples.

16. Take the 2 ml of remaining supernatant and 2 ml of 50% sucrose cushion. Pool 12-ml total volume from the three step gradients. Keep a 100-µl aliquot for counting.

17. Mix well and layer over a discontinuous sucrose gradient in a SW28.1 ultracentrifuge tube.

    2.0 ml 70% sucrose
    1.5 ml 50% sucrose
    1.5 ml 40% sucrose
    12.0 ml of centriole suspension (~25% sucrose: pooled volume from the three-step gradients).

18. Ultracentrifuge 75 min at 110,000 × *g* (25,000 rpm in SW28.1 rotor), 4°C.

19. Eliminate top of the gradient to the 40% solution. Pierce bottom of tube with an 18-G needle and collect 0.4-ml fractions manually.

Analyze gradient fractions and count centrosomes

20. Read the fractions with a refractometer. Take 5-µl aliquots from fractions between 40% and 70% sucrose to count centrosomes by immunofluorescence. Snap freeze the remaining volume of these fractions in liquid nitrogen without further aliquotting. Store at −70°C. Discard fractions at <40% and >70% sucrose.

21. Prepare one modified Corex tube for each 5-µl aliquot and one each for the samples from steps 11 and 16. Place the removable chuck on top of the bottom spacer. Place a clean 11-mm diameter coverslip on top of the chuck.

22. Mix the 5-µl aliquots each with 5 ml of PE buffer by inverting several times in a 15-ml capped tube. Pipet into the prepared Corex tube. Centrifuge 10 min at 23,600 × g (12,000 rpm in HB4 rotor), 4°C.

23. Carefully remove coverslips from the tube: use a thin spatula with a bent tip to lift the chuck out of the tube, then use forceps to transfer coverslip into cold methanol. Fix coverslips 5 min in methanol at −20°C.

    It is important to remember which side of the coverslip the centrosomes are on; maintain all of the coverslips in the same orientation.

24. Remove coverslips from fix and place in PBS/ 0.1% Triton X-100.

    For a more detailed description of the proecdure in steps 21 through 25, see UNIT 11.13, Support Protcol 4.

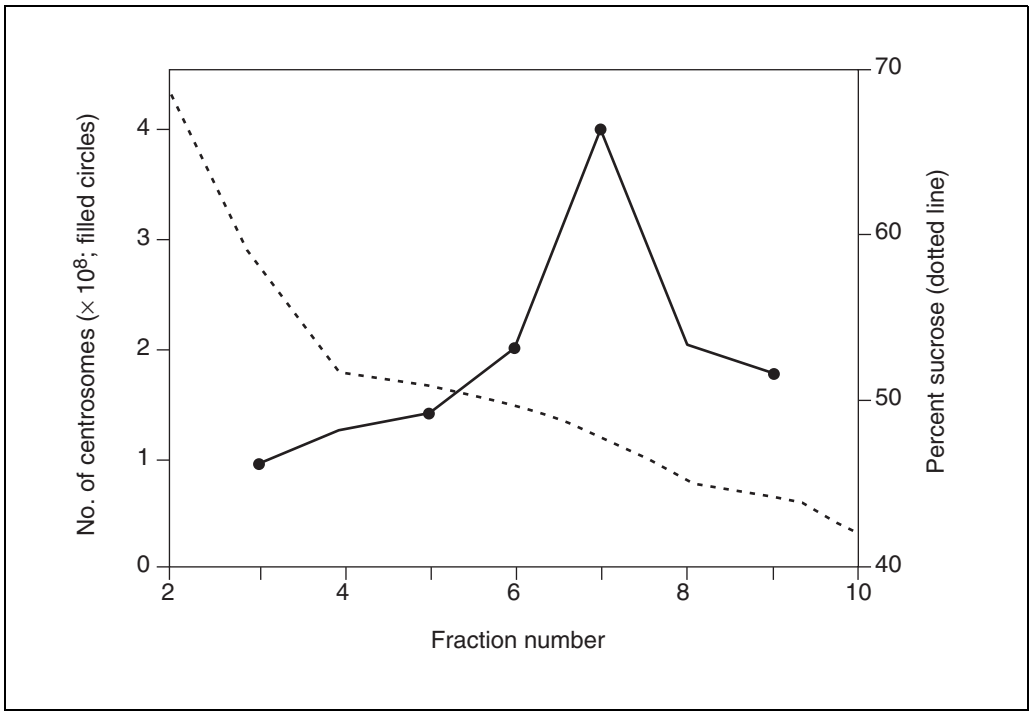


Figure 13.4.4 Fractionation of centrosomes in sucrose step gradient. A typical graph obtained after fractionation of centrosomes on a sucrose step gradient. The sucrose concentration (dotted line) of each fraction was determined using a refractometer, while the centrosome concentration (solid line) was determined as in Support Protocol 1. The peak fractions, containing centrosomes sufficiently concentrated for use in this assay, typically sediment between 45% and 50% sucrose.

25. Perform immunofluorescence using a monoclonal anti-tubulin antibody, followed by appropriate secondary antibodies (*UNIT 4.3*). Add Hoechst to secondary antibodies to check for DNA contamination.

    If possible, also use a polyclonal antibody against pericentriolar material (anti-γ-tubulin). This helps to confirm that the dots are centrosomes. Santa Cruz Biotechnology offers polyclonal anti-γ-tubulin antibody, but it has not been tested in this protocol.

26. Observe samples at a magnification where centrosomes are easily visualized (usually as pairs of dots, with some single dots). Count the total number of centrosomes in several microscope fields to get an average number. Use a stage micrometer to measure the size of the field. Extrapolate to the total cross-sectional area of the Corex tube to get the total number of centrosomes in a particular 5-µl aliquot or from intermediate steps 11 and 16 (see Fig. 13.4.4 for a typical determination of centrosome concentration from a sucrose gradient).

27. Maintain the sucrose gradient fractions of centrosomes frozen at −80°C.

    Centrosomes can be stored this way for ≥1 year. See Support Protocol 2 for subaliquotting the centrosomes and for titration in the motility assay.

TITRATION OF CONCENTRATED CENTROSOMES

SUPPORT PROTOCOL 2

The assay presented in the Basic Protocol requires standardized components so that assays performed on different days can be compared. In particular, the same concentration of centrosomes must be used from one assay to the next as the plating density of centrosomes affects assay results.

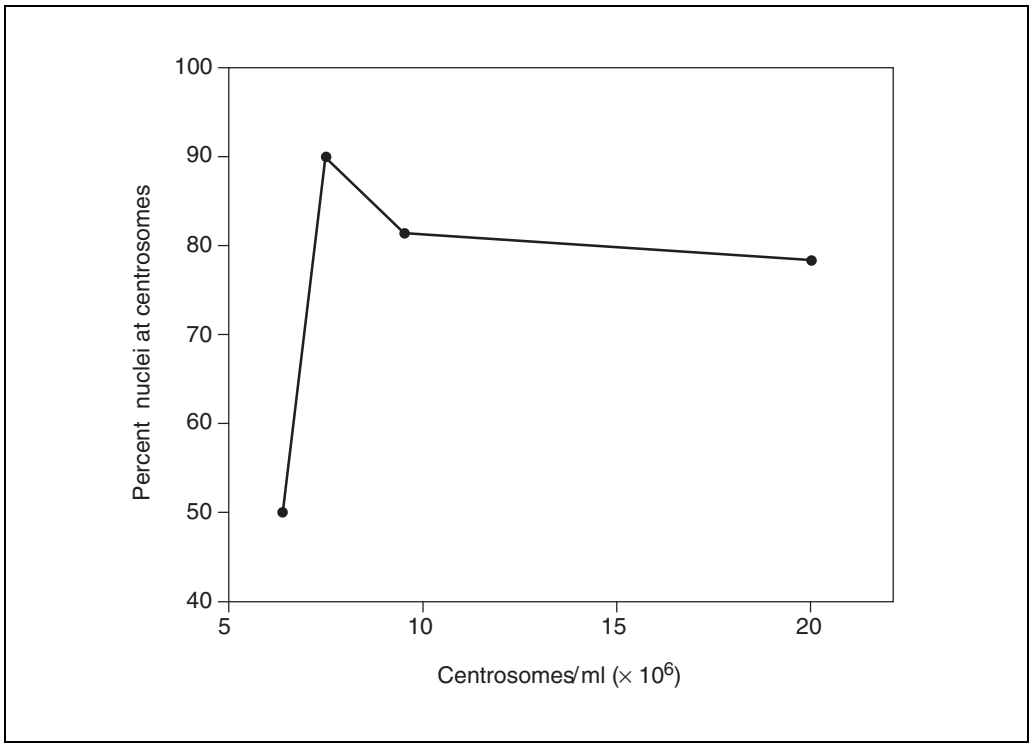


Figure 13.4.5 Titration of centrosomes in motility assay. An assay showing how centrosome concentration affects the accumulation of nuclei at centrosomes. If centrosomes are plated to sparsely, microtubules form spontaneously rather than at centrosomes preferentially, and nuclei will move in a random fashion on these microtubules rather than accumulating at centrosomes. At high centrosome concentrations, elevated concentrations beyond a certain value do not enhance accumulation of nuclei at centrosomes. Steps for titrating centrosomes for this assay are described for Support Protocol 2.

Additional Materials *(also see Basic Protocol)*

Large aliquot of centrosomes (see Support Protocol 1)
Liquid nitrogen
0.5-ml microcentrifuge tubes for aliquotting

Prepare centrosome aliquots

1. Prepare about one hundred 0.5-ml microcentrifuge tubes on ice.
2. Rapidly thaw one large 400- to 500-µl aliquot of centrosomes. Mix the aliquot well by inverting tube several times or pipetting up and down without introducing bubbles. Make 5-µl aliquots. Snap freeze in liquid nitrogen.

   It is important that the large aliquot is well mixed before dividing into smaller aliquots so that the concentration of centrosomes is the same in each tube. Since centrosomes are large particles they will sediment in the tube over time.

Titrate centrosomes

3. Prepare 8 perfusion chambers according to *UNIT 13.1*, Support Protocol 1.
4. Thaw four 5-µl aliquots of centrosomes. Resuspend each in a different volume to bracket the range of final concentrations of 5-20 $\times$ 10^6 centrosomes/ml.
5. Flow each centrosome sample into two perfusion chambers to serve as duplicates.
6. Perform Basic Protocol, steps 2 through 7 on each sample to determine which centrosome concentration yields the highest value for number of nuclei that accumulate at the centrosomes.

A typical plot is shown in Figure 13.4.5.

Empirically, a final concentration in the range of 7.5×10^6 centrosomes/ml gives consistently good values. This corresponds to a plating density of 2 to 3 centrosomes/50 μm^2 microscope field. The optimum value is a result of the competition between free nucleation of microtubules and that nucleated by centrosomes. If there are too few centrosomes, there is a lot of spontaneous nucleation of microtubules that generates random arrays. With too many centrosomes, there is little or no spontaneous microtubule assembly, but microtubules will have a shorter overall length at the 20-min time point.

PREPARING FRACTIONATED INTERPHASE EXTRACTS FOR NUCLEAR ASSEMBLY

SUPPORT PROTOCOL 3

Functional nuclei can be assembled in vitro using *Xenopus* extracts (for review see Newmeyer and Wilson, 1991; Gant and Wilson, 1997; *UNIT 11.10*). Freshly made 16,000 × *g* interphase extracts generally give the best results for nuclear assembly and can be used with the DNA-magnetic beads (stop at step 11). However, extracts that have been fractionated into membraneous and cytosolic components are useful for many types of functional studies and are therefore presented here. The cytosolic and membrane fractions are stored frozen and are recombined with template DNA to assemble chromatin and nuclei. Many published protocols for nuclear assembly use sperm nuclei. These are not appropriate for studies of nuclear motility that mimic movement of the female pronucleus along microtubules. Sperm nuclei have an associated centrosome, and microtubules nucleated from this centrosome will generate motility events in their own right (reviewed in Reinsch and Gonczy, 1998). For motility assays one can use nuclei assembled with magnetic beads coupled with plasmid DNA, or nuclei assembled around larger purified DNA such as lambda DNA. The use of magnetic beads as substrates for nuclear assembly allows rapid and gentle purification of the nuclei.

For handling of frogs and general methodologies for preparing *Xenopus* egg extracts see Murray (1991) and Newmeyer and Wilson (1991); also see *UNIT 11.10*. The following protocol uses eggs that have been collected after overnight stimulation of the frogs with human chorionic gonadotropin (HCG), and is based on a published protocol (Hartl et al., 1994; also see *UNIT 11.10*).

The general principles of this procedure are illustrated in Figure 13.4.6.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regualtions regarding the care and use of laboratory animals.

Materials

5 to 7 frogs
100 U pregnant mare serum gonadotropin (PMSG; see recipe)
500 U HCG (see recipe)
MMR (see recipe)
Dejellying solution (see recipe)
S-lysis buffer (see recipe)
S-lysis-plus buffer (see recipe)
10 mg/ml cytochalasin D stock solution in DMSO (see recipe)
1 M DTT (see recipe)
Protease inhibitors (LPC; see recipe)
2.5 M sucrose (see recipe)
Glycerol
Liquid nitrogen for freezing aliquots
S-lysis-plus/500 mM sucrose solution (see recipe)

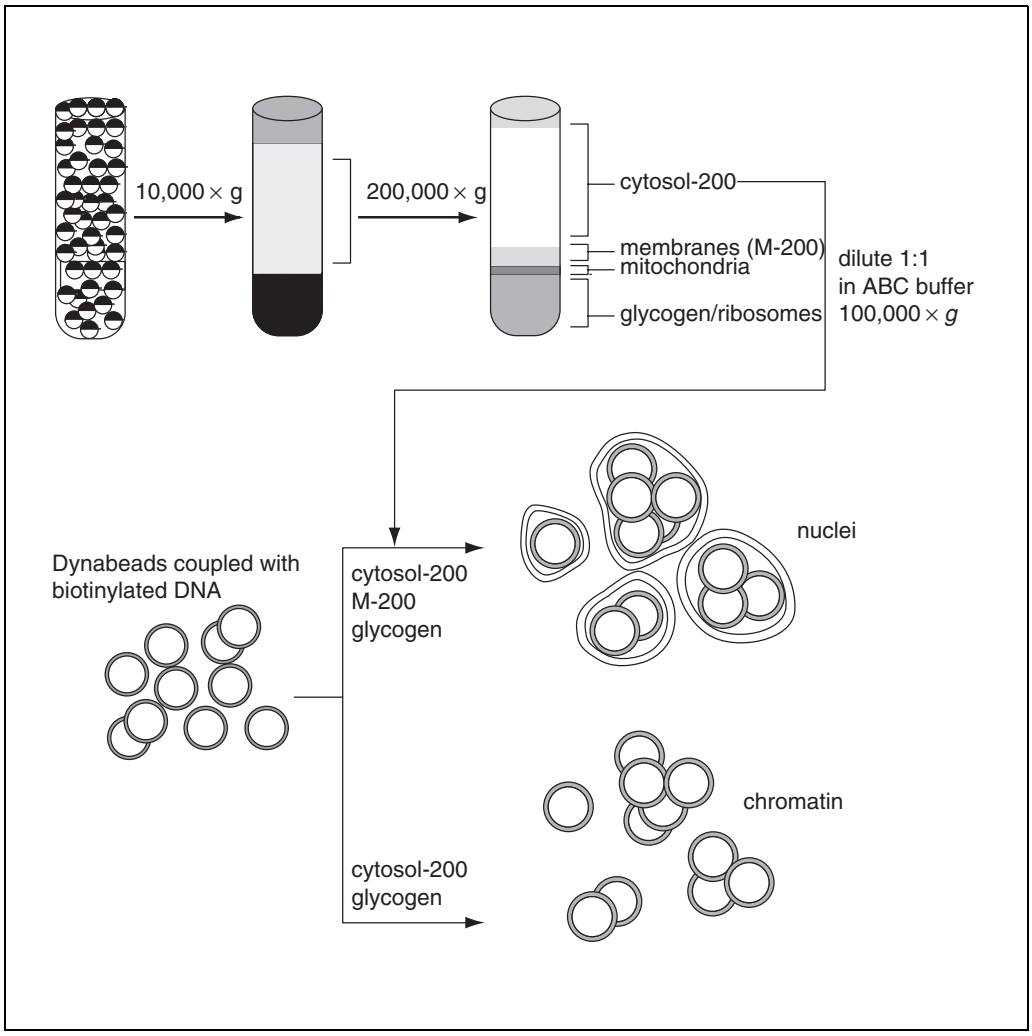


Figure 13.4.6 Nuclear assembly from fractionated extracts. Schematic showing the basic steps required to assemble synthetic nuclei using fractionated extracts. In the protocol presented here, generation of the cytosol-200 and M200 fractions are described in Support Protocol 3. These are frozen separately and then recombined with DNA-coupled Dynabeads as described in Support Protocol 5. Preparation of DNA-coupled Dynabeads is described in *UNIT 11.13* (Heald et al., 1998). If the M200 membranes are omitted during the assembly process, interphase chromatin assembles on the DNA, but full nuclear assembly requires the M200 fraction. Synthetic nuclei often contain multiple Dynabeads which behave very similarly in the assays presented here.

1-ml syringes and 27-G needles
400-ml beakers
SW50 ultraclear centrifuge tubes (Beckman)
Pasteur pipet cut to a wide-mouth bore with a file, fire polished
Sarstedt 13-ml adaptor tubes
2-ml syringe and 18-G needle
Clinical centrifuge
Sorvall RC5 centrifuge
HB-4 rotor with rubber adapters (Sorvall)
5-ml polyproponate tubes with caps
Ultracentrifuge
SW55 rotor
500-ml microcentrifuge tubes for aliquotting cytosol and membranes

NOTE: Make sure that all glassware is clean and rinsed with distilled water before use. Additionally, wet all glassware with buffer before contact with the eggs as they will stick to the glassware and activate or lyse.

Collect the eggs

1. Inject 5 to 7 frogs subcutaneously into the dorsal lymph sac with 0.5 ml of 100 U PMSG, using a 1-ml syringe and 27-G needle. Perform the injection ≥4 days (and ≤10 days) before the extract is to be prepared. Do not feed frogs after PMSG injection.
2. Twelve to 18 hr before use, inject each frog subcutaneously with 0.5 ml of 500 U HCG. Place frogs in individual containers with 500 ml MMR and maintain the frogs overnight at 16°C.
3. Collect eggs into 400-ml beakers using a separate beaker for each frog. Discard batches of eggs containing >5% of lysed, mottled, or stringy eggs.

   Keep batches of eggs separate through dejellying and even through the 16,000 × g centrifugation, if possible. Note that there is no need to activate the eggs to send them into interphase. They will spontaneously enter interphase due to lysis in the presence of Ca^{2+} ions. Cycloheximide added to the buffers will maintain the interphasic state of the extract. Any batches of eggs that lyse during dejellying, loading into tubes, or packing in the low-speed centrifuge should be discarded.

Dejelly the eggs

4. Pour off MMR and add 50 to 100 ml dejellying solution to each beaker. Swirl beaker gently and change the solution several times until the eggs start to pack (usually ~5 min).

   The eggs should be left in dejellying solution for the minimal amount of time required to completely remove the jelly coat. Further incubation seriously compromises egg quality.

5. Rinse three times with 50 to 100 ml MMR. Work quickly through the next steps, until lysis/centrifugation, as the eggs are quite fragile once the jelly coat is removed. Ensure that the eggs are always covered with buffer. Remove bad eggs (white and puffy, or dark pigment retracted or mottled).

Lyse the eggs

6. Pour off as much MMR as possible while keeping eggs immersed in a minimal quantity. Rinse two times with S-lysis buffer in the same manner.
7. Rinse once with S-lysis-plus buffer. Use a wide-bore Pasteur piper to load eggs into SW50 ultraclear centrifuge tubes containing 1 ml S-lysis-plus buffer and 10 µl of 10 mg/ml cytochalasin D stock (to 100 µg/ml final concentration). Fill tubes as full as possible and remove any buffer on top of eggs.

   Always place the tip of the pipet containing the eggs into the buffer in the tube before expelling the eggs. This ensures that the eggs do not contact air which would cause lysis.

8. Place each filled SW50 tube into a Sarstedt 13-ml adapter tube containing ~0.5 ml water in the bottom. Pack eggs by centrifuging 30 sec at low speed, 150 × *g*, room temperature, in a clinical centrifuge, then 30 sec at 700 × *g*. Remove all the buffer above the eggs and discard any tubes with significant lysis.
9. Lyse eggs by centrifuging 15 min at ~16,000 × *g* (10,000 rpm in HB-4 rotor containing rubber adapters), 4°C. Remove tubes from adapters and place upright on ice.

Collect cytoplasm

10. Attach an 18-G needle to a 2-ml syringe. Move syringe plunger back and forth once to loosen before piercing tube. Wipe sides of tube with a tissue and then pierce the tube just above the bottom of the clear cytoplasmic layer. Make sure the opening of the needle faces upwards. Withdraw the clear straw-colored cytoplasm avoiding the yellow lipid layer above and the heavier layers containing yolk, mitochondria, and cortex fragments below. Remove the needle from the syringe and expel the cytosol from the syringe into an appropriate sized polyproponate tube with a cap.

    Bracing the centrifuge tube against the inside of an ice bucket will help avoid accidents during piercing.

    One 5-ml tube of eggs will yield 1 to 2 ml of cytosol.

11. Pool cytosols that look good. Add 1/1000 volume each of cytochalasin D (final 10 µg/ml), DTT (to 1 mM) and protease inhibitors.

12. If there is any contamination of the cytosol, then repeat the 16,000 × g centrifugation to clarify cytosol of any residual yolk or heavy cortex fragments. Remove clear cytosol as above and add 2.5 M sucrose to 125 mM to the cytosol.

    This helps separate the membranes out of the cytosol in the following centrifugation.

Isolate membrane and cytosol fractions

13. Ultracentrifuge cytosol for 60 min at 200,000 × *g* (555,000 rpm in SW55 rotor), 4°C.

    There will be the following fractions from top to bottom: lipid, clear cytosol, golden membranes, grayish mitochondrial membranes, then translucent pellet consisting of glycogen and ribosomes.

14. Carefully aspirate clear lipid layer and discard. Remove the clear cytosol to a separate tube using a 200-µl tip (do not use a 1000-µl tip).

    From a 5-ml tube one should collect ~2.5 ml. It is difficult to remove the clear cytosol without pulling up some of the golden membranes. Go slowly when near the membrane layer.

15. Remove and save golden membrane fractions (they are the nuclear assembly membranes) and follow with them at step 17. Carefully avoid the gray mitochondrial layer.

16. Recentrifuge cytosol 30 min at 200,000 × *g* (55,000 rpm in TLS55 rotor), 4°C, to remove residual membranes and lipid. Remove cytosol as in step 14. To cytosol, add glycerol to a final concentration of 3%. Snap freeze in liquid nitrogen in 50-µl aliquots. Store aliquots at −70°C.

    This cytosol fraction is called cytosol-200.

17. Resuspend the golden membranes in excess S-lysis-plus (≥10 vol) and centrifuge over a 0.5-ml cushion of S-lysis-plus/500 mM sucrose for 20 min at 26,000 × *g* (14,000 rpm in SW55 rotor), 4°C. Resuspend as a 10× stock in S-lysis-plus/500mM sucrose solution and freeze in 10-µl aliquots (10× stock means 1/10th the volume of cytosol).

    This membrane stock is called M-200.

PREPARATION OF HIGH-SPEED SUPERNATANT (HSS)

SUPPORT PROTOCOL 4

The cytosol used for the motility assay is clarified of all membranes by dilution of concentrated 16,000 × *g* cytosol followed by centrifugation. The concentrated cytosol is prepared, dispensed into aliquots, and stored frozen until the day of the experiment, when the dilution and centrifugation steps are performed.

Additional Materials (see Support Protocol 3)

ABC buffer (see recipe)
Table top ultracentrifuge and TLA-100 rotor or Airfuge (Beckman) and rotor

1. Perform Support Protocol 3, steps 1 through 11. However, in step 11 lower the sucrose concentration to 100 mM rather than 125 mM.
2. Pipet the cytosol into 50-µl aliquots and snap freeze in liquid nitrogen. Store aliquots at −80°C until use.
3. On the day of the assay, thaw an aliquot of cytosol and dilute with 2 vol ABC buffer. Centrifuge for 20 min at 100,000 × *g* (15 min, 30 psi in Beckman Airfuge or 550,000 rpm in TLA-100 rotor,), 4°C, to pellet membranes.
4. Pipet the cytosol to a fresh tube and maintain on ice until use (~4 hr max).

NUCLEAR ASSEMBLY USING DNA-COATED MAGNETIC BEADS AS TEMPLATE

SUPPORT PROTOCOL 5

This protocol describes the assembly of nuclei around DNA-coated magnetic beads. The protocol for binding of DNA to the beads is presented in *UNIT 11.13* (Heald et al., 1998). The nuclei can be assembled by simply adding DNA beads to fresh 16,000 × *g* cytosol (see Support Protocol 2, step 11). However, to perform manipulations of the nuclei to test specific components of the nucleus for a role in nuclear motility, use this alternate approach. Each batch of fractionated cytosol has to be titrated for nuclear assembly using DNA-magnetic beads. Given below is a starting protocol. One may have to vary the glycogen, amount of membranes, and/or dilution of cytosol with ABC buffer to get better assembly. Assembly using beads is much trickier than assembly with either sperm nuclei or lambda DNA. The beads must have a high concentration of DNA. Only one end of the DNA should be attached to the beads (i.e., fill in only one end of the DNA with biotinylated nucleotides), and the plasmid cannot be too short. (The MCP plasmid is ~8 kb and assembles nuclei much better than a 6-kb plasmid. Much shorter plasmids do not assemble at all.) Once the parameters for assembly with the extract preparation has been optimized, then scale up to make a nuclear prep for dispensing aliquots and freezing.

Materials

Cytosol-200 (see Support Protocol 2)
ABC buffer (see recipe)
DNA-Dynabeads (for preparation see *UNIT 11.13*, Heald et al., 1998)
PBS/1% (w/v) BSA (see recipe)
150 mg/ml glycogen stock (see recipe)
M-200 (see Support Protocol 2)
50 mM Mg-ATP (see recipe)
0.5 M creatine phosphate (see recipe)
8 mg/ml creatine kinase (see recipe)
1.4 mg/ml TRITC-BSA-NLS (transport substrate; *UNIT 11.7*, Support Protocol 2)
Fix solution (see recipe)

Beckman table top ultracentrifuge and TL100 rotor (or Beckman airfuge and rotor)
Magnetic particle concentrator

Microscope slides and clean coverslips
Nail polish
Microscope equipped with epifluorescence optics

1. Thaw one 50-µl aliquot of cytosol-200.
2. Add 50 µl ABC buffer and centrifuge for 30 min at 100,000 × *g* (55,000 rpm inTL100 rotor or 15 min, 30 psi in airfuge), 4°C.
3. During spin, prepare DNA beads. Use 10 µl beads (70 µg/ml) for a 100-µl reaction. Resuspend the DNA-bead stock well before pipetting. Pipet 10 µl beads to a clean tube. Add 200 µl PBS/1% BSA and pipet beads up and down several times. Retrieve beads on the magnetic particle concentrator, carefully remove the buffer and repeat washing step.
4. Resuspend washed beads in the diluted and centrifuged ceptosol (step 2, ~100 µl). Pipet thoroughly but carefully to disrupt any aggregates. Do not introduce air bubbles into the cytosol.

   Failure to disrupt aggregates results in large masses of beads that do not properly assemble into nuclei.

5. Add and mix by pipetting:

   10 µl 150 mg/ml glycogen
   10 µl membranes (M-200)
   2 µl 50 mM Mg-ATP
   2 µl 0.5 M creatine phosphate
   1 µl 8 mg/ml creatine kinase.

   Incubate 2 to 3 hr at 20°C.

   Fully formed nuclei are first apparent at 40 min and increase progressively until 3 hr.

6. To assay for functional nuclei, remove a 10-µl aliquot and add 0.5 µl of 1.4 mg/ml TRITC-BSA-NLS. Incubate 30 min at 4°C. Pipet 2 µl of this reaction onto a clean slide. Add 5 µl of fix solution and apply a clean coverslip. Seal the edges with nail polish if retention of the sample is desired. Observe nuclei with a microscope equipped with epifluorescence optics to detect nuclei that have accumulated the transport substrate (see Fig. 13.4.7 for images of nuclear uptake).

   Since Dynabeads are autofluorescent with emissions in the red wavelengths, a nuclear uptake substrate that will emit in the green wavelengths is often preferable, such as GFP or fluorescein-labeled. If desired, prepare FITC-BSA-NLS as in UNIT 11.7, Support Protocol 2, by substituting fluorescein-5-EX succinimidyl ester (Molecular Probes) for TRITC in steps 2 to 6 of the protocol. Follow manufacturer's recommendations for coupling buffer and pH conditions. The rest of the protocol remains the same. Alternatively, prepare GFP-GST-NLS (UNIT 11.7, Support Protocol 3). Use both as indicated here for TRITC-BSA-NLS.

7. When functional nuclei are observed, then scale up the reaction 10- to 20-fold and repeat all steps including the assay step. To freeze, add glycerol to 10% (v/v), dispense 10-µl aliquots, and freeze in liquid nitrogen. Store at −70°C.

   Nuclei can be stored for several years in this state. Frozen nuclei can be thawed on ice and washed in ABC buffer, retrieved on a magnet for several minutes (on ice) and resuspended in cytosol, or other buffers. Typically, they retain ~90% activity or higher after freezing. One 10-µl aliquot of nuclei is used for each motility assay.

   If the membranes are omitted from the reaction, interphase chromatin assembles onto the DNA, but no nuclear envelope forms. The motility of the chromatin can be compared with that of bona fide nuclei (Reinsch and Karsenti, 1997).

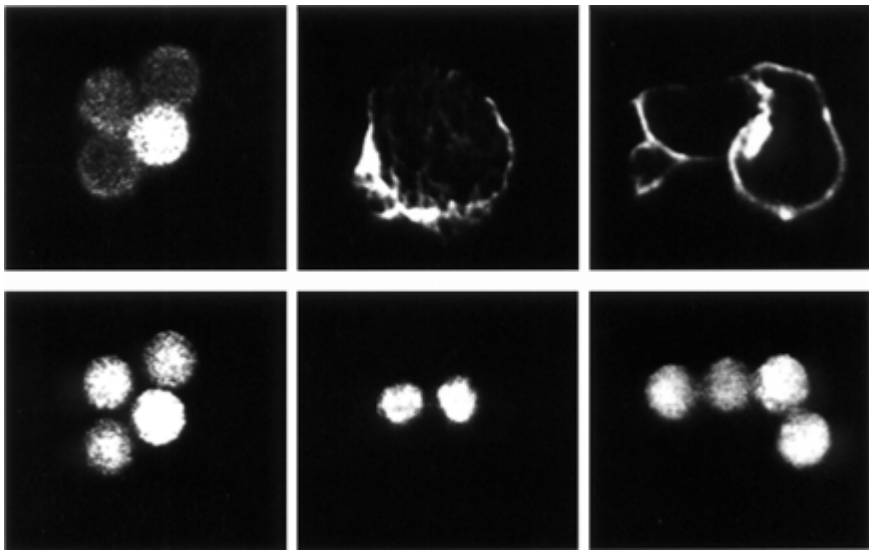


Figure 13.4.7 Nuclei assembled around magnetic DNA-beads are functional for nuclear transport and contain nuclear antigens. Confocal fluorescence images of synthetic nuclei assembled using the protocols described here. Autofluorescence of the beads is shown below (rhodamine filter), while the upper panels show the same beads in the fluorescein channel. FITC-BSA-NLS: typical fluorescence following uptake of the nuclear transport substrate FITC-BSA NLS in an unfixed sample. Staining for lamins and nuclear pores gives the typical peripheral staining pattern for these antigens.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

ABC (complete acetate buffer)

Acetate buffer (see recipe) supplemented with:
7.5 mM creatine phosphate (see recipe for 0.5 M)
1 mM MgATP
80 µg/ml creatine kinase (see recipe for 8 mg/ml)
10 mg/ml protease inhibitors
Store as 1- to 2-ml aliquots 1 year at −20°C
Thaw once and discard

Acetate buffer

To prepare 1 liter:
9.8 g potassium-acetate (100 mM final)
0.64 g magnesium-acetate (3 mM final)
1.9 g EGTA (5 mM final)
2.38 g HEPES (10 mM final, pH 7.4)
51.34 g sucrose (150 mM final)
H_2O to 950 ml
Adjust the pH to 7.4
H_2O to 1 liter
Aliquot in 50-ml tubes
Store 1 year at −20°C

Casein in PE

100 mg casein
Add 20 ml PE (see recipe)
Vortex vigorously
Incubate at 37°C with intermittent vortexing to aid solubilization of casein
Aliquot and store 1 year at −20°C

Creatine kinase, 8 mg/ml

8 mg creatine kinase
Add 475 µl distilled water
Add 20 µl 1 M HEPES-KOH, pH 7.5
Add 5 µl 1 M DTT
Add 500 µl glycerol
Snap freeze in 10-µl aliquots in liquid nitrogen
Store 1 year at −70°C

Creatine phosphate, 0.5 M

127.55 mg creatine phosphate
1 ml H_2O
Store 1 year at −20°C in 50-µl aliquots

This solution can be thawed and refrozen

Cytochalasin D, 10 mg/ml

10 mg of cytochalasin
1 ml DMSO
Store 1 year at −20°C in 50-µl aliquots

Dejellying solution (2% L-cysteine, pH 7.8)

20 g cysteine
H_2O to 970 ml
Adjust pH to 7.8 with 6 M NaOH
Prepare fresh just before use

DNaseI, 1 mg/ml

10 mg DNase I
10 ml H_2O
Store 1 week at 4°C

DTT, 1 M

0.77 g of DTT
5 ml H_2O
Keep on ice. Dissolve the DTT precipitate by moderate heating just before use.
Prepare fresh.

Fix solution

600 µl 80% glycerol
300 µl 37% formaldehyde
100 µl 10× MMR (see recipe)
0.5 µl 10 mg/ml Hoechst dye
Always prepare fresh on day of use

Glycogen, 150 mg/ml

750 mg glycogen
Dissolve in 5 ml acetate buffer (see recipe)
Aliquot and store 1 year at −20°C

Aliquots can be repeatedly thawed and refrozen

Gradient buffer

3.02 g PIPES (10 mM final)
H_2O to 950 ml
Add:
2 ml 0.5 M EDTA stock solution (1 mM final)
1 ml pure 2-β-mercaptoethanol (1% final)
10 ml of 10% Triton X-100 stock solution (0.1% final)
Bring to pH 7.2 with KOH
H_2O to 1 liter
Use immediately to prepare sucrose gradient solutions
Store remainder 1 year at −20°C

HCG, 500 U/ml

10,000 U human chorionic gonadotropin (Sigma)
Add 10 ml sterile distilled water
Keep sterile
Store at 4°C
Use within 10 days

HEPES/KOH, pH 7.5 (1 M)

59.6 g HEPES
200 ml H_2O
Adjust pH to 7.5 with concentrated KOH
H_2O to 250 ml
Filter sterilize
Store 1 year at −20°C in 5-ml aliquots

K-PIPES, pH 7.2, 0.5 M, 1 mM EDTA

7.55 g PIPES
40-ml H_2O
Add 0.1 ml 0.5 M EDTA stock solution
Bring pH to 7.2 with KOH
H_2O to 50 ml
Store 1 year at −20°C in 2-ml aliquots

Lysis buffer

0.5 ml Tris·HCl, pH 8.0, 1 M stock (*APPENDIX 2A*; 1 mM final)
490 ml H_2O
0.5 ml pure β-mercaptoethanol (0.1% v/v final)
2.5 ml pure NP-40 (0.5% v/v final)
51 µl 4.9 M $MgCl_2$ (Sigma; 0.5 mM final)
87.1 g PMSF
0.5 ml 10 mg/ml stock aprotinin
50 µl 10 mg/ml stock leupeptin
50 µl 10 mg/ml stock pepstatin
H_2O to 500 ml

This buffer can be freshly prepared or stored in aliquots at −20°C.

NOTE: *NP40 is chemically identical to IGEPAL CA-630 (Sigma).*

Mg-ATP, 50 mM

0.275 mg ATP
9 ml H_2O
0.1 g $MgCl_2$
Adjust pH to 7.5 with NaOH
H_2O to 10 ml
Store 1 year at −20°C as 500-µl aliquots

MMR, 10×

58.4 g NaCl (100 mM final)
1.49 g KCl (20 mM final)
2.04 ml of 4.9 M $MgCl_2$ stock solution (Sigma) (10 mM final)
2.94 g $CaCl_2$ (20 mM final)
372 mg EDTA (0.1 mM final)
11.9 g HEPES (50 mM final, pH 7.8)
900 ml H_2O
Adjust pH to 7.8 with 6 M NaOH
H_2O to 1 liter
Autoclave and store 6 months at room temperature

For MMR, dilute 10× stock 1:10 with water.

Nocodazole, 10 mM

To prepare 1 ml:
3 mg nocodazole
1 ml DMSO
Store 1 year at −20°C in 50-µl aliquots

PBS, 10×

14.2 g Na_2HPO_4 dissolved in 500 ml H_2O
2.67 g NaH_2PO_4 dissolved in 100 ml H_2O
Mix the 2 solutions
Add 90 g NaCl
H_2O to 1 liter
Check that solution is at pH 7.4
Store 1 year at −20°C

For PBS: Dilute 10× PBS 1:10 with water.

PBS/10 with 8% sucrose

10 ml 10× PBS (see recipe) diluted to 1 liter with H_2O (PBS/10)
80 g sucrose
Bring weight to 1 kg with PBS/10
Store 1 year at −20°C

PBS/1% (v/v) BSA

Weigh 500 mg BSA
Dissolve in 50 ml PBS (see recipe)
Filter and store 1 week at 4°C or aliquot and store 1 year at −20°C

PE buffer

To prepare 500 ml:
1.51 g K-PIPES (10 mM final; see recipe)
480 ml H_2O
Add 1 ml 0.5 M EDTA stock solution (final 1 mM)
Bring to pH 7.2 with KOH
H_2O to 500 ml
Filter
Store 3 months at 4°C

PMSG, 200 U/ml

Add 5 ml sterile water to vial containing 1000 U pregnant mare serum gonadotropin
Store 2 weeks at 4°C

Protease inhibitor stock (LPC, 10 mg/ml)

10 mg each of leupeptin, pepstatin, and chymostatin
Dissolve in 1 ml DMSO
Store 1 year at −20°C in 50-µl aliquots

S-lysis buffer

50 ml 2.5 M sucrose (250 mM final)
255 µl 4.9 M $MgCl_2$ (Sigma; 2.5 mM final)
12.5 ml 2M KCl (50 mM final)
5 ml 1 M HEPES-KOH, pH 7.5 (10 mM final)
H_2O to 500 ml
Adjust pH to 7.5 with KOH
Prepare fresh

S-lysis-plus buffer

150 ml S-lysis buffer (see recipe)
150 µl protease inhibitor stock (LPC; see recipe)
150 µl 1 M DTT (see recipe)
15 mg cycloheximide (final 100 µg/ml)
Stir until dissolved and store on ice until use

Wear gloves when handling cycloheximide.

S-lysis-plus/500 mM sucrose

3.4 g sucrose
S-lysis buffer (see recipe) to 20 ml
Incubate 37°C with intermittent vortexing until dissolved
20 µl protease inhibitor stock (LPC; see recipe)
20 µl 1 M DTT (see recipe)
20 mg cycloheximide
Store on ice until use

Wear gloves when handling cycloheximide.

Sucrose, 2.5 M (85% w/v)

85 g sucrose
Adjust to a total volume of 100 ml with H_2O
Allow overnight for dissolving (at 60°C)
Filter
Store 6 months at 4°C

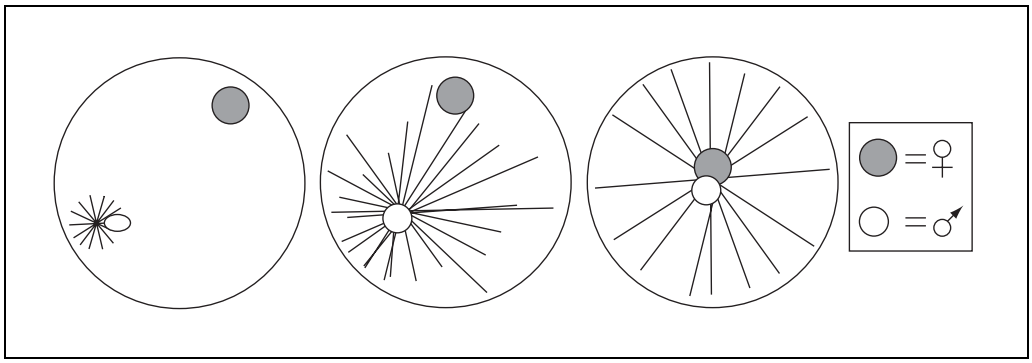


Figure 13.4.8 Movements of the male and female pronuclei in newly fertilized eggs. Schematic showing the microtubule-mediated positioning events of the male and female pronuclei in a typical metazoan early embryo. See text for details. Microtubules are represented by lines, and the male and female pronuclei by open and shaded circles respectively.

Sucrose in gradient buffer, 40% (w/w)

40 g sucrose
Gradient buffer (see recipe) to 100 g
Prepare fresh or store 6 months at −20°C

Sucrose in gradient buffer, 50% (w/w)

50 g sucrose
Gradient buffer (see recipe) to 100 g
Prepare fresh or store 6 months at −20°C

Sucrose in gradient buffer, 70% (w/w)

70 g sucrose
Gradient buffer (see recipe) to 100 g
Prepare fresh or store 6 months at −20°C

COMMENTARY

Background Information

The microtubule cytoskeleton plays a crucial role in positioning of the nucleus (reviewed in Reinsch and Gonczy, 1998). Nuclear migration processes occur in numerous cell types such as muscle, nerve, and somatic dividing cells. In newly fertilized eggs, long-range microtubule-dependent movements of the male and female pronuclei are essential for the two nuclei to meet and form the zygotic nucleus (Schatten, 1994).

During oogenesis in metazoan organisms, including *Xenopus*, the centrosome of the egg degenerates (Schatten, 1994). At fertilization, the basal body of the sperm flagellum converts to the centrosome by recruiting pericentriolar material from the egg cytosol, and nucleates a large microtubule aster. The male pronucleus (Fig. 13.4.8, open circle) remains at the center of the aster, which grows to fill the egg, thus transporting the male pronucleus to the center of the egg. The female pronucleus (Fig. 13.4.8, shaded circle) has no associated centrosome or microtubule-nucleating activity. Instead, the female pronucleus translocates along the microtubules of the sperm aster from the cell cortex to the centrosome located in the center of the aster. Thus, the two nuclei meet at the center of the aster prior to the first cleavage. Pronuclei of some species traverse hundreds of microns in a fraction of the first cell cycle (Stewart-Savage and Grey, 1982). The motility of the two nuclei differs in that the female pronucleus translocates along microtubules towards the microtubule minus-end while the movement of the entire sperm aster drives the movement of the male pronucleus.

The assay presented here recapitulates the movement of the female pronucleus in vivo and is the first system to demonstrate the translocation of nuclei along microtubules in vitro. Using this extract-based assay, it has been determined that cytoplasmic dynein is required for nuclear movements along microtubules (Reinsch and Karsenti, 1997). This system can be further used to determine how cytoplasmic

dynein is targeted to the nuclear membrane to mediate the organelle-like movement along microtubules.

Until the development of this assay, microtubule-dependent nuclear motility had only been studied in vivo because of the complexity of the process. Perturbation studies in vivo have used pharmacological agents to demonstrate the role of the microtubule cytoskeleton in pronuclear movement (Aronson, 1971; Schatten, 1982; Rouviere et al., 1994). Genetic studies in *C. elegans* have only very recently begun to address this process (Gonczy et al., 1999b). Recent experiments using RNA-mediated inhibition of expression in *C. elegans* embryos have confirmed the role of dynein and associated proteins in pronuclear migration (Gonczy et al., 1999a). These genetic experiments, thus, validate the biochemical approach presented here to demonstrate that dynein is required for the motility of synthetic nuclei along microtubules (Reinsch and Karsenti, 1997), and indicate that this approach is a valid means to identify candidate molecules involved in complex processes in the early embryo.

There were two important constraints on the choice of nuclei for the motility assay. First, it is important to use nuclei lacking an associated centrosome to resemble the female pronucleus. Nuclei purified from many tissues and from cells in culture generally have an associated centrosome whose activity could potentially generate motility events resembling those of the male rather than the female pronucleus (Reinsch and Gonczy, 1998). Second, it is important to choose a source of nuclei that would be competent for motility. Nuclei from many tissue sources may not be functional for motility if nuclear migration events are not occurring in the selected tissue at the time of purification.

Interphase *Xenopus* egg extracts spontaneously assemble nuclei from soluble and membrane components stored in the egg (Forbes et al., 1983; Newport, 1987; Newmeyer and Wilson, 1991). Upon the addition of DNA or chromatin to a lysed extract, nuclei containing double nuclear membranes, a nuclear lamina, and nuclear pores are quickly assembled (*UNIT 11.10*). These nuclei are capable of nuclear import, DNA replication, and normal mitotic disassembly. The most commonly used and convenient substrate for nuclear assembly studies is demembranated sperm nuclei. However, the sperm has an associated basal body that converts into a centrosome in the egg (or extract). Sperm nuclei are therefore not appropriate substrates for this assay. Recently, magnetic beads coupled to plasmid DNA have been demonstrated to function as artificial chromosomes capable of inducing nuclear assembly in interphase extracts and bipolar spindles in mitotic extracts (Heald et al., 1996; Reinsch and Karsenti, 1997; *UNIT 11.13*). Since DNA beads are paramagnetic, they can be simply retrieved from the extract. Centrosomes do not spontaneously assemble in these extracts and eggs contain no centrosomes. Therefore, the nuclei assembled around magnetic beads in this extract lack an associated centrosome. These synthetic nuclei provide a novel approach to studying nuclear motility since they allow rapid and gentle isolation of large numbers of nuclei in a motility competent state.

An alternative to magnetic bead nuclei, is to use DNA purified from phage lambda as a substrate for nuclear assembly. The DNA is simply added to a $16{,}000 \times g$ fresh extract and allowed to assemble (see Support Protocol 3, steps 1 through 11). However, the nuclei must then be purified over sucrose gradients (S. Reinsch, unpub. observ.). The advantage of the lambda DNA–nuclei is that they are much smaller than the DNA-bead nuclei used here and more suitable for certain analyses such as electron microscopy.

The benefits of using this extract-based assay system are numerous. This is an ideal system for the identification of the molecular components involved in nuclear motility as it is amenable to biochemical dissection of both nuclear and cytoplasmic components and allows high-resolution microscopic observation of nuclear motility.

This assay allows pronuclear migration to be studied in isolation of other simultaneous or upstream processes. Both microtubules and cytoplasmic dynein function in numerous processes that occur both prior to and after fertilization. By using this assay, the molecular components that are specific to this process as well as those which are shared with other processes can be identified. The ease of biochemical manipulation of these extracts allows experiments that are impossible in intact cells or organisms.

Nuclear motility can be observed using much cruder preparations from *Xenopus* eggs than those described here (S. Reinsch, unpub. observ.; Murray et al., 1996). However, this assay provides a means to define the minimal essential components required for nuclear motility along microtubules.

Critical Parameters and Troubleshooting

The system presented here uses largely homologous components. Only the centrosomes are purified from humans rather than frog. In theory, purified centrosomes from any source can be used. The use of homologous cytosol and nuclei is critical. Cytosol from other sources (e.g., bovine brain, HeLa cells, rat liver) does not support the movement of nuclei assembled *Xenopus* egg extracts (S. Reinsch, unpub. observ.). The use of heterologous nuclei has not been rigorously tested to determine whether heterologous nuclei will move on microtubules in *Xenopus* egg extracts. However, nuclei purified from other sources may undergo "repair" when incubated in *Xenopus* egg extracts and acquire components from the cytosol (Leno and Munshi, 1994) that may enable them to use the *Xenopus* cytosolic machinery for motility. It is conceivable then, that they will demonstrate motility events in this system. However, it is important to realize that nuclei purified from tissues or cultured cells may have associated centrosomes. Microtubules nucleated from these associated centrosomes may generate motility events in their own right that are independent of the translocation of nuclei along microtubules (reviewed in Reinsch and Gonczy, 1998).

As stated previously, nuclear motility per se can be visualized using less purified extract-based components. Freshly prepared low-speed (10,000 × *g*) extracts support both nuclear assembly and motility. Visualization of nuclear motility in this simpler system is a bit more challenging and requires the use of fluorescent components to label microtubules and nuclei. The assay presented here has been developed to allow for biochemical manipulations of both the cytosol and the nuclei to dissect the motility apparatus while simultaneously allowing visualization of motility events. It has also been optimized so that only frozen aliquotted components are used. This allows reproducibility from one assay to the next so that the effects of drugs or other added components can be determined.

For the motility assay, each component can present specific difficulties as outlined below. Once the assay has been optimized and is working well, then it is quite reproducible. To minimize difficulties, prepare larger stocks with aliquots that are the appropriate size for individual experiments. Never refreeze aliquots. Prepare a new stock (centrosomes, nuclei, HSS) well before the last aliquots are used. Try to switch out only one component at a time. Test the new batch of the component alongside the old batch to ensure that it functions properly.

Careful biochemical preparation of the individual components of the Basic Protocol is absolutely critical for the success of the quantitative motility assay. Several steps are critical in preparing extracts of *Xenopus* eggs. Please read the general references for handling *Xenopus* and extract preparation (Murray, 1991; Newmeyer and Wilson, 1991; *UNIT 11.10*). It is also worthwhile to understand the scientific literature on using *Xenopus* extracts for nuclear assembly (for review see Lohka, 1998 and *UNIT 11.10*). Eggs for preparation of nuclear assembly extracts and for HSS must be of top quality. Carefully read each support protocol and have all materials on hand before starting the preparations. Do not let the preparations sit on ice for long periods. Proceed immediately through the steps to the finished frozen product.

One of the most critical parameters in preparing fractionated extracts for nuclear assembly is to avoid contaminating the nuclear membranes with mitochondria. The mitochondria contribute to apoptotic events (Newmeyer et al., 1994; *UNIT 11.12*). The mitochondrial layer appears as grayish as opposed to the nuclear membranes, which have a golden tint. During the 200,000 × *g* centrifugation, sometimes the membranes do not band optimally and multiple bands are visible. Recentrifuge for a longer time to allow better separation. Too low a sucrose concentration in the extract can also contribute to this. The prep can be done on a smaller scale using a TLS-55 rotor instead of an SW-55 rotor.

For nuclear assembly using magnetic beads, the quality of DNA-beads is very important. Be sure to precisely follow the instructions in *UNIT 11.13* for bead preparation. By Hoechst staining, the DNA should make a uniform bright rim around the bead. There should not be a punctate pattern around the bead. Make sure to use long DNA fragments. An 8-kb fragment works very well. Shorter fragments do not assemble well into nuclei. The DNA must be filled in with biotin at only one end so that only one end attaches to the bead. This assures good chromatin assembly on the DNA bead. DNA-beads can be stored for months at 4°C. Do not freeze.

When assembling the bead-nuclei from fractionated extracts make sure that DNA-beads are well suspended in extract so that they don't clump too much. Otherwise all the nuclei will contain many beads. An average of 1 to 3 beads per nucleus is optimal for the motility assay. Glycogen is essential for assembly of

chromatin onto naked DNA (Hartl et al., 1994). The source of the glycogen is not important. Commercial oyster glycogen works quite well. The length of time for the nuclear assembly reaction is not critical and can be from 1 to 3 hr. The longer the nuclei are left in the assembly reaction, the larger they tend to get. They do not acquire more beads, but they will import proteins from the extract and become more swollen. This is not really a problem and often is helpful for visualizing uptake of the transport substrate TRITC-BSA-NLS. When performing the transport reaction, one should be able to clearly see uptake of nuclear transport substrate into 90% to 95% of nuclei. After assembly, nuclei can be stored overnight at 4°C without loss of function. Longer storage at this temperature is not advised. If one encounters difficulties in assembling nuclei from fractionated components, then try a different substrate, such as sperm nuclei (see *UNIT 11.13* for preparation) or lambda DNA to be convinced that the problem is not the DNA-bead substrate.

The centrosome preparation presented here has been optimized for high yield and high concentration of centrosomes. It is a variation of a published method (Moudjou and Bornens, 1998). Other preparations of centrosomes have not been tested for use in this assay, but would be interesting to compare (e.g., Blomberg-Wirschell and Doxsey, 1998). In theory, any centrosome preparation should be acceptable for use in this assay as long as robust microtubule asters are generated upon addition of HSS. For this particular preparation, it is essential that the lymphocytes be grown in exponential phase. The treatments with the cytoskeletal inhibitors, nocodozole and cytochalasin, separate the centrosome from the nuclear membrane so that centrosome and nucleus do not copurify. The incubation time for this treatment allows depolymerization of microtubules and actin filaments without causing cells to accumulate in mitosis. The washes with high osmotic strength buffers cause the cells to be very fragile. These steps should be done as rapidly as possible to minimize cell lysis. However, do not pipet so vigorously that bubbles are produced. The centrifugation steps are straightforward and can be adapted for other rotors and volumes. Do not leave the centrosomes unfrozen for long periods. Freeze the large aliquots as quickly as possible. Thaw only once to re-aliquot and freeze again. The size for the small aliquots should be dictated by experiment design. Do not refreeze the small aliquots.

Preparations of HSS can vary in activity largely due to egg quality. Cycloheximide is added to ensure that the extract stays in interphase. It is important to add sucrose as a cryoprotectant to the 16,000 × *g* extract before freezing. The prep is frozen as a 16,000 × *g* extract for several reasons. First, this takes up less freezer space, and the subsequent dilution and centrifugation steps to be done on the day of preparation are very reproducible. Second, preps that were carried through the subsequent dilution and centrifugation and then frozen as aliquots did not function as well in the motility assay. On the day of the motility assay, the dilution before high-speed centrifugation ensures that all of the membranes are sedimented. This allows optimal DIC imaging of nuclei without extraneous membranes. The dilution recommended here has been optimized for microtubule aster assembly. Too low a dilution causes a lot of spontaneous microtubule assembly. Sometimes asters generated in HSS become fragmented before the 20-min time point. Throw these batches of cytosol away since they will consistently give the same result, and prepare a new batch of 16,000 × *g* extract. These poor-quality batches often also show high apoptotic activity. Try keeping egg batches from different frogs separate all the way through the preparation if poor quality cytosol is a problem.

The quantitative assay for nuclear motility requires nuclei to accumulate at centrosomes. Therefore, robust aster morphology with nice spacing between the asters is crucial. Human lymphocytes assemble asters with only a few microtubules in the presence of pure tubulin. Incubating centrosomes in HSS allows pericentriolar material to be recruited to the centrosomes. After incubation in *Xenopus* egg extract, their nucleation capacity increases significantly (Buendia et al., 1992) so that robust microtubule asters are generated. Plating density of the centrosomes can affect the outcome of the assay. Steps for optimizing this parameter are outlined in Support Protocol 3. The asters should be spaced so that there are not more than 1 to 3 centrosomes/50-μm field. This allows nuclei to reach centrosomes so that quantitation is possible. If asters are too widely spaced, then there will be a lot of free microtubule nucleation and random movement of the nuclei rather than nuclei moving to and accumulating at centrosomes. Make sure the incubation to allow centrosomes to attach to the glass coverslip is consistently 5 min. Several blocking agents have been tested; casein gives satisfactory re-

sults to prevent nuclei from sticking. The casein also inhibits dynein present in HSS from binding to the coverslip. This decreases the amount of microtubule gliding that occurs.

If robust, well-spaced asters are not obtained, the problems can either be in the density of the centrosomes or more likely in the preparation of HSS as described above. Adding agents such as drugs and antibodies can also seriously affect the ability of asters to be nucleated and should only be added after the nucleation step. For example, addition of agents that inhibit dynein activity cause random microtubules to assemble, rather than preferential assembly off centrosomes (S. Reinsch, unpub. observ.). If one does not plan to use the motility assay as a quantitative assay, but only for visualizing nuclear movements without perturbing agents, then the nucleation step can be completely omitted. Many agents also compromise microtubule assembly. For example, BAPTA, a calcium chelator, completely inhibits microtubule assembly in this assay (S. Reinsch, unpub. observ.). Since the microtubules are dynamic in this assay, any factor that affects microtubule dynamics may compromise the success of the assay.

Anticipated Results

The nuclear motility assay allows clear documentation of nuclear motility events. When bead-nuclei are purified on the magnet, they do come with a considerable amount of associated endoplasmic reticulum (ER). Therefore, both nuclear movements and ER tubule elongation will be visible. In HSS, the nuclei move exclusively towards the minus-ends of the microtubules, that is, towards the centrosome. The average rate of movement of nuclei assembled in *Xenopus* extracts is 1 μm/sec with a range from 0.2 to 1.8 μm/sec. Neither DNA-beads (unassembled) nor chromatin-beads (assembled in cytosol without adding membranes) will move to the microtubule minus-ends. In the presence of inhibitors of dynein (e.g., >15 μM vanadate) minus-end directed movements are blocked. The nuclei then are moved to the aster periphery due to the action of growing microtubules. This is not plus-end directed movement. Instead nuclei appear to be pulled out by growing microtubules due to ER tubule attachment to the tips of growing microtubules (Waterman-Storer et al., 1995; Reinsch and Karsenti, 1997).

For the quantitative assay, 90% to 95% of the nuclei should accumulate at centrosomes by the 20-min time point under control conditions. One should be able to easily count between 50 and 100 nuclei in the 10-min counting interval. Dynein inhibitors significantly inhibit accumulation at centrosomes (see Fig. 13.4.3; Reinsch and Karsenti, 1997). An antibody against the intermediate chain of cytoplasmic dynein (mAb 70.1, Sigma) also inhibits nuclear motility and accumulation of nuclei at centrosomes when directly added to HSS (Reinsch and Karsenti, 1997). Not all antibodies against cytoplasmic dynein successfully inhibit nuclear motility (S. Reinsch, unpub. observ.).

Each assay should be performed at least three times to generate statistically significant data. Once a control reaction has been performed on a given batch of centrosomes/nuclei/HSS, the control reaction should be consistent from day to day and should always give 90% to 95% accumulation. This includes adding buffer to account for added experimental agents. Therefore, on a given experimental day, the control reaction can be performed once or possibly twice, to confirm that the system looks the same as on the previous experimental day, rather than having to perform multiple control experiments on each day an experiment is performed. This gives more time for experimental manipulation (see below).

The HSS should contain no membraneous materials. When incubated at room temperature, microtubule assembly should be visible within several minutes using VE-DIC optics. In the absence of centrosomes, only random microtubule assembly occurs. In the presence of centrosomes, microtubules first assemble randomly, but over the 10-min nucleation period, the centrosomes acquire pericentriolar material and nucleate more microtubules. By 10 min almost all microtubules are nucleated by centrosomes. Microtubule dynamics do occur and by VE-DIC both growing and shrinking microtubules should be visible.

The typical yield for this centrosome preparation is 30% to 50%. Therefore, a 2-liter preparation of lymphocytes (3×10^9 cells) generally yields ~10^9 centrosomes with several fractions containing centrosomes at or above the optimal concentration (~2×10^8/ml). A single centrosome preparation generates enough centrosomes for at least 1000 motility assays.

Eggs from one frog (5 ml dejellied eggs) generally yields 1 ml of 16,000 × *g* cytosol. Each milliliter of cytosol generates ~3 ml of HSS. Each assay uses 20 μl of HSS. Therefore, one frog yields enough HSS for ~150 assays.

Eggs from one frog (5 ml dejellied eggs) will generally yield ~300 μl of cytosol-200 (and

membranes). This will make a ~700-µl nuclear assembly reaction, which is aliquotted by 10 µl at the end. Each 10-µl aliquot contains nuclei for one motility assay. Therefore, eggs from one frog generate nuclei for ~70 motility assays.

Time Considerations

Once all of the support protocols have been followed to generate the components of the motility assay and the learning period has been overcome, then each motility assay takes ~40 min to perform. One to 2 hr of preparation time is required before each assay period. Therefore, only a few experimental samples can be compared on 1 day if assays are performed in triplicate with appropriate buffer controls.

The centrosome preparation requires minimal time for 10 to 14 days to grow the lymphocytes, 1 day to prepare the buffers, and 1 day to do the actual preparation. Titration of the centrosomes in the motility assay requires another full day, but should only be done once all the other components have been prepared.

Preparation of fractionated extracts for nuclear assembly requires 1 full day. Frogs must be primed 4 to 10 days in advance with PMSG and the night before the prep with HCG.

For time considerations in preparing magnetic DNA-beads, see *UNIT 11.13*. Working out the conditions for optimal nuclear assembly using DNA beads and fractionated extracts can optimistically be done in an afternoon, but often can take several tries. Actual preparative scale nuclear assembly takes half a day.

Preparation of 16,000 × *g* extracts for generation of HSS takes half a day once frogs are primed as above.

Preparation of slide chambers for motility assays takes parts of a day, during which many other activities can be undertaken simultaneously.

Time considerations for familiarization with VE-DIC and optimization for visualization of microtubules are beyond the scope of this protocol. Please refer to *UNITS 4.1 & 13.1* and to Salmon and Tran (1998) and Walker et al. (1988).

Literature Cited

Aronson, J.F. 1971. Demonstration of a colcemid-sensitive attractive force acting between the nucleus and a center. *J. Cell Biol.* 51:579-583.

Blomberg-Wirschell, M. and Doxsey, S.J. 1998. Rapid isolation of centrosomes. *Methods Enzymol.* 298:228-238.

Bornens, M., Paintrand, M., Berges, J., Marty, M.C., and Karsenti, E. 1987. Structural and chemical characterization of isolated centrosomes. *Cell Motil. Cytoskeleton* 8:238-249.

Buendia, B., Bre, M.H., Griffiths, G., and Karsenti, E. 1990. Cytoskeletal control of centrioles movement during the establishment of polarity in Madin-Darby canine kidney cells. *J. Cell Biol.* 110:1123-1135.

Buendia, B., Draetta, G., and Karsenti, E. 1992. Regulation of the microtubule nucleating activity of centrosomes in *Xenopus* egg extracts: Role of cyclin A-associated protein kinase. *J. Cell Biol.* 116:1431-1442.

Euteneuer, U. and Schliwa, M. 1985. Evidence for an involvement of actin in the positioning and motility of centrosomes. *J. Cell Biol.* 101:96-103.

Evans, L., Mitchison, T., and Kirschner, M. 1985. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* 100:1185-1191.

Forbes, D.J., Kirschner, M.W., and Newport, J.W. 1983. Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into *Xenopus* eggs. *Cell* 34:13-23.

Gant, T.M. and Wilson, K.L. 1997. Nuclear assembly. *Annu. Rev. Cell. Dev. Biol.* 13:669-695.

Gonczy, P., Pichler, S., Kirkham, M., and Hyman, A.A. 1999a. Cytoplasmic dynein is required for distinct aspects of MTOC positioning, including centrosome separation, in the one cell stage *Caenorhabditis elegans* embryo. *J. Cell Biol.* 147:135-150.

Gonczy, P., Schnabel, H., Kaletta, T., Amores, A.D., Hyman, T., and Schnabel, R. 1999b. Dissection of cell division processes in the one cell stage *Caenorhabditis elegans* embryo by mutational analysis. *J. Cell Biol.* 144:927-946.

Hartl, P., Olson, E., Dang, T., and Forbes, D.J. 1994. Nuclear assembly with lambda DNA in fractionated *Xenopus* egg extracts: An unexpected role for glycogen in formation of a higher-order chromatin intermediate. *J. Cell Biol.* 124:235-248.

Heald, R., Tournebize, R., Blank, T., Sandaltzopoulos, R., Becker, P., Hyman H., and Karsenti, E. 1996. Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts [see comments]. *Nature* 382:420-425.

Heald, R., Tournebize, R., Vernos, I., Murray, A., Hyman, T., and Karsenti, E. 1998. In vitro assays for mitotic spindle assembly and function. *In* Cell Biology. A Laboratory Handbook, Vol. 2. (J.E. Celis, ed.) pp. 326-335. Academic Press, San Diego.

Leno, G.H. and Munshi, R. 1994. Initiation of DNA replication in nuclei from quiescent cells requires permeabilization of the nuclear membrane. *J. Cell Biol.* 127:5-14.

Lohka, M.J. 1998. Analysis of nuclear envelope assembly using extracts of *Xenopus* eggs. *Methods Cell Biol.* 53:367-395.

Mack, G. and Rattner, J.B. 1993. Centrosome repositioning immediately following karyokinesis and prior to cytokinesis. *Cell Motil. Cytoskeleton* 26:239-247.

Merdes, A., Ramyar, K., Vechio, J.D., and Cleveland, D.W. 1996. A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell* 87:447-458.

Moudjou, M. and Bornens, M. 1998. Method of centrosome isolation from cultured animal cells. *In* Cell Biology: A Laboratory Handbook, Vol. 2. (J. Celis, ed.) pp. 111-119. Academic Press, San Diego.

Murray, A.W. 1991. Cell cycle extracts. *In Xenopus laevis*: Practical Uses in Cell and Molecular Biology, Vol. 36, Methods in Cell Biology (B.K. Kay and H.B. Peng, eds.) pp. 581-604. Academic Press, San Diego.

Murray, A.W., Desai, A.B., and Salmon, E.D. 1996. Real time observation of anaphase in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 93:12327-12332.

Newmeyer, D.D. and Wilson, K.L. 1991. Egg extracts for nuclear import and nuclear assembly reactions. *In Xenopus laevis*: Practical Uses in Cell and Molecular Biology, Vol. 36, Methods in Cell Biology (B.K. Kay and H.B. Peng, eds.) pp. 607-634. Academic Press, San Diego.

Newmeyer, D.D., Farschon, D.M., and Reed, J.C. 1994. Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 79:353-364.

Newport, J. 1987. Nuclear reconstitution in vitro: Stages of assembly around protein-free DNA. *Cell* 48:205-217.

Reinsch, S. and Gonczy, P. 1998. Mechanisms of nuclear positioning. *J. Cell Sci.* 111:2283-2295.

Reinsch, S. and Karsenti, E. 1997. Movement of nuclei along microtubules in *Xenopus* egg extracts. *Curr. Biol.* 7:211-214.

Rouviere, C., Houliston, E., Carre, D., Chang, P., and Sardet, C. 1994. Characteristics of pronuclear migration in *Beroe ovata*. *Cell Motil. Cytoskeleton* 29:301-311.

Salmon, E.D. and Tran, P. 1998. High resolution video enhanced differential interference contrast microscopy. *Methods Cell Biol.* 56:153-184.

Schatten, G. 1982. Motility during fertilization. *Int. Rev. Cytol.* 79:59-163.

Schatten, G. 1994. The centrosome and its mode of inheritance: The reduction of the centrosome during gametogenesis and its restoration during fertilization. *Dev. Biol.* 165:299-335.

Stewart-Savage, J. and Grey, R.D. 1982. The temporal and spatial relationships between cortical contraction, sperm trail formation, and pronuclear migration in fertilized *Xenopus* eggs. *Wilhelm Roux's Arch* 191:241-245.

Tournebize, R., Popov, A., Kinoshita, K., Ashford, A.J., Rybina, S., Poznikovsky, A., Mayer, T.U., Walczak, C.E., Karsenti, E., and Hyaman, A.A. 2000. Control of microtubule dynamics by the antagonistic activities of XMAP215 and XKCM1 in *Xenopus* egg extracts. *Nat. Cell Biol.* 2:13-19.

Walczak, C.E., Mitchison, T.J., and Desai, A. 1996. XKCM1: A *Xenopus* kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. *Cell* 84:37-47.

Walker, R.A., O'Brien, E.T., Pryer, N.K., Soboeiro, M.F., Voter, W.A., Erickson, H.P., and Salmon, E.D. 1988. Dynamic instability of individual microtubules analyzed by video light microscopy: Rate constants and transition frequencies. *J. Cell Biol.* 107:1437-1448.

Waterman-Storer, C.M., Gregory, J., Parsons, S.F., and Salmon, E.D. 1995. Membrane/microtubule tip attachment complexes (TACs) allow the assembly dynamics of plus ends to push and pull membranes into tubulovesicular networks in interphase *Xenopus* egg extracts. *J. Cell Biol.* 130:1161-1169.

Key References

Reinsch and Gonczy, 1998. See above.

This review describes the different mechanisms that drive microtubule-mediated nuclear motility events in different cell types and different organisms. Nuclear motility following fertilization is particularly emphasized.

Reinsch and Karsenti, 1997. See above.

This work demonstrates that nuclei can translocate along microtubules in Xenopus egg extracts similar to other organelles, and that cytoplasmic dynein cytoplasmic dynein drives the translocation of nuclei along microtubules.

Internet Resources

http://current-biology.com/supmat/cub/bb7325s1.mov

http://current-biology.com/supmat/cub/bb7325s2.mov

http://current-biology.com/supmat/cub/bb7325s3.mov

These movies are supplemental material for Reinsch and Karsenti (1997). They are videos of nuclei moving along microtubule asters as described in the Basic Protocol. The nuclei used in these movies were assembled as described in Support Protocols 3 and 5.

http://www.indiana.edu/~elegans/

This is the webpage for the laboratory of Susan Strome at Indiana University. Movies within this site show pronuclear migration in vivo in the nematode C. elegans.

Contributed by Sigrid Reinsch
NASA-Ames Research Center
Moffett Field, California

Measuring Dynamics of Nuclear Proteins by Photobleaching

Photobleaching techniques offer the possibility of obtaining information on molecular motion and interactions in a specific part of a cell (McNally and Smith, 2002). The three main advantages of photobleaching techniques are fast experimental turn around, good spatial and temporal resolution, and the ability to measure kinetics inside of living cells. The main disadvantage of these techniques is the requirement for fluorescently tagged proteins (in this case by creating a gene fusion with GFP). Alternatively, other genetically engineered fluorescent tags may be used for a fusion with the protein of interest—e.g., cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). Also, in some cases, addition of this moiety can functionally alter the protein. Thus, prior to proceeding with photobleaching studies, the fusion protein must be rigorously tested to ensure it has the same properties and function as its native counterpart.

Ideally, a fusion protein is tested in a functional in vivo assay. The best is a functional complementation assay, for example, in a knock-out cell line or yeast. However, for many mammalian proteins such assays are not readily available. A minimal requirement for testing GFP-fusion proteins is their stability and proper localization in cells. The stability of a GFP-fusion protein can be tested by immunoblot (*UNIT 6.2*), using either a specific antibody against the endogenous protein of interest or an antibody against GFP. An immunoblot ensures that the protein is of the expected molecular weight and that it is not degraded upon expression. Furthermore, the GFP-fusion protein must also colocalize with its endogenous counterpart. If the immunoblot shows degradation products of the protein chimera or the protein is mislocalized, the GFP-fusion protein is likely not fully functional. In this case, it is worth trying to introduce the GFP tag at the opposite end of the protein molecule. In some cases, the introduction of a longer linker between the protein and GFP is also helpful.

Three major methods of photobleaching microscopy are commonly used: fluorescence recovery after photobleaching (FRAP; see Basic Protocol), fluorescence loss in photobleaching (FLIP; see Alternate Protocol 1), and inverse fluorescence recovery after photobleaching (iFRAP; see Alternate Protocol 2). As summarized in Table 13.5.1, each of these techniques has specific characteristics permitting the determination of distinct particular parameters of protein behavior in vivo. In addition, transfection methods are given for introducing GFP-expression vectors into mammalian cells by electroporation (see Support Protocol 1) or by lipofection (see Support Protocol 2). Finally, since quantification of GFP-fusion proteins is critical, a method for the determination of the number of GFP molecules in a single, living cell is also described (see Support Protocol 3).

NOTE: The reader is referred to *UNIT 4.5* for a general discussion of confocal microscopy.

Table 13.5.1 Comparison of Photobleaching Methods for Detecting Movement of Nuclear Proteins[a]

	Mobility	Diffusion constant	Immobile fraction	Distinct pools	Compartment continuity
FRAP	+++	+++	+++	+	+
FLIP	+++	+	+	+++	+++
iFRAP	−	−	+	+++	−

[a] +++ optimal, + suitable, −not suitable.

Contributed by Miroslav Dundr and Tom Misteli
Current Protocols in Cell Biology (2003) 13.5.1-13.5.18

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP)

In a FRAP experiment, an intense focused laser beam of the appropriate wavelength bleaches a relatively small region of interest. The laser light irreversibly bleaches the fluorescent signal of molecules in the region of interest and, immediately following bleaching, the recovery of the fluorescence signal in the bleached area is monitored using an attenuated laser beam. The signal in the region of interest is measured and a fluorescence recovery curve $F_{rec}(t)$ (fluorescence intensity as a function of time after photobleaching) is generated (Fig. 13.5.1). Acquisition of a FRAP data set involves three phases: prebleach images, bleach pulse, and postbleach monitoring of fluorescence recovery.

FRAP experiments are most easily performed on any modern confocal microscope (e.g., Zeiss 510 and newer, Leica SP and newer), as these microscopes have integrated FRAP routines. Alternatively, it is possible to custom write macros for many older microscopes that will automatically perform FRAP routines (Ellenberg et al., 1998). This protocol, as well as its alternatives (see Alternate Protocols 1 and 2), are designed for the Zeiss LSM

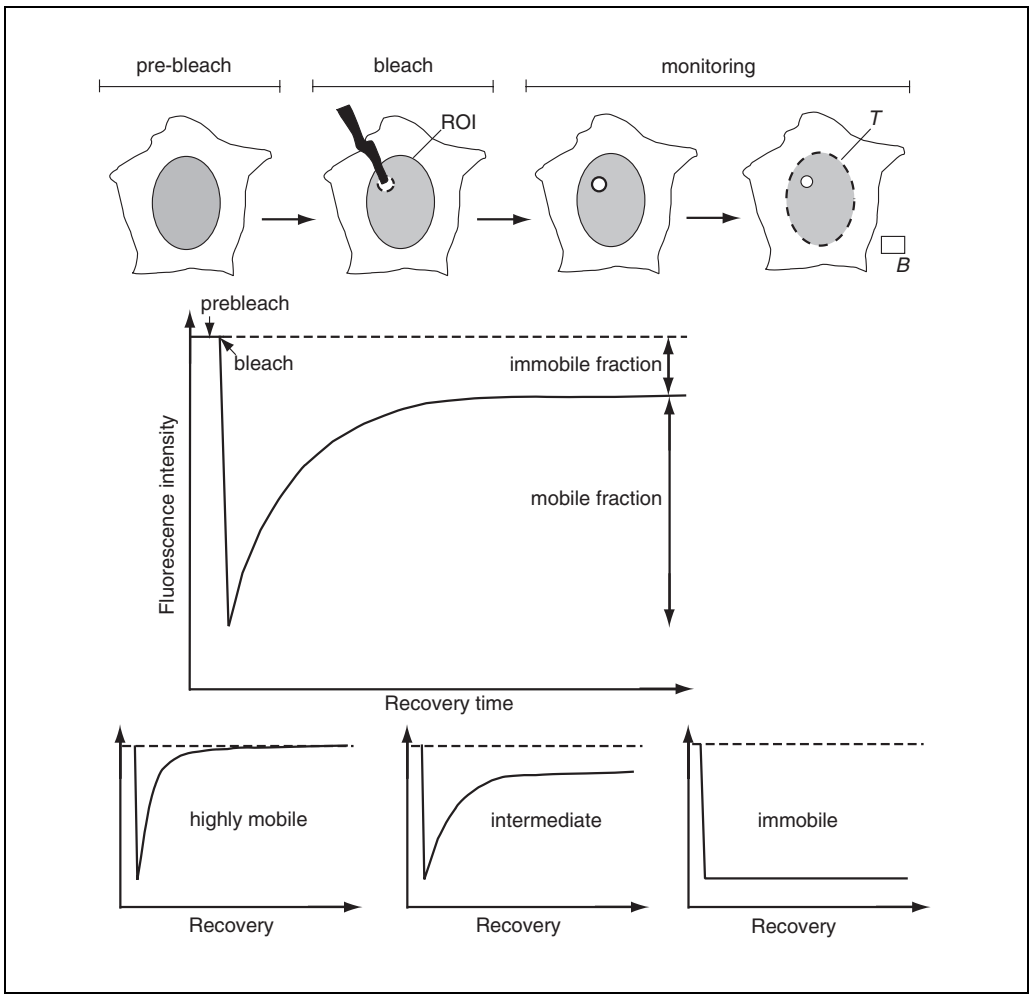


Figure 13.5.1 General scheme of a FRAP experiment. A cell is imaged before a short bleach pulse is applied to a defined region of interest (ROI). The recovery of the average fluorescence signal in the ROI is then monitored. To quantify and normalize FRAP data, the average fluorescence signal in the ROI, average total nuclear signal (*T*), and average signal in a random area outside of the cell for background correction (*B*) are measured during the entire experiment. The FRAP curve shows the prebleach intensity, depth of bleach, and recovery of the signal. The immobile fraction is determined as the difference between the prebleach signal and the signal after complete recovery. Based on different profiles of recovery curves, proteins can be considered highly mobile with virtually no immobile fraction, intermediately mobility with an immobile fraction, or immobile.

510 confocal microscope, but similar protocols can be applied to other confocal microscopes.

When FRAP experiments are carried out on confocal microscopes, the fluorescence recovery in the region of interest can be considered largely the result of two-dimensional exchange of fluorescent protein molecules within the same focal plane as the bleached area. This condition is approximately valid when objectives with high-numerical-aperture (N.A. of 1.2-1.45) lenses are used; a large cone of out-of-focus light bleaches the fluorescent molecules above and below the focal plane so that only nonbleached molecules from the focal plane participate in the recovery in the region of interest (Ellenberg et al., 1998).

There are several essential conditions that must be fulfilled for a successful FRAP experiment. (1) The fluorescent signal to be bleached must be clearly detectable over any background signal. (2) The photobleaching must be fast relative to the period of recovery to provide sufficient temporal resolution for analysis of the recovery curve and to allow measurement of the half-time of recovery. (3) The monitoring beam must be of low intensity to minimize photobleaching. To obtain a sufficiently high signal during the recovery period, the sensitivity of the photomultiplier tube may be increased.

Materials

2- or 4-chambered Lab-Tek II coverglass (Nalgene Nunc) containing cells, 50% to 70% confluent

Complete normal growth medium with 25 mM HEPES, but without phenol red (*UNIT 1.2*)

Confocal microscope

Air stream incubator (Nevtek)

1. On the stage of a confocal microscope, place a 2- or 4-chambered Lab-Tek II coverglass containing cells in complete normal growth medium with 25 mM HEPES, but without phenol red. Maintain growth temperature with a Nevtek air incubator. Examine the cells with fluorescent light of the appropriate wavelength.

2. Choose the appropriate lens (at least 63× magnification) and zoom setting to clearly observe the region of interest.

   If the same zoom is used for all observed cells, one can easily compare individual data sets without correction for bleach-area size.

3. Image a cell of interest using a low level of laser power to prevent photobleaching.

   On the Zeiss LSM 510 microscope, 20% to 50% output from a 40-mW laser with the beam attenuated to 0.1% is routinely used for the image acquisition.

4. Choose a small pinhole diameter.

   The authors recommend using a pinhole diameter in the range of 1 Airy unit (minimal resolvable distance which corresponds to the diameter of the peak of the bright spot of the point source of light; UNIT 4.1). A larger pinhole diameter improves the signal intensity, but increases the optical thickness of the collected section. This could affect monitoring of the signal in the region of interest by collecting data from a thicker optical section including unbleached fluorescent molecules. When a smaller pinhole diameter is used, the monitoring of signal recovery is restricted to the optical section most completely bleached. Therefore, it is advisable to use the smallest pinhole diameter which gives a sufficient signal-to-noise ratio.

Setup imaging sequence

5. Adjust the intensity of fluorescence signal to slightly below the saturation level by adjusting the gain on the photomultiplier tube.

   Use at least 8- but preferably 12-bit imaging. It is important to ensure that no pixels are saturated since the change in fluorescence intensity in these pixels cannot be measured accurately.

6. Adjust laser power to 100% for bleaching.

   The bleach time should be minimized. The efficiency of the bleach should be tested on a fixed sample. The average intensity in a bleached spot in a fixed sample should be reduced by at least 70% compared to its surroundings. If the bleach depth is insufficient, use several, rapid, consecutive bleach pulses. If the bleach spot in a living cell is distinct from the bleach spot in a fixed cell, do not increase the bleach time or intensity. The discrepancy is an important indication that the observed protein contains a very rapidly moving fraction. These highly mobile molecules enter the bleached spot between the time the bleach pulse is terminated and the acquisition of the first image. Recovery of freely mobile proteins in the nucleus can occur on the time scale of ≤50 msec.

7. Adjust the number of prebleach images for five to ten images and acquire five to ten prebleach images.

   It is important to acquire more than one prebleach image, as many fluorescent molecules are disproportionately bleached when first excited.

8. Adjust the number of postbleach intervals and the time between images.

   The number of intervals is determined by the resolution needed. The length of intervals should cover the curve until it reaches a plateau. Be careful not to collect an unnecessarily high number of images as this will bleach the sample.

9. Define the region to be bleached.

   The size of the bleach region is critical for quantitative analysis of FRAP recovery. The authors recommend using a small circular spot as the bleach region over the desired region of interest in the nucleus. The size of the bleach region should be small compared to the size of the nucleus. The advantage of a circular bleach spot is its symmetry, which permits finding it even when the cell moves. To check the dimensions of the bleach region, it is necessary to fix the sample, bleach the region of interest, and collect a series of optical sections through the cell. When the bleached region is monitored for signal recovery, the same size area or slightly smaller can be used for measurement of signal recovery. Using a larger monitoring area allows one to obtain the recovery data even if the bleached area moved slightly during monitoring; however, the relatively unchanged fluorescence intensity in the neighborhood of the bleached region reduces the sensitivity of the measurement.

10. Initiate data collection (i.e., prebleach images, bleach, postbleach images) and monitor the recovery of the fluorescence signal. Check the position of the cell and the focal plane to ensure that the region of interest is not moving from its original position due to focal-plane movement.

    It is essential to determine that recovery is complete—i.e., the curve should reach a clear plateau. The shape of a recovery curve should resemble the one in Figure 13.5.1. If the recovery is not complete, it is essential to increase the monitoring time of recovery by increasing the number of acquired images during monitoring or the time between images (step 8).

Perform quantitative analysis of FRAP

11. Measure the average intensity of the region of interest at each time point (I_t).

    Most confocal microscope software packages allow the researcher to perform this measurement on a whole stack of time-lapse data.

12. To correct for signal bleaching during monitoring, measure the average intensity of the entire nucleus at each time point (T_t).

13. Measure the background intensity (B_t) in a randomly selected region outside of the cell at each time point.

14. Calculate the relative intensity for each time point:

$$I_{rel} = (T_0 - B_0) \times (I_{(t)} - B_{(t)}) / (T_{(t)} - B_{(t)}) \times (I_0 - B_0)$$

where T_0 is the average intensity of the entire nucleus during prebleach and I_0 is the average intensity of the region of interest during prebleach (step 6).

This formula is derived in three steps. First, background is subtracted from all measured values, then the fluorescence loss due to monitor bleaching is normalized, and finally fluorescence intensity is normalized to one.

15. Plot I_{rel} as function of time.

For convenient calculation, it is useful to import FRAP data sheets from the microscope software to a spread sheet program such as Microsoft Excel.

16. Evaluate the individual curves and determine the average and standard deviations for each time point.

It is advisable to plot all acquired curves before determining the average. Jumps in the recovery curves or declining curves are frequently caused by cell or focal plane movement. These curves can either be discarded from analysis, or if possible, they should be manually remeasured. For many applications, it is sufficient to collect data for 10 to 20 individual cells. Typical error bars are on the order of 5% to 10% of the measured value.

Alternative normalization procedures have been published. For example, some investigators assign the value 1 to the prebleach intensity and the value 0 to the postbleach image in the bleached spot. (Kruhlak et al., 2000). This method is only appropriate for relatively slow-moving molecules where the signal in the bleached region is reduced by ≥75%. For fast moving molecules where the reduction of signal might only be ≤50%, this normalization method overestimates the recovery kinetics.

In the case of FRAP analyses of proteins which are enriched in small nuclear compartments such as the nucleolus or dot-like nuclear bodies and present at only low concentrations in the surrounding nucleoplasm, the relative fluorescent intensity in the bleached nuclear compartment can be normalized against the nonbleached nuclear compartment in the same nucleus (Chen and Huang, 2001). For each time point, the relative intensity is determined by the formula $I_{rel} = (Ne_tN1_0)/(Ne_0N1_t)$, where Ne_t and Nl_t are the average intensities of the bleached compartment and control nonbleached compartment at each time point, respectively, and Ne_0 and Nl_0 are the average intensities of the bleached compartment and control nonbleached compartment in the same nucleus during prebleach, respectively.

Determine the immobile fraction and diffusion constant

17. Calculate the immobile fraction of fluorescent molecules as the difference between the relative fluorescence intensity in the region of interest after the recovery curve has reached plateau, and the prebleach fluorescence signal intensity (normalized as 100%; Fig. 13.5.1).

Failure to recover to 100% prebleach fluorescence signal intensity even for a protein without immobile fraction such as GFP alone may represent the loss of total nuclear fluorescence due to bleaching during monitoring.

18. Determine the diffusion constant.

The mobility of a protein is characterized by its diffusion constant (D). FRAP experiments allow the determination of D assuming lateral diffusion in the focal plane (Axelrod et al.,

1976); however, it is essential to understand that the measured diffusion constant is only an apparent diffusion constant. The overall mobility of a protein is not only determined by its diffusional properties, but more significantly, by its functional properties such as incorporation into multiprotein complexes and binding to relatively immobile structures such as chromatin or a karyoskeleton (Misteli, 2001). Therefore, although a diffusion constant is a convenient method to compare the recovery kinetics of various proteins, one must be cautious in interpreting diffusion constants as a true indicator of the translational mobility of a protein. For discussion of diffusion constants see Lippincott-Schwartz et al. (2001). For determination of diffusion constants using a bleached strip of defined size see Ellenberg et al. (1998) Alternatively, diffusion constants can be determined directly from fitting the experimental data to the theoretical curve by computer simulation (Phair and Misteli, 2000; Houtsmuller and Vermeulen, 2001; Reits and Neefjes, 2001).

ALTERNATE PROTOCOL 1

FLUORESCENCE LOSS IN PHOTOBLEACHING (FLIP)

In FLIP, a region in the nucleus is repeatedly bleached and the loss of fluorescence in a monitoring region of interest some distance away from bleached region is recorded (Fig. 13.5.2). The basis of FLIP is the bleaching of mobile molecules as they pass through a bleached region. If all or a fraction of the fluorescently-tagged molecules in the nucleus are immobile, they will never enter or exit the repeatedly bleached region; therefore, they will not be bleached and cannot distribute the bleached signal elsewhere. As a consequence, the fluorescence signal in the regions surrounding the bleached spot will remain constant. In contrast, if molecules are highly mobile, they will eventually pass through the bleached region. The fluorescence signal in the nucleus will decrease. If all fluorescently-tagged molecules inside of the monitoring region of interest move freely into the bleached region, repeated bleaching will reduce the fluorescent signal in the monitoring region of interest at a constant rate. On the other hand, if multiple, kinetically distinct fractions of molecules exist, the loss curve will contain multiple distinct slopes. FLIP is also a particularly effective technique for testing continuity between nuclear compartments such as nucleoli, splicing factor compartments, Cajal bodies, or PML bodies.

FLIP is complementary to FRAP. Since in FLIP the signal in an unbleached region is measured, the technique is often used to ensure that the mobility of a protein of interest observed in FRAP experiments is not due to photodamage of the protein at the bleached spot.

See Basic Protocol for materials.

1. Set up the cells on the confocal microscope as described (see Basic Protocol, steps 1 and 2).
2. Image two cells of interest close to each other using a low level of laser power to prevent photobleaching.

   On the Zeiss LSM 510 microscope, 20% to 50% output from a 40-mW laser with the beam attenuated to 0.1% is routinely used for the image acquisition.
3. Adjust settings as described for FRAP (see Basic Protocol, steps 4 to 9).

   A successful FLIP experiment depends on the successful loss of fluorescence in regions outside the bleached region. It is therefore more practical to use bleach areas that are larger in size than the ones used in the FRAP experiments.
4. Initiate data collection (i.e., prebleach images, bleach, postbleach images) and monitor fluorescence loss. Check the position of the cell and the focal plane to be sure that the monitoring spot is not moving from the original position.

   The acquisition routine should run in a loop between bleach pulse and single image acquisition separated by the predetermined time interval.

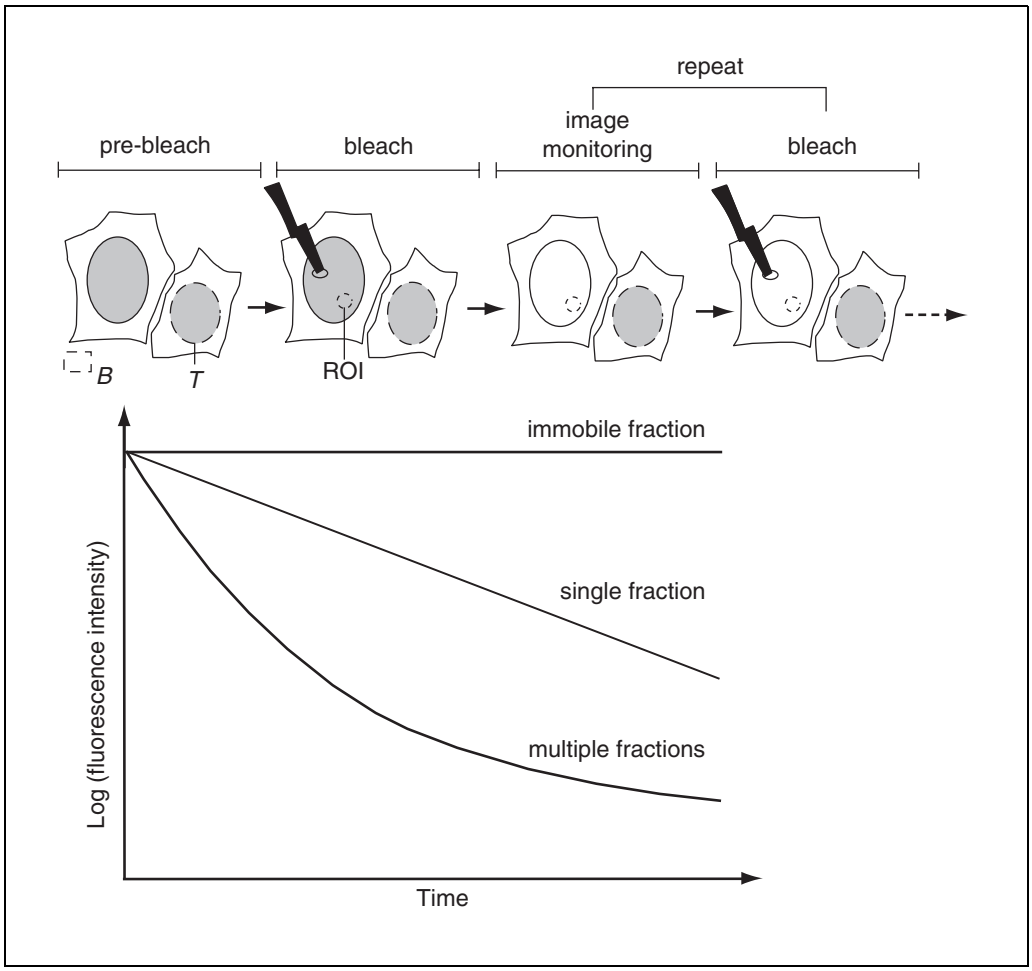


Figure 13.5.2 General scheme of a FLIP experiment. A cell is imaged before a short bleach pulse is applied to a defined bleach region. The image-bleach routine is repeated with the two steps separated by a predetermined time interval. To quantify and normalize FLIP data, measure the average fluorescence signal in a region of interest (ROI) distinct from the bleached region, measure the average total nuclear fluorescence signal (*T*) in a neighboring cell, and the average signal in a random region outside of the cell for background correction (*B*), for the duration of the experiment. When the FLIP data are plotted on a semilog scale, the shape of the curve indicates whether the GFP-fusion protein exists in the cell in a single fraction, in multiple fractions or whether it is immobile.

It is essential that the level of fluorescence signal at the end of the experiment be below 15% of the prebleach level. The fluorescence loss curve should resemble the FLIP curve in Figure 13.5.2. If the loss of fluorescence is not below 15%, it is essential to increase the monitoring time. Note that the slope of the loss curve depends on the size, intensity, and duration of the bleach pulse.

Perform quantitative analysis of FLIP

5. Measure the average intensity of the monitoring region of interest as a function of time I_t.
6. Measure the average intensity of the total nuclear area of a neighbor cell at each time point (T_t).
7. Measure the background intensity in a random region outside of the cells at each time point (B_t).
8. For each time point, calculate the relative intensity:

$$I_{rel} = (I_t - B_t)/[(I_0 - B_0) \times (T_t - B_t)]$$

where I_0 is the average intensity of the region of interest during prebleach.

This formula is derived in three steps. First, background is subtracted from all measured values, then fluorescence loss due to monitor bleaching is normalized, and finally fluorescence intensity is normalized to one.

9. Plot I_{rel} as a function of time and determine average and standard deviations as described for FRAP (see Basic Protocol, steps 16 and 17).

If all fluorescently tagged proteins in the compartment are kinetically identical, the region of interest will lose its fluorescence linearly. If the nuclear compartment contains kinetically distinct pools of proteins (e.g., due to binding of one fraction of the protein to chromatin or incorporation of a fraction into a large complex), the loss of fluorescence will be multiphasic. The number of distinct fractions can be determined by plotting the loss curve as a function of time in a semilog plot. Each kink in the semilog plot corresponds to a kinetically distinct fraction of molecules (Fig. 13.5.2).

As a negative control, fixed cells should be used. No significant loss of signal should be observed outside of the bleached region. The amount of loss that is observed corresponds to bleaching due to imaging.

ALTERNATE PROTOCOL 2

INVERSE FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (iFRAP)

iFRAP is a hybrid method between FRAP and FLIP. In iFRAP, the entire nucleus except a small region of interest is bleached using a single bleach pulse. The loss of fluorescence signal from the unbleached region of interest is then monitored over time (Fig. 13.5.3). Because the number of unbleached molecules in the region of interest is small compared to the number of bleached molecules outside the region of interest, the unbleached molecules do not contribute to new binding events because their pool is proportionally too small in comparison to the large pool of bleached molecules. Thus, iFRAP is ideally suited to provide information about off-rates of proteins from binding sites (e.g., those on chromatin) or from nuclear compartments. iFRAP is particularly well suited for proteins which are enriched in relatively small structures (e.g., replication sites, intranuclear compartments) and for proteins which are bound to a substrate for relatively long periods of time.

See Basic Protocol for materials.

1. Set up cells on the confocal microscope and image as described for FLIP (see Alternate Protocol 1, steps 1 to 2). Adjust the microscope settings as described for FRAP (see Basic Protocol, steps 4 to 6).
2. Adjust laser power to 100% for bleaching.

The bleach time should be minimized and the efficiency of the bleach should be tested on a fixed sample. The average intensity in a bleached region in a fixed sample should be reduced by at least 70% compared to the same region of the neighboring cell. If the bleach depth is insufficient, use several, rapid, consecutive bleach pulses.

3. Adjust the number of prebleach images for five to ten images.
4. Adjust the number of postbleach intervals and the time intervals.

The number of intervals is determined by the resolution needed and the length of intervals should cover the curve until it reaches a plateau. Be careful not to collect an unnecessarily high number of images as this will bleach the sample.

5. Define the region to be bleached excluding the region of interest.

This must be performed quickly as the region of interest can move while the bleached region is being outlined. The bleach has to be as fast as possible to obtain information about rapidly moving molecules in the unbleached region of interest.

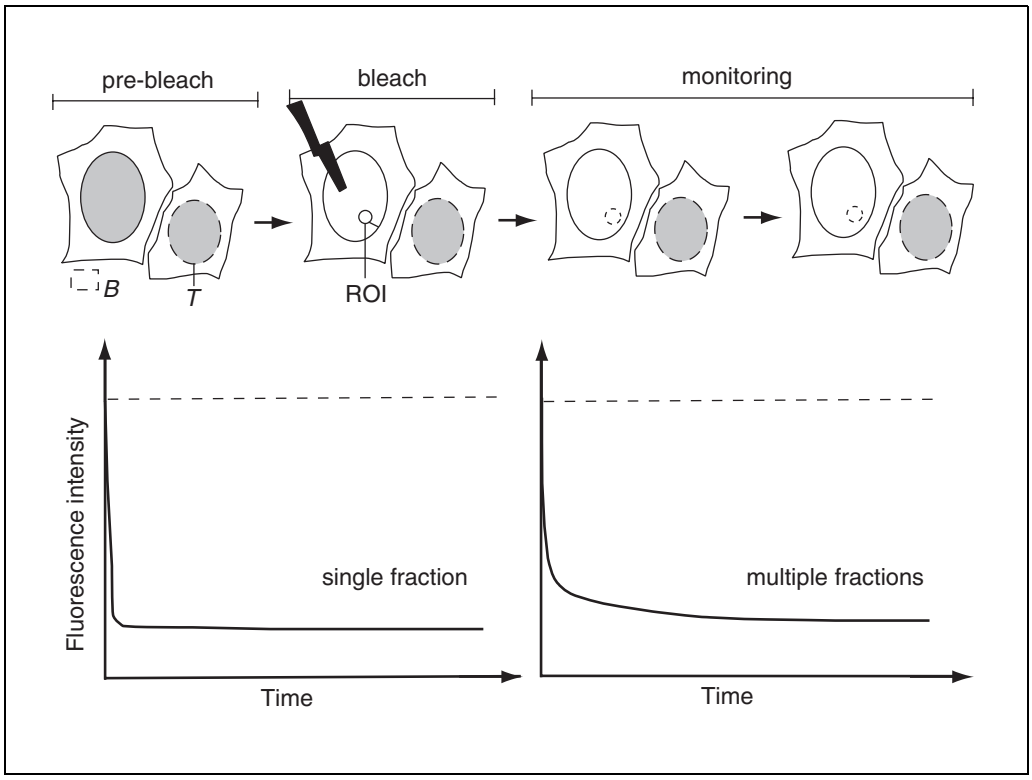


Figure 13.5.3 General scheme of an iFRAP experiment. A cell is imaged before a short bleach pulse is applied to the whole area of the nucleus except a small region of interest (ROI). The loss of fluorescence signal in the ROI is then monitored. To quantify and normalize iFRAP data, measure the average fluorescence signal in the ROI, the average total nuclear fluorescence signal (*T*) in a neighboring cell, and the average signal in random place outside of the cell for background correction (*B*), for the duration of the experiment. If the GFP-fusion protein in the ROI exists as a single population, the iFRAP curve should resemble the iFRAP curve on the left. If the GFP-fusion protein exists in the region of interest in multiple populations, the iFRAP curve should resemble the curve on right.

6. Initiate data collection (i.e., prebleach images, bleach, postbleach images) and monitor fluorescence loss in the region of interest. Check the position of the cell and the focal plane to be sure that the region of interest is not moving from the original position.

   The shape of the fluorescence loss curve should resemble the iFRAP curve in Figure 13.5.3. The region of interest has to be monitored until the loss of fluorescence signal reaches a plateau.

Perform quantitative analysis of iFRAP

7. Measure the average intensity of the region of interest at each time point (I_t).
8. To correct for the photobleaching effect due to monitoring, measure the average intensity of the entire nucleus of a neighboring cell at each time point (T_t).
9. Measure the background intensity in a randomly selected region outside of the cell at each time point (B_t).
10. Calculate the relative intensity (I_{rel}) each time point:

$$I_{rel} = \frac{(I_t - B_t) \times (T_0 - B_0)}{(I_0 - B_0) \times (T_t - B_{t)}}$$

where I_0 is the average intensity of the region of interest during prebleach (step 1) and T_0 is the average intensity of the total nuclear area of a neighbor cell during prebleach.

This formula is derived in three steps: first background is subtracted from all measured values, then fluorescence loss due to monitor bleaching is normalized, and finally fluorescence intensity is normalized to one.

11. Plot I_{rel} as a function of time and determine average and standard deviations as described for FRAP (see Basic Protocol).

It is advisable to plot all acquired curves before determining the average. Jumps in the recovery curves or declining curves are frequently caused by cell or focal plane movement. These curves can either be discarded for analysis or, if possible, they should be manually remeasured. For many applications it is sufficient to collect data for 10 to 20 individual cells. Typical error bars are on the order of 5% to 10% of the measured value.

If all GFP-fusion proteins in the compartment are fast moving components from the same pool, the fluorescent signal in the region of interest after the bleach will drop dramatically and then reach a plateau immediately (Fig. 13.5.3). If the nuclear compartment contains kinetically distinct pools of proteins (due to binding of one fraction of the protein to chromatin or substrate for longer time or incorporation of a fraction into a large complex), the curve of loss of fluorescence in the region of interest will drop dramatically and then show a linear decline until it finally reaches a plateau (Fig. 13.5.3).

As a control, fixed cells should be used. In this case the loss of fluorescence in the region of interest should show no significant loss of signal after the bleach over the monitoring.

SUPPORT PROTOCOL 1

TRANSIENT TRANSFECTION OF MAMMALIAN CELLS BY ELECTROPORATION

To study the dynamics of nuclear proteins in living cells, the specific protein-GFP chimera needs to be expressed. Ideally, a cell line stably expressing the GFP fusion protein from an integrated transgene is generated. The use of stable cell lines and inducible promoters such as the tetracycline- or ecdysone-inducible expression systems (No et al., 1996) are recommended when the overexpression of a fusion protein might be toxic or interfere with essential biological functions (Freundlieb et al., 1998). Detailed protocols for establishing stable cell lines are presented elsewhere (e.g., Stenmark and Zerial, 1998).

A quicker and often sufficient option for expression of GFP-fusion proteins in living cells is the introduction of a GFP-fusion protein by transient transfection encoded on a vector which does not integrate into the cell's genome. These vectors result in short-term, high-level expression of the fusion protein, but they will eventually be eliminated from the cell and expression will cease. Regardless of whether GFP fusion proteins are stably or transiently expressed, a vector containing the GFP-fusion protein cDNA must be introduced into cells by transfection. Two of the most effective and reliable methods of transfection are transfer of DNA via electrical current (presented here) or through one of the commercially available lipid-mediated delivery protocols (see Support Protocol 2). These protocols are for transfection of adherent cells, but both methods can also be used for transfection of suspension cells.

This protocol has been tested for the following adherent cell lines: HeLa, COS, CMT3, NIH 3T3, BHK, and CHO cells. The protocol must be optimized for other cell types. The important parameters for optimal results are the strength of applied electric field, duration of the electrical pulse, number of pulses applied, and concentrations of cells and plasmid DNA.

Materials

Cells
Complete growth medium (e.g., *UNIT 1.2*)
Plasmid DNA
Carrier DNA: sheared salmon sperm DNA
1× PBS (*APPENDIX 2A*)
0.25% (w/v) trypsin/1 mM EDTA, 37°C
Complete growth medium with 25 mM HEPES, but without phenol red

10-cm Petri dish
37°C warming tray
15-ml test tubes
Electroporation cuvettes with 2-mm gap
BTX ECM830 electroporator (BTX, a division of Genetronics, Inc.) or equivalent
Culture dish or incubation chamber (e.g., 2- or 4-chambered Lab-Tek II coverglass; Nalgene Nunc)

Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

NOTE: All solutions and materials coming into contact with cells must be sterile, and proper aseptic technique must be used.

NOTE: All cell culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise indicated.

NOTE: Avoid repeated freezing and defrosting of plasmid DNA stock solution. It may cause nicks in the plasmid DNA which leads to linearization of supercoiled DNA. Prepare aliquots of plasmid DNA and store them at −20°C.

1. Prepare an ~70% confluent monolayer of cells in a 10-cm Petri-dish (*UNIT 1.1*) in complete growth medium.
2. Add 3 to 7 µg plasmid DNA to a 1.5-ml test tube. Add sufficient carrier DNA to bring the total amount of DNA to 20 µg. Adjust the volume to 30 µl with water.
3. Wash cells once with 1× PBS.
4. Add enough 0.25% (w/v) trypsin/1 mM EDTA, 37°C to the culture to cover the adhering cell layer. Place the plate on a 37°C warming tray 1 to 2 min.
5. When cells round up, but before they detach from the substratum, add 7 ml complete growth medium. Harvest by collecting the cells and transferring them to a 15-ml centrifuge tube. Pellet cells by centrifuging 2 min at 1000 rpm in a benchtop centrifuge, room temperature.
6. Decant the aqueous layer and resuspend cells in 200 µl fresh complete medium. Pipet the cell suspension into the 1.5-ml test tube containing the prepared DNA solution (step 2). Mix well by pipetting up and down three times, let stand 2 min, and transfer to an electroporation cuvette with 2-mm gap.
7. For HeLa, COS, CMT3, NIH 3T3, BHK, or CHO cells, electroporate on a BTX ECM830 electroporator using the following settings: 150 V, 1-msec pulse, 4 pulses, and 0.5-sec interval.

   For BTX electroporator settings for other cell types refer to http://www.btxonline.com/btx and the manufacturer's lab manual at http://www.btxonline.com/products/pdfs/ECM_830/ECM_830_Manual.pdf. For electroporators from other manufacturers see the appropriate company's web pages.

8. Using a Pasteur pipet, transfer the cells from the cuvette to a well in a culture dish or to an incubation chamber.

   For HeLa, COS, CMT3, NIH 3T3, BHK, or CHO cells, one drop of cell suspension in a 35-mm well gives a confluent layer after overnight incubation. For other cell types, the amount of cells must be optimized.

9. Change the medium 8 to 10 hr after transfection.

10. Before imaging living cells (see Basic Protocol and Alternate Protocols 1 and 2), replace the medium with complete medium containing 25 mM HEPES, but no phenol red.

    Phenol red increases background fluorescence.

SUPPORT PROTOCOL 2

TRANSIENT TRANSFECTION OF MAMMALIAN CELLS USING FuGENE 6

FuGENE 6 is a transfection reagent that highly efficiently transfects a wide variety of cells including primary cultures and hard-to-transfect cell lines. The advantage of FuGENE 6 is that it demonstrates virtually no cytotoxicity even with primary cell cultures. The authors have successfully tested several adherent transformed cell lines—HeLa, COS, CMT3, NIH 3T3, CHO—and mouse primary fibroblasts. The FuGENE 6 reagent web page—*http://biochem.roche.com/techserv/fugene.htm*—has a current list of 250 successfully transfected cell lines.

Materials

Cells
Serum-containing and serum-free medium (*UNIT 1.2*)
FuGENE 6 reagent (Roche)
Plasmid DNA solution
Serum-containing medium (*UNIT 1.2*)
Serum (optional)
Glass coverslips or Lab Tek II incubation chambers (Nalgene Nunc)

Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

NOTE: All solutions and materials coming into contact with cells must be sterile, and proper aseptic technique must be used.

NOTE: All cell culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise indicated.

NOTE: Avoid repeated freezing and defrosting of plasmid DNA stock solution. It may cause nicks in the plasmid DNA which leads to linearization of supercoiled DNA. Prepare aliquots of plasmid DNA and store them at −20°C.

1. Prepare ~50% to 70% confluent monolayers on glass coverslips or in Nalgene Lab Tek II incubation chambers (*UNIT 1.1*) in serum-containing medium.

2. In a 1.5-ml sterile test tube, add 97 µl serum-free medium, then add 3 µl FuGENE6 reagent directly into this medium.

   The order of addition is critical. The serum-free medium must be aliquoted into the test tube first to avoid adversely affecting transfection efficiency by contact of the undiluted FuGENE with plastic surfaces.

3. Add 1 to 2 µg plasmid DNA solution in a volume of 0.5 to 50 µl to the diluted FuGENE 6 reagent.

   The total volume of DNA solution has to be in this range.

4. Gently tap the test tube to mix the contents, but do not vortex. Incubate a minimum of 15 min at room temperature.

   Continued incubation for up to 45 min will not affeect the transfection efficiency in most cell types.

5. Dissolve the mixture into 2 ml serum-containing medium. Add to the cells dropwise, distributing it around the well or incubation chamber. Swirl the wells or incubation chamber to ensure even dispersal.

6. Return the cells to the incubator. After 3 to 8 hr, replace the medium with serum-containing medium or add serum directly to wells. Continue incubating (12 to 20 hr) until imaging is performed (see Basic Protocol and Alternate Protocols 1 and 2).

QUANTIFICATION OF FLUORESCENTLY TAGGED PROTEIN MOLECULES IN NUCLEAR COMPARTMENTS OF SINGLE LIVING CELLS

SUPPORT PROTOCOL 3

The number of fluorescently tagged molecules in nuclear compartments in single living cells can be determined using individual fluorescently labeled virus-like particles (VLP) as a standard for the observed signals. VLPs contain a precise number of GFP molecules and the number of GFP molecules in a test volume can therefore be determined by comparing the total fluorescent intensity of VLP and the total fluorescent intensity of a nuclear volume. Since VLPs are small spherical particles, this technique is especially useful for the determination of the GFP content of small spherical cellular compartments and organelles such as Cajal bodies, PML bodies, fibrillar centers within the nucleoli, mitochondria, or vesicles.

VLPs are in vitro–assembled rotavirus particles (Charpilienne et al., 2001). Rotaviruses are large icosahedral particles, which contain three concentric capsid layers. While the outermost layer is composed of the VP4 and VP7 proteins, the intermediate capsid layer is composed of trimers of VP6. The innermost layer contains exactly 120 molecules of VP2. When VP2 fused to GFP is coexpressed with the intermediate capsid protein VP6 in a baculovirus-insect cell system, double-layered icosahedral VLP are completely assembled. Each such VLP measures ~100 nm in diameter and contains exactly 120 VP2-GFP molecules (Charpilienne et al., 2001).

Materials

Cells expressing GFP-tagged protein of interest (see Support Protocols 1 and 2)
Complete growth medium (*UNIT 1.2*)
1× PBS (*APPENDIX 2A*)
GFP-labeled VLP particles (Charpilienne et al., 2001; Dundr et al., 2002)

22 × 22–mm glass coverslips
Confocal microscope with 100× objective

Additional reagents and materials for mammalian cell culture (*UNIT 1.1*)

1. Culture cells expressing the GFP-tagged protein of interest on 22 × 22–mm glass coverslips for 16 to 20 hr (*UNIT 1.1*) in complete growth medium.

2. Wash cells twice with 1× PBS for 5 min each time.

3. Dilute purified GFP-labeled VLP particles to a concentration of 1 to 10 µg/ml in PBS.

4. Place ~30 to 40 µl diluted VLP onto the coverslip (step 1).

   It is advisable to perform a pilot experiment in which VLP particles are imaged in solution without cells. This allows for their easy detection later when they are mixed with the cells.

5. Examine cells using a confocal microscope with 100× objective.

   The powerful objective is needed because VLPs are difficult to see at low magnifications.

   Alternatively, a conventional wide-field microscope fitted with a cooled CCD camera can be used.

6. Adjust the photomultiplier tube intensity such that the fluorescence signal of the GFP fusion protein is slightly below the saturation level.

   Use at least 8- but preferably 12-bit imaging. It is important to ensure that no pixels are saturated since the fluorescence intensity in these pixels cannot be measured accurately.

7. Collect a series of 50 single images of the cells expressing the GFP-fusion protein.

8. Using identical settings, collect 50 single images of the VLP-GFP particles in a different field of view in the same specimen.

9. Measure the average area and the average intensity of at least fifty compartments of interest containing the GFP-fusion protein and at least fifty VLPs.

10. Measure the background intensity in a randomly selected area that does not contain cells or VLPs.

11. Subtract the background intensity from the average intensity value of compartments and VLPs.

12. Multiply the average area with the background-corrected average intensity to obtain average fluorescence intensities for the compartment and VLPs.

13. Calculate the number of GFP molecules per compartment:

    $$\text{no. GFP molecules/compartment} = (TI_C \times 120)/(TI_{VLP})$$

    where TI_C is the average intensity of the compartments and TI_{VLP} is the average intensity of the VLPs.

    When using a confocal microscope, not all of the imaged VLP particles will fall within the focal plane and a range of signals will be observed. To determine the average intensity of a single VLP particle, it is helpful to perform three-dimensional reconstruction of VLPs with a distance of 100 nm between optical sections. Collect the maximum number of sections possible and observe them for quantification as a maximum projection. In this case, the total intensities of VLPs should fall within a 10% range of intensities.

COMMENTARY

Background Information

It has been established that the eukaryotic cell nucleus is not a homogeneous organelle but contains several specific membrane-less compartments. Recent observations using photobleaching methods have demonstrated that many nuclear proteins are highly mobile and do not permanently reside in any specific nuclear compartment but are continuously exchanged between the compartment and the surrounding nucleoplasm (Kruhlak et al., 2000; Phair and Misteli, 2000; Chen and Huang, 2001). In this view, nuclear compartments are the consequence of the steady-state dynamic behavior of their components. Nuclear proteins are not primarily targeted to the nuclear compartments by specific targeting signals but rather retained there as a result of binding or collision with other nuclear components (i.e., protein-protein interactions; DNA-protein interactions: engagement in replication, DNA repair or RNA-protein interactions, and engagement in transcription etc.). The exchange rate of the nuclear proteins is strongly determined by the roles they play in nuclear function.

The localization of proteins within cells has traditionally been studied by indirect immunofluorescence microscopy using fluorescently labeled antibodies. This method is limited by the requirement for chemical fixation

of cells and yields only static snapshots of a protein's distribution. The development of the green fluorescent protein (GFP) as a genetically encoded fluorescent protein reporter has revolutionized the study of protein localization in living cells.

Apart from the use of GFP as a convenient and rapid method to determine where a known protein or one encoded by a newly cloned cDNA is localized, GFP has also been extensively used to visualize proteins in living cells by time-lapse microscopy. These latter approaches allow the study of dynamic aspects of protein function. GFP fusion proteins have been particularly insightful in the study of the cell nucleus. Using time-lapse-microscopy approaches, it has become clear that many nuclear compartments are highly dynamic both in interphase and during mitosis, and that many proteins continuously shuttle between the nucleus and the cytoplasm. (Ellenberg et al., 1997; Misteli et al., 1997; Boudonck et al., 1999; Dundr et al., 2000; Platani et al., 2000; Tsukamoto et al., 2000; Kamath et al., 2001). More recently, nuclear GFP fusion proteins have also been used in combination with photobleaching techniques to study the dynamic organization of nuclear compartments in vivo.

Fluorescence photobleaching techniques were originally developed in the 1970s to study the mobility of lipids and proteins in the lipid bilayer of the plasma membrane (Axelrod et al., 1976). They have now become a standard method for studying the dynamics of nuclear proteins in living cells (Ellenberg et al., 1997; Houtsmuller et al., 1999; Kruhlak et al., 2000; Phair and Misteli, 2000; Snaar et al., 2000; Misteli, 2001; Chen and Huang, 2001; Boisvert et al., 2001). The noninvasive nature of the photobleaching approach allows one to label nuclear compartments in living cells with high specificity, but often without functional interference. The GFP-protein marker is particularly useful for photobleaching experiments, because the signal is stable and does not bleach significantly at the low-intensity levels used to monitor bleach recovery. In addition, the bleaching of GFP in living cells can be considered irreversible and does not cause any detectable damage to the cell (but see Verkman, 2002).

What are the advantages of using photobleaching techniques for studying the dynamics of nuclear proteins in living cells? Recent observations using photobleaching techniques have demonstrated that many nuclear proteins are highly mobile and do not permanently reside in any specific domain of the nucleus (Kruhlak et al., 2000; Phair and Misteli, 2000; Chen and Huang, 2001). The various available photobleaching techniques can provide information about the in vivo kinetics of the protein of interest and since the kinetic behavior of a protein is often directly related to its functional status, information about a protein's biological role may also be obtained (Phair and Misteli, 2001). Furthermore, photobleaching methods can be used to determine how many different pools of a protein are present in a cell and whether an immobile statically bound fraction exists.

Critical Parameters

Transfection efficiency

Transfection efficiency plays an important role in photobleaching experiments. Very low numbers of transfected cells can affect the evaluation of the pattern of GFP-fusion protein localization and selection of optimal cells for bleaching. In the case of FLIP or iFRAP experiments, high transfection efficiency is essential so that images of two neighboring cells expressing the GFP protein can be obtained, which is required for correction of the signal due to photobleaching. Therefore special attention should be paid to optimization of transfection efficiency.

Cell type

A cell type that tolerates introduction of plasmid DNA should be selected. If there is an option, select preferentially transformed cells over primary cells, which are usually more difficult to optimize for transfection. Cells should be in logarithmic growth when used for transfection.

Quality of plasmid DNA

One critical parameter of good transfection efficiency is the quality of plasmid DNA. It should be pure and the stock DNA solution should not be repeatedly frozen and defrosted. When transfection efficiency is low, a higher concentration of plasmid DNA can be used.

Electroporation

When electroporation is used, three parameters should be varied for optimization: electrical field, duration, and number of pulses. When the electrical field is too high, the survival rate of the cells is lowered. When it is too low, the efficiency of transfection is usually poor. When

a low electric field is used, longer pulses should be applied and vice versa.

Expression levels

For photobleaching experiments, it is advisable to select only cells expressing low or moderate levels of GFP fusion protein. Overexpression of a protein of interest can dramatically affect its localization and may change its behavior as well as influence the overall metabolism of the cell.

Critical elements

Special attention must be paid to the viability of the cells during photobleaching measurements. Cells in a chambered coverglass should not be on the microscope stage for more than 1 hr. Longer exposure of cells can affect cell metabolism and can cause changes in protein mobility. After 1 hr cells should be replaced with another sample or put back in the incubator for at least 30 min.

Controls

Fixed cells should be used as a control for all photobleaching experiments. The fixed sample permits determination of the *x*, *y*, and *z* dimensions of the bleach region and depth of bleach, if the series of optical sections through the sample is collected. The size of the bleach spot should be small relative to the size of the nucleus. Typically a bleach spot 1 μm in diameter works for most applications. Bleaching a region that is larger than the compartment of interest will affect the accuracy of measurement by mixing the populations of GFP-molecules outside and inside of the compartment. Therefore, it is advisable to adjust the size of compartment relative to the size of the bleach spot by using the appropriate zoom.

Each individual recovery curve should be evaluated. Any dramatic change in the shape of the curve may indicate that the monitoring region of interest changed position or focal plane due to cell movement. These data should be discarded.

Anticipated Results

In a typical transient transfection, a fluorescent signal should be detected 12 to 20 hr after transfection, but weak signals can often be observed as early as 4 to 6 hr. The differences in expression level can vary depending on the purity of DNA, the vector and promoter used, and the cell type used in the study.

The recovery curve of the GFP fusion protein of interest contains information about the degree of mobility of a protein of interest within the nucleus. Generally, qualitative analysis of any photobleaching curves allows one to conclude whether a protein is completely mobile, mobile with a fraction of immobile molecules, or almost completely immobile. In many cases this basic information has significant ramifications for protein function. Note that coincidence of recovery curves for multiple proteins does not necessarily mean that these proteins are found in a complex and move together. Conversely, however, distinct curves of several proteins are generally a good indication that the

Table 13.5.2 Comparison of the Mobility of Nuclear Proteins

GFP-fusion protein	FRAP recovery (sec)	Diffusion coefficient ($\mu m^2/s$)	Reference
EGFP	~0.5	27	Swaminathan et al., 1997
580 kDa dextran	7	0.95	Calapez et al., 2002
GFP-ASF/SF2	20	0.24	Phair and Misteli, 2000
GFP-HMG17	30	0.45	Phair and Misteli, 2000
GFP-PABP2	7	0.6	Calapez et al., 2002
GFP-TAP	3	1.2	Calapez et al., 2002
Fibrillarin-GFP	30	0.53	Phair and Misteli, 2000
GFP-UBF1	60	0.14 (nucleolus) 0.57 (nucleoplasm)	Chen and Huang, 2001
GFP-Nucleolin	60	0.14 (nucleolus) 1.15 (nucleoplasm)	Chen and Huang, 2001
TFIIH-GFP	30	5.1	Hoogstraten et al., 2002
ERCC1-GFP/XPF	8	15 (absence of DNA damage)	Houtsmuller et al., 1999
	—	(immobile with DNA damage)	

majority of the proteins of interest are not present in a complex in vivo.

Examples of recovery times and diffusion coefficients are given in Table 13.5.2.

As a positive control for the evaluation of the mobility of GFP protein of interest, GFP alone can be used as a standard for relatively freely mobile protein. The recovery of the fluorescent signal of the GFP alone is very fast (within 1 sec) and complete recovery with virtually no immobile fraction should be observed. Convenient negative controls are GFP fusions with the core histones (H2A-GFP, H3-GFP, H4-GFP; Kimura and Cook, 2001). In this case, there should be no recovery over the monitoring period. If recovery of fluorescence is observed using either fixed cells or core histone fusion proteins, it should be ensured that the correct excitation wavelength of the laser (488 nm for GFP) is used. Similarly, if no bleaching is observed, the laser settings should be checked.

In FLIP experiments, the kinetic status of specific nuclear compartments is evaluated. Specifically it is tested whether a GFP-fusion protein resides statically or moves rapidly in and out of a compartment. If two or more compartments are in physical continuity, bleaching one will result in loss of fluorescence in the other(s).

In iFRAP experiments, loss of fluorescence in region of interest reflects predominantly how the GFP-fusion protein is lost from its binding site.

Most photobleaching experiments should be completely reproducible with a typical error among cells in a population on the order of 5% to 10% of measured values. To achieve this goal, each acquired curve has to be evaluated independently, and every curve with jumps due to cell or focal plane movement should be discarded before final averaging. Since these are single-cell experiments, variations in cell populations might be observed as relatively large errors in a population measurement. For many proteins the position of the cell in the cell cycle can affect their behavior and mobility. If large fluctuation in measurements is observed, it is recommended to use cell-cycle-synchronized cells.

Special care must be applied to minimize photobleaching due to imaging during monitoring. The number of images collected during monitoring should be a compromise between the resolution of measurements needed and the length of the interval. The decline of the signal intensity due to photobleaching during monitoring should generally not exceed ~5% to 10% of the prebleach value. If bleaching is a problem, the laser power or the interval between images can be reduced.

GFP levels can be quantified with VLPs by comparing total fluorescent intensities of VLPs with a defined number of GFP molecules and fluorescently labeled specific small nuclear compartments of interest (see Support Protocol 3). This measurement provides the average number of GFP-fusion protein molecules in the compartments in single living cells. Since in 12-bit imaging, 4096 gray levels can be distinguished and the typical background intensity is ~100 to 200 units, the observable ratio of VLP to compartment signal is ~20-fold. Given that each virus particle contains 120 GFP molecules, only compartments which contain ~2500 molecules of interest can be measured using this method. The level of GFP molecules found in many nuclear compartments is well within this range.

Time Considerations

A transient transfection can be done in 30 to 45 min and the cells can be used for microscopy 12 to 20 hr after transfection. The time for a FRAP and iFRAD experiment depends on the mobility of the GFP fusion-protein, but is typically on the order of seconds to <10 min. A FLIP experiment takes on the order of minutes up to 1 hr. Each GFP fusion protein should be tested on 15 to 20 cells. The normalization of one sheet of FRAP data using Microsoft Excel takes several minutes. Electroporation takes ~20 min and the transfection procedure using FuGENE 6 takes 25 min to 1 hr. Acquiring a sufficient number of images of cells expressing protein-GFP and VLPs requires 0.5 to 1.0 hr. Measuring the average area and the average intensity of at least fifty compartments of interest and fifty VLPs using microscope or Metamorph software requires ~2 to 3 hr.

Literature Cited

Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E., and Webb, W.W. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055-1069.

Boisvert, F.M., Kruhlak, M.J., Box, A.K., Hendzel, M.J., and Bazett-Jones, D.P. 2001. The transcription coactivator CBP is a dynamic component of the promyelocytic leukemia nuclear body. *J. Cell Biol.* 152:1099-1106.

Boudonck, K., Dolan, L., and Shaw, P.J. 1999. The movement of coiled bodies visualized in living plant cells by the green fluorescent protein. *Mol. Biol. Cell* 10:2297-2307.

Calapez, A., Pereira, H.M., Calado, A., Braga, J., Rino, J., Carvalho, C., Tavanez, J.P., Wahle, E.,

Rosa, A.C., and Carmo-Fonseca, M. 2002. The intranuclear mobility of messenger RNA binding proteins is ATP dependent and temperature sensitive. *J. Cell Biol.* 159: 795-805.

Charpilienne, A., Nejmeddine, M., Berois, M., Parez, N., Neumann, E., Hewat, E., Trugban, G., and Cohen, J. 2001. Individual rotavirus-like particles containing 120 molecules of fluorescent protein are visible in living cells. *J. Biol. Chem*. 276:29361-29367.

Chen, D., and Huang, S. 2001. Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. *J. Cell Biol*. 153:169-176.

Dundr, M., Misteli, T., and Olson, M.O.J. 2000. The dynamics of postmitotic reassembly of the nucleolus. *J. Cell Biol*. 150:433-446.

Dundr, M., McNally, J.G., Cohen, J., and Misteli, T. 2002. Quantification of GFP fusion proteins in single living cells. *J. Struct. Biol.* 140:92-99.

Ellenberg, J., Siggia, E.D., Moreira, J.E., Smith, C.L., Presley, J.F., Worman, H.J., and Lippincott-Schwartz, J. 1997. Nuclear membrane dynamics and reassembly in living cells: Targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell Biol.* 138:1193-1206.

Ellenberg, J., Chazotte, B., and Lippincott-Schwartz, J. 1998. Fluorescence photobleaching techniques. *In* Cells, A Laboratory Manual. (D.L. Spector, R.D. Goldman, L.A. Leinwand, eds). pp. 79.1-79.23. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Freundlieb, S., Baron, U., and Bujard, H. 1998. Controlling gene activities via the tetracycline regulatory systems. *In* Cell Biology: A Laboratory Handbook, 2nd Edition, Vol. 4 (J.E. Celis, ed.) pp. 230-238. Academic Press, San Diego.

Hoogstraten, D., Nigg, A.L., Heath, H., Mullenders, L.H., van Driel, R., Hoeijmakers, J.H., Vermeulen, W., and Houtsmuller, A.B. 2002. Rapid switching of TFIIH between RNA polymerase I and II transcription and DNA repair in vivo. *Mol. Cell.* 10: 1163-1174.

Houtsmuller, A.B., Rademakers, S., Nigg, A.L., Hoogstraten, D., Hoeijmakers, J.H.J., and Wim Vermeulen, W. 1999. Action of DNA repair endonuclease ERCC1/XPF in living cells. *Science* 284:958-961.

Houtsmuller, A.B. and Vermeulen, W. 2001. Macromolecular dynamics in living cell nuclei revealed by fluorescence redistribution after photobleaching. *Histochem. Cell Biol*. 115:13-21.

Kamath, R.V., Leary, D.J., and Huang S. 2001. Nucleocytoplasmic shuttling of polypyrimidine tract-binding protein is uncoupled from RNA export. *Mol. Biol. Cell* 12: 3808-3820

Kimura, H. and Cook, P.R. 2001. Kinetics of core histones in living human cells: Little exchange of H3 and H4 and some rapid exchange of H2B. *J. Cell Biol*. 153:1341-1353.

Kruhlak, M.J., Lever, M.A., Fischle, W., Verdin, E., Bazett-Jones, D.P., and Hendzel, M.J. 2000. Reduced mobility of the alternative splicing factor (ASF) through the nucleoplasm and steady state speckle compartments. *J. Cell Biol*. 150:41-51.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nature Rev. Mol. Cell Biol*. 2:444-456.

McNally, J.G. and Smith, C.L. 2002. Photobleaching by confocal microscopy. *In* Confocal and Two-Photon Microscopy: Foundations, Applications, and Advances, (A. Diaspro, ed.) pp. 525-538. Wiley-Liss, New York.

Misteli, T. 2001. Protein dynamics: Implications for nuclear architecture and gene expression. *Science* 291:843-847.

Misteli, T., Caceres, J.F., and Spector, D.L. 1997. The dynamics of a pre-mRNA splicing factor in living cells. *Nature* 387:523-527.

No, D., Yao, T.-P., and Evans, R.M. 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 93:3346-3351.

Phair, R.D. and Misteli, T. 2000. High mobility of proteins in the mammalian cell nucleus. *Nature* 404:604-609.

Phair, R.D. and Misteli, T. 2001. Kinetic modelling approaches to *in vivo* imaging. *Nature Rev. Mol. Cell Biol.* 2:1-10.

Platani, M., Goldberg, I., Swedlow, J.R., and Lamond, A.I. 2000. In vivo analysis of Cajal body movement, separation, and joining in live human cells. *J. Cell Biol*. 151:1561-1574.

Reits, E.A. and Neefjes, J.J. 2001. From fixed to FRAP: Measuring protein mobility and activity in living cells. *Nature Cell Biol*. 3:E145-E147.

Snaar S., Wiesmeijer K., Jochemsen A.G., Tanke H.J., and Dirks R.W. 2000. Mutational analysis of fibrillarin and its mobility in living human cells. *J. Cell Biol.* 151:653-662.

Stenmark, H. and Zerial M. 1998. Lipofection. *In* Cell Biology: A Laboratory Handbook, 2nd Edition, Vol. 4 (J.E. Celis, ed.) pp. 141-144. Academic Press, San Diego.

Swaminathan, R., Hoang, C.P., and Verkman, A.S.. (1997) Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cells: Cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion. *Biophys. J.* 72: 1900-1907.

Tsukamoto, T., Hashiguchi, N., Janicki, SM, Tumbar, T., Belmont, A.S., and Spector, D.L. 2000. Visualization of gene activity in living cells. *Nat. Cell Biol*. 2:871-878.

Verkman, A.S. 2002. Solute and macromolecule diffusion in cellular aqueous compartments. *Trends Biochem. Sci*. 27:27-33.

Contributed by Miroslav Dundr and Tom Misteli
National Cancer Institute
Bethesda, Maryland

Functional Characterization of Proteins Regulating Actin Assembly

The actin cytoskeleton is a dynamic array of polar helical filaments that allows a eukaryotic cell to organize its intracellular space and the traffic of organelles, as well as to react to signals from the outside world by changing shape or migrating. Directed assembly of actin filaments is at the heart of motile processes involved in cell migration, oogenesis, tissue morphogenesis and repair, embryonic development, and the immune response.

A very large repertoire of actin-binding proteins regulates the assembly dynamics and the spatial organization of actin filaments, thus orchestrating the motile behavior of the cell (Small et al., 2002). At least 50 to 60 regulatory proteins have been identified so far, yet regulatory factors endowed with new specific properties continue to be discovered. The goal of this unit is to describe a logical method and derived in vitro assays for the characterization of the function of a putative actin-binding protein. The classical basic regulatory activities are presented here as dogma, or basic themes, on which variations are played by nature to generate the full complexity of the actin response in physiological situations.

In the physiological context, actin is in a dynamic steady state between the monomeric (G, for globular; see Support Protocol 1) and polymeric (F, for filamentous) forms. A filament has two ends that differ both structurally and dynamically: a barbed end (the fast end) and a pointed end (the slow end). Actin is an ATPase: it binds MgATP, and hydrolysis of ATP is associated with incorporation of ATP-G-actin in the filament. Actin assembly at steady state is described by an ATPase cycle featuring the energetic imbalance between the two ends, which is linked to the irreversible hydrolysis of ATP. This cycle comprises three elementary steps: net depolymerization from the pointed end releases ADP-G-actin, exchange of ATP for bound ADP restores ATP-G-actin, which then undergoes net assembly at the barbed end (Fig. 13.6.1). The rate constants of all these steps determine both the concentrations of each species at steady state and the turnover of actin filaments. It is noteworthy that the concentration of unpolymerized ATP-G-actin that is maintained in this cycle is intermediate between the critical concentrations at the barbed and pointed ends.

Individual actin regulatory proteins affect the kinetic and thermodynamic parameters of actin assembly at one or the other end of the filament in various fashions. Actin exposes many functionally relevant sites for regulating assembly: side binding or end binding to the F-actin filament and binding to G-actin in ways that allow, favor, or inhibit incorporation of actin in filaments or that favor or inhibit nucleation of filaments.

A primordial question is whether a given actin-binding protein binds with a higher specificity to G-actin or F-actin. A protein that binds F-actin better than G-actin stabilizes the filaments (i.e., shifts the monomer-polymer equilibrium toward the polymer form), thus causing a decrease in G-actin concentration at steady state. Conversely, a protein that binds G-actin preferentially, also called a G-actin-sequestering factor, shifts the equilibrium toward the monomer, thus causing depolymerization of F-actin. Sedimentation assays (see Basic Protocol 1), which allow separation of F-actin (in the pellet) from free and liganded G-actin (in the supernatant) are useful to address this issue and may provide quantitative information on the protein's affinity for and molar binding ratio to actin. Changes in fluorescence of pyrenyl-labeled actin or other fluorescently labeled forms of actin (see Support Protocols 2 and 3) also provide accurate measurements of the concentration of F-actin and G-actin at the steady state of assembly.

Contributed by Maud Hertzog and Marie-France Carlier
Current Protocols in Cell Biology (2005) 13.6.1-13.6.23

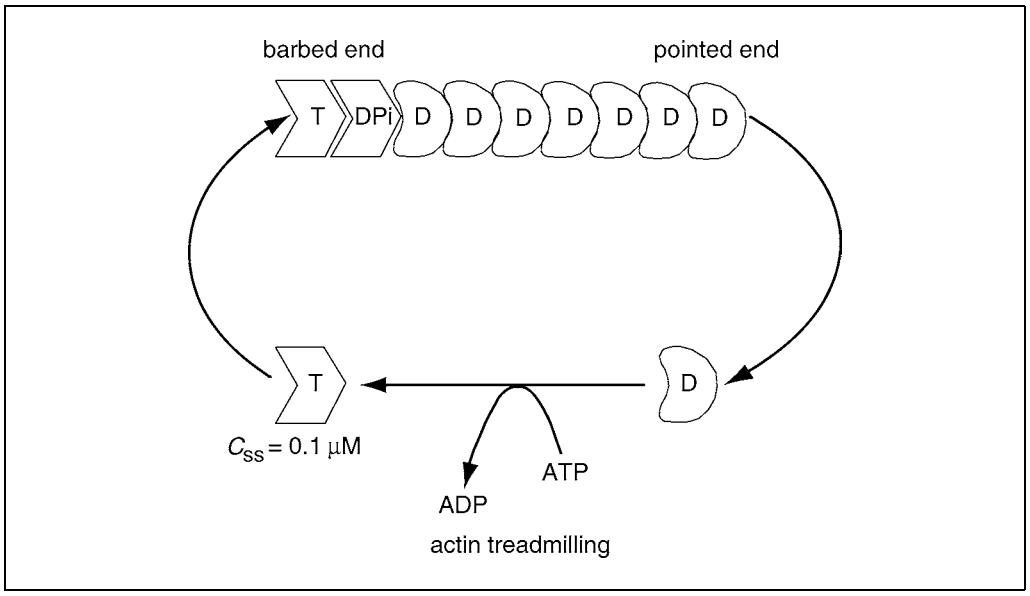


Figure 13.6.1 Treadmilling of the actin filament. This diagram emphasizes that the actin filament has a polar structure, with a barbed end and a pointed end. Under physiological ionic conditions, F-actin is at a steady state with ATP-G-actin monomers. An energetic imbalance occurs between the two ends, because of ATP hydrolysis linked to F-actin assembly. Monomer-polymer exchanges at the two ends do not sum up to zero at each end; instead the barbed ends undergo net growth, balanced by equal depolymerization at the pointed end. This flux of subunits through the filament is called treadmilling. C_{ss}, steady-state (critical) concentration; D, ADP-actin; DPi, ADP-P_i-actin; T, ATP-actin.

Because ATP hydrolysis accompanies filament assembly, a relevant question is whether an actin-binding protein shows specificity for ATP-actin or ADP-actin (in either the G or F form). Direct binding assays (solid phase or fluorometric assays; see Basic Protocols 2 and 3) are recommended. The change in tryptophan fluorescence (actin has four tryptophans) may be used as a probe of the binding of a protein to ATP- or ADP-G-actin. Alternatively, actin can be labeled on specific residues (e.g., Cys374) with a variety of fluorophores that may react to ligand binding, thus providing information on binding affinity and the mechanism of interaction through the use of rapid kinetics. Labeling may alter the affinity of the protein for actin, but this can easily be checked by competition studies with unlabeled actin. Similarly, the interaction with an actin filament can be studied in the standard ADP-F-actin state and also using the nonhydrolyzable analogs BeF_3^- and AlF_4^- in the ATP- or ADP-P_i-F-actin state (Combeau and Carlier, 1988, 1989).

Regulation of filament turnover is crucial in motility and is elicited by changing the kinetic parameters for actin association-dissociation at the two ends of the filament. A large class of actin regulatory proteins, called capping proteins, binds tightly to the barbed end of the actin filament, thus blocking all association-dissociation processes at this end. Appropriate kinetic measurements of filament growth or depolymerization at one end or the other using the change in fluorescence of pyrenyl-labeled actin is described to test the capping activity (see Basic Protocols 5 and 6). Blockage of barbed ends also results in a shift in the steady state critical concentration (C_{ss}) for assembly, increasing it up to the critical concentration at the pointed end (C_c) (see Basic Protocol 4). Most capping proteins simply block barbed ends, but leaky cappers that alter the association or dissociation of actin without blocking the end may exist.

Another way to affect the rate of filament elongation is provided by proteins that interact with G-actin to form a complex that has an association rate constant for filament ends that differs from that of free G-actin. Appropriate assays of filament growth from seeds help to evaluate this possibility. Seeds are preformed filaments with a few subunits, which are used to initiate actin elongation from the barbed or pointed end of the actin filament.

The turnover of a population of filaments in vitro is monitored using a fluorescent analog of ATP whose fluorescence changes when bound to actin. The rate at which F-actin becomes nonfluorescent following an ATP chase is an indication of the filament turnover rate (see Basic Protocol 7).

CO-SEDIMENTATION ASSAY FOR MEASURING BINDING OF A PROTEIN TO F-ACTIN (OR G-ACTIN)

BASIC PROTOCOL 1

F-actin is easily sedimented by centrifuging 20 min at 400,000 × *g* (20°C). G-actin and small complexes of G-actin with a G-actin-binding protein remain in the supernatant.

Materials

50 μM G-actin in G-buffer (see Support Protocol 1), store on ice
2 M KCl
20 mM $MgCl_2$ (*APPENDIX 2A*)
100 μM protein of interest in 10 mM Tris·Cl (pH 7.5)/1 mM dithiothreitol (DTT)
10 mM Tris·Cl (pH 7.5)/1 mM DTT
F-buffer (see recipe)
G-buffer (see recipe)

0.5-ml polycarbonate centrifuge tubes
Beckman TL100 tabletop ultracentrifuge (or equivalent)
Densitometer

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*)

1. To 1 ml of 50 μM G-actin, add 100 μl of 2 M KCl (0.2 M final) and 100 μl of 20 mM $MgCl_2$ (2 mM final). Allow to polymerize 15 min at room temperature.
2. Dilute 360 μl polymerized G-actin (i.e., F-actin) to 1.5 ml with F-buffer. Mix thoroughly and prepare ten samples of 100 μl F-actin (10 μM final) in 0.5-ml polycarbonate centrifuge tubes.
3. Centrifuge 200 μl of 100 μM protein of interest at 400,000 × *g*, 20°C, for 20 min. Prepare ten dilutions of the protein of interest ranging from 0 to 100 μM final concentration in a final volume of 100 μl using 10 mM Tris·Cl/1 mM DTT.
4. Add 100 μl protein of interest dilutions (0 to 50 μM final) to each of ten 100-μl F-actin samples (step 2; 5 μM final) and incubate 15 min at room temperature. Divide each sample between two tubes (100 μl each). Process one sample from each pair immediately (step 5). Incubate the other sample from each pair for 18 hr at room temperature before processing.

   With the longer incubation, one can monitor possible shifts in the steady state of actin assembly (changes in C_{SS}) that are induced by the protein.

5. Centrifuge 20 min at 400,000 × *g*, 20°C, using a tabletop ultracentrifuge. Remove and save supernatant, wash pellets and tube walls three times with 150 μl F-buffer, and resuspend pellets in 150 μl G-buffer.

6. Perform SDS-PAGE of the pellets and supernatants on a 12% gel to visualize full-size actin. Run samples in parallel with appropriate molecular mass standards for actin and the protein of interest (15,000 to 100,000 Da). Identify actin by its molecular weight (42 kDa).

   Under denaturing conditions, F- and G-actin migrate at the same level, but F-actin will be found in the pellet and G-actin will be in the supernatant.

7. Scan the gel and use a densitometer to evaluate the amount of protein bound to F-actin.

   If the protein binds to G-actin, it should cause depolymerization; the amount of G-actin will be greater in the supernatant after overnight incubation of the samples.

BASIC PROTOCOL 2

DIRECT BINDING TO G-ACTIN: FLUORESCENCE MEASUREMENTS

Actin can be fluorescently labeled using a variety of probes, such as 7-chloro-4-nitrobenzofurazane (NBD-Cl), pyrene, BODIPY, and *N*-iodoacetyl-*N*′-5sulfo-1-naphtyl-ethylenediamine (IAEDANS), that are differentially sensitive to local environments. Thus, binding of an actin-binding protein to the fluorescently labeled actin can lead to a change in fluorescence. The change in fluorescence (ΔF) reflects the fraction of actin in complex with the protein (Y) as follows:

$$Y = \Delta F/\Delta F_{max} = [\mathrm{PA}]/[A]_0$$

Equation 13.6.1

where ΔF_{max} is the maximal extent of change in fluorescence that is caused by the protein at a saturating actin concentration, [PA] is the concentration of the protein-actin complex, and $[A]_0$ is the total actin concentration.

Several fluorescent labels should be tried (see Support Protocols 2 and 3 for two examples), to determine which is best suited to the purpose (see Troubleshooting). This assay may be performed with ATP-G-actin or ADP-G-actin to determine which actin species is preferentially bound by the protein. For example, β-thymosins and profilin prefer ATP-actin, whereas ADF/cofilin and twinfilin prefer ADP-actin.

Materials

1.5 μM 100% fluorescently labeled G-actin in G-buffer (see Support Protocols 2 and 3)
Actin-binding protein of interest in G-buffer (see recipe)
Spectrofluorometer (time base mode) at wavelengths appropriate for label.

1. Using a spectrofluorometer, record emission and excitation spectra of 1.5 μM fluorescently labeled G-actin in the absence and in the presence of a saturating amount of an actin-binding protein of interest in G-buffer. Choose the set of excitation and emission wavelengths at which the largest change is observed upon association of actin with the protein.

   A protein is at a saturating amount when its concentration is about ten times its K_d.

   For NBD-labeled actin, $\lambda_{ex} = 475$ nm and $\lambda_{em} = 530$ nm; for IDEDANS-labeled actin, $\lambda_{ex} = 340$ nm and $\lambda_{em} = 410$ nm; and for prenyl-labeled actin, $\lambda_{ex} = 366$-nm and $\lambda_{em} = 407$.

   This protocol can also be carried out in F-buffer (see recipe) to determine the effect of ionic strength on binding.

2. Measure the fluorescence of 1.5 μM fluorescently labeled G-actin alone and in the presence of increasing amounts of the actin-binding protein across a range of 0 to 100 μM.

This protocol is suitable if the K_d is in the 0.5- to 10-μM range. If the K_d is much lower, the experiment should be carried out at a lower actin concentration (~0.1 μM) and with a lower range of protein concentrations (0 to 1 μM). For proteins with an unknown K_d, the entire range should be tested at first, with a subsequent finer resolution of solution concentrations tested once an approximate K_d is known.

3. Plot the change in fluorescence versus the protein concentration and perform data analysis.

If a simple 1:1 complex (PA) is formed between actin (A) and the protein (P), then the value of the equilibrium dissociation constant for the PA complex, K_p, can be derived from analysis of the fluorescence curve:

$$\Delta F/\Delta F_{max} = \left\{ \begin{array}{l} [A]_0 + [P]_0 + K_P \pm \\ \left(\{[A]_0 + [P]_0 + K_P\}^2 - 4[P]_0 \times [A]_0\right)^{1/2} \end{array} \right\} / 2[A]_0$$

Equation 13.6.2

where $[P]_0$ is the total protein concentration.

This method is useful to measure K_p in the range of 0.1 to 10 μM. To obtain a reliable value of K_p, the concentration of G-actin in the assay should be as close as possible to the value of K_p. A few preliminary trials at different actin concentrations are advisable.

BINDING TO ACTIN DERIVED FROM A CHANGE IN THE RATE OF NUCLEOTIDE DISSOCIATION FROM G-ACTIN

BASIC PROTOCOL 3

Many G-actin-binding proteins affect the rate of nucleotide exchange on G-actin. For instance, profilin increases the rate of nucleotide dissociation from G-actin, whereas β-thymosins and cofilin slow it down. On the condition that the protein (P) shuttles more rapidly between actin molecules (A) than nucleotide dissociates from either actin or the PA complex, the change in the observed first-order rate constant for nucleotide dissociation upon increasing the concentration of protein reflects the saturation of actin by the protein.

Dissociation of G-actin-bound ATP can be monitored by the increase in etheno-ATP (ε-ATP) fluorescence that takes place upon addition of a 10-fold molar excess of ε-ATP to the ATP-G-actin 1:1 complex. The fluorescence of ε-ATP increases 6-fold upon binding to G-actin. Because ATP dissociation from G-actin is rate limited by dissociation of the bound metal ion (the Ca^{2+} ion in G_0-buffer, which is G-buffer without ATP) and is affected by ionic strength, all measurements performed in the absence and presence of the actin-binding protein should be carried out under identical ionic conditions (i.e., the protein to be tested must be equilibrated in the G_0-buffer used for the assays).

Materials

50% suspension of Dowex-1-X8 (Sigma) *or* AG-1-X8 (Bio-Rad) strong anion exchange resin in G_0-buffer
50 μM G-actin in G-buffer (see Support Protocol 1)
G_0-buffer: G-buffer (see recipe) without ATP
1 mM etheno-ATP (ε-ATP), pH adjusted to 7.2 with NaOH, stock solution (Sigma)
100 μM protein of interest in 10 mM Tris·Cl (pH 7.5)/1 mM dithiothreitol (DTT)
Microcentrifuge, 4°C
Spectrophotometer and quartz cuvettes
Spectrofluorometer (time base mode): $\lambda_{ex} = 350$ nm, $\lambda_{em} = 410$ nm, slits = 5- to 10-nm band width

1. Add 150 µl of a 50% Dowex-1-X8 suspension in G_0-buffer to 1 ml of 50 µM G-actin in a 1.5-ml microcentrifuge tube. Incubate 30 sec on ice while rotating the tube to mix actin and Dowex-1-X8. Microcentrifuge 1 min at 10,000 × *g*, 4°C, and transfer supernatant to a clean tube.

2. Repeat step 1 with 150 µl fresh Dowex-1-X8.

   The supernatant should contain ATP-G-actin in a 1:1 complex.

3. Measure the UV spectrum in a spectrophotometer to determine whether free ATP has been removed.

   The maximum of the UV spectrum should shift from 260 nm (typical of ATP) to 280 nm (typical of protein) and should show a minimum at 250 nm.

   Most generally no dilution is required, even when using a 1-cm path cuvette. If the absorbance of the sample is larger than 3.0 at the peak, the authors generally use a smaller path cuvette (e.g. 0.2 cm) rather than diluting, and thus losing, an aliquot of the sample.

   If ATP has not been totally removed, the Dowex treatment should be repeated.

4. Use the absorbance at 290 nm to measure the concentration of ATP-G-actin 1:1 complex ($\varepsilon = 0.617$ cm^2/mg at 290 nm).

   The absorbance at 278 nm is 1.97-fold higher.

5. Dilute ATP-G-actin (1:1 complex) to 2 µM in G_0-buffer. Mix together 50 µl of 2 µM ATP-G-actin and 49 µl G_0-buffer in a 1.5-ml microcentrifuge tube. Transfer solution to a microcuvette. At time zero, add 1 µl of 1 mM ε-ATP (10 µM final) to start the exchange reaction. Monitor and record the increase in fluorescence (~50% increase) at 410 nm (excitation wavelength, 350 nm) in a spectrofluorometer.

   For G-actin in Ca^{2+}-containing buffer at a low ionic strength, dissociation of ATP follows the rate-limiting dissociation of bound metal ion. The rate of dissociation therefore varies with the concentration of free Ca^{2+} (Nowak et al., 1988; Valentin-Ranc and Carlier, 1989), and the measurement period will need to be adjusted accordingly.

6. Prepare 10 dilutions of the protein of interest using G_0-buffer to final concentrations of 0 to 50 µM in a final volume of 50 µl.

7. Repeat step 5, replacing G_0-buffer with the protein of interest diluted in G_0-buffer.

   An actin-binding protein that binds ATP-G-actin would be expected to cause an increase or a decrease in the rate of ATP association.

8. To measure the change in rate of ATP dissociation, derive the first-order rate constant (k_{obs}) at different concentrations of the protein.

   The time course of ATP dissociation is a monoexponential process. The change in k_{obs} versus the total concentration of the protein reflects the formation of the PA complex.

9. Determine *Y*, the fraction of actin in complex with the protein, as follows:

$$Y = \left(k_{obs} - k_{obs}^{0}\right) / \left(k_{obs}^{\infty} - k_{obs}^{0}\right)$$

Equation 13.6.3

where k_{obs}^{0} and k_{obs}^{∞} are the rate constants measured in the absence of protein and in the presence of a saturating amount of protein, respectively, and $[P]_0$ is the total concentration of protein. To derive K_P, plot *Y* versus $[P]_0$ as described in Equation 13.6.2 (Perelroizen et al., 1995).

STEADY-STATE MEASUREMENTS OF ACTIN ASSEMBLY: CRITICAL CONCENTRATION PLOTS

The 25-fold increase in fluorescence of pyrenyl-labeled actin linked to the G-to-F transition is the most accurate and convenient tool for measuring the critical concentration (C_{ss}) for actin polymerization at the barbed and pointed ends.

Materials

50 μM pyrenyl-labeled G-actin (see Support Protocol 2), 10% labeled
G-buffer (see recipe)
Gelsolin solution: 100 μM gelsolin (Sigma) in 10 mM Tris·Cl (pH 7.5)/1 mM dithiothreitol (DTT), for assaying actin with capped barbed ends only
Protein of interest
KM solution: 500 μl of 4 M KCl/20 μl of 1 M $MgCl_2$ (*APPENDIX 2A*)
Spectrofluorometer (time base mode): $\lambda_{ex} = 360$ nm, $\lambda_{em} = 407$ nm, slits = 5- to 10-nm band width

1a. *To assay actin with free barbed ends:* Dilute 200 μl of 50 μM pyrenyl-labeled G-actin to 1 ml with G-buffer (continue with step 2).

1b. *To assay actin with capped barbed ends:* To pretreat a 50 μM pyrenyl-labeled G-actin solution, mix together the following:

200 μl of 50 μM pyrenyl-labeled G-actin (10 μM final)
800 μl G-buffer
0.4 μl gelsolin solution (0.4 μM final).

Gelsolin needs calcium; EGTA must not be present in any solutions used for this assay.

2. To polymerize actin, add 50 μl KM solution (5%, v/v) to 1 ml untreated or gelsolin-pretreated G-actin (step 1a or 1b).

Two complementary types of assays will be carried out. In the first (step 3), the total actin concentration is varied in the presence of a constant amount of the protein of interest. In the second (step 4), actin is maintained at a given concentration, whereas the concentration of the protein of interest is varied.

3. Prepare fifteen dilutions of F-actin from 0 to 2 μM in F-buffer along with a single concentration of the protein of interest. The final volume should be 100 μl. Be sure to include control samples that do not contain the protein.

4. Prepare twelve dilutions of the protein of interest (e.g., from 0 to 10 or 20 μM) in F-buffer along with a single concentration of F-actin. The final volume should be 100 μl.

5. Incubate all samples overnight in the dark either at room temperature or at 4°C, depending on the stability of the protein of interest.

6. Add 10 μl of each protein dilution to a sample tube (step 2). Add 5 μl KM solution to all 20 tubes and incubate overnight at room temperature.

7. Read fluorescence of pyrenyl-actin from control and sample tubes in a spectrofluorometer.

8. Plot fluorescence intensity versus either total actin concentration (samples from step 3) or concentration of the protein of interest (samples from step 4) and determine C_{ss}.

The increase in pyrenyl-actin fluorescence is proportional to the amount of F-actin assembled. Fluorescence increases linearly with actin concentration. The slope is very low when actin is monomeric and 25-fold higher when actin is polymerized. C_{ss} is defined as the total actin concentration at which a large change in slope takes place (i.e., above which actin polymerizes).

Under physiological ionic conditions, a value of 0.1 μM is measured for C_{ss} when barbed ends are free, and 0.6 μM when barbed ends are capped. The value of 0.6 μM is the critical concentration (C_c) at the pointed ends. The value of 0.1 μM is slightly above the C_{ss} at the barbed ends, because pointed ends also contribute to assembly. Because the contribution of barbed ends is largely predominant, however, it is routinely considered that 0.1 μM is very close (within 15%) to the C_{ss} at the barbed ends.

BASIC PROTOCOL 5

INITIAL RATE OF FILAMENT GROWTH AT BARBED OR POINTED ENDS

Initial rates of filament growth at barbed ends are measured spectrofluorometrically using spectrin-actin seeds or F-actin filaments. Growth at pointed ends is measured using gelsolin-actin seeds [i.e., the gelsolin(actin)$_2$ (GA_2) tight complex] or gelsolin-capped filaments.

Materials

Actin seeds (choose one):
- 100 nM gelsolin-actin (for pointed-end growth): add 2.5 molar equiv G-actin to gelsolin in G-buffer
- 5 nM spectrin-actin isolated from human blood (Casella et al., 1986; for barbed-end growth) in G-buffer
- 5 μM F-actin (with or without 20 nM gelsolin)

KME solution (see recipe)

5 μM pyrenyl-labeled (10%) G-actin (see Support Protocol 2)

G-buffer (see recipe)

100 μM protein of interest in 10 mM Tris·Cl (pH 7.5)/1 mM dithiothreitol (DTT)

Microcuvettes suitable for spectrofluorometer

Spectrofluorometer (time base mode): $\lambda_{ex} = 366$ nm, $\lambda_{em} = 407$ nm, slits = 5- to 10-nm band width

Additional reagents and equipment to generate a calibration curve (see Basic Protocol 4)

1. Mix the following in a microcuvette:
   - 51 μl G-buffer
   - 4 μl actin seeds (final 4 nM for gelsolin-actin, 0.2 nM for spectrin-actin, or 0.2 μM for F-actin)
   - 5 μl KME solution
   - 40 μl 5 μM pyrenyl-labeled G-actin.
2. Immediately place the cuvette in a spectrofluorometer (time zero) and read the increase in fluorescence at 407 nm versus time for a few minutes.
3. Repeat the assay (steps 1 and 2) in the presence of the protein of interest at various concentrations (about twelve to fifteen different concentrations in a final range of 0 to 100 nM for a K_d in the nanomolar range and 0 to 100 μM for a K_d in the micromolar range), diluted from a 100 μM solution. Adjust the amount of G-buffer added to the assay to keep a final volume of 100 μl.

   For proteins with an unknown K_d, the entire range should be tested at first, with a subsequent finer resolution of solution concentrations tested once an approximate K_d is known.

4. Determine the initial rate of fluorescence increase in absorbance units (arbitrary unit of fluorescence intensity) per second (AU/sec) and convert to molar amount of F-actin assembled per second using a calibration curve (see Basic Protocol 4).

The initial rate of actin assembly or velocity (V) measured when filaments or seeds at a concentration [F] are placed in a solution of G-actin at concentration C is

$$V = k_+[F] \times \left(C - k_-\right)$$

Equation 13.6.4

$$V = k_+[F] \times \left(C - C_C\right)$$

Equation 13.6.5

Where k_+ and k_- are the rate constants for G-actin association to and dissociation from, respectively, the filament end, and C_c is the critical concentration (k_-/k_+). The effect of the protein on V provides insight into its function. Barbed-end capping and G-actin sequestering functions give different results (see Anticipated Results).

MEASUREMENT OF DILUTION-INDUCED DEPOLYMERIZATION OF FILAMENTS

BASIC PROTOCOL 6

The depolymerization rate of actin filaments is monitored by the decrease in pyrenyl fluorescence upon dilution of the solution of pyrenyl-labeled F-actin in F-buffer to a final actin concentration that is lower than the critical concentration (C_{ss}). When depolymerization from the pointed end is to be measured, gelsolin-capped filaments are used.

Materials

G-buffer (see recipe)
4 M KCl
100 mM $MgCl_2$ (*APPENDIX 2A*)
50 μM G-actin in G-buffer (see Support Protocol 1)
50 μM G-actin, 50% to 100% pyrenyl labeled in G-buffer (see Support Protocol 2)
Gelsolin solution: 100 μM gelsolin in 10 mM Tris·Cl (pH 7.5)/1 mM dithiothreitol (DTT), for assaying depolymerization from pointed ends only
100 μM protein of interest in 10 mM Tris·Cl (pH 7.5)/1 mM DTT
F-buffer (see recipe)

Microcuvettes, suitable for spectrofluorometer
Spectrofluorometer (time base mode): $\lambda_{ex} = 366$ nm, $\lambda_{em} = 407$ nm, slits = 5- to 10-nm band width

1a. *To measure total depolymerization from the actin filament:* Mix together the following in a 1.5-ml microcentrifuge tube:

865 μl G-buffer
25 μl 4 M KCl (0.1 M final)
10 μl 100 mM $MgCl_2$ (1 mM final)
50 μl 50 μM G-actin (5 μM final)
50 μl 50 μM pyrenyl-labeled G-actin (5 μM final).

Incubate 30 min at room temperature.

1b. *To measure depolymerization from the pointed end of the actin filament:* Mix together the same reagents as in step 1a but add 0.2 μl gelsolin solution (20 nM final gelsolin concentration). Incubate 1 hr at room temperature.

Gelsolin needs calcium; EGTA must not be present in any solutions used for this assay. A longer incubation allows a more complete exchange of calcium and magnesium.

Steps 1a and 1b yield 50% pyrenyl-labeled F-actin at 5 μM.

2. Dilute a 100 μM solution of the protein of interest in F-buffer as needed to generate stock solutions to add to the assay. Generate stocks that will give twelve to fifteen different concentrations in a final range of 1 to 100 nM for a K_d in the nanomolar range and 0 to 100 μM for a K_d in the micromolar range.

   For proteins with an unknown K_d, the entire range should be tested at first, with a subsequent finer resolution of solution concentrations tested once an approximate K_d is known.

3. At time zero, dilute 2 μl of the 5 μM actin solution (step 1a or 1b) 100-fold in F-buffer in a spectrofluorometer microcuvette and measure the decrease of pyrenyl fluorescence in a spectrofluorometer in the absence or in the presence of the protein of interest at various concentrations. Record the change in fluorescence for a few minutes.

   Total depolymerization occurs because the final actin concentration (50 nM) is lower than the critical concentration (0.1 μM).

4. Plot the change in fluorescence versus time. Calculate the initial rate of depolymerization from the slope of the time-dependent decrease in pyrenyl fluorescence.

5. Plot a calibration curve of the fluorescence of pyrenyl-labeled F-actin versus total actin concentration. Convert fluorescence units into micromolar F-actin and then express the initial rate of depolymerization in micromolar per second.

BASIC PROTOCOL 7

MEASUREMENTS OF THE TREADMILLING OF ACTIN FILAMENTS

The turnover of filaments is evaluated by measuring the rate at which fluorescently labeled etheno (ε)-ADP-F-actin filaments assemble from ε-ATP-G-actin subunits and become nonfluorescent ADP-F-actin filaments after addition of ATP to the medium. Turnover occurs via the consecutive steps of dissociation of ε-ADP-actin from the pointed end, exchange of ATP for bound ε-ADP on G-actin in the medium, and association of ATP-G-actin to barbed ends. The fluorescence of ε-ADP is 6-fold lower in the free state than it is in the actin-bound state.

Materials

50% (w/v) suspension of Dowex-1-X8 (Sigma) *or* AG-1-X8 (Bio-Rad) strong anion exchange resin in G_0-buffer
50 μM G-actin in G- buffer (see Support Protocol 1), store on ice
5 mM etheno-ATP (ε-ATP), pH adjusted to 7.2 with NaOH, stock solution (Sigma)
KME solution (see recipe)
G-buffer (see recipe)
100 μM protein of interest in 10 mM Tris·Cl (pH 7.5)/1 mM dithiothreitol (DTT)
200 mM ATP, pH adjusted to 7.2 with NaOH, stock solution (Sigma)

Microcentrifuge, 4°C
Spectrophotometer microcuvette, suitable for spectrofluorometer
Spectrofluorometer (time base mode; PM voltage adequate): $\lambda_{ex} = 350$ nm, $\lambda_{em} = 410$ nm, slits = 5- to 10-nm band width

1. Add 150 μl of a 50% Dowex-1-X8 suspension in G_0-buffer to 1 ml of 50 μM G-actin in a 1.5-ml microcentrifuge tube. Incubate 30 sec at 0°C. Microcentrifuge 1 min at 10,000 × *g*, 4°C, and transfer supernatant to a clean tube.

2. Repeat step 1 with 150 μl fresh Dowex-1-X8.

   The supernatant should contain ATP-G-actin in a 1:1 complex.

3. Add 50 μl of 5 mM ε-ATP (0.25 mM final). Let the solution sit overnight on ice to allow exchange of ε-ATP for bound ATP.

4. Repeat steps 1 and 2 to obtain ε-ATP-G-actin in a 1:1 complex. Measure concentration of G-actin spectrophotometrically (see Basic Protocol 3, steps 3 and 4).
5. Add 5 mM ε-ATP to make a final concentration of 50 μM free ε-ATP.
6. In a 1.5-ml microcentrifuge tube, mix together ε-ATP-G-actin (5 μM final) and 5% (v/v) KME buffer along with G-buffer as needed.

   Adding 5% (v/v) KME buffer will lead to polymerization, yielding ε-ATP-F-actin.
7. Divide the sample in 100-μl aliquots among several tubes. Add 100 μM protein of interest to individual tubes to cover a final range of 0 to 50 μM. Adjust volumes as needed with G-buffer.

   This step is used to test the effects of actin depolymerizing factor (ADF), profilin, or other proteins.
8. Transfer each sample to a spectrofluorometer microcuvette and measure its ε-ATP fluorescence intensity in a spectrofluorometer at 410 nm. At time zero, add 0.25 μl of 200 mM ATP (500 μM final).
9. Record the decrease in ε-ATP fluorescence using a time-based scan over at least 2 hr. Determine the rate of fluorescence decrease, which provides an indication of filament turnover.

ACTIN PURIFICATION FROM RABBIT MUSCLE

SUPPORT PROTOCOL 1

Actin for these protocols is purified from an acetone powder of rabbit muscle. Actin is also available commercially (Cytoskeleton). To prepare commercially purchased actin for the other protocols in this unit, follow the manufacturer's instructions.

Materials

Acetone powder of rabbit muscle (Cytoskeleton)
Extraction buffer (see recipe), 4°C
Solid KCl and 4 M KCl
Buffer D1 (see recipe)
1 M $MgCl_2$ (*APPENDIX 2A*)
G-buffer (see recipe)

500-ml beaker
Sorvall centrifuge and A641 rotor (or equivalent), 4°C
Glass wool
20°C water bath
Large (3-cm wide) dialysis bags (MWCO 14,000)
Dounce homogenizer (glass-Teflon; 20-ml working volume)
Sonicator with microtip (e.g., Vibra-cell; Sonics & Materials)
10-ml ultracentrifuge tubes
Beckman ultracentrifuge and 70.1 Ti rotor (or equivalent), 4°C
2.5 × 100–cm gel filtration column (Superdex-200 prep grade; Amersham Biosciences)
Spectrophotometer (290 nm) and quartz cuvettes

Extract actin

1. Place 9 g acetone powder in a 500 ml beaker on ice and slowly add 270 ml cold extraction buffer while gently stirring with a glass rod to thoroughly wet powder. Let sit on ice for 30 min, stirring gently once every 10 min.

   Gentle stirring minimizes α-actinin extraction.

2. Centrifuge 45 min at 25,000 × *g*, 4°C, in a Sorvall A641 rotor. Filter supernatant through glass wool into a graduated cylinder and measure volume.

3. Transfer filtrate to a beaker. Add solid KCl to 3.3 M while stirring (add KCl all at once).

   The KCl adds ~10% to the volume, which must be figured in when deciding how much KCl to add (usually 55 to 57 g for ~220 ml).

4. Place in a 20°C water bath and stir until temperature reaches 15°C. Place on ice without stirring until temperature returns to 5°C. Centrifuge 30 min at 25,000 × *g*, 4°C.

   A tight white pellet indicates the presence of actin-α-actinin networks.

5. Filter supernatant through glass wool, transfer to a large dialysis bag, and dialyze overnight at 4°C against 32 vol (usually 6 to 8 liters) buffer D1.

Release tropomyosin from F-actin

6. Add 4 M KCl to a final concentration of 0.8 M (0.22 vol of 4 M KCl) and stir for 1.5 hr at 4°C.

7. Centrifuge 3.5 hr at 100,000 × *g*, 4°C, in the Sorvall A641 rotor. Decant and discard supernatant. Dislodge pellet and transfer to a Dounce homogenizer, using 25 ml (total) extraction buffer to transfer pellet and clean centrifuge tube.

8. Homogenize pellet with 20 strokes while keeping homogenizer on ice.

9. Add 75 µl of 1 M $MgCl_2$ and 375 µl of 4 M KCl and adjust the volume to 38.6 ml with extraction buffer. Leave at 4°C overnight without stirring.

10. Add 9 ml of 4 M KCl and 2.37 ml extraction buffer and stir for 1.5 hr at 4°C. Centrifuge 3.5 hr at 100,000 × *g*, 4°C.

11. Resuspend pellets with 20 to 30 ml extraction buffer and homogenize as described (step 8).

12. Dialyze against 2 liters G-buffer for 2 days at 4°C with one change of buffer.

13. Insert microtip of a sonicator into dialysis bag, sonicate twice for 30 sec each on low-power setting. Reclose bag, change dialysis buffer, and continue dialysis one additional day.

Purify actin

14. To clarify actin, transfer to 10-ml ultracentrifuge tubes and centrifuge 2 hr at 400,000 × *g*, 4°C, in a Beckman 70.1 Ti rotor.

15. Load supernatant onto a 2.5 × 100–cm gel filtration column. Run 2 liters G-buffer through column at 1 ml/min 4°C. Collect 5-ml fractions.

16. Read A_{290} of each fraction in a spectrophotometer and pool the fractions showing an absorbance at 290 nm.

17. Determine the concentration of G-actin in the pooled fractions using A_{290} as follows:

$$C = \left(A_{290} / .0617\right) / 42$$

Equation 13.6.6

where *C* is the actin concentration in milligrams per milliliter.

18. Store actin on ice for up to 3 weeks

The typical yield of this preparation is 40 ml of 50 μM (2.1 mg/ml) G-actin (mol. wt. = 42 kDa).

PREPARATION OF PYRENYL-LABELED ACTIN

SUPPORT PROTOCOL 2

N-pyrenyliodoacetamide (NPI) is a fluorescent compound that can be used to label actin specifically on Cys374. Its fluorescence changes in response to the local environment; in particular, it increases 25-fold upon polymerization of G-actin into F-actin. Pyrenyl-labeled actin can also be used to monitor binding of proteins to actin.

Additional Materials *(also see Support Protocol 1)*

4 mg/ml (~95 μM) G-actin in G-buffer (see Support Protocol 1)
Buffer A (see recipe)
N-pyrenyliodoacetamide (NPI; Sigma)
Dimethylformamide
0.2 M dithiothreitol (DTT; *APPENDIX 2A*)
Rotating wheel, 4°C

1. Mix 10 ml of 4 mg/ml G-actin and 10 ml buffer A. Incubate 1 hr at 25°C (room temperature) to polymerize actin.
2. Transfer polymerized actin (F-actin) to a large dialysis bag and dialyze overnight against 2 liters buffer A at 4°C.

   As NPI is destroyed by the light, all labeling steps must be carried out in the dark.

3. Prepare 1 ml solution of 14 mg/ml NPI in dimethylformamide in a 1.5-ml microcentrifuge tube. Add 0.2 ml NPI solution to 20 ml F-actin (step 2) in a 50-ml conical centrifuge tube wrapped with aluminum foil. Mix by inverting the tube several times. Incubate overnight on a rotating wheel in the dark at room temperature.

   The solution becomes white after mixing because of insoluble NPI.

4. Add 50 μl of 0.2 M DTT to stop the reaction. Centrifuge 15 min at 1,000 × *g*, 4°C, to remove excess insoluble NPI.
5. Centrifuge supernatant 3.5 hr at 100,000 × *g*, 4°C in a Sorvall A641 rotor.
6. Dislodge the actin pellet and resuspend with 5 ml G-buffer. Homogenize with 20 strokes of a Dounce homogenizer on ice.
7. Continue with actin purification as described (see Support Protocol 1, steps 12 to 18), except dialyze against 1 liter G-buffer in steps 12 and 13 and perform all steps in the dark.

   G-actin thus prepared is 100% labeled. For a 10% labeled G-actin solution, 0.9× moles of regular unlabeled actin (see Support Protocol 1) is mixed with 0.1× moles of 100% pyrenyl-labeled G-actin in G-buffer.

PREPARATION OF 7-CHLORO-4-NITROBENZENO-2-OXA-1,3-DIAZOLE-LABELED ACTIN

SUPPORT PROTOCOL 3

7-Chloro-4-nitrobenzeno-2-oxa-1,3-diazole (NBD-Cl) is a fluorescent compound that can be used to label actin specifically on Lys373. Its fluorescence changes in response to the local environment. Thus, NBD-labeled actin can be used to monitor binding of proteins to actin. The fluorescence of NBD-labeled actin also increases 2.6-fold upon polymerization of G-actin into F-actin.

Additional Materials *(also see Support Protocol 1)*

0.1 M KCl (*APPENDIX 2A*)
50 μM G-actin in G-buffer (see Support Protocol 1)
100 mM *N*-ethylmaleimide (NEM), prepare fresh
20 mM dithiothreitol (DTT; *APPENDIX 2A*)
G-buffer (see recipe) with and without DTT and NaN_3
F-buffer (see recipe) without DTT and NaN_3
10 mM 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole (NBD-Cl) in dimethylformamide
15°C water bath

Label actin with NEM

1. Add 750 μl of 0.1 M KCl to 15 ml of 50 μM G-actin in G-buffer in a tube. Add 375 μl of 100 mM NEM (2.5 mM final), mix, and incubate as follows:

   15 min at room temperature
   15 min at 15°C
   3 hr at 0°C (on ice).

2. Stop the reaction by adding 375 μl of 20 mM DTT (5 mM final). Centrifuge 2 hr at 200,000 × *g*, 4°C a Beckman ultracentrifuge.

3. Discard supernatant and use 20 ml G-buffer with DTT and NaN_3 to resuspend pellet and transfer to a Dounce homogenizer. Homogenize pellet with 20 strokes on ice.

   The pellet contains NEM-F-actin.

Label NEM-actin with NBD

4. Transfer homogenized actin to a large dialysis bag and dialyze 2 days against 1 liter G-buffer without DTT and NaN_3 at 4°C.

5. Centrifuge 2 hr at 400,000 × *g*, 4°C, in the Beckman 70.1 Ti rotor.

   NEM-G-actin is in the supernatant.

6. Dilute supernatant 2-fold to a total volume of 30 ml with F-buffer without DTT and NaN_3. Let polymerize overnight at 4°C in the dark.

   The actin concentration should be at 1 to 2 mg/ml to optimize NBD labeling.

7. Add 1.2 ml of 10 mM NBD-Cl (0.4 mM final). Incubate 5 hr at 15°C.

Purify labeled actin

8. Centrifuge NBD-labeled F-actin 2 hr at 400,000 × *g*, 4°C. Homogenize pellet and dialyze as described (steps 3 and 4).

9. Centrifuge 2 hr at 20,000 × *g*, 4°C, in the Sorvall A641 rotor to eliminate aggregates.

   NBD-labeled G-actin is in the supernatant.

10. Purify actin as described (see Support Protocol 1, steps 14 to 18) except perform all steps in the dark.

    G-actin thus prepared is 100% labeled. For a 10% labeled G-actin solution, 0.9× moles of regular unlabeled actin (see Support Protocol 1) is mixed with 0.1× moles of 100% NBD-labeled G-actin in G-buffer.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. Prepare all buffers 1 day in advance. Store at 4°C for up to 1 week. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

20 ml 0.5 M Tris·Cl, pH 8 (5 mM final; *APPENDIX 2A*)
50 ml 4 M KCl (0.1 M final)
4 ml 1 M $MgCl_2$ (2 mM final; *APPENDIX 2A*)
1 ml 0.2 M $CaCl_2$ (0.1 mM final; *APPENDIX 2A*)
5 ml 0.2 M ATP (0.5 mM final)
1 ml 0.2 M dithiothreitol (DTT; 0.1 mM final; *APPENDIX 2A*)
H_2O to 2 liters

Buffer D1

2 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
1 mM $MgCl_2$ (*APPENDIX 2A*)
1 mM dithiothreitol (DTT; *APPENDIX 2A*)

Extraction buffer

2 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.5 mM ATP
0.1 mM $CaCl_2$ (*APPENDIX 2A*)
0.01% (w/v) NaN_3
1 mM dithiothreitol (DTT; *APPENDIX 2A*)

CAUTION: *Sodium azide is poisonous. Follow appropriate precautions for handling, storage, and disposal.*

F-buffer

G-buffer (see recipe) supplemented with 0.1 M KCl (*APPENDIX 2A*) and 1 mM $MgCl_2$ (*APPENDIX 2A*).

G-buffer

5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.2 mM ATP
0.1 mM $CaCl_2$ (*APPENDIX 2A*)
1 mM dithiothreitol (DTT; *APPENDIX 2A*)
0.01% (w/v) NaN_3

CAUTION: *Sodium azide is poisonous. Follow appropriate precautions for handling, storage, and disposal.*

G_0-buffer is G-buffer without ATP.

KME solution

500 µl 4 M KCl (2 M final)
20 µl 1 M $MgCl_2$ (20 mM final; *APPENDIX 2A*)
16 µl 250 mM EGTA (4 mM final)
464 µl H_2O

To polymerize actin, 0.5% (v/v) KME solution is added to G-actin (final 0.1 M KCl, 1 mM $MgCl_2$, 0.2 mM EGTA).

COMMENTARY

Background Information

G-actin-sequestering proteins

G-actin sequesterers bind G-actin in a non-polymerizable complex. β-Thymosins are the most abundant typical G-actin-sequestering proteins (Cassimeris et al., 1992; Safer and Nachmias, 1994). The β-thymosin-actin complex associates with neither the barbed nor the pointed end of the filament, and thus β-thymosins do not affect filament assembly dynamics or motility. It is often thought that these proteins buffer the free ATP-G-actin concentration in cells. On the contrary, both in vitro and in vivo evidence shows that β-thymosins react passively to changes in the steady-state concentration of free G-actin (Carlier et al., 1993; Safer and Nachmias, 1994).

Profilin and profilin-like proteins

Profilin also binds G-actin but acts differently from sequestering proteins. The profilin-actin complex associates productively with the barbed end (the dynamic end of the actin filament) but not with the pointed ends of the filaments (Pantaloni and Carlier, 1993). Thus, profilin merely sequesters actin when all barbed ends are capped but enhances barbed-end growth as soon as a few of them are not capped. Recently, other proteins like the β-thymosin repeat proteins and, more generally, the actin-binding WH2 (WASP-homology 2) domains, which are inserted in a number of proteins involved in motility, have been found to behave functionally like profilin (Egile et al., 1999; Boquet et al., 2000). As a result, profilin and its functional homologs improve the processivity of treadmilling in synergy with actin depolymerizing factor (ADF) and so enhance actin-based motility (Loisel et al., 1999; Paunola et al., 2002).

Capping proteins

Capping proteins bind tightly to the barbed end and block actin association and dissociation (Hug et al., 1995; Sun et al., 1995). Capping proteins are required for efficient motility of many cells. By blocking a large fraction of the barbed ends at the steady state of actin assembly, capping proteins funnel the treadmilling process: pointed-end depolymerization of many capped filaments feeds the growth of a few noncapped filaments, which individually grow faster than if the other filaments were not capped. Capping proteins cooperate with ADF to accelerate actin-based motility.

ADF/cofilin proteins

ADF/cofilin proteins are essential proteins in morphogenetic and motile processes. ADF/cofilin increases the turnover of actin filaments, which powers actin-based motility (Didry et al., 1998; Carlier et al., 1999). The

Figure 13.6.2 *(at right)* Functional assays of a G-actin-sequestering protein. (**A**) Steady state measurements of F-actin in the presence of increasing amounts of the sequestering protein (see Basic Protocol 4). F-actin (1.5 μM, 10% pyrenyl labeled) with free barbed ends (solid line) or gelsolin-capped barbed ends (dashed line) was supplemented by the protein as indicated. Linear depolymerization was consistent with a K_d of 2 μM. (**B**) Steady-state measurements of F-actin in the presence of the sequestering protein. Experiments were conducted as in A at 2, 1.5, and 1 μM F-actin with free barbed ends. The parallel straight lines are consistent with a K_d of 2 μM. (**C**) Critical-concentration (C_{ss}) measurements for actin alone or in the presence of 2 μM sequestering protein. The sequestering protein shifts C_{ss} for both free and capped barbed ends, in agreement with the K_d obtained in B. Solid line, free barbed ends alone; short-dashed line, capped barbed ends alone; dotted line, free barbed ends with sequestering protein; long-dashed line, capped barbed ends with sequestering protein. (**D**) Depolymerization rate measurements (see Basic Protocol 6). The solution of 2.5 μM F-actin (50% pyrenyl labeled) with free barbed ends was diluted 20-fold in F-buffer at time zero in the absence (solid line) and presence (dotted line) of 10 μM sequestering protein. The sequestering protein has no effect on actin depolymerization. (**E**) The sequestering protein inhibits pointed-end growth of actin filaments (see Basic Protocol 5). The rate of pointed-end growth was measured in the presence of 2.5 μM Mg-ATP-G-actin, 2 nM gelsolin-actin seeds, and the sequestering protein at the indicated concentration. The inhibition observed is consistent with a K_d of 2 μM. (**F**) The sequestering protein inhibits barbed-end growth of actin filaments. The rate of barbed-end growth was measured in the presence of 2.5 μM Mg-ATP-G-actin and 0.16 nM spectrin-actin seeds. The inhibition observed is consistent with a K_d of 2 μM, as in E. (**G**) Co-sedimentation assay (see Basic Protocol 1). F-actin (10 μM) was sedimented alone or in the presence of increasing amounts of the sequestering protein. The sequestering protein depolymerizes F-actin, leading to an increasing amount of G-actin in the supernatant (S) and less G-actin in the pellet (P). Lines shown in A-F represent typical experimental curves, derived from known proteins (e.g., thymosin β_4).

function of ADF/cofilin proteins is complex because under physiological ionic conditions, ADF/cofilin recognizes the ADP-bound form of both F- and G-actin with a 100-fold higher affinity than ATP-bound actin. ADF/cofilin modifies both the structure and the assembly dynamics of filaments. (1) The rate of dissociation of ADF-F-actin from the pointed ends (the rate-limiting step in treadmilling) is 30-fold higher than the rate of dissociation of F-actin. (2) The dissociation of ADP from ADF-G-actin is 10- to 20-fold slower than the dissociation of ADP from G-actin. The combination of these two properties affects actin dynamics at steady state. The physiologically relevant effect of ADF in actin-based motility is to enhance treadmilling by increasing the rate-limiting step in the ATPase cycle of actin.

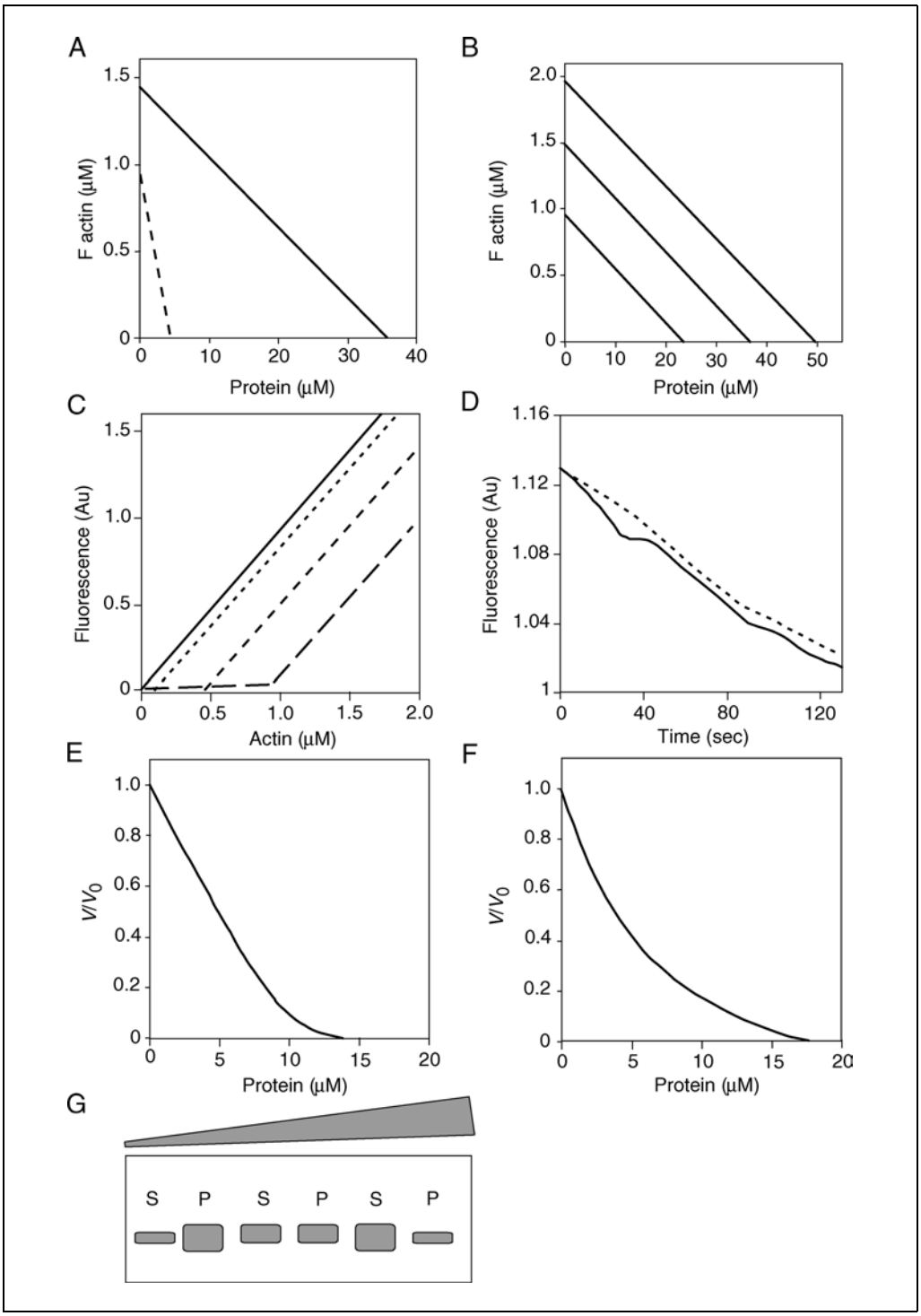


Figure 13.6.2 Legend at left.

Troubleshooting

Because in many protocols pyrenyl-labeled or NBD-labeled actin is used, it is important to check that measurements of F-actin concentrations using these probes are not biased by possible changes in fluorescence of labeled actin upon binding by the protein of interest. For instance, ADF quenches the fluorescence of NBD- and pyrenyl-F-actin, hence alternative measurements (e.g., using light scattering or sedimentation) are required to elucidate the function of this protein. Some components, such as glycerol in a buffer, could also modify the fluorescence of labeled actin.

Labeling of actin may also affect its affinity for the protein to be tested (Uyemura et al., 1978). It is recommended to carry out tests at different percentages of labeled actin and to verify that the result is independent of the percentage of labeled actin in the mix. It is also appropriate to use competition assays to determine the affinity of labeled and unlabeled actin for the protein.

The solution of F-actin should be manipulated carefully to avoid breaking of filaments.

Anticipated Results

G-actin-sequestering proteins

In the presence of β-thymosin, both β-thymosin (T) and actin filaments (A) are at equilibrium with free G-actin at the steady state (critical) concentration, C_{ss}. The double equilibrium implies that the amount of TA complex formed at equilibrium in the presence of F-actin is determined by the value of C_{ss} and the total β-thymosin concentration, $[T]_0$, as follows (Fig. 13.6.2A):

$$[\mathrm{TA}] = \left([T]_0 \times C_{SS}\right) / \left(C_{SS} + K_T\right)$$

Equation 13.6.7

where K_T is the equilibrium dissociation constant of the TA complex.

According to this equation, sequestering proteins shift the critical concentration plots to a higher apparent value. The apparent critical concentration is the sum of C_{ss} and [TA] given by Equation 13.6.7. Parallel C_{ss} plots are obtained at different values of $[T]_0$ (Fig. 13.6.2C).

Changes in C_{ss} are elicited by proteins that affect the dynamics of assembly; these changes are amplified by sequestering proteins. For instance, capping proteins increase the value of C_{ss} up to the critical concentration at pointed ends (C_c). It is easy to see (Equation 13.6.7) that when more filaments are capped, more actin is sequestered at a given concentration $[T]_0$ (i.e., F-actin depolymerizes in the cell). In contrast, when the proportion of capped filaments is lowered by creation of new barbed ends, C_{ss} becomes lower; hence the pool of sequestered actin decreases and F-actin increases (e.g., upon platelet stimulation).

The ability of sequestering proteins to depolymerize F-actin in a manner that depends on the value of C_{ss} is used to determine the value of K_T. Addition of increasing amounts of β-thymosin to a given amount of F-actin causes depolymerization because of formation of TA with a linear dependence on $[T]_0$. The (negative) slope of the plot of F-actin versus $[T]_0$ is $C_{ss}/(C_{ss} + K_T)$, which is independent of the amount of actin. Hence at different concentrations of actin, the decreases in F-actin are parallel straight lines (Fig. 13.6.2B). The

Figure 13.6.3 *(at right)* Functional assays of profilin-like proteins (barbed-end assembly-promoting factors). (**A**) Steady-state measurements of F-actin in the presence of increasing amounts of profilin-like protein. The experiment was conducted as in Figure 13.6.1A. When barbed ends are capped (dashed line), the profilin-like protein depolymerizes F-actin. Linear depolymerization was consistent with a K_d of 2 μM. When the barbed ends are free (solid line), the profilin-like protein fails to depolymerize F-actin. (**B**) Critical-concentration (C_{ss}) measurements for actin alone and in the presence of 2 μM profilin-like protein. The experiment was conducted as in Figure 13.6.2C. Solid line, free barbed ends alone; short-dashed line, capped barbed ends alone; dotted line, free barbed ends with sequestering protein; long-dashed line, capped barbed ends with sequestering protein. (**C**) Profilin-like protein inhibits pointed growth of actin filaments. The inhibition is consistent with a K_d of 2 μM. The experiment was conducted as in Figure 13.6.2E. (**D**) The profilin-like protein fails to inhibit barbed-end growth of actin filaments. The experiment was conducted as in Figure 13.6.2F. (**E**) Co-sedimentation assay in the absence or presence of profilin-like protein. The experiment was conducted as in Figure 13.6.2G. (**F**) Depolymerization rate measurements in the absence (solid line) and presence (dotted line) of 10 μM profilin-like protein. The experiment was conducted as in Figure 13.6.2D. The profilin-like proteins have no effect on actin depolymerization. Lines shown in A-D and F represent typical experimental curves derived from known proteins (e.g., profilin).

slope is steeper when filaments are capped because the value of C_{ss} is higher. Analysis of the slopes of F-actin versus $[T]_0$ yields the same value of K_T, however, when barbed ends are free or capped. This property identifies a sequestering function.

In a co-sedimentation assay, sequestering proteins cause an accumulation of actin in the supernatant, which is dependent on the value of C_{ss} (i.e., it is greater when barbed ends are capped; Fig. 13.6.2G). The sequestering proteins are always present in the supernatants.

Sequestering proteins, in forming a non-polymerizable complex with actin, cause a decrease in the rate of filament growth when tested at a given G-actin concentration (Fig. 13.6.2D,F). The equilibrium dissociation constant for the TA complex is derived from the dependence of the elongation rate at barbed ends on $[T]_0$ using the following equation,

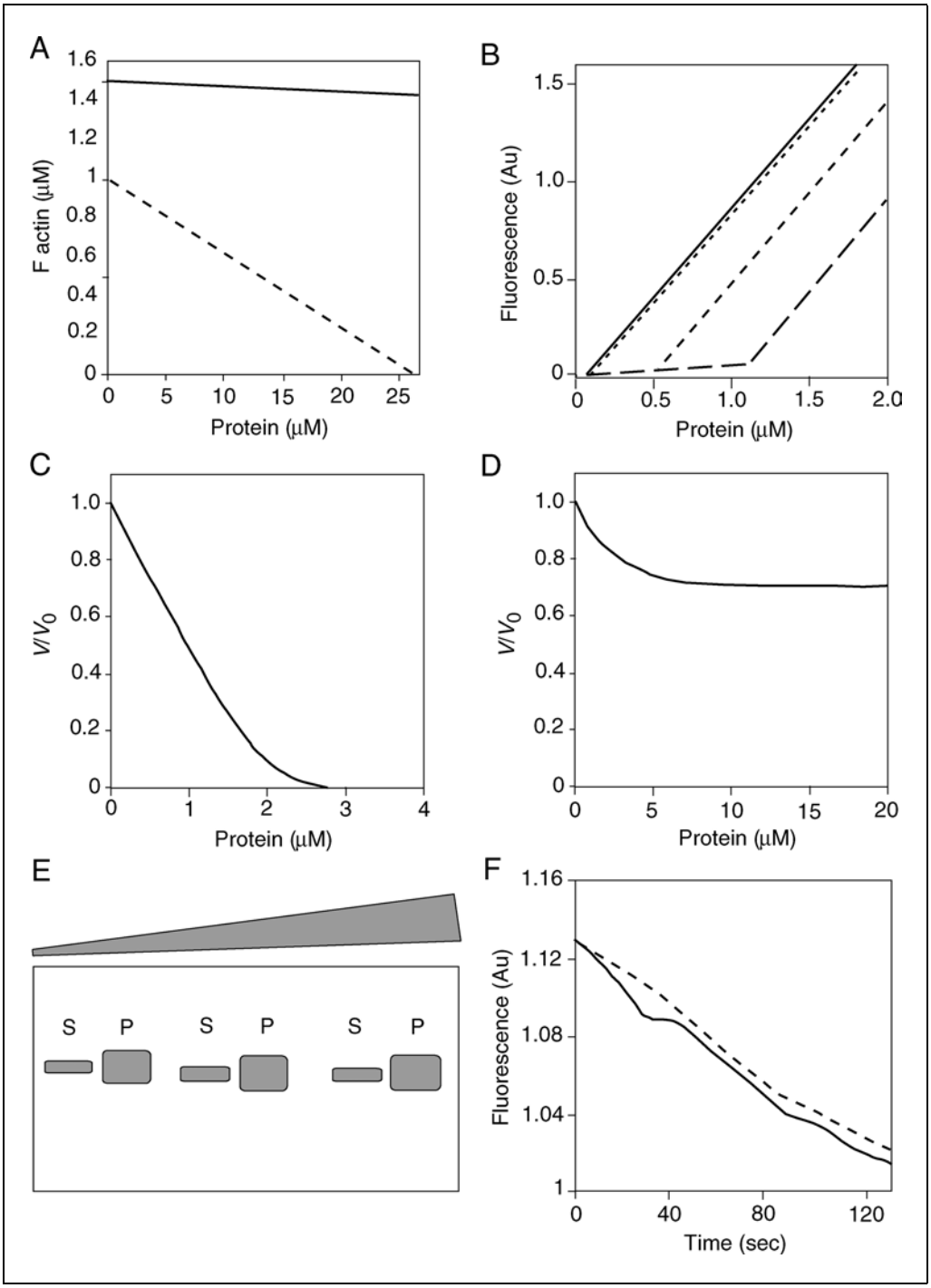


Figure 13.6.3 Legend at left.

which describes saturation of G-actin in the growth assay by the sequestering protein:

$$(V_0 - V)/V_0 = [TA]/[A]_0 = (/2[A]_0) \times \left\{ [A]_0 + [T]_0 + K_T \pm \left(\{[A]_0 + [T]_0 + K_T\}^2 - 4[A]_0[T]_0 \right)^{1/2} \right\}$$

Equation 13.6.8

where V is the initial rate of elongation measured at concentration $[T]_0$ of the sequestering protein, V_0 is the initial rate of elongation in the absence of the sequestering protein, $[A]_0$ is the total G-actin concentration (assumed for simplicity to be much higher than the critical concentration at the barbed end), and K_T is the equilibrium dissociation constant for the sequestering protein-actin complex. G-actin-sequestering proteins display no effect on the rate of depolymerization at either end of the filament (Fig. 13.6.2D).

Profilin and profilin-like proteins

At the steady state of assembly, when the barbed ends of filaments are capped, profilin-like proteins cause depolymerization of F-actin (Fig. 13.6.3). The amount of depolymerized actin increases linearly with the concentration of the protein, which is consistent with sequestration of Mg-ATP-G-actin by protein in a 1:1 complex and with an equilibrium constant as calculated with Equation 13.6.7; this behavior is typical of a sequestering protein. In contrast with sequestering proteins, however, profilin-like proteins fail to depolymerize F-actin when barbed ends are free, because the protein-actin (PA) complex participates in monomer-polymer exchanges at the barbed ends. Because both actin (A) and the PA complex contribute to stabilization of filament barbed ends, the presence of PA lowers the contribution of A (i.e., the partial critical concentration of A is lowered by profilin-like proteins; Fig. 13.6.3A,B).

Co-sedimentation assays confirm these conclusions. When barbed ends are free, profilin-like proteins fail to depolymerize F-actin, but depolymerization is readily observed if barbed ends are capped. Profilin-like proteins are present in the supernatant fraction (Fig. 13.6.3E). Finally, these proteins display no effect on the initial rate of depolymerization at either end of the filament (Fig. 13.6.3F).

In seeded growth assays, profilin-like proteins prevent association of actin to the pointed end of the filament (Fig. 13.6.3C). The equilibrium dissociation constant (0.2 μM for profilin) for the profilin-like PA complex was derived from the dependence of the elongation rate inhibition on the protein concentration with the same equation used for the sequestering protein (Equation 13.6.8).

Profilin-like proteins fail to prevent fully elongation at the barbed end of the filament because the PA complex productively associates with the barbed end. When the G-actin that is present in the growth assay is saturated by the protein, the rate constant for association of PA to the barbed end is derived from the growth rate measurements. For profilin, the value of k_+ is 30% lower than that for G-actin (Fig. 13.6.3D).

Capping proteins

In measurements of F-actin assembly at steady state, when capping protein is added

Figure 13.6.4 *(at right)* Functional assays of capping proteins. (**A**) Steady state measurements of F-actin in the presence of increasing amounts of capping protein. The experiment was conducted as in Figure 13.6.2A. When barbed ends are free (solid line), capping proteins depolymerize F-actin, partially blocking all barbed ends; the maximum amount of unpolymerized actin equals the critical concentration of the pointed end (C_c = 0.6 μM, under physiological conditions). When barbed ends are capped (dashed line), addition of another capping protein has no effect on the steady-state amount of F-actin; all barbed ends remain capped and C_{ss} remains equal to the C_c at the pointed ends. (**B**) C_{ss} measurements for actin alone or in the presence of capping proteins. The experiment was conducted as in Figure 13.6.2C. When barbed ends are capped, capping proteins have no effect on C_c. When barbed ends are free, capping proteins shift C_c to 0.6 μM, in physiological conditions. Solid line, free barbed ends alone; dotted line, free barbed ends with capping protein; dashed line, capped barbed ends alone or with capping protein. (**C**) Capping proteins do not inhibit pointed-end growth of actin filaments. The experiment was conducted as in Figure 13.6.2E. (**D**) Capping proteins inhibit barbed-end growth of actin filaments. The experiment was conducted as in Figure 13.6.2F. (**E**) Depolymerization rate measurements. The experiment was conducted as in Figure 13.6.2D. Capping proteins block depolymerization at the barbed end. Dotted line, no capping protein; solid lines, capping protein present at indicated concentrations. (**F**) Co-sedimentation assay. The experiment was conducted as in Figure 13.6.2G. At a saturating amount of capping protein, the amount of G-actin in the supernatant corresponds to the C_c at the pointed end. Lines shown in A-E represent theoretical lines in each case.

to a solution of F-actin with free barbed ends, partial depolymerization occurs until the critical concentration of the pointed ends (C_c^P) is reached. The amount of F-actin that disassembles therefore equals the difference $C_c^P - C_c^B$, at any F-actin concentration. Under physiological ionic conditions, this amount is 0.6 μM − 0.1 μM = 0.5 μM (Fig. 13.6.4A,B). When barbed ends are capped by a standard capping protein like gelsolin, addition of another capping protein does not cause further depolymerization of F-actin. The same conclusions are derived from C_c plots (Fig. 13.6.4B).

In co-sedimentation assays, capping proteins cause limited depolymerization of 0.5 μM F-actin because of the establishment of C_c^P. Capping proteins co-sediment with F-actin in a largely substoichiometric molar ratio with F-actin, but they are detectable by western blotting (Fig. 13.6.4F).

In seeded growth assays, capping proteins inhibit elongation at the barbed end, generally

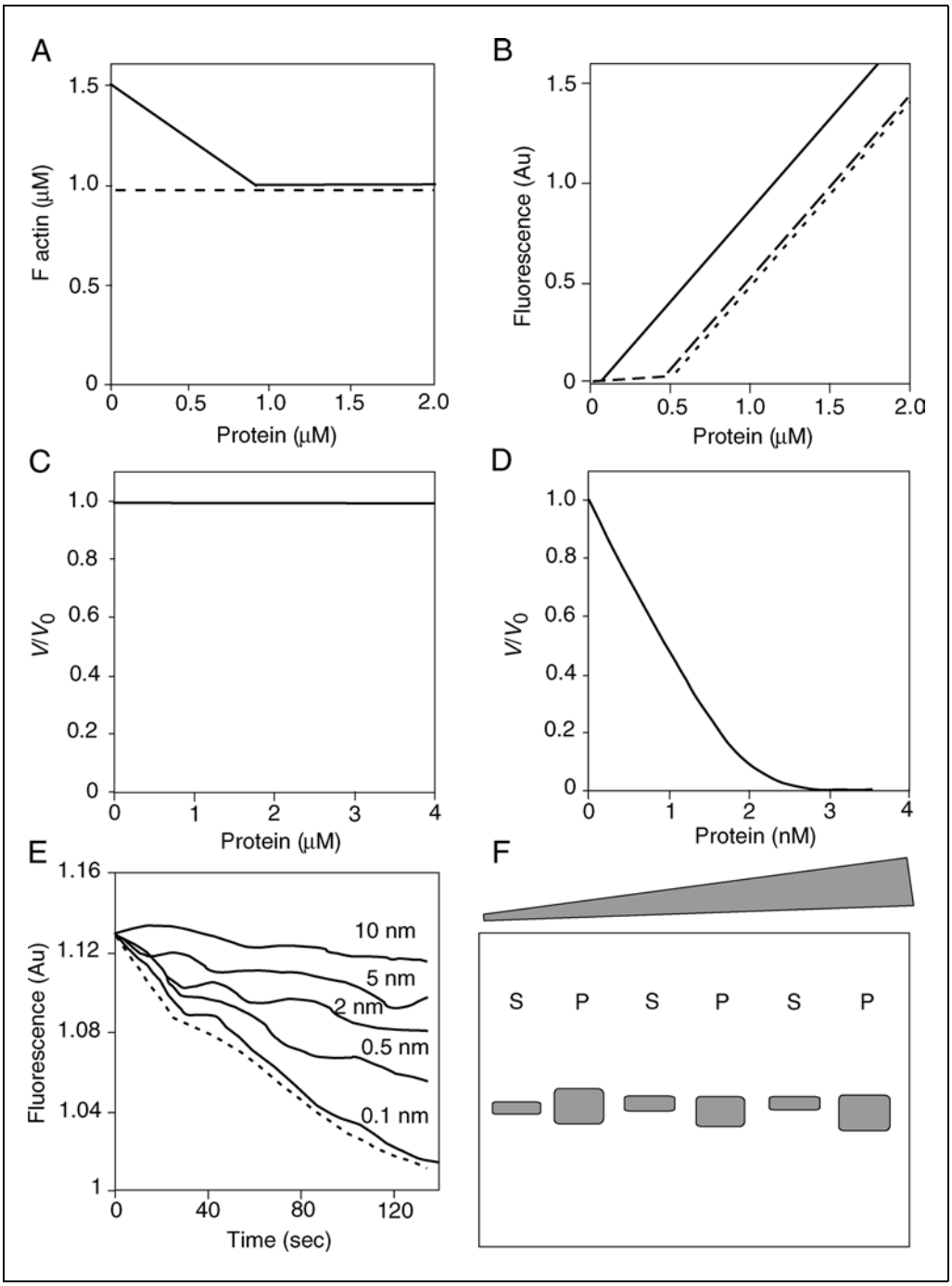


Figure 13.6.4 Legend at left.

with high affinity (nanomolar range) but not at the pointed end (Fig. 13.6.4C,D). Barbed-end growth inhibition can be described by the following equation:

$$\left(V_0 - V_\infty\right) / \left(V_0 - V\right) = 1 + K_c / C$$

Equation 13.6.9

where V_0 is the rate of elongation in the absence of the capping protein, V_∞ is the rate of elongation in the presence of a saturating amount of capping protein (corresponding to the rate of elongation of the pointed end), K_c is the equilibrium constant between the capping protein and actin, and C is the concentration of capping protein.

These proteins block dilution-induced depolymerization at the barbed end (Fig. 13.6.4E). The depolymerization rates are inhibited when the barbed ends are free. The equilibrium dissociation constant (20 nM for gelsolin) for the capping protein–actin complex can be derived from the dependence of the rate of dilution-induced depolymerization on the protein concentration using Equation 13.6.9.

Particular class of leaky cappers

The above properties characterize strong cappers that completely block reactions of both actin association to and dissociation from the barbed ends. Proteins that bind to barbed ends may still allow either growth only or depolymerization only, or may allow both reactions while remaining bound to the end, with altered kinetic parameters. Recent evidence for these activities has been reported for a new class of actin-binding proteins called formins (Pruyne et al., 2002; Wallar and Alberts, 2003). These proteins nucleate barbed-end actin assembly and can remain bound to the barbed end as it grows, thus mediating insertional polymerization under certain conditions. The steady state measurements and kinetic measurements described above allow the quantification of effects of formins on actin dynamics.

ADF/cofilin proteins

In steady-state measurements of actin assembly, ADF causes partial (limited) depolymerization; however, its behavior is different from a capper because partial depolymerization is observed in the presence as well as in the absence of capping proteins. Co-sedimentation assays show evidence for binding of ADF to F-actin, but the partial depolymerization implies that ADF also binds to G-actin. Binding studies show that ADF binds preferentially to ADP-actin and kinetic data indicate that ADF slows down nucleotide exchange, implying that at steady state a large fraction of G-actin consists of ADF-ADP-G-actin. Kinetic measurements of dilution-induced depolymerization provide evidence for faster depolymerization from the pointed ends, which is confirmed by measurements of filament turnover. Taken together, these observations help to explain the function of ADF/cofilin proteins. (for a review, Carlier et al., 1999)

Time Considerations

Purification of G-actin requires 6 days (Support Protocol 1). Labeling actin with either pyrenyl or 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole (NBD-Cl) requires 3 days (Support Protocols 2 and 3). Basic Protocols 1, 2, 3, 5, and 6 each take half of a day. Basic Protocols 4 and 7 take 2 days each.

Literature Cited

Boquet, I., Boujemaa, R., Carlier, M.F., and Preat, T. 2000. Ciboulot regulates actin assembly during *Drosophila* brain metamorphosis. *Cell* 102:797-808.

Carlier, M.F., Jean, C., Rieger, K.J., Lenfant, M., and Pantaloni, D. 1993. Modulation of the interaction between G-actin and thymosin beta 4 by the ATP/ADP ratio: Possible implication in the regulation of actin dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 90:5034-5038.

Carlier, M.F., Ressad, F., and Pantaloni, D. 1999. Control of actin dynamics in cell motility. Role of ADF/cofilin. *J. Biol. Chem.* 274:33827-33830.

Casella, J.F., Maack, D.J., and Lin, S. 1986. Purification and initial characterization of a protein from skeletal muscle that caps the barbed ends of actin filaments. *J. Biol. Chem.* 261:10915-10921.

Cassimeris, L., Safer, D., Nachmias, V.T., and Zigmond, S.H. 1992. Thymosin beta 4 sequesters the majority of G-actin in resting human polymorphonuclear leukocytes. *J. Cell. Biol.* 119:1261-1270.

Combeau, C. and Carlier, M.F. 1988. Probing the mechanism of ATP hydrolysis on F-actin using vanadate and the structural analogs of phosphate BeF_3^- and $A1F_4^-$. *J. Biol. Chem.* 263:17429-17436.

Combeau, C. and Carlier, M.F. 1989. Characterization of the aluminum and beryllium fluoride species bound to F-actin and microtubules at the site of the gamma-phosphate of the nucleotide. *J. Biol. Chem.* 264:19017-19021.

Didry, D., Carlier, M.F., and Pantaloni, D. 1998. Synergy between actin depolymerizing factor/cofilin and profilin in increasing actin filament turnover. *J. Biol. Chem.* 273:25602-25611.

Egile, C., Loisel, T.P., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P.J., and Carlier, M.F. 1999. Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J. Cell Biol.* 146:1319-1332.

Hug, C., Jay, P.Y., Reddy, I., McNally, J.G., Bridgman, P.C., Elson, E.L., and Cooper, J.A. 1995. Capping protein levels influence actin assembly and cell motility in dictyostelium. *Cell* 81:591-600.

Loisel, T.P., Boujemaa, R., Pantaloni, D., and Carlier, M.F. 1999. Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature* 401:613-616.

Nowak, E., Strzelecka-Golaszewska, H., and Goody, R.S. 1988. Kinetics of nucleotide and metal ion interaction with G-actin. *Biochemistry* 27:1785-1792.

Pantaloni, D. and Carlier, M.F. 1993. How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell* 75:1007-1014.

Paunola, E., Mattila, P.K., and Lappalainen, P. 2002. WH2 domain: A small, versatile adapter for actin monomers. *FEBS Lett.* 513:92-97.

Perelroizen, I., Carlier, M.F., and Pantaloni, D. 1995. Binding of divalent cation and nucleotide to G-actin in the presence of profilin. *J. Biol. Chem.* 270:1501-1508.

Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A., and Boone, C. 2002. Role of formins in actin assembly: Nucleation and barbed-end association. *Science* 297:612-615.

Safer, D. and Nachmias, V.T. 1994. Beta thymosins as actin binding peptides. *BioEssays* 16:473-479.

Small, J.V., Stradal, T., Vignal, E., Rottner, K. 2002. The lamellipodium: Where motility begins. *Trends Cell Biol.* 12:112-120

Sun, H.Q., Kwiatkowska, K., Wooten, D.C., and Yin, H.L. 1995. Effects of CapG overexpression on agonist-induced motility and second messenger generation. *J. Cell. Biol.* 129:147-156.

Valentin-Ranc, C. and Carlier, M.F. 1989. Evidence for the direct interaction between tightly bound divalent metal ion and ATP on actin. Binding of the lambda isomers of beta gamma-bidentate CrATP to actin. *J. Biol. Chem.* 264:20871-20880.

Wallar, B.J. and Alberts, A.S. 2003. The formins: Active scaffolds that remodel the cytoskeleton. *Trends Cell Biol.* 13:435-446.

Uyemura, D.G., Brown, S.S., and Spudich, J.A. 1978. Biochemical and structural characterization of actin from *Dictyostelium discoideum*. *J. Biol. Chem.* 253:9088-9096.

Contributed by Maud Hertzog
Istituto FIRC di Oncologia Molecolare
Fondazione Italiana per la Ricerca sul Cancro
Milano, Italy

Marie-France Carlier
Laboratoire d'Enzymologie et Biochimie Structurale Centre National de la Recherche Scientifique
Gif-sur-Yvette, France

CHAPTER 14
Signal Transduction: Protein Phosphorylation

INTRODUCTION

Cells use selective phosphorylation of proteins to regulate a vast number of intracellular processes. Enzymes termed protein kinases couple phosphate groups to tyrosine, serine, or threonine residues in specific amino acid sequence motifs of target proteins. The process can be reversed by protein phosphatases. Specificity is determined by the ability of each of the many different types of protein kinases and phosphatases to recognize specific motifs and proteins. By such site-specific regulation of phosphorylation, which affects 10% or more of all proteins, cells can switch on or modulate many major signaling and metabolic pathways, as well as regulate cell behavior in biological events such as migration and embryonic development. The importance of phosphorylation to cell biological regulation is underscored by the fact that cells have over a thousand different protein kinases.

One class of protein phosphorylations regulates enzymes, and the addition or removal of a key phosphate activates or suppresses activity of the enzyme. Other protein phosphorylations enable a protein to bind to another to form a complex—e.g., via the binding of an SH2 domain to a specific tyrosine-phosphorylated site in a target protein. Another general cellular strategy is to activate cascades of protein phosphorylation in signal-transduction pathways. For example, complex linear and interconnecting pathways of sequential phosphorylation of proteins leading to the various types of MAP kinases are important regulators of cell growth, differentiation, and gene expression. A current overview of this large field of protein phosphorylation is presented in *UNIT 14.1*, and a more specific review of MAP kinase pathways is presented in *UNIT 14.3*; both provide a number of relevant literature references.

Although phosphorylation has classically been characterized by the incorporation of ^{32}P using radioactive inorganic phosphate, a recent methodological breakthrough of particular value to cell biologists involves powerful nonradioactive approaches to the study of protein phosphorylation. Studies of complex signaling pathways in cells and tissues are now possible even for nonexperts by using immunoblotting and immunofluorescence or immunohistochemical methods. These new approaches are based on specific antibodies that recognize a phosphate group on one or more amino acids selectively—e.g., phosphotyrosine residues on any protein, or the presence of a certain type of phosphate linkage on a specific protein such as an activated MAP kinase. A wide selection of these immunological tools is now available commercially. *UNIT 14.2* provides methods for rapid direct characterization of phosphorylated proteins using a specific antibody. If antibodies of sufficiently high specificity with respect to a single protein are not available, this unit also provides a more indirect approach using immunoprecipitation by antibodies against the protein of interest, followed by anti-phosphotyrosine immunodetection. It also describes methods for antibody localization of key phosphorylated regulatory molecules. This approach permits an investigator to follow the expression patterns or intracellular movements of key phosphorylated proteins in cells, or even in various tissues of intact organisms.

Current Protocols in Cell Biology 14.0.1-14.0.3, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb1400s48

MAP kinase signaling is central to many critical cell biological regulatory events, and *UNIT 14.3* provides methods for quantitative characterization of this important signaling process. Because of the daunting complexity of signaling via MAP kinases, specific antibodies are needed. This unit starts by providing a general protocol for detecting MAP kinase activation by immunoblot analysis using specific anti-phospho-MAP kinase antibodies. It then provides an alternative approach that uses specific antibodies to isolate phosphoprotein, and then phosphorylation of a MAP kinase substrate is measured by determining amounts of incorporated ^{32}P. Because novel kinases may be involved in a specific cell biological regulatory event, *UNIT 14.3* also presents methods for detecting unknown kinases using an in-gel kinase assay. A test substrate is incorporated into an SDS-polyacrylamide gel, and electrophoretically separated crude protein extracts are evaluated for enzyme bands with ability to produce phosphorylation in vitro.

Although the recent proliferation of immunological methods for detecting specific types of protein phosphorylation has made the analysis of phosphorylation much easier, the "gold standard" for characterizing phosphorylation of individual proteins continues to be radioactive labeling with ^{32}P followed by biochemical analysis of the radiolabeled phosphoproteins. *UNIT 14.4* provides cell culture and biochemical protocols for incorporating ^{32}P-labeled inorganic phosphate and for characterizing radiolabeled phosphoproteins. *UNIT 14.5* presents current methods for unambigously identifying the specific phosporylated amino acids in individual phosphoproteins. Appropriate combinations of radioactive and immunological approaches should permit full characterization of the protein phosphorylation cascades and pathways that regulate many important cell biological functions.

Akt (protein kinase B) is another important kinase, which is regulated by phosphoinositide 3-kinase downstream of signaling cascades induced by a number of different growth factors and integrin ligands. Akt in turn regulates critical cellular functions including cell survival, growth, and proliferation, and it can play roles in cancer and other diseases. *UNIT 14.6* provides protocols for assaying the phosphorylation state of Akt that regulates its activity, as well as its dephosphorylation. It also provides a protocol for quantifying the translocation of activated Akt to the plasma membrane.

Cells generally interact with extracellular matrix molecules using integrin receptors, which trigger complex signaling cascades that regulate cell survival, growth, migration, and differentiation. These cascades involve integrin-associated kinases that include focal adhesion kinase (FAK) and c-Src. These and related kinases are both the mediators and the targets of specific tyrosine phosphorylation events that play important regulatory roles. *UNIT 14.7* provides a comprehensive series of protocols for analyzing signal transduction involving FAK and the structurally related kinase Pyk2, as well as the master regulatory kinase c-Src. Procedures are described for inducing signaling, performing immunoprecipitation analysis, measuring kinase activity, and characterizing changes in phosphorylation and activity with phospho-specific antibodies. Protocols are also provided for visualizing activated FAK, Pyk2, and Src, and there are cell biological assays for cell migration regulated by these types of kinase signaling.

Many cell biological processes are regulated by small GTPases. Cell adhesion, migration, and proliferation are strongly regulated by the Rho family of GTPases. Although this family consists of a number of members with both distinct and overlapping functions, RhoA, Rac, and Cdc42 are particularly important and implicated in a wide range of functions. *UNIT 14.8* provides methods for determining the activation of each of these key Rho GTPases using proteins that bind specifically to the activated form. Specificity of these assays is then provided by immunodetection of the specific Rho GTPase isoforms bound to these activation-detecting reagents.

The precise regulation of the activities of small GTPases is important during many biological processes. *UNIT 14.9* describes detailed protocols for assaying their key regulators, the GEFs (guanine nucleotide exchange factors) and the GAPs (GTPase activating proteins). These enzyme regulators respectively stimulate or inactivate GTPase activities. Quantification of the activities of each of these two types of regulators using these methods can provide valuable mechanistic insight into GTPase functions in cell and developmental biology.

Recent advances in biosensors and microscopy now permit researchers to image the subcellular sites of enzymatically activated Ras and Rho GTPases in living cells. These methods often use FRET (fluorescence/Förster resonance energy transfer) probes that emit fluorescence at a different wavelength when the enzyme of interest is activated and can then bind to a target molecule. In FRET, binding brings two fluorescent molecules into sufficiently close proximity that one can activate fluorescence of the other. *UNIT 14.10* describes the principles and methods for using genetically encoded, single-chain FRET biosensors for activation of Ras family GTPases. It describes strategies for developing and characterizing such FRET probes, as well as detailed information about the acquisition and processing of FRET information from living cells. *UNIT 14.11* provides detailed information about multiple different approaches to designing and using different types of biosensors. One approach uses a single fluorescent dye that detects when an enzyme changes conformation after activation, another uses FRET occurring intramolecularly, and the third functions by detecting FRET between separate proteins. Each approach requires its own type of imaging and image analysis. These two units provide a wide range of powerful tools to detect enzyme activity at specific sites within a living cell.

Another important class of GTPases besides the Rho family is the intriguing group of Arf (ADP-ribosylation factor) GTPases and Arf-like proteins. *UNIT 14.12* provides methods for determining the localization and functions of these multifunctional regulatory proteins. It also describes procedures for measuring the activation state of specific Arfs, as well as providing information on assessing functions of their regulators, the GEFs (guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins).

Kenneth M. Yamada

Overview of Protein Phosphorylation

HISTORY

Phosphorylation is the most common and important mechanism of acute and reversible regulation of protein function. Studies of mammalian cells metabolically labeled with [^{32}P]orthophosphate suggest that as many as one-third of all cellular proteins are covalently modified by protein phosphorylation. Protein phosphorylation has an important role in essentially all aspects of cell biology. Most polypeptide growth factors (platelet-derived growth factor and epidermal growth factor are among the best studied; Heldin, 1995) and cytokines (e.g., interleukin 2, colony stimulating factor 1, and γ-interferon; Ihle et al., 1994) stimulate phosphorylation upon binding to their receptors. Induced phosphorylation in turn activates cytoplasmic protein kinases, such as Raf, the activators of the mitogen-activated protein (MAP) kinases SEK and MEK, the MAP kinases ERK, JNK, and p38 (Marshall, 1995; see also *UNIT 14.3*), the Janus/JAK kinases (Ihle et al., 1994), the p21 activated kinases (PAKs) (Lim et al., 1996), and the phosphatidylinsoitil 3′-kinase-activated kinase, protein kinase B/Akt (Alessi and Cohen, 1998). Additionally, in all nucleated organisms, cell cycle progression is regulated at both the G1/S and the G2/M transitions by cyclin-dependent protein kinases (Doree and Galas, 1994; also see Chapter 8). These kinases regulate the G1/S transition by the phosphorylation of cell cycle regulators such as Rb protein (Reed, 1997) and the G2/M transition through the phosphorylation of nuclear lamins (Peter et al., 1990) and histones (Arion et al., 1988).

Differentiation and development are also controlled by phosphorylation. Development of the R7 cell in the *Drosophila* retina (Simon, 1994) and of the vulva in *Caenorhabditis elegans* (Eisenmann and Kim, 1994) are both dependent on the function of receptor and cytoplasmic protein kinases, such as the sevenless and LET-23 receptors and the RAK kinase (Perrimon et al., 1995). Additionally, adhesion is regulated by the FAK kinase (Hanks and Polte, 1997) and motility is regulated, in part, by Met, the hepatocyte growth factor/scatter factor receptor (Furlong, 1992). Finally, metabolism—in particular, the interconversion of glucose and glycogen and the transport of glucose—is regulated by phosphorylation (Cohen, 1985). Cell biologists of all stripes therefore find, often unexpectedly and occasionally reluctantly, that they must study protein phosphorylation in order to understand the regulation and function of their favorite gene and its product.

LABELING STUDIES

Protein phosphorylation is usually studied by biosynthetic labeling with ^{32}P-labeled inorganic phosphate ($^{32}P_i$). This is intrinsically quite simple—the label is just added to growth medium. It is this step of an experiment, however, that makes many investigators the most nervous, given the perceived danger of radioactive exposure and the real danger of contamination of laboratory equipment with radioactivity. Neither problem is insurmountable. With proper shielding and technique, exposure of the investigator can be limited to the hands and contamination of the laboratory can be avoided. A general protocol for biosynthetic labeling with $^{32}P_i$ that maximizes incorporation and minimizes radioactive exposure of workers in the lab and contamination of lab equipment will be provided. (For a general discussion of radiation safety consult Safe Use of Radioisotopes, *APPENDIX 1D*.)

SITES OF PHOSPHORYLATION

Most proteins are found to be phosphorylated at serine or threonine residues, and many proteins involved in signal transduction are also phosphorylated at tyrosine residues. These three hydroxyphosphoamino acids exhibit sufficient chemical stability at acidic pH that they can be recovered after acid hydrolysis and identified in a straightforward manner. Proteins that contain covalently bound phosphate at histidine, cysteine, and aspartic acid residues, either as phosphoenzyme intermediates or as stable modifications, have also been described. Each of these phosphoamino acids is chemically labile and impossible to study with the standard techniques used for the acid-stable phosphoamino acids. Indeed, they are often identified by inference or elimination. A technique for identifying phosphoserine, phosphothreonine, and phosphotyrosine by acid hydrolysis and two-dimensional thin-layer electrophoresis will be presented. Techniques for analyzing acid-labile forms of protein phosphorylation are described in Ringer, 1991; Kamps, 1991; and Duclos et al., 1991.

Contributed by Bartholomew M. Sefton

Current Protocols in Cell Biology (1998) 14.1.1-14.1.3

Phosphotyrosine is not an abundant phosphoamino acid. Its detection in samples labeled with $^{32}P_i$ is often difficult, therefore, especially if the samples contain large quantities of proteins phosphorylated at serine residues or if they are contaminated with RNA. Detection of phosphotyrosine, as well as of phosphothreonine, can be enhanced considerably by incubation of gel-fractionated samples in alkali. This hydrolyzes RNA and dephosphorylates phosphoserine, allowing visualization of minor tyrosine- and threonine-phosphorylated proteins. A simple procedure for alkaline treatment will be described.

DETECTION OF UNLABELED PHOSPHOAMINO ACIDS

If a protein is modified by phosphorylation, identification of the phosphoamino acid can often be accomplished without resorting to biosynthetic labeling. For example, tyrosine phosphorylation can be studied because proteins containing this rare phosphoamino acid can be detected with great specificity and sensitivity by antibodies to phosphotyrosine (Kamps and Sefton, 1988). Attempts to generate antibodies that recognize phosphoserine or phosphothreonine have failed to produce reagents with the required specificity and/or sufficient sensitivity to be useful. Once the primary sequence around a phosphorylation site containing phosphoserine or phosphothreonine has been determined, however, it is possible to make antibodies against synthetic phosphopeptides modeled on these phosphorylation sites (Czernik et al., 1991). Such anti-phosphopeptide antibodies have been very useful tools for monitoring phosphorylation of the parent protein at specific sites (also see *UNIT 14.2*).

More generally, because phosphorylation often alters the mobility of a protein during SDS–polyacrylamide gel electrophoresis and almost always alters its isoelectric point, the presence of phosphorylated residues in an unlabeled protein can be deduced from altered gel mobility after incubation of the protein with a phosphatase.

PROTEIN KINASES

Most protein kinases exhibit a strict specificity for phosphorylation of either serine/threonine or tyrosine residues. Protein kinases comprising a third group more closely resemble the serine/threonine kinases in their primary sequence but phosphorylate both serine/threonine and tyrosine residues. MEK, a MAP kinase activator (Crews and Erickson, 1992), and wee1, an inhibitor of cdc2 kinase (Parker et al., 1992), are examples of such dual-specificity protein kinases that phosphorylate threonine and tyrosine residues that are closely located in substrate proteins.

PROTEIN PHOSPHATASES

Many protein phosphatases also show a strict specificity for either phosphoserine/phosphothreonine or phosphotyrosine residues. Unlike kinases, however, the serine/threonine and tyrosine phosphatases are not evolutionarily related and exhibit no primary sequence homology (Shenolikar and Nairn, 1991; Charbonneau and Tonks, 1992). Acid and alkali phosphatases can also dephosphorylate phosphoproteins in vitro but share no structural homology with protein phosphatases. A new family of phosphatases, such as cdc25, CL100, and VH-1, are distantly related to the tyrosine phosphatases but dephosphorylate both phosphotyrosine and phosphothreonine in their target substrates (Fauman and Saper, 1996).

A number of potent phosphatase inhibitors have been identified in recent years. Okadaic acid and several other toxins inhibit protein (serine/threonine) phosphatase 1 and 2A, and vanadate and phenylarsenoxide inhibit tyrosine phosphatase. These compounds have implicated reversible phosphorylation as a regulatory mechanism in many physiological processes (Cohen, 1989; Hardie et al., 1991; Shenolikar and Nairn, 1991; Shenolikar, 1994); under certain conditions, they may be the dominant regulators of these cellular processes. These phosphatase inhibitors can also be used to distinguish protein dephosphorylation from proteolysis in crude tissue extracts (Cohen, 1991). By far the most important contribution of these reagents has been that they have allowed assessment of the role played by phosphorylation in cellular processes where neither the identity of the phosphoprotein involved nor that of the kinase(s) that regulates its function are known.

The units in this chapter will describe techniques that detect protein phosphorylation and identify amino acids that have been covalently modified.

LITERATURE CITED

Alessi, D.R. and Cohen, P. 1998. Mechanism of activation and function of protein kinase B. *Curr. Opin. Genet. Dev.* 8:55-62.

Arion, D., Meijer, L., Brizuela, L., and Beach, D. 1988. cdc2 is a component of the M phase-specific histone H1 kinase: Evidence for identity with MPF. *Cell* 55:371-378.

Charbonneau, H. and Tonks, N.K. 1992. 1002 protein phosphatases? *Annu. Rev. Cell Biol.* 8:463-493.

Cohen, P. 1985. The role of protein phosphorylation in the hormonal control of enzyme activity. *Eur. J. Biochem.* 15:439-448.

Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58:453-508.

Cohen, P. 1991. Classification of protein serine/threonine phosphatases: Identification and quantitation in cell extracts. *Methods Enzymol.* 201:389-398.

Crews, C.M. and Erickson, R.L. 1992. Purification of a murine protein-tyrosine/threonine kinase that phosphorylates and activates the erk-1 gene product: Relationship to the fission yeast byr1 gene product. *Proc. Natl. Acad. Sci. U.S.A.* 89:8205-8209.

Czernik, A.J., Girault, J.A., Nairn, A.C., Chen, J., Snyder, G., Kebabian, J., and Greengard, P. 1991. Production of phosphorylation state–specific antibodies. *Methods Enzymol.* 201:264-283.

Doree, M. and Galas, S. 1994. The cyclin-dependent protein kinases and the control of cell division. *FASEB J.* 85:1114-1121.

Duclos, B., Marcandier, S., and Cozzone, A.J. 1991. Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. *Methods Enzymol.* 201:10-21.

Eisenmann, D.M. and Kim, S.K. 1994. Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. *Curr. Opin. Genet. Dev.* 4:508-516.

Fauman, E.B. and Saper, M.A. 1996. Structure and function of the protein tyrosine phosphatases. *Trends Biochem. Sci.* 21:413-417.

Furlong, R.A. 1992. The biology of hepatocyte growth factor/scatter factor. *Bioessays* 14:613-617.

Hanks, S.K. and Polte, T.R. 1997. Signaling through focal adhesion kinase. *Bioessays* 19:137-145.

Hardie, D.G., Haystead, T.A.J., and Sim, A.T.R. 1991. Use of okadaic acid to inhibit protein phosphatases in intact cells. *Methods Enzymol.* 201:469-477.

Heldin, C.-H. 1995. Dimerization of cell surface receptors in signal transduction. *Cell* 80:213-223.

Ihle, J.N., Witthuhn, B.A., Quelle, F.W., Yamamoto, K., Thierfelder, W.E., Kreider, B., and Silvennoinen, O. 1994. Signalling by the cytokine receptor superfamily: JAKS and STATS. *Trends Biochem. Sci.* 19:222-227.

Kamps, M.P. 1991. Determination of phosphoamino acid composition by acid hydrolysis of protein blotted to Immobilon. *Methods Enzymol.* 201:21-27.

Kamps, M.P. and Sefton, B.M. 1988. Identification of multiple novel polypeptide substrates of the v-rsc, v-yes, v-fps, v-ros, and v-erb-B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. *Oncogene* 2:305-315.

Lim, L., Manser, E., Leung, T., and Hall, C. 1996. Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur. J. Biochem.* 242:171-185.

Marshall, C.J. 1995. Specificity of receptor tyrosine kinase signalling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80:179-185.

Parker, L.L., Atherton-Fessler, S., and Piwinica-Worms, H. 1992. p107 wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15. *Proc. Natl. Acad. Sci. U.S.A.* 89:2917-2921.

Perrimon, N., Lu, X., Hou, X.S., Hus, J.C., Melnick, M.B., Chou, T.B., and Perkins, L.A. 1995. Dissection of the Torso signal transduction pathway in Drosphila. *Mol. Reprod. Dev.* 42:515-522.

Peter, M., Nakagawa, J., Doree, M., Labbe, J.C., and Nigg, E.A. 1990. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 61:591-602.

Reed, S.I. 1997. Control of the G1/S transition. *Cancer Surv.* 29:7-23.

Ringer, D.P. 1991. Separation of phosphotyrosine, phosphoserine, and phosphothreonine by high-performance liquid chromatography. *Methods Enzymol.* 201:3-10.

Shenolikar, S. 1994. Protein serine/threonine phosphatases: New avenues for cell regulation. *Annu. Rev. Cell Biol.* 10:55-86.

Shenolikar, S. and Nairn, A.C. 1991. Protein phosphatases: Recent progress. *Adv. Second Messenger Phosphoprotein Res.* 23:1-121.

Simon, M. 1994. Signal transduction during the development of the *Drosophila* R7 photoreceptor. *Dev. Biol.* 166:431-442.

Contributed by Bartholomew M. Sefton
The Salk Institute
San Diego, California

Immunological Detection of Phosphorylation

Incorporation of phosphates into serine, threonine, and tyrosine acceptors in proteins is a common mechanism for regulating protein function. This unit presents protocols that use specific anti–phosphoamino acid (PAA) and anti-phosphoprotein antibodies to detect protein phosphorylation and protein kinase activity. The first protocol describes the use of protein blots (immunoblotting; see Basic Protocol 1) to detect protein phosphorylation using either anti-PAA or anti-phosphoprotein antibodies. This is a convenient method that usually yields impressive results. Phosphorylation can also be detected by immunoprecipitation followed by immunoblot analysis (see Basic Protocol 2) or by immunofluorescent staining (see Basic Protocol 3); these methods are typically more complicated and time consuming. All three methods have been successfully used to detect protein phosphorylation with a wide variety of antibodies and most phosphorylated proteins.

IMMUNODETECTION OF PROTEIN PHOSPHORYLATION BY IMMUNOBLOTTING

BASIC PROTOCOL 1

This method describes the immunodetection of phosphoproteins using detergent extraction of proteins followed by immunoblot analysis (also see *UNIT 6.2*). Phosphorylation of mitogen-activated protein kinase (MAP kinase, MAPK) is used here as an example; however, this immunodetection protocol can be used with most specific anti-PAA or anti-phosphoprotein antibodies. This protocol minimizes the time phosphorylated proteins are exposed to phosphatases, allowing reliable and quantitative detection of the phosphorylated proteins, and can be completed within 7 to 10 hr. This protocol describes EGF stimulation of Rat1 cells, but with minor changes can be used with almost all tissue culture cell lines, homogenized animal organs, and even whole lower organisms.

Materials

Rat1 cells
Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal bovine serum (DMEM/10% FBS; *APPENDIX 2A*)
Starvation medium: DMEM/0.1% FBS
Epidermal growth factor (EGF) buffer: phosphate-buffered saline (PBS)/0.5 mg/ml bovine serum albumin (BSA, crystalline)
50 µg/ml EGF in EGF buffer
PBS (*APPENDIX 2A*), ice cold
Homogenization buffer (see recipe), ice cold
Lysis buffer: homogenization buffer/1% Triton X-100, ice cold
Kinase buffer (see recipe), ice cold
Coomassie protein assay reagent (Pierce)
Protein standards: 5, 10, 20, 50, 100, and 200 µg/ml BSA in homogenization buffer/0.03% Triton X-100
4× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (see recipe)
Prestained protein markers (16 to 200 kDa)
12% SDS-polyacrylamide gel (12% polyacrylamide/0.32% bisacrylamide; *UNIT 6.1*)
Transfer buffer: 50 mM Tris·Cl (pH ~8.8)/50 mM glycine
Tris-buffered saline/Tween 20 (TBST; see recipe)
Blocking solution: TBST/2% BSA
Primary antibodies: monoclonal anti–active MAP kinase *and* polyclonal anti–general MAP kinase
Secondary antibodies: horseradish peroxidase (HRP)–conjugated goat anti–rabbit *and* alkaline phosphatase (AP)–conjugated goat anti-mouse

Contributed by Zhong Yao and Rony Seger
Current Protocols in Cell Biology (1998) 14.2.1-14.2.15

AP detection system (e.g., Promega)
Enhanced chemiluminescence (ECL) detection system (see recipe)

6-cm tissue culture plates
1.5-ml microcentrifuge tubes, prechilled to 4°C (four sets of six, each set labeled 1 to 6)
Stopwatch
1-ml pipet tips, prechilled to 4°C
Microcentrifuge, 4°C
96-well flat-bottomed microtiter plate
Microtiter plate reader, 595-nm wavelength
Nitrocellulose membrane cut to size of gel
Whatman 3MM filter paper, two pieces, cut to size of gel
Transfer apparatus for electroblots (e.g., Bio-Rad)
Flat container for washing nitrocellulose membrane

Additional reagents and equipment for tissue culture (*UNIT 1.1*), chromogenic and luminescent visualization (*UNIT 6.2*), and SDS-PAGE (*UNIT 6.1*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% to 10% CO_2 incubator unless otherwise specified.

Prepare cellular extracts

1. Grow six 6-cm tissue culture plates of Rat1 cells in DMEM/10% FBS to subconfluency (~0.5×10^6 cells/plate).

2. Remove culture medium, add 2 ml starvation medium to each plate, and culture a further 18 hr.

   Make sure that the plates remain flat and that the medium covers the entire plate. Serum starvation makes the cells quiescent, which can be achieved under these conditions within 14 to 24 hr. Starvation for too long or any change in temperature or pH may be stressful to the cells and may induce activation of one or more signaling pathways.

3. Add 2.5 µl of 50 µg/ml EGF to three plates (stimulated) and 2.5 µl EGF buffer to three plates (control). Return the plates to the incubator for 5, 15, and 45 min.

   Add EGF or EGF buffer first to the plates with the longest incubation, then at appropriate intervals to the plates with the next longest incubation. It is useful to make and use a time chart to ensure that stimuli will be given at the appropriate times and the cells harvested within a short period of time (5 to 10 min).

   If the influence of the stimulating agent on the particular cells being used is not yet known, a positive control should be included—for example, peroxovanadate (0.1 mM vanadate/0.2 mM hydrogen peroxide), which nonspecifically activates many signaling events in most tissue culture cells.

4. After 5, 15, or 45 min, remove medium from plates. Rinse plates twice with 5 ml ice-cold PBS and once with 5 ml ice-cold homogenization buffer.

   Since slowing and arresting of biological processes is desired at this stage, the plates should be placed on ice. Washing and harvesting each plate takes 0.5 to 1.5 min; all six plates should be harvested within 5 to 10 min.

5. Add 350 µl ice-cold lysis buffer to each plate, tilt the plate gently (on ice), and scrape the cells into the buffer using a plastic scraper or rubber policeman. Using prechilled

1-ml pipet tips, transfer the cells and buffer to prelabeled, prechilled 1.5-ml microcentrifuge tubes.

The sample being harvested may become slightly viscous; the 1-ml pipet tips can be cut to make the tip opening wider, allowing easy collection of these samples.

Detergents other than Triton X-100, such as 0.5% Nonidet P-40 (NP-40; Igepal), can also be used.

6. Continue the cell lysis for an additional 10 min on ice.

   A nonionic detergent is used for membrane disruption because it extracts proteins from membranal, cytosolic, and some nuclear fractions of the cell, and because the determination of protein concentration in such extracts is relatively easy (steps 9 to 12). Alternatively, a more stringent mix of detergents (RadioImmunoPrecipitation Assay [RIPA] buffer, see Reagents and Solutions), which causes complete extraction of proteins from most cell compartments, can be used for membrane disruption, although it might interfere with the protein determination. Cells can also be disrupted by sonication (two 7-sec, 50-W pulses for a 0.5-ml sample) on ice, which extracts proteins from the cytosolic and nuclear fractions, but not from membranes. The extraction by sonication is performed in homogenization buffer without detergents, and allows easy determination of protein concentration.

7. Microcentrifuge the cellular extracts 15 min at 15,000 × *g*, 4°C. Transfer supernatants to fresh, prechilled, and prelabeled microcentrifuge tubes.

   The supernatants contain the protein extracts to be examined for phosphorylation.

8. Transfer 5- to 10-µl aliquots of each extract to labeled microcentrifuge tubes for determination of protein concentration, and store the remainder of the extracts on ice until needed.

   The protein concentration is determined at this stage so that identical amounts of proteins from the different samples can be compared and the relative amount of phosphoproteins can be determined accurately. Comparing samples based on cell number, rather than protein concentration, can result in differences of up to 20% in the amount of protein; such large differences can cause even larger ones when phosphorylation is assessed immunologically.

Determine protein concentration

9. Add 145 µl kinase buffer to each 5-µl aliquot of cellular extract.

   High concentrations of Triton X-100 can interfere with colorimetric measurements of the Coomassie brilliant blue; therefore, dilutions of ≥1:20 are necessary. Similar or higher dilutions are required for sonicated and RIPA extracts as well, because of high detergent or protein concentrations. The Lowry method of protein determination cannot be used because of the dithiothreitol used to prevent degradative oxidation in the extraction buffer.

10. Transfer 10 µl of each of the protein standards into two wells of a 96-well flat-bottomed microtiter plate.

    Protein standards should be prepared in the same buffer as was used for cell extraction.

11. Transfer 10 µl of each of the diluted cellular extracts into two wells of the same microtiter plate. Add 200 µl Coomassie protein assay reagent to all wells.

12. Place the microtiter plate in a microtiter plate reader and measure the absorbance of the standards and samples at 595 nm (A_{595}). Use the absorbance of the standards to construct a standard curve (absorbance versus concentration). Calculate the protein concentrations of the samples by comparing the absorbance of the sample with the standard curve.

Analyze samples by SDS-PAGE

13. Based on the calculated protein concentrations, transfer a volume of each cellular extract containing 40 μg protein to a fresh 1.5-ml microcentrifuge tube.

14. Add $\frac{1}{3}$ vol of 4× SDS-PAGE sample buffer to each tube, mix the contents, and boil 3 min.

15. Load samples and prestained protein markers on 12% SDS-polyacrylamide gel.

    Load prestained markers into the first or second lane of the gel so the molecular weights of the detected proteins can be determined. These markers will also indicate whether the proteins were completely transferred from the gel onto the nitrocellulose paper during blotting.

16. Place the gel in an electrophoresis apparatus filled with appropriate buffer and run the gel at 150 V (see *UNIT 6.1*).

Immunoblot protein

17. Soak nitrocellulose membrane in transfer buffer until completely wet.

18. Once the dye front has reached the end of the gel, remove the gel from the electrophoresis apparatus, cut off the stacking (upper) gel, and carefully place the gel in a flat container with transfer buffer.

19. Fill transfer apparatus with transfer buffer. Open the inner transfer apparatus and remove air bubbles from the pads. Make a sandwich of the gel, nitrocellulose membrane, and pads by putting one piece of Whatman 3MM filter paper (wetted with transfer buffer) on the wet pad, the gel on top of the filter paper, the wet nitrocellulose membrane on top of the gel, and the second piece of wet Whatman 3MM paper on top of the nitrocellulose membrane (Fig. 6.2.1).

20. Remove any air bubbles from between the different layers of the transfer sandwich by gently rolling a 10-ml pipet over the sandwich. Place the other wet pad on top of the transfer sandwich.

    Make sure air bubbles are not trapped between the gel and the other components.

21. Place the transfer sandwich containing the gel and nitrocellulose membrane into the buffer-filled transfer apparatus, with the nitrocellulose membrane facing the side with the cathode and the gel facing the side with the anode. Connect the apparatus to a power supply and start the current (200-mA constant current, preferably with a cooling device). Run for 2 hr.

    The voltage will drop as the transfer progresses and the conductivity increases.

    To shorten the time and improve the yield of transfer, methanol or 0.05% SDS is sometimes included in the transfer buffer; their inclusion will allow higher current but will necessitate the use of a cooling device.

22. At the end of the transfer period, turn off the power supply and remove the nitrocellulose membrane from the transfer sandwich. Rinse the nitrocellulose membrane with transfer buffer to remove any adhering pieces of gel and place the membrane in a flat container.

    At this stage, the efficiency of protein transfer can be visually monitored by assessing the transfer of prestained protein markers from the gel to the nitrocellulose membrane. The total amount of protein transferred can also be detected by staining the nitrocellulose membrane with Ponceau S (Salinovich and Montelaro, 1986). The total amount of unphosphorylated protein is determined by staining with general antibodies as described later, however, and staining with Ponceau S is not essential.

23. Place nitrocellulose membrane into flat container and incubate in 30 to 50 ml blocking solution for 60 min at room temperature.

This ensures that any free nonspecific protein-binding sites on the membrane are blocked, and will not nonspecifically bind the antibodies used in this protocol.

Incubate nitrocellulose membrane with antibody

24. Dilute primary antibody (e.g., monoclonal anti–active MAP kinase antibody) in TBST according to manufacturer's instructions. Incubate nitrocellulose membrane in a flat container with 15 ml primary antibody overnight at 4°C, 30 min at 37°C, or 1 to 2 hr at room temperature.

Although anti-MAP kinase antibodies are used as an example, other anti-phosphoprotein or anti-PAA antibodies (from, e.g., Transduction Laboratories, Sigma, Santa Cruz Biotechnology, Upstate Biotechnology, New England BioLabs, or Zymed Laboratories) can be used as well, in combination with antibodies directed to the same nonphosphorylated proteins.

25. Wash nitrocellulose membrane with TBST in flat container at least three times, 15 min each time, at room temperature.

26. Dilute secondary antibody in TBST according to manufacturer's instructions. Incubate nitrocellulose membrane with secondary antibody for 45 min at room temperature.

For detection of anti–active MAP kinase antibody; the AP detection system is recommended, because it has a broader linear range than does ECL (see Commentary). Alternatively, HRP-conjugated antibodies can be used as secondary antibodies and can be detected by ECL (see below). If HRP- rather than AP-conjugated antibodies are used at this stage, then AP-conjugated antibodies should be used in step 30.

27. Wash nitrocellulose membrane with TBST at least three times, 10 min each time, at room temperature.

28. Use an AP detection protocol (*UNIT 6.2*) to detect active MAP kinase.

After detecting active (phosophorylated) MAP kinase, determine whether there is an equal amount of MAP kinase in all lanes by exposing the same nitrocellulose membrane to anti–general MAP kinase antibody and redeveloping it (steps 29 to 31).

29. Incubate the stained nitrocellulose membrane in blocking solution for 30 min at room temperature.

If there is no steric hindrance between the two antibodies, it is not necessary to strip away the antibodies used in steps 24 to 26 because two different types of antibodies are used (mouse and rabbit). To perform the second blotting add the different type of antibody and follow steps 29 to 31. Antibodies from the same species of origin (mouse or rabbit) or antibodies that interfere with each other can be used for both steps, but in that case the membrane must be stripped (UNIT 6.2) before step 29 is performed.

30. Remove blocking solution and incubate nitrocellulose membrane with the new primary antibody (e.g., polyclonal anti–general MAP kinase antibody) as in steps 24 to 25, and use HRP-conjugated goat anti–rabbit antibody as the secondary antibody (step 26).

31. Use an ECL detection protocol (*UNIT 6.2*) to visualize general MAP kinase staining. Incubate nitrocellulose membrane with ECL solution for 1 min, dry the blot with Whatman 3MM filter paper, wrap the blot in plastic wrap, and expose to X-ray film.

BASIC PROTOCOL 2

IMMUNODETECTION OF PROTEIN PHOSPHORYLATION BY IMMUNOPRECIPITATION FOLLOWED BY IMMUNOBLOTTING

This protocol, for which reagents are readily available, can be used instead of Basic Protocol 1 when antibodies against phosphorylated sequences (anti-phosphoprotein antibodies) are not available. This protocol involves immunoprecipitation (also see *UNIT 7.2*) using antibodies to the whole protein, followed by immunoblot detection with anti-PAA antibodies, which have been used effectively to detect many tyrosine-phosphorylated adapter molecules in growth factor signaling pathways.

Materials

Protein A–Sepharose beads
PBS (*APPENDIX 2A*), room temperature and ice cold
Antibodies for immunoprecipitation of the desired protein (1 to 5 µg per reaction)
Homogenization buffer (see recipe), ice cold
Cellular extract in lysis buffer (see Basic Protocol 1, step 7)
RIPA buffer (see recipe), ice cold
0.5 M LiCl solution, ice cold
0.1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
4× SDS-PAGE sample buffer (see recipe)
12% SDS-polyacrylamide gel (12% polyacrylamide/0.32% bisacrylamide; *UNIT 6.1*)

End-over-end rotator
Microcentrifuge, 4°C

Additional reagents and equipment for analysis by SDS-PAGE and immunoblotting (see Basic Protocol 1 and *UNIT 6.1*)

Prepare antibody-conjugated protein A beads

1. Place protein A–Sepharose beads (~150 µl) in a 1.5-ml microcentrifuge tube, add 1 ml PBS, and let the beads swell for 10 min at room temperature.

   Although protein A–Sepharose is recommended for this method, other commercially available protein A–conjugated resins (e.g., agarose, HiTrap) can be used. Protein G–coupled resins are required to immunoprecipitate certain types of monoclonal antibodies (Table 7.2.1).

   Some resins are supplied as ready-to-use solutions and will not require this swelling step.

2. Add 1 ml PBS to swollen beads and microcentrifuge 1 min at 15,000 × *g*, room temperature. Discard the supernatant. Repeat for a total of three washes.

3. Add 300 µl PBS and 25 µl of the antibody to be conjugated to 250 µl swollen packed beads. Rotate the mixture on an end-over-end rotator for 1 hr at 10 to 30 rounds/min, room temperature to allow antibody to bind to protein A.

   These volumes are calculated for ten reactions (usually 10 to 20 µl beads is used per reaction), but because of the density of the beads, this amount will probably only be sufficient for eight reactions. The amounts can be scaled up as long as the proportions are maintained.

   For easy handling of the resin, cut the ends of the pipet tips to make the openings larger.

4. Resuspend beads in 1 ml ice-cold PBS, then microcentrifuge 1 min at 15,000 × *g*, room temperature. Remove supernatant and add 1 ml ice-cold homogenization buffer, then repeat centrifugation two more times with homogenization buffer ending with a final addition of an equal volume of buffer.

   Antibody-conjugated beads can be stored in homogenization buffer for ≤2 days at 4°C.

Immunoprecipitate

5. Add 40 µl antibody-conjugated bead suspension (20 µl beads and 20 µl homogenization buffer) to a 300-µl sample of cellular extract containing 100 to 500 µg total protein in prechilled 1.5-ml plastic microcentrifuge tubes. Rotate on end-over-end rotator for 2 hr at 10 to 30 rounds/min, 4°C.

   See Critical Parameters for the rationale behind this particular method.

6. Microcentrifuge antibody-conjugated beads 1 min at 15,000 × *g*, 4°C. Discard supernatant, add 1 ml ice-cold RIPA buffer, and centrifuge again as before. Repeat twice with 1 ml ice-cold 0.5 M LiCl and once with 1 ml ice-cold 0.1 M Tris·Cl, pH 8.0.

   These stringent washes will remove most of the phosphorylated proteins that can nonspecifically interact with the protein A beads.

7. Add 30 µl of 4× SDS-PAGE sample buffer to each sample of washed beads, boil 5 min, and centrifuge 1 min at 15,000 × *g*, room temperature. Load supernatant and prestained markers on 12% SDS-polyacrylamide gel (see *UNIT 6.1*).

8. Perform SDS-PAGE and immunoblot analysis (see Basic Protocol 1, steps 16 to 31).

   At this stage the immunoprecipitate can also be subjected to a protein kinase assay (see UNIT 14.3).

FLUORESCENT IMMUNOSTAINING OF TISSUE CULTURE CELLS

BASIC PROTOCOL 3

This protocol uses similar antibody reagents as described in Basic Protocol 1 to allow staining of tissue culture cells, blood cells, thin tissue sections, or lower organisms. It involves fixation of the cells or tissues to be examined, brief permeabilization of the plasma membranes to allow immunological recognition of intracellular components, and fluorescent detection. This method is especially useful to determine localization of signaling events, but is less accurate in determining the level of activations. Recently, Gabay et al. (1997) used this method to identify the MAP kinase pattern of activation in the developing *Drosophila* enbryo.

Materials

Rat1 cells
DMEM/10% FBS (*APPENDIX 2A*)
Starvation medium: DMEM/0.1% FBS
PBS (*APPENDIX 2A*)
3% (w/v) paraformaldehyde in PBS
Permeabilization buffer: PBS/0.2% (v/v) Triton X-100
Primary antibody
Secondary antibodies: fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit *and* rhodamine-conjugated goat anti–mouse

6-well tissue culture plates
22-mm-square coverslips, sterile
Parafilm, two 10 × 20–cm pieces
Slide mounting medium (e.g., Polymount, Polyscience)
Confocal or fluorescent microscope

Additional reagents and equipment for tissue culture (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with cells must be aseptic, and proper aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% to 10% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

1. Place one 22-mm-square coverslip in each well of a 6-well tissue culture plate. Seed ~30,000 Rat1 cells in 2 ml DMEM/10% FBS in each well. Incubate 24 hr. Remove medium, replace with starvation medium, and incubate a further 14 hr (see Basic Protocol 1, steps 1 to 2).

   Seeding a small number of cells per well allows the cells to be well dispersed as they grow, which usually facilitates good cell staining.

   Many cell types other than Rat1 can be used with this protocol.

2. Remove starvation medium and add 2 ml DMEM/10% FBS to each well. Incubate 30 min in incubator.

   Serum-induced translocation of MAP kinases into the nucleus is much more pronounced and prolonged than the translocation induced by EGF.

3. Wash wells twice with 5 ml PBS at room temperature. Add 2.5 ml of 3% paraformaldehyde in PBS to each well for 20 min. Remove paraformaldehyde solution and wash three times with 5 ml PBS each time.

   Once the samples are fixed by the paraformaldehyde, protein degradation and dephosphorylation are minimized; therefore, the subsequent stages can be performed at room temperature. Leave coverslips in the wells for all washes.

4. Add 2 ml permeabilization buffer to each well for 5 min. Remove permeabilization buffer, add 5 ml PBS to each well, allow to sit for 2 min, then remove. Repeat PBS wash twice.

   Other methods of fixation can be used, but paraformaldehyde fixation followed by permeabilization with 0.2% Triton X-100 is a very effective method when antibodies directed against signaling components are used.

5. Stretch one 10 × 20–cm piece of Parafilm on a clean, straight, and stable surface, such as a work bench; fasten it to the surface.

6. Centrifuge primary antibody 5 min at 15,000 × *g*, room temperature. Dilute primary antibody in PBS as per manufacturer's instructions. Place six 40-µl drops of diluted primary antibody on the stretched piece of Parafilm ~5 cm apart from each other. Remove the fixed and permeabilized coverslips from the wells with fine forceps and place each, cell side down, on top of one of the drops. Incubate 45 min at room temperature.

   An antibody dilution of 1:20 to 1:500 is usually used for staining by this method. For costaining with monoclonal and polyclonal antibodies, the 40-µl drops can contain both antibodies. It is important to know which coverslip was exposed to which antibody.

   Centrifuging the antibody solution 5 min at 15,000 × g, room temperature, will remove particulate contaminants and antibody aggregates.

7. Remove each coverslip from the drop of primary antibody solution with fine forceps. Holding each coverslip with forceps, tilt it and gently touch its edge to a Kimwipe to remove all antibody solution. Place the coverslips back in the wells of the 6-well plate, cell side up. Wash the wells three times with 2 ml PBS for 10 min each.

8. Centrifuge rhodamine-conjugated secondary antibody 1 min at 15,000 × *g*, room temperature. Dilute secondary antibody 1:100 to 1:500 in PBS. Prepare a new 10 × 20–cm piece of Parafilm as in step 5 and place 40-µl drops of centrifuged secondary antibody on the Parafilm. Place coverslips on top of the drops, cell side down. Incubate 45 min at room temperature.

The type of secondary antibody will depend on the species in which the primary antibody was generated.

9. Remove coverslips from the drops of antibody and remove any remaining antibody solution with a Kimwipe as in step 7. Place coverslips back in the 6-well plate, cell side up, and wash three times with 2 ml PBS, 10 min each time.

10. Place one drop of mounting medium on each slide. Remove each coverslip from the well with fine forceps and remove remaining PBS by touching its side with a Kimwipe. Place each coverslip on the drop of mounting medium with the cell side facing the mounting medium. Store slides in the dark at least overnight.

    Keep stained slides in the dark to minimize decay of the fluorescent dye.

11. Monitor the fluorescent staining by examining the slides with either a normal or confocal fluorescent microscope. Note the amount of staining per cell and the percentage of stained cells per sample.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Enhanced chemiluminescence (ECL) system

Solution A:
2.5 mM luminol (Sigma)
400 mM *p*-coumarin (Sigma)
100 mM Tris·Cl, pH 8.5 (*APPENDIX 2A*)

Solution B:
5.4 mM H_2O_2
100 mM Tris·Cl, pH 8.5 (*APPENDIX 2A*)

Store solutions A and B up to 3 months at 4°C
Mix equal amounts of solution A and solution B just before use

The ECL system is also commercially available.

Homogenization buffer

50 mM β-glycerophosphate, pH 7.3
1.5 mM EGTA
1.0 mM EDTA
0.1 mM sodium vanadate
1.0 mM benzamidine
10 µg/ml aprotinin
10 µg/ml leupeptin
2.0 µg/ml pepstatin A
1.0 mM DTT (*APPENDIX 2A*)
Store up to 3 months at 4°C without DTT
Add DTT just before use

This buffer is derived from the work of Ahn et al. (1990) and Seger et al. (1994).

Kinase buffer

50 mM β-glycerophosphate, pH 7.3
1.5 mM EGTA
1.0 mM EDTA
0.1 mM sodium vanadate
1.0 mM dithiothreitol (DTT; *APPENDIX 2A*)
Store up to 3 months at 4°C without DTT
Add DTT just before use

This buffer is derived from the work of Ahn et al. (1990).

RadioImmunoPrecipitation Assay (RIPA) buffer

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
137 mM NaCl
10% (v/v) glycerol
0.1% (w/v) SDS (*APPENDIX 2A*)
0.5% (w/v) deoxycholate
1% (v/v) Triton X-100
2.0 mM EDTA
1.0 mM PMSF (*APPENDIX 2A*; add fresh)
20 μM leupeptin
Store up to 3 months at 4°C

SDS-PAGE sample buffer, 4×

200 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
40% (v/v) glycerol
8% (w/v) SDS (*APPENDIX 2A*)
0.2% (w/v) bromphenol blue
8% (v/v) 2-mercaptoethanol
Store up to 12 months at –20°C

TBST (Tris-buffered saline/Tween 20)

20 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
0.05% (v/v) Tween 20
Store up to 3 months at 4°C

COMMENTARY

Background Information

Protein phosphorylation, the incorporation of phosphates into serine, threonine, and tyrosine acceptors in proteins, is a common means of regulating protein function and hence many cellular processes (Krebs, 1994; Campbell et al., 1995) that has been extensively studied in recent years. More than 30% of cellular proteins are believed to be either constitutively or transiently phosphorylated by ~2000 distinct protein kinases (Hunter, 1994), and the large number of protein kinases and phosphoproteins makes detection of specific phosphorylation sites challenging. Over the years, several methods have been developed to single out and characterize particular phosphoproteins, including the traditional methods of metabolic labeling with inorganic ^{32}P and in vitro protein phosphorylation (*UNIT 14.1*). Some of these techniques, such as metabolic labeling, phosphorylation by known protein kinases, and purification of phosphorylated proteins, are covered in other units in this chapter.

Another approach is immunological (*UNIT 14.3*); a number of immunological reagents have now been developed that can be used to study many aspects of phosphorylation. Two classes of antibodies have historically been used to monitor phosphorylation. One class consists of anti–phosphoamino acid (PAA) antibodies, such as anti-phosphotyrosine (PY), -phosphothreonine (PT), and -phosphoserine (PS; see Fig. 14.2.1). The anti-PAA antibodies nonspecifically recognize many of the phosphorylated amino acids in most proteins (Fig. 14.2.2). The second class is composed of anti-phosphoprotein antibodies, i.e., antibodies against specific phosphate-containing sequences.

Of the anti-PAA antibodies, the anti-PY antibodies, which can detect membranal signaling in response to extracellular agents, are the most commonly used. Immunoblot analysis with anti-PY antibodies usually detects a dramatic increase in the amount of PY in proteins from stimulated cells, such as epidermal growth factor (EGF)–stimulated A431 cells (Frackelton et al., 1983), and insulin-stimulated Fao hepatoma cells (Pang et al., 1985). A similar growth factor–induced increase in other PAAs is also detected with anti-PT and anti-PS antibodies, but the increases detected with these antibodies are usually less dramatic. Anti-PAA antibodies are, therefore, good probes to detect bulk changes in phosphorylation that accompany signaling processes in a variety of cell types. Changes in the phosphorylation of specific pro-

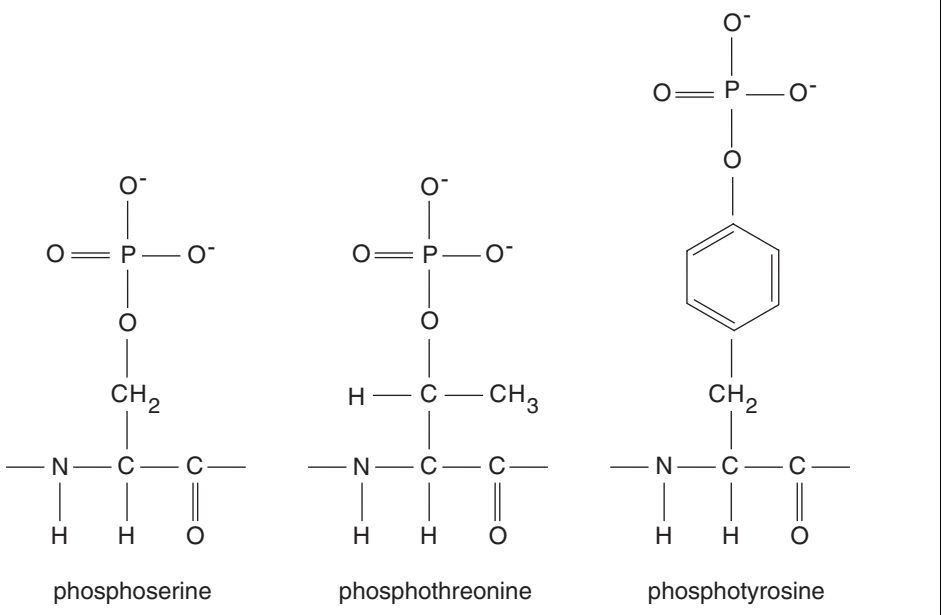


Figure 14.2.1 Schematic representation of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY).

teins can also be detected by these anti-PAA antibodies if isolated (usually immunoprecipitated) proteins are used (Fig. 14.2.2).

Anti-PAA antibodies are widely used, but they usually do not interact with all the PAAs in a given extract; the anti-PS and anti-PT antibodies appear to recognize only a small percentage of the phosphorylated residues on some proteins. Another disadvantage of using anti-PAA antibodies to monitor phosphorylation is their inability to distinguish between several similar PAAs that may appear simultaneously on a particular protein, which can sometimes preclude detection of activity-modulating site-specific phosphorylation.

Anti-PAA antibodies provide a general and nonspecific tool for monitoring phosphorylation. Anti-phosphoprotein antibodies, those with unique specificity to a certain phosphorylated sequence in a given protein, provide a specific monitoring probe. Probably the best known examples are the anti–active mitogen-activated protein kinase (MAP kinase; Seger and Krebs, 1995) antibodies called anti-diphospho-ERKs (Gabay et al., 1997; Yung et al., 1997), which are now commercially available (Sigma).

In response to mitogen stimulation, the MAP kinases (ERK1 and ERK2) are rapidly phosphorylated at up to six sites (Robbins and Cobb, 1992). Phosphorylation at two of these sites (Thr 183 and Tyr 185 in ERK2; Payne et al., 1991) can lead to full activation of the enzyme; the effects of phosphorylation at the other sites are not yet known. Antibodies directed towards the activation motif of ERK (PT–glutamic acid–PY) serve as good tools to detect the enzymatic activity of ERK. Anti-PAA antibodies, on the other hand, can detect total phosphorylation, which does not fully correlate with ERK's enzymatic activity.

An important advantage to using anti-phosphoprotein antibodies is that the procedure for their detection is shorter and simpler than that for anti-PAA antibodies, and can be achieved in a single step (immunoblotting or cell staining). Because of the specificity of anti-phosphoprotein antibodies, however, these antibodies do not always provide an overall indication of signaling events, as do anti-PAA antibodies.

Immunological reagents have been extensively used to study the involvement of phosphorylation in growth factor signaling. When growth factors bind to their receptors, the receptor-associated tyrosine kinases are activated to initiate intracellular signaling events (Krebs, 1994). These signals are then transmitted through a series of interacting proteins (Grb2, SOS, and Ras; see *UNIT 14.3*) that in turn sequentially stimulate the several groups of cytoplasmic protein serine/threonine kinases known as MAP kinase signaling cascades (Seger and

Krebs, 1995; Marshall, 1996; Robinson and Cobb, 1997). Thus, the initial burst of tyrosyl phosphorylation associated with growth factor binding is translated into serine/threonine phosphorylation, which alters the properties and activities of the target proteins (Karin and Hunter, 1995). These phosphorylation cascades eventually activate regulatory molecules and initiate cellular proliferation (Seger and Krebs, 1995).

The first antibodies that were used to study growth factor and oncogene signaling were specific anti-PY antibodies. These antibodies, which were first prepared by immunizing rabbits with azobenzyl phosphate (Ross et al., 1981), were successfully used to monitor signaling events in many cellular systems. Thousands of studies using anti-PY antibodies and various immunological techniques have been published since then. For example, affinity chromatography with anti-PY antibodies was used to isolate PY-containing proteins (Frackelton et al., 1983); immunoblot analysis and immunoprecipitation were used to detect the huge increase in protein tyrosine phosphorylation that occurs in response to cellular transformation (Comoglio et al., 1984); and immunofluorescence techniques were used to localize tyrosine kinases to adherens-type junctions and other intracellular locations (Volberg et al., 1992). The successful use of anti-PY antibodies encouraged the preparation of anti-PT and anti-PS antibodies (Heffetz et al., 1989). These antibodies are usually not as reliable tools as the anti-PY ones, however, because they are less specific.

Anti–active kinase antibodies, which are specific anti-phosphoprotein antibodies, are

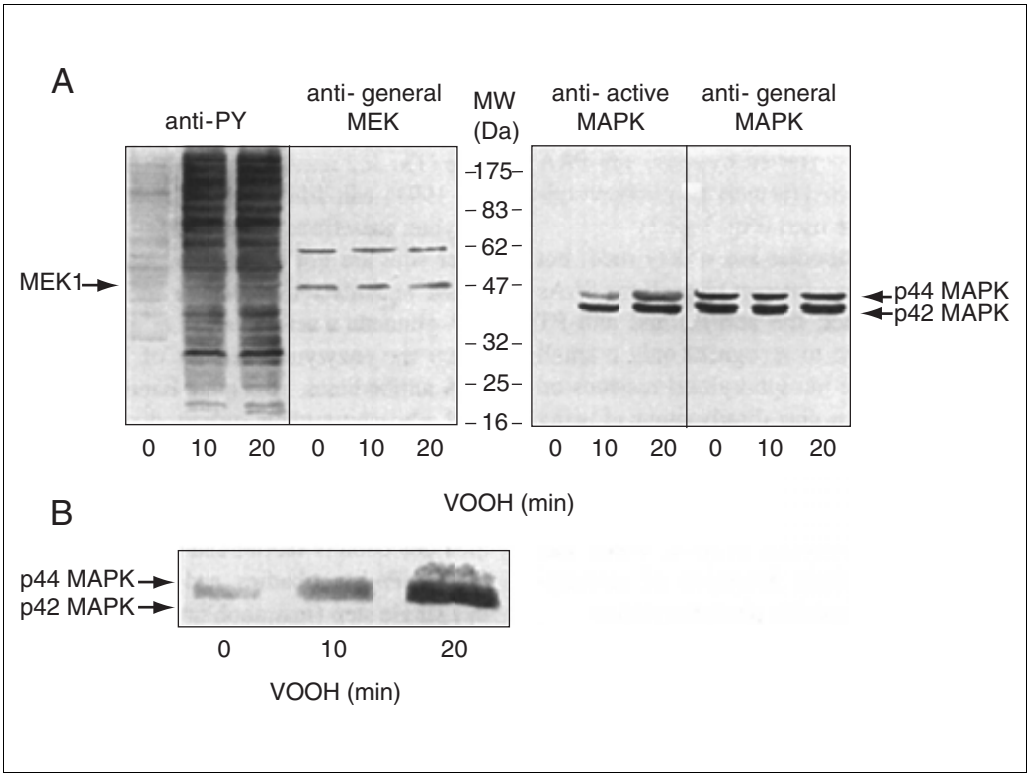


Figure 14.2.2 Immunoblotting of phosphorylated proteins. (**A**) NIH 3T3 cells were grown in 6-cm plates until subconfluency and then serum starved for 18 hr in 2 ml starvation medium. After stimulation with peroxovanadate (VOOH) for different times (0, 10, and 20 min), the cells were harvested and lysed. Aliquots (30 μg) from each sample were run on 10% SDS-polyacrylamide gels and subjected to immunoblotting. Blots were first reacted with polyclonal anti-phosphotyrosine (PY; Zymed) antibody or monoclonal anti–active mitogen-activated protein kinase (MAP kinase; Sigma) antibody and detected using an alkaline phosphatase detection system. The same membranes were blocked and reacted with monoclonal anti–general MAP kinase kinase (MEK) antibody (Transduction Laboratories) or polyclonal anti–general MAP kinase antibody (Sigma). Enhanced chemiluminescence (ECL) was used for detection. (**B**) Aliquots (200 μg) from each sample in A were immunoprecipitated with polyclonal anti-MAP kinase antibody (Santa Cruz Biotechnology). The samples were subjected to SDS-PAGE and immunoblotted with anti-PY antibody followed by detection with ECL.

also useful tools for studying cellular signaling. Many of these antibodies are directed against protein kinases that belong to the MAP kinase signaling cascades, and are activated by phosphorylation of distinct amino acids in the kinase domains. For example, antibodies specific for the doubly phosphorylated, activated form of MAP kinases are now extensively used to determine kinase activity, in place of traditional chemical methods (Fig. 14.2.2). As with anti-PY antibodies, anti–MAP kinase antibodies have been used in a variety of methods, including immunoblotting and fluorescence staining of tissue culture cells, tissue sections, and whole tissues. The latter enables the detection of MAP kinase activation in whole or sectioned organisms (see *UNIT 14.3* for more details).

Anti-phosphoprotein antibodies have also been used to efficiently detect the phosphorylation and activation of the transcription factor CREB (Ginty et al., 1993) and the phosphorylation of the brain-specific, microtubule-associated protein Tau (Biernat et al., 1992). The multitude of studies using anti-phosphoprotein antibodies indicate that these reagents are simple and reliable tools to study phosphorylation and that their use will be invaluable for studying a variety of cellular processes that involve phosphorylation and dephosphorylation.

Critical Parameters

The elements most critical to the success of the methods for immunological monitoring of phosphorylation described here are the quality and specificity of the antibodies used. Antibodies should (1) recognize the phosphorylated protein but not its unphosphorylated counterpart and (2) recognize only the desired PAA, not additional phosphorylated sites in either the same or different proteins. In addition, the amount of protein in the different samples subjected to immunoblot analysis and the dilution of antibodies should be optimized to avoid nonspecific recognition of excess proteins.

Successful detection of phosphorylation by immunological or other means also depends in large part on the efficiency of cellular extraction. Since most phosphorylated proteins are localized within cells, cellular membranes must be disrupted to physically access the desired targets. During disruption of these membranes (cellular extraction), most cellular organelles break, exposing phosphoproteins to phosphatases and proteinases (see *UNIT 14.1*), which have detrimental effects on the detection of phosphorylation (Hunter, 1995). The effect of these enzymes can be minimized by adding specific enzyme inhibitors to extraction buffers and extracting at low temperatures. Phosphatases are usually efficient enzymes, however, and extractions should be performed as fast as possible; if necessary, additional means should be used to secure phosphorylation sites.

Other considerations for the extraction of phosphoproteins are: (1) to ensure that the proteins of interest are indeed presented in the examined fraction and (2) to reduce the number of irrelevant proteins in this fraction. For example, the presence of PY-containing cytosolic proteins might be masked by an excess of activated membranal proteins, and detection of cytoskeletal-associated phosphoproteins might be difficult unless extraction is performed using RadioImmunoPrecipitation Assay (RIPA) buffer. One good cellular extraction method, which can be used for detection of phosphoproteins by either immunoprecipitation or immunoblotting, is described in Basic Protocol 1. In this method, detergent is used to disrupt the cellular membranes, and the resulting extracts should contain membranal, cytosolic, and some nuclear fractions.

Depending on the subcellular localization of the proteins of interest, however, other extraction methods can be used. For example, extracts obtained by sonication usually contain cytosolic and nuclear components, but not membranal ones; those obtained with RIPA buffer should contain most cellular components. Cellular extraction by the addition of hot SDS-PAGE sample buffer to cells is not recommended, because it frees chromatin and results in a gel that is difficult to handle. Extraction by freeze-thawing is also not recommended because molecular degradation can occur during thawing.

Several immunoprecipitation methods have been developed and used over the years (see *UNIT 7.2*). These methods vary in the order in which the antibodies and protein A are added to the cell extracts. In Basic Protocol 2, the antibodies are first conjugated to protein A beads and then added to the cellular extracts. This procedure minimizes the time the samples are incubated with the antibodies, minimizing exposure of the phosphoproteins to phosphatases and proteinases in the extracts. Furthermore, this procedure ensures that only antibodies recognized by protein A will be used for the immunoprecipitation. If the antibodies are added to the lysate prior to precipitation with the beads, antibodies that are not recognized by protein A can bind to the desired antigen, but will not be precipitated when pro-

tein A beads are added, reducing the efficiency of the immunoprecipitation.

The successful use of sequence-specific anti-phosphoprotein antibodies relies on their specificity for the phosphorylated form of the examined protein (see anti-MAP kinase antibodies below). Monoclonal antibodies, which are generally more specific than polyclonal ones, are reliable tools for distinguishing phosphorylated from unphosphorylated forms of proteins; affinity-purified polyclonal antibodies can be used as well. Both monoclonal and polyclonal antibodies can be generated by immunization with whole phosphorylated proteins or with keyhole limpet hemagglutinin (KLH)– or BSA–coupled peptides, followed by standard procedures for the isolation of the monoclonal and polyclonal antibodies. For example, immunization with whole phosphorylated protein was used to prepare monoclonal antibodies against several of the phosphorylated sites of Tau (Biernat et al., 1992). Immunization with KLH-conjugated phosphorylated peptides was used to prepare anti–active MAP kinases (Yung et al., 1997), anti–phosphorylated CREB (Ginty et al., 1993), and other anti–phosphorylated protein antibodies.

Immunofluorescent staining, another immunodetection method, can be performed with intact cells, such as those obtained from blood or tissue cultures, and on thin sections of various tissues. With immunofluorescence, degradation and dephosphorylation is less problematic because of the rapid fixation of the tissues. Although this method is very useful for detecting changes in phosphorylation in intact tissues and for localization studies, it requires highly specific antibodies, which are not always available. When appropriate highly specific antibodies are available, however, this method should be seriously considered.

For accurate comparison of the amounts of phosphoproteins, detection must be performed in the linear range of the detection system. Thus, the amount of protein loaded on the gel, the concentrations of primary and secondary antibodies, and the enhanced chemiluminescence (ECL) exposure time should be optimized in order to attain linearity. Alternatively, a standard curve can be prepared using a range of concentrations of the proteins of interest, and serial dilutions of the cellular extracts of each treatment can be loaded on the SDS-polyacrylamide gel. The blotting detection systems—e.g., ECL–^{125}I-, alkaline phosphatase (AP)–, or biotin-conjugated antibody—should be chosen carefully. ECL generally has the narrowest linear range of these systems, whereas ^{125}I-labeled antibodies have a relatively broad range. The AP detection system is convenient to use, has a moderate linear range, and is usually used for the types of experiments described here.

Anticipated Results

The desired phosphoprotein can be selectively detected with immunoblotting. For example, when anti–active MAP kinase antibodies are used (Basic Protocol 1; Yung et al., 1997), two faint bands at molecular weights of 42 and 44 kDa can be detected in the basal, nonstimulated fractions of Rat1 cells (Fig. 14.2.2). These two bands represent the small amount of active MAP kinase (ERK1 and ERK2) present in resting cells. Upon addition of EGF to these cells, the staining intensity of those bands should increase, peak at 30 min after stimulation, and decline thereafter. At later time points after EGF stimulation, a third band, at 46 kDa, usually appears; this may represent another isoform of MAP kinase. The kinetics of detection of the phosphoprotein represents the transient activation of MAP kinase by EGF in these cells. Staining the same blots with anti–general MAP kinase antibodies should result in equal staining of the 42-, 44-, and 46-kDa bands in all lanes of the blot.

Time Considerations

After cell harvesting, Basic Protocol 1 requires extraction (0.5 hr), determination of protein concentration (0.5 hr), SDS-PAGE (2.5 hr), and immunoblot analysis (6 to 8 hr). Since this procedure may take more than a working day, it can be stopped after boiling the samples in sample buffer. Alternatively, the transfer onto the nitrocellulose membrane can be performed at 40 to 50 mA overnight (instead of at 200 mA for 2 hr). Immunoprecipitation and washes (Basic Protocol 2) add ~4 to 5 hr to Basic Protocol 1. Fluorescent staining (Basic Protocol 3) takes ~3 to 4 hr, and up to 24 hr may be required to mount the stained coverslips onto the slides, depending on the mounting medium used.

Literature Cited

Ahn, N.G., Weiel, J.E., Chan, C.P., and Krebs, E.G. 1990. Identification of multiple epidermal growth factor–stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J. Biol. Chem.* 265:11487-11494.

Biernat, J., Mandelkow, E.M., Schroter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M., and Mandelkon, E. 1992. The switch of tau protein to an Alzheimer-like state includes the

phosphorylation of two serine-proline motifs upstream of the microtubule binding region. *EMBO J.* 11:1593-1597.

Campbell, J.S., Seger, R., Graves, J.D., Graves, L.M., Jensen, A.M., and Krebs, E.G., 1995. The MAP kinase cascade. *Recent Prog. Horm. Res*. 50:131-159.

Comoglio, P.M., Di Renzo, M.F., Tarone, G., Giancotti, F.G., Naldini, L., and Marchisio, P.C. 1984. Detection of phosphotyrosine-containing proteins in the detergent-insoluble fraction of RSV–transformed fibroblasts by azobenzene phosphonate antibodies. *EMBO J.* 3:483-489.

Frackelton, A.R. Jr., Ross, A.H., and Eisen, H.N. 1983. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor–stimulated cells. *Mol. Cell Biol*. 3:1343-1352.

Gabay, L., Seger, R., and Shilo, B.Z. 1997. In situ activation pattern of Drosophila EGF receptor pathway during development. *Science* 277:1103-1106.

Ginty, D.D., Kornhauser, J.M., Thompson, M.A., Bading, H., Mayo, K.E., Takahashi, J.S., and Greenberg, M.E. 1993. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science* 260:238-241.

Heffetz, D., Fridkin, M., and Zick, Y. 1989. Antibodies directed against phosphothreonine residues as potent tools for studying protein phosphorylation. *Eur. J. Biochem*. 182:343-348.

Hunter, T. 1994. 1001 protein kinases redux—Towards 2000. *Semin. Cell Biol*. 5:367-376.

Hunter, T. 1995. Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* 80:225-236.

Karin, M. and Hunter, T. 1995. Transcriptional control by protein phosphorylation: Signal transmission from the cell surface to the nucleus. *Curr. Biol*. 5:747-757.

Krebs, E.G. 1994. The growth of research on protein phosphorylation. *Trends Biochem. Sci*. 19:439.

Marshall, C.J. 1996. Ras effectors. *Curr. Opin. Cell Biol*. 8:197-204.

Pang, D.T., Sharma, B.R., Shafer, J.A., White, M.F., and Kahn, C.R. 1985. Predominance of tyrosine phosphorylation of insulin receptors during the initial response of intact cells to insulin. *J. Biol. Chem*. 260:7131-7136.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.-H., Shabanowitz, J., Hunt, D.F., Weber, M.J., and Sturgill, T.W. 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.* 10:885-892.

Robbins, D.J. and Cobb, M.H. 1992. Extracellular signal–regulated kinases 2 autophosphorylates on a subset of peptides phosphorylated in intact cells in response to insulin and nerve growth factor: Analysis by peptide mapping. *Mol. Biol. Cell* 3:299-308.

Robinson, M.J. and Cobb, M.H. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol*. 9:180-186.

Ross, A.H., Baltimore, D., and Eisen, H.N. 1981. Phosphotyrosine-containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten. *Nature* 294:654-656.

Salinovich, O. and Montelaro, R.C. 1986. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *Anal. Biochem.* 156:341-347.

Seger, R. and Krebs, E.G. 1995. The MAP kinase signaling cascade. *FASEB J.* 9:726-735.

Seger, R., Seger, D., Reszka, A.A., Munar, E.S., Eldar-Finkelman, H., Dobrowolska, G., Jensen, A.M., Campbell, J.S., Fischer, E.H., and Krebs, E.G. 1994. Over-expression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH-3T3 cells: Evidence that MAPKK's involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. *J. Biol. Chem.* 269:29876-29886.

Volberg, T., Zick, Y., Dror, R., Sabanay, I., Gilon, C., Levitzki, A., and Geiger, B. 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO J.* 11:1733-1742.

Yung, Y., Dolginov, Y., Yao, Z., Rubinfeld, H., Michael, D., Hanoch, T., Roubini, E., Lando, Z., Zharhary, D., and Seger, R. 1997. Detection of ERK activation by a novel monoclonal antibody. *FEBS Lett.* 408:292-296.

Key Reference

Gabay et al., 1997. See above.

Reports the use of anti–active MAP kinase antibodies (anti-diphospho-ERK) to follow MAP kinase (ERK) activation during different stages of Drosophila development, with immunostaining and immunoblotting both used throughout the study.

Contributed by Zhong Yao and Rony Seger
The Weizmann Institute of Science
Rehovot, Israel

The Detection of MAPK Signaling

The transmission of extracellular signals to their intracellular targets is mediated by a network of interacting proteins that relays biochemical messages and thus controls cellular processes. Several related intracellular signaling pathways (Seger and Krebs, 1995; Chen et al., 2001; Morrison and Davis, 2003; Raman and Cobb, 2003; Rubinfeld and Seger, 2004), collectively known as mitogen-activated protein kinase (MAPK) signaling cascades, have been demonstrated to play a role in many systems. Transmission of signals via these cascades is usually initiated by activation of a small G protein (e.g., Ras) and followed by a sequential stimulation of several sets of cytosolic protein kinases. Four distinct MAPK cascades (ERK, JNK, p38 and BMK; Fig. 14.3.1) have been elucidated so far. Each is named after the subgroup of its MAPK component and is composed of from three to five tiers (MAP4K, MAP3K, MAPKK, MAPK and MAPKAPK; Fig.14.3.1), where the three tiers MAP3K, MAPKK, and MAPK are considered the core cascade. One or more components in each of these tiers phosphorylates and activates components in the next level, until a downstream component phosphorylates a target regulatory molecule. These cascades can cooperate in transmitting signals from most extracellular stimuli, and thus can determine a cell's fate in response to the ever-changing environment. For detailed description see Background Information.

Because many of the MAPK cascade components are activated by phosphorylation, a convenient method to detect their activation is the use of phosphorylation-site-specific (anti-phospho) antibodies. Indeed, Basic Protocol 1 describes the use of protein blots (immunoblotting; also see *UNIT 6.2*) to detect protein phosphorylation with anti-phospho antibodies (see, e.g., Fig. 14.3.2). This method involves only three steps and usually yields impressive results. Unfortunately, the available, reliable anti-phospho antibodies are limited to some of the MAPK cascade components, and the use of these antibodies does not always reflect the kinetic parameters of the kinase activation. To overcome this problem, the unit also includes protocols that utilize the kinase activity of the MAPK components to determine their activation (Basic Protocols 2 and 3). Basic Protocol 2 describes an immunoprecipitation of desired protein kinases followed by phosphorylation of specific substrates. Basic Protocol 3 describes an in-gel kinase assay, which is mainly used when the identity of the kinase is not known, or when there are no reagents available for its determination.

STRATEGIC PLANNING

The relative intensity and duration of signals transmitted in each MAPK cascade are thought to be major determinants of signaling specificity (Marshall, 1995). Therefore, an accurate detection of the amount of signals transmitted via various MAPK cascades toward target molecules is important for studying intracellular signaling. Usually, the activity of one component of the MAPK level of each cascade (ERK, JNK, p38 and BMK) is a sufficient indicator of the transmitted signal. However, sometimes the activity of additional components at upstream or downstream levels must be determined because of a cross-talk between various cascades. For example, p38 can be activated by as many as three distinct MAPKKs (MKK 3, 4, or 6; Ono and Han, 2000), and, therefore, it is important to check which one of these MAPKKs is the immediate activator in different systems.

Most components of MAPK cascades belong to the large family of protein kinases, which in humans consists of about 520 members (Manning et al., 2002). To study protein kinases in general, and MAPK components in particular, specific detection of the activity

Contributed by Yoav Shaul and Rony Seger
Current Protocols in Cell Biology (2005) 14.3.1-14.3.34

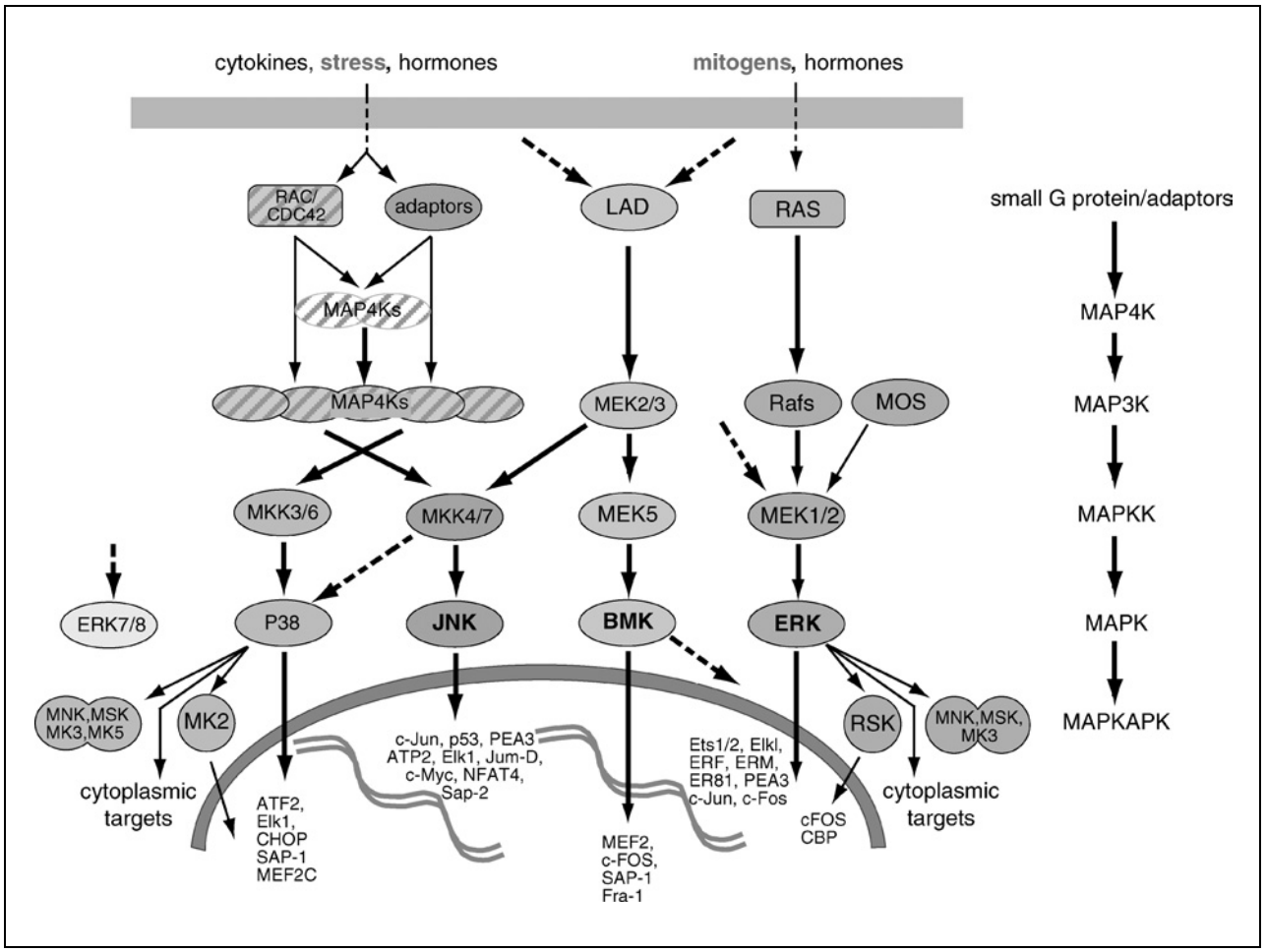


Figure 14.3.1 Schematic representation of MAPK cascades. The ERK cascade is represented in green, the JNK cascade in red, the p38 cascade in blue, and the BMK cascade in brown. Components that are shared by more than one cascade have combination of colors. The connections between components from different levels are shown by arrows; the specifics of these interactions have yet to be defined. This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.interscience.wiley.com/c_p/colorfigures.htm*.

of the desired protein kinase is essential. Singling out the activity of a particular protein kinase from a multitude of related activities that might mask its activity can be achieved in two main ways. One of them requires the use of a specific substrate that is recognized only by the desired protein kinase. This method is good for detecting kinases like MEK, which seems to specifically and selectively phosphorylate its downstream component, ERK. The other, and more common method is to isolate the protein kinase and then use a general substrate as an indicator of its activity. This unit will concentrate on the latter method of kinase activity determination, which has successfully been used in studies of MAPK cascades.

In one of the first methods used for the systematic detection of protein kinases involved in growth factor signaling, protein kinases were isolated using MonoQ fast protein liquid chromatography (FPLC; Ahn et al., 1990). This method involves stimulation of tissue culture cells, fractionating the cytosolic extracts of these cells on a MonoQ column, and examining the resulting fractions for protein kinase activity. Since fractionation with the MonoQ column is extremely reproducible, kinases that are activated upon stimulation can be detected by comparing the elution profiles of kinases from activated and nonactivated cells. The advantages of this method are: (1) the ability to identify novel protein kinases and measure their activity, (2) the ability to detect the overall activity of many protein kinases, and (3) the method's good linear range, which allows the determination of

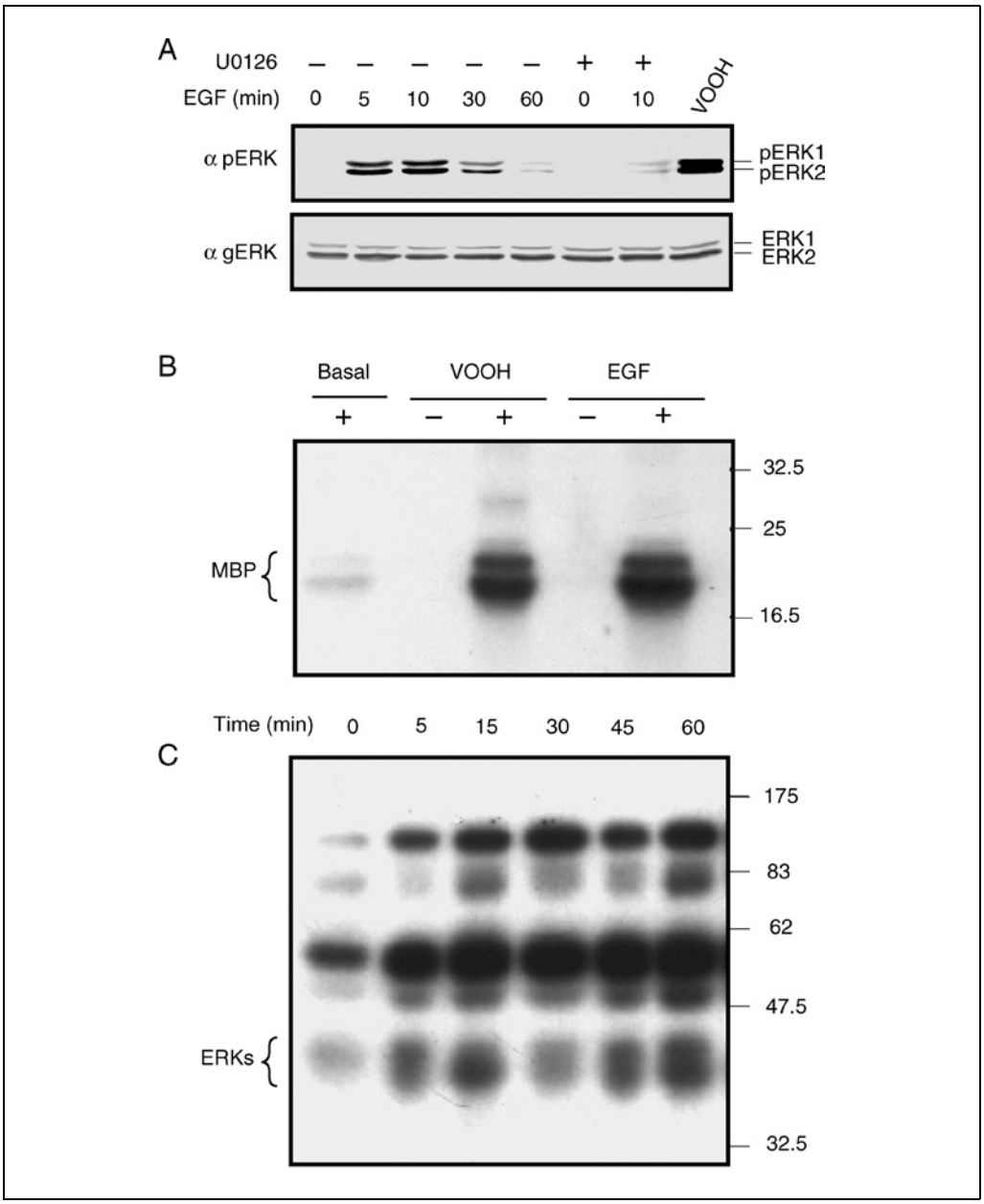


Figure 14.3.2 Detection of MAPK activity by the methods described in this unit. (**A**) Detection of ERK phosphorylation (activity) with anti–phospho ERK antibody. HeLa cells were grown in 6-cm plates until subconfluency and then starved for 18 hr in 2 ml/plate of starvation medium. Two plates (indicated) were then pretreated with the MEK inhibitor U0126 (10 μM) for 20 min while the other plates were left untreated. The cells were then either stimulated for the indicated times with EGF, peroxovanadate (0.1 mM vanadate, 0.2 mM H_2O_2), or with vehiecle control. Aliquots (30 μg) from each sample were separated by a 10% SDS-PAGE and subjected to immunoblotting with anti-pERK antibody and anti-gERK antibody (C-16; Santa Cruz Biotechnology), and detected using an AP detection system (Sigma). (**B**) Detection of ERK activity by immunoprecipitation and MBP phosphorylation. NIH-3T3 cells were grown in 6-cm plates until subconfluency and then starved as described as above. Cells were then stimulated either with VOOH (100 μM sodium orthovanadate/200 μM H_2O_2) for 15 min, with EGF (50 ng/ml) for 5 min, or left untreated (basal). Cytosolic extracts were prepared by sonication and the resulting proteins (300 μg) were incubated either with 30 μl of protein A beads conjugated with anti-ERK C-terminus antibody (C-16; Santa Cruz Biotechnology), represented by (+) or with protein A beads only, represented by (–). The phosphorylation reaction on MBP was performed as described in Basic Protocol 2. (**C**) Detection of protein kinase activities by in-gel kinase assay. MCF7 cells overexpressing ErbB-2 receptor were stimulated with EGF (50 ng/ml) for the indicated times. The in-gel kinase assay was performed as described in Basic Protocol 3. ERK1 and ERK2 bands are indicated. The identity of other bands was not determined.

the ratio between the activities of distinct protein kinases at a given time. Although this method has been good for some protein kinases, its main disadvantages are that separation of various proteins kinases is not always complete, and that it is a very laborious method.

Another method that is useful in detecting novel protein kinases is the in-gel kinase assay (Basic Protocol 3). This technique involves copolymerization of a given substrate on a sodium dodecyl sulfate (SDS)–polyacrylamide gel, electrophoresis of the samples of interest on the copolymerized gel, and in-gel phosphorylation in the presence of [γ-^{32}P]ATP. The advantage of this method is that it reveals the molecular weight of the kinases with the desired specificity, assisting in the identification of the enzymes of interest. Also, several samples can be examined simultaneously. The main disadvantages of this procedure are: (1) not all protein kinases can be renatured in the SDS gel, (2) each in-gel assay takes 2 or 3 days, and (3) there is a narrow linear range of protein kinase activities that can interfere with the detection of the fold induction of protein kinases upon stimulation.

The MonoQ fractionation and in-gel kinase assay methods are mainly used to identify or characterize novel protein kinases. However, since the resolutions of these two methods are not always adequate and they are very labor-intensive, more specific and convenient methods are recommended for the characterization of given protein kinases. Such specific methods often require the isolation of the protein kinase of interest, although a specific activator or substrate can sometimes be used (as is the case with PKA or MEK). The separation is often done by specific antibodies, which can be either directed against the phosphorylated sequence (anti-phospho antibodies) or against the whole molecule (anti-general antibodies), as described in this unit. The anti-phospho antibody becomes useful when the examined protein kinase is activated by phosphorylation and the activating phosphorylation site is known. Indeed, this seems to be the case for most MAPKs, MAPKAPKs, and MAPKKs known today, but it does not seem to be applicable for MAP3Ks and MAP4Ks, as described below. Probably the best known examples are the anti–active mitogen-activated protein kinase (MAPK; Seger and Krebs, 1995) antibodies (anti-diphospho ERK; Gabay et al., 1997; Yung et al., 1997), which are commercially available (Table 14.3.1). In response to stimulation, ERK1 and ERK2 are rapidly phosphorylated at up to six sites (Robbins and Cobb, 1992). Phosphorylation of two of these sites (threonine 183 and tyrosine 185 in ERK2; Payne et al., 1991) can lead to full activation of the enzyme, whereas the effects of phosphorylation of the other sites are not yet known. Antibodies directed towards the activation motif of ERK, which is PT-E-PY, serve as good tools for detecting its enzymatic activity, whereas anti-PAA antibodies can detect the total phosphorylation, which does not fully correlate with ERK's enzymatic activity. An important advantage of using anti-phospho antibodies is that they shorten and simplify the procedure of detection, which can be achieved in only one step (immunoblotting or cell staining). However, the antibodies do not usually serve as reliable tools to determine the exact kinetic parameters of the MAPK, as their dynamic range of detection is limited.

The successful use of sequence-specific anti-phospho antibodies relies on their specificity for the phosphorylated form of the examined protein. Monoclonal antibodies, which usually confer better specificity than polyclonal ones, are considered a reliable tool for distinguishing phosphorylated from nonphosphorylated forms of proteins, although affinity-purified polyclonal antibodies can be used as well. Both monoclonal and polyclonal antibodies can be generated by immunization with whole phosphorylated proteins or with KLH- or BSA-coupled peptides (see *UNIT 16.6*). Standard procedures for the isolation of the monoclonal and polyclonal antibodies are then employed. In this unit, the use of anti-phospho antibodies in immunoblot analysis is described. However, most of these antibodies can be used also for cell staining, which is described in *UNITS 4.3 & 14.2*.

Table 14.3.1 Antibodies Available Against MAPKs

Tier	Name	MAPK activated	Mol. wt. (kDa)	Commercially available antibodies	Supplier[a]	Reference
MAP4K	GCK	JNK	91	General	Many companies	Pombo et al. (1995)
	GLK	JNK	104	General	2, 11	Diener et al. (1997)
	GCKR	JNK	100	General	11	Shi and Kehrl (1997)
	HPK	JNK	97	General	7	Kiefer et al. (1996)
	HGK	JNK	130	General	11	Yao et al. (1999)
	KHS	JNK	95	General	11	Tung and Blenis (1997)
	SLK	JNK	210	General	2	Sabourin and Rudnicki (1999)
	MAP4K4/ NIK	JNK	100	General + phospho	Many companies	Su et al. (1997)
	SPAK	p38	64	General	2	Johnston et al. (2000)
	MST1	JNK, p38	59	General + phospho	7	Graves et al. (1998)
	TNIK	JNK	160	General	2,4	Fu et al. (1999)
	NESK	JNK	175	—	—	Nakano et al. (2000)
	MINK	JNK, p38	155	General	2, 8	Dan et al. (2000)
	PAK1	JNK, p38 (?)	68	General + phospho	11	Zhang et al. (1995)
	PAK5	JNK	90	General	11	Dan et al. (2002)
	MST4	ERK (?)	52	General	7	Lin et al. (2001)
MAP3K	ASK1	JNK, p38	155	General + phospho	Many companies	Ichijo et al. (1997)
	ASK2	JNK	145	General + phospho	—	Wang et al. (1998)
	TAK1	JNK, p38	82	General + phospho	Many companies	Moriguchi et al. (1996)
	LZK1	JNK	140	—	—	Sakuma et al. (1997)
	DLK1	JNK, p38	110	—	—	Fan et al. (1996)
	MLK1	JNK	130	General	11	Xu et al. (2001)
	MLK2	JNK, p38-? ERK1/2-?	115	General	11	Hirai et al. (1997)
	MLK3	JNK, p38 ERK-?	93	General + phospho	7, 11	Rana et al. (1996)
	MLK4	JNK, p38	120	—	—	Gallo and Johnson (2002)
	MLTK α/β	JNK, p38, ERK1/2-?, BMK-?	95/50	General	14	Gotoh et al. (2001)
	ZAK	JNK	91	General	2, 14	Yang (2002).
	MEKK1	JNK, p38. ERK1/2-?	195	General	1, 11	Yan et al. (1994)
	MEKK2	JNK, p38 BMK	70	General	11	Blank et al. (1996)
	MEKK3	JNK, p38, BMK	71	General	11	Blank et al. (1996)
	MEKK4	JNK	180	General	11	Gerwins et al. (1997)

continued

Table 14.3.1 Antibodies Available Against MAPKs, *continued*

Tier	Name	MAPK activated	Mol. wt. (kDa)	Commercially available antibodies	Supplier[a]	Reference
	TPL2	JNK, p38, ERK1/2, BMK	60 +	General + phospho	7	Salmeron et al. (1996)
	Raf-1	ERK1/2	74	General + phospho	Many companies	Kyriakis et al. (1992)
	B-Raf	ERK1/2	94	General	1, 11	Peraldi et al. (1995)
	A-Raf	ERK1/2	68	General	7	Hagemann and Rapp (1999)
	MOS	ERK1/2	39	General	1, 11	Posada et al. (1993)
	TAO1	JNK, p38	140	General	4	Hutchison et al. (1998)
	TAO2	JNK, p38	120	General + phospho	2, 9	Chen et al. (1999)
	MAP3K6/7, TAO3	?	Not charach-terized	—	—	Manning et al. (2002)
MAPKK	MEK1/2	ERK1/2	45/46	General + phospho	Many companies	Ahn et al. (1991)
	MKK3/6	p38	40/41	General + phospho	Many companies	Derijard et al. (1995)
	MKK4	JNK, p38	44	General + phospho	Many companies	Yan et al. (1994)
	MKK5	BMK	45	General	11	Zhou et al. (1995)
	MKK7	JNK	48	General + phospho	Many companies	Tournier et al. (1997)
MAPK	ERK1/2	ERK1/2	42.44	General + phospho	1-14	Ray and Sturgill (1987)
	p38 α−δ	p38	41,43.47, 54 + other forms	General + phospho	Many companies	Han et al. (1994)
	JNK1-3	JNK	46,52,54	General + phospho	Many companies	Derijard et al. (1994)
	BMK	BMK	110	General + phospho	Many companies	Zhou et al. (1995)
MAPKAPK	RSK1-3	ERK1/2	90	General + phospho	Many companies	Sturgill et al. (1988)
	MAPKAPK2	p38	45+other	General + phospho	Many companies	Stokoe et al. (1992)
	MAPKAPK3	p38, ERK1/2	43	General	3, 13	McLaughlin et al. (1996)
	MK5	p38, ERK1/2	56	—	—	Ni et al. (1998)
	MNK	p38, ERK1/2	52	General + phospho	7, 11	Waskiewicz et al. (1997)
	MSK	p38, ERK1/2	90	General + phospho	7, 13	Deak et al. (1998)
	SGK	BMK	54	General + phospho	11, 13	Hayashi et al. (2001)

[a]Suppliers: 1, Abcam (*http://www.abcam.com/*); 2, Abgent (*http://www.abgent.com*); 3, Abnova, (*http://www.abnova.com/tw*); 4, BD Biosciences; 5, Biomol; 6, Calbiochem; 7, Cell Signaling (*http://www.cellsignal.com*); 8, Novus Biologicals; 9, PhosphoSolutions (*http://www.phosphosolutions.com*); 10, Promega; 11, Santa Cruz Biotechnology; 12, Sigma; 13, Upstate Biotechnology; 14, Zymed. See *SUPPLIERS APPENDIX* for additional contact information.

Because not all MAPK components are activated by phosphorylation, and because the availability of commercial anti-phospho antibodies might be limited, the activity of the desired protein kinases may be determined by immunoprecipitation with specific antibodies directed to the C-terminal domain of the kinase (as described in Basic Protocol 2). Another use for anti-general antibodies is detection of slower migration on SDS-PAGE that occurs upon phosphorylation of regulatory residues of some MAPKs. However, this gel shift does not always correlate with enzymatic activity, as was shown for ERK and Raf1. Methods for affinity purification that do not involve antibodies can sometime be used to isolate given protein kinases (e.g., JNKs; Hibi et al., 1993). Although affinity techniques (including immunoprecipitation) are often used, it should be noted that the attachment to a solid support that occurs in this method may interfere with the accurate detection of the kinase activity. However, affinity reagents are not available for all MAPK components, and the identity of the desired protein kinase might be obscured. Therefore, the method that can be used in this case is the in-gel kinase assay as described in Basic Protocol 3. Although other methods are available, the combination of the methods described here should allow determination of the activity of most MAPK components and identification of the components that are activated by various extracellular stimuli.

IMMUNODETECTION OF MAPK ACTIVATION USING ANTI-PHOSPHO MAPK ANTIBODIES

BASIC PROTOCOL 1

This method describes the immunodetection of phosphoproteins using extraction of proteins by sonication followed by immunoblot analysis. Although phosphorylation of ERK1/2 is used here as an example, this immunodetection protocol can be used with most specific anti-phospho antibodies available for many components of the MAPK cascades (Table 14.3.1). This protocol minimizes the time phosphorylated proteins are exposed to phosphatases, which allows reliable and quantitative detection of the phosphorylated proteins, and it can be completed within 7 to 10 hr. Furthermore, this method can be used with almost all tissue culture cell lines, homogenized animal organs, and even whole lower organisms.

Materials

Rat1 cells (ATCC #CRL-2210)
DMEM containing 10% heat-inactivated FBS (see *APPENDIX 2A* and *UNIT 1.2*)
Starvation medium: DMEM containing 0.1% (v/v) heat-inactivated FBS (see *APPENDIX 2A* and *UNIT 1.2*)
50 μg/ml epidermal growth factor (EGF) in EGF buffer (0.5 mg/ml BSA in PBS)
EGF buffer: 0.5 mg/ml bovine serum albumin (BSA) in phosphate-buffered saline (PBS; *APPENDIX 2A*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
Buffer A (see recipe), ice-cold
Buffer H (see recipe), ice-cold
Protein standards: 5, 10, 20, 50, 100, and 200 μg/ml BSA in Buffer H (see recipe for buffer H)
Bradford protein assay reagent (Pierce or Bio-Rad; also see recipe for Coomassie dye reagent in *APPENDIX 3H*)
4× sample buffer for SDS-PAGE (see recipe)
1.5 M Tris·Cl, pH 8.8 (*APPENDIX 2A*)
30% acrylamide/0.8% bisacrylamide (Table 6.1.1)
10% (w/v) ammonium persulfate (prepare fresh)
Tetramethylethelendiamine (TEMED)
0.5 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
Running buffer (see recipe)
Prestained protein markers

Transfer buffer (see recipe)

Blocking solution: 2% (w/v) bovine serum albumin (BSA) in TBST (see recipe for TBST)

Primary antibodies: monoclonal mouse anti-phospho ERK antibody (Table 14.3.1) and polyclonal rabbit anti-general ERK antibody (C-terminus)

Tris-buffered saline with Tween 20 (TBST; see recipe)

Secondary antibodies: alkaline phosphatase (AP)–coupled goat anti-mouse antibody and horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibody

6-cm tissue culture dishes

1-ml pipet tips, precooled

1.5-ml microcentrifuge tubes precooled; four sets of six (each labeled 1 to 6)

Plastic (or rubber) policeman

Probe sonicator (e.g., Branson)

96-well flat-bottom microtiter plate

Microtiter plate reader capable of reading at 595 nm

95°C or boiling water bath

Gel-casting apparatus: 7 × 10–cm glass plates, 1.5-mm spacers, and 1.5-mm comb with 10 teeth (also see *UNIT 6.1*)

Gel electrophoresis apparatus and power supply (also see *UNIT 6.1*)

0.45-μm nitrocellulose membrane (e.g., Schleicher & Schuell or Millipore), cut to gel size

Whatman 3MM paper (two sheets cut to gel size)

Transfer apparatus and power supply (also see *UNIT 6.2*)

Additional reagents and equipment for cell culture (*UNIT 1.1*), SDS-PAGE (*UNIT 6.1*), and visualization of immunoblotted proteins (*UNIT 6.2 & 14.2*)

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH 7.4.

Prepare cellular extracts

1. Grow Rat1 cells in six 6-cm tissue culture dishes in 4 ml DMEM containing 10% FBS to subconfluency (~0.5×10^6 cells/dish).

   UNIT 1.1 describes basic cell culture techniques.

2. Serum-starve the Rat1 cells by removing the culture medium from each dish, replacing it with 2 ml starvation medium, and incubating 18 hr.

   During serum starvation, place the dishes in the tissue culture incubator, making sure that the dishes remain flat and that the medium covers the entire dish evenly. The aim of this starvation is to make the cells quiescent, and thereby reduce the amount of the inducible MAPK phosphatases (MKPs). Under these conditions, this can be achieved within 14 to 24 hr. Starvation for too long or any change in temperature or pH may be stressful to the cells, thereby inducing activation of one or more signaling pathways. Although this protocol describes EGF stimulation of Rat1 cells, this procedure, with minor changes, can be used for most extracellularly stimulated cells.

3. To each of three of the dishes, add 2.0 μl of 50 μg/ml EGF (stimulated samples). To each of the other three dishes, add 2.0 μl EGF buffer (control samples). Incubate one sample and control for 5 min, another sample and control for 15 min, and the third sample and control for 45 min.

Usually, the stimulus is given first to the dishes with the longest incubation, then, at appropriate intervals, to the dishes with the second longest and the shortest incubation periods. It is useful to make and use a time chart so that stimuli will be given at the appropriate times and the cells harvested within a short period of time (within 5 to 10 min for all dishes). If the influence of the stimulating agent on the particular cells is not yet known, a positive control should be included, such as a dish treated with 50 μl peroxovanadate (VOOH) solution (see recipe), for a final concentration of 100 μM sodium orthovanadate and 200 μM H_2O_2, which nonspecifically activates many signaling events in most tissue culture cells.

4. At the end of the assay, remove the medium from the dishes containing the Rat1 cells. Rinse dishes twice with 5 ml ice-cold PBS and once with 5 ml ice-cold buffer A. Be sure to remove all of the PBS after the last wash. Place dishes on ice.

   Because the objective at this stage is to arrest or slow down biological processes, the dishes should be placed on ice. Washing and harvesting of each dish should take 0.5 to 1.5 min. so that all six dishes are harvested within 5 to 10 min.

5. Add 250 μl of ice-cold buffer H to each dish, tilt the dish gently (preferably on ice), and scrape the cells into the buffer using a plastic (or rubber) policeman. Using precooled pipet tips, transfer the cells and buffer to prelabeled, precooled 1.5-ml plastic test tubes.

   Special consideration should be given to the composition of the buffer H (see Reagents and Solutions). The authors recommend using β-glycerophosphate, which serves both as a buffer and a general phosphatase inhibitor, rather than Tris or HEPES. Sodium orthovanadate is used to inhibit tyrosine phosphatases, and the mixture of pepstatin A, aprotinin, leupeptin, and benzamidine is used to inhibit proteinases. This buffer, when cold, blocks most of the phosphatase and proteinase activities in cell extracts.

6. Disrupt the cells by sonication using two 7-sec, 50-W pulses with a 20-sec interval per ~0.5-ml sample on ice.

   Over the years, several methods of protein extraction from cells have been successfully used in the study of protein kinases. In this protocol, which utilizes sonication, proteins are extracted from the cytosolic and nuclear fractions, but not from the membrane fraction, and therefore can be considered as cytosolic extract. Cellular extraction with nonionic detergents, which extract proteins from membranal, cytosolic, and some nuclear fractions of the cell, makes determination of the protein concentration somewhat difficult, but it is often used. Extraction with RIPA buffer or by freezing-thawing can also be used for some kinases.

7. Microcentrifuge the cellular extracts 15 min at 14,000 × *g*, 4°C. Transfer the resulting supernatants (cytosolic extracts), which contain the protein to be examined for phosphorylation, to fresh, precooled microcentrifuge tubes. Take a 5- to 20-μl aliquot of each extract and determine protein concentration (steps 8 to 11). Store the remainder of each cytosolic extract on ice until needed for the electrophoresis, transfer, and detection steps (steps 12 to 36).

   The protein concentration of each sample should be determined so that the amounts of proteins from the different samples can be compared, making it possible to determine the relative amounts of protein kinases in all samples accurately. If samples are compared based on cell number, differences of up to 20% in the amount of protein may result. Such differences may cause even larger ones in the following steps.

Determine protein concentration and prepare extracts for electrophoresis

8. Dilute 10 μl of each cytosolic extract sample in 190 μl buffer A (a final dilution of 1:20) in labeled tubes.

   Usually, dilutions of at least 1:20 are necessary to ensure that the samples will be in the linear range of the protein determination assay. For some Coomassie blue reagents with extended ranges, this dilution is not always necessary.

9. Put 10 μl of each of the protein standards (10, 25, 50, 75, 100, 150, and 200 μg/ml BSA in buffer H) into duplicate or triplicate wells of a flat-bottom 96-well microtiter plate.

   Protein standards should be prepared in the same buffer used for the cell extraction (in this case, buffer H).

10. Put 10 μl of each of the sample dilutions from step 8 into duplicate or triplicate wells of the same microtiter plate. Add 200 μl of Bradford protein assay reagent to all wells.

11. Place the microtiter plate in a microtiter plate reader and measure the optical density at 595 nM.

Perform electrophoresis

12. From the optical densities, calculate the protein concentrations of the samples. Take an aliquot of each extract corresponding to 40 μg of protein and place in a new 1.5-ml microcentrifuge tube.

    30 to 50 μg protein should be loaded in each lane of the gel. The authors strongly recommend using equal protein amounts for each sample to avoid inaccuracies.

13. Add 1/3 vol of 4× sample buffer to each tube, mix the contents, and place in 95°C or boiling water bath for 5 min.

14. Assemble 7 × 10–cm glass plates and 1.5-mm spacers for casting the polyacrylamide gel.

15. Prepare a 12% separating gel by mixing the following (total volume, 10.0 ml):

    3.4 ml H_2O
    2.5 ml 1.5 M Tris·Cl, pH 8.8
    4.0 ml 30% acrylamide/0.8% bisacrylamide
    100 μl 10% ammonium persulfate
    6 μl TEMED.

16. Pour the separating gel (*UNIT 6.1*). Overlay the top of the gel with water and allow it to polymerize ~30 to 45 min.

17. Prepare 3% polyacrylamide stacking gel by mixing the following (5 ml total):

    550 μl 30% acrylamide/0.8% bisacrylaimde solution
    625 μl 0.5 M Tris·Cl, pH 6.8
    3.7 ml H_2O
    120 μl ammonium persulfate
    5 μl TEMED.

18. Remove the water from the top of the polymerized separating gel. Using a Pasteur pipet, layer 2 ml of the stacking gel on top of the separating gel (*UNIT 6.1*) to fill up the apparatus. Insert comb into the stacking gel and allow it to polymerize for ~10 to 20 min.

19. Place the polymerized gel in the electrophoresis apparatus, add running buffer, and check for leaks.

20. Load 30 μl of the samples (from step 13) and 10 μl of the prestained protein markers into each well of the gel.

    Usually, prestained markers are loaded into the first or second lane of the gel so that the first lane can be located in the immunoblot and the molecular weights of the detected proteins determined. These markers will also indicate whether the proteins were completely transferred from the gel onto the nitrocellulose paper during blotting.

21. Connect the wire leads of the gel apparatus to the power supply. Turn on the power supply and run the gel at 150 V (constant voltage) until the bromphenol blue dye reaches a point 0.5 cm from the bottom of the gel (which usually takes ~1 hr).

Transfer the proteins to a membrane

22. Prewet (soak) the nitrocellulose membrane and 3MM Whatman paper in transfer buffer.

23. Once the dye front of the SDS-PAGE has reached the end of the gel, remove the gel from the apparatus, cut off the stacking gel, and place the separating gel in a container with transfer buffer.

24. Fill the transfer apparatus with transfer buffer. Open the inner transfer apparatus and remove air bubbles from the pads. Make a sandwich by putting a wet 3MM Whatman paper on the wet pad, the gel on top of the 3MM Whatman paper, the wet nitrocellulose membrane on top of the gel, and the other wet 3MM Whatman paper on top of the nitrocellulose membrane.

    Basic immunoblotting techniques are described in detail in UNIT 6.2.

25. Remove any air bubbles from between the different layers of the transfer sandwich by gently rolling a 10-ml pipet over the sandwich. Place the other wet pad on top of the transfer sandwich.

    Make sure that no air bubbles are trapped between the gel and the other components.

26. Place the sandwich containing the gel and nitrocellulose membrane into the buffer-filled transfer apparatus with the nitrocellulose membrane facing the side with the cathode and the gel facing the side with the anode. Connect the apparatus to a power supply and start the current. Transfer the proteins at 200 mA constant current for 120 min, preferably with a cooling device.

    The voltage will drop as the transfer progresses due to an increase in the conductivity. Methanol or 0.05% SDS are sometimes included in the transfer buffer; their inclusion will require different transfer conditions (see UNIT 6.2).

27. At the end of the transfer period, turn off the power supply and remove the nitrocellulose membrane from the transfer sandwich. Rinse the nitrocellulose membrane with transfer buffer to remove any adhering pieces of gel, and place the membrane in a flat container.

    At this stage, the efficiency of protein transfer can be monitored visually by the transfer of prestained protein markers to the membrane. The total amount of protein transferred can also be detected by staining the membrane with Ponceau S solution (UNIT 6.2). However, since the total amount of nonphosphorylated proteins is determined by general antibodies, as described later, staining with Ponceau S is probably not essential for this particular protocol.

Expose the membrane to antibodies

28. Incubate the nitrocellulose membrane in ~25 ml blocking solution for 60 min at room temperature.

    This will ensure that nonspecific protein binding sites on the membrane are blocked. The use of milk for blocking is not recommended because it causes nonspecific binding of some of the antibodies.

29. Incubate the blot with 10 to 20 ml of the primary antibody (e.g., monoclonal anti-phospho ERK antibody, diluted according to the supplier's recommendations in TBST) either overnight at 4°C, 30 min at 37°C, or 1 to 2 hr at room temperature.

30. Wash the blot in the flat container at least three times, each time for 15 min with 50 ml TBST at room temperature.

31. Incubate the blot with 10 to 20 ml of the secondary antibody (e.g., AP-conjugated goat anti-mouse IgG, diluted according to the supplier's instructions in TBST) for 45 min at room temperature.

For detection of the anti-phospho ERK antibody, the AP detection system is recommended because of its broader linear range compared to ECL (see Commentary). Alternatively, HRP-conjugated goat anti-rabbit antibodies can be used as secondary antibodies with detection by ECL. If HRP-rather than AP-conjugated antibodies are used at this stage, then AP-conjugated antibodies should be used in step 35.

32. Wash the blot at least three times, each time for 10 min, with 50 ml TBST.

33. Use an AP detection protocol (*UNIT 6.2*) to detect phospho ERK.

After detecting the phosophorylated (active) ERK, it is recommended that it be determined whether there is an equal amount of ERK in all lanes by exposing the same blot to polyclonal anti-general ERK antibody and redeveloping it (see steps 34 to 36).

Expose the membrane to general antibody

34. Incubate the stained nitrocellulose in blocking solution for 30 min at room temperature.

Since two different types of antibodies are used (mouse and rabbit), stripping away the antibodies used in steps 29 to 30 is not necessary, and the second blotting can be performed by simply adding the different type of antibody and following steps 35 and 36. Antibodies from the same species of origin (mouse or rabbit) can be used for both steps; however this requires a stripping step, which must be performed before continuing with step 35. Note that some of the antibodies can hinder recognition by other antibodies directed against different epitopes, and therefore also require stripping before the second type of antibody is applied.

35. Incubate the blot with 10 to 20 ml of the polyclonal rabbit anti-general ERK primary antibody, wash as described in step 30, then incubate with 10 to 20 ml of HRP-conjugated goat anti-rabbit secondary antibody.

36. Use a luminescent detection protocol for HRP (*UNIT 6.2*) to observe general ERK.

The ECL protocol involves mixing solution A (2.5 mM Luminol, 400 mM p-coumarin, 100 mM Tris·Cl, pH 8.5) with an equal amount of solution B (5.4 mM H_2O_2, 100 mM Tris·Cl, pH 8.5), incubation of the blot with the substrate solution for 1 min, drying the blot with 3MM Whatman paper, wrapping the blot in transparent plastic wrap and exposing it to X-ray film. See UNITS 6.2 & 14.2 for additional details.

BASIC PROTOCOL 2

DETERMINATION OF MAPK (ERK) ACTIVITY BY IMMUNOPRECIPITATION

This method describes determination of ERK activity by isolating the enzyme using immunoprecipitation with specific antibodies and then performing a phosphorylation reaction in vitro. Although this protocol uses ERK with its appropriate reagents, it can be performed with other components of the MAPK cascade, as antibodies are available for most components of these cascades (Table 14.3.1). This protocol allows a fast and efficient isolation of the desired protein kinase and its reliable quantitation by a phosphorylation reaction. It involves standard biochemical procedures and can be completed in 7 to 10 hr. Furthermore, this method can be used with almost all tissue culture cell lines, homogenized animal organs, and even lower organisms.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the institutional Radiation Safety Officer (also see *UNIT 7.1* and *APPENDIX 1D*).

Materials

Rat1 cells (ATCC #CRL-2210)
DMEM containing 10% heat-inactivated FBS (see *APPENDIX 2A* and *UNIT 1.2*)
Starvation medium: DMEM containing 0.1% (v/v) heat-inactivated FBS (see *APPENDIX 2A* and *UNIT 1.2*)
50 μg/ml epidermal growth factor (EGF) in EGF buffer
EGF buffer: 0.5 mg/ml bovine serum albumin (BSA) in phosphate buffered saline (PBS; *APPENDIX 2A*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
Buffer A (see recipe), ice-cold
Buffer H (see recipe), ice-cold
Protein standards: 5, 10, 20, 50, 100 and 200 μg/ml BSA in Buffer H
Bradford protein assay reagent (Pierce or Bio-Rad; also see recipe for Coomassie dye reagent in *APPENDIX 3H*)
Protein A–Sepharose beads (Amersham Biosciences; 10 to 20 μl of packed beads per reaction)
Antibody for immunoprecipitation (e.g., anti-ERK C-terminus antibody, 1 to 5 μg per reaction according to the supplier's instructions)
RIPA buffer (see recipe), ice-cold
Lithium chloride solution: 0.5 M LiCl/0.1 M Tris·Cl, pH 8.0 (see *APPENDIX 2A* for Tris·Cl), ice-cold
RM×3 (see recipe)
2 mg/ml myelin basic protein (MBP), or other appropriate phosphorylation substrate at appropriate (usually lower) concentration
4× sample buffer for SDS-PAGE (see recipe)
7 × 10–cm 15% SDS-PAGE gel with stacking gel (*UNIT 6.1*; also see Basic Protocol 1)
Prestained protein markers
Staining solution (see recipe)
Destaining solution (see recipe)
6-cm tissue culture dishes
1-ml pipet tips, precooled
1.5-ml microcentrifuge tubes precooled; four sets of six (each labeled 1 to 6)
Plastic (or rubber) policeman
Probe sonicator (e.g., Branson)
96-well flat-bottom microtiter plate
Microtiter plate reader capable of reading at 595 nm
End-over-end rotator
30°C Thermomixer (Eppendorf) or water bath
Boiling water bath
Flat container for staining/destaining gel
Additional reagents and equipment for cell culture (*UNIT 1.1*), SDS-PAGE (*UNIT 6.1*), and autoradiography or phosphor imaging (*UNIT 6.3*)

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH 7.4.

Prepare cellular extracts

1. Grow Rat1 cells in six 6-cm tissue culture dishes in 4 ml DMEM containing 10% FBS to subconfluency ($\sim 0.5 \times 10^6$ cells/dish).

   UNIT 1.1 describes basic cell culture techniques.

2. Serum-starve the Rat1 cells by removing the culture medium from each dish, replacing it with 2 ml starvation medium, and incubating 18 hr.

   During serum starvation, place the dishes in the tissue culture incubator, making sure that the dishes remain flat and that the medium covers the entire dish evenly. The aim of this starvation is to make the cells quiescent, and thereby reduce the amount of the inducible MAPK phosphatases (MKPs). Under these conditions, this can be achieved within 14 to 24 hr. Starvation for too long or any change in temperature or pH may be stressful to the cells, thereby inducing activation of one or more signaling pathways. Although this protocol describes EGF stimulation of Rat1 cells, this procedure, with minor changes, can be used for most extracellularly stimulated cells.

3. To each of three of the dishes, add 2.0 μl of 50 μg/ml EGF (stimulated samples). To each of the other three dishes, add 2.0 μl EGF buffer (control samples). Incubate one sample and control for 5 min, another sample and control for 15 min, and the third sample and control for 45 min.

   Usually, the stimulus is given first to the dishes with the longest incubation, then, at appropriate intervals, to the dishes with the second longest and the shortest incubation periods. It is useful to make and use a time chart so that stimuli will be given at the appropriate times and the cells harvested within a short period of time (within 5 to 10 min for all dishes). If the influence of the stimulating agent on the particular cells is not yet known, a positive control should be included, such as a dish treated with 50 μl peroxovanadate (VOOH) solution (see recipe), for a final concentration of 100 μM sodium orthovanadate and 200 μM H_2O_2, which nonspecifically activates many signaling events in most tissue culture cells.

4. At the end of the assay, remove the medium from the dishes containing the Rat1 cells. Rinse the dishes twice with 5 ml ice-cold PBS and once with 5 ml ice-cold buffer A. Be sure to remove all of the PBS after the last wash. Place dishes on ice.

   Because the objective at this stage is to arrest or slow down biological processes, the dishes should be placed on ice. Washing and harvesting of each dish should take 0.5 to 1.5 min. so that all six dishes are harvested within 5 to 10 min.

5. Add 250 μl of ice-cold buffer H to each dish, tilt the dish gently (preferably on ice), and scrape the cells into the buffer using a plastic (or rubber) policeman. Using precooled pipet tips, transfer the cells and buffer to prelabeled, precooled 1.5-ml plastic test tubes.

   Special consideration should be given to the composition of the buffer H (see Reagents and Solutions). The authors recommend using β-glycerophosphate, which serves both as a buffer and a general phosphatase inhibitor, rather than Tris or HEPES. Sodium orthovanadate is used to inhibit tyrosine phosphatases, and the mixture of pepstatin A, aprotinin, leupeptin, and benzamidine is used to inhibit proteinases. This buffer, when cold, blocks most of the phosphatase and proteinase activities in cell extracts.

6. Disrupt the cells by sonication using two 7-sec 50-W pulses with a 20-sec interval per ~0.5-ml sample on ice.

   Over the years, several methods of protein extraction from cells have been successfully used in the study of protein kinases. In this protocol, which utilizes sonication, proteins are extracted from the cytosolic and nuclear fractions, but not from the membrane fraction, and therefore can be considered as cytosolic extract. Cellular extraction with nonionic detergents, which extract proteins from membranal, cytosolic, and some nuclear fractions of the cell, makes determination of the protein concentration somewhat difficult, but is often used. Extraction with RIPA buffer or by freezing-thawing can also be used for some kinases.

7. Microcentrifuge the cellular extracts 15 min at 14,000 × *g*, 4°C. Transfer the resulting supernatants (cytosolic extracts), which contain the protein to be examined for phosphorylation, to fresh precooled microcentrifuge tubes. Take a 5- to 20-µl aliquot of each extract and determine protein concentration (steps 8 to 12). Store the remainder of each cytosolic extract on ice until needed for the immunoprecipitation and phosphorylation steps (steps 13 to 27).

   The protein concentration of each sample should be determined so that the amounts of proteins from the different samples can be compared, making it possible to determine the relative amounts of protein kinases in all samples accurately. If samples are compared based on cell number, differences of up to 20% in the amount of protein may result. Such differences may cause even larger ones in the following steps.

Determine protein concentration

8. Dilute 10 µl of each cytosolic extract sample in 190 µl buffer A (a final dilution of 1:20) in labeled tubes.

   Usually, dilutions of at least 1:20 are necessary to ensure that the samples will be in the linear range of the protein determination assay. For some Coomassie blue reagents with extended ranges, this dilution is not always necessary.

9. Put 10 µl of each of the protein standards (10, 25, 50, 75, 100, 150, and 200 µg/ml BSA in buffer H) into duplicate or triplicate wells of a flat-bottom 96-well microtiter plate.

   Protein standards should be prepared in the same buffer used for the cell extraction (in this case, buffer H).

10. Put 10 µl of each of the sample dilutions from step 8 into duplicate or triplicate wells of the same microtiter plate. Add 200 µl of the Bradford protein assay reagent to all wells.

11. Place the microtiter plate in a microtiter plate reader and measure the optical density at 595 nM.

12. From the optical densities, calculate the protein concentrations of the samples.

Prepare antibody-conjugated protein A–Sepharose beads

13. Place ~150 µl protein A–Sepharose beads in a 1.5-ml microcentrifuge tube and add 1 ml of PBS. Let the beads swell for 10 min at room temperature.

    Although protein A–conjugated Sepharose is recommended for this method, other commercially available protein A–conjugated resins, such as agarose, HiTrap, etc., may be used. Protein G–coupled resins are sometimes required to immunoprecipitate certain types of monoclonal antibodies (Table 7.2.1). If resins are supplied as ready-to-use suspensions, this swelling step is not necessary.

14. Microcentrifuge the swollen beads 1 min at 14,000 × *g*, room temperature, and remove the supernatant. Wash the swollen beads three times, each time by adding 1 ml PBS, centrifuging again as before, and discarding the supernatant.

15. Combine 180 µl of the swollen packed beads with 320 µl PBS. Add the recommended amount of the antibody for immunoprecipitation (e.g., ~10 µg anti-ERK C terminus). Rotate the mixture 1 hr at room temperature on an end-over-end rotator to allow the antibodies to bind to the protein A.

    Alternatively, this step can be done at 4°C for 16 hr.

    Usually, anti-C-terminal antibodies are used for the determination of kinase activity because their binding to the kinase does not interfere with the kinase activity. Ideally, the volumes listed here should be sufficient for ten reactions, but, because of the density of the beads, they will probably only be sufficient for eight reactions. Depending on the

number of reactions to be performed, the amounts given here can be scaled up as long as the proportions are maintained.

For easy handling of the resin, the ends of the pipet tips can be cut to enlarge their openings.

16. Microcentrifuge the antibody-conjugated beads 1 min at 14,000 × *g*, room temperature. Resuspend the beads in 1 ml PBS, then centrifuge again as before and remove the supernatant. Wash three times, each time by adding 1 ml ice-cold PBS, centrifuging again as before, and removing the supenatant. Resuspend the washed beads in an equal volume of ice-cold buffer A (~250 µl for ~250 µl of beads).

    Either use the antibody-conjugated beads immediately, or store at 4°C until used. It is best to use the conjugated beads within 3 days of preparation.

17. In precooled 1.5-ml microcentrifuge tubes, add 30 µl of the antibody-conjugated bead suspension from step 16 (equivalent to 15 µl packed beads) to 300 µl of cytosolic extract (from step 7) containing 50 to 500 µg total protein (determined as in steps 8 to 12) in buffer H. Rotate on an end-over-end rotator for 2 hr at 4°C.

    Several immunoprecipitation methods have been developed. These methods usually vary with respect to the order in which the antibodies and protein A are added to the cell extracts. In the protocol described here, the antibodies are conjugated to protein A beads and only then added to the cytosolic extracts. This procedure minimizes the time the samples are incubated with the antibodies, and thereby minimizes exposure of the desired kinases to phosphatases and proteinases in the extracts. Furthermore, this procedure ensures that only antibodies recognized by protein A will be used for the immunoprecipitation. Antibodies that are not recognized by protein A can bind to the desired antigen, but they will not be precipitated when protein A beads are added; therefore, such antibodies will reduce the efficiency of immunoprecipitation.

18. Centrifuge the incubation mixture 1 min at 14,000 × *g*, 4°C. Remove and discard the incubation supernatant from the antibody-conjugated beads. Add 1 ml ice-cold RIPA buffer, centrifuge again as before, and discard the supernatant. Wash the pellet twice, each time by adding 1 ml ice-cold 0.5 M lithium chloride solution, centrifuging again as before, and removing the supernatant, then wash twice, each time by adding 1 ml ice-cold buffer A, centrifuging again, and discarding the supernatant.

    These stringent washes are important because they will remove most sticky protein kinases that might nonspecifically interact with the protein A beads.

Perform phosphorylation reaction

19. After the last wash step in 18, completely remove buffer A from the conjugated beads by microcentrifuging again after the buffer has been aspirated from above the pellet (without resuspension), then gently removing the residual buffer above the beads.

20. Resuspend the bead pellet in 15 µl water.

    At this stage, prepare the bench for working with small amounts of radioactivity (see APPENDIX 1D).

21. Add 10 µl of RM×3 to each tube.

    See Reagents and Solutions for a discussion of the components of RM×3.

22. Start the phosphorylation reaction by adding 5 µl of the phosphorylation substrate (e.g., 2 mg/ml MBP) to the tube and placing the mixture in an Eppendorf Thermomixer (or water bath) at 30°C. Incubate 20 min at 30°C with either constant or frequent shaking.

    Although MBP is probably not a physiological substrate for any MAPK, it is a good general substrate for many kinases, including ERKs, in vitro. Substrates should be well phosphorylated by the desired kinases to allow accurate detection of the phosphorylation

kinetics. Therefore known substrates of the MAPK can be used as good substrates instead of MBP, but those are usually used at lower concentrations in the phosphorylation reaction.

23. End the phosphorylation reaction by adding 10 µl of 4× sample buffer to each tube.

24. Place samples in a boiling water bath for 5 min. Microcentrifuge 1 min at 14,000 × *g*, room temperature. Load the resulting supernatants on a 7 × 10–cm, 15% SDS-PAGE gel with stacking gel (*UNIT 6.1*). Load prestained markers on gel as well. Run the gel at 150 V (constant voltage) for ~1 hr or until the dye front is 0.5 cm from the bottom (*UNIT 6.1*).

    Usually, prestained markers are loaded into the first or second lane of the gel so that the first lane can be located on the dried gel and the molecular weight of the detected proteins determined.

    Since determination of enzymatic activity is not always accurate when enzymes (in this case protein kinases) are bound to beads, the kinase(s) of interest can be released from the beads at step 19 or 20 by using excess immunizing peptide. The phosphorylation reaction (steps 21 to 23) can thus be performed without the interference of the beads. Then the activity can be detected as described (step 27) or by a paper assay. For the paper assay, the phosphorylation reaction is terminated by spotting 20 µl of each sample on phosphocellulose paper squares (Whatman P81), which are immediately washed with 150 mM phosphoric acid. Phosphate incorporation is then measured using a scintillation cocktail. Gel quantitation can also be carried out by densitometry after imaging the gel as in step 27.

25. Turn the power supply off and disconnect the leads. Remove the glass plates containing the gel and remove the gel from the plate.

26. Place the gel in a flat container and stain the gel with 50 ml staining solution for 20 min at room temperature. Destain with four 30-min changes of 50 ml destaining solution.

    This extensive destaining removes excess free [γ-^{32}P]ATP, which would affect the background. In some cases, the extensive destaining is not necessary, or the proteins can be transferred to a nitrocellulose membrane and then be imaged as described below.

27. Place the gel on a sheet of Whatman 3MM paper, cover the gel with a clear plastic wrap, and dry the gel in a gel dryer (usually 1.5 hr at 80°C is sufficient). Expose the gel in a phosphor imager or autoradiograph on X-ray film (*UNIT 6.3*).

    Bands should appear at 16 to 21 kDa, corresponding to the molecular weight of the four MBP isoforms.

IN-GEL KINASE ASSAY

BASIC PROTOCOL 3

If the identity of the kinase is not known or there are no specific antibodies available for the kinase, the in-gel kinase assay may be used instead of the immunoprecipitation protocol (Basic Protocol 2). This in-gel protocol involves copolymerization of a substrate with the polyacrylamide gel followed by electrophoresis of the protein sample(s) on the gel. After several rounds of denaturation and renaturation, a phosphorylation reaction is performed with the gel, and the phosphorylated bands are visualized by autoradiography or phosphor imaging. With this method, the molecular weight of the protein kinase is revealed and unknown protein kinases can be identified (the molecular weight of most MAPK components is shown in Table 14.3.1). However, not all protein kinases can be renatured under the conditions of this protocol, and the linear range of this assay is usually limited. Therefore, this method should not be routinely used to monitor and characterize known protein kinases.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the institutional Radiation Safety Officer (also see *UNIT 7.1* and *APPENDIX 1D*).

Materials

Rat1 cells (ATCC #CRL-2210)
DMEM containing 10% heat-inactivated FBS (see *APPENDIX 2A* and *UNIT 1.2*)
Starvation medium: DMEM containing 0.1% (v/v) heat-inactivated FBS (see *APPENDIX 2A* and *UNIT 1.2*)
50 μg/ml epidermal growth factor (EGF) in EGF buffer
EGF buffer: 0.5 mg/ml bovine serum albumin (BSA) in phosphate buffered saline (PBS; *APPENDIX 2A*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
Buffer A (see recipe), ice-cold
Buffer H (see recipe) containing 1% (v/v) Triton X-100, ice-cold
Buffer H (see recipe), ice-cold
Protein standards: 5, 10, 20, 50, 100 and 200 μg/ml BSA in Buffer H (see recipe for buffer H)
Bradford protein assay reagent (Pierce or Bio-Rad; also see recipe for Coomassie dye reagent in *APPENDIX 3H*)
4× sample buffer for SDS-PAGE (see recipe)
1.5 M Tris·Cl, pH 8.8 (*APPENDIX 2A*)
30% acrylamide/0.8% bisacrylamide (Table 14.3.1)
10% (w/v) ammonium persulfate (prepare fresh)
Tetramethylethelendiamine (TEMED)
2 mg/ml myelin basic protein (MBP)
0.5 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
Running buffer (see recipe)
20% (v/v) isopropanol/50 mM HEPES, pH 7.6
Renaturation buffer**:** 50 mM HEPES, pH 7.6 containing 5 mM 2-mercaptoethanol
Renaturation buffer containing 6 M urea
Renaturation buffer containing 0.05% (v/v) Tween 20
In-gel kinase buffer (see recipe)
In-gel kinase reaction solution (see recipe)
5% (w/v) trichloroacetic acid (TCA)/1% (w/v) sodium pyrophosphate

6-cm tissue culture dishes
1-ml pipet tips, precooled
1.5-ml microcentrifuge tubes precooled; four sets of six (each labeled 1 to 6)
Plastic (or rubber) policeman
96-well flat-bottom microtiter plate
Microtiter plate reader capable of reading at 595 nm
Gel-casting apparatus: 7 × 10–cm glass plates, 1.5-mm spacers, and 1.5-mm comb with 10 teeth (also see *UNIT 6.1*)
Gel electrophoresis apparatus and power supply (also see *UNIT 6.1*)
Flat containers for washing gel
30°C water bath with proper shielding for radioactive work

Additional reagents and equipment for cell culture (*UNIT 1.1*), SDS-PAGE (*UNIT 6.1*), and autoradiography (*UNIT 6.3*)

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH 7.4.

Prepare cellular extracts

1. Grow Rat1 cells in six 6-cm tissue culture dishes in DMEM containing 10% FBS to subconfluency ($\sim 0.5 \times 10^6$ cells/dish).

   UNIT 1.1 describes basic cell culture techniques.

2. Serum-starve the Rat1 cells by removing the culture medium from each dish, replacing it with 2 ml starvation medium, and incubating 18 hr.

   During serum starvation, place the dishes in the tissue culture incubator, making sure that the dishes remain flat and that the medium covers the entire dish evenly. The aim of this starvation is to make the cells quiescent, and thereby reduce the amount of the inducible MAPK phosphatases (MKPs). Under these conditions, this can be achieved within 14 to 24 hr. Starvation for too long or any change in temperature or pH may be stressful to the cells, thereby inducing activation of one or more signaling pathways. Although this protocol describes EGF stimulation of Rat1 cells, this procedure, with minor changes, can be used for most extracellularly stimulated cells.

3. To each of three of the dishes, add 2.0 µl of 50 µg/ml EGF (stimulated samples). To each of the other three dishes, add 2.0 µl EGF buffer (control samples). Incubate one sample and control for 5 min, another sample and control for 15 min, and the third sample and control for 45 min.

   Usually, the stimulus is given first to the dishes with the longest incubation, then, at appropriate intervals, to the dishes with the second longest and the shortest incubation periods. It is useful to make and use a time chart so that stimuli will be given at the appropriate times and the cells harvested within a short period of time (within 5 to 10 min for all dishes). If the influence of the stimulating agent on the particular cells is not yet known, a positive control should be included, such as a dish treated with 50 µl peroxovanadate (VOOH) solution (see recipe), for a final concentration of 100 µM sodium orthovanadate and 200 µM H_2O_2, which nonspecifically activates many signaling events in most tissue culture cells.

4. At the end of the assay, remove the medium from the dishes containing the Rat1 cells. Rinse the dishes twice with 5 ml ice-cold PBS and once with 5 ml ice-cold buffer A. Be sure to remove all of the PBS after the last wash. Place dishes on ice.

   Because the objective at this stage is to arrest or slow down biological processes, the dishes should be placed on ice. Washing and harvesting of each dish should take 0.5 to 1.5 min, so that all six dishes are harvested within 5 to 10 min.

5. Harvest cells in 250 µl buffer H containing 1% Triton X-100 on ice, scraping the dishes with a plastic or rubber policeman and transferring the homogenate to a 1.5-ml microcentrifuge tube. Microcentrifuge 15 min at 14,000 × g, 4°C. Collect the supernatant and transfer to a new 1.5-ml tube.

   For this protocol, extraction with detergent is usually better than sonication.

Determine protein concentration

6. Dilute 10 µl of each extract from step 5 in 190 µl buffer A (a final dilution of 1:20) in labeled tubes.

   Usually, dilutions of at least 1:20 are necessary to ensure that the samples will be in the linear range of the protein determination assay. For some Coomassie blue reagents with extended ranges, this dilution is not always necessary.

7. Put 10 μl of each of the protein standards (10, 25, 50, 75, 100, 150, and 200 μg/ml BSA in buffer H) into duplicate or triplicate wells of a flat-bottom 96-well microtiter plate.

   Protein standards should be prepared in the same buffer used for the cell extraction (in this case, buffer H).

8. Put 10 μl of each of the sample dilutions from step 6 into duplicate or triplicate wells of the same microtiter plate. Add 200 μl of Bradford protein assay reagent to all wells.

9. Place the microtiter plate in a microtiter plate reader and measure the optical density at 595 nM.

10. From the optical densities, calculate the protein concentrations of the samples. Take an aliquot of each extract corresponding to 50 to 100 μg of protein and place in a new 1.5-ml microcentrifuge tube.

11. Add 1/3 vol of 4× sample buffer to each tube. Keep at 4°C, without boiling.

Electrophorese the sample

12. Assemble 7 × 10–cm glass plates and 1.5-mm spacers for casting the polyacrylamide gel.

13. Prepare a 12% separating gel containing MBP by mixing the following (total volume, 8 ml):

    0.7 ml H_2O
    2.0 ml 2 mg/ml MBP
    2.0 ml 1.5 M Tris·Cl, pH 8.8
    3.2 ml 30% acrylamide/0.8% bisacrylamide
    100 μl 10% ammonium persulfate
    6 μl TEMED.

14. Pour the separating gel (*UNIT 6.1*). Overlay the top of the separating gel with water. Allow the gel to polymerize ~30 to 45 min.

15. Prepare 3% polyacrylamide stacking gel by mixing the following (5 ml total):

    550 μl 30% acrylamide/0.8% bisacrylaimde solution
    625 μl 0.5 M Tris·Cl, pH 6.8
    3.7 ml H_2O
    120 μl ammonium persulfate
    5 μl TEMED
    5 ml total.

16. Remove the water from the top of the polymerized separating gel. Using a Pasteur pipet, layer ~2 ml of the stacking gel on top of the separating gel (*UNIT 6.1*) to fill up the apparatus. Insert comb into the stacking gel and allow it to polymerize 10 to 20 min.

17. Place the polymerized gel in the electrophoresis apparatus, add running buffer, and check for leaks.

18. Load ~40 μl of the samples (from step 13; containing ~70 μg protein per lane) and 10 μl of the prestained protein markers (according to the manufacturer's instructions) onto the gel. Electrophorese at 100 V for ~1 hr or until the dye front is ~0.5 cm from the bottom (*UNIT 6.1*).

    The gel should not be heated above 30°C, therefore, the voltage used for electrophoresis should not be higher than 100 V.

19. When electrophoresis is completed, turn the power supply off, remove the glass plates from the electrophoresis apparatus, and remove the gel from the plates.

Wash the gel and renature the proteins

20. Cut off the stacking gel. Place the separating gel in a flat container and wash twice, each time with 100 ml of 20% isopropanol/50 mM HEPES, pH 7.6, for 30 min at room temperature with shaking on a platform shaker. Wash the gel twice more, each time with 100 ml renaturation buffer for 30 min at room temperature with shaking, then wash the gel twice more, each time with 100 ml of 6 M urea (in renaturation buffer) for 15 min with shaking at room temperature. Leave the last urea wash on the gel and proceed to step 21.

    If necessary, the second wash with 20% isopropanol/50 mM HEPES can be done overnight at 4°C.

21. Place the gel in a 4°C cold room, remove 50 ml of the 6 M urea, add 50 ml renaturation buffer containing 0.05% Tween 20, and shake for 15 min on a platform shaker.

    The washing solution is now 3 M urea in renaturation buffer containing Tween 20.

22. Remove 50 ml of the washing solution, add 50 ml of renaturation buffer containing 0.05% Tween 20, and shake for additional 15 min

    This reduces the urea to 1.5 M.

23. Remove 50 ml of the washing solution once more and replace with renaturation buffer/0.05% Tween 20, so that the washing solution contains 0.75 M urea. Shake for 15 min.

24. Wash the gel twice, each time with 100 ml renaturation buffer containing 0.05% Tween 20 for 15 min with shaking at 4°C. Wash a third time with the same solution with shaking overnight in the cold room.

Detect kinase activity

25. Remove the washing buffer and incubate the gel in 30 ml in-gel kinase buffer for 30 min at 30°C. Remove the buffer and add 20 ml of in-gel kinase reaction solution.

    At this stage, the amount of radioactive material is very high, and therefore the reaction should be performed with a proper shielding. Make sure that the gel is straight in the flat container. Unequal distribution of the phosphorylation buffer may lead to wrong concentrations of the ingredients and thereby interfere with the phosphorylation reaction.

26. Wash gel carefully four times, each time with 3 ml 5% TCA/1% sodium pyrophosphate for 15 min at room temperature.

    If the gel is still very radioactive, continue washing overnight.

27. Dry the gel and subject to autoradiography (*UNIT 6.3*).

    Bands should appear where kinases are present and cause phosphorylation of the MBP copolymerized in the gel (see Fig. 14.3.2).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

50 mM β-glycerophosphate, pH 7.3
1.5 mM EGTA
1.0 mM EDTA
1.0 mM dithiothreitol (DTT)
0.1 mM sodium orthovanadate
Store up to 2 weeks at 4°C

Recipe from Ahn et al. (1990).

Buffer H

50 mM β-glycerophosphate, pH 7.3
1.5 mM EGTA
1.0 mM EDTA
0.1 mM sodium orthovanadate
Store solution with above components up to 3 months at 4°C
Immediately before use, add:
1.0 mM DTT
1.0 mM benzamidine
10 μg/ml aprotinin
10 μg/ml leupeptin
2.0 μg/ml pepstatin A

Recipe from Seger et al. (1992). "H" refers to homogenization buffer.

Destaining solution

15% (v/v) isopropanol
7% (v/v) acetic acid
Store up to 1 month at room temperature

In-gel kinase buffer

20 mM HEPES, pH 7.6
20 mM $MgCl_2$
Store up to 1 month at 4°C

In-gel kinase reaction solution

20 mM HEPES, pH 7.6
20 mM $MgCl_2$
2 mM DTT
20 μM ATP (unlabeled)
100 μCi [γ-^{32}P]ATP
Store up to 2 weeks at −20°C

Peroxovanadate (VOOH) solution

Mix 200 μl of 20 mM sodium orthovanadate with 800 μl of 10 mM H_2O_2 in PBS (see *APPENDIX 2A* for PBS). Allow mixture to incubate 15 min at room temperature before use. Prepare fresh.

RIPA buffer

137 mM NaCl
20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
10% (v/v) glycerol
1% (v/v) Triton X-100
0.5% (w/v) sodium deoxycholate
0.1% (w/v) SDS
2.0 mM EDTA
Store with above components up to 1 month at −20°C
1.0 mM phenylmethylsulfonyl fluoride (PMSF, add fresh)
20 μM leupeptin

RM×3

75 mM β-glycerophosphate, pH 7.3
30 mM $MgCl_2$
100 μM [γ-^{32}P]ATP (~4000 cpm/pmol)
0.9% (w/v) bovine serum albumin (BSA)

continued

3 mM DTT
3 mM EGTA
0.3 mM sodium orthovanadate
Store up to 3 month at −20°C

The most important components of the reaction mixture (also see Basic Protocol 2, step 21) are the Mg^{2+} and [γ-^{32}P]ATP, which are essential for the phosphorylation reaction. The authors recommend the use of 100 μM ATP with ~4000 cpm/pmol of the labeled ATP, which provides an extended linear range and reproducible results. When the enzymatic activity of the kinases is very low, which makes detection of phosphorylation difficult, the concentration of cold ATP should be reduced to 10 to 20 μM and the amount of radioactive material elevated. Addition of labeled ATP alone is not recommended because this will result in a nanomolar concentration of ATP, which is much below the K_m for ATP and may lead to nonspecific phosphorylation. The β-glycerophosphate in the reaction mixture serves as a buffer, but it can also inhibit residual phosphatases that may have nonspecifically bound to the beads. The BSA serves as a carrier protein, but when purity is required, it can be eliminated. The EGTA chelates Ca^{2+}, which may interfere with some kinase activities, DTT keeps the proteins reduced, and sodium orthovanadate inhibits tyrosine phosphatases.

Running buffer

25 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
188 mM glycine
0.1% (w/v) SDS
Store up to 1 month at room temperature

Sample buffer, 4×

200 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
40% (v/v) glycerol
8% (w/v) SDS
8% (v/v) β-mercaptoethanol
0.2% (w/v) bromphenol blue
Store up to 3 months at −20°C

Staining solution

40% (v/v) methanol
7% (v/v) acetic acid
0.005% (w/v) bromphenol blue
Store up to 3 months at room temperature

Transfer buffer

50 mM Tris·Cl, pH 8.8 (*APPENDIX 2A*)
50 mM glycine
Store up to 1 month at room temperature

Tris-buffered saline with Tween 20 (TBST)

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
150 mM NaCl
0.05% (v/v) Tween 20
Store up to 1 month at room temperature

COMMENTARY

Background Information

Sequential activation of kinases (protein kinase cascades) is a common mechanism of signal transduction in many cellular processes (Seger and Krebs, 1995). The best studied protein kinase cascades known today are mitogen-activated protein kinase (MAPK) signaling cascades, which are the topic of this unit (Seger

and Krebs, 1995; Chen et al., 2001; Morrison and Davis, 2003; Raman and Cobb, 2003; Rubinfeld and Seger, 2004). Each of these signaling cascades consists of up to five tiers of protein kinases that sequentially activate each other by phosphorylation. The similarity among the enzymes that comprise each tier in the various cascades categorizes them into a superfamily of kinases with similar structure and function.

The activation of each of these cascades is initiated either by small GTP-binding proteins or by adaptor proteins that transmit the signal to protein kinases, commonly referred to as MAPK kinase kinases (MAP3Ks). In some cases, the activation is mediated by upstream, Ste20-like kinases, which are termed MAP4Ks, although in many cases the MAP3Ks can be activated directly by the GTP binding proteins, or the MAP4Ks bypass the MAP3Ks (Fig. 14.3.1, Table 14.3.1). The signal is then transmitted downstream in the cascade by the sequential activation of MAPK kinase (MAPKK), MAPK and MAPK-activated protein kinases (MAPKAPKs), and, in a few cases, also kinases downstream of this tier. The existence of as many as three to six tiers in each of the MAPK cascades is probably essential for signal amplification, specificity determination, and tight regulation of the transmitted signal.

The four distinct MAPK cascades that are currently known are named according to the subgroup of their MAPK components. These are (1) the extracellular signal-regulated kinase (ERK) cascade; (2) the c-Jun N-terminal kinase [JNK; or stress activated protein kinase 1 (SAPK1) cascade]; (3) the p38 cascade (Freshney et al., 1994; Han et al., 1994; Lee et al., 1994); and (4) the BMK1 (ERK5) cascade (see Seger and Krebs, 1995 and Chuderland and Seger, 2005, for reviews). The identification of ERK7/8 (Abe et al., 1999) indicates that additional full MAPK cascades can emerge. The ERK cascade seems to participate mainly in the transmission of mitogenic signals, whereas the p38 and JNK cascades transmit mainly stress signals. BMK seems to be activated equally by mitogens and stress stimuli. These MAPK cascades cooperate to transmit signals to their intracellular targets, and thus to initiate cellular processes such as proliferation, differentiation, development, stress response, and apoptosis. In this section, the various MAPK cascades will be briefly reviewed.

The ERK cascade, also known as the p42, p44 MAPK cascade, was the first MAPK cascade elucidated (Seger and Krebs, 1995). This cascade is initiated in many cases by the small G-protein Ras, which, upon stimulation, causes membranal translocation and activation of the protein serine/threonine kinase, Raf1 (reviewed in Hagemann and Rapp, 1999). Once activated, Raf1 continues the transmission of the signal by phosphorylating two regulatory serine residues located in the activation loop of MEK, thus causing its full activation (Kyriakis et al., 1992). Interestingly, Raf-1 activation can be facilitated by PKC, and this can implicate the PKC as a MAP4K of the ERK cascade. Other kinases that act in the MAP3K level of the cascade under various conditions are—(1) B-Raf (Peraldi et al., 1995), which is usually activated by the small G-protein Rap1 (Hagemann and Rapp, 1999) in combination with phosphorylation by MLK3 (Chadee and Kyriakis, 2004); (2) A-Raf (Hagemann and Rapp, 1999); (3) Mos, which acts specifically in the reproductive system (Gotoh and Nishida, 1995); (4) the protooncogene TPL2 (Salmeron et al., 1996); (5) under stress conditions, possibly also MEKK1 (Lange-Carter et al., 1993) and other MAP3Ks such as MLK2 (Hirai et al., 1997), MLTK, (Gotoh et al., 2001), MLK3 (Chadee and Kyriakis, 2004), and even IRAK (MacGillivray et al., 2000), although the direct effect of these kinases is still controversial. Another kinase on this level is kinase suppressor of Ras (KSR), which seems to act mainly as a scaffold protein for some of the MAP3K and MAPKK components of the cascade (Morrison and Davis, 2003); but the role of its catalytic activity is still controversial (Kolesnick and Xing, 2004). In any case, all the indicated MAP3Ks seem to phosphorylate the same regulatory residues of MEK, which are required for its full activation (Ahn et al., 1991; Seger et al., 1992). Activated MEK is a dual-specificity protein kinase, which appears to be the only kinase capable of specifically phosphorylating and activating the next kinase in this cascade, i.e., ERK (Boulton et al., 1990, 1991). ERK activation requires phosphorylation of two regulatory residues, threonine and tyrosine, which reside in a TEY phosphorylation motif (Payne et al., 1991; Canagarajah et al., 1997). Although phosphorylation of threonine and tyrosine residues is essential for the activation of all MAPKs, in the other cascades, the identity of the middle amino acid in the TXY motif of the MAPK is different,

and this determines the specificity of the signal (Seger and Krebs, 1995).

ERK appears to be an important regulatory molecule, which, by itself, can phosphorylate regulatory targets in the cytosol such as phospholipase A_2 (PLA_2; Lin et al., 1993). It can translocate into and phosphorylate nuclear substrates such as the transcription factors Elk1 (Gille et al., 1992), cFos (Murphy et al., 2002), p53 (Milne et al., 1994), ERF (Sgouras et al., 1995), PEA-3 (O'Hagan et al., 1996), ERM (Janknecht et al., 1996), ER81 (Janknecht et al., 1996), Ets1/2 (Yang et al., 1996), SAP-1a (Strahl et al., 1996), and cJun (Morton et al., 2003), or it can transmit the signal to the MAPKAPK level. The main MAPKAPK of the ERK cascade is RSK (Sturgill et al., 1988), which can also translocate to the nucleus upon activation and phosphorylate a set of nuclear substrates different from those phosphorylated by ERK. Additional MAPKAPKs are the mitogen- and stress-activated kinase (MSK; Deak et al., 1998) and the MAPK-interacting kinases (MNKs; Fukunaga and Hunter, 1997; Waskiewicz et al., 1997), which are activated equally by the related p38 cascade. Finally, the MAPKAPK3 (McLaughlin et al., 1996) and MK5 (Ni et al., 1998) cascades are only slightly related to the ERK cascade and are mainly activated by the p38 cascade (for review see Roux and Blenis, 2004). MAPKAPKs can regulate the activity of additional kinases (e.g., Myt1; Palmer et al., 1998), but those are usually not considered to be genuine members of the MAPK cascade.

ERKs are activated primarily by mitogenic signals, whereas other MAPK cascades (p38, JNK) are activated mainly by cellular stresses such as heat shock, ischemia, UV irradiation, and cytokines (Davis, 2000); therefore they are referred to as stress-activated protein kinase (SAPK) cascades (Kyriakis and Avruch, 1996). The p38 cascade consists of MAPKs that contain a glycine residue in their activation, TXY motif (TGY; Han et al., 1994). Many kinases on the MAP3K and MAP4K levels have been implicated in the p38 cascade (Fig. 14.3.1, Table 14.3.1); however, their individual roles are not yet fully elucidated. Moreover, in spite of their sequence similarity to the Ste20 and Ste11 kinases (the MAP4K and MAP3K in *Saccharomyces cerevisiae* that operate in a sequential order; Herskowitz, 1995), the mammalian kinases do not always act in a sequential order, and may use different mechanisms to activate the rest of the cascade (Dan et al., 2001; Hagemann and Blank, 2001).

Independent of the particular MAP3K or MAP4K, the signals of the p38 cascade are funneled into two main MAPKKs: MKK3 (also known as MEK3 or p38MAPKK; Derijard et al., 1995) and MKK6 (also known as MEK6, or SAPKK3; Han et al., 1996). Similarly to MEK1/2, these are dual-specificity protein kinases that phosphorylate the regulatory threonine and tyrosine residues of the p38s to fully activate them. Although these two MKKs are unique in their ability to activate p38s and no other MAPKs, the p38s seem to be activated by MKK4 (Derijard et al., 1995) and to some extent also by MKK7 (Dashti et al., 2001), which are the main activators of the JNK cascade (as indicated below). The MAPK-level components of this cascade are the four p38s (p38α to δ, also known as SAPK2a SAPK2b, SAPK3, and SAPK4; Han et al., 1994; Goedert et al., 1997), which are normally expressed as four main proteins of 41, 43, 47, and 51 kDa, and at least six additional alternatively spliced forms with molecular masses that range between 32 and 65 kDa. Once these p38s are activated, they phosphorylate and modulate the activity of a large number of substrates in various compartments in the cells (Davis, 2000). Thus, the p38s can phosphorylate and activate various MAPKAPK-level components including MAPKAPK2 and 3 (Stokoe et al., 1992; McLaughlin et al., 1996), MSK (Deak et al., 1998), MNK (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997), and MK5 (Ni et al., 1998). Except for MAPKAPK2, all MAPKAPKs can also be activated by the ERK cascade (see above) and therefore present a point of cross-talk between the two cascades (for review see Roux and Blenis, 2004). Another important group of p38 substrates are various transcription factors that are usually activated under stress conditions, including ATF2, Elk1 (Raingeaud et al., 1996), CHOP (Wang and Ron, 1996), MEF2C (Han et al., 1997), SAP-1 (Cuenda et al., 1997), and p53 (Huang et al., 1999).

Other stress-activated MAPKs are the c-Jun amino-terminal kinases (JNKs; also named SAPK1), which comprise a third MAPK subgroup (Derijard et al., 1994; Kyriakis et al., 1994). These enzymes are distinct from the p38s mainly because they contain a TPY rather than a TGY motif in their activation loop. As the other MAPK cascades, the JNK cascade is triggered by small GTPases (Crespo et al., 1997), i.e., Rac and CDC42, or it can be activated by various adaptor molecules (Davis,

2000). Next, the signals are transmitted via MAP4K and MAP3K components that are mostly shared with the p38 cascades (Table 14.3.1). Since the p38 and JNK cascades are not always simultaneously activated, the signals must be separately regulated to allow separate cascades, and this is mostly executed by a physical segragation between the components by specialized scaffold proteins (Morrison and Davis, 2003). At the MAPKK level, the JNKs can be activated by MKK4 (MEK4, SEK1, JNKK1; Sanchez et al., 1994; Yan et al., 1994), and MKK7 (MEK7, JNKK2; Tournier et al., 1997). These MKKs are able to phosphorylate and activate the components in the MAPK level, which are JNK1-3 (SAPKs) of molecular weights 46, 54, and 52 kDa, respectively (Derijard et al., 1994; Kyriakis et al., 1994). No enzymes in the MAPKAPK level have been identified for JNKs, which appear to be major regulators of nuclear processes, in particular, transcription. Shortly after activation, JNKs translocate to the nucleus where they physically associate with and activate their targets, mostly transcription factors such as c-Jun (Hibi et al., 1993), ATF2 (Gupta et al., 1995), Elk1 (Whitmarsh et al., 1995), p53 (Milne et al., 1995), JUN-D (Kallunki et al., 1996), PEA3 (O'Hagan et al., 1996), NFAT4 (Chow et al., 1997), c-Myc (Noguchi et al., 1999), and SAP-2/Net (Ducret et al., 2000). Interestingly, the JNKs also participate in the induction of apoptosis by several proapoptotic drugs, and this is mediated by the phosphorylation of various death-related components in various cellular compartments (for review see Varfolomeev and Ashkenazi, 2004).

Another MAPK subgroup is the BMK (also known as ERK5; Zhou et al., 1995; Abe et al., 1996), with a molecular weight of 110 kDa. Like ERK1/2, BMK contains a TEY phosphorylation motif in its activation loop, but because it is not activated at all by MEK1 and 2, it forms a separate signaling cascade. BMK seems to be activated equally by stress (Zhou et al., 1995; Abe et al., 1996) and by mitogenic signals (Kato et al., 1998), and thereby participates in the regulation of a variety of cellular processes including oncogenic transformation (English et al., 1999). The cascade is thought to be activated by the adaptor protein Lad, which operates downstream of c-Src, at least in the pathway that leads to BMK activation by growth factors (Sun et al., 2003). In turn, Lad transmits the signal to protein kinases on the MAP3K level of the cascade, MEKK2 and MEKK3, which are implicated also in the activation of the p38 and JNK cascade (Chao et al., 1999; Chayama et al., 2001). The signal is then transmitted downstream the cascade to the MAPKK, MEK5 (Zhou et al., 1995), which specifically phosphorylates and activates the kinase on the MAPK level, BMK. Interestingly, MEK5 and BMK are often localized in the cell nucleus (Raviv et al., 2004), where BMK associates with several transcription factors substrates including c-Myc (English et al., 1998), MEF2 family members (Kato et al., 1997; Yang et al., 1998), c-Fos (Kamakura et al., 1999), SAP1a (Kamakura et al., 1999), Fra-1 (Terasawa et al., 2003), and possibly also NFκB (Pearson et al., 2001). Importantly, BMK also influences transcription through a direct transcriptional activity, as shown in the activation of the Nur77 gene upon calcium signals in T cells (Kasler et al., 2000). In addition to its nuclear activity, BMK mediates several cytosolic functions including phosphorylation of connexin-43 (Cameron et al., 2003) and of the serum- and glucocorticoid-inducible kinase SGK (Hayashi et al., 2001), implicating the latter as a MAPKAPK in the ERK5 cascade.

Besides these four MAPK cascades, which are composed of similar components in each level, other protein kinases and kinase cascades are activated in response to mitogenic stimulation. First, two MAPKs termed ERK7 and ERK8, which are similar to each other (Abe et al., 2001), have been identified. Although their mode of activation is not clear, they are likely to operate within an independent, fifth MAPK cascade. Another cascade is that of ERK3, which, in spite of its 50% identity to ERK1/2, is not considered a MAPK because of the absence of the TEY motif in its activation loop (Boulton et al., 1991). ERK3 is activated by an ERK3K with an unknown identity (Cheng et al., 1996), and it seems to function mainly in muscles. In addition, the NIK-IKK (Malinin et al., 1997), PI3K-PDK-AKT-GSK3 (Cohen et al., 1997), Rho-dependent pathways (Leung et al., 1995), and the PKA-phosphorylase kinase pathway (Brushia and Walsh, 1999) operate in a kinase cascade. However, because of their distinct characteristics, these pathways are not considered MAPK cascades, although they are involved in transmission of mitogenic signals. The inactivation of many components of the MAPK cascade is mediated by a large number of distinct phosphatases, including MKP, PTPs, and PPs (for review see Yao and Seger, 2004).

All the pathways mentioned are apparently activated to some extent by distinct extracellular agents, and as a result of their action in an elaborate network, determine the outcome of each stimulation. The full dimensions of this network, the mode of regulation of its components, and the mechanism by which these cascades determine cell fates in response to various stimuli, have yet to be fully elucidated.

Critical Parameters

Several points should be considered when using Basic Protocol 2 (immunoprecipitation followed by phosphorylation). One of the most important parameters for the success of this protocol depends on the method of protein extraction used. Since the MAPKs are localized within cells, the cellular membranes must be disrupted to access the desired targets. The protein kinases of interest must then be obtained and preserved in their active form, while decreasing the amount of nonrelevant kinases. For example, activated Raf-1 can be present in Golgi membranes, which might not be disrupted by some extraction procedures, but are disrupted if RIPA buffer is used for extraction. The method for cellular extraction described in Basic Protocol 1 can be effectively used for detection of most MAPKs by immunoprecipitation or other methods. Sonication disrupts the plasma membrane but does not solubilize it, and therefore the resulting extracts (referred to as cytosolic extracts) should contain both cytosolic and some nuclear fractions. Depending on the subcellular localization of the proteins of interest, other extraction methods can be applied. For example, cellular extracts obtained with Triton X-100 usually contain membrane, cytosolic, and some nuclear components, whereas cellular extracts obtained with RIPA buffer should contain solubilized proteins from most cellular compartment. Cellular extraction by addition of hot SDS-PAGE sample buffer to cells is not recommended because it frees chromatin, which causes formation of a gel that is hard to handle. Extraction by freeze-thawing is not recommended either, because of molecular degradations that might occur during the thawing phases.

Another consideration for successful detection of phosphoproteins is minimization of protein degradation and dephosphorylation. During extraction, most cellular organelles break, and thus expose phosphoproteins to phosphatases and proteinases. Addition of specific inhibitors of phosphatases and proteinases to the extraction buffers, and extraction at low temperatures, minimizes the effect of these enzymes. However, since phosphatases are usually efficient enzymes, even if these precautions are taken extractions should be performed as quickly as possible.

One of the most critical elements for the success of the basic methods for immunological monitoring of phosphorylation is the quality and specificity of the antibodies used. The antibodies employed should (1) recognize the phosphorylated protein but not its nonphosphorylated counterpart, and (2) recognize only the desired phosphorylated amino acid, not additional phosphorylated sites in either the same or different proteins. In addition, the amount of proteins in the different samples subjected to immunoblot analysis and the dilution of the antibodies should be optimized to avoid nonspecific recognition of excess proteins. The antibodies used for immunoprecipitation (Basic Protocol 2) should not interfere with the enzymatic activity of the enzymes tested. In addition, it is necessary to avoid nonspecific precipitation of contaminant kinases. However, sometimes the washings may not prevent coimmunoprecipitation of protein kinases other than those desired, and these might interfere with the phosphorylation reaction. This difficulty can be overcome by using a specific substrate or direct assay methods (i.e., in-gel kinase assay, Basic Protocol 3).

For accurate comparison of the amounts of phosphoproteins, detection should be performed in the linear range of the detection system. Thus, the amount of protein loaded on the gel, the concentration of primary and secondary antibodies, and the time of ECL exposure should be optimized in order to reach linearity. Alternatively, a standard curve with the proteins of interest can be made and serial dilutions of the cellular extracts of each treatment can be loaded onto the SDS-PAGE gel. The blot detection system, e.g., ECL-^{125}I-, AP-, or biotin-conjugated antibodies, should be chosen carefully. Usually, ECL has the narrowest linear range of these systems, whereas ^{125}I-antibodies have a relatively broad range. The AP detection system, which has moderate linear range, is usually used for the types of experiments described here, because it is a reliable method that does not necessitate the use of radioactive materials.

Other parameters that should be considered for accurate comparison of protein kinases are as follows.

1. Starvation of the cells before activation, which may interfere with the activation of the desired protein kinase or cause activation of some stress-activated protein kinases.

2. The optimal length of stimulation may vary from cell type to cell type and from one protein kinase to the other; thus, appropriate time points for each kinase should be determined.

3. For accurate comparison of protein kinase activities, detection should be performed in the linear range of the phosphorylation reaction. Thus, the amount of protein used for immunoprecipitation, the concentration of antibodies, the length of the phosphorylation reaction, and the exposure to X-ray film or to the phosphor imager should be optimized in order to reach linearity. If necessary, a standard curve with the protein kinases of interest can be made, and serial dilutions of the cytosolic extracts or a time course of the phosphorylation can be used to ensure one is working in a linear range.

Anticipated Results

With immunoblotting, the desired phosphoprotein should be selectively detected by both anti-phospho and anti-general antibodies. For example, when anti-active ERK antibodies are used (Basic Protocol 1), two faint bands at molecular weights of 42 and 44 kDa should be detected in the basal, nonstimulated fractions of the cells (Fig. 14.3.2). These two bands represent the small amount of active ERK (ERK1 and ERK2) present in resting cells. Upon addition of EGF to these cells, the intensity of staining of the same bands should increase with time, peak at 30 min after stimulation, and decline thereafter. Usually a third band at 46 kDa (ERK1b) appears at the longer time points of EGF stimulation. The kinetics of detection of the phosphoprotein result from the transient activation of ERK by EGF in these cells. Staining the same blots with anti-general ERK antibodies should result in equal staining of the 42-, 44-, and 46-kDa bands in all lanes of the blot.

In Basic Protocol 2, extracts from nonstimulated Rat-1 cells should yield very little MBP phosphorylation (Fig. 14.3.2), which represents the activity of both ERK1 and ERK2 in these cells. Upon addition of EGF to the cells, the amount of phosphate incorporated should increase with time, peaking at 30 min after stimulation and declining thereafter. The kinetics of phosphorylation represent the transient activation of ERK by EGF in these cells. When the in-gel assay is used (Basic Protocol 3), both ERK1 and ERK2 should be detected at molecular weights of 44 and 42 kDa, and the kinetics of activation of both of them should be similar to those of the MBP phosphorylation obtained with Basic Protocol 1. Typical results of Basic Protocol 3 are shown in Figure 14.3.2.

Time Considerations

After cell harvesting, Basic Protocol 1 requires extraction (0.5 hr), determination of protein concentration (0.5 hr), SDS-PAGE (2.5 hr), and immunoblot analysis (6 to 8 hr). Because this procedure may take more than 1 working day, it can be stopped after boiling the samples in sample buffer, or the transfer onto the nitrocellulose membrane can be performed overnight at 40 to 50 mA (instead of 200 to 300 mA). Immunoprecipitation and washes (Basic Protocol 2) add about 4 to 5 hr to Basic Protocol 1.

After cell harvesting, Basic Protocol 2 requires extraction (0.5 hr), immunoprecipitation and washings (3 to 4 hr), the phosphorylation reaction (0.5 to 1.0 hour), SDS-PAGE (2.5 hr), and processing of the gel (6 to 16 hr). Because this procedure usually takes more than 1 working day, it can be interrupted after boiling the samples in sample buffer or at any time during the destaining period.

The in-gel kinase assay (Basic Protocol 3) may take 2 to 3 days.

Literature Cited

Abe, J., Kusuhara, M., Ulevitch, R.J., Berk, B.C., and Lee, J.D. 1996. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. *J. Biol. Chem.* 271:16586-16590.

Abe, M.K., Kuo, W.L., Hershenson, M.B., and Rosner, M.R. 1999. Extracellular signal-regulated kinase 7 (ERK7), a novel ERK with a C-terminal domain that regulates its activity, its cellular localization, and cell growth. *Mol. Cell Biol.* 19:1301-1312.

Abe, M.K., Kahle, K.T., Saelzler, M.P., Orth, K., Dixon, J.E., and Rosner, M.R. 2001. ERK7 is an autoactivated member of the MAP kinase family. *J. Biol. Chem.* 276:21272-21279.

Ahn, N.G., Weiel, J.E., Chan, C.P., and Krebs, E.G. 1990. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J. Biol. Chem.* 265:11487-11494.

Ahn, N.G., Seger, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K., and Krebs, E.G. 1991. Multiple components in an epidermal growth factor-stimulated protein kinase cascade: In vitro activation of myelin basic protein/microtubule-associated protein-2 kinase. *J. Biol. Chem.* 266:4220-4227.

Blank, J.L., Gerwins, P., Elliott, E.M., Sather, S., and Johnson, G.L. 1996. Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3: Regulation of sequential phosphorylation pathways involving mitogen-activated protein kinase and c-Jun kinase. *J. Biol. Chem.* 271:5361-5368.

Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M.H. 1990. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* 249:64-67.

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., and Yancopoulos, G.D. 1991. ERK's: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65:663-675.

Brushia, R.J. and Walsh, D.A. 1999. Phosphorylase kinase: The complexity of its regulation is reflected in the complexity of its structure. *Front. Biosci.* 4:D618-D641.

Cameron, S.J., Malik, S., Akaike, M., Lerner-Marmarosh, N., Yan, C., Lee, J.D., Abe, J., and Yang, J. 2003. Regulation of epidermal growth factor-induced connexin 43 gap junction communication by big mitogen–activated protein kinase1/ERK5 but not ERK1/2 kinase activation. *J. Biol. Chem.* 278:18682-18688.

Canagarajah, B.J., Khokhlatchev, A., Cobb, M.H., and Goldsmith, E.J. 1997. Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell* 90:859-869.

Chadee, D.N. and Kyriakis, J.M. 2004. MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation. *Nat. Cell Biol.* 6:770-776.

Chao, T.H., Hayashi, M., Tapping, R.I., Kato, Y., and Lee, J.D. 1999. MEKK3 directly regulates MEK5 activity as part of the big mitogen-activated protein kinase 1 (BMK1) signaling pathway. *J. Biol. Chem.* 274:36035-36038.

Chayama, K., Papst, P.J., Garrington, T.P., Pratt, J.C., Ishizuka, T., Webb, S., Ganiatsas, S., Zon, L.I., Sun, W., Johnson, G.L., and Gelfand, E.W. 2001. Role of MEKK2-MEK5 in the regulation of TNF-alpha gene expression and MEKK2-MKK7 in the activation of c-Jun N-terminal kinase in mast cells. *Proc. Natl. Acad. Sci. U.S.A.* 98:4599-4604.

Chen, Z., Hutchison, M., and Cobb, M.H. 1999. Isolation of the protein kinase TAO2 and identification of its mitogen-activated protein kinase/extracellular signal-regulated kinase kinase binding domain. *J. Biol. Chem.* 274:28803-28807.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., and Cobb, M.H. 2001. MAP kinases. *Chem. Rev.* 101:2449-2476.

Cheng, M., Zhen, E., Robinson, M.J., Ebert, D., Goldsmith, E., and Cobb, M.H. 1996. Characterization of a protein kinase that phosphorylates serine 189 of the mitogen-activated protein kinase homolog ERK3. *J. Biol. Chem.* 271:12057-12062.

Chow, C.W., Rincon, M., Cavanagh, J., Dickens, M., and Davis, R.J. 1997. Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. *Science* 278:1638-1641.

Chuderland, D. and Seger, R. 2005. Protein-protein interactions in the regulation of the extracellular signal-regulated kinase (ERK). *Mol. Biotechnol.* 29:47-64.

Cohen, P., Alessi, D.R., and Cross, D.A. 1997. PDK1, one of the missing links in insulin signal transduction? *FEBS Lett.* 410:3-10.

Crespo, P., Schuebel, K.F., Ostrom, A.A., Gutkind, A.S., and Bustel, X.P. 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature* 385:169-172.

Cuenda, A., Cohen, P., Buee-Scherrer, V., and Goedert, M. 1997. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6): Comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.* 16:295-305.

Dan, I., Watanabe, N.M., Kobayashi, T., Yamashita-Suzuki, K., Fukagaya, Y., Kajikawa, E., Kimura, W.K., Nakashima, T.M., Matsumoto, K., Ninomiya-Tsuji, J., and Kusumi, A. 2000. Molecular cloning of MINK, a novel member of mammalian GCK family kinases, which is up-regulated during postnatal mouse cerebral development. *FEBS Lett.* 469:19-23.

Dan, I., Watanabe, N.M., and Kusumi, A. 2001. The Ste20 group kinases as regulators of MAP kinase cascades. *Trends Cell Biol.* 11:220-230.

Dan, C., Nath, N., Liberto, M., and Minden, A. 2002. PAK5, a new brain-specific kinase, promotes neurite outgrowth in N1E-115 cells. *Mol. Cell Biol.* 22:567-577.

Dashti, S., Efimova, R.T., and Eckert, R.L. 2001. MEK7-dependent activation of p38 MAP kinase in keratinocytes. *J. Biol. Chem.* 276:8059-8063.

Davis, R.J. 2000. Signal transduction by the JNK group of MAP kinases. *Cell* 103:239-252.

Deak, M., Clifton, A.D., Lucocq, L.M., and Alessi, D.R. 1998. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J.* 17:4426-4441.

Derijard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. 1994. JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 76:1025-1027.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I.H., Han, J., Ulevitch, R.J., and Davis, R.J. 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 267:682-685 [published erratum appears in *Science* 269:17].

Diener, K., Wang, X.S., Chen, C., Meyer, C.F., Keesler, G., Zukowski, M., Tan, T.H., and Yao, Z. 1997. Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. *Proc. Natl. Acad. Sci. U.S.A.* 94:9687-9692.

Ducret, C., Maira, S.M., Lutz, Y., and Wasylyk, B. 2000. The ternary complex factor Net contains two distinct elements that mediate different responses to MAP kinase signalling cascades. *Oncogene* 19:5063-5072.

English, J.M., Pearson, G., Baer, R., and Cobb, M.H. 1998. Identification of substrates and regulators of the mitogen-activated protein kinase ERK5 using chimeric protein kinases. *J. Biol. Chem.* 273:3854-3860.

English, J.M., Pearson, G., Hockenberry, T., Shivakumar, L., White, M.A., and Cobb, M.H. 1999. Contribution of the ERK5/MEK5 pathway to Ras/Raf signaling and growth control. *J. Biol. Chem.* 274:31588-31592.

Fan, G., Merritt, S.E., Kortenjann, M., Shaw, P.E., and Holzman, L.B. 1996. Dual leucine zipper-bearing kinase (DLK) activates p46SAPK and p38mapk but not ERK2. *J. Biol. Chem.* 271:24788-24793.

Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. 1994. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* 78:1039-1049.

Fu, C.A., Shen, M., Huang, B.C., Lasaga, J., Payan, D.G., and Luo, Y. 1999. TNIK, a novel member of the germinal center kinase family that activates the c-Jun N-terminal kinase pathway and regulates the cytoskeleton. *J. Biol. Chem.* 274:30729-30737.

Fukunaga, R. and Hunter, T. 1997. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* 16:1921-1933.

Gabay, L., Seger, R., and Shilo, B.Z. 1997. In situ activation pattern of *Drosophila* EGF receptor pathway during development. *Science* 277:1103-1106.

Gallo, K.A. and Johnson, G.L. 2002. Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat. Rev. Mol. Cell Biol.* 3:663-672.

Gerwins, P., Blank, J.L., and Johnson, G.L. 1997. Cloning of a novel mitogen-activated protein kinase kinase kinase, MEKK4, that selectively regulates the c-Jun amino terminal kinase pathway. *J. Biol. Chem.* 272:8288-8295.

Gille, H., Sharrocks, A.D., and Shaw, P.E. 1992. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature* 358:414-417.

Goedert, M., Cuenda, A., Craxton, M., Jakes, R., and Cohen, P. 1997. Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6): Comparison of its substrate specificity with that of other SAP kinases. *EMBO J.* 16:3563-3571.

Gotoh, I., Adachi, M., and Nishida, E. 2001. Identification and characterization of a novel MAP kinase kinase kinase, MLTK. *J. Biol. Chem.* 276:4276-4286.

Gotoh, Y. and Nishida, E. 1995. Activation mechanism and function of the MAP kinase cascade. *Mol. Reprod. Dev.* 42:486-492.

Graves, J.D., Gotoh, Y., Draves, K.E., Ambrose, D., Han, D.K., Wright, M., Chernoff, J., Clark, E.A., and Krebs, E.G., 1998. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *EMBO J.* 17:2224-2234.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267:389-393.

Hagemann, C. and Blank, J.L. 2001. The ups and downs of MEK kinase interactions. *Cell Signal* 13:863-875.

Hagemann, C. and Rapp, U.R. 1999. Isotype-specific functions of Raf kinases. *Exp. Cell Res.* 253:34-46.

Han, J., Lee, J.D., Bibbs, L., and Ulevitch, R.J. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265: 808-811.

Han, J., Lee, J.D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R.J. 1996. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem.* 271:2886-2891.

Han, J., Jiang, Y., Li, Z., Kravchenko, V.V., and Ulevitch, R.J. 1997. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* 386:296-299.

Hayashi, M., Tapping, R.I., Chao, T.H., Lo, J.F., King, C.C., Yang, Y., and Lee, J.D. 2001. BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of serum and glucocorticoid-inducible kinase. *J. Biol. Chem.* 276:8631-8634.

Herskowitz, I. 1995. MAP kinase pathways in yeast: For mating and more. *Cell* 80:187-197.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* 7:2135-2148.

Hirai, S., Katoh, M., Terada, M., Kyriakis, J.M., Zon, L.I., Rana, A., Avruch, J., and Ohno, S. 1997. MST/MLK2, a member of the mixed lineage kinase family, directly phosphorylates and activates SEK1, an activator of c-Jun N-terminal kinase/stress-activated protein kinase. *J. Biol. Chem.* 272:15167-15173.

Huang, C., Ma, W.Y., Maxiner, A., Sun, Y., and Dong, Z. 1999. p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *J. Biol. Chem.* 274:12229-12235.

Hutchison, M., Berman, K.S., and Cobb, M.H. 1998. Isolation of TAO1, a protein kinase that activates MEKs in stress- activated protein kinase cascades. *J. Biol. Chem.* 273:28625-28632.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. 1997. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275:90-94.

Janknecht, R., Monte, D., Baert, J.L., and de Launoit, Y. 1996. The ETS-related transcription factor ERM is a nuclear target of signaling cascades involving MAPK and PKA. *Oncogene* 13:1745-1754.

Johnston, A.M., Naselli, G., Gonez, L.J., Martin, R.M., Harrison, L.C., and DeAizpurua, H.J. 2000. SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. *Oncogene* 19:4290-4297.

Kallunki, T., Deng, T., Hibi, M., and Karin, M. 1996. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* 87:929-939.

Kamakura, S., Moriguchi, T., and Nishida, E. 1999. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases: Identification and characterization of a signaling pathway to the nucleus. *J. Biol. Chem.* 274:26563-26571.

Kasler, H. Victoria, G.J., Duramad, O., and Winoto, A. 2000. ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. *Mol. Cell Biol.* 20:8382-8389.

Kato, Y., Kravchenko, V.V., Tapping, R.I., Han, J., Ulevitch, R.J., and Lee, J.D. 1997. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* 16:7054-7066.

Kato, Y., Tapping, R.I., Huang, S., Watson, M.H., Ulevitch, R.J., and Lee, J.D. 1998. Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. *Nature* 395:713-716.

Kiefer, F., Tibbles, L.A., Anafi, M., Janssen, A., Zanke, B.W., Lassam, N., Pawson, T., Woodgett, J.R., and Iscove, N.N. 1996. HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. *EMBO J.* 15:7013-7025.

Kolesnick, R. and Xing, H.R. 2004. Inflammatory bowel disease reveals the kinase activity of KSR1. *J. Clin. Invest.* 114:1233-1237.

Kyriakis, J.M. and Avruch, J. 1996. Sounding the alarm: Protein kinase cascades activated by stress and inflamation. *J. Biol. Chem.* 271:24313-24316.

Kyriakis, J.M., App, H., Zhang, F.X., Banerjee, P., Brautigan, D.L., Rapp, U.R., and Avruch, J. 1992. Raf-1 activates MAP kinase-kinase. *Nature* 358:417-421.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369:156-160.

Lange-Carter, C.A., Pleiman, C.M., Gardner, A.M., Blumer, K.J., and Johnson, G.L. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science* 260:315-317.

Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, J.R., and Landvatter, S.W., et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372:739-746.

Leung, T., Manser, E., Tan, L., and Lim, L. 1995. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* 270:29051-29054.

Lin, J.L., Chen, H.C., Fang, H.I., Robinson, D., Kung, H.J., and Shih, H.M. 2001. MST4, a new Ste20-related kinase that mediates cell growth and transformation via modulating ERK pathway. *Oncogene* 20:6559-6569.

Lin, L.L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A., and Davis, R.J. 1993. cPLA2 is phosphorylated and activated by MAP kinase. *Cell* 72:269-278.

MacGillivray, M.K., Cruz, T.F., and McCulloch, C.A. 2000. The recruitment of the interleukin-1 (IL-1) receptor-associated kinase (IRAK) into focal adhesion complexes is required for IL-1beta-induced ERK activation. *J. Biol. Chem.* 275:23509-23515.

Malinin, N.L., Boldin, M.P., Kovalenko, A.V., and Wallach, D. 1997. MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature* 385:540-544.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. 2002. The protein kinase complement of the human genome. *Science* 298:1912-1934.

Marshall, C.J. 1995. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80:179-185.

McLaughlin, M.M., Kumar, S., McDonnell, P.C., Van Horn, S., Lee, J.C., Livi, G.P., and Young, P.R. 1996. Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J. Biol. Chem.* 271:8488-8492.

Milne, D.M., Campbell, D.G., Caudwell, F.B., and Meek, D.W. 1994. Phosphorylation of the tumor suppressor protein p53 by mitogen-activated protein kinases. *J. Biol. Chem.* 269:9253-9260.

Milne, D.M., Campbell, L.E., Campbell, D.G., and Meek, D.W. 1995. p53 is phosphorylated in vitro and in vivo by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. *J. Biol. Chem.* 270:5511-5518.

Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. 1996. A novel kinase cascade

mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.* 271:13675-13679.

Morrison, D.K. and Davis, R.J. 2003. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annu. Rev. Cell Dev. Biol.* 19:91-118.

Morton, S., Davis, R.J., McLaren, A., and Cohen, P. 2003. A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. *EMBO J.* 22:3876-3886.

Murphy, L.O., Smith, S., Chen, R.H., Fingar, D.C., and Blenis, J. 2002. Molecular interpretation of ERK signal duration by immediate early gene products. *Nat. Cell Biol.* 4:556-564.

Nakano, K., Yamauchi, J., Nakagawa, K., Itoh, H., and Kitamura, N. 2000. NESK, a member of the germinal center kinase family that activates the c-Jun N-terminal kinase pathway and is expressed during the late stages of embryogenesis. *J. Biol. Chem.* 275:20533-20539.

Ni, H., Wang, X.S., Diener, K., and Yao, Z. 1998. MAPKAPK5, a novel mitogen-activated protein kinase (MAPK)-activated protein kinase, is a substrate of the extracellular-regulated kinase (ERK) and p38 kinase. *Biochem. Biophys. Res. Commun.* 243:492-496.

Noguchi, K., Kitanaka, C., Yamana, H., Kokubu, A., Mochizuki, T., and Kuchino, Y. 1999. Regulation of c-Myc through phosphorylation at Ser-62 and Ser-71 by c-Jun N-terminal kinase. *J. Biol. Chem.* 274:32580-32587.

O'Hagan, R.C., Tozer, R.G., Symons, M., McCormick, F., and Hassell, J.A. 1996. The activity of the Ets transcription factor PEA3 is regulated by two distinct MAPK cascades. *Oncogene* 13:1323-1333.

Ono, K. and Han, J. 2000. The p38 signal transduction pathway: activation and function. *Cell Signal* 12:1-13.

Palmer, A., Gavin, A.C., and Nebreda, A.R. 1998. A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J.* 17:5037-5047.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.-H., Shabanowitz, J., Hunt, D.F., Weber, M.J., and Sturgill, T.W. 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen activated protein kinase (MAP kinase). *EMBO J.* 10:885-892.

Pearson, G., English, J.M., White, M.A., and Cobb, M.H. 2001. ERK5 and ERK2 cooperate to regulate NF-kappaB and cell transformation. *J. Biol. Chem.* 276:7927-7931.

Peraldi, P., Frodin, M., Barnier, J.V., Calleja, V., Scimeca, J.C., Filloux, C., Calothy, G., and Van Obberghen, E. 1995. Regulation of the MAP kinase cascade in PC12 cells: B-Raf activates MEK-1 (MAP kinase or ERK kinase) and is inhibited by cAMP. *FEBS Lett.* 357:290-296.

Pombo, C.M., Kehrl, J.H., Sanchez, I., Katz, P., Avruch, J., Zon, L.I., Woodgett, J.R., Force, T., and Kyriakis, J.M. 1995. Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. *Nature* 377:750-754.

Posada, J., Yew, N., Ahn, N.G., Vande-Woude, G.F., and Cooper, J.A. 1993. Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase kinase in vitro. *Mol. Cell. Biol.* 13:2546-2552.

Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B., and Davis, R.J. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell Biol.* 16:1247-1255.

Raman, M. and Cobb, M.H. 2003. MAP kinase modules: Many roads home. *Curr. Biol.* 13:R886-R888.

Rana, A., Gallo, K., Godowski, P., Hirai, S., Ohno, S., Zon, L., Kyriakis, J.M., and Avruch, J. 1996. The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. *J. Biol. Chem.* 271:19025-19028.

Raviv, Z., Kalie, E., and Seger, R. 2004. MEK5 and ERK5 are localized in the nuclei of resting as well as stimulated cells, while MEKK2 translocates from the cytosol to the nucleus upon stimulation. *J. Cell Sci.* 117:1773-1784.

Ray, L.B. and Sturgill, T.W. 1987. Characterization of insulin-stimulated microtubule-associated protein kinase: Rapid isolation and stabilization of a novel serine/threonine kinase from 3T3-L1 cells. *Proc. Natl. Acad. Sci. U.S.A.* 84:1502-1506.

Robbins, D.J. and Cobb, M.H. 1992. Extracellular signal-regulated kinases 2 autophosphorylates on a subset of peptides phosphorylated in intact cells in response to insulin and nerve growth factor: analysis by peptide mapping. *Mol. Biol. Cell* 3:299-308.

Roux, P.P. and Blenis, J. 2004. ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* 68:320-344.

Rubinfeld, H. and Seger, R. 2004. The ERK cascade as a prototype of MAPK signaling pathways. *Methods Mol. Biol.* 250:1-28.

Sabourin, L.A. and Rudnicki, M.A. 1999. Induction of apoptosis by SLK, a Ste20-related kinase. *Oncogene* 18:7566-7575.

Sakuma, H., Ikeda, A., Oka, S., Kozutsumi, Y., Zanetta, J.P., and Kawasaki, T. 1997. Molecular cloning and functional expression of a cDNA encoding a new member of mixed lineage protein kinase from human brain. *J. Biol. Chem.* 272:28622-28629.

Salmeron, A., Ahmad, T.B., Carlile, G.W., Pappin, D., Narsimhan, R.P., and Ley, S.C. 1996. Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. *EMBO J.* 15:817-826.

Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M., and

Zon, L.I. 1994. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature* 372:794-798.

Seger, R. and Krebs, E.G., 1995. The MAPK signaling cascade. *FASEB J.* 9:726-735.

Seger, R., Ahn, N.G., Posada, J., Munar, E.S., Jensen, A.M., Cooper, J.A., Cobb, M.H., and Krebs, E.G., 1992. Purification and characterization of MAP kinase activator(s) from epidermal growth factor stimulated A431 cells. *J. Biol. Chem.* 267:14373-14381.

Sgouras, D.N., Athanasiou, M.A., Beal, G.J. Jr., Fisher, R.J., Blair D.G., and Mavrothalassitis, G.J. 1995. ERF: An ETS domain protein with strong transcriptional repressor activity, can suppress ets-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. *EMBO J.* 14:4781-4793.

Shi, C.S. and Kehrl, J.H. 1997. Activation of stress-activated protein kinase/c-Jun N-terminal kinase, but not NF-kappaB, by the tumor necrosis factor (TNF) receptor 1 through a TNF receptor–associated factor 2- and germinal center kinase related-dependent pathway. *J. Biol. Chem.* 272:32102-32107.

Stokoe, D., Campbell, D.G., Nakielny, S., Hidaka, H., Leevers, S.J., Marshall, C., and Cohen, P. 1992. MAPKAP kinase-2: A novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.* 11:3985-3994.

Strahl, T., Gille, H., and Shaw, P.E. 1996. Selective response of ternary complex factor Sap1a to different mitogen-activated protein kinase subgroups. *Proc. Natl. Acad. Sci. U.S.A.* 93:11563-11568.

Sturgill, T.W., Ray, L.B., Erikson, E., and Maller, J.L. 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* 334:715-718.

Su, Y.C., Han, J., Xu, S., Cobb, M., and Skolnik, E.Y. 1997. NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. *EMBO J.* 16:1279-1290.

Sun, W., Wei, X., Kesavan, K., Garrington, T.P., Fan, R., Mei, J., Anderson, S.M., Gelfand, E.W., and Johnson, G.L. 2003. MEK kinase 2 and the adaptor protein Lad regulate extracellular signal-regulated kinase 5 activation by epidermal growth factor via Src. *Mol. Cell Biol.* 23:2298-2308.

Terasawa, K., Okazaki, K., and Nishida, E. 2003. Regulation of c-Fos and Fra-1 by the MEK5-ERK5 pathway. *Genes Cells* 8:263-273.

Tournier, C., Whitmarsh, A.J., Cavanagh, J., Barrett, T., and Davis, R.J. 1997. Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. *Proc. Natl. Acad. Sci. U.S.A* 94:7337-7342.

Tung, R.M. and Blenis, J. 1997. A novel human SPS1/STE20 homologue, KHS, activates Jun N-terminal kinase. *Oncogene* 14:653-659.

Varfolomeev, E.E. and Ashkenazi, A. 2004. Tumor necrosis factor: An apoptosis JuNKie? *Cell* 116:491-497.

Wang, X.S., Diener, K., Tan, T.H., and Yao, Z. 1998. MAPKKK6, a novel mitogen-activated protein kinase kinase kinase, that associates with MAPKKK5. *Biochem. Biophys. Res. Commun.* 253:33-37.

Wang, X.Z. and Ron, D. 1996. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science* 272:1347-1349.

Waskiewicz, A.J., Flynn, A., Proud, C.G., and Cooper, J.A. 1997. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.* 16:1909-1920.

Whitmarsh, A.J., Shore, P., Sharrocks, A.D., and Davis, R.J. 1995. Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 269:403-407.

Xu, Z., Maroney, A.C., Dobrzanski, P., Kukekov, N.V., and Greene, L.A. 2001. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol. Cell Biol.* 21:4713-4724.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R., and Templeton, D.J. 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* 372:798-800.

Yang, B.S., Hauser, C.A., Henkel, G., Colman, M.S., Van Beveren, C., Stacey, K.J., Hume, D.A., Maki, R.A., and Ostrowski, M.C. 1996. Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. *Mol. Cell Biol.* 16:538-547.

Yang, C.C., Ornatsky, O.I., McDermott, J.C., Cruz, T.F., and Prody, C.A. 1998. Interaction of myocyte enhancer factor 2 (MEF2) with a mitogen-activated protein kinase, ERK5/BMK1. *Nucl. Acids Res.* 26:4771-4777.

Yang, J.J. 2002. Mixed lineage kinase ZAK utilizing MKK7 and not MKK4 to activate the c-Jun N-terminal kinase and playing a role in the cell arrest. *Biochem. Biophys. Res. Commun.* 297:105-110.

Yao, Z. and Seger, R. 2004. The molecular mechanism of MAPK/ERK inactivation. *Curr. Genomics* 5:385-393.

Yao, Z., Zhou, G., Wang, X.S., Brown, A., Diener, K., Gan, H., and Tan, T.H. 1999. A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. *J. Biol. Chem.* 274:2118-2125.

Yung, Y., Dolginov, Y., Yao, Z., Rubinfeld, H., Michael, D., Hanoch, T., Roubini, E., Lando, Z., Zharhary, D., and Seger, R. 1997. Detection of ERK activation by a novel monoclonal antibody. *FEBS Lett.* 408:292-296.

Zhang, S., Han, J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R.J., and Bokoch, G.M. 1995. Rho family GTPases regulate p38

mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.* 270:23934-23936.

Zhou, G., Bao, Z.Q., and Dixon, J.E. 1995. Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem.* 270:12665-12669.

Contributed by Yoav Shaul and
Rony Seger
Department of Biological Regulation,
The Weizmann Institute of Science
Rehovot, Israel

Labeling Cultured Cells with $^{32}P_i$ and Preparing Cell Lysates for Immunoprecipitation

This unit describes $^{32}P_i$ labeling and lysis of cultured cells to be used for subsequent immunoprecipitation of proteins. The approach is appropriate, however, for labeling any cellular constituent with ^{32}P. This procedure is suitable for insect, avian, and mammalian cells and can be used with both adherent and nonadherent cultures. The same general approach—biosynthetic labeling with $^{32}P_i$ in medium containing a reduced concentration of phosphate—can also be applied to bacteria and yeast; however, specific techniques to accomplish this are not presented here. If this approach is used to label cellular constituents other than proteins, it will probably be necessary to modify the Basic Protocol beginning with step 9 (cell lysis).

The first procedure described (see Basic Protocol) is $^{32}P_i$ labeling of adherent or nonadherent (e.g., hematopoietic) cells with subsequent lysis in a detergent buffer containing either Nonidet P-40 (NP-40), 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), or a combination of NP-40, sodium deoxycholate and SDS (RadioImmunoPrecipitation Assay or RIPA buffer). More rigorous lysis conditions to be used for working with proteins that are difficult to solubilize are also described (see Alternate Protocol).

CAUTION: Unshielded ^{32}P will penetrate ~1 cm into flesh. Exposure to the skin and eyes is, therefore, of concern. Gloves and protective eyeware should always be worn when handling significant amounts of ^{32}P. A 1-in.-thick (2.5-cm) Plexiglas shield, tall enough to look through when seated or standing comfortably, should be used when handling samples containing ^{32}P. For more specific precautions, refer to the section on Safe Use of Radioisotopes (*APPENDIX 1D*).

LABELING CULTURED CELLS WITH $^{32}P_i$ AND LYSIS USING MILD DETERGENT

BASIC PROTOCOL

The first six steps of this protocol describe culturing and labeling procedures for cultures of adherent cells; modifications appropriate for nonadherent cells are described in alternate steps. The remaining steps describe lysis of cells in detergent buffer to prepare the sample for immunoprecipitation. For a more detailed discussion of mammalian cell culture conditions and reagents see *UNITS 1.1* and *1.2*.

Materials

Cell culture to be labeled
Labeling medium: phosphate-free tissue culture medium (e.g., DMEM, *APPENDIX 2A*) supplemented with the usual concentration of serum or serum dialyzed against phosphate-free saline, 37°C
500 mCi/ml to 1 Ci/ml $H_3{}^{32}PO_4$ in HCl (carrier free ICN)
Tris-buffered (TBS; see recipe) or Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*), cold
Mild lysis buffer or RIPA lysis buffer (see recipes)

1-in.-thick Plexiglas shield (*APPENDIX 1D*)
Plugged, aerosol-resistant pipet tips
Plexiglas box (*APPENDIX 1D*), warmed to 37°C
Screw-cap microcentrifuge tubes
Plugged disposable pipet or disposable one-piece transfer pipet

Contributed by Bartholomew M. Sefton
Current Protocols in Cell Biology (1999) 14.4.1-14.4.7

Rubber policeman

Sorvall refrigerated centrifuge with SM 24 rotor and rubber adaptors, refrigerated microcentrifuge, or equivalent, 4°C

Plexiglas sheet (10 × 10 × ¼–in.) or Plexiglas tube holder, 4°C (*APPENDIX 1D*)

Additional reagents and equipment for cell culture (*UNIT 1.1*) and gel electrophoresis (*UNIT 6.1*) or immunoprecipitation (*UNIT 6.2*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and appropriate aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Label the cell culture

1. Culture the cells to be labeled to an appropriate stage of growth.

   Phosphate transport is maximal in rapidly growing cells. Therefore, except in those cases where phosphorylation of a protein in quiescent cells is to be examined, cells to be labeled should be subconfluent (adherent cells) or at less than maximal density (nonadherent cells). It is useful to change the medium to fresh growth medium 3 to 18 hr prior to labeling.

   Brief cultivation of cells in low-phosphate medium (to reduce the phosphate pool by starvation prior to labeling) is of minimal value.

For adherent cells

2a. Remove growth medium by aspiration. Wash away any residual phosphate-containing medium by adding 37°C labeling medium supplemented with serum, but lacking the label, and removing the wash medium by aspiration.

   Phosphate-free DMEM is routinely used for labeling medium because RPMI has a very high concentration of phosphates.

3a. Add prewarmed labeling medium to cultures, using 0.5 to 1 ml per 35-mm dish, 1 to 2 ml per 50-mm dish, or 2 to 4 ml per 100-mm dish of adherent cells.

For nonadherent cells

2b. Gently centrifuge the culture 1 min at 1800 × *g*, room temperature, and aspirate the medium away from the cell pellet. Resuspend the cells in labeling medium supplemented with serum, but lacking the label. Centrifuge and remove the medium.

3b. Add 2 ml medium per 10^7 cells and transfer to an appropriate size petri dish.

4. Working behind a Plexiglas shield, use a micropipettor with plugged, aerosol-resistant pipet tips to add $^{32}P_i$ to a final concentration of 0.1 to 2 mCi/ml.

   Use of plugged, aerosol-resistant pipet tips will minimize contamination of the micropipettor. $^{32}P_i$ is usually shipped in HCl and is generally sterile, so it is not necessary to sterilize the labeling medium after adding the radioisotope. Additionally, except in the rare cases when steady-state labeling with ^{32}P is desired, the labeling interval is usually so short (<6 hr) that microbial growth resulting from added isotope is undetectable. Therefore, addition of ^{32}P to cells can be performed on the lab bench rather than in a tissue culture hood.

5. Place dishes in a warmed Plexiglas box, and put box in the incubator.

   Labeling for 1 to 2 hr is usually sufficient, but cells will tolerate as much as 2 mCi/ml for 6 hr and lower concentrations (0.1 to 0.5 mCi/ml) for 18 hr.

Wash and lyse the cells

6. At the end of the labeling period, carry the labeled cells, still in the Plexiglas box, into the cold room. Place the cells behind a Plexiglas shield.

For adherent cells

7a. Take the dish out of the box and remove the labeling medium manually, using either a plugged disposable pipet or a disposable one-piece plastic Pasteur transfer pipet. Discard the medium and pipet as radioactive wastes.

Labeling medium should be removed without using a vacuum aspirator. Vacuum aspiration generates radioactive aerosols and leaves a radioactive film on the equipment.

8a. Wash cells once with 2 to 10 ml cold TBS. Remove the wash buffer manually, as in step 7, and discard as radioactive waste.

The uptake of phosphate is efficient and often a majority of the added $^{32}P_i$ is found within the labeled cells. This necessitates continued shielding of the labeled cells following removal of the labeling medium.

9a. Add lysis buffer to cells, using 0.3 ml per 35-mm dish, 0.6 ml per 50-mm dish, or 1.0 ml per 100-mm dish of adherent cells. Dislodge adherent cells by scraping with a rubber policeman, but leave the lysate in the dish. Incubate 20 min at 4°C. With a rubber policeman, scrape the lysate of adherent cells to the side of the dish and transfer lysate to a screw-cap microcentrifuge tube.

If a low background of nonspecific contaminants is critical, use RIPA lysis buffer. If maintenance of enzymatic activity or the structure of protein complexes is critical, use a milder lysis buffer containing either 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) or Nonidet P-40 (NP-40) as the only detergent. If complete solubilization of the cells and denaturation of the protein is desired, use SDS for lysis (see Alternate Protocol).

For nonadherent cells

7b. Take the dish out of the box, transfer the cells to a screw-cap centrifuge tube, pellet them by centrifugation (1 min at 1800 × *g*), and remove the medium.

Do not use a vacuum aspirator to remove medium.

8b. Resuspend pelleted cells gently in a small volume of cold TBS, transfer to a screw-cap microcentrifuge tube, and pellet the cells by microcentrifuging 1 min at 1800 × *g*.

Continue to use shielding with cells and lysate.

9b. Add 0.5 to 1 ml lysis buffer per 10^7 cells and resuspend the pellet by gentle agitation with a disposable plastic Pasteur pipet. Incubate 20 min at 4°C.

Choose the lysis buffer as in annotation to step 9a.

10. Cap the tube, and clarify the lysate by centrifuging 30 min at 26,000 × *g* (17,000 rpm in Sorvall SM24 rotor), 4°C.

Use an ice bucket with a sheet of Plexiglas over it, a chilled Plexiglas tube holder, or a prechilled centrifuge rotor to transport the tube of lysate.

It is best to half-fill the tube to prevent spilling. A Sorvall SM24 rotor with rubber adaptors for microcentrifuge tubes is ideal for clarifying lysates. Alternatively, a refrigerated microcentrifuge can be used for 30 min at maximum speed. Do not use a nonrefrigerated microcentrifuge in the cold room because the centrifuge will warm to 20°C during a 30-min spin.

Lysates prepared with RIPA buffer often become viscous due to lysis of nuclei. If this occurs, increase the time of centrifugation to 90 min, or add 50 µl of fixed Staphylococcus aureus bacteria (Pansorbin, Calbiochem) in RIPA buffer to the lysate prior to centrifugation. Either modification will cause the solubilized DNA to pellet.

11. After centrifugation, transfer the supernatant (lysate) to a new tube and discard the tube and pellet in radioactive waste.

If the pellet is very viscous, so that it is impossible to remove the supernatant cleanly, suck part of the pellet into a micropipet tip, lift the viscous material out of the tube, and discard it in the radioactive waste. The residual liquid in the tube is the supernatant and can be used for immunoprecipitation.

12. Analyze the labeled lysate using gel electrophoresis, immunoprecipitation, or protein purification. Carry out all analytical procedures at 4°C using adequate shielding.

ALTERNATE PROTOCOL

LYSIS OF CELLS BY BOILING IN SDS

Some proteins, such as eukaryotic RNA polymerase II, are difficult to solubilize with mild lysis buffer or RIPA lysis buffer, and some analytical procedures use antibodies that recognize epitopes exposed only in denatured proteins. In these cases, it is useful to solubilize labeled cells completely in SDS and then adjust the composition of the lysate solution to match that of RIPA buffer for immunoprecipitation. To avoid the formation of spurious disulfide bonds, lysis and washing during immunoprecipitation are carried out in the presence of fresh 1 mM dithiothreitol (DTT). The protocol describes the procedure for adherent cells; modifications for working with nonadherent cells are described as alternate steps.

Additional Materials *(also see Basic Protocol)*

SDS lysis buffer (see recipe)
RIPA correction buffer (see recipe)
Immunoprecipitate wash buffer (see recipe)
Fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem; optional)
Boiling water bath

1. Label and wash cells (see Basic Protocol steps 1 to 7).

2a. *For adherent cells:* Add SDS lysis buffer using 0.1 ml for a 35-mm dish, 0.25 ml for a 50-mm dish, or 0.5 ml for an 100-mm dish. Immediately scrape off the dish with a rubber policeman and transfer the cell lysate to a screw-cap microcentrifuge tube.

2b. *For nonadherent cells:* Vortex briefly to loosen the cell pellet, add 1 ml SDS lysis buffer per 5×10^7 cells and vortex again.

3. Boil the samples 2 to 5 min, then add 4 vol RIPA correction buffer and mix well.

4. Clarify the cell lysate by centrifuging 90 min at 26,000 × *g* (17,000 rpm in Sorvall SM24), 4°C or at maximum speed, 4°C in a refrigerated microcentrifuge.

   Lysate may also be clarified by adding 50 µl fixed Staphylococcus aureus and centrifuging 30 min at 26,000 × g, 4°C.

5. Carry out immunoprecipitation as usual using immunoprecipitate wash buffer for washes.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Immunoprecipitate wash buffer for boiled sample

RIPA lysis buffer (see recipe)
1 mM DTT, added fresh

DTT is added from a 1 M stock solution stored at −20°C.

Mild lysis buffer

10 mM 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) or 1% (w/w) Nonidet P-40 (NP-40)

0.15 M NaCl
0.01 M sodium phosphate, pH 7.2 (*APPENDIX 2A*)
2 mM EDTA
50 mM sodium fluoride
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

CHAPS is a milder detergent than NP-40, but yields precipitates with a higher background and may solubilize some proteins less efficiently.

Sodium vanadate stock solution can be stored in plastic at room temperature.

RIPA (RadioImmunoPrecipitation Assay) correction buffer for boiled sample

1.25% (w/w) Nonidet P-40 (NP-40)
1.25% (w/v) sodium deoxycholate
0.0125 M sodium phosphate, pH 7.2 (*APPENDIX 2A*)
2 mM EDTA
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
50 mM sodium fluoride
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

RIPA (RadioImmunoPrecipitation Assay) lysis buffer

1% (w/w) Nonidet P-40 (NP-40)
1% (w/v) sodium deoxycholate
0.1% (w/v) SDS
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2 (*APPENDIX 2A*)
2 mM EDTA
50 mM sodium fluoride
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

SDS lysis buffer

0.5% (w/v) SDS
0.05 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM DTT, added fresh

SDS and Tris·Cl solutions can be made in advance and stored at room temperature. DTT is added from a 1 M stock solutions stored at −20°C.

Tris-buffered saline (TBS)

Dissolve the following salts in 800 to 900 ml H_2O:
8 g sodium chloride (136.8 mM final)
0.38 g potassium chloride (5.0 mM final)
0.1 g calcium chloride (anhydrous; 0.9 mM final)
0.1 g magnesium chloride hexahydrate (0.5 mM final)
0.1 g dibasic sodium phosphate (anhydrous; 0.7 mM final)
Add 25 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Add H_2O to 1 liter
Dispense 50- or 100-ml aliquots into glass bottles
Autoclave and store indefinitely at room temperature

COMMENTARY

Background Information

The extent to which a protein becomes radiolabeled via biosynthetic labeling with $^{32}P_i$ depends on the rate of transport of phosphate into the cells being labeled, abundance of the protein, stoichiometry of phosphorylation of the protein, and rate of phosphate turnover in the protein. The rate of turnover of the protein itself is less important because phosphate in protein usually turns over at a much faster rate than does the protein. Most of the phosphate incorporated into cells is not incorporated into phosphoproteins—the vast majority is incorporated into phospholipid, RNA, and DNA. The efficiency of incorporation of $^{32}P_i$ into proteins is therefore low—only ~1% of incorporated radioactivity is found in phosphoproteins.

An obvious and immediate question is how many cells and how much isotope should be used. The answer is that it depends on the protein being studied. If the protein is abundant (i.e., it constitutes 0.5% to 1% of total cellular protein) and highly phosphorylated (i.e., it contains 1 mole of phosphate per mole of protein), it can be labeled by incubating 10^6 cells with 0.2 to 0.5 mCi $^{32}P_i$ in 2 ml labeling medium for 1 hr. In contrast, if the protein is rare or nonabundant, it may be necessary to label 10^7 cells with 5 to 10 mCi of $^{32}P_i$ in 3 to 5 ml labeling medium for 4 to 6 hr. A pilot experiment can be useful: if 50 cpm are recovered in the protein of interest, use more cells and more isotope; if 200,000 cpm are recovered in the protein of interest, use fewer cells with less isotope.

Critical Parameters

Incorporation of $^{32}P_i$ during biosynthetic labeling is greatest if labeling is done in medium lacking any phosphate except the added radioisotope. Labeling can also be accomplished at a somewhat reduced efficiency in complete growth medium. Phosphate-free medium is commercially available but can also be easily formulated in-house by omitting sodium and potassium phosphate from a recipe and replacing them with either sodium chloride or potassium chloride or both. Serum dialyzed against phosphate-free saline can be used instead of complete serum to further reduce the level of phosphate in labeling medium. Dialysis of serum against phosphate-free saline reduces but never entirely eliminates the phosphate in serum. Washing or rinsing cells in reduced-phosphate medium just prior to labeling significantly increases labeling efficiency, but starvation of cells by incubation in reduced-phosphate medium prior to labeling is of very limited value.

Labeling cells with ^{32}P almost certainly induces radiation damage in the labeled cells and will affect or arrest cell-cycle progression. If phosphorylation of the protein under study varies during the cell cycle, labeling with ^{32}P may alter its phosphorylation.

At the end of the labeling interval, the radioactive medium should be discarded, except in experiments where labeled virions or labeled secreted proteins are being studied. Labeling medium should be removed *without* using a vacuum aspirator. Vacuum aspiration generates radioactive aerosols and leaves a radioactive film inside the aspirator hose. As a result the hose can become an intense source of radiation exposure. It is best to use a plugged disposable pipet or disposable plastic Pasteur pipet for removing labeling medium. The medium and pipet should be discarded immediately as radioactive wastes.

In general, labeled cells are lysed and subjected to gel electrophoresis, immunoprecipitation, or protein purification. If the labeled cells are to be centrifuged, they should be transferred to capped, disposable centrifuge tubes. Tubes should be no more than half full if they are to be spun in a fixed-angle rotor; tubes that are too full may leak radioactive lysate and contaminate the centrifuge. Screw-cap microcentrifuge tubes are ideal for centrifuging lysates. If large volumes are being handled, multiple partially filled tubes are preferable to tubes filled to the top.

An ice bucket can be used for storage and transport of samples to keep the samples cold and provide considerable shielding. Radiation exposure from the tops of the tubes can be minimized by covering the ice bucket with a ¼-in. Plexiglas sheet.

RadioImmunoPrecipitation Assay (RIPA) buffer (Brugge and Erikson, 1977) is the lysis buffer of choice because it solubilizes proteins well, gives a low background of nonspecific proteins, and is tolerated by most antigens and antibodies. It does however denature some antigens and disrupt some protein:protein complexes. If 1% (w/w) Nonidet P-40 (NP-40) is used as the only detergent, most of these problems are solved without increasing background unacceptably (Sefton et al., 1980). Some workers like to use digitonin as an extremely mild detergent for cell lysis. Digitonin sometimes is

tricky and idiosyncratic, giving erratic results and high backgrounds during immunoprecipitation. 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS; Chen et al., 1990) and Brij 96 (Osman et al., 1992) are mild detergents that appear to give more reproducible results.

Besides the usual concern about proteolysis following cell lysis, the cells must be handled carefully to prevent protein phosphorylation and dephosphorylation following lysis of the cells. Both problems can be minimized by proper formulation of the lysis buffer and by keeping the sample at 4°C. Inclusion of 2 mM EDTA in the lysis buffer will minimize phosphorylation in the lysate by chelating both Mg^{2+} and Mn^{2+}, which are essential for protein kinase activity. Addition of phosphatase inhibitors to the lysis buffer will reduce dephosphorylation significantly. For example, 50 mM sodium fluoride is used to inhibit serine and threonine phosphatases, and 0.2 mM sodium vanadate is used to inhibit all known tyrosine phosphatases. Sodium vanadate appears to lose its effectiveness if it is stored diluted in lysis buffer, so it should be added fresh each time. The concentrated stock solution should be stored in plastic at room temperature.

Anticipated Results

This protocol can be used to label phosphoproteins or other phosphorylated cellular constituents. Phosphoproteins will contain ~1% of incorporated label.

Time Considerations

Proteins can and should be labeled biosynthetically with $^{32}P_i$ and isolated by immunoprecipitation in a single day. As is the case with all ^{32}P-labeled samples, it is important to work quickly because the specific activity of the sample declines 5% per day. If it is absolutely necessary to stop during the immunoprecipitation, it is best to leave the samples as precipitated pellets after aspiration of the wash buffer. Such samples can be stored at 4°C or −20°C overnight. Freezing the cell lysate tends to increase the background. Frozen lysates should be reclarified by centrifugation prior to immunoprecipitation.

Literature Cited

Brugge, J.S. and Erikson, R.L. 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature* 269:346-348.

Chen, J., Stall, A.M., Herzenberg, L.A., and Herzenberg, L.A. 1990. Differences in glycoprotein complexes associated with IgM and IgD on normal murine B cells potentially enable transduction of different signals. *EMBO J.* 9:2117-2124.

Osman, N., Ley, S.C., and Crumpton, M.J. 1992. Evidence for an association between the T cell receptor/CD3 antigen complex and the CD5 antigen complex in human T lymphocytes. *Eur. J. Immunol.* 22:2995-3000.

Sefton, B.M., Hunter, T., and Beemon, K. 1980. Temperature-sensitive transformation by Rous sarcoma virus and temperature-sensitive protein kinase activity. *J. Virol.* 33:220-229.

Contributed by Bartholomew M. Sefton
The Salk Institute
San Diego, California

Phosphoamino Acid Analysis

It is often valuable to identify the phosphorylated residue in a protein. In the case of proteins phosphorylated at serine, threonine, or tyrosine, this is readily accomplished by partial acid hydrolysis in HCl followed by two-dimensional thin-layer electrophoresis of the labeled phosphoamino acid (see Basic Protocol). Phosphothreonine and phosphotyrosine are more stable to hydrolysis in alkali than are RNA and phosphoserine. Therefore, mild alkaline hydrolysis of protein samples can be used to enhance the detection of phosphothreonine and phosphotyrosine (see Alternate Protocol).

Although this procedure can be carried out with a protein eluted from a preparative gel and concentrated by trichloroacetic acid (TCA) or acetone precipitation, it is most easily accomplished by transfer of the protein of interest to a PVDF membrane. This technique is obviously not ideal if the protein being studied does not transfer efficiently.

NOTE: Wear gloves and use blunt-end forceps to handle membranes.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety department (also see *APPENDIX 1D*).

ACID HYDROLYSIS AND TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF PHOSPHOAMINO ACIDS

BASIC PROTOCOL

The protein to be acid hydrolyzed is transferred to a PVDF membrane using the same technique used for immunoblotting (*UNIT 6.2*) or for microsequencing. It is valuable, but not absolutely essential, to keep the filter wet following transfer. Following acid hydrolysis, phosphoamino acids are separated by two-dimensional thin-layer electrophoresis. Because electrophoresis equipment differs considerably in design, the details of the assembly and placement of the plate are not discussed here. It is assumed that a suitable apparatus is available for use by an experienced operator. Electrophoresis conditions are described for using the HTLE 7000 (CBS Scientific). They are almost certainly not correct for other equipment and will need to be altered according to the equipment manufacturer's directions.

Materials

^{32}P-labeled phosphoprotein (*UNIT 14.4*)
India ink solution: 1 µl/ml India ink in TBS (*UNIT 14.4*)/0.02% (v/v) Tween 20, pH 6.5 (prepare fresh or store indefinitely at room temperature); *or* radioactive or phosphorescent alignment markers
6 M HCl
Phosphoamino acid standards mixture (see recipe)
pH 1.9 electrophoresis buffer (see recipe)
pH 3.5 electrophoresis buffer (see recipe)
0.25% (w/v) ninhydrin in acetone in a freon (aerosol, gas-driven) atomizer/sprayer
PVDF membrane (Immobilon-P, Millipore)
110° oven
Screw-cap microcentrifuge tubes
20 cm × 20 cm × 100 µm glass-backed cellulose thin-layer chromatography plate (EM Sciences)
Large blotter: two 25 × 25–cm layers of Whatman 3MM paper sewn together at the edges, with four 2-cm holes that align with the origins on the TLC plate

Contributed by Bartholomew M. Sefton
Current Protocols in Cell Biology (1999) 14.5.1-14.5.8

Glass tray or plastic box
Whatman 3MM paper
Thin-layer electrophoresis apparatus (e.g., HTLE 7000, CBS Scientific)
Fan
Small blotters: 4 × 25–cm, 5 × 25–cm, and 10 × 25–cm pieces of Whatman 3MM paper
50° to 80°C drying oven
Sheets of transparency film for overhead projector

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and autoradiography (*UNIT 6.3*)

Prepare sample

1. Run radiolabeled phosphoprotein on a preparative SDS-polyacrylamide gel (*UNIT 6.1*).

   It is difficult to obtain good results with <100 cpm of labeled protein.

2. Transfer proteins electrophoretically to a PVDF membrane (*UNIT 6.2*). Wash the membrane several times with water. Do not let the membrane dry.

   These washes remove buffer and detergent.

3. Locate the band of interest by staining the filter 5 to 10 min in 30 to 50 ml India ink solution with shaking until bands are detectable, *or* by wrapping the filter in plastic wrap, applying radioactive or phosphorescent alignment markers, and performing autoradiography (*UNIT 6.3*).

4. Excise the piece of filter containing the band of interest with a clean razor blade. Rewet the piece of filter with methanol for 1 min and then rewet it in >0.5 ml water. Place the piece of filter paper in a screw-cap microcentrifuge tube.

   Keep the excised piece as small as possible.

Hydrolyze sample

5. Add enough 6 M HCl to submerge the piece of filter. Screw the cap on the tube tightly and incubate 60 min in 110°C oven.

6. Let cool. Microcentrifuge 2 min at maximum speed, room temperature. Transfer the liquid hydrolysate to a fresh microcentrifuge tube and dry with a Speedvac evaporator.

   Drying takes ~2 hr. Simultaneous drying of the hydrolysate and deblocked oligonucleotides in NH_4OH must be avoided, as this will generate a cloud of ammonium chloride that will collect in the centrifuge tube and render the hydrolysate unsuitable for thin-layer electrophoresis.

7. Dissolve the sample in 6 to 10 µl water by vortexing vigorously. Microcentrifuge 5 min at maximum speed.

Prepare plate for first-dimension electrophoresis

8. Spot 25% to 50% of the sample, in 0.25- to 0.50-µl aliquots, on one origin of a 20 cm × 20 cm × 100 µm glass-backed cellulose thin-layer chromatography plate (see Fig. 14.5.1 for arrangement of samples). Between each application, dry the sample spot with compressed air delivered through a Pasteur pipet plugged with cotton.

   Use long, thin plastic micropipet loading tips for loading, and do not let the tip touch the plate.

   Four samples can be analyzed simultaneously. The complete hydrolysate can be spotted on a single origin, but some streaking in the first dimension may be observed due to overloading. This problem can be avoided by using a fraction of the sample.

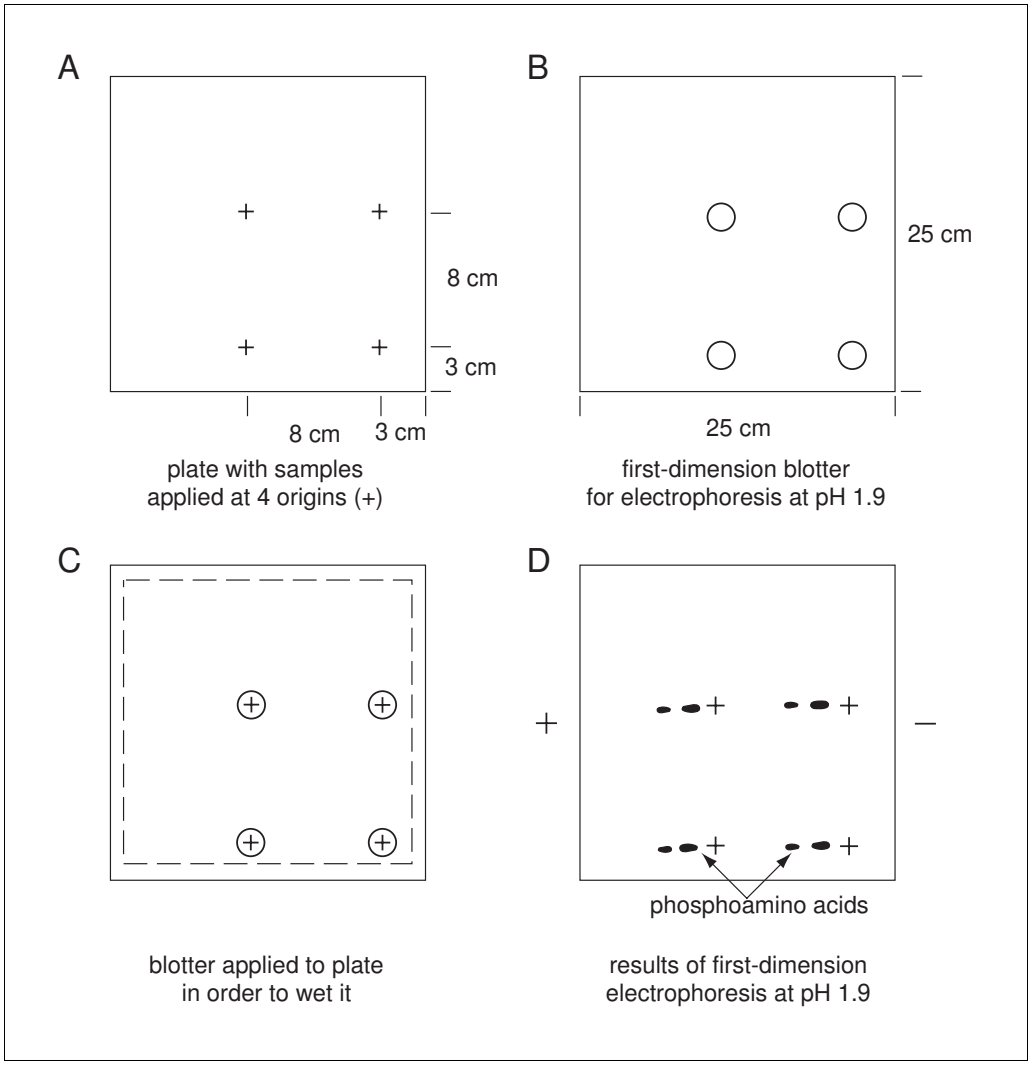


Figure 14.5.1 First-dimension electrophoretic separation of phosphoamino acids at pH 1.9. (**A**) Positions of the four origins on a single 20 × 20–cm plate; (**B**) blotter used for wetting the plate with pH 1.9 electrophoresis buffer; (**C**) placement of the blotter on the plate (underneath; indicated by dashed outline); and (**D**) orientation of the plate between the + and – electrodes with the positions of the phosphoamino acids after electrophoresis.

9. Spot 1 µl nonradioactive phosphoamino acid standards mixture (containing phosphoserine, phosphothreonine, and phosphotyrosine) on top of each sample in 0.25- to 0.50-µl aliquots as above.

10. Wet the large blotter (with four holes) by submerging it in pH 1.9 electrophoresis buffer in a large glass tray or plastic box. Briefly allow the excess buffer to drain off. Lower the wet blotter onto the prespotted plate with the origins on the plate in the centers of the four holes in the blotter (Fig. 14.5.1). Press on the blotter gently to achieve even wetting of the cellulose and concentration of the samples. When the plate is uniformly wet, remove the blotter.

    The blotter should be quite damp but not sopping wet. Excess buffer can be wicked off onto filter paper.

    Areas of the plate that are too dry can be seen through the blotter and will appear to be whiter than the rest of the plate. If this happens, dab the blotter with a Kimwipe wetted with pH 1.9 electrophoresis buffer. If there are puddles of buffer on the plate, let them dry before carrying out electrophoresis.

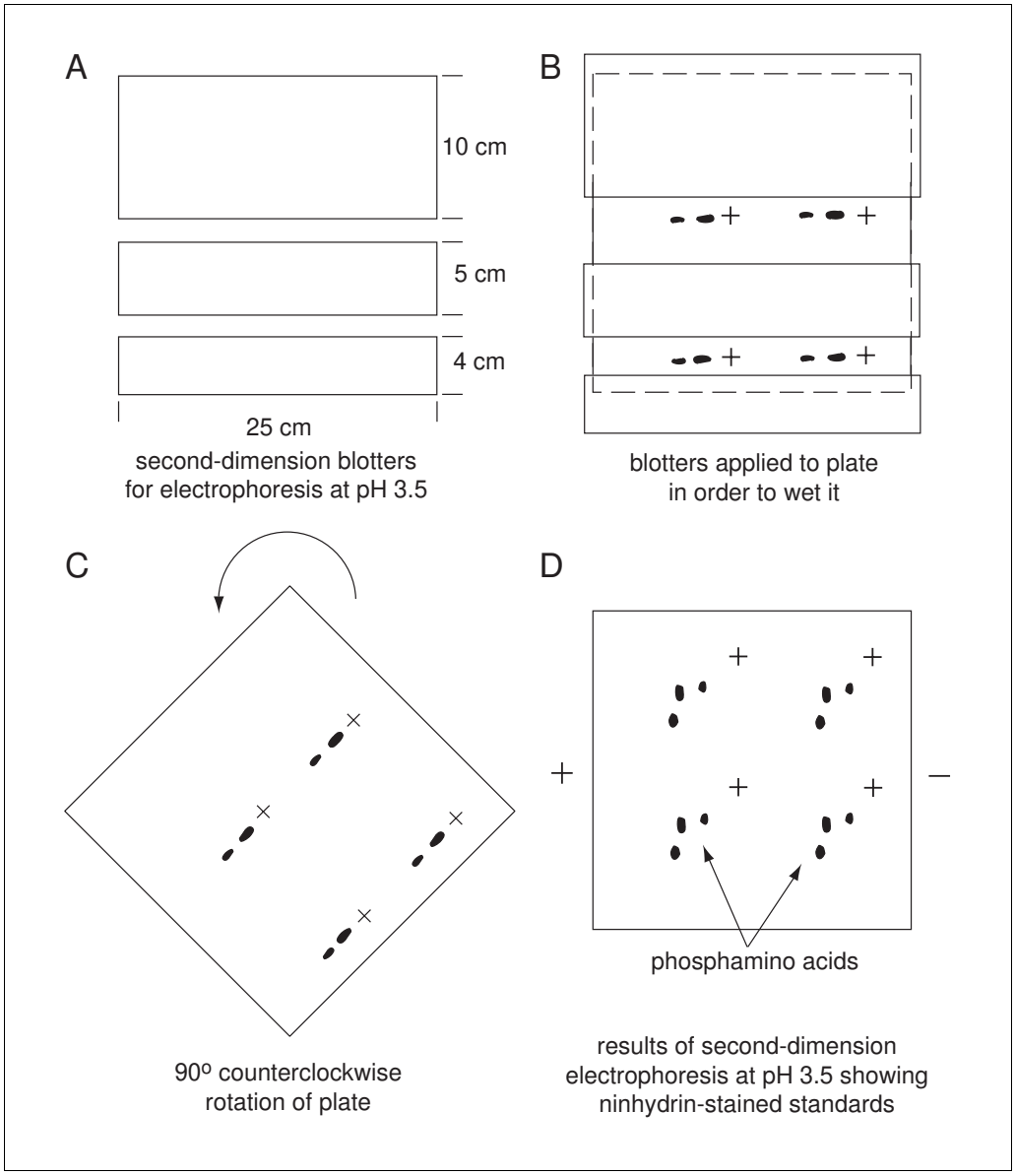


Figure 14.5.2 Second-dimension electrophoretic separation of phosphoamino acids at pH 3.5. (**A**) The three pieces of Whatman 3MM paper used for wetting the plate with pH 3.5 electrophoresis buffer; (**B**) proper placement of the blotters on the plate (underneath; indicated by dashed outline); (**C**) reorientation of the plate for electrophoresis in the second dimension; and (**D**) orientation of the plate between the + and - electrodes with the position of the phosphoamino acids after electrophoresis.

11. Place the thin-layer plate in the electrophoresis apparatus and overlap 0.5 cm of the right and left sides of the plate with wicks made of Whatman 3MM paper. If the apparatus has an air bag, be sure to inflate it. Close the cover and start electrophoresis. With an HTLE 7000, double-thickness Whatman 3MM wicks, and a plate with four samples, electrophorese 20 min at 1.5 kV.

    For the HTLE 7000 apparatus, use folded-over Whatman 3MM wicks that are 20 cm wide (the same as the plate) and not overly wet. Overly wet wicks will flood the plate and cause sample diffusion.

    For other electrophoresis apparatuses the appropriate duration of electrophoresis can be determined empirically by examining the rate of migration of the phosphoamino acid standards.

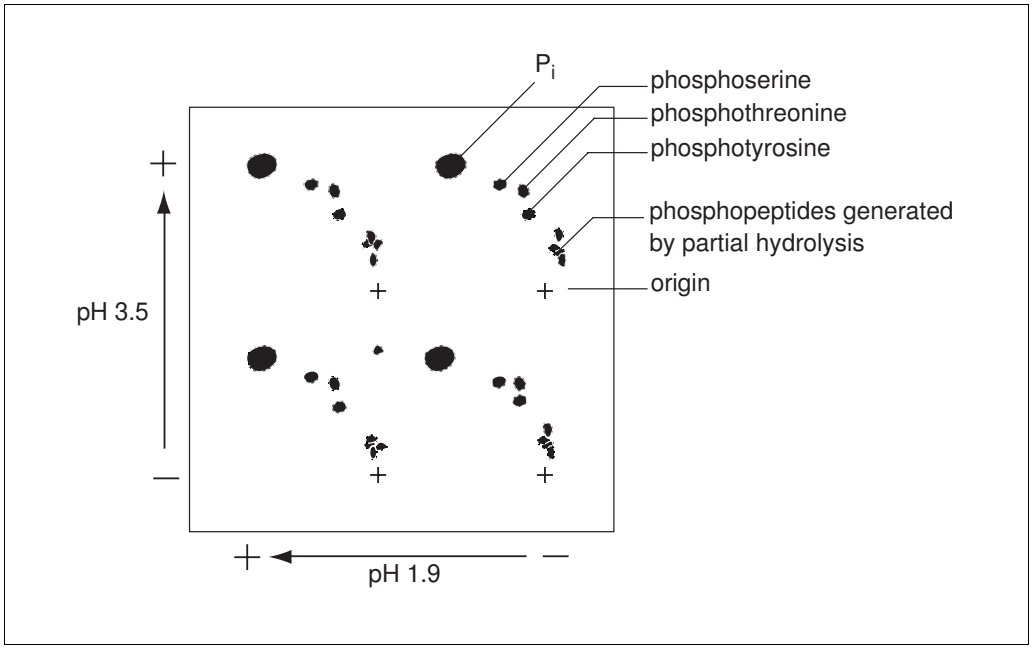


Figure 14.5.3 Hypothetical autoradiogram of a two-dimensional separation. Four samples of acid-hydrolyzed, ^{32}P-labeled proteins are applied at the origins, one in each of the four quandrants. This diagram shows the origins, the directions of electrophoresis, the positions of phosphoserine, phosphothreonine, and phosphotyrosine, the position of P_i, and the position of partially hydrolyzed fragments of the proteins for the upper right-hand sample. Every protein generates different partial hydrolysis peptide fragments.

12. Following electrophoresis, remove the plate and quickly air dry with a fan without heating.

    It takes ~20 min to dry the plate.

Perform second-dimension electrophoresis

13. Wet the small blotters in pH 3.5 electrophoresis buffer and use them to wet the plate using the method described in step 10 to achieve even wetting without puddling (Fig. 14.5.2).

    After electrophoresis at pH 1.9, phosphoamino acids are present as a streak extending from the origin towards the + electrode. Blotters are not applied directly over the phosphoamino acids to prevent sample blurring or smearing.

14. Remove the blotters, rotate the plate 90° counterclockwise, and electrophorese 16 min at 1.3 kV in pH 3.5 electrophoresis buffer if using the HTLE 7000 apparatus.

15. At the end of the electrophoresis run, remove the plate and dry 20 to 30 min in an oven at 50° to 80°C. When dry, spray with 0.25% ninhydrin in acetone, then reheat in the oven 5 to 10 min to visualize the phosphoamino acid standards.

16. Place radioactive or phosphorescent alignment marks on the plate and autoradiograph with an intensifying screen overnight to 10 days at −70°C.

17. Following autoradiography, trace the alignment markers and the stained phosphoamino acid markers onto a transparent sheet used for overhead projectors. Save this template. Align the film with the plate and identify radioactive phosphoamino acids (Fig. 14.5.3).

    Use of fluorography or autoradiography to detect the labeled phosphoamino acids is preferable to use of a phosphorimager. The image on film is precisely the same size as the thin-layer plate, which allows the transparent film to be overlaid on the plate for an unambiguous spot identification. A phosphorimager can subsequently be used for quantification.

ALTERNATE PROTOCOL

ALKALI TREATMENT TO ENHANCE DETECTION OF TYR- AND THR-PHOSPHORYLATED PROTEINS BLOTTED ONTO FILTERS

Phosphothreonine and phosphotyrosine are much more stable to hydrolysis in alkali than RNA or phosphoserine. Detection of proteins containing phosphothreonine and phosphotyrosine in impure samples containing ^{32}P-labeled RNA and serine-phosphorylated proteins can often be enhanced by mild alkaline hydrolysis of gel-fractionated samples. Although this technique was first developed for the treatment of fixed polyacrylamide gels, it is much more easily performed with proteins that have been first transferred to a PVDF membrane.

Alkaline hydrolysis does not preclude subsequent phosphoamino acid analysis. A band from a blot that has been treated with alkali can be excised and subjected to acid hydrolysis as described in the Basic Protocol.

Additional Materials *(also see Basic Protocol)*

1 M KOH
TN buffer: 10 mM Tris·Cl (pH 7.4 at room temperature)/0.15 M NaCl
1 M Tris·Cl, pH 7.0 at room temperature

Covered plastic container (e.g., Tupperware box)
55°C oven or water bath

1. Run radiolabeled sample on a preparative SDS–polyacrylamide gel and transfer proteins electrophoretically to a PVDF membrane (see Basic Protocol steps 1 and 2).

   A band containing as few as 10 cpm is detectable under optimal conditions with this technique.

   A nylon membrane may be used in place of a PVDF membrane, but in that case, the bands cannot subsequently be analyzed by acid hydrolysis, as nylon membrane will dissolve in 6 M HCl.

2. Wash membrane thoroughly with water: three 2-min incubations in 1 liter water are sufficient.

   These washes remove buffer and detergent.

3. Incubate membrane 120 min at 55°C in an oven or water bath in sufficient 1 M KOH to cover the filter in a covered Tupperware container.

4. Discard KOH. Wash membrane and neutralize remaining KOH by rinsing once for 5 min in 500 ml TN buffer, once for 5 min in 500 ml of 1 M Tris·Cl (pH 7.0), and twice for 5 min in 500 ml water. Wrap the membrane in plastic wrap and autoradiograph (*UNIT 6.3*) overnight with flashed film and an intensifying screen at −70°C.

   Identification of the band of interest is most easily accomplished by coelectrophoresis of a radioactive marker protein of known identity.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

pH 1.9 electrophoresis buffer

50 ml 88% formic acid (0.58 M final)
156 ml glacial acetic acid (1.36 M final)
1794 ml H_2O
Store indefinitely in a sealed bottle at room temperature

pH 3.5 electrophoresis buffer

100 ml glacial acetic acid (0.87 M final)
10 ml pyridine [0.5% (v/v) final]
10 ml 100 mM EDTA (0.5 mM final)
1880 ml H_2O
Store indefinitely in a sealed bottle at room temperature

Phosphoamino acid standards mixture

Prepare a solution of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) in water at a final concentration of 0.3 mg/ml each. Store in 1-ml aliquots indefinitely at −20°C.

COMMENTARY

Background Information

Phosphoamino acid analysis by the two-dimensional electrophoretic technique described in the basic protocol was first carried out with proteins isolated by elution from unfixed SDS-polyacrylamide gels (Hunter and Sefton, 1980). However, this technique is laborious, especially if it involves grinding up pieces of high-percentage acrylamide gels, and the yields can be disappointing. Additionally, because the eluted protein must be precipitated in the presence of a carrier protein, spotting the whole sample on a single origin usually yields a badly smeared pattern. The grind-and-elute technique is, however, advantageous with proteins that are very refractory to electrophoretic transfer to PVDF membranes.

The alkaline treatment of protein described in the alternate protocol was first developed by Jon Cooper and Tony Hunter, who treated fixed gels with KOH (Cooper and Hunter, 1981). The original technique is tricky because the gel becomes extremely sticky during incubation with KOH and swells. Additionally, the manipulations needed to recover proteins from the gel following treatment are very involved because the proteins are contaminated with products of the hydrolysis of polyacrylamide.

Critical Parameters and Troubleshooting

It is essential to use PVDF membranes to immobilize proteins for acid hydrolysis rather than nylon or nitrocellulose membranes, both of which dissolve in 6 M HCl. Proteins immobilized on either PVDF or nylon membranes may be subjected to alkaline hydrolysis with KOH (Contor et al., 1987), but nitrocellulose membranes are not suitable. Proteins immobilized on nylon cannot subsequently be analyzed by acid hydrolysis because nylon is dissolved by 6 M HCl.

Two-dimensional thin-layer electrophoresis is required for unambiguous identification of phosphorylated residues, as some spots after one-dimensional electrophoresis do not represent pure species. For example, uridine monophosphate, which is generated during acid hydrolysis of RNA (a frequent contaminant of phosphoproteins), comigrates with phosphotyrosine during one-dimensional electrophoresis at pH 3.5.

Streaking of the sample in the first dimension is a symptom of overloading, either with the phosphoprotein itself or with contaminants in the sample. This problem can be corrected by loading less sample. Streaking in the second dimension is usually the result of problems with wetting or running the plate and cannot be corrected by loading less sample.

Some batches of blotting paper contain calcium, which interferes with electrophoresis of phosphoamino acids (probably by precipitating them). In the author's experience Whatman 3MM paper is quite reliable; other blotting papers are probably suitable as well. Inclusion of EDTA in the pH 3.5 buffer alleviates this problem.

This unit calls for glass-backed cellulose thin-layer plates rather than the plastic-backed variety, which are lighter and less expensive. This is because plastic-backed plates can under some circumstances cause sample streaking. They are, however, probably satisfactory for most experiments. If use of plastic-backed plates results in streaking, try glass-backed plates to see if that corrects the problem.

Anticipated Results

To detect a phosphoamino acid by autoradiography, a minimum of 10 cpm must be spotted and the plate exposed for a week with flashed film and an intensifying screen. Only 15% to 20% of the radioactivity in a phospho-

protein is recovered as phosphoamino acids. The majority is present as $^{32}P_i$, which is released by dephosphorylation of phosphoamino acids, with the remainder being peptide products resulting from partial acid hydrolysis. As a result, the thin-layer plates will contain a number of radioactive spots that are not phosphoamino acids (see Fig. 14.5.3). Partial hydrolysis products remain near the origin during electrophoresis at pH 1.9, but exhibit some mobility at pH 3.5 (see Fig. 14.5.3). After two-dimensional electrophoresis, they are found above the origin and below the phosphoamino acids. $^{32}P_i$ has a high mobility at both pH 1.9 and pH 3.5 and is found in the upper left-hand corner of each quadrant of the plate (see Fig. 14.5.3). Because of these additional radioactive spots, it is essential to localize internal phosphoamino acid standards by staining with ninhydrin.

Time Considerations

After the preparative gel has been run and the protein transferred to the membrane, isolation of the membrane fragment containing the protein, followed by acid hydrolysis, takes <2 hr. Two to three hours are required for drying the sample with a Speedvac evaporator. First- and second-dimension electrophoresis and staining the internal phosphoamino acid standards takes no more than ~2 hr. Overnight autoradiographic exposure with flashed film and an intensifying screen is usually sufficient; however, exposures can be carried out for up to 10 days before the background from the screen becomes objectionable.

Detection of phosphorylated protein after alkali treatment, as described in the alternate protocol, is a very quick procedure requiring little more time than it takes to carry out the alkaline hydrolysis. In general, samples of this sort can be detected after overnight exposure with flashed film and an intensifying screen at –70°C.

Literature Cited

Contor, L., Lamy, F., and Lecocq, R.E. 1987. Use of electroblotting to detect and analyze phosphotyrosine containing peptides separated by two-dimensional gel electrophoresis. *Anal. Biochem.* 160:414-420.

Cooper, J.A. and Hunter, T. 1981. Four different classes of retroviruses induce phosphorylation of tyrosines present in similar cellular proteins. *Mol. Cell. Biol.* 1:394-407.

Hunter, T. and Sefton, B.M. 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. U.S.A.* 77:1311-1315.

Key Reference

Kamps, M.P. and Sefton, B.M. 1989. Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates the analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.* 176:22-27.

Discusses all of the variables involved in subjecting filter-bound proteins to acid and base hydrolysis.

Contributed by Bartholomew M. Sefton
The Salk Institute
San Diego, California

Determination of Akt/PKB Signaling

Akt—also known as protein kinase B (PKB)—is a central regulator of cell survival (Franke et al., 1997; Hemmings, 1997; Marte and Downward, 1997), and its activity is often used to assess the apoptotic effect of different experimental conditions. This Ser/Thr protein kinase is activated downstream of phosphoinositide 3-kinase (PI3K) in response to a wide variety of growth factors (Fig. 14.6.1). The activated form of the kinase targets a specific set of effector proteins involved in cell-survival signaling. The activation involves membrane translocation and dual phosphorylation on threonine at position 308 and serine at position 473. Because the activation of Akt depends on this phosphorylation, it is possible to assess Akt activity not only with a kinase assay but also by determining its level of phosphorylation.

This unit provides a protocol for assaying the phosphorylation and the dynamics of dephosphorylation of Akt/PKB in cultured cells (see Basic Protocol). A protocol is also provided for assaying membrane translocation in response to Akt activation (see Support Protocol).

DETERMINATION OF Akt/PKB SIGNALING IN CULTURED CELLS

BASIC PROTOCOL

This protocol can be applied to nearly all cultured cell lines with few or no modifications. In this particular example, the chosen cell system involves cells expressing or deficient in the β1 integrin because of the links between integrin signaling, Akt activation, and cell survival. This protocol describes PDGF stimulation of GD25 β1 integrin–null cells (Fassler et al., 1995) expressing wild-type β1 integrin or its mutant variant W/A, which is deficient in Akt signaling (Pankov et al., 2003). Specific treatments and time-course sampling of cultured cells are described; these allow determination of the phosphorylation level of Akt, its sensitivity to growth factor stimulation, and the mode of its inactivation by dephosphorylation. All these results can be obtained in a single nonradioactive experiment by using standard techniques such as immunoblotting (*UNIT 6.2*) and immunodetection with phosphospecific antibodies (*UNIT 14.2*). Cell cultures are serum-starved, stimulated with growth factor, and maintained for 2 hr after the stimulation for assessing the dynamics of Akt dephosphorylation. Samples are taken after each treatment, and Akt activity is determined with phosphospecific antibodies.

Materials

GD25 cells obtained from R. Fassler, Max Planck Institute of Biochemistry, Martinsried, Germany, and expressing wild-type β1 integrin (β1 cells) or integrin mutant W/A (mutant cells)

Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (DMEM/10% FBS; *APPENDIX 2A*)

Starvation medium: DMEM containing 1% bovine serum albumin (DMEM/1% BSA)

0.5 μM okadaic acid (OA; 1 mM stock solution in DMSO, *APPENDIX 1B*) in DMEM/1% BSA

Platelet-derived growth factor-BB (PDGF-BB), freshly prepared at 10 μg/ml in 10 mM acetic acid

AG 1433 (e.g., Calbiochem) PDGF kinase inhibitor, 10 mM stock solution in dimethyl sulfoxide (DMSO)

PBS (*APPENDIX 2A*), ice cold

Modified radioimmunoprecipitation assay (mRIPA) buffer (see recipe), ice cold

2× SDS sample buffer (*APPENDIX 2A*)

10% separating gels with 4% stacking gels (*UNIT 6.1*)

Contributed by Roumen Pankov

Current Protocols in Cell Biology (2004) 14.6.1-14.6.12

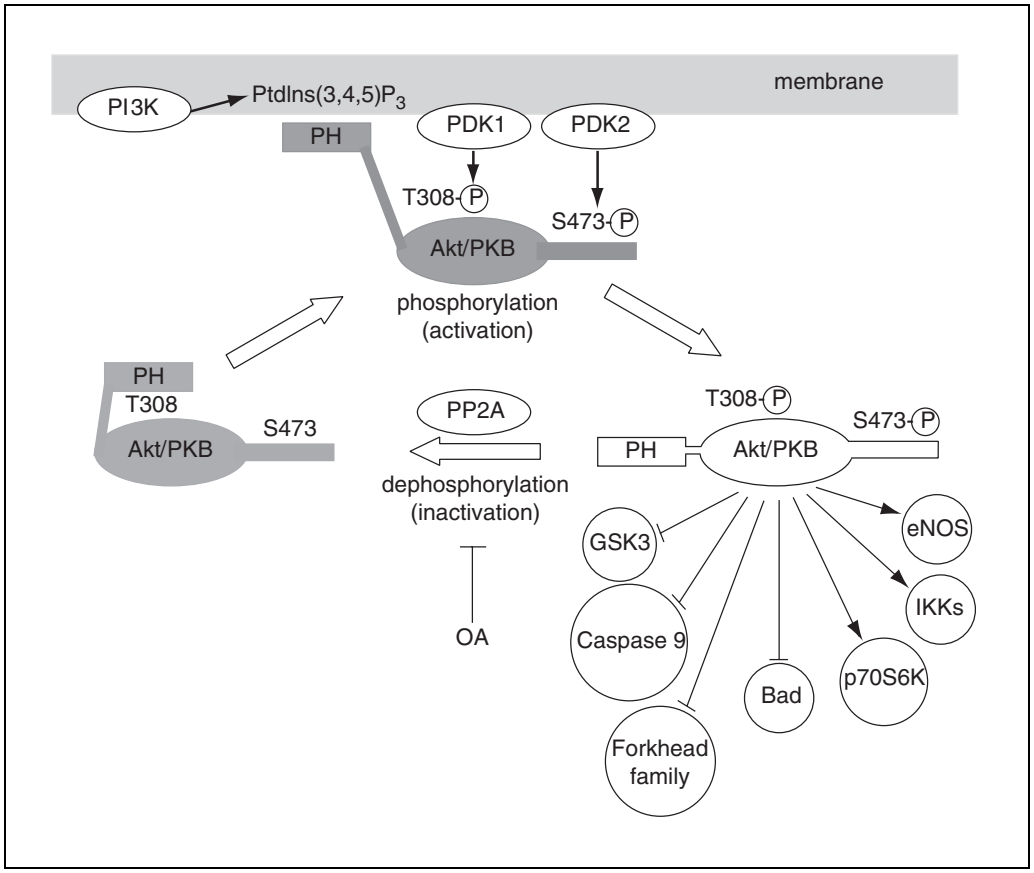


Figure 14.6.1 Activation of Akt/PKB and its effectors. Akt is activated by phosphatidylinositol 3-kinase (PI3K) products PtdIns(3,4,5)P_3 and PtdIns(3,4,)P_2. These phosphoinositides target inactive Akt to the plasma membrane via its pleckstrin homology (PH) domain. At the membrane, Akt is phosphorylated on two residues: threonine 308 (T308) by phosphoinositide-dependent kinase 1 (PDK1) and serine 473 (S473) by an unidentified PDK2. The dual phosphorylation is necessary for full activation of Akt. Activated Akt in turn phosphorylates its targets, thus modulating their function. Akt effectors include glycogen synthase kinase-3 (GSK3), caspase 9, Forkhead family of transcription factors, Bad, p70 ribosomal S6 kinase (p70S6K), IκB kinases (IKKs), and endothelial nitric oxide synthase (eNOS). Akt is inactivated through dephosphorylation by protein phosphatase 2A (PP2A), and this process can be blocked by okadaic acid (OA).

Pre-stained protein standards (e.g., Novex)
Transfer buffer (*UNIT 6.2*)
Ponceau S solution (*UNIT 6.2*)
Tris-buffered saline with 0.1% Tween 20 (TTBS, *APPENDIX 2A*)
Blocking solution: TTBS containing 5% dry nonfat milk (TTBS/milk)
Primary antibodies: polyclonal anti-phospho Akt (Ser 473) (e.g., Cell Signaling, Biosource), monoclonal anti-actin (e.g., Sigma)
Dry nonfat milk
Secondary horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse antibodies (e.g., Amersham Biosciences)
Enhanced chemiluminescence (ECL) detection reagent (*UNIT 14.2*)

35-mm tissue culture dishes
Plastic cell scraper (rubber policeman)
1.5-ml microcentrifuge tubes, prechilled
Sonicator/ultrasonic processor
Boiling water bath
Two nitrocellulose membranes cut to gel size

Four Whatman 3MM filter papers cut to gel size
SDS-PAGE/transfer apparatus (e.g., Bio-Rad, Novex)
Constant-voltage/current power supply (e.g., Bio-Rad)
Flat containers for washing gels and membranes
Shaker
Heat-sealable plastic bags
Heat sealer
X-ray film (e.g., Hyperfilm; Amersham Biosciences)
Film cassette for X-ray film
X-ray film developer

Additional reagents and equipment for tissue culture (*UNIT 1.1*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 10% CO_2 incubator. Use pre-warmed cell culture medium for all treatments.

Treat cells

1. Plate twelve 35-mm tissue culture dishes each of GD25 cells expressing wild-type β1 integrin and mutant cells (1.0×10^6 cells/dish) in 2 ml DMEM/10% FBS and allow them to attach and spread for 3 to 4 hr.

2. Wash dishes with 2 ml starvation medium two times, then add 2 ml/dish of the starvation medium and serum-starve the cells overnight.

   At this point the dishes should be ~80% confluent. Depending on the cell type being used, adjustment of the initial number of plated cells may be necessary.

3. Treat half of the dishes (six dishes of β1 cells and six dishes of mutant cells) with 0.5 μM okadaic acid (OA) (protein phosphatase inhibitor) in DMEM/1% BSA for 15 min. Label the dishes "OA". Maintain the same concentration of OA in the medium to be used to treat these dishes throughout the entire experiment. To ensure similar treatment, dispense 25 ml of pre-warmed DMEM/1% BSA and add 12.5 μl of 1 mM okadaic acid stock solution to the 25 ml of medium to be used for the "OA" sets of dishes. Add 12.5 μl of DMSO to the other 25 ml medium for the untreated sets.

   After this treatment, four sets of six samples are formed (two for each cell line). These are untreated cells of each β1 cells (β1) and W/A mutant cells (mutant), and okadaic acid–treated β1 cells (β1/OA) and mutant cells (mutant/OA).

4. Aspirate the starvation medium and stimulate cells on five dishes of each set with 20 ng/ml PDGF-BB in 2 ml DMEM/1% BSA for 15 min. Leave four dishes, one from each set (β1, β1/OA, W/A, and W/A/OA) without stimulation in fresh DMEM/1% BSA and label them as controls. To ensure similar treatment, dispense 40 ml of pre-warmed DMEM/1% BSA and add 80 μl of 10 μg/ml stock solution of PDGF-BB, mix well, divide into two 20-ml aliquots, and add 10 μl of 1 mM okadaic acid stock solution to the 20 ml of medium to be used for the "OA" sets of dishes. Add 10 μl of DMSO to the other 20 ml of PDGF-containing medium.

5. Rinse all dishes one time with DMEM/1% BSA. Put aside the control dishes and one of each PDGF-stimulated dishes (β1, β1/OA, W/A, and W/A/OA) for lysis. To the remaining four dishes of each set, add 2 ml/dish of the same medium containing 30 μM AG 1433 PDGF kinase inhibitor (four of the β1 and four of the mutant PDGF-stimulated dishes). Treat the "OA" set of dishes (four of the β1/OA and four of the W/A/OA PDGF-stimulated dishes) with the same AG 1433–containing medium supplemented with 0.5 μM okadaic acid. Incubate the AG 1433–containing dishes for 30, 60, 90, and 120 min at 37°C. Specifically, dispense 40 ml of pre-warmed

DMEM/1% BSA and add 120 µl of 10 mM stock solution of AG 1433, mix well, and divide into 20-ml aliquots and add 10 µl of 1 mM okadaic acid stock solution to the 20 ml of medium to be used for the "OA" sets of dishes. Add 10 µl of DMSO to the other 20 ml of AG 1433-containing medium.

AG 1433 is used to eliminate residual receptor kinase activity after the PDGF stimulation and to identify the rate of dephosphorylation.

Prepare cellular lysates

6. Rinse the dishes that were put aside for lysis (control and PDGF-stimulated β1, β1/OA, W/A, and W/A/OA) with 2 ml ice-cold PBS two times. Keep the dishes on ice, and use ice-cold buffers to slow down biological processes and prevent protein degradation.

7. Add 100 µl/dish of ice-cold mRIPA buffer plus inhibitors, scrape the cells with a plastic scraper or rubber policeman, and transfer the lysate into prechilled and prelabeled 1.5-ml microcentrifuge tubes. Incubate the lysates for an additional 10 min on ice.

Prepare gel samples

8. Add 100 µl/sample of 2× SDS sample buffer, sonicate (two 5-sec, 50-W pulses) on ice.

   The samples will become quite viscous after the addition of the SDS sample buffer. It is necessary to shear the released DNA by sonication to eliminate the viscosity and to allow correct loading of the samples on SDS-PAGE gels.

9. Boil samples in a water bath for 3 min or heat at 95°C for 5 min on a heating block.

10. Remove the PDGF-stimulated and AG 1433–treated β1, β1/OA, W/A, and W/A/OA dishes after 30, 60, 90, and 120 min.

11. Rinse with 2 ml ice-cold PBS two times and repeat steps 7, 8, and 9 for the samples at each timepoint.

    The samples can be stored sealed and frozen at least 1 month at −20°C.

Separate samples by SDS-PAGE

12. Cast two 10% separating gels with 4% stacking gels (*UNIT 6.1*).

    Use 15-well combs so that all 24 samples can be loaded on two gels.

13. Load 20 µl of each sample/gel lane and a separate sample containing prestained protein standards on the gel.

    Load each set of samples starting with the control sample, followed by the PDGF-stimulated sample, taken immediately after the stimulation, followed by the samples that have been kept in AG 1433–containing medium for 30, 60, 90, and 120 min for each sample set. Load the prestained protein standards into the first or the last lane so that the orientation of the gel can be identified after it is removed from the apparatus. Fill the empty lanes of the gels with 1× SDS sample buffer to prevent distortion of the separation in the adjacent lanes.

14. Run the gels at 150 V until the bromophenol blue dye reaches the bottom of the gel (see *UNIT 6.1*).

Transfer separated proteins from gel to membrane

15. When the electrophoresis is complete, remove the gels from the gel plates, cut off the stacking gels and incubate the separating portion of the gels in 50 ml transfer buffer for 15 min.

    Use gloves to handle the gels and membranes since oils from hands can block the transfer.

16. Assemble the transfer sandwich consisting of pad, Whatman 3MM filter paper, nitrocellulose membrane, equilibrated acrylamide gel, second Whatman 3MM filter paper, and second pad (Fig. 6.2.1).

    All pads, filter papers, and nitrocellulose membranes should be handled using gloves and prewetted with transfer buffer. The transfer cassette should be assembled underneath the transfer buffer to avoid trapping air bubbles. Ensure the orientation of the gel (judged by the position of the prestained protein standards) such that the correct order of the samples after transfer onto the nitrocellulose membrane is maintained.

17. Place the transfer sandwich into the electroblotting apparatus filled with transfer buffer with the nitrocellulose membrane on the cathode side of the gel. Connect the apparatus to the power supply and transfer proteins for 1 hr at 100V (constant voltage) with cooling (*UNIT 6.2*).

    Transfer time depends on the size of the proteins, acrylamide percentage, and the thickness of the gel. The completeness of the transfer can be easily judged by the transfer of the prestained protein standards.

18. At the end of the transfer, turn off the power supply and disassemble the apparatus and transfer cassette. Remove the nitrocellulose membranes and stain with 50 ml Ponceau S solution in a flat container for 5 min. Destain the membranes by rinsing several times with distilled water.

    Two membranes can be incubated in the same container by orienting them back to back.

    Staining with Ponceau S does not interfere with the subsequent antibody reactions and provides a good estimation of the protein loading, separation, and quality of the transfer. The Ponceau S solution can be reused several times.

Probe the membranes with antibodies

19. Rinse the membranes once with TTBS and incubate in 50 ml blocking solution for 30 min at room temperature with gentle shaking.

    Milk proteins in the blocking buffer are used to saturate free protein-binding sites and to prevent the nonspecific binding of the antibody. Do not incubate the membrane longer than 1 hr in this buffer since blocking buffer also has a slight stripping effect and may cause detachment of the transferred sample proteins.

20. Dilute primary anti-phospho Akt antibody according to the supplier's instructions in 10 ml TTBS containing 3% dry nonfat milk. Place the membranes in heat-sealable plastic bags, add diluted antibody, and seal the bag with a heat sealer. Incubate the membranes overnight at 4°C with gentle shaking.

    Two membranes can be incubated in the same bag by orienting them back to back. Remove all air bubbles from the bag before sealing it.

21. Remove the membranes from the bag and wash them three times, 15 min each with 50 ml TTBS in flat container with vigorous shaking.

    Do not allow the membranes to dry out after the incubation with primary antibody.

22. Dilute the secondary antibody in 10 ml TTBS, 3% dry nonfat milk according to the supplier's instructions. Place the membranes in a new heat-sealable bag, add diluted antibody and seal the bag. Incubate for 30 to 45 min at room temperature with gentle shaking.

    Either HRP- or AP-conjugated secondary antibodies can be used. HRP-conjugated secondary antibodies can be combined with the high-sensitivity ECL detection system. This system allows detection of signals from weak antibodies although attention should be paid if accurate quantification of the signal is necessary (see Commentary).

23. Repeat step 21.

24. Use the ECL immunodetection protocol (*UNIT 6.2*) to detect phosphorylated Akt. Incubate the membranes with ECL solution for 1 min, dry the excess fluid by touching the edge of the membrane to a piece of filter paper, wrap the membranes in plastic wrap, and expose to X-ray film for 1 min.

25. Incubate the membranes in 50 ml blocking buffer for 15 min at room temperature with shaking.

26. Repeat steps 20 to 24 with new primary antibody (e.g., anti-actin).

    This second reaction is used as an internal control for loading.

    If both primary antibodies are generated in the same species (e.g., both are mouse or both are rabbit) and the molecular weights of the antigens are different (e.g., 60 kDa for Akt and 45 kDa for actin) the membranes can be incubated with a mixture of the primary antibodies, and the two signals can be detected simultaneously on the same X-ray film.

SUPPORT PROTOCOL

DETERMINATION OF Akt/PKB TRANSLOCATION TO THE PLASMA MEMBRANE

An important step in Akt activation involves its targeting to the plasma membrane. This translocation is dependent on the pleckstrin homology (PH) domain localized at the N-terminus of the Akt molecule and the presence of phosphatidylinositol 3-kinase products PtdIns(3,4,5)P_3 and PtdIns(3,4,)P_2.

This protocol describes the preparation of membrane and cytosolic fractions from starved and PDGF-stimulated GD25 β1 integrin-null cells expressing wild-type β1 integrin or its mutant variant W/A that is deficient in Akt signaling. The distribution of Akt is determined in each fraction by immunoblotting and immunodetection with antibodies. The same protocol can be applied to almost all cultured cell lines with little or no modifications.

Additional Materials *(also see Basic Protocol)*

Cytosolic buffer (see recipe)
Membrane buffer (see recipe)
Dry ice
60-mm dishes

Treat cells

1. Plate two 60-mm dishes each of β1 and mutant cells (2.5×10^6 cells/dish) in DMEM/10% FBS and allow them to attach and spread for 3 to 4 hr.

2. Wash dishes with 5 ml DMEM/1% BSA (starvation medium) two times, then add 5 ml/dish of DMEM/1% BSA and serum-starve the cells overnight.

    At this point the dishes should be ~80% confluent. Depending on the cell type being used, adjustment of the initial number of plated cells may be necessary.

3. Aspirate the starvation medium and stimulate one dish from each cell line with 20 ng/ml PDGF-BB in 5 ml DMEM/1% BSA for 15 min. Leave one β1 and one mutant dish without stimulation in fresh DMEM/1% BSA and label them as controls. To ensure similar treatment, dispense 10 ml of pre-warmed DMEM/1% BSA and add 20 µl of 10 µg/ml stock solution of PDGF-BB, mix well, and add to the dishes to be stimulated.

Prepare cytosol fractions

4. Rinse the control and PDGF-stimulated dishes with 5 ml cytosolic buffer two times. Keep the dishes on ice and use ice-cold buffers to slow down the biological processes and prevent protein degradation.
5. Add 350 µl/dish of ice-cold cytosolic buffer, scrape the cells with a plastic scraper or rubber policeman, and transfer the lysate into prelabeled 1.5-ml microcentrifuge tubes.
6. Place the tubes on dry ice until frozen. Thaw the cells in a 37°C water bath. Repeat freeze-thaw cycle two additional times.

   Freeze-thaw cycles will break plasma membranes and liberate most of the cytosolic proteins.

7. Centrifuge 15 min at 19,000 × *g*, 4°C. Transfer the supernatant into prelabeled 1.5-ml microcentrifuge tubes, mix with equal volume of 2× SDS sample buffer, and leave on ice.

   This supernatant represents the cytosolic fraction.

Prepare membrane fraction

8. Resuspend the pellet in fresh 350 µl cytosolic buffer, centrifuge as in step 7, and discard the supernatant.

   This washing step clears most of the remaining cytosolic proteins from the pellet.

9. Suspend the pellet in 50 µl membrane buffer and centrifuge 15 min at 10,000 × *g*, 4°C.

   This step solubilizes cellular membranes. Pipet the pellet up and down through a micropipet tip several times, but avoid excessive foaming.

10. Transfer the supernatant into prelabeled 1.5-ml microcentrifuge tubes and mix with an equal volume of 2× SDS sample buffer.

Analyze fractions

11. Boil all the samples (supernatants from steps 7 and 10) in a boiling water bath for 3 min or heat at 95°C for 5 min on a heating block.

    The samples can be stored sealed and frozen at least 1 month at −20°C.

12. Proceed with SDS-PAGE and immunoblotting (see Basic Protocol 1, steps 12 through 24). Use anti-Akt antibody to probe the distribution of the total Akt between the cytosolic and membrane fractions.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cytosolic buffer

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
150 mM NaCl
50 µM leupeptin (*APPENDIX 1B*), add fresh
50 µM pepstatin (*APPENDIX 1B*), add fresh
1 mM PMSF (*APPENDIX 1B*), add fresh
1 mM sodium vanadate (*APPENDIX 1B*), add fresh
50 mM NaF, add fresh
Store up to 3 months at 4°C

The cystolic buffer is derived from Kobayashi et al. (2001).

Membrane buffer

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
150 mM NaCl
1% (v/v) Triton X-100
50 µM leupeptin (*APPENDIX 1B*), add fresh
50 µM pepstatin (*APPENDIX 1B*), add fresh
1 mM PMSF (*APPENDIX 1B*), add fresh
1 mM sodium vanadate (*APPENDIX 1B*), add fresh
50 mM NaF, add fresh
Store up to 3 months at 4°C

The membrane buffer is derived from Kobayashi et al. (2001).

Modified radioimmunoprecipitation assay (mRIPA) buffer

50 mM HEPES, pH.5
150 mM NaCl
10% (v/v) glycerol
0.1% (w/v) SDS (*APPENDIX 2A*)
1% (w/v) sodium deoxycholate
1% (v/v) Triton X-100
1.5 mM $MgCl_2$
1 mM EGTA
50 µM leupeptin (*APPENDIX 1B*), add fresh
50 µM pepstatin (*APPENDIX 1B*), add fresh
1 mM PMSF (*APPENDIX 1B*), add fresh
1 mM sodium vanadate (*APPENDIX 1B*), add fresh
50 mM NaF, add fresh
Store up to 3 months at 4°C

COMMENTARY

Background Information

Akt (c-Akt) is the cellular homolog of the viral oncoprotein v-Akt. It was first identified as a protein kinase with high homology to protein kinases A and C and was therefore termed PKB (protein kinase B) or RAC (related to A and C). Mammals have three closely related Akt genes that are expressed differentially at both mRNA and protein levels (Datta et al., 1999; Brazil and Hemmings, 2001). The family of Akt proteins contains a central kinase domain with specificity for serine or threonine residues in substrate proteins and a carboxyl terminus containing a hydrophobic motif (HM) and a proline-rich domain. The N-terminal region of the molecule includes a pleckstrin homology (PH) domain, which together with the HM play important roles in the Akt activation process (Scheid and Woodgett, 2003). The activation mechanism involves direct binding of PI3K

products phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate to the PH domain of Akt (Chan et al., 1999). As a consequence, Akt is localized to the plasma membrane where PI3K-generated 3′-phosphorylated phospholipids reside (Fig. 14.6.1). This translocation is now known to be an important step in Akt activation (Testa and Bellacosa, 1997). Myristylated c-Akt, which is specifically targeted to the plasma membrane, is constitutively active similarly to the oncogenic v-Akt, which is permanently targeted to the plasma membrane by the viral gag sequence, and exhibits constant kinase activity. Membrane-localized Akt undergoes conformational changes leading to the exposure and phosphorylation of two residues—Thr308 in the activation loop, proximal to the catalytic core, and Ser473 in the HM. This dual phosphorylation is necessary for the full activation of Akt. The kinase that phosphorylates Thr308 has been named 3-phosphoinositide-dependent kinase 1 (PDK1) because it also requires lipids for its activity (Vanhaesebroeck and Alessi, 2000). Phosphorylation of Akt on Thr308 causes a charge-induced change in conformation allowing substrate binding and an elevated rate of catalysis. The second important phosphorylation event associated with Akt activation occurs at Ser473. The mechanism of this phosphorylation is not completely understood and may involve autophosphorylation or distinct serine kinases like integrin-linked kinase (ILK; Persad and Dedhar, 2003). The function of phosphorylation at this site is also not fully clarified, though the necessity of this modification for Akt activity is well documented. For example, kinase activity is significantly reduced by mutations of this residue, and a similar effect is observed after ceramide-promoted dephosphorylation at this site. It has been proposed that phosphorylation at Ser473 may change the properties of the hydrophobic motif, shifting it to a docking site for PDK1 (Scheid and Woodgett, 2003). After activation, Akt appears to detach from the inner leaflet of the plasma membrane and to translocate through the cytosol to the nucleus (Andjelkovic et al., 1997; Meier and Hemmings, 1999).

Cells can inhibit or reverse the activation of Akt by several mechanisms (Hill and Hemmings, 2002). One of them includes the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10; Yamada and Araki, 2001), which decreases the levels of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate within cells, thus preventing membrane translocation of Akt. Direct inactivation of Akt occurs by dephosphorylation of Thr308 and Ser473. This process is mediated by the ubiquitous Ser/Thr protein phosphatase 2A (PP2A; see Millward et al., 1999) and can be blocked by the phosphatase inhibitor okadaic acid. A negative regulator termed CTMP (carboxy-terminal modulator protein), which attenuates the activation of Akt at plasma membrane, has also been identified (Maira et al., 2001). Thus, the level of Akt activity in steady state is the result of an equilibrium between activation and inhibition events.

Extensive research has clarified the intracellular mechanisms of Akt activation by upstream PI3K and PDK1. Less is known about the means through which Akt regulates cell growth, proliferation, and survival, even though a number of downstream Akt substrates have been identified (Fig. 14.6.1; Chan et al., 1999; Datta et al., 1999; Vanhaesebroeck and Alessi, 2000). While the Akt phosphorylation consensus sequence is defined (RXRXXS/THydrophobic), to date only a small number of Akt substrates have been positively or tentatively identified.

Ongoing studies on Akt signaling and the possibility of using this kinase as a drug target for cancer, diabetes, and stroke, make probing for Akt activity a frequently used task. This can be achieved by measuring Akt kinase activity directly or by assaying the level of phosphorylation as described in this unit. Determination of Akt kinase activity follows the general scheme applicable for most kinases (e.g., see *UNIT 14.3*) and involves immunoprecipitation of the enzyme with antibodies and then performing a phosphorylation reaction in vitro. While this approach offers a direct and relatively accurate determination of Akt activity, the method is laborious and often involves use of radioactivity. Determination of Akt activation state by assaying the level of phosphorylation after specific treatments is quicker and employs basic non-radioactive laboratory techniques. Moreover, the same samples (in some cases the same membrane used for immunoblotting with anti-Akt antibodies) can be used for determination of the activity of upstream Akt regulators and the phosphorylation level of downstream Akt substrates, making this method more versatile than the classical kinase assay.

Critical Parameters and Troubleshooting

Several parameters play critical roles for success in determination of Akt activation and

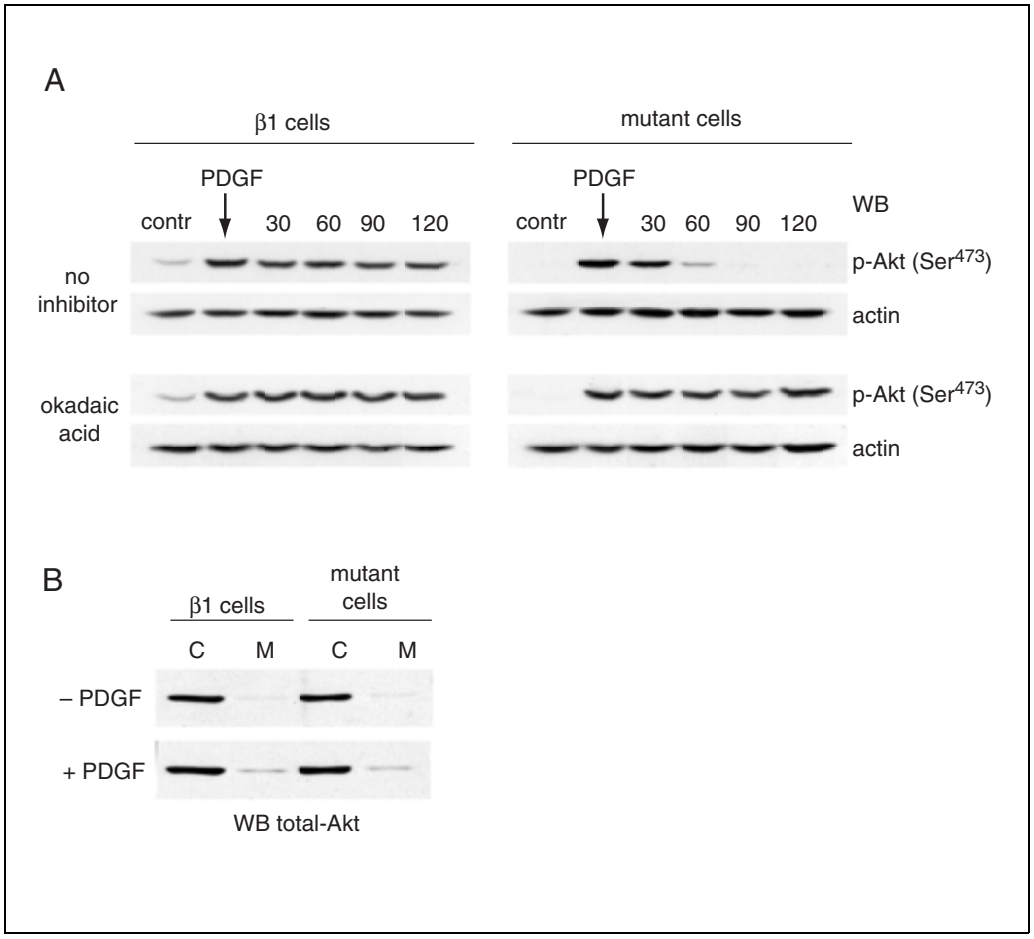


Figure 14.6.2 Probing of Akt signaling by the methods described in this unit. (**A**) Determination of Akt signaling in cultured cells. GD25 cells expressing either wild-type (β1 cells) or tryptophan mutant (mutant cells) integrins were cultured overnight in the absence of serum. Cells were then stimulated with 20 ng/ml PDGF-BB for 15 min, the growth factor was washed away, and the cells were maintained in medium without serum supplemented with 30 μM PDGF kinase inhibitor AG 1433. Samples were taken after starvation (contr), immediately after stimulation (PDGF, ↓), and at the indicated time points. The experiment was performed in the absence (no inhibitor) or presence of 0.5 μM okadaic acid. Lysates from the samples were analyzed by immunoblotting (WB) with anti-phospho Ser^{473} Akt [p-Akt (Ser^{473})] or anti-actin (actin) antibodies. Actin was used as an internal control for loading. (**B**) Determination of Akt translocation to the plasma membrane. Cells were starved overnight and stimulated with 20 ng/ml PDGF (+ PDGF) or left without stimulation (–PDGF) and used to prepare cytoplasmic (C) and membrane (M) fractions. Samples from these fractions were analyzed by immunoblotting with anti-total Akt (WB total-Akt) antibodies. Increased amount of Akt is detected in the membrane fraction of the PDGF stimulated cells.

inhibition. Initial reduction of the phosphorylation level of Akt is achieved by a period of starvation, which varies depending on the type of cells and should be determined experimentally. For some cell lines like primary human fibroblasts, withdrawal from serum for 4 hr is sufficient, while for most immortalized cell lines, longer periods of starvation work better. The length of serum starvation of the chosen cell line needs to be such that it will ensure a five-fold or higher increase of Akt phosphorylation after stimulation with growth factor.

The response to PDGF by different cell lines varies significantly. If the increase of Akt phosphorylation after stimulation is not sufficient, activation with another growth factor, e.g., EGF, may be used. If a different growth factor is applied, the corresponding growth factor receptor (GFR) kinase inhibitor should be added to the medium instead of AG 1433 (e.g., AG 1478 if EGF is utilized). Addition of a growth factor kinase inhibitor is necessary to block residual activity of the receptor after withdrawal of the growth factor. This treatment is critical, since residual GFR activity may

mask the Akt inactivation pattern by prolonging the dephosphorylation time. Experimental blocking of dephosphorylation is achieved by treatment with okadaic acid. Incubations with this potent protein phosphatase inhibitor, especially for prolonged times, may cause cell rounding and even detachment from the substrate. Okadaic acid sensitivity should be determined experimentally for each cell type to be used.

Obtaining a high signal-to-noise ratio after immunoblotting is essential for the successful determination of Akt signaling. This can be ensured by: (1) use of specific antibodies that recognize the phosphorylated Akt but not its unphosphorylated form (now offered by several companies like Cell Signaling, Biosource); (2) use of freshly added phosphatase and protease inhibitors added to the lysis buffers to prevent Akt dephosphorylation and degradation after cell disruption; (3) loading sufficient amount of proteins from the cellular lysate that will ensure trouble-free detection of the Akt by the antibodies (this is easily achieved by keeping the samples for SDS-PAGE concentrated—e.g., $>5 \times 10^5$ cells/minigel lane); (4) following the proper techniques for SDS-PAGE and immunoblotting (see *UNITS 6.1 & 6.2*).

Accurate comparison and quantification by densitometry of the amounts of phosphorylated Akt in different samples can be achieved if the detection system is kept in a linear range. The enhanced chemiluminescence (ECL) system should be optimized to obtain linearity by adjustments of the amount of the protein loaded on the gel, concentrations of primary and secondary antibody, and the X-ray film exposure time. If the weakest signal is detectable and the strongest signal is still within the linear range of the film (e.g., not saturated), then the rest of the samples are also in the linear range of the system, which can be used for quantification.

Anticipated Results

Typical results expected after probing for Akt activity (see Basic Protocol) or Akt membrane translocation (see Support Protocol) are presented in Figure 14.6.2A and B, respectively. Comparison between the starved cells (contr) and cells after PDGF stimulation (PDGF) should demonstrate a several-fold increase in the amount of phosphorylated Akt. Attenuation of this response after experimental manipulations of the cell cultures may indicate effects on the upstream pathways leading to Akt activation. Evaluation of the amount of phosphorylated Akt in the samples taken after PDGF stimulation at different time points provides information about the rate of Akt dephosphorylation (inhibition). While in the samples from β1 cells, this decrease is modest, the Akt in the samples from mutant cells is rapidly dephosphorylated. Such a result indicates activation of some of the systems for Akt inhibition (see Background Information). Performing the same experiment in the presence of a PP2A inhibitor, okadaic acid completely reverses this effect, indicating that activation of this phosphatase is involved in the observed increased dephosphorylation rate in W/A mutant cells. If OA is ineffective, additional experiments should be designed to test the role of other Akt inhibitors (see Background Information). Activation of Akt is dependent on its membrane translocation. A typical increase in membrane-bound Akt after growth factor stimulation is presented in Figure 14.6.2B. A modest but detectable increase in total Akt in the membrane fraction is observed in both cell lines after PDGF stimulation. Failure to detect such translocation may indicate defects in the function of the PH domain of Akt or insufficient phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (see Background Information).

Time Considerations

The entire procedure described in the Basic Protocol can be completed in 3 days. This period includes the time for cell attachment after plating (4 hr); starvation (12 hr); PDGF stimulation and the necessary incubations up to preparation of the SDS-PAGE samples (3 hr); SDS-PAGE and electrotransfer (6.5 hr for mini gels or 9.5 hr for normal size gels); overnight incubation with the primary antibody; and completion of the immunoreactions with ECL processing (4 hr). Since this procedure takes >1 day, it is helpful to use one night for starvation of the cells and the next night for incubation with the primary phosphospecific antibody. There are a number of points where the procedure can be interrupted: (1) after the preparation of the SDS-PAGE samples; (2) after the electrotransfer (membranes can be stored wet or dry in resealable plastic bags at 4°C); and (3) after the completion of the first immunoreaction (membranes can be stored wet in resealable plastic bags at 4°C). A similar timeframe applies for the procedure described in the Support Protocol.

Literature Cited

Andjelkovic, M., Alessi, D.R., Meier, R., Fernandez, A., Lamb, N.J.C., Frech, M., Cron, P., Cohen, P., Lucoc, J.M., and Hemmings, B.A. 1997. Role of translocation in the activation and function of protein kinase B. *J. Biol. Chem.* 272:31515-31524.

Brazil, D.P. and Hemmings, B.A. 2001. Ten years of protein kinase B signalling: A hard Akt to follow. *Trends Biochem. Sci.* 26:657-664.

Chan, T.O., Rittenhouse, S.E., and Tsichlis, P.N. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: Kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* 68:965-1014.

Datta, S.R., Brunet, A., and Greenberg, M.E. 1999. Cellular survival: A play in three Akts. *Genes Dev.* 13:2905-2927.

Fassler, R., Pfaff, M., Murphy, J., Noegel, A., Johansson, S., Timpl, R., and Albrecht, R. 1995. Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. *J. Cell Biol.* 128:979-988.

Franke, T.F., Kaplan, D.R., and Cantley, L.C. 1997. PI3K: Downstream AKT ion blocks apoptosis. *Cell* 88:435-437.

Hemmings, B.A. 1997. Akt signaling: Linking membrane events to life and death decisions. *Science* 275:628-603.

Hill, M.M. and Hemmings, B.A. 2002. Inhibition of protein kinase B/Akt. Implications for cancer therapy. *Pharmacol. Ther.* 93:243-251.

Kobayashi, S., Shirai, T., Kiyokawa, E., Mochizuki, N., Matsuda, M., and Fukui, Y. 2001. Membrane recruitment of DOCK180 by binding to PtdIns(3,4,5)P3. *Biochem. J.* 354:73-78.

Maira, S.M., Galetic, I., Brazil, D.P., Kaech, S., Ingley, E., Thelen, M., and Hemmings, B.A. 2001. Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. *Science* 294:374-380.

Marte, B.M. and Downward, J. 1997. PKB/Akt: Connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem. Sci.* 22:355-358

Meier, R. and Hemmings, B.A. 1999. Regulation of protein kinase B. *J. Recept. Signal Transduct. Res.* 19:121-128.

Millward, T.A., Zolnierowicz, S., and Hemmings, B.A. 1999. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem. Sci.* 24:186-191.

Pankov, R., Cukierman, E., Clark, K., Matsumoto, K., Hahn, C., Poulin, B., and Yamada, K.M. 2003. Specific beta 1 integrin site selectively regulates Akt/protein kinase B signaling via local activation of protein phosphatase 2A. *J. Biol. Chem.* 278:18671-18681.

Persad, S. and Dedhar, S. 2003. The role of integrin-linked kinase (ILK) in cancer progression. *Cancer Metastasis Rev.* 22:375-384.

Scheid, M.P. and Woodgett, J.R. 2003. Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett.* 546:108-112.

Testa, J.R. and Bellacosa, A. 1997. Membrane translocation and activation of the Akt kinase in growth factor-stimulated hematopoietic cells. *Leuk. Res.* 21:1027-1031.

Vanhaesebroeck, B. and Alessi, D.R. 2000. The PI3K-PDK1 connection: More than just a road to PKB. *Biochem. J.* 346:561-576.

Yamada, K.M. and Araki, M. 2001. Tumor suppressor PTEN: Modulator of cell signaling, growth, migration and apoptosis. *J. Cell Sci.* 114:2375-2382.

Contributed by Roumen Pankov
National Institute of Dental
and Craniofacial Research
National Institutes of Health
Bethesda, Maryland

Analyzing FAK and Pyk2 in Early Integrin Signaling Events

Integrins are a family of heterodimeric α/β transmembrane receptors that bind to extracellular matrix proteins (ECM) proteins, and the signals generated by activated integrins regulate cell motility (Schwartz, 2001; Hynes, 2002). As integrins do not possess intrinsic signaling activity, integrin-stimulated signaling events promoting cell motility are initiated by integrin-associated kinases, e.g., focal adhesion kinase (FAK). FAK is one of several intracellular protein-tyrosine kinases (including the Src family, Pyk2, c-Abl, and Syk) that are activated by cell adhesion to ECM proteins such as fibronectin, collagen, or vitronectin. Integrin activation of FAK promotes increased FAK tyrosine phosphorylation and leads to the formation of an FAK-Src signaling complex (Schlaepfer et al., 1999; Schlaepfer and Mitra, 2004). Fibroblasts lacking FAK spread poorly and display migration defects in response to integrin stimuli (Ilic et al., 1995). Although the FAK-related kinase Pyk2 is expressed in FAK-null fibroblasts (FAK−/−), Pyk2 is not as effective in promoting FAK−/− cell motility as the FAK-Src signaling complex (Sieg et al., 1998). This unit describes methods for culturing FAK+/+ and FAK−/− mouse embryo fibroblasts (MEFs; see Support Protocol 1) and stimulating these cells by replating onto fibronectin-coated dishes (see Basic Protocol 1). It also contains support protocols for cell starvation (see Support Protocol 2), preparation of protein cell lysates (see Support Protocol 3), and conditions and antibodies available for immunoprecipitation of FAK, Pyk2, and c-Src (see Support Protocol 4).

Cell replating onto fibronectin activates FAK, and procedures are described for analyzing FAK-associated autophosphorylation activity (see Basic Protocol 2) with alternate protocols describing assays for measuring FAK-Pyk2 phosphorylation of a peptide substrate (see Alternate Protocol 1) and changes in c-Src-associated in vitro kinase activity (see Alternate Protocol 2). These catalytic measurement assays are complemented by methods using antibodies available for evaluating activity changes in FAK, Pyk2, or c-Src using immunoblotting techniques with phospho-specific antibodies (see Basic Protocol 3). In addition, the biochemical analyses are complemented by cell biological methods to evaluate integrin-stimulated haptotaxis migration (see Basic Protocol 4) and time-lapse imaging to monitor wound closure motility in culture (see Basic Protocol 5). In these cell motility assays, transient plasmid expression vector transfection can be used to overexpress various proteins to test their effects on cell motility (Alternate Protocol 3). Finally, protocols are provided for visualizing activated FAK, Pyk2, and c-Src in cells plated onto extracellular matrix proteins (see Basic Protocol 6) with support methods for staining filamentous actin in cells (see Alternate Protocol 4), an easy means to visualize changes in cell shape and indirectly localize integrin-containing focal adhesions that are found at the ends of actin stress fibers.

NOTE: All culture incubations should be performed in a humidified 37°C, 10% CO_2 incubator unless otherwise specified. Some media, e.g., DMEM, require higher levels of CO_2 to maintain pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

Contributed by Joie A. Bernard-Trifilo, Ssang-Taek Lim, Shihe Hou, Dusko Ilic, and David D. Schlaepfer

Current Protocols in Cell Biology (2006) 14.7.1-14.7.35

BASIC PROTOCOL 1

REPLATING ASSAYS FOR SIGNALING STUDIES

Although anti-integrin antibodies can be used to facilitate integrin clustering, one of the strongest activators of integrin signaling is replating or passaging cells onto ECM-coated culture dishes. In the span of 1 hr, cells will rapidly bind to the ECM protein provided and will undergo cell spreading and morphology changes. Fibronectin binds to variety of α/β integrin pairs expressed on cells and is the strongest activator of FAK. Other ECM proteins can be used in replating assays, although the effective concentrations needed to stimulate integrin signaling are varied and dependent on cell type. This protocol describes the optimal conditions for replating FAK+/+ and FAK−/− cells on fibronectin to achieve maximal FAK or Pyk2-Src activation, respectively. It also provides a method for collecting protein lysates from these cells for further signaling studies. For comparative purposes, cell lysates are collected from adherent serum-starved cells, cells that have been held in suspension, cells replated onto fibronectin, and cells that have been replated onto a positively-charged ligand such as poly-L-lysine where rapid cell adhesion does not depend on integrin engagement.

Materials

Fibronectin (from bovine plasma; Sigma-Aldrich)
Poly-L-lysine (mol. wt. 70,000 to 150,000 or 150,000 to 300,000; Sigma-Aldrich)
Replating and migration medium (see recipe)
FAK+/+ and FAK−/− MEFs, serum-starved (Support Protocol 2)
Phosphate buffered saline (PBS; *APPENDIX 2A*)
Trypsin/EDTA: 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen)
Trypsin inhibitor solution (see recipe)

10-cm plastic tissue culture plates (Falcon)
4°C incubator
15-ml centrifuge tubes (Corning)
Tabletop centrifuge
10-ml transfer pipets, sterile
50-ml conical centrifuge tubes (Corning)
Light microscope

Coat plates and prepare cells

1a. *To prepare fibronectin plates:* Dilute fibronectin to 10 μg/ml in PBS and distribute 5 ml per 10-cm plastic tissue culture plate. Incubate at 4°C overnight.

1b. *To prepare poly-L-lysine plates:* Dilute poly-L-lysine to 50 μg/ml in PBS and distribute 5 ml per 10-cm plastic tissue culture plate. Incubate at 4°C overnight.

2. Prior to preparing cells, aspirate fibronectin or poly-L-lysine solutions, add 3 ml of replating and migration medium, aspirate, and place dishes at 37°C.

   The number of cell plates prepared depends on the number of experimental points to be evaluated. For example, to perform a time course of FAK activation, it is recommended that fibronectin and poly-L-lysine replating time points of 20, 40, 60, and 180 min be performed (see Support Protocol 3).

3. Reserve one plate of serum-starved cells for a control. Wash serum-starved cells with PBS by adding 10 ml PBS and aspirating it off with a transfer pipet. Add 2.5 ml trypsin/EDTA per dish. Tap side of plates to facilitate cell release.

4. Add 5 ml trypsin inhibitor solution warmed to 37°C. Transfer cells to a 15-ml centrifuge tube. Centrifuge 3 min at 750 × *g*, 21°C, in a tabletop centrifuge and aspirate supernatant.

Replate cells

5. Resuspend cells in 37°C replating and migration medium to 2×10^5 cells/ml. Keep cells in suspension at least 30 min and rotate tube gently at least every 5 min to keep cells from clumping, settling, and sticking to the plastic.

   It is convenient to pool all suspended cells into 50-ml conical centrifuge tubes such that subsequent cell distribution in replating assays is consistent. FAK−/− cells will sometimes clump together in suspension and forced pipetting to break apart clumps is recommended prior to replating. Keep additional cells in suspension during replating.

6. Add 5 ml cell suspension to a 10-cm plate pre-coated with fibronectin or poly-L-lysine (from step 3) and incubate at 37°C.

7. Periodically visualize replated cells under a microscope for changes in cell attachment and spreading.

   Maximal FAK activation occurs when cells have bound to fibronectin and are undergoing rapid cell spreading (between 20 and 60 min). Cells on poly-L-lysine will adhere, but will be very slow to spread.

8. Proceed with Support Protocol 3 to prepare cell lysates of control cells (adherent serum-starved cells), experimental cells (replated on fibronectin or poly-L-lysine), and unplated serum-starved cells in suspension (45 min post replating).

   The unplated serum-starved cells in suspension and serum-starved experimental cells will be very important in correctly evaluating changes in FAK activation in immunoblotting (Basic Protocol 3) and in vitro kinase assays (Basic Protocol 2).

GROWTH OF FAK+/+ AND FAK−/− FIBROBLASTS

SUPPORT PROTOCOL 1

FAK-null MEFs were derived from an E8.0-day-old mouse embryo that had null mutations in genes for FAK and p53 (Ilic et al., 1995). Therefore, the cells are genetically FAK−/− p53−/−. Control FAK+/+ cells were derived in 1995 from an E8.0-day-old mouse embryo that had null mutations in p53 but not in FAK. Therefore, the cells are genetically FAK+/+ p53−/−. Both cell lines were derived from embryos of littermates obtained by crossing FAK+/− p53−/− female and FAK+/− p53−/− male mice.

FAK+/+ and FAK−/− MEFs are grown on gelatin precoated plates because this was used during cell outgrowth from embryos. Although the fibroblasts are p53-null, the cells will become senescent (exhibited by an increase in cell size and the lack of cell proliferation) at high passage numbers.

Materials

Gelatin
Phosphate buffered saline (PBS; *APPENDIX 2A*)
FAK+/+ mouse embryo fibroblasts (ATCC CRL-2645) FAK−/− mouse embryo fibroblasts (ATCC CRL-2644): grown on plates as per ATCC product sheet
Trypsin/EDTA: 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen), 37°C
Cell growth medium (see recipe), warmed to 37°C
42°C water bath
0.22-μm GP Express Plus filter membrane (Millipore)
10-cm plastic tissue culture plates (Falcon)
10-ml transfer pipets, sterile
Light microscope
15-ml conical centrifuge tubes (Corning)

Prepare gelatin-coated plates

1. Dissolve 0.5 g gelatin in 500 ml PBS to make 0.1% (w/v) solution.
2. Heat in 42°C water bath to dissolve gelatin.
3. Sterilize by passing through a Millipore 0.22-μm GP Express Plus filter membrane. Store up to 2 weeks at 4°C.
4. Coat 10-cm plastic tissue culture plates with 5 ml sterile 0.1% gelatin solution and incubate 1 hr at 37°C
5. Aspirate gelatin solution. Do not wash plates.

Trypsinize cells

6. Aspirate growth medium from a 10-cm plate of FAK−/− or FAK+/+ mouse embryo fibroblasts.
7. Wash cells with 5 ml PBS.
8. Add 2.5 ml warm trypsin/EDTA per plate. Tap side of plates to facilitate cell release.
9. Incubate 2 to 3 min. Verify cell detachment under a microscope.

   FAK−/− cells adhere to the culture plates more firmly than FAK+/+ cells and will take longer to be released by trypsin/EDTA treatment. Senescent FAK−/− cells exhibit very strong adhesion to gelatin-coated dishes.

10. Inactivate trypsin/EDTA by adding 5 ml cell growth medium.

Replate cells

11. Wash cells off plates by pipetting, and transfer them into 15-ml conical centrifuge tubes.
12. Centrifuge cells in a tabletop centrifuge 3 min at 750 × *g*, 21°C.
13. Aspirate supernatant and discard, retaining the pellet.
14. Resuspend cells in fresh growth medium, count (see *UNIT 1.1*), and add growth medium to obtain the desired density. Add to gelatin-coated plates containing cell growth medium.

    Total volume is 8 to 10 ml medium per 10-cm plate.

    Consistent subcultivation is very important as cells that remain confluent for more than 24 hr exhibit alterations in cell morphology and motility properties. In routine passage, FAK−/− cells may proliferate faster than FAK+/+ cells, so cell counting and normalization of cell numbers is required for equivalent experimental analyses.

    Both FAK−/− and FAK+/+ cells contain neomycin resistance genes and G418 (neomycin) selection can be applied (500 μg/ml), although this is not absolutely required.

15. Passage the cells when they are confluent.

    Split cells 1:10 to 1:15 every 3 days. Do not allow cells to become overly confluent, as this can result in a premature senescent phenotype.

SUPPORT PROTOCOL 2

SERUM STARVATION OF CELLS

For the stimulation and analysis of FAK activity and phosphorylation (Basic Protocols 1, 2, and 3) and assays that involve cell replating onto extracellular matrix (ECM) proteins (Basic Protocols 4, 5, and 6), it is important to start with cells that are adherent and in a quiescent or nonproliferative state. This is accomplished by lowering the percent of serum to 0.5% in the growth medium.

Additional Materials *(also see Support Protocol 1)*

Starvation medium: prepared by making cell growth medium (see recipe) with FBS reduced to 0.5%.

1. Passage cells (see Support Protocol 1) by plating 1×10^6 cells onto gelatin-coated 10-cm dishes in growth medium.

   This is a subconfluent density, and it is important that cells do not reach confluency prior to initiation of cell motility analyses.

2. After 24 hr, wash cells with PBS.

3. Add 8 ml of starvation medium and incubate 16 to 24 hr at 37°C. Use cells immediately after starvation.

   Greater than 95% of FAK−/− and FAK+/+ cells should remain adherent under serum-starvation conditions.

PREPARATION OF CELL LYSATES

SUPPORT PROTOCOL 3

Activated FAK is associated with the cytoskeleton-rich fraction of cells that is not easily solubilized with low-detergent-containing buffers. The following lysate preclearing steps are used as the first step for immunoprecipitation, blotting, and in vitro kinase assays. All procedures are to be performed at 4°C.

Materials

Experimentally treated, control adherent, and control suspended cells (Basic Protocol 1)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), 4°C
RIPA cell lysis buffer (see recipe), 4°C
50% (w/v) Sephadex G-100 (Sigma G100-120) slurry in PBS (*APPENDIX 2A*)
Cell scraper (Corning)
Refrigerated centrifuge
1.5-ml microcentrifuge tubes
1-ml disposable syringes
21-G Luer-Lok needles
Tube rotation device (e.g., Labquake, Barnstadt-Thermolyne)
Microcentrifuge
−80°C freezer (optional)

1. Wash experimentally treated and adherent control cells with 10 ml cold PBS.

2. At 45 min after replating the experimental cells (see Basic Protocol 1), centrifuge control suspended cells 3 min at 750 × *g*, 21°C. Resuspend cells in PBS and repeat centrifugation.

3. Add 750 µl cold RIPA cell lysis buffer to each 10-cm plate (or 1×10^6 suspended cells).

4. Scrape adherent cells using cell scraper and transfer to a 1.5-ml microcentrifuge tube.

5. Using a 1-ml disposable syringe and 21-G Luer-Lok needle, pull cell lysate into syringe and shear cellular DNA by extrusion. Repeat at least five times.

   Be careful using the sharp needle and do not use too much force as this will result in excessive bubbling of the lysate.

6. Add 100 µl 50% Sephadex G-100 slurry in PBS to each tube. Rotate tubes 10 min at 4°C.

   Sephadex G-100 beads will reduce DNA contamination and non-specific protein binding for subsequent immunoprecipitation analyses.

7. Centrifuge 10 min at 16,000 × *g*, 4°C.

8. Transfer supernatant (cleared total cell lysate) into new 1.5-ml microcentrifuge tubes.

   If the bead pellet is distributed along the sidewall of the microcentrifuge tubes, rotate tube direction 180° in centrifuge rotor and centrifuge an additional 5 min.

9. Store cleared lysate indefinitely at −80°C or proceed with subsequent analyses.

SUPPORT PROTOCOL 4

IMMUNOPRECIPITATION OF FAK, Pyk2, AND c-Src

FAK is expressed at high levels in FAK+/+ fibroblasts. The FAK-related kinase, Pyk2, is expressed at low levels in FAK+/+ fibroblasts and at high levels in FAK−/− fibroblasts. Upon replating on fibronectin, both FAK and Pyk2 will form transient signaling complexes with the c-Src protein-tyrosine kinase. Replating of cells on fibronectin can also lead to the activation of c-Src through dephosphorylation of Tyr-527 located in the C-terminal domain of the regulatory region. This protocol describes standard immunoprecipitation methods used to isolate FAK, Pyk2, or multi-protein complexes with c-Src.

Materials

Primary antibody (see Table 14.7.1 and Table 14.7.2)
Lysate of treated or control cells (Support Protocol 3)
50% (w/v) protein A-agarose beads (fast flow immobilized protein A; Repligen, *http://www.repligen.com*) in PBS (*APPENDIX 2A*)
50% (w/v) protein G-Plus-agarose beads (Calbiochem) in PBS (*APPENDIX 2A*)
Triton lysis buffer (see recipe), 4°C
HNTG buffer (see recipe), 4°C
1.5-ml microcentrifuge tubes
Tube rotation device (e.g., Labquake, Barnstadt-Thermolyne)
Centrifuge
Transfer pipets

1. Add 1 to 2 µg of primary antibody to 0.75 ml lysate of treated or control cells in a microcentrifuge tube.

   If using cells from a frozen lysate, thaw at 37°C then maintain at 4°C.

   Use a FAK or Pyk2 antibody for subsequent analysis of FAK/Pyk2 kinase activity or phosphorylation. (Basic Protocol 2 or Alternate Protocol 1).

   Use an Src antibody for subsequent Src-associated analysis (Alternate Protocol 2).

2. Rotate from 3 to 14 hr at 4°C.

3. Add 30 µl of protein A– or protein G–bead slurry and rotate 60 min at 4°C.

   Protein A beads are used to capture rabbit polyclonal antibodies and protein G beads are used to bind goat polyclonal and mouse monoclonal antibodies (Table 14.7.1 and Table 7.2.1).

4. Centrifuge beads 1 min at 1000 × g, 4°C. Remove supernatant with a transfer pipet.

5. Add 800 µl Triton lysis buffer to beads, invert tube to suspend the bead pellet, and centrifuge 1 min at 1000 × *g*, 4°C. Remove supernatant with a transfer pipet and repeat step two more times.

6. Add 800 µl of HNTG buffer, invert the tube to suspend the bead pellet, and centrifuge 1 min at 1000 × *g*, 4°C. Remove supernatant with a transfer pipet and repeat step two more times.

From this point, the immunoprecipitated samples can be analyzed in either the in vitro kinase assay (Basic Protocol 2) or resolved by SDS-PAGE (UNIT 6.1) and processed by immunoblotting (UNIT 6.2 and Basic Protocol 3).

IN VITRO KINASE ASSAY

BASIC PROTOCOL 2

Integrin binding to fibronectin leads to rapid cell spreading, and FAK, Pyk2, and c-Src tyrosine kinases become activated. The process can be characterized through changes associated with in vitro kinase activity. Assays are designed to detect auto-phosphorylation or the intramolecular phosphorylation of an added substrate such as a synthetic poly (Glu:Tyr) peptide for FAK and Pyk2, or the phosphorylation of a recombinant fragment of the FAK C-terminal domain by c-Src.

Materials

Immunoprecipitated samples (Support Protocol 4, step 6)
FAK-Pyk2 kinase buffer (see recipe), 4°C
Src kinase buffer (see recipe), 4°C
10 µCi/µl [γ^{32}P]ATP (>3,000 Ci/mmol; PerkinElmer)
Magnesium/ATP cocktail (Upstate Biotechnology or see recipe)
2× Laemmli SDS buffer (see recipe)
Coomassie blue stain (see recipe)
Molecular weight marker (Precision Plus, Bio-Rad)
Destaining solution (see recipe)

Microcentrifuge
1.5-ml microcentrifuge tubes
32°C water bath
Plexiglas shielding
Gloves
Geiger counter
Plexiglas box
Micro tube cap locks (RPI 145063; *http://www.rpicorp.com*)
Whatman 3MM filter paper
Gel dryer
Immobilon PVDF membrane (Millipore IPFL 000-10)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), autoradiography (*UNIT 6.3*), and transfer of proteins to membranes for immunoblotting (*UNIT 6.2*)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

1. Wash the immunoprecipitated samples (Support Protocol 4, step 6) with 800 µl cold FAK-Pyk2 kinase buffer two times, centrifuging 2 min at 16,000 × *g*, 4°C, each time.
2. Aspirate supernatant, but leave a small amount above pellet (~20 to 30 µl).
3. For FAK-Pyk2 autophosphorylation assays, add 2.5 µl of 10 µCi/µl [γ^{32}P] ATP and 5 µl magnesium/ATP cocktail to the immune complexes. Slightly flick tubes to mix and place in 32°C water bath for 15 min.

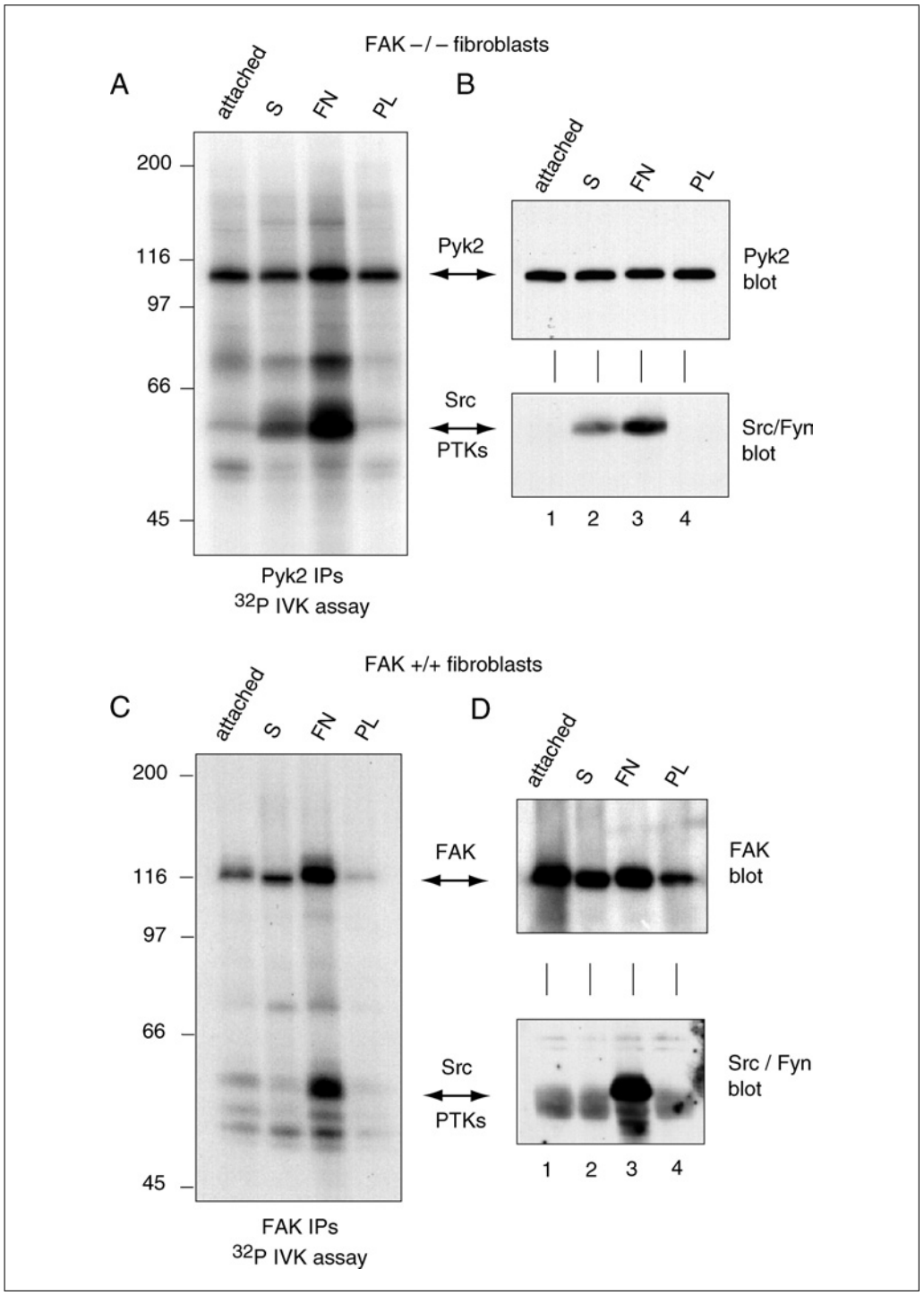


Figure 14.7.1 Measurements of Pyk2 of FAK-associated in vitro kinase activity. Lysates from serum-starved (attached), suspended (S) fibronectin-plated (FN), and poly-L-lysine-plated (PL) FAK$-/-$ cells (A and B) or FAK$+/+$ cells (C and D) were prepared and divided into equal aliquots for either Pyk2 immunoprecipitates (IPs) or FAK IPs, respectively. (**A**) Pyk2 IPs were labeled by the addition of [γ-^{32}P]ATP in an in vitro kinase (IVK) assay. ^{32}P-labeled proteins were transferred to membranes and visualized by autoradiography. (**B**) The same membrane shown in (A) was cut and analyzed by either anti-Pyk2 or anti-Src family PTK blotting. (**C**) FAK IPs were labeled by the addition of [γ-^{32}P]ATP in an IVK assay and the same membrane (**D**) was cut and analyzed by either anti-FAK or anti-Src family protein-tyrosine kinase (PTK) immunoblotting. Molecular weight standards are indicated in kDa to the left. (previously published in the *EMBO Journal*; Sieg et al., 1998)

CAUTION: *Place Plexiglas shielding around gel area to prevent radiation exposure. Remember to check gloves and work areas with a Geiger counter after the kinase assay is done. Use a Plexiglas box for moving radioactive materials within the laboratory and dispose of radioactive waste into designated storage according to institutional requirements.*

4. Add 50 µl 2× Laemmli SDS buffer to stop kinase reactions. Secure 1.5-ml tubes with micro tube cap locks.
5. Boil samples for 3 min at 100°C.
6. Microcentrifuge agarose beads 2 min at 16,000 × *g*, 21°C. Load supernatants and molecular weight marker onto a 7.5% SDS-PAGE gel (see *UNIT 6.1*).

For autoradiography

7a. After gel is run (~4 hr), cut one top corner to mark orientation. Place gel in Coomassie blue stain for 1 hr and then destain gel for 6 to 12 hr in multiple changes of destaining solution on a shaking platform. Wash a final time in water.

8a. Place gel onto Whatman 3MM filter paper and dry using a gel dryer.

9a. Expose gel to film or phosphor-imager screen (*UNIT 6.3*) to quantify ^{32}P incorporated into FAK (~116 kDa), Pyk2 (~110 kDa) or associated c-Src (~60 kDa).

See Fig. 14.7.1 A and C.

For autoradiography and subsequent immunoblotting

7b. Transfer the gel to a PVDF membrane via semidry electrophoretic transfer (see *UNIT 6.2*).

8b. Stain min with Coomassie blue stain. Destain 10 min in destaining solution and wash a final time in water.

9b. Place membrane between protective plastic sheets and expose gel to film or phosphor-imager screen to quantify ^{32}P incorporated into FAK (~116 kDa), Pyk2 (~110 kDa) or associated c-Src (~60 kDa). See *UNIT 6.3* for more information.

In this manner, subsequent immunoblotting (see Basic Protocol 3) can be performed on the kinase reactions to verify the amount of FAK or Pyk2 in the immune complex or the amount of c-Src associated with the activated FAK and/or Pyk2 complex reaction (see Fig. 14.7.1B and D).

FAK-PYK2 POLY GLU:TYR PHOSPHORYLATION

ALTERNATE PROTOCOL 1

This procedure is used to measure the autophosphorylation of FAK/Pyk2 (as in Basic Protocol 2) combined with the transphosphorylation of an added generic substrate peptide.

Additional Materials *(also see Basic Protocol 2)*

Immunoprecipitated samples (see Support Protocol 4, step 6)
10 mg/ml poly(Glu:Tyr; 4:1) mol. wt. 20,000 to 50,000: sodium salt (Sigma-Aldrich) prepared in PBS and stored up to 2 years at −20°C in 1-ml aliquots
Magnesium/ATP cocktail (Upstate Biotechnology or see recipe)
10 µCi/µl [$\gamma^{32}P$]ATP (>3,000 Ci/mmol; PerkinElmer)
FAK-Pyk2 kinase buffer
0.75% phosphoric acid
100% acetone
Scintillation fluid (Sigma)
2 × 2–cm Whatman 3MM filter paper squares

Conical 50-ml centrifuge tube
Scintillation vials
Scintillation counter

Additional reagents and equipment for preparing immunoprecipitated samples (Support Protocol 4)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

1. Wash the immunoprecipitated samples (from Support Protocol 4, step 6) with 800 µl cold FAK-Pyk2 kinase buffer two times, centrifuging 2 min at 16,000 × g, 4°C, each time.
2. Remove supernatant, but leave a small amount above pellet (~20 to 30 µl).
3. Add 5 µl 10 mg/ml poly(Glu:Tyr), 5 µl magnesium/ATP cocktail, and 2.5 µl of 10 µCi/µl [γ^{32}P]ATP (25 µCi) to the immunoprecipitated samples. Slightly flick tubes to mix and place in 32°C water bath for 15 min. Include a negative control for the phosphorylation assay, substituting 35 µl kinase buffer for the immunoprecipitated samples.

   CAUTION: *Place Plexiglas shielding around gel area to prevent radiation exposure. Remember to check gloves and work areas with a Geiger counter after the kinase assay is done. Use a Plexiglas box for moving radioactive materials within the laboratory and dispose of radioactive waste into designated storage according to institutional requirements.*

   The negative control will measure the level of background binding of [^{32}P ATP] to the assay squares.

   The total volume of immune complex reaction is ~50 µl yielding a final concentration of 50 µM ATP from the magnesium/ATP cocktail and 1 mg/ml poly Glu:Tyr.

4. Transfer a 10-µl aliquot onto the center of a labeled (with a #2 pencil) 2 × 2–cm Whatman filter paper square.

   There should be enough volume for triplicates for each immune complex reaction.

5. Allow the radiolabeled substrate to bind to filter paper for at least 1 min. Transfer filter papers to a 50-ml conical tube containing 40 ml 0.75% phosphoric acid. Gently invert tube to wash the assay squares for 5 min. Repeat three times and discard liquid as radioactive waste.

   Ten or more assays squares can be washed per 50-ml tube.

6. Wash assay squares once with 40 ml of 100% acetone for 5 min. Discard liquid waste and transfer assay squares to scintillation vial. Add scintillation fluid and evaluate using a scintillation counter.

   For information on creating a standard curve, see http://www. perkinelmer.com.

ALTERNATE PROTOCOL 2

MEASUREMENTS OF Src-ASSOCIATED KINASE ACTIVITY

As c-Src contains both stimulatory (Tyr-416) and inhibitory (Tyr-527) phosphorylation sites that act to regulate catalytic activity, measurements to analyze changes in c-Src-associated kinase activity are performed by c-Src immune complex phosphorylation of an FAK C-terminal domain fragment that contains the known c-Src phosphorylation sites, FAK Tyr-861 and FAK Tyr-925.

Additional Materials *(also see Basic Protocol 2)*

10 mg/ml GST-FAK 853-1052 (Schlaepfer lab)
Enolase (Sigma-Aldrich E-0379), optional
50 mM HCl, optional
1 M PIPES, pH 7, optional
30°C water bath, optional

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

1. Wash the immunoprecipitated samples (from step 6 in Support Protocol 4) with 800 µl cold FAK-Pyk2 kinase buffer two times, centrifuging 2 min at 16,000 × *g*, 4°C, each time.
2. Remove supernatant, but leave a small amount above pellet (~20 to 30 µl)
3. Add 5 µl of 10 mg/ml GST-FAK 853-1052, 5 µl magnesium/ATP cocktail, and 2.5 µl of 10 µCi/µl [γ^{32}P]ATP (25 µCi) to the immunoprecipitated samples. Slightly flick tubes to mix and place in 32°C water bath for 15 min.

   CAUTION: *Place Plexiglas shielding around gel area to prevent radiation exposure. Remember to check gloves and work areas with a Geiger counter after the kinase assay is done. Use a Plexiglas box for moving radioactive materials within the laboratory and dispose of radioactive waste into designated storage according to institutional requirements.*

   The total volume of immune complex reaction is ~50 µl yielding a final concentration of 50 µM ATP from the magnesium/ATP cocktail and 1 mg/ml GST-FAK 853-1052.

   Alternatively, acid-denatured enolase may be used as a substrate. Enolase must be freshly prepared: Add 10 µl of enolase (1 mg/ml) to 2 µl of 50 mM HCl and incubate 10 min at 30°C. Neutralize with 2 µl 1 M PIPES, pH 7.0, and use 2 µl acid-denatured enolase per kinase reaction.
4. Proceed with Basic Protocol 2, steps 4 through 9 to perform SDS-PAGE and autoradiography or autoradiography and immunoblotting.

IMMUNOBLOTTING WITH FAK/PYK2 PHOSPHO-SPECIFIC ANTIBODIES

BASIC PROTOCOL 3

While in vitro kinase assays will reflect changes in catalytic activity, immunoblotting with FAK/PYK2 phospho-specific antibodies can be used to assess specific phosphorylation site modification(s) within the kinases. For example, FAK and Pyk2 first become phosphorylated at their autophosphorylation sites Y397 and Y402 respectively. Upon phosphorylation and binding of c-Src in an active signaling complex, c-Src can phosphorylate other sites on FAK and Pyk2. Additionally, there are several serine-threonine sites on FAK that can be phosphorylated by other cellular kinases. Finally, phospho-specific antibodies have been developed to indirectly measure the activation state of c-Src.

Materials

2× Laemmli SDS buffer (see recipe)
Immunoprecipitated samples (Support Protocol 4, step 6)
100% and 20% methanol
Transfer buffer (see *UNIT 6.2*)
EZBlue gel staining reagent (Sigma)
BSA blocking buffer (see recipe)
Primary antibody (see Tables 14.7.1 and 14.7.2)

Tris-buffered saline with Tween (TBST, see recipe)

Secondary antibody: Horseradish peroxidase (HRP)-conjugated appropriate species (Pierce)

Enhanced chemiluminescence (ECL) western detection solution (Amersham RPN 2132)

Western blot stripping buffer (see recipe)

Immobilon PVDF membrane (Millipore)

Rotating shaker

Additional reagents and equipment for gel electrophoresis (*UNIT 6.1*) and electrophoretic transfer (*UNIT 6.2*)

Perform gel electrophoresis

1. Add 50 µl of 2× Laemmli SDS buffer to the immunoprecipitated samples from Support Protocol 4, step 6 and boil samples 3 min at 100°C.
2. Centrifuge 2 min at 16,000 × *g*, 21°C. Using the supernatants perform SDS-PAGE (*UNIT 6.1*), including molecular size markers.

Transfer proteins to PVDF membrane

3. Transfer proteins to a PVDF membrane using an electrophoretic transfer unit (*UNIT 6.2*).

   PVDF membrane should be prepared by soaking in 100% methanol followed by the appropriate transfer buffer before use.

4. After transfer is complete, stain protein bands with EZBlue gel staining reagent. Rinse membrane in water and destain in 20% methanol until background staining disappears.
5. Photocopy membrane for record keeping purposes and to note positions of protein size markers.

Probe membrane

6. Place membrane in BSA blocking buffer for 1 hr at room temperature.
7. Remove blocking buffer and incubate membranes with primary antibodies 3 hr at room temperature or overnight at 4°C.

   A 1:1000 dilution (~0.5 µg/ml in BSA blocking buffer) is a good starting point for using an antibody for the first time.

8. Wash membranes by rotational shaking with TBST for 10 min. Repeat three times using fresh TBST.
9. Dilute secondary antibody 1:5000 to 1:10,000 in TBST (e.g., 5 µl in 15 ml) and incubate membranes with the antibody 1 hr at room temperature.

   HRP-conjugated secondary antibodies commonly used are: goat anti-mouse IgG, Protein A, donkey anti-rabbit IgG, and mouse anti-goat IgG.

10. Wash membranes by rotational shaking with TBST for 10 min. Repeat three times using fresh TBST.
11. Incubate in 5 ml ECL solution for 5 min at 21°C. Expose to film 30 sec to 10 min to achieve optimal exposure.

    Although many phospho-specific antibodies can be used to probe FAK, Pyk2, or c-Src activation states in whole cell lysates, multiple immunoreactive bands detected can complicate the interpretation of results. This is not a problem when analyzing FAK, Pyk2, or c-Src by immunoprecipitation.

Table 14.7.1 Commercially Available Antibodies to FAK, Pyk2, and c-Src[a]

Antibody	Company	Source	Application	Catalog number
FAK, clone 4.47	Upstate	mouse (mAb)	WB, IP, IC, IH	05-537
FAK clone 2A7	Upstate	mouse (mAb)	IP, IC	05-182
FAK	Upstate	rabbit	WB, IP, IH	06-543
FAK, BC3	Upstate	rabbit	IP, IC	06-446
FAK	BioSource	rabbit	IP, WB	AHO0502
FAK	BD PharMingen	rabbit	WB, IP	556368
FAK	BD Transduction	mouse (mAb)	WB, IP, IC, IH	610087
FAK	Cell Signaling	rabbit	WB, IH	3285
FAK	BioSource	rabbit	WB, IP, IC	AH0502
FAK	BioSource	rabbit	WB, IP, IC	AMO0672
FAK	Chemicon	rabbit	IP	AB1605
FAK	Chemicon	mouse (mAb)	WB, IP, IC	MAB2156
FAK (H-1)	Santa Cruz	mouse (mAb)	WB, IP, IC, IH	sc-1688
FAK (A-17)	Santa Cruz	rabbit	WB, IP, IC	sc-557
FAK (C-20)	Santa Cruz	rabbit	WB, IP, IC	sc-558
FAK (C-903)	Santa Cruz	rabbit	WB, IP, IC, IH	sc-932
Pyk2	Upstate	rabbit	WB, IP, IC	06-559
Pyk2, clone 74	Upstate	mouse (mAb)	WB, IP	05-488
Pyk2	Upstate	rabbit	WB, IP, IC	07-437
Pyk2	Cell Signaling	rabbit	WB, IP	3292
Pyk2	BD Transduction	mouse (mAb)	WB, IP, IC, IH	610548
Pyk2 (H-102)	Santa Cruz	mouse (mAb)	WB, IP, IC	sc-9019
Pyk2 (N-19)	Santa Cruz	rabbit	WB, IP, IC	sc-1514
Pyk2 (C-19)	Santa Cruz	rabbit	WB, IP, IC	sc-1515
c-Src, clone GD11	Upstate	mouse	WB, IP	05-184
c-Src, clone N6L	Upstate	rabbit (mAb)	WB	05-889
c-Src, clone NL19	Upstate	rabbit (mAb)	WB, IP	05-772
c-Src	BioSource	mouse (mAb)	WB	AHO1152
c-Src	BioSource	rabbit	WB	44-655G
c-Src	BioSource	rabbit	WB	44-656G
c-Src	Chemicon	sheep	WB, IP	CB769
c-Src, clone 36D10	Cell Signaling	rabbit (mAb)	WB, IP, IC, IH	2109
c-Src, clone L4A1	Cell Signaling	mouse (mAb)	WB, IP	2110
c-Src	Cell Signaling	rabbit	WB, IP, IC, IH	2108
c-Src (H-12)	Santa Cruz	mouse (mAb)	WB, IP, IC	sc-5266
c-Src (B-12)	Santa Cruz	mouse (mAb)	WB, IP, IC	sc-8056
c-Src (N-16)	Santa Cruz	rabbit	WB, IP, IC	sc-19
c-Src (Src 2)	Santa Cruz	rabbit	WB, IP, IC	sc-18

[a]Abbreviation: mAb, monoclonal antibody; WB, western (immuno)blot; IP, immunoprecipitation; IC, immunocytochemistry; IH, immunohistochemistry.

Table 14.7.2 Commercially Available Phospho-Specific Antibodies to FAK, Pyk2, and c-Src[a]

Antibody	Company	Source	Application	Catalog number
FAK pY397	BioSource	rabbit	WB, IC, IH	44-624G
FAK pY397 clone 141-9	BioSource	rabbit (mAb)	WB, IC	44-625G
FAK pY407	BioSource	rabbit	WB, IC, IH	44-650G
FAK pY576	BioSource	rabbit	WB	44-652G
FAK pY577	BioSource	rabbit	WB, IC	44-614G
FAK pS722	BioSource	rabbit	WB	44-588
FAK pS732	BioSource	rabbit	WB	44-590G
FAK pS843	BioSource	rabbit	WB	44-594
FAK pY861	BioSource	rabbit	WB, IC	44-626G
FAK pS910	BioSource	rabbit	WB	44-596
FAK pY397 clone 14	BD/Transduction	mouse (mAb)	WB, IC	611722
FAK pY397 clone 18	BD/Transduction	mouse (mAb)	WB, IC	611806
FAK pY576/pY577	Cell Signaling	rabbit	WB	3281
FAK pY397	Santa Cruz	rabbit	WB, IC	sc-11765-R
FAK pY397	Santa Cruz	rabbit	WB, IC	sc-21868-R
FAK pY407	Santa Cruz	goat	WB, IC	sc-16664
FAK pY576	Santa Cruz	rabbit	WB, IC	sc-16563-R
FAK pY477	Santa Cruz	goat	WB, IC	sc-16665
FAK pY576/pY577	Santa Cruz	rabbit	WB, IC	sc-21831-R
FAK pY576/pY577	Santa Cruz	goat	WB, IC	sc-21831
FAK pS722	Santa Cruz	goat	WB, IC	sc-16662
FAK pY861	Santa Cruz	goat	WB, IC	sc-16663
FAK pS910	Santa Cruz	goat	WB, IC	sc-16666
FAK pY925	Santa Cruz	goat	WB, IC	sc-11766
FAK pY397	Chemicon	mouse (mAb)	WB, IC	MAB1144
FAK pY397	Upstate	rabbit	WB, IC	07-012
FAK pY576	Upstate	rabbit	WB, IC	07-157
Pyk2 pY402	BioSource	rabbit	WB, IH	44-618G
Pyk2 pY579	BioSource	rabbit	WB, IC	44-632G
Pyk2 pY579/pY580	BioSource	rabbit	WB	44-636G
Pyk2 pY580	BioSource	rabbit	WB	44-634G
Pyk2 pY881	BioSource	rabbit	WB, IH	44-620
Pyk2 pY402	Cell Signaling	rabbit	WB, IP	3291

continued

Table 14.7.2 Commercially Available Phospho-Specific Antibodies to FAK, Pyk2, and c-Src[a], *continued*

Antibody	Company	Source	Application	Catalog number
Pyk2 pY402	Santa Cruz	rabbit	WB, IC	sc-11767-R
Pyk2 pY579	Santa Cruz	goat	WB, IC	sc-16822
Pyk2 pY579/pY580	Santa Cruz	goat	WB, IC	sc-16824
Pyk2 pY580	Santa Cruz	goat	WB, IC	sc-16823
Pyk2 pY881	Santa Cruz	goat	WB, IC	sc-16825
Pyk2 pY402 clone RR102	Upstate	mouse (mAb)	IP	05-679
Active Src clone 28	BioSource	mouse (mAb)	WB, IC, IH	AHO0051
Src pY416	BioSource	rabbit	WB, IC	44-660G
Src pY527	BioSource	rabbit	WB, IH	44-662G
Src pY527 clone 31	BD/Transduction	mouse (mAb)	WB	612668
Src pY416	Cell Signaling	rabbit	WB, IC, IH	2101
Src pY416 clone 7G9	Cell Signaling	mouse (mAb)	WB, IP	2102
Non-phospho Src pY527	Cell Signaling	rabbit	WB	2107
Src pY527	Cell Signaling	rabbit	WB, IH	2105
Src pY527	Santa Cruz	goat	WB, IC	sc-16846
Src pY416 clone 2N8	Upstate	rabbit (mAb)	WB	05-857
Src pY416 clone 9A6	Upstate	mouse (mAb)	WB	05-677

[a]Abbreviations: mAb, monoclonal antibody; WB, western (immuno)blot; IP Immunoprecipitation; IC, immunocytochemistry; IH, immunohistochemistry.

Reprobe membranes

12. For sequential probing of membranes with different antibodies, strip bound antibodies from membranes by incubating 15 min in western blot stripping buffer at 65°C. Wash membrane thoroughly with water.

13. Repeat immunoblotting procedure from step 4 to step 9.

Blots can be stripped two to three times to analyze FAK and Pyk2 immune complexes with various phospho-specific antibodies. It is recommended that a different species of primary antibody be used (rabbit, mouse, or goat) in the subsequent blotting analyses to ensure that signals detected are not from the first set of antibodies used to probe the membrane.

HAPTOTAXIS MOTILITY ASSAY: MATRIX-STIMULATED MIGRATION

BASIC PROTOCOL 4

FAK is rapidly activated via tyrosine phosphorylation and binds directly to c-Src, resulting in the formation of a FAK-Src signaling complex that leads to the activation of various downstream signaling cascades. In the preceding protocols, emphasis was placed on biochemical evaluations of FAK, Pyk2, or Src activation or phosphorylation after cell binding to extracellular matrix proteins. In addition to biochemical signaling changes

inside cells, cell binding to matrix proteins such as fibronectin can result in enhanced cell spreading and the initiation of cell migration. Notably, FAK−/− cells spread poorly and exhibit refractory motility responses in response to a fibronectin stimulus. Although it has been shown that FAK reexpression within FAK−/− cells can reverse the motility defects, the molecular pathways through which FAK promotes cell motility and the inability of Pyk2 in FAK−/− cells to function as a replacement for FAK remain active areas of research. There are various assays whereby cell motility can be measured. To specifically evaluate the contribution of integrin signaling, haptotaxis (a directed response of cells in a gradient of adhesion) motility assays are performed in the absence of serum where FAK signaling plays important roles in promoting efficient cell movement towards the extracellular matrix.

Materials

FAK−/− and FAK+/+ cells, subconfluent (see Support Protocol 1)
Human plasma fibronectin (Sigma-Aldrich F2006): 2 to 10 μg/ml in replating and migration medium (see recipe), 37°C
BSA-coating control: 10 μg BSA/ml in replating and migration medium (see recipe)
Trypsin/EDTA: 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen)
Trypsin inhibitor solution (see recipe)
PBS^{++}: phosphate-buffered saline (PBS; *APPENDIX 2A*) with 0.1 g/liter $CaCl_2$ and 0.5 mM $MgCl_2$
Cell fixative: PBS with 1.85% (v/v) formaldehyde and 0.05% (v/v) glutaraldehyde
10% (w/v) crystal violet stain (Sigma) in ethanol
0.1 M sodium borate, pH 9.0
Parafilm
Millicell PCF chamber inserts, 8-μm pore (Millipore PITP01250)
Flat-tipped forceps
15-ml centrifuge tube
Tabletop centrifuge
Hemacytometer
24 well-tissue culture dishes (Costar)
Cotton swabs
Inverted light microscope
Spectrophotometer with A_{600} capability, optional
Additional reagents and equipment for serum-starving cells (Support Protocol 2) and counting cells (*UNIT 1.1*)

Starve cells

1. Twenty-four hr before the motility assay, serum starve subconfluent FAK−/− and FAK+/+ cells (see Support Protocol 2).

   Cells should be subconfluent during starvation. High-density or contact-inhibited cells do not respond well to motility stimuli during the short time period of the assay.

Prepare assay chamber

2. Pipet 100 μl of human plasma fibronectin onto Parafilm. Carefully place Millicell PCF chamber inserts one at a time on top the fibronectin-containing droplets. Prepare three chambers per experimental point and include a BSA-coating control.

   The use of flat tipped forceps is best for the handling of individual chambers.

   Be sure to cover the entire membrane and to avoid bubbles as this will result in poor ligand coverage. This method is designed to coat just the under surface of the Millicell chamber with fibronectin.

3. Incubate chambers 2 hr at room temperature. Cover the Parafilm and chambers on the bench top with a plastic container to avoid evaporation.

4. Rinse each chamber gently in PBS and let dry for at least 10 min at room temperature.

Prepare cells

5. Wash serum-starved cells with 10 ml PBS and aspirate off. Add 2.5 ml trypsin/EDTA per dish. Tap side of plates to facilitate cell release.

6. Add 5 ml trypsin inhibitor solution warmed to 37°C. Transfer cells to 15-ml centrifuge tube. Centrifuge 3 min at 750 × *g*, 21°C, in a tabletop centrifuge and aspirate supernatant.

7. Resuspend cells to ~2×10^5 cells/ml in 37°C replating and migration medium. Keep in suspension at least 30 min and rotate tube at least every 5 min to keep cells from clumping, settling, and sticking to the plastic.

8. During incubation period, count cells using a hemacytometer (*UNIT 1.1*) and dilute a suspension of cells to a density of 33×10^4 cells/ml in replating and migration medium

Perform migration assay

9. Add 400 µl replating and migration medium to each experimental well of a 24-well tissue culture dish. Carefully place chambers into wells making sure there are no bubbles.

10. Add 300 µl cell suspension to each chamber (total of 1×10^5 cells).

11. Incubate 3 to 4 hr at 37°C with 10% CO_2.

    Fibroblasts will show maximal migration within this period.

    Variations of this assay can be used to examine different types of motility. For example, chambers can be coated on the top and bottom to study random motility. Additionally, growth factors can be added to the lower chamber to investigate cell chemotaxis responses.

Fix, stain, and count cells

12. Fill two 24-well tissue culture plates with 0.5 ml PBS^{++} and one with 0.5 ml cell fixative. Transfer chambers to PBS^{++} and incubate 2 min to wash. Move chambers to fixative for 5 min then into fresh PBS^{++} for 2 min.

13. Dilute 10% crystal violet stain stock 1:100 in 0.1 M sodium borate, pH 9.0. Place chambers into 0.5 ml of 0.1% crystal violet for 30 min.

14. Remove chambers from stain and wash repeatedly in a large volume of water (e.g., in a 500 ml beaker).

15. Use a cotton swab to wipe excess cells and stain from the top side of the chamber membrane while taking care not to touch the bottom side of the chamber membrane. Let chambers dry overnight.

    Chambers are now ready for analysis. Cells that migrate to the bottom of the chamber towards the ligand will be stained purple with crystal violet while nonmigrating cells on the upper surface have been removed.

16. Count cells using a standard 10× objective on an inverted microscope. Use the lid of the 24-well plate to support the chamber. Count fields at 2, 4, 6, 8, 10, and 12 o'clock positions and in the center of the membrane.

    If there are too many cells per field to be accurately counted, the crystal violet stain can be eluted from the membrane in 10% acetic acid and values can be obtained by measuring light absorption in a spectrophotometer at 600 nm (A_{600}).

Motility values are determined by the average number of cells per field when counting three chambers per experimental point. The BSA-coated chambers should contain less than 1% of the total as migratory cells.

ALTERNATE PROTOCOL 3

ANALYZING CELL MOTILITY USING PLASMID-TRANSFECTED CELLS

The haptotaxis protocol is a good way to evaluate integrin-stimulated signaling events affecting cell migration. In many instances, there is a need to test the role of particular signaling proteins in altering cell motility responses. This is best accomplished through the transient transfection of cells with plasmid expression vectors combined with the cotransfection of a marker such as β-galactosidase (*lac Z*) to identify the plasmid-transfected cells, the assumption being that the cells that have been transfected with the *lac Z* plasmid and show β-galactosidase activity have also taken up the cotransfected FAK plasmid. This protocol is used to show that FAK re-expression can rescue the motility defects of FAK−/− cells.

Additional Materials *(also see Basic Protocol 4)*

pcDNA3.1 FAK (contact Schlaepfer lab)
FAK−/− cells (see Support Protocol 1)
pcDNA3.1 LacZ (Invitrogen)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS^{++}: phosphate-buffered saline (*APPENDIX 2A*) with 0.1 g/liter $CaCl_2$ and 0.5 mM $MgCl_2$
Opti-MEM I reduced-serum medium (Invitrogen)
PLUS reagent (Invitrogen)
Lipofectamine (Invitrogen)
Cell growth medium (see recipe) containing 20% (v/v) FBS (instead of 10%)
Starvation medium: Cell growth medium (see recipe) with FBS reduced to 0.5%
Lac Z staining solution (see recipe)
Additional reagents and equipment for bacterial transformation (Seidman et al., 1997), plasmid miniprep (Engebrecht et al., 1991), and DNA quantification (*APPENDIX 3D*)

Prepare plasmids

1. Prepare high purity supercoiled plasmid DNA from bacteria (see Seidman et al., 1997; Engebrecht et al., 1991; and *APPENDIX 3D*). Combine 5 μg of pCDNA3.1 FAK with 3 μg pcDNA3.1 LacZ for each 10-cm plate.

   Make sure the DNA is sterile by using alcohol precipitation.

   Maximum amount of DNA per 10-cm plate transfection is 8.0 μg.

Prepare cells

2. Plate 7.5×10^5 FAK−/− cells onto gelatin-coated 10-cm plates (see Support Protocol 1) and let grow overnight at 37°C.
3. Aspirate medium and wash once with PBS to remove serum.
4. Add 4 ml of Opti-MEM I reduced-serum medium and place cells in the 37°C incubator for 30 to 45 min.

Prepare transfection mix

5. Aliquot 0.5 ml Opti-MEM I reduced-serum medium into two 1.5-ml microcentrifuge tubes (label tubes A and B).
6. Add 8 μg plasmid DNA (FAK plus LacZ) to tube A and resuspend by tapping with your fingers.

7. Add 48 µl PLUS reagent to the DNA in tube A and mix by tapping.
8. Add 32 µl Lipofectamine reagent to tube B. Mix by tapping and incubate for 15 min.
9. Add contents of tube B to tube A and mix by tapping. Incubate for 30 min at 21°C.

   IMPORTANT NOTE: *Do not mix by pipetting at this step.*

Transfect cells

10. Add the DNA/lipid complexes from step 9 to the cells from step 4 and allow cell transfection to proceed for 5 hr at 37°C in the 10% CO_2 incubator.
11. Add 5 ml cell growth medium containing 20% FBS to stop transfection. Continue incubation to 24 hr without changing the medium.

Prepare cells for motility assays

12. To set up cells for motility assays, wash with 10 ml PBS and place in 10 ml serum starvation medium and incubate overnight at 37°C in 10% CO_2.
13. Use cells for motility experiments 48 hr after transfection to ensure good protein expression from plasmids.
14. Proceed with the motility assay (Basic Protocol 4, steps 2 to 11).
15. Save excess cells not used in the motility assay to make protein lysates (Support Protocol 3) and to analyze by SDS-PAGE (*UNIT 6.1*) followed by immunoblotting (Basic Protocol 3 and *UNIT 6.2*).

    This is required in order to verify transient protein overexpression (i.e., FAK and Lac Z) in the population of cells used for the motility assay.

Fix and count cells

16. After haptotaxis assays have proceeded for 3 to 4 hr, fill two 24-well tissue culture plates with 0.5 ml PBS^{++} and one with 0.5 ml cell fixative. Transfer chambers to PBS^{++} and incubate for 2 min (wash). Move chambers to fixative for 5 min then into fresh PBS^{++} for 2 min. Repeat final PBS wash.

    It is important to not leave cells in fixative for too long as this will result in less β-galactosidase activity.

17. Use a cotton swab to wipe excess cells from the top side of the chamber membrane while taking care not to touch the bottom side of the chamber membrane. Do not let chambers get too dry.
18. Add 0.5 ml Lac Z staining solution to wells of a 24-well tissue culture plate and incubate chambers 2 to 12 hr at 37°C or until blue-green color is observed.
19. Rinse chambers by gently immersing them in PBS and enumerate the blue β-gal positive cells under light microscopy.
20. To store plates fix chambers 10 min with 1 ml 10% formalin in PBS at room temperature, rinse with PBS, and store in PBS up to 1 week at 4°C.

    It is assumed that the cells that have been transfected with the Lac Z plasmid and show β-galactosidase activity have also taken up the cotransfected FAK plasmid. If the protein of interest is tagged with a fluorescent marker such as green fluorescent protein, then the direct identification of transfected cells is facilitated, making it possible to observe the behavior of such cells in time-lapse imaging studies (see Basic Protocol 5).

BASIC PROTOCOL 5

SCRATCH-WOUND HEALING ASSAY WITH TIME-LAPSE IMAGING

Wound healing assays (*UNIT 12.4*) are ideal for visualizing cellular dynamics during stimulated migration. In particular, time-lapse imaging allows for analysis of lamellipodia extension and membrane ruffling into the wounded area. Wound healing assays do not, however, address directional motility because the cells are plated at high density and movement is limited into the wound. Random migration assays can be preformed to address directional motility by plating cells at low density and following the imaging protocol outlined below.

Materials

Extracellular matrix molecule (ECM) of interest (e.g., 2 μg fibronectin/ml PBS)
70% confluent 24-hr serum-starved cells (see Support Protocol 2)
DMEM with and without 0.5 μg/ml mitomycin-C
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Serum, optional
Mitomycin-C (Sigma)
Medium 199 (Invitrogen) with 0.5 μg/ml mitomycin-C
Mineral oil (Sigma)
25-mm glass coverslips (1 oz., Fisher) and 6-well tissue culture plates *or* 35-mm Bioptechs delta-T dishes (Fisher)
Forceps
1- to 10-μl micropipet tips
Transfer pipets, sterile
Inverted microscope with 20× objective, 37°C heated stage, and acquisition/analysis software (e.g., Improvision Openlab; *https://www.improvision.com*)
Etched-grid coverslips (Bellco), optional

Coat coverslips

1. Place 25-mm round glass coverslips into wells of a 6-well tissue culture plate. Precoat glass coverslip or delta-T dishes with matrix molecule of interest (e.g., 2 μg fibronectin/ml PBS) for 2 hr at 37°C.

   Other ECM molecules may be prepared in the same concentrations in PBS as fibronectin.

   Too high a ligand concentration will inhibit cell motility. If migration is slow or nonexistent, try decreasing the ligand concentration.

Add cells

2. Prepare a cell suspension of 1×10^6 70% confluent 24 hr serum-starved cells/ml in DMEM (see Basic Protocol 1, steps 4 to 5).

   Serum starve cells for 24 hr before initiating wound healing assay.

   Cells should be ~70% confluent during starvation.

3. Wash coverslips or delta-T dishes with PBS and plate 2×10^6 cells onto coverslips in a 6-well tissue culture plate or directly onto the delta-T dish.

   Coverslips in 6-well tissue culture plates are used if subsequent procedures include an imaging chamber. Bioptechs delta-T dishes are self-contained.

4. Incubate at 37°C with 10% CO_2 for 2 hr.

   The incubation time can be adjusted to accommodate other cells types if needed. This step is to facilitate equal cell adhesion and spreading on the matrix-coated glass surface.

Wound the cultures

5. Remove plates with coverslips from incubator. Using forceps to stabilize the coverslip in the well, carefully scratch the confluent monolayer of cells down the center using a micropipet tip.

   If using a delta-T dish, use a similar technique to scratch the center of the glass insert while holding the dish steady.

   This step is critical and will require practice in order to achieve consistent wounds. In order to reliably compare results from experiment to experiment, the wound distance must be equivalent each time.

6. Rinse the coverslip/delta-T dish three times with 2 ml PBS to remove loose cells.

   If the cell monolayer is not washed effectively after scratching, loose cells can settle into the wounded area and result in an inaccurate interpretation of wound healing ability.

7. Place coverslip in fresh DMEM medium with 0.5 μg/ml mitomycin-C and incubate at 37°C with 10% CO_2 for 1 hr.

   Add 0.5 μg/ml mitomycin-C to all medium changes from this point. This will inhibit cell mitogenesis and, therefore, control for wound closure due to cell proliferation.

   Serum can be added (1% to 5% v/v, depending on cell type and required stimulation) at this point to stimulate cell motility if needed.

Assess motility

8. Aspirate medium and move coverslip into imaging chamber (if using the delta-T dishes, the imaging chamber is the unit itself). Add 1 ml of medium 199 with 0.5 μg/ml mitomycin-C (with serum addition as needed).

9. Carefully layer 0.5 ml mineral oil on top of the medium to prevent evaporation during imaging and place imaging chamber/delta-T dish in heated stage on the microscope and set to 37°C.

10. Using a 10× or 20× objective, center the wound such that the image captured will show both sides of the cell monolayer flanking the wounded region. Using Improvision Openlab or comparable acquisition software, begin the time-lapse sequence and collect one image every 2 to 5 min for 12 to 15 hr or until the wound is closed.

    Alternatively, if time-lapse software is not available, etched-grid coverslips can be used (Bellco). These coverslips have a grid system that allows tracking and measuring wound closure over time with a light microscope while leaving the coverslips in the incubator in between phase-image collection.

IMMUNOLOCALIZATION OF FOCAL ADHESION PROTEINS

BASIC PROTOCOL 6

Immunostaining is the best approach to determining whether a protein of interest is localized to integrin-enriched focal adhesions. For valid interpretation of the staining results, two controls are essential: (1) immunoblot evaluation of the antibody against the protein before immunostaining and (2) cells costained with markers for focal adhesion proteins. The antibody to be used is suitable for cell staining analyses if it yields one band by immunoblotting analyses of whole cell lysates. The presence of multiple bands, even though the main one is the strongest, easily leads to misinterpretation of results. The most commonly used focal adhesion markers are vinculin and paxillin. Some of the best markers for activated focal adhesions are antibodies that recognize tyrosine-397-phosphorylated FAK or active Src-family members.

Materials

Matrix-coating substrates: prepared according to manufacturer's directions and diluted (commonly to 10 μg/ml) in PBS (*APPENDIX 2A*)
  Fibronectin, human plasma (Roche)
  Laminin, human placenta (Sigma)
  Vitronectin, human plama (Sigma)
  Poly-L-lysine (70,000–150,000 or 150,000–300,000; Sigma)
  Collagen, Type I, human placenta (Calbiochem)
  Collagen, Type II, bovine (Calbiochem)
  Collagen, Type IV, human placenta (Calbiochem)
  Collagen, Type V, human (Calbiochem)
FAK−/− and FAK+/+ cells (see Support Protocol 1)
Replating and migration medium (see recipe), warm *or* growth medium (see recipe), 37°C
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
3.8% (w/v) paraformaldehyde fixative (see recipe)
Acetone, cold (stored at −20°C) *or*
0.5% (v/v) Triton X-100/0.05% (v/v) Tween 20/PBS *or*
0.2% Triton X-100/PBS *or*
0.2% (v/v) Triton X-100/3.8% (w/v) paraformaldehyde/PBS *or*
0.1% (v/v) Triton X-100/0.1% (w/v) sodium citrate *or*
Methanol, cold
Blocking antibody (e.g., ChromPure donkey IgG, unconjugated; Jackson Immunoresearch) *or*
2% (w/v) BSA in PBS
1 to 10 μg/ml PBS (*APPENDIX 2A*) primary antibodies for focal adhesion markers
  anti-paxillin (ZO35; Zymed/Invitrogen)
  anti-vinculin (VIN-11-5; Sigma)
  anti-FAK (clone #77; BD-Transduction)
  anti-FAK (Ab-1; LabVision)
  anti-phospho Y397FAK (BioSource)
  anti-active Src family members (clone #28; BioSource)
Secondary antibodies: e.g., fluorescein (FITC)-conjugated donkey antibodies (excitation/emission maxima 492/520 nm; Jackson Immunoresearch) *or* FITC-conjugate
  anti-mouse IgG
  anti-mouse IgM
  anti-rabbit IgG
  anti-goat IgG
  anti-rat IgG
Rhodamine X (RRX)-conjugated donkey antibodies (excitation/emission maxima 570/590 nm; Jackson Immunoresearch) *or* Rhodamine X (RRX)-conjugated
  anti-mouse IgG
  anti-mouse IgM
  anti-rabbit IgG
  anti-goat IgG
  anti-rat IgG
Hoechst 33342 (excitation/emission maxima 350/460; Molecular Probes)
Vectashield mounting medium (Vector)
Nail polish, clear

12-mm round coverslips, German glass (Bellco Glass)
4-well tissue culture plates (Nunc)
12-well tissue culture plates (Costar)
18- to 22-G needle with a slightly bent tip
Flat-ended forceps with beveled, unserrated tips, stainless steel (Millipore)

6-well tissue culture plates
Rotating platform shaker
Vacuum source
Porcelain spot plates with 12 cavities (CoorsTek)
Light microscope with fluorescence excitation and detecting capability

1. Coat 12-mm round coverslips with matrix-coating substrate (e.g., by incubating with 10 μg/ml fibronectin 45 min at room temperature).

   The most commonly used adhesion molecule to induce formation of focal contacts is fibronectin, but the same volumes and concentrations are recommended for laminin, vitronectin, poly-L-lysine, and collagen. The coating time should be extended to 2 hr for collagen, and the coverslips should be washed with PBS to neutralize the acidity of the collagen solutions.

Plate cells

2. Prepare FAK−/− and FAK+/+ cell suspension of 2×10^4 cells/ml in 37°C replating and migration medium or growth medium (see Basic Protocol 1, steps 5 to 6), and add 0.5 ml cell suspension per well of a 4-well tissue culture plate containing a coated coverslip.

   Blocking with BSA is not required because cells will not attach to the uncoated glass surface.

   At this concentration the adherent cells will be spread apart.

   If the purpose is to determine activation or localization of the protein by adhesion molecule of interest, replating and migration medium should be used, otherwise growth medium is appropriate.

   The well size of 4-well tissue culture plates is the same as in 24-well plates. However, the wells are not so deep, and it is easier to take coverslips out.

3. Incubate at 37°C for desired time.

   Activation (phosphorylation) of the focal adhesion proteins is the highest during cell spreading. For fibroblasts, the best time to capture localization of the activated proteins in focal adhesions is ∼1 hr after plating. At that time most of cells will be spread. Some cells will still be spreading and often both types can be visualized within a single field.

   Activated focal adhesion proteins at the leading edge of migrating cell are best visualized 5 to 6 hr after wounding a cell monolayer with a micropipet tip. Activation is higher in the presence of serum. The most commonly used serum concentration is 10%, but one could also see activation at much lower serum concentrations.

Fix and permeabilize cells

4. Aspirate medium and rinse briefly 2 to 3 times in ∼1 ml PBS by adding ∼1 ml PBS and aspirating or decanting it.

For most cells

5a. Add ∼1 ml 3.8% paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature and rinse with PBS as above.

6a. Transfer coverslips into a porcelain spot plate filled with cold acetone (stored at −20°C) to permeabilize the cells. Keep on ice for 10 min. Remove acetone and add ∼1 ml PBS to rehydrate for 3 to 5 min.

   Transfer of coverslips to a porcelain plate is necessary only when using acetone, which will dissolve plastic tissue culture dishes.

Fixation and permeabilization are the most critical steps for successful immunostaining. Even for the same protein, this step can differ from cell type to cell type and from antibody to antibody. Most focal adhesion proteins are detectable when cells are fixed in paraformaldehyde and permeabilized in acetone. If the staining is weak or negative, changing fixation and permeabilization should be the first step to troubleshoot.

For weakly staining cells (alternative 1)

5b. Add ~1 ml 3.8% (w/v) paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature.

6b. Remove paraformaldehyde and add ~1 ml 0.5% (v/v) Triton X-100 + 0.05% (v/v) Tween 20/PBS for 2 min to permeabilize the cells.

For weakly staining cells (alternative 2)

5c. Add ~1 ml 3.8% (w/v) paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature.

6c. Remove paraformaldehyde and add ~1 ml to 0.2% (v/v) Triton X-100/PBS for 2 min to permeabilize the cells.

For weakly staining cells (alternative 3)

5d. Add ~1 ml 0.2% (v/v) Triton X-100/3.8% (w/v) paraformaldehyde/PBS and incubate 2 min at room temperature to fix and permeabilize cells. Rinse several times with 3.8% paraformaldehyde/PBS as in step 4.

6d. Add ~1 ml 3.8% paraformaldehyde/PBS and continue fixing for 20 min at room temperature.

For weakly staining cells (alternative 4)

5e. Add ~1 ml 3.8% (w/v) paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature.

6e. Add ~1 ml 0.1% (v/v) Triton X-100/0.1% (w/v) sodium citrate and keep 2 to 5 min on ice to permeabilize the cells.

For weakly staining cells (alternative 5)

5f. Transfer coverslips into a porcelain spot plate filled with cold acetone (stored at −20°C) to fix and permeabilize cells. Keep on ice for 10 min.

In some cases in the more common protocol described in step 5a, paraformaldehyde fixation can interfere with staining. In these cases coverslips are directly exposed to acetone, which can also act as a fixative.

6f. Remove acetone and add ~1 ml PBS to rehydrate for 3 to 5 min.

Staining of actin stress fibers is impossible when fixation/permeabilization is done with ONLY acetone or methanol (alternatives 5 and 6).

For weakly staining cells (alternative 6)

5g. Add ice-cold methanol (stored at −20°C) to fix and permeabilize cells. Keep on ice for 10 min.

6g. Aspirate methanol, rinse briefly in ~1 ml PBS and leave in PBS for 3 to 5 min to rehydrate.

Staining of actin stress fibers is impossible when fixation/permeabilization is done with ONLY acetone or methanol (alternatives 5 and 6).

Block and immunostain cells

7. Pace a 30-µl drop of PBS containing blocking antibodies and primary antibodies for focal adhesion markers on the inner side of 12-well plate lid (facing up), and place the coverslip with fixed and permeabilized cells on top, with the attached cells facing the drop of antibody solution. Cover with the bottom of the plate and incubate 1 hr at room temperature or as long as overnight at 4°C, depending on convenience and the effectiveness of staining with various samples and procedures.

   Using a 30-µl drop of PBS containing blocking antibodies and primary antibodies for focal adhesion markers on the inner side of a 12-well plate lid helps prevent evaporation, ensure equal exposure, and use less antibody solution.

   Flip coverslip over the 30-µl drop of PBS containing blocking and primary antibodies using an 18- to 22-G needle with a slightly bent tip to lift and flat-ended forceps to hold and transfer the coverslip. Sharp-ended forceps tend to crack the coverslip. Alternatively, if the cell type is sensitive to flipping the coverslips for incubations and cells detach, utilize a humidified chamber (to avoid evaporation) and add 50 µl of antibody directly on top of the coverslips.

   Blocking is done with whole IgG of the host animal for the secondary antibody. For example, the primary antibody is made in mouse. If the secondary antibody is fluorescein-conjugated donkey anti-mouse, donkey whole IgG should be used for blocking. If the secondary antibody is fluorescein-conjugated goat anti-mouse, goat whole IgG should be used for blocking, etc. Blocking antibodies are sold in concentrations ~10 mg/ml. Recommended dilution for blocking purposes is 1:100 (~100 µg/ml). To keep costs low, the authors use secondary antibodies that are all made in donkey, requiring only one reagent (ChromPure donkey whole IgG) for blocking purposes.

   Alternatively, blocking can be performed with 2% (w/v) BSA in PBS. In this case, blocking is done at the same time as sample incubation with the primary antibody, saving time. BSA blocking is less specific than IgG, and in rare cases BSA might be too strong a blocking agent.

   Dilution of primary antibodies can vary and should be optimized for each antibody. Generally a range of 1 to 10 µg/ml is effective. A starting point for optimization would be a 1:100 dilution of a commercial antibody, which is usually sold in concentrations 0.1 to 1 mg/ml.

   For co-immunostaining, incubate samples with both primary antibodies and with blocking antibodies at the same time. If incubations are for several hours, samples can be left at room temperature. If incubation is performed overnight, the incubation should be at 4°C.

8. Lift the coverslip by injecting 150 µl PBS under it, and place it in a well of a 6-well tissue culture plate, attached cells facing up. Wash in three changes of ~3 ml PBS 5 min each time at ~50 rpm on rotating platform shaker.

   Smaller size wells (e.g., wells of 12- or 24-well plates) do not allow sufficient movement of PBS to wash unbound antibodies from the samples.

9. Pace a 30-µl drop of PBS containing secondary antibodies and 10 µg/ml Hoechst 33342 on the inner side of a 12-well plate lid (facing up), and place the coverslip with fixed and permeabilized cells on top with the attached cells facing the drop of antibody solution. Cover with plate bottom and incubate 40 min at room temperature.

   Hoechst 33342 binds DNA, and it is an excellent bright nuclear marker visible at UV excitation wavelengths. It does not leak to green or red channels. It is of immense help for orientation and focusing of samples during microscopy.

   For visualization of actin stress fibers, fluorescent phalloidin conjugate is added together with secondary antibody for costaining of two antigens: one is detected by the primary antibody, and the other (actin stress fibers) is detected directly by fluorophore-conjugated phalloidin.

10. Wash in three changes of ~3 ml PBS three times 5 min each time at ~50 rpm on a rotating platform shaker.

11. Mount on microscope slides in Vectashield mounting medium. Aspirate excess mounting medium under the coverslip using a Pasteur pipet attached to vacuum. To prevent sliding, fix the edge of the coverslip with clear nail polish. Wait 15 to 20 min for the nail polish to dry, then analyze samples under the microscope.

    Samples can be preserved at 4°C in the dark for varying lengths of time, depending on the strength of the staining. For example, actin staining can be preserved for a month or longer. Weak staining will fade beyond recognition within several days. The authors recommend analysis within 2 to 3 days after staining.

ALTERNATE PROTOCOL 4

IMMUNOLOCALIZATION OF ACTIN STRESS FIBERS

Visualization of actin stress fibers with fluorescent phalloidin, a toxin with high binding affinity for actin filaments, is easy and almost certainly yields dazzling results. Unlike indirect immunostaining with antibodies, phalloidin staining is strong, and the contrast between stained and unstained areas is high. Because focal adhesions are located at the end of actin stress fibers, phalloidin staining is also used as a localization marker and as a quick assay to assess cell shape. Costaining actin stress fibers with fluorescent phalloidin–conjugate and DNA-binding Hoechst dye can be a rewarding experience for a new investigator who is performing immunofluorescence cell staining for the first time.

Additional Materials *(also see Basic Protocol 6)*

0.4 to 1 U/ml (10 to 30 nM) fluorescein-conjugated phalloidin (Molecular Probes) or rhodamine-conjugated phalloidin (Molecular Probes) in PBS

Rotating platform shaker
Vacuum source

Prepare cells

1. Coat coverslips, prepare FAK−/− and FAK+/+ cell suspension, and plate the cells (Basic Protocol 6, steps 1 to 3).

2. Incubate at 37°C for desired time.

Fix and permeabilize cells

3. Aspirate medium and rinse briefly 2 to 3 times in ~1 ml PBS by adding ~1 ml PBS and aspirating or decanting it.

4. Add ~1 ml 3.8% paraformaldehyde/PBS, pH 7.4, to fix cells. Incubate 20 min at room temperature and rinse with PBS as above.

5. Transfer coverslips into a porcelain spot plate filled with cold acetone (stored at −20°C) to permeabilize the cells. Keep on ice for 10 min. Remove acetone and add ~1 ml PBS to rehydrate for 3 to 5 min.

6. Place a 30-µl drop containing 0.4 to 1 U/ml (10 to 30 nM) fluorescein-conjugated phalloidin with 10 µg/ml Hoechst 33342 in PBS on the inner side of a 12-well tissue culture plate lid (facing up), and place the coverslip with fixed and permeabilized cells on top with the attached cells facing the drop of solution. Cover with the plate bottom and incubate 30 min at room temperature.

    If dissolved as recommended by the manufacturer (Molecular Probes), 0.4 to 1 U/ml (10 to 30 nM) phalloidin is 1:200 to 1:500.

Rhodamine-conjugated phalloidin is an alternative to fluorescein-conjugated phalloidin.

If costaining with some other protein, phalloidin should be added together with the secondary antibody (see Basic Protocol 6).

7. Wash in three changes of ~3 ml PBS 5 min each time at ~50 rpm on a rotating platform shaker.

8. Mount on microscope slides in Vectashield mounting medium. Aspirate excess mounting medium under the coverslip using a Pasteur pipet attached to vacuum. To prevent sliding, fix the edge of the coverslip with nail polish. Wait 15 to 20 min for the nail polish to dry, then analyze samples under the microscope.

Samples can be preserved at 4°C in the dark for varying lengths of time depending on the strength of the staining. For example, actin staining can be preserved for a month or longer. Weak staining will fade beyond recognition within several days. The authors recommend analysis within 2 to 3 days after staining.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BSA blocking buffer

20 mM Tris·Cl, pH 7.4
150 mM NaCl
2% (w/v) BSA (Fraction V)
0.05% (v/v) Tween 20
0.05% (w/v) sodium azide
Store up to 3 months at 4°C

Cell growth medium

DMEM high-glucose with L-glutamine (Invitrogen)
10% (v/v) FBS
1 mM sodium pyruvate (Invitrogen)
0.1 mM MEM nonessential amino acids (Invitrogen)
10 U/ml penicillin and 10 μg/ml streptomycin (Invitrogen; 100× stock)
Sterilize by passing through a 0.22-μm filter
Store up to 2 weeks at 4°C

Coomassie blue stain

25% (v/v) isopropanol (2-propanol)
10% (v/v) glacial acetic acid
0.03% (w/v) Coomassie G-250 (BioRad)
Store up to 1 year at 21°C

Destaining solution

10% (v/v) isopropanol (2-propanol)
10% (v/v) glacial acetic acid
Store up to 1 year at 21°C

FAK-Pyk2 kinase buffer

20 mM HEPES, pH 7.4
10% (v/v) glycerol
10 mM $MgCl_2$
10 mM $MnCl_2$
150 mM NaCl
Store up to 6 months at 4°C

HNTG buffer

50 mM HEPES, pH 7.4
0.1% (v/v) Triton X-100
150 mM NaCl
10% (v/v) glycerol
Store up to 6 months at 4°C

Lac Z staining solution

70 ml H_2O
10 ml of 10× PBS (1× final)
10 ml of 50 mM potassium ferricyanide (5 mM final)
10 ml of 50 mM potassium ferrocyanide (5 mM final)
0.2 ml of 1 ml $MgCl_2$ (2 mM final)
Store up to 2 weeks at 4°C
Just before use, add 1 ml of 20 mg/ml Xgal per 20 ml of staining solution (1 mg/ml final). Make a stock solution of 20 mg/ml X-gal in dimethylformamide (DMF). Store at −20°C in the dark.
NOTE: *Use a glass pipet to measure DMF solutions as it will dissolve plastics.*

Laemmli SDS sample buffer (2×)

125 mM Tris·Cl, pH 6.8
20% (v/v) glycerol
4% (w/v) SDS
0.006% (w/v) bromophenol blue (Fisher Biotech)
3.5% (v/v) 2-mercaptoethanol
Store up to 1 week at 4°C or 6 months at −20°C

Magnesium/ATP cocktail

75 mM $MgCl_2$
500 μM ATP
20 mM MOPS, pH 7.2
25 mM β-glycerol phosphate
5 mM EGTA
1 mM sodium orthovanadate
1 mM dithiothreitol
Store up to 1 week at 4°C

Paraformaldehyde fixative, 3.8% (w/v)

Add 3.8 g paraformaldehyde (Sigma) to 100 ml PBS (*APPENDIX 2A*), heat, and stir to dissolve. When temperature reaches 50°C, add ~100 μl 1 N NaOH, and continue stirring until temperature reaches 60°C. Remove from the heating plate, continue stirring until it reaches room temperature and filter to remove fine precipitate. Store up to 10 days at 21°C.

Once made, paraformaldehyde fixative is ready to use for up to 10 days. The exact percentage of paraformaldehyde is not crucial; it is sufficient to be somewhere between 3.8% and 4.0%. Similarly, the exact pH does not matter, if it is somewhere in a physiological range (7.2 to 7.6). Paraformaldehyde from different manufacturers or of different purity might require different amount of NaOH to adjust the pH.

Replating and migration medium

500 ml DMEM
0.5% (w/v) BSA (add 20 ml 12.5% BSA solution)
1 mM sodium pyruvate (Invitrogen)

continued

0.1 mM MEM nonessential amino acids (Invitrogen)
Sterilize by passing through a 0.22-μm filter
Store up to 1 month at 4°C

RIPA cell lysis buffer

1% (v/v) Triton X-100
1% (v/v) sodium deoxycholate
0.1% (w/v) SDS
50 mM HEPES, pH 7.4
150 mM NaCl
10% (v/v) glycerol
1.5 mM $MgCl_2$
1 mM EGTA
10 mM sodium pyrophosphate
100 mM sodium fluoride
1 mM sodium orthovanadate (add dropwise)
10 μg/ml aprotinin
10 μg/ml leupeptin
Sterilize by passing through a 0.22-μm filter
Store up to 1 week at 4°C
Add ingredients slowly to water. Start over if buffer becomes brown after sodium orthovanadate addition.
Alternatively, a protease inhibitor cocktail (e.g. Sigma P2714) may be used instead of aprotinin and leupeptin.

Src kinase buffer

20 mM PIPES, pH 7.0
10 mM $MnCl_2$
1 mM DTT
Store up to 6 months at 4°C

Tris-buffered saline with Tween (TBST)

50 mM Tris·Cl, pH 7.5
150 mM NaCl
0.05% (v/v) Tween 20
Store up to 6 months at 4°C

Triton lysis buffer

RIPA cell lysis buffer (see recipe) without sodium deoxycholate and SDS addition. Store up to 1 week at 4°C

Trypsin inhibitor solution

500 ml DMEM
125 mg soybean trypsin inhibitor (Worthington)
Dissolve by stirring
0.25% (w/v) BSA (add 10 ml of 12.5% w/v BSA solution)
Sterilize by passing through a 0.22 μm filter
Prepare fresh

Western blot stripping buffer

100 mM Tris·Cl, pH 6.8
2% (w/v) SDS
100 mM 2-mercaptoethanol
Store up to 3 months at 21°C

COMMENTARY

Background Information

FAK was first identified in 1992 as a highly tyrosine-phosphorylated protein associated with cellular focal adhesions, i.e., the integrin-associated cell attachment points with the extracellular matrix (Parsons, 2003). At that time, it was shown that changes in cell adhesion or integrin clustering could result in the increased phosphorylation of tyrosine in FAK (Guan and Shalloway, 1992; Hanks et al., 1992; Kornberg et al., 1992; Schaller et al., 1992). Today we know that this activation event is much more complicated than the simple binding of FAK to integrins leading to intermolecular FAK phosphorylation after clustering (Schlaepfer and Mitra, 2004). For instance, evidence to date does not strongly support direct binding of FAK to integrins. Instead, FAK is associated with integrin cytoplasmic tails through binding to integrin-associated proteins such as paxillin and talin. Additionally, integrins can activate Src-family protein-tyrosine kinases in a manner that is independent of FAK. It is known that cell binding to fibronectin promotes FAK and c-Src activation and the formation of a FAK-Src signaling complex, and these facts support the conclusion that the events are related. Current research is focused on deciphering the molecular mechanisms through which FAK and c-Src are activated by integrins, and the methods outlined in this unit comprise the foundation for these studies.

Additional complexity in this integrin signaling linkage comes from the studies of the FAK-related kinase, Pyk2, which is highly expressed in FAK−/− cells (Sieg et al., 1998). Stimulation of FAK−/− cells by replating on fibronectin promotes the activation of Src-family tyrosine kinases and the enhanced tyrosine phosphorylation of Pyk2. However, neither Pyk2 nor c-Src strongly colocalize with FAK−/− cell focal adhesions, and these cells do not correctly spread and migrate like wild-type FAK+/+ fibroblasts (Sieg et al., 1999; Klingbeil et al., 2001). The localization of FAK to particular integrin signaling sites is important for fibronectin-stimulated cell motility, the molecular mechanisms of which are still under investigation.

What the authors have tried to accomplish in this unit is to consolidate related protocols on how to measure changes in FAK, c-Src, and Pyk2 activity after fibronectin-mediated integrin stimulation through the use of in vitro kinase assays, phospho-specific blotting, or immunolocalization techniques. These assays involve using FAK−/− and FAK+/+ cells. The handling or preparation of these cells prior to performing biochemical activation assays is the same as the procedures used in evaluating the properties of these cells in cell migration assays. In this manner, cause and effect relationships can be established between integrin-stimulated signaling events and the modulation of a complex biological process such as cell motility. Alternate protocols that have been successfully used by other investigators include the use of antibodies or recombinant ligands for particular integrin subunits to facilitate binding or clustering and intracellular signaling (Giancotti and Ruoslahti, 1999). As cells usually contain multiple integrin α/β pairs that can bind fibronectin, and current research has shown that sequence-specific differences between integrin cytoplasmic domains facilitates the binding or activation of distinct intracellular signaling proteins (Liu et al., 1999; de Virgilio et al., 2004), antibody/ligand activation of integrins is a powerful means to study integrin signaling. However, antibody-mediated clustering of integrins is usually a weaker stimulus compared to cell binding and spreading on matrix proteins. Therefore, if a recombinant ligand can be purified and immobilized to facilitate specific integrin engagement and cell binding, this can be effectively used to study the signaling linkage between particular integrins, the actin cytoskeleton, and cell motility.

Critical Parameters and Troubleshooting

Maintenance of FAK+/+ AND FAK−/− fibroblasts and replating assays for signaling studies

It is critical to use well maintained FAK+/+ and FAK−/− cells for all experiments in this unit. Because the fibroblasts may exhibit altered morphology and premature senescence when overly confluent or at high passage number, cells need to be subcultured consistently and used within the first 15 passages from the cells available from American Type Culture Collection (ATCC). To prevent poor attachment to the extracellular matrices when cells are replated, avoid overtrypsinization and always make fresh trypsin inhibitor solution. Further, serum starvation is an important step in allowing maximal cell response and activation of focal adhesion molecules to stimuli (e.g., fibronectin). This

is also critical for successful motility assays (Basic Protocol 4) and scratch-wound healing assays (Basic Protocol 5).

In vitro kinase assay

It is important that a control antibody is included with immunoprecipitation experiments to accurately interpret in vitro kinase assay results. For example, preimmune serum can serve as the control for a rabbit antiserum. Further, the species (e.g., rabbit, mouse, goat, rat, or sheep) of antibody used in immunoprecipitation should be carefully considered if the immunoprecipitates will be analyzed by subsequent immunoblotting. HRP-conjugated secondary antibodies will recognize the immunoprecipitating antibody as well as the primary immunoblotting antibody of the same species, resulting in very high background around the 60-kDa protein region. As this is region where c-Src is present on gels, nonspecific antibody cross-reactivity can complicate co-immunoprecipitation experiments and c-Src immunodetection.

Immunoblotting with FAK/Pyk2 phospho-specific antibodies

For the detection of FAK and Pyk2 phosphorylation with phospho-specific antibodies, the most critical issue is the specificity and quality of the antibody. Ideally, the phospho-specific antibody should recognize only the phosphorylated form of the target protein on a specific site. However, in the experience of the authors, some phospho-specific antibodies for FAK can cross-react with corresponding sites on Pyk2 and may even react with other phosphorylation sites on the target protein. It is for this reason that results should be double-checked using complementary means such as phospho-specific antibodies from various manufacturers and alternate procedures (e.g., in vitro kinase assays). When reprobing with different phospho-specific antibodies, it is critical to ensure that stripping or removal of previous antibodies is complete. This can be done by performing the ECL reaction and exposing film after every blot stripping to confirm that no residual signal remains.

Haptotaxis motility assays

As confluent cells do not respond to extracellular matrix stimuli, it is essential to maintain a low cell confluency when performing the haptotaxis assay. Additionally, chamber coating is key to a successful and interpretable motility assay. Uneven or improper chamber coating will result in experimental variability within single chambers as well as throughout the experiment. Potential causes of uneven coating include denatured or sticky extracellular proteins and the presence of bubbles underneath the chambers during coating.

Scratch-wound healing assays

After generating the wound, it is important to ensure that no residual cells settle in the scratch area. This would severely complicate interpretation of the assay and can be avoided by thorough washing after creating the scratch wound. Additionally, wound-closure measurements must be compared to the original wound distance for each individual coverslip due to experimental scratch variations.

Immunostaining

The most important issues for visualization of focal adhesion molecules with immunofluorescent staining are (1) fixation and permeabilization of cells and (2) specificity of the staining antibody. Optimized fixation allows the preservation and localization of the antigen as well as cell morphology, while proper permeabilization allows access to the antigen. To achieve specificity for the immunostaining, the primary antibody should recognize only its target antigen, and proper controls (e.g., preimmune serum or secondary antibody only) should be included to optimize the staining conditions and to reduce background. See Table 14.7.3 for troubleshooting the experiments described in this unit.

The authors do not recommend use of preimmune serum as a control for staining specificity. The more appropriate control is a Western blot of whole cell lysate made from the same cells treated in the same matter. If multiple bands are visible, the antibody is not reliable for immunostaining. A control for secondary antibody is not necessary if whole IgG of the same species in which the secondary antibody was generated is used as a blocking agent together with the primary antibody.

Anticipated Results

In attached and serum-starved FAK+/+ and FAK−/− cells, FAK and Pyk2, respectively, display high basal levels of tyrosine phosphorylation and moderate level of kinase activity. In suspended cells, phosphorylation of tyrosine in both FAK and Pyk2 is greatly reduced. Replating cells on fibronectin, but not poly-L-lysine, increases tyrosine phosphorylation on both FAK and Pyk2 in FAK+/+ and FAK−/− cells. Immunoblotting with phospho-specific antibodies reveals that FAK and Pyk2 can be phosphorylated on

Table 14.7.3 Troubleshooting Guide for Common Problems Encountered in These Assays

Problem	Possible cause	Solution
Cell replating		
Cells slow to adhere and spread on matrix	Too much trypsin used to prepare cell suspension	Use limited trypsin/EDTA treatment at 37°C and tap plates to remove cells
		Make fresh trypsin inhibitor solution
In vitro kinase assay		
No or low levels of substrate labeling	Old [γ-^{32}P]ATP	Order fresh [γ-^{32}P]ATP
	Kinase denatured or degraded	Add fresh protease inhibitor to lysis buffer. Use freshly prepared lysates for immunoprecipitation
		Avoid repeated freeze-thaw of samples
		Use polyclonal antibodies to target protein for immunoprecipitation
High background	Carryover of cell debris following preclearance	Avoid disturbing cell debris when transferring supernatant during lysate preparation
	Incomplete washing	Completely resuspend beads at each wash
Phospho-specific immunoblotting		
Phospho-specific antibody recognizing nonphosphorylated form of protein in immunoblotting	Too much antigen or antibody present	Optimize the amount of lysates and concentration of antibody used. Reduce antibody incubation time and increase washing
Haptotaxis motility assay		
Migratory cells too dense to quantify	Assay went too long	Decrease assay time
	Too many cells were plated	Plate fewer cells
Inconsistent cell migration	Improper chamber coating	Avoid bubbles underneath chamber
	Matrix protein precipitated out of solution	Confirm the pH of medium used to resuspend matrix protein
Scratch-wound healing		
Cells do not close the wound	Matrix protein concentration is too high	Decrease matrix protein concentration
	Cells are not plated as a confluent monolayer	Increase cell density
	Cells require serum stimulation	Add 1% to 5% serum during assay
Cells close the wound too quickly	Too many cells	Decrease cell density
	Elevated cell proliferation	Add mitomycin C to block cell division

continued

Table 14.7.3 Troubleshooting Guide for Common Problems Encountered in These Assays, *continued*

Problem	Possible cause	Solution
Immunolocalization		
No staining	Primary antibody is not binding to epitope	Use different primary antibody
		Try another fixation procedure
		Try a different secondary antibody
Staining looks non-specific or high background	Complications due to fixation procedure	Try another fixation procedure
	Primary antibody does not cross-react across species	Change primary antibody
	Antigen target is expressed at low levels	Increase primary antibody incubation from 1 hr to overnight.
		Increase concentration of primary antibody
		Use biotin-avidin enhancement
Identical pattern observed for multiple proteins	Primary antibodies are from same species	Change one primary antibody to a different species
Signal "leaking" through microscope filters	Too high concentration of primary or secondary antibody	Decrease antibody concentrations or increase number of washes

multiple tyrosine residues following replating onto fibronectin matrix, and, following integrin activation, Src-associated in vitro kinase assays demonstrate that FAK and Pyk2 can transiently form signaling complexes with Src.

In haptotaxis and scratch-wound healing assays, FAK−/− cells display impaired migration compared to FAK+/+ cells. The defective cell motility of FAK−/− cells cannot be compensated by the presence of high levels of Pyk2, but it can be rescued by transiently re-expressing FAK. Furthermore, FAK can be detected via immunofluorescence staining that prominently and specifically localize to focal adhesions. In fact, some of the best markers of activated focal adhesions are antibodies that recognize FAK phosphorylated on tyrosine 397. Finally, strong activation of FAK can be detected in focal adhesions about 1 hr after replating fibroblasts onto extracellular matrix as well as at the leading edge of migration 5 to 6 hr following a scratch wound.

Time Considerations

Cells need to be serum starved (Support Protocol 2) for 16 to 24 hr before replating (Basic Protocol 1), haptotaxis motility (Basic Protocol 4), and scratch-wound healing (Basic Protocol 5) assays. Cells are kept in suspension for at least 45 min or replated for 1 to 3 hr before whole-cell lysates are collected, which takes 1 hr to complete (Support Protocol 3).

FAK, Pyk2, and c-Src can be captured from whole cell lysates by immunoprecipitation in 4 to 5 hr (Support Protocol 4), and can be further analyzed through in vitro kinase assay (Basic Protocol 2) or processed by immunoblotting for their phosphorylation and activation status (Basic Protocol 3). In these protocols SDS-PAGE gel electrophoresis takes 1 to 1.5 hr, gel transfer onto membrane takes 1.5 to 2 hr, and immunoblotting is best when membranes are incubated in primary antibodies overnight. Therefore, these biochemical assays will take a total of 3 days.

Following serum starvation (16 to 24 hr), the haptotaxis assay (Basic Protocol 4) takes ~8 hr to complete. It takes 2.5 to 3 hr to prepare the chambers and 1 to 1.5 hr to prepare cells (depending on how many different experimental groups are being analyzed), but these two items can be done concurrently. Cells migrate for 3 hr followed by an additional 1 to 2 hr

to fix and stain chambers. Microscopic quantification of migratory cells can take several hours.

To transfect FAK+/+ and FAK−/− cells (Alternate Protocol 3), cells need to be plated the previous day; preparation of transfection mix requires 45 min to 1 hr, and the transfection takes 5 hr. Cells are grown for an additional 48 hr to allow maximal protein expression and need to be serum starved for 24 hr before haptotaxis assay.

For the scratch-wound healing assay (Basic Protocol 5), cells are serum starved for 16 to 24 hr, plated on a cover slip for 2 hr, scratch wounded, and incubated for 1 hr after wounding. Live images are collected for 12 to 15 hr. The scratch-wound healing assay spans 32 to 45 hrs, but requires only 2 to 3 hr of hands-on time. For immunolocalization of focal adhesion proteins (Basic Protocol 6), immunofluorescence staining can be performed in ~7 hr.

Internet Resources

http://www.cellmigration.org/index.shtml

This cell migration gateway represents a unique collaboration between the Cell Migration Consortium (CMC) and Nature Publishing Group (NPG) and is designed to facilitate navigation through the complex world of cell migration research.

http://www.signaling-gateway.org

This signaling gateway represents a unique collaboration between academia and scientific publishing and is designed to facilitate navigation of the complex world of research into cellular signaling. Information and data presented here are freely available to all.

Literature Cited

de Virgilio, M., Kiosses, W.B., and Shattil, S.J. 2004. Proximal, selective, and dynamic interactions between integrin $\alpha_{IIb}\beta_3$ and protein tyrosine kinases in living cells. *J. Cell Biol.* 165:305-311.

Engebrecht, J., Brent, R., and Kaderbhai, M.A. 1991. Minipreps of plasmid DNA. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 1.6.1-1.6.10. John Wiley & Sons, Hoboken, N.J.

Giancotti, F.G. and Ruoslahti, E. 1999. Integrin signaling. *Science* 285:1028-1032.

Guan, J.L. and Shalloway, D. 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* 358:690-692.

Hanks, S.K., Calalb, M.B., Harper, M.C., and Patel, S.K. 1992. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. U.S.A.* 89:8487-8491.

Hynes, R.O. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell* 110:673-687.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Natkatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from *fak*-deficient mice. *Nature* 377:539-544.

Klingbeil, C.K., Hauck, C.R., Hsia, D.A., Jones, K.C., Reider, S.R., and Schlaepfer, D.D. 2001. Targeting Pyk2 to β1-integrin-containing focal contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells. *J. Cell Biol.* 152:97-110.

Kornberg, L., Earp, H.S., Parsons, J.T., Schaller, M., and Juliano, R.I. 1992. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* 267:23439-23442.

Liu, S., Thomas, S.M., Woodside, D.G., Rose, D.M., Kiosses, W.B., Pfaff, M., and Ginsberg, M.H. 1999. Binding of paxillin to α4 integrins modifies integrin-dependent biological responses. *Nature* 402:676-681.

Parsons, J.T. 2003. Focal adhesion kinase: The first ten years. *J. Cell Sci.* 116:1409-1416.

Schaller, M.D., Borgman, C.A., Cobb, B.A., Vines, R.R., Reynolds, A.B., and Parsons, J.T. 1992. $pp125^{fak}$ a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. U.S.A.* 89:5192-5196.

Schlaepfer, D.D. and Mitra, S.K., 2004. Multiple connections link FAK to cell motility and invasion. *Curr. Opin. Genet. Dev.* 14:92-101.

Schlaepfer, D.D., Hauck, C.R., and Sieg, D.J. 1999. Signaling through focal adhesion kinase. *Prog. Biophys. Mol. Biol.* 71:435-478.

Schwartz, M.A. 2001. Integrin signaling revisited. *Trends Cell Biol.* 11:466-70.

Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. 1997. Introduction of plasmid DNA into cells. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 1.8.1-1.8.10. John Wiley & Sons, Hoboken, N.J.

Sieg, D.J., Ilic, D., Jones, K.C., Damsky, C.H., Hunter, T., and Schlaepfer, D.D. 1998. Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK^- cell migration. *EMBO J.* 17:5933-5947.

Sieg, D.J., Hauck, C.R., and Schlaepfer, D.D. 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J. Cell Sci.* 112:2677-2691.

Contributed by Joie A. Bernard-Trifilo,
Ssang-Taek Lim, Shihe Hou, and
David D. Schlaepfer
The Scripps Research Institute
La Jolla, California

Dusko Ilic
Stem Life Line, Inc.
San Carlos, California

Rho GTPase Activation Assays

Stéphanie Pellegrin[1] and Harry Mellor[1]

[1]Mammalian Cell Biology Laboratory, Department of Biochemistry, School of Medical Sciences, University of Bristol, United Kingdom

ABSTRACT

The Rho GTPase family of signaling proteins controls a wide range of highly dynamic cellular processes. Activation of Rho GTPases can be investigated and quantified in cell extracts using so-called pull-down assays. Proteins that bind specifically to the activated form of the Rho GTPase are used to capture it onto a bead support. Western blotting of the captured samples with specific antibodies then allows for quantification of the level of Rho GTPase activation in the sample. This unit describes the techniques for preparing the reagents required for assays of RhoA, Rac, and Cdc42 and gives practical tips for the successful application of the assay in a range of situations. *Curr. Protoc. Cell Biol.* 38:14.8.1-14.8.19. © 2008 by John Wiley & Sons, Inc.

Keywords: Rho GTPase • cytoskeleton • cell signaling

INTRODUCTION

Most Rho GTPases cycle between an active GTP-bound and an inactive GDP-bound state. When bound to GTP, Rho GTPases can interact with their effector proteins, and this nucleotide-dependent interaction forms the basis of the activation assays described below. The Rho GTPase-binding domain (RBD) of Rho GTPase effectors can be made

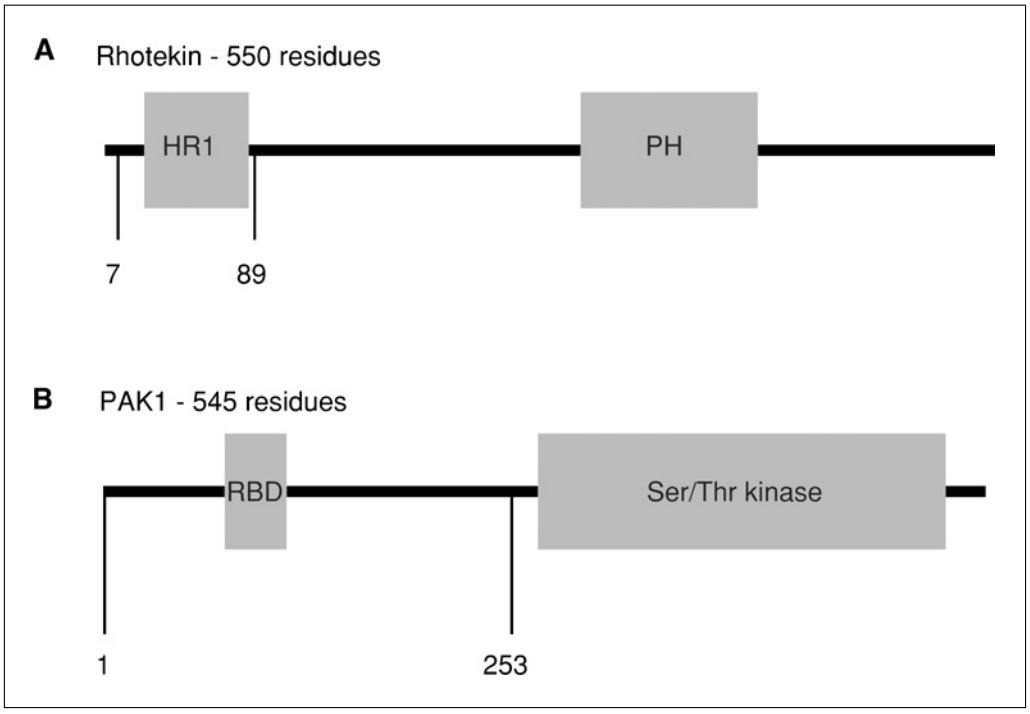


Figure 14.8.1 Diagram of Rho-binding domains used for GST pull-down assays: (**A**) Rhotekin-RBD and (**B**) PAK-CRIB. The GST-RBD constructs we have used span residues 7 to 89 of Rhotekin variant 2 (accession number NM_033046) and residues 1 to 253 of PAK1 (accession number NM_002576). When made as GST-fusion proteins, these are 35.7-kDa and 55.7-kDa respectively. Abbreviations: HR1, protein kinase C-related kinase homology region 1; PH, pleckstrin homology domain; RBD, Rho binding domain.

Current Protocols in Cell Biology 14.8.1-14.8.19, March 2008
Published online March 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1408s38

as a glutathione-*S*-transferase (GST) fusion protein, coupled to beads, and added to cell lysates to pull down the active, GTP-bound form of the Rho GTPase (see *UNIT 17.5*). The RhoA binding domain from Rhotekin has been widely used to pull down active RhoA (Ren et al., 1999; Ren and Schwartz, 2000), whereas the PAK-CRIB domain of PAK1 is favored for Rac1 and Cdc42 activation assays (Sander et al., 1998; Benard et al., 1999; Benard and Bokoch, 2002). These constructs are shown in Figure 14.8.1. The CRIB motif of WASP has also been used for Cdc42 activation assays (Haddad et al., 2001).

This unit describes how to make recombinant GST-PAK-CRIB beads for Rac1 or Cdc42 activation assays and recombinant GST-Rhotekin-RBD beads for RhoA activation assays (Support Protocol). We use the same protocol to prepare both types of GST-RBD beads; the only difference is the construct used. The activation assay itself is detailed in the Basic Protocol and Alternate Protocol 1. Additional protocols describe how to load Rho GTPases with GDP, GTP, or GTPγS (Alternate Protocol 2) and how to perform activation assays on nonadherent cells (Alternate Protocol 3). Finally, suggestions on how to design activation assays for Rho GTPases other than RhoA, Rac1, and Cdc42 are given in the Commentary.

STRATEGIC PLANNING

The first step consists of making the recombinant GST-RBD fusion proteins. These are loaded onto glutathione-coupled beads and stored with the beads at −80°C in a buffer containing 10% glycerol. Following this, a small aliquot of the GST-RBD beads is run on an SDS-PAGE gel to estimate the amount of fusion protein made and purified. All of these steps are described in the Support Protocol.

The GST-RBD beads then need to be tested to verify that they only interact with the active, GTP-bound form of the Rho GTPase of interest. Good controls for Rac1 and RhoA activation assays are described in the Basic Protocol. These include treatment of serum-starved HeLa cells (70% confluent) with 100 ng/ml epidermal growth factor (EGF) for 3 min, which induces robust Rac1 (e.g., see Figure 14.8.2) and RhoA activation. A clear increase in active Rac1 or RhoA should be seen when comparing the amount of active GTPase in starved and agonist-treated cells. In order to test GST-RBD beads before use in Cdc42 activation assays, it is easier to load Cdc42 with GTP than to use agonist stimulation because few agonists give marked activation of this small GTPase. This method is described in Alternate Protocol 2, and expected results are shown in Figure 14.8.4. Since the GST-RBD beads can be stored at −80°C for long periods of time, it is important to test them regularly to ensure they have not lost their specificity for active Rho GTPase over time.

BASIC PROTOCOL

Rac1 ACTIVATION ASSAYS ON HeLa CELLS

This protocol describes how to prepare the cell lysates to carry out Rac1 and RhoA (also see Alternate Protocol 1) activation assays on agonist-treated cells. The conditions described in this protocol (see step 2) are used as a quality control or positive control for Rac1 or RhoA activation (see Fig. 14.8.2 for an example of a Rac1 activation assay). This protocol forms the basis for activation assays that can be carried out on cells treated with other agonists or over-expressing Rho GTPase mutants (active, GTP-bound or inactive, GDP-bound mutants), inhibitory GTPase activating proteins (GAPs), or activating guanine nucleotide exchange factors (GEFs) of interest. This protocol is also valid for Cdc42 activation assays although it is unclear which agonist can be used to obtain good Cdc42 activation. In this case, the alternative control is to load endogenous Cdc42 with GDP, GTP, or GTPγS (see Alternate Protocol 2 and Figure 14.8.4).

Preparation of cell lysates for Rac1 and RhoA activation assays are identical except for the lysis buffers used—lysis buffer B and lysis buffer C (see Alternate Protocol 1),

respectively. In all cases, it is important to avoid phosphate-based buffers for washing or lysing the cells because phosphate forms a precipitate with magnesium (Ren and Schwartz, 2000). $MgCl_2$ is present in the lysis buffer to stabilize GTP-bound GTPases and to prevent nucleotide exchange.

In an experimental setting, the level of activation obtained should be quantified by normalizing the amount of GST-RBD bound GTPase (i.e., active GTPase) to the amount of total GTPase. Care must be taken when quantifying scanned images of X-ray films because they have a narrow linear range of exposure (Ren and Schwartz, 2000); it is very important to make sure that the films are not overexposed. Activation assays are usually performed at least three times so that the mean (± standard deviation) of the ratio of active over total GTPase signal can be shown.

Materials

HeLa cells: 70% confluent 10-cm plates (two plates per experiment)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
100 μg/ml epidermal growth factor (EGF; Calbiochem); store aliquots at −80°C
DMEM/0.1% (w/v) fatty-acid-free bovine serum albumin (BSA)
Tris-buffered saline (TBS): 50 mM Tris·Cl (pH 7.6; see *APPENDIX 2A*)/140 mM NaCl, ice cold
Lysis buffer B, ice cold (see recipe)
4× SDS-PAGE sample buffer (see recipe)
GST-PAK-CRIB beads in Tris wash buffer A/10% (v/v) glycerol (Support Protocol)
Tris wash buffer B (see recipe), ice cold
2× SDS-PAGE sample buffer (see recipe)
SDS-PAGE gel (see *UNIT 6.1*)

Refrigerated centrifuge, 4°C
Heating block, 95°C
0.5- to 20-μl GELoader tips (Eppendorf)

Additional reagents and equipment for performing SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Wash two 10-cm plates of 70% confluent HeLa cells twice with 10 ml PBS and serum starve overnight in DMEM/0.1% (w/v) fatty-acid-free BSA.
2. Incubate one of the plates of HeLa cells 3 min with 10 ml of 100 ng EGF/ml of DMEM/0.1% fatty-acid-free BSA (prepared by diluting the 100 μg/ml EGF in the medium).

   The second plate in each experiment is a control. It is serum-starved but not treated with agonist. It is incubated overnight in DMEM/0.1% (w/v) fatty-acid-free BSA and remains in the cell incubator while treating the other plate with agonist.

3. On ice, quickly wash the cells twice in 10 ml ice-cold TBS and drain.
4. Still on ice, extract each plate in 800 μl ice-cold lysis buffer B.

   It is important to keep the samples cold and to work quickly throughout the protocol. Over time, the GAP activity in the cell lysates can drastically reduce the amount of active GTPase (Ren and Schwartz, 2000).

5. Centrifuge the cell lysates 10 min at 25,000 × *g*, 4°C. Carefully collect the supernatant and transfer to a new tube on ice.
6. Meanwhile, thaw 400 μl GST-PAK-CRIB beads on ice.
7. Remove 30 μl from each cleared cell lysate for total Rac1 protein control and add to 10 μl of 4× SDS-PAGE sample buffer. Heat 10 min at 95°C.

Taking samples from each lysate for each time point is important because these will be used to determine the amount of total GTPase, which is used to normalize the amount of active, GTP-bound GTPase.

8. Transfer 650 µl of each cell lysate into a microcentrifuge tube with 200 µl (or the equivalent of 30 µg) of GST-PAK-CRIB beads in Tris wash buffer A/10% glycerol.
9. Rotate 45 min at 4°C.
10. Wash the beads four times with 600 µl ice-cold Tris wash buffer B: resuspend the beads gently by inversion, centrifuge 20 sec at 500 × *g*, 4°C, and remove the supernatant.
11. After the last wash, use the thin GELoader pipet tip to remove all of the remaining buffer from the beads.

    This can be done by attaching the GELoader tips to a vacuum aspirator. These tips have a smaller diameter than the beads and the beads will not be aspirated. As the buffer is removed, the beads should become whiter.

12. Elute the proteins from the beads by adding 50 µl hot 2× SDS-PAGE sample buffer/40 mM DTT and heating 10 min at 95°C.
13. Load both the samples for total (obtained in step 7) and active Rac1 (obtained in step 12) onto an SDS-PAGE gel to resolve the ~21 kDa GTPase (*UNIT 6.1*) and process for western blotting (*UNIT 6.2*).

    The amount of total and active Rac1 will vary with the conditions and cell type used, so the volume of cell lysate to load in each well should be determined accordingly. As a starting point, 20 µl of total and 10 µl of active Rac1 can be loaded for each time point (Fig. 14.8.2).

    We have used 15% Tris·Cl gels but have found that 12% Bis-Tris gels run with MES buffer give much better resolution and allow for better detection.

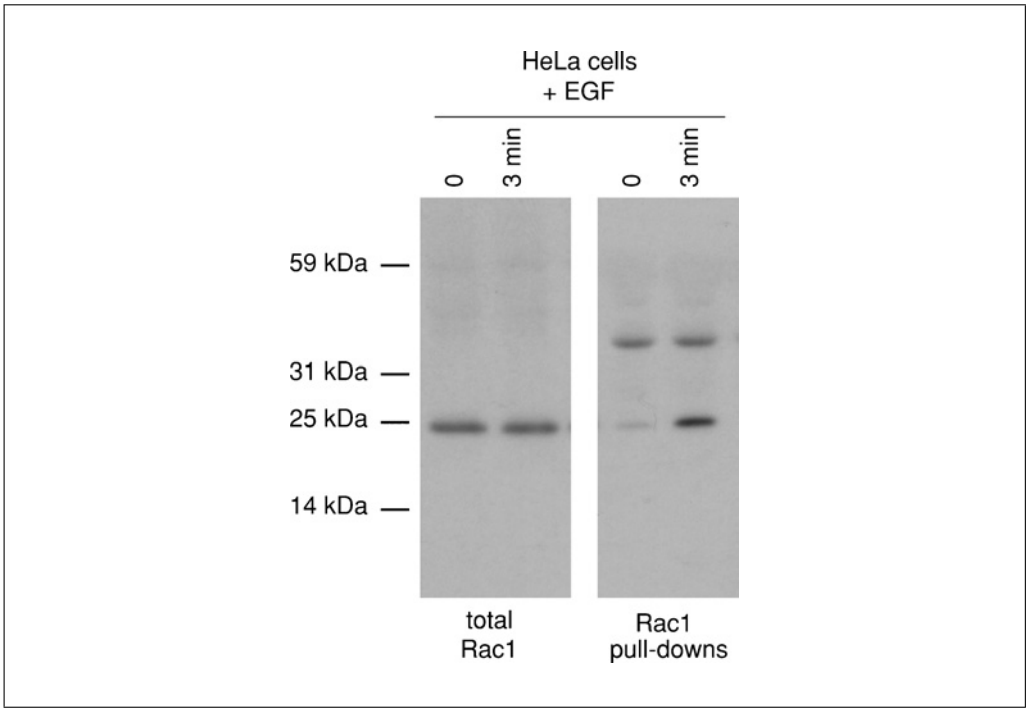


Figure 14.8.2 Rac1 activation assays carried out on HeLa cells. Total Rac1 is shown in the left panel and the activation assay in the right panel. After 3 min of EGF treatment (100 ng/ml), Rac1 is activated as seen by the appearance of Rac1 in the right-hand panel. The upper bands are nonspecific signal from the GST-RBD protein.

MAKING GST-RBD BEADS FOR ACTIVATION ASSAYS

SUPPORT PROTOCOL

The CRIB motif of PAK1 interacts with active (i.e., GTP-bound) Rac1 and Cdc42 (Burbelo et al., 1995) and has thus been used extensively to pull down active Rac1 and Cdc42 from cell lysates (Sander et al., 1998; Benard et al., 1999; Benard and Bokoch, 2002). The WASP CRIB motif has also been used to pull down active Cdc42, but this construct does not interact with Rac1 (Haddad et al., 2001). It is still unclear which of the CRIB motifs gives a more robust assay for Cdc42 activation. For RhoA, the Rho-binding domain of Rhotekin is used (Reid et al., 1996; Ren et al., 1999; Ren and Schwartz, 2000).

This protocol assumes that the construct of interest is available and already transformed into *Escherichia coli* to make recombinant protein. For activation assays on RhoA, the construct used consists of the cDNA sequence for amino acids 7 to 89 of Rhotekin cloned into a pGEX2T vector (Amersham; Fig. 14.8.1A). For Rac1 and Cdc42 activation assays, we use a construct which consists of the cDNA sequence for amino acids 1 to 253 of PAK1 cloned into the same expression vector (Ren and Schwartz, 2000; Fig. 14.8.1B). In pGEX vectors the GST-fusion protein is expressed from the tac promoter after induction with IPTG. Because these vectors also carry the *lacI^q* gene, they can be used in any *E. coli* host. We have used BL21(DE3)pLysS *E. coli*, but other hosts such as simple BL21 *E. coli* should work as well.

This protocol describes how to make beads from 4 liters of *E. coli* culture, which will yield enough GST-RBD beads for at least 40 activation assays. The method can theoretically be scaled down, but this is not recommended because using smaller volumes may cause the solutions to undergo greater temperature fluctuations during the procedure.

Materials

LB broth (*UNIT 20.2*)
100 mg/ml ampicillin stock solution in water; store small aliquots at −20°C
34 mg/ml chloramphenicol stock solution in ethanol; store small aliquots at −20°C, protected from light
E. coli: e. g., BL21(DE3)pLysS (Stratagene)
GST-RBD construct (obtained from the authors or self-constructed; see Figure 14.8.1)
1 M isopropyl-β-D-thiogalactopyranoside (IPTG); store small aliquots at −20°C
Glutathione-Sepharose 4B beads (Amersham)
Lysis buffer A (see recipe), ice cold
Tris wash buffer A (see recipe), ice cold
Tris wash buffer A (see recipe)/10% (v/v) glycerol, ice cold
4× SDS-PAGE sample buffer (see recipe)
12% SDS-PAGE gel (see *UNIT 6.1*)
Bovine serum albumin (BSA)
Coomassie blue staining solution (*UNIT 6.1*)

Shaking incubator, 37°C
2-liter flasks
Refrigerated centrifuge, 4°C
30-ml centrifuge tubes, capable of withstanding 17000 × *g* for 30 min (e. g., Oak Ridge), prechilled
Sonicator
15- and 50-ml polypropylene tubes (e. g., Falcon), prechilled
End-over-end rotator, 4°C
Heating block, 95°C
Microcentrifuge tubes, prechilled

Additional reagents and equipment for performing SDS-PAGE (*UNIT 6.1*)

Grow plasmid-bearing bacteria

1. Inoculate 5 ml LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol with a single colony of *E. coli* containing the GST-RBD construct.

   Instead of keeping glycerol stocks, it is preferred to keep the DNA at −20°C and to transform E. coli when needed. The plates can be kept for a month in the refrigerator.

   Chloramphenicol is required for maintenance of the pLysS plasmid of the BL21(DE3)pLysS E. Coli. It is not needed if other hosts are used.

2. Incubate overnight at 37°C, with shaking.
3. Inoculate 400 ml LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol with the 5-ml overnight culture.
4. Incubate overnight at 37°C, with shaking.
5. Prewarm 4 liters LB broth overnight at 37°C.

   LB broth is kept in a 37°C incubator overnight to save time the next day.

6. Dilute the overnight culture 1:10 into prewarmed 3.6 liters LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol (typically, 100 ml culture in 900 ml broth in a 2-liter flask).
7. Grow to $OD_{600} = 0.8$.

   This usually takes ∼1 hr, but the OD should be checked after 45 min.

Induce protein synthesis

8. Add IPTG to a final concentration of 0.5 mM IPTG using the 1M IPTG stock. Incubate 2 hr at 37°C.
9. Harvest by centrifuging 25 min at 2500 × *g*, 4°C.

   The culture does not have to be precooled.

10. Carefully remove the supernatant
11. Keep the bacterial pellets on ice and resuspend them completely in a total of 40 ml ice-cold lysis buffer A (i.e., 10 ml lysis buffer A per liter of bacteria culture)

    It is important to keep the solutions cold at all times. Everything should be kept on ice, and, if possible, working in a cold room is recommended.

    The bacteria should be completely resuspended before sonication (step 13). This is best achieved by first resuspending the pellet into only a small volume of lysis buffer, before adding the entire 40 ml. The suspension should be homogenous and devoid of clumps.

Sonicate bacteria

12. Transfer the suspension into two prechilled 30-ml centrifuge tubes.

    A volume of ∼20 ml is dispensed equally into each tube. These centrifuge tubes should be able to withstand centrifugation at 17,000 × g for 30 min (see step 14).

13. Sonicate on ice six to eight times for 15 sec each. Cool 2 min on ice between sonication bursts to prevent the lysates from overheating.

    It is important that the samples do not overheat and do not foam. The settings for sonication will vary from sonicator to sonicator. The samples should be checked regularly.

    Expect the lysate to first become a little viscous as the cells release their DNA—you may see a trail from the sonicator probe when you remove it from the lysate. This viscosity should disappear with further sonication as the DNA is sheared. It is important that all traces of viscous material are removed by sonication, or the lysate will not form a proper pellet of debris in the next step.

14. Centrifuge 30 min at 17,000 × *g*, 4°C.

Prepare beads

15. In the meantime, equilibrate 0.6 ml (packed bead volume) of glutathione-Sepharose 4B beads in lysis buffer A by washing them three times in lysis buffer A: add 0.8 ml lysis buffer A, resuspend the beads by inverting the tube gently, collect the beads by brief centrifugation (1 min at 500 × *g*, 4°C), and remove the supernatant without disturbing the bead pellet.

    The beads are sold as a suspension in ethanol. To obtain a reasonably accurate volume of packed beads, it is useful to first pipet 0.6 ml of lysis buffer A into a tube (e.g., a 1.5-ml microcentrifuge tube) and to draw a line at the level of the meniscus. Remove the buffer and add bead suspension until the level of packed beads meets the line on tube.

Bind GST-RBD to beads

16. Remove the clarified bacteria lysate supernatant and transfer the ~40 ml of lysate to a prechilled 50-ml polypropylene tube.

17. Add the equilibrated glutathione-Sepharose 4B beads (prepared in step 15) to the lysate.

    To make sure all the beads are transferred, resuspending the beads in a small volume of lysate is recommended. The tube that contained the beads can also be washed several times with lysate.

18. Rotate 60 min at 4°C.

19. Centrifuge 1 min at 500 × *g*, 4°C.

Recover bound beads

20. Carefully discard the supernatant without disturbing the bead pellet and add 12 ml ice-cold Tris wash buffer A.

21. Resuspend the beads by gentle inversions and transfer everything into a prechilled 15-ml polypropylene tube. Centrifuge 1 min at 500 × *g*, 4°C.

22. Wash the bead pellet another five times by discarding the supernatant, resuspending the beads in 12 ml ice-cold Tris wash buffer A by gentle inversions, and centrifuging 1 min at 500 × *g*, 4°C.

23. Wash once with 12 ml Tris wash buffer A/10% glycerol as in step 22.

24. Resuspend the beads in 8 ml Tris wash buffer A/10% glycerol.

25. Remove a 30-µl aliquot of beads in Tris wash buffer A/10% glycerol and add to 10 µl of 4× SDS-PAGE sample buffer. Heat at 95°C for 10 min and store at −70°C.

    This sample will be used to check the protein preparation on an SDS-PAGE by Coomassie blue staining (see steps 27 to 29).

26. Divide the beads into small aliquots in prechilled microcentrifuge tubes and store at −70°C.

    200 µl of beads in Tris wash buffer A/10% glycerol are generally used for each activation assay; make aliquots of 400 µl and 800 µl for two or four activation assays, respectively.

Quantify GST-RBD bound to beads

27. On a 12% SDS-PAGE gel, load 21 µl, 14 µl, and 7 µl of beads in 4× SDS-PAGE sample buffer prepared in step 25.

    This is equivalent to loading 15 µl, 10 µl, and 5 µl of beads in Tris wash buffer A/10% glycerol.

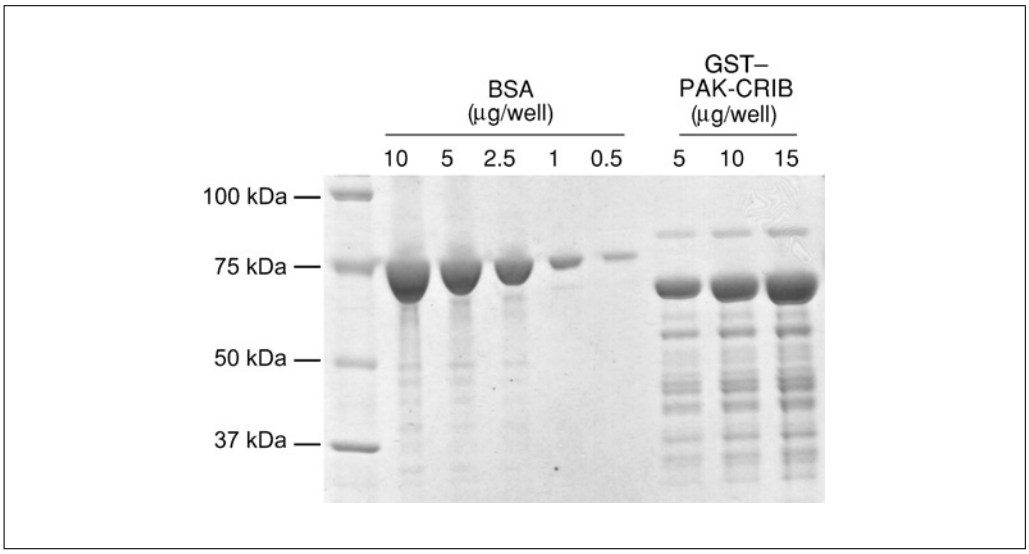


Figure 14.8.3 GST-PAK-CRIB preparation visualized on an SDS-PAGE gel stained with Coomassie blue. 45 μl of bead suspension in glycerol buffer was added to 15 μl of 4× SDS-PAGE sample buffer to give a final volume of 60 μl. Aliquots of 7 μl, 14 μl, and 21 μl (corresponding to 5, 10, and 15 μl of bead suspension respectively) were added to each well and compared to a dilution range of BSA (10, 5, 2.5, 1, and 0.5 μg per well). In this preparation 5 μl of bead suspension is equivalent to ~2.5 μg of protein, so the 200 μl of bead suspension used per assay is equivalent to ~100 μg of protein (30 μg would be fine).

28. Also load different amounts of BSA (e.g., 0.5 μg, 1 μg, 2.5 μg, 5 μg, and 10 μg of BSA per well).

29. Perform electrophoresis, staining with Coomassie blue (see *UNIT 6.1*).

30. Estimate the amount of GST-RBD protein bound to the beads by comparing band intensities to that of the known amounts of BSA.

    Usually, there is at least ~2.5 μg of GST-PAK-CRIB for 5 μl of beads in Tris wash buffer A/10% glycerol (Fig. 14.8.3). We use 30 to 100 μg of fusion protein per assay. This is considerably more than supplied by commercial assay kits, and the assay is consequently more sensitive.

    The presence of breakdown products is typical in these preparations, and they do not affect the assay (Benard and Bokoch, 2002).

ALTERNATE PROTOCOL 1

RhoA ACTIVATION ASSAYS

The only difference between preparing cell lysates for Rac1/Cdc42 activation assays and RhoA activation assays lies in the constituents of the lysis buffer. The lysis buffer used for RhoA activation assays (lysis buffer C) contains 0.1% SDS (Ren and Schwartz, 2000). This prevents nonspecific interactions in this assay but would affect Rac1 or Cdc42 binding to GST-PAK-CRIB if used in the Basic Protocol (Benard and Bokoch, 2002). As in the Basic Protocol, it is important to avoid phosphate-based buffers for washing or lysing the cells because phosphate forms a precipitate with magnesium (Ren and Schwartz, 2000). $MgCl_2$ is present in the lysis buffer to stabilize GTP-bound GTPases and to prevent nucleotide exchange. The high salt concentration has been shown to help minimize GAP activity that might otherwise lead to inactivation of Rho GTPases in the lysate (Ren and Schwartz, 2000).

***Additional Materials** (also see the Basic Protocol)*

Swiss 3T3 cells: e.g., 70% confluent 10-cm plates (two plates per experiment)
Lysis buffer C, ice cold
GST-Rhotekin-RBD beads in wash buffer A/10% (v/v) glycerol (Support Protocol)

Proceed as in the Basic Protocol with changes in the following steps:

2. Treat HeLa cells as described in the Basic Protocol, or, alternatively, treat serum-starved Swiss 3T3 cells with 10 ml of 1 μg/ml lysophosphatidic acid (LPA) for 1 min (Ren et al., 1999) or with 10% (v/v) serum for 2 to 3 min (Ren and Schwartz, 2000).

4. Still on ice, extract each plate in 800 μl ice-cold lysis buffer C.

   Lysis buffer C contains 0.1% SDS (Ren and Schwartz, 2000). This prevents nonspecific interactions, but if used for Rac1 activation assays (see Basic Protocol) would affect Rac1 or Cdc42 binding to GST-PAK-CRIB (Benard and Bokoch, 2002).

8. Transfer 650 μl of each cell lysate into a microcentrifuge tube with 200 μl (or the equivalent of 30 μg) of GST-Rhotekin-RBD beads in wash buffer A/10% glycerol.

LOADING Rac1 OR Cdc42 WITH NUCLEOTIDE

ALTERNATE PROTOCOL 2

This protocol provides an alternative control to the ones described above and is useful when no strong agonist is known (e.g., for Cdc42). The cells are lysed in a buffer devoid of magnesium. To load the endogenous GTPases with nucleotide, the cleared lysates are spiked with a magnesium-chelating agent (EDTA) and a high concentration of GDP, GTP, or GTPγS. Under these conditions, the GTPases will bind the most abundant nucleotide. Loading of endogenous GTPases with GTP rather than GTPγS gives a more realistic view of the level of activation one can expect in the cell. The exchange is stopped by adding magnesium chloride, and GST-RBD beads are added to carry out a pull-down assay.

An example of a Rac1 and Cdc42 activation assay carried out after nucleotide exchange is shown in Figure 14.8.4. An aliquot of the lysate is taken to show basal levels of the GTPase of interest before nucleotide exchange. Also recommended is taking an aliquot after nucleotide loading (but before adding the beads) to check that the GTPase has not been degraded during the incubation at 30°C. The only difference between the protocols for RhoA or Rac1/Cdc42 is the time of incubation at 30°C, during which nucleotide loading takes place. This protocol is theoretically applicable to other Rho GTPases but the conditions for nucleotide loading might have to be optimized (e.g., time and temperature of incubation).

Materials

10-cm plates of cells (two plates for each experiment)
Tris-buffered saline (TBS): 50 mM Tris·Cl (pH 7.6; see *APPENDIX 2A*)/140 mM NaCl, ice cold
Lysis buffer D (see recipe), ice cold
4× SDS-PAGE sample buffer (see recipe)
10 mM GTPγS (Calbiochem)/20 mM HEPES, pH 7.4; store aliquots at −20°C
100 mM GDP (Sigma)/20 mM HEPES, pH 7.4; store aliquots at −20°C
0.5 M EDTA, pH 8.0 (see *APPENDIX 2A*)
1 M $MgCl_2$
GST-RBD beads in Tris wash buffer A/10% glycerol (Support Protocol)
Tris wash buffer C (see recipe), ice cold
2× SDS-PAGE sample buffer (see recipe), 95°C
SDS-PAGE gel (see *UNIT 6.1*)

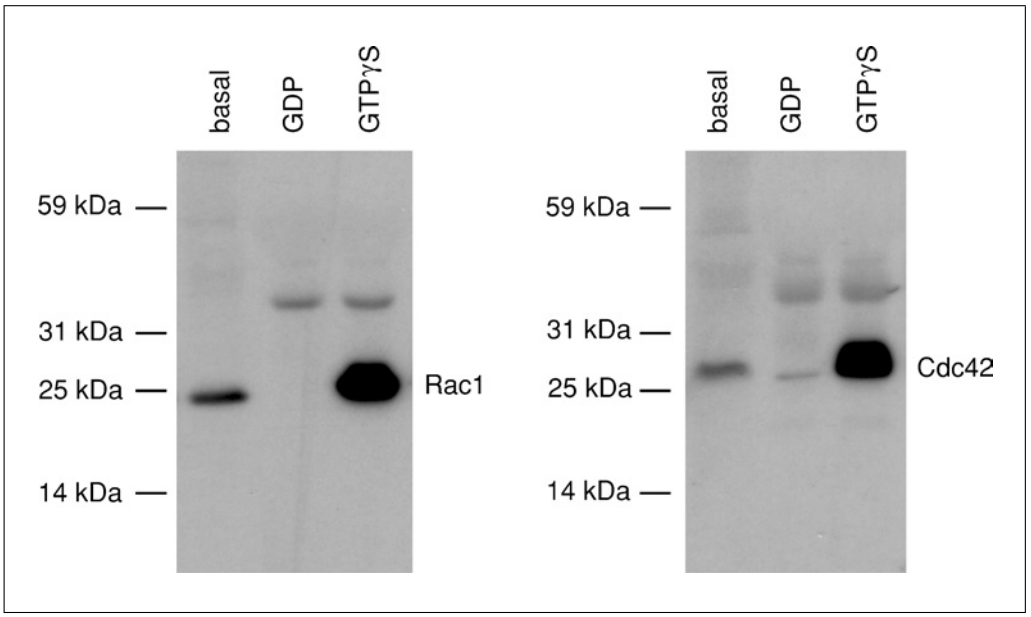


Figure 14.8.4 GDP and GTPγS loading of Rac1 and Cdc42. HeLa cells were lysed and loaded with GDP or GTPγS as described in Alternate Protocol 2, and Rac1 or Cdc42 activation assays were carried out. The amount of GTPase in lysates to which no nucleotide was added is referred to as basal.

Refrigerated centrifuge, 4°C
Shaking incubator, 30°C
Heating block, 95°C
End-over-end rotator, 4°C
0.5- to 20-µl GELoader tips (Eppendorf)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B* or *3H*) and performing SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Wash two 10-cm plates of cells twice with 10 ml cold TBS.
2. Extract each plate in 600 µl lysis buffer D.
3. Clear the lysate by centrifuging 10 min at 25,000 × *g*, 4°C.
4. Remove 60 µl of supernatant (to determine total protein; e.g., see *APPENDIX 3B or 3H*) and add 20 µl of 4× SDS-PAGE sample buffer.
5. Transfer 500 µl of each lysate to a new tube.

6a. *To load GTPγS:* Add

6 µl of 10 mM GTPγS (≥100 µM final concentration)
12 µl of 0.5 M EDTA pH 8.0 (≥10 mM final concentration).

Pipet gently to mix, using a pipet volume of ≥200 µl.

6b. *To load GDP:* Add

6 µl of 100 mM GDP (≥1 mM final concentration)
12 µl of 0.5 M EDTA pH 8.0 (≥10 mM final concentration).
Pipet gently to mix, using a pipet volume of ≥200 µl.

7. Incubate 15 min at 30°C, with gentle shaking (for Cdc42 and Rac1), or 30 min for RhoA.

8. Terminate the exchange by adding 38 µl of 1 M $MgCl_2$ to each tube (≥60 mM final concentration).
9. Add 200 µl (or 30 µg) GST-RBD beads in Tris wash buffer A/10% glycerol to each of the tubes.
10. Rotate 45 min at 4°C.
11. Centrifuge 20 sec at 500 × *g*, 4°C, and discard the supernatant.
12. Wash the beads four times in 600 µl cold Tris wash buffer C: add 600 µl cold Tris wash buffer C, invert the tube to gently resuspend the beads, and centrifuge 20 sec at 500 × *g*, 4°C, for each wash.
13. After the last wash, use the GELoader tips to remove all of the remaining buffer from the beads.
14. Elute with 50 µl of hot (95°C) 2× SDS-PAGE sample buffer.
15. Heat 10 min at 95°C.
16. Dilute again two-fold in 2× SDS-PAGE sample buffer and load 10 µl per well of an SDS-PAGE gel (*UNIT 6.1*) for immunoblotting (*UNIT 6.2*).

    The exact amount to load may vary and should be determined accordingly.

Rac1 ACTIVATION ASSAY ON NONADHERENT CELLS

ALTERNATE PROTOCOL 3

Preparing lysates from nonadherent cells to carry out an activation assay consists of spiking the cell culture medium with a concentrated solution of agonist and stopping the activation by adding an equal volume of 2× lysis buffer. This allows for rapid lysis just after agonist treatment. The protocol below is based on that described by Takesono et al. (2004). The activation assay for Rac1 is carried out on a lymphocyte cell line treated with 100 ng/ml of SDF1α for 0 sec, 30 sec, 2, 5, and 10 min. The results are shown in Figure 14.8.5.

Materials

Cultures of cells in suspension
RPMI/0.1% (w/v) fatty-acid-free BSA
RPMI/0.1% (w/v) fatty-acid-free BSA/20 mM HEPES, pH 7.4
SDF1α (R&D #350-NS)
2× lysis buffer (see recipe), ice cold
GST-PAK-CRIB beads in Tris wash buffer A/10% (v/v) glycerol (Support Protocol)
4× SDS-PAGE sample buffer (see recipe)
2× SDS-PAGE sample buffer (see recipe)
SDS-PAGE gel (see *UNIT 6.1*)

Refrigerated centrifuge, 8°C
0.5- to 20-µl GELoader tips (Eppendorf)
Heating block, 95°C

Additional reagents and equipment for performing SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

Prepare cells for assay

1. Starve the suspension cells for 4 hr in RPMI/0.1% (w/v) fatty-acid-free BSA.

   It might be possible to starve certain cell lines overnight and this could help reduce the basal level of active GTPase. The protocol can be adapted accordingly.

2. Prewarm the RPMI/ 0.1% (w/v) fatty-acid-free BSA/20 mM HEPES.

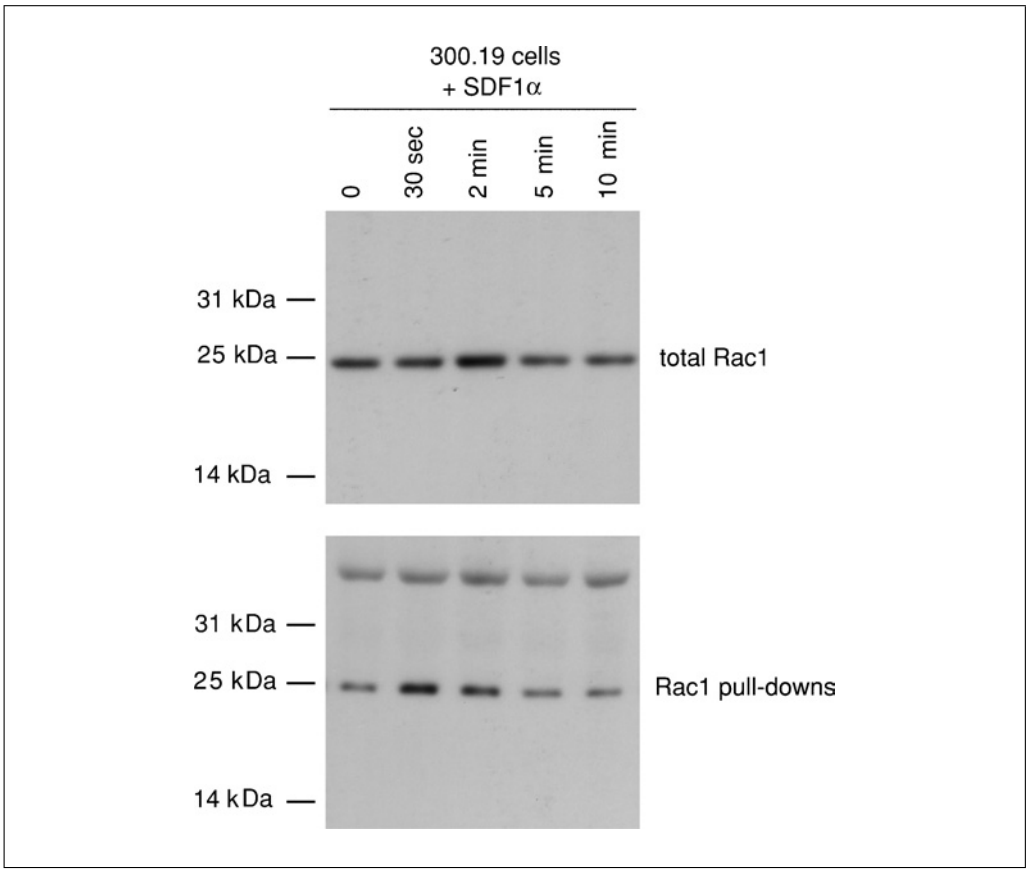


Figure 14.8.5 Rac1 activation assay carried out on nonadherent cells. Nonadherent 300.19 cells, a mouse pre-B lymphocyte cell line, were treated with SDF1α for different time points and Rac1 activation assays were carried out. The levels of total Rac1 are shown in the top panel (exposure of time 30 sec), and the bottom panel shows the levels of active Rac1 (exposure time of 1 min 30 sec). Rac1 is activated after 30 sec but the activation is lost by 5 min.

3. Resuspend the cells at 4.4×10^7 cells/ml in RPMI/0.1% (w/v) fatty-acid-free BSA/20 mM HEPES. For five time points, prepare 6.6×10^7 cells in 1.5 ml.
4. Transfer 225 µl of cells (10^7 cells) for each of the five time points into microcentrifuge tubes.
5. Prepare at least 120 µl of 1 µg/ml SDF1α in RPMI/0.1% (w/v) fatty-acid-free BSA/20 mM HEPES.

   SDF1α at 1 µg/ml is ten times more concentrated than the final SDF1α concentration to which the cells will be exposed. When added to the cells, the concentrated solution of SDF1α will be diluted 1:10 by the cell culture medium, and the final concentration of SDF1α will be 100 ng/ml.

6. To the cells corresponding to the 0 sec time point, add 25 µl of RPMI/0.1% (w/v) fatty-acid-free BSA/20 mM HEPES.

Perform assay

7. To each of the tubes for the other four time points, add 25 µl of the 1 µg/ml SDF1α to the 225-µl cell suspension prepared in step 4.

   Each tube will contain 10^7 cells in 250 µl and a final concentration of 100 ng/ml of SDF1α.

8. Stop the reaction by adding 250 µl ice-cold 2× lysis buffer. Keep the lysates on ice.

Analyze lysates

9. Clear the lysates by centrifuging 10 min at 25,000 × *g*, 4°C.
10. Meanwhile, thaw 1 ml (or the equivalent of 150 μg) of GST-PAK-CRIB beads in Tris wash buffer A/10% glycerol on ice.
11. Transfer the cleared lysates to new tubes.
12. For each time point, transfer 30 μl of cleared cell lysate to a new tube and add 10 μl of 4× SDS-PAGE sample buffer. Heat 10 min at 95°C.

    These will be the "total" Rac1 samples.

13. Add 200 μl of beads in Tris wash buffer A/10% glycerol (or the equivalent of 30 μg) to 450 μl of cleared lysate (the rest of the cleared lysate).
14. Rotate end-over-end 45 min at 4°C.
15. Wash the beads four times with 0.5 ml ice-cold 1× lysis buffer (dilute 2× lysis buffer): add 0.5 ml cold 1× lysis buffer, invert the tube to gently resuspend the beads, and centrifuge 20 sec at 500 × *g*, 4°C, for each wash.
16. After the last wash, use the thin GELoader pipet tip to remove all of the remaining buffer from the beads.
17. Elute the proteins from the beads by adding 50 μl of 2× SDS-PAGE sample buffer and heat for 10 min at 95°C.
18. Load both the samples for total (obtained on step 12) and active Rac1 (obtained in step 17) onto an SDS-PAGE gel to resolve the ~21-kDa GTPase (*UNIT 6.1*), and process for immunoblotting (*UNIT 6.2*).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Lysis buffer, 2× (for nonadherent cells)

100 mM HEPES, pH 7.4
300 mM NaCl
2% (w/v) Triton X-100
20 mM $MgCl_2$
20 μg/ml aprotinin
20 μg/ml leupeptin
Prepare fresh and keep on ice
Add 0.4 mM PMSF (use 200 mM stock; see recipe) just before use

For nonadherent cells, the lysis buffer has to be twice as concentrated (2× lysis buffer) because it will be diluted in half by the cell culture medium.

Lysis buffer A

50 mM Tris·Cl, pH 7.5 (see *APPENDIX 2 A*)
1% (w/v) Triton X-100
150 mM NaCl
5 mM $MgCl_2$
1 mM DTT
Prepare fresh and keep on ice
Add 0.2 mM PMSF (use 200 mM stock; see recipe) just before use

Lysis buffer B (for Rac1 & Cdc42 activation)

50 mM Tris·Cl, pH 7.2 (see *APPENDIX 2A*)
1% (w/v) Triton X-100
500 mM NaCl
10 mM $MgCl_2$
10 μg/ml aprotinin
10 μg/ml leupeptin
Prepare fresh and keep on ice
Add 0.2 mM PMSF (use 200 mM stock; see recipe) just before use

Lysis buffer C (for RhoA activation)

50 mM Tris·Cl, pH 7.2 (see *APPENDIX 2A*)
1% (w/v) Triton X-100
0.1% (w/v) SDS
500 mM NaCl
10 mM $MgCl_2$
10 μg/ml aprotinin
10 μg/ml leupeptin
Prepare fresh and keep on ice
Add 0.2 mM PMSF (use 200 mM stock; see recipe) just before use

Lysis buffer D

50 mM Tris·Cl, pH 7.2 (see *APPENDIX 2A*)
1% (w/v) Triton X-100
500 mM NaCl
10 μg/ml aprotinin
10 μg/ml leupeptin
Prepare fresh and keep on ice
Add 0.2 mM PMSF (use 200 mM stock; see recipe) just before use

PMSF, 200 mM

200 mM phenylmethylsulfonyl fluoride in ethanol
Store protected from light

SDS-PAGE sample buffer, 4×

0.5 M Tris·Cl, pH 6.8 (see *APPENDIX 2A*)
4% (w/v) SDS
20% (w/v) glycerol
40 mM DTT
0.02% (w/v) bromphenol blue

SDS-PAGE sample buffer, 2×

250 mM Tris·Cl, pH 6.8 (see *APPENDIX 2A*)
2% (w/v) SDS
10% (w/v) glycerol
40 mM DTT
0.01% (w/v) bromphenol blue

Note that this sample buffer also contains 40 mM DTT.

Tris wash buffer A

50 mM Tris·Cl, pH 7.5 (see *APPENDIX 2A*)
0.5% (w/v) Triton X-100
150 mM NaCl

continued

5 mM $MgCl_2$
1 mM DTT
Prepare fresh and keep on ice
Add 20 μM mM PMSF (use 200 mM stock; see recipe) just before use

Tris wash buffer B

50 mM Tris·Cl, pH 7.2 (see *APPENDIX 2A*)
1% (w/v) Triton X-100
150 mM NaCl
10 mM $MgCl_2$
10 μg/ml aprotinin
10 μg/ml leupeptin
Prepare fresh and keep on ice
Add 0.2 mM PMSF (use 200 mM stock; see recipe) just before use

Tris wash buffer C

50 mM Tris·Cl, pH 7.2 (see *APPENDIX 2A*)
1% (w/v) Triton X-100
150 mM NaCl
30 mM $MgCl_2$
10 μg/ml aprotinin
10 μg/ml leupeptin
Prepare fresh and keep on ice
Add 0.2 mM PMSF (use 200 mM stock; see recipe) just before use

COMMENTARY

Background Information

The Rho proteins belong to the Ras superfamily of low-molecular-weight GTPases (Wennerberg et al., 2005). They were first identified in 1985 (Madaule and Axel, 1985) and have now been shown to regulate a wide range of cellular processes (Jaffe and Hall, 2005). Most Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form. When bound to GTP, the active Rho proteins exert their biological functions by interacting with target or effector proteins (Bishop and Hall, 2000). In the cell, the GTPases are themselves regulated by three different classes of proteins. The guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, thereby activating the GTPase (Rossman et al., 2005). The GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho proteins, leading to inactivation (Moon and Zheng, 2003). Finally, the guanine nucleotide dissociation inhibitors (GDIs) affect membrane targeting and nucleotide exchange (DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005).

Activation assays have become fundamental to the study of small GTPase function. Originally, GTPase activity was measured by immunoprecipitating the GTPase of interest from lysates of cells that had been metabolically labeled with [^{32}P]phosphate and by identifying the associated radioactive nucleotide using thin layer chromatography (Downward et al., 1990). The first nonradioactive activation assay was developed for the small GTPase Ras. In 1996, Taylor and Shalloway used the amino acids 1 to 149 of Raf1 to pull down active Ras (Taylor and Shalloway, 1996; Taylor et al., 2001). Very soon after, De Rooij and Bos (1997) described the same approach using a smaller domain of Raf1 (amino acids 51 to 131). After RhoA was shown to interact with Rhotekin in a nucleotide dependent fashion (Reid et al., 1996), Martin Schwartz and colleagues used the Rho-binding domain of Rhotekin to pull down active RhoA from cell lysates (Ren et al., 1999; Ren and Schwartz, 2000). The CRIB motif of PAK1 interacts with active (i.e., GTP-bound) Rac1 and Cdc42 (Burbelo et al., 1995) and has thus been used extensively to pull down active Rac1 and Cdc42 from cell lysates (Sander et al., 1998; Benard et al., 1999; Benard and Bokoch, 2002). The WASP CRIB motif has also been used to pull down active Cdc42, but this construct does not interact with Rac1 (Haddad et al., 2001). It is still unclear which of the CRIB motifs gives a more robust assay for Cdc42 activation.

As shown in Figure 14.8.1A, the Rhotekin construct used to pull down RhoA usually consists of amino acids 7 to 89 (Ren and Schwartz, 2000). On the other hand, a range of PAK-CRIB constructs have been used successfully for Rac1 and Cdc42 activation assays. Collard and colleagues used amino acids 56 to 272 of PAK1B (Sander et al., 1998), whereas Bokoch's group prefers using amino acids 67 to 150 of PAK1 (Benard and Bokoch, 2002). We have used a larger portion of PAK1, namely amino acids 1 to 253 (Fig. 14.8.1B). Although the CRIB motif is only 16 amino acids long, the minimal domain for Rac1/Cdc42 binding to PAK consists of residues 75 to 105 (Thompson et al., 1998).

Activation assays for small GTPases have led to the identification of a wide range of extracellular stimuli that result in GTPase activation and initiate downstream signaling. They are also a good tool for assessing basal activation states, especially in tumor biology where they can be used to compare normal and tumor samples or cell lines. GST-RBD pull-down assays are also useful for measuring GEF-mediated activation and GAP inhibition, but it has been suggested that they will not reflect GDI-mediated inhibition (Ellerbroek et al., 2003).

Designing and testing activation assays for Rho GTPases other than RhoA, Rac1, and Cdc42

The best characterized Rho GTPases are RhoA, Rac1, and Cdc42, but over 20 human Rho GTPases have now been identified (Wherlock and Mellor, 2002; Wennerberg and Der, 2004; Boureux et al., 2007). Developing activation assays for the other Rho GTPases could prove an important tool for studying their function, assuming that a specific antibody is available for the GTPase of interest.

Certain Rho GTPases, however, do not cycle between an inactive, GDP-bound and an active, GTP-bound form. These include Rnd1/RhoE, Rnd2, and Rnd3 which are thought to be GTPase deficient and constitutively GTP-bound (Foster et al., 1996; Nobes et al., 1998; Chardin, 2006). Like the Rnd proteins, RhoH/TTF, RhoBTB1, and RhoBTB2 lack the conserved residues corresponding to G12 and Q61 (Rac1 numbering) found in other Rho GTPases; they are therefore likely to be GTPase deficient and not regulated by GDP/GTP cycling (Wennerberg and Der, 2004; Li et al., 2002). Such noncycling Rho GTPases are not amenable to activation assays. So far, their activity is thought to be regulated by their level of expression and/or by phosphorylation and could, therefore, be monitored by assessing the amount of protein present and/or the level of phosphorylation.

Among the cycling RhoGTPases, RhoB and RhoC both bind Rhotekin, and the GST-Rhotekin-RBD construct used for RhoA activation assays has also been used successfully for RhoB and RhoC (Arthur et al., 2002; Gampel and Mellor, 2002; Bellovin et al., 2006; Pan et al., 2006). In this case, it is important to ensure that the antibodies used are specific and can distinguish between the different Rho proteins (Table 14.8.1). The GST-PAK-CRIB construct has been used successfully to pull down Rac1 and Cdc42-like Rho GTPases such as active Rac2 (Benard et al., 1999) and TC10 (Tong et al., 2007). TCL has been shown to bind GST-PAK-CRIB, suggesting that this construct could be used to pull down active TCL from cell lysates (Vignal et al., 2000). Thus, the first step in designing an activation assay would be to test whether the GST-Rhotekin-RBD, GST-PAK-CRIB, or GST-WASP-CRIB constructs can be used (see Figure 14.8.1). If this is not the case, one needs to identify a protein domain which can be made as a GST fusion protein and which only interacts with the GTP-bound form of the GTPase of interest.

In order to test whether the GST-fusion protein only interacts with the active GTPase, pull-downs can be carried out on cells overexpressing the inactive, GDP-bound T17N mutant or the active, GTP-bound G12V and Q61L mutants (Rac1 numbering; Benard et al., 1999). Alternatively, endogenous GTPases can be loaded with nucleotides (Alternate Protocol 2). In our experience, not all Rho GTPases can be loaded following Alternate Protocol 2 because incubation at 30°C for 15 or 30 min can lead to degradation of the protein of interest. It might be possible to overcome this by optimizing the protocol.

Rho GTPases generally have multiple binding partners, with a range of different binding parameters. The general sense is that successful pull-down assay probes will have high affinities for the active Rho GTPase, high specificity for the active form, and a low rate of dissociation. The last parameter is important in preventing loss of the complex during the assay procedure.

Critical Parameters and Troubleshooting

When studying agonist-induced activation, it is sometimes difficult to obtain low levels of active GTPase in the control, untreated cells. Not all cell types can be starved and the length of serum starvation needed can vary from cell type to cell type; this has to be determined empirically. The level of active GTPase should be as low as possible, and this is usually achieved by long periods of serum starvation (e.g., 16 hr). However, the cells should still look healthy after serum starvation. Also, the cells should not be too confluent; 60% to 70% confluency is usually ideal. Finally, carrying out regular quality control activation assays on the GST-RBD beads is important. Beads do lose their specificity over time and can then interact with the inactive form of the GTPase. The only way to resolve this is by making new GST-RBD beads.

The major obstacle to activation assays is the residual GAP activity found in cell lysates (Ren and Schwartz, 2000). The high salt content of lysis buffers should help minimize GAP activity, but it is also important to work quickly and to keep all solutions and samples at low temperature (Ren and Schwartz, 2000). If possible, it is advisable to carry out the procedure in a cold room. Buffers should be made fresh and kept on ice. The PMSF is added just before use.

Rho GTPases are not very abundant and they can be difficult to detect by immunoblotting. Good antibodies are available commercially, and these are described in Table 14.8.1. The GTPases are only 21 kDa and the SDS-PAGE gels used should be chosen accordingly. We have used 15% Tris·Cl gels but have found that 12% Bis-Tris gels run with MES buffer give much better resolution and allow for better detection.

The kinetics of GTPase activation are not always predictable. These depend on the cell type, the agonist used, and the GTPase studied. A typical assay involves a time course of 0, 30 sec, 1, 3, 5, and 10 min. If no activation is seen over this period, it is advisable to use a wider range of time points. Activation can occur very quickly and be very short lived; alternatively, it can occur a very long time after treatment with agonist. Indeed, treatment of Schwann cells with sphingosine-1-phosphate (S1P) leads to maximal RhoA and Rac1 activation after only 15 sec (Barber et al., 2004). This activation is very rapidly downregulated; it is barely noticeable after 1 min and disappears completely after 3 min of S1P treatment (Barber et al., 2004). On the other hand, treatment of HeLa cells with EGF leads to a biphasic activation of RhoB, with an early peak in activity at 3 min, followed by a second peak that appears as late as 60 min after stimulation (Gampel and Mellor, 2002).

In an experimental setting, the level of activation obtained should be quantified by normalizing the amount of GST-RBD bound GTPase (i.e., active GTPase) to the amount of total GTPase. Care has to be taken when quantifying scanned images of X-ray films because they have a narrow linear range of exposure (Ren and Schwartz, 2000); it is very important to make sure that the films are not overexposed. Activation assays are usually performed at least three times so that the mean ($\pm$ standard deviation) of the ratio of active over total GTPase signal can be shown.

Anticipated Results

Examples of anticipated results are shown in Figures 14.8.2 to 14.8.5. The level of sensitivity of the activation assay has not been determined. It would vary hugely among cell types. Concentrations of agonists used would

Table 14.8.1 Commercially Available Antibodies for RhoA, RhoB, Rac1, Rac2, and Cdc42

GTPase	Company	Catalogue number	Species
RhoA[a]	Santa Cruz	RhoA (26C4) sc418	Mouse monoclonal
RhoB[a]	Santa Cruz	RhoB (C5) sc8048	Mouse monoclonal
Rac1	BD Trans Lab	R56220	Mouse monoclonal
Rac2	Santa Cruz	Rac2(C-11) sc96	Rabbit polyclonal
Cdc42	BD Trans Lab	610928	Mouse monoclonal

[a]The RhoA antibody is specific for RhoA, and similarly, the RhoB antibody only recognizes RhoB and not RhoA or RhoC (Gampel and Mellor, 2002). We have not checked the specificity of the other antibodies.

saturate the receptors, based on their known affinities.

Time Considerations

The most time consuming task is making the GST-RBD beads (Support Protocol). The whole procedure is carried out on the same day, starting with the inoculation of 4 liters of medium with 400 ml overnight culture and finishing with the aliquoting and freezing of the GST-RBD beads. The beads then need to be checked on an SDS-PAGE gel and tested in a quality control activation assay using known agonists or Rho GTPases loaded with nucleotide. Once a batch of beads has been made and tested, individual aliquots can be thawed on ice just before being added to cell lysates.

A specific timeline for this process follows: After the GST-RBD construct has been made or obtained, it needs to be transfected into bacteria which are left to grow overnight (day 1). A single colony can be picked the next day and grown overnight in a 5-ml culture (day 2). The 5-ml culture is used to inoculate a 400-ml culture, which is grown overnight (day 3). The GST-RBD beads (see Support Protocol) can then be made in 1 day (day 5) and frozen in small aliquots. Checking that the GST-RBD is bound to the beads can be done the next day (day 6). The beads can be tested as described in the Basic Protocol on control cell lysates to check that the beads bind the active but not the inactive form of the GTPase either on that same day (day 6) or the next (day 7).

Literature Cited

Arthur, W.T., Ellerbroek, S.M., Der, C.J., Burridge, K., and Wennerberg, K. 2002. XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. *J. Biol. Chem.* 277:42964-42972.

Barber, S.C., Mellor, H., Gampel, A., and Scolding, N.J. 2004. S1P and LPA trigger Schwann cell actin changes and migration. *Eur. J. Neurosci.* 19:3142-150.

Bellovin, D.I., Simpson, K.J., Danilov, T., Maynard, E., Rimm, D.L., Oettgen, P., and Mercurio, A.M. 2006. Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma. *Oncogene*. 25:6959-6967.

Benard, V., Bohl, B.P., and Bokoch, G.M. 1999. Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J. Biol. Chem.* 274:13198-13204.

Benard, V. and Bokoch, G.M. 2002. Assay of Cdc42, Rac, and Rho GTPase activation by affinity methods. *Methods Enzymol.* 345:349-359.

Bishop, A.L. and Hall, A. 2000. Rho GTPases and their effector proteins. *Biochem. J.* 348 Pt 2:241-255.

Boureux, A., Vignal, E., Faure, S., and Fort, P. 2007. Evolution of the Rho family of Ras-like GTPases in eukaryotes. *Mol. Biol. Evol.* 24:203-216.

Burbelo, P.D., Drechsel, D., and Hall, A. 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem.* 270:29071-29074.

Chardin, P. 2006. Function and regulation of Rnd proteins. *Nat. Rev. Mol. Cell Biol.* 7:54-62.

de Rooij, J., and Bos, J.L. 1997. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. *Oncogene*. 14:623-625.

DerMardirossian, C., and Bokoch, G.M. 2005. GDIs: Central regulatory molecules in Rho GTPase activation. *Trends Cell Biol.* 15:356-363.

Dovas, A., and Couchman, J.R. 2005. RhoGDI: Multiple functions in the regulation of Rho family GTPase activities. *Biochem. J.* 390:1-9.

Downward, J., Graves, J.D., Warne, P.H., Rayter, S., and Cantrell, D.A. 1990. Stimulation of p21ras upon T-cell activation. *Nature* 346:719-723.

Ellerbroek, S.M., Wennerberg, K., and Burridge, K. 2003. Serine phosphorylation negatively regulates RhoA in vivo. *J. Biol. Chem.* 278:19023-19031.

Foster, R., Hu, K.Q., Lu, Y., Nolan, K.M., Thissen, J., and Settleman, J. 1996. Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation. *Mol. Cell Biol.* 16:2689-2699.

Gampel, A. and Mellor, H. 2002. Small interfering RNAs as a tool to assign Rho GTPase exchange-factor function in vivo. *Biochem. J.* 366:393-398.

Haddad, E., Zugaza, J.L., Louache, F., Debili, N., Crouin, C., Schwarz, K., Fischer, A., Vainchenker, W., and Bertoglio, J. 2001. The interaction between Cdc42 and WASP is required for SDF-1-induced T-lymphocyte chemotaxis. *Blood* 97:33-38.

Jaffe, A.B. and Hall, A. 2005. Rho GTPases: Biochemistry and biology. *Annu. Rev. Cell Dev. Biol.* 21:247-269.

Li, X., Bu, X., Lu, B., Avraham, H., Flavell, R.A., and Lim, B. 2002. The hematopoiesis-specific GTP-binding protein RhoH is GTPase deficient and modulates activities of other Rho GTPases by an inhibitory function. *Mol. Cell Biol.* 22:1158-1171.

Madaule, P. and Axel, R. 1985. A novel *ras*-related gene family. *Cell* 41:31-40.

Moon, S.Y. and Zheng, Y. 2003. Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol.* 13:13-22.

Nobes, C.D., Lauritzen, I., Mattei, M.G., Paris, S., Hall, A., and Chardin, P. 1998. A new member of the Rho family, Rnd1, promotes disassembly

of actin filament structures and loss of cell adhesion. *J. Cell Biol*. 141:187-197.

Pan, Q., Bao, L.W., Teknos, T.N., and Merajver, S.D. 2006. Targeted disruption of protein kinase C (varepsilon) reduces cell invasion and motility through inactivation of RhoA and RhoC GTPases in head and neck squamous cell carcinoma. *Cancer Res*. 66:9379-9384.

Reid, T., Furuyashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P., and Narumiya, S. 1996. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. *J. Biol. Chem*. 271:13556-13560.

Ren, X.D. and Schwartz, M.A. 2000. Determination of GTP loading on Rho. *Methods Enzymol*. 325:264-272.

Ren, X.D., Kiosses, W.B., and Schwartz, M.A. 1999. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J*. 18:578-585.

Rossman, K.L., Der, C.J., and Sondek, J. 2005. GEF means go: Turning on Rho GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol*. 6:167-180.

Sander, E.E., van Delft, S., ten Klooster, J.P., Reid, T., van der Kammen, R.A., Michiels, F., and Collard, J.G. 1998. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol*. 143:1385-1398.

Takesono, A., Horai, R., Mandai, M., Dombroski, D., and Schwartzberg, P.L. 2004. Requirement for Tec kinases in chemokine-induced migration and activation of Cdc42 and Rac. *Curr. Biol*. 14:917-922.

Taylor, S.J. and Shalloway, D. 1996. Cell cycle-dependent activation of Ras. *Curr. Biol*. 6:1621-1627.

Taylor, S.J., Resnick, R.J., and Shalloway, D. 2001. Nonradioactive determination of Ras-GTP levels using activated Ras interaction assay. *Methods Enzymol*. 333:333-342.

Thompson, G., Owen, D., Chalk, P.A., and Lowe, P.N. 1998. Delineation of the Cdc42/Rac-binding domain of p21-activated kinase. *Biochemistry* 37:7885-7891.

Tong, S., Liss, A.S., You, M., and Bose, Jr., H.R. 2007. The activation of TC10, a Rho small GTPase, contributes to v-Rel-mediated transformation. *Oncogene* 26:2318-2329.

Vignal, E., De Toledo, M., Comunale, F., Ladopoulou, A., Gauthier-Rouviere, C., Blangy, A., and Fort, P. 2000. Characterization of TCL, a new GTPase of the rho family related to TC10 and Cdc42. *J. Biol. Chem*. 275:36457-36464.

Wennerberg, K. and Der, C.J. 2004. Rho-family GTPases: It's not only Rac and Rho (and I like it). *J. Cell Sci*. 117:1301-1312.

Wennerberg, K., Rossman, K.L., and Der, C.J. 2005. The Ras superfamily at a glance. *J. Cell Sci*. 118:843-846.

Wherlock, M. and Mellor, H. 2002. The Rho GTPase family: A Racs to Wrchs story. *J. Cell Sci*. 115:239-240.

In Vitro GEF and GAP Assays

Alexander Eberth[1] and Mohammad Reza Ahmadian[1]

[1]Institut für Biochemie und Molekularbiologie II, Klinikum der Heinrich-Heine-Universität, Düsseldorf, Germany

ABSTRACT

Small GTPases act as tightly regulated molecular switches governing a large variety of critical cellular functions. Their activity is controlled by two different biochemical reactions, GDP/GTP exchange and GTP hydrolysis. These very slow reactions require catalysis in cells by two kinds of regulatory proteins. While the guanine nucleotide exchange factors (GEFs) activate small GTPases by stimulating the slow exchange of bound GDP for the cellularly abundant GTP, GTPase-activating proteins (GAPs) accelerate the slow intrinsic rate of GTP hydrolysis by several orders of magnitude, leading to inactivation. There are a number of methods that can be used to characterize the specificity and activity of such regulators, to understand the effect of binding on the protein structure, and, ultimately, to obtain insights into their biological functions. This unit describes (1) detailed protocols for the expression and the purification of small GTPases and the catalytic domains of GEFs and GAPs; (2) preparation of nucleotide-free and fluorescent nucleotide-bound small GTPases; and (3) methods for monitoring of the intrinsic and GEF-catalyzed nucleotide exchange as well as intrinsic and GAP-stimulated GTP hydrolysis. *Curr. Protoc. Cell Biol.* 43:14.9.1-14.9.25. © 2009 by John Wiley & Sons, Inc.

Keywords: fluorescence spectroscopy • guanine nucleotide • mant • tamra

INTRODUCTION

A great variety of small GTPases are known, and each of these in turn interacts with a variety of regulatory proteins, including guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Analysis of the human genome sequence predicts 69 GEFs and up to 80 GAPs for Rho family GTPases, which may possibly regulate the activity of 19 Rho GTPases. To understand the biological relevance of this molecular diversity, it is important to measure the activity and to determine the specificity of these regulators in vitro. Only a sparse number of such intermolecular interactions have been investigated, primarily using radioactive ligand overlay, filter binding, or pull-down assays. These methods are often not sufficient to determine the specificity of regulation and quantify the activity of recombinant proteins. However, many of the potential interactions defined by these methods require a more detailed analysis of their kinetics by appropriate real-time methods. Fluorescent guanine nucleotides are often ideally suited to fulfill these criteria, as it is known that they do not grossly disturb the biochemical properties of the GTPase and that the fluorescence reporter group is sensitive enough to changes in the local environment to produce a sufficiently large fluorescence change (Ahmadian et al., 2002; Hemsath and Ahmadian, 2005). Furthermore, it is often sensitive to the interaction with partner proteins that happen to bind in its neighborhood. This unit describes the role of two different fluorescently labeled guanine nucleotides in the biochemical analysis of Rho GTPases (Fig.14.9.1), which can be used to evaluate the GEF-catalyzed nucleotide exchange and the GAP-stimulated GTP-hydrolysis activities, respectively. Table 14.9.1 describes the protocols presented in this unit.

Current Protocols in Cell Biology 14.9.1-14.9.25, June 2009
Published online June 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1409s43

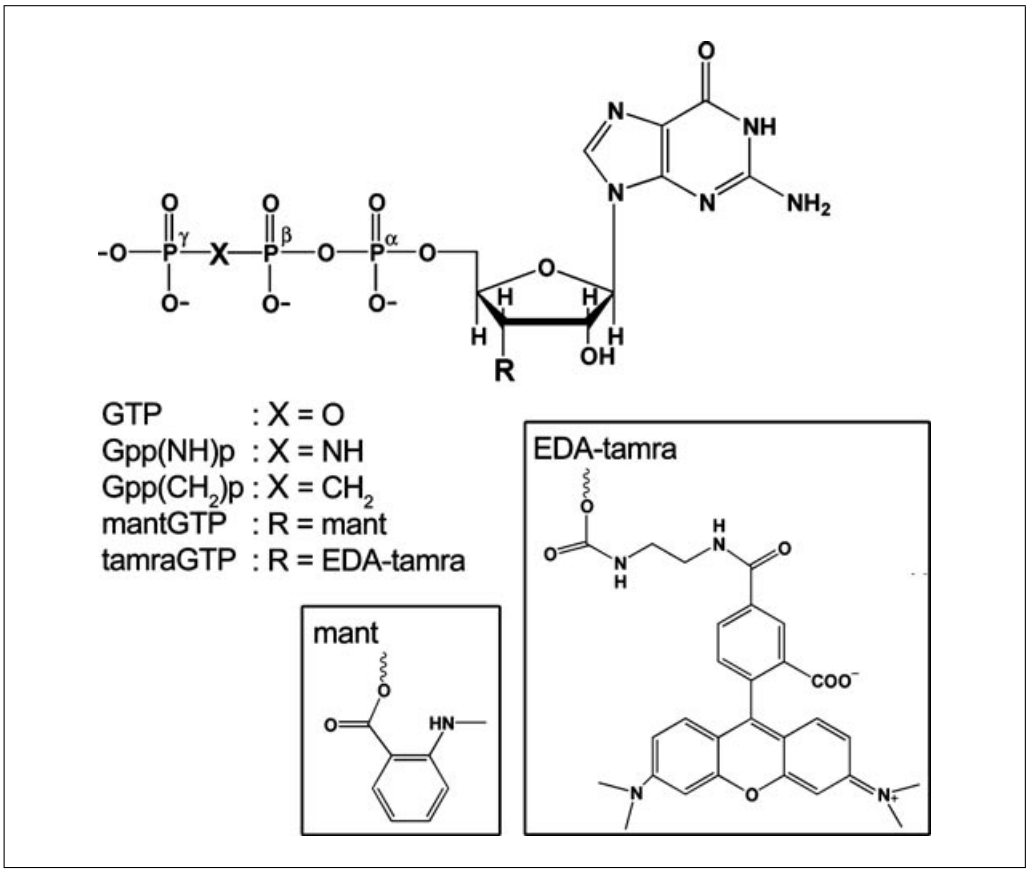


Figure 14.9.1 The chemical structures of the guanosine nucleotide derivatives used in this unit. Unlabeled fluorescent nucleotides contain an OH-group at the position R.

BASIC PROTOCOL 1

MEASUREMENT OF INTRINSIC AND SLOW GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF)–CATALYZED NUCLEOTIDE EXCHANGE REACTIONS

To obtain a detailed picture of the molecular switch function of small GTPases and their interaction with regulators and effectors, we have established fluorescence-based methods for the time-resolved monitoring and quantification of small GTPase functions and interactions with their binding partners (Ahmadian et al., 2002; Hemsath and Ahmadian, 2005).

Different procedures are available for investigating the guanine nucleotide exchange on small GTPases. The dissociation of a protein-bound nucleotide can easily be determined in real time by fluorescence spectroscopy using a fluorescent GDP. Usually, *N*-methylanthraniloyl (mant) derivatives of guanosine nucleotides, coupled at the 2′(3′) hydroxyl group of the ribose, are used.

In principle, each nucleotide-binding protein has a defined intrinsic rate of GDP release, which is often too low to be physiologically relevant. Thus, GEFs operate on these small GTPases and catalyze the generation of the active GTP-bound state from the inactive GDP-bound form. This process is often a result of the GEFs themselves being activated or recruited to the vicinity of the corresponding GTPase in response to extracellular signaling events.

Specificity and activity of GEFs can be analyzed qualitatively by comparison of intrinsic and GEF-stimulated fluorescence measurements. Usually, this is performed in a fluorescence spectrometer, since these reactions are slow (>1000 sec). Here, the bacterially expressed recombinant proteins, purified to >90% homogeneity, as well as the chemically

Table 14.9.1 Protocols for In Vitro GEF and GAP Assays

Protocol	Title
Basic Protocol 1	Measurement of Intrinsic and Slow Guanine Nucleotide Exchange Factor (GEF)–Catalyzed Nucleotide Exchange Reactions
Support Protocol 1	Preparation of mantGDP-Bound GTPases
Support Protocol 2	Preparation of Nucleotide-Free Forms of Small GTPases
Support Protocol 3	Determining Nucleotide Concentration Using HPLC
Alternate Protocol 1	Measurement of Fast GEF-Catalyzed Nucleotide Exchange Reactions
Basic Protocol 2	Measurement of GTPase-Activating Protein (GAP)–Stimulated GTP Hydrolysis by HPLC
Alternate Protocol 2	Measurement of Slow GAP-Stimulated GTP Hydrolysis Using mantGTP
Alternate Protocol 3	Measurement of Slow GAP-Stimulated GTP Hydrolysis Using tamraGTP
Alternate Protocol 4	Measurement of Fast GAP-Catalyzed GTP-Hydrolysis Using tamraGTP
Support Protocol 4	Gene Expression and Bacterial Culture Conditions
Support Protocol 5	Bacterial Lysis by Sonication
Support Protocol 6	Bacterial Lysis by a Microfluidizer
Support Protocol 7	Protein Purification for GST Fusion Proteins
Support Protocol 8	Determining Protein Concentration Using the Bradford Assay
Support Protocol 9	Determining Protein Concentration Using the Ehresmann Assay
Support Protocol 10	Concentrating a Dilute Protein Solution
Support Protocol 11	Thrombin Proteolytic Cleavage of GST Fusion Proteins
Support Protocol 12	Gel-Filtration Chromatography
Support Protocol 13	Freezing and Thawing of Proteins

synthesized pure nucleotide solution, are prepared in a cuvette. The mant-fluorescence signal in a fluorescence spectrometer is recorded using an excitation wavelength of 366 nm and an emission wavelength of 450 nm, an integration time of at least 2 sec, and a recording time for each data point of 20 sec.

GEF and also GAP assays do not need post-translationally modified GTPases. Thus, all proteins and protein domains produced in *Escherichia coli* can be used. Cleared cell lysate is not suitable in this assay for several reasons: (1) protein concentration may not be sufficient; (2) the protein of interest may exist in a complex with other proteins and may thus not be freely available; (3) the activity of other regulators may falsify the assay.

Materials

>5 μM mantGDP-bound GTPase (Support Protocol 1)
GEF buffer (see recipe), store at 25°C
>50 μM GEF protein including the catalytic domains (recombinant protein, expressed and purified as described in Support Protocols 4 to 13)
10 mM GDP (Pharma Waldhof, *http://www.pharmawaldhof.de/*), pH 7.5
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)

Fluorescence cuvettes (Suprasil quartz glass; Hellma, cat. no. 108.002F-QS)
Fluorescence spectrometer (Perkin-Elmer, Spex Instruments)
Grafit program (Erithacus Software) or alternative program packages for evaluation of the data

1. Preincubate a solution of 0.1 μM mantGDP-bound GTPase (see Support Protocol 1) in a fluorescence cuvette in GEF buffer with the GEF protein at a final volume

of 600 µl and at 25°C for at least 5 min, while measuring the fluorescence signal at 366 nm excitation and 450 nm emission.

Always use 366 nm for excitation and 450 nm for emission with mant-labeled nucleotides.

2. If the fluorescence signal is stable, add 1.2 µl of 10 mM nonfluorescent GDP solution (20 µM final GDP concentration) and mix rapidly with a pipet to start the reaction.

   With this setup, the intrinsic dissociation reaction in the absence of GEF is monitored, which, depending on the GTPase, lasts between 2 and 72 hr. This slow reaction is accelerated in the presence of GEF proteins. To determine the activity and specificity of the exchange factors in a fluorimeter, usually concentrations of 0.1 to 2 µM (depending on the specific activity of the GEF) should be used (for faster kinetics, see Alternate Protocol 1).

   For slow nucleotide dissociation reactions (>5 hr), the cuvettes should be closed with a lid and can additionally be sealed by a thin layer of silicone grease between cuvette and lid to prevent evaporation of the sample, which usually results in an artifactual increase in fluorescence.

   Alternatively, four parallel measurements can be performed simultaneously using an instrument equipped with an automated four-position turret.

   Using cleared cell lysates may disturb the proper fluorescence signal in this assay because they are often very opaque solutions and, due to the very low GEF concentrations, useless if greatly diluted.

3. Monitor the exponential decrease in fluorescence over the time course of the reaction (2 to 72 hr).

   The decrease in fluorescence is due to the mantGDP release into the aqueous solution.

4. When no further change in fluorescence can be observed, add 24 µl of 0.5 M EDTA solution (for final concentration of 20 mM) and monitor the reaction for an additional 10 min.

   This will reveal whether the nucleotide dissociation reaction is entirely complete.

   EDTA will deplete the magnesium ion, which is an essential cofactor for nucleotide binding, from the nucleotide binding site of the GTPase. This reduces the nucleotide affinity by several orders of magnitude and thus leads to a complete dissociation of the bound mant-nucleotide. However, the fluorescence baseline will not change if mantGDP dissociation is already complete.

5. Fit the data single exponentially with, e.g., the Grafit program to provide the dissociation (off) rates.

   In the case of small GTPases, the dissociation rate is usually $\sim 10^{-3}$ to 10^{-5} sec^{-1}.

SUPPORT PROTOCOL 1

PREPARATION OF mantGDP-BOUND GTPases

Loading of nucleotide-free forms of GTPases with fluorescently labeled nucleotides can be achieved by simply mixing both components and subsequently performing small-scale size-exclusion chromatography with a desalting column. The protocol described below is usually necessary for the preparation of GTPases bound to fluorescent GDP analogs. For nonhydrolyzable GTP analogs like Gpp(NH)p, the method described in Support Protocol 2 is sufficient and, additionally, steps 4 to 5 of Support Protocol 2 could be omitted (addition of phosphodiesterase is dispensable).

Materials

Standard buffer (see recipe)
Nucleotide-free GTPase (see Support Protocol 2)
mantGDP (synthesized as described in Hemsath and Ahmadian, 2005, or purchased from Jena Bioscience, *http://www.jenabioscience.com/*)

Ponceau S (*UNIT 6.2*)
HPLC buffer (see recipe) containing 20% to 25% (v/v) acetonitrile

NAP-5 column (GE Healthcare)
Nitrocellulose membrane (*UNIT 6.2*)

Additional reagents and equipment for Ponceau S staining of proteins on nitrocellulose membrane (*UNIT 6.2*) and HPLC (Support Protocol 3)

1. Equilibrate the NAP-5 column with 2 to 3 column volumes of standard buffer.
2. Mix 0.5 mg of a nucleotide-free GTPase (e.g., 50 µl from a 0.5 mM solution) with a 1.5-fold molar excess of mantGDP (e.g., 3.75 µl from a 10 mM stock solution).
3. Apply complete sample volume to the NAP-5 column and let it sink into the medium.
4. Add sufficient standard buffer for a 500-µl total buffer/sample volume and let it sink into the medium again (e.g., for the example here, add 446.25 µl).
5. Add 1 ml of standard buffer and collect fractions at 2 drops per fraction.

   Usually the protein elutes in fractions 3 to 5, which corresponds to an elution volume between 0.2 and 0.5 ml.

6. Analyze the fractions for their protein content by dotting 2 µl from each fraction on a nitrocellulose membrane and subsequently staining with Ponceau S (*UNIT 6.2*).

   This is just to determine which fractions contain the protein of interest.

   We do not use any protein standard here since this is not a quantitative analysis. It is just qualitative and should answer in which fractions the protein is localized and identify which fractions to pool.

7. Pool protein-containing fractions and determine the mantGDP-bound protein concentration by HPLC (Support Protocol 3) using an HPLC buffer containing 20% to 25% acetonitrile.
8. Store the protein aliquots at −80°C.

   It is important to note that the resulting concentration needs to be multiplied by the factor 0.6 to filter out absorption portions, which trace back to the mant-group absorption at 254 nm.

PREPARATION OF NUCLEOTIDE-FREE FORMS OF SMALL GTPases

SUPPORT PROTOCOL 2

Preparation of nucleotide-free GTPase is carried out in two steps according to John et al. (1990). In the first step, the bound GDP is degraded by alkaline phosphatase and replaced by Gpp(CH_2)p (a nonhydrolysable GTP analog, which is resistant to alkaline phosphatase but sensitive to phosphodiesterase). In the second step, after GDP is completely degraded, snake venom phosphodiesterase is added to the solution of the Gpp(CH_2)p-bound GTPase to cleave this nucleotide to GMP, G, and P_i.

Materials

Alkaline phosphatase, agarose bead-coupled (Sigma-Aldrich)
Nonhydrolyzable GTP analog Gpp(CH_2)p (Sigma-Aldrich)
GDP-bound GTPase (expressed and purified from *E. coli*; as described in Support Protocols 4 to 13)
10× exchange buffer (see recipe)
HPLC buffer (see recipe) containing 7.5% (v/v) acetonitrile
Snake venom phosphodiesterase (Sigma-Aldrich, cat. no. P3134)
Liquid nitrogen

Additional reagents and equipment for HPLC (Support Protocol 3 and Basic Protocol 2)

1. Add 0.1 to 1 U of agarose bead–coupled alkaline phosphatase and a 1.5 molar excess of Gpp(CH_2)p to 1 mg GDP-bound GTPase.

   Use a highly concentrated protein solution of 0.5 to 1 mM in order to obtain highly concentrated nucleotide-free GTPases.

2. Start the reaction by diluting the 10× exchange buffer to 1× in the prepared reaction mixture from step 1. Mix exchange buffer and protein/nucleotide-solution rapidly.

   Fast mixing of the exchange buffer should be carried out to prevent local protein precipitation due to a high ammonium sulfate concentration. Ammonium sulfate destabilizes the nucleotide binding of the GTPase and zinc chloride, and is an essential cofactor of the alkaline phosphatase.

3. Incubate the protein solution at 4°C for 2 to 16 hr (depending on the GTPase used) and analyze the GDP content by HPLC (Support Protocol 3) using an HPLC buffer containing 7.5% acetonitrile.

   The GDP peak declines in the course of the degradation progress. It disappears and GMP or G peaks appear instead. The amount of Gpp(CH_2)p remains unchanged, as it is resistant to alkaline phosphatase.

   This protocol can also be used to prepare Gpp(NH)p-bound or mantGp(NH)p-bound GTPases as described by Hemsath and Ahmadian (2005).

4. After GDP is degraded completely, add 0.002 U snake venom phosphodiesterase per mg GTPase to cleave Gpp(CH_2)p to GMP, G, and P_i. Incubate 2 to 24 hr at 4°C (depending on the GTPase and activity of the phosphodiesterase).

   This is the critical step in the procedure, since here the nucleotide is degraded and generation of a low-affinity product can lead to partial denaturation of the protein. In some cases, it might be helpful to reduce the amount of exchange buffer used or to do repeated cycles of room temperature incubation (e.g., for 20 min) followed by recooling on ice (e.g., for 10 to 15 min), which might accelerate the process. The latter method might be employed in cases where denaturation of the protein occurs due to a long incubation time (>2 days).

5. Analyze the degradation process by HPLC (Basic Protocol 2).

   Progress of the reaction can be followed by observing reduction and finally elimination of the Gpp(CH2)p peak and an increase in the guanosine peak.

6. After the degradation of Gpp(CH_2)p is complete, centrifuge the solution 2 min at 1500 × *g*, 4°C, to remove bead-coupled alkaline phosphatase and insoluble guanosine. Repeat this process two to three times to quantitatively remove all traces of alkaline phosphatase-coupled beads, since this enzyme might interfere with subsequent biochemical assays performed with the nucleotide-free GTPase.

   Depending on the GTPase, Gpp(CH_2)p degradation continues 6 to 18 hr at 4°C.

7. Inactivate the phosphodiesterase by snap freezing in liquid nitrogen and quickly defrosting at 37°C, and then repeating the process. Store the protein solution at −80°C.

   Nucleotide-free GTPases are actually in a GMP- and G-bound form that can be stored at −80°C for several months. GMP and G, which are the products of the enzymatic degradation of GDP and Gpp(CH_2)p, usually exhibit a 6– to 7–orders of magnitude lower affinity for the GTPases as shown previously for H-Ras (John et al., 1990). Nonetheless, the presence of G and GMP, which can be rapidly exchanged by GDP- and GTP-derivatives, provides for stability of the so-called nucleotide-free GTPases.

DETERMINING NUCLEOTIDE CONCENTRATION USING HPLC

SUPPORT PROTOCOL 3

HPLC allows determination of the concentration of nucleotides (nonlabeled- or mant- or tamra-labeled nucleotide) using a reversed-phase C18 column (ODS-Hypersil, 5 μM, Bischoff Chromatography) and a precolumn (Nucleosil 100 C18, Bischoff Chromatography), which separates protein–nucleotide complexes by adsorbing the denatured protein. Due to the 1:1 ratio of GTPase and nucleotide complex, this method can be used to accurately determine the concentration of a nucleotide-bound protein population.

Materials

HPLC buffer (see recipe)
50 to 100 μM GTPase (recombinant protein, expressed and purified as described in Support Protocols 4 to 13)
Nucleotide standard solutions: e.g., 20 to 400 μM GDP

Beckman Gold HPLC instrument (Beckman Coulter)
Reversed-phase C18 HPLC column: Ultrasphere ODS, 5-μM; 250 × 4, 6-mm (Beckman Coulter)
Guard column: Nucleosil 100-5-C18, 5 μM (Bischoff Chromatography; *http://www.bischoff-chrom.de/*)
20- or 50-μl sample loop (Bischoff Chromatography; *http://www.bischoff-chrom.de/*)

1. Equilibrate the pump and column of the HPLC system with HPLC buffer until a stable baseline at 254 nm is reached.

   For the separation of nonlabeled nucleotides, HPLC buffer (see Reagents and Solutions) containing 7.5% acetonitrile is used; 20% to 25% acetonitrile is used for mant- or tamra-labeled nucleotides.

2. Wash the sample loop (20- or 50-μl) with ≥1 ml deionized water.

3. Prepare ~30 or 60 μl (depending on the loop size used) of a 50 to 100 μM GTPase solution (1 to 2 mg/ml in the case of 21-kDa proteins such as Ras or Rho GTPases) in water or standard buffer, and load the entire 20- or 50-μl sample loop with it.

4. Inject the protein sample prepared in step 3 and monitor eluting nucleotides for 5 to 10 min (depending on the performance of the reversed-phase column and the flow rate; here 1.8 ml/min is used) by absorption at the appropriate wavelength.

   The absorption is detected at 254 nm for nonlabeled as well as mant-labeled nucleotides (for the latter, a factor of 0.6 is multiplied by the result, which is based upon the ratio of the extinction coefficient of nonlabeled and mant-labeled nucleotides). Molar extinction coefficients (at 254 nm) of 13,700 M^{-1} cm^{-1} for nonlabeled, 22,000 for mant-labeled, and 78,000 for tamra-labeled guanine nucleotides (measured at 546 nm) are employed. Due to ion–pair formation between the bulky hydrophobic tetrabutylammonium bromide and phosphate groups, the elution of the nucleotides is retarded with increasing phosphates. Concentrations of tamra-labeled GTPases are not determined by HPLC but spectrophotometrically at 546 nm.

5. Evaluate the nucleotide concentration using a linear absorbance profile of a nucleotide standard, e.g., 20 to 400 μM GDP. Calculate the area below a peak in the chromatogram using the HPLC integrator and correlate with the absorption of the respective probe. Use this value to determine the nucleotide concentration of the applied sample by comparison with a linear absorption profile that is generated with a nucleotide standard.

   Analogous to the calibration of the Bradford dye solution, the reversed-phase column can be calibrated using different GDP nucleotide samples of known concentration. In order to calibrate a column, GDP solutions in the range of 20 μM to 400 μM should be prepared,

and their concentrations should be verified spectrophotometrically. These samples can be then be applied to the column and their respective peak areas can be plotted versus the nucleotide concentration. Linear fitting of the data gives a regression line, and the slope represents a correlation factor between concentration and peak area.

ALTERNATE PROTOCOL 1

MEASUREMENT OF FAST GEF-CATALYZED NUCLEOTIDE EXCHANGE REACTIONS

For fast GEF-catalyzed nucleotide dissociation reactions, the time resolution of a fluorescence spectrometer is insufficient for a reliable data analysis. Instead, a stopped-flow instrument is routinely used for analysis of rapid kinetics as obtained by quantitative GEF-stimulated nucleotide exchange reactions. Here, equal volumes of two different 2× samples are automatically injected into a mixing chamber (in a single mixing mode), where the fluorescence can be detected directly after the rapid mixing (dead time ~2 to 5 msec). Five to eight identical measurements are recorded and averaged in order to obtain a higher degree of accuracy. The excitation wavelength for the mant-nucleotides is 366 nm and the fluorescence is detected with a cut-off filter mounted in front of a photomultiplier (408 nm for mant-nucleotides).

This is a fast and easy assay to obtain nucleotide exchange activities of GEFs toward the respective GTPases. Also, GEFs can be compared in respect to their specificity with different GTPases either by simply comparing nucleotide dissociation rates at given GEF and GTPase concentrations or by quantitative determination of Michaelis–Menten constant (K_m) and maximal dissociation rate (k_{max}). Such kinetic parameters can be obtained by using the above condition and increasing concentrations of the GEF proteins as described (Guo et al., 2005; Hemsath and Ahmadian, 2005).

***Additional Materials** (also see Basic Protocol 1)*

Stopped-flow instrument (Applied Photophysics SX18MV or Hi-Tech SF-61 DX2; *http://www.photophysics.com*)

NOTE: Because the samples are mixed 1:1, all stock solutions for components of the samples should be 2×.

1. Wash the drive syringes of the stopped-flow instrument several times with 5 to 10 ml GEF buffer and adjust the temperature to 25°C.
2. Prepare 2× samples in 1× GEF buffer at room temperature (~25°C) and a final volume of 1000 µl:
   a. One sample contains 0.2 µM of mantGDP-bound GTPase.

      Example: Dilute 2 µl from a 100 µM mantGDP-bound GTPase solution in 998 µl of GEF buffer to obtain a 0.2 µM solution of the respective mantGDP-bound protein.

   b. The other sample contains the GEF protein (at a concentrations ranging from 2 to 1000 µM, depending on the activity and affinity of the GEF for the respective GTPase), and 40 µM GDP (200-fold excess above mantGDP).

      A 10-fold excess of the GEF protein usually is a first choice to determine the activity of the GEF protein. Therefore, mix 20 µl from a 100 µM GEF solution (20 µM final concentration) and 4 µl from a 10 mM GDP solution (40 µM final concentration) in 976 µl with GEF buffer.
3. Load each sample into one of the two drive syringes of the stopped-flow instrument. Set the excitation wavelength for the mant-nucleotides to 366 nm and detect the fluorescence with a cut-off-filter mounted in front of a photomultiplier (408 nm for mant-nucleotides).

Signal-to-noise ratio can be considerably improved if all samples are centrifuged (>20 min, 20,000 × g, 4°C) and if the buffer is filtered and degassed.

The photomultiplier current should be adjusted manually to a value with a well balanced signal-to-noise ratio. More modern instruments are also able to set an optimal value automatically.

4. Start the measurement with the supplied stopped-flow software, which initiates pushing the contents of the two syringes containing the samples into the sample cell so that both samples join together and rapidly mix at a final volume of about 50 to 75 µl. Record the fluorescence using excitation at 366 nm and detection with a cut-off filter mounted in front of a photomultiplier at 408 nm for mant-nucleotides, for a defined time period (depending on the kinetics).

5. Repeat the mixing and fluorescence recording event up to 10 times until all volume in the drive syringe reservoir is consumed.

   Using 1000-µl samples, up to 11 identical measurements can be performed, from which the first three are required to equilibrate the sample cell. The fourth measurement can be used to reset the photomultiplier current and to determine the timescale for recording the complete reaction. Between 400 and 1000 data points should be recorded per measurement. Thus, about seven identical measurements can be repeatedly performed and averaged to obtain a mean value of all comparable curves. The single exponential data obtained corresponds to one averaged experiment at a defined GEF concentration.

6. Fit all data according to Hemsath and Ahmadian (2005) to obtain the observed rate constant (k_{obs}) for the respective concentration of the GEF protein.

MEASUREMENT OF GTPase-ACTIVATING PROTEIN (GAP)-STIMULATED GTP HYDROLYSIS BY HPLC

BASIC PROTOCOL 2

All small GTP-binding proteins, with a few exceptions, e.g., Rnd proteins, have a defined rate of GTP hydrolysis, which is often too low to be physiologically relevant. Thus, GAPs stimulate the very slow intrinsic reaction rate of GTP hydrolysis by several orders of magnitude, which is a critical step in signal transduction.

For the measurement of intrinsic and GAP-stimulated GTP-hydrolysis reaction of small GTPases, several assays have been developed so far. There are filter-binding and HPLC-based assays as well as spectrophotometric and spectrofluorometric methods available. A generally useful and accurate method is HPLC, by which concentrations of GDP and GTP can be determined to describe the reaction progress as described for Ras (Ahmadian et al., 1999) and Rho-proteins (Hemsath and Ahmadian, 2005).

HPLC needs large amounts of proteins and is not suitable for quantitative analysis of the GAP activity. In another approach, which is less material- and time-consuming, the GTPase reaction rates can be conveniently measured in real time using tryptophan fluorescence with an excitation wavelength of 295 nm and an emission wavelength of 350 nm. For example, the Ras(Y32W) mutant provides a large increase in fluorescence signal upon hydrolysis of GTP to GDP and inorganic phosphate, which has been used to study the mechanism of the intrinsic GTPase reaction (Ahmadian et al., 1999).

Stopped-flow experiments using mantGTP and mantGpp(NH)p (see Alternate Protocol 2) have provided key insights into the mechanism of the GAP-stimulated GTPase reaction of Ras in several studies (Ahmadian et al., 1997a,b, 2003). However, it should be noted that mantGTP does not work for most GTPase/GAP systems. A case in point is the interaction between Rho and RhoGAP, which, although similar in the type of enzymatic catalysis to the Ras–RasGAP interaction (Scheffzek and Ahmadian, 2005), does not show any fluorescence change upon interaction or hydrolysis.

Most recently, we have demonstrated that tetramethylrhodamine (tamra; see Alternate Protocol 4) is a powerful fluorescence reporter group to study the GTP-hydrolysis in the presence and in the absence of GAPs (Eberth et al., 2005). The intrinsic GTP-hydrolysis reaction of Ras and Rho GTPases can be detected in a fluorescence spectrometer or a stopped-flow instrument, showing a significant decrease in the fluorescence signal.

The HPLC method is an accurate way of determining the intrinsic hydrolysis rate of a GTPase and is also useful for determining a GAP protein's specific activity. The advantage is the lack of any artificial reporter group or necessity to use a GTPase mutant in this assay. Here wild-type proteins and unlabeled nucleotides can be used.

Materials

500 μM nucleotide-free GTPase (Support Protocol 2)
GAP buffer (see recipe)
10 mM GTP (Pharma Waldhof; *http://www.pharmawaldhof.de/*), pH 7.5
>50 μM GAP protein including the catalytic domains (recombinant protein, expressed and purified as described in Support Protocols 4 to 13)
Liquid nitrogen
HPLC buffer (see recipe) containing 7.5% (v/v) acetonitrile

Thermomixer/thermoblock
Beckman Gold HPLC instrument (Beckman Coulter)
Reversed-phase C18 HPLC column: Ultrasphere ODS, 5-μM; 250 × 4.6–mm (Beckman Coulter)
Guard column: Nucleosil 100-5-C18, 5-μM (Bischoff Chromatography)

1. Dilute 40 μl of 500 μM nucleotide-free GTPase with 60 μl GAP buffer and the GAP protein and preincubate it at 25°C in a thermomixer/thermoblock (the final concentration of the nucleotide-free GTPase should be 80 μM in a volume of 250 μl after addition of the GTP).

   The GAP protein can be used in a ratio of 1:100 to 1:1000 with respect to the GTPase concentration (0.8 to 0.008 μM in this example) and should be added in step 1 to the nucleotide-free GTPase before starting the reaction by addition of GTP.

2. Dilute 10 μl of 10 mM GTP stock solution with 990 μl GAP buffer (1:100 dilution) to obtain a 100 μM GTP solution, and preincubate this solution at 25°C in a thermomixer/thermoblock.

3. Add 150 μl of the 100 μM GTP solution (60 μM final concentration) and mix it with the 80 μM nucleotide-free GTPase solution by pipetting up and down.

   The total volume (in this case calculated for 8 time points) should be 250 μl, but depending on the number of data points to be taken, can be increased or even decreased.

   The timescale for the intrinsic GTP hydrolysis reaction varies from 0.5 to 6 hr, which depends on the GTPase variant used. Alternatively, a protein comprising a catalytic active GAP domain can be added before starting the reaction, which allows the determination of the activity and specificity of the GAP protein.

   Note that in the presence of the GAP, the reactions are much faster and the time scale (e.g., 1 to 5 min instead of 30 to 60 min for RhoGTPases) is much shorter. Using the conditions described here, the reaction is usually accelerated by a factor of 5 to 20 (depending on the dilution ratio of GAP with respect to GTPase and the activity of the GAP protein). In order to obtain reliable results, parameters including GAP concentration and the time points need to be optimized for the respective GTPase/GAP system.

   In the case of slow GTP-hydrolyzing GTPases (e.g., Ras or Rap) or GTP-hydrolysis, deficient Rho or Ras proteins GTP-bound proteins can be prepared and stored at −80°C for several weeks.

Depending on the sensitivity of the HPLC, the concentrations of GTPase and GTP can be reduced to 25 μM and 20 μM, respectively.

4. At defined time points, collect and immediately snap-freeze 30-μl samples in liquid nitrogen.

   Proper time points and intervals are important for an appropriate evaluation of the data to determine the rate constant of the hydrolysis reaction. These need to be adjusted to the activity of the respective GTPases. In principle, 7 to 8 time points are enough to cover a complete reaction. The intervals increase in the course of the reaction. For example 0, 1, 2, 5, 10, 15, 20, and 30 min have been used to determine the GTP hydrolysis reaction of the Rac proteins (Haeusler et al., 2003) and 0, 5, 10, 15, 20, 30, 40, 80, 150, and 200 min to determine that of H-Ras wild-type (Ahmadian et al., 1999).

5. Defrost each sample of the defined incubation interval at 95°C (using a thermoblock for about 10 sec) such that they are freshly thawed just before injecting on a reversed-phase analytical HPLC system.

   The HPLC system should be equipped with a guard column in between the injector valve and the analytical C18 reversed phase column. The buffer condition used for the separation of non-fluorescently-labeled nucleotides is HPLC buffer (see Reagents and Solutions) containing 7.5% acetonitrile. The retention time of guanine nucleotides on such an HPLC system and under the described isocratic conditions correlates with the number of phosphate groups of the nucleotide (retention order: GMP, GDP, and then GTP).

6. Determine the amount of each nucleotide immediately, using the HPLC integrator, from the area integration of the GTP- and GDP-peaks, respectively.

   These values are used to calculate the relative content of the GTP-bound GTPase species from the ratio (GTP)/(GTP) + (GDP).

   GTP content (y-axis) can be plotted versus the incubation time of the respective sample (x-axis) and fitted with a single exponential. Intrinsic GTP-hydrolysis rates of small GTPases usually are in the region of 0.0005 to 0.5 min^{-1}.

MEASUREMENT OF SLOW GAP-STIMULATED GTP HYDROLYSIS USING mantGTP

ALTERNATE PROTOCOL 2

Since there is almost no difference in the fluorescence signal between mantGTP and mantGDP, mantGTP-hydrolysis by the GTPase cannot be monitored directly. However, association of GAPs with the mantGTP-bound GTPase provides a tool to measure GAP-stimulated GTP-hydrolysis reaction, as shown for the Ras and RasGAP proteins (Ahmadian et al., 1997a). The GAP-stimulated GTP hydrolysis can be recorded with a stopped-flow instrument as a consequence of rapid association and dissociation of the GAP protein. Since this assay accounts only for the Ras/RasGAP system, here, the specific proteins Ras and the catalytic domain of p120RasGAP are mentioned as an example.

Additional Materials *(also see Basic Protocol 2 and Alternate Protocol 1)*

10 mM mantGTP (synthesized as described in Hemsath and Ahmadian, 2005 or purchased from Jena Biosciences) bound to Ras, pH 7.5
20 μM catalytic domains of RasGAP protein (e.g., $p120^{RasGAP}$) or any other GAP protein specific for Ras (expressed and purified as in Support Protocols 4 to 13)
Stopped-flow instrument (Applied Photophysics SX18MV or Hi-Tech SF-61 DX2)

NOTE: Because the samples are mixed 1:1, all stock solutions for components of the samples should be 2×.

1. Wash the drive syringes of the stopped-flow instrument several times with 5 to 10 ml GAP buffer and adjust the temperature to 25°C.

2. Charge one drive syringe with 2 μM mantGTP-bound Ras and fill the other syringe with 20 μM catalytic domains of the RasGAP protein (e. g., $p120^{RasGAP}$) in GAP buffer at 25°C.

3. Set the excitation wavelength to 366 nm (as for all mant-nucleotides) and restrict the emission light to >408 nm by a corresponding cut-off-filter.

   The same rules concerning volumes to prepare and photmultiplier settings described in Alternate Protocol 1 also apply to this assay.

4. After the initial injections to prepare the system for reliable data recording, carry out individual measurements, which usually show a rapid decrease of fluorescence.

   Change in fluorescence is, in this case, due to the GAP dissociation from the GDP-bound Ras after GTP hydrolysis. This implies a preceding fluorescence increase upon GAP association with the GTP-bound Ras, which cannot be resolved temporally by the stopped-flow machine due to the large rate constant of the association process. Depending on the rate constant of the association it is possible to observe (by a stopped-flow system with a short dead time) both association and GTP hydrolysis in one experiment, following subsequent dissociation of the Ras-RasGAP complex, which allows mechanistic studies (Ahmadian et al., 1997a,b).

   These measurements are in principle performed using the same procedure and conditions described in Alternate Protocol 1.

ALTERNATE PROTOCOL 3

MEASUREMENT OF SLOW GAP-STIMULATED GTP HYDROLYSIS USING tamraGTP

In contrast to other fluorescent nucleotide derivatives, including the mant-nucleotides, tamraGTP (a ribose hydroxyl-substituted tetramethylrhodamine derivative of GTP) enables us to measure the intrinsic and GAP-stimulated GTPase reactions of Rho and Ras proteins using fluorescence spectroscopy (Eberth et al., 2005). Besides much lower consumption of proteins and nucleotides as compared to the HPLC-based assay, the tamraGTP hydrolysis assay allows monitoring the real-time kinetics of the hydrolysis reaction of the Ras and Rho families. Intrinsic and GAP-stimulated hydrolysis reactions are recorded by a fluorescence spectrometer at an excitation wavelength of 546 nm and an emission wavelength of 583 nm, with an integration time of at least 2 sec and a recording time for each data point of 20 sec.

Additional Materials *(also see Basic Protocol 2)*

2 mM tamraGTP (synthesized as described in Eberth et al., 2005), pH 7.5
50 μM nucleotide-free GTPase (Support Protocol 2) in GAP buffer (see recipe for buffer)

Fluorescence cuvettes, Suprasil quartz glass; Hellma, cat. no. 108.002F-QS
Fluorescence spectrometer (Perkin Elmer, Spex Instruments)
Grafit program (Erithacus Software) or alternative program packages for evaluation of the data

1. Preincubate a solution of 0.1 μM tamraGTP (prepared from 2 mM stock solution) in a fluorescence cuvette in GAP buffer (stored at 25°C) with the GAP protein, at a final volume of 600 μl at 25°C for at least 5 min.

   The GAP protein would be added in step 1 to the tamraGTP before the reaction is started by addition of nucleotide-free GTPase in step 2.

2. Add 1.8 μl of 50 μM stock solution of nucleotide-free GTPase (0.15 μM final concentration), to observe complex formation with the nucleotide through a strong increase in fluorescence.

   Alternatively, four parallel measurements can be performed simultaneously using an instrument equipped with an automated four-position turret.

3. After this initial phase of nucleotide association, monitor the significant fluorescence decay as a result of GTP hydrolysis, which lasts between 0.5 and 6 hr, depending on the GTPase variant used (Eberth et al., 2005).

   Such an intrinsic reaction is significantly faster in the presence of a catalytic amount of a GAP protein (0.001 μM to 1 μM, depending on the specific activity of the GAP protein).

   TamraGTP fluorescence cannot be used for Rab, Ran, alpha-subunits of the heterotrimeric G-proteins, and elongation factors (Eberth et al., 2005).

4. Continue the measurement until no further decrease in fluorescence can be observed.

5. Evaluate data obtained by single-exponential fitting with a scientific software, e.g., the Grafit program.

MEASUREMENT OF FAST GAP-CATALYZED GTP HYDROLYSIS WITH tamraGTP

ALTERNATE PROTOCOL 4

The following protocol measures GAP-stimulated tamraGTP hydrolysis by a stopped-flow instrument as described in Alternate Protocol 1, except that an excitation wavelength of 546 nm is employed and a cut-off-filter of 570 nm in front of a photomultiplier is used to detect the fluorescence.

Additional Materials *(also see Basic Protocol 2 and Alternate Protocol 1)*

tamraGTP (synthesized as described in Eberth et al., 2005; 2 mM in deionized H_2O, pH 7.5)
Stopped-flow instrument (Applied Photophysics SX18MV or Hi-Tech SF-61 DX2)

NOTE: Because the samples are mixed 1:1, all stock solutions for components of the samples should be 2×.

1. Wash the drive syringes of the stopped-flow instrument several times with 5 to 10 ml GAP buffer and adjust the temperature to 25°C.

2. Prepare 2× samples in GAP buffer at room temperature (~25°C) and a final volume of 1000 μl:

   a. One sample contains 0.3 μM nucleotide-free GTPase and 0.2 μM tamraGTP.

   b. The other sample contains the GAP protein (at a concentration ranging from 0.2 to 200 μM, depending on the activity and affinity of the GAP for the respective GTPase).

      Example: (1) prepare the GAP solution by diluting the concentrated GAP protein to a defined concentration between 0.2 to 200 μM in GAP buffer. (2) Dilute 6 μl from a 50 μM solution of nucleotide-free GTPase (0.3 μM final concentration) in 984 μl GAP buffer and add 10 μl from a 20 μM tamraGTP solution (0.2 μM final concentration) just prior to loading the drive syringes of the stopped-flow instrument with your sample. The latter sample is prepared immediately before starting the measurement in order to avoid intrinsic tamraGTP-hydrolysis, particularly in the case of rapidly GTP-hydrolyzing proteins such as Rho GTPases.

      In the case of slow GTP-hydrolyzing GTPases (e.g., Ras or Rap) or GTP-hydrolysis-deficient Rho or Ras proteins, tamraGTP-bound proteins can be prepared and stored at −80°C for several weeks.

      Using 1000-μl samples, up to 11 identical measurements can be sequentially performed, from which the first three are required equilibrate the sample cell. The fourth measurement can be used to reset of the photomultiplier current and to determine the timescale for recording the complete reaction.

The photomultiplier current should be adjusted manually to a value with a well-balanced signal-to-noise ratio. Alternatively, modern instruments are also able to set an optimal value automatically.

The single-exponential data obtained corresponds to one experiment at a defined GAP concentration.

To obtain k_{cat} and K_d values, increase GAP concentrations as described (Eberth et al., 2005).

3. Continue as described in steps 3 to 6 of Alternate Protocol 1, except use 546 nm as excitation wavelength and a 570 nm cutoff filter mounted in front of the photomultiplier.

SUPPORT PROTOCOL 4

GENE EXPRESSION AND BACTERIAL CULTURE CONDITIONS

For the optimization of synthesis of a protein of interest in *E. coli*, various culture conditions should be examined including concentration of isopropyl-D-thiogalactopyranoside (IPTG; the inducer of lac-promoter-controlled gene expression), optical density of culture at IPTG induction, and, particularly, the temperature and culturing time. These culture-condition tests should be performed in small-scale (20 to 50 ml) pilot studies prior to scaling up to large-scale cultures for preparative protein expression. To improve the maximal recovery, we alternatively used, besides *E. coli* strain BL21 (DE3), strains containing additional plasmids such as BL21 pLys S (to improve bacterial lysis and for the expression of toxic proteins), and BL21 Codon Plus RIL or Rosetta (DE3) (to improve the codon usage).

All recombinant proteins described in this unit, including GTPases, GEFs, GAPs, and their catalytically active fragments are expressed in *E. coli* and purified using the methods described in this and the following support protocols.

Materials

Escherichia coli strain: BL21(DE3), BL21(DE3) Codon plus RIL, BL21(DE3) pLysS, or Rosetta (DE3) (Novagen) containing prokaryotic expression plasmid carrying gene for protein of interest
Terrific broth medium (TB medium; see recipe)
Appropriate selection antibiotics
Isopropyl-β-D-thiogalactopyranoside (IPTG; Gerbu Biochemicals, *http://www.gerbu.de*)
Wash buffer (see recipe)

150- to 1000-ml and 5-liter sterilized Erlenmeyer flasks
Horizontal environmental shaker incubator (Infors HT, *http://www.infors-ht.com/*)
1000-ml and 30- to 250-ml centrifuge bottles
Centrifuge: Avanti J-20 XP (Beckman Coulter) or equivalent
6-liter rotor: JLA-8.1000 (Beckman Coulter) or equivalent
50-ml plastic tubes

1. Grow 30 to 250-ml precultures of the desired *E. coli* strain in TB medium in a 150- to 1000-ml flask overnight at 37°C.

   Remember to add the required antibiotics to the TB medium to maintain transformed plasmids. If using BL21 (DE3) Codon plus RIL, BL21 (DE3) pLysS, or Rosetta (DE3) strains, chloramphenicol (25 mg/liter) needs to be added.

2. Fill each 5-liter Erlenmeyer flask to be used with 2.5 liter TB medium. Inoculate each flask with 25 ml of an overnight preculture.

   Cultivations usually are carried out in 2.5- to 20-liter scale, depending on the expression level and yield of the particular protein.

3. Place the inoculated culture flasks in a horizontal environmental shaker and let them grow at 37°C and 160 rpm.
4. When the logarithmic growth phase is reached (OD_{600} = 0.4 to 0.8), lower the temperature to the previously determined optimal expression condition (usually 18° to 30°C), and add IPTG (usually 0.05 mM to 0.5 mM; depending on pilot tests of expression conditions) to induce gene expression.

   The small GTPases as well as GEF and GAP proteins including their catalytic domains are usually expressed at an optical density of OD_{600} = 0.6 to 0.8, with 0.1 mM IPTG, and at 18° to 25°C overnight.
5. Perform the incubation either overnight, or for only 2 to 4 hr in difficult cases, depending on the results from the optimization of expression tests.
6. Transfer the cells to 1000-ml centrifuge bottles and harvest the cells by centrifugation for 15 min at 6000 × *g*, 4°C, using a 6-liter rotor if available. Repeat this step several times if the culture volume exceeds the capacity of the available rotor.
7. Wash the bacterial pellet in each rotor bottle with 20 ml wash buffer. Combine the resuspended cell pellets into a smaller (30- to 250-ml) rotor bottle (tared) and centrifuge again for 20 min at 6000 × *g*, 4°C.

   This step is carried out in order to remove residual medium.
8. Discard the supernatant and weigh the bacterial pellet–containing bottle. Determine the weight of the bacterial pellet and resuspend it in wash buffer (3 ml/g bacterial pellet) or any other proper buffer that is suitable for solubilizing and stabilizing the desired protein. Prepare aliquots in 50-ml plastic tubes.
9. Store aliquots at −20°C.

   In addition to cryopreserving the sample, freezing will also help to improve the efficiency of bacterial lysis.

BACTERIAL LYSIS BY SONICATION

SUPPORT PROTOCOL 5

An efficient bacterial lysis is an important prerequisite for the complete recovery of expressed recombinant protein. Cell walls of bacteria which have produced proteins of interest must be disrupted in order to allow access to intracellular components. Different methods have been developed to achieve this goal; they vary considerably in the severity of the disruption process, reagents needed, and the equipment available. Besides enzymatic methods using lysozyme treatment, which is suitable for analytical scale and not always reproducible, there are several mechanical methods—including glass bead, cell bomb, French press, sonication, and microfluidizer—to gently disrupt bacterial cell walls. We commonly use the latter two methods, both of which are efficient and fairly quick. This protocol describes sonication, which permits cell disruption in smaller samples (≥200 µl and ≤200 ml).

Materials

70% ethanol
Bacterial sample (Support Protocol 4)
Pefabloc (ICN Biochemicals)
Lysozyme (Sigma-Aldrich)
DNase I (Sigma-Aldrich)
Sonicator: Branson Sonifier S-450A and 3- to 19-mm titanium probe

NOTE: The protease inhibitor Pefabloc (0.02% w/v) and lysozyme (2 µg/ml suspension), as well as DNase I (10 µg/ml suspension) are added to the bacterial suspension before lysis.

1. Equip the cell sonicator with a titanium probe of 3- to 19-mm diameter (depending on the culture volume to be disrupted) and clean it before use with 70% ethanol.

   Use a 3-mm sonifier probe for volumes of 2 to 50 ml and a 19-mm probe for volumes of 50 to 500 ml.

2. Transfer the thawed bacterial suspension from all aliquots to a beaker of suitable size and place it on ice.

3. Place the sonicator probe about 0.5 to 1 cm beneath the surface into the suspension.

4. Start the sonication procedure by increasing the output control (add 5 to 10 W each time) at 10-sec intervals starting with 30 W and ending with ~95 W. Repeat this procedure eight to twelve times, and always wait 30 sec in between to prevent overheating of the sample. Keep the beaker with the bacterial solution on ice during the entire procedure.

   Optionally, the wave duty cycle function of the ultrasonic instrument can be used to reduce heat production and free radical formation.

   A color change from very milky to slightly more translucent should be observed and can act as an indicator for cell disruption.

SUPPORT PROTOCOL 6

BACTERIAL LYSIS USING A MICROFLUIDIZER

The microfluidizer is an instrument that uses high pressure to squeeze the bacterial solution through a flux cell containing a narrow channel, thereby generating high shear forces that pull the cells apart. The system permits controlled cell breakage and does not require addition of detergent or higher ionic strength. Since heat is generated during this process, the flux cell needs to be cooled. The microfluidizer system provides a convenient and efficient method for cell lysis of larger cell suspensions (≥20 ml to several liters).

Materials

Bacterial sample (Support Protocol 4)
Buffer to be used for protein purification
Pefabloc (ICN Biochemicals)
Lysozyme (Sigma-Aldrich)
DNase I (Sigma-Aldrich)
100% 2-propanol
Microfluidizer (Microfluidics Corp., *http://www.microfluidicscorp.com*)

NOTE: The protease inhibitor Pefabloc (0.02% w/v) and lysozyme (2 μg/ml suspension) as well as DNase I (10 μg/ml suspension) are added to the bacterial suspension before lysis.

1. Wash the instrument extensively with water. For a final wash step, use the standard buffer for the protein purification.

   This will remove all traces of alcohol in which the instrument is usually stored to prevent microbial growth.

2. Pour the thawed bacterial suspension into the instrument's reservoir and turn on the instrument

   Direct the flow of the instrument's outlet toward the wall of a beaker to prevent foam formation.

3. Prevent intake of air on the inlet, as this will also produce foam and lead to protein denaturation. For this, turn off the instrument before air enters the instrument's inlet.

Wash with a small volume of standard buffer and switch the instrument on again. Stop again before air enters the inlet of the instrument.

By repeating this step two to three times, nearly all of the bacterial suspension will be processed.

4. If necessary, flush the bacterial solution two to three times through the instrument, until a color change from milky to slightly more translucent is observed.

5. Wash the instrument extensively with water and finally with 2-propanol, and store it in this solution.

PROTEIN PURIFICATION FOR GST FUSION PROTEINS

SUPPORT PROTOCOL 7

The use of proteins with high purity (>95%) is a mandatory prerequisite for investigation of protein structure-functional relationships. The use of recombinant protein expression systems and the development of a variety of fusion tags has facilitated the preparation of high-purity proteins dramatically. Nevertheless, choosing the right purification strategy is still a matter of trial and error and has to be discovered for each individual protein. The GST-fusion affinity purification system is a well established technique and works successfully for purification of GTPases and their regulatory proteins.

Materials

Bacterial lysate with an overexpressed GST-fusion protein
Glutathione-Sepharose 4B FF (GE Healthcare)
Standard buffer (see recipe)
Standard buffer (see recipe) containing 500 mM KCl and 1 mM ATP
Standard buffer containing 20 mM glutathione (adjusted to pH 7.5 with NaOH again after addition of glutathione)
0.01% (w/v) sodium azide *or* 20% (v/v) ethanol

Centrifuge: Avanti J-30I (Beckman Coulter) or equivalent
Rotor: JA-30.50 or JA-17 (Beckman Coulter) or equivalent
Äkta Sytem, e.g. Äkta Prime (GE Healthcare)
XK 26/20 chromatography column chassis (GE Healthcare)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and Coomassie staining (*UNIT 6.6*)

Centrifuge lysate

1. Separate insoluble constituents from the bacterial lysate by centrifuging 40 min at 35,000 to 100,000 × *g*, 4°C.

If possible, centrifuge at 100,000 × g to remove as much insoluble cell debris as possible. If such a high-speed rotor/centrifuge is not available, a minimal force of 35,000 × g might also be sufficient.

Prepare glutathione-Sepharose column

2. Equilibrate an XK 26/20 column packed with Glutathione-Sepharose (≥25-ml bed volume) with ~3 to 4 column volumes of standard buffer until a stable baseline is reached. Monitor absorption at 280 nm using the Äkta System.

3. After centrifugation apply the cleared bacterial lysate onto the Glutathione-Sepharose column (at 4 ml/min, if using fast-flow material).

4. After all lysate is applied, wash with standard buffer until the baseline at 280 nm is reached again.

5. Wash with 2 to 4 column volumes standard buffer containing 500 mM KCl and 1 mM ATP in order to elute nonspecific proteins, especially chaperones that might

be associated with the desired protein. Afterwards, wash again with standard buffer until the baseline at 280 nm is reached.

High amounts of chaperones indicate folding difficulties with the protein of interest.

Note that the baseline increases due to 1 mM ATP and drops down after changing to the standard buffer with no ATP.

6. Elute GST-fusion proteins from the column with 100 to 150 ml standard buffer containing 20 mM glutathione. Collect fractions of 2- to 5-ml volume using the fraction collector provided with the Äkta system.

   The pH value of the standard buffer needs to be readjusted to 7.5 with NaOH after addition of glutathione.

7. Analyze peak fractions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*) and Coomassie staining (*UNIT 6.6*).

8. Regenerate the column with 50 ml 5 M guanidine hydrochloride and wash with 100 to 150 ml standard buffer afterwards.

   If the column will not be used for a long period, it should be stored in a buffer containing 0.01% sodium azide, or, alternatively, in 20% ethanol solution.

SUPPORT PROTOCOL 8

DETERMINING PROTEIN CONCENTRATION USING THE BRADFORD ASSAY

There are different colorimetric and spectrophotometric methods to determine the concentration of proteins in a solution including the Lowry, the Smith copper/bicinchoninic assay, or the Bradford dye assay. We use both the Bradford dye assay and the Ehresmann UV method (Support Protocol 9) for determination of the concentration of a protein in solution as well as HPLC in the case of nucleotide binding proteins (also see *APPENDIX 3B* and *APPENDIX 3H*).

Materials

Protein solutions: standards (e.g., BSA or γ-globulin) and test sample
Bradford reagent (Coomassie dye reagent; Sigma, Pierce, Bio-Rad, or see *APPENDIX 3H*)
Spectrophotometer

1. Mix 0.5 ml of Bradford reagent with 1 to 20 μl of a protein solution. For the standard curve, mix 0.5 ml of Bradford reagent with 1 to 2 μl of different concentrations of a standard protein (e.g., BSA or γ-globulin) covering the range of 0.25 to 2 mg/ml.

   The reagent/protein solution should shift color to blue.

2. Incubate for 5 min at room temperature.

3. Measure the absorption of the sample at 595 nm.

   The absorption should be between 0.2 and 0.8 to guarantee correlation between absorption and concentration according to the Lambert-Beer law. If the absorption is too low or too high, concentrate or dilute the protein solution and reassay.

4. Determine the protein concentration using the linear absorbance profile of the protein standard.

   The Bradford dye solution can be calibrated using different concentrations of a standard protein like BSA or γ-globulin. The absorption values of the standards at 595 nm can be plotted versus the standard protein concentrations and fit by a linear equation. The slope of this regression line will represent a correlation factor between absorbance and protein concentration and can in principle be used for the determination of protein concentrations.

DETERMINING PROTEIN CONCENTRATION USING THE EHRESMANN ASSAY

SUPPORT PROTOCOL 9

This method is rather useful for small proteins or peptides, since their staining by the Bradford dye solution does not lead to a shift of the absorption maxima from 465 nm to 595 nm comparable to that of BSA or γ-globulin used as standard protein for calibration. This method cannot be used for nucleotide binding proteins, since the bound nucleotides also absorb at the wavelengths used.

Materials

Protein solution
Quartz cuvettes
UV/VIS spectrophotometer

1. Dilute the protein sample to ~50 μg/ml in deionized water.
2. Measure the absorption (using a quartz cuvette) of the dilution at 228.5 nm and 234.5 nm, and use deionized water as a reference.
3. Calculate the concentration by the following equation: $A_{228.5} - A_{234.5}/3.154 =$ mg/ml.

No protein standard is used here since the correlation between concentration and absorption is due to light absorption of the peptide backbone and not to reactive side chains of the proteins.

CONCENTRATING A DILUTE PROTEIN SOLUTION

SUPPORT PROTOCOL 10

There are a large variety of methods that can be used for concentrating a protein solution, including dialysis, ammonium sulfate precipitation (salting out), ion exchange followed by desalting chromatography, ultrafiltration or spin-filters, and trichloroacetic acid/deoxycholate precipitation.

We often use the ultrafiltration method by employing spin filters, which accumulate the protein at a membrane with a defined molecular weight cutoff (MWCO 5 to 100 kDa) while the solvent passes through.

Materials

Protein solution
Refrigerated centrifuge
Amicon filter, MWCO 5 to 100 kDa

1. Fill the Amicon device with the diluted protein solution and centrifuge at 2900 × *g*, 4°C.
2. Continue centrifuging either until a desired concentration is reached (10 to 20 mg/ml is a proper concentration for storage) or the volume is sufficient for further purification via gel filtration ($\leq$1% of the column volume).

It is important not to centrifuge to the point where all liquid in the reservoir passes through the membrane; this would lead to drying of the protein and consequently to denaturation.

THROMBIN PROTEOLYTIC CLEAVAGE OF GST FUSION PROTEINS

SUPPORT PROTOCOL 11

The fusion tag that helps to purify a recombinant protein from crude cell extracts should be removed when the protein is intended for use in structural or biochemical analysis. There are a variety of expression vectors available which have protease-specific cleavage sites inserted between the coding sequence for the fusion tag and the multiple cloning site.

The corresponding fusion protein can thus be processed with the appropriate protease and, finally, the fusion tag can be removed by further chromatographic purification steps. While this protocol is for thrombin cleavage of GST fusion proteins, it can be adapted for other enzyme/linker combinations.

Materials

GST-fusion protein
Thrombin (Serva)
End-over-end rotator

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and Coomassie staining (*UNIT 6.6*)

1. Cleave fusion proteins (at a concentration of ≥1 mg/ml) in batch by incubation with 1 U thrombin per mg GST-fusion protein for 4 to 16 hr at 4°C with end-over-end rotation.

   Other vectors containing PreScission, factor Xa, TEV, enterokinase, or IgA protease cleavage sites can alternatively be used if thrombin does not cleave (masked cleavage site) or additional thrombin cleavage sites are within the fusion protein.

2. Take 10 µl from the reaction mix after 4 hr and after the overnight incubation and analyze the cleavage progress by SDS-PAGE (*UNIT 6.1*) and Coomassie blue staining (*UNIT 6.6*).

   Usually, an overnight incubation at 4°C is sufficient for a quantitative cleavage of the fusion protein. In the SDS-PAGE gel, a band of 26 kDa for GST and a band of the size of the fusion partner will appear, whereas the band of the fusion protein at higher molecular size will disappear.

SUPPORT PROTOCOL 12

GEL-FILTRATION CHROMATOGRAPHY

Further purification and removal of protein impurities or small components including glutathione are achieved by size-exclusion chromatography (also called gel filtration) on the scale of 16/600 or 26/600 columns (meaning columns of 16- or 26-mm diameter and 600-mm length, respectively) using Superdex 75 or Superdex 200 medium.

Materials

Concentrated protein sample (after digestion of the fusion protein with the respective protease; see Support Protocol 10 for concentration and Support Protocol 11 for digestion)
Standard buffer (see recipe)

16/600 or 26/600 columns prepacked with Superdex 75 or Superdex 200 (GE Healthcare)
Äkta System, e.g. Äkta Prime (GE Healthcare)
Prepacked 16/600 or 26/600 columns with Superdex 75 or Superdex 200 resin (GE Healthcare)
1- to 5-ml sample loop (GE Healthcare)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), Coomassie staining (*UNIT 6.6*), and concentration of protein samples (Support Protocol 10)

1. Equilibrate the column with one column volume (120 ml for 16/600 Superdex or 320 ml for 26/600 Superdex) of standard buffer using the Äkta System.

   Use a 16/600 column for protein amounts of ≤40 mg or 26/600 column for ≥40 mg but ≤100 mg; when the protein amount to be purified exceeds 100 mg, divide the sample into several portions ≤100 mg and do several consecutive column runs. Use 120 ml of buffer for the 16/600 column and 320 ml buffer for the 26/600 column.

2. Mount a 1- to 5-ml loop (GE Healthcare) on to the Äkta System, flush it with standard buffer, and load with the concentrated protein (≥10 mg/ml).

   The volume of the concentrated protein sample to be injected should not exceed 1% of the column volume to guarantee efficient separation. In case of a 16/600 column this means that no more than 1.2 ml, and in case of a 26/600 no more than 3.6 ml of protein solution should be injected.

3. Collect 1- to 3-ml fractions with the Äkta fraction collector and analyze 10-µl samples by SDS-PAGE (*UNIT 6.1*) and Coomassie staining (*UNIT 6.6*).

4. Pool and concentrate the fractions containing the desired protein to 10 to 20 mg/ml (Support Protocol 10).

   If the gel filtration does not separate the GST fractions from the desired protein, a second affinity chromatography with glutathione-Sepharose is necessary to remove the GST completely.

FREEZING AND THAWING PROTEINS

SUPPORT PROTOCOL 13

Freezing and thawing of protein solutions is a very critical step and has a large impact on protein stability. It is absolutely mandatory to freeze a protein solution in liquid nitrogen and to store it afterwards at −20°C or even −80°C for storage. For longer storage periods, the latter is recommended. Before freezing a protein, try a small-scale test to verify that the protein can be frozen in a standard buffer and to test if addition of supplements like glycerol or sucrose might help to prevent protein denaturation during freezing. Thawing of protein solutions can either be performed rapidly at 37°C or slowly on ice. For each protein, the recommended strategy has to be elucidated in trials.

Materials

50- to 500-µl aliquots of purified protein (10 to 20 mg/ml)
Liquid nitrogen

1. Snap freeze 50- to 500-µl aliquots of purified proteins in liquid nitrogen, then store in −80°C freezer.

2. Thaw all proteins except nucleotide-free GTPases on ice. Thaw nucleotide-free GTPases at 37°C and immediately store on ice when defrosted.

   It is recommended to freeze and thaw an aliquot only once; otherwise, activity of the protein might be reduced due to repeated freezing/thawing cycles. Adjust the volume of the aliquots to the volume needed for the respective application. However, we have observed that most proteins described in this unit (GTPases and GTPase-regulators like GEF- and GAP-proteins) can in fact be frozen and thawed at least five times without any detectable loss of activity.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Exchange buffer, 10×

2 M $(NH_4)_2SO_4$
10 mM $ZnCl_2$
Filter and degas
Store up to several weeks at room temperature

GAP buffer

30 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10 mM $MgCl_2$
3 mM dithiothreitol (DTT)
10 mM potassium phosphate buffer,, pH 7.4 (*APPENDIX 2A*)
Filter and degas
Store up to several weeks at room temperature

GEF buffer

30 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
5 mM $MgCl_2$
3 mM dithiothreitol (DTT)
10 mM potassium phosphate buffer, pH 7.4 (*APPENDIX 2A*)
Filter and degas
Store up to several weeks at room temperature

HPLC buffer

100 mM potassium phosphate buffer, pH 6.5 (*APPENDIX 2A*)
10 mM tetrabutylammonium bromide (e.g., Merck)
7.5% to 25% (v/v) acetonitrile as specified in protocol
Filter and degas
Store up to several weeks at room temperature

Standard buffer

30 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
5 mM $MgCl_2$
3 mM dithiothreitol (DTT)
50 mM NaCl
Filter and degas
Store up to several weeks at room temperature

Terrific broth (TB) medium

12 g/liter Bacto-tryptone (BD Difco)
24 g/liter yeast extract (BD Difco)
0.4% (v/v) glycerol
2.31 g/liter KH_2PO_4
12.54 g/liter K_2HPO_4
Sterilize by autoclaving
Store up to 2 weeks at 4°C

Wash buffer

30 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
5 mM $MgCl_2$
3 mM dithiothreitol (DTT)
100 mM NaCl
Filter and degas
Store up to several weeks at room temperature

COMMENTARY

Background Information

Fluorescence techniques to study the intrinsic properties of GTPase-like nucleotide binding, nucleotide exchange, or GTP hydrolysis have been evolving over more than two decades. They were pioneered by Toshiaki

Hiratsuka and Roger S. Goody (Alexandrov et al., 2001; Hiratsuka, 2003). Since that time, further development has been going on, with the design of new fluorescent molecules, enhanced technical instrumentation, and new applications to study biochemical reactions and processes.

Fluorescence-based GEF and GAP assays described in Basic Protocols 1 and 2 and Alternate Protocols 1 through 4 are convenient methods to study the process of nucleotide release or hydrolysis. It has been shown that the extrinsic fluorophore usually does not influence these reactions. Fluorescence-based assays allow real-time monitoring of ligand- and protein-protein interactions at submicromolar concentrations, as well as quantification of the kinetic and equilibrium constants.

Critical Parameters and Troubleshooting

Spectroscopic measurements like the fluorescence assays described in this unit require the use of very clean quartz cuvettes, filtered and degassed buffers, and protein solutions without precipitate or any other solid material. Otherwise, light dispersion will take place and the signal-to-noise ratio will deteriorate. It is, therefore, very important to centrifuge the protein solution immediately before using it in assays.

To obtain reliable and reproducible kinetic data, the protein and nucleotide quality need to be high. Thus, nucleotides with >90% purity should be used and, if necessary, additional purification steps should be carried out to obtain nucleotides and proteins of a high purity. For HPLC measurements, the performance of the guard and main columns are essential for an optimal separation of the nucleotides. Washing the system with filtered and degassed deionized water should be carried out daily to prevent valve blockage by buffer salts. Regeneration of the columns should be accomplished periodically according to the column manufacturer's instructions to maintain separation performance.

When purifying guanine nucleotide–binding proteins, it is mandatory to add both GDP and magnesium ions to the buffer; they are essential especially in the case of low-affinity GDP/GTP-binding proteins, and will increase the protein stability.

Releasing the GTPase from GDP (or GTP in the case of the constitutive mutants) and loading with fluorescent nucleotides, as described above, are prerequisites to perform fluorescence measurements. The incubation time for preparing the nucleotide-free proteins varies among GDP/GTP-binding proteins, and has to be established for every GTPase.

The amounts of proteins and (fluorescent) nucleotides required are rather dependent on the assay used. For the determination of intrinsic nucleotide dissociation or intrinsic GTP hydrolysis in a cuvette (at a final volume of 600 μl), ~10 to 20 μg of nucleotide-bound GTPase is required for three identical experiments. At least 60 μg of catalytic domains of GEF or GAP proteins (250 to 300 amino acids) is needed for one experiment to measure the specificity and activity of these regulatory proteins. A stopped-flow experiment requires 3 to 5 μg GTPase, but provides an averaged value obtained from five to seven identical measurements. A stopped-flow analysis of the specificity of GEFs or GAPs requires about 15 to 50 μg of these proteins. However, between 2 and 10 mg of the catalytic domains will be required to quantitatively analyze the GEF or GAP activities.

It is recommended to perform each experiment using a negative and a positive control sample. The negative sample, necessary in order to have correct instrument settings, is assessed by measuring free fluorescent nucleotide in the absence of GTPase. The positive sample, necessary in order to make sure that the method works, is assessed by using known proteins, for example Ras, to measure intrinsic nucleotide dissociation and GTP-hydrolysis as well as GTPases in combination with their specific GEFs (e.g., Ras/Sos1, Rac1/Tiam1, Cdc42/Asef or RhoA/p115) or GAPs (Ras/p120, Rho GTPases/p50).

Anticipated Results

The elucidation of the molecular switch mechanism of the GTPases and particularly their specificities and affinities for regulators require the dissection of such interactions at the molecular level by utilizing a sensitive biochemical assay. Fluorescence spectroscopic methods provide researchers with a number of tools for studying the intercommunication of a GTPase with nucleotides and binding partners. Compared to other qualitative assays (e.g., filter-binding assay or thin-layer chromatography, which contain between three and six time points), fluorescence methods described in this unit allow monitoring of the activity of the GEFs and GAPs in real time, where every single measurement consists of at least 400 data points per reaction trace. These assays are highly sensitive and, in principle, reproducible presuming: (1) that the proteins of

interest do not reveal the expected activity; and (2) that the proteins and reagents are carefully prepared from high-purity materials and tested for quality. Thus, optimal gene expression and protein purification as well as sufficient quality of fluorescent nucleotide-bound GTPases and other components including GEF and GAP proteins and nucleotide derivatives are prerequisites for reliable and reproducible measurements. In all assays described in this unit, a decrease in fluorescence should be monitored in a time-dependent manner. However, another important aspect to be considered is the fluorescence offset, which represents the actual fluorescence signal value before the fluorescence decay is initiated by starting the reaction. This should be relatively similar for all experiments (1) when using the same instrument settings (e.g. the same monochromator slit with in a fluorescence spectrometer and photomultiplier tension in case of the stopped-flow instrument); (2) under the same concentrations of the fluorescent nucleotides in the complex with the GTPase; and (3) independent of the GEF or GAP concentrations.

The fluorescence-based assays described in this unit are quite sensitive methods which give proper results even if using submicromolar protein and nucleotide concentrations. The fluorescence decay followed over time is usually in the range of 40% to 60% in the case of nucleotide exchange experiments (GEF assay) and 10% to 15% in the case of the fluorescence GTP hydrolysis assay using tamra-GTP.

Time Considerations

The expression of a protein in *E. coli* takes about 2 days, including inoculation of the culture and induction of gene expression. On the second day, the culture is harvested, washed, resuspended, and stored in a buffer solution. When the expression is carried out for only 2 to 4 hr, all steps should be performed in 1 day. The purification of a protein usually involves several steps. It takes about 1 day to do the affinity chromatography, to elute, to analyze by SDS-PAGE, and to concentrate the fusion protein before the overnight protease digestion. On the next day, the protein can be applied to the size-exclusion chromatography (gel filtration) and subsequently analyzed by SDS-PAGE. Optionally, a second affinity chromatography may need to be carried out to completely remove the tag; it can be performed immediately after gel filtration. Finally the protein needs to be concentrated, which may take several hours, before it can be frozen in small aliquots and stored at –80°C.

The preparation of Gpp(NH)p-bound and mantGpp(NH)p-bound GTPases takes 2 to 48 hr, and usually can be carried out overnight. The generation of a nucleotide-free GTPase involves the same step followed by the phosphodiesterase reaction for another 2 to 30 hr until $Gpp(CH_2)p$ is completely degraded. The loading of a nucleotide-free GTPase with mant-GDP can be performed in 1 to 2 hr.

GEF measurements in a fluorescence spectrometer usually take at least 16 hr before the intrinsic nucleotide dissociation in the absence of the GEF protein is recorded. GEF-accelerated processes usually take less than 1 hr. Stopped-flow experiments are performed at higher concentrations of the GEF protein and are, thus, quite fast reactions on the order of seconds or even milliseconds. For quantitative measurements and evaluation of the data, 2 to 3 hr should be allowed.

Intrinsic GTP-hydrolysis measurements for small GTPases take 0.5 to 6 hr (depending on the GTPase), whereas the presence of catalytic amounts of a GAP protein advances the reaction to a 0.5- to 30-min process. Quantitative measurements using increasing GAP concentrations are also very fast and complete after a few seconds or even milliseconds.

Literature Cited

Ahmadian, M.R., Hoffmann, U., Goody, R.S., and Wittinghofer, A. 1997a. Individual rate constants for the interaction of Ras proteins with GTPase-activating proteins determined by fluorescence spectroscopy. *Biochemistry* 36:4535-4541.

Ahmadian, M.R., Stege, P., Scheffzek, K., and Wittinghofer, A. 1997b. Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras. *Nat. Struc. Biol.* 4:686-689.

Ahmadian, M.R., Zor, T., Vogt, D., Kabsch, W., Selinger, Z., Wittinghofer, A., and Scheffzek, K. 1999. Guanosine triphosphatase stimulation of oncogenic Ras mutants. *Proc. Natl. Acad. Sci. U.S.A.* 96:7065-7070.

Ahmadian, M.R., Wittinghofer, A., and Herrmann, C. 2002. Fluorescence methods in the study of small GTP-binding proteins. *Methods Mol. Biol.* 189:45-63.

Ahmadian, M.R., Kiel, C., Stege, P., and Scheffzek, K. 2003. Structural fingerprints of the Ras-GTPase activating proteins neurofibromin and p120GAP. *J. Mol. Biol.* 329:699-710.

Alexandrov, K., Scheidig, A.J., and Goody, R.S. 2001. Fluorescence methods for monitoring interactions of Rab proteins with nucleotides, Rab escort protein, and geranylgeranyltransferase. *Methods Enzymol.* 329:14-31.

Eberth, A., Dvorsky, R., Becker, C., Beste, A., Goody, R.S., and Ahmadian, M.R. 2005.

Monitoring the real-time kinetics of the hydrolysis reaction of guanine nucleotide binding proteins. *Biol. Chem.* 386:1105-1114.

Guo, Z., Ahmadian, M.R., and Goody, R.S. 2005. Guanine nucleotide exchange factors operate by a simple allosteric competitive mechanism. *Biochemistry* 44:15423-15429.

Haeusler, L.C., Blumenstein, L., Stege, P., Dvorsky, R., and Ahmadian, M.R. 2003. Comparative functional analysis of the Rac GTPases. *FEBS Lett.* 555:556-560.

Hemsath, L. and Ahmadian, M.R. 2005. Fluorescence approaches for monitoring interactions of RhoGTPases with nucleotides, regulators and effectors. *Methods* 37:173-182.

Hiratsuka, T. 2003. Fluorescent and colored trinitrophenylated analogs of ATP and GTP. *Eur. J. Biochem.* 270:3479-85.

John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., and Goody, R.S. 1990. Kinetics of interaction with nucleotide-free H-ras p21. *Biochemistry* 29:6058-6065.

Scheffzek, K. and Ahmadian, M.R. 2005. GTPase activating proteins: Structural and functional insights 18 years after discovery. *Cell. Mol. Life Sci.* 62:3014-3038.

In Vivo Imaging of Signal Transduction Cascades with Probes Based on Förster Resonance Energy Transfer (FRET)

Takeshi Nakamura[1] and Michiyuki Matsuda[1]

[1]Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Kyoto, Japan

ABSTRACT

Genetically encoded FRET probes enable us to visualize a variety of signaling events such as protein phosphorylation and G-protein activation in living cells. This unit focuses on FRET probes wherein both the donor and acceptor are fluorescence proteins and incorporated into a single molecule, i.e., a unimolecular probe. Advantages of these probes lie in their easy loading into cells, simple acquisition of FRET images, and clear evaluation of data. We have developed FRET probes for Ras-superfamily GTPases, designated Ras and interacting protein chimeric unit (Raichu) probes. We hereby describe strategies to develop Raichu-type FRET probes, procedures for their characterization, and acquisition and processing of images. Although improvements upon FRET probes are still based on trial-and-error, we provide practical tips for their optimization and briefly discuss the theory and applications of unimolecular FRET probes. *Curr. Protoc. Cell Biol.* 45:14.10.1-14.10.12. © 2009 by John Wiley & Sons, Inc.

Keywords: FRET • unimolecular probe • CFP • YFP • Ras GTPase • Rho GTPase

INTRODUCTION

Signal transduction is an organized ensemble of dynamic state changes in signaling molecules, which is often caused by phosphorylation and guanine nucleotide exchange reactions. Needless to say, the timing and localization of these events critically influence the outcomes. To visualize such spatio-temporal changes in the activity of signaling molecules, several research groups have been developing probes based on the principle of Förster resonance energy transfer (FRET; Miyawaki, 2003; Kiyokawa et al., 2006). FRET is a radiation-less transfer of excited-state energy from a donor fluorophore to an acceptor fluorophore (Lakowicz, 2006). This unit focuses on FRET probes wherein both the donor and acceptor fluorophores are proteins and incorporated into a single molecule, i.e., genetically encoded unimolecular probes. The advantages of these probes lie in their easy loading into cells, simple acquisition of FRET images, and clear evaluation of the data (Kurokawa et al., 2004). Another type of FRET probe, namely, the bimolecular probe, is preferably used for monitoring protein-protein interactions (*UNIT 21.3*; also see *UNITS 17.1 & 17.9*).

A major application of unimolecular FRET probes is the monitoring of protein kinase activities. The activities of EGF receptor, Src, PKA, and PKC have been successfully monitored by FRET probes containing the specific substrate peptides for each kinase (Kurokawa et al., 2001; Ting et al., 2001; Zhang et al., 2001; Violin et al., 2003; Wang et al., 2005). Furthermore, FRET probes that monitor the activation-related conformational change have also been developed for PKC, Raf, Erk, and CaMKII (Braun et al., 2005; Takao et al., 2005; Terai and Matsuda, 2005; Fujioka et al., 2006). Visualization of the "on" and "off" states of Ras-superfamily GTPases is another valuable application of unimolecular FRET probes; such FRET probes developed in the authors' laboratory

Current Protocols in Cell Biology 14.10.1-14.10.12, December 2009
Published online December 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1410s45

are collectively designated Ras and interacting protein chimeric unit (Raichu) probes (Mochizuki et al., 2001).

The following procedure describes the development of Raichu-type FRET probes and the use of these probes to visualize the activation state of GTPases.

BASIC PROTOCOL 1

DEVELOPMENT OF RAICHU FRET PROBES

Raichu probes are composed of four modules: donor (CFP), acceptor (YFP), a GTPase, and the GTPase-binding domain of its binding partner (Fig. 14.10.1). In the Raichu probes for Ras-family GTPases, YFP, GTPase, GTPase-binding domain, and CFP are sequentially connected from the N-terminus by spacer peptides (Mochizuki et al., 2001). A typical mode of action of Raichu is as follows. In the inactive GDP-bound conformation, CFP and YFP are located at a distance from each other. Therefore, excitation of CFP results in emission mostly from CFP. Upon stimulation, GDP on the GTPase is exchanged for GTP, which induces an interaction between the GTP-bound GTPase and the GTPase-binding domain. This intramolecular binding brings CFP within close proximity to YFP, thereby permitting energy transfer from CFP to YFP. FRET is manifested by a quenching of CFP fluorescence and an increase in sensitized emission of YFP; therefore the fluorescence intensity ratio of YFP versus CFP can be conveniently used as a representation of FRET efficiency. This YFP/CFP value of the Raichu-expressing cells correlates with the molecular ratio of GTP-bound probes versus GDP-bound probes, which reflects the balance between guanine nucleotide exchange factors (GEFs) and GTPase-activating domains (GAPs) for the GTPase within the probe (Mochizuki et al., 2001; Yoshizaki et al., 2003). Therefore, the activities of endogenous GTPases, which are under the control of the same set of GEFs and GAPs, can be estimated by measuring the YFP/CFP ratio of Raichu-expressing cells.

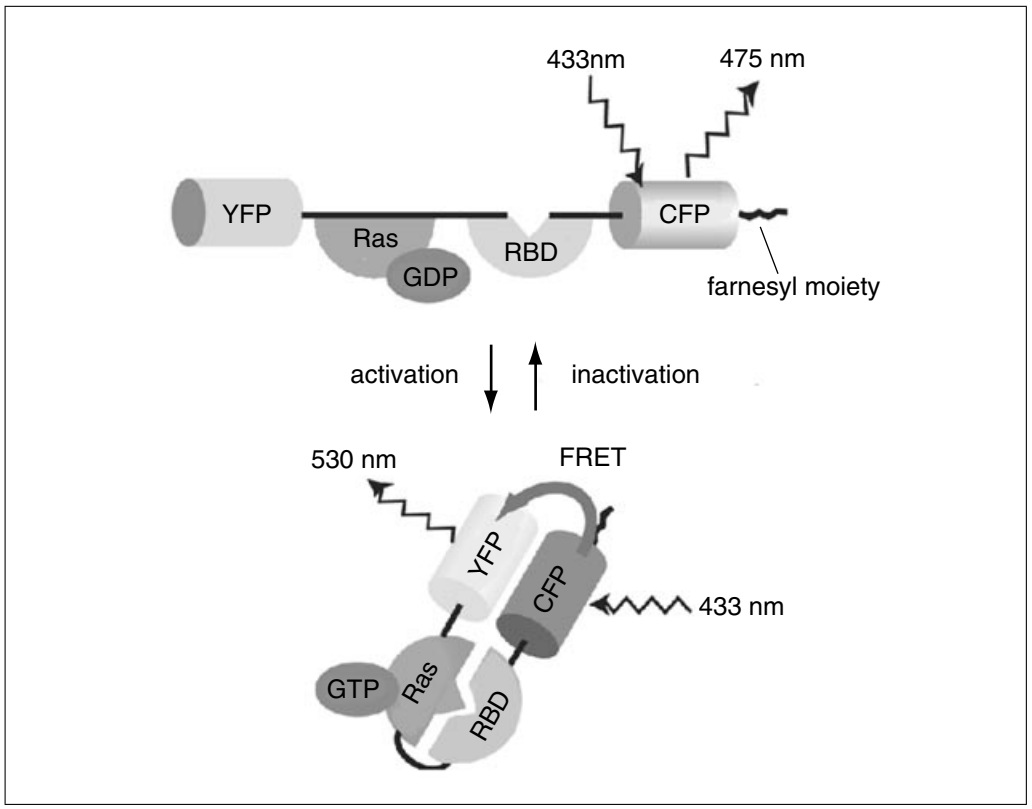


Figure 14.10.1 Basic structure of the GFP-based FRET probe for Ras GTPases (Raichu-Ras). The FRET probe for Ras activation comprises H-Ras and the Ras-binding domain (RBD) of Raf sandwiched between YFP and CFP. For plasma-membrane localization, this probe is fused to the carboxyl-terminal region of K-Ras4B. Upon stimulation, GDP on Ras is exchanged for GTP, and FRET occurs.

Materials

Needed DNA constructs:

- Plasmid for the Raichu probe (available from Matsuda Lab, *http://www.path1.med.kyoto-u.ac.jp/mm/e-phogemon/index.htm*)
- cDNA of the GTPase of interest (can either be purchased from a public depository or cloned by PCR from cDNA libraries)
- cDNA of GTPase-binding domains of effector proteins (can either be purchased from a public depository or cloned by PCR from cDNA libraries; try at least a few known effectors for initial experiments)

Ion-exchange resin for DNA purification (e.g., Qiagen; also see Ausubel et al., 2009)

Transfection reagent for calcium phosphate coprecipitation (*UNIT 20.3*)

293T cells (ATCC cat. no. CRL-11268), cultured in 100-mm-diameter collagen-coated dishes, 80% confluent

MEM (Invitrogen, cat. no. 10370) containing 10% fetal bovine serum (FBS)

Phenol-red-free MEM (Invitrogen, cat. no. 11054) containing 10% FBS

Lysis buffer (see recipe)

Fluorescence spectrophotometer (for example, JASCO FP-750) and 3-ml cuvettes

Additional reagents and equipment for basic molecular biology techniques including restriction digestion, PCR, plasmid preparation, cloning of DNA, and purification of DNA (Ausubel et al., 2009), and calcium phosphate transfection of DNA (*UNIT 20.3*)

Make candidate probes

1. Design a candidate Raichu probe.

   To achieve a wide dynamic range, it is desirable to search for a GTPase-binding domain having a moderate affinity for the GTPase (Yoshizaki et al., 2003). One explanation for this is that the GTPase-binding domain competes with GTPase-activating proteins (GAPs) in cells (Kurokawa et al., 2004). This inhibition of GAPs leads to a relatively high GTP level in the probe, even in the unstimulated state, and may therefore cause a narrowing of the dynamic range.

   Crystallographic data for the GTPase and GTPase-binding domain can help to determine the minimum regions to be incorporated into the probe. Unfortunately, crystallographic data for optimal design of a Raichu probe cannot currently be obtained in most cases. Therefore, various lengths of the GTPase and GTPase-binding domain should be tried until a satisfactory result is obtained. In addition, various combinations of the four modules, namely, YFP, CFP, GTPase, and the GTPase-binding domain, should be tested. YFP is usually placed before CFP because an excess of acceptor (YFP) does not decrease the signal-to-noise ratio by much, even when the translation of the probe is prematurely terminated.

   Eleven amino acids at the carboxyl terminus of GFP can be truncated without affecting its fluorescence profile. In most Raichu probes, we have removed the 11 carboxyl-terminal residues of YFP, hoping to reduce the flexibility between YFP and the subsequent module.

   The length and sequence of the spacers are also critical. If the FRET efficiency of a prototype probe varies to some extent upon activation, the possibility of further improvement by changing the spacer should be considered. As spacers, we usually use 1 to 6 repeats of the pentapeptide Gly-Gly-Ser-Gly-Gly. It is believed that Gly provides flexibility while Ser prevents aggregation of peptide chains. Misfolding of CFP occasionally occurs, and this can sometimes be rectified by modifying the spacer before the CFP. The ideal location for a probe in a cell has been a matter of debate. The most persuasive idea is that the probe should be colocalized with the endogenous protein; for this purpose, the GTPase's own authentic CAAX-box should be added to the C-terminus of the probe. Alternatively, the addition of the CAAX-box of K-Ras4B to the C-terminus enables the probe to be located mostly at the plasma membrane; this approach yields a high signal-to-noise ratio, especially when only a limited fraction of the GTPase is activated upon stimulation.

2. Construct a plasmid for a candidate probe.

   If you use the basic Raichu probe structure (Fig. 14.10.1), you should first perform PCR cloning of the trimmed GTPase and GTPase-binding domain with restriction enzyme recognition sites. Thereafter, insert the PCR fragments into the basic FRET vector.

   The 5′ and 3′ primers for the GTPase should have XhoI and Aor13HI restriction sites, respectively. The 5′ and 3′ primers for the GTPase-binding domain should have KpnI and NotI restriction sites, respectively. Any restriction enzymes that generate compatible ends can also be used. The primers must be designed so that the inserted cDNA is in the correct reading frame of the Raichu (http://www.path1.med.kyoto-u.ac.jp/mm/e-phogemon/index.htm). The amplified cDNAs of the GTPase and GTPase-binding domain are cleaved with the aforementioned restriction enzymes and subcloned into XhoI/Aor13HI and KpnI/NotI restriction sites, respectively. The order of the GTPase and GTPase-binding domain can be reversed by changing the restriction sites in the primers accordingly.

3. Prepare transfection-quality plasmid DNA.

   DNA purified using Qiagen ion-exchange resin (or equivalents from other manufacturers) performs well. Also see Ausubel et al. (2009).

Characterize candidate probes

4. Grow 293T cells to 80% confluence in MEM containing 10% FBS (may contain phenol red). Transfect 10 μg of a probe-encoding plasmid into 293T cells using calcium phosphate coprecipitation (*UNIT 20.3*).

   Candidate probes without lipid modification at the C-terminus are often preferable for spectral analysis, because this lipid modification generally reduces the level of probe expression.

5. At 6 hr after transfection, change the culture medium to phenol red–free MEM/10% FBS.

6. At 48 hr after transfection, harvest the cells in 1 ml lysis buffer. Microcentrifuge the lysates at 15 min at 10,000 × *g*, 4°C.

   The centrifugation serves to clear the lysate. If necessary, the cell lysates may be cleared by ultracentrifugation for 15 min at 100,000 × g, 4°C, to reduce the autofluorescence from cell debris.

7. Transfer the cleared lysates into 3-ml cuvettes and place the cuvettes in a fluorescence spectrophotometer. Next, illuminate lysates with an excitation wavelength of 433 nm, and obtain a fluorescence spectrum from 450 nm to 550 nm. Subtract the background using the spectra of cell lysates prepared without transfection.

   Fluorescence spectral analysis becomes even easier when the FreeStyle 293-F cell line (Invitrogen), a variant of the 293 cell line adapted for suspension growth, is used. This cell line is very easy to culture, transfect, and harvest, and only 1.5 ml of cell suspension is required to obtain a fluorescence spectrum.

8. Evaluate a candidate probe using the fluorescence spectrum (Fig. 14.10.2).

   For the characterization of a candidate probe, we introduce a constitutively active or inactive mutation into the GTPase in the probe for comparison with the same probe containing the wild-type GTPase. Alternatively, we cotransfect the candidate probe with guanine nucleotide exchange factor (GEF) and GAP toward the GTPase, and compare the new spectrum with those of samples transfected with the probe alone. Under our criteria, Raichu-type probes can be applicable for imaging analysis when the maximum increase (%) in the YFP/CFP ratio exceeds 30%.

   Practically, further evaluation of a probe is recommended before use in a wide range of applications: i.e., (1) whether the probe shows a near-linear correlation between its GTP loading and FRET efficiency upon cotransfection with various quantities of GEF or GAP and (2) whether the probe and its endogenous counterpart show comparable responses to physiological stimulations when examined by biochemical methods.

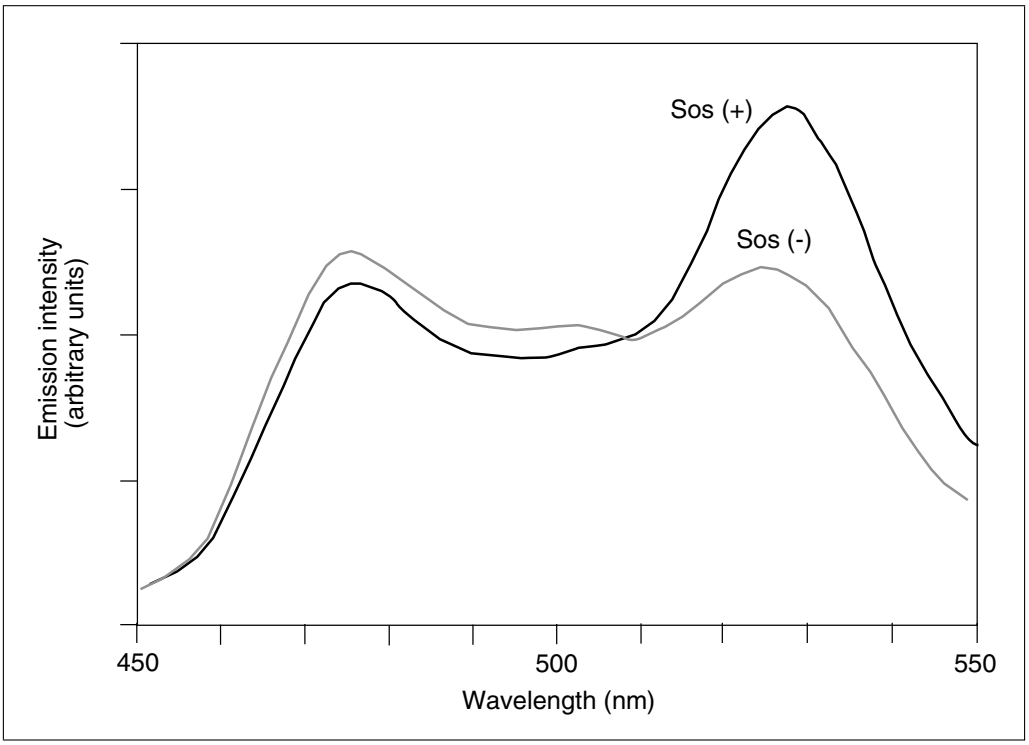


Figure 14.10.2 Fluorescence spectral analysis using 293T cells. Shown are emission spectra of Raichu-Ras expressed with the guanidine nucleotide factor Sos (black line, Sos +) or without Sos (gray line, Sos −) in 293T cells at an excitation wavelength of 433 nm.

IMAGING WITH FRET PROBES

BASIC PROTOCOL 2

Imaging with FRET probes must be performed with general live-cell imaging precautions, and should be modified depending on the specific cell type; these modifications are beyond the scope of this unit. Furthermore, the technical details differ among optical imaging systems and image analysis software. The following is only a typical example of such an analysis.

Materials

Cells for experiment [e.g., HeLa cells (ATCC cat. no. CRL-2) or COS7 cells (ATCC cat. no. CRK-1651)] and appropriate medium
Expression plasmid for FRET probe (Basic Protocol 1)
Transfection reagent
Phenol red-free medium
Mineral oil (Sigma)

35-mm glass-base dish (Asahi Techno Glass; *http://www.agc.co.jp/*) with a 10-mm-diameter glass coverslip mounted on the bottom
Temperature-controlled chamber with thermostat and/or a CO_2 controller
A fluorescence microscope with a CCD camera including:
IX81 inverted microscope
75-W xenon arc lamp
60× oil immersion objective lens, PlanApo 60×/1.4 (Olympus)
Cool SNAP-HQ cooled CCD camera (Roper Scientific)
Laser-based auto-focusing system, IX2-ZDC (Olympus)
Automatically programmable XY stage, MD-XY30100T-Meta (SIGMA KOKI).
Excitation and emission filter wheels: Filter wheel 99A354 and MAC5000 shutter controller (Ludl Electronic Products)

Filters:
XF1071 (Omega Optical; cat. no. 440AF21) excitation filter
XF2034 (Omega Optical; cat. no. 455DRLP) dichroic mirror
XF3075 (Omega Optical; cat. no. 480AF30) emission filter for CFP
XF3079 (Omega Optical; cat. no. 535AF26) emission filter for FRET
Neutral-density (ND) filters

Software for operation of the microscope and analysis of acquired images; e.g., MetaMorph software (Universal Imaging)

Additional reagents and equipment for construction of the FRET probe (Basic Protocol 1)

Prepare cells

1. Seed the cells onto a glass-base dish with a glass coverslip at the bottom.

   The coverslip at the bottom may be coated with collagen, fibronectin etc., depending on the cell types used. The cell density can be varied depending on the purpose of experiments and the transfection protocol.

2. Transfect cells with the plasmid for a FRET probe using any protocol suitable for the cells of interest.

   The transfection efficiency need not be high, because fewer than 10 cells can be time-lapse imaged in most experiments. Rather, the transfection method should be chosen to minimize cell toxicity.

3. Grow the cells, typically for 1 to 3 days, depending on the probe and cell type.

   In most cases, within 2 days the amount of probe reaches a level sufficient for imaging. In cases where the overexpression of the probes is toxic to the cells, the cells should be used 1 day after transfection.

4. On the day of the experiments, change the medium to phenol red-free medium and overlay the medium in the cell-containing dish with mineral oil.

   A reduction in the amount of serum is preferable to reduce the background fluorescence. Bovine serum albumin may be included when serum is not included. HEPES should be included in the medium unless CO_2 is supplied during imaging. In some cases, phosphate or HEPES-buffered saline may be preferable to minimize the background fluorescence. Note that some media, such as Opti-MEM, give off high fluorescence.

Acquire images of a single field of view

5. Warm the temperature control chamber to 37°C and set up fluorescence microscope.

   A large chamber that covers most of the microscope is preferable to minimize defocusing during image acquisition. However, if the microscope is equipped with an autofocusing system, the chamber may be as small as needed to keep the cells warm. Always use a neutral density (ND) filter to minimize the photobleaching of the probe during observation.

6. View the specimen on the microscope using a 60× oil-immersion objective and a filter set for CFP, and find a cell for time-lapse imaging.

   Cells to be observed should look healthy and represent the cell population. The optimal expression level varies depending on each FRET probe. Repeated experiments are required to determine the conditions suitable for the imaging.

7. Set up the image-acquisition software, with the following being a typical setup for the FRET imaging of growth factor-induced activation of signaling molecules:

   ND filter, 10%
   Camera binning, 4
   Digitizer, 10 MHz
   Gain 2 (4×)

Shutter speed for phase contrast image or differential interference contrast image, 50 msec
Shutter speed for CFP and FRET, 200 msec
Time-lapse interval, 1 min
Number of images, 60.

The conditions should be optimized for the probes and cells to be analyzed.

What should be most considered in this process is the photobleaching of YFP and phototoxicity to the cells. For an unknown reason, images obtained with shorter exposure time and brighter excitation light, which can be easily achieved by removing the ND filter, usually result in lower FRET efficiency. This is a principal reason why we prefer the conventional epifluorescence microscope to the scanning laser confocal microscope.

8. Start time-lapse imaging.

   When cells of interest become defocused, stop the image acquisition and focus the cells again. Minimize the time to focus, or YFP intensity will be decreased (YFP bleaches faster than CFP), resulting in apparent decrease in FRET.

Acquire images of multiple fields of view with autofocusing system

9. Set up the microscope as described above. Find the first cell to be monitored and focus on it. Set the offset value by using the laser-based auto-focusing system.

   Offset value is the distance from the top of the objective lens to the top of the coverslip. With a "find focus" command, the autofocusing system remembers the distance between the top of the objective lens to the top of the coverslip, and thereby corrects the z-axis coordinate prior to image acquisition. Usually, this correction is required only for the first cells at each time point.

10. Find cells to be monitored and record the coordinates.

    The number of cells to be imaged can be reduced afterwards; therefore, a large number of cells should be recorded at this stage. When the interval of time lapse is 1 min, 10 to 15 view fields can be imaged.

11. By moving the motor-driven stage with the obtained coordinates, confirm the cells to be monitored and correct the focus, if necessary.

    The order of the image acquisition can be changed to minimize the movement of the stage.

12. Start image acquisition and confirm whether a series of image acquisitions finishes within the interval time. If it goes well, continue image acquisition.

    If not, interrupt image acquisition and reduce the number of cells to be monitored, or extend the interval time. It is also strongly recommended to ensure that the hard disc contains sufficient space for the image.

Analyze the images

13. Create stack files for the acquired images and overview.

    Carefully examine for defocusing, floating bright debris, photobleaching, and phototoxic change in the cells.

14. Create background-subtracted images for CFP and FRET. First, choose region(s) for background, where no cells or debris are observed during the entire time-lapse imaging. Second, subtract the averaged value of the background region from the image. Third, repeat this background subtraction for all planes and make a background-subtracted stack file.

    The background value should be obtained for each plane. A macro should be prepared to repeat this process. Other corrections such as shading can be applied at this stage, if necessary.

15. Create ratio images either in simple pseudocolor or intensity-modulated display mode.

    The FRET/CFP value is usually used for showing the level of FRET.

    In the intensity-modulated display mode, the brightness and color of each pixel indicates the concentration of the probe and the FRET level, respectively.

16. Create video files. From the stacked ratio image, create video files either in `.avi`, `.mov`, or `.mpg` format.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Lysis buffer

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 mM NaCl
5 mM $MgCl_2$
0.5% (v/v) Triton X-100
Store indefinitely at 4°C

COMMENTARY

Background Information

FRET is a process by which a donor fluorophore in an excited state transfers its excitation energy to an acceptor nonradiatively through dipole-dipole interactions if the emission spectrum of the donor overlaps the excitation spectrum of the acceptor (Lakowicz, 2006; *UNIT 17.1*). This energy transfer manifests itself by a quenching of donor fluorescence intensity and lifetime, as well as the simultaneous increase in the emission of acceptor fluorescence. Because the efficiency of FRET decreases in proportion to the inverse sixth power of the distance between the donor and acceptor, FRET has been utilized as a spectroscopic ruler to measure the distance (<10 nm) between two sites on macromolecules. Additionally, when GFP and its derivatives are used for FRET probes, the relative orientation between the donor emission dipole and the acceptor absorption dipole has a significant impact on FRET efficiency. Based on these two properties, GFP-based FRET probes should be able to monitor protein-protein interactions and protein conformational changes in living cells (Miyawaki, 2003).

In general, GFP-based FRET probes are classified into two types, bimolecular and unimolecular probes (Miyawaki, 2003; Kurokawa et al., 2004). In the bimolecular probe, donor (CFP) and acceptor (YFP) are fused to the sensor domain and detector domain, respectively. The sensor domain changes its conformation upon modification. The detector domain binds to the sensor domain upon this conformational change. In the unimolecular probes, all four modules are combined into a single chain. In the authors' opinion, if good probes are available, using a unimolecular probe is a better way to start; in fact, the most significant hurdle of a unimolecular probe is the labor-intensive process needed to optimize its design. The reasons we chose a unimolecular probe as the basic design for detecting conformational changes are as follows. First, in the unimolecular probe, the sensor domain and the detector domain are placed within close proximity, and thus the detector domain is primed to bind the sensor domain upon stimulation. This increases the percentage of real FRET signal versus undesired signals that result from donor emission bleedthrough and direct acceptor excitation (Hailey et al., 2002; Kurokawa et al., 2004). Concurrently, the perturbation of endogenous signaling will be reduced (Miyawaki, 2003). Second, the molar ratio of CFP and YFP is a fixed constant at every pixel in the unimolecular probe; therefore, a simple ratio between the intensity of CFP fluorescence and that of YFP fluorescence suffices to represent FRET efficiency.

It should be noted that, from a general point of view, suitable applications for bimolecular and unimolecular probes are different. Thus, in practice, the type of probe is chosen depending on the aim of the experiment. In the case of monitoring an interaction

between two proteins, it is natural to select a bimolecular probe (*UNIT 17.1 & 21.3*). Correction of FRET signals obtained with a bimolecular probe is elaborate, but executable (Kraynov et al., 2000; Sekar and Periasamy, 2003). If one wishes to visualize the change of the activity of a protein, pH, Ca^{2+} concentration, etc., a unimolecular probe is preferable; in this case, one might take advantage of the merits described above.

Unimolecular FRET probes now cover a wide range of signal transduction cascades. The archetype is the Ca^{2+} sensor, cameleon (Miyawaki et al., 1997). The unimolecular cameleon has been expressed in specific cell types of genetically tractable animals, aiming at in vivo Ca^{2+} imaging (Kerr et al., 2000; Fiala et al., 2002). Concentration of other second-messenger molecules such as cyclic nucleotides and phosphoinositides can be visualized using unimolecular FRET probes (Sato et al., 2000, 2003; Zhang et al., 2001).

Indicators for the on and off states of small GTPases such as Ras, Rap1, Ral, R-Ras, RhoA, Rac1, and Cdc42 have been developed (Mochizuki et al., 2001; Itoh et al., 2002; Yoshizaki et al., 2003; Takaya et al., 2004; Pertz et al., 2006; Takaya et al., 2007). These FRET probes have been used for the investigation of ligand-induced morphological change, cell migration, cell division, neurite outgrowth, and membrane trafficking. FRET imaging of Raichu-TC10 by total internal reflection microscopy uncovered that the activity of TC10 on the exocytic vesicles drops immediately before the vesicular fusion (Kawase et al., 2006).

The largest number of unimolecular FRET probes are used to monitor protein kinase activities. In archetypical probes, an appropriate phosphorylation substrate peptide or domain and a phosphoamino acid binding domain have been linked to produce a hybrid protein, which was then flanked by CFP and YFP. This approach has yielded indicators for the activities of PKA, PKC, Src, and EGF receptor (Kurokawa et al., 2001; Ting et al., 2001; Zhang et al., 2001; Violin et al., 2003). In a simpler form, the substrate peptide is sandwiched between CFP and YFP; conformational changes induced by the phosphorylation of substrate lead to the change in FRET efficiency (Nagai et al., 2000; Yamada et al., 2005; Brumbaugh et al., 2006). Alternatively, entire protein kinases are flanked by YFP and CFP to produce a kinase-type probe. Protein kinases generally consist of the catalytic and regulatory domains, the latter of which associates with the former to hold the enzyme in a closed, inactive state. Upon stimulation, the regulatory domain dissociates from the catalytic domain to drive the enzyme into an open, active state. This conformational change has been successfully monitored as a change in FRET efficiency in the probes for PKC, Raf, Erk, and CaMKII (Braun et al., 2005; Terai and Matsuda, 2005; Takao et al., 2005; Fujioka et al., 2006).

Critical Parameters

For the development of a FRET probe, the signal gain, i.e., the difference in the FRET ratio (the lowest versus the highest value of [YFP fluorescence]/[CFP fluorescence] of the probe excited for CFP) should exceed 30%. If one uses probes in which the gain is less than 30%, it is typically difficult to observe a significant change in FRET efficiency upon stimulation.

As for image acquisition, we strongly recommend starting with the easiest experiment, using established probes, to become familiar with the imaging technique. For example, we recommend monitoring agonist-induced calcium oscillation with a recently developed calcium sensor, cameleon YC3.60, which has an extraordinarily wide dynamic range (Nagai et al., 2004). Imaging of Ras activation in EGF-stimulated COS cells with Raichu-Ras probe (Mochizuki et al., 2001) is another fairly easy example (Fig. 14.10.3).

Recently, the quantitative analysis of acquired images has become a necessary skill in live cell imaging. Therefore, it is desirable to have a good knowledge of image-processing software. For that purpose, expert training in image processing is helpful.

Troubleshooting

Table 14.10.1 provides troubleshooting information for these protocols.

Anticipated Results

Figure 14.10.3 shows the FRET images of COS-1 cells expressing Raichu-Ras following EGF stimulation. The FRET images are presented in an intensity-modulated display mode. EGF-induced Ras activation was detected at the periphery of the cell, where membrane ruffling was prominent. The increase in the YFP/CFP ratio of Raichu-Ras upon EGF stimulation is usually 30% to 50%.

Table 14.10.1 Troubleshooting Guide to In Vivo Imaging of Signaling Cascades

Problem	Possible cause	Solution
Gradual decrease in YFP/CFP ratio without stimulation	Photobleaching during imaging	Lengthen time-lapse interval or shorten exposure time
Gradual increase in YFP/CFP ratio without stimulation	1. Photobleaching during searching for the target cell. 2. Progress of folding of fluorescence proteins. In this case, both YFP and CFP intensities should increase.	Wait for 5-10 min and restart the imaging. Prolong the incubation time after probe transfection.
Defocus during imaging	Distortion of warmed microscope	Warm up microscope and chambers in advance. To avoid defocus completely, a focus-drift compensation system (e.g., Olympus IX2-ZDC) is very useful.
Higher (or lower) YFP/CFP ratio in specific side of the image	Uneven illumination to samples	Align an arc lamp precisely
Appearance of paired high and low areas of YFP/CFP ratio at the edge of cells	Misregistration (subtle displacement between CFP and YFP images)	Correct misregistration using imaging software
Cells expressing FRET probes look unhealthy	Cell toxicity due to the overexpression of probes	Choose the healthy cells expressing low levels of probes or retry the transfection with less DNA

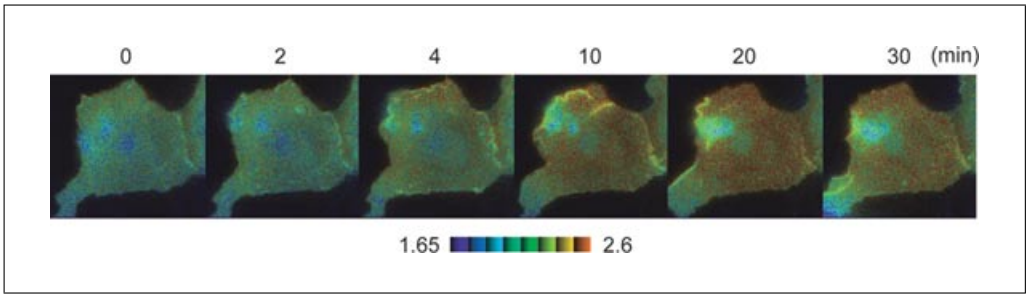


Figure 14.10.3 Time-lapse experiment of Ras activation upon EGF stimulation in COS-1 cells. Serum-starved COS-1 cells expressing Raichu-Ras were treated with 50 ng/ml of EGF and imaged every 2 min. FRET images are shown at the indicated time points. For color figure go to *http://www.currentprotocols.com/protocol/cb1410*.

Time Considerations

In some cases, more than one hundred candidate probes should be designed, constructed, and checked for FRET efficiency to obtain an ideal probe. Thus, the term needed to develop a good probe is ~3 months. This time frame significantly varies, depending on the knowledge of the target for probe development.

The entire cell-imaging procedure can be performed in 3 to 4 days from cell seeding. This period includes 1 day for expression of FRET probe after transfection, 1 day for data acquisition, and 1 day for data analysis.

Literature Cited

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. 2009. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Braun, D.C., Garfield, S.H., and Blumberg, P.M. 2005. Analysis by fluorescence resonance energy transfer of the interaction between ligands and protein kinase Cδ in the intact cell. *J. Biol. Chem.* 280:8164-8171.

Brumbaugh, J., Schleifenbaum, A., Gasch, A., Sattler, M., and Schultz, C. 2006. A dual parameter FRET probe for measuring PKC and PKA activity in living cells. *J. Am. Chem. Soc.* 128:24-25.

Fiala, A., Spall, T., Diegelmann, S., Eisermann, B., Sachse, S., Devaud, J.M., Buchner, E., and Galizia, C.G. 2002. Genetically expressed cameleon in *Drosophila melanogaster* is used to visualize olfactory information in projection neurons. *Curr. Biol.* 12:1877-1884.

Fujioka, A., Terai, K., Itoh, R.E., Aoki, K., Nakamura, T., Kuroda, S., Nishida, E., and Matsuda, M. 2006. Dynamics of the Ras/ERK MAPK cascade as monitored by fluorescent probes. *J. Biol. Chem.* 281:8917-8926.

Hailey, D.W., Davis, T.N., and Muller, E.G., 2002. Fluorescence resonance energy transfer using color variants of green fluorescent protein. *Methods Enzymol.* 351:34-49.

Itoh, R.E., Kurokawa, K., Ohba, Y., Yoshizaki, H., Mochizuki, N., and Matsuda, M. 2002. Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol. Cell. Biol.* 22:6582-6591.

Kawase, K., Nakamura, T., Takaya, A., Aoki, K., Namikawa, K., Kiyama, H., Inagaki, S., Takemoto, H., Saltiel, A.R., and Matsuda, M. 2006. GTP hydrolysis by the Rho family GTPase TC10 promotes exocytic vesicle fusion. *Dev. Cell* 11:411-421.

Kerr, R., Lev-Ram, V., Baird, G., Vincent, P., Tsien, R.Y., and Schafer, W.R. 2000. Optical imaging of calcium transients in neurons and pharyngeal muscle of *C. elegans*. *Neuron* 26:583-594.

Kiyokawa, E., Hara, S., Nakamura, T., and Matsuda, M. 2006. Fluorescence (Förster) resonance energy transfer imaging of oncogene activity in living cells. *Cancer Sci.* 97:8-15.

Kraynov, V.S., Chamberlain, C., Bokoch, G.M., Schwartz, M.A., Slabaugh, S., and Hahn, K.M. 2000. Localized Rac activation dynamics visualized in living cells. *Science* 290:333-337.

Kurokawa, K., Mochizuki, N., Ohba, Y., Mizuno, H., Miyawaki, A., and Matsuda, M. 2001. A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the CrkII adaptor protein in vivo. *J. Biol. Chem.* 276:31305-31310.

Kurokawa, K., Takaya, A., Fujioka, A., Terai, K., and Matsuda, M. 2004. Visualizing the signal transduction pathways in living cells with GFP-based FRET probes. *Acta Histochem. Cytochem.* 37:347-355.

Lakowicz, K.R. 2006. Energy transfer. *In* Principles of Fluorescence Spectroscopy, 3rd ed. (J.R. Lakowicz, ed.) pp. 443-475. Springer, New York.

Miyawaki, A. 2003. Visualization of the spatial and temporal dynamics of intracellular signaling. *Dev. Cell* 4:295-305.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. 1997. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 388:882-887.

Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. 2001. Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature* 411:1065-1068.

Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M., and Miyawaki, A. 2004. Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proc. Natl. Acad. Sci. U.S.A.* 101:10554-10559.

Nagai, Y., Miyazaki, M., Aoki, R., Zama, T., Inouye, S., Hirose, K., Iino, M., and Hagiwara, M. 2000. A fluorescent indicator for visualizing cAMP-induced phosphorylation in vivo. *Nat. Biotechnol.* 18:313-316.

Pertz, O., Hodgson, L., Klemke, R.L., and Hahn, K.M. 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* 440:1069-1072.

Sato, M., Hida, N., Ozawa, T., and Umezawa, Y. 2000. Fluorescent indicators for cyclic GMP based on cyclic GMP-dependent protein kinase Iα and green fluorescent proteins. *Anal. Chem.* 72:5918-5924.

Sato, M., Ueda, Y., Takagi, T., and Umezawa, Y. 2003. Production of $PtdInsP_3$ at endomembranes is triggered by receptor endocytosis. *Nat. Cell Biol.* 5:1016-1022.

Sekar, R.B. and Periasamy, A. 2003. Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J. Cell Biol.* 160:629-633.

Takao, K., Okamoto, K.I., Nakagawa, T., Neve, R.L., Nagai, T., Miyawaki, A., Hashikawa, T., Kobayashi, S., and Hayashi, Y. 2005. Visualization of synaptic Ca^{2+} /calmodulin-dependent protein kinase II activity in living neurons. *J. Neurosci.* 25:3107-3112.

Takaya, A., Ohba, Y., Kurokawa, K., and Matsuda, M. 2004. RalA activation at nascent lamellipodia of epidermal growth factor-stimulated Cos7 cells and migrating Madin-Darby canine kidney cells. *Mol. Biol. Cell* 15:2549-2557.

Takaya, A., Kamio, T., Masuda, M., Mochizuki, N., Sawa, H., Sato, M., Nagashima, K., Mizutani, A., Matsuno, A., Kiyokawa, E., and Matsuda, M. 2007. R-Ras regulates exocytosis by Rgl2/Rlf-mediated activation of RalA on endosomes. *Mol. Biol. Cell* 18:1850-1860.

Terai, K. and Matsuda, M. 2005. Ras binding opens c-Raf to expose the docking site for mitogen-activated protein kinase kinase. *EMBO Rep.* 6:251-255.

Ting, A.Y., Kain, K.H., Klemke, R.L., and Tsien, R.Y. 2001. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 98:15003-15008.

Violin, J.D., Zhang, J., Tsien, R.Y., and Newton, A.C. 2003. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J. Cell Biol.* 161:899-909.

Wang, Y., Botvinick, E.L., Zhao, Y., Berns, M.W., Usami, S., Tsien, R.Y., and Chien, S. 2005. Visualizing the mechanical activation of Src. *Nature* 434:1040-1045.

Yamada, A., Hirose, K., Hashimoto, A., and Iino, M. 2005. Real-time imaging of myosin II regulatory light-chain phosphorylation using a new protein biosensor. *Biochem. J.* 385:589-594.

Yoshizaki, H., Ohba, Y., Kurokawa, K., Itoh, R.E., Nakamura, T., Mochizuki, N., Nagashima, K., and Matsuda, M. 2003. Activity of Rho-family G proteins during cell division as visualized with FRET-based probes. *J. Cell Biol.* 162:223-232.

Zhang, J., Ma, Y., Taylor, S.S., and Tsien, R.Y. 2001. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc. Natl. Acad. Sci. U.S.A.* 98:14997-15002.

Key References

Miyawaki, 2003. See above.

Comprehensive review of GFP-based FRET technology.

Internet Resources

http://www.path1.med.kyoto-u.ac.jp/mm/e-phogemon/index.htm

Web site for further information about the Raichu-type FRET probe. Setup of the FRET imaging system is described.

Biosensors for Characterizing the Dynamics of Rho Family GTPases in Living Cells

Louis Hodgson,[1] Feimo Shen,[2] and Klaus Hahn[2]

[1]Gruss-Lipper Biophotonics Center, Department of Anatomy and Structural Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York
[2]University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

ABSTRACT

The biosensors developed in the authors' laboratory have been based on different designs, each imparting specific strengths and weaknesses. Here we describe detailed protocols for the application of three biosensors exemplifying different designs—first, a design in which an environmentally sensitive dye is used to report the activation of endogenous Cdc42, followed by two biosensors based on FRET, one using intramolecular and the other intermolecular FRET. The design differences lead to the need for different approaches in imaging and image analysis. *Curr. Protoc. Cell Biol.* 46:14.11.1-14.11.26.

Keywords: biosensors • Rho family GTPases • FRET • live cell imaging

INTRODUCTION

In this unit, we describe detailed protocols for the application of three biosensors developed in our laboratory, exemplifying different designs with specific strengths and weaknesses. The first biosensor described represents a design in which an environmentally sensitive dye is used to report the activation of endogenous Cdc42. Also described are two biosensors based on fluorescence resonance energy transfer (FRET; also see *UNIT 17.1*), one using intramolecular and the other intermolecular FRET. The design differences lead to the need for different approaches in imaging and image analysis.

Figure 14.11.1 shows the design of the three biosensors. MeroCBD (Fig. 14.11.1A) is a sensor for Cdc42 activation in which a fragment of Wiskott-Aldrich Syndrome protein that binds only to active Cdc42 is derivatized with an environmentally sensitive dye (Nalbant et al., 2004). In living cells, the dye undergoes a fluorescence change when the biosensor binds to endogenous, untagged Cdc42. This design is advantageous for several reasons: Cdc42 is not tagged with a fluorophore or other biosensor component, expression of exogenous Cdc42 is not needed, and the signal is substantially brighter than FRET because a bright dye is directly excited, rather than indirectly as in FRET. The major disadvantage is that the biosensor is not genetically encoded, so must be loaded in the cells, e.g., via microinjection or electroporation.

The FRET biosensor for RhoA (Pertz et al., 2006) is shown in Figure 14.11.1B. A fragment of Rhotekin is attached directly to RhoA, and fluorescent proteins undergoing FRET are built into the chain connecting the RhoA and Rhotekin fragment. The C terminus of the RhoA is purposely left free of fluorescent protein or other modifications, to maintain intact the regulation of RhoA by GDI. Unlike the Cdc42 biosensor, this biosensor is completely genetically encoded, greatly simplifying loading into cells.

Finally, the biosensor for Rac1 is shown in Figure 14.11.1C. Named Rac1 FLAIR, it is a modification of a design originally published using a covalently attached fluorescent dye

Current Protocols in Cell Biology 14.11.1-14.11.26, March 2010
Published online March 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1411s46

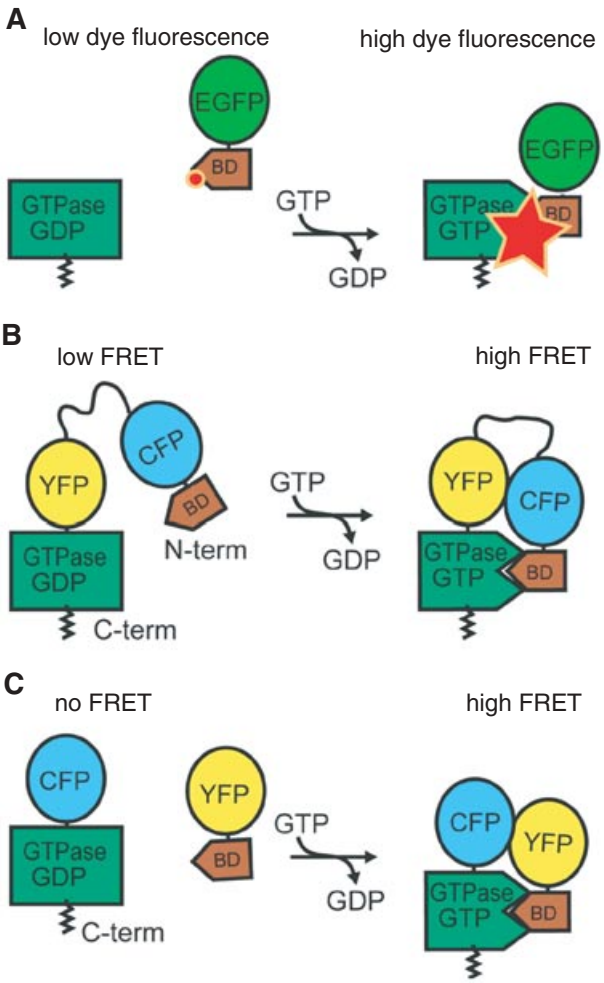


Figure 14.11.1 Fluorescent biosensor designs. (**A**) MeroCBD, biosensor of endogenous Cdc42 activation. Here, a fragment of Wiskott-Aldrich syndrome protein (WASP) that binds only to activated Cdc42 is covalently derivatized with an environmentally sensitive dye. When the WASP fragment encounters and binds to activated Cdc42, the solvation of the dye by water is reduced, leading to a fluorescence change. Advantages of this design include the ability to study endogenous protein, and enhanced sensitivity due to direct excitation of a bright, long-wavelength dye (as opposed to indirect excitation via FRET). The disadvantage is the need for microinjection, electroporation, or some other means to load the covalently tagged protein biosensor into cells. (**B**) RhoA biosensor. Here a fragment of Rhotekin that binds only to activated RhoA is attached to RhoA as part of the same protein chain. Two different fluorescent proteins undergoing FRET are in the chain between RhoA and the Rhotekin fragment, such that binding of the fragment to activated RhoA alters the distance and/or orientation between the fluorescent proteins, affecting FRET. This biosensor is fully genetically encoded, greatly simplifying loading into the cell. Because the Rhotekin fragment is attached to the RhoA, image processing is simplified relative to the dual-chain sensor shown in (C) (see text). (**C**) Rac1 FLAIR, biosensor of Rac1 activation. This design is similar to that of the RhoA biosensor, but here the PAK fragment used to bind activated Rac1 is not part of the same chain as the Rac1. The use of a dual-chain, intermolecular FRET design enhances sensitivity because, unlike the single-chain design, there is no FRET when the biosensor is in the off state. Additional image processing (bleed-through correction) is required because the biosensor components can distribute differently throughout the cell. For color figure go to *http://www.currentprotocols.com/protocol/cb1411*.

for FRET (Kraynov et al., 2000). In the modification described here, the dye has been replaced with a fluorescent protein, rendering the biosensor fully genetically encoded (Machacek et al., 2009). A fragment of p21-activated kinase (PAK) is used to bind specifically to activated Rac1. The main difference between this design and that of the RhoA biosensor is that the PAK fragment is not attached to Rac1. Rac1 FLAIR is an intermolecular FRET biosensor, frequently referred to as a dual-chain sensor.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

PRODUCTION AND USE OF meroCBD, DYE-BASED BIOSENSOR FOR CDC42

BASIC PROTOCOL 1

As discussed above, the meroCBD biosensor for Cdc42 activation is based on a fragment of WASP (the Cdc42 binding domain, CBD) that binds only to activated Cdc42. This fragment is covalently coupled to a solvent-sensitive dye that increases in fluorescence intensity when the CBD interacts with endogenous, activated Cdc42 (Toutchkine et al., 2003, 2007a,b). Because the dye responds to Cdc42 binding with an intensity change, rather than with a shift in λ_{max}, it is useful to attach a second fluorophore to the biosensor for ratio imaging.

CBD-EGFP is expressed in the form of a C-terminal 6× His fusion from the prokaryotic expression vector pET23. This vector has a strong T7 promoter, and is designed to work with BL21(DE3) strains of *E.coli* (Stratagene). It was determined experimentally that the highest levels of expression are observed when a plain T7 promoter (not T7*lac*) is used in combination with a BL21(DE3) strain, and not BL21(DE3)pLysS. The BL21(DE3) strain is more leaky, but this is not of great consequence, as CBD-EGFP shows no appreciable toxicity. The protein is best induced and expressed at room temperature (26°C), which increases the proportion of correctly folded, soluble CBD-EGFP.

Materials

Transformation-competent BL21(DE3) *E. coli* (e.g., Stratagene)
pET23-CBD-EGFP (Addgene)
LB medium and plates (*APPENDIX 2A*) containing 100 μg/ml carbenicillin
1 M IPTG (in water), store at −20°C
Lysis buffer (see recipe)
Talon resin (Co^{2+} affinity, Clontech)
Lysis buffer (see recipe but omit 2-ME and PMSF) containing 5 mM and 150 mM imidazole
50 mM Tris·Cl, pH 7.5 to 8.0 (*APPENDIX 2A*)
50 mM sodium phosphate buffer, pH 7.5 (*APPENDIX 2A*)
Storage buffer (see recipe)

2000-ml Erlenmeyer flask
Incubator with shaker
250-ml centrifuge bottles
Beckman centrifuge with JA-10 and JA-20 rotors (or equivalent)
50-ml conical polypropylene centrifuge tubes (Falcon)
Centrifuge with swinging-bucket rotor
End-over-end rotator
Ultrafree Centrifugal Filtration Device (MWCO, 5000; Fisher Scientific, cat. no. UFV5BCC25)
Slide-A-Lyzer cassettes (MWCO 3500; Pierce)

Additional reagents and equipment for transformation of bacteria and other basic molecular biological techniques (e.g., Sambrook et al., 1989; Ausubel et al., 2009), SDS-PAGE (*UNIT 6.1*), and dialysis (*APPENDIX 3H*)

NOTE: Use the buffers suggested in this unit. Apparently small changes have proven to greatly reduce yield.

Day 1: Transform the bacteria

1. Transform competent BL21(DE3) *E. coli* cells with pET23-CBD-EGFP according to standard protocols (Sambrook et al., 1989; Ausubel et al., 2009), and plate on LB plates containing 100 μg/ml carbenicillin.

   The 200-μl transformation volume should be split over two plates.

   The bacterial strain is critical. BL21(DE3) are recommended. Do not use BL21(DE3)pLysS.

2. Incubate plates at 37°C overnight.

Day 2: Grow and induce the transformed bacteria

3. Inoculate 500 ml of LB liquid medium containing 100 μg/ml carbenicillin with the colonies from the plates by adding 5 ml of medium to each plate and resuspending the cells into the medium, then transferring the cell suspension into 500 ml LB medium/carbenicillin (in a separate 2000-ml Erlenmeyer flask for each plate) and growing at 37°C with shaking at 225 rpm, to an OD_{600} of 0.8 to 0.9.

4. Briefly chill the culture on ice to 26°C and put back in the shaking incubator, with temperature reduced to 26°C.

5. Add IPTG (1 M stock in water, kept at –20°C) to a final concentration of 0.2 mM, and allow the cultures to grow for another 6 hr at 26°C at 225 rpm.

   IPTG concentrations of 0.2 to 0.5 mM have been used successfully.

6. Transfer cells to 250-ml centrifuge bottles. Collect cells by centrifuging 10 min at 6000 × *g*, 4°C, and store as a pellet at –20°C until use.

   Approximately 2.5 to 3 g of cells is usually obtained from each liter of culture.

Day 3: Prepare the lysate

7. Resuspend cells (~3 g) in 35 ml of lysis buffer in a 50-ml conical tube and lyse by sonication with a probe sonicator (four pulses, 30 sec each, on ice with 1-min intervals).

8. Centrifuge the lysates 30 min at 20,000 × *g* (13,000 rpm in a JA-20 rotor), 4°C, and carefully transfer the supernatant containing CBD-EGFP into a 50-ml conical polypropylene centrifuge tube.

Day 3: Prepare the resin

9. While the lysates are being centrifuged, transfer 2 ml of Talon resin (dry volume) into a 50-ml conical polypropylene centrifuge tube and centrifuge 3 min at 700 × *g*, 24°C, in a centrifuge with a swinging-bucket rotor. Remove supernatant.

   Do not use Ni-NTA resin.

   A ratio of 2 ml resin per 3 g cell pellet is recommended.

10. Wash Talon resin twice, each time by adding 10 volumes of lysis buffer without 2-mercaptoethanol or PMSF centrifuging 3 min at 700 × *g*, 24°C, and removing the supernatant.

Day 3: Bind the CBD-EGFP to the resin

11. Add the cell lysate to the washed Talon resin in the 50-ml tube, wrap in foil, and invert gently using an end-over-end rotator at room temperature for 40 min to 1 hr.

    The tube is wrapped in foil to avoid unnecessary exposure of EGFP to light.

12. Separate the unbound material by centrifuging 3 min at 700 × *g*, 24°C, in a centrifuge with a swinging-bucket rotor.

13. Remove and save the supernatant (unbound fraction) for SDS-PAGE analysis (*UNIT 6.1*).

    If a large portion of unbound material is present in this fraction, consider increasing the Talon resin volume by preparing two tubes of 2 ml resin and splitting the lysates into two tubes during the binding reaction.

14. Wash the resin twice, each time by adding 20 ml fresh lysis buffer without PMSF or 2-mercaptoethanol, rotating for 5 min at room temperature on an end-over-end rotator, then centrifuging as in step 12 and removing the supernatant.

15. Perform the final resin wash with 10 volumes of lysis buffer containing 5 mM imidazole (no PMSF or 2-ME).

    It is important to prepare a fresh 5 ml of 1 M imidazole stock solution in the same buffer. Always prepare the stock imidazole solution fresh.

Day 3: Elute the resin

16. Perform the elution by adding 5 ml of lysis buffer (no PMSF or 2-ME) containing 150 mM imidazole to the resin and then rotating using an end-over-end rotator for 5 min. Pellet the resin again by centrifugation for 3 min at 700 × *g*, 24°C.

17. Remove the supernatant and save (eluted fraction).

18. Concentrate the resulting 5-ml eluate with a centrifugal concentrator by centrifugation at 4°C according to the manufacturer's instructions. Check the concentration process every 20 min to ensure proper filtration.

    Do not over-concentrate; optimal final concentration should be ∼120 μM.

19. Measure the concentration of CBD-EGFP by taking a small aliquot (5 to 10 μl) and diluting 1:10 into 50 mM Tris·Cl, pH 7.5 to 8.0.

20. Determine protein concentration using the absorption at 280 with an extinction coefficient of 28,260 (cm^{-1} M^{-1}), and Equation 14.11.1:

$$[\text{CBD}-\text{EGFP}](\text{in mol/liter}) = \frac{\text{OD}_{280} \times \text{dilution factor}}{28,260}$$

Equation 14.11.1

    On average, 10 to 20 mg of CBD-EGFP is obtained per liter culture.

21a. *If dye-labeling is to be performed the following day:* Dialyze a part of the concentrated protein overnight against 2 liters of 50 mM sodium phosphate buffer, pH 7.5, at 4°C (*APPENDIX 3H*).

    Slide-A-Lyzer cassettes (Pierce) with a molecular weight cut-off of 3,500 Da can be conveniently used.

21b. *For long-term storage:* Dialyze the protein overnight against 2 liters of storage buffer at 4°C (*APPENDIX 3H*), and freeze at −80°C.

    Freezing at −80°C appears to be better than flash freezing for this protein.

SUPPORT PROTOCOL

LABELING CBD-EGFP WITH REACTIVE FLUOROPHORE

Dyes are available from the Hahn lab and should be commercially available soon. Solvent-sensitive dyes other than those that have been used to date should also respond to Cdc42 binding.

Materials

Dye: ISO-IAA (Toutchkine et al., 2003, 2007a,b)
Dimethylsulfoxide (DMSO)
CBD-EGFP (Basic Protocol 1)
2-mercaptoethanol (2-ME)
50 mM sodium phosphate buffer, pH 7.5 (*APPENDIX 2A*)
12% SDS-PAGE gel (*UNIT 6.1*)
50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)

Spectrophotometer
2-ml microcentrifuge tubes
End-over-end rotator
0.5 cm × 6 to 8 cm column packed with Sephadex G15 gel-filtration resin (GE Healthcare)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and spectrophotometric determination of protein concentration (*APPENDIX 3B*)

Prepare dye stock solution

1. Prepare a fresh solution of dye, ISO-IAA, in pure DMSO by adding ~1 mg of dye into 30 to 40 µl DMSO.

   Once dissolved in DMSO, the dye should be used immediately.

2. Determine the exact concentration of the dye spectrophotometrically at 593 nm by diluting a small aliquot of the DMSO solution 1:5,000 to 1:10,000 in additional DMSO. For ISO-IAA, assume an extinction coefficient in DMSO at maximum absorption (593 nm) of 135,000 (cm^{-1} M^{-1}):

$$[\text{ISO}-\text{IAA}](\text{in mol/liter}) = \frac{\text{OD}_{593} \times \text{dilution factor}}{135,000}$$

Equation 14.11.2

   The authors routinely obtain concentrations of 30 to 40 mM for the DMSO stock solution.

Label the protein

3. Transfer a 300-µl aliquot of fresh CBD-EGFP protein into a 2-ml microcentrifuge tube wrapped in foil to protect from light.

   For attachment of the dyes to cysteine, CBD-EGFP needs to be in 50 mM sodium phosphate buffer, pH 7.5 (APPENDIX 2A), at a protein concentration of 200 µM.

4. Add the dye to the CBD-EGFP solution in two to three aliquots to bring the final dye-to-protein molar ratio in the reaction to 6:1.

   The molecular weight of the ISO-IAA is ~1300 Da, and the molecular weight of the CBD-EGFP is ~36.5 kDa.

   This ratio can be further optimized depending on the reactivity of the dye if different dyes are used. We suggest 6:1 as a useful starting point.

Using higher dye-to-CBD-EGFP ratios (e. g. 10:1 or 15:1) in the reaction mixture can produce excessive amounts of precipitated material and "over-labeling" (dye to protein ratio in the purified covalent adduct >1.0 due to labeling of the exposed cysteine in EGFP). EGFP mutants lacking surface cysteines can be used to ameliorate this problem. The optimal dye-to-protein ratio to use in the reaction mixture depends on the reactivity of the dye, so it can vary for different dyes, or dyes from different sources/batches.

5. Vortex the protein/dye mix once briefly at the highest setting, immediately after addition of the dye stock solution.

   Avoid excess mixing as this may denature the protein.

6. Wrap the tube containing the reaction mixture in foil and gently rotate on an end-over-end rotator at room temperature for exactly 1 hr.

   Here, the time is critical; longer reaction times result in over-labeling of the protein.

7. After the reaction is complete, add 1 to 5 μl of 2–ME to stop the reaction, and incubate the tube 5 to 10 min at room temperature on the end-over-end rotator.

8. Microcentrifuge the tube 2 min at 13,000 × *g*, room temperature, to pellet insoluble material.

9. Load the supernatant on a 0.5 cm × 6 to 8 cm G15 gel-filtration column to separate the conjugate from free dye. Equilibrate and run this column in 50 mM NaH_2PO_4 buffer at pH 7.5. Collect fractions of ∼200 μl each.

   The first colored band to elute contains the dye-labeled CBD-EGFP (meroCBD).

Analyze the labeled protein

10. Analyze an aliquot (3 to 5 μl) of each fraction on a 12% SDS-PAGE gel (*UNIT 6.1*) to confirm the presence and purity of the meroCBD. Visualize the gel with fluorescent illumination using a UV transilluminator or a hand-held UV lamp.

    Fluorescence visualization of the gel ensures labeling with the dye, and can reveal a band of free dye running at much lower molecular weight, often near marker dyes used in the electrophoresis sample.

11. To evaluate the success of the procedure, and generation of an optimal biosensor, determine the dye/protein ratio once the biosensor has been freed from unattached dye.

Determine protein concentration

It is also important to know the protein concentration to optimize later microinjection in living cells.

12. Determine an approximate protein concentration rapidly by taking an absorbance spectrum of the conjugate solution. Dilute a small aliquot (5 to 10 μl) in 50 mM Tris·Cl, pH 7.5 to 8.0, and use OD_{280} and Equation 14.11.1 above (also see *APPENDIX 3B*).

    Importantly, the protein absorbance at 280 overlaps dye absorbance.

13. Correct this overlapping absorbance by subtracting, from the OD_{280}, a fraction of the dye's absorbance at its maximum (∼593 nm); however, this is variable due to the solvent sensitivity of the dye. For a more accurate measure, compare a sample of the meroCBD (dye-labeled CBD-EGFP) to unlabeled CBD-EGFP by running them on the same gel, using different known concentrations of the unlabeled protein.

    Colorimetric assays, limited to those whose readouts do not overlap dye absorbance, have proven less reliable in our hands.

Determine the dye concentration

14. Determine the dye concentration by taking an absorbance spectrum of meroCBD. Dilute 5 to 10 μl of meroCBD in 100 μl DMSO.

    DMSO as a solvent will denature and overwhelm the effect of the protein or buffer on the dye's absorbance, resulting in a consistent dye extinction coefficient.

15. Use the OD at the dye absorbance maximum of 593 nm to calculate the dye concentration, using Equation 14.11.2 above.

BASIC PROTOCOL 2

IMAGING meroCBD IN LIVING CELLS

The authors of this unit routinely microinject cells on coverslips and image meroCBD following 30 min of recovery time in a tissue culture incubator. MeroCBD can produce puncta (possibly autophagy) after 2 to 3 hr in some cells, in which case the timing between microinjection and observation must be carefully controlled.

Cells are observed in a live-cell chamber atop an inverted epifluorescence microscope outfitted with an Hg arc-lamp light source. When imaging with a single camera, excitation and emission filter wheels can be used to alternately illuminate the dye and EGFP to obtain a ratiometric image set at every time point during the time-lapse experiment. If two cameras are available, two-camera modules such as those available from Olympus (U-DPCAD module), Technical Video (Technical Video Inc.), or Optical Insights (Dual-Cam) can be used to acquire two images simultaneously using a dual band-pass excitation filter. Since the dye and EGFP will have different brightnesses, band-pass filters can be designed to pass different amounts of excitation light at each wavelength through the dual band-pass filter.

When meroCBD is made using I-SO type fluorophores (Toutchkine et al., 2003, 2007a,b), including the new dyes we currently employ (Dyes 53 and 87; manuscript in preparation), EGFP bleed-through into the dye channel is practically zero. This is made possible by using relatively narrow excitation and emission band-pass filters. We use a customized multiband-pass dichroic mirror (Fig. 14.11.2) and the following excitation and emission filters: HQ470/40 and HQ525/50 for EGFP; HQ580/30 and HQ630/40 for dye (Chroma Technology).

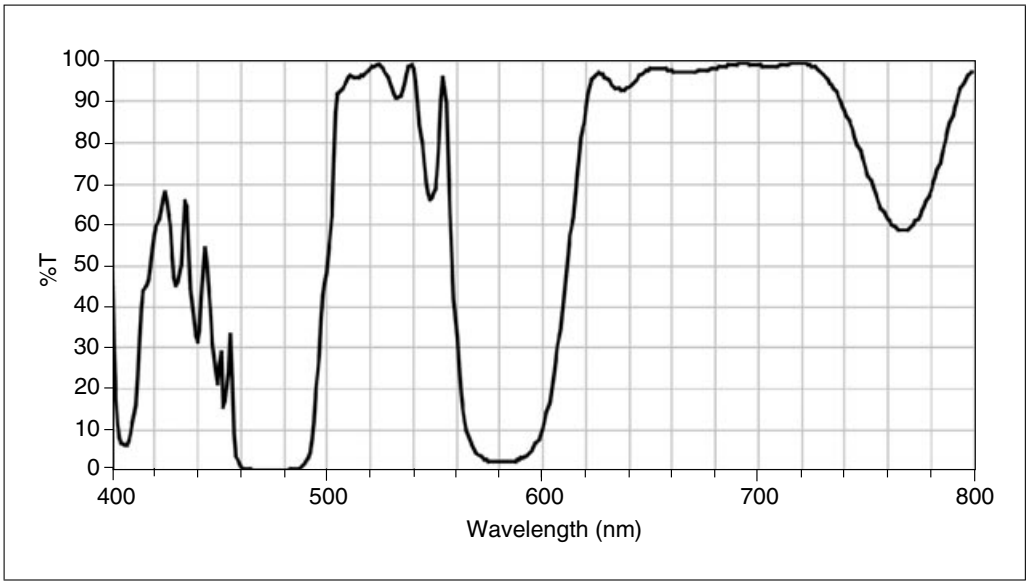


Figure 14.11.2 Transmittance spectra for the "Scripps Custom" dichroic mirror (Chroma Technology, lot no. 511111886).

The interval between images will vary depending on the images. We routinely acquire images at 10-sec to 1-min time intervals using 2 × 2 binning on a full-field 1.3k-by-1.0k cooled CCD camera. Importantly, the excitation light from the 100 W Hg arc lamp source is too bright, so it is routinely reduced using neutral-density filters for 4% to 10% transmittance (ND 1.4 to 1.0). The integration (exposure) time on a CCD camera depends on the quantum efficiency of the CCD and the noise characteristics of the camera. The dye exposure is usually 0.5× to 0.3× as long as the EGFP exposure. Dye exposure times may be reduced with some cameras; many current cameras, including those we use, are less efficient at the red wavelengths of the dyes. Users should fine-tune the exposure times depending on their optics and camera setup. Ideally, the integrated average intensity from both fluorescence channels should be similar, to maximize the dynamic range of the data. Cameras with 12-bit depth have an intensity range from 0 to 4096; we routinely fill ∼3000 of this dynamic range in a single field of view. We carefully monitor the pixel intensities near the cell periphery where the signals are usually low, aiming for approximately 100 to 200 over the background values. This translates to 2 to 3 in signal-to-noise ratio at the darkest regions.

Because different wavelengths are used to image EGFP and dye, many objectives (even apochromatic objectives) will focus on slightly different planes within the specimen. The *z*-axis difference between these two focal planes needs to be calibrated, and the objective slightly offset between the two images. We use a *z*-axis position control on our microscope to accomplish this. Typically, the *z*-distance offset is determined by focusing on the same small object at different wavelengths, using image-based autofocus (Shen et al., 2006, 2008) followed by manual fine adjustment. A script is written to execute the shifts in *z* for each color acquired during an automated time-lapse sequence. Moving the objective turret back and forth in different directions to account for these *z* offsets will not give reproducible results, due to hysteresis in the focus drive mechanism. Therefore, it is better to acquire different colors so that the objective always changes *z* position in one direction. Alternatively, a linear-encoder feedback or piezo-stage system can be installed for precise *z* positioning, to eliminate the hysteresis problem. It is important to always set the search range for autofocus to be more than twice as large as the total motions programmed in for *z* focus offset.

EXPRESSING THE RhoA SINGLE-CHAIN BIOSENSOR

BASIC PROTOCOL 3

The genetically encoded biosensor for RhoA consists of (N-terminus to the C-terminus) a small RhoA-binding domain (RBD) derived from the RhoA effector Rhotekin, ECFP, an unstructured linker of optimized length, pH-insensitive Citrine-YFP, and a full-length RhoA (Fig. 14.11.1B). Three different expression constructs for this single-chain biosensor have been developed, all available from the nonprofit distributor Addgene (*http://www.addgene.org*):

1. pTriEX mammalian expression and cloning vector (pTriEX-RhoA/amp resistance).

2. pBabe retroviral expression vector (pBabe-RhoA/amp resistance).

3. pBabe-sin-tet-CMV-puro retroviral vector for Tet-On/Off system (pBabe-sin-tet-CMV-puro-RhoA/amp resistance).

The pTriEX backbone is used for the cloning of the biosensor, and the complete biosensor cDNA can be cut out as a cassette by digesting with *Nco*I/*Xho*I. *Nco*I encodes the start codon, and *Xho*I is placed in-frame immediately following the stop codon after the RhoA GTPase. The pTriEX version of the biosensor allows overexpression in mammalian cells driven by the CMV promoter.

One must beware of the potential toxicity of GTPases upon overexpression (Pertz et al., 2006) and the importance of the relative intracellular concentrations of GTPases and upstream regulators in cells (Michaelson et al., 2001; Del Pozo et al., 2002). We have addressed these issues by using retroviral transduction to stably incorporate low copy numbers of the biosensor DNA using the pBabe expression system (Pertz et al., 2006). Transduced cells are sorted flow cytometrically to obtain low to medium expressors only, so that the biosensor expression level is comparable to endogenous GTPase. We have shown that competition with endogenous effectors of the GTPase is not a significant problem at appropriate biosensor concentrations (Pertz et al., 2006).

In order to address the toxicity of overexpressed biosensor, a tetracycline-inducible retroviral construct based on a pBabe-sin-tet-CMV-puro backbone is used.

Materials

Tet-off stable MEF/3T3 cell system (Clontech)
MEF/3T3 cells transduced with the appropriate construct
10 mg/ml doxycycline stock solution
10 mg/ml puromycin stock solution

10-cm tissue culture dishes
Coverslips coated with fibronectin: immerse glass coverslips 30 to 60 min in 10 μg/ml fibronectin (e.g., Sigma), then rinse three times with PBS (*APPENDIX 2A*) and leave immersed in PBS until use

Additional reagents and equipment for cell culture techniques including trypsinization (*UNIT 1.1*), flow cytometric cell sorting (Robinson et al., 2009), and imaging the RhoA biosensor (Basic Protocol 4)

1. Using the tet-off stable MEF/3T3 cell system (Clontech), repress infected cells using 1 μg/ml doxycycline.

   Refer to user's guide for Clontech tet-off stable MEF/3T3 cell system for this and the following step.

2. Select cells with puromycin up to 10 μg/ml, increasing the puromycin concentration gradually following the infection (2 μg/ml increase per each successive selection cycle).

   "Selection cycle" means that the cells have tolerated that particular puromycin concentration.

3. At the end of selection, induce cells for biosensor expression by removal of doxycycline and replating cells at a sparse concentration (1×10^4 cells per 10-cm tissue culture dish) for 48 hr.

4. Sort cells flow cytometrically (Robinson et al., 2009) to produce nearly 100% biosensor-positive cells. Sort for CFP and YFP using two-color system.

5. Repress the cells again by application of 1 μg/ml doxycycline.

   We routinely maintain repressed cells in 1 μg/ml doxycycline under standard tissue culture conditions.

6. For the induction of the biosensor prior to experiments, detach the cells by brief trypsinization (*UNIT 1.1*) and centrifuge 5 min at $300 \times g$, 24°C. Carefully remove the supernatant and resuspend the cells in medium without doxycycline.

7. Plate the cells sparsely (1×10^4 cells per 10-cm tissue culture dish) without doxycycline and incubate for 48 hr prior to the experiments.

8. At a time point 24 hr after induction (i.e., the plating in step 7), check the cells for fluorescence. Continue incubating the cells under doxycycline-free conditions for an additional 24 hr prior to the assay.

   The total of 48 hr after removal of doxycycline ensures that overexpressors die and only cells with a sustainable expression level survive for the experiment.

9. On the day of the experiment, detach the cells with a brief trypsinization (*UNIT 1.1*).

10. Replate the cells on coverslips coated with fibronectin on the morning of the experiment and allow to adhere for 5 hr prior to imaging (see Basic Protocol 4).

IMAGING THE RhoA BIOSENSOR

BASIC PROTOCOL 4

Imaging is performed with the cells in Ham's F-12K medium without phenol red (see recipe in Reagents and Solutions), supplemented with 2% fetal bovine serum (FBS). For a single-chain design, it is sufficient to simply take a ratio of the FRET emission over the donor emission (Pertz et al., 2006). The two fluorophores will bleach at different rates, so bleaching corrections may be required to counter a bias in the signal over time. Such photobleach corrections are covered in detail elsewhere (Hodgson et al., 2006). Correcting for bleed-through (discussed in Basic Protocol 7) is not required for single-chain biosensors, but can be used to improve dynamic range when the biosensor is bright enough to permit this additional image processing.

This biosensor design preserves upstream regulatory interactions with molecules requiring a free C terminus, such as GDI. While this is an advantage in accurately reflecting cell physiology, it requires that one maintain low, near-endogenous levels of biosensor to minimize perturbation of normal cell physiology. At these low biosensor levels one must be careful to maximize light collection. It is not possible to compensate for low biosensor concentrations simply by increasing irradiation, as this bleaches the biosensor and increases phototoxicity. We routinely use an oil-immersion 40× Olympus UIS2 DIC objective lens with a numerical aperture of 1.3, together with 2 × 2 binning on our cooled CCD camera. We find that using 60× or higher magnification objectives cuts down greatly on light collection while increasing the photobleach rate. The signal intensity scales to the 4th power of the numerical aperture and to the inverse square of the magnification. Therefore, switching from a 1.3 NA 40× DIC objective lens to a 1.42 NA 60× DIC objective lens will result in an ∼37% decrease in signal intensity. It is important to use DIC rather than phase-contrast objective lenses, as the phase objective contains a phase annulus that substantially reduces light transmittance. Neutral-density filters of 0.6 to 1.0 (25% transmittance to 10% transmittance) are used to cut the brightness of the excitation light. It is better to use longer exposure with dimmer excitation rather than shorter, more intense irradiation; this reduces both photobleaching and phototoxicity. We routinely use methods to remove oxygen from the medium to further decrease photobleaching and phototoxicity, such as including the oxygen scavenger OxyFluor (Oxyrase, Inc.), including antioxidants such as vitamin C at 1 mM concentration, and purging the assay medium with argon gas.

The authors of this unit currently use a Roper Photometrics CoolSnapESII camera (*http://www.roperscientific.com/*), which is a Sony ICX285-based interline transfer cooled CCD camera, cooled to 0°C. This camera can be obtained with $<6e^-$ read noise, and $0.1e^-$/pixel/second dark current. The quantum efficiency of the chip is ∼60% for 450- to 625-nm light, and it has a small pixel size (6.45 × 6.45 μm) for good spatial resolution. We routinely use 2 by 2 binning and expose 800 msec for CFP and 400 msec for FRET, with a 10% transmittance neutral-density filter in the excitation light path. These conditions usually result in gray values filling ∼75% of the full 12-bit

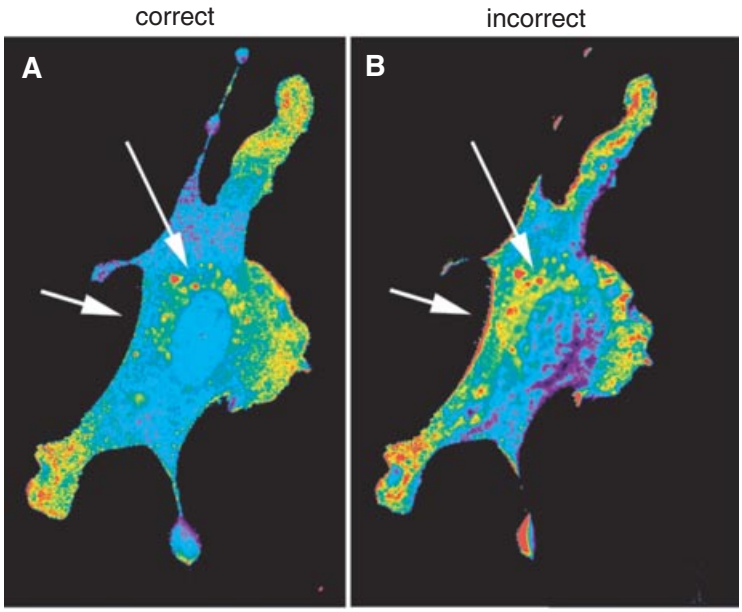


Figure 14.11.3 *x-y* translational image registration artifacts generate features on ratio images. In panel (**A**), a correctly registered ratio image of MeroCBD is shown. Panel (**B**) shows the ratio image from the same cell without registration. In the latter case, the dye image was misaligned by −5 pixels in both x and y directions. The white arrows in both figures point to regions where misalignment produces edge artifacts and other artifactual features. In (B), a lower-ratio rim can be seen on one side of the nucleus, and a higher ratio on the opposite side. Similarly, edge artifacts appear as higher ratio on predominantly one side of the cell, with an artifactually low ratio on the opposite side. For color figure go to *http://www.currentprotocols.com/protocol/cb1411*.

range of camera digitization. We do not recommend the current generation of on-chip amplification-gain CCD (EM-CCD) cameras for biosensor imaging. While these cameras can capture images under extremely dark illumination conditions, the gain circuitry introduces large amount of stochastic noise, which is problematic for ratiometric imaging (Fig. 14.11.3). When mathematical operations are performed on raw images, the stochastic noise adds greatly to the total noise levels in the resulting ratio image. While there are techniques available for signal restoration from acquisition with EM-CCD and other noisy approaches (Wang, 2007a,b), we have found conventional cooled CCD cameras to be sufficient. Other viable options may include back-thinned, back-illuminated cooled CCD cameras with high quantum efficiency. These cameras offer ultra-high quantum efficiency (>90%), but the pixel size is usually large (16 × 16 μm), reducing spatial resolution.

Two identical cameras can be mounted via a beamsplitter to simultaneously acquire the FRET and CFP channels. In such an approach, image registration can be affected by camera orientation as well as the angle of the dichroic mirror (causing rotation, *x-y* translation, image shear, mismatched scale, and mismatched focus). One must carefully mount the cameras to minimize rotational and translational effects, while accounting for the focus differences. Typically, beamsplitters are designed with an adjustable parfocal compensation device that can independently adjust the focusing distance of either camera head. This should be performed empirically to minimize focus difference between the two channels. Rotation, *x-y* translation, shear, scaling difference, and curvatures in the field of view will need to be corrected computationally based on a priori calibration, as described in Basic Protocol 7.

EXPRESSION OF Rac1 FLAIR, DUAL-CHAIN FRET BIOSENSOR FOR Rac1

BASIC PROTOCOL 5

The Rac1 biosensor currently used by the authors of this unit is a modification of an older design, wherein the FRET donor was EGFP-Rac1 and the FRET acceptor was a small binding domain derived from p21-activated kinase 1 (PAK1), labeled with the dye Alexa 546 (Kraynov et al., 2000). We modified this biosensor by replacing the EGFP with the fluorescent protein CyPet, and replaced the Alexa 546 with the fluorescent protein YPet (Fig. 14.11.1C). The new Rac1-FLAIR biosensor is wholly genetically encoded. It has been extensively validated and compared with other designs (Machacek et al., 2009). Unlike the RhoA biosensor above, the donor and acceptor fluorophores are not on the same chain. This improves the dynamic range and hence the sensitivity of the biosensor (in single-chain designs, some residual FRET is usually present even in the off state, so the change induced by activation is not as great as in dual-chain designs). The disadvantage of the dual-chain design is that the two chains do not distribute equally through the cell, necessitating careful bleed-through correction (see Basic Protocol 7). The two designs are prone to different effects on cell physiology and artifacts affecting the activation signal. Controls for the Rac1 FLAIR biosensor indicate that results have not been compromised by the separation of the chains (Machacek et al., 2009), and the sensitivity is, in fact, substantially enhanced.

Expression of the Rac1 biosensor is essentially the same as described for the RhoA biosensor above. The Rac1 biosensor is available from Addgene (*http://www.addgene.org*) in two formats:

1. Mammalian expression and cloning vectors: pECFP-C1 (Clontech) with ECFP replaced by CyPet (pCyPet-Rac1/kanamycin resistance), and pEYFP-C1 (Clontech Inc.) with EYFP replaced by YPet (pYPet-PBD/kanamycin resistance).

2. pBabe-sin-tet-CMV-puro retroviral vector for Tet-On/Off system incorporating the CyPet-Rac1 and YPet-PBD (amp resistance).

Because the donor and acceptor chains are on separate expression constructs, co-transfection/transduction of both components is required.

Materials

Mouse embryo fibroblast (MEF/3T3) cells (Clontech; tet-OFF MEF/3T3)
Rac1 biosensor vector system (see above)
Fugene6 transfection reagent (Roche)
10 mg/ml doxycycline stock solution
Coverslips coated with fibronectin: immerse glass coverslips 30 to 60 min in 10 μg/ml fibronectin (e.g., Sigma), then rinse three times with PBS (*APPENDIX 2A*) and leave immersed in PBS until use

Additional reagents and equipment for cell culture techniques (*UNIT 1.1*), flow cytometric cell sorting (Robinson et al., 2009), and imaging the Rac1-FLAIR biosensor (Basic Protocol 6)

For transient expression

1a. Transfect mouse embryo fibroblast cells (MEF/3T3) using Fugene6 following the manufacturer's protocols. Use a DNA ratio of 3:2 to transfect CyPet-Rac1 and YPet-PBD, respectively, for optimal results in MEF (or COS-7) cells.

It is important to premix the DNA solutions in the correct ratio, then add them to the Fugene-containing medium as a single aliquot. This optimizes correct mixing of both DNA constructs and results in an evenly distributed expression profile 24 hr post-transfection.

2a. Plate cells on fibronectin-coated glass coverslips at a density of 4×10^4 cells/coverslip for 3 to 4 hr prior to imaging.

For stable cell lines

Stable cell lines are preferred to reduce variability in the fluorescence profile from experiment to experiment, as well as for attaining better control of expression levels.

1b. For viral transduction using the pBabe Tet-off system, cotransduce viruses for the donor and acceptor portions of the biosensor according to the manufacturer's instructions.

2b. Select cells for stable incorporation by successively increasing the puromycin concentration, similar to the procedure described for stable RhoA biosensor cells (see Basic Protocol 3, step 2).

3b. Once the stable population is produced, sort the cells flow cytometrically (Robinson et al., 2009) for low to medium brightness, with equal CyPet and YPet fluorescence emission,

4b. Replate the collected fraction with doxycycline (1 μg/ml) to repress the expression until 48 hr prior to experiments.

Examination of varying ratios and levels of expression for each construct have not affected the results of motility studies, but can influence sensitivity and the ease of bleed-through corrections.

BASIC PROTOCOL 6

IMAGING RAC1-FLAIR

Imaging is performed in Ham's F-12K medium without phenol red (see recipe in Reagents and Solutions), supplemented with 2% fetal bovine serum. For emission ratio imaging, the following filter sets (Chroma Technology) are used (for excitation and emission, respectively): CyPet: HQ436/20 (ex), HQ470/40 (em); FRET: HQ436/20 (ex), HQ535/30 (em); YPet: HQ500/20 (ex), HQ535/30 (em). The dichroic mirror ("Quad-Custom," lot no. 511112038; spectra shown in Fig. 14.11.4) was custom manufactured by Chroma

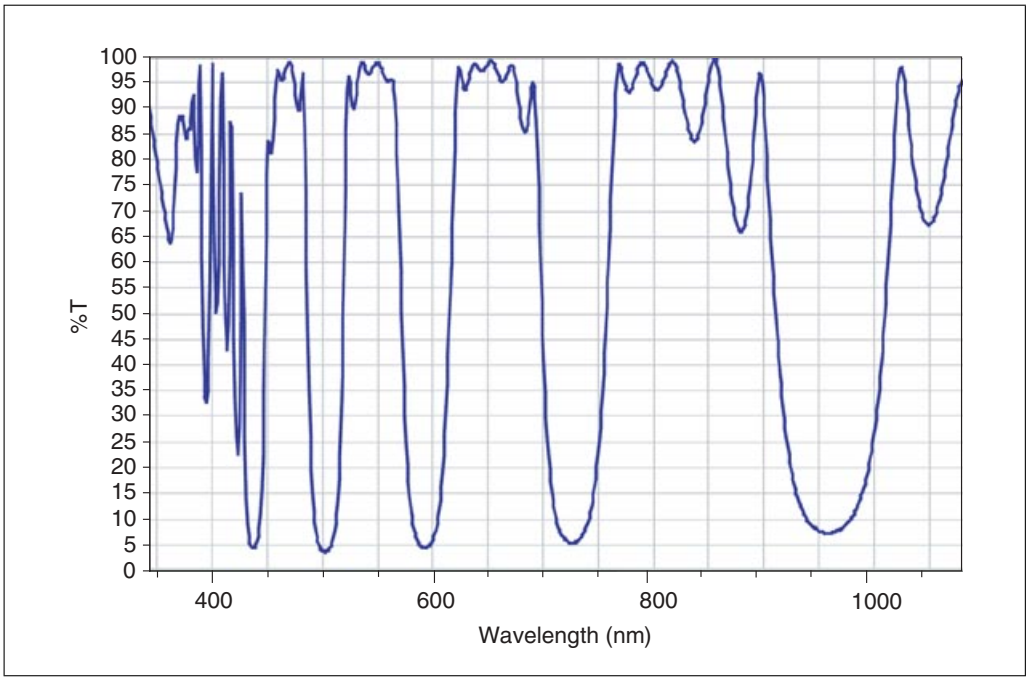


Figure 14.11.4 Transmittance spectra for the "Quad Custom" dichroic mirror (Chroma Technology, lot no. 511112038).

Technology Corp. for compatibility with all of these filter sets. A more recent sputter-coated ET series of dichroic mirrors and band-pass filters from Chroma could be used. For CyPet/YPet imaging or any other ECFP/EYFP-FRET-based imaging, either filter set 59217 (single-band exciters with a dual-band emitter) or set 89002 (all single-band filters) can be used effectively (Chroma Technology). Cells are illuminated with a 100 W Hg arc lamp through a neutral-density filter of 10% transmittance. At each time point, three images are recorded with the following exposure times, typical for the low biosensor concentrations used to minimize cell perturbation—CyPet, 900 msec; FRET (excitation of donor, observation of acceptor emission), 900 msec; YPet, 300 msec—at binning 2 × 2. The image sets have been taken at 10-sec to 10-min intervals. Ideally, one fills ~75% of the total 12-bit dynamic range of the camera for each channel. This produces a good signal-to-noise ratio and does not saturate the dynamic range should any one channel dramatically increase brightness. Imaging is detailed further in the RhoA biosensor section above.

Bleed-through correction for intermolecular biosensors is described in the following discussion of imaging procedures.

IMAGE PROCESSING PROCEDURES

BASIC PROTOCOL 7

Shading correction

The first step in image analysis is to correct for uneven illumination in the field of view. This is present in almost all images, including those taken using flat-field (Plan) corrected objective lenses. In order to correct for shading, one obtains images from fields of view that contain no samples. Images of each fluorescence channel are obtained using the same camera integration times and illumination conditions as are used for the real images that will be corrected. The images for shading correction are acquired following the acquisition of the real fluorescence image sets either by using cell-free areas within the same coverslip or by mounting a fresh coverslip with identical media and mounting conditions. In the latter case, it is convenient to mark a spot in the middle of the fresh coverslip on the side where cells would be present on an actual sample coverslip, so that one can focus on the edge of the spot to find the correct plane of focus. Usually, 20 to 30 shading images are acquired at the appropriate camera integration times and illumination settings for each fluorescence channel. The resulting image set for each channel is averaged to produce a single shade-corrected image for each fluorescence channel. The averaging process is important because each image frame contains stochastic camera noise. This noise can be reduced by averaging many frames of the same field of view. Additionally, images corrected for camera noise are required. These images are obtained by taking an average of multiple exposures (10 frames) of a field of view, but with the illumination shutter completely closed so as to prevent any light from reaching the CCD sensor. This basically captures the electronic and dark current noise associated with each acquisition condition. The noise image is then subtracted from the raw images and the shading images. Once these corrections are made, one simply divides the sample field of view by the corresponding shading image. Here, care must be taken to prevent floating-point errors from the image analysis software. Software including Metamorph (Universal Imaging Co.) and ISEE Inovision (ISee Imaging Systems) do not process floating-point data. Matlab (Mathworks) codes can be written to process images using a multiple decimal-point precision. Metamorph offers a convenient plug-in module for shade correction where a scaling factor can be specified to increase the relative pixel values so that floating-point operation is avoided. One can either scale to the maximum pixel value in the shading image or specify some fixed value. The latter is usually the better choice, as one can specify the same scaling factor (i.e., 1000) for images from both channels of fluorescence, thereby maintaining the relative intensities of the image pairs.

Background subtraction

Because of the flat-field correction, cell-free background areas within the field of view have the same intensities. One can use an area with minimal debris in the field of view as the background. The pixel intensities within such a region are averaged and subtracted from the whole image. Small variations in background can be introduced, e.g., by debris in your field of view. It is important to choose the same background position in each fluorescence image to minimize artifacts. Metamorph offers a convenient plug-in module ("Use region as background") to expedite the process. Using this utility, one can select a background region in the first fluorescence image of a ratio pair, and copy/save the region and paste/load onto the second fluorescence image. When using this Metamorph utility, it is important to note that processing of stack images requires additional considerations. If the region is selected on the top plane and the utility is run for all planes in the stack, the averaged background value from the first plane is subtracted from each of the subsequent planes. In order to work around this problem, the region on the top plane can be saved and loaded onto each of the subsequent planes, and background subtracted. Similarly, the same region can be loaded onto each plane of the fluorescence ratio pair images and processed.

Image masking

Ratio division can introduce noise and hot spots in regions outside of the cell. Furthermore, pixel intensities in the thin, peripheral regions of the cell can be near background levels, making reliable quantitative ratio calculations difficult. In order to limit the area within which ratio calculations are performed, a binary mask can be applied to the cell, setting areas outside of the cell uniformly to zero. The mask can be based on an image of a volume marker (i.e., fluorescent dextran) or a membrane marker in a fluorescence color different from that of the biosensor. This is used to unambiguously specify the true cell boundary in cases where intracellular distribution of the biosensor does not correspond to the cell shape. In the case of most GTPase biosensors, distribution of the biosensors sufficiently delineates the cell boundaries, so this is unnecessary.

To produce the binary mask, all fluorescence image stacks (dye and EGFP channels, or ECFP and FRET/Citrine-YFP channels) are intensity thresholded. If the shade correction and background subtraction have been performed correctly, this is a straightforward process (i.e., using the Threshold Image command in Metamorph). With properly processed images, the histogram distribution is such that there will be a large peak in the zero-pixel-intensity position, followed by a spread of non-zero pixels in the image histogram distribution (Fig. 14.11.5). Inclusive thresholding is manually performed by trial and error, observing which setting appropriately includes the cell pixels and excludes the surrounding pixels (i.e., using the Metamorph Threshold Image function). For a 16-bit image, the upper bound needs to be set at 65,535, the maximum value for 16-bit dynamic range. When processing a series of images from a time-lapse study, it is important to check the low-end threshold value selected using the first plane ($t = 0$ time point) against the last image plane ($t =$ end). Photobleaching will shift the low-end intensity distribution downward, resulting in under-selection of cell area at later times. In order to work around this problem, threshold values are determined through one of two methods: (1) thresholding is performed for later time points of the data stack, resulting in a relaxed thresholding for earlier time points; or (2) the data stack is histogram-equalized for the lowest intensity value using the Metamorph Equalize Light function, setting the equalization for "minimum" via the "addition" option, then producing the binary mask using the equalized data stack and then applying the resulting binary mask to the original non-equalized data stack. The latter approach produces better masking precision without resulting in relaxed selection in the earlier time points. Once the threshold values are set, a binary mask can be produced based on the selected inclusive threshold (in Metamorph, the Clip tab within the Threshold Image module is used to produce a binary mask in

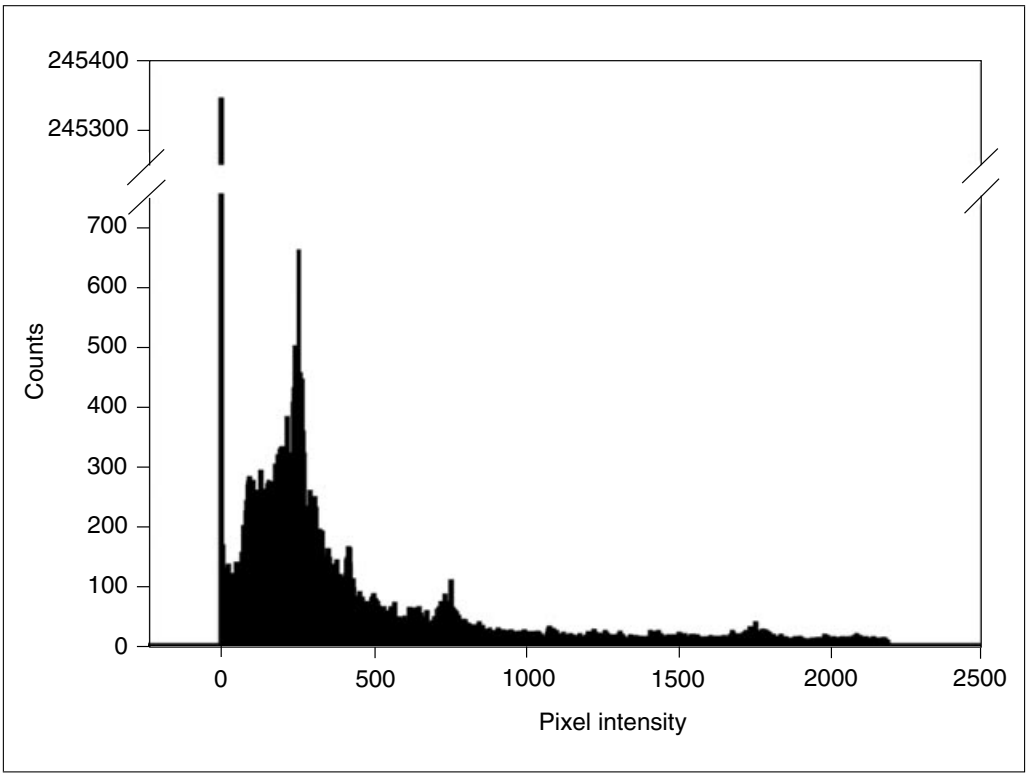


Figure 14.11.5 A representative histogram from a shade-corrected and background-subtracted image, prior to masking. The prominent histogram peak at zero intensity is followed by a continuous distribution of pixels at a range of positive intensity values. Threshold masking to remove pixels of too low an intensity is performed using this histogram. One maximizes selection of pixels from within the cell while removing pixels outside the cell, including noise around the periphery.

16-bit). This will produce a binary mask where regions outside the threshold selected area are uniformly zero, while inside is 65,535. Divide the binary mask with a constant (65,535) to produce a true binary mask containing the value one inside the masked region and zero outside (Arithmetic function in Metamorph). Binary masks produced for each fluorescence channel are multiplied into the corresponding fluorescence image stacks to produce the masked fluorescence image stacks prior to automated image registration.

Automated image registration

The ratio calculation requires a division of one image by another. It is essential that the fluorescence ratio image pair be perfectly aligned prior to division. There are a host of potential sources for misalignment: placement of optical filters, dichroic turret movement, *x-y-z* stage movement, temperature fluctuations and ambient vibration, and sub-pixel misalignment. Figure 14.11.3 indicates the result of misalignment on ratiometric analysis. The edge artifacts in the form of high ratio values on one side and low ratio values on the other, as well as similar symmetrical artifacts within the cell, provide clues that image misregistration has occurred. Sub-pixel registration routines are available in most image processing-software. For some experiments, only manual registration will suffice, but in many cases, especially when there are subcellular structures with clearly defined edges, automated registration is possible. The authors of this unit developed an automated method for image registration based on normalized cross-correlation technique (Shen and Price, 2006). A modified version of this routine, based on the Matlab program, applicable for registration of up to three channels of fluorescence image sets, is provided at *http;//www.currentprotocols.com/protocol/cb1411* (files `regAny2.m` and `regFCY2.m`).

The Matlab routine for processing the 2-channel data set is `regAny2.m` and for the 3-channel data set is `regFCY2.m`. The `regAny2.m` will take individual tiff files with the running number index and align the FRET channel with respect to the CFP channel to an accuracy of 1/20th of a pixel (maximum pixel shift allowed = 10 whole pixels). Similarly, the `regFCY2.m` will align the FRET and YFP channels against the CFP channel. In both cases, the relative displacements in *x* and *y* are recorded on a frame-by-frame basis for an entire time series, and the median values of the distance displacements in *x* and *y* are applied to the whole time series at the end of the routine. This mode of registration is based on the assumption that the majority of the channel misalignment will stay constant for the duration of a single time-lapse experiment. If individual *x-y* displacements are corrected on a frame-by-frame basis, we have observed an unacceptable level of jitter in the final movie playback. Detailed requirements for the naming convention, file formats, etc., are listed within the headers of the programs. One tip for making this program run faster and more smoothly is to crop the cell image as tightly as possible (ensuring for all timepoints that the cell stays within the region of interest selected and cropped), making sure both channels to be aligned are cropped using the same cropping factor. This will minimize the computational load required to translationally align the images, as there will be fewer total pixels to compute the cross-correlation coefficients compared to processing the entire field of view.

Image shear, rotation, and scaling correction

Two separate cameras can be used to simultaneously acquire the two images required for ratio imaging (CFP and FRET, or EGFP and dye). This can be valuable when rapid image sequences are required, or when rapid changes in cell shape or biosensor distribution can produce motion artifacts. For simultaneous imaging with two cameras, one uses a beam splitter and appropriate filters and dichroic mirror to direct different wavelengths to each camera. Differences in positioning of the two cameras can produce image misalignment other than the straightforward *x-y* translation encountered with one camera. Such additional misalignments include shear, rotation, and scaling between the two images. These problems primarily arise from imperfect mounting of the cameras and the dichroic mirrors in the beam splitter. Alignment should first be optimized manually, but an automated solution based on a priori calibration is required to sufficiently correct these effects. We present here a polynomial-based method for a priori calibration. Figure 14.11.6 shows calibration grid images and ratiometric images of RhoA activity before and after the correction. It is clear that manually aligning one corner of the grid leads to misalignment of the far corner. The software for Matlab consists of two parts (*http://cuurrentprotocols.com/protocol/cb1411*, files `morphPrep.m` and `morph.m`): (1) a priori calibration software `morphPrep.m`, used to determine the coordinate transformation control points; and (2) the transformation program `morph.m`, which applies a 3rd-order polynomial-based coordinate transformation using the calibrated control points. We find it best to apply these corrections prior to image masking and *x-y* translational alignment. For calibration, one can use a grid-type stage micrometer imaged in bright-field, or microbeads coated with fluorophores to simulate fluorescence imaging conditions. Imaging of the calibration specimen should be performed at the end of an experiment (be careful not to move the two cameras during the imaging experiment!). This correction method is not applicable to a majority of cases where a single camera and filter wheel are used to acquire multiple images in succession. However, in some cases fluorophores of very different wavelengths can scale differently due to the behavior of chromatically corrected optics (i.e., ECFP versus Cy-7). This method can be used effectively to correct for such issues if a priori calibration is performed.

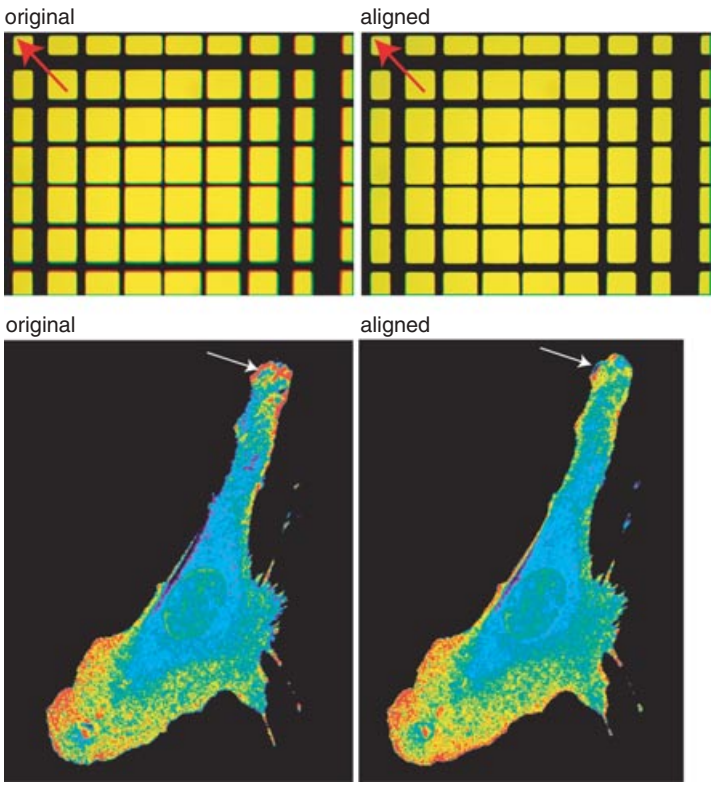


Figure 14.11.6 Image shear between two cameras creates artifacts in ratio images. In the upper panels, calibration grid images from two cameras are overlaid with and without shear correction (red arrow: two channels aligned relative to the corner indicated). In the lower panels, the ratios of RhoA biosensor readouts from the two camera channels are shown with and without shear correction. A peripheral ruffle shows a large artifactual feature when correction is not applied (white arrow). For color figure go to *http://www.currentprotocols.com/protocol/cb1411*.

Ratio calculation

The masked and registered images are divided to produce a ratio image. For meroCBD, the dye image is divided by the EGFP image. For the FRET sensors, the FRET channel is divided by the donor image. The floating point error consideration is important in this step; for software that does not carry out floating point calculations, all numbers must be multiplied by a constant. This converts decimal portions of intensity values to larger numbers, so that they are not truncated or otherwise modified prior to division. In the Metamorph Arithmetic module, a scaling factor of 1000 is specified as a multiplication factor during the ratio calculation. A smaller factor (10 to 100) can be used. However, this is not recommended since it tends to produce a non-smooth histogram distribution (Fig. 14.11.7). The same scaling factor should be used when comparing ratio calculations from different experiments with the same biosensor.

Ratio images are scaled and often displayed as a pseudocolor map to reflect the range of ratio values within the image. Many methods are used for this, and the range of values within an image cannot readily be used to determine the dynamic range of the biosensor. For example, the lowest and highest pixel intensities are often not included in the scaling, as these can be due to spurious artifacts or noise. If one chooses to eliminate the lowest and highest 1% of pixel intensities, a 99-fold change is shown within the image. Eliminating the lowest and highest 5% reduces this to a 20-fold change.

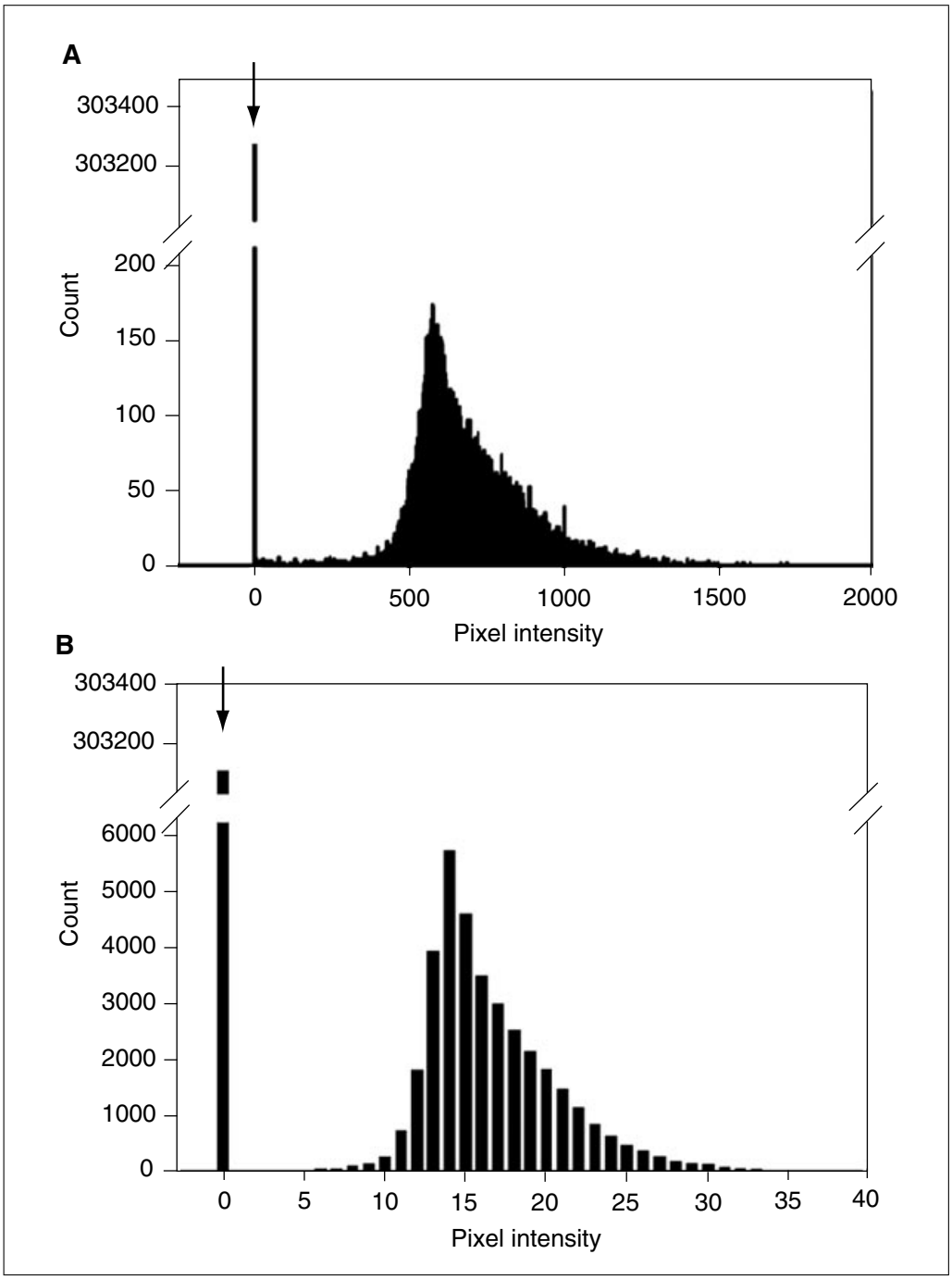


Figure 14.11.7 Effect of the multiplication factor during ratiometric calculation. In panel (**A**), using a scaling factor of 1000 produced a smooth histogram distribution in a ratio image. Panel (**B**) shows the same ratio image with the scaling factor of 25. Though the general distribution pattern is similar, the histogram distribution in (A) retains higher bit-depth resolution.

Ratio calculations for intermolecular FRET biosensors

In the case of intermolecular FRET biosensors such as the biosensor for Rac1, a bleed-through correction is required. Images for the bleed-through correction should be obtained routinely as part of each experiment, so that variations in the microscope system over time do not come into play. Control cells expressing either CyPet alone or YPet alone are used to determine how much light bleeds through into channels where it is not desirable. For example, CyPet excitation is used to generate FRET. However, when this is done, some light emitted from CyPet itself appears in the FRET emission channel.

This bleed-through must be quantified so that it can be subtracted out in real experiments. For this correction, intensity is measured using the CyPet excitation and CyPet emission channels, then measured again under the same exposure conditions using the CyPet excitation and YPet emission channels that will be used to monitor FRET. This reveals what percentage of the intensity measured using the direct CyPet fluorescence channels would also appear in the FRET channels. Bleed-through coefficients determined in this way are the coefficients α and β in the following equation:

$$R = \frac{\mathrm{FRET_t} - (\alpha \times \mathrm{CyPet}) - (\beta \times \mathrm{YPet})}{\mathrm{CyPet}}$$

Equation 14.11.3

where R is the ratio, $\mathrm{FRET_t}$ is the total FRET intensity as measured, α is the bleed-through of CyPet into FRET channel upon CyPet excitation, β is the bleed-through of YPet into FRET channel upon CyPet excitation of YPet, and CyPet is the total CyPet intensity as measured. Bleed-through into the CyPet channel (denominator) is negligibly small. By calculating the linear slope of the relationship between FRET and CyPet intensities upon CyPet excitation of cells expressing only the CyPet, the bleed-through parameter α can be determined. Similarly, by relating bleed-through into the FRET channel upon CyPet excitation of cells expressing only the YPet, the bleed-through contribution of YPet excitation by CyPet excitation into the FRET channel β can be determined. For our microscope and exposure conditions, the α parameter was found to be routinely within 0.4 and 0.5 and the β parameter to be $\sim$0.2. These are dependent on the optical configuration of the microscope used. We provide a convenient Matlab routine, `BT_AB.m` for calculating the α and β parameters from raw images and associated shading images (*http://www.currentprotocols.com/protocol/cb1411*; file `BT_AB.m`), utilizing a segmentation based on the K-means clusters method (Shen and Price, 2006). The ratio of corrected FRET over CyPet can be calculated and used as a measure of Rac1 activation.

In time-lapse experiments, CyPet and YPet photobleach at different rates. The ratio can be corrected for photobleaching as described elsewhere (Hodgson et al., 2006). Briefly, by algebraic manipulation, Equation 14.11.3 can be rearranged to:

$$R = \frac{\mathrm{FRET_t}}{\mathrm{CyPet}} - \beta \frac{\mathrm{Ypet}}{\mathrm{CyPet}} - \alpha = \Gamma - \beta \times \Psi - \alpha$$

Equation 14.11.4

where Γ is the fraction representing total FRET intensity over total CyPet intensity, and Ψ is the fraction representing total YPet intensity over total CyPet intensity, and both α and β are bleed-through constants described before. By taking double exponential fits of the decays of both Γ and Ψ, the correction function, $\bar{R}^{-1}$, can be calculated as outlined previously (Hodgson et al., 2006). We provide a convenient routine, `PB5.m`, that takes the masked and registered image sets (CyPet, YPet, and FRET) plus the a priori–determined α and β parameters, and calculates the photobleach-corrected ratio R and corrected FRET, which is represented by the numerator of Equation 14.11.3 (see *http://www.currentprotocols.com/protocol/cb1411*; file `PB5.m`). These codes are available for download from the Hahn lab Web page and from the Current Protocols Web site (*http://www.currentprotocols.com/cb1411*). Also included are the Matlab routines `SubpixShift.m`, `subalign.m`, `kmeanst.m`, and `maxArray.m`,which are required functions that are called from within the routines described above.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ham's F-12K, phenol red-free

Inorganic salts
7530.00 mg/liter NaCl
285 mg/liter KCl
106 mg/liter $MgCl_2 \cdot 6H_2O$
393 mg/liter $MgSO_4 \cdot 7H_2O$
135 mg/liter $CaCl_2$
218 mg/liter $Na_2HPO_4 \cdot 7H_2O$
59 mg/liter KH_2PO_4
2500.00 mg/liter $NaHCO_3$
0.8 mg/liter FeSO4·$7H_2O$
2 μg/liter $CuSO_4 \cdot 5H_2O$
0.14 mg/liter $ZnSO_4 \cdot 7H_2O$

Amino acids
17.8 mg/liter L-alanine
421.3 mg/liter L-arginine HCl
30 mg/liter L-asparagine·H_2O
26.6 mg/liter L-aspartic acid
70.04 mg/liter L-cysteine HCl·H_2O
0 mg/liter L-cystine
29.4 mg/liter L-glutamic acid
15 mg/liter glycine
41.9 mg/liter L-histidine HCl·H_2O
7.9 mg/liter L-isoleucine
26.2 mg/liter L-leucine
73.1 mg/liter L-lysine HCl
8.9 mg/liter L-methionine
9.9 mg/liter L-phenylalanine
69.1 mg/liter l-proline
21 mg/liter L-serine
23.8 mg/liter L-threonine
4.1 mg/liter L-tryptophan
10.9 mg/liter L-tyrosine
23.4 mg/liter L-valine

Vitamins
0 mg/ml L-ascorbic acid
0.07 mg/ml biotin
0.48 mg/ml D-calcium pantothenate
13.96 mg/liter choline chloride
1.36 mg/liter cyanocobalamin
1.32 mg/liter folic acid
18 mg/liter inositol
0.04 mg/liter nicotinamide
0.06 mg/liter pyridoxine HCl
0.04 mg/liter riboflavin
0.21 mg/liter thiamine HCl
0.21 mg/liter DL-thioctic acid

continued

Other compounds
1260.00 mg/liter glucose
0 mg/liter linoleic acid
4 mg/liter hypoxanthine·Na
0 mg/liter phenol red
0.3 mg/liter putrescine
220 mg/liter sodium pyruvate
0.7 mg/liter thymidine

Lysis buffer

50 mM sodium phosphate buffer, pH 7.6 (*APPENDIX 2A*)
300 mM NaCl
10% (w/v) glycerol
5 mM $MgCl_2$
2 mM 2-mercaptoethanol (omit where indicated in protocol)
1 mM PMSF (omit where indicated in protocol)
Store up to 12 months at 4°C

Storage buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
50 mM NaCl
5 mM $MgCl_2$
10% (w/v) glycerol
Store up to 12 months at 4°C

COMMENTARY

Background Information

Biosensors have become valued tools in cell biology, with commercial software and specifically designed microscopes greatly enhancing their accessibility and ease of use. It is important to apply this software with an understanding of the variables that can affect biological conclusions. Final images are sensitive to subtle changes in image-processing procedures, or the use of biosensors under conditions that affect cell biology. The authors of this unit hope that the procedures outlined here, for three different biosensor designs, can be a guide for proper application of new biosensors to shed light on previously invisible protein dynamics.

Rac1 FLAIR is an intermolecular FRET biosensor, frequently referred to as a dual-chain sensor. The use of a dual-chain design significantly enhances the sensitivity of the biosensor in comparison with single-chain FRET biosensors. The latter usually show substantial FRET even when the biosensor is in the off state; the two fluorophores are never fully separated. With the Rac1 sensor, the dynamic range, the difference between the on and off states, is enhanced. There is a downside to the dual-chain approach. The two components of the biosensor distribute differently in the cell, necessitating additional steps in image processing. Both single-chain and dual-chain biosensors are potentially perturbed by competition from native protein ligands; the dual-chain sensor may be more likely to produce false negatives, as it may be more sensitive to ligands that compete for the GTPase-binding fragment, while the steric bulk of the single-chain sensor may prevent it from reaching all sites normally accessed by the GTPases. The issue of competition and its effect on biosensor readout is complex and beyond the scope of this unit. We recently completed a careful study of the effects of varying the concentration of each component of the dual-chain Rac1 biosensor. This did not appreciably alter the results of our cell protrusion studies (Machacek et al., 2009).

Factors affecting FRET efficiency

FRET is sensitive to both the distance and orientation of the two fluorophores in a biosensor. When fluorophores are sufficiently far apart or have orthogonal dipole orientations, excitation of the donor leads simply to donor emission, rather than to FRET. However, when the distance is sufficiently small, and orientation enables sufficient dipole coupling, excitation energy is transferred from the donor

to the acceptor, leading to decreased donor emission and increased emission from the acceptor. This produces a characteristic FRET excitation/emission spectrum, different from that of the donor or the acceptor alone.

GFP mutants optimized for FRET in living cells have shown impressive improvements. Mutants incorporate different trade-offs between brightness, FRET efficiency, photostability, and pH dependence of fluorescence characteristics (Heikal et al., 2000; Miyawaki and Tsien, 2000). Enhanced brightness improves the overall signal-to-noise ratio in cells, but is not always an improvement if it comes at the cost of FRET efficiency or photostability (Nguyen and Daugherty, 2005). The Cyan and Yellow fluorescent proteins (CFP and YFP), and their brighter, pH-stable versions Cerulean and Venus (Miyawaki and Tsien, 2000; Rizzo and Piston, 2005), are relatively fast-maturing, bright GFP mutants that have proven useful in many FRET biosensors. More recent mutants with improved FRET efficiency (Nguyen and Daugherty, 2005) include CyPet and YPet, which exhibit 6- to 7-fold greater FRET efficiency than the original CFP-YFP pair, though more recent findings indicated that this particular pair may be more prone to dimerization (Nguyen and Daugherty, 2005; Ohashi et al., 2007).

FRET efficiency is quantified by the Förster equation:

$$R_0 = \left[8.8\times10^{23} K^2 n^{14} Q_d J\right]^{1/6}$$

Equation 14.11.5

where R_o is the Förster distance (distance at which energy transfer is 50% efficient) and K^2 is the dipole orientation factor, a function of the donor emission transition moment and the acceptor absorption transition moment. K^2 = 2/3 is generally assumed when fluorophore rotation can occur about the bond attaching the fluorophore to the protein (Lakowicz, 1999). Q_d is the fluorescence quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium, generally assumed to be 1.4 for proteins (dos Remedios and Moens, 1995), and J is the spectral overlap integral, indicating the extent of overlap between the donor fluorescence emission spectrum and the acceptor excitation spectrum (Lakowicz, 1999).

In FRET biosensors, activation of the targeted protein leads to a change in the distance and/or orientation of the fluorophores. FRET efficiency is exquisitely sensitive to distance between fluorophores (varies as the inverse 6th power of the distance between the fluorophores):

$$E = \frac{R_0^6}{\left(R_0^6 + R^6\right)}$$

Equation 14.11.6

where E is FRET efficiency, R_o is the Förster distance, and R is the actual distance (Lakowicz, 1999). Changes in the relative angular orientations of the dipoles produce a lesser but nonetheless important effect; the dipole orientation factor K^2 can be assumed to be 2/3 only when both fluorophores are free to rotate isotropically during the excited-state lifetime. A change in the fixed angle of the fluorophores in different biosensor states affect FRET because K^2 can change between 0 and 4 (Lakowicz, 1999; dos Remedios and Moens, 1995). The effects of fluorophore separation (linear displacement) versus angular reorientation cannot be readily separated in live-cell studies, so FRET changes are not used to precisely determine distances between proteins in cells. Rather, the extent of FRET produced by fully activated versus inactive target protein is determined, and these endpoints are used to interpret FRET signals in vivo.

Critical Parameters and Troubleshooting

The critical parameters for setting up a microscope system for time-lapse biosensor imaging will not be discussed here, as the relevant protocols above deal with these in detail. For successful biosensor imaging, critical components are production of clean and functional biosensor at optimal dye labeling efficiency in the case of meroCBD, and optimal expression levels and cell health in the case of genetically encoded biosensors. The proper maintenance of cells before and after microinjection will significantly affect cell survival during time-lapse imaging in the case of MeroCBD. We have found that careful attention to deoxygenating the imaging medium, using reagents including Oxyrase/Oxyfluor, has a significant effect in minimizing photobleaching as well as photodamage to cells from irradiation. To this end, use of low-intensity light for a longer duration of exposure is much preferred, rather than a short exposure with a full intensity of excitation light.

The choice of imaging medium is an important consideration when imaging FRET biosensors. Background fluorescence from the

medium is a significant issue at the wavelengths used. We have performed a quantitative comparison of various media available commercially (data not shown), and concluded that Ham's F-12K medium without phenol red (Kaighn, 1973; Robey and Termine, 1985; see Reagents and Solutions) is a good choice. Unfortunately, this medium is no longer commercially available. The published formulation can be found in Reagents and Solutions.

Anticipated Results

Biosensors for Rho family GTPases should provide sensitive and high-resolution data of activation of GTPases in living cells. Typically, only 40% to 60% of the CBD-EGFP is recovered as purified meroCBD, due to some denaturation/precipitation during labeling and loss during gel filtration. The final eluate concentration is usually 120 to 140 μM. Labeling efficiency under these conditions varies between a dye/protein ratio of 0.7 and 0.9. The meroCBD solution is divided into 15- to 20-μl aliquots and stored at –80°C. Flash-freezing using liquid N_2 or dry ice appears to increase precipitation during microinjection. Alternatively, meroCBD may be kept at 4°C for up to 1 week.

The single-chain RhoA and bimolecular Rac1 biosensor transduction using retrovirus usually results in a nearly 100% positive population following FACS sorting. FACS sorting with relatively tight gates is used to isolate a low-expression-level population that results in biosensor expression levels of ~20% to 30% of endogenous protein. For microscopy imaging, the typical time resolution can be in the order of seconds, limited only by the user's microscope system configuration. The spatial resolution can be theoretically at the optical limit of light microscopy in *x* and *y*; however, due to low levels of injection/expression that are preferred so as to not impact the biological processes of interest, binning may be required to increase sensitivity. The 2 × 2 binned dataset obtained using a 40× DIC oil-immersion objective lens will be on the order of 320-nm spatial resolution in *x* and *y*. The dynamic range of the biosensor ratio readout for meroCBD can be on the order of 1:3 to 1:6, that of genetically encoded single-chain RhoA biosensor can be on the order of 1:1.5 to 1:2, and that of genetically encoded bimolecular Rac1 biosensor can be on the order of 1:7 to 1:20. Dynamics of the resulting ratio readouts should be carefully interpreted, keeping in mind the potential sources of artifacts, including misalignment of the ratio pair and motion artifacts if using single camera-based imaging approach. These artifacts will significantly skew the resulting ratio and impact spatial as well as temporal ratiometric readouts.

Time Considerations

Preparation of meroCBD requires 3 days, including the dye-labeling reaction and post-reaction purification. Cells plated on fibronectin-coated coverslips are usually ready for microinjection within 3 hr following plating. Microinjection of live cells on coverslips is typically performed within 10 min, followed by a 30-min to 1-hr recovery prior to imaging experiments. The process of viral transduction can be performed, taking 1 week, while preparation of stable cell lines containing the genetically encoded biosensor can take up to 2 weeks, including the FACS sorting. Genetically encoded biosensors will require 48 hr after removal of doxycycline from the medium to induce expression prior to imaging experiments. Imaging experiments using the biosensors can be as long as the biological process of interest, but are usually limited to ~2 hr for meroCBD experiments, and can be extended to overnight for genetically encoded biosensors. The post-imaging data processing will require ~10 min per data set.

Literature Cited

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.). 2009. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Del Pozo, M.A., Klosses, W.B., Alderson, N.B., Meller, N., Hahn, K.M., and Schwartz, M.A. 2002. Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. *Nat. Cell Biol.* 4:232-239.

dos Remedios, C.G. and Moens, P.D. 1995. Fluorescence resonance energy transfer spectroscopy is a reliable "ruler" for measuring structural changes in proteins: Dispelling the problem of the unknown orientation factor. *J. Struct. Biol.* 115:175-185.

Heikal, A.A., Hess, S.T., Baird, G.S., Tsien, R.Y., and Webb, W.W. 2000. Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: Coral red (dsRed) and yellow (Citrine). *Proc. Natl. Acad. Sci. U.S.A.* 97:11996-12001.

Hodgson, L., Nalbant, P., Shen, F., and Hahn, K. 2006. Imaging and photobleach correction of Mero-CBD, sensor of endogenous Cdc42 activation. *Methods Enzymol.* 406:140-156.

Kaighn, M.E. 1973. Tissue Culture Methods and Applications (J. Kruse and J. Patterson, eds.) Academy Press, New York.

Kraynov, V.S., Chamberlain, C., Bokoch, G.M., Schwartz, M.A., Slabaugh, S., and Hahn, K.M. 2000. Localized Rac activation dynamics visualized in living cells. *Science* 290:333-337.

Lakowicz, J.R. (ed.). 1999. Principles of Fluorescence Spectroscopy, pp. 368-377. Kluwer Academic/Plenum Publishers, New York.

Machacek, M., Hodgson, L., Welch, C., Elliott, H., Pertz, O., Nalbant, P., Abell, A., Johnson, G.L., Hahn, K.M., and Danuser, G. 2009. Coordination of Rho GTPase activities during cell protrusion. *Nature* 461:99-103.

Michaelson, D., Silletti, J., Murphy, G., D'Eustachio, P., Rush, M., and Phillips, M.R. 2001. Differential localization of Rho GTPases in live cells: Regulation by hypervariable regions and RhoGDI binding. *J. Cell Biol.* 152:111-126.

Miyawaki, A. and Tsien, R.Y. 2000. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.* 327:472-500.

Nalbant, P., Hodgson, L., Kraynov, V., Toutchkine, A., and Hahn, K.M. 2004. Activation of endogenous Cdc42 visualized in living cells. *Science* 305:1615-1619.

Nguyen, A.W. and Daugherty, P.S. 2005. Evolutionary optimization of fluorescent proteins for intracellular FRET. *Nat. Biotechnol.* 23:355-360.

Ohashi, T., Galiacy, S.D., Briscoe, G.M., and Erickson, H.P. 2007. An experimental study of GFP-based FRET, with application to intrinsically unstructured proteins. *Protein Sci.* 16:1429-1438.

Pertz, O., Hodgson, L., Klemke, R.L., and Hahn, K.M. 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* 440:1069-1072.

Rizzo, M.A. and Piston, D.W. 2005. A high contrast method for imaging FRET between fluorescent proteins. *Biophys. J.* 88:L14-L16.

Robey, P.G. and Termine, J.D. 1985. Human bone cells in vitro. *Calcif. Tissue Int.* 37:453-460.

Robinson, J.P., Darzynkiewicz, Z., Hoffman, R., Nolan, J.P., Orfao, A., Rabinovitch, P.S., and Watkins, S. (eds.). 2009. Current Protocols in Cytometry. John Wiley & Sons. Hoboken, N.J.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Shen, F. and Price, J.H. 2006. Melanoma coculture outgrowth model for testing complete tumor contaminant ablation. *Cytometry A* 69:573-581

Shen, F., Hodgson, L., and Hahn, K. 2006. Digital autofocus methods for automated microscopy. *Methods Enzymol.* 414:620-632.

Shen, F., Hodgson, L., Price, J.H., and Hahn, K.M. 2008. Digital differential interference contrast autofocus for high-resolution oil-immersion microscopy. *Cytometry A* 73:656-666.

Toutchkine, A., Kraynov, V., and Hahn, K. 2003. Solvent-sensitive dyes to report protein conformational changes in living cells. *J. Amer. Chem. Soc.* 125:4132-4145.

Toutchkine, A., Nguyen, D.V., and Hahn, K.M. 2007a. Simple one-pot preparation of water-soluble, cysteine-reactive cyanine and merocyanine dyes for biological imaging. *Bioconjug. Chem.* 18:1344-1348.

Toutchkine, A., Nguyen, D.V., and Hahn, K.M. 2007b. Merocyanine dyes with improved photostability. *Org. Lett.* 9:2775-2777.

Wang, Y.L. 2007a. Computational restoration of fluorescence images: Noise reduction, deconvolution, and pattern recognition. *Methods Cell Biol.* 81:435-445.

Wang, Y.L. 2007b. Noise-induced systematic errors in ratio imaging: Serious artifacts and correction with multi-resolution denoising. *J. Microsc.* 228:123-131.

Analysis of Arf GTP-Binding Protein Function in Cells

Lee Ann Cohen[1] and Julie G. Donaldson[1]

[1]Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, Maryland

ABSTRACT

This unit describes techniques and approaches that can be used to study the functions of the ADP-ribosylation factor (Arf) GTP-binding proteins in cells. There are six mammalian Arfs and many more Arf-like proteins (Arls), and these proteins are conserved in eukaryotes from yeast to humans. Like all GTPases, Arfs cycle between GDP-bound, inactive and GTP-bound active conformations, facilitated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that catalyze GTP binding and hydrolysis, respectively. This unit describes approaches that can be taken to examine the localization and function of Arf and Arl proteins in cells. A simple protocol for measuring activation (GTP-binding) of specific Arf proteins in cells using a pull-down assay is also described. Approaches that can be taken to assess function of GEFs and GAPs in cells is described. *Curr. Protoc. Cell Biol.* 48:14.12.1-14.12.17. © 2010 by John Wiley & Sons, Inc.

Keywords: Arf • GTP-binding proteins • guanine nucleotide exchange factors • GTPase-activating proteins

INTRODUCTION

The ADP-ribosylation factor (Arf) family of guanine nucleotide binding proteins were originally identified and named for their ability to act as cofactors during cholera toxin–catalyzed ADP-ribosylation of Gαs. Subsequently, six mammalian and three yeast Arf proteins were identified, and Arf1 was found to specifically associate with the Golgi complex and regulate vesicle trafficking between the endoplasmic reticulum (ER) and Golgi complex (Donaldson and Jackson, 2000; D'Souza-Schorey and Chavrier, 2006). Arfs are conserved 175 to 183 amino acid G proteins that have defined and nearly identical Switch I and Switch II effector domains. All Arfs are co-translationally myristoylated at the amino terminus, and this modification is required for Arfs to interact with membrane surfaces, regulators, and effectors. Thus, addition of epitope tags onto Arf proteins must be made at the carboxyl terminus to retain biological activity. Arfs 1 through 5 reversibly associate with the Golgi complex and cycle into the cytosol during GTP-binding and GTP-hydrolysis, respectively. Arf6, on the other hand, appears to spend more time associated with membranes while GDP-bound (D'Souza-Schorey and Chavrier, 2006).

In their active, GTP-bound form, Arfs modify membrane surfaces by recruitment of coat proteins that sort membrane proteins into forming vesicles, and by stimulating the activity of lipid-modifying enzymes (e.g., phosphatidylinositol 4-phosphate 5-kinase and phospholipase D) that lead to production of phosphatidylinositol 4,5-bisphosphate and phosphatidic acid, respectively (Donaldson and Jackson, 2000; D'Souza-Schorey and Chavrier, 2006). Through these activities, Arfs mediate membrane trafficking in cells. Most of the studies on Arfs have concerned the function of Arf1 at the Golgi complex and Arf6 at the plasma membrane in standard tissue culture cell lines. Specific localization and functions for the other Arf proteins and assessment of Arf function in specialized cells and tissues have yet to be determined. Basic Protocol 1 describes ways to examine

Current Protocols in Cell Biology 14.12.1-14.12.17, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb1412s48

localization and function of Arf or Arl proteins in cells in culture. Cells expressing wild-type or mutant Arfs can be examined for effects on a cellular function of interest. If an Arf is involved in a particular physiological process or membrane trafficking step, it is often desirable to be able to determine what fraction of a particular Arf is in the active, GTP-bound state. Basic Protocol 2 describes a simple assay to determine the amount of active Arf in cells.

Arf proteins cycle between GTP-bound, active and GDP-bound, inactive forms. This cycle is regulated by GEFs that catalyze GDP release and GTP binding, and GAPs that catalyze GTP hydrolysis and return to the GDP-bound form. There are over 15 GEFs and 24 GAPs expressed in mammalian cells, and although the Arf specificities and functions for some of these regulators are known, for many, their specificities, localization, and function have not been evaluated. There are several families of Arf GEFs whose members all share the catalytic Sec7 domain and then differ in other domains. The BIG and GBF GEFs are large proteins that are localized to the Golgi and are sensitive to inhibition by the drug brefeldin A (BFA). The ARNO/Cytohesin, EFA6, and BRAG families of Arf GEFs localize to the cytosol and plasma membrane, and are not inhibited by BFA (Casanova, 2007). There are also many Arf GAP families of proteins that contain the catalytic Arf GAP domain. Arf GAPs also contain many other domains that function to regulate and recruit GAP to membranes as well as domains that serve as scaffolding platforms to recruit other Arf effectors. In this sense, Arf GAPs may be effectors as well as signal terminators (Inoue and Randazzo, 2007). Since there are many more GEFs and GAPs than there are Arfs, there is much to learn about the function of these proteins, and approaches to assessing their cellular function is presented in Basic Protocol 3.

STRATEGIC PLANNING

Although the Arf proteins are ubiquitously expressed, high-affinity, specific antibodies to examine the localization and function of endogenous proteins are not available. Hence, the transient expression of Arf proteins is a useful tool for examining Arf function in cells. Expression of the wild-type Arf protein is a practical way to determine how an Arf localizes and behaves in a cell since expression of the wild-type protein, especially at low levels, does not usually cause a strong phenotype by itself, presumably because the Arf can cycle between GDP-bound inactive and GTP-bound active forms. Due to the importance of the amino terminal myristoyl group for biological function, epitope tags, if used, should be placed at the carboxyl terminus of an Arf protein. A number of tags have been appended to the carboxyl terminus of Arf proteins including peptide tags like HA, myc, FLAG, and also fluorescent proteins like green fluorescent protein (GFP) and red fluorescent proteins (RFP). Small epitope tags are not benign, however, and in many cases they are highly charged. Thus, it is important to consider the consequence of each tag when examining Arf function in cells. Although antibodies are not available that can detect endogenous Arfs, which might be expressed at low levels, commercial antibodies are available that can detect over-expressed Arf proteins (in particular, for Arf1 and Arf6); thus, the behavior of an epitope-tagged over-expressed Arf protein can be compared to that of an untagged over-expressed Arf protein.

When over-expressed, Arfs1–5 typically have a prominent localization at the Golgi complex that can be verified by co-staining cells with antibodies to Golgi-resident proteins. Arfs1–5 also have a substantial cytosolic pool, which represents an inactive pool of the molecules. Because Arfs1–5 are released into the cytosol upon GTP hydrolysis, it is important to realize that they can be recruited to extra-Golgi sites as part of their activation cycle. There are reports of Arf1 at the plasma membrane and on peripheral endosomal structures. Arf6 is not localized to the Golgi, and instead localizes to the plasma membrane and on endosomal structures. In some cells, like HeLa cells, Arf6 is

mostly localized on membranes, whereas in other cells types, Arf6 expression shows a significant cytosolic pool. The cytosolic pool can be considered inactive, but Arf6 can localize to the membrane regardless of its GTP/GDP status, so it cannot be assumed that all Arf6 localized to membranes is GTP bound.

A useful pharmacological tool for examining Arf function in cells is the fungal metabolite BFA. BFA is an uncompetitive inhibitor of certain Arf GEFs, stabilizing a complex of the GEF with GDP-bound Arf. In this way, it inhibits activation of Arfs by that GEF. BFA inhibits Arf activation catalyzed by GBF and BIG family GEFs that contain the residues necessary to form this complex whereas other Arf GEFs, such as the cytohesin/ARNO and EFA6 families, lack these residues. An Arf-dependent process that is BFA sensitive would point to activation of the Arf through GBF1 or BIG family exchange factors. An Arf-dependent process that is not BFA-sensitive would suggest activation of Arf through other GEFs. Treating cells with BFA results in a rapid release of Arf1 and Golgi-associated coat proteins from the Golgi, reflecting a block in activation of Arf at the Golgi. At later times of BFA treatment, Golgi membranes and content fuse with the ER, and in some cells an alteration of endosomal and lysosomal membrane morphology is also observed.

Another useful approach to examining Arf function in cells is expression of mutant Arf proteins. Threonine to asparagine mutations in the nucleotide binding pocket (T31N in Arfs1–5 and T27N in Arf6) act as dominant negative proteins, used to inhibit function of an endogenous Arf. These mutants are inactive and may sequester endogenous Arf GEFs by mimicking the inactive, GDP-bound or nucleotide-free form of the Arf, preventing activation of the endogenous Arf. The first Arf exchange factor was identified as a supressor of an Arf1T31N mutant in a genetic screen in yeast. Both Arf1T31N-HA and Arf1T31N-GFP function effectively as dominant negatives when expressed in cells. Arf1T31N itself is more cytosolic, as it is in a GDP-bound state and its expression results in cytosolic distribution of Golgi coat proteins and loss of Golgi structure, similar to what is observed with acute BFA treatment (see Fig. 14.12.1).

Mutation of a glutamine residue to leucine in the guanine nucleotide binding pocket (Q71L in Arfs1–5 or Q67L in Arf6) creates GTP-hydrolysis-defective mutants of Arf proteins, which are constitutively GTP bound, and can be used to mimic a persistently activated state. Although activated Arfs can presumably function, Arfs must cycle between the GTP-bound, active and GDP-bound, inactive states for biological activity. Thus, expression of these mutants often inhibits Arf-specific processes. For instance,

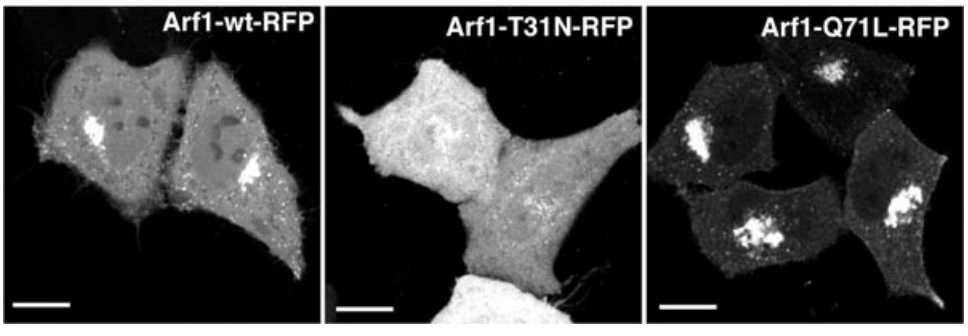


Figure 14.12.1 Expression of Arf1-RFP and its mutants in HeLa cells. Wild-type Arf1-RFP has both a prominent localization at the Golgi complex and a cytosolic pool. In cells expressing Arf1-T31N-RFP, the Arf is largely cytosolic and staining for a Golgi marker would reveal that the Golgi is largely absent (redistributed to the ER). Arf1-Q71L-RFP is almost entirely membrane bound, and causes vesiculation and swelling of the Golgi complex. Bar, 10-μm.

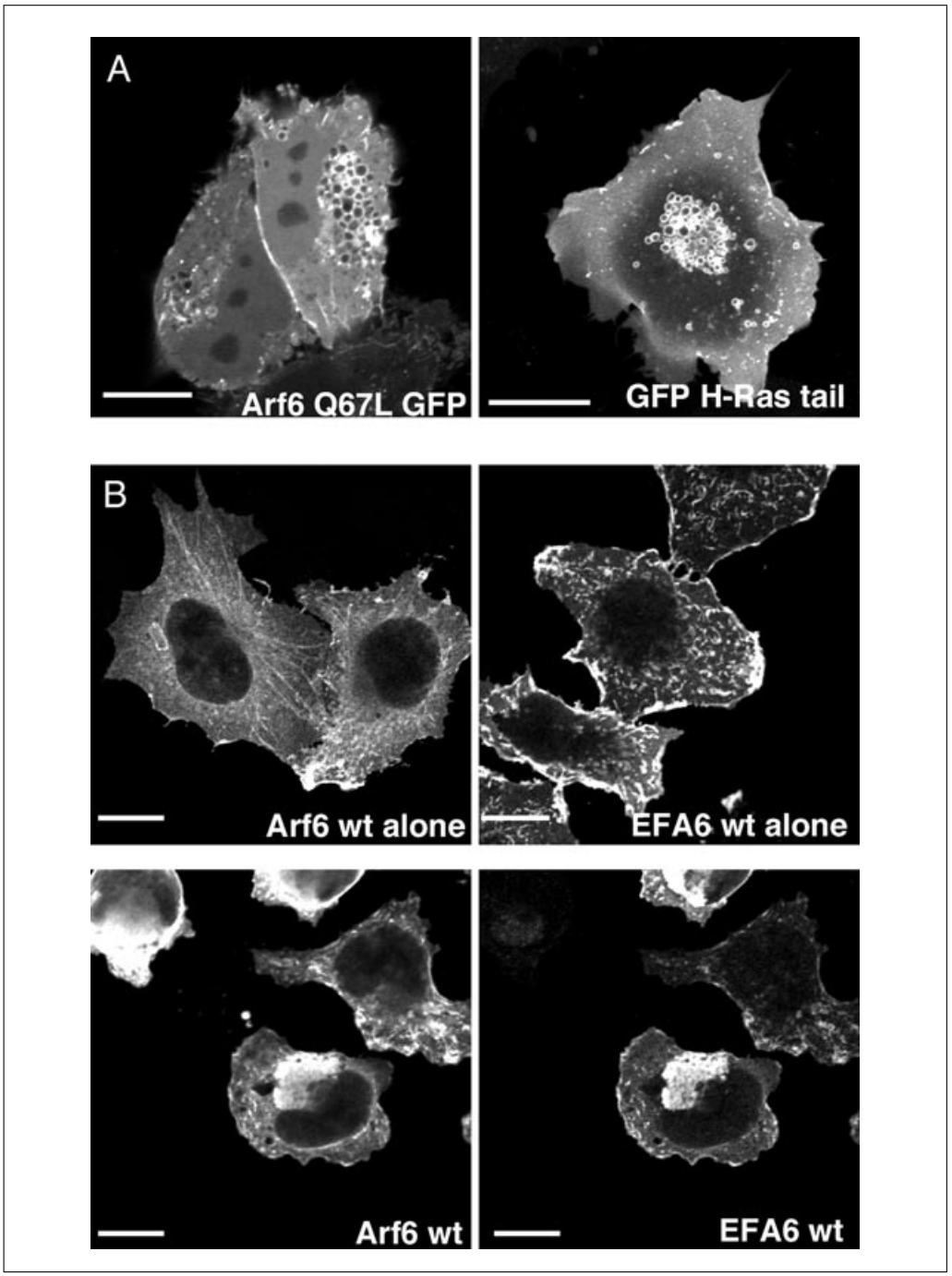


Figure 14.12.2 (**A**) Expression of Arf6-Q67L in cells induces the formation of distinctive vacuoles. The left image shows a HeLa cell expressing Arf6-Q67L-GFP, which causes accumulation of large vacuolar structures. The right image shows a COS-7 cell co-expressing Arf6 Q67L (not shown) and the carboxyl terminal sequence of H-Ras fused to GFP. The H-Ras-tail-GFP highlights the Q67L vacuole clearly. (**B**) Expression of Arf6 and EFA6 in cells promotes a phenotype similar to Arf6 Q67L. Arf6 wild-type localizes on tubular endosomes and at the plasma membrane in HeLa cells. Its GEF, EFA6, is localized mostly at the plasma membrane and causes cell ruffling. Co-expression of Arf6 and EFA6 induces the formation of vacuoles similar to expression of Arf6 Q67L. Bar, 10 μm.

expression of Arf1Q71L leads to the stable association of coat proteins with the Golgi complex causing vesiculation and swelling of Golgi cisternae (see Fig. 14.12.1), which inhibits transport through the secretory pathway. Expression of the Arf6Q67L mutant promotes cellular protrusions and also stimulates endocytosis and leads to the accumulation of endosomal membranes (see Fig. 14.12.2). This is often accompanied by a reduction of the cell footprint, especially with high levels of expression and at longer times after expression. The association of Arf6 Q67L with these membranes is difficult to discern in fixed preparations, and often it is necessary to label the endosomal structures with a marker that retains strong membrane association. The vacuole phenotype is strongest in the non-tagged version of Arf6Q67L, followed by Arf6Q67L-HA, and Arf6Q67L-GFP. Length and level of expression is also an important variable in experiments using Arf6Q67L. Cells tend to round up after long or high-level expression of this mutant.

LOCALIZATION AND FUNCTION OF SPECIFIC Arf PROTEINS IN CELLS

BASIC PROTOCOL 1

This protocol describes how to evaluate Arf function in HeLa cells by exogenous expression of wild-type and mutant Arf proteins in cells. It can easily be adapted to other systems.

Materials

1 M HCl (*APPENDIX 2A*)
70% ethanol
Culture medium: DMEM containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution (*APPENDIX 2A*)
Cells (e.g., HeLa)
Mammalian expression plasmid containing Arf construct of interest with an epitope tag (e.g., Fugene, Roche)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Drugs, growth factors, etc.
2% (w/v) formaldehyde solution in PBS
PBS/FBS: 10% (v/v) FBS in PBS and 0.02% (w/v) sodium azide
Primary antibody against the epitope tag (e.g., for an HAtag, etc.)
10% (v/v) saponin
Secondary antibody: Alexa Fluor 488 or 594 conjugated against the species of the primary antibody
Fluoromount G
Clear nail polish

12-mm no. 1 round, glass coverslips
65° to 75°C heating block/plate
100-mm and 15-cm tissue culture dishes
Watchmaker forceps
6- and 12-well tissue culture plates
Parafilm
1.2-mm thick glass slides
Small Kimwipes

Prepare cells and coverslips

1. Acid wash coverslips by incubating them in 1 M HCl for 6 to 8 hr at 65° to 75°C. Rinse coverslips four times in distilled water, and three times in 70% ethanol. Store coverslips in 70% ethanol at room temperature.

   CAUTION: *Appropriate care should be taken when heating 1 M HCl as it is corrosive. Coverslips should be acid washed in a glass beaker under a chemical fume hood.*

Acid washing coverslips helps to provide a good surface for cells to adhere to, and helps ensure reproducibility between different batches of coverslips. Coverslips can be treated in large batches in advance and stored in 70% ethanol until needed. Specific cell lines may require different coverslip treatment.

2. In a tissue culture hood, place 10 ml of prewarmed culture medium into a 100-mm tissue culture dish.

3. Remove several coverslips from 70% ethanol with watchmaker forceps, and carefully flame to sterilize and remove ethanol. Place coverslips in tissue culture dish, ensuring that they are carefully separated from one another. Gently push each coverslip down onto the bottom of the culture dish to help prevent them from moving around. Continue until the desired number of coverslips are in the dish (up to 25 coverslips can fit in onedish).

4. Remove medium from coverslips and replace with 10 ml of fresh culture medium to remove any residual ethanol.

Plate cells

5. Plate 0.5–0.75 $\times$ 10^6 cells into the dish with coverslips and incubate in a 37°C, 5% CO_2 incubator.

   Cells should be plated to be 40% to 60% confluent the day after plating. If cells are less crowded, the cells often do not transfect well. If they are more crowded, it is often hard to clearly distinguish subcellular structures by immunofluorescence. This condition could vary with different cell lines. Generally, any adherent cell lines able to be transfected can be plated and used in these experiments.

6. On the following day when cells are 40% to 60% confluent, place 2 ml of culture medium into each well of a 6-well plate. Place one to four coverslips into each well.

7. Transfect cells with 1 μg DNA for all Arf constructs except Arf6 Q67L. Transfect 0.25 μg Arf6 Q67L constructs to avoid toxicity.

   The authors routinely use Fugene from Roche to transfect HeLa cells, and obtain 70% transfection efficiency for most constructs.

8. Incubate cells for 18 hr at 37°C to allow expression of proteins from plasmids.

   Expression of Arf6Q67L causes cells to round up. This is exacerbated by high levels of expression; therefore, often a smaller amount of DNA is used and transfection is carried out for shorter times (12 hr rather than 18 hr) to induce a milder phenotype. Other Arf mutants may also have toxicity issues under different conditions and both level and length of expression may have to be varied similarly.

Fix cells (also see UNIT 4.3)

9. After the transfection period, remove medium and gently rinse cells with 2 ml of PBS, and then replace with 2 ml of culture medium.

10. Treat cells can with drugs, growth factors, etc. as desired and incubate at 37°C.

11. Fix cells by placing one coverslip each into 1 ml of 2% formaldehyde in a 12-well plate. Incubate for 10 min at room temperature.

12. Remove formaldehyde and rinse with 1 ml of PBS and then with 1 ml of PBS/FBS. Incubate for 1 hr at room temperature.

    The PBS/FBS solution is used to inhibit non-specific binding of antibodies to the cells; other blocking solutions can be used containing other proteins, e.g., BSA or gelatin. If the entire immunofluorescence protocol cannot be completed in 1 day, this is an appropriate place to stop. Cells can be stored in PBS/FBS overnight at 4°C.

Stain cells to detect Arf protein

13. Dilute primary antibody to the epitope tag of the Arf used in PBS/FBS with 0.2% saponin.

    Typically, epitope tagged antibodies are diluted to 1 to 2 μg/ml.

14. Place a piece of Parafilm in a 15-cm tissue culture dish. Place a 30-μl drop of the primary antibody onto the Parafilm. Invert coverslip (cells facing down) over the drop. Place twelve coverslips, corresponding to the 12-well plate, into each 15-cm dish. Incubate for 1 hr at room temperature.

    If using GFP-tagged proteins, the fluorescence can be observed directly or the fluorescent signal can be enhanced with an antibody against GFP, followed by Alexa 488-conjugated secondary antibodies. Because Arf1 is discretely localized to the Golgi it is often easily detected by fluorescence alone.

    To help evaluate how expression of Arf proteins affects the system under study, it is often useful to co-stain the cells with antibodies to specific cellular components. The Golgi structure can be monitored by staining with antibodies to Golgi resident proteins (e.g., GM130). Arf6-associated membrane structures can be stained with antibodies directed against endosomal protein markers such as clathrin-independent endosomal cargo proteins (Eyster et al., 2009), transferrin receptor, or co-expression of a GFP-tagged membrane marker like carboxyl terminal sequence of H-Ras (see Fig. 14.12.2).

15. Place coverslips (cells facing up) back into 1 ml PBS/FBS in a 12-well plate. Wash coverslips three times, 5 min each time, with PBS/FBS.

16. Dilute appropriate fluorescently-conjugated secondary antibodies in PBS/FBS with 0.2% saponin. Place a 30-μl drop on a piece of Parafilm in a 15-cm culture dish. Invert coverslip onto drop. Incubate for 30 min at room temperature.

    The authors typically use Alexa dye–conjugated secondary antibodies such as 488, 594, and 633.

17. Place coverslips back into the 12-well plate, cells facing up. Wash two times with 1 ml PBS/FBS and once in PBS, incubating 5 min for each wash.

18. Place a drop of glycerol-based mounting fluid such as Fluoromount G into the middle of a 1.2-mm thick glass slide. Invert coverslip on top of drop (cells facing down). Gently press down on the coverslip using three to four Kimwipes to remove excess mounting solution.

19. Allow coverslips to air-dry for 5 min and then seal coverslips around edges with clear nail polish.

20. Allow nail polish to dry (~10 min) prior to examination of slides.

    Slides can be stored in covered boxes at least 1 month at 4°C.

ASSESSMENT OF GTP-BOUND Arfs

BASIC PROTOCOL 2

In this protocol, a pull-down assay is described using a domain of an Arf-GTP interacting protein to assess the amount of GTP-bound Arf. This is a variation of the protocol first described by Santy (Santy and Casanova, 2001). Gamma-adaptin ear domain homology art binding domain (GGAs) are a family of Golgi-associated coat proteins that bind to GTP-bound Arf. The domain that binds to Arf-GTP is restricted to what is called the GGA and TOM domain (GAT) (Boman et al., 2000; Dell'Angelica et al., 2000). In vitro, this region binds to Arf-GTP with high affinity, with little binding to Arf-GDP. In addition, like many Arf effectors, GGA can bind all Arf isoforms. This protocol uses the purified recombinant VHS-GAT domains of GGA3 fused to GST to probe cellular lysates for GTP-bound Arf. The VHS-GAT domain binds to active Arf, and thus can provide an

estimate of the percentage of Arf in the cell that is active. This protocol describes using this pull-down to look at activation of endogenous Arfs. The Alternate Protocol describes an adaptation of this protocol for assessing the activation of transfected Arf proteins and the effect of co-expression of regulators.

Materials

Frozen bacterial stock for GST-VHS-GAT expression (Dell'Angelica et al., 2000)
LB/Amp (LB plus 100 μg/ml ampicillin; *APPENDIX 2A*) plates and medium
1 M isopropyl-beta-D-thiogalactopyranoside (IPTG)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice cold
2 mM EDTA
Lysozyme (lyophilized powder)
Protease inhibitors (e.g., 1 mg/ml pepstatin, 1 mM leupeptin, 5 mg/ml aproptinin, and 1 mM PMSF)
10% (v/v) Triton X-100 stock
10 U/μl DNase I
1 mg/ml RNase stock
1 M dithiothreitol (DTT; *APPENDIX 2A*)
Glutathione Sepharose 4B beads (GE Life Sciences)
Lysis buffer (see recipe)
5× SDS-PAGE sample buffer (*APPENDIX 2A*)
2× sample buffer without reducing agent
12% polyacrylamide gel or 4% to 20% gradient gel
Specific antibodies for detecting Arf proteins in immunoblots

37°C incubator
14-ml round-bottom culture tubes
Spectrophotometer
Refrigerated centrifuge
Tube rotator, 4°C
10-cm tissue culture dish
Cell scraper
1.7-ml microcentrifuge tubes
Refrigerated microcentrifuge
Microcentrifuge spin columns (e.g., Pierce Spin Cups with cellulose acetate filters)
Nitrocellulose paper

Additional reagents and equipment for SDS-PAGE gel (*UNIT 6.1*) and nitrocellulose transfer (*UNIT 6.2*)

Express GST-VHS GAT from GGA3

1. From a frozen bacterial stock, streak out GST-VHS GAT–bearing bacteria on an LB/Amp plate to obtain single colonies after growth overnight at 37°C.
2. Inoculate one colony into a 2-ml culture of LB/Amp in 14-ml tube and grow overnight at 37°C with shaking.
3. On the following morning, inoculate a 100-ml culture of LB/Amp with 100 μl of overnight culture in a 500-ml sterile flask and place in 37°C shaking incubator.
4. When the OD_{600} of the culture reaches 0.8 to 1.0 (about 3 hr), add 500 μM IPTG from a 1 M stock. Grow for 3 hr at 37°C.
5. Transfer culture to centrifuge bottles and centrifuge 10 min at 10,000 × g, 4°C, remove culture medium, and freeze pellet at −80°C.

   Pellet can be kept at −80°C for 2 to 5 days if necessary.

Purify GST-VHS-GAT

6. Thaw bacterial pellet on ice.

   This and subsequent steps are used to purify GST-VHS-GAT. Other protocols for purifying GST fusion proteins will also work.

7. Resuspend in 20 ml ice-cold PBS containing 2 mM EDTA, 1 mg/ml lysozyme, plus protease inhibitors.

8. Incubate for 30 min on ice.

9. Add 0.4 ml of 10% Triton-X 100 stock (for final concentration of 0.2%).

10. Add 30 μl DNase I (10 U/μl stock), and 70 μl of a 1 mg/ml solution of RNase.

11. Incubate 10 min in a cold room with tube rotation.

12. Add DTT to a final concentration of 1 mM (20 μl of 1 M stock).

13. Centrifuge 30 min at × *g* (10,000 rpm in a Sorvall SS-34), 4°C.

    For convenience, the resultant supernatant (lysate) may be aliquoted into single-use aliquots (5-ml) and frozen in liquid N_2 and stored at −80°C. It is best to avoid repeated rounds of freeze/thaw at this point.

14. Wash 250 μl of glutathione Sepharose beads once with 1 ml of 0.2% Triton X-100 in PBS, centrifuge beads ∼15 sec and remove supernatant.

15. Incubate glutathione Sepharose with 5 ml of bacterial lysate for 30 min at 4°C.

16. Wash beads three times with 1 ml of 0.2% Triton X-100 in PBS and then once with 1 ml lysis buffer. After each wash, centrifuge beads for 35 sec at 13,000 × *g* in a microcentrifuge. Resuspend beads at a 1:1 ratio of beads to lysis buffer.

    It can be useful to verify that 30 μl of the 1:1 suspension contains 50 to 100 mg of GST-VHS-GAT. This can be done by running a 30-μl sample on an SDS-PAGE gel with appropriate BSA standards and Coomassie gel staining (UNITS 6.1 & 6.6). The GST-VHS-GAT fusion is ∼40 kDa, and should be the major band. Adjust the amount of beads used in the assay if necessary.

Prepare cell lysates

17. Plate 0.75–1.0 × 10^6 cells in 10 ml culture medium in a 10-cm tissue culture dish, and incubate overnight at 37°C.

    The amount of cells used in this experiment will vary depending on what is being examined, the cell type used, the amount of Arf expressed, and the ability to detect an Arf by immunoblotting (UNIT 6.2). This protocol is based on looking for endogenous Arf1 or Arf6 in one 10-cm tissue culture dish that is 70% to 80% confluent. If having trouble detecting the endogenous Arf by immunoblotting, more cells may be needed or adjustments must be made to the protocol. An alternative approach is to transiently express an epitope-tagged Arf in cells to perform this assay. Specific considerations for this approach are outlined in the Alternate Protocol.

18. Treat cells according to experimental design. For example, treat cells with hepatocyte growth factor, which can activate Arf6 in some cells (D'Souza-Schorey and Chavrier, 2006).

19. Wash cells once in 10 ml ice-cold PBS. Carefully remove all excess liquid.

20. Add 250 μl lysis buffer containing protease inhibitors, and use a cell scraper to collect cells.

The amount of lysis buffer used will vary depending on the level of expression of endogenous Arf in the specific cell line used, but generally it has been found that the lysates should be kept as concentrated as possible so that the protein in the loading control is easily detected. To obtain a reliable result, it is very important that clear detection of the endogenous Arf in the whole cell lysate fraction is seen.

21. Place lysate into a 1.7-ml microcentrifuge tube, and microcentrifuge 5 min at 13,000 × *g*, 4°C.

22. Place 20 µl of lysate in 5 µl of 5× sample buffer with reducing agent and heat sample 10 min at 95°C.

Bind Arf to beads

23. Incubate 200 µl of lysate with 30 µl glutathione Sepharose beads bound with GST-VHS-GAT for 30 min on a rotating rack at 4°C.

24. Microcentrifuge beads 30 sec at 6000 × *g*, 4°C, and remove supernatant.

25. Resuspend beads in 0.75 ml lysis buffer and transfer to a microcentrifuge spin column.

26. Wash beads two times in 0.75 ml of lysis buffer at 4°C. Microcentrifuge columns 30 min at 6000 × *g*, 4°C, removing wash from collection tubes with each spin.

27. Place columns in fresh collection tubes and add 25 µl of 2× sample buffer without reducing agent, vortex briefly to mix buffer and beads, and incubate 10 min at room temperature.

28. Microcentrifuge column 1 min at 13,000 × *g*, room temperature, to collect sample.

29. Add 5 µl of 1 M DTT to sample, and boil sample for 5 min (this is the pull-down sample).

Analyze samples

30. Run the whole-cell lysate sample (from step 22) and pull-down sample (from step 29) on an SDS-PAGE gel (either 13% or a 4% to 20% Tris·Cl gradient mini gel; *UNIT 6.1*) and transfer to nitrocellulose paper (*UNIT 6.2*).

    It is useful to run a pre-stained molecular weight marker along with the samples. To obtain good resolution of Arfs, which are ~20 kDa, it is often necessary to run the dye front off the gel, and run the gel until a marker, ~5 kDa, is close to the bottom of the mini gel.

31. Conduct immunoblot using Arf-specific antibodies.

    Of commercially available antibodies, the authors have experience using the Arf1/3 antibody produced in sheep from Sigma (A4594), which can be used at 1:1000 for immunoblotting, and the Arf6 (3A-1) mouse IgG_{2b} antibody from Santa Cruz Biotechnology that can be used at 1:100.

    It is important to verify the specificity of the antibody that is being used. One convenient way to do this is to transiently express different epitope-tagged Arf isoforms and make individual cell lysates. However, be aware that the tag at the carboxyl terminus sometimes may interfere with recognition of an Arf, particularly if the epitope from the Arf is at the extreme carboxyl terminus.

32. Quantify the intensity of the bands to determine the fraction of total Arf that is GTP bound.

    In this scenario that would be the (intensity of the band from the pull down)/(10× the intensity of the whole cell lysate band).

ASSESSMENT OF Arf GTP LEVELS IN TRANSFECTED CELLS

ALTERNATE PROTOCOL

Reliable and reproducible detection of endogenous Arf proteins can be difficult, making interpretation of the pull-down assay an issue. Transient transfection of low levels of epitope-tagged wild-type Arf proteins is an alternative approach. This approach allows one to directly compare activation of different Arf isoforms using the same antibody. It can also be used to look at the effect of expression of regulatory proteins (such as GEFs and GAPs) on Arf isoforms. This protocol is a modification of Basic Protocol 2.

***Additional Materials** (also see Basic Protocol 2)*

Mammalian expression plasmids containing epitope-tagged wild type (and mutant) Arf proteins (epitope tag on carboxyl terminus)
6-well culture plates

Prepare GST-VHS GAT fusion protein

1. Follow Basic Protocol 2, steps 1 through 16.

Prepare cell lysates

2. Plate 1.0×10^5 HeLa cells into each well of a 6-well plate and incubate in a 37°C, 5% CO_2 incubator.

3. On the following day when the cells are ~50% to 60% confluent, transfect the cells with relevant plasmids.

   It is important to have a control lane in which the wild-type Arf alone is expressed, and one in which Arf and a regulator (GEFs or GAPs) is expressed. Expression of a GEF should increase Arf-GTP and a GAP should decrease Arf-GTP levels. This allows one to see a fold change in GTP-bound Arf upon expression of the regulator.

   Generally, this has worked well when transfecting 0.25 μg of the wild-type Arf, and 1.0 μg of the regulator. It is important that most of the cells transfected with the Arf also contain the regulator. To monitor whether this is happening, include one coverslip in the plate and assess the efficiency of co-transfection by immunofluorescence.

   If comparison of the specificity of a regulator for one or another Arf is desired, express Arfs with the same eptitope tag and use the same antibody to conduct the immunoblot.

4. On the following day, remove medium and add 10 ml ice-cold PBS at 4°C.

5. Carefully aspirate all of the PBS from the dish.

6. Lyse cells directly on the plate in 100 μl of lysis buffer at 4°C. Scrape cells off dish with cell scraper and collect lysate.

7. Place lysate into a 1.7-ml microcentrifuge tube, and microcentrifuge 5 min at 13,000 × *g*, 4°C.

8. Place 25 μl of lysate in microcentrifuge tube containing 5 μl of 4× sample buffer with reducing agent and heat 10 min at 95°C.

Collect Arf

9. Dilute 50 μl of lysate into 0.4 ml lysis buffer, and incubate with 30 μl glutathione beads bound with GST-VHS-GAT for 30 min on a rotating rack at 4°C.

10. Microcentrifuge beads 30 sec at 6000 × *g*, 4°C, and remove supernatant.

11. Resuspend beads in 0.75 ml of lysis buffer and transfer to a microcentrifuge spin column.

12. Wash beads two times in 0.75 ml of lysis buffer at 4°C. Microcentrifuge columns 30 sec at 6000 × *g*, 4°C, removing wash from collection tubes with each spin.

13. Place columns in fresh collection tubes and add 25 µl of 2× sample buffer without reducing agent, vortex briefly to mix buffer and beads, and incubate 10 min at room temperature.

14. Microcentrifuge column 60 sec at 13,000 × *g*, room temperature and collect sample.

15. Add 5 µl of 1 M DTT to sample, and boil sample for 5 min (this is the pull-down sample).

Analyze samples

16. Run whole-cell lysate sample (from step 8) and pull-down sample (from step 15) on an SDS-PAGE gel (either 13% or a 4% to 20% Tris·Cl gradient mini gel; *UNIT 6.1*) and transfer to nitrocellulose paper (*UNIT 6.2*).

17. Quantify the intensity of the Arf bands and calculate the fraction of Arf that is GTP bound.

    In this scenario that would be the (intensity of the band from the pull down)/(2 × the intensity of the whole-cell lysate band).

BASIC PROTOCOL 3

ASSESSMENT OF SPECIFICITY AND FUNCTION OF Arf GEFS AND GAPS

In this section strategies for evaluating the function and specificity of Arf GEFs and GAPs in cells are described. Identifying which isoform of Arf is the target of a particular GEF or GAP in cells is a challenge. While cell-free, biochemical assays often point to a preferred substrate, these assays cannot recreate the cellular environment. Furthermore, since GEFs and GAPs have catalytic activity, the expression of these regulators can reveal distinct cellular phenotypes due to activation or inactivation of particular Arf proteins.

Strategies for GEFs

The localization of endogenous GEFs is generally difficult due to the low abundance of these proteins and the lack of specific antibodies. Thus, it is advantageous to transiently over-express epitope-tagged GEFs in cells, and look at them by immunofluorescence, modifying Basic Protocol 1. The localization of the GEF can give some information about the likely specificity of the GEF. If it is localized to the Golgi, then it probably is not using Arf6 as a substrate. However, if it is localized in the cell periphery, then it cannot be assumed that it is acting on Arf6, as other Arfs may be recruited to extra-Golgi sites.

A second important consideration is whether expression of a GEF affects the localization of an Arf protein. This can be addressed by co-expressing the GEF with wild-type Arf proteins and conducting indirect immunofluorescence similar to what is outlined in Basic Protocol 1. A GEF that uses a particular Arf might co-localize with that Arf, but sometimes might change the normal distribution of the Arf. For example, co-expression of Arf1 with the Arf exchange factor ARNO can recruit Arf1 to the plasma membrane where it co-localizes with ARNO. Furthermore, a GEF would be expected to bind to and co-localize with the dominant negative mutant of the Arf it activates.

Unlike over-expression of the wild-type Arf, over-expression of the wild-type GEF sometimes creates an obvious phenotype. For instance, over-expression of the Arf6 exchange factor, EFA6A, induces plasma membrane ruffling and macropinocytosis (Brown et al., 2001), not observed in cells expressing Arf6 alone (see Fig. 14.12.2). All Arf GEFs contain a conserved glutamic acid residue within their sec7 domain that is critical to their activity as exchange factors. When this residue is mutated to lysine (E156K in ARNO or E794K in GBF1), the exchange factor is unable to activate an Arf. In some cases, these mutants have also been reported to act like dominant negatives, and inhibit a process that Arf is involved in. Over-expression of a BFA-sensitive Arf GEF can sometimes provide

protection against the effects of BFA treatment by shifting the dose-response curve to the drug, and prevent or slow loss of its substrate Arf from Golgi membranes.

Co-expression of an Arf GEF with its substrate Arf often mimics the phenotype observed for the constitutively active mutants of the Arf. For example, co-expression of Arf6 with its GEF, EFA6A, can promote a vacuolar phenotype that is similar to expression of Arf6Q67L (see Fig. 14.12.2). Finally, looking at the effect the GEF has on activation of specific Arf proteins can be assessed by using the VHS-GAT pull-down assay described in Basic Protocol 2. Comparing how different Arf proteins respond to a particular GEF when co-expressed in cells may provide some insight into specificity issues.

Strategies for GAPs

Since GAPs inactivate Arf proteins, their expression often antagonizes Arf function. The localization of an Arf GAP can give information about its specificity. If it is localized to the Golgi, then it is probably using a Golgi Arf (Arf 1–5) as a substrate. However, Arf GAPs that localize outside of the Golgi may still work on Arf 1–5 as a substrate since other Arfs can be recruited to extra-Golgi sites. In some cases, there may be antibodies available to look at the localization of endogenous ArfGAPs; however, exogenous expression of epitope-tagged GAPs can be readily performed using Basic Protocol 1.

Just like Arf GEFs, expression of Arf GAPs sometimes exhibits a distinct cellular phenotype. In this case however, an Arf GAP would be expected to mimic the Arf dominant negative and antagonize Arf function. For example, over-expression of ArfGAP1 leads to loss of the COP I coat from the Golgi and dissolution of the Golgi similar to that induced by expression of Arf1 T31N (Aoe et al., 1997). In some cases, co-expression of a GAP with the Arf substrate can ameliorate the phenotype of expression of the GAP by itself. The Arf GAP would also be expected to co-localize with the constitutively active mutant of its Arf substrate. In the VHS-GAT pull-down, co-expression of the GAP with its substrate should lead to a reduction in the amount of Arf that is GTP bound.

Inactive mutants for Arf GAPs have also been described. If expression of the wild-type GAP causes a phenotype, then expression of the inactive GAP should not cause that phenotype, if Arf activity is important in producing the phenotype. GAPs are often large proteins with many other protein domains that are important to their biological activity. Some GAPs have been reported to have phenotypes that are independent of their ability to activate Arf (Inoue and Randazzo, 2007). For example, over-expression of ASAP1, a peripheral Arf1 GAP, can induce podosome formation, but the ASAP1 mutant that lacks GAP activity (ASAP1-R495K) can also induce podosome formation suggesting that the GAP activity is not critical to this aspect of ASAP1's function (Bharti et al., 2007). For this reason, it is very important to determine whether the GEF or GAP activity of the regulator is important to the biological function being examined.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Lysis buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 mM NaCl (*APPENDIX 2A*)
2 mM $MgCl_2$ (*APPENDIX 2A*)
1% (v/v) Triton X-100 (*APPENDIX 2A*)
10% (v/v) glycerol
Store up to 1 year at 4°C

COMMENTARY

Background Information

Cell biologists have gained a great deal of information about the functions of Arf proteins in cells using transient expression of wild-type and mutant forms of the GTPase. The ability to examine phenotypes in cells expressing constitutively active and dominant negative forms of Arfs has been an effective tool for figuring out the function of Arf1 at the Golgi complex and Arf6 at the plasma membrane (Donaldson and Jackson, 2000; D'Souza-Schorey and Chavrier, 2006). This experimental tool has also been used for discovering functions of the numerous Rab proteins, which also affect membrane traffic in cells. Although there are some concerns about indirect effects of expression of some of these mutants in cells (see below), a careful evaluation of observed localization and phenotypes for expression of wild-type and mutant forms of Arfs should ensure that a reasonable understanding of Arf function can be revealed.

Studies of Arf proteins have benefited greatly from the identification of the many Arf GEFs and GAPs in the genome (Casanova, 2007; Inoue and Randazzo, 2007). By contrast, few GEFs and GAPs have been identified for the Rab GTPases. Although analysis of the function of these regulatory molecules can in themselves be complex, their anticipated actions in terms of turning on and off a particular Arf can complement the analysis of the effect of expressing the corresponding Arf-GDP versus Arf-GTP mutant. Results from studies on transfected cells expressing specific Arfs, GEFs, and GAPs should complement cell-free biochemical assays using recombinant proteins where GTP binding and hydrolysis can be measured directly.

The use of the GGA-VHS-GAT pull-down assay to determine the fraction of Arf in the GTP-bound state has been widely used to demonstrate Arf activation and inactivation in response to a physiological signal. For Arf6, EGF treatment results in an increase in Arf6-GTP (Morishige et al., 2008) whereas platelet activation leads to an immediate drop in Arf6-GTP (Choi et al., 2006). These changes in the Arf6 GTP/GDP state can be due to either stimulation or inhibition of actions of the GEFs and GAPs. The Arf investigator thus has many tools with which to investigate these effects.

In addition to the expression of mutant and wild-type Arfs, GEFs, and GAPs, another approach is to deplete cells of these proteins using siRNA protocols. These studies have many challenges and require good immunological reagents to determine the extent of depletion of the protein; however, they offer the possibility of rescuing a given phenotype by expression of an siRNA-resistant, wild-type or mutants of the protein. In addition to the classical dominant negative and constitutively active mutants of Arf proteins, other mutations have been described including effector domain mutants in Arf1 (N52R) and Arf6 (N48R) that are defective in stimulating phospholipase D (PLD), but are normal with respect to other activities (Skippen et al., 2002).

Critical Parameters

In attempting to investigate potential Arf function in a physiological process, it is important to gather reliable reagents to undertake these studies. For expression of Arfs, GEFs, and GAPs, vectors encoding these proteins for expression in mammalian cells can usually be obtained from active investigators in the field. Depending on whether antibodies are available to detect these proteins, epitope tagging of these constructs is often desirable so that commercial, anti-epitope antibodies can be used.

The choice of cell type for these studies is usually dictated by the particular biological process under study. If the process is apparent in all cells, the standard use of HeLa or Cos cells will ensure high efficiency of transfection and ease of analysis. More specialized cell types may, for example, require alternative transfection strategies. The amount of plasmid DNA to use in the transfection and also the time of transfection may also vary with the cell type and type of protein being expressed. Pilot experiments to maximize expression of protein are advisable. It is generally helpful to examine first the localization and cellular phenotype of expression of the Arfs, GEFs, and GAPs in cells by immunofluorescence prior to looking specifically at whether a particular GEF or GAP or physiological stimulus affects Arf-GTP levels in the pull-down assay. Looking at the whole cell can also alert the investigator to toxic or secondary, indirect effects. For example, when examining effects of Arf1T31N on an endosomal compartment, one should examine whether in these cells the Golgi complex is still functional. Since Arf1T31N often disassembles the Golgi complex and blocks the secretory pathway, the effect observed on the endosomal compartment could reflect a requirement for normal secretory pathway traffic. Another consideration when examining

specific Arf function in cells is possible redundancy among Arf proteins in the biological processes under study. From siRNA depletion studies, there is evidence that Arf proteins may act in pairs at particular locations (Volpicelli-Daley et al., 2005).

Troubleshooting

Described here are common problems encountered when conducting these types of experiments.

No immunoflourescence signal for epitope-tagged Arf

Check to see that a protein with the same epitope tag, which previously has been known to express fluorescence under the experimental conditions, is detectable. If no fluorescence signal is detected, it may be a more general problem in the transfection protocol, or a general problem detecting the epitope tag by immunofluorescence. If fluorescence is detected from the test protein, it suggests a specific problem expressing or detecting the Arf protein of interest. Check the quality of the plasmid DNA source, as poor quality plasmid DNA might interfere with expression. Consider using an anti-GFP antibody to boost the fluorescence signal if visualizing a GFP-fusion protein.

Vary the amount of DNA used, and the time of expression, making it either shorter or longer. Arf mutants can have toxic effects on cells, particularly when expressed for a longer period of time, and some cells may be more sensitive to these effects than others. Observing a lot of floating cells after the transfection might indicate a toxicity problem. Shortening the time of expression and decreasing the amount of DNA used may help with toxicity issues.

Low co-transfection efficiency

Sometimes Arfs or their mutants are refractory to being co-expressed with other proteins. Try varying the ratio of the Arf to the other protein of interest, and shorten the length of time of expression. Consider the experimental design when varying the ratio. For example, when using the GAT assay to look at the effect of a protein on Arf's nucleotide status, it is critical that most of the Arf is expressed in cells with the protein of interest. Therefore, the ratio should be biased to favor expression of the protein of interest. Occasionally, this is hard to overcome, but be aware that often the problem is co-transfecting the protein with a specific mutant (constitutively active or dominant negative), so information may be obtained by using the wild-type or a different mutant.

Little or no Arf GTP signal in VHS-GAT pulldown assay

Verify that the GST fusion protein is present by staining nitrocellulose membrane with Ponceau S. A prominent band at ~40 kDa should be apparent. There might also be some degradation products of the GST fusion protein present, but as long as the 40-kDa band in abundant, it should not interfere with the assay. If there is no 40-kDa band present, there is a problem with the purification or expression of the GST-fusion protein.

Verify that the GST-fusion protein can precipitate constitutively active Arf. If it cannot, there is a problem with the fusion protein or the assay. Check to see that the fusion protein is intact. For this assay to work well, it is also important once the cells containing the Arf are lysed that the assay is completed in a timely fashion (2 to 3 hr), and kept cold. If the GST-VHS GAT beads can precipitate constitutively active Arf, it suggests the fusion protein and assay are working correctly. Consider increasing the amount of lysate used in the pull-down.

Reproducibility of the VHS-GAT pull-down assay

Results for the GAT assay need to be verified by repetition of the experiment. If results are variable from one experiment to another, it suggests a problem in the assay. Consider monitoring the ability of the VHS-GAT-GST fusion to distinguish between the constitutively active and dominant negative mutants. If the constitutively active and dominant negative mutants are pulled down in the assay in similar amounts, try loading less lysate for the pull-down, or increasing the number of washing steps.

It is critical to be able to detect the Arf of interest in the whole-cell lysate. A very weak signal could make it difficult to quantify band intensity reliably, and this could add a large amount of error into the final calculation. Optimize the conditions for the immunoblot. Consider increasing the amount of total protein used in the whole-cell lysate sample. One way to do this is to reduce the volume of total lysis buffer used to make the initial sample more concentrated, or to use more cells and the same amount of lysis buffer. Although the ultimate measure in this assay is the percentage of Arf that is GTP bound, and slight differences for the level of Arf expression in the whole-cell lysate will be accounted for in this calculation,

a large difference may artificially bias the results. If protein loading between samples in the whole-cell lysate is uneven, consider quantifying total protein concentration using a Bradford or BCA assay, and correcting for differences. Sometimes when co-expressing an Arf and a regulator, the amount of Arf expressed decreases. Consider using less of the regulator.

Anticipated Results

This unit is meant to help investigators evaluate whether an Arf is involved in a biological process under study by providing basic tools and strategies to investigate Arf protein function. Investigators should be able to examine whether an Arf localizes on cellular structures of interest, or co-localizes with proteins of interest. They can also evaluate whether a particular biological process is affected by expression of dominant negative or constitutively active mutants of Arfs. They can evaluate whether an Arf is activated or inactivated during a particular treatment or process. Finally, they can address how an Arf might be regulated in their system by addressing GEF and GAP function using wild-type and mutant proteins.

Time Considerations

For localization and function of Arf proteins in cells (see Basic Protocol 1), cells are plated on day 1, transfected on day 2, and an experiment performed 12 to 24 hr after the start of transfection. After fixing the cells in formaldehyde and replacing the formaldehyde with a blocking solution, the cells may be placed for up to 24 hr at 4°C. After fixation, the immunofluorescence staining should take ~4 hr to complete.

For assessment of GTP-bound Arfs (see Basic Protocol 2), a bacterial lysate of the GST-VHS-GAT protein can be made in advance, and stored at −80°C. Allow 2 days for growth of the bacteria, and 2 hr for making the bacterial lysate. Purification of the GST-VHS-GAT fusion will take ~1 hr. The purification of the GST-VHS-GAT fusion protein should be done on the same day as the preparation of cell lysates. For preparation of cell lysates, cells are plated on day 1. The experiment is either performed on day 2, or the cells are transfected on day 2, and the experiment is performed 12 to 24 hr after transfection. It will take ~3 hr to prepare cell lyates and the pull-down portion of this experiment. Once boiled, samples can be stored at −20°C. Allow 2 additional days to finish the experiment: to run and transfer SDS-PAGE gels, and conduct the immunoblot.

Literature Cited

Aoe, T., Cukierman, E., Lee, A., Cassel, D., Peters, P.J., and Hsu, V.W. 1997. The KDEL receptor, ERD2, regulates intracellular traffic by recruiting a GTPase-activating protein for ARF1. *EMBO J.* 16:7305-7316.

Bharti, S., Inoue, H., Bharti, K., Hirsch, D.S., Nie, Z., Yoon, H.Y., Artym, V., Yamada, K.M., Mueller, S.C., Barr, V.A., and Randazzo, P.A. 2007. Src-dependent phosphorylation of ASAP1 regulates podosomes. *Mol. Cell Biol.* 27:8271-8283.

Boman, A.L., Zhang, C., Zhu, X., and Kahn, R.A. 2000. A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi. *Mol. Biol. Cell* 11:1241-1255.

Brown, F.D., Rozelle, A.L., Yin, H.L., Balla, T., and Donaldson, J.G. 2001. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.* 154:1007-1017.

Casanova, J.E. 2007. Regulation of Arf activation: The Sec7 family of guanine nucleotide exchange factors. *Traffic* 8:1476-1485.

Choi, W., Karim, Z.A., and Whiteheart, S.W. 2006. Arf6 plays an early role in platelet activation by collagen and convulxin. *Blood* 107:3145-3152.

Dell'Angelica, E.C., Puertollano, R., Mullins, C., Aguilar, R.C., Vargas, J.D., Hartnell, L.M., and Bonifacino, J.S. 2000. GGAs: A family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *J. Cell Biol.* 149:81-94.

Donaldson, J.G. and Jackson, C.L. 2000. Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* 12:475-482.

D'Souza-Schorey, C. and Chavrier, P. 2006. ARF proteins: Roles in membrane traffic and beyond. *Nat. Rev. Mol. Cell Biol.* 7:347-358.

Eyster, C.A., Higginson, J.D., Huebner, R., Porat-Shliom, N., Weigert, R., Wu, W.W., Shen, R.F., and Donaldson, J.G. 2009. Discovery of new cargo proteins that enter cells through clathrin-independent endocytosis. *Traffic* 10:590-599.

Inoue, H. and Randazzo, P.A. 2007. Arf GAPs and their interacting proteins. *Traffic* 8:1465-1475.

Morishige, M., Hashimoto, S., Ogawa, E., Toda, Y., Kotani, H., Hirose, M., Wei, S., Hashimoto, A. Yamada, A., Yano, H., Mazaki, Y., Kodama, H., Nio, Y., Manabe, T., Wada, H., Kobayashi, H., and Sabe, H. 2008. GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion. *Nat. Cell Biol.* 10:85-92.

Santy, L.C. and Casanova, J.E. 2001. Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J. Cell Biol.* 154:599-610.

Skippen, A., Jones, D.H., Morgan, C.P., Li, M., and Cockcroft, S. 2002. Mechanism of ADP ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J. Biol. Chem.* 277:5823-5831.

Volpicelli-Daley, L.A., Li, Y., Zhang, C.J., and Kahn, R.A. 2005. Isoform-selective effects of the depletion of ADP-ribosylation factors 1-5 on membrane traffic. *Mol. Biol. Cell* 16:4495-4508.

CHAPTER 15
Protein Trafficking

INTRODUCTION

As discussed in the introduction to Chapter 3, eukaryotic cells are not simply sacs of amorphous protoplasm but are instead organized into an array of membrane-bound structures known as organelles. Subcellular fractionation (Chapter 3) and microscopic analyses (Chapter 4) have revealed a wondrous diversity of subcellular organelles, including the nucleus, endoplasmic reticulum (ER), Golgi complex, lysosomes, endosomes, secretory granules, plasma membrane, mitochondria, peroxisomes, chloroplasts, and a variety of other structures found in specialized cells. Each of these organelles fulfills a distinct function, which is carried out by a specific set of organellar proteins. Genetic information directing the synthesis of most organellar proteins, as well as cytosolic and extracellular proteins, is encoded by DNA contained within the nucleus. Genomic DNA is transcribed into messenger RNA (see *UNIT 11.6*), and this in turn is translated into protein (see *UNIT 11.1*). It is at the time of their translation that proteins begin a journey that will take them to different locations within the cell, or out to the extracellular space. Some proteins are synthesized on ribosomes that exist free in the cytosol. These proteins either remain soluble in the cytosol, become incorporated into supramolecular structures such as the cytoskeleton, or are imported into the nucleus, mitochondria, peroxisomes, chloroplasts, and in some cases the ER. Other proteins are synthesized on ribosomes that are associated with the cytosolic face of the ER membrane. These proteins are translocated across that membrane and subsequently distributed to different compartments of the secretory and endocytic pathways, including the ER proper, the Golgi complex, lysosomes, endosomes, secretory granules, and the plasma membrane; in other cases they are secreted into the extracellular medium. Thus, the interior of the cell is a highly dynamic environment, in which heterogeneous yet precisely controlled protein distributions are achieved via various protein trafficking pathways.

In vitro assays for examining specific protein transport steps and their molecular mechanisms have been described in Chapter 11. However, in vitro assays are valid only if they reflect the transport processes that occur in intact cells. In addition, the itineraries followed by proteins as they traffic through different cellular compartments are often too complex to be reconstituted in vitro and are best studied in intact cells.

Chapter 15 is devoted to biochemical and morphologic assays used to study protein trafficking in vivo. The chapter begins with an overview of protein trafficking in the secretory and endocytic pathways (*UNIT 15.1*), which provides useful background information for some of the succeeding protocol units. Most proteins destined for organelles of the secretory or endocytic pathways, or for secretion into the extracellular milieu, are co-translationally modified by addition of carbohydrate moities. These moieties are progressively remodeled as the proteins traverse the different compartments of the secretory pathway. The type of carbohydrate present on a protein thus serves as a record of its itinerary through the cell. Gross analyses of the carbohydrate moities of glycoproteins can be conveniently carried out by treatment of metabolically labeled, immunoprecipitated proteins (see *UNITS 7.1 & 7.2*) with an assortment of specific glycosidases. *UNIT 15.2* presents a comprehensive description of carbohydrate modifications along the secretory pathway, and provides a series of protocols for analysis of the general structure of carbohydrate moieties of glycoproteins labeled in vivo.

Current Protocols in Cell Biology 15.0.1-15.0.5, September 2010
Published online September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb1500s48

Even after proteins reach their allotted destinations within the cells, they remain in constant flux. Indeed, most proteins are not fixed at a particular location but attain a particular steady-state distribution as a result of opposing exit and retrieval pathways. This is very neatly exemplified by some plasma membrane proteins, such as endocytic receptors, that are rapidly internalized but return to the cell surface after a sojourn in the endosomal system. *UNIT 15.3* describes a set of biochemical techniques for measuring the steady-state distribution, internalization, and recycling of the transferrin receptor, a typical endocytic receptor, using radioiodinated transferrin as a probe. Protocols are also provided to measure the steady-state distribution, internalization, and recycling of any plasma membrane protein, even those that do not have a physiologic ligand, by using radioiodinated antibodies or Fab fragments. Internalized ligands and antibodies used to tag plasma membrane proteins are often degraded in lysosomes, a process that can be measured using another protocol included in this unit. Finally, the unit also covers methods for measuring fluid-phase uptake from the medium and for inhibiting endocytosis.

Integral membrane proteins expressed at the cell surface are synthesized in the ER and transported to the plasma membrane following the secretory pathway. By the time that these proteins reach the cell surface, almost all post-translational modifications have taken place. For this reason, the carbohydrate analyses described in *UNIT 15.2* cannot be used to determine the kinetics of protein arrival at the cell surface. However, the accessibility of cell surface proteins to externally added reagents has been exploited to develop assays for biosynthetic protein transport to the plasma membrane. *UNIT 15.4* contains several protocols that combine pulse-chase metabolic labeling (see *UNIT 7.1*) with treatments that modify surface-exposed proteins on intact cells: hydrolysis of sialic acid residues with sialidase, biotinylation, protease digestion, and antibody binding. Modified surface-exposed proteins are detected by electrophoretic or immunoprecipitation techniques sensitive to the modifications introduced.

Although one often refers to the plasma membrane as if it were a single compartment, most eukaryotic cells exhibit more than one plasma membrane domain. Polarized epithelial cells are perhaps the best characterized example of cells that have specialized plasma membrane domains. In epithelial cells, these domains are known as apical and basolateral. Protein trafficking to and from these two domains follows distinct pathways, the analysis of which is the focus of *UNIT 15.5*. For analysis of polarized sorting to be possible, these cells need to be grown in special chambers fitted with porous filters. The cells form a tight monolayer in which the apical and basolateral domains are segregated. In the chambers, the apical and basolateral media are separately available for addition of reagents or collection of secreted proteins. The unit contains protocols for metabolic labeling of polarized epithelial cells and collection of proteins secreted from the apical and basolateral surfaces, stable transfection and selection of transfected clones, culture of epithelial cells on filters, determination of leakiness of epithelial monolayers, monitoring the arrival of newly synthesized proteins at both plasma membrane domains, and indirect immunofluorescence microscopy of epithelial cells grown as polarized monolayers.

UNIT 15.6 takes us back to early events in the maturation of newly synthesized proteins in the ER. Upon emergence into the lumen of the ER, nascent polypeptide chains undergo a series of post-translational modifications that lead to the development of a mature protein (see *UNIT 15.1*). Among these modifications are glycosylation (*UNIT 15.2*), folding, and disulfide bond formation. *UNIT 15.6* describes a series of protocols for the analysis of protein folding and disulfide bond formation in the ER. In these protocols, newly synthesized proteins are first labeled with radiolabeled amino acids either in intact cells (also see *UNIT 7.1*) or in cell-free (also see *UNIT 11.4*) or semi-permeabilized cell systems. Protein folding can then be followed by limited proteolytic digestion or immunoprecipitation

with conformation-specific antibodies. Disulfide bond formation can be analyzed by monitoring the disappearance of sulfhydryl groups or the appearance of faster-moving species on nonreducing SDS-PAGE (also see *UNIT 6.5*).

The subject of *UNIT 15.7* is phagocytosis—the process by which specialized cells such as macrophages and monocytes ingest particles such as bacteria, yeast, and apoptotic bodies. Because of the size and complexity of the internalized particles, phagocytosis is distinct from the receptor-mediated or fluid-phase endocytosis described in *UNIT 15.3*. The first step in phagocytosis is the recognition of specific molecules on the surface of the particle by the phagocytic cell. These molecules can be intrinsic to the particle or deposited onto the particle by the host (i.e., "opsonization"). The unit starts with microscope-based protocols to measure phagocytosis of red blood cells or latex beads opsonized with immunoglobulin G (IgG), which are internalized upon binding to Fcγ receptors on the phagocytic cells. These protocols are followed by others designed to measure complement-mediated phagocytosis of red blood cells or latex beads opsonized with complement factor C3bi, a process that is mediated by binding to complement receptors. Phagocytosis can be measured more quantitatively by flow cytometry, for which a protocol is also included in this unit. The next protocols deal with events that follow the uptake of particles, such as the acidification and maturation of the phagosome. The final protocol describes a procedure to inhibit phagocytosis with wortmannin or cytochalasin D.

UNIT 15.8 deals with protein transport to lysosomes, organelles that constitute a major site of degradation for both endogenous and exogenous biomacromolecules. The degenerative function of lysosomes depends on over 50 acidic hydrolases contained within the lumen and a similarly large set of tranmembrane proteins embedded in their limiting membrane. The transport of these resident proteins, or of proteins targeted for lysosomal degradation, can be measured by methods that take advantage of the high density of lysosomes. The best of these methods is centrifugation on self-forming Percoll gradients, in which lysosomes band near the bottom while most other organelles band near the tops of the gradients. When used in combination with metabolic labeling, pulse-chase analyses, Percoll gradient fractionation allows analysis of biosynthetic transport of proteins to lysosomes. It is also possible to combine extrinsic labeling of the plasma membrane or internalization of cargo molecules with Percoll gradient fractionation in order to monitor endocytic delivery to lysosomes. In addition to the basic protocol for Percoll gradient fractionation, *UNIT 15.8* describes a β-hexosaminidase assay to check the efficiency of the fractionation.

Another major system for the degradation of endogenous proteins relies on their covalent modification with ubiquitin followed by their targeting to proteasomes. Conjugation with ubiquitin also regulates many other cellular functions, including endocytosis, lysosomal targeting, signal transduction, transcription, and nuclear-cytoplasmic shuttling. So numerous and varied are the roles of ubiquitylation that it has been likened to another ubiquitous covalent modification, phosphorylation. *UNIT 15.9* prevents a comprehensive set of protocols to analyze protein modification with ubiquitin and other molecules (the latter as exemplified by NEDD8). Among the protocols described in this unit are procedures to produce and characterize the cascade of enzyme (i.e., E1, E2, and E3) that mediate ubiquitylation, examine ubiquitylation of proteins in vivo and in vitro, and inhibit ubiquitylation and proteasomal degradation. Protocols to study modification with NEDD8 are also described.

After internalization from the cell surface into endosomes, membrane-bound proteins can follow different fates: they can recycle back to the cell surface like the transferrin receptor, be delivered to lysosomes like the activated EGF receptor, or undergo transport to the TGN. *UNIT 15.10* deals with this latter pathway, mainly using the B subunit of Shiga

toxin (STxB) as a model cargo protein. STxB binds to cell surface glycosphingolipids and is endocytosed into early endosomes, from where it begins a journey that takes it to the TGN, the cisternae of the Golgi complex, and eventually the ER. The assays for measuring this "retrograde" transport from endosomes to the TGN rely on the addition of a sulfation site peptide to the cargo protein (e.g., STxB, the B subunit of cholera toxin, antibody to a GFP-mannose 6-phosphate receptor fusion protein) by either genetic fusion or chemical coupling, and then monitoring the incorporation of radioactive sulfate into the cargo protein either by trichloroacetic acid precipitation and scintillation counting or by SDS-PAGE and fluorography. These protocols are applicable to both live and permeabilized cells. This unit also includes a protocol for chemical coupling of horseradish peroxidase to STxB, which permits ultrastructural analysis by whole-mount electron microscopy of the intracellular compartments through which STxB transits in its retrograde path. The unit ends with protocols designed to analyze the internalization of biotinylated STxB into cells.

UNIT 15.11 deals with another important function of certain specialized cells, which is the regulated secretion of proteins and other substances into the extracellular space. The protocols in this unit make use of mast cells as a model system. These cells have secretory granules that contain a variety of mediators of inflammation, including serotonin, histamine, proteoglycans, proteases, and lysosomal hydrolases. Allergen-induced aggregation of immunoglobulin E bound to its receptor or engagement of other signaling receptors on the surface of these cells triggers the fusion of the regulated secretory granules with the plasma membrane, resulting in the release of the granule contents into the extracellular milieu. This unit describes protocols to measure regulated secretion of the soluble granule contents, serotonin and β-hexosaminidase, into the medium. In addition, granule exocytosis causes the merging of the membrane of the granules with the plasma membrane. This leads to transient changes in both the protein and lipid composition of the plasma membrane. One of these changes is a transient increase in the levels of phosphatidylserine, which, as described in another protocol, can be detected by binding of Annexin V using flow cytofluorometry. Finally, protocols are presented to indirectly detect the release of histamine in live mice by measuring passive or active anaphylactic reactions.

UNIT 15.12 continues with the topic of regulated secretion, in this case using PC12 cells as a model system. PC12 is a cell line derived from a rat pheochromocytoma, a catecholamine-producing tumor of the adrenal medulla. Like their normal counterparts, PC12 cells have dense-core (chromaffin) granules filled with proteins (e.g., granins, peptide hormones, neuropeptides) and small molecules (e.g., catecholamines, nucleotides, Ca^{2+}), which can be released into the extracellular space upon exposure of the cells to various secretagogues (e.g., cholinergic agonists, depolarizing agents, Ca^{2+} ionophores). This unit includes protocols for measuring exocytic release of a radiolabeled catecholamine, [^{3}H]-L-norepinephrine, the polypeptide human growth hormone, and the reporter protein, CgA-EAP, a chimeric protein comprising chromogranin A fused to embryonic alkaline phosphatase (EAP). Additional protocols in this unit describe how to culture and transfect PC12 cells.

The post-endocytic fate of cell surface proteins can be monitored by extracellular tagging with fluorescent ligands and visualization by fluorescence microscopy. Localization of the fluorescently tagged proteins to organelles along the pathway can be determined by costaining for organellar markers. Although informative, these microscopic analyses generally suffer from limited accuracy and low quantitative power. *UNIT 15.13* presents a set of protocols that overcome these shortcomings by exploiting the ability to measure the pH of endocytic organelles using a pH-sensitive fluorescent tag. Internalized cargo proteins are progressively exposed to increasingly acidic environments as they move from

early endosomes to late endosomes to lysosomes. Each of these organelles possesses a lumenal pH in a characteristic range of values. Thus, measurement of the pH to which the fluorescent tag is exposed over time provides a more precise assessment of the localization and transport of endocytic cargo. The basic technique, named fluorescence ratiometric image analysis (FRIA), involves labeling cell surface proteins with a primary antibody followed by a secondary antibody conjugated with fluoroscein isothiocyanate (FITC). After different times of internalization, single-cell FRIA measurements are made by fluorescence microscopy.

The epidermal growth factor (EGF) receptor (EGFR) is a prototypical example of a transmembrane signaling receptor that is down-regulated from the cell surface upon ligand binding. EGF binding to the EGFR triggers activation of signal transduction pathways that lead to changes in gene expression. Receptor activation is quickly followed by endocytosis and lysosomal delivery of the EGF-EGFR complex via the multivesicular body (MVB) pathway. Analyses of EGFR-EGF traffic and degradation are often used to assess the importance of various components of the endocytic and lysosomal/MVB targeting machinery. *UNIT 15.14* describes several protocols aimed at quantifying EGF and EGFR traffic and turnover within cells. The first two protocols are used to measure the rates of EGFR synthesis and degradation. These are followed by protocols to measure down-regulation of cell surface EGFR and internalization, recycling, and degradation of radioiodinated EGF.

Juan S. Bonifacino

Overview of Protein Trafficking in the Secretory and Endocytic Pathways

COMPARTMENTALIZATION OF EUKARYOTIC CELLS

The interior of eukaryotic cells is organized as an interconnected system of membrane-bounded compartments. The compartments that form part of the secretory and endocytic pathways include the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), the Golgi complex with its different subcompartments (*cis*, medial, and *trans* cisternae, and the *trans*-Golgi network or TGN), early and late endosomes, lysosomes, regulated secretory granules, the plasma membrane, and various types of intermediary vesicles and tubular-vesicular carriers (Fig. 15.1.1). Each of these compartments contains a characteristic set of resident proteins. Protein localization to these compartments is mediated by specific targeting information present within the proteins (see *APPENDIX 1C*). This unit is intended as a brief overview of the itinerary followed by proteins as they move through the secretory and endocytic pathways, and of the mechanisms that determine protein sorting within these pathways.

TARGETING AND TRANSLOCATION OF PROTEINS INTO THE ER

Most proteins enter the secretory pathway by translocation from the cytosol into the ER. In mammalian cells, protein translocation in the ER occurs co-translationally as the nascent polypeptide chain emerges from the ribosome. In yeast cells, some proteins utilize this cotranslational mechanism and others are translocated posttranslationally. The steps involved in cotranslational transport are shown in Figure 15.1.2. Early experiments established that the signal for translocation of a protein across the ER membrane lies in the nascent polypeptide rather than in the encoding mRNA (reviewed by Rapoport et al., 1996). As the signal sequence emerges from the ribosome, it binds to the signal-recognition particle (SRP; step 1), a complex composed of six proteins and one 7S RNA molecule. SRP arrests further elongation of the polypeptide on the ribosome and then binds the complex to the SRP receptor on the cytosolic side of the ER membrane (step 2). Both SRP and the SRP receptor bind GTP, which allows transfer of the ribosome to the translocon. Hydrolysis of GTP induces dissociation of SRP from the SRP receptor (step 3). The nascent polypeptide then moves through the central channel of the translocon, the main component of which is the multi-spanning membrane protein Sec61α (step 4). The signal peptide is cleaved cotranslationally by signal peptidase. Throughout translocation, the impermeability of the ER membrane is maintained by a complex set of events, involving the ribosome or ER-lumenal proteins such as BiP (binding immunoglobin protein). Translocation is complete either when a stop-transfer sequence in the polypeptide is encountered (step 5), leading to protein integration in the lipid bilayer (step 6), or when a stop codon is reached, in which case the protein is released into the ER lumen.

PROTEIN MODIFICATIONS IN THE ER

Once the nascent polypeptide chains emerge into the ER lumen, they undergo a series of modifications including signal peptide cleavage, glycosylation, folding, and disulfide-bond formation. Amino-terminal signal sequences are cotranslationally cleaved by signal peptidase, an enzyme complex that is closely associated with the translocon (Andrews and Johnson, 1996). In addition to signal peptidase, the oligosaccharyl transferase complex also appears to be in close proximity to the translocon. This complex is responsible for transferring the preassembled core oligosaccharide moieties to the nascent polypeptide chains at Asn-X-Ser/Thr sequences (see *UNIT 15.2*). The nascent polypeptide chain can be glycosylated when as few as fifteen amino acids have emerged from the translocon.

The nascent polypeptide chain associates with a number of chaperone proteins in the ER, including BiP, GRP94, calnexin, calreticulin, protein disulfide isomerase, and peptidyl proline *cis-trans* isomerase. BiP comprises ~5% of the ER lumenal protein content and associates transiently with a number of newly synthesized proteins (Gething and Sambrook, 1992). BiP has been postulated to sequentially bind, release, and rebind newly synthesized proteins in an ATP-dependent manner until the proteins fold correctly and their hydrophobic regions are no longer accessible to BiP. Proteins

Contributed by Caroline Enns
Current Protocols in Cell Biology (1999) 15.1.1-15.1.10

that do not fold correctly remain bound to BiP and are not transported further along the secretory pathway.

Calreticulin and calnexin are carbohydrate-binding chaperones, which are also involved in quality control in the ER (reviewed by Bergeron et al., 1994; Hammond and Helenius, 1995). Calreticulin is a lumenal protein, whereas calnexin is an integral membrane protein. Shortly after addition of the core oligosaccharide complex to the nascent polypeptide, the two terminal glucose residues of the complex are removed by glucosidases I and II (Fig. 15.1.3). This generates a monoglucosylated oligosaccharide to which calnexin or calreticulin binds. Further processing of the oligosaccharide results in the release of the newly synthesized polypeptide from either chaperone. If the protein is not folded correctly, it is re-glucosylated by a UDP-glucosyl transferase, and the

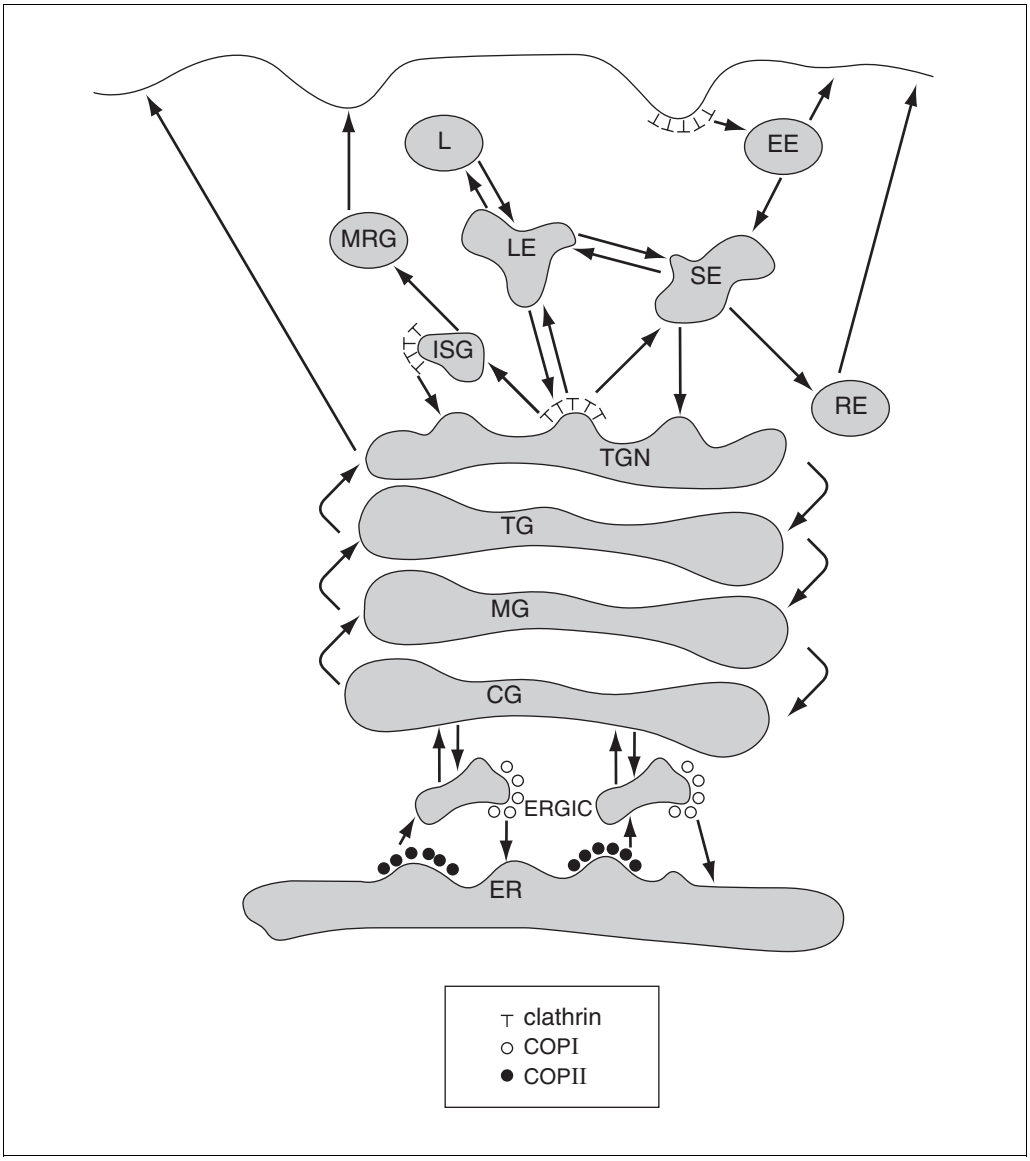


Figure 15.1.1 Compartments of the biosynthetic and endocytic pathways. The known routes in the biosynthetic and endocytic pathways are shown in this schematic diagram. The compartments include: the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), the *cis*-Golgi (CG), the medial Golgi (MG), the *trans*-Golgi (TG), the *trans*-Golgi network (TGN), the lysosome (L), the late endosome (LE), the sorting endosome (SE), the recycling endosome (RE), the early endosome (EE), the immature secretory granule (ISG), and the mature regulated granule (MRG). The extent to which each of the pathways are utilized is not known. The steps where coat proteins have been identified to be involved in vesicle formation are indicated. See Transit Between the ER and the Golgi Complex for discussion of COPI and COPII.

cycle of calnexin/calreticulin binding is repeated until the protein folds correctly and no longer interacts with the UDP-glucosyl transferase. The lumen of the ER is much more oxidizing than the cytosol, allowing the formation of disulfide bonds in a reaction catalyzed by protein disulfide isomerase.

ER-ASSOCIATED PROTEIN DEGRADATION

Quality control mechanisms of the ER not only prevent misfolded proteins from exiting the ER, but also ensure that abnormal proteins are degraded (Sommer and Wolf, 1997). Recent evidence indicates that misfolded proteins, as well as ER proteins whose levels are subject to

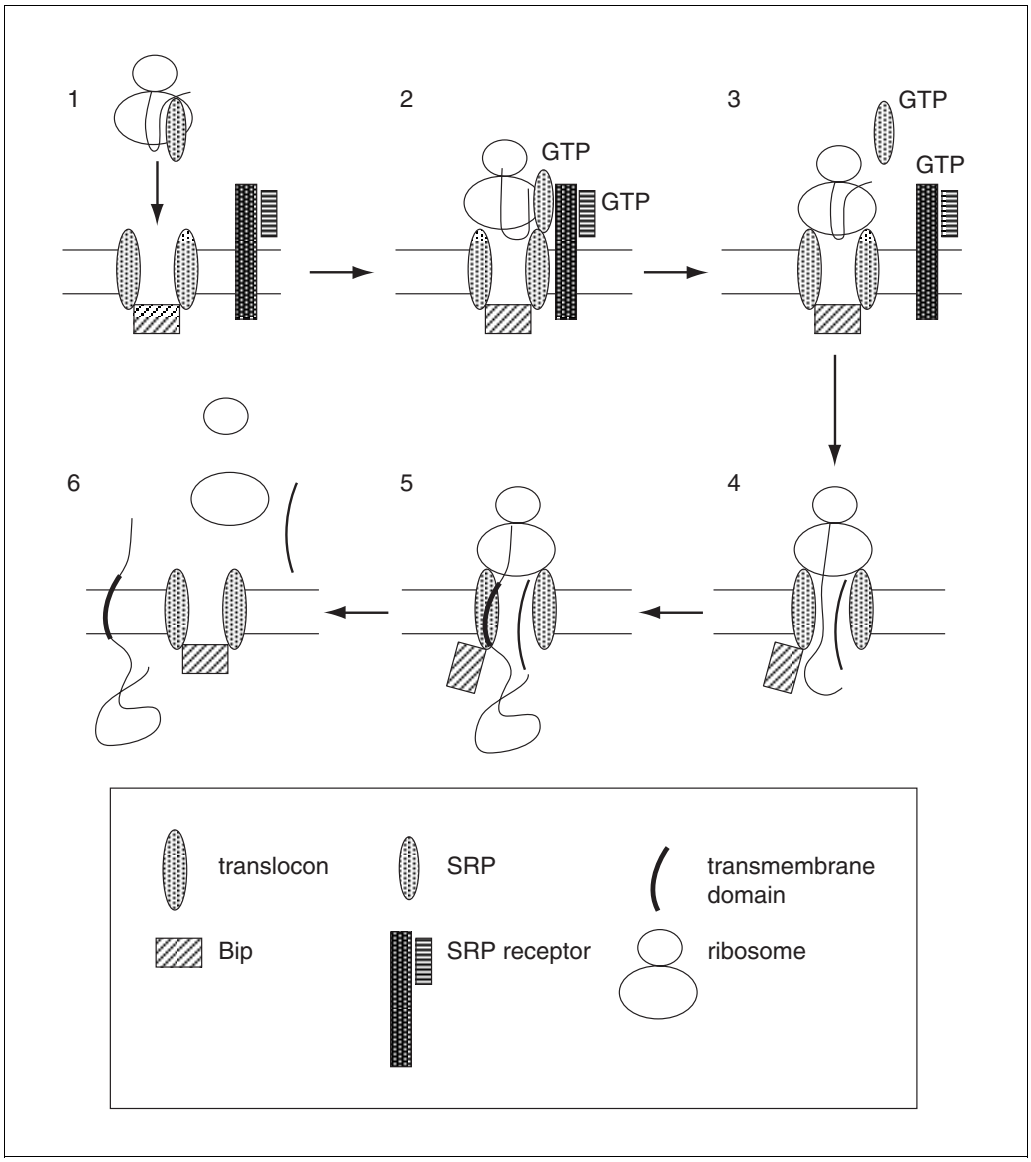


Figure 15.1.2 Cotranslational translocation of a type 1 membrane protein into the ER. (**1**) When the signal sequence emerges from the ribosome, it binds to the signal-recognition particle (SRP) in the GTP-empty form and translocation is arrested. The pore of the translocon is in the closed position, sealed by binding immunoglobulin protein (Bip). (**2**) The SRP/signal peptide/ribosome complex binds to the two subunits of the GTP-bound SRP receptor. The ribosome interacts with the translocon. (**3**) GTP is hydrolyzed, releasing the SRP from the SRP receptor. Translation is continued. (**4**) The ribosome seals the pore and when the nascent chain reaches ~70 residues Bip is released. The protein is extruded into the lumen of the ER, where the signal peptide is cleaved. (**5**) The transmembrane sequence emerges from the ribosome and interacts with different proteins in the translocon. (**6**) The protein is inserted into the bilayer by the completion of translation. The ribosome disassociates and the pore is closed by Bip.

metabolic regulation, are exported from the ER into the cytosol, where they are degraded by the ubiquitin-proteasome pathway (Sommer and Wolf, 1997). A variety of viruses use this degradative pathway to subvert the immune system. For example, viral proteins such as US2 and US11 of human cytomegalovirus appear to promote ejection from the ER and subsequent proteosomal degradation of the heavy chain of MHC class I complexes (Wiertz et al., 1996). This serves to down-regulate molecules that would otherwise stimulate an immune response against the virus.

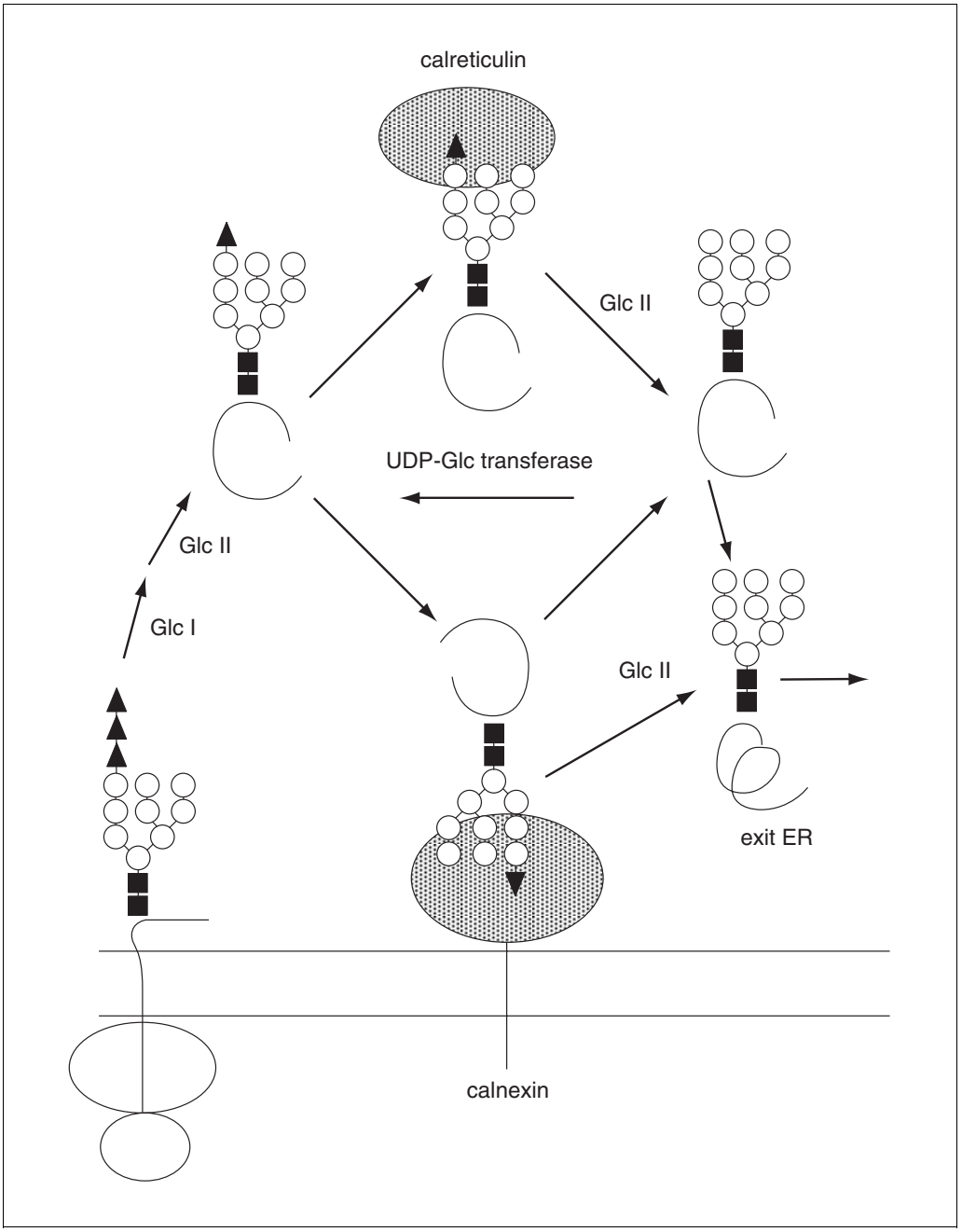


Figure 15.1.3 The calnexin/calreticulin quality control pathway in the ER. Glucosidases I and II (Glc I and Glc II) remove the two outermost glucose residues (black triangles) from the protein and allow interaction of the oligosaccharide with either calnexin or calreticulin. Glucosidase II removes the last glucose residue, and the protein no longer associates with these lectins. If the protein is folded properly, it is free to exit the ER. If it is not folded correctly, it is reglucosylated by the enzyme UDP-glucosyl transferase and rebinds to calnexin or calreticulin. Adapted from Hammond and Helenius (1995). See Figure 15.2.1 for identification of the sugar residues.

TRANSIT BETWEEN THE ER AND THE GOLGI COMPLEX

Vesicle budding in both the secretory and endocytic pathways is mediated by cytosolic proteins that form coated structures on the membranes. Two types of coat proteins—known as COPI and COPII—have been shown to be involved in trafficking of proteins between the ER and the Golgi complex (Cosson and Letourneur, 1997; Kuehn and Schekman, 1997; Fig. 15.1.1). COPII-coated vesicles mediate anterograde ER-to-Golgi transport, whereas COPI-coated vesicles mediate retrograde Golgi-to-ER transport. Controversy still exists as to whether COPI vesicles are also involved in anterograde transport. Vesicles generated from the ER are targeted to and fuse with the ER-Golgi intermediate compartment (ERGIC). ERGIC structures then migrate towards the centrosomal region of the cell and fuse with the Golgi complex.

Newly synthesized proteins were initially proposed to exit the ER via a bulk-flow mechanism that did not involve concentration into transport vesicles. However, recent evidence suggests that newly synthesized proteins destined for transport out of the ER are specifically concentrated at ER exit sites (reviewed by Bannykh et al., 1998). For example, a diacidic (D/EXD/E) sequence close to an internalization signal has been shown to be responsible for the efficient concentration of vesicular stomatitis virus G protein at ER exit sites (Nishimura and Balch, 1997). In yeast, selective packaging of glycosylated pro-α-factor for export from the ER has also been demonstrated (Kuehn and Schekman, 1997). A lectin-like molecule named ERGIC-53 (Emp47p in yeast) is thought to be involved in the transport of some soluble glycoproteins out of the ER after the glucose residues have been trimmed (reviewed by Hammond and Helenius, 1995).

RETENTION IN THE ER AND RETRIEVAL FROM THE GOLGI COMPLEX

Most soluble ER-resident proteins contain a carboxy-terminal tetrapeptide sequence, KDEL (HDEL in *S. cerevisiae*). Addition of this sequence to the carboxy terminus of soluble secreted proteins results in their accumulation in the ER, implying that this motif is sufficient for ER localization. The KDEL sequence is thought to retrieve proteins from the ERGIC or the Golgi complex to the ER. This retrieval is mediated by the membrane-bound receptor protein Erd2p.

Other signals are used to retrieve integral membrane proteins to the ER. Type I ER-resident membrane proteins have cytosolic KKXX or KXKXX (dilysine) motifs at their carboxy termini. Some type II ER-resident membrane proteins have a cytosolic ER localization signal consisting of two arginine residues (RR) located within the first five amino-terminal residues of the protein. Direct binding of COPI to the dilysine motif of ER integral membrane proteins has been implicated in the retrograde transport of these proteins to the ER (reviewed by Cosson and Letourneur, 1997).

Thus, a picture emerges in which anterograde transport of secretory cargo allows newly synthesized proteins to exit the ER selectively, and retrograde transport mediates the retrieval of ER-resident proteins, as well as transport factors necessary for a subsequent round of anterograde transport. The balance of the two transport pathways determines the steady-state distribution of proteins in each compartment.

CARBOHYDRATE MODIFICATIONS IN THE GOLGI COMPLEX

The Golgi complex is a major site of N-linked oligosaccharide modification within the secretory pathway. A highly organized set of glycosyl transferases, glycosidases, and nucleotide- or lipid-linked glycosyl donors and transporters cooperates to produce these modifications. Each component of this protein modification machinery has a characteristic distribution within the Golgi complex. For example, generation of mannose-6-phosphate residues, which serve as a signal for sorting soluble proteins to lysosomes, occurs in the *cis* region of the Golgi complex. In the biosynthesis of complex carbohydrates (*UNIT 15.2*), mannose residues are removed in the *cis*-medial Golgi complex, and *N*-acetylglucosamine residues are added in the medial Golgi complex, whereas galactose and sialic acid addition occurs in the *trans*-Golgi complex and TGN (Fig. 15.1.4). Sulfation of oligosaccharides and tyrosine residues takes place in the TGN. The extent of glycosylation of proteins traveling through the Golgi complex can be deduced by a variety of methods, including the use of glycosidases specific for certain oligosaccharide structures (see *UNIT 15.2*). The oligosaccharide composition of a mature glycoprotein can be quite heterogeneous and can depend on the cell type and metabolic state. Moreover, whether a particular consensus sequence is modified and

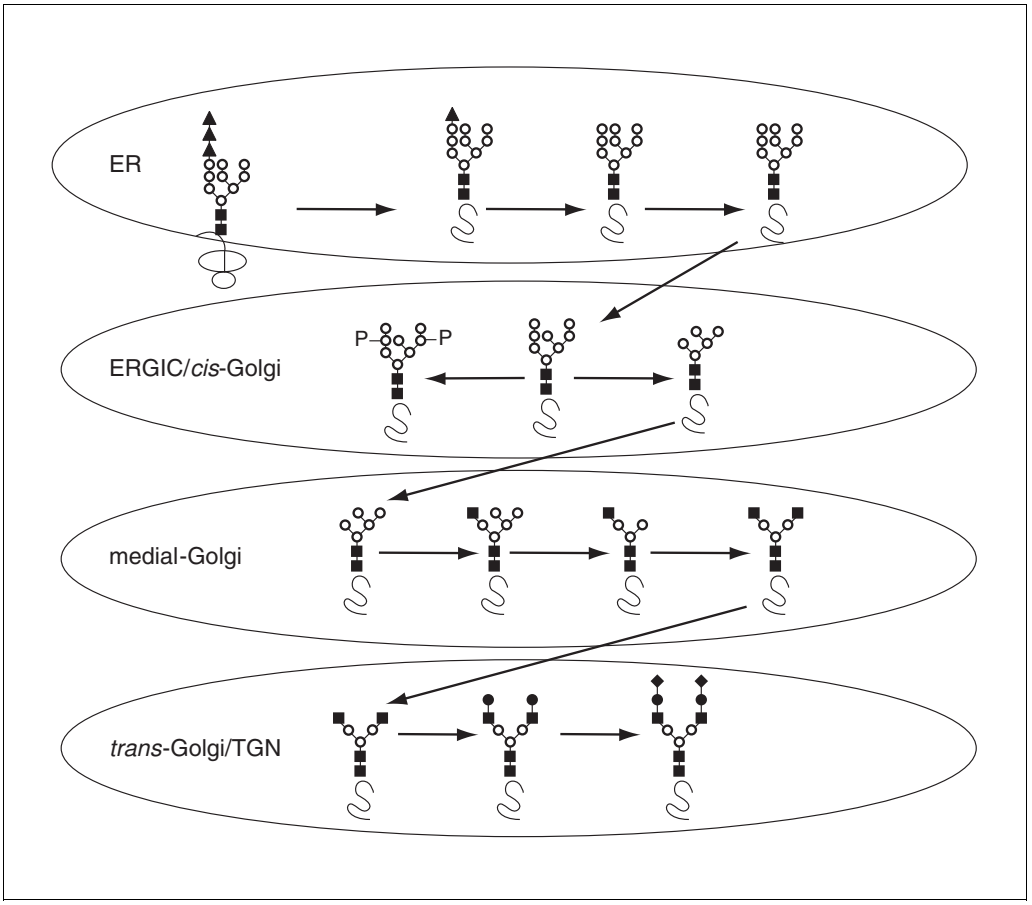


Figure 15.1.4 Processing of oligosaccharides in the Golgi. A schematic diagram of the approximate locations of oligosaccharide modification in the biosynthetic pathway. See Figure 15.2.1 for identification of sugar residues.

to what extent depend on the local conformation of the protein at each glycosylation site (Dwek, 1995).

In contrast to N-linked glycosylation, O-linked glycosylation occurs posttranslationally with the addition of a single *N*-acetylglucosamine residue to a serine/threonine residue. While there is no consensus sequence for O-linked glycosylation, it often occurs in the proximity of proline and close to transmembrane regions (Hounsell et al., 1996; see *UNIT 15.2*).

MODELS OF PROTEIN RETENTION IN AND TRANSPORT THROUGH THE GOLGI COMPLEX

How are secretory proteins transported through the Golgi complex, and how are resident components retained in the Golgi in the face of this continuous transport? At present, no single mechanistic model has been embraced by all cell biologists (Mellman and Simons, 1992; Mironov et al., 1997; Glick and Malhotra, 1998).

A widely accepted model for Golgi protein transport is by vesicular traffic (Rothman, 1994). This model views the Golgi complex as comprised of a series of distinct and stable subcompartments. Secretory cargo moves between subcompartments in a vectorial direction (*cis* to *trans*) by being packaged into transport vesicles that specifically target acceptor membranes. According to this model, membrane association of COPI provides the mechanochemical force for driving vesicle budding from donor membranes. Golgi enzymes are thought to be organized into large, immobile aggregates in Golgi membranes to avoid being packaged into the forward-moving transport vesicles, which deliver secretory cargo by bulk flow. Evidence in favor of this model includes the findings that cisternae exhibit relatively distinct and stable enzymatic composition, that protein transport can be reconstituted in vitro between biochemically isolated donor and acceptor Golgi compartments, and that COPI-coated Golgi vesicles accumulate in cells where pro-

tein transport has been inhibited with GTPγS (a GTP analog that prevents COPI dissociation from membranes; Rothman, 1994).

A second model for Golgi protein transport that is gaining popularity is transport by cisternal maturation (Mironov et al., 1997; Bonfanti et al., 1998; Pelham, 1998). In this model, Golgi cisternae themselves act as carriers through the Golgi stack. The observation that ER-derived vesicles carrying protein into the Golgi complex undergo a process of fusion and maturation into relatively large (200- to 500-nm), pleiomorphic intermediates that translocate into the Golgi region is consistent with this nonvesicular transport model (Presley et al., 1998). According to the maturation hypothesis, as pre-Golgi intermediates arrive at the *cis* face of the Golgi stack, they form a new *cis* cisternae replacing preexisting *cis* cisternae. This replacement of one cisternae for another continues distally through the stack. The size of the stack is kept constant by the continued consumption of *trans* cisternae through the formation of post-Golgi transport intermediates. To account for the observed constancy of cisternal enzyme distribution by this model, the anterograde shift of each cisterna would be coupled with retrograde relocation of its enzymes into the next proximal cisterna. Morphological observations of the transport of supramolecular structures through the Golgi complex—including casein submicelles, apolipoprotein E, and procollagen—are consistent with this possibility (Bonfanti et al., 1998). These structures are detected throughout Golgi cisternae, but are consistently absent from Golgi vesicles. As these secretory products are simply too large to be packaged into transport vesicles, progression of Golgi cisternae seems reasonable. The observation that Golgi enzymes are highly mobile within the Golgi stack (Cole et al., 1996) and undergo continuous recycling (Cole et al., 1998) is also consistent with this model. A problem with this model, however, is that it cannot explain how different cargo passes through the Golgi complex at distinct rates.

A third model for protein transport through the Golgi complex is by directed diffusion within a single compartment that is interconnected by tubules (Mironov et al., 1997). Tubules are a well characterized feature of the Golgi complex, with both the *cis*- and *trans*-most Golgi elements largely tubular in character. Moreover, the central part of the Golgi complex is comprised of stacks of cisternae that are interlinked by tubular-reticular networks. The finding that there is rapid diffusion of enzymes within the intact Golgi complex of living cells (Cole et al., 1996) is consistent with the possibility that protein transport through the Golgi occurs by directed diffusion within an interconnected structure. Different cargo would move through this system at distinct rates based on their rate of diffusion within the extended membrane system. Directional, *cis*-to-*trans* flow would arise because of continuous membrane addition at the proximal end (*cis* face) of this system, and membrane removal at the distal end (*trans* face). One difficulty with this model is that it requires unknown mechanisms for maintaining distinct membrane domains within an interconnected system. It also requires alternative functions for COPI coat proteins, which previously have been thought of only in the context of vesicle formation.

The above models for Golgi protein transport may not be mutually exclusive; instead, a spectrum of interrelated mechanisms may be involved. For example, recycling of Golgi enzymes may occur by directed diffusion within a continuous bilayer or by vesicles. Intermittent tubular connections might allow fast forward traffic of cargo, while large structures progress by the slower cisternal maturation pathway. Future work in this area will undoubtedly involve more systematic study of key aspects of each of the above models. Distinguishing between these models is nevertheless important for characterizing the exact roles of molecular machinery identified in in vitro transport assays and genetic screens (Schekman and Orci, 1996), and for understanding the relationship between protein traffic and Golgi structure/function.

PROTEIN SORTING IN POST-GOLGI COMPARTMENTS

The TGN is the major sorting compartment for newly synthesized proteins destined for endosomes, lysosomes, regulated secretory granules, or different domains of the plasma membrane (Fig. 15.1.1; reviewed by Keller and Simons, 1997; Traub and Kornfeld, 1997). The signals for sorting integral membrane proteins to endosomes, lysosomes, and the basolateral plasma membrane of polarized epithelial cells are found in the cytosolic domain of the proteins (see *APPENDIX 1C*). Two types of signal, referred to as tyrosine-based and dileucine-based signals, have been shown to play multiple roles in sorting to these compartments (Kirchhausen et al., 1997). Both types of signal interact with the heterotetrameric adaptor protein complexes AP-1, AP-2, and AP-3. AP-1 is com-

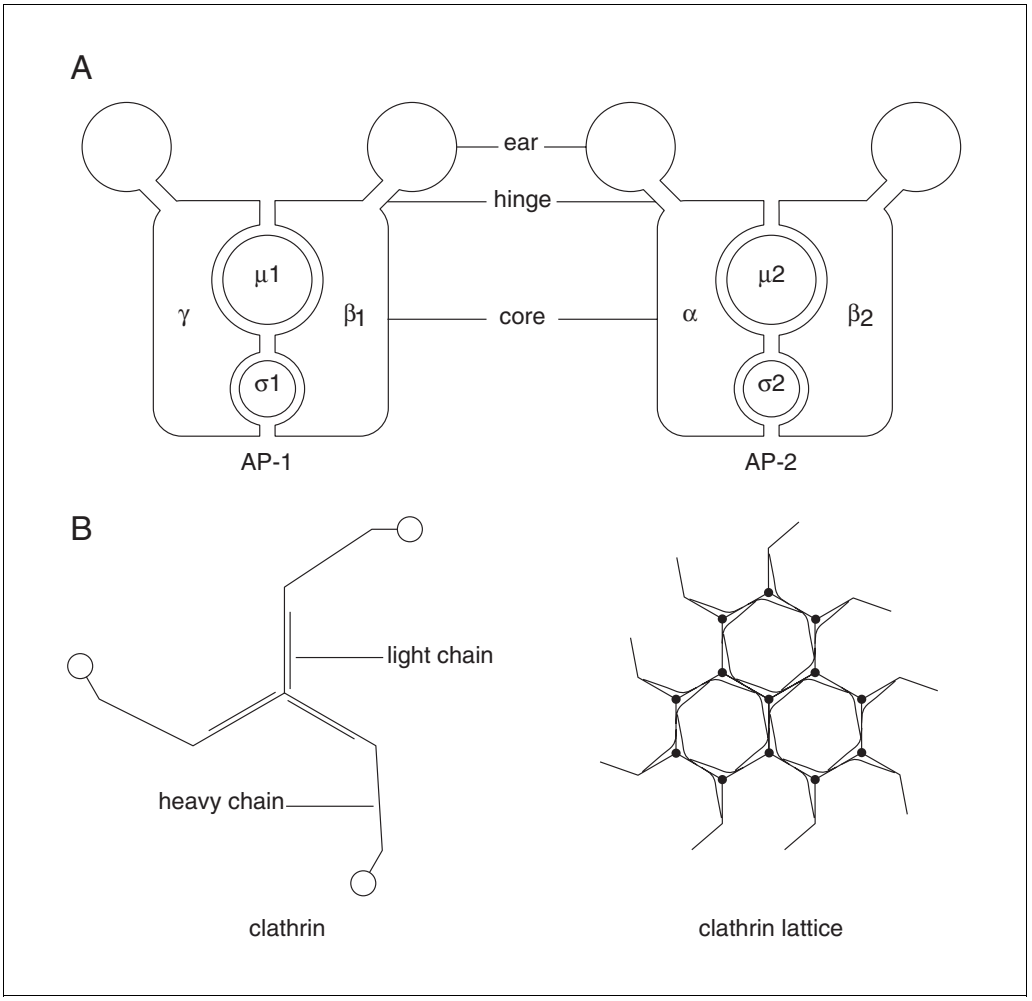


Figure 15.1.5 Clathrin and associated proteins. (**A**) Subunit composition and overall structure of AP-1, which is associated with the TGN, and of AP-2, which is associated with the plasma membrane. (**B**) Clathrin is composed of three heavy chains and three light chains forming a triskelion (left). Clathrin oligomerizes to form two-dimensional lattices (right) or three-dimensional clathrate structures (not pictured).

posed of γ, β1, μ1, and σ1 subunits; AP-2 of α, β2, μ2, and σ2 subunits; and AP-3 of δ, β3, μ3, and σ3 subunits (Fig. 15.1.5A). AP-1 localizes to the TGN and endosomes, whereas AP-2 is found on the plasma membrane. AP-3 has been proposed to exist in association with the TGN and/or endosomes. Both AP-1 and AP-2 associate with clathrin, a protein composed of three heavy chains (~190 kDa) and three light chains (~30 kDa). Electron microscopy of purified clathrin shows a three-legged structure termed a triskelion (Fig. 15.1.5B). The triskelion can self-associate into both planar lattices and three-dimensional clathrate structures. Both AP-1 and AP-2 promote the formation of these structures. AP-3 also interacts with clathrin in vitro, although it is currently unclear whether this interaction is required for AP-3 function in vivo.

Lysosomal integral membrane proteins, as well as lysosomal lumenal proteins bound via mannose-6-phosphate residues to specific receptors, are concentrated into AP-1/clathrin-coated areas of the TGN. The resulting coated vesicles transport these proteins to early or late endosomes, where they are in turn delivered to lysosomes. The cytosolic domain of the proteins targeted to lysosomes by this pathway—e.g., lysosome associated protein 1 (LAMP-1) and the mannose-6-phosphate receptors—appear to interact directly with AP-1. The mannose-6-phosphate receptors are subsequently recycled to the TGN by virtue of sorting information contained within the cytosolic tail. Recent studies suggest that the AP-3 adaptor complex may also play a role in transport of lysosomal membrane proteins to lysosomes.

Transport of proteins to the plasma membrane occurs either by default or by information contained within the cytosolic domains of the proteins. All plasma membrane proteins were originally thought to be targeted directly from the TGN. However, recent evidence suggests that at least a fraction of proteins intersect with the endocytic pathway en route to the cell surface (Fig. 15.1.1). In polarized epithelial cells, proteins can be targeted to either the basolateral or the apical plasma membrane. Basolateral targeting of integral membrane proteins appears to be dominant over apical targeting and depends on information found in the cytosolic domain of the proteins. Truncation of the cytosolic domain results in distribution to both the basolateral and apical membranes or only to the apical membrane. Proteins may be directed to the apical region by concentration into membrane domains rich in sphingolipids and cholesterol, or by interaction of N-linked oligosaccharides with putative sorting lectins in the TGN (Keller and Simons, 1997).

Exocrine, endocrine, and neuronal cells, as well as certain other cell types, have specialized vesicles whose secretion is regulated. Two models have been proposed for the biogenesis of these vesicles. One model proposes that proteins aggregate in the environment of the TGN, and that nonaggregated proteins are sorted from the condensed proteins in the immature vesicles that pinch off from the TGN. A second model proposes that the proteins bind to a putative receptor in a manner similar to the mechanism used by soluble lysosomal proteins (reviewed in Keller and Simons, 1997).

ENDOCYTOSIS

Most plasma membrane proteins are subject to internalization. However, only a subset of plasma membrane proteins are internalized at a very rapid rate. In most cases, rapid internalization occurs at areas of the plasma membrane that are coated with clathrin and the AP-2 adaptor complex (i.e., clathrin-coated pits). Concentration of integral membrane proteins within clathrin-coated pits is mediated by cytosolic tyrosine-based or dileucine-based signals similar to those that mediate sorting at the TGN. These signals interact directly with proteins within the clathrin-coated pits. Rapid internalization of endocytic receptors can occur either constitutively or upon ligand binding. For example, the transferrin receptor and the low-density lipoprotein receptor are internalized by a constitutive mechanism, even in the absence of ligand. In contrast, the rate of endocytosis of the epidermal growth factor receptor increases several fold upon ligand binding (Wiley, 1988).

Clathrin-coated pits become invaginated and eventually pinch off as clathrin-coated vesicles. Various accessory molecules such as dynamin, amphiphysin, and Eps15 participate in the formation of clathrin-coated vesicles (reviewed in Schmid, 1997). The clathrin-coated vesicles are subsequently uncoated by the HSP70 chaperone in an ATP-dependent reaction and fuse with early endosomes. In this compartment, internalized proteins are either sorted to late endosomes and lysosomes (e.g., activated epidermal growth factor receptors) or recycle to the plasma membrane (e.g., transferrin receptors, low-density lipoprotein receptors).

BIOCHEMICAL ANALYSES OF PROTEIN TRAFFICKING

The localization of proteins to different compartments of the secretory and endocytic pathways described above can be determined by immunofluorescence and immunoelectron microscopy (see Chapter 4). In addition, direct visualization of protein movement in these pathways is now possible through the use of chimeras containing green fluorescent protein (GFP). However, quantitative analyses of protein trafficking in cell populations require the use of biochemical assays. These assays rely on the acquisition of posttranslational modifications (e.g., glycosylation, disulfide-bond formation, proteolytic cleavage), accessibility to externally added reagents (e.g., biotinylating agents, glycosidases), or detection by specific probes (e.g., labeled ligands or antibodies). The following units in this chapter describe a series of protocols based on these principles.

LITERATURE CITED

Andrews, D.W. and Johnson, A.E. 1996. The translocon: More than a hole in the ER membrane? *Trends Biochem. Sci.* 21:365-369.

Bannykh, S.I., Nishimura, N., and Balch, W.E. 1998. Getting into the Golgi. *Trends Cell Biol.* 8:21-25.

Bergeron, J.J., Brenner, M.B., Thomas, D.Y., and Williams, D.B. 1994. Calnexin: A membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.* 19:124-128.

Bonfanti, L., Mironov, A.J., Martinez-Menarguez, J.A., Martella, O., Fusella, A., Baldassarre, M., Buccione, R., Geuze, H.J., Mironov, A.A., and Luini, A. 1998. Procollagen traverses the Golgi stack without leaving the lumen of cisternae: Evidence for cisternal maturation. *Cell* 95:993-1003.

Cole, N.B., Sciaky, N., Marotta, A., Song, J., and Lippincott-Schwartz, J. 1996. Golgi dispersal

during microtubule disruption: Regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. *Mol. Biol. Cell* 7:631-650.

Cole, N.B., Ellenberg, J., Song, J., DiEuliis, D., and Lippincott-Schwartz, J. 1998. Retrograde transport of Golgi-localized proteins to the ER. *J. Cell Biol.* 140:1-15.

Cosson, P. and Letourneur, F. 1997. Coatomer (COPI)-coated vesicles: Role in intracellular transport and protein sorting. *Curr. Opin. Cell Biol.* 9:484-487.

Dwek, R.A. 1995. Glycobiology: Towards understanding the function of sugars. *Biochem. Soc. Trans.* 23:1-25.

Gething, M.J. and Sambrook, J. 1992. Protein folding in the cell. *Nature* 355:33-45.

Glick, B.S. and Malhotra, V. 1998. The curious status of the Golgi apparatus. *Cell* 95:883-889.

Hammond, C. and Helenius, A. 1995. Quality control in the secretory pathway. *Curr. Opin. Cell Biol.* 7:523-529.

Hounsell, E F., Davies, M.J., and Renouf, D.V. 1996. O-linked protein glycosylation structure and function. *Glycoconj. J.* 13:19-26.

Keller, P. and Simons, K. 1997. Post-Golgi biosynthetic trafficking. *J. Cell Sci.* 110:3001-3009.

Kirchhausen, T., Bonifacino, J.S., and Riezman, H. 1997. Linking cargo to vesicle formation: Receptor tail interactions with coat proteins. *Curr. Op. Cell Biol.* 9:488-495.

Kuehn, M.J. and Schekman, R. 1997. COPII and secretory cargo capture into transport vesicles. *Curr. Opin. Cell Biol.* 9:477-483.

Mellman, I. and Simons, K. 1992. The Golgi complex: In vitro veritas? *Cell* 68:829-840.

Mironov, A.A., Weidman, P., and Luini, A. 1997. Variations on the intracellular transport theme: Maturing cisternae and trafficking tubules. *J. Cell Biol.* 138:481-484.

Nishimura, N. and Balch, W.E. 1997. A di-acidic signal required for selective export from the endoplasmic reticulum. *Science* 277:556-558.

Pelham, H.R. 1998. Getting through the Golgi complex. *Trends Cell Biol.* 8:45-49.

Presley, J.F., Smith, C., Hirschberg, K., Miller, C., Cole, N.B., Zaal, K.J., and Lippincott-Schwartz, J. 1998. Golgi membrane dynamics. *Mol. Biol. Cell* 9:1617-1626.

Rapoport, T.A., Jungnickel, B., and Kutay, U. 1996. Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* 65:271-303.

Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature* 372:55-63.

Schekman, R. and Orci, L. 1996. Coat proteins and vesicle budding. *Science* 271:1526-1533.

Schmid, S.L. 1997. Clathrin-coated vesicle formation and protein sorting: An integrated process. *Annu. Rev. Biochem.* 66:511-548.

Sommer, T. and Wolf, D.H. 1997. Endoplasmic reticulum degradation: Reverse protein flow of no return. *FASEB J.* 11:1227-1233.

Traub, L.M. and Kornfeld, S. 1997. The trans-Golgi network: A late secretory sorting station. *Curr. Opin. Cell Biol.* 9:527-533.

Wiertz, E.J., Jones, T.R., Sun, L., Bogyo, M., Geuze, H.J., and Ploegh, H.L. 1996. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* 84:769-779.

Wiley, H.S. 1988. Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system. *J. Cell Biol.* 107:801-810.

Contributed by Caroline Enns
Oregon Health Sciences University
Portland, Oregon

Use Of Glycosidases To Study Protein Trafficking

Carbohydrate chain modifications are often used to monitor glycoprotein movement through the secretory pathway (*UNIT 15.1*). This is because stepwise sugar-chain processing is unidirectional and generally corresponds to the forward or anterograde movement of proteins. This unit offers a group of techniques that will help analyze the general structure of carbohydrate chains on a protein and, therefore, oligosaccharide processing mileposts. The minimum requirements are that the protein can be labeled metabolically (*UNIT 7.1*) and immunoprecipitated (*UNIT 7.2*) and clearly seen on a gel or blot (*UNIT 6.2*). The sugar chains themselves are not analyzed, but their presence and structure are inferred from gel mobility differences after one or more enzymatic digestions. This approach is most often used in combination with [^{35}S]Met pulse-chase metabolic labeling protocols, but they can be applied to any suitably labeled protein (e.g., biotinylated or ^{125}I-labeled). As the oligosaccharide chains mature, they become either sensitive or resistant to highly specific glycosidases. Some of these enzymes cleave intact oligosaccharide chains from the protein—e.g., endo H, endo F_2, endo F_3, peptide:N-glycosidase F (PNGase F), endo D, and O-glycosidase. Others strip only terminal sugars (e.g., sialidase) or degrade a selected portion of the chain (e.g., endo-β-galactosidase). The techniques can be adapted to count the number of N-linked oligosaccharide chains on a protein. One unusual protease (O-sialoglycoprotease) degrades only proteins containing tight clusters of O-linked sialylated sugar chains. These techniques work best on average size proteins (<100 kDa) that contain a few percent carbohydrate by weight, where a gel shift of 1 kDa can be seen. A summary of the enzymes and their applications is shown in Table 15.2.1.

The ever-changing view of the organization and structure of the secretory pathway, and of protein trafficking through it, presents formidable conceptual challenges, but these will not be covered here. Instead, this unit provides information on how to measure changes in carbohydrate structure and how these changes relate to protein trafficking. Fortunately,

Table 15.2.1 Enzymes Described in This Unit

Enzyme	Indications and uses	Monitors[a]
Endo D	Transient appearance of highly processed, sensitive forms prior to addition of GlcNAc by GlcNAc transferase I	*Cis* to medial Golgi
Endo F_2	Presence of biantennary chains ± core fucose	Medial Golgi
Endo F_3	Presence of core fucosylated biantennary α chains and/or triantennary chains ± core fucosylation	Medial Golgi
Endo-β-galactosidase	Presence of polylactosamines	*Trans*-Golgi and TGN
Endo H	Conversion of high mannose to complex type N-linked chains	*Cis*- to medial Golgi
O-Glycosidase	Presence of Galβ1,3GalNAc-α-Thr/Ser O-linked chains	*Cis*/medial Golgi
PNGase F	Presence of N-linked chains cleaves; nearly all N-linked chains; only enzyme that cleaves tetrantennary chains	Medial Golgi
Sialidase	Acquisition of sialic acids	*Trans*-Golgi and TGN
O-Sialoglycoprotease	Presence of mucin-like proteins with cluster of sialylated oligosaccharides	*Trans*-Golgi and TGN

[a]Abbreviation: TGN, *trans*-Golgi network.

Contributed by Hudson H. Freeze
Current Protocols in Cell Biology (1999) 15.2.1-15.2.26

the techniques are independent of mechanistic views, although it should be borne in mind that the organization and distribution of many of these indicator enzymes are cell type dependent.

The starting material for these protocols is assumed to be [^{35}S]Met-labeled, immunoprecipitated protein bound to ~20 µl of protein A–Sepharose beads (as described in *UNIT 7.2*). The trace amount of protein is eluted by heating in a small volume of 0.1% SDS, diluted in the appropriate buffer and then digested with one or more enzymes in a small volume. The digest is analyzed on an appropriate SDS-PAGE system that can detect a 1- to 2-kDa size change. A change in the mobility of the protein after digestion is evidence that the carbohydrate chain was sensitive to the enzyme and therefore, that the protein had encountered a certain enzyme in the processing pathway. Alternatively, the analysis can be done by two-dimensional isoelectric focusing (IEF)/SDS-PAGE or two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE (*APPENDIX 3*) to see the loss of charged sugar residues or of anionic oligosaccharide chains. The same digestions and SDS-PAGE analysis also apply to proteins that are radioiodinated or biotinylated, or to immunoprecipitates derived from subcellular fractions separated on sucrose or Percoll gradients.

It is important to present the glycosylation pathways, as a detailed description of the pathways is needed to appreciate how they will be used in this unit. A single protein can have more than one kind of oligosaccharide (N-linked and O-linked), and each individual N-linked chain can mature into a different final form. The same is true for O-linked chains. Each is described below.

THE N-LINKED PATHWAY

The N-linked oligosaccharide maturation pathway is most frequently used for tracking protein movement through the Golgi complex. A common feature of all N-linked chains is the core region pentasaccharide shown in Figure 15.2.1, which consists of three mannose units and two *N*-acetylglucosamine units. The mannose units comprise the trimannosyl core, and two of these residues are α-linked to the only β-linked mannose in the molecule. The β-linked mannose is bound to one of the two *N*-acetylglucosamines. Because they are β1-4 linked to each other, resembling the polysaccharide chitin, this is called a chitobiose disaccharide. Initially, all *N*-glycosylated proteins begin life when a preformed, lipid-associated oligosaccharide is transferred within the lumen of the endoplasmic reticulum (ER) to Asn of proteins having an Asn-X-Thr/Ser sequence. This precursor oligosaccharide contains three glucose (Glc), nine mannose (Man), and two *N*-acetylglucosamine (GlcNAc) sugar residues, and has the structure shown in Figure 15.2.1. There are several ways to depict this structure. The short-hand symbol method used in Figure 15.2.1 is the most convenient, but be sure to note the linkages of the individual sugars, as they are important. The α and β symbols denote the anomeric configuration of the sugar, and the number indicates which hydoxyl group of the next sugar is involved in the glycosidic linkage. In all cases, the anomeric position is 1, except in sialic acid where it is 2.

The details of the pathway are presented in Figures 15.2.2 and 15.2.3, along with the sensitivity to each endoglycosidase or glycoamidase. The figures show the steps between high-mannose and hybrid types (Fig. 15.2.2) and complex types (Fig. 15.2.3). The three Glc residues (filled triangles) are removed from properly folded proteins within the ER by two different oligosaccharide-processing α-glucosidases. The first α1-2Glc is cleaved by α-glucosidase I, and the next two α1-3Glc residues by α-glucosidase II (Fig. 15.2.2, step 1). An ER-associated α-mannosidase removes one Man residue (open circle; Fig.

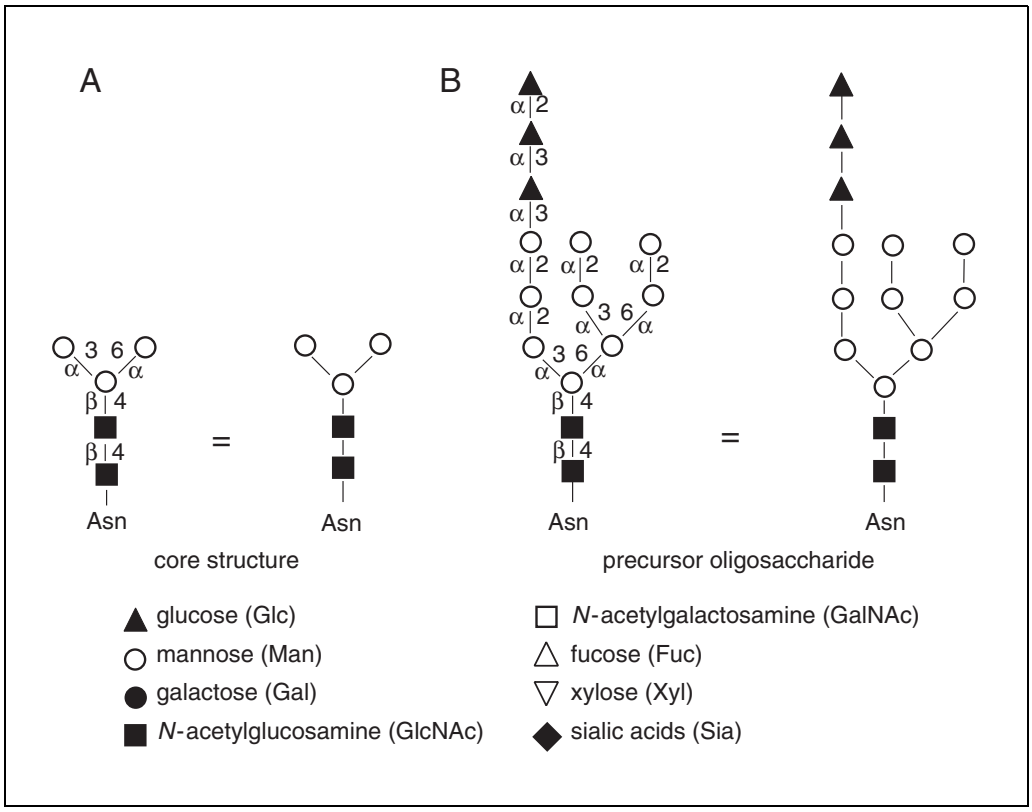


Figure 15.2.1 Symbol structures for the core region and precursor of N-linked sugar chains. Each sugar is given a symbol and abbreviation at the bottom of the figure. Each one except sialic acid uses its anomeric carbon (C-1) for linking to other sugars. Sialic acid uses C-2 for glycosidic linkage to other sugars. Glycosidases and glycosyltransferases are anomeric specific and distinguish α or β configurations of each sugar. The core structure (**A**) is common to all N-linked chains and is composed of three Man and two GlcNAc residues. The α or β configuration of each sugar is indicated, and the OH group to which that sugar is linked is shown on the bar linking the two symbols. Thus, GlcNAcβ1-4GlcNAcβ is represented by two filled squares with β and 4 between them. When a structure is first presented, it will have full display such as that on the left side; if it is repeated, only the symbols will be used as shown immediately to the right. The precursor oligosaccharide (**B**) for all N-linked chains is synthesized in the ER and transferred cotranslationally to the peptide containing an available Asn-X-Thr/Ser sequon.

15.2.2, step 2). The protein then moves on to the first step in Golgi-localized processing—the removal of the three remaining α1-2 Man units by Golgi α-mannosidase I to produce $Man_5GlcNAc_2$ (Fig. 15.2.2, steps 3 and 4). Many proteins have only high-mannose-type oligosaccharides with five to nine Man residues, and no further processing occurs. Alternatively, one to five GlcNAc residues (filled squares) can be added to the trimannosyl core, and these are usually extended with galactose (Gal; filled circles) and sialic acid (Sia; filled diamonds) residues. These extensions, called antennae, are the hallmarks of complex-type oligosaccharides. The transformation of the precursor sugar chain into various high-mannose or complex types is called oligosaccharide processing (Kornfeld and Kornfeld, 1985).

$Man_5GlcNAc_2$ is an important intermediate because it can have several fates. The first is the well-established addition of one GlcNAc residue by GlcNAc transferase I (Fig. 15.2.2, step 6). This is the first step toward the formation of complex chains. However, simply adding Gal and Sia to the terminal GlcNAc of this oligosaccharide forms a hybrid structure (Fig. 15.2.2, steps 12 and 13), where the left side of the molecule looks like a complex chain having one antenna, and the right side still resembles a high-mannose chain. The

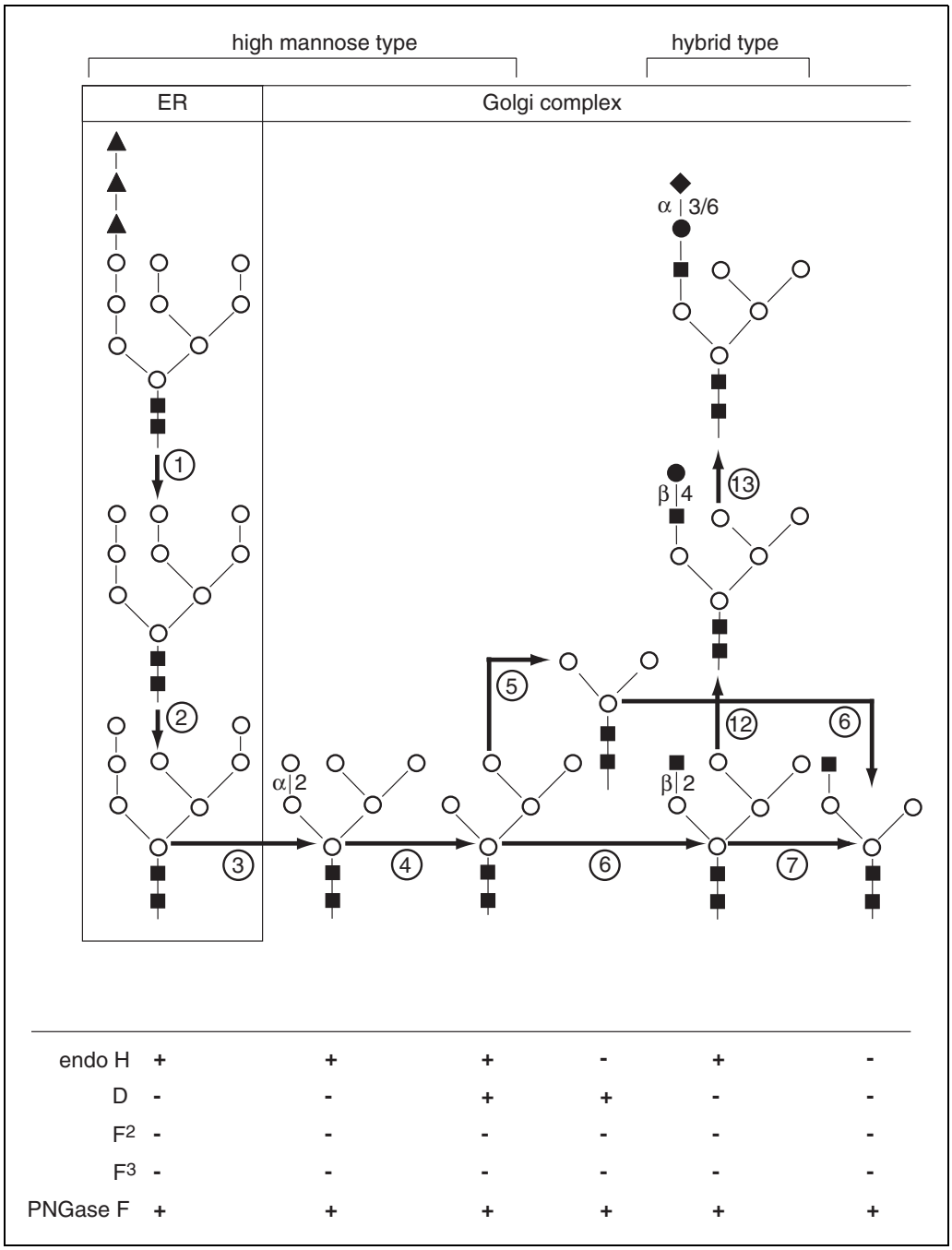


Figure 15.2.2 N-linked oligosaccharide maturation pathway for high-mannose and hybrid types, and sensitivities to various enzymes (see Fig. 15.2.1 for key). Brackets (top) show the structures designated as high-mannose and hybrid chains. The boxes indicate ER or Golgi localization.The pathway begins with the precursor oligosaccharide (see Figure 15.2.1). Each successive numbered step in circles represents a glycosidase or glycosyl transferase that generates a new sugar chain with different sensitivities to the various endoglycosidases or PNGase F. (**1**) precursor oligosaccharide is trimmed by α-glucosidases I and II, removing three Glc. (**2**) ER mannosidase removes one Man. (**3**) α-Mannosidase I in Golgi complex removes two Man to make $Man_6GlcNAc_2$, with a single remaining α1-2Man. (**4**) The final α1-2Man is removed by a Golgi complex α-mannosidase I. (**5**) α-Mannosidase III removes the α1-3 and α1-6Man units to make $Man_3GlcNAc_2$. (**6**) GlcNAc transferase I adds GlcNAc to either $Man_5GlcNAc_2$ or $Man_3GlcNAc_2$. (**7**) α-Mannosidase II removes the α1-3 and α1-6Man units to make $GlcNAc_1Man_3GlcNAc_2$. Sensitivity to various enzymes (bottom) changes when moving from left to right, but remains the same within vertical columns. *NOTE:* This continued maturation to form complex chains is shown in Figure 15.2.3. Additionally, these figures are not comprehensive; many glycosylation steps have not been included, but they do not affect the sensitivities to the enzymes listed.

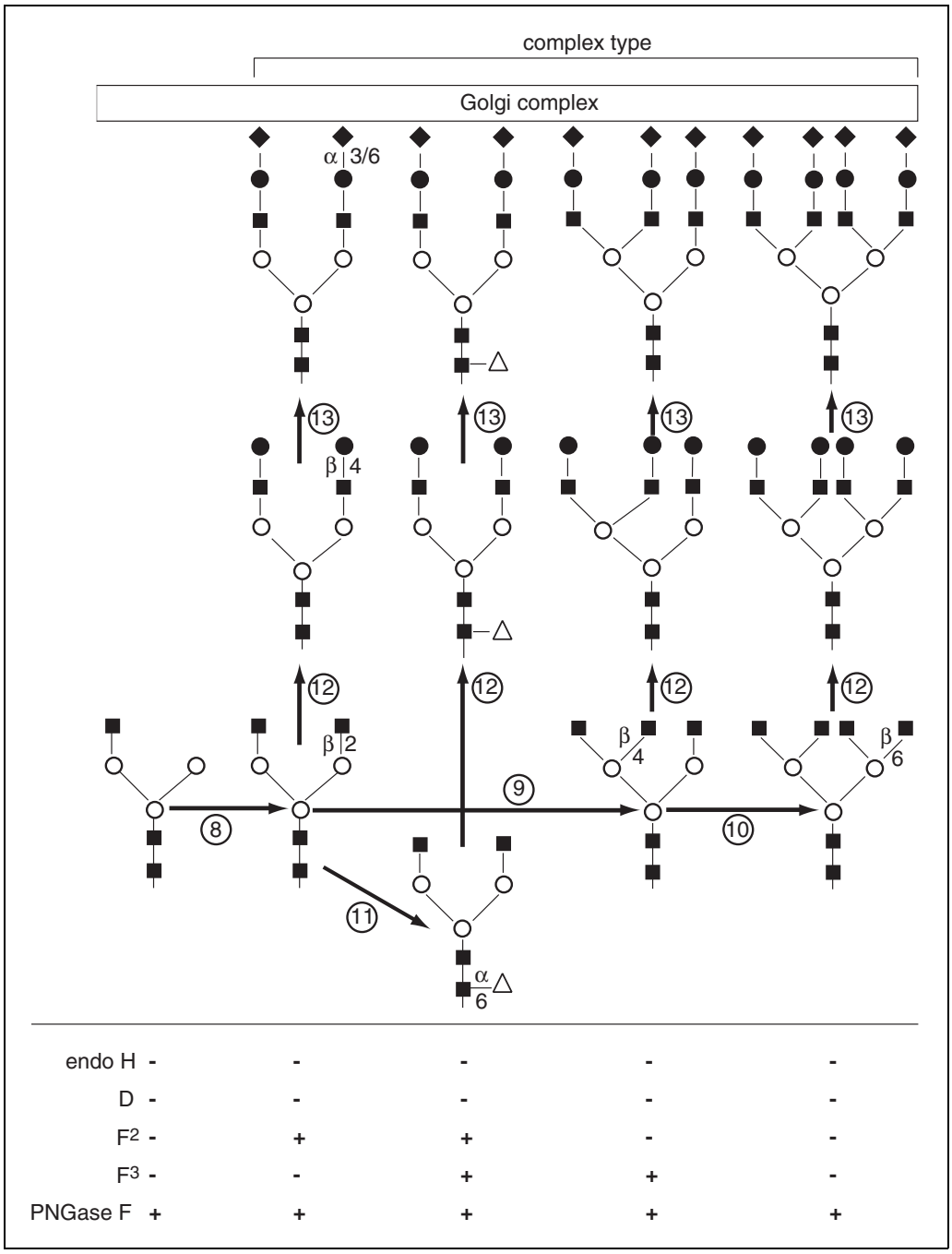


Figure 15.2.3 N-linked oligosaccharide maturation pathway for complex types, and sensitivities to various enzymes (see Fig. 15.2.1 for key; see Fig. 15.2.2 for additional details). (**8**) GlcNAc transferase II adds a second GlcNAc to initiate a biantennary chain. (**9**) GlcNAc transferase IV adds a third GlcNAc to initiate a triantennary chain. (**10**) GlcNAc transferase V adds a fourth GlcNAc to initiate a tetraantennary chain. (**11**) Fucosyltransferase adds α1-6Fuc to the core region of complex chains. (**12**) β1-4Gal is added to available GlcNAc residues of hybrid and complex chains. (**13**) α2-3 or α2-6Sia is added to Gal residues of hybrid and complex chains.

$GlcNAc_1Man_5GlcNAc_2$ structure is the *required* substrate for α-mannosidase II, which removes the two terminal Man units from the upper branch of the chain (i.e., the α1-3Man and α1-6 Man units; Fig. 15.2.2, step 7). This enzyme only works after the addition of the first GlcNAc.

The other fate for the $Man_5GlcNAc_2$ chain is to act as a substrate for the newly identified α-mannosidase III (Chui et al., 1997), which removes the same two Man units as α-mannosidase II, but does not require the prior addition of the first GlcNAc (Fig. 15.2.2, step 5). α-Mannosidases II and III show partial overlap in the Golgi complex, but α-mannosidase III may occur preferentially in an earlier compartment. The $Man_3GlcNAc_2$ product of α-mannosidase III is also a substrate for GlcNAc transferase I. Thus, the same product, $GlcNAc_1Man_3GlcNAc_2$ can be formed in two ways: first, by the sequential action of α-mannosidase III and GlcNAc transferase I (Fig. 15.2.2, steps 5 and 6) or, second, by GlcNAc transferase I and α-mannosidase II (Fig. 15.2.2, steps 6 and 7). Sensitivity to specific enzyme digestions (endo H and endo D) can distinguish which route was taken (Fig. 15.2.2).

GlcNAc transferase II now adds a second GlcNAc to the α1-6-linked Man (Fig. 15.2.3, step 8). This molecule can also have several fates. First, fucose (Fuc) can be added to GlcNAc residue linked to the Asn of the protein (Fig. 15.2.3, step 11). Second, one to three more GlcNAc residues can be added to the core mannose residues to initiate tri- and tetraantennary chains (Fig. 15.2.3, steps 9 and 10), and even pentaantennary chains (not shown). GlcNAc additions are considered to occur in the medial Golgi regions. Each GlcNAc-based branch can be individually modified, but they are usually extended by one Gal (Fig. 15.2.3, step 12) and terminated by a Sia (Fig. 15.2.3, step 13). Both of these sugars are usually thought to be added in *trans*-Golgi cisternae or in the *trans*-Golgi network (TGN). Sometimes selected antennae are also fucosylated in the TGN. One or more terminal Gal residues can be extended by variable-length polylactosamines (Galβ4-GlcNAc repeats) capped by a Sia. GlcNAc and Gal can be sulfated as a late, perhaps even final, step of processing. These extensions/modifications are thought to occur in the late Golgi complex and TGN, but their order and compartmental segregation are not well understood. Other modifications of N-linked sugar chains are known, but there are fewer tools available to analyze their biosynthetic localization.

THE O-LINKED PATHWAY

For practical purposes, only a portion of the O-linked pathway—i.e., the addition of the first few sugars—will be presented. However, it is very important to remember that some of the same outer chain structures such as Sia, polylactosamines, and Fuc residues are common to both N- and O-linked oligosaccharides.

α-*N*-acetylgalactosamine (α-GalNAc; open square) is the lead-off sugar for the O-linked pathway (Fig. 15.2.4; also see Fig. 15.2.1 for symbols). It is added to Ser/Thr residues that occur in the proper configuration, generating a broad variety of acceptor sequences. These sequences often cluster as repeats within mucin-like domains. GalNAc is added in the earliest parts of the Golgi complex, not cotranslationally. GalNAc can be further extended by at least six different sugars. The most common is the addition of a β1-3Gal (Fig. 15.2.4, step 1), forming a disaccharide that is one of the few O-linked chains that can be diagnosed by enzymatic digestions. This disaccharide is often capped by a Sia (Fig. 15.2.4, step 2). Additional sugars such as Sia (Fig. 15.2.4, step 3) or GlcNAc followed by Gal (Fig. 15.2.4, steps 4 and 5) can be added. Structural analysis can be done by sequential exoglycosidase digestion, but given the complexity and heterogeneity of the sugar chains, such analysis is not a very useful indicator for tracking protein movement through the Golgi complex. Many O-linked chains have terminal Sia residues and, when

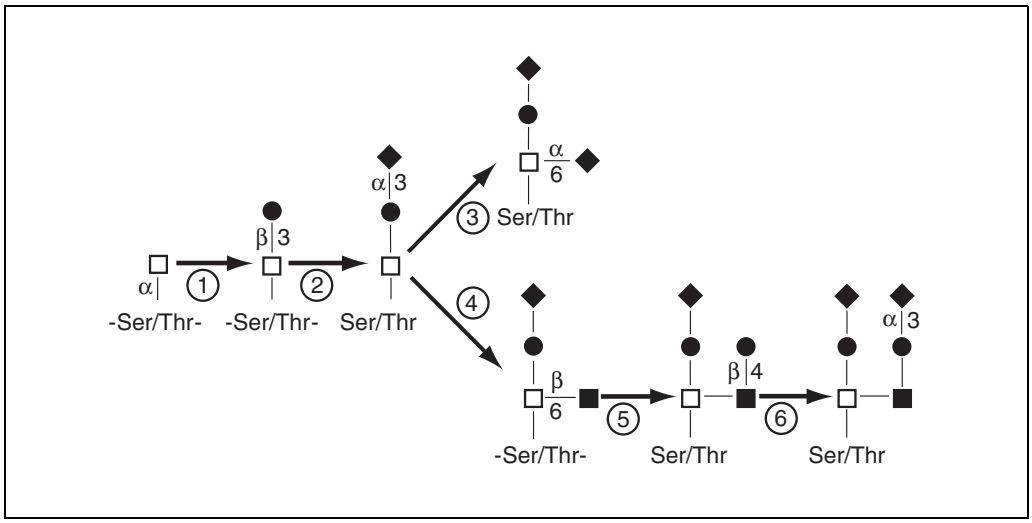


Figure 15.2.4 A small portion of the O-GalNAc pathway (see Fig. 15.2.1 for key). The first step of the O-linked pathway occurs in the early Golgi complex with the addition of α-GalNAc. There are at least six other sugars that can be added at this point in this complex pathway. Often β1-3Gal is added (**1**), quickly followed by α2-3Sia (**2**). The presence of these structures can be detected with a combination of O-glycosidase and sialidase. Additional sugars can be added as shown. α2-6Sia (**3**) or β1-6GlcNAc (**4**) followed by β1-4Gal (**5**) and α2-3Sia (**6**) on Gal. Each of these sugars must be removed before O-glycosidase can cleave the disaccharide.

tightly clustered on Ser/Thr residues, these chains promote proteolysis by O-sialoglycoprotease regardless of the structure of the underlying sugar chain.

Another type of O-linked glycosylation is the addition of glycosaminoglycan (GAG) chains to form proteoglycans. This occurs by a different pathway than the α-GalNAc linkage. Instead, the chains begin by addition of a β-Xylose (Xyl; open inverted triangle) residue to Ser and are then elongated by two Gal residues and a glucuronic acid (GlcA; half-filled diamond) residue. This core structure can be further elongated by the addition of GlcAβ1-3GalNAcβ disaccharides to form the backbone of chondroitin/dermatan sulfate chains, or by GlcAβ1-3GlcNAcα to form the backbone of heparan sulfate chains. Biosynthesis and movement of these proteins have also been followed through the Golgi complex. Initiation begins in late ER/early Golgi complex, and the core tetrasaccharide is probably finished within the medial Golgi, but the addition of chondroitin chains appears to be confined to the TGN. In addition to the well-known O-linked GAG chains, there is clear evidence for the existence of a class of N-linked GAG chains.

ENDOGLYCOSIDASE H DIGESTION

BASIC PROTOCOL 1

Endoglycosidase H (endo H) cleaves N-linked oligosaccharides between the two *N*-acetylglucosamine (GlcNAc) residues (Fig. 15.2.5) in the core region of the oligosaccharide chain (Fig. 15.2.1) on high-mannose and hybrid, but not complex, oligosaccharides. In this protocol, a fully denatured protein is digested with endo H to obtain complete release of sensitive oligosaccharides.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M sodium citrate, pH 5.5
1% (w/v) phenylmethylsulfonyl fluoride (PMSF) in isopropanol

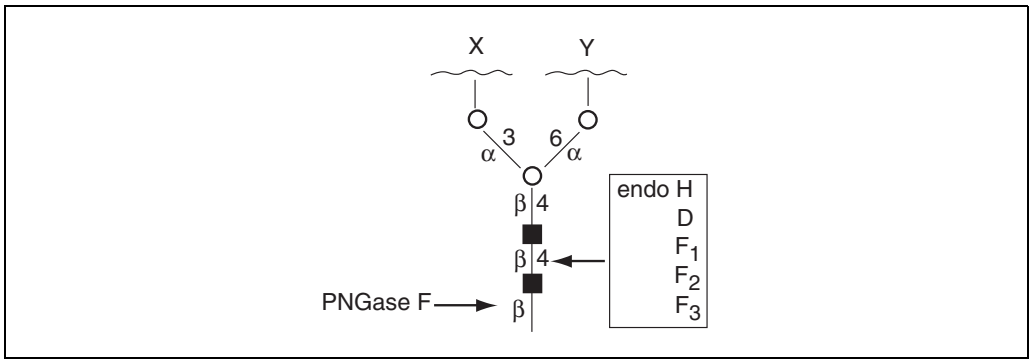


Figure 15.2.5 PNGase F and endoglycosidase-sensitive bonds in the core of N-linked oligosaccharides (see Fig. 15.2.1 for key). PNGase F is a glycoamidase that severs the bond between GlcNAc and Asn, liberating the entire sugar chain and converting Asn into Asp. The endoglycosidases (H, D, and Fs) cleave the bond between the two GlcNAc residues in the core region, leaving one GlcNAc still bound to the protein. The differential specificity of the endoglyosidases is based on the structure of the sugar chain in a fully denatured protein. Incomplete denaturation may not expose all sensitive linkages. X and Y are unspecified sugar residues.

0.5 U/ml endoglycosidase H (endo H; natural or recombinant; Sigma, Glyko, or Boehringer Mannheim)
10× SDS-PAGE sample buffer (*APPENDIX 2A*)
Water baths, 30° to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Add 20 to 30 µl of 0.1 M 2-ME/0.1% SDS to immunoprecipitate in a microcentrifuge tube, mix well, and heat denature 3 to 5 min at 90°C.

   Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

   Protein solubilization in nonionic detergents such as Triton X-100 or Nonidet P-40 is not always sufficient to completely expose all susceptible cleavage sites. Only strong denaturation with SDS exposes all sites for maximum cleavage.

2. Cool and microcentrifuge for 1 sec at 1000 × *g* to collect condensed droplets in the bottom of the tube.

3. Place 10-µl aliquots of solubilized, denatured protein (supernatant) in each of two clean microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   6 µl 0.5 M sodium citrate, pH 5.5
   20 µl H_2O
   2 µl 1% PMSF (in isopropanol)
   1 µl 0.5 U/ml endo H (enzyme digest only; substitute with water in control).

   The PMSF prevents proteolysis. Nonionic detergent is not required to prevent inactivation of endo H as long as high-purity SDS is used.

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate overnight at 30°C to 37°C.

6. Immediately prior to electrophoresis, inactivate endo H by adding 4 µl of 10× SDS-PAGE sample buffer and heating 5 min at 90°C.

7. Analyze protein by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

   The presence of high mannose and/or hybrid N-linked oligsaccharide chains will be evidenced by increased mobility of the digested proteins on SDS-PAGE.

ENDOGLYCOSIDASE D DIGESTION

BASIC PROTOCOL 2

Like endo H, endo D also cleaves between the two GlcNAc residues in the core of the N-linked sugar chains (Fig. 15.2.5). However, its narrow substrate specificity makes it useful for detecting the transient appearance of just a few early processing intermediates. It requires that the 2 position of the α1-3-linked core Man be unsubstituted. This intermediate arises after processing by either α-mannosidase I or III, but prior to addition of the first GlcNAc or action of α-mannosidase II (see Fig. 15.2.2, steps 3 to 5). Cells with a defect in GlcNAc I transferase (e.g., Lec 1 CHO cells) do not add the first GlcNAc residue (Fig. 15.2.2, step 6), and N-linked oligosaccharides will remain sensitive to endo D because they cannot modify the α1-3Man residue.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M NaH_2PO_4, pH 6.5
10% (w/v) Triton X-100 (*APPENDIX 2A*) or Nonidet P-40 (NP-40)
0.5 U/ml endoglycosidase D (endo D; Boehringer Mannheim)
10× SDS-PAGE sample buffer (*APPENDIX 2A*)
Water baths, 37° and 90°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating for 3 to 5 min at 90°C.

   Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge at 1000 × *g* for 1 sec to collect condensed droplets in the bottom of the tube.

3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   2 µl 0.5 M NaH_2PO_4, pH 6.5
   5 µl H_2O
   1 µl 1 IU/ml endo D (enzyme digest only; substitute with water in control).

   The 20-fold excess of nonionic detergent is essential to prevent inactivation of endo D by SDS.

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because

they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C to 95°C.
7. Analyze protein by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

Endo D sensitivity is detected by increased electrophoretic mobility of the digested proteins on SDS-PAGE.

BASIC PROTOCOL 3

ENDOGLYCOSIDASE F_2 DIGESTION

Endo F_2, like endo H and endo D, cleaves between the two GlcNAc residues in the chitobiose core (Fig. 15.2.5). It preferentially releases biantennary complex-type oligosaccharide chains from glycoproteins, but does not cleave tri- or tetraantennary chains.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M sodium acetate, pH 4.5
10% (w/v) Triton X-100 (*APPENDIX 2A*) or Nonidet P-40 (NP-40)
0.1 M 1,10-phenanthroline in methanol
200 mU/ml endoglycosidase F_2 (endo F_2; Glyko)
4× SDS-PAGE sample buffer (*APPENDIX 2A*)
Water baths, 30° to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating for 3 to 5 min at 90°C.

Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge at 1000 × *g* for 1 sec to collect condensed droplets in the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

   15 µl 0.5 M sodium acetate, pH 4.5
   3 µl 0.1 M 1,10-phenanthroline in methanol
   2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   1 µl 200 mU/ml endo F_2 (enzyme digest only; substitute 0.5 M sodium acetate in control).

A 10- to 20-fold excess of nonionic detergent is required to stabilize the enzyme.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate the mixture at 30°C to 37°C overnight.

   Some inactivation of the enzyme occurs at 37°C, even with nonionic detergent present; however, if the enzyme is present in sufficient excess, incubation can generally be carried out successfully at 37°C.

6. Immediately before electrophoresis, inactivate by adding 8 µl of 4× SDS-PAGE sample buffer and heating for 5 min at 90°C to 95°C.

7. Analyze the protein by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

   Sensitivity to endo F_2 is detected by increased electrophoretic mobility on SDS-PAGE.

ENDOGLYCOSIDASE F_3 DIGESTION

BASIC PROTOCOL 4

Endoglycosidase F_3 (endo F_3) is another endoglycosidase with a narrow substrate range and, therefore, high specificity: it cleaves triantennary chains, but not high-mannose, hybrid, nonfucosylated biantennary or tetraantennary chains. A core-fucosylated biantennary chain is the only other demonstrated substrate. When both endo F_3 and endo F_2 digestions are done in parallel on a sample, it can provide evidence for chain branching and core fucosylation. The approach is essentially the same as for the other endoglycosidases.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M sodium acetate, pH 4.5
10% (w/v) Triton X-100 (*APPENDIX 2A*) or NP-40
0.1 U/ml endoglycosidase F_3 (endo F_3; Glyko)
10× SDS-PAGE sample buffer (*APPENDIX 2A*)
Water baths, 37° and 90°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS and heat denature 3 to 5 min at 90°C.

   Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge for 1 sec at 1000 × *g* to collect condensed droplets at the bottom of the tube.

3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   4 µl 0.5 M sodium acetate, pH 4.5
   5 µl H_2O
   1 µl 0.1 U/ml endo F_3 (enzyme digest only; substitute with water in control).

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C to 95°C.
7. Analyze by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

Sensitivity to endo F_3 is detected by increased mobility on SDS-PAGE.

BASIC PROTOCOL 5

PEPTIDE: N-GLYCOSIDASE F DIGESTION

PNGase F is a glycoamidase that cleaves the bond between the Asp residue of the protein and the GlcNAc residue that joins the carbohydrate to the protein (Fig. 15.2.5). Because it liberates nearly all known N-linked oligosaccharides from glycoproteins, it is the preferred enzyme for complete removal of N-linked chains. It is the only enzyme that releases tetra- and pentaantennary chains. The glycoprotein sample must be denatured and digested with PNGase F to remove N-linked oligosaccharides completely.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M Tris·Cl, pH 8.6 determined at 37°C (*APPENDIX 2A*)
10% (w/v) Triton X-100 (*APPENDIX 2A*) or Nonidet P-40 (NP-40)
200 to 250 mU/ml peptide:N-glycosidase F (PNGase F; Sigma or Glyko)
10× SDS sample buffer (*APPENDIX 2A*)
Water baths, 30° to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 90°C.

   Use the larger amount of reagent ≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge for 1 sec at 1000 × *g* to collect condensed droplets in the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

   3 µl 0.5 M Tris·Cl, pH 8.6
   5 µl H_2O
   2 µl 10% NP-40 or Triton X-100
   5 µl 200 to 250 mU/ml PNGase F (enzyme digest only; substitute with 0.5 M Tris·Cl in control).

   Sodium phosphate or HEPES buffer, pH 7.0, can be used instead of Tris·Cl. Avoid potassium buffers because these may cause precipitation of a potassium SDS salt. Use of a nonionic detergent is essential, because SDS inactivates PNGase F. A 10-fold weight excess of any of the above nonionic detergents over the amount of SDS will stabilize the enzyme.

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate overnight at 30°C to 37°C.

6. Immediately prior to electrophoresis, inactivate the enzyme by adding 2.5 µl of 10× SDS-PAGE sample buffer and heating 3 to 5 min at 90°C.

7. Analyze the protein by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

   The presence of N-linked oligosaccharide chains will be evidenced by increased electrophoretic mobility on SDS-PAGE.

ESTIMATING THE NUMBER OF N-LINKED OLIGOSACCHARIDE CHAINS ON A GLYCOPROTEIN

SUPPORT PROTOCOL

One widely used application of endo H or PNGase F digestion is estimation of the number of N-linked oligosaccharide chains on a given glycoprotein. This is done by creating a ladder of partially digested molecules that each differ by only one N-linked sugar chain. The number of separate bands in a one-dimensional polyacrylamide gel (less one for the totally deglycosylated protein) provides an estimate of the number of N-linked chains. The conditions used to generate partially deglycosylated protein must be determined for each protein studied, because the sensitivity of each chain may be different, even when all of them are completely exposed by denaturation. For this protocol, either the incubation time or the amount of enzyme can be varied to determine the best conditions to produce a ladder of partial digests. Usually five or six points are enough to provide a reasonable estimate (Fig. 15.2.6). Of course, it is important to use enough enzyme to obtain complete deglycosylation. This is best done by monitoring the effects of endo H or PNGase F on newly synthesized [^{35}S]Met pulse-labeled protein just after synthesis, but before any N-linked oligosaccharide processing has occurred. Pulse labeling of protein for 10 min with [^{35}S]Met followed by digestion is the best way to be sure that all chains are removed.

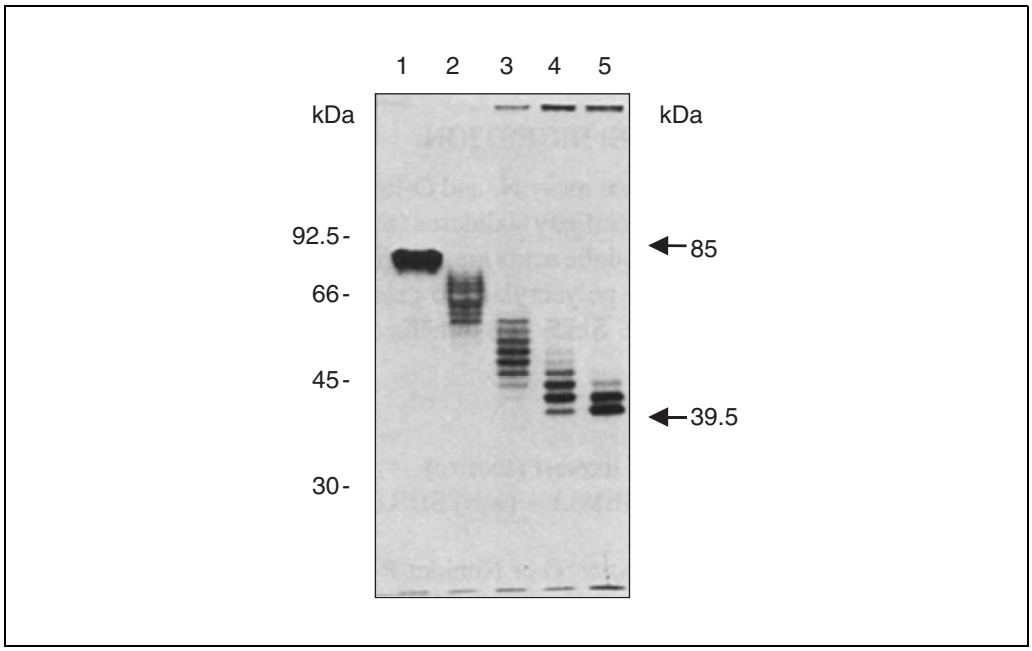


Figure 15.2.6 Data from the estimation of the number of glycosylation sites on lysosome-associated membrane protein 1 (LAMP-1; Viitala et al., 1988). LAMP-1 contains eighteen potential N-linked sites. Graded digestion with increasing amounts of PNGase F was used to generate this ladder of glycoforms. Each band contains at least one less N-linked chain than the band above it. An average N-linked carbohydrate chain has an apparent mass of ~1.5 to 3 kDa. Lysosomal membrane glycoprotein was immunoprecipitated from [^{35}S]Met-labeled cells and the sample was digested with PNGase F for 0 min (lane 1), 5 min (lane 2), 20 min (lane 3), 45 min (lane 4), and 24 hr (lane 5). Figure courtesy of Dr. Minoru Fukuda.

1. Add 0.1 M 2-ME/0.1% SDS solution to the total volume of immunoprecipitated protein required and heat denature by incubating 3 to 5 min at 90°C.

   Each digestion reaction requires 20 µl of immunoprecipitate. Thus, 120 to 140 µl is sufficient for one control plus five or six digests.

2. Cool and centrifuge for 1 sec at 1000 × *g* to collect condensed droplets at the bottom of the tube.

3. Aliquot 10 µl supernatant to the number of microcentrifuge tubes required to cover the concentration range (e.g., 0.01 to 1 mU/ml PNGase F) or incubation times (e.g., 5 to 60 min) plus one for an undigested control.

4. Add remaining reagents as specified for endo H (see Basic Protocol 1, step 4) or PNGase F (see Basic Protocol 5, step 4), adjusting the enzyme concentration as desired.

5. Incubate at 30°C for the desired length of time.

   High enzyme concentration (10 mU/ml) and prolonged incubation (16 hr) must be among the conditions included, in order to ensure that there is a data point for maximum deglycosylation.

   For varying enzyme concentrations, incubate for the same amount of time, but the duration of incubation should be shorter than what would give complete digestion because the goal is to obtain increasing extent of incomplete cleavage.

6. After the desired incubation time, inactivate enzyme by adding 0.1 volume of µl of 10× SDS-PAGE sample buffer and heating 5 min at 90° to 95°C.

7. Analyze the sample from each concentration/time point, including undigested sample, by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

   Most newly formed N-linked chains will have a molecular weight in the range of 1500 to 2200, and loss of one chain is sufficient to change the migration of a protein. This procedure has been used to count up to eighteen N-linked sites on one molecule. A sample result is shown in Figure 15.2.6.

BASIC PROTOCOL 6

SIALIDASE (NEURAMINIDASE) DIGESTION

Sialic acids are the terminal sugars on many N- and O-linked oligosaccharides. The great majority are released with broad-specificity sialidases (neuraminidases) such as that from *Arthrobacter ureafaciens*. Because sialic acids are charged, their loss usually changes the mobility on one-dimensional SDS polyacrylamide gels, but it will always change the mobility on a two-dimensional gel. Since one-dimensional analysis is easier, it can be tried first.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
10% (w/v) Triton X-100 (*APPENDIX 2A*) or Nonidet P-40 (NP-40)
0.5 M sodium acetate, pH 5.0(*APPENDIX 2A*)
1 IU/ml neuraminidase from *Arthrobacter ureafaciens* (Sigma or Glyko)
10× SDS sample buffer (*APPENDIX 2A*)
Water baths, 37° and 90°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), IEF/SDS-PAGE, or NEPHGE/SDS-PAGE (*APPENDIX 3*), and for autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 90°C.

   Denaturation is less important here, because the sialic acids are exposed at the ends of the sugar chains. In most instances, the denaturation step can probably be omitted and the digestion done while the protein is still bound to the beads.

2. Cool and microcentrifuge for 1 sec at 1000 × *g* to collect condensed droplets in the bottom of the tube.

3. Transfer 10-µl aliquots of supernatant to two clean microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   4 µl 0.5 M sodium acetate, pH 5.0
   5 µl H_2O
   1 µl 1 IU/ml neuraminidase (enzyme digest only; substitute with water for control).

   This amount of neuraminidase should be in great excess. Addition of nonionic detergent is not needed if the digestion is done while the protein was still bound to the beads.

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.

   This time can be shortened to 2 hr, if necessary, but longer incubations are better.

6. Immediately prior to electrophoresis, inactivate the enzyme by adding 2 µl of 10× SDS sample buffer and heating 3 to 5 min at 90°C.

   If the protein will be analyzed by IEF or NEPHGE, addition of sample buffer is replaced by lysis buffer used for these techniques.

7. Analyze the protein on the appropriate one-dimentional SDS-PAGE system (*UNIT 6.1*) or by a two-dimentional IEF/SDS-PAGE or NEPHGE/SDS-PAGE system (*APPENDIX 3*), and detect by autoradiography (*UNIT 6.3*).

   Removal of sialic acids usually results in a decrease in apparent molecular weight on one-dimentional gel analysis, or an increase in the isoelectric point of the protein analyzed by two-dimentional gel analysis.

ENDO-β-GALACTOSIDASE DIGESTION

BASIC PROTOCOL 7

The endo-β-galactosidase from *Bacillus fragilis* degrades polylactosamine chains $(Gal\beta1\text{-}4GlcNAc\beta1\text{-}3)_n$ found on both N- and O-linked oligosaccharides. The variable length of these repeating units usually causes the protein to run as a broad band or a smear on the gel. Although not all linkages are equally cleaved by this enzyme (see Fig. 15.2.7), sensitive proteins that often run as broad bands or smears on gels—e.g., lysosome-associated membrane protein 1 (LAMP-1)—produce both sharper bands and lower molecular weight species after digestion.

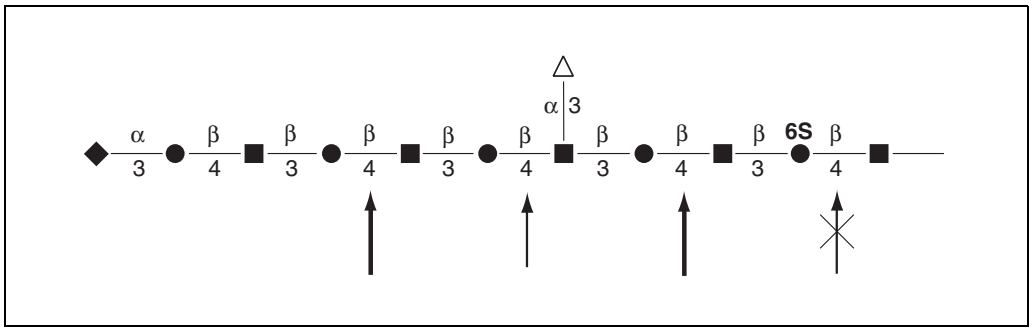


Figure 15.2.7 Endo-β-galactosidase-sensitive linkages in polylactosamines (see Fig. 15.2.1 for key). Linear, unsubstituted polylactosamine units (GlcNAcβ1-3Galβ1-4) are sensitive to digestion with endo-β-galactosidase, while substitutions—such as sulfate esters (S) or branches starting with GlcNAc (not shown)—completely block digestion. Substitution of neighboring GlcNAc with Fuc or sulfate esters slows the rate, but does not block cleavage. Sensitive sites are shown with bold arrows, slowly hydrolyzed sites with a lighter arrow, and resistant bonds are struck out. Various substitutions are possible, leading to broad bands on gels. This will create variable sensitivities, but even partial sensitivity should give a sharper, more defined band.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M sodium acetate buffer, pH 5.8
10% (w/v) Triton X-100 (*APPENDIX 2A*) or Nonidet P-40 (NP-40)
100 mU/ml endo-β-galactosidase (Boehringer Mannheim, Oxford GlycoSystems, Sigma)
10× SDS-PAGE sample buffer (*APPENDIX 2A*)
Water baths, 37° and 95°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.

   Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge 1 sec at 1000 × *g* to collect condensed droplets in the bottom of the tube.

3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

   2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   4 µl 0.5 M sodium acetate, pH 5.8
   5 µl H_2O
   1 µl 100 mU/ml endo-β-galactosidase (enzyme digest only; substitute with water in control).

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 3 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C.
7. Analyze protein by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

If the protein has polylactosamine chains, its mobility should increase after digestion.

ENDO-α-*N*-ACETYLGALACTOSAMINIDASE DIGESTION

BASIC PROTOCOL 8

This enzyme (also known as O-glycosidase or O-glycanase) has limited utility because it is highly specific for cleaving only one O-linked disaccharide, Galβ1-3GalNAcα-Ser/Thr. Adding any more sugars, including sialic acid, renders the molecule resistant to cleavage and requires removal of each residue before the enzyme will work. Prior sialidase digestion is sometimes used (see Basic Protocol 6), and this can be done while the protein is still bound to the immunoprecipitation beads.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M sodium citrate phosphate buffer, pH 6.0, containing 500 µg/ml BSA (complete buffer supplied with enzyme)
10% (w/v) Triton X-100 (*APPENDIX 2A*) or Nonidet P-40 (NP-40)
300 mU/ml endo-α-*N*-acetylgalactosaminidase (5× concentrate from Glyko; use according to directions)
10× SDS-PAGE sample buffer (*APPENDIX 2A*)
Water bath, 37° and 95°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.

   Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge 1 sec at 1000 × *g* to collect condensed droplets in the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

   2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
   4 µl 0.5 M sodium citrate phosphate buffer, pH 6.0, with 500 µg/ml BSA
   3 µl H_2O
   1 µl 300 mU/ml endo-α-*N*-acetylgalactosidase (enzyme digest only; substitute with water in control).

   The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C.
7. Analyze protein by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

If the protein contains the disaccharide unit, the mobility of the protein should increase. Presence of only a single unit (mol. wt. ~400 Da) may be difficult to detect unless a high-resolution gel is used.

BASIC PROTOCOL 9

O-SIALOGLYCOPROTEASE DIGESTION

Digestion with O-sialoglycoprotease requires that the substrate have a tight cluster of sialylated O-linked oligosaccharides. Proteins with a single O-linked chain or a few widely spaced chains will not be cleaved. This property makes the enzyme a valuable diagnostic tool.

Materials

Immunoprecipitated protein of interest (*UNIT 7.2*)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.4 M HEPES buffer, pH 7.4
10% (w/v) Triton X-100 (*APPENDIX 2A*) or Nonidet P-40 (NP-40)
2.4 mg/ml O-sialoglycoprotease (O-sialoglycoprotein endoglycoprotease; Accurate Chemical & Scientific; reconstituted according to directions)
10× SDS-PAGE sample buffer (*APPENDIX 2A*)
Water baths, 37° and 95°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.

Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge 1 sec at 1000 × *g* to collect condensed droplets in the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
4 µl 0.4 M HEPES buffer, pH 7.4
5 µl H_2O
2 µl 2.4 mg/ml O-*s*ialoglycoprotease (enzyme digest only; substitute with water in control).

O-Sialoglycoprotein endopeptidase is a partially purified enzyme, and the specific activity is relatively low. A quantity of 1.0 µg of this enzyme preparation will cleave 5 µg of sensitive substrate per hour at 37°C. Human glycophorin A can serve as a positive control.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2.5 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C.
7. Analyze protein by one-dimensional SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

If the digestion was successful, the target protein will be undetectable or may be cleaved into small fragments.

COMMENTARY

Background Information

The results of digestion of a hypothetical protein with two N-linked carbohydrate chains as it moves through the ER and Golgi complex with various enzymes are shown in Figure 15.2.8. At 0 min, both *N*-linked chains are high-mannose type. They have lost their Glc residues and one Man residue in the ER. Both are sensitive to endo H and PNGase F digestion, yielding a protein with only two remaining GlcNAc residues in the case of endo H digestion, and no carbohydrate at all in the case of PNGase F digestion. These sugar chains are resistant to the other enzyme digestions.

At 45 min, the protein is in the medial Golgi complex and both sugar chains have been processed by Golgi α-mannosidase I. However, one of the chains (left) has been partially processed

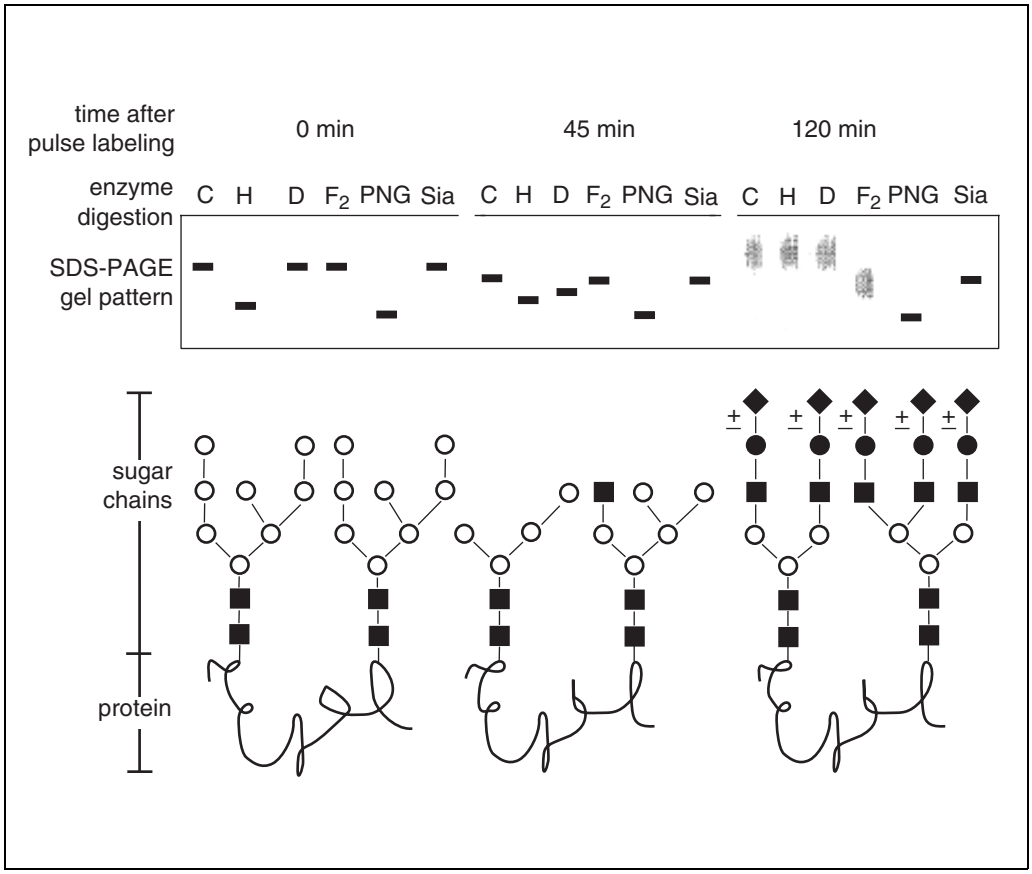


Figure 15.2.8 Schematic diagram showing results that could be obtained for a hypothetical protein with two N-linked glycosylation sites as it moves through the Golgi complex. Assume that the protein has been biosynthetically labeled with an amino acid precursor (such as [^{35}S]Met) for 10 min and chased in the absence of label for 45 min and 120 min. The protein is then precipitated with a specific antibody. At each time point, equal amounts of the sample are analyzed by flourography after one-dimensional SDS-PAGE, either without any digestion (control; C) or following digestion with endo H (H), endo D (D), endo F_2 (F_2), PNGase F (PNG), or sialidase (Sia). Oligosaccharide structures consistent with the banding patterns are shown below the schematic gel pattern.

by Golgi α-mannosidase III to an endo H–resistant/endo D–sensitive chain. The other chain (right) has received a GlcNAc residue from GlcNAc transferase I, but has not encountered Golgi α-mannosidase II. This chain is still endo H sensitive and endo D resistant. Both chains are sensitive to PNGase F, but neither is sensitive to endo F_2 or sialidase digestion.

At 120 min, both chains have fully matured to complex-type chains. One is a sialylated biantennary chain and the other is a sialylated triantennary chain. Note that each sialic acid is marked ±, indicating that not all molecules are fully sialylated, accounting for the broader bands. Neither sugar chain is sensitive to endo H or endo D digestion. The biantennary chain (left) is sensitive to endo F_2 cleavage, leaving one GlcNAc residue on the protein, but the triantennary chain (right) is not sensitive to this enzyme. Both chains are cleaved by PNGase F. All sialic acids are removed by sialidase to produce a sharp band, but the underlying sugar chains remain.

Endoglycosidase H

Endo H from *Streptomyces plicatus* cleaves the bond between the two GlcNAc residues in the core of N-linked oligosaccharides. One GlcNAc residue remains attached to the protein or peptide and the remainder of the chain is released as an intact unit (Tarentino et al., 1989). The oligosaccharide structures cleaved by endo H are shown schematically in Figure 15.2.5. Substrates for endo H include all high-mannose oligosaccharides and certain hybrid types, but not bi-, tri-, tetra- or pentaantennary (complex) chains. Endo H also cleaves oligosaccharides that have a1-6 fucose residues bound to the reducing (protein to carbohydrate linkage) GlcNAc residue (Tarentino et al., 1989).

Endo H sensitivity is the most common way to trace the movement of newly synthesized glycoproteins from the endoplasmic reticulum (ER) into the Golgi complex. Proteins remain sensitive to endo H while they are in the ER and in early regions of the Golgi complex; they become endo H–resistant after they are processed by enzymes located in the medial Golgi complex. Endo H cleaves the N-linked oligosaccharides from proteins as long as they have not lost the α1-3Man residue cleaved by Golgi mannosidase II or mannosidase III (Fig. 15.2.2). After removal of that mannose, the oligosaccharide becomes endo H resistant. Endo H is the best enzyme for identifying high-mannose and hybrid chains and their general transition toward complex chains.

Using endo H alone gives an incomplete map of subcellular trafficking. The resolution of this picture can be enhanced by combining it with endo D digestions. In general, N-linked chains become endo H resistant at about the same time they become transiently sensitive to endo D. However, the discovery of the alternative pathway for complex *N*-glycan processing using α-mannosidase III now gives a different perspective. Both α-mannosidase II and III are expressed in most mouse tissues except for hematopoietic cells, which seem to have only α-mannosidase II. So it is conceivable that some proteins, and even different N-linked chains on a single protein, can be processed by α-mannosidase II and others by α-mannosidase III, yielding complex digestion patterns and kinetics of endo H and endo D sensitivity.

Endoglycosidase D

The narrow substrate specificity of endo D makes it useful for detecting a very restricted set of processing intermediates, particularly for distinguishing α-mannosidase II from α-mannosidase III processing. When the results of endo D digestion are combined with the results of an endo H digestion of the same sample, it can help determine which of the two alternate processing pathways is being used. The key requirement for endo D cleavage is an unsubstituted 2 position on the α1-3Man that forms the trimannosyl core (Fig. 15.2.1). This window of endo D sensitivity occurs only *after* removal of the α1-2Man residue by α-mannosidase I and *before* the addition of a GlcNAc residue to this same position by GlcNAc transferase I (Beckers et al., 1987; Davidson and Balch, 1993). The immediate precursor of both pathways is $Man_6GlcNAc_2$, which is endo D resistant/endo H sensitive. The better-known α-mannosidase II pathway involves the removal of the last α1-2Man residue by α-mannosidase I (making the oligosaccharide endo D sensitive/endo H sensitive), and then the addition of GlcNAc via GlcNAc transferase I (making it endo D resistant/endo H sensitive). This product, and only this one, is the substrate for α-mannosidase II, which removes two of the remaining five Man residues, specifically the α1-3Man and α1-6Man that form the upper branch. Once this occurs, this endo D–resistant molecule also becomes endo H resistant. This structure is also a substrate for GlcNAc transferase II on the way to forming a biantennary chain.

The second pathway involves α-mannosidase III, which was identified as a functionally

important enzyme in nearly all tissues when the α-mannosidase II pathway was ablated in mice (Chui et al., 1997). After α-mannosidase I removes the final α1-2Man from $Man_6GlcNAc_2$ to generate $Man_5GlcNAc_2$, α-mannosidase III cleaves the same two Man residues as α-mannosidase II; however, α-mannosidase III cleavage does not require addition of the first GlcNAc that α-mannosidase II requires. α-Mannosidase III creates the endo D–sensitive/endo H–resistant trimannosyl core. This sugar chain serves as an acceptor for GlcNAc transferase I to form an endo D–resistant/endo H–resistant molecule that can be modified by GlcNAc transferase II, and then on to biantennary chains. As summarized in Figure 15.2.2, using the two endoglycosidases together will distinguish whether the oligosaccharide was processed by α-mannosidase II or α-mannosidase III. This may be important since there is evidence that distribution of the two enzymes in the Golgi complex is different with α-mannosidase III in an earlier compartment. It is important to point out that all chains of even a single protein are not necessarily processed using all the same enzymes.

Mutant cell line CHO Lec1 lacks GlcNAc transferase I activity and cannot synthesize either complex or hybrid chains. These cells can be used to measure the kinetics of acquiring endo D sensitivity (Beckers et al., 1987). This cell line can be obtained through ATCC (CRL-1735) . The chains become permanently endo D sensitive, because they lack GlcNAc transferase I and cannot be converted back to an endo D–resistant form. These chains will also remain endo H sensitive, because α-mannosidase II requires GlcNAc transferase I. However, if any chains are processed by α-mannosidase III, they would be become endo H resistant. Again, the combination of endo D and endo H digestions can reveal which pathway was used.

CHO Lec1 cells are also useful for tracking the movement of a protein from the ER into the earliest Golgi compartment where α-mannosidase I is located. Acquisition of endo D sensitivity requires the action of this enzyme. The advantage of using Lec1 cells is that the proteins remain permanently endo D sensitive and there is no risk of kinetically missing that small window of sensitivity before the sugar chain might become endo D resistant once again. Even if α-mannosidase III acts on the protein, it would still remain endo D sensitive, and no further processing would occur.

Endoglycosidase F and peptide N-glycosidase F

Elder and Alexander (1982; Alexander and Elder, 1989) made a landmark discovery when they identified an enzyme in culture filtrates of the bacterium *Flavobacterium meningosepticum* that cleaved N-linked oligosaccharides from glycoproteins. This preparation had a broad substrate specificity. The endoglycosidase activity in this preparation (endo F) was actually due to a set of enzymes, each with a more restricted substrate range. Like endo H, the endo F enzymes cleave the sugar chains between the two core GlcNAc residues (Fig. 15.2.5). Endo F was originally thought to be a single enzyme, but it is now known that each of the three enzymes has a distinct specificity (Plummer and Tarentino, 1991). The specificity of endo F_1 is very similar to that of endo H, while endo F_2 prefers biantennary chains, and endo F_3 will cleave core fucosylated biantennary and triantennary, but not tetraantennary, chains (Fig. 15.2.2 and Fig. 15.2.3). All are commercially available from Glyko.

Plummer et al. (1984) carefully analyzed *Flavobacterium* filtrates and found that the very broad substrate range was actually due to a glycoamidase activity rather than an endoglycosidase activity. The glycoamidase releases the entire carbohydrate chain from the protein by cleaving the Asp-GlcNAc bond (Fig. 15.2.5). The enzyme is called by various names, including peptide:N-glycosidase F (PNGase F), glycopeptidase F, and N-glycanase (previously available from Genzyme), but the proper name is peptide *N*-4(*N*-acetyl-β-glucosaminyl)asparagine amidase F. PNGase F has the broadest specificity, and it releases most of the N-linked oligosaccharide chains from proteins.

Endo F_2 and Endo F_3

Endo F_2 prefers biantennary chains over high-mannose chains by ~20 fold. Thus, endo H and PNGase F are better choices for broadly distinguishing high-mannose from complex chains as described above in the endo H protocol (Tarentino and Plummer, 1994).

Many proteins have core-fucosylated N-linked glycans, and the addition of fucose can be used as an additional trafficking marker. Endo F_3 will hydrolyze triantennary chains, but endo F_2 will not. Endo F_2 hydrolyzes biantennary chains; however, endo F_3 will also hydrolyze core-fucosylated biantennary chains only a bit more slowly than it does triantennary chains. Thus, if all the chains on a protein are

sensitive to endo F_2 (biantennary) and to endo F_3, this is evidence for the presence of a core fucose on those chains.

The enhancement of endo F_3 activity on biantennary chains with a core α1-6Fuc points out that some specificities are really a matter of relative rates of cleavage. If both endo F_2 (biantennary) and endo F_3 (triantennary and biantennary with core fucose) cleave the protein, they may be acting on different chains on the same molecule. If there is only a single chain, repeat the experiment under the same conditions using 10- to 20-fold dilutions of each enzyme. If both still cleave the chain about equally, it is evidence for core fucosylation of a biantennary chain.

PNGase F

PNGase F has the broadest specificity of all the enzymes that cleave N-linked oligosaccharides. It is indifferent to all extended structures on the chains, such as sulfate, phosphate, polylactosamines, polysialic acids, and even the occasional glycosaminoglycan chain. Most of the modifications in the $Man_3GlcNAc_2$ core region also make no difference in chain cleavage. The only oligosaccharide structural feature that confers PNGase F resistance is the presence of an α1-3Fuc on the GlcNAc bound to Asn (Tretter et al., 1991). This modification is commonly found in plants and in some insect glycoproteins, but it is rare in most mammalian cell lines. However, caution is warranted, as there is evidence that some mammalian cells do have the critical α1-3Fuc transferase, and some studies show that a majority of N-linked chains of bovine lung are actually PNGase F resistant! It is not known how common this resistance may be. It is thus important to document N-glycosylation with proteins still in the ER (see Support Protocol) before they might be processed to a PNGase F–resistant form.

Sialidase

Sialidases are also called neuraminidases because the most common form of sialic acid is *N*-acetyl neuraminic acid. The sialic acids are a family with over forty different members, but fortunately the very great majority of them can be removed from the oligosaccharides by the broad-spectrum sialidase from *Arthrobacter ureafaciens* (AUS). It can even digest polysialic acids, a rare modification found on only a few proteins such as neuronal cell adhesion molecule (NCAM). Sialidases with selected specificities from other sources are available but would not usually be needed. AUS has an optimum pH of 5.0, with ~30% of maximum activity at pH 7.

Because sialic acids are charged, they affect gel mobility of proteins more than would be expected from their nominal molecular weight. The magnitude of the gel shift depends on the number of residues. It is difficult to estimate their number by sialidase digestion, but the mobility change is usually sufficient if there are several sialic acid residues. On the other hand, if a protein has only one sialic acid, its presence could be missed using standard one-dimensional SDS-PAGE. To be certain of the effects of sialidase, the sample can be analyzed by a two-dimensional system, using IEF or NEPHGE in the first dimension. The loss of even a single sialic acid will be evident because it changes the isoelectric point.

AUS will remove sialic acids from both N- and O-linked chains, so the type of chain carrying them must be determined independently using PNGase F or possibly O-glycosidase in combination with sialidase. A protein will generally be partially or completely resistant to O-glycosidase because the required disaccharide, Galβ1-3GalNAc, is usually extended and often sialylated. Until the sialic acid is removed, it will be resistant.

The presence of sialic acid (sialidase sensitivity) is often used as an indication of the transport of a protein into the *trans*-Golgi network (TGN). This may be true in general, but it is important to remember that the distribution of Golgi enzymes is cell type dependent. For instance, α-mannosidases I and II, which are typically considered *cis*/medial Golgi enzymes, are strongly expressed on the brush border of enterocytes—hardly a Golgi compartment. There are other similar examples of various distributions of sialyl transferase. Moreover, there are different sialyl transferases and each may have its own unique distribution. Although one should be cautious, it is probably safe to place sialyl transferase in the late Golgi compartment rather than an early one.

Endo-α-N-acetylgalactosaminidase

This enzyme from *Dipplococcus pneumoniae* also goes by various names, including O-glycosidase and O-Glycanase. The last name is a trade name from Genzyme, which no longer sells enzymes; the enzyme is now available from Glyko. This enzyme has a narrow substrate range and cleaves only Galβ1-3GalNAcα-Ser/Thr. These are only the first two sugars added in the diverse O-linked pathway that can produce glycans with a dozen or more

sugar units. A portion of the pathway is shown in Figure 15.2.4. Fortunately, many, but far from all, O-linked chains have the simple trisaccharide structure and would be sensitive to cleavage after removing the Sia. Thus, sequential individual digestions or mixed digestions can be used. As both Gal and GalNAc (and probably Sia) are added in the early Golgi complex, sensitivity to the enzyme shows that the protein carries O-linked chains, but matching enzyme sensitivity and a Golgi compartment to further chain extension is difficult. Combining a battery of exoglycosidases (sialidase, α-fucosidase, α-*N*-acetylgalactosaminidase, and β-hexosaminidase) with endo-α-*N*-acetylgalactosaminidase will probably remove most O-linked sugar chains, except sulfated ones. The bottom line is that it is easy to use the enzyme in combination with sialidase to show that a protein has simple O-linked chains, but it is difficult to conclude much more concerning either the structure of the sugar chain or intracellular trafficking.

Endo-β-galactosidase

Bacterioides fragilis endo-β-galactosidase is one of several enzymes that specifically degrade polylactosamines by cleaving linear chains of GlcNAcβ1-3Galβ1-4 repeats at the Galβ1-4 linkage. Any substitution on the galactose itself blocks cleavage; however, modifications of the neighboring sugars can slow hydrolysis (Fig. 15.2.7). For instance, fucosylation and/or sulfation of nearby GlcNAc slows cleavage, but chain branching or sulfation at Gal block it. Even with these potential complexities, digestion with endo-β-galactosidase will sharpen a broad band even if it does not cleave every linkage. The repeating GlcNAcβ1-3Galβ1-4 units can be found on both N- and O-linked chains, so sensitivity to PNGase F digestion can potentially distinguish the location. Lysosome-associated membrane protein 1 (LAMP-1) has polylactosamine repeats on N-linked chains. Remember that glycosylation is not template driven, so oligosaccharides often exist as a continuum of different structures on individual proteins. For example, heavily sulfated polylactosamine repeats are also known as keratan sulfate and are degraded by keratanases.

O-Sialoglycoprotease

O-Sialoglycoprotease (also called O-glycoprotease or O-sialoglycoprotein endopeptidase; Mellors and Lo, 1995) is a neutral metalloprotease produced by *Pasteurella haemolytica*. This enzyme requires clusters of sialylated oligosaccharides on Ser or Thr residues (Norgard et al., 1993). Having a single sialylated O-linked sugar chain on a protein will not lead to degradation, nor will having a nonsialylated sugar chain. Therefore, this enzyme can be used in a typical pulse-chase experiment to indicate whether there are tightly grouped O-linked chains and when they are sialylated. Adding the initial α-GalNAc in the early Golgi complex is insufficient to cause proteolysis; sialylation is specifically required, and this may occur in a later Golgi compartment. Proteolysis can generate smaller fragments of a target protein that are still visible on gels, or the fragments may be so small that they are not even seen on the gels. It depends on the protein. Many leukocyte antigens such as the P-selectin ligand, or others such as CD43, CD44, CD45, and CD34 found on hematopoietic stem cells, are all substrates. As sialic acids are found on both N- and O-linked chains (in clusters and not), sequential digestions using PNGase F, sialidase, and O-sialoglycoprotease endopeptidase in different orders can reveal different kinds of sialylated glycans. If used as part of a pulse-chase protocol, they can reveal different kinetic subcompartments. Not all sialyl transferases are necessarily within the same Golgi compartment.

Critical Parameters

For nearly all of the digestions, complete denaturation of the protein can be important, as maximum deglycosylation occurs only when the sugar chains of glycoproteins are completely exposed. This is not important for sialidase digestions since sialic acids are exposed. Usually endo H digestions will work without full denaturation, but the unprocessed high-mannose chains remain unprocessed because they are often less exposed to the processing enzymes. This may or may not be true of the target protein, but it is better to be safe and denature it completely before digestion. Heating the immunoprecipitate with 0.1 M 2-mercaptoethanol/0.1% SDS is the best way to release the protein in a denatured state. Assuming that the immunoprecipitate contains <10 μg of protein, 30 μl of 0.1% SDS still provides a three-fold excess over the protein. Adding SDS presents another problem: free SDS may denature the digesting enzyme before it has a chance to finish its job. For most enzymes, be sure that the free SDS concentration is <0.01%. The best way to do this is to add a 10- to 20-fold weight excess of nonionic detergent with a low critical

micellar concentration (e.g., Triton X-100 or NP-40). These detergents will form mixed micelles with the free SDS and keep it from denaturing the added enzymes.

The amount of enzyme and the incubation time recommended in the protocols are in excess and should be sufficient to cleave any of the sensitive linkages. The incubation times can be shortened, if necessary, but it is better to keep the enzyme concentration as indicated.

Many of the digestions (e.g., sialidase, O-sialoglycoprotease) can be adapted for use on membrane preparations or on live cell surfaces by simply omitting the ionic and nonionic detergents and decreasing the incubation time. The problem is that some linkages may not be exposed and/or sensitive to the digestion. Thus, the usefulness of this approach needs to be determined on a case-by-case basis.

Endo H

Endo H has a broad pH optimum between 5.5 and 6.5, and phosphate or citrate/phosphate buffers can be used in place of citrate. Endo H is very stable to proteases, freezing and thawing, and prolonged incubations. No additives are required for storage of the enzyme. At concentrations below 5 to 10 µg/ml (200 to 400 mU/ml), endo H will bind to glass, so it should be stored in plastic vials (e.g., screw-cap microcentrifuge tubes).

Endo D

Endo D has a broad pH optimum of 4 to 6.5. One unit of enzyme activity will cleave 1 µmole of a $Man_5GlcNAc_3$ glycopeptide per min at 37°C. It is supplied from Boehringer Mannheim as a powder containing 0.1 U of activity. Adding 0.1 ml of water gives a 20 mM phosphate buffer, pH 7, 0.05% sodium azide, and 5 mg/ml BSA. It is stable for 3 months at 4°C or at −20°C, but freezing and thawing should be avoided. There may be a slight contamination (<0.2%) with β-*N*-acetylglucosaminidase activity.

Endo F

Commercial endo F preparations are mixtures of endo F_1, F_2, and F_3. Endo F preparations should not be used for routine deglycosylation or to draw conclusions about the structure of the released oligosaccharides unless the specificity is clearly defined.

Endo F_2

Endo F_2 has a broad pH optimum of 4 to 6 and retains >50% of its activity at pH 7. The enzyme is sensitive to SDS, but adding nonionic detergents prevents denaturation of the enzyme by SDS. Although the enzyme is stable at 4°C for months, it can be frozen in aliquots at −70°C as long as repeated freeze/thaw cycles are avoided. The 1,10-phenanthroline can be used to inhibit a trace of a zinc metalloprotease that may be present.

PNGase F

The pH optimum for PNGase F is 8.6, but 80% of full activity occurs between 7.5 to 9.5 with a range of buffers including phosphate, ammonium bicarbonate, Tris·Cl, and HEPES. Borate buffers inhibit the enzyme. Commercial PNGase F is endo F free and is stable for 6 months at 4°C, or indefinitely at −70°C. However, it should be stored in small aliquots and repeated freeze/thaw cycles should be avoided. PNGase F will bind to glass and plastic surfaces and should not be stored in dilute solutions (<0.1 mU/ml). All of the unit activities of commercial preparations are based on cleavage of dansylated glycopeptides; they are expressed in nmoles/min, which are actually mU, not true International Units (1 International Unit = 1 µmole/min). SDS inactivates PNGase F, but adding a ten-fold weight excess of nonionic detergents protects the enzyme (Tarentino et al., 1989; Tarentino and Plummer, 1994).

Sialidase

AUS is available as a lyophilyzed powder from typical commercial sources such as Sigma, Boehringer Mannheim, and Glyko. It should be reconstituted in water at 1 to 10 mU/µl according to manufacturer's directions. It is stable for 6 months at 4°C. Treatment with sialidase is also used in assays of protein transport to the cell surface.

Endo-α-N-acetylgalactosaminidase (O-glycosidase)

Endo-α-*N*-acetylgalactosaminidase has a pH optimum of 6.0 and has 50% activity at 5.5 to 7.0. The thiol inhibitor parachloromercuric benzoate (PCMB; 1 mM) inactivates the enzyme, and 1 mM EDTA inhibits it (63%), as do Mn^{2+} and Zn^{2+} (50%). Chloride also inhibits the enzyme, so HCl-containing buffers should be avoided. The enzyme will have full access to the sugar chains only after denaturating the protein with SDS, but the excess SDS needs to be removed by forming mixed micelles with nonionic detergents. The enzyme is stable at 4°C and at −20°C, but freeze/thaw cycles should be avoided.

Endo-ß-galactosidase

The enzyme is supplied by several commercial sources, including Glyko and Sigma. The enzyme is free of contaminating endo- and exoglycosidases. It has a pH optimum of 5.8 and should be stored at −20°C, but is stable for ≥2 months at 4°C. Glyko supplies the enzyme as a lyophilyzed powder with BSA for stability. EDTA, Ca^{2+}, Mn^{2+}, and Mg^{2+} do not affect stability or activity, but PCMB inactivates it.

O-Sialoglycoprotease

The partially purified enzyme is supplied by Accurate Chemical and Scientific as a lyophilyzed powder containing nonsubstrate bovine serum proteins and HEPES buffer. The enzyme should be reconstituted according to the manufacturer's instructions, divided into aliquots appropriate for a single use, and stored at −20°C. Freeze/thaw cycles inactivate the enzyme, and it is inhibited by EDTA or 1,10-phenanthroline. It is possible to check the activity with a positive control of glycophorin A, which is available through Sigma.

Troubleshooting

Most of the procedures should work as described, but there is a chance that the enzyme is inactive because of a variety of factors such as age, poor storage, or excess SDS. To check activity, it is worthwhile to run a positive control digestion using the same solutions including SDS and nonionic detergents as for the samples. Since the positive controls are simply glycoproteins that are visualized by Coomassie or silver staining, this requires running a separate gel for staining. This should not be required on a routine basis if the enzymes are used and stored as directed. The most likely culprit in failed digestions is using SDS solutions that are too old or too impure.

Anticipated Results

If the digestions are effective, the labeled band will usually show increased mobility on the gel. In rare instances, digestions can actually decrease mobility. The amount of change will depend on the contribution of that component to the overall mass of the protein. As mentioned before, a gel system that allows visualization of a 1-kDa change should be used. Proteins that are >100 kDa may cause problems for fine resolution. Here are a few numbers to keep in mind.

1. The smallest N-linked chain ($Man_3GlcNAc_2$) will have a mass of ~0.9 kDa.

2. Two sialic acids on a single N-linked biantennary chain will have a mass of ~0.6 kDa, but their loss may appear larger. If they occur on clustered O-linked chains (sialoglycoprotease sensitive), the apparent size difference will be even larger.

3. Most polylactosamines are three or more repeats, and therefore their mass would be ~1 kDa. The protein will probably run as a heterogeneous smear or broad band before digestion.

4. A single O-glycosidase-sensitive disaccharide (0.4 kDa) may be below detection limits.

Time Considerations

All digestions can be done overnight for convenience, but the amount of enzymes should be sufficient for complete digestion in less time. The gels are run the next day, but the time needed for the development of autoradiograms will depend on the strength of the signal. A low-abundance protein labeled for 10 to 30 min with [^{35}S]Met may give a weak signal and require long exposures (e.g., 2 weeks). Trafficking of abundant glycoproteins such as viral coat proteins require only short exposure times (e.g., a few hours).

Literature Cited

Alexander, S. and Elder, J.H. 1989. Endoglycosidases from *Flavobacterium meningosepticum*: Application to biological problems. *Methods Enzymol.* 179:505-518.

Beckers, C.J.M., Keller, D.S., and Balch, W.E. 1987. Semi-intact cells permeable to macromolecules: Use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell* 50:523-534.

Chui, D., Oh-Eda, M., Liao, Y.-F., Panneerselvam, K., Lal, A., Marek, K.W., Freeze, H.H., Moremen, K.W., Fukuda, M.N., and Marth, J.D. 1997. Alpha-mannosidase-II deficiency results in dyserythropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. *Cell* 90:157-167.

Davidson, H.W. and Balch, W.E. 1993. Differential inhibition of multiple vesicular transport steps between the endoplasmic reticulum and trans Golgi network. *J. Biol. Chem.* 268:4216-4226.

Elder, J.H. and Alexander, S. 1982. Endo-β-*N*-acetylglucosaminidase F: Endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high mannose and complex glycoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 79:4540-4544.

Kornfeld, R. and Kornfeld, S. 1985. Assembly of asparagine-linked qoligosaccharides. *Annu. Rev. Biochem.* 54:631-664.

Mellors, A. and Lo, R.Y. 1995. *O*-Sialoglycoprotease from *Pasteurella haemolytica*. *Methods Enzymol.* 248:728-740.

Norgard, K.E., Moore, K.L., Diaz, S., Stults, N.L., Ushiyama, S., McEver, R.P., Cummings, R.D.,

and Varki, A. 1993. Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated *O*-linked oligosaccharides. *J. Biol. Chem.* 268:12764-12774.

Plummer, T.H. Jr. and Tarentino, A.L. 1991. Purification of the oligosaccharide-cleaving enzymes of *Flavobacterium meningosepticum*. *Glycobiology* 1:257-263.

Plummer, T.H. Jr., Elder, J.H., Alexander, S., Phelan, A.W., and Tarentino, A.L. 1984. Demonstration of peptide:*N*-glycosidase F activity in endo-β-*N*-acetylglucosaminidase F preparations. *J. Biol. Chem.* 259:10700-10704.

Tarentino, A.L. and Plummer, T.H. Jr. 1994. Enzymatic deglycosylation of asparagine-linked glycans: Purification, properties, and specificity of oligosaccharide-cleaving enzymes from *Flavobacterium meningosepticum*. *Methods Enzymol.* 230:44-57.

Tarentino, A.L., Trimble, R.B., and Plummer, T.H. Jr. 1989. Enzymatic approaches for studying the structure, synthesis, and processing of glycoproteins. *Methods Cell Biol.* 32:111-139.

Tretter, V., Altmann, F., and März, L. 1991. Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F cannot release glycans with fucose attached α1→3 to the asparagine-linked *N*-acetylglucosamine residue. *Eur. J. Biochem.* 199:647-652.

Viitala, J., Carlsson, S.R., Siebert, P.D., and Fukuda, M. 1988. Molecular cloning of cDNAs encoding lamp A, a human lysosomal membrane glycoprotein with apparent Mr approximately equal to 120,000. *Proc. Natl. Acad. Sci. U.S.A.* 85(11):3743-3747.

Key References

Beckers et al., 1987. See above.

Describes the use of Lec 1 CHO cells and endo D to study processing.

Chui et al., 1997. See above.

Demonstrates the importance of α-mannosidase III.

Kornfeld and Kornfeld, 1985. See above.

Landmark review of processing.

Tarentino and Plummer, 1994. See above.

Best and most recent review of the use of these enzymes.

Contributed by Hudson H. Freeze
The Burnham Institute
La Jolla, California

Endocytosis: Biochemical Analyses

Cells internalize a large variety of macromolecules from the extracellular milieu (e.g., growth hormones, nutrients, viruses) by the process of endocytosis (Mellman, 1996; Mukherjee et al., 1997). In addition, endocytic membrane trafficking plays an important role in the constitutive and regulated targeting of proteins among cellular membrane compartments such as the plasma membrane, endosomes, and the *trans*-Golgi network (TGN). Thus, endosomal trafficking includes the internalization of membrane from the cell surface, intracellular traffic among membrane compartments, and the recycling of membrane from endosomes back to the cell surface. This unit describes biochemical methods for analyzing the kinetics of endocytic trafficking. One set of assays describes the use of radio-iodinated transferrin to analyze trafficking of the transferrin receptor. The other set describes the use of radio-iodinated antibody to analyze trafficking of any membrane protein that cycles through the plasma membrane.

The transferrin receptor is the most commonly used probe of endocytic trafficking (Dautry-Varsat et al., 1983; Klausner et al., 1983). Extracellular diferric transferrin binds to transferrin receptor on the cell surface (Fig. 15.3.1). This complex is rapidly internalized through clathrin-coated pits into endosomes. Iron is released from transferrin at the acidic lumenal pH of endosomes. The iron-free transferrin remains bound to the receptor because the pH of endocytic recycling compartments is mildly acidic. Iron-free transferrin is recycled back to the plasma membrane with transferrin receptor. At the neutral extracellular pH, iron-free transferrin is released from the receptor. The fact that transferrin remains bound to the receptor for the complete endocytic cycle makes transferrin an ideal ligand for studies of intracellular trafficking and recycling.

Basic Protocol 1 is a method for measuring the steady-state surface-to-internal distribution of the transferrin receptor, and Alternate Protocol 1 is a method for measuring the steady-state surface-to-internal distribution of other proteins that cycle through the plasma membrane. Support Protocol 1 describes how to load transferrin with iron, and Support Protocol 2 is a method for radio-iodinating transferrin. Basic Protocol 2 is a method for measuring the internalization rate of the transferrin receptor, Alternate Protocol 2 measures internalization of other proteins, and Alternate Protocol 3 measures internalization in cells grown in suspension. Basic Protocol 3 is a method for measuring the recycling rate of the transferrin receptor. Basic Protocol 4 is a method for measuring the recycling rate of a membrane protein using an antibody as a probe. Basic Protocol 5 is a method for measuring the proteolysis of internalized proteins. Basic Protocol 6 is a method for measuring fluid-phase uptake. Finally, two methods for inhibiting endocytosis by clathrin-coated pits are described in Support Protocol 3 and Support Protocol 4.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX ID*).

MEASURING THE STEADY-STATE SURFACE-TO-INTERNAL DISTRIBUTION OF THE TRANSFERRIN RECEPTOR

BASIC PROTOCOL 1

The steady-state distribution of a membrane protein between the surface and cell interior is dependent on its rate of internalization and its rate of recycling. This distribution is measured for the transferrin receptor by incubating cells with radio-iodinated transferrin and determining the amounts of transferrin on the cell surface and inside cells at steady state. There are two methods for measuring surface-bound label. In one method, transfer-

Contributed by Timothy E. McGraw and Agathe Subtil
Current Protocols in Cell Biology (1999) 15.3.1-15.3.23

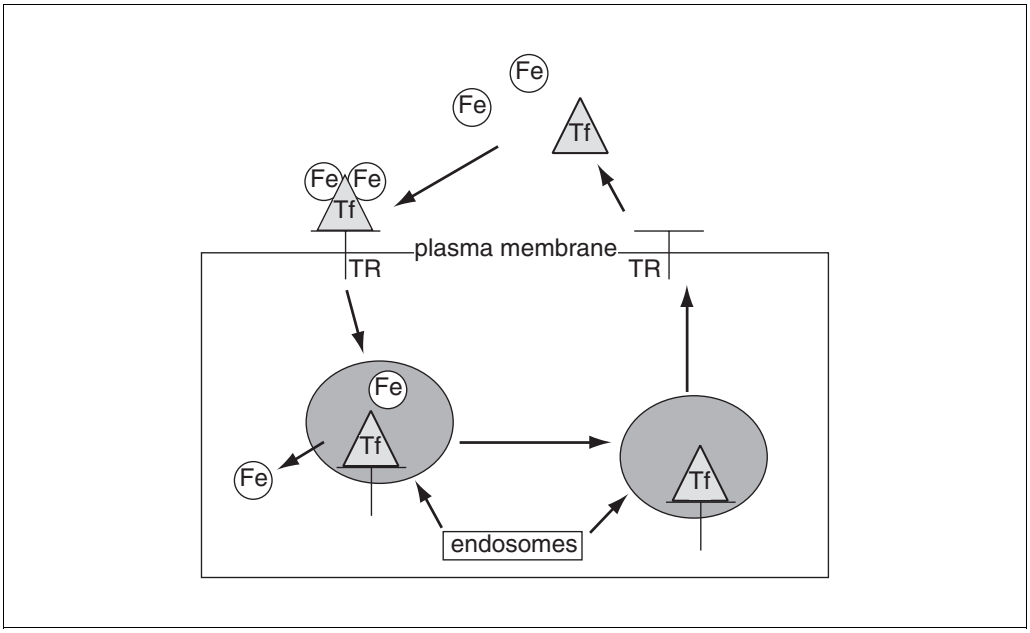


Figure 15.3.1 Transferrin and transferrin receptor cycle. At the near-neutral extracellular pH, diferric transferrin binds to the transferrin receptor with high affinity ($k_d \approx 10^{-9}$), whereas at neutral pH, iron-free transferrin has ~10-fold lower affinity for the receptor. The transferrin receptor–transferrin complex is concentrated in clathrin-coated pits and rapidly internalized into endosomes. At the mildly acidic pH of endosomes, Fe is released from transferrin. However, at this pH iron-free transferrin remains bound to the receptor. Because the endosomal recycling compartments are mildly acidic, the iron-free transferrin remains bound to the receptor until the complex is recycled back to the cell surface. When the iron-free transferrin is exposed to the near-neutral extracellular pH, it is released form the receptor. The unoccupied receptor can bind diferric transferrin and mediate another round of transferrin internalization. The iron-free transferrin binds two atoms of iron, and then binds the transferrin receptor. The transferrin receptor is constitutively cycled regardless of occupancy with ligand. For details see Dautry-Varsat et al. (1983). Tf, transferrin; TR, transferrin receptor.

rin released by an acidic pH wash is measured. In the second method, the amount of transferrin bound to cells at 4°C is measured. Measurement of surface transferrin receptor by incubating cells at 4°C usually gives more consistent results and is the preferred method. Since transferrin does not alter the trafficking of the transferrin receptor, either method can be used. If, however, ligand binding to its receptor changes trafficking of the receptor (e.g., induces internalization), then surface binding should be measured by collecting the acid wash and the two neutral pH washes (step 7).

Materials

Cells of interest
SF medium (see recipe)
^{125}I-labeled human diferric transferrin (~500 cpm/ng; see Support Protocol 2)
Unlabeled (nonradioactive) human diferric transferrin
Neutral pH buffer (see recipe), ice cold
pH 2.0 buffer (see recipe), ice cold
Solubilization solution: 1% (w/v) Triton X-100 in 0.1 N NaOH
10 mg/ml BSA

6-well tissue culture plates
γ counter and tubes

Prepare cells

1. Plate 1×10^5 cells/well in SF medium in two 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency ($\sim 5 \times 10^5$/well) on the day of the experiment.

   Bicarbonate-buffered medium is used if the experiment is performed in a CO_2 incubator. HEPES-buffered medium is used if the experiment is performed in air.

Measure intracellular transferrin

2. Wash one 6-well plate of cells with 2 ml/well SF medium.
3. Add 1 ml SF medium containing a saturating concentration (3 μg/ml) of ^{125}I-labeled transferrin to four wells (total radioactivity wells). Add 1 ml SF medium containing 3 μg/ml ^{125}I-labeled transferrin and a 200-fold excess of unlabeled diferric transferrin to the other two wells (nonspecific radioactivity wells).
4. Incubate at 37°C for a sufficient time for all transferrin receptors to be occupied with ^{125}I-labeled transferrin.

   This time is determined empirically (see Basic Protocol 4). For most cell lines, a 120-min incubation is sufficient.

5. Place plate on ice and wash six times with 2 ml/wash prechilled neutral pH buffer.

   Endocytosis is inhibited at 4°C. The neutral pH washes will not remove transferrin bound to the transferrin receptor.

   CAUTION: *Dispose of radioactive waste appropriately.*

6. Incubate cells with 1 ml prechilled pH 2.0 buffer for 5 min at 4°C.

   This incubation removes transferrin bound to the cell surface. Internal transferrin is not released from the cells.

7. Remove and discard the pH 2.0 buffer. Wash the cells twice with prechilled neutral pH buffer.
8. Add 1 ml solubilization solution to each well and transfer the solubilized cells to tubes suitable for γ counting. Rinse wells once with 1 ml water, and pool with the solubilization solution.
9. Determine the amount of ^{125}I-labeled transferrin per tube using a γ counter.

   These values are the amount of intracellular transferrin.

Measure cell-surface transferrin

10. Wash the second 6-well plate with SF medium. Discard wash and incubate in fresh SF medium at 37°C for the same time as in step 4.

    This incubation can be performed concurrently with the incubation in step 4.

11. Place cells on ice and wash three times with 2 ml/wash prechilled neutral pH buffer.
12. Add 1 ml of prechilled (4°C) neutral pH buffer containing a saturating concentration (3 μg/ml) of ^{125}I-labeled transferrin (3 μg/ml) and 1 mg/ml BSA to four wells (total radioactivity wells). Add 1 ml of the same solution containing a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific radioactivity wells).
13. Incubate the cells at 4°C for 2 hr.

    Endocytosis is blocked at 4°C; therefore, the amount of transferrin bound at 4°C is a measure of the amount of receptor on the cell surface.

14. Place plate on ice and wash the cells six times with 2 ml/wash prechilled neutral pH buffer.

    CAUTION: *Dispose of radioactive waste appropriately.*

15. Solubilize cells and determine the amount of ^{125}I-labeled transferrin per tube as described in steps 8 and 9.

    These values are a measure of the amount of transferrin receptor on the cell surface at steady state.

Analyze data

16. Calculate the specific radioactivity of both intracellular and surface transferrin by subtracting the average of the two nonspecific radioactivity wells from the average of the four total radioactivity wells.

17. Present data as the surface-to-internal ratio or as the amount of transferrin on the surface as percent of the total.

    In CHO cells, the steady-state surface-to-internal ratio for the transferrin receptor is ~0.5 (~35% on the surface; Johnson et al., 1994).

    Another method for determining the surface-to-internal distribution of the transferrin receptor is to collect and count the pH 2.0 buffer and neutral buffer washes of steps 6 and 7. In this method, the surface-to-internal ratio for each well is determined after correcting for nonspecific radioactivity. However, the authors find that the procedure outlined in Basic Protocol 1 yields more reproducible results.

ALTERNATE PROTOCOL 1

MEASURING THE STEADY-STATE SURFACE-TO-INTERNAL DISTRIBUTION OF OTHER MEMBRANE PROTEINS

The surface-to-internal ratio of any membrane protein that constitutively cycles between intracellular compartments and the plasma membrane can be measured using Basic Protocol 1 and a radio-iodinated antibody to the extracellular domain of the protein. Iodinated Fab fragments should be used, because bivalent antibodies cause cross-linking and therefore may modify the protein's trafficking (Weissman et al., 1986). Fab fragments can be prepared using the ImmunoPure Fab preparation kit (Pierce) and iodinated using Support Protocol 2. An excess of unlabeled antibody is used to determine nonspecific binding.

The procedures are the same as described in Basic Protocol 1, except that ^{125}I-labeled Fab fragments (2 to 10 μCi/μg) are used in place of ^{125}I-labeled transferrin. Some antibodies are not efficiently stripped from the surface with a single incubation in pH 2.0 buffer. If this is the case, several rounds of alternating acid/neutral washes can be used to increase the stripping efficiency. Some antibodies are resistant to the acid stripping. In these cases, incubation with 1 mg/ml proteinase K or pronase at 4°C for 30 min (or more) can be used to release surface-bound antibody.

BASIC PROTOCOL 2

MEASURING THE KINETICS OF TRANSFERRIN INTERNALIZATION

Internalization of the transferrin receptor is measured by monitoring the internalization of ^{125}I-labeled transferrin. Cells are washed with an acidic pH buffer to distinguish internalized transferrin from transferrin bound to receptors on the cell surface. The initial transferrin receptor internalization rate constant is the slope of a plot of the ratio of internalized transferrin to surface transferrin versus time.

Materials

Cells of interest
SF medium (see recipe)
^{125}I-labeled human diferric transferrin (~500 cpm/ng; see Support Protocol 2)
Unlabeled (nonradioactive) human diferric transferrin
Neutral pH buffer (see recipe), ice cold
pH 2.0 buffer (see recipe), ice cold
Solubilization buffer: 1% (w/v) Triton X-100 in 0.1 N NaOH
10 mg/ml BSA

6-well tissue culture plates
γ counter and tubes

Prepare cells

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency (~5×10^5/well) on the day of the experiment.

   Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

   One 6-well plate is required for each time point and an additional plate is required to measure steady-state surface transferrin binding. Typically four time points are used, in which case a total of five 6-well plates are required.

Measure internalized transferrin

2. On the day of the experiment, wash the cells from plates for specific time points once with 2 ml SF medium and incubate in SF medium for 1 hr at 37°C.

   During this incubation, any cell-associated transferrin from the growth medium will be recycled and dissociated from the cells, ensuring that the receptors will be unoccupied at the beginning of the experiment.

3. Starting with a single 6-well plate, add 1 ml SF medium containing a saturating concentration (3 μg/ml) of ^{125}I-labeled transferrin to four wells (total radioactivity wells) and add 1 ml SF medium containing ^{125}I-labeled transferrin and a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific radioactivity wells).

4. Incubate the plate for the desired time at 37°C.

   Incubation time points of <10 min are typically used (e.g., 2, 4, 6, and 8 min). Internalized transferrin is eventually returned to the cell surface; therefore, it is important to use incubations times in which little of the transferrin is recycled.

5. Remove the plate from the incubator, place on ice, and wash six times with 2 ml/wash prechilled neutral pH buffer.

   Endocytosis is inhibited at 4°C. The neutral pH washes will remove transferrin not specifically bound to the transferrin receptor.

   CAUTION: *Dispose of radioactive waste appropriately.*

6. Incubate the cells with 1 ml/well prechilled pH 2.0 buffer for 5 min at 4°C.

   This incubation removes transferrin bound to the cell surface. Internal transferrin is not released from the cells.

7. Remove and discard the pH 2.0 buffer. Wash the wells twice with prechilled (4°) neutral pH buffer.

8. Repeat steps 3 to 7 with the plates for the remaining time points (but not with the plate for steady-state surface binding). Treat each time point individually.

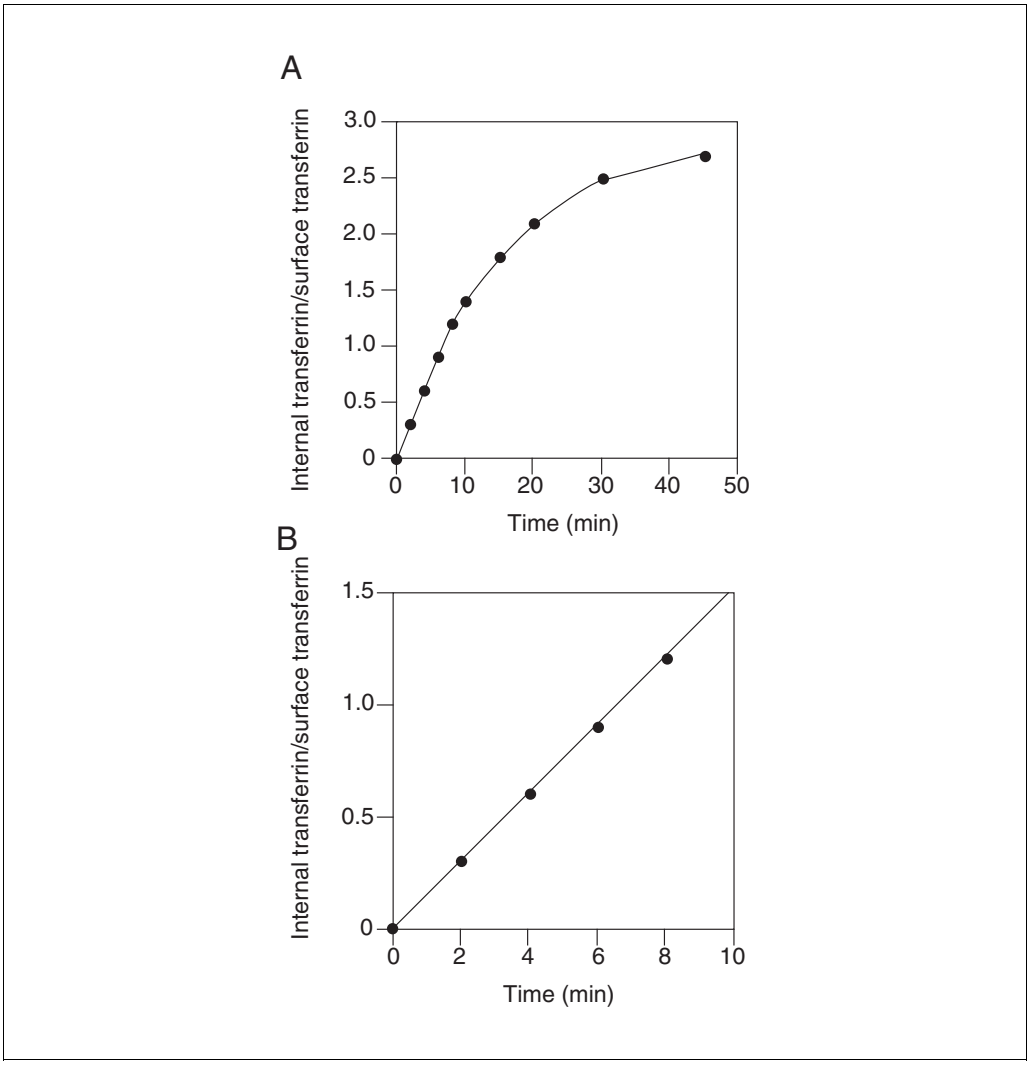


Figure 15.3.2 Data from an idealized transferrin internalization assay are shown. (**A**) Data are shown for an assay carried out for a time sufficient to reach steady state. Data from the early time points are replotted on an expanded time scale in (**B**). In both graphs the ratios of internal transferrin to steady-state surface transferrin are plotted as a function of time.

9. Add 1 ml of solubilization solution to each well. Remove the solubilized cells by trituration and transfer to tubes suitable for γ counting. Rinse wells once with 1 ml water, and pool with the solubilization solution.
10. Determine the amount of ^{125}I-labeled transferrin per tube using a γ counter.

These values are the amount of transferrin internalized during the incubation at 37°C.

Measure steady-state surface transferrin

11. Wash the steady-state plate with 2 ml SF medium. Discard wash and incubate in fresh SF medium for 1 hr at 37°C.

This incubation can be performed concurrently with the incubations of step 4.

12. Place cells on ice and wash three times with 2 ml/wash prechilled neutral pH buffer.
13. Add 1 ml prechilled neutral pH buffer containing a saturating concentration of ^{125}I-labeled transferrin (3 μg/ml) and 1 mg/ml BSA to four wells (total radioactivity wells). Add 1 ml of the same solution containing a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific radioactivity wells).

14. Incubate the cells at 4°C for 2 hr.

    Endocytosis is blocked at 4°C; therefore, the amount of transferrin bound at 4°C is a measure of the receptors on the cell surface.

15. Place the plate on ice and wash the cells six times with 2 ml/well neutral pH buffer.

    CAUTION: *Dispose of radioactive waste appropriately.*

16. Solubilize the cells and quantify the amount of ^{125}I-labeled transferrin as in steps 9 and 10.

    This value is the amount of transferrin receptor on the cell surface at steady state.

Analyze data

17. Calculate the specific radioactivity of intracellular and surface transferrin by subtracting the average of the two nonspecific radioactivity wells from the average of the four total radioactivity wells.

18. Calculate the internal-to-surface transferrin ratio and plot versus time (see Fig. 15.3.2).

    The data should yield a straight line extrapolating through zero. The slope of this line is the internalization rate constant (Wiley and Cunningham, 1982). In CHO cells, the internalization rate constant of the transferrin receptor is ~0.15 min^{-1} (Johnson et al., 1994). If the slope decreases at the later time points (i.e., if the line flattens), then shorter time points must be used. The internal-to-surface plot will begin to flatten as internalized transferrin is recycled back to the cell surface.

    Another method for determining the amount of transferrin bound to the surface is to collect and count the pH 2.0 buffer and neutral buffer washes of steps 7 and 8. For each time point, the total radioactivity removed by the acid wash and the total cell-associated radioactivity are individually corrected for nonspecific radioactivity, and the internal-to-surface ratio is determined for each well. The average of these values for each time point is plotted as a function of time. However, the authors find that the procedure described in Basic Protocol 2 yields more reproducible results.

MEASURING THE KINETICS OF MEMBRANE PROTEIN INTERNALIZATION USING ^{125}I-LABELED ANTIBODIES

ALTERNATE PROTOCOL 2

For membrane proteins with no known ligand, antibodies against the extracellular domain can be used to measure internalization. For these experiments an ^{125}I-labeled antibody against the extracellular domain is used. Fab fragments should be used, because bivalent antibodies cause cross-linking and therefore may modify the protein's trafficking (Weissman et al., 1986). Fab fragments can be prepared using the ImmunoPure Fab preparation kit (Pierce) and labeled using Support Protocol 2. An excess of unlabeled antibody is used to determine nonspecific binding.

The procedures are the same as that described in Basic Protocol 2, except that iodinated Fab antibody fragments are used instead of ^{125}I-labeled transferrin. Some antibodies are not efficiently stripped from the surface with a single incubation in pH 2.0 buffer. If this is the case, several rounds of alternating acid/neutral wash can be used to increase the stripping efficiency. Some antibodies are resistant to the acid stripping. In these cases, incubation with 1 mg/ml proteinase K or pronase at 4°C for 30 min (or more) can be used to release surface-bound antibody.

ALTERNATE PROTOCOL 3

MEASURING THE KINETICS OF TRANSFERRIN INTERNALIZATION IN CELLS GROWN IN SUSPENSION

For cells grown in suspension (e.g., K562 human erythroleukemia cells), the procedure is basically the same, but the washes are more tedious because they require centrifugation. To avoid handling too many tubes, each time point is a single determination. Nonspecific labeling is determined on two of the time points, and the results are averaged and subtracted from every time point.

Additional Materials *(also see Basic Protocol 2)*

Suspension culture of interest
HEPES-buffered SF medium (see recipe)
pH 2.0 medium, ice cold: medium formulation used to grow cells (e.g., RPMI, DMEM) with 25 mM sodium acetate, adjusted to pH 2.0 with HCl
pH 11.0 medium, ice cold: medium formulation used to grow cells (e.g., RPMI, DMEM) with 25 mM Tris base, adjusted to pH 11.0 with NaOH

Prepare cells

1. For this assay, ~1×10^5 cells are used per time point. Transfer the number of cells sufficient for all time points to a 15-ml conical centrifuge tube. Pellet the cells by centrifuging 5 min at 500 × *g*, room temperature.

   Typically five time points are used, so a total of 5×10^5 cells would be sufficient.

2. Aspirate medium, wash cells once with 10 ml HEPES-buffered SF medium, and centrifuge 5 min at 500 × *g*, room temperature.

3. Incubate in 10 ml SF medium for 30 min at 37°C.

   During this incubation, any cell-associated transferrin from the growth medium will be recycled and released from the cells, ensuring that the receptors will be unoccupied at the beginning of the experiment.

4. Centrifuge cells (5 min at 500 × *g*, room temperature). Resuspend in 0.8 ml SF medium and place at 37°C.

Perform timed internalization

5. Transfer 0.2 ml cell suspension to a microcentrifuge tube and add 0.6 mg/ml unlabeled transferrin to the 0.2-ml aliquot. Place tube in a 37°C water bath.

   These cells are for nonspecific labeling (see step 8).

6. For $t = 0$, add 3 µg/ml ^{125}I-labeled transferrin to the 15-ml conical tube containing 0.6 ml cell suspension. Place tube at 37°C.

7. At the appropriate time points (e.g., 1, 2, 3, 4, and 5 min), resuspend the cells in the 15-ml tube (containing labeled transferrin only) by gently inverting the tube. Transfer 100 µl of cell suspension into a new 1.5-ml tube containing 1 ml prechilled neutral pH buffer on ice. Repeat for each time point.

   Diluting the cells in a large volume of cold neutral buffer ensures that endocytosis is stopped at once. Internalized transferrin is eventually returned to the cell surface, therefore it is important to use incubations times in which little of the transferrin is recycled. In K562 cells, incubation times <5 min are commonly used.

8. Add 3 µg/ml of ^{125}I-labeled transferrin to the tube containing 0.2 ml cell suspension and 0.6 mg/ml transferrin (step 5). Place the tube at 37°C. At the appropriate time points (e.g., 2 and 5 min), invert the tube to resuspend the cells and transfer 100 µl of the suspension to a new 1.5-ml conical tube containing 1 ml prechilled neutral buffer on ice. Repeat the procedure for the second time point.

   These are the nonspecific uptake samples.

9. Wash the time point three times with 1 ml/wash prechilled neutral pH buffer. Centrifuge each wash for 5 min at 500 × *g*, 4°C.

   The authors have also successfully used shorter centrifugations at higher speed (e.g., 15,000 × g for 20 sec).

   CAUTION: *Dispose of radioactive waste appropriately.*

10. Incubate cells with 0.2 ml prechilled pH 2.0 medium for 2 min at 4°C.

    This incubation removes transferrin bound to the cell surface.

11. Add 0.2 ml prechilled pH 11.0 medium to bring the cell to a neutral pH, and repeat centrifugation.

12. Transfer supernatant to a tube suitable for γ counting (surface radioactivity). Resuspend cell pellet in 200 µl water and transfer to another tube suitable for counting (intracellular radioactivity).

13. Determine the amount of ^{125}I-labeled transferrin in each tube.

Analyze data

14. Calculate the average surface radioactivity of the two tubes containing nonradioactive transferrin (nonspecific surface radioactivity). Subtract this value from the surface radioactivity of each of the sample time points to give the specific surface radioactivity.

15. Similarly, calculate the average nonspecific intracellular radioactivity and subtract this from the intracellular radioactivity of each of the sample time points.

16. Express the results as the ratio of internal to surface label versus time.

MEASURING THE KINETICS OF TRANSFERRIN RECEPTOR RECYCLING

BASIC PROTOCOL 3

The recycling of the transferrin receptor from endosomes back to the cell surface is examined by measuring the release of transferrin into the medium. For these studies, cells are incubated with ^{125}I-labeled transferrin to achieve steady-state occupancy of the transferrin receptor with transferrin. Transferrin bound to the cell surface is removed, and the release of internal transferrin from cells is then monitored as a function of time. Because transferrin remains bound to the transferrin receptor in intracellular endosomal compartments, the release of previously endocytosed transferrin into the medium reflects the return of transferrin receptors from endosomes to the plasma membrane. The iron-free transferrin returned to the plasma membrane with the recycled transferrin receptor is released from the receptor at the extracellular pH.

Materials

Cells of interest
SF medium (see recipe)
^{125}I-labeled human transferrin (see Support Protocol 2)
Unlabeled (nonradioactive) human diferric transferrin
pH 5.0 buffer (see recipe), prewarmed to 37°C
Efflux medium (see recipe), prewarmed to 37°C
Solubilization solution: 1% (w/v) Triton X-100 in 0.1 N NaOH

6-well tissue culture plates
γ counter and tubes

Prepare cells

1. Plate 1.5×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Prepare one plate for each time point. Allow the cells to reach ~80% confluency on the day of the experiment.

   Six time points are typically used. The time points used depend on the rate of recycling of the cell line being studied. In CHO cells, which have a half-time for transferrin receptor recycling of ~2 min, time points of 2.5, 5, 10, 15, 20, and 30 min are used.

   Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

2. Wash cells with 2 ml SF medium.

Remove surface transferrin

3. Add 1 ml SF medium containing a saturating concentration (3 μg/ml) of ^{125}I-labeled transferrin to four wells (total radioactivity wells). Add 1 ml of SF medium containing a saturating concentration of ^{125}I-labeled transferrin and a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific wells).
4. Incubate at 37°C for a time sufficient to reach steady-state occupancy of the transferrin receptor with transferrin (~20 min for most cell types).
5. Remove the first plate from the incubator, wash the cells with 2 ml SF medium, and incubate for 2 min at room temperature with 1 ml prewarmed (37°C) pH 5.0 buffer.

   Either the citrate- or the MES-based pH 5.0 buffer can be used.

   CAUTION: *Dispose of radioactive waste appropriately.*

6. Discard the pH 5.0 buffer and wash the wells three times over the course of 1 min at room temperature with prewarmed (37°C) efflux medium.

   Incubation in pH 5.0 buffer followed by washes with efflux medium promotes the release of transferrin bound to receptors on the cell surface.

Measure recycled transferrin

7. Add 1 ml prewarmed efflux medium to each well and incubate at 37°C for the desired amount of time (see time points in step 1).

   The unlabeled transferrin and the iron chelator desferrioxamine in the efflux medium ensure that the released radioactive iron-free transferrin is not reloaded with iron, and therefore cannot rebind the transferrin receptor.

8. Repeat steps 5 through 7 on each six-well plate (i.e., time point).
9. At the appropriate time, remove the plate from the incubator and transfer the efflux medium to a suitable tube for γ counting. Wash the cells (rapidly) with 1 ml efflux medium and add this wash to efflux medium in the tube.
10. Add 1 ml solubilization solution to each well. Remove the solubilized cells by trituration and transfer to tube suitable for γ counting. Rinse the wells once with 1 ml water, and pool with the solubilization solution.
11. Determine the amount of ^{125}I-labeled transferrin in each tube of efflux medium and solubilized cells.

Analyze data

12. At each time point, correct the radioactivity of ^{125}I-labeled transferrin in each individual vial of efflux medium and solubilization solution for nonspecific binding by subtracting the average of the radioactivity in the two nonspecific radioactivity wells.

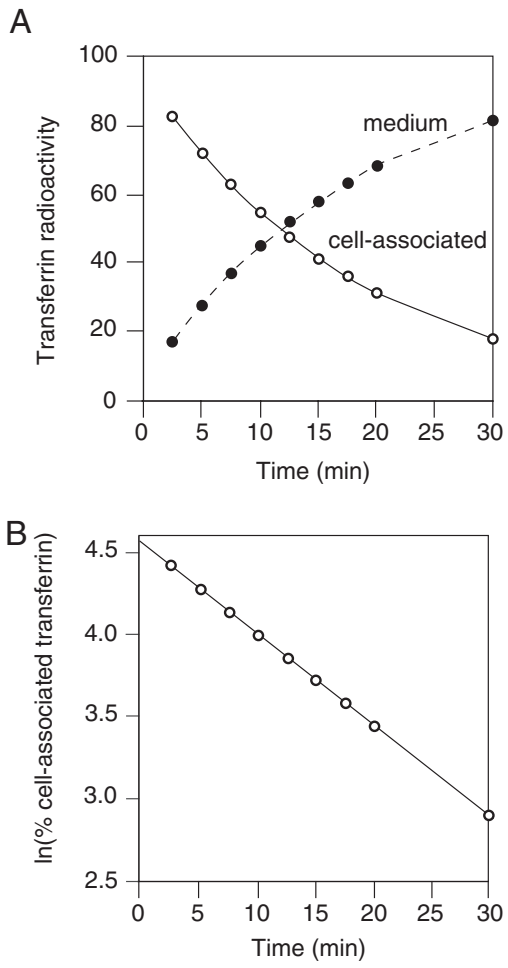


Figure 15.3.3 Transferrin recycling assay. (**A**) Data from an idealized transferrin recycling assay are shown. Over time, the amount of transferrin in the medium increases, while the cell-associated transferrin decreases as iron-free transferrin is released from cells. (**B**) The recycling rate constant is the slope of a graph of the natural logarithm of the percent-cell-associated transferrin versus time.

13. Calculate the percent cell-associated transferrin for each well, and average the four values per time point.

14. Plot percent cell-associated transferrin as a function of time (see Fig. 15.3.3).

In most cell types, transferrin is recycled by an apparent first-order process; therefore, the decrease in cell-associated transferrin should fit an exponential decay. The externalization rate constant can be calculated by determining the slope of a plot of the natural logarithm of the percent cell-associated versus time. In CHO cells, transferrin receptor is recycled at a rate of ~0.06 min^{-1} (Johnson et al., 1994).

IRON-LOADING TRANSFERRIN

SUPPORT PROTOCOL 1

This protocol describes the method for preparing diferric transferrin for use in transferrin recycling experiments (Basic Protocols 1 to 3). Its subsequent radiolabeling is described in Support Protocol 2.

Materials

Apo-transferrin (iron-free transferrin; available from a number of commercial sources)
PBS (see recipe)
1 mg/ml ferric ammonium citrate solution in 10 mM $NaHCO_3$/20 mM HEPES acid, pH 7.7
PD-10 columns (pre-packed Sephadex G-25 columns; Pharmacia Biotech) or any other desalting column
0.2-µm sterile syringe filter

1. Dissolve 200 mg apo-transferrin (iron-free) in 6 ml PBS.
2. Add 4 ml ferric ammonium citrate solution.

   The solution will be an ochre color.
3. Incubate at 37°C for 10 min.

   The solution will turn a dark rust color.
4. During this incubation, equilibrate five prepacked PD-10 columns with PBS.

   Any desalting column can be used.
5. Run 2 ml iron-loaded transferrin over each column.
6. Collect the orange product (iron-loaded transferrin). Pool the orange fractions from each column.
7. Measure the OD of the sample at 280 and 465 nm.

   The OD_{280}/OD_{465} ratio should be 0.0496 if 100% of the transferrin is loaded with iron.
8. Filter sterilize the iron-loaded transferrin with a 0.2-µm filter and store at 4°C.

   The transferrin is stable for at least 6 months at 4°C.

SUPPORT PROTOCOL 2

RADIO-IODINATION OF DIFERRIC TRANSFERRIN

There are a number of methods for radio-iodinating transferrin. The chloramine T method is described below. This method is suitable for use with any protein that contains tyrosine residues. In addition, it is important to test that the radio-iodination procedure has not grossly altered the affinity of transferrin for the transferrin receptor. This is done by measuring the ability of unlabeled transferrin to compete for the binding of ^{125}I-labeled transferrin to cells.

CAUTION: This procedure must be performed in a hood certified for radio-iodination. All personnel performing iodinations or using radioactive materials must be properly trained.

Additional Materials *(also see Basic Protocol 1)*

PBS (see recipe)
Human diferric transferrin (see Support Protocol 1)
37 MBq/ml $Na^{125}I$ (100 mCi/ml; NEN Life Science Products)
4 mg/ml chloramine T in PBS (prepare fresh)
8 mg/ml sodium bisulfite in PBS (prepare fresh)
1 mg/ml BSA
100% (w/v) trichloroacetic acid (TCA)
5×10^5 cells/well plated in 6-well tissue culture plates (see Basic Protocol 1, step 1)
PD-10 column (pre-packed Sephadex G-25 column; Pharmacia Biotech) or any other desalting column

Radiolabel transferrin

1. Equilibrate a PD-10 column with three bed volumes of PBS.
2. Add 2 mg human diferric transferrin in 0.2 ml PBS to a vial containing 1 mCi $Na^{125}I$. Mix by pipetting up and down.
3. Add 25 µl freshly prepared 4 mg/ml chloramine T in PBS. Mix by pipetting up and down. Incubate in the hood for 2 min.
4. To stop the reaction, add 25 µl freshly prepared 8 mg/ml sodium bisulfite in PBS. Mix by pipetting up and down.
5. Transfer solution to the equilibrated PD-10 column and collect 1-ml fractions.

   With a PD-10 column, the protein will be in fractions 3 and 4. It is not necessary to collect more than six fractions.

Analyze labeling

6. Quantify radioactivity in 10-µl aliquots of each fraction.
7. To confirm that the counts in the excluded fractions are ^{125}I-labeled protein, dilute a 10-µl aliquot of each fraction with 100 µl of 1 mg/ml BSA solution. Add 15 µl of 100% TCA, mix, and incubate at 4°C for 30 min.
8. Microcentrifuge samples at maximum speed for 10 min at room temperature. Quantify radioactivity in the supernatant and pellet separately.

   Radioactivity in the pellet is ^{125}I-labeled protein, and radioactivity in the supernatant is free ^{125}I.

9. Determine the specific activity (radioactivity per ng of protein).

   The procedure yields transferrin with a specific activity of 300 to 500 cpm/ng.

   CAUTION: *Dispose of radioactive waste appropriately.*

Test affinity of radiolabeled transferrin

10. Chill 6-well plate of cells (5×10^5 cells/well) to 4°C and wash each well with 2 ml prechilled neutral pH buffer.
11. Add 3 µg/ml ^{125}I-labeled transferrin and various amounts of unlabeled transferrin to each sample and incubate for 2 hr at 4°C in SF medium.
12. Wash cells with 2 ml neutral pH buffer (4°C).
13. Solubilize the cells and measure cell-associated radioactivity (see Basic Protocol 1, steps 8 and 9, for details).

    A 200-fold excess of unlabeled transferrin reduces ^{125}I-labeled transferrin binding to less than 10%.

14. Store radio-iodinated transferrin for up to 1 month at 4°C in a lead container.

TIME COURSE FOR STEADY-STATE OCCUPANCY OF MEMBRANE PROTEIN WITH ANTIBODY

BASIC PROTOCOL 4

It is not possible to use a modification of Basic Protocol 3 to measure the recycling of a membrane protein using a ^{125}I-labeled antibody, because the antibody remains bound to the membrane protein when it is returned to the cell surface, and the methods used to strip antibodies are not compatible with studies in living cells. One method for determining the recycling rate of a membrane protein using an antibody is to incubate cells at 37°C with ^{125}I-labeled antibody and monitor the amount of cell-associated antibody as a function of incubation time. The protein on the surface at the start of the experiment will be rapidly bound by the antibody, and will remain bound throughout the course of the

experiment (i.e., is it recycles between the cell surface and endosomes). Cell-associated label, however, will increase over time as the pool of protein that was inside the cell at the beginning of the experiment (and therefore unoccupied) is recycled back to the cell surface. Cell-associated label will plateau (reach a steady-state value) when all the cycling protein has bound antibody (i.e., is trafficked to the cell surface). Thus, the rate of approach to the steady-state value is the rate at which the intracellular pool is cycled to the surface. Note that this method for measuring recycling of a membrane protein requires that the antibody remain associated with the protein during its cycle, that the antibody does not modify the trafficking of the protein, and that the amount of protein synthesized over the course of the experiment is negligible compared to the amount of recycled protein.

Materials

Cells of interest
SF medium (see recipe)
^{125}I-labeled antibody (Fab fragments; 2 to 10 μCi/μg)
Unlabeled (nonradioactive) antibody
Neutral pH buffer (see recipe), room temperature
Solubilization solution: 1% (w/v) Triton X-100 in 0.1 N NaOH

6-well tissue culture plates
γ counter and tubes

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Use one plate for each time point. Allow cells to reach ~80% confluency on the day of the experiment.

   Eight time points are typically sufficient.

   Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator. HEPES-buffered medium is used if the experiment is to be performed in air.

2. Wash cells with 2 ml SF medium.
3. Add 1 ml SF medium containing 1 to 5 nM ^{125}I-labeled antibody to four wells (total radioactivity wells). Add 1 ml SF medium containing the same concentration of ^{125}I-labeled antibody and a 200-fold excess of unlabeled antibody (nonspecific radioactivity wells) to the other two wells.
4. Incubate the plates for variable times at 37°C (e.g., 2.5, 5, 10, 15 min).

   The time points used are empirically determined.

5. At the appropriate times, remove the plates from the incubator and wash three times with 2 ml neutral pH buffer.

   CAUTION: *Dispose of radioactive waste appropriately.*

6. Add 1 ml solubilization solution to each well. Remove the solubilized cells by trituration and transfer to tubes suitable for γ counting. Rinse the wells once with 1 ml water, and pool with the solubilization solution.
7. Determine the amount of ^{125}I-labeled antibody per tube.
8. Repeat steps 5 to 7 are for each plate after the appropriate incubation time.
9. For each plate (time point), average the values of the four total radioactivity wells and subtract the average of the two nonspecific radioactivity wells.
10. Plot the amount of cell-associated antibody (specific radioactivity) as a function of incubation time.

    This curve should asymptotically approach a plateau level. The increase in cell-associated antibody over time reflects the recycling of unoccupied membrane protein receptors from

endosomes to the surface. The recycling rate constant is calculated from this curve (Johnson et al., 1993).

DETECTING DEGRADATION OF INTERNALIZED LIGANDS

BASIC PROTOCOL 5

Most ligands internalized by endocytosis are delivered to late endosomal or lysosomal compartments where they are degraded into peptides. The degradation of ^{125}I-labeled ligand is examined by measuring the release of these peptides into the medium. The medium is collected and proteins are precipitated with trichloroacetic acid (TCA). The radioactivity in the pellet is intact protein, and the radioactivity in the supernatant is degraded ligand. This protocol describes the detection of degraded peptide in the extracellular medium after various times of endocytosis.

Materials

Cells of interest
SF medium (see recipe)
Neutral pH buffer (see recipe), ice cold
^{125}I-labeled ligand of interest (Support Protocol 2)
Unlabeled (nonradioactive) ligand
10 mg/ml BSA
100% (w/v) trichloroacetic acid (TCA) solution

6-well tissue culture plate
γ counter and tubes

Prepare cells

1. Plate 1×10^5 cells/ml in SF medium in three wells of a 6-well tissue culture plate 2 days before the experiment. Prepare one plate (three wells) per time point. Allow cells to reach ~80% confluency on the day of the experiment.

   Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator. HEPES-buffered medium is used if the experiment is to be performed in air.

2. On the day of the experiment, wash the cells once with 2 ml SF medium and incubate in SF medium for 1 hr at 37°C.

   During this incubation, most of the cell-associated ligand from the growth medium will be released from the cells, ensuring that the receptors will be unoccupied at the beginning of the experiment.

3. At the end of this incubation, place the cells on ice and wash two times with 2 ml/well prechilled neutral pH buffer.

Internalize ligand

4. Add 1 ml prechilled (4°C) neutral pH buffer containing a saturating concentration of ^{125}I-labeled ligand and 1 mg/ml BSA to two wells (total radioactivity wells). Add 1 ml of the same solution containing a 200-fold excess of unlabeled ligand to the other well (nonspecific radioactivity well).

   The concentration of ligand depends on the ligand used; refer to appropriate literature.

5. Incubate the cells at 4°C for 2 hr.

6. Wash cells four times with 2 ml neutral pH buffer at 4°C.

   CAUTION: *Dispose of radioactive waste appropriately.*

   The neutral pH wash removes unbound ligand from the cells.

7. Add 1 ml SF medium to each well and incubate the plate at 37°C.

   At 37°C, receptor-bound ligands are internalized.

Collect/precipitate degraded and intact ligand

8. At various time points (e.g., 10, 20, 30, 60, 90, and 120 min) transfer the incubation medium from the three walls into separate microcentrifuge tubes on ice.
9. Solubilize the cells and determine the radioactivity (see Basic Protocol, steps 8 and 9).

   This is the cell-associated radioactivity.

10. Add 10 mg/ml BSA to the incubation medium at a final concentration of 2 mg/ml.

    Adding BSA will increase the efficiency of precipitation of the intact ligand.

11. Add 100% (w/v) TCA at a final concentration of 15%.
12. Incubate 30 min at 4°C.
13. Centrifuge at 15,000 × *g*, 4°C, for 20 min.
14. Collect the supernatant and count the radioactivity in each separately.
15. At the same time, test the efficiency of the precipitation procedure by precipitating a 5-µl aliquot of labeled ligand diluted to 1 ml in SF medium as described in steps 9 to 13.

    Typically, more than 90% of the ligand should be precipitable.

Analyze data

16. Correct all the data for nonspecific radioactivity by subtracting the radioactivity in the nonspecific sample from the average of the radioactivity in the other two samples. Determine the radioactivity in the 15% TCA supernatant (ligand released into the medium in degraded form) and in the 15% TCA pellet (ligand released in the undegraded or intact form). Plot the three values—cell-associated radioactivity (step 9), the released degraded ligand, and the released intact ligand—as per cent of the total (the sum of the three values at that time point) versus time.

    An alternative protocol is to incubate the cells at 37°C in the presence of ^{125}I-labeled ligand (pulse), wash the cells, and measure the appearance of degraded ligand in the medium after various chase times (Brown and Goldstein, 1976), which is more sensitive because it does not rely on a single round of internalization.

BASIC PROTOCOL 6

MEASURING FLUID-PHASE UPTAKE

Any molecule in the extracellular medium can be internalized by bulk fluid uptake inside newly endocytosed vesicles. To measure this non-concentrated uptake, cells are incubated with a protein that does not have a specific membrane receptor and accumulation of this protein inside cells over time is determined.

Materials

Cells of interest
SF medium (see recipe)
5 mg/ml horseradish peroxidase (HRP) in SF medium
Neutral pH buffer (see recipe), ice cold
0.01% (w/v) Triton X-100
6-well tissue culture plates

Additional reagents and equipment for colorimetric assay (see Steinman et al., 1976) and protein assay (*APPENDIX 3*)

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Use three wells for each time point. Allow the cells to reach ~80% confluency on the day of the experiment.

   The time points used are determined empirically.

   Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator. HEPES-buffered medium is used if the experiment is to be performed in air.

2. Wash the cells once with 2 ml SF medium, and incubate the cells in 5 mg/ml HRP in SF medium at 37°C for various times (e.g., 5, 10, 15, 20, 30, 60 min).

   ^{125}I-labeled BSA or other detectable protein can be used instead of HRP.

3. At the appropriate times, remove the plate from the incubator, place on ice, and wash six times with 2 ml/wash prechilled neutral pH buffer.

   Endocytosis is inhibited at 4°C. More than six washes may be needed to remove HRP adsorbed to the cell surface. To test this, incubate 6 wells with 5 mg/ml HRP at 4°C for 2 hr. Measure the number of washes necessary to remove essentially all cell-associated HRP activity as determined in step 4. The surface HRP can also be removed by incubation in 0.1% pronase in PBS. This procedure also removes the cells from the plate; the cells are collected by centrifugation 3 min at 15,000 × g.

4. Lyse cells in 1 ml of 0.01% Triton X-100. Take a 100-µl aliquot to measure HRP activity in the lysate using a colorimetric assay (see Steinman et al., 1976). Measure HRP activity of a 5 mg/ml HRP solution as a standard for normalization.

5. Measure protein content in the lysate (*APPENDIX 3)*.

   A number of kits are also available to measure protein content.

6. Express the results as the amount of HRP with respect to the total protein content for each well (i.e., ng HRP/mg cell protein). Calculate the average of these values for each time point, and plot as a function of time.

INHIBITION OF CLATHRIN-MEDIATED ENDOCYTOSIS BY POTASSIUM DEPLETION

SUPPORT PROTOCOL 3

All endocytic mechanisms are energy and temperature dependent, and are blocked at 4°C. Clathrin-mediated endocytosis (e.g., transferrin endocytosis) is also inhibited by two other methods: intracellular potassium depletion (Larkin et al., 1983) and cytosol acidification (Sandvig et al., 1987; see Support Protocol 4). The potassium depletion assay is easy to perform, but inhibits clathrin-dependent endocytosis only in certain cell types (e.g., human and chick embryo fibroblasts, Hep 2 cells; Moya et al., 1985). These assays are used to establish that internalization is by clathrin-mediated endocytosis.

Materials

Cells of interest
SF medium (see recipe)
Hypotonic medium: 1:1 (v/v) SF medium/water
K^+-depleted buffer: 100 mM NaCl, 50 mM HEPES acid, pH 7.4
6-well tissue culture plates

Additional reagents and equipment for measuring internalization (see Basic Protocol 2 or Alternate Protocol 2)

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency (~5×10^5/well) on the day of the experiment. Prepare one plate for each time point and one plate for surface labeling.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

2. On the day of the experiment, wash the cells once with 2 ml SF medium.
3. Incubate cells for 5 min at 37°C in 2 ml hypotonic medium.
4. Wash cells with 2 ml K^+-depleted buffer.
5. Incubate cells for 30 min at 37°C in K^+-depleted buffer.
6. Measure internalization as described (see Basic Protocol 2, steps 3 to 17, or see Alternate Protocol 2), but perform internalization incubations (step 3) in K^+-depleted buffer.

The inhibition is fully reversible within 15 min of adding back 10 mM KCl in the K^+-depleted buffer. Incubating cells in hypertonic media (e.g., 0.45 M sucrose) also inhibits clathrin-dependent endocytosis, but may affect other types of endocytosis as well (Sandvig et al., 1989).

SUPPORT PROTOCOL 4

INHIBITION OF CLATHRIN-MEDIATED ENDOCYTOSIS BY CYTOSOL ACIDIFICATION

Only one of a number of methods for acidifying the cytosol are discussed below (Sandvig et al., 1987). Excessive acidification of the cytosol may also inhibit clathrin-independent endocytosis, as measured following fluid-phase endocytosis.

Materials

Cells of interest
SF medium (see recipe)
SF medium supplemented with 25 mM NH_4Cl
140 mM KCl/1 mM amiloride/40 mM HEPES acid, pH 7.0
6-well tissue culture plates

Additional reagents and equipment for measuring internalization (see Basic Protocol 2 or Alternate Protocol 2)

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency (~5×10^5/well) on the day of the experiment. Prepare one plate for each time point and one for surface labeling.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

2. On the day of the experiment, wash the cells once with 2 ml SF medium.
3. Incubate for 30 min at 37°C in SF medium supplemented with 25 mM NH_4Cl.

The optimal NH_4Cl concentration that inhibits clathrin-mediated entry without affecting other cellular processes may vary with the cell line, and different concentrations (e.g., 10 to 50 mM) should be tested.

4. Incubate cells for 2 min at 37°C in 140 mM KCl/1 mM amiloride/40 mM HEPES acid, pH 7.0.
5. Measure internalization (see Basic Protocol 2, steps 3 to 17, or see Alternate Protocol 2), but perform internalization incubations (step 4) in the amiloride-containing buffer.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Either concentrated HCl or concentrated NaOH is used to pH all buffers.

Efflux medium

SF medium (see recipe) containing:
0.1 mM desferrioxamine (Sigma; from 10 mM stock in H_2O)
3 µg/ml human diferric transferrin
Prepare fresh

Neutral pH buffer

8.8 g NaCl (final 150 mM)
0.37 g KCl (5 mM)
0.11 g $CaCl_2$ (1 mM)
0.20 g $MgCl_2$ (1 mM)
4.77 g HEPES acid (20 mM)
Adjust to 1 liter with distilled water
Adjust pH to 7.4
Store up to 6 months at 4°C

pH 2.0 buffer

29.22 g NaCl (final 500 mM)
28.57 ml glacial acetic acid (0.2 N)
Adjust to 1 liter with distilled water
Adjust pH to 2.0
Store up to 6 months at 4°C

pH 5.0 buffer

8.76 g NaCl (final 150 mM)
9.74 g 2-(*N*-morpholino)ethanesulfonic acid (MES; 50 mM) *or* 5.88 g sodium citrate (20 mM)
Adjust to 1 liter with distilled water
Adjust pH to 5.0
Store up to 6 months at 4°C

Phosphate-buffered saline (PBS)

8 g NaCl (final 137 mM)
0.2 g KCl (2.6 mM)
2.2 g Na_2HPO_4 heptahydrate (8 mM)
0.2 g KH_2PO_4 (1.5 mM)
Adjust to 1 liter with distilled water
Adjust pH to 7.4
Store up to 6 months at room temperature

Serum-free (SF) medium

If the experimental incubations are to be performed in a CO_2 incubator, the SF medium is the medium formulation used to grow cells (e.g., Ham's F12, DMEM, RPMI) supplemented with the amount of sodium bicarbonate recommended for that particular medium formulation, and 10 mM HEPES acid (pH 7.2). A number of manipulations using this medium are performed at room temperature, and the HEPES is included to ensure that the pH of the bicarbonate-buffered medium does not alkalinize during these manipulations. If the experiment is to be performed in an air incubator, omit the sodium bicarbonate from the SF medium.

COMMENTARY

Background Information

The term endocytic trafficking is used to describe the processes of internalization from the cell surface into endosomes, trafficking among various intracellular compartments, and recycling back to the surface. Endocytosis through clathrin-coated pits is the major mechanism for receptor-mediated internalization of extracellular macromolecules. Ligands internalized by endocytosis include peptide hormones (e.g., insulin, epidermal growth factor), nutrient-carrying proteins (e.g., transferrin, low-density lipoprotein), viruses, and both plant and animal toxins. The distribution of receptors between intracellular compartments and the cell surface is determined by the rates of internalization and recycling. Endocytic trafficking also plays a fundamental role in the maintenance of intracellular organelle identity because, in many instances, the localization of membrane proteins to specific compartments involves retrieval and retargeting through the endosomal system. In addition, a number of specialized and regulated trafficking processes overlap, at least in part, with the general endosomal system (e.g., insulin-regulated trafficking of the glucose transporter GLUT4). For reviews of constitutive and regulated endocytic trafficking, see Watts and Marsh (1992), Sorkin and Waters (1993), Holman et al. (1994), Gruenberg and Maxfield (1995), and Mukherjee et al. (1997).

Transferrin receptor trafficking has been extensively studied in many different cell types. The are a number of advantages in using the transferrin receptor to monitor endocytic trafficking. First, transferrin remains bound to its receptor until it is recycled back to the cell surface. Therefore, recycling of the receptor can be followed directly using labeled transferrin. Second, transferrin receptors are expressed in most cell types. Third, transferrin is commercially available and readily labeled for use in biochemistry and microscopy experiments. Fourth, human transferrin has a high affinity for transferrin receptors from other species, making human transferrin a versatile reagent.

Using transferrin as a probe, the kinetics of clathrin-coated-pit-mediated internalization (see Basic Protocol 2) and the kinetics of constitutive recycling (see Basic Protocol 3) can be measured. Since transferrin receptor is constitutively cycled between endosomes and the plasma membrane, the rates of internalization and recycling determine the steady-state surface-to-internal distribution of transferrin receptor (see Basic Protocol 1; Johnson et al., 1994). Thus, when characterizing the behavior of the transferrin receptor, the ratio of the recycling to internalization rate constants should be equal to the measured surface-to-internal ratio. The transferrin receptor assays described in this unit (and slight modifications of these assays) have been used successfully in conjunction with molecular genetic approaches (e.g., expression of dominant negative mutants; Bucci et al., 1992; van der Sluijs et al., 1992) and chemical inhibitors (Schonhorn and Wessling-Resnick, 1994; Martys et al., 1996) to further understand the molecular mechanisms of endocytosis.

The antibody-based trafficking assays described in this unit can be used to monitor the behavior of any membrane protein for which an antibody to the extracellular domain is available (see Alternate Protocols 1 and 2, and Basic Protocol 4). Although many proteins are internalized through clathrin-coated pits, there are other non-clathrin mechanisms for internalization. Methods for inhibiting endocytosis—via potassium depletion (see Support Protocol 3) or cytosol acidification (see Support Protocol 4)—have proven useful in determining the internalization pathways of membrane proteins (e.g., Moya et al., 1985; Sandvig et al., 1987; Subtil et al., 1994). However, conclusions must be drawn cautiously, as these treatments are not specific and the extent of inhibition varies greatly between cell types (Moya et al., 1985).

When characterizing the trafficking of a membrane protein, one can determine whether the protein is recycled or if it trafficks to lysosomes and is degraded (see Basic Protocol 5). If the protein is recycled, then the approach to steady-state occupancy (Basic Protocol 4) can be used to measure the recycling rate. As is the case for the transferrin receptor, if a membrane protein is constitutively recycled, then the ratio of the rate of internalization to the rate of recycling should be equal to the steady-state surface-to-internal distribution. In the case of antibody-based trafficking studies, the internalization rate and the surface-to-internal ratio can be directly measured, and a value for the recycling rate can be determined by multiplying the internalization rate by the surface-to-internal ratio.

In addition to the biochemical assays described in this unit, similar quantitative fluorescence microscopy assays have been used to

characterize endocytic trafficking (e.g., Mayor et al., 1993; Presley et al., 1993). A description of these powerful approaches is beyond the scope of this unit. Quantitative studies can also be performed by flow cytometry using fluorescent ligands or antibodies (Marks et al., 1996).

Critical Parameters

The sensitivity of the biochemical assays depends on the number of receptors (or molecules of membrane protein being studied) per cell and the specific activity of the probe. If a protein expressed by transfection is being studied, highly expressing clones can be used. However, it is imperative to demonstrate that the behavior of the protein is not dependent on the level of expression, as would be the case if a critical trafficking step is saturated at high levels of expression (Marks et al., 1996). Increasing the specific activity of the ^{125}I-labeled probe can compensate for a low number of receptors, but too high incorporation of ^{125}I may result in a loss of specificity (see Troubleshooting).

Another critical parameter is the efficient release of ligand from the cell surface, because this is used to discriminate between surface and internal ligand. The efficiency of the acid stripping is assessed by stripping ligand bound to the surface of cells at 4°C. When it works, acid stripping is the method of choice (see Basic Protocol 1). Several rounds of alternating acid/neutral washes can be performed to increase the stripping efficiency. Some antibodies are resistant to release by incubation in acidic buffers. In those cases, incubation with 1 mg/ml proteinase K or pronase at 4°C for 30 min (or more) can be tried to release surface-associated radioactivity.

To minimize experiment-to-experiment variation, it is important to use cells at the same degree of confluency.

Troubleshooting

The quality of the ^{125}I-labeled ligand (transferrin, other ligand, or antibody) is critical. If the ligand is over-derivatized (too high specific activity), specific binding to receptor/antigen can be compromised, which will result in a reduced signal-to-noise ratio (i.e., the nonspecific radioactivity will be a large percentage of the total radioactivity). In addition, the trafficking of an over-derivatized ligand can be altered. For example, over-derivatized transferrin often aggregates, and these aggregates are trafficked to lysosomes and degraded rather than recycled. On the other hand, if the specific activity is too low, there may not be enough signal. The optimal specific activity must be determined empirically for each ligand and each cell type. There are a number of different methods for iodinating proteins. If problems are encountered with the degree of derivatization using one method, one of the other methods should be tried.

If the nonspecific radioactivity is a large fraction of the total, the amount of unlabeled ligand used for competition can be increased, and the number of washes can also be increased. Adding 1 mg/ml BSA during the incubation with the radio-iodinated probe can help lower background.

When using an antibody as a probe, verify (if possible) that the antibody does not modify trafficking of the protein (Subtil et al., 1994). Fab fragments should be used because bivalent antibodies cause cross-linking and therefore may modify the protein's trafficking (Weissman et al., 1986). Also, antibodies should be aliquoted and frozen immediately after radiolabeling to avoid freeze/thaw cycles. Aliquots may be kept at 4°C for several days.

Anticipated Results

The methods discussed in this unit (and variations of these methods) have been widely used. Examples of results obtained using these methods can be found in Weissman et al. (1986), Backer et al. (1991), Pelchen-Matthews et al. (1991), Herbst et al. (1994), Johnson et al. (1994), Schonhorn and Wessling-Resnick (1994), and Subtil et al. (1997).

The values for transferrin receptor trafficking in cultured fibroblast cells lines are:

Internalization rate constant: 0.15 to 0.2 min^{-1}
Recycling rate constant: 0.05 to 0.07 min^{-1}
Percent on surface at steady state: 30% to 40%.

Data from an idealized transferrin internalization assay are shown Figure 15.3.2. In panel A, data are shown for an assay carried out for a time sufficient to reach steady-state. During short incubation times, the ratio of the internal transferrin to surface transferrin increases linearly (panel B). The slope of this line is the internalization rate constant. After longer incubations, the rate of increase in ratio of internal-to-surface transferrin slows down as transferrin is recycled. To accurately measure the internalization rate constant it is important to use time points in the linear (early) portion of the curve. Ideally, the line should extrapolate through zero.

Data from an idealized transferrin recycling assay are shown in Figure 15.3.3. At the start

of the experiment, the great majority of transferrin is inside the cells because the pH 5 wash followed by neutral pH washes removes transferrin from the surface of cells. Over time the amount of transferrin in the medium increases and the amount of transferrin inside cells decreases as iron-free transferrin is recycled back to the cell surface. The recycling rate constant is the slope of a plot of the natural logarithm of the percent cell-associated transferrin versus time.

Time Considerations

Each individual experiment is readily performed within a single day.

Literature Cited

Backer, J.M., Shoelson, S.E., Haring, E., and White, M.F. 1991. Insulin receptors internalize by a rapid, saturable pathway requiring receptor autophosphorylation and an intact juxtamembrane region. *J. Cell Biol.* 115:1535-1545.

Brown, M.S. and Goldstein, J.L. 1976. Analysis of a mutant strain of human fibroblasts with a defect in the internalization of receptor-bound low density lipoprotein. *Cell* 9:663-674.

Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. 1992. The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* 70:715-728.

Dautry-Varsat, A., Ciechanover, A., and Lodish, H.F. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 80:2258-262.

Gruenberg, J. and Maxfield, F.R. 1995. Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol.* 7:552-563.

Herbst, J.J., Opresko, L.K., Walsh, B.J., Lauffenburger, D.A., and Wiley, H.S. 1994. Regulation of postendocytic trafficking of the epidermal growth factor receptor through endosomal retention. *J. Biol. Chem.* 269:12865-12873.

Holman, G.D., Lo Leggio, L., and Cushman, S.W. 1994. Insulin-stimulated GLUT4 glucose transporter recycling. A problem in membrane protein subcellular trafficking through multiple pools. *J. Biol. Chem.* 269:17516-17524.

Johnson, L.S., Dunn, K.W., Pytowski, B., and McGraw, T.E. 1993. Endosome acidification and receptor trafficking: Bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. *Mol. Biol. Cell* 4:1251-1266.

Johnson, L.S., Presley, J.F., Park, J.C., and McGraw, T.E. 1994. Slowed receptor trafficking in mutant CHO lines of the End1 and End2 complementation groups. *J. Cell Physiol.* 158:29-38.

Klausner, R.D., Ashwell, G., van Renswoude, J., Harford, J.B., and Bridges, K.R. 1983. Binding of apotransferrin to K562 cells: Explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. U.S.A.* 80:2263-266.

Larkin, J.M., Brown, M.S., Goldstein, J.L., and Anderson, R.G. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell* 33:273-285.

Marks, M.S., Woodruff, L., Ohno, H., and Bonifacino, J.S. 1996. Protein targeting by tyrosine- and di-leucine-based signals: Evidence for distinct saturable components. *J. Cell Biol.* 135:341-354.

Martys, J.L., Wjasow, C., Gangi, D.M., Kielian, M.C., McGraw, T.E., and Backer, J.M. 1996. Wortmannin-sensitive trafficking pathways in Chinese hamster ovary cells. Differential effects on endocytosis and lysosomal sorting. *J. Biol. Chem.* 271:10953-10962.

Mayor, S., Presley, J.F., and Maxfield, F.R. 1993. Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J. Cell Biol.* 121:1257-1269.

Mellman, I. 1996. Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* 12:575-625.

Moya, M., Dautry-Varsat, A., Goud, B., Louvard, D., and Boquet, P. 1985. Inhibition of coated pit formation in Hep2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. *J. Cell Biol*. 101:548-559.

Mukherjee, S., Ghosh, R.N., and Maxfield, F. R. 1997. Endocytosis. *Physiol. Rev.* 77:759-803.

Pelchen-Matthews, A., Armes, J.E., Griffiths, G., and Marsh, M. 1991. Differential endocytosis of CD4 in lymphocytic and nonlymphocytic cells. *J. Exp. Med.* 173:575-587.

Presley, J.F., Mayor, S., Dunn, K.W., Johnson, L.S., McGraw, T.E., and Maxfield, F. R. 1993. The End2 mutation in CHO cells slows the exit of transferrin receptors from the recycling compartment but bulk membrane recycling is unaffected. *J. Cell Biol*. 122:1231-1241.

Sandvig, K., Olsnes, S., Petersen, O.W., and van Deurs, B. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* 105:679-689.

Sandvig, K., Olsnes, S., Petersen, O.W., and van Deurs, B. 1989. Control of coated-pit function by cytoplasmic pH. *Methods Cell Biol.* 32:365-382.

Schonhorn, J.E. and Wessling-Resnick, M. 1994. Brefeldin A down-regulates the transferrin receptor in K562 cells. *Mol. Cell. Biochem.* 135:159-169.

Sorkin, A. and Waters, C.M. 1993. Endocytosis of growth factor receptors. *BioEssays* 15:375-382.

Steinman, R.M., Brodie, S.E., and Cohn, Z.A. 1976. Membrane flow during pinocytosis. *J. Cell Biol.* 68:665-687.

Subtil, A., Hemar, A., and Dautry-Varsat, A. 1994. Rapid endocytosis of interleukin 2 receptors when clathrin-coated pit endocytosis is inhibited. *J. Cell Sci.* 107:3461-3468.

Subtil, A., Delepierre, M., and Dautry-Varsat, A. 1997. An alpha-helical signal in the cytosolic domain of the interleukin 2 receptor beta chain mediates sorting towards degradation after endocytosis. *J. Cell Biol.* 136:583-595.

van der Sluijs, P., Hull, M., Webster, P., Male, P., Goud, B., and Mellman, I. 1992. The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. *Cell* 70:729-740.

Watts, C. and Marsh, M. 1992. Endocytosis: What goes in and how? *J. Cell Sci.* 103:1-8.

Weissman, A.M., Klausner, R.D., Rao, K., and Harford, J.B. 1986. Exposure of K562 cells to anti-receptor monoclonal antibody OKT9 results in rapid redistribution and enhanced degradation of the transferrin receptor. *J. Cell Biol.* 102:951-958.

Wiley, H.S. and Cunningham, D.D. 1982. The endocytotic rate constant. A cellular parameter for quantitating receptor-mediated endocytosis. *J. Biol. Chem.* 257:4222-4229.

Contributed by Timothy E. McGraw and Agathe Subtil
Weill Medical School of Cornell University
New York, New York

Determining Protein Transport to the Plasma Membrane

Many integral membrane proteins, synthesized in the endoplasmic reticulum (ER), ultimately arrive at the cell surface to contact the cell's environment. During export from the ER, and transport through the Golgi subcompartments and *trans*-Golgi network (TGN), proteins may undergo a number of posttranslational modifications that affect the molecular weight and/or charge. These post-translational modifications include N-linked glycan modifications, O-linked glycan modifications, sulfation, phosphorylation, lipid modifications, and proteolytic trimming/cleavage (*UNITS 15.1 & 15.2*).

Since localization of the enzymes involved in these alterations is well known, posttranslational modifications are the most convenient tool to monitor intracellular transport processes. However, these modifications have gone to completion when the proteins arrive at the plasma membrane. The appearance of a particular protein at the cell surface can then be visualized by pulse-labeling the protein and removing the posttranslational modifications in intact cells using various enzymes (see Basic Protocol 1). Alternatively, cell-surface appearance can be assayed by selectively labeling the cell-surface pool of pulse-labeled proteins using biotin (see Alternate Protocol 1) or antibodies (see Alternate Protocol 3), or by removing the surface-protein pool with proteases (see Alternate Protocol 2). Various techniques for measuring cell-surface appearance based on these principles are also described. Changes in molecular charge can be assessed by one-dimensional isoelectric focusing (IEF; see Support Protocol 2). Fab fragments of antibodies can be biotinylated (see Support Protocol 1) and used to detect surface proteins. It is also possible to measure transport to endosomes before appearance at the plasma membrane (see Basic Protocol 2).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

MEASURING ARRIVAL AT THE CELL SURFACE BY DIGESTION WITH SIALIDASE

BASIC PROTOCOL 1

This assay is based on the fact that, in the TGN, glycoproteins acquire one or more sialic-acid residues at the end of their N- and O-linked carbohydrate chains. This sialylation alters not only the total molecular weight (although this will only be observed by SDS-PAGE when many sialic acids are added; see Fig. 15.2.6), but also the charge or isoelectric point of the glycoprotein (*UNIT 15.2*). Addition of sialic acids can be most readily observed when isolated proteins are separated by isoelectric point, i.e., using one-dimensional IEF (see Support Protocol 2) or two-dimensional IEF/SDS-PAGE (*UNIT 6.4*). The sialic acids can be easily and quantitatively removed from intact cells by incubation with sialidase, resulting in a more basic isoelectric point and a shift of the glycoprotein to the position on IEF corresponding to that of the glycoprotein prior to sialylation. Cell-surface appearance of glycoproteins can thus be determined by combining a pulse-chase experiment (*UNIT 7.1*) with incubations of the intact cells in the presence or absence of sialidase (*UNIT 15.2*). Cell-surface appearance is then determined by comparing the sialic-acid content of a glycoprotein prior to and after sialidase treatment. A glycoprotein has arrived at the plasma membrane when it becomes a substrate for the sialidase treatment of intact cells at low temperature.

Contributed by Jacques Neefjes
Current Protocols in Cell Biology (2000) 15.4.1-15.4.14

Materials

Cells
Amino acid–free medium containing 8% to 10% (v/v) FBS
Radiolabeled amino acid
PBS (*APPENDIX 2A*), ice cold
PBS containing 1 mM $CaCl_2$
Sialidase Type V from *Clostridium perfringens* (Sigma) dissolved in PBS and stored in 10-µl 1 to 10 U/ml aliquots up to several years at −20°C
PBS containing 10% (v/v) fetal bovine serum (FBS)
Lysis mixture (see recipe)
Fetuin

Additional reagents and equipment for pulse-labeling (*UNIT 7.1*), TCA precipitation (*UNIT 7.1*), immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*) or one-dimensional IEF (see Support Protocol 2), and autoradiography (*UNIT 6.3*)

1. Culture cells in amino acid-free medium containing 8% to 10% FBS. Pulse label ~5 × 10^6 cells per sample with a ~50 µCi radiolabeled amino acid and chase for various times. Chase times can be 0, 1, 2, 3, 5, 10, and 30 min for early events, and 0, 15, 30, 60, 120, and 240 min for following most stages in intracellular transport. Label two sets of cells for each chase time point: one for sialidase treatment and one as a control sample.

   Usually cells are labeled with 10 to 50 µCi radioactive amino acids (UNIT 7.1), but they can also be labeled with radioactive sugars (Warmerdam et al., 1996).

2a. Collect nonadherent cells at each chase point by low-speed centrifugation (3 min at 1500 × g, 4°C).

2b. Collect adherent cells at each chase point by detaching from the plate with 10 mM EDTA in PBS at <15°C (to stop intracellular transport).

   Alternatively, the sialidase digestion can be performed with the cells attached to the plate.

3. Wash cells two times with 1 ml PBS at 4°C. Collect nonadherent or detached cells between washes by centrifuging 3 min at 1500 × *g*, 4°C. Resuspend the pellet by gently tapping the bottom of the tube before adding PBS.

4. Suspend the final pellet from the control sample in 100 µl PBS containing 1 mM $CaCl_2$ and the other sample in 100 µl PBS containing 1 mM $CaCl_2$ and 0.1 IU sialidase. Incubate cells on ice for 1 hr.

   Both the time of sialidase treatment and the concentration of sialidase may be varied to ensure full desialylation.

   Sialidase from other sources, e.g., Arthrobacter ureafaciens, can also be used.

5. Remove sialidase by washing the cells three times with 1 ml PBS containing 10% FBS.

6. Lyse cells in 1 ml lysis mixture containing 1 to 10 mg fetuin to inhibit postlysis desialylation.

   It is advisable to confirm the conditions for complete sialic acid removal that may result in postlysis desialylation. This can be done by mixing cells containing radiolabeled surface proteins with unlabeled cells incubated with sialidase prior to lysis.

7. To ensure the isolation of proteins from equal numbers of radioactively labeled cells, determine the amount of incorporated radioactivity per lysate by TCA precipitation of 10 µl of each cell lysate (see *UNIT 7.1*).

8. Isolate the proteins from equal amounts of radioactivity by immunoprecipitation (*UNIT 7.2*).

9. Analyze immunoprecipitates by SDS-PAGE (*UNIT 6.1*) or one-dimensional IEF (see Support Protocol 2) followed by autoradiography (*UNIT 6.3*).

   Sialic acid addition/removal may sometimes result in an altered apparent molecular weight on SDS-PAGE (UNIT 6.1; Fig. 15.2.6). This can be most easily visualized by loading the control sample next to the sialidase-treated sample. If this does not give proper resolution, however, proteins have to be separated on the basis of isoelectric point, either by one-dimensional IEF (see Support Protocol 2) or two-dimensional IEF/SDS-PAGE (UNIT 6.4). SDS-PAGE as well as one-dimensional IEF allows separation of many samples in a single run and direct side-by-side comparison.

MEASURING ARRIVAL AT THE CELL SURFACE BY BIOTINYLATION OF CELL-SURFACE MOLECULES

ALTERNATE PROTOCOL 1

Biotinylation of surface proteins employs monovalent, water-soluble cross-linkers with an additional biotin group to allow isolation by avidin or streptavidin coupled to Sepharose. Only proteins at the plasma membrane are substrates for this reaction, provided that the reaction is performed at temperatures <15°C. Arrival of newly synthesized proteins at the cell surface can be analyzed by performing biotinylation of cells after a pulse-chase protocol (see *UNIT 7.1*). The experimental design is simple but not quantitative. Generally, between 0% and 25% of the surface-protein pool is biotinylated. As most cross-linkers react with amine groups, including those from serum components, cells should be extensively washed with PBS before biotinylation.

Materials

Cells
Wash buffer: PBS (*APPENDIX 2A*) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$
15 mg/ml sulfo-*N*-hydroxysuccinimde-biotin (sulfo-NHS-biotin; Pierce) in PBS, prepared fresh prior to use
Wash buffer containing 25 mM lysine monohydrochloride
Lysis buffer containing 50 mM glycine
0.5% (w/v) SDS
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Antibody specific for protein of interest
Avidin- or streptavidin-Sepharose beads

Additional reagents and equipment for pulse labeling (*UNIT 7.1*), immunoprecipitation (*UNIT 7.2*), and SDS-PAGE (*UNIT 6.1*)

1. Perform a pulse-chase experiment (see *UNIT 7.1*).

   The number of cells required depends on the experiment and expression level of the protein. The cells can be either suspension or adherent cells.

2. At the respective chase times, wash a sample/dish of cells carefully with wash buffer three times.

3. Incubate cells 30 min at 4°C in a small volume of wash buffer (enough to cover the cells) containing sulfo-NHS-biotin to a final concentration of 1 mg/ml.

   Other water-soluble, biotin-coupled cross-linkers can be used as well. Since the reagent reacts with amine groups, use of Tris-based buffers should be avoided.

   If the biotinylation is inefficient, the concentration of the reagent can be increased up to 10 mg/ml.

4. Wash cells twice with wash buffer containing 25 mM lysine monohydrochloride.

   Lysine contains two reactive amino groups for inactivation of the NHS group.

5. Lyse cells in an appropriate lysis buffer containing 50 mM glycine.

   Lysis buffer also usually contains Triton X-100; however, when unstable interactions occur use octylglucoside, digitonin, or CHAPS (e.g., see recipe for lysis mixture).

6. Determine the radioactivity, as above (see Basic Protocol 1, step 7). Immunoprecipitate (*UNIT 7.2*) the protein of interest from equal amounts of TCA-precipitable radioactivity.

7. Split the immunoprecipitate in two equal portions. Analyze one portion by SDS-PAGE (*UNIT 6.1*) directly after immunoprecipitation.

8. Denature the other portion by boiling 5 min in 100 µl of 0.5% SDS. Dilute the SDS by adding 900 µl of 50 mM Tris·Cl, pH 7.5. Centrifuge 5 min at 10,000 × *g*, 4°C. Transfer the supernatant to a new microcentrifuge tube. Take the supernatant and reprecipitate the biotinylated pool of immunoisolated proteins with ~10 µl packed avidin- or streptavidin-Sepharose beads. Analyze by SDS-PAGE (*UNIT 6.1*).

   Comparison of the total and biotinylated protein pool (run on the same gel) will show the kinetics of plasma membrane appearance.

ALTERNATE PROTOCOL 2

MEASURING ARRIVAL AT THE CELL SURFACE USING PROTEASE SENSITIVITY

Proteases can also be used to selectively degrade plasma-membrane proteins and thus to measure their arrival at the cell surface. Various proteases can be used depending on the susceptibility of the protein of interest. The optimal concentration of the protease for complete digestion has to be determined first. It should be below protease concentrations that break up cells.

Materials

Cells
PBS (*APPENDIX 2A*)
Protease: fresh 10 mg/ml stock solutions of either trypsin, chymotrypsin, or pronase in PBS
PBS containing 5% (v/v) fetal bovine serum (FBS)
Protease inhibitors, freshly prepared stocks:
  for trypsin: 100 mM (1000×) phenylmethylsulfonyl fluoride (PMSF) in methanol and 1 mg/ml (100×) trypsin inhibitor in PBS
  for chymotrypsin: 100 mM PMSF and 2 mg/ml (1000×) aprotinin in PBS
  for pronase: 100 mM PMSF and 10 mM (1000×) *N*-tosyl-lysine chloromethyl ketone (TLCK) in PBS
Lysis mixture (see recipe)
Antibody specific for protein of interest

Additional reagents and equipment for biotinylation of cell-surface proteins (Alternate Protocol 1), immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), pulse labeling (*UNIT 7.1*), and autoradiogarphy (*UNIT 6.3*)

Determine appropriate protease concentration

1. Label the protein pool at the plasma membrane by biotinylation (see Alternate Protocol 1).
2. Incubate labeled cells (usually one dish per condition or ~1 to 5×10^6 cells per condition) 30 min on ice in ~5 ml PBS with varying concentrations of proteases (0 to 10 mg/ml).

   Note that adherent cells will become detached by the protease treatment.
3. Remove soluble proteases by washing cells at least three times with 1 ml PBS containing 5% FBS each. Collect cells after each wash by centrifuging 3 min at 1500 × *g*, 4°C.

   Centrifugation is necessary because the cells will be detached by the protease treatment.
4. After the final wash, resuspend cells in a small volume of FBS (5×10^6 cells in 250 µl) and inactivate the proteases by adding the respective protease inhibitors at the correct 1× concentrations. Incubate the cells 10 min on ice. Centrifuge cells 5 min at 1500 × *g*, 4°C. Remove supernatant.

   Pronase should be removed by extensive washing.

   Adding protease inhibitors directly from stock solutions involves very small volumes (~0.25 µl); it is best if they are serially diluted to allow for more accurate pipetting.
5. Lyse cells with 1 ml lysis mixture containing the appropriate protease inhibitors at indicated 1× concentrations.
6. Isolate the desired protein by immunoprecipitation from equal numbers of cells (*UNIT 7.2*).
7. Analyze samples by SDS-PAGE (*UNITS 6.1*) followed by immunoblotting (*UNIT 6.2*) and incubation of the filters with avidin or streptavidin-coupled to peroxidase.
8. Detect the proteins with peroxidase-catalyzed reactions, preferably by chemiluminescence (e.g., enhanced chemiluminescence; ECL).

   This assay will determine the optimal concentration and the appropriate protease that will allow quantitative removal of surface protein without substantial cell destruction.

Measure protein arrival

9. Perform a pulse-chase experiment (*UNIT 7.1*).
10. Incubate cells from every chase time in the presence or absence of proteases under conditions determined above (steps 2 to 4).
11. Lyse cells (step 5), then immunoprecipitate proteins from equal numbers of cells (*UNIT 7.2*).
12. Analyze samples by SDS-PAGE (*UNIT 6.1*).
13. Fix gel and detect surface appearance by autoradiography (*UNIT 6.3*).

    Newly arrived protein will be trimmed (migrate faster by SDS-PAGE) or disappear due to protease activity.

ALTERNATE PROTOCOL 3

MEASURING ARRIVAL AT THE CELL SURFACE USING ANTIBODIES

Cell-surface appearance of proteins can also be analyzed by combining a pulse-chase experiment (*UNIT 7.1*) with incubation of antibodies with intact cells (Burke et al., 1984). After removal of unbound antibodies, the surface-protein population can be isolated and analyzed. Postlysis binding of intracellular proteins to the antibodies (which have two or more antigen-combining sites) should be prevented, and a control for this should be included in the analysis. To avoid this problem, it is preferable to use biotinylated Fab fragments instead of complete antibodies.

Materials

Cells
PBS (*APPENDIX 2A*), ice cold
Biotinylated Fab fragments of antibody specific for protein of interest (see Support Protocol 1) or complete antibody
PBS containing 5% (v/v) fetal bovine serum (FBS)
Unlabeled cells or antigen
Protein A–Sepharose *or* avidin- or streptavidin-coupled beads

Additional reagents and equipment for pulse labeling (*UNIT 7.1*), immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), and autoradiography (*UNIT 6.3*)

1. Perform a pulse-chase experiment (*UNIT 7.1*); include at least one early chase-point at which the protein has not yet reached the plasma membrane (e.g., 10 min).

2. At each chase time, wash cells once with 1 ml ice-cold PBS, suspend cells in a small volume (sufficient to cover the cells) of PBS or culture medium with antibodies or biotinylated Fab fragments, and incubate 30 min on ice.

   Antibody or Fab should be added at a saturation concentration as determined by preliminary experiments.

   Biotinylated Fab fragments are preferred because they have only one binding site, but complete antibodies can be used. In the latter case, controls for postlysis binding should be performed (see step 4).

3. Remove unbound antibodies/Fab fragments by washing at least three times at 4°C with 1 ml PBS containing 5% FBS.

   If using biotinylated Fab fragments, proceed directly to step 5.

4. *If using intact antibodies:* Add a 10-fold excess of unlabeled cells or a 10- to 100-fold excess of antigen before lysis to prevent postlysis binding of intracellular radiolabeled proteins to the antibody.

   Most intact antibodies have two antigen-binding sites (up to ten for IgM), of which only one may be occupied by the surface protein. The others may thus capture antigen postlysis. Competition with nonradioactive antigen added exogenously is not necessary when Fab fragments are used.

   Control (and exclude) postlysis binding of antigen through the analysis of at least one early chase point (see step 1) that should not give precipitation of radiolabeled surface proteins.

5. Lyse (see Basic Protocol 1, step 6), clear by centrifuging 10 min at 10,000 × *g*, 4°C, and add 20 µl protein A–Sepharose (when using antibodies) or streptavidin- or avidin-coupled beads (when using biotinylated Fab fragments) to the lysate. Follow a normal immunoprecipitation protocol (*UNIT 7.2*).

6. Analyze immunoprecipitates by SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*).

PREPARATION OF BIOTINYLATED FAB FRAGMENTS

The production of biotinylated Fab antibody fragments requires some time investment. However, the investment is easily rewarded because they can be used for multiple surface-appearance studies (see Alternate Protocol 3) and internalization experiments. Papain appears to be an ideal protease, cleaving between the Fc and Fab portions of the antibody. Since papain is a cysteine protease, it first has to be activated (by reducing the active-site cysteine) and then titrated to establish the optimal conditions for Ig digestion (also see *UNIT 16.4*).

Materials

23 mg/ml papain in 70% ethanol
25 and 40 mM sodium acetate buffer, pH 4.5 (*APPENDIX 2A*)
2-Mercaptoethanol
100 mM sodium acetate buffer, pH 5.5 (*APPENDIX 2A*)
Protein A– or G–purified antibody (*UNIT 16.3*)
150 mM NaCl/5 mM EDTA/100 mM Tris·Cl, pH 8.0
Dithiothreitol (DTT)
Iodoacetamide (store protected from light and moisture)
50 mM $NaHCO_3$ buffer, pH 8.5
Sulfo-*N*-hydroxysuccinimidyl-6-biotinamido-hexanoate (sulfo-NHS-SS-biotin; Pierce)
10 mM Na_3PO_4, pH 7.4/150 mM NaCl buffer

Sephadex G-25 column
Centricon-30 (Amicon)

Additional reagents and equipment for dialysis (*APPENDIX 3C*), SDS-PAGE (*UNIT 6.1*), and standard anion or cation exchange chromatography (*UNIT 16.4*)

Activate papain

1. Dilute 23 mg/ml papain stock with 25 mM sodium acetate buffer, pH 4.5, to a final papain concentration of 8 mg/ml.

   Ready-to-use kits for activation of papain are available from Pierce.

2. Add 6 µl of 2-mercaptoethanol to 200 µl diluted papain and 400 µl of 40 mM sodium acetate buffer, pH 4.5. Incubate 20 to 30 min at 4°C.

   2-ME is added to activate the papain.

3. Equilibrate and elute a 10-ml Sephadex G-25 column with 20 ml of 100 mM sodium acetate buffer, pH 5.5.

4. Run the activated papain solution over the column to remove various impurities from papain. Pool fractions with the highest protein concentration by measuring the absorbance at 280 nm.

Digest antibody

5. Dialyze protein A– or G–purified antibody against three changes of 200 ml of 150 mM NaCl/5 mM EDTA/100 mM Tris·Cl, pH 8.0.

6. Add 1 to 2 mM DTT prior to digestion.

7. Mix antibody and activated papain. Incubate at 4°C or room temperature.

   The efficiency of cleavage depends on the antibody isotype. It is thus best to vary the digestion period, the temperature, and the concentration of papain to determine optimal conditions for digesting the antibody of interest.

   The ratio of papain to antibody is usually between 1:50 and 1:200 for time points between 1 and 24 hr.

8. Stop reaction by adding iodoacetamide to a final concentration of 2 mM.
9. Check the digestion by analyzing an aliquot 12% SDS-PAGE (*UNIT 6.1*).

Isolate Fab fragments

10. Separate the Fab fragment from the Fc fragment by standard anion or cation exchange chromatography (*UNIT 16.4*).

    The Fc fragment will be selectively retained by an anion-exchange column, whereas the Fab fragment will be retained by a cation-exchange column and will only elute with high salt.

11. Check separation by SDS-PAGE under reducing and nonreducing conditions (*UNIT 6.1*).

    The Fc fragment contains an intrachain disulfide bridge and contaminations can best be visualized under nonreducing conditions.

Biotinylate Fab fragments

12. Dialyze the Fab fragments with three changes of 200 ml of 50 mM $NaHCO_3$ buffer, pH 8.5.
13. Add a ten-fold molar excess of sulfo-NHS-SS-biotin and incubate 2 hr at 4°C.
14. Remove unreacted biotin by centrifugation through a Centricon-30 concentrator according to manufacturers' instructions.
15. Dilute the sample in 10 ml of 10 mM Na_3PO_4, pH 7.4/150 mM NaCl buffer and centrifuge two successive times through the Centricon-30 concentrator to remove unreacted biotin.
16. Store labeled Fab at a concentration of 10 mg/ml in 50- to 100-µl aliquots at −80°C.

BASIC PROTOCOL 2

MEASURING TRANSPORT TO ENDOSOMES BEFORE PLASMA MEMBRANE APPEARANCE

Some proteins, like class II molecules of the major histocompatibility complex, first enter the endocytic pathway before they appear at the plasma membrane (Neefjes et al., 1990). A variation on the sialidase protection assay, described in Basic Protocol 1, has been used to visualize this on intact cells. Plasma membrane, but not endosomal, glycoproteins can be desialylated by sialidase treatment at 4°C on intact cells, whereas both protein pools are digested by sialidase that has entered the endocytic pathway by fluid-phase endocytosis at 37°C. A comparison of the number of sialic acids on glycoproteins from cells incubated under these conditions will allow determination of the localization of proteins in the ER/Golgi (not sialylated in the control situation), the TGN (sialylated under all conditions), endosomes (desialylated by endocytosed sialidase only), and the plasma membrane (desialylated by sialidase at 4°C and in the medium). Combined with a pulse-chase experiment, this will allow the positioning of the protein in the respective compartments at any time during the chase period. Entry in endosomes *before* arrival at the plasma membrane is indicative of direct transport from TGN to endosomes (see Neefjes et al., 1990). However, very rapid transit of the protein over the plasma membrane is still possible, and chase points should be selected carefully for validating the direct transport (without intersection with the plasma membrane) from TGN to endosomes.

Materials

Cells
Sialidase Type V from *Clostridium perfringens* (Sigma), dissolved in PBS and stored in aliquots at −20°C
Normal culture medium with 0.1% (w/v) BSA and no FBS
PBS (*APPENDIX 2A*) containing 10% (v/v) fetal bovine serum (FBS), ice-cold
Lysis mixture (see recipe)
Fetuin

Additional reagents and equipment for pulse labeling and TCA precipitation (*UNIT 7.1*), immunoprecipitation (*UNIT 7.2*), one-dimensional IEF (see Support Protocol 2), SDS-PAGE (*UNIT 6.1*), and autoradiography (*UNIT 6.3*)

1. Perform a pulse-chase experiment (*UNIT 7.1*); follow a 10- to 15-min labeling period with chases of 0, 15, 30, 60, 120, and 240 min.

   In this experiment, three conditions are tested at each chase point: (1) cells will be cultured after the radioactive labeling and analyzed at each chase point without further treatment to determine entry in the TGN; (2) cells will be treated with sialidase at 4°C to show appearance at the plasma membrane; (3) and cells will be cultured after the pulse in the presence of sialidase that will enter the endocytic pathway and thus desialylate endosomal and plasma-membrane proteins.

   For adherent cells, label three plates per chase point. Use ~5×10^6 cells per chase point per condition.

2. Split the cell culture at the beginning of the chase into two aliquots or use different dishes for the various conditions. Culture one set of cells at 37°C in culture medium containing 0.1% BSA and sialidase at a final concentration of 1 IU/ml to allow entry of sialidase in the endocytic route. Culture the other half of the cells in culture medium with 0.1% BSA alone.

   The culture medium should not contain fetal bovine serum (which has many sialylated proteins), but should contain 0.1% (w/v) BSA (which is not glycosylated).

   Cells like B lymphocytes that endocytose only small volumes may require higher concentrations of sialidase.

3. At every chase point divide the sample of the cells cultured in the medium/BSA alone (step 2) into two equal portions and incubate 1 hr at 4°C (or use two dishes), wash the cells, add PBS/$CaCl_2$ with or without 1 IU/ml sialidase. Also incubate the sample that was cultured in the presence of sialidase (step 2) for an additional 1 hr at 4°C.

   Include in the assay one sample that is not radiolabeled but is cultured in the presence of sialidase. These cells will be mixed with control radiolabeled cells before washing and lysis to verify complete removal of sialidase activity (because the sialidase should not act postlysis).

4. Remove sialidase by washing the cells three times with 1 ml ice-cold PBS containing 10% FBS.

5. Lyse cells in 1 ml lysis mixture containing 1 to 10 mg/ml fetuin.

   Fetuin is highly sialylated (for further inhibition of sialidase) and has a neutral pH. Thus, unlike free sialic acids, it will not increase the background during the immunoprecipitation procedure.

6. Determine the amount of radioactivity incorporated by TCA precipitation of 10 µl of each lysate (*UNIT 7.1*). Isolate proteins by immunoprecipitation (*UNIT 7.2*) from lysates containing equal amounts of TCA-precipitable radioactivity.

7. Analyze immunoprecipitates by one-dimensional IEF (see Support Protocol 2) or SDS-PAGE (*UNIT 6.1*).

   For best comparison, analyze the three conditions (control, sialidase 4°C, and sialidase 37°C) side by side on the gel. The protein has entered the TGN when the carbohydrate acquires terminal sialic acids that are not removed by the different sialidase treatments. The protein has entered endosomes when desialylated by the sialidase added to the chase medium, but not by the sialidase treatment at 4°C. Finally, the protein has reached the plasma membrane when it is a substrate for sialidase both in the culture medium and at 4°C.

SUPPORT PROTOCOL 2

SEPARATION OF PROTEINS BY CHARGE USING DENATURING ONE-DIMENSIONAL ISOELECTRIC FOCUSING

For one-dimensional IEF a 4% polyacrylamide gel is used to avoid separation by size (Neefjes et al., 1986). This requires careful handling of the gels during the preparation, run, and fixation. This protocol is for gels of ~55 ml.

Materials

0.8% (w/v) agarose in water
Urea
10% (v/v) Triton X-100 or Igepal CA-630 (Sigma)
30% (w/v) acrylamide/1.6% (w/v) bisacrylamide solution
Ampholytes (Pharmacia Biotech)
10% (w/v) ammonium persulfate
N,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)
Isoelectric focusing sample buffer (see recipe)
Immunoprecipitates (see Basic Protocol 1 or see Basic Protocol 2)
20 mM H_3PO_4
Bromphenol blue
50 mM NaOH

30 × 19–cm glass plates
1-mm spacers
20-well Teflon comb
Vertical gel chamber

Additional reagents and equipment for gel electrophoresis (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

Prepare IEF gel

1. Assemble electrophoresis apparatus using 30 × 19–cm glass plates with 1-mm spacers. To prevent the gel from slipping between the glass plates, place spacers slightly tilted towards each other with the narrow side toward the bottom. Apply 0.8% (w/v) agarose outside spacers, between the glass plates, to prevent leakage.

2. Prepare 55 ml gel solution by mixing the following reagents. Dissolve urea by gently shaking the solution under hot tap water.

   30.25 g urea
   11 ml 10% Triton X-100 or Igepal CA-630
   8.25 ml 30% acrylamide/1.6% bisacrylamide solution
   12.1 ml H_2O.

   Do not dissolve urea in a microwave or by boiling as this can result in evaporation and crystalization of the urea after polymerization of the gel.

3. Add ampholytes after solubilization of the urea.

   The mixture of ampholytes determines the pH gradient over the gel. The author generally uses 2.2 ml of pH 5.7 ampholytes, 550 µl of pH 3.5 to 10 ampholytes, and 220 µl of pH 7 to 9 ampholytes.

4. Polymerize the gel by adding 200 µl of 10% ammonium persulfate and 100 µl TEMED.

5. Pour gel mixture between the preassembled plates and insert a 20-well Teflon comb. Allow to solidify.

   The gel usually takes ~30 min to solidify.

6. Place gel in a vertical gel chamber. Fill lower chamber with 20 mM H_3PO_4.

Prepare samples and run gel

7. Incubate immunoprecipitates ≥5 min at room temperature with isoelectric focusing sample buffer and load samples.

8. Before addition of the upper buffer, load a small layer of 3-fold diluted isoelectric focusing sample buffer with ~1 (% or mg/ml) bromphenol blue.

   This is to prevent hydrolysis of asparagine and glutamine side chains by the NaOH in the upper gel chamber.

   The bromphenol blue will indicate the extent of the run but is not strictly necessary.

9. Carefully fill the upper chamber with 50 mM NaOH.

10. Run the gel overnight at 1000 V. Limit power such that the run starts at ~300 to 400 V and increases to a maximum of 1000 V.

    This will prevent heating and melting of the gel. The voltage will swiftly rise due to increased resistance. Smaller gels should be run at a lower limiting voltage; e.g., a gel 15 cm wide should be run at ~600 V with the power set such that the run starts at ~200 V.

11. Fix gel and expose for autoradiography (*UNIT 6.3*).

    The gel is very sloppy. Do not take the gel off the glass plates, but rather turn the glass plates around over the fixative. The gel will then fall into the fixative. Perform a similar procedure for transfer of the gel to Whatman 3MM paper prior to drying the gel.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Isoelectric focusing sample buffer

9.5 M urea
2% (w/v) Triton X-100 or Igepal CA-630 (Sigma)
2% (v/v) ampholytes (Pharmacia Biotech), pH 3.5 to 10
5% (v/v) 2-mercaptoethanol
Store 1-ml aliquots at −20°C

Lysis mixture

50 mM Tris·Cl, pH 7.5AS (*APPENDIX 2A*)
150 mM NaCl
5 mM $MgCl_2$
0.5% (w/v) detergent
Store at 4°C

The choice of detergent depends on the experiment. For stable protein complexes the author routinely uses 0.5% Igepal CA-630 (Sigma) or Triton X-100. For more unstable protein complexes, 0.5% digitonin or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) should be used.

COMMENTARY

Background Information

Plasma-membrane proteins are synthesized in the ER. Here they have to fold properly and assemble before they are allowed to pass the "quality control system" of the ER and enter the Golgi complex. The folding and assembly steps are rate limiting and can vary considerably between different proteins, although some variability in transport rates through the Golgi complex occurs as well. Consequently, the rate of surface appearance can vary from 10 to 15 min up to many hours, even for proteins belonging to the same family such as MHC class I alleles (Neefjes and Ploegh, 1988). These differences cannot be predicted and can only be experimentally determined.

Many endosomal and lysosomal proteins are transported to their final destination by transient trafficking through the plasma membrane. Because proteins are only briefly at the plasma membrane before endocytosis, it is very hard to distinguish between direct sorting at the TGN for the endocytic pathway and an indirect route involving transport over the plasma membrane. In addition, the two routes are not mutually exclusive. For example, the process of sorting at the TGN appears to be saturable (Marks et al., 1996) and overexpression of endosomal proteins may easily result in transient cell-surface appearance. Transient transport over the plasma membrane can best be visualized by slowing or inhibiting endocytosis in combination with the techniques described in this unit for detection of cell-surface appearance. However, easy and reproducible techniques for selectively inhibiting endocytosis have not been developed yet.

Several different techniques to selectively tag the surface population of glycoproteins have been described in this unit. All of these techniques consist of combinations of pulse-chase experiments and chemical or enzymatic modification on intact cells. These techniques require that the modifications are quantitative and readily inhibitable. In addition, optimal conditions for the modifications should be determined prior to the experiment. Internal controls excluding postlysis modifications should always be included in the experiments. The different experimental protocols all have advantages and disadvantages as discussed below.

Biotinylation of intact cells to tag the cell-surface protein pool is an efficient way to visualize cell-surface arrival (see Alternate Protocol 1). Postlysis biotinylation does not occur. Since the modification is not quantitative, the unmodified pool of proteins contains intracellular as well as cell-surface proteins. The assay employing sialidase to remove the sialic acids at the plasma membrane (see Basic Protocol 1) is quantitative and thus makes it possible to distinguish the intracellular from the cell-surface pool. However, the sialidase treatment protocol is more laborious. Furthermore, the effect of desialylation is not always obvious upon analysis by SDS-PAGE and often requires analysis by one-dimensional IEF or two-dimensional IEF/SDS-PAGE, techniques that are more time consuming.

Protease removal of cell-surface proteins (Alternate Protocol 2) is another alternative, but the usefulness of this technique depends on the sensitivity of the target protein. The optimal concentration and type of protease have to be defined first. If proteolysis requires high concentrations of protease, cell death and DNA release may be observed, which may complicate the interpretation of the experiment. In addition, some remnants of proteolytic activity in the lysate may obscure the results. Unless the protein is sensitive and accessible to a particular protease, this assay will not easily result in quantitative removal of cell-surface proteins.

Finally, biotinylated Fab fragments are an easy tool to follow specifically the surface appearance of proteins by combining a pulse-chase protocol with surface binding (Alternate Protocol 3). However, the generation and biotinylation of Fab fragments is laborious. Complete (bivalent) antibodies may be a more convenient alternative for immunoprecipitation of proteins expressed at the cell surface, but postlysis binding of antigen should be excluded.

Critical Parameters and Troubleshooting

All the protocols presented in this unit require a pulse-chase experiment in combination

with detection of cell-surface appearance. When early events (folding or assembly) are followed, label for 5 min and chase 0, 5, 10, 20, 30, and 60 min. Early folding events may be traced by labeling for 1 min and chasing for 0, 1, 2, 4, 7, and 10 min. For transport processes to the plasma membrane, the author usually labels cells for 15 min followed by chases of 0, 15, 30, 60, 120, and 240 min. Proteins should be sufficiently labeled so as to effectively visualize them after each incubation step. If they are not, use more cells for the pulse labeling, increase the pulse time, and/or add more radioactive amino acid. The concentration of radioactive amino acids during the pulse may be increased by simply performing the pulse in a smaller volume. Starve the cells for 30 to 45 min prior to the pulse in culture medium devoid of the amino acid used for radioactive labeling of the cells. Include 10% fetal bovine serum in this starvation medium; the cells will then be more viable and will incorporate higher amounts of radioactivity. [^{35}S]Methionine and/or cysteine are usually employed for biosynthetic labeling. If proteins are poor in these amino acids, consider pulse labeling the cells with other amino acids (see *UNIT 7.1*).

It is essential to keep the cells viable and intact during the different incubation and washing steps. Gentle centrifugation (e.g., $400 \times g$) during the washing procedures improves the intactness and recovery of the cells. Cells can best be resuspended after removal of the supernatant by simply tapping against the microcentrifuge tube. Addition of 0.1% (w/v) bovine serum albumin or 5% to 10% (v/v) fetal bovine serum to the washing buffer may also increase viability and recovery of the cells.

Biotinylation of intact cells (Alternate Protocol 1) to label surface proteins is a convenient assay although it is not quantitative. However, this protocol is useful for establishing when and at what rate proteins appear at the plasma membrane, provided that identical conditions are used to treat the samples. The decrease in intracellular protein pool cannot be easily determined when, for example, 20% of the surface proteins are biotinylated. Higher concentrations of the biotinylation reagent increase the efficiency of labeling, but such experiments require relatively large amounts of reagent and are very expensive. When biotin labeling is poor, wash cells more carefully to remove competing proteins from the medium.

The sialidase treatment of intact cells (Basic Protocol 1) to detect surface appearance requires establishing the conditions for full desialylation. The conditions for inactivation/removal of sialidase should be internally controlled. The conditions described usually result in quantitative removal of sialic acids on nonadherent cells. Adherent cells can be detached from the culture plates before the sialidase treatment to ensure complete desialylation. Although this is usually not necessary, it ensures complete desialylation. Free sialic acids should not be included in the lysis buffers for inactivation of sialidase because this acidifies the buffer and thus increases the background of the immunoprecipitation. Fetuin (which is a highly sialylated glycoprotein) inhibits sialidase without changing the pH of the lysis buffer.

One-dimensional IEF to separate different sialylated forms of a protein (Support Protocol 2) is a powerful but difficult technique. Always place the spacers on the sides slightly tilted towards each other to prevent the gel from slipping into the lower chamber. Also be careful not to allow direct contact between the lower and upper gel chambers, e.g., along the spacers. If this happens, the gel will become very hot and the glass plates may break. The author often adds a small drop of agarose to the corners of the gel contacting the spacers to prevent leakage and direct contact between the upper and lower buffer. One-dimensional IEF gels are very fragile and can easily break. Handle gels carefully and never pick them up with your hands. Most fixatives will not make the gel stronger. One clear exception is fixation in dimethyl sulfoxide/2,5-diphenyloxazole (DMSO/PPO), but this is no longer regularly used.

The use of proteases (Alternate Protocol 2) also requires titration to determine the optimal conditions. Often, cell destruction is observed and the resulting released DNA interferes with the experiments. This protocol is not suitable for all proteins, as many proteins are very resistant to proteases or are only degraded under conditions that also destroy the intact cell. If so, try to use other proteases that more selectively degrade the surface protein without resulting in significant cell damage. Most commercial proteases have selective and efficient inhibitors. However, this may be a problem for protease cocktails like pronase, which should not be used for cell-surface appearance experiments.

The production of the biotinylated Fab fragments (Support Protocol 1) is somewhat time consuming and requires isolation of reasonably large quantities of antibodies before the papain digestion can be performed. Ready-to-use kits

are available for this. Biotinylated Fab fragments may result in easy isolation of the surface pool of proteins. The unbound fraction may not necessarily be the intracellular fraction unless it is shown that *all* surface proteins were captured by the Fab fragment. To test this, take a late chase point (e.g., 6 hr) and show experimentally that the radioactive protein is quantitatively recovered. It is preferable to not use intact multivalent antibodies because they may induce internalization by cross-linking two or more antigens, and they may easily bind antigens postlysis.

Anticipated Results

Sialidase treatment is the most sensitive and quantitative of the assays described and allows analysis of surface appearance when the chase times are correctly chosen. SDS-PAGE may show a shift in apparent molecular weight. Otherwise, one-dimensional IEF or two-dimensional IEF/SDS-PAGE should be used. Combining a pulse-chase experiment with treatment of cells at 4°C and 37°C (see Basic Protocol 2) allows biochemical detection of the localization of the glycoprotein in pre-Golgi, TGN, endosomes, and plasma membrane.

The biotinylation protocol does not quantitatively modify all proteins at the plasma membrane, but it is still a very practical way to determine cell-surface appearance. Quantitative biotinylation is not required. The isolates can be analyzed by normal SDS-PAGE and surface appearance can be efficiently seen.

Protease treatment of intact cells to remove the plasma-membrane protein pool is possible for protease-sensitive proteins, but it is difficult to verify that the digested protein is obtained from equal numbers of cells/proteins.

Cell-surface immunoisolation by Fab fragments is an efficient way to show cell-surface appearance. The pulse-chase can be analyzed by SDS-PAGE prior to and after Fab isolation and the rate of cell-surface appearance of the protein of interest can be easily determined.

Time Considerations

Most assays described in this unit are relatively laborious. Pulse-chase experiments combined with protease/sialidase/biotin treatment, TCA precipitation for quantitation and immunoprecipitation, followed by SDS-PAGE or one-dimensional IEF take ≥3 days before the result can be seen. The preparation of the biotinylated Fab fragments is time consuming (~2 to 3 days) but clearly worth the effort when used for multiple experiments.

Literature Cited

Burke, B., Matlin, K., Bause, E., Legier, G., Peyrieras, N., and Ploegh, H. 1984. Inhibition of N-linked oligosaccharide trimming does not interfere with surface expression of certain integral membrane proteins. *EMBO J.* 3:551-556.

Marks, M.S., Woodruff, L., Ohno, H., and Bonifacino, J.S. 1996. Protein targeting by tyrosine- and di-leucine-based signals: Evidence for distinct saturable components. *J. Cell Biol.* 135:341-354.

Neefjes, J.J. and Ploegh, H.L. 1988. Allele and locus-specific differences in cell surface expression and the association of HLA-class I heavy chain with β_2-microglobulin: Differential effects of inhibition of glycosylation on class I subunit association. *Eur. J. Immunol.* 18:801-810.

Neefjes, J.J., Breur-Vriesendorp, B.S., Van Seventer, G.A., lvanyi, P., and Ploegh, H.L. 1986. An improved biochemical method for the analysis of HLA-class I antigens. Definition of new HLA class I subtypes. *Hum. Immunol.* 16:169-181.

Neefjes, J.J., Stollorz, V., Peters, P.J., Geuze, H.L., and Ploegh, H.L. 1990. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell* 61:171-181.

Warmerdam, P.A.M., Long, E.O., and Roche, P.A. 1996. lsoforms of the invariant chain regulate transport of MHC class II molecules to antigen processing compartments. *J. Cell Biol.* 133:281-291.

Contributed by Jacques Neefjes
The Netherlands Cancer Institute
Amsterdam, The Netherlands

Analysis of Membrane Traffic in Polarized Epithelial Cells

Spatial asymmetry is fundamental to the structure and function of most eukaryotic cells (Drubin and Nelson, 1996). A basic aspect of this polarity is that the cell's plasma membrane is divided into discrete domains. The best studied and simplest example of this occurs in epithelial cells, which line exposed body surfaces (Matter and Mellman, 1994; Mostov et al., 1999, 2000). Epithelial cells have an apical surface facing the outside world and a basolateral surface contacting adjacent cells and the underlying connective tissue. These surfaces have completely different compositions and are separated by tight junctions, which block movement of plasma membrane proteins between the apical and basolateral surfaces and also prevent diffusion of extracellular material between cells. Epithelial cells use two pathways to send proteins to the cell surface. Newly made proteins can travel directly from the trans-Golgi network (TGN) to either the apical or basolateral surface. Alternatively, proteins can be sent to the basolateral surface and then endocytosed and transcytosed to the apical surface. A schematic view of a simple polarized epithelial cell is given in Figure 15.5.1.

Studies on membrane traffic in polarized epithelial cells have been greatly facilitated by the use of cell lines grown as a well-polarized monolayer. The most widely utilized of these is the Madin-Darby canine kidney (MDCK) cell line. Other cell lines that have been used for studying polarized membrane traffic include Caco-2 (human intestinal cancer) and FRTL (Fischer rat thyroid; Weimbs et al., 1997). Table 15.5.1 summarizes the cell lines most commonly used in studies of membrane traffic in polarized epithelial cells. Generally these cell lines are maintained by growing adherent cells in standard cell culture plastic dishes or flasks; however, for the actual experiments in which polarized traffic is assessed, the cells are usually grown on porous filters. Such filters are available mounted in holders sold by several manufacturers, including Corning, Falcon, and Nunc. These devices come in several sizes and are available with pores of different diameters. Most of the work done by the authors has been with 12-mm Corning Transwells with filters containing pores of 0.4-μm diameter. Table 15.5.2 lists the main types of units that are commercially available.

The usual type of study is to examine the biosynthetic delivery of a membrane or secreted protein to the apical or basolateral surface (see Basic Protocol 2). This sort of experiment is quite similar to studying the delivery of proteins to the surface of nonpolarized cells, as described in *UNIT 15.4*, with the principal difference being that delivery to either the apical or basolateral surface is measured, as opposed to the plasma membrane as a whole. Although MDCK cells contain numerous endogenous apical and basolateral plasma membrane proteins, these are usually expressed at very low levels, making it difficult to study the trafficking of any individual protein. Additionally, one often wishes to examine the polarized localization or trafficking of a protein that may not be expressed endogenously in MDCK cells; therefore, one typically begins a project by transfecting cDNA for the protein of interest into MDCK cells in order to overexpress the protein for study (see Basic Protocol 1). Most of the authors' experience is with the calcium phosphate-based transfection system (see Basic Protocol 1; Breitfeld et al., 1989). Cells can be transfected by other methods, including various commercially available lipid-based transfection reagents (e.g., Lipofectamine 2000) or by electroporation. Although transient transfection and expression systems can be used, in many cases the fraction of cells expressing the protein of interest is very low, and the cells that express the transfected protein tend to be less well polarized in part because the process of polarization takes 4

Contributed by Joshua H. Lipschutz, Lucy Erin O'Brien, Yoram Altschuler, Dana Avrahami, Yen Nguyen, Kitty Tang, and Keith E. Mostov

Current Protocols in Cell Biology (2001) 15.5.1-15.5.18

to 7 days, whereas transient protein expression generally peaks at 48 hr and is often gone by 72 hr; therefore, in most cases it is advisable to generate stable clones (see Support Protocol 1). These clones are then expanded for further use by growing on standard tissue culture dishes. For studies of membrane traffic the cells must be cultured on filter units (see Support Protocol 2). It is important to check that the cells form a tight, well-polarized monolayer on the filter. Several methods are available to do this, and a particularly easy method is presented in this unit (see Support Protocol 3). A cell surface biotinylation assay to measure delivery of proteins is presented (Basic Protocol 3; Luton and Mostov, 1999). As the study of polarized membrane traffic is fundamentally the study of the localization of proteins, it is extremely useful to visualize the location of proteins in the epithelial cell. In addition, a fixation method to use for laser scanning confocal immunofluorescence

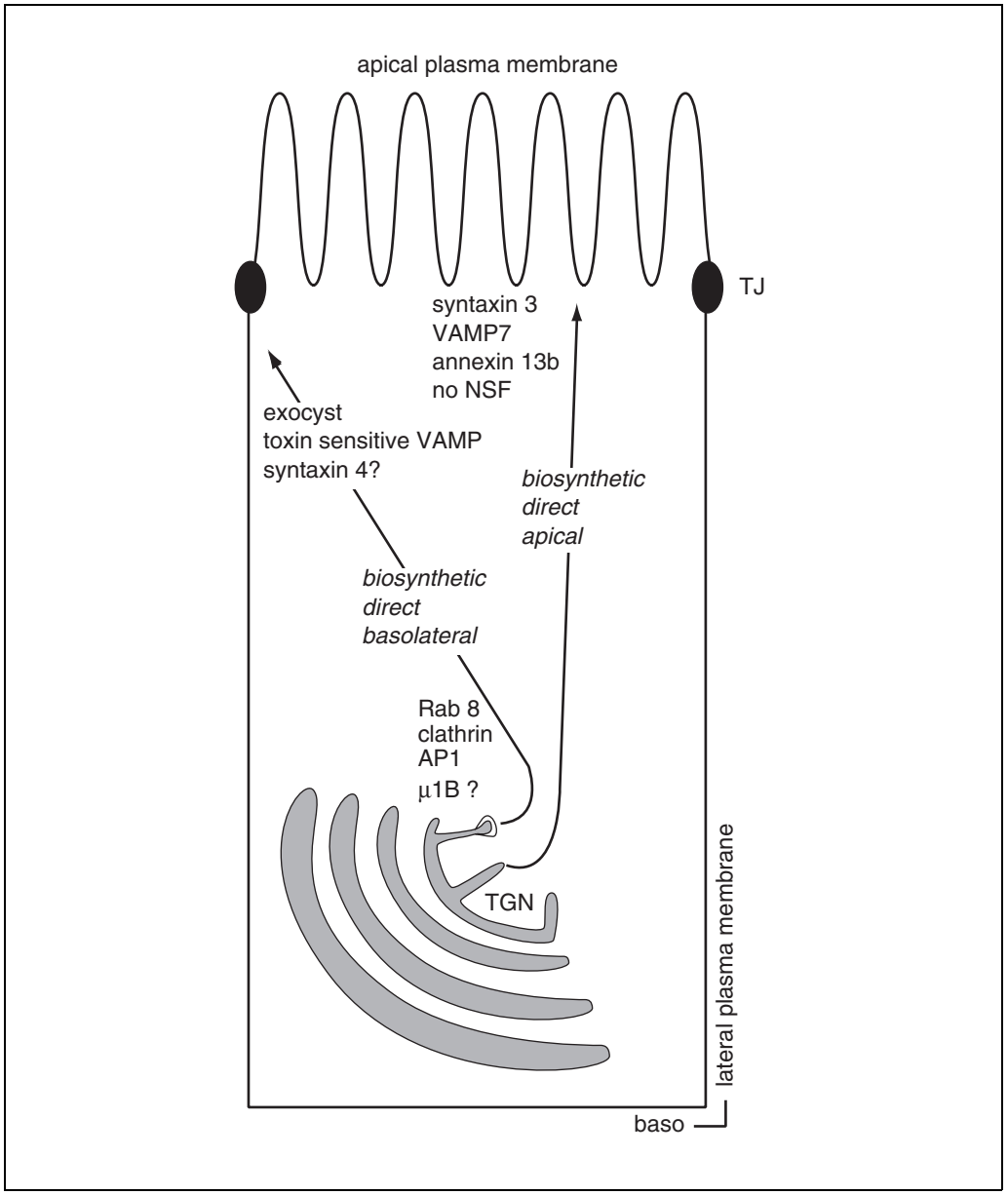


Figure 15.5.1 Schematic diagram of a polarized epithelial cell. Only the major biosynthetic pathways leading directly from the TGN to the apical and basolateral surfaces are shown. Components of the membrane traffic machinery that are likely to be involved in each of these two pathways are also indicated. For further details, see Mostov et al. (2000). Abbreviations: TGN, trans-Golgi network; TJ, tight junction.

Table 15.5.1 Cell Lines Commonly Used to Study Epithelial Membrane Traffic in Polarized Epithelial Cells

Cell line	Origin	Comments	ATCC#[a]
MDCK	Canine kidney	Most widely used. Two major categories, types I and II, though many clones with specific phenotypes.	CCL-34
Caco-2	Human intestine	Although derived from human colon cancer, more closely resembles small intestine. Slow growing.	HTB-37
FRTL	Rat thyroid	Some glycolipids and GPI-anchored proteins are found at the basolateral surface, unlike other epithelial cells.	CRL-1468

[a]The ATCC home page can be reached at *http://www.atcc.org.*

Table 15.5.2 Commercially Available Filters for Growth of Epithelial Cells

Material	Manufacturer	Comments
Polycarbonate	Corning Millipore BD Biosciences Labware Nalge Nunc International	Most widely used
PTFE/FP	Millipore Corning BD Biosciences Labware	Good optics. Coating with extracellular matrix (ECM) is usually required; can be purchased precoated
PET	BD Biosciences Labware	Available in different pore densities, with low density having better optical properties, but lower porosity. Available precoated with several types of ECM
Anopore	Nalge Nunc International	Very low intrinsic fluorescence, well-suited for immunofluorescence microscopy
Cellulose esters	Millipore	High protein binding
Polyester	Corning	Good optics

microscopy of proteins in polarized epithelial cells is also presented (see Basic Protocol 4; Luton and Mostov, 1999).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly (e.g., use of a sterile hood).

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH.

BASIC PROTOCOL 1

TRANSFECTION OF POLARIZED EPITHELIAL CELLS IN SUSPENSION AND SELECTION OF RESISTANT CLONES

The goal of this protocol is to transfect a plasmid containing the gene of interest into mammalian epithelial cells under a suitable promoter (i.e., a CMV promoter). This protocol provides the basis for the other experimental protocols described.

Materials

Confluent cells grown on 10-cm tissue culture dish
HEPES buffered saline (HeBS), pH 7.05 (see recipe; also see *APPENDIX 2A*)
20 µg plasmid DNA in 1 to 20 µl H_2O
2 M $CaCl_2$: filter sterilize and store up to 6 months at 4°C
CMF-DPBS (*APPENDIX 2A*)
Trypsin/EDTA solution—e.g., 0.25% (w/v) trypsin/0.2% (w/v) EDTA (*UNIT 1.1*)
MEM medium (*APPENDIX 2B*) with and without 5% (v/v) FBS (*APPENDIX 2A*)
20 mM chloroquine in water: filter sterilize and store up to 6 months at 4°C
15% (w/v) glycerol in HeBS: filter sterilize and store up to 6 months at 4°C
Selection medium: MEM/5% FBS containing eukaryotic antibiotic (e.g., G418; see recipe)
Nontransfected cells
10-cm dishes

Additional reagents and equipment for picking clones (see Support Protocol 1)

Transfect cells

1. Split cells grown on a 10-cm dish 1:10 on the day prior to transfection in order to yield ~30% confluency on the day of transfection.
2. To 0.5 ml HEPES buffered saline (HeBS), pH 7.05, add 20 µg plasmid DNA in 1 to 20 µl water, and 31 µl of 2.0 M $CaCl_2$. Flick the tube for 20 sec.

   The pH of the HeBS has to be exact for a good precipitate to form.

   This step should be done in a sterile hood.
3. Incubate the plasmid at room temperature for 30 min, avoiding any unnecessary shaking of the sample.
4. Trypsinize one subconfluent (i.e., ~30% confluent or ~1×10^7 cells) 10-cm dish of cells by adding 1 to 2 ml of trypsin/EDTA solution after washing with CMF-DPBS.

   For trypsinization of MDCK cells, use 0.25% (w/v) trypsin/0.2% (w/v) EDTA. The desired concentration of cells is ~0.5 to 1 × 10^6 cells/ml.
5. Centrifuge cells for 3 min at 300 × *g*, room temperature. Resuspend in 5 ml MEM medium with 5% FBS.
6. Transfer 1 ml of the cell suspension into a 10-cm dish.
7. Slowly add 500 µl DNA-Ca^{2+} coprecipitate (step 3) while simultaneously agitating the 10-cm dish. Incubate at room temperature for 15 to 20 min.
8. Add 50 µl of 20 mM chloroquine (200 µM final) to 3.5 ml MEM/5% FBS. Add the MEM/FBS/chloroquine mixture to the dish and agitate to spread cells.
9. Incubate 6 to 18 hr (e.g., overnight) at 37°C in order to allow the cells to attach to the dish.

Plate transfected cells

10. Remove the medium. Wash once with chloroquine-containing medium (see step 8).
11. Add 2 ml of 15% glycerol in HeBS gently. Incubate exactly 1 min at 37°C.
12. Remove glycerol by washing gently twice with MEM without FBS, and then adding 10 ml MEM/5% FBS.
13. Allow cells to grow 2 to 3 days at 37°C, until dish is confluent.

    Don't allow the cells to grow >3 days, even if the dish is not yet confluent as this will result in an overgrowth of nontransfected cells. The important thing is to allow the cells to divide several times.

14. Trypsinize the cells (step 4), and resuspend in 10 ml selection medium. As a control, use nontransfected cells.

Clone cells

15. Split the cells into 7 different dilutions in 10-cm dishes:

Cell suspension (ml)	0.025	0.05	0.1	0.2	0.4	0.8
Selection medium (ml)	9.975	9.95	9.9	9.8	9.6	9.2

    Shake to distribute cells evenly.

Select stably transfected cells

16. Incubate cells at 37°C for 16 to 21 days (i.e., until the colonies are big enough to pick), adding fresh selection medium every 4 to 6 days.

    Depending on the stability of the selection agent, it may not be necessary to change the medium.

    Cells will start to die after 3 to 5 days and colonies should be seen within 10 to 14 days, but will not be big enough to pick until after 16 to 21 days.

17. Clones should generally be picked from the dishes with the highest dilution possible (see Support Protocol 1).

    Dilutions in which 0.4 or 0.8 ml cells were used can usually serve as the pooled clones.

PICKING STABLY TRANSFECTED CLONES

SUPPORT PROTOCOL 1

The goal in this protocol is to identify and stably propagate epithelial cell clones that express the gene of interest.

Additional Materials *(also see Basic Protocol 1)*

~16-day-old 10-cm dishes containing diluted transfected and nontransfected cells in selection medium (see Basic Protocol 1)
Calcium- and magnesium-free DPBS (CMF-DPBS; *APPENDIX 2A*)
Medium-sized glass cloning ring, sterile (Bellco Glass)
0.5% (w/v) SDS lysis buffer
12% (w/v) slurry of CL-2B beads
12-well tissue culture plates

1. Examine ~16-day-old 10-cm dishes containing diluted transfected and nontransfected cells in selection medium, looking for single colonies. With a marker, draw a circle around the single colonies on the outside bottom of the dishes.

If the original 10-cm dishes (i.e., the one which was split into seven different dilutions; see Basic Protocol 1, step 15) was ~30% confluent, single colonies are generally found in the dilutions in which 0.025 to 0.2 ml cells were used.

2. Wash dishes with 10 ml CMF-DPBS.
3. Use a Pasteur pipet attached to a vacuum device to aspirate a "dry" ring (i.e., devoid of liquid) around a single colony.

   The "dry" ring will prevent trypsin from leaking under the cloning ring in step 4. Do this procedure for each colony individually, as the cells will dry out if more than one colony is harvested at a time.

4. Place a medium-sized sterile glass cloning ring around the colony. Add trypsin/EDTA solution until the cloning ring is filled. Repeat steps 3 and 4 for all colonies to be picked.

   For epithelial cells (e.g., MDCK), use 0.25% trypsin and 0.2% EDTA.

   At least 15 clones should be picked, though more is preferable (i.e., 50 if possible).

5. After all the cloning rings have been placed, incubate for ~20 min at room temperature in a sterile hood.
6. Remove the trypsin containing the loose cells and add to the well (i.e., 1 colony per well) of a 12-well tissue culture plate containing 2 ml selection medium. Repeat for all colonies.
7. Resuspend cells by pipetting up and down several times. Transfer 1 ml from each well to the corresponding well of a new 12-well plate (one plate will be for protein collection and the other for colony expansion).
8. Incubate to confluency (i.e., ~5 to 7 days) at 37°C.

   It is generally not necessary to change the medium if the cells are harvested as soon as they are confluent.

   The length of time to confluency depends on how many loose cells are transferred in good condition to the wells. This can vary significantly from well to well.

9. Add 500 µl 0.5% SDS lysis buffer, boil the samples 5 min at 100°C. Vortex samples 10 min. Preclear the samples by adding 20 µl of a 12% slurry of CL-2B beads. Mix several times. Microcentrifuge briefly at maximum speed. Collect supernatant and use an aliquot for immunoblotting (*UNIT 6.2*).
10. For the colonies that show expression, use the second identical well to expand the cells.

    The authors recommend keeping the cells under selection while expanding the colony size.

SUPPORT PROTOCOL 2

CULTURE OF EPITHELIAL CELLS ON FILTERS

The goal in this protocol is to allow epithelial cells to become fully polarized. This is accomplished by culturing epithelial cells on porous filters that allow the cells to access medium both apically and basolaterally.

Additional Materials *(also see Basic Protocol 1 and Support Protocol 1)*

10-cm dish of confluent epithelial cells (Table 15.5.1)
12-mm Transwell filters and appropriate dishes
IEC clinical centrifuge with 12 × 15 rotor

Additional reagents and equipment for determining the tightness of epithelial monolayers (see Support Protocol 3)

1. Wash a 10-cm dish of confluent epithelial cells with 10 ml CMF-PBS.
2. Add 3 ml trypsin/EDTA solution (e.g., 0.25% trypsin/0.2% EDTA for MDCK cells) and incubate at 37°C for 15 min.
3. Tap dish to agitate cells. When all cells are detached, add 8 ml MEM/5% FBS.

   This step is done in order to neutralize the trypsin.
4. Transfer to an appropriate tube and centrifuge cells 3 min at 300 × *g* (e.g., 1000 rpm in an IEC 12 × 15 rotor), room temperature. Discard the supernatant.
5. Resuspend cells in 10 ml MEM/5% FBS and mix thoroughly by pipetting up and down ~10 times. Add 0.5 ml of this solution to the apical chamber (i.e., inside) of a 12-mm Transwell filter. Then add an additional 0.4 ml MEM/5% FBS to the apical chamber for a total of 0.9 ml.

   This corresponds to ~2.5×10^5 cells/well.
6. Add 1 ml MEM/FBS to the basal chamber (i.e., area surrounding the Transwell filter).
7. Incubate at 37°C, replacing 1 ml apical and 1 ml basolateral medium every day for 3 to 5 days until cells form a tight monolayer (see Support Protocol 3).

   IMPORTANT NOTE: *It is important to first remove the basal medium and then remove the apical medium, and to replace in the opposite order. If the basal medium is present and the apical medium is absent then the cells can be pushed off the filter.*

   The cells are fully polarized and ready to examine after 4 to 7 days of growth on the filter.

DETERMINING THE LEAKINESS OF A MONOLAYER OF CELLS GROWN ON A FILTER

SUPPORT PROTOCOL 3

When grown on a Transwell or similar filter support, epithelial cells should form a confluent monolayer; however, with most types of filter materials, it is difficult or impossible to clearly visualize the cells themselves. Hence, it is not easy to determine if they have formed a confluent tight monolayer. Several methods have been devised to measure how intact the monolayer is. Classically, electrical resistance across the monolayer is measured; however, type II MDCK cells typically have a rather low electrical resistance, on the order of 100 ohms/cm^2, and so electrical resistance is rather insensitive to defects in the monolayer. Thus, the authors devised an extremely simple method to monitor the integrity of the monolayer. Although it is qualitative, it has been found to be a very reliable predictor of the integrity of the monolayer and the success of the experiment in the authors' laboratory.

Additional Materials *(also see Support Protocol 2)*

Pasteur pipet connected to a vacuum system

1. Culture cells on filters, as described (see Support Protocol 2).
2. The day before the experiment (i.e., typically after 3 to 6 days of growth on the filter), add normal cell culture medium to the apical well to fill it to overflowing.

   This support protocol is usually performed as part of the daily replacement of apical and basolateral media.

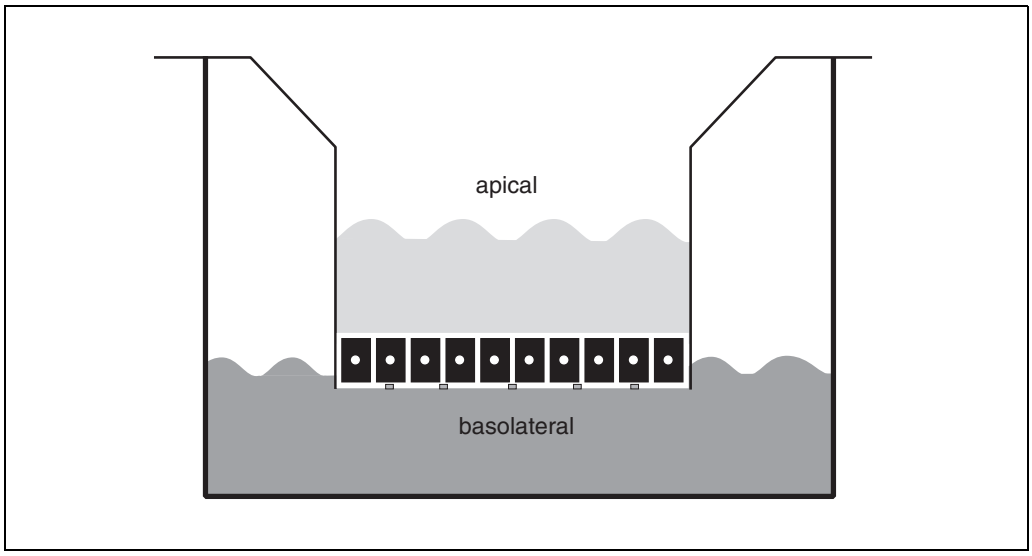


Figure 15.5.2 Schematic diagram of polarized epithelial cells growing in a Transwell. Cells are black rectangles with small white circles indicating nuclei. The apical and basolateral medium are gray. The fluid level of the apical medium is higher than the basolateral medium, as occurs during Support Protocol 3.

3. Use a sterile Pasteur pipet connected to a vacuum system to aspirate most of the medium from the basolateral chamber, leaving just enough medium to contact the entire basal surface of the filter.
4. Incubate the Transwell filter, still in the multiwell plate, at 37°C overnight (i.e., typically 8 to 16 hr).
5. Determine the tightness of the monolayer by assessing the fluid level of the apical chamber.

   If the cell monolayer is intact, the fluid level inside the apical chamber will not go down overnight—i.e., the apical chamber will remain filled to the brim (Figure 15.5.2). If, on the other hand, the fluid level leaks, the monolayer is not tight and the cells should be discarded.

BASIC PROTOCOL 2

PULSE-CHASE EXPERIMENTS IN POLARIZED EPITHELIAL CELLS

The goal of this protocol is to study the trafficking of apical and basolateral secreted proteins in transfected polarized mammalian epithelial cells. This is accomplished utilizing a radioactive amino acid as a tracer molecule (Lipshutz et al., 2000).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimentor and surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

Materials

4- to 7-day-old epithelial cell cultures growing on 12-mm Transwell filters (see Support Protocol 2)
Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*)
Starvation medium: MEM medium (*APPENDIX 2B*) lacking the radioactive tracer amino acid
Radioactive amino acid (e.g., 1175 Ci/mmol [^{35}S]methionine)
MEM medium
0.5% (w/v) SDS lysis buffer (see recipe)

Parafilm
Humid box: plastic box with 2 charcoal bags and a piece of wet Whatman filter paper
12-well Transwell tissue culture plate (Table 15.5.2)
Phosphorimager
Scalpel

Additional reagents and equipment for gel electrophoresis (Chapter 6)

Label cells

1. Obtain 4- to 7-day-old epithelial cell cultures growing on 12-mm Transwell filters.
2. Wash cells three times with 37°C Dulbecco's phosphate-buffered saline (DPBS).
3. Add 500 µl starvation medium apically and basolaterally. Incubate at 37°C, 5% CO_2 for 15 min.

   For example, if [^{35}S]methionine is to be used as the radioactive amino acid, then the starvation medium is medium lacking methionine.

4. Place 25-µl spots containing radioactive amino acid (e.g., 4 µl of 1175 Ci/mmol [^{35}S]methionine and 21 µl starvation medium) on a Parafilm sheet inside a humid box.

   The humid box is a plastic box to which two bags containing charcoal are attached to the top inside surface of the box in order to trap the aerosolized [^{35}S]methionine. A piece of Whatman filter paper is placed on the bottom of the plastic box and soaked with water in order to prevent the cells from drying out. Finally, a piece of Parafilm is placed on top of the damp Whatman filter paper.

5. Remove the basal medium while leaving the apical medium in place. Carefully, set one filter on top of each spot for basolateral exposure of cells to the radioactive amino acid.
6. Incubate (i.e., allow labeling to occur) for 15 to 20 min at 37°C. Analyze labeled protein.

   If one is interested in analyzing the synthesis of secretory proteins, carry out steps 11 and 12 at this point.

7. Prepare a new 12-well plate containing fresh MEM medium. Transfer transwells to the new plate and wash cells three times apically and basolaterally with 37°C DPBS.
8. Add 300 µl MEM medium apically and 500 µl basolaterally. Incubate for the desired amount of time (e.g., 1 hr) at 37°C.

   In the case of incubation in an environment without CO_2, 20 mM HEPES is added to the medium as a buffer.

9. Collect apical medium. Transfer transwells to a new 12-well plate, and collect the basolateral medium.
10. Analyze aliquots of the secreted apical and basolateral medium on an appropriate gel by electrophoresis (Chapter 6), and determine amounts of radioactivity using a phosphorimager.

    Immunoprecipitation (UNIT 7.2) of specific secretory proteins can be done before gel electrophoresis.

11. Cut out the filters with a scalpel and collect the cells. Lyse cells by placing the filters in 500 µl of 0.5% SDS-lysis buffer.
12. Determine the amounts of intracellular protein radioactivity by analyzing aliquots on an appropriate gel (*UNIT 6.1*) followed by phospoimaging (*UNIT 6.3*).

    Again, immunoprecipitation (UNIT 7.2) can be performed before gel electrophoresis.

BASIC PROTOCOL 3

BIOTINYLATION OF NEWLY SYNTHESIZED EPITHELIAL CELL SURFACE PROTEINS

The goal in this protocol is to study the trafficking of newly synthesized apical and basolateral epithelial cell surface proteins using a combination of a radioactive amino acid tracer molecule and sulfo-NHS-biotin, an exogenous cell surface marker. Sulfo-NHS-biotin is used to label all cell surface proteins. Individual surface proteins are then isolated by immunoprecipitation (*UNIT 7.2*). This method is adapted from one developed by E. Rodriguez-Boulan and colleagues (LeBivic et al., 1989).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimentor and surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

Materials

Radioactively labeled amino acid
4-day-old epithelial cultures growing on 12-mm Transwell filters (see Support Protocol 2)
Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), 4°C
20 mg/ml sulfo-NHS-biotin (Pierce) in anhydrous DMSO: prepare just before use
10 mM Tris buffered saline, pH 7.4 (TBS; *APPENDIX 2A*)
0.5% (v/v) SDS lysis buffer (see recipe)
2.5% (v/v) Triton dilution buffer (see recipe)
Protein A–Sepharose beads without and with rabbit antimouse and the monoclonal antibody of choice, or rabbit polyclonal antibodies
Streptavidin beads (e.g., Pierce)
Mixed micelle wash buffer (see recipe)
Final wash buffer (see recipe)
5% (w/v) SDS
2× loading buffer (see recipe)

Platform rocker
Orbital shaker (e.g., Bellco)
Phosphorimager

Additional equipment and reagents for radioactively labeling epithelial cultures (see Basic Protocol 2, steps 1 to 6) and denaturing (SDS) gel electrophoresis (*UNIT 6.1*)

Label cells

1. Add radioactively labeled amino acid to 4-day-old epithelial cultures growing on 12-mm Transwell filters as described for pulse-chase experiments (see Basic Protocol 2, steps 1 to 6), labeling for 15 min.
2. Remove medium and wash cells three times with cold HBSS.
3. Just before using prepare 20 mg/ml sulfo-NHS-biotin in anhydrous DMSO and then dilute it 1:100 with HBSS to a final concentration of 200 µg/ml. Add either 400 µl apically or 800 µl basolaterally to cells.

   Sulfo-NHS-biotin binds tightly to primary amines on cell surface proteins via the NHS ester.

4. Incubate 30 min at 4°C on a platform rocker.
5. Remove biotin solution and wash five times with 10 mM Tris buffered saline.

Prepare lysate

6. Cut out filters and place in 500 µl of 0.5% SDS lysis buffer in a microcentrifuge tube. Boil 5 min, vortex 15 min, and microcentrifuge 5 min at 13,000 rpm. Transfer supernatant to new microcentrifuge tube.

   The filters are most easily cut out of the plastic ring with a surgical scalpel (i.e., Bard-Parker no. 11 blade).

7. Add an equal volume of 2.5% Triton dilution buffer to give final concentrations of 0.25% SDS lysis buffer/1.25% Triton dilution buffer.

Immunoprecipitate target protein

8. Preclear the lysate by incubating with 4 µl protein A–Sepharose beads free of antibody on an orbital shaker for 30 min at 4°C. Microcentrifuge 20 min at 13,000 rpm, 4°C. Transfer supernatant to a new microcentrifuge tube.

9. Add protein A–Sepharose precoated with rabbit antimouse and the monoclonal antibody of choice, or rabbit polyclonal antibodies, to 10 mg/ml. Incubate 12 hr at 4°C.

10. Block streptavidin beads by incubating in equal amounts of 0.5% SDS and 2.5% Triton dilution buffer for 1 to 12 hr.

    This mixture of SDS and Triton buffers is also called lysis buffer.

11. Wash protein A–Sepharose-antibody complexes three times with mixed micelle wash buffer and one time with final wash buffer by diluting with buffer and then microcentrifuging.

12. To recover immunoprecipitated biotinylated antigens, boil the beads in 40 µl of 5% (w/v) SDS for 5 min.

    Boiling in 5% (w/v) SDS will separate the biotinylated antigens from the antibody-protein A complex. The biotin, however, will remain attached to the antigen (surface protein of interest).

13. Dilute with 460 µl of 0.5% SDS/2.5% Triton dilution buffer and microcentrifuge 1 min at 13,000 rpm. Transfer the supernatant to a fresh microcentrifuge tube.

14. Add 13 µl streptavidin beads (i.e., 13 µl per filter) and immunoprecipitate overnight on an orbital shaker at 4°C.

15. Wash streptavidin-bead-antibody complex three times with mixed micelle wash buffer and one time with final wash buffer as described above (step 11).

16. Add 8 µl of 2× loading buffer, boil 5 min, and analyze by denaturing SDS gel electrophoresis (*UNIT 6.1*).

    The DTT (dithiothreitol; present in the loading buffer) is a reducing agent that will disrupt the S-S bond in the sulfo-NHS-biotin allowing for recovery of the protein.

17. Determine phosphorimager counts according to the manufacturer's instructions.

    This procedure identifies newly synthesized proteins of interest that reach the apical or basolateral surface, depending on which was exposed to biotin.

BASIC PROTOCOL 4

INDIRECT IMMUNOFLUORESCENCE OF PROTEINS IN POLARIZED EPITHELIAL CELLS

The goal of this protocol is to identify the location of proteins in polarized epithelial cells. This is accomplished using a primary antibody directed against the protein of interest and a secondary antibody containing an immunofluorescent marker directed against the species in which the primary antibody was made.

Materials

5- to 7-day-old cells growing on 12-mm Transwell filters (see Support Protocol 2)
DPBS (*APPENDIX 2A*), ice cold
16% or 40% (v/v) paraformaldehyde
Quenching solution, fresh (see recipe)
Permeabilizing solution, fresh (see recipe)
Primary antibody
Secondary antibody coupled to fluorophore
DPBS/0.1% (v/v) Triton: add 250 µl of 20% (v/v) Triton X-100 to 49.75 ml DPBS (*APPENDIX 2A*), store up to 6 months at 4°C
Antifade mounting solution

Metal board
Orbital shaker (e.g., Bellco)
Humid box: plastic box with a piece of damp Whatman filter paper
Aluminum foil
Scalpel
Inverted fluorescent microscope with appropriate glass slide and cover slip

1. Cool 5- to 7-day-old cells growing on 12-mm Transwell filters to 4°C by placing on a metal board on ice.
2. Prepare 4% (v/v) paraformaldehyde in ice-cold DPBS from either a 16% or 40% paraformaldehyde stock.
3. Wash Transwell filters twice with 1 ml ice-cold DPBS, both apically and basally.
4. Fix cells with 4% paraformaldehyde in DPBS by adding 0.5 ml to the basal and apical surfaces. Allow cells to fix by incubating 20 min on a slowly rotating orbital shaker (e.g., setting 3 on a Bellco Glass orbital shaker) in a container on ice.
5. Wash Transwell filters three times with 1 ml DPBS to remove paraformaldehyde.
6. Add 1 ml fresh quenching solution both apically and basally, and rotate slowly for 10 min at room temperature.

   The glycine in the quenching solution will bind to the remaining paraformaldehyde.
7. Wash once with 1 ml DPBS.
8. Add 1 ml fresh permeabilizing solution both apically and basally. Incubate 15 min in a 37°C water bath.
9. Dilute primary antibody in permeabilizing solution as appropriate (i.e., 1:100) in 150 µl. Place 40 µl as a drop on a square of Parafilm inside a humid box and place the Transwell filter on the drop (i.e., add basally) and add 110 µl to the apical surface of the Transwell filter.

   The concentration of primary antibody depends on the properties of each individual antibody. A rough rule is that this technique will require five to ten times the amount required for immunoblotting.

10. Incubate 1 hr at 37°C on an orbital shaker.
11. Transfer Transwell filter back to the plate. Wash four times with 1 ml permeabilizing solution, incubating on an orbital shaker for 5 min each time.
12. Dilute secondary antibody coupled to fluorophore 1:100 (generally) with permeabilizing solution. Add to the filter as described for the primary antibody solution (see step 9). Wrap the humid box in aluminum foil and incubate on a platform rocker 30 min at 37°C.

    The samples in the following steps should be protected from light as much as possible.

13. Wash four times with 1 ml permeabilizing solution for 5 min each on an orbital shaker.
14. Rinse once with 1 ml DPBS.
15. Wash twice for 3 min each with 1 ml DPBS/0.1% Triton.
16. Rinse once with 1 ml DPBS.
17. Post-fix with 4% paraformaldehyde in DPBS for 15 min at room temperature (see step 4).
18. Rinse once with 1 ml DPBS.
19. Cut out filter with a scalpel and place on slide containing a drop of antifade mounting solution. Add a coverslip and dry overnight at room temperature in the dark.

    Mount the filter cell-side-up so that the coverslip can be placed directly on an inverted fluorescent microscope.

20. Analyze on an inverted fluorescent or confocal microscope to detemine the location of the protein of interest, or store in the dark at −20°C to prevent fading of the fluorophore.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Final wash buffer

37.5 ml 4 M NaCl (150 mM final; *APPENDIX 2A*)
20 ml 1 M triethanolamine chloride (TEA·Cl), pH 8.6 (20 mM final)
10 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.001% v/v final)
2.0 ml 10% NaN_3 (0.02% w/v final)
Adjust volume to 1 liter with H_2O
Store up to 6 months at 4°C

Geneticin (G418)

Prepare G418 as a 100 mg/ml stock in 200 mM HEPES (sodium salt), pH 7.9. Filter sterilize and store at −20°or −80°C. For every new batch (new lot number of G418 powder), determine the effective G418 concentration which kills cells by performing killing curves (i.e., expose the cells to different G418 concentrations and determine the minimal drug concentration needed to kill the entire cell population in the dish in a 14-day test).

For more information concerning geneticin, see APPENDIX 1B.

HEPES buffered saline (HeBS), pH 7.05

For 500 ml HeBS, add 2.5 g HEPES (final concentration 20 mM) to DPBS and adjust the pH to 7.05 with 5 M NaOH (*APPENDIX 2A*).

Loading buffer, 2×

0.3 ml 1 M Tris·Cl, pH 6.8 (15 mM; *APPENDIX 2A*)
2.0 ml 10% SDS (1% w/v; *APPENDIX 2A*)
0.05 ml 100 mM EDTA, pH 7.0 (25 mM; *APPENDIX 2A*)
0.2 g DTT (65 mM)
0.5 ml 1% bromophenol blue dye (0.025% w/v)
1.2 ml 100% glycerol or sucrose (6% w/v)
5.75 ml H_2O
10 ml total volume
Store up to 6 months at 4°C

Concentrations in parentheses are for a 1× solution.

Mixed micelle wash buffer

37.5 ml 4 M NaCl (150 mM final; *APPENDIX 2A*)
20 ml 1 M TEA·Cl, pH 8.6 (20 mM final)
10 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.001% v/v final)
2.0 ml 10% NaN_3 (0.02% w/v final)
60 ml 65% (w/w), 85% (w/v), or 39 g sucrose
942 ml H_2O
50 ml 20% Triton X-100 (1% v/v final)
20 ml 10% SDS (0.2% v/v final; *APPENDIX 2A*)
Total volume 1 liter
Store up to 6 months at 4°C

Permeabilizing solution

3.5 g of cold-water fish skin gelatin (0.7% w/v final; Sigma-Aldrich)
0.8 ml 10% (w/v) saponin in H_2O (0.016% final)
Bring to 500 ml with PBS (*APPENDIX 2A*)
Prepare fresh on the day of the experiment; do not store

This is a sufficient amount of solution for the preparation of twelve 12-mm Transwell filters.

Quenching solution

1.5 ml 1 M NH_4Cl (75 mM final)
0.4 ml 1 M glycine (20 mM final)
18.1 ml PBS (*APPENDIX 2A*)
20 ml total volume
Prepare fresh on the day of the experiment; do not store

SDS lysis buffer, 0.5% (w/v)

12.5 ml 4 M NaCl (100 mM final; *APPENDIX 2A*)
25 ml 1 M TEA·Cl, pH 8.1 (50 mM final; *APPENDIX 2A*)
5 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.2% v/v final)
1.0 ml 10% NaN_3 (0.02% w/v final)
25 ml 10% SDS (0.5% w/v final; *APPENDIX 2A*)
475 ml H_2O
500 ml total volume
Store up to 6 months at room temperature

Triton dilution buffer, 2.5% (v/v)

12.5 ml 4 M NaCl (100 mM final; *APPENDIX 2A*)
50 ml 1 M TEA·Cl, pH 8.6 (100 mM final; *APPENDIX 2A*)
5 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
5.0 ml Trasylol (1% v/v final)
1.0 ml 10% NaN_3 (0.02% w/v final)
Add H_2O to 436 ml
Add 62.5 ml of 20% Triton X-100 (2.5% v/v final)
Total volume 500 ml
Store up to 6 months at 4°C

COMMENTARY

Background Information

Growing cells on porous (i.e., Transwell) filters has two major advantages. First, polarized epithelial cells usually obtain most of their nutrients from their basolateral surface, which in vivo is exposed to the blood. When grown on a typical impermeable plastic culture dish, the cells cannot obtain nutrients from the overlying culture medium in contact with the apical surface because the tight junctions prevent nutrients from reaching the basolateral surface. This forces the cells to become partially depolarized in order to survive. In contrast, when grown on permeable filters, the cells can obtain nutrients from the culture medium that underlies the filter, which is in contact with the basolateral surface via the pores in the filter.

Second, the cells form a continuous monolayer covering the filter, and the tight junctions between cells largely prevent diffusion between them. Thus, the culture medium in contact with the apical surface of the cells cannot exchange with medium in contact with the basolateral surface. In the Transwell or similar device, the filter is mounted at the end of a short plastic tube, which is suspended in the well of a multiwell tissue culture plate. This effectively provides two chambers holding two potentially different pools of culture medium: an apical chamber overlying the apical surface of the cells on top of the monolayer, and a basolateral chamber underlying the filter and in contact with the basolateral surface of the cells.

Many early studies on biosynthetic protein sorting utilized viral membrane proteins. Typically in these studies, cells were infected with a virus and the transport of the envelope glycoproteins to the surface was studied. This provided a large amount of one or two viral glycoproteins, which could easily be followed. The most widely used examples were the hemagglutinin (HA) protein of influenza, which goes apical and the G protein of vesicular stomatitis virus (VSG-G) which goes basolateral (Brewer and Roth, 1991); however, infection with these and other viruses has significant toxic repercussions to the cell. Thus, expression of these proteins from the actual virus has largely been supplanted by expression from transfected vectors (e.g., Brewer and Roth, 1991). VSV-G has the added advantage that a temperature sensitive form (i.e., ts-045) accumulates in the rough endoplasmic reticulum (RER) at 40°C, but is transported through the biosynthetic pathway at ≤30°C. Additionally, green fluorescent protein (GFP)–fusions with this and other proteins are being used to follow transport in live cells.

This unit focuses on biosynthetic pathways leading to the cell surface. One can also study endocytosis from either the apical or basolateral surface. The approaches used are similar to those presented in *UNIT 15.4*. Additionally, one can study the fate of material endocytosed from either surface (*UNIT 15.3*). This can include recycling to the original surface from which the material was endocytosed, transcytosis to the opposite surface, or degradation. One difficulty is that there are relatively few endogenous receptors in Madin-Darby canine kidney (MDCK) cells that can be conveniently utilized to follow receptor-mediated endocytosis. When confluent and well polarized, MDCK cells express relatively few receptors for many nutrients (e.g., transferrin or low-density lipoprotein). The endogenous transferrin receptors work very poorly with human transferrin, although they do work considerably better with canine transferrin, which is available commercially from Sigma-Aldrich. Most endogenous endocytotic receptors are located on the basolateral surface, so finding a receptor system for studying apical endocytosis is more difficult. Most studies on endocytosis and post-endocytic sorting in epithelial cells have utilized transfected exogenous receptors, expressed at a high level. For instance, the authors' labora-

tory has primarily utilized the transfected polymeric immunoglobulin receptor.

Critical Parameters and Troubleshooting

Clonal variation of MDCK cells

The original MDCK cell line, available from the American Type Culture collection (*http://www.atcc.org*; ATCC #CCL-34), is quite heterogeneous. Several investigators have isolated individual clonal lines from this heterogeneous population. Clones of MDCK cells can be broadly divided into two categories, type I and type II. Generally, type I have a high electrical resistance (i.e., >1000 ohms/cm^2), whereas type II have a low electrical resistance (~100 ohms/cm^2). In reality, MDCK clones can be thought of as forming a nearly continuous spectrum in terms of their electrical resistance as well as other properties. Almost all of the work done by the authors has utilized a nominally type II clonal line originally isolated by Daniel Louvard at the European Molecular Biology Laboratory (EMBL) in Heidelberg, which is sometimes referred to as Heidelberg MDCK (Louvard, 1980).

When transfected cDNA is expressed in MDCK cells, and a stable expressing clone is isolated, the cell line is effectively subcloned. The authors have found that each subclone of MDCK can have unique and quite distinct properties; therefore, it is important to characterize each one. For example, an easy test for the ability of a subclone to form a tight monolayer is described (see Support Protocol 3). Moreover, the authors have found that the polarized trafficking of any particular protein can vary considerably among subclones. For this reason, when studying the trafficking of a newly transfected protein, the authors generally examine at least three independent subclones (Aroeti et al., 1993). If clones show different trafficking properties it is likely due to clonal variation and not a direct result of the exogenous protein being expressed. Only if three separate clones give the same result can one assume that the effect is due to the truncated gene/protein.

In addition to MDCK cells, several other epithelial cell lines have often been used for studies of polarized membrane traffic, including the major ones listed in Table 15.5.1.

Instability and heterogeneity of expression of exogenous proteins

In many cases, expression of an exogenous protein in MDCK cells appears to be detrimental to the long-term growth of the cell. In severe cases, the protein can be so toxic that it is difficult or impossible to recover clones that express a detectable level of the exogenous protein. In less severe cases, expression of the protein is lost during successive passages of the clonal cell line (Barth et al., 1997). Presumably, this is due to a growth advantage of spontaneously arising cell variants, which do not express the protein. Moreover, when the expression of the exogenous protein is observed in a population of MDCK cells by immunofluorescence microscopy, the level of expression can appear to vary from cell to cell. One strategy to deal with these problems is to freeze numerous vials of early passage cells, which are then thawed at frequent intervals and used for only a limited period before the cells are discarded. In some cases, the newly thawed cells are used for only one experiment. This helps to ensure that the cells used in every experiment are consistent.

Another approach is to repeatedly subclone the cells. In principle this should yield a clone with uniform expression of the exogenous protein; however, it is often observed that despite repeated subcloning, expression of exogenous proteins is not uniform. This suggests that variation in expression is an inherent feature of MDCK cells.

Type of filter material and support unit

Most of the authors' experience is with polycarbonate filters containing 0.4-μm nominal size pores, as part of Transwell units from Corning. Filters are available in a number of materials and support units, from several manufacturers (Table 15.5.2). Some filters are available in pore sizes >1 μm; however, these are not recommended for growth of polarized epithelial monolayers as the cells can migrate into and through the pores. Indeed, filters with pores of 3 μm or larger are used for cell migration studies. In general, filters that have more pores and/or larger pores will have optical transmission properties which are more poor, though it is not necessary to be able to visualize live cells on the filter for most experiments.

Anticipated Results

Ideally, one will find that the protein of interest is delivered directly to one surface of the cell or the other and resides at that surface. In this case, there will be a clear-cut agreement between the steady-state localization determined by immunofluorescence microscopy, and the site of delivery, as measured by the pulse-chase and biotinylation protocols. An ex-

ample of results from immunofluorescence microscopy is presented in Figure 15.5.3; however, it should be kept in mind that no protein is ever delivered 100% to only one surface. Even in the best cases, typically only ~90% of a protein is delivered to one surface, with the balance reaching the other surface. Furthermore, proteins can differ in the stability at the surface. Some, such as Na-K-ATPase, can reside at the basolateral surface for hours. Others, such as the polymeric immunoglobulin receptor, are delivered to the basolateral surface, but are then endocytosed and transcytosed to the apical surface within minutes.

Time Considerations

It takes ~4 hr to carry out a pulse-chase labeling experiment to assess trafficking. Transfection of the cells requires ~2 hr plus 2 to 3 weeks to select clones. Picking clones takes ~2 hr. It takes 7 to 14 days to expand the clones. Plating cells on Transwell filters requires ~1 hr, but 4 to 7 days are required to grow confluent monolayers of polarized cells. It takes one complete day to assess a culture for leakiness. Biotinylation of newly synthesized proteins and their analysis requires ~8 hr. Immunofluorescence experiments require ~6 hr to complete.

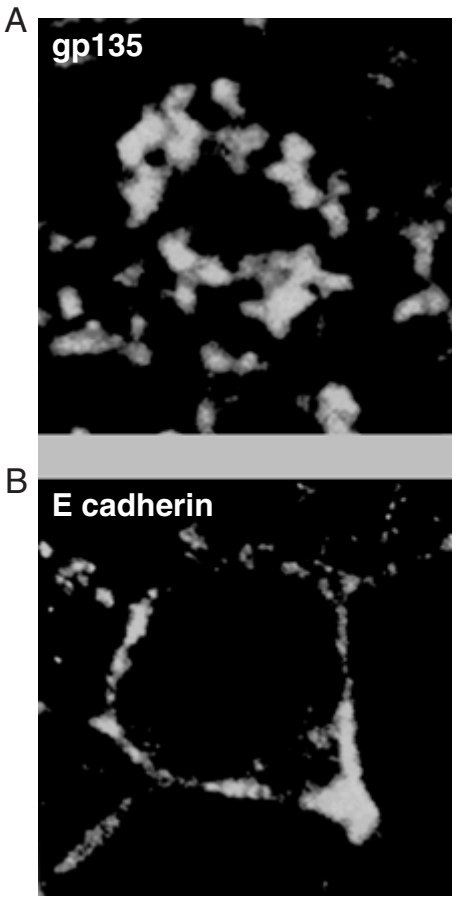


Figure 15.5.3 Immunofluorescence micrographs of polarized epithelial cells grown on a Transwell. Samples were prepared as in Basic Protocol 4, and then viewed with a Bio-Rad 1024 confocal microscope. (**A**) Staining using a monoclonal antibody directed against an apical plasma membrane protein, gp135 (kindly provided by George Ojakian, SUNY Downstate Medical Center, Brooklyn, NY), as a primary antibody. The micrograph was taken at the level of the apical surface. Staining appears irregular, due to microvilli and other irregularities of the apical surface. It is not easy in this view to discern the outline of individual cells. (**B**) A sample prepared using a primary antibody against a basolateral surface marker, E-cadherin (Chapter 9). The micrograph was taken at a level below the apical surface, approximately midway between the apical and basal poles of the cell. The E-cadherin largely outlines the lateral borders of the cell, though some intracellular staining is also seen.

Literature Cited

Aroeti, B., Kosen, P.A., Kuntz, I.D., Cohen, F.E., and Mostov, K.E. 1993. Mutational and secondary structural analysis of the basolateral sorting signal of the polymeric immunoglobulin receptor. *J. Cell Biol.* 123:1149-1160.

Barth, A.I.M., Pollack, A.L., Altschuler, Y., Mostov, K.E., and Nelson, W.J. 1997. NH_2-terminal deletion of β-catenin results in stable colocalization of mutant β-catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J. Cell Biol.* 136:693-706.

Breitfeld, P., Casanova, J.E., Hsarris, J.M., Simister, N.E., and Mostov, K.E. 1989. Expression and analysis of the polymeric immunoglobulin receptor. *Methods Cell Biol.* 32:329-337.

Brewer, C.B. and Roth, M.G. 1991. A single amino acid change in the cytoplasmic domain alters the polarized delivery of influenza virus hemagglutinin. *J. Cell Biol.* 114:413-421.

Drubin, D.G. and Nelson, W.J. 1996. Origins of cell polarity. *Cell* 84:335-344.

LeBivic, A., Real, F.X., and Rodriguez-Boulan, E. 1989. Vector targeting of apical and basolateral proteins in human adenocarcinoma cell line. *Proc. Natl. Acad. Sci. U.S.A.* 86:9313-9317.

Lipshutz, J.H., Guo, W., O'Brien, L.E., Nguyen, Y.H., Novick, P., and Mostov, K.E. 2000. The exocyst is involved in epithelial cytogenesis and tubulogenesis and acts by modulating syntheses and delivery of basolateral plasma membrane and secretory proteins. *Mol. Cell Biol.* 11:4259-4275.

Louvard, D. 1980. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 77:4132-4136.

Luton, F. and Mostov, K.E. 1999. Transduction of basolateral-to-apical signals across epithelial cells: Ligand stimulated transcytosis of the polymeric immunoglobulin receptor requires two signals. *Mol. Biol. Cell* 10:1409-1427.

Matter, K. and Mellman, I. 1994. Mechanisms of cell polarity: Sorting and transport in epithelial cells. *Curr. Opin. Cell Biol.* 6:545-554.

Mostov, K.E., ter Beest, M.B.A., and Chapin, S.J. 1999. Catch the μ1B train to the basolateral surface. *Cell* 99:121-122.

Mostov, K.E., Verges, M., and Altschuler, Y. 2000. Membrane traffic in polarized epithelial cells. *Curr. Opin. Cell Biol.* 12:483-490.

Weimbs, T., Low, S.-H., Chapin, S.J., and Mostov, K.E. 1997. Apical targeting in polarized epithelial cells: There's more afloat than rafts. *Trends Cell Biol.* 7:393-399.

Contributed by Joshua H. Lipschutz, Lucy Erin O'Brien, Yoram Altschuler, Dana Avrahami, Yen Nguyen, Kitty Tang, and Keith E. Mostov
University of California, San Francisco
San Francisco, California

Analysis of Protein Folding and Oxidation in the Endoplasmic Reticulum

Proteins that traverse the eukaryotic secretory pathway are co-translationally inserted through the endoplasmic reticulum (ER) membrane into the lumen. Upon emergence of the protein into the lumen, a series of important maturation steps begin co-translationally and finish post-translationally before the protein exits from the ER. Among these processes are protein folding and the formation of disulfide bonds, which is supported by the oxidizing ER environment. This unit describes protocols for analyzing the folding and oxidation of proteins in the ER.

Protein oxidation can be analyzed either co-translationally (Basic Protocol 1) or post-translationally (Alternate Protocol 1) by monitoring the disappearance of free cysteine thiols or appearance of faster-migrating oxidative forms on nonreducing SDS-polyacrylamide gels. Protein folding can be followed by analyzing a protein's increased resistance to proteolysis during the course of maturation (Basic Protocol 2) or by immunoprecipitation with conformation-specific antibodies (Basic Protocol 4). The transient association of folding or oxidative intermediates with chaperone proteins can also be studied using a co-immunoprecipitation procedure (Basic Protocol 3 and Alternate Protocol 2). These procedures can be applied to study protein maturation in the ER using a variety of experimental systems including: a cell-free system composed of rough ER–derived microsomes and an in vitro translation system (Basic Protocol 1), adherent cell monolayers (Basic Protocol 5), cells in suspension (Alternate Protocol 3), and an in vitro translation system that targets proteins to intact ER from semipermeabilized (SP) cells (Support Protocol). To choose the most effective system(s) for a given study, the inherent advantages and disadvantages of each system must be weighed.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter, instruments, and surroundings. Carry out the experiments and the disposal of the wastes in the appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*). In addition, some extra precautions should be taken when using ^{35}S-labeled amino acids (*UNIT 7.1*).

ANALYSIS OF DISULFIDE BOND FORMATION IN ROUGH ENDOPLASMIC RETICULUM–DERIVED MICROSOMES BY ALKYLATION AND NONREDUCING SDS-PAGE

BASIC PROTOCOL 1

Disulfide bond formation can be followed by translating proteins under oxidizing conditions in the presence of rough ER–derived microsomes. The polypeptide chain is co-translationally translocated across the ER membrane into the lumen where disulfide bond formation occurs. Because free cysteine thiols form disulfide bonds with the aid of an oxidizing agent, the disappearance of these free thiols can be used to monitor disulfide bond formation. The modification of free thiols with an alkylating agent can be used to trap oxidative intermediates and cause a mobility shift proportional to the number of free thiols modified (Fig. 15.6.1). Common alkylating reagents for this technique are *N*-ethylmaleimide (NEM; 125 Da) and 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid disodium salt (AMS; 536 Da).

As proteins fold and form disulfide bonds, they become more compact. This generally causes the protein to migrate faster by nonreducing SDS-PAGE compared with its fully reduced form (Fig. 15.6.1). Therefore, a mobility comparison of the reduced and oxidized

Contributed by Edwin Francis, Robert Daniels, and Daniel N. Hebert
Current Protocols in Cell Biology (2002) 15.6.1-15.6.29

protein on nonreducing SDS-PAGE can be used to monitor the formation of disulfide bonds. These studies can also be used in some cases to identify oxidative intermediates. In this protocol, a time course for protein oxidation is performed and the immunoprecipitates or lysates are analyzed by nonreducing SDS-PAGE to detect changes in mobility over time due to disulfide bond formation.

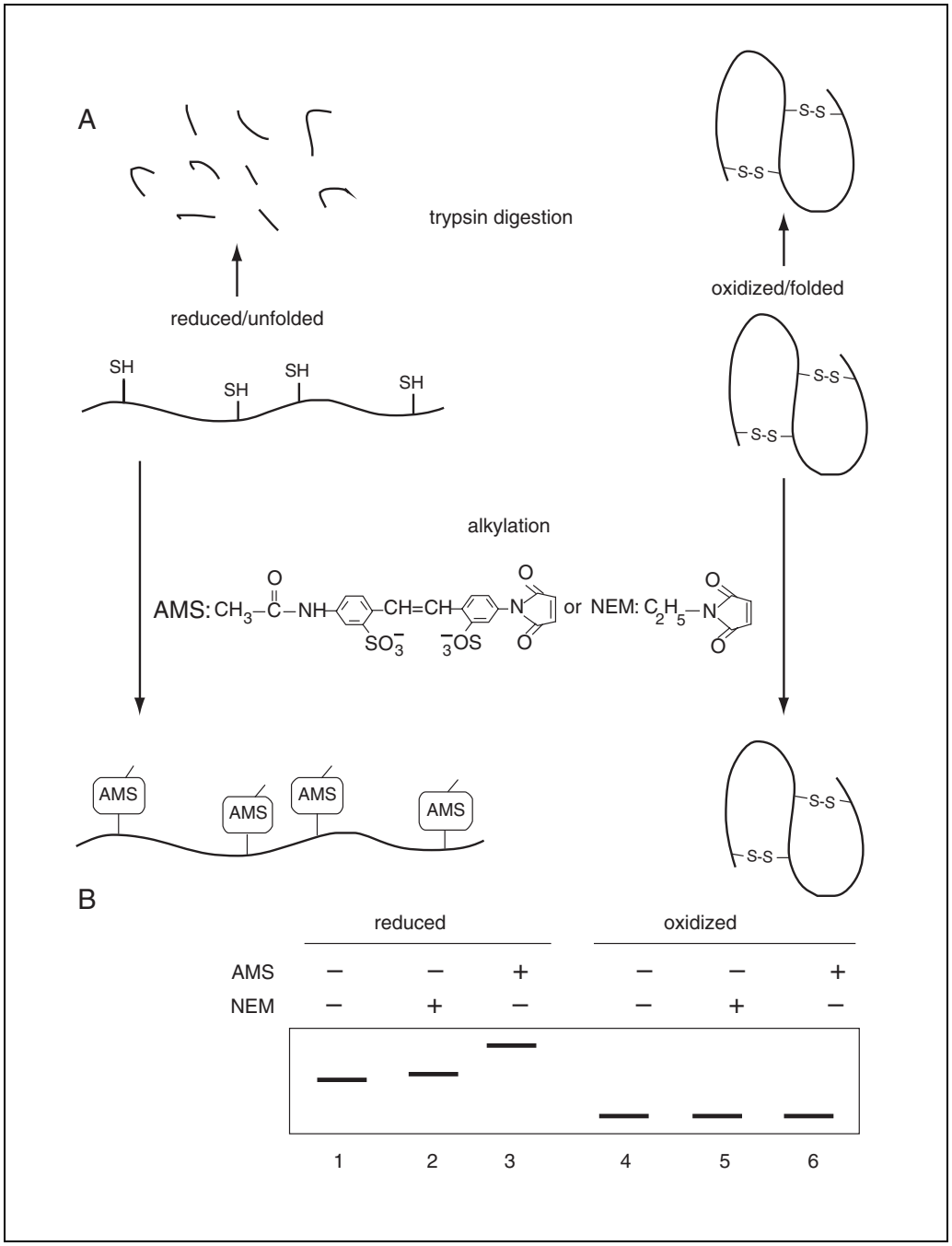


Figure 15.6.1 The effects of disulfide bond formation in proteins. (**A**) Schematic demonstration of the sensitivity of a reduced and oxidized polypeptide chain to protease digestion (top) and alkylation (bottom). (**B**) Resulting mobility changes visualized by SDS-PAGE. When a reduced or unfolded polypeptide containing free thiols is alkylated with 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid disodium salt (AMS), a bulk alkylating agent, or *N*-ethylmaleimide (NEM), a shift in protein mobility on an SDS-polyacrylamide gel is observed (compare lanes 2 and 3 to lane 1). The oxidized (disulfide bonded) or folded protein is not sensitive to alkylation with either AMS or NEM as fewer or no free cysteines are available (lanes 4 to 6). The reduced or unfolded protein is more accessible to proteolytic digestion with trypsin than the oxidized or folded protein.

This protocol uses ^{35}S-labeled proteins generated by translating an mRNA (*UNIT 11.2*) for the protein of interest with a rabbit reticulocyte lysate translation system along with [^{35}S]methionine and [^{35}S]cysteine in the presence of rough ER–derived microsomes. This system is advantageous for studying protein maturation in the ER as it provides optimum flexibility in the control and manipulation of experimental conditions. One manipulation is the addition of the oxidizing agents oxidized glutathione (GSSG) or flavin adenine dinucleotide (FAD) during translation. Both FAD and GSSG provide conditions that mimic the normal physiological environment under which proteins acquire their native disulfide bonds in the cell. Therefore, these reagents, when present during translation, permit disulfide bond formation to commence co-translationally or vectorially from the N terminus to C terminus of the protein and to be completed post-translationally.

Materials

In vitro translation reagents, including:
- 1 equivalent/µl nuclease-treated canine pancreatic microsomes (*UNIT 11.4*) or 1 × 10^5 cells/µl semipermeabilized (SP) cells (see Support Protocol)
- Rabbit reticulocyte lysate treated with ATP-regenerating system and nucleases (e.g., Promega)
- 1 mM amino acid mixture lacking methionine and cysteine
- 11 mCi/ml [^{35}S]methionine and [^{35}S]cysteine (1175 Ci/mmol; e.g., PE Biosystems)
- 40 U/µl RNase inhibitor (e.g., RNasin; Promega)
- 100 mM GSSG (see recipe) or 2.5 mM FAD (see recipe)
- 100 mM dithiothreitol (DTT; e.g., Promega or *APPENDIX 2A*)
- 2.5 M KCl
- 1 µg/µl mRNA for the protein of interest (e.g., *UNIT 11.2*)
- Nuclease-free H_2O

50 mM cycloheximide (see recipe)
120 mM *N*-ethylmaleimide (NEM) (see recipe)
20 mM AMS, prepared fresh (see recipe)
0.5% and 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) in HeBS (CHAPS/HeBS, see recipe), ice cold
2× nonreducing sample buffer (see recipe)
10% (w/v) protein A–Sepharose (see recipe)
Antibodies raised against the protein of interest
PBS (*APPENDIX 2A*), optional
2% (w/v) salicylate (see recipe)

27°C water bath
1.5-ml microcentrifuge tubes, RNase free
Tube rotator (capable of end-over-end inversions), 4°C
Microcentrifuge with a fixed-angle rotor (Eppendorf 5415C or equivalent), 4°C and room temperature
Vacuum aspirator
Microcentrifuge tube rack vortex mixer (e.g., Tommy MT-360; Tomy Tech USA Inc.), 4°C
95°C heating block
Whatman 3MM filter paper

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) minigel with Laemmli buffers (*UNIT 6.1*), Coomassie blue staining and destaining (*UNIT. 6.6*), and gel autoradiography (*UNIT 6.3*)

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

Carry out in vitro translation

1. Thaw in vitro translation reagents rapidly in a 27°C water bath and then place on ice.

   Canine pancreatic microsomes should be stored in aliquots of working volumes (multiples of 6 µl). Freeze-thawing of rabbit reticulocyte lysate more than twice should be avoided as each freeze-thaw cycle results in a decrease in translation and translocation efficiency. All other reagents should be frozen rapidly in liquid nitrogen after use and stored at −70°C.

2. Assemble reaction mixture in a 1.5-ml RNase-free microcentrifuge tube at room temperature (~92.0 µl total volume) as follows:

   6 µl of 1 equivalent/µl nuclease-treated canine pancreatic microsomes *or* 12 µl of 1×10^5 cells/µl SP cells (1.3×10^4 cells/µl final)
   52 µl treated rabbit reticulocyte lysate
   2 µl of 1 mM amino acid mixture lacking methionine and cysteine
   8 µl of 11 mCi/ml [^{35}S]methionine and [^{35}S]cysteine
   4 µl of 40 U/µl RNase inhibitor
   3.7 µl of 100 mM GSSG (4 mM final) or 2.5 mM FAD (100 µM final concentration)
   3 µl of 100 mM DTT
   3.2 µl of 2.5 M KCl
   4 µl of 1 µg/µl mRNA for the protein of interest
   6.1 µl nuclease-free H_2O.

   This reaction mix is sufficient for nine samples (i.e., three time points) and should be adjusted proportionately as needed.

   One equivalent per microliter of microsomes is equal to 50 A_{280} U/ml. A range of 6 to 12 µl of 1 eq/µl of microsomes or 0.5 to 2.5 × 10^4 cells/µl of SP-cells provides optimum translocation for a 92-µl translation mix.

   The authors have found that ~4.0 µg mRNA per 92-µl reaction is optimal for their transcripts, but this is transcript dependent and should be optimized individually. The KCl and microsome concentrations in the translation also have to be optimized for individual mRNAs. A final KCl concentration range of 30 to 90 mM is a good starting point. In general, increasing the amount of microsomes in the reaction increases the proportion of polypeptides that are processed, but it may also reduce the amount of total polypeptide synthesized.

   For oxidation, usually 4 mM GSSG or 100 µM FAD with 1.1 mM DTT can provide optimal redox conditions, but a titration may be required to provide maximal results. If more than one mRNA is used or conditions are varied, it is advantageous to aliquot the mix and make the adjustments prior to mRNA addition.

3. Mix the lysate gently but thoroughly by pipeting up and down a few times and incubate 1 hr at 27°C.

   To avoid air bubbles during mixing, the pipettor should be set to less than the total volume of the mixture. If necessary, the sample should be centrifuged briefly to bring the mixture to the bottom of the tube.

4. Add 2 µl of 50 mM cycloheximide (1 mM final) and pipet up and down to stop the translation reaction.

   Cycloheximide is a protein synthesis inhibitor.

Alkylate proteins

5. Transfer 10 µl lysate into each of three 1.5-ml microcentrifuge tubes. Return the remaining lysate to the 27°C water bath for further oxidation and maturation.

   These samples represent the first time point. For each time point, three fractions will be generated: control, no alkylation; alkylation with NEM; and alkylation with AMS.

6. Set one of the three tubes aside on ice for the control, nonalkylated sample.

7. To the second tube, add 2.0 µl of 120 mM NEM (20 mM final) to alkylate free thiols. Place on ice for 10 min.

8. To the third tube, add 25 µl of 20 mM AMS (14 mM final) to alkylate free thiols and incubate 1 hr at room temperature in the dark.

   Because AMS is a membrane-impermeable alkylating agent, the membranes have to be lysed. Therefore, the solution containing AMS also contains SDS for membrane solubilization. AMS is sensitive to light; the bottle containing the solution should be wrapped with aluminum foil, and a freshly prepared stock solution should be used.

9. After appropriate incubation times, repeat steps 5 to 8 for remaining time points.

10a. *For immunoprecipitation:* Add 500 µl ice-cold 2% CHAPS/HeBS to each sample and continue with step 11.

10b. *For direct analysis of total proteins translated:* Add 2× nonreducing sample buffer to the three samples as follows and continue with step 19:

   10 µl to nonalkylated sample
   12 µl to NEM-treated sample
   35 µl to AMS-treated sample.

   Adjust the sample volume with 1× nonreducing sample buffer to 70 µl to keep the protein concentration constant for quantification purposes.

Immunoprecipitate proteins

11. Add 100 µl of 10% protein A–Sepharose to each tube to preclear immunoprecipitation samples. Rotate samples end over end on a tube rotator for 1 hr at 4°C.

    Preclearing with protein A–Sepharose should decrease the amount of background associated with the use of protein A. More details on immunoprecipitation can be found in UNIT 7.2.

12. Microcentrifuge samples 5 min at 2900 × *g* (6000 rpm), 4°C, and transfer the supernatant to a new 1.5-ml microcentrifuge tube containing 100 µl of 10% protein A–Sepharose and the appropriate amount of antibodies raised against the protein of interest.

    A titration with antiserum may be required to obtain optimal results. Excess antibodies may give nonspecific binding or saturate the protein A–Sepharose.

13. Rotate samples end over end for 4 to 8 hr or overnight at 4°C.

14. Microcentrifuge samples 5 min at 2900 × *g*, 4°C, to pellet the protein-antibody immune complexes.

15. Place samples on ice, and use a vacuum aspirator to remove supernatant.

16. Add 500 µl of 0.5% CHAPS/HeBS and vortex in a microcentrifuge tube rack vortex mixer at medium speed for 10 min at 4°C, to wash pellet.

For in vitro translated proteins, only a single wash is needed. However, cellular studies (see Basic Protocol 5 and see Alternate Protocol 3) may require multiple washes to reduce background.

17. Microcentrifuge samples 5 min at 2900 × *g*, 4°C, and aspirate supernatant.
18. Resuspend samples in 40 µl of 2× nonreducing sample buffer by vortexing for 5 min.

Electrophorese proteins

19. Heat samples 5 min in a 95°C heating block.

    A boiling water bath may be used instead of the heating block.

20. Microcentrifuge samples 5 min at 16,000 × *g* (14,000 rpm), room temperature, to pellet the Sepharose beads prior to loading on the gel.

    Take care not to clog the pipet tip by pipetting beads.

21. For reduced samples, place 13.5 µl nonreduced sample into a fresh 1.5-ml microcentrifuge tube. Add 1.5 µl of 1 M DTT (100 mM final) and heat 5 min at 95°C. Centrifuge briefly to concentrate sample at the bottom of the tube.

    Reducing and nonreducing conditions are used to identify the disulfide bonded forms of the protein that migrate faster in the nonreducing gel compared to the reducing gel.

22. Prepare a 1- or 0.75-mm-thick SDS-polyacrylamide separating and stacking minigel with a 10- or 15-well comb (*UNIT 6.1*).

    Separating-gel acrylamide concentration depends on the molecular weight of the proteins to be resolved.

23. Load 10 µl nonreduced sample or 11.1 µl reduced sample into each well and bring all lanes, including the empty ones, up to 11.1 µl with 1× sample buffer.

    Each well must be intact and straight. If nonreduced and reduced samples are to be placed on the same gel, care must be taken to ensure that the DTT from the reduced samples does not reduce the nonreduced samples in the gel. This can be avoided by leaving two empty lanes between nonreduced and reduced samples and filling these lanes with nonreducing sample buffer. If nonreduced and reduced samples need to be loaded next to each other, samples should be cooled, and NEM added to a final concentration of 100 mM to both sets of samples to alkylate excess DTT. Samples should be loaded carefully using a gel loading pipet tip.

24. Run the gel for ~2 hr at 20 to 25 mA until the dye front is at the bottom of the gel.
25. Stain and destain the gel, including stacking gel, with Coomassie blue stain and destain (*UNIT 6.6*)

    Stacking gels often contain aggregated proteins.

26. Wash gel with distilled water or PBS by rocking gently. Repeat two or three times. Check that the pH has reached neutrality with pH paper.

    Salicylate (step 27) is inactivated by acidic pH.

27. Incubate gel 10 min with gentle agitation in 2% salicylate.

    Salicylate is used to enhance the radioactive signal 3- to 5-fold (UNIT 6.3).

28. Dry gel onto Whatman 3MM filter paper and expose gel to radiographic film for autoradiography (*UNIT 6.3*).

    An exposure of 6 to 12 hr is generally sufficient for total protein lysate samples. Immunoprecipitated samples may require longer exposure time.

ANALYSIS OF POST-TRANSLATIONAL DISULFIDE BOND FORMATION IN ROUGH ENDOPLASMIC RETICULUM–DERIVED MICROSOMES

ALTERNATE PROTOCOL 1

The formation of disulfide bonds can be inhibited by the addition of a powerful reducing agent such as dithiothreitol (DTT) while protein synthesis, translocation, signal sequence cleavage, and glycosylation continue. Here, disulfide bond formation is initiated post-translationally by the removal of the reducing agent or the addition of oxidizing agents. This post-translational method allows for synchronous initiation of disulfide bond formation. In addition, the isolation of the oxidation and folding steps in the post-translational procedure permits the characterization of factors required in this process independent of other processes involved in protein maturation such as translocation and glycosylation (Braakman et al., 1992).

This protocol is similar to Basic Protocol 1 (and uses the same list of materials) with the exception that the radiolabeled protein is synthesized under reducing conditions to inhibit disulfide bond formation (Fig. 15.6.2). Synthesis is then terminated and oxidizing conditions are restored by adding oxidizing agents such as oxidized glutathione (GSSG) or flavin adenine dinucleotide (FAD) to initiate disulfide bond formation. An intact endoplasmic reticulum (ER) source can also be used here by substituting semipermeabilized (SP) cells (see Support Protocol) for the microsomes. For cellular studies, the reducing agent (5 mM DTT final concentration) is added during the radioactive pulse and removed to initiate oxidation because intralumenal oxidizing conditions will be rapidly restored upon returning the cells to medium lacking reducing agents (Braakman et al., 1992).

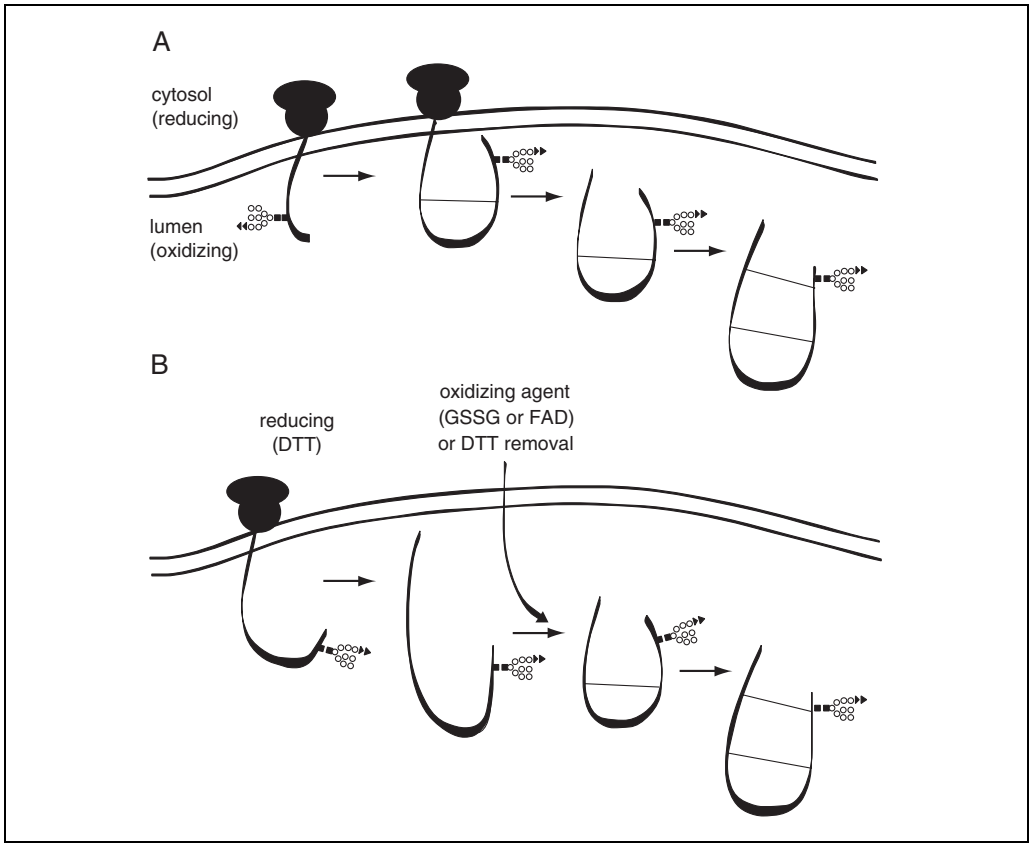


Figure 15.6.2 Formation of disulfide bonds. (**A**) Co-translational bond formation. (**B**) Post-translational bond formation. Under normal cellular redox conditions, the cytosol is reducing and the endoplasmic reticulum (ER) lumen is oxidizing. Disulfide bond formation and glycan addition can take place co-translationally (A) as the C terminus of the protein is being completed by the ribosome. Synchronous oxidation of the completed glycosylated and reduced chain can be initiated post-translationally (B) by synthesizing the protein under reducing conditions (e.g., DTT), inhibiting protein synthesis, and then adding oxidizing agents, such as oxidized glutathione (GSSG) or flavin adenine dinucleotide (FAD), post-translationally.

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

1. Carry out in vitro translation using either canine pancreatic microsomes or SP cells as described (see Basic Protocol 1, steps 1 to 4) with the omission of the oxidizing agent GSSG or FAD in step 2. Adjust the reaction volume with nuclease-free water accordingly.
2. Add 3.7 µl of 100 mM GSSG (4 mM final) or 3.7 µl of 2.5 mM FAD (100 µM final) to initiate oxidation. Mix and incubate for an appropriate length of time at 27°C.

   The incubation time(s) will depend on the experimental design.
3. Alkylate the remaining free cysteines with NEM and/or AMS to arrest further oxidation, and analyze proteins as described (see Basic Protocol 1, steps 5 to 28).

BASIC PROTOCOL 2

ANALYSIS OF PROTEIN FOLDING BY PROTEOLYTIC SENSITIVITY

The folded state and stability of proteins synthesized in vitro and in vivo can be analyzed by proteolysis. Limited proteolysis is a powerful tool for probing the globular structure of folded proteins. While a variety of promiscuous proteases can be employed for these studies, the most commonly used is trypsin. Trypsin cleaves the peptide bonds C-terminal to the basic amino acids lysine and arginine. Peptide regions buried in the protein core and in the secondary structural elements are less accessible to the enzyme (usually in the folded conformation), therefore they are not cleaved as quickly (Fig. 15.6.1). Here, in vitro translated, ^{35}S-labeled protein is allowed to fold under oxidizing conditions in the presence of canine pancreatic microsomes (see Basic Protocol 1). Microsomal membranes containing the ^{35}S-labeled protein are isolated from the lysate and solubilized in a digestion buffer. The folded state of the protein is analyzed by trypsin digestion. Early oxidation or folding forms will be easily digested by the protease while mature forms will be more protease resistant. The digested samples are analyzed directly by reducing SDS-PAGE and autoradiography. This technique can also be used with semipermeabilized (SP) cells (see Support Protocol), or it can easily be adapted for intact cell studies (see Basic Protocol 5 and see Alternate Protocol 3).

Materials

1× sucrose cushion (see recipe)
1% and 10% digestion buffer: 1% and 10% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) in HeBS (see recipe for HeBS)
50 µg/ml trypsin (prepared from 1 mg/ml stock; stored at 4°C up to 6 months)
50 µg/ml soybean trypsin inhibitor (prepared from 1 mg/ml stock; stored at 4°C up to 6 months)
150 mM PMSF (see recipe)
2× reducing sample buffer: 10% (v/v) 1 M DTT (*APPENDIX 2A*; 100 mM final) in 2× nonreducing sample buffer (see recipe)
Beckman Airfuge, 4°C, and 5 × 20–mm ultraclear centrifuge tubes (Beckman)
Additional reagents and equipment for in vitro translation using either canine pancreatic microsomes or SP cells, analyzing proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and gel autoradiography (see Basic Protocol 1)

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions

with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

Carry out in vitro translation

1. In vitro translate the protein of interest as described (see Basic Protocol 1, steps 1 to 3) in the presence of GSSG or FAD.

   The number of samples should be determined and the translation reaction scaled up accordingly.

2. Add 50 mM cycloheximide to a final concentration of 1 mM to stop the translation reaction.

   For a 92-µl reaction, 2 µl of 50 mM cycloheximide is added.

Alkylate proteins

3. Transfer 10 µl lysate to a new 1.5-ml microcentrifuge tube containing 2 µl of 120 mM NEM (20 mM final) to alkylate free cysteines, and place on ice for 10 min.

4. Return remaining lysate to the 27°C water bath for further oxidation and maturation.

5. After appropriate incubation times, repeat steps 3 and 4 for remaining time points.

Isolate membranes

6. Add 50 µl of 1× sucrose cushion to the bottom of a 5 × 20–mm ultraclear centrifuge tube. Overlay 12 µl NEM-alkylated lysate on the top of the sucrose cushion and centrifuge 10 min in a Beckman Airfuge at 20 psi, 4°C.

7. Discard supernatant and resuspend pellet containing the ^{35}S-labeled protein in 20 µl of 1% digestion buffer.

   Adherent cells or cells in suspension can be applied to this assay after this step. For adherent cells perform steps 1 to 10, Basic Protocol 5, or for cells in suspension perform steps 1 to 11, Alternate Protocol 3. Then immunoprecipitate the protein (UNIT 7.2) and resuspend the pellet in 20 µl of 1% digestion buffer and continue with step 8.

   Care must be taken not to create air bubbles while resuspending the pellet. Make sure that no pellet clumps are left undissolved.

8. Transfer 1 µl sample to pH paper to check the pH. If necessary, add 2 to 3 µl of 10% digestion buffer to increase the buffer strength and recheck the pH.

   The optimal pH range for trypsin is 7.5 to 8.0.

Digest with trypsin

9. Add 0.4 to 0.8 µl of 50 µg/ml trypsin (1 to 2 µg/ml final) to the sample (step 7) and incubate 30 min at 27°C.

   Control samples without added enzyme should be set aside for all time points. Set aside half of the sample (~10 µl) from step 8 for a control.Trypsin concentration may need to be titrated for optimal digestion. The incubation time and temperature can also be varied for optimal digestion.

10. Add 2 µl of 50 µg/ml trypsin inhibitor and 1 µl of 150 mM PMSF to stop the digestion. Mix thoroughly by pipetting up and down. Leave the sample at room temperature for 2 min.

Electrophorese proteins

11. Add 25 µl of 2× reducing sample buffer and mix thoroughly. Immediately heat 5 min at 95°C and microcentrifuge 5 min at 16,000 × *g* (14,000 rpm), room temperature.

12. Prepare and run an SDS-polyacrylamide gel and analyze proteins by gel autoradiography as described (see Basic Protocol 1, steps 22 to 28), but load 10 µl sample in step 23.

The remaining sample can be stored up to 2 months at −20°C.

SUPPORT PROTOCOL

ISOLATION OF SEMIPERMEABILIZED CELLS FOR ANALYSIS OF PROTEIN FOLDING

To study the oxidation of a protein in an intact cellular ER while maintaining the manipulative advantage of the in vitro translation system, proteins translated in vitro can be translocated into the ER of semipermeabilized cells (SP cells). Here, essentially any eukaryotic cell type can be used as the ER source. The plasma membrane is permeabilized with the detergent digitonin while the endomembranes remain intact. The SP-cell membranes can then be substituted for the microsomal membranes as discussed in Basic Protocol 1.

Materials

Cells in 75-cm^2 tissue culture flask in appropriate medium
PBS (*APPENDIX 2A*)
Trypsin/EDTA solution (see recipe), room temperature
KHM-STI buffer (see recipe), ice cold
0.4% (w/v) trypan blue solution (Sigma-Aldrich)
KHM buffer (see recipe), ice cold
20 mg/ml digitonin in dimethyl sulfoxide (DMSO), stored in 1-ml aliquots up to 2 years at −20°C
HEPES/potassium acetate buffer (see recipe), ice cold
100 mM $CaCl_2$ (*APPENDIX 2A*)
15,000 U/ml micrococcal nuclease, from *Staphylococcal aureus* (Boehringer Mannheim), stored in working-volume aliquots (e.g., 5 µl or multiples of 5 µl) at −80°C
250 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), adjusted to pH 7.4 with NaOH (stored up to 2 years at −20°C)

Vacuum aspirator
15-ml sterile polystyrene centrifuge tubes, prechilled on ice
Beckman GPR centrifuge (or equivalent), 4°C
Microcentrifuge, 4°C

Additional reagents and equipment for counting cells (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Isolate cells

1. Grow cells in 75-cm^2 tissue culture flask in appropriate medium to ~80% confluency.

Chinese Hamster Ovary (CHO) cells commonly yield 0.6 to 2 × 10^6 SP-cells from a single 75-cm^2 flask. For the 12 µl of SP-cells used in the 92 µl translation mix, two flasks are sufficient, scale up the number of flasks with respect to the size of the experiment.

2. Remove medium from flask, rinse twice with ~10 ml PBS, and use a vacuum aspirator to remove the PBS.
3. Add 4 ml trypsin/EDTA solution, prewarmed at room temperature. Quickly tilt flask back and forth so that the whole flask is covered. Remove trypsin solution immediately and incubate flask 5 min at room temperature.
4. Slap the bottom of the flask to loosen cells, add 6 ml ice-cold KHM-STI buffer, and suspend cells by pipetting up and down until there are no visible cell clumps. Place flask on ice.

   NOTE: *Cells should be kept on ice for all remaining steps except where indicated.*

5. Rinse flask with an additional 2 ml KHM-STI buffer for a total volume of 8 ml.
6. Transfer 10 µl suspended cells to a 1.5-ml microcentrifuge tube and add 10 µl of 0.4% trypan blue solution. Mix thoroughly and count cells. Note percent of dyed cells at this stage (*UNIT 1.1*).
7. Transfer cell suspension (step 5) to a prechilled 15-ml sterile polystyrene centrifuge tube ($\leq 5 \times 10^7$ cells/tube) and place on ice.

   The cell suspension should be divided among multiple tubes as needed.

8. Centrifuge 5 min at 250 × *g* (1200 rpm), 4°C, in a Beckman GPR centrifuge, to pellet cells.

Permeabilize cells

9. Remove supernatant and add 100 µl ice-cold KHM buffer at the bottom of the tube and tap the bottom of the tube to resuspend cells. Add 5.9 ml ice-cold KHM buffer and invert tube two to three times.
10. Add 6 µl of 20 mg/ml digitonin in DMSO (20 µg/ml final) to each tube and mix immediately. Incubate 5 min on ice.
11. Add 8 ml ice-cold KHM buffer and mix by inverting two or three times. Centrifuge 5 min at 250 × *g*, 4°C. Discard supernatant.
12. Resuspend cells in 14 ml ice-cold HEPES/potassium acetate buffer and incubate 10 min on ice. Centrifuge 5 min at 250 × *g*, 4°C. Discard supernatant.
13. Resuspend cells in 1 ml ice-cold KHM buffer and transfer cells into a 1.5-ml microcentrifuge tube.

    All cell pellets from different tubes ($\leq 4 \times 10^7$ cells) should be pooled in 1 ml KHM buffer.

14. Transfer 10 µl cell suspension to a 1.5-ml microcentrifuge tube and dilute 1:5 in ice-cold KHM buffer. Transfer 10 µl of this dilution to a new 1.5-ml tube and count cells and test for permeabilization efficiency with trypan blue (step 6).

    At this step, there are usually about half of the total number of cells that were present in step 6.

15. Microcentrifuge cells (step 13) 2 min at 5,200 × *g* (8,000 rpm), 4°C. Aspirate supernatant and resuspend cells in 100 µl ice-cold KHM buffer (for $2–4 \times 10^6$ cells).

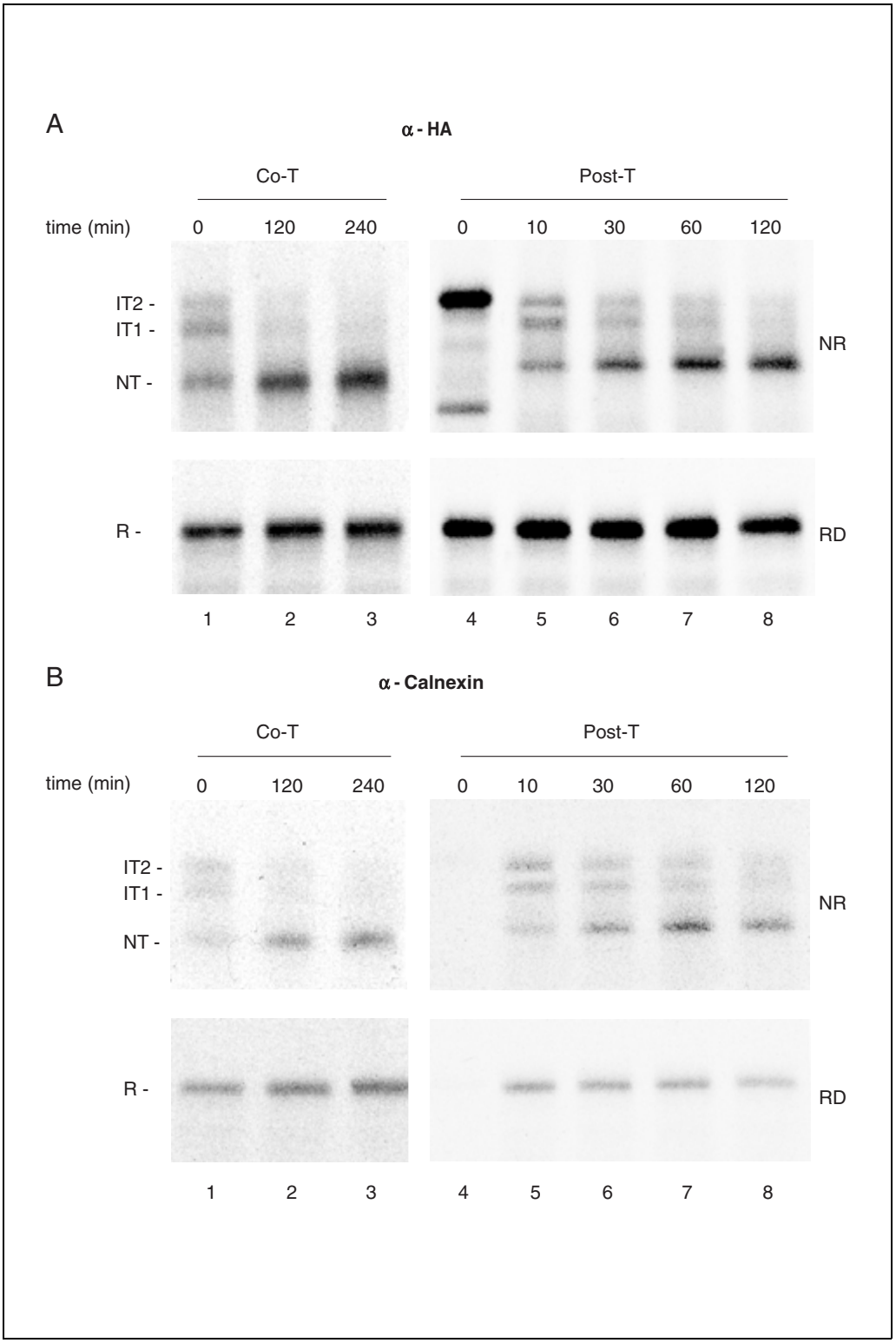


Figure 15.6.3 Disulfide bond formation and folding of hemagglutinin (HA). (A) Co-translational and post-translational formation of disulfide bonds. (B) Calnexin binding. ^{35}S-labeled HA was in vitro translated with rabbit reticulocyte lysate at 27°C for 1 hr in the presence of canine pancreatic endoplasmic reticulum (ER)–derived microsomes under oxidizing (co-translational; Co-T) or reducing (post-translational; Post-T) conditions. Cycloheximide was added to inhibit protein synthesis and protein samples were removed after the indicated chase times and subjected to immunoprecipitation with antisera raised against the whole influenza virus (A) and resolved by nonreducing (NR) and reducing (RD) SDS-PAGE. For post-translational analysis, oxidation was initiated at time zero and followed. The same samples were used to identify interactions with calnexin by the co-immunoprecipitation of ^{35}S-labeled HA with anti-calnexin antiserum (B). Protein forms are as follows: IT1, intermediate 1; IT2, intermediate 2; NT, native; R, reduced.

Treat with nuclease

16. Add 1 µl of 100 mM $CaCl_2$ (1 mM final) and 2 µl of 15,000 U/ml (300 U/ml final) micrococcal nuclease. Mix and incubate 12 min at room temperature.

    Micrococcal nuclease is a calcium-dependent nuclease that will digest endogenous mRNA.

17. Add 1.6 µl of 250 mM EGTA (4 mM final) to chelate calcium and inactivate the nuclease.

18. Microcentrifuge cells 2 min at 5,200 × *g*, 4°C. Aspirate supernatant and resuspend SP-cell pellet with ice-cold KHM buffer to a final concentration of 1×10^5 cells/µl.

    SP cells should be used as soon as they are prepared.

ANALYSIS OF FOLDING-INTERMEDIATE BINDING TO MOLECULAR CHAPERONES IN ROUGH ENDOPLASMIC RETICULUM–DERIVED MICROSOMES

BASIC PROTOCOL 3

Protein folding in the cell is a highly assisted process. Molecular chaperones and foldases play important roles in aiding the folding or stabilization of nascent proteins. In the endoplasmic reticulum (ER), proteins that help in the folding process include BiP (GRP78; an hsp70 family member), GRP94 (an hsp90 family member), the lectin chaperones calnexin and calreticulin, and the protein disulfide isomerase family members PDI, ERp57, and ERp72. The transient binding of these folding factors to the nascent chain can be studied through a co-immunoprecipitation procedure if the interactions persist during the solubilization and immunoprecipitation protocol.

In this protocol, a co-immunoprecipitation procedure is described that permits the characterization of transient interactions of folding factors. Co-immunoprecipitations (Fig. 15.6.3) require modifications to the general immunoprecipitation protocol described previously (see Basic Protocol 1). First, the detergent used in cell or microsomal lysis must enable the persistence of the native interaction of the chaperones with the substrate. Common nondenaturing detergents used for this purpose include Triton X-100, digitonin, and CHAPS (*UNIT 7.2*). In addition, antibodies against the chaperones or folding factors are required. Finally, if folding-factor binding is regulated by ATP binding (as found with BiP), it may be necessary to deplete ATP during the solubilization step to trap the interaction with the nascent chain. Chemical cross-linking can also be performed to provide a covalent bond to trap interactions between the chaperone and its substrate prior to lysis (see Alternate Protocol 2).

Materials

Antiserum raised against protein of interest
Antiserum raised against chaperone of interest

Additional reagents and equipment for in vitro translation using canine pancreatic microsomes or SP cells, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and gel autoradiography (see Basic Protocol 1)

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

1. In vitro translate the protein of interest as described (see Basic Protocol 1, steps 1 to 3, when using microsomes or Support Protocol, steps 1 to 18 for SP-cells), adjusting the volume of any control reactions with nuclease-free water.

The number of time points should be determined and the translation reaction scaled up accordingly. For each time point, three samples will be needed: lysate (for total protein analysis), immunoprecipitation with antibodies directed against the maturing protein, and immunoprecipitation with antibodies directed against the chaperone(s). A reaction mixture of 30 µl is appropriate for each time point when only one chaperone immunoprecipitation is performed. For each additional chaperone immunoprecipitation increase the reaction mixture by 10 µl. Keeping the DTT concentration constant (1.1 mM) for these reactions, titrate GSSG from 2 to 6 mM or FAD from 50 to 200 µM to determine optimum oxidation conditions.

A control reaction without microsomes or SP-cells should be performed to eliminate the possibility that non-specific binding is occurring int he chaperone binding assays.

2. Transfer 30 µl lysate into a 1.5-ml microcentrifuge tube. Return the remaining lysate to the 27°C water bath for further oxidation and maturation.
3. Add 6 µl of 120 mM NEM to 30-µl sample to arrest oxidation and incubate 10 min on ice to stop the translation.

   Cycloheximide at a final concentration of 1 mM can also be included to stop the translation if further maturation is to be followed in the absence of protein synthesis.

4. Divide the sample into three 12-µl aliquots.
5. To the first, add 12 µl of 2× nonreducing sample buffer and 16 µl of 1× nonreducing sample buffer, store samples at −20°C until all samples are prepared. To prepare the samples heat 5 min at 920°C and analyze total proteins (see Basic Protocol 1, steps 22 to 28).
6. To the remaining two aliquots, add 500 µl ice-cold 2% CHAPS/HeBS to lyse the samples.
7. Immunoprecipitate both samples as described (see Basic Protocol 1, steps 11 to 18), using an antibody raised against the protein of interest in one sample to measure total protein level and an antibody raised against the interacting molecular chaperone in the other.
8. Electrophorese and analyze proteins by SDS-PAGE and gel autoradiography as described (see Basic Protocol 1, steps 19 to 28).

   Table 15.6.1 is a list of chaperones and their molecular weights; this can be used to calculate the molecular weight change in complexes.

Table 15.6.1 Approximate Molecular Weights of Endoplasmic Reticulum Chaperones

Chaperone[a]	Molecular weight (kDa)
BiP (GRP78)	75
CNX	57[b]; 90[c]
CRT	48[b]; 60[c]
ERp57	57
ERp72	72
GRP94	94
PDI	55

[a]Abbreviations: CNX, calnexin; CRT, calreticulin; ER, endoplasmic reticulum; GRP, glucose-regulated protein; PDI, protein disulfide isomerase.
[b]Calculated molecular weight.

MONITORING TRANSIENT CHAPERONE INTERACTIONS USING CROSS-LINKING AND DENATURING IMMUNOPRECIPITATIONS

In some cases, the interactions between maturing proteins and molecular chaperones may be weak, therefore disruption can occur during the immunoprecipitation procedure. These types of interactions may require the additional stability offered through chemical cross-linking. Several types of membrane-permeable chemical cross-linkers are commercially available with reactivity towards free sulfhydryls, amines, carboxyls, or other functional groups, while others are UV excitable and can trap a broader range of functional groups (Table 15.6.2).

Once a cross-link is recognized by a molecular weight shift following SDS-PAGE, it can be identified using denaturing immunoprecipitation with antiserum raised against the molecular chaperone adduct. To minimize background cross-linking, the microsomes are isolated from the translation system by centrifugation and resuspended in an isotonic resuspension buffer prior to cross-linking.

Additional Materials *(also see Basic Protocol 3)*

Isotonic sucrose cushion (see recipe)
Isotonic resuspension buffer (see recipe)
10 mM BMH (see recipe) or alternative cross-linker
Quenching solution: 100 mM 2-mercaptoethanol (stored up to 1 month at 4°C)
20% (w/v) SDS (*APPENDIX 2A*)
Denaturing immunoprecipitation buffer (see recipe)
Beckman Optima TLX ultracentrifuge and TLA 120.2 rotor, 4°C, and 7 × 20–mm ultracentrifuge tubes *or* Beckman Airfuge, 4°C, and 5 × 20–mm ultraclear centrifuge tubes

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

1. In vitro translate the protein of interest as described (see Basic Protocol 3 steps 1 to 3), but when using a thiol-reactive cross-linker, such as BMH (step 4), do not add NEM. Adjust samples to 1 mM cycloheximide and incubate 10 min on ice to terminate translation.

   NEM will block reactive groups on thiol-reactive cross-linkers (e.g., BMH).

Table 15.6.2 Commonly Used Cross-Linkers for Monitoring Chaperone Interactions

Cross-linker[a]	Reactivity
BASED	Nonselective, photoreactive
BMH	Sulfhydryls
DSP	Amines
EDC	Amines and carboxyls
SMCC	Amines and sulfhydryls

[a]Available from Pierce Chemical. Abbreviations: BASED, bis[β-(4-azidosalicylamido)ethyl] disulfide; BMH, bismaleimidohexane; DSP, dithiobis(succinimidyl propionate); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; SMCC, succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate.

For interactions with some folding factors, it is essential that the translation be performed under oxidizing conditions, as this will enable the chaperones to remain in their native oxidized state. Nonnative reducing conditions may reduce a critical disulfide bond in the chaperone thereby destroying its ability to interact with other substrates (for example, calnexin and calreticulin's lectin-binding properties are diminished under reducing conditions; Fig. 15.6.3B, lane 4).

2. Carefully layer a 10-µl sample on a 30-µl isotonic sucrose cushion (1:3 sample/cushion ratio) within a 7 × 20–mm ultracentrifuge tube or a 5 × 20–mm ultraclear centrifuge tube, and centrifuge 10 min either at 157,000 × *g* (60,000 rpm), 4°C, using a Beckman Optima TLX ultracentrifuge with a TLA 120.2 rotor or at 20 psi, 4°C, using a Beckman Airfuge, respectively.
3. Keeping samples on ice, aspirate the supernatant. Resuspend pellet by pipetting up and down in 10 µl isotonic resuspension buffer, and transfer to a new 1.5-ml microcentrifuge tube.

   It is important that the microsomal pellet is fully resuspended and the samples are kept on ice.

   Removal of the reticulocyte lysate by centrifugation ensures that cross-links will not occur with reticulocyte lysate proteins.
4. Add 1.1 µl of 10 mM BMH (1 mM final) and incubate 10 min at 27°C.

   It may be necessary to increase or decrease the cross-linker concentration to obtain the optimal results.
5. Add 1.2 µl quenching solution and place samples on ice for 10 min.

   While the authors describe a protocol for BMH, a thiol-reactive cross-linker that is quenched with alkylating agents, a variety of cross-linkers (Table 15.6.2) should be tested for optimal cross-linking. The quenching agent employed will depend on the cross-linker chemistry.
6. Add 0.65 µl of 20% SDS to a 12.2 µl sample (1% final) and heat 5 min at 95°C.

   This will denature all noncovalent interactions and ensure that only cross-linked adducts are visualized,
7. Add 500 µl denaturing immunoprecipitation buffer to quench SDS.
8. Complete immunoprecipitation as described (see Basic Protocol 3, steps 7 to 8), but use 500 µl denaturing immunoprecipitation buffer in place of 500 µl of 0.5% CHAPS/HeBS for the wash step (see Basic Protocol 1, step 16).

   Antibodies to predicted adducts can be used here (Table 15.6.2; Affinity BioReagents, Stressgen). Once adducts are observed, their molecular weights can be estimated by reducing SDS-PAGE. Antibodies to molecular chaperones with similar molecular weights (Table 15.6.1) can then be used to identify protein adducts. StressGen Biotechnologies and Affinity BioReagents are excellent sources for antibodies against ER folding factors.

BASIC PROTOCOL 4

MONITORING PROTEIN FOLDING USING CONFORMATION-SPECIFIC ANTIBODIES

The recognition of protein determinants (epitopes) by antibodies can be conformation dependent. This enables the use of conformation-specific antibodies as a powerful tool for the characterization of the folding process (Goldberg, 1991). The acquisition or disappearance of specific epitopes can be used as a probe to monitor the folding reaction. On occasion, monospecific antibodies that recognize a single epitope can be obtained from polyclonal sources by generating antipeptide antibodies or by antibody purification. However, more commonly, monoclonal antibodies (mAbs) have been found to be the most effective for these studies.

This protocol describes the use of conformation-specific antibodies to characterize the folding process in rough endoplasmic reticulum (ER)–derived microsomes. It is similar to Basic Protocol 3, which explains the analysis of chaperone binding with the use of co-immunoprecipitation studies. Here, it is also important to lyse the microsomes and perform the antibody binding and washing under gentle nondenaturing conditions to ensure that the protein conformation is not disrupted. The antibodies employed are mAbs raised against the folding substrate that recognize individual epitopes. In short, the folding reaction is initiated with the radiolabeled protein as in Basic Protocol 1 (co-translationally) or Alternate Protocol 1 (post-translationally), with samples being removed at various times and analyzed by immunoprecipitation with the mAbs, and then the radiolabeled precipitate is resolved by SDS-PAGE and autoradiography.

Materials

50% (w/v) protein G–Sepharose or protein A–Sepharose bead (Sigma-Aldrich) slurry in PBS/0.1% (w/v) BSA/0.01% (w/v) sodium azide

Conformation-specific monoclonal antibody (mAb) against protein of interest

Control antibodies (preimmune control and antibody control that recognizes all conformations of the protein of interest)

Additional reagents and equipment for in vitro translation using canine pancreatic microsomes, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and gel autoradiography (see Basic Protocol 1)

CAUTION: Sodium azide is poisonous; follow appropriate precautions for handling, storage, and disposal.

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

NOTE: All solutions should be ice cold and procedures should be carried out at 4°C or on ice unless otherwise indicated.

1. In vitro translate the protein of interest as described (for cotranslational analysis see Basic Protocol 1, steps 1 to 3; for post-translational analysis see Alternate Protocol 1, steps 1 to 3).

   The number of time points should be determined and the translation reaction scaled up accordingly. For each time point three 12-µl samples will be needed: lysate (for total protein analysis), immunoprecipitate with antibody that is not conformation dependent, and immunoprecipitate with antibody that is conformation dependent.

2. Transfer 30 µl lysate into a 1.5-ml microcentrifuge tube. Return the remaining lysate to the 27°C water bath for further oxidation and maturation.

3. Add 6 µl of 120 mM NEM to 30-µl sample and incubate on ice 10 min to alkylate proteins and arrest disulfide bond formation.

4. Add 500 µl ice-cold 2% CHAPS/HeBS and vortex in a microcentrifuge tube rack vortex mixer at medium speed for 10 min at 4°C to lyse ER microsomes.

5. Add 100 µl of 50% protein G (or A)–Sepharose bead slurry and rotate end over end on a tube rotator for 1 hr at 4°C, to preclear sample.

   For a discussion and chart on the use of protein A versus protein G, see UNIT 7.2 (Table 7.2.1).

6. Centrifuge sample 5 min at 2900 × *g*, 4°C, and divide sample among three 1.5-ml microcentrifuge tubes containing 100 µl of 50% protein G (or A)–Sepharose and 2 to 10 µl of either a conformation-specific mAb against the protein of interest, a preimmune control antibody, or a control antibody that recognizes all conformations of the protein of interest.

   A titration with antiserum may be required to obtain optimal results. Excess antibodies may give nonspecific binding or saturate the protein G (or A)–Sepharose.

7. Immunoprecipitate and analyze the proteins using nonreducing and reducing SDS-PAGE and gel autoradiography as described (see Basic Protocol 1, steps 13 to 28).

BASIC PROTOCOL 5

ANALYSIS OF PROTEIN FOLDING AND DISULFIDE BOND FORMATION IN CELLS GROWN IN INTACT MONOLAYERS (ADHERENT CELLS)

Co-translational and post-translational folding of a protein can be followed in cells grown in monolayers in tissue culture dishes (Braakman et al., 1991). This procedure allows multiple rapid wash steps, facilitating experiments that require short pulse-chase times. Adherent-cell studies are also ideal for experiments requiring frequent changes of medium. The disadvantage of these studies is that large volumes of medium can be required. When low total volume of medium is desired (e.g., when expensive reagents are needed for the experiment), the analysis can be performed with cells in suspension (see Alternate Protocol 3).

For a detailed explanation of metabolic labeling procedures, see *UNIT 7.1*. In brief, cellular proteins are metabolically labeled in tissue culture dishes with [^{35}S]methionine and [^{35}S]cysteine (the pulse). The labeling medium is removed by aspiration and replaced by medium containing cold (i.e., unlabeled) amino acids, and the cells are incubated (the chase). Samples are removed at various times and oxidative intermediates trapped with chilled buffer containing alkylating agents. Because all cellular proteins are labeled by this procedure, analysis of a specific protein requires isolation by immunoprecipitation (*UNIT 7.2* or see Basic Protocol 1). The isolated protein can then be analyzed for disulfide bond formation (Basic Protocol 1), protease sensitivity (Basic Protocol 2), chaperone binding (Basic Protocol 3), or folding with conformation-specific antibodies (Basic Protocol 4).

Materials

Adherent cells grown to 80% to 90% confluency in a 60-mm tissue culture dish
Depletion medium: cysteine- and methionine-free tissue culture medium, 37°C
Labeling medium: depletion medium containing 0.125 to 0.75 mCi/ml [^{35}S]methionine and [^{35}S]cysteine (prepared fresh), 37°C
Chase medium (see recipe), 37°C
Stop buffer: 20 mM NEM (see recipe for 1 M stock) in PBS, prepared just before use and kept on ice
Lysis buffer (see recipe), ice cold

Aspirator
Cell scraper

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2* or see Basic Protocol 1) and analysis of disulfide bond formation (see Basic Protocol 1), protein folding by proteolytic sensitivity (see Basic Protocol 2), chaperone binding (see Basic Protocol 3), *or* protein folding with conformation-specific antibodies (see Basic Protocol 4)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: For the analysis of co-translational protein folding no reducing agents are added to either the pulse or chase medium. Chase medium should contain 1 mM cycloheximide in order to stop protein synthesis. In the case of post-translational protein folding and disulfide bond formation, reducing agent (5 mM DTT final) is added to the pulse medium. When the reducing agent is removed after pulse labeling, redox conditions are restored that support the formation of disulfide bonds.

NOTE: Separate cell culture dishes will be required for each condition and time point.

Perform metabolic labeling

1. Use an aspirator to remove culture medium from adherent cells grown to 80% to 90% confluency in a 60-mm tissue culture dish, and wash dish twice by gently swirling with 10 ml prewarmed depletion medium, aspirating medium after each wash.

   Prior to the experiment, the number of chase time points should be determined. Depending on the cell type, a confluent 60-mm dish will contain 1.8–7 × 10^6 cells, or enough for approximately one time point. Other dish sizes can be used. The volumes should be adjusted accordingly based on the surface area of the dish.

2. Add 5 ml prewarmed depletion medium and incubate 15 min in a humidified 37°C, 5% CO_2 incubator to deplete intracellular pools of methionine and cysteine.

3. Remove medium from cells, add 450 µl labeling medium, and incubate 10 to 60 min in a CO_2 incubator.

   It is critical that the plates sit on a perfectly horizontal surface during incubation to avoid drying of the cell monolayer.

   Mammalian translation rates are on the order of five amino acids per second. Labeling time will depend on the length and the expression level of the protein of interest, as well as the number of methionine and cysteine residues in the protein.

4. Remove and discard labeling medium.

Perform chase

5a. *For 0-min chase:* Add 2 ml chase medium and gently rock solution over cells. Rapidly aspirate chase medium, immediately add 2.5 ml ice-cold stop buffer, and place dish on ice.

   The chase medium contains the protein synthesis inhibitor cycloheximide (1 mM final).

5b. *For other chase times:* Add 2 ml chase medium and gently rock solution over cells. Aspirate medium, replace with 2 ml fresh chase medium, and incubate for desired times in the CO_2 incubator. Rapidly aspirate chase medium, immediately add 2.5 ml ice-cold stop buffer, and place dish on ice.

   Chase times are dependent upon the time it takes the protein of interest to oxidize, fold, and exit the endoplasmic reticulum (ER).

Prepare lysate

6. Remove stop buffer and add 2.5 ml fresh stop buffer. Aspirate dish dry.

7. Add 600 µl ice-cold lysis buffer.

8. Scrape off cells using a cell scraper and collect in a 1.5-ml microcentrifuge tube.
9. Microcentrifuge 5 min at 11,800 × *g* (12,000 rpm), 4°C, to pellet nuclei.
10. Transfer post-nuclear supernatant to a new 1.5-ml microcentrifuge tube.
11. Analyze disulfide bond formation (see Basic Protocol 1, steps 5 to 28), protein folding by proteolytic sensitivity (see Basic Protocol 2, steps 7 to 12), chaperone binding (see Basic Protocol 3, steps 7 to 8), or protein folding with conformation-specific antibodies (see Basic Protocol 4, steps 5 to 7). Immunoprecipitate the protein of interest (*UNIT 7.2*) if needed.

To analyze the disulfide bond formation (Basic Protocol 1) use ice-cold PBS without NEM for the stop buffer (steps 5b and 6 above). Divide the sample appropriately after step 10 and alkylate with either NEM or AMS. Keep one sample that is not alkylated on ice as a control.

When analyzing for proteolytic sensitivity immunoprecipitate (UNIT 7.2) the 600 µl of post nuclear lysate from step 10 prior to performing steps 7 to 12 of Basic Protocol 2.

ALTERNATE PROTOCOL 3

ANALYSIS OF PROTEIN FOLDING AND DISULFIDE BOND FORMATION IN SUSPENDED (NONADHERENT) CELLS

When cells do not adhere adequately or when it is desirable to use small volumes of reagents for analysis (i.e., when expensive drugs are used), cells in suspension may be used to analyze protein folding and disulfide bond formation. The advantage with this approach is that samples at different chase intervals can be divided from the same population of ^{35}S-labeled cells. Cells are collected in 15- or 50-ml polystyrene conical centrifuge tubes and the pulse-chase is performed in the tube. However, the exchange of media and buffers requires centrifugation of cells. Each chase time point should have ~1 × 10^6 cells. Co-translational and post-translational folding can be followed using appropriate reducing and oxidizing conditions in the pulse and chase medium (see Alternate Protocol 1) and analyzed for disulfide bond formation (Basic Protocol 1), protease sensitivity (Basic Protocol 2), chaperone binding (Basic Protocol 3), or protein folding with conformation-specific antibodies (Basic Protocol 4).

Additional Materials (also see Basic Protocol 5)

Cell suspension
Suspension labeling medium: depletion medium containing 25 to 50 µCi/ml [^{35}S]methionine and [^{35}S]cysteine (prepared fresh), 37°C
15-ml sterile polystyrene conical centrifuge tubes
Beckman GPR centrifuge or equivalent
37°C water bath
Additional reagents and equipment for immunoprecipitation (*UNIT 7.2* or see Basic Protocol 1) and for analysis of disulfide bond formation (see Basic Protocol 1), protein folding by proteolytic sensitivity (see Basic Protocol 2), chaperone binding (see Basic Protocol 3), *or* protein folding with conformation-specific antibodies (see Basic Protocol 4)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: Keep cells in suspension by periodically swirling the tube during incubations.

Collect cells

1. Collect cell suspension in a 15-ml sterile polystyrene conical centrifuge tube.

   Prior to the experiment, the minimum volume in which the cells are to be suspended, the number of chase time points, and the desired sample volume should be determined. Approximately 1×10^6 cells should be used per time point. The volumes should be scaled up accordingly; a 50-ml conical centrifuge tube should be used for larger volumes.

   The cells can also be collected by treating adherent cells with sterile 0.25% (w/v) trypsin/0.2% (w/v) EDTA at room temperature and resuspending in regular culture medium. (Some cell types require EDTA for detachment while others do not. This must be determined in each case.) Cells are resuspended in normal growth medium by pipetting up and down two to three times.

2. Centrifuge cell suspension 5 min at $250 \times g$ (1200 rpm), room temperature, in a Beckman GPR centrifuge and discard the medium.

   The pelleting efficiency may vary with different cell types.

3. Resuspend cells in 0.5 ml per time point depletion medium and centrifuge 5 min at $250 \times g$, room temperature.

   For resuspending cells, a minimal volume of depletion medium should be used.

4. Discard supernatant and resuspend cells in 0.5 ml per time point depletion medium and incubate 30 to 60 min in a 37°C water bath to deplete intracellular methionine and cysteine. Resuspend the cells periodically by gently swirling the tube.

5. Centrifuge cells 5 min at $250 \times g$, room temperature, and aspirate supernatant.

Perform pulse-chase labeling

6. Resuspend cells in 2 ml suspension labeling medium. Cap tube tightly and incubate for the desired pulse time at 37°C. Resuspend the cells periodically by swirling the tube.

   A 15- to 30-min pulse is usually sufficient. However, labeling time will depend on the length and expression level of the protein of interest, as well as the number of methionine and cysteine residues in the protein.

7. Centrifuge cells 5 min at $250 \times g$, room temperature, and aspirate supernatant. Resuspend cells in 0.5 ml per time point chase medium and centrifuge again. Aspirate supernatant and resuspend cells in 0.5 ml per time point chase medium.

   Chase medium contains a protein synthesis inhibitor (1 mM cycloheximide).

   For experiments with multiple time points, a single population of cells is labeled, and then aliquots (each containing ~1×10^6 cells) are removed at appropriate chase time points. Because the cells settle to the bottom of the tube during the pulse and chase intervals, the tube should be swirled gently at regular intervals and the cells mixed thoroughly by pipeting up and down several times before dividing.

8a. *For 0-min chase:* Immediately take an aliquot of cells (~10^6 cells) and add an equal volume of ice-cold lysis buffer to the aliquot, mix well, and place on ice.

   Alternatively, 14 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid disodium salt (AMS) can be used instead of NEM in the lysis buffer. When analyzing disulfide bond formation AMS provides a larger molecular weight shift than NEM.

8b. *For other chase times:* Incubate cell suspension for the desired chase time at 37°C. At the end of each of the appropriate chase times, take an aliquot of ~10^6 cells and add an equal volume ice-cold lysis buffer, mix well, and place on ice.

   Alternatively, 14 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid disodium salt (AMS) can be used instead of NEM in the lysis buffer.

Prepare lysate

9. Transfer sample to a 1.5-ml microcentrifuge tube, and vortex sample 10 min in a microcentrifuge tube rack vortex mixer at full speed, 4°C, to ensure complete cell lysis.
10. Microcentrifuge 10 min at 11,800 × *g*, 4°C.
11. Discard nuclear pellet. Transfer postnuclear supernatant to a new 1.5-ml microcentrifuge tube.
12. Analyze disulfide bond formation (see Basic Protocol 1, steps 5 to 28), protein folding by proteolytic sensitivity (see Basic Protocol 2, steps 7 to 12), chaperone binding (see Basic Protocol 3, steps 7 to 8), or protein folding with conformation-specific antibodies (see Basic Protocol 4, steps 5 to 7). Immunoprecipitate the protein of interest (see *UNIT 7.2*) if needed.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid disodium salt (AMS), 20 mM

5.36 mg AMS (Molecular Probes; 20 mM final)
40 µl 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*; 80 mM final)
25 µl 20% (w/v) SDS (*APPENDIX 2A*; 1% final)
H_2O to 0.5 ml
Prepare fresh daily, keep on ice, and avoid exposure to light

AMS is sensitive to light and hydrolysis.

Bismaleimidohexane (BMH), 100 mM

2.76 mg bis-maleimidohexane (BMH; Pierce Chemical)
100 µl 50% (v/v) dimethyl sulfoxide (DMSO)
Store up to 6 months at −20°C
Dilute as needed in 50% (v/v) DMSO

Chase medium

2 ml 1 M HEPES buffer (see recipe), pH 7.3 (20 mM final)
0.5 ml 1 M methionine (see recipe; 5 mM final)
0.5 ml 1 M cysteine (see recipe; 5 mM final)
2 ml 50 mM cycloheximide (see recipe; 1 mM final)
Tissue culture medium to 100 ml
Prepare fresh daily

The tissue culture medium should be appropriate for the cells of interest.

Cycloheximide, 50 mM

0.28 g cycloheximide
H_2O to 10 ml
Store 1-ml aliquots up to 2 years at −20°C

Cysteine, 1 M

1.212 g L-cysteine
H_2O to 10 ml
Store 1-ml aliquots up to 2 years at −20°C

Denaturing immunoprecipitation buffer

5 ml 1 M Tris·Cl, pH 7.6 (*APPENDIX 2A*; 10 mM final)
70 ml 1 M NaCl (140 mM final)
2 ml 250 mM EDTA, pH 8.0 (*APPENDIX 2A*; 1 mM final)
5 ml Triton X-100 (1% final)
H_2O to 500 ml
Store up to 6 months at 4°C

Flavin adenine dinucleotide (FAD), 25 mM

0.207 g FAD
H_2O to 10 ml
Store in 1-ml aliquots up to 2 years at −20°C

Glutathione (GSSG), 100 mM, pH 7.0

0.656 g oxidized GSSG
9 ml H_2O
Adjust pH to 7.0 with 1 N NaOH
H_2O to 10 ml
Store 1-ml aliquots up to 2 years at −20°C

NaOH is used here because K^+ concentrations are more critical in maintaining the fidelity of the translation system.

HEPES buffer

260.3 g *N*-2-hydroxyethylpiperazine-(*N*′2)-2-ethanesulfonic acid (HEPES), sodium salt
H_2O to 800 ml
Adjust pH to 7.2, 7.3, or 7.5 with 10 N KOH
H_2O to 1 liter
Store up to 6 months at 4°C

HEPES-buffered saline (HeBS)

50 ml 1 M HEPES buffer (see recipe), pH 7.5 (50 mM final)
11.69 g NaCl (200 mM final)
H_2O to 1 liter
Store up to 6 months at 4°C

HEPES/potassium acetate buffer

18 ml 1 M HEPES buffer (see recipe), pH 7.2 (90 mM final)
10 ml 1 M potassium acetate, pH 7.2 (50 mM final)
H_2O to 200 ml
Store up to 6 months at 4°C

Isotonic resuspension buffer

2.5 ml 1 M sucrose (250 mM final)
1 ml 1 M potassium acetate (100 mM final)
50 µl 1 M magnesium acetate (5 mM final)
0.5 ml 1 M HEPES buffer (see recipe), pH 7.5 (50 mM final)
H_2O to 10 ml
Store up to 6 months at 4°C

Isotonic sucrose cushion

2.5 ml 1 M sucrose (250 mM final)
0.5 ml 1 M potassium acetate (500 mM final)
50 µl 1 M magnesium acetate (5 mM final)
0.5 ml 1 M HEPES buffer (see recipe), pH 7.5 (50 mM final)
H_2O to 10 ml
Store up to 6 months at 4°C

KHM buffer

55 ml 1 M potassium acetate (110 mM final)
1 ml 1 M magnesium acetate (2 mM final)
10 ml 1 M HEPES buffer, pH 7.2 (see recipe; 20 mM final)
H_2O to 500 ml
Store up to 6 months at 4°C

KHM-STI buffer

100 ml KHM buffer (see recipe)
200 µl 50 mg/ml soybean trypsin inhibitor
Prepare fresh daily and store at 4°C

Lysis buffer

0.2 ml 10% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) in HeBS (see recipe; 2% final)
26.7 µl 150 mM PMSF (see recipe; 0.4 mM final)
10 µl 10 mg/ml leupeptin (10 µg/ml final)
80 µl 1.25 mg/ml aprotinin (10 µg/ml final)
0.2 ml 1 M NEM (see recipe; 20 mM final)
PBS or similar buffer to 10 ml
Add PMSF, leupeptin, aprotinin, and NEM from concentrated stocks just before use

Methionine, 1 M

1.49 g L-methionine
H_2O to 10 ml
Store 1-ml aliquots up to 2 years at −20°C

N-ethylmaleimide (NEM), 1 M

1.25 g NEM
10 ml 100% (v/v) ethanol
Store in 1-ml aliquots up to 2 years at −20°C protected from light
Dilute as needed with 100% (v/v) ethanol

NEM is sensitive to hydrolysis in water and light. The number of freeze thaws should be limited as the NEM will precipitate out and the solution will turn yellow.

Nonreducing sample buffer, 2×

4.5 g SDS (9% final)
7.5 ml glycerol (15% final)
1.5 ml 1 M Tris·Cl, pH 7.8 (*APPENDIX 2A*; 30 mM final)
25 mg bromphenol blue (0.05% final)
H_2O to 50 ml
Store up to 1 year at room temperature

Phenylmethylsulfonyl fluoride (PMSF), 150 mM

2.613 g PMSF
10 ml DMSO
Store in 500-µl aliquots up to 2 years at −20°C

PMSF is highly unstable in water: the half life is ~30 min at 37°C and a few hours on ice.

Protein A–Sepharose, 10%

0.5 g protein A–Sepharose
20 ml 0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) in HeBS (see recipe for HeBS)
Store up to 6 months at 4°C

Salicylate, 2%

2 g sodium salicylate
30 ml methanol
H_2O to 100 ml
Cover the bottle with aluminum foil and store up to 6 to 12 months at room temperature

CAUTION: Gloves should be worn at all times; sodium salicylate can elicit allergic reactions and is readily absorbed through the skin.

Sucrose cushion, 1×

5 ml 1 M sucrose (0.5 M final)
76.1 µl 98% (w/v) triethanolamine (50 mM final)
H_2O to 10 ml
Store up to 6 months at 4°C

Trypsin/EDTA solution

90 ml 1× S-MEM (from 10× S-MEM; Life Technologies) containing 0.23% (w/v) $NaCO_3$ mix and adjust to pH 4.1 to 4.2
10 ml 2.5% trypsin/2% EDTA (Life Technologies; 0.25% final trypsin and 0.2% final EDTA)
Prepare fresh daily

COMMENTARY

Background Information

The early maturation events for a protein in the secretory pathway involve protein folding and oxidation in the endoplasmic reticulum (ER). The folding process commences co-translationally as the protein is vectorially inserted into the ER lumen and is generally completed post-translationally once the protein is released from the ribosome (Braakman et al., 1991; Chen et al., 1995; Fig. 15.6.2). Protein folding can be monitored by using antibodies for immunoprecipitation that recognize specific epitopes that appear or disappear during the folding process (see Basic Protocol 4). In addition, as a protein folds and stabilizes, it becomes more resistant to protease digestion. Domains that are cleaved by the protease in the unfolded state become concealed within the protein core making them inaccessible to proteases (see Basic Protocol 2).

The ER lumen provides an intracellular compartment that is topologically equivalent to the extracellular space. This allows the preparation of proteins that will be secreted and will reside outside the cell. The ER lumen is an oxidizing environment that supports the formation of disulfide bonds. Disulfide bonds can form within a protein (intramolecular) or between two different polypeptide chains (intermolecular). Intramolecular disulfide bond formation can begin co-translationally and is often completed post-translationally. Usually, disulfide bond formation results in a more compact structure that can be resolved following nonreducing SDS-PAGE as an increase in protein mobility (see Basic Protocol 1). As a general rule, intermolecular disulfides form after the folding and oxidation of individual polypeptides. Once this occurs, the two subunits can come together to form an intermolecular covalent bond causing a decrease in protein mobility on nonreducing SDS-polyacrylamide gels.

Besides providing an environment for disulfide bond formation, the ER also contains a variety of resident proteins that assist in the protein maturation process. These proteins include covalent modifiers that add glycans, remove carbohydrates or signal sequences, and catalyze the formation of disulfide bonds as well as molecular chaperones that assist in translocation, protect vulnerable nascent chains from aggregation, and retain misfolded proteins. These proteins play a central role in defining the ER, retaining misfolded or misassembled proteins so they do not travel through the secretory pathway and ensuring the high success rates of nascent protein maturation observed in the cell.

Pulse-chase experiments in live cells permit the biogenesis of a protein to be followed after sufficient radiolabel has been incorporated for visualization (see Critical Parameters, discussion of protein radiolabeling). Currently this methodology provides the most sensitive meth-

Table 15.6.3 Troubleshooting Guide for Protein Folding Analyses[a]

Problem	Possible causes	Solutions
Little or no protein translation	Ionic strength not optimized for transcript	Perform a K^+ titration, or purify mRNA to remove residual buffer salts and then perform K^+ titration
	Oxidation conditions are inhibitory	Perform a titration with GSSG or FAD to determine best oxidation and translation conditions
	Microsome or SP-cell concentrations are inhibitory (i.e., too high)	Perform a microsomal or SP-cell titration to optimize translation and translocation
	mRNA degradation	Check mRNA on agarose gel for degradation; monitor working environment and supplies more carefully to prevent RNase degradation while performing a new transcription
	Transcript too large	Translate individual domains of the protein
Little or no translocation	Microsome or SP-cell concentrations not optimized	Perform a microsomal or SP-cell titration
	SP cells not permeabilized	Make sure cells are not overconfluent. Use an appropriate amount of digitonin after counting cells for optimum permeabilization
	Poor signal sequence	Replace old signal sequence with a powerful ER-targeting signal sequence
	SRP degraded or absent	If using wheat germ, switch to reticulocyte lysate or add purified SRP
No post-translational oxidation	Too much oxidizing agent	Check nonreducing gel for aggregates at the top and within the stacker; if there are a lot, decrease oxidizing agent concentration
	Insufficient oxidizing agent	Perform titration with GSSG or FAD
Inability to co-immunoprecipitate protein	Detergent is denaturing	Use less or change detergent in the lysis buffer to a more native condition (e.g., digitonin, Triton X-100)
	Not enough antibody	Perform antibody titration
	Binding too transient	Cross-link prior to immunoprecipitation
		Deplete ATP with apyrase before adding antibody
Trypsin overdigestion	Too much trypsin	Perform a trypsin titration
	Incubation too long	Incubate at 4°C for shorter time
		Pick the best concentration and perform a time course for digestion

continued

Table 15.6.3 Troubleshooting Guide for Protein Folding Analyses[a], *continued*

Problem	Possible causes	Solutions
No trypsin digestion	Incubation temperature too high or low	Pick the best digestion time and perform a digestion at various temperatures
	Microsomes or SP cells not lysed	Use a higher concentration of lysis buffer
	Incubation time too short	Pick the best concentration and perform a time course for digestion
	Trypsin not the appropriate protease for the protein	Try proteinase K, chymotrypsin, or another protease
Inability to cross-link	No reactive groups available in the protein	Check protein sequence, or structure if available, to determine the best cross-linker
		Try a nonspecific broad-range UV cross-linker
	Too much cross-linking	Check stacker and top of gel for aggregates; if present, try decreasing the amount of cross-linker
Nonreducing gel is blurry	pH of resolving buffer incorrect	Test pH of the resolving buffer to make sure it is 8.8
	Stacking gel too small	Increase size of the stacker and decrease current when running sample through the stacker
	Gel too thick	Decrease thickness of the gel

[a]Abbreviations: ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; GSSG, oxidized glutathione; SP, semipermeabilized.

ods to monitor the synchronous maturation of nascent proteins in the complexity of the live cell. The pulse-chase approach can also be used with an in vitro translation system coupled with canine pancreatic microsomes to study the maturation of proteins that can be controlled and manipulated to a higher degree than intact cells. Originally, this system was used to study the translocation of secretory-pathway proteins across the ER membrane (Blobel and Dobberstein, 1975). More recently, it has been shown to support the maturation of proteins from their earliest folding intermediate to the mature oligomer through careful control of the redox conditions (Hebert et al., 1996). The in vitro translation system can also be coupled with semipermeabilized (SP) cells to combine the facilitated production of radiolabeled proteins with the maturation in an intact ER (Wilson et al., 1995). Together, these various biological systems can be combined with the protocols described to dissect the protein folding and oxidation pathway in the ER.

Critical Parameters

Protein radiolabeling

All the methods described in this unit involve the generation of a radiolabeled protein, which permits the monitoring of its maturation. In choosing a labeling reagent, the amino acid composition of the protein should be considered so that an adequate number of radiolabeled sites will be incorporated. Most commonly, a mixture of ^{35}S-labeled methionine and cysteine is used, but other radiolabeled amino acids are also available for use with proteins lacking an adequate number of cysteines or methionines. The number of radiolabeled sites, the length of the protein, and the level of protein expression will all be important for determining the length of the radioactive pulse. Generally with cells, a 15- to 30-min radioactive pulse is sufficient for visualization of a moderately expressed protein by autoradiography. In vitro translation studies require a longer pulse period because the translation rate is three to five times slower and the optimal temperature for translation is lower at ~27°C. A pulse time of 1 hr is sufficient for most transcripts. Chase times are highly

protein dependent, and a variety of durations (1 to 5 hr) should be explored.

In vitro translation systems

Success with the in vitro translation systems used in Basic Protocols 1 to 4, and Alternate Protocols 1 and 2 is dependent on optimizing the protein translation, translocation, and maturation processes. For specific details for the optimization of each step, see Table 15.6.3. In short, translation levels rely on the integrity of the transcript used, as well as salt concentrations. It is also common for different reticulocyte lysate or wheat germ preparations (*UNIT 11.2*) to translate a given transcript with varying levels of success. The efficiency of protein targeting to and translocation into the ER membrane source (ER-derived microsomes or SP cells) is highly dependent on the membrane concentration. Care must also be taken to ensure that a lumenal redox environment that supports disulfide bond formation is maintained using the oxidizing agents GSSG or FAD. However, the extralumenal environment cannot be overly oxidizing as this will inhibit translation. Therefore, a thorough titration with the oxidizing agent is needed, and in many cases this has to be repeated for every batch of reticulocyte lysate or wheat germ or membrane preparation employed. When SP cells are used as membranes, the cell concentration may be varied for efficient translation and translocation.

Cell studies

Because pulse-chase experiments in cells involve the production of a lysate containing thousands of radiolabeled proteins, an immunoprecipitation procedure is essential to monitor a single individual protein. This often results in a high background. To optimize the signal-to-background ratio, a number of wash buffers at various temperatures and different numbers of wash steps should be tested (*UNIT 7.2*).

Care must also be taken to ensure that the adherent cells remain attached to the cell dish during the many wash steps performed with medium changes (see Basic Protocol 5). The presence of Ca^{2+} and Mg^{2+} in the wash buffer aids in cell adherence.

Monitoring protein maturation

To characterize the protein folding and oxidation process, the maturation process has to be stopped and the protein conformation maintained during the extraction and analysis procedures. An alkylating agent such as NEM, AMS, iodoacetamide, or iodoacetic acid can be used to prevent further formation of disulfide bonds. To preserve the protein's conformation during the extraction procedure, nondenaturing detergents such as CHAPS, Triton X-100, digitonin, Nonidet P-40, or octyl glucoside should be used for membrane and protein solubilization (*UNIT 5.1*). In some cases, these gentle detergents may also preserve the interactions with ER resident molecular chaperones and therefore can be used to monitor these interactions.

Anticipated Results

Regardless of the system used for the studies (adherent, suspended, or SP cells or ER-derived microsomes), a similar maturation pathway should be identified for a given protein. However, because the rate of translation with the in vitro translation system is 3- to 5-fold slower than in live cells, the time frame for maturation may vary. As a protein is oxidized, it will generally migrate faster on nonreducing SDS-polyacrylamide gels (Fig. 15.6.1). For the influenza glycoprotein hemagglutinin (HA), two folding intermediates have been identified that are designated intermediate 1 and 2 (IT1 and IT2; Fig. 15.6.3). The native (NT) protein, with all its disulfides in place, migrates at an even faster rate. Overall, a shift that corresponds to ~15 kDa is observed between the reduced (R) and completely oxidized (NT) protein on nonreducing SDS-polyacrylamide gels. On a reducing gel, all oxidative forms migrate with a similar mobility, indicating that the molecular mass of the oxidative form is the same and that the differences in mobility are due solely to disulfide bond formation. Oxidation can be followed under normal physiological conditions (co-translationally; Figs. 15.6.2 and 15.6.3), or synchronous oxidation can be monitored by accumulating the protein under reducing conditions and initiating oxidation post-translationally (Figs. 15.6.2 and 15.6.3).

The alkylation of free thiols with alkylating agents can also be used to accentuate mobility shifts observed for oxidative forms of a protein by SDS-PAGE. The larger the number of free thiols, the more alkylating groups should attach to the polypeptide, producing an enhanced shift on reducing SDS-polyacrylamide gels (Fig. 15.6.1).

Chaperone binding to the oxidative intermediates can be probed by co-immunoprecipitation studies. Interactions between the lectin chaperone calnexin and HA were analyzed by immunoprecipitating with anti-calnexin antibodies (Fig. 15.6.3B). Calnexin binds to proteins possessing monoglucosylated glycans

(*UNIT 15.1*; Fig. 15.1.3). Calnexin bound to all oxidative forms of HA under oxidizing conditions. However, under reducing conditions, the lectin-binding properties of calnexin were inhibited by the reduction of a calnexin intramolecular disulfide bond (Fig. 15.6.3B, compare lanes 4 and 5; Hebert et al., 1995). In the microsomal system at 27°C (Fig. 15.6.3), binding to calnexin persists even after 120 min of post-translational oxidation. Release from the chaperone and oligomerization can be observed if the chase temperature is increased to 32°C (Hebert et al., 1996).

Under reducing conditions, one protein band should be observed with cell studies; however, with systems that use in vitro translations, two bands should be generated for glycosylated substrates. Because protein targeting and translocation are not 100% efficient with the in vitro translation systems, a common artifact is the production of an untranslocated product. This protein retains its signal sequence and does not receive glycosylations. Therefore it migrates faster than the glycosylated and translocated form (Fig. 15.6.3).

Time Considerations

In vitro translation experiments require 1 hr for translation with chase times varying depending on the protein. For cell studies, the cells are starved for 2 hr prior to the pulse-chase experiment, which can take an additional 1 to 8 hr depending upon the chase times. SP-cell preparation requires 2 to 3 hrs. Processing of samples using both systems requires 90 min followed by an overnight (or 4 to 6 hr) incubation with antibodies for immunoprecipitations. Sample preparation, including cross-linking and protease sensitivity studies, requires ≥90 min after the antibody binding step. Immunoprecipitates can then be analyzed immediately by SDS-PAGE or frozen for future analysis.

Literature Cited

Blobel, G. and Dobberstein, B. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* 67:852-862.

Braakman, I., Hoover-Litty, H., Wagner, K.R., and Helenius, A. 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. *J. Cell Biol.* 114:401-411.

Braakman, I., Helenius, J., and Helenius, A. 1992. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. *EMBO J.* 11:1717-1722.

Chen, W., Helenius, J., Braakman, I., and Helenius, A. 1995. Cotranslational folding and calnexin binding of influenza hemagglutinin in the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 92:6229-6233.

Goldberg, M.E. 1991. Investigating protein conformation, dynamics and folding with monoclonal antibodies. *Trends Biochem. Sci.* 16:358-362.

Hebert, D.N., Foellmer, B., and Helenius, A. 1995. Glucose trimming and reglucosylation determines glycoprotein association with calnexin. *Cell* 81:425-433.

Hebert, D.N., Foellmer, B., and Helenius, A. 1996. Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. *EMBO J.* 15:2961-2968.

Wilson, R., Allen, A.J., Oliver, J., Brookman, J.L., High, S., and Bulleid, N.J. 1995. The translocation, folding, assembly and redox-dependent degradation of secretory and membrane proteins in semi-permeabilized mammalian cells. *Biochem. J.* 307:679-687.

Contributed by Edwin Francis,
Robert Daniels, and Daniel N. Hebert
University of Massachusetts
Amherst, Massachusetts

Measurements of Phagocytosis and Phagosomal Maturation

Invading microorganisms and other foreign particles, as well as apoptotic bodies, are eliminated in the organism by a process known as phagocytosis (Aderem and Underhill, 1999; Greenberg and Grinstein, 2002). The elimination process can be conceptually divided into two stages: an initial uptake phase that involves ingestion of the target particle into a plasma membrane–derived vacuole, known as the phagosome, and a secondary phase in which the particle is degraded and the debris are cleared. The nascent phagosome, which is generated by invagination of the plasmalemma, lacks the machinery required for particle degradation and clearance. These capabilities are acquired subsequently by an exquisitely coordinated sequence of membrane fusion and fission events collectively termed phagosomal maturation (Vieira et al., 2002). The objective of this unit is to briefly overview the processes of phagosome formation and maturation, and to provide protocols to assess and quantify their occurrence.

Phagocytosis is initiated by the interaction of receptors on the plasma membrane of the effector cell, or phagocyte, with their cognate ligands on the surface of the target particle. The ligands can be endogenous components of the target particle, such as lipopolysaccharides of the bacterial wall, or the phosphatidylserine that becomes exposed on the outer monolayer of apoptotic cells. However, particles lacking intrinsic ligands can also be targeted for phagocytosis by a process called opsonization, which entails coating of their surface with host-derived proteins that are themselves recognized by phagocytic receptors. Such coating host proteins, termed opsonins, include immunoglobulins, components of the complement cascade, and other serum proteins such as thrombospondin. Different receptors are responsible for the recognition of these opsonins, and they, in turn, differ from the receptors that mediate nonopsonic phagocytosis. The responses triggered by these different receptors are not identical, and one must therefore appreciate that phagocytosis is not a single phenomenon, but a term of convenience used to encompass a collection of different, yet related, processes.

At the molecular level, the sequence of events that result in particle engulfment has only been studied in some detail for a couple of receptor types. Briefly, engagement by their ligands on the particle surface induces the clustering and activation of the receptors. Though none of the phagocytic receptors studied to date possess intrinsic kinase activity, phosphorylation has been identified as the most proximal response to receptor clustering. Tyrosine kinases of the Src family are an integral part of these early events, which are rapidly followed by extensive remodeling of phospholipids in the immediate vicinity of the phagocytic cup. Kinases and lipases that target phosphoinositides are activated during the early stages of phagocytosis, as are phospholipases D and A2. Jointly, the products of tyrosine phosphorylation and lipid metabolism recruit and activate other effectors that direct two principal responses: the extensive remodelling of the actin cytoskeleton and the focal fusion of endomembranes with the membrane of nascent and early phagosomes. The cytoskeletal reorganization is complex and incompletely understood, but early activation of Rho-family GTPases is believed to be essential. In contrast, little is known about the determinants of membrane fusion and fission that initiate phagosomal maturation.

Phagosomal maturation resembles, yet is not identical to, the progression of internalized fluid or solutes along the endocytic pathway. Both processes involve sequential fusion with components of the early and late endosomal compartments and culminate in coalescence with lysosomes. It is thought that the lysosomal hydrolases play a central

Contributed by Chung-Wai Chow, Gregory P. Downey, and Sergio Grinstein
Current Protocols in Cell Biology (2004) 15.7.1-15.7.33

role in microbial killing and in the degradation and disposal of foreign particles in general. The lumen of the hybrid organelles, formed as the phagosome merges with components of the endocytic pathway, undergoes a gradual acidification that is similarly important for the microbicidal and degradative functions of the phagosome.

Simple protocols are included for the assessment of FcγR-mediated phagocytosis (see Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1), for the assessment of complement-mediated phagocytosis (see Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3), and for flow cytometric analysis of phagocytosis (see Basic Protocol 3). These protocols include steps for opsonization of target particles and for the assessment of the efficiency of phagocytosis. Additional protocols are provided for assessment of phagosomal acidification (see Basic Protocol 4 and Alternate Protocol 4) and maturation (see Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6). Finally, a protocol is provided for assessment of inhibition of phagocytosis (see Basic Protocol 6). Phagocytosis assays are illustrated schematically in Figure 15.7.1.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

ASSESSMENT OF Fcγ RECEPTOR-MEDIATED PHAGOCYTOSIS

Particles opsonized by immunoglobulins are recognized by Fcγ receptors (FcγR), that specifically interact with the Fc portion of IgG (McKenzie and Schreiber, 1998; Heyman, 2000). Focal clustering of FcγR upon binding to IgG-opsonized materials results in receptor activation and initiation of phagocytosis. There are three general classes of FcγR (FcγRI, II, and III) that vary in their molecular structure, affinity for different IgG isotypes, and signaling ability. It is noteworthy that not all FcγR are equally capable of eliciting phagocytosis. A subgroup of Fc receptors, typified by FcγRIIB, are unable to support phagocytosis and in fact generate inhibitory signals.

The protocols below detail several different methods to opsonize particles with IgG and to estimate FcγR-mediated phagocytosis. Two types of model particles, namely latex beads and red blood cells (RBC), have been extensively used to assess phagocytosis. The advantages and limitations of these systems are described below. Labeling of the particles with fluorescent probes is recommended in some of the protocols listed below to locate the particles and to quantify their uptake. A wide range of fluorophores is available for these purposes; consult the manufacturers' handbooks to select the probes with the appropriate spectral features that will best suit the particular microscopic setup and experimental design (also see *APPENDIX 1E*).

BASIC PROTOCOL 1

Assessment of FcγR-Mediated Phagocytosis Using IgG-Opsonized Red Blood Cells (RBCs)

This section describes a method to coat RBCs with IgG and to assess phagocytosis of the resulting opsonized particles. A procedure for labeling such IgG-coated RBCs by conjugation with an amine-reactive fluorescent dye to facilitate detection is included. The protocol is written for assessment of phagocytosis of the opsonized particles by adherent cells, but it can easily be adapted for use with suspended phagocytic cells.

RBCs are very well suited for the study of phagocytosis, for several reasons: (1) their size is remarkably homogeneous; (2) normal, unopsonized RBCs are rarely internalized by

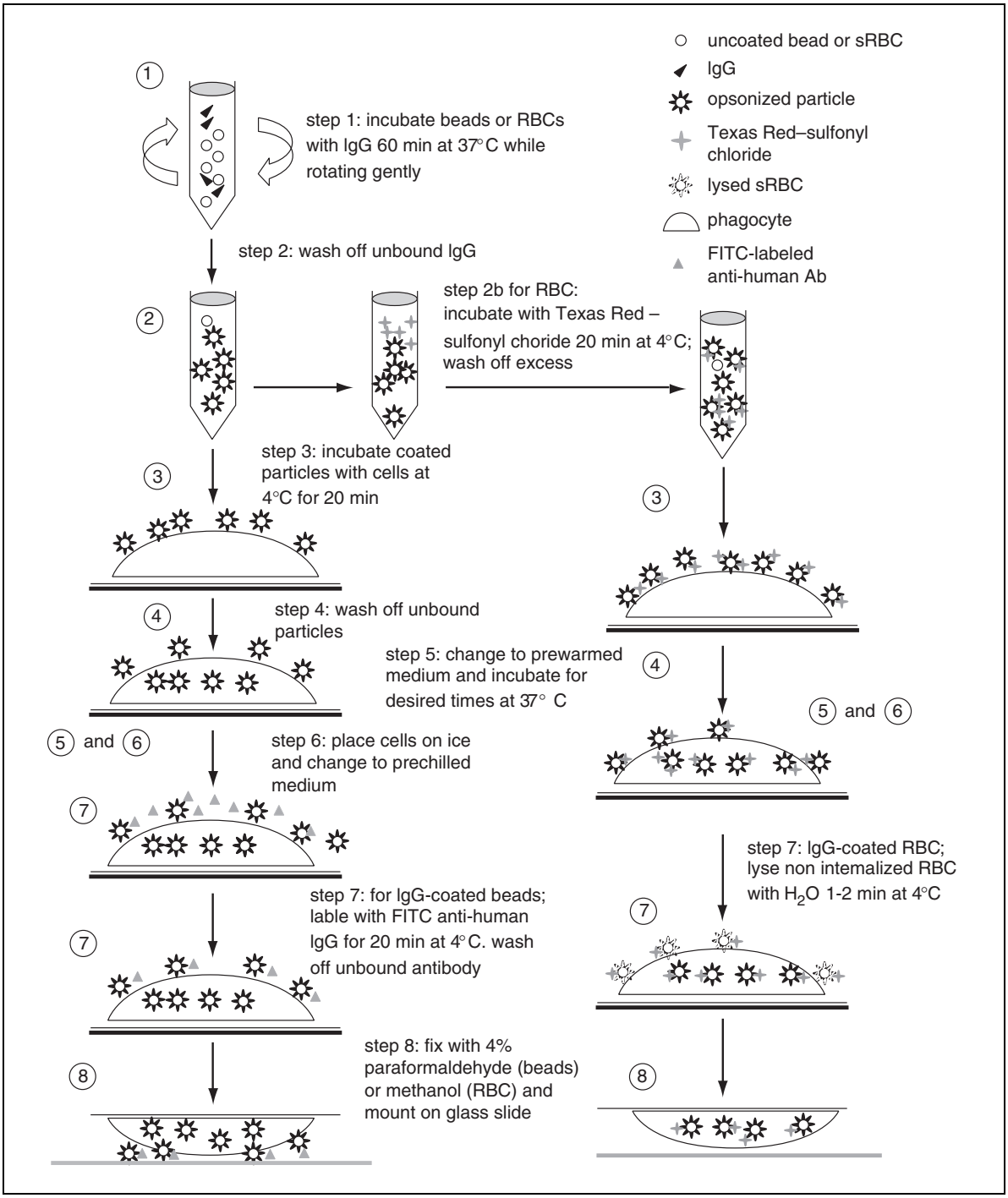


Figure 15.7.1 Schematic representation of the protocol for phagocytosis of IgG-opsonized particles. The procedure outlines the steps involved in the preparation of IgG-coated red cells or latex beads, for the identification of intracellular versus extracellular particles or for elimination of the latter prior to analysis. Note that Texas-Red labeled red cell ghosts that remain adherent (extracellular) after hypotonic lysis will be observable by fluorescence microscopy but not by bright field because they have lost hemoglobin.

phagocytosis, contributing a very low background; (3) the light absorbance of hemoglobin facilitates their detection by light microscopy; and (4) adherent, noninternalized RBCs are susceptible to osmotic lysis, while those protected within phagosomes are not. This provides a simple means of identifying internalized particles. On the other hand, RBCs are malleable and can undergo constriction and fragmentation during phagocytosis, which may lead to an underestimation of the number of phagocytic events.

Materials

Primary monocytes or macrophages (*UNIT 2.2*), or monocytic cell lines (e.g., RAW 264.7 cells ATCC #TIB-71 or J774 cells, ATCC #HB-197).

Culture medium: e.g., DMEM with or without 10% FBS (*APPENDIX 2B*); nominally bicarbonate-free, HEPES-buffered media can be used to obviate the need for a CO_2 atmosphere

Sheep red blood cells (sRBCs; 10% v/v suspension, ICN Biomedicals)

Phosphate-buffered saline (PBS; without Ca^{2+} or Mg^{2+}; see recipe)

4% (v/v) paraformaldehyde in PBS (optional)

PBS (see recipe) containing 100 mM glycine

Rabbit IgG stock: reconstitute rabbit antibody (IgG fraction) to sRBC (ICN Biomedicals) with Milli-Q water to 5 mg/ml (store in aliquots at –20°C; store thawed aliquots up to 1 month at 4°C)

HEPES-buffered RPMI 1640 (e.g., Life Technologies)

Texas Red–sulfonyl chloride (Molecular Probes, 1 mg per ampule)

Methanol, prechilled to –20°C

Fluorescence mounting medium (Dako)

6-well tissue culture plates containing sterile glass coverslips (see recipe)

End-over-end rotator

Glass microscope slides

Epifluorescence microscope with bright-field capabilities and appropriate filters (see *UNITS 4.1 & 4.2* and *APPENDIX 1E*)

Additional reagents and equipment for cell culture and counting cells (*UNIT 1.1*) and microscopy (*UNITS 4.1 & 4.2*)

Plate phagocytes (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will to reach ~75% confluence at the time of the experiment.

   The phagocytic index and rate are easier to quantitate when cells are subconfluent.

Opsonize sRBCs with IgG (day 2)

2. Prewarm the end-over-end rotator within an incubator to 37°C. Keep all reagents on ice until step 8.
3. Place a 200-µl aliquot of 10% sRBC suspension into a 1.5-ml microcentrifuge tube.
4. Sediment the sRBCs by microcentrifuging 5 to 30 sec at maximum speed.
5. Aspirate supernatant and resuspend cells in 1 ml ice-cold PBS.
6. Wash two more times with ice-cold PBS as in steps 4 to 5.
7. *Optional:* Fix sRBCs with 4% paraformaldehyde prior to opsonization.

   This has been found empirically to enhance phagocytosis, most likely by exposing ligands to receptors other than FcγR.

   a. Resuspend the sRBCs in 1 ml of 4% paraformaldehyde in PBS.
   b. Incubate overnight at 4°C in end-over-end rotor.
   c. Sediment and wash fixed cells as in steps 4 to 5. Repeat two additional times.
   d. Resuspend the fixed sRBCs in 1 ml PBS containing 100 mM glycine and incubate 20 to 30 min at 4°C to quench free aldehyde groups.
   e. Sediment fixed cells using the microcentrifuge. Aspirate supernatant. Wash twice with 1 ml PBS as in steps 4 to 5.

8. Resuspend the washed sRBCs in 200 µl PBS.

9. Dilute 1 µl of rabbit IgG stock into 50 µl of PBS. Add 4 µl of the diluted rabbit IgG to the washed sRBCs.

   The protocol has been optimized for the sRBCs and rabbit IgG from ICN Biomedicals. However, for other suppliers of sRBCs or IgG it may be necessary to optimize the opsonization ratio by varying the dilution of IgG.

10. Incubate sRBC-rabbit IgG mixture at 37°C for 1 to 1.5 hr in the prewarmed end-over-end rotator (see step 2).

To perform phagocytosis assay using unlabeled opsonized sRBCs

11a. Wash opsonized sRBCs from step 10 three times with ice-cold PBS as in steps 4 to 5, and resuspend in 500 µl ice-cold PBS. Keep on ice until use.

12a. Count the opsonized sRBCs using a Coulter counter or hemocytometer (*UNIT 1.1*) and resuspend in cold HEPES-buffered DMEM at 5–7 × 10^8 cells/ml.

13a. Proceed to phagocytosis assay (step 14).

To perform phagocytosis assay using opsonized sRBCs labeled with Texas Red–sulfonyl chloride

11b. Take one ampule of Texas Red–sulfonyl chloride and dissolve in 0.5 ml ice-cold PBS in a 1.5-ml microcentrifuge tube.

   Covered with aluminum foil and stored at 4°C, this stock solution will keep for a several days.

12b. Add 100 µl of the diluted Texas Red–sulfonyl chloride to 0.5 ml of opsonized sRBCs (from step 10). Incubate on ice for 20 min. Mix by inverting the tube every 5 min.

   Texas Red–sulfonyl chloride is an amine-reactive dye that labels proteins covalently. As an alternative, IgG prelabeled with the fluorescent dye of choice can be used for opsonization.

13b. Wash the IgG-sRBC-dye mixture three times with 1 ml cold PBS, or until the supernatant is clear, using the technique described in step 4 to 5. Resuspend in 0.6 ml cold HEPES-buffered DMEM as in step 12a. Proceed to phagocytosis assay (step 14).

Perform phagocytosis assay (day 2)

14. Place the tissue culture plates with the prepared cells (from step 1) on ice.

   Optionally, replace the culture medium with 1 ml fresh, prechilled medium to accelerate the cooling process.

15. For each well to be assayed, prewarm 2 ml of HEPES-buffered RPMI 1640 medium to 37°C.

16. Add 50 to 100 µl of prepared IgG-sRBCs (from step 13a or b) to each well and incubate on ice for 10 to 20 min.

   Ideally, the ratio of IgG-sRBC to phagocytic cells should be between 5:1 and 10:1.

   Phagocytosis does not proceed at 4°C. This step will therefore standardize and synchronize the binding of IgG-sRBC to the cells and allow for comparison of different treatment conditions or at different time points.

17. Wash cells gently once with cold medium to remove unbound IgG-sRBC.

18. To initiate phagocytosis, remove cells from ice, aspirate the cold medium, and add 2 ml prewarmed (37°C) DMEM medium per well. Place in 37°C incubator.

    The duration of incubation at 37°C will depend on the stage of phagocytosis and phagosomal maturation of interest. For assessment of phagocytic efficiency, 30 min is a convenient point for evaluation.

19. Stop phagocytosis by placing the cells back on ice. Wash with ice-cold PBS.

20. *Optional:* To remove the IgG-sRBCs that have not been internalized, lyse the sRBCs by incubating with 1 ml of water for 2 min at 4°C.

    Depending on the cell type being assessed, incubation with water may affect cell morphology and/or integrity. When evaluating different phagocytes, a preliminary time course experiment may be necessary to optimize the duration of hypotonic lysis of sRBC.

21. Wash again with cold PBS. Keep on ice.

    Wet mounts of the preparation can be analyzed directly or the cells can be fixed for subsequent analysis. For direct analysis proceed to step 23. For fixation see step 22.

Fix cells (days 2 to 3)

22. Aspirate PBS and add 1 ml per well of prechilled methanol. Incubate on ice 20 min. Wash twice with PBS.

    It is important to aspirate gently and add the methanol slowly to the side of the well to avoid dislodging any cells. The authors recommend prechilling the methanol to –20°C. An alternative to fixation with methanol is to fix with 1% to 2% glutaraldehyde. When using sRBCs, do not use paraformaldehyde for fixation because retention of hemoglobin is poor.

Mount coverslips and score phagocytosis

23. Mount coverslips containing cells onto slides with Dako fluorescence mounting medium. Allow to dry at room temperature for several hours prior to visualization.

    It is preferable to dry the slides overnight before analysis.

    Dab the coverslip on tissue wipe to remove excess fluid before mounting. This will decrease the time required for the slide to dry. To minimize air bubbles in the mounted slide, place a drop of the mounting medium on the glass slide first, then slowly ease the coverslip onto the slide. Slides can be stored for months at –20°C with little loss of fluorescence.

24. Score phagocytosis under microscope.

    Only the internalized sRBC remain intact and can be readily identified by bright-field (phase or differential interference contrast) microscopy. Typically, phagocytic efficiency is scored as the number of sRBC internalized per 100 phagocytic cells counted.

    Fluorescence microscopy and automated image analysis can be used if the SRBC are coupled to Texas-Red.

ALTERNATE PROTOCOL 1

Assessment of FcγR-Mediated Phagocytosis Using IgG-Opsonized Latex Particles

A second approach to evaluating FcγR-mediated phagocytosis utilizes inert latex beads opsonized with IgG. The advantages of beads over sRBC include their uniform (spherical) shape, which allows for ready identification of phagosomes on visual inspection or when using automated image analysis programs, and the availability of varying bead sizes. However, uniform-size latex beads that are appropriate for phagocytosis experiments are considerably more expensive than RBCs. Furthermore, unlike sRBCs, extracellular beads are not amenable to hypotonic lysis for elimination. Therefore, if it is necessary to distinguish internalized versus noninternalized adherent beads by alternative means, the experiment will consume additional time and reagents. Lastly, plain, unopsonized latex beads can be recognized and engulfed by phagocytes at significant rates, complicating the analysis of the contribution of FcγR.

Additional Materials *(also see Basic Protocol 1)*

10% slurry of latex beads, ~3-μm diameter (Bangs Laboratories cat no. PS05N or equivalent from other manufacturers)

Human IgG stock solution: reconstitute IgG from human serum (Sigma) to 50 mg/ml in PBS (see recipe) or Milli-Q purified H_2O (store in aliquots at –20°C; store thawed aliquots up to 1 month at 4°C)

Fluorescently labeled anti-human IgG secondary antibody (e.g., Cy3- or FITC-labeled anti-human IgG $F(ab)_2$, Jackson Laboratories), diluted 1/500 in PBS (see recipe) containing 1% (w/v) BSA and 5% (v/v) goat serum

15-ml polystyrene tubes, sterile

End-over-end rotator

Tabletop centrifuge

Prepare cells (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will to reach ~75% confluence at the time of the experiment.

Opsonize beads with IgG (day 2)

2. Place the following in a sterile 15-ml polystyrene tube:

   3 ml PBS
   60 μl bead suspension
   60 μl human IgG stock solution.

   The volumes given are sufficient for an experiment using one 6-well plate. They can be scaled up or down depending on the number of wells being assessed.

3. Incubate 60 min at 37°C, rotating gently in an end-over-end rotator.

4. Sediment beads by centrifuging 10 min at 750 × *g* (2500 rpm), 4°C, in a tabletop centrifuge. Aspirate supernatant, being careful not to aspirate the beads.

5. Resuspend beads in 5 ml cold PBS, centrifuge again as in step 4, and aspirate supernatant. Repeat this washing procedure a second time.

6. Resuspend beads in 3 ml cold HEPES-buffered DMEM. Keep on ice until ready to use.

Perform phagocytosis assay (day 2)

7. Prewarm culture medium at 37°C (to be used in step 12).

8. Put prepared plate of cells (from step 1) on ice.

   One may wish to exchange the culture medium with prechilled medium just prior to this step.

9. Add 0.5 ml of the IgG-opsonized bead suspension (from step 6) per well.

10. Incubate on ice for 20 min to allow beads to bind.

11. Wash off excess beads with cold PBS.

    The excess beads can be washed three times with PBS and reused. When stored at 4°C with 0.01% sodium azide, the beads can be used for 2 to 3 months.

12. Remove the plate from ice. Aspirate medium and add 2 ml of 37°C prewarmed culture medium per well.

13. Incubate at 37°C for the desired time periods.

14. Stop phagocytosis by placing plates on ice and changing to ice-cold medium or PBS.

Label extracellular opsonized beads

15. To label beads that are adherent to the cells but not internalized, incubate 20 min on ice with a fluorescently labeled anti-human IgG secondary antibody.

    Cover the plate with foil to minimize bleaching of the fluorophore during the incubation procedure.

    The authors typically use Cy3- or FITC-labeled anti-human IgG $F(ab)_2$ (Jackson Laboratories). Dilute antibody 1:500 in PBS (see recipe) containing 1% (w/v) BSA and 5% (v/v) goat serum.

Fix cells (days 2 to 3)

16. Wash three times with PBS.

17. Fix the cells by covering them with 4% paraformaldehyde in PBS. Incubate 20 min at room temperature. Wash three times with PBS.

18. *Optional:* Quench free aldehyde groups with PBS containing 100 mM glycine in PBS for 20 min. Wash three times with PBS.

19. Mount coverslips containing cells onto slides with Dako fluorescence mounting reagent. Allow to dry at room temperature for several hours prior to visualization.

    Slides can be stored at –20°C in the dark for months with little loss of fluorescence intensity.

20. Score phagocytosis under microscope.

    The phagocytic index is generally defined as the number of particles internalized per 100 cells located.

SUPPORT PROTOCOL 1

Assessment of Surface Expression and Binding Capacity of Fcγ

The surface expression and the binding affinity of the FcγR for their ligands will determine the efficiency and rate of phagocytosis. For many experiments (e.g., comparison of phagocytic rates under different treatment conditions or in phagocytes with different genotypes), it is important to determine the surface expression and binding capacity of the FcγR in order to draw valid conclusions. Receptor density and phagocytic efficiency can then be directly compared.

Additional Materials *(also see Basic Protocol 1 and Alternate Protocol 1)*

FcγR-specific antibody: e.g., rat anti-mouse CD 16/CD32 (BD Bioscience)
Fluorescently labeled secondary antibody (Jackson Immunoresearch Labs)

To assess Fcγ receptor expression and binding using IgG-opsonized sRBC

1a. Follow steps 1 to 16 of Basic Protocol 1.

2a. After the 20-min incubation on ice, wash wells twice with ice-cold PBS.

3a. Fix with methanol and mount as in Basic Protocol 1, step 22-24.

4a. Score the number of bound sRBCs per phagocytic cell.

To assess Fcγ receptor expression and binding using IgG-opsonized beads

1b. Follow steps 1 to 12 of Alternate Protocol 1.

2b. Fix with 4% paraformaldehyde as in Alternate Protocol 1, steps 17 to 19.

3b. Score the number of bound beads per cell.

To assess Fcγ receptor expression and binding using FcγR-specific antibodies

1c. Label surface receptors by incubating 60 min at 4°C with 0.5 ml of FcγR-specific antibody diluted 1:10 PBS containing 1% BSA.

Antibodies directed against several classes of human as well as murine FcγR are commercially available and can be used to assess surface expression of the these receptors. If using these antibodies to label surface receptors, perform the incubations at 4°C to prevent internalization of the receptors by endocytosis.

2c. Wash twice with PBS.

3c. Mount using Dako fluorescent mounting medium and evaluate expression by counting the traction of fluorescently labeled cells using microscopy or flow cytometry.

ASSESSMENT OF COMPLEMENT-MEDIATED PHAGOCYTOSIS

Activation of the complement cascade leads to cleavage of the normally inactive complement proteins into several functional fragments. Some of these complement fragments function as opsonins which attach to microorganisms or particles, promoting their internalization via complement receptor (CR)–mediated phagocytosis. Complement-dependent phagocytosis is mediated primarily by the complement receptor CR3 (also known as CD11b/CD18 or Mac1). Unlike FcγR-mediated phagocytosis, where binding of the ligand to its cognate receptor is sufficient to initiate phagocytosis, CR3-mediated phagocytosis requires an additional stimulus to render the CR competent for phagocytosis (Newman et al., 1985; Aderem and Underhill, 1999). Success of experiments involving complement-opsonized particles depends on the optimal deposition of the C3bi component of complement onto the particles and on suitable priming of the phagocytic cells to capacitate the CR.

Assessment of Complement-Mediated Phagocytosis Using C3bi-Opsonized Erythrocytes

BASIC PROTOCOL 2

The protocol below describes a method to attach C3bi to RBCs. This method relies on binding of IgM to activate the complement cascade on the particle surface by the classical pathway. Under the conditions described, C3b fragments are rapidly deposited onto RBCs and are almost completely converted to C3bi (Newman and Mikus, 1985).

Materials

Primary monocytes or macrophages (*UNIT 2.2*) or monocytic cell lines (e.g., RAW 264.7 cells ATCC #TIB-71 or J774 cells, ATCC #HB-197)

Culture medium: e.g., DMEM with or without 10% FBS (*APPENDIX 2B*); nominally bicarbonate-free, HEPES-buffered media can be used to obviate the need for a CO_2 atmosphere

Sheep red blood cells (sRBCs; 10% v/v suspension, ICN Biomedicals)

Phosphate-buffered saline (without Ca^{2+} or Mg^{2+}; see recipe)

Gelatin veronal buffer (GVB; Sigma)

Rabbit anti-sheep erythrocyte IgM (Cedarlane or Accurate Chemical): reconstitute lyophilized antibody in 1 ml H_2O (final concentration 1 mg/ml) and store aliquots at –20°C (avoid repeated freeze-thaw cycles)

C5-deficient human serum (Sigma)

GVB (Sigma) supplemented with 1% (w/v) BSA and 7.5% (v/v) goat serum (optional)

Goat anti-C3bi antibody (optional; Sigma)

GVB (Sigma) supplemented with 5% (w/v) BSA and 7.5% (v/v) donkey serum (optional)

FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Labs; optional)
HEPES-buffered DMEM (e.g., Life Technologies)
20 mM phorbol 12-myristate 13-acetate (PMA) in DMSO
Methanol, prechilled to –20°C
Fluorescence mounting medium (Dako)

6-well tissue culture plates containing sterile glass coverslips (see recipe)
End-over-end rotator
Glass microscope slides
Epifluorescence microscope with bright-field capabilities and appropriate filters to visualize FITC fluorescence (see *UNITS 4.1 & 4.2* and *APPENDIX 1E*)

Additional reagents and equipment for cell culture and counting cells (*UNIT 1.1*) and microscopy (*UNITS 4.1 & 4.2*)

Plate phagocytes (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will to reach ~75% confluence at the time of the experiment.

Opsonize sRBCs with IgG (day 2)

2. Prewarm the end-over-end rotator within an incubator to 37°C. Keep all reagents on ice until step 8.
3. Place a 200-µl aliquot of 10% sRBC suspension into a 1.5-ml microcentrifuge tube.
4. Sediment the sRBCs by microcentrifuging 5 to 30 sec at maximum speed.
5. Aspirate supernatant and resuspend cells in 1 ml ice-cold PBS.

   It is best to use freshly opsonized sRBC for the phagocytosis assay. Keep all reagents on ice until step 8.

6. Wash two more times, first with ice-cold PBS and then with 1 ml of cold GVB, using the technique described in steps 4 to 5. After the last wash, resuspend the sRBCs in 200 µl GVB. Count cells using a hemocytometer (*UNIT 1.1*).

   Use of GVB minimizes aggregation and spontaneous lysis of the cells.

7. Dilute 1 µl of rabbit anti-sheep IgM stock in 50 µl of GVB. Add 2 µl of the diluted IgM to the sRBC suspension.

   It may be necessary to determine the optimal sub-agglutinating dilution for individual lots of IgM.

8. Incubate sRBC-rabbit IgM at 37°C for 1 hr in the prewarmed end-over-end rotator (see step 2).
9. Wash sRBCs three times with ice-cold GVB using the technique described in steps 4 to 5, then resuspend in 225 µl cold GVB. Keep on ice.
10. Add 25 µl of C5-deficient human serum (for a final concentration of 10% v/v) and incubate at 37°C for 20 min. Wash three times in GVB using the technique described in steps 4 to 5.

    Under these conditions, the Fc region of the IgM pentamer activates the classical pathway of the complement cascade and deposits C3b to the IgM-coated sRBCs, where it is rapidly converted to C3bi (Newman and Mikus, 1985). While IgG-coated RBCs can also be used to opsonize particles with C3bi under comparable conditions, IgG is not an efficient activator of the complement cascade because two adjacent Fc regions are required to initiate the first step of the cascade. Furthermore, the presence of IgG could confound subsequent interpretation of phagocytosis data due to concomitant engagement of the Fcγ receptors. Use of C5-deficient serum prevents lysis of the red blood cells.

11. *Optional:* Assess opsonization efficiency using immunofluorescence.

    This step is recommended for each lot of sRBC and IgM used.

    a. Take a small aliquot of the opsonized sRBC and resuspend in 200 µl of GVB supplemented with 1% BSA and 7.5% donkey serum.

    b. Add goat anti-C3bi antibody to a final dilution of 1:100. Incubate for 20 min on end-over-end rotator at 4°C. Wash three times with GVB.

    c. Counterstain with a 1:100 dilution of FITC-conjugated donkey anti-goat IgG in GVB containing 5% bovine serum albumin and 7.5% donkey serum for 20 min.

    d. Observe under a fluorescence microscope using the appropriate filters for the fluorochrome (*APPENDIX 1E*).

    A positive reaction will yield obvious, bright fluorescence.

12. Resuspend the opsonized sRBC in HEPES-buffered DMEM to yield 5–7 × 10^8 cells/ml

Perform phagocytosis assay (day 2)

13. Add 50 to 100 µl of C3bi-opsonized sRBC to each well of the tissue culture plates with the cells (step 1) and allow binding to proceed for 20 min at 37°C. Use 5 to 10 C3bi-coated sRBCs per phagocytic cell for optimal assessment of phagocytosis.

    C3bi binds to CR3 poorly at 4°C. At 37°C, resting cells will bind C3bi but will not initiate CR3-mediated phagocytosis.

14. To activate CR3 and initiate phagocytosis, add PMA (as 20 mM stock) to each well for a final concentration of 150 ng/ml and incubate at 37°C for the desired time period.

    In resting leukocytes, CR3 is inactive, although capable of binding C3bi, and requires an additional signal for activation and initiation of phagocytosis. A number of different stimuli can activate CR3. Phorbol esters such as PMA activate CR3 by stimulation of protein kinase C (Wright et al., 1983). Other stimuli that lead to activation of CR3 include certain cytokines and chemokines, attachment to extracellular matrix proteins, and glucan binding.

15. Stop phagocytosis by placing the cells on ice. Wash with ice-cold PBS.

    The duration of incubation at 37°C will depend on the stage of phagocytosis and phagosomal maturation that are of interest.

16. *Optional:* To remove the C3bi-sRBCs that have not been internalized, lyse the sRBC by incubating with 1 ml of water for 2 min.

17. Wash again with cold PBS. Keep on ice.

Fix cells

18. Aspirate PBS and add 1 ml per well of prechilled methanol. Incubate on ice for 20 min. Wash twice with PBS.

19. Mount coverslips containing cells onto slides with Dako fluorescence mounting medium. Allow to dry at room temperature for several hours prior to visualization.

    It is preferable to dry the slides overnight before analysis.

    Dab the coverslip on tissue wipe to remove excess fluid before mounting. This will decrease the time required for the slide to dry. To minimize air bubbles in the mounted slides, place a drop of the mounting medium on the glass slide first, then slowly ease the coverslip onto the slide. Slides can be stored for months at –20°C with little loss of fluorescence.

20. Score phagocytosis under microscope.

    Only the internalized sRBC remain intact and can be readily identified by bright-field (phase or differential interference contrast) microscopy. Typically, phagocytic efficiency is scored as the number of sRBC internalized per 100 phagocytic cells counted.

ALTERNATE PROTOCOL 2

Assessment of Complement-Mediated Phagocytosis Using C3bi-Opsonized Latex Beads

CR-mediated phagocytosis can also be assessed using complement-opsonized latex beads. This protocol and Alternate Protocol 3 below describe the opsonization and fluorescent labeling of beads for assessment of phagocytosis.

Coating of latex beads with C3bi is less complicated than the similar procedure using erythrocytes (see Basic Protocol 2). Furthermore, the uniform sizes of the beads allows for easy identification of phagosomes and, because lysis is not a concern, C5-deficient serum is not required. However, latex beads are significantly more expensive than sRBCs.

Additional Materials *(also see Basic Protocol 2)*

10% slurry of latex beads, ~3-μm diameter (Bangs Laboratories cat no. PS05N or equivalent from other manufacturers)
1 mg/ml human IgM stock (Sigma; store in aliquots at –20°C)
Human or mouse serum, freshly isolated, diluted 1:1 in PBS (see recipe for PBS)
Hanks' balanced salt solution (HBSS; see recipe)
20 mM phorbol 12-myristate 13-acetate (PMA) in DMSO
Goat anti-human C3bi antibody (Sigma)
PBS (see recipe) supplemented with 1% (w/v) BSA and 7.5% (v/v) goat serum (optional)
FITC-conjugated donkey anti-goat IgG (optional)
4% (v/v) paraformaldehyde in PBS (optional)
PBS (see recipe) supplemented with 1% (w/v) BSA and 7.5% (v/v) donkey serum (optional)
PBS (see recipe) containing 100 mM glycine (optional)
15-ml polystyrene tubes, sterile

Plate phagocytes (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will to reach ~75% confluence at the time of the experiment.

Opsonize beads (day 2)

2. Place the following in a sterile 15-ml polystyrene tube:

   3 ml PBS
   60 μl bead suspension
   60 μl IgM stock solution.

3. Incubate 60 min at 37°C, rotating gently in an end-over-end rotator.
4. Sediment beads by centrifuging 10 min at 750 × *g* (2500 rpm), 4°C, in a tabletop centrifuge. Aspirate supernatant, being careful not to remove the beads.
5. Resuspend beads in 5 ml cold PBS, centrifuge again as in step 4, and aspirate supernatant. Repeat this washing procedure a second time.
6. Resuspend beads in freshly isolated human or mouse serum diluted 1:1 in PBS. Incubate at 37° for another 20 min.

   During this step, the Fc region of IgM will initiate the classical pathway of complement cascade and fix C3b to the beads where it is quickly and completely converted to C3bi (Newman and Mikus, 1985).

7. At the end of the incubation period, collect beads by microcentrifugation and wash with HBSS using the technique described in steps 4 and 5. Resuspend in 3 ml HEPES-buffered DMEM.

Perform phagocytosis assay (day 2)

8. Add 500 µl of the C3bi-beads from step 7 to each well of the tissue culture plates with the cells (see step 1) and allow binding to proceed for 20 min at 37°C.
9. To activate CR3 and initiate phagocytosis, add PMA (as 20 mM stock) to each well for a final concentration of 150 ng/ml and incubate at 37°C for the desired time period.
10. Stop phagocytosis by placing the cells on ice. Wash with ice-cold PBS.
11. *Optional:* To identify C3bi-beads that are bound but not internalized, label the external beads with anti-C3bi antibodies on ice as follows:
    a. Incubate each well with a 1:100 dilution of goat anti-C3bi antibody in 200 µl PBS supplemented with1% BSA and 7.5% donkey serum for 20 min.
    b. Wash three times with ice-cold PBS.
    c. Incubate with a 1:100 dilution of FITC-conjugated donkey anti-goat IgG in 200 µl PBS with 1% BSA and 7.5% donkey serum for 20 min
    d. Wash three times with ice-cold PBS.

Fix cells and score phagocytosis (day 2)

12. Aspirate PBS and add 1 ml of 4% paraformaldehyde in PBS per well. Incubate on ice for 20 min. Wash twice with PBS.
13. *Optional:* Add 1 ml of PBS containing 100 mM glycine to each well and incubate 15 to 30 min at 4°C to quench the free aldehyde groups. Wash three times with PBS.
14. Mount cells using fluorescent mounting medium and score phagocytosis efficiency by counting the fraction of fluorescently labeled cells using microscopy or flow cytometry.

C3bi Opsonization of Beads

ALTERNATE PROTOCOL 3

It is possible to skip the IgM adsorption step and deposit C3bi onto the beads by incubating directly with fresh human or mouse serum. This requires a longer incubation period in serum to allow for the complement cascade and fixation of C3bi to the beads to proceed. It is recommended that efficiency of C3bi deposition be verified by immunofluorescence before proceeding with the phagocytosis assay.

Additional Materials *(also see Alternate Protocol 2)*

Human or mouse serum, freshly isolated

At step 2 of Alternate Protocol 2, place the following in a sterile 15-ml polystyrene tube instead of the items indicated in that protocol:

1.5 ml PBS
1.5 ml human or murine serum
60 µl bead suspension.

Incubate 60 min at 37°C with rotation. At the end of the incubation period in step 3, continue with Alternate Protocol 2, starting from step 7.

BASIC PROTOCOL 3

ASSESSMENT OF PHAGOCYTOSIS USING FLOW CYTOMETRY

In addition to the conventional microscope-based techniques described above, phagocytosis of microorganisms and particles can be measured using flow cytometry (Bassoe and Solberg, 1984; Santos et al., 1995; Oben and Foreman, 1988; Lehmann et al., 2000). The principle of the flow cytometric technique for assessment of phagocytosis is relatively straightforward. After allowing phagocytic cells to ingest fluorescent particles (e.g., labeled microorganisms or particles), cell-associated fluorescence is quantified using flow cytometry. If the fluorescent phagocytic "prey" is evenly labeled, the amount of cell-associated fluorescence is directly proportional to the number of ingested particles, thus allowing a calculation of the phagocytic index. This technique has several advantages over the conventional techniques (Harvath and Terle, 1999), but also some limitations that are discussed below, along with several strategic considerations.

Distinguishing Particle Attachment to the Cell Surface From Internalization ("In" From "On")

A key technical issue is the necessity to quantify only internalized particles, while excluding extracellular adherent ones. This is generally accomplished by minimizing extracellular fluorescence using impermeant quenching agents. Many fluorophores can be quenched by compounds such as trypan blue, ethidium bromide, or iodoacetate that, under optimal conditions, are able to dampen surface fluorescence by up to 95% without penetrating the plasma membrane (Steinkamp et al., 1982; Bassoe and Solberg, 1984). In addition, many fluorophores including FITC, Alexa Fluor 488, BODIPY FL, Oregon Green, tetramethylrhodamine, Texas Red, and Cascade Blue dyes can be quenched by specific antibodies. It should be emphasized that quenching is not an all-or-none phenomenon and that proper controls must be carried out to ensure that there is satisfactory separation of the internalized and adherent particle populations. It is also important to note that not all fluorophores can be quenched in this manner, and it is essential to ascertain that untested dyes can be quenched efficiently. In addition, the florescence of synthetic beads that are synthesized with the fluorophore incorporated within the polymer cannot be quenched in this manner.

Sensitivity To pH

It is important to consider that many fluorophores are sensitive to environmental factors—notably intracellular pH—that can affect the signal of the fluorescent probe. In the case of phagocytosis, this is particularly important because, as the phagosome matures, the luminal pH decreases, thus quenching the fluorescence of dyes such as FITC. This can result in artifactual underestimation of the phagocytic efficiency. Fluorophores such as those of the Alexa and BODIPY series, tetramethylrhodamine, and Texas Red exhibit much less quenching over a wide pH range (from pH 4 to 9), and they are much less susceptible to artifactual quenching.

On the other hand, one can take advantage of the pH sensitivity of some probes to discriminate between internalized and externally adherent particles. As described in more detail elsewhere, it is also possible to tag phagocytic targets with pH-sensitive dyes such as FITC, SNARF, SNAFL, or Oregon Green (Molecular Probes) that undergo spectral shifts or intensity changes at the acidic pH found within phagosomes. If the appropriate pH-sensitive wavelength is selected for detection, the fluorescence intensity will be proportional to phagocytosis and can be used to quantify internalized particles by flow cytometry.

Selection of Phagocytic Targets

Phagocytic targets suitable for flow cytometry include bacteria (*E. coli* and *Staphylococcus aureus*), zymosan (*Saccharomyces cerevisiae*), fungi (*Aspergillus*), and latex beads. These targets are usually opsonized with IgG or complement fragments (see Basic Protocols 1 and 2 and Alternate Protocols 1 and 2), but many present endogenous ligands such as β-glucan and other complex carbohydrates that can initiate or at least influence phagocytosis. These factors must be taken into consideration when selecting the most appropriate target and when comparing the efficiency of phagocytosis between different targets.

The protocol below delineates several different methods to assess phagocytosis by flow cytometry using bacteria, yeast, or synthetic beads.

Materials

Zymosan A (from *S. cerevisiae*; Sigma)
Phosphate-buffered saline (PBS; see recipe), pH 7.4
0.1 M sodium carbonate, pH 9.3
1 mg/ml solution of reactive fluorophore, e.g., fluorescein isothiocyanate (FITC) *or* Oregon Green 514 succinimidyl ester, in 0.1 M sodium carbonate, pH 9.3
Bacteria of interest as phagocytic target (e.g., *E. coli* K-12 strain)
1 mg/ml fluorescein isothiocyanate (FITC) in PBS (see recipe), pH 8.0
1% (v/v) glutaraldehyde in PBS (see recipe for PBS)
PBS (see recipe) containing 100 mM glycine
Phagocytic cells of interest: cultured cell lines or primary isolates of peripheral blood neutrophils or monocytes (e.g., *UNIT 2.2*)
PBS (see recipe) containing 0.02% EDTA
PBS (see recipe) containing 1.25 mg/ml trypan blue
0.25% trypsin/EDTA *or* nonenzymatic cell dissociation solution (both available from Sigma)
Quenching antibody to fluorophore (*e.g.,* anti-FITC/Oregon Green Cat# A-889; anti-Alexa Fluor 488 Cat # A-11094 Molecular Probes)

Bath sonicator (e.g., Bransonic 1200 Ultracleaner, Branson)
Rotating shaker
Polypropylene tubes for use with flow cytometer
24- or 96-well tissue culture plates (Polypropylene Cluster, Costar)
Flow cytometer

Additional reagents and equipment for flow cytometry (e.g., Robinson et al., 2003)

Prepare fluorophore-coupled phagocytic particles

For zymosan A

1a. Suspend 10 to 20 mg of dried zymosan A in 10 ml PBS. Vortex vigorously and boil for 10 min.

2a. Wash three times in 1 ml PBS by resuspension followed by centrifugation 1 min at 3000 × *g* and resuspend in PBS. Sonicate in bath sonicator to disperse aggregates.

3a. Sediment zymosan A by centrifuging 1 min at 3000 × *g*, 4°C, remove supernatant, and resuspend pellet in 0.1 M sodium carbonate, pH 9.3, at a final concentration of 50 mg/ml.

4a. Initiate labeling reaction by adding 0.1 mg of fluorophore (as 1 mg/ml solution) to 1 mg of suspended zymosan particles. Allow reaction to proceed 4 hr at room temperature with constant mixing.

Reactive fluorophores (e.g., fluorescein isothiocyanate and the succinimidyl ester of Oregon Green 514) are dissolved in 0.1 M sodium carbonate at 1 mg/ml.

5a. Remove unbound probe by centrifugation for 1 min at 3000 × *g*, 4°C, discard the supernatant, and resuspend product in 0.5 ml of dimethylsulfoxide with 3 drops of PBS, pH 7.4. Sonicate to disperse the zymosan. Repeat centrifugation, resuspension, and sonication 2 to 3 more times while adding progressively more PBS until the supernatant is completely clear.

6a. Dissolve the final pellet in 1 ml PBS.

This can be frozen in aliquots or stored at 4°C in 2 mM sodium azide for several weeks (make certain to wash off the azide before using for phagocytosis assays, as azide inhibits phagocytosis).

For bacteria

1b. Transfer 5.0×10^8 bacteria from overnight culture to a microcentrifuge tube.

2b. Sediment by microcentrifuging 1 min at maximum speed and wash once by resuspension in PBS and resedimentation.

3b. Resuspend cells in 1 ml of 1 mg/ml FITC in PBS, pH 8.0. Incubate at room temperature on an end-over-end rotator for 25 min.

4b. Wash in PBS as in 2b and fix in 1 ml of 1% glutaraldehyde in PBS for 20 min.

5b. Quench free aldehyde groups by incubating in 0.5 ml of PBS containing 100 mM glycine for 10 min at room temperature.

6b. Wash as in 2b and resuspend in 500 µl sterile PBS. Keep on ice until use the same day.

Initiate phagocytosis

Using the labeled zymosan A particles or bacteria from step 6a or 6b, respectively, proceed with steps 7a to 11a if studying phagocytosis by cells in suspension (e.g., neutrophils or monocytes) or proceed with steps 7b to 1b if studying phagocytosis by adherent cells.

Phagocytosis assays for cells in suspension

7a. To a 1.5-ml microcentrifuge tube, add 100 µl of a suspension of 5×10^6 phagocytic cells/ml, prewarmed to 37°C. Next, mix with 100 µl of a suspension of 5×10^7 particles/ml of the labeled phagocytic target from step 6a or b.

It is crucial to ensure good contact between the phagocytes and their prey, bearing in mind that while adherent phagocytes can crawl, suspended cells cannot swim.

8a. To bring phagocytic cells and target particles into close proximity, cosediment the cells and beads by a rapid microcentrifugion (15 sec at 70% maximum speed or ~8000 rpm).

The cell-to-particle ratio should be optimized for the specific cell type, but it is usually ~1:10 (range 1:5 to 1:100).

9a. Incubate the cells at 37°C for the appropriate length of time. Terminate phagocytosis by adding 1 ml ice-cold PBS containing 0.02% EDTA. Keep samples on ice.

The incubation time for maximum phagocytosis should be optimized for the individual cell type and can vary from 10 to 15 min for human neutrophils to 30 to 40 min for monocytes.

10a. Dilute samples 1:5 with PBS containing 1.25 mg/ml trypan blue. Incubate at 4°C for at least 2 min to allow efficient quenching of fluorophore. Maintain the samples at 4°C in trypan blue–containing solution until analysis.

11a. Harvest cells using a micropipettor and transfer to a polypropylene tube suitable for use in the flow cytometer.

If there will be a delay before analysis, fix cells with 2% paraformaldehyde. Fluorescence will be stable for at least 24 to 48 hr in fixed cells.

For adherent cells

7b. Add labeled particles (from step 6 a or b; 5 to 10 per cell) to adherent phagocytes growing in wells of a 24-well tissue culture plate. Incubate for 10 to 60 min at 37°C.

8b. Terminate phagocytosis by adding 200 µl per well of ice-cold PBS containing 0.02% EDTA.

9b. Remove the cells from the surface using 0.25% trypsin/EDTA or nonenzymatic cell dissociation solution. Pipet repeatedly yet gently to ensure dispersal into a single-cell suspension.

10b. If needed, filter through a mesh filter to remove clumps of cells

One risks the wrath of the flow cytometry facility if one clogs up the aspiration port of the cytometer.

11b. Keep samples on ice and dilute 1:5 with PBS containing 1.25 mg/ml trypan blue. Transfer to a polypropylene tube suitable for use with the flow cytometer and analyze immediately.

If there will be a delay, fix cells with 2% paraformaldehyde. Fluorescence will be stable for at least 24 to 48 hr in fixed cells.

Analyze by flow cytometry

12. Aspirate cells into the chamber of the flow cytometer and collect a minimum of 10,000 cells in the viable cell gate (also see Robinson et al., 2003).

Flow cytometers with one or several lasers are available from several commercial sources. the authors use either a BD Biosciences FACSCalibur or a Beckman-Coulter Elite.

The fluorescence measurements collected in the individual fluorescence channels are displayed on log scales. The proportion of cells that performed phagocytosis is estimated as the percentage of viable cells that have target fluorescence exceeding the background values (van Eeden et al., 1999).

When using evenly labeled fluorescent beads, the mean number of beads per phagocytic cell can be calculated by dividing the mean target fluorescence in the appropriate gate by the fluorescence of a single extracellular bead. The latter value is estimated separately using pure beads.

DETERMINATION OF PHAGOSOMAL pH

Acidification of the phagosomal lumen is apparent shortly after sealing of the phagosomal cup. As in the case of endosomes, accumulation of H^+ is thought to result from vectorial pumping by vacuolar-type (V) ATPases (Demaurex, 2002), which are delivered to the membrane of the maturing phagosome via fusion with acidic endomembrane compartments (i.e., endosomes and lysosomes). What determines the progressive acidification is unclear, but increased density of pumps, reduced leakage of H^+ (equivalents), and/or increasing counterion conductance have all been contemplated. All V-type ATPases are exquisitely sensitive to inhibition by macrolide antibiotics such as bafilomycin and

concanamycin (folimycin), and these drugs are routinely used to prevent or dissipate the phagosomal acidification and to validate the measurements of luminal pH. Two general approaches have been used to detect and quantify phagosomal pH, as described in Basic Protocol 4 and Alternate Protocol 4, below.

BASIC PROTOCOL 4

Measuring Phagosomal pH with Permeant Reagent

The simplest method for determining phagosomal pH involves the use of absorbing or fluorescent weak bases that can permeate biological membranes in their unprotonated state. Such compounds readily traverse the surface and phagosomal membranes to reach the phagosomal lumen where they become protonated because of the prevailing low pH. Because the resulting protonated species is much less permeant than its unprotonated precursor, the total concentration of the molecule in the phagosome increases beyond that of the medium and cytosol, in direct proportion to $[H^+]$. This accumulation, an indirect measure of pH, can then be detected spectroscopically either by the increased absorbance or fluorescence of the probe, or by spectral shifts that are a consequence of the formation of excimers at high fluorophore concentrations. Acridine orange, quinacrine, and LysoTracker are fluorescent weak bases that can be used to detect phagosomal acidification.

Materials

Cells of interest (e.g., RAW264.7 macrophages; ATCC #TIB-71)
Na^+-rich medium (see recipe), 37°C
Fluorophore-coupled phagocytic particles (see other protocols in this unit)
1 mM stock of LysoTracker Red DND 99 (Molecular Probes) in DMSO

6-well tissue culture plates containing sterile glass coverslips (see recipe)
Leiden chambers and thermostatted holder for microscope stage
Fluorescence imaging microscope system with rhodamine filter set
Light microscope with differential interference contrast (DIC) capability (*UNIT 4.1*)

Additional reagents and equipment for light and fluorescence microscopy (*UNIT 4.1*)

Prepare cells

1. Seed cells onto sterile glass coverslips contained in 6-well plates (prepared as in Reagents and Solutions) and grow to desired level of confluence.
2. Remove coverslip from well, place in Leiden chamber and immediately cover with prewarmed (37°C) Na^+-rich medium.

Allow cells to carry out phagocytosis and accumulate LysoTracker

3. Initiate phagocytosis by incubation with desired particles (see other protocols in this unit).
4. Wash off excess particles and allow maturation to proceed for desired time.
5. Add to the medium 50 to 100 nM LysoTracker Red DND 99 (as 1 mM stock) and allow a minimum of 10 min for complete equilibration.
6. Visualize fluorescence using a fluorescence microscope with a conventional rhodamine filter set.
7. Confirm the identity of phagosomes by visualizing the same field by differential interference contrast (DIC) microscopy.

Quantitative pH Determinations by Fluorescence Ratio Microscopy

More quantitative measurements can be made by ratio fluorimetry. This approach is based on the use of pH-sensitive fluorophores that are trapped in the phagosome along with the particle. It is best to attach the probe covalently to the particle, since the rapid remodeling of the luminal contents during maturation results in rapid clearance of soluble material that is trapped during phagosome formation. Very accurate determinations can be obtained by calculating the ratio of fluorescence at two different wavelengths that behave differentially when pH is altered. This normalization procedure corrects for overall changes in emission due to photobleaching or to alterations in focal plane, a common occurrence in the case of phagosomes, which are comparatively mobile organelles. Derivatives of fluorescein have been used extensively for this purpose.

Additional Materials *(also see Basic Protocol 4)*

Fluorophore-coupled zymosan particles (see Basic Protocol 3, steps 1a to 6a)
K^+-rich medium, pH 7.5 and lower pHs for calibration (see recipe)
1 mg/ml nigericin stock in ethanol
Ratio imaging setup (see Support Protocol 2)

Prepare cells

1. Grow cells, load in a Leiden chamber, and overlay with Na^+-rich medium (see Basic Protocol 4, steps 1 and 2).
2. Place chamber on thermostatted holder on stage of imaging microscope.

Allow cells to phagocytose particles

3. Allow the cells to ingest fluorophore-coupled zymosan particles (see Basic Protocol 3).

   Particles can be opsonized if desired by incubation with anti-zymosan antibodies (Molecular Probes), as described for red cells in Basic Protocol 1. Use a ratio of 5 to 10 particles per cell for effective phagocytosis

4. Wash off excess particles and allow maturation to proceed for desired time.
5. Acquire images, alternating the excitation between 440 nm and 490 nm and directing the light to the samples with a 510 nm dichroic mirror. Record emitted light using a 535BP25 nm filter.

Calibrate the pH

6. For calibration, replace Na^+-rich medium with K^+-rich medium, pH 7.5. Add 5 µg/ml nigericin (from 1 mM stock) and allow 3 to 5 min for equilibration.
7. Repeat image acquisition as in step 5.
8. Replace K^+-rich medium, pH 7.5, with similar medium of lower pH, and nigericin as in step 6.
9. Allow equilibration and repeat image acquisition cycle
10. Repeat steps 8 to 9 for all calibration solutions.

    At least four calibration points in the range of pH 5 to 7.5 are recommended.

11. Construct a calibration curve plotting fluorescence ratio versus pH. Interpolate experimental values obtained in step 5 to calculate the absolute pH from the calibration curve.

If using the Metamorph software, these steps can be expedited by using the QUICK TITRATION feature.

Calibration can be done, alternatively, by gently permeabilizing the plasma and phagosomal membranes using 0.1% Triton X-100, while ensuring that the phagocytic particle(s) of interest remain in the field of view. The K^+-rich media of varying pH can then be introduced sequentially as above, but nigericin can be omitted. Ensure that the particle being calibrated is not displaced during solution changes.

SUPPORT PROTOCOL 2

Ratio Imaging Setup

A complete imaging system appropriate for use in quantitative pH determination via ratio imaging (see Alternate Protocol 4) consists of an excitation source, microscope with appropriate filter sets and filter-positioning devices, camera, computer, and software (Demaurex et al., 1998). Several "turn-key" complete systems are available for fluorescence imaging. On the other hand, a large variety of setups can be assembled using individual components from different manufacturers. The system that the authors have assembled in their laboratory consists of a Leica DMR IB fluorescence microscope equipped with a PL Fluotar 100×/1.3 N.A. oil-immersion objective. A Sutter wheel and controller position the excitation filters in front of a mercury lamp. Neutral-density filters are often used to minimize dye bleaching and photodynamic damage to the cells. Emitted fluorescence is captured by an Orca ER cooled charge-coupled device camera. Image acquisition is controlled by the Metafluor software (Universal Imaging Corp.) operating on a Dell computer.

The authors' system includes a modification that makes it possible to continuously monitor the cells by bright-field (differential interference contrast) microscopy (also see *UNIT 4.1*). To this end, the sample is continuously illuminated at >620 nm by placing a red filter in front of the transmitted incandescent source. By placing an additional 660-nm dichroic mirror in the light path, the red light can be directed to a separate video camera (Dage MTI).

ASSESSMENT OF PHAGOSOMAL MATURATION

The composition of the nascent phagosome is very similar to that of the plasma membrane. However, both the limiting membrane and luminal contents of the phagosome undergo graded changes that result from progressive and finely coordinated rounds of fusion with endomembranes. These fusion events, together with the concomitant membrane budding and vesiculation steps that maintain the phagosomal area nearly constant, are collectively termed phagosomal maturation. Maturation confers upon the phagosome its microbicidal and lytic capabilities. Table 15.7.1 summarizes some of the characteristics of phagosomes at different stages of maturation.

The following protocols, outline methods to assess the occurrence of phagosomal maturation and to identify some of the intermediate stages of the process, using reagents that are readily available. It should be noted, however, that the rate and extent of maturation, and consequently the fate of the internalized particles, most likely depend on the number and type of receptors engaged. Therefore, the design and interpretation of individual experiments need to be tailored to the particular phagocytic stimulus under investigation.

Assessment of the Presence of Transferrin Receptors in Early Phagosomes

Transferrin (Tfn) receptors, which are abundant in early/recycling endosomes, are present on the phagosomal membrane at the earliest stages of maturation. The following are steps assess transferrin distribution in cells.

Materials

Primary monocytes or macrophages (*UNIT 2.2*), or monocytic cell lines (e.g., RAW 264.7 cells, ATCC #TIB-71 or J774 cells, ATCC #HB-197)
DMEM with 10% FBS (*APPENDIX 2B*)
Phosphate-buffered saline (PBS; see recipe)
Serum-free DMEM (e.g., Life Technologies)
Fluorophore-conjugated transferrin (Tfn; Molecular Probes; store in small aliquots at −20°C)
HEPES-buffered DMEM (e.g., Life Technologies)

6-well tissue culture plates containing sterile glass coverslips (see recipe)
Fluorescence microscope with appropriate filters (*UNIT 4.2* and *APPENDIX 1E*)

Additional reagents and equipment for phagocytosis assay using sRBCs (see Basic Protocol 1 or Alternate Protocol 1) or latex beads (see Alternate Protocol 1 or 2) and fluorescence microscopy (*UNIT 4.2*)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will to reach ~75% confluence at the time of the experiment.
2. Wash cells with PBS, then incubate in serum-free DMEM for 1 hr in a CO_2 incubator at 37°C.

   This step will deplete the cells of endogenous transferrin.
3. While the cells are incubating, proceed with opsonization of sRBC or latex beads (see Basic Protocol 1 or Alternate Protocol 1) in preparation for the phagocytosis assay (step 6 below).

Table 15.7.1 Markers of Phagosomal Maturation

Stage	Time	pH	Interacting organelle	Markers[a]
Early phagosome	~5 min	6.0	Sorting endosomes, endoplasmic reticulum (?)	Transferrin receptor[b], EEA1[b], Rab5[b], PI(3)P, syntaxin 13
Late phagosome	~20 min	5.0–6.0	Late endosomes, multivesicular bodies	Mannose-6-phosphate receptor[b], LAMP1/2[b], Rab7[b], Rab9[b], syntaxin 7, lyso(bis)phosphatidic acid
Phagolysosome	~40 min	4.5–5.0	Lysosomes	LAMP1/2[b], mature cathepsin D[b], fluid-phase markers chased for = 2 hr

[a]Abbreviation: PI(3)P, phosphatidylinositol 3-phosphate.
[b]Antibodies directed against these antigens are commercially available.

4. Prepare a solution of 50 µg/ml fluorophore-conjugated Tfn in HEPES-buffered DMEM. Overlay cells with this solution and incubate for 1 hr at 37°C to label the endosomes.

   Molecular Probes sells human transferrin conjugated to a number of different fluorophores. One should choose the most appropriate fluorophore for one's application. Reconstitute according to manufacturer's directions and store in the dark in small aliquots at –20°C.

5. Wash off excess unbound Tfn with PBS.

6. Proceed with phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1).

7. Assess the presence of fluorescently labeled Tfn on the phagosomal membrane using fluorescence microscopy.

   Tfn should be present in phagosomes at early times (5 to 15 min), but not thereafter.

   Confocal microscopy is optimal for evaluation, particularly when latex beads are employed, since such beads are prone to birefringence of fluorescent light emitted outside the focal plane.

ALTERNATE PROTOCOL 5

Assessment of the Delivery of Fluid-Phase Markers to the Phagosome

The protocol detailed below takes advantage of the uptake and accumulation in lysosomes of biologically inert fluorophore-conjugated dextrans to identify phagolysosomes. For these experiments, the authors recommend the use of latex particles to avoid the conflicting fixative requirements of sRBC and the lysine-fixable dextran.

Materials

Primary monocytes or macrophages (*UNIT 2.2*), or monocytic cell lines (e.g., RAW 264.7 cells, ATCC #TIB-71 or J774 cells, ATCC #HB-197)
DMEM with 10% FBS (*APPENDIX 2B*)
Fluorophore-conjugated dextran, MW 3000, lysine fixable (Molecular Probes; store in dark in small aliquots at –20°C)

6-well tissue culture plates containing sterile glass coverslips (see recipe)
Fluorescence microscope with appropriate filters (*UNIT 4.2* and *APPENDIX 1E*)

Additional reagents and equipment for phagocytosis assay using sRBCs (see Basic Protocol 1 or Alternate Protocol 1) or latex beads (see Alternate Protocol 1 or 2) and fluorescence microscopy (*UNIT 4.2*)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will to reach ~75% confluence at the time of the experiment.

2. Add the fluorophore-conjugated dextran at 250 µg/ml. Incubate 30 to 45 min at 37°C.

   Molecular Probes sells dextran (mol. wt. 3000, lysine fixable) conjugated to a number of different fluorophores. One should choose the most appropriate fluorophore for one's application. Reconstitute according to manufacturer's directions and store in the dark in small aliquots at –20°C. The "fixable" probes have lysine residues incorporated into the dextran which allow them to be fixed by paraformaldehyde and other aldehyde fixatives.

3. Wash off excess dextran with two changes of medium.

4. Incubate for an additional 3 to 4 hr at 37°C in DMEM with 10% FBS.

5. While the cells are incubating, proceed with opsonization of sRBC or latex beads (see Basic Protocol 1 or Alternate Protocol 1) in preparation for the phagocytosis assay (step 6).
6. Proceed with phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1).
7. Assess the colocalization of fluorescently labeled dextran with the internalized latex beads by microscopy.

   Dextran will be found in phagosomes only at advanced stages of maturation (>30 min).

   Fresh mounts or samples fixed with 4% paraformaldehyde can be used. Confocal microscopy (UNIT 4.5) is optimal for evaluation, particularly when latex beads are employed.

Assessment of Phagosomal Maturation by Immunostaining of Endogenous Organellar Markers

ALTERNATE PROTOCOL 6

As it matures, the phagosome acquires and sheds a number of different membrane and soluble proteins, characteristic of the compartments it interacts with. Antibodies directed against many of these proteins are commercially available and can be used to assess their presence in phagosomes by immunofluorescence.

It should be noted, however, that these markers are not always discretely localized to one single compartment (e.g., LAMP1 and LAMP2 are abundant in both late endosomes and lysosomes and can be present at lower density in the plasma membrane and early endosomes). Therefore, the authors recommend the use of multiple markers to validate the stage of phagosomal maturation

The following is a generic protocol that describes the immunofluorescence staining procedure following a phagocytosis assay. The species and concentrations of the primary and secondary antibodies will depend on the specific antigen under investigation. Some of the antibodies, such as those directed against some types of LAMP, are only effective in cells fixed with methanol. Please consult the specifications provided by the manufacturer for the appropriate antibody dilutions, blocking and permeabilizing conditions, and fixative to be used.

Materials

Permeabilization buffer: e.g., 0.1% (v/v) Triton X-100 in PBS or 2% (w/v) saponin in PBS (see recipe for PBS)
Blocking buffer: PBS (see recipe) containing 1% (w/v) BSA and 7.5% (v/v) of serum in which primary antibody was raised)
Primary antibody directed against the protein marker of interest (Table 15.7.1)
Fluorophore-conjugated secondary antibody
Fluorescence mounting medium (Dako)
Additional reagents and equipment for phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1) and fluorescence microscopy (*UNIT 4.2*)

1. Perform phagocytosis assay (see Basic Protocol 1, steps 1 to 21, or Alternate Protocol 1, steps 1 to 15).
2. Fix with methanol (see Basic Protocol 1, step 21) or paraformaldehyde (see Alternate Protocol 1, step 17). Quench with 100 mM glycine in PBS (see Alternate Protocol 1, step 17) if paraformaldehyde is used.
3. Wash fixed cells three times with PBS.

4. Permeabilize fixed cells for 20 min with the appropriate permeabilization buffer at 4°C.

   Detergent-containing permeabilization buffers are required if paraformaldehyde is used to fix the cells but not if methanol is used.

5. Block for 1 hr at room temperature in blocking buffer.
6. Incubate with primary antibody diluted in blocking buffer for 1 hr at room temperature.
7. Wash three times with PBS.
8. Incubate with fluorophore-conjugated secondary antibody diluted in blocking buffer for1 hr at room temperature.
9. Wash three times with PBS.
10. Mount in Dako fluorescence mounting medium and analyze by fluorescence microscopy.

BASIC PROTOCOL 6

INHIBITION OF PHAGOCYTOSIS

Because phagocytosis is a complex phenomenon, it can be arrested at a number of steps, all of which are essential for completion of particle engulfment (Aderem and Underhill, 1999). A variety of conditions and agents have been reported to impair phagocytosis, but their detailed mode of action is not known in every instance. The simplest, most readily reversible means of preventing phagocytosis is to cool the cells. For mammalian phagocytes, temperatures below 15°C virtually eliminate particle ingestion. The precise critical step(s) affected by reducing the temperature has not been identified, and it is likely that multiple events of the phagocytic reaction are impaired simultaneously. Because it is harmless and reversible, cooling is used extensively to synchronize phagocytosis. This approach is based on the notion that binding of phagocytic receptors to their ligands is much less temperature sensitive than the consequent signaling and effector functions that lead to particle internalization.

The requirement for extensive rearrangement of the actin cytoskeleton during the uptake of large particles is universally acknowledged. For this reason, many laboratories have used cytochalasins or other antagonists of actin remodelling, such as latrunculin, to inhibit phagocytosis (also see *APPENDIX 1B*). Under these conditions, the phagocytes still associate with the target particles, but fail to extend pseudopods and do not engulf their prey. It is important to note that the effectiveness of antagonists of actin remodeling as inhibitors of phagocytosis is proportional to the size of the particle. Uptake of particles of ≤1 μm is only modestly affected, if at all, whereas engulfment of large targets such as apoptotic bodies is fully arrested. This divergence highlights the notion, introduced earlier, that phagocytosis is not a single phenomenon but rather a collection of phenotypically related events.

Inhibitors of phosphatidylinositol 3 kinase, such as wortmannin and compound LY294002 (*APPENDIX 1B*), also effectively impair the uptake of large particles. These drugs inactivate both class I and class III phosphatidylinositol 3 kinases at similar concentrations, and their use alone cannot distinguish the contribution of these enzyme types. However, experiments using inhibitory antibodies as well as studies of targeted gene disruption indicate that the class I, but not class III kinases are required for phagocytosis. Conversely, class III phosphatidylinositol 3 kinase is essential for normal maturation of the phagosome. Cells pretreated with wortmannin bind the target particles and initiate pseudopod extension, but the process is arrested before the particle becomes entirely trapped, resulting in the sustained formation of phagosomal cups, rich in F-actin. The critical step impaired by phosphatidylinositol 3 kinase inhibitors may be the detachment of actin from the base of the phagocytic cup, limiting the availability of actin and

associated proteins for the continued extension of the pseudopods. This may result, for instance, from sustained stimulation of Rho-family GTPases that are unable to inactivate in a timely manner. Alternatively (or additionally), phosphatidylinositol 3 kinase blockers may prevent the targeted delivery of endomembranes to the site of phagocytosis, which may be required for extension of the pseudopodia. As in the case of actin antagonists, inhibition by wortmannin and related compounds is particle size dependent. Phagocytosis of particles smaller than 2 to 3 μm can proceed to varying degrees despite inhibition of the kinase, while the uptake of larger particles is virtually eliminated.

Materials

20 to 50 μM wortmannin stock solution in DMSO *or* 1 to 2 mM cytochalasin D stock solution in ethanol (see *APPENDIX 1B* for more information on these reagents)

Additional reagents and equipment for phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1)

1. Prepare cells for phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1).
2. Prior to addition of phagocytic target particles, incubate cells in medium of choice containing either 50 to 100 nM wortmannin or 5 to 10 μM cytochalasin D. Incubate ≥10 min.
3. Add phagocytic particles.

   Note that cytochalasin needs to be maintained in the medium during phagocytosis, as it is a reversible agent. In contrast, wortmannin is thought to alkylate and irreversibly modify phosphatidylinositol 3 kinase and, in principle, need not be present after the preincubation period is completed.
4. Mount cells and score phagocytosis (see Basic Protocol 1 or Alternate Protocol 1).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Hanks balanced salt solution (HBSS)

5.4 mM KCl
0.3 mM Na_2HPO_4
0.4 mM KH_2HPO_4
4.2 mM $NaHCO_3$
1.3 mM $CaCl_2$
0.5 mM $MgCl_2$
0.5 mM $MgSO_4$
137 mM NaCl
5.6 mM D-glucose
Adjust pH to 7.4.
Store up to 1 month at 4°C

K^+-rich media

143 mM KCl
1 mM $MgCl_2$
1 mM $CaCl_2$
5 mM glucose
Store up to 1 month at 4°C.

Titrate to desired pH between 5.0 and 7.5 using PIPES, MES, or HEPES, as appropriate.

Na^+-rich medium

140 mM NaCl
3 mM KCl
1 mM $MgCl_2$
1 mM $CaCl_2$
5 mM glucose
20 mM HEPES, titrated to pH 7.3 at 37°C
Store up to 1 month at 4°C.

Phosphate-buffered saline (PBS)

140 mM NaCl
10 mM KCl
8 mM monobasic sodium phosphate
2 mM dibasic potassium phosphate
Check that pH is 7.4
Store up to 6 months at 4°C

Tissue culture plates, 6-well, containing sterile glass coverslips

Hold glass 25-mm round glass coverslips with 5-in. stainless steel tissue forceps and dip in a beaker containing 70% ethanol to sterilize. Gently shake off the excess ethanol and place coverslip on an angle in the well of the 6-well plate. When all six wells have been fitted with sterile coverslips, loosely place the lid over the plate and allow it to air dry in a culture hood for 10 to 15 min. Lower the lid securely onto the plate and gently tap the plate to allow the coverslips to settle to the bottom of the well. Seed cells as usual (*UNIT 1.1*).

COMMENTARY

Background Information

FcγR-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

The principal immunoglobulin (Ig) that functions as an opsonin is IgG. The constant (Fc) region of IgG binds to its cognate Fcγ receptor (FcγR). Binding of other classes of immunoglobulins, such as IgA and IgE, to their cognate Fcα and Fcε receptors can also support particle engulfment. However, phagocytosis is not the primary function of these receptors and is not discussed in this unit.

Internalization of IgG-opsonized materials is mediated by some but not all Fcγ receptors, including FcγRI, FcγRIIA, and FcγRIIIA. The cell expression and binding specificities of individual FcγR are listed in Table 15.7.2. FcγRI and FcγRIIIA are heterotrimeric proteins composed of a single α chain (which contains the Fc binding region) and two associated γ chains that contain the immunoreceptor tyrosine activation motif (ITAM). FcγRII is a monomeric transmembrane protein containing both the Fc-binding and the signal transduction motifs. FcγRIIA, expressed exclusively on human leukocytes, contains an ITAM motif in the cytosolic region of the protein and is able to support phagocytosis.

FcγRIIB, on the other hand, has an inhibitory ITIM (immunoreceptor tyrosine inhibitory motif) in place of the ITAM motif and is a negative (inhibitory) receptor. FcγRIIIB is a GPI-linked protein expressed in granulocytes. It can initiate calcium signaling and actin polymerization but it was omitted from Table 15.7.2 because its role in phagocytosis is ill defined.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

Complement (C) comprises a group of proteins that are constitutively produced and secreted as inactive precursors by macrophages and hepatocytes. Complement is an important component of the innate immune response. It is not antigen-specific and can be activated

rapidly in the presence of microorganisms or foreign bodies to induce phagocytosis and cell lysis.

Activation of the complement cascade results in the cleavage of the inactive complement precursor proteins, some of which, in turn, become proteases that cleave other complement components. There are two complement cascades. The classical cascade is activated by the Fc (constant) region of antigen-bound IgG and IgM, and it is the pathway utilized in the above protocols to deposit C3bi onto erythrocytes and latex beads. The alternative complement cascade is activated by cell-surface molecules of bacteria, including the lipopolysaccharide (LPS) of gram-negative organisms, teichoic acid from gram-positive bacteria, and components from fungal and yeast cell walls.

As indicated, some fragments such as C3bi are opsonins that target coated materials for degradation. Particles that have been opsonized by complement fragments can be internalized by several complement receptors. Table 15.7.3 summarizes the different complement receptors (CR) that support phagocytosis, the receptor-binding specificities of each receptor, and the cell types where they are found. CR1 is a large monomeric glycoprotein that binds primarily C3b fragments and promotes phagocytosis and clearance of antigen-antibody complexes in conjunction with Fc receptors. CR3 and CR4 are integrin heterodimers which bind specifically to C3bi. CR3, also known as Mac1, $\alpha_M\beta2$, or CD11b/CD18, binds to C3bi with the highest affinity and is commonly considered to be the primary receptor involved in complement-mediated phagocytosis. The binding specificities of the receptors for C3bi are as follows: CR3>CR1>CR4. Ligation of C3bi-coated material is insufficient to initiate phagocytosis via CR3 and CR4, which, like other integrins, require a second activation (priming) stimulus such as phorbol esters (e.g., PMA), glucan binding, or adhesion of the cells to extracellular matrix proteins. For professional phagocytes such as neutrophils, cell adhesion may provide a sufficient stimulus to initiate CR3-mediated phagocytosis.

As discussed in the introduction to this unit, cell model systems using heterologous transfection of receptors have been used extensively to study phagocytosis. If using such model systems to study complement receptor–mediated phagocytosis, bear in mind that, when expressed exogenously, most integrins are in the active state. Therefore, a priming stimulus may not be necessary to elicit phagocytosis.

Flow cytometry of phagocytosis (Basic Protocol 3)

Flow cytometric analysis offers many potential advantages over microscopic assays of phagocytosis. These include: (1) the ability to assess large numbers of cells (tens of thousands), thus facilitating statistical analysis; (2) the availability of new fluorophores and multiparametric analysis that enable simultaneous measurements of structural (e.g., change in cell size or shape) and/or functional (e.g., calcium flux, oxidant production, surface adhesion molecule expression) properties of suspended cells rapidly and quantitatively (Trinkle et al, 1987); and (3) the capacity to use small volumes of whole blood. This method is particularly well suited for analysis of human samples from individuals suspected of having defects in the innate immune system, or for analysis of rodent (murine or rat) blood samples for which

Table 15.7.2 Main Types of FcγR

Receptor	Cell type	Binding affinity	Ligands	Function
FcγRI	Neutrophil, macrophage	High	IgG2a	Phagocytosis
FcγRIIA	Human neutrophil, macrophage	Low	IgG1, IgG2a, and IgG2b	Phagocytosis
FcγRIIB	Murine neutrophil, macrophage, B cell, mast cell	ND[a]	IgG1, IgG2a, and IgG2b	Inhibitory receptor
FcγRIIIA	Macrophage	High	IgG1, IgG2a, and IgG2b	Phagocytosis

[a]Abbreviation: ND, not determined

Table 15.7.3 Complement Receptor (CR)-Mediated Phagocytosis

Receptor	Cell type	Ligands
CRI (CD35)	Monocytes, neutrophils, dendritic cells	C3b, C3bi, C4b, C4bi
CR3 (Mac1, $\alpha_M\beta_2$ CD11b/CD18)	Monocytes, neutrophils, natural killer cells, dendritic cells	C3bi
CR4 (p150.95, $\alpha_V\ \beta_2$ integrin,CD11c/CD18)	Macrophages, neutrophils, natural killer cells, dendritic cells	C3bi
CR5aR	Macrophages, neutrophils	C5a

only small volumes are available. The method is compatible with the use of whole blood, which reduces the possibility of artifactual changes (e.g., priming or activation) caused by even the most gentle of cell purification procedures, and may be more reflective of the behavior of leukocytes in vivo.

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

The addition of labeled weak bases to assess the pH of acidic organelles is convenient and has therefore been used extensively. While rapid and convenient, this method is not exactly quantitative, because factors other than the transmembrane proton gradient contribute to the accumulation and retention of the bases. Binding of the probes to the contents of the probe can skew the determinations and limit their reversibility.

The more quantitative measurements using covalently labeled particles are far superior, though technically more demanding. The fact that fluorescence ratios are determined greatly reduces the risk of misinterpretation or miscalculation due to bleaching or defocusing. Nevertheless, the experimenter must be cautious to ensure that the appropriate background is subtracted separately from each one of the signals acquired at different wavelengths and that any changes, such as oxidation of the dye that can occur in phagosomes, affects both wavelengths to a comparable extent.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

The primary purpose of internalization of opsonized particles is their ultimate destruction by a combination of low pH, degradative enzymes, and cytotoxic mediators. To achieve this goal, the phagosome acquires proteases and other hydrolases, high concentrations of H^+ ions, a variety of bactericidal peptides, and other factors as it matures. A detailed review of phagosome maturation has recently been published (Vieira et al, 2002).

In the maturation protocols, advantage is taken of the progressive acquisition of specific markers as the phagosome interacts with defined intracellular compartments to identify the stage of phagosomal maturation. Assessments of maturation can be complemented with measurement of phagosomal pH.

Inhibition of phagocytosis (Basic Protocol 6)

Pharmacological tools have been the source of a great deal of knowledge in the field of phagocytosis. While they are deservedly criticized for their imperfect specificity, they can be most useful when used under optimal conditions (i.e., the concentration range where their effect is most specific) and while bearing in mind their potential limitations. Molecular approaches, such as transfection of dominant negative constructs, gene ablation, or silencing using interference RNA technology are in principle more specific. However, these normally take many hours or days to develop within the cell or animal, and they can therefore generate a plethora of secondary, even developmental defects that are only distantly related to phagocytosis. Thus, pharmacology and molecular approaches are complementary. Continued improvement of the selectivity of pharmacological agonists and antagonists will surely be an

ongoing source of information about phagosome formation and maturation.

Critical Parameters and Troubleshooting

FcγR-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

Optimal adsorption of IgG onto sRBC and latex beads is critical for the success of the phagocytosis assays. The authors highly recommend that pilot experiments be performed with serial dilutions of IgG to find the concentrations required for optimal opsonization of individual lots of reagents.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

For both Basic Protocol 2 and Alternate Protocol 2, the most critical parameter is the coating of the erythrocytes or latex beads with C3bi. The authors have indicated their preferred sources for some of the critical reagents in the respective protocols. It is recommended that initial experiments with serial dilutions of IgM be performed to find the optimal sub-agglutinating titers of individual lots of antibody, as well as for sRBC and latex beads from different sources.

The incubation period in C5-deficient medium is also critical. In studies of IgG- and IgM-coated erythrocytes, it has been shown that excessively long incubation periods (>20 min) lead to loss of surface-bound C3bi (Newman and Mikus, 1985).

Flow cytometry of phagocytosis (Basic Protocol 3)

The success of these experiments will depend on several factors: optimal condition of the phagocytes to be assessed, proper opsonization of phagocytic targets, and a signal-to-noise ratio that will allow clear discrimination of particles that are internalized from those that are adherent to the cell surface ("in" from "on"). For flow cytometry, this means efficient quenching of extracellular fluorescence of surface-bound particles. In studies using trypan blue to quench extracellular fluorescence, it is important not to wash the trypan blue away, or else the quenching of fluorescence will be lost.

Because of the automated nature of the procedure and the fact that the cells are never directly visualized during the course of flow cytometry, there are several potential artifacts that need to be avoided. These include false positive scoring of particles that are adherent to the cell surface rather than internalized and enclosed within a phagosome. This may result when quenching of the fluorescence of extracellular particles is incomplete. In addition, because the technique involves the analysis of cells in suspension, it is limited to cells that are naturally suspended or to adherent cells that can be detached easily from their substrate to form single-cell suspensions. Lifting adherent neutrophils or macrophages without incurring damage is not simple, especially after the cells have performed phagocytosis. Finally, because clumps of cells are either removed by filters designed to collect debris or excluded from analysis by gating, phagocytosis by cell types that are prone to aggregate after stimulation, such as neutrophils, might be underestimated if the most responsive and likely most avidly phagocytic cells are excluded from analysis.

There are several key parameters that require attention during flow cytometric analysis:

1. *Gating.* Cells are gated on a forward versus side scatter plot to exclude debris and broken cells. Forward scatter is proportional to the size of cells and side scatter is proportional to their granularity and shape. If there is a mixed cell population (e.g., when using whole blood or buffy coat preparations), it is critical that relatively pure populations of the cells of interest be included in the gate. It is usually possible to separate neutrophils, monocytes, and lymphocytes into distinct populations based on their light scattering. However, there is some overlap between small monocytes and neutrophils, and complete separation of these two cell types requires the use of a fluorescently labeled antibody that binds to a unique surface epitope on one cell type (e.g., CD15a for neutrophils and CD14 for monocytes). A minimum of 10,000 cells are counted in the viable cell gate

2. *Coincidence.* This term refers to the simultaneous appearance through the laser beam of the cytometer of more than one cell or free target particle, and it can lead to inaccuracies in the assessment of phagocytosis. Several parameters impact on coincidence, including the flow rate of the sheath fluid, the concentration of phagocytic cells and targets in the sample, and the ratio of targets per cell (Lehmann et al., 2000).

3. *Fluorescence channels.* Each channel is designed to collect emitted light within a defined range of wavelengths. However, each fluorochrome emits light with wider emission spectra than the limits of the single channel. This gives rise to the possibility of spectral overlap, an issue that is of critical importance when two or more fluorochromes are used (e.g., FITC-labeled phagocytic particles and a phycoerythrin-labeled anti-CD15 antibody to allow identification of human neutrophils). This potential problem can be overcome by choosing fluorochromes that have minimal spectral overlap and by electronic color compensation (a discussion of this issue is beyond the scope of this unit).

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

In the case of partitioning probes, such as weak bases, a range of different compounds are available with varying spectral properties. For experiments where multiple fluorophores need to be used, pH-sensitive probes that absorb light in the ultraviolet, blue, or green parts of the spectrum are commercially available.

It is essential to select probes that are most pH-sensitive in the range of interest. The pK_a of the probe chosen for analysis must ideally be in the mid-range of the measurements to be performed to provide optimal linearity. In the case of macrophage phagosomes, the pH generally becomes more acidic than in neutrophils, where the oxygen radicals generated by the NADPH oxidase can scavenge protons and thereby make the lumen more alkaline.

These oxygen radicals can greatly complicate the measurements by inducing acute chemical transformation of the fluorescent probes. When the contribution of the oxidase is not important to the measurements, it is convenient, indeed advisable to inhibit the flavoprotein component of the enzyme using diphenylene iodonium. Alternatively, the contribution of peroxidases can be minimized by addition of azide, bearing in mind that this compound will block mitochondrial ATP generation.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

None of the procedures outlined will, by itself, identify the stage of phagosome maturation unambiguously. Therefore, the authors suggest that two or more different markers or methods be used to confirm the precise stage of maturation. Acquisition of defined isoforms of Rab (e.g., Rab5 versus Rab7), and differentiation between the mature and immature forms of lysosomal enzymes, such as cathepsins, can be most informative.

The second critical parameter is the choice of fluorophores used for the colocalization experiments. Care must be taken to avoid cross-talk between fluorophores when multiple determinations are performed simultaneously.

Inhibition of phagocytosis (Basic Protocol 6)

The duration of the (pre)treatment with inhibitors should be kept to the minimum required to achieve the inactivation of the desired target. Longer than necessary treatments should be avoided as they can result in secondary, indirect effects that can complicate the interpretation of the data. Extended pretreatment with wortmannin will impair vesicular traffic along the endocytic and secretory pathways and may impact negatively on the composition of the plasma membrane or on the ability of endosomes to be delivered to the surface upon activation. Similarly, prolonged exposure to cytochalasins will grossly distort cell morphology and function and can ultimately lead to detachment of the cells from the substratum. For non-adherent cells like blood neutrophils, cytochalasins have been reported to have a "priming" effect that potentiates some responses like granule secretion. The possible consequences of such priming must be borne in mind.

Anticipated Results

FcγR-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

Particles coated with IgG are expected to be ingested avidly by both macrophages and neutrophils and by monocytic cell lines like J774 and RAW264.7. The vast majority of the cells in any one culture will prove to be competent to internalize one or more particles over the course of 5 to 10 min.

The simplest and least ambiguous way to differentiate between extracellular adherent particles and those that have been internalized is to lyse hypotonically those on the outside. Visualization by bright-field microscopy will only detect the engulfed, intact red cells that retained their hemoglobin. Bear in mind that while fluorescently labeled red cell ghosts that remain adherent (extracellular) after hypotonic

lysis will be observable by fluorescence microscopy they will not be observable by bright-field microscopy because they have lost hemoglobin.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

The efficiency of complement-mediated phagocytosis is, in the authors' experience, considerably lower that that of Fc-mediated phagocytosis. Therefore, expect only a fraction of the cells (typically 30% to 50%) to ingest one or a couple of the particles. Moreover, the kinetics of ingestion appears to be slower, perhaps because it is mediated by Rho, unlike Fc-dependent phagocytosis that involves Rac and Cdc42. The morphology of the phagocytic site also differs: complement-coated particles appear to "sink" into the cells and do not reveal the florid pseudopods seen in Fc-dependent internalization.

Flow cytometry of phagocytosis (Basic Protocol 3)

If the protocols outlined above are followed and if the phagocytic cells are healthy, the use of flow cytometry to assess phagocytosis is straightforward. For professional phagocytes such as neutrophils, phagocytosis should be very efficient, with over 70% of cells ingesting one or more phagocytic particles. By comparison, in cell lines such as COS or Chinese hamster ovary cells transfected with phagocytic receptors, phagocytosis may be much less efficient, with only 20% to 30% of cells ingesting particles.

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

Phagosomes formed by macrophages are expected to become acidic very rapidly, reaching steady state after 10 to 15 min at pH as low as 5.0. In human neutrophils the pH change is smaller and has been reported to be biphasic, with some authors reporting a transient alkalinization due to proton consumption by superoxide. The latter can be obviated and the acidification accentuated by inhibition of the NADPH oxidase with diphenylene iodonium.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

Under normal circumstances, phagosomes formed by macrophages acquire early endosomal markers almost immediately. In fact, delivery of endosomal components to the nascent (unsealed) phagosome has been detected, accounting for the increased membrane area measured by electrophysiological capacitance determinations. Late endosomal markers appear as early as 10 min after ingestion and bona-fide lysosomal contents are detects after 45 to 60 min of phagocytosis.

Neutrophils contain unique secretory organelles that are delivered to phagosomes with different kinetics. Secretory vesicles, primary, secondary, and tertiary granules are all thought to deliver their constituents to the phagosome, although the signals that mediate fusion and the kinetics of delivery vary. Bear in mind that some granulocytic cell lines, such as HL-60 cells, fail to generate and/or accumulate some of the secretory compartments, which will therefore not contribute to phagosomal maturation.

Inhibition of phagocytosis (Basic Protocol 6)

As intimated in the introductory section of this unit, phagocytosis is a generic term that describes multiple, related but not identical processes. Even in the case of a single type of receptor and ligand, the properties of the phagocytic event may vary. Thus, phagocytosis of large particles is much more sensitive to wortmannin than is the ingestion of smaller ones (≤3 μm). Similarly, cytochalasin effectively inhibits the uptake of particles larger than 1–2 μm, but it is not nearly as effective in preventing the uptake of smaller particles. Much less is known for phagocytosis mediated by other receptors, so the reader should be prepared to find variations in the effectiveness of drugs when testing new systems.

Time Considerations

FcγR-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

Growth, expansion, and plating of the cells on the appropriate substratum is the most time-consuming part of the procedure. The phagocytosis protocol proper can be performed within 1 day. Therefore, in cases where primary cells can be isolated the same day, the entire experiment can be completed in a regular working day. It is not advisable to store opsonized red cells for extended periods.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

The preparation of complement-opsonized particles is more laborious than that of IgG-coated particles. Nevertheless, it can and should be performed the day of the experiment. As in the case of Fc-mediated phagocytosis experiments, the most time-consuming part of the procedure is the growth, expansion, and plating of the cells on the appropriate substratum. Bear in mind that priming with phorbol esters is required for optimal complement-mediated phagocytosis, and this increases the duration of the experiments.

Flow cytometry of phagocytosis (Basic Protocol 3)

When studying primary cells, the entire experiment will take 5 to 6 hr if the phagocytic targets are prepared ahead of time. When working with cultured cells, the entire experiment will take 2 to 3 days, with the majority of the bench work on day 2.

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

Qualitative assessment of phagosomal pH using fluorescent weak bases can be accomplished in under 1 hr, if the reagents are prepared in advance and the microscope is available. In contrast, the ratiometric determinations are more laborious, requiring several hours from start to finish, even when the labeled particles are prepared in advance. A sizable fraction of the time is invested in the calibrations, which last longer than the pH determinations themselves. Nevertheless, if the cells are available at the beginning of the day, multiple measurements can be accommodated in a single working day.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

The delivery of fluid-phase markers to phagosomes can be assessed within hours, provided the cells and opsonized particles are prepared in advance. The marker solutions are generally stable for considerable periods and can be prepared in advance.

Assessment of maturation by immunostaining is more time-consuming, requiring long fixation, blocking, staining, and mounting steps. From start to finish, an immunolabeling experiments can be completed within 2 days.

Inhibition of phagocytosis (Basic Protocol 6)

Pretreatment with most inhibitors is generally short (under 1 hr). Therefore, assessment of their effects on phagocytosis should not take much longer than the measurements of phagocytosis themselves (see above).

Literature Cited

Aderem, A. and Underhill, D.M. 1999. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 17:593-623.

Bassoe, C.F. and Solberg, C.O. 1984. Phagocytosis of *Staphylococcus aureus* by human leukocytes: Quantitation by a flow cytometric and a microbiological method. *Acta Pathol. Microbiol. Immunol. Scand.* 92:43-53.

Demaurex, N. 2002. pH Homeostasis of cellular organelles. *News Physiol Sci.* 17:1-5.

Demaurex, N., Romanek, R., Rotstein, O., and Grinstein, S. 1998. Measurement of cytosolic pH in single cells by dual excitation fluorescence imaging. Simultaneous visualization using differential interference contrast optics. *In* Cell Biology: A Laboratory Handbook, 2nd edition. (J. Celis, ed.) pp. 380-386. Academic Press, San Diego.

Greenberg, S. and Grinstein, S. 2002. Phagocytosis and innate immunity. *Curr. Opin. Immunol.* 14:136-45.

Harvath, L. and Terle, D.A. 1999. Assay for phagocytosis. *Methods Mol. Biol.* 115:281-289.

Heyman, B. 2000. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu. Rev. Immunol.* 18:709-737.

Lehmann, A.K., Sornes, S., and Halstensen, A. 2000. Phagocytosis: Measurement by flow cytometry. *J. Immunol. Methods* 243:229.

McKenzie, S.E. and Schreiber, A.D., 1998. Fc gamma receptors in phagocytes. *Curr. Opin. Hematol.* 5:16-21.

Newman, S.L. and Mikus, L.K. 1985. Deposition of C3b and iC3b onto particulate activators of the human complement system. Quantitation with monoclonal antibodies to human C3. *J. Exp. Med.* 161:1414-1431.

Newman, S.L., Becker, S., and Halme, J. 1985. Phagocytosis by receptors for C3b (CR1), iC3b (CR3), and IgG (Fc) on human peritoneal macrophages. *J. Leukoc. Biol.* 38:267-278.

Oben, J.A. and Foreman, J.C. 1988. A simple quantitative fluorimetric assay of in vitro phagocytosis in human neutrophils. *J. Immunol. Methods* 112:99-103.

Robinson, J.P., Darzynkiewicz, Z., Dean, P.N., Dressler, L.G., Orfao, A., Rabinovitch, P.S., Stewart, C.S., Tanke, H.J., and Wheeless, L.L. (eds.) 2003. Current Protocols in Cytometry. John Wiley & Sons, New York.

Santos, J.L., Montes, M.J., Gutierrez, F., and Ruiz, C. 1995. Evaluation of phagocytic capacity with a modified flow cytometry technique. *Immunol. Lett.* 45:1-14

Steinkamp, J.A., Wilson, J.S., Saunders, G.C., and Stewart, C.C. 1982. Phagocytosis: Flow cytometric quantitation with fluorescent microspheres. *Science* 215:64-67.

Trinkle, L.S., Wellhausen, S.R., and McLeish, K.R. 1987. A simultaneous flow cytometric measurement of neutrophil phagocytosis and oxidative burst in whole blood. *Diagn. Clin. Immunol.* 5:62-68.

van Eeden, S.F., Klut, M.E., Walker, B.A., and Hogg, J.C. 1999. The use of flow cytometry to measure neutrophil function. *J. Immunol. Methods* 232:23-31.

Vieira, O.V., Botelho, R.J., and Grinstein, S. 2002. Phagosome maturation: Aging gracefully. *Biochem. J.* 366:689-704.

Wright, S.D., Craigmyle, L.S., and Silverstein, S.C. 1983. Fibronectin and serum amyloid P component stimulate C3b- and C3bi-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 158:1338-134.

Contributed by Chung-Wai Chow
and Gregory P. Downey
University of Toronto and Toronto
General Hospital
Toronto, Canada

Sergio Grinstein
University of Toronto
Toronto, Canada

Analysis of Protein Transport to Lysosomes

Lysosomes are membrane-bound degradative organelles that are found in all higher eukaryotic cells. The limiting membranes of lysosomes contain a characteristic set of transmembrane glycoproteins that are transported to lysosomes by virtue of lysosomal targeting motifs found in the proteins' cytoplasmic tails (Bonifacino and Dell'angelica, 1999) The lumens of lysosomes contain an assortment of acidic hydrolases. These enzymes are synthesized in the endoplasmic reticulum (ER), where they become core-glycosylated. They are then transported into the Golgi complex, where they acquire a mannose-6-phosphate recognition tag in a two-step enzymatic process mediated by a phosphotransferase and an uncovering enzyme. The tag is then recognized by cation-dependent and cation-independent mannose-6-phoshate receptors in the *trans*-Golgi network (TGN). The resulting enzyme-receptor complexes are transported onward to endosomes, where the acidic pH induces dissociation of the enzymes from the receptors. The enzymes are then transported to lysosomes, while the receptors recycle back to the TGN to facilitate further rounds of transport. Failure of the receptors to recycle results in their misrouting to (and subsequent degradation in) lysosomes. The return of these receptors from endosomes to the TGN depends on specific amino acid residues (including specific cysteine residues that undergo reversible palmitoylation) in the cytoplasmic tails of receptor molecules. The final delivery of lysosomal enzymes to lysosomes has been proposed to occur by a fusion-fission process, referred to as "kiss and run," involving late endosomes and lysosomes (Luzio et al., 2003).

In this unit, the authors describe a method for analyzing the transport of proteins to lysosomes using subcellular fractionation on Percoll gradients. As an example, the authors have used this method to analyze how mutations in known signal sequences in the cytoplasmic tail of the cation-dependent mannose-6-phosphate receptor affect the routing of this protein to lysosomes (Nair et al., 2003). In brief, a mutation in which the residues in a diaromatic motif in the cytoplasmic tail of the receptor are replaced by other amino acid residues leads to varying degrees of receptor mislocalization to lysosomes. Using Percoll density gradients, it is possible to determine the amount of receptor mislocalized to lysosomes within a given period of time, and this allows validation of the strict requirement for the critical diaromatic motif to be present to ensure proper intracellular sorting of the receptor. The homogeneity of the fractions obtained following density gradient centrifugation can be tested using enzymatic activity assays and immunoblotting (*UNIT 6.2*) for marker proteins.

The Basic Protocol described in this unit is aimed at determining the amount of a protein of interest in lysosomes. Additionally, a comparison of two different homogenization methods and a description of the conditions to be optimized for the cell line being used are provided later in the unit. Furthermore, methods of fraction analysis are described in detail in the Support Protocol.

FRACTIONATION OF CELLS USING A SELF-FORMING PERCOLL DENSITY GRADIENT

BASIC PROTOCOL

Sedimentation on self-forming Percoll gradients can be used to separate dense lysosomes from lower-density organelles such as endosomes, Golgi apparatus, plasma membrane, and ER. Separation is based on the sedimentation of particles to their isopycnic position—i.e., the position where the gradient density is equal to the density of the particle. The organelles are thus separated solely on the basis of differences in density, irrespective of their size. The gradient described in this protocol is a continuous gradient that forms

Contributed by Beat E. Schaub, Prashant Nair, and Jack Rohrer
Current Protocols in Cell Biology (2005) 15.8.1-15.8.12

upon centrifugation of a homogeneous Percoll suspension. This is in contrast to preformed gradients, which can be generated using various types of gradient makers (*UNIT 5.3*).

The method described here can be used for the analysis of transport of proteins to lysosomes. As an example, the authors study the misrouting of the cation-dependent mannose-6-phosphate receptor (CD-MPR) in a mouse L fibroblast cell line that lacks the cation-independent mannose-6-phosphate receptor (CI-MPR). The cell line used is transfected either with wild-type bovine CD-MPR or with a mutant form lacking the diaromatic endosomal sorting motif in its cytoplasmic tail. The effect of this mutation on the localization of the construct is analyzed by assessing levels of the construct in each fraction obtained upon gradient centrifugation. In order to detect receptor that has been mislocalized to lysosomes, the transfected cells are incubated with lysosomal protease inhibitors prior to homogenization.

In brief, cells are prepared, harvested, homogenized, and then fractionated on the Percoll gradient. A cushion and nine subsequent fractions are collected from the bottom of the gradient tube and pooled as follows: fractions 1 to 3 are combined to give pool I (containing 70% to 80% of lysosomal enzyme activity); fractions 4 to 6 are combined to give pool II (containing intermediate-density membranes); and fractions 7 to 9 are combined to give pool III (containing low-density membranes, including endosomes, Golgi apparatus, plasma membrane, and ER). Fraction analysis is described in the Support Protocol.

Materials

Mouse D9 L (Rec$^-$) fibroblast cell line (CRL-2648 mouse L cells available from ATCC; for mutant strain, contact Dr. Stuart Kornfeld at *skornfel@im.wustl.edu*) or other cell line of choice (e.g., HeLa)
Growth medium: e.g., Invitrogen α-minimal essential medium supplemented with 10% v/v fetal bovine serum, with or without 500 μg/ml geneticin
Growth medium containing 100 μM pepstatin A (from 30 mM stock in dimethyl sulfoxide; Sigma) and 100 μM leupeptin (from 30 mM stock in H_2O; Sigma)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
10× and 1× homogenization buffer (see recipe)
1× homogenization buffer/17.5% (v/v) Percoll solution (see recipe)
10% (v/v) Triton X-100
500× protease inhibitor cocktail (see recipe)
3× protein gel sample buffer (see recipe)
10-cm-diameter Falcon tissue culture dishes (BD Biosciences)
Rubber or plastic cell scraper
Refrigerated centrifuge with swinging-bucket rotor suitable for 15-ml tubes (e.g., Heraeus Cryofuge M7000)
Ball-bearing homogenizer (clearance, 16 μm; Isobiotec) or 25-G needle attached to 1-ml syringe
16 × 76–mm Ultra-Clear centrifuge tubes with metal screw caps (Beckman Coulter)
Refrigerated ultracentrifuge with Beckman Ti50 rotor (or equivalent) and Beckman TLA 100.3 rotor (or equivalent)
20-G needles
3-ml thick-walled polycarbonate ultracentrifuge tubes (Beckman Coulter)
1.5-ml polyallomer microcentrifuge tubes (Beckman Coulter) with suitable adaptors
Adaptors for centrifuging 1.5-ml microcentrifuge tubes in Beckman TLA 100.3 rotor (or equivalent)

NOTE: After cell culturing, perform all steps on ice, and cool all centrifuge parts to 4°C before use.

Prepare cells

1. Several days before fractionation is to be performed, plate the cells of interest on 10-cm-diameter tissue culture dishes (one dish of cells per sample to be analyzed). Grow the cells (*UNIT 1.1*) so that they reach 100% confluence 2 to 3 days before the start of the fractionation process. If the protein of interest is expected to undergo degradation in the lysosomes, add 10 ml growth medium supplemented with 100 μM pepstatin A and 100 μM leupeptin to the confluent cells 24 hr before performing step 2.

Homogenize cells

2. Aspirate growth medium from culture dish. Wash cells twice, each time by adding 10 ml ice-cold PBS and then aspirating.

   Process each culture dish separately throughout the steps that follow.

3. Add 2 ml ice-cold 1× homogenization buffer (HB) to the culture dish.

4. Scrape cells off of the dish using a rubber or plastic scraper, and then transfer the contents to a separate 15-ml centrifuge tube.

   It may be useful to hold the dish up to the light to check for parts of the cell lawn that might still be stuck to the surface following scraping.

5. To ensure that cell harvesting is complete, rinse the dish with 2 ml of 1× HB, scrape the surface of the dish with a rubber or plastic scraper, and then pool the contents of the dish with the material in the 15-ml tube from step 4.

6. Centrifuge the harvested cell suspension 5 min at ∼260 × *g*, 4°C, in a swinging-bucket rotor.

7. Aspirate the supernatant. Add 850 μl of 1× HB to the pellet, resuspend by trituration, and transfer the resulting suspension to a 1.5-ml microcentrifuge tube.

8. Homogenize the suspension using twelve strokes in a ball-bearing homogenizer with a clearance of 16 μm (optimal clearance for L, HeLa and HepG2 cells). Transfer the homogenized suspension to a 15-ml centrifuge tube.

   Alternatively, the cell suspension can be homogenized using twelve passes through a 1-ml syringe attached to a 25-G needle.

   The appropriate homogenizer clearance (and thus the diameter of choice for the ball bearing in the homogenizer) depends on the cell line being fractionated. Therefore, homogenizer clearance needs to be optimized empirically for the cell line of interest. In this optimization process, homogenization efficiency can be assessed by using special dyes (e.g., Evans blue) to stain intact nuclei and comparing the number of free nuclei after homogenization with the number of cells present before homogenization.

   Special care of the homogenizer is crucial to the success of every fractionation. Rust on the ball bearing and particles adhering to the tunnel walls can result in clearance aberrations, thereby creating suboptimal homogenization conditions. Rust and particle adherence can be prevented by cleaning the homogenizer with an appropriate brush, making sure not to use any detergent.

   It is important not to introduce air bubbles into the suspension during homogenization, as the presence of air bubbles can make passage through the homogenizer difficult and result in improper homogenization.

Fractionate cells

9. Add 850 μl of 1× HB to the homogenized suspension, and then centrifuge 10 min at ∼370 × *g*, 4°C, in a swinging-bucket rotor. Collect the supernatant (i.e., the

upper liquid phase) and transfer it to a separate 1.5-ml microcentrifuge tube kept on ice.

Following centrifugation, two liquid phases will be visible. The upper phase (referred to as the postnuclear supernatant, or PNS), which contains the cell membranes, will have a turbid appearance; this is the phase that should be collected. The lower phase, which contains cell nuclei, should be discarded.

10. Prepare a Percoll gradient on which to fractionate the postnuclear supernatant (PNS) collected in step 9.

    a. Add 1.2 ml of 10× HB to a 16 × 76–mm Beckman Ultra-Clear centrifuge tube.

       This 10× HB will serve as a sucrose cushion, ensuring that organelles are not pelleted by centrifugation.

    b. Using a pipet filler and pipet, carefully layer 8.5 ml of 1× HB/17.5% Percoll on top of the 10× HB sucrose cushion in the tube.

11. Using a pipet filler and pipet, carefully layer the PNS on top of the Percoll gradient. When PNS layering is complete, cap the Percoll gradient tube and centrifuge 30 min at ∼26,500 × *g*, 4°C, in an ultracentrifuge equipped with a Beckman Ti50 rotor or equivalent.

    If more than one gradient is being centrifuged, add 1× HB as necessary to ensure that all tubes are carrying the same weight.

12. After centrifugation, collect fractions in the following way.

    a. Uncap the tube, and then punch a hole in its bottom using a 20-G needle attached to a syringe.

       Uncapping of the centrifuge tube is critical, as this will prevent the subsequent introduction of air bubbles into the liquid in the tube.

    b. Draw 1 ml of liquid (i.e., the sucrose cushion) into the syringe from the bottom of the tube. Discard this liquid, and then use the syringe to collect nine more 1.2-ml fractions from the bottom of the tube. Transfer each collected fraction to a separate microcentrifuge tube.

       Beckman Ultra-Clear centrifuge tubes are soft and should therefore not be squeezed during fraction collection, as doing so can lead to turbulence and mixing of fractions.

13. Pool fractions 1 to 3 (pool I), fractions 4 to 6 (pool II), and fractions 7 to 9 (pool III). Use each of these fraction pools to fill a separate thick-walled 3-ml centrifuge tube, and save the remainder of each pool for use in step 15. Add PBS as necessary to ensure that all tubes are carrying the same weight.

    Alternatively, the collected fractions can be kept separate and analyzed individually.

    Sample tubes are adjusted to equal weight with the aid of a sensitive balance (±10 mg). Such a balance should also be used to equalize tube weights in steps 15 and 17.

    If larger tubes are available, each fraction pool can be transferred in its entirety to a separate tube and centrifuged in a single step (i.e., steps 14 and 15 can be skipped).

14. Centrifuge the pooled fractions 20 min at 300,000 × *g*, 4°C, in an ultracentrifuge equipped with a Beckman TLA 100.3 rotor or equivalent.

15. Remove and discard 0.5 to 1 ml of the supernatant from each centrifuge tube, add the rest of each fraction pool (i.e., what was saved in step 13) to the appropriate tube, and then add PBS as necessary to ensure that all tubes are carrying the same weight.

16. Centrifuge the pooled fractions once more, this time for 30 min at 300,000 × *g*, 4°C, in an ultracentrifuge equipped with a Beckman TLA 100.3 rotor or equivalent.

    After this centrifugation step, each tube will contain a white cell membrane band settled on top of a clear Percoll layer. Of the three fraction pools, pool III will have the highest membrane content; after centrifugation, membranes should be visible as a tight clump in this pool.

17. Using a micropipettor with a trimmed pipet tip, collect the entire white membrane band from each centrifuge tube, taking care to obtain as little excess liquid as possible. Carefully transfer each collected band to a separate 1.5-ml polyallomer microcentrifuge tube. Add PBS as necessary to ensure that all of the microcentrifuge tubes are carrying the same weight.

    It is advisable to cut the pipet tip before collecting the membranes. This prevents loss of membrane material during collection.

18. Centrifuge the collected membrane layers 30 min at 200,000 × *g*, 4°C, in an ultracentrifuge equipped with a TLA 100.3 rotor (or equivalent) fitted with suitable adaptors.

    This additional centrifugation step removes residual Percoll from the collected membranes. Although Percoll is inert and does not penetrate organelles, it can hamper the smooth running of polyacrylamide gels during fraction analysis.

19. Using a micropipettor with a trimmed pipet tip, collect the membrane layer from each tube (leaving the clear Percoll layer behind) and transfer it to a clean 1.5-ml microcentrifuge tube. Add PBS as necessary to adjust the amount of liquid in each tube, using a sensitive balance to ensure that all tubes are carrying the same weight.

20. Add 1/20 volume of 10% Triton X-100 and 1 μl of 500× protease inhibitor cocktail (PIC) to each tube and vortex.

    Triton X-100 solubilizes membranes, thereby releasing hydrolytic enzymes. Among the hydrolytic enzymes released are proteases, which are inhibited by the subsequent addition of protease inhibitors.

    Some antibody-antigen interactions are affected by Triton X-100, and therefore, it may be necessary to use a different detergent, depending on the protein being analyzed.

    If membranes do not dissolve after the addition of Triton X-100, it will be necessary to sonicate the membrane samples.

Analyze fractions

21. Use the contents of the tubes from step 20 to perform the β-hexosaminidase assay (Support Protocol).

22. If the results of the β-hexosaminidase assay are satisfactory, prepare samples for further analysis by combining material from step 20 with an appropriate volume of 3× protein gel loading buffer and then boiling 3 min. Use these samples to perform SDS-PAGE (*UNIT 6.1*) followed by immunoblotting with the antibody of choice (*UNIT 6.2*).

β-HEXOSAMINIDASE ASSAY TO CHECK THE EFFICIENCY OF FRACTIONATION

SUPPORT PROTOCOL

This assay is used to determine how the activity of the lysosomal marker enzyme β-hexosaminidase activity is distributed among the different pools obtained at the end of fractionation in the Basic Protocol. Therefore, the results of the assay indicate, to a certain extent, the purity or homogeneity of the lysosomal fractions obtained using Percoll gradient fractionation. Ideally, pool I should show >70% of all β-hexosaminidase activity; such a finding would indicate that the lysosomal fraction is acceptably pure and

that the other fractions are mostly free of lysosomes. It is worth mentioning that certain lysosomal enzymes are partially activated in late endosomes (Hunziker and Geuze, 1996). The assay presented here has been used successfully in several studies for the assessment of fraction purity (Rohrer et al., 1995; Nair et al., 2003).

Materials

Percoll gradient fractions/pools (see Basic Protocol)
β-Hexosaminidase substrate mix (see recipe)
0.2 M Na_2CO_3
Spectrophotometer capable of reading at 400 nm

1. Transfer 5 μl of each fraction/pool to a separate 1.5-ml microcentrifuge tube. To each tube, add distilled water to a total volume of 80 μl. Prepare a blank sample by adding 80 μl distilled water to another 1.5-ml tube.
2. Add 200 μl β-hexosaminidase substrate mix to each tube (including the blank) and incubate for 15 to 30 min at 37°C.

   To maximize the likelihood of staying within the linear range of the assay, do not incubate samples longer than 30 min.

   Samples turn turbid in this step, because their pH is lowered by the addition of substrate mix.
3. Stop the β-hexosaminidase-catalyzed reaction by adding 1 ml of 0.2 M Na_2CO_3 to each tube (including the blank).

   Samples (aside from the blank) should begin turning yellow upon the addition of 0.2 M Na_2CO_3.
4. Set the spectrophotometer wavelength to 400 nm, zero the spectrophotometer using the blank sample, and then obtain an optical density (OD) reading for each experimental sample.

   If the OD for a given sample is not within the linear range (0.1 to ~1.5 OD units), prepare a new sample (step 1) using a smaller or larger aliquot of the fraction/pool used to prepare the original sample, and then repeat steps 2 to 4 using this new sample. The OD reading yielded by the new sample must be normalized before it can be directly compared with other OD readings. This is done by multiplying the new OD reading by 5/v, where v is the volume (in μl) of the fraction/pool aliquot used to prepare the new sample.
5. For each Percoll gradient used in the Basic Protocol, calculate the percentage of β-hexosaminidase activity attributable to each of the fractions/pools obtained from that gradient.

   The calculations in this step are made under the following assumptions: (1) each OD reading is proportional to the amount of β-hexosaminidase activity in the corresponding fraction/pool; and (2) the amount of activity in pool I, the amount of activity in pool II, and the amount of activity in pool III (or, equivalently, the activity values observed in fractions 1 to 9) add up to 100%.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

β-Hexosaminidase substrate mix

For one sample:
50 μl of 0.01 M *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma)
15 μl of 10 M sodium citrate, pH 4

continued

3 μl of 10% (v/v) Triton X-100
132 μl H_2O
Prepare fresh before each use, and scale recipe up according to the number of samples to be tested

Homogenization buffer (10× stock)

2.5 M sucrose
10 mM EDTA
Store for several months at 4°C

Percoll solution, 17.5% (v/v)

To make enough material for four gradients, add 7 ml of 90% (v/v) Percoll solution (see recipe) to 29 ml of 1× homogenization buffer (see recipe for 10× stock). Store for several months at 4°C.

Percoll solution, 90% (v/v)

90 ml Percoll
10 ml of 10× homogenization buffer (see recipe)
Store for several months at 4°C

Protease inhibitor cocktail (500× stock)

To a 3:2 (v/v) mixture of ethanol and dimethyl sulfoxide, add:
5 mg/ml benzamidine
1 mg/ml pepstatin A
1 mg/ml leupeptin
1 mg/ml antipain
1 mg/ml chymostatin
Store for several months at −20°C

Protein gel sample buffer (3× stock)

1.135 g Tris base (0.1875 M final)
3 g SDS (6% w/v final)
15 g glycerol (30% w/v final)
5 mg bromphenol blue (0.003% w/v final)
2.31 g dithiothreitol (0.3 M final; where required)
Add H_2O to 40 ml and then adjust pH to 6.8 with 1 M HCl
Add H_2O to 50 ml
Store for several months at −20°C

COMMENTARY

Background Information

Lysosomes are degradative organelles that contain numerous acid hydrolases capable of catabolizing proteins, lipids, nucleic acids, and carbohydrates. Among the plethora of lysosomal functions are turnover of cellular constituents, breakdown of damaged macromolecules, autophagy, and antigen processing. Proper synthesis and delivery of the acid hydrolases are crucial to the physiological functioning of this organelle. Defects in lysosomal enzyme synthesis, functioning, or targeting can lead to one of the many known lysosomal storage disorders (Varki et al., 1982; Wilcox, 2004; Vellodi, 2005). The limiting membrane of the lysosome has several important functions, such as acidification and sequestration of lysosomal contents, maintenance of lysosomal stability and integrity, and export of the degradation products resulting from catabolic activity (Peters and von Figura, 1994). The proper transport of lysosomal membrane glycoproteins is thus equally crucial. The membrane is composed of several transient and resident glycoproteins (Williams and Fukuda, 1990; Peters and von Figura, 1994), including LAP (lysosomal acid phosphatase), LAMP-1 (lysosome-associated membrane protein 1), LAMP-2 (lysosome-associated membrane protein 2),

and LIMP-2 (lysosomal integral membrane protein 2).

The biogenesis of lysosomes results from the convergence of the endocytic and biosynthetic pathways. The soluble lysosomal hydrolases are transported initially by the biosynthetic pathway to the *trans*-Golgi network (TGN), by the cation-dependent mannose-6-phosphate receptor (CD-MPR) and the cation-independent mannose-6-phosphate receptor (CI-MPR) from the TGN to endosomes, and subsequently by the endocytic pathway to lysosomes. Lysosomes are thought to mature by a process of fusion and fission with the penultimate organelle in the biosynthetic pathway, referred to as the late endosome, thereby acquiring the lysosomal hydrolases (Storrie and Desjardins, 1996). However, lysosomes are devoid of mannose-6-phosphate receptors, which get retrieved by the *trans*-Golgi network because they possess signals for escaping lysosomal delivery.

The lysosomal membrane proteins are transported in much the same way, with their inclusion in the lysosome being attributable to signals that direct lysosomal delivery. The cytoplasmic tails of LAMP-1, LAMP-2, and LAP all contain tyrosine-based signals for endocytosis and delivery to the lysosome (Peters et al., 1990; Williams and Fukuda, 1990; Lehmann et al., 1992). Kinetic analysis of the delivery of lysosomal membrane proteins has revealed that the median time ($t_{1/2}$) for delivery of LAMP-1, LAMP-2, and LIMP-2 to lysosomes is ~30 to 45 min, while LIMP-1 is delivered to lysosomes with a $t_{1/2}$ of ~90 min (Barriocanal et al., 1986; D'Souza and August, 1986; Gottschalk et al., 1989). However, other lysosomal membrane proteins, such as the precursor of LAP, are delivered rather slowly to dense lysosomes. LAP undergoes, on average, 20 to 50 cycles of transport between endosomes and the plasma membrane before being delivered to lysosomes (Braun et al., 1989). This indicates that the sorting signal directing the transport of LAP from endosomes to lysosomes is not very efficient.

Density gradient centrifugation has long been the method of choice for the separation of intracellular organelles. The position of an organelle in a density gradient depends on the lipid-to-protein ratio in the organelle's membranes, the organelle's contents, and the density of the particles potentially attached to the organelle. Percoll is a modified form of colloidal silica that was introduced by Amersham Pharmacia Biotech in 1977 as a medium for density gradient centrifugation. It provides a useful density range for separation of organelles, is nontoxic, possesses physiological ionic strength and pH, does not penetrate into the organelles, is osmotically inactive, and is easily removed from purified organelles. Furthermore, self-forming Percoll gradients can be generated by a single centrifugation step.

Percoll density gradients have long been used as a reliable method for studying transport of proteins to lysosomes. The combination of Percoll gradient fractionation with pulse–chase methods greatly increases the potential of this technique for studying the kinetics of lysosomal protein transport. The transport of newly synthesized LAMP-1 from Golgi to lysosomes, for example, was studied by isolating lysosomes using isosmotic Percoll gradient fractionation followed by immunoprecipitation of LAMP-1 from lysosomal fractions (Green et al., 1987). Furthermore, lysosomal structures containing LAMP-1 and the lysosomal enzyme β-glucuronidase were analyzed by electron microscopic examination of frozen sections of lysosomal fractions purified using a Percoll gradient (Green et al., 1987). Percoll density gradient fractionation has also been used to show that the targeting of LAMP-1 to lysosomes is dependent on the spacing of LAMP-1's tyrosine-based sorting motif with respect to the membrane (Rohrer et al., 1996). Likewise, this technique has been used to demonstrate that the correct localization of the CD-MPR and its function in lysosomal enzyme sorting are dependent on the reversible palmitoylation of cysteine-34, which resides in the cytoplasmic tail of the CD-MPR (Schweizer et al., 1996). Furthermore, Percoll gradient fractionation has been used to show that correct endosomal sorting of the human mannose receptor depends on a diaromatic motif in the receptor's cytoplasmic tail, with this motif ensuring that the receptor escapes from lysosomal delivery (Schweizer et al., 2000).

Critical Parameters

The success of every fractionation experiment depends on the optimization of specific experimental steps (see Basic Protocol) for the cell line being used. Key among these are the culturing and homogenization of the cells. The cell lines used in the protocol described here were kept confluent for 2 days before homogenization. In the authors' experience, lysosomes appear to be denser in confluent cells, thus facilitating their separation.

Table 15.8.1 Troubleshooting Guide for Percoll Gradient Fractionation

Problem	Possible cause	Solution
Less than 70% of β-hexosaminidase activity found in pool I, or nonlysosomal markers found in pool I	Improper separation	Verify confluence of cells, adjust centrifugation conditions, and check density of Percoll solution using density marker beads (Amersham)
No membranes pelleted by gradient centrifugation, or too little reporter protein detected in immunoblot	Inadequate homogenization	Optimize homogenization process using guidelines listed in Critical Parameters; measure amount of protein in postnuclear supernatant before loading gradient

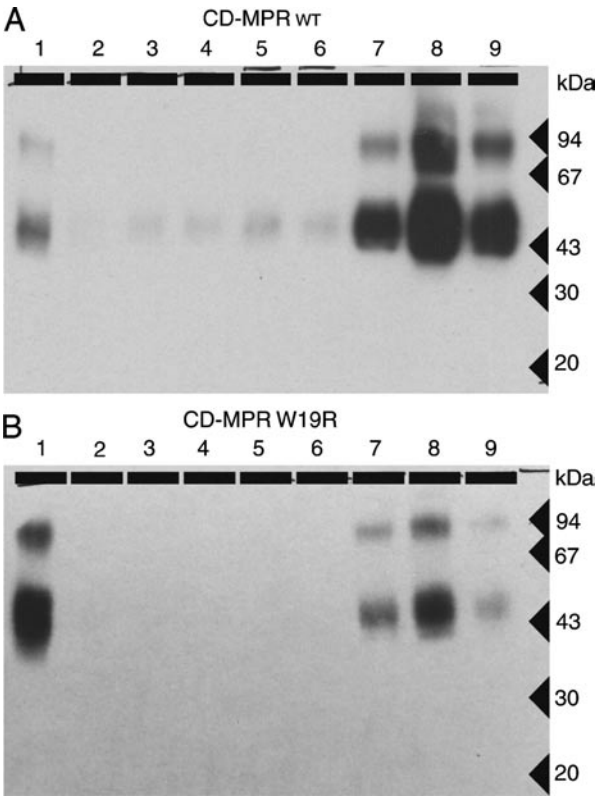


Figure 15.8.1 Immunoblots showing the distribution of wild-type (wt) and mutant cation-dependent mannose-6-phosphate receptor (CD-MPR) among the individual fractions obtained following Percoll density gradient centrifugation. (**A**) Most of the wild-type CD-MPR is recovered in fractions 7, 8, and 9, which contain low-density membranes such as the endoplasmic reticulum (ER), the Golgi membrane, and the plasma membrane. Very little wild-type CD-MPR is detected in fractions 1, 2, and 3, which contain the dense lysosomes and late endosomes. (**B**) A significant amount of CD-MPR mutant W19R is misrouted to lysosomes and subsequently detected in fraction 1. Anti-CD-MPR monoclonal antibody 22D4 was a kind gift from Don Messner.

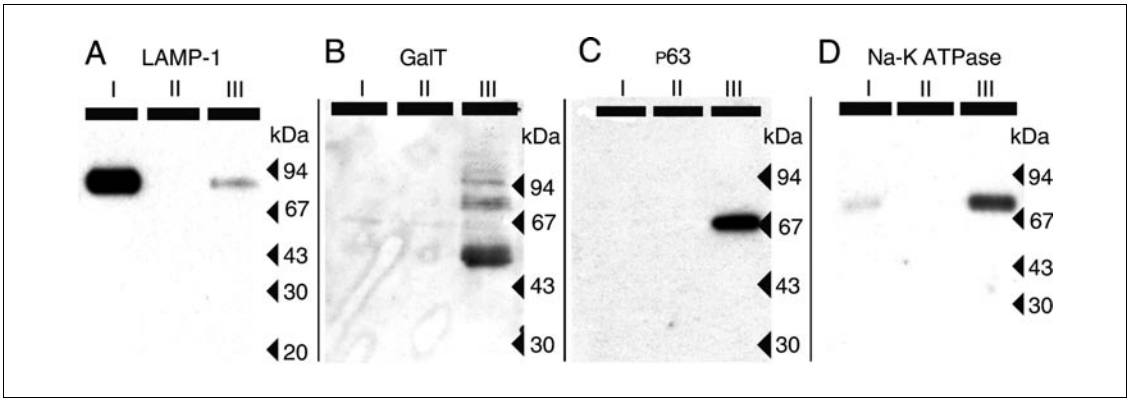


Figure 15.8.2 Immunoblots showing the distributions of four marker proteins from different cell compartments separated by Percoll density gradient centrifugation. (**A**) LAMP-1 is found primarily in pool I (fractions 1 to 3), which contains the dense lysosomes. (**B**) Galactosyltransferase (GalT), a Golgi marker, is found exclusively in pool III (fractions 7 to 9), which contains low-density membranes. (**C**) p63, a marker protein for the endoplasmic reticulum (ER), is also found exclusively in pool III. (**D**) The sodium-potassium (Na-K) ATPase, a marker of the plasma membrane, is found in pool III as well. Antibodies to LAMP-1 and sodium-potassium ATPase were generously provided by Hans-Peter Hauri, and the antibody to GalT was a kind gift from Eric G. Berger.

Excessive homogenization can result in disruption of organelles, while incomplete homogenization leads to poor recovery. It is important to treat all of the samples in each experiment identically during homogenization, in order to reduce experimental variability. Thus, the number of strokes applied with a ball-bearing homogenizer or syringe should be kept constant within an experiment in order to generate reproducible results.

Gradient formation is a function of the percentage of Percoll used. A self-forming Percoll gradient containing 17.5% (v/v) Percoll is used in the Basic Protocol. However, the optimal Percoll concentration depends on the cell line used and the organelle being isolated. The conditions for centrifugation also affect the linearity of gradient formation. For instance, rotor geometry has been shown to affect gradient shape (*UNIT 3.1*). Specifically, as the angle of the tube in the rotor approaches vertical, the path length for gradient formation becomes shorter and the gradient forms more rapidly. The speed and time of centrifugation also affect the shape of the gradient and thus have to be optimized for each experimental setup.

Harvesting the gradient is the final crucial step to be considered. Care should be taken not to introduce air bubbles into the gradient, as this leads to gradient mixing and hampers separation. The flow rate of fraction collection is another critical factor; if the gradient is fractionated too rapidly, mixing could occur.

Troubleshooting

See Table 15.8.1 for a guide to solving problems encountered in Percoll gradient fractionation.

Anticipated Results

The gradient centrifugation method described in this unit has been published previously (Rohrer et al., 1996; Schweizer et al., 1996; 1997; 2000). An example of an analysis of the amount of wild-type versus mutant cation-dependent mannose-6-phosphate receptor in lysosomes is shown in Figure 15.8.1. In their experiments, the authors observed that the lysosomal marker LAMP-1 is found primarily in the fraction containing lysosomes and late endosomes (Fig. 15.8.2). The presence of LAMP-1 on the membranes of late endosomes had been documented previously, confirming the homogeneity of the fraction obtained (Griffiths et al., 1988). Galactosyltransferase, a Golgi marker, was detected exclusively in pool III, where Golgi membranes are expected to band. The same was also true for p63, a marker protein for the rough endoplasmic reticulum (ER). The cation-dependent mannose-6-phosphate receptor, a protein found in late endosomes, early endosomes, the plasma membrane, and the Golgi, also was detected primarily in pool III, as was expected. The sodium-potassium ATPase, a plasma membrane marker, was also found predominantly in pool III (Fig. 15.8.2).

Table 15.8.2 Comparison of Various Experimental Conditions and Their Effects on the Distribution of β-Hexosaminidase Activity

Experimental parameter	% β-hexosaminidase activity in pool I
Cell confluence[a]	
Dense cells	71.1–76.3
Subconfluent cells	38.9–46.8
Homogenization instrument[b]	
Ball-bearing homogenizer	75.3–81.3
Syringe	47.7–68.9
Protease inhibitor treatment of cells[c]	
No	67.7–82.3
Yes	72.8–85.9

[a]Cells were either kept confluent for 2 to 3 days (*dense cells*) or harvested at subconfluent levels (*subconfluent cells).*
[b]Cells were homogenized using either a ball-bearing homogenizer or a 1-ml syringe equipped with a 25-G needle.
[c]Cells were cultured with or without being treated with 100 μM pepstatin A and 100 μM leupeptin for 24 hr.

Comparison of dense versus subconfluent cultures of HeLa cells showed that the denser cultures resulted in better Percoll gradient separation of lysosomes from the other fractions (Table 15.8.2). Furthermore, denser cultures made membrane collection following gradient centrifugation easier. A comparison of the two homogenization methods revealed that homogenization tends to be more variable when using a syringe as opposed to a ball-bearing homogenizer. In addition, the separation obtained after using a ball-bearing homogenizer was found to be superior as revealed by the β-hexosaminidase assay (Table 15.8.2). A comparison of two different cell lines, HeLa and murine D9 fibroblast cells, showed that while a 17% (v/v) Percoll gradient resulted in optimal separation of fractions from HeLa cells, a 17.5% (v/v) Percoll gradient provided better separation of fractions from D9 cells.

Time Considerations

Each individual experiment (assuming a total of four gradients per experiment) takes ~11 hr, according to the following breakdown: preparation of extract for loading onto the gradient, 2 hr; gradient formation, separation, and collection, 1.5 hr; Percoll removal, 3 hr; and fraction analysis, 4 to 5 hr. However, the exact time required needs to be determined empirically for each experimental setup and will depend on the number of gradients to be run and the number of centrifuges available, among other factors.

Literature Cited

Barriocanal, J.G., Bonifacino, J.S., Yuan, L., and Sandoval, I.V. 1986. Biosynthesis, glycosylation, movement through the Golgi system, and transport to lysosomes by an N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *J. Biol. Chem.* 261:16755-16763.

Bonifacino, J.S. and Dell'angelica, E.C. 1999. Molecular bases for the recognition of tyrosine-based sorting signals. *J. Cell. Biol.* 145: 923-926.

Braun, M., Waheed, A., and von Figura, K. 1989. Lysosomal acid phosphatase is transported to lysosomes via the cell surface. *EMBO J.* 8:3633-3640.

D'Souza, M.P. and August, J.T. 1986. A kinetic analysis of biosynthesis and localization of a lysosome-associated membrane glycoprotein. *Arch. Biochem. Biophys.* 249:522-532.

Gottschalk, S., Waheed, A., Schmidt, B., Laidler, P., and von Figura, K. 1989. Sequential processing of lysosomal acid phosphatase by a cytoplasmic thiol proteinase and a lysosomal aspartyl proteinase. *EMBO J.* 8:3215-3219.

Green, S.A., Zimmer, K.P., Griffiths, G., and Mellman, I. 1987. Kinetics of intracellular transport and sorting of lysosomal membrane and plasma membrane proteins. *J. Cell. Biol.* 105:1227-1240.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. 1988. The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* 52:329-341.

Hunziker, W. and Geuze, H.J. 1996. Intracellular trafficking of lysosomal membrane proteins. *Bioessays* 18:379-389.

Lehmann, L.E., Eberle, W., Krull, S., Prill, V., Schmidt, B., Sander, C., von Figura, K., and Peters, C. 1992. The internalization signal in the cytoplasmic tail of lysosomal acid phosphatase consists of the hexapeptide PGYRHV. *EMBO J.* 11:4391-4399.

Luzio, J.P., Poupon, V., Lindsay, M.R., Mullock, B.M., Piper, R.C., and Pryor, P.R. 2003. Membrane dynamics and the biogenesis of lysosomes. *Mol. Membr. Biol.* 20:141-154.

Nair, P., Schaub, B.E., and Rohrer, J. 2003. Characterization of the endosomal sorting signal of the cation-dependent mannose 6-phosphate receptor. *J. Biol. Chem.* 278:24753-24758.

Peters, C. and von Figura, K. 1994. Biogenesis of lysosomal membranes. *FEBS Lett.* 346:108-114.

Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A., and von Figura, K. 1990. Targeting of a lysosomal membrane protein: A tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. *EMBO J.* 9:3497-3506.

Rohrer, J., Schweizer, A., Johnson, K.F., and Kornfeld, S. 1995. A determinant in the cytoplasmic tail of the cation-dependent mannose 6- phosphate receptor prevents trafficking to lysosomes. *J. Cell. Biol.* 130:1297-1306.

Rohrer, J., Schweizer, A., Russell, D., and Kornfeld, S. 1996. The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. *J. Cell. Biol.* 132:565-576.

Schmidt, B., Selmer, T., Ingendoh, A., and von Figura, K. 1995. A novel amino acid modification in sulfatases that is defective in multiple sulfatase deficiency. *Cell* 82:271-278.

Schweizer, A., Kornfeld, S., and Rohrer, J. 1996. Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. *J. Cell. Biol.* 132:577-584.

Schweizer, A., Kornfeld, S., and Rohrer, J. 1997. Proper sorting of the cation-dependent mannose 6-phosphate receptor in endosomes depends on a pair of aromatic amino acids in its cytoplasmic tail. *Proc. Natl. Acad. Sci. U.S.A.* 94:14471-14476.

Schweizer, A., Stahl, P.D., and Rohrer, J. 2000. A di-aromatic motif in the cytosolic tail of the mannose receptor mediates endosomal sorting. *J. Biol. Chem.* 275:29694-29700.

Storrie, B. and Desjardins, M. 1996. The biogenesis of lysosomes: Is it a kiss and run, continuous fusion and fission process? *Bioessays* 18:895-903.

Varki, A., Reitman, M.L., Vannier, A., Kornfeld, S., Grubb, J.H., and Sly, W.S. 1982. Demonstration of the heterozygous state for I-cell disease and pseudo-Hurler polydystrophy by assay of N-acetylglucosaminylphosphotransferase in white blood cells and fibroblasts. *Am. J. Hum. Genet.* 34:717-729.

Vellodi, A. 2005. Lysosomal storage disorders. *Br. J. Haematol.* 128:413-431.

Wilcox, W.R. 2004. Lysosomal storage disorders: The need for better pediatric recognition and comprehensive care. *J. Pediatr.* 144:3-14.

Williams, M.A. and Fukuda, M. 1990. Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell. Biol.* 111:955-966.

Contributed by Beat E. Schaub, Prashant Nair, and Jack Rohrer
University of Zurich
Zurich, Switzerland

Studies of the Ubiquitin Proteasome System

The ubiquitin conjugating system, initially described in the late 1970's and early 1980's, was first associated with the targeting of proteins for degradation in proteasomes. While this is clearly the best-studied role for ubiquitylation, it is now appreciated that modification of proteins with ubiquitin has a large number of other functions unrelated to proteasomal targeting (Fig. 15.9.1). These include roles in endocytosis and targeting to lysosomes/vacuoles, in activation of kinase signaling pathways, and in regulating transcription and nuclear-cytoplasmic shuttling. It is no exaggeration that ubiquitylation is now implicated, in one way or another, in essentially all cellular processes in eukaryotes.

There are also a number of ubiquitin-like molecules that are linked to proteins through enzymatic cascades similar to ubiquitylation. Like ubiquitin, these are attached to other proteins by isopeptide linkages between glycines at their carboxyl termini and primary amines on target proteins. Well characterized ubiquitin-like molecules include SUMO1-3, NEDD8, ISG15, and APG12. Among these, NEDD8 is associated primarily with activation of cullin-containing ubiquitin protein ligases; therefore, aspects of the neddylation system are included in this unit (Basic Protocols 14, 15, 16, and Support Protocols 1 and 2).

In general, modification of proteins with ubiquitin (Ub) involves a multienzyme process that utilizes enzymes known as ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2), and ubiquitin protein ligases (E3). There is a single E1, eleven E2s in yeast and over 35 in mammals, and well over 500 different substrate-specific ubiquitin ligases.

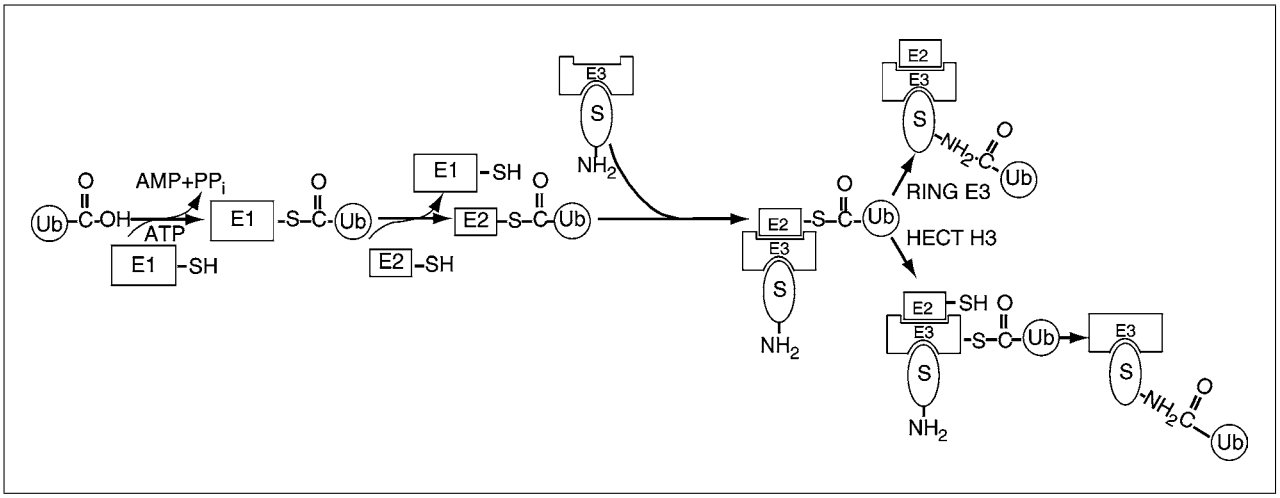


Figure 15.9.1 The ubiquitylation pathway. Free ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s, which might or might not have substrate already bound. For HECT domain E3s, ubiquitin is next transferred to the active-site cysteine of the HECT domain followed by transfer to substrate (S) (as shown) or to a substrate-bound multi-ubiquitin chain. For RING E3s, current evidence indicates that ubiquitin might be transferred directly from the E2 to the substrate. Reproduced with permission from *Nature Reviews Molecular Cell Biology* (A.M. Weissman. 2001. 2:169-178.) copyright 2001 Mcmillan Magazines Ltd (*http://www.nature.com/reviews*).

Contributed by Kevin L. Lorick, Yili Yang, Jane P. Jensen, Kazuhiro Iwai, and Allan. M. Weissman
Current Protocols in Cell Biology (2006) 15.9.1-15.9.85

The details of ubiquitylation are discussed in depth elsewhere (Weissman, 2001; Fang and Weissman, 2004); however, the essentials include invariant steps beginning with the ATP-dependent activation of ubiquitin by E1 where a thiolester linkage between the active-site cysteine of E1 and the C-terminus of ubiquitin is formed. Next the transesterification of ubiquitin to the active-site cysteine of an E2 occurs, followed by the association of E2 with members of the various families of E3s.

While there are exceptions, E3s can be divided into two classes. The first of these is the HECT-domain E3s which have large, highly conserved 350-amino acid catalytic domains and, like E1 and E2, form thiolester intermediates with ubiquitin through conserved cysteine residues as part of the process leading to substrate modification. The second class of E3s is the RING-finger and related E3s. In addition to the RING-finger E3s, this family includes the PHD-finger E3s, which like the RING-finger E3s, coordinate two zinc ions. The U-box E3 conforms to a cross-brace structure in a manner similar to the RING and PHD fingers, but without apparent metal coordination. RING-finger E3s include proteins in which the RING finger and the substrate-recognition element exist on the same polypeptide as well as more complex E3s in which the RING finger is but one subunit of a multi-subunit assembly with a RING finger–containing core associating with various substrate-recognition elements. Most notable among these are the cullin-containing E3s, all of which include a small RING-finger protein.

An important aspect of ubiquitylation is the propensity to form multi-ubiquitin chains on proteins, involving linkages between one of the seven lysines on one ubiquitin and the C terminal of the next ubiquitin in the chain. Chains of four or more ubiquitins linked through K48 represent potent proteasomal targeting signals for modified proteins, while other linkages, or the presence of a single ubiquitin, are associated with other functions. The 26S proteasome is a highly developed and regulated molecular machine that includes a multicatalytic cylindrical 20S proteolytic core. This is capped at either end by a highly complex 19S cap that includes, among other things, multiple ATPases, subunits that recognize ubiquitin and proteins having ubiquitin-like domains, and several distinct deubiquitylating activities (Glickman and Ciechanover, 2002). Akin to the dynamic nature of phosphorylation and dephosphorylation, ubiquitylation is actively opposed by deubiquitylation (Wilkinson, 1997). There are a large number of different deubiquitylating enzymes (DUbs) of at least three distinct classes. These are ubiquitously present and highly active. A major challenge to researchers is detecting ubiquitylation with the ongoing and competing processes of deubiquitylation and proteasomal degradation. Accordingly, in the protocols below, ways to overcome these issues are repeatedly emphasized.

The number of techniques and reagents in common use that are related to ubiquitylation is rapidly growing; thus, the methods described below are by no means inclusive. However, they provide basic techniques to generate tools for determining if a particular protein is modified by ubiquitin or plays a role in the ubiquitylation process and to evaluate the nature of ubiquitin linkages. Further they provide important initial approaches for assessing the fate of ubiquitylated proteins. See Table 15.9.1 for a guide to the protocols included in this unit.

Table 15.9.1 Summary of Protocols Described in this Unit

Assay	Protocol	Authors
E1 and E2 activity		Jensen, Yang, Lorick, Iwai, and Weissman
E1-ubiquitin thiolester formation	Basic Protocol 1	
E2-ubiquitin thiolester formation	Basic Protocol 2	
Binding of ubiquitin-proteasome proteins		Lorick, Jensen, Yang, and Weissman
E2 binding to E3	Basic Protocol 3	
Ubiquitin substrate binding to E3	Alternate Protocol 1	
Ubiquitylation (E3) assays		Lorick, Jensen, Yang, and Weissman
E3 auto-ubiquitylation	Basic Protocol 4	
Auto-ubiquitylation vs. pseudo-substrate ubiquitylation	Basic Protocol 5	
Ubiquitylation of E3 enzymes in in vitro translation	Basic Protocol 6	
Determination of ubiquitin chain variant phenotype	Alternate Protocol 2	
Chelation of zinc from RING- and PHD-finger E3s	Basic Protocol 7	
Inhibition of HECT-domain E3s	Alternate Protocol 3	
Substrate ubiquitylation in vitro		Lorick, Yang, Jensen, Iwai, and Weissman
Substrate ubiquitylation in solution	Basic Protocol 8	
Substrate ubiquitylation after E3 binding	Alternate Protocol 4	
Substrate ubiquitylation by multiple subunit E3s	Basic Protocol 9	
Detection of E3 activity in immunoprecipitates	Basic Protocol 10	
Ubiquitin modification of substrate after GST pull-down	Alternate Protocol 5	
Detection of ubiquitylation in vivo		Yang, Lorick, Jensen, and Weissman
Lysis and immunoprecipitation	Basic Protocol 11	
Detection with tagged ubiquitin	Alternate Protocol 6	
Inhibition of ubiquitylation and degradation		Yang, Lorick, Jensen, and Weissman
Use of dominant negative ubiquitin protein	Basic Protocol 12	
Use of small interfering RNAs	Alternate Protocol 7	
Localizing degration to the proteasome	Basic Protocol 13	

continued

Table 15.9.1 Summary of Protocols Described in this Unit, *continued*

Assay	Protocol	Authors
NEDD8 conjugation		Iwai and Lorick
Purification of APP-BPI/UBA3	Basic Protocol 14	
Batch purification	Support Protocol 1	
Column purification	Support Protocol 2	
Production of UBC12 and NEDD8	Basic Protocol 15	
In vitro neddylation	Basic Protocol 16	
Labeling ubiquitin and detection		Jensen, Lorick, Yang, and Weissman
Labeling with ^{32}P	Basic Protocol 17	
Labeling with ^{125}I	Alternate Protocol 8	
Labeling with nonradioactive tags	Alternate Protocol 9	
Generating tagged ubiquitin plasmids	Alternate Protocol 10	
Immunoblotting with anti-ubiquitin	Basic Protocol 18	
Generation and purification of rabbit anti-ubiquitin		Jensen, Yang, Lorick, and Weissman
Preparation of antigen	Basic Protocol 19	
Preparation of ubiquitin affinity column	Support Protocol 3	
Generation of reagent-grade E1		Jensen, Iwai, Lorick, Yang, and Weissman
Preparation of wheat E1 in *E. coli*	Basic Protocol 20	
Preparation of mouse E1 in insect cells	Basic Protocol 21	
Preparation of rabbit E1	Alternate Protocol 11	
Generation of empty bacterial lysates	Support Protocol 4	
Generation of E2		Jensen, Iwai, Lorick, Yang, and Weissman
Expression of E2 in *E. coli* without affinity tag	Basic Protocol 22	
Purification of ubiquitin from *E. coli* using an affinity tag	Alternate Protocol 12	
Purification of E2 from *E. coli* using anti-affinity tag antibodies	Alternate Protocol 13	
In vitro transcription/translation of E2	Basic Protocol 23	
In vitro production of E3s		Lorick, Iwai, Jensen, Yang, and Weissman
Expression of RING-finger E3s in bacterial lysates	Basic Protocol 24	
Expression of HECT and U-box E3s in bacterial lysates	Alternate Protocol 14	

continued

Table 15.9.1 Summary of Protocols Described in this Unit, *continued*

Assay	Protocol	Authors
Expression of unstable E3s in bacterial lysates	Alternate Protocol 15	
Expression of E3s in eukaryotic in vitro translation systems	Basic Protocol 25	
Expression of multi-subunit E3s in using a baculovirus expression system	Basic Protocol 26	
Batch purification of multimeric E3s	Support Protocol 5	
Column purification of multimeric E3s	Support Protocol 6	

ASSAYS OF E1 AND E2 ACTIVITY

For large amounts of E1 and E2, consider producing them in house (see Table 15.9.1). Purchasing these reagents is a viable alternative when performing only a few assays. No matter the source, reagents should be tested before using them extensively for assays involving E3s and substrates. Assays for E1 and E2 activity are simple, and consist of detecting thiolester bonds between Ub and the active-site cysteine of the ubiquitin enzyme. The importance of validating reagents in this way cannot be overstated. In the authors' experience, different preparations of E1 can have vastly different activities, due either to amount of time in the freezer or to variations in reducing agent concentrations. In addition, because a particular E3 will show activity only in the presence of a subset of E2 enzymes, the researcher has to be aware of the relative activity of the E2 before concluding that a particular E2/E3 pair has no activity or that an E3 functions preferentially with a particular E2. The protocols provided make use of radiolabeled ubiquitin or cold ubiquitin with anti-ubiquitin antibody immunodetection.

Strategic Planning

Before beginning, be aware of the E1 that is being used. The authors have found E1-thiolesters that are more easily detectable with commercial rabbit E1 antibodies than mouse or wheat E1 generated in the laboratory. This may be a function of some combination of activity and concentration. Where E1 does not exhibit discernable thiolester formation on its own, an E1/E2 combination should be tested as in Basic Protocol 2.

For Basic Protocol 2, make sure that a suitable positive control is used. Wheat E1 works well with mammalian type I E2s, those with only a catalytic core domain. It should not be assumed that it functions well with all mammalian type II, III, and IV E2s, where there are N-terminal, C-terminal, or N-and C-terminal extensions beyond the core domain. On the other hand, mammalian E1s appear to work well with all E2s. The authors find that bacterially expressed UbcH5B is a good positive control that functions with E1s of all sources.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

Thiolester Formation Between Rabbit E1 and Ubiquitin

This protocol is used generally to test the activity of E1 before doing more complex assays. It can be the primary assay used in screens of compounds to be used as E1 inhibitors.

Materials

E1: rabbit E1 (Calbiochem, Boston Biochem, Affiniti), bacterially expressed wheat E1 (Basic Protocol 20), or mouse E1 (Basic Protocol 21)
100 mM HEPES, pH 7.5
10× thiolester buffer (see recipe)
[^{32}P]ubiquitin (Basic Protocol 17) *or* 10 μg/μl "cold" (unlabeled) ubiquitin (Sigma) for immunoblot detection
4× nonreducing SDS-PAGE sample buffer (see recipe)
4× reducing SDS-PAGE sample buffer (see recipe)
6% to 8% SDS-PAGE minigels (*UNIT 6.1*)
Anti-ubiquitin antibody (Basic Protocol 19; for immunoblot detection)
Boiling water bath
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*) *or* immunoblotting (Basic Protocol 18)

1. Dilute commercial E1 to 100 ng/μl in 100 mM HEPES, pH 7.5, or use undiluted recombinant mouse or wheat E1s prepared in house.
2. Set up two identical thiolester reactions in 1.5-ml tubes as follows:

   1.2 μl E1 prepared as in step 1
   1.2 μl 10× thiolester buffer
   1 μl [^{32}P]ubiquitin *or* 1 μl of 10 μg/μl "cold" ubiquitin if immunodetection is used
   H_2O to 12 μl.

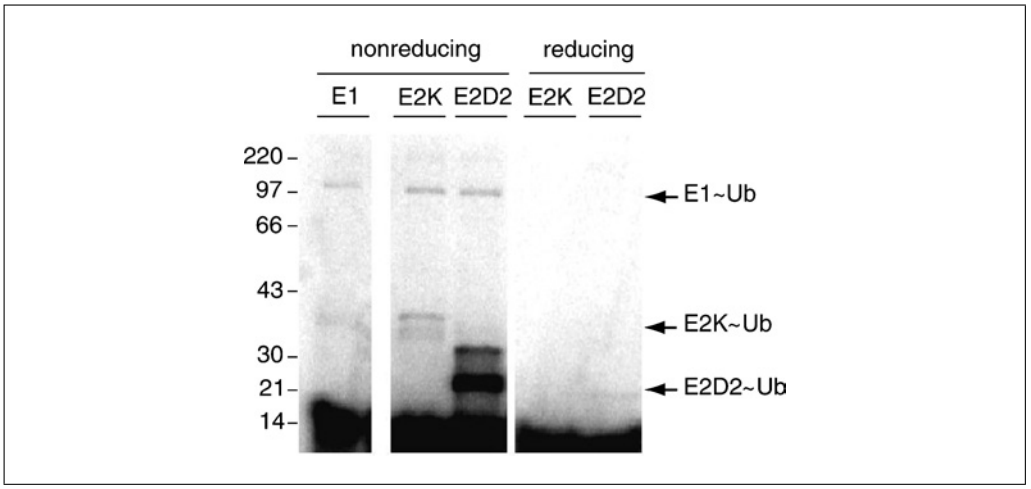


Figure 15.9.2 Thiolester formation between E1 and ubiquitin (E1∼Ub) or one of two E2s and ubiqutin (E2K∼Ub or E2D2∼Ub). 100 ng of mouse E1 alone and with 100 ng of E2K (E2-25K, HIP2) or ∼100 ng E2D2 (UbcH5B) were incubated 5 min at room temperature in the presence of ATP and ^{32}P-labeled ubiquitin (see Basic Protocol 17). The samples were then separated on 4% to 20% gradient SDS-PAGE gels in the absence (nonreducing) or presence (reducing) of 2-mercaptoethanol. Upon reduction, the higher-molecular-weight bands corresponding to ubiquitin-thiolester-modified proteins disappear (see Basic Protocols 1 and 2). Here, mouse E1 was expressed in a baculovirus system (Basic Protocol 21) and purified on nickel affinity resin. E2D2 was expressed in bacteria and used without further purification (see Basic Protocol 22). E2K was expressed as a GST-fusion in bacteria and purified on glutathione Sepharose column with thrombin cleavage (see Alternate Protocol 12; K.L. Lorick, unpub. observ.).

Incubate at room temperature for 5 min.

3. Add 4 μl of 4× nonreducing sample buffer to one tube and add 4 μl of 4× reducing sample buffer to the second tube.
4. Heat samples 5 min in a boiling water bath.
5. Resolve the entire sample on 6% to 8% SDS-PAGE minigels (*UNIT 6.1*).

   Make sure when running the gel that the reduced and nonreduced samples are separated by several lanes or run on separate gels. Gels should be run rapidly to preclude spontaneous reduction of thiolester linkages. See Figure 15.9.2 for results.

6a. *If radiolabeled ubiquitin is used:* Dry the gel and expose for autoradiography (*UNIT 6.3*).

6b. *If unlabeled ubiquitin is used:* Perform anti-ubiquitin immunoblotting (see Basic Protocol 18).

Thiolester Formation Between E2 and Ubiquitin

BASIC PROTOCOL 2

This protocol is used generally to test the activity of E2 before doing more complex assays. It can, however, be the primary assay used in drug screens for E2 inhibitors.

Materials

E1: rabbit E1 (Calbiochem, Boston Biochem, Affiniti,), bacterially expressed wheat E1 (Basic Protocol 20), or mouse E1 (Basic Protocol 21)
100 mM HEPES, pH 7.5
E2: crude (Basic Protocol 22 or 23) or purified (Alternate Protocol 12 or 13) *or* commercially available
10× thiolester buffer (see recipe)
[^{32}P]ubiquitin (Basic Protocol 17)
4× nonreducing SDS-PAGE sample buffer (see recipe)
4× reducing SDS-PAGE sample buffer (see recipe)
18% to 20% SDS-PAGE minigels (*UNIT 6.1*)
Anti-ubiquitin antibody (Basic Protocol 19; optional)
Boiling water bath
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*) *or* immunoblotting (Basic Protocol 18)

1. Dilute commercial E1 to 100 ng/μl in 100 mM HEPES, pH 7.5, or use undiluted recombinant mouse or wheat E1s prepared in house.
2. Dilute commercial E2 to 50 ng/ml in 100 mM HEPES, pH 7.5, or use undiluted crude or purified E2 prepared in house.
3. Set up two identical thiolester reactions for each E2 in 1.5-ml tubes as follows:

   1.2 μl E1 prepared as in step 1
   1.2 μl E2 (prepared as in step 2) or 100 mM HEPES, pH 7.5 as negative control
   1.2 μl 10× thiolester buffer
   1 μl [^{32}P]ubiquitin *or* 1 μl "cold" ubiquitin if immunodetection is used
   H_2O to 12 μl.

   Incubate at room temperature for 5 min.

   If using a crude bacterial preparation of E2, an "empty" bacterial lysate (Support Protocol 4) should be used as the negative control.

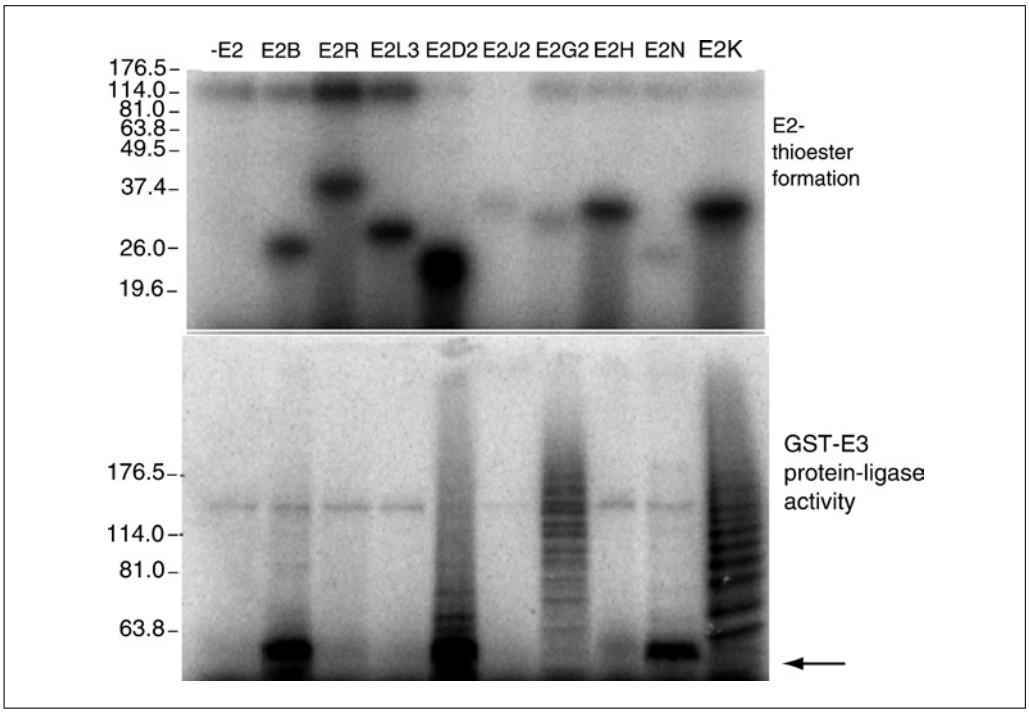


Figure 15.9.3 [^{32}P] Ubiquitin-thioester formation with various E2 enzymes and corresponding ubiquitin ligase activity (See Basic Protocol 4) with GST-SNURF protein. The arrow indicates mono-ubiquitylated protein (From Häkli et al., 2004).

4. Add 4 μl of 4× nonreducing sample buffer to one tube and add 4 μl 4× reducing sample buffer to the second.
5. Heat tubes 5 min in a boiling water bath.
6. Resolve the entire sample on high-percentage (18% to 20%) SDS-PAGE minigels (*UNIT 6.1*).

   Make sure when running the gel that the reduced and nonreduced samples are separated by several lanes or run on separate gels. Gels should be run rapidly to preclude spontaneous reduction of thiolester linkages.

7a. *If radiolabeled ubiquitin is used:* Dry the gel and expose for autoradiography (*UNIT 6.1*).

7b. *If unlabeled ubiquitin is used:* Perform anti-ubiquitin immunoblotting (see Basic Protocol 18).

   Active E2 enzymes will produce a band 8 kDa above their own molecular weight under nonreducing conditions when using radiolabeled ubiquitin or blotting for anti-ubiquitin (see Figure 15.9.3 for example results). Under reducing conditions, the labeled band should disappear and run with the free ubiquitin. If blotting for anti-E2, two bands appear under nonreducing conditions, one 8 kDa above the other; upon reduction the higher band should disappear.

BINDING OF UBIQUITIN-PROTEASOME PROTEINS

This section describes assays to determine binding of a soluble E2 (Basic Protocol 3) or ubiquitylation substrate (Alternate Protocol 1) to glutathione-Sepharose-immobilized GST-E3 or other E2 binding sites. E2s and E3s that work together to ubiquitylate a substrate by definition must interact. However, detection of stable E2-E3 interactions varies considerably when assessed using a standard coimmunoprecipitation or GST pull-down assay (*UNIT 17.5*). In the authors' experience, the easiest E2-E3 interaction to

detect is that between UbcH5B (Ube2d2) and AO7 (Lorick et al., 1999). Other physical interactions, such as that between BRCA1 and UbcH5C (Ube2d3), have only been detected by NMR (Brzovic et al., 2003). The binding assays described here are amenable to detecting E3 substrate interactions and are employed for later substrate ubiquitylation assays (Alternate Protocol 4).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

Binding of E2s to E3s

BASIC PROTOCOL 3

The experiment described here is presented with a number of alternatives for testing binding in a single protocol. The experiments can also be done in a reciprocal manner if the E3 is easily translated using in vitro systems or purified. The E2 can be bound to either glutathione-Sepharose (GS) beads or Ni-NTA agarose.

Materials

E3 or other putative E2 binding site of interest expressed as a GST fusion protein (see Basic Protocol 24 and Alternate Protocols 14 and 15)
Glutathione-Sepharose 4B beads (50% slurry; GE Healthcare)
Phosphate-buffered saline (PBS; see recipe), 4°C
Binding buffer (see recipe), 4°C
Purified native E2 (see Alternate Protocols 12 and 13; for detection by Coomassie blue staining or immunoblotting), E2 fusion with GST, His or other tag (Wu et al., 2003; for detection by Coomassie blue staining or immunoblotting), *or* ^{35}S-labeled E2 (see Basic Protocol 23; for detection by autoradiography)
4× nonreducing SDS-PAGE sample buffer (see recipe)
4× reducing SDS-PAGE sample buffer (see recipe)
18% to 20% SDS-PAGE minigels (*UNIT 6.1*)
10% (v/v) acetic acid/25% methanol
Anti-E2 or anti-tag antibody (Calbiochem, Boston Biochem, Affinity; for immunoblotting)
End-over-end rotator
Boiling water bath
Gel densitometer
Additional reagents and equipment for Coomassie blue staining (*UNIT 6.6*), autoradiography and phospher imaging (*UNIT 6.3*), *or* immunoblotting (*UNIT 6.2*)

Prepare glutathione-Sepharose-immobilized E3

1. On the day that the binding experiment is to be set up, prepare E3 beads by combining 25 µl packed beads and a volume of E3 crude extract containing 100 pmol GST-E3 in a 1.5-ml tube and incubating 1 to 2 hr at 4°C on an end-over-end rotator.

2. Microcentrifuge 30 sec at 14,000× *g*, 4°C, and aspirate the supernatant. Wash four times, each time by adding 1 ml cold PBS, microcentrifuging again as before, and aspirating the supernatant.

Perform binding assay

Include negative controls with glutathione-Sepharose beads only, and glutathione-Sepharose beads plus GST.

3a. *For detection by Coomassie blue staining:* Add 200 µl binding buffer and 1.2 µg (100 pmol) E2 to each tube of E3-GS beads. Incubate overnight at 4°C on an end-over-end rotator.

3b. *For detection by ^{35}S autoradiography:* Add 200 µl binding buffer and 100,000 cpm of in vitro translated [^{35}S]E2. Incubate overnight at 4°C on an end-over-end rotator.

3c. *For detection by immunoblotting:* Add 200 µl binding buffer and 150 to 200 ng E2. Incubate overnight at 4°C on an end-over-end rotator.

4. Wash four times, each time by microcentrifuging 30 sec at 14,000 × *g*, 4°C, removing the supernatant, adding 1 ml cold binding buffer (with gentle mixing after first two washes), centrifuging again as before, and removing the supernatant.

5. Add 30 µl of 4× reducing SDS-PAGE buffer to the final pellet of washed beads, heat 5 min in a boiling water bath, then microcentrifuge briefly at maximum speed to collect the sample (supernatant).

6. Resolve the supernatant on high-percentage (16% to 18%) minigels. Also include one sample that contains ~10% of the amount of the E2 input into each sample, without any additional protein.

Analyze gels

7a. *For staining:* Stain the gel with Coomassie Brilliant Blue R-250 as described in *UNIT 6.6*.

7b. *For autoradiography:* Wash the gel 10 min in 10% (v/v) acetic acid/25% methanol, dry the gel and detect bound protein by autoradiography (*UNIT 6.3*).

7c. *For immunoblotting:* Transfer to Immobilon P (Millipore) or nitrocellulose membrane and carry out immunoblotting (Basic Protocol 18) using anti-E2 or anti-tag antibody.

8. Compare the input band (10%) to the amount of bound material using phosphor imaging or densitometry to determine percentage of input material bound to the protein of interest.

The success of the experiment depends upon what results are expected. For some protein-protein interactions, 1% of the input material bound may be considered positive, 1% to 10% bound is a moderate interaction, and >10% bound is a strong interaction. The interaction of AO7 with Ube2D2 is strong enough that 20% to 40% of the E2 will bind to the E3. The interaction of E2g2 with the tail of gp78 is even stronger. However, in all cases, one has to make sure that the amount of binding observed is not the result of nonspecific interactions of the E2 to either beads alone or to the GST moiety of the E3 protein. Many if not most E2-E3 interactions, while real and significant, can only be detected via much more sensitive methods such as NMR or surface plasmon resonance (UNIT 17.6).

ALTERNATE PROTOCOL 1

Binding of Ubiquitin Substrates to E3s

This protocol is an adaptation of Basic Protocol 3 for determining binding to any substrate that can be expressed in a mammalian translation system. Because of the possibility that the substrate can be degraded by components of the ubiquitin-proteasome system, it may be necessary to add MG132 or other proteasome inhibitor during translation. Here, only the binding of ^{35}S-labeled substrate is described; however, any protein may be detected with appropriate adaptation.

Additional Materials *(also see Basic Protocol 3)*

In vitro–translated ^{35}S-labeled substrate protein (Basic Protocol 25)
50 mM MG132 (Calbiochem, Boston Biochem, or Biomol) in DMSO
10% (v/v) acetic acid/25% (v/v) methanol

1. Bind 20 pmol E3 to 25 μl beads as described in Basic Protocol 3, steps 1 to 2.
2. To each 1.5-ml tube of E3-GS beads, add:

   200 μl binding buffer
   Up to 2.0 μl of 50 mM MG132
   100,000 cpm of in vitro–translated ^{35}S substrate.

   Include negative controls with beads only, and beads plus GST.

3. Incubate overnight at 4°C on an end-over-end rotator.
4. Microcentrifuge 30 sec at 14,000× *g*, 4°C, and aspirate the supernatant. Wash four times, each time by adding 1 ml cold binding buffer, microcentrifuging again as before, and aspirating the supernatant.
5. To washed, pelleted beads, add 30 μl of 4× reducing buffer and heat 5 min in a boiling water bath.
6. Resolve on SDS-PAGE minigels (*UNIT 6.1*) appropriate to the substrate being studied.

   One sample on the gel must include 5% to 10% of the input radiolabeled material in order to determine the percentage of input material bound.

7. Wash the gel 10 min in 10% acetic acid/25% methanol, dry the gel, and detect bound protein by autoradiography (*UNIT 6.3*).

   A detectable amount of binding is usually ~1% of the total input, moderate binding is 1% to 10%, and strong binding will show 10% to 100% of the input. The final determination of the amount bound requires subtracting any background binding found in the GST alone or GS beads samples.

IN VITRO UBIQUITYLATION (E3) ASSAYS

The first protocols in this section test E3s for activity. These protocols lay the groundwork for additional protocols in which an active E3 is used to modify a substrate with ubiquitin. With few exceptions, E3s can ubiquitylate themselves in vitro in the presence of E1 and an appropriate E2. Therefore auto-ubiquitylation is a good indicator of activity. Basic Protocol 4 describes testing bacterially expressed GST fusions of E3 bound to glutathione-Sepharose. In Basic Protocol 5, the GST moiety is cleaved, allowing for an assessment of the specificity of auto-ubiquitylation sites. Basic Protocol 6 describes ubiquitylation in solution of E3s expressed in in vitro translation systems. Alternate Protocol 2 covers the use of ubiquitin variants to assess the nature of the ubiquitin chain formed. Basic Protocol 7 describes chemical inhibition of E3 activity of Zn-binding proteins such as PHD- or RING-finger proteins by the chelation of zinc. Alternate Protocol 3 describes the chemical inhibition of HECT-domain E3s using alkylating agents.

Strategic Planning

When working with proteins for which E3 activity is yet to be established, the use of several E2 enzymes is important to determine which is optimal for ubiquitylation. The authors recommend inclusion of UbcH5 (Ube2d) family members in such evaluations, because, generally, at least one member of this family is functional with almost all E3s, the exception being the TRAF family. A literature search for previous studies on

the E3 being used or those that bear amino acid similarity in the ubiquitin ligase and surrounding region can help make informed choices regarding E2s to test. Once activity has been established, the E3 can be used to ubiquitylate suspected substrate proteins (Basic Protocols 8, 9, and 10), or investigated in vivo (Basic Protocols 11, 12, and 13).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

BASIC PROTOCOL 4

E3 Auto-Ubiquitylation

This simple protocol has several advantages. First, all of the components are expressed in bacteria, which lack the ubiquitin-proteasome system (UPS). Thus, there is no interference from endogenous UPS components. Second, GST oligomerizes. This allows dimerization, which may facilitate detection of E3 activity. Third, immobilization on GS beads allows free ubiquitin to be removed before electrophoresis. The protocol utilizes radiolabeled ubiquitin (Basic Protocol 17) but can easily be adapted to using unlabeled ubiquitin and anti-ubiquitin immunoblotting (Basic Protocol 18).

Materials

Glutathione-Sepharose (GS) beads (GE Healthcare)
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Putative E3 expressed as a GST fusion in bacteria (GST-E3; Basic Protocol 25)
Empty bacterial lysate (Support Protocol 4)
Wheat E1 (Basic Protocol 20), mouse E1 (Basic Protocol 21), or rabbit E1 (Calbiochem)
E2 (Basic Protocol 22)
10× PCK (see recipe)
10× ubiquitylation buffer 1 (see recipe)
[^{32}P]ubiquitin (Basic Protocol 17)
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
4× reducing SDS-PAGE sample buffer (see recipe)
10% (v/v) acetic acid/25% methanol

30°C Thermomixer (Eppendorf), or other heated shaker
Boiling water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

Bind GST protein to GS beads

1. For each E3 sample to be tested, add 50 µl of a 50% slurry of GS beads to two 1.5-ml tubes.
2. Microcentrifuge the beads 30 sec at 8000 × *g*, room temperature, and remove the supernatant. Wash beads three times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, microcentrifuging again as before, and removing the supernatant.
3. Add 20 pmol GST-E3 protein to the beads.

   The E3 does not have to be purified at this point, as the binding to glutathione-Sepharose and subsequent washing will serve as the purification step.

4. Adjust sample volumes to at least 200 µl with 50 mM Tris·Cl, pH 7.5.
5. Allow protein to bind at least 20 min at room temperature on an end-over-end rotator.

6. Rinse the bound material three times, each time with 1 ml of 50 mM Tris·Cl, pH 7.5, using the technique described in step 2.

7. Remove residual fluid using a syringe or gel loading pipet tip.

Set up the in vitro ubiquitylation

8. Make two reaction mixtures for each E3 to be tested as follows:

   2 μl (100 ng) wheat E1
   2 μl (100 ng) E2 *or* empty bacterial lysate as control
   3 μl 10× PCK
   3 μl 10× ubiquitylation buffer 1
   1 μl [^{32}P]Ub
   19 μl H_2O.

9. Add 30 μl of reaction mix either with or without E2 to each E3 sample. Incubate samples 1.5 hr at 30°C, with shaking.

Wash samples (optional)

10. Microcentrifuge beads 30 sec at 14,000 × *g*, 4°C, and remove supernatant (dispose of radioactive material properly).

    Removal of the supernatant allows for a cleaner gel. The supernatant may be run separately to determine the amount of ubiquitylated material that is in that fraction.

11. Wash beads five times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, centrifuging 30 sec at 14,000 × *g*, 4°C, and removing the supernatant.

12. Remove residual fluid. Add 30 μl 50 mM Tris·Cl, pH 7.5.

Carry out electrophoresis

13. Add 8 μl of 4× reducing SDS-PAGE sample buffer to each tube. Heat samples 5 min in a boiling water bath.

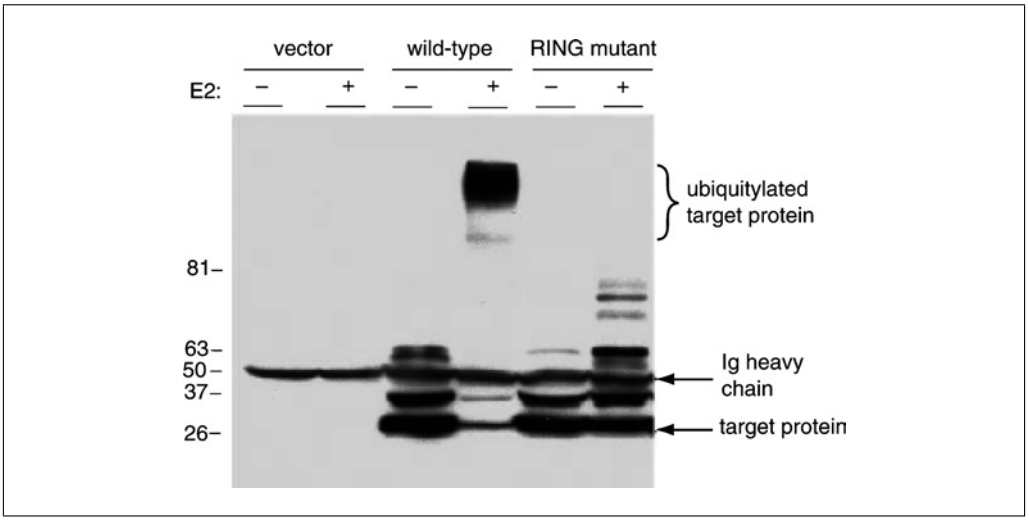


Figure 15.9.4 In vivo ubiquitylation of a typical RING-finger protein in U20S cells. Cells in 6-well plates were transfected with 2 μg Flag-tagged RING-finger protein, a mutant version of the RING-finger protein, or vector alone. In addition, 1 μg of Ube2D2 was transfected in the indicated lanes to enhance the amount of ubiquitylated species. After 24 hr, the cells were lysed in RIPA buffer. 150 μl of lysate was immunoprecipitated with mouse-anti-FLAG antibody and immunoprecipitates were resolved on 4% to 20% SDS-PAGE gels. The protein was transferred to PVDF membrane and immunoblotted with anti-FLAG antibody (Y. Yang, unpub. observ.).

14. Resolve samples on appropriate percentage SDS-PAGE to allow for resolution of species migrating above the unmodified form of the putative E3.

E3s vary in molecular weight from 12,000 to >300,000 kDa. The percentage gel will therefore be very specific to the individual experiment. One needs to resolve one to four bands separated by 8 kDa above the molecular weight of the particular E3 protein. For example, MDM2 migrates at 90 kDa, MDM2 plus Ub migrates at 98 kDa, MDM2 plus 2 Ub migrates at 106 kDa, and MDM2 plus 3 Ub migrates at 114 kDa; this would be separated on an 8% gel.

15. Wash the gel 10 min in 10% acetic acid/25% methanol, dry the gel and detect bound protein by autoradiography (*UNIT 6.3*).

A single ubiquitin modification will increase the weight of the protein of interest by 8 kDa. A protein can be modified with monoubiquitin or polyubiquitin chains or by multiple events on different lysines. Therefore, the autoradiogram may appear as a single band 8 kDa larger than the GST-E3, as a series of bands each 8 kDa above the previous one, or as a smear of radioactive signal that may run to the top of the gel (see Fig. 15.9.4).

BASIC PROTOCOL 5

Determination of Auto-Ubiquitylation versus Pseudo-Substrate Ubiquitylation

This protocol is designed to give a preliminary determination of how much auto-ubiquitylation of a GST-E3 fusion is on the E3 itself and how much is on GST. The procedure is the same Basic Protocol 4, with an added thrombin-cleavage step. The bound material (glutathione-*S*-transferase, GST) is separated from the cleaved material (E3 protein) and both fractions are electrophoresed. This protocol requires that the E3 be expressed in a GST vector that has a cleavage site. Thrombin is the most commonly used cleavage enzyme.

Materials

Putative E3 expressed as a GST fusion in bacteria (Basic Protocol 25)
Glutathione-Sepharose (GS) beads (GE Healthcare)
Empty bacterial lysate (Support Protocol 4)
Wheat E1 (Basic Protocol 20), mouse E1 (Basic Protocol 21), *or* rabbit E1 (Calbiochem)
E2 (Basic Protocol 22)
10× PCK (see recipe)
10× ubiquitylation buffer 1 (see recipe)
[^{32}P]ubiquitin (Basic Protocol 17)
50 mM Tris·Cl, pH 7.5
20× thrombin stock solution (see recipe)
Phosphate-buffered saline (PBS; see recipe)
Ubiquitylation buffer 2 for E3 assays (see recipe; optional)
Thermomixer, or other heated shaker
Additional reagents and equipment for in vitro ubiquitylation (Basic Protocol 5), SDS-PAGE (*UNIT 6.1*), and autoradiography (*UNIT 6.3*).

Carry out ubiquitylation and wash beads

1. Perform in vitro ubiquitylation (see Basic Protocol 4, steps 1 to 9).

2. Wash the ubiquitylated GST-E3 fusion protein on GS beads four to five times, each time by centrifuging 30 sec at 14,000 × *g*, 4°C, removing the supernatant (dispose of radioactive material properly), adding 1 ml of 50 mM Tris·Cl, pH 7.5, centrifuging again as before, and removing the supernatant. Remove residual fluid from final pellet.

Removal of the supernatant allows for a cleaner gel. The supernatant may be run separately to determine the amount of ubiquitylated material that is in that fraction.

Cleave the E3 away from the GST moiety

3. Prepare 1× thrombin by diluting 20× thrombin in PBS. Add 30 µl of 1× thrombin to bead pellet. Incubate at room temperature 30 min.
4. Microcentrifuge 30 sec at 14,000 × *g*, 4°C. Retain supernatant and transfer to a new tube. Repeat thrombin cleavage as described in the previous step and microcentrifuge again as before.
5. Combine supernatants and add 16 µl of 4× reducing SDS-PAGE sample buffer.
6. Wash beads three times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, microcentrifuging 30 sec at 14,000 × *g*, 4°C, and removing the supernatant. Carefully remove residual fluid from the final bead pellet.
7. Add 60 µl 50 mM Tris·Cl and 16 µl of 4× reducing sample buffer to each GS bead pellet.
8. Heat both the supernatants and the pellets (which now contain 1× reducing SDS-PAGE sample buffer) 5 min in a boiling water bath.

Perform electrophoresis and autoradiography

9. Resolve one-half of each sample by SDS-PAGE (*UNIT 6.1*) on gels of a percentage such that species migrating above the molecular weight of the protein of interest can be discerned (see Basic Protocol 4, step 14 annotation).

   Unmodified GST migrates at 28 kDa.

   No more than half of the sample will fit in the wells of the average gel.

10. Wash the gel 10 min in 10% acetic acid/25% methanol, dry the gel, and detect evaluate for ^{32}P signal by autoradiography (*UNIT 6.3*).

    If the E3 under study is modifying itself with ubiquitin, then the supernatant portion of the sample should contain a significant amount of the radioactive signal beginning at the molecular weight of the cleaved protein plus ubiquitin. That is, if the GST-E3 fusion is 68 kDa, following cleavage the GST will run at 28 kDa, and signal from the radiolabeled ubiquitin plus GST will start at 36 kDa and migrate higher from there. All of this would be seen in the bead-bound fraction. The E3 after cleavage would migrate at 40 kDa, and signal from radiolabeled ubiquitin plus E3 would start at 48 kDa and migrate higher from there. All of this would be found in the supernatant fraction. While not definitive, the experiment speaks to the ability of the E3 to modify heterologous substrates with ubiquitin.

Ubiquitylation of E3 Enzymes Expressed in In Vitro Translation Systems

BASIC PROTOCOL 6

The protocol for auto-ubiquitylation of in vitro–translated proteins is similar to Basic Protocol 4; however, eukaryotic in vitro translation systems contain proteasomes. Therefore, unless degradation is desired, inhibitors may need to be included in the reaction. In addition, ubiquitin aldehyde may be added to inhibit deubiquitylation. The protocol described here takes advantage of the fact that the E3 is selectively radiolabeled with ^{35}S-labeled methionine, and evidence of ubiquitylation is manifest as higher-molecular-weight forms of the putative E3 when appropriate controls are included.

Materials

50 mM MG132 (Calbiochem, Boston Biochem, or BioMol) in DMSO
30 µM ubiquitin aldehyde (Calbiochem, Boston Biochem, or BioMol) in 1 M HEPES, pH 8.0
50 ng/µl E1 (Basic Protocols 21 and 22)

50 ng/μl E2 (e.g., Ube2d2/UbcH5B; Basic Protocol 22)
10× PCK (see recipe)
10× ubiquitylation buffer 1 (see recipe) *or* 20× ubiquitylation buffer 2 for E3 assays (see recipe)
10 mg/ml ubiquitin (Sigma)
Suspected E3 protein, expressed with ^{35}S label in reticulocyte or wheat germ lysates (25,000 to 100,000 cpm; Basic Protocol 23)
4× reducing SDS sample buffer (see recipe)
10% (v/v) acetic acid/25% methanol
30°C Thermomixer (Eppendorf), or other heated shaker
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

1. Prepare in vitro ubiquitylation reaction as follows:

   0.5 μl 50 mM MG132
   0.5 μl 30 μM ubiquitin aldehyde
   2.0 μl E1
   2.0 μl E2
   3.0 μl 10× PCK
   3.0 μl 10× ubiquitylation buffer 1 *or* 20× ubiquitylation buffer 2 for E3 assays
   1.0 μl 10 mg/ml ubiquitin
   25,000 to 100,000 cpm ^{35}S-labeled E3
   H_2O to 30 μl.

   Incubate the mixture 90 min at 30°C with shaking.

   Control reactions lacking E1, E2, or ubiquitin add to the clarity and specificity of the experiment.

   See Critical Parameters and Troubleshooting for discussion of the choice between 10× ubiquitylation buffer 1 and 20× ubiquitylation buffer 2 for E3 assays.

2. Add 10 μl 4× reducing SDS-PAGE sample buffer and heat samples 5 min in a boiling water bath. Resolve entire sample on SDS-PAGE minigels (*UNIT 6.1*), using a gel percentage appropriate to the E3.

   The high concentration of protein in plant and rabbit in vitro translation systems can result in formation of aggregates upon heating which can both trap the protein of interest and cloud the final gel. There are a number of ways to prevent this. These include pelleting of material after translation, heating to 65° to 70°C rather than in a boiling water bath before electrophoresis, or heating to lower temperature for longer times, for example 37°C for 30 min.

3. Wash the gel 10 min in 10% acetic acid/25% methanol, dry the gel and detect bound protein by autoradiography (*UNIT 6.3*).

   Unlike experiments using labeled ubiquitin, when E3 is labeled there should be a band corresponding to the unmodified form of the protein, and one band for every ubiquitin molecule added.

   Fixing gels in 10% acetic acid 25% methanol for 10 min before drying also helps to remove free label that may migrate above the dye front; this will therefore provide cleaner results.

Determination of Ubiquitin Chain Variant Phenotype

Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63). Thus, there is the potential for substantial heterogeneity in ubiquitin chain formation. Additionally, some ubiquitin modifications may consist of a single molecule (mono-ubiquitylation). This protocol utilizes recombinant ubiquitins in which lysines have been mutated to evaluate chain formation with different E2-E3 combinations. While there are a number of mutants to assess, for initial experiments lysine-to-arginine mutations of either lysine 48 or lysine 63 (K48R and K63R) represent a good starting point. For controls, wild-type ubiquitin and a ubiquitin with no lysines (ubiquitin K0) should be included. This experiment may be done with labeled or unlabeled ubiquitin. However, using radiolabeled ubiquitin in these types of experiments can be messy due to the large number of radiolabeling reactions required.

The following is a protocol using unlabeled lysine mutants; detection of modified proteins is accomplished by anti-ubiquitin immunoblotting. The experiment will help determine if a particular type of ubiquitin chain can be formed using a particular E2-E3 combination. It does not address whether the chains are formed under physiological conditions. Such information may be determined using gas chromatography and mass spectrometry (GC-MS) following protease digestion of ubiquitin-modified proteins isolated form cells.

Additional Materials *(also see Basic Protocol 4)*

20× ubiquitylation buffer 2 for E3 assays (see recipe)
Mutant ubiquitins: lysine-less (K0), K48R, K63R, K48 only, K63 only (BioMol, Boston Biochem, or made in house)
Wild-type ubiquitin as reference
Additional reagents and equipment for anti-ubiquitin immunoblotting (Basic Protocol 18)

1. For each type of ubiquitin used, set up and incubate an auto-ubiquitylation reaction, then wash the beads and treat with reducing SDS-PAGE buffer (Basic Protocol 4, steps 8 to 13) with the following variations:

   a. Omit the 10× ubiquitylation buffer 1 and the 10× PCK from the reaction mixture in step 8 of Basic Protocol 4.

   b. Add 1.5 μl of 20× ubiquitylation buffer 2 to that reaction mix in place of the two abovementioned reagents.

   c. Add 1 μg of the ubiquitin to be tested to each reaction.

   d. Bring the final volume of each reaction to 30 μl with water.

2. Perform electrophoresis on all samples (Basic Protocol 4, steps 14 to 15). Perform anti-ubiquitin immunoblotting on the resulting gels (Basic Protocol 18).

3. Determine type of ubiquitin chain formed by comparing ubiquitin patterns of mutants compared to those obtained with the wild type ubiquitin.

   Wild-type ubiquitin is used as a reference for the complete ubiquitin pattern that may be obtained. K(0) ubiquitin should indicate how many lysines are modified (each ubiquitin band above the unmodified protein represents a lysine that may be modified. When multi-ubiquitinylation above the K(0) pattern is detected with K48R or K63R, this indicates that lysines at these positions are not required to form Ub chains. When multi-ubiquitinylation above the K(0) pattern is detected with K48-Ub or K63-Ub only, this indicates that the lysines are used for multi-Ub chain formation when using the E2 and E3 pair employed in the experiment.

BASIC PROTOCOL 7

Chelation of Zinc from RING- and PHD-Finger E3s

When dealing with RING- and PHD-finger proteins in vitro, E3 activity can be eliminated by chelation of zinc from the E3 before binding of substrate or the addition of the ubiquitylation mix. The requirement for zinc can be confirmed by adding it back to restore activity. The following protocol should be employed for any RING- or PHD-finger protein that will be used extensively. The protocol helps provide what can be essential biochemical data about such proteins. For example, the authors have observed E3s that, despite losing activity due to mutation of zinc coordination sites, cannot lose zinc through chelation because the binding is so strong. At the other extreme, some proteins with zinc removed cannot refold in the presence of added zinc.

Materials

Putative E3 expressed as a GST fusion in bacteria (Basic Protocol 24).
Glutathione-Sepharose (GS) beads (GE Healthcare)
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Zinc chelating agents (Sigma): 5 to 10 mM EDTA, 5 to 10 mM EGTA, 5 to 10 mM DTPA, 1 to 5 mM TPEN, *or* 1 to 5 mM *o*-phenanthroline in 50 mM Tris·Cl, pH 7.5
5 mM $ZnCl_2$
Wheat E1 (Basic Protocol 20), mouse E1 (Basic Protocol 21), *or* rabbit E1 (Calbiochem)
E2 (Basic Protocol 22)
Empty bacterial lysate (Support Protocol 4)
10× PCK (see recipe)
10× ubiquitylation buffer 1 (see recipe)
[^{32}P]ubiquitin (Basic Protocol 17)
4× reducing SDS-PAGE sample buffer (see recipe)
End-over-end rotator
Gel-loading pipet tips
30°C Thermomixer (Eppendorf), or other heated shaker
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

Bind E3 to GS beads

1. For each sample, prepare two 1.5-ml microcentrifuge tubes containing the following (combine ingredients in the indicated order):

   20 pmol bacterially produced GST-E3
   50 µl 50% GS bead slurry
   50 mM Tris·Cl, pH 7.5, to 200 µl.

   Incubate 20 min at room temperature on an end-over-end rotator.

2. Microcentrifuge 30 sec at 14,000 × *g*, 4°C, and remove supernatant. Wash three times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, microcentrifuging again as before, and removing the supernatant.

3. Incubate the bead-bound E3 three times, each time for 1 hr at room temperature on an end-over-end rotator with 5 to 10 mM EDTA, 5 to 10 mM EGTA, 5 to 10 mM DTPA, 1 to 5 mM TPEN, or 1 to 5 mM *o*-phenanthroline in 50 mM Tris·Cl, pH 7.5, microcentrifuging 30 sec at 14,000× *g*, 4°C, removing the supernatant and replacing with fresh solution between incubations. Also include one sample incubated in buffer alone as a control.

4. Microcentrifuge beads 30 sec at 14,000 × *g*, room temperature. Wash three times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, microcentrifuging again as before, and removing the supernatant.

Perform zinc add-back (optional)

5. Add 1 ml 5 mM $ZnCl_2$ and incubate for 1 hr at room temperature with rotation.
6. Wash twice 50 mM Tris·Cl, pH 7.5, using the technique described in step 4. Remove any residual fluid with a gel-loading pipet tip.

Perform E3 reaction

7. Add 30 µl ubiquitylating mix to each tube:

   2 µl (100 ng) wheat E1
   2 µl (100 ng) E2 *or* empty bacterial lysate as control
   3 µl 10× PCK
   3 µl 10× ubiquitylation buffer 1
   1 µl [^{32}P]Ub
   19 µl H_2O.

   Incubate 1.5 hr at 30°C.

8. Stop the reaction by adding 8 µl of 4× reducing SDS-PAGE sample buffer. Heat each sample 5 min in a boiling water bath.
9. Using a gel appropriate to the E3, analyze the entire sample on SDS-PAGE minigels (*UNIT 6.1*) followed by autoradiography (*UNIT 6.3*) or immunoblotting (Basic Protocol 18).

   If zinc binding is critical to E3 activity, incubation with TPEN should abrogate E3 activity as seen with buffer alone. The other chelating agents have weaker ability to chelate zinc; however, if they too are able to abrogate E3 activity, this indicates that the E3 requires zinc but does not bind it tightly. Cases where zinc add-back can restore E3 activity provide positive proof of the zinc requirement. Where zinc cannot reconstitute activity, two possibilities arise. The first is that the protein is irreversibly denatured when zinc is removed. The second is that the protein actually binds a divalent cation other than zinc. The second possibility is easier to rule out when TPEN alone destroys E3 activity, as this has a high specificity for zinc, while EDTA has high affinity for most divalent cations.

Inhibition of HECT Domain E3s by Alkylation of Active-Site Cysteines

ALTERNATE PROTOCOL 3

While Zn^{2+} chelation is required for RING-finger activity and is often reversible, inhibition of HECT domain proteins requires irreversible modification of the active-site cysteine. This protocol deals briefly with such inhibition.

Additional Materials *(see Basic Protocol 4)*

Alkylating agents: 10 mM iodoacetamide (IAA) *or* 5 mM N-ethylmaleamide (NEM) in Tris·Cl, pH 7.5 (see *APPENDIX 2A* for buffer)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*) *or* immunoblotting (*UNIT 6.2*)

Bind GST-E3 to beads and perform alkylation reaction

1. Bind GST-E3 to GS beads as described in Basic Protocol 4, steps 1 to 5.
2. Microcentrifuge 30 sec at 14,000 × *g*, room temperature. Wash beads three times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, microcentrifuging again as before, and removing the supernatant.

3. Incubate the bead-bound E3 1 hr in 1 ml of 50 mM Tris·Cl containing 5 mM NEM or 10 mM IAA.

4. Wash three times with 50 mM Tris·Cl, pH 7.5, using the technique described in step 2.

Perform ubiquitylation reaction

5. Add 30 μl ubiquitylating mix to each sample:

   2 μl (100 ng) wheat E1
   2 μl (100 ng) E2 *or* empty bacterial lysate as control
   3 μl 10× PCK
   3 μl 10× ubiquitylation buffer 1
   1 μl [^{32}P]Ub
   18 μl H_2O.

   Incubate 1.5 hr at 30°C

6. Using a gel appropriate to the E3, analyze the entire sample on SDS-PAGE minigels (*UNIT 6.1*) followed by autoradiography (*UNIT 6.3*) or immunoblotting (*UNIT 6.2*).

   If the E3 under study contains an active-site cysteine, increasing amounts of alkylating agents should completely abrogate the ability of the protein to facilitate the formation of ubiquitin modifications. In contrast to RING-finger proteins, the inhibition should be irreversible.

SUBSTRATE UBIQUITYLATION IN VITRO

Once activity has been established for an E3, potential substrates can be evaluated. In Basic Protocol 8, bacterially expressed E3 and radiolabeled in vitro–translated substrate are assessed. In Alternate Protocol 4, the E3 and substrate are bound and unbound material is removed before the ubiquitylation mixture is added.

Strategic Planning

Basic Protocol 8 is useful when interactions between substrate and E3 are not of sufficient affinity to withstand washing. Where interactions are more stable, Alternate Protocol 4 is preferable. As with other protocols in this unit, these experiments can be done either using radiolabeled material for autoradiography or appropriate antibodies for immunoblotting. Until one has a sense of the stability of the expressed substrate, it is best that the in vitro translation reaction and binding of substrate to E3 be carried out before freezing the translated product (preferentially the same day).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

BASIC PROTOCOL 8

Substrate Ubiquitylation in Solution

This protocol is used when there is no information concerning the affinity of substrate for E3 or when this affinity is too low to withstand washing. This protocol is, in most cases, more indicative of the physiological conditions under which E3 and substrate normally interact.

Materials

50 μM MG132 (Calbiochem, Boston Biochem, or Biomol) in DMSO
30 μM ubiquitin aldehyde (Calbiochem, Boston Biochem, or BioMol) in 1 M HEPES, pH 8.0
50 ng/μl E1 (Basic Protocol 20 or 21)
50 ng/μl E2 (Basic Protocol 22 or 23)
10× PCK (see recipe)
10× ubiquitylation buffer 1 (see recipe)
10 mg/ml ubiquitin
Substrate, expressed with ^{35}S-label in reticulocyte or wheat germ lysates (25,000 to 100,000 cpm; see Basic Protocol 23)
E3, expressed as a GST fusion in bacteria (Basic Protocol 24)
4× reducing SDS-PAGE sample buffer (see recipe)
20× ubiquitylation buffer 2 for E3 assays (see recipe; optional)
30°C Thermomixer (Eppendorf), or other heated shaker
Boiling water bath
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

Set up in vitro ubiquitylation

1. For each E3 to be tested mix the following components in a microcentrifuge tube on ice:

   0.5 μl 50 mM MG132
   0.5 μl 30 μM ubiquitin aldehyde
   2 μl (100 ng) E1
   2 μl (100 ng) E2
   3 μl 10× PCK
   3 μl 10× ubiquitylation buffer 1
   1.0 μl 10 mg/ml ubiquitin
   25,000 to 100,000 cpm ^{35}S-labeled substrate
   H_2O to 30 μl.

2. Add 30 μl of substrate-containing reaction mix to each E3 sample and adjust volume to 30 μl.

   The E3 does not have to be purified at this point, provided that the volume of the E3 and substrate together is less than 18 μl. The reaction may also be carried out with E3 immobilized on glutathione-Sepharose beads, in which case the E3 contributes no volume to the 30-μl reaction.

   Be sure to include one sample of the input material that has been incubated with the ubiquitylation mixture in the absence of any E3 as well as input material that has had no ubiquitylation mixture added as controls.

3. Incubate samples 1.5 hr at 30°C with shaking.

Carry out electrophoresis

4. Terminate reaction by adding 8 μl of 4× reducing SDS-PAGE sample buffer. Heat 5 min in a boiling water bath.

5. Resolve samples on appropriate percentage SDS-PAGE gel so as to allow for resolution of high-molecular-weight forms of substrate (*UNIT 6.1*).

6. Perform autoradiography (*UNIT 6.3*).

If the reaction is successful, there will be a significant shift in the migration of ^{35}S-labeled substrate on the gel. Its migration should shift from that of the unreacted material to a minimum of 8 kDa higher. The pattern will vary from a single band shift to a ladder to a smear, depending upon whether the substrate is mono-ubiquitylated, poly-ubiquitylated, or ubiquitylated on various lysine residues.

ALTERNATE PROTOCOL 4

Substrate Ubiquitylation After E3 Binding

This protocol allows for cleaner results than Basic Protocol 8. However, good results depend upon a detectable binding of the substrate to the E3 (see Alternate Protocol 1). The cleaner results are obtained by removal of material not bound to the GST-E3 fusion protein. For this protocol the E3 should be immobilized before substrate binding.

Additional Materials *(also see Basic Protocol 8)*

Glutathione-Sepharose (GS) beads (GE Healthcare)
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Binding buffer (see recipe)
1× SDS-PAGE sample buffer: 4× sample buffer (see recipe) diluted to 1× with binding buffer (see recipe)
End-over-end rotator
30°C Thermomixer (Eppendorf), or other heated shaker

Bind GST protein to GS beads

1. For each sample, add 50 µl of a 50% GS bead slurry to two 1.5-ml tubes.
2. Microcentrifuge 30 sec at 8000 × *g*, room temperature. Wash beads three times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, centrifuging again as before, and removing the supernatant.
3. Add 20 pmol GST-E3 protein to the beads.

   The E3 does not have to be purified at this point, as the binding to GS and subsequent washing will serve as the purification step.

4. Adjust the volume of the sample to at least 200 µl with 50 mM Tris·Cl, pH 7.5.
5. Allow protein to bind at least 20 min on an end-over-end rotator at room temperature.
6. Wash the bead-bound material three times with 50 mM Tris·Cl, pH 7.5, using the technique described in step 2. Remove residual fluid using a syringe or gel loading pipet tip.

Bind substrate to washed E3 beads

7. Wash the beads once by adding 1 ml binding buffer, microcentrifuging 30 sec at 14,000 × *g*, room temperature, and removing the supernatant.
8. Add 200 µl binding buffer and 1 µl of 50 µM MG132 to each tube.
9. Add 25,000 to 100,000 cpm of substrate protein and allow binding at 4°C overnight on an end-over-end rotator.
10. Wash the beads three times with binding buffer using the technique described in step 7.
11. For each E3 to be tested mix the following components. Keep on ice:

    0.5 µl 50 mM MG132
    0.5 µl 30 µM ubiquitin aldehyde
    2 µl (100 ng) E1

2 μl (100 ng) E2
3 μl 10× PCK
3 μl 10× ubiquitylation buffer 1
1.0 μl ubiquitin
25,000 to 100,000 cpm ^{35}S-labeled substrate
H_2O to 30 μl.

12. Add 30 μl of substrate-containing reaction mix to each bead-bound E3 sample. Incubate 90 min at 30°C with shaking.

Analyze samples

13. Remove residual fluid and add 25 μl of 1× sample buffer to each tube. Heat samples 5 min in a boiling water bath.

14. Resolve samples on appropriate percentage SDS-PAGE to allow for resolution of high-molecular-weight forms of substrate (*UNIT 6.1*).

    Include a sample containing 10% of the input radiolabeled material.

15. Process gel for autoradiography (*UNIT 6.3*).

    If the reaction is successful, there will be a significant shift in the migration of ^{35}S-labeled substrate on the gel. Its migration should shift from that of the unreacted material to a minimum of 8 kDa higher. The pattern will vary from a single band shift, to a ladder to a smear, depending upon whether the substrate is mono-ubiquitylated, poly-ubiquitylated, or ubiquitylated on various lysine residues.

Substrate Ubiquitylation by Multisubunit E3s

BASIC PROTOCOL 9

The authors have established several in vitro ubiquitylation systems mediated by multisubunit E3s. α subunits of hypoxia-inducible factors (HIF-α) are known to be ubiquitylated by a cullin-containing E3 known as VBC-Cul2. As suggested by its designation, this complex consists of von Hippel–Lindau tumor suppressor protein (pVHL), elongin B, elongin C, Rbx1, and Cul2. Ubiquitylation of HIF-α is provoked by hydroxylation of specific prolines in the 22-kDa oxygen-dependent degradation (ODD) domain. Proline hydroxylation is mediated by egg laying defective nine (EGLN) hydroxylases in response to normal oxygen conditions. The following is a protocol for ubiquitylation of the (ODD) domain of HIF-2α (the substrate) by VBC-Cul2 (the E3). The NEDD8 modification system (Basic Protocols 14, 15, 16, and 26) is added because neddylation of cullins enhances the ubiquitin ligase activity of cullin-containing E3 complexes (Kawakami et al., 2001; Amir et al., 2002).

Materials

VBC-Cul2 E3 protein complex expressed and purified using baculoviral expression system (Basic Protocol 26)
Mouse E1 (Basic Protocol 21)
E2-UbcH5B (Basic Protocol 22)
GST-ubiquitin (Alternate Protocol 10)
Human APP-BP1/Uba3 (NEDD8 E1; Basic Protocol 14)
Ubc12 (E2 for NEDD8), expressed and purified by using bacterial expression system (Basic Protocol 15)
NEDD8, expressed and purified by using bacterial expression system (Basic Protocol 15)
MBP-HIF2α-ODD (Megumi et al., 2005) expressed and purified by using bacterial expression system

His_6-EGLN3 (proline hydroxylases of HIF-α; Megumi et al., 2005), expressed and purified by using bacterial expression system (note that this is necessary for proline hydroxylation of MBP-ODD)
10× ATP and ATP-regenerating system (see recipe)
10× ubiquitylation buffer 2 for multisubunit E3s and neddylation (see recipe)
40 mM sodium 2-oxoglutarate (α-ketoglutarate, sodium salt; Sigma)
40 mM ascorbic acid (Sigma)
Anti-MBP antibody (Santa Cruz Biotechnology)

Boiling water bath
Thermomixer, or other heated shaker

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

Set up in vitro ubiquitylation

1. Mix reagents as follows (20 μl per reaction):

   800 ng VBC-Cul2
   100 ng E1
   200 ng E2 (UbcH5c)
   5 μg GST-ubiquitin
   50 ng APP-BP1/Uba3
   160 ng Ubc12
   1 μg NEDD8
   200 ng MBP-ODD
   1 μg EGLN3
   2 μl ATP and ATP-regenerating system
   2 μl 10× ubiquitylation buffer 2 for E3s and neddylation
   1 μl 40 mM sodium 2-oxoglutarate
   1 μl 40 mM ascorbic acid

   As a control omit the E2.

   Note that EGLNs require both 2-oxoglutarate and ascorbic acid.

2. Incubate experimental and control samples 120 min at 37°C.

3. Terminate reactions by addition of 7 μl of 4× reducing SDS-PAGE sample buffer to each tube.

Carry out electrophoresis

4. Heat samples 5 min in a boiling water bath.

5. Run the entire sample on appropriate percentage SDS-PAGE gel so that the MBP-ODD (67 kDa) fusion band runs to the bottom of the gel.

6. Transfer and immunoblot with anti-MBP antibody at 1 μg/ml (*UNIT 6.2*).

   As with all ubiquitylation reactions, results depend upon the nature of the substrate's ubiquitin modification. However, a successful reaction will always have bands that migrate at least 8 kDa above the molecular weight of the unmodified substrate. There may be a single band, a ladder of bands separated by 8 kDa, or a smear of bands indicating multiple ubiquitylation events on different lysines. Under physiological conditions, ODD is degraded by the ubiquitin proteasome system, so there should be at least one band corresponding to a tetra-ubiquitin chain (32 kDa) + the ODD (67 kDa) = 99 kDa. There may be additional bands representing mono-, di-, and tri-ubiquitin modifications.

Detection of E3 Activity in Immunoprecipitated Protein

This protocol combines in vivo protein modification and complex formation with the in vitro ubiquitylation assay described in Basic Protocol 8. The obvious advantage is that ubiquitylation can be carried out using both E3 and substrate proteins that are translated, modified, and folded in the exact system of interest. The protocol can be used to find substrates for a particular E3 or to compare the activity of different E3s on a single substrate. However the reactions rely on the presence of strong binding between the E3 and substrate. Because this experiment is designed to look for E3 activity from cell lysates, those lysates must be generated under nondenaturing conditions. In addition, while the proteasome must be inhibited, there is no need to inhibit deubiquitylating enzymes until the ubiquitylation reaction is set up. The authors have employed a similar technique for the ubiquitin modification of p53 by Hdm2 (Fang et al., 2000). In Alternate Protocol 5, the substrate is pulled out of solution by an excess of bead-bound GST-E3 (Fang et al., 2000).

Materials

Adherent cells expressing the substrate protein and E3 of interest (e.g., U2OS cells for p53 and MDM2), growing in 100-mm dishes
50 mM MG132 (Calbiochem, Boston Biochem, or Biomol) in DMSO *or* other proteasome inhibitor (e.g., lactacystin; Calbiochem or Biomol)
Phosphate-buffered saline (PBS; see recipe)
Nondenaturing Triton X-100 lysis buffer (see recipe) containing 1× phosphatase inhibitors and 50 μM MG132 (or lactacystin)
Anti-E3 or anti-substrate antibody
Nondenaturing Triton X-100 wash buffer (see recipe)
Tris-buffered saline (TBS; *APPENDIX 2A*; optional)
30 μM ubiquitin aldehyde (Calbiochem, Boston Biochem, or BioMol) in 1 M HEPES, pH 8.0
E1: rabbit (Calbiochem, Boston Biochem, Affiniti,), bacterially expressed wheat E1 (Basic Protocol 20), *or* mouse E1 (Basic Protocol 21)
E2: crude (Basic Protocol 22 or 23) or purified (Alternate Protocol 12 or 13)
20× ubiquitin buffer 2 for E3 assays (see recipe)
10 mg/ml ubiquitin
4× reducing sample buffer
30°C Thermomixer (Eppendorf), or other heated shaker
Boiling water bath
Additional reagents and equipment for cell culture (*UNIT 1.1*), immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Grow adherent cells expressing the substrate protein and E3 of interest to confluency in 100-mm dishes (*UNIT 1.1*).

   The cells used may be selected because they have high endogenous levels of the proteins of interest or cDNAs for these proteins may be transfected into cells where they are expressed well.

2. Expose the cells to proteasome inhibitor (e.g., 5 to 50 μM MG132 or 5 to 20 μM lactacystin) in order to build up high levels of the substrate.

   The amount of inhibitor will depend upon the half-life of the protein and the sensitivity of the cells to the inhibitor used. These must be determined empirically.

3. Aspirate supernatant. Wash cells by adding 5 ml PBS, then aspirating the PBS. Lyse cells on the plate by adding 250 μl of nondenaturing Triton X-100 lysis buffer containing 1× phosphatase inhibitors and 50 μM MG132 or lactacystin.

4. Immunoprecipitate the protein of interest (*UNIT 7.2*) with 1 μg per sample of anti-E3 or anti-substrate antibody.

   The antibody used must be carefully chosen so that it does not interfere with the protein-protein interaction, the active site of the E3, or the target lysine of the substrate. Often, using a FLAG, HA, or similar epitope tag on the E3 will eliminate this problem by allowing the corresponding anti-tag antibody to be used.

5. Wash the protein precipitate three times, each time by adding 1 ml nondenaturing Triton X-100 wash buffer, centrifuging 30 sec at 3000 × *g*, 4°C, and aspirating the supernatant. Perform a final wash in PBS or TBS using the same technique, to remove wash buffer components. Remove any residual fluid with a gel-loading pipet.

6. Set up a 30-μl ubiquitylation reaction mixture for each immunoprecipitate by combining the following:

   0.5 μl 30 μM ubiquitin aldehyde
   0.5 μl 50 mM MG132 or lactacystin
   2.0 μl E1 (100 ng)
   2.0 μl E2 (100 ng)
   1.5 μl 20× ubiquitin buffer 2
   1.0 μl 10 mg/ml ubiquitin
   22.5 μl H_2O.

   Add the 30-μl reaction mixture to the immunoprecipitate and incubate 1.5 hr at 30°C.

7. Add 4× reducing sample buffer to the incubated reaction mixture for a final concentration of 1× and heat 5 min in a boiling water bath. Analyze the reaction mixture by SDS-PAGE (*UNIT 6.1*) and anti-substrate immunoblotting (*UNIT 6.2*).

   It should be possible to detect higher-molecular-weight bands above the predicted molecular weight of the substrate protein. In order to ensure that the higher-molecular-weight bands are indeed due to added ubiquitin, the immunoblot may be stripped of antibody and reblotted with anti-ubiquitin antibody (Basic Protocol 18).

ALTERNATE PROTOCOL 5

Detection of Ubiquitin Modification of Substrates After GST Pull-Down

This protocol combines Basic Protocols 8 and 10. The benefit of this protocol is that the substrate is generated in cells in what should be the natural state. Here the E3 is generated in bacteria as a GST fusion protein in order to guarantee high levels of the E3. This should be used when expression of the substrate in in vitro translation systems is difficult and when antibodies to the E3 are unavailable or interfere with E3-substrate interaction.

Additional Materials *(also see Basic Protocols 8 and 10)*

Glutathione-Sepharose (GS) beads (GE Healthcare)
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
End-over-end rotator

Bind GST protein to GS beads

1. For each sample, add 50 μl of a 50% slurry of GS beads to two 1.5-ml tubes.

2. Microcentrifuge 30 sec at 8000 × *g*, room temperature, and remove the supernatant. Wash three times, each time by adding 1 ml of 50 mM Tris·Cl, pH 7.5, microcentrifuging again as before, and removing the supernatant.

3. Add 20 pmol (in a volume of up to 1 ml) of GST-E3 protein to the beads.
4. Adjust the volume of the sample to at least 200 μl with 50 mM Tris·Cl, pH 7.5.
5. Allow protein to bind at least 20 min at room temperature on an end-over-end rotator.
6. Wash the bead-bound material three times as in step 2. Remove any residual fluid using a syringe or gel loading pipet tip.

Pull down substrate from cell lysate

7. Grow cells expressing the substrate protein and E3 of interest to confluency in 100-mm dishes (*UNIT 1.1*).

   The cells used may be selected because they have high endogenous levels of the proteins of interest or cDNAs for these proteins may be transfected into cells where they are expressed well. For example, U2OS cells express wild-type p53, a substrate for MDM2 E3 activity.

8. Expose the cells to proteasome inhibitor (e.g., 5 to 50 μM MG132) in order to build up high levels of the substrate.
9. Lyse cells in 250 μl of nondenaturing Triton X-100 lysis buffer containing 1× phosphatase inhibitors and 50 μM MG132 or lactacystin.
10. Combine the lysate with the GS-bound E3 and incubate at 4°C overnight on an end-over-end rotator.
11. Microcentrifuge 30 sec at 14,000 × *g*, 4°C. Wash beads once by adding 1 ml nondenaturing Triton X-100 wash buffer to the bead pellet, microcentrifuging again as before, and removing the supernatant. Perform a second wash in PBS or TBS using the same technique, to remove wash buffer components. Remove any residual fluid with a gel loading pipet.

Set up the E3 reaction

12. Set up a 30-μl ubiquitylation reaction mixture for each sample of the bead-bound lysate:

    0.5 μl 30 μM ubiquitin aldehyde
    0.5 μl 50 mM MG132 or lactacystin
    2.0 μl E1 (100 ng)
    2.0 μl E2 (100 ng)
    1.5 μl 20× ubiquitin buffer 2
    1.0 μl 10 mg/ml ubiquitin
    22.5 μl H_2O.

    Add the 30-μl reaction mixture to the pellet of bead-bound lysate and incubate 1.5 hr at 30°C.

13. Analyze the reaction mixture by SDS-PAGE (*UNIT 6.1*) and anti-substrate immunoblotting (*UNIT 6.2*).

    It should be possible to detect higher-molecular-weight bands above the predicted molecular weight of the substrate protein. In order to ensure that the higher-molecular-weight bands are indeed due to ubiquitin, the immunoblot may be stripped of antibody and reblotted with anti-ubiquitin antibody (Basic Protocol 18).

DETECTION OF UBIQUITYLATION IN VIVO

This section provides an introduction to detecting and evaluating the ubiquitylation system through analysis of proteins produced in mammalian cells. There are a number of permutations of each of the protocols described below. Variables to consider depend on the system and on the question being asked. These variables include the type of lysis buffer, the need for addition of proteasome or lysosome inhibitors to cells, and the need to inactivate DUbs upon cell lysis. Furthermore, inhibition of DUbs may involve using alkylating agents that will also inactivate E1s, E2s, and some E3s. Additionally, there are an increasing number of tools that can be used to enhance detection of ubiquitylated species. These include various anti-ubiquitin antibodies and tagged ubiquitins, which come with the added benefit of enhancing the prevalence of ubiquitylated species. One particularly important point common to almost all approaches is that cells, once harvested, and particularly upon lysis, should be kept cold.

BASIC PROTOCOL 11

Lysis and Immunoprecipitation of Ubiquitylated Protein

The fundamental issues in lysis and immunoprecipitation of potentially ubiquitylated proteins are similar to those for any other proteins. There are myriad of different lysis conditions used by investigators. Some methods include using detergents with a wide range of characteristics; other methods involve mechanical disruption of cell membranes. The protocol presented below uses detergent solubilization. When deciding on a detergent-containing lysis buffer, considerations include the cellular compartments to be examined, the importance of either disrupting or maintaining protein-protein interactions, the need to inhibit deubiquitylating enzymes or ubiquitylating enzymes, whether immunoprecipitation will be carried out, and, if so, the effect of the lysis buffer on the ability of antibody to recognize the target protein. For example if one is interested in looking at ubiquitylated proteins in a multi-protein complex, detergent (and salt) conditions should be chosen that maintain the interactions. Generally, one should start with a relatively mild nonionic detergent such as Triton X-100 or NP-40. On the other hand, if the complex to be examined is nuclear or otherwise not easily solubilized, such buffers may not be optimal. At the other extreme, one might consider direct lysis in SDS-PAGE sample buffer. This is the case if protein-protein interactions are not a concern and the plan is to directly lyse and analyze a protein for evidence of higher-molecular-weight forms by immunoblotting. SDS-PAGE sample buffer also aids in maximum inhibition of deubiquitylating enzyme activity during cell lysis. However, lysis into buffers containing high concentrations of SDS is not generally amenable to immunoprecipitation and will require dilution with nonionic detergents prior to immunoprecipitation. Also, because of release of DNA from chromatin, depending on the cell type and number it may be necessary to treat these samples with DNase.

Another major consideration is which inhibitors to add to the lysis buffer. A cocktail of protease inhibitors is strongly advised. Among these are alkylating agents that serve as cysteine protease inhibitors and also prevent spurious disulfide bond formation during cell lysis. Alkylating agents also inhibit most deubiquitylating enzymes. The authors generally include 10 mM iodoacetamide in the lysis buffer unless the experiment involves functional evaluation of components of the ubiquitin system. However, while E1, E2s, and HECT E3s are inactivated by iodoacetamide, this is not necessarily the case for all E3s. N-ethylmaleamide (NEM) is also commonly used for alkylation, and there may be cases where use of both is desirable. Additionally, it may be helpful to add ubiquitin aldehyde to the lysis buffer to further inhibit deubiquitylation; this reagent is more costly than the alkylating agents, so before committing to routine use it might be worth assessing whether it adds to the value of alkylating agents.

Materials

Protein A–Sepharose CL-4B beads (50% slurry; GE Healthcare)
Tris-buffered saline (TBS; *APPENDIX 2A*), 4°C
Specific antibody for the protein of interest and isotype-matched control antibody, e.g., irrelevant or preimmune serum, purified
Tris-buffered saline/Tween 20 (TBST; see recipe), 4°C
Cells growing in culture (*UNIT 1.1*)
Phosphate-buffered saline (PBS; see recipe), ice cold
Nondenaturing Triton X-100 lysis buffer (see recipe), ice cold
2× reducing SDS-PAGE sample buffer (see recipe for 4×)
Anti–specific antigen antibody
Anti-ubiquitin antibody

End-over-end rotator
Boiling water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (Basic Protocol 18 and *UNIT 6.2*)

Coat beads with antibodies

1. Add 40 µl of 50% suspension of protein A–Sepharose CL-4B beads to a 1.5 ml microcentrifuge tube.
2. Wash once by adding 1 ml 4°C TBS, microcentrifuging 30 sec at 3000 × *g*, 4°C, and removing the supernatant. Resuspend beads in 200 µl TBS.
3. Add 0.5 to 1.0 µg of purified specific antibody or control antibody to the beads.
4. Incubate the tubes 2 hr at 4°C on an end-over-end rotator.
5. Microcentrifuge 5 sec at 3000 × *g*, 4°C, and aspirate supernatant.
6. Wash the beads three times, each time by adding 1 ml 4°C TBST, microcentrifuging 30 sec at 3000 × *g*, 4°C, and aspirating the supernatant.

Prepare cell lysate

7. Collect cells and wash once in 1 ml cold PBS using methods appropriate for the cell type.

   This protocol will work for adherent or suspension cells. Usually start with 10^6 cells, such as U2OS and 293T, for each immunoprecipitation.

8. Add 0.5 to 1.0 ml cold nondenaturing Triton X-100 lysis buffer to cells. Incubate at least 10 min at 4°C or on ice.

   If the cells are already pelleted in a microcentrifuge tube, resuspend them in lysis buffer by vortexing. If lysis was carried out on tissue culture plates, quantitatively transfer the lysates to microcentrifuge tubes.

9. Centrifuge 15 min at 14,000 × *g*, 4°C. Either transfer lysate to a fresh tube and keep on ice or immediately transfer to antibody-coated beads as in step 10.

Perform immunoprecipitation, SDS-PAGE, and immunoblotting

10. Transfer 500 µl cell lysate to each sample of antibody-coated beads. Incubate 1 hr to overnight at 4°C depending on the antibody.
11. Microcentrifuge 30 sec at 4000 × *g*, 4°C. Remove supernatant. Resuspend beads in 1 ml nondenaturing Triton X-100 wash buffer. Wash beads three times, each time by adding 1 ml nondenaturing Triton X-100 wash buffer, microcentrifuging again as before, and removing the supernatant.

12. Add 40 µl of 2× reducing SDS-PAGE sample buffer, mix thoroughly, and heat for 3 min in a boiling water bath.
13. Centrifuge the beads 2 min at 14,000 × *g*, room temperature
14. Divide the supernatant into two equal parts and resolve by SDS-PAGE (*UNIT 6.1*) on separate gels.

    The gel percentage will depend upon protein of interest; however, the band representing unmodified protein should run toward the bottom of the gel. In this way it is possible to resolve multiple ubiquitin modifications more easily.

15. Carry out immunoblotting (Basic Protocol 18) using anti–specific antigen with one membrane and anti-ubiquitin antibody with the other membrane.

    If the target protein is ubiquitylated, high-molecular-weight species are expected on the blot. These may appear as a single band 8 kDa above the unmodified protein, or as multiple discrete bands separated from the unmodified protein by 8 kilodaltons (the pattern looks like a ladder or a raccoon's tail). If multiple lysines are modified with ubiquitin chains, the higher-molecular-weight species appear as a smear running to the top of the gel.

ALTERNATE PROTOCOL 6

Detection of Protein Ubiquitylation Using Tagged Ubiquitin

This alternate protocol uses epitope-tagged ubiquitin expressed by transient transfection (see Alternate Protocol 10 for generation of the plasmid) to detect ubiquitylation of proteins of interest. The ubiquitylated proteins are isolated from the cell lysates using anti-tag antibodies and immunoblotted using a specific antibody against the protein of interest. The experiment can also be done in the reciprocal manner with immunoprecipitation of the substrate and blotting with anti-tag antibody to detect ubiquitylated species. The protocol is easily modified to look for differences in ubiquitin patterns when different E2 proteins are cotransfected (see Basic Protocol 2). It employs the calcium phosphate precipitation method of transfection (also see *UNIT 20.3*), which works well for most adherent cell types and is inexpensive. Lipid-based (*UNIT 20.6*) or other proprietary fusion-based transfection methods may work better for particular DNAs or cells. Electroporation (*UNIT 20.5*) or viral infection is generally the method of choice for most nonadherent cells.

Additional Materials *(also see Basic Protocol 11)*

Cells to be transfected
1 M $CaCl_2$
Mammalian expression plasmid for ubiquitin fused to FLAG, HA, His_6, myc-tag, GFP, or GST tag (Alternate Protocol 10, or obtained by request from other investigators)
Transfection efficiency control plasmid, e.g., GFP, RFP, β-galactosidase
2× HEPES-buffered saline (HeBS; see recipe)
Anti– tag epitope antibody *or* anti-substrate antibody (e.g., Sigma for anti-FLAG; Santa Cruz- Biotechnology for other antibodies)
6-well tissue culture plates
Boiling water bath
Additional reagents and equipment for cell culture (*UNIT 1.1*)

Plate cells for transfection

1. Plate cells the day before use at 40% to 50% confluency in a 6-well plate.

   Typically use 4 × 10^5 cells per well for large cells such as HeLa, 3T3, COS or CHO or 6 × 10^5 cells per well for smaller cells such as HEK293.

2. At a time point 4 hr before transfection, replace the medium on the cells with 1.0 ml fresh medium.

Transfect cells with tagged-ubiquitin expression plasmid

3. For each well of the 6-well plate to be transfected, make up a transfection mix in two parts in two separate microcentrifuge tubes:

   Tube 1:
   18 μl 1 M $CaCl_2$
   5 μg total DNA consisting of 4.5 to 4.9 μg ubiquitin expression plasmid and 0.1 to 0.5 μg transfection efficiency control (e.g., GFP)
   H_2O to 150 μl.
   Tube 2: 150 μl 2× HeBS.

4. Attach a hose with an inline filter to the compressed air line inside the sterile hood and attach a sterile plastic tissue culture pipet to the end. Set the valve to a very low pressure. Place the end of the pipet into tube 2 and gently bubble the solution.

   There should be a gentle bubbling of the solution. The bubbling can be achieved with a Pasteur pipet, but this becomes awkward in the next step.

5. Using a Pasteur pipet, add the solution in tube 1 dropwise to tube 2 while continuing the bubbling. Allow one drop to mix before adding the next.

   This should take ~2 min. The mixture should become cloudy.

6. Remove the bubbler, incubate the mixture 30 min at room temperature, then add the DNA/$CaPO_4$ solution dropwise to the cells (without removing the medium).

Prepare for ubiquitin detection

7. If using anti-tag or anti-substrate antibody for the initial protein precipitation, coat protein A–Sepharose beads with the appropriate antibody (Basic Protocol 11, steps 1 to 6).

8. Precipitate the protein using the antibody-coated beads as described in Basic Protocol 11, steps 10 to 11.

Perform immunoblotting

9. Add 40 μl of 2× reducing SDS-PAGE sample buffer, mix thoroughly, and heat for 3 min in a boiling water bath. Analyze the ubiquitylated proteins by SDS-PAGE (*UNIT 6.1*) and immunoblotting with appropriate antibody (*UNIT 6.2*).

   If the target protein is ubiquitylated, high-molecular-weight species are expected in the blot.

INHIBITION OF UBIQUITYLATION AND PROTEIN DEGRADATION IN VIVO

It is often useful to be able to inhibit the ubiquitylation/degradation process at several steps. A cell line with a temperature-sensitive mutation in the E1 gene (TS85) can inhibit the whole system. Mutation of a specific E2, where several E2s may activate an E3, can help to discern pathways. Mutating an E3 or suspected E3 can confirm localized E3 activity to a particular part of the molecule. Inhibiting proteasomes can preserve a substrate or demonstrate that a different degradation system is required.

Basic Protocol 12 describes mutation of E2s and E3s. Alternate Protocol 7 describes inhibition of ubiquitylation via the use of small interfering RNAs (siRNAs). Finally, Basic Protocol 13 addresses a method for determining if the protein degradation observed is indeed taking place in the proteasome.

BASIC PROTOCOL 12

Use of Dominant Negative Ubiquitin Proteins in Cells

For E2s and HECT-domain E3s, abrogation of ubiquitylating activity is achieved by mutation of single active-site cysteine residues. For RING-finger and PHD-finger proteins, mutation of one or two of eight zinc-binding residues eliminates nearly all activity. When overexpressed in cells, these mutants may act as dominant negative proteins against endogenous wild-type proteins. The mutation of E3s may result in both reduced auto-ubiquitylation and reduction of substrate ubiquitylation and degradation.

Materials

Expression plasmids for mutant and wild-type E2 or E3
Cultured cells to be transfected (e.g., U2OS or 293T)
Nondenaturing Triton X-100 lysis buffer (see recipe), ice cold
Specific antibody to the protein of interest and matched control antibody, e.g., irrelevant antibody or serum from unimmunized rabbits
Antibodies to mutated/wild-type protein *or* substrate *or* ubiquitin
Additional reagents and equipment for transfection (Alternate Protocol 6), SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and immunoprecipitation (Basic Protocol 11)

Transfect and harvest cells

1. Use the transfection protocol in Alternate Protocol 6 with the appropriate plasmids to express the mutant and wild-type E2 or E3 proteins.
2. Lyse 10^6 transfected cells per immunoprecipitation reaction with ice-cold nondenaturing Triton X-100 lysis buffer by incubating 10 min at 4°C.
3. Perform SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*) or immunoprecipitate with appropriate antibody attached to protein A–Sepharose beads and perform immunoblotting, as described in Basic Protocol 11.
4. Analyze total E2, E3, or substrate levels as appropriate by immunoblotting (Basic Protocol 18) with antibodies specific to the protein of interest.

   Pay attention to higher-molecular-weight bands that correspond to ubiquitylated species. A single ubiquitin will add 8 kDa to the mass of the protein that it is modifying. Most proteins will be modified by more than one ubiquitin, resulting in the presence of an 8-kDa ladder or a smear of higher-molecular-weight bands beginning 8 kDa above the molecular weight of the protein of interest and extending sometimes to the top of the gel. Expect less ubiquitin modification of substrate and E3 as well as higher absolute levels of these proteins due to lack of degradation. Be aware that inhibition may not be detected if the protein of interest may be ubiquitin modified by any of several E2-E3 combinations.

ALTERNATE PROTOCOL 7

Use of Small Interfering RNAs (siRNAs) to Reduce Expression of Ubiquitin-Proteasome System Proteins in Cells

The use of small interfering RNAs (siRNA) against E2 or E3 proteins complements the use of active-site mutant forms of these same proteins. While active-site mutants may prevent the degradation of a substrate, they can still influence the overall degradation process by binding to substrates, proteasomes, or other components of the ubiquitin proteasome system. siRNAs, on the other hand, when effective, can remove all influence of a particular E2 or E3. This allows researchers to determine if other E2s or E3s may compensate for, or overcome, the effects of the ablated protein. Two standard approaches have been employed for knocking down protein expression through siRNA. The original approach is to generate complementary oligonucleotides specific to a 19-bp segment of the gene of interest. The oligos are annealed to one another or cotranscribed in the same

tube, forming a double-stranded RNA. The RNA is then transfected into cells using lipid-based reagents, such as Invitrogen's Oligofectamine. The second approach is to place an oligonucleotide with a hairpin loop and self-complementary region into a plasmid vector. This is more correctly referred to as shRNA for short hairpin RNA. The plasmid is transfected into cells and expressed. The RNA transcribed forms a self-complementary hairpin loop structure. The hairpin is processed in the cell to give a double-stranded RNA similar to that introduced in the original method. While the oligonucleotide method (siRNA) is often more effective and has the advantage of rapid knockdown of RNA, the plasmid method (shRNA) is less expensive and more versatile, as it can be stably introduced or placed under an inducible promoter. The investigator should study the benefits of each for a particular system to see which is best. Kits for the design and production of siRNA oligos are available from several companies including Ambion, Qiagen, and Invitrogen. Plasmid-based shRNA kits and protocols are available from Ambion or Oligo Engine.

Once a plasmid or oligo pair has been chosen, transfect cells with shRNA plasmid as described in Alternate Protocol 6 or introduce siRNA oligo pairs by using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Analyze cell lysates for levels of the ablated protein as well as ubiquitin modification of the protein of interest by immunoblotting as in Basic Protocol 18.

If a particular E2 or E3 is absolutely required for a ubiquitin modification to occur, use of siRNAs targeting these proteins should eliminate 80% or more of the modification seen when these enzymes have not been ablated. Where the siRNA is effective in reducing the levels of protein, if no loss of ubiquitin modification or degradation is observed, this should be an indication that the protein of interest is targeted for ubiquitin modification by multiple E3s.

Localizing Degradation to the Proteasome

BASIC PROTOCOL 13

Degradation of proteins can occur through several different systems. When a target protein that is degraded in response to a particular stimulus is identified, the following protocol will help to determine if the proteasome is responsible. The protocol utilizes selective inhibitors of various protein degradation pathways. Inhibiting the system responsible for degrading the protein of interest should increase the overall levels of that protein. The inhibition of other pathways should have no effect. The authors use ALLN (calpain inhibitor I), a peptide aldehyde inhibitor of both calpains and proteasomes, ALLM (calpain inhibitor II), a specific inhibitor of calpains only, MG132, a more potent version of ALLN, lactacystin, a specific inhibitor of proteasomes, and ammonium chloride, which inhibits lysosomal proteases. There are other available inhibitors for these processes, including epoxomicin, clasto-lactacystin, and MG101 for the proteasome and calpain inhibitors III and IV for calpains, so do not feel bound to those mentioned here.

Materials

Cultured cells to be transfected (e.g., U2OS or 293T)
Inhibitors (Calbiochem, Boston Biochem, Biomol):
  Calpain inhibitor I (CPI, also known as LlnL or ALLN), soluble in DMSO
  Calpain inhibitor II (CPII, also known as ALLM), soluble in DMSO
  MG132, soluble in DMSO
  Lactacystin, soluble in DMSO or water, but unstable in H_2O
  NH_4Cl in H_2O
Nondenaturing Triton X-100 lysis buffer (see recipe)
4× reducing SDS-PAGE sample buffer (see recipe)
Specific antibody against protein of interest
6-well tissue culture dishes

Additional reagents and equipment for cell culture (*UNIT 1.1*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

1. Plate adherent cells in 6-well dishes at 50% to 80% confluency (typically 0.8–1.5 $\times$ 10^6 cells/well).
2. Expose different wells to appropriate concentration(s) of the various inhibitors for 4 to 24 hr:

   50 μM CPI
   5 to 50 μM CPII
   5 to 50 μM MG132
   5 to 20 μM lactacystin
   10 to 20 mM NH_4Cl.

   All of the drugs above are listed at their final concentrations in cell culture. A range of concentrations is specified for some because some cell lines are more sensitive to the effects of these inhibitors. The time of exposure and concentration will have to be determined empirically. The inhibitors are available from several manufacturers including Calbiochem, Boston Biochem, and Biomol.

   The length of exposure depends upon the half- life of the protein of interest. Be sure that at least one protein half-life has passed.

3. Harvest and lyse cells as follows:

   a. Remove medium and rinse cells with 1 ml PBS. Aspirate PBS.
   b. Add 250 μl nondenaturing Triton X-100 lysis buffer. Incubate 10 min on ice.
   c. Transfer lysate to 1.5-ml microcentrifuge tubes and microcentrifuge 10 min at 14,000 $\times$ *g*, 4°C. Transfer supernatant to a new microcentrifuge tube.
   d. Prepare samples for SDS-PAGE by adding 10 μl of 4× reducing SDS-PAGE sample buffer to 30 μl lysate.
   e. Freeze remaining lysate at –80°C for future studies.

4. Analyze lysates by SDS-PAGE (*UNIT 6.1*) and immunoblot for the target protein using the appropriate antibody (*UNIT 6.2*).

   Proteins that are degraded exclusively in the proteasome will be stabilized significantly in cells exposed to CPI, MG132, and lactacystin, but not NH_4Cl or CPII. If proteins are degraded in the lysosome, NH_4Cl will cause stabilization. If calpains are responsible for the degradation, CPI and CPII, but not lactacystin or MG132, will stabilize the proteins. Some proteins, such as EGFR, require both the lysosome and the proteasome for degradation, and stabilization may be significant with both types of inhibitor.

THE NEDD8 CONJUGATION SYSTEM

There exist ubiquitin-like protein modifiers that conjugate and regulate function of conjugated proteins. One example is the NEDD8 conjugation system.

NEDD8, like ubiquitin, is a 76–amino acid modifier. It modifies cullins in cullin-based ubiquitin ligase complexes and regulates their E3 activity. NEDD8 conjugation, termed neddylation, is catalyzed by several enzymes that are similar, though not identical to, those involved in ubiquitylation. NEDD8 is activated by a heterodimeric E1-like enzyme, the APP-BP1 and Uba3 complex, and is then transferred to an E2-like enzyme, Ubc12. Here, methods are described to express and purify APP-BP1/Uba3 complex by using a baculovirus expression system (Basic Protocol 14) and Ubc12 and NEDD8 by bacterial expression (Basic Protocol 15). The techniques employed for expressing and testing NEDD8 conjugating system proteins are similar to those used for the ubiquitin system

described in the previous sections. The similarities and differences between this system and the ubiquitin system may help later on when working with other ubiquitin-like modifiers, such as SUMO family members or ISG15. The main differences in these systems appear to be the use of a single subunit E1 for ubiquitin and ISG15 and heterodimeric E1 complexes for NEDD8 or SUMO (Tanaka et al., 1998). In addition, the ubiquitin system appears to require an E3. An absolute requirement for E3 has not been shown for the ubiquitin-like molecules, though the PIAS proteins appear to enhance SUMO1 transfer (Johnson and Gupta, 2001).

Purification of APP-BP1/Uba3 Complex (E1 for NEDD8) Expressed Using the Baculovirus Expression System

BASIC PROTOCOL 14

The baculovirus expression system is well suited to the production of the heterodimeric NEDD8 E1. This is because of the large size of the dimer, along with its requirement for multimeric assembly. In addition, the eukaryotic insect cells provide post-translational modifications that are not detected in bacteria and that may be necessary for proper folding or function of the enzyme.

Materials

*Sf*9 cells or *Sf*21 cells (Invitrogen)
Complete Grace's insect medium (see recipe)
cDNAs encoding APP-BP1 and Uba3
Baculovirus transfer vectors (see Murphy et al., 2004)
Hypotonic lysis buffer (see recipe)
1 M imidazole, pH 7.5 (adjusted with HCl)
Ni-NTA beads (50% slurry; Qiagen)
25-cm^2 culture flasks (Nunc) or 100-mm tissue culture dishes
27°C incubator without enhanced CO_2
175-cm^2 tissue culture flasks
15-ml centrifuge tubes
Refrigerated centrifuge
15-ml Dounce homogenizer with tight-fitting pestle
15-ml and 50-ml centrifuge tubes
Additional reagents and equipment for maintenance of insect cell cultures and generation of recombinant baculovirus (Murphy et al., 2004) and batch (Support Protocol 1) or column (Support Protocol 2) purification of APP-BP1 and Uba3

Culture insect cells

1. Plate 2×10^6 *Sf*9 as adherent cells in 25-cm^2 flasks or 3×10^6 cells in 100-mm dishes in complete Grace's insect medium. Grow in a 27°C incubator without enhanced CO_2.

   Murphy et al. (2004) contains general protocols for working with baculovirus. See Table 15.9.2 for recommendations for other culture configurations. Both Sf9 and Sf21 cells, derived from Spodoptera frugiperda (army moth) ovarian cells, are commonly used to isolate and propagate recombinant AcMNPV (baculovirus) stocks and produce the recombinant protein of interest. Sf9 was isolated as a clone of Sf21. It was reported that Sf21 cells might express more protein than Sf9 cells with some constructs (Heitz et al., 1997; Wang et al., 1997). The cells are grown as adherent cells in culture flasks or dishes.

2. When cells have just reached confluence, split 1:3, dislodging the cells either by streaming medium over the monolayer or by hitting the flask against the palm of the hand. Continue incubation at 27°C.

Table 15.9.2 Plating Recommendations for *Sf*9 Cells

Dish/flask size	Volume medium	Plating density
60-mm	3	2×10^6
100-mm	10	4×10^6
25-cm^2	5	3×10^6
75-cm^2	12	9×10^6
175-cm^2	24	21×10^6

Construct recombinant baculovirus

3. Subclone cDNAs encoding APP-BP1 and Uba3 into baculovirus transfer vectors.

   The authors use His_6-tagged APP-BP1 and T7-tagged Uba3 viral vectors. Any kits and general protocols (e.g., Murphy et al., 2004) for making baculovirus constructs should work well for APP-BP1 and Uba3. However, baculovirus transfer vectors must be suitable to the kits chosen.

Infect the cells

4. Seed 21×10^6 log-phase *Sf*9 cells in 24 ml complete Grace's medium in a 175-cm^2 flask. Allow to adhere at room temperature for >1 hr, then remove medium from cells.

   The baculovirus expression system is suitable for production of heterodimeric proteins because simple coinfection of the two viruses results in the production of heterodimeric protein complexes.

5. Infect His_6-APP-BP1 and T7-Uba3 recombinant baculoviruses at an MOI of 1 to 5 (i.e., 2.1–10.5 $\times$ 10^7 pfu in 5 ml medium from recently titered (see Basic Protocol 21 and Murphy et al., 2004) baculovirus stock. Include at least one flask with a "mock" infection (5 ml medium containing no recombinant baculovirus) as a negative control. Prepare flasks in triplicate.

   The amount of His_6-APP-BP1 baculovirus should be less than Uba3 baculovirus, to avoid contamination of APP-BP1 monomer.

6. Incubate the infected cells 1 hr at 27°C, rocking gently every 15 min to make sure cells are covered with virus supernatant.

7. Remove virus solution and add 24 ml complete Grace's insect medium.

8. Incubate 60 to 72 hr at 27°C (no CO_2).

Purify protein

9. From the triplicate 175-cm^2 flasks, harvest cells by pipetting medium up and down over the monolayer and transfer to 50-ml centrifuge tubes. Centrifuge 5 min at 300 $\times$ *g*, 4°C, and remove the supernatant.

10. Wash twice, each time by adding 10 ml ice-cold PBS, centrifuging 5 min at 300 $\times$ *g*, 4°C, and removing the supernatant.

11. Swell cells by adding 3 to 10 pellet volumes of hypotonic lysis buffer and leaving on ice for 10 min.

12. Lyse cells by 30 to 60 strokes of a 15-ml Dounce homogenizer with a tight-fitting pestle on ice. Transfer lysate to a 15-ml centrifuge tube.

13. Centrifuge 15 min at 20,000 × *g*, 4°C. Transfer supernatant to 15-ml tube.
14. Add 1 M imidazole for a final concentration of 0.2 mM imidazole.
15. Add 0.2 to 0.5 ml Ni-NTA beads as 0.4 to 1.0 ml of the 50% bead slurry supplied by the manufacturer.
16. Incubate at 4°C for 60 min on an end-over-end rotator.
17. Proceed with batch (Support Protocol 1) or column (Support Protocol 2) purification.

Batch Purification of APP-BP1 and Uba3

SUPPORT PROTOCOL 1

Following lysis and binding of the NEDD8 E1 components to Ni-NTA agarose, two common methods are employed to purify the protein. The first is a batch purification employing a centrifuge and the second is a column purification (Support Protocol 2). Normally, the batch purification is best suited to small amounts of protein. For larger quantities of NEDD8 E1, column purification may be easier and give better results.

Materials

NEDD8 E1 on Ni-NTA agarose beads (Basic Protocol 14)
Wash buffer 1 for His_6-tagged proteins (see recipe)
Wash buffer 2 for His_6-tagged proteins (see recipe)
Elution buffer for His_6-tagged proteins (see recipe)
Dialysis buffer (see recipe; include DTT)
Glycerol

Refrigerated centrifuge
1.5-ml screw-cap microcentrifuge tubes
End-over-end rotator
8000-MWCO dialysis membrane

Additional reagents and equipment for dialysis (*APPENDIX 3C*)

NOTE: Perform all steps at 4°C.

Wash the beads

1. Centrifuge the 15-ml tube containing the supernatant and Ni-NTA-agarose 5 min at 300 × *g*, 4°C. Remove supernatant.
2. Wash the Ni-NTA-agarose six times, each time by adding 12 ml wash buffer 1 for His_6-tagged proteins, centrifuging again as in step 1, and removing the supernatant.
3. *Optional:* Wash two times, each time with 12 ml wash buffer 2 for His_6-tagged proteins using the technique described in the previous step.
4. Transfer Ni-NTA-agarose to a 1.5-ml screw-cap microcentrifuge tube.

Elute the protein

5. Elute protein by adding 0.6 ml of elution buffer for His_6-tagged proteins and rotating 10 min at 4°C on an end-over-end rotator.
6. Microcentrifuge 2 min at 800 × *g*, 4°C. Transfer supernatant to a new 1.5-ml screw-cap tube.
7. Repeat steps 5 and 6 and combine the supernatants.

Dialyze and store the purified protein

8. Dialyze the eluate against 1 liter dialysis buffer at 4°C overnight using a 8000-MWCO dialysis membrane, changing the buffer twice at intervals at least 4 hr apart (*APPENDIX 3C*).
9. Remove the dialysate and divide into aliquots in 1.5-ml tubes. Add an equal volume of glycerol to each tube and mix thoroughly.
10. Rapidly freeze samples in liquid nitrogen, then store at −80°C.

SUPPORT PROTOCOL 2

Column Purification of APP-BP1 and Uba3

Loss of Ni-NTA agarose may occur during centrifugation because these beads do not pack down completely and may be aspirated along with the wash buffer. Using a column method, all of the beads are retained on a membrane or filter support, ensuring no loss of protein.

Materials

NEDD8 E1 on Ni-NTA agarose beads (Basic Protocol 14)
Wash buffer 1 for His_6-tagged proteins (see recipe)
Wash buffer 2 for His_6-tagged proteins (see recipe)
Elution buffer for His_6-tagged proteins (see recipe)
Dialysis buffer for ubiquitylating and neddylating enzymes (see recipe; include DTT)
Glycerol
2-ml Poly-Prep disposable chromatography column (Bio-Rad)
8000-MWCO dialysis membrane
Additional reagents and equipment for dialysis (*APPENDIX 3C*)

NOTE: Perform all steps at 4°C.

Elute complex from the beads

1. Transfer Ni-NTA beads to a 2-ml Poly-Prep column.
2. Wash the beads in the column with 10 bed volumes of wash buffer 1 for His_6-tagged proteins.
3. *Optional*: Wash beads with 2 to 3 bed volumes of wash buffer 2 for His_6-tagged proteins.
4. Elute protein with 1 bed volume of elution buffer for His_6-tagged proteins and collect the eluate.
5. Repeat elution step again and combine the eluates.

Dialyze and store the purified protein

6. Dialyze the eluate against 1 liter of dialysis buffer at 4°C overnight using an 8000-MWCO dialysis membrane, changing the buffer twice at intervals at least 4 hr apart.
7. Remove the dialysate and divide into aliquots in 1.5-ml tubes. Add an equal volume of glycerol to each tube and mix thoroughly.
8. Rapidly freeze samples in liquid nitrogen, then store at −80°C.

Production of Ubc12 and NEDD8 in Bacterial Expression Systems

The authors usually use N-terminally His_6-tagged constructs for Ubc12 and NEDD8. This is because purified Ubc12 and NEDD8 are solely used for in vitro assays. The small His_6 tag has little effect on the function of these molecules in such assays. N-terminally His_6-tagged human Ubc12 cDNA or NEDD8 cDNA lacking C-terminal extension (NEDD8-GG) is subcloned into the pT7-7 bacterial expression vector (Studier et. al., 1990; pT7 plasmids are derivatives of pET vectors), or pET vector, respectively. In many cases, E2 can be used without purification, but that requires changing the sonication buffer.

Materials

Ubc12 or NEDD8 plasmids (Kawakami et al., 2001)
E. coli strain BL21(DE3) (Invitrogen)
2×YT + G medium (see recipe) with 50 μg/ml ampicillin
1 M IPTG (dissolve 1 g IPTG in 4.2 ml H_2O; store at −20°C)
Sonication buffer (see recipe) *or* Ni-NTA purification buffer (see recipe)
Liquid N_2
1 M imidazole, pH 7.5 (adjusted with HCl)
Ni-NTA beads (Qiagen)
Wash buffer 1 for His_6-tagged proteins (see recipe)
Wash buffer 2 for His_6-tagged proteins (see recipe)
Elution buffer for His_6-tagged proteins (see recipe)
Dialysis buffer for ubiquitylating and neddylating enzymes (see recipe; include DTT)
Glycerol
37°C shaking incubator or water bath
Refrigerated centrifuge
Probe sonicator
15-ml centrifuge tubes
End-over-end rotator
Screw-cap 1.5-ml microcentrifuge tubes
8000-MWCO dialysis membrane
Additional reagents and equipment for transforming bacteria with plasmids (Seidman et al., 1997) and dialysis (*APPENDIX 3C*)

Prepare transformed cultures

1. Transform Ubc12 or NEDD8 plasmids into *E. coli* strain BL21(DE3) (Seidman et al., 1997).
2. Grow BL21(DE3) cells carrying appropriate plasmids in 2.5 ml of 2×YT + G medium containing 50 μg/ml of ampicillin at 37°C overnight with shaking.
3. Inoculate 2 ml of overnight culture into 1 liter of 2×YT + G with 50 μg/ml of ampicillin. Grow at 37°C with shaking for ~4 to 5 hr (until OD_{600} is 0.7 to 0.8).

Induce expression

4. Add 400 μl of 1 M IPTG to the 1-liter culture (for a final IPTG concentration of 0.4 mM).
5. Culture at 37°C with shaking for 2 to 5 hr.

6. Transfer cells to centrifuge bottles and harvest by centrifuging 10 min at 4000 × *g*, 4°C, and removing the supernatant.

7a. *For further purification on nickel:* Resuspend cells in 25 ml of 20 mM Tris·Cl (pH 7.5)/10 mM 2-ME and protease inhibitors.

In general, the authors recommend this additional purification step.

7b. *If no further purification on nickel is required:* Resuspend cells in 25 to 40 ml of sonication buffer.

Further purification is not necessary in pilot studies where the objective is to determine if a particular protein is neddlyated.

Lyse cells

8. Lyse cells on ice by sonication using a probe sonicator at setting 7, 60% duty cycle, for 90 sec.

9. Centrifuge lysate 20 min at 27,000 × *g*, 4°C. Transfer supernatant to new 15-ml tube and further purify (steps 10 to 21), or divide into 0.1- to 1-ml aliquots, freeze rapidly in liquid nitrogen, and store at −80°C.

Bind protein to Ni-NTA beads

10. Add 1 M imidazole solution to the supernatant to make final imidazole concentration of 2 mM.

11. Add 0.7 ml Ni-NTA beads. Incubate at 4°C for 60 min on an end-over-end rotator.

12. Centrifuge 15-ml tube containing the supernatant and Ni-NTA beads 5 min at 300 × *g*, 4°C. Remove supernatant.

13. Wash the Ni-NTA beads six times, each time by adding 12 ml wash buffer 1, centrifuging again as in the previous step, and removing the supernatant.

14. Wash the Ni-NTA beads twice, each time by adding 12 ml wash buffer 2, centrifuging again as before, and removing the supernatant.

Elute protein

15. Transfer the Ni-NTA beads to a screw-cap 1.5-ml tube

16. Elute protein by adding 1.0 ml of elution buffer and incubating 10 min at 4°C on an end-over-end rotator.

17. Centrifuge 2 min at 800 × *g*, 4°C. Transfer supernatant to a new tube.

18. Repeat steps 15 to 17 once and combine the supernatants.

Dialyze and store the purified protein

19. Dialyze the eluate against dialysis buffer with DTT at 4°C overnight using an 8000-MWCO dialysis membrane, changing the buffer twice at intervals at least 4 hr apart (*APPENDIX 3C*).

20. Remove the dialysate and divide into aliquots into 1.5-ml tubes. Add an equal volume of glycerol to each tube and mix thoroughly.

21. Freeze aliquots rapidly in liquid nitrogen, then store at −80°C.

In Vitro Neddylation

So far, the reported targets of neddylation are the cullins found in cullin-based multimeric E3s. NEDD8 modification of cullin enhances the E3 activity of these complexes. This protocol describes an in vitro neddylation assay for Cul2 in VBC-Cul2 E3 complexes.

Materials

VBC-Cul2 E3 protein complex expressed and purified by using baculoviral expression system (Basic Protocol 14)
Human APP-BP1/Uba3 (NEDD8 E1), expressed and purified by using baculoviral expression system (Basic Protocol 14)
Ubc12 (E2 for NEDD8; Basic Protocol 15)
NEDD8 (Basic Protocol 15)
10× ATP and ATP-regenerating system (see recipe)
10× ubiquitylation buffer 2 for E3s and neddylation (see recipe)
4× nonreducing SDS-PAGE sample buffer (see recipe)
Antibody to detect Cul2 (e.g., anti-myc antibody; Covance)
37°C Thermomixer (Eppendorf), or other heated shaker
Boiling water bath
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Mix the following in a total volume of 20 µl per reaction:

   800 ng VBC-Cul2
   50 ng APP-BP1/Uba3
   160 ng Ubc12
   1 µg NEDD8
   2 µl ATP and ATP-regenerating system
   2 µl 10× ubiquitylation buffer 2 for E3s and neddlyation.

   As a control, omit the E2 for NEDD8.

2. Incubate experimental and control reaction mixtures 60 min at 37°C.
3. Terminate reactions by adding 7 µl of 4× nonreducing SDS-PAGE sample buffer to each tube. Heat samples 5 min in a boiling water bath.
4. Run samples on 5% SDS-PAGE gel so that the band of Cul2 runs to the bottom of the gel (*UNIT 6.1*).
5. Immunoblot (*UNIT 6.2*) with antibody suitable for detecting Cul2.

   The authors use anti-myc antibody because their baculoviral-expressed Cul2 has an N-terminal myc tag.

6. Evaluate the immunoblot for a slowly migrating signal that reacts with antibody detecting Cul2.

   The slowly migrating signal will be ~8 to 9 kDa above the cul2 signal, which is at ~76 kDa.

LABELING AND DETECTION OF UBIQUITIN

In this section, protocols are provided for labeling ubiquitin to be used in in vitro biochemical assays. The methods of detection vary, and may involve radiolabeling with ^{32}P or ^{125}I followed by autoradiographic detection, labeling with nonradioactive labels such as biotin or fluorescein, or immunoblotting using anti-ubiquitin antibodies.

Strategic Planning

There are several basic considerations in deciding on an approach for detecting ubiquitin in in vitro experiments. Anti-ubiquitin antibodies vary in quality and may differ in their capacity to recognize ubiquitin depending on whether or not it is in chains; antibody binding may also be dependent on the nature of the linkage between ubiquitin molecules. Radiolabeling, while providing a strong signal, is not practical for some laboratories. Nonradioactive methods can be safer and more versatile, but reagents may be less available, more expensive, or otherwise impractical.

In some cases, plasmid constructs that are not commercially available are used in the protocols described; therefore, advance planning is required. However, people in the ubiquitin field are generally responsive to requests for plasmids.

BASIC PROTOCOL 17

Radiolabeling Ubiquitin with ^{32}P

This protocol utilizes a plasmid construct encoding a glutathione-*S*-transferase (GST) tag followed by a thrombin cleavage site and a cAMP-dependent protein kinase phosphorylation site N-terminal to ubiquitin. Radiolabeling is carried out using [γ-^{32}P]ATP and the catalytic subunit of protein kinase A. The protein can be used as a labeled GST fusion or can be cleaved resulting in ubiquitin having a small N-terminal extension modified with ^{32}P.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

Materials

E. coli BL21(DE3) or other protease-deficient bacterial strain (Novagen or Invitrogen)
pGEX-2TK-ubiquitin plasmid (purchase from GE Healthcare or prepare in house as in Alternate Protocol 10)
1 M IPTG (dissolve 1 g IPTG in 4.2 ml H_2O, store at −20°C)
Sonication buffer (see recipe)
Glutathione-Sepharose beads (GE Healthcare)
Phosphate-buffered saline (PBS; see recipe)
4× reducing SDS-PAGE sample buffer (see recipe)
12% SDS-PAGE gel (*UNIT 6.1*)
10× kinase buffer (see recipe)
Protein kinase A catalytic subunit from bovine heart (259 U; Sigma), resuspend lyophilized powder in 25 μl of 40 mM DTT on ice, allow to solubilize 15 min; prepare fresh
[γ-^{32}P]ATP (GE Healthcare) 6000 Ci/mmol
20× thrombin stock solution (see recipe)
Benzamidine-Sepharose (GE Healthcare)
Eco-Scint A (National Diagnostics)
Elution buffer for GST proteins (see recipe)

Refrigerated centrifuge and centrifuge bottles
Probe sonicator (e.g., Misonix Sonicator 3000)
Refrigerated centrifuge
Platform shaker
End-over-end rotator
Nitrocellulose filter circles
4-ml scintillation vials
β scintillation counter

Additional reagents and equipment for transforming bacteria with plasmids (Seidman et al., 1997), SDS-PAGE (*UNIT 6.1*), and staining of gels with Coomassie blue (*UNIT 6.6*)

Generate recombinant GST-thrombin-kinase site-ubiquitin

1. Transform BL21(DE3) with the pGEX-2TK-Ub plasmid (Seidman et al., 1997) and grow in a 1-liter culture with shaking at 37°C to an OD_{600} of 0.6 to 1.0.
2. Induce cultures by adding 1 M IPTG to a final concentration of 0.2 mM IPTG and incubating 1 to 2 hr at 37°C. When incubation is completed, place on ice.
3. Transfer culture to centrifuge bottle and centrifuge 20 min at 3000 × *g*, 4°C. Discard supernatant.
4. Add 40 ml cold sonication buffer to the pellet and resuspend completely by pipetting up and down.

Harvest the protein

5. Sonicate using probe sonicator for a total of 30 sec, either in a cold room or on ice, using three 10-sec pulses at 60% to 70% of the microtip limit setting (3.5 on Misonix Sonicator 3000).

   For smaller volumes (down to 10 ml lysate for a 250-ml culture) linearly reduce sonication time. When volumes of less than l0 ml are used, the sonicator will change pitch when lysis is complete. This can be used to find an optimal stopping point for sonication.

6. Centrifuge the sonicate 20 min at 15,000 × *g*, 4°C, and collect clarified sonicate.

Analyze the sonicate

7. Wash 66 μl of a well mixed glutathione-Sepharose slurry twice to remove the ethanol preservative, each time by microcentrifuging 30 sec at 14,000 × *g*, 4°C, removing the supernatant, adding 10 vol PBS, microcentrifuging again as before, and removing the supernatant.
8. Bind 50 to 500 μl of the sonicate to 50 μl of washed glutathione-Sepharose beads 15 min at room temperature either on an end-over-end rotator or a platform shaker.
9. Wash beads four times, each time by microcentrifuging 15 sec at 14,000 × *g*, 4°C, removing the supernatant, adding 10 vol PBS, microcentrifuging again as before, and removing the supernatant.
10. Add 40 μl of 4× SDS-PAGE reducing buffer to the bead pellet. Heat 3 min in a boiling water bath.
11. Resolve 40 μl of sample on a 12% SDS-PAGE gel (*UNIT 6.1*) that also includes lanes with known amounts of BSA ranging from 0.5 to 20 μg to serve as standards for quantification. Stain gel with Coomassie blue and quantify bands (*UNIT 6.6*). Divide sonicate (generated in step 6) into 1-ml aliquots and store in −80°C freezer.

Purify GST-Ub away from bacterial proteins

12. Add 150 μl of well mixed glutathione-Sepharose slurry to a 1.5-ml microcentrifuge tube.

    This will yield a final packed volume of 100 μl.

13. Wash the beads three times, each time by microcentrifuging briefly at 14,000 × *g*, 4°C, pouring off the supernatant, adding 1 ml PBS, microcentrifuging again as before and removing the supernatant. Remove final wash with a syringe or gel loading pipet.

14. Add 1 ml post-sonication supernatant from step 6. Allow to bind by incubating 30 to 60 min at room temperature on and end-over-end rotator.

15. Wash three times, each time with 1 ml 1× PBS using the technique described in step 13, then wash twice, each time with 0.5 ml 1× kinase buffer, using that same technique. Remove final wash with a syringe or gel loading pipet.

Label GST-ubiquitin

16. Add the following to the GST-Ub-bound beads from the previous step:

    5 µl 10× kinase buffer
    2 µl protein kinase A catalytic subunit
    2 µl [γ-^{32}P]ATP, 6000 Ci/mmol
    41 µl distilled H_2O.

17. Incubate on ice for 30 min, occasionally resuspending beads.

18. To remove unincorporated label, wash beads four to five times, each time by microcentrifuging 30 sec at 14,000 × *g*, 4°C, removing the supernatant, adding enough PBS to fill the tube, and removing the supernatant. Remove final wash with a syringe or gel loading pipet.

19. Proceed with thrombin cleavage (steps 20a to 26a) or skip to step 20b to produce purified labeled GST-ubiquitin.

For purified, labeled ubiquitin

20a. Prepare 800 µl of 1× thrombin by mixing 760 µl PBS with 40 µl of 20× thrombin stock, for a final concentration of 0.05 U/µl. Add to the labeled ubiquitin bound to the beads.

21a. Incubate 2 to 3 hr at room temperature (or overnight at 4°C) on an end-over-end rotator.

22a. Microcentrifuge 1 min at 14,000 × *g*, 4°C. Transfer the labeled Ub (supernatant) to a fresh tube.

23a. Wash benzamidine-Sepharose beads with PBS using the technique described in step 7. Add 100 µl PBS-washed benzamidine-Sepharose beads to the supernatant containing the labeled Ub. Incubate 30 to 60 min at room temperature on an end-over-end rotator.

24a. Microcentrifuge the beads 1 min at 14,000 × *g*, room temperature, and transfer the thrombin-depleted [^{32}P]Ub (supernatant) to a fresh tube.

25a. Spot 1 to 2 µl of the supernatant onto a nitrocellulose filter circle. Submerge the nitrocellulose in EcoScint A in a 4-ml scintillation vial. Count in a β scintillation counter.

Expect 10,000 to 30,000 cpm/µl.

26a. Store supernatant with the labeled Ub at 4°C for up to 4 weeks.

As the [^{32}P]Ub ages, aggregates may form which may obscure smaller protein+Ub conjugates resolved on gels. These aggregates may be removed by centrifuging the preparation for 30 to 60 min at 25,000 × g, 4°C.

For purified, labeled GST-ubiquitin fusion protein

20b. Elute the labeled protein from step 19 with 150 µl PBS elution buffer for GST proteins by incubating 10 min at 4°C.

21b. Microcentrifuge briefly at 14,000 × *g*. Transfer the eluate (supernatant) to a new tube.

22b. Repeat steps 20b and 21b twice. Combine the eluates (supernatants).

23b. Microcentrifuge briefly at 14,000 × *g*, and transfer supernatant to a new tube to remove any remaining beads.

24b. Spot 1 to 2 μl of the supernatant onto a nitrocellulose filter circle. Submerge the nitrocellulose in EcoScint A in a 4-ml scintillation vial. Count in a β scintillation counter.

Expect 10,000 to 40,000 cpm /μl.

25b. Store supernatant at 4°C until needed, up to 4 weeks.

Radiolabeling Ubiquitin with ^{125}Iodine

ALTERNATE PROTOCOL 8

^{125}I[ubiquitin] is available from GE Healthcare. With the availability of commercially available iodinated ubiquitin, the authors do not recommend labeling in-house unless the laboratory is set up with safety hoods, exhaust systems, and scientists trained to deal with non-bound ^{125}I.

Labeling Ubiquitin with Nonradioactive Tag (Biotin)

ALTERNATE PROTOCOL 9

Nonradioactive labeling techniques include biotinylation, labeling with fluorochromes, or labeling with chemiluminescence reagents. All of these require chemical cross-linking to ubiquitin. The authors recommend kits such as Pierce's EZ-Link. Again, the issue of modification of the ubiquitin itself should be considered. Here, the authors specifically present the labeling of ubiquitin with biotin. With this label, modified proteins in subsequent ubiquitylation assays may be detected with avidin–horseradish peroxidase (avidin-HRP).

Materials

20 mg/ml bovine ubiquitin (Sigma-Aldrich) in coupling buffer
Coupling buffer: 50 mM sodium borate, pH 8.5
10 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in coupling buffer (prepare immediately prior to use)
1 M Tris Cl, pH 8.5 (*APPENDIX 2A*)
20 mM Tris Cl, pH 7.4 (*APPENDIX 2A*)
PD-10 column prepacked with Sephadex G-25 medium (GE Healthcare)
2000-MWCO dialysis membrane

1. Dissolve 25 mg bovine ubiquitin in 1.25 ml coupling buffer.
2. Combine the ubiquitin solution with 1 ml of freshly prepared 10 mg/ml Sulfo-NHS-LC-Biotin in coupling buffer. Incubate 30 min at room temperature.
3. Add 0.25 ml of 1 M Tris·Cl, pH 8.5. Incubate 30 min at room temperature.
4. Apply the entire 2.5 ml of reaction mixture to a prepacked PD-10 Sephadex G-25 column. Do not collect flowthrough. After the mixture has entered the column bed, add 3.5 ml of 20 mM Tris·Cl, pH 7.4/150 mM NaCl, and collect the eluate for dialysis.
5. Dialyze (*APPENDIX 3C*), using a 2000-MWCO dialysis membrane, against 1 liter of 20 mM Tris·Cl, pH 7.4, overnight at 4°C. Over the course of the next day, change buffer twice at intervals of several hours.
6. Divide dialysate into 200 μl to 1 ml aliquots and store at −80°C.

ALTERNATE PROTOCOL 10

Generation of Tagged Ubiquitin Expression Plasmids

If ubiquitin plasmids are not available, or if a specific tag is required, it is possible to generate reagents in house using basic molecular biology techniques. Ubiquitin is encoded in several genes, including a polyubiquitin gene that is cleaved by deubiquitylating enzymes (DUbs) to form ubiquitin monomers, and as fusions with certain ribosomal proteins that are later cleaved. In all cases, mature, active ubiquitin includes a C-terminal diglycine that is important for activation by E1. Sources of ubiquitin DNA are plentiful. Ubiquitin only varies in two amino acids between yeast and human, and ubiquitin from any source works well in all organisms. When adding tags to ubiquitin, it is crucial that the C-terminus not be modified, so as to allow activation by E1. Also, large tags, e.g., GST, GFP, or MBP, are more likely to hinder ubiquitin function than are small ones, e.g., His_6, FLAG, HA, or myc. Be aware that the FLAG epitope contains lysines that may themselves be ubiquitylated. The protocol described here uses PCR to generate a plasmid containing ubiquitin fused to GST, which may be used in the labeling protocol above. It may be modified as appropriate for plasmids that are available and to suit specific needs.

Materials

Whole RNA from any eukaryote, or total yeast DNA

Primer for reverse transcription (if starting with whole eukaryotic RNA): RT 3′ reverse: ATATAGAATTCCTATCCTCCTCTCAGGCGAAGGACCAGGT 3′ (synthesized at 100 nM scale, and desalted, by IDT, *http://www.idtdna.com*, Invitrogen, or similar oligonucleotide synthesis facility)

Kit for first-stand cDNA synthesis (e.g., RT-PCR kit; Fermentas)

Kit for PCR amplification of DNA (e.g., RT-PCR kit, Fermentas, or Ready-To-Go PCR beads, GE Healthcare)

Primers for PCR :
5′ forward: ATATAGGATCCCAGATCTTCGTGAAGACCCT
3′ reverse: ATATAGAATTCCTATCCTCCTCTCAGGCGAAGGACCAGGT

QIAquick PCR Purification Kit (Qiagen)

*Bam*HI and *Eco*R1 restriction endonucleases and 10× *Eco*RI buffer

pGEX-2TK vector (GE Healthcare)

1% agarose gels with and without ethidium bromide (Voytas, 2000)

Molecular weight markers

Calf intestinal alkaline phosphatase (CIAP; Roche)

QIAquick Gel Extraction Kit (Qiagen)

T4 DNA ligase kit (Takara)

TOP10 chemically competent *E. coli* strain DH5α (Invitrogen) or other suitable host bacterial strain

SOC medium (Digene)

LB medium (*APPENDIX 2A*) with ampicillin

100-mm LB agar plates containing 100 μg/ml ampicillin (*APPENDIX 2A*)

Ready-To-Go PCR kit (containing PCR tubes premixed PCR reagents; GE Healthcare)

DNA size markers

14°, 42°C. 56° and 65°C water baths

96-well microtiter plate

15-ml snap-cap culture tubes (e.g., Falcon 2059)

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000), transformation of bacteria (Seidman et al., 1997), DNA minipreps (Engebrecht et al., 1991), and DNA sequencing (Chapter 7 in Ausubel et al., 2006)

Generate cDNA for ubiquitin

1a. *If using whole RNA:* Perform first-strand synthesis using the RT 3′ primer and the protocol in the Fermentas RT-PCR kit.

1b. *If using yeast DNA:* Proceed directly to step 2.

This procedure is appropriate for yeast DNA, which does not contain introns, but not for other eukaryotic DNA which does contain introns.

2. Use 1 to 3 μg of the first-strand cDNA or yeast DNA template with 25 pmol forward and reverse PCR primers in a total volume of 25 μl to perform PCR for the generation of ubiquitin double-stranded cDNA containing restriction sites that are compatible and in frame for ligation into pGEX-2TK.

The 5′ primer has a BamHI site, the 3′ primer an EcoRI site. Use either the RT-PCR kit or the GE Healthcare Ready-To-Go PCR Beads with the appropriate protocols and reaction conditions for either kit. Also see APPENDIX 3F *for general PCR protocols.*

3. Clean up the PCR reaction using a Qiagen QIAquick PCR Purification Kit, eluting the PCR product in 50 μl.

4. Digest 10 μl of the purified PCR product with 20 U each of *Bam*HI and *Eco*RI in 1× *Eco*RI buffer at 37°C for 2 hr in a total volume of 50 μl.

5. Run 5 μl of the restriction digest on a small 1% agarose gel (Voytas, 2000) with molecular weight markers to quantify the PCR product.

The PCR fragment will be 219 nucleotides plus restriction sites and other exogenous primer sequence. Larger fragments most likely contain polyubiquitin.

Create the ubiquitin fusion protein plasmid

6. Digest 5 μg pGEX-2TK with 50 U each of *Bam*HI and *Eco*RI in 1× *Eco*RI buffer.

Cloning sites may be viewed at http://www.amersham.com.

7. Dephosphorylate the vector DNA by adding 1 U CIAP, incubating 15 min at 37°C, and then incubating 15 min at 56°C. Add another 1 U CIAP and repeat the successive incubations at the two different temperatures. Heat inactivate the phosphatase by incubating at 65°C for 10 min.

8. Purify the digested vector by running on a 1% agarose gel containing ethidium bromide (Voytas, 2000), cut out the band at ∼5 kb, and purify using a Qiagen QIAquick Gel Extraction Kit.

9. Run 5 μl on a small 1% agarose gel (Voytas, 2000) with molecular weight markers to quantify.

10. Combine three molar equivalents of digested PCR fragment with 1 equivalent of vector DNA (30 to 100 ng total) in a 20-μl reaction with T4 ligase and buffer from the Takara T4 DNA ligase kit, according to the kit protocol.

11. Incubate 2 to 14 hr at 14°C or 15 to 30 min at room temperature.

Expand plasmid

12. Use all 20 μl of the ligated DNA to transform 100 μl TOP10 competent bacteria (Seidman et al., 1997) by heat-shock as follows. Incubate 15 min on ice, then 30 sec at 42°C, then 2 min back on ice, then add 400 μl SOC medium. Incubate with shaking at 37°C for 1 hr, microcentrifuge briefly at maximum speed to pellet bacteria, bring up in 100 μl with LB medium, and plate the entire mixture onto 100-mm LB ampicillin plates containing 100 μg/ml ampicillin. Incubate overnight at 37°C.

13. Select eight to twelve large, well isolated colonies to screen by PCR and to prepare a DNA miniprep for further verification by DNA sequencing by touching each colony with a toothpick, then transferring first into 50 μl LB medium with ampicillin in a well on a 96-well plate, and then into 2 ml LB medium with ampicillin in a 15-ml snap-cap culture tube.

14. Grow the bacteria in the 96-well plate, which has been sealed with tape, at 37°C for 1 hr while shaking. Grow the bacteria in the 15-ml tube by shaking at 225 rpm at 37°C until an OD_{600} of 0.6 to 1.0 is reached.

15. Prepare sufficient PCR cocktail for each colony picked plus one (22 μl per sample plus an additional 22 μl):

    0.1 μl 5′ primer at 100 pmol/μl
    0.1 μl 3′ primer at 100 pmol/μl
    H_2O to 22 μl.

    Multiply the above by the number of colonies picked plus 1.

16. Using one Ready-To-Go PCR tube per culture, add 22 μl of the above cocktail to each tube. Add 3 μl of each 1-hr culture from the 96-well plate to an individual PCR tube.

17. Carry out PCR reaction according to the protocol enclosed in the Ready-To-Go PCR kit.

18. Run 5 μl of each PCR reaction on an agarose gel (Voytas, 2000) with size markers to evaluate.

    Correct bands should be ~220 bp plus a little extra for added nucleotides.

19. Prepare minipreps from the appropriate 2-ml cultures (Engebrecht et al., 1991) and verify clones by sequencing miniprep DNA.

    Positive clones may be used to produce larger amounts of DNA. Cultures containing these clones may be stored as glycerol stocks, or the isolated DNA may be stored at –20°C.

BASIC PROTOCOL 18

Immunoblotting with Anti-Ubiquitin

An immunoblot probed with anti-ubiquitin will reveal both native ubiquitin and any protein to which ubiquitin has been conjugated, either as a monomer, or as multiple couplings. This can be used to study both in vivo interactions and to evaluate in vitro ubiquitination.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

Materials

Protein samples to be analyzed
0.5% (v/v) glutaraldehyde (Sigma) in 0.1 M sodium phosphate, pH 7.0 (*APPENDIX 2A*)
Phosphate-buffered saline (PBS; see recipe)
TBST/BSA (see recipe for TBST)
1.5 μg/ml polyclonal rabbit anti-ubiquitin (commercially available or prepared as in Basic Protocol 19) or monoclonal anti-ubiquitin in TBST/BSA

TBST buffer (see recipe)

[^{125}I]protein A (MP Biomedicals Life Science Division, or see recipe) *or* HRP-conjugated anti–rabbit Ig and chemiluminescent detection reagents (SuperSignal West Pico, Pierce, or ECL Plus, GE Healthcare)

Biomax MR film (Kodak)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), blotting from gels to PDVF or nitrocellulose membranes (*UNIT 6.2*), and autoradiography (*UNIT 6.3*)

Analyze and transfer protein

1. Resolve samples generated with unlabeled ubiquitin on SDS-PAGE gel (*UNIT 6.1*) and transfer to Immobilon (PVDF) or nitrocellulose membrane using established protocols (*UNIT 6.2*).

2a. *For PVDF membrane:* Fix the membrane by incubating in a solution of 0.5% glutaraldehyde for 20 min at room temperature. Wash three times, each time with 20 ml PBS.

2b. *For nitrocellulose membrane:* Fix by autoclaving the membrane on a wet cycle.

Expose membrane

3. Block nonspecific binding by incubating membrane with TBST/BSA for at least 1 hr at room temperature or overnight at 4°C.

4. Incubate with rabbit polyclonal anti-Ub (1.5 μg/ml in TBST/BSA) for at least 1 hr at room temperature with shaking, or overnight at 4°C.

5. Wash extensively with TBST at room temperature.

Detect bound antibody

To detect with ^{125}I

6a. Expose membrane to 0.5 to 1 μCi/ml [^{125}I]protein A in TBST/BSA for 1 hr at room temperature. Wash extensively with TBST buffer.

7a. Evaluate by autoradiography (*UNIT 6.3*).

To detect with enhanced chemiluminescence (ECL)

6b. Expose membrane to a 1/20,000 dilution of HRP-conjugated rabbit IgG secondary antibody in TBST/BSA for 1 hr.

Use HRP anti-mouse IgG at 1/10,000 when primary antibody is mouse monoclonal.

7b. Wash extensively with TBST buffer.

8b. Mix equal parts of SuperSignal West Pico Stable Peroxide Solution and Luminol/Enhancer Solution, or follow the directions for the ECL Plus kit.

About 2 ml total is sufficient for a membrane of 48 cm^2.

9b. Place the membrane on a piece of plastic wrap and cover with the SuperSignal mixture. After a few minutes drain the membrane onto the plastic wrap and place the opposite side of the membrane on top of the pool.

10b. Drain onto a paper towel, wrap in plastic wrap, and expose to Kodak Biomax MR film in a darkroom for varying times, usually 5 sec to a few minutes.

A band of ~8 kDa is native, unbound ubiquitin. A known protein that is even multiples of 8 kDa higher than expected is either mono-ubiquitylated or multi-ubiquitylated. An upward smear represents various multiples of ubiquitinations, either as multiple mono-ubiquitins, or various combinations of multi-ubiquitin chains.

GENERATION AND PURIFICATION OF RABBIT ANTI-UBIQUITIN ANTIBODIES

Often, the use of tagged or radiolabeled ubiquitin is undesirable. This is either because the epitope tags may interfere with ubiquitin modification or because the availability of radiolabeled ubiquitin or the licenses required for this type of work are problematic. In this case, it is desirable to use anti-ubiquitin antibodies to detect the modification of proteins.

There are an increasing number of anti-ubiquitin reagents commercially available, including monoclonal antibodies that will differentially recognize multi-ubiquitin chains. A problem in the field has been the quality of commercially available reagents. This is in part due to quality-control issues, but it is made more difficult by the conservation of ubiquitin between species and its consequent poor immunogenicity. The authors have had success in producing high-quality polyclonal antibodies useful for immunoblotting using commercial ubiquitin as a source. However, because of low yield and high background, affinity purification is necessary. In general, yields after purification are on the order of 0.2 to 1.2 mg antibody from 25 ml of serum. Other potential sources of antigen include recombinant ubiquitins, such as GST-ubiquitin and synthetic ubiquitin peptides. Each of these has potential advantages and disadvantages. The protocol described here uses commercial ubiquitin linked to a carrier to generate rabbit polyclonal antibodies.

Even though affinity purification will eventually be carried out, the authors recommend that investigators obtain a number of preimmune sera from rabbits that can potentially be immunized and select those that have relatively little background when used to probe a cell lysate.

BASIC PROTOCOL 19

Generation and Purification of Anti-Ubiquitin Antibodies

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Chicken albumin or ovalbumin, KLH, or other suitable hapten
Ubiquitin (from bovine erythrocytes; Sigma)
0.1 M potassium phosphate buffer, pH 7.0 (*APPENDIX 2A*)
3% (v/v) glutaraldehyde in potassium phosphate buffer, pH 7.0
Phosphate buffered saline (PBS; see recipe)
10% (w/v) SDS
Rabbits
Complete Freund's adjuvant (CFA, Sigma)
Incomplete Freund's adjuvant (IFA, Sigma)
Sodium azide
Ubiquitin-agarose affinity column (see Support Protocol 3)
0.2 M glycine, pH 2.7
2 M Tris base
PBS (see recipe) containing 0.02% (w/v) sodium azide
1 M KCl

15-ml tube
10,000-MWCO dialysis membrane
90°C water bath
Tabletop and refrigerated centrifuges

Additional reagents and equipment for preparing antigens with adjuvants (*UNIT 16.1*), immunoblotting (Basic Protocol 18), and dialysis (*APPENDIX 3C*)

Prepare the ubiquitin/hapten antigen

1. Combine the following in a 15-ml tube:

   15 mg chicken albumin or ovalbumin
   3.8 mg bovine erythrocyte ubiquitin
   2.2 ml of 0.1 M potassium phosphate buffer, pH 7.0.

   Vortex at room temperature until homogeneous.

2. To the above mixture, add 80 µl of 3% glutaraldehyde, 20 µl at a time at 10-min intervals. Vortex between additions.

3. Dialyze (*APPENDIX 3C*) overnight against 4 liters PBS using a 3500-MWCO dialysis membrane. Change the buffer at least once after 4 hr.

4. To the dialysate, add sufficient 10% SDS for a final concentration of 2% (w/v). Heat 10 min at 90°C.

5. Dilute 1:10, then divide into aliquots containing ~0.2 mg of Ub each.

Immunize rabbits

6. Obtain an ~20- to 30-ml bleed from the ear vein of unimmunized rabbits for preimmune negative controls. Store at 4°C overnight and remove coagulate by centrifuging 5 min at 750 × *g*, room temperature. Collect the serum.

   This should provide 10 to 15 ml of serum.

   The authors strongly recommend using a reputable commercial laboratory for animal care, immunization, and bleeding.

7. For each rabbit to be immunized, emulsify 0.2 to 0.5 mg of the albumin-conjugated ubiquitin 1:1 with complete Freund's adjuvant (*UNIT 16.1*). Inject the rabbit subcutaneously at multiple sites with 0.2 to 0.5 mg of this mixture.

   Booster injections can be done at single sites but the comfort of the animal should always be considered.

Collect antisera

8. After 1 month, collect a test bleed of 2 ml and prepare serum as for the pre-bleed.

9. Every 2 weeks, collect a test bleed of 2 ml and boost with 0.12 to 0.25 mg conjugated Ub diluted and emulsified at a 1:10 ratio with incomplete Freund's adjuvant.

10. Test the bleeds by immunoblotting (Basic Protocol 18) on 1, 10, and 100 ng of GST-ubiquitin or free ubiquitin and on cell lysate from cells treated with and without proteasome inhibitor (Basic Protocol 13). Use an initial serum dilution of 1:200.

11. After the test bleed indicates that the rabbit has produced a significant amount of antibody, continue boosting and set up regular bleeding schedule of ~ 25 ml serum every 2 weeks.

Affinity purify anti-ubiquitin antibodies

12. Clarify sera by centrifuging 30 min at 15,000 × *g*, 4°C, to remove debris. Add sodium azide to the supernatant for a final concentration of 0.02% (w/v).

    If there is significant lipid in the cleared serum, filter through gauze or cheesecloth. Lipid is readily detected by eye and characterized by the presence of a milky appearing white suspension in serum sample.

13. Wash ubiquitin affinity columns with 20 to 50 ml of PBS.

14. Bind antibodies to the column by passing 50 ml serum through the column twice. Save the flowthrough.

    It generally takes ~1 hr per pass of 50 ml.

14. Wash the column extensively with at least 100 ml PBS, monitoring the OD_{280} of the flowthrough until it is <0.005.

    If OD_{280} is >0.005 after 100 ml, wash with an additional 50 ml PBS and recheck. If the OD is not dropping at all and is <0.010, proceed with elution; if not continue washing.

15. Elute antibody using 0.2 M glycine, pH 2.7, collecting eluate fractions 1 ml at a time into tubes containing 50 µl of 2 M Tris base.

16. After collecting fifteen 1-ml fractions, determine the OD_{280} of each using the a mixture of 1 ml of 0.2 M glycine and 50 µl of Tris base as a blank.

17. Pool the tubes with significant levels of material (OD_{600} > 0.010) and dialyze (*APPENDIX 3C*) against 4 liters PBS with 0.02% sodium azide (0.02%) at 4°C overnight using a 10,000-MWCO dialysis membrane. Perform two additional changes of buffer over the course of the following day.

18. Regenerate column by passing 10 to 15 ml PBS through it, followed by 10 ml of 1 M KCl. Run an additional 50 ml of PBS through the column and store at 4°C in PBS/0.02% NaN_3.

SUPPORT PROTOCOL 3

Preparation of Ubiquitin Affinity Column

Crude serum or protein A–clarified antibodies often give good results in immunoblotting and other applications. However, the use of antibody purified on specific affinity columns avoids detection of nonspecific bands that are often seen with the more crude preparations.

Affinity columns for use in Basic Protocol 19 can be made using kits available from Pierce, Amersham, and other companies. The authors use Affi-gel-10 from Bio-Rad, which couples basic and neutral proteins such as ubiquitin through primary amine groups.

Materials

Affi-Gel-10 slurry (Bio-Rad)
4 mg/ml ubiquitin (Sigma) in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0
1 M ethanolamine, pH 8.0
0.2 M glycine, pH 4.0 (adjusted with HCl)
Phosphate-buffered saline (PBS; see recipe)
1 M KCl
1% (w/v) sodium azide

Fritted-glass filter, coarse
50-ml conical centrifuge tubes

1. Wash 25 ml of Affi-Gel-10 slurry in water over a clean coarse fritted-glass filter.

2. Remove the slurry from filter into a 50-ml conical centrifuge tube. Add 9 to 10 ml of 4 mg/ml ubiquitin in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0.

3. Incubate on an end-over-end rotator overnight at 4°C or for 1 to 2 hr at room temperature. Recover supernatant by centrifuging 5 min at 3750 × *g*, 4°C.

4. Block unreacted sites by incubating at room temperature for 1 hr with an equal bed volume of 1 M ethanolamine, pH 8.0.

5. Wash three times over fritted-glass filter alternately with 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0, and 0.2 M glycine, pH 4, using 1 bed volume for each wash.

   Six washes are thus performed, three with the bicarbonate/NaCl solution and three with the glycine solution.

6. Follow up by washing on filter with 10 bed volumes of PBS followed by 10 bed volumes of 1 M KCl two to three times.

7. Finish by washing slurry into 10 vol PBS, add 1 M sodium azide to 0.02% final, and store at 4°C.

GENERATION OF REAGENT-GRADE UBIQUITIN ACTIVATING ENZYME (E1)

When studying the ubiquitin system, it is necessary to have the means to produce partially pure or fully purified components. Many components of the ubiquitin system are available commercially; however, it is often cost effective to produce these proteins in house.

In this section, the authors describe the production of E1 or ubiquitin activating enzyme, the first protein in the multi-step process of ubiquitin chain formation. Subsequent sections address the production of other ubiquitin pathway components.

Any E1 purchased or generated in the laboratory will have to be tested both for its own activity and for its ability to interact with specific ubiquitin conjugating enzymes or E2s (see Basic Protocol 2). Wheat E1 works well with many E2s, but it has been shown to be inactive with HIP2 (E2-25K), for example. If wheat E1 works in the system of interest, producing it is comparatively inexpensive and easy (Basic Protocol 20). For most purposes, mouse and rabbit E1s work very well with any E2. They are, however, more difficult to produce or more expensive, depending on the source. Mouse E1 can be produced in a baculovirus system and will show good activity during long-term storage in glycerol (Basic Protocol 21). If the laboratory does few ubiquitin assays, it may be worth considering purchasing rabbit E1 from a commercial source.

Preparation of Wheat E1 in *E. coli*

BASIC PROTOCOL 20

Wheat E1 is inexpensive and easy to produce because it can successfully be obtained in an active form from *E. coli*. It generally works with most E2s, but in some cases mammalian E2 will require mammalian E1.

The bacterially expressed E1 that the authors use, which is cloned from wheat by Richard Vierstra and colleagues, University of Wisconsin at Madison (Hatfield et al., 1990), is in pET3a. pET vectors are supplied by Novagen. The pET vectors are transformed into BL21 competent cells (Novagen) according to the manufacturer's protocol. Appropriate bacterial strains may also be grown in the laboratory and made competent for transformation.

Materials

pET E1 plasmid (request from R. Vierstra, University of Wisconsin at Madison, or A. Weissman)
E. coli strain BL21 transformation-competent bacteria (Novagen)
SOC medium (Digene)
LB plates (*APPENDIX 2A*) containing the appropriate selection antibiotic
LB medium (*APPENDIX 2A*) containing the appropriate selection antibiotic
1 M IPTG (dissolve 1 g IPTG in 4.2 ml water; store at −20°C)
Sonication buffer (see recipe)
E2 of choice

42°C water bath
Platform shaker
Refrigerated centrifuge
Probe sonicator (e.g., Misonix Sonicator 3000)

Transform BL21 with pET E1 construct

1. Add 1 μl ubiquitin-containing plasmid to 50 μl of transformation-competent cells which have been thawed on ice.
2. Incubate on ice for 30 min, heat shock for 45 sec at 42°C, and return to ice for 2 min.
3. Add 450 μl of room temperature SOC medium. Incubate 1 hr at 37°C with shaking on a platform shaker.
4. Microcentrifuge 15 min at 14,000 × *g*, 4°C. Remove all but 100 μl of medium, resuspend cells in this residual medium, and spread onto an LB plate with appropriate antibiotic. Incubate at 37°C.
5. The following day start a 2-ml culture in LB medium with appropriate antibiotic from a single colony. Incubate 8 hr at 37°C with shaking.
6. Use the 2-ml culture to start a larger culture (100 ml to 1 liter) with antibiotic. Grow the larger culture at 37°C until the OD_{600} is 0.6 to 1.0.

Express protein in bacteria

7. Add 1 M IPTG to the culture for a final concentration of 0.2 mM IPTG and continue incubation 1 to 2 hr at room temperature.
8. Centrifuge cells 15 min at 300 × *g*, 4°C, and remove the supernatant. Freeze pellet at −80°C or sonicate immediately.

Prepare lysate

9. For each 50 ml of culture, add 1 ml sonication buffer to the cell pellet. Sonicate 30 sec on ice at 60% to 70% of the microtip limit (setting 3.5 on Misonix Sonicator 3000).
10. Centrifuge 15 min at 15,000 × *g*, 4°C, to pellet debris.

    The supernatant is the crude lysate, which generally contains 100 to 150 ng recombinant protein/μl crude lysate.

11. Divide supernatant into aliquots sufficient for individual experiments and store at −80°C.
12. Assay for function by thiolester assay with E2 of choice (Basic Protocol 2).

BASIC PROTOCOL 21

Preparation of Mouse E1 in Insect Cells Using a Baculovirus System

Certain E2s require a mammalian E1, and for some E3 ubiquitylation assays the robustness of ubiquitylation is enhanced by using a mammalian E1. For poorly understood reasons, active mouse E1 is not readily expressed in *E. coli*, but it can be successfully expressed in insect cells. *Sf*9 cells, derived from *Spodoptera frugiperda* (army moth) ovarian cells, are commonly used to isolate and propagate recombinant AcMNPV (baculovirus) stocks and produce the recombinant protein of interest. The authors' mouse E1 baculovirus stock was generated in the Iwai laboratory (Iwai et al., 1999). Stratagene and Promega also have kits and protocols for making these baculovirus constructs. The following describes a generic method for working with baculovirus and producing mouse E1 protein in insect cells.

Materials

*Sf*9 cells (Invitrogen)
Complete Grace's insect medium (see recipe)
Baculovirus (AcMNPV) stock encoding His_6-tagged E1, recently titered
Baculovirus lysis buffer 2 (see recipe)
5% (w/v) agarose (SeaKem from FMC or Baculovirus Agarose from Invitrogen) in H_2O
Grace's insect medium (Invitrogen), serum free
1% (w/v) neutral red (Sigma)
175-cm^2 tissue culture flasks and 60-mm tissue culture dishes, plus other appropriate tissue culture vessels (Table 15.9.2)
50-ml conical polypropylene centrifuge tubes
60°C and 47°C water baths
Refrigerated centrifuge
Probe sonicator (e.g., Misonix Sonicator 3000)
Additional reagents and equipment for maintenance of insect cell cultures and generation of recombinant baculovirus (Murphy et al., 2004)

Culture cells

1. Plate *Sf*9 cells as adherent cells in flasks or dishes in complete Grace's insect medium (see Table 15.9.2).
2. Grow cells at 27°C without enhanced CO_2 just to confluence, then split 1:3, dislodging the cells either by streaming medium over the monolayer or by hitting the flask against the palm of the hand. Continue incubation at 27°C.

Produce virus

3. Seed a 175-cm^2 tissue culture flask with 21×10^6 log-phase *Sf*9 cells. Incubate at room temperature for >1 hr, then remove medium.
4. Infect cells at MOI of 0.1, i.e., 21×10^5 pfu in 5 ml medium from recently titered baculovirus stock.
5. Incubate the infected cells 1.25 hr, rocking gently every 15 min.
6. Remove virus solution and add 24 ml complete Grace's medium. Incubate 5 days at 27°C (no CO_2).
7. Transfer the culture to a 50-ml centrifuge tube. Centrifuge 5 min at $500 \times g$, room temperature.
8. Collect the supernatant, which is the high-titer virus stock. Store at 4°C, wrapped in foil to protect from light.
9. Titer by serial dilution to determine the pfu/ml.

Perform plaque assay to purify recombinant virus or titer virus

10. Seed 2×10^6 log-phase *Sf*9 cells in 3 ml complete Grace's medium in 60-mm Nunc dishes. Set up two dishes for each dilution to be used. Include a dish for one uninfected control.

    If cell number is limited, set up only one dish per dilution.

11. Let cells adhere at room temperature at least 1 hr.

12. During the incubation in the previous step, prepare agarose overlay as follows:
    a. Autoclave 0.5 ml of 5% agarose per dish, then cool to 60°C in a water bath.
    b. Heat 4.5 ml complete Grace's medium to 60°C per dish.
    c. Mix the medium with the agarose and cool to 40°C.
13. Prepare serial ten-fold dilutions of virus stock from 10^{-2} to 10^{-10} in complete Grace's medium. For duplicate dishes, make the 1:100 dilution by mixing 30 µl of stock with 3 ml medium, then for the rest take 240 µl of this and add to 2.2 ml Grace's medium.
14. Remove medium from cells and add 1 ml of viral stock dilution to each dish. Incubate at room temperature for 1.25 hr, rocking plates every 15 min.
15. Remove virus inocula from cells and add 4 ml of agarose overlay by slowly streaming down the side of the dish. Let solidify.
16. Incubate, top-side-up, in a 27°C humidified incubator for 5 to 8 days.
17. Count plaques when clearly visible. If plaques are difficult to see, enhance with neutral red as follows:
    a. Add 5 ml 2.5% agarose at 47°C to 24 ml serum-free Grace's insect medium at 47°C. Place in 47°C water bath.
    b. Add 0.8 ml of 1% neutral red (Sigma).
    c. Add 2 ml of this mixture per plate and let stand 3 hr at room temperature.
18. Calculate pfu/ml as follows:

$$\text{Average pfu/dish} \times \text{dilution}^{(-1)} = \text{pfu/ml}.$$

For example, if plates with 10^{-5} dilutions have an average of 200 plaques per dish, pfu/ml = 200×10^5 or 2×10^7/ml.

BD Biosystems is offering a new technology for titering baculovirus stocks, based on an ELISA assay, with results available in a few days rather than almost 2 weeks. It is called BacPAK Baculovirus Rapid Titer Kit.

Infect Sf9 cells with recombinant baculovirus to produce protein

19. Seed 21×10^6 *Sf*9 cells in 24 ml complete Grace's medium in a 175-cm^2 flask. Allow to adhere at room temperature for > 1 hr.
20. Remove medium from cells, add 5 ml virus solution containing 10 to 50 MOI, i.e., 21×10^7 to 1×10^8 pfu per 5 ml. Incubate at room temperature for 1.25 hr, rocking flask every 15 min.
21. Remove virus inoculum from cells and add back 24 ml complete Grace's medium Incubate at 27°C for 2 to 3 days. Include at least one flask with a "mock" infection (medium only) to use as a negative control.
22. Harvest cells by centrifuging 5 min at $750 \times g$, 4°C. Add 1 ml baculovirus lysis buffer 2 to the pellet per 8×10^6 cells (~1.5 ml buffer per 175-cm^2 flask).
23. Sonicate three times, each time for 20 sec using Misonix sonicator at 3.5 setting, on ice. Centrifuge 20 min at $12{,}000 \times g$, 4°C. Collect the supernatant, which is the crude E1 extract.
24. Store in small aliquots at −80°C, or purify by chromatography.

Preparation of Rabbit E1

ALTERNATE PROTOCOL 11

Mammalian E1 from rabbit may be purchased commercially from Calbiochem. The authors dilute the Calbiochem E1 1:5 in 50 mM HEPES, pH 7.6, which yields 100 ng/μl. The diluted E1 is stored up to 6 months at −80°C.

Generation of Empty Bacterial Lysates

SUPPORT PROTOCOL 4

For many of the protocols described, ubiquitin system enzymes are generated in bacteria as crude lysates. As a proper control in many of these reactions, it is necessary to add bacterial proteins that contain none of the ubiquitin-conjugating enzymes, i.e., "empty" lysates. This protocol is essentially identical to Basic Protocol 20. Briefly, pET vectors without a protein sequence are transformed into BL21 competent cells according to the manufacturer's protocol.

Additional Materials *(also see Basic Protocol 20)*

PET3a, pET15b or other vector for expression of proteins in *E. coli* from a T7 promoter but without a protein sequence

Transform BL21 with pET E1 construct

1. Transform BL21 with pET vector that has not had a cDNA cloned into its multiple cloning region and spread onto a plate with appropriate antibiotic. Incubate at 37°C.
2. The following day, start a 2-ml culture in LB medium with appropriate antibiotic from a single colony. Grow 8 hr at 37° with shaking.
3. From the 2-ml start a larger culture (100 ml to 1 liter) in LB medium with antibiotic. Grow at 37°C with shaking until the OD_{600} is 0.6 to 1.0.

Produce protein in bacteria and generate lysate

4. Add 1 M IPTG to the culture for a final concentration of 0.2 mM and continue incubation 1 to 2 hr at 37°C.
5. Pellet cells by centrifuging 15 min at 300 × *g*, 4°C. Discard the supernatant. Freeze pellet at −80°C or sonicate immediately.
6. For each 50 ml of culture, add 1 ml sonication buffer to the cell pellet. Sonicate 30 sec on ice at 60% to 70% of the microtip limit (setting 3.5 on Misonix Sonicator 3000).
7. Centrifuge 15 min at 15,000 × *g*, 4°C, to pellet debris.
8. Divide supernatant into aliquots sufficient for individual experiments and store at −80°C.

GENERATION OF UBIQUITIN-CONJUGATING ENZYMES (E2s)

In this section, protocols are included for the generation of members of the second class of enzymes of the ubiquitylation pathway, ubiquitin-conjugating enzymes, or E2s. While there is only a single mammalian E1, there are approximately 34 different E2s. Protocols are provided for production of both crude and purified forms of E2.

Strategic Planning

A major issue in working with E2s (Ubc's or Ube's) is making sure one is working with the correct enzyme. While some E2s from higher eukaryotes match the names of their yeast counterparts, in many cases they do not. Table 15.9.3 clarifies some of the confusion (Lorick et al., 2005a, b). Accession numbers provided are current GenBank reference sequences where available.

Table 15.9.3 Current E2 Nomenclature

Common name(s) of human E2/E2 variant	Official name	Refseq/Genbank ID	Nearest yeast E2 homolog
E2s for ubiquitin or ubiquitin-like molecules			
Apollon, BRUCE, BIR6	BIRC6	NP_057336	ubc7p/qri8p
HHR6A, RAD6A	Ube2A	NP_003327	rad6p/ubc2p
HHR6B, RAD6B	Ube2B	NP_003328	rad6p/ubc2p
UbcH10	Ube2C	NP_008950	ubc11p
UbcH5A	Ube2D1	NP_003329	ubc4p or ubc5p
UbcH5B	Ube2D2	NP_003330	ubc4p or ubc5p
UbcH5C	Ube2D3	NP_003331	ubc4p or ubc5p
UbcH5D, hBUCE1	Ube2D4	NP_057067	ubc4p or ubc5p
UbcH6	Ube2E1	NP_003332	ubc5p or ubc4p
UbcH8	Ube2E2	NP_689866	ubc5p or ubc4p
UbcH9, UbcM2, E2-23K	Ube2E3	NP_872619, NP_006348	ubc4p or ubc5p
Ubc7, E2-17K, HH C. elegans Ubc7	Ube2G1	NP_003333	ubc7p/qri8p
HH mouse Ubc7, MmUbc7	Ube2G2	NP_003334	ubc7p/qri8p
UbcH2, E2-20K, ubcH8	Ube2H	NP_003335	ubc8p
UbcH9/sumo1 conjugating enzyme	Ube2I	NP_003336, NP_919235, NP_919236, NP_919237	ubc9p
NCUBE1, UBC6 homolog E, HSUBC6e, CGI-76, HSPC153/HSPC205	Ube2J1	NP_057420, NP_057105	ubc6p, some 3′ homology to IRA2
NCUBE2,UBC6 homolog	Ube2J2	NP_919296	ubc6p
E2-25K, HsUBC1, HIP2	Ube2K	NP_005330	ubc1p, ubc-X or pex4p
UbcH7, E2-18K, UbcM4, E2-F1, L-UBC	Ube2L3	NP_003338	ubc4p or ubc5p
UbcH8, RIG-B	Ube2L6	NP_004214	ubc4p or ubc5p
UbcH12, Nedd8-conjugating enzyme	Ube2M1	NP_003960	ubc12p
Similar to E2M, UbcH12, Nedd8 conjugating enzyme	Ube2M2	XP_497504	ubc12p
NEDD-conjugating enzyme	Ube2M3	NP_542409	ubc12p or ubc5p or ubc4p or ubc13p
UbcH13, bendless homolog	Ube2N1	NP_003339	ubc13p
UBE2NL, similar to ubcH13	Ube2N2	XP_372257	ubc13p
Ube2Q	Ube2Q	NP_060052	ubc11p or ubc3/cdc34p
LOC92912	Ube2Q2	NP_775740	ubc3p/cdc34
cdc34, E2-32K	Ube2R1	NP_004350	ubc3p/cdc34 or ubc7p
UBC3B	Ube2R2	NP_060281	ubc3p/cdc34

continued

Table 15.9.3 Current E2 Nomenclature, *continued*

Common name(s) of human E2/E2 variant	Official name	Refseq/Genbank ID	Nearest yeast E2 homolog
E2-EPF5, E2-24K	Ube2S	NP_055316	ubc13p
E2-24K-2	Ube2S2	XP_496186	ubc13p
Hypothetical, HSPC150	Ube2T	NP_054895	ubc13p
Hypothetical, LOC148581, MGC31530	Ube2U	NP_689702	rad6p
Hypothetical, LOC55284, FLJ11011	Ube2W	NP_001001481	ubc7p/qri8p
KIAA1734, ortholog of mouse E2-230K	E2-230K	NP_071349	rad6p
HOYS7, LOC65264	FLJ13855	NP_075567	rad6p
Similar to Drosophila CG4502	CG4502	XP_059689	ubc3p/cdc34
E2 variants/UEVs			
MMS2, CROC-1, IKKactivator, UEV1a	Ube2V1	NP_068823	Mms2p
MMS2, EDAF/EDPF-1, DDVit 1, Enterocyte differentiation associated/promoting factor, Vitamin D3 inducible protein	Ube2V2	NP_003341	Mms2p
UEV3	Ube2V3	NP_060784	STP22p
LOC441372, Similar to E2V1	Ube2V4	XP_496988	Mms2p
Fts, Ftl, Fused toes homolog	FTH	NP_071921	ubc8p
TSG101	TSG101	NP_006283	STP22p

Before embarking on expressing E2s, it may be worth checking whether the increasing number of commercially available E2s suits one's needs.

Expression of E2s in *E. coli* Without a Purification Tag

BASIC PROTOCOL 22

For most applications, a crude bacterial lysate containing expressed protein is sufficient for use in ubiquitylation assays. Bacteria do not have a ubiquitylation system, and therefore the only ubiquitin-related protein in the crude lysate will be the mammalian enzyme expressed by the bacteria.

Materials

pET expression vector (Novagen) containing cDNA for E2 of interest

Additional reagents and equipment for transforming bacteria with pET plasmid and preparing crude lysates of bacteria (Basic Protocol 20), purification of E2 (Alternate Protocol 12 or 13), and testing E2 activity (Basic Protocol 2)

1. Transform bacteria with pET plasmid containing cDNA for the E2 of interest and grow the cells (see Basic Protocol 20).
2. Prepare crude lysates of bacterial transformed with E2 plasmid (see Basic Protocol 20).

3. Store E2 in small aliquots (10 μl) or proceed to purification (Alternate Protocols 12 and 13).

4. Test E2 activity before use (Basic Protocol 2).

   E2s have varied requirements for reducing agent. Some will have maximum activity with no DTT added; some may require as much as 10 mM. This will have to be determined empirically. The authors typically start with 5 mM DTT in the lysis buffer.

ALTERNATE PROTOCOL 12

Purification of E2s from *E. coli* Using an Affinity Tag

When purified E2 is required, small tags such as His_6, T7, FLAG, or HA may be added by cloning into commercially available vectors. These tags may often be used for rapid purification without affecting overall function. All tags, especially larger ones, such as GFP or GST, have to be tested in the specific application to make sure they do not alter or eliminate activity of the E2. Most of the tagged E2 constructs used in the authors' laboratory have a His_6 tag and their purification is described below. Purification of other tags may be carried out using an agarose-conjugated anti-tag antibody or other affinity matrices (Alternate Protocol 13). In all cases, a protease cleavage site may be inserted between the tag and the E2 allowing for purification of untagged E2. This type of purification is described in Basic Protocol 17 for GST fusions.

Materials

Affinity matrix (Ni-NTA agarose from Qiagen; Talon from Clontech; or equivalent product from Invitrogen)
50 mM sodium phosphate, pH 8.0 (*APPENDIX 2A*)
Bacterial lysate containing E2 protein (Basic Protocol 22)
1 M imidazole, pH 7.5 (adjusted with HCl)
Wash buffer 1 for His_6-tagged proteins (see recipe)
Elution buffer for His_6-tagged proteins (see recipe)
Dialysis buffer for ubiquitylating and neddylating enzymes (see recipe)
Glycerol
3500-MWCO dialysis membrane

Additional reagents and equipment for dialysis (*APPENDIX 3C*) and testing E2 activity

Prepare affinity matrix

1. Add 50 μl affinity matrix to a 1.5-ml microcentrifuge tube.

2. Rinse the beads twice, each time by adding 1 ml of 50 mM sodium phosphate, microcentrifuging 30 sec at 6000 × *g*, room temperature, and removing the supernatant.

   Agarose conjugates are susceptible to matrix crushing, so higher speeds are not recommended.

Bind and wash the lysate

3. Add 1.0 ml (up to 500 μg of the protein to be purified, depending on the capacity of the matrix) crude lysate to the beads and adjust the concentration of the solution to 8 mM imidazole by adding 8 μl of 1 M imidazole. Remove a 10-μl aliquot for electrophoresis.

4. Bind the protein to the matrix beads by incubating 2 hr at 4°C on an end-over-end rotator.

5. Centrifuge the tube 30 sec at 6000 × *g*, 4°C. Remove the depleted supernatant, reserving a 10-μl aliquot for electrophoresis.

6. Wash the beads three times, each time by adding 1 ml wash buffer 1, centrifuging again as before, and removing the supernatant.
7. Elute the protein from the beads by adding 500 µl elution buffer and incubating 10 min at room temperature.
8. Centrifuge 30 sec at 6000 × *g*. Collect the supernatant into a fresh 1.5-ml microcentrifuge tube on ice.
9. Repeat the elution and pool the supernatants.

Dialyze and store the purified protein

10. Dialyze the eluate against 4 liters dialysis buffer overnight at 4°C using a 3500-MWCO dialysis membrane, changing the buffer twice at intervals at least 4 hr apart.
11. Remove the dialysate and divide into two tubes. Add an equal volume of glycerol to each tube and mix thoroughly. Store at −20°C to −80°C.
12. Test E2 activity before use (Basic Protocol 2).

Purification of E2s from *E. coli* Using Anti–Affinity Tag Antibodies

ALTERNATE PROTOCOL 13

This protocol can be carried out using centrifugation as described here, but it is easily modified for column purification. The latter option should be considered when working with larger volumes or when the matrix needs to be reused. However, success in purifying with antibodies varies greatly with the affinity of the antibody and the quality of the antibody-agarose conjugate, so materials should be reused only when necessary. In addition, the activity of the E2 purified in this way may be questionable. Therefore it may be most useful for producing antigen for antibody production or as a positive control for immunoblotting. Alternatively, protease cleavage may be employed to avoid the harsh acid elution (see Basic Protocol 17 for thrombin cleavage of GST fusions).

Materials

Antibody-agarose conjugates (Santa Cruz Biotechnology; 50% w/v slurry)
50 mM sodium phosphate, pH 7.0
Bacterial lysate containing E2 protein (Basic Protocol 22)
50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
200 mM glycine, pH 2.8
1 M Tris base
Dialysis buffer (see recipe)
Glycerol
0.1 M NaOH (optional)
20% ethanol or 50 mM sodium phosphate (pH 7.0)/0.02% (w/v) sodium azide (optional)
3500-MWCO dialysis membrane
Additional reagents and equipment for dialysis (*APPENDIX 3C*) and testing E2 activity (Basic Protocol 2)

Prepare affinity matrix

1. Add 25 µl of a 50% slurry of agarose-conjugated antibody to a 1.5-ml tube.
2. Microcentrifuge 30 sec at 6000 × *g*, 4°C. Wash beads twice, each time by adding 1 ml of 50 mM sodium phosphate, microcentrifuging again as before, and removing the supernatant.

Bind and wash the lysate

3. Add 1.0 ml crude lysate (containing up to 500 μg protein, depending on the capacity of the matrix) to the beads. Remove 10 μl for electrophoresis.
4. Bind the protein to the matrix beads by incubating 2 hr at 4°C on an end-over-end rotator.
5. Pellet beads by centrifuging 30 sec at 6000 × *g*. Remove the depleted supernatant, retaining a 10-μl aliquot for electrophoresis.
6. Wash beads three times, each time by adding 1 ml Tris·Cl, pH 7.4, centrifuging 30 sec at 6000 × *g* to pellet the beads, and removing the supernatant.
7. Elute protein from beads by adding 500 μl 100 mM glycine, pH 2.8, and incubating 10 min at room temperature.
8. Microcentrifuge 30 sec at 6000 × *g*. Collect the supernatant into a fresh tube on ice containing 50 μl 2 M unbuffered Tris base.
9. Repeat the elution (steps 7 and 8) into a second tube containing 50 μl of 2 M Tris base and pool the supernatants. Retain beads for regeneration (steps 13 to 15).

Dialyze and store the purified protein

10. Dialyze the eluate (*APPENDIX 3C*) against 4 liters dialysis buffer at 4°C overnight using a 3500-MWCO dialysis membrane, changing the buffer twice at intervals of at least 4 hr.
11. Remove the dialysate and divide into two tubes. Add an equal volume of glycerol to each tube and mix thoroughly. Store up to 6 months at −20°C or indefinitely at −80°C.
12. Test E2 activity before use (Basic Protocol 2)

Regenerate the antibody matrix (optional)

13. If the matrix needs to be regenerated, after elution, add 500 μl of 0.1 M NaOH, Microcentrifuge 30 sec at 6000 × *g*, and remove the supernatant.
14. Wash beads with 500 μl 50 mM sodium phosphate, pH 7.0, using the technique described in the previous step.
15. Store in 20% ethanol or 50 mM sodium phosphate (pH 7.0)/0.02% sodium azide for later use.

BASIC PROTOCOL 23

Expression of E2s with In Vitro Transcription and Translation Systems

The large amount of protein produced at relatively low cost and lack of endogenous E2s make bacterial expression systems preferable for many studies of E2 activity. However, several factors may make it advantageous to produce a particular E2 in a higher eukaryotic expression system. In particular, the authors have found lower background in E2-E3 binding assays using radiolabeled in vitro–translated E2 material. Other factors include cross-species incompatibility of E2 for an E3, requirements for post-translational modifications, or the need for an unknown co-factor not present in prokaryotic systems. In such cases, in vitro translation (IVT) in wheat germ extracts or rabbit reticulocyte lysates is very convenient (*UNIT 11.2*). In addition, when attempting to detect the interaction of E2s with other proteins, it is often necessary to label the E2. These in vitro systems are particularly well suited for this purpose. For some proteins, researchers may find best results when producing their own RNA and using it in one of these systems. However, because most E2s are very small and easily expressed, the authors recommend using commercially available coupled transcription and translation systems.

Strategic planning

The ubiquitin conjugating system was first discovered in rabbit reticulocyte lysates (Ellison and Hochstrasser, 1991). Components of the ubiquitylation system are enriched in reticulocytes because of the high level of ongoing protein degradation required for transformation into an erythrocyte. This can be an advantage in some cases and a detriment in others. In one case, the protein of interest could be tagged with ubiquitin and prematurely degraded before one has the chance to analyze its function. With E2s, in particular, care must be taken to make sure that effects seen with reticulocyte-expressed proteins are the result of the expressed E2 and not of those proteins endogenous to reticulocytes. Often, a mammalian protein that is degraded in reticulocytes will not be degraded when translated in wheat germ extracts, due to incompatibilities between plant E1s and some mammalian E2s. In many cases, the use of proteasome inhibitors, such as MG132, allows for the production of ubiquitin system components with minimal degradation. This is especially important for producing E3 proteins or substrates for E3s, but may also be an issue in E2 production. Proteins produced using IVT systems may, of course, also be expressed with affinity tags and purified as described in Alternate Protocol 13.

When labeling the IVT product with radioactive amino acids, amino acid mixtures added to the reaction must be complete except for the radioactive component. Nonradioactive labels are also available for amino acids. For unlabeled protein, use a complete amino acid mixture.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

Materials

10× transcription buffer (Promega)
20 to 40 U/μl ribonuclease inhibitor (e.g., RNasin; Promega)
10 mM NTP mix (10 mM each ATP, CTP, GTP, UTP; Promega)
0.5 + 2μg/μl plasmid vector containing cDNA for E2 of interest under the control of a bacteriophage RNA polymerase, e.g., T7, SP6, or T3 (Promega)10 to 20 U/μl RNA polymerase appropriate to vector (SP6, T3, or T7)
0.7% agarose gel in MOPS-formaldehyde (optional; Brown et al., 2004)
10× in vitro translation (IVT) buffer (Promega)
Rabbit reticulocyte lysate or wheat germ extract (Promega)
Amino acid mixture (Promega) lacking methionine (−Met), cysteine (−Cys) or lysine (−Lys) depending on amino acid of choice for radiolabeling
50 mM MG132 (Calbiochem, Boston Biochem, or Biomol) in DMSO
15 μCi/μl [^{35}S]methionine, [^{35}S]cysteine, or [^{15}N]lysine (1000 Ci/mmol, NEN)
10% (w/v) trichloroacetic acid (TCA; Sigma), cold
EcoScint scintillation fluid (National Diagnostics)
30°C water bath
0.45-μM HA filters (Millipore)
Additional reagents and equipment for spectrophotometric determination of RNA concentration (*APPENDIX 3D*) *or* agarose-formaldehyde gel electrophoresis of RNA (Brown et al., 2004) and SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

Generate RNA for E2s

1. In a 1.5-ml tube, mix:

   5 μl 10× transcription buffer
   2 μl 20 to 40 U/μl ribonuclease inhibitor

4 μl 10 mM NTP mix
2 μg plasmid DNA (from 0.5 to 2μg/μl stock)
2 μl of appropriate RNA polymerase for the vector (SP6, T3, or T7; typically at 10 to 20 U/μl)
H_2O to 50 μl.

Incubate at 30°C for 1.5 hr

2. Measure the RNA content in a spectrophotometer (*APPENDIX 3D*) or by agarose gel electrophoresis (typically using a 0.7% agarose gel in MOPS-formaldehyde; Brown et al., 2004).

Use 100 ng to 1 μg RNA for translation; the remainder of the RNA may be frozen in aliquots at −80°C for later use.

Translate RNA to protein

3. To a 1.5-ml tube add:

5 μl 10× IVT buffer
25 μl reticulocyte lysate
4 μl amino acid mixture (lacking the amino acid of choice for radiolabeling)
2 μl 20 to 40 U/μl ribonuclease inhibitor
100 ng to 1 μg RNA for E2 of interest (from step 2)
Up to 1 μl 50 mM MG132 or other proteasome inhibitor (optional).
2 μl 15 μCi/μl [^{35}S]methionine (or radiolabeled amino acid of choice).

4. Microcentrifuge briefly at maximum speed to mix the sample and collect the solution at the bottom of the tube.
5. Incubate 1.5 hr at 30°C.
6. Test the quality of the protein via SDS-PAGE (*UNIT 6.1*), detecting radioactive proteins by autoradiography (*UNIT 6.3*).

Most E2s have molecular weights between 14 and 35 kDa so 16% or 18% gels are generally appropriate.

7. *Optional:* Further check radioactive proteins by precipitating 1 μl of the sample from step 6 in 10% cold TCA and spotting on 0.45-μm filters. Place filter in 5 ml scintillation fluid and measure cpm using a liquid scintillation counter.
8. Store protein up to 1 month at −20°C.

IN VITRO PRODUCTION OF UBIQUITIN-PROTEIN LIGASES (E3s)

Ubiquitin ligases (E3s) are the ultimate arbiters of substrate specificity in ubiquitylation. E3s fall into two broad classes defined by the nature of their ubiquitin ligase domains. The first class is the HECT (Homologous to E6-AP C-Terminus) domain family. These enzymes are characterized by a ∼350 amino acid, highly conserved catalytic domain and by the formation of obligate catalytic intermediates between a C-terminal cysteine in the HECT domain and ubiquitin. The second, and by far larger class, consists of the RING-finger E3s and RING-finger-like E3s. The RING finger is characterized by a three-dimensional structure defined by the presence of two Zn ions coordinated by a total of eight cysteine and histidine residues in a cross-braced type of pattern. This forms a platform for E2-Ub and facilitates the transfer of ubiquitin from E2 to heterologous substrates or to the E3 itself. The PHD finger is a variation on the RING finger that differs in the distribution of coordinating residues. The more distantly related U-box takes on the same general three-dimensional structure as the RING finger, but its stability relies

on salt bridges and charged residues, rather than on Zn binding (Aravind and Koonin, 2000; Hatakeyama et al., 2001).

The RING finger E3s may exist as a multi-subunit complex in which multiple components are necessary for both E3 activity and substrate specificity. These are characterized by a small RING finger, a member of the cullin family of proteins and other linkers, regulatory components, and substrate recognition elements that are required for activity. Such multi-subunit E3s include the SCF complexes, the anaphase-promoting complex, or cyclosome, and the CBC E3s (Iwai et al., 1999; Skowyra et al., 1999; Chan et al., 2001). For most RING-finger proteins, it is currently thought that the RING finger and substrate-recognition element are encoded on a single polypeptide (Lorick et al., 2005b).

Strategic Planning

There are a variety of ways to generate recombinant ubiquitin ligases. For the most part, the authors produce these either as GST or His_6 fusions in *E. coli* or in in vitro translation systems in much the same way as E2s (see above). At other times, the authors express and purify E3s from cells (see Basic Protocol 10, for example). Regardless, negative controls are important in evaluating function. While samples lacking added E1 or E2 serve such a role for in vitro ubiquitylation assays, it is also helpful to generate catalytically inactive versions of the putative E3. These can be particularly useful when trying to determine whether a low level of activity is real or background. With HECT domain proteins, simple mutation of the active-site cysteine is sufficient to abrogate E3 activity. For RING- or PHD-finger proteins, mutation of a single Zn-coordinating Cys or His will eliminate most activity. However, it is the authors' experience that some RING-finger E3s have the potential to show residual activity with mutation of a single residue; thus it is reasonable to consider mutating two residues—one involved in the coordination of each Zn. For U-box proteins, mutation of specific residues required for the structure will destroy E3 activity (see Hatekayama et al., 2001). Alternatively, the authors generate inactive forms through truncations or by treating with alkylating agents to inactive HECT E3s—this will also inactivate some RING-finger proteins.

Nearly all E3s that have been tested by the authors are able to ubiquitylate themselves in vitro to a greater or lesser degree. This is referred to as either auto- or self-ubiquitylation. Consequently, when produced in eukaryotic translation systems, one potentially needs to deal with ubiquitylation and proteasomal degradation during synthesis or when a ubiquitylation reaction is carried out. This is especially true when a reticulocyte lysate is being used, as this material is rich in ubiquitin-proteasome components.

When generating RING-finger proteins, the presence of sufficient Zn is critical to proper folding and E3 activity, so consider addition of Zn either during production or purification. Inhibition of RING-finger E3s by the use of metal-chelating agents to remove zinc is discussed in Basic Protocol 7. For production of most E3s, and particularly HECT proteins, maintaining a reducing environment is desirable. Multi-subunit E3s are a challenge to produce because all necessary components may not be known or be available, or they may need to be simultaneously produced in an insect cell system (see Basic Protocol 26). Expression in IVT systems is essentially identical to that described above for E2 production. Most of the protocols discussed here deal specifically with Zn-containing E3s expressed in bacteria. GST tagging has the advantage of dimerization, which appears to be necessary for the activity of many E3s. As is the case with other enzymes, some E3s are more stable in an unpurified form in a cell lysate than after purification. The authors have found several E3s to be particularly difficult to maintain in an active form after undergoing freeze-thaw cycles, regardless of their purification. Prominent among these is Hdm2/Mdm2. Alternate Protocol 15 provides one way in which this problem may be avoided.

Expression of RING-Finger E3s in Bacterial Lysates

The protocol described here takes advantage of affinity tags for purification of E3. Unlike E2, E3s often require at least partial purification before they can be detected by Coomassie blue staining. In most cases, however, RING-finger E3s expressed in bacteria possess high levels of activity.

Materials

E. coli, transformation-competent strain BL21(DE3) pLysS (Novagen)
E3 plasmid construct (typically pET vectors from Novagen or pGex vectors from GE Healthcare containing E3 cDNA alone or as a tagged fusion protein)
LB agar plates and liquid medium containing appropriate antibiotic (*APPENDIX 2A*)
2×YT medium (see recipe) containing appropriate antibiotic and up to 250 μM $ZnCl_2$
1 M IPTG (dissolve 1 g IPTG in 4.2 ml water; store at −20°C)
Sonication buffer (see recipe)
Glutathione-Sepharose (GS) or Ni-NTA agarose (Ni-NTA) beads
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
2 μg/μl stock solution of bovine serum albumin (BSA; Pierce)
Platform shaker
Spectrophotometer
Probe sonicator (e.g., Misonix Sonicator 300)
Refrigerated centrifuge and round-bottom centrifuge tubes
Additional reagents and equipment for transforming bacteria with plasmids (Seidman et al., 1997), SDS-PAGE (*UNIT 6.1*), and staining of gels (*UNIT 6.6*)

Transform bacteria

1. Transform competent BL21(DE3) pLysS *E. coli* with a prokaryotic plasmid vector expressing the E3 of interest (Seidman et al., 1997).

2. Spread the transformants onto LB agar plates containing appropriate antibiotic.

Start culture for protein production

3. The next morning, select a single colony and use it to inoculate 4 ml of LB liquid medium containing the appropriate antibiotic. Incubate with shaking at 37°C for 8 hr.

4. Transfer 10 μl of this starter culture to 100 ml of 2×YT medium containing appropriate antibiotic and up to 250 μM $ZnCl_2$. Incubate overnight with shaking at 25° to 37°C.

   Larger proteins are often made more efficiently at lower temperatures. The addition of zinc chloride is meant to increase the folding and therefore the activity of the RING finger. Temperature and zinc addition must be determined empirically for each E3 studied.

Induce protein expression in bacteria

5. The next morning, check OD_{600}. If between 0.6 and 1.0, proceed with the induction. If the $0D_{600}$ is below 0.6, allow cultures to grow at 37°C until an $0D_{600}$ of 0.6 has been reached. If the reading is above 1.0, dilute culture below 0.6 and allow to reach 0.6 to 1.0.

6. Induce protein expression by adding 10 μl of 1 M IPTG to the 100-ml culture. Incubate with shaking for the duration and at the temperature determined empirically for each fusion.

Collect lysate

7. Centrifuge bacterial culture 20 min at 3500 × *g*, 4°C. Remove supernatant.
8. Resuspend bacterial pellet in 4 ml cold sonication buffer per 100 ml of original culture.
9. Sonicate with a probe sonicator, either in a cold room or on ice, using three 10-sec pulses at 60% to 70% of the microtip limit setting (3.5 on a Misonix Sonicator 3000).

   For smaller volumes (down to 10 ml lysate for a 250-ml culture), linearly reduce the sonication time. The sonicator will change pitch when lysis of volumes less than 10 ml is complete. This can be used to find an optimal stopping point for sonication.

10. Transfer lysate to a round-bottom centrifuge tube and centrifuge 30 min at 20,000 × *g*, 4°C.
11. Collect the supernatant. Reserve 200 µl for quantitation.

    If the protein of interest does not form inclusion bodies, the supernatant will contain the crude protein lysate and the pellet may be discarded.

12. Divide lysate into aliquots and store at −80°C, reserving 200 µl for quantitation as in the remaining steps of this protocol, or proceed with purification by Basic Protocol 17 for GST-tagged proteins or by Alternate Protocol 12 for His_6-tagged proteins. If using the latter, add $ZnCl_2$ to buffers at 25 µM final to maintain RING-finger structure.

Measure crude protein content

13. Bind 200 µl of lysate to 50 µl GS or Ni-NTA beads by incubating them together for 20 min at room temperature on an end-over end rotator.
14. Microcentrifuge 30 sec at 14,000 × *g*, and remove supernatant. Wash by adding 1 ml of 50 mM Tris·Cl, pH 7.5, centrifuging again.
15. Wash the beads three more times with 50 mM Tris·Cl.
16. Analyze by SDS-PAGE (*UNIT 6.1*). Also load BSA standards ranging from 1 to 10 µg on the gel, and compare to 10 to 500 µl of unbound lysate. Stain gel with Coomassie blue (*UNIT 6.6*).

    If no protein is detected in the supernatant, it may be in inclusion bodies in the pellet. Protein may be extracted from inclusion bodies using SDS-PAGE sample buffer or using commercial kits (Pierce).

Expression of HECT and U-Box E3s in Bacterial Lysates

ALTERNATE PROTOCOL 14

The protocol for expression of E3s that do not coordinate Zn is identical to that for RING- and PHD-finger E3s described in Basic Protocol 24, *except* that addition of Zn to buffers is not necessary. To ensure that the conditions used are suitable to maintain the critical free cysteines for HECT domain proteins and otherwise maintain E3 structure, reducing agents and pH should be optimized for each protein.

Expression of Unstable E3s in Bacterial Lysates

ALTERNATE PROTOCOL 15

Several E3s including Hdm2/Mdm2 do not provide consistently reproducible results from frozen stocks. To circumvent this problem, the authors aliquot induced bacteria in amounts suitable for individual experiments (typically 50 ml of bacterial culture at OD_{600} of 0.6 to 1.0). Again the IPTG concentration, time, and temperature for induction must be determined empirically for each protein prepared. These aliquots are pelleted by

centrifuging 20 min at 3500 × *g*, 4°C, to produce the bacterial pellet, and the supernatant is removed. The pellets are stored at −80°C. When needed, the cells from an individual aliquot are lysed in 400 µl sonication buffer (see recipe), using the technique described in Basic Protocol 24.

BASIC PROTOCOL 25

Expression of E3s in Eukaryotic In Vitro Translation Systems

Expression of E3s in in vitro translation systems follows the same protocol as expression of E2s (Basic Protocol 23) and the same considerations apply. E3s have the potential to utilize E1 and E2 intrinsic to reticulocyte and wheat germ lysates. Therefore, consider the possibility that the E3 may undergo auto-ubiquitylation and proteasomal degradation while synthesis is taking place. If this is an issue, addition of proteasome inhibitor (e.g., 500 µM MG132) may be required. Unlike expression in bacterial systems, adjustment of pH and the presence Zn are generally not required.

BASIC PROTOCOL 26

Expression of Multi-Subunit E3s Using a Baculovirus Expression System

E3s can be divided into single-subunit E3s and E3s in which the substrate recognition element is on a distinct subunit from the ubiquitin-ligase domain. Most of the latter E3s contain a member of the cullin family as a central element. For example, Cul1-based SCF E3s are composed of four subunits. These include elements common to all of the SCF E3s, Cul1, Skp1, and Rbx1/ROC1/Hrt, and one of a number of F-box proteins that serve to recognize substrate. Baculoviral expression systems are well suited to express and purify multi-subunit E3s because coinfection with the multiple viruses that encode the individual E3 subunits results in the assembly of the multimeric protein complex. The authors generally add a His_6 tag to the subunits (i.e., F-box proteins) that are heterogeneous and recognize substrates so as to facilitate purification of E3 complexes. MOI of the viruses should be determined carefully to minimize incomplete protein complexes.

Materials

*Sf*9 cells or *Sf*21 cells (Invitrogen)
Complete Grace's insect medium (see recipe)
Baculovirus lysis buffer 1 (see recipe)
1 M imidazole, pH 7.5 (adjust with HCl)
Ni-NTA beads (Qiagen)
25-cm^2 culture flasks (Nunc) or 100-mm tissue culture dishes
27°C incubator without enhanced CO_2
175-cm^2 tissue culture flasks
15- and 50-ml centrifuge tubes
Additional reagents and equipment for maintenance of insect cell cultures and generation of recombinant baculovirus (Murphy et al., 2004) and batch (Support Protocol 5) or column (Support Protocol 6) purification of multimeric E3s

Culture insect cells

1. Plate 2 × 10^6 *Sf*9 as adherent cells in 25-cm^2 flasks or 3 × 10^6 cells in 100-mm dishes in complete Grace's insect medium.

   Murphy et al. (2004) contains general protocols for working with baculovirus. See Table 15.9.2 for recommendations for other culture configurations. Both Sf9 and Sf21 cells, derived from Spodoptera frugiperda (army moth) ovarian cells, are commonly used to isolate and propagate recombinant AcMNPV (baculovirus) stocks and produce the recombinant protein of interest. Sf9 was isolated as a clone of Sf21. It was reported that Sf21 cells might express more protein than Sf9 cells with some constructs (Heitz et al., 1997; Wang et al., 1997). They are grown as adherent cells in culture flasks or dishes.

2. Grow just to confluence in a 27°C incubator without enhanced CO_2, then split 1:3, dislodging the cells either by streaming medium over the monolayer or by hitting the flask against the palm of the hand.

Construct recombinant baculovirus

3. Subclone cDNAs encoding subunits of the E3s into baculovirus transfer vectors.

   Any kits and general protocols (e.g., Murphy et al., 2004) for making baculovirus constructs should work well for E3s. However, baculovirus transfer vectors must be suitable to the kits chosen.

Infect cells

4. Seed a 175-cm^2 with 21×10^6 log-phase *Sf*9 cells in 24 ml complete Grace's medium. Allow cells to adhere at room temperature for >1 hr, then remove medium from cells.

5. Infect cells with recombinant baculoviruses encoding E3 subunits at MOI of 1 to 5 virus particles per cell (i.e., $2.1–10.5 \times 10^7$ pfu) in 5 ml medium from recently titered (see Basic Protocol 21 and Murphy et al., 2004) baculovirus stock. Include at least one flask with a "mock" infection (medium only) as a negative control.

   The amount of His_6-tagged baculovirus should be at a lower MOI than other baculoviruses to minimize partial complexes.

6. Incubate the infected cells 1 hr at 27°C, rocking gently every 15 min to make sure that cells are covered with virus supernatant.

7. Remove virus solution and add 24 ml complete Grace's insect medium.

8. Incubate 60 to 72 hr at 27°C (no CO_2).

Lyse cells

9. Harvest cells by pipetting medium up and down. Transfer cells to a 50-ml centrifuge tube.

10. Centrifuge 4 min at $350 \times g$, 4°C, to pellet cells. Remove supernatant.

11. Wash twice, each time by adding 30 ml ice-cold PBS, centrifuging 4 min at $350 \times g$, 4°C, and removing the supernatant.

12. Lyse cells by adding 3 to 10 vol baculovirus lysis buffer 1 and incubating on ice for 20 min.

13. Centrifuge 15 min at $20{,}000 \times g$, 4°C. Transfer supernatant to new 15-ml tubes.

Purify the complex

14. Add appropriate amount of 1 M imidazole stock solution for a final concentration of 0.2 mM imidazole.

15. Add 0.2 to 0.4 ml of Ni-NTA beads for every ten 175-cm^2 flasks. Incubate 60 min at 4°C on an end-over-end rotator.

    The authors usually use ten 175-cm^2 flasks for purification of the E3 complex.

16. Purify the multimeric E3s by batch purification (Support Protocol 5) or column purification (Support Protocol 6).

SUPPORT PROTOCOL 5

Batch Purification of Multimeric E3s

Multimeric E3s can be purified either in batch or by column purification. The batch method is usually used by the authors.

Materials

E3 complexes bound to Ni-NTA beads (Basic Protocol 26)
Wash buffer 1 for His_6-tagged proteins (see recipe)
Wash buffer 2 for His_6-tagged proteins (see recipe)
Elution buffer for His_6-tagged proteins (see recipe)
Dialysis buffer 1 for ubiquitylating and neddylating enzymes (see recipe)
Glycerol

Refrigerated centrifuge
1.5-ml screw-cap microcentrifuge tubes
End-over-end rotator
8000-MWCO dialysis membrane

Additional reagents and equipment for dialysis (*APPENDIX 3C*)

Elute complexes

1. Centrifuge 15-ml tube containing the supernatant of Ni-NTA-agarose 5 min at 350 × *g*, 4°C. Remove supernatant.
2. Wash the Ni-NTA-agarose six times, each time by adding 12 ml wash buffer 1 for His_6-tagged proteins, centrifuging again as in step 1, and removing the supernatant.
3. *Optional:* Wash twice, each time with 12 ml wash buffer 2 for His_6-tagged proteins using the technique described in step 1.
4. Transfer Ni-NTA-agarose to a 1.5-ml screw-cap microcentrifuge tube.
5. Elute protein by adding 0.6 ml of elution buffer for His_6-tagged proteins and rotating 10 min at 4°C on an end-over-end rotator.
6. Microcentrifuge 2 min at 800 × *g*, 4°C. Transfer supernatant to a new 1.5-ml screw-cap tube.
7. Repeat steps 5 and 6 and combine the supernatants.

Dialyze and store the purified protein

8. Dialyze the eluate against 1 liter of dialysis buffer overnight at 4°C, using a 8000-MWCO dialysis membrane, changing the buffer twice at intervals at least 4 hr apart (*APPENDIX 3C*).
9. Remove the dialysate and divide into aliquots in 1.5-ml tubes. Add an equal volume of glycerol to each tube and mix thoroughly.
10. Rapidly freeze samples in liquid nitrogen, then store at −80°C.

SUPPORT PROTOCOL 6

Column Purification of Multimeric E3s

Column purification of E3 complexes is an alternative to batch purification.

Materials

E3 complexes bound to Ni-NTA beads
Wash buffer 1 for His_6-tagged proteins (see recipe)
Wash buffer 2 for His_6-tagged proteins (see recipe)
Elution buffer for His_6-tagged proteins (see recipe)

Dialysis buffer for ubiquitylating and neddylating enzymes (see recipe)
Glycerol

2-ml Poly-Prep disposable chromatography column (Bio-Rad)
8000-MWCO dialysis membrane

Additional reagents and equipment for dialysis (*APPENDIX 3C*)

Elute complex from the beads

1. Transfer Ni-NTA beads to a 2-ml Poly-Prep column.
2. Wash the beads in the column with 10 column volumes of wash buffer 1 for His_6-tagged proteins.
3. *Optional:* Wash beads with 2 to 3 column volumes of wash buffer 2 for His_6-tagged proteins.
4. Elute protein with one bed volume of elution buffer for His_6-tagged proteins.
5. Repeat elution step again and combine the eluates. Dialyze and store the purified protein
6. Dialyze the eluate against 1 liter of dialysis buffer overnight at 4°C using a 8000-MWCO dialysis membrane, changing the buffer twice at intervals at least 4 hr apart.
7. Remove the dialysate and divide into aliquots in 1.5-ml tubes. Add an equal volume of glycerol to each tube and mix thoroughly.
8. Rapidly freeze samples in liquid nitrogen, then store at −80°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

[^{125}I]protein A for immunoblotting

Purchase from ICN Biochemicals; dilute 1/1000 (for a final concentration of 0.5 to 1 μCi/ml) in PBS/0.1% (v/v) Tween 20. Prepare fresh.

2×YT medium

16 g Bacto tryptone (Invitrogen)
10 g Bacto yeast extract (Invitrogen)
5 g NaCl
H_2O to 1 liter
Autoclave
Store up to 1 year at room temperature

2×YT + G medium

16 g Bacto tryptone (Invitrogen)
10 g Bacto yeast extract (Invitrogen)
5 g NaCl
20 g glucose
H_2O to 1 liter
Autoclave
Store up to 1 month at room temperature

ATP, and ATP-regenerating system, 10×

5 mM ATP
100 mM creatine phosphate
100 μg/ml creatine phosphokinase
40 mm DTT (for ubiquitinylation only)
Prepare just before use

ATP and creatine phosphate are stored at −20°C.

Baculovirus lysis buffer 1

1% (v/v) Triton X-100
50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
10 mM 2-mercaptoethanol
1× protease inhibitors (e.g., Complete Protease Inhibitor Cocktail from Roche Diagnostics)
Prepare fresh; keep at 4°C during use

Baculovirus lysis buffer 2

1% (v/v) NP-40
100 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
100 mM NaCl
1 mM dithioerthyritol (DTE; Sigma)
10% (v/v) glycerol
1× protease inhibitors (e.g., Complete Protease Inhibitor Cocktail from Roche Diagnostics)
Prepare fresh; keep at 4°C during use

Binding buffer

5 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*; 25 mM final)
2 ml 5 M NaCl (50 mM final)
0.154 g DTT (add immediately before use; 5 mM final)
1 ml NP-40 (0.5% v/v final)
H_2O to 200 ml
Store indefinitely at room temperature without DTT

Complete Grace's insect medium

Grace's insect medium (Invitrogen) supplemented with:
10% (v/v) fetal bovine serum
2 mM L-glutamine
100 IU/ml penicillin
100 μg/ml streptomycin
Store up to 1 month at 4°C

Dialysis buffer

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.5 mM DTT

DTT is not required for ubiquitin or NEDD8 production.

Elution buffer for GST proteins

20 mM Tris·Cl pH 7.5 (*APPENDIX 2A*)
20 mM reduced glutathione
Store up to 8 months at −20°C

Elution buffer for His_6-tagged proteins

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
300 mM imidazole
Prepare fresh

HEPES-buffered saline (HBS), 2×

16.4 g NaCl (280 mM final)
11.9 g HEPES acid (50 mM final)
0.21 g anhydrous Na_2HPO_4 (14.8 mM final)
Add H_2O to 800 ml
Adjust pH to 7.05 with NaOH
Add H_2O to 1 liter
Store in 50-ml aliquots up to 1 year at −20°C

The pH of 2× HBS and $CaCl_2$ used in transfections is critical. A narrow range of 7.05 to 7.12 is acceptable. Including glucose up to 15 mM helps maintain health of cell cultures where glucose is normally present. Addition of phenol red provides a pH indictor.

Hypotonic lysis buffer

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10 mM 2-mercaptoethanol
1× protease inhibitors (e.g., Complete Protease Inhibitor Cocktail from Roche Diagnostics)
Prepare fresh

Kinase buffer for ubiquitin labeling, 10×

200 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 M NaCl
120 mM $MgCl_2$
10 mM DTT
Store up to 1 year at −20°C

Ni-NTA purification buffer

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10 mM 2-mercaptoethanol
2 mM PMSF
Prepare fresh

Nondenaturing Triton X-100 lysis buffer for detection of ubiquitylation

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
300 mM NaCl
5 mM EDTA (optional)
0.5% (v/v) Triton X-100
Store with above ingredients indefinitely at 4°C
Immediately before use, add:
1 mM PMSF
2 μg/ml aprotinin
5 μg/ml leupeptin
1× phosphatase inhibitors (optional; see recipe for 10×)

Do not substitute PMSF with AEBSF, the nonhydrolyzable analog, as AEBSF may inhibit certain enzymatic reactions.

Nonreducing SDS-PAGE sample buffer, 4×

2.5 ml 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*; 250 mM final)
0.8 g SDS (8% w/v final)
4 ml glycerol (40% v/v final)
2 mg bromphenol blue (0.2% w/v final)
H_2O to 10 ml
Store in 0.5- to 1-ml aliquots up to 8 months at room temperature

PCK 10×

(Calbiochem, 5000 U), reconstitute in 1 ml 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*).

Phosphatase inhibitors, 10×

4 mM sodium orthovanadate
4 mM EDTA
100 mM sodium fluoride
100 mM sodium pyrophosphate
Store stock solutions up to 1 year at 4°C

Phosphate-buffered saline (PBS,) pH 7.4

1.9 mM NaH_2PO_4
8.1 mM Na_2HPO_4
154 mM NaCl
H_2O to 900 ml
Adjust pH to 7.4 with NaOH or HCl as necessary
H_2O to 1 liter
Store indefinitely at room temperature

Reducing SDS-PAGE sample buffer, 4×

2.5 ml 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*; 250 mM final)
0.4 ml 2-mercaptoethanol (4% v/v final)
0.8 g SDS (8% w/v final)
4 ml glycerol (40% v/v final)
2 mg bromphenol blue (0.2 % w/v final)
H_2O to 10 ml
Store in 0.5- to 1-ml aliquots up to 8 months at room temperature

Sonication buffer

50 mM Tris·Cl, pH 8 (*APPENDIX 2A*)
1 mM EDTA
1 % (v/v) Triton X-100
Store with above components up to 1 year at 4°C
10 mM 2-mercaptoethanol or 5 mM DTT (add just before use)
2 mM PMSF (add just before use)

Thiolester buffer, 10×

200 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
500 mM NaCl
50 mM ATP
50 mM $MgCl_2$
Store up to 1 year at −20°C

Thrombin stock solution, 20×

Dissolve 500 U lyophilized thrombin (GE Healthcare) in 0.5 ml cold PBS (*APPENDIX 2A*). Stored in 100-µl aliquots up to 6 months at −80°C.

Tris-buffered saline containing bovine serum albumin (TBS/BSA)

25 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
0.5% (w/v) bovine serum albumin (BSA)
Store up to 1 month at 4°C

Tris-buffered saline(TBS) containing Tween 20 (TBST)

25 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
0.05% to 0.1% (v/v) Tween 20
Store indefinitely at room temperature

Ubiquitin buffer 2 for E3 assays, 20×

1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
40 mM ATP
100 mM $MgCl_2$
40 mM DTT

The 1× buffer contains 25 mM Tris·Cl, 5 mM ATP, 4 mM $MgCl_2$, and 0.1 mM DTT.

Ubiquitylation buffer 1 for E3, 10×

500 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
2 mM ATP
5 mM $MgCl_2$
1 mM DTT
10 mM creatine phosphate (Sigma; 45 mg/10 ml)
Store up to 1 year at −20°C

Ubiquitylation buffer 2 for multi-subunit E3s and neddylation, 10×

200 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
50 mM $MgCl_2$
20 mM DTT
Prepare fresh

Wash buffer 1 for His_6-tagged proteins

20 mM Tris·Cl pH 7.5 (*APPENDIX 2A*)
5 mM imidazole
Prepare fresh

Wash buffer 2 for His_6-tagged proteins

20 mM Tris·Cl pH 7.5 (*APPENDIX 2A*)
10 mM imidazole
Prepare fresh

COMMENTARY

Background Information

Thiolester assays

Thiolester bonds formed between ubiquitin and E1 or E2 are evaluated in Basic Protocols 1 and 2. Thiolester bonds are relatively stable to heat but sensitive to reducing agents. Formation of a thiolester linkage of E1 or E2 with ubiquitin results is a change in migration of E1 or E2 by 8 kDa under nonreducing conditions. Upon reduction, the E1 or E2 migrates at its unnmodified molecular weight, while the ubiquitin is observed at 8 kDa (assuming the resolving gel distinguishes this from the dye front).

In vitro ubiquitylation (E3) assays

In this unit, protocols are included for determining whether a putative E3 can transfer ubiquitin in an E2-dependent manner. In Basic Protocol 8 a GST-E3 fusion serves as the predominant substrate for ubiquitylation.

Basic Protocol 5 is used to demonstrate that the E3 may modify itself when separated from the GST. Basic Protocol 6 addresses the inherent artificial nature of the system by removing the E3 from beads and eliminating the dimerization that is forced by the GST moiety. A number of E3s have been shown to function as dimers or to form dimers, e.g., as c-Cbl, Brca1-Bard1, and Rad5-Rad23. Alternate Protocol 2 addresses the potential for E2-E3 combination to result in various types of ubiquitin chains or to preferentially stimulate the addition of ubiquitin monomers. Of course it is important to be cognizant that chains preferentially formed in vitro using a substrate that may or may not be a bona fide intracellular substrate may not reflect the situation in a cell.

Substrate ubiquitylation in vitro

The purpose of this set of protocols is to establish, as clearly as possible, the ability of an E3 to modify a particular substrate with ubiquitin. To achieve this, the authors have generally used recombinant proteins to avoid obscuring the results with other ubiquitin-proteasome system proteins (see Basic Protocol 8 and Alternate Protocol 4). However, for multi-subunit E3s, use of eukaryotic systems is necessary (see Basic Protocol 9). These types of assays in which the E3 components are either expressed in bacteria or overexpressed may not, of course, reflect the physiological system. A missing cofactor could be required for substrate modification. In such cases, the use of in vivo protocols is recommended. Alternatively, use of eukaryotic systems such as insect cells may result in the unwitting copurification of a factor necessary for activity. In fact, this was the case for Rbx1 which was not originally appreciated as a component of cullin-containing E3s.

When substrate is expressed using in vitro systems, it is useful to add ubiquitin aldehyde along with proteasome inhibitor to the reaction mixture to inhibit deubiquitylation and proteasome activity, respectively.

An additional theme of this section is the precipitation of proteins. The authors use two general methods to study ubiquitylation of proteins from cell lysates. The first is detection of ubiquitin modification in precipitated proteins (Basic Protocol 11). The second is using precipitated proteins as the source of substrate for in vitro ubiquitylation reactions (Basic Protocol 10, Alternate Protocol 5). The authors precipitate proteins in two ways. The first (Basic Protocol 10) is using a specific antibody to the protein of interest. This can be used to pull down a ubiquitin-modified protein or an E3-substrate complex (coimmunoprecipitation). The second (Alternate Protocol 5) is pull-down of substrate using a GST-fusion of the E3. The second technique is perhaps most useful when subsequent activity assays will be performed.

Detection of ubiquitylation in vivo

The protocols in this section are designed to look at a "snapshot" of ubiquitylated species in living tissue. Under ideal conditions, the substrate will be of high abundance and will be easily detected in unstimulated cells. This is also the case for the ubiquitin-modified form of the protein. However, the activity of proteasomes, proteases, and DUbs may result in the protein being rapidly degraded, of low abundance, or deubiquitylated. The use of chemical inhibitors (Basic Protocol 13) may aid in overcoming these problems. In addition, anti-substrate antibodies may not recognize the ubiquitin-modified forms. Also, commercial anti-ubiquitin antibodies are notoriously variable. However, this may be overcome by the use of tagged ubiquitins (Alternate Protocol 6). These have the advantage that they can be detected by inexpensive, high-quality, well-defined antibodies. In addition, tagged ubiquitin may be less susceptible to the activity of DUbs.

Inhibition of ubiquitylation of protein and degradation

Each of these protocols (Basic Protocol 12, Alternate Protocol 7, Basic Protocol 13) takes a different approach to inhibiting target protein modification or degradation. Before employing a particular protocol, one must consider whether the answer needed will best be obtained by inhibiting the ubiquitin proteasome pathway at the E2, E3, or proteasome level.

NEDD8 conjugation

The protocols for NEDD8 conjugation (neddylation; Basic Protocols 14 to 16 and Support Protocols 1 and 2) are a prototype for conjugation of a ubiquitin-like modifier. NEDD8 modifies cullins in cullin-based ubiquitin ligase complexes and regulates their E3 activity. Although p53 and pVHL have recently been shown to be conjugated by NEDD8, cullins in cullin-based ubiquitin ligase complexes are the major targets of neddylation. Neddylation is catalyzed by several enzymes that are similar, though not identical to those involved in ubiquitylation. NEDD8 is activated by a heterodimeric E1-like enzyme,

the APP-BP1 and Uba3 complex, and is then transferred to an E2-like enzyme, Ubc12. Although the ubiquitin system appears to require an E3, an absolute requirement for E3 has not been shown for the ubiquitin-like molecules. However, Rbx1, a RING-finger protein, which is the catalytic center of ubiquitylation mediated by cullin-based E3s, also appears to function as an E3 for NEDD8 (Kamura et al., 1999).

E2 production

Expression of proteins in bacteria has been carried out for a number of years and has been used to express every kind of protein imaginable. However, this strategy is not well suited to every protein, as issues of solubility, premature transcriptional termination, and post-translational modification have arisen. In nearly all cases, small proteins, such as E2s, have been easy to express in large quantities in bacteria (Basic Protocol 22 and Alternate Protocols 12 and 13). Where bacteria can be used with satisfactory results, proteins are produced quickly, in large quantities, and at low cost. When expressing components of the ubiquitin proteasome system, bacteria have an added advantage–they possess none of these components endogenously. This permits the use of crude bacterial extracts containing the expressed E2 in ubiquitylation reactions with little or no purification (Basic Protocols 2 and 3). This is also true of bacterial extracts containing E1, E3, or ubiquitin (Basic Protocols 17, 20, and 24).

When bacterial expression of E2s is problematic, the use of eukaryotic in vitro transcription and translation systems provides for a more physiological setting (Basic Protocol 23). These systems provide proteins that are more likely to be folded properly and contain post-translational modifications, thereby maximizing the opportunity to obtain active E2. In most cases, the in vitro–translated protein should be used soon after synthesis in order to avoid the many complications caused by ubiquitin conjugation or degradation. This is because the proteasome machinery is also present in these systems. In addition, therefore, experiments employing IVT E2 should include both wild-type and active-site-mutant forms of the E2. This guarantees that the E2 of interest and not an endogenous wheat or rabbit E2 is responsible for observed effects.

E2s, particularly, can be very sensitive to freeze/thaw cycles and to redox conditions. Therefore, when using crude lysates stored at −80°C, aliquotting in the smallest volume convenient for an average experiment (10 to 20 μl) is wise. The authors find that 5 mM DTT works well to regulate the redox condition of most E2s, but they have seen maximum activity vary with DTT concentrations from 0 mM to 10 mM.

E3 production

E3s were originally considered to be substrate-recognition elements that facilitated transfer of ubiquitin from E2. The discovery of HECT-domain proteins, with active-site cysteines, provided evidence that E3s themselves could be catalytic. The subsequent description of RING-finger E3s seemed to indicate that the original concept was correct for these E3s, as RING fingers apparently do not form intermediates with ubiquitin. There is evidence to suggest, however, that they are not merely docking sites for E2s. (Lorick et al., 2005b).

Critical Parameters and Troubleshooting

Thiolester assays

Different E2s prepared under identical conditions may have different activities as assessed by ubiquitin thiolester bonds. This may reflect differing requirements for reducing agents during purification. If there are large differences in activity among E2s, it may be necessary to experiment with varying the DTT (or other reducing agent) level between 0 and 10 mM for production and purification. Redox potential of the E2s or E1 may also be affected by pH so this may similarly need to be varied from ~6.8 to 8.0.

E2s and E1s can lose activity after freeze/thaw cycles from −80°C. This can be avoided either purifying the protein and storing it at −20°C in 50% glycerol or by distributing it in small, single-use aliquots and storing at −80°C.

To guarantee that the amount of thiolester formation is equal when comparing various E2s, test all of them together using Basic Protocol 2. Use the results from this to normalize the thiolester formation potential and run a second assay. One problem that has arisen from this practice is that too much E2 added to the assay will result in decreased activity compared to when less material is used. While the authors are not certain of the biological reasons for this, it may be that inactive E2 molecules have a dominant-negative effect on interactions between E1 and active E2s.

Binding of ubiquitin-proteasome proteins

Generally, washing more than four or five times will not reduce background. If background persists, consider increasing detergent or altering the NaCl concentration. The concentration of NaCl determines the stringency of the binding. A range of 50 to 200 mM is reasonable to evaluate most interactions. Different protein interactions vary in the optimal NaCl for assessing binding.

In vitro ubiquitylation (E3) assays

The protocols described are fairly straightforward. One problem that has arisen in the authors' hands is the decay of radiolabeled ubiquitin. ^{32}P has a 2-week half-life. In addition, the ubiquitin is labeled to high specific activity. This causes loss of signal both due to radioactive decay and radiodegradation of the ubiquitin. Labeling ubiquitin every 3 weeks is usually sufficient to maintain signal (see Basic Protocol 17).

Occasionally the authors find a lack of activity in their bacterial preparations of E3. For RING-finger E3s, this may be obviated under certain circumstance by addition of Zn to the bacterial culture when RING- and PHD-finger proteins are being evaluated (see Basic Protocol 25).

When using Basic Protocol 6, 20× ubiquitin buffer 2 may be used in place of the 10× ubiquitin buffer 1. The former buffer replaces the ATP-regenerating system of the latter with much higher levels of $MgCl_2$ and ATP, and increases the reduction potential as a consequence of increased DTT. A similar buffer has been used by Huibregeste et al. (1993) which also includes NaCl. Occasionally, this has resulted in gels with aberrant migration, and may interfere with the activity of a particular E3 when compared to the buffer without added salt. The lack of salt in the buffers described here may be advantageous when using rabbit reticulocytes in in vitro translation systems. This is because the net salt concentration is above physiological. The 10× buffer is recommended for initial assays, but consider trying the 20× high-ATP system or the Huibregeste buffer to see if it increases ubiquitylation.

When generating mutant ubiquitin, be aware that bacterial codon usage is a particular problem in Alternate Protocol 2. This is regardless of whether radiolabeled or unlabeled ubiquitin is employed. Because bacteria have few of the mammalian codons for arginine, they may incorporate lysines into mutant ubiquitins where a lysine was thought to have been eliminated. Use of codon-optimized bacteria strains such as Stratagene CodonPlus RP or RIL strains may overcome the problem. In addition, gas chromatography–mass spectrometry (GC-MS) techniques should be employed to purify or confirm the proper translation of a mutant ubiquitin if amino acid misincorporation is a potential issue.

Zinc chelation also provides variable results. Some RING-finger E3s never lose activity even when exposed to the strongest zinc chelators (Lorick et al., 1999). Others cannot reconstitute activity when zinc is added back.

As an alternative to chelation of zinc or alkylation of active site cysteines, similar information may be obtained by mutation of critical RING-finger domain or HECT-domain residues.

Substrate ubiquitylation in vitro

The use of proteasome inhibitors increases the chance of detecting the substrate protein. Additionally, substrate generated in mammalian systems will be subject to the activity of DUbs. This activity may be inhibited by the addition of ubiquitin aldehyde during in vitro ubiquitylation. Addition of ubiquitin aldehyde before the in vitro reaction, however, may artificially increase the amount of ubiquitin-modified substrate detected.

Caution must be exercised when precipitating proteins that will be used in enzymatic assays. Lysis of cells should be carried out under relatively gentle conditions using nonionic detergents such as Triton X-100 or NP-40. Alkylating agents such as N-ethyl maleimide or iodoacetamide must be avoided so as to prevent inactivation of the ubiquitylation machinery.

When immunoprecipitating, consider that protein A and protein G have differential preferences for antibody subtypes and species (Table 7.2.1). If there is concern about antibody binding, protein G is the safer bet, but protein A is considerably less expensive. For further discussion of immunoprecipitation see *UNIT 7.2* and Coligan et al. (2006).

Detection of ubiquitylation in vivo

To detect steady-state ubiquitin modification, the presence of DUbs warrants harsher lysis conditions. Alkylating agents and harsh detergents or chaotropic salts will help to inhibit DUbs and allow for the detection of ubiquitylation. Working quickly will also limit the amount of time the substrate of interest is exposed to DUbs.

Inhibition of ubiquitylation of protein and degradation

Mutation of E3 (or E2) active sites, may not give complete inhibition of E3 activity. The reason for this, first of all, is that loss of a particular E3 may be compensated by other E3s in the cell. Additionally, it is the authors' impression that RING-finger proteins sometimes have residual activity, even with one of their zinc-binding residues is mutated. For this reason the authors frequently mutate two or more coordinating residues for the zinc ligand.

Be careful, when interpreting results, to take into account all of the protein in the sample. Inhibiting the proteasome will increase the total level of any protein degraded in a proteasome-dependent manner. The inhibition may increase the fraction of target protein that is ubiquitin modified. This makes the target appear depleted at its unmodified molecular weight. Therefore, any increase or decrease in protein level has to be determined by looking at both ubiquitin-modified and unmodified protein levels.

Some substrate proteins are known to give slightly ambiguous results in proteasome inhibition assays. Foremost among these is the epidermal growth factor receptor (EGFR), which appears to be degraded in the lysosome, but requires proteasome activity, possibly associated with Cbl proteins, for its degradation. Also, consider that there is at least one example of ubiquitin-independent proteasomal degradation—ornithine decarboxylase (Murakami et al., 1992, 1993; Mahaffey and Rechsteiner 1999; Newman et al., 2004).

Some researchers have used quantitative northern blotting or RT-PCR to demonstrate knockdown of expression. However, just because RNA levels may be reduced, it is impossible to know intrinsically how translation may compensate using even a small amount of remaining RNA. Therefore, the use of antibody to detect the ablated protein is greatly preferred in most experiments.

NEDD8 conjugation

The protocols for expressing and testing NEDD8 conjugating system proteins resemble those outlined for the ubiquitin system. Namely, the purification protocol for APP-BP1/Uba3 resembles that of mouse E1 or VBC-Cul2 E3. Also, the protocol for production of NEDD8 or Ubc12 resembles that of ubiquitin or E2s for ubiquitin, respectively. The protocol for in vitro neddylation resembles that for in vitro ubiquitination. Therefore, critical parameters and troubleshooting are almost identical to those for the previously described protocols. However, there are a couple of points that should be stressed.

The baculovirus expression system is a suitable system to express multimeric protein complexes. Since E1 for NEDD8 is the APP-BP1/Uba3 heterodimeric complex, use a baculovirus expression system to express them. To avoid contamination of incomplete complex, the MOI of His_6-APP-BP1 baculovirus infection should be higher than that of Uba3. NEDD8 is a 76-amino acid protein. However, NEDD8 cDNA encodes additional amino acids at the C-terminus of the protein (GGLRQ in human). Although this extension is cleaved by C-terminal hydroxylase to produce mature NEDD8 in mammalian cells, *E. coli* has no C-terminal hydroxylase for NEDD8. Therefore, C-terminal extension of NEDD8 must be removed in a bacterial expression plasmid for NEDD8. Because both APP-BP1/Uba3 and Ubc12 are extremely sensitive to oxidation, reducing agents should be added to the system during purification. However, strong reducing agents such as DTT are not suitable for the purification of His_6-tagged proteins by Ni-NTA column because they may reduce the nickel ions and thereby prevent them from binding to His_6-tagged proteins. Therefore, add 10 to 20 mM of 2-mercaptoethanol (2-ME) to lysis buffer.

Labeling and detection of ubiquitin

If care is taken in planning, few problems arise in labeling ubiquitin. However, there are a few caveats. Thrombin, used in the cleavage of GST-Ub, can be dissolved in either PBS or TBS; however, NaCl must be present in the buffer. Thrombin can be finicky; hence, once a reliable source is identified, one should stick to it. Removal of thrombin with benzamidine-Sepharose can be incomplete, so this step may need to be repeated if other proteins integral to the assays have potential thrombin cleavage sites. There is always the potential for beads to be carried over after cleavage of GST-Ub or from the benzamidine-Sepharose. It is also possible for a small amount of non-bead-associated, uncleaved GST-Ub to be present. One way to prevent contaminating any ubiquitylation assay with ^{32}P-labeled GST-ubiquitin is to add a small amount of glutathione-Sepharose to the tube of labeled ubiquitin to absorb this material.

Both [^{32}P] or [^{125}I]ubiquitin can be prone to radiodegradation. For [^{32}P]ubiquitin it is best to label at least every 3 weeks.

Because of its high degree of conservation, ubiquitin is not very antigenic; thus, generation of high-affinity antibodies can be problematic. Indeed, lots of commercially available polyclonal anti-ubiquitin antibodies are highly variable. Partially because of issues of immunoreactivity, scientists in the field have historically taken extra steps to increase the ability of antibodies to detect their proteins. This is the reason for recommending glutaraldehyde fixation of PVDF or autoclaving of nitrocellulose in the immunoblotting protocol. At times, the authors have achieved good results without glutaraldehyde treatment. However, it is recommended that such fixation be initially employed; further judgments can then be made as to the need for this step. Blocking of membrane should be done in BSA or casein, not milk, because milk can potentially include ubiquitylated protein. When planning an experiment that involves immunoblotting with anti-ubiquitin to determine whether an immunoprecipitated protein, for example, is ubiquitylated, it is essential to have appropriate negative controls. Because ubiquitylation is such a prevalent modification and such a low percentage of any protein is found ubiquitylated at steady state, care must be exercised that ubiquitylated species represent the protein of interest and not nonspecifically associated material.

Serum preparation and construction of a ubiquitin affinity column

It is important to test serum bleeds soon after they are collected. Many antigens are toxic to rabbits, and rabbits have been known to die before any appreciable amount of serum has been collected. When purifying the antibodies, remember to store every fraction that contains detectable antibody. Often, the highest affinity antibodies come off of a purification column after the bulk of the material has been eluted.

Preparation of E1

Most E1s, whether laboratory generated wheat E1 or commercially purchased rabbit E1, are extremely sensitive to freeze/thaw cycles; therefore, they should be stored in small aliquots at −80°C after their initial thaw and never be reused. Baculovirus-expressed mouse E1, however, can be stored in 50% glycerol at −20°C for long periods of time (months) with little or no loss of activity.

E2 production

When expressing proteins in bacteria, major considerations are what plasmids and bacterial strains to use. Most bacterial expression systems take advantage of the lac operon genes. This permits a gene under the control of the lac promoter to be repressed by the lacIq gene product (the lac repressor) until an inducer, such as IPTG, is added. The lac repressor element has been employed to repress genes not under the control of the lac promoter. These include the tac promoter in pGEX vectors and a hybrid trp/lac promoter in pTrc vectors. Perhaps the most widely used system currently is that employed by Novagen's pET vectors. This system uses a variation of the lac operon in which the lac repressor reduces expression of T7 from the DE3 plasmid found in certain BL21 strains. In addition, lacIq represses expression of a transgene under the control of the T7 promoter. In this way, unwanted or leaky expression is suppressed both at the promoter and by controlled expression of the driving RNA polymerase. The key to any of these systems is the presence of an intact lacIq gene, and in the case of the pET system, the DE3 plasmid. While many *E. coli* strains can be used to express proteins using the lac operon, the BL21 strain is generally recommended for its reduced protease activity. BL21 cells are also readily available with DE3. Other variations of BL21 available compensate for leaky expression from T7 (pLysS strains) by degrading T7 polymerase until induction. These cells are recommended for longer proteins, as the leaky expression will cause the production of a large number of incomplete transcripts and truncated proteins. Another source of incomplete protein production is the bacterial scarcity of tRNAs for GC-rich codons found in cDNAs from eukaryotic species. Stratagene offers two strains of BL21 that have mammalian codon bias, BL21(DE3) Codon plus RP or Codon plus RIL. The mammalian codon bias is provided by plasmids that encode GC-rich arginine and proline tRNAs or arginine, isoleucine, and leucine tRNAs (Sambrook et. al., 1989).

When producing IVT proteins, a major consideration is the presence of excess protein in the wheat or rabbit machinery. These proteins tend to form aggregates upon heating. The aggregates can sequester free radioactive material, and the result is clouding at the top and bottom of the gel upon autoradiography. If the extent of clouding is high, it may conceal or obscure the signal from the desired translation product. Heating to 65° to 70°C rather than boiling samples before electrophoresis or heating to lower temperature for longer times, for example 37°C for 30 min, may help this

problem. In addition, spinning out insoluble material after translation, immunoprecipitating the products, or fixing the gel for 30 min in 10% acetic acid/25% methanol will further reduce or eliminate this problem. If no translation product is seen, purifying or linearizing the plasmids is recommended. The authors find that commercial wheat germ extracts or rabbit reticulocytes do not translate well even after a single freeze/thaw cycle. Even when using quick systems where the only necessary addition to the reaction is DNA and radioactivity, the authors have found that addition of ribonuclease inhibitor improves the overall quality of translation products.

When using tagged proteins that will be purified, a C-terminal tag will allow for purification of the full-length protein away from truncated or degraded proteins, but the presence of the tag will need to be checked for effects on function. Generally, the authors find that placement of tags at N-termini has little effect on E2 activity. In all cases, the tagged protein must be tested for function. Where the tag interferes with function, purification may require using untagged E2s and employing more complex chromatographic methods.

The levels of inducing agent, bacterial density, time, and temperature may all be varied in order to maximize protein yield and complete protein translation. IPTG may be varied from 0.1 to 1 mM with proteins that express well; usually smaller ones require less. The OD_{600} of the cultures may be varied from 0.6 to 1.0; the key is to have the bacteria in logarithmic growth phase. The time of induction may be varied from 30 min to 3 hr; this generally depends upon the OD_{600} of the culture and the ability of the plasmid to express protein well. Longer incubation generally does not help if bacteria have entered stationary phase or if only a small amount of protein is needed. Larger proteins should be induced earlier and for a longer period of time. Finally, most *E. coli* machinery works best at 37°C. However the bacteria also contain proteases, so larger proteins may be produced with less degradation if their cultures are induced at 22° to 30°C. In some cases optimal induction temperature may be as low as 16°C. Smaller proteins generally produce well at the higher temperature.

E3 production

When expressing E3s in bacteria, one often encounters expression or solubility issues that do not generally arise with E2 production. This derives in part form the larger size of E3s and their potential to be sequestered into inclusion bodies. This can be assessed by evaluating a small amount of post-lysis protein pellet by SDS-PAGE and comparing it to pellet from untransfected cells. If the protein of interest is found in the inclusion body, it will be necessary to extract protein from the inclusion body using buffers containing a high concentration of SDS, urea, or guanidine, followed by refolding. Commercial kits are available for inclusion body extraction and refolding. There is no guarantee that this approach will yield active E3. The second approach is to clone the protein with a solubility tag such as NusA, or MBP. A trial-and-error approach will be necessary to determine the optimal tag. Low levels of expression also often correlate with protein size, with larger proteins expressing more poorly than smaller ones. Often, this is caused by differences in codon usage between bacteria and eukaryotes. Certain bacterial strains, such as Stratagene's CodonPlus BL21 strains, compensate for this codon preference by expressing cDNAs for the less abundant tRNAs. For all E3s, obtaining active protein can also be an issue. For RING-finger proteins, activity can often be improved by optimizing zinc levels. For HECT-domain E3s, maintaining the active site cysteine in a free form by avoiding alkylating agents and maintaining an optimal reducing environment are crucial to activity.

When performing IVT of E3s, other types of proteasome inhibitors may be substituted for MG132, which like ALLN is a peptide aldehyde. Other inhibitors to be considered include members of the vinyl sulfone family (Mc Cormack et al., 1997; Naujokat et al., 2000; Groll and Huber, 2004) and lactacystin (Imajoh-Ohmi et al., 1995; Maki et al., 1996), which continues to be considered a relatively specific inhibitor of proteasomes. Another approach to inhibiting proteasomes is use of the nonhydrolyzable ATP analog ATP-γ-S. Its use has declined with the discovery of inhibitors of specific proteasome inhibitors.

Anticipated Results

Activity assays

When testing E1 or E2s in thiolester formation assays with ubiquitin, activity is determined by the presence of a band on SDS-PAGE migrating at 8 kDa above the molecular weight of the unmodified protein. These bands are detected either as a radiolabeled [^{32}P]ubiquitin signal by autoradiography or as an anti-ubiquitin immunoreactive band. The samples are run under nonreducing conditions

in order to detect the ubiquitin thiolester. When a similar sample is resolved under reducing conditions, i.e. in the presence of 2-ME or DTT, the thiolester band should disappear (see Fig. 15.9.2). As the ubiquitin is often added in excess, the proportion of ubiquitin in the higher-molecular-weight form should be relatively low. On the other hand, the proportion of E1 or E2 in the higher-molecular-weight form should be relatively high. The activity of each E1 batch or each E2 will be relative to the percentage of active protein that was produced. The molecular weight of unmodified E1 from most sources is about 114 to 118 kDa; therefore expect the E1-ubiquitin thiolester to migrate at 122 to 126 kDa. While there is at least one E2 (Birc6) as large as 528 kDa, the molecular weight of E2s is typically 14 to 34 kDa. Therefore, bands representing E2 ubiquitin thiolesters run in the 22- to 42-kDa range. It appears in some cases that E2s may form multi-ubiquitin changes in the absence of E3, ube2K being the best example of this, and that some E2s may be self-modifying. Therefore, when looking at E2 activity, look carefully at all of the bands in the gel and consider which are disappearing (active-site thiolesters) and which cannot be reduced (ubiquitin chains or nonreducible ubiquitin modification of the E2).

E3 activity assays differ in that one is generally testing the ability of the E3 to be permanently modified. The ability of the E3 to modify itself with ubiquitin is determined by several factors. These include whether the E3 is folded properly and which E2 is present. For RING fingers, proper folding is often determined by the amount of zinc present. Most E3 proteins demonstrate some ability to modify themselves when employing ube2d1, ube2d2, or ube2d3 as the ubiquitin-conjugating enzyme; however, this is not always the case. When one has found a productive E2-E3 pair, the E3 may be modified by a single ubiquitin (monoubiquitylation), by a single ubiquitin on multiple lysines (multi-monoubiquitylation), or by ubiquitin chains (multi-ubiquitylation). Monoubiquitylation is indicated by a band migrating 8 kDa above the molecular weight of the unmodified E3. Multi-monoubiquitylation is determined by a ladder of bands separated by 8 kDa with the first band migrating 8 kDa above the molecular weight of the unmodified E3. Modification by multi-ubiquitin chains may resemble multiple monubiquitins or may result in a smear of ^{32}P or anti-Ub signal running up to the top of the gel. Which pattern is obtained sometimes depends upon the amount of E3 present. Less E3 will result in a smear, while an excess results in the laddering pattern.

Inhibition of ubiquitylation of protein and degradation

Mutation of the active site of E3s will often result in the stabilization of substrate, as is the case for p53 when Hdm2 is mutated. It will also stabilize the E3 itself. This is seen with Hdm2 and Siah1. However, the authors have not seen appreciable stabilization of AO7 with RING mutation.

siRNA knockdown of a protein can be detected by immunoblotting. An effective knockdown will show about 80% to 100% loss of the targeted protein. Using a plasmid-based method, knockdown may take up to 3 days. Using oligonucleotides, loss of protein may be detected in a matter of 8 hr. This is based upon using Ambion's pSilencer vector in combination their Silencer oligonucleotide kit. This does not, however, mean that all genes will act the same way. Regardless of the method used, the loss of the gene product should be monitored for several days to determine optimal time required for maximum effect.

If the degradation that is observed is truly the result of 20S proteasome activity, expect to see an increase in protein level with samples exposed to ALLN, MG132, and lactacystin. Ammonium chloride and ALLM should show no difference when compared to untreated control samples. If lysosomal proteins are involved, only ammonium chloride is expected to increase the level of the target protein. If calpains are responsible for degrading the target protein, expect to see increased protein levels with ALLN, ALLM, and MG132, but not with lactacystin.

E1 production

When producing E1 in a baculovirus system, three 175-cm^2 flasks of *Sf*9 cells should produce 4.5 to 5 ml of crude lysate. After purification, this should produce 3.5 to 5 ml of eluate and 7 to 10 ml of glycerol stock. The amount of protein produced will vary, but should be in the range of 40 to 50 μg E1 per ml of glycerol stock. An important factor to keep in mind is that not all of the protein will be active; therefore a small amount should be tested to determine its relative activity.

E2 production

From a 100-ml culture of a 28-kDa protein, e.g., glutathione-*S*-transferase, expect as much as 250 μg of protein. For a larger protein,

e.g., E2-230K, a 100-ml culture may produce closer to 2.5 μg. Any purification will generally reduce the overall yield. For expression of E2s in IVT systems, incorporation of radioactivity will depend upon the number of methionines in the protein. For example E2D2 (UbcH5B) contains four methionines in a 148-amino acid protein (2.7%), while E2G2 (MmUbc7) contains nine in a 166-amino acid protein (5.4%). However, the authors generally see about 10,000 to 20,000 cpm/μl for E2D2 representing 1 to 2 fmol/μl of radiolabeled E2, assuming that radioactive methionine is incorporated evenly into each molecule. A sample calculation is shown below. It assumes that the scintillation counter detects methionine with 100% efficiency, e.g., radioactive methionine is (1000 Ci/mmol) (2.2×10^6 dpm/μCi) = 2.2×10^{15} dpm/mmol. 10,000 cpm/2.2×10^{15} dpm/mmol = 4.54×10^{-12} mmol or 4.54 fmol methionine incorporated. However, this represents total incorporated methionine. With four methionines/molecule, the total number of labeled molecules will be 25% of the total or (4.54 fmol)(25 %) = 1.14 fmol of radioactive E2.

E3 production

Results for E3 production in bacteria are expected to be similar to those for E2 production. The single most significant factor determining yields of E3 appears to be the size of the protein. Larger proteins often require longer induction, lower temperatures, and more inducing agent in order to get yields comparable to much smaller proteins. For example, it is possible to purify c-cbl, a 90-kDa protein, at 1 μg/ml from a 100 ml culture. AO7, a 50-kDa protein, can be purified at 20 μg/ml.

When expressed in in vitro translation systems, radiolabeled E3s may be produced at a rate proportional to their size and the number of naturally occurring methionine residues in the protein. Typically 1 μl of a 50 μl in vitro translation mixture will incorporate between 10,000 cpm and 100,000 cpm of [^{35}S]methionine.

Time Considerations

Most of the activity assays described in this unit require up to 2 hr to prepare the E3s on glutathione-Sepharose, 1 to 3 hr to carry out the reaction, 1 to 2 hr to perform gel electrophoresis, 1 hr to dry gels, and 5 min to overnight to detect radioactive signals. Therefore, a typical in vitro E3 reaction will require ~1 day. Less time is required when performing E1 or E1-E2 thiolester assays because there is no E3 preparation time and the reactions are incubated only 5 min. Thiolester formation can be done in less than 4 hr. Expression of proteins in bacteria is by far the most common method used in the authors' laboratory. Typically, researchers should schedule a week ahead if they will require fresh as opposed to thawed proteins. Preparation of radiolabeled proteins by in vitro translation can require as little as 3 hr if using a commercial kit or 8 hr if it is necessary to make the RNA first. Baculovirus preparation of E1 or multi-subunit E3s is by far the most time-consuming method that the authors use. This may require 17 days total with 5 days for virus production, 8 days for virus titering, 3 days for infection to produce protein, and an additional day to lyse cells, collect protein lysates, and purify the protein of interest.

For detecting protein ubiquitylation in cells, direct immunoblotting can be done in 1 day. If immunoprecipitation is required, an additional 1 day is needed to let antibody bind to protein A or G beads (2 to 4 hr) and to let target protein bind to immobilized antibody (1 to 4 hr). When transfection of E2, E3, or ubiquitin is involved, an additional 2 days are need to seed the cells and allow transfected proteins to be expressed in cells.

Literature Cited

Amir, R.E., Iwai, K., and Ciechanover, A. 2002. The NEDD8 pathway is essential for SCF(beta-TrCP)-mediated ubiquitination and processing of the NF-kappa B precursor p105. *J. Biol. Chem.* 277:23253-23259.

Aravind, L. and Koonin, E.V. 2000. The U box is a modified RING finger: A common domain in ubiquitination. *Curr. Biol.* 10: R132-R134.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. 2006. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Brown, T., Mackey, K., and Du, T. 2004. Analysis of RNA by northern and slot blot hybridization. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 4.9.1-4.9.19. John Wiley & Sons, Hoboken, N.J.

Brzovic, P.S., Keeffe, J.R., Nishikawa, H., Miyamoto, K., Fox, D. 3rd, Fukuda, M., Ohta, T., and Klevit, R. 2003. Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc. Natl. Acad. Sci. U.S.A.* 100:5646-5651.

Chan, A.H., Lee, S.M., Chim, S.S., Kok, L.D., Waye, M.M., Lee, C.Y., Fung, K.P., and Tsui, S.K. 2001. Molecular cloning and characterization of a RING-H2 finger protein, ANAPC11, the human homolog of yeast Apc11p. *J. Cell Biochem.* 83:249-258.

Coligan, J.E., Bierer, B., Marguiles, D.H., Shevach, E.M., and Strober, W., eds.) 2006. Current Protocols in Immunology. John Wiley & Sons, Hoboken, N.J.

Ellison, M.J. and Hochstrasser, M. 1991. Epitope-tagged ubiquitin. A new probe for analyzing ubiquitin function. *J. Biol. Chem.* 266:21150-21157.

Engebrecht, J., Brent, R., and Kaderbhai, M.A. 1991. Minipreps of plasmid DNA. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 1.6.1-1.6.10. John Wiley & Sons, Hoboken, N.J.

Fang, S., Jensen, J.P., Ludwig, R.L., Vousden, K.H., and Weissman, A.M. 2000. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J. Biol. Chem.* 275:8945-8951.

Fang, S. and Weissman, A.M. 2004. A field guide to ubiquitylation. *Cell Mol. Life Sci.* 61:1546-1561.

Glickman, M. H. and Ciechanover, A. 2002. The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. *Physiol. Rev.* 82:373-428.

Groll, M. and Huber, R. 2004. Inhibitors of the eukaryotic 20S proteasome core particle: A structural approach. *Biochim. Biophys. Acta* 1695:33-44.

Häkli M, Lorick, K.L., Weissman, A.M., Janne, O.A., and Palvimo, J.J. 2004. Transcriptional coregulator SNURF (RNF4) possesses ubiquitin E3 ligase activity. *FEBS Lett.* 560:56-62.

Hatakeyama, S., Yada, M., Natsumoto, M., Ishida, N., and Nakayama, K.I. 2001. U box proteins as a new family of ubiquitin-protein ligases. *J. Biol. Chem.* 276:33111-33120.

Hatfield, P.M., Callis, J., and Vierstra, R.D. 1990. Cloning of ubiquitin activating enzyme from wheat and expression of a functional protein in *Escherichia coli*. *J. Biol. Chem.* 265:15813-15817.

Heitz, F., Nay, C., and Guenet, C. 1997. Expression of functional human muscarinic M2 receptors in different insect cell lines. *J. Recept. Signal Transduct. Res.* 17: 305-317.

Huibregtse, J.M., Scheffner, M., and Howley, P.M. 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol. Cell Biol.* 13:775-784.

Imajoh-Ohmi, S., Kawaguchi, T., Sugiyama, S., Tanaka, K., Omura, S., and Kikuchi, H. 1995. Lactacystin, a specific inhibitor of the proteasome, induces apoptosis in human monoblast U937 cells. *Biochem. Biophys. Res. Commun.* 217:1070-1077.

Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R.C., Conaway, J.W., Klausner, R.D., and Pause, A. 1999. Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc. Natl. Acad. Sci. U.S.A* 96:12436-12441.

Johnson, E.S. and Gupta, A.A. 2001. An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* 106:735-744.

Kamura, T., Conrad, M.N., Yan, Q., Conaway, R.C., and Conaway, J.W. 1999. The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes Dev.* 13:2928-933.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., Omata, M., and Tanaka, K. 2001. NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J.* 20:4003-4012.

Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S., and Weissman, A.M. 1999. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl. Acad. Sci. U.S.A.* 96:11364-11369.

Lorick, K. L., Jensen, J.P., and Weissman, A.M. 2005a. Expression, purification and properties of the Ubc4/5 family of E2 enzymes. *Methods Enzymol.* 398:54-68.

Lorick, K.L., Tsai, Y.-C., Yang, Y., and Weissman, A.M. 2005b. RING fingers and relatives: Determinators of protein fate. *In* Protein Degradation. Vol. 1: Ubiquitin and the Chemistry of Life (R.J. Mayer, A.J. Ciechanover, and M. Rechsteiner, eds.) pp. 4-101. Wiley-VCH, Weinheim, Germany.

Mahaffey, D. and Rechsteiner, M. 1999. Discrimination between ubiquitin-dependent and ubiquitin-independent proteolytic pathways by the 26S proteasome subunit 5a. *FEBS Lett.* 450:123-125.

Maki, C.G., Huibregtse, J.M., and Howley, P.M. 1996. In vivo ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Res.* 56:2649-2654.

Mc Cormack, T., Baumeister, W., Grenier, L., Moomaw, C., Plamondon, L., Pramanik, B., Slaughter, C., Soucy, F., Stein, R., Zuhl, F., and Dick, L. 1997. Active site-directed inhibitors of Rhodococcus 20 S proteasome: Kinetics and mechanism. *J. Biol. Chem.* 272:26103-26109.

Megumi, Y., Miyauchi, Y., Sakurai, H., Nobeyama, H., Lorick, K., Nakamura, E., Chiba, T., Tanaka, K, Weissman, A.M., Kirisako, T., Ogawa, O., and Iwai, K. 2005. Multiple roles of Rbx1 in the VBC-Cul2 ubiquitin ligase complex. *Genes Cells* 10:679-691.

Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. 1992. Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* 360:597-599.

Murakami, Y., Matsufuji, S., Tanaka, K., Ichihara, A., and Hayashi, S. 1993. Involvement of the proteasome and antizyme in ornithine decarboxylase degradation by a reticulocyte lysate. *Biochem J.* 295:305-308.

Murphy, C.I., Piwnica-Worms, H., Grünwald, S., Romanow, W.G., Francis, N., and Fan, H.-Y. 2004. Maintainance of insect cell cultures

and generation of recombinant baculoviruses. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 16.10.1-16.10.19. John Wiley & Sons, Hoboken, N.J.

Naujokat, C., Sezer, O., Zinke, H., Leclere, A., Hauptmann, S., and Possinger, K. 2000. Proteasome inhibitors induced caspase-dependent apoptosis and accumulation of p21WAF1/Cip1 in human immature leukemic cells. *Eur. J. Haematol.* 65:221-236.

Newman, R.M., Mobascher, A., Mangold, U., Koika, C., Diah, S., Schmidt, M., Finley, D., and Zeiter, B.R. 2004. Antizyme targets cyclin D1 for degradation: A novel mechanism for cell growth repression. *J. Biol. Chem.* 279:41504-41511.

Sambrook, J.F., Frisch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y.

Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. 1997. Introduction of plasmid DNA into cells. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 1.8.1-1.8.10. John Wiley & Sons, Hoboken, N.J.

Skowyra, D., Koepp, D.M., Kamura, T., Conrad, M.N, Conaway, R.C., Conaway, J.W., Elledge, S.J., and Harper, J.W. 1999. Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. *Science* 284:662-665.

Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60-89.

Tanaka, K., Suzuki, T., and Chiba, T. 1998. The ligation systems for ubiquitin and ubiquitin-like proteins. *Mol. Cells* 8:503-512.

Voytas, D. 2000. Agarose gel electrophoresis. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 2.5A.1-2.5A.9. John Wiley & Sons, Hoboken, N.J.

Wang, W., Yum, L., and Beinfeld, M.C. 1997. Expression of rat pro cholecystokinin (CCK) in bacteria and in insect cells infected with recombinant baculovirus. *Peptides* 18:1295-1299.

Weissman, A. M. 2001. Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell Biol.* 2:169-178.

Wilkinson, K.D. 1997. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J.* 11:1245-1256.

Wu, P.Y., Hanlon, M., Eddins, M., Tsui, C., Rogers, R.S., Jensen, J.P., Matunis M.J., Weissman, A.M., Wolberger, C.P., and Pickart, C.M. 2003. A conserved catalytic residue in the ubiquitin-conjugating enzyme family. *EMBO J.* 22:5241-5250.

Contributed by Kevin L. Lorick, Yili Yang, Jane P. Jensen, and Allan. M. Weissman
National Cancer Institute
Frederick, Maryland

Kazuhiro Iwai
Osaka City University
Osaka, Japan

Measuring Retrograde Transport to the *Trans*-Golgi Network

After internalization from the plasma membrane, most molecules are delivered to early/recyling endosomes. From here, two principle routes have been described: (1) the molecules return to the plasma membrane, or (2) they are targeted to late endosomes/lysosomes for degradation. Some molecules can escape recycling and degradation, however, and reach other compartments in the cell, such as the Golgi apparatus, the endoplasmic reticulum (ER), and in some instances the cytosol. This pathway is termed the retrograde route (Johannes and Goud, 1998).

Shiga toxin was the first protein that was convincingly shown to be transported from the plasma membrane to the ER (Sandvig et al., 1992). The initial morphological observation was later confirmed using protein biochemistry (Johannes et al., 1997). Similar observations were described for the protein toxins ricin (Rapak et al., 1997) and cholera toxin (Fujinaga et al., 2003). Some endogenous proteins can also traffic between endosomes and the *trans*-Golgi network (TGN), and in the case of TGN38 and the mannose-6 phosphate receptors, evidence was provided that they, at least in part, use the same pathway as Shiga toxin (Ghosh et al., 1998; Mallard et al., 1998; Saint-Pol et al., 2004).

The receptor-binding non-toxic B-subunit of Shiga toxin (STxB) has proven a valuable marker for the retrograde route. Indeed, STxB-based tools permit morphological and biochemical characterization of retrograde transport. In a previous methods report (Mallard and Johannes, 2003), the authors described experimental set-ups for the purification of STxB and quantification of its endocytosis and retrograde transport to the TGN and the ER. In this unit, the authors describe the extension of STxB methodology to the generalized study of cargo-protein transport between early/recycling endosomes and the TGN (sulfation-site peptide coupling to model cargo proteins; see Support Protocol 1). Basic Protocol 1 describes the measure of early/recycling endosomes-to-TGN transport in intact cells, using the sulfation assay. The Alternate Protocol details a method to study early/recycling endosomes-to-TGN transport on permeabilized cells. Support Protocol 2 describes the preparation of Tris-tricine mini gels, which are used to analyze low-molecular-weight protein substrates. Support Protocol 3 elaborates on the chemical coupling scheme by introducing full-size horseradish peroxidase (HRP) onto STxB for whole mount ultrastructural analysis of the Shiga toxin–containing early/recycling endosome (Saint-Pol et al., 2004). Finally, an improvement of the STxB endocytosis assay is explained in Basic Protocol 2.

QUANTITAVE ASSAY TO MEASURE RETROGRADE TRANSPORT OF CARGO PROTEINS IN INTACT CELLS

BASIC PROTOCOL 1

The STxB-based sulfation assay for the retrograde route, as described previously (Johannes et al., 1997; Mallard et al., 1998), is based on the use of a STxB variant to which a tandem of protein sulfation recognition sites is added by genetic fusion. Bacteria lack sulfotransferase activity, and STxB-Sulf2 is purified as a non-sulfated protein. Upon retrograde transport to the TGN in higher eukaryotic target cells, STxB-Sulf2 becomes the substrate of a *trans*-Golgi/TGN localized sulfotransferase that catalyzes the post-translational transfer of radioactive sulfate from the medium onto a tyrosine residue within the sulfation recognition sites. After cell lysis, immunoprecipitation, and gel electrophoresis, sulfated STxB-Sulf2 can be detected and quantified.

Contributed by Mohamed Amessou, Vincent Popoff, Belèn Yelamos, Agnès Saint-Pol, and Ludger Johannes

Current Protocols in Cell Biology (2006) 15.10.1-15.10.21

The sulfation reaction on sulfation-site-modified cargo protein allows the detection and quantification of its arrival in the TGN, following binding to cells and uptake at the plasma membrane. The sulfation reaction protocol on intact cells is described below. The Alternate Protocol describes a permeabilized cell assay for the study of early/recycling endosomes-to-TGN transport step.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

Materials

HeLa cells
DMEM3+ (see recipe)
DMEM without sulfate (Invitrogen), cold and 37°C
Sulfation substrate: e.g., STxB-Sulf2, CTxB-Sulf2, *or* anti-GFP-Sulf2 (see Support Protocol 1)
Radioactive sulfate ($^{35}SO_4^{2-}$; Amersham Pharmacia Biotech, specific activity of at least 50 μCi/μl)
RIPA buffer (see recipe)
Protease inhibitor cocktail (PIC; see recipe)
13C4 monoclonal anti-STxB antibody (hybridoma from ATCC #CRL 1794)
Protein G–Sepharose beads 4 fast flow (Amersham Pharmacia Biotech) equilibrated with RIPA buffer
Streptavidin agarose beads (Pierce) equilibrated with RIPA buffer
Washing buffer: 50 mM Tris·Cl, pH 8.0
1.5× SDS sample buffer
Tris-tricine mini-gels (see Support Protocol 2)
Fixation buffer: 40% ethanol, 10% acetic acid

24-well tissue culture dishes
37°C, 5% CO_2 incubator
1.5-ml tubes
End-over-end rotator
Glass microfiber GF/C filters
Microbeta scintillation counter
Thermal cycler
Micropipettor
Gel dryer
PhosphorImager and appropriate screens

Prepare cells

1. Prior to starting the experiment (12 to 15 hr before start), plate 1×10^5 HeLa cells in 1 ml DMEM3+ in each well of a 24-well tissue culture dish.
2. To deplete endogenous sulfate, wash cells three times with 0.5 ml DMEM without sulfate per well.
3. Replace the medium with 0.5 ml DMEM without sulfate per well and incubate cells 90 min in a 37°C, 5% CO_2 incubator.

Perform sulfation

4. Place cells on ice and replace the medium with 0.25 ml ice-cold DMEM without sulfate and containing a sulfation substrate, e.g., 1 μM STxB-Sulf2, 1 μM CTxB-Sulf2, or 15 μg/ml anti-GFP-Sulf2. Incubate cells 30 min on ice.
5. Discard medium and wash three times with 0.5 ml cold DMEM without sulfate.

6. To allow internalization, replace medium with 0.25 ml of 37°C DMEM without sulfate and containing 0.5 mCi/ml $^{35}SO_4^{2-}$. Incubate in a 37°C, 5% CO_2 incubator (typically 20 to 40 min).

7. Put cells on ice and wash three times with 0.5 ml of ice-cold DMEM.

Immunoprecipitate the marker

8. Lyse cells as follows:

   a. Place 24-well tissue culture dishes on ice.

   b. To each well, add 0.5 ml of ice-cold RIPA buffer containing 1× PIC.

   c. Use a pipet tip to scrape off cells and transfer lysates to 1.5-ml tubes.

   d. To each well, add another 0.5 ml of ice-cold RIPA buffer containing 1× PIC and transfer to 1.5-ml tubes.

9. For STxB, add 10 μg/ml 13C4 monoclonal anti-STxB and 80 μl protein G–Sepharose beads to the cell lysate. For anti-GFP-Sulf2, add 80 μl protein G–Sepharose beads to the lysates. For CTxB, add 100 μl strepavidin agarose. Incubate with end-over-end rotation for ≥90 min at 4°C.

   The used marker is immunoprecipitated. STxB-Sulf2 is immunoprecipitated with anti-STxB antibody and protein G-Sepharose beads, whereas, anti-GFP-Sulf2 or CTxB-Sulf1, which contains biotin, is directly immunoprecipitated by protein G-Sepharose beads or streptavidin agarose beads, respectively.

10. Centrifuge tubes 2 min at 800 × *g*, 4°C.

11. Aspirate supernatant, add 1 ml of washing buffer, gently resuspend beads, and centrifuge again as in step 10.

Determine total sulfation counts

12. Precipitate 900 μl of supernatant by adding 100 μl of 100% trichloroacetic acid (TCA). Incubate at least 1 hr at 4°C.

13. Filter on glass microfiber GF/C filters.

14. Count filters in a Microbeta scintillation counter.

    At this step, the supernatant contains all of the proteins that have not been immunoprecipitated, i.e., endogenous proteins. Among these proteins, some, such as the proteoglycans, are natural substrates for sulfotransferase and thus have been labeled by radioactive sulfate during the assay. Precipitating and filtrating the supernatant permits measurement of the amount of radioactivity of endogenous proteins. It defines the total sulfation level. If it is assumed that this level is equal in all conditions (only the retrograde transport and thus the sulfation of the marker should be affected), it can be used to normalize the results. It compensates for the variation of sulfotransferase and cell number that could be induced by cell treatments (e.g., siRNA, drugs).

Analyze sulfated substrate

15. Dry beads by removing the fluid around the beads with a microsyringe, and immediately add 25 μl of 1.5× SDS sample buffer without reducing agent.

16. Resuspend beads by briefly vortexing, heat 5 min to 95°C in a thermal cycler.

17. Load the samples (all 25 μl) on Tris-tricine mini gels using a microsyringe.

18. Connect the power supply to the electrophoresis cell and run for 1.5 hr at 25 mA for each 1-mm gel.

19. Fix gel for 20 min in fixation buffer.

20. Dry the gel (2 hr at 70°C) and expose it in a PhosphorImager cassette.

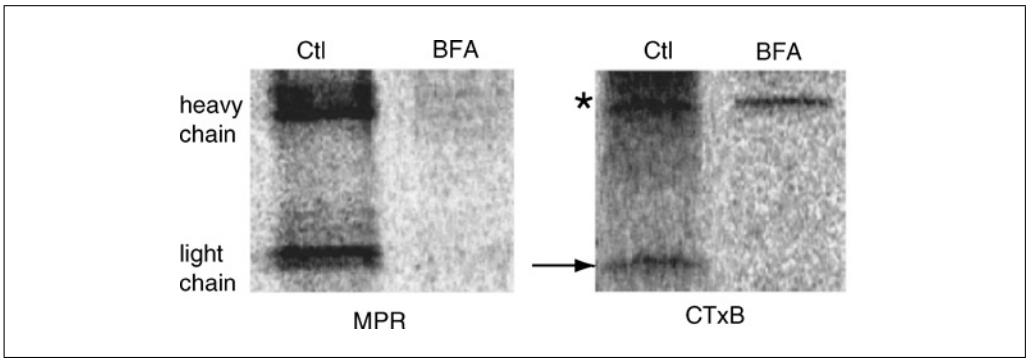


Figure 15.10.1 Sulfation analysis using sulfation site peptide–tagged CTxB and anti-GFP antibody. Note that brefeldin A (BFA) treatment prevents sulfation. The arrow indicates the sulfation product of sulfation-site peptide–tagged CTxB. The asterisk indicates high-molecular-weight material that has not entered the gel.

21. Quantify bands corresponding to sulfated STxB-Sulf$_2$ or other sulfated substrate.

Figure 15.10.1 shows a typical sulfation analysis experiment using CTxB-Sulf2 or anti-GFP-Sulf2. Both proteins are sulfated when incubated with HeLa cells. In the case of anti-GFP-Sulf2, HeLa cells were used that stably express GFP-tagged MPR300 (Waguri et al., 2003). Brefeldin A strongly alters Golgi structure and inhibits retrograde transport (Mallard et al., 1998). Under these conditions, an inhibition of CTxB and anti-GFP sulfation is observed.

ALTERNATE PROTOCOL

PERMEABLIZED CELL ASSAY FOR MEASURING TRANSPORT OF CARGO PROTEINS FROM EARLY/RECYCLING ENDOSOMES TO THE TGN

This protocol describes a permeabilized cell assay that reconstitutes early/recycling endosomes-to-TGN transport of STxB under conditions that allow modification of the activity of the cytosol using macromolecular tools (Mallard et al., 2002). In this assay, STxB-Sulf2 is internalized into early/recycling endosomes of HeLa cells at low temperature (19.5°C), conditions under which retrograde transport to the TGN is inhibited. After permeabilization of the plasma membrane using streptolysin O (SLO), cells are shifted to 37°C in the presence of exogenous cytosol, an energy regeneration system, radioactive sulfate, and tools that allow modification of the activity of the cytosol (e.g., antibodies, mutant proteins, peptides). At the end of the sulfation reaction, cells are lysed and radiolabeled sulfated STxB-Sulf2 is analyzed by SDS-PAGE.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

Additional Materials *(also see Basic Protocol 1)*

HeLa S3 cells for cytosol preparation
Phosphate-buffered saline (PBS)
HK buffer (see recipe)
ICT/DTT buffer (see recipe), ice cold and at 37°C
Streptolysin O (SLO; see recipe)
ATP-regenerating system (see recipe)

Potter-Elvehjam homogenizer
Ultracentrifuge
15- and 50-ml tubes
Dialysis membrane (10 kDa MWCO)
1.5-ml microcentirifuge tubes

Prepare HeLa cell cytosol

1. Pellet at least 10^9 HeLa S3 cells 5 min at 300 × *g*, 4°C.
2. Wash cells three times with 50 ml PBS centrifuging as in step 1.
3. Resuspend the cells in ice-cold HK buffer supplemented with protein inhibitor cocktail (PIC): 1 vol buffer for 1 vol cells.
4. Homogenize cells using a bead homogenizer (e.g., from HGM) or a Dounce homogenizer. Homogenize up and down ~25 times on ice. Check the completeness of homogenization by trypan blue exclusion (*UNIT 1.1*). Transfer the homogenate to a 15-ml tube.
5. Centrifuge the homogenate 5 min at 3000 × *g*, 4°C. Transfer the supernatant to a 15-ml tube.
6. Centrifuge the supernatant 45 min at 100,000 × *g*, 4°C. Collect the supernatant.
7. Using a 10-kDa dialysis membrane, dialyze the supernatant three times against 2 liters of ICT/DTT buffer at 4°C. Change dialysis buffer every 2 hr. Freeze at −80°C.

Collect and treat cells for in vitro transport assay

8. Before the start of the experiment (16 hr before), plate 1.2×10^5 cells/well in 24-well tissue culture plates.
9. Wash cells three times with 0.5 ml of sulfate-free DMEM using a Pasteur pipet to remove the medium.
10. Incubate cells in sulfate-free DMEM 90 min in a 37°C, 5%$C0_2$ incubator.

    This is the sulfate starvation step.

Bind STxB-Sulf2 to cells

11. Place cells on ice and incubate 30 min with 1 μM STxB-Sulf2 in sulfate-free DMEM.

    This allows cell-surface binding of STxB-Sulf2.

12. Shift cells to 19.5°C for 40 min.

    At this temperature, STxB accumulates in early/recycling endosomes.

13. Place cells on ice again and wash three times with 0.5 ml of 4°C ICT/DTT buffer.

Permeabilize cells

14. To each well, add 250 μl SLO at 2 μg/ml in ICT/DTT buffer. Incubate 10 min on ice.

    The SLO reagent binds to the plasma membrane. After the shift to 37°C (step 16), the plasma membrane is permeabilized. Endogenous cytosol diffuses out of the cell.

15. Maintain cells at 4°C and wash cells three times with 0.5 ml of 4°C ICT/DTT buffer.

    This will remove the free SLO.

16. Add 0.5 ml of pre-warmed ICT/DTT buffer at 37°C to each well, and then transfer plate to 37°C for 10 min.

Carry out transport assay

17. Add transport mix containing ATP-regenerating system, 1 mCi/ml (^{35}S)sulfate, 3 mg/ml of HeLa cell cytosol, and STxB-Sulf2 in ICT/DTT buffer.
18. Incubate cells 30 min at 37°C.

    In this condition, the STxB-Sulf2 will be transported to TGN and sulfated.

19. Place cells on ice and gently wash each well once with 0.5 ml of ice-cold ICT/DTT.

Collect and analyze labeled proteins

20. Lyse cells two times with 0.5 ml RIPA/PIC buffer and transfer lysed cells to 1.5-ml microcentrifuge tubes containing: 10 μg/ml 13C4 anti-STxB antibody and 80 μl of protein G–sepharose beads equilibrated in RIPA buffer.

21. Continue experiment as described in Basic Protocol 1, steps 15 to 21.

SUPPORT PROTOCOL 1

CHEMICAL COUPLING OF PROTEINS WITH SULFATION SITES INTO A PROTEIN OF INTEREST

The following protocol describes an adaptation of the sulfation methodology to other cargo proteins of the retrograde route. The genetic fusion approach is replaced by a chemical coupling procedure that allows the introduction of peptides with sulfation sites onto purified proteins. In some instances, the model cargo has free sulfhydryl groups, and the sulfation peptide can be coupled in a one-step reaction. In other instances, it is necessary to first introduce free sulfhydryls onto the molecule of interest, followed by sulfation-site peptide coupling. This protocol uses the *N*-succinimidyl *S*-acetylthioacetate (SATA) reagent for introducing sulfhydryl groups onto proteins. The advantages are: (1) SATA reacts with molecules in nondenaturing conditions; (2) it introduces stable and covalent links through the interaction of its *N*-hydroxysuccinimide function with primary amines of the protein to yield an amide bond; and (3) the sulfhydryl group introduced is protected, which reduces the risk of intra- or inter-chain disulfide bonding. This protocol is written for two proteins that do not have natural free sulfhydryl groups. The first protein is the B-subunit of cholera toxin. Cholera toxin has the same subunit structure as Shiga toxin. Its homopentameric B-subunit (CTxB) binds to the glycosphingolipid GM1. GM1-bound CTxB is targeted from the plasma membrane to the ER, via the TGN/Golgi apparatus (Fujinaga et al., 2003), as described for STxB. The second protein is an antibody directed against the GFP moiety of a fusion protein between GFP and the cation-independent mannose 6-phosphate receptor (CI-MPR). A cell line has been described that stably expresses GFP-CI-MPR (Waguri et al., 2003). A recent work showed that this protein follows the retrograde route (Saint-Pol et al., 2004).

The experimental procedures of the coupling strategy are described below and are illustrated in Figure 15.10.2.

Materials

N-Succinimidyl *S*-acetylthioacetate (SATA; Pierce)
Dimethyl sulfoxide (DMSO)
Protein of interest; e.g., CTxB and anti-GFP antibody
50 mM sodium phosphate/1 mM EDTA buffer, pH 7.5
Bromoacetylated sulfation site peptide with or without biotin group (see Fig. 15.10.1)
10× deacetylation solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Dialysis cassettes: 10-kDa molecular weight cutoff (MWCO; Pierce), optional
PD-10 desalting columns (Amersham Pharmacia Biotech), optional
Additional reagents and equipment for measuring protein concentration using the Bradford assay (*APPENDIX 3H*) and SDS-PAGE (*UNIT 6.1*)

NOTE: Should the cargo protein naturally contain free sulfhydryl groups, such as in STxB/Cys, start at step 6.

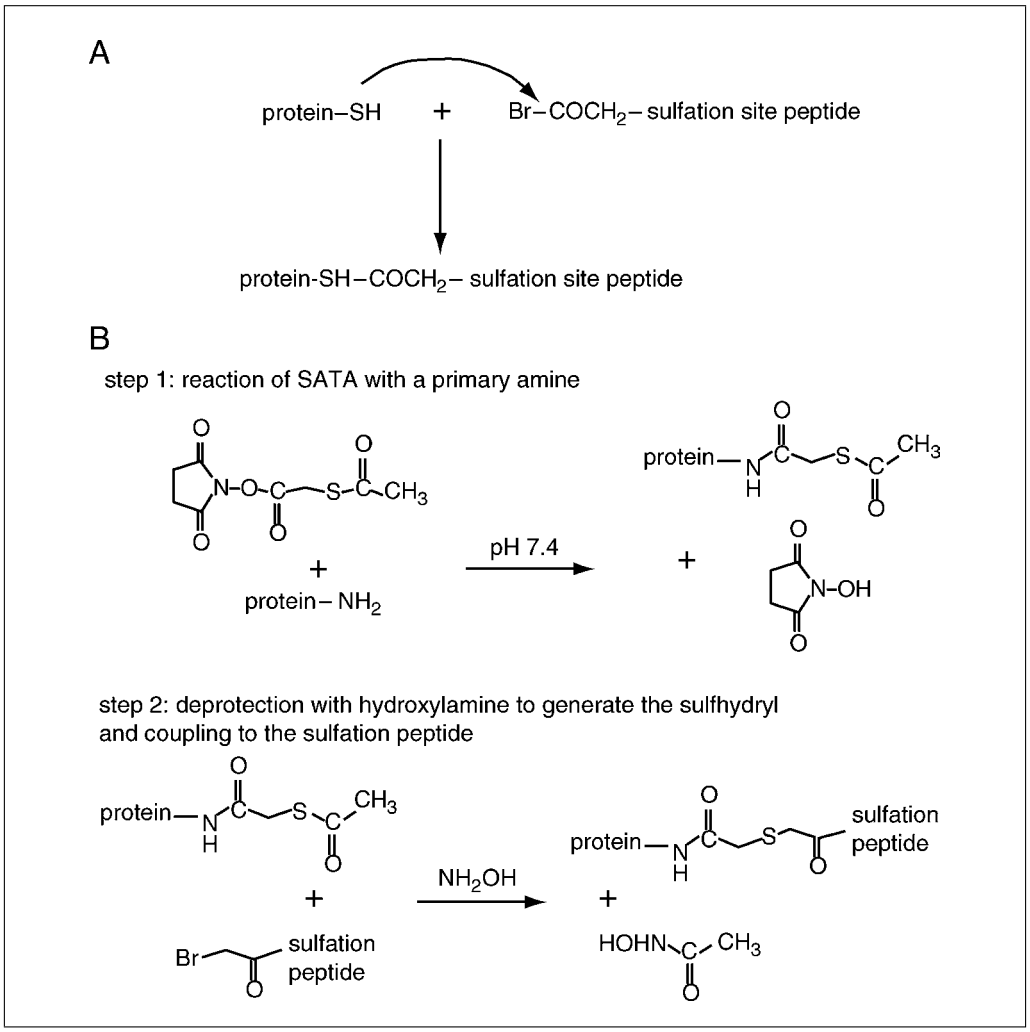


Figure 15.10.2 Experimental strategies for coupling sulfation site peptides to cargo proteins. (**A**) One-step reaction of bromoacetylated peptides with cargo proteins carrying free sulfhydryl groups (e.g., STxB/Cys). (**B**) Reaction scheme for introducing protected sulfhydryl groups onto cargo proteins (step 1) followed by concomitant deprotection and sulfation site peptide coupling (step 2). Sulfation peptide sequence: BrCOCH2-TSEEPEYGEEEPEYGEE(±biotin).

Modify sulfhydryl group

1. Prepare SATA stock solution at 100 mM in DMSO.
2. Prepare the protein of interest in 50 mM sodium phosphate/1 mM EDTA buffer, pH 7.5, at a concentration of 50 to 100 μM.

   For CTxB and anti-GFP antibody, the authors have worked with solutions at 100 μM and 50 μM, respectively. Typical volumes of coupling reactions are 20 to 200 μl.
3. Add the SATA reagent to the protein sample.

   In general, use a ten-fold molar excess of the SATA reagent over the protein when the protein concentration is >5 mg/ml. If the protein concentration is <5 mg/ml, try to concentrate it using Nanosep 10-kDa microconcentration chambers (PallFiltron), otherwise add 20- to 50-fold-molar excess of SATA reagent.

   For CTxB and anti-GFP antibody, the concentration is ~5 mg/ml.

   Typically, the volume of DMSO should not exceed 10% of the final reaction volume.

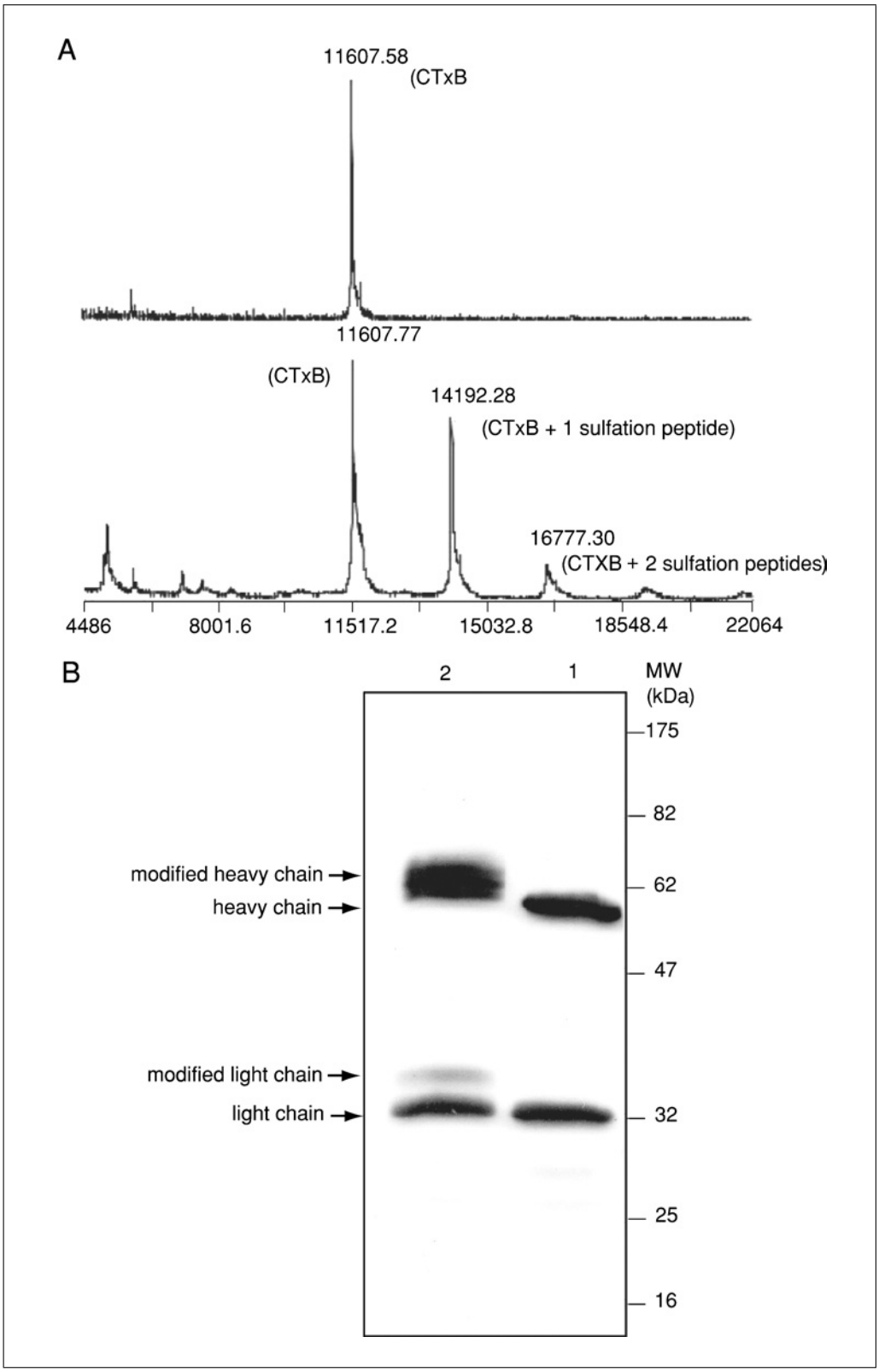


Figure 15.10.3 Analysis of sulfation-site peptide coupling to CTxB and anti-GFP antibody. (**A**) MALDI-TOF analysis of uncoupled CTxB (upper panel) and sulfation-site peptide coupled CTxB (lower panel). Note that after sulfation-site peptide coupling, some B-chains remain unmodified, some carry one sulfation-site peptide, and exceptionally also two sulfation-site peptides. Because CTxB is a homopentamer of B-chains, it is reasonable to assume that statistically, at least one sulfation site coupled B-chain is present per pentamer. (**B**) Western analysis of uncoupled (lane 1) and sulfation site peptide–coupled (lane 2) anti-GFP antibody. Unmodified and sulfation site peptide–modified heavy and light chains are indicated by arrows.

4. React for 30 min at room temperature gently shaking in a rotary incubator.
5. Remove uncoupled SATA reagent by dialysis three times, 1 hr each time, against 0.5 liters of 50 mM sodium phosphate/1 mM EDTA buffer, pH 7.5, with 10-kDa MWCO dialysis cassettes or by gel filtration through a PD-10 desalting column equilibrated with 25 ml of 50 mM sodium phosphate/1 mM EDTA buffer, pH 7.5.

Couple bromoacetylated sulfation-site peptide

6. Measure the concentration of activated protein using the Bradford assay (*APPENDIX 3H*), and incubate with ten-fold molar excess of the bromoacetylated sulfation-site peptide in presence of 1× deacetylation solution.

   In the case of CTxB, use biotin-tagged bromoacetylated sulfation-site peptide.

   Should the cargo protein naturally contain free sulfhydryl groups, the deacetylation solution can be omitted.

7. React 2 hr at room temperature gently shaking in a rotary incubator.
8. Then dialyze against PBS buffer to remove hydroxylamine and free peptide.
9. Determine the yield of the conjugation reaction either by MALDI-TOF mass spectroscopy for small proteins (typically <25 kDa) or by SDS-PAGE (*UNIT 6.1*) analysis for large proteins such as antibodies.
10. Snap freeze proteins in liquid nitrogen and store at −20°C.

    Figure 15.10.3A presents the MALDI-TOF analysis of sulfation site peptide coupling to CTxB. The authors estimate that about two B-chains per pentamer are sulfation-site peptide modified. Figure 15.10.3B presents the results of sulfation-site peptide coupling to anti-GFP antibody. The sulfation-site peptide–modified antibody is indicated by an arrow.

PREPARING TRIS-TRICINE GELS

SUPPORT PROTOCOL 2

Tris-tricine gels are adapted for low-weight proteins. They are used to separate sulfated STxB or CTxB from radioactive contaminants such as free radiolabeled sulfate or sulfated endogenous proteins remaining after immunoprecipitation.

Materials

Acrylamide solution A (see recipe)
Tris-tricine gel buffer (see recipe)
Glycerol
10% (w/v) ammonium persulfate (APS) solution in water
Tetramethyl-1-,2-diaminomethane (TEMED)
Acrylamide solution B (see recipe)
10× cathode buffer (see recipe)
10× anode buffer (see recipe)
Glass plates and electrophoresis apparatus (BioRad)
Pasteur pipets
Teflon combs
Glass microfiber GF/C filters (Whatman)

Prepare separating Tris-tricine gels

1. Assemble the glass-plate sandwich of the electrophoresis apparatus according to manufacturer's instructions.

2. Prepare the separating gel. For two BioRad gel chambers:

   1.7 ml acrylamide solution A
   1.7 ml Tris-tricine gel buffer
   0.7 g glycerol
   1.1 ml water
   Mix and add 50 μl of 10% APS solution and 5 μl TEMED.

3. Immediately apply separating gel solution to gel chamber, using a Pasteur pipet.
4. Slowly cover the top of the gel with water.

   The overlay provides an oxygen-free environment that favors polymerization.

5. Allow the gel to polymerize 30 to 60 min at room temperature.
6. Pour off the water.

Prepare stacking gel

7. Prepare the stacking gel solution as follows:

   0.24 ml acrylamide solution B
   0.78 ml Tris-tricine gel buffer
   2 ml water
   Mix and add 24 μl of 10% APS solution and 2.4 μl TEMED.

8. Immediately pour stacking gel solution into gel chamber, above the separating gel, using a Pasteur pipet.
9. Insert a Teflon comb. If necessary, add additional stacking gel to fill gel chamber completely.
10. Allow the stacking gel solution to polymerize 30 to 60 min at room temperature.

    The gel can be stored for 1 day at 4°C wrapped in humidified tissue.

11. Carefully remove the Teflon comb. Rinse wells with water. Mount gel in the apparatus.
12. Fill the internal buffer chamber with 10× cathode buffer and fill the external buffer chamber with 10× anode buffer.

SUPPORT PROTOCOL 3

CHEMICAL COUPLING OF HRP TO STxB

This protocol describes a method for the chemical coupling of horseradish peroxidase (HRP) to STxB for use in whole-mount experiments. The whole-mount technique allows an ultrastructural analysis of membrane systems that have been fixed from inside using an HRP-catalyzed polymerization reaction. Due to the possibility of removing endogenous cytosol before general fixation, no ultrafine sectioning is required, and cells can be analyzed, as a whole, under the electron microscope. Immunolabeling permits the detection of antigens on the cytosolic side of membranes. For the endocytic membrane system, the study by Stoorvogel and colleagues (Stoorvogel et al., 1996) was a milestone. The authors have recently adopted the whole-mount technique to the Shiga toxin–containing early/recycling endosomes (Saint-Pol et al., 2004).

For this purpose, a STxB variant is used in which a cysteine residue is placed at the C-terminus to avoid the formation of inter-chain disulfide bonds. This variant is termed STxB/Cys (Haicheur et al., 2003). The coupling reaction between HRP and STxB/Cys is performed in two steps using the heterobifunctional crosslinking agent

MBS (maleimidobenzoyl-*N*-hydroxusuccinimide ester). In the first step, MBS is reacted via its *N*-hydroxysuccinimide function with primary amines of HRP. After removal of free crosslinker, activated HRP reacts with STxB/Cys via its maleimide group. The coupling product is purified by gel filtration and immunoaffinity chromatography.

Materials

Horseradish peroxidase (HRP; Sigma-Aldrich)
Water-soluble crosslinker: sulfo-MBS (Pierce)
100 mM HEPES buffer, pH 7.4
PD-10 desalting columns (Sephadex G-25, Amersham-Pharmacia Biotech)
PBS/10 mM EDTA buffer, pH 8
100 μM STxB/Cys
1 M NH_4Cl
Superdex 200 (GE Bioscience)
13C4 monoclonal anti-STxB antibody (hybridoma from ATCC #CRL 1794)
Glycine buffer, pH 2.2

Nanosep 10-kDa microconcentration chambers for 0.1-0.5 ml (PallFiltron)
Gel filtration chromatography columns

Activate HRP

1. Reconstitute 10 mg of HRP in 500 μl HEPES buffer.
2. Dissolve 1 mg sulfo-MBS in 50 μl HEPES buffer. Immediately transfer to 450 μl HRP at 20 mg/ml in HEPES buffer.
3. React for 30 min at room temperature.
4. Equilibrate PD-10 desalting column with 25 ml PBS/10 mM EDTA buffer, pH 8.
5. Add the reaction volume onto the column. Elute in 0.5-ml aliquots using PBS/10 mM EDTA buffer, pH 8.
6. Monitor the protein elution using the Bio-Rad protein assay.

   The maleimide-activated HRP should elute in fractions six to nine.

7. If necessary, concentrate maleimide-activated HRP to 10 mg/ml, using the nanosep 10-kDa microconcentration chambers.

Couple HRP to STxB/Cys

8. Add 400 μl of 100 μM STxB/Cys in PBS/10 mM EDTA buffer, pH 8, to the maleimide-activated HRP. Incubate the reaction for 16 hr at room temperature in the dark.

   In the coupling reaction, the latter protein is in 5 molar excess over STxB/Cys.

9. Quench the reaction by adding 50 mM NH_4Cl.
10. Using HPLC chromatography, separate conjugated STxB/Cys-HRP from uncoupled STxB/Cys on a Superdex 200 gel filtration column. Remove uncoupled HRP using a 13C4 immunoaffinity column (monoclonal anti-STxB antibody). Elute this column with glycine buffer, pH 2.2 into tubes containing Tris·Cl, pH 7.4, and sodium hydroxide to neutralize the acidic eluate. Adjust pH after elution if necessary.
11. Characterize the coupling product by gel electrophoresis and western blotting.

    An example of whole-mount analysis using STxB/Cys-HRP in HeLa cells is represented in Figure 15.10.4.

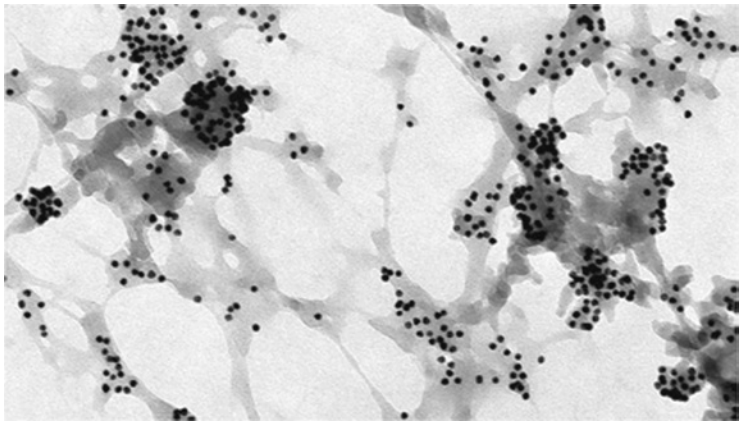


Figure 15.10.4 Whole-mount analysis of clathrin localization on early/recycling endosomes containing STxB-HRP. STxB-HRP-containing tubular membranes appear dark, due to the formation of the polymerization product. 10-nm gold particles indicate immunolocalization of clathrin.

BASIC PROTOCOL 2

STxB INTERNALIZATION ASSAY

This protocol describes a recent improvement to a previously described endocytosis assay for STxB (Mallard and Johannes, 2003).

A STxB variant, termed STxB-K3, which carries three lysine residues at the C-terminus, is biotinylated using cleavable NHS-SS-biotin reagent, yielding STxB-K3-SSbiot (see Support Protocol 4). This protein is bound on ice to plasma membrane Gb3, the cellular Shiga toxin receptor. After a shift to 37°C for variable times, cells are placed on ice again and treated, or not, with the membrane-impermeable reducing agent MESNA. After cell lysis, biotinylated STxB is revealed using ELISA. The ratio of the signal obtained on MESNA-treated cells over total signal in MESNA-untreated cells reflects the endocytosis of STxB at each time point. The endocytosis of transferrin is tested in parallel on the same cells as a marker for clathrin-dependent endocytosis.

Materials

HeLa cells
PBS++ (see recipe)
DMEM without serum, 37°C
Phospahte-buffered saline (PBS; *APPENDIX 2A*)
PBS/2mM EDTA
PBS++ supplemented with 5 mM glucose, room temperature and ice cold
STxB-K3, biotinylated (STxB-K3-SSbiot; see Support Protocol 4)
Transferrin, biotinylated (transferrin-SSbiot)
MESNA buffer (see recipe)
300 mM iodoacetamide (Sigma) in TNB buffer (see recipe for TNB buffer)
Blocking buffer (see recipe)
13C4 monoclonal antibody anti-STxB (hybridoma from ATCC #CRL 1794) *or* polyclonal anti-transferrin antibody (Les Makin)
Saturation buffer (see recipe)
Streptavidin-conjugated HRP (Roche)
Hydrogen peroxide solution (H_2O_2, Sigma)
O-Phenylenediamine tablets (OPD, Sigma)
Citrate buffer (see recipe)
6 N hydrogen sulfate (H_2SO_4)

15-cm petri dishes
37°C, 5% CO_2 incubator
PD-10 columns desalting (Sephadex G-25, Pharmacia Biotech)
Centrifuge
1.5-ml microcentrifuge tubes
96-well ELISA dishes
Microtiter plate reader

Additional reagents and equipment for cell counting (*UNIT 1.1*)

Prepare cells for assay

1. Plate 1×10^6 HeLa cells/dish 2 days before the experiment. Allow the cells to reach 80% confluency (5×10^6 cells/dish) on the day of the experiment.
2. Wash two dishes of cells two times with 10 ml of PBS++ per dish, then add 15 ml of pre-warmed DMEM without serum to each dish of cells. Incubate 1 hr in a 37°C, 5% CO_2 incubator.

   This is the serum starvation step.

3. Wash cells two times with 10 ml PBS and once with 8 ml PBS/2 mM EDTA. Incubate 10 min in 1 ml PBS/2 mM EDTA at 37°C.
4. Suspend detached cells in 10 ml of PBS++ supplemented with 5 mM glucose and count (*UNIT 1.1*).
5. Centrifuge 3×10^6 cells for 5 min at $300 \times g$, 4°C. Wash cells two times with 10 ml PBS++ supplemented with 5 mM glucose.

Label cell surfaces

6. Resuspend the cells in 300 µl ice-cold PBS++ supplemented with 5 mM glucose containing 0.5 µM STxB-K3-SSbiot and 200 nM transferrin-SSbiot. Incubate 30 min at 4°C. Mix cells gently every 5 min to prevent sedimentation.

   This is the cell surface binding step.

7. Dilute the cells in 12 ml PBS++ supplemented with 5 mM glucose and wash two times with 12 ml PBS++ supplemented with 5 mM glucose, centrifuging 5 min at $300 \times g$, 4°C.

   This step allows for the removal of unbound STxB-K3-SSbiot and unbound transferrin-SSbiot.

Perform endocytosis assay

8. Resuspend cells in 500 µl PBS++ supplemented with 5 mM glucose and transfer 50-µl aliquots to 1.5-ml microcentrifuge tubes, eight per time point.

   Typically, endocytosis is tested in duplicate at 0, 5, 10, 20, and 40 min. For a typical experiment (with or without MESNA buffer, control versus experimental condition, each point in duplicate), eight tubes per time point are used.

9. Incubate cells at 30°C or 37°C for the indicated times, then place tubes on ice.

   Endocytosis is slower at 30°C, which may allow for easier detection of differences between conditions.

10. To half of the tubes, add 50 µl MESNA buffer. Incubate 30 min on ice.

    MESNA will cleave the disulfide bond between the biotin group and STxB-K3 or transferrin. It will act on cell surface–exposed proteins but not on internalized proteins.

11. Inactivate MESNA by adding 100 µl of 300 mM ioadoacetamide in TNB buffer to the MESNA tubes. Incubate cells 30 min on ice.

12. Centrifuge cells 20 sec at 300 × *g*, 4°C. Discard the supernatant using a Pasteur pipet.

Detect by ELISA

13. Lyse cells in 400 µl blocking buffer.

14. Load the cell lysates in 96-well ELISA dishes, 200 µl in a 13C4 anti-STxB antibody–coated well and 200 µl in an anti-transferrin antibody–coated well. Incubate dishes overnight at 4°C.

15. Wash wells two times with 400 µl PBS, and once with 400 µl saturation buffer decanting each wash by inverting plates. Incubate 5 min at room temperature.

16. Wash again two times with 400 µl PBS.

17. Add 200 µl of 1:5000 (v/v) streptavidin-conjugated HRP diluted in saturation buffer, to each well, and incubate dishes 1 hr at room temperature in the dark.

18. Wash again, as described in step 15.

19. Add 10 µl of hydrogen peroxide solution to freshly dissolved OPD tablet in 25 ml citrate buffer, pH 5.5. Transfer 200 µl to each well.

20. Incubate until yellow color appears (~5 min), then stop the reaction by adding 50 µl of 6 N H_2SO_4.

21. Read the absorbance values at 490 nm on a microtiter plate reader.

Analyze data

22. To determine the percentage of internalized STxB and transferrin at each time point and in each experimental condition, divide the signal obtained on the +MESNA sample (internalized) by the signal obtained on the –MESNA sample (total cell associated).

A typical result is shown in Figure 15.10.5.

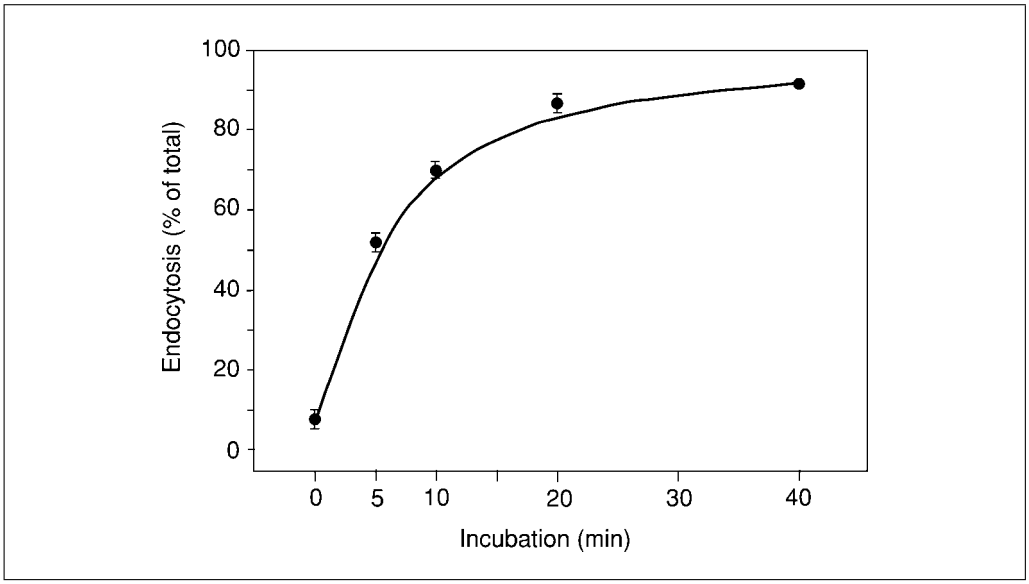


Figure 15.10.5 Internalization kinetics of STxB in HeLa cells at 30°C.

BIOTINYLATION OF STxB-K3

Proteins are typically labeled with biotin to tag them for later recovery or for detection with biotin-binding proteins, such as avidin or streptavidin. Some applications of this approach include identification and purification, measurement of ligand internalization, and temporal changes in protein distribution inside cells using light or electron microscopy. To measure the internalization kinetics of STxB, the authors use a biotinylated STxB variant termed STxB-K3, which carries three additional lysine residues at its C-terminus (Johannes et al., 2003).

Materials

STxB-K3 protein
PBS (*APPENDIX 2A*)
1 M carbonate buffer, pH 9.5
NHS-SS-biotin in DMSO
1 M NH_4Cl
Dialysis membrane (10-kDa)

1. Dialyze 0.5 ml of purified STxB-K3 protein (1 mg/ml) two times for 1 hr each time, against PBS buffer at 4°C using a 10-kDa membrane to remove traces of interfering buffer components that may have been introduced during purification.

   Avoid buffers containing primary amines (e.g., Tris or glycine) as they compete with the biotin-labeling reaction.

2. Add 18 µl of 1 M carbonate buffer, pH 9.5.

3. Add 60 µl of freshly prepared NHS-SS-biotin in DMSO (1 mg/ml) to the protein.

   Sulfo-NHS-SS-biotin is a thiol-cleavable amine-reactive biotinylation reagent that contains an extended spacer arm to reduce steric hindrances associated with avidin binding

4. Incubate 30 min on ice. Mix occasionally.

5. Terminate the reaction by adding 1 M NH_4Cl (50 mM final concentration), and keep the reaction 15 min on ice.

6. Remove unreacted biotin by dialysis three times for 1 hr each against PBS buffer at 4°C using a 10-kDa membrane.

   Alternatively, excess biotin may be removed by gel filtration chromatography using PD-10 columns. Collect at least ten fractions, each containing 0.5 ml.

7. Read the absorbance of the biotinylated protein by Bradford assay at 595 nm (A_{595}).

   If a gel filtration column is used, pool fractions that correspond to the peak of absorbance.

8. Store the biotinylated STxB-K3 protein for several years at –80°C.

9. Verify biotinylation by immunoblot analysis of electrotransferred protein using streptavidin-HRP (*UNIT 6.2*) or by MALDI-TOF.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acrylamide solution A

Add 1.135 g bisacrylamide to 100 ml of protogel solution (30% acrylamide/0.8% bisacrylamide); store up to 6 months at 4°C.

Acrylamide solution B

Add 0.138 g bisacrylamide to 100 ml of protogel solution (30% acrylamide/0.08% bisacrylamide); store up to 6 months at 4°C.

Anode buffer, 10×

242 g Tris
800 ml H_2O
Adjust to pH 8.9 with HC1
Add H_2O to 1 liter
Store up to 6 months at room temperature

ATP-regenerating system

10 mM HEPES buffer, pH 7.2
1 mM ATP
0.2 mM GTP
0.05 mM UTP (Bohringer)
5 mM creatine phosphate (Sigma)
15 μg/ml creatin kinase (Bohringer)
Store up to 6 months at −80°C

Blocking buffer

1% (v/v) Triton X-100
0.1% (w/v) SDS
0.2% (w/v) BSA
50 mM NaCl
1 mM EDTA
10 mM Tris·Cl, pH 7.4
Store up to 2 weeks at 4°C

Cathode buffer, 10×

121 g Tris
179.5 g Tricine
800 ml H_2O
50 ml 20% (w/v) SDS
Adjust to pH 8.25 with HC1
Store up to 6 months at room temperature

Citrate buffer

50 mM Na_2HPO_4
30 mM citric acid, pH 5
Store up to 6 months at 4°C

Deacetylation solution, 10×

50 mM sodium phosphate
25 mM EDTA
0.5 M hydroxylamine-HCl, pH 7.5
Prepare fresh

DMEM3+

Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with:
10% (w/v) fetal calf serum
0.01% (w/v) penicillin/streptomycin (GIBCO)

continued

5 mM pyruvate
4 mM glutamine
Store up to 1 month at 4°C.

HK buffer

90 mM KCl
50 mM HEPES, pH 7.1
Store up to 6 months at room temperature

ICT/DTT buffer

78 mM KCl
4 mM $MgCl_2$
8.4 mM $CaCl_2$
10 mM EGTA
50 mM HEPES/KOH, pH 7.2
1 mM DTT
Store up to 6 months at −20°C

MESNA buffer

100 mM 2-mercaptoethanesulfonic acid (MESNA; Sigma)
TNB buffer (see recipe)
Prepare fresh

PBS++

1× phosphate-buffered saline (PBS; *APPENDIX 2A*)
0.5 mM $CaCl_2$
1 mM $MgCl_2$
Store up to 6 months at 4°C

Protease inhibitor cocktail (PIC)

In DMSO, add:
1 mg/ml aprotinin
1 mg/ml leupeptin
1 mg/ml pepstatin
1 mg/ml antipaïn
1 M benzamidin
40 mg/ml PMSF
Store up to 6 months at −20°C

RIPA buffer

1× PBS (*APPENDIX 2A*)
1% (v/v) Nonidet P40 (NP-40)
0.5% (v/v) deoxycholic acid
0.5% (w/v) SDS
Store up to 6 months at 4°C

Saturation buffer

10 mM Tris·Cl (*APPENDIX 2A*)
50 mM EDTA
0.2% (w/v) BSA
0.1% (w/v) SDS
1% (v/v) Triton X-100, pH 7.4
Store up to 6 months at 4°C

Streptolysin O (SLO)

200 μg/ml streptolysin O in potassium acetate buffer (115 μM) containing:
1 mg/ml BSA
2 mM DTT
Store up to 5 years at –80°C

TNB buffer

50 mM Tris·Cl (*APPENDIX 2A*)
100 mM NaCl
0.2% (w/v) BSA, pH 8.60
Store up to 2 weeks at 4°C

Tris-tricine gel buffer

91 g Tris
800 ml H_2O
Adjust to pH 8.5 with HCl
Add 15 ml 20% (w/v) SDS
Bring up to 1 liter with H_2O
Store up to 6 months at room temperature

COMMENTARY

Background Information

Many studies on the molecular mechanisms of retrograde transport were done with Shiga toxin. This toxin and the related verotoxins (or Shiga-like toxins) form a family of structurally and functionally related proteins that are associated with disease in humans and animals. Shiga toxin is produced by *Shigella dysenteriae*, whereas verotoxins are produced by enterohemorrhagic *Escherichia coli* strains. These toxins are composed of two subunits, A and B, which associate in a noncovalent manner. The A-subunit is a ribosomal RNA *N*-glycosidase that inhibits protein biosynthesis by removing a specific adenine base from a highly conserved region of the 28S rRNA molecule. The receptor-binding nontoxic B-subunit is a homo-pentamer. In the case of Shiga toxin, the B-subunit (abbreviated STxB for Shiga toxin B-subunit) binds with high affinity to the glycosphingolipid globotriaosyl ceramide (Gb3 or CD77). In the absence of the A-subunit, the STxB conserves its pentameric structure, the capacity to bind Gb3, and the intracellular trafficking characteristics of the holotoxin.

Using morphological and biochemical tools based on STxB, it was shown that retrograde transport between early/recycling endosomes and the TGN involves two SNARE complexes around the heavy chain tSNAREs syntaxin 16 (Mallard et al., 2002) and syntaxin 5 (Tai et al., 2004), the GTPase Rab6a′ (Mallard et al., 2002), the tethering molecule Golgin 97 (Lu et al., 2004), and possibly also the conserved oligomeric Golgi (COG) tethering complex (Zolov and Lupashin, 2005). Retrograde sorting on early/recycling endosomes requires clathrin (Lauvrak et al., 2004; Saint-Pol et al., 2004), dynamin (Lauvrak et al., 2004), and the clathrin-binding protein epsinR (Saint-Pol et al., 2004). Furthermore, STxB association with membrane microdomains of the raft-type was correlated with efficient retrograde transport (Falguières et al., 2001). Other proteins such as the GARP complex in yeast (Conibear et al., 2003) or the retromer complex in yeast and mammals (Arighi et al., 2004; Seaman, 2004) were also involved in retrograde transport between endosomes and the TGN, but their implication in Shiga toxin transport remains to be shown. It should also be pointed out that the early/recycling endosomes-to-TGN pathway of Shiga toxin is different from the classical mannose-6-phosphate receptor recycling pathway between late endosomes and the TGN, as described by the Pfeffer group (Diaz and Pfeffer, 1998).

A critical question is whether the trafficking machinery that was identified for retrograde STxB transport at the early/recycling endosomes-TGN interface also controls retrograde transport of other exogenous and endogenous proteins. Based on the STxB-based retrograde trafficking assays, the protocols in this unit describe a generalized methodology

that allows addressing this question in a quantitative manner. Furthermore, the permeabilized cell assay that is described in this unit complements the intact cell approaches. In both cases, specific limitations exist, such as adaptation phenomena in intact cell approaches if one uses long treatments required for overexpression or RNAi, or permeabilization artifacts in the reconstitution assays. However, it is unlikely that the same limitations apply in each case, and it seems reasonable to assume that concomitant use of several approaches (single-cell and population-based intact cell techniques and reconstitution assays) allows a maximum of confidence in the conclusions that are reached as a result of each experimental series.

It should be kept in mind that retrograde transport efficiency of specific cargo molecules varies between cell types. A well-studied example is Shiga toxin. While it appears that retrograde transport is quantitative in cancer cell lines such as HeLa cells (Johannes et al., 1997; Mallard et al., 1998), other cells do not sort Shiga toxin to the retrograde route, as for example observed in human monocyte-derived macrophages and dendritic cells (Falguières et al., 2001), and these cells are totally resistant to the toxin (Falguières and Johannes, 2006). In the mouse dendritic cell line D1, Shiga toxin is partly targeted to late endosomes/lysosomes, and partly to the retrograde route, thus representing an intermediate situation (Haicheur et al., 2003).

Critical Parameters

For the sulfation assay, cells other than Hela cells can also be used, e.g., A431, Vero, and others. The general requirements are that cells should express Gb3, and STxB should be transported to the TGN.

For the sulfation peptide–coupling methodology, the availability of a recognition molecule (e.g., natural ligand, antibody) is an indispensable requirement, and it must be verified that such recognition does not alter the trafficking characteristics of the cellular cargo molecule (lipid or protein), which is under analysis. If the recognition molecule is itself the cargo that is to be analyzed (e.g., bacterial or plant toxins), it also needs to be verified that sulfation site tagging does not alter its activities. The nature of such tests may depend on the cargo molecule itself. In the case of protein toxins, cell intoxication is a convenient independent way of testing trafficking and activity.

As with any biochemical technique, the sulfation analysis is prone to experimental artifacts. These include, but are not limited to, modification of sulfotransferase activity itself and a possible relocalization of the enzyme. Sulfotransferase activity is tested by measuring the level of endogenous sulfation, and can thus be controlled for within some limitations. The relocalization of the enzyme is more difficult to assess, because relatively small changes could have major effects. It is therefore indispensable, as noted above, to combine several approaches. Indeed, the relocalization problem is unlikely to apply to the reconstitution assay, if one considers the short incubation time (30 min). Furthermore, analysis of cargo trafficking to the TGN/Golgi using immunofluorescence and/or electron microscopy techniques at the single-cell level yields valuable information on where the cargo protein is blocked in a given experimental situation, and at the same time, this indirectly controls for possible (and as yet hypothetical) sulfotransferase relocalization.

Data obtained with the sulfation assay are relative measures, i.e., sulfation efficiency in a given experimental condition, compared to sulfation efficiency under control conditions. Thus, it is necessary to verify that experimental conditions do not change the number of cell-associated cargo molecules, which would have a direct impact on the sulfation signal.

The sensitivity of the sulfation assay depends on several factors, such as expression levels of the cargo molecule (or its receptor), the stoichiometry of the sulfation site modification, the efficiency of retrograde transport, possibly the residence time in the TGN, and other factors. At this stage, it is impossible to propose an algorithm for predicting feasibility, which must be established experimentally.

The immunoprecipitation step in the sulfation protocol permits the concomitant analysis of retrograde transport of several cargo proteins at the same time on the same cells. On a practical note, ensure that the sensitivity of detection for each component is compatible with this approach.

The protein coupling protocol, as described in this unit for HRP, can also be applied to other proteins. The authors' experience suggests that any protein can be coupled, provided that it is soluble in biological buffers and that it does not aggregate. Successful coupling examples include bovine serum albumin, ovalbumin, and antigenic proteins from HIV and human cancers. The reaction conditions (stoichiometry of

SMBS over STxB/Cys, incubation times, etc.) need to be optimized in each case.

The STxB endocytosis assay, as described in this unit, yields more robust results than the previous version (Mallard and Johannes, 2003), and it is possible to analyze more conditions at the same time. Furthermore, several markers may be analyzed at the same time on the same cells, e.g., STxB and transferrin.

Troubleshooting

For the sulfation assay, only sulfate with high specific activity should be used (typically at least at 50 μCi/μl). As mentioned above, tests need to be performed so that chemical cross-linking does not change the activity of the retrograde transport ligands and receptors. It should also be kept in mind that some cell lines may not express given receptors (e.g., Gb3 expression is very heterogeneous between cell lines), and that overlap between Gb3 and GM1 expression varies significantly between cell lines as well. In its current format for STxB trafficking, the permeabilized cell assay is sensitive to reaction conditions (cytosol, cells in good shape, quality of SLO batch), in that even in control conditions, the sulfation signal is rapidly lost if the conditions are suboptimal. The endocytosis assay critically depends on the quality of the 13C4 antibody preparation.

Anticipated Results

The methods presented in this unit have been developed in the authors' group (Johannes et al., 1997; Mallard et al., 1998; Falguières et al., 2001; Mallard et al., 2002; Saint-Pol et al., 2004), but they are now also used by other laboratories (Lauvrak et al., 2004; Lu et al., 2004; Natarajan and Linstedt, 2004). Using STxB in the sulfation assay, robust signals can be detected on 1×10^5 cells, and sulfation becomes detectable ~10 to 20 min after uptake from the plasma membrane (Mallard et al., 1998). The exact kinetics differs between cell types. For sulfation analysis with CTxB or anti-GFP on GFP-MPR HeLa cells, 1×10^6 cells should be used. The anticipated results for the endocytosis assay are shown in Figure 15.10.5.

Time Considerations

Sulfation-site peptide and HRP coupling can be done in 2 to 3 days, including MALDI-TOF or immunoblot analysis of the coupling product. The sulfation assay on intact cells takes 3 days starting with establishing the cells in culture (day 1), performing the assay (day 2), and developing the autoradiogram (day 3). The endocytosis assay can be done in 3 days, starting with establishing the cells in culture (day 1), performing the assay (day 2), and finishing the ELISA test on day 3.

Acknowledgements

The authors would like to thank Danièle Tenza and Graça Raposo for the whole-mount figure, Wolfgang Faigle for mass spectrometry analysis, and Christophe Lamaze for critical reading of the manuscript. This work was supported by grants from the Ligue Nationale contre le Cancer, Association de Recherche Contre le Cancer (n°5177 and n°3105), Fondation de France, Action Concertée Incitative – Jeunes chercheurs (n°5233).

Literature Cited

Arighi, C.N., Hartnell, L.M., Aguilar, R.C., Haft, C.R., and Bonifacino, J.S. 2004. Role of the mammalian retromer in sorting of the cation-independent mannose 6-phosphate receptor. *J. Cell Biol.* 165:123-133.

Conibear, E., Cleck, J.N., and Stevens, T.H. 2003. Vps51p mediates the association of the GARP (Vps52/53/54) complex with the late Golgi t-SNARE Tlg1p. *Mol. Biol. Cell* 14:1610-1623.

Diaz, E. and Pfeffer, S.R. 1998. TIP47: A cargo selection device for mannose 6-phosphate receptor trafficking. *Cell* 93:433-443.

Falguières, T. and Johannes, L. 2006. Shiga toxin B-subunit binds to the chaperone BiP and the nucleolar protein B23. *Biol. Cell.* 98:125-134.

Falguières, T., Mallard, F., Baron, C.L., Hanau, D., Lingwood, C., Goud, B., Salamero, J., and Johannes, L. 2001. Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent resistant membranes. *Mol. Biol. Cell* 12:2453-2468.

Fujinaga, Y., Wolf, A.A., Rodighiero, C., Wheeler, H., Tsai, B., Allen, L., Jobling, M.G., Rapoport, T., Holmes, R.K., and Lencer, W.I. 2003. Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to the endoplasmic reticulum. *Mol. Biol. Cell* 14:4783-4793.

Ghosh, R.N., Mallet, W.G., Soe, T.T., McGraw, T.E., and Maxfield, F.R. 1998. An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. *J. Cell Biol.* 142:923-936.

Haicheur, N., Benchetrit, F., Amessou, M., Leclerc, C., Falguières, T., Fayolle, C., Bismuth, E., Fridman, W.H., Johannes, L., and Tartour, E. 2003. The B-subunit of Shiga toxin coupled to full-size protein elicits humoral and cellular immune responses associated with a TH1 dominant polarization. *Int. Immunol.* 15:1161-1171.

Johannes, L. and Goud, B. 1998. Surfing on a retrograde wave: How does Shiga toxin reach the endoplasmic reticulum? *Trends Cell Biol.* 8:158-162.

Johannes, L., Pezo, V., Mallard, F., Tenza, D., Wiltz, A., Saint-Pol, A., Helft, J., Antony, C., and Benaroch, P. 2003. Effects of HIV-1 Nef on retrograde transport from the plasma membrane to the endoplasmic reticulum. *Traffic* 4:323-332.

Johannes, L., Tenza, D., Antony, C., and Goud, B. 1997. Retrograde transport of KDEL-bearing B-fragment of Shiga toxin. *J. Biol. Chem.* 272:19554-19561.

Lauvrak, S.U., Torgersen, M.L., and Sandvig, K. 2004. Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin. *J. Cell Sci.* 117:2321-2331.

Lu, L., Tai, G., and Hong, W. 2004. Autoantigen Golgin-97, an effector of Arl1 GTPase, participates in traffic from the endosome to the trans-golgi network. *Mol. Biol. Cell* 15:4426-4443.

Mallard, F. and Johannes, L. 2003. Shiga toxin B-subunit as a tool to study retrograde transport. *Methods Mol. Med.* 73:209-220.

Mallard, F., Tang, B.L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W.J., Goud, B., and Johannes, L. 2002. Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. *J. Cell Biol.* 156:653-664.

Mallard, F., Tenza, D., Antony, C., Salamero, J., Goud, B., and Johannes, L. 1998. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of Shiga toxin B-fragment transport. *J. Cell Biol.* 143:973-990.

Natarajan, R. and Linstedt, A.D. 2004. A cycling cis-Golgi protein mediates endosome-to-Golgi traffic. *Mol. Biol. Cell* 15:4798-4806.

Rapak, A., Falnes, P.O., and Olsnes, S. 1997. Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. *Proc. Natl. Acad. Sci. U.S.A.* 94:3783-3788.

Saint-Pol, A., Yélamos, B., Amessou, M., Mills, I., Dugast, M., Tenza, D., Schu, P., Antony, C., McMahon, H.T., Lamaze, C., and Johannes, L. 2004. Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. *Dev. Cell* 6:525-538.

Sandvig, K., Garred, O., Prydz, K., Kozlov, J.V., Hansen, S.H., and van Deurs, B. 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* 358:510-512.

Seaman, M.N. 2004. Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. *J. Cell Biol.* 165:111-122.

Stoorvogel, W., Oorschot, V., and Geuze, H.J. 1996. A novel class of clathrin-coated vesicles budding from endosomes. *J. Cell Biol.* 132:21-33.

Tai, G., Lu, L., Wang, T.L., Tang, B.L., Goud, B., Johannes, L., and Hong, W. 2004. Participation of syntaxin 5/Ykt6/GS28/GS15 SNARE complex in transport from the early/recycling endosome to the TGN. *Mol. Biol. Cell* 15:4011-4022.

Waguri, S., Dewitte, F., Le Borgne, R., Rouille, Y., Uchiyama, Y., Dubremetz, J.F., and Hoflack, B. 2003. Visualization of TGN to endosome trafficking through fluorescently labeled MPR and AP-1 in living cells. *Mol. Biol. Cell* 14:142-155.

Zolov, S.N. and Lupashin, V.V. 2005. Cog3p depletion blocks vesicle-mediated Golgi retrograde trafficking in HeLa cells. *J. Cell Biol.* 168:747-759.

Contributed by Mohamed Amessou, Vincent Popoff, Belèn Yelamos, Agnès Saint-Pol, and Ludger Johannes
Institut Curie
Paris, France

Assays for Regulated Exocytosis of Mast Cell Granules

The mast cell has long been known as an important effector cell in allergy and asthma (Galli et al., 2005a). These cells, however, are also becoming increasingly recognized for their ability to participate in both innate and adaptive immune responses (Galli et al., 2005b). A key feature of mast cells is that they contain in their cytoplasm numerous (up to 1000) secretory granules that hold a vast array of inflammatory mediators and biologically active substances such as histamine, proteoglycans, proteases, and lysosomal hydrolases. Upon appropriate stimulation, mast cells release most of their granular content into the surrounding environment. This is made possible by their unique ability to activate inter-granular fusion events in addition to granule-to-plasma membrane fusion (compound exocytosis; Röhlich et al., 1971). This process of regulated exocytosis is therefore often also termed degranulation. Degranulation can be triggered by allergen-induced aggregation of allergen-specific IgE bound to high-affinity IgE receptors (FcεRI) expressed on the surface of the mast cell (Galli et al., 2005b). Degranulation is also triggered by non-IgE-mediated physiological and pathophysiological stimuli through a wide variety of receptors, which couple to distinct early signaling proteins such as nonreceptor tyrosine kinases or heterotrimeric GTP-binding proteins. Moreover, degranulation can also be triggered by various pharmacological agents (such as phorbol esters in combination with calcium ionophores) that bypass early receptor-mediated signals. This unit describes protocols that can be used to assay regulated exocytosis in mast cells both in vitro and in vivo. For the purpose of clarity, the authors focus on IgE-mediated stimulation protocols for rodent cells. However, in principle, all methods are adaptable to the use of other stimulants and human cells.

The assays described herein can be classified as follows:

I. Detection of released soluble mediators stored in cytoplasmic granules using assays based on in vitro colorimetric detection (β-hexosaminidase; Basic Protocol 1), radiolabel detection (serotonin; Alternate Protocol 1), or antibody detection (histamine; Alternate Protocol 2). These assays are frequently used in many laboratories specialized in the analysis of mast cell exocytosis.

II. Detection of the exposure of phosphatidylserine at the cell surface as a marker of degranulation (single cell assay; Basic Protocol 2).

III. In vivo assays for mast cell degranulation in murine models—plasma histamine (Basic Protocol 3) and body-temperature measurements (Alternate Protocol 3).

DETECTION OF SOLUBLE MEDIATORS

For ease of data comparison and to avoid the variability introduced by differing cell numbers or by differences in the absolute amount of granule content between various batches of cells, the extent of degranulation for most soluble mediators is usually expressed as the percent release of total content. Spontaneous release (that detected in the absence of cell stimulation) is usually subtracted to report the (net) stimulated degranulation. Total degranulation should be reported in situations where differences in experimental conditions or in the genetic makeup of the cells might not allow direct comparison or could differentially alter the spontaneous release. Regardless, spontaneous release (%) should be always be reported, because it provides an assessment of the resting state or viability of the mast cells. Therefore, for every experiment in which soluble mediators

Contributed by Ulrich Blank and Juan Rivera
Current Protocols in Cell Biology (2006) 15.11.1-15.11.18

are measured, the design of an experimental protocol should include determination of the following parameters: (1) released soluble mediator content in test supernatants after cell stimulation; (2) soluble mediator content in the test supernatants in the absence of cell stimulation; and (3) soluble mediator content both in the test supernatants and cell lysates as a measurement of total cellular content. Percent net specific release is then calculated according to the formula:

$$\%\text{Net specific release} = \frac{\text{release}_{\text{stimulated}} - \text{release}_{\text{spontaneous}}}{\text{content}_{\text{total}} - \text{release}_{\text{spontaneous}}} \times 100$$

Equation 15.11.1

BASIC PROTOCOL 1

Measurement of Histamine Release

Histamine (β-imidazole-ethylamine) is produced by the decarboxylation of histidine by the enzyme l-histidine decarboxylase. Mast cells and basophils represent the most important pool of cells capable of storing and promptly releasing histamine from their granules upon stimulation (MacGlashan, 2003). In vitro measurement of the histamine released from mast cells is therefore an excellent correlate of granule mediator release. Histamine exerts potent effects on various target tissues and is involved in neurotransmission, various brain functions, secretion of pituitary hormones, regulation of gastrointestinal and circulatory functions, and inflammatory reactions (Dy and Schneider, 2004). Its effects on smooth muscle, which are profound during anaphylactic shock, are well known. Histamine receptors (H1, H2, H3, H4) have been identified on a wide variety of cells and tissue with distribution in the immune system as well as in the central nervous system (MacGlashan, 2003; Dy and Schneider, 2004). Histamine is actively released from mast cells upon allergen interaction with cell-bound allergen-specific IgE or when cells are stimulated by various other physiological and pharmacological agents. Several methods have been developed for the in vitro measurement of histamine. Among these, the assay of histamine by fluorometry is an accurate and sensitive method for detection of this biologically active molecule (Siraganian, 1974). This method employs the coupling of histamine with *o*-phthalaldehyde at high alkaline pH to form a fluorescent product that can be easily detected by fluorometric means. Although sensitive, this method requires specialized instrumentation that is not available to all laboratories, and thus will not be detailed here.

A method of choice for most laboratories is the use of a competitive enzyme immunoassay. This assay is based on competition between the histamine in the sample to be assayed and an enzyme conjugate, histamine–alkaline phosphatase, used as a tracer for binding to a limited amount of antibody that recognizes acylated histamine. This assay can be used for measurement of histamine in plasma, urine, cell supernatants, cell lysates, and other liquid or solid biological samples. Because of its commercial availability (manufactured by Immuno Biological Laboratories, Hamburg, Germany, and distributed by Research Diagnostics Inc. in the United States), this assay has become the most commonly used assay for quantitative measurement of histamine release. The detailed procedure is provided with the assay kit from the manufacturer, and is briefly outlined in Basic Protocol 3. Several important points must be considered:

1. The monoamine histamine is too small to completely occupy the binding site on the antibody, and the antibody used in this assay was raised against a modified (acylated) histamine. Thus the histamine in the sample must be acylated in the same manner as the conjugated histamine. An effective and complete acylation reaction is key to obtaining reproducible results from experiments conducted on different days. One key to consistency in acylation of histamine is to dissolve or thaw the acylation reagent

immediately before use and keep aliquots (for one reaction) stored at −20°C. Do not freeze/thaw repeatedly.

2. The assay must be conducted within the linear range of the standards; thus, samples may require dilution to be within the range of concentrations of the standard. It is recommended that multiple dilutions and replicates be run within an experiment to determine what dilution(s) of the sample fall within the linear range of the assay.

3. The wash instructions should be followed explicitly. Two key points in obtaining reproducible results are to avoid the drying of the wells during the wash steps and to effectively remove all unbound components by filling the wells to the rim.

Measurement of Radiolabeled Serotonin Release

ALTERNATE PROTOCOL 1

Serotonin (5-hydroxytryptamine or 5-HT) is a major component of rodent mast cell granules. Methods to measure its release take advantage of the rapid incorporation of radiolabeled serotonin (5-[1,2-^{3}H(N)]-hydroxytryptamine binoxalate) in a selective manner into mast cell granules. This method can be used for cell lines, cultured bone marrow–derived mast cells (Support Protocol), or ex vivo–derived mast cells (such as peritoneal mast cells). It requires preincubation with the radiolabeled 5-HT prior to cell stimulation. This is a quantitative method, but one must keep in mind that it does not measure the total amount of serotonin in granules. It measures the secreted fraction of exogenously incorporated serotonin and is thus reported as the percent of total incorporated 5-HT. The procedure described is for nonadherent cells, but can be adapted as an adherent cell assay (using RBL-2H3 cells), as incorporation of 5-HT, cell stimulation, supernatant recovery, and determination of total cellular 5-HT can also be done on adherent cells. It can also be adapted to a 96-well microwell format.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Materials

RBL-2H3 (ATCC no. CRL-2256) *or* bone-marrow mast cells (BMMC; see Support Protocol), in late log phase (3 to 4 days after feeding)
Tyrode's buffer (see recipe)
5-[1,2-^{3}H(N)]-hydroxytryptamine binoxalate (sp. act., 28.5 Ci/mmol; ViTrax Radiochemicals; *http://www.vitrax.com/*)
DNP-specific murine IgE (Sigma-Aldrich) *or* TNP-specific murine IgE (BD PharMingen)
Antigen: dinitrophenol conjugated to human serum albumin (DNP_{36}-HSA; Sigma-Aldrich), trinitrophenol conjugated to ovalbumin (TNP-OVA; Biosearch Technologies), or trinitrophenol conjugated to bovine serum albumin (TNP-BSA; Biosearch Technologies)
Tyrode's buffer–E (see recipe)
1% (v/v) Triton X-100
Scintillation fluid, water soluble

Centrifuge
Scintillation vials

Harvest and wash cells

1. Harvest 5×10^6 cells by centrifuging 10 min at $400 \times g$, room temperature, and removing the supernatant.

   Centrifugation force varies depending on cell type; for bone marrow mast cells (BMMC), 400 × g should be used.

2. Wash the cell pellet once by adding 25 ml prewarmed (37°C) Tyrode's buffer, centrifuging again 10 min at 400 × *g*, room temperature, and removing the supernatant.

Treat with radiolabeled serotonin and IgE

3. Gently resuspend cells at 0.5×10^6 cells/ml in 10 ml prewarmed (37°C) Tyrode's buffer containing 1 μCi/ml 5-[1,2-^{3}H(N)]-hydroxytryptamine binoxalate and 1 μg/ml DNP-specific murine IgE.

4. Incubate cells for 1 hr at 37°C with occasional mixing.

5. Centrifuge 10 min at 400 × *g*, room temperature, and remove the supernatant. Wash cells twice, each time by adding 10 ml Tyrode's buffer, centrifuging again as before, and removing the supernatant.

   The washings remove unincorporated 5-[1,2-^{3}H(N)]-hydroxytryptamine binoxalate and unbound DNP-specific IgE.

6. Gently resuspend cells to 0.5×10^7 cells/ml in 1.0 ml Tyrode's buffer (prewarmed to 37°C).

Perform degranulation reaction in presence of antigen

7. Prepare ten 1.5-ml microcentrifuge tubes containing duplicate doses of antigen (e.g., 0, 0.1., 10, and 100 ng/ml antigen) in 0.4 ml of 37°C Tyrode's buffer (with the 0 mg/ml tubes containing only the 0.4 ml of prewarmed Tyrode's buffer). To each tube, add a 0.1-ml aliquot of the mast cell suspension prepared in step 6.

   Antigen concentrations can be varied to determine suboptimal, optimal, or supraoptimal thresholds for degranulation; the concentrations depend on the molar ratio of hapten to the molecule used for conjugation (e.g., HSA). For DNP_{36} -HSA, the optimal dose for degranulation is in the range of 10 to 30 ng/ml for BMMC, peritoneal, and RBL 2H3 cells. Tubes without antigen are used to determine spontaneous release.

   The subscript 36 in DNP_{36}-HSA represents the average number of DNPs (dinitrophenyl) conjugated per HSA (human serum albumin) molecule.

8. Incubate cells at 37°C for 30 to 60 min.

   Degranulation of BMMC and peritoneal mast cells occurs more rapidly than RBL-2H3 cells and is complete in <30 min. RBL-2H3 cells require longer incubation times (45 min). Kinetic studies can be done on all cell types with detectable stimulated release as early as 1 min post-stimulation for BMMC and peritoneal mast cells.

9. To stop reaction, add 0.3 ml ice-cold Tyrode's buffer–E to each tube and place tubes on ice.

Collect cells and supernatant

10. Microcentrifuge cells 10 min at 400 × *g*, 4°C.

11. Remove 0.25 ml of the supernatant from the top of each tube and place on ice.

    The supernatants will contain radiolabeled 5-HT, released spontaneously or as a consequence of cell stimulation. These will be counted at step 15 to determine spontaneous release (blanks) or stimulated release (IgE/antigen-treated cells). It is possible to centrifuge the aliquots of supernatant 5 min at 1000 × g, 4°C, to ensure removal of any cells not pelleted in the initial centrifugation. This is usually unnecessary if tubes are removed gently from the centrifuge and the supernatant to be recovered is removed immediately.

12. Carefully aspirate remaining supernatant (avoiding the pellet) and discard as radioactive waste.

13. Add 0.25 ml of 1% Triton X-100 to the cell pellet and incubate for 15 min on ice to solubilize cells.

14. Microcentrifuge Triton X-100 solubilized cells 15 min at 12,000 × *g*, 4°C.

Determine total incorporated 5-HT, spontaneous release, and stimulated release

15. Take duplicate or triplicate 0.05-ml aliquots from each tube, transfer to scintillation vials each containing 5 ml scintillation fluid, and count on scintillation counter.

16. Calculate total incorporated 5-HT ($content_{total}$), spontaneous release ($release_{spontaneous}$), and stimulated release ($release_{stimulated}$).

    Total incorporated 5-HT is the sum of the cpm in supernatants and cell pellets.

    Spontaneous release is the cpm found in the supernatant of nonstimulated (no antigen) cells.

    Stimulated release is the cpm found in the supernatant of cells treated with antigen.

17. Calculate the net percent specific (stimulated) release using Equation 15.11.1

Measurement of β-Hexosaminidase Release

ALTERNATE PROTOCOL 2

β-hexosaminidase (EC 3.2.1.52) is a lysosomal enzyme that hydrolyzes terminal non-reducing *N*-acetyl-D-hexosamine residues in *N*-acetyl-β-D-hexosaminides (Aronson and Kuranda, 1989). It acts on glucosides, galactosides, and several oligosaccharides. Mast cell granules contain large quantities of this enzyme due to the lysosomal nature of their granules, which are also called secretory lysosomes. This results from the close connection between the endocytic and exocytic pathway in secretory cells of hematopoietic origin (Andrews, 2000). β-hexosaminidase release after stimulation correlates well with the release of histamine and can thus be used to measure degranulation (Schwartz et al., 1979). In comparison to direct or indirect measurements of released histamine or serotonin, this assay is relatively inexpensive, requires no specialized equipment, is highly reproducible, and does not require use of radioactive tracers. It has therefore become the method of choice in many applications. However, due to the ubiquitous nature of lysosomal hydrolases, it is not suitable for accurate measurement of mast cell/basophil exocytosis in a mixture of cells, e.g., in the measurement of degranulation of blood basophils, where measurement of histamine is the method of choice.

The assay exploits the hydrolytic property of β-hexosaminidase and measures catalysis of the following reaction:

$$p\text{-nitrophenyl acetyl-D glucosamine (pNAG)} + H_2O \xrightarrow{\text{enzyme}} p\text{-nitrophenol} + .$$

$$N\text{-acetyl-D-glucosamine}$$

Equation 15.11.2

The amount of *p*-nitrophenol formed is directly proportional to the amount of β-hexosaminidase in the supernatant or cell lysates, provided that the assay is performed in its linear range. This can be easily determined by assaying serial dilutions of the supernatant or cell lysates using the conditions detailed below. *p*-Nitrophenol absorbs light at 410 nm

Materials

RBL-2H3 cells (ATCC no. CRL-2256) *or* bone-marrow mast cells (BMMC; see Support Protocol), in late log phase (3 to 4 days after feeding)
Culture medium for RBL-2H3 cells (see recipe) *or* culture medium for BMMC (see recipe; if BMMC are used)
DNP-specific murine IgE (Sigma-Aldrich)
Tyrode's buffer (see recipe), room temperature and 37°C

Antigen: dinitrophenol conjugated to human serum albumin (DNP_{36}-HSA; Sigma-Aldrich)
0.5% (v/v) Triton X-100
pNAG substrate (see recipe)
Carbonate buffer (see recipe)
Culture medium for RBL-2H3 cells (see recipe) without IL-3

Flat-bottom 96-well microtiter plates
Refrigerated centrifuge with microtiter plate carrier
Microtiter plate reader

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

For RBL-2H3 cells

1a. Seed 200 µl of RBL-2H3 cells at 2.5×10^5 cells/ml in flat-bottom 96-well plates in culture medium for RBL-2H3 cells. Add IgE-anti-DNP for a final concentration of 0.5 µg/ml. Incubate for 1 hr (overnight incubation is also possible).

This protocol is optimized for the RBL-2H3 cell line and stimulation through the IgE receptor following sensitization with anti-DNP-IgE and triggering with specific antigen (DNP-HSA). Use of other mast cell lines or BMMC requires some variations in cell number and stimulation conditions that should be determined for each cell type prior to any specific experiment.

In this protocol, a commercially available DNP-specific IgE (Sigma-Aldrich) is used. However, various clones producing DNP-specific IgE exist and are described in the literature (Kitaura et al., 2005). IgE derived from these clones may be obtained from their originators and may also be used in this protocol.

2a. Centrifuge cells 5 min at 300 × g, room temperature, in a centrifuge with a microtiter plate carrier. Remove the medium from all wells.

3a. Add 200 µl/well of room temperature Tyrode's buffer and centrifuge 5 min at 300 × g, room temperature. Remove the supernatant from all wells. Repeat this wash a second time to remove unbound IgE.

4a. Add 100 µl/well of Tyrode's buffer prewarmed to 37°C and containing appropriate concentration of antigen (DNP_{36}-HSA, to wells for determination of total content) or solvent (to wells for determination of spontaneous release). Incubate 45 min at 37°C.

Maximal degranulation is usually achieved at antigen concentrations ranging from 10 to 30 ng/ml when DNP_{36}-HSA is used. Conditions for optimal degranulation may differ if the antigen is more or less haptenated.

The subscript 36 in DNP_{36}-HSA represents the average number of DNPs (dinitrophenyl) conjugated per HSA (human serum albumin) molecule.

5a. Stop the reaction by putting the plates on ice.

6a. To determine total cellular content, add 5 µl of 10% Triton X-100 (final concentration 0.5%) to the wells that were set up for this purpose. Incubate 5 min at room temperature, occasionally pipetting up and down to lyse cells.

7a. Centrifuge plate with cells 10 min at 300 × g, 4°C.

8a. Slightly incline 96-well plate and, using a pipettor and 100-µl pipet tip, transfer 50 to 70 µl of supernatant (taking care to not transfer any cells) or lysate to a 96-well plate for storage at −20°C or for immediate processing and measurement of β-hexosaminidase content (step 9a).

9a. In a well of a flat-bottom 96-well plate add 25 μl of supernatant (test, spontaneous, or total) to 50 μl of pNAG substrate. Cover and incubate 90 min in a 37°C incubator.

To verify that the assay is in the linear range, two-fold dilutions of total content can be made and tested in parallel.

10a. Stop the reaction by adding 150 μl carbonate buffer.

11a. Read the absorbance in a microtiter plate reader at 405 nm. To automatically subtract background noise, use a dual-wavelength setting of 405 nm and 630 nm.

The color is stable for a few hours at room temperature. Developed plates can also be stored at −20°C if the measurement cannot be done until a later time.

12a. Calculate results according to Equation 15.11.1.

For BMMC

1b. Harvest 0.5×10^7 BMMC (per sample) by centrifuging 10 min at 400 × g, room temperature.

2b. Resuspend cells in 0.5 ml culture medium for RBL-2H3 cells (omit IL-3) containing 0.5 μg/ml DNP-specific IgE and incubate 3 hr at 37°C.

3b. Centrifuge 10 min at 400 × *g*, room temperature, and remove supernatant. Wash cells twice, each time by adding 1 ml of room temperature Tyrode's buffer, centrifuging again as before, and removing the supernatant. Resuspend at a concentration of 2×10^6 cells/ml in prewarmed (37°C) Tyrode's buffer and incubate 5 to 10 min at 37°C.

4b. Distribute 250-μl aliquots of the cell suspension into 1-ml microcentrifuge tubes containing the appropriate concentration of stimulant (antigen, DNP-HSA) or solvent as control.

Refer to Alternate Protocol 1, step 7, for appropriate concentration ranges. Prepare concentration points in duplicate; also prepare duplicate 250-μl aliquots of Tyrode's buffer alone as no-antigen controls.

5b. Incubate for 15 to 30 min at 37°C.

6b. Stop the reaction by placing the tubes on ice for 5 min. Centrifuge 10 min at 400 × *g*, 4°C.

7b. Transfer supernatants to microcentrifuge tubes and place on ice while incubating remaining pelleted cells with 500 μl of 0.5% Triton X-100 to lyse the cells (for determination of total content).

8b. After 15 min on ice, transfer cell lysates to microcentrifuge tubes and microcentrifuge cell lysates and supernatants 1 min at 12,000 × *g*, 4°C.

9b. Transfer 200 μl of each supernatant and lysate to a new microcentrifuge tube.

Samples can be snap frozen on dry ice and stored at −20°C if they cannot be processed immediately.

10b. Transfer 20 μl of each sample to a 96-well plate and add 20 μl of 1 mM pNAG substrate. Incubate for 1 hr at 37°C.

11b. Add 200 μl carbonate buffer to stop the reaction.

12b. Read the absorbance at 405 nm in a microtiter plate reader. To automatically subtract background noise, use a dual-wavelength setting of 405 nm and 630 nm.

13b. Calculate results according to Equation 15.11.1.

MEASUREMENT OF REGULATED EXOCYTOSIS BY ANNEXIN V BINDING

Eukaryotic cells maintain an asymmetric distribution of lipids across the bilayer plasma membrane (Bretscher, 1972), and the phospholipid phosphatidylserine (PS) is found almost exclusively in the inner leaflet of the plasma membrane. Under certain physiological conditions, notably apoptosis, this asymmetry is lost and PS is translocated from the inner to the outer leaflet of the plasma membrane. Stimulation of mast cells (cell lines as well as primary mouse and human mast cells) also leads to the externalization of PS at the plasma membrane in a manner that is highly correlated with inflammatory mediator release (Martin et al., 2000). While PS exposure during apoptosis is irreversible, PS externalization during mast cell degranulation is reversible and thus not related to physiological cell death. Quantitative determination of PS at the plasma membrane represents, therefore, a convenient means of evaluating degranulation. It can be measured by annexin V binding using flow cytometry. Annexin V is a 35-kDa protein that specifically binds PS in a Ca^{2+}-dependent manner. It can be conjugated to the typical fluorophores used in cytofluorometry. As flow cytometry determines surface labeling of individual cells, this assay allows measurement of degranulation activity in a single mast cell, which is not possible in the assays measuring release of soluble mediators.

Materials

RBL-2H3 (ATCC no. CRL-2256) *or* bone-marrow mast cells (BMMC; see Support Protocol), in late log phase (3 to 4 days after feeding)
Culture medium for RBL-2H3 cells (see recipe) *or* culture medium for BMMC (see recipe; if BMMC are used)
1 M HEPES, pH 7.3
DNP- *or* TNP-specific murine IgE (Sigma-Aldrich)
Tyrode's buffer (see recipe)
Antigen: DNP-HSA *or* TNP-HSA (Sigma-Aldrich)
10× annexin V binding buffer (see recipe), ice cold
Biotinylated annexin V (Invitrogen)
0.1 mg/ml streptavidin-phycoerythrin (streptavidin-PE; Invitrogen) or streptavidin-allophycocyanin (streptavidin-APC; Invitrogen)
50-ml conical centrifuge tubes
Platform shaker
12 × 15–mm round bottom FACS analysis test tubes (Becton Dickinson)
Fluorescence activated cell sorter (FACS; Becton Dickinson)

Prepare cells

1. Collect required amounts of mast cells (~1×10^6 cells per final assay point) and wash them once in the appropriate culture medium.

   Adherent mast cells (e.g., RBL-2H3) are trypsinized before collection. Trypsinization may affect certain biological properties of cells, or eventually, also activate them. Therefore, it is advisable to check whether this procedure affects established stimulation parameters. In the case of IgE-dependent stimulation, it is known that trypsin does not affect the binding capacity of the FcεRI receptor. However, IgE is affected by trypsin, and, thus, cells have to be IgE sensitized after trypsinization.

2. In a 50-ml tube, resuspend cells in the appropriate culture medium, to a final density of 1×10^6 cells/ml.

3. Add IgE-anti-DNP to a final concentration of 0.5 μg/ml.

4. Incubate for 1 hr at room temperature with continuous shaking on a platform shaker. Take care that cells do not settle.

5. Centrifuge cells 5 min at 300 × *g*, room temperature, and remove the supernatant.

Wash cells

6. Wash cells twice, each time by adding 20 ml Tyrode's buffer, centrifuging 5 min at 300 × *g*, room temperature, and removing the supernatant.

   These washings are needed to remove unbound IgE.

7. Resuspend cells in Tyrode's buffer at 1×10^6 cells/ml. Transfer 1 ml per test sample into 12 × 15–mm round bottom FACS analysis tubes. Incubate the cells 5 min at 37°C.

Stimulate cells

8. Stimulate the cells by adding the appropriate concentration of antigen (DNP-HSA or TNP-HSA) or solvent.

   The solvent controls are for determination of PS exposure in unstimulated cells.

9. Stop the reaction by adding 3 ml of ice-cold 1× annexin V binding buffer and put tubes on ice.

10. Centrifuge cells 5 min at 300 × *g*, 4°C, and remove the supernatant.

11. Wash cells twice, each time by adding 3 ml of ice-cold 1× annexin V binding buffer, centrifuging again as before, and discarding the supernatant.

Treat with biotinylated annexin V

12. Resuspend cell pellet in 50 µl of 1× annexin V binding buffer containing 2 µg/ml biotinylated annexin V. Incubate 30 to 45 min on ice.

13. Centrifuge cells 5 min at 300 × *g*, 4°C, and discard the supernatant.

14. Wash the cells twice, each time by adding 3 ml of ice-cold 1× annexin V binding buffer, centrifuging again as before, and discarding the supernatant.

Detect with streptavidin-conjugated reagent and analyze by flow cytometry

15. Resuspend cell pellet in 50 µl of a 1:200 dilution of 0.1 mg/ml streptavidin-PE or streptavidin-APC stock in 1 × annexin V binding buffer. Incubate 30 min on ice

    Use the concentration of streptavidin conjugate recommended by the supplier.

    For the purpose of higher sensitivity, an indirect staining procedure using a PE- or APC-labeled secondary reagent is preferred over direct staining. However, if after preliminary determination staining with annexin-PE or annexin-APC suffices, direct staining with annexin-V-PE or annexin-V-APC can be performed.

16. Centrifuge cells 5 min at 300 × *g*, 4°C, and remove the supernatant.

17. Wash cells twice, each time by adding with 3 ml of ice-cold 1× annexin V binding buffer, centrifuging again as before, and removing the supernatant.

    It is advisable that cells also be treated with 10 µg/ml propidium iodide (Sigma-Aldrich) when using streptavidin-APC or with 0.01 µM Sytox Green nucleic acid stain (Invitrogen) when using streptavidin-PE before the last wash step, to distinguish dead cells that expose PS from degranulating PS-exposing cells. This can be done by adding 500 µl of annexin V binding buffer containing propidium iodide (10 µg/ml) or Sytox Green (0.01 µM) for 5 min at 4°C before the two washes described in this step.

18. Resuspend stained cell pellets in 500 µl of ice cold 1× annexin V binding buffer. Keep all tubes on ice until analyzed by flow cytometry.

19. Determine the mean fluorescence intensity (MFI) of live cells and calculate the relative expression levels as the ratio of MFI of annexin V staining between unstimulated and stimulated cells.

SUPPORT PROTOCOL

BONE MARROW EXTRACTION FOR PREPARATION OF BONE MARROW-DERIVED CULTURED MAST CELL (BMMC)

Materials

Mice, 8- to 12-weeks old
70% ethanol
Culture medium for BMMC (see recipe)
Culture medium for BMMC (see recipe) without IL-3 (but containing SCF)
Culture medium for BMMC (see recipe) without IL-3 or SCF

Dissection instruments
3-cc syringe with 30-G needle
Centrifuge
75-cm^2 tissue culture flasks

Additional reagents and equipment for euthanasia of mice (Donovan and Brown, 2006a)

1. Euthanize an 8- to 12-week-old mouse by CO_2 asphyxiation (Donovan and Brown, 2006a).
2. Wet fur with 70% ethanol and cut skin off legs (one at a time).
3. Remove foot by cutting below the ankle joint and cut leg off above hip.
4. Under a biological hood, trim away the muscle and cut off ends of bone.
5. Pass 3-ml of culture medium for BMMC through each end of the bone using a 3-cc syringe with a 30-G needle. Collect the medium in a 50-ml conical centrifuge tube.
6. Centrifuge 10 min at 400 × *g*, room temperature.
7. Wash three times with 20 ml culture medium for BMMC, resuspend in 10 ml of the same medium, and transfer to a 75-cm^2 tissue culture flask. Incubate.
8. Before BMMCs are used for assays, culture overnight in culture medium for BMMC without SCF (if that was used in the medium). At a time point 3 hr before experimental use, replace the medium with culture medium for BMMC without IL-3 or SCF and continue incubation until ready to perform experiment.

   The use of both SCF and IL-3 results in a mast cell with characteristics of connective tissue type mast cells. BMMC can be grown in medium containing IL-3 alone, resulting in a cell type with similarities to mucosal mast cells.

BASIC PROTOCOL 3

MEASUREMENT OF REGULATED EXOCYTOSIS USING THE PASSIVE SYSTEMIC ANAPHYLAXIS (PSA) METHOD

The following method is an adaptation of an established acute systemic anaphylaxis model (Pivniouk et al., 1999). The in vivo phase of this procedure is done within the confines of a procedure room in the animal facility in which the test animals are housed, and the procedure is not performed until IACUC approval of the method is provided in writing.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Mice
15 μg/ml IgE anti-DNP antibody (Sigma-Aldrich) in sterile PBS (*APPENDIX 2A*)

2.5 mg/ml dinitrophenol conjugated to human serum albumin (DNP-HSA; Sigma-Aldrich) in sterile PBS (*APPENDIX 2A*)

Phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile

Histamine enzyme immunoassay kit (Beckman Coulter) containing:
- Anti–histamine antibody–coated microtiter plate
- Zero standard
- Histamine standards
- Control
- Histamine–alkaline phosphatase conjugate
- Conjugate diluent H
- Acylation reagent
- Release buffer
- 20× wash solution
- Substrate buffer (diethylamine)
- *p*-nitrophenylphosphate substrate tablets
- Stop solution (NaOH)

Gauze pads, sterile

Mouse cages

Heat lamp

1-ml syringes with 26-G, 1/2 in. needles

1-ml heparinized syringes with 23-G, 1-in. needles

500-µl EDTA-coated microtainer tubes

Microtiter plate reader

Semi-logarithmic graph paper

Additional reagents and equipment for injection of the mouse (Donovan and Brown, 2006b), euthanasia of the mouse (Donovan and Brown, 2006a), and blood collection via cardiac puncture (Donovan and Brown, 2006c)

Day 1: Sensitize mice

1. Organize mice into two groups of at least six for each phenotype to be tested.

   The first group will receive antigen challenge (see day 2) while the second group will receive vehicle challenge.

   An equal number of animals should be used for the two groups to allow for statistical comparison, and at least six animals should be used per group to minimize the standard error and increase the accuracy of the statistical evaluation.

   Different strains of mice can be used for this procedure. While the extent of the response may differ (i.e., Sv129 or BALB/c mice are stronger responders than C57/BL6 mice), all strains undergo an increase in circulating histamine. Optimally, mice should be between 6 and 12 weeks of age, but older mice still respond, though non-stimulated levels of circulating histamine are slightly increased with aging. No apparent gender differences are observed in the anaphylactic response.

2. Put each group of animals into a separate cage. Place each mouse under a heat lamp for 1 to 2 min prior injection to help visualize the tail vein.

3. To sensitize, inject mice intravenously in the tail vein (Donovan and Brown, 2006b) with 0.2 ml of 15 µg/ml mouse IgE anti-DNP antibody using a 1-ml syringe and a 26-G, 1/2 in. needle. After the injection, compress the site with sterile gauze to stop any bleeding and prevent the sample from leaking out of the injection site.

Day 2: Challenge and sacrifice mice and obtain blood samples

4. After 24 hr, challenge mice by injecting intravenously in the tail vein (Donovan and Brown, 2006b) with 0.2 ml of 2.5 mg/ml DNP-HSA (in sterile PBS) or sterile PBS (controls) as in step 3.

5. Sacrifice the animals 1.5 min following injection by asphyxiation in a sealed chamber using compressed CO_2 (Donovan and Brown, 2006a).

6. Immediately collect blood samples for determination of serum histamine levels by cardiac puncture (Donovan and Brown, 2006c) using a 1-ml heparinized syringe connected to a 23-G, 1-in. needle.

7. Transfer the blood sample into a 500-µl EDTA-coated microtainer tube and place immediately on ice to preserve the histamine in the sample.

   The microtainer can be microcentrifuged directly or placed inside a larger microcentrifuge tube which is in turn placed in the microcentrifuge.

8. Microcentrifuge the sample 15 min at 900 × *g*, 4°C, to isolate the plasma.

9. Using a sterile pipet, transfer the plasma to a clean microcentrifuge tube and immediately place on dry ice to freeze the sample for later analysis.

Analyze histamine content

For histamine measurement, follow the assay procedure, step 8, in the kit instruction manual.

10. Prepare serial dilutions of the plasma for histamine enzyme immunoassay measurement.

    The dilution factors usually range from 1:500 to 1:10,000.

11. Acylate 100 µl of histamine standards or diluted sera with 25 µl acylation reagent.

    Acylation of standards and samples is a rapid reaction that requires no incubation.

12. Add 50 µl of each acylated standard and sample to each well of an anti–histamine antibody–coated plate. Add 200 µl histamine–alkaline phosphatase conjugate. Incubate overnight at 4°C.

13. Wash wells three times, each time by filling all wells completely with 1× wash solution (prepared by diluting the 20× wash solution from the kit with distilled water), then manually tapping the solution out on clean absorbent paper placed on the benchtop.

14. Add 200 µl *p*-nitrophenylphosphate (pNPP). Incubate 30 min at room temperature.

15. Read the absorbance at 405 nm in a microtiter plate reader to detect the enzymatic activity bound to the plate.

    The color will be inversely proportional to the concentration of histamine in the plasma.

16. Plot a standard curve of absorbance at 405 nm versus histamine concentration of standards on semi-log paper.

17. Determine concentration of histamine by interpolation.

ALTERNATE PROTOCOL 3

DETECTION OF REGULATED EXOCYTOSIS BY PASSIVE SYSTEMIC ANAPHYLAXIS

Instead of measuring histamine release, it is possible to measure anaphylaxis by dye extravasation into tissues or by monitoring body temperature (Dombrowicz et al., 1993, 1997).

Additional Materials *(also see Basic Protocol 3)*

250 to 500 µg/ml IgE anti-DNP antibody (Sigma-Aldrich) or IgE anti-TNP (BD Pharmingen) in sterile PBS (*APPENDIX 2A*)

Antigen in 2% (w/v) Evans Blue (see recipe)
Formamide

Rectal temperature probe connected to a digital recorder (Cole-Parmer)
70-μm nylon cell strainer (BD Falcon)
Spectrophotometer

Day 1: Sensitize mice

1. Sensitize mice for IgE-induced passive systemic anaphylaxis by intravenous injection into the tail vein (Donovan and Brown, 2006b) of 50 to 100 μg antigen-specific IgE in 200 μl PBS.

   Control animals receive PBS only. Note that the injected dose of antigen-specific IgE is higher than that used in Basic Protocol 3; this is necessary in order to induce enough tissue edema to efficiently observe the extravasation of the dye. The larger dose of IgE also causes a larger drop in body temperature.

Day 2: Challenge animals

2. After 24 hr, measure the animal's body temperature using a rectal probe connected to a digital thermometer, then challenge the animals by intravenous injection into the tail vein (Donovan and Brown, 2006b) of 0.2 ml of antigen in 2% Evans Blue.

   Referring to the recipe for antigen in 2% Evans Blue (see Reagents and Solutions), DNP-HSA or DNP-KLH is injected when anti-DNP IgE is used; TNP-OVA or TNP-BSA is injected when anti-TNP IgE is used.

3. Monitor body temperature at various time points (e.g., every 10 min over 60 to 90 min) following antigen injection using a rectal probe connected to a digital thermometer.

   Anaphylactic shock causes decreased blood pressure, making body temperature difficult to maintain. Thus, loss of body temperature is a reasonable measure of the in vivo response to a systemic anaphylactic challenge, and these changes can be readily measured even at antigen challenge doses where death from anaphylactic shock is not an endpoint.

Measure vascular leakage using Evans Blue extravasation

4. At a time point 90 min following antigen challenge, euthanize animals who did not die during the course of the anaphylactic reaction by CO_2 asphyxiation (Donovan and Brown, 2006a).

   Death (number of mice that die out of a given number of mice challenged) can be an endpoint for anaphylactic shock, as it demonstrates the most severe reaction to the antigen challenge. Temperatures recorded on mice that die from anaphylactic shock can be used in the analysis up to the time of death. Alternatively, the challenge dose of antigen can be lowered to allow for temperature monitoring, recovery, and survival following shock.

5. Remove the ears and mince into 2 × 2–mm pieces. Transfer to a 1-ml microcentrifuge tube.

6. Extract the dye from the ear tissue by incubating overnight 1 ml formamide at 55°C.

7. Eliminate hairs and tissue fragments by rapid gravity filtration through a 70-μm nylon cell strainer, read the absorbance at 620 nm.

   IgG-induced PSA can be obtained using a similar procedure except that the concentration of antigen-specific IgG_1 must be 10 to 20 times higher than that of IgE to obtain a reaction of a similar magnitude.

   Absorbance at 620 nm is close to the peak absorbance of Evans Blue dye and reflects the extent of mast cell degranulation, which causes tissue edema leading to dye extravasation. Absorbance values are reported.

ALTERNATE PROTOCOL 4

DETECTION OF REGULATED EXOCYTOSIS BY ACTIVE SYSTEMIC ANAPHYLAXIS

Animals can also be immunized under conditions favoring an IgE-mediated immune response to a specific antigen. The anaphylactic response to antigen is measured 3 weeks after immunization. This protocol uses DNP-KLH as a specific antigen, but it may be adapted in principle to any other antigen for which an IgE-mediated immune response can be elicited (Dombrowicz et al., 1993, 1997).

Additional Materials *(also see Basic Protocol 3 and Alternate Protocol 3)*

1.5 μg/ml *Bordetella pertussis* toxin (Invitrogen) in PBS (*APPENDIX 2A*)
2.5 mg/ml DNP-KLH and 0.5% (w/v) aluminum hydroxide (Rehydragel, Reheis Inc.; *http://www.reheis.com*) in PBS (*APPENDIX 2A*)
0.5% (w/v) aluminum hydroxide (Rehydragel, Reheis Inc.; *http://www.reheis.com*) in PBS (*APPENDIX 2A*)

1. For active systemic anaphylaxis, inject control and experimental mice intravenously in the tail vein (Donovan and Brown, 2006b) with 200 μl of 1.5 μg/ml *Bordetella pertussis* toxin (i.e., 300 ng toxin) in PBS.
2. After 48 hr, inject animals intraperitoneally (Donovan and Brown, 2006b) with 500 μg DNP-KLH and 0.5% aluminum hydroxide in 0.2 ml PBS. Inject control animals with 200 μl of 0.5% aluminum hydroxide without DNP-KLH.
3. Challenge animals 3 weeks later as described in Basic Protocol 3, step 4 or Alternate Protocol 3, step 2.

   The injection of DNP-KLH in the presence of aluminum hydroxide causes the production of high titers of DNP-specific IgE; thus, the mice should respond to the challenge with any multivalent DNP-conjugated protein.
4. Measure histamine (Basic Protocol 3) if challenge was administered as in Basic Protocol 3, step 4, or hypothermia (Alternate Protocol 3) and vascular leakage (Alternate Protocol 3) if challenge was administered as in Alternate Protocol 3, step 2.

ALTERNATE PROTOCOL 5

DETECTING REGULATED EXOCYTOSIS BY PASSIVE CUTANEOUS ANAPHYLAXIS

Mast cells are present in high numbers in the skin, and mast cell degranulation in response to antigen can also be elicited by local sensitization with antigen-specific IgE followed by challenge by intravenous injection of specific antigen (Dombrowicz et al., 1993).

For materials, see Basic Protocol 3 and Alternate Protocol 3

1. Sensitize mice for IgE-induced passive cutaneous anaphylaxis (PCA) by injecting 20 μl of 2.5 μg/ml antigen-specific (e.g., DNP-specific) IgE (50 ng IgE) intradermally into the ear pinna. Inject the control ear with 20 μl PBS.
2. At a time point 24 to 48 hr later, challenge animals by intravenous injection of 100 μg of the cognate multivalent antigen (e.g., DNP_{36}-HSA) in 200 μl of 2% Evans Blue in PBS.
3. Measure dye extravasation (Alternate Protocol 3).

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antigen in 2% Evans Blue

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing:
5 mg/ml antigen: DNP-HSA (Sigma-Aldrich), DNP-KLH (Calbiochem), TNP-OVA (Biosearch Technologies), or TNP-BSA (Biosearch Technologies), depending on antibody used for sensitization
2% (w/v) Evans Blue (Sigma-Aldrich)
Prepare fresh

DNP-HSA or DNP-KLH is injected when anti-DNP IgE is used for sensitization; TNP-OVA or TNP-BSA is injected when anti-TNP IgE is used for sensitization (see Alternate Protocol 3).

Annexin V binding buffer, 10×

100 mM HEPES
NaOH, pH 7.3
1.5 M NaCl
50 mM KCl
10 mM $MgCl_2$
18 mM $CaCl_2$
Store up to 6 months at 4°C

Carbonate buffer, 0.1 M, pH 9.0

1.06 g Na_2CO_3
0.840 g $NaHCO_3$
Bring to 100 ml with distilled H_2O
Filter sterilize through 0.22-μm nylon filter
Store up to 6 months at room temperature

Citrate buffer, 0.1 M, pH 4.5

Prepare the following stock solutions:
0.1 M citric acid: dissolve 1.05 g citric acid in 50 ml H_2O (store up to 6 months at 4°C)
0.1 M sodium citrate: dissolve 1.47 g sodium citrate in 50 ml H_2O (store up to 6 months at 4°C)
Mix 26.5 ml of 0.1 M citric acid with 23.25 ml of 0.1 M sodium citrate. Filter sterilize and store up to 2 weeks at 4°C

Culture medium for BMMC

500 ml RPMI 1640 medium without glutamine (Biofluids)
6 ml 100× Penicillin-Streptomycin-Glutamine (Invitrogen)
12.5 ml 1 M HEPES (Invitrogen)
5 ml 100× nonessential amino acids (NEAA; Biofluids)
5 ml 100 mM sodium pyruvate (Biofluids)
175 μl 1% (v/v) 2-mercaptoethanol (Sigma-Aldrich)
60 ml fetal bovine serum (FBS)
10 ng/ml interleukin 3 (IL-3; PeproTech; omit where directed in protocols)
10 ng/ml stem cell factor (SCF; PeproTech; omit where directed in protocols)

Culture medium for RBL-2H3 cells

500 ml Earle's Minimal Essential Medium (EMEM; Biofluids)
100 ml fetal bovine serum (FBS)
6 ml 100× Penicillin-Streptomycin-Glutamine (Invitrogen)

pNAG substrate

Prepare 1 mM *p*-nitrophenyl acetyl-D-glucosamine (Sigma) in citrate buffer (see recipe), vortexing and warming to 37°C to dissolve. Store at −20°C in 5-ml aliquots (sufficient for one 96-well plate).

Tyrode's buffer

135 mM NaCl
5 mM KCl
5.6 mM glucose
1.8 mM $CaCl_2$
1 mM $MgCl_2$
0.5 mg/ml BSA Fraction V
20 mM HEPES
Adjust pH to 7.3 with NaOH
Store without BSA up to 6 months at 4°C

Tyrode's buffer–E

135 mM NaCl
5 mM KCl
5.6 mM glucose
1.5 mM EDTA
0.5 mg/ml BSA Fraction V
10 mM HEPES
Adjust pH to 7.3 with NaOH
Store without BSA up to 6 months at 4°C

COMMENTARY

Background Information

Measurement of exocytosis from mast cell granules represents a rapid and convenient means for evaluating the activation of mast cells and blood basophils. Although mast cells can be activated without concomitant degranulation, in particular when the stimulus is suboptimal (Gonzalez-Espinosa et al., 2003), most physiological stimuli activate these cells to release the mediators stored in their cytoplasmic granules. The appearance of exocytosed products occurs within a few minutes of cell activation and can be highly specific for these cells (see below). In contrast, release of cytokines and chemokines from mast cells requires hours following cell activation and is usually less specific, as most cytokines and chemokines can also be released by other cells. Interestingly, mast cells have been reported to represent one of the few types of cells, if not the only one in the body, that stores the inflammatory cytokine TNF in secretory granules from which it can be rapidly released (Beil et al., 1996). Additionally, mast cells and platelets are unique in secreting copious amounts of sphingosine-1-phosphate (S1P), a lipid ligand known to regulate the chemotactic response of various hematopoietic cells (Olivera and Rivera, 2005). Although not detailed here, the rapid appearance of TNF or S1P in body fluids might therefore be considered another possible indicator of mast cell activity in vivo.

Regardless, measurement of granule exocytosis is the starting point of most studies that evaluate the activation of mast cells. For in vitro studies with cultured cells, several methods have been developed in parallel. Mast cells and basophils represent the most important pool of cells capable of storing and promptly releasing histamine from their granules upon stimulation (MacGlashan, 2003). Measurement of histamine, therefore, represents an excellent and highly specific correlate of granular mediator release, and it is the

method of choice when one wants to determine mast cell activation in vivo, in a mixture of cells, or when measuring the activation of basophils in human blood. Using the described assay, histamine can be measured in plasma, urine, cell supernatants, cell lysates, or in any other liquid or solid biological sample. For in vivo experiments, the immediate consequences of the mobilization of histamine, such as the drop in body temperature or blood vessel permeability (dye extravasation), may also be monitored as a faithful correlate of mast cell activation. While not applicable for cell mixtures, other described assays correlate with the liberation of histamine and have frequently been used as a measure of mast cell degranulation. For example, measurement of externalization of PS at the plasma membrane using flow cytometry is a relatively new assay (Martin et al., 2000). As flow cytometry determines surface labeling of individual cells, this assay allows one to measure degranulation activity in a single mast cell, which is not possible in the assays measuring release of soluble mediators. Although it has not yet been reported, measurement of PS exposure may also allow determination of exocytosis in a mixture of cells by identifying the mast cell population via a specific surface marker (such as FcεRI c-kit). However, although PS accumulates specifically at the sites of granule exocytosis (Demo et al., 1999), the exact relationship between the exocytosis and PS exposure is still unknown. Notably, it is not known whether exposure simply represents the externalization of the inner leaflet of granule membrane or whether it corresponds to phospholipid scrambling that takes place due to the extensive mobilization of cellular membranes during the degranulation process.

Critical Parameters and Troubleshooting

All described assays (except for the in vivo experiments, which require experience with the handling of animals) are straightforward. The method of choice may be based on individual preference and, importantly, on the cost of reagents and equipment. However, for cell mixtures, the only accepted approach is the direct measurement of histamine. The histamine release observed from cell mixtures can be normalized by flow cytometric measurement of IgE receptor (FcεRI)– and c-kit-positive cells, indicating the relative numbers of mast cells in the mixture. Also, as mast cells and basophils contain high levels of histamine (relative to other cells), normalization to total histamine content may also be appropriate. To adequately measure spontaneous and stimulated release, it is critically important to carefully remove supernatants without removing any of the pelleted cells. Inclusion of cells in the analysis may modify results, especially when the stimulated release is low compared to total cellular content. All assays should be performed at 37°C, as lower temperatures alter the kinetics of release and can block exocytosis.

It is known that the percent of release varies between individual clones or cell lines. Subcloning of the RBL-2H3 mast cell line may yield clones that release between 10% and 90% of total granular content. Thus, in studies where individual clones are analyzed (for example in transfection experiments), care should be taken to analyze a number of clones to allow unambiguous interpretation of the results. Primary cultures of mouse and human mast cells may also show differences in releasing capacity, depending on background strain or individual. When IgE and antigen-dependent stimulation is used to induce degranulation in experiments comparing cells from different sources or that are genetically altered, the cell surface expression level of IgE receptors should be verified prior to conducting the experiment to assess whether stimulus strength may be a factor. Loading of equivalent numbers of receptors with IgE would then allow a fair comparison under circumstances where receptor numbers may differ.

Anticipated Results

When appropriately stimulated, mast cells can release up to 90% of total granular content. However, values can vary widely depending on whether cell lines, individual cloned lines, cultured primary cells, or ex vivo primary cells are used. Normally, values in the range of 30% to 60% of total granular content are considered satisfactory.

Time Considerations

For in vivo assays, an exact time scheme is given in the protocols. The in vitro degranulation assays or the annexin V binding assays can easily be performed within 1 day. Sensitization of cells with IgE requires 1 hr, but it can also be performed overnight. The subsequent washing step, antigen stimulation, and recovery of supernatants and total cell lysates require between 1 and 2 hr for all degranulation assays. The quantification of the released products usually requires another 2 hr for β-hexosaminidase measurements, 15 to 30 min for determination of released radiolabeled

serotonin, and 2 to 3 hr for determination of histamine. Supernatants and cell lysates can be rapidly frozen (in a dry ice/acetone bath), stored at –20°C or lower, and analyzed later. Samples should not be stored for prolonged periods (i.e., for weeks or longer). Flow cytometric analysis for the annexin V binding assay requires between 2 and 3 hr and should be performed immediately following cell stimulation.

Literature Cited

Andrews, N.W. 2000. Regulated secretion of conventional lysosomes. *Trends Cell Biol.* 10:316-321.

Aronson, N.N. Jr. and Kuranda, M.J. 1989. Lysosomal degradation of Asn-linked glycoproteins. *FASEB J.* 3:2615-2622.

Beil, W.J., Login, G.R., Aoki, M., Lunardi, L.O., Morgan, E.S., Galli, S.J., and Dvorak, A.M. 1996. Tumor necrosis factor alpha immunoreactivity of rat peritoneal mast cell granules during early secretion induced by compound 48/80: An ultrastructural immunogold morphometric analysis. *Int. Arch. Allergy Immunol.* 109:383-389.

Bretscher, M.S. 1972. Asymmetrical lipid bilayer structure for biological membranes. *Nat. New Biol.* 236:11-12.

Demo, S.D., Masuda, E., Rossi, A.B., Throndset, B.T., Gerard, A.L., Chan, E.H., Armstrong, R.J., Fox, B.P., Lorens, J.B., Payan, D.G., Scheller, R.H., and Fisher, J.M. 1999. Quantitative measurement of mast cell degranulation using a novel flow cytometric annexin-V binding assay. *Cytometry* 36:340-348.

Dombrowicz, D., Flamand, V., Brigman, K.K., Koller, B.H., and Kinet, J.P. 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain. *Cell* 75:969-976.

Dombrowicz, D., Flamand, V., Miyajima, I., Ravetch, J.V., Galli, S.J., and Kinet, J.P. Absence of Fc epsilonRI alpha chain results in up regulation of gammaRIII-dependent mast cell degranulation and anaphylaxis: Evidence of competition between Fc epsilonRI and Fc gammaRIII for limiting amounts of FcR beta and gamma chains. 1997. *J. Clin. Invest.* 99:915-925.

Donovan, J. and Brown, P. 2006a. Euthanasia. *In* Current Protocols in Immunology (J.E. Coligan, B.E. Bierer, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.8.1-1.8.4. John Wiley & Sons, Hoboken, N.J.

Donovan, J. and Brown, P. 2006b. Parenteral injections. *In* Current Protocols in Immunology (J.E. Coligan, B.E. Bierer, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.6.1-1.6.10. John Wiley & Sons, Hoboken, N.J.

Donovan, J. and Brown, P. 2006c. Blood collection. *In* Current Protocols in Immunology (J.E. Coligan, B.E. Bierer, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.7.1-1.6.9. John Wiley & Sons, Hoboken, N.J.

Dy, M. and Schneider, E. 2004. Histamine-cytokine connection in immunity and hematopoiesis. *Cytokine Growth Factor Rev* 15:393-410.

Galli, S.J., Nakae, S., and Tsai, M. 2005a. Mast cells in the development of adaptive immune responses. *Nat. Immunol.* 6:135-142.

Galli, S.J., Kalesnikoff, J., Grimbaldeston, M.A., Piliponsky, A.M., Williams, C.M., and Tsai, M. 2005b. Mast cells as "tunable" effector and immunoregulatory cells: Recent advances. *Annu. Rev. Immunol.* 23:749-786.

Gonzalez-Espinosa, C., Odom, S., Olivera, A., Hobson, J.P., Martinez, M.E., Oliveira-Dos-Santos, A., Barra, L., Spiegel, S., Penninger, J.M., and Rivera, J. 2003. Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J. Exp. Med.* 197:1453-1465.

Kitaura, J., Eto, K., Kinoshita, T., Kawakami, Y., Leitges, M., Lowell, C.A., and Kawakami, T. 2005. Regulation of highly cytokinergic IgE-induced mast cell adhesion by Src, Syk, Tec, and protein kinase C family kinases. *J. Immunol.* 174:4495-4504.

MacGlashan, D., Jr. 2003. Histamine: A mediator of inflammation. *J. Allergy Clin. Immunol.* 112:S53-S59.

Martin, S., Pombo, I., Poncet, P., David, B., Arock, M., and Blank, U. 2000. Immunologic stimulation of mast cells leads to the reversible exposure of phosphatidylserine in the absence of apoptosis. *Int. Arch. Allergy Immunol.* 123:249-258.

Olivera, A. and Rivera, J. 2005. Sphingolipids and the balancing of immune functions: Lessons from the mast cell. *J. Immunol.* 174:1153-1158.

Pivniouk, V.I., Martin, T.R., Lu-Kuo, J.M., Katz, H.R., Oettgen, H.C., and Geha, R.S. 1999. SLP-76 deficiency impairs signaling via the high-affinity IgE receptor in mast cells. *J. Clin. Invest.* 103:1737-1743.

Röhlich, P., Anderson, P., and Uvnäs, B. 1971. Electron microscope observations on compounds 48-80-induced degranulation in rat mast cells: Evidence for sequential exocytosis of storage granules. *J. Cell Biol.* 51:465-483.

Schwartz, L.B., Austen, K.F., and Wasserman, S.I. 1979. Immunologic release of beta-hexosaminidase and beta glucuronidase from purified rat serosal mast cells. *J. Immunol.* 123:1445-1450.

Siraganian, R.P. 1974. An automated continuous-flow system for the extraction and fluorometric analysis of histamine *Anal. Biochem.* 57:383-394.

Contributed by Ulrich Blank
Bichat Medical School
Paris, France

Juan Rivera
National Insitutes of Health
Bethesda, Maryland

Analysis of Regulated Secretion Using PC12 Cells

Laurent Taupenot[1,2]

[1]University of California at San Diego, La Jolla, California
[2]Veterans Affairs San Diego Healthcare System, San Diego, California

ABSTRACT

The catecholamine-secreting PC12 cell line derived from the rat adrenal medulla has long been considered a model system for neurosecretion and neuronal differentiation. PC12 cells contain a large number of secretory granules (otherwise known as large dense-core vesicles) for storage of small molecules, processing enzymes, neuropeptides, and peptide hormones. Secretory granule exocytosis in PC12 cells is tightly regulated by calcium and occurs in response to a secretagogue. This unit provides protocols for maintenance and transfection of PC12 cells. Several secretion assays are described to measure the release of secretory granule cargo molecules by detection of radioactive catecholamine, or by immunochemical or chemiluminescence detection of transfected regulated secretory proteins. *Curr. Protoc. Cell Biol.* 36:15.12.1-15.12.13. © 2007 by John Wiley & Sons, Inc.

Keywords: granule • PC12 • exocytosis • chromogranin • catecholamine

INTRODUCTION

Following its establishment by Greene and Tischler in 1976, the PC12 line of rat pheochromocytoma cells has been widely considered as a model of choice to study mechanisms underlying regulated exocytosis, secretory organelle biogenesis, secretory protein trafficking, catecholamine metabolism, and neurotrophin action. This unit provides protocols for maintenance and transfection of PC12 cells, and describes assays to monitor exocytosis of chromaffin secretory granules by radiodetection of preloaded radioactive norepinephrine (Basic Protocol 1), or by chemiluminescence (Basic Protocol 2), or immunochemical detection (Alternate Protocol) of ectopic regulated secretory proteins. There are also protocols for the routine culture (Support Protocol 1) and transfection (Support Protocol 2) of PC12 cells.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

MEASUREMENT OF RADIOLABLED CATECHOLAMINE RELEASE

BASIC PROTOCOL 1

Catecholamines are a major component of dense-core secretory granules (sometimes referred to as catecholamine storage vesicles) found in PC12 cells. These secretory organelles can be labeled with [^{3}H]-L-norepinephrine by a mechanism involving cell surface norepinephrine transporters (NET) and the catecholamine uptake system of the secretory granule membrane (VMAT). This procedure (and variants thereof) has been widely used to assess regulated secretion from chromaffin cells in culture and sympathetic neurons. The method is quantitative but measures the relative (%) rather than the absolute amount of catecholamine ([^{3}H]-norepinephrine) release. Thus, relative [^{3}H]-L-norepinephrine secretion (Sec) is calculated as a percentage of total radioactivity present

Current Protocols in Cell Biology 15.12.1-15.12.13, September 2007
Published online September 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1512s36

in the cells before stimulation, according to the following equation:

$$\text{Sec} = \frac{\text{Cpm}_{\text{rel}}}{\text{Cpm}_{\text{rel}} + \text{Cpm}_{\text{cell}}} \times 100$$

Equation 15.12.1

Total radioactivity is the sum of the amount released (Cpm_{rel}) plus the amount remaining in the cells (Cpm_{Cell}). The protocol described below is for PC12 cells but can be adapted to other catecholamine-secreting cells, including primary cultures of chromaffin cells and sympathetic neurons.

Materials

Early-passage PC12 cells or ATCC-PC12 cells (#CRL-1721)
Poly-L-lysine (Sigma, cat. no. P6282)
Rat tail collagen Type I (Upstate, cat. no. 08-115)
DMEM or F12K basal and complete culture media (see recipe)
Levo-[ring-2,5,6-^{3}H]-norepinephrine (specific activity 40 to 80 Ci/mmol; PerkinElmer Life and Analytical Sciences)
Calcium saline buffer (CaSB; see recipe)
Stimulation buffer (see recipe)
Scintillation fluid, water soluble
Triton X-100

6-well tissue-culture treated polystyrene plates (e.g., Falcon, Costar)
37°C, CO_2 incubator
Motorized pipetting aid
Scintillation vials
Orbital shaker
Liquid scintillation beta-counter

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Grow PC12 cells in multi-well tissue culture plates

1. Seed PC12 cells onto poly-L-lysine-coated 6-well plates (early passage PC12 cells) or poly-L-lysine- and rat collagen-coated 6-well plates (ATCC-PC12 cells) at a 1:3 subcultivation ratio, using appropriate complete culture medium (see Support Protocol 1).

   Measurement of radiolabeled norepinephrine release involves several washing maneuvers that depend on good attachment of the cells to poly-L-lysine and collagen substrates. Thus, seeding early-passage PC12 cells on poly-L-lysine-coated 6-well plates is preferable to enhance the moderate adhesion of the cells to the tissue culture plastic. In the authors' experience, best results are obtained when cells are seeded 48 hr prior to the beginning of the secretion assay.

2. Grow the cells in a CO_2 incubator at 37°C, until confluency reaches 70% to 80%.

Load cells with [^{3}H]-L-norepinephrine

3. On the day of the study, remove old medium by tilting the plate and aspirating culture medium. Add 1.0 ml of prewarmed Levo-[ring-2,5,6-^{3}H]-norepinephrine ([^{3}H]-L-norepinephrine) labeling solution (0.7 μCi/ml in complete culture medium) to each well of the plate.

4. Incubate cells for 90 min in CO_2 incubator at 37°C.

   A 90-min incubation allows equilibration between intracellular and extracellular pools of [^{3}H]-L-norepinephrine.

5. Using a motorized pipetting aid, wash the cells twice with 2.0 ml prewarmed CaSB at 2-min intervals.

   This and subsequent washing steps to remove unincorporated [^{3}H]-L-norepinephrine can be performed outside a laminar-flow hood. By slightly tilting the plate, the washing buffer should be carefully added to the edge of each well to limit cell detachment.

6. Add 2 ml of basal culture medium per well, incubate cells in CO_2 incubator for 30 min at 37°C.

   This incubation step will further remove unincorporated [^{3}H]-L-norepinephrine. Incubation is performed in a CO_2 incubator to maintain the pH of the basal culture medium. During this period prepare CaSB solutions containing secretagogues ± antagonists/modulators.

7. Aspirate basal culture medium, wash the cells twice with 2.0 ml prewarmed CaSB at 2-min intervals.

   This last washing step will ensure complete removal of free [^{3}H]-L-norepinephrine.

Measure [^{3}H]-L-norepinephrine secretion

8. Using a pipettor add 1 ml of control buffer (CaSB) or stimulation solution to each well, incubate 15 min at 37°C.

   At 10-sec intervals, replace the content of each well with mock (control) solution to assess the basal (unstimulated) release, or with stimulation buffer [e.g., 60 μM nicotine in CaSB, 2 mM Ba^{2+} (BaSB; see recipe), or 55 mM KCl (KSB; see recipe)] to assess regulated secretion. When all the wells have the appropriate solution, incubate without further change for a total of 15 min. When exchanging buffers, do not allow the cells to become dry. Each condition is performed at least in triplicate. Maximum stimulation is usually achieved within 15 min, since few agonists stimulate release past this time period.

9. Terminate secretion by collecting the supernatant from each well into separate scintillation vials. Add 5 ml of scintillation fluid, and count for 2 min.

   After 15 min incubation, collection of released media should be done at the same pace as in the previous incubation step (i.e., every 10 sec) to ensure that each well has been exposed to mock or secretagogue for an equal amount of time (i.e., 15 min total).

10. To determine cellular content of [^{3}H]-L-norepinephrine, add 1 ml of 0.1% Triton X-100 (in CaSB). Incubate 10 min at room temperature with gentle rotation. Collect lysates in separate scintillation vials. Add 5 ml of scintillation fluid, and count for 2 min.

Calculate amount secreted

11. For each well express the amount of [^{3}H]-L-norepinephrine secreted by dividing the radioactivity of the release medium by the sum of the radioactivity present in the cell lysate and release medium according to Equation 15.12.1.

ROUTINE GROWTH AND MAINTENANCE OF PC12 CELLS

SUPPORT PROTOCOL 1

This protocol describes two routine methods to maintain PC12 cells in culture. A widely used clone of PC12 cells may be readily obtained from the ATTC's repository, and is derived from a single-cell clonal line established from a rat adrenal pheochromocytoma, an adrenal medullary tumor (Greene and Tischler, 1976). One characteristic of this line of PC12 cells is that the cells adhere poorly to plastic and tend to grow in small patches of loosely attached cells. Alternatively, the authors routinely use an early passage (passage 8)

of the original PC12 cell line generated by Greene and Tischler (referred to in this article as early-passage PC12 cells). In contrast to ATCC-PC12 cells, early-passage PC12 cells adhere fairly well to tissue culture plastic, and tend to form confluent monolayers.

Materials

Early-passage PC12 cells or ATCC-PC12 cells (#CRL-1721)
DMEM or F12K complete culture media (see recipe)
100 μg/ml poly-L-lysine hydrobromide solution (Sigma, no. P6282)
100 μg/ml rat tail collagen Type I (Upstate, no. 08-115) in PBS (*APPENDIX 2A*)

100-mm cell culture dishes, tissue-culture treated polystyrene (e.g., Becton Dickinson-Falcon, Corning-Costar)
37°C, 6% CO_2, humidified incubator

Propagation of early-passage PC12 cells

1a. Grow cells in 100-mm cell culture dishes in 10 ml complete DMEM culture medium, in a humidified incubator, 6% CO_2, 37°C.

2a. Passage cells as necessary from a subcultivation ratio of 1:3 up to a 1:7, as needed for experiments.

Cells' doubling time is ~48 hr. Cells may be passaged when the confluency reaches ~70% to 80%. No trypsinization is necessary as PC12 cells can be easily detached from the tissue culture plastic by gently pipetting up and down in 10 ml fresh complete DMEM.

Propagation of ATCC-PC12 cells

1b. Coat 100-mm cell culture dishes by adding sufficient 100 μg/ml poly-L-lysine solution to cover the surface and incubate 60 min at 37°C. Remove poly-L-lysine solution and add sufficient 100 μg/ml rat tail collagen Type I solution to cover the surface and incubate 120 min at room temperature.

ATCC-PC12 cells adhere poorly to plastic; it is thus critical to coat the tissue culture dish with adhesion substrata. ATCC-PC12 cells attach reasonably well to a tissue culture substratum containing both poly-L-lysine and collagen.

2b. Wash the cell culture dishes two times, each time with 5 ml sterile water.

Coated dishes can be sealed with parafilm and stored for up to 3 weeks at 4°C.

3b. Grow cells in coated 100-mm culture dishes containing 10 ml of complete F12K culture medium, in a humidified incubator, 6% CO_2, 37°C.

4b. Passage as necessary to a subcultivation ratio of 1:3 up to a 1:5, as needed for your experiments.

ATCC-PC12 cells' proliferation rate is lower than the early-passage PC12 cells, with an estimated doubling time of ~72 hr. Although ATCC-PC12 cells never form a confluent monolayer, the formation of large clumps and loss of attachment to the tissue culture substratum is generally a sign of over-confluency, and thus of a need to passage the cells. For instance, ATCC-PC12 cells subcultivated at a ratio of 1:3 in a 100-mm dish are typically passaged twice a week by pipetting up and down in 10 ml fresh complete F12K medium.

BASIC PROTOCOL 2

MEASURING EXOCYTOSIS AFTER TRANSIENT TRANSFECTION OF THE REGULATED SECRETORY REPORTER PROTEIN CgA-EAP

As noted below, high-efficiency transfection of PC12 cells, and sympathoadrenal cells in general, is relatively difficult to achieve. Thus, one might find it difficult to study the influence of an exogenous, transiently (transfection) expressed protein on the exocytotic machinery of PC12 cells by merely assessing the secretion of catecholamine from [^{3}H]-L-norepinephrine-labeled cells. Indeed, this approach would provide information largely

determined by the exocytotic response of nontransfected cells, rather than from the cells expressing the protein of interest. This potential limitation of the [^{3}H]-L-norepinephrine labeling technique may be circumvented by co-transfecting a reporter protein specifically targeted to secretory granules, allowing the investigation of the effect of a co-expressed protein on the secretory pathway, in the same cell.

This protocol describes the use of CgA-EAP (Taupenot et al., 2005, Courel et al., 2006), a regulated secretory chimeric protein comprising an engineered form of secreted embryonic alkaline phosphatase (EAP) fused in-frame to the carboxy-terminus of chromogranin A (CgA), a member of the granin family of regulated secretory proteins targeted to the cores of biogenic amine- and peptide-containing secretory granules of neuroendocrine and neuronal cells, including chromaffin granules (Taupenot et al., 2003). Expression of CgA-EAP is under the control of the highly active human cytomegalovirus promoter (pCMV-CgA-EAP(N2)]. Detection of the EAP chimeric protein enzymatic activity is achieved by chemiluminescence with the substrate 3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′chloro)tricyclo[3.3.1.1(3,7)]decan]-4-yl) phenyl phosphate (CSPD). The assay is commercially available from Applied Biosystems (Phospha-Light SEAP) or Clontech (GreatEscAPe SEAP). The detection limit of EAP by chemiluminescence is ~0.1 pg/ml. Human growth hormone (hGH) may be used as an alternative reporter to analyze regulated secretion in PC12 cells (see Alternate Protocol).

The relative secretion rate (*Sec*) is calculated as a percentage of total exogenous regulated secretory protein (EAP enzymatic activity or hGH amount) present in the cells before stimulation, according to the following equation:

$$\text{Sec} = \frac{\text{RSP}_{\text{rel}}}{\text{RSP}_{\text{rel}} + \text{RSP}_{\text{cell}}} \times 100$$

Equation 15.12.2

Total exogenous regulated secretory protein (RSP) is defined as the sum of the amount released (RSP_{rel}) plus the amount remaining in the cells (RSP_{cell}).

Volumes indicated in this protocol are given for one well of a 6-well cell culture plate. The procedure can be scaled up or down to other vessel sizes (for instance, 12-well cell culture plate) by adjusting volumes in proportion to the surface area. Secretagogue-stimulated release assay of transfected reporter proteins may be performed outside a laminar-flow hood.

Materials

Early-passage PC12 cells or ATCC-PC12 cells (see Support Protocol 1)
pCMV-CgA-EAP(N2) [available from the authors]
Expression plasmid containing transgene of interest
Calcium saline buffer (CaSB; see recipe)
Stimulation buffer (see recipe)
0.1% (v/v) Triton X-100 in CaSB
Secreted Alkaline Phosphatase Chemiluminescence assay kit (e.g., Phospha-Light, Applied Biosystem, no. T1017) containing:
  Assay Buffer
  Reaction Buffer Diluent
3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′chloro)tricyclo[3.3.1.1(3,7)]decan]-4-yl) phenyl phosphate (CSPD)
37°C incubator
Cell scraper

Ethanol/dry ice or liquid N_2 baths
Luminometer (tube or plate)
Absorbance microplate reader

Additional reagents and equipment for co-transfecting PC12 cells (Support Protocol 2)

Transfect reporter protein

1. Co-transfect PC12 cells with the plasmid encoding the reporter protein [pCMV-CgA-EAP(N2)] together with an expression plasmid containing a transgene of interest (see Support Protocol 2).

   Each condition is studied at least in triplicate, including mock-transfected cells for subsequent assay background determination. Design of co-transfection experiments should include a control group where cells are co-transfected with an empty vector, preferably containing a similar promoter (e.g., CMV, SV40) to that mediating the expression of the protein of interest.

Perform secretagogue-stimulated secretion assay of reporter protein

2. Wash the cells twice with 2.0 ml of prewarmed CaSB at 2-min intervals.

   The secretagogue-stimulated release assay for the reporter proteins is typically performed 24 to 48 hr post-transfection, depending on the transfection efficiency and the sensitivity of the assay used to detect expression of the transgene. The washing steps remove any excess of serum that may contribute to high assay background. By slightly tilting the plate, aspirate the medium and add washing buffer to the edge of each well to limit cell detachment.

3. Add 700 µl of prewarmed CaSB or stimulation buffer to each well, incubate 15 min at 37°C.

   At 10-sec intervals, replace the content of each well with mock (control) solution to assess the basal (unstimulated) release, or with stimulation buffer [e.g., 60 µM nicotine in CaSB, 2 mM Ba^{2+} (BaSB; see recipe), or 55 mM KCl (KSB; see recipe)] to assess regulated secretion. When all the wells have the appropriate solution, incubate without further change for a total of 15 min. When exchanging buffers, do not allow the cells to become dry. Each condition is performed at least in triplicate. Maximum stimulation is usually achieved within 15 min, since few agonists stimulate release past this time period.

4. Terminate secretion by collecting the release medium from each well into individual microcentrifuge tubes. Centrifuge 3 min at 1700 × *g*, 4°C, to pellet any detached cells. Keep the tubes on ice until further processing.

   After 15 min incubation, collection of release medium should be done at the same pace as in the previous incubation step (i.e., every 10 sec) to ensure that each well has been exposed to mock or secretagogue for an equal amount of time (i.e., 15 min total). Microcentrifuge tubes should be kept on ice until further processing.

5. Add 700 µl of 0.1% Triton X-100 (in CaSB) to each well, and harvest cells using a cell scraper.

6. Transfer cell suspension into individual microcentrifuge tubes, and further disrupt cells by two cycles of quick freeze/thaw (using ethanol/dry ice or liquid N_2 bath). Clear lysates from cell debris by centrifuging 5 min at ~20,000 × *g*, 4°C. Save supernatants and keep on ice until further processing.

   Preparation of cell lysates in steps 5 and 6 allows the determination of the cellular content of reporter protein.

Detect CgA-EAP release by chemiluminescence

This protocol is an adaptation of Applied Biosystem's instructions for Phospha-Light chemiluminescence assay kit, which includes a heat inactivation step at 65°C in the presence of phosphatase inhibitors. Such a maneuver is designed to substantially

reduce nonspecific chemiluminescence due to endogenous alkaline phosphatases while preserving the enzymatic activity of EAP, which is heat stable and resistant to several phosphatase inhibitors (Berger et al., 1988). In PC12 cells, the assay background due to endogenous alkaline phosphatase is typically low, and it is not substantially reduced after heat inactivation and differential inhibition. Thus inactivation of endogenous alkaline phosphatases may not be required when using PC12 cells. Nevertheless, a control group of mock-transfected PC12 cells should be included in the design of every experiment to estimate the background (nonspecific chemiluminescence) of the assay (see step 13). The following volumes are given for one sample.

7. Equilibrate 50 µl of Assay Buffer to room temperature.
8. Prepare 50 µl of reaction buffer by adding 2.5 µl CSPD stock to 47.5 µl of Reaction Buffer Diluent.
9. Add 50 µl sample from step 4 (release medium) or step 6 (cell lysate) into a vessel (tube or microtiter plate) suitable for luminometry.

   A standard curve can be made with known amounts of purified placental alkaline phosphatase provided with the kit to determine the high end detection limit. The assay must be conducted within its linear range. Therefore, samples may be diluted with CaSB to remain within the range of concentration of the standard.

10. Add 50 µl Assay Buffer, mix, incubate for 5 min at room temperature.
11. Add 50 µl of reaction buffer, mix, incubate for 30 min at room temp.

    Perform incubation in the dark as CSPD substrate is sensitive to light.

12. Read chemiluminescence light output for 5 to 10 sec (expressed as relative light units, RLU) in the luminometer.
13. Calculate CgA-EAP relative (%) secretion. For each well express the amount of EAP activity secreted by dividing relative light units (RLU) of the secreted medium by the sum of RLU present in the secreted medium plus the cell lysate, according to Equation 15.12.2.

    ~10% to 20% of the chemiluminescence measured in the release medium from CgA-EAP expressing PC12 cells may be due to assay background (that is from the nonspecific chemiluminescent background of the assay reagents alone), and should be subtracted from RSP_{rel} prior calculation of the relative secretion rate of CgA-EAP. In contrast, the chemiluminescence background of cellular lysate from naive PC12 cells is negligible as compared to the 1000 to 10,000 higher chemiluminescence output of EAP enzymatic activity in CgA-EAP expressing cells.

MEASUREMENT OF HUMAN GROWTH HORMONE RELEASE

ALTERNATE PROTOCOL 1

Human growth hormone (hGH) may be used as an alternative reporter to analyze regulated secretion in PC12 cells. When expressed in PC12 cells, hGH is sorted to the regulated pathway of secretion and released by exocytosis in response to a secretagogue (Wick et al., 1993; for review see Sugita, 2004). Vectors allowing expression of hGH in PC12 cells include pXGH5 plasmid where the expression of the hormone is driven by the mouse metallothionein I promoter (Wick et al., 1993).

Several methods based on the use of monoclonal or polyclonal antibodies have been developed for in vitro measurement of hGH, including immunoradiometric (IRMA), colorimetric enzyme-linked immunosorbent (ELISA), and chemiluminescence enzyme immunoassays (CLEIA). All these assays are readily available as commercial kits. ELISA has become a method of choice for quantitative measurement of hGH, with the clear advantage that it is nonradioactive, and therefore does not require tedious procedures

associated with handling of radioactive substances. The authors currently use Roche Applied Science's colorimetric microplate hGH ELISA kit, which has a linear range of detection of ~5 to 400 pg hGH/ml.

The experimental design for the analysis of the secretagogue-stimulated exocytosis of hGH from transiently transfected PC12 cells is identical to that of CgA-EAP (Basic Protocol 2, steps 1 to 6) and can be summarized as follow:

Additional materials (also see Basic Protocol 2)

Human growth hormone ELISA kit (e.g., Roche Applied Science, no. 11585878001)

1. Co-transfect PC12 cells with a plasmid encoding hGH (e.g., pXGH5) together with an expression plasmid expressing the protein of interest (see Basic Protocol 2, step 1).
2. Perform secretagogue-stimulated secretion assay, then collect secreted media and cell lysates.
3. Quantify hGH in the secreted media and cell lysates in a microplate reader. The detailed procedure for quantification of hGH by ELISA is provided by the assay kit from the manufacturer (e.g., Roche Applied Science hGH ELISA, cat. no. 11585878001).
4. Calculate the relative (%) secretion of hGH. For each well express the amount of hGH secreted by dividing the absorbance at 405 nm of the release medium by the sum of the absorbance at 405 nm of the release medium plus cell lysate according to Equation 15.12.2.

SUPPORT PROTOCOL 2

TRANSFECTION OF PC12 CELLS USING CATIONIC LIPIDS

Many commercial cationic lipid–based transfection reagents are available for transient and stable transfection of DNA in PC12 cells. However, one should keep in mind that high-efficiency transfection of PC12 cells is relatively difficult to achieve. The following protocol describes the transfection of PC12 cells using the cationic lipid transfection reagent GenePORTER 2 (Genlantis). The authors found that this product provides a substantially higher transfection efficiency than several other popular lipid-based transfection reagents. For instance, transfection efficiency evaluated by the expression of the transgene enhanced GFP (EGFP) 48 hr post-transfection typically reaches 15% to 20%.

Masses and volumes indicated in the protocol are given for one well of a 6-well cell culture plate. Transfection can be scaled up or down to other vessel sizes by adjusting the amounts of plasmid DNA transfection reagent, and culture medium in proportion to the surface area. All of the procedures should be performed in a laminar-flow hood to maintain sterility of the cell culture.

Materials

Early-passage PC12 cells or ATCC-PC12 cells (#CRL-1721)
Poly-L-lysine (Sigma, no. P6282)
DMEM or F12K complete culture medium (see recipe)
Rat tail collagen type I (Upstate, cat no. 08-115)
Cationic lipid reagent GenePORTER 2 (Genlantis)
Plasmid DNA, purified by anion-exchange chromatography (e.g., Qiagen)
DNA diluent (Genlantis)

6-well cell culture plates, tissue-culture treated polystyrene (e.g., Becton Dickinson-Falcon, Corning-Costar)
37°C CO_2 incubator
Clear polystyrene plastic snap tubes (e.g., Becton Dickinson Falcon)

Prepare cells and reagents for transfection

1. At a time point 24 hr before transfection, split nearly confluent PC12 cells onto poly-L-lysine coated 6-well plates (early passage PC12 cells) or poly-L-lysine- and rat collagen-coated 6-well plates (ATCC-PC12 cells) at a 1:4-1:5 subcultivation ratio, using appropriate complete culture medium (see Support Protocol 1).
2. Culture cells overnight in a CO_2 incubator at 37°C so as to achieve ~40% to 50% confluency.

   Transfection efficiency is influenced by culture confluency. Best results are typically obtained when transfection is performed in PC12 cells cultured at a moderate density, i.e., in a mid-log growth phase.
3. Add 12.5 µl of GenePORTER 2 reagent to 50 µl of appropriate serum-free culture medium (DMEM or F12K) in a polystyrene tube. Mix by gently flicking the tube 4 to 5 times.
4. Dilute 2.5 µg of DNA into 62.5 µl of Genlantis' DNA diluent in a polystyrene tube. Mix by gently flicking the tube 4 to 5 times, and leave at room temperature for no more than 5 min.

   For co-transfection of several plasmids, DNA can amount to a total of 5 µg per well of a 6-well cell culture plate. In that case, maintain the ratio of GenePORTER 2 reagent (5 µl/µg of plasmid), Genlantis' DNA diluent (25 µl/µg of DNA), and basal culture medium diluent (4 µl/µl of GenePORTER 2 reagent).
5. Combine DNA solution with the diluted GenePORTER reagent, and incubate for 10 min at room temperature to allow complex formation.
6. Add 875 µl of prewarmed complete culture medium to the tube containing the DNA/GenePORTER 2 solution.

Transfect the cells

7. Aspirate culture medium from the well, and replace with 1.0 ml of DNA/GenePORTER solution.
8. Incubate cells with the transfection complex for 5 hr in a 5% CO_2 incubator at 37°C.
9. Remove transfection solution from the cells, and add 2.0 ml of appropriate fresh prewarmed complete culture medium.
10. Grow cells in a CO_2 incubator at 37°C in preparation for assay of the reporter gene (see Basic Protocol 2 and Alternate Protocol).

    Depending on the promoter activity, the translation of the transgene and its intracellular targeting, the assay for the reporter gene may be performed 24 to 72 hr post-transfection.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Barium stimulation buffer (BaSB)

150 mM NaCl
5 mM KCl
2 mM $BaCl_2$ (no calcium)
10 mM HEPES
Adjust to pH 7.4 with HCl
Store up to 1 month at 4°C

Calcium saline buffer (CaSB)

150 mM NaCl
5 mM KCl
2 mM $CaCl_2$
10 mM HEPES
Adjust to pH 7.4 with HCl
Store up to 1 month at 4°C

CaSB may be supplemented with secretagogues (e.g., nicotine 60 μM, PACAP 250 nM, ATP 100 μM) to stimulate the exocytotic response of PC12 cells.

F12K medium

410 ml F12K medium (Invitrogen, cat. no. 21127)
75 ml heat-inactivated horse serum (Gemini Bio-Products, cat. no.100-508)
12.5 ml heat-inactivated fetal bovine serum (Gemini Bio-Products, cat. no. 100-106)
5 ml 100× penicillin-streptomycin-glutamine (Invitrogen, cat. no. 10378-016)
Filter sterilize with 0.22-μm pore-size unit (e.g., Nalge Nunc, Corning)
Store up to 1 month at 4°C

This medium is used for culturing ATCC-PC12 cells.

DMEM medium

500 ml DMEM medium (Invitrogen, cat. no. 11965)
50 ml heat-inactivated horse serum (Gemini Bio-Products, cat. no. 100-508)
25 ml heat-inactivated fetal bovine serum (Gemini Bio-Products, cat. no. 100-106)
6 ml 100× penicillin-streptomycin-glutamine (Invitrogen, cat. no. 10378-016)
Filter sterilize with 0.22-μm pore-size unit (e.g., Nalge Nunc, Corning)
Store up to 1 month at 4°C

This medium is used for culturing early passage PC12 cells.

Potassium stimulation buffer (KSB)

100 mM NaCl
55 mM KCl
2 mM $CaCl_2$
10 mM HEPES
Adjust to pH 7.4 with HCl
Store up to 1 month at 4°C

COMMENTARY

Background Information

The constitutive and regulated secretory pathways represent the classical routes for secretion of proteins (Kelly, 1985). The constitutive pathway exists in every cell type, allowing rapid transport of proteins in small vesicles originating from the *trans*-Golgi network, and destined for rapid fusion with the plasma membrane. In contrast, the regulated secretory pathway found in endocrine, neuroendocrine, and neuronal cells is characterized by the sorting and concentration of a pool of secretory proteins into specialized intracellular organelles with a typical electron-dense appearance on transmission electron microscopy, prompting the morphologic term dense-core secretory granules. These granules may remain in the cell for an extended period of time after their formation, until the cell is stimulated for Ca^{2+}-dependent exocytotic fusion with the plasma membrane by a secretagogue characteristic of a particular cell type.

PC12 cells contain dense-core (chromaffin) granules that store small molecules (e.g., catecholamine, nucleotides, ascorbic acid, and Ca^{2+}), and peptide hormones and neuropeptides, including chromogranin-secretogranins (granins; Fig. 15.12.1). PC12 cells have become an important model to study the mechanisms of regulated neuroendocrine secretion,

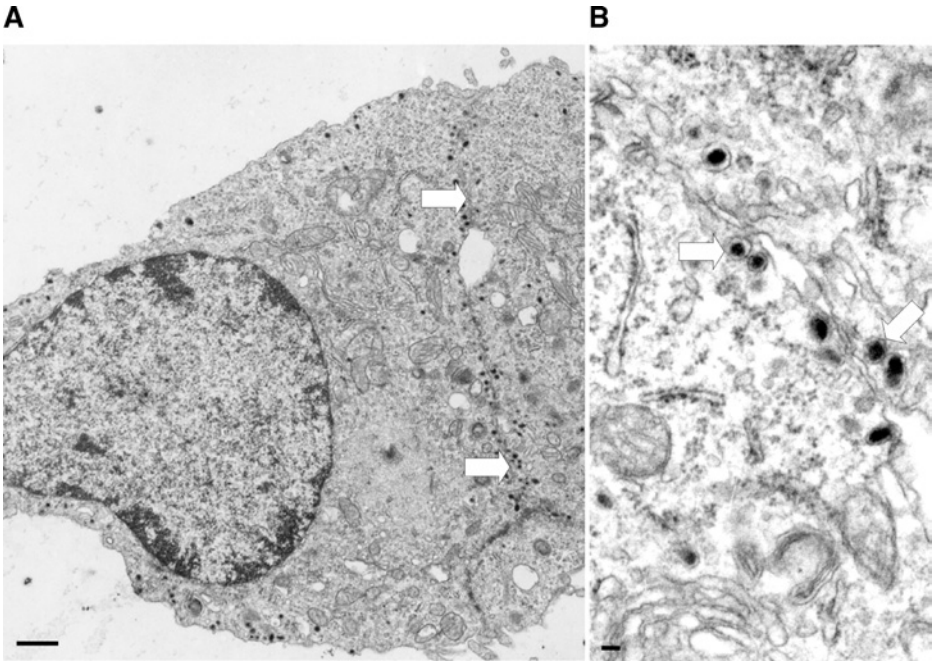


Figure 15.12.1 Electron micrograph of early passage PC12 cells. Dense-core secretory granules (arrows) are seen in the vicinity of or docked at the plasma membrane (**A**). Scale bar = 1 μm. High magnification electron micrograph in (**B**) shows the presence of electron-dense, membrane-delimited vesicles of ~100 to 130 nm in diameter (arrows). Scale bar = 100 nm. Cells were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde and post-fixed in 1% OsO_4.

allowing the discovery of several components of the machinery that controls the various stages of the regulated exocytotic pathway (for review see Martin and Grishanin, 2003). A rise in intracellular Ca^{2+} is an essential requirement for the initiation of dense-core granule exocytosis, which may be stimulated in PC12 cells by a variety of extracellular triggers including secretagogues stimulating physiological pathways [60 μM nicotine (acting as a nicotinic cholinergic agonist), 250 nM pituitary adenylyl cyclase polypeptide (PACAP), 100 μM ATP], as well as nonphysiological stimuli [2 mM $BaCl_2$, Ca^{2+} ionophores (e.g., 10 μM A23187)], or by a depolarizing concentration of potassium (55 mM KCl; Mahata et al., 1997; Taupenot et al., 1999).

Critical Parameters and Troubleshooting

Procedures to maintain PC12 cells in culture are straightforward and require a basic knowledge of techniques for mammalian cell tissue culture (*UNIT 1.1*).

All the secretagogue-stimulated assays should be performed on healthy cells, growing at subconfluency to limit cell detachment during washing and buffer exchange. Buffers should be prewarmed to 37°C, as lower temperatures may reduce exocytosis.

Careful washing to remove unincorporated [^{3}H]-L-norepinephrine is critical to adequately measure basal (unstimulated) versus secretagogue-stimulated release, particularly if stimulated release of [^{3}H]-L-norepinephrine is low relative to its cellular content. For the same reason, it is important to limit cell detachment into the secreted medium during the stimulation step. In a typical experiment, basal release of [^{3}H]-L-norepinephrine from PC12 cells should be ~5% of total cellular content, and secretagogue stimulation should yield between 2 to 6 times basal release, depending on the secretagogue.

As noted earlier, studying the influence of a protein on the exocytotic response of [^{3}H]-L-norepinephrine-labeled PC12 cells may be limited by a low transfection efficiency, which is often observed with lipid-based transfection reagents. However, a modest expression of CgA-EAP or hGH is usually sufficient to report on the regulated secretory pathway in PC12 cells after stimulation with potent secretagogues such as Ba^{2+}, KCl, or ATP, since the proteins can readily be detected in the secreted medium and cell lysate with high sensitivity by

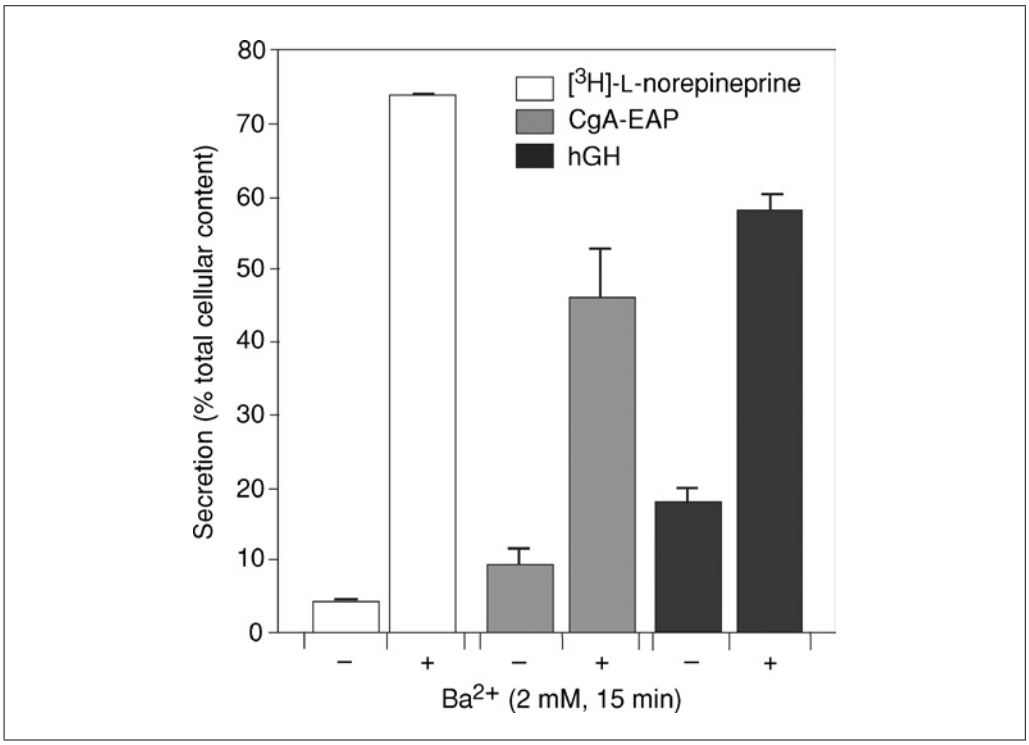


Figure 15.12.2 Detection of secretagogue stimulated exocytosis of secretory granules in PC12 cells. Cells labeled with [^{3}H]-norepinephrine, or transfected for 48 hr with CgA-EAP or hGH, were incubated for 15 min in secretion medium alone (CaSB, *mock*) or with 2 mM $BaCl_2$.

chemiluminescence or ELISA. Basal (unstimulated) release of CgA-EAP from PC12 cells is typically low at ~5% to 8% of total cellular content. On the other hand, basal secretion of hGH may be elevated when overexpressed in PC12 cells (reaching up to 20% of total hormone cellular content), which may suggest some degree of entry of the hormone into the constitutive (secretagogue-insensitive) pathway of secretion (Fig. 15.12.2; Sugita et al., 2004; Taupenot et al., 2005; Courel et al., 2006).

Anticipated Results

Following a 15-min stimulation period, PC12 cells can release up to 80% of total [^{3}H]-L-norepinephrine cellular content, but only up to 50% of the total cellular content of regulated secretory protein reporter, in response to the same stimulation. While [^{3}H]-L-norepinephrine primarily labels mature secretory granules, the expressed reporter must first route through several intracellular compartments of the early secretory pathway of PC12 cells (endoplasmic reticulum, *cis* and *trans* Golgi), prior storage into dense-core secretory granules. Hence, the total cellular content of regulated secretory protein reporter includes an intracellular pool of protein unavailable for exocytotic release. Consequently, the *relative* (%) secretion of a regulated secretory protein in response to a secretagogue is typically lower than that of [^{3}H]-L-norepinephrine, although the *absolute* exocytotic rate is expected to be the same. Shown in Figure 15.12.2 are representative secretory responses to Ba^{2+} stimulation (2 mM, 15 min) of PC12 cells labeled with [^{3}H]-L-norepinephrine, or transfected for 48 hr with CgA-EAP or hGH.

Time Considerations

Cell culture. Plating the cells on substratum-coated culture plates is done 24 to 48 hr prior transfection and usually requires up to 3 hr, including the time needed to coat the tissue culture dish with collagen and/or poly-L-lysine.

Transfection. Transfection is typically performed in the morning. Depending on the number of conditions being tested, the initial transfection procedure may require up to 2 hr, and will be followed by incubation with the transfection complex, which will be replaced with fresh complete culture medium after 5 hr. Thus the total procedure can be performed within one working day.

Radiolabeled catecholamine secretion assay. Depending on the number of conditions being tested, labeling of cells and completing

the secretagogue-stimulated release of [^{3}H]-L-norepinephrine assay requires between 4 and 5 hr.

Reporter secretory granule protein release assay. This procedure is typically performed 48 hr post-transfection and takes <2 hr. Secreted media and cell lysates may be processed the same day for chemiluminescence determination (CgA-EAP; Basic Protocol 2) or immunoassay (hGH; Alternate Protocol). Alternatively, samples may be quickly frozen, stored at −20°C or lower, and analyzed later.

Literature Cited

Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B.R. 1988. Secreted placental alkaline phosphatase: A powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* 66:1-10.

Courel, M., Rodemer, C., Nguyen, S.T., Pance, A., Jackson, A.P., O'Connor, D.T., and Taupenot, L. 2006. Secretory granule biogenesis in sympathoadrenal cells: Identification of a granulogenic determinant in the secretory prohormone chromogranin A. *J. Biol. Chem.* 281:38038-38051.

Greene, L.A. and Tischler, A.S. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* 73:2424-2428.

Kelly, R.B. 1985. Pathways of protein secretion in eukaryotes. *Science* 230:25-32.

Mahata, S.K., O'Connor, D.T., Mahata, M., Yoo, S.H., Taupenot, L., Wu, H., Gill, B.M., and Parmer, R.J. 1997. Novel autocrine feedback control of catecholamine release. A discrete chromogranin a fragment is a noncompetitive nicotinic cholinergic antagonist. *J. Clin. Invest.* 100:1623-1633.

Martin, T.F. and Grishanin, R.N. 2003. PC12 cells as a model for studies of regulated secretion in neuronal and endocrine cells. *Methods Cell Biol.* 71:267-286.

Sugita, S. 2004. Human growth hormone cotransfection assay to study molecular mechanisms of neurosecretion in PC12 cells. *Methods* 33:267-272.

Taupenot, L., Mahata, M., Mahata, S.K., and O'Connor, D.T. 1999. Time-dependent effects of the neuropeptide PACAP on catecholamine secretion: Stimulation and desensitization. *Hypertension* 34:1152-1162.

Taupenot, L., Harper, K.L., and O'Connor, D.T. 2003. The chromogranin-secretogranin family. *N. Engl. J. Med.* 348:1134-1149.

Taupenot, L., Harper, K.L., and O'Connor, D.T. 2005. Role of H^+-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: Evidence for a vesiculogenic function. *J. Biol. Chem.* 280:3885-3897.

Wick, P.F., Senter, R.A., Parsels, L.A., Uhler, M.D., and Holz, R.W. 1993. Transient transfection studies of secretion in bovine chromaffin cells and PC12 cells. Generation of kainate-sensitive chromaffin cells. *J. Biol. Chem.* 268:10983-10989.

Internet Resources

http://medicine.ucsd.edu/hypertension

UCSD chromaffin and PC12 cell biology online protocols.

Analysis of Endocytic Trafficking by Single-Cell Fluorescence Ratio Imaging

Herve Barriere[1] and Gergely L. Lukacs[1]

[1]McGill University, Department of Physiology, Montreal, Quebec, Canada

ABSTRACT

The post-endocytic sorting of internalized membrane proteins plays a critical role in numerous physiological processes, including receptor desensitization, degradation of non-native plasma membrane proteins, and cell surface retrieval of receptors from early endosomes upon ligand dissociation. Here, we describe a fluorescence ratiometric image analysis (FRIA) method used to determine the post-endocytic fate and transport kinetics of transmembrane proteins based on the pH measurement of internalized cargo-containing compartments in living cells. The method relies on the notion that the pH of a cargo-containing transport vesicle (vesicular pH, pH_v) could be taken as an indicator of its identity, considering that endocytic organelles (e.g., sorting endosome, recycling endosome, late endosome/MVB, and lysosome) have characteristic pH_v. The pH-sensitive FITC-conjugated secondary antibody is attached to the cargo via a primary antibody, recognizing the cargo extracellular domain. The pH_v is determined by single-cell FRIA. Internalized cargo colocalization with organellar markers, as well as pH_v measurement of recycling endosome, lysosome, and the TGN are discussed to validate the technique and facilitate data interpretation. *Curr. Protoc. Cell Biol.* 40:15.13.1-15.13.21. © 2008 by John Wiley & Sons, Inc.

Keywords: post-endocytotic sorting • pH • fluorescence ratiometric image analysis • endocytic organelles

INTRODUCTION

Endocytosis entails the cellular uptake of macromolecules (e.g., hormones, nutrients, and viruses) from the extracellular space, as well as polypeptides and lipids from the plasma membrane (Mukherjee et al., 1997). Endocytosis is critical for preserving homeostasis at the cellular, organ, and organism level by regulating cell surface receptor density and cell polarity and motility, providing nutrient uptake, and antigen presentation, just to mention a few examples (Mukherjee et al., 1997). Trafficking pathways of incoming cargo molecules, regardless of their specific entry route, seem to converge at early endosomes (Mukherjee et al., 1997; Sharma et al., 2003). Subsequent maturation of early endosomes coincides with their progressive acidification by vacuolar H^+-ATPase and formation of the tubular endosomal network (TEN), one of the critical structures where endocytic cargo sorting occurs (Mukherjee et al., 1997; Sun-Wada et al., 2004; Bonifacino and Rojas, 2006).

The biochemical and functional subcompartmentalization of the TEN, in concert with coat protein assembly at the cytoplasmic surface, ensure the concentration and packaging of transmembrane cargo molecules according to their destination (Bonifacino and Traub, 2003). Three major destinations have been delineated from the TEN (Mukherjee et al., 1997; Katzmann et al., 2002). Numerous membrane proteins recycle back to the plasma membrane by bulk flow that traverses the recycling endosomal compartment that represents the default trafficking route. Recent evidence, however, suggests the existence of a signal-dependent recycling pathway as well (Bonifacino and Traub, 2003; Maxfield and McGraw, 2004; von Zastrow and Sorkin, 2007). Another subset of cargo molecules are

Current Protocols in Cell Biology 15.13.1-15.13.21, September 2008
Published online September 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1513s40

routed to late endosome/lysosome and lysosome-like organelles relying on a variety of lysosomal sorting signals [e.g., Tyr-, di-Leu- or ubiquitin (Ub)-based motifs; Marks et al., 1996; Janvier and Bonifacino, 2005). The third possible destination is represented by the trans-Golgi network (TGN; Humphrey et al., 1993; Ghosh et al., 2003) and mediated by retrograde transport (Bonifacino and Traub, 2003; Bonifacino and Rojas, 2006).

Immuno-colocalization of cargo with markers of early and recycling endosomes, late endosomes-MVB, lysosomes, and TGN provide valuable information regarding the steady-state, as well as dynamic distribution of transmembrane proteins and the regulation of their post-endocytic sorting at the light and electron microscopy level. The post-endocytic fate of selected cargo molecules can also be monitored by biochemical assays taking advantage of covalent modification of sugar residues (e.g., sialic acid) or proteolytic processing by organelle-specific proteases (e.g., cathepsin D, metalloproteinases). In this unit, we describe an alternative method for determining the post-endocytic fate and transport kinetics of membrane proteins based on ratiometric pH_v measurements of internalized cargo-containing vesicles.

Since the pH_v of sorting endosomes, recycling endosomes, late endosomes/MVB, lysosomes, and the *trans*-Golgi network (TGN) have relatively well-defined and characteristic pH (see Table 15.13.1 and references therein), the pH_v of cargo-containing transport vesicles could be taken as an indicator of cargo localization. The pH_v is determined by single-cell fluorescence ratiometric image analysis (FRIA). The FRIA technique was originally described for lysosomal pH determination following the labeling of the lysosomal compartment with the fluid-phase marker, dextran, conjugated to the pH-sensitive fluorescein isothiocyanate (FITC; Ohkuma and Poole, 1978).

Transmembrane cargo labeling with the pH-sensitive fluorophore is usually accomplished by primary antibody (Ab) and FITC-conjugated secondary Fab capture in live cells. The primary Ab recognizes the extracellular domain of the cargo (Basic Protocol). As an alternative approach, high-affinity FITC-conjugated ligand (e.g., FITC-transferrin) could be employed if the transmembrane cargo has ligand-binding capacity. The advantage of the FRIA is its sensitivity and capacity to monitor cargo partitioning among multiple cellular compartments simultaneously. The major weakness is its inability to discriminate cargo distribution between organelles with overlapping pH_v (see Table 15.13.1). Therefore, validation of results is required to discriminate between endocytic cargo accumulation in the TGN and recycling endosomes by morphological and/or biochemical techniques (Support Protocol 5.).

The Basic Protocol is a method for determining the destination and sorting kinetics of model membrane proteins following their synchronized internalization in complex with FITC-labeled Ab. This protocol is recommended for cargo molecules that exhibit relatively high cell surface density. If the plasma membrane density of the cargo precludes the labeling of internal compartments by synchronized internalization, continuous Ab capture is used to increase the sensitivity of the assay, as described in the Alternate Protocol. We used these methods to uncover the Ub configuration that is necessary and sufficient as a lysosomal sorting signal in mammalian cells (Barriere et al., 2007). Determination of the fluorescence intensity ratio as a function of vesicular pH provides the in situ calibration for FRIA experiments and is described in Support Protocol 1. Considering that cell-specific variations may occur in the steady-state pH_v of organelles, pH_v measurements of recycling endosomes, lysosomes, and the TGN are included in Support Protocols 2, 3, and 4, respectively. Finally, additional control experiments are included to rule out premature dissociation of Abs from cargo molecules during live-cell imaging (Support Protocol 6).

Table 15.13.1 Summary of Organellar pH Determinations

Cellular compartment	pH_v	Reference
Sorting endosome	5.9-6.0	Presley et al. (1993); Mukherjee et al. (1997)
Recycling endosome	6.4-6.7	Yamashiro et al. (1984); Presley et al. (1993); Mukherjee et al. (1997)
Late endosome	5.4; 5.0-6.0	Tycko and Maxfield (1982); Yamashiro et al. (1984); Mukherjee et al. (1997)
Lysosome	5.0-5.5	Tycko and Maxfield(1982); Mukherjee et al. (1997)
TGN	6.25; 6.45; 6.58; 6.3; 6.2-6.8; 6.2; 6.4	Seksek et al. (1995); Kim et al. (1996); Llopis et al. (1998); Farinas and Verkman (1999); Wu et al. (2000, 2001); Machen et al. (2003)

MONITORING SYNCHRONIZED ENDOCYTIC TRAFFICKING OF TRANSMEMBRANE PROTEINS BY FLUORESCENCE RATIOMETRIC IMAGE ANALYSIS OF VESICULAR pH

BASIC PROTOCOL

To monitor the role of Ub in the post-endocytic sorting of transmembrane cargoes, the truncated CD4 consisting of the extracellular and transmembrane domains (CD4Tl) and its chimeric variants (CD4Tl fused to Ubs) are expressed in HEK 293 and COS-7 cells (Barriere et al., 2007). Due to the autonomous and transferable nature of sorting signals, this methodology has been widely used to identify sorting motifs in the context of different transmembrane reporter molecules (Maxfield, 1982; Presley et al., 1993; Lukacs et al., 1997; Demaurex et al., 1998). Fusing unextendable, wild-type or tetrameric mono-Ub variants to CD4Tl, this experimental design permits dissection of the role of Ub configuration as a lysosomal sorting signal in mammalian cells.

This protocol illustrates the distinct post-endocytic sorting of CD4Tl and CD4Tl-Ub following synchronized internalization. First, the anti-CD4 primary and FITC-conjugated secondary Fab are bound to CD4Tl and CD4Tl-Ub-expressing HEK 293 cells on ice. Then internalization is initiated by raising the temperature to 37°C for 1 to 30 min, followed by FRIA using an inverted epifluorescence microscope (Fig. 15.13.1). Cells are illuminated alternately with 495 nm and 440 nm fluorescent light and the emitted fluorescence is detected at 535 nm with a cooled CCD camera. Since FITC fluorescence at 495 nm excitation, but not at 440 nm, is sensitive to pH, normalization ensures that the fluorescence light intensity ratio ($495_{nm}/440_{nm}$) is primarily pH-dependent and independent of the dye concentration (Geisow, 1984).

Materials

HEK 293 cells expressing CD4Tl and CD4Tl-Ub (Invitrogen; see Barrier et al., 2006 for a description of cell lines)
DMEM-F: DMEM supplemented with 10% (v/v) fetal bovine serum (FBS)
COS-7 cells (ATCC, CRL-1651), optional
Fugene 6 (Roche)
H-DMEM-F: DMEM without bicarbonate and phenol-red (Invitrogen) supplemented with 5% (v/v) bovine serum (BS) and 15 mM HEPES

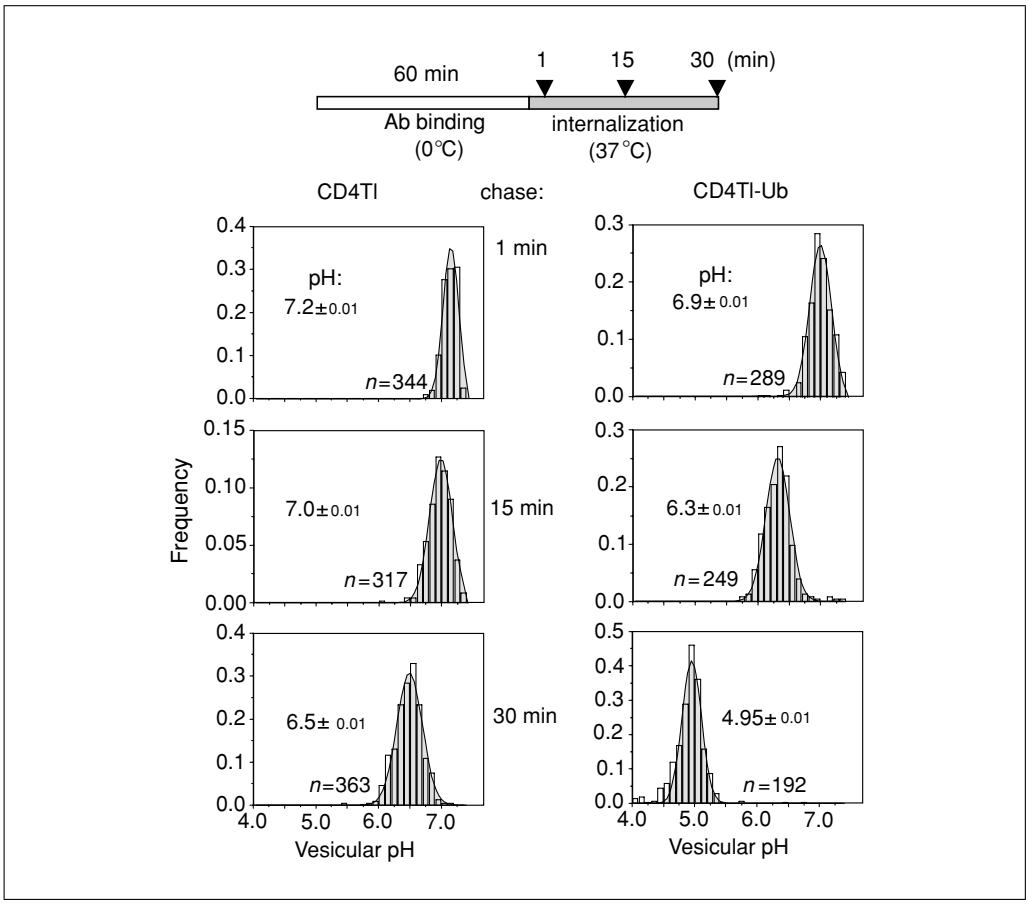


Figure 15.13.1 The lysosomal delivery kinetics of CD4TI-Ub. Anti-CD4 and FITC-conjugated secondary Fab was bound to HEK 293 cells transfected with CD4TI or CD4TI-Ub for 1 hr at 0°C. The temperature was then raised to 37°C for 1, 15, or 30 min and the pH_v was measured by FRIA. The mean pH_v (±SEM) and the number of vesicles analyzed are indicated.

Anti-CD4 antibody OKT4 (Harlan Bioproducts)
FITC-conjugated goat anti–mouse secondary Fab (Jackson ImmunoResearch Laboratories)
PBS++ solution (see recipe)
Modified Ringer's solution (see recipe)
K^+-rich buffer (see recipe)
Nigericin (Sigma-Aldrich, cat. no. N-7143)
Monensin (Sigma-Aldrich, cat. no. M-5273)

25-mm glass coverslips with standard no. 1 or no. 1.5 thickness, sterile, coated with poly-L-lysine
6-well culture plates (Falcon, cat. no. 353046)
Tissue culture incubator at 37°C with 5% CO_2
35-mm tissue culture dish
Perfusion chamber (MSC-TD, Warner Instruments)
Thermostated coverslip holder: Bipolar temperature controller (Med Systems, TC-202)
Microscope: inverted fluorescence microscope (e.g., Zeiss Axiovert 100 TV) equipped with:
Planachromat objective (63× NA 1.4, Carl Zeiss MicroImaging)
Objective heater (TempControl mini, Carl Zeiss MicroImaging)
Cooled CCD camera (e.g., Hamamatsu ORCA-ER CCD)

Filter set for BCECF: excitation filters, D495/10 and D440/20 and emission filter, D535/25 (Chroma)
Lambda 10-2 filter wheel (Sutter Instrument), containing the D495/10 and D440/20 excitation filters and used during image acquisition in step 10
Lamp: X-Cite 120 fluorescence illumination system (EXFO) allowing the adjustment of the Hg-lamp intensity (EXFO Life Sciences Division)
MetaFluor (MDS Analytical Technologies) Imaging System (for ratiometric fluorescence ion measurements and run on a PC computer)
OriginPro7 software
Ice bucket [used for cooling the cells at 4°C (e.g., steps 2, 3, 5, and 8)]
Vacuum and perfusion [used for replacing medium (see step 11)]

Prepare cells

1a. *For stably expressing cells:* At least 24 hr before the experiment, seed HEK 293 cells stably expressing CD4 chimera on poly-L-lysine-coated glass cover slips. Use 6-well tissue culture plates and 2 ml DMEM supplemented with 10% (v/v) FBS. Prepare mock transfected cells as well to verify that the fluorescent signal is negligible.

To prepare poly-L-lysine-coated coverslips follow the instructions provided by the manufacturer (Sigma, cat. no. P8920).

By the time of the FRIA, cells should be at 60% to 70% confluency.

1b. *For transiently expressing cells:* If CD4 chimeras are expressed transiently, transfect HEK 293 or COS-7 cells with the CD4 plasmid using Fugene 6 according to the manufacturer's instructions 24 to 48 hr before the measurement.

Label with the pH-sensitive probe

2. Working on ice, rinse the cells twice, each time with 2 ml ice-cold H-DMEM-F gently to avoid cell loss.

3. Bind the primary anti-CD4 antibody OKT4 (1:400 to 1:800 dilution) in 0.8 ml H-DMEM-F for 1 hr on ice.

Optimize the antibody concentration to maximize the fluorescence signal and minimize the background fluorescence. This could be achieved by using serial dilutions of the primary and secondary antibodies on transfected and nontransfected cells in pilot studies in concert with quantitative fluorescence video image analysis.

4. Rinse the cells three times, each time with 2 ml ice-cold H-DMEM-F.

5. Bind FITC-conjugated goat anti–mouse Fab (1:500 dilution) in 0.8 ml H-DMEM-F for 1 hr on ice.

6. Rinse the cells three times, each time with 2 ml ice-cold H-DMEM-F.

Internalization

7. Induce endocytosis of cargo complexed with FITC-labeled Ab by adding 2 ml prewarmed DMEM-F and transferring the plate into the tissue culture incubator (37°C) for 1 to 30 min.

8. To terminate the internalization rinse the cells with 2 ml ice-cold PBS++ solution and place the coverslip in a 35-mm tissue culture dish on ice in 2 ml modified Ringer's solution.

Acquire fluorescent ratio images

9. Mount the coverslip in the perfusion chamber and add 0.8 ml of modified Ringer's solution (25°C). Place the chamber in the thermostated coverslip holder prewarmed to 25°C. Maintain the microscope objective temperature at 25°C with a dedicated heater.

10. Acquire image pairs at 495 and 440 nm excitation wavelengths by selecting at least 10 to 15 areas on the coverslip. Do this step as quickly as possible to avoid significant cargo processing.

    It is advised that the first coverslip be used to adjust the exposure time, the camera gain and the fluorescence light intensity, and/or the lamp intensity to avoid photobleaching and saturation of the CCD camera. This is particularly important when cargo molecules partition between lysosomes and recycling endosomes in the same experiment.

Perform single-point in situ pH calibration

After completing image acquisition, single-point calibration is performed to verify that the microscope optical characteristics correspond to that observed by multi-point calibration.

11. Aspirate the modified Ringer's solution and add 0.8 ml of freshly prepared K^+-rich medium containing 10 μM nigericin and 10 μM monensin (pH 6.5, 25°C).

12. To ensure that vesicular H^+ concentration is equilibrated with the extracellular medium pH, replace the K^+-rich medium (0.8 ml) once more.

    The equilibration time required for clamping the intracellular pH to 6.5 may vary according to the vesicles and cells analyzed. Therefore, it is important to ascertain that the fluorescence ratio reaches a steady-state value by repeated image acquisition during a ~5 min time period.

13. Record the fluorescence ratio as in step 10. If the fluorescence ratio is different from that observed by the multi-point calibration technique, recalibrate the system (see Support Protocol 6).

Vesicular pH analysis

14. Setup the Metafluor program to obtain the average fluorescence intensity values of selected regions of interest (ROI) at 495 nm and 440 nm excitation wavelength in an Excel table.

    The average fluorescence intensity represents the integrated fluorescence intensity divided by the area of the ROI.

15. Select all of the ROIs corresponding to labeled vesicles in the first cell.

    Use the image obtained at 495 nm excitation wavelengths for the selection since the signal-to-noise ratio is better at 495 nm than at 440 nm. Identify as many vesicles as possible. Avoid analyzing vesicles that are in the proximity of the plasma membrane.

16. In the same cells also select three to six cytoplasmic ROIs that are free of labeled vesicles.

    These ROIs with comparable size to that of selected in step 15 will be used to calculate the background fluorescence.

17. Acquire the mean fluorescence intensity value of ROIs for all cells repeating steps 15 and 16.

18. The Excel file contains the mean fluorescence intensity value for each ROI at 495 nm and 440 nm excitation wavelengths and the background signal for each cell. Calculate the mean background fluorescence intensity at 495 nm and 440 nm excitation wavelengths for each cell. Subtract the mean background fluorescence values from the mean fluorescence intensity of each ROI at 495 nm and 440 nm excitation wavelengths to obtain the specific fluorescent signal.

    The mean fluorescence intensity of ROIs of the labeled vesicles should be at least 2-fold higher than the background fluorescence intensity.

19. Calculate the fluorescence ratio intensity values by dividing the mean fluorescence intensity at 495 nm by the mean fluorescence intensity at 440 nm for each ROI. The intensity values are corrected with the respective background fluorescence intensity. Based on the multi-point calibration curve, convert the fluorescence ratio values of ROIs to pH_v values (see Support Protocol 6).

    It is recommended to use a macro routine for executing background subtraction and transforming the ratio values into pH_v if a large number of vesicles are analyzed.

20. Plot the frequency distribution of pH_v values with the OriginPro 7.0 software using single- or multi-component Gaussian distribution. Calculate the mean pH_v for the individual peak.

    Partitioning of the cargo molecule into various organelles can be estimated based on the fractional distribution of vesicles in the individual peak relative to the total number of vesicles.

MONITORING POST-ENDOCYTIC TRAFFICKING OF TRANSMEMBRANE PROTEINS FOLLOWING CONTINUOUS LABELING OF CARGO WITH pH-SENSITIVE FLUOROPHORE

ALTERNATE PROTOCOL

If the steady-state expression of the cargo molecule at the cell surface is insufficient for synchronized labeling and endocytosis (see the Basic Protocol), association of Ab with a larger cohort of membrane proteins can be achieved by continuous labeling in the presence of extracellular primary and FITC-labeled secondary Fab at 37°C. This protocol describes the determination of CD4Tl, CD4Tl-Ub, and CD4Tl-UbR post-endocytic sorting after 1 hr labeling and 30 min chase at 37°C by the FRIA technique. Although the temporal resolution of this assay is reduced as compared to that described in the Basic Protocol, the lysosomal targeting of CD4Tl-Ub and the highly efficient recycling of CD4Tl and CD4Tl-UbR can be readily demonstrated (see Fig 15.13.2). This assay uses transiently transfected cells, but similar results are obtained in stable cell lines as well (Barriere et al., 2007).

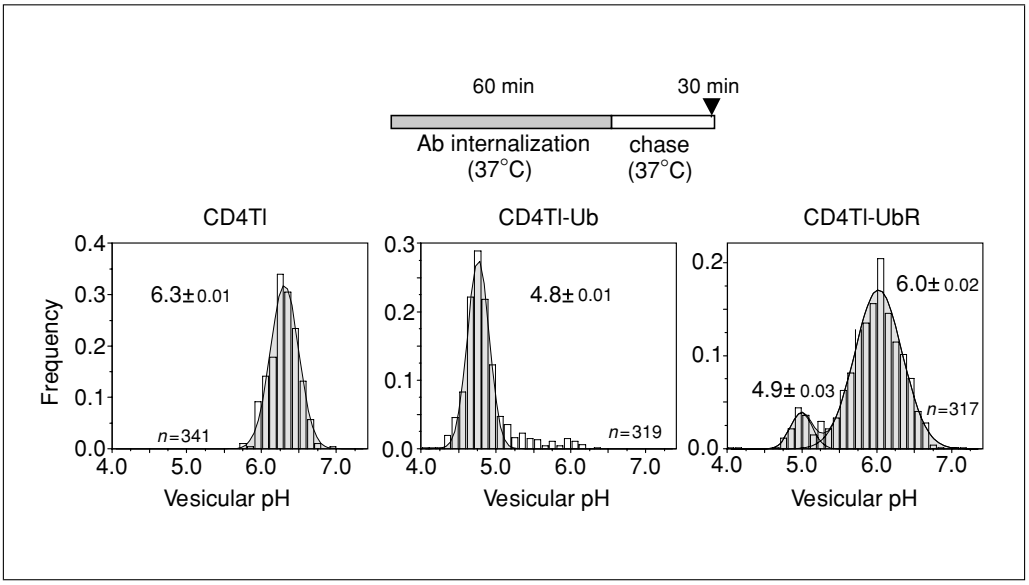


Figure 15.13.2 CD4Tl-, CD4Tl-Ub-, and CD4Tl-UbR-expressing HEK 293 cells were allowed to internalize anti-CD4 primary Ab and FITC-conjugated secondary Fab for 1 hr at 37°C and chased for 30 min in Ab-free medium before FRIA. The mean pH_v (±SEM) and the number of vesicles analyzed are indicated. Similar results were obtained in COS-7 cells.

Additional Materials *(also see Basic Protocol)*

COS-7 *or* HEK 293 cells, 70% confluent in 60-mm dishes
pCDNA3 expression vector encoding CD4Tl, CD4Tl-Ub, or CD4Tl-UbR
Fugene 6 (Roche)

Prepare cells

1. Transfect <70% confluent COS-7 (or HEK 293) cells with the pCDNA3.1 expression vector encoding CD4Tl, CD4Tl-Ub, or CD4Tl-UbR using Fugene6 according to the manufacturer's instruction. Prepare mock-transfected cells for control measurements.

   These constructs have been described in Barriere et al. (2006).

2. After 24 hr of transfection, seed the cells on glass coverslips as described in the Basic Protocol, step 1a.

Label chimeras with pH-sensitive probe

3. Incubate the cells on each coverslip with anti-CD4 antibody and FITC-conjugated goat anti–mouse Fab for 1 hr in 1 ml DMEM-F at 37°C in tissue culture incubator.

   Antibody concentrations are identical to the concentrations used in Basic Protocol steps 3 and 5.

Chase and image

4. Thoroughly wash away the extracellular Ab with 2 ml PBS++ solution at room temperature and resume the incubation of cells in 2 ml DMEM-F for 30 min at 37°C in the tissue culture incubator.

   Different chase periods would provide information about the transport kinetics of the cargo.

5. Terminate the chase and image the coverslip as described in the Basic Protocol, step 10.

6. Carry out single-point pH calibration (see the Basic Protocol, steps 11 to 13).

7. Perform data analysis (see the Basic Protocol, step 14).

SUPPORT PROTOCOL 1

PERFORMING MULTI-POINT IN SITU pH CALIBRATION

To calibrate the fluorescence ratio values as a function of pH_v, FRIA is performed after clamping the luminal pH of intracellular organelles loaded with primary Ab and FITC-conjugated secondary Fab. To optimize signal-to-noise ratio, calibration is usually, but not exclusively, performed on cells expressing a cargo molecule that is retained intracellularly (e.g., CD4Tl-Ub). If the cargo recycles with high efficiency, plasma membrane bound Abs should be removed by an acid wash (see Support Protocol 6 and Troubleshooting). Following the loading of intracellular vesicles with FITC-conjugated Fab, the luminal pH of intracellular compartments is clamped to the extracellular pH in the presence of monensin and nigericin, ionophores that mediate H^+/Na^+ and H^+/K^+ exchange (Harold and Baarda, 1968; Tartakoff et al., 1978). A multi-point calibration curve is obtained by conducting FRIA at various extracellular pH in the range of 7.4 to 4.5. The theoretical basis of this method has been described in Geisow (1984). For each pH value, ~300 vesicles are determined, and the mean pH_v is calculated, based on single-peak Gaussian distribution of pH_v (Fig. 15.13.3A,B). Considering that the pH sensitivity of FITC is reduced at pH <5.4 (pK_a ~6.4), the methodology at pH_v <5.4 can be validated using a mixture of Oregon-Green-dextran (70%) and FITC-dextran (30%). The combination of dyes increases the pH-sensitivity at acid pH, since the Oregon-Green pK_a is ~4.7 (Delmotte and Delmas, 1999). The Oregon-Green/FITC-dextran loading protocol provides similar lysosomal pH_v values to those obtained with FITC-dextran

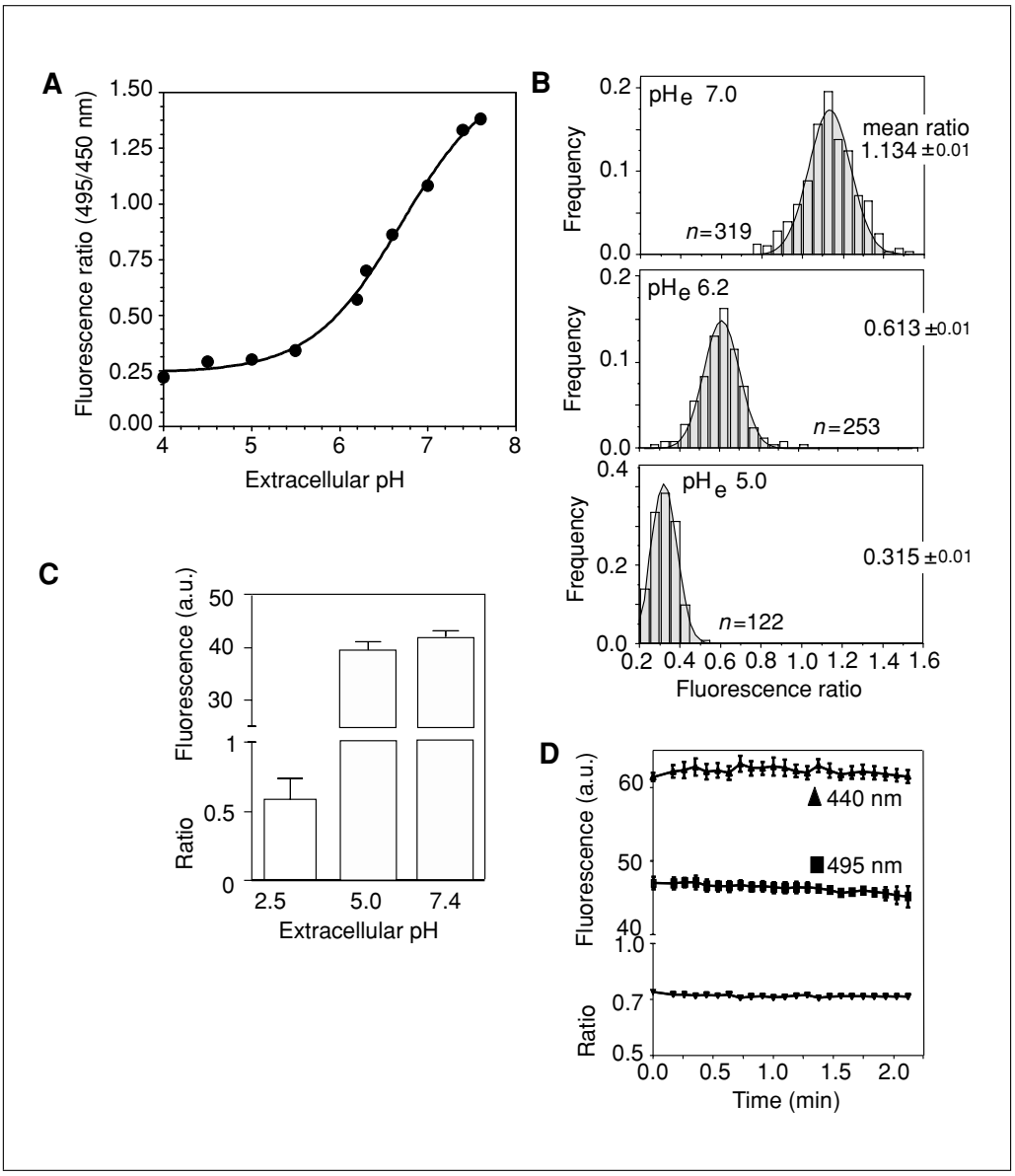


Figure 15.13.3 Calibration curve using CD4TI-Ub transfected HEK 293 cells. (**A**) Anti-CD4 primary Ab and FITC-conjugated secondary Fab are internalized for 1 hr at 37°C and chased for 30 min before FRIA. Calibration is performed as described in Support Protocol 1. (**B**) Distributions of fluorescence ratios of intracellular vesicles after clamping the cells at the indicated extracellular pH (pH_e). (**C**) Measurement of antibody-cargo dissociation in acidic environment. The cell surface antibody binding is determined as described in Support Protocol 6 at the indicated extracellular pH. (**D**) Example of photobleaching control experiment. Fluorescence intensities at 495 nm and 440 nm excitation wavelengths and the measured ratio are recorded every 5 sec for 2-min period in HEK 293 CD4TI-expressing cells. Internalization of Ab was performed as described in the Basic Protocol. Data were obtained on 25 vesicles (means ± SEM).

alone, suggesting that the extrapolating procedure for pH_v <5.4 in the presence of FITC alone is fairly reliable (Barriere et al., 2007).

Additional Materials *(also see Basic Protocol)*

Prism4 software

Prepare cells

1. Prepare the same cell type (stably or transiently expressing) used for cargo trafficking studies in the Basic Protocol (step 1a or 1b).

Prepare calibration solutions

2. Prepare 50-ml aliquots of K^+-rich medium and adjust the pH to 4.5, 5.0, 5.5, 6.0, 6.3, 6.6, 6.9, 7.2, and 7.4. Add nigericin (10 μM) and monensin (10 μM) from 10 mM stocks before the experiment.

   Note that the buffer composition of the calibration medium should be changed according to its final pH. Use 20 mM MES and 20 mM HEPES for medium with pH ≤ 5.5 and pH >5.5, respectively. The pH of the medium should be adjusted with 1 M KOH at 25°C and filter sterilized. Do not store buffers >4 weeks at 4°C and check the medium pH prior to use at 25°C.

Label chimeras with pH-sensitive probe

3. Internalize anti-CD4 primary IgG and FITC-conjugated secondary Fab in complex with CD4Tl-Ub as described in the Alternate Protocol.

   The internalization time could be extended to increase the fluorescence signal-to-noise ratio.

4. Wash the cells three times, each time with 2 ml PBS++ solution at room temperature.

Acquire fluorescence ratio images

5. Place the coverslips in the perfusion chamber and add 0.8 ml K^+-rich calibration medium containing 10 μM nigericin and 10 μM monensin at 25°C.

6. Incubate for 1 to 2 min and replace the medium to facilitate fast equilibration of the intracellular and extracellular pH (identical volume as step 5).Wait an additional 2 to 3 min and proceed with image acquisition by selecting 10 to 20 areas on the coverslip.

   It is critical to use identical or very similar acquisition parameters, including exposure time, light intensities, and camera gain, during the calibration and the FRIA experiment. If the emitted fluorescence light intensities are either below or above the detection limit, adjust the illumination light intensity, camera gain, and/or exposure time appropriately.

Determine the relationship between the fluorescence ratio value and the pH_v in situ

Calculation of the intracellular vesicle pH is based on the relationship between fluorescence ratio and pH_v values, established by the in situ multi-point calibration technique, as described in steps 1 to 5 (Fig. 15.13.3A).

7. Fit the fluorescence ratios of intracellular vesicles clamped to pH 4.5 to 7.4 by nonlinear regression analysis according to a modified version of the Henderson-Hasselbalch equation, as described originally in Geisow (1984):

$$pH_i = pK_a - \log_{10}\left(\frac{R_{max} - R_i}{R_i - R_{min}}\right)$$

   where R_i is the fluorescence ratio ($I_{495\ nm}/I_{440\ nm}$) after background subtraction and R_{max} and R_{min} are the asymptotes of minimum and maximum ratio values at highly acidic and alkaline pH, respectively. The apparent dissociation constant of FITC is indicated by pK_a, and pH_i is the intracellular pH. Rearranging the equation for defining R yields (Erdahl et al., 1995):

$$R_i = \frac{R_{min} + R_{max}10^{(pH_i - pK_a)}}{10^{(pH_i - pK_a)} + 1}$$

8. Based on measured ratio values at different pH, iterate the R_{max} and R_{min} values with the Prism4 (GraphPad Software) software. Calculate the cargo-containing pH_v with Excel, using the extrapolated constants and the calculated R value.

MEASURING THE pH OF RECYCLING ENDOSOMES

SUPPORT PROTOCOL 2

To correlate the pH_v of cargo-containing vesicles with organelle identity in your favorite cellular model, determination of the luminal pH of specific organellar compartments is recommended prior to the FRIA. Support Protocols 2, 3, and 4 describe the pH measurements of recycling endosomes, lysosomes, and the TGN, respectively. Since the recycling pathway of internalized transferrin receptor (TfR) is well documented and ~80% of the cellular TfR pool is confined to recycling endosomes at steady-state (Mukherjee et al., 1997), FITC-transferrin (Tf) is used to monitor the pH of recycling endosomes (Fig. 15.13.4A).

Additional Materials *(also see Basic Protocol)*

FITC-Transferrin f (FITC-Tf; Molecular Probes)
Holotransferrin Sigma, cat. no T-0665)
Ovalbumin (Sigma, cat. no. A-5503)

Prepare cells

1. Prepare the same cell type (stably or transiently expressing) used for cargo trafficking studies in the Basic Protocol (step 1a or 1b).

Load cells with FITC-transferrin

2. Deplete endogenous Tf by incubating COS-7 or HEK 293 cells in 2 ml serum-free DMEM for 45 min at 37°C in a tissue culture incubator.

This protocol could be adapted to any cell lines expressing the Tf receptor.

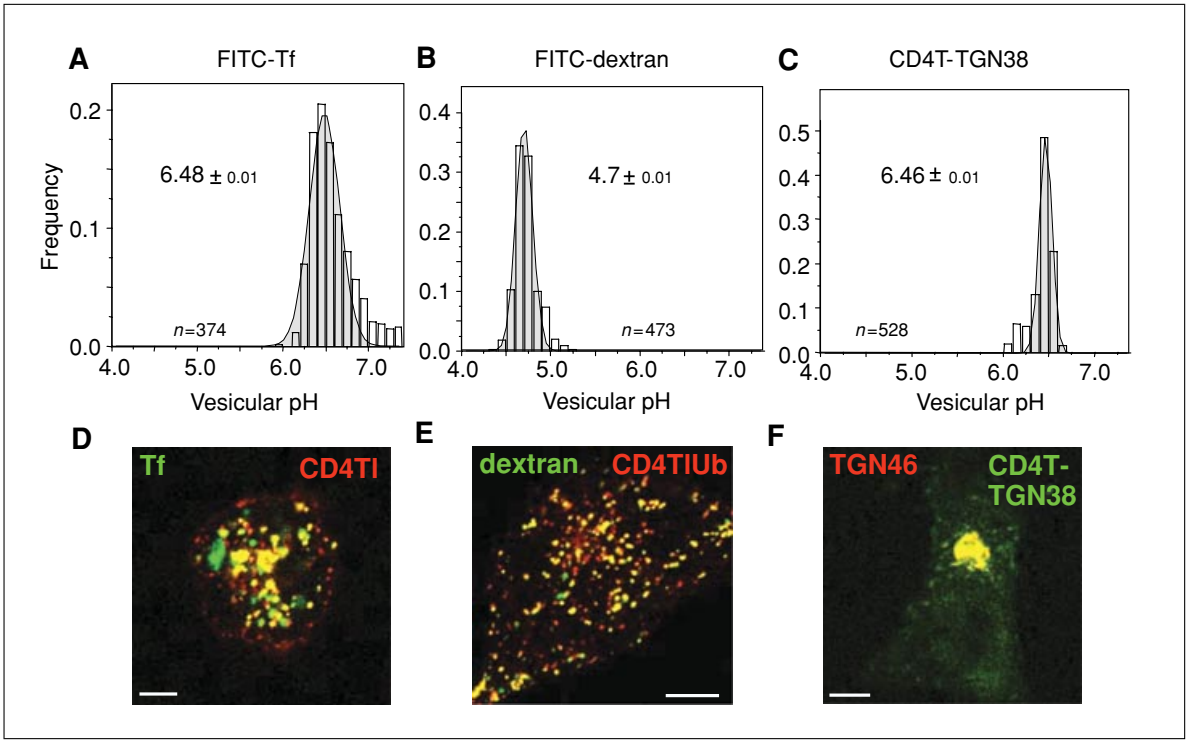


Figure 15.13.4 Colocalization and luminal pH determination studies for recycling endosomes, lysosomes, and the TGN. FRIA was carried out for recycling endosomes (**A**), lysosomes (**B**), and the TGN (**C**) using FITC-conjugated Tf, dextran, and FITC-Ab labeled CD4 as described in Support Protocols 2, 3 and 4 respectively. Subcellular colocalization of internalized CD4TI (**D**), CD4TI-Ub (**E**), and CD4T-TGN38 (**F**) chimeras with the respective organellar markers in HEK 293 cells was performed as described in the Support Protocol 5. Single optical sections were obtained by laser confocal fluorescence microscopy. Bar = 10 μm. For color version of this figure see *http://currentprotocols.com.*

3. Load the cells in 1 ml serum-free DMEM containing 5 μg/ml FITC-Tf for 1 hr at 37°C in the tissue culture incubator.
4. Transfer the cells to ice and rinse four times, each time with 2 ml of H-DMEM-F containing 1 mg/ml ovalbumin and 0.1 mg/ml holotransferrin.

Chase

5. Chase the cells for 2 min to decrease the cell-surface amount of FITC-Tf in 2 ml H-DMEM-F at 37°C.
6. Acquire fluorescence ratio images (see the Basic Protocol, steps 9 to 10).
7. Carry out single-point pH calibration (see the Basic Protocol, steps 11 to 13).
8. Analyze vesicular pH (see the Basic Protocol, steps 14 to 20).

SUPPORT PROTOCOL 3

MEASURING THE pH OF LYSOSOMES

Selective labeling of the lysosomal compartment can be achieved by the fluid-phase marker, FITC-dextran (Lencer et al., 1990; Poet et al., 2006). Following the FITC-dextran uptake, an extended chase in dextran-free medium ensures that early and late endosomes are free of the fluorophore. Colocalization of FITC-dextran loaded lysosomes with Lamp1 can validate the specificity of the dextran labeling (Falcon-Perez et al., 2005; Barriere et al., 2007).

Additional Materials *(also see Basic Protocol)*

FITC-Dextran (mol. wt. 10 kDa anionic; Molecular Probes)
Dextran (mol. wt. 68.8 kDa; cat. no. D4876)

Prepare cells

1. Prepare the same cell type (stably or transiently expressing) used for cargo trafficking studies in the Basic Protocol (step 1a or 1b).

Label lysosomes with FITC-dextran

2. Incubate cells with 5 μg/ml FITC-dextran overnight in 2 ml DMEM-F in the tissue culture incubator at 37°C.

   Filter sterilize the dextran solutions using a 0.22-μm disposable sterile filter unit to avoid contamination of the cells.

3. Rinse the cells three times, each time with 2 ml PBS++ solution.
4. Incubate the cells in 2 ml DMEM-F containing 100 μg/ml dextran for 3 hr in the tissue culture incubator at 37°C.
5. Acquire fluorescence ratio images (see the Basic Protocol, steps 9 to 10).
6. Calibrate the single-point in situ pH (see the Basic Protocol, steps 11 to 13).
7. Analyze vesicular pH (see the Basic Protocol, steps 14 to 20).

   Representative results of pH_v determination of FITC-dextran loaded lysosomes are illustrated in Figure 15.13.4B, top panel.

SUPPORT PROTOCOL 4

MEASURING THE pH OF THE *TRANS*-GOLGI NETWORK (TGN)

Considering the potential overlap in the pH of recycling endosomes and the TGN (Table 15.13.1), it is important to establish the luminal pH of the TGN in the expression system used for the FRIA. In case of overlapping pH_v, immunolocalization studies are required to distinguish between the recycling and retrograde transport route of cargo molecule (see Support Protocol 5).

Internalized TGN38 and furin are targeted via retrograde transport to the TGN from early endosomes. The localization signal of TGN38 and furin has been identified in the cytoplasmic tails using transmembrane reporter molecules (Bosshart et al., 1994; Molloy et al., 1994; Mallet and Maxfield, 1999). Chimeras bearing the TGN localization signal are instrumental in measuring the pH_v of the TGN (Humphrey et al., 1993; Demaurex et al., 1998; Machen et al., 2003). Relying on a similar strategy, we constructed a CD4T-TGN38 chimera to specifically label the TGN with primary anti-CD4 and FITC-conjugated secondary Fab.

Additional Materials *(also see Basic Protocol)*

Expression plasmid encoding the CD4T-TGN38 chimera, pCDNA3.1 (Invitrogen)
Fugene 6 (Roche)

1. Transfect <70% confluent COS-7 (or HEK 293) cells with the pCDNA3.1 expression vector encoding the CD4T-TGN38 chimera using Fugene 6 according to the manufacturer's instruction. Prepare mock-transfected cells for control FRIA measurements.

   The cytoplasmic tail of the TGN38 was fused to the extracellular and transmembrane domain of CD4 (CD4T-TGN38) as described for the Tac-TGN38 chimera (Bosshart et al., 1994). The CD4T construct was described in Barriere et al. (2006). Significant colocalization of the CD4T-TGN38 chimera with the endogenous TGN46 verified that the chimera was accumulated in the TGN (Figure 15.13.4C,F, H. Barriere, unpub. observ.).

2. After 24 hr of transfection, seed the cells on glass coverslips as described in the Basic Protocol, step 1a.

3. Incubate the cells with anti-CD4 primary Ab (1:500 dilution) and FITC-conjugated goat anti–mouse Fab (1:500 dilution) for 4 to 6 hr in 1 ml DMEM-F to label the TGN.

   Extended labeling is necessary due to the limited expression and slow exocytosis/endocytosis of the chimera.

4. To eliminate the Ab from the cell surface, wash the cells twice, each time with 2 ml PBS++ solution and incubate with 2 ml Ab-free DMEM-F at 37°C for 1 hr.

5. Acquire fluorescence ratio images (see the Basic Protocol, steps 9 to 10).

6. Perform single-point pH calibration (see the Basic Protocol, steps 11 to 13).

7. Analyze vesicular pH (see the Basic Protocol, steps 14 to 20).

   Representative results of the TGN pH_v determination are illustrated in Figure 15.13.4C.

VERIFYING THE POST-ENDOCYTIC FATE OF INTERNALIZED MEMBRANE PROTEIN BY IMMUNOLOCALIZATION

SUPPORT PROTOCOL 5

Subcellular distribution of internalized cargo, determined by FRIA, should be validated by colocalization studies using organelle markers. To selectively visualize the internalized cargo pool, endocytosed molecule is labeled with the Ab capture assay as described for the FRIA experiment (Alternate Protocol). Although both recycling endosomes and lysosomes could be labeled with FITC-Tf and FITC-dextran uptake as described in Support Protocols 2 and 3, respectively, these organelles, similar to the TGN, could be also identified by indirect immunostaining. Detection of organelle-specific antigens, e.g., TfR or rab11 (recycling endosomes), Lamp1, Lamp2 and CD63 (MVB/lysosomes), and TGN46 or TGN38 (TGN), can be performed by commercially available primary and secondary antibodies. Use identical internalization and chase period as for the FRIA studies to ascertain that colocalization results are comparable to that of pH_v measurements.

Additional Materials *(also see Basic Protocol)*

Antibodies:
Anti-TGN46 (AHP500; AbD Serotec)
TRITC-conjugated goat anti-mouse Fab (Jackson ImmunoResearch Laboratories)
TRITC-Tf (Jackson ImmunoResearch Laboratories)
4% (v/v) paraformaldehyde solution (see recipe)
1 M glycine containing PBS++ solution (see recipe)
Mounting medium (e.g., Vectashield H-1200, Vector Laboratories)
Laser confocal fluorescence microscope (e.g., LSM 510 Carl Zeiss)

Prepare cells

1. Prepare the relevant cells and transiently transfect (as described in the Basic Protocol, step 1b) with the CD4T-TGN38 expression construct.

Label internalized cargo by Ab capture

2. Internalize the relevant primary and secondary Abs complexed with cargo molecule of interest as described in the Alternate Protocol.

   The use of secondary TRITC- or Cy3-conjugated Fab is recommended to minimize photobleaching. If only mouse primary Ab is available for organelle identification, immunostaining should be performed with rabbit anti-CD4 Ab.

3. Load recycling endosomes and lysosomes with FITC-Tf and FITC-dextran as described in Support Protocols 2 and 3.

   It's possible to label two different cargoes simultaneously. For example during the 45 min TRITC-Tf (5 μg/ml) loading period, CD4Tl labeling can be carried out for the first 15 min with anti-CD4 Ab.

Chase

4. Incubate the cells in 2 ml Ab-free medium for 30 min or as performed in the FRIA experiment.

Fix the cells

5. Wash the cells twice, each time with 2 ml ice-cold PBS++ solution.
6. Fix the cells in 1 ml 4% paraformaldehyde (PFA) solution for 10 min at room temperature.
7. Wash the cells three times (5 min each), each time with 2 ml PBS++ solution, then once with 0.1 M glycine containing PBS++ solution to quench the PFA.
8. Mount the cells in mounting medium.

Acquire fluorescence confocal images

9. Collect single optical sections with the laser confocal fluorescence microscope.
10. Analyze the extent of colocalization quantitatively using an appropriate software program (e.g., Volocity, PerkinElmer).

SUPPORT PROTOCOL 6 DETERMINING THE ANTIBODY-CARGO COMPLEX STABILITY IN ACIDIC ENVIRONMENTS

Ab dissociation from cargo molecules in the acidic environment of the endosomal compartment may compromise the validity of the FRIA. To assess the primary and secondary Ab dissociation from CD4 chimeras (or other cargo) in an acidic environment mimicking the late endosomes, cell surface Ab binding is measured at different extracellular pH by an ELISA-based immunoperoxidase assay with AmplexRed as the fluorescent substrate.

The results can also be informative regarding the necessary and sufficient extracellular H^+ concentration to strip excessive Ab binding from the plasma membrane in FRIA studies.

Additional Materials *(also see Basic Protocol)*

HRP-conjugated anti–mouse Fab antibody (Jackson ImmunoResearch Laboratories)
Acetic acid buffer adjusted to pH 2.5 and 5.0
Recovery buffer (see recipe)
AmplexRed kit (Molecular Probes, cat. no. A22170)
BCA assay kit (BioRad)

12-well culture plates
96-well black plates (Greiner)
Fluorescent plate reader (e.g., POLARstar OPTIMA; BMG Labtech) with 560 and 590 nm excitation and emission wavelengths, respectively
Spectrophotometer

Prepare cells

1. Seed ($\sim 2 \times 10^5$ cells/well) mock- and CD4Tl-transfected HEK 293 cells with 1 ml DMEM-F in a 12-well plate 24 hr before the start of the experiment to obtain an 80% to 90% confluent culture the next day.

   Each measurement is done in triplicate. Nonspecific binding of the primary and secondary Ab is measured in mock-transfected cells.

Label cell surface resident cargo molecules with antibody

2. Rinse cells twice, each time with 1.5 ml ice-cold H-DMEM-F.

   All rinses should be gentle to avoid the loss of cells.

3. Add anti-CD4 Ab (1:500 dilution) in 0.6 ml H-DMEM-F and incubate the cells on ice for 60 min.

4. Wash cells four times, each time with 1.5 ml ice-cold PBS++ solution.

5. Incubate with HRP-conjugated secondary Fab (1:3000 to 1:10,000 dilution) in 0.6 ml H-DMEM-F for 60 min on ice.

   The dilution of the secondary antibody should be optimized in pilot studies to maximize the signal-to-noise ratio.

6. Wash cells six times, each time with 1.5 ml ice-cold PBS++ solution.

Perform an acid wash

7. Incubate the cells in 1 ml ice-cold acetic acid buffer at pH 5.0 and 2.5 and in H-DMEM-F for 2 min.

8. Wash twice, each time with 1.5 ml ice-cold PBS++ solution.

9. Incubate cells 3 min in 1 ml cold recovery buffer.

10. Repeat steps 7 to 9 three to four times.

Measure the cell surface bound antibody

11. Aspirate the remaining buffer and add 250 µl of the AmplexRed assay working solution prepared according to the manufacturer's description.

12. Incubate the reaction on ice for 5 to 20 min.

    Wrap the plate with aluminum foil to avoid photobleaching.

13. Transfer 100 µl of the reaction mixture to a 96-well black plate.

14. Determine the fluorescence signal in the 96-well plate using a fluorescence plate reader at 560 nm excitation and 590 nm emission wavelengths.
15. Determine the cellular protein content in each well using the BCA assay kit according to the manufacturer's instruction.
16. Normalize the fluorescence intensity by the cellular protein content.
17. Calculate the specific Ab binding. Subtract the background fluorescence, measured on mock-transfected cells, from the total fluorescence signal.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetic acid buffer

0.2 M acetic acid
0.2 M NaCl
Adjust to pH 2.5 and 5.0 with 1 N NaOH
Store up to 5 days at 4°C

K^+-rich buffer (for in situ pH calibration)

10 mM NaCl
135 mM KCl
10 mM glucose
1 mM $CaCl_2$
1 mM $MgCl_2$
20 mM MES for pH $\leq$5.5
20 mM HEPES for pH >5.5
Adjust pH with 1 N KOH
Store up to 3 weeks at 4°C

Modified Ringer's solution

140 mM NaCl
5 mM KCl
10 mM HEPES
10 mM glucose
1 mM $CaCl_2$
1 mM $MgCl_2$
Adjust the pH to 7.3 using 1 N NaOH
Store up to 3 weeks at 4°C

Paraformaldehyde solution 4%

Add 1g paraformaldehyde (PFA; Sigma Aldrich) to 20 ml PBS
Heat to 65°C dissolve
Add 750 µl of 1 M sucrose
Adjust volume to 25 ml with PBS
Filter and store the solution in the dark up to 1 week at 4°C

PBS++ solution

PBS 1× (*APPENDIX 2A*)
1 mM $CaCl_2$
1 mM $MgCl_2$
5% BSA
Store up to 2 weeks at 4°C

Recovery buffer

150 mM NaCl
20 mM HEPES
1 mM $CaCl_2$
5 mM KCl
1 mM $MgCl_2$
Adjust to pH 7.4 with 1 N NaOH
Store up to 3 weeks at 4°C

COMMENTARY

Background Information

Each of the endo-lysosomal and biosynthetic compartments has a characteristic luminal pH that is essential for its physiological function (Mukherjee et al., 1997). The luminal pH of these organelles can be monitored after selective loading with pH-sensitive fluorophores (e.g., FITC), using quantitative fluorescence ratio image analysis (Ohkuma and Poole, 1978; Maxfield, 1989). In light of the tightly regulated pH_v of organelles, measuring the pH_v of cargo-containing vesicles can reveal the trafficking pathway of internalized membrane proteins. Either FITC-conjugated antibody or a specific ligand (e.g., Tf and EGF) with high affinity binding to the extracellular domain of cargo molecules can serve as a selective label. If the cell surface density of the cargo molecule is high, direct labeling with FITC-conjugated primary IgG or monovalent Fab is recommended to minimize cargo oligomerization. If monovalent primary antibodies are not available, the possible side effect of cross-linking has to be evaluated by comparing the antibody complexed cargo trafficking to that of the native molecule. These experiments help to rule out the possibility that oligomerization provokes lysosomal targeting as described for the cross-linked TfR (Weissman et al., 1986). If the plasma membrane cargo density is low and the intracellular pool is relatively small, amplification of the fluorescence signal intensity can be achieved by indirect staining with primary and FITC-conjugated secondary Fab, as described in the Basic and Alternate Protocols. Since complex formation of OKT4 and secondary Ab with CD4 had no influence on the internalization and trafficking of CD4 (Barriere et al., 2007), we choose to use the indirect labeling approach to amplify the fluorescent signal. The pH-sensitive fluorophore can also be attached to high-affinity ligands (e.g., Tf and EGF), as illustrated in Support Protocol 2.

Endosomal acidification induces the dissociation of a subset of ligands from their receptors, followed by the receptor delivery to the cell surface or TGN and the ligand into lysosomes (DiPaola and Maxfield, 1984; Davis et al., 1987). Similarly, Ab dissociation from cargo molecule may take place in acidic endosomes, compromising the validity of the assay. Therefore, the stability of FITC-conjugated Ab association with cargo has to be verified under acidic conditions. Support Protocol 6 describes the assessment of the stability of Ab-cargo complex at an acidic pH. Another caveat of the FRIA technique is that the pH_v of the TGN and the recycling endosomes may overlap (pH_v ~6.2 to 6.8), depending on the cell type and pH detection method (see Table 15.13.1). Therefore, assessment of the pH_v of the TGN and recycling endosomes is recommended in the specific experimental model. If the pH_v of recycling endosomes and the TGN pH_v overlap, co-immunolocalization of internalized cargo molecules with markers of recycling endosomes (e.g., TfR and rab11) and the TGN (e.g., TGN46) is necessary to distinguish between cargo destinations (Support Protocol 5). As a complementary approach, the recycling rate of cargo molecules can be measured as described for CD4-Ub chimeras (Barriere et al., 2007).

Critical Parameters and Troubleshooting

The sensitivity of the FRIA technique depends on the receptor/cargo cell surface density, internalization and recycling rate, copy number in the individual vesicle, the signal intensity of the fluorescent probe, and the fluorescence detection efficiency of the imaging system. Since the fluorescence excitation light intensity should be kept at a minimum to avoid photobleaching, it is important to use a cooled CCD camera with high quantum efficiency for image acquisition. Considering the possibility that overexpression may interfere with the intracellular itinerary of cargo by saturating the relevant sorting machinery, the cargo expression should be carefully optimized in

heterologous expression systems (Marks et al., 1996). Comparison of exogenous and endogenous protein trafficking is necessary to validate data obtained by overexpression and to demonstrate that the sorting pathway is independent of the expression level. The adverse effect of transient overexpression can be minimized by selecting stably transfected cell lines with appropriate transgene level. Control experiments should verify that heterologous expression does not interfere with the sorting pathway and kinetics of other transmembrane polypeptides with different destinations (e.g., TfR and Lamp1).

Besides enhancing cargo expression level to some extent, the detection sensitivity can be increased by adjusting the camera gain and/or extending the exposure time to fluorescence excitation light. The former method is preferred, considering that extensive illumination results in the bleaching of FITC and the loss of pH sensitivity. If the recycling efficiency is limited, the internal cargo pool can be enhanced by extending the internalization time as described in the Alternate Protocol. Selecting the Ab with the highest specific fluorescence label or ligand with the lowest nonspecific binding also improves the signal-to-noise ratio. Ab should be stored in 50% glycerol at −20°C, if not otherwise recommended. If the Ab is sensitive to glycerol, freeze the Ab in small aliquots. After thawing, the antibody can be stored for up to 5 days at 4°C. Finally, stored Ab may aggregate, and that could lead to a nonspecific signal. Aggregates can be cleared by centrifugation in a benchtop microcentrifuge at maximum speed before labeling the cell.

Reliable data acquisition requires at least a two-fold higher signal above the background fluorescence. A number of approaches can enhance the low signal-to-noise ratio. (1) Optimization of Ab dilution in pilot experiments should help to select the primary and secondary Ab concentrations and medium composition to maximize the specific signal over the background fluorescence. (2) The concentration dependence of various serum preparations (e.g., FBS and BS) and other blocking agents (e.g., BSA and ovalbumin) should be evaluated to curtail nonspecific antibody or ligand binding. (3) The medium should be free of phenol red to reduce autofluorescence. (4) Increasing the cell surface expression of cargo molecule within a reasonable limit (but see possible adverse effects above) can be accomplished by using a transient expression system and/or nonspecific transcriptional activators e.g., by pre-incubating the cells in the presence of Na-butyrate (1 to 4 mM) for 24 hr (Loffing et al., 1999; Moyer et al., 1999). (5) Increasing internalization time may increase the fluorescence signal by enhancing the labeled cargo pool size, although this would diminish the kinetic resolution of the FRIA. (6) Photo-damage of the excitation fluorescence filter set diminishes the signal-to-noise ratio. Regular visual examination of filters and replacing them in a timely manner should address this problem. (7) Proper adjustment of the camera gain, excitation light intensity, and exposure time may contribute to obtaining high-quality images. (8) Finally, it is important to work with cell lines that are free of contamination. Even latent mycoplasma infection can dramatically reduce transfectability and specific Ab binding of the cell.

Although FITC is relatively sensitive to photobleaching, the pK_a of FITC renders this fluorophore one of the best candidates for monitoring the pH of early and late endocytic compartments. However, precautions should be taken to avoid FITC photobleaching. To this end, the illumination light intensity and the exposure time of the sample should be minimized, while the cooled CCD gain is increased. Narrowing the excitation filter bandwidth and/or application of neutral density filters could also reduce the excitation light intensity and photobleaching. Verification that photobleaching is not a confounding factor during the course of the experiment should be demonstrated by successive image acquisition of the same field in a relatively short period of time, and plotting the fluorescence light intensities at 440 and 495 nm excitation wavelengths, as well as the fluorescence ratio values (Fig. 15.13.3D).

When using antibody labeling as a fluorescent probe, verify that the Ab does not modify the trafficking pathway of the respective cargo using e.g., coimmunolocalization and metabolic pulse-chase experiment (Subtil et al., 1994), since Ab-mediated cross-linking may direct plasma membrane proteins for lysosomal degradation. If this is the case, application of FITC-labeled monovalent Fab may rectify the problem (Weissman et al., 1986).

Anticipated Results

The FRIA method discussed in this unit has been used previously to follow the post-endocytic fate of various plasma membrane proteins in living cells (Piguet et al., 1999; Sharma et al., 2004; Barriere et al. 2007). We have utilized FRIA to demonstrate profound

changes in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel endocytic sorting by the most prevalent CF-causing mutation, the deletion of the F508 amino acid residue (Sharma et al., 2004). Although the mutant CFTR is largely trapped in the ER, small amounts can be accumulated at the cell surface at reduced temperatures in the presence of chemical chaperone. Monitoring the intracellular trafficking of CFTR by FRIA clearly showed that the mutation provoked the efficient lysosomal accumulation of the internalized channel, in contrast to its wild-type counterpart that was targeted back to the cell surface (Sharma et al., 2004). In these studies CFTR was labeled with anti-HA primary and FITC-conjugated secondary antibody, taking advantage of the 3HA exofacial epitope in CFTR (Sharma et al., 2004). Predominant accumulation of the mutant in a highly acidic vesicular compartment was indicated by the single peak Gaussian distribution and mean pH_v (5.5 $\pm$ 0.1) of labeled CFTR-containing vesicles, as anticipated based on its accelerated metabolic turnover at the cell surface (Sharma et al., 2004). Supporting evidence for MVB/lysosomal accumulation was provided by the mutant channel coimmunolocalization with lysosomal markers and its severely attenuated recycling (Sharma et al., 2004). In sharp contrast, endocytosed wild-type CFTR was detected in a vesicular compartment with mean pH_v of 6.5 $\pm$ 0.1, consistent with its sorting into the recycling endosomes. This inference and the lack of accumulation of the wild-type CFTR in the TGN was substantiated by immunocolocalization with transferrin, a marker of the recycling endosomes and by direct determination of the fast recycling of internalized wild-type CFTR (Sharma et al., 2004).

Other examples for the utility of FRIA shown in this unit and published by Barriere et al. (2007) demonstrate that the technique was instrumental in elucidating the molecular requirement of ubiquitination as a lysosomal sorting signal. This experimental paradigm took advantage of the autonomous and transferable nature of lysosomal sorting signals (Bonifacino and Traub, 2003), enabling us to generate CD4 chimeras with unextendable mono-Ub, poly-Ub chain, and tetrameric mono-Ub, but it could be applied for other endocytic sorting signals as well. Rapid and high-fidelity lysosomal accumulation of the CD4 reporter molecule as detected by FRIA was indicated by the single peak Gaussian pH_v distribution and mean pH_v (4.9$\pm$0.01) for chimeras harboring poly-Ub chain or the tetrameric mono-Ub (Fig. 15.13.1 and Fig. 15.13.2; Barriere et al., 2007). In contrast, the CD4 chimera with unextendable mono-Ub accumulated in the recycling compartment, suggested by the mean pH_v (6.0$\pm$0.02, Fig. 15.13.2; Barriere et al., 2007). As pointed out in preceding paragraphs, the mildly acidic pH_v ($\sim$6.2 to 6.6) could indicate cargo accumulation in recycling endosomes or the TGN (see Table 15.13.1). Therefore, complementary assays were required to distinguish between cargo destination to TGN and/or recycling endosomes. Co-immunolocalization with organelle markers (see Support Protocol 5) and direct monitoring of the recycling efficiency of the CD4-UbR demonstrated that the chimera with unextendable Ub was predominantly routed toward the recycling compartment (Barriere et al., 2007). These examples provide compelling evidence that the FRIA technique in concert with complementary methods can serve to elucidate endocytic cargo destinations, as well as the molecular composition of autonomous sorting signals. Finally, using the synchronized labeling protocol and the FRIA technique, valuable information can be obtained for interorganellar transport kinetics of endocytic cargo as described in the Basic Protocol (Fig. 15.13.1). Frequent sampling with FRIA during the chase period can reveal the kinetics of cargo transfer from early endosomes (pH_v $\sim$5.9 to 6.0) to late endosomes (pH_v $\sim$5.4 to 6.0) and subsequent accumulation in multivesicular bodies/lysosomes (pH_v <5.5; Fig. 15.13.2).

Time Considerations

Each individual experiment is easily performed within 1 day if the cell line constitutively expresses the cargo. A half-day should be reserved for data analysis.

Literature Cited

Barriere, H., Nemes, C., Lechardeur, D., Khan-Mohammad, M., Fruh, K., and Lukacs, G.L. 2006. Molecular basis of oligoubiquitin-dependent internalization of membrane proteins in Mammalian cells. *Traffic*. 7:282-297.

Barriere, H., Nemes, C., Du, K., and Lukacs, G.L. 2007. Plasticity of Poly-Ubiquitin Recognition as Lysosomal Targeting Signals by the Endosomal Sorting Machinery. *Mol. Biol. Cell.* 18:3952-3965.

Bonifacino, J.S. and Rojas, R. 2006. Retrograde transport from endosomes to the trans-Golgi network. *Nat. Rev. Mol. Cell Biol.*. 7:568-579.

Bonifacino, J.S. and Traub, L.M 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biochem.* 72:395-447.

Bosshart, H., Humphrey, J., Deignan, E., Davidson, J., Drazba, J., Yuan, L., Oorschot, V., Peters, P.J., and Bonifacino, J.S. 1994. The cytoplasmic domain mediates localization of furin to the trans-Golgi network en route to the endosomal/lysosomal system. *J. Cell Biol.* 126:1157-1172.

Davis, C.G., Goldstein, J.L., Sudhof, T.C., Anderson, R.G., Russell, D.W., and Brown, M.S. 1987. Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature*. 326:760-765.

Delmotte, C. and Delmas, A. 1999. Synthesis and fluorescence properties of Oregon Green 514 labeled peptides. *Bioorg. Med. Chem. Lett.* 9:2989-2994.

Demaurex, N., Furuya, W., D'Souza, S., Bonifacino, J.S., and Grinstein, S. 1998. Mechanism of acidification of the trans-Golgi network (TGN). In situ measurements of pH using retrieval of TGN38 and furin from the cell surface. *J. Biol. Chem.* 273:2044-2051.

DiPaola, M. and F.R. Maxfield. 1984. Conformational changes in the receptors for epidermal growth factor and asialoglycoproteins induced by the mildly acidic pH found in endocytic vesicles. *J. Biol. Chem.* 259:9163-9171.

Erdahl, W.L., Chapman, C.J., Taylor, R.W., and Pfeiffer, D.R. 1995. Effects of pH conditions on Ca2 +transport catalyzed by ionophores A23187, 4-BrA23187, and ionomycin suggest problems with common applications of these compounds in biological systems. *Biophys. J.*. 69:2350-2363.

Falcon-Perez, J.M., Nazarian, R., Sabatti, C., and Dell'Angelica, E.C. 2005. Distribution and dynamics of Lamp1-containing endocytic organelles in fibroblasts deficient in BLOC-3. *J. Cell Sci.* 118:5243-5255.

Farinas, J. and Verkman, A.S. 1999. Receptor-mediated targeting of fluorescent probes in living cells. *J. Biol. Chem.* 274:7603-7606.

Geisow, M.J. 1984. Fluorescein conjugates as indicators of subcellular pH. A critical evaluation. *Exp. Cell Res.* 150:29-35.

Ghosh, P., Dahms, N.M., and Kornfeld, S. 2003. Mannose 6-phosphate receptors: New twists in the tale. *Nat. Rev. Mol. Cell Biol.* 4:202-212.

Harold, F.M. and Baarda, J.R. 1968. Effects of nigericin and monactin on cation permeability of Streptococcus faecalis and metabolic capacities of potassium-depleted cells. *J. Bacteriol.* 95:816-823.

Humphrey, J.S., Peters, P.J., Yuan, L.C., and Bonifacino, J.S. 1993. Localization of TGN38 to the trans-Golgi network: Involvement of a cytoplasmic tyrosine-containing sequence. *J. Cell Biol.* 120:1123-1135.

Janvier, K. and Bonifacino, J.S. 2005. Role of the endocytic machinery in the sorting of lysosome-associated membrane proteins. *Mol. Biol. Cell.* 16:4231-4242.

Katzmann, D.J., Odorizzi, G., and Emr, S.D.. 2002. Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell Biol.* 3:893-905.

Kim, J.H., Lingwood, C.A., Williams, D.B., Furuya, W., Manolson, M.F., and Grinstein, S. 1996. Dynamic measurement of the pH of the Golgi complex in living cells using retrograde transport of the verotoxin receptor. *J. Cell Biol.* 134:1387-1399.

Lencer, W.I., Weyer, P., Verkman, A.S., Ausiello, D.A., and Brown, D. 1990. FITC-dextran as a probe for endosome function and localization in kidney. *Am. J. Physiol.* 258:C309-C317.

Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G., and Tsien, R.Y. 1998. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. U.S.A.* 95:6803-6808.

Loffing, J., Moyer, B.D., Reynolds, D., and Stanton, B.A.. 1999. PBA increases CFTR expression but at high doses inhibits Cl(-) secretion in Calu-3 airway epithelial cells. *Am. J. Physiol.* 277:L700-L708.

Lukacs, G.L., Segal, G., Kartner, N., Grinstein, S., and Zhang, F. 1997. Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation. *Biochem. J.*. 328:353-361.

Machen, T.E., Leigh, M.J., Taylor, C., Kimura, T., Asano, S., and Moore, H.P. 2003. pH of TGN and recycling endosomes of H+/K+-ATPase-transfected HEK-293 cells: Implications for pH regulation in the secretory pathway. *Am. J. Physiol. Cell Physiol.*. 285:C205-C214.

Mallet, W.G. and Maxfield, F.R. 1999. Chimeric forms of furin and TGN38 are transported with the plasma membrane in the trans-Golgi network via distinct endosomal pathways. *J. Cell Biol.* 146:345-359.

Marks, M.S., Woodruff, L., Ohno, H., and Bonifacino, J.S.. 1996. Protein targeting by tyrosine- and di-leucine-based signals: Evidence for distinct saturable components. *J. Cell Biol.* 135:341-354.

Maxfield, F.R. 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.* 95:676-681.

Maxfield, F.R. 1989. Measurement of vacuolar pH and cytoplasmic calcium in living cells using fluorescence microscopy. *Methods Enzymol*. 173:745-771.

Maxfield, F.R. and McGraw, T.E. 2004. Endocytic recycling. *Nat. Rev. Mol. Cell Biol.* 5:121-132.

Molloy, S.S., Thomas, L., VanSlyke, J.R., Stenberg, P.E., and Thomas, G. 1994. Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *EMBO J*. 13:18-33.

Moyer, B.D., Loffing-Cueni, D., Loffing, J., Reynolds, D., and Stanton, B.A. 1999. Butyrate increases apical membrane CFTR but reduces chloride secretion in MDCK cells. *Am. J. Physiol.* 277:F271-F276.

Mukherjee, S., Ghosh, R.N., and Maxfield, F.R. 1997. Endocytosis. *Physiol. Rev.* 77:759-803.

Ohkuma, S. and Poole, B. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. U.S.A.* 75:3327-3331.

Piguet, V., Gu, F., Foti, M., Demaurex, N., Gruenberg, J., Carpentier, J.L., and Trono, D. 1999. Nef-induced CD4 degradation: A diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes. *Cell*. 97:63-73.

Poet, M., Kornak, U., Schweizer, M., Zdebik, A.A., Scheel, O., Hoelter, S., Wurst, W., Schmitt, A., Fuhrmann, J.C., Planells-Cases, R., Mole, S.E., Hubner, C.A., and. Jentsch, T.J. 2006. Lysosomal storage disease upon disruption of the neuronal chloride transport protein ClC-6. *Proc. Natl. Acad. Sci. U.S.A*. 103:13854-13859.

Presley, J.F., Mayor, S., Dunn, K.W., Johnson, L.S., McGraw, T.E., and Maxfield, F.R. 1993. The End2 mutation in CHO cells slows the exit of transferrin receptors from the recycling compartment but bulk membrane recycling is unaffected. *J. Cell Biol.* 122:1231-1241.

Seksek, O., Biwersi, J., and Verkman, A.S. 1995. Direct measurement of trans-Golgi pH in living cells and regulation by second messengers. *J. Biol. Chem.* 270:4967-4970.

Sharma, D.K., Choudhury, A., Singh, R.D., Wheatley, C.L., Marks, D.L., and Pagano, R.E. 2003. Glycosphingolipids internalized via caveolar-related endocytosis rapidly merge with the clathrin pathway in early endosomes and form microdomains for recycling. *J. Biol. Chem.* 278:7564-7572.

Sharma, M., Pampinella, F., Nemes, C., Benharouga, M., So, J., Du, K., Bache, K.G., Papsin, B., Zerangue, N., Stenmark, H., and Lukacs, G.L. 2004. Misfolding diverts CFTR from recycling to degradation: Quality control at early endosomes. *J. Cell Biol.* 164:923-933.

Subtil, A., Hemar, A., and Dautry-Varsat, A. 1994. Rapid endocytosis of interleukin 2 receptors when clathrin-coated pit endocytosis is inhibited. *J. Cell Sci.* 107:3461-3468.

Sun-Wada, G.H., Wada, Y., and Futai, M. 2004. Diverse and essential roles of mammalian vacuolar-type proton pump ATPase: Toward the physiological understanding of inside acidic compartments. *Biochim. Biophys. Acta.* 1658:106-114.

Tartakoff, A., Vassalli, P., and Detraz, M. 1978. Comparative studies of intracellular transport of secretory proteins. *J. Cell Biol.* 79:694-707.

Tycko, B. and Maxfield, F.R. 1982. Rapid acidification of endocytic vesicles containing alpha 2-macroglobulin. *Cell*. 28:643-51.

von Zastrow, M. and Sorkin, A. 2007. Signaling on the endocytic pathway. *Curr. Opin. Cell Biol.* 19:436-445.

Weissman, A.M., Klausner, R.D., Rao, K., and Harford, J.B.. 1986. Exposure of K562 cells to anti-receptor monoclonal antibody OKT9 results in rapid redistribution and enhanced degradation of the transferrin receptor. *J. Cell Biol.* 102:951-958.

Wu, M.M., Llopis, J., Adams, S., McCaffery, J.M., Kulomaa, M.S., Machen, T.E., Moore, H.P., and Tsien, R.Y. 2000. Organelle pH studies using targeted avidin and fluorescein-biotin. *Chem. Biol.* 7:197-209.

Wu, M.M., Grabe, M., Adams, S., Tsien, R.Y., Moore, H.P., and Machen, T.E. 2001. Mechanisms of pH regulation in the regulated secretory pathway. *J. Biol. Chem.* 276:33027-33035.

Yamashiro, D.J., Tycko, B., Fluss, S.R., and Maxfield, F.R. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell*. 37:789-800.

Quantitative Analysis of Endocytosis and Turnover of Epidermal Growth Factor (EGF) and EGF Receptor

Alexander Sorkin[1] and Jason E. Duex[1]

[1]Department of Pharmacology, University of Colorado Denver Medical School, Aurora, Colorado

ABSTRACT

Binding of epidermal growth factor (EGF) to the EGF receptor (EGFR) initiates signal transduction, ultimately leading to altered gene expression. Ligand-activated EGFR is also rapidly internalized and then targeted to lysosomes for degradation or recycled back to the plasma membrane. Endocytosis is a major regulator of EGFR signaling. Therefore, elucidation of the mechanisms of EGFR endocytosis is essential for a better understanding of EGFR biology. In order to achieve a comprehensive analysis of these mechanisms, reliable methods for measuring the rates of EGFR protein turnover and the rate parameters for individual steps of EGFR endocytic trafficking must be employed. The protocols in this unit describe methodologies to measure the rates of EGFR synthesis and degradation, to monitor EGF-induced down-regulation of surface EGFR, to measure the kinetic rate parameters of internalization, recycling, and degradation of radiolabeled EGF, and to perform radioiodination of EGF by the chloramine T method. *Curr. Protoc. Cell Biol.* 46:15.14.1-15.14.20. © 2010 by John Wiley & Sons, Inc.

Keywords: EGF receptor • endocytosis • recycling • degradation • synthesis

INTRODUCTION

At the molecular level, binding of a growth factor to EGFR triggers several signal-transduction cascades, ultimately leading to altered gene expression. There are at least six ligands of EGFR, with EGF being the best characterized. Upon ligand binding, activated EGFR is rapidly internalized through clathrin-dependent and -independent pathways of endocytosis. After internalization to early endosomes, ligand-receptor complexes are either retained in endosomes during their maturation into late endosomes or recycled back to the plasma membrane. Acceleration of internalization and degradation of EGFR by EGF results in dramatic decreases in the amount of EGFR at the cell surface (termed ligand-induced receptor down-regulation) and an eventual decrease of total cellular EGFR protein. The efficiency of receptor targeting to late endosomes and subsequent degradation by lysosomal enzymes determines the overall rate of ligand-induced receptor down-regulation.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Current Protocols in Cell Biology 15.14.1-15.14.20, March 2010
Published online March 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1514s46

BASIC PROTOCOL 1

MEASURING EGFR PROTEIN SYNTHESIS

The turnover rate of a protein is a function of its rates of biosynthesis and degradation. Transmembrane proteins like EGFR are synthesized by ribosomal complexes that are associated with the endoplasmic reticulum (ER), allowing for the simultaneous translocation of the nascent transmembrane protein through the ER membrane. EGFR is N-glycosylated in the Golgi complex and then delivered to the plasma membrane (Todderud and Carpenter, 1989). Fully glycosylated EGFR protein runs on SDS-PAGE as a ~170-kDa band.

Measurement of the rate of EGFR biosynthesis relies on determining the rate of incorporation of radiolabeled amino acids (methionine and cysteine) into EGFR (see *UNIT 7.1*). These two amino acids are the only ones that contain sulfur and, therefore, can be labeled with the ^{35}S isotope. ^{35}S offers a stronger radioactivity and other advantages as compared to ^{14}C, ^{3}H, and other isotopes used for radiolabeling of proteins in the research laboratory. Methionine and cysteine are rapidly transported into the cell by a concentration gradient–dependent mechanism. The cellular concentration of labeled amino acids is, therefore, maintained constant during the assay if the concentration of these amino acids is constant in the medium. Hence, the amount of the label incorporated into EGFR per unit of time can be used as a measure of the rate of EGFR biosynthesis. Since all newly synthesized proteins incorporate labeled amino acids, EGFR has to be isolated from cell lysates by immunoprecipitation (*UNIT 7.2*) followed by SDS-PAGE (*UNIT 6.1*). The amount of radioactivity associated with EGFR bands is then measured at each time point.

Materials

EGFR-expressing cells (most cultured cells of epithelial and fibroblast origin express EGFR at levels from 5,000 to 2,000,000 per cell)
Complete DMEM (*UNIT 1.1*; use 5% to 10% FBS) or other appropriate cell culture medium
DMEM or other appropriate culture medium free of L-methionine, L-cysteine, and L-glutamine (Invitrogen)
EasyTag ^{35}S-labeled amino acids (PerkinElmer)
Phosphate-buffered saline (DPBS; Invitrogen
TGH lysis buffer (see recipe) containing 150 mM NaCl
EGFR antibody mAb528 (mouse hybridoma, ATCC #HB8509)
20% (v/v) protein A–Sepharose resin (Sigma) in 50 mM HEPES
TGH lysis buffer (see recipe) containing no NaCl and 500 mM NaCl
2× sample buffer for SDS-PAGE (see recipe for 1×)
7.5% acrylamide gel (*UNIT 6.1*)
EGFR antibody 1005 (Santa Cruz Biotechnology)

60-mm plastic culture dishes
Rubber policemen
Platform rotator (e.g., Nutator from Clay Adams)
Image analysis software: e.g., ImageJ (NIH freeware)

Additional reagents and equipment for cell culture (*UNIT 1.1*), determining protein concentration (*APPENDIX 3H*), SDS-PAGE (*UNIT 6.1*), immunoblotting and immunodetection (*UNIT 6.2*), and phosphor imaging (*UNIT 6.3*)

Label cells

1. Seed EGFR-expressing cells at ~ 0.5–1.0 × 10^6 per 60-mm dish in 3 ml of complete DMEM (with 5% to 10% FBS depending on the cell line). Grow the cells for 2 days or until the cells are confluent.

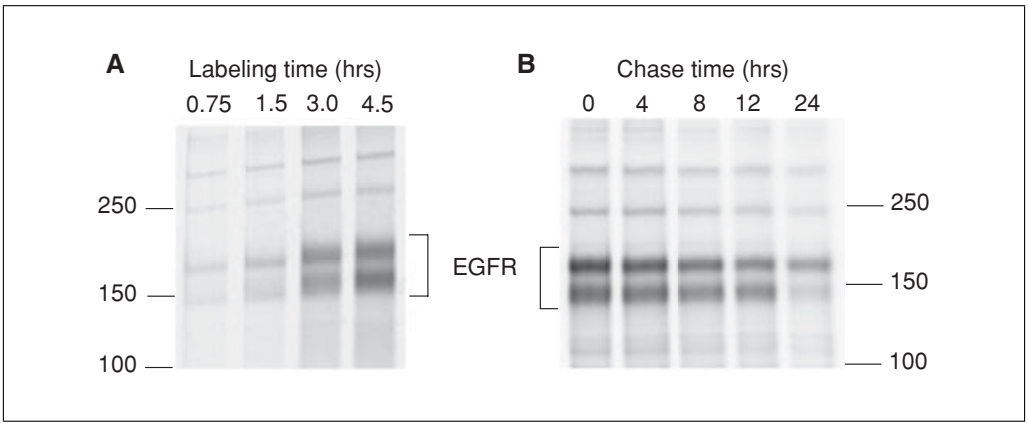


Figure 15.14.1 Examples of EGFR biosynthesis and degradation experiments. (**A**) Squamous cell carcinoma (SCC2) cells (~10^6 EGFR/cell) were incubated with the [^{35}S]amino acid labeling mix in the methionine-free medium containing 5% dialyzed FBS for 45 min to 4.5 hr. The cells were solubilized and EGFR-immunoprecipitated as described in Basic Protocol 1. (**B**) SCC2 cells were incubated with the [^{35}S]amino acid labeling mix in the methionine-free medium containing 5% dialyzed FBS for 4 hr. After washing, the cells were incubated in complete medium containing 5% FBS for 1 hr before initiating chase times of 0 to 24 hr. The cells were solubilized and EGFR immunoprecipitated as described in Basic Protocol 1. Note that EGFR immunoprecipitated from SCC2 cells runs as a doublet of 170- and 150-kDa (probably degradation-product) bands.

A basic experiment will require a minimum of four time points for each experimental condition, with two dishes per time point. Repeat the entire experiment a minimum of two times.

The SCC2 cells used in Figure 15.14.1 are grown in 5% FBS, whereas many other cell types require 10% FBS.

The amount of cells to use depends on the abundance of EGFR in the particular cell line. This protocol is geared towards cell lines that express high levels of EGFR (>100,000 receptors per cell).

2. Wash cells once with 3 ml DMEM lacking FBS, L-methionine, L-cysteine, and L-glutamine.
3. Add 3 ml of freshly prepared ^{35}S protein labeling medium to each dish and incubate the cells at 37°C for appropriate periods of time (e.g., 0.75, 1.5, 3.0, and 4.5 hr, as in Fig. 15.14.1A).
4. After each desired labeling time, place the cells on ice, collect radiolabeled medium, and dispose of it according to proper institutional procedures for radioactive waste.

   Properly dispose of all radiolabeled medium, subsequent washes, extracts and disposable labware.

5. Wash the cells three times with 3 ml ice-cold PBS. Aspirate as much as possible of the PBS after the last wash. Store dishes with washed monolayers at −70°C until further analysis.

   All dishes from the different time points of the pulse-chase are eventually frozen. Subsequent thawing and solubilization, and all remaining steps of the protocol, are performed simultaneously to minimize sample variability.

Lyse the cells

6. Place the frozen dishes on ice to thaw.
7. Add 300 µl of freshly prepared ice-cold TGH lysis buffer (with 150 mM NaCl) to each dish. Scrape the cells using a rubber policeman and transfer the lysates to 1.5-ml tubes.

8. Carry out the lysis for 10 min by gently rotating tubes on a platform rotator at 4°C.

9. Clear the lysates by microcentrifugation for 10 min at 15,000 × *g*, 4°C, and transfer the supernatants to new tubes.

10. Determine protein concentration (BradFord or Lowry assay; *APPENDIX 3H*) for each sample to assure that an equal amount of protein is added to each immunoprecipitation reaction.

11. Save an 10-µl aliquot of each cell extract for future analysis of immunoprecipitation efficiency.

Immunoprecipitate EGFR

12. Add 20 µg of affinity-purified mouse monoclonal EGFR antibody 528 (mAb528) to each tube containing an equal amount of protein and mix the tubes gently on a platform rotator for 2 hr or longer at 4°C (this amount of mAb528 is sufficient for cells with >100,000 receptors per cell, e.g., human epidermal carcinoma A-431 and human head-and-neck squamous cell carcinoma SCC2 cells).

    The amount of mAb528 needed will vary depending upon the abundance of EGFR in a particular cell line. Other antibodies that efficiently precipitate EGFR can be used. The dose of antibodies necessary for efficient immunoprecipitation should be determined in preliminary experiments using unlabeled cell lysates and immunoblotting.

13. Add 200 µl of protein A–Sepharose (20% v/v suspension in 50 mM HEPES) and continue the incubation for 1 hr at 4°C.

14. Pellet the protein A–Sepharose and bound EGFR immunoprecipitates by microcentrifugation for 2 min at 2000 × *g*, 4°C.

15. Collect supernatants and save 10-µl aliquots for future analysis of immunoprecipitation efficiency.

16. Wash the immunoprecipitates (pellets) three times with 1 ml of TGH lysis buffer containing decreasing amounts of NaCl (500 mM, 150 mM, and finally 0 mM NaCl).

17. Mix washed immunoprecipitates with 2× sample buffer for SDS-PAGE at a ratio of 1:1. Mix aliquots of cell extracts and supernatants from immunoprecipitation reactions at a ratio of 1:4 with 4× sample buffer for SDS-PAGE. Heat all samples at 95°C for 5 min, and subject to SDS-PAGE in a 7.5% acrylamide gel (*UNIT 6.1*).

Analyze labeling

18. Cut out the very bottom part of the gel (2 cm) containing most of the unincorporated low-molecular-weight radioactivity, and dispose of it in a designated ^{35}S waste container.

19. Transfer the portion of the gel that contains aliquots of cell extract input and output to nitrocellulose for subsequent analysis by immunoblotting with EGFR antibody 1005 and an appropriate loading control (see *UNIT 6.2*).

20. Carefully place the portion of the gel that contains the immunoprecipitation reactions onto chromatography paper and vacuum dry (*UNIT 6.3*).

21. For quantification of radioactivity using a phosphor imager, place the dried portion of the gel containing the immunoprecipitation reactions against an appropriate phosphor imaging screen in a phosphor imaging cassette. Expose for the necessary time (typically 12 to 48 hr), being careful not to generate a saturating signal.

22. Perform analysis of the phosphor imaging screen on an imager such as a Typhoon8600.

23. Quantify signal intensities using software such as ImageJ.

MEASURING EGFR PROTEIN DEGRADATION

ALTERNATE PROTOCOL 1

After synthesis and delivery to the cell surface, EGFR is turned over, typically with half-lives ranging from 8 to 24 hr, or longer, depending on the cell type and the level of EGFR expression. These turnover rates can be dramatically accelerated if EGFR is activated by EGF or other ligands. Measurement of the degradation rate involves pre-labeling of cells with [^{35}S]methionine and cysteine, followed by a chase incubation in the presence of an excess of the corresponding unlabeled amino acids. Since it takes several minutes for the newly synthesized receptor to acquire post-translational modifications in the Golgi complex and reach the plasma membrane, the pre-labeled cells are incubated without the label for ~30 min prior to the beginning of the chase incubation. This allows most of the newly labeled synthesized receptors to complete glycosylation and move to the plasma membrane. As in the biosynthesis assay, EGFR is immunoprecipitated before and during the chase incubation, electrophoresis is performed, and the amount of radioactivity in each EGFR band is measured. In contrast to the biosynthesis assay, the amount of labeled EGFR decreases during the chase incubation due to degradation. An example of a degradation experiment is shown in Figure 15.14.1B.

Additional Materials *(also see Basic Protocol 1)*

Experimental compounds of interest (e.g., EGF)

1. Prepare the cell cultures as in Basic Protocol 1, steps 1 and 2.

2. Add 3 ml of freshly prepared [^{35}S]protein labeling medium to each dish and incubate the cells at 37°C for 4 hr or longer.

   Incubation of cells with labeling medium for less than 4 hr may result in labeling of only a small pool of EGFR, since the half-life of EGFR is typically 8 to 24 hr. However, long incubations of some cells (in particular, cancer cells with enhanced metabolism) in methionine-cysteine-free medium and dialyzed serum may cause a substantial toxicity.

3. After an appropriate incubation time, dispose of the radioactive medium properly and rinse the cells with 3 ml of DMEM lacking FBS, L-methionine, L-cysteine, and L-glutamine.

4. Add 4 ml of fresh, complete medium with 10% (v/v) FBS to each dish. Incubate the cells at 37°C for 30 min to allow newly synthesized EGFR to reach the plasma membrane.

5. Following the 30-min incubation time, add any desired experimental compounds (for instance, EGF at 1 to 200 ng/ml dosage) and chase the cells for appropriate times at 37°C (e.g., 0, 4, 8, 12, and 24 hr as in Fig.15.14.1B).

   The range of chase incubations is discussed below in Commentary.

6. At the end of each chase incubation period, wash the appropriate dishes three times with 3 ml ice-cold PBS and store at −70°C for later analysis.

7. Thaw and lyse the labeled cultures, immunoprecipitate, and analyze the samples by following steps 6 through 23 of Basic Protocol 1.

MEASURING EGFR DOWN-REGULATION

Calculation of the number of EGFR molecules at the cell surface is based on measuring the number of EGF binding sites per intact cell. Because the stoichiometry of the EGF-EGFR interaction is 1:1, radiolabeled [^{125}I]EGF with known specific activity can be used to determine the number of EGF binding sites or surface EGFR per cell. This [^{125}I]EGF binding assay is performed at 4°C to avoid the uptake of [^{125}I]EGF inside the cell due to endocytosis. For an example of results of a down-regulation experiment, see Figure 15.14.2.

Materials

EGFR-expressing cells (most cultured cells of epithelial and fibroblast origin express EGFR at levels from 5,000 to 2,000,000 per cell)
Complete DMEM (*UNIT 1.1*; use 5% to 10% FBS) or other appropriate cell culture medium
Recombinant human culture-grade EGF (BD Biosciences, cat. no. 354052)
Binding medium; DMEM or other medium containing 0.1% (w/v) BSA
Sodium acetate buffer, pH 4.5: 0.2 M sodium acetate, pH 4.5 (*APPENDIX 2A*)/0.5 M NaCl
1 to 200 ng/ml [^{125}I]EGF (sp. act. ~200,000 cpm/ng; Support Protocol)
1 N NaOH
12-well plastic culture plates

Additional reagents and equipment for cell culture (*UNIT 1.1*)

Prepare cells

1. Seed EGFR-expressing cells at ~0.125–0.250 × 10^6 cells per well of a 12-well dish in 2 ml of complete DMEM (with 5% to 10% FBS depending on the cell line). Grow the cells for 2 days or until the cells are confluent.

   A basic experiment will require a minimum of four time points for each experimental condition, with four wells of a 12-well dish per time point. Repeat the entire experiment a minimum of two times. Three additional wells are used for cell counting.

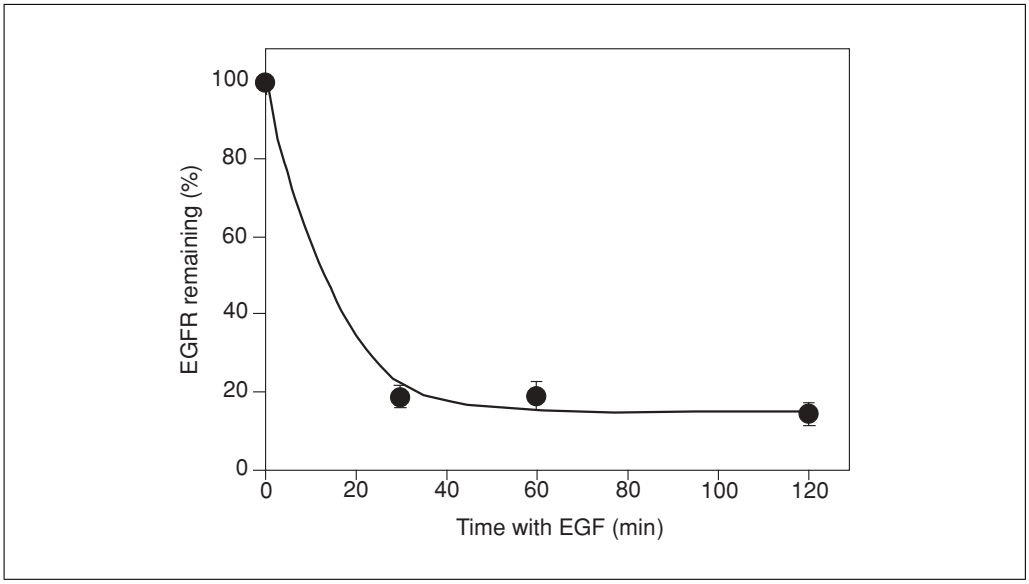


Figure 15.14.2 Example of EGFR down-regulation experiment. Porcine aortic endothelial cells stably expressing EGFR were incubated with 100 ng/ml EGF for 30 to 120 min at 37°C, acid washed, and further incubated with 50 ng/ml [^{125}I]EGF for 1 hr at 4°C. The cells were solubilized in NaOH. The specific radioactivity of cell lysates was determined and expressed as percent of the specific radioactivity associated with cells that were not incubated with unlabeled EGF (mean ± SE).

A confluent monolayer helps to maximize specific binding of [^{125}I]EGF to cells and minimize nonspecific binding of [^{125}I]EGF to plastic surfaces of culture dishes.

Incubate cells with and without EGF

2. Incubate the cells with or without unlabeled EGF in binding medium (1 ml/well of 1 to 200 ng/ml) for various lengths of time at 37°C starting from the longest incubation time.

   The start of shorter incubations is delayed to make it possible harvest all cells in the 12-well plate in the same time.

   Possible lengths of incubations with EGF and EGF concentrations are discussed in Commentary.

3. At the end of the longest incubation time, place the cells on ice and rinse once with 1 ml ice-cold medium to remove unbound EGF.

4. Strip surface-bound, unlabeled EGF by incubating the cells with sodium acetate buffer, pH 4.5, for 2.5 min (1 ml/well) at 4°C, then washing the cells twice, each time with 1 ml ice-cold culture medium, to neutralize the acid.

   This is an important step that has been omitted in many studies. If cold EGF is not stripped from receptors, binding of [^{125}I]EGF at step 5 will be underestimated. The mild acidic wash does not affect EGFR properties.

Incubate the cells with labeled EGF

5. Incubate the cells in 1 ml of binding medium containing saturating concentrations of [^{125}I]EGF (up to 200 ng/ml) alone (specific binding) or together with a 50-fold molar excess of unlabeled EGF (controls for nonspecific binding) at 4°C for 1 hr.

6. Rinse the wells three times with 1 ml ice-cold DMEM or other medium to remove unbound [^{125}I]EGF. Aspirate the medium as much as possible after the last wash.

   There are high- and low-affinity EGF binding sites in most studied cells. The mechanisms determining the EGF binding affinity are unknown. If [^{125}I]EGF is used at low concentrations it may predominantly bind to high-affinity receptors that would yield misleading results. The number of high-affinity sites may not be proportional to the total receptor number under various experimental conditions.

Measure radioactivity

7. Lyse the cells in 1 ml of 1 N NaOH for 1 hr or longer at 37°C to determine cell-bound radioactivity.

8. Measure nonspecific binding for each time point in the presence of unlabeled EGF (see step 5).

   Typically nonspecific binding is not more than 10% to 20% of the total [^{125}I]EGF binding.

Make calculations

9. Calculate the specific binding by subtracting the nonspecific radioactivity from total bound radioactivity for each time point of the time course.

10. Translate cpm values into the number of EGFR molecules per well (number of surface EGFRs) based on the specific activity of each [^{125}I]EGF preparation.

    No. of EGFRs per well = [(cpm/well)/(spec.act. – cpm/ng)] × 10^{11}.

11. Divide the number of surface EGFR per well by the number of cells in the well to obtain a number of EGFRs per cell.

    No. of EGFRs per cell = (EGFR/well)/(no. of cells/well).

MEASURING INTERNALIZATION OF [^{125}I]EGF

Binding of EGF to EGFR at the cell surface results in acceleration of internalization of receptors. After internalization into early endosomes, EGF-receptor complexes are either recycled back to the plasma membrane or sorted to late endosomes and lysosomes where both EGF and EGFR are degraded. Availability of EGF, its high level of stability, and the ease in preparing fully active labeled forms of EGF has led to wide use of various EGF conjugates for the quantitative analysis of EGFR endocytosis. Endocytosis assays that use radiolabeled EGF ([^{125}I]EGF) remain the gold standard of quantitative endocytosis assays because ^{125}I-radioactivity detection is highly sensitive and linear within a large range of radioactivity concentrations.

The internalization assay is based on short incubations of cells with [^{125}I]EGF followed by separation of surface bound [^{125}I]EGF from intracellular (internalized) [^{125}I]EGF. Internalization is highly temperature-dependent and, therefore, this assay is performed at 37°C. The internalization is stopped by placing cells on ice followed by the stripping of surface [^{125}I]EGF. The most reliable method to strip [^{125}I]EGF bound to surface receptors without affecting intracellular [^{125}I]EGF is a low-pH treatment (pH 2.5 to 2.8; Haigler et al., 1980). The stripped [^{125}I]EGF is then quantitated as the surface-bound [^{125}I]EGF, whereas acid-wash-resistant [^{125}I]EGF corresponds to internalized [^{125}I]EGF. The ratio of internalized to surface [^{125}I]EGF (I/S) plotted against incubation time is considered to be a measure of the apparent internalization rates. Internalization is considered to be a first-order kinetics process. Therefore, the specific rate of internalization depends on the concentration of EGF-receptor complexes at the cell surface. The precise and easy calculation of the internalization rate constant k_e can be performed if the [^{125}I]EGF concentration at the cell surface remains constant during the time-course of [^{125}I]EGF uptake. Under these conditions, the I/S ratio displays a linear dependence on time, and, therefore, k_e corresponds to the linear regression coefficient of I/S dependence on time. See Wiley and Cunningham (1982) for detailed explanations.

NOTE: Rapid handling of all steps of this procedure, particularly washes, is critical because of the short incubations of cells with [^{125}I]EGF.

Materials

EGFR-expressing cells (most cultured cells of epithelial and fibroblast origin express EGFR at levels from 5,000 to 2,000,000 per cell)
Complete DMEM (*UNIT 1.1*; use 5% to 10% FBS) or other appropriate cell culture medium
Binding medium; DMEM or other medium containing 0.1% (w/v) BSA
0.1 to 200 ng/ml [^{125}I]EGF (sp. act., ~200,000 cpm/ng; Support Protocol)
Recombinant human culture-grade EGF (BD Biosciences, cat. no. 354052)
Sodium acetate buffer, pH 2.8: 0.2 M acetic acid/0.5 M NaCl, pH 2.5 to 2.8
1 N NaOH

12-well plastic cell culture plates
γ-counter vials
γ counter

Additional reagents and equipment for cell culture (*UNIT 1.1*)

Prepare cells and binding medium

1. Seed EGFR-expressing cells at 0.125 to 0.25 × 10^6 cells/well in 12-well plates in 2 ml of DMEM containing 5% to 10% FBS and grow for 2 days or until confluent.

Typically, each experiment includes at least five time points, each represented by two to four wells of a 12-well dish. The time-course experiment needs to be repeated at least twice to obtain statistically significant data.

The use of confluent monolayers helps to minimize nonspecific binding of [^{125}I]EGF to plastic surfaces of a culture dish.

UNIT 1.1 describes basic cell culture techniques.

2. Prepare [^{125}I]EGF-containing binding medium (see Commentary for consideration of [^{125}I]EGF concentrations) and the same [^{125}I]EGF-containing medium supplemented with 50× to 100× molar excess of unlabeled human recombinant EGF ("nonspecific" medium).

   For each 12-well plate, 3 ml each of "specific" (not containing unlabeled EGF) and "nonspecific" medium are needed and should be warmed to 37°C prior to the assay.

   IMPORTANT NOTE: *Prepare 6.5 ml of [^{125}I]EGF-containing medium and then add unlabeled EGF to 3 ml of this medium. After the experiment, the remaining ~0.5 ml is used to measure the specific activity of [^{125}I]EGF.*

3. Place the plate in a water bath at 37°C.

Bind [^{125}I]EGF to the cells

4. Quickly wash two wells with 1 ml of warm (37°C) binding medium without labeled or unlabeled EGF. To these wells, add 0.5 ml of specific or nonspecific [^{125}I]EGF-containing medium (one well each).

   These cells correspond to a 6-min time point.

   It is advisable to add specific and nonspecific medium to the two wells simultaneously using a pipettor in each hand.

5. Repeat the same procedure (step 4) with the other five pairs of wells at 1-min intervals while the dish is kept at 37°C.

   In this way each pair of wells (specific and nonspecific) receives the radioactive medium at precisely 1-min intervals.

Remove unbound [^{125}I]EGF

6. After the last pair of wells has incubated for 1 min, place the dish on ice and rapidly aspirate the radioactive medium and discard as radioactive waste.

7. Rapidly wash the monolayers three times with the ice-cold medium by adding ~1 ml per well, followed by vacuum aspiration to remove as much unbound [^{125}I]EGF as possible after the last wash.

   This step is performed as quickly as possible to minimize the dissociation of [^{125}I]EGF from surface receptors during washes.

Strip the surface-bound [^{125}I]EGF

8. Strip surface-bound [^{125}I]EGF from all wells by incubating cells for 5 min with sodium acetate buffer, pH 2.8, at 1 ml/well, 4°C. Collect this acid wash in γ-counter vials, perform another short rinse with 0.5 ml/well of acetate buffer, pH 2.8, and combine with the original wash in the vial to determine the amount of surface-bound [^{125}I]EGF by gamma counting.

Measure internalized [^{125}I]EGF

9. Lyse the cells in 1 ml of 1 N NaOH for 1 hr at 37°C to determine the amount of internalized [^{125}I]EGF by gamma counting.

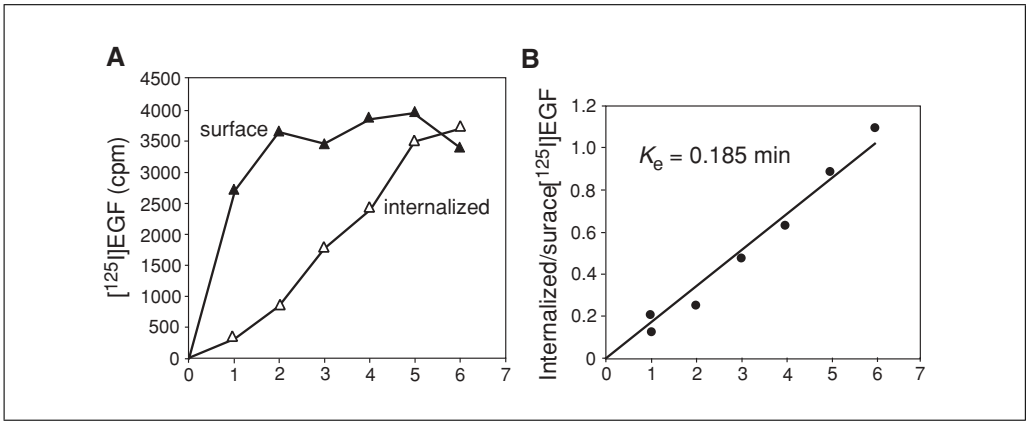


Figure 15.14.3 Example of the [^{125}I]EGF internalization assay. NIH 3T3 cells stably expressing EGFR tagged with GFP were incubated with 1 ng/ml [^{125}I]EGF for 1 to 6 min according to Basic Protocol 3. (**A**) The specific [^{125}I]EGF radioactivity of acid-wash (surface) and acid-resistant [^{125}I]EGF (internalized) are plotted against time. (**B**) The ratio of internalized to surface [^{125}I]EGF is plotted against time. The linear regression coefficient was calculated to obtain the k_e value.

Perform calculations

10. Calculate the specific radioactivity of the acid-sensitive (surface) and acid-insensitive (internalized) fractions by subtracting nonspecific counts from the corresponding counts of specific wells (Figure 15.14.3A).

    Nonspecific radioactivity is typically not more than 3% to 10% of the total counts.

    Internalization is considered to be a first-order kinetics process. Therefore, the specific rate of internalization depends on the concentration of EGF-receptor complexes at the cell surface.

11. To estimate the rate of internalization, plot the ratio of specific internalized to specific surface radioactivity (I/S) against time (Figure 15.14.3B).

    The slope of this plot corresponds to the apparent rate of internalization. If this slope is linear, it can be used to calculate the rate constant of internalization (k_e). k_e is the linear regression coefficient of the dependence of I/S ratio on time. Such a simple calculation of the values is, however, possible only if the amount of occupied surface receptors stays relatively constant during the time course of [^{125}I]EGF uptake.

    Figure 15.14.3 shows an example of internalization data from one 12-well plate. In Figure 15.14.3A the amount of surface [^{125}I]EGF is relatively constant (does not decrease due to EGF-induced down-regulation of surface EGFR). This results in a near linear dependence of I/S [^{125}I]EGF ratio on time and, therefore, allows calculation of the k_e value (Fig. 15.14.3B).

BASIC PROTOCOL 4 MEASURING [^{125}I]EGF RECYCLING AND DEGRADATION

The rationale to use [^{125}I]EGF for estimating both the recycling and degradation rates of EGFR is underscored by two important facts. First, EGF, which is sorted at the early endosome for degradation in the lysosome, does not significantly dissociate from the internalized EGFR until it reaches the lysosome (Sorkin et al., 1988). Second, degradation of [^{125}I]EGF results in a product that can be biochemically separated from intact [^{125}I]EGF which has been recycled or remains internalized. The recycling/degradation assay is based on loading early endosomes with [^{125}I]EGF-receptor complexes by allowing cells to endocytose [^{125}I]EGF. The endocytosis is then stopped at 4°C, and [^{125}I]EGF that has not been internalized is stripped from the cell surface by the mild acidic wash treatment. When cells are returned to 37°C, a pool of [^{125}I]EGF-receptor complexes are recycled back to the cell surface, where [^{125}I]EGF is released from the receptor or

re-internalized. Another pool of internalized [^{125}I]EGF-receptor complexes are sorted from early endosomes to late endosomes and lysosomes where [^{125}I]EGF-receptor complexes are degraded by proteolytic enzymes. This degradation results in the release of mono- and di-[^{125}I]iodotyrosines, which readily penetrate membranes and accumulate in the extracellular medium. This form of ^{125}I radioactivity is not precipitable by trichloroacetic acid (TCA) and is used as a measure of the rate of [^{125}I]EGF degradation. Only negligible amounts of TCA-soluble ^{125}I can be detected in the cells. On the other hand, intact [^{125}I]EGF (TCA-precipitable) detected in the medium and at the cell surface (acid-wash stripped) after the chase incubation of [^{125}I]EGF-loaded cells corresponds to [^{125}I]EGF-receptor complexes that have recycled from endosomes to the plasma membrane.

Recycling and degradation can also be considered first-order processes. This means that their rates are proportional to the concentration of [^{125}I]EGF in early/sorting endosomes. However, the calculation of specific rate constants is difficult due to heterogeneity of endosomal compartments and the decrease in the concentration of [^{125}I]EGF in early/sorting endosomes during the chase time. Also, the presence of unlabeled EGF (Basic Protocol 4) and re-internalization of [^{125}I]EGF-receptor complexes (Alternate Protocol 2) make calculations of specific rate constants challenging. Therefore, it is important to interpret the results of the recycling/degradation assay described in these protocols only as apparent rates. In theory, kinetic rate parameters can be calculated by using computational modeling that takes into account all EGF-receptor association-dissociation parameters and apparent trafficking rates (see, for example, Resat et al., 2003). For a schematic of the assay and example of results, see Figure 15.14.4.

Materials

EGFR-expressing cells (most cultured cells of epithelial and fibroblast origin express EGFR at levels from 5,000 to 2,000,000 per cell)
Complete DMEM (*UNIT 1.1*; use 5% to 10% FBS) or other appropriate cell culture medium
Binding medium: DMEM or other medium containing 0.1% (w/v) BSA
1 to 200 ng/ml [^{125}I]EGF (sp. act., ~200,000 cpm/ng; Support Protocol)
Recombinant human culture-grade EGF (BD Biosciences, cat. no. 354052)
Sodium acetate buffer, pH 4.5: 0.2 M sodium acetate buffer, pH 4.3 to 4.5 (*APPENDIX 2A*)/0.5 M NaCl
Sodium acetate buffer, pH 2.8: 0.2 M acetic acid/0.5 M NaCl, pH 2.5 to 2.8
1 N NaOH
10% (w/v) trichloroacetic acid (TCA)/2% (w/v) phosphotungstic acid (PTA) in H_2O

35-mm plastic cell culture dishes
γ-counter vials
γ counter
Refrigerated centrifuge

Additional reagents and equipment for cell culture (*UNIT 1.1*)

Prepare cells

1. Seed 0.25 to 0.50 $\times$ 10^6 cells in 35-mm culture dishes in 2 ml of DMEM containing 5% to 10% FBS and grow for 2 days or until confluent.

   UNIT 1.1 describes basic cell culture techniques.

   Typically, at least five time points are required, each represented by two to three dishes. The entire time-course experiment should be repeated at least twice.

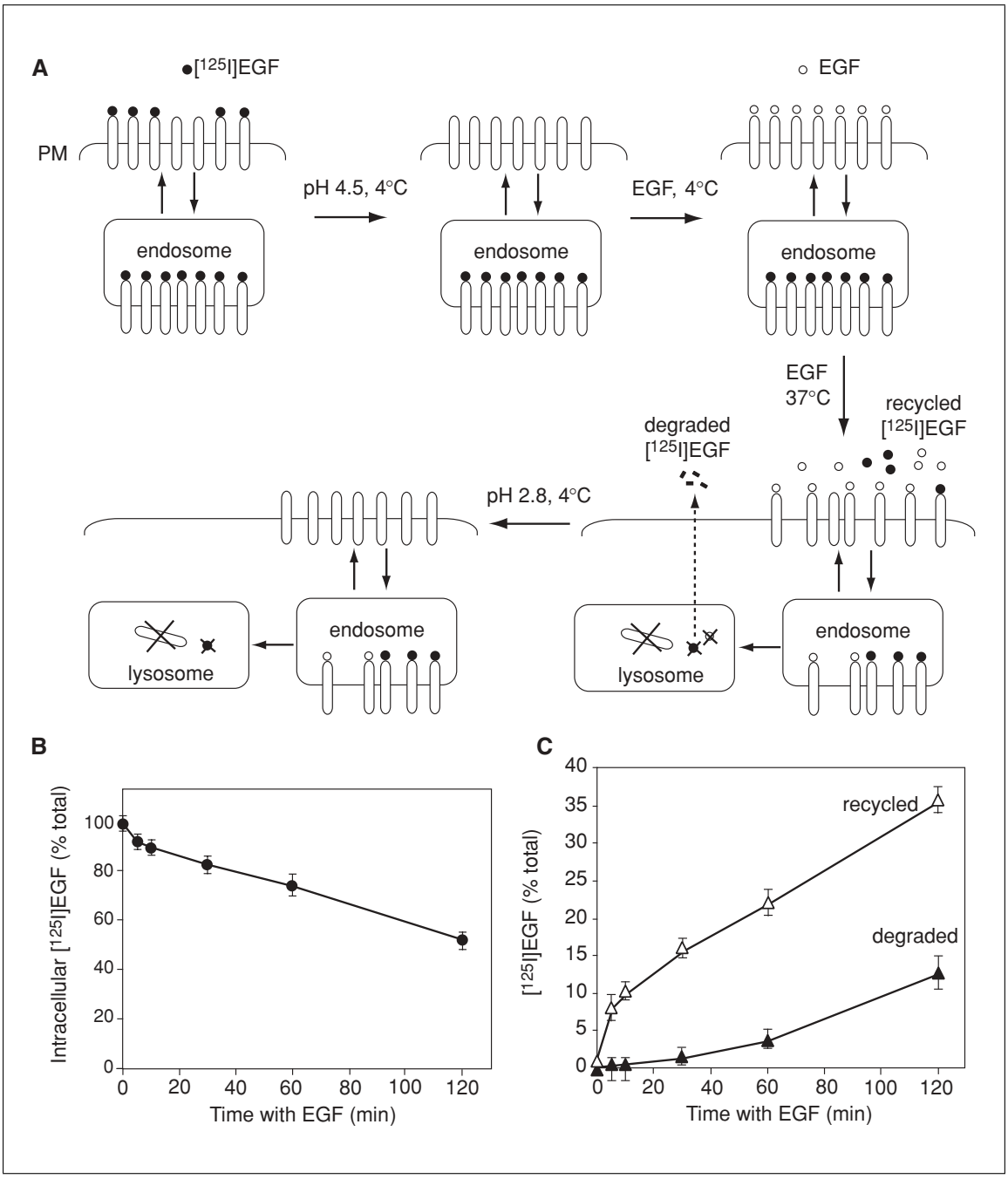


Figure 15.14.4 [^{125}I]EGF recycling/degradation assay (Basic Protocol 4). (**A**) Schematic representation of the main steps of the assay. PM, plasma membrane. (**B**) and (**C**) Representative example of the [^{125}I]EGF recycling/degradation experiment. Mouse embryonic fibroblasts were incubated with 5 ng/ml [^{125}I]EGF for 5 min at 37°C, and [^{125}I]EGF that has not been internalized was stripped by the acid wash. [^{125}I]EGF-loaded cells were incubated with unlabeled EGF at 4°C and then chased with unlabeled EGF for 0 to 120 min according to the protocol. The specific radioactivities of all fractions were determined and expressed as percent of the total radioactivity associated with cells and present in the medium (mean ±SE). The amount of intracellular [^{125}I]EGF (NaOH lysates) plotted against the chase time is shown in (B), whereas the time course of [^{125}I]EGF recycling (surface plus medium trichloroacetic acid/phosphotungstic acid-insoluble [^{125}I]EGF) and degradation (trichloroacetic acid/phosphotungstic acid ^{125}I-radioactivity) is shown in (C).

2. Incubate the cells at 37°C with 1 ml [^{125}I]EGF in binding medium for 5 to 10 min (see Commentary for consideration of [^{125}I]EGF concentration and incubation times) to allow for accumulation of [^{125}I]EGF-receptor complexes in early endosomes. Incubate control dishes with the same [^{125}I]EGF-containing medium supplemented with a 50-fold molar excess of unlabeled EGF to determine nonspecific radioactivity.

   Because handling of multiple dishes in this type of experiments is difficult, it is recommended to use two "specific" dishes and one "nonspecific" dish for each time point of the chase incubation (step 7 below). The entire time-course experiment can be repeated to ensure the statistical significance of the data.

3. After 37°C incubation, place the dishes on ice and then rapidly wash three times with 1 ml of ice-cold DMEM. Briefly rinse cells with DMEM (2 ml/dish) to neutralize the acid.

4. Incubate the cells with 1 ml/dish of sodium acetate buffer, pH 4.5, for 2.5 min, remove supernatants, and then rinse briefly with 1 ml of the same buffer to remove [^{125}I]EGF that had not been internalized during the 37°C incubation.

5. Combine both of the acidic washes from step 4 to determine the amount of [^{125}I]EGF at the cell surface by gamma counting.

   At this point the cells contain[^{125}I]EGF only in endosomes and are referred to as "[^{125}I]EGF-loaded cells."

6. Incubate the [^{125}I]EGF-loaded cells in binding medium containing 200 ng/ml unlabeled EGF for 40 min at 4°C to occupy surface EGFRs, and aspirate the medium.

   This EGF concentration is sufficient to occupy >90% of surface receptors in all types of cells, including cells expressing the highest levels of EGFR.

7. Add 1 ml fresh pre-warmed (37°C) medium containing 200 ng/ml EGF. Rapidly place the cells in the water bath or incubator (for incubations longer than 15 min) at 37°C to allow trafficking of [^{125}I]EGF-receptor complexes in loaded cells. Keep three dishes (two "specific" medium and one nonspecific medium) on ice for the zero chase time point.

   The lengths of chase incubations are discussed in the Commentary, below.

   An excess of unlabeled EGF bound to surface receptors and in the medium minimizes re-internalization of recycled [^{125}I]EGF-receptor complexes and rebinding of dissociated [^{125}I]EGF from the medium.

8. While other dishes are incubating at 37°C, add 1 ml prewarmed medium to zero-time dishes and immediately collect the medium in γ-counter vials.

   This step is necessary to have the zero-time dishes exposed to medium that is the same as that used for the chase incubation of other dishes in step 7.

9. At the end of each of the chase incubations, place the dishes on ice, add 1 ml medium, and collect the medium into γ-counter vials.

10. Strip surface-bound [^{125}I]EGF from all wells by incubating cells for 5 min with sodium acetate buffer, pH 2.8, at 1 ml/dish, 4°C. Collect this acid wash in γ-counter vials, perform another short rinse at 0.5 ml/dish, and combine this rinse with the original wash to determine the amount of surface-bound (recycled) [^{125}I]EGF by gamma counting.

11. Solubilize the cells in 1 N NaOH for 1 hr to measure the amount of intracellular [^{125}I]EGF.

12. Mix the medium collected at step 8 with 0.3 ml of 10% (w/v) TCA/2% (w/v) PTA and incubate 1 hr or longer at 4°C. Centrifuge 10 min at 5000 × *g*, 4°C.

    Because of its small size, EGF polypeptide is not efficiently precipitated by TCA alone. Therefore, addition of PTA is important for complete precipitation of [^{125}I]EGF.

13. Transfer supernatants to new γ-counter tubes to determine the amount of degraded [^{125}I]EGF by gamma counting.

14. Dissolve the pellets in 1 ml of 1 N NaOH to determine the amount of [^{125}I]EGF by gamma counting.

Make calculations

15. First, obtain specific counts by subtracting nonspecific radioactivity of all fractions from the counts of the same fractions of corresponding specific dishes.

16. Calculate the total amount of [^{125}I]EGF specifically associated with cells for each dish as the sum of the second (pH 2.8) acid wash (surface [^{125}I]EGF, step 10), intracellular [^{125}I]EGF (step 11), intact-medium [^{125}I]EGF (TCA-precipitated radioactivity, step 12), and degraded-medium [^{125}I]EGF (step 13).

17. Calculate the amount of recycled [^{125}I]EGF by summing the surface [^{125}I]EGF and intact medium [^{125}I]EGF.

18. Calculate the percent of each [^{125}I]EGF pool (intracellular, recycled, and degraded) relative to the total cell-associated [^{125}I]EGF for each time point.

    The example of the dynamics of recycled, degraded and intracellular [^{125}I]EGF is presented in Figure 15.14.4B and C.

19. Calculate the ratios of the percent of recycled and degraded [^{125}I]EGF to percent of intracellular [^{125}I]EGF and plot against time.

    The slopes of these graphs correspond to the apparent recycling and degradation rates, respectively.

ALTERNATE PROTOCOL 2

MEASURING [^{125}I]EGF RECYCLING/DEGRADATION IN THE ABSENCE OF EXCESS UNLABELED EGF

This assay for recycling/degradation differs from Basic Protocol 4 at the step of incubation of [^{125}I]EGF-loaded cells at 37°C, during which no unlabeled EGF is added.

For materials, see Basic Protocol 4.

1. Follow steps 1 through 4 of Basic Protocol 4 to prepare cells and load the cells with labeled EGF.

2. Initiate trafficking of [^{125}I]EGF-receptor complexes in loaded cells by incubating the cells in fresh binding medium with neither labeled nor unlabeled EGF at 37°C for 0 to 60 min or longer.

    See Commentary for considerations in the choice of the chase incubation time.

3. Follow steps 7 to 19 in Basic Protocol 4 to measure the various EGF pools in the cells.

SUPPORT PROTOCOL

PREPARATION OF [^{125}I]EGF

For the binding and trafficking of EGF studies, it is necessary to have [^{125}I]labeled EGF. This protocol describes the chloramine T method for labeling proteins.

Materials

0.05 M sodium phosphate buffer, pH 7.5 (*APPENDIX 2A*) containing 0.075 M NaCl
20 mg/ml BSA in 0.05 M sodium phosphate buffer, pH 7.5 (*APPENDIX 2A*) containing 0.075 M NaCl
Receptor-grade mouse EGF (BD Biosciences, cat. no. 354010)
0.5 M sodium phosphate buffer, pH 7.5 (*APPENDIX 2A*)
0.1 mCi/μl Na[^{125}I] (PerkinElmer; cat. no. NEZ33A)
2 mg/ml chloramine T (Sigma) in 0.05 M sodium phosphate buffer, pH 7.5 (see *APPENDIX 2A* for buffer); prepare fresh
4 mg/ml sodium metabisulfite in 0.05 M sodium phosphate buffer, pH 7.5 (see *APPENDIX 2A* for buffer); prepare fresh

PD-10 (G-25) Sepharose disposable column (GE Healthcare)
γ counter

NOTE: The entire procedure is carried out at room temperature.

Prepare the column

1. Wash a PD-10 (G-25) Sepharose disposable column with 50 ml Milli-Q water and equilibrate with 50 ml 0.05 M sodium phosphate buffer, pH 7.5. containing 0.075 M NaCl.

2. Load 1 ml of 20 mg/ml BSA in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.075 M NaCl onto the column.

Label the EGF

3. In a 1.5-ml microcentrifuge tube, mix 24 μl of 0.5 M sodium phosphate buffer, pH 7.5, 28 μl of 0.05 sodium phosphate buffer, pH 7.5, and 5 μg of receptor-grade mouse EGF (5 μl of 1 mg/ml EGF in water).

   This gives a precisely 100 mM final sodium phosphate concentration in the mixture.

   The following steps are performed in a designated fume hood.

4. Add 10 μl of 0.1 mCi/μl Na[^{125}I] (1 mCi) to the EGF mixture from step 3 and initiate the reaction by adding 10 μl of 2 mg/ml chloramine T in the dark.

5. After a 40-sec incubation in the dark, stop the reaction by adding 20 μl of 4 mg/ml sodium metabisulfite.

   Both the chloramine T and sodium metabisulfite solutions should be freshly prepared.

Separate the ^{125}I-labeled EGF

6. Mix 100 μl of 20 mg/ml BSA in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.075 M NaCl into the reaction mixture (final volume ~200 μl) and load onto the PD-10 column. Allow the column to equilibrate for 3 min.

7. After 3 min of equilibration, start gel filtration by eluting with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.075 M NaCl.

8. Allow the first 1.8 ml to flow through.

9. Collect subsequent fractions at the rate of 10 drops per tube into 10 tubes, each containing 50 μl of 20 mg/ml BSA.

   BSA serves as a protein carrier to minimize binding of [^{125}IEGF] to the tube walls.

10. Measure the amount of radioactivity in 2 μl from each tube in the γ counter and plot the number of counts against the tube number.

11. Based on the curve, combine the first peak (usually tubes 2 to 5 or 2 to 6) and mix in a larger plastic tube.

12. Measure the specific activity of the final [^{125}I]EGF preparation in a 2-μl sample of the pool.

13. Divide the final [^{125}I]EGF preparation into 0.1-ml aliquots and store at −20°C.

Make calculations

14. Calculate the specific activity of the preparation.

Considering that the approximate yield of EGF in this procedure is 90%, then the total amount of [^{125}I]EGF is ∼4.5 μg. Typically, the volume of the peak is ∼2 ml, which results in the final concentration of [^{125}I]EGF being ∼2.25 ng/μl. The amount of radioactivity is usually ∼450,000 cpm per μl in our experiments. Therefore, the specific activity of [^{125}I]EGF is 450,000 cpm/2.25 ng = 200,000 cpm/ng.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Sample buffer for SDS-PAGE, 1× (prepare at 2× or 4× concentration as indicated for protocol)

50 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
10% (v/v) glycerol
2% (w/v) SDS
5% (v/v) 2-mercaptoethanol
Bromphenol blue (a few small crystals per 15 ml)
Store up to 1 year at room temperature

[^{35}S]Protein labeling medium

DMEM free of L-methionine, L-cysteine, and L-glutamine (Invitrogen) supplemented with:
5% to 10% (v/v) dialyzed FBS (typically)
300 μCi Easy-Tag ^{35}S-labeled amino acids
Prepare fresh

TGH lysis buffer

1% (v/v) Triton X-100
10% (v/v) glycerol
50 mM HEPES, pH 7.4
150 mM NaCl (or 0 mM or 500 mM as specified in protocol)
2 mM EGTA
2 mM EDTA
1 mM phenylmethylsulfonyl fluoride
10 ng/μl aprotinin
10 ng/μl leupeptin
Store up to 2 to 3 months at 4°C

COMMENTARY

Background Information

EGFR is a member of the ErbB tyrosine kinase family. EGFR is an important regulator of eukaryotic development and is involved in regulation of proliferation, survival, and differentiation of many types of cells in adult organisms. EGFR and other ErbBs are frequently overexpressed in various types of human carcinoma, glioblastoma, and glioma. In recent years, EGFR became an important diagnostic and prognostic marker in several carcinomas. Several EGFR inhibitors have been

developed and approved for clinical use as anti-cancer targeted therapies.

Endocytosis and lysosomal degradation of active EGFR is thought to serve as the negative feedback loop in the regulation of EGFR signaling. However, endocytic trafficking regulates more than just receptor down-regulation. Several lines of evidence suggest that EGF-receptor complexes continue to signal after endocytosis and that signaling from endosomes may generate outcomes different from those triggered from the plasma membrane. Thus, mechanisms of EGFR endocytosis and its role in signaling has been the subject of more than 1000 publications. However, the large amount of data in the field is impossible to reconcile into a single reasonable model. One of the major reasons for such a slow pace towards the complete understanding of EGFR trafficking is the use of incomparable and often inadequate methodologies by different laboratories. This underscores the importance of standardizing the methodological approaches to monitoring EGFR endocytosis, specifically the methodologies that allow for reliable quantification of kinetics rates. Therefore, we focus this article on describing standard quantitative methods used to measure the rates of EGFR turnover (synthesis and degradation), EGFR down-regulation, and EGF internalization, both recycling and degradation.

Methods to study EGF and EGFR endocytosis using [^{125}I]EGF were the first to be introduced and remain the most quantitative and sensitive. Most of these methodologies are designed to study active, EGF-occupied receptors. However, similar methods using ^{125}I-labeled antibodies have been developed to study the kinetics of endocytosed EGFR which is not occupied by ligand (Wiley et al., 1991). It is important to be aware that cross-linking of EGFR by antibodies can alter trafficking parameters. Therefore, Fab fragments are the most appropriate in this type of experiment. Also, methods should use only those antibodies that do not interfere with any functions of EGFR. Other conventional methods of measuring surface receptor density and receptor endocytosis include those using surface biotinylation and reversible biotinylation (Shtiegman et al., 2007). These methods can also be used to study the endocytic trafficking of EGFR. However, they involve multiple steps, including pull-downs and SDS-PAGE. Each of these steps increases the chances of experimental error while not being as sensitive and quantitative as the methods using [^{125}I]EGF.

Critical Parameters and Troubleshooting

Consideration of EGFR expression levels

The design and interpretation of the quantitative analysis of EGFR turnover and trafficking must take into consideration the expression levels of EGFR in the cell line to be studied. Normal mammalian cells express from 5,000 (most epithelial and fibroblast cells) to 200,000 (hepatocytes) EGFR per cell. Cancer cells express up to 2–3 $\times$ 10^6 EGFR/cell. Recombinant EGFR can be stably expressed in cells at different levels to directly analyze the dependency of the parameters of the EGFR biosynthesis, endocytosis, recycling, and degradation on the EGFR concentration in the cell.

Synthesis of EGFR

In Basic Protocol 1 it is important to use the shortest time course possible because ^{35}S-labeled amino acids can be rapidly depleted from the medium. A significant decrease in available free [^{35}S]amino acids will result in nonlinear incorporation, and thus an inaccurate determination of the rate of EGFR biosynthesis. Different cell lines have different rates of metabolism, and, therefore, different rates of methionine/cysteine incorporation. Control experiments using variable concentrations of labeled amino acids and incubation times should be performed to confirm linear incorporation of radiolabeled methionine/cysteine for the desired concentration and pulse time. For most cell lines, this will be in the 1- to 4-hr range. Alternatively, unlabeled methionine/cysteine may be simultaneously added to extend the half-life of labeled methionine/cysteine in the medium.

EGFR degradation

Two types of methods have been used to determine the rates of EGFR degradation. In the first method, the amount of EGFR is determined by immunoblotting (*UNIT 6.2*) of lysates from cells incubated without or with EGF and in the presence of an inhibitor of protein synthesis, such as cycloheximide. This assay relies on the assumption that cycloheximide completely inhibits protein synthesis and does not have other, unrelated effects.

The second method, which is described in the Alternate Protocol 1, involves radiolabeling of cellular proteins followed by chasing the cells in the absence of label and subsequent immunoprecipitation of EGFR at various time points. In this method, inhibition of synthesis is not necessary. The amount of time

cells are incubated with labeled amino acids in these experiments should be sufficient to label a significant pool of EGFR. This is especially important in cells with high levels of EGFR expression, in which the turnover of the receptor is very slow.

Down-regulation assay

Typically, the effect that low amounts of EGF have on EGFR surface expression is difficult to detect in cells expressing moderate and high levels of EGFR. Under these conditions, there is not a significant number of EGFR being internalized in these cells. However, low EGF concentrations can be used in experiments with cells expressing low EGFR levels. When measuring the number of [^{125}I]EGF binding sites at the cell surface, the time of incubation with [^{125}I]EGF at 4°C must be 1 hr or longer. This time is necessary to reach a binding equilibrium for [^{125}I]EGF at 4°C in various types of cells.

Internalization

It has been demonstrated that EGF-induced internalization of EGFR via the clathrin-dependent pathway is saturated if too many EGF-receptor complexes are present at the cell surface. This is typically observed with [^{125}I]EGF concentrations higher than 1 to 2 ng/ml when [^{125}I]EGF occupies more than 10,000 to 15,000 receptors per cell. Therefore, these low concentrations of [^{125}I]EGF are recommended for experiments comparing clathrin-dependent endocytosis of EGFR mutants or the effects of inhibitors and modulators of this process.

One caveat of this type of internalization experiment is the possible underestimation of the internalization rate because of rapid recycling of internalized [^{125}I]EGF. Thus, the internalization assay must be performed using a short time course so that the bulk of internalized [^{125}I]EGF does not have time to return to the cell surface.

It is also important to note that Basic Protocol 3 uses a low-pH wash with acetate to strip [^{125}I]EGF from the cell surface. This treatment removes ~95% of surface-bound [^{125}I]EGF. In fact, a 1-min treatment with the pH 2.5 to 2.8 acetate buffer is sufficient to strip 90% of surface [^{125}I]EGF. However, other low-pH buffers can also be used. For instance, glycine-Cl buffer has been used in other studies (Wiley et al., 1991). Also, addition of 2 M urea further improves the efficiency of stripping (Wiley et al., 1991).

Recycling and degradation

In the recycling/degradation assay, it is important to load the cells with sufficient concentrations of [^{125}I]EGF for a sufficient amount of time to obtain a substantial concentration of [^{125}I]EGF in early endosomes. These parameters should be chosen based on both EGFR cellular levels and internalization rates for each particular cell type. The initial incubation time should be 5 to 10 min in most cell types and not longer than 15 min in cells with the slowest endocytic trafficking. This helps ensure that most endosomal [^{125}I]EGF is still in early endosomes and has not been sorted to late compartments or back to the plasma membrane. Similarly, it is critical to keep the loading time consistent between all samples in the experiment to obtain comparable pools of endosomal [^{125}I]EGF. Thus, it is advisable to limit the number of variants in each experiment, depending upon the experience level of the investigator with this type of experiments.

This report offers two versions of the recycling/degradation assay (Basic Protocol 4 and Alternate Protocol 2). Basic Protocol 4 is designed to prevent any rebinding and reinternalization of [^{125}I]EGF after one round of recycling because of the presence of an excess of unlabeled EGF during the chase incubation. However, the caveat of this protocol is that unlabeled EGF-receptor complexes compete with [^{125}I]EGF-receptor complexes during sorting to recycling and degradation pathways. This is especially important for estimating the degradation rates of [^{125}I]EGF, because the degradation pathway has been shown to be saturable (Herbst et al., 1994). On the other hand, Alternate Protocol 2 is designed to avoid interference of unlabeled EGF, although recycled [^{125}I]EGF can dissociate and rebind as well as re-internalize before dissociation from the receptor at the cell surface. Typically, degradation products of [^{125}I]EGF (TCA-soluble) can be detected after about 30 min of chase incubation in [^{125}I]EGF-loaded cells. However, it is necessary to extend the time of chase incubation to 2 to 4 hr to enable detection of significant amounts of degraded [^{125}I]EGF. Thus, in experiments aiming to measure recycling rates, short chase incubations are recommended (1 to 15 min). In experiments focusing on degradation rates, a time course of 30 to 240 min is recommended. Finally, it should be noted that as an alternative to trichloroacetic acid/phosphotungstic acid precipitation, a G-25 gel filtration procedure

can be used to separate intact [^{125}I]EGF and low-molecular-weight products of [^{125}I]EGF degradation (Wiley et al., 1991).

Iodination

The protocol for EGF iodination using chloramine T was developed and optimized in the laboratories of Stanley Cohen and Graham Carpenter (Carpenter and Cohen, 1976). This protocol is designed to carry out iodination under mild conditions, avoid excessive exposure to chloramine T, and avoid formation of highly reactive [^{125}I]EGF products that can covalently cross-link to the plasma membrane. It is critical to limit the time of iodination to 40 sec. Alternative protocols of EGF iodination can also be used. For instance, EGF can be iodinated using Iodobeads (Pierce). In our experience, the specific activity of [^{125}I]EGF made using Iodobeads is lower as compared with preparations obtained using the chloramine T protocol.

Anticipated Results

Biosynthesis of EGFR

In the biosynthesis assay, the amount of labeled EGFR should correlate in a linear fashion with the time of label incorporation. The apparent rate of the label incorporation into newly synthesized EGFR varies depending on the cell type.

EGFR degradation

The half-life of EGFR ranges from 8 to 24 hr (low to high levels of receptor respectively) and even longer in other cells. The half-life of EGFR is dramatically shortened following EGF stimulation, to as low a value as $t_{1/2} =$ 1 hr in cells expressing low levels of the receptor. Examples of EGFR biosynthesis and degradation analysis performed in our laboratory have recently been published (Duex and Sorkin, 2009).

EGFR down-regulation

Significant EGFR down-regulation in cells treated with high EGF concentrations (20 to 100 ng/ml) is observed beginning at 15 to 20 min after EGF stimulation. The amount of surface EGFR usually stabilizes after 30 to 60 min of EGF stimulation. Therefore, a complete dynamics of the down-regulation process can be observed within the range of incubation times ranging from 15 to 120 min.

EGF internalization

The values of k_e measured in various types of cells using low [^{125}I]EGF concentrations (clathrin-mediated pathway) are typically within the range of 0.15 to 0.40 min^{-1}. This range in values is quite similar to the rate constants measured for other clathrin-coated pit-internalized cargo, such as the transferrin receptor. k_e values measured using high EGF concentrations (resulting in a significant contribution from clathrin-independent pathways) are typically below 0.1 min^{-1} and can be as low as 0.03 min^{-1}. Such values correspond to the rates of constitutive endocytosis of membrane proteins.

EGF recycling and degradation

In both Basic Protocol 4 and Alternate Protocol 2, recycling of [^{125}I]EGF is evident immediately when the [^{125}I]EGF-loaded cells are chased at 37°C. The protocols can produce similar kinetics of [^{125}I]EGF recycling if a short time course is used for the chase (5 to 10 min). However, in Alternate Protocol 2, re-internalization of [^{125}I]EGF results in a slight underestimation of the recycling rates, especially, at longer chase times. Typically, as much as 50% of endosomal [^{125}I]EGF in [^{125}I]EGF-loaded cells is recycled during a 2-hr chase if re-internalization is prevented (Alternate Protocol 2). In contrast, under conditions of this protocol, only about 20% to 25% of endosomal [^{125}I]EGF is recycled. In both protocols, degradation products of [^{125}I]EGF can be detected after about 30 min of chase incubation of [^{125}I]EGF-loaded cells. At maximum, 10% to 20% of endosomal [^{125}I]EGF is degraded after 2 to 4 hr of chase incubation in the presence of excess cold EGF (Basic Protocol 4), whereas up to 40% to 50% of internalized [^{125}I]EGF is degraded in the absence of unlabeled EGF during the same time (Alternate Protocol 2).

Iodination

Typically, the specific activity of [^{125}I]EGF is within the range of 120,000 to 200,000 cpm/ng.

Time Considerations

Synthesis of EGFR

The incubation of cells with radiolabeled methionine/cysteine is up to 6 hr. Since the cells are frozen at −70°C at the end of the incubation, the experiment can be interrupted at this step and continued later. Preparation of cell lysates takes ∼1 hr. A minimal time of incubation of lysates with primary antibodies is 2 hr, although the incubation can be run overnight. Subsequent incubation with protein A–Sepharose, washes and loading the samples

for SDS-PAGE take ~2 hr. Thus, the minimal length of the entire assay until starting electrophoresis is ~11 hr.

EGFR degradation

The cells are labeled with the [^{35}S]amino acid mix for 4 to 16 hr. Subsequent washes and a 30 to 60 min preincubation take ~1 to 1.5 hr. The longest chase incubation of cells in unlabeled medium should extend to at least 4 hr for cells with low levels of EGFR and up to 30 hr for cells with high EGFR levels. If the goal of the experiment is to measure the degradation rates of EGF-activated EGFR, the length of the longest chase incubation can be reduced to 2 and 10 hr in cells with low and high EGFR levels, respectively. The time required for cell lysis and EGFR immunoprecipitation is the same as described above for the EGFR biosynthesis assay. Therefore, the length of the entire experiment until SDS-PAGE can be from 12 hr at minimum and up to 2 days.

EGFR down-regulation

A typical experiment includes a 2-hr incubation with unlabeled EGF, 15-min cell washing, 1-hr incubation with [^{125}I]EGF, 5-min washing, and about 1-hr solubilization with NaOH. Therefore, the entire experiment with two to three 12-well plates, including radioactivity counting, can be completed in 5 hr.

EGF internalization

One internalization experiment (one 12-well plate) is performed in about 20 min followed by a 1-hr cell solubilization in NaOH. More internalization experiments can be carried out during solubilization of the first set of cells. Practically, internalization measurements in four 12-well plates can be accomplished within 3 hr, including radioactivity counting on a γ-counter.

EGF recycling and degradation

Loading of cells with [^{125}I]EGF including the first acid wash in Basic Protocol 4 and Alternate Protocol 2 takes ~20 min. In Basic Protocol 4, pre-incubation of loaded cells with unlabeled EGF and the following chase can take together up to 5 hr. In Alternate Protocol 2, the manipulations with loaded cells are ~1 hr shorter. The acid wash treatment of the last time point takes only 10 min, but the trichloroacetic acid/phosphotungstic acid precipitation procedure and separation of the soluble from the insoluble radioactivity takes at least 2 hr. Therefore, a degradation/recycling experiment can last up to 7.5 hr, although it can be done in a significantly shorter period of time if the main goal is to measure the recycling rates.

Iodination

The total length of the iodination procedure is ~3 hr.

Acknowledgments

This work was supported by NCI grant CA089151(A.S.) and NCI/NRSA grant F32CA126344 (J.E.D.).

Literature Cited

Carpenter, G. and Cohen, S. 1976. ^{125}I-Labeled human epidermal growth factor: Binding internalization, and degradation in human fibroblasts. *J. Cell Biol.* 71:159-171.

Duex, J.E. and Sorkin, A. 2009. RNA interference screen identifies Usp18 as a regulator of EGF receptor synthesis. *Mol. Biol. Cell* 20:1833-1844.

Haigler, H.T., Maxfield, F.R., Willingham, M.C., and Pastan, I. 1980. Dansylcadaverine inhibits internalization of ^{125}I-epidermal growth factor in BALB 3T3 cells. *J. Biol. Chem.* 255:1239-1241.

Herbst, J.J., Opresko, L.K., Walsh, B.J., Lauffenberger, D.A., and Wiley, H.S. 1994. Regulation of postendocytic trafficking of the epidermal growth factor receptor through endosomal retention. *J. Biol. Chem.* 269:12865-12873.

Resat, H., Ewald, J.A., Dixon, D.A., and Wiley, H.S. 2003. An integrated model of epidermal growth factor receptor trafficking and signal transduction. *Biophys. J.* 85:730-743.

Sorkin, A., Teslenko, L., and Nikolsky, N. 1988. The endocytosis of epidermal growth factor in A431 cells: A pH of microenvironment and the dynamics of receptor complexes dissociation. *Exp. Cell Res.* 175:192-205.

Shtiegman, K., Kochupurakkal, B.S., Zwang, Y., Pines, G., Starr, A., Vexler, A., Citri, A., Katz, M., Lavi, S., Ben-Basat, Y., Benjamin, S., Corso, S., Gan, J., Yosef, R.B., Giordano, S., and Yarden, Y. Defective ubiquitinylation of EGFR mutants of lung cancer confers prolonged signaling. *Oncogene* 2007. 26:6968-6978.

Todderud, G. and Carpenter, G. 1989. Epidermal growth factor: The receptor and its function. *BioFactors* 2:11-15.

Wiley, H.S. and Cunningham, D.D. 1982. The endocytotic rate constant: A cellular parameter for quantitating receptor-mediated endocytosis. *J. Biol. Chem.* 257:4222-4229.

Wiley, H.S., Herbst, J.J., Walsh, B.J., Lauffenberger, D.A., Rosenfeld, M.G., and Gill, G.N. 1991. The role of tyrosine kinase activity in endocytosis, compartmentalization and down-regulation of the epidermal growth factor receptor. *J. Biol. Chem.* 266:11083-11094.

Documenting GLUT4 Exocytosis and Endocytosis in Muscle Cell Monolayers

Shuhei Ishikura,[1] Costin N. Antonescu,[1] and Amira Klip[1]

[1]Program in Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada

ABSTRACT

The elevated blood glucose following a meal is cleared by insulin-stimulated glucose entry into muscle and fat cells. The hormone increases the amount of the glucose transporter GLUT4 at the plasma membrane in these tissues at the expense of preformed intracellular pools. In addition, muscle contraction also increases glucose uptake via a gain in GLUT4 at the plasma membrane. Regulation of GLUT4 levels at the cell surface could arise from alterations in the rate of its exocytosis, endocytosis, or both. Hence, methods that can independently measure these traffic parameters for GLUT4 are essential to understanding the mechanism of regulation of membrane traffic of the transporter. Here, we describe cell population–based assays to measure the steady-state levels of GLUT4 at the cell surface, as well as to separately measure the rates of GLUT4 endocytosis and endocytosis. *Curr. Protoc. Cell Biol.* 46:15.15.1-15.15.19. © 2010 by John Wiley & Sons, Inc.

Keywords: glucose • internalization • insulin • muscle contraction • regulated membrane traffic

INTRODUCTION

Glucose is the primary energy source used in mammals. The elevated blood glucose levels that occur after a meal are rapidly returned to normal through enhanced glucose uptake into muscle and adipose tissues. In muscle fibers and adipocytes, glucose is stored as glycogen or triglycerides, respectively, and only a fraction is immediately oxidized to produce energy through oxidative phosphorylation and anaerobic glycolysis. It is precisely the entry into muscle and fat cells that is the rate-limiting step in the metabolism of glucose (Klip et al., 1982; D'Amore and Lo, 1986; Cline et al., 1998). This transport step is mediated by the insulin-regulated glucose transporter, GLUT4 (Zisman et al., 2000). In the basal (unstimulated) state, GLUT4 constitutively cycles to and from the plasma membrane (Fig. 15.15.1) and the steady-state distribution of GLUT4 favors intracellular compartments due to its fast endocytosis and slow exocytosis (Zaid et al., 2008). Insulin, released in response to the rising blood glucose concentration, stimulates glucose uptake into muscle and fat tissues by increasing the amount of GLUT4 at the cell surface.

The dynamic nature of this process is, however, missed by most biochemical and cell biological methods that simply document the net change in surface GLUT4. A gain in cell surface GLUT4 could conceivably be due to either an increase in the rate of exocytosis or a decrease in the rate of endocytosis of the transporter, or both. Hence, kinetic analyses that can differentiate between these two membrane traffic parameters are required to understand the mechanism of regulation of GLUT4 traffic. The purpose of this unit is to describe enzyme-linked immunosorbent assay (ELISA)–based methods to measure the amount of cell surface GLUT4. To separately determine the exocytic and endocytic rates of GLUT4, we describe the methods for measuring the amount of cell surface GLUT4 at steady-state (Basic Protocol 1), GLUT4 endocytosis (Basic Protocol 2), and GLUT4 exocytosis (Basic Protocol 3).

Current Protocols in Cell Biology 15.15.1-15.15.19, March 2010
Published online March 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1515s46

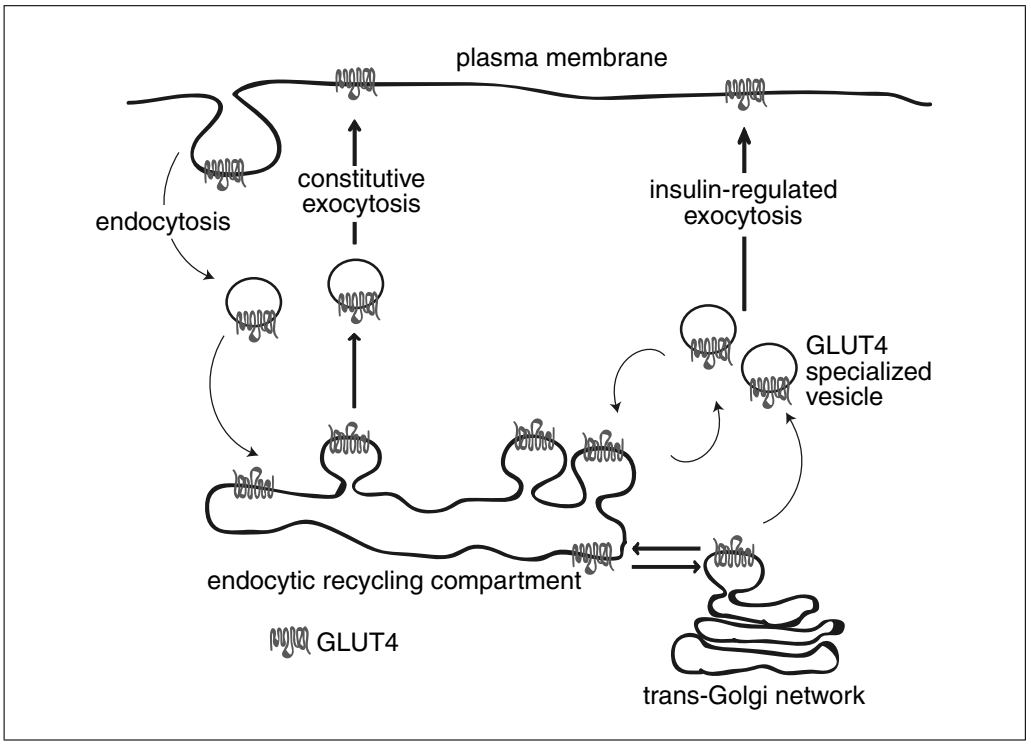


Figure 15.15.1 Schematic representation of GLUT4 traffic in muscle and fat cells. GLUT4 cycles between the intracellular compartments and the plasma membrane. Approximately half of intracellular GLUT4 is located in GLUT4 specialized vesicles, which are segregated from the endocytic recycling compartment and the trans-Golgi network. In the absence of stimuli, the steady-state distribution of GLUT4 favors intracellular compartments due to fast endocytosis and slow exocytosis. Insulin increases cell surface GLUT4 mainly by stimulating exocytosis from the GLUT4 specialized vesicle compartment.

Cell line stably expressing epitope-tagged GLUT4

Measurements of GLUT4 membrane traffic kinetics are vastly aided by introduction of epitopes (*myc* or HA) into exofacial portions of the transporter (GLUT4*myc* or GLUT4-HA). Using such chimeric transporters, GLUT4 molecules present at the cell surface can be distinguished from those within the cell by labeling intact cells with anti-HA or anti-*myc* antibodies (Fig. 15.15.2). When the epitope-tagged GLUT4 cDNA constructs are transiently transfected in muscle or fat cells, assays for detection of cell surface GLUT4 must account for the vast disparity in expression levels of the exogenous transporters between cells. Hence, following transient expression of GLUT4*myc* or HA-GLUT4, one is limited to implementing single cell–based immunofluorescence assays, and the relative amount of cell surface GLUT4 must be normalized to the expression level of the exogenous transporter in each cell. Therefore, these methods are time- and resource-consuming, and require knowledgeable implementation of quantitative tools to analyze fluorescence images (Antonescu et al., 2008a). Alternative strategies to document the traffic kinetics involve the generation of cells that stably express epitope-tagged GLUT4. Stable expression ensures that all cells express the same amount of epitope-tagged GLUT4, allowing direct comparison of the relative amount of cell surface GLUT4 between conditions without requiring normalization to total epitope-tagged GLUT4 expressed in each cell.

L6 cells are originally derived from rat skeletal muscle, and propagate as mononucleated myoblasts, but differentiate into multinucleated myotubes upon incubation in low-serum-containing medium (Yaffe, 1968; Richler and Yaffe, 1970; Mandel and Pearson, 1974; Yaffe and Saxel, 1977). The myotubes express several proteins typical of skeletal muscle,

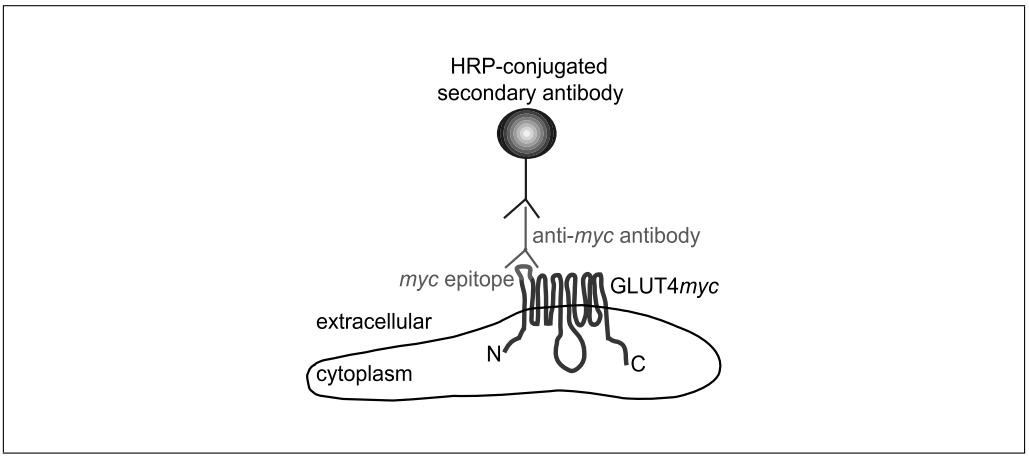


Figure 15.15.2 Detection of cell surface GLUT4*myc* in intact cells by antibody labeling of exofacial epitope coupled to colorimetric assay. Diagram depicting a plasma membrane–inserted GLUT4 molecule with *myc*-epitope engineered into the first exofacial loop of the transporter. Anti-*myc* antibodies bound to GLUT4*myc* are detected by HRP-conjugated secondary antibodies.

and insulin stimulates glucose uptake in the myotubes to a similar extent as mature skeletal muscle (Antonescu et al., 2005a). To create a GLUT4 molecule with an exofacially accessible epitope, a DNA sequence coding for a fragment of the human c-*myc* epitope (AEEQKLISEEDLLK) was inserted into the first ectodomain of the rat GLUT4 cDNA (Kanai et al., 1993). The corresponding cDNA construct was used to create L6 muscle cell lines in culture stably expressing *myc*-tagged GLUT4*myc* (L6-GLUT4*myc*; Wang et al., 1998), which have been extensively characterized (Huang et al., 2002, 2005; Niu et al., 2003; JeBailey et al., 2004, 2007; Foster et al., 2006; Diaz et al., 2007; Antonescu et al., 2008b) . By allowing measurement of the dynamic availability of the *myc*-epitope to the extracellular milieu (Fig. 15.15.2), L6 cells are an ideal model in which to study GLUT4 traffic in a skeletal muscle background. These assays can be performed on either confluent L6 myoblasts or differentiated L6 myotubes.

MEASURING STEADY-STATE CELL SURFACE GLUT4*myc*

BASIC PROTOCOL 1

Measurement of the amount of cell surface GLUT4*myc* at steady state is achieved through detection of the exofacial *myc* epitope in intact L6 muscle cells in culture (Fig. 15.15.2). Cells are labeled with anti-*myc* antibodies, and subsequently labeled with secondary antibodies coupled to horseradish peroxidase (HRP; Fig. 15.15.3A). The amount of cell-surface bound HRP activity is detected by the amount of chromogen formed from the HRP substrate *o*-phenylenediamine (OPD; Fig. 15.15.3B), which is proportional to the amount of cell-surface GLUT4*myc*. Importantly, this reaction remains linear with respect to the amount of HRP present over a large range of enzyme concentrations (Fig. 15.15.3C), indicating that absorbance is linearly related to the amount of cell surface GLUT4*myc*.

Materials

GLUT4*myc* cells [L6-GLUT4*myc*; Wang et al., 1998; L6 cell lines can be obtained from ATCC (*http://www.atcc.org*, cat. no. CRL-1458); these cells can be made to stably express epitope-tagged GLUT4 as desired; for further inquiries regarding currently existing L6-GLUT4*myc* stable cell lines, please contact Dr. Amira Klip (*amira@sickkids.ca*)]

L6 growth medium (see recipe) *or* L6 differentiation medium (see recipe)

α-modification of Eagle's medium (α-MEM; Wisent Bioproducts; *http://www.wisent.ca/*)

Stimulatory agents: e.g., human insulin (Humulin R, Lilly), sucrose (Sigma-Aldrich), or platelet-derived growth factor (PDGF; Sigma-Aldrich)

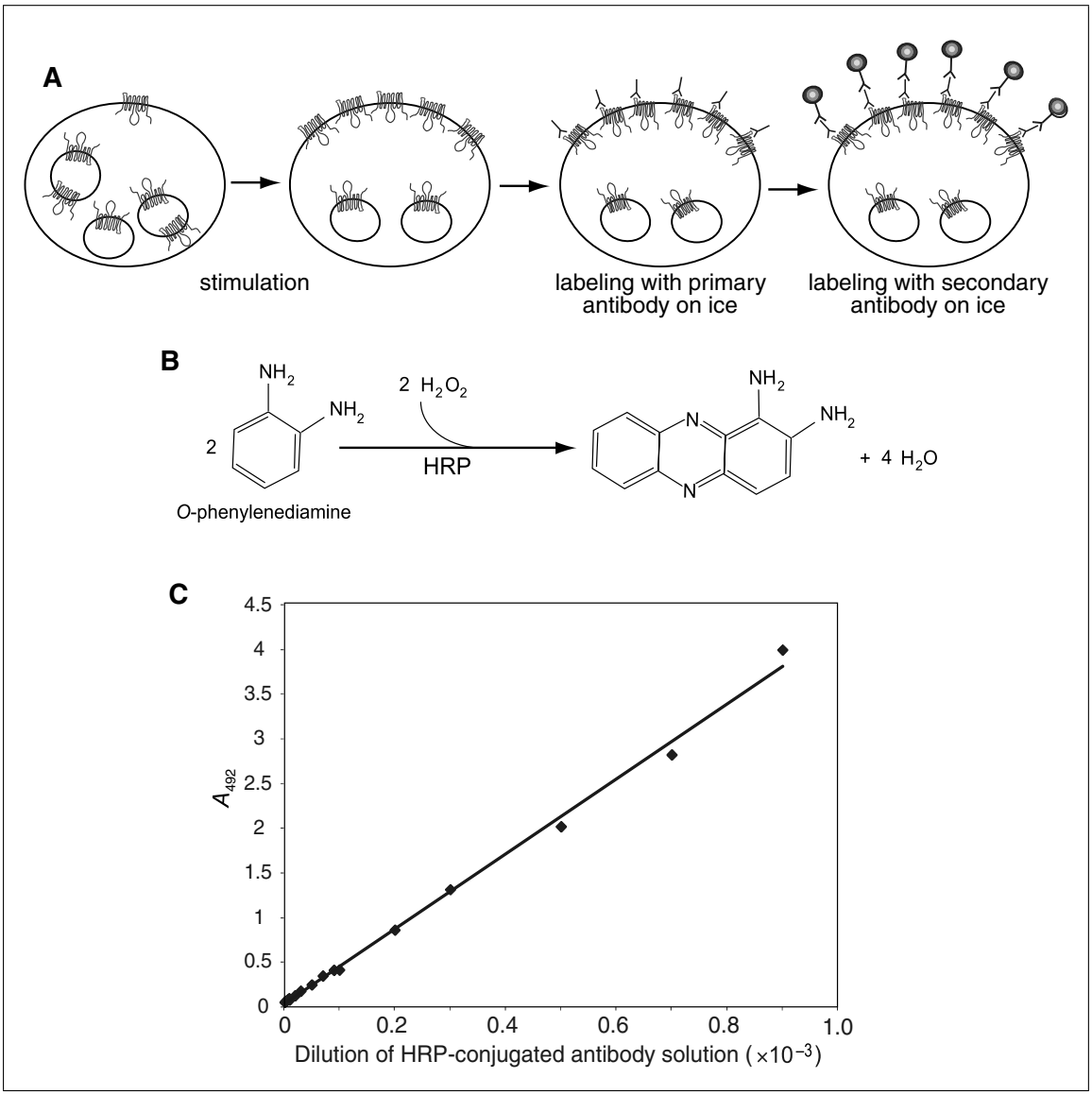


Figure 15.15.3 Measurement of steady-state cell surface GLUT4*myc* in intact cells. (**A**) Diagram depicting the labeling of cell surface–exposed GLUT4*myc* in intact cells with anti-*myc* antibodies, while intracellularly located transporters remain unlabeled. Upon stimulation, there is an increase in cell surface transporters at the expense of the intracellular content of GLUT4*myc*, reflected by an increase in cell surface-bound *myc*-antibodies. (**B**) Diagram of chemical reaction by which *o*-phenylenediamine (OPD) is converted to a chromogenic product by HRP. (**C**) 200 μl of OPD assay solution was incubated with various amounts of HRP-conjugated secondary antibody, as indicated. Each reaction was stopped simultaneously by addition of 50 μl of 3 M HCl, followed by measurement of absorbance at 492 nm. Shown are the absorbance values of each HRP-conjugated secondary antibody concentration.

PBS+: phosphate-buffered saline (PBS; see recipe) supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$

Blocking solution: PBS+ (see above) 5% (v/v) goat serum (Sigma-Aldrich)

Anti-*myc* rabbit polyclonal antibody (Sigma-Aldrich)

4% (w/v) paraformaldehyde in PBS+, ice cold

PBS+ (see above) containing 100 mM glycine

Horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibody (Jackson Immunoresearch)

OPD assay solution (see recipe)

3 N HCl or H_2SO_4

24-well culture plates (BD Biosciences)
96-well microtiter plate
Spectrophotometer with microtiter plate reader

Plan the experiment and seed cells

1. Seed GLUT4*myc* cells at a density of 2.0–3.0 $\times$ 10^4 cells per well of a 24-well plate in 0.5 ml/well L6 growth medium or L6 differentiation medium.

   For experiments on myoblasts, cells are subsequently grown to confluence, typically for 36 to 48 hr in L6 growth medium. For experiments on myotubes, cells are placed in L6 differentiation medium at the time of seeding and grown for ~7 days to allow for myoblast differentiation into myotubes.

2. Set up the cultures so that each condition to be assayed is done in triplicate (three wells/condition) with a well for an (unlabeled) control for each condition.

   Thus, a 24-well plate can be used to assay at most six experimental conditions.

Measure steady-state cell surface GLUT4myc

3. Aspirate medium from cells and wash cells once by adding and then removing 0.5 ml per well prewarmed serum-free α-MEM. Replace medium with serum-free α-MEM (at least 250 μl/well) and incubate for 3 to 5 hr at 37°C.

4. Stimulate cells as required by incubating cells for the appropriate amount of time in 250 μl of serum-free α-MEM containing the appropriate agent.

5. Subsequently, place cells on ice and wash thee times, each time by adding and then removing 0.5 ml per well ice-cold PBS+.

   These washes are performed to remove residual medium.

6. Aspirate PBS+ from cells and place the intact cells in 250 μl/well of blocking solution for 20 min on ice.

7. Prepare ≥5 ml/plate of a solution of ice-cold blocking solution containing 1 μg/ml rabbit polyclonal anti-*myc* antibody (*myc*-labeling solution).

8. Aspirate blocking solution from cells and add 250 μl of the *myc*-labeling solution to each well, except for unlabeled control wells. Instead, add 250 μl of blocking solution to the control wells. Incubate cells on ice for 1 hr.

9. Aspirate *myc*-labeling solution from cells and wash eight times, each time with 0.5 ml per well ice-cold PBS+. Aspirate medium from each well for the first three washes; in subsequent washes dump the wash solution by inverting the plate to remove solution from wells.

10. Fix cells for 10 min in 0.5 ml/well ice-cold PBS+ containing 4% PFA.

11. Aspirate fixative solution and quickly wash cells in 0.5 ml per well PBS+. Quench fixative in 0.5 ml/well PBS+ containing 100 mM glycine (quenching solution) for 10 min on ice.

12. Aspirate quenching solution. Incubate cells in 0.5 ml/well blocking solution for 15 min at room temperature.

13. Prepare 7 ml/plate of ice-cold blocking buffer containing a 1:750 dilution of horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibody (secondary labeling solution).

14. Aspirate blocking buffer, add 250 μl of secondary labeling solution to each well, and incubate for 1 hr on ice.

15. Aspirate solution from cells and wash six times, each time by adding and then removing 0.5 ml per well ice-cold PBS+.

Detect bound antibody

16. Prepare 12.5 ml/plate OPD assay solution immediately before use.

17. Aspirate any remaining PBS+ from cells and quickly add 500 µl of OPD assay solution to each well.

    It is essential that a minimal amount of time elapses between addition of the reagent to the first and last wells, as the amount of chromogenic product formed depends on the length of incubation of cells with the OPD reagent and should be as close to constant for all wells as possible.

18. After sufficient color develops (i.e., yellow is visible by eye in each well to which anti-*myc* antibody solution was added, typically after 5 to 20 min), add 125 µl of 3 N HCl (or equivalent normality of H_2SO_4 or other adequate strong acid) to halt the OPD chromogenic reaction.

    Note that 3 N HCl solution should be added to wells in the same order as was used for addition of the OPD assay solution to ensure constant time of incubation for each well.

Quantify labeling

19. Transfer 200 µl from each well to designated wells of a 96-well microtiter plate and read absorbance at 492 nm.

20. Determine the relative amount of cell surface GLUT4*myc* (csG4) for each stimulated condition (*s*) following normalization to that of the basal state (also see Anticipated Results):

$$\mathrm{csG4}(s) = \frac{A_{492}(s) - A_{492}\,(\text{unlabeled control }(s))}{A_{492}(basal) - A_{492}\,(\text{unlabeled control }(basal))}$$

BASIC PROTOCOL 2

MEASURING GLUT4*myc* ENDOCYTOSIS

Measurement of the rate of GLUT4*myc* endocytosis is achieved through first labeling the exofacial *myc* epitope in intact L6 muscle cells in culture and then measuring the loss of label from the cell surface as GLUT4*myc* undergoes endocytosis. Following labeling of intact cell surface GLUT4*myc* with anti-*myc* antibodies on ice, cells are rewarmed to 37°C to allow GLUT4*myc* internalization (Fig. 15.15.4). The amount of *myc*-antibody-bound GLUT4*myc* remaining at the cell surface is detected by labeling with secondary antibodies coupled to HRP. Cell-surface HRP-bound GLUT4*myc* is subsequently detected as described in Basic Protocol 1.

For materials, see Basic Protocol 1.

Plan the experiment and seed cells

1. Seed GLUT4*myc* cells at a density of 2.0–3.0 × 10^4 cells per well of a 24-well plate in 0.5 ml/well L6 growth medium or L6 differentiation medium.

   For experiments on myoblasts, cells are subsequently grown to confluence, typically for 36 to 48 hr in L6 growth medium. For experiments on myotubes, cells are placed in L6 differentiation medium at the time of seeding and grown for 7 days to allow for myoblast differentiation into myotubes.

2. To prepare for the experiment, note that a separate 24-well plate is required for each timepoint of GLUT4*myc* endocytosis to be assayed. Also note that a separate

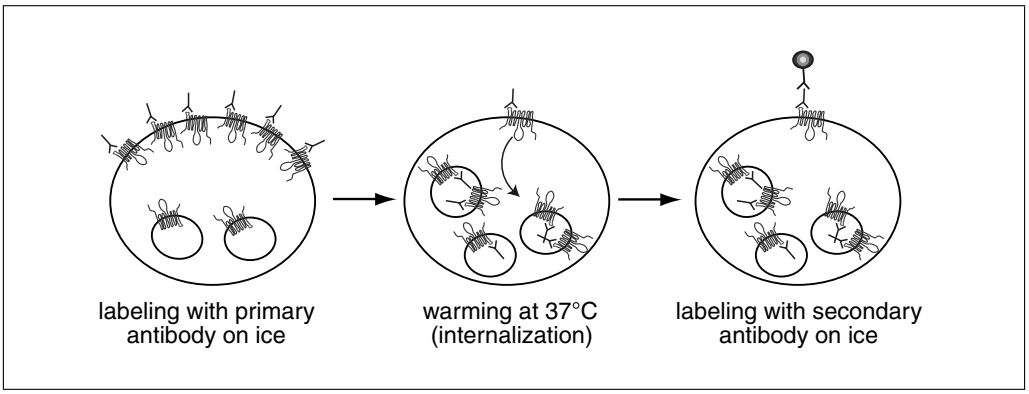


Figure 15.15.4 Measurement of GLUT4*myc* endocytosis in intact cells. Diagram depicting measurement of GLUT4*myc* endocytosis: cells are first stimulated at 37°C as required then placed on ice and labeled with anti-*myc* antibodies at 4°C. Subsequently, cells are rewarmed to 37°C for fixed amounts of time, thereby allowing internalization of some anti-*myc* antibody bound GLUT4*myc* molecules.

24-well plate without internalization (0 min, termed control without internalization) is required for each experiment.

Typically, GLUT4myc endocytosis is measured at 2, 5, and 10 min (Antonescu et al., 2008b), although more timepoints can be assayed if desired.

3. Also note that each condition to be assayed must be done in triplicate (three wells/condition) for each timepoint, with a well for an (unlabeled) control for each condition.

Thus, each 24-well plate (representing a single timepoint) can be used to assay at most six experimental conditions.

Prepare cells for GLUT4 assay

4. Aspirate medium from cells and wash cells once by adding and then removing 0.5 ml/well prewarmed serum-free α-MEM. Replace medium with serum-free α-MEM and incubate for 3 to 4 hr at 37°C.

5. Stimulate cells as required by incubating cells for the appropriate amount of time in 250 µl of serum-free α-MEM containing the appropriate agent.

6. Subsequently, place cells on ice and wash three times, each time by adding and then removing 0.5 ml/well ice-cold PBS+.

These washes are performed to remove residual medium.

Block and label with antibody

7. Aspirate PBS+ from cells and place the intact cells in 250 µl/well of blocking solution for 20 min on ice.

8. Prepare ≥5 ml/plate of a solution of ice-cold blocking solution containing 1 µg/ml rabbit polyclonal anti-*myc* antibody (*myc*-labeling solution).

9. Aspirate blocking solution from cells and add 250 µl of the *myc*-labeling solution to each well except for unlabeled control wells. Instead, add 250 µl of blocking solution to the control wells. Incubate cells on ice for 1 hr.

10. Aspirate *myc*-labeling solution from cells and wash five times, each time with 0.5 ml/well ice-cold PBS+. Aspirate medium from wells for the first three washes; in subsequent washes dump the wash solution by inverting the plate to remove solution from wells.

Measure GLUT4 endocytosis

11. For the 24-well plates to be used to measure internalization for 2, 5, and 10 min (or other time points, except the 0 min control without internalization), quickly wash cells twice in 0.5 ml/well PBS+, prewarmed to 37°C. For control without internalization, wash cells twice in 0.5 ml/well ice-cold PBS+ and retain these cells on ice until step 13 (bypass step 12 for this control).

12. Quickly aspirate solution from cells and add 0.5 ml/well prewarmed α-MEM containing the appropriate stimulus. Incubate cells in a 37°C incubator for 2, 5, or 10 min.

    It is essential to perform steps 11 to 12 rapidly to ensure that a minimum of time elapses between rewarming of cells in PBS+ (step 11) and addition of stimulus-containing α-MEM (step 12).

13. Subsequently, quickly replace cells on ice and wash all plates twice by adding and removing 0.5 ml/well ice-cold PBS+. Also perform this and all subsequent steps for the "control without internalization" plate of cells.

Fix cells

14. Fix cells for 10 min in 0.5 ml/well ice-cold PBS+ containing 4% PFA.

15. Aspirate fixative solution and quickly wash cells in 0.5 ml/well PBS+. Quench fixative in 0.5 ml/well PBS+ containing 100 mM glycine (quenching solution) for 10 min on ice.

16. Aspirate quenching solution. Incubate cells in 0.5 ml/well blocking solution for 15 min on ice.

Label with secondary antibody

17. Prepare 7 ml/plate of ice-cold blocking buffer containing a 1:750 dilution of horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibody (secondary labeling solution).

18. Aspirate blocking buffer, add 250 μl of secondary labeling solution to each well, and incubate for 1 hr on ice.

19. Aspirate solution from cells and wash six times, each time by adding and then removing ml/well ice-cold PBS+.

Detect labeling

20. Prepare 12.5 ml/plate OPD assay solution (see Reagents and Solutions) immediately before use.

21. Aspirate any remaining PBS+ from cells and quickly add 500 μl of OPD assay solution to each well.

    It is essential that a minimal amount of time elapses between addition of the reagent to the first and last wells, as the amount of chromogenic product formed depends on the length of incubation of cells with the OPD reagent and should be as close to constant for all wells as possible.

22. After sufficient color develops (i.e., yellow is visible by eye in each well to which anti-*myc* antibody solution was added, typically after 5 to 20 min), add 125 μl of 3 N HCl (or equivalent normality of H_2SO_4 or other adequate strong acid) to halt the OPD chromogenic reaction.

    Note that 3 N HCl solution should be added to wells in the same order as was used for addition of the OPD assay solution to ensure constant time of incubation for each well.

Quantify labeling

23. Transfer 200 µl from each well to designated wells of a 96-well microtiter plate and read absorbance at 492 nm.

24. Express the rate of GLUT4 endocytosis in two different ways (Fig. 15.15.6B):

    a. *GLUT4myc not internalized* (G4NI; i.e., remaining at the cell surface) at each time point (t) and each stimulus condition (s) is determined as follows (also see Anticipated Results):

$$\text{G4NI}\ (t,s) = \frac{A_{492}(t,s) - A_{492}\ (\text{unlabeled control}\,(t,s))}{A_{492}(0\ \text{min},s) - A_{492}\ (\text{unlabeled control}\ (0\ \text{min},s))}$$

    b. *GLUT4myc internalized* (G4I) at each time point (t) and each stimulus condition (s) is determined as follows (also see Anticipated Results):

$$\text{G4I}\ (t,s) = 1 - G4\text{NI}\ (t,s)$$

MEASURING GLUT4*myc* EXOCYTOSIS

BASIC PROTOCOL 3

Measurement of the rate of GLUT4*myc* exocytosis is achieved in two stages (Fig. 15.15.5). During the first stage, GLUT4 at the cell surface is labeled with anti-*myc* antibodies (on ice), followed by extensive washing to remove unbound anti-*myc* antibodies. GLUT4 thus labeled at the cell surface is then allowed to internalize from the cell surface for 2 hr at 37°C, achieving homogenous mixing of labeled transporters with all pools of intracellular GLUT4 (Fig. 15.15.1). In the second stage, the rate of arrival of previously *myc*-antibody-labeled GLUT4 molecules to the cell surface is measured over time. This measurement is achieved via detection of anti-*myc* labeling with secondary antibodies coupled to HRP at saturating concentrations. Cell-surface HRP-bound GLUT4*myc* is subsequently detected as described in Basic Protocol 1.

For materials, see Basic Protocol 1.

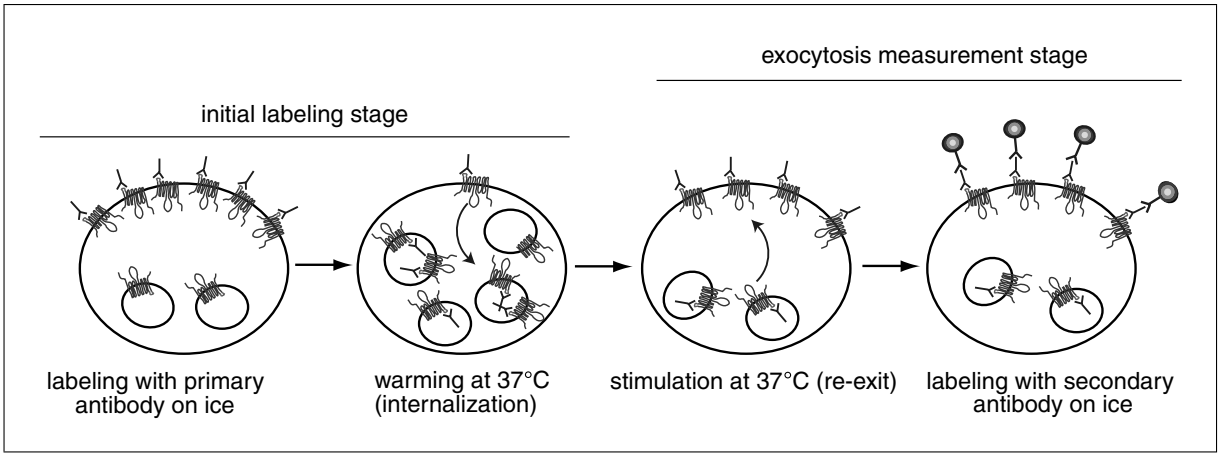


Figure 15.15.5 Measurement of GLUT4*myc* exocytosis in intact cells. Diagram depicting measurement of GLUT4*myc* exocytosis: cells are placed on ice and labeled with anti-*myc* antibodies at 4°C. Subsequently, cells are warmed to 37°C for 2 hr, thereby allowing internalization of anti-*myc* antibody–bound GLUT4*myc* molecules. After internalization, cells are treated with stimuli, thereby allowing re-exit of antibody-bound GLUT4*myc*. Finally, anti-*myc* antibody bound GLUT4*myc* are detected by HRP-conjugated secondary antibodies.

Plan experiment and seed cells

1. Seed GLUT4*myc* cells at a density of 2.0–3.0 × 10^4 cells per well of a 24-well plate in 0.5 ml/well medium.

   For experiments on myoblasts, cells are subsequently grown to confluence, typically for 36 to 48 hr in L6 growth medium. For experiments on myotubes, cells are placed in L6 differentiation medium at the time of seeding and grown for 7 days to allow for myoblast differentiation into myotubes.

2. To prepare for the experiment, note that a separate 24-well plate is required for each timepoint of GLUT4*myc* exocytosis to be assayed. Also required for each experiment is a separate 24-well plate for total amount of GLUT4 without exocytosis (termed control without exocytosis).

   Typically, GLUT4myc exocytosis is measured at 5, 10, and 20 min, although other timepoints can be assayed if desired.

3. Also note that each condition to be assayed must be done in triplicate (three wells/condition) for each timepoint, with a well for an (unlabeled) control for each condition.

   Thus, each 24-well plate (representing a single timepoint) can be used to assay at most six experimental conditions. Since all values measured are typically expressed relative to basal (unstimulated) cells, this condition must be included in every experiment.

Perform initial labeling of GLUT4myc with anti-myc antibodies

4. Aspirate medium from cells and wash cells once by adding and then removing 0.5 ml/well prewarmed serum-free α-MEM. Replace medium with 0.5 ml/well serum-free α-MEM and incubate for 2 hr at 37°C.

5. Stimulate cells with 250 µl of serum-free α-MEM (per well) containing 100 nM insulin for 30 min.

   This stimulation with insulin increases the amount of GLUT4myc that becomes labeled with anti-myc antibody at step 9.

6. Subsequently, place cells on ice and wash three times, each time by adding and then removing 0.5 ml/well ice-cold PBS+.

   These washes are performed to remove residual medium.

7. Aspirate PBS+ from cells and place the intact cells in 250 µl/well of blocking solution for 20 min on ice.

8. Prepare ≥5 ml/plate of a solution of ice-cold blocking solution, containing 1 µg/ml rabbit polyclonal anti-*myc* antibody (*myc*-labeling solution).

9. Aspirate blocking solution from cells and add 250 µl of *myc*-labeling solution to each well except for unlabeled control wells. Instead, add 250 µl of blocking solution to the control wells. Incubate cells on ice for 1 hr.

10. Aspirate *myc*-labeling solution from cells and wash five times in 0.5 ml/well ice-cold PBS+. Aspirate medium from wells for the first three washes; in subsequent washes dump the wash solution by inverting the plate to remove solution from wells.

11. For the 24-well plates to be used to measure exocytosis, quickly wash cells twice in 0.5 ml/well PBS+ prewarmed to 37°C.

Measure GLUT4myc exocytosis

12. Quickly aspirate solution from cells and add 0.5 ml/well prewarmed α-MEM. Incubate cells at 37°C for 2 hr.

13. Subsequently, quickly aspirate solution from cells and immediately treat cells by incubating cells in 250 µl of serum-free α-MEM containing the appropriate stimulus

for the desired timepoints for exocytosis measurement. Do not perform this step for the "control without exocytosis" plate. For this plate, skip step 13 and proceed directly to step 14.

14. Subsequently, quickly replace cells on ice and wash all plates twice with 0.5 ml/well ice-cold PBS+.

Fix cells

15. Fix cells for 10 min in 0.5 ml/well ice-cold PBS+ containing 4% PFA.

16. Aspirate fixative solution and quickly wash cells in 0.5 ml/well PBS+. Quench fixative in 0.5 ml/well PBS+ containing 100 mM glycine (quenching solution) for 10 min on ice.

17. Aspirate quenching solution. Incubate cells in 0.5 ml/well blocking solution for 15 min on ice.

Label with secondary antibody

18. Prepare 7 ml/plate of ice-cold blocking buffer containing a 1:750 dilution of horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibody (secondary labeling solution).

19. Aspirate blocking buffer, add 250 µl of secondary labeling solution to each well, and incubate for 1 hr on ice.

20. Aspirate solution from cells and wash six times in 0.5 ml/well ice-cold PBS+.

Detect labeling

21. Prepare 12.5 ml/plate OPD assay solution (see Reagents and Solutions) immediately before use.

22. Aspirate any remaining PBS+ from cells and quickly add 500 µl of OPD assay solution to each well.

    It is essential that a minimal amount of time elapses between addition of the reagent to the first and last wells, as the amount of chromogenic product formed depends on the length of incubation of cells with the OPD reagent and should be as close to constant for all wells as possible.

23. After sufficient color develops (i.e., yellow is visible by eye in each well to which anti-*myc* antibody solution was added, typically after 5 to 20 min), add 125 µl/well of 3 N HCl (or equivalent normality of H_2SO_4 or other adequate strong acid) to halt the OPD chromogenic reaction.

    Note that 3 N HCl solution should be added to wells in the same order as was used for addition of the OPD assay solution to ensure constant time of incubation for each well.

Quantify labeling

24. Transfer 200 µl from each well to designated wells of a 96-well microtiter plate and read absorbance at 492 nm.

25. Express the rate amount of antibody-bound GLUT4*myc* undergoing exocytosis (G4Exo) following time (t) of exocytosis in any stimulated condition (s) relative to that observed in the control without exocytosis condition:

$$\text{G4Exo}\ (t,s) = \frac{A_{492}(t,s) - A_{492}(\text{unlabeled control})}{A_{492}(\text{control without exocytosis}) - A_{492}(\text{unlabeled control})}$$

Additionally, the amount of exocytosis in a stimulated condition can be normalized to that in the basal state (see Anticipated Results).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

L6 differentiation medium

α-modification of Eagle's medium (α-MEM; Wisent Bioproducts; *http://www.wisent.ca/*) supplemented with:
2% (v/v) fetal bovine serum (HyClone)
1% (v/v) antibiotic solution (Wisent Bioproducts)
If sterilized, store up to 4 months at 4°C; otherwise use immediately

L6 growth medium

α-modification of Eagle's medium (α-MEM; Wisent Bioproducts; *http://www.wisent.ca/*) supplemented with:
10% (v/v) fetal bovine serum (HyClone)
1% (v/v) antibiotic solution (Wisent Bioproducts)
If sterilized, store up to 4 months at 4°C; otherwise use immediately

OPD assay solution

Dissolve one 20 mg tablet of *O*-phenylenediamine (OPD) and 20 µl of 30% H_2O_2 per 50 ml of 0.05 M phosphate-citrate buffer, pH 5.0 (see recipe).

Phosphate-buffered saline (PBS)

0.137 M NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4, pH 7.4
Store indefinitely at room temperature but verify pH following extensive storage.

Phosphate/citrate buffer, pH 5.0

Add 25.7 ml of 0.2 M dibasic sodium phosphate to 24.3 ml of 0.1 M citric acid and 50 ml of water, and adjust the pH to 5.0, as necessary. Store indefinitely at room temperature, but verify pH following extended storage.

COMMENTARY

Background Information

The cell biology of GLUT4 traffic

Insulin stimulates glucose uptake into muscle and adipose tissues by increasing the amount of GLUT4 at the cell surface. In these cells, GLUT4 exists in multiple subcellular compartments including the plasma membrane, endosomal network, and a post-endosomal membrane compartment (Fig. 15.15.1). GLUT4 constitutively traverses the plasma membrane and these intracellular compartments, and this process is regulated by insulin, resulting in an increase in cell surface GLUT4 (termed GLUT4 translocation). Importantly, in insulin-resistant states, as in type 2 diabetes, GLUT4 translocation is impaired. Therefore, understanding the itinerary of GLUT4 traffic and its regulatory inputs is of fundamental importance.

In the absence of stimuli, the steady-state distribution of GLUT4 favors intracellular compartments due to its fast endocytosis and slow exocytosis. In the basal state, 5% to 10% of GLUT4 is found on the cell surface in L6 myoblasts, myotubes, and 3T3-L1 adipocytes (Li et al., 2001; Zeigerer et al., 2002). Using strategies such as those outlined in this chapter to measure the distinct kinetic parameters of exocytosis and endocytosis in L6 muscle cells, 3T3-L1 adipocytes, and rat adipocytes, it has consistently been found that insulin increases the rate of exocytosis of GLUT4 (reviewed by Zaid et al., 2008). While insulin also effects a small and debated decrease in GLUT4 endocytosis in 3T3-L1 adipocytes (Blot and McGraw, 2006), the hormone is without effect on the endocytosis of the transporter in L6 muscle cells (Li et al., 2001; Antonescu et al., 2008b,

2009). Hence, insulin acts to increase cell surface GLUT4 primarily by increasing its rate of exocytosis.

The mechanism by which insulin promotes exocytosis of GLUT4-containing vesicles has been increasingly documented (reviewed by Zaid et al., 2008). Upon binding to its receptor, insulin initiates phosphatidylinositol-3-kinase (PI3K)-dependent activation of the serine/threonine kinase Akt, which in turn phosphorylates the Akt substrate of 160 kDa (AS160). Phosphorylation of AS160 is thought to inactivate its Rab-GAP activity, thereby promoting GTP-loading of selective Rab proteins that impinge on GLUT4 membrane traffic (Ishikura et al., 2007). In L6 muscle cells, regulation of Rabs 8A and 14 via AS160 is required for insulin-stimulated GLUT4 exocytosis (Ishikura et al., 2007), while in 3T3-L1 adipocytes, Rab10 participates instead (Sano et al., 2007). Rab proteins are thought to affect membrane traffic by binding to certain effector molecules preferentially in the GTP-bound state; GTP-Rab8A may regulate GLUT4 exocytosis by binding to myosin Vb (Ishikura and Klip, 2008; Randhawa et al., 2008).

It remains unknown whether these Rab proteins function within endosomal compartments or at the plasma membrane in order to increase GLUT4 exocytosis (see Fig. 15.15.1). However, insulin increases the rate of fusion of GLUT4 vesicles with the plasma membrane (reviewed by Zaid et al., 2008). Insulin-stimulated GLUT4-containing vesicles fuse with the plasma membrane in a manner that requires the SNARE proteins VAMP2, SNAP23, and syntaxin 4 (Foster et al., 1998; Kawanishi et al., 2000), and that is regulated by Munc18c (Tamori et al., 1998; Thurmond et al., 2000; D'Andrea-Merrins et al., 2007). Insulin also engages other cellular elements in GLUT4 membrane traffic (reviewed by Zaid et al., 2008), including release of GLUT4 from TUG (Bogan et al., 2003; Yu et al., 2007; Schertzer et al., 2008), binding of GLUT4 to α-actinin-4 (Talior-Volodarsky et al., 2008), and participation of the dynamic actin cytoskeleton (Tsakiridis et al., 1999; Patel et al., 2003; JeBailey et al., 2004; Torok et al., 2004), and several motor proteins (Imamura et al., 2003; Semiz et al., 2003; Bose et al., 2004; Randhawa et al., 2008). Thus, insulin regulates GLUT4 traffic through diverse input at multiple stages, which in sum achieves an increase in the rate of GLUT4 exocytosis and hence in the net amount of cell surface GLUT4 (reviewed by Zaid et al., 2008).

Aside from insulin, other physiologically important stimuli also increase cell surface GLUT4. Muscle contraction, as occurs during exercise, causes an increased energy demand in skeletal muscle which is accommodated by an increase in cell surface GLUT4 (reviewed by Wijesekara et al., 2006). Due to technical limitations of achieving contraction in L6 cells in culture, it is not possible to directly observe the regulation of GLUT4 membrane traffic in this state. However, exogenous activation of the signaling pathways initiated by muscle contraction, specifically a rise in intracellular Ca^{2+} and activation of AMP-activated protein kinase (AMPK), can be emulated by treatment of cells with dinitrophenol (DNP), which accordingly also provokes a gain in surface GLUT4. Interestingly, in this case, using the strategies described in this unit, it is found that the regulation occurs largely through a reduction in the rate of GLUT4 endocytosis (Antonescu et al., 2008b). By analogy, muscle contraction may increase cell surface GLUT4 through a reduced rate of GLUT4 endocytosis, although this requires experimental verification in mature, contracting muscle fibers. Such experiments await the availability of methods to accurately detect GLUT4 traffic in intact muscle fibers, ideally expressing tagged GLUT4.

Unlike the regulation of GLUT4 exocytosis by insulin, much less is known about the mechanism by which muscle contraction may regulate GLUT4 endocytosis. GLUT4 internalization occurs by both clathrin-dependent and -independent mechanisms in 3T3-L1 adipocytes and L6 muscle cells (Blot and McGraw, 2006; Antonescu et al., 2008b, 2009). Stimulation of L6 muscle cells with DNP reduces GLUT4 internalization via the clathrin-independent route, suggesting that the signaling pathways involving intracellular Ca^{2+} and AMPK activation target this route of internalization to augment cell surface GLUT4 (Antonescu et al., 2008b). The molecules participating in the clathrin-independent internalization in L6 muscle cells remain poorly understood, but they do not involve caveolae and require the GTPase dynamin. These characteristics are also shared by the internalization of the interleukin 2 receptor β subunit (IL-2Rβ) (Antonescu et al., 2008b, 2009). Further investigation of the molecular machinery of this route of internalization will aid in understanding the mechanism by which muscle contraction regulates GLUT endocytosis.

Critical Parameters and Troubleshooting

Cell culture prior to seeding

L6 muscle cells are grown and cultured as myoblasts prior to seeding for these experiments. Experiments can then be done on L6 myoblasts or following differentiation of L6 cells into myotubes by incubation of confluent myoblasts in low-serum medium (containing 2% FBS) for approximately 5 to 7 days. Of note, L6 cells can begin to partially differentiate into myotubes even in regular growth medium (containing 10% FBS) upon attaining confluence. Once differentiation is thus initiated, L6 cells exhibit altered morphology and other parameters relative to undifferentiated L6 myoblasts, although they remain mononuclear. Furthermore, these partly differentiated cells are less efficient at completing differentiation into multinucleated myotubes upon seeding in low-serum conditions. Hence, it is important to ensure that myoblasts never reach more than ~50% confluence prior to seeding for experiments. Typically, this is best achieved by passaging cells every 48 hr, and never less frequently than every 72 hr.

Cell density for seeding

Upon seeding cells, it is important to follow the indicated cellular density. This will allow experiments to be done on myoblasts (typically 48 hr after seeding) on a confluent monolayer, and allow optimal differentiation into myotubes (over ~7 days). Performing experiments on myoblasts that are not fully confluent may result in detachment of cells from the 24-well plate during the experiment. This is a particularly important consideration in measurement of endocytosis (Basic Protocol 2) and exocytosis (Basic Protocol 3), which require extensive cellular manipulation during the assays.

Note that when intending to perform experiments on myoblasts, care must also to taken not to seed more cells than indicated because, when cells reach confluence too rapidly, differentiation is initiated, which could result in early, partial differentiation. This could produce measurements that are difficult to interpret. Hence, seeding cells at the indicated density is critical to ensure consistent, reproducible measurements of GLUT4 traffic parameters.

Transfection or infection of L6 cells prior to assays

The assays described in this unit all allow measurement of GLUT4 traffic parameters following various transfection protocols. However, care must be taken when considering such strategies to ensure that a vast majority of cells are successfully transfected. Due to the fact that the ELISA-based assays described here measure the relative amount of cell surface GLUT4 in a population of cells, transfection methods that achieve less than ~70% efficiency will likely not allow detection of a possibly altered GLUT4 traffic phenotype.

Several methods to transfect L6 myoblasts with siRNA have been used in L6 myoblasts and myotubes, including Oligofectamine (Invitrogen) and CellPhect (GE Life Science; Jebailey et al., 2004; Antonescu et al., 2005b, 2008a; Huang et al., 2005). Currently, no lipid cationic method for transient transfection of L6 myoblasts with longer oligonucleotides encoding cDNAs or shRNAs has been identified that achieves sufficiently elevated transfection efficiency for use with the methods described in this unit. However, exogenous expression of various proteins can be achieved by either creating stably transfected cell lines or by infection with adenoviral or lentiviral strategies, a strategy we previously employed to express dominant-negative mutants of p38MAPK α and β (Antonescu et al., 2005b). Of note, should transfection efficiency remain limited to a small percentage of L6 cells for a particular circumstance, we refer the reader to single-cell, fluorescence-based assays for measuring GLUT4 traffic parameters that we have previously described (Antonescu et al., 2008a), which, although resource- and labor-intensive, are suitable under those conditions.

Normalization to eliminate differences due to cell density between conditions

Some experimental treatments, such as siRNA knockdown of certain proteins, or prolonged treatment with particular inhibitors, can result in differences in cell growth or attachment. This can, in turn, result in differences in cell density between conditions when performing the experiment. Thus, it is important to normalize for cell density during such experiments in order to accurately determine the relative differences in cell surface GLUT4. This can be achieved by seeding an extra well per condition (of a 24-well plate) that is used to measure total protein concentration, followed by normalization of the amount of cell surface GLUT4 to protein concentration in each condition.

Obtaining a source of optimal antibody

Prior to beginning the experiments described in this unit, it is essential to obtain

a source of anti-*myc* antibodies that provides an appropriate strength of signal relative to background. Importantly, not all commercially available antibodies are suitable for this assay. This can be done by performing the steady-state cell surface GLUT4*myc* assay described in Basic Protocol 1 simultaneously on L6 myoblasts that express GLUT4*myc* (L6-GLUT4*myc*) and on cells that do not (wild-type cells). Appropriate antibodies for the experiments described in this unit will result in cell surface GLUT4*myc* labeling at least ten times greater than that observed on wild-type cells. After this initial characterization of the anti-*myc* antibody, it may be necessary to determine the labeling of wild-type cells in each subsequent assay in order to obtain a value for background, nonspecific anti-*myc* antibody labeling. This value is then subtracted from the value of cell surface GLUT4*myc* measured in all conditions.

Appropriate washing of excess antibodies

Following labeling with anti-*myc* and HRP-conjugated antibodies, it is essential to remove all unbound antibodies before proceeding to the next stages of the assay. This is done by washing the cells with ice-cold PBS+ for the indicated number of times. Importantly, aspiration of wash instead of dumping excess PBS+ (by turning 24-well plate upside down) allows complete removal of antibodies. This in turn results in lower volume retained between washes, and decreased nonspecific labeling of each antibody. Hence, performing the washing stages as indicated ensures optimal signal-to-noise of antibody labeling.

Timing during endocytosis and exocytosis experiments

For the experiments described in Basic Protocol 2 (GLUT4*myc* endocytosis) and Basic Protocol 3 (GLUT4*myc* exocytosis), timing is crucial during particular stages of the assay. For GLUT4*myc* endocytosis, the time for which cells are rewarmed must be precisely followed. Following washing to remove anti-*myc* antibody in ice-cold PBS+, a minimum of time should elapse from the first wash in 37°C PBS+ and addition of 37°C α-MEM containing appropriate stimulus. Furthermore, immediately after the desired time of internalization has elapsed, cells must be rapidly washed in ice-cold PBS+ and quickly placed in fixative solution. Failure to add fixative solution immediately after the desired time of internalization has elapsed may result in continued internalization of GLUT4*myc* beyond the desired timepoint. For the GLUT4myc exocytosis protocol, similarly to the measurement of GLUT4myc endocytosis, a minimum of time should elapse during and between the stages that require a change in temperature of the cells (from 4° to 37°C or vice versa).

Anticipated Results

The colorimetric ELISA-based methods to measure the amount of cell surface GLUT4 described in this unit have been used in previously publications (Wang et al., 1998; Huang et al., 2002; Niu et al., 2003; JeBailey et al., 2004, 2007; Huang et al., 2005; Foster et al., 2006; Diaz et al., 2007; Antonescu et al., 2008b). An example of measurement of cell surface GLUT4 at steady-state (Basic

Figure 15.15.6 *(continued on next page)* Results of cell surface GLUT4*myc* assays. (**A**) L6 myoblasts were stimulated with α-MEM containing either 100 nM insulin for 7 or 20 min, with platelet-derived growth factor (PDGF) for 7 min or 450 mM (hypertonic) sucrose, or left unstimulated (control). Cell surface GLUT4*myc* was measured as described in Basic Protocol 1. Shown are the mean ± SE for each condition. *, $p < 0.05$ relative to control. (**B**) L6 myoblasts were stimulated with α-MEM containing 100 nM insulin for 20 min, or left unstimulated (basal). GLUT4*myc* internalization was measured as described in Basic Protocol 2. Shown are the means ± SE of GLUT4*myc* remaining at the cell surface (i) or GLUT4*myc* internalized (ii) at various times in the continued presence or absence of insulin, as indicated. Also shown for each condition is a single exponential decay (i) or association (ii) curve determined by nonlinear regression analysis. (**C**) In L6 myoblasts, GLUT4*myc* internalization was measured for 120 min following initial labeling, as described in Basic Protocol 2. Shown in panel i are the mean ± SE for each condition of GLUT4 remaining at the cell surface for the indicated times. In parallel, GLUT4*myc* exocytosis was measured in the presence (closed markers) or absence (open markers) of insulin as described in Basic Protocol 3 in cells in which internalization of surface-labeled GLUT4*myc* had proceeded for 120 min. Shown on the same graph (i) are the means ± SE of GLUT4 exocytosis (depicted by bar marked exocytosis). GLUT4 exocytosis measurements in the presence of insulin at each timepoint were normalized to the corresponding timepoint of the basal state and shown ± SE in panel ii. Note that in panel ii, $t = 0$ corresponds to $t = 120$ of panel i, that of initiation of measurement of GLUT4 exocytosis.

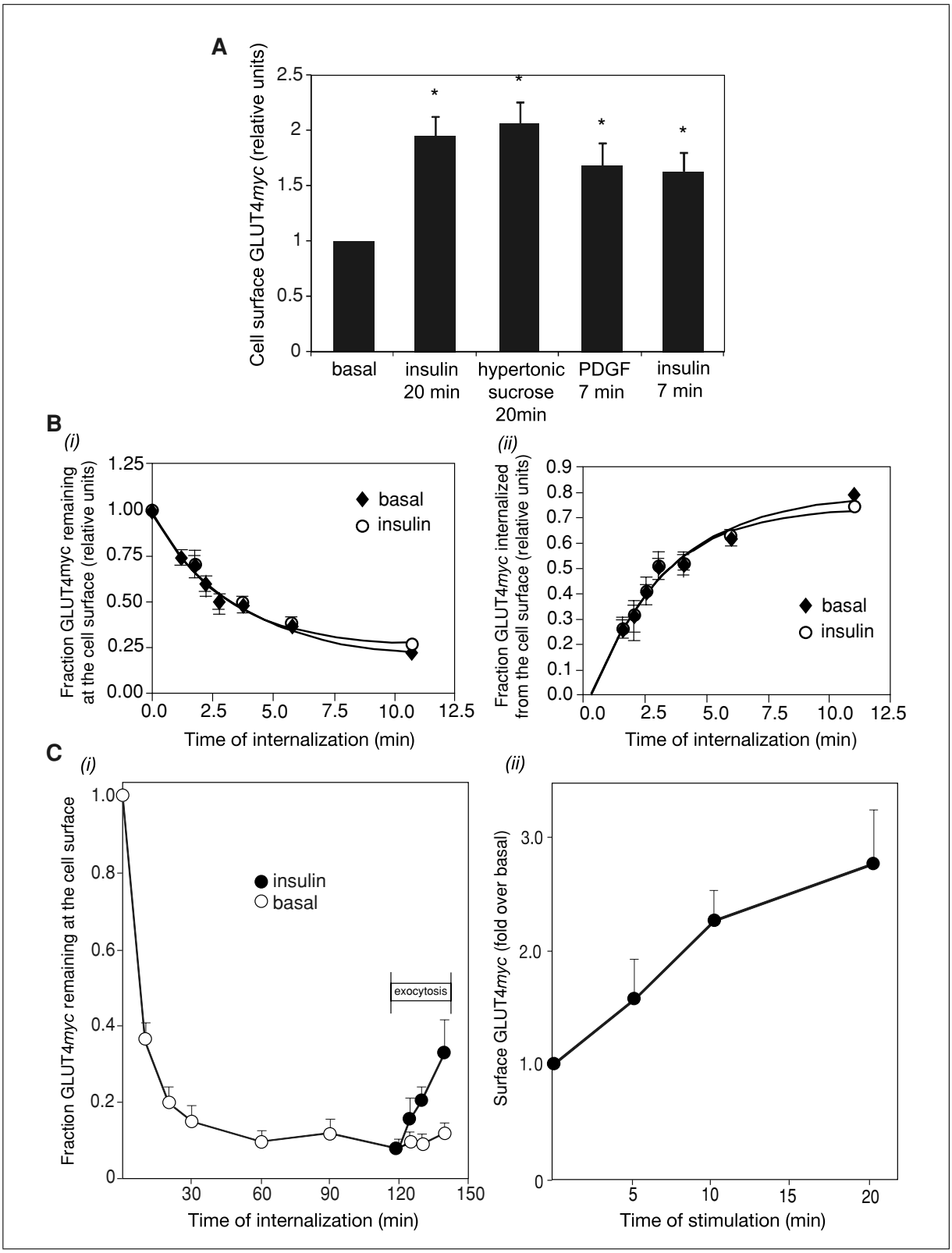


Figure 15.15.6 *(legend on previous page)*

Protocol 1) is shown in Figure 15.15.6A. Upon stimulation with insulin, cell surface GLUT4*myc* content is increased at ~2-fold. In addition to insulin, hypertonic sucrose and platelet-derived growth factor (PDGF) are also shown to elevate cell surface GLUT4 levels.

Representative data from a GLUT4*myc* endocytosis experiment (Basic Protocol 2) are shown in Figure 15.15.6B. Measurements of GLUT4*myc* internalization can be depicted as the amount of cell surface GLUT4*myc* remaining at the cell surface at various times of internalization (Fig. 15.15.6B,i). Alternatively, the same data can be depicted as the amount of *myc*-antibody-labeled GLUT4*myc* that has become inaccessible to detection by secondary antibodies, reflecting the amount of internalized GLUT4*myc* at various times (Fig. 15.15.6B,ii). In muscle cells, insulin does not affect the rate of GLUT4*myc* endocytosis.

Representative data from a GLUT4*myc* exocytosis experiment are shown in Figure 15.15.6C. We first demonstrate that GLUT4 initially labeled with *myc*-antibody at the cell surface internalizes and achieves a new steady state whereby the labeled transporter is located predominantly intracellularly. Internalization of GLUT4 from the cell surface is determined as described in Basic Protocol 2 for a period of 120 min (Fig. 15.15.6C,ii). After internalization for 120 min, ~10% of *myc*-antibody-labeled GLUT4*myc* localizes at the plasma membrane. Also performed in parallel (i.e., in the same experiment) is a measurement of GLUT4 exocytosis in cells that had internalized surface-labeled antibody-bound GLUT4 for 120 min (as described in Basic Protocol 3). The values of these measurements are also shown in Figure 15.15.6C,ii, and distinguished by a bar marked "exocytosis." The same values of GLUT4 exocytosis in the presence of 100 nM insulin were normalized to that in basal (unstimulated) cells (Fig 15.15.6C, ii). Upon stimulation with 100 nM insulin for 20 min, *myc*-antibody-labeled GLUT4*myc* content at the cell surface is increased at 2.7-fold over basal.

Time Consideration

Each individual experiment is readily performed within a single day. A possible modification of the assay to shorten the total time of each assay involves serum starvation of cells in medium containing 0.1% FBS overnight, prior to performing the experiment. Under such conditions, insulin achieves a similar increase in cell surface GLUT4 as when serum starvation is performed for 2 to 3 hr in α-MEM without FBS (JeBailey et al., 2007). However, the suitability of this modification to the assay for each condition to be assayed should first be verified by directly comparing the resulting effect on GLUT4*myc* traffic parameters between the different serum-starvation conditions.

Acknowledgements

Shuhei Ishikura and Costin N. Antonescu made equal contributions to this work. This work was supported by grant MOP-7307 from CIHR to A.K.

Literature Cited

Antonescu, C.N., Thong, F.S.L., Niu, W., Karnieli, E., and Klip, A. 2005a. To be or not to be: Regulation of the intrinsic activity of GLUT4. *Curr. Med. Chem. Immunol. Endo. Metab. Agents* 5:175-187.

Antonescu, C.N., Huang, C., Niu, W., Liu, Z., Eyers, P.A., Heidenreich, K.A., Bilan, P.J., and Klip, A. 2005b. Reduction of insulin-stimulated glucose uptake in L6 myotubes by the protein kinase inhibitor SB203580 is independent of p38MAPK activity. *Endocrinology* 146:3773-3781.

Antonescu, C.N., Randhawa, V.K., and Klip, A. 2008a. Dissecting GLUT4 traffic components in L6 myocytes by fluorescence-based, single-cell assays. *Methods Mol. Biol.* 457:367-378.

Antonescu, C.N., Diaz, M., Femia, G., Planas, J.V., and Klip, A. 2008b. Clathrin-dependent and independent endocytosis of glucose transporter 4 (GLUT4) in myoblasts: Regulation by mitochondrial uncoupling. *Traffic* 9:1173-1190.

Antonescu, C.N., Foti, M., Sauvonnet, N., and Klip, A. 2009. Ready, set, internalize: Mechanisms and regulation of GLUT4 endocytosis. *Biosci. Rep.* 29:1-11.

Blot, V. and McGraw, T.E. 2006. GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin. *EMBO J.* 25:5648-5658.

Bogan, J.S., Hendon, N., McKee, A.E., Tsao, T.S., and Lodish, H.F. 2003. Functional cloning of TUG as a regulator of GLUT4 glucose transporter trafficking. *Nature* 425:727-733.

Bose, A., Robida, S., Furcinitti, P.S., Chawla, A., Fogarty, K., Corvera, S., and Czech, M.P. 2004. Unconventional myosin Myo1c promotes membrane fusion in a regulated exocytic pathway. *Mol. Cell Biol.* 24:5447-5458.

Cline, G.W., Jucker, B.M., Trajanoski, Z., Rennings, A.J., and Shulman, G.I. 1998. A novel 13C NMR method to assess intracellular glucose concentration in muscle, in vivo. *Am. J. Physiol.* 274:E381-E389.

D'Amore, T. and Lo, T.C. 1986. Hexose transport in L6 rat myoblasts. I. Rate-limiting step, kinetic properties, and evidence for two systems. *J. Cell Physiol.* 127:95-105.

D'Andrea-Merrins, M., Chang, L., Lam, A.D., Ernst, S.A., and Stuenkel, E.L. 2007. Munc18c interaction with syntaxin 4 monomers and SNARE complex intermediates in GLUT4 vesicle trafficking. *J. Biol. Chem.* 282:16553-16566.

Diaz, M., Antonescu, C.N., Capilla, E., Klip, A., and Planas, J.V. 2007. Fish glucose transporter (GLUT)-4 differs from rat GLUT4 in its traffic characteristics but can translocate to the cell surface in response to insulin in skeletal muscle cells. *Endocrinology* 148:5248-5257.

Foster, L.J., Yeung, B., Mohtashami, M., Ross, K., Trimble, W.S., and Klip, A. 1998. Binary interactions of the SNARE proteins syntaxin-4, SNAP23, and VAMP-2 and their regulation by phosphorylation. *Biochemistry* 37:11089-11096.

Foster, L.J., Rudich, A., Talior, I., Patel, N., Huang, X., Furtado, L.M., Bilan, P.J., Mann, M., and Klip, A. 2006. Insulin-dependent interactions of proteins with GLUT4 revealed through stable isotope labeling by amino acids in cell culture (SILAC). *J. Proteome Res.* 5:64-75.

Huang, C., Somwar, R., Patel, N., Niu, W., Torok, D., and Klip, A. 2002. Sustained exposure of L6 myotubes to high glucose and insulin decreases insulin-stimulated GLUT4 translocation but upregulates GLUT4 activity. *Diabetes* 51:2090-2098.

Huang, C., Thirone, A.C., Huang, X., and Klip, A. 2005. Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in l6 myotubes. *J. Biol. Chem.* 280:19426-19435.

Imamura, T., Huang, J., Usui, I., Satoh, H., Bever, J., and Olefsky, J.M. 2003. Insulin-induced GLUT4 translocation involves protein kinase C-lambda-mediated functional coupling between Rab4 and the motor protein kinesin. *Mol. Cell Biol.* 23:4892-4900.

Ishikura, S. and Klip, A. 2008. Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. *Am. J. Physiol. Cell Physiol.* 295:C1016-C1025.

Ishikura, S., Bilan, P.J., and Klip, A. 2007. Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. *Biochem. Biophys. Res. Commun.* 353:1074-1079.

JeBailey, L., Rudich, A., Huang, X., Di Ciano-Oliveira, C., Kapus, A., and Klip, A. 2004. Skeletal muscle cells and adipocytes differ in their reliance on TC10 and Rac for insulin-induced actin remodeling. *Mol. Endocrinol.* 18:359-372.

JeBailey, L., Wanono, O., Niu, W., Roessler, J., Rudich, A., and Klip, A. 2007. Ceramide- and oxidant-induced insulin resistance involve loss of insulin-dependent Rac-activation and actin remodeling in muscle cells. *Diabetes* 56:394-403.

Kanai, F., Nishioka, Y., Hayashi, H., Kamohara, S., Todaka, M., and Ebina, Y. 1993. Direct demonstration of insulin-induced GLUT4 translocation to the surface of intact cells by insertion of a c-myc epitope into an exofacial GLUT4 domain. *J. Biol. Chem.* 268:14523-14526.

Kawanishi, M., Tamori, Y., Okazawa, H., Araki, S., Shinoda, H., and Kasuga, M. 2000. Role of SNAP23 in insulin-induced translocation of GLUT4 in 3T3-L1 adipocytes. Mediation of complex formation between syntaxin4 and VAMP2. *J. Biol. Chem.* 275:8240-8247.

Klip, A., Logan, W.J., and Li, G. 1982. Hexose transport in L6 muscle cells: Kinetic properties and the number of [^{3}H]cytochalasin B binding sites. *Biochim. Biophys. Acta* 687:265-280.

Li, D., Randhawa, V.K., Patel, N., Hayashi, M., and Klip, A. 2001. Hyperosmolarity reduces GLUT4 endocytosis and increases its exocytosis from a VAMP2-independent pool in l6 muscle cells. *J. Biol. Chem.* 276:22883-22891.

Mandel, J.L. and Pearson, M.L. 1974. Insulin stimulates myogenesis in a rat myoblast line. *Nature* 251:618-620.

Niu, W., Huang, C., Nawaz, Z., Levy, M., Somwar, R., Li, D., Bilan, P.J., and Klip, A. 2003. Maturation of the regulation of GLUT4 activity by p38 MAPK during L6 cell myogenesis. *J. Biol. Chem.* 278:17953-17962.

Patel, N., Rudich, A., Khayat, Z.A., Garg, R., and Klip, A. 2003. Intracellular segregation of phosphatidylinositol-3,4,5-trisphosphate by insulin-dependent actin remodeling in L6 skeletal muscle cells. *Mol. Cell Biol.* 23:4611-4626.

Randhawa, V.K., Ishikura, S., Talior-Volodarsky, I., Cheng, A.W., Patel, N., Hartwig, J.H., and Klip, A. 2008. GLUT4 vesicle recruitment and fusion are differentially regulated by Rac, AS160, and Rab8A in muscle cells. *J. Biol. Chem.* 283:27208-27219.

Richler, C. and Yaffe, D. 1970. The in vitro cultivation and differentiation capacities of myogenic cell lines. *Dev. Biol.* 23:1-22.

Sano, H., Eguez, L., Teruel, M.N., Fukuda, M., Chuang, T.D., Chavez, J.A., Lienhard, G.E., and McGraw, T.E. 2007. Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. *Cell Metab.* 5:293-303.

Schertzer, J.D., Antonescu, C.N., Bilan, P.J., Jain, S., Huang, X., Liu, Z., Bonen, A., and Klip, A. 2008. A transgenic mouse model to study GLUT4myc regulation in skeletal muscle. *Endocrinology.* 150:1935-1940.

Semiz, S., Park, J.G., Nicoloro, S.M., Furcinitti, P., Zhang, C., Chawla, A., Leszyk, J., and Czech, M.P. 2003. Conventional kinesin KIF5B mediates insulin-stimulated GLUT4 movements on microtubules. *EMBO J.* 22:2387-2399.

Talior-Volodarsky, I., Randhawa, V.K., Zaid, H., and Klip, A. 2008. Alpha-actinin-4 is selectively required for insulin-induced GLUT4 translocation. *J. Biol. Chem.* 283:25115-25123.

Tamori, Y., Kawanishi, M., Niki, T., Shinoda, H., Araki, S., Okazawa, H., and Kasuga, M. 1998.

Inhibition of insulin-induced GLUT4 translocation by Munc18c through interaction with syntaxin4 in 3T3-L1 adipocytes. *J. Biol. Chem.* 273:19740-19746.

Thurmond, D.C., Kanzaki, M., Khan, A.H., and Pessin, J.E. 2000. Munc18c function is required for insulin-stimulated plasma membrane fusion of GLUT4 and insulin-responsive amino peptidase storage vesicles. *Mol. Cell Biol.* 20:379-388.

Torok, D., Patel, N., JeBailey, L., Thong, F.S., Randhawa, V.K., Klip, A., and Rudich, A. 2004. Insulin but not PDGF relies on actin remodeling and on VAMP2 for GLUT4 translocation in myoblasts. *J. Cell Sci.* 117:5447-5455.

Tsakiridis, T., Tong, P., Matthews, B., Tsiani, E., Bilan, P.J., Klip, A., and Downey, G.P. 1999. Role of the actin cytoskeleton in insulin action. *Microsc. Res. Tech.* 47:79-92.

Wang, Q., Khayat, Z., Kishi, K., Ebina, Y., and Klip, A. 1998. GLUT4 translocation by insulin in intact muscle cells: Detection by a fast and quantitative assay. *FEBS Lett.* 427:193-197.

Wijesekara, N., Tung, A., Thong, F., and Klip, A. 2006. Muscle cell depolarization induces a gain in surface GLUT4 via reduced endocytosis independently of AMPK. *Am. J. Physiol. Endocrinol. Metab.* 290:E1276-E1286.

Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. U.S.A.* 61:477-483.

Yaffe, D. and Saxel, O. 1977. A myogenic cell line with altered serum requirements for differentiation. *Differentiation* 7:159-166.

Yu, C., Cresswell, J., Loffler, M.G., and Bogan, J.S. 2007. The glucose transporter 4-regulating protein TUG is essential for highly insulin-responsive glucose uptake in 3T3-L1 adipocytes. *J. Biol. Chem.* 282:7710-7722.

Zaid, H., Antonescu, C.N., Randhawa, V.K., and Klip, A. 2008. Insulin action on glucose transporters through molecular switches, tracks and tethers. *Biochem. J.* 413:201-215.

Zeigerer, A., Lampson, M.A., Karylowski, O., Sabatini, D.D., Adesnik, M., Ren, M., and McGraw, T.E. 2002. GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. *Mol. Biol. Cell* 13:2421-2435.

Zisman, A., Peroni, O.D., Abel, E.D., Michael, M.D., Mauvais-Jarvis, F., Lowell, B.B., Wojtaszewski, J.F., Hirshman, M.F., Virkamaki, A., Goodyear, L.J., Kahn, C.R., and Kahn, B.B. 2000. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat. Med.* 6:924-928.

CHAPTER 16
Antibodies as Cell Biological Tools

INTRODUCTION

Monoclonal and polyclonal antibodies are powerful tools for addressing cell biological questions. These immunological reagents can be used to detect and analyze proteins and carbohydrates, characterize the subcellular distribution of proteins, and purify proteins. In addition, when taken up by cells, antibodies can be used as tags to monitor the intracellular pathways of proteins and to inhibit protein activity. These diverse applications of antibody technology have been facilitated by advances in the understanding of the molecular genetics of antibody molecules and their three-dimensional structures. The development of methods for measuring antibody binding activity and for isolating antibodies has contributed to their widespread use and extensive range of applications.

The development of monoclonal antibodies, unique and powerful reagents for detecting and measuring interactions with specific protein epitopes, has revolutionized the use of antibodies in cell biological research. These antibodies are obtained by fusing immune B cells from the spleen with tumor cells to produce hybridomas. Hybridomas each secrete a single type of antibody—the monoclonal antibody—that has precise specificity and often high affinity (*UNIT 16.1*). Because the cloned hybridoma cell line is immortal, with appropriate care it can be maintained indefinitely and the antibodies it produces can be supplied in essentially limitless quantities. Preparations containing monoclonal antibodies include hybridoma supernatants, ascites fluid from a mouse inoculated with the hybridoma, and purified monoclonal antibody. *UNIT 16.1* describes how to produce monoclonal antibodies, including basic protocols for immunization, cell fusion, and hybridoma selection. The unit also includes methods for antigen preparation, suggestions for troubleshooting to ensure production of antibodies at high titer, and approaches for increasing the specificity of the antibody.

Polyclonal antibodies (*UNIT 16.2*) take less effort to prepare than monoclonal antibodies. The process consists simply of immunizing an animal of any one of a variety of species (including goat, horse, rat, mouse, and rabbit) with purified antigen and then, after the animal develops an immune response, isolating antibodies from its serum. Because polyclonal antibodies are essentially a collection of monoclonal antibodies with different epitope specificities and affinities, they are useful for analyses of denatured forms of a protein, for immunoprecipitation, and for immunoblotting. These types of analyses are often not successful with monoclonal antibodies because the single epitope recognized by the monoclonal antibody preparation may be destroyed during protein denaturation.

The choice of animal species for immunization depends in part on the amount of antiserum required for subsequent experiments. Mice, rats, and guinea pigs yield relatively low volumes of antiserum compared to rabbits and other larger animals. For this reason, rabbits are often the animals of choice. It is often desirable to produce polyclonal antibodies in other species, however, especially when an experiment requires two distinct types of antibodies that recognize different proteins (as is often the case in indirect immunofluorescence assays; see *UNIT 4.3*). *UNIT 16.2* contains a detailed protocol for immunizing animals to produce polyclonal antibodies, with discussion of the kinetics of the antibody response, when to boost, and the use of different adjuvants; advice on how to generate the highest-titer

Contributed by Jennifer Lippincott-Schwartz
Current Protocols in Cell Biology (2002) 16.0.1-16.0.2

antiserum is also included. Using these techniques, investigators can have polyclonal antibodies in hand for use in experiments within 6 to 8 weeks.

Purified antibody preparations are crucial when accurate concentrations of antibody are needed. For example, when chemical or structural modifications of antibody are being made for antibody binding studies. *UNIT 16.3* describes how to prepare purified forms of monoclonal and polyclonal antibodies. The purification methods that are discussed include ammonium sulfate precipitation, protein A and protein G affinity chromatography, ion-exchange chromatography, and combinations of these. Rapid assays for monitoring the activity of antibody are described for ensuring that antibody activity is not lost during purification. Commercial kits for purification and fragmentation of antibodies that are available for researchers who are unable to prepare antibodies themselves are also discussed in terms of their reliability and relative cost.

UNIT 16.4 describes how to cleave antibodies into functionally distinct subunits using proteolytic enzymes such as papain. Monovalent fragments of IgG produced by papain digestion are known as Fab fragments. These fragments usually retain the binding specificity of the intact IgG molecule. They are, therefore, desirable in binding studies since the Fc portion of IgG may cause artifacts due to binding to cells that bear the Fc receptor. In addition to describing papain digestion of IgGs, the unit discusses pepsin and ficin digestion methods which produce bivalent $F(ab')_2$ fragments and allow discrimination of different IgG subclasses. Both pilot and large-scale fragmentation protocols are described.

UNIT 16.5 describes how to conjugate antibodies with fluorescent dyes, with biotin, and with enzymes. Antibodies that are directly conjugated in this manner have proven to be essential for detecting multiple structures or functions within a specimen simultaneously. The alternative method for staining multiple epitopes is by using unconjugated primary antibodies detected with labeled secondary antibodies, but this usually results in significant cross-reactivity. *UNIT 16.5* describes several simple methods for directly conjugating antibodies so that their biological activity is retained. A basic method involves derivation of the amino group(s) on the antibody with dye, biotin, or enzyme. The methods are simple to perform and have high rates of success. Alternative, more complex methods are presented; these include attaching the label at a carbohydrate site or sulfhydryl group of the antibody. The Commentary includes a detailed discussion of the types of fluorophores and enzymes and their advantages and uses for conjugation. In addition there is discussion of the critical aspects of conjugation, including the choice of conjugating species and the optimal extent of labeling to preserve antibody affinity.

A powerful new approach to elucidating the important but bewilderingly complex signal transduction pathways of cells is to use antibodies that detect specific sites on proteins only after they become phosphorylated. For example, such antibodies can recognize with high sensitivity the addition of phosphate to specific tyrosine residues in a particular signaling or structural protein—if such phosphorylation regulates activation or inhibition of functional activity, the antibodies can be used to monitor activation, e.g., in homogenates or by immunofluorescence. *UNIT 16.6* provides methods for producing either polyclonal or monoclonal antibodies against specific phosphopeptides. In order to isolate polyclonal antibodies specific only for a unique tyrosine phosphopeptide, multistep affinity chromatography is applied to achieve appropriate specificity. Similarly, monoclonal antibodies are screened in several steps to identify a clone with sufficient specificity. Although these protocols focus on sites containing phosphorylated tyrosine, similar approaches can be used for producing antibodies that recognize specific serine- and threonine-phosphorylated peptides.

Jennifer Lippincott-Schwartz

Production of Monoclonal Antibodies

Highly specific antibodies can be obtained by fusing immune splenic B cells from the spleen with tumor cells to produce hybridomas, each of which will then secrete a single antibody. The desired antibody-producing hybridoma can be identified by a screening process. If this hybridoma is subjected to a cloning step in which clones are selected, such that all progeny are derived from a single cloned parental cell, a monoclonal antibody is obtained. Monoclonal antibodies have high specificity and can be produced in large quantities. Thus, these biological reagents have been used extensively as probes in a wide range of systems including the characterization of novel cell-surface and soluble proteins and carbohydrates, as enzyme catalysts, and for targeting in immunotherapy (see Commentary).

This unit describes the production of monoclonal antibodies beginning with basic protocols for immunization (see Basic Protocol 1) and cell fusion and selection (see Basic Protocol 2). Support protocols are provided for screening primary hybridoma supernatants for antibodies of desired specificity (see Support Protocol 1), establishment of stable hybridoma lines (see Support Protocol 2), cloning of these B cell lines by limiting dilution to obtain monoclonal lines (see Support Protocol 3), and preparation of cloning/expansion medium (thymocyte-conditioned medium; see Support Protocol 4). Figure 16.1.1 summarizes these stages and notes the protocols in this and subsequent units in which they are detailed. A major commitment of time and labor is necessary but, if successful, the monoclonal antibody may be an extremely valuable reagent that will be available in large quantities.

Submission of monoclonal antibodies to the American Type Culture Collection (ATCC) for distribution to the scientific community is encouraged. Moreover, the ATCC serves as a repository for cell lines should the line be lost in the investigator's laboratory due to unforeseen circumstances.

NOTE: All solutions and equipment coming into contact with living cells must be sterile and appropriate aseptic techniques should be used accordingly.

IMMUNIZATION TO PRODUCE MONOCLONAL ANTIBODIES

BASIC PROTOCOL 1

A wide variety of antigen preparations have been used successfully to produce monoclonal antibodies (see Critical Parameters discussion of antigen preparation). The following protocol provides an immunization schedule for the production of most antibodies, although several different schedules can be used. In this protocol, emulsified antigen is injected intraperitoneally into the species of choice. A booster injection is administered 10 to 14 days after the primary immunization. Three days after the booster injection, the animals' spleens are ready for cell fusion (Basic Protocol 2).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Antigen
Complete Freund's adjuvant (CFA; Sigma)
Animal: pathogen-free mouse, rat, or hamster (Armenian hamsters from Cytogen Research are recommended); see Critical Parameters for discussion of animal choice
Incomplete Freund's adjuvant (IFA; Sigma), optional

Contributed by Wayne M. Yokoyama
Current Protocols in Cell Biology (1999) 16.1.1-16.1.17

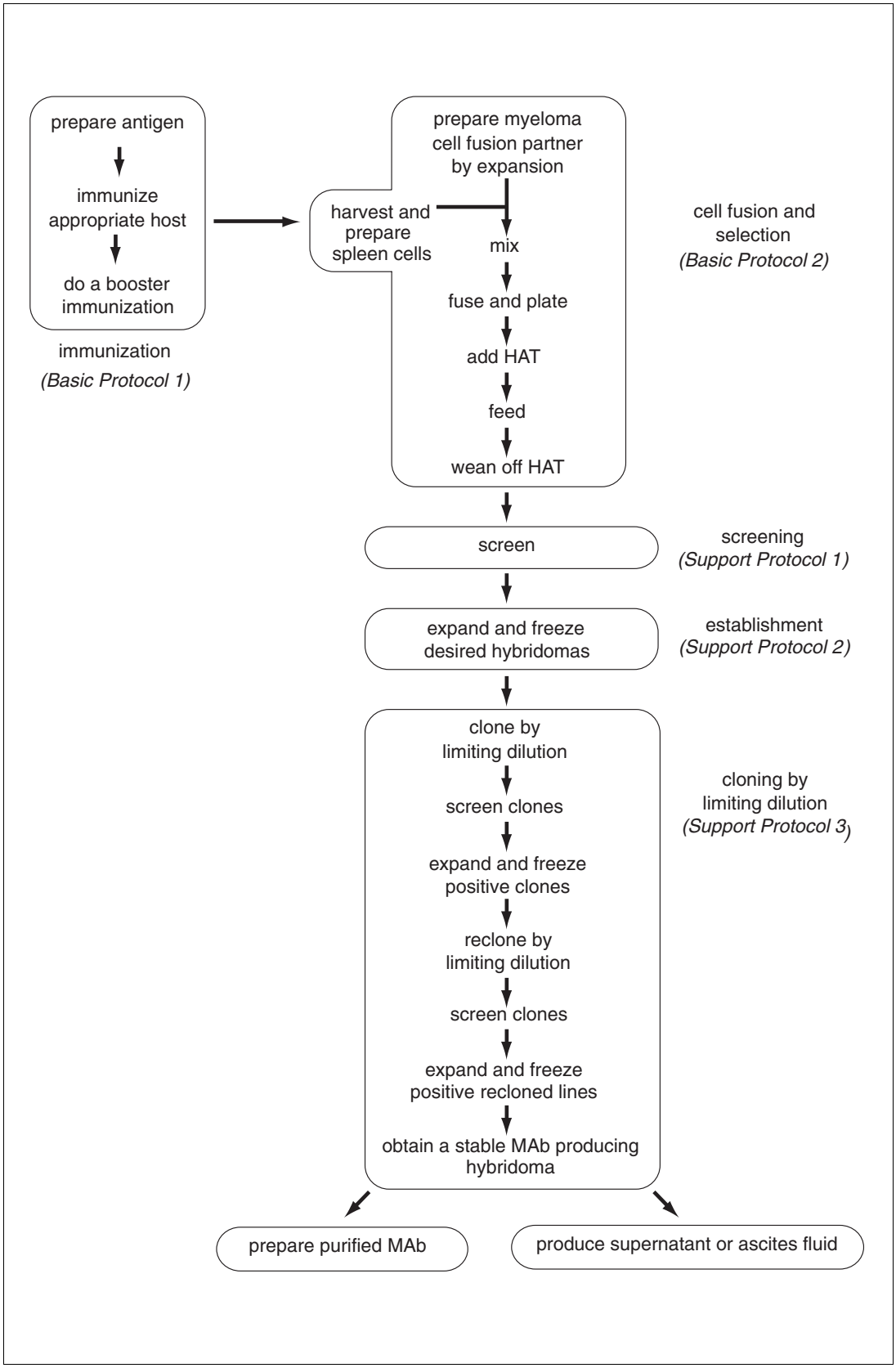


Figure 16.1.1 Stages of monoclonal antibody production, with references to the basic and support protocols in this unit that describe the steps.

1- to 2-ml glass syringes with Luer-Lok tips, sterile
3-way stopcock
20- and 22-G needles, sterile

CAUTION: CFA is an extremely potent inflammatory agent, and is hazardous to the investigator, particularly if introduced intradermally or into the eyes. Profound sloughing of skin or loss of sight may occur. Self-injection can cause a positive TB skin test and lead to a granulomatous reaction. Use gloves and protective eyewear when handling CFA.

1. Prepare antigen using 2×10^6 to 5×10^7 cells or 1 to 50 µg protein or peptide per animal to be immunized in normal saline.

   The antigen may be in any of several different forms depending on the desired property of the MAb and the method of screening (see Critical Parameters for discussion of antigen preparation and screening assays). If cells are the immunogen, wash three times in serum-free medium before immunization. Plan the immunization of several animals (enough for several fusions) so that primed and boosted animals will be ready 3 days before fusion (see Basic Protocol 2).

   To minimize the risk of introducing a pathogen into the rodent colony, screen cells for pathogens by antibody-production assay (Donovan and Brown, 1995).

2. Draw up antigen into a sterile 1- to 2-ml glass syringe with a Luer-Lok tip. Connect syringe to a 3-way stopcock.

3. Completely resuspend CFA to disperse the *Mycobacterium tuberculosis* bacilli which settle to the bottom of the container with time. Draw up a volume of CFA equal to the antigen volume in a syringe and connect to the antigen-containing syringe.

4. Emulsify antigen and CFA by discharging antigen into CFA, then discharging back and forth until a thickened mixture results. Test whether the emulsion is stable—a stable emulsion will not disperse when a drop of it is placed in water.

   Figure 16.2.1 illustrates the double-syringe device.

5. Transfer all of the CFA/antigen emulsion to one syringe and remove the other syringe and stopcock. Attach a sterile 20-G needle to the syringe containing the emulsion.

6. Inject emulsion intraperitoneally into the animal using <0.2 ml per mouse, 0.5 to 1 ml per rat, or 0.2 to 0.4 ml per hamster.

   Be careful not to force the syringe plunger since excessive pressure may dislodge the needle and spray the emulsion. Introduce the needle through the skin and tunnel the needle between the skin and peritoneal wall before entering the peritoneal cavity at a site distant from the dermal puncture site. Twirl needle before withdrawal to minimize leakage.

   Rats are generally anesthetized whereas mice and hamsters can be manipulated with one hand and do not require anesthetic.

7. Boost animal after 10 to 14 days with approximately the same dose of antigen as in step 5. If cell fusion is planned for 3 days after boosting, immunize with antigen alone in aqueous solution, or intact cells in suspension. If a fusion is not immediately planned, boost the animal with antigen emulsified in IFA (which does not contain *Mycobacterium tuberculosis* bacilli).

   Do not use CFA for the booster immunizations as this will cause intense inflammation and increased anti-TB antibody response.

   If desired, antibody titers can be assayed by ELISA or immunoprecipitation (UNIT 7.2), 7 to 10 days after the primary and booster immunizations.

BASIC PROTOCOL 2

CELL FUSION AND SELECTION OF HYBRIDOMAS

While animals should be immunized as soon as the decision has been made to produce a monoclonal antibody and the antigen has been prepared, do not perform cell fusion until the screening assay (Support Protocol 1) has been perfected. Artifactual results that may arise from conditioned media must be identified before cell fusion, because after a fusion there is only a finite amount of time available to assay for the desired monoclonal antibody.

Prior to cell fusion, the partner (myeloma) cell line is expanded and a booster injection of antigen is administered to the primed animals. On the day of fusion, the spleens are harvested. Spleen cells and partner cells are washed, harvested, and mixed. Cell fusion is performed at 37°C in the presence of polyethylene glycol (PEG). The resulting pellet is harvested and plated into tissue culture plates. After incubation with hypoxanthine, aminopterin, and thymidine (HAT) medium and feeding over ~2 weeks, the hybridomas are ready for screening (Support Protocol 1).

Materials

SP2/0-Ag14 myeloma cell line (drug-marked, nonsecretory; ATCC #CRL 1581)
Complete DMEM-10 and -20 media with 10 mM HEPES and 1 mM sodium pyruvate
Primed animal; mouse, hamster, or rat (10 to 14 days after primary immunization; see Basic Protocol 1)
Complete DMEM medium (*APPENDIX 2A*), serum-free
50% polyethylene glycol (PEG), sterile (see recipe)
Ammonium chloride solution (see recipe)
Complete DMEM-20 with 10 mM HEPES, 1 mM sodium pyruvate, and 1× HAT or 1× HT supplement (see recipe)

175-cm^2 flasks
Fine-mesh metal screen
50-ml conical polypropylene centrifuge tubes
Beckman TH-4 rotor or equivalent
96-well flat-bottom microtiter plates

Additional reagents and equipment for counting cells and assessing cell viability by Trypan blue exclusion (*UNIT 1.1*)

NOTE: All culture incubations should be performed in a humidified 37°C, 5%, CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Prepare myeloma cells (1 week before fusion)

1. One week before fusion, begin expansion of SP2/0-Ag14 myeloma cell line (the fusion partner cell line) in complete DMEM-10/HEPES/pyruvate (see Critical Parameters). By the day cell fusion is to be performed, the following total number of myeloma cells must be available (in multiple 175-cm^2 flasks containing 100 ml each), depending upon the source of the primed animal: mouse spleen, 1×10^8 cells in two or three flasks; hamster spleen, 2×10^8 cells in three or four flasks; and rat spleen, $5\text{-}10 \times 10^8$ cells in ten flasks.

   Two mouse or hamster spleens, or one rat spleen, will provide enough cells for the fusion (see step 7).

Boost primed animal (3 days before fusion)

2. Three days before fusion, boost primed animal(s) (see Basic Protocol 1, step 7).

Prepare reagents and split myeloma cells (1 day before fusion)

3. One day before fusion, prepare all reagents and media, particularly 50% PEG.
4. One day before fusion, split SP2/0-Ag14 myeloma cells (from step 1) into fresh complete DMEM-10/HEPES/pyruvate medium.

   Vigorous growth of the SP2/0-Ag14 cells is generally required for good fusion.

Check myeloma cells and prewarm reagents (day of fusion)

5. Use an inverted microscope to check the SP2/0-Ag14 myeloma cells to make sure they are growing vigorously (refractile and not pyknotic), they are not contaminated (no obvious bacteria or fungi), and there are enough cells for the fusion.

   It is better to postpone the fusion than to perform an ill-advised fusion, since the entire selection and screening effort will take ~3 weeks.

6. Prewarm the following in a 37°C water bath:

   Three 400- and three 600-ml beakers, each containing ~100 ml H_2O
   20 ml sterile complete serum-free DMEM
   5 ml sterile 50% PEG solution.

Harvest spleen and prepare cells

7. Sacrifice boosted animal(s) and aseptically harvest spleen(s).

   Do not use anesthetics for sacrifice. Instead, use cervical dislocation for mouse, or CO_2 asphyxiation for mouse, hamster, or rat to avoid introducing an anesthetic into the blood stream and therefore, into the cultures.

8. Transfer spleen to a sterile 100-mm-diameter petri dish filled with 10 ml sterile complete serum-free DMEM.

   Perform all subsequent steps in a laminar flow hood.

9. Tease spleen into a single-cell suspension by squeezing with angled forceps or by chopping with fine-tipped dissecting scissors. Remove debris and disperse cells further by passage through a fine-mesh metal screen.
10. Transfer spleen cell suspension to a sterile 50-ml conical centrifuge tube and fill with sterile complete serum-free DMEM.

    Do not use protein- or HEPES-containing medium because the PEG will precipitate proteins and HEPES can be toxic to cells during fusion.

11. Centrifuge 5 min in TH-4 rotor at 1500 rpm ($500 \times g$), room temperature, and discard supernatant.
12. Lyse red blood cells (RBC) by resuspending pellet in 5 ml ammonium chloride solution. Let stand 5 min at room temperature.
13. Add 45 ml sterile complete serum-free DMEM, and centrifuge as in step 11.
14. Resuspend pellet in 50 ml sterile complete serum-free DMEM. Centrifuge as in step 11. Repeat DMEM addition and centrifuging once (each repeat is a wash).
15. While spleen cells are being washed, separately harvest the SP2/0-Ag14 myeloma cells (from step 5) by transferring the cells to 50-ml conical centrifuge tubes. Centrifuge as in step 11. Resuspend myeloma cells in DMEM and pool all cells into one 50-ml conical centrifuge tube. Wash myeloma cells three times as in step 14.
16. Separately resuspend the spleen and myeloma cells in 10 ml complete serum-free DMEM. Count cells and assess viability in each cell suspension using a hemacytometer and trypan blue exclusion (*UNIT 1.1*).

    There should be nearly 100% viability of both suspensions.

17. On basis of cell counts (from step 16), calculate the amount of complete DMEM-20/HEPES/pyruvate needed to plate cells at ~16.1×10^6 total cells/ml. Prewarm this amount of complete DMEM-20/HEPES/pyruvate in 37°C water bath. Prepare 96-well flat-bottom plates by labeling them sequentially: one plate is required for each 10 ml of final cell suspension.

Perform cell fusion

18. Mix SP2/0-Ag14 myeloma and spleen cells at a 1:1 ratio in a 50-ml conical centrifuge tube. Fill the tube with complete serum-free DMEM.

    Other cell ratios work. Successful fusions have been performed with a ratio of myeloma/spleen cells as low as 1:20.

19. Centrifuge cell mixture 5 min at $500 \times g$, room temperature.

20. While cells are in the centrifuge, prepare three 37°C double-beaker water baths in the laminar flow hood by placing a 400-ml beaker (from step 6) containing 100 ml of 37°C water into 600-ml beaker containing 75 to 100 ml of 37°C water. Place the tubes of prewarmed 50% PEG solution and complete serum-free DMEM (from step 6) into two of the 37°C water baths in the hood.

21. Aspirate and discard supernatant from the mixed-cell pellet (from step 19).

22. Perform the cell fusion at 37°C by placing the tube containing the mixed-cell pellet in one of the double-beaker water baths in the laminar flow hood.

23. Using a 1-ml pipet, add 1 ml prewarmed 50% PEG to the mixed-cell pellet drop-by-drop over 1 min, stirring the cells with the pipet tip after each drop. Stir for an additional minute.

24. Using a clean pipet, add 1 ml prewarmed complete serum-free DMEM to the cell mixture drop-by-drop over 1 min, stirring after each drop. Repeat once with an additional 1 ml of prewarmed complete serum-free DMEM.

25. With a 10-ml pipet, add 7 ml prewarmed complete serum-free DMEM drop-by-drop over 2 to 3 min.

    Macroscopic clumps of cells should be obvious at this point.

26. Centrifuge 5 min at $500 \times g$, room temperature.

27. While the cells are in the centrifuge, rewarm the beaker water baths to 37°C and place in the hood. Place prewarmed complete DMEM-20/HEPES/pyruvate (from step 17) in the beaker water bath.

28. Discard the supernatant (from step 26). Place tube in the beaker water bath.

29. With a clean 10-ml pipet, forcefully discharge 10 ml prewarmed complete DMEM-20/HEPES/pyruvate to the cell pellet.

30. Repeat step 29 until the total volume of prewarmed complete DMEM-20/HEPES (calculated in step 17) is added. If necessary, allow clumps to settle and disrupt with the pipet tip. Further warming of cell suspension is no longer required.

    If the total volume exceeds 50 ml, gently aspirate and transfer to another sterile container such as a tissue culture flask.

31. Gently aspirate 10 ml of cell suspension with a 10-ml pipet. Add 2 drops (100 to 125 µl) of suspension to each well of a 96-well flat-bottom plate (continue until entire suspension is plated). Incubate overnight in a humidified 37°C, 5% CO_2 incubator.

Vigorous pipetting of the cell suspension should be avoided at this point, as the newly formed hybrids are unstable. Moreover, the vigorous addition of cells to the wells with repeating micropipettor is not advised. Use a pipet aid and hold the 10-ml pipet at a 45° angle with the tip 1 to 2 cm above the well, bracing the pipet with a finger from the opposite hand. To avoid introducing contaminants, do not hold hands above the plate. A steady, even flow of drops from the pipet will allow the most efficient delivery of cell suspension or medium to the wells. Use a fresh pipet to withdraw additional cell suspension.

As an optional step to minimize fibroblast overgrowth, permit the fibroblasts in bulk-fused cell suspension to adhere overnight to tissue culture flasks before seeding the 96-well plates.

Many investigators select their hybridomas under bulk conditions—i.e., they incubate large numbers of cells per well in larger plates or flasks. This makes feeding easier, but allows fast-growing hybridomas to overgrow the others. Since nonproducing hybridomas tend to grow faster, especially in the hamster-mouse fusions, hybridomas are isolated initially in multiple small wells in this protocol. The primary hybridomas tend to be monoclonal. This is especially important when screening procedures are used that require differential reactivities, e.g., to different cell lines by flow cytometry analysis or to different antigen preparations. In those cases, multiple hybridomas per well will obscure the reactivity of the MAb of interest.

Monitor and feed cells

32. After one day of incubation, check wells under an inverted microscope. If seeded with the appropriate number of cells, there should be a nearly confluent monolayer of highly viable cells on the bottom and obvious clumps of cells.

33. Add 2 drops complete DMEM-20/HEPES/pyruvate/HAT to each well with a 10-ml pipet (see step 31). Place in humidified 37°C, 5% CO_2 incubator.

    Use a separate pipet for each microtiter plate and keep the same covers with each plate to ensure that each plate remains a separate unit and to avoid spreading contamination. It cannot be overemphasized that it takes practice and meticulous attention to possible sources of contaminants to keep these plates sterile during the subsequent 2-to 3-week feeding and monitoring schedule.

    If plates become contaminated, discarding them is advised. Alternatively, contamination in one or two wells may be treated by aspirating the contents of the contaminated well with a sterile Pasteur pipet attached to a vacuum flask, rinsing the well with 70% ethanol, and wiping with a sterile cotton swab. Wash twice with ethanol. Finally, blot the well dry with the sterile cotton swab and blot the appropriate area of the cover with a sterile cotton swab soaked in 70% ethanol. Do not open contaminated plates while other plates are in the hood.

34. On days 2, 3, 4, 5, 7, 9, and 11, aspirate half the volume of each well using a sterile, short Pasteur pipet attached to a vacuum flask, holding pipet at a 45° angle and touching tip to surface of supernatant at the point where the liquid meets the opposite wall of the well. Feed the cells by adding 2 drops complete DMEM-20/HEPES/pyruvate/HAT from a 10-ml pipet (see steps 31 and 33) to each well, and return to humidified 37°C, 5% CO_2 incubator.

    Use a separate Pasteur pipet for each plate to minimize the spread of contamination. Since the frequency of successful viable hybridoma formation is $\leq 10^{-5}$, when HAT is added, profound cell death should be apparent at days 2 and 3 and the remaining viable cells should not be readily apparent until they have expanded. By day 7 to 9 for mouse-mouse fusions, day 11 for rat-mouse fusions, and day 14 for hamster-mouse fusions, clusters of hybridoma cells should become visible under the inverted microscope. If profound cell death is not apparent on days 2 and 3, check the medium containing HAT and the parental cell line by incubating an aliquot of the parental myeloma line with the medium containing HAT.

    The feeding schedule is not rigid except for the first 4 days, when it is necessary to remove the toxic products of cell death. Thereafter, feedings will depend on the actual number of

cells deposited in the wells, efficiency of fusion, and appearance and growth of hybridomas. Do not allow medium to become yellow (acidic) for more than a day. Examine plates daily, even if cells are not scheduled to be fed, and feed plates if acidic wells are noted.

35. On day 14, repeat feeding protocol outlined in step 34 except use complete DMEM-20/HEPES/pyruvate/HT to feed cells. Return to 37°C, 5% CO_2 incubator.

    Cells do not require more than one change of complete DMEM-20/HEPES/pyruvate/HT. After this change, the aminopterin (from prior addition of HAT medium) is apparently diluted out enough so that the cells can survive without additional HT.

36. On day 15 and subsequently, feed wells as noted using complete DMEM-20/HEPES/pyruvate without HAT or HT. The hybridomas are ready for screening when most of the wells containing growing cells demonstrate 10% to 25% confluence and when those with denser populations turn yellow within 2 days after feeding (see Support Protocol 1).

    If the screening assay requires a [^{3}H]thymidine incorporation assay, be aware that the large amount of thymidine in complete DMEM-20/HEPES/pyruvate with HAT and HT will serve as a cold-label inhibitor of [^{3}H]thymidine incorporation. At least 3 to 4 changes of complete DMEM-20/HEPES/pyruvate without HT are required to dilute out excess thymidine.

SUPPORT PROTOCOL 1

SCREENING PRIMARY HYBRIDOMA SUPERNATANTS

The vast majority of wells will not contain the desired antibody or may contain nonproducing hybridomas. The purpose of screening is to discover which wells (<1% to 5%) contain hybridomas that secrete the antibody of desired specificity (note that the antibody is not yet monoclonal.) Screening should be performed when most of the growing wells demonstrate 10% to 25% confluence when viewed with an inverted microscope or when some of the denser wells begin to turn yellow within 2 days after feeding. This point may be reached 10 to 14 days after a mouse-mouse or rat-mouse fusion, and 14 to 21 days after a hamster-mouse fusion. Although the most dense wells can be screened first and the less dense wells when they become more dense, this duplicates the effort required and is not recommended. Thus, the wells are fed and 2 days later, aliquots of the supernatants are tested in the screening assay for the presence of the desired antibody.

Additional Materials *(also see Basic Protocol 2)*

Growing hybridomas (Basic Protocol 2)

Additional reagents and equipment for ELISA (*APPENDIX 3*) and indirect immunofluorescence (*UNIT 4.3*)

1. Estimate the number of wells with growing hybridomas using an inverted microscope. Determine whether it will be more efficient to screen all wells or only the wells that contain hybridomas.

2. Allow hybridomas in the viable wells to grow in a humidified 37°C, 5% CO_2 incubator without feeding for ≥2 days.

    This is usually enough time to build a saturating titer of antibody in the culture supernatant.

3. Remove 100 µl from each well to be tested and use in a screening assay, such as an ELISA or indirect immunofluorescence (see Critical Parameters).

    A micropipettor with disposable sterile tips is convenient. Use a new pipet tip with each well. If all wells are screened, a multichannel pipet is convenient for transfer to another 96-well assay plate.

    For each sample, keep track of the plate number and well by its row letter and column number. This is frequently the origin of the MAb's name.

4. After harvesting one entire plate, feed wells with fresh complete DMEM-20/HEPES before harvesting the next plate (Basic Protocol 2, step 33).

ESTABLISHMENT OF HYBRIDOMA LINES

SUPPORT PROTOCOL 2

Once candidate hybridomas are identified (see Support Protocol 1), they are expanded and fed, then the cells are both frozen and cloned by limiting dilution (see Support Protocol 3). This additional work ensures that viable antibody-producing hybridomas are available after the screening process. Unfortunately, this must be done for all candidate lines before the monoclonal antibody specificity is fully characterized. To limit the amount of extra work involved, select the twenty best candidate wells. All twenty should be frozen and limiting-dilution plates set up while the supernatants are checked.

Additional Materials *(also see Basic Protocol 2)*

Growing hybridomas (Basic Protocol 2)
Cloning/expansion medium (Support Protocol 4)
24-well microtiter plates

Additional reagents and equipment for cryopreservation of cells (*UNIT 1.1*)

1. When the growing hybridoma is 25% to 50% confluent in the 96-well plate (master well), expand the candidate hybridoma to a well in a 24-well plate by resuspending the cells in the master well with a sterile pipet (a micropipettor set at 100 µl is convenient). Transfer the entire contents of the master well to a well in the 24-well plate.

   Sufficient numbers of cells will remain to serve as backup to the expanded cells.

   If a small number of cells are transferred, they may not expand. However, if there are fibroblasts in the master well, the hybridoma cells should be transferred as soon as possible to avoid fibroblast overgrowth in the well (if necessary, transfer to another 96-well plate). When transferring cells from a well with fibroblasts, be careful not to scrape the bottom of the well (which will loosen the fibroblasts).

2. Feed cells in the master well with 3 drops complete DMEM-20/HEPES/pyruvate from a 1-ml pipet (Basic Protocol 2, step 33). Incubate cells in humidified 37°C, 5% CO_2 incubator.

3. Use a fresh pipet and feed cells in 24-well plate with 1 to 1.5 ml cloning/expansion medium. Incubate 2 to 3 days in humidified 37°C, 5% CO_2 incubator.

   Remember that there are only two wells in the world that contain this hybridoma and that each is a backup to the other in case of contamination or other undesirable circumstance. Therefore, once established, each is treated as an individual entity and fed with medium from different bottles and different pipets.

4. When cells in the 24-well plate are 25% to 50% confluent (2 to 3 days), they are ready to be used for cloning by limiting dilution (see Support Protocol 3).

   Repeat steps 1 to 3 as necessary when cells are 25% to 50% confluent in master well.

5. After taking cells for limiting-dilution cloning, transfer remainder of cells in the 24-well plate to a 4-ml sterile capped tube and feed the cells in the 24-well plate with complete DMEM-20/HEPES/pyruvate.

6. Centrifuge the cells in the 4-ml tube 5 min at 500 × *g*, room temperature. Keep supernatant for further characterization of the antibody and as a control, and freeze cell pellet (*UNIT 1.1*).

   The supernatant usually contains sufficient antibody that could be reassayed in the original screening test and/or in any confirmatory test. The cell line is not established until stable

clones can be identified, frozen, and successfully thawed. If the limiting-dilution plate does not yield an antibody-producing line, the original cells from the 24-well plate can be thawed, seeded back into a 24-well plate, grown overnight, used to seed another series of limiting-dilution plates, and refrozen for safekeeping.

SUPPORT PROTOCOL 3

CLONING BY LIMITING DILUTION

Monoclonal antibodies are secreted by the progeny of a single cell that can produce only a single antibody (assuming a nonsecretory fusion-partner line). Cloning is required to ensure that the problems of polyspecificity are avoided and the risk of overgrowth by nonproducing cells is minimized. Although cloning can also be performed by the soft-agar technique, clones derived by this technique must be adapted to liquid culture before the supernatants can be tested (Coffino et al., 1972). Since cloning by limiting dilution allows direct testing of the supernatants, this method is much more advantageous (hence, the soft-agar technique will not be discussed here).

Additional Materials *(also see Basic Protocol 2)*

Candidate hybridoma line (Support Protocol 2)
Cloning/expansion medium (Support Protocol 4)

1. Resuspend the candidate hybridoma line (Support Protocol 2, step 4) in their wells and count and assess viability of a small aliquot (50 µl) of cells using a hemacytometer and Trypan blue (*UNIT 1.1*).
2. Prepare 10 ml of cells at 50 viable cells/ml and 10 ml at 5 viable cells/ml in cloning/expansion medium.

   The degree of dilution is usually very large and thus serial dilution may be needed.
3. Seed a 96-well plate with the cell suspensions at 200 µl/well. Incubate 7 to 10 days in a humidified 37°C, 5% CO_2 incubator.

   There will be enough to seed half of the wells in the plate with each dilution at final concentrations of 10 cells/well and 1 cell/well.
4. Determine which dilution was optimal for monoclonal growth by determining the number of wells that show growing hybridomas.

   Usually hybridoma growth is obvious by macroscopic visualization of the well bottoms. A microplate reading mirror (Flow Laboratories or Dynatech) is useful to avoid the necessity of holding the plate above one's head.

   Poisson statistics indicate that if <22% of the wells have growing cells (the proportion expected if the cells were seeded at 0.3 per well and had a cloning efficiency of 100%), then 88% of these wells have only one clone. However, primary hybridomas generally have a poor "plating efficiency" and thus, more cells need to be seeded in each well to derive a reasonable number of growing clones. Use the wells seeded with 1 cell/well if there are "growing" wells and, if necessary, the wells seeded with 10 cells/well. The frequency of antibody-producing clones is dependent on the time after the initial testing that the cloning plates were set up and the MAb species. Generally, the longer the wait before plating the primary hybridomas and the more phylogenetically distant from the mouse the source of spleen cells, the less frequent the positive clones.
5. Inspect wells for monoclonality with an inverted microscope before feeding cells, looking for tight single clusters of cells as evidence of monoclonal growth. Polyclonal growth is evidenced by more than one cluster of cells; if possible, do not use these wells.
6. Use the screening assay that was used in the initial identification of the master well (see Critical Parameters) to test the monoclonal wells for desired antibody activity on day 7 to 14. Use an aliquot of the original hybridoma supernatant (Support Protocol 2, step 6) as a positive control.

Mouse-mouse hybridomas could be checked as early as 7 days after plating, whereas hamster-mouse hybridomas may require up to 14 days for growth. If any of the wells begin to turn yellow, they should be tested. Since the wells were not fed, any well with growing cells should have readily detectable antibody if the cells produce the desired MAb. Wait 2 days before testing if the wells were fed.

7. When the desired clone is identified, expand and freeze the well as for the primary hybridoma (Support Protocol 2).
8. Reclone one of the positive hybridoma clones as in steps 1 to 3; seed two new 96-well plates at 0.3 cells/well (60 viable cells in 40 ml cloning medium).
9. Repeat steps 4 to 6.
10. Expand and freeze the recloned hybridoma (Support Protocol 2).
11. Wean the recloned cells to complete DMEM-10/HEPES/pyruvate by splitting the cells 1:2 every day (for 3 days) with complete DMEM-10/HEPES/pyruvate. At this point, the desired hybridoma should be stable as a cell line.

    Freeze multiple vials on different days with different aliquots of freezing medium (UNIT 1.1). Thaw representative vials and check cells for growth and supernatant for MAb activity in appropriate assay. The hybridoma can then be used for ascites production and for large-scale production of hybridoma supernatants. The isotype of the MAb can now be determined.

    Even recloned hybridomas have an unstable phenotype, especially some hamster-mouse hybridomas, which may require additional recloning. Prolonged cultures in vitro may result in loss of MAb production. To minimize this problem, frozen aliquots of cells known to produce the MAb are necessary and should be verified as sources of viable cells.

    An occasional cloned hybridoma will not tolerate complete DMEM-10/HEPES/pyruvate and will require higher concentrations of FBS. It may be necessary to wean cells first to DMEM-15 before weaning to DMEM-10. Mycoplasma contamination should be considered (see UNIT 1.5).

PREPARATION OF CLONING/EXPANSION MEDIUM

SUPPORT PROTOCOL 4

Many investigators add feeder cells (i.e., peritoneal washout cells, splenocytes, or thymocytes) to produce conditioned media that seem to enhance hybridoma growth and cloning. The direct addition of irradiated, freshly isolated cells to wells, however, sometimes results in contamination. Therefore, cell-free, sterile-filtered supernatants of all suspensions are recommended to enhance hybridoma cloning efficiencies.

The following procedure describes the preparation of a cell-free thymocyte-conditioned medium from mice. After sacrificing several mice and removing each thymus, a single cell suspension is prepared and grown for several days. The supernatant is harvested, filter sterilized, and stored at −20°C.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

1. Sacrifice 5 or 6 mice, avoiding use of anesthetics (see Support Protocol 2, step 7, annotation).

   Mice (e.g, BALB/c) should be 4 to 6 weeks old. Obtain pathogen-free mice from a reliable supplier that screens for mycoplasma contamination.

2. Aseptically remove the thymus. Tease thymus into a single-cell suspension (Basic Protocol 2, steps 8 to 11). Resuspend cells in 20 ml of complete DMEM-20/HEPES/pyruvate.
3. Add 10 ml thymus cells to a 75-cm^2 flask. Add complete DMEM-20/HEPES/pyruvate

medium to final amount of ~20 ml medium per thymus (maximum 60 ml cell suspension/flask). Incubate flask 4 to 5 days in upright position in a humidified 37°C, 5% CO_2 incubator.

4. Transfer suspension to a sterile 50-ml conical centrifuge tube. Centrifuge suspension 5 min at 1000 × *g*, room temperature, and harvest supernatant.
5. Filter sterilize supernatant through 0.45-µm filter. Freeze at –20°C in 10-ml aliquots.
6. Thaw and use at 10% to 20% final concentration in desired medium.

An alternative to feeder cells and thymocyte-conditioned medium is the use of a source of IL-6 (plasmacytoma growth factor), such as liposaccharide-stimulated P388D$_1$ supernatant.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ammonium chloride solution

0.02 M Tris·Cl, pH 7.2
0.14 M NH_4Cl
Store up to 6 months at 4°C

Complete DMEM-20/HEPES/pyruvate/HAT (or HT)

To complete DMEM-20 medium (*APPENDIX 2A*) containing 10 mM HEPES and 1 mM pyruvate, add 100× HAT (hypoxanthine/aminopterin/thymidine) or 100× HT supplement (*APPENDIX 2B*) to 1× final. Store at 4°C for up to 1 month.

100× HAT and 100× HT supplement are available commercially (e.g., Sigma).

50% polyethylene glycol (PEG)

Autoclave 10 g PEG 4000 (Merck or ATCC) in a Wheaton glass bottle and cool. Before it solidifies (at ~55°C), add 10 ml complete serum-free DMEM. This makes enough for ~20 fusions. The solution may be kept at room temperature for several months; it will become alkaline but this does not affect its performance.

Be sure not to use protein-containing medium because PEG precipitates proteins.

COMMENTARY

Background Information

Since the original description of a technique to produce monoclonal antibodies (MAbs) of defined specificity (Köhler and Milstein, 1975), MAbs have proven to be powerful tools to analyze a myriad of biological systems. As a testimony to the broad utility of this technique, the original authors noted above received the Nobel Prize for medicine in 1984. Furthermore, a search of Medline (computerized medical literature data) for 1975 to 1999 shows that more than 90,000 citations referred to MAb.

To produce MAb, a suitable host is immunized with an antigen and antibody-secreting B cells are immortalized by the fusion of the host immune B cells with a nonsecretory, drug-marked myeloma cell line. Since the unfused normal B cells cannot survive long in an in vitro culture, they derive immortality by fusion to a partner tumor cell line. The tumor line is resistant to the purine analogue 6-thioguanine because of deficiency of hypoxanthine-guanine phosphoribosyl transferase (HGPRT). This deficiency results in lethal sensitivity to aminopterin, which blocks de novo synthesis of purines. The normal B cell is not sensitive to aminopterin when hypoxanthine and thymidine are supplied, since it possesses the salvage pathways utilizing HGPRT which are necessary for survival. Thus, only hybridomas (normal B cells fused to tumor cells) will survive selection in HAT. If a hybridoma that produces the antibody of interest is identified and subjected to a cloning procedure that results in monoclonality (i.e., derivation of progeny cells

from a single cell), a MAb of desired specificity is produced.

Although monoclonal antibodies are powerful tools, it cannot be overemphasized that a major commitment is necessary to identify a MAb of interest. The work is tedious and labor intensive. Moreover, in some cases, specific polyclonal antisera, which are generally easier to produce, may be as suitable as—and in certain instances superior to—MAb. For example, specific antisera against defined synthetic peptides that will immunoprecipitate cell-surface antigens of interest can be made. Such antisera frequently will be useful in immunoblots whereas MAb, unless directly screened for such a purpose, may not recognize determinants on denatured antigens. Nevertheless, if the desired MAb is produced, this reagent has a wide variety of uses, particularly for the characterization of novel molecules, and as a specific antagonist or agonist of ligand-receptor interactions. Moreover, large quantities of the MAb can be readily purified.

There is a large body of literature on MAb production. More specific elaboration of the methods described here are discussed by Goding (1996) and Harlow and Lane (1999). Furthermore, many reports describing refinements of the basic technique are frequently published in the *Journal of Immunological Methods*. With general basic skills in animal handling, tissue culture, and screening assays, the novice should be able to produce MAb using the protocols in this unit. The major obstacle, as with any screening effort, is identification of the MAb of desired specificity. Because the technique produces many MAb-secreting hybridomas, the temptation exists to keep all or selected hybridomas even though they are not producing the MAb of initial interest. A large number of hybridomas secreting antibodies to unique antigens may be isolated, but the extra effort required is considerable. Nevertheless, it is a strategy that may prove useful while the attempt to identify the desired MAb proceeds. Many antibodies described in the literature are such by-products.

Critical Parameters

There are several major factors, other than technical, that should be considered before any MAb production project is begun.

While several fusion partners are available, the SP2/0-Ag14 myeloma is a good general-purpose cell line. The best sources of the cell line are ATCC or a laboratory actively producing hybridomas. Some sublines do not fuse well, perhaps due to some genetic variation or to undetected contaminants such as mycoplasma.

The SP2/0-Ag14 cells should grow in suspension with minimal adherence to the tissue culture flask. The cells should not be overgrown and the medium should not become acidic (yellow). Healthy cells will be refractile and none of the cells should be pyknotic when viewed with an inverted microscope. In general, the cells should be diluted (split) into fresh medium (≥1:5 if the cells will be used on the following day) when the cells in an undisturbed flask form a monolayer on the bottom of the flask ($1\text{-}2 \times 10^6$ cells/ml). If the cells do not appear healthy even when grown at lower densities, mycoplasma contamination should be suspected.

Freeze multiple large aliquots of cells (*UNIT 1.1*) so that only a few days of expansion after thawing will be necessary for future fusions. If planned correctly, a primed animal can be boosted, cells thawed on the same day, and a fusion performed 3 days later.

Choice of animal for immunization

The protocol on immunization (Basic Protocol 1) outlines the production of MAb by in vivo immunization and concentrates on the use of rodents for immunization. Specialized work may require the use of splenic injections, in vitro immunizations, or human monoclonals; these techniques are much more difficult to implement successfully and are not recommended for the novice. Other protocols designed to eliminate unwanted MAb against particularly immunogenic epitopes, involve the use of cytotoxic agents immediately after the initial immunization with the unwanted antigens and should be considered only after some experience has been gained (Sharpe et al., 1985).

Four animal species (mouse, rat, hamster, and rabbit) can be used for MAb production. The spleen cells from a given species must be able to produce stable MAb-producing hybridomas with a rodent myeloma cell line. Although several rat and mouse tumor cell partners exist and all have been successfully used, the SP2/0-Ag14 mouse cell line from ATCC is recommended because it is a drug-marked, nonsecretory myeloma that does not constitutively produce either light or heavy chains. Hybridomas derived from fusion to SP2/0-Ag14 will not make chimeric MAb. This cell line also forms stable hybridomas with mouse, rat, and hamster B cells. Rabbit MAb have been described (Raybould and Takahashi, 1988) but little collective experience has accumulated.

It is technically more difficult to produce stable MAb-producing hybridomas from animals that are phylogenetically distant from the mouse. First attempts at producing MAb should use either the mouse or rat. Of the two, the mouse is the best choice for most xenogeneic antigens, such as human antigens, because many more antibodies have been produced in the mouse. Isotype-matched MAbs to defined antigens are available commercially, and can be used as controls in the assays of interest. Moreover, the mouse is easier to handle, anti-Ig reagents specific for each mouse Ig isotype are more commonly available, and generally mouse MAb are easier than rat MAb to purify. If a mouse is to be immunized, the best choice is a BALB/c mouse, because the hybridomas resulting from fusion to SP2/0-Ag14 will be entirely of BALB/c origin and thus should grow in a BALB/c host for ascites fluid production. Moreover, BALB/c spleens are generally larger than spleens from other mouse strains.

In contrast, immunization of mice would not be appropriate for most mouse antigens unless an allotypic difference is known. Fortunately, the rat is a reasonable choice for many mouse antigens because rat MAb will frequently recognize framework determinants on mouse proteins. Most of the commonly available rat strains can be used, although this laboratory usually employs Lewis rats. If rat MAb are produced, the mouse anti-rat κ MAb, MAR 18.5 (ATCC) is useful, since MAR 18.5 recognizes rat κ light chains (present in 90% of rat MAbs) and binds protein A, and thus is easy to purify. Moreover, the hybridoma produces high titers of MAb in culture supernatants, making MAR 18.5 useful in detection and purification of rat MAbs.

Although there are mouse MAb that recognize many major, functionally important human cell-surface and soluble antigens, it has proven difficult, despite considerable effort, to produce rat MAb to several of the homologous mouse antigens. It is possible that these mouse molecules are not antigenic in rat. Some of these mouse molecules may be more antigenic in hamsters, presumably due to the greater phylogenetic distance involved (Schreiber et al., 1985). However, hamster MAb are more difficult to produce because of fibroblast overgrowth and instability of hybridomas. Hamsters are therefore not ideal choices for the novice until experience with several fusions is acquired. The Armenian hamster strain is the best suited for hybridoma production because the hybridomas are more stable and fibroblast overgrowth is less than with other available hamster strains. Unfortunately some Armenian hamster MAb are nonreactive with standard, commercially available anti-hamster immunoglobulin antibodies. However, many hamster MAb are reactive with protein A and/or with the mouse anti-rat κ MAb, RG7/7.6, which cross-reacts with hamster κ chains (Sanchez-Madrid et al., 1983). Finally, there are few serologic reagents available for hamster pathogens and thus it is wise to quarantine hamsters—and the hybridomas derived from them—in case there are occult infections that could be spread to other rodents (Donovan and Brown, 1995).

The sex of the host animal does not appear to be important. It is usually advisable to immunize young adult animals because the immunization schedule may be prolonged. Fusions of spleens from older hamsters (>6 months old) tend to have more fibroblasts.

Antigen preparation and immunization

Many types of antigen preparations have been used successfully including whole cells, partially purified lymphokines and cytokines, solubilized cell membranes and protein bands isolated from SDS-polyacrylamide gels (*UNIT 6.1*). The nature of the antigen preparation to be used is dependent on several factors, particularly the ease in preparing the antigen, the screening assay, and the desired specificity and property of the MAb.

Although it is desirable to immunize with a purified antigen to increase the frequency of hybridomas secreting the desired antibodies, in contrast to polyclonal antisera production, this is not a major requirement.

It is important to note that MAb are quite specific. It is possible to immunize with impure antigens or with multiple antigens, and with a highly specific screening assay, pick the MAb that identifies a specific antigen. In many instances, this is exactly the protocol used. For example, animals are usually immunized with whole cells to derive MAb that recognize a specific cell-surface antigen. The major advantage to whole-cell immunization is that the proteins will be in their native conformation and thus, the MAb produced can recognize these antigens in their native form. The major disadvantage to whole-cell immunization is the production of MAb to many other antigens, particularly those that have been previously produced (since they tend to be the most commonly made); immunization of rats with whole mouse T cell suspensions will produce ≥25% anti-Thy-1 MAb (W. M. Y., unpub. observ.).

Nevertheless, collective experience with whole-cell immunizations suggest that although the antigen preparation may be impure, it is important to consider the nature of the antigen relative to its native form.

Because protein purification frequently denatures molecules and synthetic peptides usually do not achieve native conformations, immunization with gel-purified proteins and synthetic peptides generally has resulted in the production of MAb that recognize the antigen in its denatured form. While such MAb may be useful for immunoprecipitation and immunoblot studies, often they are not useful for flow cytometry analysis of cell-surface antigens or functional assays that require binding of the antigen in its native conformation.

There are many immunization protocols used to produce MAb. The major requirements appear to be a primary immunization with an adjuvant and fusion 3 to 4 days after the boost. However, there are notable exceptions. For example, a single primary immunization and fusion 4 to 5 days later have been successfully used to produce MAb against cell-surface antigens (Logdberg et al., 1985). It is generally agreed that a successful fusion requires the presence of activated B cells.

Most injections can be given intraperitoneally with good results. Many investigators prefer to boost intravenously with antigen without adjuvant. While this can be done via tail vein in the mouse, this vein is inaccessible in the rat or Armenian hamster and intravenous injections are generally not used in these animals. *Be aware that intravenous injections may occasionally result in fatal systemic reactions.*

Many investigators screen their immunized animals for serum antibody titers before a final boost and fusion. This depends on the purity of the antigen and an assay that is not influenced by serum. However, many MAb have been successfully produced against cell-surface antigens without screening the sera before fusion even if whole cells are used to immunize.

Screening assays

The cell-fusion protocol outlined in this unit should yield hybridomas in ~50% of the wells. The purpose of the screening assay (Support Protocol 1) is to exclude all hybridomas that are unlikely to produce the MAb of interest and yet include all likely candidates. In that regard, a reliable assay that will detect a few false positives but no false negatives will help decrease the number of hybridomas from several hundred to ~20. Depending on the difficulty of the assay and the availability of the antigen, all wells can be screened for reactivity or—especially if most wells do not contain hybridomas—the wells can be screened first for visual evidence of growing hybridomas. Visual screening can be problematic since it requires inspection with an inverted microscope and a significant amount of bookkeeping to record which wells contain hybridomas (and which do not). More troublesome is the possibility of identifying a candidate hybridoma only to clone and save the wrong master well due to a bookkeeping error.

Screening assays should be perfected before the fusion is done. The assay should be rapid, with results available within 1 to 2 days, reliable, sensitive, and simple to perform in large numbers (hundreds to thousands of wells). In the protocol described here, the assay must be performed with 100-µl volumes. Alternatively, the supernatants from several wells could be pooled to screen fewer samples. One clever screening approach is to pool 50 µl from each well in each horizontal row and then separately pool 50 µl from wells in each vertical column of a single plate. The well containing the desired hybridoma can be pinpointed by the positive row and column. Because each supernatant is tested twice, the percentage of false positives is diminished. This technique should not be used for low-sensitivity assays as the supernatants are diluted significantly. Many different screening assays and techniques have been described. ELISA (*APPENDIX 3*) and radioimmunoassay (RIA; *APPENDIX 3*) have been popular because of the above considerations.

Because MAb are exquisitely specific, those which recognize the same antigen may bind to slightly different epitopes and thus have different functional properties. For example, if a MAb to a cell-surface antigen that inhibits ligand-receptor interaction is required, flow cytometry analysis may not be the best choice for the initial screening assay because a MAb that binds well by flow cytometry analysis may not be functionally active and vice versa. However, if the desired property of the MAb could be improved by a change of the heavy chain, it is possible to isolate isotype-switch variants that may improve the usefulness of a particular MAb. Such studies have been performed with isotype-switch variants of anti-CD3 MAb (van Lier et al., 1987). Nevertheless, the ideal screening assay should identify MAb with the desired property, not just the desired specificity.

Troubleshooting

The production of MAb is a prolonged procedure. Success requires the optimization of several steps. The antigen preparation and immunization protocol must be adequate; this can be checked by serum titers after immunization. If no hybridomas are produced, then the immunization protocol may not have produced activated B cells, an unlikely situation if an adjuvant was used. Alternatively, hybridoma formation and growth may be inadequate, particularly if the cells (spleen or fusion partner) were contaminated with mycoplasma or if the lot of fetal bovine serum (FBS) does not support hybridoma growth. Obtain the fusion partner from a reliable source, either a lab that is successfully producing hybridomas or from ATCC. Freeze aliquots of these cells as soon as possible. Obtain animals from specific pathogen-free suppliers and cage them in uncontaminated rooms. The FBS lot can be tested by assaying the cloning efficiency of stable hybridoma lines and using a lot that supports high-efficiency cloning.

If viable hybridomas are produced at the anticipated level but the desired MAb is not found, assay the master wells for other antibodies that should react with the immunogen. For example, if whole cells were used as immunogens and the original assay was a functional one (e.g., inhibition of proliferation), several wells should be screened for antibodies that recognize cell-surface antigens by flow cytometry; under these conditions, 25% to 50% of growing wells will produce antibodies that recognize cell-surface antigens. This result would prove that MAb were produced but that the desired specificity was not found. Unfortunately, this is a typical result and the source of the major commitment in time and labor that MAb production requires. The solution to the problem is to perform more fusions and screening. It is useful to consider alternatives to the immunization, choice of animals, and screening procedure.

During the cloning procedures, there are several problems that may be encountered. If none of the cloned hybridomas secrete the desired MAb, either the hybridoma was not producing the MAb initially or the clones have lost the ability to produce the MAb. An aliquot of supernatant from an expanded master well can be tested in confirmatory assays. If none of the clones produce the MAb of interest, the frozen master-well cells should be carefully thawed, reseeded in multiple cloning plates, and then refrozen. Beware that mycoplasma contamination will affect cloning efficiency adversely.

Anticipated Results

With the cell-fusion protocol outlined in this unit (Basic Protocol 2), hybridoma growth should be apparent on the days mentioned and ≥50% of the master wells will contain hybridomas. Depending on the purity of the antigen used and the immunization protocol, ≤1% to 5% of the wells will contain the desired hybridoma. If the screening assay demonstrates many positive wells, it is likely that these are false positives.

Cloning by limiting dilution of the primary hybridomas (Support Protocol 3) should yield ≥10 to 50 "growing" wells/plate when seeded as described. The second cloning under more stringent conditions should yield ≥10 wells when seeded at 0.3 cells/well. Remember that the cloning efficiency of the primary hybridomas is relatively poor. If no viable clones are derived, seed additional wells at higher density and/or use a source of IL-6 (plasmacytoma growth factor; Nordan and Potter, 1986; Bazin and Lemieux, 1989).

Time Considerations

The initial immunization to the final cloning of the desired MAb requires ≥2 to 3 months if the MAb is produced and identified in the first fusion. Usually, several fusions are required and thus, a fusion every 3 weeks may be necessary. During much of this time, especially during the 3 weeks after fusion, daily or every-other-day tasks are required. An entire day's work is required when the actual cell fusion and screening assays are performed. Feedings require 5 to 10 min/plate.

Literature Cited

Bazin, R. and Lemieux, R. 1989. Increased proportion of B cell hybridomas secreting monoclonal antibodies of desired specificity in cultures containing macrophage-derived hybridoma growth factor (IL-6). *J. Immunol. Methods* 116:245-249.

Coffino, P., Baumal. R., Laskov, R., and Scharff, M.D. 1972. Cloning of mouse myeloma cells and detection of rare variants. *J. Cell. Physiol.* 79:429-440.

Donovan, J. and Brown, P. 1995. Animal health assurance. In Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.1.1-1.1.3. John Wiley & Sons, New York.

Goding, J.W. 1996. Monoclonal Antibodies: Principles and Practice 3rd ed., Academic Press, San Diego.

Harlow, E. and Lane, D. 1999. Using Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Köhler, G. and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495-497.

Logdberg, L., Gunter, K.C., and Shevach, E.M. 1985. Rapid production of monoclonal antibodies to T lymphocyte functional antigens. *J. Immunol. Methods* 79:239-249.

Nordan, R.P. and Potter, M. 1986. A macrophage-derived factor required by plasmacytomas for survival and proliferation in vitro. *Science* 233:566-568.

Raybould, T.J.G. and Takahashi, M., 1988. Production of stable rabbit-mouse hybridomas that secrete rabbit MAb of defined specificity. *Science* 240:1788-1790.

Sanchez-Madrid, F., Szklut, P., and Springer, T.A. 1983. Stable hamster-mouse hybridomas producing IgG and IgM hamster monoclonal antibodies of defined specificity. *J. Immunol.* 130:309-317.

Schreiber, R.D., Hicks, R.D., Celada, A., Buchmeier, N.A., and Gray, P.W. 1985. Monoclonal antibodies to murine γ-interferon which differentially moderate macrophage activation and antiviral activity. *J. Immunol.* 134:1609-1618.

Sharpe, R.J., Schweizer, R.T., Krisiunas, L., Mihalyo, M.A., and Poow, L.M. 1985. Efficient production of T cell-specific monoclonal antibodies through initial tolerance induction to nonspecific antigens. *Transplant. Proc.* 17:2757-2759.

van Lier, R.A., Boot, J.H., Verhoeven, A.J., de Groot, E.R., Brouwer, M., and Aarden, L.A., 1987. Functional studies with anti-CD3 heavy chain isotype switch-variant monoclonal antibodies. Accessory cell-independent induction of interleukin-2 responsiveness in T cells by epsilon-anti-CD3. *J. Immunol.* 139:2873-2879.

Key References

Goding, 1996. See above.

An in-depth discussion of MAb production.

Köhler and Milstein, 1975. See above.

The first description of MAb, for which the authors were awarded the Nobel Prize.

Oi, V.T. and Herzenberg, L.A. 1980. Immunoglobulin-producing hybrid cell lines. *In* Selected Methods in Cellular Immunology (B.B. Mishell and S.M. Shiigi, eds.) pp. 351-372. W.H. Freeman, New York.

This reference is the basis for this protocol and for the development of many MAb in the literature.

Contributed by Wayne M. Yokoyama
Washington University School of Medicine
St. Louis, Missouri

Production of Polyclonal Antisera

Production of good antisera depends in large part upon the quality, purity, and amount of available antigen as well as on the specificity and sensitivity of the assay. For protein antigens, if possible, the material should be biochemically homogeneous and, depending on the intended use, should be in either a native or denatured conformation. Be aware that minor contaminants are often (unfortunately) more antigenic than the immunogen of interest, and antisera resulting from immunization may have more activity against the contaminants than against the protein of interest. Antisera to be used for screening bacterial expression cDNA libraries or for immunoblots are best made against denatured protein, whereas those to be used for screening cDNAs expressed in eukaryotic transfection systems or for immunoprecipitation of native-cell-synthesized structures might best be made against native protein.

Although the advances offered by the development of monoclonal antibody techniques have revolutionized the specificity, uniformity, and quantity of antibodies, there remain many circumstances in which polyclonal antibodies are more desirable than monoclonal antibodies. Production of polyclonal antisera takes less time and effort than production of monoclonal antibodies, requires relatively simple and readily available equipment, and produces reagents that can be used for immunoprecipitation, immunoblotting, and enzyme-linked immunosorbent assays (ELISAs; *APPENDIX 3*). The protocols describe the production of polyclonal antisera specific for protein antigens in rabbits, rats, mice, and hamsters using complete Freund's adjuvant (CFA; see Basic Protocol) or other adjuvants (see Alternate Protocol). In addition, the unit presents a method for preparing serum from blood (see Support Protocol). Polyclonal antipeptide antisera can be produced by substituting carrier-conjugated peptides for the purified protein antigens.

Choice of animal for the production of antibodies depends upon the amount of antiserum desired, the evolutionary distance between the species from which the protein of interest has been derived and the species of the animal to be immunized, and prior experience with the immunogens. Rabbits are the usual animal of choice because they are genetically divergent from the human and mouse sources of the proteins most often studied. Rabbits, depending on their size, provide as much as 50 ml of serum from each bleed without significant harmful effects. For smaller-scale experiments, or for those that rely on precisely defined antibody specificities, inbred mouse strains may be the system of choice. Because mice are smaller, the volume of antigen suspension used for immunization is significantly less and the amount of serum that can be obtained from a single bleed does not exceed 0.5 ml. Rats and hamsters may be used when larger amounts of serum are needed, or when the greater evolutionary distance is advantageous. With repeated bleeding, as much as 5 ml of serum can be obtained from these species. Additional discussion of the choice of species for the production of monoclonal antibodies is given in *UNIT 16.1*.

The choice of adjuvant for in vivo animal use has become problematic in recent years. Freund's adjuvant has been reliably and widely used for over fifty years (Freund et al., 1937). However, there is a degree of distress and discomfort to the animal associated with its use which requires that the responsible investigator explore alternatives (McWilliam and Niemi, 1988). Most studies that have compared available alternatives to Freund's adjuvant have indicated that Freund's adjuvant gives antibody titers that are far superior to the commonly used commercially available adjuvants (Johnston et al., 1991; Deeb et al., 1992; Smith et al., 1992). However, in some instances TiterMax (Bennett et al., 1992) and Ribi Adjuvant Systems (Mallon et al., 1991) have performed as well as Freund's adjuvant. The use of these adjuvant systems as alternatives to Freund's adjuvant is described in the Alternate Protocol.

Contributed by Helen M. Cooper and Yvonne Paterson

Current Protocols in Cell Biology (1999) 16.2.1-16.2.8

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL

IMMUNIZATION TO PRODUCE POLYCLONAL ANTIBODIES USING FREUND'S ADJUVANT

In the presence of adjuvant, the protein antigen is injected intramuscularly, intradermally, or subcutaneously into an animal of the chosen species. Booster immunizations are started 4 to 8 weeks after the priming immunization and continued at 2- to 3-week intervals. Prior to the priming immunization, following the primary and each booster immunization, the animal is bled and serum prepared from whole blood (see Support Protocol).

Factors important in preparing specific high-titer antisera, procedures for modifying protein antigens to enhance their immunogenicity, and choice of host animal are discussed in the Commentary. Protocols for immunization prior to production of monoclonal antibodies (*UNIT 16.1*) should be reviewed for these purposes.

Materials

Rabbit, rat, mouse, or hamster of appropriate strain
Complete Freund's adjuvant (CFA; Sigma)
1 to 2 mg/ml purified protein antigen in PBS (*APPENDIX 2A*)
Incomplete Freund's adjuvant (IFA; Sigma)

50-ml disposable polypropylene centrifuge tubes
3-ml glass syringes with 19-, 21-, and 22-G needles
Double-ended locking hub connector (Luer-Lok, Becton Dickinson) *or* plastic 3-way stopcock

CAUTION: CFA is an extremely potent inflammatory agent, particularly if introduced intradermally or into the eyes and may cause profound sloughing of skin or loss of sight. Self-injection can cause a positive TB test and lead to a granulomatous reaction. Use gloves and protective eyewear when handling CFA.

1. Bleed the animal prior to immunization and collect blood sample in a 50-ml centrifuge tube. Prepare serum from blood and assay and store (see Support Protocol).

   This preimmune bleed is critical as a control to ensure that the antibody activity detected in later bleeds is due to the immunization.

2. Shake CFA to disperse insoluble *Mycobacterium tuberculosis* bacilli. Add 2 ml CFA to 2 ml of 0.25 to 0.5 mg/ml purified protein antigen in PBS at 4°C.

   These volumes produce immunogen sufficient to immunize 4 rabbits or up to 80 mice. Do not use Tris-based buffers for generating the emulsion.

   An effective and simple method for preparing purified protein antigen is by preparative SDS-PAGE (UNIT 6.1). If a standard-size 1.5-mm slab gel is used with a large-toothed comb, as much as 2 mg of a homogeneous protein can be loaded across the entire gel. Following electrophoresis, an edge can be sliced off with a razor blade, fixed and stained, and used to identify the region containing the protein band. The gel slice containing the protein may then be directly added to several milliliters of PBS (APPENDIX 2A) and emulsified as described below with an equal volume of CFA. The acrylamide serves as an additional component for the protein depot provided by the adjuvant.

3. Draw up the CFA/antigen mixture into a 3-ml glass syringe with a 19-G needle. Remove needle, expel as much air as possible, and attach syringe to the double-ended locking hub connector or the plastic 3-way stopcock (see Fig. 16.2.1). Attach an empty 3-ml glass syringe at the other end and force the mixture back and forth from

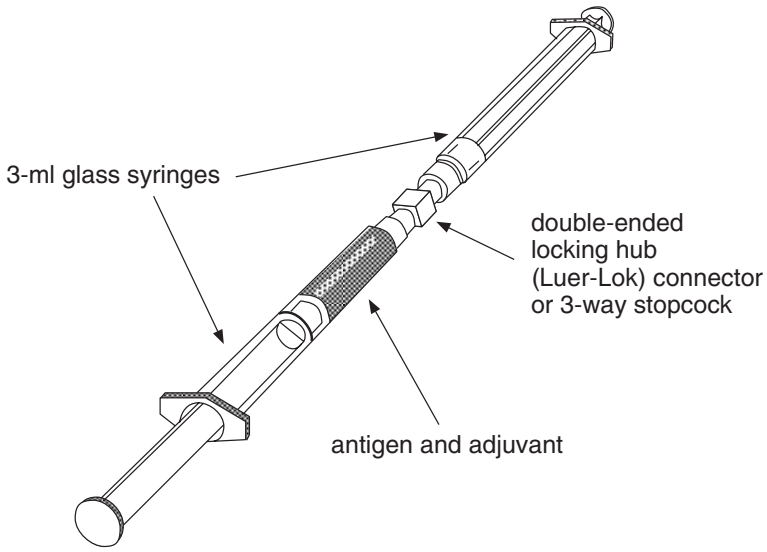


Figure 16.2.1 Double-syringe device for preparation of antigen-adjuvant emulsions.

one syringe to the other repeatedly. When the mixture is homogeneous and white, disconnect the connector or stopcock, attach a 21-G needle, and test whether the emulsion is stable by extruding a small drop onto the surface of 50 ml cold water in a 100-ml beaker. A good oil-in-water emulsion should hold together as a droplet on the surface of the water. If the drop disperses, mix the antigen using the hub-connected syringes until it forms an emulsion.

Heat will be generated by this procedure. Chill on a bed of ice from time to time to keep the mixture as close to 4°C as possible.

4. Transfer all of the adjuvant-antigen emulsion to one syringe and remove the connector or stopcock. Attach a 22-G needle to the syringe and remove air bubbles.
5. Restrain the animal and inject the adjuvant/antigen emulsion into multiple intramuscular (i.m.), intradermal (i.d.), or subcutaneous (s.c.) sites.

   For intradermal immunizations, use no more than 50 μl per site. For subcutaneous immunizations, use 100 μl per site. For intracellular inoculation of rabbits, use 300 μl in three sites in one leg muscle or 500 μl in each lumbar muscle.

   Discard the unused immunogen. For extremely valuable antigens, the emulsion may be stored at 4°C for several weeks and reemulsified before use. However, denaturation of protein antigens may take place under these conditions. For immunization of small rodents (e.g., mice), it is often better to carry out injections intraperitoneally (i.p.).

6. Bleed the animal 10 to 14 days following the priming immunization and collect blood sample. Prepare serum from blood (see Support Protocol).
7. Prepare antigen for booster immunizations, following steps 2 to 4. When CFA is the primary adjuvant, use IFA as the adjuvant for all subsequent immunizations.
8. Administer the first booster immunization 4 to 8 weeks after the priming immunization, bleed the animal 10 to 14 days later, and collect blood sample. Prepare serum from blood (see Support Protocol).

   Some investigators will administer the first booster immunization as early as 2 weeks after the primary immunization.

9. Administer further booster immunizations at 2- to 3-week intervals. Bleed animal 10 to 14 days after each boost and collect blood sample. Prepare serum from blood (see Support Protocol).

Repeated intradermal immunization should be avoided as it can cause skin ulceration. Following primary intradermal or subcutaneous immunization, it is preferable to boost with intramuscular injections for the rabbit. Some investigators prefer primary intramuscular injections with boosters at other sites.

ALTERNATE PROTOCOL

IMMUNIZATION TO PRODUCE POLYCLONAL ANTISERUM USING OTHER ADJUVANTS

For highly immunogenic antigens the use of Freund's adjuvant can certainly be avoided. For other immunogens it may be necessary to test a number of adjuvant systems. The use of two commercially available adjuvants is described in this protocol.

Additional Materials *(also see Basic Protocol)*

TiterMax #R-1 (CytRx; store <24 months at 4°C) *or* Ribi Adjuvant System (RAS; Ribi ImmunoChem; store at 2° to 8°C and do not freeze)
1-ml plastic syringes

1a. *Using TiterMax:* Emulsify aqueous antigen with TiterMax adjuvant (see Basic Protocol; follow steps 1 through 5, except use 0.5 ml antigen and 0.5-ml vial TiterMax in step 2 and plastic syringe in step 3).

TiterMax contains microparticulate silica coated with block copolymer CRL-8941, sorbitan mono-oleate, and squalene.

Although glass syringes are recommended for Freund's adjuvant emulsions, all-plastic syringes should be used with TiterMax.

Each reconstituted 0.5-ml vial will immunize 20 mice, 10 rabbits, or 1 goat. Unused antigen/adjuvant emulsion can be stored at 4°C, –20°C, or –70°C for as long as the antigen is stable. It may be necessary to re-emulsify before using.

1b. *Using Ribi Adjuvant System:* Warm the vial of RAS 5 to 10 min at 40° to 45°C. Add 2 ml antigen in PBS directly through the rubber stopper using a syringe with a 21-G needle. Vigorously vortex the vial 3 min at room temperature with the cap seal in place. The final volume of adjuvant/antigen is 2 ml containing 2% squalene oil.

Each vial of RAS contains 0.5 mg each of monophosphoryl Lipid A (MPL), synthetic trehalose dicorynomycolate (TDM), and cell wall skeleton (CWS) in 44 µl squalene and Tween 80.

Each reconstituted vial will immunize 10 mice or 2 rabbits or goats. Unused adjuvant/antigen emulsion can be stored several months at 4°C. However, if the entire vial will not be used initially, it is better to reconstitute to 1.0 ml with saline alone, store at 4°C, and mix aliquots 1:1 with antigen in saline as needed.

2. Transfer the antigen emulsion to a 1-ml syringe, attach a 22-G needle to the syringe, and remove air bubbles.

3. Restrain the animal and inject the adjuvant/antigen emulsion.

Rabbits should receive 40 µl TiterMax/antigen emulsion i.m. in each thigh. High antibody titers have been obtained with 30 to 50 µg of antigen per rabbit. Rabbits should be immunized with 1.0 ml RAS containing 50 to 250 µg of antigen in multiple sites: 0.05 ml i.d. at six sites, 0.3 ml i.m. in each thigh and 0.1 ml s.c. in the neck.

4. Bleed the animal and prepare antigen for booster immunization (see Basic Protocol, steps 6 and 7).

Serum antibody responses have been reported to be slower for both RAS and TiterMax than for Freund's adjuvant (Smith et al., 1992).

5. Administer booster immunizations at 4, 8, and 12 weeks. Bleed the animal 10 to 14 days after each booster immunization. Prepare serum from blood (see Support Protocol) and cease immunization when high antigen-specific titers have been achieved.

   Boosting with TiterMax may not be necessary for all antigens. If a second immunization is necessary, use soluble antigen in place of antigen/adjuvant at 4 weeks. If titers are still low after 10 to 14 days, a booster dose of antigen/TiterMax adjuvant can be given immediately. Increasing the amount of antigen may also help.

   Ribi ImmunoChem strongly recommends that booster injections of RAS adjuvant/antigen be repeated no more frequently than every 4 weeks.

PREPARATION OF SERUM FROM BLOOD

SUPPORT PROTOCOL

Each blood sample is allowed to stand 4 hr at room temperature and overnight at 4°C until a clot forms. After removal of the clot and debris, the serum is assayed and stored at –20°C.

Additional Materials *(also see Basic Protocol)*

Blood samples (see Basic Protocol)
Sorvall H-1000B rotor or equivalent

Additional reagents and equipment for immunoblotting (*UNIT 6.2*), immunoprecipitation (*UNIT 7.2*), ELISA (*APPENDIX 3*), or double-immunodiffusion assay in agar (*APPENDIX 3*)

1. Allow blood to stand in the 50-ml centrifuge tube 4 hr at room temperature to allow clot to form, then place overnight at 4°C to allow clot to retract.
2. Gently loosen the clot from the sides of the tube with a wooden applicator stick (do not break up the clot), then remove the clot from tube with the applicator.

   If a clot has not formed, initiate clotting by placing a wooden applicator stick into the tube containing the collected blood, then begin again at step 1.

3. Transfer serum to a 50-ml centrifuge tube. Pellet any remaining blood cells and debris by centrifuging 10 min in H-1000B rotor at 4000 rpm (2700 × *g*), 4°C, and save supernatant.
4. Assay antibody titer by the appropriate method: immunoprecipitation, immunoblotting, ELISA, or double-immunodiffusion assay in agar.
5. Store serum in aliquots in screw-top tubes at –20°C.

   Some sera lose activity on repeated freezing/thawing. Sera can be stored for up to 6 months at 4°C. If storing sera at 4°C for long periods of time, 0.02% sodium azide should be added to prevent bacterial growth.

COMMENTARY

Background Information

The kinetics of development of a specific antibody response upon immunization of a rabbit with antigen are illustrated in Figure 16.2.2. After the primary immunization, naive B cells are stimulated to differentiate into antibody-secreting plasma cells. For most soluble protein antigens, specific antibody begins to appear in the serum 5 to 7 days after the animal is injected. The antibody concentration (titer) continues to rise and peaks around day 12, after which it decreases. Similar kinetics are observed with mice, rats, hamsters, and rabbits.

In addition to differentiating into antibody-forming cells, the antigen-stimulated B cells proliferate to form a large population of mem-

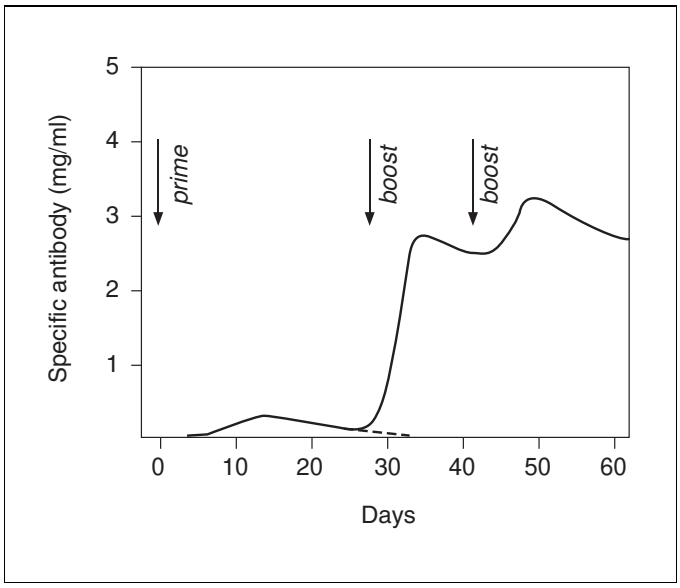


Figure 16.2.2 Kinetics of development of the specific antibody response. Arrows indicate when priming and boosting immunizations were administered. Actual amounts of specific antibody produced will vary considerably depending on immunogenicity of the protein.

ory B cells, which quickly become activated after the booster injection is administered. Thus, the lag period before the appearance of the specific antibody is much shorter after a booster injection than that observed for the initial immunization. In addition, a significantly higher titer of specific antibody is achieved and sustained for a longer period of time. The peak of antibody production occurs 7 to 14 days after boosting. As a consequence of the existence of the memory B cells, less antigen is required to stimulate a strong secondary response. Memory B cells are long-lived; therefore, a specific antibody response can be elicited as much as 6 months to a year after the last booster. Finally, the average affinity and degree of specificity of the antibody population for the antigen increase with repeated immunizations (Klinman and Press, 1975).

Adjuvants greatly enhance the specific antibody titer, as they allow the antigen to be released slowly, thus ensuring the continual presence of antigen to stimulate the immune system. Freund's adjuvant has been used extensively in the preparation of antigen because it induces a high, long-lasting antibody titer that is often still measurable 25 weeks or more after boosting. The presence of mycobacteria in complete Freund's adjuvant (CFA) activates the T cell population, providing necessary lymphokines for B cell stimulation and maturation. CFA may cause granuloma and subsequent necrotic abscesses, so it should be used only for primary immunization. Incomplete Freund's adjuvant (IFA) is adequate for booster injections. In the past decade, adjuvant research has concentrated on the production of effective adjuvants that minimize animal trauma. Wherever possible, to reduce animal discomfort, less noxious adjuvants should be used (see Alternate Protocol). In addition some countries are restricting the use of CFA in laboratory animals. Published comparisons of commercial adjuvants with the basic CFA/IFA protocol vary widely in their conclusions. In most cases, however, CFA/IFA produces higher titers of higher-affinity antibodies in a shorter time period (Johnston et al., 1991; Deeb et al., 1992; Smith et al., 1992). Conflicting results (Mallon et al., 1991; Bennett et al., 1992) may reflect differences in the immunogenicity of the antigens used.

Critical Parameters

New Zealand red or white rabbits are generally the best source of specific antisera because 30 to 50 ml of whole blood can be obtained at each bleed, depending on their body weight. The life span of a rabbit is 5 to 6 years, so a continual source of specific antiserum can be provided over a period of time by one rabbit after booster injections. In this regard, the recommended times between booster injections are not critical; the animal may be rested for several months between subsequent boosters, after the primary and secondary booster injections. Blood collection, however, must take place 10 to 14 days after each booster to ensure a high titer.

Preimmune serum from the same animal is the preferred negative control. If additional control serum is required, either immune serum from animals immunized with totally unrelated antigens or pooled serum from naive animals will be adequate. Occasionally, spurious anti-

body activities from nonimmunized animals may mimic the activity of the immune serum.

Antibody specificity may vary widely between individual animals with respect to the dominant antigenic epitopes recognized on a given protein antigen. Therefore, antiserum from a single animal should be used throughout a study. If more than one animal must be used for particular antisera, the antisera should be pooled. Large-scale production of antisera can be carried out in goats, sheep, and horses with appropriate veterinary guidance. If serum is taken from inbred animal strains, the variability in antibody specificity, as observed in outbred rabbits, is less of a problem.

The most important factor in producing a highly specific polyclonal antiserum is the purity of the antigen preparation used for immunization. The immune system is very sensitive to the presence of foreign proteins. Any contaminating proteins in the antigen preparation can potentially induce a strong immune response when injected in the presence of adjuvant. When antisera are employed in sensitive techniques such as immunoblotting or the screening of cDNA or genomic libraries, significant antibody titers to protein contaminants can be a major problem. Thus, the antigen preparation should contain no significant contaminating proteins. Ideally, there should be no visible contaminating bands when 10 to 20 μg are analyzed on an SDS-polyacrylamide gel stained with Coomassie brilliant blue (*UNITS 6.1*).

If the antiserum is to be used in functional assays, extra care must be taken to ensure that the immunizing antigen is in its native form, because antibodies directed to denatured forms of the protein antigen will interact weakly, if at all, with the antigen in its native conformation. On the other hand, antibodies used in immunoblots, immunoprecipitation of primary in vitro translation products, and immunoscreening of λgt11 expression libraries may be most effective if generated against a denatured protein with reduced and carboxymethylated disulfide bonds.

Troubleshooting

Inability to attain high-titer antiserum after several booster injections may be due to a variety of factors as described below.

Use of inappropriate adjuvant. Some experimentation may be necessary to optimize the antigen/adjuvant ratio for different antigens. If the alternate protocol still fails to produce a good antibody titer after three immunizations, switch to the Basic Protocol.

Inadequate antigen emulsification. If the emulsion fails the drop-on-water test described in the basic protocol (step 3), repeat the emulsification process. Be sure to use phosphate-buffered saline. Avoid plastic syringes and Tris-based buffers with CFA and IFA.

The antigen is a poor immunogen. In general, the immunogenicity of a protein is related to the degree to which it differs from "self" proteins (Benjamin et al., 1984). Large bacterial or viral proteins such as hemagglutinin or bacterial-coat proteins are highly immunogenic, whereas proteins from mammalian sources, such as polypeptide hormones or cell- surface receptors, may be poorly immunogenic due to tolerance. Protein antigens can be made more immunogenic in two ways. First, they can be chemically linked to a carrier protein that is known to be a good immunogen. Common carrier proteins include keyhole limpet hemocyanin (KLH), fowl immunoglobulin, and bovine serum albumin (BSA). Coupling peptides to carrier proteins is described in Maloy et al. (1991); the same protocols can be used to couple the protein antigen of interest to the desired carrier. Second, the immunogenicity of an antigen may be enhanced by its polymerization into large aggregates via a cross-linking agent such as glutaraldehyde. The protocol in Maloy et al. (1991) for the coupling of peptide antigens to a carrier protein with glutaraldehyde can also be used to polymerize any protein antigen. With both the coupling and polymerization procedures, any insoluble antigen complexes formed should be removed prior to immunization by centrifuging 10 min at 15,000 × *g*, 4°C.

Host animal's immune system may be compromised by bacterial or viral infection. Refer to Donovan and Brown (1995) for discussion of the consequences of poor animal husbandry. Utilize animals from reliable, pathogen-free sources and maintain them in appropriate infection-free facilities.

Only a few animals have been immunized. Because of the vagaries of immune-response genes in outbred animals such as rabbits, some antigens may not induce a good antibody response in a significant proportion of randomly selected animals. Thus, it is best to immunize several different animals and to screen the sera for the best responder. Obviously, this is less of a problem in homozygous inbred strains, but with a new antigen it is wise to test several strains for their antibody response.

An insufficient amount of antigen was used. Although recommended concentrations of antigen for rabbits are 0.25 to 0.5 mg/ml injected

into multiple sites, for a total of 1 to 2 ml in the same animal, good results can be obtained with $^1/_{10}$ to $^1/_{20}$ of the concentration in the same volume. It is always tempting to use less of a precious antigen, but often too low a dose leads to too low a response.

Anticipated Results

For large or nonevolutionarily related proteins, a titer of 5 to 10 mg/ml of serum can be expected after repeated boosts (hyperimmunization). When immunizing with small or highly conserved protein species, a titer of 1 to 2 mg/ml of specific antibodies is more likely. Antibody titers and affinity for the antigen will be low after primary immunization and the first booster immunization, but both titer and affinity will increase with subsequent immunizations.

Time Considerations

Preparation of the immunogen and immunization will take up to 3 hr on each occasion. Collection of antisera will take 1 to 2 hr, depending on the number and species of animals.

Collection of antiserum after the primary immunization will be at 10 to 14 days. This will be a low-titer, low-affinity serum. The first booster normally is given 4 to 8 weeks after the primary immunization but can be given as early as 2 weeks after the primary if time is critical. Ideally, there should be at least 19 days between the primary and the secondary bleeds. A second booster is given at 6 weeks with a bleed on day 52 to 59. This will usually be the first high-titer bleed. If a titer of <1 mg/ml of specific antibody is obtained, subsequent boosting immunization will be necessary.

Literature Cited

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E., and Wilson, A.C. 1984. The antigenic structure of proteins: A reappraisal. *Annu. Rev. Immunol.* 2:67-101.

Bennett, B., Check, I.J., Olsen, M.R., and Hunter, R.L. 1992. A comparison of commercially available adjuvants for use in research. *J. Immunol. Methods* 153:31-40.

Deeb, B.J., DiGiacomo, R.F., Kunz, L.L., and Stewart, J.L. 1992. Comparison of Freund's and Ribi adjuvants for inducing antibodies to the synthetic antigen (TG)-AL in rabbits. *J. Immunol. Methods* 152:105-113.

Donovan, J. and Brown, P. 1995. Animal health assurance. In Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.1.1-1.1.3. John Wiley & Sons, New York.

Freund, J., Casals, J., and Hismer, E.P. 1937. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. *Proc. Soc. Exp. Biol. Med.* 37:509.

Johnston, B.A., Eisen, H., and Fry, D. 1991. An evaluation of several adjuvant emulsion regimens for the production of polyclonal antisera in rabbits. *Lab Anim. Sci.* 41:15-21.

Klinman, N.R. and Press, J. 1975. The B cell specificity repertoire: Its relationship to definable subpopulations. *Transplant. Rev.* 24:41-83.

Mallon, F.M., Graichen, M.E., Conway, B.R., Landi, M.S., Leonard, T.B., and Hughes, H.C. 1991. A comparison of antibody response using a synthetic adjuvant system and Freund's complete adjuvant in rabbits. *Am. J. Vet. Res.* 52:1503-1506.

Maloy, W.L., Coligan, J.E., and Paterson, Y. 1991. Production of antipeptide antibodies. In Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 9.4.1-9.4.11. John Wiley & Sons, New York.

McWilliam, A. and Niemi, S.M. 1988. Freund's adjuvant. Canadian Council on Animal Care Resource. 12:1.

Smith, D.E., O'Brien, M.E., Palmer, V.J., and Sadowski, J.A. 1992. The selection of an adjuvant emulsion for polyclonal antibody production using a low-molecular-weight antigen in rabbits. *Lab. Anim. Sci.* 42:599-601.

Key Reference

Harlow, E. and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

A comprehensive methods book with many modern techniques.

Contributed by Helen M. Cooper
Ludwig Institute for Cancer Research
Melbourne, Australia

Yvonne Paterson
University of Pennsylvania
Philadelphia, Pennsylvania

Purification of Immunoglobulin G

For many applications, both monoclonal and polyclonal antibodies may be used in impure form: monoclonal antibodies may be used either as ascites fluid or as tissue culture supernatant, and polyclonal antibodies may be used as antiserum. Such unpurified antibodies are perfectly suitable for use in indirect flow cytometry assays, for most ELISAs, or for cytotoxicity studies if the concentration of antibody is not important. Purified antibodies must be used, however, when accurate concentrations are required, when chemical modifications such as labeling with fluorescent and radioactive probes for binding studies are required, or when structural modifications such as removal of the Fc portion to produce bivalent $F(ab')_2$ or monovalent Fab fragments are required. IgM can be modified and the single bivalent subunit (IgMs) of the pentamer produced as a low-molecular-weight alternative to intact IgM. Bivalent $F(ab')_2\mu$ fragments of IgM are more difficult to produce but are sometimes required. Detailed methods for production of these fragments are provided in *UNIT 16.4*.

Choice of procedure for antibody purification depends on the intended use of the antibodies and on the available resources. This unit presents purification methods that can be tailored to the laboratory's resources. If a highly purified product is required, an assay for antibody activity and a reliable means of assessing the degree of protein contamination are essential. The method of choice for determining purity is SDS-PAGE (*UNIT 6.1*). The antibody assay must be rapid and accurate to monitor activity throughout the purification. Some preferred assays are: (1) labeling of cell-surface antigens with the antibody, followed by incubation with a fluorescent anti-Ig of the correct specificity and microscopic (*UNIT 4.3*) or flow cytometry analysis of the sample; (2) ELISA; (3) complement-mediated lysis of the appropriate cells; (4) immunodiffusion; (5) radioimmunoassay (RIA; Cooper and Paterson, 1993); and (6) inhibition of purified fluorescent or radiolabeled antibody binding to its appropriate ligand. Assays will generally not provide a measure of contaminating proteins that remain in the preparation at different stages of the purification. These can be detected by SDS-PAGE.

Several companies produce kits for the purification and fragmentation of antibodies derived from all common animal species. These kits are primarily designed for IgG and IgM classes of antibody, but there is also a kit for purification of egg yolk–derived IgY. The kits mentioned here are the most reliable in the opinion of the authors, but they may not be the least expensive option, so classical methods of purification and fragmentation are described in these units.

IgG can be purified by ammonium sulfate precipitation followed by size-exclusion (SE) chromatography (see Basic Protocol 1). This is the least expensive option available for purification of antibodies. Protein A– and protein G–affinity chromatography (see Basic Protocol 2 and Alternate Protocol 1) are the fastest methods for purifying antibodies, but they are not effective for all subclasses of rat antibody. Affinity chromatography using anti-rat antibody can be used to purify rat antibodies (see Alternate Protocol 2). Ion-exchange (IEX) chromatography (see Basic Protocol 3) can also be used to purify intact monoclonal and polyclonal antibodies and antibody fragments. All these methods give a product with a high degree of purity. Ammonium sulfate precipitation followed by IEX chromatography can be applied to any type of antibody. However, affinity chromatography, either as described in Basic Protocol 2 and Alternate Protocol 1 or carried out using a commercially produced kit, is much more efficient and less tiresome to carry out. There are many commercial kits available for purifying IgG (see Table 16.3.1). The T-Gel purification kit (thiophilic gel; Pierce) produces results similar to those of ammonium

Contributed by Sarah M. Andrew and Julie A. Titus
Current Protocols in Cell Biology (2000) 16.3.1-16.3.12

Table 16.3.1 Commercial Kits for Purifying Antibody

Kit	Supplier[a]	Features
HiTrap protein A and protein G columns[b]	Amersham Pharmacia Biotech, Sigma	Ready-packed columns in a variety of sizes for use with syringe loading or a peristatic pump
Immunopure (A) and Immunopure (G) IgG[c]	Pierce	Protein A and protein G purification
Immunopure mouse IgG_1[c]	Pierce	Mild elution buffer kit for purification of mouse IgG_1 on protein A
Immunopure and Immunopure *Plus* (A/G) IgG	Pierce	Binds all IgGs that bind to both protein A and protein G; contains sufficient gel to purify 6 or 16 mg, respectively
MAb Trap GII[b]	Amersham Pharmacia Biotech	A complete protein G kit with buffers, column, and syringe
Protein A and Protein G Superose columns[b]	Amersham Pharmacia Biotech	Ready-made, reusable columns for use with the Pharmacia FPLC system; good for purifying large amounts of antibodies
Antibodies of other types/classes or general kits		
T-Gel[c]	Pierce	One-step purification by adsorption to a thiophilic gel; the gel has broad specificity for immunoglobulins of any type or subclass derived from various animal species; used to purify antibodies from serum, ascites fluid, or culture supernatant
HiTrap IgY[b]	Amersham Pharmacia Biotech	Concentrates IgY antibodies from egg yolk by centrifugation in a liquid linear polymer
HiTrap IgM Purification Column	Amersham Pharmacia Biotech	Prepacked column containing thiophilic adsorption medium coupled to Sepharose High Performance. Interacts with IgM in hydrophilic-hydrophobic interaction. Kit of buffers provided.

[a]See *SUPPLIERS APPENDIX*.

[b]These kits may require specialized equipment or preparation of additional reagents.

[c]These kits come complete or reagents may be purchased separately.

sulfate precipitation because it has a broad specificity toward immunoglobulins derived from various animal species, irrespective of the type or subclass. The E-Z-SEP system (Pharmacia Biotech) consists of a group of kits with applications for all types of antibodies—e.g., ascites IgG, ascites IgM, bioreactor medium IgM, serum-free tissue culture IgG, serum polyclonal antibodies.

BASIC PROTOCOL 1

AMMONIUM SULFATE PRECIPITATION AND SIZE-EXCLUSION CHROMATOGRAPHY

Ammonium sulfate precipitation coupled with size-exclusion (SE) chromatography is a method for purifying proteins of all types and may be the procedure of choice for purifying certain antibodies. Ammonium sulfate precipitation can be used to purify all subclasses of mouse antibodies as well as antibodies of other species. This method can be used to purify IgM, IgG, and IgA of all species. Although more time-consuming than affinity chromatography (see Basic Protocol 2), it has a wider range of applications.

After removing cell debris from ascites fluid or a monoclonal antibody supernatant, ammonium sulfate is added to precipitate the proteins. The precipitate is recovered by centrifugation and dissolved in PBS or borate buffer, then dialyzed and concentrated. It is purified by SE chromatography and the pure IgG is analyzed.

Materials

Ascites fluid or MAb supernatant
PBS (see recipe)
Saturated ammonium sulfate (SAS; *APPENDIX 2A*)
Borate-buffered saline (optional; see recipe)
Sephacryl S-200 Superfine (Amersham Pharmacia Biotech)
PBS containing 0.02% sodium azide (optional)

Glass wool (Polysciences)
Sorvall centrifuge and SS-34 rotor (or equivalent)
26 × 900–mm column (Amersham Pharmacia Biotech)

Additional reagents and equipment for protein dialysis (*APPENDIX 3C*), column chromatography (*APPENDIX 3*), concentrating proteins (*APPENDIX 3C*), and reducing and nonreducing PAGE (*UNIT 6.1 & APPENDIX 3*)

1a. *For ascites:* Remove lipid from (5 to 12 ml) ascites fluid by placing enough glass wool into a funnel to cover the opening, pouring ascites through, rinsing glass wool with PBS, and squeezing glass wool gently with gloved fingers to obtain all the sample. Centrifuge filtered ascites 30 min at 20,000 × *g* (13,000 rpm in SS-34 rotor) either 4°C or room temperature. Decant and save the supernatant and discard membranous material and cell debris remaining in the pellet.

Wear gloves when handling glass wool.

1b. *For MAb supernatant:* Centrifuge (~100 ml) MAb supernatant 30 min at 20,000 × *g*, either 4°C or room temperature. Decant and save the supernatant.

2. Add SAS slowly with stirring to the ascites or tissue culture supernatant to 45% (v/v). Leave 1 to 2 hr or overnight at 4°C to ensure precipitation of all the protein.

3. Centrifuge 1 hr at 20,000 × *g*, either 4°C or room temperature, and save precipitate to use in step 4. Save the supernatant to check for antibody activity.

4. Dissolve precipitate in a minimum volume of PBS or borate buffer (10 to 20 ml is usually suitable).

5. Place the dissolved precipitate in dialysis tubing. Dialyze against ≥20 vol PBS or borate buffer for 24 to 48 hr total at 4°C. Change the dialysis buffer four to six times during dialysis.

Generally, accurate molecular weight cutoffs are not required and it is not necessary to boil the tubing; merely soak for a few minutes in distilled water to soften.

The solution in the dialysis tubing will turn from a yellowish liquid to a cloudy or clear solution.

6. Concentrate the protein solution to ≤5 ml (*APPENDIX 3C*).

7. Prepare a 26 × 900–mm Sephacryl S-200 Superfine column and load the concentrated protein solution onto it. Elute protein with PBS, PBS containing 0.02% sodium azide, or borate buffer, and collect 100 fractions (1% of the column volume).

Monitor the protein fractions with a UV spectrophotometer at 280 nm. Alternatively, a precalibrated column can be used.

8. Check the purity of the fractions with A_{280} values >0.5 on nonreducing and reducing 10% polyacrylamide gels (*APPENDIX 3 & UNIT 6.1*).

In nonreducing PAGE, a band at ~150 kDa indicates IgG; in reducing PAGE, two bands at ~55 kDa (heavy chain) and ~25 kDa (light chain) indicate IgG. IgG_{2b} has asymmetric

Table 16.3.2 Extinction Coefficients and Molecular Size of Immunoglobulins and Their Fragments

Molecule	A_{280} (1 mg/ml solution)	Molecular weight (kDa)	
		Nonreduced	Reduced[a]
IgG	1.43	150	50, 25
IgM	1.18	900	78, 25
IgM subset	1.18	180	78, 25
Fab	1.53	50	25[b]
F(ab′)$_2$	1.48	100-110	25[b]
F(ab′)$_2$μ	1.38	135	44, 25
F(ab′)μ	1.38	65	44, 25

[a]Numbers to the left of the comma represent molecular weights of the heavy chain of immunoglobulins, and numbers to the right are weights of the light chain of immunoglobulins.

[b]May appear as a doublet on SDS-PAGE.

glycosylation of the heavy chains and will therefore appear as a doublet on the gel (Table 16.3.2). If the fractions are not sufficiently concentrated for detection by SDS-PAGE, concentrate as described in APPENDIX 3C).

9. Assess IgG concentration spectrophotometrically at A_{280} (Table 16.3.2). Pool the eluates containing pure IgG.

   Antibodies purified by ammonium sulfate precipitation followed by SE chromatography are of sufficient purity for any manipulation. They may be used for fragmentation after dialysis to the desired buffer (APPENDIX 3C), for ELISA, or for labeling with fluorescein isothiocyanate (FITC) or biotinylation.

10. Adjust IgG concentration to 0.1 to 30 mg/ml in borate buffer or PBS with 0.02% (w/v) sodium azide and store at 4°C up to several years. For back-up frozen stocks, freeze IgG at −70°C.

    Thawing and freezing once from −70°C is usually fine, although as a general rule this should be avoided. Do not store antibodies at −20°C for >1 month. Do not repeatedly freeze/thaw from −20°C.

BASIC PROTOCOL 2

AFFINITY CHROMATOGRAPHY USING PROTEIN A–SEPHAROSE

This protocol describes the purification of antibody using protein A–Sepharose affinity chromatography. Protein A can be used to isolate monoclonal and polyclonal IgG from ascites, serum, and tissue culture and bioreactor supernatants. Protein A purification is recommended for human (except IgG3; mouse IgG_1 may bind only weakly), rabbit, guinea pig, and pig antibodies (see Table 7.2.1). The protocol requires addition of the antibody to a protein A–Sepharose column at pH 8.0, followed by elution at a lower pH. The antibody is then dialyzed back against PBS.

Materials

Ascites fluid or MAb supernatant
PBS, pH 8.0 and pH 7.3 (see recipe)
1 M NaOH
Protein A–Sepharose CL-4B, hydrated (Amersham Pharmacia Biotech or Sigma)
0.1 M citric acid at pH appropriate for subclass of antibody (see step 5)
Borate-buffered saline (optional; see recipe)
3 M potassium thiocyanate, filtered

continued

Sorvall centrifuge and SS-34 rotor (or equivalent)
0.45-µm filter
1.5 × 10–cm column
HiTrap protein A column (Amersham Pharmacia Biotech or Sigma; optional)

Additional reagents and equipment for dialysis (*APPENDIX 3C*) and column chromatography (*APPENDIX 3*)

For ascites fluid

1a. Clarify ascites fluid and remove lipids (see Basic Protocol 1, step 1a).

2a. Dilute ascites fluid 10-fold with PBS, pH 8.0.

For MAb supernatant

1b. Centrifuge MAb supernatant at 20,000 × *g* (13,000 rpm in SS-34 rotor), 4°C, and filter through a 0.45-µm filter.

2b. Adjust MAb supernatant to pH 8.0 by dialysis against PBS, pH 8.0, or by adding 1 M NaOH.

It is important to have the protein sample at pH 8.0 if IgG1 is to be purified. Other subclasses should bind to protein A at pH 7.4.

3. Prepare protein A–Sepharose column and attach to fraction collector. Equilibrate column with PBS, pH 8.0, at either 4°C or room temperature. Layer antibody solution onto resin bed.

Volumes of 1 ml to several liters can be loaded onto a protein A–Sepharose column. Use a peristaltic pump or gravity to assist in loading large volumes.

There is a limit to the binding capacity for immunoglobulin (~5 mg mouse IgG, and 8 mg human IgG, per milliliter resin). The expected concentrations can be assessed from the yields (see Anticipated Results). The antibody activity of the unbound fraction can be tested to check for overloading. Use a flow cytometry assay or ELISA for assessing activity of the eluate.

The HiTrap protein A column is ready-to-use. Alternatively, a column can be prepared in a 10-ml syringe. Add glass wool to the syringe before adding hydrated protein A–Sepharose CL-4B.

4. Wash column with several volumes PBS, pH 8.0.

Eluate should have an A_{280} at baseline before proceeding to the next step.

5. Elute with 0.1 M citric acid at suitable pH (bring to appropriate pH with 1 M NaOH): for mouse IgG_1 use pH 6.5, for IgG_{2a} use pH 4.5, and for IgG_{2b} and IgG_3 use pH 3.0.

It is thought that standing in low pH may damage the antibody; therefore, 50 µl of 2 M Tris base buffer (Boehringer-Mannheim)/ml of eluate may be placed in the fraction collector tubes prior to elution. Reverse elution is undertaken by reversing the leads of the column so that the pump is pushing buffer up the column in the opposite direction to that in which the column was loaded. Samples that do not occupy the entire capacity of the column can be eluted at a higher concentration by this method. Reverse elution can concentrate the antibody if the protein A–Sepharose column is underloaded.

6. Pool protein-containing fractions, place eluates in dialysis tubing, and dialyze eluates against 1 liter PBS, pH 7.3, with or without 0.02% (w/v) sodium azide, at 4°C. Change the dialysis buffer twice. Store samples in PBS or borate-buffered saline at 4°C.

If desired, check purity by SDS-PAGE (UNIT 6.1).

7. Clean column with 1 column volume of filtered 3 M potassium thiocyanate before reequilibrating it in PBS, pH 7.3. Store at 4°C.

   Wash the column with several volumes of PBS, pH 7.3, to remove residual potassium thiocyanate, which absorbs at 280 nm and could interfere in later purifications.

   Antibodies purified by protein A–Sepharose affinity chromatography will be of similar purity to those obtained by ammonium sulfate precipitation and can be used for the same purposes (see Basic Protocol 1).

ALTERNATE PROTOCOL 1

AFFINITY CHROMATOGRAPHY USING PROTEIN G–SEPHAROSE

Affinity chromatography using protein G-Sepharose is useful for purifying antibody from serum, ascites fluid, tissue culture supernatant, and bioreactor supernatant. Protein G (Akerstrom and Bjorck, 1986) has a binding profile opposite to that of protein A with respect to pH: antibodies bind better at a low pH and badly at high pH. However, some antibodies (mouse IgG_1, and rabbit and human antibodies) do remain bound to protein G at high pH (8 to 10), so it is best to bind the antibody at pH 5 and elute at pH 2.8. This method is useful for mouse IgG_1, rat (most subclasses bind weakly although IgG_{2b} may not), monkey, rabbit, cow, goat, horse, and sheep antibodies (see Table 7.2.1). As with protein A purification, there is the possibility of some loss of antibody binding ability due to low-pH elution.

Additional Materials *(also see Basic Protocol 2)*

0.1 M sodium acetate, pH 5.0
0.1 M glycine·HCl, pH 2.8
HiTrap protein G column (Amersham Pharmacia Biotech or Sigma)

1. Prepare ascites fluid or MAb supernatant (see Basic Protocol 2, step 1a or 1b).
2. Dilute with 0.1 M sodium acetate, pH 5.0.

   Dilute >2-fold for tissue culture supernatant or 10-fold for ascites fluid and bioreactor supernatant.

3. Equilibrate HiTrap protein G column in 0.1 M sodium acetate, pH 5.0. Layer antibody solution onto protein G gel.

   Protein G has a higher capacity for IgG than protein A: ~10 mg protein/ml of gel with some species variation.

4. Wash column with several column volumes of 0.1 M sodium acetate, pH 5.0.
5. Elute bound antibody with 0.1 M glycine·HCl, pH 2.8.

   To minimize exposure to low pH, add 50 µl Tris-based buffer (Boehringer-Mannheim) per milliliter of eluate to each tube of the fraction collector..

6. Pool protein-containing fractions and dialyze (*APPENDIX 3C*).
7. Recycle the column by washing it with 0.1 M glycine·HCl, pH 2.8, then reequilibrating it to pH 5.0 with 0.1 M sodium acetate, pH 5.0.

AFFINITY CHROMATOGRAPHY USING ANTI–RAT κ CHAIN MONOCLONAL ANTIBODY COUPLED TO SEPHAROSE

ALTERNATE PROTOCOL 2

Occasionally, a monoclonal antibody (particularly one derived from the rat) cannot be purified by either protein A– or protein G–Sepharose chromatography, either because the antibody fails to bind the protein A or protein G, or because the elution conditions are too harsh for the retention of activity. In such a case, a column consisting of an anti-rat-Ig light-chain monoclonal antibody coupled to Sepharose can be used to bind the rat monoclonal antibody from a tissue culture supernatant or from ascites fluid. A series of buffers of decreasing pH can be used to assess the mildest conditions for elution of the rat antibody from the anti-Ig column. This protocol describes the production of such a column and the conditions for binding and elution from it.

Additional Materials *(also see Basic Protocol 2)*

Mouse anti-rat κ MAb: MAR 18.5 (ATCC TIB 216) purified using protein A–Sepharose (see Basic Protocol 2)
CNBr–Sepharose CL-4B (Amersham Pharmacia Biotech)
Binding buffer: 0.05 M Tris·Cl/0.15 M NaCl/0.02% (w/v) NaN_3, pH 8.6
Crude rat antibody solution to be purified (MAb supernatant or ascites fluid)
pH 7.0 elution buffer: 0.05 M sodium phosphate/0.15 M NaCl/0.02% (w/v) NaN_3, pH 7.0
pH 5.5 elution buffer: 0.05 M sodium citrate/0.15 M NaCl/0.02% (w/v) NaN_3, pH 5.5
pH 4.3 elution buffer: 0.5 M sodium acetate/0.15 M NaCl/0.02% (w/v) NaN_3, pH 4.3
pH 2.3 elution buffer: 0.5 M glycine/0.15 M NaCl/0.02% (w/v) NaN_3, pH 2.3
Additional reagents and equipment for preparation of antibody-Sepharose (*APPENDIX 3*)

Prepare column

1. Covalently couple ≥10 mg purified MAR 18.5 antibody to the CNBr–Sepharose 4B.

   Coupling ratio should be ~10 mg MAR 18.5/ml wet gel. Such a column will have a capacity to bind ~1 mg of protein.

2. Prepare the column and wash extensively with the binding buffer at 4°C or room temperature.

Purify antibody

3. Clarify ascites fluid or MAb supernatant (see Basic Protocol 2).
4. Load the column with ~10 ml ascites or ~100 ml MAb supernatant of crude rat antibody solution.
5. Wash the column extensively with 10 to 15 column volumes of binding buffer. Monitor the A_{280} to be certain the absorbance returns to baseline. Set up a fraction collector to collect all fractions from steps 6 to 9.
6. Elute with 5 column volumes of pH 7.0 elution buffer, watching the UV monitor.

   For most antibodies, this does not elute the bound antibody and serves as a preliminary wash step. Be sure A_{280} has returned to baseline before beginning the next step.

7. Elute with 5 column volumes of pH 5.5 elution buffer, watching the UV monitor.

   Some antibodies will elute under these mild conditions. Be sure A_{280} has returned to baseline before beginning the next step.

8. Elute with 5 column volumes of pH 4.3 elution buffer, watching the UV monitor.

   Most antibodies will elute under these conditions. Be sure A_{280} has returned to baseline before beginning the next step.

9. Elute with 5 column volumes of pH 2.3 elution buffer, watching the UV monitor.

   All antibodies will elute at pH 2.3. This also serves as a final wash step. Be sure A_{280} has returned to baseline before beginning the next step.

10. Equilibrate column with binding buffer by washing with ≥10 column volumes. Store column wrapped in Parafilm at 4°C.

11. Identify eluted protein peaks (including the unbound initial fractions), pool, assay for antibody activity, and concentrate if necessary (see Basic Protocol 1, step 9).

BASIC PROTOCOL 3

DE52 ION-EXCHANGE CHROMATOGRAPHY WITH TRIS·Cl

DE52 ion-exchange (IEX) chromatography can be used to purify antibodies from a tissue culture supernatant, ascites fluid, and serum or ammonium sulfate precipitates derived from any of these antibody-containing fluids. The protocol may also be used as a second step following purification by size exclusion (SE) chromatography (see Basic Protocol 1 and Alternate Protocols 1 and 2). The major contaminant protein in all these preparations is albumin, which binds DE52 tightly under conditions of low-to-moderate ionic strength. Antibody either fails to bind to DE52, in which case it elutes in the void volume as the column is loaded, or it binds loosely, and can be eluted with a gentle salt or pH gradient. Fractions eluted are assayed first by A_{280} and then by either ELISA (*APPENDIX 3*) or SDS-PAGE (*UNIT 6.1*).

Caution should be exercised in that different monoclonal antibodies as well as antibody fractions from different immunized animals' sera elute from DE52 under different conditions. In this protocol, antibody in 0.01 M Tris·Cl at pH 8.6 is passed over the DE52 column and bound antibody is eluted with a gradient in the same buffer approaching 0.5 M NaCl.

Materials

DE52 powder (Whatman)
0.01 M Tris·Cl, pH 8.6 (*APPENDIX 2A*)
0.5 M NaCl/0.01 M Tris·Cl, pH 8.6 (see recipe)
Antibody sample (ascites fluid, tissue culture supernatant, immune serum, or ammonium sulfate precipitate)
1.5 × 50–cm column

Additional reagents and equipment for column chromatography (*APPENDIX 3*), dialysis (*APPENDIX 3C*), and ELISA (*APPENDIX 3*) or SDS-PAGE (*UNIT 6.1*)

1. Swell DE52 powder in 0.01 M Tris·Cl, pH 8.6, and remove fine particles.

   It is very important to adjust the pH prior to pouring the DE52 gel into the column.

2. Pour a 1.5 × 50–cm DE52 column and equilibrate with 0.01 M Tris·Cl, pH 8.6.

   The size of the column is dependent upon the total amount of protein in the crude antibody sample. The capacity of DE52 is ≥100 mg total protein/ml hydrated gel, so a column of 1 to 5 ml is commonly sufficient.

3. Place antibody sample in dialysis tubing. Dialyze twice against ≥20 times the sample volume of 0.01 M Tris·Cl, pH 8.6, overnight at 4°C.

4. Load the dialyzed antibody sample onto the column.
5. Elute column with 0.01 M Tris·Cl, pH 8.6, until all of the protein that does not bind to DE52 at this pH comes through the column (detected by monitoring A_{280} with a UV monitor). Collect the first ten fractions (~12 ml each).

   Some antibodies elute at this stage (i.e., they do not bind DE52 in 0.01 M Tris·Cl, pH 8.6). They will thus be free of the major contaminant, albumin, which remains on the column. However, the great majority of antibodies will adsorb to the column under these conditions.
6. Elute the remaining material using a 200- to 250-ml linear gradient of 0.01 M Tris·Cl, pH 8.6, to an equal volume of 0.5 M NaCl/0.01 M Tris·Cl, pH 8.6. Collect 2-ml fractions.
7. Monitor column fractionation as follows: (1) A_{280}, to measure protein concentration; (2) conductivity, as an indication of the progress of the gradient as the concentration changes; (3) activity of the antibody by ELISA (*APPENDIX 3*); and (4) protein structure by SDS-PAGE (*UNIT 6.1*).

   A typical example of the elution profile of a mouse MAb is shown in Figure 16.3.1. The first peak that comes off as the ionic strength is increased is the IgG. This is confirmed by SDS-PAGE or ELISA.

   IEX chromatography is also useful for separating Fab fragments. Following size-exclusion chromatography of the digestion mixture to remove intact IgG (APPENDIX 3), the 50-kDa fraction is dialyzed (APPENDIX 3C) exhaustively against 0.01 M Tris·Cl, pH 8.6, and separated as described in this protocol using DE52. Elution profiles can vary greatly

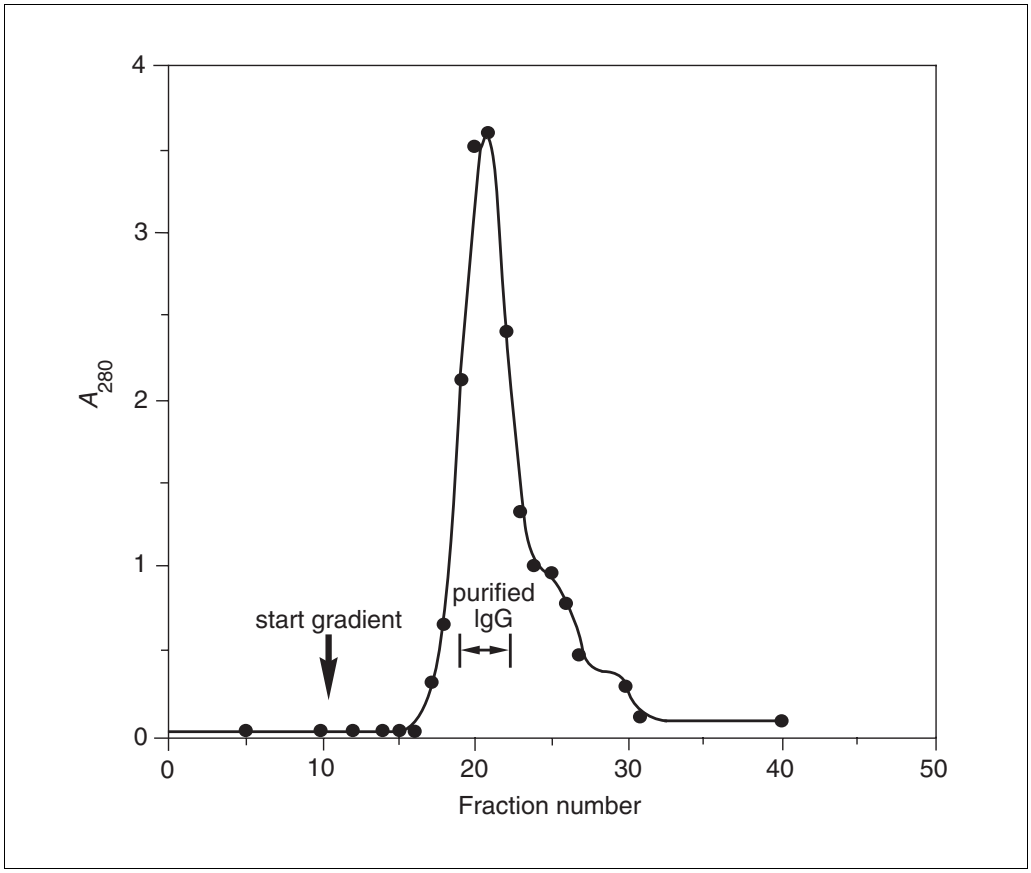


Figure 16.3.1 IEX chromatography elution profile from a DE52 column (1.5 × 50 cm). The IgG fractions from an ACA-34 column were dialyzed against 0.01 M Tris·Cl, pH 8.6, and applied to the column. Ten fractions (12 ml) were collected using the starting buffer, then a linear gradient of 0 to 0.5 M NaCl in Tris·Cl, pH 8.6, was started. Fractions 19 to 22 contained pure IgG as determined by SDS-PAGE (*UNIT 6.1*).

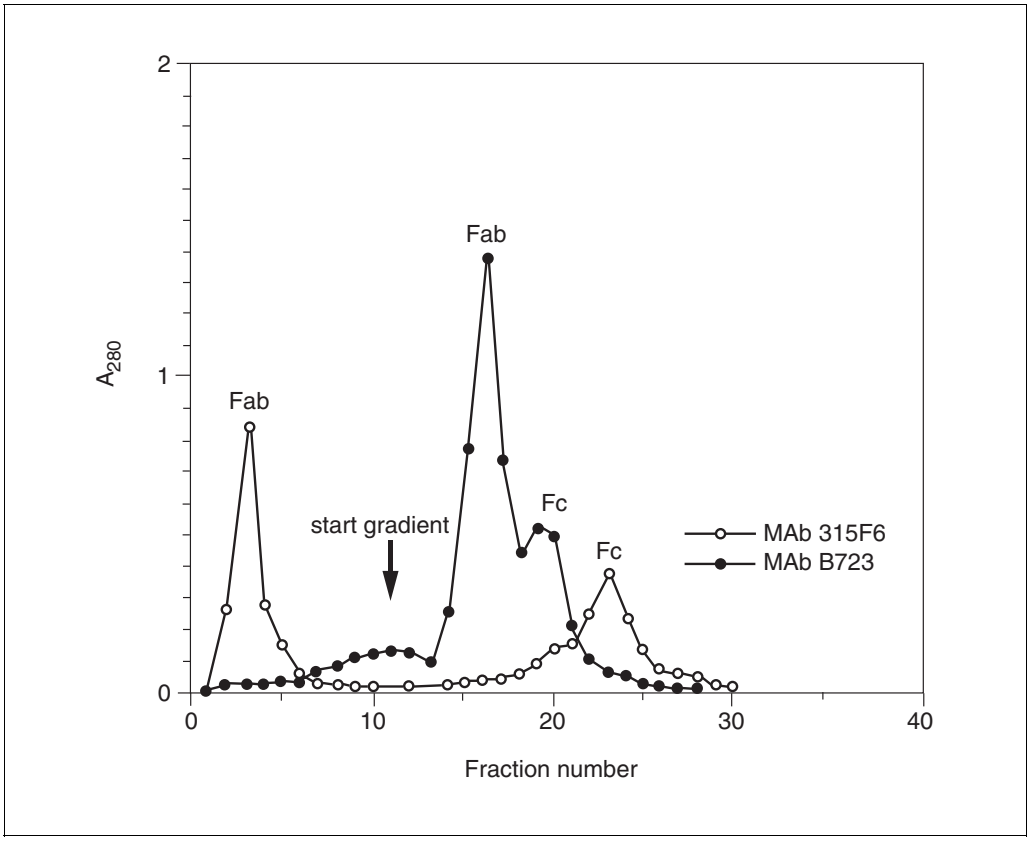


Figure 16.3.2 IEX chromatography elution profile of Fab from two different antibodies using a DE52 column (1.5 × 50 cm). Elution conditions and gradient were the same as for the example in Figure 16.3.1. For MAb 315F6 (open circles), the Fab fragment eluted in the starting buffer and the Fc portion much later in the gradient. Fab fragments from MAb B72.3 (closed circles) eluted at the beginning of the gradient.

between antibodies as shown in Figure 16.3.2. For one of the antibodies (open circles), the Fab elutes at 0.01 M Tris·Cl, pH 8.6. In contrast, the Fab from the other antibody (closed circles) elutes 40 ml after the salt gradient is started. These examples reinforce the need to monitor the location of the protein during the purification process.

REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2D**; for suppliers, see **SUPPLIERS APPENDIX**.*

Borate-buffered saline

0.015 M sodium borate
0.15 M NaCl
Adjust to pH 8.5 with 1 M NaOH and filter sterilize
Store indefinitely at room temperature

0.5 M NaCl/0.01 M Tris·Cl, pH 8.6

1.21 g Tris base
29 g NaCl
800 ml H_2O
Adjust pH to 8.6 with HCl and add H_2O to 1 liter

Phosphate-buffered saline (PBS)

0.23 g NaH_2PO_4 (anhydrous, 1.9 mM final)
1.15 g Na_2HPO_4 (anhydrous, 8.1 mM final)

continued

9 g NaCl (154 mM final)
Add H_2O to 900 ml
Adjust desired pH using 1 M NaOH or 1 M HCl
Add H_2O to 1 liter
Store indefinitely at room temperature

COMMENTARY

Background Information

Before purifying antibody from any preparation, consideration should be given to the potential use of the final product. If the antibody is to be used in an assay with internal controls or to saturate cell-surface antigen, then impure ascites fluid or a dialyzed ammonium sulfate precipitate will usually suffice. At the other extreme, biochemical modifications such as conjugation to drugs or certain fluorochromes (e.g., phicobiliproteins) may require an extremely pure product. In this case ion-exchange (IEX) chromatography (see Basic Protocol 3) should be used in addition to ammonium sulfate precipitation and affinity chromatography. For uses such as conjugation to fluorescein isothiocyanate (FITC), biotin or radioisotopes, antibody that has been purified by protein A or G is perfectly adequate.

Protein A is a cell wall component produced by several strains of *Staphylococcus aureus*; it is a single polypetide chain with a molecular weight of 42,000 Da. Protein A binds specifically to the Fc region of immunoglobulin molecules, especially IgG for which it has four high-affinity binding sites. It is heat stable and retains its native conformation even after exposure to denaturing reagents such as 3 M thiocyanate, or in acidic conditions. Thus antibody can be stripped from protein A and the reagent used again for purification. Not all IgG molecules bind to protein A; rat IgGs bind particularly weakly, and many mouse IgG_1 subclass antibodies will not bind. Some of these antibodies can be affinity purified with the similar reagent, protein G.

Protein G is a bacterial cell wall protein isolated from group G streptococci. Protein G also binds IgG molecules through their Fc portions. There are two binding sites for IgG in native protein G; it also has binding sites for Fab regions of antibody, albumin, and cell membranes. Sigma sells a recombinant form of protein G which has been truncated so the sites for Fc binding remain, but the Fab-, albumin-, and membrane-binding sites have been removed. Protein G is particularly useful for purification of human IgG3 and rat IgG_{2b}; although protein G can be used for other rat IgG molecules, the binding is frequently weak, especially in the case of IgG_1. Protein G is usually better than protein A for purification of mouse IgG_1.

Rat antibody can be problematic to purify, and an anti-rat affinity column (Alternate Protocol 2) may be required. The authors have had little success with protein G purification for rat monoclonal antibodies.

Elution profiles for IEX chromatography are different for each antibody or antibody fragment. The activity of eluted antibodies should be monitored using an ELISA (*APPENDIX 3*) in addition to the absorbance (A_{280}) profile.

Table 16.3.1 describes kits available for antibody purification. On first inspection, these may not seem to be the least expensive option, but it is important to remember that the reliability of kits and their excellent performance in terms of yield usually make them quite economic in the long term. It is a false economy to spend many hours of staff time obtaining a decreased percentage yield for the sake of saving a few dollars at the outset.

Critical Parameters

Because activity of the purified product is of prime importance, this should be evaluated for the impure antibody before purification is started and throughout the steps of purification. Some investigators report that the acid conditions required to elute from protein A can damage the antibody. Care should be taken to avoid excessively long periods of incubation at low pH. Elution from protein A– or protein G–Sepharose can also be accomplished using 3 M potassium thiocyanate, high ionic strength, or high pH. For particular antibodies these conditions may be more gentle, but in the authors' experience the best results are obtained with the methods described here.

Ammonium sulfate precipitation is a more gentle procedure than protein A purification, but it is more time-consuming. It can sometimes be difficult to remove samples from protein G. Use lower pH and increased salt concentration if problems are encountered.

Protein A columns can be used at flow rates of ~1 ml/min, while SE columns should be limited to ≤0.5 ml/min. Optimal results for SE are obtained using samples containing 5 to 100 mg of antibody. Protein A should be loaded within the limits given.

The antibody-binding capacity of protein A is variable between species. For mouse IgG, 1 ml of hydrated protein A will bind ~5 mg of antibody; for human IgG, 1 ml of hydrated protein A will bind ~8 mg of antibody.

For IEX (DE52) chromatography, the pH is very important and buffers should be checked on a pH meter immediately before starting. All column fractions should be retained until SDS-PAGE and antibody activity assays have been performed. In some cases, antibody may elute from the IEX column in the same fractions as serum albumin, in which case SE chromatography should be used.

DE52 has an enormous capacity for binding protein, as estimated by the manufacturer at 130 mg/ml bed volume. Thus, dilute antibody preparations can be concentrated effectively by this method.

Anticipated Results

Different batches of ascites fluid and monoclonal antibody supernatant can vary widely in the amount of antibody they contain. Generally, 1 ml of ascites should yield 1 to 4 mg of purified antibody and 1 ml of MAb supernatant should yield 0.5 to 50 μg of purified product. If cells are grown in automated culture systems that recycle the medium (bioreactors), yields equivalent to those of ascites fluid can be obtained. The lowest yields are usually from ammonium sulfate precipitation and the highest yields are usually from IEX or affinity methods.

Yields significantly lower than these should be a warning that the antibody-producing hybridomas are being overgrown by nonproducers. This can be remedied by returning to an earlier freeze of cells, or by recloning (and rescreening) the hybridoma.

Time Considerations

Procedures such as dialysis and running columns can be lengthy, but large amounts of antibody can be purified in 2 to 3 days. FPLC (fast peptide, protein, and polynucleotide liquid chromatography) systems are expensive, but can reduce the time taken to obtain purified antibody.

Quickest results can be obtained when culture supernatant or ascites is purified by one of the affinity methods described here (Basic Protocol 2): pure antibody can be obtained in <1 day. The ammonium sulfate, size-exclusion, or ion-exchange methods will take longer.

Literature Cited

Akerstrom, B. and Bjorck, L. 1986. A physiochemical study of protein G molecule with unique immunoglobulin G–binding properties. *J. Biol. Chem.* 261:10240-10247.

Cooper, H.M. and Paterson, Y. 1993. Determination of the specific antibody titer. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 11.16.1-11.16.13. John Wiley & Sons, New York.

Key References

Hardy, R.R. 1986. Purification and characterization of monoclonal antibodies. *In* Handbook of Experimental Immunology, Vol. 1: Immunochemistry (D.M. Weir, ed.) pp. 13.1-13.13. Blackwell Scientific, Oxford.

An excellent and detailed review of methods for purifying antibodies that includes an extensive list of current literature.

Harlow, E., and Lane, D. (ed.) 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

The complete text on antibodies; everything you want to know and more.

A Technical Guide to Antibody/Protein Purification. 1995. Pierce, Rockford, Ill.

A highly informative supplement to the Pierce antibody-purification kits.

Monoclonal Antibody Purification Handbook. 1994. Pharmacia Biotech, Piscataway, N.J.

This handbook contains the computer program MAb Assistant.

Contributed by Sarah M. Andrew
Chester College
Chester, United Kingdom

Julie A. Titus
National Cancer Institute
Bethesda, Maryland

Fragmentation of Immunoglobulin G

To study the distinct regions of an immunoglobulin, it is useful to cleave them with proteolytic enzymes into various fragments (see Fig. 16.4.1 for a schematic of the structure of antibody molecules and their proteolytic fragments). The Fc portion of the antibody molecule is responsible for certain biological effects of antibodies, such as binding to Fc receptors on cells, mediating antibody-dependent cellular cytotoxicity, and complement fixation. It is also thought to be the more immunogenic portion of the molecule in vivo. For experiments concerning effects mediated by specific binding of antibody to cells in vitro or localization of antibodies to certain organs in vivo, it may be necessary to eliminate the nonspecific effects of Fc-receptor binding. The affinity of antibody for an antigen on the surface of viable cells can only be measured accurately using monovalent antigen-binding fragments (Fab).

Classically, fragmentation to the monovalent Fab fragment is carried out using papain digestion (see Basic Protocols 1 and 2), and the bivalent $F(ab')_2$ fragment is obtained by pepsin digestion (see Basic Protocols 3 and 4). Although papain digestion produces Fab fragments from IgG of all subclasses from all species, pepsin is not so universally useful. Alternative methods of fragmentation to $F(ab')_2$ include use of papain that is preactivated with cysteine (see Alternate Protocol 1) and use of the enzyme ficin (see Alternate Protocol 2). These alternate methods are particularly useful for mouse IgG_1 antibodies. When using pepsin and papain for the first time on an antibody, pilot experiments are performed to determine the correct reaction conditions; with preactivated papain and ficin these pilot experiments are unnecessary.

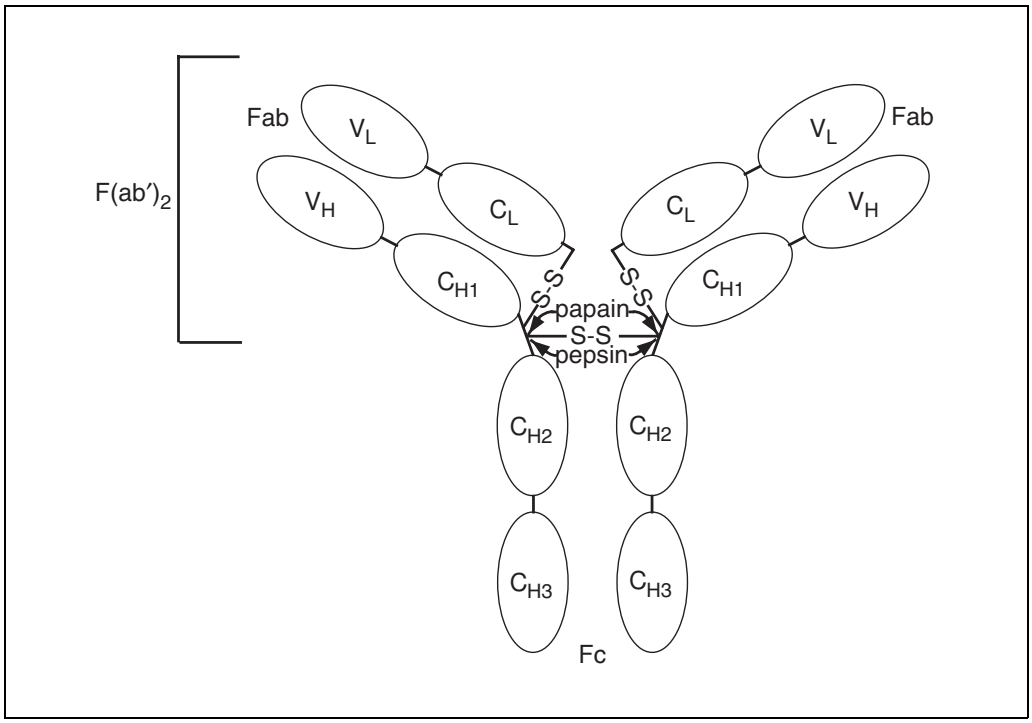


Figure 16.4.1 Proteolytic digestion fragments of immunoglobulins. Structural domains of the variable and constant regions of the light chain (V_L, C_L) and heavy chain (V_H, C_{H1}, C_{H2}, C_{H3}), as well as interchain disulfide bonds (S-S), are indicated. Monovalent fragments of IgG produced by papain digestion are known as Fab, while bivalent papain fragments are $F(ab)_2$. $F(ab')_2$ refers to the bivalent fragments of IgG produced by pepsin digestion; those derived from IgM are called $F(ab')_2\mu$. Bivalent subunits of IgM with both Fab and Fc are known as IgMs (subunit), and fragments of IgG containing a single antibody-binding site and an intact Fc portion are Fab/c.

Contributed by Sarah M. Andrew and Julie A. Titus

Current Protocols in Cell Biology (2000) 16.4.1-16.4.10

PILOT FRAGMENTATION OF IgG TO Fab USING PAPAIN

Papain digestion of IgG produces Fab fragments. This protocol is designed to determine conditions for large-scale fragmentation, and can be used for IgG from mouse (all subclasses), rat, human, goat, sheep, horse, chicken, guinea pig, and cow. Once the method for a particular antibody is established, it will be reproducible for each fragmentation.

Purified monoclonal antibody is digested with papain in the presence of the reducing agent cysteine. To determine the optimal conditions, both the concentration of papain and the time of digestion are varied. After terminating the digestions, the fragments are dialyzed and analyzed by SDS-PAGE. By assessing the gel results, optimal conditions are chosen. These conditions are used in the large-scale fragmentation of IgG to Fab (see Basic Protocol 2).

Materials

2 mg/ml purified IgG (*UNIT 16.3*) in PBS (*UNIT 16.3*)
Papain (2× crystallized suspension; Sigma)
Digestion buffer (see recipe)
0.3 M iodoacetamide (prepare fresh from crystalline material; Sigma) in PBS
PBS (*UNIT 16.3*)

Additional reagents and equipment for nonreducing gel electrophoresis (*UNIT 6.5*) and dialysis of proteins (*APPENDIX 3C*)

1. Pipet 100 µl of 2 mg/ml purified IgG in PBS into each of 24 numbered microcentrifuge tubes.

2. Prepare two papain solutions in 5 ml digestion buffer—one at 0.1 mg/ml papain and the other at 0.02 mg/ml.

   Because papain is a suspension, mix well without vortexing before pipetting.

3. Add 100 µl of 0.1 mg/ml papain to eight of the IgG tubes. Add 100 µl of 0.02 mg/ml papain to a second group of eight IgG tubes. Finally, add 100 µl digestion buffer *without* papain to the remaining eight tubes. Close the lids on all of the tubes.

   Thus, reactions have been set up with enzyme/antibody (E/A) ratios of 1:20 and 1:100, and control tubes with no enzyme.

4. Prepare a digestion time curve by incubating tubes in a 37°C water bath and removing them at different times in groups of three—one tube each of E/A at 1:20 and 1:100, and one control tube. Remove the first group after 1 hr, and the subsequent groups at 2, 4, 6, 12, 18, 24, and 48 hr. As each group of three tubes is removed from the water bath, terminate the reaction by adding 20 µl of 0.3 M iodoacetamide in PBS and then vortexing.

   The final concentration of iodoacetamide (0.03 M) irreversibly inactivates papain.

5. Dialyze the reaction mixture (*APPENDIX 3C*).

   Dialysis can be performed using a homemade microdialysis chamber or commercial microdialysis cassettes (e.g., Slide-A-Lyzer Dialysis Cassettes, Pierce).

6. Analyze digestion products by loading an 80-µl aliquot of the dialyzed mixture from each tube onto a 10% nonreducing polyacrylamide gel (*UNIT 6.5*).

   Antibodies that have been digested with papain in the presence of cysteine to produce Fab fragments show a major protein band on a nonreducing polyacrylamide gel at a molecular weight of ~50 kDa, and a second band at 27 kDa which is the remaining Fc portion. There are usually a couple of smaller bands at 24 and 15 kDa which are other unimportant products of digestion. Obviously, undigested antibody will appear as a band at 150 kDa. Frequently, effects of cysteine alone are seen in antibody which has not been treated with papain, causing a protein band on the gel at 120 kDa. This 120-kDa band is also seen in

papain, causing a protein band on the gel at 120 kDa. This 120-kDa band is also seen in the digestion mix at short incubation times (up to 4 hr). The digestion is complete when no protein bands of 150 kDa or 120 kDa are seen in the gel lanes containing the digestion mixture. The time of complete digestion will vary between antibodies.

The effect of incubation with cysteine alone will be seen in the control lanes, probably because cysteine causes some unfolding of the antibody molecule, thus altering its electrophoretic mobility. Fab fragments have a molecular weight of 50 kDa (see Table 16.3.2). At early time points some intact antibody is expected on the gel, with the reaction moving towards completion as incubation times increase.

The amounts of antibody used in this method are suitable for analysis by SDS-PAGE using standard 150 × 1.5–mm gels. If a minigel apparatus is available (e.g., the Phast system from Pharmacia), modify the amounts accordingly and use less antibody.

7. Choose the optimal conditions to obtain Fab from the IgG by assessing the gel results and use these conditions for Basic Protocol 2.

LARGE-SCALE FRAGMENTATION OF IgG TO Fab USING PAPAIN

BASIC PROTOCOL 2

Exact conditions for optimum yield of Fab from each antibody should be assessed from the pilot experiment (see Basic Protocol 1); those conditions are then scaled up in this procedure. The procedure is similar to Basic Protocol 1 where papain is used to digest the antibody and the digested antibody is dialyzed. The dialyzed antibody fragment is then purified by protein A–Sepharose affinity chromatography (*UNIT 16.3*) and can be further purified by size-exclusion chromatography (*APPENDIX 3*). The purity of the product is assessed by nonreducing gel electrophoresis (*UNIT 6.5*).

Materials

Papain (2× recrystallized suspension; Sigma)
Digestion buffer (see recipe)
≥1 mg/ml IgG (≥5 mg total; *UNIT 16.3*) in PBS
Iodoacetamide crystals
PBS, pH 8.0 (*UNIT 16.3*)
Protein A–Sepharose CL-4B
Sephacryl S-200 Superfine (Pharmacia Biotech)

5 × 100–mm column (Bio-Rad)
26 × 900–mm column (Pharmacia Biotech)

Additional reagents and equipment for protein dialysis (*APPENDIX 3C*), column chromatography (*UNIT 16.3 & APPENDIX 3*), concentrating protein solutions (*APPENDIX 3C*), and nonreducing gel electrophoresis (*UNIT 6.5*)

1. Dissolve the required amount of papain in a volume of digestion buffer equal to the volume of antibody solution to be digested; use optimum amounts of enzyme and antibody as well as incubation times from the results of the pilot fragmentation in Basic Protocol 1.

   Digestion buffer is made at twice the required final strength so that when added to the antibody solution in equal volume, the mixture will be at the correct molarity with respect to EDTA and cysteine. Because papain is a suspension, mix well without vortexing before pipetting.

2. Add the papain buffer solution to the ≥1 mg/ml IgG in PBS, mix, and incubate for the required time (determined from Basic Protocol 1) in a 37°C water bath.

3. Stop the reaction by adding crystalline iodoacetamide to 0.03 M final. Mix carefully to ensure that the iodoacetamide dissolves completely.

4. Transfer to dialysis tubing and dialyze the mixture against 2 liters PBS, pH 8.0, for 12 to 20 hr at 4°C.

   More detailed procedures for large-volume dialysis, and selection and preparation of dialysis membranes can be found in APPENDIX 3C.

5. Prepare a 5 × 100–mm protein A–Sepharose CL-4B column (*UNIT 16.3 & APPENDIX 3*) and load dialyzed mixture onto it. Collect unbound fraction containing the Fab fragment and enzyme. If necessary, wash column with PBS to completely recover Fab fragments.

   The remaining intact antibody will bind to the column.

6. Concentrate the mixture containing the Fab fragments to ≤5 ml (*APPENDIX 3C*).

7. Prepare a 26 × 900–mm Sephacryl S-200 Superfine size-exclusion column (*APPENDIX 3*) and load concentrated mixture onto it. Collect fractions corresponding to a molecular weight of 50 kDa.

   Fractions can be collected and their molecular weight assessed by SDS-PAGE or a precalibrated size-exclusion column.

8. Check the purity of the final product (1 to 80 µl) on a 10% nonreducing polyacrylamide gel (*UNIT 6.5*).

9. Assess the concentration of the Fab fragments at A_{280} (Table 16.3.2). Store fragments in borate buffer (*UNIT 16.3*) at 4°C or in PBS containing 0.02% NaN_3 at −70°C.

BASIC PROTOCOL 3

PILOT FRAGMENTATION OF IgG TO F(ab′)$_2$ USING PEPSIN

Pepsin fragmentation of IgG to produce F(ab′)$_2$ is similar to the pilot fragmentation with papain (see Basic Protocol 1) except that the pH and time of digestion are varied. The optimum pH for pepsin digestion is ~2; however, this low pH may damage the antibody, so a pH of ~4 is the minimum recommended. Two different pH values are tested in this pilot experiment, as some antibodies will fragment to Fab or smaller fragments at lower pH values. Because pepsin treatment can be slightly harsher than papain, an enzyme/antibody (E/A) ratio of no higher than 1:20 is recommended, as it is possible that pepsin could damage the binding site of the antibody molecule.

NOTE: F(ab′)$_2$ fragments cannot be obtained from IgG_{2b}. Treatment of IgG_{2b} with pepsin will result in fragments of about the correct molecular weight (120 kDa), but these will be Fab/c fragments (consisting of one Fab fragment and the Fc portion; Fig. 16.4.1).

Materials

3 mg/ml purified IgG (*UNIT 16.3*)
Acetate buffer, pH 4.0 and 4.5 (see recipe)
0.1 mg/ml pepsin (Sigma) in pH 4.0 and pH 4.5 acetate buffers
2 M Tris base
PBS (*UNIT 16.3*)

Additional reagents and equipment for protein dialysis (*APPENDIX 3C*) and nonreducing gel electrophoresis (*UNIT 6.5*)

1. Dialyze 2 ml of 3 mg/ml purified IgG against 200 ml acetate buffer, pH 4.0, and another 2 ml of 3 mg/ml purified IgG against 200 ml acetate buffer, pH 4.5, both for 4 hr at 4°C.

2. Determine the concentrations of both dialyzed IgG solutions by A_{280} readings using the acetate buffer of corresponding pH as the blank (Table 16.3.2).

3. Adjust each dialyzed antibody solution to a concentration of 2 mg/ml in the acetate buffer of corresponding pH.

4. Pipet 100 µl of the 2 mg/ml IgG, pH 4.0, into each of 16 numbered microcentrifuge tubes. Into another 16 numbered microcentrifuge tubes, pipet 100 µl of the 2 mg/ml IgG, pH 4.5.

   Thus, there are two groups of 16 tubes—one group at pH 4.0 and the other at pH 4.5.

5. Add 100 µl of 0.1 mg/ml pepsin in acetate buffer, pH 4.0, to eight tubes in the IgG, pH 4.0 group. To the other eight tubes in this group, add 100 µl of acetate buffer, pH 4.0. Add 100 µl of 0.1 mg/ml pepsin in acetate buffer, pH 4.5, to eight tubes in the IgG, pH 4.5 group. To the other eight tubes in this group, add 100 µl of acetate buffer, pH 4.5.

   Thus, there are two groups of 16 tubes with pepsin at an E/A ratio of 1:20 at two different pH values, and control tubes without enzyme.

6. Incubate the 32 tubes in a 37°C water bath, removing one tube from each group of eight tubes after 1, 2, 4, 6, 12, 24, and 48 hr. As each tube is removed, stop the reaction by adding 40 µl of 2 M Tris base.

7. Transfer to dialysis tubing and prepare microdialysis chamber (*APPENDIX 3C*). Dialyze the samples against 1 liter PBS for 4 hr at 4°C.

8. Analyze 80-µl aliquots of the fragmentation products on a 10% nonreducing polyacrylamide gel (*UNIT 6.5*).

   $F(ab')_2$ has a molecular weight of 110 kDa. Bands of this molecular weight should be seen in the fractions treated with pepsin. Lower-molecular-weight bands (~50 kDa) are probably the Fab′ fragment of the antibody, a further degradation product from pepsin. Amounts of $F(ab')_2$ and Fab′ produced will vary among antibodies, depending on the fragmentation conditions and individual susceptibilities.

9. Choose the best conditions to obtain maximum yield of $F(ab')_2$ by assessing the gel results and use these conditions in the following basic protocol.

LARGE-SCALE FRAGMENTATION OF IgG TO $F(ab')_2$ USING PEPSIN

BASIC PROTOCOL 4

As with the papain large-scale protocol, optimal fragmentation conditions should be assessed for each antibody from the pilot fragmentation (Basic Protocol 3). Purification is by protein A–Sepharose affinity chromatography (Basic Protocol 2). It is important to remember that IgG_{2b} generally cannot be fragmented to $F(ab')_2$.

Materials

≥1 mg/ml purified IgG (*UNIT 16.3*)
Acetate buffer at appropriate pH (see Basic Protocol 3 and recipe)
0.1 mg/ml pepsin in acetate buffer at appropriate pH (see Basic Protocol 3)
2 M Tris base
PBS, pH 8.0 (*UNIT 16.3*)
Protein A–Sepharose CL-4B
Sephacryl S-200 Superfine (Pharmacia Biotech)

5 × 100–mm column (Bio-Rad)
26 × 900–mm column (Pharmacia Biotech)

Additional reagents and equipment for protein dialysis (*APPENDIX 3C*), concentrating protein solutions (*APPENDIX 3C*), column chromatography (*UNIT 16.3 & APPENDIX 3*), and reducing and nonreducing gel electrophoresis (*UNIT 6.5*)

Digest IgG

1. Dialyze ≥1 mg/ml IgG against acetate buffer at the appropriate pH.
2. Determine the concentration at A_{280}.
3. Add 0.1 mg/ml pepsin dissolved in acetate buffer at appropriate pH to give an E/A ratio of 1:20 (see Basic Protocol 3, step 5). Incubate the reaction mixture for the required time (see Basic Protocol 3, step 6) in a 37°C water bath.
4. Stop the reaction by adding ~50 µl of 2 M Tris base/ml reaction. Check with pH paper that pH = 8.0 and add more Tris base if necessary.
5. Transfer the mixture to dialysis tubing and dialyze against 1 liter PBS, pH 8.0, at 4°C (*APPENDIX 3C*).

Purify F(ab′)$_2$

6. Prepare a 5 × 100–mm protein A–Sepharose CL-4B column (*UNIT 16.3 & APPENDIX 3*) and load dialyzed mixture onto it. Collect the unbound fraction.

   At this stage, the F(ab′)$_2$ fragment can be reduced to Fab′ if desired. Add cysteine to a concentration of 0.01 M, mix well and incubate 2 hr at 37°C. Cysteine should reduce and alkylate the inter–heavy chain disulfide bonds. If further alkylation is thought necessary, make the mixture 0.15 M with respect to iodoacetamide. Purify the Fab′ using the steps below.

7. Concentrate the mixture to ≤5 ml.
8. Prepare a 26 × 900–mm Sephacryl S-200 Superfine size-exclusion column (*APPENDIX 3*) and load concentrated mixture onto it. Collect fractions corresponding to a molecular weight of 110 kDa.

   Measure the protein eluting from the column with a UV monitor or spectrophotometer. Assess their contents by SDS-PAGE or use of a precalibrated column.

9. Check the purity of the final product (1 to 20 µl) on a 10% SDS–polyacrylamide gel under reducing and nonreducing conditions (*UNIT 6.5*).

   F(ab′)$_2$ will give a single band at 110 kDa on a nonreducing gel and a single band or a doublet at 25 kDa on a reducing gel.

10. Assess the concentration of the F(ab′)$_2$ fragments at A_{280} (Table 16.3.2). Store fragments in borate buffer (*UNIT 16.3*) at 4°C or in PBS containing 0.02% NaN_3 at –70°C.

ALTERNATE PROTOCOL 1

FRAGMENTATION OF IgG USING PREACTIVATED PAPAIN

Mouse IgG_1 has shown significant resistance to pepsin digestion. The antibody is missing a leucine at residue 234; this restricts the hinge region and causes pepsin resistance. This method can be used to obtain F(ab)$_2$ fragments from IgG_1. Additionally, Fab fragments can be obtained from mouse IgG_{2a} and IgG_{2b} by this cleavage. It is a gentler procedure and can give a more stable fragmentation and a high yield of fragments. The procedure is time-consuming but incubation periods are usually less critical. The conditions used here are typical of large-scale fragmentation and purification. It is not necessary to carry out a pilot fragmentation experiment to assess conditions when using this procedure, although the type of fragment obtained varies between subclasses.

***Additional Materials** (also see Basic Protocol 4)*

10 mg IgG (*UNIT 16.3*) in 2 to 5 ml PBS
Acetate/EDTA buffer (see recipe)
2 mg/ml papain in acetate/EDTA buffer
0.05 M cysteine (free base, crystalline; Sigma)
Iodoacetamide crystals

PD-10 column (Pharmacia Biotech)
26 × 900–mm column

Additional reagents and equipment for protein dialysis and concentration (*APPENDIX 3C*), column chromatography (*UNIT 16.3 & APPENDIX 3*), and SDS-PAGE (*UNIT 6.1*)

Prepare papain

1. Dialyze 10 mg IgG in 2 to 5 ml PBS in acetate/EDTA buffer (*APPENDIX 3C*).
2. Determine the concentration at A_{280} with the acetate/EDTA buffer as a blank.
3. Incubate 2 mg/ml papain and 0.05 M cysteine for 30 min in a 37°C water bath.

   Papain requires a free sulfhydryl group for its catalytic activity. In the native crystalline form, this group is blocked and the enzyme exhibits extremely low proteolytic activity. Cysteine activates the group and activation is optimal in the presence of a heavy-metal binding agent such as EDTA.

4. Equilibrate a PD-10 column with 20 ml of acetate/EDTA buffer (*APPENDIX 3*).
5. Apply papain/cysteine (from step 3) to the PD-10 column. Collect ten 1-ml fractions, eluting with acetate/EDTA buffer.

   This process removes the cysteine from preactivated papain. Use preactivated papain within 2 hr of preparation and keep on ice until use.

6. Assay the fractions at A_{280} and pool the two or three fractions containing papain. Calculate the preactivated papain concentration using the following formula:

   $A_{280}/2.5$ = mg preactivated papain/ml.

Digest IgG

7. Add 0.5 mg preactivated papain to dialyzed, 10-mg IgG solution (from step 2) and vortex. Incubate 6 to 12 hr in a 37°C water bath.
8. Stop reaction by adding crystalline iodoacetamide to a final concentration of 0.03 M.
9. Dialyze against 1 liter PBS, pH 8.0, at 4°C for 6 to 12 hr (*APPENDIX 3*).

Purify fragments

10. Prepare a protein A–Sepharose column equilibrated in PBS, pH 8.0 (*UNIT 16.3*), and apply reaction mixture. Collect unbound 2-ml protein fractions and pool those represented by the first peak.

    The antibody fragments will elute in the first protein peak. Assess this using a UV monitor or spectrophotometer.

11. Concentrate the unbound fractions to ≤5 ml (*APPENDIX 3C*).
12. Prepare a size-exclusion (SE) column (*APPENDIX 3*) and load the sample. Collect 100 fractions (1% of the column volume). Assess the fractions containing the appropriate molecular weight by SDS-PAGE (*UNIT 6.1*) or on a precalibrated column. Store in borate buffer (*UNIT 16.3*) at 4°C or in PBS containing 0.02% NaN_3 at −70°C.

    Monitor the fractions with a UV spectrophotometer at A_{280} nm. The fragments obtained from this procedure are Fab from IgG_{2a} and IgG_{2b}, and $F(ab)_2$ from IgG_1.

ALTERNATE PROTOCOL 2

FRAGMENTATION OF MOUSE IgG_1 TO $F(ab')_2$ USING FICIN

IgG_1 can also be fragmented to $F(ab')_2$ by treatment with ficin in the presence of cysteine. Ficin is a thiol protease isolated from fig latex.

Additional Materials *(also see Basic Protocol 4)*

10 mg mouse monoclonal IgG_1 (*UNIT 16.3*) in 2 to 5 ml PBS
50 mM Tris/2 mM EDTA, pH 7
Ficin solution: 1 mg/ml ficin (Sigma) in 50 mM Tris/2 mM EDTA, pH 7
Cysteine
100 mM *N*-ethylmaleimide (Sigma)
PBS (*UNIT 16.3*)

Additional reagents and equipment for protein dialysis (*APPENDIX 3C)* and column chromatography (*UNIT 16.3 & APPENDIX 3*)

1. Dialyze 10 mg mouse monoclonal IgG_1 in PBS against 1 liter of 50 mM Tris/2 mM EDTA, pH 7, for 6 hr at 4°C.
2. Determine the concentration of the antibody by reading the A_{280} with the dialysis buffer as a blank.
3. Add ficin solution to give a 1:30 enzyme/immunoglobulin ratio.
4. Add solid cysteine to the mixture to give a final cysteine concentration of 1 mM.

   Cysteine is required to activate the enzyme.
5. Incubate 4 to 5 hr in a 37°C water bath with gentle shaking.

   The incubation time is not critical.
6. Stop reaction by adding 1/10 vol of 100 mM *N*-ethylmaleimide.
7. Dialyze the mixture against PBS, then purify the fragments by protein A–Sepharose and size-exclusion (SE) chromatography (see Alternate Protocol 1, steps 10 to 12 and *UNIT 16.3 & APPENDIX 3*).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetate buffer, pH 4.0 and 4.5

Bring 0.2 M sodium acetate to pH 4.0 and 4.5 (as required) with glacial acetic acid.

Acetate/EDTA buffer, pH 5.5

Bring 0.1 M sodium acetate to pH 5.5 with glacial acetic acid and add EDTA to 0.003 M.

Digestion buffer

PBS (UNIT 16.3) containing:
0.02 M EDTA (disodium salt)
0.02 M cysteine
Prepare fresh; store <10 hr on ice

COMMENTARY

Background Information

Immunoglobulins can be cleaved by proteolytic enzymes into functionally distinct subunits (Fig. 16.4.1). Generally, fragments that bind antigens are of interest. Fab and $F(ab')_2$ fragments contain the antibody-binding portion of the molecule; the Fc fragment is generally only of interest if studying the crystal structure of antibodies. The Fab or $F(ab')_2$ portion of the antibody may be required in certain experiments. For instance, in mixed lymphocyte response (MLR) and cytolytic T lymphocyte (CTL) assays, the Fc portion may cause artifacts because it binds to cells bearing the Fc receptor. It has also been suggested that the Fc portion of the antibody may be more immunogenic in vivo (Hellstrom and Hellstrom, 1985). The Fc portion of IgG_{2a} and Ig_{G3} can invoke antibody-dependent cell-mediated cytotoxicity responses in vivo and in vitro.

The monoclonal antibodies routinely used for in vitro and in vivo experiments are from the mouse, and the protocols of this unit are designed for use with mouse antibodies. Fragmentation of antibodies from other species can be evaluated and optimized using the pilot protocol provided. Fragmentation with papain is similar for many species.

Subclass-dependent susceptibility of mouse antibodies to proteolytic cleavage has been widely reported (Parham, 1986). The order of sensitivity to proteolytic enzymes has been found to be IgG_{2b}>Ig_{G3}>IgG_{2a}>IgG_1. Not all antibodies, however, fall into this ranking; IgG_{2a} can be extremely sensitive to the action of papain in the presence of cysteine. If such sensitivities are found, then the method in which cysteine is removed from the enzyme following preactivation is recommended (see Alternate Protocol 1; Parham et al., 1982).

It must be stressed that all monoclonal antibodies are different and, for this reason, standard conditions for fragmentation are not given for papain and pepsin digestion. A pilot experiment should always be performed to assess optimal conditions before carrying out a large-scale fragmentation. The pilot experiment is very similar to the large-scale procedure. Microdialysis (Overall, 1987) is recommended so that the conditions from small- to large-scale are identical.

IgG can be fragmented to yield monovalent Fab fragments using the proteolytic enzyme papain in the presence of the reducing agent cysteine. IgG subclasses (e.g., IgG_1 and IgG_2) have different susceptibilities to papain digestion. IgG_1, IgG_{2a}, and IgG_3 can be digested to bivalent $F(ab')_2$ fragments using pepsin. Although IgG_1 may be sensitive to pepsin degradation, a more stable digestion is achieved using papain in the absence of cysteine. Ficin is also useful for digestion of IgG_1 to $F(ab')_2$ (Mariani et al., 1991). It is a thiol protease purified from fig latex. It requires activation with cysteine; the concentration of cysteine determines the extent of digestion. Generally, IgG_{2b} cannot be fragmented to $F(ab')_2$. Prior to fragmentation, a pilot experiment should be undertaken to determine individual conditions for fragmentation of each antibody. Separation of fragments can be carried out very efficiently by ion-exchange (IEX) chromatography on DE52 (*UNIT 16.3 & APPENDIX 3*); other methods for separation have been described in the protocols.

Pepsin cleavage of mouse IgG subclasses results in two sites of proteolytic sensitivity, one on either side of the inter–heavy chain disulfide bonds as suggested by Parham (1983). Primary cleavage may occur at a site on the COOH-terminal side of the bridges, giving $F(ab')_2$, followed by secondary cleavage on the NH_2-terminal side, giving Fab′. In the case of IgG_{2b}, the relative sensitivities of these two sites is reversed. Because the heavy chains of IgG_{2b} are asymmetrically glycosylated and the products of pepsin digestion of this subclass are Fab and Fab/c, $F(ab')_2$ has a molecular weight close to that of Fab/c. $F(ab')_2$, however, has rarely been reported to be obtained from IgG_{2b}, and therefore is not a feasible product of this subclass.

Pierce produces several kits for production of Fab and $F(ab')_2$ fragments from mouse (and other species) antibodies. These kits have been used with great success by the authors. Such kits are strongly recommended to those with the funds to purchase them. Each kit comes complete with all buffers and instructions.

Critical Parameters

Because each monoclonal IgG is different, if one antibody of a particular subclass is cleaved under a certain set of conditions, it cannot be expected that all other antibodies of that subclass will behave in the same manner. A pilot experiment should always be carried out when a new antibody is to be fragmented. It is probably better to choose a reaction time that will leave some antibody unfragmented rather than risk damaging the antibody-binding site by excessive exposure to proteolytic en-

zymes. Intact antibody can be easily removed from the fragmentation mixture by passage through a protein A–Sepharose column. For those antibodies that do not bind well to protein A, protein G–Sepharose (*UNIT 16.3*) or ion-exchange chromatography (*APPENDIX 3*) are reasonable alternatives.

Troubleshooting

Optimum reaction conditions may vary after scaling up from the pilot fragmentation. Occasionally the incubation times appear shorter in the small-scale procedure, so it may be necessary to increase the incubation time a little when scaling up.

Another possible difficulty is that the fragmentation does not proceed at all. Probably, this is related to the enzyme. Ascertain that there are no enzyme inhibitors, such as azide, present in the antibody solution (*UNIT 16.3*) before ordering new enzyme.

Anticipated Results

Yields are frequently subclass-dependent. A 100% yield of Fab fragment would be twice the number of moles of intact antibody in the starting mixture, and a 100% yield of $F(ab')_2$ would be equal to the number of moles of antibody in the starting mixture. Typically, yields of 45% to 55% are achieved, although some investigators report yields as high as 70% to 80%.

Time Considerations

Dialysis is usually carried out overnight, although 4 hr is sufficient for samples up to 100 ml. Running a protein A–Sepharose column takes ~4 hr, and running a size-exclusion column takes overnight.

Dialysis and running of columns are time-consuming procedures. The total time required to obtain fragments from a new antibody—beginning with the pilot experiment to the end of the large-scale production—is 4 to 5 days for Fab fragments and 6 to 8 days for $F(ab')_2$ fragments.

Literature Cited

Hellstrom, K.E. and Hellstrom, I. 1985. Monoclonal anti-melanoma antibodies and their possible use. *In* Monoclonal Antibodies for Cancer Detection and Therapy (R.W. Baldwin and V.S. Byers, eds.) pp. 17-51. Academic Press, London.

Mariani, M., Cauragra, M., Tarditi, L., and Seccariani, E. 1991. A new enzymatic method to obtain high-yield $F(ab')_2$ suitable for clinical use from mouse IgG_1. *Mol. Immunol.* 28:69-77.

Overall, C.M. 1987. A microtechnique for dialysis of small volume solutions with quantitative recoveries. *Anal. Biochem.* 165:208.

Parham, P. 1983. On the fragmentation of monoclonal IgG_1, IgG_{2a}, and IgG_{2b} from BALB/c mice. *J. Immunol.* 131:2895-2902.

Parham, P. 1986. Preparation and purification of active fragments from mouse monoclonal antibodies. *In* Handbook of Experimental Immunology, Vol.1: Immunochemistry (D.M. Wier, ed.) pp. 14.1-14.23. Blackwell Scientific, Oxford.

Parham, P., Androlewicz, M.J., Brodsky, F.M., Holmes, N.J., and Ways, J.P. 1982. Monoclonal antibodies: Purification, fragmentation, and application to structural and functional studies of class I MHC antigens. *J. Immunol. Methods* 53:133-147.

Key Reference

Parham, 1983. See above.

A lucid and comprehensive guide to the production of fragments from mouse monoclonal antibodies.

Contributed by Sarah M. Andrew
Chester College
Chester, United Kingdom

Julie A. Titus
National Cancer Institute
Bethesda, Maryland

Antibody Conjugates for Cell Biology

Antibody conjugates are extremely useful reagents for probing many biologically and chemically important molecules in vitro or in vivo. This unit presents some basic protocols for conjugating antibodies with fluorescent dyes, with biotin (see Basic Protocol 1), and with enzymes (see Basic Protocol 2). These protocols have been selected because they are relatively easy to perform and have high rates of success. The basic approach for conjugating antibodies is to derivatize amino groups on the antibody with the dye or biotin derivative of choice. Conjugation at amine sites is simple to perform, and, with a fluorescent dye, it generally yields brightly fluorescent conjugates. The conjugates retain high levels of biological activity, notwithstanding the fact that the labels react with random amines on the antibody molecule, and they may even react near or at the antigen-binding site. Labeling of the amines is the most widely used commercial method to tag antibodies. In this unit, suggestions are presented for variations on these basic protocols that are useful when quantities of available antibody are limited. Methods for estimating antibody concentration are also included (see Support Protocol).

Alternate protocols for attaching label at the carbohydrate site of the Fc portion or at the sulfhydryl groups of the hinge region, which join the two heavy chains of the antibody molecule (Fig. 16.5.1), are also possible (see Alternate Protocol 1). These alternate protocols, which entail more steps, are more complex and require more meticulous optimization. Furthermore, they are difficult to perform when <1 mg of antibody is available. Among these alternate protocols, a general procedure is described for conjugating antibodies (see Alternate Protocol 2) or enzyme (see Alternate Protocol 3) at the carbohydrate site. Although this alternate protocol is presented for antibody-enzyme conjugates only, the basic principles can easily be adapted for conjugation of antibodies with fluorophores or biotin, as detailed in the Background section. Background Information also includes references for labeling antibodies at the hinge region.

CONJUGATING ANTIBODIES TO FLUOROPHORES OR BIOTIN

BASIC PROTOCOL 1

The procedural steps for antibody-fluorophore conjugation and for antibody-biotin conjugation are quite similar. These steps consist of mixing the dye or the biotin-derived reagent at an appropriate molar ratio and pH with the antibody solution and, after incubation, purifying the conjugate from the reaction mixture by size-exclusion chromatography (*UNIT 5.5*) or dialysis (*APPENDIX 3C*). The antibody-dye or antibody-biotin conjugate can then be analyzed to determine the degree of labeling, i.e., the number of moles of dye or biotin per mole of antibody. See Critical Parameters for helpful hints.

Materials

Antibody to label
1.0 M sodium bicarbonate (see recipe)
Phosphate-buffered saline (PBS; see recipe)
Probe (fluorophore or biotin)
Anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO; see recipe)
1.5 M hydroxylamine, pH 8.0 (see recipe)
Size-exclusion chromatography matrix: MATREX Cellufine GH25 (Amicon/Millipore), BioGel P30 (BioRad Laboratories) or equivalent matrix
ToyoPearl HW-40F (TosoHaas)
Reaction buffer, pH 7.5 (see recipe)
Appropriate TLC solvent (see Table 16.5.1, optional)

continued

Contributed by Rosaria P. Haugland
Current Protocols in Cell Biology (2000) 16.5.1-16.5.22

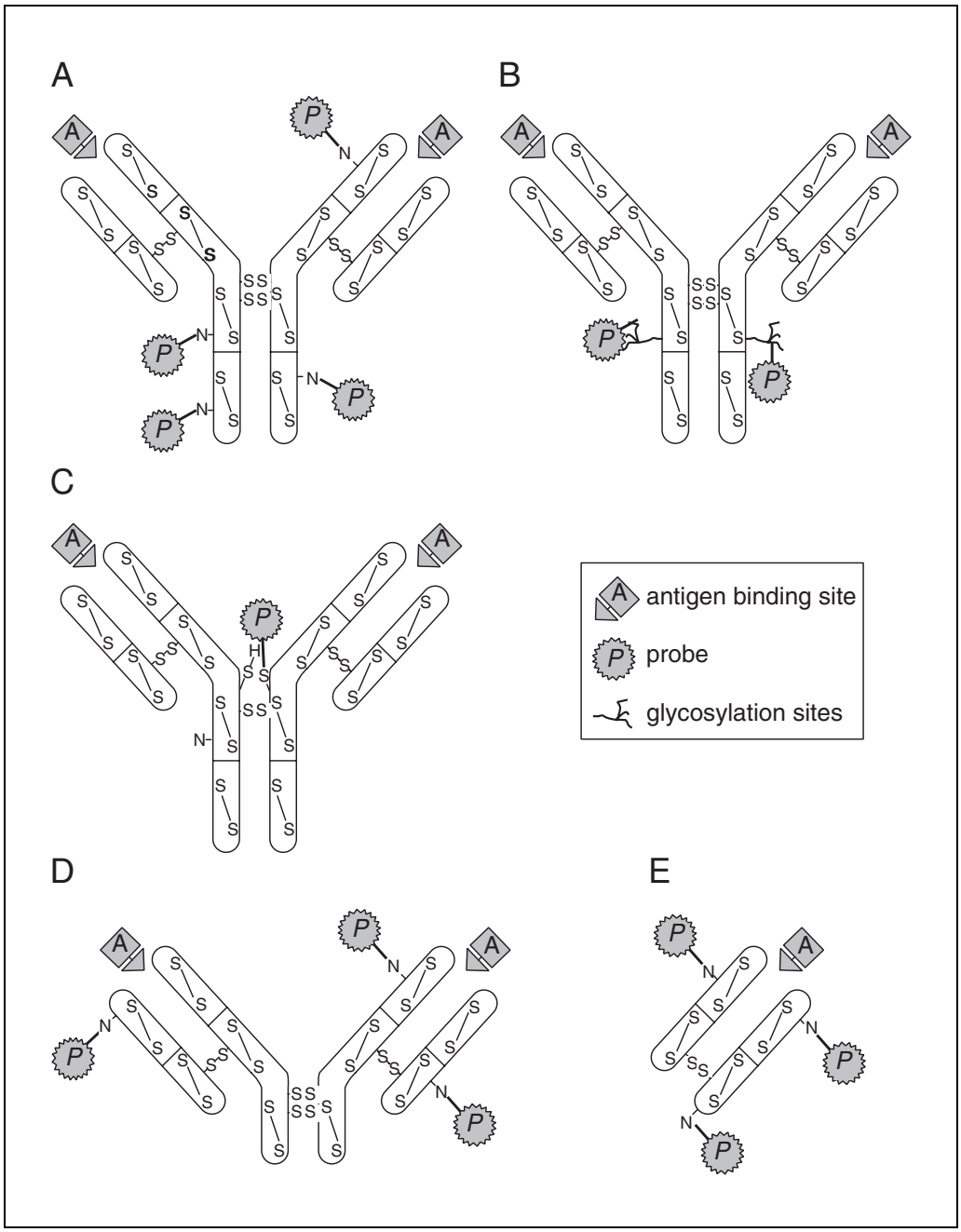


Figure 16.5.1 Schematic representation of possible modes of conjugation of antibodies or their fragments to probes. An antibody or Fab fragment is shown conjugated to one or more probe molecules. The probe symbol indicates a fluorophore, biotin, or an enzyme. (**A**) An antibody conjugated to a probe at several amine groups; (**B**) antibody conjugated at carbohydrate groups in the Fc regions; (**C**) antibody conjugated at thiol group in hinge region; (**D**) Fab fragment conjugated at amine groups; (**E**) $F(ab')_2$ fragment conjugated at amine groups. The molecular weight of intact IgG molecules (with all four subunits) is 140 to 160 kDa; where heavy chains are 50 to 75 kDa, and light chains are each ~25 kDa (A-C). Papain digestion of intact IgG yields Fab fragments of ~50 kDa, consisting of one light chain and a slightly larger fragment of the heavy chain, still attached by a disulfide bond (E). Cleavage of intact IgG antibodies with pepsin yields bivalent $F(ab')_2$ fragments of ~105 kDa (D); and subsequent reduction of the disulfides in the hinge region of $F(ab')_2$ yields two Fab' fragments (not shown), each ~53 kDa.

Table 16.5.1 TLC Solvents and Suggested Incubation Molar Ratios of Probe to Antibody for Several Commonly Used Fluorophores[a]

Fluorophore	MR (Ab at 1-3 mg/ml)	MR (Ab at 4-10 mg/ml)	MW (Da)	Probe (mg/ml)	TLC solvent
Alexa Fluor 350	15-25	10-15	410	10	H_2O:AcCN :: 20:80
Alexa Fluor 430	15-20	8-10	623	10	H_2O:AcCN :: 20:80
Alexa Fluor 488	20-25	15-20	643	10	H_2O:AcCN :: 20:80
Alexa Fluor 532	14-18	10-12	724	10	H_2O:AcCN :: 20:80
Alexa Fluor 546	12-15	8-10	1079	10	H_2O:AcCN :: 20:80
Alexa Fluor 568	25-30	10-20	792	10	H_2O:AcCN :: 20:80
Alexa Fluor 594	20-30	10-20	819	10	H_2O:AcCN :: 20:80
BODIPY FL	15-30	10-15	641	10	C:M:A :: 70:25:5
Cascade Blue	15-20	10-15	607	10	D:H_2O:P:NH_4 :: 50:16:19:15
Cy3 Bis SE	15-28	5-15	944	10	C:M:A :: 70:25:5
Cy5 Mono SE	10-20	5-10	792	10	C:M:A :: 70:25:5
Eosin	50-70	40-60	705	10	C:M:A :: 70:25:5
FITC	40-60	30-60	389	10	C:M:A :: 70:25:5
Fluorescein-EX	20-25	15-20	590	10	C:M:A :: 70:25:5
Marina Blue	8-12	6-10	367	10	C:M:A :: 70:25:5
Oregon Green 488	15-20	10-15	509	10	C:M:A :: 70:25:5
Oregon Green 514	15-20	10-15	609	10	C:M:A :: 70:25:5
Rhodamine Red-X	8-10	5-8	771	5	C:M :: 85:15
Rhodol Green	10-15	5-10	472	10	C:M:A :: 70:25:5
Tetramethyl-rhodamine (TMR)	15-20	8-10	528	10	C:M:A :: 70:25:5
Texas Red-X	5-8	5-8	817	5	C:M:A :: 70:25:5
Biotin					
Biotin-XX	20-25	10-20	568	10	

[a]MR = Incubation molar ratio of fluorophore to antibody, when antibody is at 1 to 3 mg/ml (column 2) or at 4-10 mg/ml (column 3); SE = succinimidyl ester. Solvents: C = chloroform; M = methanol; A = acetic acid; D = dioxane; P = isopropyl alcohol; NH_4 = ammonia; AcCN = acetonitrile.

10 mM 4′-hydroxyazobenzene-2-carboxylic acid (HABA) in 10 mM NaOH (for biotinylation)
Assay buffer: 50 mM sodium phosphate/150 mM NaCl, pH 6.0 (for biotinylation)
0.5 mg/ml avidin in assay buffer (for biotinylation)
0.25 mM biotin in assay buffer (for biotinylation)

Thin layer chromatography (TLC) silica gel/aluminum plates (EM Science; optional)
TLC chamber (optional)
Dialysis tubing
Chromatography column of appropriate size (see step 7)
Ultrafrel centrifugal filter devices, Biomax-50K or-100K (Amicon/Millipore; optional)

Additional reagents and equipment for size exclusion chromatography (*UNIT 5.5*) and dialysis (*APPENDIX 3C*)

Prepare the antibody for conjugation

1. Dissolve the antibody, if lyophilized, at 5 to 10 mg/ml or as concentrated as possible, in 0.1 M sodium bicarbonate (obtained by 10-fold dilution of a 1 M stock). If conjugating with isothiocyanates or sulfonyl chlorides of fluorophores, adjust the pH

of the bicarbonate solution to 9.0. If the antibody to be conjugated is already in solution in 10 to 20 mM phosphate, 0.15 M NaCl (PBS) to obtain the appropriate pH by simply adding 1/10 of the volume of 1 M bicarbonate, with pH unmodified, or pH 9.0 as needed. If the antibody is in a buffer containing Tris, glycine or other amines, dialyze the antibody against PBS or 0.1 M bicarbonate to "exhaustion," because the probes will react with any available free amine.

There is no need to modify the pH when reacting with succinimidyl esters.

Prepare probe solution

2. Calculate the volume of 10 mg/ml solution of probe (fluorophore or biotin) required to react with the antibody to be labeled, as follows:

   ml of 10 mg/ml probe = {[(mg of Ab) / (10 mg/ml of probe)] / (MW of Ab)} × (MR × MW of probe)

   where Ab = antibody; MW = molecular weight; and MR = incubation molar ratio of probe to antibody.

   For example, for labeling 5 mg of antibody with a 1:10 incubation molar ratio of carboxytetramethylrhodamine succinimidyl ester (TAMRA, SE; MW 430.5):

   volume of TAMRA, SE at 10 mg/ml = [(5/10)/(145,000)] × (10 × 430.5) = 0.0148 ml.

3. Immediately prior to starting the reaction, prepare a 10 mg/ml solution of the probe by weighing an amount of probe appropriate for the precision of the balance, for example, 3 mg, and dissolve it in 0.3 ml of DMF or DMSO.

   Sulfonyl chlorides, such as Texas Red sulfonyl chloride and Lissamine rhodamine B sulfonyl chloride, should be dissolved in DMF, because they react with DMSO.

4. Briefly, vortex or sonicate the reactive probe until it dissolves completely.

   In general, reactive probes are very sensitive to hydrolysis; thus, solutions of any reactive probe should not be stored. However, in some cases, the loss of reactivity during storage at ~20°C may be within an acceptable range.

Label antibody

5. While stirring, slowly add the probe solution to the antibody solution, in the amounts determined in step 2. Mix thoroughly.

6. Incubate the reaction mixture 60 to 90 min at room temperature with gentle stirring.

7. (*Optional*) Stop the reaction by adding 1/10 vol of 1.5 M hydroxylamine hydrochloride that has been adjusted to pH 8.0 with NaOH and incubating an additional 20 to 30 min.

   Treatment with hydroxylamine is potentially useful for removing the dye or biotin from unstable conjugates with hydroxyl-containing amino acids (Wong, 1991).

Purify conjugated antibody

8. Purify the antibody from unreacted dye or biotin by size-exclusion chromatography (*UNIT 5.5*) using PBS or reaction buffer.

   The size of the column should be selected based on the amount and concentration of the antibody. A 10 × 400–mm column is adequate for 5 to 10 mg of antibody at 5 to 10 mg/ml, while a 5 × 200– to 250–mm column can be used for 1 to 5 mg of antibody at ≥2 or more mg/ml. For dyes that are relatively hydrophobic, such as rhodamines or Texas Red, it is very useful to layer a small amount of ToyoPearl (a 2- to 3-cm layer) over the matrix used in the column, to help retain the unreacted dye.

If the antibody concentration is <2 mg/ml, purify the conjugate from excess probe by dialysis (APPENDIX 3C) to "exhaustion," i.e., until no free probe is detectable by TLC analysis (see step 9). Although it is more time consuming, dialysis is a very easy method to purify small, diluted amounts of antibody conjugates, avoiding the further dilution inevitable with size-exclusion chromatography performed with free-standing columns.

Alternatively, the solution of the antibody conjugate can be concentrated using a centrifugal spin filter device, with a mwco of 30 to 50 kDa. For amounts of antibody <1 mg, at the concentration of ≥1 mg/ml, separation from unreacted probe can be achieved by centrifuging the antibody, in volumes of ≤0.2 ml, on disposable spin columns, following the manufacturer's directions. Spin columns do not cause appreciable dilution of the sample, and they offer a rapid method to obtain small amounts of purified conjugates. To avoid denaturation, dilute solutions of antibody conjugates (<1 mg/ml) should be stabilized by adding bovine serum albumin (BSA) or gelatin to a final concentration of 1 mg/ml.

9. Identify the fractions from the fluorophore-antibody column chromatography.

For dye conjugates, the antibody conjugate is the first colored fraction to elute from the column, while the unreacted fluorophore is retarded. A clear band should be visible between the conjugate and the unreacted probe. Depending on the type of fluorophore and the type of size-exclusion matrix used, the unreacted dye could appear as a single band or multiple bands, because the succinimidyl ester might be only partially hydrolyzed and/or the dye might consist of isomers. Neither the band of unreacted biotin on the top of the column nor the biotin-antibody conjugate in the eluate from a spin column can be seen directly. Consequently, the biotin conjugate eluting from the column must be identified by measuring the A_{280}.

Analyze conjugates for purity

10. *For fluorescent antibodies only:* Analyze the purity of the eluted antibody conjugate by TLC. Spot 1 to 2 µl of sample on a small strip (~1.5 × ~10 cm) of a silica gel thin layer chromatography sheet and run it in a small, saturated chamber in the appropriate solvent (see Table 16.5.1), in parallel with the dye as a control. Remove the strip when the solvent front reaches ~3/4 of the strip height, and let it dry.

The antibody-conjugate will remain at the origin, while the contaminating unreacted dye will migrate towards the solvent front. The spots can be seen when excited by the long wavelength light of a hand-held UV lamp. If unreacted dye is present, the conjugate can be further purified by dialysis or by spinning it on a new spin column, according to the conjugate volume. This method is not applicable to antibody-biotin conjugates.

Determine the degree of antibody labeling by the probe

For antibody-fluorophore conjugates

11a. Measure the absorbance of the conjugate at the wavelength at which the dye absorbance is maximal (A_{Dye}) and at 280 nm (A_{280}). Then calculate the dye-to-antibody ratio using the following equation:

$$(A_{Dye}/\varepsilon^{M}_{Dye}) \div [(A_{280} - A_{Dye} \times CF)/\varepsilon^{M}A_{280}] = (\text{mol of dye/mol of antibody})$$

where A_{Dye} = dye absorbance at peak wavelength; ε^{M}_{A280} = 203,000 $cm^{-1}M^{-1}$, the approximate molar extinction coefficient of the antibody (IgG) at 280 nm; ε^{M}_{Dye} = molar extinction coefficient of the dye at the same wavelength as A_{Dye}. CF is a correction factor equal to A_{280}(for dye)/A_{Dye}.

Note that, while this formulation applies to fluorophore conjugates with intact IgG molecules, it can approximately apply as well to conjugates with antibody fragments, Fab, Fab′, and F(ab′)$_2$ (see Fig. 16.5.1), when used with the ε^{M}_{A280} appropriate for the antibody fragment. In this case, the ε^{M}_{A280} of the antibody fragment can be calculated from the measured absorbance of antibody solution at 1.0 mg/ml.

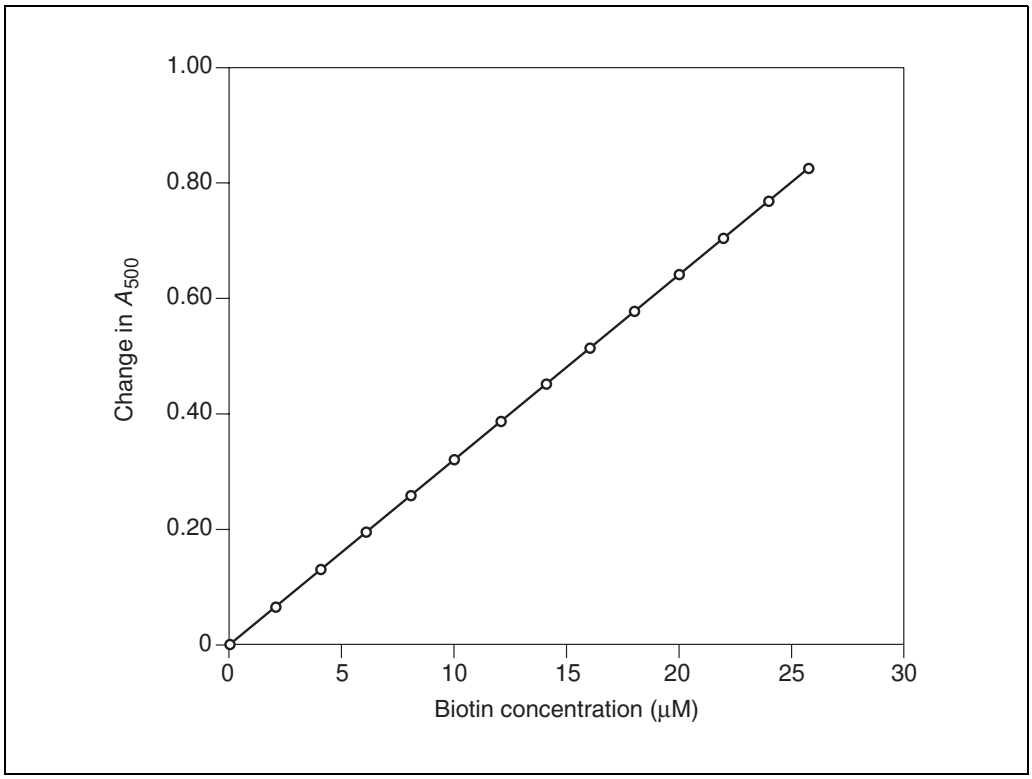


Figure 16.5.2 Standard curve of biotin concentration versus ΔA_{500}. The standard curve for biotin assay with avidin-HABA reagent relates measured difference in absorption at 500 nm to biotin concentration.

For antibody-biotin conjugates

The normal degree of biotinylation of antibodies can be determined by taking advantage of the high affinity of biotin for avidin. The dye, HABA, interacts with avidin, yielding a complex with an absorption maximum at 500 nm. Because of its higher affinity, biotin will displace HABA, decreasing the absorbance at 500 nm (A_{500}) by an amount proportional to the amount of biotin present in the assay. This relation can be quantified in a standard curve.

11b. Prepare the standard curve by adding 0.25 ml of HABA to 10 ml of the 0.5 mg/ml avidin solution. Incubate 10 min at room temperature.

12b. Distribute 0.9 ml of the avidin/HABA solution into each of seven tubes. Add 0.1 ml buffer, pH 6.0, to one tube (reference) and 0.005 to 0.1 ml biotin solution to the other six tubes. Bring the final volume to 1.0 ml (when necessary) with assay buffer, mix well and incubate 10 min at room temperature.

13b. Measure the A_{500} for each concentration point, using assay buffer as a blank, and subtract it from the value obtained from the reference absorption.

14b. Plot a standard curve of the nanomoles of biotin versus the decrease in A_{500}.

An example of such a standard curve is shown in Figure 16.5.2.

15b. To measure the degree of biotinylation of the sample, add an aliquot of biotinylated antibody of known concentration to 0.9 ml avidin-HABA complex. For example, add 0.05 to 0.1 ml of biotinylated antibody at 1 mg/ml to 0.9 ml of avidin-HABA mixture. Bring the volume to 1.0 ml with assay buffer (when necessary), incubate for 10 min, and measure the decrease in A_{500}.

16b. Using the standard curve, determine the nanomoles of biotin corresponding to the observed change in A_{500}.

The ratio between nanomoles of biotin used to displace HABA and nanomoles of antibody represents the degree of biotinylation. For example, the calculation for an IgG conjugate is:

(15 nmol biotin × 145,000 g/mol) ÷ (4.35 mg/ml antibody × 0.1 ml) = 5 mol of biotin per mol of antibody

where 145,000 is the MW of the IgG antibody, and 0.1 ml is the volume of 1 mg/ml of biotinylated antibody sample.

CONJUGATION OF FLUOROPHORES OR BIOTIN WITH F(ab′)$_2$ OR Fab ANTIBODY FRAGMENTS

ALTERNATE PROTOCOL 1

As described in *UNIT 16.4* of this series, antibody fragments that retain antibody binding activity can be generated by selective enzymatic digestion of intact antibodies. Conjugates made with antibody fragments may offer enhanced signals because their smaller size can permit penetration and reaction with the more recessed antigen sites or more extensive binding to closely located binding sites (see Critical Parameters for helpful notes).

Alternative conjugation procedure for fluorophores or biotin. In cases where conjugation with fluorophores or biotin at lysines appears to interfere with antibody binding, labeling can be performed with biotin hydrazides or fluorophore hydrazides at the carbohydrate prosthetic group, located in the Fc portion of the molecule (see Fig. 16.5.1), as described in the protocol for conjugation of antibodies with enzymes (see Basic Protocol 2). The hydrazide of the chosen fluorophore or biotin should be used in place of the enzyme, and purification of the conjugate performed as described (see Basic Protocol 2). As for the succinimidyl ester forms, biotin hydrazides are available with one (X) or two (XX) aminohexanoic acid spacers.

METHODS TO ESTIMATE ANTIBODY CONCENTRATION

SUPPORT PROTOCOL

The antibody concentration can be estimated by three different methods:

Correct the A_{280} of the conjugate for the A_{280} due to the dye, using the correction factor of the specific dye given in Table 16.5.2 and the value, $\varepsilon^{M}_{A280} = 203{,}000\ cm^{-1}\ M^{-1}$, for the IgG antibody. For example, conjugation of a goat anti-guinea pig IgG antibody with Alexa Fluor 488 succinimidyl ester yielded the following data, after 1:10 dilution: $A_{488} = 0.533$; $A_{280} = 0.314$; $\varepsilon^{M}_{Dye} = 71{,}000\ cm^{-1}M^{-1}$ (Table 16.5.2). In this case, the degree of labeling is

$$0.533/71{,}000\ cm^{-1}M^{-1} \div [(0.314 - 0.533 \times 0.11)/203{,}000\ cm^{-1}M^{-1}] =$$
$$5.96 \text{ moles of dye per mole of antibody}$$

where 0.11 is the CF for the Alexa Fluor 488 dye (from Table 16.5.2).

Estimate the protein concentration from the initial amount, assuming a loss of 15% to 20%, or more if some precipitation occurs during purification.

Use a protein assay method (*APPENDIX 3B*) to measure the concentration of the conjugate. This can be problematic, however, because most protein assays suffer from interference by fluorophores.

Table 16.5.2 Optical Properties of Commonly Used Fluorophores for Antibody Labeling

Dye	Abs/Em[a]	ε^{Mb}	CF[c]	Notes
Alexa Fluor 350	347 nm/442 nm	19,000	0.19	Blue-fluorescent labeling dye with higher fluorescence per conjugated dye than AMCA.
AMCA-X	350 nm/448 nm	18,000	0.15	Widely used blue-fluorescent labeling dye. Compact structure
Marina Blue	365 nm/460 nm	20,000	0.20	Blue-fluorescent dye. Compact structure. pH-insensitive at pH >6.
Cascade Blue	400 nm 420 nm	28,000	0.65	Resistant to quenching upon protein conjugation. Exhibits better spectral separation from FITC than does AMCA. Water soluble.
Cascade Yellow	402 nm/545 nm	20,000	0.61	Large Stokes shift. Useful for multicolor analysis in combination with blue-fluorescent dyes.
Pacific Blue	405 nm/455 nm	30,000	0.20	Bright blue fluorescence emission. Longer-wavelength alternative to AMCA and Marina Blue dye.
Lucifer yellow	428 nm/536 nm	11,900	0.30	Water soluble. Available as Lucifer yellow iodoacetamide.
Alexa Fluor 430	431 nm/541 nm	16,000	0.28	Large Stokes shift. One of relatively few dyes that absorb between 400 and 450 nm and have appreciable fluorescence beyond 500 nm.
Fluorescein	494 nm/518 nm	68,000	0.30	Widely used green-fluorescent labeling dye (FITC). Absorption overlaps the 488- nm spectral line of the argon-ion laser. Prone to photobleaching. pH-sensitive from pH 5 to 8.
Alexa Fluor 488	495 nm/519 nm	71,000	0.11	Best fluorescein substitute for immunofluorescence and other labeling applications. Exceptionally photostable and pH-insensitive from pH 4 to 10. Produces brighter conjugates than FITC. Ideal for 488-nm excitation.
Oregon Green 488	496 nm/524 nm	70,000	0.12	Fluorescein substitute. pH-insensitive at pH >6.
BODIPY FL	505 nm/513 nm	68,000	0.04	Insensitive to solvent polarity and pH from pH 4 to 8. Narrow spectral bandwidth. Neutral dye. Succinimidyl ester with a cysteic acid spacer is the preferred reactive form for protein conjugation.
Oregon Green 514	511 nm/530 nm	70,000	0.19	Very photostable. pH-insensitive at pH >6. Absorption matched for 514-nm excitation.

continued

BASIC PROTOCOL 2

CONJUGATION OF ANTIBODIES WITH ENZYMES

Conjugation of antibodies with enzymes involves crosslinking of the two proteins in successive procedural steps. The method involves (1) derivatization of the antibody with a bifunctional reagent that introduces reactive maleimide groups into the molecule and (2) derivatization of the enzyme with a thiol group that can form a stable thioether bond with the maleimide group on the antibody. This approach can be tailored to obtain either heteroconjugates consisting of one molecule of antibody and one molecule of enzyme or conjugates consisting of one antibody labeled with multiple enzyme molecules. The most commonly used enzymes are horseradish peroxidase (HRPO), alkaline phosphatase (APase), β-galactosidase (βGase), and glucose oxidase (GO).

Table 16.5.2 Optical Properties of Commonly Used Fluorophores for Antibody Labeling, continued

Dye	Abs/Em[a]	ε^{M}[b]	CF[c]	Notes
Eosin	524 nm 544 nm	90,000	0.25	Useful photooxidizer of diaminobenzidine (DAB). Phosphorescent (lifetime ~1 msec.) Useful for phosphorescence aniosotropy measurements. Efficient FRET[d] acceptor from fluorescein, BODIPY FL dye, dansyl and coumarin.
Alexa Fluor 532	531 nm/554 nm	81,000	0.09	Bright and photostable dye with spectra intermediate between those of fluorescein and tetramethylrhodamine. Water soluble. Excellent protein-labeling dye.
Tetramethyl-rhodamine (TMR)	555 nm/580 nm	65,000	0.30	Widely used orange-fluorescent labeling dye (TRITC). pH-insensitive. Very photostable. May form nonfluorescent aggregates when attached to proteins.
Cy3	553 nm/570 nm	150,000	0.08	Orange fluorescing, bright and water soluble cyanine dye.
Alexa Fluor 546	556 nm/573 nm	104,000	0.12	Very bright and photostable tetramethylrhodamine (TAMRA or TRITC) and Cy3 dye substitute. Ideal for 546-nm excitation. Water soluble.
Rhodamine Red-X	570 nm/590 nm	120,000	0.17	Rhodamine Red-X succinimidyl ester generally yields higher fluorescence per attached dye than Lissamine rhodamine B sulfonyl chloride and is more stable in water.
Alexa Fluor 568	578 nm/603 nm	91,300	0.46	Bright and photostable Lissamine rhodamine B substitute. Ideal for 568-nm laser excitation. Excellent protein-labeling dye. Water soluble.
Alexa Fluor 594	590 nm/617 nm	73,000	0.56	Bright and photostable Texas Red dye substitute. Ideal for 594-nm laser excitation. Excellent protein-labeling dye. Water soluble.
Texas Red	595 nm/615 nm	80,000	0.20	Good spectral separation from fluorescein. Texas Red-X succinimidyl ester typically yields higher fluorescence per attached dye than Texas Red sulfonyl chloride and is more stable in water.
Cy5	650 nm/667 nm	250,000	0.05	Excitable by 632-nm line of the HeNe laser.
LaserPro	807 nm/812 nm	229,000	0.12	Relatively low quantum yield. Emission is not visible to the eye.

[a]Abs/Em are the absorbance and emission maxima of the dye.
[b]ε^{M} is the molar extinction coefficient of the dye in $cm^{-1}M^{-1}$.
[c]CF is the percentage correction factor determined by the following formula: CF = A_{280} for dye/A_{max} for dye.
[d]FRET, fluorescence resonance energy transfer.

It should be emphasized that βGase contains a large number of free thiols (8 to 12 or more, according to the different preparations of the enzyme) and need not be modified; rather, it can be reacted directly in its native form with the maleimide derivatives of antibodies.

Some protein-to-protein crosslinking kits, adaptable to the conjugation of antibodies with enzymes, are commercially available (Molecular Probes, Pierce, Prozyme).

Materials

HRPO, APase, GO, βGase (Boehringer Mannheim, Sigma)
Reaction buffer, pH 7.5 (see recipe)
TEA buffer (see recipe; for APase only)
Succinimidyl 3-(2-pyridyldithio)propionate (SPDP, MW 312)
Anhydrous dimethylsulfoxide (DMSO) or dimethylformamide (DMF)
BIO-GEL P-30 (Bio-Rad) or Cellufine GH-25 (Amicon/Millipore)
Dithiothreitol (DTT)
Antibody to label
Succinimidyl *trans*-4-(*N*-maleimidylmethyl)cyclohexane-1-carboxylate (SMCC, MW 334)
Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP, MW 287; Molecular Probes, Pierce Chemical)
50 mM *N*-ethylmaleimide (MW 125.13)
Sephacryl S-200 for HRPO conjugates (Amersham Pharmacia Biotech or Sigma Chemical)
BIO-GEL A-0.5m for APase conjugates (Bio-Rad Laboratories)
BIO-GEL A-1.5m for BGase conjugates (Bio-Rad Laboratories)

Chromatography column of appropriate size (see step 4)
Ultrafrel centrifugal filter devices, Biomax-50K or-100K (Amicon/Millipore)

Additional reagents and equipment for dialysis (*APPENDIX 3C*) and size exclusion chromatography (*UNIT 5.5*)

Prepare the thiolated enzyme

1. Dissolve or dialyze HRPO (or GO) in reaction buffer at a concentration of 10 mg/ml. Dialyze APase in TEA buffer.

2. Weigh 3 mg of SPDP, and dissolve in 0.3 ml DMF to obtain a 10 mg/ml solution. Prepare this solution fresh immediately before using.

3a. *HRPO:* To 10 mg of HRPO (0.25 µmol) in 1 ml reaction buffer, add, while stirring, 40 µl of SPDP solution, to obtain a molar ratio of reagent to HRPO of 5. Incubate 1 hr at room temperature.

3b. *APase:* To 10 mg of APase (0.07 µmol) in 1 ml of pH 7.6 TEA buffer, add 10 µl of the SPDP solution and incubate 1 hr at room temperature.

3c. *GO:* To 10 mg of GO (0.063 µmol) in 1 ml reaction buffer, add 10 µl of the SPDP solution and incubate 1 hr at room temperature.

APase and GO have many more amines available for thiolation compared to HRPO, which has only 4 to 6 amines (Welinder, 1979). Thus the molar ratio of reagent to enzyme is ~5, notwithstanding the disparity in molecular weight between the two proteins.

4. Purify the thiolated enzyme by molecular exclusion column chromatography, using BIO-GEL P-30 or Cellufine GH-25, in reaction buffer for HRPO or TEA buffer, pH 7.6, for APase.

A column bed size of ~10 × 400–mm is required for 10 to 20 mg enzyme.

Determine the degree of thiolation

5. Prepare a 100 mM solution of DTT by dissolving 7.7 mg of DTT in 0.5 ml of distilled water.

6. Transfer 1 ml of 0.5 to 1 mg/ml enzyme solution to a spectrophotometer cuvet. Record the A_{280} and A_{343} of the solution.

7. Add 50 µl of DTT solution. Mix well, incubate 3 to 5 min, and record the A_{280} and A_{343} again.

8. Using an extinction coefficient of 8080 $cm^{-1}M^{-1}$ at 343 nm (Carlsson et al., 1978), calculate the amount of pyridine-2-thione liberated during the reduction.

   The amount of this product is equivalent to the number of thiols introduced in the protein.

9. To determine the enzyme concentration, correct the A_{280} of the enzyme solution for the absorbance contributed by 2-pyridyldithio propionate at this wavelength, according to the following equation:

$$A_{280} \text{ of enzyme} = A_{280} \text{ of conjugate} - (\Delta A_{343} \times 5100 \text{ cm}^{-1}\text{M}^{-1}/8080 \text{ cm}^{-1}\text{M}^{-1})$$

   where $\Delta A_{343} \times 5100 \text{ cm}^{-1}\text{M}^{-1}/8080 \text{ cm}^{-1}\text{M}^{-1}$ reflects the amount of pyridine-2-thione generated during the reaction.

   For HRPO, the enzyme concentration can be determined at 403 nm using the extinction coefficient shown in Table 16.5.2, with no correction. The number of thiols per mole of enzyme can be calculated using the following equation:

   moles of -SH groups per mole of enzyme $= (\Delta A_{343}/8080\ cm^{-1}M^{-1}) \div \{[A_{280} - (\Delta A_{343} \times 5100\ cm^{-1}M^{-1}/8080\ cm^{-1}M^{-1})]/\varepsilon^{M}_{enzyme}$

   or, for HRPO:

   $(\Delta A_{343}/8080\ cm^{-1}M^{-1}) \div (A_{403}/\varepsilon^{M}_{enzyme})$.

10. From the preceding equations, determine the average number of moles of antibody that can be reacted with each mole of enzyme.

    To obtain a 1:1 enzyme-antibody conjugate, the enzyme should be derivatized with 1.2 to 1.5 pyridyldithiol residues per mole. Thiolated enzymes remain stable for a few weeks when stored refrigerated in presence of 2 mM azide or, for HRPO, 0.01% (w/v) thimerosal, because azide inhibits the activity of this enzyme.

Prepare the antibody-maleimide derivative

11. Dissolve or dialyze the antibody in reaction buffer.

    The antibody should be as concentrated as possible to increase both the yield and the efficiency of the conjugation reaction. Ideally, the concentration should be 5 to 10 mg/ml; however, modifications of the labeling procedures can accommodate lower concentrations, especially when labeling small amounts of antibody (0.1 to 1.0 mg). Thus, the antibody concentration may be 2 to 10 mg/ml.

12. Weigh 3 to 5 mg of SMCC and immediately before use dissolve it in DMSO or DMF to obtain a 10 mg/ml solution. Vortex or sonicate to assure complete dissolution.

13. While stirring, add 15 µl of SMCC solution to each 5 mg of antibody to obtain a molar ratio of reagent to antibody of ~15.

    The amount of SMCC should be tailored to the concentration of the antibody. For example: 5 mg of antibody at the concentration of 2 mg/ml would require 15 to 17 µl of the above SMCC solution, while antibody at 10 mg/ml would yield a better conjugate when using a molar ratio of SMCC of 10.

14. Incubate 1 hr at room temperature with gentle stirring.

15. Dialyze 24 hr in the cold with four changes of 1 liter or more reaction buffer.

Deprotect the enzyme thiol groups

NOTE: It is essential that this procedure be carried out simultaneously to the labeling of the antibody with maleimide groups, because both the deprotected thiols of the enzyme and the maleimide groups of the antibody are unstable in water and are best used within a day from their preparation.

Deprotect with DTT

16a. Add 8.0 mg of DTT to each milliliter of the enzyme solution. Incubate 15 to 20 min at room temperature.

17a. Dialyze the thiolated enzyme 24 hr in the cold against 2 liters reaction buffer, with at least four changes of buffer to eliminate as much of the DTT as possible.

Deprotect with TCEP

16b. Calculate the volume of 1 mg/ml TCEP stock solution needed to obtain an incubation molar ratio of 5 moles of TCEP per mole of enzyme

$$\mu l \text{ of TCEP} = (\text{mg of enzyme})/(1 \text{ mg/ml TCEP}) \times 286.7/(\text{MW of enzyme}) \times 5 \times 1000$$

where 286.7 is the MW of TCEP, 5 is the incubation molar ratio, and 1000 is a unit conversion factor. Weigh out 3 to 5 mg of TCEP powder, and dissolve it in 3 to 5 ml reaction buffer to make a 1 mg/ml solution of TCEP.

Alternatively, TCEP can be used in place of DTT to deprotect sulfhydryl groups (Getz et al., 1999). TCEP does not react with maleimides. Thus, one need not eliminate excess TCEP from the reaction mixture, avoiding the need to dialyze to exhaustion. TCEP is very reactive and should be used at an incubation molar ratio of 5 TCEP per mole of enzyme.

17b. Add the appropriate amount of TCEP stock solution to the thiolated enzyme solution, and mix well. Incubate the mixture 10 to 15 min at room temperature.

Synthesize the antibody-enzyme conjugate

18a. *For HRPO, APase, and GO:* Transfer the antibody and the HRPO or APase from the dialysis tubes into two separate test tubes. Slowly drip the antibody solution into the enzyme solution with stirring. Incubate 1 hr at room temperature and overnight at 4°C.

18b. *For βGase:* For each 5 mg of antibody dissolve 15 mg of enzyme in 1.5 ml reaction buffer and proceed as in step 18a.

19. Stop the reaction for HRPO, GO, and APase conjugates with the addition of *N*-ethylmaleimide (NEM) at a final concentration of 50 μM, by diluting the NEM solution 1:1000 in the conjugate reaction mixture. Incubate 30 min at room temperature.

The conjugate is now ready to be purified by column chromatography, immediately or after overnight storage at 4°C.

The reaction of βGase cannot be stopped because of the large number of –SH groups of this enzyme.

Purify the antibody-enzyme conjugate

20. Concentrate the antibody-enzyme conjugate in a centrifugal filter device at the speed recommended by the manufacturer.

21. Pack appropriate size columns (e.g., 1 × 60–cm for 10 mg of protein in the conjugate reaction mixture) with the appropriate matrix.

    For purification of HRPO-antibody conjugate use Sephacryl S-200 in reaction buffer. For GO and APase-antibody conjugates use Bio-Gel A-0.5m in 3 M NaCl/30 mM TEA/1 mM $MgCl_2$, pH 7.6; for BGase Bio-Gel A-1.5m in reaction buffer, with the addition of 3 mM $MgCl_2$ and 2 mM 2-mercaptoethanol.

22. Load the conjugate onto the column, elute with the buffer used to pack the column, collecting 1-ml fractions.

    The first peak to elute, measurable by absorbance at 280 nm, or 403 nm for HRPO conjugates, corresponds to the conjugate. However, each fraction should be checked for antibody binding and enzymatic activity in an ELISA-type (APPENDIX 3) or other appropriate assay.

    An alternative method to purify antibody-enzyme conjugates from the unreacted enzyme relies on the affinity of the Fc portion of the antibody for nickel chelates. An affinity column based on this principle is available from Pierce Chemical. The Fc region complexes with nickel on the column while the unconjugated enzyme passes through. The bound antibody-enzyme conjugate can be eluted by lowering the pH, by increasing the salt concentration, or by simply adding EDTA to the elution buffer. A detailed procedure for using this affinity column accompanies the product. This column cannot be used for $F(ab')_2$ or Fab fragments, as they are devoid of the Fc region (Hermanson, 1996a).

CONJUGATION AT THE CARBOHYDRATE SITE OF THE ANTIBODY

ALTERNATE PROTOCOL 2

The sugar constituents of glycoproteins contain vicinal hydroxyl groups that can be oxidized to dialdehydes by treatment with periodic acid or its salts. The dialdehydes formed easily react with amines, including the hydrazide form of fluorophores, the ε-amino group of lysines, and the amino terminus of proteins. The carbohydrate-containing portion of an antibody molecule is located in the Fc region; thus, the periodate oxidation method of conjugating antibodies allows labeling at a specific location, remote from the antigen binding site.

CAUTION: Some monoclonal antibodies are not glycosylated and cannot, therefore, be conjugated by this procedure.

Materials

Antibody to label in solution (see Basic Protocol 1, step 1)
0.1 M acetate buffer, pH 6.0
20 mM sodium metaperiodate in acetate buffer, pH 6.0, ice cold
Reaction buffer, pH 7.5 (see recipe)
100 mM aqueous sodium cyanoborohydride, freshly prepared
HRPO, APase, βGase, GO
Column chromatography matrices (see Basic Protocol 2)

NOTE: Protect the sample from light during the entire procedure.

1. Dialyze the antibody at 2 to 10 mg/ml in 0.1 M acetate buffer, pH 6.0, at 4°C.
2. Keeping the dialyzed antibody on ice, add an equal volume of ice-cold metaperiodate solution. Incubate 2 hr in the dark at 4°C.
3. Purify the oxidized antibody: If the antibody is dilute (<1 mg/ml), dialyze it against the same buffer, protecting it from light. If the antibody is concentrated, purify it on a desalting column (Bio-Gel P-30 or equivalent) in free-standing or spin-column form (Basic Protocol 1, step 8) to remove the iodate that is produced during the oxidation.

4. Prepare a solution of the enzyme of choice in reaction buffer in the amount needed to obtain an incubation molar ratio of enzyme to antibody of 2 to 10, according to the type of conjugate desired.

   For instance, if a 1:1 ratio of probe to antibody is desired, use an incubation molar ratio of 2; otherwise, the higher the desired enzyme-antibody ratio, the higher will be the necessary incubation molar ratio.

5. Slowly add the antibody to the enzyme solution with stirring and incubate the mixture for 2 hr at room temperature with gentle stirring.

6. (*Optional*) To stabilize the imine bond into a substituted hydrazide, add sodium cyanoborohydride to a final concentration of 10 mM, adding 1/10 vol of a 100 mM stock solution of sodium cyanoborohydride in distilled water. Incubate for 2 hr at 4°C.

   Some researchers consider the imine bond between the sugars on the antibody and the amines on the enzymes to be relatively unstable.

7. Purify the conjugate by any of the chromatography methods (see Basic Protocol 2, step 21).

ALTERNATE PROTOCOL 3

CONJUGATION AT THE CARBOHYDRATE SITE OF THE ENZYME

Since HRPO and GO are glycoproteins, Alternate Protocol 2 can be modified by oxidizing the sugar on the enzyme and, following removal of excess reagent, adding it to the native antibody solution. The steps, molar ratios, and times suggested in Alternate Protocol 2 can be applied to this "reversed" version of the procedure. GO is a flavoenzyme consisting of two 80-kDa subunits that are rich in carbohydrates and joined by a disulphide bond (O'Malley and Weaver, 1972). The conjugation method of choice for GO involves oxidizing the enzyme with periodate at its carbohydrate site. The antibody can then be conjugated to GO through one or more of its amino groups (Rodwell et al., 1986; Husain and Bieniarz, 1994; Hage et al., 1997).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Hydroxylamine, I.5 M, pH 8.0

4.2 g hydroxylamine, hydrochloride
25 ml H_2O
Adjust the pH to 8.0 with 5 M NaOH
Add H_2O to 40 ml
Prepare fresh

Phosphate-buffered saline (PBS)

1.1 g K_2HPO_4
0.43 g KH_2PO_4
9 g NaCl
900 ml H_2O
Adjust the pH to 7.2 or 7.4, if necessary, with 1 M sodium bicarbonate (see recipe)
Add H_2O to 1 liter
Store 1 week at 4°C

The final concentrations are 0.01 M potassium phosphate, 0.15 M NaCl, pH 7.2.

Reaction buffer

11.5 g Na_2HPO_4
2.6 g NaH_2PO_4
5.8 g NaCl
900 ml H_2O
Adjust the pH to 7.5 with 5 M NaOH
Add H_2O to 1 liter
Store 2 weeks at 4°C

For labeling IgM, adjust the pH to 7.2.

Sodium bicarbonate, 1.0 M

8.3 g $NaHCO_3$
100 ml H_2O
Store 2 weeks at 4°C

The resulting pH is generally 8.3 to 8.5

This bicarbonate solution is used to raise the pH of PBS.

TEA buffer

For each liter of buffer, weigh 175 g of NaCl and dissolve it in 750 ml H_2O. Add 4.08 ml of TEA (98%, $\rho = 1.12$ g/ml). Prepare a stock solution of 0.5 M $MgCl_2$ and 0.05 M $ZnCl_2$ by weighing 10.16 g of $MgCl_2$ and 0.68 g of $ZnCl_2$ and dissolving them in distilled water to obtain a final volume of 100 ml. Add 2.0 ml of this stock to the NaCl-TEA solution. Adjust the pH to 7.6 with 3 M HCl, and bring the volume to 1.0 liter with H_2O. Store at 4°C. For prolonged storage (>1 week), add azide to 2 mM final concentration.

This buffer is used for APase only.

Final concentrations are 3.0 M NaCl, 30 mM TEA, 1.0 mM $MgCl_2$, 1.0 mM $ZnCl_2$, pH 7.6.

COMMENTARY

Background Information

Antibodies

The use of antibodies as probes has become widespread in the biosciences. In immunocytochemical applications, the use of labeled secondary antibodies is limited by the number of targets to be detected by different antibodies. This is due to the fact that secondary antibodies, even when purified by immunoadsorption against related species, nonetheless exhibit significant residual cross-reactivity when more than two antibodies are used to stain multiple features of the same sample. Thus, to detect multiple structures or functions simultaneously, direct labeling of primary antibodies with fluorophores, biotin, or enzymes is essential. Biotinylation of antibodies allows detection with fluorescently labeled avidin (or analogous molecules), generally without interference from the secondary antibody or antibodies required in the experimental procedure. Investigators new to the field might refer for general information on the theory of fluorescence and applications of fluorescent probes to the introduction to the topic by Johnson (1996; see also *UNIT 4.2*) and for avidin-biotin technology to the review by Wilchek and Bayer (1990).

For procedures employing fluorescent labeling of primary antibodies, it is important to select probes with the highest quantum yield, because signals from these antibodies lack the amplification obtained with the secondary antibodies. Signal amplification can be achieved by conjugating antibodies with enzymes that use fluorogenic or colorimetric substrates. These substrates should be of the precipitating type for histological preparations or soluble for ELISA and other microplate assays.

Very useful antibody labeling kits, containing detailed instructions, premeasured reactive fluorophores or biotin are now available from a few commercial sources (Amersham Pharmacia Biotech, Molecular Probes, Pierce). These kits generally allow conjugations of 0.2 to 10 mg of an antibody. They are ideal for researchers who need to perform labeling procedures only occasionally or do not have the necessary equipment (e.g., separation columns and solvent for the la-

beling reagent) readily available. They also represent a good starting step for researchers who want to familiarize themselves with the labeling procedure before acquiring larger amounts of the materials and reagents needed for the conjugation.

Fluorophores

The most commonly used amine-labeling reagents belong to three classes of acylating compounds:

1. Isothiocyanates. The isothiocyanate derivatives of fluorophores are relatively stable in aqueous solution. They react with amines at pH 9 to 9.5 to yield thiourea bonds. In this class, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are the most widely used.

2. Sulfonyl halides. The most commonly used probes in this category are the sulfonyl chloride of Texas Red and that of Lissamine rhodamine B. This reactive form of fluorophores is extremely labile in water. Consequently, in conjugation with proteins the reaction rate is strongly counteracted by the hydrolysis rate of the dye, making reproducibility of the reaction difficult. Also sulfonyl chlorides require relatively basic conditions, pH 9.0 to 9.5, to react with amines.

3. Succinimidyl esters. Reagents of this form react more selectively with aliphatic amines than the isothiocyanates by forming stable carboxamide bonds at pH 7.5 to 8.5 (Haugland, 1996a). Most fluorophores, including fluorescein, rhodamine, Cy3, Cy5, and the new Alexa Fluor dyes, are available as succinimidyl esters (Haugland, 1999). These are highly preferred because of their greater selectivity for amino groups (Wong, 1991), relatively slow hydrolysis rate (Wong, 1991), and high reactivity at lower pH (Hermanson, 1996b), closer to physiological pH. Because of their still high reactivity at pH 7.2, succinimidyl esters can be used efficiently to label IgM antibodies, which tend to denature when the pH is above 7.4.

The author strongly recommends using fluorescein, tetramethylrhodamine, and Texas Red in their succinimidyl ester forms. In the past, fluorescein and tetramethylrhodamine were available only as isothiocyanates, broadly known in their abbreviated forms as FITC and TRITC, respectively. Texas Red was available only as its sulfonyl chloride, which has an extremely rapid rate of hydrolysis in buffer. The new, succinimidyl ester forms of these dyes are preferable, because they will generally yield more reproducible and stable antibody conjugates (Banks and Paquette, 1995; Lefevre et al., 1996).

In determining the degree of antibody labeling in fluorophore conjugates, one should note that the values, both of the extinction coefficients and of the correction factors described in Table 16.5.2, are only approximate. These values are not corrected for the change in extinction coefficients that may occur when the dyes are conjugated to proteins. This change in extinction coefficient can vary from protein to protein, and it can vary with the number of dyes reacted with the protein, due to interference between dye molecules (Ravdin and Axelrod, 1977). Thus, determination of the degree of labeling is relative and approximate. Nonetheless, it is invaluable when comparing different preparations of the same antibody conjugate and for determining the relative optimal number of moles of fluorophores per mole of antibody for particular biological applications.

Phycobiliproteins

This unit does not include protocols for conjugation of antibodies with phycobiliproteins—large, highly fluorescent pigments that are isolated from the photosynthetic apparatus of algae. The conjugation procedure of phycobiliprotein to antibodies is very similar to Basic Protocol 2. Molecular Probes offers a protocol for conjugation with phycobiliproteins, and details are published (Haugland, 1996b). A kit that greatly facilitates phycobiliprotein conjugation is available from Prozyme, although it does not lend itself to optimizing the procedure. The Protein-to-Protein Crosslinking Kit from Molecular Probes could be used for this purpose. However, obtaining the phycobiliprotein of interest, R-phycoerytin (R-PE) or allophycocyanin (APC), already derivatized with 1 to 2 pyridyldisulfide groups per molecule is strongly recommend. This is more economical than preparing and optimizing the activated protein.

Other fluorophores

Fluorophores with enhanced water solubility and fluorescence output have been developed by introducing sulfonic groups in the rings of their molecule. This procedure also confers negative charges that help to decrease interaction between fluorophores and to minimize the loss of probe fluorescence that generally occurs upon conjugation to biological molecules. The excitation wavelengths for these sulfonated fluorophores span the entire spectrum from excitation in the UV range to the 633-nm excitation of the HeNe laser. They include the Alexa Fluor dyes, prepared by sulfonating coumarins or rhodamines (Panchuk-Voloshina et al., 1999), Cy dyes, prepared by sulfonating carbocyanines (Mujumdar et al.,

1989, 1993, 1996), and Cascade Blue, which is a pyrenetrisulfonic acid derivative (Whitaker et al., 1991). Each of these dyes generally offers enhanced signal-to-noise ratios. Many of these dyes form conjugates that tolerate a higher number of moles of dyes per mole of antibody in comparison with the non-sulfonated ones, while avoiding quenching of the fluorescence or precipitation of the antibody (Panchuk-Voloshina et al., 1999). In rare cases the negative charges on these dyes may cause nonspecific interaction with positive charges of the cells under investigation. In these cases, which unfortunately can be determined only experimentally, the nonsulfonated form of the dye (e.g., TAMRA, instead of Alexa Fluor 546, or Texas Red-X, instead of Alexa Fluor 594) should be used. Fluorescein itself carries two negative charges, and in cases of background problems the neutral BODIPY FL can be tried as an alternative.

Biotin

Biotin is a small, ubiquitous molecule that acts as a coenzyme of carboxylases. Due to its strong affinity for avidin and avidin-like molecules (K_d = 1.3×10^{-15} M at pH 5.0), it is broadly used as a label that can be probed with fluorescent or enzyme-linked avidin or streptavidin. Avidin (MW = 66 kDa) and streptavidin (MW = 60 kDa), both tetrameric proteins with ~33% homology in their amino acids composition, can each bind four molecules of biotin (Green, 1975).

Iminobiotin, a modified biotin with lower affinity for avidin, is occasionally used in place of biotin to prepare conjugates that can be separated by affinity chromatography. The succinimidyl ester of iminobiotin can be used to effect labeling of a tag only transiently because the iminobiotin-avidin complex dissociates at low pH (Hoffman et al., 1980; Orr, 1981). Affinity purification of biotinylated conjugates is also possible with monomeric avidin, which has a lower affinity for biotin than the tetrameric form and is now commercially available (Pierce Chemical). Nitroavidin, another form of monovalent avidin with a lower affinity for biotin, can be prepared by selectively nitrating three of the tyrosines, each located at the active site of avidin or streptavidin subunits, leaving just one site available for biotin binding (Morag et al., 1996). Another commercially available form of reactive biotin is the DNP biocytin succinimidyl ester, which carries the colorimetric properties of dinitrophenyl (DNP) and the avidin-binding properties of biotin (Briggs and Panfili, 1991; Haugland, 1996c). With this reagent, direct spectrophotometric measurement of the number of moles of biotin per mole of antibody can be achieved by using the molar extinction coefficient of DNP (15,000 $cm^{-1}M^{-1}$ at 364 nm). This reagent (MW = 862 Da) is larger than most common fluorophores and the reactive biotins described above. Thus, more reagent is necessary to obtain an efficient incubation molar ratio of reagent to antibody during the reaction, because larger molecules react more easily with surface groups and have difficulty reaching internal lysines. However, the presence of the DNP group on the conjugate offers a second tag on the target and the conjugate can be further probed with an anti-DNP antibody (Haugland, 1996c).

Enzymes

Antibody-enzyme conjugates are probably the most widely prepared antibody-based reagents because they are broadly used in diagnostic clinical tests. HRPO conjugates represent ~70% to 80% of the antibody-enzyme conjugates in common use, while APase makes up for most of the remainder; βGase and GO represent ~2%, and they are used primarily in research. The major applications of enzyme-conjugated antibodies lie in immunocytochemical studies of cells or tissue slices and in enzyme-linked immunosorbent assays (ELISA) for quantifying and detecting a wide variety of analytes. The activity of the conjugated enzyme causes the accumulation of measurable enzyme product in the test system, offering amplification of the signal and high assay sensitivity.

It is important to consider the experimental application for which the antibodies are to be conjugated with the enzymes. For cytological or histological studies, conjugates comprised of one enzyme and one antibody molecule are preferable, because, in general, larger conjugates are accompanied by higher background signals. The MW of IgG is ~150 kDa, and IgM is often >850 kDa. Enzymes can also be rather large (e.g., glucose oxidase (GO, MW = 160 kDa), alkaline phosphatase (APase, MW = 140 kDa), β-galactosidase (βGase, MW = 540 kDa)). Consequently, the mass of the antibody-enzyme conjugate can be considerable, especially when multiple enzyme molecules are bound to one antibody molecule. In contrast, for ELISA-based tests, in which only the total signal is essential, larger enzyme-antibody aggregates often increase the sensitivity of the assay.

The simplest method (and one of the oldest) to prepare enzyme-antibody conjugates involves mixing the two conjugate components and adding a "condensation" reagent, generally glutaralde-

hyde, to crosslink the two proteins at random amines. This method presents problems, however. Although conditions can be determined for obtaining an optimal yield of the enzyme-antibody conjugate, nonetheless, unwanted homopolymers of enzyme or of antibody are invariably obtained. Furthermore, this method is impractical for obtaining antibodies labeled with only one enzyme molecule.

Another commonly used method relies on the reaction of a maleimide-labeled enzyme (such as with SMCC, as described in Basic Protocol 2 for derivatizing the antibody) with an antibody or $F(ab')_2$ fragment. The maleimide groups interact with sulfhydryl groups in the hinge region after the disulfide bonds have been reduced by 2-mercaptoethylamine, in the presence of EDTA. Optimizing this procedure involves minimizing the disruption of other disulfide bridges within the antibody molecule and at the same time reducing a sufficient fraction of the hinge disulfides to give an optimal yield of conjugate. 2-Mercaptoethylamine is chosen for this reduction because it is more controllable than dithiothreitol. Details for this method can be found in Hermanson (1996c).

The basic approach described in this unit for preparing antibody-enzyme conjugates is based on forming thioether bonds between a maleimide group on the antibody and a free thiol on the enzyme. These two reactive groups are obtained by derivatizing the conjugate components with the heterobifunctional crosslinking reagents, SMCC and SPDP. This approach is "controllable" and easier to optimize than are other methods. Similar methods that involve reaction of the maleimide crosslinking reagent to the enzyme and conjugation of the sulfhydryl crosslinking reagent to the antibody have been also reported. This "reverse" method, however, is recommended only when *S*-acetylthioglycolic acid *N*-hydroxysuccinimide ester (SATA) is used as the thiolating reagent, because iminothiolane yields very unstable free thiol groups, and SPDP entails reduction with DTT or TCEP, which might disrupt the disulfide groups of the antibody. Furthermore, because deprotection of the thiol in SATA requires only exposure to hydroxylamine (Duncan et al., 1983), the antibody molecule is less likely to be altered. However, one should also consider that assaying for the free thiols generated with either iminothiolane or SATA requires a relatively large amount of antibody. In contrast, the release of pyridyl-2-thione from SPDP reduction permits one to promptly determine the number of free thiols obtained per molecule of enzyme by measuring absorption (Carlsson et al., 1978), yielding the precise number of reactive sites. This offers two advantages: (1) the enzyme can be derivatized with only 1 to 2 sulfhydryl groups in order to obtain mostly heteroconjugates of one enzyme per antibody molecule, and (2) the activity of the enzyme can be disrupted to a lesser degree when only 1 or 2 amines are derivatized.

The protocols presented in this unit for linking enzymes to antibodies can be tailored to the antibody-specific application by modulating the number of reactive crosslinking groups with which the antibody or the enzyme is modified. When free thiols are available, as with βGase, no chemical modification of the enzyme is necessary. Enzymes derivatized with only 1 to 1.5 thiol and antibodies carrying only a few maleimide groups generally yield heteroconjugates, most of which have one enzyme and one antibody. By introducing a larger number of thiols on the enzyme or a larger number of maleimide groups on the antibody, it is possible to obtain small aggregates consisting of one or more antibody molecule conjugated to two or more enzyme molecules.

Critical Parameters

Preparation of the antibody for conjugation

Before being conjugated to a probe, antibodies should be as free as possible of other proteins. Association with extraneous proteins can arise from inadequate purification of the antibody or from stabilizers added to diluted antibody preparation such as bovine serum albumin (BSA) or gelatin. The proper buffer and pH for the antibody conjugation can be obtained by dialysis or by adding 1 M bicarbonate when the antibody is dissolved in PBS. When antibodies are in PBS solution, the pH can be easily changed by adding 1/10 volume of 1 M sodium bicarbonate at pH 8.3 or 9.0, depending on which probe is used. This obviates the need for dialysis, accompanied by losses of material during manipulation of the antibody solution. One should note, however, that the buffer in which the antibody is dissolved should contain no primary amines (e.g., TRIS, glycine, or traces of ammonium sulfate), because they will react with the amine-reactive probe.

Solubility, storage, and stability

Most dyes, biotin, and cross-linking reagents are poorly soluble in buffers, and they require solubilization in an organic solvent such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO). Some of the most recently introduced dyes that exhibit greater fluorescence output and photostability (Cy and Alexa Fluor series) have

improved or good solubility in water, because of the sulfonic groups introduced in the ring of their molecule (Panchuk-Voloshina et al., 1999). Some labeling reagents are available as sulfosuccinimidyl esters, with a sulfonic acid as part of the reactive group, which increases water solubility but may decrease the overall conjugation efficiency. However, in the author's experience, dissolving the labeling reagent as a concentrated solution in DMF or DMSO (10 to 20 mg/ml) and then adding it to the antibody solution in amounts such that the solvent does not exceed 10% of the reaction mixture, does not generally decrease the antibody binding activity. The presence of the solvent, in fact, helps to maintain the labeling reagent in solution and favors the efficiency of the conjugation reaction.

All labeling reagents should be stored desiccated at −20°C. Antibody conjugates of fluorophores, biotin, or enzymes can be stored frozen in small aliquots, avoiding repeated freezing and thawing. The defrosted aliquots should be stable at 4°C for ≥1 week. Labeled antibodies generally maintain the storage requirements of their native form. However, introduction of dyes or biotin to the molecule, with concomitant loss of the positive charges of the tagged lysines or addition of enzyme molecules, may favor aggregation. Therefore, it is good practice to centrifuge antibodies and particularly antibody conjugates briefly in a microcentrifuge before use. Antibodies at concentrations <1 mg/ml are often unstable and should be stored in presence of 1 to 10 mg/ml BSA to avoid denaturation.

Appropriate degree of labeling

The appropriate number of labels per antibody molecule depends on the probe. In general, the number of labels that a protein tolerates before losing its biological activity is proportional to its molecular weight. In the author's experience, a 10- to 20-kDa protein preserves its activity when only one probe molecule per protein molecule is conjugated to the ε-amino group of a lysine. Thus 4 to 8 moles of probe per mole of an IgG antibody are possible in the case of relatively small or hydrophilic molecules (such as fluorescein, biotin, or sulfonated dyes) or 2 to 4 moles, in the case of hydrophobic probes, such as rhodamines. Excessive labeling may present problems: the antibody conjugate might aggregate or precipitate, lose binding activity to the protein, exhibit low fluorescence output due to quenching, or exhibit high background fluorescence in biological applications. The latter phenomenon can arise from a loss of specificity due to structural changes in the antibody caused by the high number of fluorophores substituting the positive charged amines. The Fab fragment (Fig. 16.5.1E), tolerates two or three fluorescein or Alexa Fluor 488 molecules well, but only one Texas Red or rhodamine. $F(ab')_2$ fragments (Fig. 16.5.1D) can be labeled with three to four fluorescein molecules or one to two Texas Red molecules. Consequently, when using Fab fragments, one should use smaller molar ratios of reactive probe to antibody than those recommended in Table 16.5.1 for native antibodies. Precipitated antibodies generally cannot be recovered.

Choosing the appropriate fluorophore

The choice of fluorophore for a particular application should be based on the following considerations. When the fluorescence microscope is the detection instrument, for single-color experiments the choice should be dictated by the spectral properties of the dye relative to the excitation source, the brightness of the probe, and its photostability. Photostability is less essential in flow cytometry experiments. For multiple-color experiments, minimal spectral overlap among the chosen fluorophores is essential. For samples with pronounced autofluorescence, longer wavelength emission dyes are better than the green fluorescence–emitting ones because the autofluorescence background will be lower. Table 16.5.2 describes the optical properties of the most commonly used fluorophores for the labeling of antibodies (also see *APPENDIX 1E*).

Choosing the appropriate incubation molar ratio of fluorophore or biotin to antibody

The factors that determine the number of moles of probe per mole of antibody (degree of labeling) that will be present in the purified conjugate are:

(1) Reactivity of the dye. This varies from dye to dye; it is pH and temperature dependent, and it must be taken into consideration when planning the reaction molar ratio of reagent to protein to use. (2) Concentration of the antibody. As in any chemical reaction, the reagent concentrations determine the rate and efficiency of the reaction and will generate higher yields of conjugate at higher concentrations. (3) Structure of the antibody. Although the Fc portion of the antibody molecule is relatively preserved, the amino acid composition of the remaining regions is quite varied and contains lysine residues in different numbers and positions. Consequently, the reactivity of each antibody is different. When possible, three or more molar incubation ratios of dye, biotin, or crosslinking reagent to antibody should be tried

Table 16.5.3 Performance of Commercially Available Anti-Photobleaching Reagents[a,b]

Dye	Reagent						
	PBS	Slow-Fade	SlowFade Light	ProLong	Vecta-Shield	Cyto-seal	Others[b,c]
Alexa Fluor 350	− − −	+ + +	− − −	+ + +	− − −	− − −	***
Alexa Fluor 430	− − −	− − −	+ + +	+ + +	− − −	− − −	***
Alexa Fluor 488	− − −	+ + +	− − −	+ + +	+ + +	− − −	***
Alexa Fluor 532	− − −	− − −	− − −	+ + +	+ + +	− − −	***
Alexa Fluor 546	− − −	− − −	− − −	− − −	− − −	− − −	***
Alexa Fluor 568	− − −	− − −	− − −	+ + +	+ + +	− − −	***
Alexa Fluor 594	− − −	+ + +	− − −	+ + +	− − −	− − −	***
BODIPY FL	− − −	+ + +	− − −	− − −	− − −	+ + +	nt
BODIPY TR-X	− − −	− − −	nt	+ + +	nt	− − −	nt
Carboxytetramethylrhodamine	− − −	+ + +	− − −	+ + +	+ + +	− − −	nt
Cascade Blue	− − −	+ + +	+ + +	+ + +	− − −	+ + +	***
Cy3	− − −	+ + +	− − −	+ + +	+ + +	− − −	nt
Cy5	− − −	+ + +	− − −	+ + +	+ + +	− − −	nt
Fluorescein	− − −	+ + +	− − −	+ + +	+ + +	− − −	nt
Oregon Green 488	− − −	− − −	nt	+ + +	nt	+ + +	nt
Oregon Green 514	− − −	− − −	nt	+ + +	− − −	+ + +	nt
Rhodamine Red-X	− − −	− − −	nt	+ + +	nt	− − −	nt
Rhodol Green	− − −	+ + +	nt	+ + +	nt	+ + +	nt
Texas Red	− − −	− − −	nt	+ + +	+ + +	− − −	nt
Texas Red-X	− − −	− − −	nt	+ + +	nt	− − −	nt

[a]Fading was measured for 90 sec.

[b]Abbreviations: nt: not tested; PBS: phosphate-buffered saline.

[c]Notes: In addition to testing anti-photobleaching reagents listed in the table, the authors tested the performance of the following anti-photobleaching reagents when used with the dyes in the Alexa Fluor series: Crystal Mount, Gel Mount, Immu-Mount, Fluor-Save, Clarion, Aqua-Poly Mount, Mowiol. Aqua-Poly Mount and Mowiol worked well with Alexa Fluor 546.

to obtain a degree of labeling of the probe-antibody conjugate that will work best in the specific application. Table 16.5.1 gives guidelines of suggested incubation molar ratios of probe to antibody for different concentrations of antibody. The guidelines are very general and individual antibodies may require customized variations. As previously mentioned, it is desirable to try three different degrees of labeling or, if that is not possible, aim for a moderate degree of labeling of three to four fluorophore moles per mole of antibody. Also note that it is possible to label an antibody for the second time, but it is not possible to reduce the moles of dye per antibody following a conjugation because they are covalently attached.

Choosing the appropriate biotin-binding protein

Avidin is a highly positively charged glycoprotein, rich in mannose and glucosamine. Thus, it may interact nonspecifically with negatively charged molecules within the cell or with sugar receptors of the cell membrane. For this reason, a chemically modified avidin, deglycosylated, and with a lower isoelectric point has been developed. It is commercially available in both unconjugated and conjugated forms under the trademark, NeutrAvidin (Haugland, 1999). Streptavidin is not glycosylated, and it generally yields less nonspecific binding than avidin. However, streptavidin contains the amino acid sequence Arg-Gly-Asp (RYD) that mimics the binding peptide of fibronectin (RGD), a universally recognized domain of the extracellular matrix. Thus, although it is rare, nonspecific interaction is possible also with streptavidin (Alon et al., 1990), in which cases, avidin or its NeutrAvidin form may be preferable.

Choosing the appropriate biotinylation reagent

As described for conjugations with fluorophores, the most broadly used reactive biotin for conjugation with antibodies is the succinimidyl ester form. Three types of succinimidyl esters are available: either biotin derivatized directly at the carboxyl with the reactive group or with a 7- or

14-atom spacer between the carboxyl and the reactive group (called biotin-X and biotin-XX, respectively). The addition of a spacer favors the efficiency of formation of the complex between avidin and the biotinylated antibody, possibly by facilitating the interaction of the biotin group with the hydrophobic crevice of the avidin binding site (Gretch et al., 1987; Hnatowich et al., 1987). Biotin-X and biotin-XX can be used interchangeably, although in this laboratory the authors have found that biotin-XX gives a somewhat higher signal in ELISA (Haugland and You, 1998). Sulfosuccinimidyl esters of biotin, which are highly water soluble are also commercially available. However, this form is generally less reactive and requires a higher molar incubation ratio of reagent to antibody.

Fluorescence intensity in histological preparations

For detection by microscopy, either conventional or confocal, the resistance of the fluorophores to photobleaching is a critical concern. It is very frustrating to see an image disappear as soon as it is in focus. There is considerable variation in the performance of anti-photobleaching products with different fluorophores. The author has tried many of the commercially available anti-photobleaching reagents in cellular preparations stained with the most commonly used fluorophores. The results from this study are reported in Table 16.5.3.

Anticipated Results

The protocols in this unit are established and have a high probability of success. The exact degree of labeling depends on the antibody to be labeled and the application for which it is used. The reactions may be scaled up or down.

Time Considerations

Fluorophore or biotin conjugation and purification of the conjugated antibody will take ~3 hr. Checking the degree of labeling will take 30 to 60 min. Longer times (~2 days) are required when working with very dilute antibodies that require dialysis for purification. Conjugation with enzymes and purification of the conjugated antibody will take ~5 days.

Literature Cited

Alon, R., Bayer, E.A., and Wilchek, M. 1990. Streptavidin contains an RYD sequence which mimics the RGD receptor domain of fibronectin. *Biochem. Biophys. Res. Commun.* 170:1236-1241.

Banks, P.R. and Paquette, D.M. 1995. Comparison of three common amine-reactive fluorescent probes used for conjugation to biomolecules by capillary zone electrophoresis. *Bioconj. Chem.* 6:447-458.

Briggs, J. and Panfili, P.R. 1991. Quantitation of DNA and protein impurities in biopharmaceuticals. Anal. Chem. 63:850-859.

Carlsson, J., Drevin, H., and Axen, R. 1978. Protein thiolation and reversible protein-protein conjugation. *N*-Succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. *Biochem. J.* 173:723-737.

Duncan, R.J., Weston, P.D., and Wrigglesworth, R. 1983. A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal. Biochem.* 132:68-73.

Getz, E.B., Xiao, M. Chakrabarty, T., Cooke, R., and Selvin, P.R. 1999. A comparison between the sulfhydryl reductants Tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. *Anal. Biochem.* 273:73-80.

Green, N.M. 1975. Avidin. In Advances in Protein Chemistry (C.B. Anfinsen, J.T. Edsall, and F.M. Richards, eds.) pp. 85-133. Academic Press, New York.

Gretch, D.R., Suter, M., and Stinski, M.F. 1987. The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate Herpes virus hydrophobic proteins or glycoproteins. *Anal. Chem.* 163:270-277.

Hage, D.S., Wolfe, C.A., and Oates, M.R. 1997. Development of a kinetic model to describe the effective rate of antibody oxidation by periodate. *Bioconj. Chem.* 8:914-920.

Haugland, R.P. 1996a. Introduction to amine modification. *In* Handbook of Fluorescent Probes and Research Chemicals, 6th ed. (M.T.Z. Spence, ed.) p. 12. Molecular Probes, Eugene, Ore.

Haugland, R.P. 1996b. Coupling of monoclonal antibodies with enzymes. *In* Methods in Molecular Biology (W.C. Davis, ed.) pp. 235-243. Humana Press, Totowa, N.J.

Haugland, R.P. 1996c. Biotins and haptens. *In* Handbook of Fluorescent Probes and Research Chemicals, 6th ed. (M.T.Z. Spence, ed.) pp. 82-88. Molecular Probes, Eugene, Ore.

Haugland, R.P. 1999. Handbook of Fluorescent Probes and Research Chemicals, 7th ed. (CD-ROM). Molecular Probes, Eugene, Ore.

Haugland, R.P. and You, W.W. 1998. Coupling of monoclonal antibodies with biotin. *In* Methods in Molecular Biology (W.C. Davis, ed.) pp. 173-184. Humana Press, Totowa, N.J.

Hermanson, G.T. 1996a. Preparation of antibody-enzyme conjugates. *In* Bioconjugate Techniques, p. 486. Academic Press, San Diego.

Hermanson, G.T. 1996b. Amine-reactive chemical reactions. *In* Bioconjugate Techniques, pp. 139-140. Academic Press, San Diego.

Hermanson, G.T. 1996c. Preparation of antibody-enzyme conjugates. *In* Bioconjugate Techniques, pp. 463-465. Academic Press, San Diego.

Hnatowich, D.J., Virzi, F., and Rusckowski, M. 1987. Investigations of avidin and biotin for imaging applications. *J. Nucl. Med.* 28:1294-1302.

Hoffman, K., Wood, S.W., Brinton, C.C., Montibeller, J.A., and Finn, F.M. 1980. Iminobiotin affinity columns and their application to retrieval of streptavidin. *Proc. Natl. Acad. Sci. U.S.A.* 77:4666-4668.

Husain, M. and Bieniarz, C. 1994. Fc site–specific labeling of immunoglobulins with calf intestinal alkaline phosphatase. *Bioconj/ Chem.* 5:482-490.

Johnson, I.D. 1996. Introduction to fluorescence techniques. *In* Handbook of Fluorescent Probes and Research Chemicals, 6th ed. (M.T.Z. Spence, ed.) pp. 1-6. Molecular Probes, Eugene, Ore.

Lefevre, C., Kang, H.C., Haugland, R.P., Malekzadeh, N., Arttamangkul, S., and Haugland, R.P. 1996. Texas Red-X and Rhodamine Red-X, new derivatives of sulforhodamine 101 and Lissamine rhodamine B with improved labeling and fluorescence properties. *Bioconj. Chem.* 7:482-489.

Morag, E., Bayer, E.A., and Wicheck, M. 1996. Reversibility of biotin-binding by selective modification of tyrosine in avidin. *Biochem. J.* 316:193-199.

Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., and Waggoner, A.S. 1989. Cyanine dye labeling reagents containing isothiocyanate groups. *Cytometry* 10:1-9.

Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., Lewis, C.J., and Waggoner, A.S. 1993. Cyanine dye labeling reagents: Sulfoindocyanine succinimidyl esters. *Bioconj. Chem.* 4:105-111.

Mujumdar, S.R., Mujumdar, R.B., Grant, C.M., and Waggoner, A.S. 1996. Cyanine labeling reagents: Sulfobenzindocyanine succinimidyl esters. *Bioconj. Chem.* 7:356-362.

O'Malley, J.J. and Weaver, J.L. 1972. Subunit structure of glucose oxidase from *Aspergillus niger*. *Biochemistry* 12:3527-3532.

Orr, G.A. 1981. The use of the 2-iminobiotin-avidin interaction for the selective retrieval of labeled plasma membrane components. *J. Biol. Chem.* 256:761-766.

Panchuk-Voloshina, N., Haugland, R.P., Bishop-Stewart, J., Bhalgat, M.K., Millard, P.J., Mao, F., and Leung, W.Y. 1999. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J. Histochem. Cytochem.* 47:1179-1188.

Ravdin, P. and Axelrod, D. 1977. Fluorescent tetramethylrhodamine derivatives of alpha-bungarotoxin: Preparation, separation, and characterization. *Anal. Biochem.* 80:585-592.

Rodwell, J.D., Alvarez, V.L., Lee, C., Lopes, A.D., Goers, J.W., King, H.D., Powsner, H.J., and McKearn, T.J. 1986. Site-specific covalent modification of monoclonal antibodies: In vitro and in vivo evaluations. *Proc. Natl. Acad. Sci. U.S.A.* 83:2632-2636.

Welinder, K.G. 1979. Amino acid sequence studies of horseradish peroxidase. *Eur. J. Biochem.* 96:483-502.

Whitaker, J.E., Haugland, R.P., Moore, P.L., Hewitt, P.C., Resse, M., and Haugland, R.P. 1991. Cascade Blue derivatives: Water soluble, reactive, blue emission dyes evaluated as fluorescent labels and tracers. *Anal. Biochem.* 198:119-130.

Wilchek, M. and Bayer, E.A. 1990. Introduction to avidin-biotin technology. *Methods Enzymol.* 184:5-13.

Wong, S.S. 1991. Reactive groups of proteins and modifying agents. *In* Chemistry of Protein Conjugation and Crosslinking, pp. 37. CRC Press, Boca Raton, Fla.

Key References

Aslam, M. and Dent, A. 1998. Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences. Macmillan Reference, London.

Hermanson, G.T. 1996. Bioconjugate Techniques. Academic Press, San Diego.

These two citations are excellent reference sources covering concepts behind all the procedures discussed in this unit.

Haugland, 1996b, 1999. See above. (The 7th edition of this Handbook (1999) is available free on CD-ROM from Molecular Probes and at *www.probes.com*).

Contains excellent background material for all procedures, as well as chemical structures, spectra, and other product information for many of the probes discussed in this article.

Internet Resources

http:// www.probes.com

This Molecular Probes site is a searchable, complete handbook and catalog of fluorescent probes, protein conjugates, and other probes for research. It contains downloadable spectra, structures, images, product information sheets, material safety data sheets, full scientific references, and link to contact the manufacturer.

http://www.piercenet.com

This Pieerce Chemical Company site is also a searchable handbook and catalog with technical tips and selection guides, literature, application notes, and link to contact the manufacturer.

Contributed by Rosaria P. Haugland
Molecular Probes, Inc.
Eugene, Oregon

Production of Antibodies That Recognize Specific Tyrosine-Phosphorylated Peptides

It is possible to produce anti-phosphopeptide antibodies (i.e., antibodies recognizing phosphorylated peptides) that recognize a protein only in its phosphorylated state, and that do not cross-react with either the cognate unphosphorylated protein or other phosphoproteins. Because the state of phosphorylation is often indicative of a protein's functional state or activity, such antibodies provide a convenient probe of the functional state of a protein. Thus, unlike conventional antibodies, anti-phosphopeptide antibodies provide information regarding not only the abundance of a protein but also its activity. Unlike general anti–phosphoamino acid (e.g., anti-phosphotyrosine) antibodies, which have broad reactivity, anti-phosphopeptide antibodies may have unique specificity toward the cognate proteins. Such reagents not only facilitate conventional in vitro analysis of phosphoproteins, but also allow heretofore impossible applications—e.g., differential isolation of species of a particular protein that have been phosphorylated at individual phosphorylation sites, as well as analysis of the functional state of a protein in situ by immunohistochemical techniques.

Basic Protocol 1 describes the production of polyclonal anti-phosphopeptide antibodies and Basic Protocol 2 describes the production of monoclonal anti-phosphopeptide antibodies. Both of these procedures are based upon immunizing animals with oligopeptides that have been synthesized containing phosphorylated tyrosine; hence, a knowledge of the sequence and phosphorylation site of the protein of interest is a prerequisite for either protocol. Such an immunization will generate an immune response with at least four components: (1) anti–carrier protein reactivity, (2) general anti-phosphotyrosine reactivity, (3) phosphorylation-independent anti-peptide reactivity, and (4) phosphorylation-dependent anti-peptide reactivity. For the production of polyclonal antibodies (see Basic Protocol 1), a multiple-step affinity chromatographic purification with several negative-selection steps is carried out to produce a final antibody preparation having the desired reactivity. For production of monoclonal antibodies (see Basic Protocol 2), ELISAs are performed to screen candidate hybridoma supernatants against the cognate phosphopeptide, as well as against related phosphorylated and nonphosphorylated peptides, until one is found with the desired reactivity and specificity. Although monoclonal antibodies have a number of advantages, production of polyclonal antibodies is likely to be more predictable, as in the authors' experience a monoclonal hybridoma clone of stringent specificity may occur with very low frequency (see Critical Parameters). Production of monoclonal antibodies is also generally more time-consuming and expensive. The relative merits of monoclonal and polyclonal antibodies are discussed in the Chapter 16 introduction and in general antibody guides (see Key References). Support Protocols are provided for the coupling of peptides (see Support Protocol 2) and phosphotyrosine (see Support Protocol 3) to the affinity matrix (Affi-Gel 10); BSA-agarose affinity matrix is commercially available.

This unit describes production of antibodies against tyrosine-phosphorylated peptides, with which the authors have the most expertise, but the principles discussed here also apply to peptides phosphorylated on serine and threonine (see Key References).

Contributed by Michael P. DiGiovanna, Robert R. Roussel, and David F. Stern
Current Protocols in Cell Biology (2002) 16.6.1-16.6.18

PRODUCTION OF POLYCLONAL ANTI-PHOSPHOPEPTIDE ANTIBODIES

Strategic Planning

The first step of the planning phase is design and synthesis of the required peptides (see Support Protocol 1 and Background Information). The authors employ a peptide containing fifteen amino acids with the phosphorylation site Tyr in the center (residue 8). This peptide consists of fourteen amino acids from the actual sequence of the protein plus an N-terminal Lys to enhance coupling of the peptide to a carrier protein (BSA is used as a carrier in this protocol). The peptide, coupled to BSA, is then used to immunize rabbits. The cognate phosphopeptide is also immobilized on an affinity-chromatography column support (see Support Protocol 2); this affinity matrix will ultimately be used to purify the antibody from the rabbit serum. In addition, a series of negative-selection affinity-chromatography columns are used to adsorb antibodies from the serum that cross-react with epitopes other than the cognate phosphopeptide. These columns are composed of matrix coupled to various synthetic peptides—principally the cognate nonphosphorylated peptide (to remove phosphorylation-independent antibodies) and phosphotyrosine itself (to remove indiscriminant anti-phosphotyrosine reactivity; see Support Protocol 3 for preparation of phosphotyrosine affinity columns). Additional negative-selection steps may be used if needed, depending upon the particular target chosen, to remove antibodies that cross-react with phosphorylation sites of other proteins having known homology to the target site. This cross-reactivity occurs particularly when the target protein is a member of a family of homologous proteins—e.g., a subgroup of related receptor tyrosine protein kinases. If such cross-reactivity is anticipated, it is possible to synthesize not only the cognate phosphorylated and nonphosphorylated peptides, but also phosphopeptides based upon the related homologous sequences, to be used for negative selection. The authors also pass the serum over a column consisting of immobilized carrier protein (commercially available BSA-agarose) to remove the majority of the antibodies generated against the much larger carrier protein. Thus, a typical purification scheme might consist of the following:

Negative-selection affinity-purification columns

- BSA (carrier)
- phosphotyrosine
- cognate nonphosphopeptide
- homologous phosphopeptides (if desired)

Positive-selection affinity-purification column

- cognate phosphopeptide.

The second step in planning is to raise and test antisera in rabbits. To prepare the phosphopeptide-carrier conjugate for immunization of rabbits, the authors couple a 30-fold molar excess of phosphopeptide (14.4 mg dissolved in water and neutralized with 1 N NaOH) to BSA (20 mg dissolved in 0.4 M sodium phosphate buffer, pH 7.5), using glutaraldehyde as the cross-linking reagent as described in Doolittle (1986). The reaction is allowed to proceed 30 min at room temperature; the development of a yellow color in the coupling solution is an indication that the glutaraldehyde has reacted. Conjugation can be confirmed by performing an anti-phosphotyrosine immunoblot on a small test aliquot of the conjugate (although a smear should be expected on the blot, resulting from variation in the amount of peptide coupled per BSA molecule as well as from possible multimers of BSA). Guidelines for immunization of rabbits are provided in *UNIT 16.2*, and many institutions have core facilities for the immunization of rabbits and production of antisera. The authors immunize with 1 mg immunogen (the peptide-BSA conjugate

prepared above, dissolved in PBS) in 1 ml of a 50% emulsion with complete Freunds adjuvant. This is administered subcutaneously at multiple sites, followed by four boosts at 2-week intervals using the same quantity of immunogen in incomplete Freunds adjuvant. An adequate immune response can be seen as early as week 6.

The specificity of the final antibody is demonstrated most simply by immunoblot analysis (*UNIT 6.2*) using a panel of relevant phosphorylated and nonphosphorylated proteins. Electrophoresis is carried out with purified cognate protein in both its phosphorylated and unphosphorylated states, as well as with any proteins toward which there could conceivably be cross-reactivity. These are most easily isolated by immunoprecipitation using conventional antibodies. The blot is probed with the purified anti-phosphopeptide antibody to demonstrate appropriate reactivity. This immunoblot assay may also be used after each chromatographic step to monitor the success of the purification.

An assay also must be devised for estimating the titer of the immune response in the sera of the immunized rabbits. Convenient screening assays for this purpose include tests for the ability of crude sera to immunoprecipitate the target phosphoprotein from a cell lysate known to contain it, as well as immunoblotting of purified phosphorylated target protein with the crude serum.

The relationship among the above procedures is illustrated by the flow chart in Figure 16.6.1.

Purification Method

The purification of polyclonal antibodies consists of multiple affinity-chromatography steps for the negative and positive selection of antibodies of the appropriate reactivity (see Fig. 16.6.1). All of the following chromatographic steps may be carried out at room temperature, with the solutions applied to the columns by gravity.

Materials

BSA-agarose affinity matrix (Sigma) packed (see Support Protocol 2) in a 10-ml bed volume column

Phosphotyrosine affinity matrix column (10-ml bed volume; see Support Protocol 3)

Crude serum from rabbit immunized with phosphopeptide-BSA conjugate (refer to Strategic Planning, above)

PBS/azide: PBS (*APPENDIX 2A*) containing 0.02% (w/v) sodium azide (store indefinitely at 4°C or room temperature)

Cognate nonphosphopeptide affinity matrix column (3-ml bed volume; see Support Protocols 1 and 2 and Strategic Planning)

3 M NaSCN

Homologous phosphopeptide affinity matrix columns (optional; 3-ml bed volume; see Support Protocols 1 and 2 and Strategic Planning)

Positive-selection phosphopeptide affinity matrix column (3-ml bed volume; see Support Protocols 1 and 2 and Strategic Planning)

3.5 M and 4.5 M $MgCl_2$ (optional; see Critical Parameters)

Spectrophotometer (optional)

Dialysis tubing (MWCO 12,000 to 14,000; 10 mm width, 6.4 mm diameter; e.g., Spectra/Por 4 from Spectrum)

Additional reagents and equipment for dialysis (*APPENDIX 3C*), protein quantitation (*APPENDIX 3B*), and analysis of antibodies by ELISA (*APPENDIX 3A*) or immunoblotting (*UNIT 6.2*)

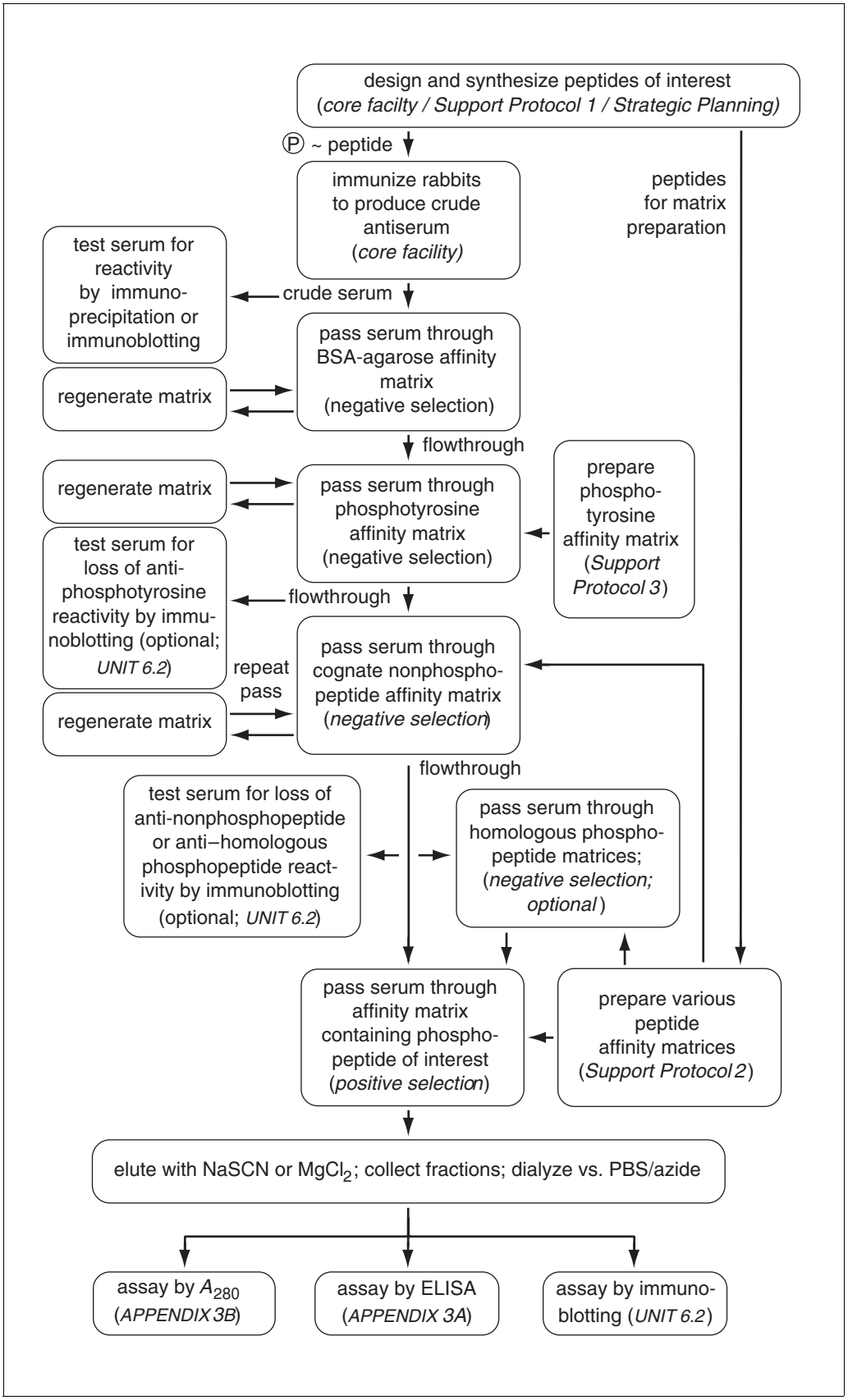


Figure 16.6.1 Flow chart for production of polyclonal anti-phosphopeptide antibodies.

Deplete reactivity of serum toward phosphotyrosine and carrier protein

1. Connect the washed BSA-agarose and phosphotyrosine affinity matrix columns in series.

2. Pass ~15 ml crude serum through the two columns, allowing it to flow through by gravity. Wash with PBS/azide until all of the yellow color of the serum has passed through the columns (or monitor the A_{280} of the column effluent spectrophotometrically until baseline absorbance is reached), then wash with an additional 5 to 10 ml PBS/azide, collecting all washings in the flowthrough fraction, which will contain the antibody of interest.

   Aliquots of the crude serum as well as this first flowthrough may be saved for subsequent analysis and comparison of individual purification fractions. The serum may be analyzed at this time for elimination of anti-phosphotyrosine reactivity by performing immunoblotting (UNIT 6.2) of samples containing a variety of phosphotyrosyl proteins; alternatively, it is possible to save an aliquot for analysis and proceed to the next step (see Troubleshooting).

   Expect that with each passage through a column, the volume of the serum will increase, which will prolong the amount of time needed for subsequent columns. Note that if flowthrough is monitored by the passage of the yellow color of the serum, this will become more difficult as the serum becomes more dilute.

   After the serum passes through, regenerate the columns by washing with 10 bed volumes of 3 M NaSCN followed by 10 bed volumes of PBS/azide, and store at 4°C in PBS/azide for future use.

Deplete reactivity of serum toward cognate nonphosphopeptide

3. Pass the flowthrough serum through the cognate nonphosphopeptide affinity matrix column by gravity as many times as necessary to deplete cross-reactivity, regenerating the column between passes by washing with 10 bed volumes of 3 M NaSCN followed by 10 bed volumes PBS/azide.

   A 15-ml starting serum sample may have to be passed over this column several times to quantitatively deplete cross-reactivity (see Critical Parameters). The serum may be analyzed after each or several passes, or an aliquot can be saved for analysis at a later time.

4. If cross-reactivity with homologous phosphoprotein(s) is anticipated, pass the flowthrough serum through the homologous phosphopeptide affinity matrix column(s) using the methodology described in step 3.

   Refer to Strategic Planning, above, for discussion of cross-reactivity with homologous phosphoproteins.

Purify antibodies by positive-selection affinity chromatography and dialysis

5. Hydrate and wash ~25-cm strips of dialysis tubing in advance for collection of fractions from the positive-selection affinity purification. Secure one end of each length of tubing with a dialysis clamp and check for leaks (APPENDIX 3C). Also prepare 6 liters PBS/azide and cool to 4°C in advance to use as the dialysis solution.

   To minimize the time that the antibodies are exposed to the elution solution, fractions (~3 ml each) from the positive-selection affinity column will be collected directly into preprepared dialysis tubing.

6. Pass the flowthrough serum from the previous chromatography step through the positive-selection phosphopeptide affinity column three times, without washing between passes.

   Three passes are performed to maximize the interaction of the antibody with the affinity matrix.

7. Collect the flowthrough serum from the final pass, then wash the column with 5 to 20 ml PBS/azide (depending upon the precolumn volume and column bed volume) and combine the washings with the flowthrough serum. Wash with an additional 20 ml PBS/azide, and collect the washings separately as the "wash" fraction.

8. Elute with chaotropic agent of choice: either 20 ml of 3 M NaSCN, or 10 ml of 3.5 M $MgCl_2$ followed by 10 ml of 4.5 M $MgCl_2$. Upon starting the elution, immediately begin collecting ~3-ml fractions directly into the dialysis bags prepared in step 5, securing the proximal end of the tubing with dialysis clamps and dropping the bag immediately into the PBS/azide dialysis solution. Collect at least six fractions.

   Alternatively, ~3-ml fractions can be collected in tubes and the liquid immediately placed into dialysis tubing upon completion of each fraction. Most of the antibody will elute in the first three fractions from a column of 2- to 3-ml bed volume. Regenerate the column as in step 3.

   See Critical Parameters for a discussion of the relative merits of NaSCN versus $MgCl_2$ as eluants.

9. Dialyze all fractions collected in step 8 exhaustively against PBS/azide at 4°C (*APPENDIX 3C*).

Analyze and store purified antibody

10. Determine protein concentration of dialyzed fractions by measuring absorbance at 280 nm or by a colorimetric protein assay (*APPENDIX 3B*) and calculate yield.

    The authors have generally recovered ≥1 mg of purified antibody from a 15-ml serum sample.

11. Store the antibody in aliquots at −70°C; store working aliquot at 4°C.

12. Assay the reactivity and cross-reactivity of the final samples, as well as any aliquots saved from previous steps, using ELISA (*APPENDIX 3A*) or immunoblotting (*UNIT 6.2*).

BASIC PROTOCOL 2

PRODUCTION OF MONOCLONAL ANTI-PHOSPHOPEPTIDE ANTIBODIES

Strategic Planning

As in the production of polyclonal anti-phosphopeptide antibodies (see Basic Protocol 1), the first step of the planning phase for anti-phosphopeptide monoclonal antibodies is the design and synthesis of the required peptides (see Support Protocol 1 and Background Information). Here too, the authors employ a peptide containing fifteen amino acids with the phosphorylation site Tyr in the center (residue 8), consisting of fourteen amino acids from the actual sequence of the protein plus an N-terminal Lys to enhance coupling to a carrier protein. A different carrier protein must be employed for immunization than will be used as substrate for the ELISA screen, to avoid detection of anti-carrier antibodies in the ELISA (see Purification Method, below). The authors use keyhole limpet hemocyanin (KLH) as the carrier for immunization and BSA as the carrier for the ELISA substrate. The cognate phosphopeptide can also be immobilized to an affinity-chromatographic column support (see Support Protocol 2) and used ultimately to affinity-purify the antibody produced by the desired hybridoma clone. A number of additional peptides must be produced for use in ELISA screening of candidate hybridoma supernatants, to screen both for the presence of the desired reactivity and the absence of undesired cross-reactivity. For ELISA screening, the peptides are coupled to a carrier protein different from the one used for immunization. Bovine serum albumin (BSA) is used for screening, because many of the clones are likely to react against the carrier used in the immunization of the

mice (KLH) and because unconjugated peptides have not been found to function well as ELISA substrates (Doolittle, 1986). In addition, because antibodies may be generated against the cross-linking reagent, the method used for coupling the phosphopeptide to the BSA to produce the substrate for ELISAs should be different from that used to link peptides to the KLH carrier for immunization (Doolittle, 1986; Czernik et al., 1991).

The second step of planning is to raise antibodies in mice. General guidelines for the immunization of mice and the production of monoclonal antibodies are described in *UNIT 16.1*, and many institutions have core facilities that will perform that task. The authors have immunized BALB/c mice by intraperitoneal injection with 1 mg/ml of immunogen (dissolved in PBS) in a 50% emulsion with complete Freunds adjuvant on day 1 and boosted intraperitoneally on days 15 and 37 with immunogen in incomplete Freunds adjuvant. Test bleeds from day 47 were analyzed by ELISA. On day 57 the mouse with the best titer was boosted intravenously, and its spleen was harvested on day 60. Freshly harvested spleen cells were prepared for cell fusion to generate hybridoma lines, which were subsequently screened by ELISA (*APPENDIX 3A*). The purpose of the ELISA screening is to identify clones that react with the cognate phosphopeptide and to eliminate clones that cross-react with other epitopes in addition to the cognate phosphopeptide. Epitopes screened by ELISA generally consist of the cognate nonphosphorylated peptide (to eliminate clones with phosphorylation-independent reactivities) and at least one unrelated phosphopeptide (to eliminate clones with indiscriminant anti-phosphotyrosine reactivity). In addition, depending upon the particular target chosen, it may be necessary to screen for reactivity with phosphopeptides from proteins of known homology. This occurs particularly when the target protein is a member of a family of homologous proteins—e.g., the subgroups of related receptor tyrosine protein kinases. If such cross-reactivity is anticipated, it is essential to synthesize not only the cognate phosphorylated and nonphosphorylated peptides and at least one unrelated phosphopeptide, but also phosphopeptides based upon the related homologous sequences. Thus, a typical ELISA screening scheme might consist of the following steps.

1. Screen for clones that exhibit reactivity against the cognate phosphopeptide. These clones, which will likely be <10% of all the clones, are potential candidates for the desired clones.
2. Screen for clones with reactivity against the cognate nonphosphopeptide. These clones, which are phosphorylation state–independent, are then eliminated.
3. Screen for clones with reactivity against at least one unrelated phosphopeptide. These clones, which are likely to represent indiscriminant anti-phosphotyrosine reactivity, are also eliminated.
4. Screen for and eliminate clones with reactivity against potential cross-reacting homologous peptides, if any are anticipated.

In the ELISAs performed in the authors' laboratory, 96-well polyvinyl chloride assay plates are generally used and bound monoclonal antibody is detected using biotinylated horse anti-mouse secondary antibody followed by horseradish peroxidase (HRP)–conjugated Avidin D (Vector Labs) and 4-aminoantipyrine (Sigma)/H_2O_2 as the substrate. This gives a deep red color in positive wells and absence of color in negative wells, allowing easy visual scoring without the need for quantitative measurements. The clones that pass all the screening steps are candidates for desirable hybridoma cell lines. These are expanded, frozen down as a pool, subcloned by limiting dilution, then rescreened against cognate phosphopeptide to ensure continued production of antibody through the early passages. Those continuing to produce are expanded further and a larger quantity of either culture supernatant or ascites fluid is screened in further immunoassays. Reactivity is

demonstrated most simply by immunoblot analysis using a panel of relevant phosphorylated and nonphosphorylated proteins. Electrophoresis is carried out with purified (generally by immunoprecipitation using conventional antibodies; *UNIT 7.2*) cognate protein in both its phosphorylated and unphosphorylated states, as well as with any proteins toward which there could conceivably be cross-reactivity. The blot is probed with the monoclonal antibody preparation to demonstrate appropriate reactivity. The antibody preparation can ultimately be purified on a cognate phosphopeptide affinity column.

The relationship among the above procedures is illustrated by the flow chart in Figure 16.6.2.

Purification Method

This protocol presents procedures for isolating hybridoma clones of the desired reactivity, starting from the point at which candidate hybridoma clones have been seeded in 96-well culture plates (see Fig. 16.6.2). Candidate hybridoma supernatants are screened in ELISA assays against a series of peptides (as described in Strategic Planning). Identified clones are expanded, subcloned by limiting dilution, and further purified.

Materials

Candidate hybridoma cell lines from fusion (refer to Strategic Planning and *UNIT 16.1*)
HT medium; prepare as for HAT medium (*UNIT 1.2*) except omit aminopterin
Screening diluent (see recipe)
BSA-conjugated cognate phosphopeptide (for use as ELISA antigen; refer to Strategic Planning and Support Protocol 1
Preimmune serum from mouse used to produce hybridoma line (negative control)
Immune serum from mouse used to produce hybridoma line (positive control)
BSA-conjugated cognate nonphosphopeptide (for use as ELISA antigen; refer to Strategic Planning and Support Protocol 1
BSA-conjugated noncognate phosphotyrosyl peptide (for use as ELISA antigen; refer to Strategic Planning and Support Protocol 1
BSA-conjugated homologous phosphopeptides (optional, for use as ELISA antigens; refer to Strategic Planning and Support Protocol 1

96-well polystyrene tissue culture plates
96-grid note sheets

Additional reagents and equipment for cloning by limiting dilution, ELISA, and freezing and recovery of hybridoma cell lines (*APPENDIX 3A*)

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper asceptic technique should be used accordingly.

Identify and sample conditioned media from candidate hybridomas

1. Seed hybridoma cells from fusion at a low density (aiming for one cell per three wells) in multiple 96-well polystyrene tissue culture plates using HT medium. Examine at ~2 weeks and identify all single-colony hybridoma culture wells as potential candidates by recording the number of colonies per well on a 96-grid note sheet. Mark the underside of each well containing one colony for easy identification.

   The supernatant fluid from wells possessing a single colony will be screened for reactivity.

2. Using sterile technique, remove an aliquot of supernatant (e.g., 100 µl from a 200-µl culture) from each potential candidate well and transfer to a separate 96-well polystyrene plate (the "screen plate"). Record the original plate number and well position and the corresponding plate number and well position for the screen plate (e.g., on a grid sheet representing the screen plate, record the original plate number

and well position from which the supernatant in each well was transferred). Replace the aliquot removed from each original well with 100 µl 37°C fresh HT medium.

A brightly colored dot sticker the size of one well, attached to the tissue culture hood surface, will help to keep the plate in register, as the plate may be moved one well at a time along the sticker. If the supernatants are not to be tested immediately, store the screen plates at 4°C, wrapped in plastic wrap.

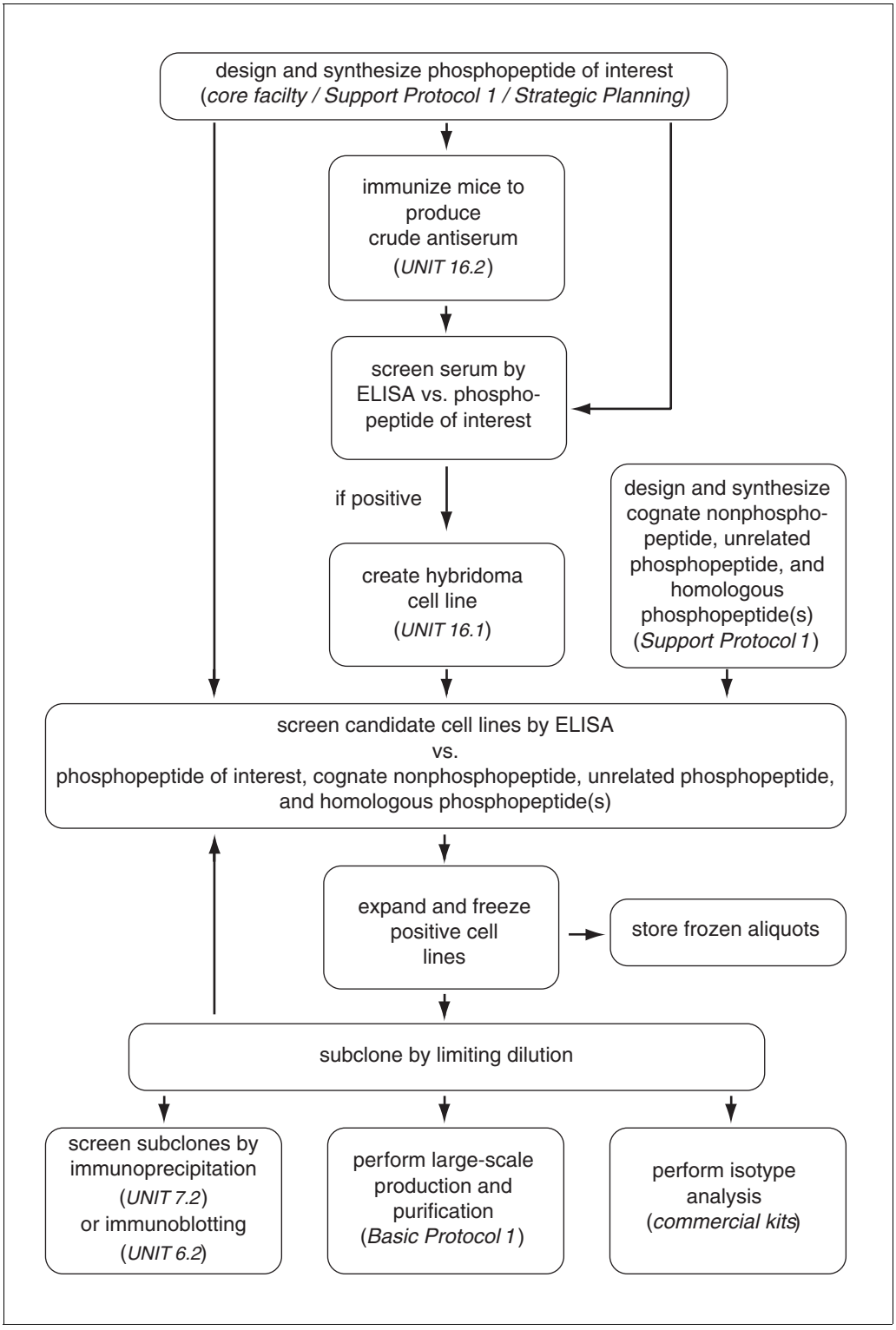


Figure 16.6.2 Flow chart for production of monoclonal anti-phosphopeptide antibodies.

3. To each supernatant in the screen plates, add 150 µl screening diluent

   The components of the screening diluent prevent microbial contamination; this reagent also serves to expand the volume should multiple rounds of screening be required.

Screen candidate hybridomas by ELISA

4. Screen an aliquot (e.g., 50 µl) of each candidate hybridoma supernatant from the screen plate in an ELISA using the BSA-conjugated cognate phosphopeptide as antigen. Record the screen-plate number and well position and corresponding to original culture plate number and well position for each positive sample. Also assay preimmune serum from the mouse used for fusion (negative control) and immune serum from the same mouse (positive control), each at a 1:100 dilution in a 1:1 mixture of HT medium and screening diluent. Include an ELISA with screening diluent only (additional negative control).

   It is important to use a different carrier protein in the ELISAs (BSA) than that used for the immunizations (KLH), to avoid detecting reactivity against the carrier (refer to Strategic Planning, above).

5. Screen an aliquot of each positive sample from step 4 in an ELISA against the BSA-conjugated cognate nonphosphopeptide to eliminate clones with phosphorylation-independent reactivity, against the unrelated BSA-conjugated phosphotyrosylpeptide to eliminate clones with indiscriminant phosphotyrosine reactivity, and, if cross-reactivity with homologous phosphoprotein(s) is anticipated, against phosphopeptide-BSA conjugates corresponding to any potentially cross-reacting homologous phosphoproteins.

   These screens may be done sequentially or, if the number of candidate hybridomas has diminished sufficiently, simultaneously. Isolation of clones with phosphorylation-independent cognate peptide specificity may also be useful.

6. Expand clones that satisfy all screening requirements and freeze aliquots as backup; use remainder for subcloning by limiting dilution.

7. Screen culture supernatants from subclones as in steps 4 and 5 to check for continued production and specificity of the antibody.

   Keep representative independent subclones from each original parental colony, as it cannot be predicted whether each parental monoclonal antibody will recognize the cognate holophosphoprotein or function in all types of immunoassays desired in the future. Candidate subclones should then also be expanded and frozen. It is a good idea to continue to check for production of the appropritate antibody after several rounds of serial passaging and after freezing, thawing, and reexpansion.

8. To characterize the subclones further and identify the most useful clones, assay the culture supernatants for reactivity with cognate holophosphoprotein in individual immunoassays (e.g., immunoprecipitations, as described in *UNIT 7.2*, or immunoblot analyses, as described in *UNIT 6.2*).

   Antibody from the desired clones can be produced on a large scale by harvesting large volumes of culture supernatant and performing affinity purification (see Basic Protocol 1), or by producing ascites fluid, which can be purified by ammonium sulfate precipitation followed by affinity purification. Isotype analysis can be performed by using commercially available kits (Pierce or Sigma).

SYNTHESIS OF PEPTIDES

SUPPORT PROTOCOL 1

Many institutions have core facilities for synthesis of peptides using automated solid-phase peptide synthesizers. For phosphotyrosine-containing peptides, the authors originally used standard Merrifield solid-phase procedures with *t*-butyloxycarbonyl (Boc) amino acids and Boc-*O*-(dibenzylphosphono)-L-tyrosine as the phosphorylated residue (Barany and Merrifield, 1979). For cleavage of phosphopeptide from the resin, 1 g phosphopeptide resin was stirred 10 min at 0°C with 2 ml dimethyl sulfide; then an ice-cold mixture of 4 ml trifluoromethanesulfonic acid (TFMSA) and 10 ml trifluoroacetic acid was added slowly with stirring. The resulting reaction mixture was stirred under a nitrogen atmosphere 4 hr at 0°C and the cleaved phosphopeptide precipitated with methyl *t*-butyl ether at −30°C. The precipitate was dissolved in 40% acetic acid and lyophilized several times from water. This original method suffers from low yields. Currently, most phosphotyrosyl-containing peptides are synthesized with 9-fluorenylmethyloxycarbonyl (Fmoc) phosphotyrosine. The lack of a side chain protecting group on the phosphotyrosine (Kitas et al., 1994) allows standard Fmoc synthesis and cleavage procedures to be employed (Chang and Meienhofer, 1978). However, a higher molar excess of activated Fmoc amino acids than that used in standard procedures may be required for efficient coupling of amino acids after unprotected phosphotyrosine has been added. In some circumstances, a double coupling procedure may be employed to add the few amino acids following phosphotyrosine to the growing peptide chain. This alternative requires the sequential, repeated addition of the next particular amino acid to be added to the peptide. A support protocol for the coupling of peptides to an affinity matrix is provided (see Support Protocol 2).

COUPLING OF PEPTIDES TO AFFI-GEL 10 AFFINITY MATRIX

SUPPORT PROTOCOL 2

This protocol describes coupling of phosphopeptides or nonphosphopeptides to Affi-Gel 10 affinity matrix for use in affinity column chromatographic purification of antibodies. Anhydrous coupling, detailed here, is the most efficient coupling method for peptides. The steps here result in production of 3 ml final bed volume of affinity resin; 3 mmol of peptide is coupled per milliliter Affi-Gel 10. According to the manufacturer's specifications, Affi-Gel 10 resin contains ~15 µmol of active ester per milliliter of gel, and the gel has a capacity for 35 mg of protein or 15 to 20 µmol of a low-molecular-weight ligand per milliliter of gel. Note that these capacities are dependent upon the molecular weight and nature of the protein. After coupling, the matrix may be poured into columns of the appropriate size.

Materials

Synthetic oligopeptide for coupling (see Support Protocol 1)
Dimethyl sulfoxide (DMSO)
N-methylmorpholine (99% purity; Acros Organics)
Affi-Gel 10 (Bio-Rad) or equivalent activated support matrix
Ethanolamine
0.1 M ethanolamine·HCl, pH 8.0
High-salt/high-pH solution: 0.5 M NaCl/0.4% (w/v) sodium bicarbonate
High-salt/low-pH solution: 0.5 M NaCl/100 mM sodium acetate, pH 4.2
PBS/azide: PBS (*APPENDIX 2A*) containing 0.02% (w/v) sodium azide
0.5 M NaCl
3 M NaSCN

Polypropylene screw-cap centrifuge tubes (do not use polystyrene tubes with DMSO)
End-over-end rotator
IEC Clinical centrifuge (or equivalent)
Vacuum aspirator
Glass chromatography columns, ≥5-ml capacity

Prepare peptide solution

1. Dissolve oligopeptide in DMSO at a volume ~0.5 to 4 times the desired bed volume.

   For a 3-ml bed volume, dissolve a total of 9 μmol peptide, which for an average 15-mer peptide of mol. wt. ~1700 would be 15.3 mg.

2. Neutralize the peptide solution by titration as follows: add peptide synthesis–grade *N*-methylmorpholine (full-strength or diluted to 1:1 to 1:9 in DMSO) in 1 to 2 μl increments, remove a 2- to 3-μl aliquot after each addition, dilute this with 50 μl water, then spot it onto a strip of pH paper. Repeat this process until a pH in the range of 7 to 8 is reached.

   Titrate carefully, as it is very easy to overshoot the desired pH. Typically 7 to 8 μl of N-methylmorpholine is required per 3 μmol peptide, although the precise amount will depend greatly upon the particular amino acid sequence. The aliquots must be mixed with water because pH is a measure of hydrogen ion concentration, and hence the pH of a nonaqueous solution cannot be measured.

Add peptide to Affi-Gel 10

3. Mix contents of the bottle of Affi-Gel 10 matrix well to resuspend the resin, then transfer the required amount of resin to a screw-cap polypropylene centrifuge tube and wash three times in DMSO, each time by centrifuging 5 min at 700 × *g* (2000 rpm in an IEC Clinical centrifuge, setting 5), room temperature, aspirating the supernatant by vacuum, resuspending the resin in 5 vol DMSO, then centrifuging again at 700 × *g*.

   Be sure to take at least twice as much Affi-Gel 10 as ultimately will be needed, as the volume will shrink. The resin is supplied as a 50% slurry; therefore, it is necessary to take at least four times the required bed volume. Avoid centrifugation at higher than recommended speed as this may result in collapse of the resin.

4. Aspirate the excess DMSO from the resin and add the peptide solution prepared in step 2. Incubate overnight at room temperature with end-over-end rotation.

Quench and wash the resin

5. Add 2 μl pure ethanolamine per milliliter resin and incubate 2 hr at room temperature with end-over-end rotation.

   Ethanolamine is added directly to the coupling reaction to quench unreacted ester groups.

6. Wash twice in DMSO as in step 3.

7. Wash twice in 0.1 M ethanolamine·HCl, pH 8.0, as in step 3 (performing the first wash on ice as a great deal of heat will be generated). Remove the 0.1 M ethanolamine·HCl from the second wash and replace with fresh, incubate tube overnight at 4°C with end-over-end rotation, then centrifuge at low speed and aspirate the supernatant.

8. Wash three times with high-salt/high-pH solution using the technique described in step 3.

9. Wash three times with high-salt/low-pH solution using the technique described in step 3.

10. Wash three times with PBS/azide using the technique described in step 3 and store washed resin at 4°C until ready to pack in column.

11. Mix matrix into a slurry with 0.5 M NaCl and pour into glass chromatography columns.

Commercially available glass chromatography columns can be used; alternatively, 5-ml plastic syringes with 1-cc plugs of silanized glass wool (UNIT 5.6) in the bottom may function as columns.

12. Wash each column with 10 column volumes 3 M NaSCN followed by 10 column volumes PBS/azide.

COUPLING OF PHOSPHOTYROSINE TO AFFI-GEL 10 AFFINITY MATRIX

SUPPORT PROTOCOL 3

This protocol describes coupling of phosphotyrosine to Affi-Gel 10 affinity matrix for use in affinity column chromatographic purification of antibodies. Phosphotyrosine is insoluble under the anhydrous conditions used for coupling peptides (see Support Protocol 2); therefore, coupling is done under aqueous conditions. The steps here result in production of 10 ml final bed volume of affinity resin, as for peptides, 3 μmol phosphotyrosine is coupled per milliliter of Affi-Gel 10. According to the manufacturer's specifications, Affi-Gel 10 resin contains ~15 μmol active ester per milliliter gel, and the gel has a capacity for 35 mg of protein per milliliter gel or 15 to 20 μmol of a low-molecular-weight ligand per milliliter gel. Note that these capacities may vary depending upon the molecular weight and nature of the protein. Phosphotyrosine-agarose affinity matrix is now also available commercially from Sigma.

Additional materials *(also see Support Protocol 2)*

Phosphotyrosine
0.4% (w/v) sodium bicarbonate
1 M NaOH (optional)

Fritted-glass funnel and vacuum aspirator
Glass chromatography column, ≥14-ml capacity

Additional reagents and equipment for coupling peptides to Affi-Gel 10 affinity matrix (see Support Protocol 2)

Prepare phosphotyrosine solution

1. Dissolve phosphotyrosine in 0.4% sodium bicarbonate at a volume ~0.5 to 4 times the desired bed volume.

   For a 10-ml bed volume, dissolve a total of 30 μmol phosphotyrosine. Based on its molecular weight of 261, this amounts to 7.8 mg, although it is acceptable (and may be easier) to use an excess, as phosphotyrosine is relatively inexpensive.

2. Check that the pH is in the range of 7 to 8 using pH paper. If adjustment is necessary, neutralize the solution by titration—i.e., adding small amounts of 1 M NaOH, removing a 2- to 3-μl aliquot after each addition, then spotting the aliquots onto a strip of pH paper. Repeat this process until a pH in the range of 7 to 8 is reached.

Add phosphotyrosine to Affi-Gel 10

3. Mix contents of the bottle of Affi-Gel 10 matrix well to resuspend resin, then transfer the required amount of resin to a fritted-glass funnel attached to a vacuum aspirator. Wash three times, each time pouring cold distilled water over the resin in the funnel and aspirating, then perform one final wash in the same manner using ice-cold 0.4% sodium bicarbonate.

   Be sure to take at least twice as much Affi-Gel 10 as ultimately will be needed, as the volume will shrink. The resin is supplied as a 50% slurry; therefore, it is necessary to take at least four times the required bed volume. Do not allow >20 min to elapse from the time the resin is removed from the bottle to the time it is mixed with the phosphotyrosine solution.

4. Remove most of the excess liquid from the gel by vacuum aspiration without letting it dry completely, transfer it to a screw-cap centrifuge tube, and add the phosphotyrosine solution from step 2.

5. Incubate overnight at 4°C with end-over-end rotation.

Quench and wash resin

6. Add 2 µl pure ethanolamine per milliliter resin and incubate 2 hr at room temperature with end-over-end rotation.

   Ethanolamine is added directly to the coupling reaction to quench unreacted ester groups.

7. Wash resin twice, each time by centrifuging at low speed (see Support Protocol 2, step 3), aspirating the supernatant, resuspending the resin in 5 vol of 0.4% sodium bicarbonate, then centrifuging again at low speed.

8. Wash resin twice with 0.1 M ethanolamine·HCl, pH 8.0, as in step 7. Remove the 0.1 M ethanolamine from the second wash and replace with fresh. Incubate tube overnight at 4°C with end-over-end rotation, then centrifuge at low speed and aspirate the supernatant.

9. Wash, store, and pack resin in columns (see Support Protocol 2, steps 8 to 12).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Screening diluent

6.18 g boric acid (100 mM final)
9.54 g sodium borate (47 mM final)
4.38 g NaCl (75 mM final)
10 g bovine serum albumin (BSA; 1% w/v final)
1 g sodium azide (1% w/v final)
H_2O to 1 liter

The pH of the solution will be 8.4 to 8.5.

COMMENTARY

Background Information

Conventional antibodies have proven to be invaluable tools in numerous techniques for the biochemical analysis of proteins. In the past antibodies with specificity toward the phosphorylated forms of proteins were produced serendipitously. Using techniques such as those described in this unit, such antibodies may now be produced by design. Whereas phosphorylated holoproteins might in general be poor candidates for immunogens in the production of such antibodies as a result of their susceptibility to dephosphorylation, it has been suggested that short phosphopeptides are relatively resistant to phosphatases (Czernik et al., 1991), thereby providing a better chance of success.

A dramatic advance in the analysis of protein tyrosine phosphorylation and the regulation of signal transduction pathways by such phosphorylation occurred with the production of polyclonal and monoclonal anti-phosphotyrosine antibodies (Frackelton et al., 1983; Ross et al., 1981; reviewed in Stern, 1991). These antibodies proved capable of recognizing phosphorylated tyrosine residues in the context of virtually any flanking peptide sequence, and were shown to be exquisitely specific in the requirement for a phosphorylated tyrosine yet at the same time remarkably promiscuous in their acceptance of any peptide sequence. Techniques using polyclonal and monoclonal anti-phosphotyrosine antibodies have supplanted the standard methodology of metabolically labeling proteins with $^{32}P_i$, which was cumbersome, time-consuming, hazardous, and of relatively lower sensitivity.

Antibodies that recognize a specific tyrosyl-phosphorylated peptide as described in this unit represent a marriage of anti-phosphotyrosine technology with the stringent sequence-dependent specificity of conventional antibodies (Roussel, 1990; Bangalore et al., 1992; Epstein et al., 1992; DiGiovanna and Stern, 1995). Conceptually, they may be thought of either as anti-phosphotyrosine antibodies that have strict sequence specificity, or as conventional antibodies possessing the additional specificity of phosphorylation dependence.

Anti-phosphotyrosine antibodies have been most useful in the analysis of tyrosine phosphorylation of proteins in a technique using a combination of immunoprecipitation and immunoblotting (known as the "IP-western" method). In this procedure, a protein typically is immunoprecipitated either with conventional anti-protein antibody or with anti-phosphotyrosine antibody, then immunoblotted with whichever of these two antibodies was not used for the immunoprecipitation. Because anti-phosphopeptide antibodies recognize the cognate protein, but only when it is phosphorylated, they are capable of performing both functions in a single step—i.e., either an immunoprecipitation alone or an immunoblot alone is sufficient to supply the desired information.

The most useful functions for such antibodies are likely to be those that in fact are not possible using traditional reagents. For example, using anti-phosphopeptide antibodies, it is possible to identify and isolate distinct phosphorylated species of a phosphoprotein containing multiple phosphorylation sites. Thus, phosphorylation at each site can be examined independently, and a preparative separation of individual phosphospecies of a holoprotein can be achieved. Another unique application is analysis of the abundance and phosphorylation state of individual proteins in situ in preparations of cells or tissues. Because phosphorylation is a major mode of regulation of protein function, the phosphorylation state is often an indicator of the functional status of a protein. The mere identification of a protein in a cell or tissue specimen gives no indication of its functional status. The ability of anti-phosphopeptide antibodies to demonstrate the phosphorylation state, and by extrapolation the functional state, of a single protein with high specificity places these reagents in a unique class. Hence, identity and functional status may be probed simultaneously using one simple assay. In tissue sections, one may probe to determine whether a particular protein is present, and, if present, in what functional state it is found. The authors have employed this strategy in immunohistochemical staining of formalin-fixed, paraffin-embedded human breast tumors using antibody to the phosphorylated form of the receptor tyrosine kinase Neu (DiGiovanna and Stern, 1995), and were able to demonstrate that Neu is phosphorylated, and therefore functionally active, in only a subset of the tumors that overexpress this protein.

Anti-phosphopeptide antibodies possess unique properties that render them capable of performing functions not possible with conventional reagents. From the technical considerations regarding their production, it would follow that antibodies with specificity for the non-phosphorylated state should also be achievable, and the production of such antibodies has also been reported (Nairn et al., 1982; Roussel, 1990; Czernik et al., 1991; Epstein, 1995; Tzartos et al., 1995; Kawakatsu et al., 1996).

Critical Parameters

In the production of polyclonal anti-phosphopeptide antibodies, a major challenge is the depletion of cross-reactivity. The first consideration in achieving this is the anticipation of potential cross-reacting proteins in the planning phase of the procedure. Cross-reactivity is most likely to occur when the target protein is a member of a family of closely related homologous proteins—e.g., of the subfamilies of tyrosine protein kinases. The authors have produced a monoclonal anti-phosphopeptide antibody with specificity for the Tyr-1248 autophosphorylation site of Neu (DiGiovanna and Stern, 1995). A homologous site exists in the epidermal growth factor receptor (EGFR). The antibody produced in the authors' laboratory does not cross-react with the homologous EGFR site in spite of having identical residues in seven of the 14 amino acids of the peptide sequence (five of which are N-terminal to the phosphotyrosine, one C-terminal, and the phosphotyrosine itself). Thus, specificity is achievable even with at least up to 50% identity. Nevertheless, such a close relationship among the phosphorylation sites of different proteins highlights the importance of considering all known related proteins.

The second consideration in depleting cross-reactivity is the quantity of cross-reactive antibodies in a given aliquot to be purified relative to the capacity of the peptide columns used for the negative selection. Because of the expense of the peptides, the authors have gen-

erally prepared small columns (with 2- to 3-ml bed volumes) and passed the sera over the columns multiple times, empirically determining the number of passes necessary to quantitatively deplete cross-reacting material. In these columns, 3 μmol of ligand is coupled per milliliter of Affi-Gel 10. The manufacturer states that the resin contains ~15 μmol active ester per milliliter gel, and that the gel has a capacity for 35 mg of protein or 15 to 20 μmol of a low-molecular-weight ligand per milliliter gel. The BSA and phosphotyrosine columns are relatively inexpensive to produce; thus, a single large column of each is practical and sufficient.

Because the authors elute the antibodies from the positive-selection affinity column in strongly chaotropic solutions that are potentially deleterious to the stability of the antibody (3 M NaSCN or 3.5 M followed by 4.5 M $MgCl_2$), care is taken to collect the fractions in preprepared dialysis tubing so that they may be immediately placed into the PBS dialysate, thus minimizing the time that the antibodies are exposed to the eluting solutions. Although $MgCl_2$ is thought to be "gentler," the authors have found that gravity-driven flow rates from phosphopeptide columns eluted with this salt quickly become extremely slow, possibly because of precipitation of the salt in the column or an interaction of the Mg^{2+} ion with the phosphate groups. Thus use of NaSCN is preferred, and this salt appears to permit recovery of comparable activity.

In the production of monoclonal anti-phosphotyrosyl peptide antibody, which the authors have carried out once, a major technical hurdle was the low frequency with which clones of the desired specificity were produced (DiGiovanna and Stern, 1995). In that work, in which antibody was produced to the phosphorylated form of the receptor tyrosine kinase Neu, >1200 hybridomas (obtained from a single fusion) were screened to obtain a single clone that satisfied all requirements. The authors found 68 candidate clones that recognized the cognate phosphopeptide, of which only 20 were unreactive toward the cognate nonphosphopeptide. Of those, seven cross-reacted with an unrelated phosphopeptide (i.e, exhibited indiscriminant phosphotyrosine activity), and of the remainder three cross-reacted with the homologous EGFR. The remaining ten were subcloned by limiting dilution, after which only five continued to produce antibody. Of these five, only one reliably detected the phosphorylated holoprotein in immunoblots and immunoprecipitations. Thus, the "hit rate" for an antibody that satisfied all requirements was <1 in 1000 clones. As this is the only monoclonal anti-phosphotyrosyl peptide antibody produced by the authors to date, it is impossible be sure whether this low frequency will be a general phenomenon. It is crucial to devise a convenient and reliable ELISA assay for such extensive screening. Other assays may require extensive optimization. For example, the monoclonal antibody produced in the above project performed poorly in immunoblotting when any detection system other than a very sensitive chemiluminescence-based assay was used. This was possibly due to a low affinity of the antibody for the cognate protein. In addition, extensive optimization, particularly aimed toward enhancing the strength of the signal, was required for use of the antibody in immunohistochemistry. Finally, it is important to use different carrier proteins for the immunizing conjugate and the ELISA conjugate, so that antibodies to the carrier used for the immunization will not give positive reactions in the ELISA. Some authorities also advocate using different cross-linking agents, as antibodies against the cross-linker itself could theoretically be generated as well (Doolittle, 1986; Czernik et al., 1991).

Troubleshooting

In the production of polyclonal anti-phosphopeptide antibodies, the most common unfavorable outcomes are persistence of cross-reactivity, loss of specific reactivity, and poor yield. Although it may be tempting, in the interest of saving time, to perform the entire purification and postpone all analyses until the end (a reasonable course of action once a scheme is known to work), it may be prudent, especially with a new protocol, to analyze the serum at each step. For example, the authors have produced antibody to phosphorylated EGFR (M. Digiovanna, M.A. Lerman, and D. Stern, unpub. observ.). The crude antiserum cross-reacted with the related receptors Neu and HER-4, which had been anticipated. The purification was monitored by probing, after each column, an immunoblot consisting of four lanes containing lysates from cells overexpressing (1) phosphorylated EGFR, (2) nonphosphorylated EGFR, (3) phosphorylated Neu, and (4) phosphorylated HER-4. In following the depletion of cross-reactivity, such stepwise analysis demonstrates whether undesired cross-reactivity has been eliminated or whether further passes through a particular negative-selection column are necessary.

If loss of specific reactivity is also a problem, stepwise analyses can also demonstrate where the loss is occurring. Loss of specific reactivity can occur for three major reasons. The first possible reason is that the antibody is not stable over the course of the purification, which may be the case if the procedure is very prolonged. If this is suspected, the pace of the purification may be hastened or the procedure may be carried out at 4°C instead of room temperature. With prolonged purifications, it is especially important to include sodium azide in the PBS used to dilute the serum, to prevent microbial growth. Activity may also be lost nonspecifically as a result of the multiple manipulations in a protocol requiring multiple columns and/or multiple passes through each column. In this case, fraction analysis may indicate which columns are absolutely essential, and also indicate the minimum necessary number of passes through each column. Also, creating larger columns with greater capacity should reduce the number of times the serum will need to be passed over each to deplete cross-reactivity. Finally, specific activity may be lost as a result of the presence in one of the negative-selection columns of a cross-reacting peptide that is so similar to the peptide of interest that essentially any antibody that recognizes the cognate phosphopeptide will recognize this phosphopeptide as well. In this last theoretical scenario, ultimate unique specificity may not be achievable.

The columns used in the purifications can be regenerated and used repeatedly if stored at 4°C in sodium azide–containing buffer to prevent microbial contamination and consequent damage to the matrix. A theoretical consideration is that with repeated use, phosphatases in serum may eventually cleave enough phosphate groups from the peptide moieties on the column to render it ineffective as a phosphopeptide column. Thus, it may be prudent to periodically prepare fresh columns. An alternative would be to add phosphatase inhibitors to the serum prior to purification. Similarly, proteases in serum may eventually cleave peptides from the resin, and this may be prevented by adding protease inhibitors to the serum.

As discussed in Critical Parameters, the main technical hurdle in the production of monoclonal antibodies appears to be the large number of clones that it may be necessary to screen to find one with the desired reactivity. Here, time and persistance are the primary defenses. Another obstacle may be the failure of hybridoma cells to continue to produce antibody upon subcloning, which is likely due to the continuing genetic instability of hybridomas at early passage. If the parental hybridoma colony has been expanded and frozen prior to subcloning, this will serve as a potential source for additional subclones, although it is possible that these additional daughter clones may also be nonproducing. The use of the final monoclonal antibody in a variety of immunoassays is more likely to require potentially extensive optimization of protocols and higher concentrations (because of lower affinities) than the use of polyclonal antibodies.

Anticipated Results

In the production of polyclonal antibodies, the authors have obtained yields of ~1 mg of purified anti-phosphopeptide antibody from a single 15-ml serum sample (M. Digiovanna, M.A. Lerman, and D. Stern, unpub. observ.). These antibodies have been used in the immunoprecipitation of phosphoproteins from both denaturing and nondenaturing solutions. They have also been used in immunoblotting, in immunofluorescence on fixed cultured cells, and in immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections. Specificity can be demonstrated when such assays are inhibited by cognate phosphopeptide but not by cognate nonphosphopeptide or unrelated phosphopeptides.

Through the production of monoclonal antibodies a permanent supply of a uniform reagent is ensured. Many milligrams can be obtained from the ascites produced by a single mouse. The authors have used such an antibody in all of the same assays as described above for polyclonal antibodies. More extensive optimization of protocols is likely to be required with monoclonal antibodies, and these may be expected to have a lower affinity than typical polyclonal antibodies. Isotype analysis can easily be performed by use of commercially available kits (Pierce or Sigma).

Time Considerations

For both protocols, the principal time-consuming step is the production of an immune response in the immunized animals. For the production of polyclonal antibodies, the authors have obtained high titers of reactive sera as early as 6 to 8 weeks in animals that have been boosted every 2 weeks. Later bleeds with continued boosts have yielded even higher titers. Once adequate bleeds have been obtained and the necessary affinity columns prepared, purification can generally be carried out within 1 week, with the exact length of time depending

upon the number of columns and the necessity of analyzing the product after each step.

In the production of monoclonal antibodies, the authors have harvested splenocytes at ~2 months from the initial immunization, with several boosts in between. The screening of hybridoma supernatants can take 1 to 2 weeks, and their expansion and subcloning several weeks more.

Literature Cited

Bangalore, L., Tanner, A.J., Laudano, A.P., and Stern, D.F. 1992. Antiserum raised against a synthetic phosphotyrosine-containing peptide selectively recognizes $p185^{neu/erbB-2}$ and the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. U.S.A.* 89:11637-11641.

Barany, G. and Merrifield, B. 1979. Solid phase peptide synthesis. *In* The Peptides, Vol. I (E. Gross and J. Meienhofer, eds.) pp. 1-284. Academic Press, New York.

Chang, C.D. and Meienhofer, J. 1978. Solid-phase peptide synthesis using mild base cleavage of *N* alpha-fluorenylmethoxycarbonylamino acids, exemplified by a synthesis of dihydrosomatostatin. *Int. J. Pept. Protein Res.* 11:246-249.

Czernik, A.J., Girault, J.-A., Nairn, A.C., Chen, J., Snyder, G., Kebabian, J., and Greengard, P. 1991. Production of phosphorylation state–specific antibodies. *Methods Enzymol.* 201:264-283.

DiGiovanna, M.P. and Stern, D.F. 1995. Activation state–specific monoclonal antibody detects tyrosine phosphorylated $p185^{neu/erbB-2}$ in a subset of human breast tumors overexpressing this receptor. *Cancer Res.* 55:1946-1955.

Doolittle, R.F. 1986. Of urfs and orfs: A primer on how to analyze derived amino acid sequences. University Science Books, Mill Valley, Calif.

Epstein, R.J. 1995. Preferential detection of catalytically inactive c-erbB-2 by antibodies to unphosphorylated peptides mimicking receptor tyrosine autophosphorylation sites. *Oncogene* 11:315-323.

Epstein, R.J., Druker, B.J., Roberts, T.M., and Stiles, C.D. 1992. Synthetic phosphopeptide immunogens yield activation-specific antibodies to the c-erbB-2 receptor. *Proc. Natl. Acad. Sci. U.S.A.* 89:10435-10439.

Frackelton, A.R. Jr., Ross, A.H., and Eisen, H.N. 1983. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor–stimulated cells. *Mol. Cell. Biol.* 3:1343-1352.

Kawakatsu, H., Sakai, T., Takagaki, Y., Shinoda, Y., Saito, M., Owada, M.K., and Yano, J. 1996. A new monoclonal antibody which selectively recognizes the active form of src tyrosine kinase. *J. Biol. Chem.* 271:5680-5685.

Kitas, E., Kung, E., and Bannwarth, W. 1994. Chemical synthesis of *O*-thiophosphotyrosyl peptides. *Int. J. Pept. Protein Res.* 43:146-153.

Nairn, A.C., Detre, J.A., Casnellie, J.E., and Greengard, P. 1982. Serum antibodies that distinguish between the phospho- and dephospho-forms of a phosphoprotein. *Nature* 299:734-736.

Ross, A.H., Baltimore, D., and Eisen, H.N. 1981. Phosphotyrosine-containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten. *Nature* 294:654-656.

Roussel, R. 1990. Synthetic phosphopeptides modeled on the carboxyl terminus of $pp60^{C\text{-}SRC}$: Specific antibodies, binding and effects on kinase activity. M.S. thesis, University of New Hampshire, Durham.

Stern, D.F. 1991. Antiphosphotyrosine antibodies in oncogene and receptor research. *Methods Enzymol.* 198:494-501.

Tzartos, S.J., Kouvatsou, R., and Tzartos, E. 1995. Monoclonal antibodies as site-specific probes for the acetylcholine-receptor δ-subunit tyrosine and serine phosphorylation sites. *Eur. J. Biochem.* 228:463-472.

Key References

Bangalore et al., 1992. See above.

A description of the production of polyclonal antiphosphopeptide antibody by the method described in this unit.

Czernik et al., 1991. See above.

A general discussion of the production of phosphorylation-dependent antibodies.

DiGiovanna and Stern, 1995. See above.

A description of the production of monoclonal antiphosphopeptide antibody by the method described in this unit.

Doolittle, 1986. See above.

A general discussion of the analysis of protein sequences and the production of anti-peptide antibodies.

Epstein et al., 1992. See above.

A description of the production of polyclonal antiphosphopeptide antibody by a variation of the method described in this unit.

Harlow, E. and Lane, D. 1988 Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

A comprehensive text describing the production and utilization of antibodies.

Contributed by Michael P. DiGiovanna
Yale University School of Medicine
New Haven, Connecticut

Robert R. Roussel
Dartmouth College
Hanover, New Hampshire

David F. Stern
Yale University School of Medicine
New Haven, Connecticut

CHAPTER 17
Macromolecular Interactions in Cells

INTRODUCTION

The capacity of proteins to perform different functions within cells is essentially conferred by information provided within the proteins themselves. That is, the function of the protein is determined by the one-dimensional sequence of amino acids encoded within the mRNA from which the protein is translated. However, in order for proteins to accomplish the complex tasks required for cell viability, this information must direct not only the three-dimensional folding and post-translational processing of the protein itself, but also the interaction of the protein with other molecules of the cell. These interactions may include binding to small ligands, enzyme substrates, nucleic acids, or other proteins. It is therefore not surprising that elucidating the interactions of proteins with other cellular components has become a major task in transforming molecular biological knowledge of protein sequences into full understanding of protein functions within cells.

Many traditional methods for detecting protein-protein interactions have relied upon biochemical co-purification of tightly associated protein complexes. In addition to conventional column chromatography, rapid affinity methods for co-purification have been developed (*UNIT 17.5*). These methods typically rely upon purification using engineered expression constructs producing proteins with tags that allow retention on an affinity matrix or upon purification by immunoprecipitation (*UNIT 7.2*). After isolation of the protein of interest through these methods, associated proteins can be detected by protein staining (*UNIT 6.6*) of the purified fraction. Alternatively, the interaction of known proteins can be assayed through Western blotting (*UNIT 6.2*), and novel interactions can be documented through methods based on mass spectrometry and bioinformatics (*UNIT 17.5*). These methods can be further extended through the use of chemical cross-linkers, particularly for analysis of multiprotein complexes (*UNIT 17.10*). Cross-linking strategies are particularly valuable when interactions between complex components are relatively transient or lose specificity outside of their native contexts. Moreover, because cross-linkers act at very close proximity, they provide information about the relative spatial distribution of complex subunits with respect to each other.

Alternative methods to identify protein-protein interactions through modified ligand blotting protocols have also been developed (*UNIT 17.2*). In such far-western blotting methods, a protein of interest is immobilized on a membrane support and probed with another non-antibody protein. This method has been particularly useful in examining the interaction between proteins within complex mixtures and in examining proteins whose insolubility or biochemical behavior make them ill-suited for traditional approaches.

A number of methods have been developed for broad-based genetic screening to find novel interactions. The two-hybrid system or interaction trap screening (*UNIT 17.3*) allows evaluation of peptides encoded within large cDNA libraries for interactions with a protein of interest. Interactions between the protein of interest and peptides encoded within the library result in transcriptional activation that can be assayed using simple selection criteria. Moreover, these protocols can also be adapted to test specific association between two proteins for which there is a prior reason to expect an interaction,

Contributed by Mary Dasso
Current Protocols in Cell Biology (2006) 17.0.1-17.0.3

again by measuring the extent of transcriptional activation. Smaller epitopes involved in protein-protein interactions can be found using phage-display combinatorial peptide libraries (*UNIT 17.4*). In this method, peptides are fused to the capsid proteins of the filamentous M13 bacteriophage, such that they remain exposed after the assembly of viable phage particles. Peptides are selected by affinity of the phage particles to immobilized proteins of interest. Through multiple rounds of affinity selection, it is possible to isolate phage expressing fused peptides that are characteristic of optimal ligand preferences or protein interaction modules. Peptides found through this method either can be utilized directly for study of the protein of interest or they can be used to search databases in order to identify potential protein-protein interaction partners.

Once interaction partners have been identified, a number of biophysical methods offer the capacity for detailed characterization of binding affinities and kinetics. One attractive method that has become increasingly valuable during the past decade is the use of optical biosensors (*UNIT 17.6*). Optical sensors provide precise measurement of the refractive index near the surface of a sensor. When a protein of interest is immobilized on the sensor, its interactions with partners in the solution above can be monitored. Analysis of steady-state interactions using biosensors can provide equilibrium binding constants over a wide range of affinities. If set up appropriately, optical biosensors can also provide accurate real-time measurements of association, from which chemical on- and off-rates can be deduced.

Precise information about the thermodynamics of a broad variety of macromolecular interactions can be obtained through isothermal titration calorimetry (ITC; *UNIT 17.8*). Changes in macromolecular interactions, for instance the binding of two proteins, are accompanied by the release (exothermic reaction) or absorption (endothermic reaction) of heat. The heat thus produced or consumed is monitored in ITC using a heat-flux calorimeter, which measures the amount of power required to maintain a constant temperature difference between the reaction cell and a reference cell. This measurement can be used to calculate the Gibbs energy, enthalpy, entropy, and heat capacity of the reaction under study.

The obvious limitation of the methods discussed above is that they assay protein interactions outside of the normal context of intact cells. The absence of a cellular context frequently causes a loss of spatial or temporal resolution regarding how macromolecular interactions occur in vivo. Chromatin immunoprecipitation (*UNIT 17.7*) is frequently used to monitor the association between proteins and DNA within intact cells. In this method, native interactions are preserved by formaldehyde cross-linking of protein-DNA and protein-protein complexes. After the DNA is fragmented into shorter pieces, complexes are precipitated with antibodies against proteins of interest, resulting in co-precipitation of the DNA sequences that are directly or indirectly associated with individual proteins. The cross-links can then be chemically reversed, allowing analysis of the DNA sequences by direct methods such as PCR. Recently, methods have also been developed to assay protein-protein interactions directly within cells via the use of fluorescence resonance energy transfer (FRET; *UNIT 17.1*). FRET is a process through which a donor fluorophore transfers energy to an acceptor fluorophore through dipole-dipole coupling. Protein-protein interactions can be detected through this photophysical process by covalently coupling donor and acceptor fluorophores with appropriate excitation and emission spectra to two proteins of interest. Due to the sixth-order distance dependence of the FRET process, efficient FRET occurs only when the fluorophores are in immediate proximity. FRET can be measured effectively by donor quenching through acceptor photobleaching. These powerful new techniques will complement more traditional biochemical methods and extend the study of interactions between molecules in their true biological context.

In an era of rapidly expanding databases of nucleic acid and protein sequences, a true appreciation of how macromolecules function within cells will require not only data about the expression of these molecules, but also knowledge about their localization and relationship to other cellular components. Thus, techniques for determining how macromolecules recognize and interact with each other will provide a key element of cell biological studies, essential for opening the door between genomic information and a deeper understanding of biology.

Mary Dasso

Imaging Protein-Protein Interactions by Fluorescence Resonance Energy Transfer (FRET) Microscopy

Specific protein-protein interactions, whether induced by covalent protein modifications or not, are generally considered to mediate cellular signaling and function. Detection of these processes has long been restricted to bulk biochemical methods such as immunoprecipitation (*UNIT 7.2*) and immunoblotting (*UNIT 6.2*). These approaches have proven invaluable, e.g., in uncovering the major signal transduction pathways by delineating the hierarchy of protein-protein interactions and kinase-substrate relationships in the different phosphorylation cascades. However, immunoblotting techniques lack spatial information and the interactions that are detected depend on the stability of the complex during the experimental conditions that exist outside the cell in homogenates. Detection of proteins using immunofluorescence provides spatial information on the micrometer scale, and it is therefore not possible to infer protein-protein interactions.

Limited information on the phosphorylation status of proteins can be obtained by the application of phospho-specific antibodies against specific phosphorylated residues in a given protein (see *UNIT 14.2*). The use of these antibodies is, however, restricted by availability and specificity. Only a small number of antibodies against phosphoproteins exist and phosphotyrosine residues have proven to produce the most specific antibodies thus far, leaving a large number of modifications undetectable by microscopy.

This unit describes the preparation and execution of a typical fluorescence resonance energy transfer (FRET) experiment. The following procedures will specifically refer to the use of a GFP-tagged protein as donor and Cy3-labeled antibodies as acceptor. As an example, covalent modification by tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) is determined by FRET between C-terminal GFP-tagged EGFR and Cy3-labeled anti-phosphotyrosine antibodies (Wouters and Bastiaens, 1999). FRET is measured by release of donor quenching through acceptor photobleaching. An advantage of this method is the convenient setup; it does not require specialized equipment but can be performed using common microscopy equipment, preferably a confocal laser scanning microscope (Bastiaens and Jovin, 1996; Bastiaens et al., 1996; Wouters et al., 1998). The method is quantitative, and by simple relationships, a FRET efficiency is obtained. The sample preparation described in this unit is identical for other FRET microscopy techniques or other donor-acceptor pairs. As an illustration, the result of a more advanced FRET measurement using fluorescence lifetime imaging microscopy (FLIM), which is based on the change in GFP fluorescence lifetime (see Background Information), is also included (Bastiaens and Squire, 1999; Ng et al., 1999; Wouters and Bastiaens, 1999).

This unit describes FRET microscopy based on release of quenched donor fluorescence after acceptor photobleaching (see Basic Protocol), microinjection of reagents into the nucleus or cytosol (see Support Protocol 1), and labeling antibodies with Cy3 (see Support Protocol 2).

FRET MICROSCOPY OF FIXED CELLS

BASIC PROTOCOL

A number of quantitative FRET microscopy techniques are available at this time. A technique based on the release of quenched donor fluorescence after acceptor photobleaching will be described in this unit. This technique requires a pixel-by-pixel reference image to be created by photobleaching of the acceptor through continuous illumination at the absorption maximum of the acceptor. The time scale of the pho-

Contributed by Fred S. Wouters and Philippe I.H. Bastiaens
Current Protocols in Cell Biology (2000) 17.1.1-17.1.15

tobleaching process is on the order of minutes. In a lifetime-based FRET approach, the photobleaching step is used at the end of a time-lapse sequence of measurements to introduce an intracellular reference in live cells. In the donor intensity approach (unquenching), described here in detail, the time scale of photobleaching restricts the technique to fixed cells but has the advantages of being relatively simple and quantitative.

Materials

Cells of interest
Plasmid for GFP-tagged protein
Transfection reagent (e.g., Fugene 5 from Boehringer Mannheim, Lipofectin from Life Technologies, Effectene from Qiagen, or Superfect from Qiagen)
Serum-free medium
Low-background fluorescence CO_2-independent medium (Life Technologies, or see recipe)
Phosphate-buffered saline (PBS; see recipe), pH 7.4
4% (w/v) formaldehyde fixative solution (see recipe)
Quench solution: 50 mM Tris·Cl (pH 8.0)/100 mM NaCl (*APPENDIX 2A*)
0.1% (v/v) Triton X-100 in PBS
Antibody (e.g., PY72 monoclonal anti-phosphotyrosine antibody) labeled with Cy3 (see Support Protocol 2)
1% (w/v) bovine serum albumin (BSA, fraction V) in PBS
Mowiol mounting medium (see recipe)

6- and 12-well tissue culture plates
Coverslips
Microscope slides
Confocal laser scanning microscope (e.g., Zeiss LSM 510), equipped with argon (488 nm) and He/Ne (543 nm) lasers selected by the HFT 488/543 double dichroic filter, GFP fluorescence selected by the NFT 545 dichroic and BP 505-530 emission filter, and Cy3 fluorescence selected by the LP560 emission filter
Imaging software package (e.g., NIH-image or IPLab Spectrum from Scanalytics)

Additional reagents and equipment for transfection of mammalian cells (*APPENDIX 3A*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Prepare cells

1. Seed the cells onto culture dishes containing coverslips.

   For live-cell FRET experiments, seed the cells onto glass-bottom MatTek culture dishes.

   The cells should be adherent and grow in a monolayer.

2. Transfect cells with the plasmid for GFP-tagged protein of interest using calcium phosphate precipitation, lipofection, or the activated dendrimer reagent, Superfect.

   For cells that are difficult to transfect, due to resistance to transfection (e.g., primary cell lines), or due to cytotoxicity of the transfection reagent, the DNA for the GFP-tagged protein can be introduced by nuclear microinjection (see Support Protocol 1). The latter method is also particularly useful when the expression levels of the protein of interest have to be controlled accurately.

3. Grow the cells, typically 1 to 2 days post transfection, until expression levels of the GFP-tagged protein are high enough to detect with fluorescence microscopy. When needed, starve the cells overnight with serum-free medium to make them semi-quiescent.

   For cells that undergo apoptosis upon serum deprivation, lower the serum concentration in the medium to 0.5%.

4. On the day of the experiment, transfer the coverslips to a 12-well tissue culture plate.

   Subject the cells to the desired experimental conditions (e.g., growth factor/hormone stimulation, or incubation with drugs or inhibitors).

Fix cells

5. Wash the cells with ice-cold PBS, aspirate, and add 1 ml 4% formaldehyde fixative solution. Allow cells to fix at room temperature for 10 min.

   The common alternative fixation protocol, where cells are incubated in −20°C methanol for 5 min, is not advised. Strictly speaking, this treatment does not fix the tissue, but precipitates the cellular proteins. Protein-protein interactions are therefore likely to be affected.

6. Quench excess fixative with quench solution by briefly washing the cells, then changing to fresh quench solution and incubating 5 min at room temperature.

   In a first short wash, the excess fixative is removed. In a second 5-min incubation, the remaining aldehyde groups are allowed to react with the primary amino group on the Tris molecule. Alternative, equally effective, quench solutions are 0.1 M hydroxylamine or 0.1 M glycine.

7. Permeabilize cellular membranes by incubating 5 min with 0.1% Triton X-100 to allow penetration of acceptor-labeled molecules into fixed cells.

   In this treatment, a compromise is made between optimal morphology and permeabilization. A milder detergent treatment with 0.1% saponin in PBS or a harsher permeabilization with −20°C methanol for 5 min can be considered if morphology or permeabilization, respectively, is compromised.

8. Wash the cells with PBS to remove the permeabilization solution.

Add antibody

9. Dilute Cy3-labeled antibodies appropriately in 1% BSA/PBS. Incubate the coverslips with appropriately diluted Cy3-labeled antibodies for 1 hr, at room temperature.

   Typical concentrations are 0.1 to 10 μg/ml, but it is advised that a titration series be performed to determine the antibody concentration where epitope binding saturates.

   To minimize the amount of antibody needed, press a sheet of Parafilm to the bench with some water, place 25-μl drops of antibody solution on the Parafilm, place the coverslips cells-down on these drops, and incubate 1 hr. To facilitate handling of the coverslips at the end of the 1-hr incubation, place a pipet to the edge of a coverslip and add ~100 μl PBS underneath it. This will lift the coverslip so that it can be easily picked up with jeweler's forceps.

10. Transfer the coverslips to 6-well tissue culture plates and wash four times, each time with 3 ml PBS, to remove excess antibody.

11. Blot off excess PBS with tissue and mount the coverslips on slides with ~10 μl Mowiol mounting solution. Allow Mowiol to harden overnight at 4°C before imaging.

If necessary, cells can be imaged after a short drying period of 1 hr if the coverslip is attached to the slide by painting the edge of the coverslip with molten agarose or rubber cement at four points. Do not use nail polish since this has been shown to quench GFP fluorescence.

Examine cells and calculate results

12. View the specimen on a confocal microscope using a 63× or 100× oil-immersion objective. Acquire an image in the GFP channel (excitation, 488 nm; emission, NFT 545, BP505-530). Either take an image of the entire field of vision or select a region of interest containing the cell that is to be imaged.

    GFP fluorescence is selected by the NFT 545 dichroic and BP 505-530 emission filter.

    Do not use the full dynamic range of the detector, since unquenching will result in added fluorescence. Depending on the make of the confocal microscope, make sure that a second image can be made at exactly the same location. Do not adjust the settings (i.e., pinhole size, contrast, brightness, laser power, or averaging) for the GFP channel.

    This acquisition provides the FRET-quenched donor image (F_{DA}), since the acceptor is present throughout the cell, causing FRET with the GFP donor.

    Minimize GFP photobleaching at this point by limiting the illumination.

13. Change to the Cy3 channel (excitation, 543 nm; emission, LP560) and take an image of the cell, minimizing Cy3 photobleaching. Select a portion of the cell in which the FRET efficiency is to be determined.

    Cy3 fluorescence is selected by the LP560 emission filter.

    This region is where the acceptor will be photobleached, thereby revealing the unquenched donor intensities enabling FRET calculation.

    Consequently, any portion of the cell where the acceptor is not photobleached serves as control [i.e., $1 - F_{DA}/F_{DA} = 0$]. This area in the same cell will provide an essential control to judge the effects of photobleaching GFP, lateral movement, or focal mismatch between the two consecutive donor images.

14. Photobleach the selected acceptor region by repeated scanning with the 543-nm He/Ne laser line at full power. Follow the progress of photobleaching by monitoring the intensities of the respective images (emission: NFT 545, BP505-530). Continue until there is no more discernible Cy3 intensity.

    Depending on the staining intensity and the area that is bleached, this will typically take 1 to 20 min.

    When selecting the photobleaching region on the Zeiss LSM 510 confocal microscope, crop the acceptor image to this region (and note the zoom settings to return to the original image after bleaching) instead of selecting a region of interest (ROI). In the latter option, the entire area will be scanned but the laser will only switch on in the ROI. Consequently, the duty cycle is low and bleaching will take considerably longer.

15. Return to the GFP channel and make the second acquisition, using identical settings and location of the prebleached image.

    This provides the F_D reference in the region where the acceptor was photobleached.

16. To calculate the FRET efficiency in the bleached area, use an appropriate imaging software package. Subtract the prebleach donor image from the post-bleach donor image (i.e., $F_D - F_{DA}$). Divide this image by the postbleach donor image: $(F_D - F_{DA})/F_D$; this is identical to $1 - (F_{DA}/F_D) = E$, the image of FRET efficiencies.

    A software package capable of performing the abovementioned image processing is NIH Image. This package is freely available from http://rsb.info.nih.gov/nih-image/ and ver-

sions are available for Apple Macintosh, Windows (Scion image) and even a Java version is available that runs on any platform (Image J). A more versatile commercial package is IPLab Spectrum.

Image arithmetic for calculating the FRET efficiency should be performed on a region of interest obtained by thresholding the images in order to prevent the amplification of background noise. Therefore, thresholds should be chosen in such a way that the background is omitted in the calculation. Accuracy can be improved by subtracting the average background intensity from the images before calculating the FRET efficiency.

NUCLEAR AND CYTOSOLIC MICROINJECTION

SUPPORT PROTOCOL 1

The following is a generic protocol for introduction of DNA or labeled proteins into a cell. DNA is microinjected directly into the nucleus to achieve controlled and high expression of the protein of interest. Labeled proteins are injected into the cytosolic region next to the nucleus since the cell is at its highest here, facilitating injection.

Materials

Cells of interest, cultured in MatTek glass-bottom 35-mm dishes (MatTek)
DNA (e.g., human EGFR cDNA in the Clontech pEGFP-N1 expression vector; *APPENDIX 3A*)
HPLC-grade water

Millex-GV4 0.22-μm filtration unit
GELloader tips (Eppendorf)
Needles for microinjection (e.g., Femtotip from Eppendorf)
Microinjector (e.g., Eppendorf model 5244)
Micromanipulator (e.g., Eppendorf model 5170)
Inverted microscope with 10× and 40× air objectives

Additional reagents and equipment for preparation of DNA (*APPENDIX 3A*)

1. Prepare DNA, to be microinjected, of the highest possible quality.

   In the authors' experience, double cesiumchloride–banded DNA and DNA purified by Qiagen ion-exchange resin (Qiaprep midi/maxiprep columns) perform equally well.

   Cy3-labeled protein (e.g., antibody against protein of interest; see Support Protocol 2) may also be microinjected.

2a. *For optimal expression a few hours after microinjection:* Dilute DNA in HPLC-grade water to 100 μg/ml.

2b. *For expression overnight:* Dilute DNA in HPLC-grade water to 1 μg/ml.

3. Clear the DNA solution to prevent blocking of the glass needle during microinjection as follows. Place a 0.22 μm Millex filtration unit in a 0.5-ml microcentrifuge tube and place the entire unit in a 1.5-ml microcentrifugation tube to enable centrifugation. Filter 10 μl of the DNA solution by microcentrifugating 1 min at maximum speed, room temperature.

   Since these membranes have low-protein-binding characteristics, they can also be used for clearing Cy3-labeled proteins (see Support Protocol 2).

   The recovery of Cy3-labeled antibodies when cleared this way is also generally very high. Alternatively, microcentrifuge the Cy3-labeled protein for 20 min at maximum speed. Sacrifice a small amount of solution to prevent disturbing the pellet of aggregated protein.

4. Load 2 µl of DNA solution (or Cy3-protein solution) using GELloader tips into the capillary glass needle of the microinjector.

   Commercially available needles (Femtotip from Eppendorf) can be used. These needles fit directly into the needle holder of the Eppendorf microinjection device. Carefully remove the pipet tip that protects the needle. In the authors' experience, this is most easily performed by holding the needle pointing downwards and loosening the tip by rotation until it falls to the ground. If access is available to a needle-pulling device, make sure that the diameter of the needle opening is ~0.25 µm. The diameter of the needle opening can be estimated using a simple syringe-operated micropipet bubble meter (Clark Electronic Instruments) by measuring the air pressure required to expel air bubbles from the pipet into a liquid (Mittman et al., 1987).

5. On an inverted microscope, microinject the DNA solution (or Cy3-protein solution) into the nucleus (or perinuclear cytosol) of cells grown in MatTek culture dishes.

   Typical settings are: 0.3 sec, 150 to 400 hPa injection pressure with 20-hPa back-pressure to prevent medium from entering the needle.

   The injection pressure may be varied according to the needle opening and cell type.

   No major movement of the nucleus (or cell organelles) should be observed. A visual indication for excessive pressure is the separation of the nucleus from the surrounding cellular material (i.e., light ring around the nucleus) and leakage into the cytosol, visible by movement of the cellular organelles.

   Restrict the microinjection procedure to a maximum of 10 min. In the authors' laboratory, microinjection is performed at room temperature in normal CO_2-dependent medium. After 10 min, the medium starts to acidify significantly (i.e., purple medium). CO_2-independent (or HEPES-buffered) media can be used for longer periods.

SUPPORT PROTOCOL 2

PROTEIN LABELING WITH Cy3

Proteins are labeled on unprotonated free amino groups (i.e., α-amino terminus or ε-amino groups on lysine side chains) by the succinimide esters of the fluorescent sulfoindocyanine (Cy) dyes (also see *UNIT 16.5*). Alkaline labeling conditions ensure deprotonation of amino groups. Cy-dyes are water soluble and have high extinction coefficients, making them particularly useful for sensitive detection of proteins with minimal disturbance of protein function. The following protocol describes the labeling of antibodies with Cy3, a suitable donor for GFP in a FRET experiment, and also explains how to remove stabilizing compounds (e.g., gelatin, BSA), which are often added to prolong shelf life, as these contain amino groups that would compete with the labeling reaction.

Materials

Antibody (PY72 monoclonal anti-phosphotyrosine antibody)
1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
10 mM and 100 mM Bicine/NaOH, pH 8.0
100 ml citric acid/NaOH, pH 2.8
1 M Bicine/NaOH, pH 9.0
1 M NaCl (*APPENDIX 2A*)
Labeling buffer: 100 mM Bicine/NaOH (pH 8.0)/100 mM NaCl
Cy3.29–OSu monofunctional sulfoindocyanine succinimide ester (Amersham Pharmacia Biotech)
Dimethylformamide (DMF) dried by addition of 10 to 20 mesh 3-Å pore diameter molecular sieve dehydrate (Fluka)
1-ml Protein G HiTrap columns (Amersham Pharmacia Biotech)
Centricon YM30 concentrators (Amicon)

Biogel P6DG Econopac prepacked size-exclusion columns (5.5 × 1.5–cm, ~10 ml; Bio-Rad)

1-ml and 10-ml syringes with HPLC Luer-Lok fitted tubing

Additional reagents and equipment for spectrophotometric protein determination (*APPENDIX 3B*)

Prepare antibody solution

1. Resuspend antibody to 1 mg/ml in PBS.

   Excess stabilizing agents containing free amino groups (e.g., BSA and gelatin) in commercially available antibody preparations compete for the labeling reagent and have to be removed by Protein A or Protein G affinity chromatography (UNIT 16.3). If the protein solution to be labeled is free of additional amino groups, proceed directly to step 10. A number of suppliers of antibodies, e.g., Transduction Laboratories and New England Biolabs, can provide their products free of these compounds. Request that the antibodies be provided at 1 mg/ml concentration in PBS.

2. Prepare a syringe-operated 1-ml protein G HiTrap column. Equilibrate column with 10 ml PBS at a maximum flow of 4 ml/min.

   All fluid handling is performed manually using appropriately sized syringes. These columns are easy to use and result in minimal loss of protein.

   A number of subclasses of IgG molecules do not bind to protein A. When using protein G or A chromatography, make sure that the antibodies are compatible (see Table 7.2.1).

3. Add 0.1 vol 1 M Tris·Cl, pH 8.0, to the antibody solution.

   Commercial antibody solution is typically 0.5 ml at 0.1 mg/ml IgG.

4. Load antibody solution onto the column and wash column with 10 ml of 100 mM Bicine/NaOH, pH 8.0. Collect run-through.

5. Wash column with 10 ml of 10 mM Bicine/NaOH, pH 8.0. Collect run-through.

6. Elute column with 5 ml of 100 mM citric acid/NaOH, pH 2.8, and collect 0.5-ml fractions (i.e., 8 drops) in 1.5-ml microcentrifuge tubes containing 100 µl of 1 M Bicine/NaOH, pH 9.0, to neutralize the pH. Mix immediately and store on ice.

7. Determine the A_{280} of the eluted fractions using a spectrophotometer (*APPENDIX 3B*) and pool the fractions that contain protein.

   Under the given conditions, the protein will typically elute in the first four fractions.

8. Add 0.1 vol of 1 M NaCl. Concentrate the solution in a YM30 Centricon to ~200 µl by centrifuging at 5000 × *g*, 4°C.

9. Redilute to 2 ml with labeling buffer and repeat the concentration and redilution steps (steps 8 and 9).

10. Concentrate to ~50 to 100 µl as in step 8 and collect the concentrated protein solution.

    When labeling proteins from solutions containing relatively high concentrations (i.e., millimolar) of compounds that contain free amino groups (e.g., Tris, glycine, gluthathione), repeat the concentration-redilution cycle more often. Each cycle dilutes the compound ~10 fold. Allow a maximum of 10% of contaminating primary amino groups, compared to the protein to be labeled.

    For example, the protein concentration in a 0.5 mg/ml antibody solution is 3.3 µM, assuming a molecular mass of 150 kDa. If this solution contains 50 mM Tris buffer (a 1.5 × 10^4 fold excess of free amino groups), a 1.5 × 10^5-fold dilution, corresponding to 5.2 concentration cycles, is needed to reach the 10% contamination level.

When labeling proteins other than antibodies, a Bicine concentration of 50 mM is recommended in the labeling buffer. Additional compounds that are needed to maintain the function of the protein to be labeled should be included. At this point, no compounds containing free amino groups should be added. High concentrations of reducing agents are also known to inhibit the labeling reaction. It is recommended that these be included in the chromatography step following labeling to prevent these compounds from interfering with the labeling reaction. Choose the cut-off value of the Centricon carefully to ensure retention of the protein of interest.

Prepare dye

11. Reconstitute Cy3.29–OSu in 20 µl dry DMF to give a ~10 mM Cy3 solution. Determine the exact concentration by measuring the absorption of a 10^4-fold diluted solution in PBS. From the ε_{550} of 150 $mM^{-1}cm^{-1}$, calculate the concentration.

    Cy3.29–OSu is supplied as a desiccated pellet in microcentrifuge tubes.

    DMF is dried by addition of hygroscopic beads to the container.

    The ε_{650} of Cy5 is 250 $mM^{-1}cm^{-1}$.

Perform labeling reaction

12. Determine the protein concentration of the antibody (or protein) solution to be labeled based on A_{280} reading (*APPENDIX 3B*).

    Antibody concentration at A_{280} = 1.0 is typically 1 mg/ml. At low protein concentrations (i.e., <0.1 mg/ml), or for smaller proteins (where the A_{280} = 1.0 = 1 mg/ml protein relation does not hold true), the protein concentration can be determined using the Bio-Rad Coomassie-based protein determination assay.

    Antibodies are known to give a lower apparent concentration when BSA is used as a standard in this assay. Multiply the concentration found by a factor of two or use an IgG standard.

13. Slowly add a 10- to 20-fold molar excess of dye to the protein solution while simultaneously stirring the solution with the pipet tip.

    The added volume of Cy3/DMF should not exceed 10% of the total volume, to prevent protein denaturation by DMF.

14. Incubate 30 min at room temperature, shaking the tube gently every 10 min.

Remove unconjugated dye

15. Remove the unconjugated dye by size-exclusion chromatography on a Bio-Rad P6DG Econopac (6-kDa exclusion size) column. Equilibrate the column with 30 ml PBS (or a buffer that is specifically formulated for the protein to be labeled).

16. Load the labeling reaction mixture onto the column and wash with a small amount (i.e., ~200 µl) of PBS (or equilibration buffer). Discard the first 2.5 ml (void volume ~3.3 ml).

17. Collect the colored protein fraction (or 2 ml of eluate if the staining is too weak to be visible) in a YM30 Centricon concentrator and concentrate to approximately the volume of the labeling reaction.

Determine labeling ratio

18. Estimate the labeling ratio by either of the following formulas:

$A_{554} \times M/[A_{280} - (0.05 \times A_{554}) \times 150]$ for Cy3

$A_{650} \times M/[A_{280} - (0.05 \times A_{650}) \times 250]$ for Cy5

where A_x is the absorption at wavelength x and M is the molecular weight of the protein in kDa.

The extinction coefficients (in units of $mM^{-1}cm^{-1}$) 150 and 250 are for Cy3 and Cy5, respectively. These formulas assume the A_{280} = 1.0 = 1 mg/ml relationship as found for larger proteins (i.e., >50 kDa) and correct for the 5% absorption of Cy3 at 280 nm. For smaller proteins or lower protein concentrations (i.e., <0.1 mg/ml) that cannot be reliably estimated from the A_{280}, determine the protein concentration by the Bio-Rad protein assay and estimate the labeling ratio from:

$A_{554} \times M/(P \times 150)$ for Cy3

$A_{650} \times M/(P \times 250)$ for Cy5

where P is the protein concentration in mg/ml as determined by Bio-Rad assay.

As previously mentioned, IgG concentration obtained with BSA as standard has to be multiplied by 2.

Using mass spectrometry, the authors have observed that ~50% of the Cy3 molecules in the preparation provided by Amersham are reactive. The molar excess is thus 7.5-fold for a 15-fold excess and, the typical labeling ratio of 2 to 3 that is reached under these conditions is therefore reasonably efficient.

19. Verify covalent labeling of the antibody by SDS-PAGE of the labeled product (*UNIT 6.1*). Use transillumination with a UV source (302 nm) to visualize labeled protein.

Ensure that there is no fluorescence at the running front of the gel; this indicates contamination with unconjugated dye. Where applicable, verify the specific activity of the protein and compare to the specific activity before labeling to ensure that labeling did not interfere with the biological function of the protein.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Formaldehyde fixative, 4% (w/v)

Dissolve 4 g of paraformaldehyde (Sigma) in 50 ml water. Add 1 ml 1 M NaOH solution and stir gently on a heating block (>60°C) until the paraformaldehyde is dissolved. Add 10 ml of 10× PBS (see recipe) and allow to cool to room temperature. Adjust the pH to 7.4 using 1 M HCl (~1 ml). Adjust to 100 ml with water and filter through a Millipore 0.45-µM filter using a syringe to remove traces of undissolved paraformaldehyde. Store up to several months at −20°C.

Low-background-fluorescence CO_2-independent medium

Adjust the formulation of the standard medium by omitting the pH indicator phenol red, the antibiotics penicillin and streptomycin, folic acid, and riboflavin. Before use, supplement the medium with 50 mM HEPES/NaOH, pH 7.4. Store 1 to 2 months at 4°C.

Mowiol mounting medium

Mix 6 ml glycerol, 2.4 g Mowiol 4-88 (Calbiochem), and 6 ml water. Shake for 2 hr. Add 12 ml of 200 mM Tris·Cl, pH 8.5 (*APPENDIX 2A*), and incubate at 50°C with occasional mixing until the Mowiol dissolves (i.e., ~3 hr). Filter through 0.45-µM Millipore filtration unit and store in aliquots up to several weeks at 4°C or up to several months at −20°C.

Phosphate-buffered saline, 10×

68 g NaCl
18.8 g Na_2HPO_4
2 g KH_2PO_4
H_2O to 1 liter

COMMENTARY

Background Information

Fluorescence resonance energy transfer (FRET) is a photophysical process that can be exploited to obtain information on protein-protein interactions and protein modification in addition to location (Clegg, 1996). Its usefulness lies in the ability to sense the presence of acceptor fluorophores in the extreme vicinity of a donor fluorophore with a maximum separation distance that is in the order of magnitude of single protein molecules. FRET is a radiationless process whereby an excited donor fluorophore transfers energy to an acceptor by dipole-dipole coupling (Förster, 1948). Due to the 6th-order distance dependence of the FRET efficiency, two discrete states of the fluorophores can be discriminated: exhibition of efficient FRET when donor and acceptor are in close proximity or no occurrence of FRET due to distance.

The transfer of energy (FRET) from an excited donor fluorophore to a nearby acceptor fluorophore has a number of consequences with respect to the fluorescent properties of both fluorophores and this can be exploited to measure the efficiency of this process.

In the methods presented in this unit, FRET is determined by measuring the donor fluorescence emission exclusively (Bastiaens and Squire, 1999). The acceptor fluorophore is excluded from the measurement by the choice of optical filters. As a consequence, a large excess of acceptor can be used and, when using antibodies, specificity can be sacrificed to gain higher occupancy.

In photophysical terms, FRET provides an extra channel of nonradiative decay by which the excited state of the donor fluorophore is depopulated. This results in a reduced quantum yield (Q), the ratio of emitted photons over absorbed photons. The reduction in quantum yield can be determined in two ways: (1) by the decrease in steady-state fluorescence emission; or (2) the decrease in fluorescence lifetime (τ), which characterizes the duration of the excited state of the fluorophore. The measurement of the change in quantum yield by steady-state emission has to be calibrated due to its dependence on concentration and light path. In contrast, the fluorescence lifetime is proportional to Q and is independent of concentration and light path. Fluorescence lifetimes are measured by fluorescence lifetime imaging microscopy (FLIM; Lakowicz and Berndt, 1991; Gadella et al., 1993; Gadella and Jovin, 1995) and can be performed sufficiently fast to enable real-time live cell experiments. FLIM requires a specialized microscopy setup enabling high-frequency modulation of the excitation light and the gain on the detector. This technique can be combined with acceptor photobleaching to provide an internal lifetime reference, e.g., at the end of a live-cell time-lapse sequence. A detailed description of a frequency domain FLIM set-up is given by Squire and Bastiaens (1999). This type of imaging system is gradually being made commercially available by companies such as LaVision or Lambert Instruments (see *SUPPLIERS APPENDIX*).

Calibration in the intensity-based FRET method is achieved by photobleaching the acceptor to provide the unquenched donor (Bastiaens et al., 1996; Bastiaens and Jovin, 1998; Wouters et al., 1998). In this method, an exposure of the donor fluorescence emission is made (F_{DA}, fluorescence emission of donor in presence of the acceptor). Removal of the acceptor fluorophore from the sample by photobleaching enables the acquisition of an unquenched donor emission image when the exposure is taken with settings identical to those used for the original image (F_D, fluorescence emission of the donor in absence of the acceptor). This essentially recovers that part of the fluorescence intensity that is lost to FRET. A pixel-by-pixel FRET efficiency map is obtained by simple image arithmetic: $E = 1 - (F_{DA}/F_D)$. The specificity of the acceptor photobleaching techniques lies in the steep edge at the long-wavelength (i.e., red edge) of the absorption spectrum of the donor, enabling exclusive photobleaching of the acceptor. Essential to the success of this technique is that the photoproduct of the acceptor does not exhibit residual absorption and does not fluoresce at donor emission wavelengths. Cy3 meets both

criteria to act as a proper acceptor for GFP; the same is the case for Cy5 acting as acceptor for Cy3. Since photobleaching of the acceptor occurs on a minute time scale, this type of FRET determination is restricted to fixed cells.

FRET microscopy can be used to detect protein-protein interactions in single cells as if one were performing an immunoprecipitation experiment. There is, however, no need to isolate the complex or remove it from its physiological environment prior to investigation, and the experiment can be performed in living cells. A number of approaches can be followed: (1) the donor molecule can be purified, labeled with Cy3, and introduced into cells by microinjection (Bastiaens and Jovin, 1996); (2) the donor molecule can be detected by a Cy3-labeled antibody or Fab fragment (provided the antibody is highly specific) on fixed cells (Ng et al., 1999); (3) the donor protein can be fused to one of the mutants of the intrinsically fluorescent green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (Tsien, 1998), genetically encoded, and expressed; (4) acceptor proteins (mostly antibodies or Fab fragments) can be labeled with Cy5 (for Cy3 donors), or labeled with Cy3 (for GFP donors) and introduced by microinjection or incubation in the case of antibodies; and (5) the recently discovered and commercial available red fluorescent protein from the *Anthozoa* sp. (Matz et al., 1999) can be employed. The latter opens up a wide array of possibilities since an ideal acceptor for GFP can be coexpressed in the same cell, obviating the need for exogenous labeling and microinjection.

Alternative methods for measuring FRET on the basis of fluorescence intensities are available. In these methods FRET is not determined from the donor fluorescence exclusively, but also from the sensitized emission of the acceptor, excited by receiving energy from the donor. These approaches fall into two categories (1) those based on changes in donor/acceptor emission ratio (Adams et al., 1991; Miyawaki et al., 1997); and (2) those based on acceptor sensitized emission (Day, 1998; Gordon et al., 1998; Mahajan et al., 1998). These approaches are attractively simple to perform but may suffer from a number of drawbacks that cannot always be corrected for.

In the ratiometric approach, the decrease in donor emission and concomitant increase in acceptor emission are imaged by calculating the ratio of these intensities at each pixel. Since this is a relative measure, a change in FRET can only be observed in live cells where the ratio changes over time. A decrease in the donor/acceptor emission ratio can be taken as an indication for the occurrence of FRET. However, this ratio is also dependent on the local concentrations of the donor and acceptor molecules measured in each pixel, which can complicate the interpretation by differential translocation of the biomolecules conjugated to donor and/or acceptor fluorophores. This problem does not occur when the donor and acceptor fluorophores are present on the same molecule (e.g., the "chameleon" Ca^{2+} biosensors; Miyawaki et al., 1997), since the relative concentrations are identical at each location in the cell.

Measuring FRET from the sensitized emission intensity alone (by using a "FRET filter set") is the most widely used method. Emission from the acceptor would be an exclusive readout for FRET when contamination with donor fluorescence (i.e., donor bleed-through) and direct excitation of the acceptor did not complicate the approach. Correction for these effects by extensive control measurements, some of which have to be performed in different samples where the acceptor is absent, is described in Gordon et al. (1998). Here, both donor-quenching and acceptor-emission information are used to derive the sensitized emission contribution, which is proportional to the FRET efficiency. However, sensitized emission is also proportional to the concentration of the acceptor. Therefore, relative populations of associated molecules cannot be determined. Molecules that are not participating in FRET are not detected. In contrast to donor-based measurements, no estimation can be made of the bound/unbound fractions from the sensitized emission alone. Since excitation spectra generally exhibit an extensive tail at the lower wavelength (blue-edge) the acceptor can easily be excited at the wavelength used for excitation of the donor. Direct excitation will be especially problematic when the recently discovered red fluorescent protein from the *Anthozoa* sp. is used as an acceptor, since its absorption spectrum shows multiple absorption peaks at the excitation optima for the green and yellow fluorescent protein.

Critical Parameters and Troubleshooting

FRET measurements are prone to false negative results. While the finding of FRET by unquenched fluorescence is highly unlikely to be caused by artifactual processes or nonspecific proximity of donor and acceptor, the absence of FRET does not provide proof for the

absence of an interaction for a number of reasons.

The presence of a GFP moiety on the protein of interest might interfere with its function or targeting, thus affecting the interaction with the target protein. Cloning vectors for the construction of GFP fusion proteins routinely contain a multiple cloning site that has been optimized for a maximum number of restriction sites to facilitate cloning. As a consequence, the random amino acid residues that they translate into can adopt an unfortunate secondary structure that hinders the proper behavior of the fused protein. In the case of the EGFR-GFP, the amino acid residues encoded by the cloning site were replaced by a flexible six-glycine linker between the GFP moiety and the EGFR to prevent the GFP from affecting EGFR function (Wouters and Bastiaens, 1999). Another problem might arise when the separation distance between the GFP moiety on the donor protein and the Cy3 groups on the antibody is too large (i.e., >10 nm) for energy to be transferred efficiently. This might be overcome by using antibodies raised against a different epitope, or the entire protein, thus increasing the chance of a favorable orientation of the antibody to the donor fluorophore.

The labeling ratio of the antibodies is a common reason for failure to detect FRET. The labeling ratio should exceed 1 to prevent unlabeled antibody from competing with the labeled antibodies. A higher labeling ratio is beneficial for FRET detection since the R_0 distance (i.e., characteristic value for a given donor-acceptor pair, the distance at which 50% of the excited state energy is transferred by FRET) is effectively increased by a larger number of acceptor molecules per donor molecule. In the authors' experience, labeling ratios up to 5 do not significantly affect the specificity of the antibody. However, this should be verified by using the Cy3-labeled antibody in an immunofluorescence experiment and judging the staining pattern as compared to unlabeled antibody. Remember that absolute specificity is not a requirement in the FRET assay, because only the donor photophysical properties are used. However, in extreme cases, the highly labeled antibody could become nonspecific to a point where significant amounts bind to the donor.

It is essential that the donor images before and after acceptor photobleaching be taken under identical conditions. Any change in parameters that influence the collection efficiency of donor fluorescence (i.e., brightness, contrast, laser power, and averaging) will influence the calculation of the FRET efficiency. This will affect the entire image, and the control region in the *E* map will no longer be distributed around 0 but will be uniformly shifted. This also occurs with donor photobleaching. When the laser is used at high intensity, or when too many scans are made for averaging, a substantial amount of the GFP becomes photobleached. This will lead to a shift to negative *E* values in the control region. Furthermore, it is essential to verify that the sample has not moved and that the same focal plane is imaged in both donor images. Movement causes structures in the control region in the *E* map with positive values at the leading edge and negative values at trailing edges. Movement is most often caused by temperature differences between the slide and the objective but can also be caused by drift in the slide holder. To correct for translation of the image after acceptor photobleaching, the maximum in the correlation function between pre- and post-bleaching images gives the shift between the two images (Bastiaens and Jovin, 1998). The correlation function of the two images is obtained by Fourier transformation on both images, followed by multiplication of the conjugate of the Fourier transform of one image by the Fourier transform of the other, and performing an inverse Fourier transform on the resulting image. Another source of structured contrast in the control region in the *E* map can be caused by focal mismatch between the two images, originating from the apparent appearance (positive *E*) or disappearance (negative *E*) of structures. This problem can be prevented by observing the *z* position of the stage between exposures. A larger pinhole can reduce these problems by decreasing the depth of focus but at the cost of *z* resolution. When using pre- and post-*z* sections rather than single-focus plane images, the maximum in a three-dimensional correlation function can be used for registration of the two data sets and a correct three-dimensional *E* map can be obtained (Bastiaens et al., 1996).

Anticipated Results

After photobleaching of the acceptor, an increase in donor emission intensity can be observed when substantial FRET is present. Image arithmetic should produce FRET efficiencies that are closely distributed around zero in the portion of the cell that was not photobleached. Deviations from this condition indicate donor bleaching, lateral movement, or focal mismatch. With the occurrence of FRET, a separate efficiency distribution is expected in

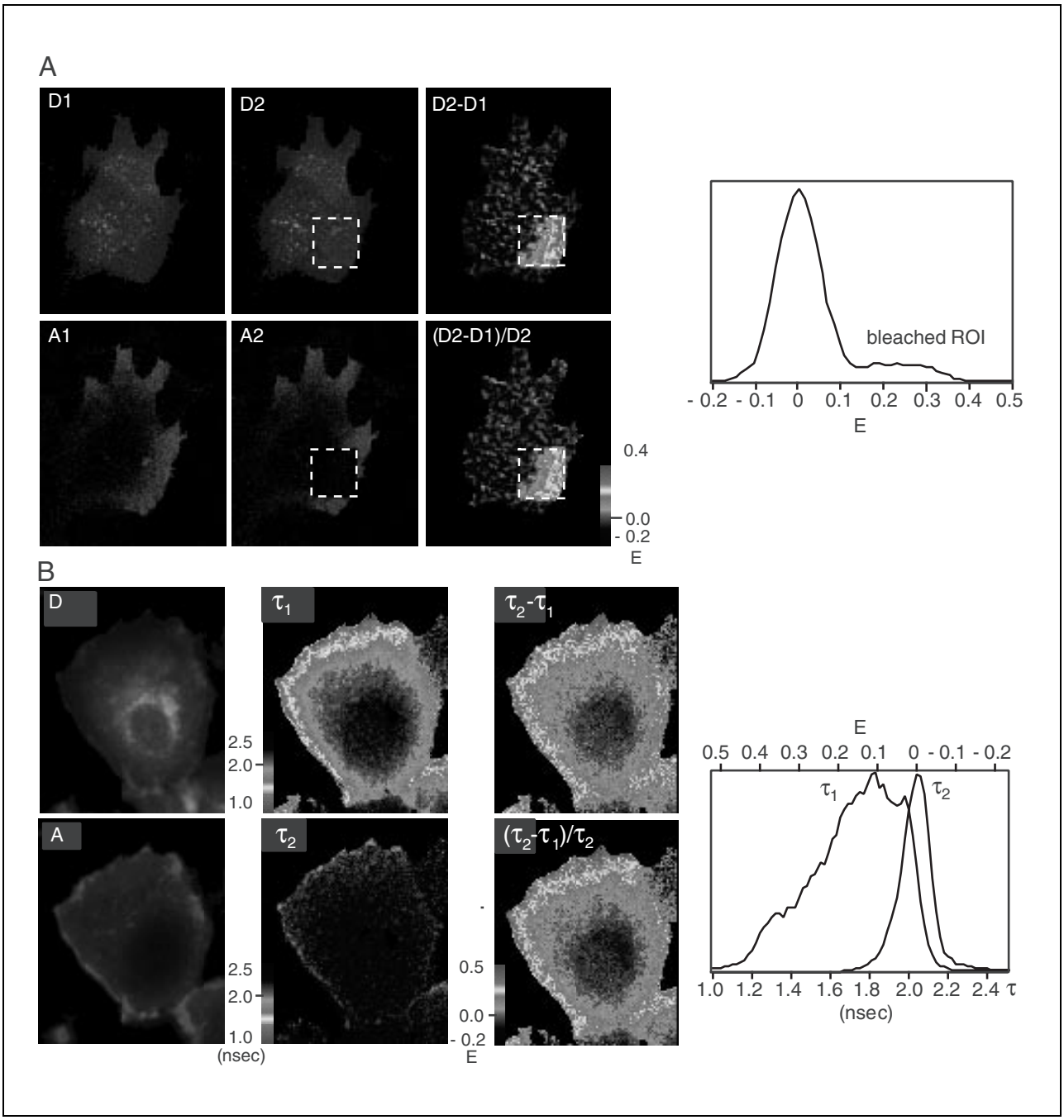


Figure 17.1.1 (**A**) Cy3-PY72 photobleaching releases FRET-quenched EGFR-GFP emission. The histogram shows the distribution of calculated FRET efficiencies in the cell. (**B**) FRET measured by fluorescence lifetime imaging microscopy. The histogram shows the distribution of measured lifetime (nsec) and corresponding calculated FRET efficiencies prior and after photobleaching of the acceptor. **See color figure**.

the photobleached area. The heterogeneity in this distribution contains information about variability in the formation of complexes of the proteins carrying the donor and acceptor fluorophores.

Figure 17.1.1 shows an MCF-7 mammary carcinoma cell expressing EGFR-GFP that was stimulated with 100 ng/ml EGF for 5 min. This cell was incubated with Cy3-labeled anti-phosphotyrosine antibodies (e.g., 10 µg/ml PY72 monoclonal) and EGFR-GFP tyrosine phosphorylation was measured by FRET using the unquenching of GFP fluorescence by acceptor photobleaching (Fig. 17.1.1A) and FLIM (Fig. 17.1.1B). The EGFR-GFP distribution is shown in the D1 image. Most EGFR-GFP is localized at the plasma membrane in addition to internalized (punctate) EGFR-GFP. The cor-

responding antibody-staining A1 shows phosphorylation at the plasma membrane. A rectangular region was subjected to acceptor photobleaching (white box). The GFP fluorescence in this region increased after photobleaching as shown in the difference image (D2 − D1), demonstrating positive FRET efficiencies [(D2 − D1)/D2]. Highest FRET efficiencies can be observed in the plasma membrane corresponding to fully phosphorylated receptor at this location. The large peak in the energy-transfer efficiency histogram that is distributed around zero originates from the area outside the photobleached region and indicates proper image registration. The additional population, ranging from 15% to 35% efficiency, corresponds to the photobleached region. Figure 17.1.1B shows the analogous result of a fluorescence lifetime measurement. The steady-state fluorescence distribution of EGFR-GFP is shown in (D) and the corresponding lifetime map in (τ_1). The anti-phosphotyrosine Cy3 immunofluorescence (A) is photobleached to obtain an intracellular reference lifetime in absence of acceptor (τ_2). As can be seen in the fluorescence-lifetime histogram, these values are homogeneously distributed around an average of ~2.0 nsec. The decrease in lifetime due to FRET is shown in the difference image ($\tau_2 - \tau_1$). The energy-transfer efficiency is given by the normalization of this difference to the reference EGFR-GFP lifetime [$E = (\tau_2 - \tau_1)/\tau_2$] and again shows highly phosphorylated receptor in the plasma membrane.

Time Considerations

From cell seeding, the entire procedure can be performed in 3 to 4 days. This period includes 1 to 2 days for expression of the GFP construct after transfection or microinjection; 1/2 day for treatment, fixation, and antibody incubation of cells; 1/2 day of data acquisition; and 1/2 day for data analysis. Removal of gelatin/BSA from commercial antibody takes ~3 hr. The most time-consuming step in the procedure for labeling antibodies is the buffer exchange using a Microcon concentration device. Depending on the amount of contaminating free amino groups in the original buffer, this can take between 2 and 5 hr. The labeling reaction and subsequent gel filtration chromatography takes ~1 hr.

There are a number of points where the procedure can be interrupted: (1) optimal expression after overnight incubation rather than a 3- to 4-hr incubation post microinjection can be achieved by lowering the concentration of DNA; (2) after fixation and permeabilization, the cells can be stored overnight in PBS at 4°C before antibody incubation; (3) after mounting, the cells can be viewed immediately rather than the following day, by application of rubber cement instead of Mowiol; and (4) the samples can be stored at −20°C for weeks without appreciable loss of antibody staining or FRET.

Literature Cited

Adams, S.R., Harootunian, A.T., Buechler, J., Taylor S.S., and Tsien, R.Y. 1991. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* 349:694-697.

Bastiaens, P.I.H. and Jovin, T.M. 1996. Microspectroscopic imaging tracks the intracellular processing of a signal-transduction protein: Fluorescent labeled protein kinase C beta I. *Proc. Natl. Acad. Sci. U.S.A.* 93:8407-8412.

Bastiaens, P.I.H. and Jovin, T.M. 1998. Fluorescence resonance energy transfer microscopy. *In* Cell Biology a Laboratory Handbook, Vol. 3 (J.E. Celis, ed.), pp. 136-146. Academic Press, New York.

Bastiaens, P.I.H. and Squire, A. 1999. Fluorescence lifetime imaging microscopy: Spatial resolution of biochemical processes in the cell. *Trends Cell Biol.* 9:48-52.

Bastiaens, P.I.H., Majoul, I.V., Verveer, P.J., Soling, H.D., and Jovin, T.M. 1996. Imaging the intracellular trafficking and state of the AB(5) quaternary structure of cholera-toxin. *EMBO J.* 15:4246-4253.

Clegg, R.M. 1996. Fluorescence resonance energy transfer spectroscopy and microscopy. *In* Fluorescence Imaging Spectroscopy and Microscopy (X.F. Wang and B. Herman eds.) pp. 179-251. John Wiley & Sons, New York.

Day, R.N. 1998. Visualization of Pit-1 transcription factor interactions in the living cell nucleus by fluorescence resonance energy transfer microscopy. *Mol. Endocrinol.* 12:1410-1419.

Förster, T. 1948. Zwischenmolekulare Energiewanderung und Fluoreszenz. *Ann. Phys.* 2:55-75.

Gadella, T.W.J. and Jovin, T.M. 1995. Oligomerization of epidermal growth-factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy—a stereochemical model for tyrosine kinase receptor activation. *J. Cell Biol.* 129:1543-1558.

Gadella, T.W.J., Jovin, T.M., and Clegg, R.M. 1993. Fluorescence lifetime imaging microscopy (FLIM)—spatial resolution of microstructures on the nanosecond time-scale. *Biophys. Chem.* 48:221-239.

Gordon, G.W., Berry, G., Huan Liang, X., Levine, B., and Herman, B. 1998. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys. J.* 74:2702-2713.

Lakowicz, J.R. and Berndt, K. 1991. Lifetime-selective fluorescence imaging using an rf phase-sensitive camera. *Rev. Sci. Instrum.* 62:1727-1734.

Mahajan, N.P., Linder, K., Berry, G., Gordon, G.W., Heim, R., and Herman, B. 1998. Bcl-2 and bax interactions in mitochondria probed with green fluorescent protein and fluorescence energy transfer. *Nature Biotechnol.* 16:547-552.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., and Lukyanov, S.A. 1999. Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nature Biotechnol.* 17:969-973.

Mittman, S., Flaming, D.G., Copenhagen, D.R., and Belgum, J.H. 1987. Bubble pressure measurement of micropipette tip outer diameter. *J. Neurosci. Methods* 22:161-166.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. 1997. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 388:882-887.

Ng, T., Squire, A., Hansra, G., Bornancin, F., Prevostel, C., Hanby, A., Harris, W., Barnes, D., Schmidt, S., Mellor, H., Bastiaens, P.I.H., and Parker, P.J. 1999. Imaging protein kinase C alpha activation in cells. *Science* 283:2085-2089.

Squire, A. and Bastiaens, P.I.H. 1999. Three dimensional image restoration in fluorescence lifetime imaging microscopy. *J. Microsc.* 193:36-49.

Tsien, R.Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 76:509-538.

Wouters, F.S. and Bastiaens, P.I.H. 1999. Fluorescence lifetime imaging of receptor tyrosine kinase activity in cells. *Curr. Biol.* 9:1127-1130.

Wouters, F.S., Bastiaens, P.I.H., Wirtz, K.W.A., and Jovin, T.M. 1998. FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nsL-TP) with fatty acid oxidation enzymes in peroxisomes. *EMBO J.* 17:7179-7189.

Contributed by Fred S. Wouters
Imperial Cancer Research Fund
London, United Kingdom

Philippe I.H. Bastiaens
European Molecular Biology Laboratory
Heidelberg, Germany

Identification of Protein Interactions by Far Western Analysis

This unit describes far western blotting, a method of identifying protein-protein interactions. In a far western blot, one protein of interest is immobilized on a solid support membrane, then probed with a non-antibody protein. Far western blots can be used to identify specific interacting proteins in a complex mixture of proteins (see Basic Protocol). They are particularly useful for examining interactions between proteins that are difficult to analyze by other methods due to solubility problems or because they are difficult to express in cells. This method is performed totally in vitro, and the proteins of interest can be prepared in a variety of ways. Peptides can be used to determine the effects of specific residues or post-translational modifications on protein-protein interactions (see Alternate Protocol 2). In addition, many different detection techniques, either radioactive or nonradioactive, can be used. For example, the protein probe may be detected indirectly with an antibody, rather than being labeled radioactively (see Alternate Protocol 1). Thus, techniques and reagents already in hand can frequently be adapted for use with this assay.

CAUTION: Appropriate safety precautions must be taken when working with radioactive materials. Information on proper handling and disposal of radioactive compounds can be found in *APPENDIX 1D* and may be obtained from local radiation safety officials. Specific information on handling ^{35}S-labeled compounds can be found in *UNIT 7.1* and in *APPENDIX 1D*.

FAR WESTERN ANALYSIS OF A PROTEIN MIXTURE

BASIC PROTOCOL

The following is a basic method for detecting protein-protein interactions by far western blotting when one protein is contained within a simple or complex mixture of proteins. First, the protein sample is fractionated on an SDS-PAGE gel (*UNIT 6.1*). After electrophoresis, the proteins are transferred from the gels onto a solid support membrane by electroblotting (*UNIT 6.2*). Transferred membranes may be stained with Ponceau S to facilitate location and identification of specific proteins. Nonspecific sites on the membranes are blocked with standard blocking reagents, and the membranes are then incubated with a radiolabeled non-antibody protein probe. After washing, proteins that bind to the probe are detected by autoradiography (*UNIT 6.3*).

Materials

Samples to be analyzed
1× SDS sample buffer (*APPENDIX 2A*)
Ponceau S staining solution (see recipe)
Blocking buffer I: 0.05% (w/v) Tween 20 in 1× PBS (see recipe for PBS); prepare fresh
Blocking buffer II: dissolve 1 g bovine serum albumin (BSA; fraction V) in 100 ml 1× PBS (see recipe for PBS); prepare fresh
Phosphate-buffered saline (PBS; see recipe), pH 7.9
cDNA encoding protein of interest cloned into an in vitro expression vector
In vitro transcription/translation kit (Promega)
10 mCi/ml ^{35}S-methionine (1000 Ci/mmol)
Probe purification buffer (see recipe)
Probe dilution buffer (see recipe)

Contributed by Diane G. Edmondson and Sharon Y. Roth
Current Protocols in Cell Biology (2000) 17.2.1-17.2.10

Polyvinyldifluoridine (PVDF) or nitrocellulose membrane for protein transfer

Microfiltration centrifuge columns (e.g., Gelman Nanosep, Pall Filtron, or Millipore Microcon)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), electrophoretic transfer of proteins to a support membrane (*UNIT 6.2*), in vitro translation (*UNIT 11.2*), and autoradiography (*UNIT 6.3*)

NOTE: Always handle support membranes with gloves or membrane forceps.

Prepare protein blot

1. Prepare the protein sample to be analyzed by resuspending it in 1× SDS sample buffer.

   UNIT 6.1 gives instructions on preparation of samples and amount of samples to load. In general, ~50 to 100 µg can be loaded in each lane for a complex mixture of proteins. A smaller amount, i.e., 10 to 20 µg, is loaded for less complex protein samples. The amount loaded may also need to be adjusted for the size of gel. (Usually 30 µg/mm^2 loading surface can be resolved without smearing.)

2. Separate the samples on an SDS-polyacrylamide gel (*UNIT 6.1*).

3. Transfer the proteins from the gel to a solid support membrane (e.g., PVDF or nitrocellulose) by semidry electroblotting (*UNIT 6.2*).

   Either nitrocellulose or PVDF membranes can be used with good results. PVDF membranes are easier to handle and tend to give a slightly higher signal-to-noise ratio, probably due to increased protein retention by the membrane.

Stain with Ponceau S

4. After transfer, stain the membrane for 5 min in ~100 ml freshly diluted 1× Ponceau S staining solution. Stain the membrane in a plastic container large enough to hold the blot and use sufficient Ponceau S to cover the membrane completely.

   This step is optional. When the protein samples consist of a few proteins, or when there are clearly visible bands that facilitate orientation of the blot, staining with Ponceau S can provide helpful landmarks. One can unequivocally identify interacting bands, mark the position of molecular weight standards, and trim away excess membrane more exactly.

5. Destain the membrane washing in several changes of deionized water until the proteins are clearly visible. Place light pencil marks adjacent to important protein bands to mark them for future reference. Trim away excess membrane.

   The stain fades quickly so the marks must be placed immediately.

6. Destain an additional 5 min in water until the red staining fades.

Block membrane

7. Block blot for 2 hr in 200 ml blocking buffer I at room temperature with gentle agitation.

8. Decant and add 200 ml blocking buffer II. Incubate as in step 7.

9. Decant blocking buffer II and rinse the membrane briefly in 100 ml of 1× PBS.

   At this point, the blot may be probed immediately or may be wrapped in plastic wrap and stored for up to 2 weeks at 4°C.

Prepare the probe

10. Following manufacturer's procedures, prepare a radiolabeled in vitro–translated probe of the protein of interest using ^{35}S methionine (also see *UNIT 11.2*).

    The probe can be conveniently prepared during the blocking steps.

    The authors routinely use the Promega TnT quick-coupled transcription/translation system for producing probes. For a small blot (e.g., ≤9 × 9–cm), one half of a standard in vitro transcription/translation reaction (i.e., 25 µl) is sufficient for the probe. For larger blots, an entire 50-µl reaction may be used. In the authors' laboratory it is considered essential that the transcription/translation lysate not be repeatedly frozen and thawed.

11. After translation, dilute the probe with 500 µl probe purification buffer, and purify by microcentrifuging 15 to 30 min at 10,000 × *g,* room temperature, in a microfiltration column. Save aliquots of the purified probe for analysis by SDS-PAGE (i.e., 2 µl), and for scintillation counting (i.e., 2 to 5 µl).

    Check microfiltration column manufacturer's procedure for exact centrifugation times required by different columns. In practice, it is not always necessary to purify the probe through a microfiltration column; many probes give good signals without purification. However, if signal-to-noise ratio is low, probe purification may improve results. In addition, it is possible to quantitate the proportion of probe bound if the probe has been purified.

Bind probe

12. Preincubate blot for 10 min in 50 ml of 1× probe dilution buffer (without probe) by gently agitating at room temperature.

13. Dilute the translated probe with 1× probe dilution buffer in a volume sufficient to cover the membrane to be probed (typically 3 ml).

    For small blots, i.e., ≤9 × 9–cm, a 50-ml conical tube makes a convenient incubation chamber. A volume of 3 ml is enough solution to cover the blot and tubes can be rotated on a mechanical rotator. In addition, the conical tube makes the radiolabeled probe easy to contain and dispose of. Larger blots can be incubated on a Nutator or orbital shaker in a heat-sealable bag, or rotated in a hybridization oven adjusted to room temperature.

14. Add the probe to the membrane and incubate 2 hr at room temperature. Rotate the tubes or agitate bags throughout the binding reaction.

Wash the membrane

15. Transfer the membrane to a plastic dish and wash the membrane with 200 ml 1× PBS for 5 min, room temperature. Repeat for a total of four washes.

    Background is generally quite low and extended washing does not substantially reduce background.

16. Air dry the membrane and expose to X-ray film (autoradiography) or phosphor imager screen (see *UNIT 6.3* for both techniques).

    Do not cover the blots with plastic wrap as this will quench the ^{35}S signal. Overnight exposure to X-ray film is usually sufficient to detect positive interactions.

DETECTING INTERACTING PROTEINS BY IMMUNOBLOTTING

ALTERNATE PROTOCOL 1

In vitro–translated probes have the advantages of being quickly produced, easily detected, and quantitated to give an estimate of relative binding. In addition, mutations in the protein probe can be generated by simple cloning procedures and can provide information on binding domains and their critical residues. A disadvantage of in vitro–translated probes is the need for multiple methionine or cysteine residues to obtain a well labeled probe. For the same reasons, small peptide fragments are often not suitable for use as in

vitro–translated probes. [^{14}C]leucine and [^{3}H]leucine can also be used for in vitro translation of proteins; however, in the authors' laboratory [^{14}C]leucine has not yielded probes suitable for use as far western probes.

There are many other ways to generate probes for far western blots. The protein probe may be labeled in vitro with ^{125}I (Schumacher and Tsomides, 1995) or enzymatically with ^{32}P (Kimball, 1998). Biotin-labeled probes may be detected with strepavidin-biotin detection schemes (Luna, 1996; Grulich-Henn et al., 1998; Kimball et al., 1998). Protein binding may be detected indirectly as well. If an antibody to the interacting protein is available, then an unlabeled protein probe can be bound to the blots as usual and then detected by western (immunoblot) analysis. This is especially useful when a tagged recombinant protein and antibody to the tag are available. The following procedure describes detection of an unlabeled protein probe with specific antibody. Many variations of immunoblotting exist; additional information and procedures may be found in *UNIT 6.2*.

Additional Materials *(also see Basic Protocol)*

Recombinant protein or unlabeled in vitro translated–protein for probe
5% (w/v) non-fat instant dry milk in 1× TBST (see recipe for TBST)
Primary antibody specific for protein probe
TBST (see recipe)
Alkaline phosphatase (AP)–conjugated secondary antibody against Ig of species from which specific antibody was obtained
Alkaline phosphatase buffer (see recipe)
Developing solution (see recipe)
100 mM EDTA, pH 8.0 (*APPENDIX 2A*)

Prepare blot and probe

1. Prepare and block the blot (see Basic Protocol, steps 1 to 9).
2. Prepare the probe protein by diluting in vitro–translated or recombinant protein in 3 ml of 1× probe dilution buffer.

   The amount of recombinant protein must be empirically determined for each protein. Various researchers have used from 0.5 to 20 μg recombinant protein/ml of probe dilution buffer.

Expose blot to probe

3. Bind the probe to blot and wash (see Basic Protocol, steps 12 and 14 to 15). Do not dry the membrane after washing.
4. Incubate blot in 200 ml of 5% non-fat milk in 1× TBST for 1 hr, room temperature, with gentle rotation on an orbital shaker.

Expose to antibodies

5. Dilute the primary antibody in 5% milk in 1× TBST. Incubate blot in 5 to 10 ml diluted antibody at room temperature with gentle agitation to ensure blot is evenly covered with the antibody solution.

   Incubations are usually carried out in heat-sealed plastic bags or hybridization bottles to minimize the volume necessary to completely cover the blot. A volume of 5 to 10 ml of diluted antibody is sufficient to cover most blots. Appropriate antibody concentrations vary for each antibody and must be determined empirically.

6. Wash for 10 min in ≥200 ml 1× TBST by agitating on an orbital shaker. Repeat an additional two times.

7. Dilute the AP-conjugated secondary antibody in 5 to 10 ml of 5% milk in 1× TBST and incubate blot for 1 hr as in step 4.

   Suppliers generally provide an estimate of appropriate dilution for the secondary antibody.

8. Wash blot six times for 5 min each in ≥200 ml TBST, with agitation.

Detect antibodies

9. Briefly, rinse blot in 50 ml alkaline phosphatase buffer.

10. Incubate blot in 20 ml developing solution for 1 to 15 min and rinse blot with 100 ml water.

11. Wash blot for 5 min with 100 ml of 100 mM EDTA, pH 8.0, to stop the development reaction. Rinse with 100 ml water, dry, and photograph.

    An example of a far western blot of proteins is shown in Figure 17.2.1. Lane 1 shows a Coomassie blue stain of a protein sample enriched for histone proteins separated on a 22% SDS-PAGE gel. Lane 2 shows a far western autoradiogram of a parallel lane probed with the yeast Tup1 protein according to this protocol. Lane 3 shows a parallel lane probed with an unlabeled Tup1 protein and detected with antibody specific to Tup1 as in Alternate Protocol 2. Both protocols yield the same result—Tup1p interacts with H3 and H4 but not with H2A or H2B. Lanes 4 and 5 show immunoblots of parallel lanes using anti-H3 and anti-H4 antibodies to identify histories H3 and H4 unequivocally.

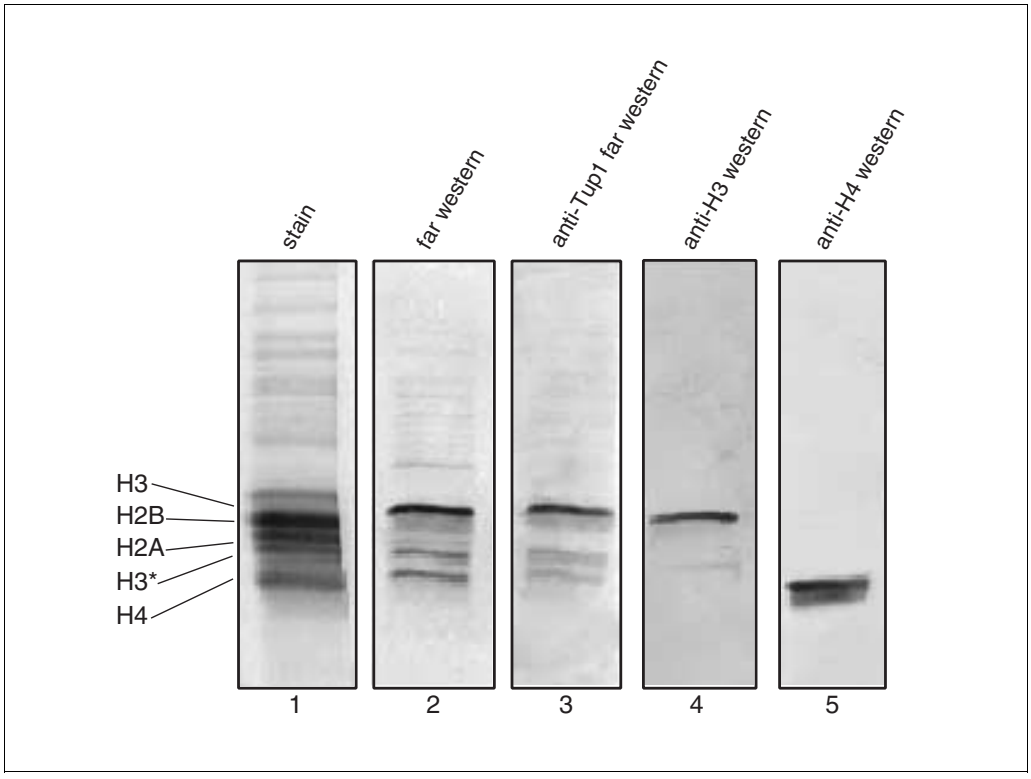


Figure 17.2.1 Far western of blotted SDS-PAGE gel. Lane 1, Coomassie blue–stained gel showing locations of histone bands. Lane 2, far western of a parallel lane using radiolabeled in vitro–translated probe. Lane 3, far western using unlabeled probe detected with probe-specific antibody. Lane 4, western blot using anti-histone H3 specific antibody. Lane 5, western blot using anti-histone H4 specific antibody.

ALTERNATE PROTOCOL 2

USING PEPTIDES TO IDENTIFY SPECIFIC INTERACTING SEQUENCES IN A FAR WESTERN BLOT

Synthetic peptides can also be used in far western analyses. The use of peptides enables the identification of specific interacting sequences. Specific post-translational modifications can be examined for their effect on protein-protein interactions. Peptides as small as 11 amino acids have been used successfully as far western targets.

Peptide far westerns differ from other far westerns only in the preparation of the blots. Peptide dilutions are prepared, then dot or slot blotted onto the support membrane. Blocking and probing of peptide blots are identical to procedures used for traditional far westerns. Duplicate blots are stained to verify that comparable quantities of different peptides have been loaded. Because Ponceau S staining is temporary, staining of duplicate blots with India ink is used to provide a permanent record for peptide blots.

In the authors' experience, only peptides that have been synthesized on MAP resins have worked well for peptide blots. MAP resins consist of branched lysine chains whose chemically active groups have been blocked. Although peptides prepared in other ways do give reproducible results, the peptide concentrations required are several orders of magnitude higher than those required for MAP peptides, making these blots very costly to perform. The reason why increased peptide is needed is unclear, but perhaps the MAP resin "presents" the peptide in such a way that it is more accessible for interaction.

Additional Materials *(also see Basic Protocol)*

Peptides
0.4% Tween 20/PBS (see recipe)
India ink solution (see recipe)
Slot or dot blot apparatus (e.g., Bio-Rad Bio-Dot SF or Schleicher & Schuell Minifold II)

1. Make dilutions of peptides between 5 ng and 5 µg in a final volume of 100 to 200 µl of distilled water.
2. Prepare slot or dot blotter and support membrane (PVDF or nitrocellulose) as described by the manufacturer. Load the peptide dilutions into wells. Prepare duplicate blots, one for far western and one for India ink staining. After all samples are loaded, apply vacuum to draw the peptide samples through the manifold device and onto the support membrane.
3. Block, bind, wash, and autoradiograph one blot for far western (see Basic Protocol, steps 7 to 16).
4. Incubate the second blot in 100 ml of 0.4% Tween 20/PBS for 5 min at room temperature with gentle agitation. Repeat incubation.
5. Stain blot by incubating 15 min to overnight with 100 ml India ink solution at room temperature.
6. Wash the filter for 2 hr in 4 changes of 1× PBS. Dry and store the membrane.

This stain is permanent.

Figure 17.2.2 shows an example of results from this alternate protocol, using peptides as a substrate for a far western. The right-hand panel is a blot stained with India ink verifying that comparable quantities of peptide were loaded on the blot. The left-hand panel is a far western of a duplicate blot demonstrating the effect of acetylation of lysine residues of histone peptides on Tup1p/histone interaction.

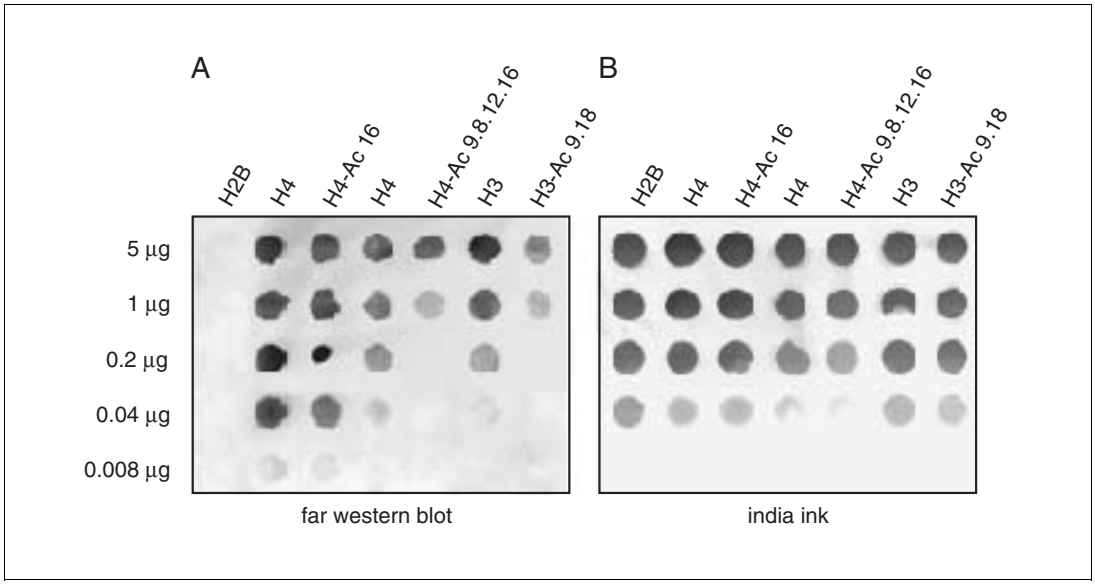


Figure 17.2.2 Far western blot of peptides (**A**) dot blotted onto PVDF membrane and (**B**) stained with India ink. Figure reproduced with permission of Cold Spring Harbor Laboratory Press.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Alkaline phosphatase buffer

100 ml 1 M Tris·Cl, pH 9.5 (*APPENDIX 2A*)
20 ml 5 M NaCl (*APPENDIX 2A*)
5 ml 1 M $MgCl_2$ (*APPENDIX 2A*)
Add H_2O to 1 liter
Store up to 1 year at room temperature

5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution

Dissolve 0.5 g of BCIP in 10 ml of 100% dimethylformamide. Store at 4°C or in small aliquots at −20°C. Discard when solution turns color.

Developing solution

Add 66 µl of NBT stock (see recipe) to 10 ml of alkaline phosphatase buffer (see recipe). Mix well. Add 33 µl of BCIP stock solution (see recipe) and mix again. Prepare fresh.

India ink solution

Add 100 µl India ink (Pelikan or Higgans) to 100 ml 0.4% Tween 20/PBS (see recipe). Prepare fresh.

Nitroblue tetrazolium chloride (NBT) stock solution

Dissolve 0.5 g of NBT in 10 ml of 70% dimethylformamide. Store at 4°C or in small aliquots up to 6 months at −20°C.

Phosphate-buffered saline (PBS), 10×

80 g NaCl
2.2 g KCl
9.9 g Na_2HPO_4
2.0 g K_2HPO_4
Add H_2O to 1 liter

continued

Adjust pH to 7.4
Store indefinitely at room temperature
Prior to use, dilute to 1× by mixing 1 part 10× PBS with 9 parts water

Leftover 1× PBS should be stored at 4°C to discourage bacterial growth.

Ponceau S staining solution, 10×

2 g Ponceau S
30 g trichloroacetic acid
30 g sulfosalicylic acid
Add H_2O to 100 ml
Store indefinitely at room temperature
Just prior to use dilute to 1× by mixing 1 part 10× Ponceau S with 9 parts water

Probe dilution buffer, 10×

3.0 g bovine serum albumin (BSA)
10 ml normal goat serum
10 ml 10× PBS (see recipe)
H_2O to 100 ml
Store indefinitely at −20°C
Just prior to use, dilute to 1× by mixing 1 part 10× stock with 9 parts 1× PBS

Probe purification buffer

400 µl 1 M HEPES, pH 7.4,
400 µl 1 M dithiothreitol (DTT)
9.2 ml H_2O
Prepare fresh

TBST, 10×

90 g NaCl
100 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10 g Tween 20
Add H_2O to 1 liter
For 1× TBST dilute 1 part 10× TBST with 9 parts water prior to use
Store indefinitely at room temperature

Tween 20/PBS, 0.4% (w/v)

Dissolve 0.4 g Tween 20 in 100 ml 1× PBS (see recipe). Store up to 1 week at room temperature.

COMMENTARY

Background Information

The far western blot (also called a west western and a ligand blot) has been widely used to examine the interactions of many diverse proteins. For example, it has been used to examine the interactions between the subunits of eukaryotic initiation factors (Kimball et al., 1998), to look at interactions between basic helix-loop-helix DNA-binding proteins (Chaudhary et al., 1997), and to examine the interactions of keratin intermediate filaments with desmosomal proteins (Kouklis et al., 1994). Far westerns have been particularly useful in examining interactions of histones with regulatory proteins. Far westerns have been used to look at interactions of WD repeat proteins with histones (Edmondson et al., 1996; Palaparti et al., 1997), the interaction of Epstein-Barr virus nuclear antigen 2 with histone H1 (Grasser et al., 1993), and the interaction of histones with the *Xenopus* oocyte protein N1 (Kleinschmidt and Seiter, 1988). In addition, far westerns have been used to study receptor-ligand interactions and to screen libraries for interacting proteins (Grulich-Henn et al., 1998; Hsiao and Chang, 1999).

Sometimes, the nature of the proteins being examined is such that standard methods of studying protein-protein interactions are not possible. For example, some proteins are diffi-

cult to solubilize or to extract from cells except under conditions that disrupt protein-protein interactions and therefore, are difficult to assay by immunoprecipitation. Other proteins cannot be expressed in bacteria or yeast due to toxicity problems, thus making the production of recombinant proteins or the use of two-hybrid assays impossible. Far westerns are particularly useful in such cases. Since far westerns are performed totally in vitro, they circumvent these types of problems.

Another advantage of the far western blot is its flexibility. Proteins prepared in a variety of ways can be used for the assay. Cell extracts, recombinant proteins, and peptides can all be used as both probe and target proteins. For example, Palaparti et al. (1997) used cell extracts to probe a semipurified histone sample and detected bound proteins of interest with antibodies. Hsiao and Chang (1999) used phage-expressed proteins immobilized on filter lifts for a far western library screen. Unpurified *E. coli* extracts containing recombinant protein have been successfully used as probes (Fischer et al., 1997).

In addition, many different detection techniques, either radioactive or nonradioactive, can be used. Kimball and co-workers used recombinant protein probes that were labeled radioactively with kinases and recombinant proteins labeled with biotin and subsequently detected with a strepavidin-biotin detection scheme (Grulich-Henn et al., 1998; Kimball et al., 1998). Thus, techniques and reagents already in hand can frequently be adapted for use with this assay.

Finally, the far western can be modified to define the protein domains and amino acid residues that are important in protein-protein interactions. Mutagenized clones can be used to produce variants of protein probes. A single SDS-PAGE gel can be run with identical lanes and cut into strips, and a different in vitro–translated probe can be used for each strip. In this way, multiple variants of a protein can be tested simultaneously for their ability to interact with a target protein.

Non-SDS polyacrylamide gels can also be used to separate proteins for far westerns. For example, acid urea gels, which separate on the basis of both size and charge, have been successfully employed. Finally, peptides corresponding to specific interacting sequences can be synthesized with specific post-translational modifications to test their effects on protein-protein interactions.

Critical Parameters

Blocking nonspecific binding sites on the membranes is critical to achieving good results with far westerns. Too little blocking results in high background, while extended time in blocking solutions results in weakened or lost signal. The reason for the diminished signal is unclear, but protein renaturation apparently takes place during the blocking step, so an optimal renaturation may require limited blocking. The best time may well be different for each protein and require empirical optimization. In addition, different lots of BSA appear to result in diminished signal. Therefore, it is important to purchase high-quality BSA from a reputable manufacturer.

An important consideration is the inclusion of appropriate controls to rule out nonspecific interactions that might result in false positives. Suitable controls should be furnished for both the target proteins and the protein probe. When using a complex mixture of proteins, such as cell extracts, as target, "negative" control proteins are already present. However, when using a mixture of only a few proteins, it is important to provide a protein that does not interact to serve as a negative control for nonspecific binding. The ideal negative control should be similar to the protein of interest in charge and size. Appropriate controls should be subjected to SDS-PAGE and blotted in parallel with the samples of interest. Another important control is the use of an unprogrammed translation lysate as a probe. Translation of an unrelated protein as a control probe is also often helpful.

Troubleshooting

Precise conditions for far westerns vary from procedure to procedure and probably reflect the nature of the individual proteins being examined. Optimal conditions for each protein may need to be determined empirically. If background staining is too high, there are several possible remedies. The probe may be diluted or the sample concentration lowered. Other "blocking" reagents may be tested.The blocking reagent, nonfat dry milk, ranging in concentration from 1% to 5%, has been used successfully. Other detergents such as NP-40 and Triton X-100 are commonly employed for this procedure and may help to decrease background. Also, increasing the length of the blocking step may aid in background reduction. Most procedures that call for extended blocking times suggest incubation at 4°C.

If no specific staining is observed, confirm the quality of the radiolabeled probe by SDS-

PAGE and autoradiography. Make sure the reticulocyte lysate has not been repeatedly frozen and thawed. If using a recombinant protein/antibody scheme, confirm the affinity of that interaction by immunoblotting.

Sometimes a decrease in blocking time or use of a different blocking reagent will increase signal. Some proteins may interact more readily following a denatuation/renaturation cycle. In this case, the membrane is incubated for 1 hr in PBS-buffered 7 M guanidine or 8 M urea and renatured overnight. Renature in blocking buffer I (see Basic Protocol) without agitation at 4°C with several changes of buffer. Finally, the length of the binding reaction can be increased.

Anticipated Results

Sensitivity of the far western blot is dependent on the affinity of the protein-protein interactions being investigated and on the quality of the probe. Thus, extra attention given to the preparation of a high-quality probe is almost invariably worthwhile. When using a radiolabeled probe, a positive interaction can typically be visualized after overnight exposure to X-ray film.

Time Considerations

The basic protocol can be performed in 2 days. An SDS-PAGE gel can be set up and run in 4 to 6 hr. The rest of the procedure can be performed in 8 to 10 hr. It is often convenient to set up the SDS-PAGE gels on one day and run them slowly overnight. The rest of the basic protocol can then be performed the next day. Semidry transfer requires ~2 hr, the blocking steps take ~4 hr, binding of the probe takes ~2 hr, washing, drying, and setting up the autoradiography cassette takes ~1 hr. Detection of interactions using antibodies to detect the probe protein requires an additional day. Peptide blots can be performed in one day.

It is possible to store the blots after the blocking steps for up to 2 weeks at 4°C. The blots should be stored in airtight containers or wrappings so they do not dry out. In addition, in vitro–translated probes may be produced ahead of time and stored unpurified at –20°C, although the efficiency may be reduced for some probes.

Literature Cited

Chaudhary, J., Cupp, A.S., and Skinner, M.K. 1997. Role of basic-helix-loop-helix transcription factors in Sertoli cell differentiation: Identification of an E-box response element in the transferrin promoter. *Endocrinology* 138:667-675.

Edmondson, D.G., Smith, M.M., and Roth, S.Y. 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. *Genes & Dev.* 10:1247-1259.

Fischer, N., Kremmer, E., Lautscham, G., Mueller-Lantzsch, N., and Grasser, F. A. 1997. Epstein-Barr virus nuclear antigen 1 forms a complex with the nuclear transporter karyopherin alpha2. *J. Biol. Chem.* 272:3999-4005.

Grasser, F. A., Sauder, C., Haiss, P., Hille, A., Konig, S., Gottel, S., Kremmer, E., Leinenbach, H. P., Zeppezauer, M., and Mueller-Lantzsch, N. 1993. Immunological detection of proteins associated with the Epstein-Barr virus nuclear antigen 2A. *Virology* 195:550-560.

Grulich-Henn, J., Spiess, S., Heinrich, U., Schonberg, D., and Bettendorf, M. 1998. Ligand blot analysis of insulin-like growth factor-binding proteins using biotinylated insulin-like growth factor-I. *Horm. Res.* 49:1-7.

Hsiao, P. W., and Chang, C. 1999. Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. *J. Biol. Chem.* 274:22373-22379.

Kimball, S. R., Heinzinger, N. K., Horetsky, R. L., and Jefferson, L. S. 1998. Identification of interprotein interactions between the subunits of eukaryotic initiation factors eIF2 and eIF2B. *J. Biol. Chem.* 273:3039-3044.

Kleinschmidt, J. A., and Seiter, A. 1988. Identification of domains involved in nuclear uptake and histone binding of protein N1 of *Xenopus laevis*. *EMBO J.* 7:1605-1614.

Kouklis, P. D., Hutton, E., and Fuchs, E. 1994. Making a connection: Direct binding between keratin intermediate filaments and desmosomal proteins. *J. Cell Biol.* 127:1049-1060.

Luna, E.J. 1996. Biotinylation of proteins in solution and on cell surfaces. *In* Current Protocols in Protein Science. (J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, and P.T. Wingfield, eds.) pp. 3.6.1-3.6.15. John Wiley & Sons, New York.

Palaparti, A., Baratz, A., and Stifani, S. 1997. The Groucho/transducin-like enhancer of split transcriptional repressors interact with the genetically defined amino-terminal silencing domain of histone H3. *J. Biol. Chem.* 272:26604-26610.

Schumacher, T.N.M. and Tsomides, T.I. 1995. In vitro radiolabeling of peptides and proteins. *In* Current Protocols in Protein Science (J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, and P.T. Wingfield. eds.) pp. 3.3.1-3.3.19. John Wiley & Sons, New York.

Contributed by Diane G. Edmondson and Sharon Y. Roth
M.D. Anderson Cancer Center
Houston, Texas

Interaction Trap/Two-Hybrid System to Identify Interacting Proteins

To understand the function of a particular protein, it is often useful to identify other proteins with which it associates. This can be done by a selection or screen in which novel proteins that specifically interact with a target protein of interest are isolated from a library. One particularly useful approach to detect novel interacting proteins—the two-hybrid system or interaction trap (see Figs. 17.3.1 and 17.3.2)—uses yeast as a "test tube" and transcriptional activation of a reporter system to identify associating proteins (see Background Information). This approach can also be used specifically to test complex formation between two proteins for which there is a prior reason to expect an interaction.

In the basic version of this method (see Fig. 17.3.2), the plasmid pEG202 or a related vector (see Fig. 17.3.3 and Table 17.3.1) is used to express the probe or "bait" protein as a fusion to the heterologous DNA-binding protein LexA. Many proteins, including transcription factors, kinases, and phosphatases, have been successfully used as bait proteins. The major requirements for the bait protein are that it should not be actively excluded from the yeast nucleus, and it should not possess an intrinsic ability to strongly activate transcription. The plasmid expressing the LexA-fused bait protein (see Table 17.3.1) is used to transform yeast possessing a dual reporter system responsive to transcriptional activation through the *LexA* operator. In one such example, the yeast strain EGY48 (see Table 17.3.2) contains the reporter plasmid pSH18-34. In this case, binding sites for LexA are located upstream of two reporter genes. In the EGY48 strain, the upstream activating sequences of the chromosomal *LEU2* gene—required in the biosynthetic pathway for leucine (Leu)—are replaced with *LexA* operators (DNA binding sites). pSH18-34 contains a *LexA* operator–*lacZ* fusion gene. These two reporters allow selection for transcriptional activation by permitting selection for viability when cells are plated on medium lacking Leu, and discrimination based on color when the yeast is grown on medium containing Xgal.

In Basic Protocol 1, EGY48/pSH18-34 transformed with a bait is characterized for its ability to express protein (Support Protocol 1), growth on medium lacking Leu, and for the level of transcriptional activation of *lacZ* (see Fig. 17.3.2A). A number of alternative strains, plasmids, and strategies are presented which can be employed if a bait proves to have an unacceptably high level of background transcriptional activation.

In an interactor hunt (Basic Protocol 2), the strain EGY48/pSH18-34 containing the bait expression plasmid is transformed (along with carrier DNA made as described in Support Protocol 2) with a conditionally expressed library made in the vector pJG4-5 (see Fig. 17.3.6 and Table 17.3.3). This library uses the inducible yeast *GAL1* promoter to express proteins as fusions to an acidic domain ("acid blob") that functions as a portable transcriptional activation motif (act) and to other useful moieties. Expression of library-encoded proteins is induced by plating transformants on medium containing galactose (Gal), so yeast cells containing library proteins that do not interact specifically with the bait protein will fail to grow in the absence of Leu (see Fig. 17.3.2B). Yeast cells containing library proteins that interact with the bait protein will form colonies within 2 to 5 days, and the colonies will turn blue when the cells are streaked on medium containing Xgal (see Fig. 17.3.2C). The DNA from interaction trap positive colonies can be analyzed by polymerase chain reaction (PCR) to streamline screening and detect redundant clones in cases where many positives are obtained in screening (see Alternate Protocol 1). The plasmids are isolated and characterized by a series of tests to confirm specificity of the interaction with the initial bait protein (Support Protocols 3 to 5). Those found to be specific are ready for further analysis (e.g., sequencing).

Contributed by Erica A. Golemis, Ilya Serebriiskii, Russell L. Finley, Jr., Mikhail G. Kolonin, Jeno Gyuris, and Roger Brent

Current Protocols in Cell Biology (2000) 17.3.1-17.3.42

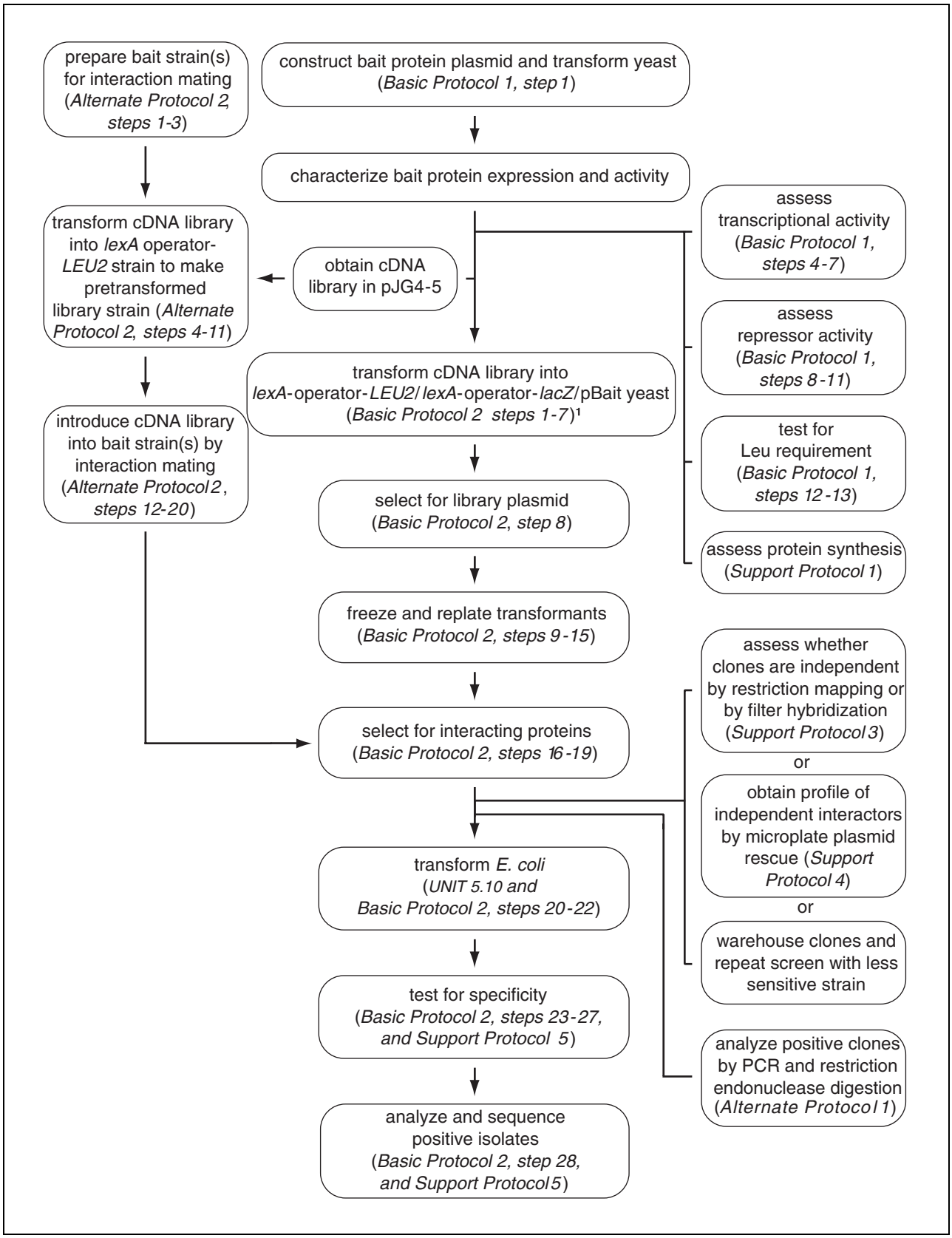


Figure 17.3.1 Flow chart for performing an interaction trap.

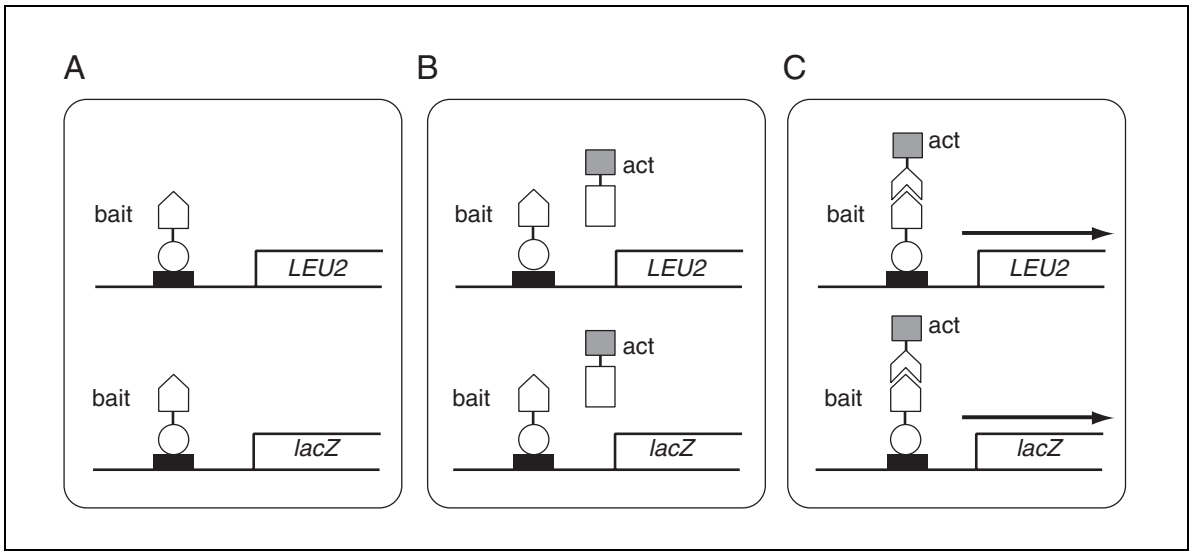


Figure 17.3.2 The interaction trap. (**A**) An EGY48 yeast cell containing two *LexA* operator–responsive reporters, one a chromosomally integrated copy of the *LEU2* gene (required for growth on –Leu medium), the second a plasmid bearing a *GAL1* promoter–*lacZ* fusion gene (causing yeast to turn blue on medium containing Xgal). The cell also contains a constitutively expressed chimeric protein, consisting of the DNA-binding domain of LexA fused to the probe or bait protein, shown as being unable to activate either of the two reporters. (**B**) and (**C**), EGY48/pSH18-34/pbait-containing yeast have been additionally transformed with an activation domain (act)–fused cDNA library in pJG4-5, and the library has been induced. In (B), the encoded protein does not interact specifically with the bait protein and the two reporters are not activated. In (C), a positive interaction is shown in which the library-encoded protein interacts with bait protein, resulting in activation of the two reporters (arrow), thus causing growth on medium lacking Leu and blue color on medium containing Xgal. Symbols: black rectangle, *LexA* operator sequence; open circle, LexA protein; open pentagon, bait protein; open rectangle, library protein; shaded box, activator protein (acid blob).

When more than one bait will be used to screen a single library, significant time and resources can be saved by performing the interactor hunt by interaction mating (see Alternate Protocol 2). In this protocol, EGY48 is transformed with library DNA and the transformants are collected and frozen in aliquots. For each interactor hunt, an aliquot of the pretransformed EGY48/library strain is thawed and mixed with an aliquot of a bait strain transformed with the bait expression plasmid and pSH18-34. Overnight incubation of the mixture on a YPD plate results in fusion of the two strains to form diploids. The diploids are then exposed to galactose to induce expression of the library-encoded proteins, and interactors are selected in the same manner as in Basic Protocol 2. The advantage to this approach is that it requires only one high-efficiency library transformation for multiple hunts with different baits. It is also useful for bait proteins that are somewhat toxic to yeast; yeast expressing toxic baits can be difficult to transform with the library DNA.

CHARACTERIZING A BAIT PROTEIN

BASIC PROTOCOL 1

The first step in an interactor hunt is to construct a plasmid that expresses LexA fused to the protein of interest. This construct is transformed into reporter yeast strains containing *LEU2* and *lacZ* reporter genes, and a series of control experiments is performed to establish whether the construct is suitable as is or must be modified, and whether alternative yeast reporter conditions should be used. These controls establish that the bait protein is made as a stable protein in yeast, that it is capable of entering the nucleus and binding *LexA* operator sites, and that it does not appreciably activate transcription of the *LexA* operator–based reporter genes. This last is the most important constraint on use of this system. The LexA-fused bait protein must not activate transcription of either re-

Table 17.3.1 Interaction Trap Components[a,b]

Plasmid name/source[c]	Selection		Comment/description
	In yeast	In *E. coli*	
LexA fusion plasmids			
pEG202[d,e]	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses LexA followed by polylinker
pJK202[e]	*HIS3*	Ap^r	Like pEG202, but incorporates nuclear localization sequences between LexA and polylinker; used to enhance translocation of bait to nucleus
pNLexA[e]	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses polylinker followed by LexA; for use with baits where amino-terminal residues must remain unblocked
pGilda[d,e]	*HIS3*	Ap^r	Contains a *GAL1* promoter that expresses same LexA and polylinker cassette as pEG202; for use with baits whose continuous presence is toxic to yeast
pEE202I	*HIS3*	Ap^r	An integrating form of pEG202 that can be targeted into *HIS3* following digestion with *Kpn*I; for use where physiological screen requires lower levels of bait to be expressed
pRFHM1[e,f] (control)	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses LexA fused to the homeodomain of bicoid to produce nonactivating fusion; used as positive control for repression assay, negative control for activation and interaction assays
pSH17-4[e,f] (control)	*HIS3*	Ap^r	*ADH* promoter expresses LexA fused to GAL4 activation domain; used as a positive control for transcriptional activation
pMW101[f]	*HIS3*	Cm^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pMW103[f]	*HIS3*	Km^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pHybLex/Zeo[f,g]	Zeo^r	Zeo^r	Bait cloning vector compatible with interaction trap and all other two-hybrid systems; minimal ADH promotor expresses LexA followed by extended polylinker
Activation domain fusion plasmids			
pJG4-5[c,d,e,f]	TRP1	Ap^r	Contains a *GAL1* promoter that expresses nuclear localization domain, transcriptional activation domain, HA epitope tag, cloning sites; used to express cDNA libraries
pJG4-5I	*TRP1*	Ap^r	An integrating form of pJG4-5 that can be targeted into *TRP1* by digestion with *Bsu*36I (New England Biolabs); to be used with pEE202I to study interactions that occur physiologically at low protein concentrations
pYESTrp[g]	*TRP1*	Ap^r	Contains a *GAL1* promoter that expresses nuclear localization domain, transcriptional activation domain, V5 epitope tag, multiple cloning sites; contains f1 ori and T7 promoter/flanking site; used to express cDNA libraries (Invitrogen)
pMW102[f]	*TRP1*	Km^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
pMW104[f]	*TRP1*	Cm^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
LacZ reporter plasmids			
pSH18-34[d,e,f]	*URA3*	Ap^r	Contains 8 *LexA* operators that direct transcription of the *lacZ* gene; one of the most sensitive indicator plasmids for transcriptional activation
pJK103[e]	*URA3*	Ap^r	Contains two *LexA* operators that direct transcription of the *lacZ* gene; an intermediate reporter plasmid for transcriptional activation
pRB1840[e]	*URA3*	Ap^r	Contains 1 *LexA* operator that directs transcription of the *lacZ* gene; one of the most stringent reporters for transcriptional activation
pMW112[f]	*URA3*	Km^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW109[f]	*URA3*	Km^r	Same as pJK103, but with altered antibiotic resistance marker

continued

Table 17.3.1 Interaction Trap Components[a,b], continued

Plasmid name/source[c]	Selection		Comment/description
	In yeast	In *E. coli*	
pMW111[f]	*URA3*	Km^r	Same as pRB1840, but with altered antibiotic resistance marker
pMW107[f]	*URA3*	Cm^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW108[f]	*URA3*	Cm^r	Same as pJK103, but with altered antibiotic resistance marker
pMW110[f]	*URA3*	Cm^r	Same as pRB1840, but with altered antibiotic resistance marker
pJK101[e,f] (control)	*URA3*	Ap^r	Contains a *GAL1* upstream activating sequence followed by two *lexA* operators followed by *lacZ* gene; used in repression assay to assess bait binding to operator sequences

[a]All plasmids contain a 2µm origin for maintenance in yeast, as well as a bacterial origin of replication, except where noted (pEE202I, pJG4.5I).

[b]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Plasmids with altered antibiotic resistance markers (all pMW plasmids) were constructed at Glaxo in Research Triangle Park, N.C. (Watson et al., 1996). Plasmids and strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (pEG202); J. Kamens, BASF, Worcester, Mass. (pJK202); cumulative efforts of I. York, Dana-Farber Cancer Center, Boston, Mass. and M. Sainz and S. Nottwehr, U. Oregon (pNLexA); D.A. Shaywitz, MIT Center for Cancer Research, Cambridge, Mass. (pGilda); R. Buckholz, Glaxo, Research Triangle Park, N.C. (pEE2021, pJG4-51); J. Gyuris, Mitotix, Cambridge, Mass. (pJG4-5); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH17-4); R.L. Finley, Wayne State University School of Medicine, Detroit, Mich. (pRFHM1); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH18-34); J. Kamens, BASF, Worcester, Mass. (pJK101, pJK103); R. Brent, The Molecular Sciences Institute, Berkeley, Calif. (pRB1840). Specialized plasmids not yet commercially available can be obtained by contacting the Brent laboratory at (510) 647-0690 or *brent@molsci.org*, or the Golemis laboratory, (215) 728-2860 or *EA_Golemis@fccc.edu*.

[c]Maps, sequences, and links for some of the plasmids are available at *http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html*.

[d]Plasmids commercially available from Clontech and OriGene; for Clontech pEG202 is listed as pLexA, pJG4-5 as pB42AD, and pSH18-34 as p8op-LacZ.

[e]Plasmids and strains available from OriGene.

[f]In pMW plasmids the ampicillin resistance gene (Ap^r) is replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r) from pBC SK(+) and pBK-CMV (Stratagene), respectively. The choice between Km^r and Cm^r or Ap^r plasmids is a matter of personal taste; use of basic Ap^r plasmids is described in the basic protocols. Use of the more recently developed reagents would facilitate the purification of library plasmid in later steps by eliminating the need for passage through KC8 bacteria, with substantial saving of time and effort. Ap^r has been maintained as marker of choice for the library plasmid because of the existence of multiple libraries already possessing this marker. These plasmids are the basic set of plasmids recommended for use.

[g]Plasmids commercially available from Invitrogen as components of a Hybrid Hunter kit; this kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

porter—the EGY48 strain (or related strain EGY191) that expresses the LexA fusion protein should not grow on medium lacking Leu, and the colonies should be white on medium containing Xgal. The characterized bait protein plasmid is used for Basic Protocol 2 to screen a library for interacting proteins.

Materials

DNA encoding the protein of interest

Plasmids (see Table 17.3.1): pEG202 (see Fig. 17.3.3), pSH18-34 (see Fig. 17.3.4), pSH17-4, pRFHM1, and pJK101 for basic characterization; other plasmids for specific circumstances as described (Clontech, Invitrogen, OriGene, or R. Brent)

Yeast strain EGY48 (*ura3 trp1 his3 3LexA*-operator-*LEU2*), or EGY191 (*ura3 trp1 his3 1LexA*-operator-*LEU2*; Table 17.3.2)

Complete minimal (CM) medium dropout plates (see recipe), supplemented with 2% (w/v) of the indicated sugars (glucose or galactose), in 100-mm plates:
- Glu/CM, −Ura, −His
- Gal/CM, −Ura, −His
- Gal/CM, −Ura, −His, −Leu

Z buffer (see recipe) with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal)

Gal/CM dropout liquid medium (see recipe) supplemented with 2% Gal

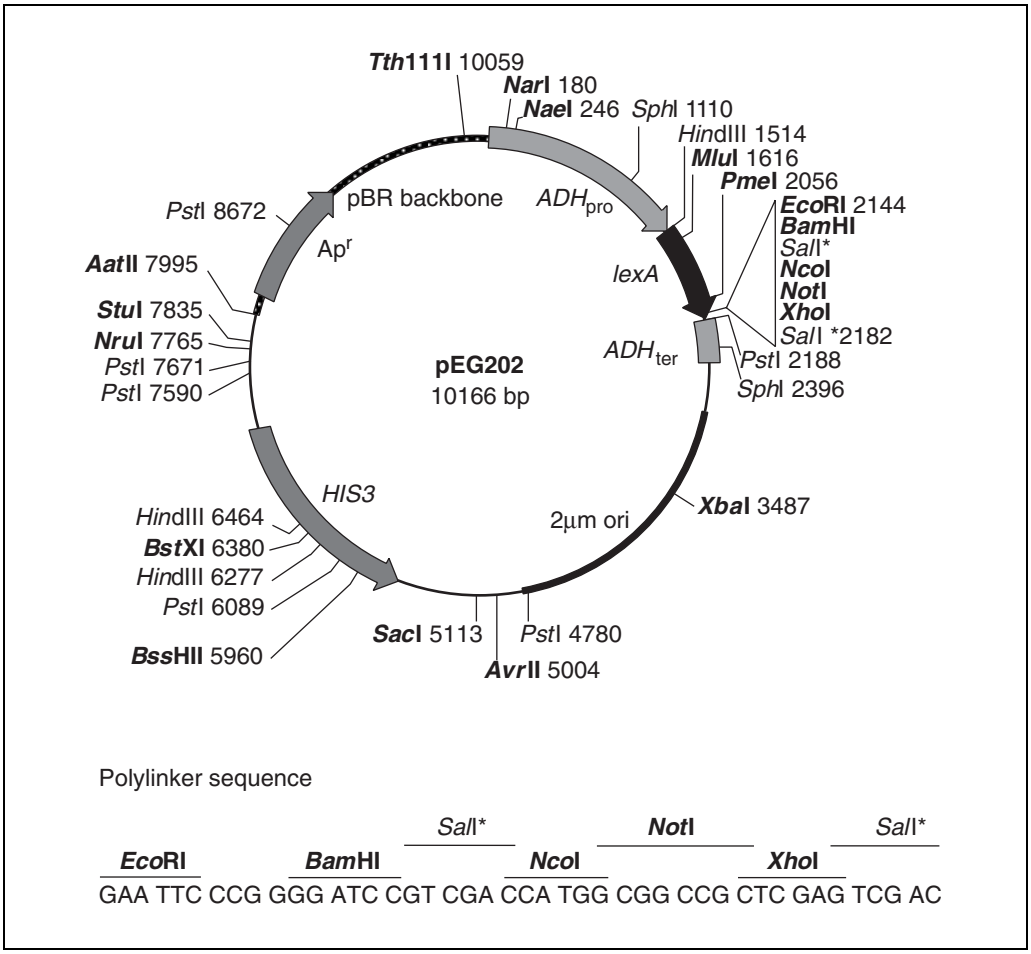


Figure 17.3.3 LexA-fusion plasmids: pEG202 (sequence: accession number U89960). The strong constitutive *ADH* promoter is used to express bait proteins as fusions to the DNA-binding protein LexA. Restriction sites shown in this map are based on recently compiled pEG202 sequence data and include selected sites suitable for diagnostic restriction endonuclease digests. A number of restriction sites are available for insertion of coding sequences to produce protein fusions with LexA; the polylinker sequence and reading frame relative to LexA are shown below the map with unique sites marked in bold type. The sequence 5′-CGT CAG CAG AGC TTC ACC ATT G-3′ can be used to design a primer to confirm correct reading frame for LexA fusions. Plasmids contain the *HIS3* selectable marker and the 2μm origin of replication to allow propagation in yeast, and the Ap^r antibiotic resistance gene and the pBR origin of replication to allow propagation in *E. coli*. In the recently developed LexA-expression plasmids pMW101 and pMW103, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r), respectively. Alternative bait plasmids are listed in Table 17.3.1, and maps, sequences, and links for some of them are available at *http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html*.

Antibody to LexA or fusion domain: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

H_2O, sterile

30°C incubator

Nylon membrane

Whatman 3MM filter paper

Additional reagents and equipment for subcloning DNA fragments, lithium acetate transformation of yeast, liquid assay for β-galactosidase (*APPENDIX 3*), preparation of protein extracts for immunoblot analysis (see Support Protocol 1), and immunoblotting and immunodetection (*UNIT 6.2*)

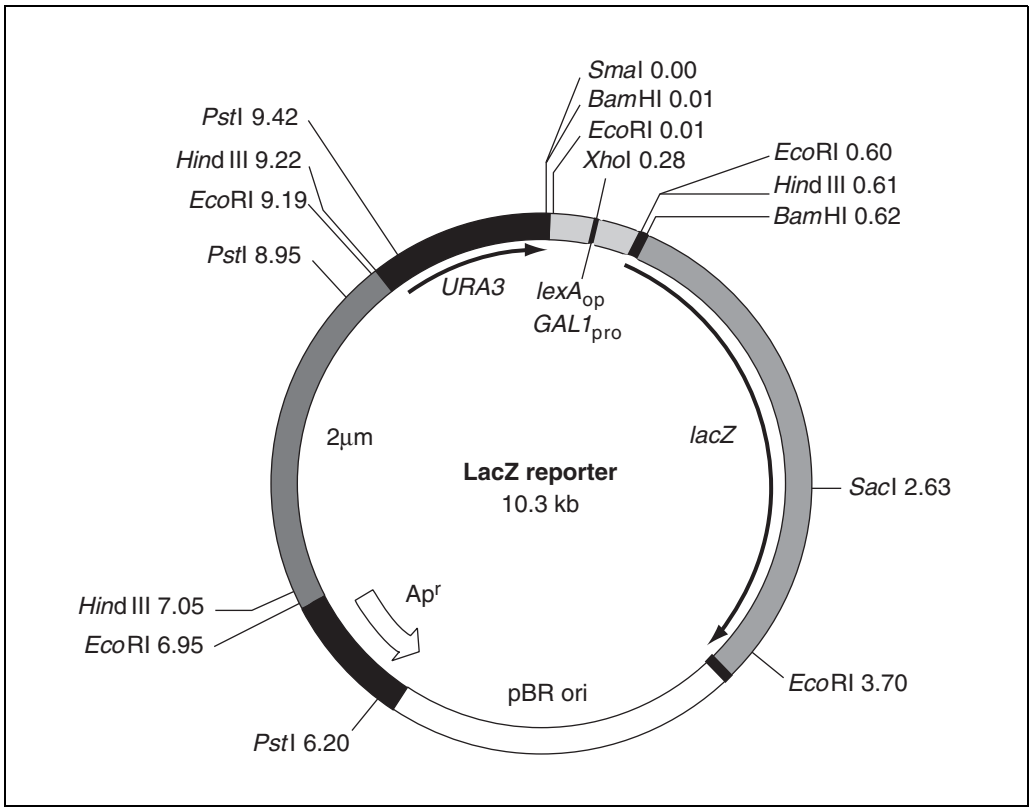


Figure 17.3.4 LacZ reporter plasmid. pRB1840, pJK103, and pSH18-34 are all derivatives of LR1Δ1 (West et al., 1984) containing eight, two, or one operator for LexA (*LexA*$_{op}$) binding inserted into the unique *Xho*I site located in the minimal *GAL1* promoter (*GAL1*$_{pro}$; 0.28 on map). The plasmid contains the *URA3* selectable marker, the 2μm origin to allow propagation in yeast, the ampicillin resistance (Ap^r) gene, and the pBR322 origin (ori) to allow propagation in *E. coli*. Numbers indicate relative map positions. In the recently developed derivatives, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol or kanamycin resistance genes.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

Transform yeast with the bait protein plasmid

1. Using standard subcloning techniques (*APPENDIX 3*), insert the DNA encoding the protein of interest into the polylinker of pEG202 (see Fig. 17.3.3) or other LexA fusion plasmid to make an in-frame protein fusion.

   The LexA fusion protein is expressed from the strong alcohol dehydrogenase (ADH) promoter. pEG202 also contains a HIS3 selectable marker and a 2μm origin for propagation in yeast. pEG202 with the DNA encoding the protein of interest inserted is designated pBait. Uses of alternative LexA fusion plasmids are described in Background Information.

2. Perform three separate lithium acetate transformations (*APPENDIX 3*) of EGY48 using the following combinations of plasmids:

   pBait + pSH18-34 (test)
   pSH17-4 + pSH18-34 (positive control for activation)
   pRFHM1 + pSH18-34 (negative control for activation).

   Use of the two LexA fusions as positive and negative controls allows a rough assessment of the transcriptional activation profile of LexA bait proteins. pEG202 itself is not a good negative control because the peptide encoded by the uninterrupted polylinker sequences is itself capable of very weakly activating transcription.

Table 17.3.2 Interaction Trap Yeast Selection Strains[a]

Strain	Relevant genotype	Number of operators	Comments/description
EGY48[b,c,d]	*MAT*α *trp1*, *his3*, *ura3*, lexAops-LEU2	6	*lexA* operators direct transcription from the *LEU2* gene; EGY48 is a basic strain used to select for interacting clones from a cDNA library
EGY191[c]	*MAT*α *trp1*, *his3*, *ura3*, lexAops-LEU2	2	EGY191 provides a more stringent selection than EGY48, producing lower background with baits with instrinsic ability to activate transcription
L40[c]	MATα *trpl*, *leu*2, *ade*2, GAL4, *lexAops-HIS3*	4	Expression driven from *GAL1* promoter is constitutive in L40 (inducible in EGY strains); selection is for *HIS* prototrophy.
	MATα *trpl*, *leu*2, *ade*2, GAL4, *lexAops-lacZ*	8	Integrated *lacZ* reporter is considerably less sensitive than pSH18-34 maintained in EGY strains

[a]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (EGY48, EGY191); A.B. Vojtek and S.M. Hollenberg, Fred Hutchinson Cancer Research Center, Seattle, Wash. (L40).

[b]Strains commercially available from Clontech.

[c]Strains commercially available from Invitrogen as components of a Hybrid Hunter kit; the kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

[d]Strains commercially available from OriGene.

pSH18-34 contains a 2μm origin and a URA3 selectable marker for maintenance in yeast, as well as a bacterial origin of replication and ampicillin-resistance gene. It is the most sensitive lacZ reporter available and will detect any potential ability to activate lacZ transcription. pSH17-4 is a HIS3 2μm plasmid encoding LexA fused to the activation domain of the yeast activator protein GAL4. This fusion protein strongly activates transcription. pRFHM1 is a HIS3 2μm plasmid encoding LexA fused to the N-terminus of the Drosophila protein bicoid. This fusion protein has no ability to activate transcription.

3. Plate each transformation mixture on Glu/CM −Ura, −His dropout plates. Incubate 2 days at 30°C to select for yeast that contain both plasmids.

   Colonies obtained can be used simultaneously in tests for the activation of lacZ (steps 4 to 7) and LEU2 (steps 12 to 13) reporters.

Assay lacZ gene activation by β-galactosidase assay

4. Streak a Glu/CM −Ura, −His master dropout plate with at least five or six independent colonies obtained from each of the three transformations in step 3 (test, positive control, and negative control) and incubate overnight at 30°C.

   The filter assay described in Steps 5a to 7a (based on Breeden and Nasmyth, 1985) provides a rapid assay for β-galactosidase transcription. Alternatively, a liquid assay (APPENDIX 3) or a plate assay (described in Steps 5b to 7b) may be used. More information on β-galactosidase assays is available on http://www.fccc.edu/research/labs/golemis/betagal.html.

Perform filter assay for β-galactosidase activity:

5a. Lift colonies by gently placing a nylon membrane on the yeast plate and allowing it to become wet through. Remove the membrane and air dry 5 min. Chill the membrane, colony side up, 10 min at −70°C.

   Whatman 3MM filters can be cut to the size of the yeast plate as a more economical alternative to nylon membranes for performing lifts. In addition, two or three 5-min temperature cycles (−70°C to room temperature) can be used instead of a single cycle to promote better lysis; this may be worth doing if there is difficulty visualizing blue color.

6a. Cut a piece of Whatman 3MM filter paper slightly larger than the colony membrane and soak it in Z buffer containing 1 mg/ml Xgal. Place colony membrane, colony side up, on Whatman 3MM paper, or float it in the lid of a petri dish containing ~2 ml Z buffer with 1 mg/ml Xgal.

Acceptable results may be obtained using as little as 300 μg/ml Xgal.

7a. Incubate at 30°C and monitor for color changes.

It is generally useful to check the membrane after 20 min, and again after 2 to 3 hr. Strong activators will produce a blue color in 5 to 10 min, and a bait protein (LexA fusion protein) that does so is unsuitable for use in an interactor hunt using this lacZ reporter plasmid. Weak activators will produce a blue color in 1 to 6 hr (compare versus negative control pRFHMI which will itself produce a faint blue color with time) and may or may not be suitable. Weak activators should be tested using the repressor assay described in steps 8 to 11.

Perform Xgal plate assay for lacZ activation:

5b. Prepare Z buffer Xgal plates.

For activation assays, plates should be prepared with glucose as a sugar source. For repression assays (steps 8 to 11), galactose should be used as a sugar source. In our experience, when patching from a master plate to Xgal plates, sufficient yeast are transferred that plasmid loss is not a major problem even in the absence of selection; this is balanced by the desire to assay sets of constructs on the same plate to eliminate batch variation in Xgal potency. Hence, plates should be made either with complete minimal amino acid mix, or by dropping out only uracil (–Ura), to make the plates universally useful.

6b. Streak yeast from master plate to Xgal plate and incubate at 30°C.

7b. Examine plates for color development at intervals over the next 2 to 3 days.

Strongly activating fusions should be visibly blue on the plate within 12 to 24 hr; moderate activators will be visibly blue after ~2 days.

When a bait protein appreciably activates transcription under these conditions, there are several recourses. The first and simplest is to switch to a less sensitive lacZ reporter plasmid; use of pJK103 and pRB1840 may be sufficient to reduce background to manageable levels. If this fails to work, it is frequently possible to generate a truncated LexA fusion that does not activate transcription.

Confirm fusion-protein synthesis by repression assay

For LexA fusions that do not activate transcription, confirm by performing a repression assay (Brent and Ptashne, 1984) that the LexA fusion protein is being synthesized in yeast (some proteins are not) and that it is capable of binding *LexA* operator sequences (Fig. 17.3.5). The following steps can be performed concurrently with the activation assay.

8. Transform EGY48 yeast with the following combinations of plasmids (three transformations):

   pBait + pJK101 (test)
   pRFHM1 + pJK101 (positive control for repression)
   pJK101 alone (negative control for repression).

9. Plate each transformation mix on Glu/CM –Ura, –His dropout plates or Glu/CM –Ura dropout plates as appropriate to select yeast cells that contain the indicated plasmids. Incubate 2 to 3 days at 30°C until colonies appear.

10. Streak colonies to a Glu/CM –Ura, –His or Glu/CM –Ura dropout master plate and incubate overnight at 30°C.

11. Assay β-galactosidase activity of the three transformed strains (test, positive control, and negative control) by liquid assay (using Gal/CM dropout liquid medium), filter

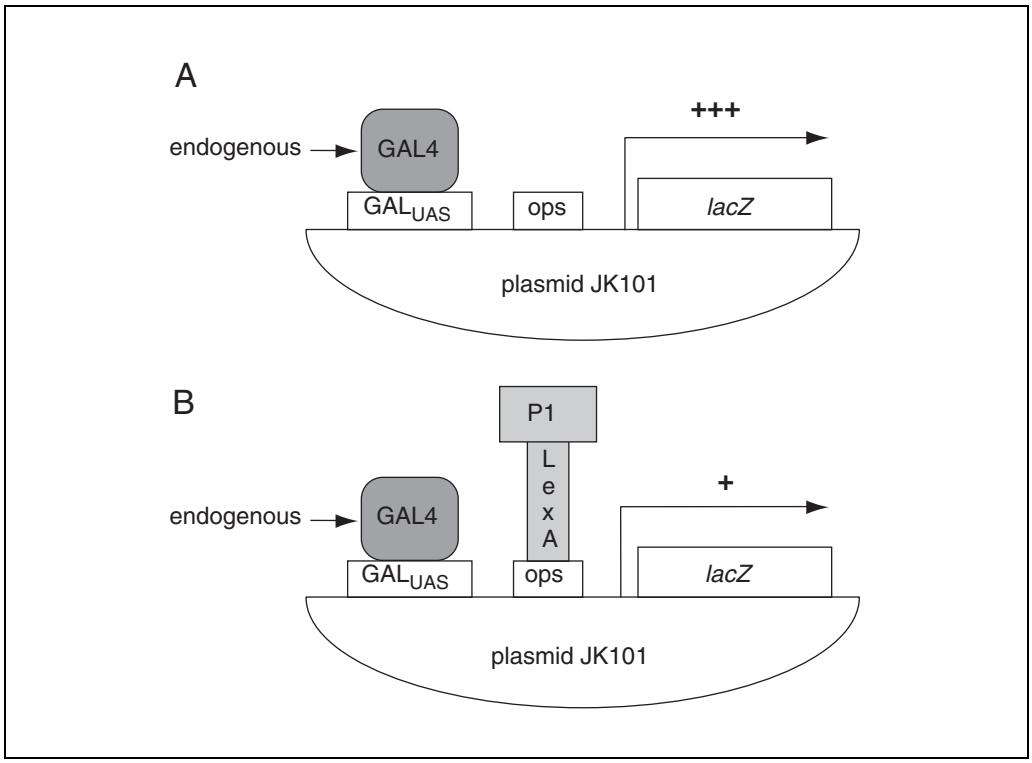


Figure 17.3.5 Repression assay for DNA binding. (**A**) The plasmid JK101 contains the upstream activating sequence (UAS) from the *GAL1* gene followed by *LexA* operators upstream of the *lacZ* coding sequence. Thus, yeast containing pJK101 will have significant β-galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS} (**B**). LexA-fused proteins (P1-LexA) that are made, enter the nucleus, and bind the *LexA* operator sequences (ops) will block activation from the GAL_{UAS}, repressing β-galactosidase activity (+) 3- to 5-fold. On glucose/Xgal medium, yeast containing pJK101 should be white because GAL_{UAS} transcription is repressed.

assay (steps 5a to 7a, first restreaking to Gal/CM plates to grow overnight), or plate assay (steps 5b to 7b, using Gal/CM −Ura XGal plates).

This assay should not be run for more than 1 to 2 hr for membranes, or 36 hr for Xgal plates, as the high basal lacZ activity will make differential activation of pJK101 impossible to see with longer incubations. Use of Xgal plates, and inspection 12 to 24 hr after streaking, is generally most effective.

The plasmid pJK101 contains the galactose upstream activating sequence (UAS) followed by LexA operators upstream of the lacZ coding sequence. Thus, yeast containing pJK101 will have significant β-galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS}. LexA-fused proteins that are made, enter the nucleus, and bind the LexA operator sequences block activation from the GAL_{UAS}, repressing β-galactosidase activity 3- to 20-fold. Note that on Glu/Xgal medium, yeast containing pJK101 should be white, because GAL_{UAS} transcription is repressed.

12. If a bait protein neither activates nor represses transcription, perform immunoblot analysis by probing an immunoblot of a crude lysate with antibodies against LexA or the fusion domain to test for protein synthesis (see Support Protocol 1).

Even if a bait protein represses transcription, it is generally a good idea to assay for the production of full-length LexA fusions, as occasionally some fusion proteins will be proteolytically cleaved by endogenous yeast proteases. If the protein is made but does not repress, it may be necessary to clone the sequence into a LexA fusion vector that contains a nuclear localization motif, e.g., pJK202 (see Table 17.3.1), or to modify or truncate the

fusion domain to remove motifs that target it to other cellular compartments (e.g., myristoylation signals).

Test for Leu requirement

These steps can be performed concurrently with the *lacZ* activation and repression assays.

13. Disperse a colony of EGY48 containing pBait and pSH18-34 reporter plasmids into 500 µl sterile water. Dilute 100 µl of suspension into 1 ml sterile water. Make a series of 1/10 dilutions in sterile water to cover a 1000-fold concentration range.

14. Plate 100 µl from each tube (undiluted, 1/10, 1/100, and 1/1000) on Gal/CM −Ura, −His dropout plates and on Gal/CM −Ura, −His, −Leu dropout plates. Incubate overnight at 30°C.

    There will be a total of eight plates. Gal/CM −Ura, −His dropout plates should show a concentration range from 10 to 10,000 colonies and Gal/CM −Ura, −His, −Leu dropout plates should have no colonies.

    Actual selection in the interactor hunt is based on the ability of the bait protein and acid-fusion pair, but not the bait protein alone, to activate transcription of the LexA operator-LEU2 gene and allow growth on medium lacking Leu. Thus, the test for the Leu requirement is the most important test of whether the bait protein is likely to have an unworkably high background. The LEU2 reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits, so it is possible that a bait protein that gives little or no signal in a β-galactosidase assay would nevertheless permit some level of growth on −Leu medium. If this occurs, there are several options for proceeding, the most immediate of which is to substitute EGY191 (see Table 17.3.2), a less sensitive screening strain, and repeat the assay.

    As outlined in this protocol, the authors recommend the strategy of performing the initial screening using the most sensitive reporters and then, if activation is detected, screening with increasingly less sensitive reporters (see Critical Parameters for further discussion).

PERFORMING AN INTERACTOR HUNT

BASIC PROTOCOL 2

An interactor hunt involves two successive large platings of yeast containing LexA-fused probes and reporters and libraries in pJG4-5 (Fig. 17.3.6, Table 17.3.3) with a cDNA expression cassette under control of the *GAL* promoter. In the first plating, yeast are plated on complete minimal (CM) medium −Ura, −His, −Trp dropout plates with glucose (Glu) as a sugar source to select for the library plasmid. In the second plating, which selects for yeast that contain interacting proteins, a slurry of primary transformants is plated on CM −Ura, −His, −Trp, −Leu dropout plates with galactose/raffinose (Gal/Raff) as the sugar source. This two-step selection is encouraged for two reasons. First, a number of interesting cDNA-encoded proteins may be deleterious to the growth of yeast that bear them; these would be competed out in an initial mass plating. Second, it seems likely that immediately after simultaneous transformation and Gal induction, yeast bearing particular interacting proteins may not be able to initially express sufficient levels of these proteins to support growth on medium lacking Leu. Library plasmids from colonies identified in the second plating are purified by bacterial transformation and used to transform yeast cells for the final specificity screen.

A list of libraries currently available for use with this system is provided in Table 17.3.3. The protocol outlined below describes the steps used to perform a single-step screen that should saturate a library derived from a mammalian cell. For screens with libraries derived from lower eukaryotes with less complex genomes, fewer plates will be required.

Occasionally, baits that seemed well-behaved during preliminary tests produce unworkably high backgrounds of "positives" during an actual screen (see Background Informa-

tion and Critical Parameters). To forestall the waste of time and materials performing a screen with such a bait would entail, an alternative approach is to perform a scaled-back screen when working with a new bait (e.g., 5 rather than 30 plates of primary transformants). The results can be assessed before doing a full screen; it is then possible to switch to lower-sensitivity reporter strains and plasmids, if appropriate. Although individual baits will vary, the authors' current default preference is to use the *lacZ* reporter pJK103 in conjunction with either EGY48 or EGY191. Polymerase chain reaction (PCR) can also be used in a rapid screening approach that may be preferable if a large number of positions are obtained in a library screen (see Alternate Protocol 1).

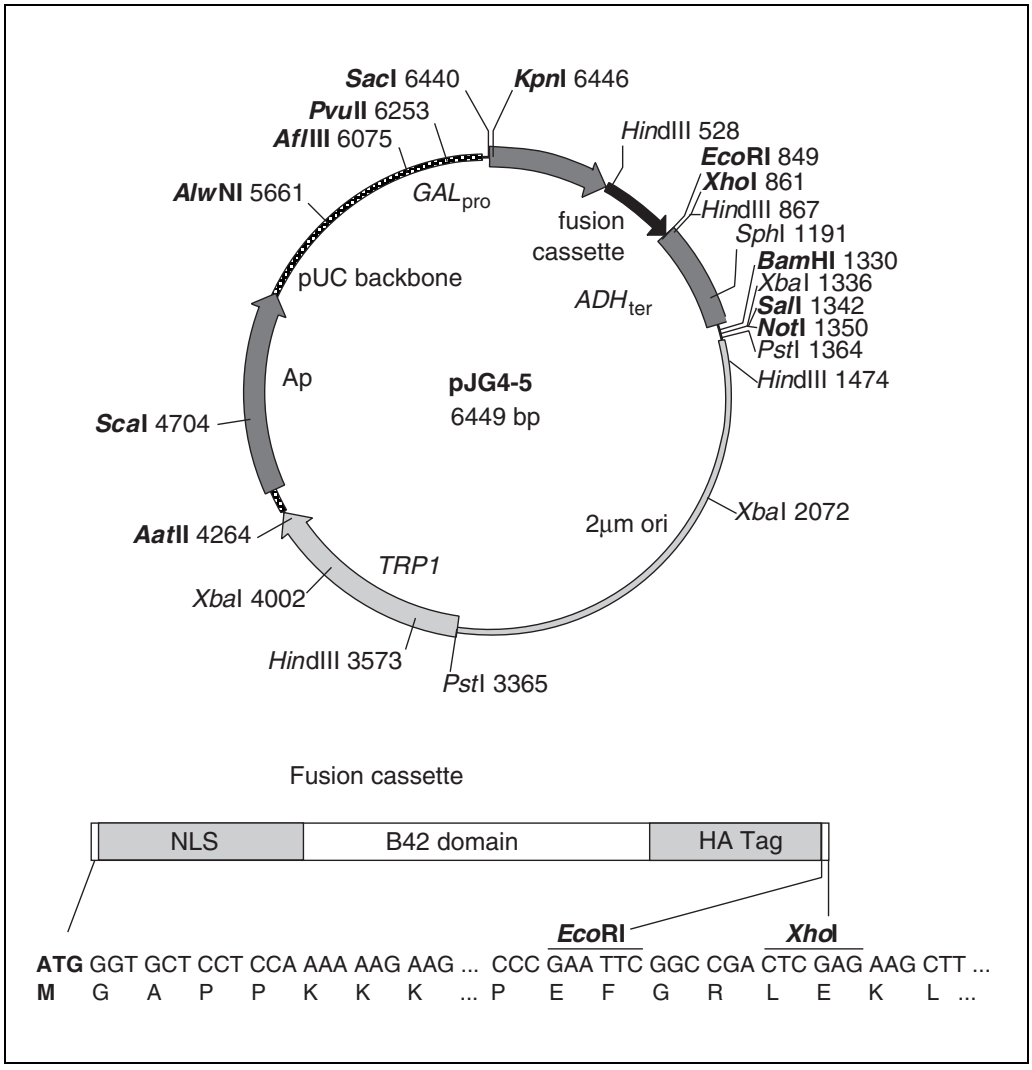


Figure 17.3.6 Library plasmids: pJG4-5 (sequence: accession number U89961). Library plasmids express cDNAs or other coding sequences inserted into unique *Eco*RI and *Xho*I sites as a translational fusion to a cassette consisting of the SV40 nuclear localization sequence (NLS; PPKKKRKVA), the acid blob B42 domain (Ruden et al., 1991), and the hemagglutinin (HA) epitope tag (YPYDVPDYA). Expression of cassette sequences is under the control of the *GAL1* galactose-inducible promoter. This map is based on the sequence data available for pJG4-5, and includes selected sites suitable for diagnostic restriction digests (shown in bold). The sequence 5′-CTG AGT GGA GAT GCC TCC-3′ can be used as a primer to identify inserts or to confirm correct reading frame. The pJG4-5 plasmid contains the *TRP1* selectable marker and the 2µm origin to allow propagation in yeast, and the antibiotic resistance gene and the pUC origin to allow propagation in *E. coli.* Unique sites are marked in bold type. Alternative library plasmids are listed in Table 17.3.1 and maps, sequences, and links for some of them are available at *http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html*.

Materials

Yeast containing appropriate combinations of plasmids (see Table 17.3.1 and Table 17.3.2):
- EGY48 containing *LexA*-operator-*lacZ* reporter and pBait (see Basic Protocol 1)
- EGY48 containing *LexA*-operator-*lacZ* reporter and pRFHM-1
- EGY48 containing *LexA*-operator-*lacZ* reporter and any nonspecific bait

Complete minimal (CM) dropout liquid medium (see recipe) supplemented with sugars (glucose, galactose, and/or raffinose) as indicated [2% (w/v) Glu, or 2% (w/v) Gal + 1% (w/v) Raff]:
- Glu/CM −Ura, −His
- Glu/CM −Trp
- Gal/Raff/CM −Ura, −His, −Trp

H_2O, sterile

TE buffer (pH 7.5; *APPENDIX 2A*)/0.1 M lithium acetate

Library DNA in pJG4-5 (Table 17.3.3 and Fig. 17.3.6)

High-quality sheared salmon sperm DNA (see Support Protocol 2)

40% (w/v) polyethylene glycol 4000 (PEG 4000; filter sterilized)/0.1 M lithium acetate/TE buffer (pH 7.5)

Dimethyl sulfoxide (DMSO)

Complete minimal (CM) medium dropout plates (see recipe) supplemented with sugars and Xgal (20 μg/ml) as indicated [2% (w/v) Glu, and 2% (w/v) Gal + 1% (w/v) Raff]:
- Glu/CM −Ura, −His, −Trp, 24 × 24–cm (Nunc) and 100-mm
- Gal/Raff/CM −Ura, −His, −Trp, 100-mm
- Gal/Raff/CM −Ura, −His, −Trp, −Leu, 100-mm
- Glu/Xgal/CM −Ura, −His, −Trp, 100-mm
- Gal/Raff/Xgal/CM −Ura, −His, −Trp, 100-mm
- Glu/CM −Ura, −His, −Trp, −Leu, 100-mm
- Glu/CM −Ura, −His, 100-mm
- Gal/CM −Ura, −His, −Trp, −Leu, 100-mm

TE buffer (pH 7.5), sterile (optional)

Glycerol solution (see recipe)

E. coli KC8 (*pyrF leuB600 trpC hisB463*; constructed by K. Struhl and available from R. Brent)

LB/ampicillin plates: LB plates (*APPENDIX 2A*) with 50 μg/ml ampicillin

E. coli DH5α or other strain suitable for preparation of DNA for sequencing

Bacterial defined minimal A medium plates: 1× A medium plates containing 0.5 μg/ml vitamin B1 supplemented with 40 μg/ml each Ura, His, and Leu

30°C incubator, with and without shaking

Low-speed centrifuge and rotor

50-ml conical tubes, sterile

1.5-ml microcentrifuge tubes, sterile

42°C heating block

Glass microscope slides, sterile

Additional reagents and equipment for rapid miniprep isolation of yeast DNA, transformation of bacteria by electroporation, miniprep isolation of bacterial DNA, restriction endonuclease digestion (optional), and agarose gel electrophoresis (optional; *APPENDIX 3*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

Table 17.3.3 Libraries Compatible with the Interaction Trap System[a,b]

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[c]	Contact information
Cell lines				
HeLa cells (human cervical carcinoma)	JG	9.6×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
HeLa cells (human cervical carcinoma)	Y	3.7×10^6	0.3-1.2 kb	Invitrogen
WI-38 cells (human lung fibroblasts), serum-starved, cDNA	JG	5.7×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, OriGene
Jurkat cells (human T cell leukemia), exponentially growing, cDNA	JG	4.0×10^6	0.7-2.8 kb (1.5 kb)	R. Brent
Jurkat cells (human T cell leukemia)	Y	3.2×10^6	0.3-1.2 kb	Invitrogen
Jurkat cells (human T cell leukemia)	Y	3.0×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Jurkat cells (human T cell leukemia)	JG	5.7×10^6	(>1.3)	OriGene
Jurkat cells (human T cell leukemia)	JG	2×10^6	0.7-3.5 kb (1.2 kb)	S. Witte
Be Wo cells (human fetal placental choriocarcinoma)	Y	5.4×10^6	0.3-0.8 kb	Invitrogen
Human lymphocyte	JG	4.0×10^6	0.4-4.0 kb (2.0 kb)	Clontech
$CD4^+$ T cell, murine, cDNA	JG	$>10^6$	0.3-2.5 kb (>0.5 kb)	R. Brent
Chinese hamster ovary (CHO) cells, exponentially growing, cDNA	JG	1.5×10^6	0.3-3.5 kb	R. Brent
A20 cells (mouse B cell lymphoma)	Y	3.11×10^6	0.3-1.2 kb	Invitrogen
Human B cell lymphoma	JG	—	—	H. Niu
Human 293 adenovirus–infected (early and late stages)	JG	—	—	K. Gustin
SKOV3 human Y ovarian cancer	Y	5.0×10^6	(>1.4 kb)	OriGene
MDBK cell, bovine kidney	JG	5.8×10^6	(>1.2 kb)	OriGene
MDCK cells	JG	—	—	D. Chen
HepG2 cell line cDNA	JG	2×10^6	—	M. Melegari
MCF7 breast cancer cells, untreated	JG	1.0×10^7	(>1.5 kb)	OriGene
MCF7 breast cancer cells, estrogen-treated	JG	1.0×10^7	(>1.1 kb)	OriGene
MCF7 cells, serum-grown	JG	1.0×10^7	0.4-3.5 kb	OriGene
LNCAP prostate cell line, untreated	JG	2.9×10^6	(>0.8 kb)	OriGene
LNCAP prostate cell line, androgen-treated	JG	4.6×10^6	(>0.9 kb)	OriGene
Mouse pachytene spermatocytes	JG	—	—	C. Hoog
Tissues				
Human breast	Y	9×10^6	0.4-1.2 kb	Invitrogen
Human breast tumor	Y	8.84×10^6	0.4-1.2 kb	Invitrogen
Human liver	JG	$>10^6$	0.6-4.0 kb (>1 kb)	R. Brent
Human liver	Y	2.2×10^6	0.5-4 kb (1.3 kb)	Clontech
Human liver	JG	3.2×10^6	0.3-1.2 kb	Invitrogen
Human liver	JG	1.1×10^7	(> 1 kb)	OriGene
Human lung	Y	5.9×10^6	0.4-1.2 kb	Invitrogen
Human lung tumor	Y	1.9×10^6	0.4-1.2	Invitrogen
Human brain	JG	3.5×10^6	0.5-4.5 kb (1.4 kb)	Clontech
Human brain	Y	8.9×10^6	0.3-1.2 kb	Invitrogen
Human testis	Y	6.4×10^6	0.3-1.2 kb	Invitrogen
Human testis	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human ovary	Y	4.6×10^6	0.3-1.2 kb	Invitrogen

continued

Table 17.3.3 Libraries Compatible with the Interaction Trap System[a,b], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[c]	Contact information
Human ovary	JG	4.6×10^6	(>1.3 kb)	OriGene
Human ovary	JG	3.5×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Human heart	JG	3.0×10^6	0.3-3.5 kb (1.3 kb)	Clontech
Human placenta	Y	4.8×10^6	0.3-1.2 kb	Invitrogen
Human placenta	JG	3.5×10^6	0.3-4.0 kb (1.2 kb)	Clontech
Human mammary gland	JG	3.5×10^6	0.5-5 kb (1.6 kb)	Clontech
Human peripheral blood leucocyte	JG	1.0×10^7	(>1.3 kb)	OriGene
Human kidney	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human fetal kidney	JG	3.0×10^6	(>1 kb)	OriGene
Human spleen	Y	1.14×10^7	0.4-1.2 kb	Invitrogen
Human prostate	Y	5.5×10^6	0.4-1.2 kb	Invitrogen
Human normal prostate	JG	1.4×10^6	0.4-4.5 kb (1.7 kb)	Clontech
Human prostate	JG	1.4×10^6	(>1 kb)	OriGene
Human prostate cancer	JG	1.1×10^6	(>0.9 kb)	OriGene
Human fetal liver	JG	3.5×10^6	0.3-4.5 kb (1.3 kb)	Clontech
Human fetal liver	Y	2.37×10^6	0.3-1.2 kb	Invitrogen
Human fetal liver	JG	8.6×10^6	(>1 kb)	OriGene
Human fetal brain	JG	3.5×10^6	0.5-1.2 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
Mouse brain	JG	6.1×10^6	(>1 kb)	OriGene
Mouse brain	JG	4.5×10^6	0.4-4.5 kb (1.2 kb)	Clontech
Mouse breast, lactating	JG	1.0×10^7	0.4-3.1 kb	OriGene
Mouse breast, involuting	JG	1.0×10^7	0.4-7.0 kb	OriGene
Mouse breast, virgin	JG	1.0×10^7	0.4-5.5 kb	OriGene
Mouse breast, 12 days pregnant	JG	6.3×10^6	0.4-5.3 kb	OriGene
Mouse skeletal muscle	JG	7.2×10^6	0.4-3.5 kb	OriGene
Rat adipocyte, 9-week-old Zucker rat	JG	1.0×10^7	0.4-5.0 kb	OriGene
Rat brain	JG	4.5×10^6	0.3-3.4 kb	OriGene
Rat brain (day 18)	JG	—	—	H. Niu
Rat testis	JG	8.0×10^6	(>1.2 kb)	OriGene
Rat thymus	JG	8.2×10^6	(>1.3 kb)	OriGene
Mouse liver	JG	9.5×10^6	(>1.4 kb)	OriGene
Mouse spleen	JG	1.0×10^7	(>1 kb)	OriGene
Mouse ovary	JG	4.0×10^6	(>1.2 kb)	OriGene
Mouse prostate	JG	3.7×10^6	0.3-4.0 kb	OriGene
Mouse embryo, whole (19-day)	JG	1.0×10^5	0.2-2.5 kb	OriGene
Mouse embryo	JG	3.6×10^6	0.5-5 kb (1.7 kb)	Clontech
Drosophila melanogaster, adult, cDNA	JG	1.8×10^6	(>1.0 kb)	OriGene
D. melanogaster, embryo, cDNA	JG	3.0×10^6	0.5-3.0 kb (1.4 kb)	Clontech
D. melanogaster, 0-12 hr embryos, cDNA	JG	4.2×10^6	0.5-2.5 kb (1.0 kb)	R. Brent
D. melanogaster, ovary, cDNA	JG	3.2×10^6	0.3-1.5 kb (800 bp)	R. Brent
D. melanogaster, disc, cDNA	JG	4.0×10^6	0.3-2.1 kb (900 bp)	R. Brent
D. melanogaster, head	JG	—	—	M. Rosbash

continued

Table 17.3.3 Libraries Compatible with the Interaction Trap System[a,b], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[c]	Contact information
Miscellaneous				
Synthetic aptamers	pJM-1	$>1\times 10^9$	60 bp	R. Brent
Saccharomyces cerevisiae, S288C, genomic	JG	$>3\times 10^6$	0.8-4.0 kb	R. Brent
S. cerevisiae, S288C, genomic	JG	4.0×10^6	0.5-4.0 kb	OriGene
Sea urchin ovary	JG	3.5×10^6	(1.7 kb)	Clontech
Caenorhabditis elegans	JG	3.8×10^6	(>1.2 kb)	OriGene
Arabidopsis thaliana, 7-day-old seedlings	JG	—	—	H.M. Goodman
Tomato (*Lycopersicon esculentum*)	JG	8×10^6	—	G.B. Martin
Xenopus laevis embryo	JG	2.2×10^6	0.3-4 kb (1.0 kb)	Clontech

[a]Most libraries are constructed in either the pJG4-5 vector or the pYESTrp vector (JG or Y in the Vector column); the peptide aptamer library is made in the pJM-1 vector. Libraries available from the public domain were constructed by the following individuals: (1) J. Gyuris; (3) C. Sardet and J. Gyuris; (4) W. Kolanus, J. Gyuris, and B. Seed; (39) D. Krainc; (50-52) R. Finley; (55) P. Watt; (54) P. Colas, B. Cohen, T. Jessen, I. Grishina, J. McCoy, and R. Brent (Colas et al., 1996). All libraries mentioned above were constructed in conjunction with and are available from the laboratory of Roger Brent, (510) 647-0690 or *brent@molsci.org*. The following individual investigators must be contacted directly: (18) J. Pugh, Fox Chase Cancer Center, Philadelphia, Pa.; (8, 9) Vinyaka Prasad, Albert Einstein Medical Center New York, N.Y.; (57, 58) Gregory B. Martin, *gmartin@dept.agry.purdue.edu*; (11) Huifeng Niu, *hn34@columbia.edu*; (16) Christer Hoog, *christer.hoog@cmb.ki.se*; (12) Kurt Gustin, *kgus@umich.edu*; (6) Stephan Witte, *Stephan.Witte@nimbus.rz.uni-konstanz.de.*

[b]The most updated list of Interaction Trap compatible libraries is available at *http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html*.

[c]Insert size ranges for pJG4-5 based libraries originally constructed in the Brent laboratory, which are now commercially available from Clontech, were reestimated by the company.

Transform the library

1. Grow an ~20-ml culture of EGY48 or EGY191 containing a *LexA*-operator-*lacZ* reporter plasmid and pBait in Glu/CM −Ura, −His liquid dropout medium overnight at 30°C.

   For best results, the pBait and lacZ reporter plasmids should have been transformed into the yeast within ~7 to 10 days of commencing a screen.

2. In the morning, dilute culture into 300 ml Glu/CM −Ura, −His liquid dropout medium to 2×10^6 cell/ml (OD_{600} = ~0.10). Incubate at 30°C until the culture contains ~1×10^7 cells/ml (OD_{600} = ~0.50).

3. Centrifuge 5 min at 1000 to 1500 × *g* in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 ml sterile water and transfer to 50-ml conical tube.

4. Centrifuge 5 min at 1000 to 1500 × *g*. Decant supernatant and resuspend cells in 1.5 ml TE buffer/0.1 M lithium acetate.

5. Add 1 µg library DNA in pJG4-5 and 50 µg high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5-ml microcentrifuge tubes. Add 50 µl of the resuspended yeast solution from step 4 to each tube.

   The total volume of library and salmon sperm DNA added should be <20 µl and preferably <10 µl.

   A typical library transformation will result in 2 to 3 × 10^6 primary transformants. Assuming a transformation efficiency of 10^5/µg library DNA, this transformation requires a total of 20 to 30 µg library DNA and 1 to 2 mg carrier DNA. Doing transformations in small aliquots helps reduce the likelihood of contamination, and for reasons that are not clear, provides significantly better transformation efficiency than scaled-up versions.

   Do not use excess transforming library DNA per aliquot of competent yeast cells because each competent cell may take up multiple library plasmids, complicating subsequent analysis.

6. Add 300 µl of sterile 40% PEG 4000/0.1 M lithium acetate/TE buffer, pH 7.5, and invert to mix thoroughly. Incubate 30 min at 30°C.

7. Add DMSO to 10% (~40 µl per tube) and invert to mix. Heat shock 10 min in 42°C heating block.

8a. *For 28 tubes:* Plate the complete contents of one tube per 24 × 24–cm Glu/CM −Ura, −His, −Trp dropout plate and incubate at 30°C.

8b. *For two remaining tubes:* Plate 360 µl of each tube on 24 × 24–cm Glu/CM −Ura, −His, −Trp dropout plate. Use the remaining 40 µl from each tube to make a series of 1/10 dilutions in sterile water. Plate dilutions on 100-mm Glu/CM −Ura, −His, −Trp dropout plates. Incubate all plates 2 to 3 days at 30°C until colonies appear.

The dilution series gives an idea of the transformation efficiency and allows an accurate estimation of the number of transformants obtained.

Collect primary transformant cells

Conventional replica plating does not work well in the selection process because so many cells are transferred to new plates that very high background levels inevitably occur. Instead, the procedure described below creates a slurry in which cells derived from $>10^6$ primary transformants are homogeneously dispersed. A precalculated number of these cells is plated for each primary transformant.

9. Cool all of the 24 × 24–cm plates containing transformants for several hours at 4°C to harden agar.

10. Wearing gloves and using a sterile glass microscope slide, gently scrape yeast cells off the plate. Pool cells from the 30 plates into one or two sterile 50-ml conical tubes.

This is the step where contamination is most likely to occur. Be careful.

11. Wash cells by adding a volume of sterile TE buffer or water at least equal to the volume of the transferred cells. Centrifuge ~5 min at 1000 to 1500 × *g*, room temperature, and discard supernatant. Repeat wash.

After the second wash, pellet volume should be ~25 ml cells derived from 1.5×10^6 transformants.

12. Resuspend pellet in 1 vol glycerol solution, mix well, and store up to 1 year in 1-ml aliquots at −70°C.

Determine replating efficiency

13. Remove an aliquot of frozen transformed yeast and dilute 1/10 with Gal/Raff/CM −Ura, −His, −Trp dropout medium. Incubate with shaking 4 hr at 30°C to induce the *GAL* promoter on the library.

Raffinose (Raff) aids in growth without diminishing transcription from the GAL1 promoter.

14. Make serial dilutions of the yeast cells using the Gal/Raff/CM −Ura, −His, −Trp dropout medium. Plate on 100-mm Gal/Raff/CM −Ura, −His, −Trp dropout plates and incubate 2 to 3 days at 30°C until colonies are visible.

15. Count colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

In calculating yeast concentrations, it is useful to remember that 1 OD_{600} unit = ~2.0×10^7 yeast cells. In general, if the harvest is done carefully, viability will be greater than 90%. Some intrepid investigators perform this step simultaneously with plating out on Leu selective medium (steps 16 and 17).

Screen for interacting proteins

16. Thaw the appropriate quantity of transformed yeast based on the plating efficiency, dilute, and incubate as in step 13. Dilute cultures in Gal/Raff/CM −Ura, −His, −Trp, −Leu medium as necessary to obtain a concentration of 10^7 cells/ml (OD_{600} = ~0.5), and plate 100 µl on each of as many 100-mm Gal/Raff/CM −Ura, −His, −Trp, −Leu dropout plates as are necessary for full representation of transformants. Incubate 2 to 3 days at 30°C until colonies appear.

    Because not all cells that contain interacting proteins plate at 100% efficiency on −Leu medium (Estojak et al., 1995), it is desirable that for actual selection, each primary colony obtained from the transformation be represented on the selection plate by three to ten individual yeast cells. This will in some cases lead to multiple isolations of the same cDNA; however, because the slurry is not perfectly homogenous, it will increase the likelihood that all primary transformants are represented by at least one cell on the selective plate.

    It is easiest to visually scan for Leu^+ colonies using cells plated at ~10^6 cfu per 100-mm plate. Plating at higher density can contribute to cross-feeding between yeast, resulting in spurious background growth. Thus, for a transformation in which 3×10^6 colonies are obtained, plate ~2×10^7 cells on a total of 20 selective plates.

17. Carefully pick appropriate colonies to a new Gal/Raff/CM −Ura, −His, −Trp, −Leu master dropout plate. Incubate 2 to 7 days at 30°C until colonies appear.

    A good strategy is to pick a master plate with colonies obtained on day 2, a second master plate (or set of plates) with colonies obtained on day 3, and a third with colonies obtained on day 4. Colonies from day 2 and 3 master plates should generally be characterized further. If many apparent positives are obtained, it may be worth making master plates of the much larger number of colonies likely to be obtained at day 4 (and after). See Critical Parameters and annotation to step 19 for additional information about appropriate colony selection for the master plate.

    If no colonies appear within a week, those arising at later time points are likely to be artifactual. Contamination that has occurred at an earlier step (e.g., during plate scraping) is generally reflected by the growth of a very large number of colonies (>500/plate) within 24 to 48 hr after plating on selective medium.

    Some investigators omit use of a Gal/Raff/CM −Ura, −His, −Trp, −Leu master plate, restreaking directly to a Glu/CM −Ura, −His, −Trp master plate as in step 19.

Test for Gal dependence

The following steps test for Gal dependence of the Leu^+ insert and lacZ phenotypes to confirm that they are attributable to expression of the library-encoded proteins. The *GAL1* promoter is turned off and −Leu selection eliminated before reinducing.

18. Restreak from the Gal/Raff/CM −Ura, −His, −Trp, −Leu master dropout plate to a 100-mm Glu/CM −Ura, −His, −Trp master dropout plate. Incubate overnight at 30°C until colonies form.

19. Restreak or replica plate from this plate to the following plates:

    Glu/Xgal/CM −Ura, −His, −Trp
    Gal/Raff/Xgal/CM −Ura, −His, −Trp
    Glu/CM −Ura, −His, −Trp, −Leu
    Gal/Raff/CM −Ura, −His, −Trp, −Leu.

    At this juncture, colonies and the library plasmids they contain are tentatively considered positive if they are blue on Gal/Raff/Xgal plates but not blue or only faintly blue on Glu/Xgal plates, and if they grow on Gal/Raff/CM −Leu plates but not on Glu/CM −Leu plates.

    The number of positives obtained will vary drastically from bait to bait. How they are processed subsequently will depend on the number initially obtained and on the preference

of the individual investigator. If none are obtained using EGY48 as reporter strain, it may be worth attempting to screen a library from an additional tissue source. If a relatively small number (≤30) are obtained, proceed to step 20. However, sometimes searches will yield large numbers of colonies (>30 to 300, or more). In this case, there are several options. The first option is to warehouse the majority of the positives and work up the first 30 that arise; those growing fastest are frequently the strongest interactors. These can be checked for specificity, and restriction digests can be used to establish whether they are all independent cDNAs or represent multiple isolates of the same, or a small number, of cDNAs. If the former is true, it may be advisable to repeat the screen in a less sensitive strain background, as obtaining many different interactors can be a sign of low-affinity nonspecific background. Alternatively, if initial indications are that a few cDNAs are dominating the positives obtained, it may be useful to perform a filter hybridization with yeast (see Support Protocol 3) using these cDNAs as a probe to establish the frequency of their identification and exclude future reisolation of these plasmids. The second major option is to work up large numbers of positives to get a complete profile of isolated interactors (see Support Protocol 4). A third option is to temporarily warehouse the entire results of this first screen, and repeat the screen with a less sensitive strain such as EGY191, on the theory that it is most important to get stronger interactors first and a complete profile of interactors later. Finally, some investigators prefer to work up the entire set of positives initially obtained, even if such positives number in the hundreds. Particularly in this latter case, it is most effective to use Alternate Protocol 1 as a means to identify unique versus common positives.

Isolate plasmid from positive colonies by transfer into E. coli

20a. Transfer yeast plasmids directly into *E. coli* by following the protocol for direct electroporation (*APPENDIX 3*, Alternate Protocol 2). Proceed to step 22.

20b. Isolate plasmid DNA from yeast by the rapid miniprep protocol (*APPENDIX 3*) with the following alteration: after obtaining aqueous phase, precipitate by adding sodium acetate to 0.3 M final and 2 vol ethanol, incubate 20 min on ice, microcentrifuge 15 min at maximum speed, wash pellet with 70% ethanol, dry, and resuspend in 5 µl TE buffer.

Cultures can be grown prior to the miniprep using Glu/CM −Trp to select only for the library plasmid; this may increase the proportion of bacterial colonies that contain the desired plasmid.

21. Use 1 µl DNA to electroporate (*APPENDIX 3*) into competent KC8 bacteria, and plate on LB/ampicillin plates. Incubate overnight at 37°C.

Electroporation must be used to obtain transformants with KC8 because the strain is generally refractory to transformation.

22. Restreak or replica plate colonies arising on LB/ampicillin plates to bacterial defined minimal A medium plates containing vitamin B1 and supplemented with Ura, His, and Leu but lacking Trp. Incubate overnight at 37°C.

Colonies that grow under these conditions contain the library plasmid.

The yeast TRP1 gene can successfully complement the bacterial trpC-9830 mutation, allowing the library plasmid to be easily distinguished from the other two plasmids contained in the yeast. It is helpful to first plate transformations on LB/ampicillin plates, which provides a less stringent selection, followed by restreaking to bacterial minimal medium to maximize the number of colonies obtained (E. Golemis, unpub. observ.).

23. Purify library-containing plasmids using a bacterial miniprep procedure (*APPENDIX 3*).

Some investigators are tempted to immediately sequence DNAs obtained at this stage. At this point, it is still possible that none of the isolated clones will express bona fide interactors, and it is suggested that the following specificity tests be completed before committing the effort to sequencing (also see annotation to step 28).

Because multiple 2μm plasmids with the same marker can be simultaneously tolerated in yeast, it sometimes happens that a single yeast will contain two or more different library plasmids, only one of which encodes an interacting protein. The frequency of this occurrence varies in the hands of different investigators and may in some cases account for disappearing positives if the wrong cDNA is picked. When choosing colonies to miniprep, it is generally useful to work up at least two individual bacterial transformants for each yeast positive. These minipreps can then be restriction digested (APPENDIX 3) with EcoRI + XhoI to release cDNA inserts, and the size of inserts determined on an agarose minigel (APPENDIX 3) to confirm that both plasmids contain the same insert. An additional benefit of analyzing insert size is that it may provide some indication as to whether repeated isolation of the same cDNA is occurring, generally a good indication concerning the biological relevance of the interactor. See Background Information for further discussion.

Assess positive colonies with specificity tests

Much spurious background will have been removed by the previous series of controls. Other classes of false positives can be eliminated by retransforming purified plasmids into "virgin" *LexA*-operator-*LEU2*/*LexA*-operator-*lacZ*/pBait–containing strains that have not been subjected to Leu selection and verifying that interaction-dependent phenotypes are still observed. Such false positives could include mutations in the initial EGY48 yeast that favor growth on Gal medium, library-encoded cDNAs that interact with the LexA DNA-binding domain, or proteins that are sticky and interact with multiple biologically unrelated fusion domains.

24. In separate transformations, use purified plasmids from step 23 to transform yeast that already contain the following plasmids and are growing on Glu/CM −Ura, −His plates:

    EGY48 containing pSH18-34 and pBait
    EGY48 containing pSH18-34 and pRFHM-1
    EGY48 containing pSH18-34 and a nonspecific bait (optional).

25. Plate each transformation mix on Glu/CM −Ura, −His, −Trp dropout plates and incubate 2 to 3 days at 30°C until colonies appear.

26. Create a Glu/CM −Ura, −His, −Trp master dropout plate for each library plasmid being tested. Streak adjacently five or six independent colonies derived from each of the transformation plates. Incubate overnight at 30°C.

27. Restreak or replica plate from this master dropout plate to the same series of test plates used for the actual screen:

    Glu/Xgal/CM −Ura, −His, −Trp
    Gal/Raff/Xgal/CM −Ura, −His, −Trp
    Glu/CM −Ura, −His, −Trp, −Leu
    Gal/CM −Ura, −His, −Trp, −Leu.

    True positive cDNAs should make cells blue on Gal/Raff/Xgal but not on Glu/Xgal plates, and should make them grow on Gal/Raff/CM −Leu but not Glu/CM −Leu dropout plates only if the cells contain LexA-bait. cDNAs that meet such criteria are ready to be sequenced (see legend to Fig. 17.3.3 for primer sequence) or otherwise characterized. Those cDNAs that also encode proteins that interact with either RFHM-1 or another nonspecific bait should be discarded.

    It may be helpful to cross-check the isolated cDNAs with a database of cDNAs thought to be false positives. This database is available on the World Wide Web as a work in progress at http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html. cDNAs reported to this database are generally those isolated only once in a screen in which obviously true interactive partners were isolated multiple times, cDNAs that may interact with more than one bait, or cDNAs for which the interaction does not appear to make biological sense

in the context of the starting bait. Although some proteins in this database may ultimately turn out in fact to associate with the bait that isolated them, they are by default unlikely to possess a unique and interesting function in the context of that bait if they are well represented in the database.

28. If appropriate, conduct additional specificity tests (see Support Protocol 5). Analyze and sequence positive isolates.

    The primer sequence for use with pJG4-5 is provided in the legend to Figure 17.3.4.

    DNA prepared from KC8 is generally unsuitable for dideoxy or automated sequencing even after use of Qiagen columns and/or cesium chloride gradients. Library plasmids to be sequenced should be retransformed from the KC8 miniprep stock (step 23) to a more amenable strain, such as DH5α, before sequencing is attempted.

RAPID SCREEN FOR INTERACTION TRAP POSITIVES

ALTERNATE PROTOCOL 1

Under some circumstances, it may be desirable to attempt the analysis of a large number of positives resulting from a two-hybrid screen. One such hypothetical example would be a bait with a leucine zipper or coiled coil known to dimerize with partner "A" that is highly expressed. In order to identify the rare novel partner "B", it is necessary to work through the high background of "A" reisolates. This protocol uses the polymerase chain reaction (PCR) in a strategy to sort positives into redundant (multiple isolates) and unique classes prior to plasmid rescue from yeast, thus greatly reducing the number of plasmid isolations that must be performed. An additional benefit is that this protocol preidentifies positive clones containing one or multiple library plasmids; for those containing only one library plasmid, only a single colony needs to be prepared through KC8/DH5α.

Additional Materials *(also see Basic Protocol 2)*

Yeast plated on Glu/CM −Ura, −His, −Trp master plate (see Basic Protocol 2, step 19)
Lysis solution (see recipe)
10 µM forward primer (FP1): 5′-CGT AGT GGA GAT GCC TCC-3′
10 µM reverse primer (FP2): 5′-CTG GCA AGG TAG ACA AGC CG-3′

Toothpicks or bacterial inoculating loops, sterile
96-well microtiter plate
Sealing tape, e.g., wide transparent tape
150- to 212-µm glass beads, acid-washed (*UNIT 3.8*)
Vortexer with flat plate

Additional reagents and equipment for performing an interactor hunt (see Basic Protocol 2), PCR amplification of DNA, agarose gel electrophoresis, restriction endonuclease digestion, electroporation, and miniprep isolation of bacterial DNA (*APPENDIX 3*)

1. Perform an interactor hunt (see Basic Protocol 2, steps 1 to 19).

2. Use a sterile toothpick or bacterial inoculating loop to transfer yeast from the Glu/CM, −Ura, −His, −Trp master plate into 25 µl lysis solution in a 96-well microtiter plate. Seal the wells of the microtiter plate with sealing tape and incubate 1.5 to 3.5 hr at 37°C with shaking.

    The volume of yeast transferred should not exceed ~2 to 3 µl of packed pellet; larger quantities of yeast will reduce quality of the DNA. DNA can be efficiently recovered from master plates that have been stored up to 1 week at 4°C. If yeast have been previously gridded on master plates, transfer to microtiter plates can be facilitated by using a multicolony replicator.

3. Remove tape from the plate, add ~25 µl acid-washed glass beads to each well, and reseal with the same tape. Firmly attach the microtiter plate to a flat-top vortexer, and vortex 5 min at medium-high power.

   The microtiter plate can be attached to the vortexer using 0.25-in (0.64-cm) rubber bands.

4. Remove the tape and add ~100 µl sterile water to each well. Swirl gently to mix, then remove sample for step 5. Press the tape back firmly to seal the microtiter plate and place in the freezer at −20°C for storage.

5. Amplify 0.8 to 2.0 µl of sample by standard PCR (*APPENDIX 3*) in a ~30-µl volume using 3 µl each of the forward primer FP1 and the reverse primer FP2. Perform PCR using the following cycles:

Initial step:	2 min	94°C
31 cycles:	45 sec	94°C
	45 sec	56°C
	45 sec	72°C.

   These conditions have been used successfully to amplify fragments up to 1.8 kb in length; some modifications, such as extension of elongation time, are also effective.

6. Load 20 µl of the PCR reaction product on a 0.7% low melting temperature agarose gel (*APPENDIX 3*) to resolve PCR products. Based on insert sizes, group the obtained interactors in families, i.e., potential multiple independent isolates of identical cDNAs. Reserve gel until results of step 7 are obtained.

   No special precautions are needed for storing the gel. Since HaeIII digests typically yield rather small DNA fragments, running the second gel does not take a lot of time. Usually, the delay does not exceed 45 to 60 min, during which time the first gel may be stored in a gel box at room temperature or wrapped in plastic wrap at 4°C.

7. While the gel is running, use the remaining 10 µl of PCR reaction product for a restriction endonuclease digestion with *Hae*III in a digestion volume of ~20 µl (*APPENDIX 3*). Based on analysis of the sizes of undigested PCR products in the gel (step 6), rearrange the tubes with *Hae*III digest samples so that those thought to represent a family are side by side. Resolve the digests on a 2% to 2.5% agarose gel (*APPENDIX 3*).

   Most restriction fragments will be in the 200-bp to 1.0-kb size range so using a long gel run is advisable. This analysis should produce a distinct fingerprint of insert sizes and allow definition of library cDNAs as unique isolates or related groups.

   A single positive yeast will sometimes contain multiple library plasmids. An advantage of this protocol is the ready detection of multiple library plasmids in PCR reactions; thus, following subsequent bacterial transformations, only a single TRP1 colony would need to be analyzed unless multiple plasmids were already known to be present.

8. Isolate DNA fragments from the low melting temperature agarose gel (step 6).

   If inspection of the banding pattern on the two gels suggests that a great many reisolates of a small number of cDNAs are present, it may be worthwhile to immediately sequence PCR products representative of these clusters, but it is generally still advisable to continue through specificity tests before doing so. If the PCR products are sequenced, the FP1 forward primer works well in automated sequencing of PCR fragments, but the FP2 primer is only effective in sequencing from purified plasmid.

   In general, priming from the AT-rich ADH terminator downstream of the polylinker/cDNA in library plasmid is less efficient than from upstream of the cDNA, and it is hard to design effective primers in this region.

9. Remove the microtiter plate of lysates from the freezer, thaw it, and remove 2 to 4 µl of lysed yeast for each desired positive. Electroporate DNA into either DH5α or KC8 *E. coli* as appropriate, depending on the choice of bait and reporter plasmids (see

Table 17.3.1 and see Background Information for further information). Refreeze the plate as a DNA reserve in case bacteria fail to transform on the first pass.

KC8 E. coli should be used for electroporation when the original reagents pEG202/pJG4-5/pJK101 are used for the interaction trap.

An additional strength of this protocol is that it identifies redundant clones before transfer of plasmids to bacteria, thus reducing the amount of work required in cases where plasmid identity can be unambiguously assigned. However, although restriction endonuclease digestion and PCR analysis are generally highly predictive, they are not 100% certain methods for estimating cDNA identity. Thus, if there is any doubt about whether two cDNAs are the same, investigators are urged to err on the side of caution.

10. Prepare a miniprep of plasmid DNA from the transformed bacteria (*APPENDIX 3*) and perform yeast transformation and specificity assessment (see Basic Protocol 2, steps 24 to 28).

PERFORMING A HUNT BY INTERACTION MATING

ALTERNATE PROTOCOL 2

An alternative way of conducting an interactor hunt is to mate a strain that expresses the bait protein with a strain that has been pretransformed with the library DNA, and screen the resulting diploid cells for interactors (Bendixen et al., 1994; Finley and Brent, 1994). This "interaction mating" approach can be used for any interactor hunt and is particularly useful in three special cases. The first case is when more than one bait will be used to screen a single library. Interaction mating allows several interactor hunts with different baits to be conducted using a single high-efficiency yeast transformation with library DNA. This can be a considerable savings, since the library transformation is one of the most challenging tasks in an interactor hunt. The second case is when a constitutively expressed bait interferes with yeast viability. For such baits, performing a hunt by interaction mating avoids the difficulty associated with achieving a high-efficiency library transformation of a strain expressing a toxic bait. Moreover, the actual selection for interactors will be conducted in diploid yeast, which are more vigorous than haploid yeast and can better tolerate expression of toxic proteins. The third case is when a bait cannot be used in a traditional interactor hunt using haploid yeast strains (see Basic Protocol 2) because it activates transcription of even the least sensitive reporters. In diploids the reporters are less sensitive to transcription activation than they are in haploids. Thus, the interaction mating hunt provides an additional method to reduce background from transactivating baits.

In the protocol described below, the library DNA is used to transform a strain with a *LEU2* reporter (e.g., EGY48). This pretransformed library strain is then frozen in many aliquots, which can be thawed and used for individual interactor hunts. The bait is expressed in a strain of mating type opposite to that of the pretransformed library strain, and also bearing the *lacZ* reporter. A hunt is conducted by mixing the pretransformed library strain with the bait strain and allowing diploids to form on YPD medium overnight. The diploids are then induced for expression of the library-encoded proteins and screened for interactors as in Basic Protocol 2.

NOTE: Strain combinations other than those described below can also be used in an interaction-mating hunt. The key to choosing the strains is to ensure that the bait and prey strains are of opposite mating types and that both have auxotrophies to allow selection for the appropriate plasmids and reporter genes. Also, once the bait plasmid and *lacZ* reporter plasmid have been introduced into the bait strain, and the library plasmids have been introduced into the library strain, the resulting bait strain and library strain must each have auxotrophies that can be complemented by the other, so that diploids can be selected.

Additional Materials *(also see Basic Protocols 1 and 2)*

Yeast strains: either RFY206 (Finley and Brent, 1994), YPH499 (Sikorski and Hieter, 1989; ATCC #6625), or an equivalent *MATa* strain with auxotrophic markers *ura3*, *trp1*, *his3*, and *leu2*

YPD liquid medium (*UNIT 1.6*)

Glu/CM –Trp plates: CM dropout plates –Trp (see recipe) supplemented with 2% glucose

pJG4-5 library vector (Fig. 17.3.6), empty

100-mm YPD plates (*UNIT 1.6*)

Additional reagents and equipment for lithium acetate transformation of yeast (*APPENDIX 3*)

Construct the bait strain

The bait strain will be a *MATa* yeast strain (mating type opposite of EGY48) containing a *lacZ* reporter plasmid like pSH18-34 and the bait-expressing plasmid, pBait.

1. Perform construction of the bait plasmid (pBait; see Basic Protocol 1, step 1).

2. Cotransform the *MATa* yeast strain (e.g., either RFY206 or YPH499) with pBait and pSH18-34 using the lithium acetate method (*APPENDIX 3*). Select transformants on Glu/CM –Ura,–His plates by incubating plates at 30°C for 3 to 4 days until colonies form. Combine 3 colonies for all future tests and for the mating hunt.

   The bait strain (RFY206/pSH18-34/pBait or YPH499/pSH18-34/pBait) can be tested by immunoblotting to ensure that the bait protein is expressed (see Support Protocol 1). Synthesis and nuclear localization of the bait protein can also be tested by the repression assay (see Basic Protocol 1, steps 8 to 12).

3. *Optional:* Assay *lacZ* gene activation in the bait strain (see Basic Protocol 1, steps 4 to 7).

   If the bait activates the lacZ reporter, a less sensitive lacZ reporter plasmid (Table 17.3.1), or an integrated version of the lacZ reporter should be tried. A bait that strongly activates the lacZ reporters usually cannot be used in a hunt based on selection of interactors with the LEU2 reporter, because the LEU2 reporters are more sensitive than the lacZ reporters. However, both reporters are less sensitive to activation by a bait in diploid cells, as compared to haploid cells. Thus, a more important test of the transactivation potential of a bait is to test the leucine requirement of diploid cells expressing it, as described in steps 6 to 20, below.

Prepare the pretransformed library strain (EGY48 + library plasmids)

4. Perform a large-scale transformation of EGY48 with library DNA using the lithium acetate method (see Basic Protocol 2, steps 1 to 8, except start with EGY48 bearing no other plasmids). To prepare for transformation, grow EGY48 in YPD liquid medium. Select library transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.

5. Collect primary transformants by scraping plates, washing yeast, and resuspending in 1 pellet vol glycerol solution (see Basic Protocol 2, steps 9 to 12). Freeze 0.2- to 1.0-ml aliquots at −70° to −80°C.

   The cells will be stable for at least 1 year. Refreezing a thawed aliquot will result in loss of viability. Thus, many frozen aliquots should be made, so that each thawed aliquot can be discarded after use.

Prepare the pretransformed control strain (EGY48 + pJG4-5)

6. Transform EGY48 grown in YPD liquid medium with the empty library vector, pJG4-5, using the lithium acetate method (*APPENDIX 3*). Select transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.

7. Pick and combine three transformant colonies and use them to inoculate 30 ml of Glu/CM –Trp medium. Incubate 15 to 24 hr at 30°C (to OD_{600} >3).

8. Centrifuge 5 min at 1000 to 1500 × *g*, room temperature, and remove supernatant. Resuspend in 10 ml sterile water to wash cells.

9. Centrifuge 5 min at 1000 to 1500 × *g*, room temperature, and remove supernatant. Resuspend in 1 pellet vol glycerol solution and freeze 100-µl aliquots at –70° to –80°C.

Determine plating efficiency of pretransformed library and pretransformed control strains

10. After freezing (at least 1 hr) thaw an aliquot of each pretransformed strain (from step 5 and step 9) at room temperature. Make several serial dilutions in sterile water, including aliquots diluted 10^5-fold, 10^6-fold, and 10^7-fold. Plate 100 µl of each dilution on 100-mm Glu/CM –Trp plates and incubate 2 to 3 days at 30°C.

11. Count the colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

    The plating efficiency for a typical library transformation and for the control strain will be ~1 × 10^8 cfu per 100 µl.

Mate the bait strain with the pretransformed library strain and the pretransformed control strain

In steps 12 through 20, an interactor hunt is conducted concurrently with testing *LEU2* reporter activation by the bait itself. For most baits, this approach will be the quickest way to isolate interactors. However, for some baits, such as those that have a high transactivation potential, or those that affect yeast mating or growth, steps 12 through 20 will serve as a pilot experiment to determine the optimal parameters for a subsequent hunt.

12. Grow a 30-ml culture of the bait strain in Glu/CM –Ura,–His liquid dropout medium to mid to late log phase (OD_{600} = 1.0 to 2.0, or 2 to 4 × 10^7cells/ml).

    A convenient way to grow the bait strain is to inoculate a 5-ml culture with approximately three colonies from a plate and grow it overnight at 30°C with shaking. In the morning, measure the OD_{600}, dilute into a 30-ml culture to a final OD_{600} = 0.2, and grow at 30°C with shaking. The culture should reach mid to late log phase before the end of the day.

13. Centrifuge the culture 5 min at 1000 to 1500 × *g*, room temperature, to harvest cells. Resuspend the cell pellet in sterile water to make a final volume of 1 ml.

    This should correspond to ~1 × 10^9 cells/ml.

14. Set up two matings. In one sterile microcentrifuge tube mix 200 µl of the bait strain with 200 µl of a thawed aliquot of the pretransformed control strain from step 9. In a second microcentrifuge tube mix 200 µl of the bait strain with ~1 × 10^8 cfu (~0.1 to 1 ml) of the pretransformed library strain from step 5.

    The library mating should be set up so that it contains a ~2-fold excess of bait strain cfu over pretransformed library strain cfu. Because the bait strain was harvested in log phase, most of the cells will be viable (i.e., cells/ml = ~cfu/ml), and the number of cfu can be sufficiently estimated from optical density (1 OD_{600} = ~2 × 10^7 cells/ml). Under these conditions, ~10% of the cfu in the pretransformed library strain will mate with the bait

strain. Thus, a complete screen of 10^7 library transformants will require a single mating with at least 10^8 cfu of the pretransformed library strain and at least 2×10^8 cfu of the bait strain.

To screen more library transformants, set up additional matings. The number of pretransformed library transformants to screen depends on the size of the library and the number of primary transformants obtained in step 5. If the size of the library is larger than the number of transformants obtained in step 5, the goal will be to screen all of the yeast transformants. In this case, complete screening of the library will require additional transformations of EGY48 and additional interactor hunts. If the size of the library is smaller than the number of transformants obtained in step 5, the goal will be to screen at least a number of transformants equivalent to the size of the library.

15. Centrifuge each cell mixture for 5 min at 1000 to 1500 × *g*, pour off medium, and resuspend cells in 200 µl YPD medium. Plate each suspension on a 100-mm YPD plate. Incubate 12 to 15 hr at 30°C.

16. Add ~1 ml of Gal/Raff/CM –Ura, –His, –Trp to the lawns of mated yeast on each plate. Mix the cells into the medium using a sterile applicator stick.

17. Transfer each slurry of mated cells to a 500-ml flask containing 100 ml of Gal/Raff/CM –Ura, –His, –Trp dropout medium. Incubate with shaking 6 hr at room temperature to induce the *GAL1* promoter, which drives expression of the cDNA library.

18. Centrifuge the cell suspensions 5 min at 1000 to 1500 × *g*, room temperature, to harvest the cells. Wash by resuspending in 30 ml of sterile water and centrifuging again. Resuspend each pellet in 5 ml sterile water. Measure OD_{600} and, if necessary, dilute to a final concentration of $\sim 1 \times 10^8$ cells/ml.

    This is a mixture consisting of haploid cells that have not mated and diploid cells. Under a microscope, the two cell types can be distinguished by size (diploids are ~1.7× bigger than haploids) and shape (diploids are slightly oblong and haploids are spherical). Because diploids grow faster than haploids, this mixture will contain ~10% to 50% diploid cells. The actual number of diploids will be determined by plating dilutions on –Ura, –His, –Trp medium, which will not support the growth of the parental haploids.

19. For each mating make a series of $^1/_{10}$ dilutions in sterile water, at least 200 µl each, to cover a 10^6-fold concentration range. Plate 100 µl from each tube (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution) on 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Plate 100 µl from the 10^{-4}, 10^{-5}, and 10^{-6} tubes on 100-mm Gal/Raff/CM –Ura, –His, –Trp plates. Incubate plates at 30°C. Count the colonies on each plate after 2 to 5 days.

20. For the mating with the pretransformed library, prepare an additional 3 ml of a 10^{-1} dilution. Plate 100 µl of the 10^{-1} dilution on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Also plate 100 µl of the undiluted cells on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Incubate at 30°C. Pick Leu^+ colonies after 2 to 5 days and characterize them beginning with step 17 of Basic Protocol 2.

    The number of Leu^+ colonies to pick to ensure that all of the pretransformed library has been screened depends on the transactivation potential of the bait protein itself. The transactivation potential is expressed as the number of Leu^+ colonies that grow per cfu (Leu^+/cfu) of the bait strain mated with the control strain, as determined in step 19 of this protocol. It can be calculated as the ratio of the number of colonies that grow on Gal/Raff/CM –Ura,–His, –Trp, –Leu to the number of colonies that grow on Gal/Raff/CM –Ura, –His, –Trp for a given dilution of the mating between the bait strain and the control strain. A bait with essentially no transactivation potential will produce less than 10^{-6} Leu^+/cfu. For a bait to be useful in an interactor hunt it should not transactivate more than 10^{-4} Leu^+/cfu.

To screen all of the pretransformed library, it will be necessary to pick a sufficient number of Leu^+ colonies in addition to background colonies produced by the transactivation potential of the bait itself. Thus, the minimum number of Leu^+ colonies that should be picked in step 20 of this protocol is given by:

(transactivation potential, Leu^+/cfu) × (# library transformants screened).

For example, if 10^7 library transformants were obtained in step 2 (and at least 10^8 cfu of these transformants were mated with the bait strain in step 14, since only ~10% will form diploids), and the transactivation potential of the bait is 10^{-4} Leu^+/cfu, then at least 1000 Leu^+ colonies must be picked and characterized. In other words, if the rarest interactor is present in the pretransformed library at a frequency of 10^{-7}, to find it one needs to screen through at least 10^7 diploids from a mating of the library strain. However, at least 1000 of these 10^7 diploids would be expected to be Leu^+ due to the bait background if the transactivation potential of the bait is 10^{-4}. The true positives will be distinguished from the bait background in the next step by the galactose dependence of their Leu^+ and $lacZ^+$ phenotypes.

PREPARATION OF PROTEIN EXTRACTS FOR IMMUNOBLOT ANALYSIS

SUPPORT PROTOCOL 1

To confirm that the bait fusion protein constructed in Basic Protocol 1 is synthesized properly, a crude lysate is prepared for SDS-PAGE and immunoblot analysis (*UNITS 6.1 & 6.2*). The presence of the target protein is detected by antibody to LexA or the fusion domain.

Materials

Master plates with pBait-containing positive and control yeast on Glu/CM −Ura, −His dropout medium (see Basic Protocol 1, step 4)

Glu/CM −Ura, −His dropout liquid medium: CM dropout plates −Ura, −His (see recipe) supplemented with 2% glucose

2× Laemmli sample buffer (see recipe)

Antibody to fusion domain or LexA: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

30°C incubator

100°C water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting and immunodetection (*UNIT 6.2*)

1. From the master plates, start a 5-ml culture in Glu/CM −Ura, −His liquid medium for each bait being tested and for a positive control for protein expression (i.e., RFHMI or SH17-4). Incubate overnight at 30°C.

   For each construct assayed, it is a good idea to grow colonies from at least two primary transformants, as levels of bait expression are sometimes heterogenous.

2. From each overnight culture, start a fresh 5-ml culture in Glu/CM −Ura, −His at OD_{600} = ~0.15. Incubate again at 30°C.

3. When the culture has reached OD_{600} = 0.45 to 0.7 (~4 to 6 hr), remove 1.5 ml to a microcentrifuge tube.

   For some LexA fusion proteins, levels of the protein drop off rapidly in cultures approaching stationary phase. This is due to a combination of the diminishing activity of the ADH1 promoter in late growth phases and the relative instability of particular fusion domains. Thus, it is not a good idea to let cultures become saturated in the hopes of obtaining a higher yield of protein.

4. Microcentrifuge cells 3 min at 13,000 × *g*, room temperature. When the pellet is visible, remove the supernatant.

   Inspection of the tube should reveal a pellet ~1 to 3 µl in volume. If the pellet is not visible, microcentrifuge another 3 min.

5. Working rapidly, add 50 µl of 2× Laemmli sample buffer to the visible pellet in the tube, vortex, and place the tube on dry ice.

   Samples may be frozen at −70°C.

6. Transfer frozen sample directly to a boiling water bath or a PCR machine set to cycle at 100°C. Boil 5 min.

7. Microcentrifuge 5 sec at maximum speed to pellet large cellular debris.

8. Perform SDS-PAGE (*UNIT 6.1*) using 20 to 50 µl sample per lane.

9. To detect the protein, immunoblot and analyze (*UNIT 6.2*) using antibody to the fusion domain or LexA.

SUPPORT PROTOCOL 2

PREPARATION OF SHEARED SALMON SPERM CARRIER DNA

This protocol generates high-quality sheared salmon sperm DNA (sssDNA) for use as carrier in transformation (Basic Protocol 2). This DNA is also suitable for other applications where high-quality carrier DNA is needed (e.g., hybridization). This protocol is based on Schiestl and Gietz (1989). For more details of phenol extraction or other DNA purification methods, consult *APPENDIX 3*.

Materials

High-quality salmon sperm DNA (e.g., sodium salt from salmon testes, Sigma or Boehringer Mannheim), desiccated
TE buffer, pH 7.5 (*APPENDIX 2A*), sterile
TE-saturated buffered phenol
1:1 (v/v) buffered phenol/chloroform
Chloroform
3 M sodium acetate, pH 5.2 (*APPENDIX 2A*)
100% and 70% ethanol, ice cold

Magnetic stirring apparatus and stir-bar, 4°C
Sonicator with probe
50-ml conical centrifuge tube
High-speed centrifuge and appropriate tube
100°C and ice-water baths

1. Dissolve desiccated high-quality salmon sperm DNA in TE buffer, pH 7.5, at a concentration of 5 to 10 mg/ml by pipetting up and down in a 10-ml glass pipet. Place in a beaker with a stir-bar and stir overnight at 4°C to obtain a homogenous viscous solution.

   It is important to use high-quality salmon sperm DNA. Sigma Type III sodium salt from salmon testes has worked well, as has a comparable grade from Boehringer Mannheim. Carrier DNA is also commercially available from Clontech. Generally it is convenient to prepare 20- to 40-ml batches at a time.

2. Shear the DNA by sonicating briefly using a large probe inserted into the beaker.

   The goal of this step is to generate sheared salmon sperm DNA (sssDNA) with an average size of 7 kb, but ranging from 2 to 15 kb. Oversonication (such that the average size is closer to 2 kb) drastically decreases the efficacy of carrier in enhancing transformation. The

original version of this protocol (Schiestl and Gietz, 1989) called for two 30-sec pulses at three-quarter power, but optimal conditions vary between sonicators. The first time this protocol is performed, it is worthwhile to sonicate briefly, then test the size of the DNA by running out a small aliquot alongside molecular weight markers on an agarose gel containing ethidium bromide. The DNA can be sonicated further if needed.

3. Once DNA of the appropriate size range has been obtained, extract the sssDNA solution with an equal volume of TE-saturated buffered phenol in a 50-ml conical tube, shaking vigorously to mix.
4. Centrifuge 5 to 10 min at 3000 × *g*, room temperature, or until clear separation of phases is obtained. Transfer the upper phase containing the DNA to a clean tube.
5. Repeat extraction using 1:1 (v/v) buffered phenol/chloroform, then chloroform alone. Transfer the DNA into a tube suitable for high-speed centrifugation.
6. Precipitate the DNA by adding $\frac{1}{10}$ vol of 3 M sodium acetate and 2.5 vol of ice-cold 100% ethanol. Mix by inversion. Centrifuge 15 min at ~12,000 × *g*, room temperature.
7. Wash the pellet with 70% ethanol. Briefly dry either by air drying, or by covering one end of the tube with Parafilm with a few holes poked in and placing the tube under vacuum. Resuspend the DNA in sterile TE buffer at 5 to 10 mg/ml.

   Do not overdry the pellet or it will be very difficult to resuspend.
8. Denature the DNA by boiling 20 min in a 100°C water bath. Then immediately transfer the tube to an ice-water bath.
9. Place aliquots of the DNA in microcentrifuge tubes and store frozen at −20°C. Thaw as needed.

   DNA should be boiled again briefly (5 min) immediately before addition to transformations.

   Before using a new batch of sssDNA in a large-scale library transformation, it is a good idea to perform a small-scale transformation using suitable plasmids to determine the transformation efficiency. Optimally, use of sssDNA prepared in the manner described will yield transformation frequencies of >10^5 colonies/µg input plasmid DNA.

YEAST COLONY HYBRIDIZATION

SUPPORT PROTOCOL 3

This protocol is adapted from a modification of the classic protocol of Grunstein and Hogness (1975; Kaiser et al., 1994). It is primarily useful when a large number of putative interactors has been obtained, and initial minipreps and restriction digests have indicated that many of them derive from a small number of cDNAs; these cDNAs can then be used as probes to screen and eliminate identical cDNAs from the pool.

Materials

Glu/CM −Trp plates: CM dropout plates −Trp (see recipe) supplemental with 2% glucose
Master dropout plate of yeast positive for Gal dependence (see Basic Protocol 2, step 18)
1 M sorbitol/20 mM EDTA/50 mM DTT (prepare fresh)
1 M sorbitol/20 mM EDTA
0.5 M NaOH
0.5 M Tris·Cl (pH 7.5)/6× SSC (*APPENDIX 2A*)
2× SSC (see recipe)
100,000 U/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma)

82-mm circular nylon membrane, sterile
Whatman 3MM paper
80°C vacuum oven *or* UV cross-linker

Additional reagents and equipment for bacterial filter hybridization (*APPENDIX 3*)

1. Place a sterile nylon membrane onto a Glu/CM −Trp dropout plate. From the master dropout plate of Gal-dependent positives, gently restreak positives to be screened onto the membrane and mark the membrane to facilitate future identification of hybridizing colonies. Grow overnight (~12 hr) at 30°C.

   Growth for extended periods of time (i.e., 24 hr) may result in difficulty in obtaining good lysis. It is a good idea to streak positive and negative controls for the cDNAs to be hybridized on the membrane.

2. Remove membrane from plate. Air dry briefly. Incubate ~30 min on a sheet of Whatman 3MM paper saturated with 1 M sorbitol/20 mM EDTA/50 mM DTT.

   Optionally, before commencing chemical lysis, membranes can be placed at −70°C for 5 min, then thawed at room temperature for one or more cycles to enhance cell wall breakage.

3. Cut a piece of Whatman 3MM paper to fit inside a 100-mm petri dish. Place the paper disc in the dish and saturate with 100,000 U/ml β-glucuronidase diluted 1:500 in 1 M sorbitol/20 mM EDTA (2 µl glucuronidase per ml of sorbitol/EDTA to give 200 U/ml final). Layer nylon membrane on dish, cover dish, and incubate up to 6 hr at 37°C until >80% of the cells lack a cell wall.

   The extent of cell wall removal can be determined by removing a small quantity of cells from the filter to a drop of 1 M sorbitol/20 mM EDTA on a microscope slide and observing directly with a phase-contrast microscope at ≥60× magnification. Cells lacking cell wall are nonrefractile.

4. Place membrane on Whatman 3MM paper saturated with 0.5 M NaOH for ~8 to 10 min.

5. Place membrane on Whatman 3MM paper saturated with 0.5 M Tris·Cl (pH 7.5)/6× SSC for 5 min. Repeat with a second sheet of Whatman 3MM paper.

6. Place membrane on Whatman 3MM paper saturated with 2× SSC for 5 min. Then place membrane on dry Whatman paper to air dry for 10 min.

7. Bake membrane 90 min at 80°C in vacuum oven or UV cross-link.

8. Process as for bacterial filter hybridization (*APPENDIX 3*), hybridizing the membrane with probes complementary to previously isolated cDNAs.

   When selecting probes, either random-primed cDNAs or oligonucleotides complementary to the cDNA sequence may be used. If the cDNA is a member of a protein family, it may be advantageous to use oligonucleotides to avoid inadvertently excluding genes related but not identical to those initially obtained.

SUPPORT PROTOCOL 4

MICROPLATE PLASMID RESCUE

In some cases, it is desirable to isolate plasmids from a large number of positive colonies (Basic Protocol 2, steps 18 and 19). The protocol described below is a batch DNA preparation protocol developed by Steve Kron (University of Chicago, Chicago, Ill.) as a scale-up of a basic method developed by Manuel Claros (Laboratoire de Génétique Moleculaire, Paris, France).

Materials

2× Glu/CM −Trp liquid medium: 2× CM −Trp liquid medium (see recipe) supplemented with 4% glucose
Master plate of Gal-dependent yeast colonies (see Basic Protocol 2, step 18)
Rescue buffer: 50 mM Tris·Cl (pH 7.5)/10 mM EDTA/0.3% (v/v) 2-mercaptoethanol (prepare fresh)
Lysis solution: 2 to 5 mg/ml Zymolyase 100T/rescue buffer *or* 100,000 U/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma) diluted 1:50 in rescue buffer
10% (w/v) SDS
7.5 M ammonium acetate (*APPENDIX 2A*)
Isopropanol
70% ethanol
TE buffer, pH 8.0 (*APPENDIX 2A*)

24-well microtiter plates
Centrifuge with microplate holders, refrigerated
Repeating micropipettor
37°C rotary shaker

Grow yeast cultures

1. Aliquot 2 ml of 2× Glu/CM −Trp medium into each well of a 24-well microtiter plate. Into each well, pick a putative positive colony. Grow overnight with shaking at 30°C.

   The 2× minimal medium is used to maximize the yield of yeast. Four plates can generally be handled conveniently at once, based on the number that can be centrifuged simultaneously.

2. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant with a snap and return the plate to upright.
3. Swirl or lightly vortex the plate to resuspend cell pellets in remaining liquid. Add 1 ml water to each well and swirl lightly.

   Cell pellets can most easily be resuspended in residual liquid before adding new solutions. Addition of liquid can be accomplished using a repeating pipettor.

4. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant and resuspend pellet. Add 1 ml rescue buffer.
5. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant and resuspend pellet in the small volume of liquid remaining in the plate.

Lyse cells

6. To each well, add 25 µl lysis solution. Swirl or vortex to mix. Incubate (with cover on) on a rotary shaker ~1 hr at 37°C.

   Lysis solution need not be completely dissolved before use. By 1 hr, lysis should be obvious as coagulation of yeast into a white precipitate.

   Susceptibility of yeast strains to lytic enzymes varies. If lysis occurs rapidly, then less lytic enzyme should be used. If the lysis step is allowed to go too far, too much of the partially dissolved cell wall may contaminate the final material. Lysis can be judged by examining cells with a phase-contrast microscope. Living cells are white with a dark halo and dead cells are uniformly gray. Lysis leads to release of granular cell contents into the medium. Once cells are mostly gray and many are disrupted, much of the plasmid should have been released.

7. To each well, add 25 µl of 10% SDS. Mix gently by swirling to completely disperse the precipitates. Allow plates to sit 1 min at room temperature.

   At this point, the wells should contain a clear, somewhat viscous solution.

Purify plasmid

8. To each well, add 100 µl of 7.5 M ammonium acetate. Swirl gently, then incubate 15 min at −70°C or −20°C until frozen.

   Addition of acetate should result in the formation of a massive white precipitate of cell debris and SDS. The freezing step appears to improve removal of inhibitors of E. coli transformation.

9. Remove plate from freezer. Once it begins to thaw, centrifuge 15 min at 3000 × *g*, 4°C. Transfer 100 to 150 µl of the resulting clear supernatants to clean 24-well plates.

   In general, some contamination of the supernatant with pelleted material cannot be avoided. However, it is better to sacrifice yield in order to maintain purity.

10. To each well, add ~0.7 vol isopropanol. Mix by swirling and allow to precipitate 2 min at room temperature.

    A cloudy fine precipitate should form immediately after isopropanol is added.

11. Centrifuge 15 min at 3000 × *g*, 4°C. Shake off supernatant with a snap.

12. To each well, add ~1 ml cold 70% ethanol. Mix by swirling, centrifuge 5 min at 3000 × *g*, 4°C. Shake off supernatant with a snap, invert plates and blot well onto paper towel. Allow plates to air dry.

13. To each well, add 100 µl TE buffer. Swirl well and allow to rest on bench several minutes, until the pellets appear fully dissolved. Transfer preps to microcentrifuge tubes or 96-well plates for storage at −20°C.

    One to five microliters of each of the resulting preparations can be used to transform competent E. coli: for KC8, electroporation should be used (see Basic Protocol 2, step 21). Sometimes, the yield of transformants is low if E. coli carrying plasmids are not permitted time to increase the plasmid copy number above a critical threshold before the cells are placed on selective medium. Allow plenty of time for cells to express antibiotic resistance or the TRP1 gene before plating.

    If insufficient numbers of colonies are obtained by this approach, the final plasmid preparation can be resuspended in 20 µl instead of 100 µl TE buffer to concentrate the DNA stock.

SUPPORT PROTOCOL 5

ADDITIONAL SPECIFICITY SCREENING

The three test plasmids outlined (pSH18-34, pRFHM1, and pEG202; see Basic Protocol 2, step 24) represent a minimal test series. If other LexA-bait proteins that are related to the bait protein used in the initial library screen are available, substantial amounts of information can be gathered by additional specificity tests. For example, if the initial bait protein was LexA fused to the leucine zipper of c-Fos, specificity screening of interactor-hunt positives against the leucine zippers of c-Jun or GCN4 in addition to that of c-Fos might allow discrimination between proteins that are specific for fos versus those that generically associate with leucine zippers.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

A medium

5 g $(NH_4)_2SO_4$
22.5 g KH_2PO_4
52.5 g K_2HPO_4
2.5 g sodium citrate·$2H_2O$

continued

Before they are used, concentrated media should be diluted to 1× with sterile water and the following sterile solutions, per liter:

1 ml 1 M $MgSO_4 \cdot 7H_2O$
10 ml 20% carbon source (sugar or glycerol)
and, if required:
0.1 ml 0.5% vitamin B1 (thiamine)
5 ml L amino acids to 40 µg/ml *or*
DL amino acids to 80 µg/ml
Antibiotic

Complete minimal (CM) dropout medium

1.3 g dropout powder (Table 17.3.4)
1.7 g YNB −AA/AS
5 g $(NH_4)_2SO_4$
20 g dextrose
(Alternatively, replace last three ingredients with 27 g minimal medium premix)

For CM plates, add 20 g agar and a pellet of NaOH per liter.

CM dropout powder, also known as minus or omission powder, lacks one or more nutrients but contains the other nutrients listed in Table 17.3.4. Complete minimal (CM) dropout medium is used to test for genes involved in biosynthetic pathways and to select for gene function in transformation experiments. To test for a gene involved in histidine biosynthesis one would determine if the yeast strain in question can grow on CM minus histidine (−His) or "histidine dropout" plates. It is convenient to make several dropout powders, each lacking a single nutrient, to avoid weighing each component separately for all the different dropout plates required in the laboratory.

It may be preferable to use a 10× solution of dropout powder (i.e., 13 g of dropout powder in 100 ml water) that has been "sterilized" separately (and added to the other ingredients after autoclaving) to improve the growth rate in this medium.

Glycerol solution

65% (v/v) glycerol, sterile
0.1 M $MgSO_4$
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Store up to 1 year at room temperature

Laemmli sample buffer, 2×

10% (v/v) 2-mercaptoethanol (2-ME)
6% (w/v) SDS
20% (v/v) glycerol
0.2 mg/ml bromphenol blue
0.025× Laemmli stacking buffer (see recipe; optional)
Store up to 2 months at room temperature

This reagent can conveniently be prepared 10 ml at a time.

Laemmli stacking buffer, 2.5×

0.3 M Tris·Cl, pH 6.8
0.25% (w/v) SDS
Store up to 1 month at 4°C

Lysis solution

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10 mM EDTA
0.3% (v/v) 2-mercaptoethanol (2-ME), added just before use

continued

Table 17.3.4 Nutrient Concentrations for Dropout Powders[a]

Nutrient[b]	Amount in dropout powder(g)[c]	Final conc. in prepared media (μg/ml)	Liquid stock conc. (mg/100 ml)[d]
Adenine (hemisulfate salt)	2.5	40	500
L-arginine (HCl)	1.2	20	240
L-aspartic acid[e]	6.0	100	1200
L-glutamic acid (monosodium salt)	6.0	100	1200
L-histidine	1.2	20	240
L-leucine[f]	3.6	60	720
L-lysine (mono-HCl)	1.8	30	360
L-methionine	1.2	20	240
L-phenylalanine	3.0	50	600
L-serine	22.5	375	4500
L-threonine[e]	12.0	200	2400
L-tryptophan	2.4	40	480
L-tyrosine	1.8	30	180[f]
L-valine	9.0	150	1800
Uracil	1.2	20	240

[a]CM dropout powder lacks one or more nutrients but contains the other nutrients listed in this table. Nomenclature in this manual refers to, e.g., a preparation that omits histidine as histidine dropout powder. Conditions from Sherman et al., 1979.
[b]Amino acids not listed here can be added to a final concentration of 40 μg/ml (40 mg/liter).
[c]Grind powders into a homogeneous mixture with a clean, dry mortar and pestle. Store in a clean, dry bottle or a covered flask.
[d]Use 8.3 ml/liter of each stock for special nutritional requirements. Store adenine, aspartic acid, glutamic acid, leucine, phenylalanine, tyrosine, and uracil solutions at room temperature. All others should be stored at 4°C.
[e]While these amino acids can be used reliably when included in autoclaved media, they supplement growth better when added after autoclaving.
[f]Use 16.6 ml/liter for L-tyrosine nutritional requirement.

2% (v/v) β-glucuronidase from *Helix pomatia* (Type HP-2; Sigma), added just before use

SSC (sodium chloride/sodium citrate), 20×

3 M NaCl (175 g/liter)
0.3 M Na_3citrate·$2H_2O$ (88 g/liter)
Adjust pH to 7.0 with 1 M HCl (*APPENDIX 2A*)

Z buffer

16.1 g $Na_2HPO_4 \cdot 7H_2O$ (60 mM final)
5.5 g $NaH_2PO_4 \cdot H_2O$ (40 mM final)
0.75 g KC1 (10 mM final)
0.246 g $MgSO_4 \cdot 7H_2O$ (1 mM final)
2.7 ml 2-mercaptoethanol (50 mM final)
Adjust to pH 7.0 and bring to 1 liter with H_2O. Do not autoclave.

COMMENTARY

Background Information

Interaction-based cloning is derived from three experimental observations. In the first, Brent and Ptashne (1985) demonstrated that it was possible to assemble a novel, functional transcriptional activator by fusing the DNA-binding domain from one protein, LexA, to the activation domain from a second protein, GAL4. This allowed the use of a single reporter system containing a single DNA-binding motif, the *LexA* operator, to study transcriptional activation by any protein of interest. In the second, Ma and Ptashne (1988) built on this work to demonstrate that the activation domain could be brought to DNA by interaction with a DNA-binding domain. In the third, Fields and Song

Table 17.3.5 Two-hybrid System Variants[a,b]

System	DNA-binding domain	Activation domain	Selection	Reference
Two-hybrid	*GAL4*	*GAL4*	Activation of *lacZ*, *HIS3*	Chien et al., 1991
Interaction trap	*LexA*	*B42*	Activation of *LEU2*, *lacZ*	Gyuris et al., 1993
"Improved two-hybrid"	*GAL4*	*GAL4*	Activation of *HIS3*, *lacZ*	Durfee at al., 1993
Modified two-hybrid	*LexA*	VP16	Activation of *HIS3*, *lacZ*	Vojtek at al., 1993
KISS	*GAL4*	VP16	Activation of *CAT*, *hyg*^{r}	Fearon et al., 1992
Contingent replication	*GAL4*	VP16	Activation of T-Ag, replication of plasmids	Vasavada et al., 1991

[a]Abbreviations: *CAT*, chloramphenicol transferase gene; *hyg*^{r}, hygromycin resistance gene; T-Ag, viral large T antigen.
[b]An extended version of this table is available on *http://www.fccc.edu/research/labs/golemis/betagal.html*.

(1989), working independently of Ma and Ptashne, used two yeast proteins, SNF1 and SNF4, to make an SNF1 fusion to the DNA-binding domain of GAL4 and an SNF4 fusion to the GAL4 activation domain. They demonstrated that the strength of the SNF1-SNF4 interaction was sufficient to allow activation through a GAL4 DNA-binding site. From this, they suggested the feasibility of selecting interacting proteins by performing screens of cDNA libraries made so that library-encoded proteins carried activating domains.

Several groups have developed cDNA library strategies along these lines, with some systems using LexA and others using GAL4 as the DNA-binding domain (Table 17.3.5). LexA and GAL4 each have different properties that should be considered when selecting a system. LexA is derived from a heterologous organism, has no known effect on the growth of yeast, possesses no residual transcriptional activity, can be used in GAL4$^+$ yeast, and can be used with a Gal-inducible promoter. Because GAL4 is an important yeast transcriptional activator, it has the disadvantage that experiments must be performed in gal4$^-$ yeast strains to avoid background due to activation of the reporter system by endogenous GAL4. Such gal4$^-$ strains are frequently less healthy and more difficult to transform than wild-type strains, and either libraries must be constitutively expressed or alternate inducible systems must be used. By contrast, the GAL4 DNA-binding domain may be more efficiently localized to the nucleus and may be preferred for some proteins (for a review of GAL4-based systems, see Bartel et al., 1993). Whichever system is used, it is important to remember that the bait protein constitutes a novel fusion protein whose properties may not exactly parallel those of the original unfused protein of interest. Although systems using the two-hybrid paradigm have been developed in mammalian cells (see Table 17.3.5), these have not been used effectively in library screens. It seems likely that the organism of choice for two-hybrid identification of novel partner proteins will remain yeast.

cDNAs that pass specificity tests are referred to as positives, or "true positives." In interactor hunts conducted to date, anywhere from zero to practically all isolated plasmids passed the final specificity test. If no positives are obtained, the tissue source for the library originally used may not be appropriate, and a different library may produce better results. However, there are some proteins for which no positives are found. Various explanations for this are provided below. Conversely, some library-encoded proteins are known to be isolated repeatedly using a series of unrelated baits, and these proteins demonstrate at least some specificity. One of these, heat shock protein 70, might be explained by positing that it assists the folding of some LexA-fused bait proteins, or alternatively, that these bait proteins are not normally folded. This example illustrates the point that the physiological relevance of even quite specific interactions may sometimes be obscure.

Because the screen involves plating multiple cells to Gal/CM −Ura, −His, −Trp, −Leu dropout medium for each primary transformant obtained, multiple reisolates of true positive cDNAs are frequently obtained. If a large number of specific positives are obtained, it is generally a good idea to attempt to sort them into classes—for example, digesting minipreps of positives with *Eco*RI, *Xho*I, and *Hae*III will

generate a fingerprint of sufficient resolution to determine whether multiple reisolates of a small number of clones or single isolates of many different clones have been obtained. The former situation is a good indication that the system is working well.

An important issue that arises in an interactor hunt is the question of how biologically relevant interacting proteins that are isolated are likely to be. This leads directly to the question of what K_d of association two molecules must have to be detected by an interactor hunt. In fact, this is not at all a simple issue. For the system described here, most fusion proteins appear to be expressed at levels ranging from 50 nM to 1 μM (Golemis and Brent, 1992). Given the strength of the *GAL* promoter, it is likely that many library-encoded proteins are expressed at similarly high levels, ≥1 μM in the nucleus (Golemis and Brent, 1992). At this concentration, which is in considerable excess over the nuclear concentration of operator-bound bait protein, a cDNA library–encoded protein should half-maximally occupy the DNA-bound bait protein if it possesses a K_d of 10^{-6} M, making it theoretically possible that very-low-affinity interactions could be detected. Such interactions have been observed in some cases. In contrast, some interactions that have been previously established using other methods and are predicted by known K_d to be easily detected by these means, either are not detected or are detected only weakly (Finley and Brent, 1994; Estojak et al., 1995). Because of the conservation of many proteins between lower and higher eukaryotes, one explanation for this observation is that either one or both of the partners being tested is being sequestered from the desired interaction by fortuitous association with an endogenous yeast protein. A reasonably complete investigation of the degree of correlation between in vitro determinations of interaction affinity and apparent strength of interaction in the interaction trap is included in Estojak et al. (1995). The result of this investigation suggests it is important to measure the affinity of detected interactions under different conditions, using a second assay system, rather than to draw conclusions about affinity based on detection in the interaction trap.

A number of different plasmids can be used for conducting an interactor hunt. Their properties are summarized in Tables 17.3.1 and 17.3.2. Because of the generous and open scientific exchange between investigators using the system, the number of available plasmids and other components has greatly expanded since the appearance of the initial two-hybrid reagents, facilitating the study of proteins inaccessible by the original system.

The original parent plasmid for generating LexA fusions, pEG202 is a derivative of 202 + PL (Ruden et al., 1991; see Fig. 17.3.3) that contains an expanded polylinker region. The available cloning sites in pEG202 include *Eco*RI, *Bam*HI, *Sal*I, *Nco*I, *Not*I, and *Xho*I, with the reading frame as described in the legend to Figure 17.3.3. Since the original presentation of this system, a number of groups have developed variants of this plasmid that address specialized research needs. Those currently available, as well as purposes for which they are suited, are listed in Table 17.3.1. pGilda, created by David A. Shaywitz, places the LexA-fusion cassette under the control of the inducible *GAL1* promoter, allowing expression of the bait protein for limited times during library screening, reducing the exposure of yeast to toxic baits. pJK202, created by Joanne Kamens, adds nuclear localization sequences to pEG202, facilitating assay of the function of proteins lacking internal nuclear localization sequences. pNLexA, created by Ian York, places LexA carboxy-terminal in the fusion domain, allowing assay of interactions that require an unblocked amino-terminus on the bait protein. pEE202I, created by Mike Watson and Rich Buckholz, allows chromosomal integration of a pEG202-like bait, thus reducing expression levels so they are more physiological for bait proteins normally present at low levels intracellularly. All of these have been extensively tested by numerous researchers. pGilda, pJK202, and pEE202I work with complete reliability. pNLexA works effectively with ~50% of the fusion domains tried, but synthesizes only very low levels of protein (relative to expression of the same fusion domain as a pEG202 fusion) with the remaining 50%. Attachment of fusion domains amino-terminal either to *LexA* or *GAL4* has been generally problematic in the hands of many investigators; it may be that appending additional protein sequences to the amino termini of these proteins is destabilizing, although the problem has not been rigorously investigated.

A series of *lacZ* reporters of differing sensitivity to transcriptional activation can be used to detect interactions of varying affinity (see Table 17.3.2). These plasmids are *LexA* operator–containing derivatives of the plasmid

LR1Δ1 (West et al., 1984). In LR1Δ1, a minimal *GAL1* promoter lacking the *GAL1* upstream activating sequences (GAL_{UAS}) is located upstream of the bacterial *lacZ* gene. In pSH18-34, eight *LexA* operators have been cloned into an *Xho*I site located 167 bp upstream of the *lacZ* gene (S. Hanes, unpub. observ.). pJK103 and pRB1840 contain two and one operator, respectively.

pJK101 is similar to pSH18-34, except that it contains the *GAL1* upstream activating sequences (GAL_{UAS}) upstream of two *LexA* operator sites. A derivative of del20B (West et al., 1984), it is used in the repression assay (Brent and Ptashne, 1984; see Fig. 17.3.5) to assess LexA fusion binding to operator.

pSH17-4 is a *HIS3* 2μm plasmid that encodes LexA fused to the activation domain of the yeast activator GAL4. EGY48 cells bearing this plasmid will produce colonies in overnight growth on medium lacking Leu, and yeast that additionally contain pSH18-34 will turn deep blue on plates containing Xgal. This plasmid serves as a positive control for the activation of transcription.

pRFHM1 is a *HIS3* 2μm plasmid that encodes LexA fused to the N-terminus of the *Drosophila* protein bicoid. The plasmid has no ability to activate transcription, so EGY48 cells that contain pRFHM1 and pSH18-34 do not grow on –Leu medium and remain white on plates containing Xgal. pRFHM1 is a good control for specificity testing, because it has been demonstrated to be sticky—that is, to associate with a number of library-encoded proteins that are clearly nonphysiological interactors (R. Finley, Wayne State University, Detroit, Mich., unpub. observ.).

This protocol uses interaction libraries (Table 17.3.3) made in pJG4-5 or its derivatives (see Fig. 17.3.6). pJG4-5 was developed to facilitate isolation and characterization of novel proteins in interactor hunts (Gyuris et al., 1993). The pJG4-5 cDNA library expression cassette is under control of the *GAL1* promoter, so library proteins are expressed in the presence of galactose (Gal) but not glucose (Glu). This conditional expression has a number of advantages, the most important of which is that many false-positives obtained in screens can be easily eliminated because they do not demonstrate a Gal-dependent phenotype. The expression cassette consists of an ATG to start translation, a nuclear localization signal to extend the interaction trap's range to include proteins that are normally predominantly localized in the cytoplasm, an activation domain (acid blob; Ma and Ptashne, 1987), the hemagglutinin epitope tag to permit rapid assessment of the size of encoded proteins, *Eco*RI-*Xho*I sites designed to receive directionally synthesized cDNAs, and the alcohol dehydrogenase (*ADH*) termination sequences to enhance the production of high levels of library protein. The plasmid also contains the *TRP1* auxotrophy marker and 2μm origin for propagation in yeast. A derivative plasmid, pJG4-5I, was created by Mike Watson and Richard Buckholz to facilitate chromosomal integration of the activation domain fusion expression plasmid.

A series of recently developed derivatives of pEG202, pJG4-5, and *lacZ* reporter plasmids (MW101 to MW112) alter the antibiotic resistance markers on these plasmids from ampicillin (Ap^r) to either kanamycin (Km^r) or chloramphenicol (Cm^r; Watson et al., 1996). Judiciously mixing and matching these plasmids in conjunction with Ap^r libraries would considerably reduce work subsequent to library screening, because the KC8 transformation, which involves *trpC* complementation in bacteria, could be omitted.

EGY48 and EGY191 (see Table 17.3.2) are both derivatives of the strain U457 (a gift of Rodney Rothstein, Columbia University, New York, N.Y.) in which the endogenous *LEU2* gene has been replaced by homologous recombination with *LEU2* reporters carrying varying numbers of *LexA* operators, using a procedure detailed in Estojak et al. (1995).

Interaction Trap–compatible reagents have recently become commercially available; Clontech and Invitrogen were the first to market such reagents and have recently been joined by OriGene. All suppliers use systems with the most sensitive reporters (EGY48 and pSH18-34), and provide their own positive and negative controls for testing activation or interaction between defined proteins. For expression of bait and library proteins, the Clontech Matchmaker LexA two-hybrid system and the OriGene Duplex-A system use some of the basic set of plasmids described here (see Table 17.3.1 for availability). Forward sequencing primers for bait and library plasmids are included in the Clontech kit, and Insert Screening Amplimer Sets for both plasmids can be acquired separately. Additional related products from Clontech include KC8 competent cells, anti-LexA monoclonal antibodies, a yeast transformation system, a yeast plasmid isolation kit, and an EGY48 partner strain for yeast

mating to facilitate the analysis of interaction specificity. OriGene has a generally similar product line to Clontech. In contrast, Invitrogen has substantially modified the Interaction Trap core reagents to develop its own bait and library plasmids. pHybLex/Zeo, a novel bait plasmid, is ~50% smaller than the original pEG202 (making it easier to clone into), and it has an enriched polylinker. Significantly, it replaces both the *Ap*^{r} and *HIS3* genes with a novel gene that confers resistance to the antibiotic Zeocin (supplied with the kit), which provides selection in both bacteria and yeast. This elimination of auxotrophic selection for the bait plasmid renders the LexA-fusion construct usable with libraries and strains from all existing two-hybrid systems and additionally facilitates the direct selection of library plasmid in strains other than KC8. Some changes, which are designed to make the vector easier to use, have also been introduced in the library vector pYESTrp (e.g., it uses a V5 epitope tag for protein detection). The Invitrogen kit, termed Hybrid Hunter, includes the bait/library/reporter plasmids and EGY48 yeast strain as noted, and additionally includes primer sets for bait and library plasmids and the L40 yeast strain, should an investigator wish to use a *HIS3* auxotrophy selection. Additional related products from Invitrogen include antibodies for detection of bait and prey fusion proteins (anti-LexA and anti-V5), pJG4-5 library vector primers, and a Transformation Kit.

A significant advantage of the entry of commercial entities into the Interaction Trap field is the rapid increase in the number of compatible cDNA libraries. A list of currently available premade libraries available from these companies is presented in Table 17.3.3, and custom-made libraries are also available upon request. Because new libraries and other related reagents are being constantly added to the line of two-hybrid related products, it is advisable to contact the companies or visit their Web sites (*www.clontech.com, www.invitrogen.com,* and *www.origene.com*) for the latest information.

Finally, over the last several years, a number of groups have adapted basic two-hybrid strategies to more specialized applications, and they have devised strategies to broaden their basic functionality. Interaction Mating (Finley and Brent, 1994) has been used to establish extended networks of targeted protein-protein interaction. In this approach, a panel of LexA-fused proteins is transformed into a *MATa* haploid selective strain (such as RFY206), a panel of activation-domain fused proteins is transformed into a suitable MATα haploid (such as EG448), and the two panels are cross-gridded against each other for mating. Selected diploids are then screened by replica plating to selective medium. This approach complements library screening in large-scale applications, such as proposed definition of interaction maps for entire genomes (Bartel et al., 1996).

Interaction mating has also provided the basis for an alternative two-hybrid hunt protocol (see Alternate Protocol 2), useful in cases when a single library will be screened with different baits. In this approach (Bendixen et al., 1994; Finley and Brent, 1994: Kolonin and Finley, 1998), a library is introduced into a single strain, like EGY48, and aliquots are stored frozen. To conduct a hunt, an aliquot is thawed and mated with a strain expressing a bait. This allows one to avoid repeated high-efficiency transformations, since a single library transformation can provide enough pretransformed yeast to conduct dozens of interactor hunts. Moreover, some yeast strains pretransformed with libraries are becoming commercially available, which may eliminate altogether the need to conduct a high-efficiency library transformation for some researchers.

Two-hybrid approaches have been shown to be effective in identifying small peptides with biological activities on selected baits (Yang et al., 1995; Colas et al., 1996), which may prove to be useful as a guide to targeted drug design. Rapid screening protocols have been devised using custom-synthesized libraries expressing sheared plasmid DNA to facilitate rapid mapping of interaction interfaces (Stagljar et al., 1996). Osborne and coworkers have demonstrated the effectiveness of a tribrid (or tri-hybrid) approach, in which an additional plasmid expresses a tyrosine kinase to specifically modify a bait protein, allowing detection of SH2-domain-containing partner proteins that recognize specific phosphotyrosine residues (Osborne et al., 1995). A variety of more elaborate tribrid approaches, in which a DNA-binding domain fused protein is used to present an intermediate nonprotein compound for interaction with a library, have been developed and proven effective. These approaches have allowed the identification of proteins binding specific drug ligands (Chiu et al., 1994; Licitra and Liu, 1996), as well as the identification of proteins binding to RNA sequences (SenGupta et al., 1996; Wang et al., 1996). It is expected that the range of utility of these systems will continue to expand.

Critical Parameters and Troubleshooting

To maximize chances of a successful interactor hunt, a number of parameters should be taken into account. Before attempting a screen, bait proteins should be carefully tested to ensure that they have little or no intrinsic ability to activate transcription. Bait proteins must be expressed at reasonably high levels and must be able to enter the yeast nucleus and bind DNA (as confirmed by the repression assay). Optimally, integrity and levels of bait proteins should be confirmed by immunoblot analysis, using an antibody to either LexA or the fused domain. In particular, at this time, bait proteins that have extensive transmembrane domains or are normally excluded from the nucleus are not likely to be productively used in a library screen. Proteins that are moderate to strong activators will need to be truncated to remove activating domains before they can be used.

If a protein neither activates nor represses, the most likely reason is that it is not being made. This can be determined by immunoblot analysis of a crude lysate protein extract of EGY48 (*UNIT 6.2*; Samson et al., 1989) containing the plasmid, using anti-LexA antibodies as primary antiserum. If the full protein is not made, it may be possible to express truncated derivatives of the protein. If the protein is made, but still does not repress, it may not enter the yeast nucleus effectively, although this appears to be a relatively rare problem. In this case, introducing the coding sequence for the fused moieity into a LexA fusion vector containing a nuclear localization motif (e.g., pJK202; J. Kamens, BASF, Worcester, Mass., unpub. observ.) may solve the problem.

The test for the leucine (Leu) requirement is extremely important to determine whether the bait protein is likely to yield an unworkably high background. The *LEU2* reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits (Estojak et al., 1995). Therefore, it is possible that a bait protein demonstrating little or no signal in a β-galactosidase assay may nevertheless permit some growth on –Leu medium. If this occurs, there are several options. First, a less sensitive strain can be used, as described in the text. Second, background can sometimes be reduced further by making the EGY strain diploid (e.g., D. Krainc, Harvard Medical School, Boston, Mass.; R. Finley and R. Brent, unpub. observ.) or by performing the hunt by interaction mating as described in Alternate Protocol 2. A third option is to attempt to truncate the bait protein to remove activating function. In general, it is useful to extrapolate from the number of cells that grow on –Leu medium to the number that would be obtained in an actual library screen, and determine if this is a background level that can be tolerated. For example, if two colonies arise from 100,000 plated cells on –Leu medium, 200 to 400 would be expected in an actual screen of 10^6 cDNAs. Although this is a high initial number of positives, the vast majority should be eliminated immediately through easily performed controls. This is a judgment call. Finally, very rarely it happens that a bait that appears to be well behaved and negative for transcriptional activation through all characterization steps will suddenly develop a very high background of transcriptional activation following library transformation. The reason for this is currently obscure, and no means of addressing this problem has as yet been found: such baits are hence inappropriate for use in screens.

The protocols described in this unit use initial screening with the most sensitive reporters followed by substitution with less sensitive reporters if activation is detected. An obvious question is, why not start out working with extremely stringent reporters and know immediately whether the system is workable? In fact, some researchers routinely use a combination of pJK103 or pRB1840 with EGY191, and obtain proteins that to date appear to be biologically relevant partners from library screens. However, extensive comparison studies using interactors of defined in vitro affinity with different combinations of *LacZ* and *LEU2* reporters (Estojak et al., 1995) have indicated that although the most sensitive reporters (pSH18-34) may in some cases be prone to background problems, the most stringent reporters (EGY191, pRB1840) may miss some interactions that certainly are biologically relevant and occur inside cells. In the end, the choice of reporters devolves to the preference of individual investigators: the bias of the authors is to cast a broad net in the early stages of a screen, and hence to use more sensitive reporters when practicable.

It is important to move expeditiously through characterization steps and to handle yeast transformed with bait plasmids with care. In cases where yeasts have been maintained on plates for extended periods (e.g., 4 days at room temperature or >2 to 3 weeks at 4°C), unex-

pected problems may crop up in subsequent library screens.

The transformation protocol is a version of the lithium acetate transformation protocol described by Schiestl and Gietz (1989) and Gietz et al. (1992) that maximizes transformation efficiency in *Saccharomyces cerevisiae* and produces up to 10^5 colonies/μg plasmid DNA. In contrast to *Escherichia coli*, the maximum efficiency of transformation for *S. cerevisiae* is ~10^4 to 10^5/μg input DNA. It is extremely important to optimize transformation conditions before attempting an interactor hunt. Perform small-scale pilot transformations to ensure this efficiency is attained and to avoid having to use prohibitive quantities of library DNA. In addition, as for any effort of this type, it is a good idea to obtain or construct a library from a tissue source in which the bait protein is known to be biologically relevant.

In practice, the majority of proteins isolated by interaction with a LexA fusion turns out to be specific for the fused domain; a smaller number are nonspecifically sticky, and to date there appears to have been only one isolation from a eukaryotic library of a protein specific for LexA. However, it is generally informative to retest positive clones on more than one LexA bait protein; ideally, library-derived clones should be tested against the LexA fusion used for their isolation, several LexA fusions to proteins that are clearly unrelated to the original fusion, and if possible, several LexA fusions that there is reason to believe are related to the initial protein (e.g., if the initial probe was LexA-Fos, a good related set would include LexA-Jun and LexA-GCN4).

Colony selection for master plate production is one of the more variable parts of the procedure. For strong interactors, colonies will grow up in 2 days. However, if plates are left at 30°C, new colonies will continue to appear every day. Those that appear rapidly are most likely to reflect interactors that are biologically relevant to the bait protein. Those that appear later may or may not be relevant. However, many parameters can delay the time of colony formation of cells that contain valid interactions, including the strength of the interaction and the level of expression of the library-encoded protein.

Anticipated Results

Depending on the protein used as bait, anywhere from zero to hundreds of specific interactors will be obtained from 10^6 primary transformants.

Time Considerations

If all goes well, once the required constructions have been made it will take ~1 week to perform yeast transformations, obtain colonies, and determine whether bait proteins are appropriate. It will take a second week to perform library transformations, replate to selective medium, and obtain putative positives. A third week will be required to rescue the plasmid from the yeast, passage it through *E. coli*, transform fresh yeast, and confirm specificity.

Literature Cited

Bartel, P.L., Chien, C.-T., Sternglanz, R., and Fields, S. 1993. Using the two-hybrid system to detect protein-protein interactions. *In* Cellular Interactions in Development: A Practical Approach (D.A. Hartley, ed.) pp. 153-179. Oxford University Press, Oxford.

Bartel, P.L., Roecklein, J.A., SenGupta, D., and Fields, S. 1996. A protein linkage map of *Escherichia coli* bacteriophage T7. *Nature Genet.* 12:72-77.

Bendixen, C., Gangloff, S., and Rothstein, R. 1994. A yeast mating-selection scheme for detection of protein-protein interactions. *Nucl. Acids Res.* 22:1778-1779.

Breeden, L. and Nasmyth, K. 1985. Regulation of the yeast HO gene. *Cold Spring Harbor Symp. Quant. Biol.* 50:643-650.

Brent, R. and Ptashne, M. 1984. A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene. *Nature* 312:612-615.

Brent, R. and Ptashne, M. 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* 43:729-736.

Chien, C.-T., Bartel, P.L., Sternglanz, R., and Fields, S. 1991. The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. U.S.A.* 88:9578-9582.

Chiu, M.I., Katz, H., and Berlin, V. 1994. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc. Nat. Acad. Sci. U.S.A.* 91:12574-12578.

Colas, P., Cohen, B., Jessen, T., Grishina, I., McCoy, J., and Brent, R. 1996. Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. *Nature* 380:548-550.

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H., and Elledge, S.J. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Dev.* 7:555-569.

Estojak, J., Brent, R., and Golemis, E.A. 1995. Correlation of two-hybrid affinity data with in vitro measurements. *Mol. Cell. Biol.* 15:5820-5829.

Fearon, E.R., Finkel, T., Gillison, M.L., Kennedy, S.P., Casella, J.F., Tomaselli, G.F., Morrow, J.S., and Dang, C.V. 1992. Karyoplasmic interaction

selection strategy: A general strategy to detect protein-protein interactions in mammalian cells. *Proc. Nat. Acad. Sci. U.S.A.* 89:7958-7962.

Fields, S. and Song, O. 1989. A novel genetic system to detect protein-protein interaction. *Nature* 340:245-246.

Finley, R.L., Jr., and Brent, R. 1994. Interaction mating reveals binary and ternary connections between *Drosophila* cell cycle regulators. *Proc. Natl. Acad. Sci. U.S.A.* 91:12980-12984.

Gietz, D., St. Jean, A., Woods, R.A., and Schiestl, R.H. 1992. Improved method for high-efficiency transformation of intact yeast cells. *Nucl. Acids Res.* 20:1425.

Golemis, E.A. and Brent, R. 1992. Fused protein domains inhibit DNA binding by LexA. *Mol. Cell Biol.* 12:3006-3014.

Grunstein, M., and Hogness, D.S. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* 72:3961-3965.

Gyuris, J., Golemis, E.A., Chertkov, H., and Brent, R. 1993. Cdi1, a human G1- and S-phase protein phosphatase that associates with Cdk2. *Cell* 75:791-803.

Kaiser, C., Michaelis, S., and Mitchell, A. 1994. Methods in Yeast Genetics, a Cold Spring Harbor Laboratory Course Manual, pp.135-136. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Kolonin, M.G. and Finley, R.L., Jr. 1998. Targeting cyclin-dependent kinases in *Drosophilia* with peptide aptamers. *Proc. Natl. Acad. Sci. U.S.A. In press.*

Licitra, E.J. and Liu, J.O. 1996. A three-hybrid system for detecting small ligand-protein receptor interactions. *Proc. Nat. Acad. Sci. U.S.A.* 93:12817-12821.

Ma, J. and Ptashne, M. 1987. A new class of yeast transcriptional activators. *Cell* 51:113-119.

Ma, J. and Ptashne, M. 1988. Converting an eukaryotic transcriptional inhibitor into an activator. *Cell* 55:443-446.

Osborne, M., Dalton, S., and Kochan, J.P. 1995. The yeast tribrid system: Genetic detection of trans-phosphorylated ITAM-SH2 interactions. *Bio/Technology* 13:1474-1478.

Ruden, D.M., Ma, J., Li, Y., Wood, K., and Ptashne, M. 1991. Generating yeast transcriptional activators containing no yeast protein sequences. *Nature* 350:426-430.

Samson, M.-L., Jackson-Grusby, L., and Brent, R. 1989. Gene activation and DNA binding by *Drosophila* Ubx and abd-A proteins. *Cell* 57:1045-1052.

Schiestl, R.H. and Gietz, R.D. 1989. High-efficiency transformation of intact yeast cells using single-stranded nucleic acids as a carrier. *Curr. Genet.* 16:339-346.

SenGupta, D.J., Zhang, B., Kraemer, B., Pochart, P., Fields, S., and Wickens, M. 1996. A three-hybrid system to detect RNA-protein interactions in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 93:8496-8501.

Sikorski, R.S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* 122:19-27.

Stagljar, I., Bourquin, J.-P., and Schaffner, W. 1996. Use of the two-hybrid system and random sonicated DNA to identify the interaction domain of a protein. *BioTechniques* 21:430-432.

Vasavada, H.A., Ganguly, S., Germino, F.J., Wang, Z.X., and Weissman, S.M. 1991. A contingent replication assay for the detection of protein-protein interactions in animal cells. *Proc. Nat. Acad. Sci. U.S.A.* 88:10686-10690.

Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74:205-214.

Wang, Z.F., Whitfield, M.L., Ingledue, T.C.3, Dominski, A., and Marzluff, W.F. 1996. The protein that binds the 3′ end of histone mRNA: A novel RNA-binding protein required for histone pre-mRNA processing. *Genes & Dev.* 10:3028-3040.

Watson, M.A., Buckholz, R., and Weiner, M.P. 1996. Vectors encoding alternative antibiotic resistance for use in the yeast two-hybrid system. *BioTechniques* 21:255-259.

West, R.W.J., Yocum, R.R., and Ptashne, M. 1984. *Saccharomyces cerevisiae GAL1-GAL10* divergent promoter region: Location and function of the upstream activator sequence UASG. *Mol. Cell Biol.* 4:2467-2478.

Yang, M., Wu, Z., and Fields, S. 1995. Protein-peptide interactions analyzed with the yeast two-hybrid system. *Nucl. Acids Res.* 23:1152-1156.

Key Reference

Gyuris et al., 1993. See above.

Initial description of interaction trap system.

Internet Resources

http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork/html

http://cmmg.biosci.wayne.edu/finlab/finlabhomeb.html#Places

Sources of two-hybrid information, protocols, and links; database for false positive proteins detected in interaction trap experiments; analysis of two-hybrid usage.

http://www.clontech.com

http://www.invitrogen.com

http://www.origene.com

Commercial sources for basic plasmids, strains, and libraries for interaction trap experiments.

EA_Golemis@fccc.edu

Sources of interaction trap plasmids for specialized interactions.

http://xanadu.mgh.harvard.edu/brentlabhome page4.html

Database of interaction trap protocols and related issues.

Contributed by Erica A. Golemis and
Ilya Serebriiskii
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Russell L. Finley, Jr. and Mikhail G. Kolonin
(hunt by interaction mating)
Wayne State University School of Medicine
Detroit, Michigan

Jeno Gyuris
Mitotix, Inc.
Cambridge, Massachusetts

Roger Brent
The Molecular Sciences Institute
Berkeley, California

Mapping Protein-Protein Interactions with Phage-Displayed Combinatorial Peptide Libraries

This unit describes the process and analysis of affinity selecting bacteriophage M13 from libraries displaying combinatorial peptides fused to either a minor or major capsid protein. The first protocol provided in this unit describes direct affinity selection using target protein bound to a microtiter dish, followed by purification of selected phage by ELISA (see Basic Protocol). A bead-based affinity selection using fusion protein targets is also described (see Alternate Protocol). With either protocol, one can readily isolate peptide ligands that bind to a target protein of interest and use the consensus sequence to search proteome databases for putative interacting proteins.

AFFINITY SELECTION USING PROTEIN TARGETS IN MICROTITER DISHES

BASIC PROTOCOL

In this method, a target protein is immobilized on a microtiter plate and a phage-display combinatorial peptide library is added to the wells to select for phage that bind to the protein target. Selected (bound) phage are then recovered and amplified. The selection is repeated two additional times to enrich for the binding phage and reduce the recovery of nonspecifically bound phage. After the third selection, the binding specificity of individual clones is examined by ELISA using wells containing target protein and a negative control. Binding is detected with an HRP-conjugated anti-phage antibody and a chromogenic substrate. The sequences from specifically bound clones can then be analyzed by DNA sequencing and the predicted peptide ligand can be compared to available databases.

While peptides have been displayed on all six of the capsid proteins of bacteriophage M13, proteins III and VIII are the most popular fusion partners. Protein III (pIII) is a minor capsid protein, present in five copies at one end of the filamentous particle, and functions in infecting bacteria. Fortunately for molecular biologists, display of peptides (or proteins) at the N terminus of the mature protein generally does not interfere with the function of its three globular domains. Protein VIII (pVIII) is the major capsid protein, with ~2500 copies per particle. Due to the crystalline nature of this protein on the virus surface, mature pVIII tolerates only short (i.e., 9-mer) peptides at its N terminus. While pIII and pVIII display libraries are both useful in selecting peptide ligands that can be used to predict protein-protein interactions, peptides isolated from pIII libraries typically have low-micromolar (1 to 10 μM) dissociation constants, whereas peptides isolated from pVIII libraries have mid-micromolar (10 to 100 μM) dissociation constants. This quantitative difference reflects the difference in valency of the displayed peptides as well as the resulting avidity effects that occur when binding microtiter plate wells are densely coated with target. Libraries of phage displaying combinatorial peptide libraries at the N terminus of pIII can be purchased from New England Biolabs. Libraries of phage displaying combinatorial peptide libraries at the N terminus of pVIII can be obtained by request (see Felici et al., 1991).

Materials

Purified protein target(s) to be analyzed
0.2 M sodium bicarbonate buffer (Na_2HCO_3), pH 8.5
Control targets (see Troubleshooting)
Blocking buffer: 1% (w/v) BSA in PBS (see recipe for PBS)
Wash buffer: 0.05% (v/v) Tween 20 in PBS

Contributed by Brian K. Kay and Luisa Castagnoli
Current Protocols in Cell Biology (2003) 17.4.1-17.4.9

Combinatorial phage-display library (New England Biolabs or Felici et al., 1991)
Acid elution buffer: 50 mM glycine·HCl, pH 2
Neutralization buffer: 0.2 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Bacteria: fresh overnight culture of DH5αF′ (*APPENDIX 3A*)
2× YT culture medium and top and bottom agar (see recipe)
Negative control protein (fusion partner)
Anti–bacteriophage M13 monoclonal antibody coupled to horseradish peroxidase (HRP; Amersham Pharmacia Biotech), diluted 1:5000 (v/v) in wash buffer
Chromogenic substrate (see recipe)

ELISA-ready 96-well microtiter plates (Costar or Immunolon, high capacity)
Aerosol-resistant pipet tips
5-ml sterile tubes
Sterile toothpicks
Spectrophotometer capable of reading microtiter plates

Additional reagents and equipment for DNA purification and sequencing (*APPENDIX 3A*)

Coat microtiter plate wells

1. Dilute 1 to 3 µg of purified protein target into 0.5 ml of 0.2 M sodium bicarbonate, pH 8.5, and add 150 µl to three wells of a 96-well microtiter plate. Repeat for each protein target to be tested and for each control. Incubate without shaking for 2 hr at room temperature or, for less stable proteins, overnight at 4°C.

   To prevent evaporation, keep the wells sealed with tape during all incubations.

2. Add 200 µl blocking buffer to each well and incubate for 30 min at room temperature.
3. Flick the liquid into a sink and slap the plate two times against a paper towel.
4. Add 200 µl wash buffer to each well, flick out the contents, and slap against a clean paper towel. Repeat two additional times.

Select interacting phage

5. Dilute combinatorial phage-display library to 1000 library equivalents per 150 µl wash buffer and then add 150 µl diluted phage particles to each test and control well. Incubate for 2 hr at room temperature.

   To ensure selection of rare peptide sequences, the authors typically use 1000 library equivalents for the first selection step with a target protein. Thus, if a library has a complexity of 10^9 members, 10^{12} particles should be added to 150 µl wash buffer.

   To minimize the contamination of pipettors with phage particles, the authors strongly recommend using aerosol-resistant pipet tips when transferring liquids containing phage.

6. Flick the liquid into a sink and slap the plate two times against a paper towel.
7. Add 200 µl wash buffer to each well, flick out the contents, and slap the plate against a clean paper towel. Repeat two additional times.

Recover bound phage

8. Add 150 µl acid elution buffer to the washed, empty wells and incubate 10 min at room temperature. Pool the liquid from triplicate wells into a single clean 5-ml plastic tube containing 150 µl neutralization buffer.

   Generally, 10^3 to 10^8 phage particles are recovered from each well. Phage can also be eluted from the wells with 0.2 M ethanolamine, pH 12, and neutralized as above.

9. Add 200 µl bacteria (fresh overnight culture) and incubate for 10 min at room temperature.

10. Add 3 ml of 2× YT top agar and pour onto a 100 × 15–mm petri dish containing 2× YT bottom agar (*APPENDIX 3A*). Allow plate to harden 5 min at room temperature, and then leave the plate overnight in a 37°C incubator.

    Instead of plate amplification, it is also possible to amplify the phage in a liquid culture. To do this, transfer the solution containing the infected bacteria to 5 ml of 2× YT broth and incubate in a 50-ml tube, with vigorous aeration, overnight at 37°C.

11. Elute the phage particles from the top agar by covering with 5 ml wash buffer and incubating overnight at 4°C without shaking.

12. Transfer ~500 µl phage suspension to a clean tube and centrifuge 10 min at 16,100 × *g* (13,200 rpm) to pellet the bacteria. Transfer the supernatant to a clean tube.

    If desired, the phage can be concentrated by precipitation. Add 400 µl of 20% (w/v) PEG/2.5 M NaCl to 1.6 ml of the cleared supernatant. Keep on ice for at least 15 min to allow precipitation of phage particles, centrifuge 5 min as above, and resuspend the pellet in 200 µl of wash buffer.

Repeat affinity selection process

13. Perform two additional rounds of screening by repeating steps 1 to 12 two times using the amplified phage supernatant from step 12. As in the first round, set up triplicate wells for selection (step 1) and pool the recovered phage before amplification (step 8).

    The phage mixtures typically have titers of 10^{11} plaque-forming units per milliliter (pfu/ml) after each round of selection and amplification.

14. After the third round of selection, make several serial dilutions at 1:100 in PBS. Then set up several sterile 5-ml tubes for infection (one for each dilution). To each tube add:

    200 µl overnight culture of DH5αF′ bacteria
    1 to 10 µl diluted phage particles
    3 ml 2× YT top agar.

15. Plate out each dilution on 2× YT bottom agar and incubate overnight at 37°C.

16. With sterile toothpicks, transfer phage from 20 well-isolated plaques to separate 5-ml sterile tubes containing 50 µl overnight culture of DH5αF′ bacteria and 1 ml of 2× YT culture medium. Grow overnight at 37°C with vigorous aeration.

    Repeat steps 14 and 15 at different dilutions if there are not twenty well-isolated plaques on one or more of the plates.

Perform ELISA

17. Coat one column of wells in a 96-well microtiter plate with the target protein and another column with a negative control protein (i.e., fusion partner), as described in steps 1 through 4. Set up enough column pairs to test the desired number of clones from step 16.

18. To each pair of wells (target and control), add 100 µl wash buffer and 25 µl of a culture supernatant prepared from a single bacteriophage clone (step 16). Incubate for 2 hr at room temperature.

19. Wash the wells as described in steps 6 and 7.

20. To each well, add 100 µl of anti-phage monoclonal antibody (HRP-conjugated) diluted 1:5000 in wash buffer. Incubate for 1 hr at room temperature.

21. Wash the wells as described in steps 6 and 7.

22. To each well, add 100 µl chromogenic substrate. Incubate ~30 min.

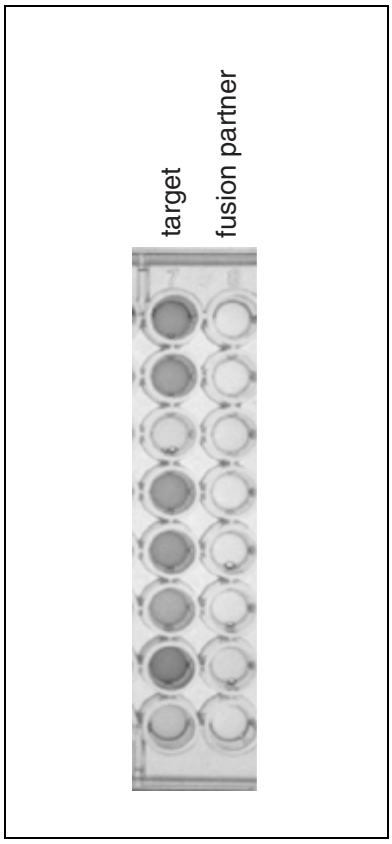


Figure 17.4.1
Photograph of a phage ELISA result. Using eight different clones that were isolated with GST-SrcSH3 domain as the target, 25 μl of culture supernatant were added to pairs of microtiter plate wells containing GST-SrcSH3 or GST. Retention of the phage in the wells was detected with anti-phage antibodies (coupled to HRP) through a colorimetric assay. Six of the clones were verified to bind the SrcSH3 domain specifically.

23. Measure the optical absorbance of the wells at 405 nm with a microtiter plate spectrophotometer.

    Wells with bound phage yield optical density values of >0.6, while wells without bound phage generate optical density values of ≤0.2. See Figure 17.4.1 for typical results.

    Typically, many of the output phage from the third round of selection bind to the target protein under these conditions. If not, one can perform an additional round of selection, perform filter lifts to identify binding clones in the output of the third round of selection (Sparks et al., 1996), or repeat the selection experiment with an alternative method of immobilizing the target (for example, see Alternate Protocol).

Characterize sequence of interacting phage

24. Purify and sequence the DNA inserts of binding phage, using either double-stranded DNA (prepared from the replicative form of DNA) or single-stranded DNA (prepared from secreted virus particles).

    Purification kits for either application are commercially available (e.g., from Qiagen). Also see APPENDIX 3A for purification and sequencing methods.

    Suitable sequencing primers upstream and downstream of the gene III cloning site are 5′-ATT CAC CTC GAA AGC AAG CTG-3′and 5′-CTC ATA GTT AGC GTA ACG-3′, respectively. Suitable sequencing primers upstream and downstream of the gene VIII cloning site are 5′-GTT CCG ATG CTG TCT TTC GCT-3′and 5′-AAC CGA TAT ATT CGG TCG CTG AGG-3′, respectively.

    The authors often sequence from ten to twenty clones. If one sequence predominates, a heteroduplex mobility assay can be used to identify nonsibling clones for sequencing (Fack et al., 2000).

25. Convert the coding sequence to peptide sequence.

In the NNK coding scheme, there are 32 codons that encode one stop codon (TAG), which is suppressed in DH5αF′ with a glutamine, and all 20 amino acids, which are represented one (C, D, E, F, H, I, K, M, N, Q, W, Y), two (A, G, P, V, T), or three times (L, R, S).

AFFINITY SELECTION USING FUSION PROTEIN TARGETS ON BEADS

ALTERNATE PROTOCOL

If a fusion protein is used as a target, phage can be affinity selected with the target protein attached to a resin. In the authors' experience, glutathione-*S*-transferase (GST) fusion proteins can successfully select phage when bound to glutathione-Sepharose resin, which is commercially available. To avoid carry over of contaminant proteins that are bound nonspecifically to the resin, it is best to elute the fusion protein off the column with gluthathione, dialyze the sample, and adsorb it to a fresh batch of glutathione-Sepharose resin. Other types of tags (i.e., maltose-binding protein or six-histidine tags) can be used to link the target protein to an affinity matrix; however, the corresponding affinity resin tends to have a background of nonspecifically adsorbed phage that is five to ten times higher than that obtained with glutathione-Sepharose. In addition, metal-chelating resins used for immobilized metal affinity chromatography tend to select for peptides rich in histidines from the phage-displayed combinatorial peptide libraries.

Additional Materials *(also see Basic Protocol)*

50% (w/v) slurry of glutathione-Sepharose resin 4B (Amersham Pharmacia Biotech, Sigma) in 0.5 M NaCl/20% (v/v) ethanol (capacity 5 mg bound protein/ml resin)
PBS (see recipe) with 0%, 1%, and 3% (w/v) BSA
Glutathione-*S*-transferase (GST) fusion protein of interest (target protein)
TBS (optional): 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*), with 150 mM NaCl

Bind fusion protein target to resin

1. Microcentrifuge ~40 µl of a 50% glutathione-Sepharose resin 4B slurry for 1 min at 800 × *g*, room temperature. Remove the supernatant.
2. Wash the pelleted resin by adding 200 µl PBS, microcentrifuging as in step 1, and removing the supernatant.
3. Resuspend beads in 200 µl PBS, add 10 µg GST fusion protein (~10^{14} molecules), and allow it to bind for 1 hr at 4°C with gentle mixing.
4. Wash the beads three times with 200 µl ice-cold wash buffer (see step 2).
5. Resuspend the beads in 300 µl PBS containing 3% BSA. Mix gently for at least 4 hr at 4°C to saturate the nonspecific binding sites on the resin.

   Usually, it is convenient to prepare enough target-bound resin for the entire experiment (i.e., through the third round of selection in step 13). Prepared resin should be kept in PBS with 3% (w/v) BSA for no longer than 1 week at 4°C.

Bind phage to resin

6. Microcentrifuge the beads as above and add 100 µl PBS (or TBS) containing 1% BSA and 10^{10} pfu of phage library.

   This amount of phage particles represents 20 library equivalents of a library with a complexity of 5 × 10^8 clones.

7. Incubate for at least 1 hr at 4°C while mixing gently.

   For convenience, overnight is also acceptable.

8. Centrifuge the beads as above and discard the supernatant.

9. Wash the beads five times with 1 ml wash buffer. While the resin beads are in the final wash, transfer the suspension to a fresh tube, centrifuge the beads, and remove the wash buffer.

   The beads are transferred to a fresh tube to avoid carry-over of phage bound to the surface of the plastic tube.

Recover bound phage

10. Elute the bound phage by adding 200 µl acid elution buffer. Let sit for 10 min at room temperature.
11. Microcentrifuge beads 30 sec at 800 × *g* (2500 rpm), transfer the supernatant to a clean tube, and neutralize the solution by adding 50 µl neutralization buffer.
12. Keep 10 µl eluted phage in a sealed tube in the refrigerator (at 4°C) as a stock of affinity-selected phage. Amplify the titer of the remaining 100 µl of bound phage as described above (see Basic Protocol, steps 9 to 12).

    If necessary, the phage supernatant can be kept at 4°C with 0.02% (w/v) NaN_3. Chloroform should not be used as this type of phage is sensitive to chloroform.

Repeat affinity selection

13. Perform two additional rounds of selection by repeating steps 6 to 12. Use 100 µl (~10^{10} pfu) for the second round of selection. Add to a fresh 40-µl aliquot of glutathione-Sepharose beads with the GST fusion protein bound to it.

    To improve the isolation of high-affinity peptide ligands, the target protein can be adsorbed to the affinity resin at a much lower concentration (2 µg total protein) and up to ten washes can be used.

    This titer (10^{11} pfu/ml) is obtained consistently. The titer should only need to be checked when looking for the cause of a failure.

Characterize phage isolates

14. Perform a phage ELISA and sequence inserts of interest as described above (see Basic Protocol, steps 17 to 25).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Chromogenic substrate

Dissolve 220 mg of 2′,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) in 50 mM citric acid (10.5 g citrate monohydrate in 1 liter of sterile water), and adjust the pH to 4 with ~6 ml of 10 M NaOH. Filter sterilize the solution and store at 4°C (stable for at least 6 months). Immediately before use, add 30% H_2O_2 to a final concentration of 0.05%.

Phosphate-buffered saline (PBS), 10×

80 g NaCl
2.2 g KCl
9.9 g Na_2HPO_4
2.0 g K_2HPO_4
Add H_2O to 1 liter
Store up to 2 weeks at room temperature
Dilute to 1× before use

YT culture medium and top and bottom agar, 2×

Make up 2× YT medium:
10 g tryptone
10 g yeast extract
5 g NaCl
Bring to 1 liter with H_2O
Autoclave
Store up to 2 weeks at 4°C
For bottom agar: Before autoclaving, add Bacto-agar to a final concentration of 1.5% (w/v)
For top agar: Before autoclaving, add Bacto-agar to a final concentration of 0.8% (w/v)

COMMENTARY

Background Information

In phage display, a protein or peptide library is encoded within a phage genome and is expressed on the surface of the phage as a fusion with the phage coat protein. Phage may then be selected on the basis of the functionality of their surface proteins. This method allows one to screen large numbers of sequences based on protein function. In this unit, phage-displayed peptides are screened based on their ability to bind to a protein of interest (i.e., the target protein). Phage-display combinatorial peptide libraries are typically screened by affinity selection with a particular target protein. In many cases, it is possible to identify, from the affinity-selected peptides, members with a sequence that closely resembles segments (epitopes) of a natural interacting partner of the protein of interest. A practical consequence of the phenomenon known as convergent evolution (Kay et al., 2000) is that one can search whole genome databases for proteins containing segments that match consensus sequences shared by the selected peptides, and then determine experimentally whether or not the identified proteins interact with the target (Smothers and Henikoff, 2001). For example, combinatorial peptide libraries have proven useful in defining the optimal ligand preferences of protein interaction modules, such as EH (Salcini et al., 1997), PDZ (Songyang et al., 1997), SH2 (Songyang et al., 1993), SH3 (Tong et al., 2002), and WW domains (Linn et al., 1997), the heterodimeric G protein βγ subunit (Scott et al., 2001), the catalytic subunit of protein phosphatase 1 (PP1c; Zhao and Lee, 1997), the estrogen receptor (Paige et al., 1999), and the ubiquitin ligase DM2 (Kay et al., 1998). Thus, a fruitful means of mapping protein-protein interactions is to isolate peptide ligands to various prokaryotic proteins and identify candidate interacting proteins in the sequenced genome by computer analysis.

Critical Parameters

The most critical aspect of a successful experiment is to ensure that the protein targets retain their ability to interact with other proteins when immobilized (Stevens, 2001). In the Basic Protocol, the authors recommend immobilizing the protein target on microtiter plate wells. However, because the protein sometimes denatures when affixed to the plastic surface and fails to yield any target-binding phage, one frequently isolates plastic-binding phage instead (Adey et al., 1995). Thus, if the necessary reagents are available, the investigator should always confirm that the immobilized target protein retains its ability to interact with other protein. On the other hand, if the protein target is a GST fusion protein, the Alternate Protocol is recommended.

Troubleshooting

The authors recommend the following targets (with their motif among selected peptides) that are commercially available and can serve as positive controls: FLAG mAb M2 (DYKXXD), streptavidin [HP(Q/M)], Src SH3 domain (RxLPxLP or PxxPxR), and troponin C [(V/L)(D/E)xLKxxLxxLA]. If the suggested positive control targets do not yield binding phage, the titer of selected and amplified phage should be checked. Titers of 10^{11} pfu/ml are expected after amplification. The pH of elution and neutralization solutions should also be checked, as phage titers drop at low pH.

Anticipated Results

The authors have successfully isolated peptide ligands to ~50% of the target proteins tested. The K_d values of peptides recovered by

phage display, when chemically synthesized and tested in solution, range from 10 μM to 500 nM (Grøn and Hyde-DeRuyscher, 2000; Hyde-DeRuyscher et al., 2000), and the peptides bind as surrogates (Pillutla et al., 2001) of interacting protein partners (i.e., the peptides compete off the interacting partner).

By inspecting the sequences of the selected peptides, one often finds a consensus or motif with which one can search sequence databases for potentially interacting proteins. Two Web sites that are useful for searching proteomes with short peptide motifs are *http://www.proteome.com/databases/* and *http://www.arraygenetics.com/about_seqit.php3*. Of course, given the large size of GenBank, a large number of computer matches will generally be found; thus, one must use some judgment in sifting through the best matches to choose the ones that might make biological sense. Such predictions can first be tested by chemically synthesizing peptide segments (i.e., 12-mers) corresponding to the matching segments and testing whether or not they bind in vitro. If antibodies are available to the candidate interacting proteins, positive results can then be followed by co-immunoprecipitation experiments. Thus, much like yeast two-hybrid screening (Fields and Song, 1989; Phizicky and Fields, 1995; *UNIT 12.1*), phage-displayed combinatorial peptide libraries can help in mapping protein-protein interactions (Tong et al., 2002).

Time Considerations

With a single target, it takes 1 to 2 weeks to complete three rounds of selection with a phage-displayed combinatorial peptide library. With a modest investment of time and effort, one can apply these relatively straightforward protocols toward isolating peptide ligands for many different targets. Much more time is spent in analyzing the selected peptides, evaluating their possible biological significance, and testing the hypotheses generated.

Literature Cited

Adey, N.B., Mataragnon, A.H., Rider, J.E., Carter, J.M., and Kay, B.K. 1995. Characterization of phage that bind plastic from phage-displayed random peptide libraries. *Gene* 156:27-31.

Fack, F., Deroo, S., Kreis, S., and Muller, C.P. 2000. Heteroduplex mobility assay (HMA) pre-screening: An improved strategy for the rapid identification of inserts selected from phage-displayed peptide libraries. *Mol. Divers.* 5:7-12.

Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R., and Cesareni, G. 1991. Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J. Mol. Biol.* 222:301-310.

Fields, S. and Song, O. 1989. A novel genetic system to detect protein-protein interactions. *Nature (London)* 340:245-246.

Grøn, H. and Hyde-DeRuyscher, R. 2000. Peptides as tools in drug discovery. *Curr. Opin. Drug Discov. Dev.* 3:636-645.

Hyde-DeRuyscher, R., Paige, L.A., Christensen, D.J., Hyde-DeRuyscher, N., Lim, A., Fredericks, Z.L., Kranz, J., Gallant, P., Zhang, J., Rocklage, S.M., Fowlkes, D.M., Wendler, P.A., and Hamilton, P.T. 2000. Detection of small-molecule enzyme inhibitors with peptides isolated from phage-displayed combinatorial peptide libraries. *Chem. Biol.* 7:17-25.

Kay, B.K., Kurakin, A., and Hyde-DeRuyscher, R. 1998. From peptides to drugs via phage-display. *Drug Discov. Today* 3:370-378.

Kay, B.K., Kasanov, J., Knight, S., and Kurakin, A. 2000. Convergent evolution with combinatorial peptides. *F.E.B.S. Lett.* 480:55-62.

Linn, H., Ermekova, K.S., Rentschler, S., Sparks, A.B., Kay, B.K., and Sudol, M. 1997. Using molecular repertoires to identify high-affinity peptide ligands of the WW domain of human and mouse YAP. *Biol. Chem.* 378:531-537.

Paige, L.A., Christensen, D.J., Grøn, H., Norris, J.D., Gottlin, E.B., Padilla, K.M., Chang, C.Y., Ballas, L.M., Hamilton, P.T., McDonnell, D.P., and Fowlkes, D.M. 1999. Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. *Proc. Natl. Acad. Sci. U.S.A.* 96:3999-4004.

Phizicky, E. and Fields, S. 1995. Protein-protein interactions: Methods for detection and analysis. *Microbiol. Rev.* 59:94-123.

Pillutla, R.C., Hsiao K.C., Brissette, R., Eder, P.S., Giordano, T., Fletcher, P.W., Lennick, M., Blume, A.J., and Goldstein, N.I. 2001. A surrogate-based approach for post-genomic partner identification. *B.M.C. Biotechnol.* 1:6.

Salcini, A.E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P.G., and Di Fiore, P.P. 1997. Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. *Genes Dev.* 11:2239-2249.

Scott, J.K., Huang, S.F., Gangadhar, B.P., Samoriski, G.M., Clapp, P., Gross, R.A., Taussig, R., and Smrcka, A.V. 2001. Evidence that a protein-protein interaction 'hot spot' on heterotrimeric G protein betagamma subunits is used for recognition of a subclass of effectors. *EMBO J.* 20:767-776.

Smothers, J.F. and Henikoff, S. 2001. Predicting in vivo protein peptide interactions with random phage display. *Comb. Chem. High Throughput Screen.* 4:585-591.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., et al. 1993.

SH2 domains recognize specific phosphopeptide sequences. *Cell* 72:767-778.

Songyang, Z., Fanning, A.S., Fu, C., Xu, J., Marfatia, S.M., Chishti, A.H., Crompton, A., Chan, A.C., Anderson, J.M., and Cantley, L.C. 1997. Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* 275:73-77.

Sparks, A., Adey, N., Cwirla, S., and Kay, B. 1996. Screening phage-displayed random peptide libraries. *In* Phage Display of Peptides and Proteins: A Laboratory Manual (B.K. Kay, J. Winter, and J. McCafferty, eds.). Academic Press, San Diego.

Stevens, F.J. 2001. Caveat receptor: Proteomes on display. *Comb. Chem. High Throughput Screen.* 4:599-602.

Tong, A.H., Drees, B., Nardelli, G., Bader, G.D., Brannetti, B., Castagnoli, L., Evangelista, M., Ferracuti, S., Nelson, B., Paoluzi, S., Quondam, M., Zucconi, A., Hogue, C.W., Fields, S., Boone, C., and Cesareni, G. 2002. A combined experimental and computational strategy to define protein interaction networks for peptide recognition modules. *Science* 295:321-324.

Zhao, S. and Lee, E.Y. 1997. A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. *J. Biol. Chem.* 272:28368-28372.

Contributed by Brian K. Kay
Argonne National Laboratory
Argonne, Illinois

Luisa Castagnoli
University of Rome Tor Vergata
Rome, Italy

Protein-Protein Interactions Identified by Pull-Down Experiments and Mass Spectrometry

The discovery of new interacting partners for a protein of interest can provide significant clues to its functional role. Identification of a binding protein with a defined cellular role can often present immediate new research directions. Several techniques have been developed to identify protein-protein interactions. Generally, these use a "bait" protein of interest to search a pool of cellular proteins for an interacting partner, coupled with some form of detection mechanism. One commonly used technique is the yeast two-hybrid system (*UNIT 17.3*), which utilizes transcriptional activation of a reporter gene in yeast as a readout of protein-protein interaction.

This unit describes the use of glutathione *S*-transferase (GST) fusion protein "pull-down" approaches to isolate interacting proteins and to then identify them by SDS-PAGE and mass spectrometry (MS). For more background on the use of GST fusion proteins and the pull-down technique, see Commentary. Protein-protein interaction partners are identified in a way that involves (and is dependent on) direct protein-protein interaction. The detection mechanism is protein staining of SDS-PAGE gels and is therefore not dependent on any secondary process, such as a cellular event or activation of enzyme activity. MS techniques for protein identification enable sensitive identification of proteins by comparison of spectral data obtained from tryptic protein digests of an unknown protein with theoretical in silico digests of entries within a DNA or protein sequence database.

The methods in this unit can be performed in a number of ways, and the advantages and disadvantages of some of these variations are presented in Strategic Planning, along with the options that are available within the protocols themselves. Some attention is given to long-term planning, after the pull-down experiments, to confirm and further study the protein-protein interactions identified. The first protocol presented (see Basic Protocol 1) details the use of GST fusion proteins coupled to glutathione (GSH)-agarose beads (as a support matrix) for affinity purification of interacting proteins from a tissue or cell lysate. The method involves the binding of GST fusion protein–coupled beads to proteins in a tissue lysate, recovery of the beads, washing away nonspecific proteins, elution of the bound proteins, and analysis by SDS-PAGE. The protocol details the application of spin columns to improve assay speed and reproducibility. A separate protocol (see Basic Protocol 2) describes MS-compatible methods of gel staining after SDS-PAGE, and sample preparation for analysis by MS. Methods of MS analysis are beyond the scope of this unit and are not described here (see Chapter 16 in Coligan et al., 2003, for an in-depth treatment of mass spectrometry; also see *UNIT 5.6* in this manual). However, the samples are prepared to a stage that is suitable for shipping to an in-house or commercial MS service for analysis. One specific application of the pull-down approach (described in the Alternate Protocol) is for the identification of nucleotide-dependent binding partners of small GTPases, critical regulators of cellular signaling and function.

Three support protocols are described that detail preparatory procedures that are necessary before performing the pull-down experiment. Support Protocol 1 describes a method for preparation of GST fusion protein–coupled beads and Support Protocol 2 provides detail on cross-linking procedures to covalently couple the GST fusion protein to the GSH beads. Procedures for large-scale tissue lysate production are provided in Support Protocol 3, with a focus on sheep brain as a large-scale tissue source. Support Protocol 4 describes recycling procedures for the reuse of the large quantities of GSH-agarose beads that are necessary for the pre-clearing steps in tissue lysate preparation.

Contributed by Adam Brymora, Valentina A. Valova, and Phillip J. Robinson
Current Protocols in Cell Biology (2004) 17.5.1-17.5.51

STRATEGIC PLANNING

This unit comprises several component protocols, some of which have alternative methods. This Strategic Planning section describes the planning steps required to link these together into a cohesive approach for performing pull-down experiments combined with mass spectrometry. A flow chart of the strategic planning and design stages involved, and how these link to the experimental steps, is shown in Figure 17.5.1. The left-hand column (design and analysis) is a representation of this Strategic Planning section; how it is linked to the experimental protocols is illustrated on the right. Some initial consideration must be given to the approach to be taken, based on the protein of interest, its cellular function, enzymatic or other activity, domain structure, and the properties to be investigated.

Achieving Specificity

One of the first considerations should be the question of how specificity can be achieved in the pull-down experiment. The conditions to be compared within the experiment should be carefully considered, as vector construction and mutation may be required well in advance of the experiment. Optimally, a pull-down experiment should encompass the target fusion protein as well as a set of truncations, mutations, deletions, or even a set of related proteins from other genes. For example, such an approach for a new SH3 domain might be to use five different SH3 domains (including the new one) to isolate binding proteins for each and determine which are unique or common. This may reveal which binding proteins are specific to the SH3 domain of interest. Another example might be the use of a set of characterized mutations from a particular disease to analyze differential protein-protein interactions in various pathological states. Another approach might use just one protein but in a number of different activation states. This produces internal controls for specificity and enables a more confident assessment of the significance of the results obtained. An example of the latter approach might be a phosphorylated protein with its phosphorylation site serine or threonine residue mutated to alanine (incapable of being phosphorylated) and/or glutamic acid (pseudophosphorylation). All three would be analyzed simultaneously to isolate potential phosphorylation-dependent binding proteins. Another example might be a GST-tagged small GTPase bound to GDP or GTP (as described in the Alternate Protocol) to isolate activation state–dependent binding proteins (effectors). It is clear from these examples that the choice of constructs required will be dependent on the protein of interest and its properties to be investigated in the pull-down experiment. Using truncations may have the extra advantage of narrowing down the protein-protein interaction site on the protein of interest and simultaneously increasing the specificity in identifying interacting proteins.

Appropriate external controls also need to be established for the constructs that will be used in the pull-down experiment. These will be analyzed by SDS-PAGE in parallel with the eluted proteins from the pull-down. The minimum set of controls includes a separate set of beads containing recombinant GST, as well as a pull-down with GSH beads alone (both to be used concurrently in the pull-down with lysate). The GSH bead pull-down control can be obtained by taking an aliquot of beads from those used to pre-clear the tissue lysates in Support Protocol 3 and Basic Protocol 1. Additionally, it may be necessary to concurrently analyze GST fusion protein beads that have not been combined with lysate—this is an essential control when the GST fusion protein beads have not been cross-linked.

Design of Expression Constructs

It is worth considering how recombinant fusion proteins will be attached to the beads, especially when designing the expression vectors from scratch. The main consideration is how the protein of interest will be fused to the tag. First, it needs to be determined if it will be an amino- or a carboxy-terminal tag. This choice will depend on where the functional regions of the protein of interest are located within the protein, as tagging close

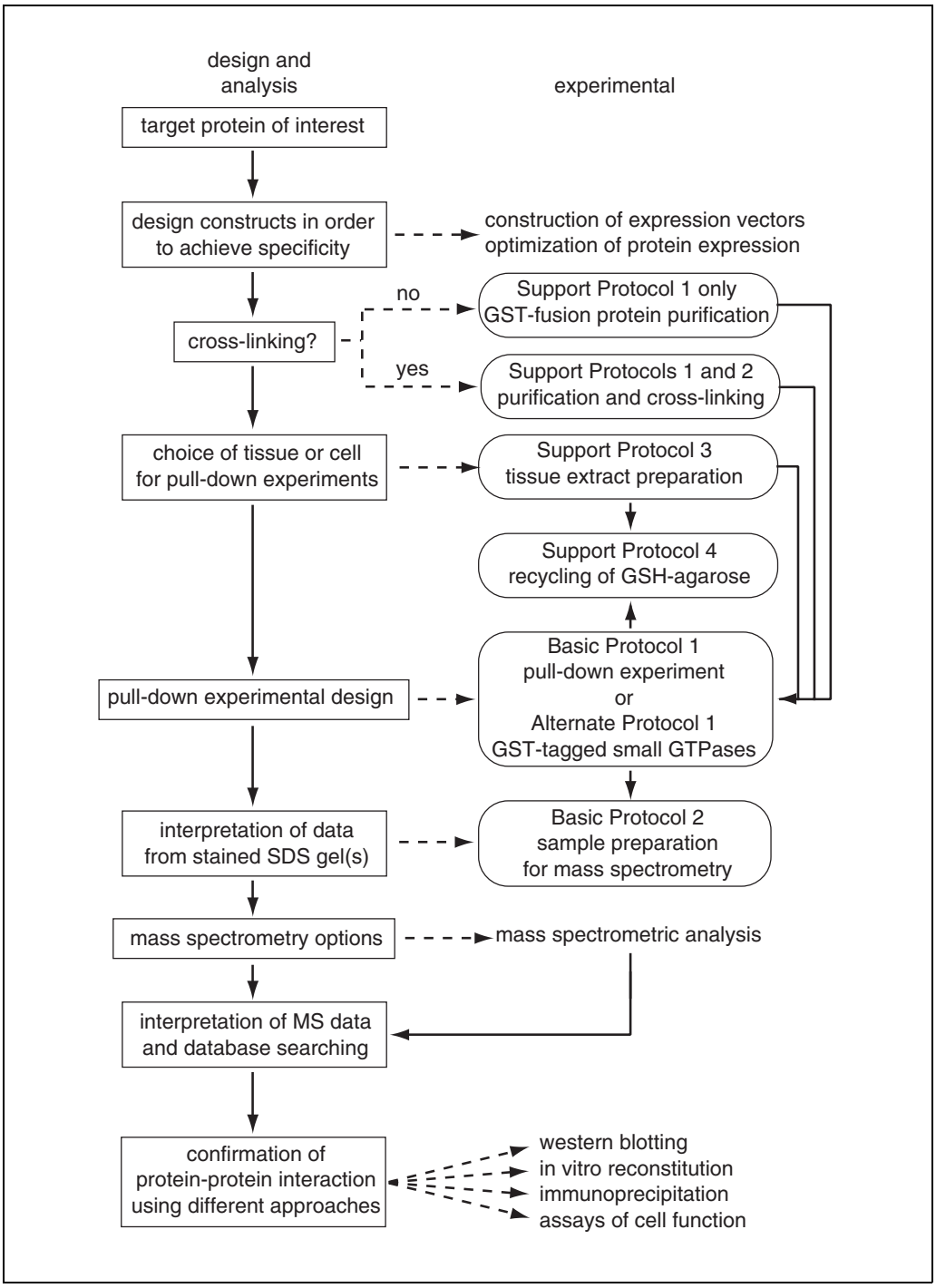


Figure 17.5.1 Flowchart for strategic design and the experimental processes described in this unit, showing how the planning and analysis stages (left-hand side; a summary of the Strategic Planning section) are linked with the experimental protocols (right-hand side). It is also a reference for how the protocols relate to one another.

to such regions could potentially interfere with the protein's function and thus with binding sites for other proteins. Secondly, the linker region between the fusion tag and the protein of interest may be significant. If this region is extensive or hydrophobic, the expressed protein could be insoluble (leading to poor yields in purification), and it may bind many proteins nonspecifically in pull-down experiments.

While this protocol is mainly concerned with GST fusion proteins, alternative tags can be used since the concepts are essentially the same. Alternatives include maltose binding protein (MBP) tags; purification of recombinant protein by whatever means, followed by coupling to a reactive matrix such as Affi-gel (Bio-Rad), may also be used. Some fusion protein tags are particularly unsuitable for use in pull-down experiments, particularly the six-histidine tag that is commonly used for recombinant protein purification as metal-affinity beads are known to bind many proteins nonspecifically. Whether or not cross-linking will be necessary needs to be considered, as recombinant proteins cannot be cross-linked to maltose or metal-affinity columns with DSS (as in Support Protocol 2) because both types of beads lack primary amine groups.

Cross-Linking

Next, consider whether to cross-link the protein of interest to the bead support. GST fusion proteins can be covalently attached to immobilized GSH-agarose beads using cross-linking reagents such as DSS (Support Protocol 2). Cross-linking a fusion protein of interest to the bead support is not essential, and significant results can be achieved without it. The advantage of coupling to the beads is the ability to elute only the interaction partners, leaving the GST fusion protein "bait" covalently attached to the bead. Thus, it will not be present in the subsequent extract or SDS acrylamide gel. The absence of GST fusion protein in the gel can be significant, because the presence of large amounts of fusion protein can make identification of specific binding proteins difficult, simply because of the number and size of the GST fusion protein–related bands that are present on the gel. This problem gets worse as the molecular weight of the fusion protein increases because many of the fusion protein–related bands migrate at smaller molecular weights than the fusion protein itself. Additionally, the presence of large quantities of GST fusion protein bands on the gel can sometimes seriously interfere with the identification of other proteins in the sample by MS peptide mass mapping (see below)—recombinant GST may seem to be present in every band, regardless of molecular weight, making identification of other proteins very difficult. Presumably, the large amount of fusion protein used does not resolve fully, and while the majority of the protein may be present as indicated by protein stain at the appropriate molecular weight, the traces that are present throughout the lane interfere with peptide mass mapping techniques. Other MS techniques of protein identification (e.g., peptide sequencing) may not be as susceptible to interference by the presence of GST fusion protein contamination in protein bands (see below). Non-MS techniques, such as Edman degradation, will also be relatively immune to this effect. While GST fusion protein contamination is common, it often proves to be inconsequential to achieving results, even from peptide mass mapping techniques like MALDI-TOF MS (see Anticipated Results and Brymora et al., 2001b).

While there are distinct advantages to using cross-linking in GST fusion protein pull-down experiments, there are also some caveats to consider. Loss of some or all biological activity of specific proteins (particularly enzymes) may occur as a result of the chemical modification that occurs during cross-linking. This loss of activity may be the consequence of a conformational change in the protein when primary amine groups (i.e., lysine residues) on the surface of the molecule react with an amine-directed cross-linking reagent (such as DSS). Alternatively, loss of activity may occur when specific lysine groups involved in binding a protein interaction partner or substrate (in the case of an enzyme) are modified

by the cross-linker. Therefore, it may be necessary to fully evaluate the influence of cross-linking reagents on any known functions of a protein of interest before using them in the pull-down approaches described herein. Alternatively, a pull-down experiment may be performed both with and without cross-linking of the GST fusion protein beads, particularly if the protein of interest has no known functions that can be tested outside of the pull-down. It may thus be possible to establish if cross-linking has any adverse influence on the protein-binding characteristics of a target protein.

Considerations in Tissue Source for Interacting Proteins

The source of protein for isolation of interacting proteins is often an obvious choice, decided on the basis of the investigator's research interests, but several factors should be kept in mind. Concerning mammalian systems, the tissue where the target protein is predominantly expressed can be an ideal starting point; presumably, if the protein is expressed there, then the interacting partner proteins must also be present. Also, a tissue could be chosen where the target protein is known to have a specific function, with the aim of determining the mechanism of how the protein carries out that function. However, the concentration of interacting partners may be higher in a tissue other than the tissue of interest (thus enabling easier isolation and identification) and yet may still represent a protein-protein interaction that is valid within the tissue of interest. In this way, it may initially be a useful approach to use a panel of different tissue sources (e.g., brain, lung, liver, kidney, and muscle) and establish which interacting proteins are unique to a particular tissue and which are common to all, and in which tissue they are present in the greatest abundance.

Likewise, it is worth considering a rational approach to how a lysate is to be prepared for use in pull-down approaches. This unit provides options for the preparation of whole-tissue lysate, as well as cytosolic, peripheral membrane, and membrane protein extracts (see Support Protocol 3). The whole tissue lysate, while having a higher concentration of potential interaction partners (and therefore being more likely to isolate them), is also more prone to higher levels of nonspecific protein binding. Fractionating proteins into the three described subcellular compartments reduces nonspecific binding but risks dilution of a protein of interest between three times the volume of lysate. It is usually also necessary to analyze the three extracts at once, which may lead to experiments that are too large and impractical (as three times the number of columns would be required). It is, however, possible that extracting proteins specifically from one of the subcellular compartments will concentrate that protein in that fraction and provide a clearer result than the use of a whole-tissue lysate. Given all of these factors, it can be worth considering the initial use of a panel of different tissue extracts to determine which gives the clearest result for isolation of interacting proteins.

Another consideration in the choice of tissue source is the abundance of endogenous GST proteins that can interfere with interpretation of results from pull-down experiments. After purification of recombinant GST fusion protein beads, the GSH beads still have free binding sites for other GSTs, and these can be specifically isolated on GSH beads if they are not fully removed from a tissue lysate. Some lysates contain high levels of endogenous GSTs, particularly cytosolic extracts and lysates from testis and liver. Further pre-clearing may be required for these lysates (see Critical Parameters and Troubleshooting).

The use of cultured cells as a source of interacting proteins has less utility for the procedures described in this unit, due to the limited quantity (and relatively high cost) of protein lysate that can be obtained. However, cells can be particularly of use in situations where a nonmammalian system is used as a source of interacting proteins (e.g., yeast), or when studying particular mammalian cell types (as opposed to whole tissues), provided

that enough lysate can be obtained. Cultured cells (e.g., thirty 7.7-ml flasks) can also be useful for a single pull-down experiment, without stringent control experiments. Any binding proteins identified may then be specifically targeted for more stringent analysis in scale-up experiments from larger tissue lysates. In this case, limited or no subcellular fractionation of cultured cells is recommended. A total cell lysate (using Triton X-100) is used in order to maximize protein recovery. Cultured cells may also be useful for further characterization of a protein-protein interaction detected from a tissue lysate (see below).

The final consideration for mammalian protein studies relates to scaling up tissue lysate size by switching to a physically larger species (e.g., ovine or bovine). The protein products of the same gene usually function similarly within different species including binding to similar proteins. Mass spectrometric methods for protein identification do not have any major difficulty adapting between species, since the vast majority of a particular protein's sequence is often identical between species (Larsen and Roepstorff, 2000). Mass spectrometric methods which rely on peptide mass mapping (see below) are thus usually little affected (except perhaps for small proteins that digest to only a few peptides), since the majority of peptides in a proteolytic digest are of identical molecular weight between species. Methods that directly provide primary sequence data for digested peptides are similarly little affected since amino acid sequence data can be used directly in database searches across species.

Mass Spectrometry Techniques

Modern advances in mass spectrometry (MS) have led to an increasingly complex number of approaches and types of instrumentation that can be utilized for identification of a protein of interest. While the sample preparation techniques are common to all (see Basic Protocol 2), the methods of analysis and the style of data obtained can vary widely between different techniques. The different instruments can also vary considerably in sensitivity, availability, and price, and often the identification of isolated proteins must be achieved using whatever instrument is available to the researcher. Proteins are generally identified by either one of two methods: peptide mass mapping (also known as peptide mass fingerprinting) or peptide sequencing (Coligan et al., 2003, Chapter 16).

Almost all types of MS instrumentation are capable of generating data suitable for analysis by peptide mass mapping. Peptide mass mapping uses a computer program to analyze a peptide mass spectrum for peaks that fit suitability criteria (e.g., of resolution and intensity). The masses of these peaks are then compared against a theoretical proteolytic digest of every protein in a selected database to identify a statistically significant match within a specified mass tolerance range (e.g., ±0.01 Da, or 50 ppm). This whole procedure can be completely automated by software, where the mass spectrum is the input and the protein identification (ranked by some kind of statistical score) is the output. The power of this approach is that it is very fast and can lead to a positive identification even in the face of changes in some peptides (e.g., post-translational modification or species differences). The main disadvantage of this technique is its dependence on the purity of the initial sample—if there is more than one protein in the gel piece being analyzed, it becomes more difficult to reliably match the data to one specific protein. While it is possible to identify one or more proteins out of such a peptide mixture, it is generally more difficult to achieve reliable results from such samples. Thus, gel resolution and dexterity in band cutting become critical to ensure that only a single protein is present in a gel piece (see Basic Protocol 2). The most suitable instrument for peptide mass mapping (despite the fact that most mass spectrometers can be used) is the matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS). This suitability arises from the simplicity, cost, and speed of the MALDI-TOF technique—it is possible to analyze (and thereby identify) hundreds of prepared protein samples each day

using this instrument (with varying success rates). Therefore, the MALDI-TOF mass spectrometer is usually the instrument of first choice for protein identification.

Peptide sequencing is a self-descriptive title that applies to the ability of some of the higher-end mass spectrometers to isolate a specific peptide from a spectrum and fragment it, yielding data that can be interpreted to give a primary amino acid sequence for that selected peptide (often called "tandem MS" or "MS/MS"). This peptide sequence, combined with the peptide mass, is then used to search against a protein sequence database, enabling identification of the protein. These instruments are available in many different combinations of components, providing versatile methods of sample delivery and analysis; one example is electrospray ionization tandem MS (ESI-MS/MS), for liquid samples, perhaps in combination with a liquid chromatography step. This technique can therefore identify a protein or proteins that are present in a sample, irrespective of whether the sample is contaminated with other proteins. It is a very powerful technique and probably the best suited for unequivocal identification of proteins. Generally speaking, however, the technique is slow (a maximum of around five samples per day) and is perhaps more suited to a secondary role as an adjunct to a high-throughput peptide mass mapping technique such as MALDI-TOF MS. Protocols for protein identification by this method, further background, and comparisons of MS techniques can be found in *UNIT 5.6* (also see Coligan et al., 2003).

If it is not possible to perform mass spectrometric analysis in the laboratory (due to lack of access or instrumentation), a commercial service can be used. Alternatively, more traditional protein sequencing techniques can also be used, but they are generally limited to samples of high abundance. Edman sequencing of tryptic peptides (described in Coligan et al., 2003) is one possibility. Sample preparation for this technique is identical to the approach presented in Basic Protocol 2. Sequencing results can be compared directly to a protein sequence database.

Confirmation of Protein-Protein Interactions

After the pull-down experiments have been concluded and the specific protein-protein interaction partners have been identified, it is important to verify the obtained results with other complimentary techniques. These are required to confirm that the discovered interaction is not an artifact of the experimental conditions and that the protein identification is accurate, as well as to determine whether the interaction is direct. The following are all approaches that may be indicated for confirmation of an interaction:

1. Immunoblots (*UNIT 6.2*) may be performed with specific antibodies raised against the new interacting partner to probe smaller-scale pull-down experiments (e.g., from cultured cell lysates) and thus confirm identification. Also, probing with antibodies against known interaction partners of the target protein (if these are available) can show that the system is working as it should.

2. The protein-protein interaction may be reconstituted in vitro with expressed recombinant protein, in pull-down experiments where only the two interacting proteins are used. These experiments demonstrate that the interaction is direct and does not involve some other factor that is present in the original tissue lysate. Thorough controls are necessary, as artifacts arising from nonspecific binding are more common in a reconstituted system using recombinant proteins than in one using proteins from a tissue or cell lysate. This is often due to recombinant protein not being correctly folded or post-translationally modified in the same manner as the tissue-derived protein.

3. Coimmunoprecipitation experiments may be performed to cosediment the two proteins complexed to one another from a cell or tissue lysate. This can also be done from cells that are overexpressing epitope-tagged versions of the two proteins (with different tags on each), enabling immunoprecipitation of the tag. This approach eliminates the need to raise or obtain immunoprecipitating antibodies, particularly if the proteins in question are poorly characterized.

4. Genetic approaches (e.g., reporter gene assays) can also be used, particularly in a nonmammalian system like yeast.

5. Overexpression of mutations or truncations in the protein of interest in cells that are deficient in the protein-protein interaction in question may be exploited in the hope of eliciting a change in cellular response through alterations in the activity of the interacting protein.

BASIC PROTOCOL 1

THE PULL-DOWN EXPERIMENT

The large-scale approach to pull-down experiments described in this protocol uses recombinant GST fusion protein coupled to GSH agarose beads, with or without cross-linking (purified as described in Support Protocols 1 and 2) and tissue lysates (prepared as described in Support Protocol 3). It utilizes small spin columns for some of the steps, as these improve the efficiency. Specifically, improved assay speed, protein recovery, reproducibilty, and decreased elution volumes are achieved when compared to traditional microcentrifuge tube approaches using centrifugation and aspiration of supernatant (Brymora et al., 2001a). There are several sizes of spin columns available that are more or less suitable for this protocol, the best being the MicroSpin columns from Amersham Biosciences. The different types, as well as the advantages and disadvantages of each, are described in Table 17.5.1.

It is possible (and often preferable) to perform the entire pull-down experiment (binding, washing, and elution steps) completely within the spin column (a schematic representation of this is shown in Fig. 17.5.2). This has the advantage of reducing any bead loss caused by repeated transfers between tubes, but it is dependent on the volume of lysate that is intended (as the columns are generally quite small: 0.5- to 1.5-ml) and the probable abundance of any interaction partners in that volume. Alternatively, the binding steps can be performed in larger volumes of tissue lysates (e.g., a 50-ml tube per pull-down), and the beads can later be transferred carefully to the spin columns for washing and elution. This latter approach is described in detail in this protocol, since it is more likely to yield successful results when combined with mass spectrometry. However, for subsequent characterization of protein-protein interactions, particularly for analysis by immunoblotting, smaller-scale pull-down experiments using the former technique are often preferable.

How much of the beads and recombinant GST-protein should be used? It is necessary to have an excess of recombinant protein relative to anything that may be in the lysate, but not so much that it will distort everything in the final analysis on the SDS acrylamide gel (if cross-linking is not used). The optimal amount of GST fusion protein to use needs to be determined empirically for a particular experiment.

For GST fusion proteins not cross-linked to GSH beads, an approximate place to start is 5 µg recombinant protein on beads per 1 g of tissue in a whole tissue lysate.

For GST fusion proteins cross-linked to GSH beads, use 10 to 50 µg recombinant protein on beads per 1 g of tissue in a whole tissue lysate.

Table 17.5.1 Advantages and Disadvantages of Different Types of Spin Columns

Name	Supplier	Maximum vol. (ml)	Advantage (+), Disadvantage (-)
MicroSpin Columns	Amersham Biosciences	0.6	+Minimal dead volume under frit +Reuseable (wash out beads with water and soak in 70% ethanol until needed; centrifuge tubes dry)
Micro Bio-Spin Chromatography Columns	Bio-Rad Laboratories	1	+Larger volume in a small profile that fits most microcentrifuges –Large dead volume in hollow plug (Luer-lok fitting) –Small volumes of elution buffers may need to be manually removed from plug after heating. Minimized by rapidly removing plug (within a few seconds) after removal from water bath. –Single-use only
Bio-Spin Chromatography Columns	Bio-Rad Laboratories	1.5	+Large volumes can be added (e.g., lysates) –Large dead volume in hollow plug (Luer-lok fitting), but this can be alleviated with custom-made plugs that penetrate into Luer fitting –Need to confirm that centrifuge has sufficient clearance above rotor to house spin column when it is sitting in its collection tube –Single-use only

The overall bead volume does not matter greatly, but it needs to be as small as possible (20 to 100 µl). A larger bead volume will wash less efficiently and will bind more nonspecific proteins from the tissue lysate. The beads sometimes have a very high concentration of bound recombinant GST fusion protein; in these cases, dilute the beads with bead storage buffer (see Support Protocol 1), so that the small volumes can be pipetted accurately. Swirl the bead slurry or vortex very gently between each pipetting step to ensure uniform suspension.

When using multiple GST fusion proteins on different beads, the final bead volume used for each pull-down must be normalized or equalized. For example, if using five different GST constructs with 25 µg of each attached to varying amounts of beads (as is normally the case), it is necessary to dilute all the beads with GSH agarose so they all match the same volume. In other words, use the same bead volume as well as the same protein amount in each pull-down that is intended to be used on the same gel. This ensures that any nonspecifically binding proteins are relatively constant between the different conditions and are therefore not mistaken for specific binding proteins.

Materials

Tissue lysate (see Support Protocol 3)
Bead storage buffer: 20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/50% (v/v) glycerol
GSH-agarose beads (e.g., glutathione-Sepharose 4B; Amersham Biosciences)
Wash buffers 1 *or* 2, and 3 (see recipes)
1× SDS sample buffer (*UNIT 6.1*)

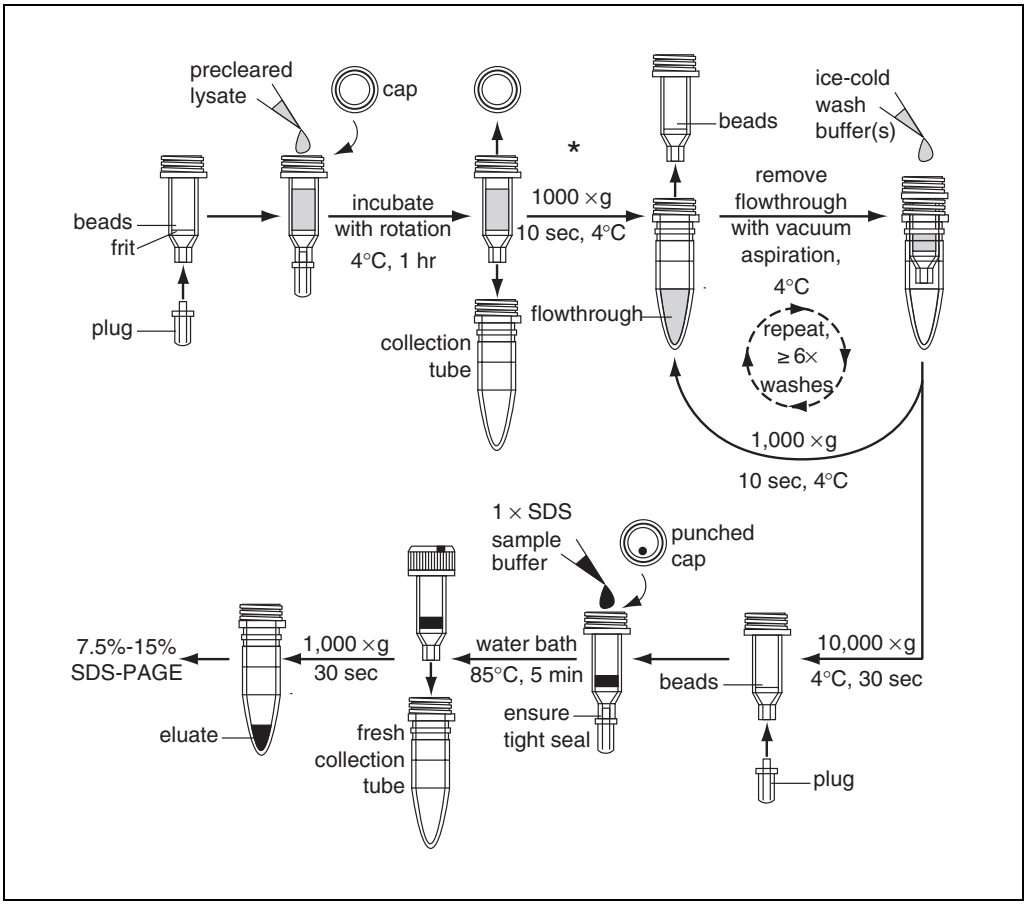


Figure 17.5.2 The spin column–based pull-down experiment. An illustrative description of the approach taken when an entire pull-down experiment is performed within the spin column. This differs from the protocol presented in Basic Protocol 1 and the Alternate Protocol, as it is designed for smaller-scale experiments. The large-scale experiments in the text intersect with this figure at the point marked with an asterisk (*), corresponding to step 12 in Basic Protocol 1 and the Alternate Protocol.

Benchtop centrifuge, 4°C
Appropriately sized tubes that can be centrifuged
End-over-end rotator
Empty plastic column (e.g., Poly-Prep from Bio-Rad; to suit lysate volume) *or* Miracloth (Calbiochem)
MicroSpin columns (Amersham Biosciences), or other spin columns (see Table 17.5.1)
85°C water bath

Additional reagents and equipment for preparing GST fusion proteins attached to GSH-agarose beads (see Support Protocol 1), cross-linking of fusion proteins (optional; see Support Protocol 2), preparing tissue lysate (see Support Protocol 3), and SDS-PAGE (*UNIT 6.1*)

Prepare GST fusion proteins and tissue lysates

1. Prepare recombinant GST fusion proteins attached to GSH-agarose beads (see Support Protocol 1). Cross-link if desired (see Strategic Planning and Support Protocol 2). Store at −20°C in bead storage buffer for up to 6 months.

2. Prepare a tissue lysate (see Support Protocol 3 or use other method).

   Ensure that lysate is free from even small traces of particulate contamination. These will only block the columns later and result in large amounts of nonspecific protein on the gel. If present, it is very important to remove them by centrifugation at high speed (e.g., 18,000 × g) for 30 min, even if this has already been performed earlier.

Pre-clear lysates with GSH beads

If using tissue lysates prepared according to Support Protocol 3, the lysates have already been precleared with GSH-agarose: skip steps 3 to 8 and proceed starting with step 9. If using a different source of lysate, then continue with step 3.

3. Wash a batch of fresh or recycled GSH-agarose beads that have no recombinant protein attached as follows:

   a. For 2 g tissue use 100 µl of beads; for 200 g tissue use about 2 ml GSH beads.

   b. Add 1 ml (for 2 g tissue) or 20 ml (for 200 g tissue) of wash buffer (i.e., the base buffer that was used for tissue lysis in Support Protocol 3, or wash buffer 1) to the beads and centrifuge in a benchtop centrifuge 5 min at 500 × *g*, 4°C. Remove supernatant and collect the beads in a minimal volume of buffer.

   Avoid centrifuging beads (until the elution step) much faster than 2000 × g, or the beads will be crushed.

4. Pre-clear the tissue lysate with the washed GSH beads for removal of endogenous GSTs present in the lysate and nonspecifically binding proteins, by combining the washed GSH beads (from step 3) with the tissue lysate and incubating at 4°C on an end-over-end rotator for 30 min.

   This will remove most of the endogenous cellular GST. The addition of a large volume of GSH-agarose still does not fully remove all endogenous GST proteins from a tissue lysate (~80% are removed with each clearing step).

5. Sediment the beads by centrifuging the mix in a benchtop centrifuge, 5 min at 500 × *g*, 4°C.

6. Carefully pour off the supernatant into fresh, cooled tubes on ice. Keep the supernatant as "precleared tissue lysate" for addition to the GST fusion protein beads at step 10.

   Optionally, keep an aliquot (of an equivalent bead volume to that of the recombinant protein beads to be added to the lysate) of these beads to run in parallel on the gel in the final analysis. The remaining bulk GSH beads can later be recycled (see Support Protocol 4).

7. Repeat this pre-clearing twice more, if required, to remove more endogenous tissue GST proteins.

   This is especially important for tissue lysates that are rich in endogenous GSTs (particularly cytosolic extracts and lysates of specific tissues, e.g., testis or liver).

8. Pass the total precleared tissue lysate through an empty, clean plastic column—e.g., a Bio-Rad Poly-prep column of an appropriate size for the volume of lysate used: use a 10-ml column (10-cm length, 20-mm top diameter) for ≤100 ml lysate—or four layers of Miracloth.

   This will remove traces of beads that may have been missed earlier but which cannot readily be seen. It is essential to prevent even minor contamination of individual samples with even a small number of beads (as they likely have bound large amounts of endogenous GSTs). A larger column (e.g., 40-ml) will be faster than a smaller column; with larger volumes of lysate, the Miracloth is preferable.

Bind tissue lysates to the GST fusion protein beads

9. Divide the precleared tissue lysate into equal portions, depending on the number of conditions to be examined.

10. Add from 0.5 to 50 ml of lysate to 10 to 200 µl of GST fusion protein beads (from step 1) in appropriately sized tubes that can be later centrifuged. Cap the tubes and incubate 1 hr at 4°C on an end-over-end rotator (preferably in a cold room).

    The beads should not be allowed to settle.

    If concerned about adding any of the components of the bead storage buffer to the tissue lysates, then centrifuge the beads 15 to 20 sec at 1000 × g, 4°C, carefully remove the supernatant, and resuspend them in the base buffer of the lysate before adding them to the precleared tissue lysate.

Recover and wash beads

11. Centrifuge the lysate and bead mixture 5 min at 500 × *g*, 4°C, then carefully decant (or remove by vacuum aspiration) most (but not all) of the supernatant, leaving a minimal volume of lysate above the bead pellet, without disturbing the bead bed.

    IMPORTANT NOTE: *Be extremely careful not to disturb the bed of beads. From here on, it is essential to recover as much of the beads as possible, minimizing accidental losses. It is important to inspect the lid and sides of the tube before centrifugation to ensure that the beads are within the liquid. Beads that are trapped on the walls or lid (not in the liquid) are not adequately pelleted by the low-speed centrifugation and will result in bead loss. Ensure that all these beads are collected by thorough washing in step 13 below.*

12. Transfer the beads plus remaining small volume of tissue lysate to a prechilled MicroSpin column and microcentrifuge the remaining tissue lysate into a collection tube for 10 sec at 1000 × *g*, 4°C.

    Try not to centrifuge beads so fast or so long that they totally dry out, as determined by visual inspection of the beads. The beads should be visible, but should not appear to be loose and crystalline.

    Note that Figure 17.5.2 is marked with an asterisk (*) at the point equivalent to step 12, so the remaining steps can be followed diagrammatically.

13. Remove the tissue lysate in the collection tube by vacuum aspiration, replace the MicroSpin column, and add 0.5 ml of ice-cold wash buffer 1 to the tube that had originally contained the lysate. Rinse the tube well to recover any traces of beads. Transfer this wash to the MicroSpin column.

    If the lysate used above was a membrane or whole-tissue lysate, substitute wash buffer 2 for wash buffer 1 in this step and in step 15.

    The aim is to recover any residual beads from the bottom and sides of the tube and pool them with the others that are now in the MicroSpin column. This eliminates variability between the various conditions. Visually inspect the initial tube for beads; if any are present, repeat this step.

14. Spin the wash into the collection tube by microcentrifuging for 10 sec at 1000 × *g*, 4°C. Remove the flowthrough in the collection tube to waste by vacuum aspiration, then replace the column in the collection tube.

    This step is faster if the collection tubes are left in the prechilled rotor.

15. Wash the beads (now containing the target binding proteins) six times, each time by adding 0.5 ml of ice-cold wash buffer 1 (or 2, see annotation to step 13), microcentrifuging 10 sec at 1000 × *g*, 4°C, and removing flowthrough from the collection tube to waste by vacuum aspiration.

16. Wash three additional times with 0.5 ml of wash buffer 3, using the technique described in step 15.

17. For the very last wash, spin the beads dry by microcentrifuging 30 sec at 10,000 × *g*, 4°C.

Elute proteins from MicroSpin columns for SDS-PAGE analysis

18. Plug the bottom of the column thoroughly to ensure a tight seal. Add 1× SDS sample buffer to the beads.

    Use a volume of 1× SDS sample buffer appropriate for the bead volume and analysis by SDS-PAGE, e.g., for 50 µl of bead volume use 50 to 80 µl of sample buffer. Try to keep the volume to a minimum. It is essential to dilute the SDS sample buffer to a final 1× (i.e., 2% SDS) before use; do not use the concentrated SDS sample buffer found in most laboratories until it is diluted.

19. Cap the MicroSpin column with a punched cap and place in an 85°C water bath for 5 min.

    A single hole in the cap, punched with a heavy-gauge needle, is essential to prevent pressure buildup during heating, which would cause the sample to leak out the bottom. Under no circumstances should the tube be heated at 100°C, or the white plastic frit may buckle, causing leakage of sample and beads. If using columns other than the MicroSpin, ensure in advance that they will tolerate heating at 85°C.

20. After heating, dry the outside of the column with a tissue, remove the bottom plug, and centrifuge the 1× SDS sample buffer (now containing the proteins for analysis) into a fresh collection tube by microcentrifuging 30 sec at maximum speed. Store the samples on ice (if loading gels immediately) or in the freezer (if the gel will be run at a later date).

    If small volumes of 1× SDS (relative to the bead volume) are used, the elutions will likely lose some blue color. This is acceptable, as the beads retain bromophenol blue dye. Load gel using the normal technique (UNIT 6.1).

21. Analyze the samples by SDS-PAGE (*UNIT 6.1*) on a long gradient gel run overnight at 10°C.

    For optimal protein resolution (essential for analysis by mass spectrometry) the gels must be long gels (e.g., 20-cm gels; preferably gradient gels of 7.5% to 15% acrylamide), not minigels. Additionally, the gels should be 1 mm in thickness for optimal strength and ease of processing for mass spectrometry. Gels of 1.5-mm thickness are too thick for in-gel digestion (see Basic Protocol 2), and 0.75-mm gels tear far too easily. The controls suggested above (see Strategic Planning) should be run on the same gel.

22. Stain the gel by an appropriate technique suitable for mass spectrometry (see Basic Protocol 2).

Analyze results

23. Compare bands between the lanes of the gel for differential binding between the conditions used and controls. Refer to Anticipated Results for more detail.

ALTERNATE PROTOCOL 1

EFFECTOR ISOLATION WITH GST-TAGGED SMALL GTPASES

This protocol is specifically aimed at the study of nucleotide-dependent binding proteins (e.g., Ras, Rab, or Rho family GTPases). For other GST fusion proteins, such as isolated domains or enzymes, use Basic Protocol 1. Small GTPases bind guanine nucleotides (GTP and GDP) in a nucleotide-binding pocket on their surface. In so doing they undergo changes in conformation which allow them to bind to specific proteins in a GTP- or GDP-dependent manner (Takai et al., 2001). The GTP-bound form of the small GTPase is termed the active state, and GTP-dependent binding proteins are termed effectors since activation of many signaling pathways leads to increased GTP binding of the small GTPase, promoting further signaling. These proteins are particularly significant in cell biology investigations because of their role as regulators of a multitude of cellular processes. In humans there are around 150 small GTPases known to be expressed.

The main differences between this alternate protocol and Basic Protocol 1 are the presence of Mg^{2+} in the buffers (for maintenance of nucleotide binding and stability of the small GTPase) and the additional nucleotide-binding steps; there are also additional small changes in other steps. It is advisable to be familiar with the details of Basic Protocol 1 before following this protocol.

For small GTPases, it is most interesting to isolate specific binding proteins for each of the two states of activation, i.e., to load the GST–small GTPase columns respectively with GDP and GTP. Depending on the intrinsic GTP hydrolysis rate of the small GTPase to be used, it may be necessary to use a GTP analog such as GTPγS [guanosine 5′-*O*-(3-thiotriphosphate)] instead of GTP. For instance, the small GTPase Rac1 has a GTP hydrolysis rate that is 40 times higher than H-Ras (Menard et al., 1992). The authors have used GTP quite successfully for the isolation of effectors for the small GTPase RalA (Brymora et al., 2001b). This is possible because RalA has a relatively low GTPase activity (Frech et al., 1990). It may be preferable to use GTP rather than costly GTPγS for purely economical reasons, but it must be justified according to the criteria above.

Additional Materials *(also see Basic Protocol 1)*

1 M $MgCl_2$ (*APPENDIX 2A*)
Bead storage buffer (20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/50% (v/v) glycerol) containing 2.5 mM $MgCl_2$ (added from 1 M $MgCl_2$ stock; *APPENDIX 2A*)
Small GTPase loading buffer (see recipe)
100 mM GDP and GTP (or GTPγS) stock solutions (prepare fresh on the day of the experiment and keep cold):
100 mM GDP (mol. wt. 463.2) = 1 mg in 21.6 µl H_2O
100 mM GTP (mol. wt. 567.1) = 1 mg in 17.6 µl H_2O
100 mM GTPγS (mol. wt. 627.2) = 1 mg in 15.9 µl H_2O
Wash buffers 4 *or* 5, and 6 (see recipes)

Prepare GST fusion proteins and tissue lysate

1. Prepare recombinant GST-tagged small GTPase proteins attached to GSH-agarose (Support Protocols 1 and 2) with the following modifications:

   a. Purify small GTPases with 1 to 5 mM Mg^{2+} present in all buffers (added from 1 M $MgCl_2$ stock), including those used in the cross-linking steps.

   b. Omit EDTA from any buffers that come into contact with the small GTPase throughout the protocol (apart from the small GTPase loading buffer) and replace with 2.5 mM $MgCl_2$ (added from 1 M $MgCl_2$ stock).

   c. Store at −20°C in bead storage buffer (including 2.5 mM $MgCl_2$ added from 1 M $MgCl_2$ stock) for up to 4 to 6 months.

2. Prepare tissue lysate (Support Protocol 3) with addition of 2.5 mM $MgCl_2$ (added from 1 M $MgCl_2$ stock) to all buffers. Preclear with GSH-agarose (Basic Protocol 1, steps 3 to 8), if necessary.

   Ensure that the tissue lysate is free from even small traces of particulate contamination.

   If using tissue lysates prepared according to Support Protocol 3, the lysates have already been precleared with GSH-agarose; in this case proceed to nucleotide loading (step 3 of this protocol). If using a different source of lysate, the lysate must be precleared with GSH-agarose beads.

Load GST fusion protein beads with the appropriate nucleotides

If the pre-clearing steps are being performed, the beads may be loaded with nucleotides while the pre-clearing beads are being incubated (see Basic Protocol 1, step 4). The following steps are based on the protocol used by Cantor et al. (1995), which works on the principle that, in the absence of Mg^{2+}, the small GTPases bind nucleotides with low affinity and cannot hydrolyze GTP. Therefore, brief incubation in EDTA (present in the small GTPase loading buffer) promotes nucleotide exchange, and subsequent addition of an excess of Mg^{2+} loads the small GTPase with whichever guanine nucleotide is present. It is essential to load the GST–small GTPase beads with nucleotides as close as possible to the point at which they will be added to the tissue lysate.

3. Pipet the required amount of GST fusion protein beads (as a well suspended slurry) into two MicroSpin columns (or use a microcentrifuge tube if the binding steps are not going to be performed within the spin column): two equal lots for GDP versus GTP.

   It is important to use precisely equal amounts of beads for the different nucleotide loading procedures.

4. Remove the storage buffer (i.e., remove most of the Mg^{2+}) by microcentrifuging the MicroSpin column 10 sec at 1000 × *g* into a collection tube. Plug the base of the column.

5. Add an equal volume (or 50 µl, if the bed volume is <50 µl) of small GTPase loading buffer; ensure that there is an excess of EDTA present, so as to chelate all the Mg^{2+} in the protein beads (i.e., if not using MicroSpin columns, increase the added EDTA to 5 mM, for a final concentration of 2.5 mM).

6. Add the required nucleotide (GDP, GTP, or GTPγS) from a 100 mM stock, to a final concentration of 2 mM.

   GTP stocks must be prepared fresh, as GTP hydrolyzes to GDP in solution.

7. Mix the GST fusion protein beads by gently flicking the tube (avoid vigorous vortexing and try to avoid spreading the beads on the sides of the tube), then incubate at 37°C for 20 min in a water bath to induce nucleotide binding and exchange.

8. Cool the tubes on ice. After ~1 min, add 1 M $MgCl_2$ to achieve a final concentration of 10 mM Mg^{2+} (1.2 µl for a 100-µl reaction), and gently mix.

   The beads are now ready for pull-down experiments. They should be kept on ice and not left too long before use (not more than 30 min). The buffer that is currently in the beads (nucleotide and Mg^{2+}) can remain.

Bind tissue extracts to the GST fusion protein

9. Divide the precleared tissue lysate into precisely equal portions, depending on the controls included (at minimum this is three aliquots: one for GST beads and one each for GDP and GTP). Add GDP or GTP to the appropriate aliquot, from a 100 mM stock, to achieve a final concentration of 0.1 mM.

   There should already be Mg^{2+} present; if not, add a sufficient quantity of 1 M $MgCl_2$ to achieve 2.5 mM.

   The addition of nucleotides to the precleared tissue lysate will reduce competition and exchange with the preloaded nucleotides on the GST-small GTPase beads to be added in the next step.

10. Directly add the nucleotide-loaded beads to the precleared tissue lysate, including the buffer in which they are contained (anywhere from 10 to 200 µl of beads are added to 0.5 to 50 ml of lysate in suitably sized capped tubes). Incubate at 4°C for 1 hr on an end-over-end rotator (in a cold room).

Recover and wash beads

11. Centrifuge the lysate and bead mixture 5 min at 500 × *g*, 4°C, then carefully decant (or remove by vacuum aspiration) most (but not all) of the supernatant, leaving a minimal volume of lysate above the bead pellet, without disturbing the bead bed.

    IMPORTANT NOTE: *Be extremely careful not to disturb the bed of beads. From here on, it is essential to recover as much of the beads as possible, minimizing accidental losses. It is important to inspect the lid and sides of the tube before centrifugation to ensure that the beads are within the liquid. Beads that are trapped on the walls or lid (not in the liquid) are not adequately pelleted by the low-speed centrifugation, and will result in bead loss. Ensure that all these beads are collected by thorough washing in step 13 below.*

12. Transfer the beads plus remaining small volume of tissue lysate to a prechilled MicroSpin column and microcentrifuge the remaining tissue lysate into a collection tube for 10 sec at 1000 × *g*, 4°C.

    Try not to centrifuge the beads so fast or so long that they totally dry out, as determined by visual inspection of the beads. The beads should be visible but should not appear to be loose and crystalline.

    Note that Figure 17.5.2 is marked with an asterisk (*) at the point equivalent to step 12, so the remaining steps can be followed diagrammatically.

13. Remove the tissue lysate in the collection tube by vacuum aspiration, replace the MicroSpin column, and add 0.5 ml ice-cold wash buffer 4, to the tube that had originally contained the lysate. Rinse the tube well to recover any traces of beads. Transfer this volume to the MicroSpin column.

    If membrane or whole tissue lysates were used above, substitute wash buffer 5 in this step and step 15.

    The aim is to recover any residual beads and pool them with the others that are now in the MicroSpin column. This eliminates variability between the samples (particularly between GDP- and GTP-loaded samples). Visually inspect the initial tube for beads; if any are present, repeat this step.

14. Spin the wash into the collection tube by microcentrifuging for 10 sec at 1000 × *g*, 4°C. Remove the flowthrough in the collection tube to waste by vacuum aspiration, then replace the column in the collection tube.

    This step is faster if the collection tubes are left in the prechilled rotor.

15. Wash the beads (now containing the target binding proteins) six times, each time by adding 0.5 ml of ice-cold wash buffer 4 (or 5, see annotation to step 13), microcentrifuging 10 sec at 1,000 × *g*, 4°C, and removing the flowthrough from the collection tube to waste by vacuum aspiration.

16. Wash three additional times with 0.5 ml of ice-cold wash buffer 6, using the technique described in step 15.

17. For the very last wash, spin the beads dry by microcentrifuging 30 sec at 10,000 × *g*, 4°C.

Elute proteins from MicroSpin columns for SDS-PAGE analysis

18. Plug the bottom of the column thoroughly to ensure a tight seal. Add 1× SDS sample buffer to the beads.

    Use a volume of 1× SDS sample buffer appropriate for the bead volume and analysis by SDS-PAGE, e.g., for 50 µl of bead volume use 50 to 80 µl of sample buffer. Try to keep the volume to a minimum. It is essential to dilute the SDS sample buffer to a final 1× (i.e., 2% SDS) before use; do not use the concentrated SDS sample buffer found in most laboratories until it is diluted.

19. Cap the MicroSpin column with a punched cap and place in an 85°C water bath for 5 min.

    A single hole in the cap, punched with a heavy-gauge needle, is essential. to prevent pressure buildup during heating, which would cause the sample to leak out the bottom. Under no circumstances should the tube be heated at 100°C, or the white plastic frit may buckle, causing leakage of sample and beads. If using columns other than the MicroSpin, ensure in advance that they will tolerate heating at 85°C.

20. After heating, dry the outside of the columns with a tissue, remove the bottom plug, and centrifuge the 1× SDS sample buffer (now containing the proteins for analysis) into a fresh collection tube by microcentrifuging 30 sec at maximum speed. Store the samples on ice (if loading gels immediately) or in the freezer (if the gel will be run at a later date).

    If small volumes of 1× SDS (relative to the bead volume) are used, the elutions will likely lose some blue color. This is acceptable, as the beads retain bromophenol blue dye. Load gel using the normal technique.

21. Analyze the samples by SDS-PAGE (*UNIT 6.1*) on a long-gradient gel run overnight at 10°C.

    For optimal protein resolution (essential for analysis by mass spectrometry) the gels must be long gels (e.g., 20-cm gels; preferably gradient gels of 7.5% to 15% acrylamide), not minigels. Additionally, the gels should be 1 mm in thickness for optimal strength and ease of processing for mass spectrometry. Gels of 1.5-mm thickness are too thick for in-gel digestion (see Basic Protocol 2), and 0.75-mm gels tear far too easily. The controls suggested above (see Strategic Planning) should be run on the same gel.

22. Stain the gel by an appropriate technique suitable for mass spectrometry (see Basic Protocol 2).

Analyze results

23. Compare the protein stain in both lanes (GDP-loaded versus GTP-loaded) to find the proteins that bind in a nucleotide-dependent manner, and which do not bind to the various controls. See Anticipated Results for more details.

SUPPORT PROTOCOL 1

GST FUSION PROTEIN PURIFICATION

This procedure describes a technique for expression and purification of GST fusion proteins. There are many different protocols available for purification of GST fusion proteins, and the one provided here is a fairly standard protocol that works well for many GST fusion proteins. It is not, however, universally applicable, and modifications may be required to increase yield of functional product. Such modifications are many and varied (there are almost as many different protocols as there are GST fusion proteins), and as such they will not be discussed in this unit, since there are many other suitable references available that specifically cover such optimization procedures (see, e.g., Harper and Speicher, 1997). Some of these are provided by the manufacturers of expression vectors or GSH-agarose beads (e.g., see The Recombinant Protein Handbook form Amersham Biosciences).

Materials

Glycerol stock of *E. coli* cells containing GST fusion protein expression vector (see *APPENDIX 3A* and Coligan et al., 2003)
LB medium and LB agar plates (see *APPENDIX 2A*) containing 100 μg/ml ampicillin (or other appropriate antibiotic selection for GST expression vector used)
100 mM isopropyl-1-thio-β-D-galactoside (IPTG), filter sterilized
Bleach (e.g., Clorox)
Bacterial lysis buffer: 20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/250 mM NaCl
100 mg/ml lysozyme
100 mM PMSF (*APPENDIX 2A*)
20 mg/ml leupeptin
Liquid nitrogen and appropriate storage canister
1 mg/ml DNase I
10% (v/v) Triton X-100 (*APPENDIX 2A*)
Bacterial lysis buffer (see above) containing 1% (v/v) Triton X-100
1% (w/v) SDS
Wash buffer 1 (see recipe)
Bead storage buffer: 20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/50% (v/v) glycerol
1× and 3× SDS sample buffer (*UNIT 6.1*)
Bovine serum albumin (BSA; optional)

Shaking bacterial incubator
Culture tubes, sterile
1-liter conical flasks (Pyrex or autoclavable plastic), sterilized by autoclaving or baking
Bugstoppers (Whatman), sterilized by autoclaving, *or* other sterile, gas-permeable flask closures
Probe sonicator (e.g., Branson)
Refrigerated high-speed centrifuge and benchtop centrifuge
15-ml and 50-ml polypropylene screw-cap tubes (e.g., Falcon)
Pipet tip cut to increase opening to >1 mm
85°C water bath
MicroSpin columns (Amersham Biosciences)

Additional reagents and equipment for growing bacterial cells (see *APPENDIX 3A*), SDS-PAGE (*UNIT 6.1*), Coomassie blue staining of gels (*UNIT 6.6*)

Culture bacterial cells and induce protein expression

1. *Day 1 (evening):* Streak out glycerol stock of an *E. coli* strain containing GST fusion protein expression vector on an LB agar plate containing 100 µg/ml ampicillin (or other appropriate antibiotic selection for GST-expression vector), and grow overnight (~16 hr) at 37°C.

   See APPENDIX 3A for references on growing bacterial cells.

2. *Day 2 (morning):* Remove plates from incubator and store inverted at 4°C for up to a month.

3. *Day 2 (evening):* Inoculate an appropriate volume of LB medium containing 100 µg/ml ampicillin (or other appropriate antibiotic) with a single colony from the LB agar plate. Incubate overnight at 37°C with shaking at 200 to 250 rpm.

   Typically, a 1:50 to 1:100 dilution into 0.5 to 2 liters will be required the next day, so ensure that sufficient overnight culture is inoculated.

4. *Day 3 (morning):* Dilute 7.5 ml of the overnight culture into 500 ml of LB medium containing 100 µg/ml ampicillin (or other appropriate antibiotic) in a 1-liter conical flask, and cap flask with an autoclaved Bugstopper (or another suitable gas-permeable closure). Incubate at 37°C with shaking at 200 to 250 rpm for 3 to 4 hr. Measure the optical density (OD) at 600 nm against LB medium (or water) as a blank.

   Induce the bacteria (step 5) when the OD is ~0.8 to 1.2 (up to 2).

5. Remove a 1-ml aliquot of the OD 0.8 to 1.2 bacterial culture to a sterile 1.5-ml microcentrifuge tube and grow in parallel with the remainder of the culture (this is the noninduced control sample, "–IPTG"). To the remainder of the culture, add 100 mM IPTG for a final concentration of 0.1 mM to induce protein expression, and incubate 2 to 6 hr at 25° to 30°C.

   The optimal induction temperature, OD, and duration will vary depending on the fusion protein of interest and may need to be determined systematically on a small scale (e.g., in 5- to 10-ml cultures) prior to large-scale protein production. Lower-temperature induction and shorter induction times at higher OD may increase the yield of folded, soluble protein (also see Coligan et al., 2003).

6. At the end of the incubation, place a 1-ml aliquot of the culture in a microcentrifuge tube (this is the induced sample, +IPTG). Centrifuge the bulk culture for 20 min at 18,000 × *g* (or maximum speed in a high-speed centrifuge), room temperature. Add bleach for a concentration of 2% to 4% (v/v) to the supernatant in the flasks, let stand for a minimum of 2 hr, and discard.

   Some plastic flasks may not be tolerant to bleach; these should be washed well in detergent and disinfected by autoclaving. Do not bleach Bugstoppers, or soak them in detergent; instead, autoclave them.

7. Resuspend bacterial pellet in a volume of bacterial lysis buffer equal to 5% of the total induced culture volume (i.e., 50 ml per liter of culture), and freeze this bacterial suspension at −20°C overnight or until purification. Microcentrifuge the 1-ml −IPTG and +1PTG control samples at maximum speed, carefully remove and discard the supernatant, and freeze these control pellets at −20°C.

   The samples can be stored in this way for up to 6 months.

Purify GST fusion proteins

8. Thaw the bacterial suspension (use cool water if necessary, but do not allow the samples to warm above ~4°C). Once thawed, add a final concentration of 1 mg/ml lysozyme (from 100 mg/ml stock), 1 mM PMSF (from 100 mM stock), and 20 µg/ml leupeptin (from 20 mg/ml stock), and vortex well. Incubate on ice for ~5 min.

9. Pass the bacterial suspension through two freeze/thaw cycles by sequential dropping of the tubes into liquid nitrogen for about 20 to 30 sec (or longer on dry ice if liquid nitrogen is not available), removing with tongs, and thawing in cool water (again ensuring that the samples do not warm much beyond 4°C).

   CAUTION: *When handling liquid nitrogen, ensure adequate eye protection and ventilation. Also, ensure that the tubes can withstand liquid nitrogen without shattering before dropping in any samples. There may be a slight color change (creamy white to a slightly more yellow/brown color) and a viscosity change after the freeze/thaw cycles, indicating that lysis has occurred.*

10. Sonicate on ice for 30 sec with a probe sonicator to further rupture the cells and break up any DNA.

    Do not oversonicate, as this may denature the proteins. This step can be omitted if protein degradation or denaturation occurs.

11. Add 1 mg/ml DNase I stock to a final concentration of 5 µg/ml and 10% Triton X-100 to a final concentration of 1% (v/v). Incubate at 4°C (in a cold room), with slow rotation on an end-over-end rotator for 30 min.

12. Centrifuge samples in a high-speed centrifuge 30 min at 18,000 × *g*, 4°C.

13. While centrifugation is in progress, pipet an appropriate quantity of well-suspended GSH-agarose slurry (as supplied by the manufacturer) into a 50-ml polypropylene screw-cap tube using a pipet tip that has been cut to increase the opening to >1 mm.

    How much of the GSH-agarose beads should be used? The manufacturers of GSH-agarose usually evaluate each batch of beads for binding capacity (and this is usually around 8 mg/ml for GST). Some prior estimation of protein expression level is useful, and an initial small-scale purification can be useful to assess this. For 0.5 to 1 liter of bacterial culture, a 0.5- to 1-ml bed volume of GSH-agarose yields a relatively high incorporation of protein to the beads (~1 to 2 mg/ml bed volume or greater). Some highly expressing soluble proteins may require even more GSH-agarose (e.g., the GST that is used for controls).

14. Centrifuge in a benchtop centrifuge 5 min at 500 × *g*, then remove and discard the supernatant (preferably by vacuum aspiration), being extremely careful not to lose any beads. Add 10 bed volumes of bacterial lysis buffer including 1% Triton X-100, resuspend the beads well and centrifuge 5 min at 500 × *g*, then carefully remove the supernatant and discard.

    Do not centrifuge beads much faster than 2000 × g at any point, or the beads may be crushed. Crushed beads are harder to isolate and bind more nonspecific proteins.

15. When the centrifugation of the bacterial lysate (from step 12) is finished, mark the level of the liquid in the centrifuge tube with a marker (the soluble bacterial fraction is the supernatant). Take a 20-µl sample (for gel; label as soluble), then decant the rest of the supernatant into the 50-ml tube containing the beads (step 14). Incubate at 4°C for 1 hr with rotation on an end-over-end rotator (preferably in a cold room) to bind the GST fusion proteins to the beads.

    The beads should remain suspended at all times during incubation.

16. While the beads are binding, resuspend the pellet from the centrifugation of the bacterial lysate in a volume of 1% SDS solution equivalent to that indicated by the mark on the centrifuge tube. When it is relatively homogenous, take a 20-µl sample (for the gel; label as pellet) and discard the remainder.

    Considerable agitation (vortexing and trituration) will be required to disrupt the pellet. It will likely not fully solubilize, the aim is to homogenize and reduce the particle size. Full solubilization is not required, as it is intended as a control on SDS-PAGE—the heating and additional SDS added will make it homogenous enough for the gel.

17. After the binding step (step 15) is complete, centrifuge the 50-ml tube 5 min at 500 × *g*, 4°C, to pellet the beads. Carefully decant the supernatant from the beads, taking care not to lose any beads. Ideally, leave a small volume of lysate behind at the end to avoid disturbing the bead bed. Take a 20-µl sample of the supernatant (for the gel; label as through).

18. Add 10 to 20 bed volumes of wash buffer 1 to the beads, then centrifuge 5 min at 500 × *g*, 4°C, and carefully remove the supernatant by decanting or vacuum aspiration. Repeat this wash three to four times, and in the process carefully transfer all of the beads to a 15-ml tube for storage, ensuring that all beads are transferred.

    One of the best tubes for storage of beads are 15-ml Falcon 2059 polypropylene tubes, as the beads do not adhere to the walls and resuspend easily from the round base.

19. Wash the beads once by resuspending them in 5 bed volumes of bead storage buffer, centrifuging 5 min at 1000 × *g*, 4°C, and removing the supernatant.

20. Add 2 bed volumes of bead storage buffer to the bead volume to make a 33% (v/v) slurry, and store at −20°C.

    The beads should never be allowed to freeze, which will cause the beads to crack. The presence of 50% glycerol in the bead storage buffer is essential to prevent this.

21. Perform SDS-PAGE (*UNIT 6.1*) and stain with Coomassie blue (*UNIT 6.6*) to analyze purification and quantitate amount of protein bound to GSH beads. Prepare samples and lay out gel as follows:

    a. *Molecular weight standards:* Serially dilute to provide a reference for estimation of protein on beads after gel is stained (see step 22). Alternatively, load four lanes with 2, 5, 10, and 20 µg BSA as a standard.

    b. *−IPTG and +IPTG induction controls:* Resuspend control pellets (step 7) in 80 µl of 1× SDS sample buffer, heat to 85°C for 5 min, and microcentrifuge 1 min at maximum speed. Load 10 µl of the supernatant on the gel.

    c. *Soluble, pellet, and through samples:* Add 10 µl of 3× SDS sample buffer to the 20 µl of sample, vortex, and heat to 85°C for 5 min. Load 10 µl on the gel.

    d. *Beads:* Using a cut-off pipet tip with an opening 1 to 2 mm in diameter, pipet 5, 10, or 20 µl (or all three to quantitate more accurately) of the slurry prepared in step 20 (ensuring the beads are well suspended by swirling or vortexing gently for ~10 sec) into a fresh MicroSpin column. Place in a collection tube and microcentrifuge 30 sec at 10,000 × *g* to remove bead storage buffer. Cap the bottom of the column well, add 30 µl of 1× SDS sample buffer, and cover with a punched cap. Heat at 85°C in a water bath for 5 to 10 min. Dry the outside of the column, and place in a fresh microcentrifuge tube. Microcentrifuge 30 sec at maximum speed to collect sample buffer and eluted protein. Load the entire volume on the gel.

Normalize the protein between different GST fusion proteins

Prior to performing the pull-down experiment (in Basic Protocol 1 or the Alternate Protocol) or the cross-linking (Support Protocol 2) it is necessary to approximate the amount of protein attached to the beads (milligram protein per milliliter beads).

22. Analyze the gel bands from 5, 10, and 20 µl of bead slurry on the gel (see step 21) by visual (or densitometric) comparison with the serially diluted markers to estimate the protein concentration on the beads.

    This is important even for a single GST fusion protein, but it is essential for multiple GST fusion proteins. When comparing a number of different GST fusion proteins in the same pull-down experiment, the same amount of protein must be used for each condition on the same amount of beads (see Basic Protocol 1).

23. Before performing pull-down experiments (Basic Protocol 1 or the Alternate Protocol) or cross-linking (Support Protocol 2), dilute each of the batches of beads to be used in the experiment with GSH beads that have been freshly washed as in step 13, to adjust them all to about the same protein concentration per milliliter of beads. If desired, confirm equal protein concentration by loading an equal small volume of all beads on SDS-PAGE (*UNIT 6.1*).

    The target is around 1 mg/ml, however the range can be rather large. A protein concentration of 0.05 mg/ml (5 µg on 100 µl beads) is about the minimum, while there is probably no maximum concentration; once the bed volume added to a pull-down becomes too small, it can become difficult to recover the beads from a large volume (unless they never leave the column, such as in the spin column–based pull-down experiment in Figure 17.5.2).

SUPPORT PROTOCOL 2

CROSS-LINKING GST FUSION PROTEINS TO GSH-AGAROSE

Perform this protocol only if the intention is to covalently couple a GST fusion protein to GSH beads. Cross-linking has a number of benefits and caveats, and these are discussed in Strategic Planning. The main chemical cross-linking agents used for this purpose are disuccinimidyl suberate (DSS) and bis(sulfosuccinimidyl) suberate (BS3). DSS is a water-insoluble, homobifunctional *N*-hydroxysuccinimide ester (NHS-ester), and BS3 is its water-soluble analog (both are available from Pierce). The main targets for NHS esters are primary amines. For proteins, cross-linking usually involves reaction with the side chains of amino acids. Only the ε-amine of lysine reacts significantly with NHS esters, as the N-terminal α-amine groups on a protein are not available for cross-linking. A covalent amide bond is formed when the NHS ester reacts with the primary amine, releasing N-hydroxysuccinimide. When cross-linking GST fusion proteins to GSH beads, the reaction must be between the primary amine of GSH (the α-amine of the γ-glutamyl residue is the only one available) and lysine residues in either recombinant GST or the attached fusion protein. Due to the length of the DSS molecule (11.4 Å), the former seems more likely, though the latter is not impossible. Due to reactivity with amines, cross-linking reactions should be performed in non-amine buffers such as phosphate, carbonate/bicarbonate, HEPES, or borate buffers to maximize coupling efficiency. Other buffers can also be used provided they do not contain primary amines; Tris buffers contain primary amines and must be avoided. A large excess of Tris at neutral-to-basic pH is added at the end of the reaction to quench it.

Since DSS is insoluble in aqueous solvent, it is first dissolved in an organic solvent (e.g. DMSO), then added to the aqueous reaction mixture. Cross-linkers fall out of aqueous solutions at the concentrations used for cross-linking, and a milky, turbid solution can be observed. Cross-linking will still proceed effectively. Storage of stock solutions for long periods (hours) is not recommended for either class of compounds, because NHS esters hydrolyze rapidly. The solvent DMSO is hygroscopic and absorbs water, which will

promote hydrolysis. BS3 is a more efficient cross-linker than DSS, primarily because it is not insoluble in aqueous solution; however, it is considerably more expensive.

This procedure is designed for the coupling of an ~0.3- to 1.0-ml bed volume of GST fusion protein beads.

Materials

GST fusion protein beads (prepared as in Support Protocol 1, with the protein concentration determined)
PBS (*APPENDIX 2A*)
Disuccinimidyl suberate (DSS), 13.2 mg in screw-cap tube (see recipe)
bis(sulfosuccinimidyl) suberate (BS3; see recipe)
Dimethylsulfoxide (DMSO; H_2O-free, i.e., fresh)
Quenching buffer: 1 M Tris·Cl, pH 8.2 (*APPENDIX 2A*), room temperature
Glutathione wash buffer (see recipe), room temperature
Wash buffer 1 (see recipe), ice-cold
Cross-linking wash buffer: 25 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)/1.5 M NaCl, ice-cold
Bead storage buffer: 20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/50% (v/v) glycerol
Appropriately sized columns (e.g., Bio-Rad 2-ml Bio-Spin columns or 10-ml Poly-Prep columns)

Cross-link beads

1. Pipet the required volume of GST fusion protein beads to be cross-linked into a suitably sized plastic column (which can also be capped at both ends, e.g., Bio-Rad Bio-Spin 2-ml column), and allow to drain. Keep a 10-µl bed volume aliquot of starting beads for SDS-PAGE analysis of cross-linking efficiency at the end of the protocol (step 14).

   If the beads have been stored in 50% glycerol (i.e., bead storage buffer in Support Protocol 1), the column will not drain, due to the viscosity. It can be assisted by the use of a vacuum line or gentle centrifugation into a collection tube (5 min at 500 to 1000 × g). Make sure that the beads do not dry out.

2. Wash the column with four 2-ml washes of PBS. Let all the solution drain out.

   This is required to thoroughly remove any traces of amines, such as Tris.

3. Plug the bottom of the column and add 0.5 ml PBS. Flick gently to resuspend the beads.

4. Dissolve 13.2 mg of DSS in 1 ml of dry DMSO to make a 35.8 mM solution. Dilute this 1 ml of DSS solution into a clean tube containing 1.5 ml of PBS at room temperature.

   The DSS solution must be made fresh, immediately before use; do not store this reagent.

   The 35.8 mM DSS stock solution is a good place to start in most cases, but it may not always be ideal. Generally, if the exact amount of protein is known, a 20- to 50-fold molar excess of the cross-linker is appropriate. The final cross-linker concentration in the reaction should be between 0.25 and 5 mM.

   BS3 is added to a cross-linking reaction at the same molar concentration as DSS. Due to its aqueous solubility, it can be added directly to a protein solution. If desired, a 35.8 mM stock solution can be prepared by dissolving 20.5 mg of BS3, immediately before use, in 1 ml of 5 mM sodium citrate, buffer pH 5.0. This stock solution should be added in a dropwise fashion to the reaction.

5. Immediately add the diluted DSS solution to the prepared GST fusion protein bound to the column (step 3), at room temperature. Plug both ends of the column and suspend the beads by repeated gentle inversions and by tapping the gel-bed end of the column (alternatively, gently mix with a fine spatula). Incubate 45 min at room temperature, with continuous mixing or inversion.

   When DSS is added to the aqueous buffer it may become very cloudy. This is normal, and cross-linking will still proceed.

6. After the reaction is complete, remove the top cap first, then the bottom cap. Place the column inside a collection tube. Drain the column, but do not allow the beads to run completely dry, then plug the bottom.

Block amine-reactive sites on beads

7. Add 2 ml quenching buffer to the column. Mix it in and gently invert the column for 10 min at room temperature.

   This blocks all amine-reactive sites that may still be present after the cross-linking reaction, preventing any other proteins from being covalently coupled to the beads.

8. Drain the column, but do not allow the gel to run completely dry, then plug the bottom.

9. Wash the column four times, each time by adding 3 ml freshly prepared glutathione wash buffer at room temperature, and allowing it to pass through. Keep an aliquot of the eluate to run on a gel later (step 14).

   This step removes GST fusion protein that is not covalently coupled to the glutathione beads. Keeping the eluate allows the assessment of any eluted GST fusion protein, and thus gives a crude index of the coupling efficiency.

Wash beads

Switch back to 4°C for the washing steps.

10. Wash the column by adding 4 ml of ice-cold wash buffer 1 and allowing it to pass through.

    The column can be on the laboratory bench (at room temperature) for the washing, but keep the buffers ice cold.

11. Wash the column twice, each time with 4 ml of ice-cold cross-linking wash buffer.

12. Wash the column twice, each time with 4 ml of ice-cold wash buffer 1.

13. Add twice the bed volume of bead storage buffer, resuspend the beads by gently flicking the column, and store capped at −20°C up to 4 to 6 months.

14. Analyze 10 µl of starting beads, 20 µl of the GSH elutions (step 9), and 10 µl of the final cross-linked beads on an SDS-PAGE mini-gel.

    Ideally, there should be no or little protein on the gel from the GSH elutions, and no protein from the final cross-linked beads. Practically, there are often faint bands in the elutions and occasionally a trace in the final beads. This level of protein can still be acceptable for use in pull-down experiments, depending on the initial reason for performing the cross-linking. Greater levels of protein in either the elutions or the final beads indicates that the reaction was inefficient and probably insufficient for use.

PREPARATION OF LARGE-SCALE TISSUE LYSATES

This method is for preparation of two liters of sheep brain cytosol, peripheral membrane, and integral membrane lysates for use with GST fusion–protein pull-down experiments. Alternatively, a whole-tissue lysate (Triton X-100 extract) can be prepared. The samples are prepared and frozen as fifty 40-ml aliquots in 50-ml screw-cap tubes, ready to thaw, centrifuge, and then use immediately. The aliquots provide a rapid source of protein that can be stored in the freezer until required. Lysates are stored at −20°C for up to 6 months and could be stored longer at −70°C. Generally, it is best to use the lysates when they are as fresh as possible. For other tissues, this method may need to be adapted or scaled up or down. However the protocol below gives a good indication of the optimal tissue-derived protein amounts required for identification of the major proteins that bind to a particular GST fusion protein. Using less tissue will work, but this amount is optimal for obtaining the largest number of interacting proteins.

Materials

200 g sheep brain (see recipe)
Buffers A, B, C, and D (see recipes; prepare in advance and cool overnight to 4°C)
GSH-agarose beads (e.g., glutathione-Sepharose 4B; Amersham Biosciences)
10% (v/v) Triton X-100

Colander or tea strainer
1-liter plastic beakers
Ultra-Turrax tissue homogenizer (IKA Labortechnik) with 25-mm-diameter blade/tip
Refrigerated centrifuge and 0.5-liter centrifuge bottles
Miracloth (Calbiochem)
50-ml screw-cap plastic tubes

NOTE: Sometimes precipitates accumulate during freeze/thaw of these samples, and this is particularly true for the peripheral membrane extract (see note above). All frozen aliquots must be centrifuged after defrosting at 35,000 × *g* (or maximum speed) for 30 min at 4°C, before incubation with recombinant GST fusion protein beads. It is essential to clear any debris before adding the extract to the beads. It may also be useful to pre-clear the cytosol extracts a third time before use, to remove any remaining traces of endogenous GST (this is optional; see Basic Protocol 1 or the Alternate Protocol). All volumes of beads in this protocol refer to bed volumes.

Extract cytosolic protein

1. Defrost 200 g frozen sheep brain by placing the pieces in 1 liter of buffer A that is being stirred at 10°C**,** (add the brains to the buffer, rather than vice versa) and leaving them until they start to sink (after ~7 min). Strain the brains with a colander or tea strainer and place them in 2 liters of ice-cold buffer B.

   Sheep brain can be replaced with rat brain (one rat brain weights ~1.85 g; 40 rats = 74 g), but at considerable expense.

   Alternatively, for a whole-tissue lysate, add 0.5 liter buffer D (instead of buffer B) and skip directly to step 17. Make sure the homogenate is free of particulate matter by an additional centrifugation in step 17, followed by filtration through Miracloth, before proceeding to step 18.

2. Homogenize brains in four 500-ml lots of buffer B (in 1-liter plastic beakers) with an Ultra-Turrax (i.e., an homogenizer with sharp blades, not a Teflon/glass homogenizer), using a 25-mm diameter blade/tip and a speed setting of 19,000 rpm.

   This should be performed in 1-liter plastic beakers (to minimize risk of breaking glass).

3. Centrifuge homogenate in 0.5-liter centrifuge bottles 30 min at 18,000 × *g*, 4°C.

   If the available centrifuge cannot manage large volumes at 18,000 × g, then use the maximum speed wherever this is indicated in this protocol.

4. Collect the supernatant and filter through Miracloth to remove floating particulate matter.

   Keep the pellet on ice for step 10.

5. Centrifuge the filtrate again as in step 3 and filter through fresh Miracloth again.

6. Wash 8 ml of GSH-agarose beads in a 50-ml screw-cap tube by centrifuging 5 min at 500 × *g*, 4°C, and carefully remove the supernatant. Resuspend the pellet in 50 ml of buffer B and centrifuge again 5 min at 500 × *g*, 4°C. Discard the supernatant, and pre-clear the cytosol extract by incubating 20 min with 2 ml of the prewashed beads at 4°C with continuous stirring. Filter the pre-cleared cytosol extract through clean Miracloth.

   Do not use a stir bar to stir the extract with beads, as the beads may be crushed and will then pass through the Miracloth.

7. Repeat the pre-clearing step with an additional 2 ml of fresh GSH-agarose beads.

   The multiple pre-clearing steps are performed to remove the enormous amounts of endogenous GST protein from the brain (particularly the brain cytosol), which would interfere with the subsequent pull-down experiments.

8. Filter the precleared cytosolic protein extract through clean Miracloth to collect the GSH-beads.

   Pool the GSH beads with the previous volume for later recycling (see Support Protocol 4).

9. Divide the cytosol into fifty 40-ml aliquots in 50-ml plastic tubes with screw-cap lids, freeze on dry ice, and store up to 6 months at −20°C.

Extract peripheral membrane protein

10. *Wash 1:* Rehomogenize the pellet from step 4 in 2 liters of buffer B (see step 2) and centrifuge (see step 3).

    This is simplified by leaving the homogenate in the same centrifuge bottle and rehomogenizing within that bottle.

11. *Wash 2:* Remove and discard the supernatant, then repeat the homogenization and centrifugation.

12. Remove and discard the supernatant. Rehomogenize the pellet in 500 ml of buffer C using the technique described in step 2. Stir in a beaker in the cold room for 20 min, then centrifuge 30 min at 35,000 × *g*, 4°C. Remove the supernatant and centrifuge it again for 30 min at 35,000 × *g*, 4°C.

13. Collect the supernatant as peripheral membrane extract and dilute to 2 liters with buffer B. Add 10% Triton X-100 solution to a final concentration of 0.1%.

    Keep the pellet for step 16.

    Since no extra salt is added beyond that in the buffer C that was added in step 12, the final NaCl concentration is reduced to 125 mM, which is more appropriate for pull-downs than 0.5 M. The addition of Triton X-100 is required to prevent the formation of excessive protein precipitates that occur with this peripheral membrane extract during pre-clearing steps and during pull-down experiment incubations. This precipitation only occurs with this extract and seems to be triggered by freeze/thawing and incubation with beads. It is not removed by prior centrifugation, but it is prevented by addition of trace amounts of Triton X-100 (0.1% v/v final concentration). It is worth keeping in mind that the presence of this detergent may interfere with some protein-protein interactions.

14. *Pre-clearing:* Incubate the peripheral membrane extract with 2 ml prewashed (step 6) GSH-agarose beads for 20 min at 4°C with continuous stirring. Filter the precleared peripheral membrane protein extract through Miracloth.

    For the peripheral membrane, it is not necessary to repeat the pre-clearing, since there is much less endogenous GST in this fraction than there is in the cytosol.

    Pool the GSH beads with the others (from steps 6 to 8) for recycling.

15. Divide the peripheral membrane extract into fifty 40-ml aliquots in 50-ml tubes with screw-cap lids, freeze on dry ice, and store at −20°C for up to 6 months.

Extract membrane protein

16. Wash the pellet from step 13 once with 1 liter buffer C by rehomogenizing (as in step 2) and centrifuging as in step 3. Discard the supernatant.

17. Rehomogenize the pellet in 500 ml of buffer D using the technique described in step 2. Stir in the cold room for 20 min in a beaker, then centrifuge 30 min at 35,000 × *g*, 4°C.

18. Collect the supernatant as membrane extract and immediately dilute to 2 liters with buffer B, to match the volume of the cytosol. Discard the pellet.

    Since no extra Triton X-100 is added beyond that present in the buffer D, the Triton X-100 is diluted to 0.25%.

19. *Pre-clearing:* Incubate the membrane extract with 2 ml pre-washed (step 6) GSH-agarose beads for 20 min at 4°C (keep stirring). Filter the precleared peripheral membrane protein extract through clean Miracloth to collect the beads.

    For the membrane, it is not necessary to repeat the pre-clearing, since there is much less endogenous GST in this fraction than there is in the cytosol.

    Pool the GSH beads with those from the other extracts, and recycle them for reuse as described in Support Protocol 4.

20. Divide the membrane extract into fifty 40-ml aliquots in 50-ml tubes with screw-cap lids, freeze on dry ice, and store up to 6 months at −20°C.

SAMPLE PREPARATION FOR MASS SPECTROMETRY

BASIC PROTOCOL 2

Enzymatic in-gel digestion of SDS-PAGE-separated proteins followed by mass spectrometry (MS) analysis and database searching is now a common approach for identifying unknown proteins from a semipurified system such as a pull-down experiment. The following is a step-by-step protocol to take protein samples from an unstained SDS-PAGE gel through proteolytic digestion to a peptide mixture ready for analysis by MS. First, an SDS-PAGE gel, prepared and run in Basic Protocol 1 or the Alternate Protocol, is stained and analyzed for protein bands that bind specifically to the target protein (see Anticipated Results). The bands of interest are excised and completely destained to remove any stain that might interfere with subsequent proteolysis and MS analysis. Treatment with protease (usually trypsin) digests the protein within the gel piece, and the resulting peptides are extracted, concentrated, and analyzed by MS. Techniques for performing MS analysis are not described here, as they are beyond the scope of this unit (some techniques are described in *UNIT 5.6;* see Chapter 16 in Coligan et al., 2003, for a full treatment). It is envisaged that many researchers would utilize a service (institutional or commercial) for their MS analysis; therefore, suitable methods for shipping SDS acrylamide gels and gel pieces are outlined.

Two different MS-compatible options are presented for staining of SDS acrylamide gels: colloidal Coomassie blue and zinc-imidazole stain. Either staining technique is particularly suitable for routine use in this protocol. There are other MS-compatible stains not described in this protocol. One of these is the silver stain, a highly sensitive stain that enables detection of low nanogram quantities of protein. One must ensure that the chosen silver stain protocol does not contain a glutaraldehyde fixation step, as this interferes heavily with MS analyzes. The most widely used compatible silver stain is that described by Shevchenko et al. (1996). It is important to convert the silver in the excised gel piece to a soluble form and remove it from the gel piece completely for MS analysis. Another staining method utilizes the fluorescent dye SYPRO Ruby, which is highly suitable for MS as it requires no destaining (Berggren et al., 2002; *UNIT 6.6*). This has the disadvantage that it requires specialized imaging equipment to observe the stained bands, but it is a very simple technique that is highly sensitive (comparable with silver stain).

The colloidal Coomassie blue stain (Neuhoff et al., 1988) is a rapid gel staining method that is slightly superior in sensitivity and reliability of quantitation when compared with traditional noncolloidal Coomassie brilliant blue stain (which is usually dissolved in acetic acid/methanol or trichloroacetic acid). The colloidal stain is faster than other stains since there is no need for strong destaining steps. In fact, destaining of the whole gel is not required at all, though it does help to better visualize the lower-intensity protein bands. Destaining is performed only briefly in high-quality water, thus avoiding acids and improving protein identification by mass spectrometric techniques. Acidic residues of proteins can become methylated when SDS-PAGE gels are kept for prolonged periods in acetic acid, making it harder to identify the protein. Additionally, colloidal Coomassie can be easily destained from a small gel piece. Coomassie blue has a distinct appearance in mass spectra, a variable peak of m/z ~800 to 850 Da (often 804 or 832 Da). If this is a strong peak in the spectra, it can significantly interfere with detection of weaker samples, particularly in MALDI-TOF MS. Additionally, the presence of Coomassie in a gel piece can inhibit trypsin's proteolytic activity, so that digestion may not proceed efficiently. Therefore, effective destaining of a Coomassie-stained gel piece is essential for analysis by MS.

The zinc-imidazole negative staining method is fast, simple, sensitive, and highly compatible with MS (Castellanos-Serra and Hardy, 2001). It is also gentle, as there are no acids, dyes or chemical modifiers. The sensitivity of the method (>10 ng protein) is better than Coomassie blue staining (>100 ng protein) and approaches that of silver staining (1 to 10 ng protein/band). Therefore, this negative stain often reveals bands not stained with Coomassie (Fernandez-Patron et al., 1995). It is also completely reversible. The stain is a negative, or reverse stain: it relies on precipitation of a zinc salt in the part of the gel matrix that is not occupied by proteins. Imidazole reacts with Zn^{2+} to produce insoluble zinc imidazolate, which is a deep white. Proteins inhibit the formation of zinc imidazolate and therefore appear clear. Protein bands are visualized by placing the gel above a dark surface, e.g., black paper or plastic. Negative staining techniques do not offer as much contrast as a positively stained gel, and they are therefore not ideal for quantification purposes. Also, it should be noted that the zinc-imidazole stain is not retained when the gel is dried. On the positive side are the great speed of this method and the fact that no stain is attached to protein, which avoids unwanted chemical modifications. Finally, one should note that not all staining methods will detect all proteins, and some may be missed by any of these approaches.

It is also possible to excise and in-gel digest bands from dried gels for analysis by MS (but not from zinc-imidazole negatively stained gels). However, a small reduction in peptide recovery after in-gel tryptic digestion is observed when compared with wet gels

that have not been dried. Therefore, it is recommended that wet gels be used as a first preference for the procedures described in this protocol. If dried gels are to be used, begin from step 10, carefully excising the band with a sharp scalpel blade. The dried gel piece will reswell upon washing with water (step 13a or b). At this point it is important to remove any and all pieces of cellulose membrane (commonly used for gel drying procedures), which will detach from the gel as it swells, then proceed with destaining and in-gel digestion in the protocol as described.

The protocols for in-gel protein digestion are well established, fairly simple, and straightforward. Very often, key handling procedures during the sample preparation steps are overlooked because they seem easy and nonchallenging. Weeks and months of work can easily be jeopardized because the sample was contaminated or wasted in the final steps before MS analysis. Therefore, several general experimental considerations apply to all stages in this protocol. Precautions need to be taken, mainly to avoid contamination of the samples with various substances that will interfere with MS analysis. These precautions are:

1. Lab coats and powder-free gloves must be worn at all times. Use the highest-grade purity chemicals available, including the highest-grade laboratory water.

2. Take particular care to avoid protein contamination from skin, hair, and saliva (one of the most common interfering substances for MS protein identification is keratin) at all steps, including scanning and drying of SDS acrylamide gels and cutting and washing of stained bands. Avoid talking, coughing, sneezing, or breathing directly over the sample.

3. Keep samples covered or capped at all times unless being processed. Use cleaned tools and containers for anything that will come in contact with the samples.

4. Always use high-quality microcentrifuge tubes and pipet tips to avoid polymer contamination and loss of peptide sample through binding to plastic. Either siliconized, hydrophobically coated or highly polished plastics are recommended.

5. Heating gel pieces or peptide samples should be avoided. When using the Speedvac evaporator, the heat setting is not recommended.

6. Check with the mass spectrometry service first, to obtain additional advice on how to prepare, store, and ship the samples for analysis, and strictly follow their instructions.

Two different protocols for staining SDS acrylamide gels are presented, the colloidal Coomassie and zinc-imidazole negative stain. Either protocol is appropriate for the purposes of this unit, so both are described.

Materials

Unstained SDS acrylamide gel (Basic Protocol 1 or Alternate Protocol)
Colloidal Coomassie stain (see recipe)
1% (v/v) acetic acid
Zinc stain solutions I and II (see recipes)
50% (v/v) and 100% acetonitrile
25 and 50 mM ammonium bicarbonate (prepare from 1 M stock; see recipe)
25 mM ammonium bicarbonate in 50% (v/v) acetonitrile (prepare from 1 M ammonium bicarbonate stock; see recipe)
Zinc destain solution (see recipe)
Digestion buffer (see recipe), ice-cold
5% (v/v) formic acid
50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA)

Sealable plastic containers of appropriate size for gel
Platform shaker
Flatbed scanner (preferably with transparency adapter for Coomassie stain)
Computer running graphics software, e.g., Adobe Photoshop
Light box
Scalpel (with new blades) or fine-gauge needles
Siliconized, hydrophobically coated, or highly polished plastic microcentrifuge tubes (e.g., Axygen Maxymum recovery clear 0.6-ml tubes), pipet tips (Axygen Maxymum recovery pipet tips), and gel-loading tips (e.g., Sorenson Multi Miniflex round sterile gel loading pipet tips, 0.1 to 10 µl)
Stainless steel mortar and pestle (e.g., Quantum Scientific)
Heat-sealable plastic sleeves and heat sealer
Pieces of cardboard and large sturdy flat box to accommodate gels
50-ml screw-cap tubes
Heavy-gauge needle
Thermomixer/shaker (to fit microcentrifuge tubes)
Bath sonicator

Stain the gel

For colloidal Coomassie staining

1a. After running the gel, immediately rinse the whole gel with water at room temperature at least three times (each time for 5 min) before proceeding with the stain.

This step is to remove any traces of SDS remaining on the gel, which could lead to high background and reduced protein staining.

2a. In a container with a cover that can be sealed, cover gel with enough colloidal Coomassie stain so that gel can float freely. Seal the container (to avoid methanol evaporation or contamination) and incubate 1 hr to 3 days with gentle shaking or rocking at room temperature.

The gel must float freely, or it may tear during staining.

Most proteins are detectable after ~1 hr, even while still in the stain solution. Weaker bands show up better within a few hours (normally 3 hr). For quantitative staining, the gel should be left in the solution for ~24 hr (up to 3 days), but this is not usually required.

It is not possible to overstain the gel, and over 1 to 3 days the staining increases slightly in sensitivity.

3a. Destain by immersing in two to three changes of Milli-Q purified water for 15 to 30 min each.

Faint bands often become much clearer after destaining. Very little background staining is encountered with this protocol.

4a. If destaining goes too far, or if the gel was not left in the initial stain for >3 hr, restain as in step 2a.

In many cases restaining actually enhances band detection after a second round of staining. Restaining can be done at any time.

5a. Store gels up to 2 weeks in water at 4°C (to prevent bacterial or fungal growth) or for longer periods in 1% (v/v) acetic acid.

It is highly desirable to excise the target protein bands within a few days of gel staining (see step 10) to avoid artifacts arising from storage.

For zinc-imidazole negative staining of SDS gels

1b. After running the gel, immediately rinse it twice in Milli-Q purified water for up to 5 min each time.

Free glycine can bind to zinc and interfere with the staining process.

2b. Cover the gel with zinc stain solution I and leave on a shaker for 15 min.

3b. Pour off the solution and rinse once quickly with distilled water for 10 to 30 sec.

This rinse step must be very brief. Do not leave the gel in the rinse even for a couple of minutes or the stain will wash out.

4b. Cover the gel with in zinc stain solution II for 35 sec.

The gel background will rapidly appear as a milky precipitate. If the gel is left in this solution longer than 35 sec the stain will begin to fade. To prevent overstaining, proceed quickly to the next step. The timing here also depends somewhat on the acrylamide percentage. Thus, at low acrylamide (~7.5%), <35 sec may be slightly better, while for higher acrylamide (~15%), 40 to 45 sec may be better.

5b. Pour off the solution and immediately rinse three times in Milli-Q purified water (each time for ~1 min), then leave the gel in Milli-Q purified water.

A transparent container will enable the bands to be visualized when placed above a dark background like black paper.

Analyze the stained gel

6. Search the gel for bands that appear to be specific for a particular condition and are absent from the controls, as described in Anticipated Results.

Scan the stained gel

7. For documentation purposes, obtain a quality scan (or a photograph) of gels before excising the bands of interest. For Coomassie-stained gels, use a flatbed scanner (preferably with a transparency adaptor, i.e., a light box in the lid) to record the gel stain. For zinc-imidazole negative staining, record an image by placing the gel onto a flatbed scanner and covering it with a black piece of paper or plastic.

For some scanners it is necessary only to simply leave the lid of the scanner open while recording the zinc image.

For large gels, scan at least at 600 dpi, TIFF format, in 8-bit color. Use LZW compression to keep the file size to a minimum.

8. Using graphics software like Photoshop, convert a copy of the TIFF file to the much smaller JPEG format and then insert the JPEG image into a drawing program (e.g., Microsoft Powerpoint, or CorelDraw). Draw rectangular shapes around the bands of interest and place a simple, unique label next to each one. Use exactly the same labels on the microtubes with the samples as in the computer file. Cover the labels with tape so the writing does not rub off during subsequent steps.

This serves as a laboratory record, and a copy can be sent to an MS service as well. If it is intended to send the whole, intact gel to an MS service, skip to step 13a.

Excise bands

9. Inspect the hydrophobically coated or highly polished pipet tips and microcentrifuge tubes for dust prior to use. If unsure, rinse the microcentrifuge tubes with 50% acetonitrile, followed by water, prior to use.

10. Rinse the whole gel with water and (wearing powder-free gloves) place on a cleaned glass plate over a light box.

For band cutting, work in a clean filtered forced-air-flow hood if possible. If not, clean the bench area thoroughly with ethanol or methanol.

11. Excise the band of interest with a clean scalpel or needle, cutting as close to the edge of the band as possible.

Remember to change scalpels and needles between samples to avoid cross-contamination. Alternatively, rinse the scalpel after each band first with ethanol, then with water.

It is important to reduce the volume of the background gel that is excised. Keep the amount of excess acrylamide included around the band to a minimum. For very large or fat bands, a piece from the center or side is often all that is required.

12. Carefully transfer the gel piece to a clean, labeled microcentrifuge tube.

Store and ship intact gels and gel pieces

It is preferable to not store gels or gel pieces for prolonged periods, but the following guidelines are provided to prevent unwanted spoilage if this is necessary. Additionally, if the researcher is using a commercial or institutional MS service, these often require that the intact gels or excised gel pieces are shipped to them for analysis. Therefore, appropriate handling techniques for shipping these are described here, and the remainder of this protocol does not need to be followed. If it is intended to perform in-gel protein digestion and peptide extraction, then continue with step 14.

13a. *For intact gels:* Store intact gels at 4°C submerged in water for no more than 2 weeks, in a covered container to avoid drying out or contamination. Do not store the gels on the bench or they will grow mold. Alternatively, heat seal them in a strong thoroughly rinsed plastic sleeve for storage at 4°C. Ship intact gels in a few milliliters of water in a heat-sealed plastic bag, packaged in such a way as to avoid tearing or having the gel fold over on itself—e.g., place the heat-sealed bag between rigid pieces of cardboard to keep the gel flat, then place the assembly in a large sturdy flat box.

13b. *For excised gel pieces:* Store excised gel pieces (sometimes referred to as gel plugs) in sealed microcentrifuge tubes either at 4°C submerged in a small volume of water or at −20°C without water for months. Ship gel pieces, each in a separately labeled microcentrifuge tube, submerged in a small volume of water (e.g., 50 µl) to prevent the gel slices from drying out; place the microcentrifuge tubes in a 50-ml screw-cap tube to protect them from being crushed in transit.

Wash and destain the excised gel pieces

For destaining colloidal Coomassie–stained gel pieces

14a. Discard the storage solution. Rinse gel pieces three times, each time by adding 100 µl water and incubating 10 min at room temperature with shaking, then removing the liquid.

Coomassie blue interferes with the MS analysis and inhibits tryptic digestion. Therefore it is removed prior to tryptic digestion. The number and the duration of the following washing steps depends on the sample itself. Some bands destain easily and may only need a few rinses with water and a single step with 100% acetonitrile, while others are more stubborn and may need several rounds of ammonium bicarbonate/acetonitrile (CH_3CN). Under no circumstances should one proceed with trypsin digestion until the gel pieces are completely clear and colorless.

The use of a mixer/shaker (suitable for microcentrifuge tubes) or occasional vortexing at room temperature is recommended for all washing and destaining steps. Use gel-loading tips, to prevent aspirating the gel particles when removing and discarding the liquid after each change of solution during the wash steps. Change tips between different samples. A

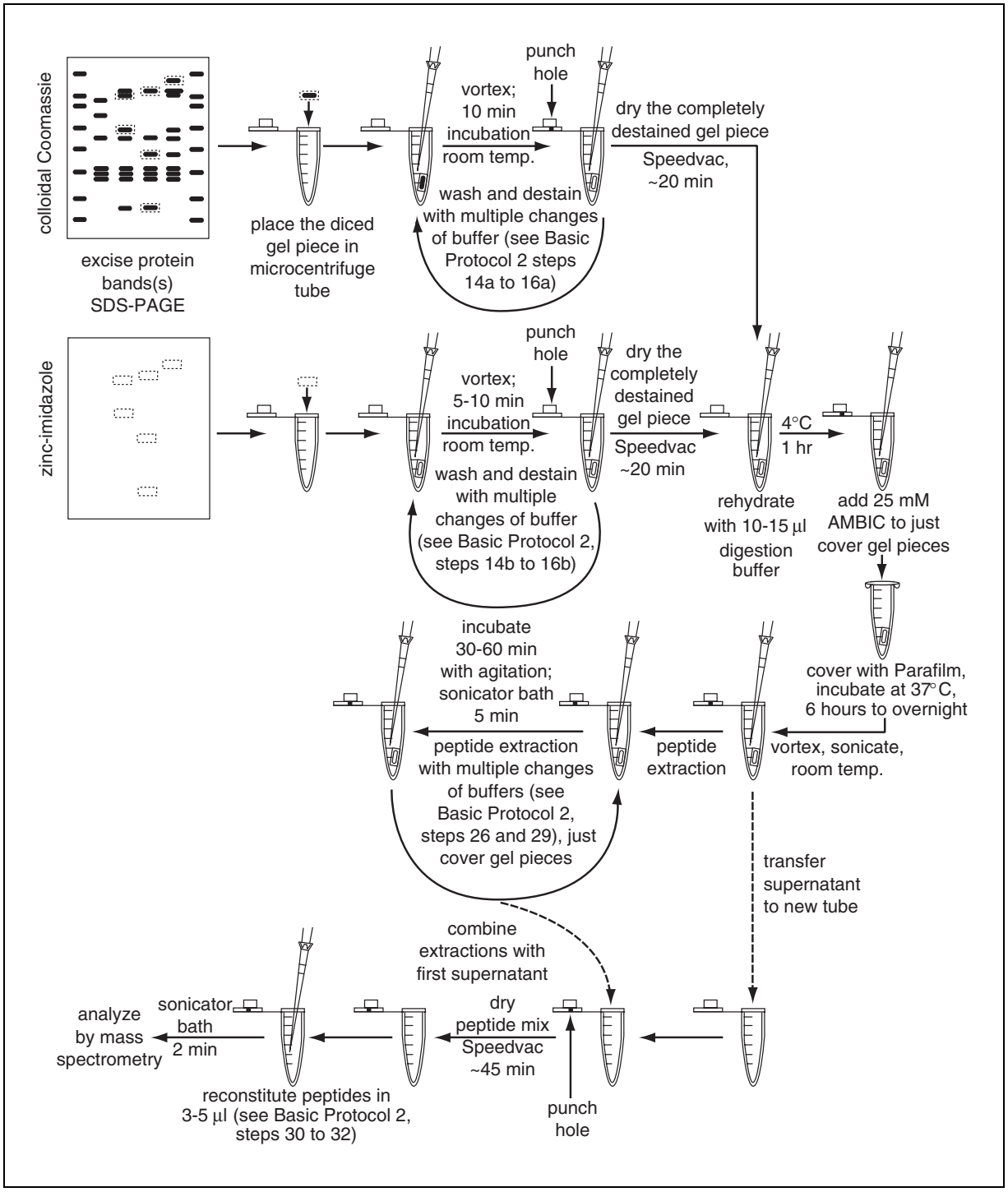


Figure 17.5.3 Schematic of Basic Protocol 2 describing the in-gel digestion technique and peptide extraction steps. For graphical reasons, the gel pieces are depicted as whole (to show the state of the stain), but would in practice be diced. Refer to the main body of the text for more details. Abbreviation: AMBIC, ammonium bicarbonate, pH 8.0.

schematic diagram of the remainder of this protocol can be found in Figure 17.5.3, though it is best to use the figure while paying close attention to the text of the following steps.

15a. Add 100 µl of 25 mM ammonium bicarbonate in water to equilibrate the gel particles. Incubate for 30 min to overnight with shaking. Remove liquid. Wash gel pieces three times, each time by adding 100 µl of 25 mM ammonium bicarbonate in 50% acetonitrile, incubating 10 min at room temperature with shaking, then removing the liquid.

16a. Add enough 100% acetonitrile to just cover the gel particles (which should become white and stick together). Change the acetonitrile once after 5 min. Remove the liquid. Add 100 µl of 50 mM ammonium bicarbonate, leave for 5 min, then add an equal volume of 100% acetonitrile (to make a final concentration of 25 mM ammonium bicarbonate in 50% acetonitrile). Incubate for an additional 5 to 10 min with shaking. Remove liquid.

In this step the gel particles are completely dehydrated with neat acetonitrile to allow the subsequent aqueous solution to better access the center of the gel piece, giving a better chance of removing any stain.

For destaining zinc-imidazole negative stained gel pieces

14b. Discard the storage solution. Rinse gel pieces three times, each time by adding 100 µl water and incubating 10 min at room temperature with shaking, then removing the liquid.

15b. Add 200 µl of zinc destain solution per gel piece, vortex, and incubate with shaking for 5 to 10 min at room temperature until gel piece is completely clear. Remove the liquid.

A zinc chelator is required to destain the gel. Glycine (present in the zinc destain solution) is suitable for this purpose. The pH must be kept in the neutral to basic range to avoid precipitation of the protein in the gel.

16b. Wash gel pieces four times, each time by adding 1 ml water and incubating 10 min at room temperature with shaking, then removing the liquid.

This step is to dilute the glycine, which will interfere with subsequent staining or analysis.

Grind and dehydrate the gel pieces

17. Using a clean stainless steel mortar and pestle, grind each of the excised bands into smaller particles inside the tube. Alternatively, use a long needle to carefully cut it into cubes (~1 mm to a side) inside the tube.

This step is to increase the surface area in contact with buffer and to minimize the volumes of the solutions used in the subsequent in-gel digestion and extraction of the resulting peptide mixture.

18. Add sufficient 100% acetonitrile to just cover the gel particles and leave for 5 min to dehydrate. Remove the liquid.

19. Pierce a hole, if one is not already present, in the lid of the microcentrifuge tube using a heavy-gauge needle to allow for evaporation. Completely dry the gel particles in a Speedvac evaporator (~20 min).

The gel pieces should shrink, become white and stick together. At this point the destained gel pieces can be stored at 4°C for months.

Perform in-gel tryptic digestion

20. Rehydrate the dried gel particles in 10 to 15 µl of freshly prepared ice-cold digestion buffer (use just enough to cover the sample).

Gel pieces should expand and change from white dry pellets back to transparent gel pieces.

21. Incubate the samples at 4°C (sitting on ice) for about 1 hr.

This will allow the trypsin to enter the gel pieces, while it remains inactive at 4°C.

22. After 1 hr, if the initial liquid is fully absorbed, add 10 to 15 µl of 25 mM ammonium bicarbonate in water (without trypsin), or just enough to cover the gel particles.

It is necessary to have a small excess of solution covering the gel piece, so that the gel piece does not dry out during the subsequent 37°C overnight incubation (step 23).

23. Cap the tubes and wrap the tops with Parafilm to seal them. Transfer the tubes to an incubator at 37°C for overnight digestion.

    At 37°C, the trypsin (which had previously entered the gel piece during rehydration) is active and digests the proteins from within the gel piece.

    Most proteins should be completely digested by trypsin after 4 to 6 hr. However samples are usually incubated overnight for convenience. The extraction of the resulting peptide mixture could then take place on the following day.

Extract the peptides

24. Next day, microcentrifuge the tubes briefly at maximum speed to get the condensation to the bottom of the tubes. Vortex the tubes or sonicate (in a mild sonicator bath) for 1 to 5 min.

    This step is to assist the peptides to diffuse from the gel piece into the solution.

25. Microcentrifuge briefly at maximum speed (to force all the sample to the bottom of the tube) and transfer the supernatant to a fresh microcentrifuge tube.

    The supernatant should contain most of the tryptic peptides. If performing MS on-site, then take a small aliquot (0.5 µl) for immediate analysis.

    This first extraction step has a lower extraction efficiency but is quick, and there is less sample loss because the samples are not dried in a Speedvac evaporator. Subsequent extractions in organic phase are needed for recovery of hydrophobic or long peptides.

26. Wash gel pieces by adding ~25 µl (or enough) 25 mM ammonium bicarbonate in water to just cover them, then removing the liquid and combining it with the supernatant from step 25.

27. Add 20 µl of 5% formic acid, incubate 10 min, then add 20 µl of 100% acetonitrile (final concentration of 2.5% v/v formic acid and 50% v/v acetonitrile) to the gel pieces and incubate for 20 to 30 min at room temperature with shaking.

28. Vortex or sonicate the gel particles and supernatant for 1 to 5 min. Microcentrifuge briefly at top speed and collect the supernatant. Pool with the previous extract from step 25.

29. Add 20 µl of 100% acetonitrile for a single extraction of 20 min with shaking. Vortex or sonicate the gel particles and supernatant for 1 to 5 min. Microcentrifuge briefly at maximum speed, collect the supernatant, and pool it with the previous extracts.

30. Concentrate the pooled extracts in an Speedvac evaporator to near-complete dryness (5 µl).

    The aim is to reduce the sample volume and to remove volatile salts (such as ammonium bicarbonate).

31. Subject the samples to MS analysis (see remaining steps) or freeze them and store at −20°C for shipment to an MS facility.

    Alternatively, lyophilize the samples and store them at −80°C for long periods of time.

Reconstitution of tryptic peptides

32a. *For MS analysis by MALDI-TOF MS:* Resuspend the dried peptides in 3 to 5 µl of 50% acetonitrile/0.1% TFA.

32b. *For analysis by ESI-MS:* Resuspend the dried peptides in 0.1% v/v formic acid.

    The choice of reconstitution solvent system depends on the MS method to be used (see Strategic Planning). Some solvents (e.g., TFA) may not be universally compatible between approaches.

33. Sonicate for 2 to 5 min by immersion of the tubes in a bath sonicator, then microcentrifuge the tubes briefly (1 min) at maximum speed.

The optimal resuspension conditions may vary, depending on the chosen method of MS analysis (see Strategic Planning). The above techniques are suitable for general resuspension of dried peptides.

34. Analyze the reconstituted peptides by the MS method of choice (see, e.g., Coligan et al., 2003, Chapter 16).

SUPPORT PROTOCOL 4

RECYCLING OF GSH AGAROSE

GSH-agarose beads can be regenerated and reused, preferably for the same purpose (for example, preclearing of tissue lysates). In this support protocol, a method is described which removes bound endogenous GSTs from the GSH beads. Since large amounts of these beads are used in the preclearing steps (see Basic Protocol 1, the Alternate Protocol, and Support Protocol 3), it can be economical to recycle them for use in the next preparation of tissue lysates. It should be noted, however, that it is not recommended to recycle any beads that have had their bound proteins eluted for analysis by SDS-PAGE.

It is also worth noting that the GSH-agarose regeneration protocol described by the manufacturer (Amersham Biosciences) proved ineffective at removing the large quantities of bound proteins that are present after preclearing tissue lysates. The authors have found that many other treatments will remove bound endogenous GSTs from GSH agarose, including reduced glutathione, acidic glycine, and denaturants (such as SDS or guanidine). All of these treatments will remove an equivalent amount of GST from the beads (~95% after two washes); however, the order in which they are applied influences the ability to remove the remaining bound protein. Specifically, if denaturants are used initially, the protein remaining on the beads is rendered insensitive to removal by glutathione. Therefore, the optimal protocol is described herein—reduced GSH, followed by acidic glycine. After many cycles of reuse, the beads may lose some binding capacity from accumulated protein and hydrophobically bound material, so additional steps are included to remove these substances using detergents.

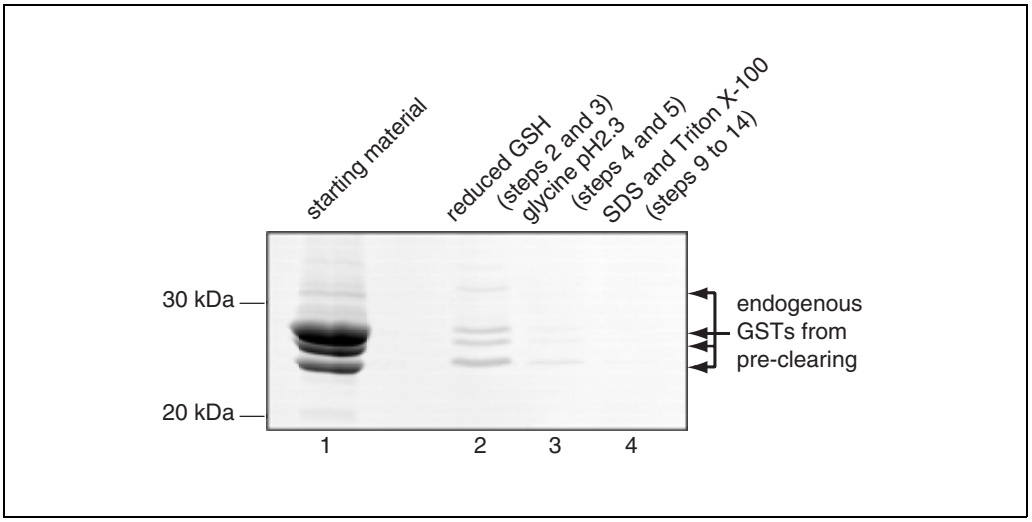


Figure 17.5.4 Regeneration of GSH agarose. The expected results from Support Protocol 4 are shown. Lane 1 shows the stained endogenous GST proteins present bound to a batch of GSH agarose that has been used for preclearing a tissue lysate. Lanes 2 to 4 show the sequential treatments which remove protein from the beads, and enable their reuse for preclearing procedures.

The sequential removal of bound endogenous GSTs that occurs from the utilization of this protocol is illustrated in Figure 17.5.4. After all the washes are performed, almost none of the endogenous GST protein remains bound to the beads.

Materials

Beads for recycling (from pre-clearing steps in Basic Protocol 1, the Alternate Protocol, or Support Protocol 3)
Glutathione wash buffer (see recipe), room temperature
Bead recycling buffer: 100 mM glycine hydrochloride, pH 2.3, room temperature
Phosphate buffered saline (PBS; *APPENDIX 2A*), room temperature
20% (v/v) ethanol
1% (w/v) SDS (see *APPENDIX 2A*)
0.1% (v/v) Triton X-100 (see *APPENDIX 2A*)
End-over-end rotator

1. Estimate or measure the bed volume empirically from the settled mass of beads. Take a small sample of the beads (20 to 40 µl bed volume) before recycling for later analysis by SDS-PAGE.
2. Elute the GST proteins from the GSH beads by adding ten times the bed volume of glutathione wash buffer and incubating 1 hr at room temperature on an end-over-end rotator. Centrifuge 5 min at 500 × *g*, room temperature, in a benchtop centrifuge, then carefully remove the supernatant by vacuum aspiration or decanting.

   This is a competitive elution of GST with glutathione.
3. Repeat step 2, for another 1-hr incubation with glutathione wash buffer.
4. Repeat step 2, but use bead recycling buffer in place of glutathione wash buffer.

   This step uses pH 2.3 glycine to effect greater elution. The authors have noted that bound GSTs are not eluted from GSH beads at pH 2.5, hence attention to pH is essential.
5. Repeat step 4, for another 1-hr incubation with bead recycling buffer.
6. Wash the GSH beads by resuspending them in ten bed volumes of room temperature PBS. Centrifuge 5 min at 500 × *g*, room temperature, in a benchtop centrifuge, then carefully remove the supernatant by vacuum aspiration or decanting. Repeat two additional times.
7. Wash once more as in step 6, but use ten bed volumes of 20% ethanol in place of the PBS.

   The ethanol is included as a preservative.
8. Store at 4°C in 20% ethanol as a 25% to 50% slurry.

   At this point the beads are largely clear of specifically bound endogenous GSTs. However, it is useful to analyze the beads for the absence of bound proteins by taking a small sample (20- to 40-µl bed volume) and analyzing the bound proteins, both before and after recycling by SDS-PAGE and Coomassie staining. If the beads show no protein bands (or very faint bands) after the above steps, they are suitable for reuse. If significant protein remains bound to the beads, continue with step 9.

   After multiple cycles of recycling, the GSH beads may lose some binding capacity and become unable to efficiently extract endogenous GSTs from tissue lysates. This may be due to an accumulation of precipitated, denatured or nonspecifically bound proteins and hydrophobically bound substances. If this is found to be the case, carry out the remaining steps of this protocol to remove these.

9. Incubate the beads twice, for 1 hr each (as in step 2), but with 10 bed volumes of 1% SDS.

   Note that GSH-agarose beads are stable to 1% SDS for up to 14 days at room temperature, without any loss in binding capacity.

10. Wash briefly with ten bed volumes of PBS, centrifuge 5 min at 500 × *g*, room temperature, and remove supernatant.

11. Wash beads as in step 6, but use 0.1% Triton X-100 (in water) in place of PBS.

    The Triton X-100 wash is included to remove more hydrophobic material, and to complex with any residual SDS that is present on or around the beads.

12. Wash the GSH-agarose beads three times with PBS, using the technique described in step 6 except without the 1-hr incubation.

13. Wash once more as in step 6 (without the incubation), but use ten bed volumes of 20% v/v ethanol in place of the PBS.

14. Store at 4°C in 20% ethanol as a 25% to 50% slurry.

    It may be necessary to re-evaluate the amount of protein bound to the beads by taking a sample (20- to 40-µl bed volume) and analyzing by SDS-PAGE.

REAGENTS AND SOLUTIONS

*Use Milli-Q purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Ammonium bicarbonate, 1 M

Prepare a 1 M stock solution, pH 8.0, by dissolving 0.79 g of ammonium bicarbonate in 10 ml water; there is no need to adjust the pH because it should be pH 8.0.

Store at room temperature for up a few weeks.

Bis(sulfosuccinimidyl) suberate (BS3)

Weigh out 20.5-mg aliquots of bis(sulfosuccinimidyl) suberate (B53; Pierce) into screw-capped tubes, flush with N_2 gas, and cap tightly. Store desiccated at −20°C. Immediately before use, dissolve one 20.5-mg aliquot in 1 ml of 5 mM sodium citrate buffer, pH 5.0 (38.5 mM final). Add the solution dropwise to the reaction.

Buffer A

20 mM Tris·Cl, pH 7.5
1 mM EGTA

Prepare 10 liters by dissolving 24.22 g Tris base in 10 liters water and adding 50 ml of 200 mM EGTA stock (see recipe), then adjusting the pH to 7.5 with concentrated HCl. Prepare fresh before use, store at 4°C.

Buffer B

20 mM Tris·Cl, pH 7.5
1 mM EGTA
7 µg/ml leupeptin
1 mM PMSF

To 9 liters buffer A (see recipe), add 3.15 ml of 20 mg/ml leupeptin stock. Prepare fresh and store at 4°C without PMSF. Add 5 ml of 100 mM PMSF stock (*APPENDIX 2A*) per 500-ml volume to each aliquot of buffer immediately before use.

PMSF is added immediately before use because it does not last more than 45 min in solution.

Buffer C

20 mM Tris·Cl, pH 7.5
500 mM NaCl
1 mM EGTA
7 µg/ml leupeptin
1 mM PMSF

To 1.5 liters buffer A (see recipe), add 43.8 g NaCl, 525 µl of 20 mg/ml leupeptin stock. Prepare fresh before use and store at 4°C without PMSF. Add 5 ml of 100 mM PMSF stock (*APPENDIX 2A*) per 500-ml volume to each aliquot of buffer immediately before use.

Buffer D

20 mM Tris·Cl, pH 7.5
1% v/v Triton X-100
1 mM EGTA
7 µg/ml leupeptin
1 mM PMSF

To 500 ml buffer A (see recipe), add 50 ml of 10% (v/v) Triton X-100 (*APPENDIX 2A*) and 175 µl of 20 mg/ml leupeptin stock. Prepare fresh before use and store at 4°C without PMSF. Add 5 ml of 100 mM PMSF stock (*APPENDIX 2A*) immediately before use.

Colloidal Coomassie Blue stain

Mix the following components in the order indicated. Weigh 170 g ammonium sulfate into a beaker. Add 340 ml of methanol, then 36 ml of concentrated phosphoric acid, and bring to 1 liter with water. Dissolve completely using magnetic stirring and heating to 60° to 70°C for 30 min or more (it is essential to fully dissolve the ammonium sulfate before proceeding). Add 1 g of Coomassie G-250 and again stir well to dissolve (to retain the colloidal dye particles this solution must not be filtered). Store up to 6 months at room temperature.

It is extremely helpful to let this stain settle in the bottle (i.e., by leaving the bottle to sit on the bench) for a day or more before use, and then to slowly decant the stain to a clean bottle, leaving the insoluble material behind. This removes/reduces the fine particulate matter which can adhere to the gel during staining. Do not recycle this stain, discard after each use.

Final concentrations: 17% (w/v) $(NH_4)_2SO_4$, 3% (v/v) phosphoric acid, 34% (v/v) methanol, and 0.1% (w/v) Coomassie Brilliant Blue G-250.

Digestion buffer

Reconstitute lyophilized trypsin (sequencing-grade porcine trypsin or Trypsin Gold MS grade, Promega) in 50 mM acetic acid. Divide into aliquots and store reconstituted trypsin at −70°C.

For short periods of time, the reconstituted trypsin may be stored at −20°C, with no more than five freeze/thaw cycles.

Final concentrations: 25 mM ammonium bicarbonate, pH 8.0/12.5 ng/µl trypsin.

Disuccinimidyl suberate (DSS)

Weigh out 13.2-mg aliquots of disuccimidyl suberate (DSS; Pierce) into screw-cap tubes, flush with N_2 gas, and cap tightly. DSS is moisture-sensitive, so store it desiccated at 4°C. Warm vials to room temperature before opening. Once opened, store under nitrogen gas (stable up to 2 years, but only if stored correctly). Dissolve DSS in DMSO (see Support Protocol 2, step 4) immediately before use, and do not keep in solution for more than 1 hr.

EGTA, 200 mM

Combine 76.08 g EGTA (acid form; mol. wt. 380.4) with 1 liter of water (or 15.22 g with 200 ml of water). Adjust pH to 7.4 with ~26 ml saturated Tris base.

The EGTA takes a long time to dissolve and to equilibrate at the correct pH; EGTA will not fully dissolve until pH is increased.

To prepare saturated Tris base, note that maximum Tris solubility at 0°C is 2.4 M, or 290.6 g/liter.

Glutathione wash buffer

Dissolve 184 mg of reduced glutathione (GSH; mol. wt. 307.3) in 8 ml TBS (see recipe for wash buffer 1), adjust to pH 8.0 with ~300 µl saturated Tris base, and make up to 12 ml with TBS (final glutathione concentration, 50 mM). Make fresh before use.

To prepare saturated Tris base, note that maximum Tris solubility at 0°C is 2.4 M, or 290.6 g/liter.

Sheep brain

One sheep brain is ~76 g. The authors normally collect about 12 brains (900 g) on each abattoir visit. At the abattoir, brains must be obtained within 1 min of sacrifice, rinsed very briefly in cold PBS (*APPENDIX 2A*), diced into 2- to 5-g cubes (~1 to 2 cm per side) on a cutting board, and frozen immediately in liquid nitrogen, before returning to the laboratory. Store frozen at –70°C and use as soon as possible (within ~3 months).

Small GTPase loading buffer

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
2 mM EDTA
25 mM NaCl

The small GTPase loading buffer must be made fresh the same day it is to be used. It is not stable whether frozen or kept at room temperature for these experiments, as the EDTA breaks down in solution (Glebska et al., 2002).

Wash buffer 1 (TBS)

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
150 mM NaCl
Store up to 1 year at room temperature

Wash buffer 2

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
150 mM NaCl
0.2% (v/v) Triton X-100
Store up to 1 year at room temperature

Wash buffer 3

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Store up to 1 year at room temperature

Wash buffer 4

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
150 mM NaCl
2.5 mM $MgCl_2$
Store up to 1 year at room temperature

Wash buffer 5

20 mM Tris·Cl pH, 7.4 (*APPENDIX 2A*)
150 mM NaCl
0.2% v/v Triton X-100
2.5 mM $MgCl_2$
Store up to 1 year at room temperature

Wash buffer 6

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
2.5 mM $MgCl_2$
Store up to 1 year at room temperature

Zinc destain solution

25 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
192 mM glycine

Combine 3.03 g of Tris base and 14.4 g glycine and make up to 1 liter with water. Do not adjust pH, it will be pH 8.3.

Zinc stain solution I

Add 10 ml of 10% (w/v) SDS (*APPENDIX 2A*) to 13.62 g of imidazole, and make up to 1 liter with water. Store up to 1 year at room temperature.

Final concentrations: 0.2 M imidazole, 0.1% (w/v) SDS.

Zinc stain solution II

Bring 57.51 g $ZnSO_4 \cdot 7H_2O$ to 1 liter with water (final concentration, 0.2 M). Store up to 1 year at room temperature.

COMMENTARY

Background Information

Glutathione (γ-glutamyl cysteinyl glycine; GSH) contains a free thiol group that provides protection for cells against oxidative stress by maintaining a reducing intracellular environment. In conjunction with glutathione *S*-transferases (GSTs), glutathione also participates in detoxification of many reactive exogenous chemical substances by forming adducts through the thiol group. Glutathione-agarose (GSH-agarose, or GSH beads) are prepared by immobilizing glutathione through the thiol group (S-linked) onto beaded agarose, using a 12-atom carbon spacer to link the bead to the glutathione. GSTs bind glutathione with high affinity, and this is the basis for the use of *Schistosoma japonicum* GST in recombinant fusion proteins. A simple purification procedure therefore utilizes the GST fusion protein's specific coupling to GSH agarose, isolation of beads, washing, and competition from GSH beads with free glutathione for elution. This is commonly used as a method for purification of GST fusion proteins, but the affinity of GST fusion proteins for GSH agarose has also been employed as a way to couple a protein of interest to a solid support for affinity chromatographic approaches.

Pull-down experiments are based on affinity chromatography. The use of GST fusion proteins for this technique is common. As an affinity chromatography technique, therefore, the pull-down experiment is ideally suited to specifically isolate protein-protein interaction

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments

Problem	Probable/possible causes	Possible solutions
Many or all bands are identified as glutathione *S*-transferase (*Schistosoma japonicum*)	Very large amount of GST recombinant protein used as bait	Cross-link the GST fusion protein to the GSH beads with DSS or BS3 (see Support Protocol 2). Refer to Strategic Planning for more details. Reduce the amount of GST fusion protein used.
Intense staining of four to five proteins of 20-30 kDa (in all lanes), which interfere with the detection of other proteins	The very large amount of endogenous tissue GST present in tissue lysates, binding to the remaining free glutathione sites of the GSH beads (multiple mammalian isoforms give rise to the band pattern); see Figures 17.5.4 and 17.5.5 for examples of brain GSTs	Rigorously preclear lysates with GSH beads (at least three times). Alternatively, if the GST fusion protein is cross-linked to the beads (Support Protocol 2): wash the beads (after binding to lysate) 2 to 3 times with glutathione wash buffer, followed by three washes of wash buffer 2 to specifically elute the GSTs before elution in SDS sample buffer. *CAUTION:* Washing with GSH may also elute other specifically binding proteins, due to its reducing potential, and should therefore be carefully tested.
No specific binding proteins are observed for the protein of interest relative to control	Bacterially produced GST fusion proteins are not correctly folded or post-translationally modified	Refer to literature on protein of interest and on bacterial expression systems, and change expression system accordingly. Use a nonbacterial expression system (e.g., baculovirus, in vitro translation), as long as sufficient GST fusion protein for pull-downs can be obtained.
	Tissue source for lysate inappropriate for protein of interest	Change tissue source. Use a tissue that is known to express the protein of interest, or screen several tissues and analyze simultanously by SDS-PAGE, to compare binding proteins from different tissues. This has the advantage of revealing binding proteins that are specific to the function of the protein of interest in a particular tissue as contrasted with other tissues. Scale up chosen tissue 10-fold. Try tissues isolated from animals of different developmental stages.
	Washing or binding buffers may be too stringent (salt, detergent concentration interferes with binding)	Dilute out salt or detergent further (for peripheral membrane and membrane extracts). Homogenize pellets in smaller volumes (e.g., 250 ml instead of 500 ml), and then dilute to 2 liters with buffer B. Alternatively, use buffers for tissue extraction that have different properties (e.g., CHAPS, NP-40, or saponin).

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
Spin columns blocked and any added solutions will not flow through	Particulate contamination is present in lysate, which blocks the pores of the column's frit (10-µm pore size)	Ensure that the lysate used is free of particulate contamination. Sometimes the particles cannot be seen with the naked eye, so it is important to thoroughly centrifuge the lysate before use at maximum speed in a high-speed centrifuge, ≥10,000 × *g* for 30 min at 4°C, even if this has already been done. It is possible to obtain temporary flow in the column by the use of a thin, flat spatula to stir the beads within the column. However, such samples do not usually wash efficiently and are not likely to yield useful results.
SDS sample buffer leaks out of MicroSpin columns during heating in the 85°C water bath, resulting in lost samples	A sufficiently tight seal was not obtained when the bottom of the column was plugged	Ensure that: (1) The plug is not loose. It may be necessary to exchange and try different plugs until one is sufficiently tight. It can help to maintain a small collection of washed plugs, in case one cannot be found in the current batch that adequately seals the column. (2) The column is not cracked or unevenly broken at the base. Care must be taken early on when snapping the columns from the plugs that the plastic does not break unevenly or crack the column. It may be necessary, if it is too uneven, to trim the base of the column with a scalpel blade to ensure a flush seal.
	The top cap for the column was not punched with a needle. When heated, pressure built up inside the column, forcing the SDS sample buffer out through the bottom	Punch the top screw caps of the columns with a heavy gauge needle (22-G or wider). Keep a collection of punched caps (they never contact the sample) to avoid having to punch them every time.
Large amounts of nonspecific proteins are eluted from GST fusion protein beads after pull-down (particularly from membrane protein extracts)	Hydrophobic (largely membrane) proteins may adhere to the beads when washed without detergent	After the binding step, wash beads with wash buffer 1 plus 0.1% Triton X-100 for the first round of washing. It is essential to subsequently wash thoroughly (at least three times) with wash buffer 2 (no Triton) to eliminate the chance of SDS-PAGE distortions caused by Triton X-100. This detergent also can interfere with MS analysis if present in the sample loaded onto the gel.
High-molecular-weight bands appear in samples eluted from non-cross-linked GST fusion protein beads after storage (e.g., the bands marked G on Figure 17.5.5)	Many GST fusion proteins (by unknown mechanisms) form polymers upon prolonged storage at −20°C that are resistant to reducing SDS-PAGE	Purify fresh GST fusion–protein beads, and perhaps cross-link beads to prevent recurrence.

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
Beads stick to the walls of the tubes and cannot be easily removed by washing	Agarose beads often stick to walls of tubes, particularly when using small volumes of bead/liquid mix in large tubes	Polypropylene tubes (particularly 15-ml Falcon 2059 tubes) bind fewer beads than equivalent tubes of other plastics. Use tubes which are of a suitable volume for the volume containing the beads, or increase volume to match the tubes. The inclusion of a detergent (e.g., Triton X-100) may prevent beads adhering to the walls of the tubes. With small volumes of bead slurry, avoid vigorous vortexing when resuspending the settled beads.
In the Alternate Protocol: Known GTP-dependent binding proteins (effectors) do not bind, or bind in a fashion that is not GTP-dependent	The GTP used for preloading of GST-small GTPase beads has been hydrolyzed to GDP, or GST-small GTPase beads were inefficiently loaded with nucleotides	Ensure that: (1) GTP stock is sufficiently fresh (sometimes it is hydrolyzed before the expiration date). (2) All the Mg^{2+} was complexed with freshly prepared EDTA in the loading step. Any free Mg^{2+} that is present will promote hydrolysis of GTP at 37°C. Use a nonhydrolyzable analog of GTP, such as GTPγS, instead of GTP.
There is still GST fusion protein eluted from the beads after chemical cross-linking, which interferes with pull-down experiment	The cross-linking reaction was incomplete or inefficient, or cross-linker has degraded	Ensure that sufficient cross-linker is used; requantitate the level of protein bound to GSH beads. Wash more extensively with PBS before cross-linking to ensure absence of amine buffers. Cross-link for longer durations (up to 2 hr). Use freshly purchased DSS (if stored for some time). Switch to the more soluble cross- linker BS3.
	GSH washing to remove small amounts of non-cross-linked fusion protein was inadequate, or inefficient	Wash the column after cross-linking with two 4-ml washes of bead recycling buffer, and allow the solution to pass through. Note that 100 mM glycine at pH 2.3 is relatively harsh, and may damage some proteins. At this pH it elutes GST from GSH, and thus can be a useful adjunct to the GSH wash. Keep the eluate (pH-neutralized) for a gel to see if any protein eluted.
Poor digestion (seen as few peptides in mass spectrum, i.e., poor sequence coverage)	Protein substrate has many oxidized cysteine residues, and/or is internally disulfide-bonded	Reduction and alkylation of the substrate can lead to improved sequence coverage (an example protocol is found in *UNIT 5.6*).
	The protein of interest is not a good trypsin substrate (i.e., too few Lys and Arg residues)	Chose a different protease, such as LysC. This is particularly necessary for highly basic, or particularly small (<15-20-kDa) proteins with few Arg or Lys residues.

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
	Too short or too long incubation	Need to optimize digestion time. Most proteins are completely digested after 4-6 hr. Exhaustive digestion of up to 48 hr, with an addition of a second batch of digestion buffer, could be performed in some cases to achieve a higher sequence coverage.
	Trypsin has been stored or reconstituted inappropriately, has expired, or has lost activity	Check storage conditions and expiration date Wrong reconstitution buffer. Resuspend trypsin according to manufacturer's recommendations. Multiple freeze/thaw will affect enzyme activity. Check pH of digestion buffer. Trypsin works best at pH 7-9. May need different enzyme-to-substrate ratio: add more digestion buffer for larger (more intensely stained) protein bands.
	Coomassie Blue stain interferes with digestion. (In this case it will also appear in the spectra, most likely as the strongest peak, m/z = 800-850 Da.)	More thorough destaining required. Under no circumstances should one proceed with trypsin digestion unless Coomassie-stained gel pieces are completely clear and colorless.
No solution remains around the gel piece after the digestion period	Insufficient rehydration with digestion buffer, before 37°C incubation	Add more (10-20 µl) of 25 mM ammonium bicarbonate, pH 8.0, before 37°C incubation Incubate longer at 4°C before digestion.
	The temperature difference between the bottom of the tube and the lid may result in evaporation of the liquid	Use of a 37°C oven or incubator is better than a heating block or water bath.
Polymers, detergents, and other impurities lead to poor quality MS spectra	Check all stages of experimental and sample preparatory protocols for possible sources of contamination	In general, minimize or completely avoid the use of detergents throughout all protocol steps. Triton X-100 particularly interferes with MS. This detergent may even be present from the gel piece at sufficient levels to interfere with MS analysis. Ensure that Triton X-100 is not present in any samples for SDS-PAGE (it may also originate from an adjacent lane in the same gel). Remake all buffers from scratch.
	Many brands of plastics may leach small amounts of contaminants that interfere with high-sensitivity MS techniques	Use high-quality plastics: siliconized, highly polished, or hydrophobic-coated microcentrifuge tubes and pipet tips. Avoid screw-cap microcentrifuge tubes, as the rubber O-ring may leach plasticizers. Colored tubes and tips may also leak dyes. Pierce holes on the top of the microcentrifuge tube from the inside out to avoid contamination of the sample with plastic fragments from the lid.
	Salt and detergents in sample	Use Millipore Zip-Tips or other microcolumns to concentrate and purify the peptide samples. Follow the manufacturer's protocols.

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
Trypsin autolytic products interfere with and suppress other peaks in mass spectrum (note that peaks at 842 Da and 2211 Da are expected autolytic products of trypsin)	Some autolytic peaks are expected; excessive peaks may be the result of incorrect trypsin use	Sequencing-grade trypsin is modified for maximal specificity. When stored according to instructions, it is highly stable and resistant to autolysis, as the Lys residues have been reduced and methylated. Autolytic products are expected as a result of a autocleavage at Arg residues, which are not protected. Use sequencing-grade trypsin (see recipe for digestion buffer).
	Too much trypsin during digestion. Excessive volume of digestion buffer used (inappropriate for small gel pieces)	Use less: 10-15 µl or just enough to cover gel particles. Change trypsin-to-substrate ratio. 12.5 ng/µl is usually enough for an average-sized protein band that has been diced to pieces and properly destained.
	Trypsin stored inappropriately, or stock freeze/thawed too often	Store according to manufacturer's recommendations. Aliquot trypsin stock and freeze/thaw no more than five times.
	Wrong buffer used to reconstitute the trypsin	Use 50 mM acetic acid, or the optimal buffer recommended by the manufacturer.
MALDI-TOF MS sample spots do not dry well, forming a glossy surface; crystallization is poor	Impurities in the sample, such as detergents or glycine from zinc-imidazole stained gels interferes with crystallization	More extensive gel washing steps are required to thoroughly remove glycine before preparing gel bands for MS analysis. Use Millipore Zip-Tips or other microcolumns to purify the peptides from the impurities. Follow the manufacturer's protocols.
High-intensity peak in mass spectrum of 832 Da suppresses all other peptide signals	The peak is most likely Coomassie blue	Destain gel piece more thoroughly before proceeding to tryptic digestion. Samples can be recovered by micropurification using MilliPore Zip-Tips or other microcolumns. Follow manufacturer's protocols.
Many or all bands are identified by MS as keratin	Common contamination from skin, hair, or dust	Review and follow the recommendations and precautions as described in the introduction to Basic Protocol 2.

Protein-Protein Interactions Identified by Pull-Down Experiments

partners with a GST fusion of a protein of interest. Traditionally, the pull-down experiment has been used as a secondary confirmatory approach to some other protein-protein interaction detection technique, such as a yeast two-hybrid screen. Partly this has been due to the lack of a sufficiently sensitive detection technique for the identification of stained bands on an SDS-PAGE gel. Immunoblots are sensitive enough for this purpose, so once an interaction is discovered, it is relatively simple to confirm it using a pull-down experiment and a specific antibody. Rapid advances in mass spectrometry (MS) techniques have led to it becoming standard institutional or laboratory instrumentation. The sensitivity of these instruments is now sufficient to identify the proteins in a stained gel piece at the sensitivity of the staining technique. Therefore, the pairing of GST fusion protein pull-down experiments with MS creates a new and powerful technique that is readily able to identify protein-protein interaction partners from SDS acrylamide gels (Graves and Haystead, 2002).

Some of the main advantages of the pull-down experiment in combination with MS as a means of identifying protein-protein interactions are:

1. The isolation of a protein-protein interaction partner roughly correlates to the strength of the particular protein-protein interaction. That is, the more intense a stained, identified band on SDS-PAGE, the greater the likely affinity of the interaction. It can therefore give some notion of the major (highest-affinity) interaction in a particular lysate, based on the

intensity of band staining. This is only an approximate correlation, since the intensity of staining may also be influenced by the abundance of the binding protein, or its activation state.

2. Isolation and identification of the protein-protein interaction are not dependent on any third-party detection system (e.g., transcriptional activation of a reporter gene, or selection by cellular viability).

3. The target gene is, by the nature of the approach, cloned into a suitable GST vector, and thus is immediately available for further experiments to map the interaction site using deletion or site-directed mutagenesis.

4. The assay can be internally controlled for specificity, and can simultaneously isolate protein-protein interaction partners dependent on target protein structure and/or function. This is determined by the experimental design (see Strategic Planning for more details).

5. The use of MS techniques draws the identification of a protein-protein interaction partner from an entire sequence database, and it is possible to use protein sequence databases (e.g., nonredundant or Swiss-Prot) or translated DNA sequence databases (nucleotide, EST, or even genomic) for this purpose. Therefore, if one database does not contain a match to the obtained data, other possibilities can be entertained through comparison with other databases, particularly if a binding protein is a novel protein product.

As described in Strategic Planning, MS-based techniques for protein identification are largely species-independent, at least within a group of closely related species (e.g., mammals).

Some of the limitations/disadvantages of the pull-down experiment are:

1. It is dependent on MS analysis (therefore initially expensive and technically demanding); however, results may be achieved collaboratively with MS labs or through the use of a commercial MS service.

2. The entire approach is dependent on recombinant GST fusion proteins and therefore requires optimization of expression, solubility within the bacteria, and achieving appropriate folding. Not all proteins will be compatible for expression of functional product in bacteria.

3. Not all protein-protein interactions are stable enough, or are of sufficient affinity, to be isolated by pull-down experiment. Interference can also arise from detergents, salts, and pre-existing cellular complexes, among other factors.

4. The approach can perform poorly for identification of low-abundance protein-protein interaction partners. This is particularly true for some nuclear proteins (e.g., telomerase), which exist at very low levels in cells. In this case, a good interaction may be achieved, but it may not be detected by stained SDS-PAGE gels.

5. Not all proteins isolated are direct binding partners of the protein of interest. It is common to isolate entire complexes of proteins in the pull-down experiment, where only a subset of the constituent proteins interact directly with the GST fusion protein. An example of this can be observed in Figure 17.5.5, where the entire exocyst complex is isolated with GTP-loaded GST-RalA (see Anticipated Results for more detail). It was later shown that two out of the eight exocyst complex proteins, Sec5 and Exo84, are the binding partners for RalA (Moskalenko et al., 2002; Sugihara et al., 2002; Moskalenko et al., 2003). The authors have observed several other examples where binding proteins of the partners of the target protein are isolated by pull-down experiment. Additional experiments are required to clarify the mode of binding for proteins which are isolated in this way (see Strategic Planning).

Critical Parameters and Troubleshooting

There are many factors that will contribute towards a successful outcome when using the methods in this unit. Most of these are discussed throughout the protocols in the appropriate steps. The pull-down experimental design is heavily dependent on the Support Protocols in this unit, which are the preparatory techniques required for achieving successful outcomes. The commonly encountered problems, their possible cause, and possible solutions are outlined in Table 17.5.2, in the form of a troubleshooting guide. The solutions described are remedies for the major interfering factors in these experiments; however, since this unit contains a relatively complex set of protocols, this list is by no means exhaustive. Considerations that are specific to a particular protein of interest should also be taken into account when troubleshooting any problems that are encountered. Other sources of reference should also be consulted, particularly for troubleshooting GST fusion protein expression and purification, a subject that is better and more comprehensively covered elsewhere (see, e.g., Harper and Speicher, 1997).

Anticipated Results

The intention of this unit is to isolate specific binding proteins that are specific to a protein of interest through the use of GST fusion proteins coupled to GSH-agarose beads and to prepare them for identification by mass spectrometry. An example of the type of results that can be achieved is shown in Figure 17.5.5, which illustrates a sample pull-down experiment using GST-RalA to isolate nucleotide-specific binding proteins, by the approach described in the Alternate Protocol. Pull-down experimental results can often be interpreted in more than one way, so there are several possibilities that should be considered.

Generally speaking, the most important results are those protein bands that bind specifically to the internal controls (e.g., to one mu-

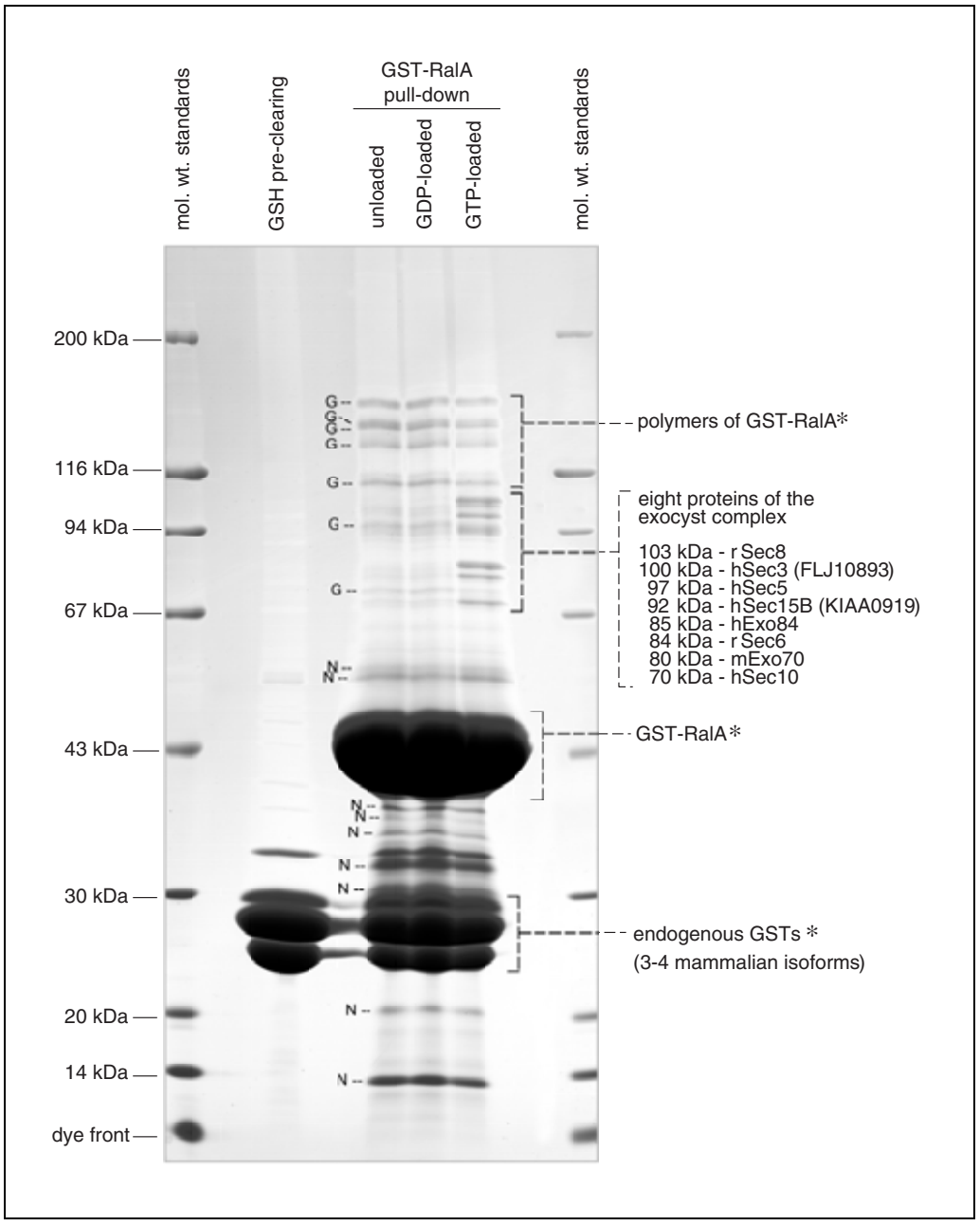


Figure 17.5.5 Sample results from a GST-RalA pull-down experiment. Shown is a Coomassie blue–stained gel of a pull-down from sheep brain cytosol using GST-tagged RalA. For a full discussion, see Anticipated Results. Stained protein bands that have been identified by MALDI-TOF MS are indicated on the right. Features marked with an asterisk (*) are interfering species. Bands that have been identified as erroneously migrating GST-RalA are marked G. The bands marked N are either nonspecific sheep proteins, fragments of GST-RalA, copurified bacterial contaminants, or real nucleotide-independent binding proteins.

Table 17.5.3 Time Considerations for GST Fusion Protein Pull-Down Experiments

Protocol	Step(s)	Action	Total time	Hands-on time
Support Protocol 1	1–2	Streak out glycerol stock	Overnight[a]	20 min
	3	Inoculate overnight culture	Overnight	20 min
	4–7	Dilute bacteria and induce GST- fusion–protein expression	1 day[a]	1–2 hr
	8–21	Purification of recombinant GST fusion proteins	1 day (including SDS-PAGE)	1 day (with breaks)
	22–23	Normalize protein concentration on beads	3–4 hr (including SDS- PAGE)[a]	1–2 hr
Support Protocol 2	All	Cross-linking of beads	5–6 hr (including SDS- PAGE)[a]	3–4 hr (with incubations)
Support Protocol 3	—[b]	Abattoir and tissue collection	1–2 hr	1–2 hr
	1–20	Tissue lysate preparation	1 (long) day, less with help[a]	1 day (with some breaks)
Basic Protocol 1 or Alternate Protocol	All, except gels	Pull-down experiment	1 day[a]	1 day (with breaks)
	21 (Basic Protocol 1 and Alternate Protocol)	Large-format SDS-PAGE	Overnight	1 hr
Basic Protocol 2	1a–5a	Staining gels with colloidal Coomassie stain	1–3 days	20 min
	1b–5b	Staining gels with zinc-imidazole negative stain	1 hr	30 min
	6–12	Gel scanning and band excision	2–4 hr[a]	2–4 hr
13a–19a	12a–18	Coomassie gel piece destaining	2–3 hr[a]	1–2 hr
13b–19b	12b–18	Zinc-imidazole gel piece destaining	1–2 hr[a]	1 hr
	20–23	In-gel digestion	6 hr to overnight	1–2 hr
	24–30	Peptide extraction	2–4 hr[a]	1–3 hr
Support Protocol 4	All	Recycling GSH beads	6–7 hr (including SDS-PAGE)	2–3 hr

[a]Product can be stored for prolonged period of time at conclusion of steps.
[b]See recipe for sheep brains in Reagents and Solutions.

tant/domain/activation state of the protein relative to others), but at the same time do not bind to the external controls (e.g., GSH or GST beads). These proteins are likely to be functionally relevant protein-protein interaction partners, since their interaction is specific to one state of the GST fusion protein under study. In Figure 17.5.5, the most striking results are the eight bands that bind specifically to GTP-loaded GST-RalA beads, between 70 and 103 kDa. These were identified by MALDI-TOF MS as the eight components of the mammalian exocyst complex (Brymora et al., 2001b). The proteins were identified by peptide mass mapping and comparison with the mammalian nonredundant database, and it can be seen that this approach has identified the proteins—regardless of the fact that they are all are from sheep lysate—as being identical to exocyst sequences from human, rat, and mouse sources. Two of the identified proteins were identified as novel human proteins (FLJ10893 and KIAA0919) and subsequently through bioinformatic analysis as new exocyst complex proteins (hSec3 and hSec15B, respectively). It is apparent that the pull-down approach, combined with the power

of mass spectrometric analysis, enabled the identification of a new, GTP-dependent binding complex for the small GTPase RalA. It should also be noted that even though this gel lacks most of the required external controls (e.g., recombinant GST beads, GST-RalA beads alone), it is still possible to assign specificity of binding to the exocyst bands, since they do not bind to the other internal states of GST-RalA (i.e., unloaded and GDP-loaded GST-RalA).

Proteins that bind to all states of the GST fusion protein beads, but not the external controls, may also be relevant binding proteins that are not regulated under the conditions used in the pull-down experiment. Also, the issue may simply be that the protein-protein interaction is a more stable, less dynamic interaction that fulfills a different cellular purpose. This type of interaction requires more characterization to establish its relevance (see Strategic Planning). It is possible that the correct conformational change for an alteration in binding could not be achieved in vitro with the GST fusion protein, but it might be achieved in a different system. It is also possible that they are nonspecifically binding proteins, so it is necessary to run sufficient controls to determine if this is so. In Figure 17.5.5, the protein bands marked "N" are a good example of where insufficient controls limit data interpretation. It is impossible to determine from this experiment (without using MS analysis to identify every protein band) if they represent nonspecific binding of sheep brain proteins to the recombinant GST part of GST-RalA, fragments or polymers of GST-RalA (e.g., the bands marked G), copurified bacterial contaminants, or specifically binding proteins that bind in a nucleotide-independent manner. This experiment could have been strengthened considerably (for the interpretation of these bands) by the addition of a recombinant GST with lysate control (to eliminate recombinant GST–binding proteins), and also by running an equal amount of lysate-untouched GST-RalA (to see which bands originate from GST-RalA or bacterial contamination). After these control conditions have been taken into account, it probably could be determined whether these bands are specific binding proteins.

Proteins that are present throughout all lanes (external controls and pull-down lanes) are not likely to be genuine interactions and are most likely to be nonspecific. In Figure 17.5.5, the bands marked as endogenous GSTs are a prime example of this (the level of endogenous GST in this experiment could have been reduced significantly by repeated preclearing; see Critical Parameters and Troubleshooting). However, if there is a difference in the intensity of staining of these nonspecific bands that is disproportionate with the level of GST fusion protein coupled to the beads, there may also be a specifically binding protein that is comigrating on SDS-PAGE with the nonspecific protein. Identification of such bands requires careful analysis.

Additionally, it should be noted that identification of specific types of proteins should be viewed with caution. Cytoskeletal proteins and chaperones—notably tubulin, actin, myosin heavy chain, and hsc70—are often found in pull-down experiments to some degree. Be careful when ascribing specific binding to any of these proteins, as they are commonly present in pull-down experiments and are mostly bound nonspecifically or trapped in the spin column by filtration.

Time Considerations

An estimation of the time required for each group of steps is described in detail in Table 17.5.3. Several steps have been given a relatively wide time estimate (e.g., 4 to 6 hr), due to the number of samples being processed, experience of the investigator, and other experimental variables. Time taken to perform these experiments may actually be longer than described herein when particularly large numbers of samples need to be processed. Steps that include the time required for performing SDS-PAGE (indicated) can be shortened within 1 day by ~2 hr by freezing the samples in SDS sample buffer at −20°C and running the gels at a later time.

Literature Cited

Berggren, K.N., Schulenberg, B., Lopez, M.F., Steinberg, T.H., Bogdanova, A., Smejkal, G., Wang, A., and Patton, W.F. 2002. An improved formulation of SYPRO Ruby protein gel stain: Comparison with the original formulation and with a ruthenium II tris (bathophenanthroline disulfonate) formulation. *Proteomics* 2:486-498.

Brymora, A., Cousin, M.A., Roufogalis, B.D., and Robinson, P.J. 2001a. Enhanced protein recovery and reproducibility from pull-down assays and immunoprecipitations using spin columns. *Anal. Biochem.* 295:119-122.

Brymora, A., Valova, V.A., Larsen, M.R., Roufogalis, B.D., and Robinson, P.J. 2001b. The brain exocyst complex interacts with RalA in a GTP-dependent manner: Identification of a novel mammalian Sec3 gene and a second Sec15 gene. *J. Biol. Chem.* 276:29792-29797.

Cantor, S.B., Urano, T., and Feig, L.A. 1995. Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. *Mol. Cell. Biol.* 15:4578-4584.

Castellanos-Serra, L. and Hardy, E. 2001. Detection of biomolecules in electrophoresis gels with salts of imidazole and zinc II: A decade of research. *Electrophoresis* 22:864-873.

Coligan, J.E., Dunn, B.M., Speicher, D.W., and Wingfield, P.T. (eds.). 2003. Current Protocols in Protein Science. John Wiley & Sons, New York.

Fernandez-Patron, C., Hardy, E., Sosa, A., Seoane, J., and Castellanos, L. 1995. Double staining of Coomassie blue-stained polyacrylamide gels by imidazole-sodium dodecyl sulfate-zinc reverse staining: Sensitive detection of Coomassie blue–undetected proteins. *Anal. Biochem.* 224:263-269.

Frech, M., Schlichting, I., Wittinghofer, A., and Chardin, P. 1990. Guanine nucleotide binding properties of the mammalian RalA protein produced in *Escherichia coli*. *J. Biol. Chem.* 265:6353-6359.

Glebska, J., Grzelak, A., Pulaski, L., and Bartosz, G. 2002. EDTA loses its antioxidant properties upon storage in buffer. *Anal. Biochem.* 311:87-89.

Graves, P.R. and Haystead, T.A. 2002. Molecular biologist's guide to proteomics. *Microbiol. Mol. Biol. Rev.* 66:39-63.

Harper, S. and Speicher, D.W. 1997. Expression and purification of GST fusion proteins. *In* Current Protocols in Protein Science (J.E. Coligan, B.M. Dunn, D.W. Speicher, and P.T. Wingfield, eds.) pp. 6.6.1-6.6.21. John Wiley & Sons, New York.

Larsen, M.R. and Roepstorff, P. 2000. Mass spectrometric identification of proteins and characterization of their post-translational modifications in proteome analysis. *Fresenius J. Anal. Chem.* 366:677-690.

Menard, L., Tomhave, E., Casey, P.J., Uhing, R.J., Snyderman, R., and Didsbury, J.R. 1992. Rac1, a low-molecular-mass GTP-binding-protein with high intrinsic GTPase activity and distinct biochemical properties. *Eur. J. Biochem.* 206:537-546.

Moskalenko, S., Henry, D.O., Rosse, C., Mirey, G., Camonis, J.H., and White, M.A. 2002. The exocyst is a Ral effector complex. *Nat. Cell Biol.* 4:66-72.

Moskalenko, S., Tong, C., Rosse, C., Camonis, J., and White, M.A. 2003. Ral GTPases regulate exocyst assembly through dual subunit interactions. *J. Biol. Chem.* 278:51743-51748.

Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9:255-262.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 68:850-858.

Sugihara, K., Asano, S., Tanaka, K., Iwamatsu, A., Okawa, K., and Ohta, Y. 2002. The exocyst complex binds the small GTPase RalA to mediate filopodia formation. *Nat. Cell Biol.* 4:73-78.

Takai, Y., Sasaki, T., and Matozaki, T. 2001. Small GTP-binding proteins. *Physiol. Rev.* 81:153-208.

Contributed by Adam Brymora,
Valentina A. Valova,
and Phillip J. Robinson
Children's Medical Research Institute
Westmead NSW, Australia

Measuring Protein Interactions by Optical Biosensors

Optical evanescent wave biosensors, such as surface plasmon resonance, resonant mirror, or waveguide biosensors, have been increasingly used during the last several years for the characterization of protein-protein interactions (Margulies et al., 1993, 1996; Malmborg and Borrebaeck, 1995; Garland, 1996; van der Merwe and Barclay, 1996; Schuck, 1997a). This includes both the determination of equilibrium binding constants, which reveal the magnitude of the binding energy, as well as the determination of the bimolecular rate constants describing the kinetics of the interaction.

Studying protein interactions at a surface, as opposed to solution methods, has at least two fundamental advantages. First, the magnitude of the signal involved is independent of the affinity of the interaction over a wide range. Because one binding partner is confined to the surface (in the following referred to as ligand), the number of free binding sites that are under observation in an experiment is determined predominantly by the surface immobilization procedure (requiring usually only very small amounts of material). Therefore, the sensitivity of the method is limited essentially by the availability of the soluble binding partner (the analyte) in a well-defined state, at concentrations in the order of the inverse affinity constant. This allows studies of interactions with affinities spanning a range of at least 10^5 to 10^{10} M^{-1}, and makes this method very attractive, in particular, for high-affinity interactions. Second, the separation of the surface-immobilized sites from the mobile binding partners in solution, if combined with an appropriate fluidic handling system, is virtually ideally suited for the real-time observation of the kinetics of the binding processes. Chemical on-rate constants of up to 10^5/Msec to 10^6/Msec and off-rate constants of between 10^{-5}/sec and 10^{-1}/sec are generally accessible. Information on the chemical on-rate constants, and on the lifetime of the complexes formed, can be of great value, for example, in the study of interactions involved in cellular signaling processes.

However, these fundamental advantages are gained at the expense of additional experimental difficulties intrinsic to surface binding studies. These include the need for immobilization of one reactant at a relatively high local surface density (and the execution of binding studies under these conditions), the problems of nonspecific surface binding, possible multiple attachments to the surface through multivalent interactions, the problem of mass transport in kinetic experiments, and finally the possibility of surface potentials influencing the thermodynamics of the interaction (Leckband et al., 1995; Leckband, 1997).

Although the different commercial instruments for optical biosensing are based on slightly different physical principles, they all exploit surface-confined electromagnetic fields for the precise and real-time measurement of the refractive index of the medium in the immediate vicinity of the sensor surface (Garland, 1996; Knoll, 1998; Lukosz, 1991). When ligands are immobilized to the sensor surface, reversibly interacting analytes will accumulate at the sensor surface when brought into the solution above. By virtue of the difference of the refractive index increments of proteins and most aqueous buffer solutions, an increase of the total refractive index at the surface will then be reported (Fig. 17.6.1). Removal of the analyte from the solution causes dissociation of the reversibly bound molecules from the surface sites, which is accompanied by a decrease in the refractive index at the sensor surface, generating a decrease in the signal. Since the changes in signal are, to a good approximation, proportional to the changes in the mass of surface-bound analyte, the steady-state signals can be analyzed in terms of the law of

Contributed by Peter Schuck, Lisa F. Boyd, and Peter S. Andersen
Current Protocols in Cell Biology (2004) 17.6.1-17.6.22

mass action and Langmuir isotherms. The time course of the signal can be interpreted in the context of chemical binding kinetics. This basic principle, and consequently the derived strategies for studying protein-protein interactions, as well as most of the experimental procedures and potential complications, are the same in the different commercial instruments. Among the manufacturers are Biacore (*http://www.biacore.com*), Affinity Sensors (*http://www.affinity-sensors.com*), Intersens Instruments (Amersfoort, Netherlands), BioTul (*http://www.biotul.com*), and Artificial Sensing Instruments

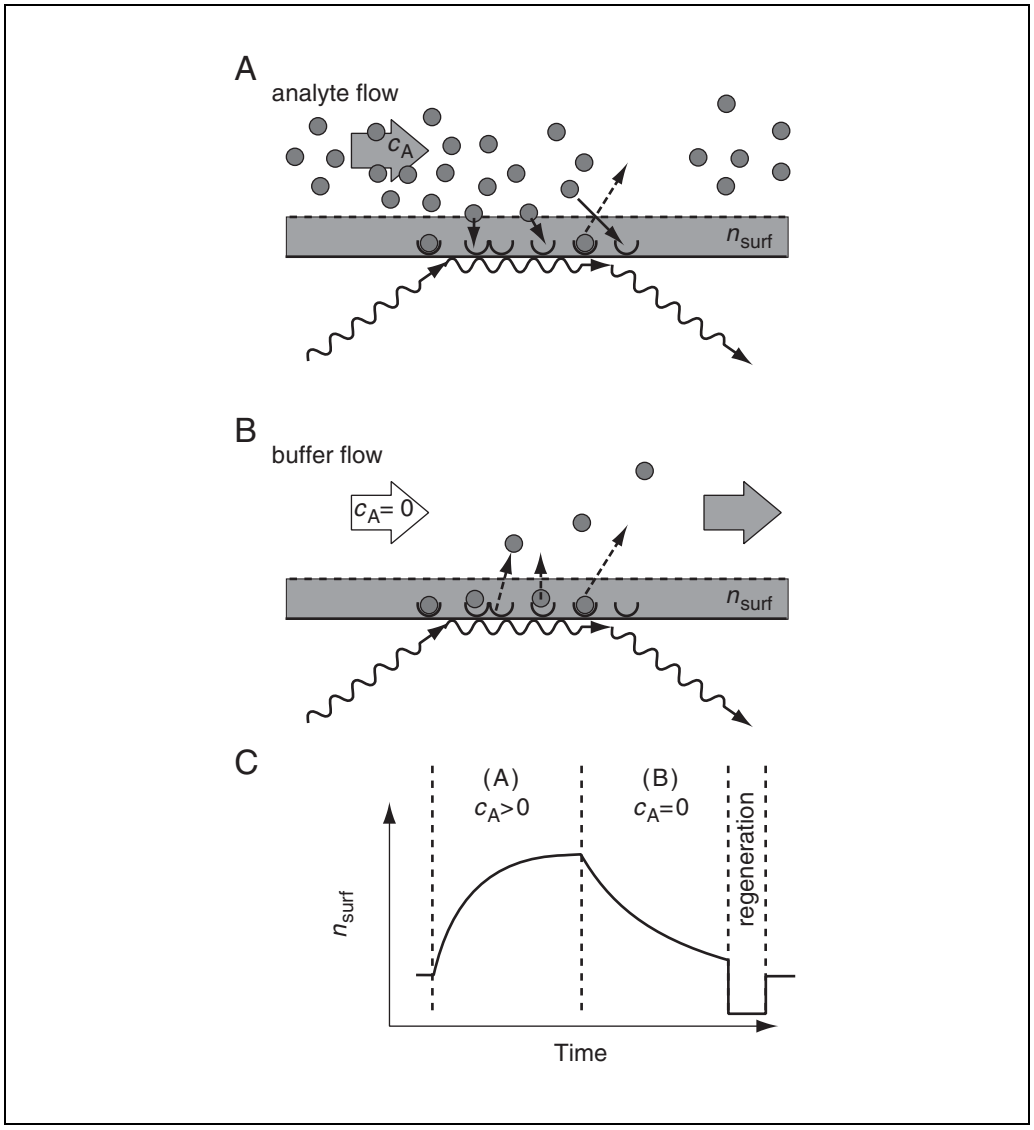


Figure 17.6.1 Schematic presentation of a typical optical biosensor experiment. Light is coupled into a structure that allows generation of surface-confined electromagnetic waves (e.g., surface plasmons in a gold film, or modes of a planar waveguide), which are sensitive to the refractive index of the solution in the range of the evanescent field, n_{surf}. Typical penetration depths of the sensitive volume into the solution are in the order of 100 nm. Ligands are attached to the sensor surface, as indicated by half-circles. (**A**) When analytes (full circles) are introduced into the solution above the surface, reversible interactions with the ligand lead to association events at a rate governed by $k_{on}c_A c_{L,free}$ (solid arrows), and dissociation events at a rate $c_{complex}k_{off}$ (dashed arrows). In the association phase, association events outnumber dissociation events until a steady state is reached. (**B**) When the surface is washed with running buffer in the absence of analyte, only dissociation events take place. (**C**) Signal obtained from probing the refractive index n_{surf} during the sequential application of the configurations depicted in A and B, given in arbitrary units. Following the association phase (A) and the dissociation phase (B), usually a regeneration procedure is applied for removing all remaining analyte from the surface before a new experimental cycle takes place.

(ASI AG, Zürich, Switzerland). The purpose of this unit is to provide a brief introduction to these techniques for the quantitative characterization of biomolecular interactions.

At the time of the introduction of optical biosensors in biomedical research, these instruments were thought to offer a particularly simple technique for the rapid quantitative measurement of equilibrium binding constants as well as binding kinetics. The reader is cautioned that the apparent simplicity and rapidity of the method is deceptive, and that the ease of using some of the commercial data analysis software packages can be highly misleading. The reliability of many of the initially used approaches in this field could not be verified in subsequent critical assessments of the methods. However, the technique has evolved significantly during the last few years. This is mainly due to the refinement and increasing variety of immobilization strategies, the identification of sources of artifacts, the use of control experiments, and the maturation of the analytical techniques (most notably global modeling). Emphasis is given in this unit to introducing the reader to robust approaches that will result in reliable results. Although the technique is frequently presented as being label-free, one of the interacting proteins has to be attached in an active form to the sensor surface. Most frequently, this is accomplished through covalent coupling, which can introduce all the problems well-known in chromophoric labeling and related covalent protein modifications. For all these reasons, the use of this technique requires careful planning and the execution of a number of different experiments, with time and effort more or less comparable to any of the other techniques available for the study of protein-protein interactions.

Optical biosensors are very versatile for the study of protein interactions. However, as described below (see Strategic Planning), the choice of the experimental strategy is usually dictated by the proteins under investigation. Only general guidelines can be given for this stage of the experiment. Detailed procedures for some of the most commonly employed immobilization procedures are described, followed by an introduction to the different strategies for the binding studies, and a brief description of some of the most commonly encountered problems. Next, the need for control experiments is emphasized, and the execution of a variety of such control experiments is described. Finally, some ideas are given for simple troubleshooting. Obviously, this introductory unit cannot be even remotely complete, or cover many of the more advanced techniques such as assembly of protein complexes (Andersen et al., 1999; Schuster et al., 1993). For many of these, the reader will be referred to the specialized literature cited throughout this unit.

STRATEGIC PLANNING

The first step in planning a biosensor experiment for the measurement of the interaction of two proteins requires the choice of the binding partner to be immobilized and the type of binding experiment that will be employed (steady-state, kinetic, or competition experiment, see Binding Experiments and Data Analysis). This involves the consideration of several factors related to size, purity, and chemical properties of the protein, as well as the stoichiometry of the interaction. In general, this will require the protein samples to be well characterized with respect to these properties.

Size, Concentration, and Volume

Optical biosensors detect changes in the refractive index at the sensor surface due to analyte binding. This signal is superimposed by an offset from the bulk refractive index differences of the buffers used). Because the refractive index increment of proteins is very similar in units of weight concentration, the signal obtained from saturation of a given number of surface sites will increase with increasing molar mass of the soluble protein

(or other analyte). In some of the commercial instruments, the binding of an analyte with a molar mass of only 1000 Da can be detected without difficulty, given the commonly achievable surface densities of sites. There is virtually no upper limit for the size of the analyte. Size consideration is important before setting up the experiment if the two binding partners to be studied are of significantly different size. The choice of a larger species as the analyte is generally preferable because this provides a larger signal. However, the immobilization of a very small peptide is usually difficult to control, and in practice frequently leads to unsuitably high density of surface sites, which can introduce problems of steric hindrance and mass transport limitation (see discussion of Mass Transport Limitation). In the absence of any other considerations, it seems advantageous to immobilize the larger binding partner, in order to diminish the probability of steric hindrance problems brought about by binding to surface sites in close vicinity. This will also help to minimize the probability of the immobilization affecting the conformation of the binding epitope. On the other hand, peptide ligands often are far more resistant to the denaturing effects of repeated cycles of binding and regeneration, and can be expected to provide a more stable binding surface than most proteins.

If one of the proteins under study is available only in small quantities, it is advantageous to immobilize this species, because during the course of a study usually only a few functionalized surfaces are needed, each requiring usually only a few micrograms of material. The analytes are used in larger amounts; in one complete set of kinetic experiments volumes of the order of 100 µl at concentrations of ten times the K_D are typically required. In competition experiments, similar quantities of the immobilized species are additionally required. The active concentration of the analyte should be well known, because errors in this value will directly translate into proportional errors of both the binding constant K_D and the chemical on-rate constant k_{on}, resulting from analysis of the experiment (see Binding Experiments and Data Analysis).

Purity

The sample purity requirements in biosensor experiments can be relatively low. In principle, the criterion for sufficient purity is the absence of cross-reaction between the two samples, aside from the interaction of the proteins under study—i.e., that no binding occurs between any contaminating species in the ligand sample and in the analyte sample. In practice, this means that at least either one of the samples should be highly purified. However, very strict requirements have to be met to ensure the absence of contaminating multimeric aggregates of the mobile species (see discussion of Analyte Aggregates, below, and Davis et al., 1998).

Oligomeric Structure, Stoichiometry, and Multivalent Binding

It is important to avoid multiple attachment of a single analyte molecule to two or more surface sites. If this occurs, the lifetime of the surface-bound complex is greatly enhanced. This avidity effect depends strongly on the local density of surface sites (Muller et al., 1998), and the binding data obtained under these conditions are governed to a large extent by the specific properties of the functionalized surface, much more than the surface binding properties of the analyte. (It cannot be assumed that the avidity effect of multivalent binding to the sensor surface is similar to possible avidity effects in binding to cell surface receptors.) Therefore, if the proteins under study are subject to multimeric binding, the multivalent species must be chosen for immobilization. For example, in antibody-antigen interactions, the antibody should be immobilized and the antigen should be the mobile analyte (Fig. 17.6.2). For unknown stoichiometries, appropriate control experiments should be performed that can identify the presence of multivalent species. For the same reason, the presence of even very small amounts of reversibly formed

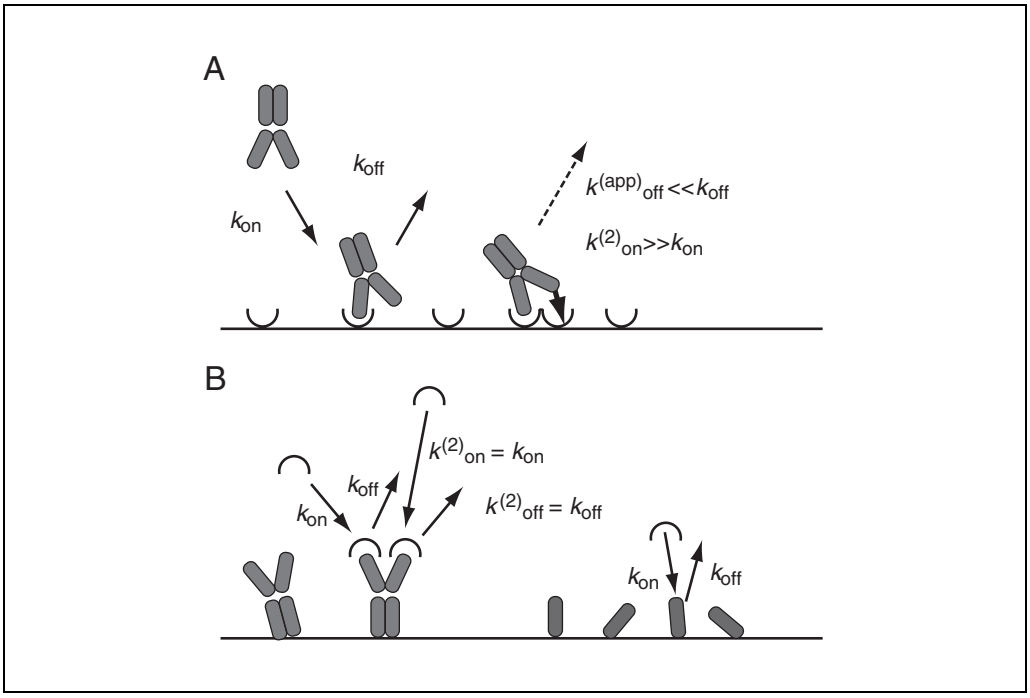


Figure 17.6.2 (**A**) The binding of a bivalent analyte can take place through the interaction with a single ligand molecule (left), or with two ligand molecules (right), depending on the local ligand density. If an unoccupied immobilized ligand molecule is within an accessible distance to an already singly bound analyte molecule, then the on-rate constant of the second interaction is strongly enhanced because of the entropic colocalization constraints. The overall dissociation rate constant of the double attached analyte is substantially reduced (usually by orders of magnitude), because it requires the virtually simultaneous dissociation of both interactions, which is much more improbable than a single dissociation event. The overall binding kinetics obtained from multivalent analytes is highly complex. (**B**) If the configuration is reversed, with the bivalent binding partner immobilized to the sensor surface, then both valencies act independently (left), and (in the absence of cooperativity of the sites) lead to binding kinetics that are identical to that of the monovalent interactions (right).

oligomers (self-association) or irreversibly formed oligomers can significantly interfere with the analysis of surface binding equilibria or kinetics. Because of the difficulties in detecting relatively weak or rapidly reversible oligomerization, solution techniques like analytical ultracentrifugation can be preferable to chromatographic or electrophoretic techniques.

Reactive Properties: Susceptibility to Immobilization, Nonspecific Binding, and Regeneration

The key to successful biosensor experiments is the immobilization of one protein species in an active, and, if possible, uniformly oriented state to the sensor surface. The ease of immobilization can be the major consideration for the choice of the immobilized species. Examples are the presence of biotin, histidine, or other tags on the molecule that allow specific capture, or the presence of a single accessible cysteine suitable for employing specific cross-linking chemistry. If a protein is produced by recombinant methods, appropriate tags can frequently be introduced.

Analogously, the protein that exhibits less nonspecific surface binding is the preferable choice for an analyte. Many sensor surfaces are slightly hydrophobic, and it is highly recommended to measure the extent of binding of both proteins to the nonfunctionalized surface at the maximal concentrations to be used in the binding experiments (e.g., at concentrations equal to 10-fold the expected dissociation equilibrium constant, K_D).

Significant degrees of nonspecific binding can make the analysis of the surface binding kinetics very difficult.

Most analytical strategies require a series of multiple association/dissociation cycles to be executed with the same surface. Therefore, conditions need to be established that allow removal of all surface-bound analyte, without permanently altering the conformation and activity of the immobilized ligand (but tolerating possible destruction of the analyte). Naturally, the choice of the regeneration procedure depends very much on the proteins. Frequently, helpful hints can be obtained from the procedure used to purify the ligand—e.g., conditions employed during ion-exchange or affinity chromatography. Examples of common regeneration procedures include exposure to low-pH glycine or HCl buffer for 1 or 2 min. For some sensitive ligands that interact weakly with their respective analytes, an extended washout with the binding buffer is the gentlest and most reliable regeneration procedure. After selection of a regeneration procedure, control experiments for the integrity of the surface and for the reproducibility of the observed signal should be performed. These should consist of several identical association/dissociation/regeneration cycles. Insufficient regeneration can be identified by residual signal increase after each cycle and/or a negative drift of the baseline. The loss of binding sites through too-harsh regeneration conditions is indicated by a maximal signal during the association phase, which decreases with each cycle. Regeneration is not required for the equilibrium-titration techniques (Myszka et al., 1998; Schuck et al., 1998).

IMMOBILIZATION PROTOCOLS

The goal of immobilization is the stable coupling of the ligand to the sensor surface in an active form. In addition to the obvious functional test of analyte binding during the course of the interaction study, probing the conformation with monoclonal antibodies against ligand epitopes can be highly useful. If possible, it is desirable to reverse the role of ligand and analyte to rule out artifacts of immobilization on the interaction. It is important to note that the immobilization methods differ in their requirement for the exposure of the ligand to relatively harsh conditions, such as pH values below the pI in the commonly employed amine coupling. A related consideration in the choice of the immobilization strategy is the orientation of the surface-immobilized ligand. Random coupling techniques, such as amine coupling, may create different subpopulations of differently reactive ligand molecules at the surface, while specific orientation techniques (e.g., through specific cysteines or through specifically attached biotin moieties) create uniform surfaces. An elegant method for allowing uniform surface attachment is the introduction of specific tags (such as cysteine or histidine) into recombinantly produced or synthetic ligands.

Below are basic descriptions of three of the most commonly used immobilization techniques, which can be applied to the most commonly employed carboxymethylated dextran sensor surfaces. In any of these methods it is very important to control the number of surface sites, since several surface-related artifacts are invoked if the surface density of the proteins under study is too high. The density of immobilized protein can be measured as the difference of the signal before and after immobilization, which can be transformed into the maximal analyte binding capacity by multiplication with the molar mass ratio of the analyte and ligand. For kinetic experiments, generally a relatively low density of immobilized protein should be obtained—i.e., a binding capacity of 50- to 200-fold of the instrumental noise [~50 to 200 units (RU) on a Biacore system]. For competition and for certain control experiments (see Competition Analysis), a larger binding capacity can be desirable. In practice, for initial orientation on the behavior of a particular system of interacting proteins, it is best to prepare several different surfaces of different ligand densities and to compare the analysis of each. This will be helpful for

diagnosing the regimes where mass transport limitations may be present (see Mass Transport Limitations), or where high surface coverage may lead to steric hindrance of analyte binding to neighboring surface sites (leading to biphasic surface binding kinetics).

Instruments that allow the on-line observation of a reference surface can be optimally utilized if the reference surface is treated as similarly as possible to the functionalized surface. This is important predominantly because of possible nonspecific interactions of the analyte with the sensor surface and the possible changes in surface properties due to immobilization. For amine coupling, this can include the application of the activation/deactivation cycle to the reference surface, but without cross-linking of any protein, or with cross-linking of a nonfunctional control protein (for example an unrelated antibody of the same isotype). For the same reason, if using biotin-streptavidin coupling, it is advantageous to immobilize streptavidin to both surfaces.

A variety of additional immobilization techniques are available in addition those described below, such as hydrazide coupling (O'Shannessy et al., 1992), Ni^{2+} chelate coupling (Gershon and Khilko, 1995; O'Shannessy et al., 1995; Sigal et al., 1996), coupling of hydrophobic groups (Stein and Gerisch, 1996), the use of self-assembled monolayers (Sigal et al., 1996) or supported lipid or hybrid bilayers (Plant et al., 1995; Ramsden et al., 1996), and immobilization to aminosilane-derivatized surfaces (Edwards et al., 1995; Buckle et al., 1993). For details on chemical modifications of the proteins, see Hermanson (1996). Additionally, capturing strategies can be used for reversible attachment of the ligand. More specific information on commercially available sensor surfaces, and step-by-step protocols for the corresponding immobilization techniques, can be found in the commercial instrument handbooks, such as the Biacore applications handbook or the IAsys applications notes.

Amine Coupling

Amine coupling is most frequently carried out using a sensor surface that is modified with carboxymethylated dextran. It relies on the electrostatic preconcentration of the ligand to the sensor surface, and therefore requires that the proteins be positively charged. The coupling buffer should be 1 to 2 pH units below the pI of the protein, of low ionic strength (to avoid charge screening), and not contain any primary amines. Effective pre-concentration can be tested before the activation of the surface. It is frequently necessary to test immobilization with several different buffers of slightly different pH; an adjustment by only one half pH unit can be crucial for a successful immobilization.

In a typical protocol, the carboxyl groups of the sensor surface are activated by exposure for 5 to 7 min with 50 mM *N*-hydroxysuccinimide (NHS) and 200 mM *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC). Next, the ligand is injected in 10 mM sodium acetate pH 4.5 buffer. To control the amount of cross-linked ligand, it is usually better to adjust the reaction time as compared to adjustment of the ligand concentration. Repeated injection/wash cycles can be used to differentiate the covalently attached ligand from electrostatically surface-bound ligand. Finally, the surface is deactivated with 1 M ethanolamine·HCl, pH 8.5, for 7 min. Biacore and IAsys provide kits and prepared buffers for this procedure. The NHS and EDC should be kept frozen separately, in aliquots, at −20°C until use.

Avidin- or Streptavidin-Biotin Coupling

Covalent immobilization of avidin or streptavidin allows the capture of biotinylated proteins. This method avoids the need to expose the protein to a pH lower than the pI, and therefore can better preserve activity. Procedures for biotinylating the analyte are found in Hermanson (1996). The affinity of biotin-avidin interaction is very high and leads to a

very stable surface. It should be noted that streptavidin-coated surfaces tend to be more sticky and lead to an increased level of nonspecific binding of the analyte to the surface. Therefore, good blank control surfaces are important.

The amine coupling protocol described above and a carboxymethylated dextran surface can be used for this procedure. The surface is activated with NHS/EDC (see discussion of Amine Coupling), the streptavidin (~100 μg/ml in buffer of pH 4.5 to 5.0) or avidin (~50 μg/ml in pH 4.5 buffer) is immobilized, and the surface is deactivated with ethanolamine. Note that, if commercially prepared streptavidin surfaces are used, this activation step can be omitted. The biotinylated protein is then injected; usually concentrations of 10 to 100 μg/ml and low flow rates are sufficient for efficient capture. Typical times for exposure of the surface are 10 to 30 min.

Thiol-Disulfide Exchange Coupling

This immobilization technique is frequently used for acidic proteins, for small peptides, and for cases when immobilization in a specific orientation is important. It also serves as a highly efficient coupling procedure that can be used if amine coupling or avidin-biotin coupling is unsuccessful. In this method, the immobilization is accomplished either by the introduction of an active disulfide group onto the sensor surface, which can react with thiols on the protein to be immobilized, or, vice versa, by the modification of the sensor surface with a thiol group, which then can react with active disulfides on the protein. The choice between these alternative procedures is dependent on the protein, i.e., the presence of accessible thiol groups, or the potential for introducing activated disulfide groups. The latter procedure may be accomplished, e.g., using 2-(2-pyridinyldithio)ethaneamine (PDEA) to derivatize carboxyl groups or succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT) for derivatizing amino groups. It should be noted that, in general, thiol-disulfide exchange coupling cannot be used for studies in the presence of reducing agents. However, the use of covalent thioester coupling—e.g., with succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), sulfo-SMCC, or *N*-(γ-maleimidobutyryloxy)succinimide ester (GMBS)—produces a surface that is stable against reducing agents, and allows one to specifically chose the site of coupling. In addition, the latter technique provides a small spacer between the protein and the surface, which may allow better accessibility (Khilko et al., 1995).

BINDING EXPERIMENTS AND DATA ANALYSIS

Optical biosensors provide data on the time course of analyte binding, and allow for a number of different experimental and analytical strategies for interaction analysis. Most rely on a repeated cycle of association, dissociation, and surface regeneration (Fig. 17.6.1). Among the most important analysis strategies are the kinetic analysis, which takes advantage of the real-time observation to obtain the kinetic rate constants of the reaction; the steady state analysis for measuring the affinity of the reaction; and the competition analysis for measuring the affinity of the interaction in solution. The choice may be constrained by the affinity and kinetics of the proteins under study. If possible, multiple approaches should be taken and the results should be compared for their consistency. Their optimal application requires approximate knowledge of the order of magnitude of the affinity of the interaction. This may make it necessary to perform these studies in several steps.

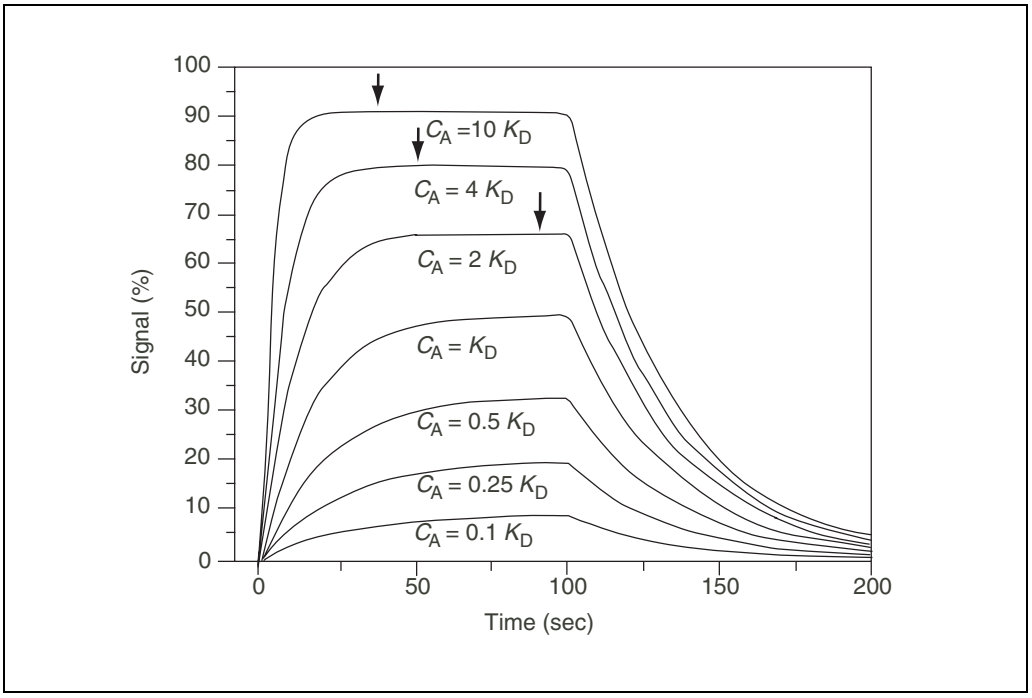


Figure 17.6.3 Schematic presentation of an ideal 1:1 pseudo first-order reaction. Shown is a superposition of the binding progress curves that should be obtained in a series of experiments at different analyte concentrations. The data are given in percent of the signal at maximal binding, but the units of the signal do not affect the results of the data analysis. The arrows on the curves at the higher concentrations indicate that steady-state binding is attained, whereas, for the lower curves, longer association times would be required as part of a steady-state analysis.

Kinetic Analysis

Experimental

The experiment consists of repeated injections of the analyte at different concentrations (Fig. 17.6.3). It should be conducted at relatively low surface density of sites, to avoid kinetic artifacts arising from mass transport limitation. An analyte concentration range as wide as possible should be covered (at least from 1/10 of the K_D to 10 times the K_D), using two-fold or three-fold dilutions. The time allowed for the surface binding phase (injection time) should be long enough for the observation of significant curvature in the binding progress, since it is only the curvature that contains the information about the reaction rate constants. It is highly desirable that steady-state binding be attained, at least for the highest concentrations used. The observation time for the dissociation phase should at least be similar to the injection time.

Data analysis

If the analyte-ligand interaction follows a pseudo first-order reaction, L + A $\Leftrightarrow$ LA then the measured binding progress (proportional to [LA]) is described by:

$$\frac{dR}{dt} = k_{on} c_A (R_{max} - R) - k_{off} R$$

Equation 17.6.1

where c_A denotes the analyte concentration, R denotes the signal (in arbitrary units), and R_{max} denotes the signal at maximum saturation of the surface sites. The goal is the calculation of the chemical on-rate and off-rate constants, k_{on} and k_{off}, which relate to the equilibrium constant as:

$$K_D = \frac{k_{off}}{k_{on}}$$

Equation 17.6.2

While it is possible, in principle, to transform the data as dR/dt versus R in the association phase and as $\log(R)$ versus time in the dissociation phase, where $k_{obs} = (k_{on}c_A + k_{off})$ and k_{off}, respectively, would represent the slopes of the obtained straight lines, this procedure is very unreliable. Instead, it is state-of-the-art procedure to directly fit Equation 17.6.1, or its integrated form:

$$R_{assoc}(t) = \frac{R_{max}}{1 + \frac{k_{off}}{k_{on}c_A}}[1 - \exp(k_{on}c_A + k_{off})t]$$

Equation 17.6.3

$$R_{dissoc}(t) = R_{assoc}(t_d)\exp[-k_{off}(t - t_d)]$$

Equation 17.6.4

directly to the data (with t_d denoting the end of the association and the beginning of the dissociation phase, where $c_A = 0$; O'Shannessy et al., 1993). This should be done without truncation of the data beyond possible regions of artifacts from buffer changes or injections. Global analysis should be employed, which allows one to analyze all experimentally observed curves at all analyte concentrations simultaneously. This results in unambiguous best-parameter estimates for the binding constants, and provides residuals that should be inspected for comparison of the model and the data. Software needed for global analysis can be obtained from the manufacturer or as shareware. It should be noted that exclusion of specific data regions that may be poorly described by the model will introduce a bias into the analysis.

If the global analysis cannot be performed, and separate analyses of the individual binding curves of each experiment according to Equations 17.6.3 and 17.6.4 are applied, then tests for consistency of the results are crucial (Schuck and Minton, 1996a). Important tests are: (1) the estimated extrapolated values of R_{assoc} ($t \rightarrow \infty$) at different concentrations should be consistent with a Langmuir isotherm for the estimated K_D (e.g., approaching half-saturation at $c_A = K_D$; see Figure 17.6.2); and (2) the extrapolated values of ($k_{assoc}c_A + k_{off}$) from the association phases should be consistent with the value of k_{off} as estimated in the dissociation phase. An analysis of the accuracy of the kinetic rate constants can be found in Ober and Ward (1999a).

Possible problems

The data may be only poorly described by Equations 17.6.3 and 17.6.4 (leading to a poor fit and systematically distributed residuals) and instead show multiphasic behavior. Although it is in principle possible to apply global modeling with multiple binding-sites models, this is not recommended, because it is very difficult and can easily lead to very large errors (up to several orders of magnitude). Such modeling is usually not very robust, and many different models may fit the same data equally well (Glaser and Hausdorf, 1996). The reader is also advised to be extremely cautious with some of the implemented models in commercial or shared software, because they may not be valid descriptions of real surface binding processes (for example, mass transport models or models for bivalent analytes). Frequently, the deviations of the data from the single exponential binding

progress predicted in Equations 17.6.3 and 17.6.4 are signatures of experimental artifacts, such as heterogeneity of the ligand (subpopulations of ligand with different binding properties through nonspecific immobilization), steric hindrance of binding to neighboring sites, or the presence of mass transport limitations or multivalent aggregates of the analyte (see discussion of Common Experimental Obstacles).

Steady-State Experiments and Binding Isotherm Analysis

This approach can yield both equilibrium constants and, if appended by a dissociation analysis, kinetic constants. Its main advantage with respect to the previously described technique (see discussion of Kinetic Analysis) is that it is much more robust and reliable because it is less affected by problems of potential multiphasic binding.

Experimental

If in the course of the association experiments a steady-state signal is observed, which is generally possible for interactions with relatively high k_{off}, then a binding isotherm can be constructed (Figs. 17.6.3 and 17.6.4). In an experimental procedure similar to the kinetic analysis, a concentration range as wide as possible should be covered, with two-fold concentration steps starting from both far below (≤1/10) K_D up to almost complete saturation of the surface sites, far above the K_D (≥ 10 times the K_D). The injection times required to attain steady state at the lower concentrations are longer (see Equations 17.6.3 and 17.6.4), and exponential extrapolation may be used, in particular in the Biacore system, since the association times may be limited in the by the limited volume in the injection loop of the microfluidics. Exponential extrapolation is not advised, however, for concentrations above K_D.

For interactions that do not yield steady-state binding within a tolerable time interval for the association phase, or where the ligand does not withstand a regeneration procedure, equilibrium titration represents an experimental variant (Fig. 17.6.4). In this approach, the analyte concentration is increased in two-fold steps, allowing for attainment of steady state in each step, avoiding regeneration between the steps. This can be achieved by addition of a small aliquot of the analyte into a fixed volume of solution above the sensor surface (in cuvette-based sensors; Hall and Winzor, 1997), into a loop of recycling sample (slightly modified Biacore X; Schuck et al., 1998), or into the running buffer reservoir of a standard Biacore (Myszka et al., 1998). The fixed-volume techniques require far less sample volume.

After the highest analyte concentration has been applied, the dissociation phase can be observed and analyzed in terms of Equation 17.6.4. This can give an estimate of k_{off}, which can be used in conjunction with Equation 17.6.2 to calculate k_{on} as k_{off}/K_D.

Data analysis

Although the data analysis could in principle be performed by Scatchard analysis, this is not recommended for statistical reasons. A better method is a plot of $R_{steady\text{-}state}$ versus $\log(c_A)$, in which the K_D can be easily estimated as the inflection point at half saturation of the typical sigmoid isotherm (Fig. 17.6.4). The functional form for modeling is:

$$R_{steady-state}(c_A) = \frac{R_{max}}{1 + K_D/c_A}$$

Equation 17.6.5

Even if the data cannot be fitted well with this expression, the 50% saturation will still represent a good estimate of the order of magnitude of an average K_D. It is important, however, in such an approach, to have enough data points at high analyte concentrations

for a reliable estimate of R_{max} and the 50% saturation level, respectively. Then the robustness will be greater than that of the kinetic analysis of Equations 17.6.3 and 17.6.4.

Competition Analysis

Competition experiments can be performed in different variations, but conceptually all use a calibration of the sensor signal as a function of free analyte concentration, followed by experiments with mixtures of analyte and soluble ligand in different molar ratios. The basic presumption is that the soluble ligand competes with the immobilized ligand for the

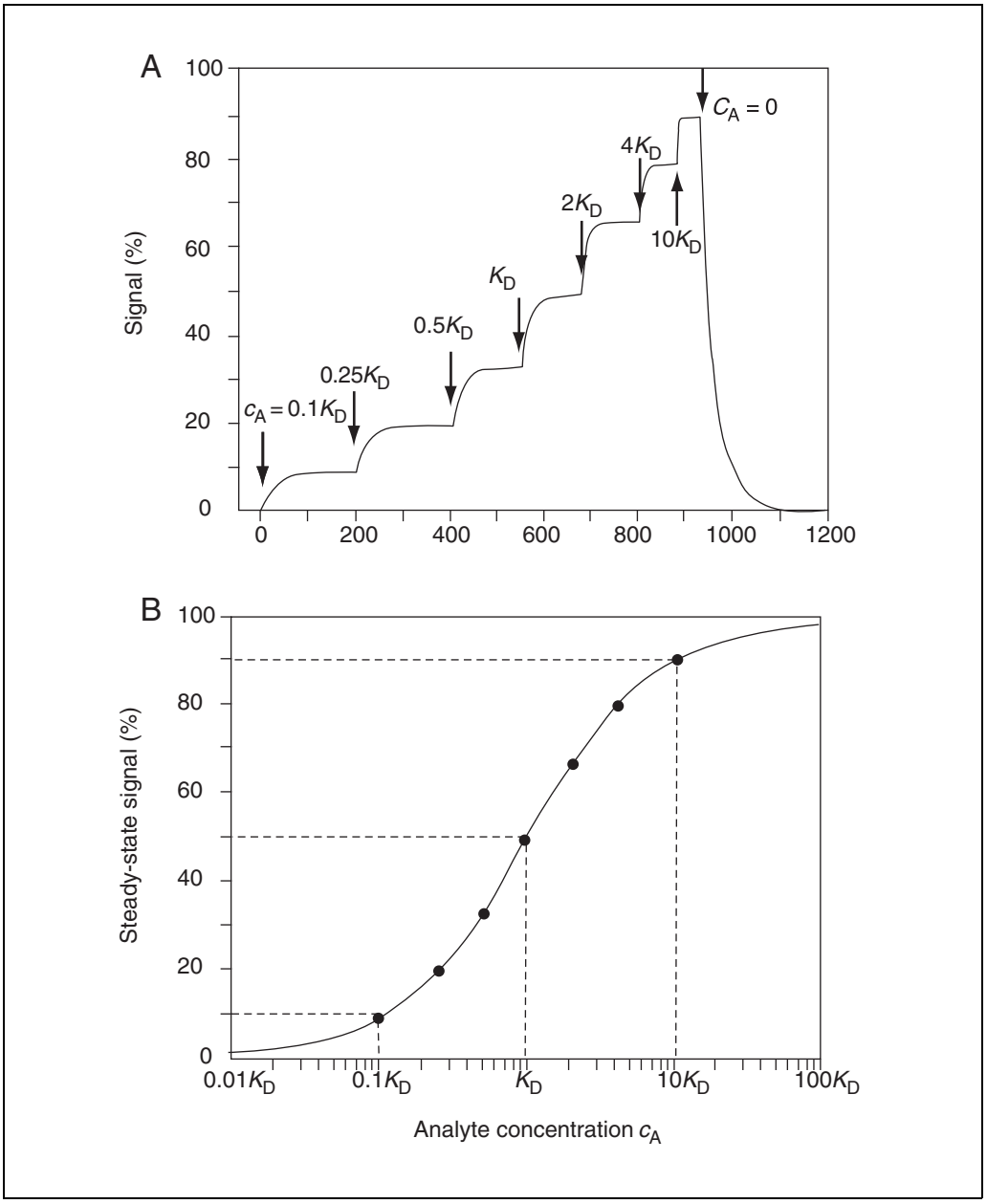


Figure 17.6.4 (**A**) Time course of an equilibrium titration experiment. Arrows indicate the time-points of the step-wise increase in the analyte concentration. (**B**) Steady-state binding analysis according to Equation 17.6.5. In a plot of the steady-state signal versus the base-10 logarithm of the analyte concentration, the binding isotherm exhibits a typical sigmoid shape. The inflection point at 50% saturation determines K_D, and the width of the curve is characterized by ~10% saturation at 1/10 K_D and ~90% saturation at 10 K_D (dotted lines). Visible inspection of the data in this representation already allows a robust parameter estimation.

free analyte, which leads to a reduction of the surface binding. This gives information on the solution affinity of ligand and analyte, while the freedom from the need to explicitly model the surface binding drastically diminishes the influence of potential surface artifacts on the results (Nieba et al., 1996; Schuck et al., 1998). This analysis can be extended to any other competitor that completely inhibits ligand binding of the analyte when bound to the analyte.

Experimental

For low-affinity systems with high chemical off-rate constants, in general, steady-state conditions can be achieved. Accordingly, the binding isotherm can be taken as a "calibration" of the sensor signal as a function of the free analyte concentration. Experiments to derive the surface binding constant can be conducted as described above (see Steady-State Experiments and Binding Isotherm Analysis). In a second variation, for high-affinity systems, mass-transport limited conditions are established by using a large immobilization density. Under these conditions, the initial binding rate constant will be directly proportional to the free analyte concentration ($dR/dt = k_t c_A$). The initial binding rate constant should be measured in equidistant concentration intervals for analyte concentrations up to a few times (two-fold) the K_D. After either one of these sets of calibration experiments, a set of experiments is performed using a constant concentration of analyte, $c_{A,tot}$ (which should be taken in the range of K_D), premixed and equilibrated with different concentrations of the soluble form of the ligand. The ligand concentrations, c_L, should be taken in two-fold steps, spanning the range from far below to far above the K_D. Choice of the fixed analyte concentration is important, since too high a value for $c_{A,tot}$ would show competition only in the range of stoichiometric binding. For systems that do not withstand regeneration, a recycling competitive titration procedure has been developed (Schuck et al., 1998).

Data analysis

For the high-affinity variant of the experiment with a linear initial rate of binding, $dR/dt = k_t c_A$, a plot of dR/dt versus c_A can be used to graphically calculate the amount of free analyte, $c_{A,free}$, during the competition experiment, or $c_{A,free}$ is calculated according to the analytical inversion $c_{A,free}(c_L) = dR/dt(c_L)/k_t$. Similarly, in place of the linear function, an empirical calibration function for $dR/dt(c_A)$ could be used. The obtained concentration of free analyte $c_{A,free}$ in experiments at different soluble ligand concentrations c_L can be plotted as $c_{A,free}$ versus $\log(c_L)$, which gives a competition isotherm with the characteristic sigmoid shape. It can be fitted with Equation 17.6.6, the solution of the quadratic equation of the mass action law combined with mass balance:

$$c_{A,free} = c_{A,tot} - \frac{1}{2}\left(c_{A,tot} + c_L + K_D^{sol} - \sqrt{(c_{A,tot} + c_L + K_D^{sol})^2 - 4c_{A,tot}c_L}\right)$$

Equation 17.6.6

which gives the solution binding constant of analyte and ligand, K_D^{sol}. If the low-affinity variant of the experiment was used, the steady-state analysis of the sensor response in the absence of soluble ligand can be performed according to Equation 17.6.5, giving R_{max} and $K_D^{surface}$. This can be used in a direct analysis of the competition steady-state response using:

$$R(c_L) = \frac{R_{max}}{1 + K_D^{surf} / \left[c_{A,tot} - \frac{1}{2}\left(c_{A,tot} + c_L + K_D^{sol} - \sqrt{(c_{A,tot} + c_L + K_D^{sol})^2 - 4c_{A,tot}c_L}\right)\right]}$$

Equation 17.6.7

Alternatively, both titrations can be analyzed simultaneously in a global fit to both isotherms, assuming the binding constants to the surface sites and in solution to be identical.

COMMON EXPERIMENTAL OBSTACLES

Two of the most important experimental problems are mass transport limitations and the effect of aggregates on the binding kinetics. The first difficulty is found most frequently (but not exclusively) in systems of medium to high affinity with high k_{on}, whereas the second is observed predominantly in systems with lower affinity, where higher analyte concentrations are required. Other obstacles can be steric hindrance effects of binding to adjacent surface sites (O'Shannessy and Winzor, 1996), or the contribution of second-order kinetics to the binding process in cuvette systems (Edwards et al., 1998). Most of these processes scale with the immobilization level of the ligand. For this reason, a low ligand density is generally advantageous in minimizing potential problems. Special considerations for working at very low ligand densities are described in Ober and Ward (1999a,b). The use of control experiments (e.g., comparison of the K_D from solution competition experiments with the K_D obtained from surface binding kinetic measurements) is very useful in verifying the absence of artifacts. The role of mass transport and analyte aggregates will be explained in detail below.

Mass Transport Limitation

If the supply of analyte to the sensor surface is the limiting factor in the surface binding rate, the observed binding kinetics are mass transport limited. This will depend directly on both the density of the immobilized sites, and on the chemical on-rate constant of the interaction. In the association phase, this will produce a zone of depleted analyte concentration in the vicinity of the surface, while in the dissociation phase, this will lead to a zone of nonvanishing free analyte concentration, which is subject to rebinding-retention effect (Silhavy et al., 1975; Fig. 17.6.5). The quantitative influence of this process on the observed surface binding kinetics is governed by a highly complex reaction/diffusion/convection process (Yarmush et al., 1996; Schuck, 1996). Although the processes in the association and dissociation phase are conceptually closely related, it cannot be assumed that the ratio of the "apparent" rate constants still reflects a good estimate of the equilibrium constant. Also, it is strongly recommended that the nonspecialist reader refrain from modeling using mass-transport-corrected kinetic models.

Instead, the primary goal is to identify mass transport limitations, and to establish experimental conditions avoiding transport effects. The following is a list of diagnostic indicators, in the order of reliability, for mass transport limited binding.

1. *Dependence on surface capacity.* This is a very good indicator of mass transport limitation, since the transport limit always directly scales with the density of active surface sites. For this reason, variations of the immobilization density of the ligand are recommended as routine tests demonstrating the absence of transport limitation and other surface-related artifacts. This test can fail in the presence of significant non-specific analyte binding to the surface.
2. *Increased dissociation rate when a soluble form of the ligand is injected (Fig. 17.6.5D).* This always strongly indicates rebinding (mass transport limitation) if detected. Since the ligand by itself should not interact with the surface, its effect is the binding to analyte near the sensor surface, preventing it from rebinding and allowing diffusion and washout from the surface (Fig. 17.6.5C). Unfortunately, this

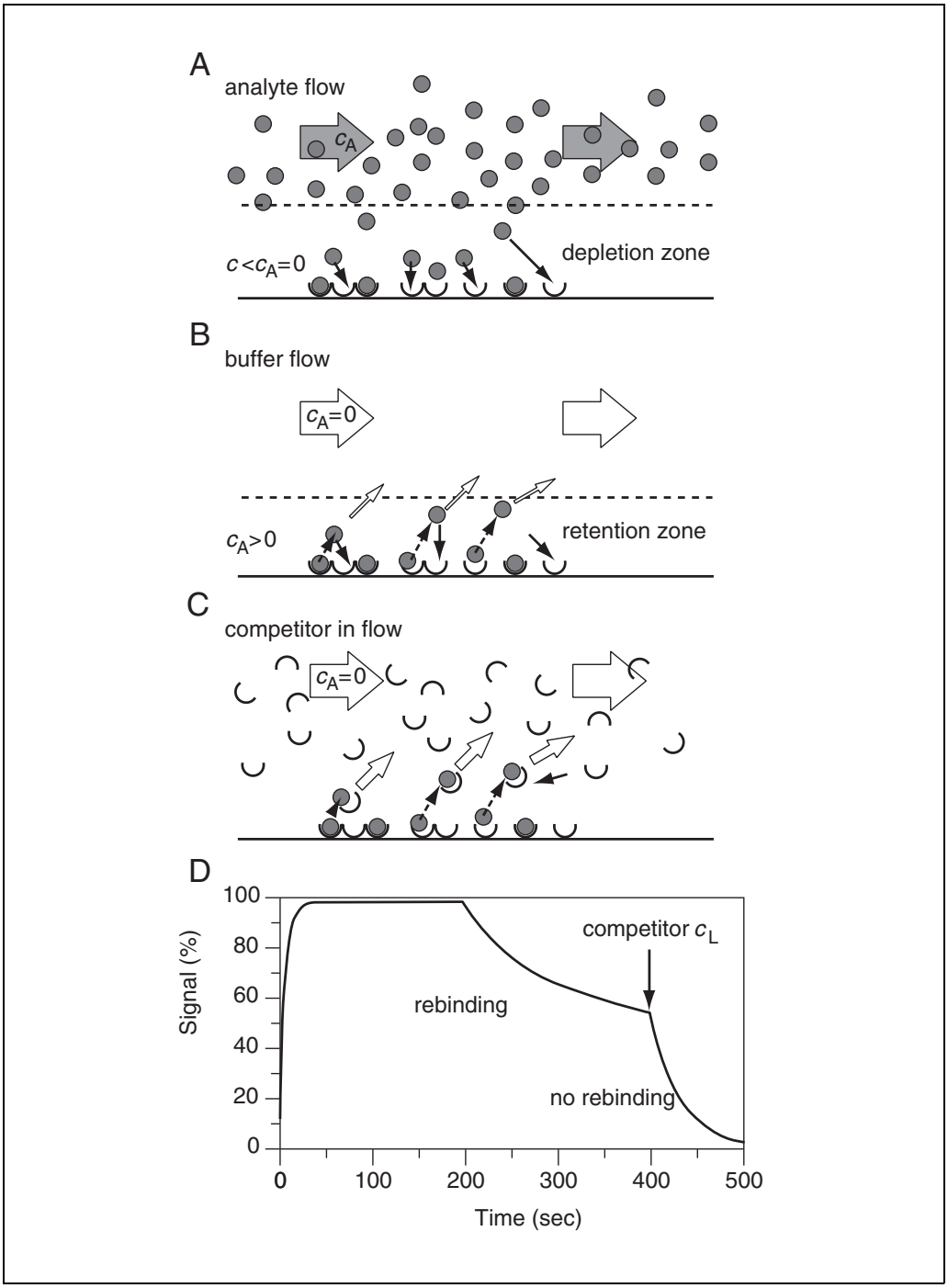


Figure 17.6.5 Mass transport effects on the surface binding process. (**A**) In the association phase, insufficient transport does not fully replenish the free analyte in a zone near the sensor surface (depletion zone). The inability to maintain the concentration c_A in the depletion zone leads to a limited surface binding rate. (**B**) The corresponding effect of mass transport limitation in the dissociation phase is that the surface is insufficiently washed and dissociated analyte is insufficiently removed. This generates a zone near the surface in which free analyte (after dissociation from the ligand) can rebind to empty ligand sites before diffusing into the bulk flow. This zone is indicated as retention zone. (**C**) Introduction of an excess of the soluble form of the ligand as a competitor into the buffer of the dissociation phase helps prevent rebinding to the surface. Soluble ligand can bind to dissociated analyte before rebinding takes place, and the soluble complex can diffuse into the buffer flow. This can allow the measurement of k_{off} free of mass transport effects. (**D**) Time course of dissociation during a mass transport induced rebinding situation (as depicted in panel B), and after introduction of a soluble competitor into the washing buffer (arrow). In the presence of the competitor, the signal reflects the chemical off-rate constant k_{off}, while in the rebinding situation, the apparent rate governing the signal is reduced.

effect may not be present for large ligands or ligands/analytes with high nonspecific binding if they exhibit limited diffusivity.

3. *Double exponential dissociation phase*. This will be seen only after dissociation from more than 50% occupation of all available surface sites.

4. *Weak dependence on the flow rate (stirring speed in cuvette-based systems).* Since the transport parameters only change with the cube root of the flow rate, i.e., generating only a factor of 2 when changing the flow rate by a factor of 10, this flow rate dependency can be difficult to detect. This is particularly true if the flow rate is only varied by a factor of 2, and the reaction is only partially transport influenced. Then, only a ~10% change in the apparent k_{off} would be expected in cases where the true k_{off} is 100% larger than the apparent k_{off}.

5. *Linear association phase*. This effect may not always be present, in particular at substantial transport limitation (Schuck, 1996; Schuck, 1997b; Schuck and Minton, 1996b).

Generally, tests 1 and 2 are the most reliable. They also lead to experimental techniques that can be utilized to reduce or eliminate mass transport artifacts. The most effective way for reducing mass transport influence is lowering the surface density of the immobilized ligand. Higher flow rates give only comparatively very small improvements, but are connected with strongly increased sample volume requirements. If the surface density of the ligand cannot be reduced further without leading to an insufficient signal-to-noise ratio, then switching from kinetic experiments to steady-state or competition steady-state experiments is the best solution. This will give information on the equilibrium constant. The kinetic rate constants can then be estimated best from a saturation experiment (approaching complete saturation of all surface sites), followed by a dissociation phase during which soluble ligand is coinjected. The soluble ligand will minimize rebinding and allow the estimation of the chemical off-rate constant, from which the chemical on-rate constant can be determined via Equation 17.6.3.

Analyte Aggregates

Oligomeric aggregates of analyte can be troublesome in biosensor experiments in two different ways. First, if trace amounts of higher oligomers are present in the analyte sample, this will lead, in the association phase, to a slow accumulation at the sensor surface, which can be visible as a slower second phase of binding. As depicted in Figure 17.6.2, these multimeric analyte aggregates can have multiple interactions with immobilized ligand molecules, and therefore they will dissociate much more slowly than the monomeric analyte. Consequently, they will appear in the dissociation phase as a submoiety with a very low off-rate constant (Davis et al., 1998). The troublesome trace amounts of oligomers can be eliminated by careful chromatographic purification, or their influence can be minimized by exchanging the role of analyte and ligand (Davis et al., 1998; Andersen et al., 1999). This is illustrated in the example of Figure 17.6.6, which demonstrates the importance of both the sample preparation and the choice of ligand and analyte.

The second potentially problematic form of aggregation is a surface-induced multimerization of the analyte. Because the local macromolecular concentrations at the sensor surface are very high (e.g., in the order of 10 mg/ml at a signal of 1000 RU in Biacore instruments), local crowding effects combined with non-specific interactions of the analyte can promote oligomer formation at the sensor surface (Fig. 17.6.7A; Minton, 1995, 1998). As with the influence of preformed aggregates, this process will lead to biphasic association and dissociation profiles, with the slower phase resulting from oligomer accumulation and dissociation, respectively. This process will be favored by

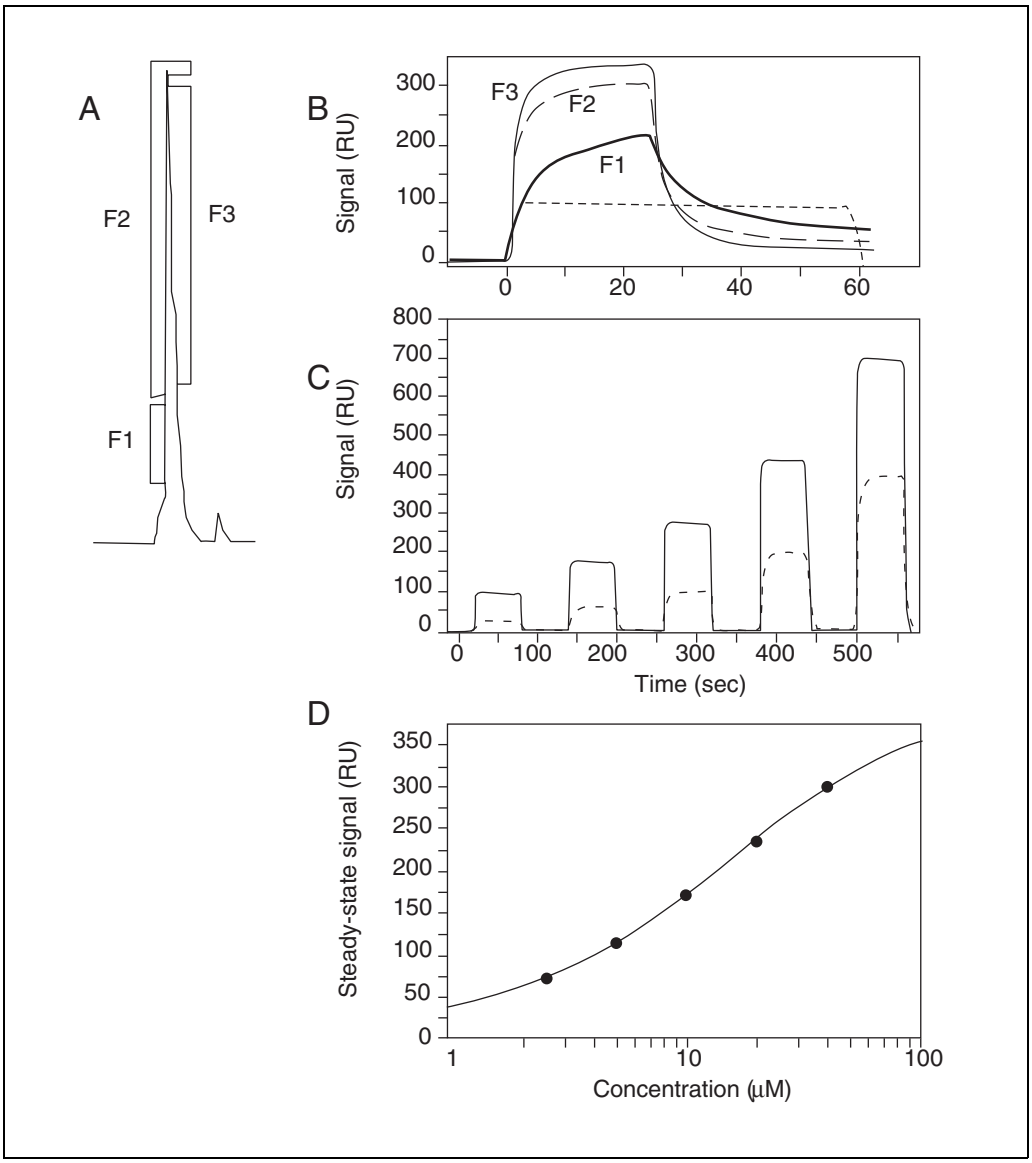


Figure 17.6.6 Exemplary data of the interaction of TCR with superantigen (for details of this interaction see Andersen et al., 1999). (**A**) Elution profile of the TCR from size-exclusion chromatography, indicating the fractions used for kinetic analysis of the interaction with immobilized superantigen, as shown in (**B**). It should be noted that fraction 1 (bold solid line), fraction 2 (dashed line), and fraction 3 (solid line) all exhibit multiphasic binding, with different relative magnitudes of the slower component. It should also be noted that despite the lower concentration of fraction 1, which results in the lowest response in the association phase, the signal in the dissociation phase is highest. This slower kinetic component in the binding progress curve reflects different amounts of aggregates bound to the sensor surface. The aggregates have a slower dissociation because of their multivalent attachment. For comparison, the same interaction is shown in the reverse orientation, with immobilized TCR and soluble superantigen (dotted line). (**C**) Sequence of association-dissociation curves at different superantigen concentrations, binding to immobilized TCR. In this orientation, the association and the dissociation is much faster, virtually monophasic, and the binding is completely reversible, which provides further evidence that the slow kinetic components introduced in the different fractions of the soluble TCR sample in Panel B are artifacts. For quantitative analysis, the signal measured at a nonfunctionalized surface (dotted line) is subtracted in order to remove the bulk refractive index contribution of the analyte. (**D**) A plot of the net steady-state binding signal versus superantigen concentration allows the measurement of the affinity of the interaction free from artifacts due to TCR aggregation that would be introduced in the configuration of Panel B.

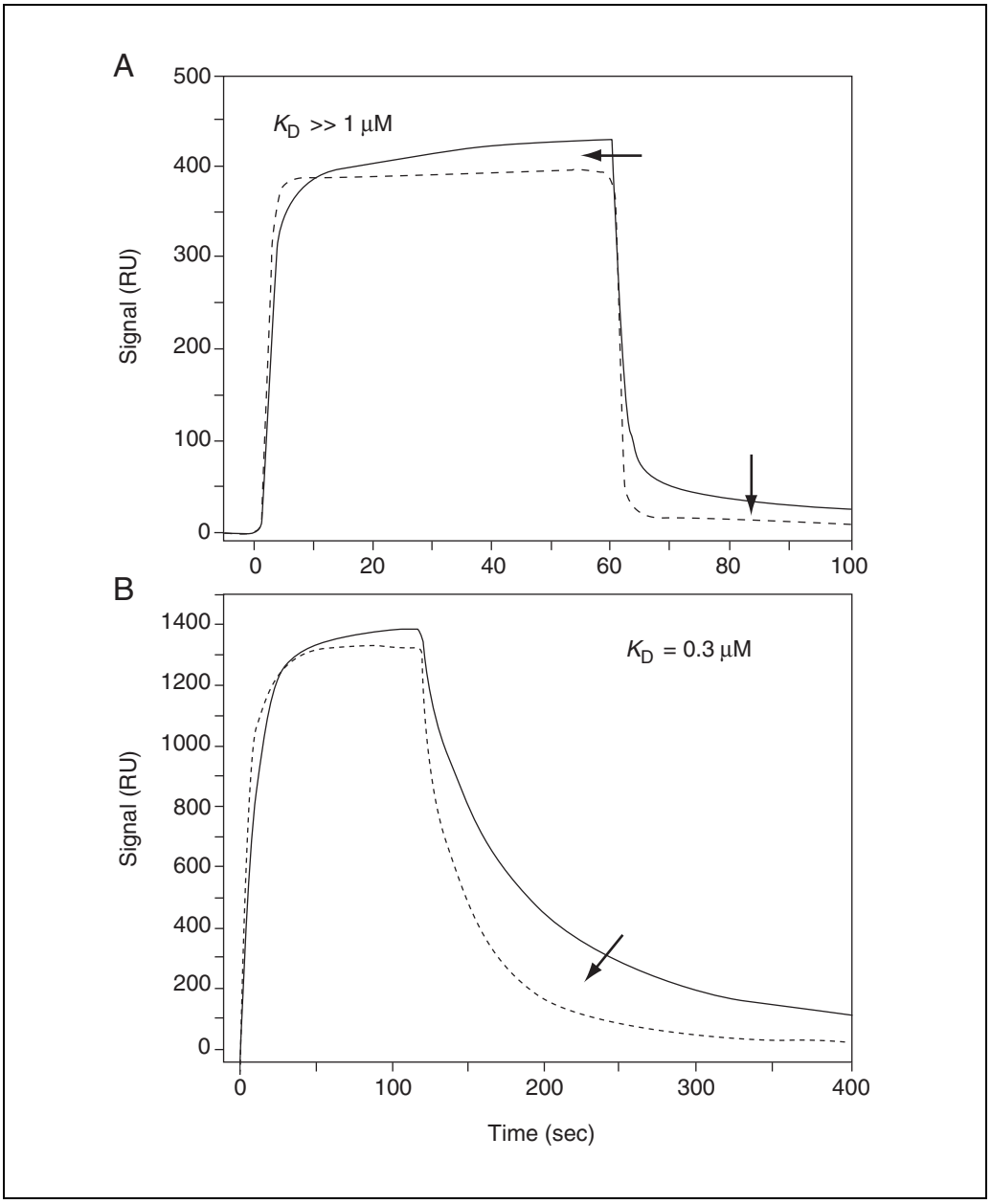


Figure 17.6.7 Effects of the ligand density on the binding kinetics for interactions with relative low affinity (**A**) and medium affinity (**B**). Each panel shows the interaction of immobilized superantigen with single-chain TCR, observed at high ligand surface density (1700 RU, solid lines) and low ligand surface density (700 RU, dashed lines). For easier comparison, the signal obtained at the lower density surface was scaled proportionally. In panel A, a slow phase of binding in the association phase and a residual binding (possible slow dissociation of multivalently bound aggregates) is introduced at high ligand density, under otherwise identical conditions. For the interaction in panel B, the chemical off-rate constant is smaller than for the low-affinity interaction shown above. Nevertheless, from comparison of the binding curves at different ligand surface densities (under otherwise identical conditions) it is obvious that an increased ligand density has significant effects on the surface binding kinetics. This observation could be due to aggregation or to mass transport limitations, both of which are more likely at higher ligand densities.

Table 17.6.1 Troubleshooting Guide for Measuring Protein Interactions by Optical Biosensors

Problem	Solution
No electrostatic preconcentration achieved; poor immobilization	Desalt protein (e.g., using spin column or microdialysis); decrease pH of buffer used for immobilization (should be below pI of protein).
Nonspecific binding is high	Analyte may be too hydrophobic, or there may be electrostatic interaction with surface. Increase salt or detergent concentration in running buffer.[a]
High signal from buffer; refractive index changes	If this does not result from high analyte concentrations, dialyze the analyte against running buffer, or use a spin column for buffer exchange.
Analyte does not come off after regeneration	Use increasingly harsher conditions for regeneration; test procedures used in affinity chromatography; check for strong nonspecific binding of the analyte; check for possible incomplete blocking of activated surface sites after immobilization.
No binding of biotinylated sample to streptavidin surface	Check for presence of free biotin in sample. Biotin on the analyte may not be accessible by surface-immobilized streptavidin; in this case try biotinylated linker.
Kinetics does not follow 1:1 binding	Check for mass-transport artifacts; check for possible traces of aggregates and for formation of aggregates at the surface; change immobilization method (avoid random coupling, avoid large surface densities). If this is not successful, go to steady-state analysis methods.
No steady-state binding is reached in a flow system	Use longer injections by increasing injection volume and/or decreasing flow rate. If sample volume is limiting, try an equilibrium titration.
Mass transport limitation	Decrease immobilization density.[b] If immobilization density cannot be lowered further, go to steady-state analysis, combined with establishment of lower limit of k_{dis} from dissociation after saturation.
Increasing slope in the association phase	Potential signature of mass transport; lower the immobilization density.
Increasing signal in the dissociation phase	Signature of mass transport limitation; lower the immobilization density.

[a]Be aware of effects of nonspecific binding on the binding kinetics, which substantially decreases the diffusivity of the analyte across the sensor surface, potentially leading to mass transport artifacts that cannot be detected through change of the ligand density.

[b]Increasing the flow rate affects transport much less, but consumes much more sample.

higher surface concentrations—i.e., higher density of immobilized ligand, and by higher ligand affinity (Fig. 17.6.7). As with mass transport limitations, they can be detected by variation of the surface density of the ligand. They also can be reduced by lowering the surface density of the immobilized ligand, by size-exclusion chromatography immediately prior to the experiment, or by exchanging the role of ligand and analyte.

Troubleshooting

As with other complex biophysical techniques and all investigations involving biological samples, it is impossible to give guidelines which are even nearly complete or generally helpful for troubleshooting. Nevertheless, Table 17.6.1 presents a small list of possible solutions to potential problems, given in the hope that some readers may find them helpful.

SUMMARY

Binding studies with optical biosensors can be very powerful and versatile. Among the most important virtues are their high sensitivity and utility for a broad range of affinities, real-time detection allowing studies of binding kinetics, and relatively low requirements of sample volume. We have outlined some general strategies and described some of the most commonly used techniques. The work with protein interactions at a surface can introduce additional experimental difficulties as compared to solution methods. As a general rule in experiments with optical biosensors, it is highly recommended that analyses be performed in different ways so that the consistency of the results can be tested. Biosensors can be an excellent tool in the study of protein interactions, and become particularly powerful if combined with other methods.

ACKNOWLEDGMENT

The authors are grateful for helpful comments by D. Margulies in the preparation of the manuscript. They acknowledge D. Kranz and K. Karjalainen for providing the samples used for some of the experiments shown. Peter Andersen acknowledges grant support by the Danish Natural Sciences Research Council.

LITERATURE CITED

Andersen, P.S., Lavoie, P.M., Sekaly, R.P., Churchill, H., Kranz, D.M., Schlievert, P.M., Karjalainen, K., and Mariuzza, R.A. 1999. Role of the T cell receptor alpha chain in stabilizing TCR-superantigen-MHC class II complexes. *Immunity* 10:473-483.

Buckle, P.E., Davies, R.J., Kinning, T., Yeung, D., Edwards, P.R., Pollard-Knight, D., and Lowe, C.R. 1993. The resonant mirror: A novel optical sensor for direct sensing of biomolecular interactions. Part II: Applications. *Biosens. Bioelectron*. 8:355-363.

Davis, S.J., Ikemizu, S., Wild, M.K., and van der Merwe, P.A. 1998. CD2 and the nature of protein interactions mediating cell-cell recognition. *Immunol. Rev*. 163:217-236.

Edwards, P.R., Gill, A., Pollard-Knight, D.V., Hoare, M., Buckle, P.E., Lowe, P.A., and Leatherbarrow, R.J. 1995. Kinetics of protein-protein interactions at the surface of an optical biosensor. *Anal. Biochem*. 231:210-217.

Edwards, P.R., Maule, C.H., Leatherbarrow, R.J., and Winzor, D.J. 1998. Second-order kinetic analysis of IAsys biosensor data: Its use and applicability. *Anal. Biochem*. 263:1-12.

Garland, P.B. 1996. Optical evanescent wave methods for the study of biomolecular interactions. *Q. Rev. Biophys*. 29:91-117.

Gershon, P.D. and Khilko, S. 1995. Stable chelating linkage for reversible immobilization of oligohistidine tagged proteins in the Biacore surface plasmon resonance detector. *J. Immunol. Methods*. 183:65-76.

Glaser, R.W. and Hausdorf, G. 1996. Binding kinetics of an antibody against HIV p24 core protein measured with real-time biomolecular interaction analysis suggest a slow conformational change in antigen p24. *J. Immunol. Methods.* 189:1-14.

Hall, D.R. and Winzor, D.J. 1997. Use of a resonant mirror biosensor to characterize the interaction of carboxypeptidase A with an elicited monoclonal antibody. *Anal. Biochem*. 244:152-160.

Hermanson, G.T. 1996. Bioconjugate Techniques. Academic Press, San Diego.

Khilko, S.N., Jelonek, M.T., Corr, M., Boyd, L.F., Bothwell, A.L.M., and Margulies, D.H. 1995. Measuring interactions of MHC class I molecules using surface plasmon resonance. *J. Immunol. Methods*. 183:77-94.

Knoll, W. 1998. Interfaces and thin films as seen by bound electromagnetic waves. *Annu. Rev. Phys. Chem*. 49:569-638.

Leckband, D.E. 1997. The influence of protein and interfacial structure on the self-assembly of oriented protein arrays. *Adv. Biophys*. 34:173-190.

Leckband, D.E., Kuhl, T., Wang, H.K., Herron, J., Muller, W., and Ringsdorf, H. 1995. 4-4-20 anti-fluorescyl IgG Fab′ recognition of membrane bound hapten: Direct evidence for the role of protein and interfacial structure. *Biochemistry* 36:11467-11478.

Lukosz, W. 1991. Principles and sensitivities of integrated optical and surface plasmon sensors for direct affinity sensing and immunosensing. *Biosens. Bioelectronics*. 6:215-225.

Malmborg, A.C. and Borrebaeck, C.A. 1995. Biacore as a tool in antibody engineering. *J. Immunol. Methods*. 183:7-13.

Margulies, D.H., Corr, M., Boyd, L.F., and Khilko, S.N. 1993. MHC Class I/peptide interactions: Binding specificity and kinetics. *J. Mol. Recognit*. 6:59-69.

Margulies, D.H., Plaksin, D., Khilko, S.N., and Jelonek, M.T. 1996. Studying interactions involving the T-cell antigen receptor by surface plasmon resonance. *Curr. Opin. Immunol*. 8:262-270.

Minton, A.P. 1995. Confinement as a determinant of macromolecular structure and reactivity. 2. Effects of weakly attractive interactions between confined macrosolutes and confining structures. *Biophys. J*. 68:1311-1322.

Minton, A.P. 1998. Molecular crowding: Analysis of effects of high concentrations of inert cosolutes on biochemical equilibria and rates in terms of volume exclusion. *Methods Enzymol.* 295:127-149.

Muller, K.M., Arndt, K.M., and Plückthun, A. 1998. Model and simulation of multivalent binding to fixed ligands. *Anal. Biochem*. 261:149-158.

Myszka, D.G., Jonsen, M.D., and Graves, B.J. 1998. Equilibrium analysis of high affinity interactions using Biacore. *Anal. Biochem*. 265:326-30.

Nieba, L., Krebber, A., and Plückthun, A. 1996. Competition Biacore for measuring true affinities: Large differences from values determined from binding kinetics. *Anal. Biochem*. 234:155-165.

Ober, R.J. and Ward, E.S. 1999a. The influence of signal noise on the accuracy of kinetic constants measured by surface plasmon resonance experiments. *Anal. Biochem*. 273:49-59.

Ober, R.J. and Ward, E.S. 1999b. The choice of reference cell in the analysis of kinetic data using Biacore. *Anal. Biochem.* 271:70-80.

O'Shannessy, D.J. and Winzor, D.J. 1996. Interpretation of deviations from pseudo-first-order kinetic behavior in the characterization of ligand binding by biosensor technology. *Anal. Biochem*. 236:275-283.

O'Shannessy, D.J., Brigham-Burke, M., and Peck, K. 1992. Immobilization chemistries suitable for use in the Biacore surface plasmon resonance detector. *Anal. Biochem*. 205:132-136.

O'Shannessy, D.J., Brigham-Burke, M., Soneson, K.K., Hensley, P., and Brooks, I. 1993. Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: Use of nonlinear least squares analysis methods. *Anal. Biochem*. 212:457-468.

O'Shannessy, D.J., O'Donnell, K.C., Martin, J., and Brigham-Burke, M. 1995. Detection and quantitation of hexa-histidine-tagged recombinant proteins on western blots and by a surface plasmon resonance biosensor technique. *Anal Biochem*. 229:119-124.

Plant, A.L., Brigham-Burke, M., Petrella, E.C., and O'Shannessy, D.J. 1995. Phospholipid/alkanethiol bilayers for cell-surface receptor studies by surface plasmon resonance. *Anal. Biochem.* 226:342-348.

Ramsden, J.J., Bachmanova, G.I., and Archakov, A.I. 1996. Immobilization of proteins to lipid bilayers. *Biosens. Bioelectronics* 11:523-528.

Schuck, P. 1996. Kinetics of ligand binding to receptor immobilized in a polymer matrix, as detected with an evanescent wave biosensor. I. A computer simulation of the influence of mass transport. *Biophys. J*. 70:1230-1249.

Schuck, P. 1997a. Use of surface plasmon resonance to probe the equilibrium and dynamic aspects of interactions between biological macromolecules. *Annu. Rev. Biophys. Biomol. Struct*. 26:541-566.

Schuck, P. 1997b. Reliable determination of binding affinity and kinetics using surface plasmon resonance biosensors. *Curr. Opin. Biotechnol.* 8:498-502.

Schuck, P. and Minton, A.P. 1996a. Minimal requirements for internal consistency of the analysis of surface plasmon resonance biosensor data. *Trends Biochem. Sci*. 252:458-460.

Schuck, P. and Minton, A.P. 1996b. Analysis of mass transport limited binding kinetics in evanescent wave biosensors. *Anal. Biochem*. 240:262-272.

Schuck, P., Millar, D.B., and Kortt, A.A. 1998. Determination of binding constants by equilibrium titration with circulating sample in a surface plasmon resonance biosensor. *Anal. Biochem*. 265:79-91.

Schuster, S.C., Swanson, R.V., Alex, L.A., Bourret, R.B., and Simon, M.I. 1993. Assembly and function of a quaternary signal transduction complex monitored by surface plasmon resonance. *Nature* 365:343-347.

Sigal, G.B., Bamdad, C., Barberis, A., Strominger, J., and Whitesides, G.M. 1996. A self-assembled monolayer for the binding and study of histidine-tagged proteins by surface plasmon resonance. *Anal. Chem*. 68:490-497.

Silhavy, T.J., Szmelcman, S., Boos, W., and Schwartz, M. 1975. On the significance of the retention of ligand by protein. *Proc. Natl. Acad. Sci. USA.* 72:2120-2124.

Stein, T. and Gerisch, G. 1996. Oriented binding of a lipid-anchored cell adhesion protein onto a biosensor surface using hydrophobic immobilization and photoactive crosslinking. *Anal. Biochem*. 237:252-259.

van der Merwe, P.A. and Barclay, A.N. 1996. Analysis of cell-adhesion molecule interactions using surface plasmon resonance. *Curr. Opin. Immunol*. 8:257-261.

Yarmush, M.L., Patankar, D.B., and Yarmush, D.M. 1996. An analysis of transport resistances in the operation of Biacore; implications for kinetic studies of biospecific interactions. *Mol. Immunol*. 33:1203-1214.

KEY REFERENCES

Davis et al, 1998. See above.

Contains a detailed description of analyte aggregation effects on the measured surface binding.

Nieba et al., 1996. See above.

Demonstration how competition approaches can be used to circumvent kinetic artifacts.

O'Shannessy et al., 1992. See above.

Collection of immobilization techniques.

Schuck, 1997b. See above.

General review of the method and its application.

INTERNET RESOURCES

http://www.biacore.com

Web site for Biacore; extensive list of published biosensor applications

http://www.affinity-sensors.com

Web site for Affinity Sensors; extensive list of published biosensor applications.

Contributed by Peter Schuck and
Lisa F. Boyd
National Institutes of Health
Bethesda, Maryland

Peter S. Andersen
University of Maryland
Rockville, Maryland

Chromatin Immunoprecipitation for Determining the Association of Proteins with Specific Genomic Sequences In Vivo

Chromatin immunoprecipitation (ChIP) is a powerful and widely applied technique for detecting the association of individual proteins with specific genomic regions in vivo. In this technique, live cells are treated with formaldehyde to generate protein-protein and protein-DNA cross-links between molecules in close proximity on the chromatin template in vivo. A whole-cell extract is prepared, and the cross-linked chromatin is sheared by sonication to reduce average DNA fragment size to ~500 bp. The resulting material is immunoprecipitated with an antibody against a desired protein, modified (e.g., acetylated, phosphorylated, methylated) peptide, or epitope (in situations where the protein of interest is epitope-tagged). DNA sequences that directly or indirectly cross-link with a given protein (or modified variant) are selectively enriched in the immunoprecipitated sample. Thus, the method is not restricted to sequence-specific DNA-binding proteins. Reversal of the formaldehyde cross-linking by heating permits the recovery and quantitative analysis of the immunoprecipitated DNA. The amounts of specific genomic regions in control and immunoprecipitated samples are determined individually by quantitative PCR. The fold enrichment of certain chromosomal sequences (e.g., presumed binding sites) relative to other chromosomal sequences (e.g., presumed nonbinding sites) provides quantitative information about the relative level of association of a given protein with different genomic regions. Protein association with specific genomic regions can be performed under a variety of conditions (e.g., environmental change, cell-cycle status) and/or in wild-type versus mutant strains. Furthermore, as formaldehyde inactivates cellular enzymes essentially immediately upon addition to cells, ChIP provides snapshots of protein-protein and protein-DNA interactions at a particular time point, and hence is useful for kinetic analysis of events occurring on chromosomal sequences in vivo. In addition, ChIP can be combined with microarray technology to identify the location of specific proteins on a genome-wide basis (see Commentary). This unit describes the ChIP protocol for cells of the baker's yeast *Saccharomyces cerevisiae* (see Basic Protocol); however, it is also applicable to other organisms, although some organism-specific modifications related to cell lysis and sonication are necessary. A protocol for eluting immunoprecipitated protein-DNA complexes is also provided (see Alternate Protocol 1). As an alternative to gel electrophoretic analysis of the PCR products, a quantitative PCR analysis in real time with SYBR Green is also provided (see Alternate Protocol 2).

CHROMATIN IMMUNOPRECIPITATION

BASIC PROTOCOL

Materials

Saccharomyces cerevisiae cells to be studied
37% formaldehyde: store up to 1 year at room temperature
2.5 M glycine, heat sterilized
TBS (*APPENDIX 2A*), ice cold
FA lysis buffer with and without 2 mM PMSF (see recipe), ice cold
ChIP elution buffer (see recipe)
20 mg/ml Pronase (Roche) in TBS; store up to 1 year at −20°C
TE buffer, pH 7.5 (*APPENDIX 2A*)
20 mg/ml DNase-free RNase A (see recipe)
10× loading buffer (see recipe)

Contributed by Oscar Aparicio, Joseph V. Geisberg, and Kevin Struhl
Current Protocols in Cell Biology (2004) 17.7.1-17.7.23

Primary antibody against protein or epitope of interest
50% (v/v) protein A-Sepharose beads (Amersham Pharmacia Biotech) or equivalent in TBS
FA lysis buffer (see recipe), room temperature
FA lysis buffer (see recipe)/0.5 M NaCl
ChIP wash buffer (see recipe)
Primers (see Critical Parameters and Troubleshooting)
3000 Ci/mmol [^{32}P]dATP (optional; see annotation to step 30)

2-ml screw-cap microcentrifuge tubes with (relatively) flat bottoms
~0.5-mm-diameter silica-zirconia (BioSpec; preferred) or glass beads
Mini bead beater (BioSpec; preferred) or individual or multivortexer
5-ml syringe
15-ml conical tubes, disposable
25-G needles
Sonicator with microtip probe (e.g., Branson Sonifier 250)
End-over-end rotator
0.5-ml PCR tube
Spin-X centrifuge-tube filter (e.g., Corning)
65°C water bath
PCR-purification spin column (Qiagen)
Software for analyzing PCR primers and products

Additional reagents and equipment for growth of *Saccharomyces cerevisiae* cultures (*APPENDIX 3A*), phenol/chloroform extraction and ethanol precipitation (*APPENDIX 3A*), PCR (*APPENDIX 3F*), agarose gel electrophoresis (*APPENDIX 3A*), and nondenaturing acrylamide gel electrophoresis (*UNIT 6.5*)

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Cross-link protein-DNA complexes in vivo

1. For each sample, grow 200 ml *Saccharomyces cerevisiae* to $OD_{600} = 0.6$ to 0.8.

   CAUTION: *Keep cultures covered or work in a fume hood to avoid noxious formaldehyde fumes.*

   The volumes of culture can be reduced (20 ml is a reasonable minimum) or increased depending on need. Typically, 20 to 40 ml yeast is used for an individual immunoprecipitation, so the 200-ml volume permits multiple immunoprecipitations from the same cells. This is particularly useful for experiments involving the analysis of multiple factors or for carrying out independent immunoprecipitations involving the same factor for data reproducibility.

2. Add 5.5 ml of 37% formaldehyde (1% final). Cross-link 15 to 20 min at room temperature by occasionally swirling flask or shaking slowly on a platform.

3. Add 30 ml heat-sterilized 2.5 M glycine and incubate an additional 5 min at room temperature.

   Glycine stops the cross-linking by reacting with formaldehyde.

Harvest cells

4. Centrifuge cells 5 min at 2500 × *g*, 4°C. Discard supernatant into a chemical waste container and resuspend pellet in 50 to 200 ml ice-cold TBS. Repeat once.

5. Centrifuge cells for a third time 5 min at 2500 × g, 4°C. Discard supernatant and resuspend cells in 10 ml ice-cold FA lysis buffer.

6. Pellet cells by centrifuging in a benchtop centrifuge 5 min at 3000 rpm, 4°C. Discard supernatant.

   The cells can remain on ice for a few hours while other samples are being collected so that all samples may be processed as a group from this point onward. Alternatively, the cells may be frozen in liquid nitrogen or a dry ice/ethanol bath and stored up to several months at −80°C. This is particularly helpful if multiple samples are being generated during a time-course experiment. If cells are frozen, they must be thawed on ice before continuing with the procedure.

Lyse cells and isolate chromatin

For lysis using a mini bead beater (preferred)

7a. Resuspend the cell pellet in 1 ml ice-cold FA lysis buffer/2 mM PMSF. Fill three-quarters of a 2-ml flat-bottomed screw-cap microcentrifuge tube with ∼0.5-mm-diameter silica-zirconia or glass beads. Add cells, taking care to avoid introduction of bubbles, and screw the cap on tightly. Make sure there are no leaks.

   The mini bead beater is recommended because it is more efficient at breaking cells (multiple samples can be broken simultaneously). Silica-zirconia beads are more efficient at breaking cells than glass beads and are also recommended. To facilitate cell breakage with the mini bead beater, it is important that the final suspension nearly fill the tube. Do not break >160 OD_{600} units of cells (i.e., <5 × 10^9 cells) in a single 2-ml tube; for larger cultures, split the cells into multiple tubes.

8a. Lyse cells 3 min with a mini bead beater at maximum speed. Remove sample and incubate 1 min in an ice-water bath. Repeat five times for a total breakage time of 18 min.

   This step assumes breakage with silica-zirconia beads. The cell breakage time with glass beads may be longer.

For lysis using an individual or multivortexer

7b. Resuspend in 250 µl FA lysis buffer/2 mM PMSF. Add 350 µl silica-zirconia or glass beads to a 2-ml microcentrifuge tube with relatively flat bottom. Add cells.

   When using a multivortexer (or standard vortexer), it is important to keep the volume small as this improves cell breakage. Do not break >160 OD_{600} units of cells (i.e., <5 × 10^9 cells) in a single 2-ml tube; for larger cultures, split the cells into multiple tubes.

8b. Vortex continuously on an individual or multivortexer 30 min at full speed, 4°C.

   Success and reproducibility of the ChIP procedure is aided by complete (or near-complete) breakage of cells. In this regard, formaldehyde-cross-linked cells are considerably harder to break than untreated cells. The use of 1.5-ml microcentrifuge tubes with conical bottoms should be avoided because the narrow shape constricts bead movement, resulting in unequal lysis among samples. The 2-ml microcentrifuge tubes have a nearly flat bottom that allows the beads to vortex vigorously. The indicated vortexing or bead-beating conditions should be tested if a different device is used.

Isolate lysate

9. Cut a 5-ml syringe ∼1 cm below the flared opening (i.e., where the plunger is inserted) with a razor. Insert the smaller portion into a 15-ml disposable conical tube so that the flared portion of the truncated syringe rests on top of the conical tube opening, forming a microcentrifuge-tube holder.

10. Invert the sample tube and punch a hole in the bottom with a 25-G needle. Place the sample tube into the syringe/conical tube and punch a hole in the top cover with the same needle.

11. Spin the assembly in a benchtop centrifuge 1 min at 1000 rpm, 4°C. Place the conical tube on ice. Discard the 2-ml centrifuge tube containing the dry beads after confirming the sample has been transferred to the 15-ml tube.

    Occasionally, beads will clog the pierced hole and prevent complete transfer of the sample. If this occurs, pierce the tube one or two more times and repeat the step in the same 15-ml tube. No additional buffer should be added.

Shear DNA

12. Transfer the sample to a standard 1.5-ml microcentrifuge tube. Microcentrifuge 15 min at maximum speed, 4°C. Discard the supernatant and add 1 ml ice-cold FA lysis buffer to the pellet.

    The pellet contains the cross-linked chromatin, cell debris, and unbroken cells. The purpose of this centrifugation step is to remove soluble protein, most of which is not cross-linked to DNA, as it might contribute to nonspecific background in the subsequent immunoprecipitations step. There is no need to resuspend the pellet at this point.

13. Holding the microtip probe near the bottom of the tube to prevent foaming, sonicate the sample 30 sec at 4°C using a continuous pulse at a power output of 20%. Cool in an ice-water bath >1 min. Repeat two more times.

    Take great care that the sample does not get too hot.

    If a different sonication device is used, empirically determine the conditions necessary to achieve the desired level of DNA shearing. The shear size is determined as described below (see Critical Parameters and Troubleshooting).

14. Microcentrifuge 30 min at maximum speed, 4°C. Transfer the supernatant to a fresh 15-ml disposable conical tube, add 4 ml ice-cold FA lysis buffer, and gently mix by inversion. Remove 250 µl for checking DNA fragment size and freeze the remaining chromatin solution in 800-µl aliquots in liquid nitrogen.

    Upon sonication, the cross-linked chromatin is solubilized and purified away from the pelleted material which contains cell debris and unbroken cells. The resulting chromatin solution constitutes the input sample for the subsequent immunoprecipitation. The frozen aliquots are stable for many months when stored at −70°C and are suitable for immunoprecipitations.

Check chromatin-fragment size

15. Add 250 µl ChIP elution buffer and 20 µl of 20 mg/ml Pronase in TBS to the 250-µl chromatin aliquot. Incubate 2 hr at 42°C, followed by 6 hr at 65°C. Phenol extract and ethanol precipitate sample (*APPENDIX 3A*).

    While it is convenient to perform the reaction in a PCR machine overnight, it could just as easily be done in heat blocks or water baths. The same is true of the incubation described in step 26.

16. Resuspend in 30 µl TE buffer, pH 7.5, add 1 µl of 20 mg/ml DNase-free RNase A, and incubate 15 min at 37°C. Add 3 µl of 10× loading buffer and electrophoretically separate the material on a 1.5% agarose gel (*APPENDIX 3A*).

    Fragments should be between 100 to 1000 bp, with an average length of 400 to 500 bp.

    It is important to shear DNA fragments down to an average length of 400 to 500 bp. Longer fragments will increase the background and will decrease the resolution of the region to which the protein associates (see Commentary).

Immunoprecipitate

17. Incubate 800 µl chromatin solution with 10 µl primary antibody against the protein or epitope of interest and 20 µl of 50% (v/v) protein A–Sepharose beads in TBS on an end-over-end rotator 90 min at room temperature.

The actual amount of antibody needed has to be empirically determined and can vary considerably. The idea is to have an excess of antibody to efficiently precipitate at least 50% of the antigen in question. One way to assess the efficiency of antigen immunoprecipitation is to determine the amount of antigen present in the sample before and after the immunoprecipitation. An aliquot of 30 µl chromatin solution, taken before and after immunoprecipitation, is usually sufficient to visualize the protein of interest via immunoblotting (UNIT 6.2) and standard chemiluminescent detection; however, the samples have to be boiled in SDS/PAGE sample buffer for 30 min prior to loading in order to reverse the formaldehyde cross-links. The immunoprecipitation conditions can be varied (e.g., time, temperature, salt concentration, presence of detergents) if necessary.

Protein A–Sepharose beads are used here because they work well with most monoclonal and polyclonal sera derived from mouse and rabbit, respectively. In some cases, the use of other beads (e.g., protein G–Sepharose) may improve binding of some antibodies, including rat IgG (see Table 7.2.1).

18. Microcentrifuge beads 1 min at 3000 rpm, room temperature. Transfer 300 µl supernatant into a 0.5-ml PCR tube labeled "INPUT." Discard the rest of the liquid.

Wash beads

19. Resuspend beads in 700 µl FA lysis buffer, room temperature, and transfer mixture into a Spin-X centrifuge-tube filter.

The use of Spin-X filters aids in the recovery of the beads after washes and results in better uniformity between different samples. The procedure is also substantially faster with the filters, particularly when multiple samples are processed simultaneously. Alternatively, one could use conventional microcentrifuge tubes for the washes and aspirate the supernatant with a narrow-bore pipet tip after each spin.

20. Place the filter into a 1.5-ml microcentrifuge tube and mix sample 3 min on an end-over end rotator. Microcentrifuge 2 min at 3000 rpm, room temperature. Discard the flow-through liquid at the bottom of the tube.

21. Add 700 µl FA lysis buffer, room temperature, to the beads and repeat step 20.

Elute protein from beads

22. Wash beads for 3 min each with 700 µl FA lysis buffer/0.5 M NaCl, 700 µl ChIP wash buffer, and finally 700 µl TE.

For many polyclonal antibodies, the more stringent washes in this step result in a cleaner signal, while gentle washes frequently lead to an unacceptably high background. For some antibodies (e.g., monoclonal against peptide epitopes; see Alternate Protocol 1), repeated washes with FA lysis buffer, which are gentler, might be more appropriate.

23. Place filter unit containing the beads into a new 1.5-ml microcentrifuge tube and add 100 µl of ChIP elution buffer. Gently pipet up and down two or three times in order to dislodge beads from the filter. Incubate 10 min in a 65°C water bath.

A water bath is used instead of other heating apparatuses in order to improve heat transfer.

24. Microcentrifuge beads 2 min at 3000 rpm, room temperature. Discard filter with beads. Transfer the eluate into a 0.5-ml PCR tube labeled "IP."

Reverse cross-links and purify DNA

25. Add 80 µl TE and 20 µl Pronase in TBS to the IP tube. Combine 20 µl INPUT material (step 18), 100 µl ChIP elution buffer, 60 µl TE, and 20 µl TBS into a new 0.5-ml PCR tube.

26. To reverse cross-links, place tubes into a PCR machine. Incubate 2 hr at 42°C, followed by 6 hr at 65°C. Store samples at 4°C until use.

The incubation at 42°C allows for Pronase digestion of cross-linked polypeptides, while the 65°C incubation results in a reversal of the formaldehyde cross-links.

27. Purify DNA using a Qiagen PCR-purification spin column as per manufacturer's instructions.

    This will require double loading of the spin column (i.e., 600 μl spin through and then repeat).

    Alternatively, add 20 μl of 4 M LiCl and purify by extracting with 25:24:1 phenol/chloroform/isoamyl alcohol, followed by extraction with chloroform and ethanol precipitation (APPENDIX 3A). It is useful to add 2 μl of Pellet Paint (Novagen) prior to the addition of ethanol, as this aids both the ethanol precipitation and visualization of the very small pellet.

28. Resuspend in 300 μl TE and store at −20°C.

    DNA pellets stored in this fashion should be stable for years.

Perform quantitative PCR

29. Design primer pairs for the desired genomic regions to be examined.

    Success in obtaining high-quality data is critically dependent on good primer design (see Critical Parameters and Troubleshooting). In general, primers should be 20 to 30 bases long with a T_m of 55° to 60°C. The design of good primers is greatly facilitated by commercially available software packages such as Oligo 6.6 (see http://www.oligo.net) or Primer Express 1.5 (see http://www.appliedbiosystems.com). Most primers require no purification or special treatment prior to PCR. Amplification products should be 75 to 350 bp; longer products should be avoided, as the amplification efficiency is substantially lower. A final primer concentration of 1 μM works well for most primers, but in some instances, improved product specificity may be obtained by lowering the final primer concentration 5 to 10 fold. Refer to APPENDIX 3F for more information.

30. Dilute INPUT DNA (obtained from step 18) in three separate tubes by a factor of 5, 10, and 20. Set up standard PCR reactions (*APPENDIX 3F*) with 2 μl DNA sample, primers at 1 pmol/μl, and total reaction volumes of 10 to 50 μl. If PCR products will be detected by radioactivity, add 1 μCi of 3000 Ci/mmol [^{32}P]dATP.

    For a typical measurement, the three dilutions of input DNA are tested along with duplicate immunoprecipitated samples (or undiluted and 5-fold diluted immunoprecipitated samples). This permits an assessment of whether the assay is being performed in the linear range as well as of the reproducibility of the PCR reaction. The immunoprecipitated DNA is typically used without dilution, although it is useful to analyze different amounts to ensure that it is also in the linear range.

    There are several key parameters for achieving an optimum PCR reaction. For example, it is very important to have a quality repeat pipettor that can reproducibly dispense 2 μl DNA. Pipetting inaccuracies at this stage will lead to greater well-to-well variability and poorer reproducibility among identical samples. Additionally, multiple primer pairs (up to 4 to 5) can be included in the same reaction, provided that the PCR products can be unambiguously resolved from each other by gel electrophoresis. This permits simultaneous and internally controlled analysis of multiple genomic regions in a single reaction. However, it is critical to ensure that there is no competition between the different primer pairs and PCR products. Also, comparable results are obtained when PCR reactions are performed in volumes between 10 to 50 μl; using smaller volumes reduces the cost and facilitates loading of the reaction products on gels. See Critical Parameters and Troubleshooting for a discussion of primer choice.

    Detection of PCR products by [^{32}P]label is recommended over detection by ethidium bromide or SYBR Green (see Alternate Protocol 2) staining as it improves the sensitivity and extends the linear range of detection; however, it necessitates using the usual precautions in working with radioactivity.

31. Carry out hot-start PCR using the following thermal cycling parameters.

Initial step:	10 min	95°C	(denaturation)
26 cycles:	30 sec	95°C	(denaturation)
	30 sec	55°C	(annealing)
	1 min	72°C	(extension)
Final step:	4 min	72°C	(final extension).

These conditions are generally appropriate for most situations. The annealing temperature may have to be adjusted if the melting temperatures of the primers is substantially above or below 55°C. The number of cycles might also have to be adjusted in some cases if reactions are not in the linear range. See Critical Parameters and Troubleshooting for more details.

Analyze PCR products

32. Add the appropriate loading buffer to the PCR products, and analyze by electrophoresis on nondenaturing polyacrylamide (*UNIT 6.5*) or agarose gels (*APPENDIX 3A*).

The gels should be stained either with ethidium bromide or SYBR Green dyes, or analyzed by autoradiography or PhosphorImager.

33. Quantitate the relative amount of PCR products using appropriate software for the accompanying instrument.

34. Calculate the apparent immunoprecipitation efficiency for a specific fragment by dividing the amount of PCR product obtained with the immunoprecipitated DNA by the amount obtained with the input DNA.

A volume of 2 μl immunoprecipitated DNA sample (1/150 total immunoprecipitated material) contains ~200 times the number of cell equivalents as 2 μl INPUT sample that has been diluted 5-fold (1/30,000 of the original aliquot that was immunoprecipitated). Thus, if the amount of PCR product in the immunoprecipitated sample is equal to the amount of PCR product in the 5-fold diluted INPUT sample, the apparent immunoprecipitation efficiency is 0.5%. The apparent immunoprecipitation efficiency for the background signal is typically ~0.025% to 0.05%, and it should not be higher than 0.1%.

SPECIFIC PEPTIDE ELUTION OF PROTEIN-DNA COMPLEXES IMMUNOPRECIPITATED FROM CROSS-LINKED CHROMATIN

ALTERNATE PROTOCOL 1

Peptide elution represents an alternative method for removal of immunoprecipitated protein-DNA complexes from beads. In this procedure, beads containing the immunoprecipitated complexes are incubated with high concentrations of a peptide recognized by the antibody used in the immunoprecipitation. The added peptide competes with the protein antigen of interest for binding to the antibody, and specifically liberates the protein-DNA complexes from the beads. The high specificity of peptide elution reduces the nonspecific background (typically by a factor of 2 to 4), which makes it the method of choice, particularly for applications where the expected immunoprecipitation signal is low. Peptide elution is especially useful for chromatin immunoprecipitation experiments involving proteins that are tagged with the HA or myc epitopes (in single or multiple copies); however, it would also be appropriate in cases where the antibody used for the immunoprecipitation was generated against a defined peptide sequence. Peptide elution is slightly more expensive than conventional elution, due to the cost of the peptide. In general, peptide elution should be used in conjunction with gentle washes during the immunoprecipitation procedure described below, which minimizes antigen leaching. Stringent washes, such as those employed in the main method (see Basic Protocol), will often result in signals that are several-fold lower, with little or no improvement in background. Finally, peptide elution may vary in quality depending on factors such as the number of epitopes in the antigen and the relative stability of the antibody-antigen interaction.

Additional Materials *(also see Basic Protocol)*

1 mg/ml peptide (e.g., myc, HA) in TBS (see *APPENDIX 2A* for buffer)

For this protocol, follow steps 1 to 21 of the main method (see Basic Protocol), replace steps 22 to 25 with the following, and continue with step 26 onwards.

22. Repeat FA lysis buffer wash (see Basic Protocol, steps 20 and 21) three additional times for a total of five washes.

    Repeated washes with FA lysis buffer are much more gentle than the single washes with FA lysis buffer/0.5 M NaCl, ChIP wash buffer, and TE used in the Basic Protocol and result in higher signal-to-background ratios.

23. Place the Spin-X centrifuge-tube filter unit containing the beads into a new 1.5-ml microcentrifuge tube and add 100 µl of 1 mg/ml peptide (typically myc or HA) in TBS. Gently pipet up and down two or three times in order to dislodge beads from the filter. Incubate 30 min at 30°C.

24. Microcentrifuge beads 2 min at 3000, room temperature. Discard filter with beads. Transfer the eluate into a 0.5-ml PCR tube suitable for PCR labeled "IP."

25. Add 150 µl TE, pH 7.5, 250 µl of ChIP elution buffer, and 20 µl of 20 mg/ml Pronase in TBS.

ALTERNATE PROTOCOL 2

ANALYSIS OF CHROMATIN IMMUNOPRECIPITATION EXPERIMENTS BY REAL-TIME QUANTITATIVE PCR WITH SYBR GREEN

Quantitative PCR (QPCR) analysis in real time with SYBR Green has several advantages over the analysis of PCR reactions by gel electrophoresis (see Basic Protocol, step 32). First, the method saves considerable time because no gels are involved and because quantitative values are obtained directly from the data curves and do not require densitometry or phosphor imager analysis. As a consequence, this approach permits very rapid analysis of much larger numbers of chromatin immunoprecipitation samples than can be performed with the Basic Protocol. Using standard 96-well instruments, it is a straightforward procedure to analyze 100 to 200 samples/day (in replicates of three) with only 1 to 2 hr of total setup time. With newer 384-well instruments and automated robotics equipment, sample throughput can be further increased to thousands per week. Second, the data generated by this procedure are more accurate and reproducible, because quantitative values are determined from continuous sampling throughout the PCR reaction rather than a single end-point determination. Furthermore, the quality and "linear range" of every PCR reaction are directly visualized. Third, the procedure is significantly safer for the researcher, as no radioactive materials or toxic acrylamide are used. The major disadvantage of this procedure is that the measurements are performed individually and hence are not internally controlled, whereas the Basic Protocol permits the simultaneous analysis of multiple genomic regions in a single PCR reaction (provided the individual primer pairs function independently). As such, the Basic Protocol is more useful for analyzing the same small set of genomic regions under multiple experimental conditions and for simultaneous analysis of electrophoretically distinguishable alleles of a given genomic region.

SYBR Green is a sensitive and highly selective double-stranded DNA (dsDNA)–binding dye that remains associated even at the high temperatures normally used for PCR template extension. Real-time PCR reactions involving SYBR Green are performed with standard oligonucleotide primers, and hence are much less expensive than real-time PCR reactions using fluorophore-conjugated oligonucleotides (e.g., TaqMan or Lux probes). Measurements of SYBR Green fluorescence at the polymerase extension step of PCR, when

plotted against PCR cycle number, provide both a qualitative assessment of the progress of the PCR and a way to quantitate the relative amount of DNA template initially present in the reaction. Typical real-time QPCR graphs feature the plot of the $\log_{10}$(Net fluorescence) on the *y* axis versus the PCR cycle number on the *x* axis, and usually contain three well-defined stages: (1) baseline, (2) linear, and (3) plateau. In the baseline stage, the amount of DNA product formed is still below the sensitivity threshold of SYBR Green, so product formation is undetectable. This part of the curve is typically used as a baseline for SYBR Green signal drift. The linear part of the curve is the most important from the analytical standpoint, because it is at this stage that the rate of PCR product accumulation is both constant on a per-cycle basis and readily detectable by increased SYBR Green fluorescence. Finally, as all of the SYBR Green in the reaction becomes bound to the recently synthesized PCR products, the amount of fluorescence stays constant from cycle to cycle and the reaction reaches a plateau.

In the protocol described below, PCR is performed under special conditions that minimize the inhibitory effects of SYBR Green on *Taq* activity and maximize the linear range of product detection. After amplification is complete, raw data are stripped of outliers and exported in a format readable by a spreadsheet program such as Microsoft Excel. Finally, data points from replicate samples are averaged, and mean values are further manipulated and ultimately compared to some internal reference or control.

Additional Materials *(also see Basic Protocol)*

Input DNA (see Basic Protocol, step 28)
Immunoprecipitated fragments ("IP" sample; see Basic Protocol, step 23)
2× SYBR Green *Taq* mix (see recipe)

Real-time PCR machine and corresponding software (e.g., ABI)
96-well PCR plates (ABI, cat. no. 4306737) and optical adhesive covers
Centrifuge with swinging-bucket rotor and microtiter plate adapter
Spreadsheet program (e.g., Microsoft Excel)

Set up PCR reactions

1. Dilute input DNA to an approximate equivalent of 1×10^6 cells/ml in TE buffer, pH 7.5.

   If immunoprecipitations were performed as described in the Basic Protocol, then a 1:25 dilution of the input sample from step 28 will result in 1:1000 overall dilution and will correspond to ~5×10^8 to 1×10^9 cell equivalents.

2. If necessary, resuspend immunoprecipitated fragments in TE buffer, pH 7.5, so that the approximate cell equivalent is 1×10^9 cells/ml.

   Immunoprecipitated DNA derived from the IP sample obtained by the Basic Protocol (step 23) is appropriately diluted and needs no further treatment.

3. Prepare PCR primer stocks by mixing each primer pair at a final concentration of 3.3 μM in TE buffer, pH 7.5.

   It is critical to test newly obtained primer pairs for amplification specificity and performance under conditions that will be used for real-time PCR with SYBR Green (see Critical Parameters and Troubleshooting). SYBR Green can inhibit PCR reactions, and primer pairs that are appropriate for quantitative PCR analysis in the absence of SYBR Green may not work well in the presence of SYBR Green. High-quality primer pairs should result in ~1.9-fold amplification/cycle (this can be determined from quantitative analysis of raw fluorescence data for each cycle, which is generally available on commercial instruments). Amplified material at the completion of the PCR should contain only one band

(as assayed on high-percentage agarose or polyacrylamide gels). Specificity information may also be obtained by running dissociation curves on reactions following the conclusion of the PCR run. Typically, samples are melted for 15 min at 95°C, cooled to 60°C, and then slowly heated back up to 95°C over a period of 20 min. Plotting the first derivative of the fluorescence against the temperature allows for simple visual identification of sample heterogeneity. Some instrument-specific software packages have built-in modules for dissociation curve analysis.

4. Select and label the wells to be used in the run.

   In general, individual samples should be run in triplicate. Obvious outliers occur with some frequency, generally at <5%. Triplicate analysis of samples permits removal of those outliers while still allowing for inclusion of two accurate measurements for each sample. While this reduces the number of different samples that can be run at any given time, the resulting data is much more reliable and accurate.

   For each primer pair examined, the input DNA samples should be run alongside the immunoprecipitated samples. Amplification efficiencies among different primer pairs vary slightly on a per-cycle basis, but those slight variations in efficiency translate into substantially different amounts of amplified material in the cycle range used for analysis. Precise quantitation of relative binding cannot be accurately performed without primer pair–specific input signal.

   Detailed instructions on the use of the real-time PCR machine and general issues, e.g., calibration and camera exposure settings, are addressed in the documentation that accompanies the instruments.

5. Program the real-time PCR machine as follows:

1 cycle:	10 min	95°C	(initial denaturation)
40 cycles:	30 sec	95°C	(denaturation)
	30 sec	53°C	(annealing)
	30 sec	72°C	(extension).

   Collect the data only at 72°C.

   The annealing temperature may have to be adjusted if the melting temperatures of the primers are substantially above or below 53°C. If the desired amplification product is >500 bp (this is not recommended), the extension time at 72°C should be increased to 1 min. See Critical Parameters and Troubleshooting for more details.

6. Using a small-volume automatic pipettor (20-µl capacity), place a 2-µl aliquot of each DNA template into the appropriate wells of a 96-well PCR plate. Gently tap the plate to allow the sample droplets to fall to the bottoms of the wells.

   It is very important to have a quality repeating pipettor that can reproducibly dispense small volumes of sample into the wells. Pipetting inaccuracies at this stage will lead to greater well-to-well variability and poorer reproducibility among identical samples.

7. Using a small-volume automatic pipettor (20-µl capacity), place a 3-µl aliquot of primer mix (see step 3) into the relevant wells and tap the plate a few times to settle the contents.

   On many real-time PCR machines, results from 10-µl reactions are virtually indistinguishable from those of 25- and 50-µl reactions in their accuracy and reproducibility. The use of 10-µl reactions provides substantial savings in reagent costs. On some machines, the minimal reaction volume needed for accurate and reproducible results may be greater.

8. With a larger automatic pipettor (100-µl capacity), add 5 µl of 2× SYBR Green *Taq* Mix to every assayed well. Place microtiter plate into appropriate microtiter plate adapter and centrifuge 1 min at 200 × *g*, room temperature, in a swinging-bucket rotor.

The 2× SYBR Green Taq Mix contains a variety of components that considerably reduce the inhibitory effect of SYBR Green, thereby resulting in more reproducible signals that require fewer amplification cycles. Comparable mixes containing proprietary buffers can be obtained commercially. It is critical that quantitative PCR reactions containing SYBR Green be performed under conditions of efficient amplification (e.g., 1.9-fold amplification/cycle).

9. Seal plate with clear optical adhesive covers, overlay foam compression pad with gold side facing up, and place into the real-time PCR machine. Secure lid.

   The details of this step may differ, depending on the machine.

10. Start the PCR protocol (see step 5). After completion, save the run for future analysis.

DATA analysis

11. Open the file containing the real-time data according to the manufacturer's instructions for the instrument.

    Although the specific protocol will depend on the software and instrument, the overall logic and approach to the analysis of real-time data is generally applicable.

12. Look at the different curves and set the baseline as needed.

    Generally, the baseline should be set from cycle 3 to the cycle just prior to where the curves start increasing in a linear fashion. It is desirable to have at least 10 cycles for the calculation of the baseline, as this results in increased accuracy in the subsequent calculations of the threshold cycle.

13. Change the value in the Threshold box to be about halfway up in the linear range, and apply changes to the data set.

    The threshold cycle is defined to be the PCR cycle at which the fluorescence is 10 times (10 is the default multiplier) the standard deviation obtained in the baseline calculation. When the multiplier is set to 10, the fluorescence at the threshold cycle is considered the lowest fluorescence value that is significantly above the background. In practice, this number frequently lies in the nonlinear range of many of the curves. For later calculations, it is easier to manually set the fluorescence value used to calculate the threshold cycles to 0.04. At this value, all the curves should be in the linear range and well above the baseline, allowing for far more accurate comparisons of the threshold cycles. On occasion, however, it will be necessary to adjust this value either up or down to better reflect the linear range of net fluorescence for most of the curves.

14. Manually select one group of triplicates and visually inspect their amplification plots. If curves are essentially superimposable and the threshold cycle (C_T) values are close to each other (maximal and minimal replicates within 1 cycle, preferably within 0.5 cycles), proceed to the next triplicate sample. Otherwise, remove the outlier and continue to the next triplicate.

    Decisions regarding the removal of some outliers could either be straightforward or judgment calls, depending on the circumstances. In cases where two out of three curves are superimposable while the third is clearly off by more than a cycle, it is a fairly easy decision to consider the third replicate an outlier. If the curves are closer, the decision on which one to eliminate, if any, becomes much more difficult. As a general guide only, if the spread between the lowest and highest C_T values is less than 0.5, it is probably safe to average all the C_T values (see step 12). If the C_T range is <1 but >0.5, the data are less reliable and the decision to remove any data points should probably be made on a case-by-case basis. It is highly recommended that the PCR be repeated for samples where the C_T ranges are >1 with no two curves superimposable.

15. Proceed to analyze the data for all triplicates in the manner described above. Save the results in a different file.

16. Export the data to a spreadsheet program such as Microsoft Excel by using built-in filters. The file should not contain omitted wells (see step 14) and should be in a column format containing well positions, descriptors, and C_T values for each selected well.

    Final calculations are most easily handled in a spreadsheet but could also be performed with a scientific calculator.

17. Open the exported file. Proceed to average triplicate measurements for each sample in a new column ($^{AVERAGE}C_T$).

    For some samples, there may be only two measurements left as a result of the removal of outliers in step 14.

18. For each primer pair, subtract the $^{AVERAGE}C_T$ (INPUT) from $^{AVERAGE}C_T$ (IP) in a new column. This number is the $^{NET}C_T$.

    This value represents the difference in cycles between the immunoprecipitated sample and the input DNA.

19. Subtract $^{NET}C_T$ for one primer pair (experimental; EXPT) from the $^{NET}C_T$ of another primer pair that serves as a reference or a control (CTRL) in a new column. The resulting value is $^{NET}C_T{}^{EXPT} - CTRL$. Repeat $^{NET}C_T$ subtraction of control primer for all other experimental primers.

    It is very desirable to have a control primer pair that can be used to assess the relative cross-linking efficiency at promoters of interest. Frequently, the control primer pair could be specific for a DNA region that does not bind to the immunoprecipitated protein of interest. The signal from the control primer pair could then be considered the background, and the binding efficiency of the protein to different promoter regions could be expressed as fold over background binding.

20. Evaluate the expression POWER ([mean primer slope], $[- {}^{NET}C_T{}^{EXPT} - CTRL]$), where the [mean primer slope] is the base and $[- {}^{NET}C_T{}^{EXPT} - CTRL]$ is the exponent. Repeat the process with other primers by using the different $^{NET}C_T{}^{EXPT} -$ CTRL values calculated in step 19.

    The actual value calculated in the POWER expression above is the degree of occupancy of the immunoprecipitated protein at the sequence of interest relative to that of a control (or background) DNA region.

    Perfectly efficient PCR, in which the number of amplified molecules doubles every cycle, has a primer slope of 2. As defined, this value is independent of primer pair sequence, target sequence length, and other variables that under normal circumstances may adversely affect the efficiency of amplification. In practice, however, the mean primer slope is almost always <2 and is slightly variable from primer pair to primer pair, mostly due to differences in primer sequence and other parameters (e.g. GC content and length of amplified sequence). For the majority of primers designed to amplify S. cerevisiae promoter sequences, the mean slope is 1.9 ± 0.06, and this value can be safely used in the calculations above. However, it is still good practice to calculate representative slopes for every newly synthesized primer pair; any substantial deviation from 1.9 (± 0.06), especially to the downside, should be viewed suspiciously. Frequently, a slope that substantially deviates from the normal range is indicative of problems in the amplification.

    The mean primer slope for a given primer pair is most easily calculated by performing linear regression on the linear portion of the amplification plot (log net fluorescence versus PCR cycle). In order to perform this calculation, it is first necessary (if possible) to export a file containing the fluorescence values for all the wells in use at every PCR cycle (see software manuals for more information). It is then possible to use linear regression to estimate the slope within the linear range (i.e., by using the MS Excel function LINEST). Since it is rather time-consuming to calculate slopes for an entire 96-well plate one-by-one, it may well be worthwhile to write a macro (or a stand-alone program) to automate this process.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ChIP elution buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10 mM EDTA (*APPENDIX 2A*)
1% (w/v) SDS (*APPENDIX 2A*)
Store up to 1 year at room temperature

ChIP wash buffer

10 mM Tris·Cl pH 8.0 (*APPENDIX 2A*)
0.25 M LiCl
1 mM EDTA (*APPENDIX 2A*)
0.5% (v/v) Nonidet P-40
0.5% (w/v) sodium deoxycholate
Store up to 1 year at room temperature

FA lysis buffer with and without 2 mM PMSF or 0.5 M NaCl

For FA lysis buffer:
50 mM HEPES: adjust pH to 7.5 with KOH
150 mM NaCl
1 mM EDTA (*APPENDIX 2A*)
1% (v/v) Triton X-100
0.1% (w/v) sodium deoxycholate
0.1% (w/v) SDS
Store up to 1 year at room temperature

For FA lysis buffer/2 mM PMSF add 100 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol (store up to 1 year at −80°C) to a final concentration of 2 mM just before use. For FA lysis buffer/0.5 M NaCl, change the amount of NaCl added to 0.5 M. Store up to 1 year at room temperature

Loading buffer, 10×

20% (w/v) Ficoll 400
0.1 M disodium EDTA, pH 8 (*APPENDIX 2A*)
1.0% (w/v) sodium dodecyl sulfate
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol (optional; runs ∼50% as fast as bromphenol blue and can interfere with visualization of bands of moderate molecular weight, but can be helpful for monitoring very long runs)

RNase A stock solution (DNase-free), 2 mg/ml

Dissolve RNase A (e.g., Sigma) in DEPC-treated H_2O (*APPENDIX 2A*) to 2 mg/ml. Boil 10 min in a 100°C water bath. Store ≤1 year at 4°C.

SYBR Green Taq mix, 2×

12 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
50 mM KCl
5 mM $MgCl_2$

continued

150 mM trehalose (Sigma)
100 mM betaine (Aldrich)
0.2% (v/v) Surfact-Amps 20 (Pierce; active ingredient, Tween 20)
0.2 mg/ml nonacetylated BSA (Sigma, B8667)
1 μM 5(6)-carboxy-X-rhodamine (ROX; Helix Research)
0.133× SYBR Green (Molecular Probes; final dilution 1:75,000)
Store solution with above components indefinitely at −80°C or up to several months at 4°C in the dark
Add the following immediately prior to use
0.5 mM each dATP, dTTP, dCTP, dGTP
0.2 U/μl hot-start *Taq* DNA polymerase

The amount of Taq DNA polymerase may need to be slightly adjusted to account for batch/activity variations among different manufacturers.

COMMENTARY

Background Information

Direct protein-DNA contacts and indirect protein-DNA interactions regulate fundamental chromosomal functions such as DNA replication, gene expression, and chromosome segregation. Thus, knowledge about the distribution of particular proteins on specific chromosomal DNA sequences can provide important insights into the mechanisms that govern chromosomal functions, structure, and organization. In vivo footprinting methods provide high-resolution mapping of protein-DNA interactions but cannot directly identify the chromatin-associated protein(s) responsible for the footprint. Chromatin immunoprecipitation, by contrast, is ideally suited for determining the identity of proteins associated with specific DNA sequences in vivo, albeit with lower resolution (≤1 kbp).

Two groups, Gilmour and Lis and Solomon and Varshavsky, independently pioneered cross-linking and immunoprecipitation methods for in vivo chromatin analysis (Gilmour and Lis, 1984; Solomon and Varshavsky, 1985; Solomon et al., 1988; Gilmour et al., 1991). These methods exploited cross-linking to conserve in vivo chromatin structures and permit their isolation under the stringent conditions necessary to isolate soluble chromatin. Cross-linked protein-DNA complexes were purified by cesium chloride centrifugation (a time-consuming step) and subjected to immunoprecipitation. Their methods were distinguished primarily by the cross-linking agent: Gilmour and Lis employed UV irradiation, while Solomon and Varshavsky used formaldehyde. The biochemical characteristics of each method have been discussed extensively (Gilmour and Lis, 1984; Solomon and Varshavsky, 1985; Orlando et al., 1997, and references therein). In short, UV irradiation cross-links only protein-DNA complexes in direct contact, which limits its use. Formaldehyde reacts with primary amines on amino acids and DNA and RNA bases, reversibly forming a covalent adduct between two primary amines in close proximity to each other (≤2 Å). Because protein-protein adducts are formed in addition to protein-DNA adducts, chromatin-associated proteins not directly bound to DNA can be cross-linked to DNA via other proteins such as histones, significantly broadening the applicability of this procedure. Cross-linking with formaldehyde is also more easily accomplished than UV irradiation, especially with larger culture volumes.

Formaldehyde-based chromatin immunoprecipitation was simplified and adapted for use in other experimental systems, including budding yeast, where it was first used to assess the association of differentially acetylated histones with the silent mating-type loci (Dedon et al., 1991; Braunstein et al., 1993). This method involved fractionation of cell extracts to isolate a chromatin fraction before immunoprecipitation. A closely related method was used to assess the composition of the budding yeast centromere (Meluh and Koshland, 1997). The protocol presented here (see Basic Protocol; Fig. 17.7.1) is for a simpler procedure derived by Hecht and Grunstein in which immunoprecipitations were performed with whole-cell extracts to assess the spatial distribution of SIR proteins on telomere-proximal DNA regions (Hecht et al., 1996; Strahl-Bolsinger et al., 1997). The Basic Protocol has also been applied to characterize the spatial and temporal associations of DNA replication proteins with chromatin at replication origins (Aparicio et al., 1997; Tanaka et al., 1997), the

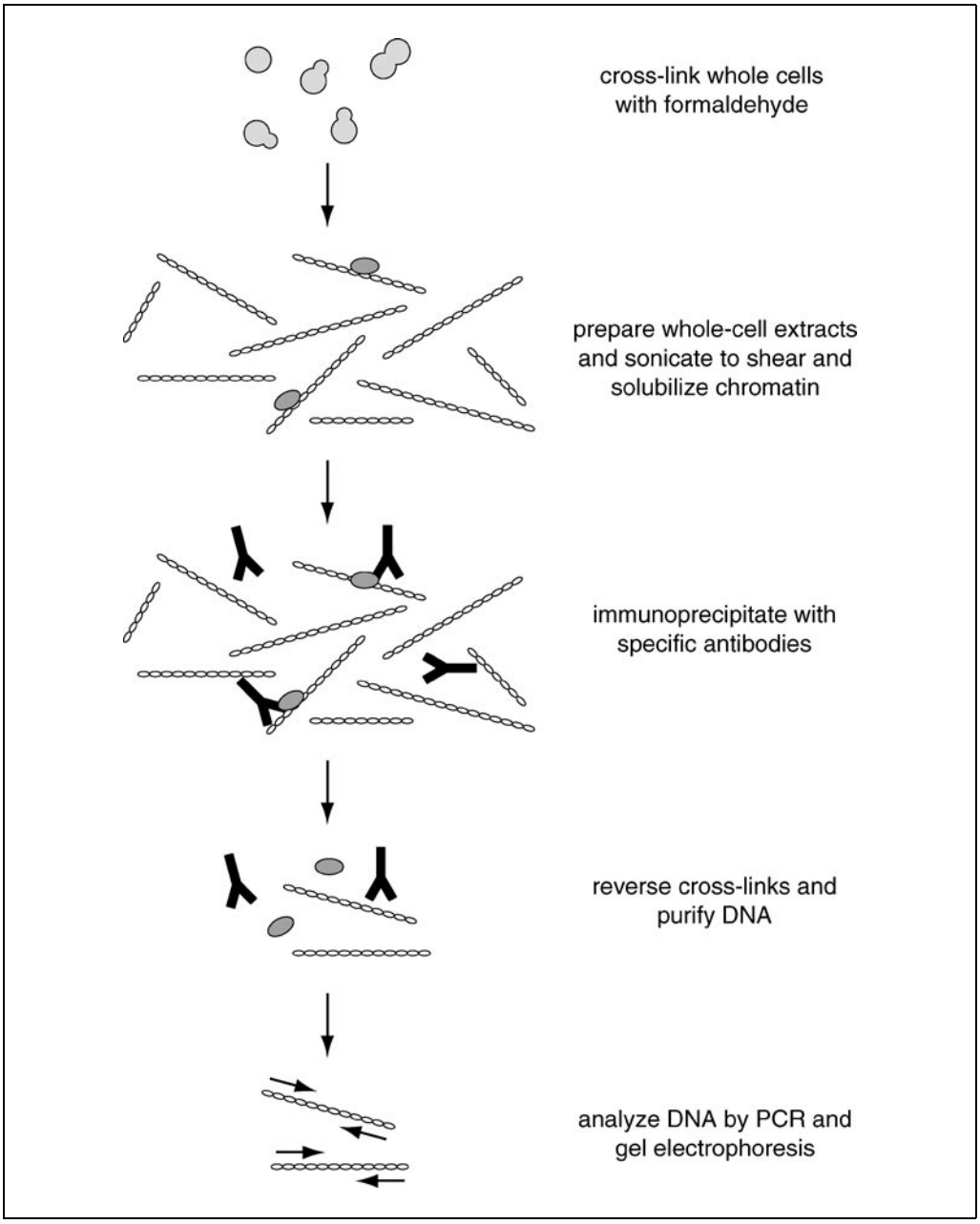


Figure 17.7.1 Scheme for chromatin immunoprecipitation from yeast whole-cell extracts.

association of general transcription factors at promoters (Kuras and Struhl, 1999; Li et al., 1999), and the dynamics of DNA-binding proteins and chromatin-modifying activities associated with a cell-cycle and developmentally regulated promoter (Cosma et al., 1999). In each of these latter studies, protein association with relevant DNA sequences was examined using PCR amplification.

ChIP can also be used to specifically follow the genomic association of mutant proteins that are unable to support cell growth (Mencia and Struhl, 2001). This involves a strain containing both an epitope-tagged version of the mutant protein and an untagged version of the wild-type protein, which supports cell growth; ChIP is performed with an antibody against the epitope. Lastly, ChIP can be combined with microarray technology to identify the location of specific proteins on a genome-wide basis (Ren et al., 2000; Iyer et al., 2001). The immunoprecipitated DNA is PCR amplified with linkers, and the resulting material hybridized to microarrays containing the complete set of intergenic regions in *Saccharomyces cerevisiae*. Such "genome-wide location" or "ChIP-chip" is particularly powerful, because it allows one to identify novel regions of protein association, without any previous knowledge.

Critical Parameters and Troubleshooting

Controls

There are two basic types of controls for a standard ChIP experiment. One control is mock immunoprecipitation to determine the specificity of an observed signal. This is accomplished by performing parallel immunoprecipitations of a given cross-linked chromatin sample with the antibody of interest and with an irrelevant antibody (or simply not providing any antibody). Alternatively, when antibodies against epitope-tagged proteins are employed (e.g., anti-HA, anti-myc), a similar comparison can be made with parallel immunoprecipitations (primary antibody included) of chromatin samples from strains expressing epitope-tagged or untagged versions of the protein of interest. However, in such experiments, it is often observed that the apparent immunoprecipitation efficiency for any irrelevant genomic region is about 2- to 3-fold higher in the experimental sample than in the control sample. This probably reflects nonspecific, and perhaps artifactual, association of the protein of interest with chromatin; hence it is not indicative of specific protein association with the genomic region. For proteins that generally and strongly associate with the entire genome (e.g., histones), the apparent immunoprecipitation efficiency in the experimental sample will be much higher than the control sample.

The second control, which is usually more meaningful, is to examine a given pair of input and immunoprecipitated samples for multiple genomic regions. Control genomic regions (i.e., those not interacting with the protein) should all give the same background level of apparent immunoprecipitation efficiency (typically ~0.025% to 0.05%). Fragments bound by the protein of interest will give higher apparent immunoprecipitation efficiencies, and the difference can be expressed as fold enrichment over the background level. In the best cases, enrichments can be >100-fold, but even a factor of two can be meaningful if the experiment is repeated enough times and the experimentally determined error is sufficiently low. The advantage of this approach is that the identical samples are used to directly determine relative protein association to different genomic regions. Furthermore, differences in fold enrichments for different genomic regions represent relative quantitative measurements of protein association in vivo.

Additional controls may be used depending on the specific application. For example, where binding to a putative binding site is being tested, a mutation in the binding site is a critical control (especially if such a mutation previously was shown to eliminate binding in vitro). In a related manner, it might be useful to determine the protein association in mutant strains or under particular environmental conditions that are suspected to be important for the protein of interest.

Cross-linking

The extent of formaldehyde cross-linking is an important variable that in principle may be modified by changing the duration of cross-linking, the concentration of formaldehyde, or the temperature at which the cross-linking is performed. The use of 1% (final concentration) formaldehyde for 15 min at temperatures ranging from 12°C to 37°C usually works well; however, at temperatures above 30°C, background sometimes increases. Therefore, when fixation at a higher temperature is required, reducing the duration of cross-linking or the formaldehyde concentration may be helpful. Excessive cross-linking can interfere with cell breakage by bead beating and effective fragmentation and solubilization of the DNA by sonication (see below). For some applications where protein cross-linking is particularly efficient (e.g., histones), it might be useful to decrease the cross-linking time or formaldehyde concentration. In particular, histone tails have a number of lysine residues that are likely to be modified by formaldehyde, and such modified lysines may interfere with the binding of antibodies against specific peptides corresponding to modified histones (e.g., by acetylation, phosphorylation, methylation).

Cell lysis

Although complete lysis of all cells is not absolutely necessary (and may be difficult to achieve), it is very important that lysis be as efficient as possible. Efficient lysis is important to obtain a reproducible degree of cell breakage among a group of samples to reliably compare results. Significant differences in cell lysis efficiency will result in immunoprecipitations with different ratios of antibody to chromatin, which will possibly alter immunoprecipitation efficiency. Cell breakage by a mini bead beater is generally more efficient than breakage by a multi-vortexing apparatus, although both methods work. In both cases, it is important to use flat-bottomed 2-ml microcentrifuge tubes. When using the mini bead beater, the sample and beads should nearly fill the tube, whereas for vortexing it is important

to maintain a small volume. The extent of cell breakage may be monitored microscopically by comparing the number of intact cells (determined by counting on a hemacytometer) in small, diluted aliquots of the sample taken before and after vortexing. In addition, the size of the remaining pellet (unbroken cells and debris) obtained in the first centrifugation following sonication (see Basic Protocol, step 14) is a good general indicator of the extent of lysis. The size of this pellet should be routinely checked (by rapid visual inspection) to compare the extent of lysis among samples.

The final yield of genomic DNA in the extract is also an important indicator of the extent of cell breakage, although the DNA yield is also dependent on the solubilization of chromatin by sonication (see below). Poor or variable cell breakage may result from excessive cross-linking that toughens the yeast cell wall and other structures. The procedure for lysis of *Saccharomyces cerevisiae* is appropriate for other yeast species. However, modified procedures are necessary for breaking mammalian cells.

Sonication

Shearing DNA to a small size (~500 bp average) by sonication is the critical factor in achieving resolution between a DNA sequence where a particular protein is bound and a nearby (*cis*-)DNA sequence that does not bind that protein. In addition, fragmentation of the chromatin is essential for its solubilization from the ruptured cells. As indicated above, the ability to fragment and solubilize the chromatin depends on the extent of chromatin cross-linking. In general, more cross-linking results in larger fragment size and lower solubility, resulting in lower yield (Orlando et al., 1997). Because of the importance of this variable, the shear size of the DNA should be assessed to confirm that the desired degree of fragmentation has been achieved, and it should be reassessed if fixation conditions are altered. The shear size is determined by electrophoresing DNA from step 16 of the Basic Protocol on a 1.5% to 2.0% agarose gel and visualizing with ethidium bromide. A smear of DNA should be apparent with an average size of 500 bp and most of the DNA (>90%) should be in the size range of 100 to 1000 bp.

As an alternative to sonication, DNA fragment size can be reduced by treatment of the cross-linked chromatin with micrococcal nuclease. Micrococcal nuclease preferentially cleaves DNA located in the linker regions between nucleosomes. By varying the concentration of micrococcal nuclease, it is possible to generate samples in which average DNA size varies. The minimal useful size is about 150 bp, which corresponds to a mononucleosome. However, cleavage to mononucleosome-sized fragments may also result in a preferential loss of certain genomic regions due to the sequence-specificity of micrococcal nuclease.

Immunoprecipitation

The success of this procedure relies on the use of an antibody that will specifically and tightly bind its target protein in the buffer and wash conditions used. In addition, antibody should be present in excess with respect to its target protein so that differences in the amounts of the protein-DNA complexes of interest will be accurately measured. Perform preliminary experiments to confirm avid immunoprecipitation and determine an approximate amount of antibody to use. Chromatin extracts should be prepared without prior cross-linking of the cells and subjected to immunoprecipitation with varying concentrations of antibody (20 μg/ml antibody may be a good starting point). The amount of the protein of interest in the extracts before and after immunoprecipitation should be analyzed by immunoblotting (*UNIT 6.2*) to determine the lowest antibody concentration that depletes >90% of the protein of interest from the extract. This antibody concentration is a good starting point for the full protocol and may later be modified to maximize the signal-to-noise ratio (see Anticipated Results). With cross-linked chromatin, immunodepletion of the target protein is less efficient (~50%), presumably due to masking or modification of the epitopes, and a significant amount of the protein remains refractory to immunoprecipitation even with higher antibody concentrations. Thus, the ideal antibody concentration is ultimately determined empirically to maximize the yield of specific coprecipitated DNA while minimizing precipitation of nonspecific DNA.

Both monoclonal and polyclonal antibodies have been used in this procedure. The monoclonal antibodies 12CA5 (anti-HA), 17D09 (anti-HA), and 9E10 (anti-myc) have been used successfully in different laboratories. In general, triple-HA epitope tags work well (Hecht et al., 1996; Aparicio et al., 1997; Tanaka et al., 1997), and larger multi-myc epitope tags have also been successful (e.g., myc-9, myc-18; Tanaka et al., 1997). Protein G–Sepharose, Protein A–Sepharose, and

anti-mouse immunoglobulin-coupled magnetic beads have all been used to precipitate the immune complexes, although it should be noted that certain classes of mouse and rat immunoglobulins are not strongly bound by protein A (Harlow and Lane, 1988; see Table 7.2.1).

For optimal results, it is critical to minimize the background level of material that inevitably comes down during the immunoprecipitation. The procedures described here work well with a diverse set of antibodies, but it might be necessary to modify the binding and elution conditions in specific cases. Peptide elution is clearly preferred over heat elution, as it is more specific and results in lower experimental backgrounds and hence higher-fold inductions. However, peptide elution is only possible for experiments using antibodies against peptides (typically for analyzing epitope-tagged proteins, but analysis of native proteins should also be possible). In performing peptide elution, it is important to add enough peptide such that the protein-DNA complexes are efficiently eluted from the beads.

Another consideration is that the epitope of interest in the chromatin-bound protein might be inaccessible to the antibody due to associated proteins or DNA structures. In such a case, one might obtain a false-negative result. Whereas the majority of a given protein may be efficiently immunoprecipitated from the cross-linked cells, the fraction that is actually cross-linked might be undetectable. The use of polyclonal antibodies (which often recognize multiple determinants within a protein) or epitope-tagged proteins (the epitope is unlikely to have a specific interaction with other proteins or DNA sequences, particularly if the epitope does not affect the biological function as determined by genetic complementation) minimizes, but does not eliminate this concern. Because of this caveat, negative results should be interpreted cautiously and alternative methods (e.g., in vitro DNA binding or association of the protein with bulk chromatin) should be tried. This concern is particularly relevant when a protein of interest does not appear to interact with any genomic sequence. However, if a protein selectively associates with some genomic sequences, this concern is significantly reduced—i.e., it is unlikely that epitope masking will occur at some loci, but not others.

PCR strategy

The choice of primers depends on the experimental goals. If binding to a specific site is being tested, a primer pair that flanks the site and at least one control primer pair recognizing a DNA sequence not expected to bind the protein of interest are the minimal requirements (see Fig. 17.7.2). When choosing primers, it is important to remember that resolution between adjacent sequences is limited by the shear size of the DNA. For an average DNA size of 500 bp, a significant fraction of the DNA molecules will be in the 500 to 1000 bp

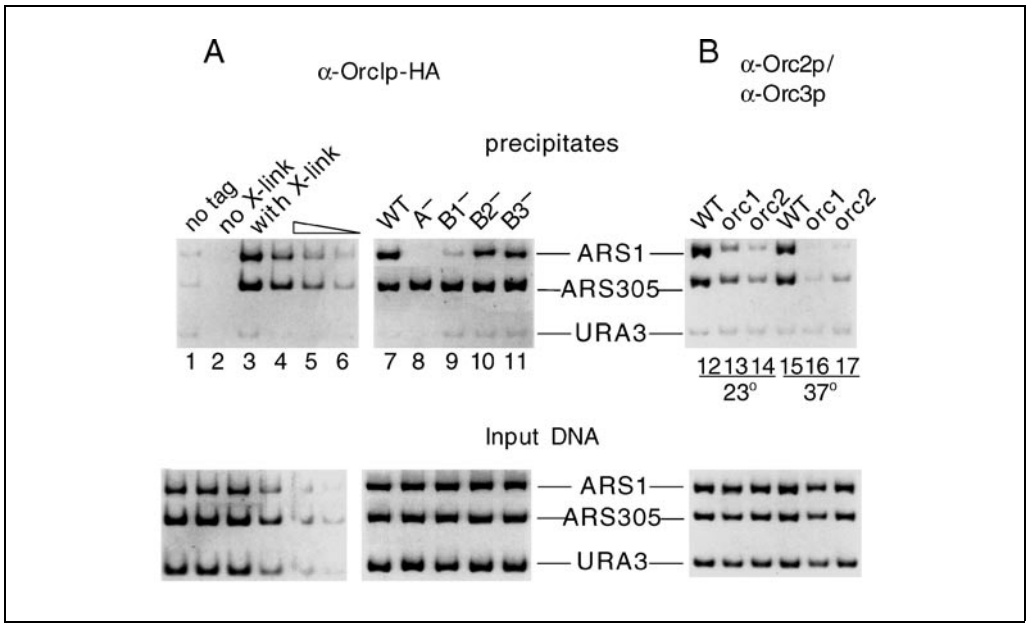


Figure 17.7.2 Anticipated results from chromatin immunoprecipitation analysis of origin recognition complex (ORC) with replication origin and nonorigin DNA sequences.

range, and hence DNA sequences 1000 bp distal from the actual protein binding site may be coprecipitated. Therefore, primer pairs used as controls should amplify a region of DNA that is far enough away from the expected binding site (e.g., >1 kbp) that coprecipitation of adjacent DNA is not detected. A good strategy is to design multiple sets of primers at increasing distances from a suspected binding site. Such a strategy has also been used to probe the "spreading" and "movement" of proteins on chromatin (Hecht et al., 1996; Aparicio et al., 1997; Strahl-Bolsinger et al., 1997).

Success in obtaining high-quality quantitative data is critically dependent on good primer design! In general, primers should be 20 to 30 bases long with a T_m of 55° to 60°C. Most primers require no purification or special treatment prior to PCR. Amplification products should be 75 to 300 bp. Longer PCR products should be avoided, because the amplification efficiency is substantially lower, and DNA fragments that do not bind to both primers will not be amplified (this can be a significant problem since the size of DNA fragments in the samples averages ~500 bp and ranges between 100 to 1000 bp). A final primer concentration of 1 μM works well for most primers, but in some instances, improved product specificity may be obtained by lowering the final primer concentration 5 to 10 fold.

The design of good primers is greatly facilitated by commercially available software packages such as Oligo 6.6 or Primer Express 1.5. These packages allow for extensive customization of many different parameters, including T_m, oligonucleotide length, GC content, and more. While the success of each individual primer pair in the specific amplification of its target sequence is dependent on many variables, special care must be taken to minimize primer-dimers and hairpins. Finally, it is a good idea to check primers for hybridization to other genomic sequences through the use of a web-based program such as BLAST.

Newly obtained primer pairs must be tested for amplification specificity and performance under the conditions that will be used in quantitative PCR. Primer pairs that are suitable for reactions performed by the Basic Protocol might not be suitable for real-time PCR reactions using SYBR Green, because SYBR Green can inhibit *Taq* polymerase. It is particularly informative to analyze input DNA amplification by the primers in question on high-percentage agarose or polyacrylamide gels after completion of the PCR. The presence of multiple product bands indicates poor specificity and will invariably lead to unreliable results.

For the Basic Protocol, the best test for quality of a given primer pair is to carry out a standard curve using different dilutions of DNA. For a high-quality primer pair, the amount of PCR product should be directly proportional to the amount of DNA, with an error of less than ±20%. The number of PCR cycles is determined empirically. Usually, 25 to 28 cycles is appropriate. More than 28 cycles can result in detection of nonspecifically precipitated sequences and/or lead to variable results due to inactivation of *Taq* polymerase. Multiple primer pairs can be used in combination if the PCR products are separable by gel electrophoresis (as many as five have been used), but some combinations interfere with efficient amplification of one or more products. It is essential to test primer pairs singly and in combination, with titrations of template DNA, to determine if this is a problem. The advantage of using multiple primer pairs is that individual reactions can generate data for multiple genomic regions in an internally controlled manner. In addition, the Basic Protocol can be used to simultaneously analyze two alleles of a given locus in an internally controlled manner, provided the individual alleles result in different-sized PCR products.

When quantitative PCR will be performed in real time using SYBR Green (see Alternate Protocol 2), high-quality primer pairs should result in ~1.9-fold amplification/cycle. Such amplification efficiency can be determined from quantitative analysis of raw fluorescence data for each cycle. Amplification efficiencies <1.8 are likely to cause problems, particularly if detection of the PCR product requires 30 cycles or more. Specificity information may also be obtained by running dissociation curves on reactions following the conclusion of the PCR run.

Quantitation

For the Basic Protocol, PCR products are analyzed by gel electrophoresis and detected by staining with ethidium bromide or SYBR Green or by radioactivity (typically by including a small amount of [^{32}P]dATP in the reactions). DNA staining has the advantage of not requiring radioactivity, but the linear detection range is relatively limited. In this regard, SYBR Green is more sensitive than ethidium bromide and is preferred. Radioactive detection is more sensitive and has a larger linear range than detection by DNA staining. Whatever detection method is employed, it is

essential to use a high-quality imager to accurately quantitate the amounts of PCR product in the various reactions.

When quantitative PCR is performed in real time using SYBR Green (Alternate Protocol 2), the linear range is directly visualized and the quality of the reactions can be directly assessed. For reactions involving a given primer, the curves should be superimposable with respect to shape, and they should differ only in the number of cycles needed to reach the threshold (C_T). Amplification efficiencies should be ~1.9-fold/cycle. If the curves have different shapes and/or amplification efficiencies are <1.8, the reactions are not equivalent and accurate quantitation is impossible.

Data interpretation

In most experiments, it is presumed that the protein of interest associates specifically with certain genomic regions and associates only nonspecifically with other genomic regions. In general, it is very difficult to distinguish true nonspecific association from experimental background of the cross-linking procedure. In this regard, immunoprecipitations with the antibody of interest generally give 2- to 3-fold higher immunoprecipitation efficiencies than immunoprecipitations with control (or no) antibodies, but it is unclear whether this effect is physiologically meaningful or an experimental artifact.

For this reason, the best way to interpret the data for most experiments is to compare the immunoprecipitation efficiencies for different genomic regions from the same INPUT and IP samples. The immunoprecipitation efficiency is calculated by the amount of PCR product in the IP sample divided by the amount of PCR product in the INPUT sample. A typical background level for DNA fragments that do not associate with the protein of interest is 0.025% to 0.05%. However, background levels can vary, depending on the antibody used and the elution method. In general, monoclonal antibodies give lower background signals than polyclonal antibodies. Peptide elution is preferred over heat elution for the same reason, although this can only be employed for ChIP experiments involving antibodies against defined peptide epitopes. By definition, the background level should be the same for all DNA fragments that do not specifically associate with the protein of interest. In many cases, the choice of suitable negative control regions is based on expectation from other lines of evidence (e.g., the middle of protein-coding regions are unlikely to bind general transcriptional initiation factors). In cases where there is no previous knowledge, the background level can only be based on multiple regions having similar immunoprecipitation efficiencies that are roughly at the level of a typical background immunoprecipitation efficiency. In this regard, it is particularly useful to use proteins tagged with a standard epitope (e.g., HA or myc), as there is considerable information on background levels in such cases.

DNA fragments that display immunoprecipitation efficiencies significantly above the background are indicative of protein association to those genomic regions in vivo. Moreover, for a given pair of INPUT and IP samples, the fold enrichment of a given genomic region over the background is directly related to the level of protein association in vivo. It is useful to define "relative protein occupancies" for different regions by subtracting the background from the observed immunoprecipitation efficiencies. For example, if the background level is arbitrarily defined as 1 occupancy unit, fragment A that shows 6-fold enrichment over background will have 5 occupancy units, whereas fragment B that shows 21-fold enrichment over background will have 20 occupancy units. Thus, one can conclude that the protein association with fragment B is 4-fold greater than with fragment A. Without further considerations (see below), this conclusion is only relevant for the particular pair of INPUT and IP samples because absolute immunoprecipitation efficiencies and fold enrichments can vary among repeated trials of the same experiment.

Absolute immunoprecipitation efficiencies and fold enrichments depend on multiple factors. First, the number and physical location of amino acid and nucleotide residues within the interacting protein surfaces that react with formaldehyde vary considerably among protein-protein and protein-DNA interactions. Second, proteins directly interacting with DNA can be cross-linked by a single event, whereas proteins that indirectly associate with DNA need multiple cross-linking events. In this regard, proteins directly binding DNA (e.g., specific DNA binding proteins and general transcription factors) typically give higher fold enrichments than other proteins (e.g., components of chromatin-modifying complexes). Third, some proteins might stably associate with genomic DNA sequences (maximally 100% occupancy), whereas association of other proteins might be transient. Fourth, the absolute immunoprecipitation efficiency depends on the quality of the specific

antibody-antigen interaction as well as the antibody concentrations, and the fold enrichment depends on both the absolute immunoprecipitation efficiency and on the background. Thus, absolute immunoprecipitation efficiencies and fold enrichments cannot be used to compare binding characteristics of different proteins. Furthermore, ChIP experiments do not provide absolute measurements of protein occupancy on specific genomic regions or relative stoichiometry of factors on a given sequence. Despite these limitations, ChIP experiments do provide direct quantitative information on the relative levels of protein association on different genomic sequences.

As mentioned above, absolute immunoprecipitation efficiencies and fold enrichments can vary among repeated trials of the same experiment due to potential differences in overall immunoprecipitation efficiency and experimental background. To account for these experimental variations, it is useful to arbitrarily define occupancy units for a specific protein-DNA association. In the example above, one might arbitrarily define fragment A to have 4 occupancy units. In independent experiments, association with fragment A will always be defined as having 4 occupancy units, and association with other fragments will be defined relative to that of fragment A in the same pair of INPUT and IP samples. In this manner, it is possible to accurately determine the relative level of protein association to multiple genomic regions even though the absolute immunoprecipitation efficiencies and fold enrichments might vary in different repeats of the same experiment. However, in actual practice, differences in absolute immunoprecipitation efficiencies and fold enrichments should not show significant sample-to-sample variation.

A related issue occurs when comparing the level of protein association under different physiological conditions (e.g., different growth conditions or strains). The ideal way to handle this situation is to analyze a "positive control" region that is predicted to be unaffected by the growth condition or genetic constitution. For example, in analyzing association of general transcription factors under a particular environmental condition, it would be useful to examine promoters that are regulated and promoters that are not. In some cases, such a control genomic region is not available, in which case one must rely on simple sample-to-sample reproducibility from independent trials of the same experiment.

ChIP can be used to determine the relative occupancy levels of different proteins at genomic regions (Kuras et al., 2000). Ideally, this is accomplished by performing parallel immunoprecipitations with different antibodies on the same cross-linked chromatin sample. Alternatively, immunoprecipitations can be performed on different samples (this often occurs when using epitope-tagged strains or when multiple proteins are examined). To determine occupancy ratios for two different factors (e.g., X and Y), occupancy units for X and Y are calculated independently as described above. The X:Y occupancy ratios are then calculated for all genomic regions examined. The resulting occupancy ratios are defined in arbitrary units, but the relative occupancy ratios for the different genomic regions are valid. To account for potential sample-to-sample variations among repeats of the same experiment, a given X:Y occupancy ratio should be defined for a specific genomic region and ratios at all other genomic regions calculated in relative terms. Using this rationale, it has been shown that the relative associations of TBP and the general transcription factors TFIIA and TFIIB are essentially constant at all promoters, whereas the TAF:TBP occupancy ratios vary considerably (Kuras et al., 2000). Importantly, however, occupancy ratios determined from such experiments cannot address whether two proteins co-occupy a given genomic region or mutually compete for the same genomic region.

For some experiments, particularly those involving histone modifications, it is inappropriate to analyze the data in terms of occupancy units and specific versus nonspecific binding sites. Histones associate with essentially all genomic regions, and the level of a particular chromatin modification typically occurs in a continuum. Thus, it is very difficult to determine whether a given region is devoid of a particular modification, although information in this regard can be obtained in control immunoprecipitations using an irrelevant antibody. For these reasons, quantitative analysis of the relative level of a given histone modification is best presented using simple immunoprecipitation efficiencies. Again, to account for sample-to-sample variations, a specific genomic region should be given an arbitrarily defined value, which is used to determine the relative levels of all other genomic regions.

Anticipated Results

Figure 17.7.2 shows the results of chromatin immunoprecipitation of protein subunits of the origin recognition complex (ORC)

and relevant controls (Aparicio et al., 1997). In panel A, immunoprecipitation of Orc1p-HA was shown to specifically coprecipitate the replication origin sequences *ARS1* and *ARS305* but not the nonorigin DNA sequence *URA3*. Enrichment of *ARS1* and *ARS305* (~0.4% precipitated relative to total) compared to *URA3* (~0.01% precipitated) was ~40-fold and depended on formaldehyde cross-linking and on the epitope-tagged Orc1p (lanes 1 to 3). Mutation of DNA sequences (A and B1) in the *ARS1* replication origin (required in vivo for origin activity and in vitro for binding of ORC to origin DNA) greatly reduced or eliminated association of Orc1p-HA with *ARS1*. The continued association with the wild-type *ARS305* origin served as an additional control (lanes 7 to 9). Analysis of the totals demonstrated that the origin and nonorigin DNA sequences were equally represented in the whole-cell extract (lower panel, "input DNA"). In panel B, origin association of ORC was tested in strains with temperature-sensitive alleles of *ORC1* or *ORC2* demonstrating loss of ORC-origin DNA binding under nonpermissive conditions (lanes 15 to 17). Nevertheless, it should not be assumed that a mutation in a protein of interest would necessarily result in loss of its chromatin association.

Time Considerations

The Basic Protocol may be completed in a 2- or 3-day period. On the first day, cells are fixed with formaldehyde and harvested (1 hr). For convenience, or if preparation of the cells for cross-linking will require an extended period of time beforehand (e.g., induction of expression, cell cycle synchronization), the cells may be frozen and stored at −80°C as described (see Basic Protocol, step 6). Preparation of chromatin extracts (2 hr) and immunoprecipitations (primary antibody incubation, ~2 hr; incubation with secondary-coupled beads, ~1 hr; washing and elution, 2 hr) may be carried out in 1 day, after which the samples are placed at 65°C overnight to reverse the cross-links. If necessary, immunoprecipitation with the primary antibody or bead-coupled secondary antibody may be extended overnight; however, it is most efficient to perform the ≥6 hr cross-link reversal step overnight. On the final day, the DNA is purified (<4 hr, including a 2-hr incubation with proteinase K), PCR amplified (<3 hr, including 2 hr for the PCR program), and analyzed by gel electrophoresis (<2 hr, including 1 hr of gel running time).

For Alternate Protocol 2, setup time is <30 min, thermal cycling takes ~2 hr, and data analysis takes <30 min.

Literature Cited

Aparicio, O.M., Weinstein, D.M., and Bell, S.P. 1997. Components and dynamics of DNA replication complexes in *S. cerevisiae*: Redistribution of MCM proteins and Cdc45p during S phase. *Cell* 91:59-69.

Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D., and Broach, J.R. 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.* 7:592-604.

Cosma, M.P., Tanaka, T., and Nasmyth, K. 1999. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle-and developmentally regulated promoter. *Cell* 97:299-311.

Dedon, P.C., Soults, J.A., Allis, C.D., and Gorovsky, M.A. 1991. A simplified formaldehyde fixation and immunoprecipitation technique for studying protein-DNA interactions. *Anal. Biochem.* 197:83-90.

Gilmour, D.S. and Lis, J.T. 1984. Detecting protein-DNA interactions in vivo: Distribution of RNA polymerase on specific bacterial genes. *Proc. Natl. Acad. Sci. U.S.A.* 81:4275-4279.

Gilmour, D.S., Rougvie, A.E., and Lis, J.T. 1991. Protein-DNA cross-linking as a means to determine the distribution of proteins on DNA in vivo. *Meth. Cell Biol.* 35:369-381.

Harlow, E. and Lane, D. 1998. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Hecht, A., Strahl-Bolsinger, S., and Grunstein, M. 1996. Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature* 383:92-96.

Iyer, V.R., Horak, C.E., Scafe, C.S., Botstein, D., Snyder, M., and Brown, P.O. 2001. Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature* 409:533-538.

Kuras, L. and Struhl, K. 1999. Binding of TBP to promoters *in vivo* is stimulated by activators and requires Pol II holoenzyme. *Nature* 399:609-612.

Kuras, L., Kosa, P., Mencia, M., and Struhl, K. 2000. TAF-containing and TAF-independent forms of transcriptionally active TBP *in vivo*. *Science* 288:1244-1248.

Li, X.-Y., Virbasius, A., Zhu, X., and Green, M.R. 1999. Enhancement of TBP binding by activators and general transcription factors. *Nature* 399:605-609.

Meluh, P.B. and Koshland, D. 1997. Budding yeast centromere composition and assembly as revealed by in vivo cross-linking. *Genes Dev.* 11:3401-3412.

Mencia, M. and Struhl, K. 2001. A region of TAF130 required for the TFIID complex to associate with promoters. *Mol. Cell. Biol.* 21:1145-1154.

Orlando, V., Strutt, H., and Paro, R. 1997. Analysis of chromatin structure by in vivo formaldehyde cross-linking. *Methods* 11:205-214.

Ren, B. et al. 2000. Genome-wide location and function of DNA binding proteins. *Science* 290:2306-2309.

Solomon, M.J. and Varshavsky, A. 1985. Formaldehyde-mediated DNA-protein crosslinking: A probe for in vivo chromatin structures. *Proc. Natl. Acad. Sci. U.S.A.* 82:6470-6474.

Solomon, M.J., Larsen, P.L., and Varshavsky, A. 1988. Mapping protein-DNA interactions in vivo with formaldehyde: Evidence that histone H4 is retained on a highly transcribed gene. *Cell* 53:937-947.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev.* 11:83-93.

Tanaka, T., Knapp, D., and Nasmyth, K. 1997. Loading of an MCM protein onto DNA replication origins is regulated by cdc6p and CDKs. *Cell* 90:649-660.

Key References

Hecht et al., 1996. See above.

Describes the technique from which the Basic Protocol was adapted.

Orlando et al., 1997. See above.

Describes formaldehyde cross-linking and immunoprecipitation for chromatin analysis in Drosophila, and discusses various parameters of the technique.

Solomon et al., 1988. See above.

Describes original formaldehyde cross-linking and immunoprecipitation technique for mapping protein-DNA interactions.

Solomon and Varshavsky, 1985. See above.

Characterizes formaldehyde cross-linking, cross-link reversal, and sensitivity of cross-linked protein-DNA complexes to proteases and endonucleases.

Contributed by Oscar Aparicio
University of Southern California
Los Angeles, California

Joseph V. Geisberg and Kevin Struhl
Harvard Medical School
Boston, Massachusetts

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) is the only technique that can directly measure the binding energetics of biological processes, including protein-ligand binding, protein-protein binding, DNA-protein binding, protein-carbohydrate binding, protein-lipid binding, and antigen-antibody binding. ITC has the ability to precisely determine the Gibbs energy, enthalpy, entropy, and heat capacity changes associated with binding.

In this unit several protocols are presented, from the basic ones that are aimed at characterizing binding of moderate affinity (see Basic Protocols 2 and 3) to the advanced ones that are for determining very high or very low affinity (see Alternate Protocols 1 and 2). Also, alternate protocols for special cases (homodimeric proteins and unstable proteins; see Alternate Protocols 3 and 4) and additional information obtainable by ITC (heat capacity and proton transfer coupled to binding; see Alternate Protocols 5 and 6) are presented.

PRINCIPLES OF THE TECHNIQUE

The ITC instrument is a heat-flux calorimeter operating according to the dynamic power compensation principle, i.e., it measures the amount of power (μcal/sec) required to maintain a constant temperature difference (close to zero) between the sample and the reference cell (see Basic Protocol 1). Initially, the feedback system continuously applies a small power to the sample cell, which determines the baseline level. Each injection of the syringe solution (usually termed as ligand) triggers the binding reaction and, depending on the binding affinity and the concentration of reactants (macromolecule, M, and ligand, L) in the cell, a certain amount of macromolecule/ligand (ML) complex is formed. The formation of complex is accompanied by the release (exothermic reaction) or the absorption (endothermic reaction) of heat that causes a difference in temperature between the two cells. Then, the feedback system either lowers or raises the thermal power applied to compensate such temperature unbalance. After each injection, the system reaches equilibrium and the temperature balance is restored. Therefore, the recorded signal shows a typical deflection pattern in the form of a peak (Fig. 17.8.1). Integrating the area under the peak, assuming the baseline as reference, provides the amount of heat associated with the injection. As the reactant in the cell becomes saturated, the heat signal diminishes until only the background heat, due to an unspecific phenomena (e.g., ligand dilution, liquid friction), is observed.

INSTRUMENTATION

Two identical coin-shaped cells, sample and reference, are enclosed in an adiabatic shield (jacket) as illustrated in Figure 17.8.1. The temperature difference between the reference cell and the jacket is continuously monitored to maintain a constant temperature. A feedback control system monitors the difference in temperature between the two cells through a semiconductor Peltier sensor device sandwiched between them. This temperature difference is kept constant and as close to zero as possible at any time. The feedback signal is the measured signal.

One of the reactants is placed in the sample cell and the other one in the injection syringe. During the course of an experiment, the syringe is used to add the titrant reactant to the sample cell in a stepwise fashion and, at the same time, stir the solution in the sample cell to achieve fast mixing. The reference cell serves only as a temperature reference.

Contributed by Adrián Velázquez-Campoy, Hiroyasu Ohtaka, Azin Nezami, Salman Muzammil, and Ernesto Freire

Current Protocols in Cell Biology (2004) 17.8.1-17.8.24

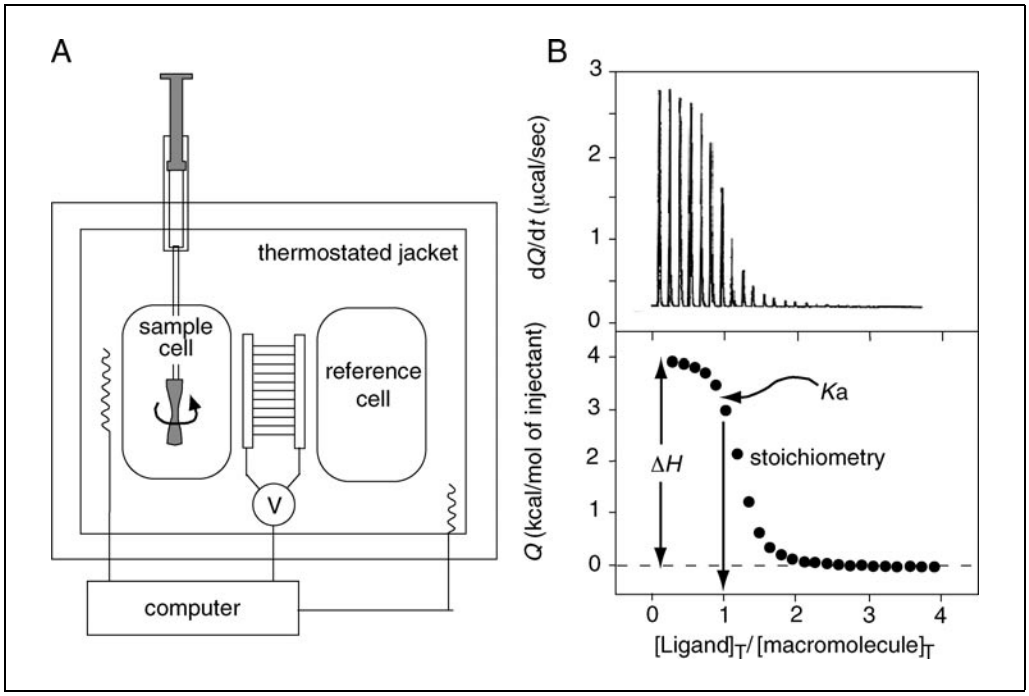


Figure 17.8.1 (**A**) Basic schematic illustration of the ITC instrument, showing the two cells (sample and reference) surrounded by the thermostated jacket, the injection syringe that also works as stirring device, and the computer-controlled thermostatic and feedback systems (using Peltier and resistor devices as sensor and actuator subsystems). (**B**) Example of a typical ITC experiment. The top panel shows the sequence of peaks, each one corresponding to each injection of the solution in the syringe. The monitored signal is the additional thermal power needed to be supplied or removed at anytime to keep a constant temperature in the sample cell and as close as possible to the reference cell temperature. This example corresponds to an endothermic binding. The bottom panel shows the integrated heat plot. The areas under each peak, calculated and normalized per mole of ligand injected in each injection, are plotted against the molar ratio (quotient of the total concentrations of ligand and macromolecule in the sample cell). From this plot, and applying the appropriate model, the thermodynamic parameters of the binding can be obtained: binding affinity, binding enthalpy, and stoichiometry.

BASIC PROTOCOL 1

ISOTHERMAL TITRATION CALORIMETRY

The protocols differ only in the way the experiment is planned and not in the procedure for using the instrument. This protocol consists of a detailed description of setting up and using the ITC instrument. The time required for performing a complete ITC experiment (setting up the instrument, running the experiment, and analyzing the results) is ~2 hr.

This protocol should not be considered a substitute for the instrument manual, where more detailed and specific explanations are provided. This protocol is written based on the VP-ITC MicroCalorimeter (Microcal LLC) and it can serve as a guide for other models.

Materials

Reactant solutions: macromolecule and ligand
Methanol

VP-ITC calorimeter (e.g., Microcal LLC or equivalent)
Vacuum pump
2.5-ml long-needle syringe (e.g., Hamilton)
12 × 75–mm and 6 × 50–mm glass tubes

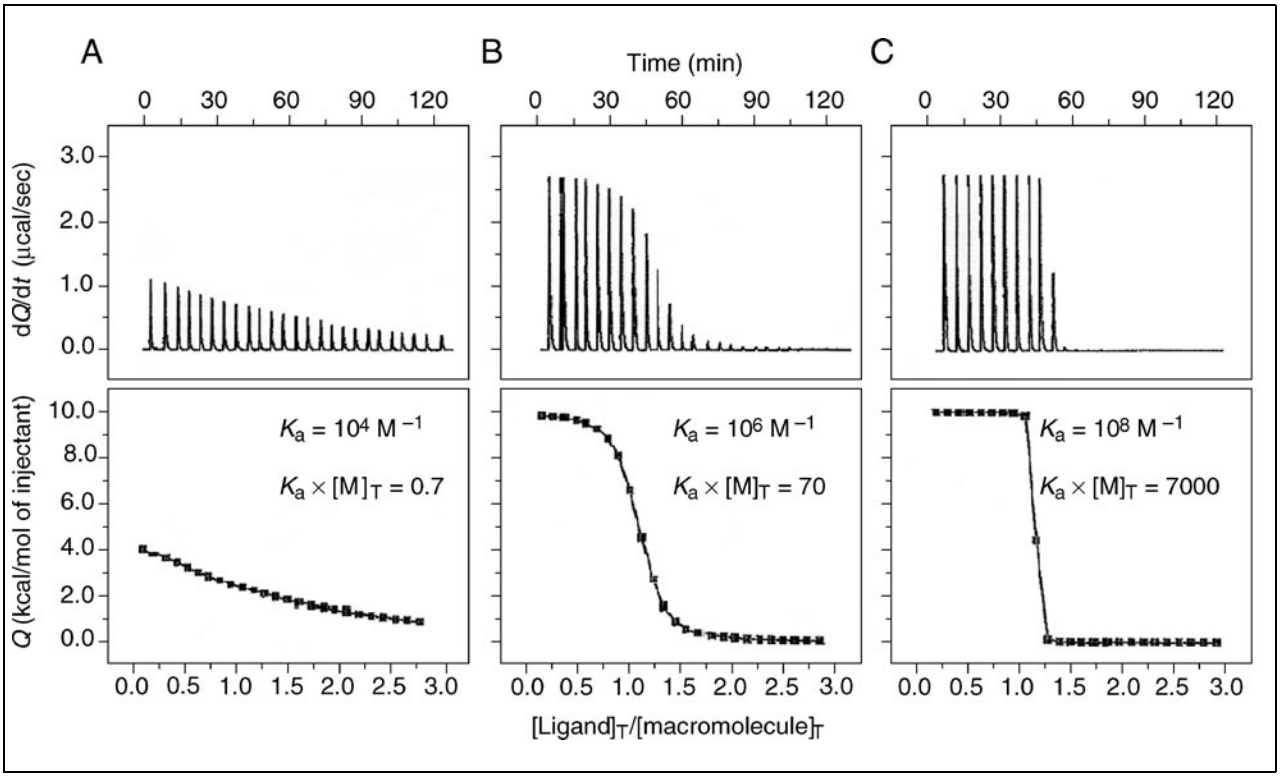


Figure 17.8.2 Illustration of the effect of the association constant value on the shape of a titration curve. The plots represent three titrations simulated using the same parameters (concentrations of reactants and binding enthalpy), but different binding affinities. Low (**A**), moderate (**B**), and high affinity (**C**) binding processes are shown. To obtain accurate estimates of the binding affinity an intermediate case is desirable (**B**, $1 < c = K_a \times [M]_T < 1000$). When the parameter c is large enough, a good estimate of the enthalpy can be determined from the *y*-axis intercept of the curve (if the heat effect associated with dilution is used as a reference). Such an intercept value is given by $(c/c+1) \times \Delta H_a$ (Indyk and Fisher, 1998).

Select concentration of reactants

1. Determine the appropriate concentrations of reactants.

   Typically, the concentrations of reactants are in the micromolar range; if the heat associated with the reaction is significant, however, the concentrations can be lowered.

   An important requirement for an ITC experiment is having appropriate concentrations of interacting molecules such that the heat associated with a given binding reaction is within the calorimetric determination range. Sensitivity of the instrument varies depending on brand and model. The Microcal VP-ITC instrument used in all the experiments described in this unit has a limiting sensitivity of 0.1 μcal. Therefore, to accurately determine the change of heat involved in the interaction between molecules, each injection should have a minimum of 1 μcal of associated heat.

   The affinity of the interaction should also be considered in choosing the required concentrations of the interaction components. A dimensionless constant, c, describes the practical window of the instrument to accurately determine the binding constants (Wiseman et al., 1989) in Equation 17.8.1:

$$c = nK_a[M]_T$$

Equation 17.8.1

   where, K_a is the binding constant, $[M]_T$ the total macromolecular concentration in the cell, and n is the stoichiometry of interaction. As illustrated in Figure 17.8.2, a large c value corresponds to very tight binding, resulting in an isotherm that is rectangular in shape and is devoid of data points in the transition region of binding, therefore, making the estimation of the binding affinity unreliable, although the binding enthalpy can still be obtained accurately. Conversely, a very low c value corresponds to weak binding and

the resultant isotherm is featureless, making the determination of both the binding affinity and enthalpy unreliable. Keeping other parameters constant (K_a, ΔH, etc), the c value could be reduced or increased by changing the $[M]_T$. ITC data with c values between 1 and 1000 can be used for determination of binding constants.

To obtain a complete binding isotherm within the specified number of injections, the ligand solution in the syringe should be more concentrated than the macromolecule in the cell, so that at the end of the experiment, the molar ratio of ligand to macromolecule inside the cell is 2 to 3 (for 1:1 stoichiometry). Considering the volume of the sample cell (1.4 ml), the typical injection volume (10 μl), and the typical number of injections (~30), it is advisable to use a concentration of ligand in the syringe 10 to 20 times higher than the solution in the cell. This will guarantee that the reaction reaches the neutralization point after 7 to 13 injections.

Prepare samples

2. Choose experimental conditions, taking into account the stability and the solubility of the reactants and the biological considerations of the system under study. Prepare reactant solutions for the cell and the syringe under identical conditions and with the same composition.

   For the reactant solutions, buffer type, buffer concentration, pH, ionic strength, and co-solvents must be the same. In an ITC assay, consistency between the exact composition of the buffer in the cell and the syringe is of crucial importance to prevent dominance of unspecific heat effects. The composition, concentration, pH, and ionic strength of the buffer all affect the thermodynamic parameters, and the quality of the experiment depends on maintaining a perfect match of the buffer in the cell and syringe samples. One effective way of achieving this goal is to dialyze the macromolecule against the desired buffer and then using the filtered dialysis buffer to prepare the ligand solution. Certain additives such as DMSO, which increases the solubility of hydrophobic ligands, have an enormous effect on the ITC signal, therefore, utmost care should be taken to keep the concentration of DMSO in the cell and syringe nearly the same.

3. Prepare the reactant solutions in the appropriate volumes.

   Although the apparent volume of the sample cell is 1.4 ml, the volume of solution required for proper filling of the cell is 2.2 ml. The volume required for the syringe is 0.5 ml.

4. Degas all solutions (reactants and buffer solution used for rinsing the cell) for 10 to 20 min (stirring and temperature control are optional) to avoid formation of bubbles in the sample cell during the experiment.

5. To prevent long equilibration delays, lower the calorimeter thermostat setting slightly below the running temperature (with a difference of 0.5° to 2°C).

Load solutions

6. Place degassed, distilled water or buffer solution in the reference cell using a 2.5-ml, long-needle syringe.

7. Rinse the sample cell several times with buffer solution, then remove all liquid.

8. Fill the cell with macromolecule solution, taking care to prevent the appearance of bubbles in the cell.

   The most common configuration is one in which a macromolecule is placed in the reaction cell and a low-molecular-weight ligand is placed in the injecting syringe. For convenience, the injected reactant located in the syringe is referred to as "ligand" and the one in the reaction cell is referred to as "macromolecule," throughout this unit. The reader should be aware that other configurations are possible.

9. Fill the calorimeter syringe with ligand solution according to the maufacturer's instructions. Rinse off excess ligand solution on the surface of the needle with water and then carefully blot the surface of the needle with a paper towel.

A special plastic syringe is used to fill the calorimeter injecting syringe. A purge-refill cycle may be performed to ensure the absence of air bubbles inside the syringe.

Set instrument settings

10. Set the total number of injections to 30.
11. Set measurement temperature to °C.
12. Set reference power to 10 μcal/sec.

    The reference power can be modified if a large exothermic or endothermic reaction is expected.

13. Set the initial delay (time between the start and the first injection) to 200 sec.
14. Set the syringe ligand concentration to millimolar (mM).
15. Set the cell macromolecule concentration to millimolar (mM).

    During the experiment and analysis, the concentrations are always expressed as millimolar.

16. Set the stirring speed ~500 rpm.

    This speed is selected to ensure good mixing, but it can be modified depending on the sample (e.g., high viscosity, tendency to generate foam, etc.).

17. Set feedback mode/gain to high (high sensitivity and small response time).
18. Set injection volume to 10 μl.

    A different injection volume can be set, for example, if the heat of reaction is expected to be very large or very small. However, if the ligand concentration can be varied, then it is preferable to adjust its concentration in the injecting syringe rather than the injection volume.

19. Set duration for injection to 20 sec.

    This setting is automatically assigned according to the injection volume.

20. Set spacing between injections to 400 sec (usually).

    The spacing might be increased depending on peak size or the reaction kinetics.

    The user should allow enough time between peaks in order to keep the next injection from starting before the signal from the previous injection returns to baseline. Typically, there should be ~200 sec between the end of an injection and the beginning of the next one.

21. Set filter period (the signal-average time period) to 2 sec.
22. Set the volume of the first injection to ≤3 μl. Do not include the heat associated with the first injection in the data analysis.

    Due to inter-diffusion of the solutions during the insertion of the syringe or the equilibration stage, the first injection is not useful in the analysis, therefore, the volume of the first injection should be set to ≤3 μl to minimize the amount of ligand wasted.

Start measurement and troubleshoot the baseline (noise, drift, level)

23. After gently setting the injection syringe in place, initiate the experiment. If fast equilibration is initially selected, both thermal and mechanical equilibration will proceed simultaneously.

    The equilibration level will be slightly less than the reference power value entered in the parameter setting due to the power generated by stirring.

24. Check the quality of the baseline in a full scale of 1 μcal/sec. When the baseline becomes flat, with no significant drift or noise, start the injection sequence.

If the baseline is of poor quality, several possibilities should be considered. (1) Small irregular noise is probably due to air bubbles in reference or sample cell. In the case of bubbles in the sample cell, stop measurement, remove syringe, and check cell content. In the case of bubbles in the reference cell, replace the content of the cell. (2) Strong regular noise and/or drift indicates that the injection syringe is bent. Stop measurement and check the needle of the syringe by rolling it on a flat surface. (3) A high baseline level indicates low liquid level or air bubbles in the reference cell. (4) A low baseline level indicates that the viscosity of the cell solution is high or the cell is dirty.

25. Collect data.

    Once the sequence of injections is initiated, the titration should be continued until saturation is observed.

Clean the ITC

26. Wash the injection syringe with ≥150 ml water. Frequently, and especially for low solubility reactants, wash the syringe with 50 ml methanol (or water-organic co-solvent) and 300 ml water. Dry syringe for ≥10 min.

27. Wash the plunger tip of the syringe injector with water and dry it.

28. Wash the sample cell with water (ten times or more) using the long-needle syringe.

    For periodic deep cleansing or when using a different macromolecule or in cases where the sample precipitated or aggregated in the sample cell, the sample cell should be washed with a cleaning solution (compatible with the material the cells are made of) according to the manufacturer's instructions.

    CAUTION: *Do not leave any methanol or organic solvent in the injection syringe or the cells.*

SUPPORT PROTOCOL

DATA ANALYSIS FOR ITC EXPERIMENTS

The following section is an outline of the foundations for data analysis, including the basic equations describing the binding equilibrium and its implementation. Data analysis is usually performed by non-linear regression using fitting functions defined in the control and analysis software provided by the manufacturer.

Adjust baseline and integrate peaks

1. Carefully check the quality of the automatic baseline and, if applicable, manually modify the baseline and the integration interval.

    The application automatically creates a baseline and integrates each peak when reading the data file. The baseline should follow a smooth path, clearly define a peak signal that relaxes and asymptotically merges with the baseline, and go through the noise when the reaction is finished. The appropriate integration interval should cover the entire peak, taking care to include all the area corresponding to the relaxation stage.

Enter concentration values

2. Enter the values of the ligand and macromolecule concentrations, as well as the sample cell volume to ensure the accuracy of the calculations.

Estimate heat of dilution

3. Note the heat observed after saturation; it reflects different unspecific phenomena, and it is referred to as "heat of dilution." If the fitting procedure does not account for the heat of dilution through an adjustable parameter, make an estimation of such value, e.g., by averaging the values of the last injections or by performing a blank experiment injecting reactant into a buffer solution (see below). If estimated, subtract the heat of dilution from the heat associated with each injection.

Select fitting function

4. Determine the model for binding to be used.

   Although traditional data analysis methods based on linearization of the binding equations have been commonly used, a non-linear regression is a more appropriate methodology for analyzing ITC data. A suitable model, according to the system under study, needs to be considered when selecting a fitting function to use in the non-linear least square regression analysis. The description for different models can be found in the literature (Cantor and Schimmel, 1980; Freire et al., 1990; Wyman and Gill, 1990; Van Holde et al., 1998; Jelesarov and Bosshard, 1999; Sigurskjold, 2000).

5. Express the binding equations in terms of total concentrations. The total concentration of each reactant inside the calorimetric cell is known and after each consecutive injection, *i*, is given by Equation 17.8.2:

$$[\mathrm{M}]_{\mathrm{T},i} = [\mathrm{M}]_o\left(1-\frac{v}{V}\right)^i$$

$$[\mathrm{L}]_{\mathrm{T},i} = [\mathrm{L}]_0\left(1-\left[1-\frac{v}{V}\right]^i\right)$$

Equation 17.8.2

   where $[\mathrm{M}]_0$ is the initial concentration of macromolecule in the cell, $[\mathrm{L}]_0$ is the concentration of ligand in the syringe, V is the cell volume, v is the injection volume and $1-(v/V)$ is the factor that accounts for the change in the concentration of reactants due to the dilution that takes place upon each injection.

   Since the concentration of each chemical species (free reactants and complex) is not known, the binding equations must be expressed in terms of total concentrations.

6. Determine the heat released or absorbed due to each ligand injection, q_i, i.e., the heat associated with the formation of complex in injection *i* according to Equation 17.8.3:

$$q_\mathrm{i} = V\Delta H_\mathrm{a}\left([\mathrm{ML}]_i - [\mathrm{ML}]_{i-1}\left[1-\frac{v}{V}\right]\right)$$

Equation 17.8.3

   where ΔH_a is the binding or association enthalpy. Again, the dilution factor indicates that the heat change in each injection corresponds to different reactant concentrations.

7. Using the mass action law and the conservation of mass for each species, the expression for the concentration of complex after each injection becomes Equation 17.8.4:

$$[\mathrm{ML}]_i = \frac{1+[\mathrm{M}]_{\mathrm{T},i}K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i} - \sqrt{(1+[\mathrm{M}]_{\mathrm{T},i}K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i})^2 - 4[\mathrm{M}]_{\mathrm{T},i}K_\mathrm{a}^2[\mathrm{L}]_{\mathrm{T},i}}}{2K_\mathrm{a}}$$

Equation 17.8.4

8. It is customary to include in this equation a factor *n*, the stoichiometry, which can be interpreted in two ways: (1) as the generalization for a macromolecule with *n*

equivalent and independent binding sites or (2) to account for the possibility of having a percentage of the macromolecule in a non-competent binding conformation:

$$[\mathrm{ML}]_i = \frac{1 + n[\mathrm{M}]_{\mathrm{T},i} K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i} - \sqrt{(1 + n[\mathrm{M}]_{\mathrm{T},i} K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i})^2 - 4n[\mathrm{M}]_{\mathrm{T},i} K_\mathrm{a}^2 [\mathrm{L}]_{\mathrm{T},i}}}{2K_\mathrm{a}}$$

Equation 17.8.5

Equations 17.8.4 and 17.8.5 constitute the formalism to analyze binding experiments in which a ligand binds a macromolecule with n equivalent and independent binding sites. Given the initial concentrations of reactants, $[M]_0$ and $[L]_0$, the binding affinity, K_a, the binding enthalpy, ΔH_a, and the stoichiometry, n, it is possible to estimate the heat involved in each injection, which will be the dependent variable used in the analysis, and incorporate that calculation in a fitting function. The thermodynamic parameters (K_a, ΔH_a, and n) will be estimated as adjustable parameters in the fitting procedure.

All examples presented in this unit can be analyzed with built-in functions embedded in Origin version 5.0 or 7.0.

The previous formalism can be applied to other cases (see Basic Protocol 2) by appropriately modifying Equations 17.8.3 and 17.8.4.

BASIC PROTOCOL 2

MACROMOLECULE/LIGAND INTERACTION WITH MODERATE AFFINITY

Ligands with moderate binding affinities to their target macromolecule constitute the majority of the cases in biological systems. However, ligands with very low or very high affinities are also encountered. Conducting ITC assays for a complete thermodynamic characterization of the binding of such ligands requires modified versions of the basic ITC protocol and will be explained later. When the affinity between the macromolecule and the ligand is moderate ($10^4 < K_\mathrm{a} < 10^8\ \mathrm{M}^{-1}$), it is possible to determine the affinity and the enthalpy of binding simultaneously in a single experiment.

This protocol uses commercially available ribonuclease A (RNase A), a well-studied endonuclease of 13.7 kDa, and its commercially available inhibitor 2′-cytidine-monophosphate (2′CMP) of 323 Da, to familiarize first-time users with the setup, procedure, and data analysis of isothermal titration calorimetry. The binding of 2′CMP to RNase A is an example of an exothermic binding reaction, and the binding constant for this reaction is within the range measurable by conventional ITC (Wiseman et al., 1989; Straume and Freire, 1992). It is highly recommended that every attempt at ITC begin with this simple protocol and that conducting experiments with other samples only take place after mastering the technique and analysis explained in this protocol. This protocol presents an efficient way of keeping the conditions in the ITC cell and syringe as close to each other as possible.

Materials

RNase A, lyophilized powder (Sigma)
15 mM potassium acetate buffer, pH 5.5
2′CMP, lyophilized powder (Sigma)

12 × 75–mm and 6 × 50–mm glass tubes
6-kDa MWCO dialysis membrane
0.22-μm filter

Prepare samples

1. Prepare RNase A stock solution in a 12 × 75–mm glass tube. Dissolve the protein in 15 mM potassium acetate buffer, pH 5.5, to a final concentration of 200 to 300 μM.

   RNase A is commercially available as a lyophilized powder. A final volume of 5 ml of this stock solution should be sufficient to conduct several experiments.

2. Dialyze RNase A stock solution against ∼1 liter 15 mM potassium acetate buffer, pH 5.5, overnight at 4°C using a dialysis membrane with a ∼6.0-kDa MWCO.

3. After dialysis, filter the RNase A solution through a 0.22-μm filter. Determine the exact concentration of the stock solution by measuring its absorbance at 278 nm using an extinction coefficient of 9800 $M^{-1}cm^{-1}$. Store the dialyzed stock of RNase A up to several weeks at 4°C.

4. Prepare 2′CMP stock solution in a 12 × 75–mm glass tube. Filter 15 mM potassium acetate buffer, pH 5.5, used for dialysis using a filter with a 0.22-μm pore size. Dissolve the lyophilized 2′CMP (or 5′CMP) in the filtered buffer to a final concentration of 5 mM. Determine the exact concentration of the stock solution by measuring its

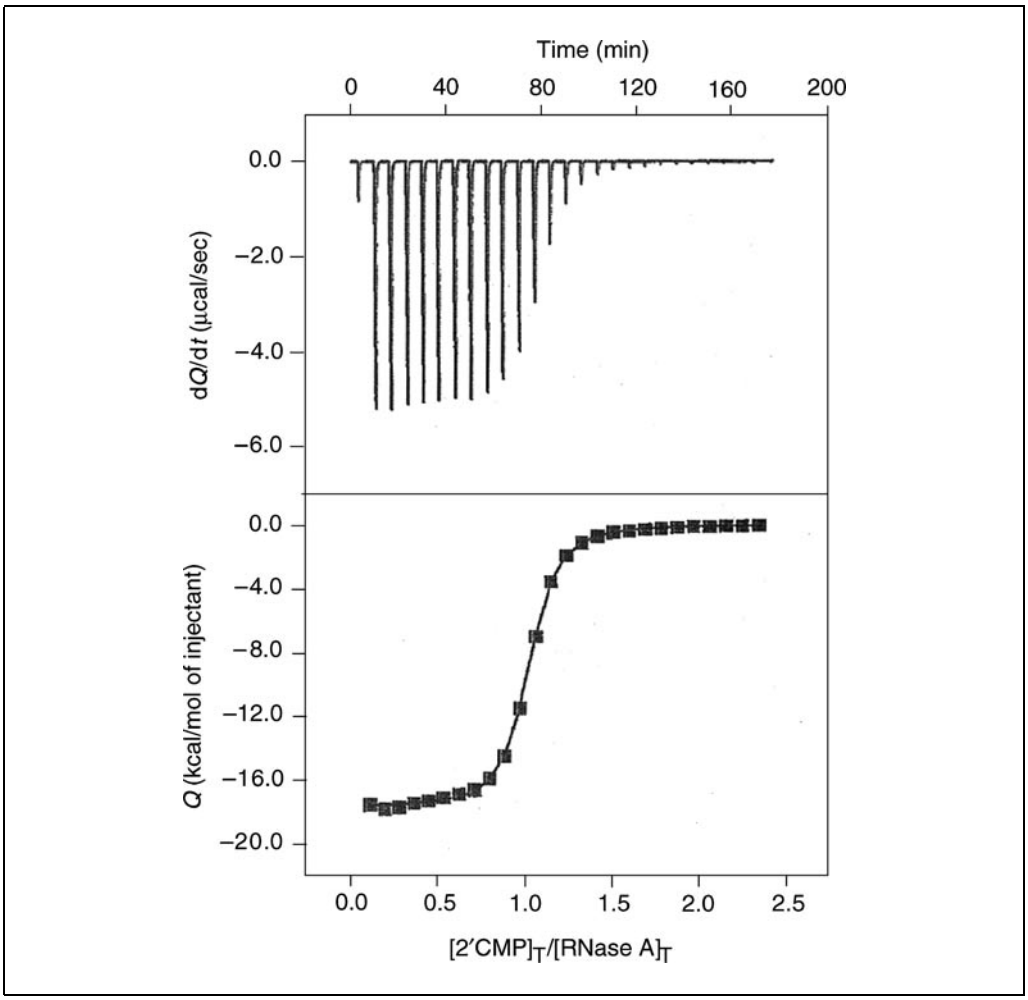


Figure 17.8.3 Titration of RNase A with 2′CMP. The experiment was performed in 15 mM potassium acetate, pH 5.5, at 25°C. The concentration of reactants are 76 μM RNase A and 1.13 mM 2′CMP. The solid line corresponds to the theoretical curve with $n = 1.02$, $K_a = 2.9 \times 10^6$ M^{-1} and $\Delta H = -19.3$ kcal/mol.

absorbance at 260 nm using an extinction coefficient of 7400 $M^{-1}cm^{-1}$ in 100 mM phosphate buffer, pH 7.0.

A final volume of 1 ml should be sufficient to conduct several experiments.

5. Dilute the dialyzed RNase A stock to a final concentration of 50 to 100 μM in 15 mM potassium acetate buffer, pH 5.5.
6. Prepare cell sample (2.2 ml of diluted RNase A) to ensure proper loading of the cell.
7. Dilute the 2′CMP stock to a final concentration of 0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5, depending on the concentration of RNase A.
8. Prepare syringe sample (0.5 ml of diluted 2′CMP) to ensure proper loading of the syringe.
9. Degas 10 ml of the same buffer employed, cell sample solution, and syringe sample solution 20 to 30 min.

   This buffer solution will be used for rinsing the sample cell.

10. After preparation, transfer the syringe solution to a 6 × 50–mm glass tube to fill the syringe.
11. Set the instrument settings, run the experiment, and analyze the data (see Basic Protocol 1 and Support Protocol).

    Figure 17.8.3 shows the result from an experiment in which 2′CMP binds to the enzyme RNase A. The thermodynamic parameters obtained from this experiment are: $K_a = 2.0 \times 10^6\ M^{-1}$, $\Delta H_a = -17.8$ kcal/mol, $n = 0.99$.

BASIC PROTOCOL 3

MACROMOLECULE/MACROMOLECULE INTERACTION WITH MODERATE AFFINITY

The same equations above (see Support Protocol) apply to macromolecule/macromolecule binding. The formalism can be applied to this case by considering the macromolecule in the syringe as the ligand. As in every situation, the less soluble reactant should be placed in the calorimetric cell.

This protocol uses commercially available porcine pancreatic trypsin (PPT), a well-studied serine protease of 23.8 kDa, and its commercially available inhibitor, soybean trypsin inhibitor (STI), of 20.0 kDa. The binding of STI to PPT is an example of an endothermic binding reaction, and the binding constant for this reaction is within the range measurable by conventional ITC (El Harrous and Parody-Morreale, 1997).

Materials

Porcine pancreatic trypsin (PPT), lyophilized powder (Sigma)
25 mM potassium acetate/10 mM calcium chloride, pH 4.5
Soybean trypsin inhibitor (STI), lyophilized powder (Sigma)

10-kDa MWCO dialysis tubing
0.22-μm filter

Prepare samples

1. Dissolve PPT in 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, to a final concentration of ∼400 μM.

   A final volume of 1 ml of this PPT stock solution should be sufficient.

2. Dissolve STI in 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, to a final concentration of ~30 μM.

   A final volume of 5 ml of this STI stock solution should be sufficient.

3. Dialyze PPT and STI against ~1 liter of 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, overnight at 4°C using a dialysis membrane with a ~10-kDa MWCO.

4. After dialysis, filter the solutions through 0.22-μm pore filters.

5. Determine the exact concentration of the PPT and STI stock solutions by measuring their absorbances at 280 nm using extinction coefficients of 35,700 and 18,200 $M^{-1}cm^{-1}$, respectively. Store the dialyzed stocks for several weeks at 4°C.

6. Prepare cell sample. Prepare 2.2 ml of STI at a concentration of ~30 μM.

   Since STI has very low solubility compared to PPT, the former will be placed in the calorimetric cell.

7. Prepare syringe sample. Prepare 0.5 ml of PPT at a concentration of ~400 μM.

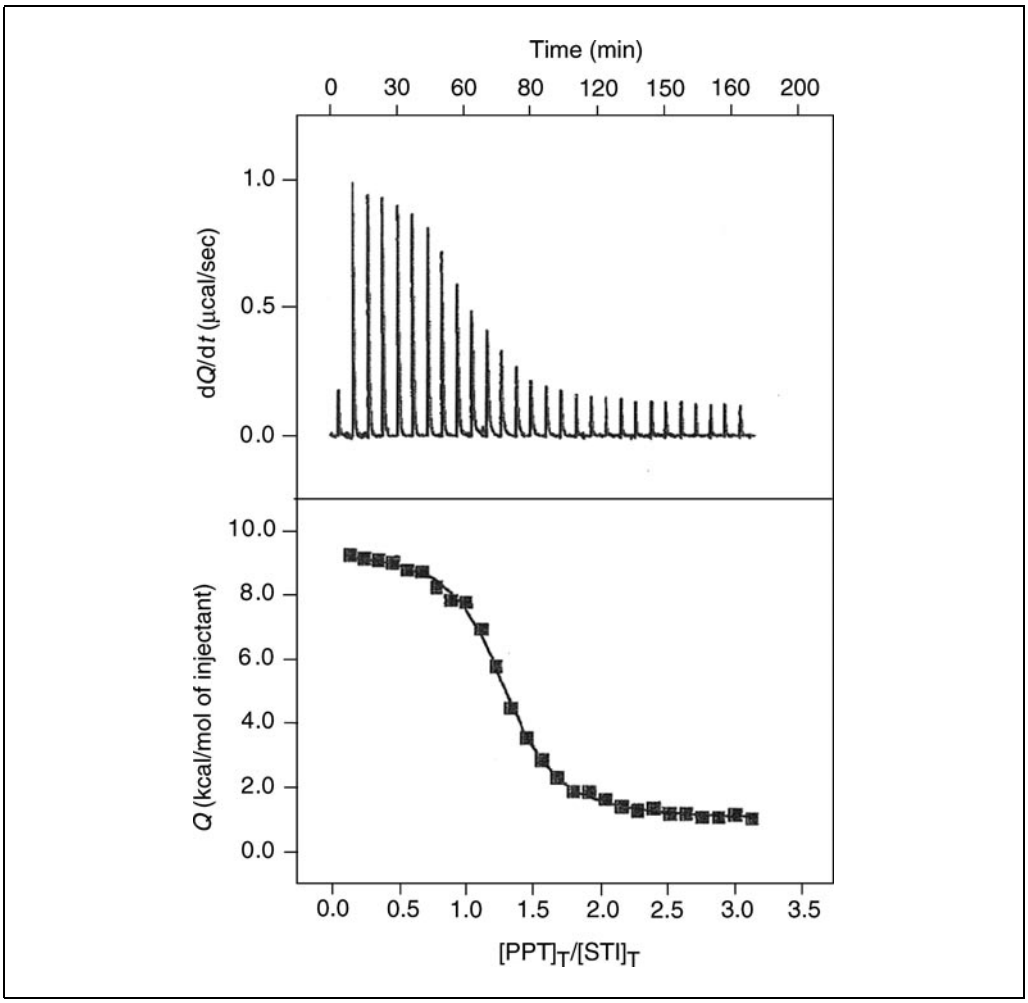


Figure 17.8.4 Titration of STI with PPT. The experiment was performed in 25 mM potassium acetate, pH 4.5/10 mM calcium chloride, at 25°C. The concentrations of reactants are 21 μM STI (in cell) and 312 μM PPT (in syringe). The inhibitor was placed in the calorimetric cell due to its low solubility. The solid line corresponds to theoretical curve with $n = 1.26$, $K_a = 1.5 \times 10^6$ M^{-1} and $\Delta H_a = 8.4$ kcal/mol.

8. Set instrument settings, run the experiment, and analyze the data (see Basic Protocol 1 and Support Protocol).

Figure 17.8.4 shows the result from an experiment in which STI binds to PPT. The thermodynamic parameters obtained from this experiment are: $K_a = 1.5 \times 10^6\ M^{-1}$, $\Delta H_a = 8.4$ kcal/mol, $n = 1.26$.

ALTERNATE PROTOCOL 1

MACROMOLECULE/LIGAND BINDING WITH HIGH AFFINITY

If the affinity between the reactants is too high ($K_a > 10^8\ M^{-1}$), the affinity of binding cannot be reliably measured. To determine the affinity directly, the concentrations of the reactants need to be lowered to levels below the sensitivity limit of the calorimeter. However, in the case of tight binding ligands, the binding enthalpy can be measured very accurately.

Traditionally, several methods have been employed to determine high affinity. All of them rely on changing the experimental conditions (temperature, pH) to reduce the apparent affinity to a measurable value (Doyle et al., 1995; Doyle and Hensley, 1998). The disadvantages are important: (1) appropriate equations (e.g., van't Hoff relationship) have to be used in order to extrapolate (with cumulative errors) to the initially intended experimental conditions; and (2) changing experimental conditions can compromise the stability or the solubility of the reactants.

A method that does not require a change in the experimental conditions is the displacement method (Sigurskjold, 2000). In this method, the ligand solution is placed in the syringe and a solution of the macromolecule pre-bound to a weaker, competitive ligand is placed in the cell. In this way, the apparent affinity of the potent ligand is reduced because it has to displace the weak ligand from the binding site to bind to the macromolecule, therefore, incurring in an energy penalty. Thus, the thermodynamic parameters for the binding of the weak ligand and also the linkage equations for the binding of the potent ligand in the presence of the weak one should be well known.

The same reasoning as in the basic case is made: from the mass conservation equations and the mass action law, it is possible to reach a cubic equation from which the concentration of all species, free and bound, can be obtained and the analysis can be done in an exact manner. The details are explained elsewhere (Sigurskjold, 2000).

However, even if the exact analysis is always the one to be performed, an approximation can be considered when the weak ligand concentration is much higher than the macromolecule concentration, so that the concentration of free weak ligand is assumed to be equal to the concentration of total weak ligand (Zhang and Zhang, 1998). In this approximation, the apparent binding parameters in the displacement binding are related to the individual binding parameters as in Equations 17.8.6 and 17.8.7:

$$K_{a,A}^{app} = \frac{K_{a,A}}{1 + K_{a,B}[B]}$$

Equation 17.8.6

$$\Delta H_{a,A}^{app} = \Delta H_{a,A} - \Delta H_{a,B} \frac{K_{a,B}[B]}{1 + K_{a,B}[B]}$$

Equation 17.8.7

where A and B are the potent and the weak ligands, $K_{a,A}$ and $\Delta H_{a,A}$ are the affinity and enthalpy of binding of the potent ligand, and $K_{a,B}$ and $\Delta H_{a,B}$ are the affinity and enthalpy

of binding of the weak ligand, and the free concentration of ligand B can be approximated by its total concentration.

One of the most important applications of ITC is in the field of structure-based drug design. The goal in any drug design process is to obtain inhibitors with very tight binding affinities to the desired target, while minimizing the binding of the inhibitor to unwanted targets. Another goal is to design inhibitors that are able to adapt to genetic polymorphisms in their target. Genetic polymorphisms can arise as a result of natural population polymorphisms or mutations that cause drug resistance in viral, microbial, and parasitic targets.

Understanding the detailed mechanism and thermodynamic differences in the binding of various inhibitors allows the development of guidelines essential to the design of highly specific drugs that are able to adapt to genetic polymorphisms in their target. The first structure-based drugs were targeted against HIV-1 protease, an aspartic protease essential for the processing of the HIV virus. Measuring the thermodynamic parameters of the binding of HIV-1 protease inhibitors to their target is compounded by the very tight binding of these inhibitors ($K_a > 10^8$ M^{-1}), which makes direct titration impossible. A displacement assay, in which a tight-binding inhibitor competes with a weaker binding inhibitor ($K_a = 10^6 - 10^7$ M^{-1}), allows the measurement of all thermodynamic parameters in a single ITC experiment. This protocol uses HIV-1 protease, a well-studied aspartic protease of 21.6 kDa, and its commercially available inhibitors nelfinavir and acetyl-pepstatin. Unlike acetyl-pepstatin, the affinity of the binding of nelfinavir to HIV-1 protease is beyond the higher limit of measurable range by conventional ITC.

Three titrations should be made: two direct titrations with nelfinavir and acetyl-pepstatin binding to HIV-1 protease, respectively, and one displacement titration of nelfinavir binding to protease prebound to acetyl-pepstatin.

Materials

Acetyl pepstatin (Bachem)
9 mM NaOH
Nelfinavir (Viracept; or any other clinical or experimental inhibitor)
100% DMSO
HIV-1 protease (Todd et al., 1998 and Velazquez-Campoy et al., 2002)
10 mM sodium acetate, pH 5.0/2% (v/v) DMSO

Prepare samples

1. Prepare acetyl-pepstatin stock solution: dissolve the lyophilized acetyl-pepstatin in 9 mM NaOH (this compound is very insoluble in pure water) to a concentration of 8 to 9 mM.

2. Determine the concentration by analytical quantitative nitrogen content determination (Jaenicke, 1974; Pace et al., 1995).

   Acetyl-pepstatin shows no absorbance at 280 nm.

3. Prepare nelfinavir stock solution at >15 mM in 100% DMSO.

   Nelfinavir is not water soluble. The solution in pure DMSO should be very concentrated (15 to 40 mM). When DMSO is diluted in aqueous buffer, the concentration of nelfinavir is closer to 200 to 300 μM.

4. Prepare cell sample for first titration by purifying HIV-1 protease according to Todd et al. (1998) and Velázquez-Campoy et al. (2002) and storing at ∼20°C at a

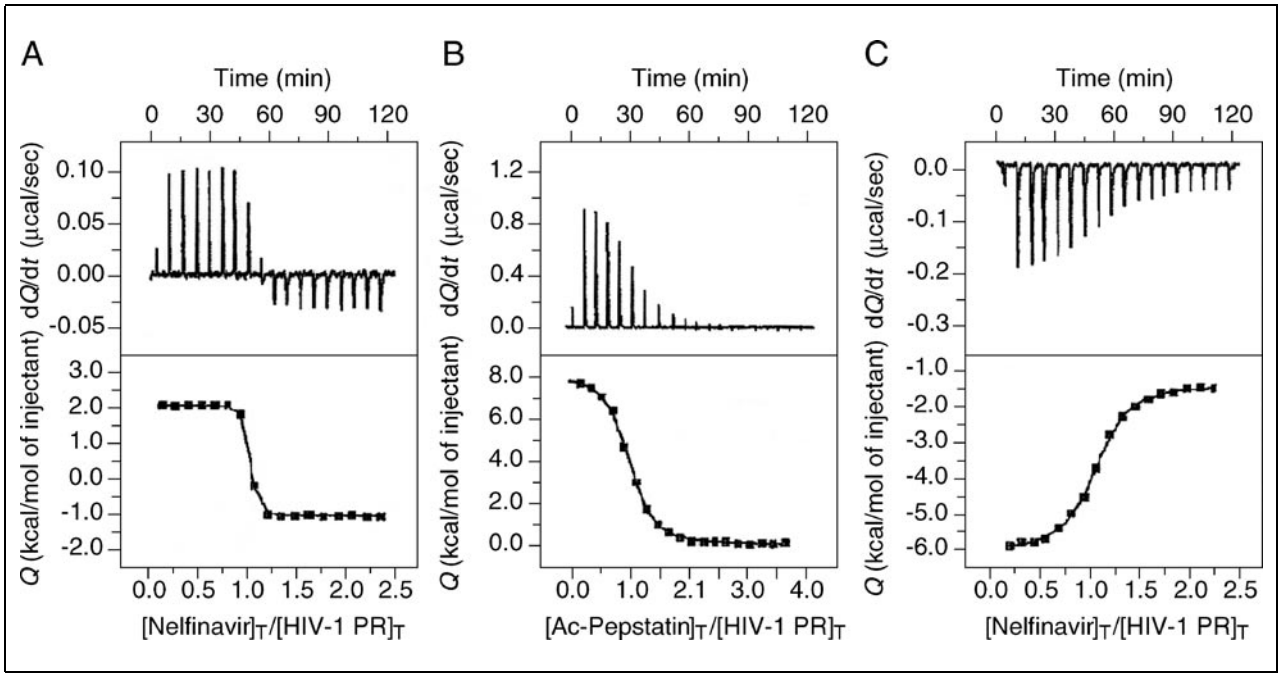


Figure 17.8.5 Set of calorimetric titrations corresponding to the implementation of the displacement protocol for the estimation of very high affinity. The concentrations of reactants are: (**A**) HIV-1 protease 11 μM in cell and nelfinavir 130 μM in syringe; (**B**) HIV-1 protease 19 μM in cell and acetyl-pepstatin 300 μM in syringe; and (**C**) HIV-1 protease 10 μM and acetyl-pepstatin 200 μM in cell, and nelfinavir 130 μM in syringe. The thermodynamic parameters obtained from these experiments are: nelfinavir binding to protease: $\Delta H_a = 3.1$ kcal/mol, $n = 1.02$, and K_a cannot be reliably obtained; acetyl-pepstatin binding to protease: $K_a = 2.3 \times 10^6$ M^{-1}, $\Delta H_a = 8.0$ kcal/mol, $n = 0.98$; nelfinavir binding to protease pre-bound to acetyl-pepstatin $K_a = 2.2 \times 10^9$ M^{-1}, $\Delta H_a = 3.3$ kcal/mol, $n = 0.99$.

concentration of >2.5 mg/ml. Dissolve the protein to a final concentration of 20 μM in 10 mM sodium acetate, pH 5.0/2% DMSO.

5. Determine the exact concentration of the cell sample by measuring its absorbance at 280 nm using an extinction coefficient of 25,500 M^{-1} cm^{-1}.
6. Prepare syringe sample for first titration: 2.2 ml of nelfinavir stock diluted to a concentration of ~300 μM in 10 mM sodium acetate, pH 5.0. If necessary, add DMSO to a final concentration of 2% to ensure that the inhibitor is dissolved in the aqueous buffer.
7. Perform the first titration with HIV-1 protease in the cell and nelfinavir in the syringe.
8. Prepare cell sample for second titration. Repeat step 4.
9. Prepare syringe sample for second titration: 0.5 ml of acetyl-pepstatin diluted to a concentration of ~300 μM in 10 mM sodium acetate, pH 5.0. Add DMSO to a final concentration of 2%.
10. Perform the second titration with HIV-1 protease in the cell and acetyl-pepstatin in the syringe.
11. Prepare cell sample for third titration. Repeat step 4 adding acetyl-pepstatin to the HIV-1 protease in the sample cell solution to a final concentration of 200 μM.
12. Prepare syringe sample for third titration. Repeat step 6.
13. Perform third titration.

Figure 17.8.5 shows the results from an experimental scheme aimed at determining the thermodynamic parameters for the binding of nelfinavir to HIV-1 protease. The thermodynamic

parameters obtained from these experiments are: nelfinavir binding to protease: ΔH_a = 3.1 kcal/mol, n = 1.02; acetyl-pepstatin binding to protease: $K_a = 2.3 \times 10^6\ M^{-1}$, ΔH_a = 8.0 kcal/mol, n = 0.98; nelfinavir binding to protease prebound to acetyl-pepstatin: $K_a = 2.2 \times 10^9\ M^{-1}$, ΔH_a = 3.3 kcal/mol, n = 0.99 (applying the exact equations for the displacement experiment in the data analysis).

MACROMOLECULE/LIGAND BINDING WITH LOW AFFINITY

ALTERNATE PROTOCOL 2

If the affinity between the reactants is too low ($K_a < 10^4\ M^{-1}$), neither the affinity nor the enthalpy of binding can be reliably determined.

One way to determine both is again by using the displacement strategy (Zhang and Zhang, 1998). In this case, the binding parameters for the weak ligand are obtained from the change in the binding parameters of the potent ligand binding to the free macromolecule and its binding to the macromolecule pre-bound to the weak ligand. The same considerations and analysis procedure explained in the case of very high affinity ligands applies here.

Many natural ligands show very low affinity ($K_a < 10^4\ M^{-1}$) for their biological targets. To measure such affinity, the displacement protocol can be implemented again.

In the case of the CMP inhibitors of RNase A, a change in the position of the hydroxyl group from 2′ to 5′ position causes a dramatic decrease in the strength of the interaction. This example illustrates the effect of minor modifications in the ligand on the thermodynamic parameters of binding.

To estimate the binding affinity for 5′-cytidine-monophosphate (5′CMP) binding to RNase A, a similar set of titrations as in the previous section should be done: two direct titrations with 2′CMP and 5′CMP binding to RNase A and one displacement titration of 2′CMP binding to RNase A prebound to 5′CMP.

For materials, see Basic Protocol 2.

Prepare samples

1. Prepare RNase A and inhibitors 2′CMP and 5′CMP stock solutions following instructions in the Basic Protocol 2.
2. Prepare cell sample for first titration: 2.2 ml of RNase A diluted to a concentration of 50 to 100 μM in 15 mM potassium acetate buffer, pH 5.5.
3. Prepare syringe sample for first titration: 0.5 ml of 2′CMP diluted to a concentration of ∼0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5.
4. Prepare cell sample for second titration. Repeat step 2.
5. Prepare syringe sample for second titration: 0.5 ml of 5′CMP diluted to a concentration of ∼0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5.
6. Prepare cell sample for third titration. Repeat step 2 adding 5′CMP to the solution to a concentration of 0.6 mM.
7. Prepare syringe sample for third titration. Repeat step 3.

Figure 17.8.6 shows the results from an experimental scheme aimed at determining the thermodynamic parameters for the binding of 5′CMP to RNase A. Three titrations are shown: 2′CMP binding to RNase A, 5′CMP binding to RNase A, and 2′CMP binding to RNase A pre-bound to 5′CMP. In the first titration, the binding parameters are obtained as described earlier. It is not possible to obtain a reliable value for either the affinity or the enthalpy of binding from the second titration. On the other hand, applying the exact analysis

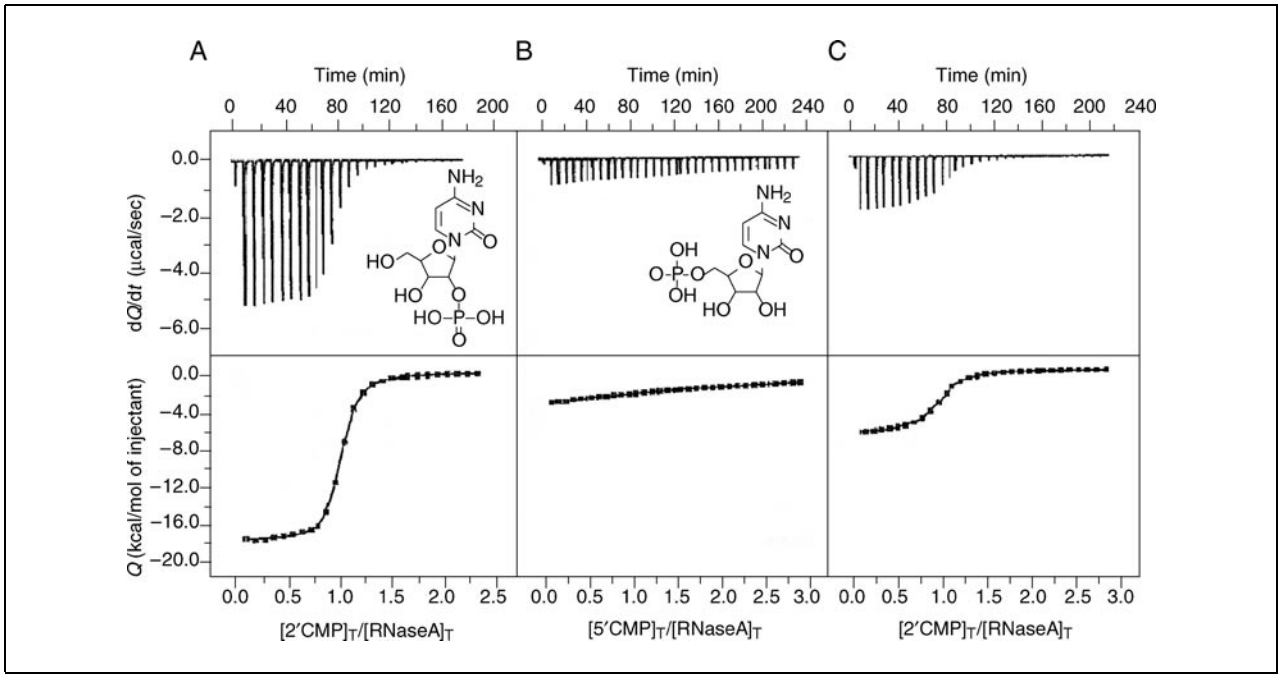


Figure 17.8.6 Set of calorimetric titrations corresponding to the implementation of the displacement protocol for the estimation of very low affinity. The concentrations of reactants are: (**A**) RNase A 76 μM in cell and 2′CMP 1.13 mM in syringe; (**B**) RNase A 76 μM in cell and 5′CMP 1.07 μM in syringe; and (**C**) RNase A 76 μM and 5′CMP 600 μM in cell, and 2′CMP 1.13 mM in syringe. The first titration corresponds to the one shown in Figure 17.8.1. In the second titration neither the binding affinity nor the binding enthalpy can be estimated. In the third titration, the thermodynamic parameters for the binding of 5′CMP obtained applying the exact analysis are: $K_a = 4250\ M^{-1}$, $\Delta H_a = -16.3$ kcal/mol, $n = 0.99$. If using the approximation method, the values obtained for the binding of 5′CMP are: $K_a = 4300\ M^{-1}$, $\Delta H_a = -15.7$ kcal/mol, $n = 0.98$, which are in agreement with the previous ones.

to the third titration yields the following thermodynamic parameters for the binding of 5′CMP: $K_a = 4250\ M^{-1}$, $\Delta H_a = -16.3$ kcal/mol, $n = 0.99$. Using the approximation method, the values obtained for the binding of 5′CMP are: $K_a = 4300\ M^{-1}$, $\Delta H_a = -15.7$ kcal/mol, $n = 0.98$, which are in reasonable agreement with the exact analysis.

ALTERNATE PROTOCOL 3

BINDING OF HOMODIMERIC PROTEINS

When the objective is to determine the thermodynamic parameters for the formation of homodimers, Basic Protocols 1 and 2 are not useful since they require the physical separation between reactants. However, it is still possible to modify the standard procedure towards that goal. In this case, the only reactant is placed in the syringe and the cell is filled with the appropriate buffer solution. The experiment consists of performing a series of injections of the reactant solution into the calorimetric cell. Since the concentration of reactant in the syringe is constant throughout the experiment, the fraction of monomers and dimers within the syringe remains constant. However, the dilution of the reactant in the cell upon injection promotes the dissociation of dimers into monomers. In these circumstances, the area under the peak associated with each injection is simply the heat released or absorbed during the dissociation reaction. Details and examples can be found in the literature (Burrows et al., 1994; Lovatt et al., 1996).

ALTERNATE PROTOCOL 4

BINDING OF UNSTABLE PROTEINS

Sometimes the unique nature of some macromolecules impedes the implementation of Basic Protocol 1 for ITC binding studies. Certain proteins tend to denature or aggregate under the vigorous stirring conditions of the calorimetric cell, which makes conducting standard ITC assays impossible.

However, it is possible to modify the protocol to determine the enthalpy of binding of ligands to such proteins. A solution of the ligand is placed in the calorimetric cell and a solution of the macromolecule is placed in the syringe. Concentrations of reactants should be chosen so that, when injecting, all of the macromolecule binds to the ligand. Therefore, all the heat associated with the injection can be assigned to the binding of all injected macromolecules to the ligand. To obtain the enthalpy of binding, it is necessary to estimate how much heat is coming from binding and how much from unspecific processes (e.g., injection, friction, dilution). Blank experiments, in which buffer solution alone (without ligand) is placed in the cell, are needed to evaluate the non-binding heat. The binding enthalpy is obtained by subtracting such contribution from the observed heat (see Equation 17.8.8):

$$\Delta H_a = \frac{Q_T - Q_{Blank}}{v[S]_0}$$

Equation 17.8.8

where Q_T is the heat associated with the injection of macromolecule into the ligand solution, Q_{Blank} is the heat associated with the injection of macromolecule into the buffer solution, v is the injection volume, and $[S]_0$ is the concentration of reactant in the syringe.

Similar experiments can also be used in Basic Protocol 1 to estimate the heat produced by ligand dilution and to determine if the ligand self-associates or aggregates at high concentration in the syringe. Details and examples can be found in the literature (Nezami et al., 2002, 2003).

MEASURING THE HEAT CAPACITY CHANGE ASSOCIATED WITH BINDING

ALTERNATE PROTOCOL 5

A single ITC assay can provide information regarding the binding constant, the Gibbs free energy of binding, the binding enthalpy, the binding entropy, and the stoichiometry of the binding reaction. In addition to this information, the change in heat capacity upon binding and the change in ionization state upon binding can be obtained by repeating the experiment with varying temperature or buffer conditions.

The heat capacity change, at constant pressure, is simply the temperature derivative of the enthalpy change (see Equation 17.8.9):

$$\Delta C_P = \left(\frac{\partial \Delta H}{\partial T} \right)_P$$

Equation 17.8.9

Therefore, one can determine its value by measuring the enthalpy change of a binding reaction at different temperatures. Plotting ΔH versus temperature would yield ΔC_P as its slope. The heat capacity of binding reflects the burial of polar and non-polar surfaces as a consequence of the binding reaction (Privalov and Makhatadze, 1992; Murphy and Freire, 1992; Gómez et al., 1995).

ΔC_P is usually determined by measuring the enthalpy of binding from 15° to 35°C at 5° intervals under identical buffer and pH conditions. Figure 17.8.7 shows the temperature dependence of the binding enthalpy of amprenavir and TMC-126, a clinical and an experimental inhibitor of HIV-1 protease, respectively (Ohtaka et al., 2002).

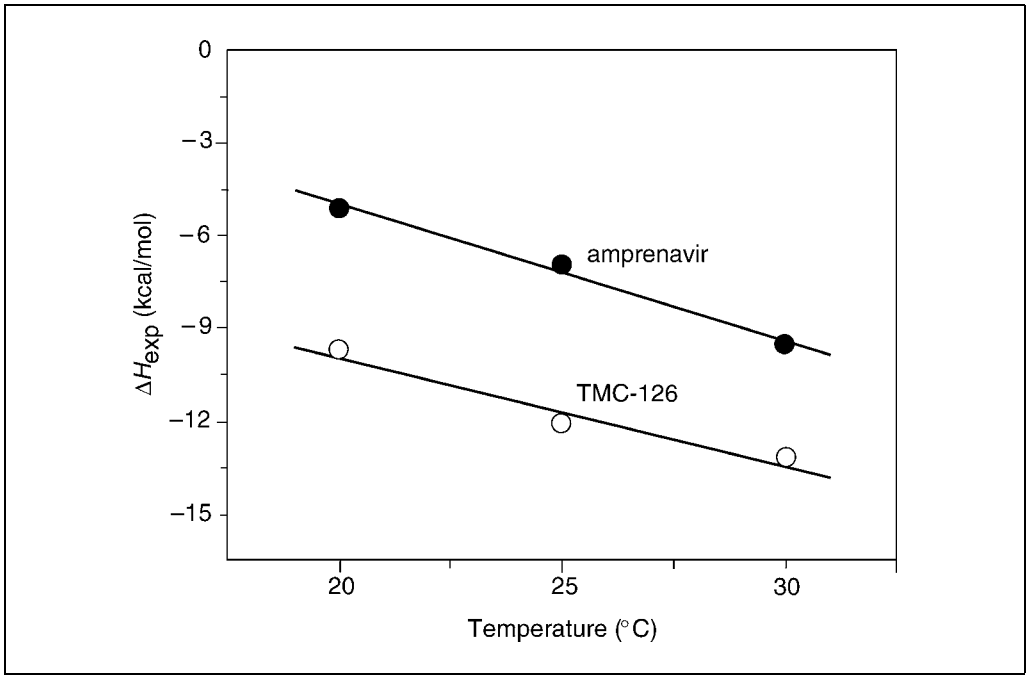


Figure 17.8.7 Temperature dependence of the binding enthalpy for amprenavir and TMC-126 binding to HIV-1 protease (Ohtaka et al., 2002). The experiments were done in 10 mM sodium acetate, pH 5.0/2%DMSO. The slope of the plot is equal to the heat capacity change upon binding and it is related to the burial of accessible surface areas from both the protein and the ligand. The values of the binding heat capacity for amprenavir and TMC-126 are −440 cal/K·mol and −350 cal/K·mol, respectively.

The heat capacity change of binding processes between a protein and a ligand is usually negative and <1 kcal/K·mol in absolute value. However, the binding of two macromolecules can be associated with a higher heat capacity change, which is indicative of a larger burial of solvent-accessible surface area upon binding or the structuring of some regions of the macromolecule.

ALTERNATE PROTOCOL 6

MEASURING PROTONATION/DEPROTONATION PROCESSES COUPLED TO BINDING

The change in the protonation state of certain residues involved in the binding process produces a proton transfer between the complex and the bulk solution. This phenomenon can be studied by detecting the heat effect produced by the protons exchanged between the protein and the buffer as a consequence of the inhibitor binding (Gómez and Freire, 1995; Baker and Murphy, 1996, 1997). In this case, the experimental enthalpy of binding, ΔH_{exp}, is the sum of the intrinsic enthalpy of the binding reaction, $\Delta H_{binding}$, independent of the buffer used and a term proportional to the enthalpy of ionization of the buffer (see Equation 17.8.10):

$$\Delta H_{exp} = \Delta H_{binding} + N_{H^+} \Delta H_{ion}$$

Equation 17.8.10

where the proportionality constant, N_{H^+}, is the number of protons that are exchanged between the complex and the bulk solution, and ΔH_{ion} is the ionization enthalpy of the buffer. Experiments are done under the same pH and temperature, using buffers with different ionization enthalpies. Figure 17.8.8 shows the protonation/deprotonation effect associated with the binding of amprenavir and TMC-126 to HIV-1 protease (Ohtaka et al., 2002). The coupling of protonation/deprotonation processes to the binding of a

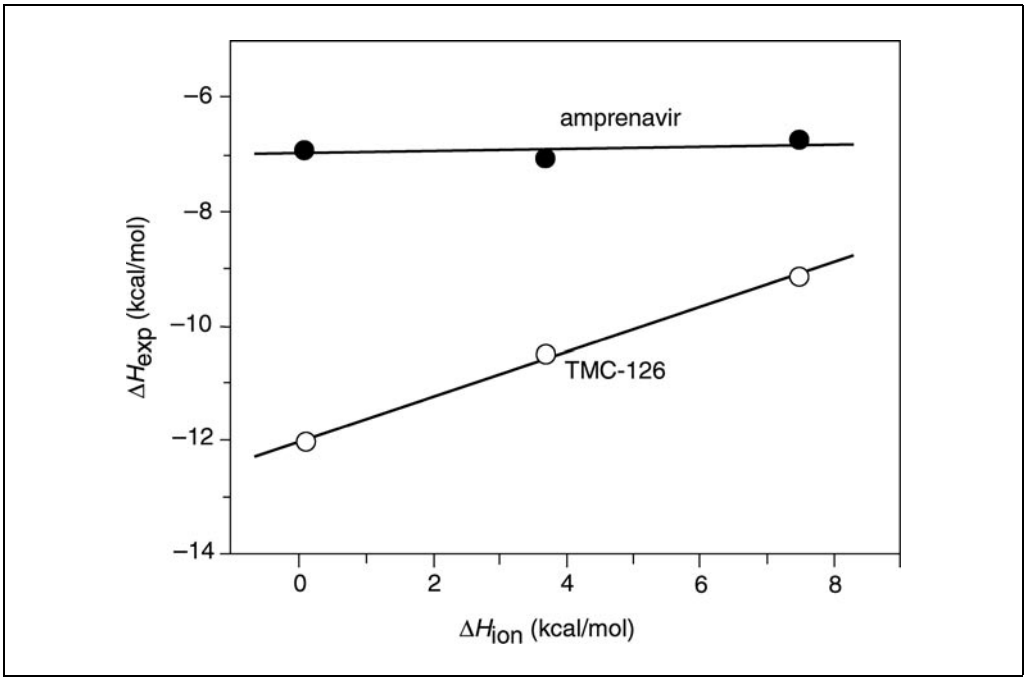


Figure 17.8.8 Dependence of the binding enthalpy for amprenavir and TMC-126 binding to HIV-1 protease on the ionization enthalpy of the buffer used in the experiment (Ohtaka et al., 2002). The experiments were done at pH 5.0 in 2% DMSO and 10 mM buffer concentration with buffers of different ionization enthalpy (acetate 0.12 kcal/mol, MES 3.72 kcal/mol, ACES 7.51 kcal/mol). The number of protons exchanged (slope) for amprenavir and TMC-126 is 0.02 and 0.39, respectively. The buffer-independent binding enthalpy (intercept with *y*-axis) for amprenavir and TMC-126 is −6.9 kcal/mol and −12.0 kcal/mol, respectively. Unlike in the case of amprenavir, where there is no net proton exchange between the ML complex and bulk solution upon binding, for TMC-126 there is a significant proton transfer.

ligand could make the experimental enthalpy measured directly, ΔH_{ion}, very different (even opposite) from the actual binding enthalpy, $\Delta H_{binding}$, if either N_{H^+} or ΔH_{ion} is large enough.

COMMENTARY

Background Information

Isothermal titration calorimetry (ITC) is a technique that directly measures the energetics associated with the binding of two components. ITC is the only biophysical method that directly determines the enthalpy (ΔH) associated with binding and provides a complete thermodynamic profile of the interaction, including binding affinity (K_a), number of binding sites (n), entropy change (ΔS), and Gibbs energy change (ΔG). ITC can be utilized for determining very low (10^3 M^{-1}) to very high binding affinities (10^{12} M^{-1}). Besides providing fundamental information about binding, ITC does not require immobilization of the binding components and takes very little time, unlike other techniques [e.g., surface plasmon resonance (*UNIT 17.6*) and ultra-centrifugation]. Moreover, since heat is universally generated or absorbed during any molecular interaction, ITC has become a more common and general detection method without the need of reporter labels. An ITC instrument has a simple setup consisting of two identical cells, a sample cell that contains a solution of one of the reactants and a reference cell that serves as a temperature reference. The instrument is equipped with a syringe that contains the second binding component and adds aliquots stepwise to the sample cell. In the last two decades, ITC has become an important technique in determining the binding energetics of various biochemical processes (Wiseman et al., 1989; Freire at al., 1990; Doyle, 1997; El Harrous and Parody-Morreale, 1997; Jelesarov and Bosshard, 1999; Leavitt and Freire, 2001; Ward and Holdgate, 2001; Ladbury, 2001), including protein-ligand binding, protein-protein binding, DNA-protein binding, protein-carbohydrate binding,

protein-lipid binding, and antigen-antibody binding. Moreover, with the ability to precisely determine the enthalpic and entropic components of binding, ITC has become an important component of the drug discovery process.

Thermodynamics of binding

Consider a binding reaction at equilibrium where a biological macromolecule M binds another molecule L (ligand; see Equation 17.8.11):

$$M + L \leftrightarrow ML$$

Equation 17.8.11

Assuming that there is a single binding site, the binding affinity or association constant, K_a, which is the inverse of the dissociation constant, K_d, determines the partition of the reactants into free and bound species, is given by (see Equation 17.8.12):

$$K_a = [ML]/[M][L]$$

Equation 17.8.12

The binding affinity is related to the free energy of binding, ΔG (see Equation 17.8.13):

$$\Delta G = -RT \ln K_a$$

Equation 17.8.13

where R is the gas constant and T the absolute temperature. The free energy of binding can be expressed in terms of the enthalpy (ΔH) and entropy (ΔS) of binding (see Equation 17.8.14):

$$\Delta G = \Delta H - T\Delta S$$

Equation 17.8.14

and therefore (Equation 17.8.15):

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S$$

Equation 17.8.15

As it can be seen from Equation 17.8.15, enthalpy (ΔH) and entropy (ΔS) are the two determinants of the binding affinity. The binding enthalpy primarily reflects the strength of interactions between the ligand and the target (non-covalent interactions, e.g., van der Waals, hydrogen bonds, electrostatics) relative to those existing with the solvent. On the other hand, the entropy change mainly reflects two contributions, changes in solvation entropy, ΔS_{solv}, and changes in conformational entropy, ΔS_{conf} (Lee at al., 1994; D'Aquino et al., 1996; D'Aquino et al., 2000). Other energetic contributions, such as those due to the loss of translational degrees of freedom are similar for all bimolecular associations and amount to 8 to 10 cal/K-mol (Murphy et al., 1994; Amzel, 1997, 2000). As the ligand binds to the macromolecule, desolvation occurs, and water molecules are released from the binding site and the ligand, producing a significant increase in degrees of freedom. This desolvation process is favorable to binding as it increases the entropy of the system. At the same time, however, the ligand and certain groups in the protein lose conformational freedom as they bind to each other (relative to their conformational degree of freedom in water). Therefore, this loss in conformational entropy, ΔS_{conf}, is usually unfavorable for the binding process, unless the binding reaction is coupled to the loss in structure of distal regions from the binding site. Since the enthalpy and entropy changes are the manifestation of different types of inter- and intra-molecular interactions, two ligands that exhibit different enthalpic and entropic contributions to the Gibbs energy of binding reflect different binding mechanisms, even if their binding affinities might be the same (Myzska et al., 1997; Parker et al., 1999; Velázquez-Campoy et al., 2000a,b; Velázquez-Campoy et al., 2001). Additional information and more advanced formalism and developments about binding thermodynamics can be found in the literature (Cantor and Schimmel, 1980; Wyman and Gill, 1990; Luque and Freire, 1998; Van Holde et al., 1998).

Applications

ITC has evolved to become a very useful technique in determining the energetics of a wide variety of chemical and biochemical interactions. Furthermore, ITC can be utilized as a standard technique in determining the precise binding affinities of components from nanomolar range to micromolar range and coupling of the binding reaction to protonation/deprotonation processes. The implementation of displacement titration experiments has extended range for affinity determination, as explained below.

Ligand binding energetics

The useful range for an accurate binding affinity measurement by standard ITC is between 10^4 and 10^8 M^{-1}. However, the practical range of binding affinities experimentally accessible can be expanded to 10^3 to 10^{12} M^{-1} by using an appropriate well-characterized secondary ligand (ITC displacement technique). This is particularly useful in the drug design

process, where the ability to accurately measure binding affinities in the sub-nanomolar range is crucial during the lead optimization stage. ITC displacement experiments will be discussed in detail in later sections.

Drug development

ITC has been utilized in the characterization and optimization of lead compounds as viable drug candidates as it provides complete information of the binding affinity of a ligand to a target in terms of its thermodynamic components ΔG, ΔH, ΔS, and ΔC_P. Current strategies for lead identification and optimization depend almost exclusively on binding affinities or Gibbs energy of binding. The Gibbs energy of binding is a result of a delicate balance between the enthalpic and the entropic contributions, and different combinations of ΔS and ΔH can give rise to the same ΔG values and, therefore, elicit the same binding affinity. A higher affinity can be achieved by two means: (1) obtaining a more favorable (negative) enthalpy change, i.e., improving ligand-protein interactions over those with the solvent, and (2) obtaining a more favorable (positive) entropy change, which in turn can be achieved by making a ligand more hydrophobic in order to maximize the solvation entropy, and/or pre-shaping the ligand in order to minimize the loss of conformational entropy. Traditional drug-design methods rely on hydrophobicity and pre-shaping compounds through the use of conformational constraints, therefore, giving rise to entropically optimized compounds (Todd et al., 2000; Velázquez-Campoy et al., 2002; Ohtaka et al., 2002). Such an approach exhibits several pitfalls, the most important ones being the lack of specificity, the susceptibility to mutations in the target, and the impossibility to achieve extremely high affinity. For example, in the case of HIV-1 protease inhibitors, there is not a single reported case of an entropically driven inhibitor with an affinity stronger than 0.3 nM (Todd et al., 2000; Ohtaka et al., 2002). Therefore, ITC can be utilized not only to characterize existing ligands but also to provide a step-wise guide in the design process by permitting a direct evaluation of the energetic effects of specific chemical modifications.

Evaluation of mutations effects

Genetic diversity and mutations associated with drug resistance are the two major causes of failure of drug therapy in various bacterial and viral diseases, e.g., HIV infection. These two phenomena involve changes in the drug binding sites of the target molecules. Since drug molecules developed under the traditional drug-design paradigm, based on the lock-and-key concept, are conformationally constrained and pre-shaped to the geometry of the target molecule, they cannot effectively adapt to changes in binding site geometry due to naturally occurring polymorphisms or drug resistance mutations (Velázquez-Campoy et al., 2002; Ohtaka et al., 2002). As a result, they lose significant binding affinity. Therefore, a major challenge in drug design is the integration of binding site heterogeneity in the design process. An ideal molecule would be one that is able to adapt to the variability in the target while simultaneously displaying high binding affinity and specificity. ITC being the only technique to provide the individual components of the Gibbs free energy of binding (ΔH, ΔS, and ΔC_P), is able to determine subtle changes in the binding process arising from polymorphisms or mutations in the target. Therefore, ITC can be utilized in the design process of an adaptive ligand that will overcome the current deficiency in the drug design process against variable targets (Velázquez-Campoy et al., 2001; Velázquez-Campoy and Freire, 2001; Freire, 2002; Nezami et al., 2003).

Critical Parameters and Toubleshooting

For a titration experiment to be successful and well designed, the following critical points should be considered.

1. ITC measures the global heat effect associated with the binding of two molecules. This includes the actual heat of binding (binding enthalpy) and all other heat effects originated from non-specific events (dilution of reactants, friction of injected liquid, etc.). Therefore, it is important to ensure that these contributions are minimized or considered in the analysis. For example, in the case of the dilution of reactants, the last peaks in the experiment after saturation can be an estimate of the heat effect due to such unspecific phenomena. Also, a titration of the reactant in the syringe into the buffer solution gives an estimation of the dilution effect of the titrant. In the case of friction of the injected liquid, small injection volumes (between 3 and 10 μl) and the recommended rate of injection (0.5 μl/sec) should be employed.

2. Regarding the possibility of aggregation or association of the reactants, the reactant with the lower solubility should be placed in the calorimetric cell.

3. As explained above, the appropriate range of concentrations and the proper ratio of ligand/macromolecule concentrations needs to be employed to guarantee completion of titration, reaching the saturation point in a reasonable number of injections (<30). If saturation is achieved in fewer injections (<5), then, the ligand/macromolecule concentration ratio should be lowered. On the contrary, if saturation is hardly reached, then the ratio should be increased.

4. A perfect match between the buffers has to be achieved to avoid spurious heat effects due to protonation of different species and mixing of different components that could be larger than the actual heat effect associated with the binding reaction. Accordingly, extreme care should be taken when using organic co-solvents (e.g., DMSO), because a mismatch in the concentration of these components between the solutions in the cell and the syringe will cause large heat effects.

5. From a practical point of view, it should always be emphasized that a clean cell and a perfectly straight syringe are decisive to avoid spurious results and to give excellent baselines with high signal-to-noise ratio.

Anticipated Results

Different titration experiments and representative examples are shown in each protocol.

Typical titration experiments in the range of moderate affinity ($K_a \sim 10^6$ M^{-1}) corresponding to exothermic and endothermic binding processes are shown in Figures 17.8.3 and 17.8.4, respectively. The following are several features that will indicate the quality of the experiment:

1. The curvature of the titration curve is appropriate to obtain a reliable estimation of the binding affinity.

2. The saturation point has been achieved and the final molar ratio is ~2.5 to 3.

3. There is a good signal-to-noise ratio. The data in Figure 17.8.3 is better than that of Figure 17.8.4 because the concentrations of reactants used in the titration are ten times higher. A good signal-to-noise ratio will guarantee small errors in the integration stage in data analysis.

4. In terms of thermal power between the peaks before and after the titration, there is a 50-fold decrease (5 μcal/sec compared to 0.1 μcal/sec, in absolute value) and a five-fold decrease (1 μcal/sec compared to 0.2 μcal/sec, in absolute value) in Figure 17.8.3 and 17.8.4, respectively. The standard deviation of the noise is 0.002 μcal/sec and 0.004 μcal/sec in Figure 17.8.3 and 17.8.4, respectively, much lower than the height of the peaks before and after saturation.

Time Considerations

The time required for a complete ITC experiment can be divided into different steps. Preparing sample solutions requires ~30 min. Running the ITC experiment takes ~2 to 3 hr. Cleaning the ITC instrument requires ~20 min. Analyzing the results takes ~30 min.

Literature Cited

Amzel, L.M. 1997. Loss of translational entropy in binding, folding, and catalysis. *Proteins* 28:144-149.

Amzel, L.M. 2000. Calculation of entropy changes in biological processes: Folding, binding, and oligomerization. *Methods Enzymol.* 323:167-177.

Baker, B.M. and Murphy, K.P. 1996. Evaluation of linked protonation effects in protein binding using isothermal titration calorimetry. *Biophys. J.* 71:2049-2055.

Baker, B.M. and Murphy, K.P. 1997. Dissecting the energetics of a protein-protein interaction: The binding of ovomucoid third domain to elastase. *J. Mol. Bio.* 268:557-569.

Burrows, S.D., Doyle, M.L., Murphy, K.P., Franklin, S.G., White, J.R., Brooks, I., McNulty, D.E., Scott, M.O., Knutson, J.R., Porter, D., Young, P.R., and Hensley, P. 1994. Determination of the monomer-dimer equilibrium of interleukin-8 reveals it is a monomer at physiological concentrations. *Biochemistry* 33:12741-12745.

Cantor, C.R. and Schimmel, P.R. 1980. Biophysical Chemistry: The Behavior of Biological Macromolecules. W.H. Freeman & Co. New York.

D'Aquino, J.A., Gómez, J., Hilser, V.J., Lee, K.H., Amzel, L.M., and Freire, E. 1996. The magnitude of the backbone conformational entropy change in protein folding. *Proteins* 25:143-156.

D'Aquino, J.A., Freire, E., and Amzel, L.M. 2000. Binding of small organic molecules to macromolecular targets: Evaluation of conformational entropy changes. *Proteins Suppl* 4:93-107.

Doyle, M.L. 1997 Characterization of binding interactions by isothermal titration. *Curr. Opin. Biotech.* 8:31-35.

Doyle, M.L. and Hensley, P. 1998. Tight ligand binding affinities determined from thermodynamic linkage to temperature by titration calorimetry. *Methods Enzymol.* 295:88-99.

Doyle, M.L., Louie, G.L., Dal Monte, P.R., and Sokoloski, T.D. 1995. Tight binding affinities determined from linkage to protons by titration calorimetry. *Methods Enzymol.* 259:183-194.

El Harrous, M. and Parody-Morreale, A. 1997. Measurement of biochemical affinities with a Gill titration calorimeter. *Anal. Biochem.* 254:96-108.

Freire, E. 2002. Designing drugs against heterogeneous targets. *Nature Biotech.* 20:15-16.

Freire, E., Mayorga, O.L., and Straume, M. 1990. Isothermal titration calorimetry. *Anal. Chem.* 62:950A-959A.

Gómez, J. and Freire, E. 1995. Thermodynamic mapping of the inhibitor site of the aspartic protease endothiapepsin. *J. Mol. Biol.* 252:337-350.

Gómez, J., Hilser, V.J., and Freire, E. 1995. The heat capacity of proteins. *Proteins* 22:404-412.

Indyk, L. and Fisher, H.F. 1998. Theoretical aspects of isothermal titration calorimetry. *Methods Enzymol.* 295:350-364.

Jaenicke, L. 1974. A rapid micromethod for the determination of nitrogen and phosphate in biological material. *Anal. Biochem.* 61:623-627.

Jelessarov, I. and Bosshard, H.R. 1999. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J. Mol. Recog.* 12:3-18.

Ladbury, J.E. 2001. Isothermal titration calorimetry: Application to structure-based drug design. *Thermochimica Acta* 380:209-215.

Leavitt, S. and Freire, E. 2001. Direct measurement of protein binding energetics by isothermal titration calorimetry. *Curr. Opin. Struct. Biol.* 11:560-566.

Lee, K.H., Xie, D., Freire, E., and Amzel, L.M. 1994. Estimation of changes in side chain configurational entropy in binding and folding: General methods and application to helix formation. *Proteins* 20:68-84.

Lovatt, M., Cooper, A., and Camilleri, P. 1996. Energetics of cyclodextrin-induced dissociation of insulin oligomers. *Euro. Biophys. J.* 24:354-357.

Luque, I. and Freire, E. 1998. A system for the structure-based prediction of binding affinities and molecular design of peptide ligands. *Methods Enzymol.* 295:100-127.

Murphy, K.P. and Freire, E. 1992. Thermodynamics of structural stability and cooperative folding behavior in proteins. *Adv. Protein Chem.* 43:313-361.

Murphy, K.P., Xie, D., Garcia, K.C., Amzel, L.M., and Freire, E. 1993. Structural energetics of peptide recognition: Angiotensin II/antibody binding. *Proteins* 15:113-120.

Murphy, K.P., Xie, D., Thompson, K.S., Amzel, L.M., and Freire, E. 1994. Entropy in biological binding processes: Estimation of translational entropy loss. *Proteins* 18:63-67.

Myszka, D.G, Sweet, R.W., Hensley, P., Brigham-Burke, M., Kwong, P.D., Hendrickson, W.A., Wyatt, R., Sodroski, J., and Doyle, M.L. 1997. Energetics of the HIV gp120-CD4 binding reaction. *Proc. Natl. Acad. Sci. U.S.A.* 97:9026-9031.

Nezami, A., Luque, I., Kimura, T., Kiso, Y., and Freire, E. 2002. Identification and characterization of allophenylnorstatine-based inhibitors of plasmepsin II, an antimalarial target. *Biochemistry* 41:2273-2280.

Nezami, A., Kimura, T., Hidaka, K., Kiso, A., Liu, J., Kiso, Y., Goldberg, D.E., and Freire, E. 2003. High-affinity inhibition of a family of plasmodium falciparum proteases by a designed adaptive inhibitor. *Biochemistry* 42:8459-8464.

Ohtaka, H., Velázquez-Campoy, A., Xie, D., and Freire, E. 2002. Overcoming drug resistance in HIV-1 chemotherapy: The binding thermodynamics of amprenavir and TMC-126 to wild-type and drug-resistant mutants of the HIV-1 protease. *Prot. Sci.* 11:1908-1916.

Pace, C.N., Vaidos, F., Fee, L., Grimsley, G., and Gray, T. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* 4:2411-2423.

Parker, M.H., Lunney, E.A., Ortwine, D.F., Pavlovsky, A.G., Humblet, C., and Brouillette, C.G.. 1999. Analysis of the binding of hydroxamic acid and carboxylic acid inhibitors to the stromelysin-1 (matrix metalloproteinase-3) catalytic domain by isothermal titration calorimetry. *Biochemistry* 38:13592-13601.

Privalov, P.L. and Makhatadze, G.I. 1992. Contribution of hydration and non-covalent interactions to the heat capacity effect on protein unfolding. *J. Mol. Bio.* 224:715-723.

Sigurskjold, B.W. 2000. Exact analysis of competition ligand binding by displacement isothermal titration calorimetry. *Anal. Biochem.* 277:260-266.

Straume, M. and Freire, E. 1992. Two-dimensional differential scanning calorimetry: Simultaneous resolution of intrinsic protein structural energetics and ligand binding interactions by global linkage analysis. *Anal. Biochem.* 203:259-268.

Todd, M.J., Semo, N., and Freire, E. 1998. The structural stability of the HIV-1 protease. *J. Mol. Bio.* 283:475-488.

Todd, M.J., Luque, I., Velázquez-Campoy, A., and Freire, E. 2000. Thermodynamic basis of resistance to HIV-1 protease inhibition: Calorimetric analysis of the V82F/I84V active site resistant mutant. *Biochemistry* 39:11876-11883.

Van Holde, K.E., Johnson, W.C., and Ho, P.S. 1998. Principles of Physical Biochemistry. Prentice Hall. Upper Saddle River, New Jersey.

Velázquez-Campoy, A. and Freire, E. 2001. Incorporating target heterogeneity in drug design. *J. Cell. Biochem.* S37:82-88.

Velázquez-Campoy, A., Todd, M.J., and Freire, E. 2000a. HIV-1 protease inhibitors: Enthalpic versus entropic optimization of the binding affinity. *Biochemistry* 39:2201-2207.

Velázquez-Campoy, A., Luque, I., Todd, M.J., and Freire, E. 2000b. Thermodynamic dissection of the binding energetics of KNI-272, a potent HIV-1 protease inhibitor. *Prot. Sci.* 9:1801-1809.

Velázquez-Campoy, A. Kiso, Y., and Freire, E. 2001. The binding energetics of first- and second-generation HIV-1 protease inhibitors: Implications for drug design. *Arch. Biochem. Biophys.* 390:169-175.

Velázquez-Campoy, A., Vega, S., and Freire, E. 2002. Amplification of the effects of drug-resistance mutations by background polymorphisms in HIV-1 protease from African subtypes. *Biochemistry* 41:8613-8619.

Ward, W.H. and Holdgate, G.A. 2001. Isothermal titration calorimetry in drug discovery. *Prog. Med. Chem.* 38:309-376.

Wiseman, T., Williston, S., Brandts, J.F., and Nin, L.N. 1989. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* 179:131-137.

Wyman, J. and Gill, S.J. 1990. Binding and Linkage: Functional Chemistry of Biological Macromolecules. University Science Books Mill Valley California.

Zhang, Y.-L. and Zhang, Z.-Y. 1998. Low-affinity binding determined by titration calorimetry using a high-affinity coupling ligand: A thermodynamic study of ligand binding to protein tyrosine phosphatase 1B. *Anal. Biochem.* 261:139-148.

Contributed by Adrián Velázquez-Campoy, Hiroyasu Ohtaka, Azin Nezami, Salman Muzammil, and Ernesto Freire
Johns Hopkins University
Baltimore, Maryland

Rational Design and Evaluation of FRET Experiments to Measure Protein Proximities in Cells

Defining the repertoire of interactions that a particular protein can undergo is crucial for understanding its function and regulation. Characterizing where and when these interactions occur is a major goal of cell biology. Although biochemical approaches (e.g., immunoprecipitation, pull-down assays, and cross-linking) are indispensable for identifying protein-protein interactions, they do not provide spatial and temporal information in the context of an intact cell. Conversely, immunofluorescence localization or genetically encoded fluorescent tags such as green fluorescent proteins (GFP) can provide spatial information regarding an individual protein, but little insight into its interacting partners. While co-localization of two proteins (e.g., in the same organelle) is a prerequisite for their interaction, an interaction cannot be concluded just because two proteins are co-localized by fluorescence microscopy. Clearly, a combination of fluorescence-based visualization of proteins (in their cellular context) that simultaneously provides subnanometer resolution of their proximities (i.e., whether they can physically interact) is highly desirable in nearly all areas of cell biology. For this reason, numerous approaches have been developed to meet these demands.

Because a protein's localization is one of its most basic features, there are enormous numbers of reagents for visualizing individual proteins by fluorescence microscopy. These include an ever-growing collection of fluorescent protein–tagged constructs as well as high-affinity mono-specific antibodies suitable for immunofluorescence. Given the wide range of color variants of both fluorescent proteins and fluorescent dyes, visualizing two or more proteins simultaneously is now routine. To convert this basic methodology to additionally report on close (subnanometer) proximities of the fluorescently marked proteins, one needs to employ fluorescence resonance energy transfer (FRET). In essence, measurement of FRET between two appropriately labeled proteins containing fluorophores with suitable properties can be used to infer the spatial and temporal characteristics of protein interactions in their native cellular environment.

How does this work? FRET refers to the nonradiative transfer of energy from one fluorescent molecule (the donor) to another fluorescent molecule (the acceptor; *UNITS 4.14 & 17.1*). Hence, energy that is captured by the donor upon its excitation is transferred to the acceptor. This results in the donor *failing* to emit a photon, while the acceptor emits a photon at its characteristic wavelength (despite the fact it was not directly excited). Although a wide variety of parameters influences the probability of FRET (see Matyus, 1992; Clegg, 1995; Wouters et al., 2001 for detailed discussions), the most important are the distance separating the donor and acceptor, and their respective fluorescence spectra. Multiple experimental methods and instruments exist for measuring FRET (Jares-Erijman and Jovin, 2003). Selecting the appropriate method and instrumentation can be daunting, even for experienced fluorescence microscopists. Each of the techniques has particular advantages and disadvantages, and the appropriateness of a technique depends on the nature of the hypothesis being tested.

The method that can be most widely and simply implemented, quantified, and interpreted is the acceptor-photobleaching FRET technique (also see *UNIT 17.1*). In this method, the presence of FRET between a donor and acceptor is revealed upon destruction (by photobleaching) of the acceptor. If the donor fluorescence now gets brighter, one can infer that it had been in sufficiently close proximity to the acceptor to undergo FRET. The extent of increase is a quantitative and direct measure of FRET efficiency. As described below, acceptor photobleaching represents a robust technique that can be exploited to detect changes in the composition and organization of subunit proteins within a multiprotein complex and even to gain insight into relative stoichiometries of proteins within the complex.

To obtain high-quality FRET data, care must be taken to select appropriate controls, maximize the signal-to-noise ratio, and

Contributed by Erik L. Snapp and Ramanujan S. Hegde
Current Protocols in Cell Biology (2006) 17.9.1-17.9.20

perform sufficient numbers of measurements for the intended questions. In addition, subtleties of FRET theory have implications for accurate interpretation of experimental results. In this unit, strategies, tools, and background for designing and interpreting acceptor-photobleaching FRET experiments in cells are described. As mentioned above, the proteins of interest can be labeled in many ways: fluorescent antibodies, genetically encoded fluorescent protein tags, direct conjugation to dyes, or even fluorescent ligands. Different combinations of all of these methods have been exploited in various FRET methods. For this unit discussion will be limited to two proteins both labeled with fluorescent antibodies. However, the principles, particularly those related to the planning and interpretation stages of the experiment, can be applied easily to other methods of labeling. The basic protocols for cell fixation, labeling, dye-labeling of antibodies, and acceptor photobleaching are provided in *UNIT 17.1* and will be referred to as appropriate.

BACKGROUND INFORMATION

Fluorescence resonance energy transfer (FRET) refers to the nonradiative transfer of energy from an excited donor fluorescent molecule to an acceptor molecule. Multiple parameters influence the probability of FRET (see Matyus, 1992; Clegg, 1995; Kenworthy, 2001; Wouters et al., 2001; Wallrabe and Periasamy, 2005; and *UNITS 4.14 & 17.1* for detailed discussions). The most important parameters are the distance separating the donor and acceptor and their respective fluorescence spectra. Because FRET efficiency is inversely dependent on the sixth power of the distance separating the donor and acceptor, it is a highly sensitive measure of even small (subnanometer) changes in the relative proximities of the dyes. For a single donor and acceptor fluorophore, the probability of FRET upon excitation of the donor is $1/[1 + (r/R_0)^6]$, where r is the distance separating the fluorophores, and R_0 is the distance at which a 50% probability of FRET is observed (the so-called Förster distance; Förster, 1948).

The applications of FRET are numerous. For the cell biologist, FRET has been used to create biosensors of ions (e.g., the calcium-sensing cameleon fluorescent indicators; Miyawaki et al., 1997) or the active state of a protein, measure protein proximities, and measure changes in organization or composition of a protein complex. FRET biosensor assays have been well characterized and typically are measured using sensitized emission (see the Commentary in *UNIT 17.1*; Miyawaki and Tsien, 2000; Van Rheenen et al., 2004) or fluorescence lifetime imaging microscopy (FLIM; *UNIT 4.14*). These FRET assays benefit from the presence of both FRET dyes on the same molecule, which obviates the need to separately express donor and acceptor molecules at comparable levels. In contrast, studies of complexes containing multiple proteins are more theoretically and technically complex. The appropriate design and interpretation of such FRET experiments depend upon a careful consideration of the theoretical expectations.

A critical yet often overlooked concept in understanding FRET measurements is that FRET is a stochastic, all-or-nothing phenomenon. In other words, for any given donor molecule and acceptor molecule, FRET either happens or it does not happen; there is no such thing as partial transfer of energy. If FRET is an all-or-nothing phenomenon, why aren't reported FRET values either 0% or 100%, but something in between? The short answer is that FRET measurements in cells and solutions reflect the *averaged probability* of energy transfer between a very large number of donor and acceptor molecules in the sample. This means that a FRET value is the mean detected energy transfer efficiency for multiple FRET events. Furthermore, each measurement also reflects whether FRET occurs for all of the fluorophore molecules in each pixel of an image. A fluorescence image is a collection of fluorescence photon intensity values for each pixel (Michalet et al., 2003). A single pixel can contain multiple fluorophores. The intensity value of a pixel also reflects the time for collecting photons at that point, either the dwell time of a scanning laser in a confocal microscope or the detection time for a charge-coupled device (CCD) on a wide-field microscope. Therefore, a typical FRET measurement for each pixel in a cell is an ensemble measurement that averages numerous FRET events. For this reason, FRET measurements are often described as per cent energy transfer efficiency. Thus, a measurement reflects how frequently FRET events occur for a population of fluorophores under the given conditions.

Often, investigators focus on the Förster distance of a donor/acceptor pair in FRET studies, the rapid drop in energy transfer efficiency with distance, and the power of FRET measurements as a "spectroscopic ruler" (Stryer and Haugland, 1967). In the case of single-molecule studies or well defined and homogeneous biochemical samples, FRET

can indeed be used to measure absolute distances between fluorophores. However, interpretation of FRET measurements between pairs of proteins expressed in cells is complicated by the number of proteins being assayed and by how the donor and acceptor proteins are labeled.

For this unit, it is assumed that the investigator will label the proteins of interest with at least one antibody and either a variant fluorescent protein (i.e., GFP), a small dye (FlAsh and ReAsh), or another antibody. The dimensions of the antibody probes (~14 nm) are substantially larger than the distances over which FRET occurs, and the number and distribution of dyes on the antibody surface are random. Furthermore, the antibodies, as well as the dyes conjugated to them, are flexible enough to substantially influence their absolute positions. These and other variables complicate the relationship between the observed FRET and the distance separating the antigens to which donor and acceptor antibodies are bound (Dewey and Hammes, 1980; Haas and Steinberg, 1984). The consequences of these properties are that FRET cannot be used as a "spectroscopic ruler" for measuring absolute distances of native cellular proteins. This is not to imply that FRET can't be used to detect discrete changes in protein proximity, but rather to emphasize the difference between measuring absolute versus comparative distances. For most cell biologists, the *absolute* FRET values are far less important to the interpretation of the results than the *relative* differences obtained for direct comparisons. For example, the absolute FRET values are generally not used to calculate or draw conclusions regarding distances between components; rather, it is the *changes in FRET that are used to infer changes in complex organization or structure*.

OPTIMIZATION OF ACCEPTOR-PHOTOBLEACHING FRET

Acceptor-Photobleaching FRET in Cells

There are several ways to measure FRET (Jares-Erijman and Jovin, 2003) in cells including sensitized emission (Miyawaki and Tsien, 2000), FLIM (Deniz et al., 2001; Haj et al., 2002; *UNIT 4.14*), fluorescence anisotropy (Krishnan et al., 2001; Rizzo and Piston, 2005), and acceptor photobleaching (Kenworthy and Edidin, 1998; Haj et al., 2002; Snapp et al., 2004; *UNIT 17.1*). Acceptor-photobleaching FRET has several advantages that make it suitable for studying protein interactions in cells. First, FLIM and anisotropy measurements require access to instruments that often must be custom designed and built by the user. In contrast, suitable laser scanning confocal microscopes that can be used for acceptor-photobleaching experiments are both commercially available and accessible at most institutions. In addition, even a standard fluorescence microscope with a mercury lamp can be used to perform acceptor photobleaching (Kenworthy and Edidin, 1998). Second, sensitized emission depends on acceptor emission and often suffers from signal bleed-through from the donor, requires multiple correction factors, and provides no information about the relative populations of associated proteins (see Commentary in *UNIT 17.1*). In contrast, acceptor photobleaching can be quantitated with a simple arithmetic equation, is unaffected by bleed-through, and can be used to gain insights into associated and unassociated populations of proteins. A noteworthy disadvantage of acceptor-photobleaching FRET is that it requires destruction of the probe, which prevents more than one measurement in a region of a cell. In addition, photobleaching to background levels of fluorescence intensity is often slow and either requires cells to be fixed or the proteins of interest to be relatively immobile.

An important consideration for experimental design is whether the goal of the study is to distinguish between two distinct readout states or a continuum of states. It is often not feasible to synchronize multiple protein complexes in their dynamic changes. In contrast, measuring the change between a treated and untreated sample is more experimentally tractable for samples in cells. The measurement of discrete states permits cells to be fixed. While the aesthetic and intellectual appeal of live cell data is undeniable, the actual requirement for using live cells is worth considering. If the experimental readout is treated versus untreated cells, then cells in the two states can be fixed and assayed. Fixation of cells for 15 min in 3.7% formaldehyde retains substantial cell structure and does not significantly promote nonspecific protein interactions (Jackson, 1999; Metz et al., 2004; Snapp et al., 2004). Formaldehyde is a remarkably specific cross-linker and has been used for years in chromatin immunoprecipitation (ChIP; *UNIT 17.7*) assays to identify proteins that bind to unique DNA sequences in the nucleus (Jackson, 1999). It is still possible to perform live-cell acceptor-photobleaching FRET studies, though the investigator must now contend with the issue of diffusion.

Many proteins are not immobile (Lippincott-Schwartz et al., 2001). Because complete photobleaching of a fluorophore can take several seconds to minutes, acceptor photobleaching benefits substantially from immobilizing proteins with fixation.

Based on the background information above, acceptor-photobleaching FRET of native proteins in cells is most appropriate for the following types of questions. Does a pair of proteins interact to a significant degree in a particular region of a cell? Does a protein complex undergo changes in composition? Do protein subunits undergo changes in their proximity or organization in a protein complex? Are two different protein subunits present at equivalent amounts in a complex? In contrast, acceptor photobleaching is poor at detecting small subpopulations of interacting proteins (e.g., only 10% of a protein is in a complex, and the complexes are homogenously distributed throughout the cell), quantitating absolute distances between protein subunits, and detecting small continuous changes in a mixed population of protein complexes.

Before investing the time and resources into performing FRET experiments, the investigator will benefit from critical analyses of the questions to be addressed, the available reagents, and anticipated outcomes under idealized conditions. For example, a failure to consider expression levels when choosing which of two proteins to label as the donor, or attempting to measure absolute distances between proteins with antibody-based FRET will result in low probabilities of success. To enhance the probability of detecting the highest possible FRET signal for a pair of proteins, several conditions need to be empirically optimized.

Determine Which Protein Will Be the Donor and the Acceptor

In acceptor-photobleaching experiments, this choice can significantly impact the observed FRET value. There are two key points to consider. First, unpaired donors (i.e., those not in complex with the acceptor) dilute the detectable FRET signal. Second, unpaired acceptors have little effect on acceptor-photobleaching FRET values, and are thus effectively invisible in this assay format. The percent of unpaired molecules can be minimized by labeling the protein of lower stoichiometry (if known from independent studies) with the donor fluorophore. Note that if the stoichiometry of the two proteins is not known, a discrepancy in FRET values upon exchanging donor and acceptor status can provide insight into this issue (see Reciprocal FRET section).

Select an Appropriate Labeling Scheme

The method of labeling the proteins of interest will directly affect the ability of FRET to detect changes in protein proximities. For this unit it is assumed that proteins of interest will be proteins expressed in the cell. These proteins can be labeled either with fluorescent dye-labeled antibodies, variant fluorescent proteins (i.e., GFP) or newer fluorescent tags including FlAsh and ReAsh (Adams et al., 2002). After designating which protein will be the donor and which will be the acceptor, the investigator must choose a suitable labeling scheme. If a donor protein is present at low levels and overexpression changes a cellular phenotype, then antibody labeling is likely to be better than using a fluorescent fusion protein. Not only will the antibody boost the fluorescent signal due to multiple dyes on the antibody, but the multiple dyes can also enhance the probability of detecting FRET (see Fig. 17.9.1). If the donor and acceptor are abundant proteins and the donor remains functional when fused to a fluorescent protein, such as GFP, then addition of a fluorescent protein tag or epitope tag may provide flexibility in experimental design. It is not recommended to have an antibody donor and a fluorescent protein acceptor. In this case, there would be multiple dyes on the antibody that might fail to exhibit FRET with a single fluorophore acceptor. Again, the key principle is to avoid situations with excess donor molecules or limiting acceptor molecules.

If using antibodies to label the protein(s) of interest, either monoclonal or mono-specific polyclonal antibodies (raised against short peptide sequences of ~8 to 20 amino acids) are preferable to broad specificity antibodies. A single protein epitope is likely to ensure that only a single antibody molecule will bind any given copy of a protein. This is an especially useful quality when an investigator is trying to determine whether a complex contains more than one copy of a protein. A single epitope will provide more specific information concerning protein organization and can aid in experimental design. For example, an epitope against the cytoplasmic domain of a membrane protein has a higher probability of undergoing FRET with the cytoplasmic epitope of a partner protein.

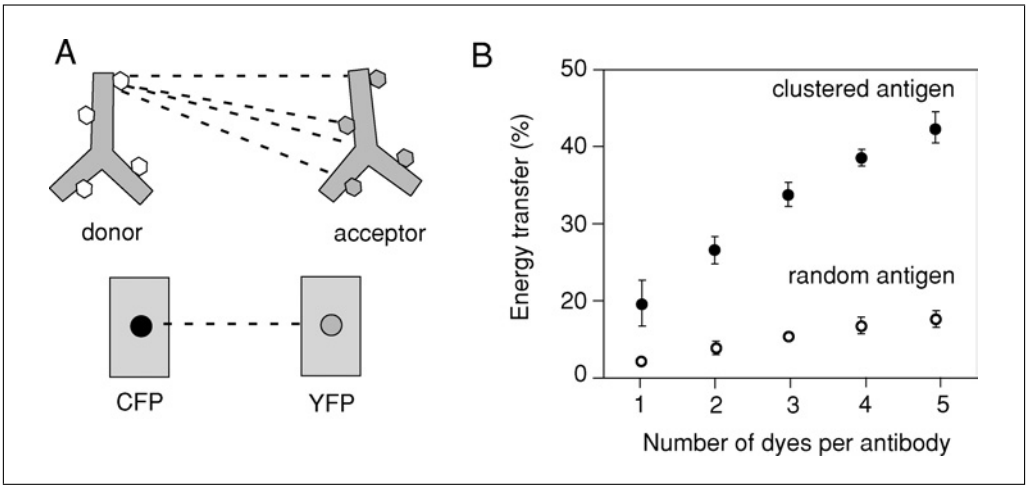


Figure 17.9.1 Illustration of the advantage of using antibody probes labeled with multiple dyes. (**A**) Cartoon illustrates how each donor dye on an antibody has the potential to transfer energy to four different acceptor dyes on an acceptor antibody. In contrast, a cyan fluorescent protein (CFP) molecule can only potentially transfer energy to one yellow fluorescent protein (YFP) acceptor. The consequence is that multiple dye–labeled acceptor antibodies enhance the probability of detecting FRET. (**B**) The relationship of dye number on each donor and acceptor antibody as plotted with simulated data for antibodies bound to antigens that are either assembled in a multiprotein complex (clustered) or distributed in a random manner. See Snapp et al. (2004) for details concerning the simulation parameters. Note that the value calculated for a single dye will not be equivalent to the CFP-YFP pair because the simulated dye placement on the antibodies was random and could include distances up to three times greater than for the fluorescent fusion proteins.

Select the Donor and Acceptor Fluorophores

The donor should be a fluorophore that is excited at a lower wavelength than the acceptor, and with an emission spectrum that overlaps significantly with the excitation spectrum of the acceptor. The degree of spectral overlap is a key determinant of the efficiency of FRET. A useful measure of the suitability of a donor-acceptor pair for FRET is known as the Förster value: the distance at which the probability of FRET between the donor and acceptor is 50%. Optimal FRET pairs are constantly being updated. A popular and well characterized donor-acceptor pair is the dyes Cy3 and Cy5, with a Förster distance of ~5 nm (Bastiaens and Jovin, 1996). Another useful FRET pair in cells is GFP and Cy3 with a Förster distance of 6 nm (Haj et al., 2002). A second parameter important in FRET efficiency is the brightness and stability of the fluorophores. Photostability is a desirable quality in the donor and less desirable for the acceptor. For example, Alexa fluorophores photobleach poorly and will thus be poor choices as acceptor, but they are reasonable choices for donors.

Optimize Antibody Dye Labeling Ratios

See *UNIT 17.1* for the Cy3/Cy5 dye labeling protocol. Addition of dyes to antibodies requires balancing of three properties: maximal FRET signal, antibody intensity quenching, and antibody binding inhibition. For Cy3 and Cy5 dyes, four dye molecules per antibody generally give excellent results (Snapp et al., 2004). This number of dye molecules tends to maximize the fluorescent signal and FRET efficiency while minimizing the disruption of binding activity. It goes without saying that it should be confirmed that the directly conjugated antibody recognizes antigen with specificity similar to the unconjugated starting antibody.

Identify Relevant Positive and Negative Controls

The importance of the choice of controls cannot be overstated. Quite simply, controls define the limits and scale for interpretation of experimental FRET values. Regardless of the predicted Förster distance for a FRET pair, actual FRET data is very much dependent on the properties of the system and must take into account the geometry of the protein complex, cellular autofluorescence, the method of FRET measurement, the size of the fluorescent label, and effects of the cellular environment on fluorophore properties. Therefore, the investigator should select controls that: (1) use the same FRET pair of fluorophores; (2) mimic the spatial environment of the proteins of

interest (i.e., if membrane proteins are being investigated, the controls should also be membrane proteins); (3) label cells with similar fluorescence intensities relative to the experimental labeling; and (4) display co-localization in immunofluorescence images (i.e., in the same organelle) for both positive and negative controls. This last point is obvious for a positive control because proteins that do not co-localize at the level of light microscopy will not exhibit FRET. However, using co-localized proteins for the negative control is also important because the goal of the experiment is to get spatial resolution that is higher than light microscopy can deliver. Thus, it is important to demonstrate that noninteracting proteins close enough to co-localize in the cell do not give significant FRET. For acceptor-photobleaching FRET using antibodies, a simple but nice positive control is an acceptor-labeled primary antibody bound by a donor-labeled secondary antibody. In this instance, all donor-labeled antibodies are necessarily adjacent to an acceptor-labeled antibody, and should necessarily yield highly efficient FRET.

Empirically Determine Antibody Labeling Conditions

It is assumed that the investigator is familiar with the basic operation of a confocal microscope. The investigator should understand both the concept and the operation of scan speed, zoom, detector gain, laser power, photobleaching, and collection of a time series on a laser scanning confocal microscope. A series of simple immunofluorescence experiments should be performed to determine the several imaging and labeling conditions (see below). Cells can be assayed with a fluorescence microscope fitted with a charge-coupled device (CCD) or a confocal microscope with a photomultiplier tube (PMT). It is important to collect the data and quantitate intensities using software provided by the microscope maker or a program such as NIH Image, ImageJ, or Metamorph. The human eye is remarkably poor at quantifying subtle differences in intensity. Also carefully consider the method used for monitoring intensities. If the proteins of interest localize to a discrete structure or only a few dispersed structures, it will be more informative to assay a region of interest that includes only the structure of interest. Large regions of interest that include large unlabeled regions will average out differences in the fluorescence intensities of structures.

Initially, fix, permeabilize, and label cells separately with donor and acceptor antibodies to separately optimize each to maximize signal intensity and reduce nonspecific labeling. Typically, the donor antibody is used at ~0.2 to 1.0 μg/ml and the acceptor is used at ~2 to 4 μg/ml (this refers to the concentration of the specific antibody; hence, crude IgG from a polyclonal serum would be used at ~10-fold higher concentrations since only ~10% is specific antibody). A donor/acceptor ratio at or below 1:4 (usually ~1:8) will help maximize FRET efficiency without making the donor fluorescence intensity too dim to easily visualize. Incubation times will vary depending on antibody affinity and will need to be determined for each antibody. In general, 60 to 120 min is sufficient for optimal antibody labeling, as assessed by maximal fluorescence intensity and minimal nonspecific staining. Each of these modifications increases the efficiency of FRET as would be expected if the occupancy of antigens were improved. The goal is to maximize occupancy of the antigens bound by the acceptor antibody and have a sufficiently bright but highly specific labeling of the donor. Recall that excess acceptor is effectively invisible to FRET, as measured by acceptor photobleaching, so some nonspecific binding by the acceptor is acceptable. In contrast, optimization of conditions that give highly specific donor labeling is critical.

Another important control is to label cells separately with donor followed by acceptor, acceptor followed by donor, and donor and acceptor simultaneously and then measure mean fluorescence intensities of the whole cell or relevant structures. This control will reveal whether the antibodies sterically affect accessibility of protein epitopes and potentially affect labeling. A similar control experiment that tests the same parameter is to systematically change the concentration of one antibody and determine whether the efficiency of labeling by the other antibody of the donor-acceptor pair in cells is affected.

Identify Optimal Photobleaching Conditions for the Acceptor

Photobleaching the acceptor by high-intensity laser illumination is influenced by laser power, magnification, dwell time of the laser, number of bleach iterations, and sample preparation. The goal should be to optimize conditions where more than 90% of acceptor fluorescence can be bleached. Dyes such as Cy5 may require hundreds of iterations

of laser photobleaching to deplete acceptor fluorescence to background levels. If a large area is to be photobleached or the viewing field is at a low magnification, then the time required to achieve sufficient photobleaching will become burdensome. So choose the highest magnification that allows the regions of interest to be visualized completely. Also, perform the optimization at different magnifications (optimum conditions will differ) so that in the future, a complete set of bleach conditions for whatever application might arise has been obtained. Once optimized, be sure that the selected imaging conditions do not result in photobleaching of the cell outside of the photobleach region of interest.

Make and Quantify FRET Measurements

Once the above parameters are optimized, the samples (along with the predetermined positive and negative controls) can be prepared in which both donor and acceptor are labeled. To make measurements, find some appropriate cells or regions of interest. This is best done by visualization of the cells using the absolute lowest illumination as possible to prevent premature partial bleaching of the sample. One can use phase-contrast microscopy to find suitable cells, or if fluorescence microscopy is used, illuminate only the donor while searching. Once the area is focused and imaging/bleaching conditions are set, the procedure is to take pre-bleach images of the donor and acceptor fluorescence, photobleach the acceptor in a region of interest, and take a second set of post-bleach images of the donor and acceptor. It is critical to avoid sample movement, change in focal plane, or any change in imaging conditions between the pre- and post-bleach images. Once completed, there should be four images for the measurement: pre- and post-bleach donor images, and pre- and post-bleach acceptor images. Although only the donor images are absolutely required for the calculation of FRET efficiency, capture and save the acceptor images because they contain additional information that aids in the interpretation.

The calculation of FRET efficiency from these images can be done manually, or automated using relatively straightforward custom macros for a program like NIH image (see section on automated image analysis). To manually calculate FRET, measure the donor intensity in both the pre- and post-bleach images within the region that was bleached (referred to here as D_{pre} and D_{post}). Subtract background intensity (i.e., the value obtained in an area where there are no cells) from both intensity values. Once these values are obtained, the % FRET (also referred to in various publications as % energy transfer, $\%E$, or just E) can be calculated as:

$$\%E = (D_{post} - D_{pre})/D_{post}$$

All that has been done is to calculate the percent of total donor fluorescence that had been quenched in the presence of the acceptor. It is important to confirm that in an area of the image that was NOT subject to photobleaching, the donor intensity does not change significantly between the pre- and post-bleach images. If it does, it means that the sample may have moved or changed focus between the two images, or that perhaps the acceptor was partially bleached unintentionally between capturing the two donor images. If conditions have been optimized as outlined above, this should not happen (except for occasional sample movement or change in focus).

An increase in donor intensity selectively in the region of acceptor photobleaching is indicative of FRET (with numerous caveats outlined in the next section). Instead of doing the manual calculation on the entire bleached region as a whole, one can calculate FRET for subregions of the sample to gain insight into the spatial distribution of FRET (which could for example reflect differences in protein-protein interaction in different regions of the cell). Although this can be done manually, it is cumbersome. The macro the authors developed for NIH image does this automatically on individual 8×8–pixel regions throughout the image and displays the results in a color-coded map of FRET. This can be extremely useful for visualizing spatial differences in FRET measurements in different areas of the cell. The macro may be obtained by sending an e-mail request to *esnapp@aecom.yu.edu*.

INTERPRETATION OF RESULTS

Of equal importance to the actual collection of FRET data is a rational interpretation of the results (also see *UNIT 17.1*). This is critical to ensure that any observed FRET is actually due to the proximities of the proteins of interest. Sometimes it is easy to forget that the FRET values are indirect measurements based on a series of assumptions (such as specificity of the antibodies) that may or may not have been thoroughly validated. Furthermore, other potential artifacts must be taken into account and excluded to maximize the likelihood of correct interpretation. Some of these issues are listed below.

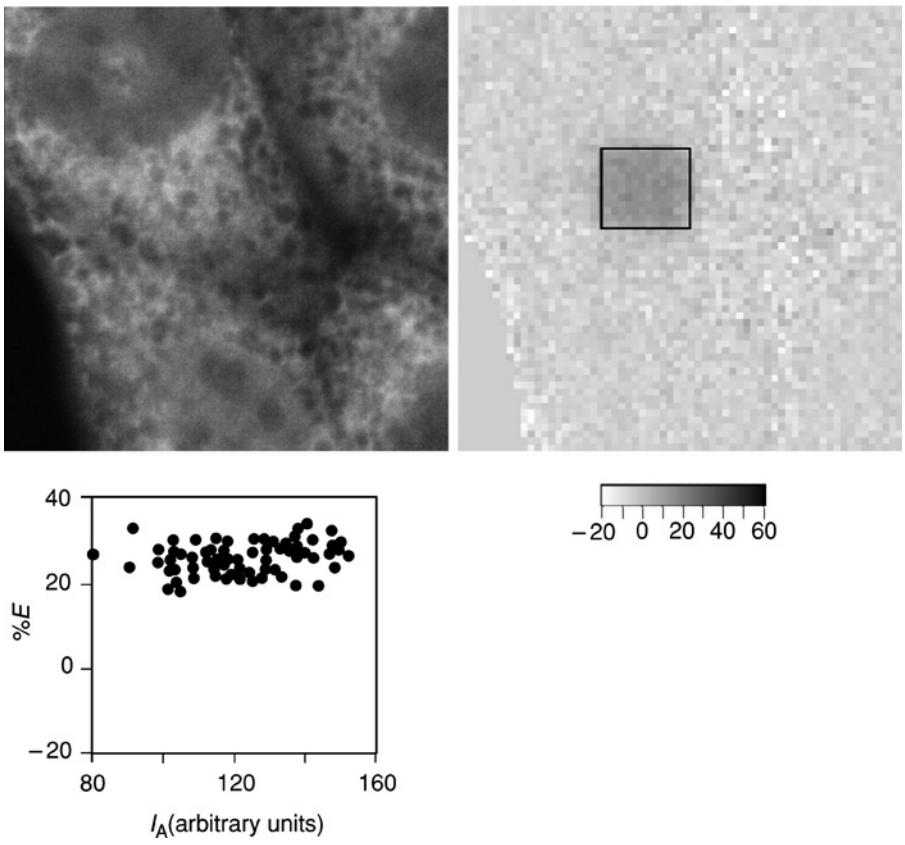


Figure 17.9.2 Control for acceptor density dependence. The top left panel is an image of the acceptor-labeled cell prior to photobleaching. The top right panel shows an energy transfer efficiency map with the extent of energy transfer revealed by acceptor photobleaching in the box. The scale indicated below the map is the energy transfer efficiency (%*E*). The plot in the lower left panel displays %*E* as a function of acceptor-fluorophore intensity (I_A). Note that similar FRET values (%*E*) are observed over a broad range of acceptor intensities, demonstrating that FRET is not acceptor density dependent for this experiment.

Restrict Quantitative Comparisons to Experiments Performed at the Same Time

Experimental FRET values can be affected by batch-to-batch differences including variations in cell density, cell cycle, alignment of the photobleaching laser (which will affect completeness of the photobleach), and antibody degradation. While the overall trends and relationships in the data should be robustly repeatable, the absolute values in the data will tend to vary by as much as 20% between experiments. This observation also emphasizes the importance of including a positive control with every experiment to assist in detecting batch-to-batch variability.

Be Aware of Artifactual Reasons for Changes in Donor Fluorescence

It was recently reported that upon photobleaching, the Cy5 fluorophore is photoconverted into a fluorescein-like fluorophore that has the potential to confound FRET analyses using the acceptor-photobleaching method (Nichols, 2003; Snapp et al. 2004). This is because the photoconverted Cy5 has a fluorescence spectrum that overlaps with the Cy3 spectrum and therefore interferes with its quantitative measurement. This is particularly relevant if the Cy3 fluorescence is extremely dim relative to Cy5 and/or if the Cy3 excitation light intensity or Cy3 detector gains are set at very high levels. Because of the potential to substantially influence the apparent FRET that is observed, it is important and worthwhile to carefully consider this phenomenon in interpreting the results.

The artifactual FRET signal due to photoconversion shows a direct dependence on Cy5 intensity (e.g., Nichols, 2003; Snapp et al. 2004). A useful control is to measure the effect of decreasing the donor concentration (with constant acceptor concentration) on FRET efficiency. All other things being equal, FRET

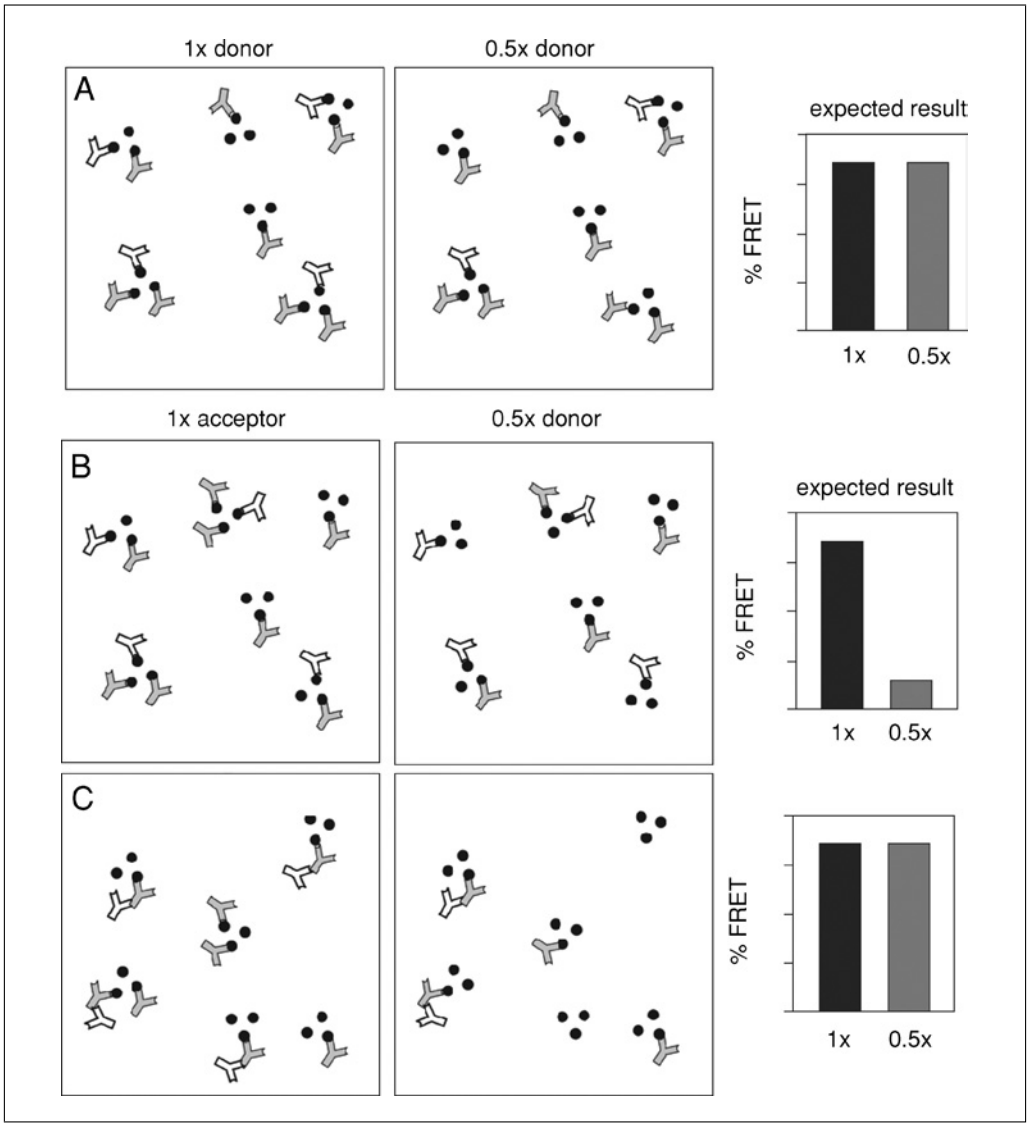


Figure 17.9.3 Relationship of donor and acceptor concentrations to FRET. (**A**) Schematic diagram illustrating the effect of donor concentration on FRET. Oligomers of antigens (black dots) are shown randomly labeled with donor (white) and acceptor (gray) antibodies. The right diagram indicates one half the amount of donor antibody than that in the left panel. Although the donor fluorescence is expected to be lower for the right panel, the proximity of each donor to acceptor antibodies predicts that FRET efficiency should stay the same. The situation is very different for acceptor antibody concentration. (**B** and **C**) Antibody distributions are illustrated for donor (white) and acceptor (gray) antibodies on a hypothetical clustered three-antigen oligomer (black dots). Panel (B) shows the situation where the donor and acceptor antibodies bind their antigens, but do not interact with each other. Panel (C) shows the situation where the acceptor binds its antigen, but the donor antibody interacts with the acceptor antibody. In both cases, the right panel shows the consequence of reducing the acceptor concentration by one half, and the predicted effect on FRET.

efficiency should be independent of donor intensity. If photoconversion is contributing significantly to the Cy3 fluorescence measurements, halving the donor concentration will substantially increase the apparent FRET signal. This is because the photoconverted product continues to contribute the same amount of fluorescence to the Cy3 measurements; however, the starting donor intensity is decreased. Thus, the photoconverted product will cause the Cy3 measurements to increase by a much higher percent upon Cy5 photobleaching, resulting in erroneously high FRET values. By contrast, genuine FRET without interference from photoconversion should be largely independent of donor intensity and occupancy. Indeed, a useful analysis of one's FRET data is to plot *%E* vs. absolute donor intensity. The

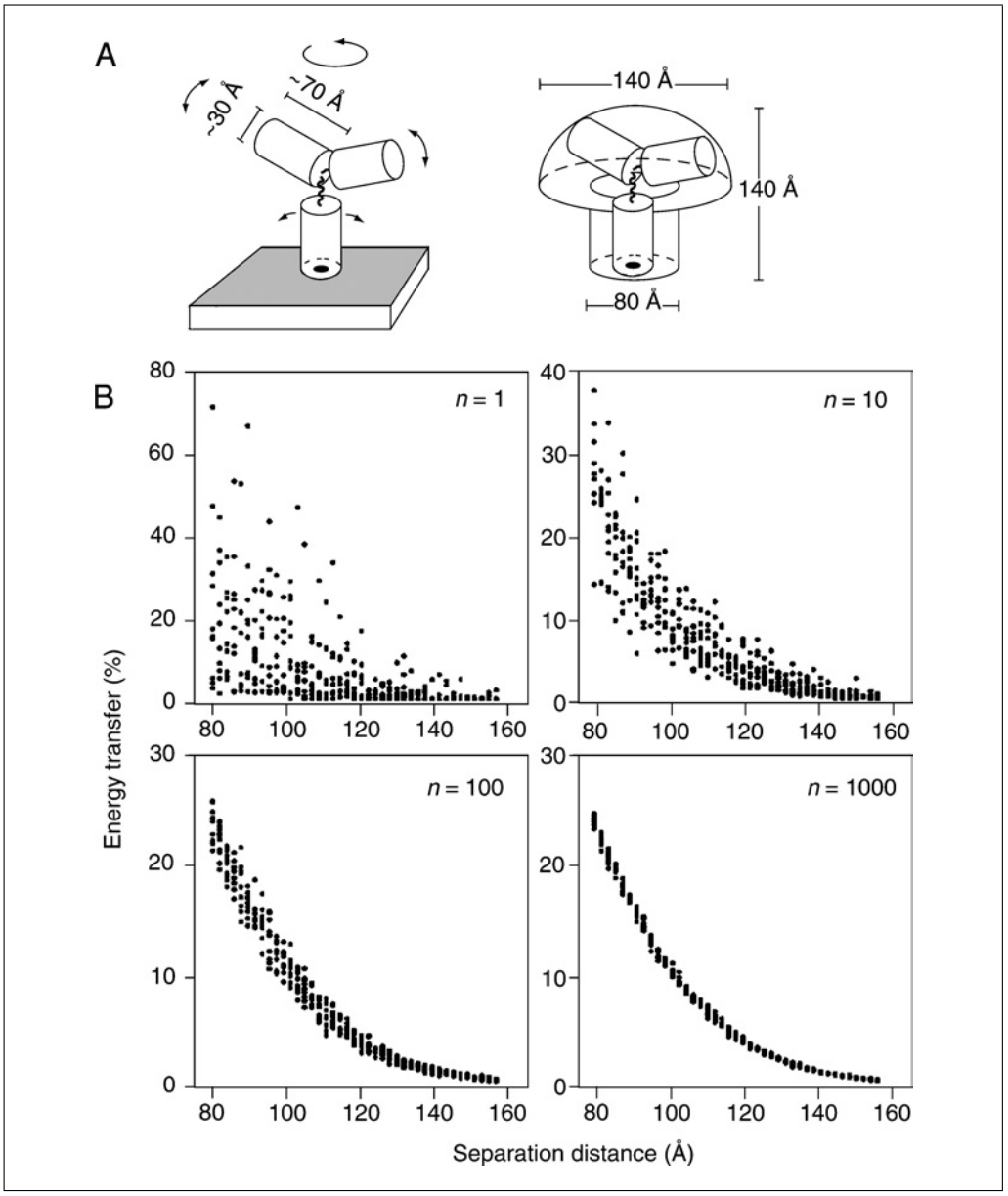


Figure 17.9.4 Sampling size and the resolving power of antibody-mediated FRET. (**A**) Diagram of a model IgG molecule bound to an antigen on the membrane surface (left). The Fc and each Fab domain are modeled as a cylinder of diameter 30 Å, height 70 Å, connected by flexible hinges. Arrows indicate directions of allowed rotational flexibility. The range of potential positions that can be occupied by dyes conjugated to the antibody surface is indicated on the right. Dyes are allowed to be on the surface of the stalk of the mushroom-shaped space, and anywhere in the volume of the head. (**B**) A simulated donor and acceptor labeled antibody (4 dyes/IgG, randomly distributed as described in panel (A) were bound to antigens separated by distances of between 80 and 160 Å, and the % energy transfer between the dyes calculated and plotted. Each datum represents the average of between 1 and 1000 such simulations as indicated (*n*) on each graph. Custom macros (available upon request from the authors) were written for NIH Image 1.62 to perform the antibody-mediated FRET simulations. In essence, an algorithm was designed to simulate the stochastic binding of a mixture of donor- and acceptor-labeled antibodies to a set of antigens on a membrane surface, followed by a calculation of the FRET between the randomly distributed dyes on all of the bound antibodies. The algorithm encompassed the following steps:

continued

relationship should be horizontal line. If FRET efficiency increases as the donor intensity decreases, then the investigator should suspect photoconversion of the acceptor fluorophore to a donor-like spectral emission.

One approach to this problem is to correct for photoconversion by using a standard curve in which defined intensities of the acceptor are correlated with the respective amounts of photoconverted product upon photobleaching (Nichols, 2003). However, data correction is often not necessary in cases where photoconversion contributes less than ~5% of the value of observed FRET signals. This phenomenon can be monitored by plotting $\%E$ as a function of acceptor intensity for different regions of an image (Fig. 17.9.2). Additionally, a substantial contribution from photoconversion can be ruled out if it is demonstrated that the FRET signal is lost solely by changing the choice of donor antibody to one that recognizes an unrelated antigen. An alternative solution to the photoconversion problem is to use different donor/acceptor pairs. This can be difficult if one has already invested substantial time and effort in one FRET pair, but may be feasible early in the experimental design or project.

Ensure That the Antibodies Do Not Interact Directly with Each Other

It is assumed that antibodies do not directly interact with each other to generate FRET, but instead simply mark the positions of the antigens against which they are directed. Thus, a FRET signal between antibodies is taken to reflect the proximities of the antigens to which the antibodies are bound, and not to nonspecific interactions among the antibodies themselves. It is therefore critical to the interpretation of the results that the antibodies not interact with each other. FRET between directly interacting antibodies is largely insensitive to changes in concentration of the acceptor antibody (Fig. 17.9.3A). This is because the only donor labeling that occurs is via binding to an acceptor. Thus, although reduced labeling occurs due to the reduced acceptor concentration, all donors are still adjacent to an acceptor and therefore generate a high FRET signal (see Fig. 17.9.3A).

In marked contrast, FRET between the antibodies as a consequence of the fact that their antigens are in close proximity is highly sensitive to acceptor concentration (Fig. 17.9.3B). Here, the donor antibodies still bind to their antigens, but some of the acceptor antigens will now be unoccupied by acceptor antibodies (Fig. 17.9.3C). The presence of donor antibodies unaccompanied by nearby acceptors reduces the FRET signal and helps rule out inappropriate interactions between antibodies.

For an additional control, measure FRET between the donor and acceptor antibodies with and without a peptide competitor corresponding to the antigen for the acceptor antibody. Inclusion of the peptide during the antibody incubation should result in both decreased labeling of cells by the acceptor antibody (but not the donor antibody), and a loss of the FRET signal. Taken together, these controls help confirm that FRET between dye-labeled antibodies is due to the proximities of the antigens to which the antibodies bind, and not due to interactions between the antibodies themselves.

Figure 17.9.4 *(at left)* (1) The *x-y* positions of the appropriate number of antigens were distributed on a hypothetical surface of defined area (usually 0.5×0.5 μm) at the indicated density and configuration (either randomly distributed, or in clusters of three). Clusters were not allowed to overlap, and the minimal distance separating adjacent antigens was limited to 8 nm, as determined by the steric hindrance of bound IgG molecules. (2) Each antigen was randomly assigned to either be unoccupied, bound by a donor antibody, or bound by an acceptor antibody. The relative probabilities of each assignment were determined by the desired occupancy and donor/acceptor ratio. (3) The *x-y-z* positions of dyes were randomly chosen relative to each antigen by the criteria outlined in the text. (4) Once the *x-y-z* positions for all of the donor and acceptor dyes were set, the summed FRET efficiency that would be expected for this distribution of dyes was calculated according to previously established equations (Förster, 1948; Dewey and Hammes, 1980).

Optimize Sample Size

Part of the power of FRET methods is the ability to quantitate small changes in protein proximities. However, achieving this quantitation requires sufficient statistical sampling. The amount of sampling dramatically increases as the investigator attempts to monitor single nanometer changes. At a minimum, perform multiple, at least $n \geq 10$, measurements to permit statistical analysis to identify significant changes in FRET efficiency values.

Expression levels of the proteins of interest will affect both the cell and the sensitivity of the planned measurements. Proteins, such as kinases or transcription factors, tend to be expressed at nanomolar concentrations (Huang and Ferrell., 1996). This translates to a few hundred copies of a protein or less per cell, in some cases. Consider that a homogeneously distributed fluorescent protein must be present at 200 nM to be visualized over background cellular fluorescence (Niswender et al., 1995). Thus, experimental questions involving cellular expression of a fluorescently tagged protein may require unnaturally high expression levels of a protein that may affect a cell phenotype. In contrast, dye-labeled antibodies rarely require overexpression of a protein of interest and also avoid problems related to whether a fluorescent fusion protein is functional (*UNIT 21.4*). Measurement sensitivity will be dependent on the available number of proteins to be assayed in a region of interest. Too few proteins will result in a low signal-to-noise ratio and low sampling sizes.

For purposes of experimental design, measuring absolute states of associated and nonassociated proteins should be readily discernable even for low protein sampling sizes. This is illustrated in Figure 17.9.4. The first graph of Figure 17.9.4B ($n = 1$) shows a scatter plot of the range of FRET values obtained for any single antibody pair separated by various distances. At a separation distance of 80 Å, the FRET efficiencies ranged broadly from less than 5% to nearly 80% (Fig. 17.9.4B; $n = 1$). This tremendous variability reflects the stochastic distributions of the donor and acceptor dyes over a large volume, combined with the extreme sensitivity of FRET to small changes in the distances separating them. Indeed, it has been shown previously that if the number of sampled molecules is small, such dramatic fluctuations can be anticipated (Haas and Steinberg, 1984).

Although a trend is observed in which increased separation distance between the antibodies results in lower FRET, a single interaction cannot be used to discriminate different antigen positions (except to say that antigens are either within ~15 nm of each other, or further away). However, the resolving power increases substantially as more antibody pair interactions are sampled and averaged (Fig. 17.9.4B; $n = 10$ through $n = 1000$). At a sampling size of 1000, differences in separation distance of between 0.2 and 0.4 nm can be resolved with confidence. Thus, subnanometer changes in antigen separation can be resolved using FRET between dye-conjugated antibodies despite the highly flexible and large nature of the probes, the stochastic distribution of the dyes bound to them, and the complex relationships for FRET between ensembles of donor and acceptor fluorophores.

Create a Model for the FRET Experiment

If sufficient information about the proteins of interest is available, it is worthwhile to model and simulate FRET experiments to assist with experimental design and expectations for results. Simulated results serve to illustrate the capabilities, sensitivity, and specificity of an approach and provide boundaries for the type of questions that can be addressed. In particular, two issues are of direct relevance to most studies of protein complex assembly: the discrimination of assembled from disassembled multiprotein (or oligomeric) complexes and the discrimination of small changes in the structure of a complex that remains assembled in the same general configuration.

Monte Carlo simulations, rather than theoretical calculations using simplifying assumptions, can provide insight into not only the expected FRET efficiency, but also the degree of variability that can be anticipated from measurement to measurement due to the stochastic nature of many of the variables involved (e.g., see Haas and Steinberg, 1984). For example, FRET between subunits of a membrane protein complex containing three subunits was modeled (Fig. 17.9.5A). Donor and acceptor antibodies labeled with four dyes at random positions were simulated in binding to the proteins with varying ratios. A visual representation of the relative proximities of subunit proteins and fluorescent dyes in the disassembled and assembled states for a small section of membrane is shown in Figure 17.9.5B as a two-dimensional illustration. Then the expected FRET efficiency for each ensemble of fluorophores was calculated according to previously established equations (Dewey and Hammes, 1980).

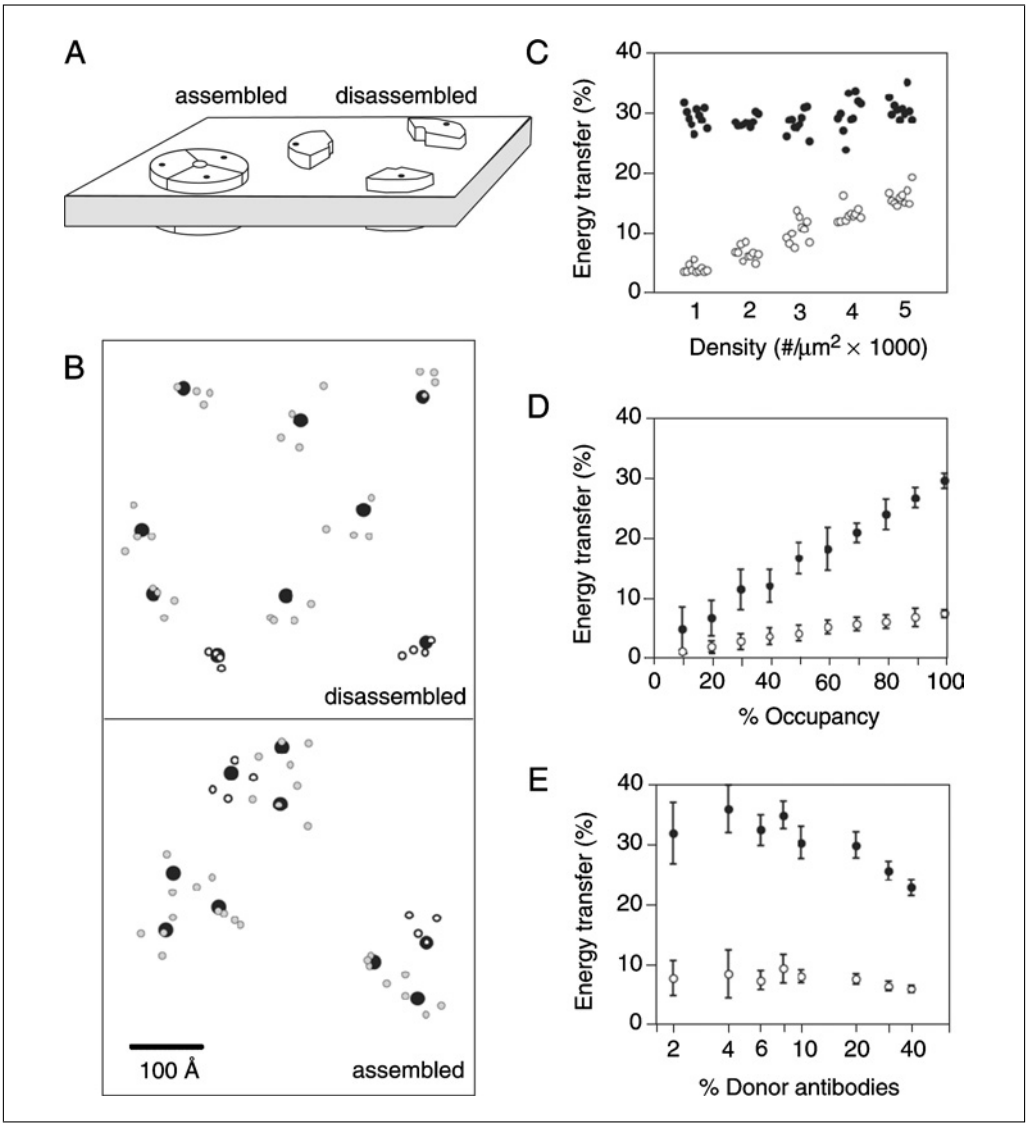


Figure 17.9.5 Simulations of FRET for assembled versus unassembled oligomers. (**A**) Idealized configurations of an oligomeric channel in the membrane in an assembled and disassembled configuration. The positions of a putative antigen present as three copies in the channel are indicated with a black dot. (**B**) Surface view of a 70 × 70–nm section of membrane containing antigens (black dots) at 2000 copies per mm^2 in assembled and disassembled configurations as in (A). The two dimensional projection of the positions of donor (clear outlined) and acceptor (gray) dyes on antibodies bound to these antigens is also indicated. An antibody was assigned a 20% probability of containing donor dyes. (**C**) Monte Carlo simulations were used to calculate the FRET efficiencies in a 0.25 μm^2 section of membrane containing donor and acceptor labeled antibodies (at a donor/acceptor ratio of 1:4, each containing 4 dye molecules per IgG) bound to antigens (with 100% occupancy) at densities of 1000 to 5000 copies per μm^2. The antigens were distributed in the membrane surface either randomly (open circles) or assembled into clusters of three (closed circles). The simulation was repeated ten times for each condition, with each point representing the FRET from a single simulation. Note that FRET in the nonclustered configuration displays a clear density-dependence that is not seen in the clustered configuration. (**D**) Monte Carlo simulations of FRET as a function of the degree of occupancy of the antigen by antibody. Randomly distributed antigens (open circles) were compared to clustered antigens (closed circles). Note that %*E* is always higher for the clustered situation regardless of % occupancy. (**E**) Simulation of FRET as a function of the proportion of antibodies that carry donor dyes. Note that the best discrimination between random (open circles) and clustered (closed circles) antigens is seen at donor antibody values below 20%. In (D) and (E), the mean ± SD of ten simulations is plotted for each condition. When not being specifically varied, antibodies contained four dyes each, 20% of antibodies were donors, antigen occupancy was 100%, and antigen density was 2000 copies per μm^2.

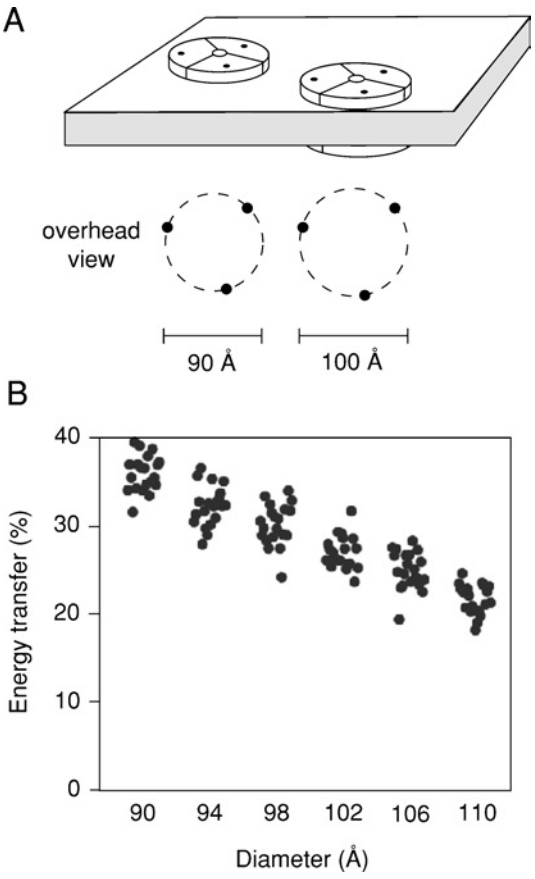


Figure 17.9.6 Simulations of FRET for multiprotein complexes undergoing a conformational change. (**A**) Scale diagram of a complex of 90- versus 100-Å diameter. The modeled complex is of three antigens around a circle of the indicated diameter. (**B**) Clusters of three antigens in circles of diameters ranging from 9 to 11-nm were randomly distributed in 0.25 μm^2 areas, and the FRET between the bound antibodies calculated by Monte Carlo simulations as in Figure 17.9.5. Each set of 20 measurements was statistically significant from an adjacent set. These findings illustrate that with sufficient sampling even modest changes (in the subnanometer range) to the configuration of a complex can be detected using FRET.

The putative assembled versus disassembled states of a protein complex represent the extremes of configurations, thereby making them easily distinguishable by antibody-mediated FRET. Because the active versus inactive configurations of the complex may instead represent a more subtle change in arrangement of its components, the authors used the simulations to ask whether such small changes could also be distinguished. As seen in Figure 17.9.4, even such small changes can be distinguished given sufficient sampling. Under these conditions, twenty areas of 0.25 μm^2, each containing assemblies at a density of ~667 per μm^2 (Fig. 17.9.6), was sufficient to distinguish a 0.4-nm difference. This modest sampling size (e.g., a total of ~5 μm^2) represents the lower limit for distinguishing such small changes using idealized conditions. Thus, *changes* to a multiprotein structure, whether it is simple or complex, are likely to be detectable with antibody-mediated FRET. While different geometries of a protein complex can produce different absolute FRET values compared to an idealized complex, changes within the confines of a different structure are still detectable with antibody-mediated FRET (Snapp et al., 2004). These simulations were in fact very useful in concluding that antibody-mediated FRET is a feasible method to monitor both gross and subtle changes in complex structure in cells.

The details of the simulation model system benefit from data on the size, structure, and abundance of the complex to be studied. Parameters to test include: simulated FRET between fluorophores with varying parameters such density of antigens (Fig. 17.9.5C), percent occupancy of antigens by antibodies

(Fig. 17.9.5D), relative ratios of donor to acceptor antibodies (Fig. 17.9.5E), proximities of protein subunits, and configuration of antigens (e.g., randomly distributed versus assembled into clusters of defined size). It is beyond the scope of this unit to provide a detailed guide for model design and simulation. For more background on designing molecular simulations, the investigator is referred to Frenkel and Smit (2001). Examples of simulation design are described in the supplemental data sections for Snapp et al. (2004) and Sharma et al. (2004) and the appendix in Kenworthy and Edidin (1998).

RECIPROCAL FRET

When a protein complex contains more than two different proteins, determining the relative stoichiometries of the protein subunits can be valuable. The choice of acceptor and donor will significantly affect the FRET values if one protein is present at lower levels of expression (Fig. 17.9.7). The inherently asymmetric relationship between donor and acceptor molecules during acceptor-photobleaching FRET measurements makes it possible to assess whether protein subunits are present at comparable stoichiometries. While acceptors adjacent to a donor are essential for FRET to occur, the presence or absence of *additional* acceptor molecules that are unaccompanied by an adjacent donor does not change the overall FRET efficiency. In contrast, the presence of donor molecules that are unaccompanied by an adjacent acceptor causes an overall reduction of FRET efficiency. This is because the excess donors increase the overall donor fluorescence intensity without changing the absolute amount of energy that is transferred to the acceptor, thereby resulting in a net decrease in the *percent* of total energy transferred. That is, excess acceptors are effectively invisible and do not affect FRET values, while excess donors decrease FRET values.

A schematic illustration of this principle is shown in Figure 17.9.7. In panel 17.9.4A, all copies of two interacting components (represented by squares and circles) are paired with each other, with neither containing excess copies. The FRET efficiency in this scenario should be equivalent regardless of which component serves as the donor or acceptor. In

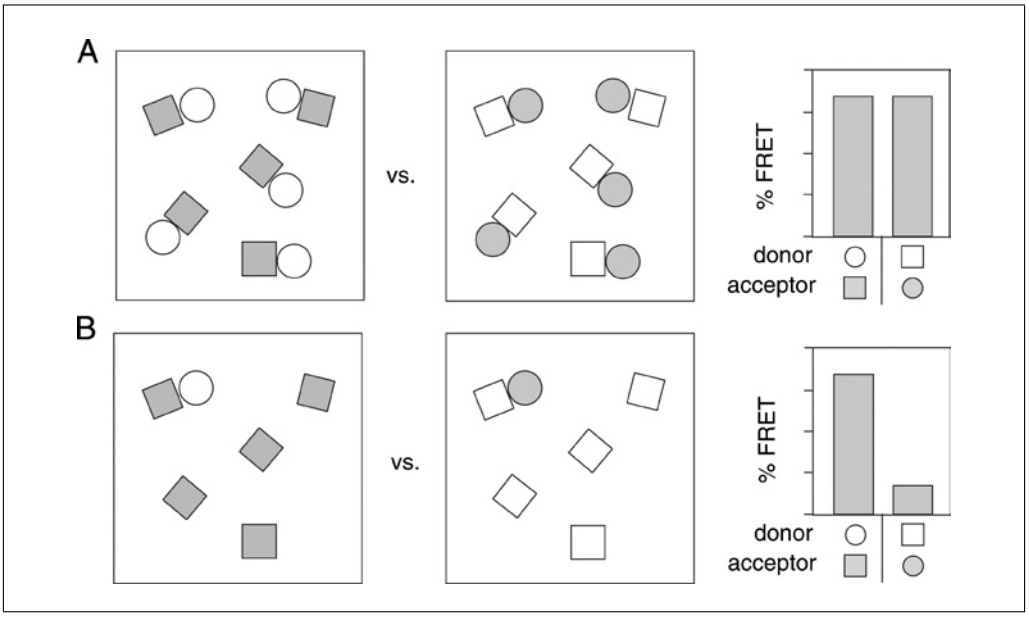


Figure 17.9.7 FRET-based analysis of multiprotein complex component interactions and stoichiometry. (**A** and **B**) Cartoon of reciprocal FRET measurements between a hypothetical interacting pair of molecules (squares and circles) at different stoichiometries. The two fields in each panel represent situations in which each shape is labeled as donor (open) or acceptor (gray). The histograms to the right represent relative FRET efficiencies for each situation. Equal numbers of paired squares and circles (A) yields equivalent FRET values regardless of which protein is the donor or acceptor. In contrast, a substoichiometric number of circles, with excess unpaired squares, results in a discordance in FRET between the reciprocal measurements (B). These findings illustrate that by changing the labeling scheme systematically it is theoretically possible to gain insight into the relative stoichiometry between two interacting proteins.

Figure 17.9.7B, however, one of two interacting components is present in excess (i.e., the squares). In this case, the FRET efficiency will be substantially lower if the squares serve as the donor than if the circles serve as the donor. Thus, a greater than 2-fold discordance in the FRET efficiencies upon switching the donor and acceptor molecules can be used to infer that at least some copies of one of the components exists in the absence of the other component. Reciprocal FRET will not necessarily reveal the precise stoichiometry, as the FRET relationships are not inherently linear. Nonetheless, the method should enable the investigator to distinguish between proteins present as single or multiple copies in a complex, and whether this changes in response to some treatment or manipulation. In general, it is not appropriate to perform reciprocal FRET when one of the proteins is labeled with a fluorescent fusion protein. Expressing a fluorescent fusion protein may significantly increase the levels of the protein of interest and result in protein that isn't incorporated into complexes or overexpression-related changes in cell behavior (*UNIT 21.4*).

To perform reciprocal FRET, use the optimized acceptor-photobleaching protocol established in the acceptor-photobleaching FRET strategy with the following modifications:

1. When using antibodies to label donor and acceptor proteins, the experimental protocol is reversed, though the labeling protocol for the antibodies needs to be optimized. Determine labeling conditions (see Empirically Determine Antibody Labeling Conditions). Specifically, identify appropriate antibody labeling ratios for each antibody, maintain a ratio of ~1:4 to 1:8 donor/acceptor antibodies, and optimize amounts of antibodies to achieve maximal labeling intensity without causing nonspecific labeling or a decrease in FRET due to excess donor. In addition, confirm that order of addition of antibodies does not affect labeling efficiency and that FRET values are independent of donor intensities.

2. Using a fluorescent microscope, perform acceptor-photobleaching FRET for each combination of donor and acceptor, and then switch the labeling of each protein to make the initial donor now the acceptor and the initial acceptor now the donor. If one protein is present at significantly lower concentration than the other or if one protein is always bound to a partner while the other protein is only sometimes bound, a significant difference in FRET under the two conditions will be observed. Because the absolute FRET efficiencies can vary somewhat depending on the number of dyes per antibody and labeling conditions, it is suggested that only discordance differences more than 2-fold be considered meaningful.

AUTOMATED IMAGE ANALYSIS

To process multiple data sets, automation of FRET analysis can be readily performed and one such scheme and macro are described. The authors have created an acceptor-photobleaching FRET analysis macro for NIH Image 1.62 (freely available from the authors by sending an e-mail request to *esnapp@aecom.yu.edu*). The macro quantitates FRET within the photobleached area and generates an energy transfer map. Briefly, the macro performs the following operations in sequence: (1) The pre-bleach and post-bleach images are registered to optimal alignment. (2) The area of photobleaching is identified. (3) The percent change in donor intensity (after background subtraction) is calculated in each 8 × 8–pixel region (0.56 × 0.56 μm) of the image and drawn as a pseudocolored map. (4) The average change within the entire photobleached and non-bleached regions is calculated. The user is free to either write her own or modify the authors' macro as necessary for specific applications. To use the macro, the user must perform the following steps:

1. Name image files as 101000.tif, 101001.tif, 102000.tif, 102001.tif. for consecutive files. Note that the nomenclature is as follows: the first digit is the sample number (or the chamber number to indicate which chamber of an 8-well chambered coverslip is being used); the next two digits are the trial number within this sample; and the last three digits are necessarily "000" to indicate a pre-bleach image and "001" to indicate a post-bleach image. Each image must be an 8-bit 512 × 512–pixel RGB file in which the red channel contains the acceptor image and the green channel contains the donor image. The photobleached region must be a 75 × 75–pixel region; the portion of the image that contains the bleach box does not matter (the macro automatically finds this).

2. Place all images in a single folder. Within that folder, the user must create a new folder entitled "processed images."

3. Load the macro into NIH image and run. Before the macro starts, the user is asked a series of questions as follows. The user will

be asked how many chambers. This is usually between 1 and 8. The user is then asked how many experiments are in a chamber (e.g., how many trials for each sample). Enter the number of experiments from 1 to 99. The user is next asked for a threshold (background value). The default is 10, but the user can decrease or increase this value as necessary based on the imaging conditions used. The user is asked the size of the sampling square. The default value is 8. This means that the FRET calculations are performed on each 8 × 8–pixel square within the image and the average FRET value within this area is plotted in the map. The averaging helps reduce image noise. It also affects the speed of the program. A smaller sampling square will slow the analysis because more pixels must be individually calculated.

4. When the user is asked to open the first image of the set, select OK. Then NIH image will open a browsing window to find the folder containing the images. Open this folder, select the first image, and select Open. This points the program to the place where the data set of interest is stored. The program will now run until all of the images in this folder have been processed.

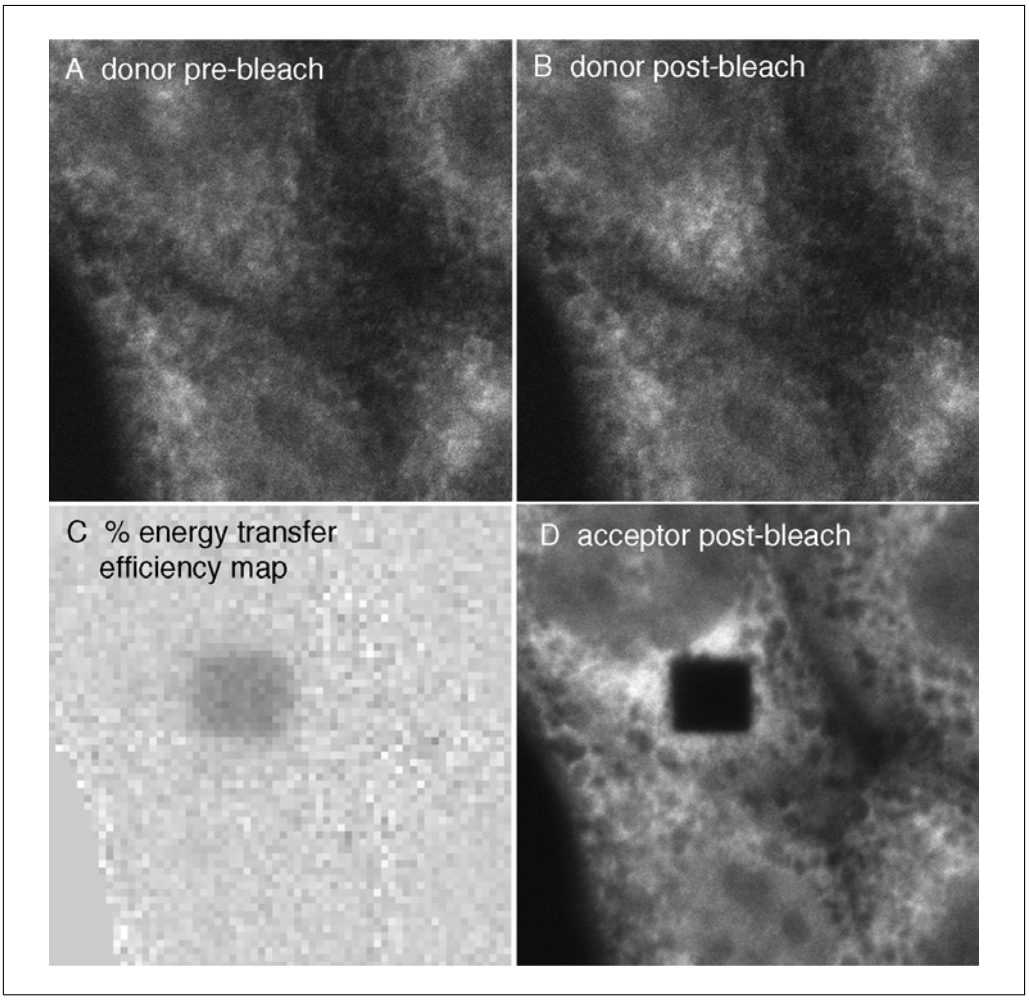


Figure 17.9.8 Output from the macro developed by the authors to assist in data interpretation (see Automated Image Analysis section). In this experiment, two proteins known to interact were labeled with Cy3-labeled donor and Cy5-labeled acceptor antibodies. Shown are the images from a typical FRET measurement. (**A**) Image of the donor fluorescence prior to photobleaching of the acceptor. (**B**) Image of the donor fluorescence immediately after photobleaching of the acceptor fluorescence in a small square near the center of the image (see panel D). Note that the donor fluorescence has selectively increased in intensity in this region. (**C**) Quantitation of the change in donor fluorescence between pre-bleach and post-bleach images (i.e., the %*E* map). The darker gray region near the center of the image indicates an area of significantly increased donor fluorescence relative to the remainder of the image, and reflects a positive FRET signal. (**D**) Image of the acceptor fluorescence after photobleaching a square region near the center of the image.

Table 17.9.1 Troubleshooting Guide for Acceptor-photobleaching FRET

Problem	Possible cause	Solution
FRET efficiency low, even for positive control	Acceptor fluorophore photobleached before performing FRET measurement	Do not image labeled cells with a fluorescent lamp, which will cause significant photobleaching; instead use low intensity laser power, using the donor fluorescence to find cells and focus them. Keep imaging time to a minimum before making the FRET measurement. Any loss of acceptor fluorescence will reduce the difference between the quenched and unquenched donor fluorophore.
	Acceptor fluorophore incompletely photobleached	Be sure to identify photobleaching conditions to reduce fluorescence of the acceptor to background levels; otherwise, the donor will not be completely dequenched.
High and low FRET observed along edges of fluorescent structures	Pre- and post-bleach images not registered, causing a slight shift of positions of areas with low fluorescence in a pre-bleach image to areas with significant fluorescence and resulting in an apparent increase in the donor channel	Align pre- and post-bleach images in an image manipulation program (e.g., Photoshop, ImageJ or NIH Image; then perform FRET analysis with the modified images. Note that the authors' macro includes a registration step.
FRET detected when using fluorescent fusion proteins as negative controls (if the proteins used authentically do not interact)	Fused fluorescent proteins dimerizing (may occur at high concentrations; see Zacharias et al., 2002 and Snapp et al., 2003)	Be sure to make fluorescent fusion proteins with fluorescent proteins that contain monomerizing mutations.
FRET observed between negative control proteins in a fluorescence intensity-dependent manner	FRET due to density-dependent clustering of proteins most likely caused by (1) nonspecificity of an antibody or (2) labeled proteins present at such high concentrations that their proximities are sufficiently close for FRET to occur	Confirm specificity by preincubating antibody with peptide that antibody was raised against; restrict analysis to regions of cells in which FRET occurs in an intensity independent manner (problem may be unavoidable in cases where the protein is concentrated within an organelle); use smaller fluorescent probes instead of antibodies; or consider other FRET methods including FLIM and fluorescence anisotropy (see supplementary data and appendix in Kenworthy and Edidin, 1998 and Sharma et al., 2004).

At the end of the program, the processed data file will include data images and a quantitation text file. The data images will contain the pre- and post-bleach donor images, an energy map, and the post-bleach acceptor image, in that order (Fig. 17.9.8).

The quantitation file can be opened in a spreadsheet such as Microsoft Excel. The first column is the chamber number, the second column is the experiment number, the third column is the background subtracted post-bleach intensity of the donor, and the fourth column is the background-subtracted pre-bleach donor intensity for the bleach region of interest. The final column is the calculated percent energy transfer.

CONCLUSION

The strategies described in this unit and *UNIT 17.1* should permit the investigator to design, carry out, and interpret an acceptor-photobleaching FRET experiment to study

protein interactions in cells. The investigator can use the same methodology to gain insight into comparative stoichiometry of proteins in a complex. Finally, the modeling methods described allow the investigator to simulate potential results to optimize the experimental setup and interpretation. Table 17.9.1 discusses methods to troubleshoot acceptor-photobleaching FRET.

LITERATURE CITED

Adams, S.R., Campbell, R.E., Gross, L.A., Martin, B.R., Walkup, G.K., Yao, Y., and Tsien, R.Y. 2002. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: Synthesis and biological applications. *J. Am. Chem. Soc.* 124:6063-6076.

Bastiaens, P.I. and Jovin, T.M. 1996. Microspectroscopic imaging tracks the intracellular processing of a signal transduction protein: Fluorescent-labeled protein kinase C beta I. *Proc. Natl. Acad. Sci. U.S.A.* 93:8407-8412.

Clegg, R.M. 1995. Fluorescence resonance energy transfer. *Curr. Opin. Biotechnol.* 6:103-110.

Deniz, A.A., Laurence, T.A., Dahan, M., Chemla, D.S., Schultz, P.G., and Weiss, S. 2001. Ratiometric single-molecule studies of freely diffusing biomolecules. *Annu. Rev. Phys. Chem.* 52:233-253.

Dewey, T.G. and Hammes, G.G. 1980. Calculation on fluorescence resonance energy transfer on surfaces. *Biophys. J.* 32:1023-1035.

Förster, T. 1948. Zwischenmolekulare energiewanderung und fluoreszenz. *Ann. Phys.* 2:57-75.

Frenkel, D. and Smit, B. 2001. Understanding Molecular Simulations. Academic Press, San Diego.

Haas, E. and Steinberg, I.Z. 1984. Intramolecular dynamics of chain molecules monitored by fluctuations in efficiency of excitation energy transfer. A theoretical study. *Biophys. J.* 46:429-437.

Haj, F.G., Verveer, P.J., Squire, A., Neel, B.G., and Bastiaens, P.I. 2002. Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. *Science.* 295:1708-1711.

Huang, C.Y. and Ferrell, J.E. 1996. Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.* 93:10078-10083.

Jackson, V. 1999. Formaldehyde cross-linking for studying nucleosomal dynamics. *Methods* 17:125-139.

Jares-Erijman, E. and Jovin, T.M. 2003. FRET imaging. *Nat. Biotechnol.* 21:1387-1395.

Kenworthy, A.K. 2001. Imaging protein-protein interactions using fluorescence resonance energy transfer microscopy. *Methods* 24:289-296.

Kenworthy, A.K. and Edidin, M. 1998. Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of <100 Å using imaging fluorescence resonance energy transfer. *J. Cell Biol.* 142:69-84.

Krishnan, R.V. Varma, R., and Mayor, S. 2001. Fluorescence methods to probe nanometer-scale organization of molecules in living cell membranes. *J. Fluor.* 11:211-226.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* 2:444-456.

Matyus, L. 1992. Fluorescence resonance energy transfer measurements on cell surfaces. A spectroscopic tool for determining protein interactions. *J. Photochem. Photobiol. B.* 12:323-337.

Metz, B., Kersten, G.F.A., Hoogerhout, P., Brugghe, H.F., Timmermans, H.A.M., de Jong, A., Meiring, H., ten Hove, J., Hennink, W.E., Crommelin, D.J.A., and Jiskoot, W. 2004. Identification of formaldehyde-induced modifications in proteins—reactions with model peptides. *J. Biol. Chem.* 279:6235-6243.

Michalet, X., Kapanidis, A.N., Laurence, T., Pinaud, F., Doose, S., Pflughoefft, M., and Weiss, S. 2003. The power and prospects of fluorescence microscopies and spectroscopies. *Annu. Rev. Biophys. Biomol. Struct.* 32:161-182.

Miyawaki A. and Tsien, R.Y. 2000. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.* 327:472-500.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. 1997. Fluorescent indicators for Ca^{2+} based on green fluorescent protein and calmodulin. *Nature* 388:882-887.

Nichols, B.J. 2003. GM1-containing lipid rafts are depleted within clathrin-coated pits. *Curr. Biol.* 13:686-690.

Niswender, K.D., Blackman, S.M., Rohde, L., Magnuson, M.A., and Piston, D.W. 1995. Quantitative imaging of green fluorescent protein in cultured cells: Comparison of microscopic techniques, use in fusion proteins and detection limits. *J. Microsc.* 180:109-116.

Rizzo, M.A. and Piston, D.W. 2005. High-contrast imaging of fluorescent protein FRET by fluorescence polarization microscopy. *Biophys. J.* 88:L14-L16.

Sharma, P., Varma, R., Sarasij, R.C., Ira, Gousset, K., Krishnamoorthy, G., Rao, M., and Mayor, S. 2004. Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell.* 116:577-589.

Snapp, E., Hegde, R., Francolini, M., Lombardo, F., Colombo, S., Pedrazzini, E., Borgese, N., and Lippincott-Schwartz, J. 2003. Formation of stacked cisternae by low affinity protein interactions. *J. Cell Biol.* 163:257-269.

Snapp, E., Reinhart, G., Bogert, B., Lippincott-Schwartz, J., and Hegde, R. 2004. The organization of engaged and quiescent translocons in

the endoplasmic reticulum of mammalian cells. *J. Cell Biol.* 164:997-1007.

Stryer, L. and Haugland, R.P. 1967. Energy transfer: A spectroscopic ruler. *Proc. Natl. Acad. Sci. U.S.A.* 58:719-726.

Van Rheenen, J., Langeslag, M., and Jalink, K. 2004. Correcting confocal acquisition to optimize imaging of fluorescence resonance energy transfer by sensitized emission. *Biophys. J.* 86:2517-2529.

Wallrabe, H. and Periasamy, A. 2005. Imaging protein molecules using FRET and FLIM microscopy. *Curr. Opin. Biotech.* 16:19-27.

Wouters, F.S., Verveer, P.J., and Bastiaens, P.I. 2001. Imaging biochemistry inside cells. *Trends Cell Biol.* 11:203-211.

Zacharias, D.A., Violin, J.D., Newton, A.C., and Tsien, R.Y. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science*. 296:913-916.

Contributed by Erik L. Snapp
Albert Einstein College of Medicine
Bronx, New York

Ramanujan S. Hegde
NICHD, National Institutes of Health
Bethesda, Maryland

Identification and Analysis of Multiprotein Complexes Through Chemical Crosslinking

Completion of many genome projects in conjunction with the development of proteomic tools has enabled the large-scale isolation of multiprotein complexes and the identification of their subunit composition (see *UNIT 17.5*). In contrast, relatively little information exists about the structural organization of these complexes, i.e., the stable interaction of subunits with each other as well as their transient interactions with cellular proteins. Techniques such as GST pull-downs, co-immunoprecipitations, or two-hybrid screens can give information about an interaction of two polypeptides outside of their physiological multiprotein context, i.e., in purified form in the absence of the other subunits of the multiprotein complex. They can also provide information about interactions involving intact multiprotein complexes without information on which of the polypeptides within the multiprotein complexes directly interact. The main advantage of crosslinking studies is that they can be performed in the context of intact protein complexes. This is important because subunits of multiprotein complexes often interact synergistically with several polypeptides simultaneously, and subunit interactions often lose specificity outside of their native contexts (Melcher, 2004).

Chemical crosslinking is the covalent linkage of molecules in very close proximity to each other. In the case of proteins, this linkage can be achieved by incubation with small molecules (crosslinkers) that (1) efficiently and constitutively react with free amino, carboxy, or sulfhydryl groups on amino acid side chains, (2) upon light induction form short-lived intermediates that insert relatively nonspecifically into proteins, or (3) upon oxidation generate highly reactive radicals within aromatic side chains. Most crosslinkers have two terminal reactive groups separated by spacers that allow attack and covalent linkage of two amino acid side chains in close proximity within the same polypeptide (intramolecular crosslinking) or between interacting proteins (intermolecular crosslinking). Versatility of crosslinking applications is often further extended by introducing labeled groups or affinity handles into crosslinkers or by localizing crosslinkers to a bait protein. The bait protein is protein from the multiprotein complex under investigation that has been modified with a covalently linked crosslinker. It is used to probe other proteins (targets) in the multiprotein complex for a possible interaction (see Bait-localized crosslinkers in the Strategic Planning section). Most of these studies are performed in vitro although some crosslinkers can be employed in vivo (see Crosslinking Chemistries in the Strategic Planning section).

Assessments of protein interaction data in which structures of the protein complexes are known have revealed that crosslinking studies are less prone to artifacts than two-hybrid screens (which typically use highly overexpressed bait and prey proteins; see *UNIT 17.3*), or pull-down experiments (*UNIT 17.5*) with recombinant or in vitro translated proteins (Edwards et al., 2002). However, crosslinking methods also have a propensity for false negatives (failure to identify specific interactions) and false positives (interactions that do not occur in native complexes in vivo). This unit compares different crosslinking approaches and discusses strategies for maximizing the number of trapped physiological interactions and minimizing the occurrence of false positive and false negative interactions. This general section is followed by two protocols with examples of crosslinking methods that the authors consider particularly useful for the analysis of large multiprotein complexes (see Basic Protocols 1 and 2).

Contributed by Karsten Melcher and Hung-Ta Chen
Current Protocols in Cell Biology (2006) 17.10.1-17.10.29

STRATEGIC PLANNING

Chemical crosslinking does not represent a single distinct technique but rather a smorgasbord of techniques that greatly differ in both chemistry and scope. This section guides the reader through the complexities of crosslinking to find the most suitable approach for a given biological question. This general section will be complemented with sample protocols for two localized, inducible, crosslinking methods that the authors consider to be particularly useful for the analysis of large multiprotein complexes.

Crosslinking Chemistries

Amine-reactive crosslinkers

Lysine residues are the amino acids that are most frequently targeted by chemical crosslinking. They are present in large numbers on the surfaces of most proteins, and the ε-NH_2 side groups of lysine residues react efficiently under physiological conditions with various crosslinking reagents to yield amide or amine bonds. Typical lysine-reactive crosslinkers are N-hydroxysuccinimide (NHS)-esters and imidoesters (Fig. 17.10.1).

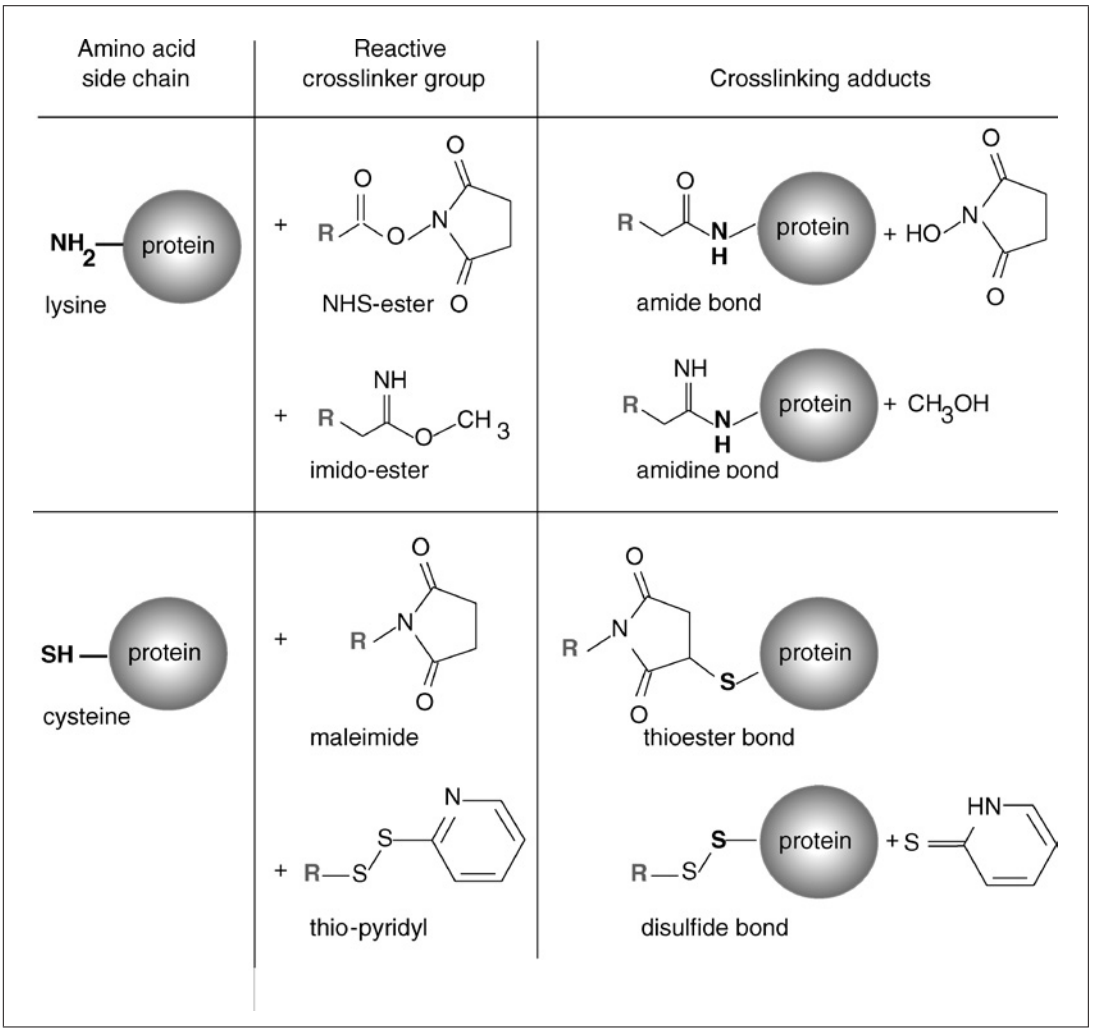


Figure 17.10.1 Crosslinker groups that react with lysine or cysteine residues on proteins of interest and the resulting crosslinking adducts. R is the nonreactive moiety of the crosslinker.

Sulfhydryl-, carboxyl- and carbohydrate-reactive crosslinkers

The second most frequently targeted groups are sulfhydryl groups on cysteine residues. Cysteine is present less frequently on the surfaces of proteins but is often introduced site-specifically into proteins by in vitro mutagenesis. Commonly used sulfhydryl-reactive crosslinkers are maleimides, which form thioester bonds with cysteines, and thiopyridyl compounds, which undergo disulfide exchange reactions (Fig. 17.10.1). Thiopyridyl-protein crosslinking can be reversed by using reductants like dithiothreitol (DTT). Other groups targeted in proteins include carboxyl groups and the carbohydrate moieties of glycoproteins (see the Pierce Web site at *http://www.perbio.com/upload/Literature_1601191%20XLinking%20handbook.pdf* for an excellent overview on different crosslinkers and crosslinking chemistries).

Photoactivatable crosslinkers

Amine- and sulfhydryl-reactive crosslinkers must be chemically relatively stable in an aqueous environment. This stability, however, comes at the price of a relatively low chemical reactivity towards their target groups. Therefore, these reagents need to be incubated with proteins for periods of 30 min to more than 1 hr. In contrast, photoactivatable crosslinkers remain largely nonreactive until induced by exposure to UV light to undergo photolysis, after which they require a much shorter crosslinking time. The most commonly employed photoactivatable groups are aryl azides, which upon photolysis form highly reactive, short-lived nitrenes (see Fig. 17.10.2A). These activated species

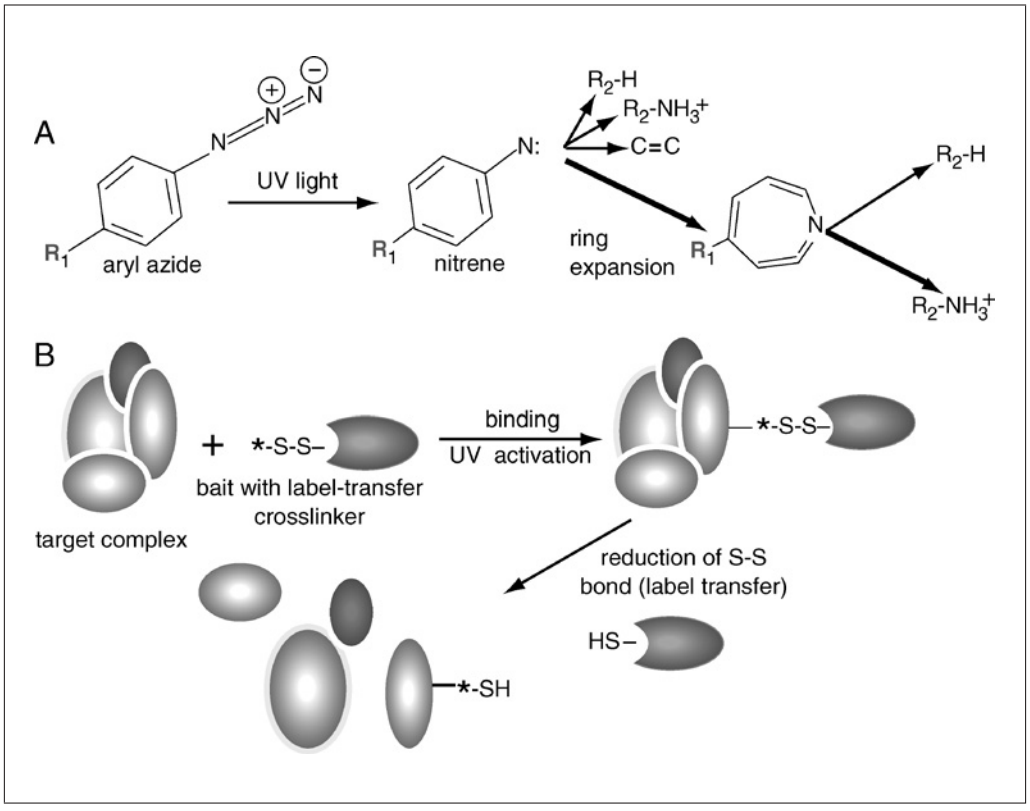


Figure 17.10.2 Photoactivatable crosslinking and label transfer. (**A**) Aryl azide photoactivation and formation of crosslinking adducts. UV illumination of aryl azides triggers photolysis to form short-lived nitrenes that either directly, or after ring expansion, attack chemical bonds in target proteins. The most frequently attacked groups are primary amines (thick arrows). R_1, nonreactive moiety of the crosslinker; R_2, target protein. (**B**) Label-transfer crosslinking. A labeled (∗) photoactivatable crosslinker with a cleavable disulfide (S-S) bond is first covalently attached to a bait protein that is subsequently incubated with a target complex. UV illumination triggers crosslinking with directly interacting target subunits. Reducing agents cleave the disulfide bond, thereby transferring the label to the target polypeptide.

can insert nonspecifically into chemical bonds, initiate addition reactions into double bonds, or react with nucleophiles like primary amines. Aryl azide–containing crosslinkers are therefore not limited to only one specific side group that may not be available at an appropriate distance on the surfaces of interacting proteins, and they require crosslinking times of only 1 to 15 min.

Disulfide crosslinking

Exposure of proteins to mildly oxidative conditions can trigger the reversible formation of nonphysiological disulfide bonds between appropriately spaced cysteine residues. Disulfide bond formation has been exploited in combination with the insertion of site-specific cysteine substitution mutants to map domain interactions within a protein (e.g., Cai et al., 2003) or between proteins (e.g., Bonner et al., 2000; van der Sluis et al., 2002).

Metal-catalyzed oxidative crosslinkers

In the presence of transition metals complexed with strong donor ligands, relatively mild oxidative conditions can lead to the formation of highly reactive, short-lived radicals in aromatic or sulfhydryl side chains of proteins. These in turn efficiently form covalent bonds with interacting proteins (Kodadek et al., 2005). Reactions are thought to proceed via oxidation of metals to highly electrophilic, high-valency species that subtract electrons from nearby aromatic side chains. Proton release then generates radicals that rapidly form adducts with nearby oxidizable amino acids or the peptide backbone (Fig. 17.10.3; Melcher, 2004; Kodadek et al., 2005).

This principle is exploited in localizing a crosslinking reaction to a bait protein that has been genetically tagged with a sequence of six histidine residues (His6; Fancy et al., 1996) or an N-terminal Gly-Gly-His sequence (GGH; Brown et al., 1998). Ni(II) complexed by a His6 or GGH tag can be oxidized by magnesium monoperphtalate (MMPP) to Ni(III) to induce formation of highly reactive radicals within the tagged protein. In contrast to heterobifunctional localized crosslinkers, bait proteins do not have to be isolated and chemically modified, but instead can be expressed under physiological conditions and allowed to assemble into native complexes in vivo.

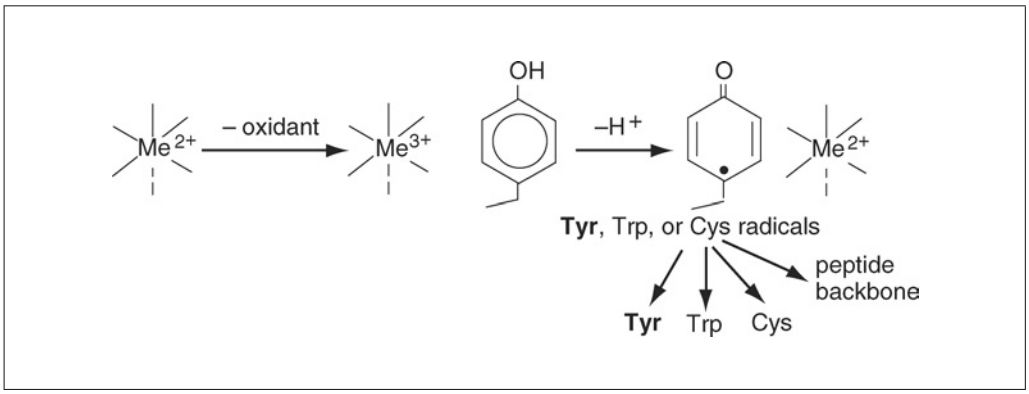


Figure 17.10.3 Oxidative crosslinking and formation of crosslinking adducts. Complexed transition metal ions (Me^{2+}), e.g., Ni^{2+} chelated by hexahistidine (His6) and Ru^{2+} chelated by tris(2,2′-bipyridyl), can be oxidized to highly electrophilic Me^{3+} species that subtract electrons from oxidizable amino acid side chains (e.g., Tyr, Trp, Cys) within the tagged protein. Tyrosine (**Tyr**) is the amino acid side chain shown in the figure and the most efficient electron donor for His6-mediated crosslinking. Subsequent proton release generates highly reactive radicals that can form adducts with susceptible amino acid residues in side chain or backbone components of the target protein.

Another application of oxidative crosslinking is nontargeted, photo-induced crosslinking. Visible light irradiation of proteins in the presence of tris(2,2′-bipyridyl)ruthenium(II) ions [Ru(II)$(bpy_2)_3^{2+}Cl_2$] and ammonium persulfate can efficiently crosslink proteins within less than a second (Fancy and Kodadek, 1999). The remarkable speed of this reaction minimizes the chances of false positive interactions (see False Positive and False Negative Results) and makes this method suitable for kinetic analyses of protein-protein interactions.

Organization of Reactive Groups

Homobifunctional and heterobifunctional crosslinkers

Most crosslinkers are bifunctional, i.e., they posses two reactive groups separated by a spacer. Homofunctional crosslinkers contain identical reactive groups while heterofunctional crosslinkers consist of reactive groups with specificities towards different amino acid side chains (most often lysine and cysteine; see Fig. 17.10.4 for examples). Spacer lengths in both homo- and heterobifunctional crosslinkers typically range from 4 Å to >20 Å. Without prior knowledge of the distance between reactive amino acid side chains in proteins of interest, it is important to employ a series of crosslinkers with different spacer lengths.

Bait-localized heterobifunctional crosslinkers

Instead of being distributed randomly over the surface of a bait protein, heterobifunctional crosslinkers can be localized to a specific amino acid side chain of the bait. Crosslinkers like N-succinimidyl 3-(2-pyridylthio)propionate (SPDP; Fig. 17.10.4), which has one cysteine-reactive and one lysine reactive group, can first be attached under defined conditions to cysteine residues (ideally a single cysteine engineered into an appropriate location on the protein) of an isolated bait protein. After removal of excess crosslinker, the activated adduct can be incubated with other proteins to determine interactions by crosslinking with accessible surface lysine residues on the other proteins. This approach minimizes the risk of inactivating the bait protein through excessive modification and

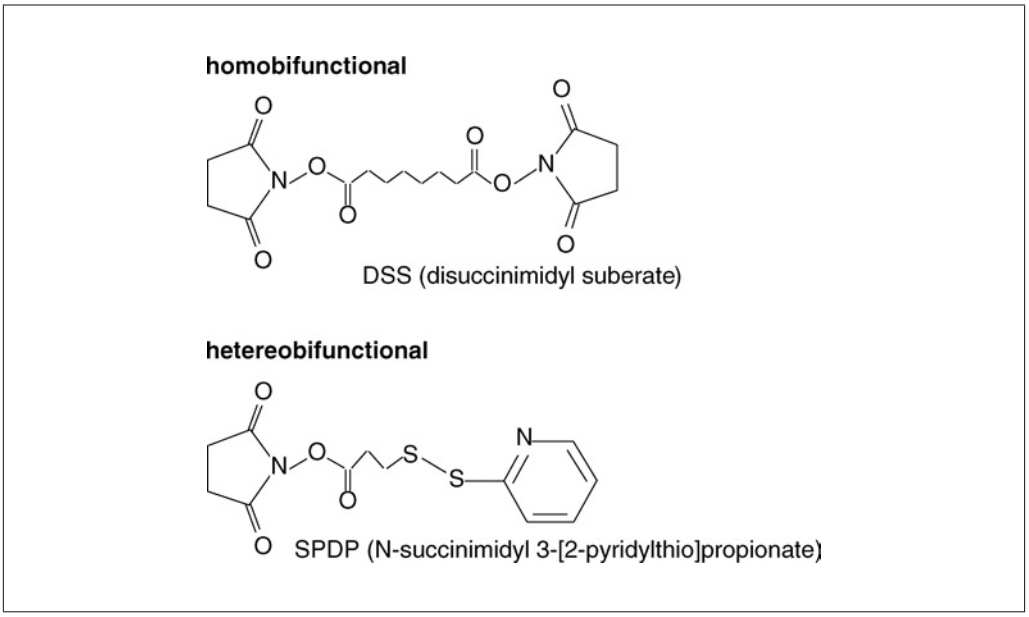


Figure 17.10.4 Structures of the lysine-targeting homobifunctional crosslinker disuccinimidyl suberate (DSS) and the heterobifunctional crosslinker *N*-succinimidyl 3-(2-pyridylthio)propionate (SPDP). SPDP consists of a lysine-targeting NHS group that is separated by a cleavable disulfide bond from a cysteine-targeting pyridyldisulfide group.

localizes the crosslinker the bait protein. Localization reduces the degree of formation of difficult-to-analyze high-molecular-weight multimeric adducts and minimizes the number of chemically distinct crosslinking adducts between the same two polypeptides, which could complicate subsequent analysis by mass spectrometry or immunodetection (Rappsilber et al., 2000). It also greatly facilitates analysis of individual crosslinking adducts (see Detection of Crosslinking Adducts and Fig. 17.10.5).

Label-transfer reagents

A photoactivatable label-transfer crosslinker consists of a labeled photoactivatable group that is separated from a sulfhydryl- or amide-coupling group by a cleavable disulfide bond. These crosslinkers are first attached to isolated bait proteins that are subsequently incubated with interacting proteins to form covalent protein-bait adducts upon exposure to UV light. The disulfide bond within the crosslinker can then be cleaved by addition of reducing agents to result in transfer of the label from the bait protein to the interacting protein (see Fig. 17.10.2B and Fig. 17.10.8). Photoactivation can lead to significant nonspecific protein labeling due to label release during photoactivation, a risk that is reduced by using heterotrifunctional crosslinkers. These reagents consist of a sulfhydryl- or amide-coupling group, a labeled group, and a photoactivatable group; the physical separation from a highly reactive photoactivated group stabilizes the labeled group.

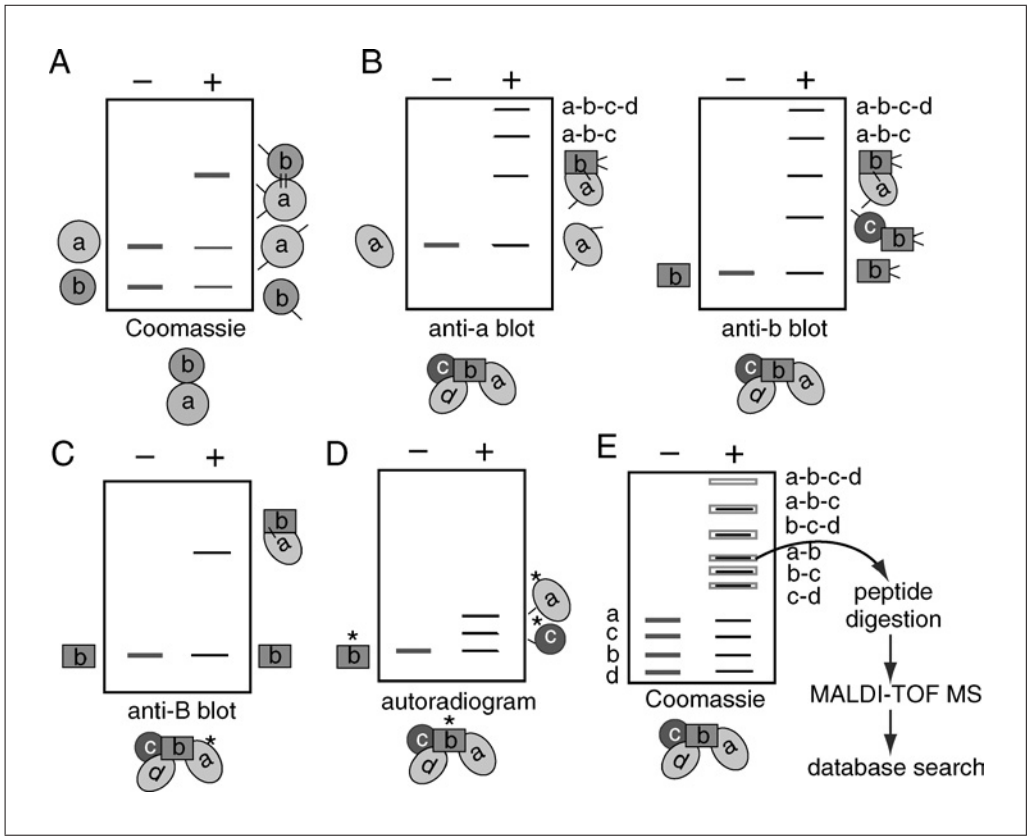


Figure 17.10.5 Cartoon comparing different methods for detection and analysis of crosslinking adducts. A protein complex consisting of subunits (a, b, c, and d; panel A contains only two subunits, a and b) is subjected to crosslinking. Reactions are separated by SDS-PAGE and analyzed by Coomassie staining (**A**), immunoblotting (**B**) and (**C**), autoradiography (**D**), and MALDI-TOF MS (**E**). Samples have either been treated with diffusible crosslinkers (panels A, B, and E), with localized crosslinkers (panel C), or with-label transfer crosslinkers (panel D). Please note that bifunctional crosslinkers (depicted by thin bars) can either covalently connect two amino acid side chains within or between subunits or modify a single amino acid chain. See text for further details. Abbreviation: $*$, radioactive label; $-$, without crosslinking; $+$, with crosslinking.

Labels can be radioactive ^{125}I (see Basic Protocol 2 and Fig. 17.10.8), fluorophores, or biotin. Recently, a variant of localized oxidative crosslinking has been developed for biotin labeling of proteins that interact with His6- or GGH-tagged baits (Amini et al., 2002); it allows localized labeling reactions within native, in vivo–assembled protein complexes.

Detection of Crosslinking Adducts

Coomassie or silver staining

Crosslinking reaction products are usually separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*). If crosslinking is performed with purified homomultimeric or heterodimeric protein complexes, separated proteins and protein adducts can simply be stained with Coomassie brilliant blue or silver (*UNIT 6.6*) and distinguished by their electrophoretic mobilities. Crosslinking will result in reduced band intensities for the monomeric subunits and the corresponding appearance of covalently linked crosslinking adducts (see Fig. 17.10.5A). These adducts typically (but not always) migrate close to their calculated molecular weight positions.

Immunodetection

The composition of crosslinking adducts can be confirmed by immunoblotting (*UNIT 6.2*). While immunoblotting obviously requires the availability of antibodies against the subunits of the protein complex under analysis, it does have the advantage of allowing performance of crosslinking reactions with more complex mixtures of proteins. In the example of the heterotetrameric complex in Figure 17.10.5B, crosslinking would generate three new bands recognized by an antibody against subunit a. The sizes of two of these bands correspond to higher-order crosslinking adducts (i.e., adducts that consist of more than two subunits and that are therefore more difficult to analyze) while the mobility of one of the bands would be consistent with a dimeric a-b crosslinking product. This assumption can be further substantiated by reprobing the blot with an antibody against subunit b, which needs to bind and detect the same position on the blot. However, it is important to realize that the same position could potentially represent two fortuitously co-migrating crosslinking adducts (e.g., a-*x* and b-*y*) rather than a genuine a-b heterodimer. This lack of resolution will be strongly aggravated with increasing subunit complexity. Thus immunodetection in reactions with standard nonlocalized crosslinkers is in most instances not suitable for the mapping of large multiprotein complexes.

In contrast, immunodetection can unambiguously determine the composition of dimeric crosslinking adducts in instances where crosslinking is strictly localized to a single modified subunit: these adducts must contain both the modified subunit as well as the immunologically detected subunit (Fig. 17.10.5C).

Radioactive, fluorescent, and chemiluminescent labeling

Label-transfer crosslinking allows the identification of labeled interacting proteins (rather than adducts) by size (Fig. 17.10.5D), or, often in combination with affinity purification, by mass spectrometry (see below). Labeled species within SDS-PAGE-separated crosslinking reactions are visualized by autoradiography, fluorography, or by streptavidin horseradish peroxidase–catalyzed chemiluminescence. Detection by size requires crosslinking reactions with limited complexity for unambiguous identification. The bait modified with the labeled crosslinker is typically the most strongly labeled protein in the reaction. Target proteins should therefore clearly differ in size both from the bait protein as well as from other subunits (i.e., other potential targets) of the protein complex under investigation.

Mass spectrometry

The most attractive method for determining the composition of crosslinking adducts is the use of mass spectrometry (MS), particularly MALDI-TOF MS. MS can unambiguously identify proteins in adducts separated by SDS-PAGE (*UNIT 6.1*) or by liquid chromatography without the need of highly specific antibodies (which may not be available) or epitope tags introduced into target proteins (which may interfere with protein function). Also, MS usually allows the unbiased identification of polypeptides in adducts without prior knowledge of their involvement, and it even has the potential to map crosslink sites within adducts of low-complexity crosslinking reactions (e.g., intramolecular crosslinking or homo- or hetero-dimeric crosslinking reactions).

Purified complexes of low to medium complexity can be directly crosslinked, fractionated by SDS-PAGE (*UNIT 6.1*), and stained with Coomassie blue or silver (*UNIT 6.6*). Protein bands produced as a result of crosslinking can be excised from the gel and their compositions determined by peptidase digestion and MS analysis of the resulting peptide mixture (Fig. 17.10.5E). This approach has been successful for the analysis of several dimeric protein complexes and also for a few multimeric complexes, employing either several different bifunctional crosslinkers (Rappsilber et al., 2000) or metal-catalyzed oxidative crosslinking (Denison and Kodadek, 2004). The major limitation of this approach is its dependence on clearly separated dimeric crosslinking adducts. Large multimeric complexes form a multitude of crosslinking adducts, resulting in excised bands that contain more than two proteins (Denison and Kodadek, 2004). This problem is further aggravated by the formation of different crosslinking adducts between the same two polypeptides, which migrate differently due to variations in protein modifications (Denison and Kodadek, 2004).

False Positive and False Negative Results

False negative results

The occurrence of crosslinking depends on a suitable position and distance of reactive groups on the surfaces of two interacting polypeptides. Interactions between proteins that lack such correctly spaced groups will not be identified (or "trapped") by crosslinking, resulting in reactions that erroneously suggest a lack of interaction (false negative interaction). This risk is particularly high for sulfhydryl- and amide-reactive bifunctional crosslinkers with defined spacer lengths; therefore, it is imperative to evaluate a variety of different crosslinkers both in terms of crosslinking chemistry as well as spacer length (Rappsilber et al., 2000). Spacer lengths are much less of a concern for photoactivatable aryl azides, which depend on chemistries with very broad substrate specificities, or for oxidative crosslinking reagents, which function without spacers and which also have relaxed substrate specificity. Still, it is important to realize that photoactivatable and oxidative reagents are also biased towards particular amino acid side chains and will therefore trap protein-protein interactions with variable efficiencies.

False positive results

The occurrence of false positive results is more difficult to understand than that of false negative results. False positive results are defined as the formation of crosslinking adducts between polypeptides that, under physiological conditions, do not interact with each other. In the simplest case, they are due to random collisions between two polypeptides in solution, particularly during extended incubation at high concentrations of polypeptides and crosslinkers. This background can usually be eliminated by titration of crosslinkers and bait and target proteins as well as by incorporation of appropriate controls (e.g., reactions with noninteracting variants or proteins). In the case of stable multiprotein complexes crosslinked using bifunctional reagents with long spacers, false positive results

can also be due to crosslinking of subunits that are close to each other in space, but not in direct contact. In this case it may be necessary to test identified polypeptides in isolation for direct interaction by methods such as GST pull-downs or co-immunoprecipitation assays. However, these methods will not be able to confirm interactions that exclusively occur in the context of an intact physiological multiprotein complex.

However, the most serious concern is the alteration of protein-protein interactions due to protein modifications during crosslinking reactions. Incubation of protein complexes with nonlocalized crosslinkers typically results in extensive protein modifications, as well as intramolecular crosslinking, before crosslinkers covalently connect two interacting polypeptides. Modifications by amide-reactive crosslinkers can be particularly disruptive because they neutralize the positive charge of lysine residues, which often serve as important determinants of interaction specificities.

Modifications by sulfhydryl-reactive crosslinkers may be less critical for protein-protein interactions because cysteine residues are less frequently directly involved in protein-protein interactions. However, cysteines often have catalytic roles, or they can stabilize structural domains; therefore, modifications to cysteine residues can inactivate proteins and disrupt subunit-subunit interactions, causing subunit dissociations or rearrangements within complexes. This in turn can result in the crosslinking of subunits that, under physiological conditions, would not touch each other, and it is very likely the reason for the high rates of false positives when multiprotein complexes are analyzed using standard nonlocalized bifunctional crosslinkers (Edwards et al., 2002; Denison and Kodadek, 2004).

Use of localized bifunctional or trifunctional crosslinkers largely limits the risk for disruptive modifications to the coupling reaction of the crosslinker to the bait protein. That is because the subsequent step, binding of bait-localized crosslinkers to target proteins, directly results in covalent bait-target coupling. In other words, any modification of target proteins would occur as a consequence of bait-target crosslinking rather than preceding it. The risk of inactivating modifications occurring during the initial bait-crosslinker coupling can be minimized by generating a panel of bait protein variants by in vitro mutagenesis, with each variant having a single cysteine residue at a nonessential amino acid position. Sulfhydryl-targeted conjugation of the crosslinker to the bait protein will then result in an array of bait proteins with crosslinkers localized at different single positions (e.g., Brown et al., 2001; Chen and Hahn, 2003; Fishburn et al., 2005; see also Basic Protocol 2).

While oxidative crosslinking conditions can also modify proteins (e.g., by inducing the formation of nonphysiological disulfide bonds), ultra-fast photoinduced oxidative crosslinking appears to cause very few, if any, false positive crosslinking interactions (Denison and Kodadek, 2004).

Crosslinking Reactions Involving Large Multiprotein Complexes

Analyses of large multiprotein complexes are the most demanding crosslinking applications. Reactions should be fast, lack extensive modifications of target proteins, and allow the unambiguous identification of the composition of crosslinking adducts. Photoactivatable label-transfer crosslinking, particularly in combination with affinity purification and mass spectrometry, is probably the most appropriate and most established method for determining interactions between an isolated bait protein and intact protein complexes. Basic Protocol 2 provides an example of this method.

Label-transfer crosslinking is less suited for the analysis of subunit interactions within stable complexes because bait proteins are derivatized in vitro and hence cannot assemble in vivo into native complexes. Analysis of inter-subunit interactions is therefore best

performed using localized crosslinking that involves genetically introduced tags (His6- or GGH-mediated crosslinking) or nonlocalized crosslinking in combination with mass spectrometry. Currently the best method using nonlocalized crosslinking appears to be photo-induced metal-catalyzed oxidative crosslinking because of its fast reaction time, very high reactivity, and low rate of false positive interactions (Denison and Kodadek, 2004), although nonlocalized crosslinking/MS has also been successful in combination with standard bifunctional crosslinkers (Rappsilber et al., 2000). His6-mediated localized crosslinking overcomes the limitations on the analysis and separation of very complex mixtures of crosslinking adducts; however, experience with His6-mediated crosslinking is limited and it has so far not been used in combination with mass spectrometry. Detailed protocols for photo-induced metal-catalyzed oxidative crosslinking have been published recently (Fancy and Kodadek, 1999; Kluger and Alagic, 2004), and Basic Protocol 1 provides a sample laboratory protocol for His6-mediated nickel-catalyzed oxidative crosslinking.

BASIC PROTOCOL 1

HIS6-MEDIATED CROSSLINKING

The crosslinking method described in this protocol is based on the introduction of a Ni^{2+}-binding His6 tag into a bait protein. His6-Ni(II) complexes can be induced by addition of diffusible oxidizers, such as magnesium monoperphtalate (MMPP), to trigger electron subtraction and formation of highly reactive radicals in nearby oxidizable amino acid side chains, in particular tyrosine residues (see Fig. 17.10.3 and Strategic Planning, Crosslinking Chemistries). Baits can either be specific subunits of multiprotein complexes or polypeptides transiently interacting with multiprotein complexes. Since targets are identified immunologically, this protocol depends on either the availability of target-specific antibodies or the introduction of epitope tags into targets.

While a standard His6-tagged bait is sufficient for crosslinking studies, the use of composite tags allows for the purification or enrichment of multiprotein complexes prior to crosslinking and the analysis of crosslinking adducts by analytical proteolysis. This protocol employs a composite tag (His6-GST-TEVcs) consisting of His6 for crosslinking the bait with the complexing proteins; glutathione-*S*-transferase (GST) for immobilizing the bait to a glutathione-Sepharose bead and enriching the interacting protein complexes by GST pull-down and one- or two-step affinity enrichment (along with His6; see step 17); and a tobacco etch virus protease cleavage site (TEVcs) to enhance crosslinking efficiency (see step 26) and allow proteolytic tag removal to verify presence of the bait in crosslinking adducts (see step 33; Fig. 17.10.7).

This protocol has been developed to identify subunits of large, multiprotein coactivator complexes that directly interact with transcriptional activator proteins. Some of the protocol steps may have to be modified for use with other proteins and in other organisms. Because targets are identified immunologically, this protocol depends on either the availability of target-specific antibodies or the introduction of epitope tags into targets.

Materials

Saccharomyces cerevisiae cultures: source of multiprotein complexes, either wild-type cells or cells in which putative target polypeptides are genomically epitope-tagged (see Klein et al., 2003)
YEPD medium (see recipe)
Extract buffer (see recipe)
0.5-mm glass beads, (e.g., Scientific Instruments), prechilled to −20°C
Siliconized glass wool, sterile (see recipe)

Ammonium sulfate, finely ground with mortar and pestle or with coffee grinder
1 M KOH
Dialysis buffer (see recipe)
Glutathione-Sepharose immobilized proteins (see recipe) in A50 buffer (see recipe)
  ~80 μg His6-GST-TEVcs-bait protein
  ~20 μg His6-GST-TEVcs (control)
A50 buffer (see recipe)
Wash buffer: A50 buffer (see recipe) without DTT
4× and 2× SDS sample buffer (see recipe)
12 mM nickel acetate
12 mM magnesium monoperphtalate (MMPP; Aldrich): prepare fresh just before use
TEV protease cleavage buffer (see recipe)
TEV protease (Invitrogen or USB)

Visible light spectrophotometer
50-ml centrifuge tube
Refrigerated centrifuge
30-ml round-bottom Corex tube
10-ml syringe
Ultracentrifuge (e.g., Beckman 70.1Ti) and corresponding ~10-ml ultracentrifuge tubes, precooled to 4°C
25-ml beakers
Magnetic stir plate and stir bars
Dialysis tubing or dialysis cassettes (10,000 to 12,000 kDa MWCO; e.g. Slide-A-Lyzer cassettes from Pierce)
0.5-ml microcentrifuge tubes
Nutator

Additional reagents and equipment for determining protein concentration (*APPENDIX 3H*), performing SDS-PAGE (*UNIT 6.1*), and detecting proteins by immunoblotting (*UNIT 6.2*)

Grow and harvest yeast

1. Grow *Saccharomyces cerevisiae* in 800 ml of YEPD medium at 30°C, with shaking at 200 to 250 rpm to ~1 OD_{600}.

   Preparation of extracts is based on Wootner et al., 1991.

2. Centrifuge cells 6 min at 6000 × *g*, 4°C. Discard the supernatant and resuspend cells in ~20 ml cold water. Transfer cells to a preweighed 50-ml centrifuge tube on ice, and centrifuge 4 min at 3000 × *g*, 4°C.

3. Decant the supernatant and weigh the tube containing the cells. Subtract the weight of the empty tube to determine the weight of the cells. Resuspend the cells in ~1 ml/g cells of cold extract buffer and transfer to a 30-ml Corex tube on ice.

   Alternatively, cell pellets may be frozen and stored up to several months at –80°C.

   Round-bottom glass Corex tubes are preferred because they work well for cell breakage using the glass bead methods describe in steps 4 and 5, but other round-bottom tubes may be used as well.

Lyse cells

4. Add 0.5-mm prechilled glass beads to the cell suspension to a final fill height of about two-thirds of the suspension.

5. Vortex at least ten times for 30 sec each at full speed, with 30 sec cooling on ice between each vortex cycle.

   Hold tubes as close to the top as possible to achieve maximal vortex amplitude. Instead of a vortex, yeast cells can be efficiently broken by the glass bead method using a specialized bead beater (e.g., BioSpec) following the manufacturer's recommendations.

Collect whole-cell lysate

6. For each extract plug the end (where the needle is inserted) of one 10-ml syringe with a small amount of sterile, siliconized glass wool.

7. Separate extract from glass beads by pouring the suspension into the glass wool–plugged syringe and pressing out the extract with the plunger directly into a precooled ultracentrifuge tube.

8. Wash the remaining glass beads with 1 to 2 ml of additional extract buffer, pass through syringe, and combine with the first pass-through from step 7.

9. Adjust the extract buffer to 4.5 ml/g cell pellet and centrifuge 75 min at 300,000 × *g*, 4°C.

Precipitate proteins

10. Carefully pipet supernatants in to 25-ml beakers on ice. Record volume, add stir bars, and stir on ice.

    Stirring on ice can be done most conveniently by placing an ice bucket with beaker directly onto a magnetic stir plate. Alternatively, ammonium sulfate precipitation can be done in a cold room.

11. Add 0.337 g finely ground ammonium sulfate powder per milliliter of supernatant slowly spaced equally over the course of 1 hr. Also add a total of 10 µl of 1 M KOH per gram ammonium sulfate in six aliquots every 10 min. Stir for an additional 30 min on ice.

    To avoid high local concentrations of ammonium sulfate, it is important to add ammonium sulfate continuously in as small amounts as possible over the whole period of one hour.

12. Transfer the mix to 10-ml centrifuge tubes on ice and centrifuge 30 min at 20,000 × *g*, 4°C.

Dialyze precipitated proteins

13. Completely remove supernatant including the lipid phase with a pipet and resuspend the pellet in 1 ml dialysis buffer.

14. Thoroughly rinse and equilibrate dialysis tubing or casettes for at least 5 min in dialysis buffer.

15. Transfer the resuspended pellet to dialysis tubing or casettes and dialyze for at least two 2-hr changes against 1 liter dialysis buffer at 4°C.

16. Remove dialysate from dialysis tubing or casettes. Use a small amount to determine protein concentration (*APPENDIX 3H*) and store whole cell extract (WCE; ~1.5 ml) in 100-µl aliquots up to 1 year at –80°C.

    The dialysate is the whole cell extract that is used as a source of target protein complexes for crosslinking. Typical yield: ~ 400 mg total protein.

Enrich bait-interacting complexes

17. Place 500 µl glutathione-Sepharose immobilized proteins in A50 buffer into each of five 0.5-ml microcentrifuge tubes on ice: one control tube containing ~20 µg of

His6-GST-TEVcs and four reaction tubes each containing ~20 μg of the His6-GST-TEVcs-bait protein.

The required amount of glutathione-Sepharose-immobilized protein depends on the affinity of the bait-target interaction and may need to be optimized for a particular target protein. The amount of immobilized bait protein used in this protocol is suitable for interactions with a dissociation constant, K_D, in the range of 10^{-7} M. An interaction with a ten times lower K_D would require an approximately ten times lower amount of bait protein.

Total bead volume should be sufficiently large to minimize the chance of bead losses during washes, but low enough to minimize unspecific binding or precipitation. A total volume of 5 to 10 μl packed beads per reaction generally works well. If protein concentrations vary, add free glutathione-Sepharose beads to samples with high concentration of immobilized protein until all samples contain about the same volume of total beads.

Note that the efficiency of the enrichment of bait-target protein complexes can be increased by a two-step procedure, in which complexes are first selectively bound to Ni-agarose and eluted with imidazole prior to the GST pull-down (see Melcher, 2000). This additional step is not required if crosslinking adducts are analyzed by immunoblotting (as described in this protocol), but would be important for analysis by mass spectrometry.

18. Centrifuge the glutathione-Sepharose immobilized protein suspensions 30 sec at 16,000 × *g*, 4°C, and discard supernatants.

19. Add 200 μl cold A50 buffer plus whole cell extract (WCE; from step 16) corresponding to ~0.5 mg total protein to the glutathione-Sepharose immobilized protein pellet. Keep an aliquot of the WCE as input control.

20. Nutate at 4°C for 1 hr.

    Ideally it is best to keep the reaction cold at all times. However, the authors use a nonrefrigerated microcentrifuge for the remaining centrifugations, immediately removing the tubes from the centrifuge after the short centrifugation step and placing them on ice. This way the samples are kept sufficiently cold without the need for a refrigerated microcentrifuge.

21. Centrifuge 20 sec at 10,000 × *g*, 4°C, and carefully remove supernatants with a pipettor.

22. Resuspend each bead pellet in 550 μl cold wash buffer, centrifuge 20 sec at 10,000 × *g*, 4°C, and carefully remove supernatants with a pipettor. Repeat this step two more times for a total of three washes.

23. Resuspend the pellets in 550 μl wash buffer. Remove 120 μl of each suspension and centrifuge 30 sec at 16,000 × *g*, 4°C. Remove supernatant and resuspend beads in 12 μl 2× SDS sample buffer.

    These reserved samples will serve as pull-down controls.

Crosslink bait and target proteins

24. Centrifuge the remaining bead suspension from step 23 for 30 sec at 16,000 × *g*. Remove supernatants (~430 μl each) with a pipettor and allow the beads to remain in a residual volume of 20 to 25 μl.

    To estimate a volume of 20 to 25 μl, compare to the volume of two reference tubes filled with 20 and 25 μl buffer, respectively.

25. Add 1.5 μl of 12 mM nickel acetate (final concentration: 0.6 to 0.8 mM Ni-acetate) and nutate 10 min at room temperature.

    The indicated amount of Ni-acetate should be sufficient to saturate Ni(II) complexation by His6 tags. However, if crosslinking efficiency is low, the concentration of the Ni-acetate solution may be titrated over a range of 4 to 60 mM.

26. Create the crosslinking and control reaction mixes by adding:

    a. 1.5 µl 12 mM MMPP (600 µM final concentration) to His6-GST-TEVcs reaction tube (control)
    b. 1.5 µl water to a His6-GST-TEVcs -bait protein reaction tube (mock reaction)
    c. 1.5 µl 4 mM MMPP to a His6-GST-TEVcs -bait protein reaction tube
    d. 1.5 µl 12 mM MMPP to a His6-GST-TEVcs -bait protein reaction tube
    e. 1.5 µl 40 mM MMPP to a His6-GST-TEVcs -bait protein reaction tube.

    The three reactions treated with different MMPP concentrations identify optimal crosslinking conditions. Once established, a single crosslinking reaction will be sufficient.

    The optimal amount of MMPP is critical for the success of the protocol. Because high concentrations of oxidant may destabilize protein complexes during crosslinking (see False positive results in the Strategic Planning section), it is important to use the minimal concentration of oxidant that allows efficient crosslinking. Complex integrity will be tested by analyzing both beads and buffer supernatants of crosslinking reactions as well as a mock reaction prior to denaturation (see step 23). In some cases, MMPP concentration may need to be tested over a broader range (100 µM to 10 mM final MMPP concentration).

    Crosslinking efficiency depends on the position of the His6 tag relative to an appropriately spaced oxidizable amino acid, such as tyrosine, and the central tyrosine residue in the cleavage site for TEV protease (ENLYFQG) has been shown to increase crosslinking yield in the context of the His6-GST-TEVcs composite tag (Fancy and Kodadek, 1998; Klein et al., 2003).

27. Nutate 5 min at room temperature.

28. Centrifuge 20 sec at 16,000 × *g*, 4°C. Add supernatants to 6 µl of 4× SDS sample buffer to use as a complex integrity control.

    If target protein dissociated from the complex during crosslinking, small amounts of target protein will appear in the supernatant of the crosslinking reaction, but not the supernatant of the mock reaction, in which case crosslinking conditions must be modified.

29. Divide each bead sample (suspended in the residual supernatant) into two aliquots.

30. Add 6 µl of 2× SDS sample buffer to one half of the beads crosslinking reaction.

    This aliquot will constitute the crosslinking reaction without TEV protease treatment.

Cleave crosslinking adducts

31. Resuspend the other half of the beads in 550 µl cold TEV protease cleavage buffer. Centrifuge 20 sec at 10,000 × *g*, 4°C or room temperature and carefully remove supernatants with pipettor.

32. Repeat step 31 two more times for a total of three wash steps.

    These steps will remove remaining traces of MMPP.

33. Resuspend washed beads in 10 µl cleavage buffer and add 1 µl TEV protease. Incubate 30 min at room temperature.

    TEV protease cleaves its recognition sequence with exquisitely high specificity under a variety of different incubation conditions (Dougherty et al., 1988; Polayes et al., 1994; http://mcl1.ncifcrf.gov/waugh_tech/faq/tev.pdf).

    Treatment with TEV protease is recommended as a control to confirm presence of the tagged bait protein in crosslinking adducts. Treating part of the crosslinking reaction with and part without TEV protease will generate crosslinking adducts that do or do not contain the relatively large (~27 kDa) His6-GST-TEVcs tags. These indicative size differences between treated and untreated crosslinking adducts will only occur if adducts contain the cleavable tag and therefore serve as direct evidence for the presence of the tagged bait protein (see Fig. 17.10.6 and 17.10.7C).

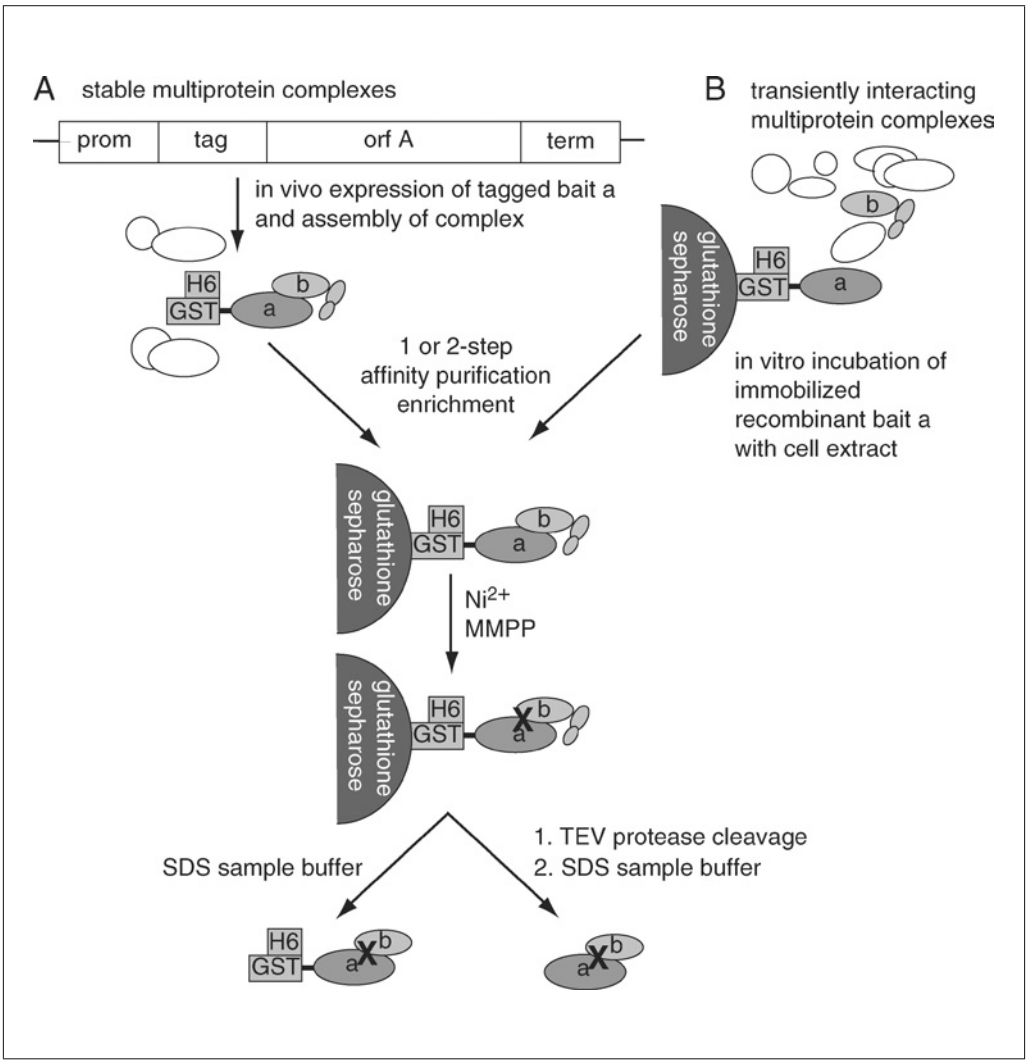


Figure 17.10.6 Cartoon depicting steps in isolating interacting proteins using a bait protein. (**A**) Baits that form subunits of stable protein complexes can be allowed to assemble in vivo prior to affinity purification of the bait-containing protein complex. (**B**) Baits that transiently interact with protein complexes can be first purified as glutathione Sepharose bead–immobilized recombinant bait proteins and allowed to interact in vitro with cell extracts. The bait (a) containing complexes are enriched or isolated by affinity purification, and the interacting protein (b) is crosslinked and analyzed (with or without removal of the His6-GST-TEVcs-tag) by SDS-PAGE. Abbreviations: H6, His6 tag; GST, glutathione-*S*-transferase tag; MMPP, magnesium monoperphtalate. Crosslinking is indicated by the large, bold X, and the cleavage site for TEV protease is indicated by a thick bar.

The authors produce their own recombinant TEV protease. Commercial suppliers of TEV protease include Invitrogen and USB.

34. Centrifuge 30 sec at 16,000 × *g*, 4°C. Add the supernatant to 6 µl of 4× SDS sample buffer.

Analyze samples

35. Heat all samples (from steps 30 and 34) 4 min at 95°C, separate by SDS-PAGE (*UNIT 6.1*), and detect proteins of interest (target proteins) by immunoblotting (*UNIT 6.2*) with appropriate antibodies.

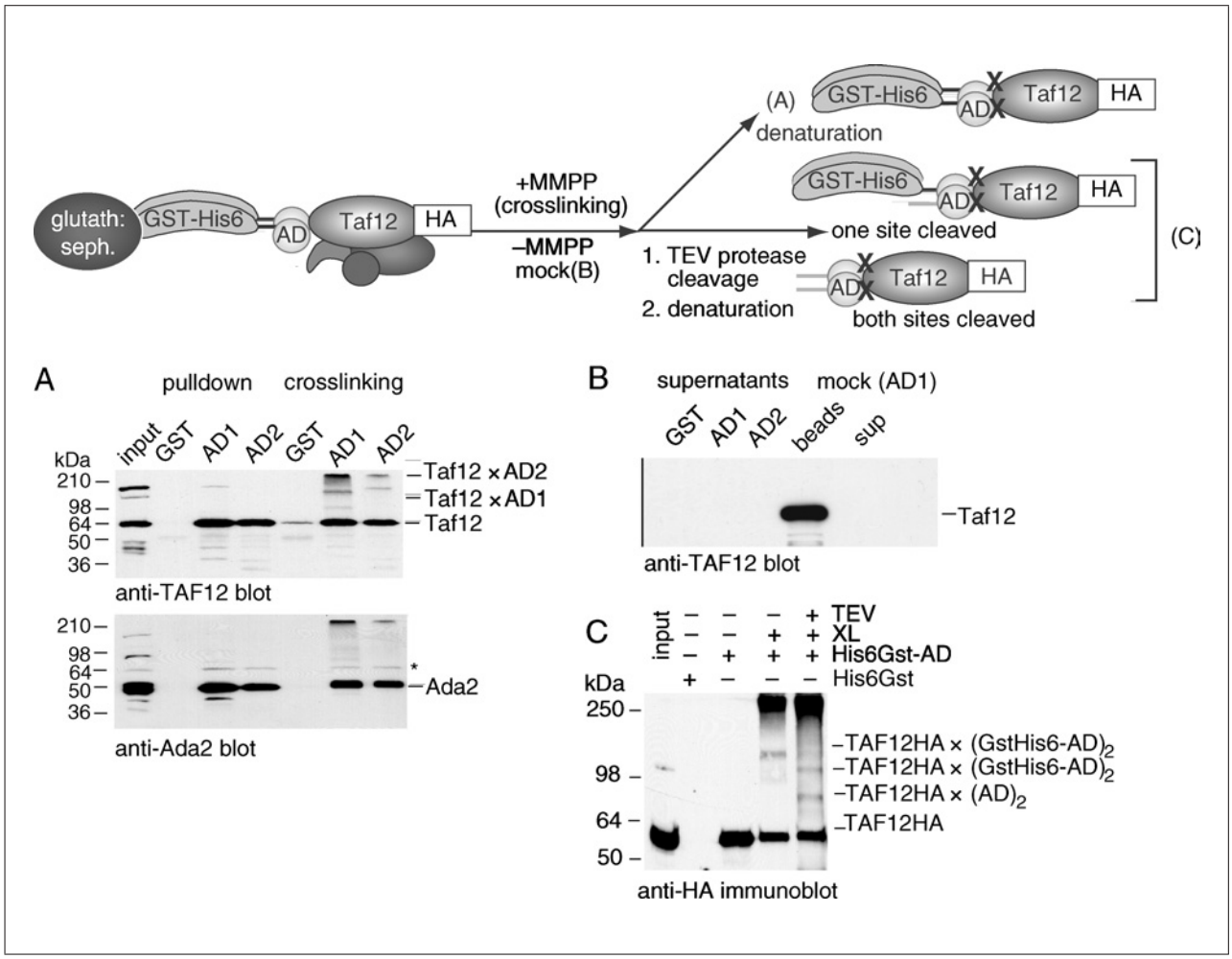


Figure 17.10.7 Crosslinking of two acidic activation domains (ADs) with Taf12, TAF12 is a subunit of both coactivators SAGA (the example used for the gels shown in this figure) and TFIID (the example illustrating Basic Protocol 2), and the complex depicted at the top of the figure can represent either. Immobilized His6-GST-TEVcs-AD fusion proteins of two different sizes (AD1 and AD2) were incubated with yeast whole cell extract. Native complexes bound to the bait proteins were crosslinked, and the crosslinking reaction mix was separated by SDS-PAGE. (**A**) Top: immunoblot with anti-Taf12 antibodies. A, bottom: the same blot stripped and reprobed with an antibody against a different SAGA subunit, Ada2. The asterisk (∗) indicates residual TAF12 signal after stripping. (**B**) Immunoblot of supernatants of the crosslinking reaction and a mock reaction. (**C**) Part of the crosslinking reaction was treated with TEV protease prior to denaturation and immunoblotting with anti-HA (heme agglutinin epitope tag) antibody. The cleavage site for TEV protease is indicated by a thick bar. Modified from Klein et al., 2003; see Klein et al., 2003 for details.

BASIC PROTOCOL 2

BAIT-LOCALIZED PHOTOACTIVATABLE CROSSLINKING

This protocol describes the bait-localized photoactivatable crosslinking (photocrosslinking) method suitable for elucidating the binding targets of a protein of interest within a transient multiprotein complex. The strategy for using a photocrosslinker is depicted in Figure 17.10.8. First, the heterobifunctional photocrosslinker, N-[(2-pyridyldithio)ethyl]-4-azidosalicylamide (PEAS; Chen et al., 1994, Ebright et al., 1996) is iodinated at its phenyl ring with radioactive iodine (^{125}I). Second, the radiolabeled photocrosslinker ^{125}I-PEAS is conjugated to a unique cysteine on the bait protein through a disulfide bridge. Third, the modified bait protein sample is added into an in vitro multiprotein complex formation assay to react with its binding target(s). Fourth, the resulting complex is irradiated with UV light of wavelength ~350 nm to activate covalent bond formation (crosslinking) between the azido group of the crosslinker and its nearest neighboring protein. Finally, the radiolabeled crosslinker is transferred to the crosslinked target(s) in

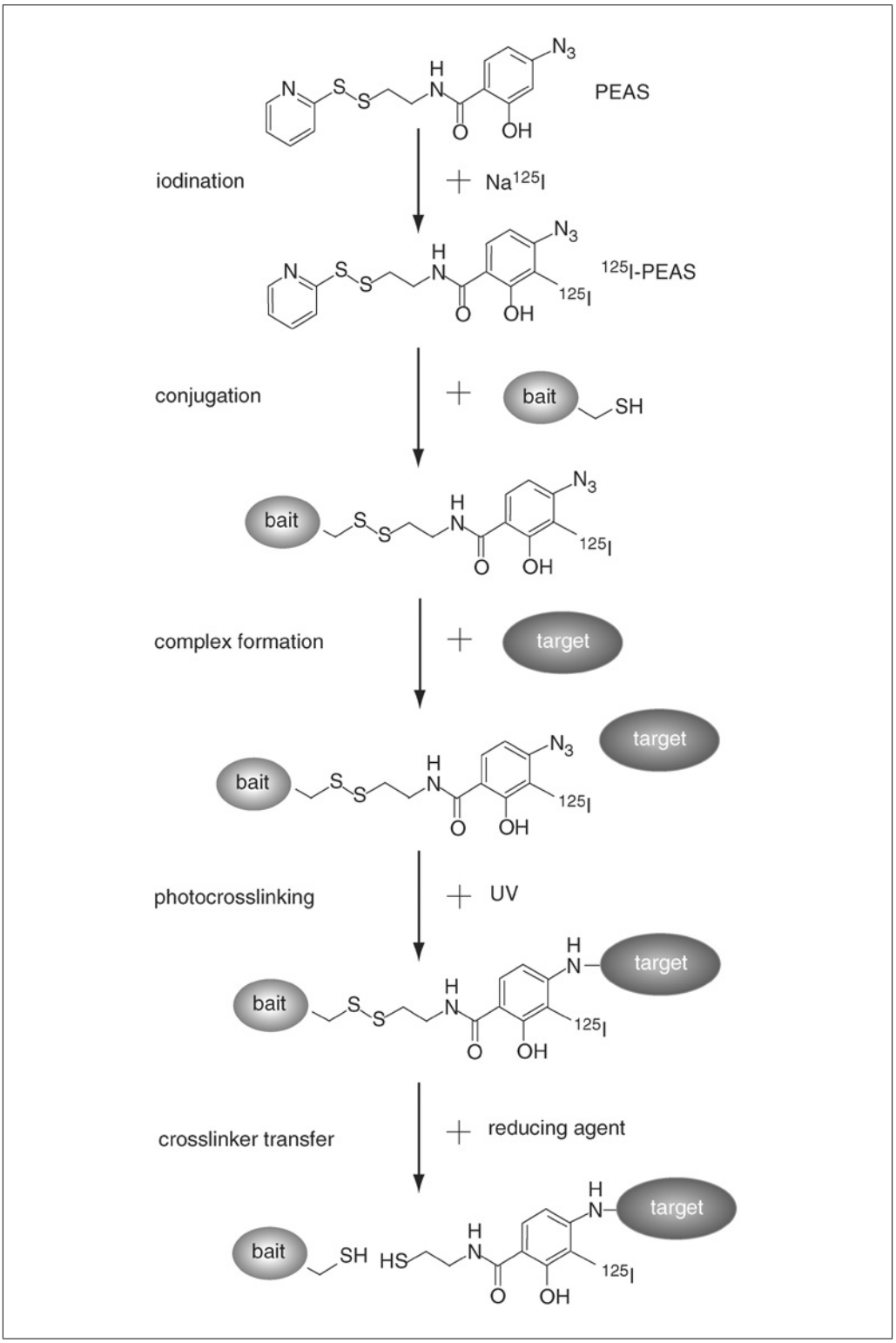


Figure 17.10.8 Flow chart demonstrating a strategy for the use of protein-protein photocrosslinking with radiolabeled bait protein to identify the protein binding target.

the presence of a reducing agent (i.e., DTT or 2-mercaptoethanol). The crosslinked proteins are analyzed by SDS-PAGE electrophoresis (*UNIT 6.1*) followed by autoradiography (*UNIT 6.3*).

Generally, all endogenous surface cysteines of the bait protein are genetically altered to non-cysteine residues and a unique cysteine residue is inserted on the protein surface of interest for incorporation of the photocrosslinker. The protein is bacterially expressed to allow chromatographic purification of a large quantity of the protein sample. In this protocol, general procedures are provided for iodination and conjugation reactions with reagents and parameters specific for conjugating an example protein, purified yeast transcription factor TFIIB. Because the conditions for assembling multiprotein complexes differ greatly in various cases and must be optimized for each protein, a detailed description of a specific protein complex formation assay is not practical for the purpose of this protocol; therefore, only a procedure for photocrosslinking on a preassembled multiprotein complex is described. This procedure uses the PEAS-conjugated TFIIB, yeast nuclear extract, and DNA template immobilized on magnetic beads to form the RNA polymerase II pre-initiation complex (PIC; see Chen and Hahn, 2003). Even this procedure and the parameters listed below will require certain modifications for adaptation in specific cases because the properties of proteins and the biological contexts differ in various multiprotein complexes.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

CAUTION: Use of the radioisotope ^{125}I requires appropriate shielding and special handling, including the use of a fume hood.

Materials

600 mM N-[(2-pyridyldithio)ethyl]-4-azidosalicylamide (PEAS) stock solution: prepare by dissolving the solid compound (Invitrogen) in DMSO; store up to 2 years at −70°C in an amber bottle
0.1 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*)
Na^{125}I in NaOH solution, pH 8.0-12.0 (e.g., Amersham Biosciences, specific activity >0.6 TBq/mg iodide, 15 Ci/mg iodide)
50 mM tyrosine in water
7.5 μM purified yeast transcription factor TFIIB: pass the purified protein sample through a desalting column (Chen and Hahn, 2003) to remove the reducing agent in the purification buffer and elute in TFIIB conjugation buffer (see recipe)
TFIIB conjugation buffer
Dry Ice
Multiprotein complex: e.g., RNA polymerase II pre-initiation complex (PIC) on beads formed using the immobilized template assay (Chen and Hahn, 2003)
Acetate transcription buffer (see recipe)
Reducing agent: dithiothreitol or 2-mercaptoethanol

IODO-GEN precoated iodination tube (Pierce)
Desalting column: NAP-5 Sephadex G-25 column (Amersham Biosciences)
Scintillation counter for gamma counting
−70°C freezer
Magnet
UV irradiation device: ∼350-nm wavelength
Additional reagents and equipment for performing SDS-PAGE (*UNIT 6.1*) and autoradiography (*UNIT 6.3*)

Radiolabel photocrosslinker with ^{125}I

1. Pipet 1 µl PEAS stock solution (in DMSO) into 0.6 ml 0.1 M sodium phosphate (pH 7.4) buffer to a final concentration of 1 mM in a 1.5-ml microcentrifuge tube.

   Ideally, the entire experimental process from this point to photocrosslinking is conducted in the dark or reduced lighting to prevent activation of crosslinker with residual UV irradiation from laboratory fluorescent light. However, it has also been reported that there is no difference between results obtained from experiments performed under normal laboratory lighting and in the dark (Zecherle et al. 1992).

2. Pipet ~24 µl of $Na^{125}I$ (10 mCi; 3.8 nmole), 36 µl PEAS (36 nmole), and 0.1 M sodium phosphate buffer, pH 7.4 to a total volume of 90 µl in an IODO-GEN pre-coated iodination tube. Incubate the solution 10 min at room temperature.

   CAUTION: *The labeling experiment should be conducted inside a chemical fume hood to prevent any vaporized radioactive iodine from getting into the laboratory space.*

   The protocol provided here is designed to prepare ^{125}I-PEAS for conjugating with multiple protein samples with a combined total quantity of 18 nmole if the molar ratio of crosslinker to protein in the subsequent conjugation reaction is 2; therefore, the amounts of $Na^{125}I$ and PEAS should be adjusted according to the total quantity of protein samples.

   At pH above 7, ^{125}I remains as iodide ion in aqueous solution. The amount of ^{125}I used in this reaction is substoichiometric (1/10 of PEAS). Lower amounts (≤5 mCi) of ^{125}I may also be used with satisfactory results.

   The use of the IODO-GEN pre-coated tube is to supply iodous ions ($^{125}I^+$), which is generated by oxidation of iodide ($^{125}I^-$) by IODO-GEN iodination reagent. The iodous ion can subsequently undergo electrophilic attack at the ortho position of the phenyl ring of the crosslinker.

3. Transfer the iodination solution to a 1.5-ml microcentrifuge tube containing 4 µl of 50 mM tyrosine to quench the iodination reaction.

   The radiolabeled photocrosslinker ^{125}I-PEAS is now ready for subsequent conjugation with the protein sample or can be temporarily stored on ice before conjugation with protein. Tyrosine is used to scavenge the unincorporated iodide that can later react with certain aromatic residues (tyrosine or histidine) of proteins. The extent of iodination can be assessed by TLC chromatography analysis (Zecherle et al., 1992). Because the amount of ^{125}I is substoichiometric and the iodination reaction is rapid, ^{125}I is likely to be completely incorporated into PEAS. Therefore, the use of tyrosine is optional. The concentration of the resulting ^{125}I-PEAS is 400 µM (400 pmole/µl).

Conjugate radiolabeled photocrosslinker to bait protein

4. *Recommended:* Pass the purified bait protein through a desalting column to remove the reducing agent in the purification buffer and elute it in TFIIB conjugation buffer.

   Alternatively, dialyze the purified bait protein.

5. Pipet 15 µl of ^{125}I-PEAS (from step 3) and 400 µl of the bait protein in TFIIB conjugation buffer into a 1.5-ml microcentrifuge tube for conjugation. Incubate the reaction mixture 30 min on ice or at room temperature.

   Ideally, the conjugation buffer is the same as the buffer used for protein purification except that the reducing agent is omitted. Alternatively, the conjugation could also be the same as the reaction buffer used in assembling the multiprotein complex.

   The conjugation procedure is designed for conjugating 15 µl of the ^{125}I-PEAS (400 pmole/µl) to a protein sample (i.e., TFIIB; see above) with the concentration of 7.5 µM (7.5 pmole/µl) and the sample volume of 400 µl; therefore, the molar ratio of the radiolabeled crosslinker to the protein is 2 in the reaction mixture.

A conjugation reaction at a higher temperature and/or with a longer incubation can provide higher conjugation efficiency; however, certain proteins could be destabilized with elevated temperature and prolonged incubation. These two reaction parameters should therefore be adjusted to achieve optimal conjugation efficiency. Due to the subsequent step for removal of the unincorporated ^{125}I-PEAS using the NAP-5 desalting column, the maximum reaction volume is limited to the maximal sample volume (i.e., 500 μl) of the desalting column.

6. Transfer the conjugation mixture to a desalting column, which is pre-equilibrated with 10 ml TFIIB conjugation buffer.

7. Elute the modified bait protein from the desalting column with 1000 μl TFIIB conjugation buffer. Measure radioactivity of γ radiation of a 100-μl aliquot in a scintillation counter.

   The modified protein sample is expected to have a concentration of 3 μM based on the assumption of 100% recovery of the protein sample. The sample should have a radioactivity of ~1 mCi (specific radioactivity 0.33 μCi/pmole).

8. Pipet 10 μl of the eluted modified bait protein to each of two 1.5-ml microcentrifuge tubes.

   These two samples will be analyzed by SDS-PAGE electrophoresis in step 14 in the presence and absence of the reducing agent to assess the formation of the disulfide linkage between the crosslinker and the cysteinyl SH group of the protein.

9. Dispense 0.2-ml aliquots of the modified bait protein sample into microcentrifuge tubes and freeze the tubes on dry ice. Store up to 6 months at −70°C.

Assemble and crosslink protein complex

10. Add 2.4 μl of modified bait protein (the conjugated TFIIB in this example) to 100 μl PIC multiprotein complex on beads in a 1.5-ml microcentrifuge tube and incubate for 40 min.

    Because the conditions for assembling protein complexes vary greatly, specific parameters and reagents vary for the protein assembly assay. For forming the PIC, see Chen and Hahn, 2003. Other examples for assembling multiprotein complexes and photocrosslinking can be found in Cai et al. (2001) and Chen et al. (1994).

    It is important to note that the reaction buffer and protein samples should not contain any reducing agent to avoid cleavage of the disulfide linkage between the crosslinker and the bait protein before photocrosslinking. Generally, the amount of each protein in the reaction mixture ranges from 0.1 pmole to 1 nmole, and the total reaction volume ranges from 10 to 1000 μl; consequently, the protein concentration is 0.1 nM to 100 μM. Small reaction volumes could allow direct addition of the gel sample buffer for SDS-PAGE; in contrast, large volumes will require concentration of the proteins by methods such as affinity pull-down (UNIT 17.5) or TCA precipitation.

11. Place the sample tube on a magnet to precipitate the beads with bound PICs and remove the supernatant. Wash beads three times with 400 μl acetate transcription buffer, remove the supernatant, and resuspend beads in 100 μl acetate transcription buffer. Split the sample into two by pipetting one half of the sample into a new microcentrifuge tube.

    One of the samples will be used as a negative photocrosslinking control to which the UV irradiation is not applied.

12. Apply UV irradiation (~365 nm) to one of the samples from step 11 for 1 to 20 min to allow effective photolysis. Generally, place the sample tube at a distance of 5 to 10 cm from the irradiation source.

As reaction conditions and types of proteins samples vary, a survey for an effective photocrosslinking condition is required. Most of the common UV equipment can achieve maximal crosslinking within minutes. For the authors' photocrosslinking experiment, the optimal time length is 2 min with a UV lamp that provides an intensity of 21,700 μW/cm^2. Various equipment for UV irradiation have been used in the literature, including a hand-held UV lamp, a UV transilluminator, a Spectrolinker UV Crosslinker (Spectronics), and a Rayonet photochemical reactor (Southern New England Ultraviolet).

13. Place the sample tube on a magnet to precipitate the beads with bound PICs. Remove the supernatant.

Transfer radiolabeled photocrosslinker

14. Pipet 15 μl 1× SDS-PAGE sample buffer into the UV-irradiated sample and the negative control (the non-irradiated sample). For the UV-irradiated sample, include reducing agent (i.e., DTT or 2-mercaptoethanol) in the sample buffer to permit transfer of the radiolabeled crosslinker to the crosslinked protein.

The resulting samples can be frozen on dry ice and stored overnight at −70°C before SDS-PAGE.

15. Analyze photocrosslinking by SDS-PAGE (*UNIT 6.1*) on a 4% to 12% gel. After electrophoresis, dry gel and expose to autoradiography film or storage phosphor screen (Phosphorimager) for autoradiography (*UNIT 6.3*).

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

A50 buffer

25 mM Tris·HCl, pH 7.5
50 mM KCl
5 mM $MgCl_2$
5% (v/v) glycerol
0.1% (v/v) Triton X-100
Autoclave and store up to several years at 4°C
Immediately before use add:
DTT to 1 mM
Phenylmethyhlsulfonyl fluoride (PMSF; see *APPENDIX 2A*) to 0.1 mM
Leupeptin (Roche or Calbiochem) to 1 μg/μl
Pepstatin (Roche or Calbiochem) to 1 μg/μl

Acetate transcription buffer, 5×

100 mM HEPES, pH 7.6
500 mM potassium acetate
5 mM EDTA
25 mM magnesium acetate
Store up to 2 years at −20°C
Dilute to 1× with H_2O just before use

Dialysis buffer

20 mM HEPES
10 mM Mg-acetate
20% (v/v) glycerol
1 mM EDTA
Adjust to pH 7.5 with 1 M KOH
Prepare fresh and cool to 4°C

continued

Immediately before use add:
DTT to 5 mM
PMSF (phenylmethylsulfonyl fluoride; see *APPENDIX 2A*) to 0.1 mM

Extract buffer

200 mM Tris·base
390 mM $(NH_4)_2SO_4$
10 mM Mg-acetate
20% (v/v) glycerol
1 mM EDTA
Titrate with acetic acid to pH 7.9
Autoclave and store up to several years at 4°C
Immediately before use add:
DTT to 1 mM
PMSF (phenylmethylsulfonyl fluoride; see *APPENDIX 2A*) to 0.1 mM
Leupeptin (Roche or Calbiochem) to 1 μg/μl
Pepstatin to (Roche or Calbiochem) 1 μg/μl

Glutathione-Sepharose immobilized proteins

Introduce open reading frames into plasmid pKM263 (Melcher, 2000; obtainable from EUROSCARF at *http://web.uni-frankfurt.de/fb15/mikro/euroscarf/*). Express the His6-GST-TEVcs tagged proteins in *E. coli* and purify by binding to glutathione-Sepharose using standard published protocols (e.g., Melcher, 2000). Store up to 2 weeks at 4°C.

Plasmid pKM263 is a pET vector (Novagen) derivative that contains the His6-GST-TEVcs composite tag. Open reading frames cloned into pKM263 will be expressed as His6-GST-TEVcs fusion proteins.

For expression in other organisms to allow in vivo assembly into complexes, amplify the tag-encoding region by PCR and clone into appropriate expression vectors in A50 buffer.

SDS sample buffer, 4×

130 mM Tris·Cl, pH 6.8
10% (w/v) SDS
0.5% (w/v) bromphenol blue
50% (v/v) glycerol
Store up to several years at 4°C
Immediately before use mix 4 volumes of this buffer with 1 volume of 2-mercaptoethanol.
For 2× SDS sample buffer: Dilute 4× SDS sample buffer (containing 2-mercaptoethanol) 1:1 with water.

Siliconized glass wool

Place small loose heap of glass wool (e.g., Riedel-deHaen, #18421) together with a beaker containing ~1 ml of 5% dimethyldichlorosilane in heptane (silanization solution I, Fluka) in desiccator. Briefly apply vacuum, then close connection to vacuum pump and allow silanization solution to completely evaporate and coat glass wool. Bake for 2 hr at 180°C. Store up to several years at room temperature.

TEV protease cleavage buffer

20 mM sodium phosphate, pH 7.3
50 mM NaCl
1 mM DTT
0.5 mM EDTA
Prepare fresh and cool to 4°C

TFIIB conjugation buffer

20 mM Tris·Cl, pH 7.8
300 mM potassium acetate
10 % (v/v) glycerol
Store up to 1 year at 4°C

This buffer is specifically used for conjugating the TFIIB protein.

YEPD medium

1% (w/v) yeast extract
2% (w/v) peptone
2% (w/v) glucose
Autoclave
Store up to 1 month at room temperature

COMMENTARY

Background Information

Early crosslinking methods employed diffusible, nonlocalized crosslinkers in combination with size separation of unlabeled adducts. This approach was, and still is, very useful for the analysis of interactions within low-complexity, typically heterodimeric or homomultimeric, complexes. The two protocols described in this unit are capable of extending the application of crosslinking to the identification of interacting polypeptide subunits in the context of highly complex multimeric assemblies because the methods incorporate bait-localized crosslinkers as well as labels or immunological reagents.

Critical Parameters

Basic Protocol 1

Controls. The authors routinely use two types of controls: one is used to confirm correct pull-down and crosslinking conditions, the other to confirm identity and composition of crosslinking adducts.

Controls to confirm pull-down and crosslinking reactions. To detect any potential bead loss during GST pull-down, it is advisable to fractionate part of each pull-down reaction by SDS-PAGE and stain the gel with Coomassie blue stain. The presence of higher-order crosslinking adducts (adducts that contain multiple subunits) on blots serves as control for successful crosslinking. This analysis is important to exclude any failure to detect direct bait-target adducts due to inefficient crosslinking. Where an interactor is already known for a given bait, blots with crosslinking reactions can also be reprobed with antibodies to the known interactor to confirm correct execution of the crosslinking protocol.

Controls to confirm identity and composition of crosslinking adducts. This control is a mock reaction, in which samples are treated without MMPP. Crosslinking adducts can then be identified as bands on immunoblots that are visible in the crosslinking reaction, but not in the mock reaction (compare Fig. 17.10.7 panels A and B). All crosslinking adducts should contain the immunologically detected protein and the tagged bait protein. In addition to absolute size, the presence of the bait protein can be confirmed by using differently sized variants of the bait (e.g., 60-kDa bait AD1 versus 70-kDa bait AD2 in Fig. 17.10.7A) and by TEV protease cleavage of crosslinking adducts (Fig. 17.10.6 and 17.10.7C). Both TEV protease cleavage and crosslinking with differently sized bait variants result in diagnostic mobility shifts.

The final control is the immunological analysis of supernatants after crosslinking. The presence of immunoblot bands in supernatants of crosslinking reactions, but not of mock reactions, would indicate complex destabilization during crosslinking. While the authors have not seen complex dissociation or rearrangement at the low levels of oxidizer recommended in the protocol, if they do happen they could artificially put proteins that under physiological conditions would not touch each other in direct contact and may therefore result in false positive interactions.

Choice of buffers. Crosslinking is quenched by oxidizable buffers like HEPES and PIPES, and oxidizable buffer additives, such as DTT and β-mercaptoethanol (Brown et al., 1995). Where possible, these substances should be avoided in all parts of the protocol. Otherwise, buffers have to be exchanged immediately prior to crosslinking. It is not recommended

to overcome the reducing power of buffer additives by simply increasing the concentration of the oxidizer MMPP because high concentrations of MMPP may destabilize protein complexes. Similarly, Ni-chelators such as EDTA should be avoided or removed prior to the addition of nickel acetate.

Amino acid compositions of bait and target proteins. As with conventional crosslinkers, chemical crosslinking efficiencies vary for different proteins. For instance, when crosslinking transcriptional activation domains to co-activator complexes, the authors have experienced higher crosslinking efficiencies for baits containing the activation domains of yeast Gal4p and Herpes simplex VP16 than for baits that contain the activation domain of a different, slightly less potent activator, yeast Gcn4p. Similarly, the Swi/Snf co-activator complex crosslinks less efficiently to Gal4- and VP16-containing baits than the TFIID and SAGA complexes (J. Klein, S. Hauptmann, and K. Melcher, unpub. observ.). A weak, but specific, crosslinking signal may therefore be just as meaningful as a strong signal, and lack of a crosslinking signal cannot be taken as evidence for lack of interaction.

Crosslinking of large proteins. Detection of crosslinking adducts depends on the electrophoretic separation of the free (monomeric) target protein from bait-target adducts. Target proteins that migrate at the exclusion limit of SDS-PAGE, such as the 435-kDa Tra1p subunit of the SAGA co-activator complex, can therefore not be analyzed by His6-mediated crosslinking in combination with immunoblotting.

Basic Protocol 2

Cysteine mutations. To avoid potential background crosslinker conjugation at unwanted positions, extensive mutagenesis of the bait protein is required to alter the endogenous cysteines to non-cysteine amino acids. However, it is not necessary to completely mutate all endogenous cysteines. The cysteine residues that are buried or involved in thiol-metal coordination are generally inert for crosslinker conjugation; thus, these non-reactive endogenous cysteines can be kept unchanged.

Selection of a suitable position in the bait protein for inserting the cysteine residue requires knowledge of the structure and function of the protein. Residues that are directly involved in binding target proteins are likely not suitable for cysteine mutation; in contrast, residues located near the interface of interaction are ideal for mutation. Frequently, the functionally important protein residues and surfaces can also be inferred from genetic studies.

Photocrosslinkers. PEAS photocrosslinker contains a flexible ethyl spacer group between the end-moieties pyridyldithio and azidosalicylamido groups to allow crosslinking within a diameter of ~14 Å from the Cα of the cysteine residue of the bait protein to the photoreactive atom (Chen et al., 1994). Another similar photocrosslinker, N-[4-(p-azidosalicylamido)butyl]-3′(2-pyridyldithio)propionamide (APDP; Pierce), contains a longer spacer group that can provide a larger crosslinking diameter of ~21 Å (Zecherle et al., 1992).

Choice of desalting columns. The choice of a desalting column for removing the unincorporated crosslinker from the modified protein sample is dependent on the properties of the proteins. An ideal desalting column should have relatively low affinity for the protein and small size to permit high recovery and minimal dilution of the resulting protein sample.

Efficiency of crosslinker conjugation. Although the formation of disulfide bonds can take place under mild conditions (4°C to 25°C) in a biological buffer, the conjugation efficiency is highly dependent on the structural location of the cysteine residue on the protein. If the unique cysteine is not completely exposed to react with the photocrosslinker, conjugation may require a longer incubation time to provide satisfactory efficiency. Raising the conjugation temperature or the photocrosslinker concentration could also achieve higher efficiency. However, conjugation with prolonged incubation, elevated temperature, or higher crosslinker concentration can result in inactive proteins, possibly caused by non-specific crosslinker-protein interactions that destabilize the protein. It is important to conduct several parallel experiments to determine an optimal reaction condition.

The disulfide bond formation between the crosslinker and the cysteinyl SH group of the bait protein is analyzed by SDS-PAGE in the presence of the reducing agent DTT or 2-mercaptoethanol. As the disulfide linkage is cleavable by the reducing agent, a complete loss of radioactivity is indicative of successful conjugation (see Fig. 17.10.9A). More accurate measurement of conjugation efficiency could be obtained through spectroscopic analysis (Zecherle et al., 1992) of the conjugation adduct.

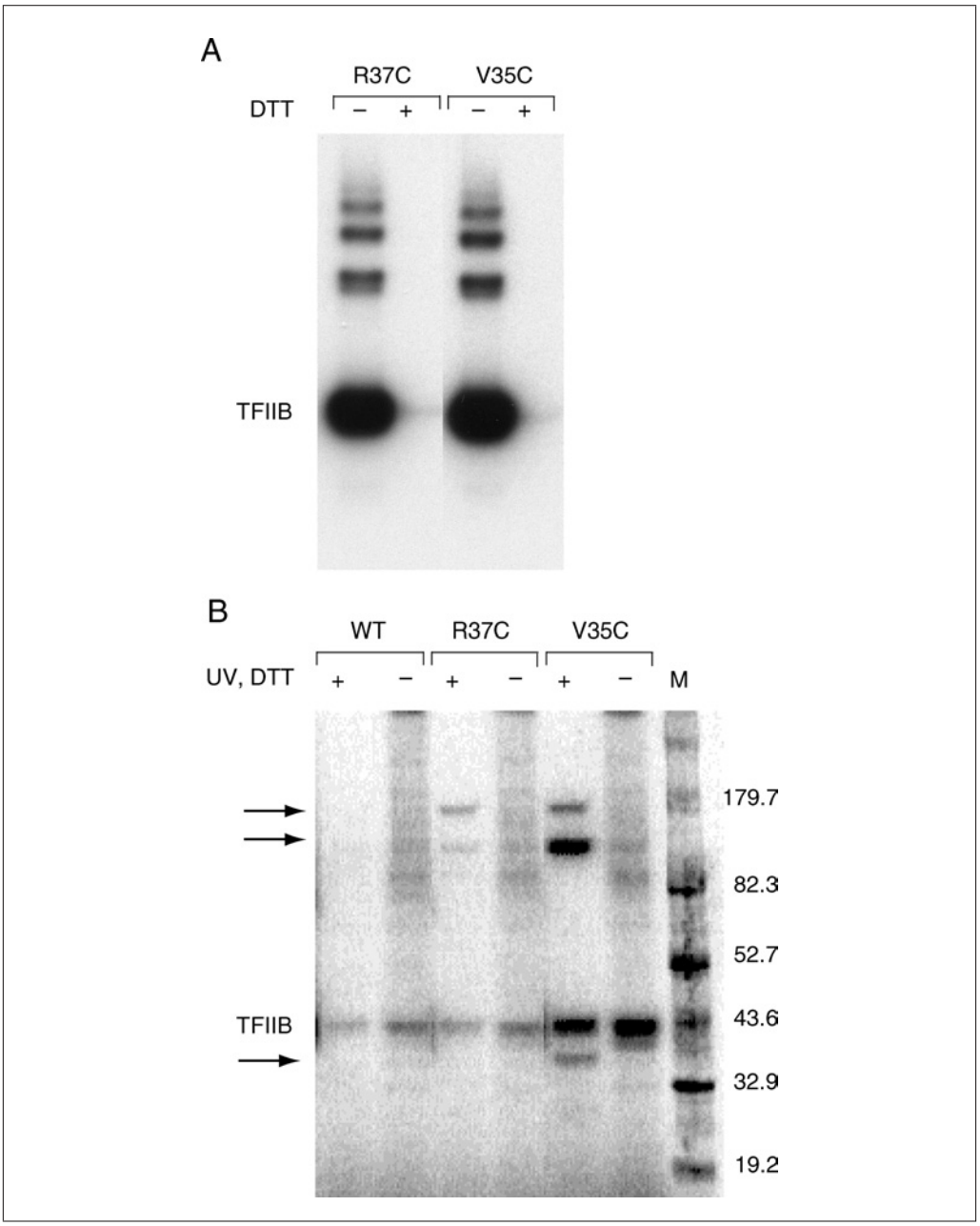


Figure 17.10.9 Autoradiograms of radiolabeled crosslinker conjugation and photocrosslinking products from TFIIB complexes. (**A**) Protein complexes formed using two TFIIB mutants (R37C and V35C) with surface cysteine residues conjugated to ^{125}I-PEAS and analyzed by SDS-PAGE electrophoresis in the presence (+) and absence (−) of DTT. A complete loss of radiolabeling of the modified protein sample in the presence of 50 mM DTT indicates cleavage of the disulfide linkage between the crosslinker and the inserted surface cysteine residue of the protein. Each lane contains 10 μl of protein sample from the conjugation reaction. (**B**) Protein complexes formed using two TFIIB mutants (R37C and V35C) with surface cysteine residues conjugated to ^{125}I-PEAS as well as a control protein (WT, wild type) without a surface cysteine in the formation of the polymerase II transcription pre-initiation complex (PIC). Photocrosslinking samples treated with UV irradiation and DTT are indicated by +. Arrows point to the crosslinked polypeptides. Lane M contains ^{14}C molecular weight markers.

Controls. Proper control experiments are critical for analyzing photocrosslinking, as the background radioactive signals, likely due to crosslinkings from non-specific interactions, are generally high. In addition to the negative control without UV-irradiation, it is necessary to conduct the same procedure for a non-cysteine mutant that contains no surface cysteine.

Because nonspecific interactions are unlikely to be avoided, additional measures can be employed to reduce the problem. One of the popular approaches is to engineer affinity tags to components involved in the complex assembly. The affinity tag permits application of the pull-down process with concomitant stringent washes to remove non-specific bindings, and thus allows purification of the complex assembly that retains mostly high-affinity specific bindings within the complex.

Identification of the crosslinker polypeptides

Identification of the crosslinking targets relies on determining the approximate molecular weight from the gel mobility of the radiolabeled crosslinked protein band on the autoradiogram. However, for a complex assembly that contains a large number of polypeptides with similar molecular weight, it becomes impossible to determine the identity of the targets with this method alone. Additional analyses such as immunostaining (Western analysis) of the SDS-PAGE resolved proteins or mass spectrometry (e.g., Cai et al., 2001) could be used to provide further information on the crosslinked proteins. Another effective method is to incorporate an epitope tag to individual polypeptide candidate and repeat the photocrosslinking experiment using the tagged polypeptide. The correct crosslinked candidate, therefore, can be determined based on the altered gel mobility of the crosslinked protein caused by the tag (Chen and Hahn, 2003).

Troubleshooting

See Table 17.10.1 for troubleshooting information for chemical crosslinking.

Anticipated Results

Figure 17.10.7 presents the results from the crosslinking of two immobilized transcriptional activation domain (AD) baits to complexes in extract. AD1 is the 34-amino acid core activation domain from yeast Gal4p; AD2 the 78-amino acid activation domain from Herpes simplex VP16. Reactions were separated by SDS-PAGE and probed by immunoblotting for the presence of Taf12, a shared subunit of the co-activator complexes SAGA and TFIID. The top blot of panel A shows that Taf12-containing complexes were specifically retained by both immobilized His6-GST-TEVcs-AD bait proteins (AD1 and AD2), but not by His6-GST-TEVcs alone. In the lanes containing the AD crosslinking reactions, three bands were detected by anti-Taf12 antibody: one corresponding in size to monomeric Taf12 (61 kDa), one corresponding to the expected sizes of the Taf12-(His6-GST-TEVcs-AD)$_2$ adducts (120 kDa for AD1 and 130 kDa for AD2), and one band that did not enter the gel corresponding to a high-molecular-weight complex.

Only the band corresponding in size to monomeric Taf12 was detected in the bead fraction of the mock reaction (Fig. 17.10.7B) indicating that the two other bands contained crosslinking adducts. Formation of the high-molecular-weight band is indicative of crosslinking to multiprotein complexes and thus can be taken as a crosslinking control. These bands are believed to result from higher-order crosslinks due to radicals that move through more than one protein in the complex. The size difference between AD1 and AD2 is reflected in the difference of mobilities of the AD1-Taf12 and AD2-Taf12 adducts and thus serves as an additional control for the correct assignment of crosslinking adducts.

The second, more generally applicable control for the presence of bait proteins in crosslinking adducts is the TEV protease digest shown in panel C. Incubation with TEV protease resulted in the disappearance of the presumed Taf12-(His6-GST-TEVcs-AD2)$_2$ adduct and the formation of two distinct new bands with mobilities corresponding to adducts with either one or both of the His6-GST tags cleaved (Fig. 17.10.7C).

The bottom blot in Figure 17.10.7A shows the same membrane after stripping and reincubation with an antibody against a different SAGA subunit, Ada2p. Ada2p was also retained by both ADs (pull-down lanes), consistent with Taf12 and Ada2p being subunits of the same intact complex. However, the same samples and same reactions that contained direct Taf12-AD adducts did not form bands of sizes corresponding to direct Ada2p-AD adducts, suggesting that the two ADs bound the SAGA complex without directly contacting Ada2p. Probing of multiple subunits in the same reaction ensures that

Table 17.10.1 Troubleshooting Guide for Chemical Crosslinking (Basic Protocol 1)

Problem	Possible Cause	Solution
Absent or weak crosslinking bands (including high-molecular-weight bands)	Inactive or insufficient MMPP (MMPP can be labile in certain environments)	Store MMPP under argon or nitrogen
		Increase concentration of MMPP
		Reduce amount of protein
	High concentrations of bait or target protein or MMPP may cause formation of multiple diffused crosslinking bands, rather than defined bands	Increase complexity of protein mixture to reduce unspecific interactions
		Increase concentration of salt or nonionic detergent
		Titrate MMPP concentration
	Amino acid composition of bait-target interaction surface may not support crosslinking	Analyze interaction by different crosslinking chemistry; test crosslinking efficiency by Coomassie-staining gel with His6-GST-TEVcs tag control. If a His6-GST-TEVcs tag dimer fails to become visible after crosslinking, check possible causes above.
Very high concentration of MMPP or Ni^{2+} required	Inactive MMPP	Store MMPP under argon or nitrogen
	Presence of oxidizable buffer components or additives; presence of Ni chelators	Omit reductants and chelators or exchange buffers prior to crosslinking by multiple washes
Nonspecific crosslinking	Artificial interactions due to high local concentrations of bait and target subunits upon complex destabilization	Titrate MMPP concentration
		Include stabilizers such as glycerol
		Reduce MMPP and/or Ni^{2+} incubation times

differences in the formation of crosslinking adducts are not due to differences in crosslinking conditions. Unspecific antibody cross-reactivity could be essentially excluded by detecting Taf12-HA in crosslinking adducts by both anti-Taf12 antibodies (Fig. 17.10.7A and 17.10.7B) as well as anti-HA antibodies (Fig. 17.10.7C).

Figure 17.10.7B contains an anti-Taf12 blot with supernatants of mock and crosslinking reactions. The absence of Taf12 in the supernatant of the mock reaction indicates that interaction between Taf12-containing complexes and the immobilized AD baits was stable during the course of the experiment. Finally, the absence of target complex subunits in crosslinking reactions suggests that complex integrity was not affected by MMPP treatment and that Taf12-AD adducts were not formed upon artificial contacts due to Taf12 dissociation, rather than by specific TAF12-AD interaction.

Another example is from a photocrosslinking study to identify binding targets for the general transcription factor TFIIB within the yeast (*Saccharomyces cerevisiae*) RNA polymerase II (Pol II) transcription pre-initiation complex (PIC; similar to the closed complex in the bacterial RNA polymerase transcription initiation) on a yeast promoter (Chen and Hahn, 2003). Individually, this tightly regulated transient complex is assembled at the promoter region of a Pol II-specific gene in the nucleus of a eukaryotic cell and is poised to start mRNA synthesis upon the supply of nucleotides.

In the crosslinker-protein conjugation experiment, individual recombinant TFIIB

cysteine mutants were conjugated with ^{125}I-PEAS to generate the modified bait proteins, which were analyzed by SDS-PAGE in the presence and absence of DTT to provide evidence of disulfide bond formation between the crosslinker and the protein. This analysis is illustrated in Figure 17.10.9A in which a complete loss of radiolabeling (^{125}I-crosslinker) occurred in the presence of the reducing agent, DTT.

In the multiprotein assembly assay, referred to as the immobilized template assay, 240 μg yeast nuclear extract (containing all essential transcription factors and Pol II), 120 ng (3 pmole) modified TFIIB from ^{125}I-PEAS conjugation, and 1.1 pmole promoter DNA (PCR-generated 595-bp DNA containing the yeast *HIS4* promoter) attached to magnetic beads were used to assemble the PIC in solution. After stringent washes to remove excess proteins, UV-irradiation was applied to the complex to permit crosslinking between the crosslinker and the nearest polypeptides, i.e., the TFIIB binding targets. From a parallel experiment, the amount of TFIIB assembled into the PIC was estimated to be 0.15 pmole based on the immunoblot analysis.

As demonstrated in Figure 17.10.9B from a photocrosslinking experiment of the Pol II immobilized template assay, the crosslinked proteins, which appear as radioactive gel bands, can easily be distinguished by comparing with bands from the negative controls. Generally, a dried SDS-PAGE gel with 10 samples produces approximately 1000 cpm with gamma counting. The autoradiogram was obtained from 2-day exposure to a Phosphorimager storage phosphor screen.

Time Considerations

After initial optimization, Basic Protocol 1 can be completed within 3 days. On the first day extracts are prepared and dialyzed and may be stored at −80°C for several months. Pull-downs (about 3 hr), crosslinking (1 hr), TEV protease cleavage (1 hr), and SDS-PAGE (2 to 3 hr) can all be completed on the same day. Blotting of proteins can be done either the same day (semi-dry blotting, about 1 hr) or over night (tank blotting). On the final day membranes are blocked (about 2 hr or overnight), incubated with primary and secondary antibodies (4 to 8 hr including wash steps), and incubated with detection solution and exposed (30 min to 2 hr).

The procedure for conjugation of the photocrosslinker can be finished within 1 day, and the modified protein sample can be stored for later use. The protein complex formation assay and photocrosslinking can be conducted in 1 day. After SDS-PAGE, it requires overnight or up to a week exposure to a storage phosphor screen to generate a high-intensity autoradiogram. The length of exposure is dependent on the efficiency of crosslinker conjugation and the yield of photocrosslinking.

Literature Cited

Amini, F., Kodadek, T., and Brown, K.C. 2002. Protein affinity labeling mediated by genetically encoded peptide tags. *Angew Chem. Int. Ed. Engl.* 41:356-359.

Bonner, J.J., Chen, D., Storey, K., Tushan, M., and Lea, K. 2000. Structural analysis of yeast HSF by site-specific crosslinking. *J. Mol. Biol.* 302:581-592.

Brown, K.C., Yang, S.-H., and Kodadek, T. 1995. Highly specific oxidative cross-linking of proteins mediated by a nickel-peptide complex. *Biochemistry* 34:4733-4739.

Brown, K.C., Yu, Z., Burlingame, A.L., and Craik, C.S. 1998. Determining protein-protein interactions by oxidative cross-linking of a glycine-glycine-histidine fusion protein. *Biochemistry* 37:4397-4406.

Brown, C.E., Howe, L., Sousa, K., Alley, S.C., Carrozza, M.J., Tan, S., and Workman, J.L. 2001. Recruitment of HAT complexes by direct activator interactions with the ATM-related Tra1 subunit. *Science* 292:2333-2337.

Cai, K., Itoh, Y., and Khorana, H. 2001. Mapping of contact sites in complex formation between transducin and light-activated rhodopsin by covalent crosslinking: use of a photoactivatable reagent. *Proc. Natl. Acad. Sci. U.S.A.* 98:4877-4882.

Cai, S.J., Khorchid, A., Ikura, M., and Inouye, M. 2003. Probing catalytically essential domain orientation in histidine kinase EnvZ by targeted disulfide crosslinking. *J. Mol. Biol.* 328:409-418.

Chen, H.T. and Hahn, S. 2003. Binding of TFIIB to RNA polymerase II: Mapping the binding site for the TFIIB zinc ribbon domain within the preinitiation complex. *Mol. Cell* 12:437-447.

Chen, Y., Ebright, Y.W., and Ebright, R.H. 1994. Identification of the target of a transcriptional activator protein by protein-protein photocrosslinking. *Science* 265:90-92.

Denison, C. and Kodadek, T. 2004. Toward a general chemical method for rapidly mapping multiprotein complexes. *J. Proteome Res.* 3:417-425.

Dougherty, W.G., Carrington, J.C., Cary, S.M., and Parks, T.D. 1988. Biochemical and mutational analysis of a plant virus polyprotein cleavage site. *EMBO J.* 7:1281-1287.

Ebright, Y.W., Chen, Y., Kim, Y., and Ebright, R.H. 1996. S-[2-(4-azidosalicylamido)ethylthio]-2-thiopyridine: Radioiodinatable, cleavable,

photoactivatible cross-linking agent. *Bioconjug. Chem.* 7:380-384.

Edwards, A.M., Kus, B., Jansen, R., Greenbaum, D., Greenblatt, J., and Gerstein, M. 2002. Bridging structural biology and genomics: assessing protein interaction data with known complexes. *Trends Genet.* 18:529-536.

Fancy, D.A. and Kodadek, T. 1998. A critical role for tyrosine residues in His6Ni-mediated protein cross-linking. *Biochem. Biophys. Res. Commun.* 247:420-426.

Fancy, D.A. and Kodadek, T. 1999. Chemistry for the analysis of protein-protein interactions: rapid and efficient cross-linking triggered by long wavelength light. *Proc. Natl. Acad. Sci. U.S.A.* 96:6020-6024.

Fancy, D.A., Melcher, K., Johnston, S.A., and Kodadek, T. 1996. New chemistry for the study of multiprotein complexes: the six-histidine tag as receptor for a protein crosslinking reagent. *Chem. Biol.* 3:551-559.

Fishburn, J., Mohibullah, N., and Hahn, S. 2005. Function of a eukaryotic transcription activator during the transcription cycle. *Mol. Cell* 18:369-378.

Klein, J., Nolden, M., Sanders, S.L., Kirchner, J., Weil, P.A., and Melcher, K. 2003. Use of a genetically introduced cross-linker to identify interaction sites of acidic activators within native transcription factor IID and SAGA. *J. Biol. Chem.* 278:6779-6786.

Kluger, R. and Alagic, A. 2004. Chemical cross-linking and protein-protein interactions-a review with illustrative protocols. *Bioorg. Chem.* 32:451-472.

Kodadek, T., Duroux-Richard, I., and Bonnafous, J.C. 2005. Techniques: Oxidative cross-linking as an emergent tool for the analysis of receptor-mediated signalling events. *Trends Pharmacol. Sci.* 26:210-217.

Melcher, K. 2000. A modular set of prokaryotic and eukaryotic expression vectors. *Anal. Biochem.* 277:109-120.

Melcher, K. 2004. New chemical crosslinking methods for the identification of transient protein-protein interactions with multiprotein complexes. *Curr. Prot. Pept. Sci.* 5:287-296.

Polayes, D.A., Goldstein, A., Ward, G., and Hughes, A.J. 1994. TEV protease, recombinant: a site-specific protease for efficient cleavage of affinity tags from expressed proteins. *Focus* 16:2-5.

Rappsilber, J., Siniossoglou, S., Hurt, E.C., and Mann, M. 2000. A generic strategy to analyze the spatial organization of multi-protein complexes by cross-linking and mass spectrometry. *Anal. Chem.* 72:267-275.

van der Sluis, E.O., Nouwen, N., and Driessen, A.J., 2002. SecY-SecY and SecY-SecG contacts revealed by site-specific crosslinking. *FEBS Lett.* 527:159-165.

Wootner, M., Wade, P.A., Bonner, J., and Jaehning, J.A. 1991. Transcriptional activation in an improved whole-cell extract from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 11:4555-4560.

Zecherle, G.N., Oleinikov, A., and Traut, R.R. 1992. The C-terminal domain of Escherichia coli ribosomal protein L7/L12 can occupy a location near the factor-binding domain of the 50S subunit as shown by cross-linking with N-[4-(p-azidosalicylamido)butyl]-3-(2′-pyridyldithio)propionamide. *Biochemistry* 31:9526-9532.

Contributed by Karsten Melcher
University of Ulster
Coleraine, United Kingdom

Hung-Ta Chen
Fred Hutchinson Cancer Research Center
Seattle, Washington

Visualization of RNA Using Fluorescence Complementation Triggered by Aptamer-Protein Interactions (RFAP) in Live Bacterial Cells

Maria Valencia-Burton[1] and Natalia E. Broude[1]

[1]Center for Advanced Biotechnology, College of Engineering, Department of Biomedical Engineering, Boston University,Boston, Massachusetts

ABSTRACT

This unit describes a method allowing RNA visualization in live cells. The method is based on fluorescent protein complementation regulated by RNA-aptamer/RNA-binding protein interactions. Based on these two principles, a fluorescent ribonucleoprotein complex is assembled inside the cell only in response to the presence of the aptamer sequence on the target RNA. *Curr. Protoc. Cell Biol.* 37:17.11.1-17.11.20. © 2007 by John Wiley & Sons, Inc.

Keywords: protein complementation • aptamer-protein interactions • RNA localization • fluorescent proteins • eukaryotic initiation factor 4A • bacterial cells

INTRODUCTION

RNA is an indispensable, multifunctional molecule that acts as a key player in the complex and tightly regulated process of gene expression. In eukaryotic cells, gene expression includes synthesis and processing of RNA within the nucleus, export from the nucleus, transport through the cytoplasm, and translation within ribosomes. Additionally, an ever-growing class of noncoding RNA molecules participate in a variety of post-transcriptional and post-translational events that take place in different cellular compartments. Noncoding RNAs play a role in editing and modifications of other RNAs as well as in RNA quality control, DNA methylation, and protein modifications (Kiss, 2002, Mattick, 2003; Huttenhofer et al., 2005). To perform these multiple roles, RNA must be present in the right place at the right time. Thus, spatial and temporal localization of RNA molecules within the cell has emerged as an important mechanism in cell biology. It has been realized that localization of RNA not only regulates protein synthesis, but also creates a gradient of morphogens and determines formation of organelles and cell lineages (for review, see Kloc et al., 2002).

The unstable nature of RNA on the one hand and its complex functions linked to its dynamic behavior on the other has generated great interest, and has also posed a challenge for many scientists. Despite the difficulties in studying RNA in vivo, the last decade has yielded much information about mechanisms of RNA localization, functional ribonucleoprotein complex formation, and the movements of such complexes within the cell (see Anderson and Kedersha, 2006; Czaplinski and Singer, 2006, for reviews). The vast majority of these new data have been acquired using fluorescent methods of RNA labeling.

This unit describes a new method for in vivo RNA visualization, which is based on two principles: fluorescent protein complementation and interaction of an RNA-binding

Current Protocols in Cell Biology 17.11.1-17.11.20, December 2007
Published online December 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1711s37

protein with its interacting sequence, an RNA aptamer. Based on these two principles, a fluorescent ribonucleoprotein complex is assembled inside the cell only in response to the presence of the aptamer sequence on the RNA of interest (target RNA). In the absence of target RNA, cell fluorescence is very low. In this approach, the RNA-binding protein is the eukaryotic initiation factor 4A (eIF4A), consisting of two globular domains. The eIF4A protein is dissected into two fragments, which are separately fused with two inactive fragments of a marker protein, the enhanced green fluorescent protein (EGFP). Simultaneous binding of the eIF4A fragments to an RNA aptamer inserted into a target RNA brings the two inactive fragments of EGFP in close proximity, and results in the reassembly of the functional protein, reconstitution of fluorescence, and detection of the RNA of interest (Fig. 17.11.1). Using this approach, we visualized an aptamer-tagged noncoding RNA, an mRNA, and 5S ribosomal RNA in bacteria. As a result, we observed distinct localization patterns reflecting the nature of each transcript (Valencia-Burton et al., 2007).

This unit contains three basic protocols which describe key steps for the labeling of any RNA in bacteria. First, the three components of the complementation complex are cloned into appropriate prokaryotic expression vectors (Basic Protocol 1). Second, the constructs are transformed and coexpressed in bacteria (Basic Protocol 2), and, finally, fluorescence is analyzed by flow cytometry (Basic Protocol 3) and/or microscopy (Basic Protocol 4).

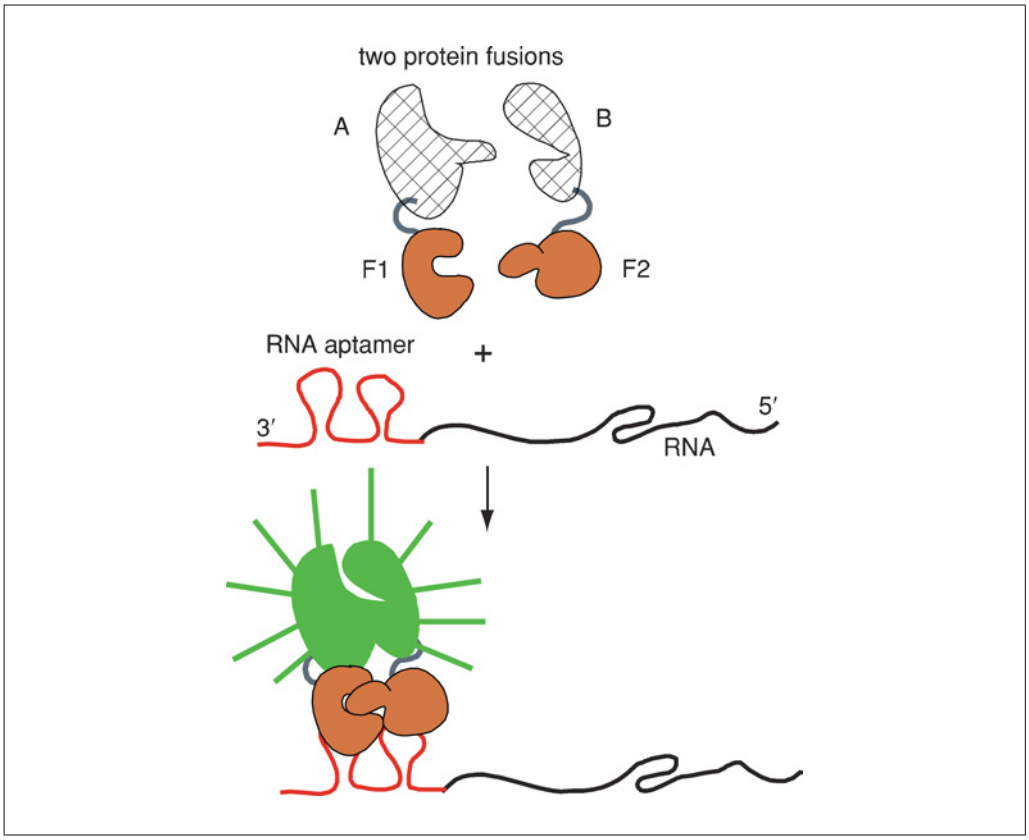


Figure 17.11.1 Diagram of RFAP assay for localization and detection of RNA in vivo. Two fragments (A and B) of the enhanced green fluorescent protein (EGFP) are fused to two fragments (F1 and F2) of eukaryotic initiation factor 4A (eIF4A), an RNA-binding protein. These protein fusions are coexpressed in the presence of an RNA target modified with eIF4A-interactive aptamer sequence inserted into the 3′ UTR of the gene. Binding of F1 and F2 fragments of eIF4A to the aptamer motif brings the EGFP fragments in close proximity to reconstitute a functional fluorescent protein. This RNP complex generates a signal that can be used to track RNA in real time.

DESIGN AND CLONING OF DNA CONTRUCTS FOR THE EXPRESSION OF PROTEIN AND RNA COMPONENTS OF THE COMPLEMENTATION COMPLEX

This protocol describes the design and cloning of two interacting protein fusions and target RNA tagged with the aptamer. The first protein component consists of a C-terminal fusion of amino acids 1 to 158 of EGFP (A), with the N-terminal fragment of eIF4A comprising the first 215 amino acids (F1). These fragments are separated by a 10-amino-acid flexible linker to aid in the refolding of the fragments and reconstitution of fluorescence upon RNA interaction. The second interacting protein fusion similarly consists of amino acids 159 to 238 of EGFP (B) fused at the C-terminus of eIF4A, with amino acids 216 to 406 (F2) connected via a 10-amino-acid-long flexible linker (Fig. 17.11.2). Both fusions are cloned into a vector designed for the coexpression of two genes, e.g., the Duet vector series from Novagen. The target RNA is modified to include a 58-nucleotide-long aptamer sequence with high affinity for eIF4A. The binding of F1 and F2 fragments of eIF4A in the presence of the aptamer sequence brings together the inactive EGFP fragments, generating a fluorescence signal that can be used to track RNA in live cells. To clone these components of the complementation complex, we have used both conventional and nonconventional techniques.

Materials

ThermoPol reaction buffer (New England Biolabs)
10 mM dNTP solution (containing all four dNTPs; see *APPENDIX 3F*, but prepare at 10 mM)
10 mM primers (see Table 17.11.1)
EGFP template: DNA encoding EGFP (Clontech)
Vent exo$^-$ DNA polymerase (New England Biolabs)
eIF4AI template: DNA encoding mouse eIF4AI (plasmid pGEX-4AI; available from Chris Proud, University of Dundee, U.K., *c.g.proud@dundee.ac.uk*)
1% agarose gel (Voytas, 2000)
PCR purification kit (QIAquick PCR Purification Kit, Qiagen)
Appropriate restriction enzymes (*Nco*I, *Bam*HI, *Sal*I, *Not*I, *Dpn*I, *Xbo*I, *Xho*I; New England Biolabs)
DNA purification kit (QIAprep Spin Miniprep Kit, Qiagen)
Cloning vectors: pACYCDuet 1 or pETDuet-1 (Novagen)
T4 DNA ligase and 10× buffer
Competent *E. coli* cells (also see Seidman et al., 1997): e.g., XL-10 (Stratagene), DH5αPRO (Clontech)
LB plates and liquid medium (*APPENDIX 2A*) containing appropriate antibiotic
Thermostable DNA polymerase appropriate for amplifying long templates (e.g., DNA polymerase from Expand Long Template PCR System; Roche Diagnostics) and corresponding 10× buffer
Pfu Turbo DNA Polymerase (Stratagene) and corresponding 10× buffer

Thermal cycler
16° and 65°C water baths

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000), transformation of *E. coli* (Seidman et al., 1997), DNA miniprep from bacteria by alkaline lysis (Wilson, 2001), and gel purification of DNA (Moore et al., 2002)

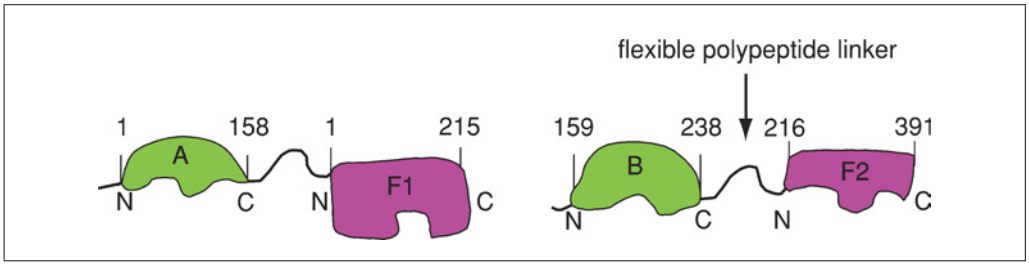


Figure 17.11.2 Schematics of the design of RNA-interacting protein fusions. Carboxy-terminal fusions of EGFP fragments to eIF4A fragments were made with an intervening linker sequence of 10 amino acid residues.

Table 17.11.1 Primers for Amplifying Gene Fragments for Cloning

Primers	Sequence (5′ to 3′)[a]
GFP-A1	CCCGACCATGGTGAGCAAGGGCGAGGAGCTGTTC
GFP-A2	CCCGAGGATCCCTGCTTGTCGGCCATGATATAGAC
GFP-B1	CCCGACCATGGGCAAGAACGGCATCAAGGTGAAC
GFP-B2	CCGAGGATCCCTTGTACAGCTCGTCCATGCCGA
eIF4A-F1-1	CCCGAGTCGACATGGAGCCGGAAGGCGTCATCGA
eIF4A-F1-2	CGAGCGGCCGCTCAAGGGTCTCTCATAAATTTCTT
eIF4A-F2-1	CCCGAGTCGACATTCGGATTCTTGTCAAGAAGGAAG
eIF4A-F2-2	CGAGCGGCCGCTCAAATGAGGTCAGCAACGTTGAG

[a]Underlined bases correspond to restriction enzyme sites used for cloning into appropriate vectors.

Clone EGFP fragments A and B and eIF4A fragments F1 and F2 into prokaryotic expression vectors

In the steps below, each protein fragment is amplified by PCR and cloned separately into either pACYCDuet-1 or pETDuet-1 vectors. Fragments A and F1 and fragments B and F2 are cloned in the same vector but at different sites (Fig. 17.11.3) so that a fusion of these fragments can be accomplished later on using the method of Vasl et al. (2004).

1. In a sterile microcentrifuge tube, mix the following, starting with distilled water and then proceeding in the order below:

   5 µl 10× ThermoPol reaction buffer
   1 µl 10 mM dNTPs
   1 µl 10 µM primer GFP-A1
   1 µl 10 µM primer GFP-A2
   1 µl (10 to 50 ng) EGFP DNA template
   1 µl (1 U) Vent exo$^-$ DNA polymerase
   H_2O to 50 µl final.

   This reaction will amplify the A fragment of EGFP.

2. Set up another reaction as above, but this time using primers GFP-B1 and GFP-B2.

   This will amplify the B fragment of EGFP.

3. Set up two more reactions identical to that in step 1, but replace the template for EGFP with a DNA template encoding the eIF4AI gene and use a combination of primers eIF4A-F1-1 and eIF4A-F1-2 in one reaction for amplifying the F1 domain of eIF4AI and a combination of primers eIF4A-F2-1 and eIF4A-F2-2 in the other reaction for amplifying the F2 domain of eIF4AI.

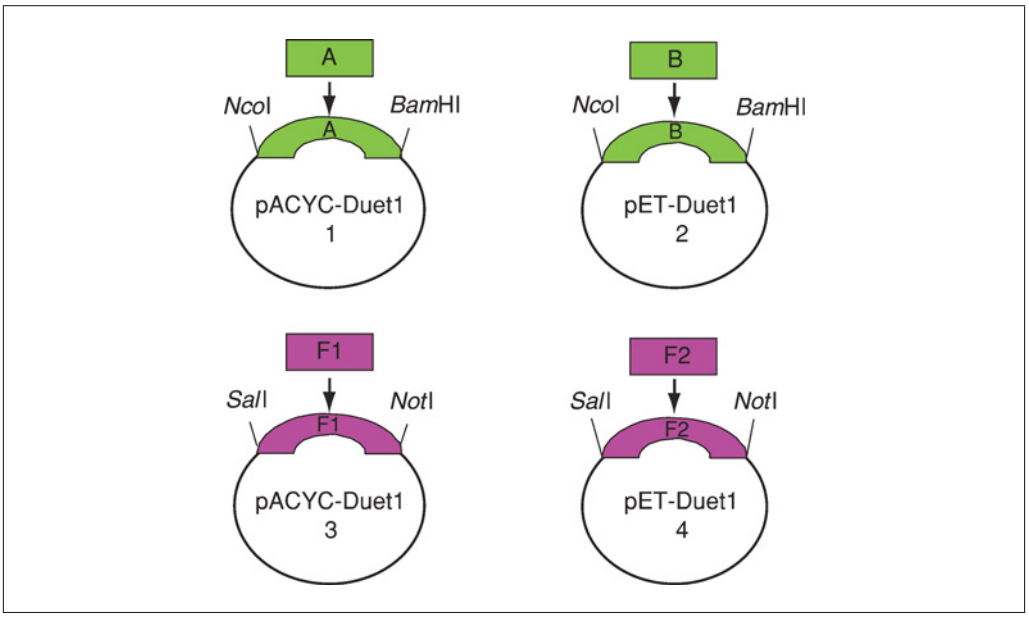


Figure 17.11.3 Step-wise cloning of EGFP and eIF4A fragments into prokaryotic expression vectors. Each fragment is PCR-amplified and cloned into two different vectors used for the co-expression of one or more genes in bacteria. The A and F1 fragments are cloned between *Nco*I-*BamH*I and *Sal*I-*Not*I sites of pACYCDuet-1, respectively. Fragments B and F2 are cloned in a similar manner, but in vector pETDuet-1. These constructs are later used in the fusion of these fragments as in Basic Protocol 1 (also see Figure 17.11.4).

4. Perform PCR on each of the reaction mixes to amplify the respective fragment, in a thermal cycler using the following reaction conditions:

1 cycle:	1 min	95°C	(initial denaturation)
	30 sec	95°C	(denaturation)
	30 sec	52°C	(annealing)
	1 min	72°C	(extension)
25 to 30 cycles:	30 sec	95°C	(denaturation)
	30 sec	52°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	5 min	72°C	(final extension)
	indefinite	4°C	(hold).

5. Remove an aliquot (3 to 5 μl) from each reaction mixture and analyze it by electrophoresis on a 1% agarose gel (Voytas, 2000).

   A successful PCR should produce a clearly visible band of the expected size: e.g., ~470 bp for fragment A; ~290 bp for B; ~650 bp for F1; and ~530 bp for F2.

6. Clean up each of the amplified products using a PCR purification kit according to the manufacturer's instructions.

7. Set up restriction enzyme digest reactions of the PCR products and the cloning vector plasmids, as directed by the supplier. Digest fragment A and cloning vector pACYCDuet-1 with restriction enzymes *Nco*I and *Bam*HI as outlined in Fig. 17.11.3. Similarly, cut fragment B and plasmid pETDuet-1 with *Nco*I and *Bam H*I. Cut fragments F1 and F2 with *Sal*I and *Not*I to be cloned into pACYC and pET vectors, respectively, that have also been digested with *Sal*I and *Not*I enzymes.

8. Clean up the products of the digest reactions using a DNA purification protocol or kit, e.g., using the PCR purification kit from Qiagen.

   We recommend treating the cut vectors with a phosphatase to increase the efficiency of cloning, and then gel purifying the treated vectors to remove traces of the enzyme.

9. Ligate each fragment with its corresponding plasmid vector as outlined in Figure 17.11.3, setting up a ligation reaction in a microcentrifuge tube as follows:

   50 to 100 ng cut/dephosphorylated vector
   200 to 300 ng amplified DNA
   1× ligase buffer (added from 10× buffer)
   1 U T4 DNA ligase
   H_2O to 20 μl final.

   As a control, set up a reaction that contains all of the reagents except for the amplified DNA.

10. Incubate the ligation mixture for 4 hr at 16°C.

11. Use 1 to 4 μl of the ligation mixture to transform chemically competent *E. coli* cells (Seidman et al., 1997). Plate the transformed cultures on LB plates supplemented with 34 μg/ml chloramphenicol for ligations with the pACYC vector and 100 μg/ml ampicillin for ligations with the pET vector. As a control, transform *E. coli* cells with the self-ligated vector.

12. Pick several recombinant colonies and grow cultures in liquid LB medium supplemented with the appropriate antibiotic. Confirm the presence of the cloned fragment by restriction enzyme digest and/or PCR. Perform DNA minipreps (Wilson, 1997, or use QIAprep Spin Miniprep Kit) to obtain the plasmids containing each fragment, to be used later in step 14.

Prepare A-F1 and B-F2 fusions with a flexible polypeptide linker

This method describes the generation of chimeric genes without the need for subcloning (Vasl et al., 2004). For this approach to work, it is critical that the genes to be fused be cloned in the same or in a related vector. Next, two phosphorylated primers are designed, with one placed at the end of one gene and the second placed at the beginning of the other gene. These primers have opposite orientations and their 5′ ends are at the site of junction between both genes (Fig. 17.11.4). A linker sequence is added to the 5′ end of the primer, and each plasmid is cleaved at a site near the 5′ end of each primer. This creates linear templates with homologous sequences that can be annealed during PCR and then extended to a final length. The designed primers help in the amplification of this new DNA molecule. Template plasmids are removed by treatment with the enzyme *Dpn*I, and the new plasmid is circularized via blunt-end ligation.

13. Design two types of 5′-phosphorylated primers as follows:

    a. A reverse primer containing the sequence encoding the amino acids GSSGSS and the *last* 20 to 22 bp of the A or B fragment (primers Chimeric-P1 and Chimeric-P3, Table 17.11.2).

    b. A forward primer containing the sequence encoding amino acids GSGS and the *first* 20 to 22 bp of the F1 or F2 fragments (primers Chimeric-P2 and Chimeric-P4, Table 17.11.2).

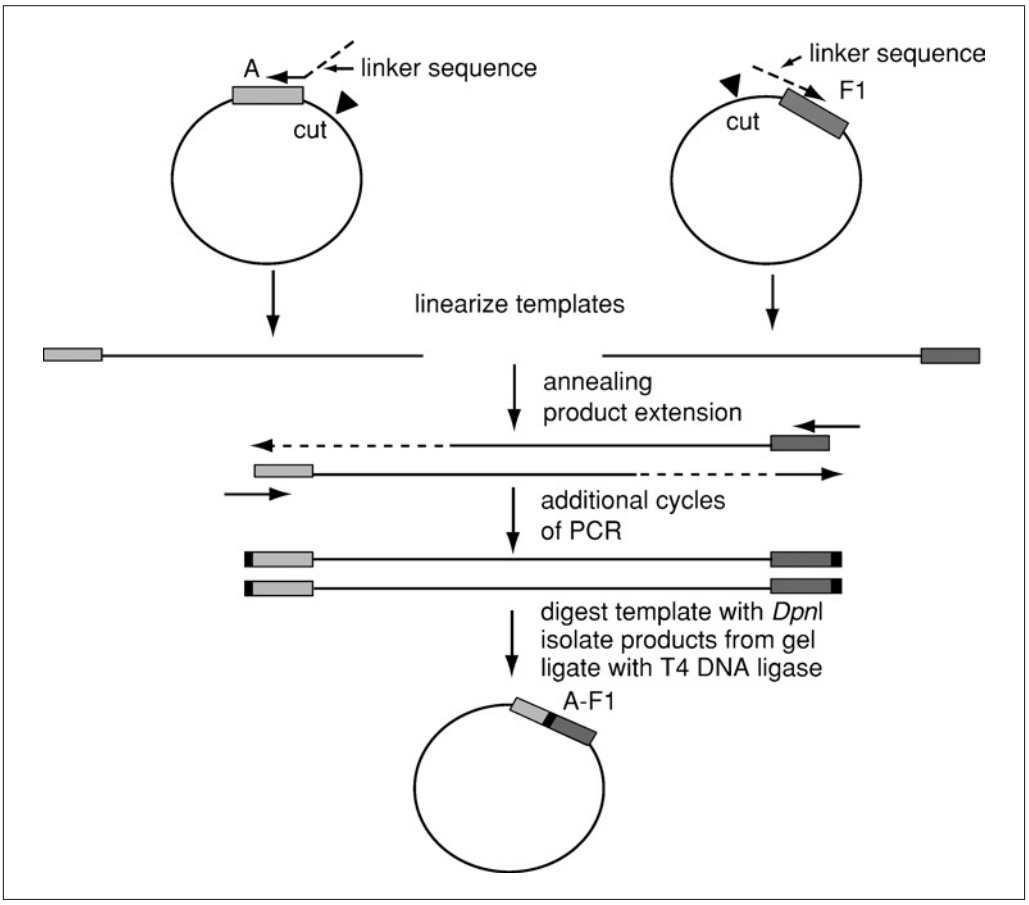


Figure 17.11.4 Outline of the procedure followed to create A-F1 and B-F2 fusions without the need for subcloning (Vasl et al., 2004). This procedure consists of four basic steps: (1) linearization of template plasmids using different restriction enzymes; (2) PCR using linearized templates and phosphorylated primers; (3) isolation of product and removal of template plasmids; (4) ligation and transformation of product into competent *E. coli* cells.

Table 17.11.2 Primer Sequences for Creating Gene Fusions Separated by a Polypeptide Linker

Primers	Sequence (5′ to 3′)[a,b]
Chimeric-P1	pCGAAGATCCAGAGGATCC*CTGCTTGTCGGCCATGATATAG*
Chimeric-P2	pGGTTCTGGTAGC*ATGGAGCCGGAAGGCGTCATCGA*
Chimeric-P3	pCGAAGATCCAGAGGATCC*CTTGTACAGCTCGTCCATGCC*
Chimeric-P4	pGGTTCTGGTAGC*ATTCGGATTCTTGTCAAGAAGGA*

[a]In primers Chimeric-P1 and Chimeric-P3, sequences in italics correspond to sequences complementary to the last 20 bp of the A and B gene, respectively. In primers Chimeric-P2 and Chimeric-P4, sequences in italics correspond to the first 20 bp of F1 and F2 genes, respectively. The rest of the sequence corresponds to the complementary sequence encoding GSSGSS in primers Chimeric-P1 and Chimeric-P3, and homologous sequences encoding the amino acid stretch GSGS in primers Chimeric-P2 and Chimeric-P4.
[b]The letter "p" represents the 5′ phosphate.

14. Linearize the plasmids carrying the fragments to be fused (step 12) with a restriction enzyme that cuts 5′ with respect to the site where the primers anneal (Fig. 17.11.4).

15. After digestion, heat-inactivate the restriction enzyme by incubating at 65°C for 20 min, and take 1 to 2 µl for use in the next PCR.

16. Set up the following PCR:

    10 µl 10× thermostable DNA polymerase buffer
    2 µl 10 mM dNTPs
    2 µl 10 µM primer Chimeric-P1
    10 µM primer Chimeric-P2
    100 ng linearized plasmid 1
    100 ng linearized plasmid 2
    1 µl (1 U) Expand Long Template DNA polymerase
    H_2O to 100 µl final.

    For this reaction, we recommend the use of a thermostable DNA polymerase that can efficiently and accurately amplify long templates, e.g., DNA polymerase from the Long Template PCR kit (Roche Diagnostics).

17. Amplify the fragments in a thermal cycler using the following reaction conditions:

1 cycle:	3 min	94°C	(initial denaturation)
	1 min	94°C	(denaturation)
	30 sec	60°C	(annealing)
	10 min	68°C	(extension)
30 cycles:	1 min	94°C	(denaturation)
	30 sec	60°C	(annealing)
	10 min	68°C	(extension)
	11 min	68°C	(final extension)
	indefinite	4°C	(hold).

18. Add 10 U of *Dpn*I to the PCR mixture and incubate the reaction at 37°C for 3 hr to remove any methylated template DNA.

19. Remove an aliquot (3 to 5 µl) from the reaction mixture and analyze it by electrophoresis on a 1% agarose gel (Voytas, 2000).

    The size of the expected product band corresponds to the size of the vector plus the size of the fused fragments.

20. Gel purify this product (Moore et al., 2002) and circularize it via the 5′ phosphorylated ends by setting up a DNA ligase reaction as in step 9.

21. Use 2 to 4 µl of the circularized product to transform chemically competent *E. coli* cells (Seidman et al., 1997). Perform a DNA miniprep (Wilson, 1997) on the recombinant colonies to obtain the plasmids containing A-F1 or B-F2 to be used in step 23. Screen the plasmids by restriction enzyme digest and PCR. Confirm by sequencing.

Clone B-F2 fragment in the same plasmid containing A-F1 by PCR-mediated integration (Geiser et al., 2001)

22. Design primers to amplify a B-F2 fragment with 20- to 25-bp vector flanking sequences (Table 17.11.3).

    Flanking sequences correspond to the second multiple cloning site (MCS) of the pACYC vector backbone. Sequences were chosen so that B-F2 will be integrated between the NdeI and XhoI sites of pACYC.

23. Set up a PCR as in step 1 to 4 using the primers designed in step 22 and a plasmid containing B-F2 sequences (generated in steps 13 to 21). Analyze and purify PCR product from this reaction as in steps 5 to 6.

Table 17.11.3 Primer Sequences to Amplify B-F2 Fragment

Primers	Sequence (5′ to 3′)[a]
20-Beta*Nde*I	*GTATAAGAAGGAGATATACATATG*GCAGATCTCAAGAACGGCATCAAG
F2*Xho*I	CCAGACTCGAGGGTACCGACTCAAATGAGGTCAGCAACGTTGAG

[a]Sequences in italics correspond to vector sequences. The rest of the sequence is gene-specific B-F2 sequence.

24. Take 200 to 300 ng of this amplified product and 50 to 100 ng of the recipient vector and set up the following amplification reaction:

    5 µl 10× thermostable DNA polymerase buffer
    1 µl 10 mM dNTPs
    300 ng amplified product (step 23)
    100 ng recipient vector containing A-F1 (from step 21)
    1 µl (1 U) *Pfu* Turbo DNA polymerase
    H_2O to 50 µl final.

    The amplified product serves as the primer for polymerization of the entire plasmid and fragment sequence.

25. Amplify the new plasmid in a thermal cycler using the following reaction conditions:

1 cycle:	30 sec	95°C	(initial denaturation)
	30 sec	95°C	(denaturation)
	30 sec	55°C	(annealing)
	2 min/kb recipient DNA	68°C	(extension)
18 cycles:	30 sec	95°C	(denaturation)
	30 sec	55°C	(annealing)
	2 min/kb recipient DNA	68°C	(extension)
	indefinitely	4°C	(hold).

26. Add 10 U of *Dpn*I and incubate the reaction at 37°C for 3 hr to remove any methylated/template plasmid.

27. Clean up the amplified product by using a DNA purification kit or protocol (e.g., Q1A quick PCR purification Kit).

28. Use 3 to 6 µl of the amplified and purified plasmid to transform chemically competent *E. coli* cells (Seidman et al., 1997).

29. Perform DNA minipreps (Wilson, 1997) of the recombinant colonies or transformants. Analyze the DNA by restriction digest or PCR.

    The correct plasmid should contain both A-F1 and B-F2 sequences. The new plasmid will be used for the coexpression of proteins and RNA in Basic Protocol 2, step 1.

Preparation of a target gene modified with an aptamer sequence

To label RNA in vivo, the target gene is modified at the 3′ end by inserting the eIF4A aptamer recognition sequence. Tagging of the gene at the 3′ end has proven to be more advantageous, since it does not seem to interfere with the level of gene expression (M. Valencia-Burton, unpub. observ.) and RNA localization and dynamics in the cell (Bertrand et al., 1998; Beach et al., 1999; Corral-Debrinski et al., 2000; Beach and Bloom, 2001). If the RNA target contains a 3′ UTR sequence that is essential for localization of the message, we recommend cloning this sequence right after the aptamer tag (see Critical Parameters). The eIF4A aptamer consists of a 58-nucleotide-long sequence (Oguro et al.,

Table 17.11.4 Primer Sequences for Amplifying Aptamer

Primers	Sequence (5′ to 3′)[a]
IF4Aapt 30-R	p*GCCTCACTCACATGTGGGGCGCGGTCCCCTG*TCTAGAGGGGAATTGTTATCCGCTCAC (reverse)
IF4Aapt 30-F	p*CGAAACGTAGATTCGACAGGAGGCTCACAAC*CCTAGGCTGCTGCCACCGCTGAG (forward)
pET-*Bcl*I	CCAGCGGATAGTTAATGATAGCCCAC (forward)
pET-*Bgl*II	GCGCTCGGCCCTTCCGGCTG (reverse)

[a]Sequences in italics correspond to half of the aptamer sequence (primers IF4Aapt 30-R and IF4Aapt 30-F) while the rest of the primer consists of vector sequences. In this case it is pETDuet (Novagen).

2003) with affinity for eIF4A in the nanomolar range. This strong affinity will provide the binding force necessary for the reassembly of the labeling complex on RNA.

30. Design two phosphorylated primers, one forward and one reverse (primers 1 and 2, Table 17.11.4) each containing half of the aptamer sequence (31 bp) plus vector sequences directly upstream and downstream from the cloning site or site of insertion. Also, design two 20-mer to 22-mer oligonucleotides (primers 3 and 4) in the opposite orientation from the phosphorylated primers to generate two fragments that will later be ligated via 5′ phosphorylated ends.

31. Run two standard PCRs as in steps 1 to 4 with primers IF4Aapt 30-R and pET-*Bcl*I and primers IF4Aapt 30-F and pET-*Bgl*II (Table 17.11.4), using a vector that can be used for coexpression along with pACYCDuet-1 as the DNA template.

    Because the plasmid carrying the protein components (pACYCDuet vector, Novagen) contains a p15A origin of replication and the cat gene (chloramphenicol resistance), the RNA component should be expressed from a vector with a different replicon and selection marker—e.g., ColE1 bla gene (ampicillin resistance) as is the case with pETDuet-1.

32. Ligate the two fragments obtained in step 31 using equimolar amounts of each product (see reaction mixture described in step 9) and incubate the reaction for 4 hr at 16°C.

33. Use 1 to 2 μl of the ligation mixture to reamplify the resulting ligated product with primers 3 and 4 (Table 17.11.4).

    For reaction mixture and cycling program, see steps 1 to 4.

34. Digest the product and the cloning vector pETDuet-1 with restriction enzymes *Xbo*I and *Xho*I and follow steps 8 to 12 for cloning and screening transformants containing plasmid coding for eIF4A-binding aptamer sequence.

BASIC PROTOCOL 2

EXPRESSION OF RNA LABELING COMPONENTS IN *E. COLI*

Once protein and RNA-expressing constructs are made, they need to be introduced into an appropriate bacterial strain designed for expression from the strong T7 promoter. Since both protein and RNA are under the control of the same promoter, only one inducer molecule, IPTG, needs to be added to the culture to assemble the RNA labeling complex in vivo (see Critical Parameters under "Time and temperature," for growth conditions).

Materials

Competent *E. coli* cells (also see Seidman et al., 1997): e.g., BL21(DE)3 (Stratagene)
Plasmid encoding protein fusions (Basic Protocol 1, step 29)
Plasmid encoding tagged RNA (Basic Protocol 1, step 34)

LB plates (*APPENDIX 2A*) containing appropriate antibiotic
LB medium (*APPENDIX 2A*) containing appropriate antibiotic
LB medium (*APPENDIX 2A*) containing 1 mM IPTG

Additional reagents and equipment for transformation of *E. coli* (Seidman et al., 1997)

1. Cotransform bacterial cells, e.g., BL21(DE)3, with the plasmid expressing the fusion proteins and the plasmid expressing the tagged RNA.

   The strain should be able to activate the promoters driving the expression of all parts of the complex.

   Protocols for transformation of E. coli are presented in Seidman et al. (1997).

2. Plate transformed cultures on LB plates supplemented with antibiotics needed for the selection of both plasmids (100 μg ampicillin and 34 μg/ml chloramphenicol).

3. Pick a single colony from this transformation and start a day culture in 3 ml LB supplemented with appropriate antibiotics (100 μg ampicillin and 34 μg/ml chloramphenicol) by incubating the cultures at 37°C for 3 hr with vigorous shaking.

4. Dilute cultures 1:250 to 1:500 into 3 ml LB medium containing 1 mM IPTG and the abovementioned antibiotics. Grow cells at room temperature (25°C) for 14 to 16 hr or until cultures reach an optical density of 0.5 to 0.6.

   It is critical that cells be allowed to grow in the presence of the inducer for more than 3 to 4 hr for fluorescence to be detected. Growth of cells at 25°C seems to favor proper maturation of the fluorophore and formation of a robust signal (see Critical Parameters under time and temperature).

ANALYSIS OF CELLS BY FLOW CYTOMETRY

BASIC PROTOCOL 3

This procedure constitutes the first assessment of the formation of the fluorescent RNP complex in vivo. This method also allows for the quantification of the efficiency of the RNA-labeling complex formation by comparison with an uninduced sample or a sample lacking the RNA component. A successful experiment should result in cell fluorescence, which may range 5- to 20-fold above background levels. Background fluorescence is determined by the signal that comes from cells expressing only the protein fragments or from uninduced cells containing the complex. Another control experiment is to measure fluorescence of cells expressing protein fusions and an RNA without the interacting aptamer sequence. If fluorescence of such cells is several-fold lower than fluorescence of the cells expressing the tagged RNA, then the experiment can be considered successful, and cell fluorescence is the result of protein complementation dependent on the presence of the target RNA. The signal-to-background ratio depends on the nature of the RNA. Structured RNAs (e.g., tRNA) consisting of complex hairpins and bulges may produce higher background and lower signal to background ratio than less structured mRNAs. We believe this is the case because of the nature of eIF4A protein, which interacts with RNA via secondary structures. Thus, we found that aptamer-tagged *lacZ* RNA usually produces a better signal-to-background ratio of 4- to 5-fold, compared to aptamer-tagged 5S rRNA, where signal-to-background ratio was about 2-fold.

For cells expressing only protein fusions, the authors use a modification of Basic Protocol 2, step 1, where BL21(DE)3 cells are transformed only with plasmid encoding protein fusions (from Basic Protocol 1, step 29). For cells expressing an untagged RNA, we cloned the coding sequence for the β-galactosidase gene (*lacZ*) from the pBAD/His/lacZ plasmid (Invitrogen) as a *Bam*HI-*Xho*I fragment into cloning vector pACYC following a procedure similar to that of Basic Protocol 1, steps 1 to 12.

Materials

Cells expressing protein and RNA components of the complementation complex (Basic Protocol 2, step 1)
Cells expressing only protein fusions (Control 1; Basic Protocol 2, step 29)
Cells where protein expression have not been turned on (lacking IPTG, Control 2; perform steps 1 to 4 of Basic Protocol 1, but omit IPTG in step 4)
Cells expressing protein fusions and an untagged RNA template (pET plasmid, Novagen)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Flow cytometer with 488-nm excitation filter for collection of green fluorescence

1. Allow all cells to grow to 0.5 to 0.6 OD_{600} units. Pellet cells by microcentrifuging 0.5 ml of cell suspension 1 min at 10,000 rpm at 4°C, and removing the supernatant. Wash cells once by resuspending in 0.5 ml PBS, centrifuging again as before, and removing the supernatant. Resuspend each cell pellet in 0.5 ml PBS.
2. Analyze cell fluorescence on the flow cytometer. Excite fluorescence with a 488 nm argon laser and a 515 to 545 nm emission filter (FL1).
3. Measure the fluorescence of 100,000 cells in each sample at a low flow rate. Select a small forward scatter and side scatter gate to decrease fluorescence variation due to cell size.
4. Compare fluorescence of induced cells with those of uninduced cultures. Also, measure fluorescence of cells transformed only with the plasmid expressing fusion proteins in the absence of interacting RNA. To further determine the specificity of the signal, measure cell fluorescence of samples expressing the protein fusions and an untagged RNA.

BASIC PROTOCOL 4

ANALYSIS OF CELLS BY MICROSCOPY

The RFAP assay allows for direct visualization of RNA particles in the cell via complementation of fluorescent protein fragments induced by aptamer-eIF4A interactions. Moreover, the localization of these fluorescent complexes in a bacterial cell changes depending on the nature and function of the target RNA (Valencia-Burton et al., 2007). Specifically, localization of three different RNAs—untranslated RNA, mRNA, and 5S ribosomal RNA—was distinctly different and consistent with the functions of these RNA molecules (Valencia-Burton et al., 2007). In all cases, the target RNA was modified with an aptamer sequence, which was introduced into the 3′ UTR right after the stop codon (in the case of the *lacZ* gene and 5S RNA gene). The cells expressing the entire complementation complex and corresponding controls should be grown and induced as in steps 3 to 4 of Basic Protocol 2.

Materials

Agarose
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Cells expressing protein and RNA component of the complementation complex (Basic Protocol 2, steps 3 to 4)
Cells expressing only protein fusions (Control 1; Basic Protocol 1, step 29)
50°C water bath
Vacuum aspirator
15-well multi-test microscope slides (e.g., MP Biomedicals)
Long (e.g., 24 × 50–mm) coverslips
Inverted fluorescence microscope allowing excitation at 490 to 500 nm and emission detection at 525 to 535 nm, equipped with neutral density (ND) filters

Additional reagents and equipment for fluorescence microscopy (*UNIT 4.2*) and differential interference contrast microcopy (*UNIT 4.1*)

1. Prepare a 1% (w/v) agarose solution in PBS by heating up the solution in a microwave oven at high power for 15-sec increments until the agarose is melted (solution appears clear). Keep solution warm at 50°C in a water bath prior to use.
2. Place 20 μl of agarose solution into each of 15 wells of a multi-test slide.

   Do not make the agarose pads too tall, as they will then prop open the coverslip.

3. Immediately dispense a 10- to 20-μl aliquot of cell culture (0.5 to 0.6 OD units) over its corresponding well (enough to cover the agarose).

   It is important not to wait too long or the agarose pads will dry up.

4. Allow the cells to settle on the pads for 4 min at room temperature.
5. Carefully aspirate excess liquid covering the pads using a vacuum aspirator. Take care not to disrupt or remove pads from the slide.
6. Cover the slide with a long coverslip.
7. Image cells using an inverted fluorescence microscope with a 100× oil immersion objective and an epifluorescence system allowing excitation at 490 to 500 nm and emission detection at 525 to 535 nm (also see *UNIT 4.2*).
8. Compare fluorescence images with differential interference contrast images (*UNIT 4.1*) to examine cell morphology and growth state. Also, compare these images with those obtained with cells expressing only fusion proteins and with cells expressing full-length EGFP.

COMMENTARY

Background Information

The study of RNA in live cells is a challenging and exciting field that promises a better understanding of mechanisms of cell function. In bacteria, it is especially a challenge due to the small size and the apparent lack of compartmentalization in these cells.

Recently, two groups performed RNA kinetic studies in *E. coli* (Golding and Cox, 2004; Golding et al., 2005; Le et al., 2005) using a strategy that was originally developed to study RNA in live eukaryotic cells (Bertrand et al., 1998; Beach et al., 1999; Takizawa and Vale, 2000; Beach and Bloom, 2001). This strategy is based on the interaction of an RNA-binding protein with protein-binding motifs introduced in the target RNA. To make the resulting RNP complex fluorescent, the RNA-binding protein is fused with a fluorescent protein. This technique represents the first successful attempt to combine fluorescent proteins with RNA detection in vivo. However, a considerable limitation of this approach is high background signal in the absence of the target RNA produced by the full-length fluorescent protein fused to the RNA-binding protein (Golding and Cox, 2004; Le et al., 2005). Here, specific precautions should be taken to exclude possibilities of protein aggregation that would produce a signal similar to that of RNA. An alternative technique using RNA-specific prelabeled probes has also been used for RNA localization in live cells (Bratu et al., 2003, Santangelo et al., 2004, Silverman and Kool, 2005, Dirks and Tanke, 2006). This approach, however, requires invasive methods of probe delivery into the cell.

The new method allowing RNA visualization in live cells is rooted in two techniques: protein fragment complementation and binding of RNA aptamers with RNA-binding proteins. Both components are produced within the cell, giving this new technique specificity and strength. Application of protein fragment complementation substantially reduces nonspecific background fluorescence and thus increases signal-to-background ratio. The high affinity of an aptamer for its corresponding protein provides great specificity for detection of the target RNA.

Protein fragment complementation

The protein complementation assay (PCA) is a comparatively new method developed for

protein interaction studies both in vivo and in vitro (for review, see Remy and Michnick, 2007). In PCA, a marker protein is split into two inactive fragments in such a way that they cannot reassemble into functional protein by themselves. Proper folding and restoration of the activity of the marker protein takes place only when proteins fused to inactive marker fragments interact and bind (Johnsson and Varshavsky, 1994; Pelletier et al., 1998; Remy and Michnick, 1999; Paulmurugan et al., 2002). Refolding of the marker protein from two fragments is a cooperative process, which means that the signal generated by complementation has a larger dynamic range as compared, for example, with fluorescence resonance energy transfer (FRET; Michnick, 2003; de Virgilio et al., 2004).

PCA also has great advantages over other methods used to study protein-protein interactions (Michnick, 2003; Remy and Michnick, 2007) such as the yeast- and bacterial two-hybrid systems. Unlike these methods that rely on transcriptional induction, PCA is capable of detecting direct protein-protein interactions as well as interactions that take place in cellular compartments outside the nucleus. PCA is now being used to study changes in protein interactions caused by chemical or physical perturbations in the cell (Remy and Michnick, 2001), as well as for high-throughput drug screening (MacDonald and Westwick, 2007; MacDonald et al., 2006). This assay also makes it possible to study transient and weak protein interactions (de Virgilio et al., 2004, Remy et al., 2004; Remy and Michnick, 2006; Schmidt et al., 2006), including membrane proteins participating in the secretory pathway (Nyfeler et al., 2005). Other recent applications include detection of duplex DNA formation in vitro (Demidov et al., 2006) and determination and monitoring of DNA-protein interactions (Ooi et al., 2006), as well as analysis of the methylation state of DNA (Stains et al., 2006).

Different proteins have been used as markers in PCA. Among them are β-lactamase, dihydrofolate reductase, luciferases, and fluorescent proteins. The use of fluorescent proteins in PCA has substantially extended the range of applications for this technique. (Ghosh et al., 2000; Paulmurugan et al., 2002; Hu and Kerppola, 2003; Remy and Michnick, 2004; Magliery et al., 2005). For example, it has allowed the simultaneous study of different protein-protein interactions by using fluorescent proteins with unique spectral characteristics (Hu and Kerppola, 2003; Hu et al., 2002).

PCA has also been applied to the study of RNA-protein interactions in vivo (Rackham and Brown, 2004). In this assay, the RNA binding protein of interest is fused to a portion of a split fluorescent protein, while the second protein fusion consists of another fragment of a split fluorescent protein and the MS2 coat protein. The target RNA contains the MS2 binding motif, which tethers one fragment of the fluorescent protein to the RNA via the MS2 protein-motif interaction. Protein complementation occurs when an RNA binding protein fused with the complementary fluorescent protein fragment interacts with a specific sequence adjacent to the MS2 binding motif (Rackham and Brown, 2004). Fluorescent signal in this case is indicative of the interaction between the protein of interest and a specific sequence on the target RNA.

Despite its advantages, fluorescent protein complementation has limitations stemming from the slow rate of chromophore maturation and the stability of the reassembled fluorescent protein complex. Thus far, it is assumed based on in vitro studies that re-assembled fluorescent proteins can persist in vivo even after the supporting interactions are gone (Hu et al., 2005; Magliery et al., 2005). This presumed stability can make experiments designed to measure kinetics difficult to interpret (Zhang et al., 2002; Magliery et al., 2005; Nyfeler et al., 2005; Remy and Michnick, 2007). Complex stability, however, can prove to be beneficial for the detection of transient and relatively weak protein interactions (Nyfeler et al., 2005; Remy and Michnik, 2006).

Interaction of RNA aptamers with RNA-binding proteins

The second component in the design of a fluorescent RNA complex capable of labeling RNA within the cell is the interaction between an RNA aptamer and an RNA-binding protein. Aptamers are relatively short RNA or DNA oligonucleotides, which bind ligands and are isolated in vitro using selection procedure called SELEX (Systematic Evolution of Ligands by Exponential enrichment; Ellington and Szostak, 1990; Tuerk and Gold, 1990). Because the selection procedure is driven by binding of ligands, aptamers bind their cognate target with high affinity and adopt secondary structures, which are optimized for ligand binding (Herman and Patel, 2000). In this respect, aptamers resemble antibodies

by selectively binding a corresponding ligand from complex chemical or biological mixtures. High specificity of aptamer interaction with cognate ligand makes them an attractive tool in molecular engineering.

When expressed in the cell, RNA aptamers function as predicted from experiments in vitro, and their expression does not seem to interfere with other cellular functions (Choi et al., 2006; Hafner et al., 2006; Mi et al., 2006).

Characteristics of RNA visualization through fluorescence complementation triggered by aptamer-protein interactions (RFAP)

Combining both of the above principles, we were able to label and detect three different RNAs in live bacterial cells. This technique does not seem to interfere with localization of the message, as each transcript showed a unique distribution pattern in the cell. Furthermore, we observed interesting fluorescence changes over time that could be related to dynamics of gene expression. The application of this technique in RNA studies presents several advantages over existing fluorescent RNA labeling techniques. First, we can independently induce protein and RNA components of the complementation complex. In this way, the synthesis of fluorescent protein fragments can be done in advance to allow enough time for chromophore formation. Once proteins are synthesized and folded, RNA expression could be induced, and its fate followed by fluorescence measurements in real time. Second, one protein marker and one RNA-binding protein could be used in studies of different RNAs, thus providing a universal approach for RNA studies in vivo.

Other useful features of the new approach are the direct visualization of the target RNA in its natural cellular environment; there is no need to perform any additional procedures such as permeabilization or fixing of cells. The low background fluorescence allows simple analysis of RNA concentration and localization by either flow cytometry or fluorescence microscopy. Finally, interpretation of the data does not require post-acquisition image processing.

The application of this technique in quantitative kinetic studies, however, should be done with more caution. The issue in this case is the stability of the fluorescent complex in in vitro experiments in which the disassembly of the complex is practically irreversible (Magliery et al., 2005). There are still not enough data supporting such stability of the reassembled EGFP in vivo. More experiments are needed to reveal the stability of the reassembled complex in vivo.

Critical Parameters and Troubleshooting

Design of ribonucleoprotein complementation complex to visualize RNA in vivo

To express fluorescent RNA in vivo, the RNA of interest should contain an aptamer tag capable of interacting with the RNA-binding protein. At the same time, introduction of the aptamer sequence within RNA should not change RNA function and localization pattern. The RNA-binding protein must consist of two dissectible domains, so that each protein fragment can be fused with fragments of a split fluorescent protein. All three components of the complementation complex should be co-expressed in the cell.

Splitting of the marker protein

Splitting of fluorescent protein into two fragments could be performed in different ways (see *UNIT 21.3*). We split EGFP within the loop formed by amino acids 153 to 161, according to the scheme previously used in many protein complementation studies (Ozawa et al., 2000; Ghosh et al., 2000; Ozawa et al., 2001a,b; Hu et al., 2002; Hu and Kerppola, 2003; Remy and Michnick, 2004; Magliery et al., 2005). This splitting has been shown to result in fragments that are not capable of spontaneous self assembly, both in vitro and in vivo (Ghosh et al., 2000; Hu et al., 2002; Magliery et al., 2005; Demidov et al., 2006).

Splitting of eIF4A as an RNA-binding protein

Eukaryotic initiation factor 4A (eIF4A) is a small, 29-kDa protein that acts as an ATP-dependent RNA helicase working in a complex with other initiation factors (4B, 4H, 4G, 4F). This RNA-binding protein consists of two globular domains, and its crystal structure resembles a dumbbell—the compact N-terminal and C-terminal domains are connected via a flexible linker consisting of 11 amino acids (Caruthers et al., 2000). These structural features make eIF4A a useful candidate for domain dissection. Furthermore, recent studies reported the isolation of several high-affinity aptamers that bind eIF4A with a binding constant in the nanomolar range (Oguro et al., 2003). Oguro et al. also showed that the aptamer sequence could be as short as 58 nt while

still preserving high-affinity binding. Furthermore, these authors showed, using a nitrocellulose filter assay, that neither of the dissected eIF4A domains can bind aptamer alone, but rather both domains of eIF4A need to be present for tight RNA binding to occur (Oguro et al., 2003). The affinity of the eIF4A-aptamer interaction is in the same range as that of the MS2 coat protein-MS2 RNA interaction used to monitor RNA within the cell (Bertrand et al., 1998; Beach et al., 1999; Beach and Bloom, 2001; Golding and Cox, 2004; Chubb et al., 2006).

Fusion polarity and linkers

To create fusion proteins containing fragments of fluorescent protein with fragments of the RNA-binding protein, one can use different schemes (see for example *UNIT 21.3*). In our work, we used C-terminal fusions—EGFP fragment A (amino acids 1 to 158) was fused with the N-terminus of the F1 fragment of eIF4A through its C-terminus, and the C-terminus of the EGFP fragment B (amino acids 159 to 238) was fused with the N-terminus of the eIF4A F2 fragment. However, a different scheme where only N-terminal fusions are done or where one fusion is C- and the other N-terminal is also acceptable.

In each particular case, self-assembly of protein fusions should be tested. If there is some spontaneous reassembly resulting in notable fluorescence, several steps can be undertaken to reduce fluorescent background: (1) to change polarity of fusions; or (2) to decrease concentration of protein fusions by using a plasmid with a lower copy number, lower promoter strength, or lower concentration of the inducer.

The linkers connecting the two protein domains in each fusion should allow enough flexibility for interaction with the mutually complementary protein partner and RNA aptamer. We used a 10-amino-acid-long glycine-serine $(GSS)_2(GS)_2$ linker in all fusions (Valencia-Burton et al., 2007), since similar linker sequences have been successfully used in other PCAs (Remy and Michnick, 2001).

Position of the aptamer sequence within RNA

To visualize RNA in the cell, it should be modified to contain an aptamer tag, which will be recognized by the protein complementation complex. To reliably use this system for RNA studies, one needs to make sure that incorporation of the aptamer tag does not interfere with RNA synthesis and function.

Previous studies showed that aptamer sequences can be incorporated within 5′ or 3′ untranslated regions (UTR) without interfering with RNA synthesis (Hanson et al., 2003; Nickens et al., 2003), although it may affect the efficiency of translation, especially if the aptamer interacts with a translational regulator such as tetracycline (Hanson et al., 2003). Our results showed that in *Saccharomyces cerevisiae*, incorporation of the eIF4A-specific aptamer in the 3′ UTR (right after the stop codon) of a marker gene did not affect its expression as compared with the untagged gene control. However, incorporation of the aptamer sequence in the 5′ UTR substantially reduced the level of expression of the marker protein. These results showed that the 3′ UTR is a preferred location for insertion of the aptamer sequence in this tag. Other RNA localization studies employing the MS2 coat protein-motif interaction have also placed these binding motifs in the 3′ UTR of a gene without altering gene expression or RNA localization (Bertrand et al., 1998; Beach et al., 1999; Fusco et al., 2003; Golding et al., 2005).

Coexpression of protein fusions and RNA tagged with aptamer

To assemble a fluorescent ribonucleoprotein complex within the cell, several important conditions must be fulfilled. First, there should be equimolar amounts of the fusion proteins for efficient re-assembly to occur. The best way to accomplish this is to express both fusions from the same vector and under the control of the same promoter. In our experiments, we did this by cloning our protein fusions in the Duet vector, pACYCDuet-1 from Novagen, which can coexpress two messages from two independent T7 promoters. Second, concentration of proteins should exceed that of RNA, to allow efficient and robust binding of RNA molecules. Finally, the RNA should be expressed from a compatible vector, e.g., pETDuet-1 (Novagen), with a promoter strength equivalent to that of the protein-expressing construct. In our case, both protein and RNA were expressed from the strong T7 promoter that is activated by the addition of IPTG to the culture medium. Uncoupling of protein and RNA expression should be done with an inducible promoter of similar or equal strength.

Time and temperature

For fluorescence development, both protein and RNA constructs need to be coexpressed for at least 12 to 14 hr at room temperature

in the presence of the inducer IPTG. For this, we recommend that cultures be diluted 1:600 to 1:1000 in the presence of the inducer and that they be grown overnight at room temperature (20° to 25°C). Usually, cultures grown under these conditions will reach an optical density of 0.4 to 0.6 at the time of examination, which is ideal for microscopic and flow cytometric analysis. We failed to observe fluorescence above background levels when culture was incubated at 30° or 37°C, even after the cultures reached an optical density of 1.

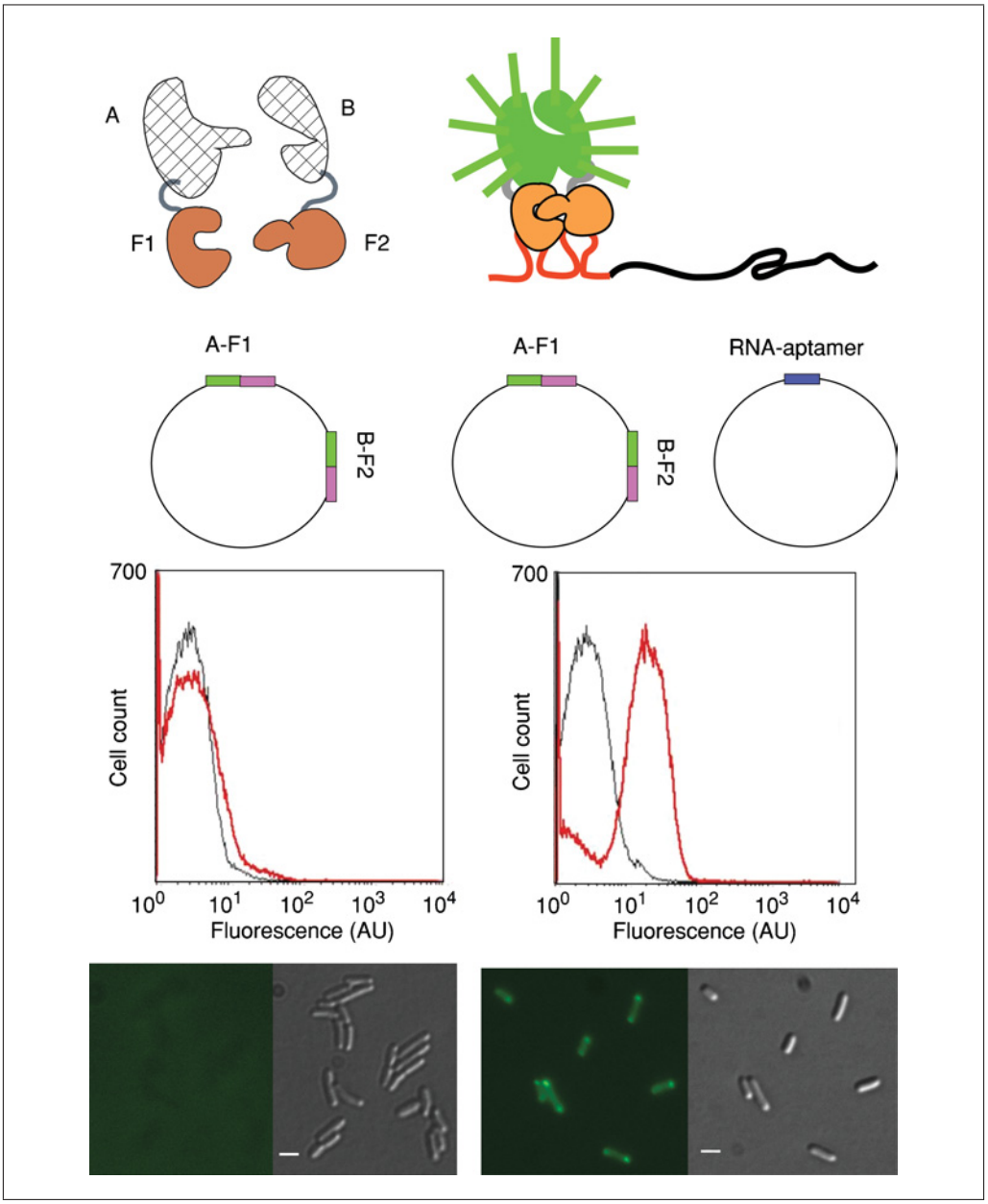


Figure 17.11.5 Localization of untranslated RNA in live bacterial cells using the RFAP method. The left-hand side of the figure illustrates expression of two protein fusions, each containing a fragment of a split eIF4A and a split EGFP, that does not result in a fluorescent signal. The right-hand side of the figure illustrates coexpression of two protein fusions and the RNA transcript with aptamer, which results in a fluorescent signal often localized to the cell. Top row, molecular constructs expressed in *E.coli*; second row, plasmids expressing components of the complementation complex; third row, fluorescence distributions of cells expressing EGFP-complementing complexes, obtained by flow cytometry—black, before IPTG induction, red, after IPTG induction; and bottom row, fluorescence micrographs of *E. coli* cells expressing corresponding components of the complementing complexes. Scale bar = 2 μm. For the color version of this figure go to *http://www.currentprotocols.com*.

In the protocols described above, we simultaneously induced both components of the detection system, proteins and RNA. Therefore, in this system it is impossible to determine the optimal time to observe a signal, because two critical parameters, the time necessary for chromophore formation and the RNA concentration, increase after the start of transcription and cannot be measured independently.

To determine optimal temperature and time conditions for maximal fluorescent signal, a system allowing separate induction of protein and RNA is necessary. Such a system would be also preferable in RNA kinetic studies.

Anticipated Results

The RFAP technique allows RNA detection and localization within live bacterial cells. The efficiency of the RNA-labeling complex formation can be assessed by comparing fluorescence of the cells expressing the entire complex with an uninduced sample or a sample lacking the RNA component. A successful experiment should result in cell fluorescence, which may range 5- to 20-fold above background levels. A useful control includes cells expressing target RNA without the aptamer tag. If fluorescence of cells with untagged RNA is several-fold lower than fluorescence of the cells expressing the tagged RNA, then the experiment can be considered successful, and cell fluorescence is the result of protein complementation dependent on the presence of the target RNA. Figure 17.11.5 exemplifies an experiment with localization of a short untranslated RNA that, in most cells, is visible as a bright particle at one or both poles.

As we mentioned above, the signal-to-background ratio depends on the nature of the RNA. Structured RNAs (e.g., tRNA) consisting of complex hairpins and bulges may produce higher background and lower signal-to-background ratio than less structured mRNAs. We believe this is the case because of the nature of eIF4A protein, which interacts with RNA via secondary structures. Thus, we found that aptamer-tagged *lacZ* RNA usually produces a better signal-to-background ratio of 4- to 5-fold, compared to aptamer-tagged 5S rRNA where signal-to-background ratio was about 2-fold (Valencia-Burton et al., 2007).

Time Considerations

The preparation of DNA constructs expressing protein fusions and RNA tagged with an aptamer sequence may take from 2 to 4 weeks. Transformation of these constructs into a protein-expression bacterial strain will only take a day. The coexpression of protein and RNA will take 2 days, since cells need to grow overnight at room temperature (20° to 25°C) to allow enough time for chromophore formation and maturation. Analysis of the fluorescence signal by flow cytometry or microscopic analysis takes 1 day. When cells are analyzed using fluorescence microscopy, enough time should be set aside for cell examination and image acquisition. Depending on the experiment, time-lapse photographs every 10 or 30 min can be taken to study the dynamic behavior of the signal and/or its distribution over time. Also, depending on the nature of the RNA being examined, we recommend a careful examination of cells in the field, since it can reveal unique distribution and localization patterns. In all, the time-limiting steps for this protocol are those for the construction of plasmids. Once the constructs are made, the cytological analysis mentioned above takes 1 week, including all the necessary controls that can and should be done simultaneously.

Literature Cited

Anderson, P., and Kedersha, N. 2006. RNA granules. *J. Cell Biol.* 172:803-808.

Beach, D.L., and Bloom, K. 2001. ASH1 mRNA localization in three acts. *Mol. Biol. Cell.* 12:2567-2577.

Beach, D.L., Salmon, E.D., and Bloom, K. 1999. Localization and anchoring of mRNA in budding yeast. *Curr. Biol.* 9:569-578.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S.M., Singer, R.H., and Long. R.M. 1998. Localization of ASH1 mRNA particles in living yeast. *Mol. Cell.* 2:437-445.

Bratu, D.P., Cha, B.J., Mhlanga, M.M., Kramer, F.R., and Tyagi S. 2003. Visualizing the distribution and transport of mRNAs in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 100:13308-13313.

Caruthers, J.M., Johnson, E.R., and McKay, D.B. 2000. Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase. *Proc. Natl. Acad. Sci. U.S.A.* 97:13080-13085.

Corral-Debrinski, M., Blugeon, C., and Jacq, C. 2000. In yeast, the 3′ untranslated region or the presequence of ATM1 is required for the exclusive localization of its mRNA to the vicinity of mitochondria. *Mol. Cell Biol.* 20:7881-7892.

Czaplinski, K. and Singer, R.H. 2006. Pathways for mRNA localization in the cytoplasm. *Trends Biochem. Sci.* 31:687-693.

Choi, K.H., Park, M.W., Lee, S.Y., Jeon, M.Y., Kim, M.Y., Lee, H.K., Yu, J., Kim, H.J., Han, K., Lee, H., Park, K., Park, W.J., and Jeong, S. 2006. Intracellular expression of the T-cell factor-1 RNA aptamer as an intramer. *Mol. Cancer Ther.* 5:2428-2434.

Chubb, J.R., Trcek, T., Shenoy, S.M., and Singer, R.H. 2006. Transcriptional pulsing of a developmental gene. *Curr. Biol.* 16:1018-1025.

Demidov, V.V., Dokholyan, N.V., Witte-Hoffmann, C., Chalasani, P., Yiu, H.W., Ding, F., Yu, Y., Cantor, C.R., and Broude, N.E. 2006. Fast complementation of split fluorescent protein triggered by DNA hybridization. *Proc. Natl. Acad. Sci. U.S.A.* 103:2052-2056.

de Virgilio, M., Kiosses, W.B., and Shattil, S.J. 2004. Proximal, selective, and dynamic interactions between integrin alphaIIbbeta3 and protein tyrosine kinases in living cells. *J. Cell Biol.* 165:305-311.

Dirks, R.W. and Tanke, H.J. 2006. Advances in fluorescent tracking of nucleic acids in living cells. *Biotechniques* 40:489-496.

Ellington, A.D. and Szostak, J.W. 1990. In vitro selection of RNA molecules that bind specific ligands. *Nature* 346:818-822.

Fusco, D., Accornero, N., Lavoie, B., Shenoy, S.M., Blanchard, J.M., Singer, R.H., and Bertrand E. 2003. Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr. Biol.* 13:161-167.

Johnsson, N. and Varshavsky, A. 1994. Ubiquitin-assisted dissection of protein transport across membranes. *EMBO J.* 13:2686-2698.

Geiser, M., Cebe, R., Drewello, D. and Schmitz, R. 2001. Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *Biotechniques* 31:88-92.

Golding, I. and Cox, E.C. 2004. RNA dynamics in live *Escherichia coli* cells. *Proc. Natl. Acad. Sci. U.S.A.* 101:11310-11315.

Golding, I., Paulsson, J., Zawilski, S.M., and Cox, E.C. 2005. Real-time kinetics of gene activity in individual bacteria. *Cell* 123:1025-1036.

Ghosh, I., Hamilton, A.D., and Regan, L. 2000. Antiparallel leucine zipper-directed protein reassembly: Application to green fluorescent protein. *J. Am. Chem. Soc.* 122:5658-5659.

Hafner, M., Schmitz, A., Grune, I., Srivatsan, S.G., Paul, B., Kolanus, W., Quast, T., Kremmer, E., Bauer, I., and Famulok, M. 2006. Inhibition of cytohesins by SecinH3 leads to hepatic insulin resistance. *Nature* 444:941-944.

Hanson, S., Berthelot, K., Fink, B., McCarthy, J.E., and Suess, B. 2003. Tetracycline-aptamer-mediated translational regulation in yeast. *Mol. Microbiol.* 49:1627-1637.

Hermann, T. and Patel, D.J. 2000. Adaptive recognition by nucleic acid aptamers. *Science* 287:820-825.

Hu, C.D. and Kerppola, T.K. 2003. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat. Biotechnol.* 21:539-545.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. 2002. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell.* 9:789-798.

Huttenhofer, A., Schattner, P., and Polacek, N. 2005. Non-coding RNAs: Hope or hype? *Trends Genet.* 21:289-297.

Kiss, T. 2002. Small nucleolar RNAs: An abundant group of noncoding RNAs with diverse cellular functions. *Cell* 109:145-148.

Kloc, M., Zearfoss, N.R., and Etkin, L.D. 2002. Mechanisms of subcellular mRNA localization. *Cell* 108:533-544.

Le, T.T., Harlepp, S., Guet, C.C., Dittmar, K., Emonet, T., Pan, T., and Cluzel, P. 2005. Real-time RNA profiling within a single bacterium. *Proc. Natl. Acad. Sci. U.S.A.* 102:9160-9164.

MacDonald, M.L. and Westwick, J.K. 2007. Exploiting network biology to improve drug discovery. *Methods Mol. Biol.* 356:221-232.

MacDonald, M.L., Lamerdin, J., Owens, S., Keon, B.H., Bilter, G.K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S.W., and Westwick, J.K. 2006. Identifying off-target effects and hidden phenotypes of drugs in human cells. *Nat. Chem. Biol.* 2:329-337.

Magliery, T.J., Wilson, C.G., Pan, W., Mishler, D., Ghosh, I., Hamilton, A.D., and Regan, L. 2005. Detecting protein-protein interactions with a green fluorescent protein fragment reassembly trap: Scope and mechanism. *J. Am. Chem. Soc.*127:146-157.

Mattick, J.S. 2003. Challenging the dogma: The hidden layer of non-protein-coding RNAs in complex organisms. *Bioessays* 25:930-939.

Mi, J., Zhang, X., Rabbani, Z.N., Liu, Y., Su, Z., Vujaskovic, Z., Kontos, C.D., Sullenger, B.A., and Clary, B.M. 2006. H1 RNA polymerase III promoter-driven expression of an RNA aptamer leads to high-level inhibition of intracellular protein activity. *Nucleic Acids Res.* 34:3577-3584.

Michnick, S.W. 2003. Protein fragment complementation strategies for biochemical network mapping. *Curr. Opin. Biotechnol.* 14:610-617.

Moore, D., Dowhan, D., Chory, J., and Ribaudo, R.K. 2002. Isolation and purification of large DNA restriction fragments from agarose gels. *Curr. Protoc. Mol. Biol.* 59:2.6.1-2.6.12.

Nickens, D.G., Patterson, J.T., and Burke, D.H. 2003. Inhibition of HIV-1 reverse transcriptase by RNA aptamers in *Escherichia coli*. *RNA.* 9:1029-1033.

Nyfeler, B., Michnick, S.W., and Hauri, H.P. 2005. Capturing protein interactions in the secretory pathway of living cells. *Proc. Natl. Acad. Sci. U.S.A.* 102:6350-6355.

Oguro, A., Ohtsu, T., Svitkin, Y.V., Sonenberg, N,, and Nakamura, Y. 2003. RNA aptamers to initiation factor 4A helicase hinder cap-dependent translation by blocking ATP hydrolysis. *RNA* 9:394-407.

Ooi, A.T., Stains, C.I., Ghosh, I., and Segal, D.J. 2006. Sequence-enabled reassembly of beta-lactamase (SEER-LAC): A sensitive method

for the detection of double-stranded DNA. *Biochemisry* 45:3620-3625.

Ozawa, T., Nogami, S., Sato, M., Ohya, Y., and Umezawa, Y. 2000. A fluorescent indicator for detecting protein-protein interactions in vivo based on protein splicing. *Anal. Chem.* 72:5151-5157.

Ozawa, T., Takeuchi, T.M., Kaihara, A., Sato, M., and Umezawa, Y. 2001a. Protein splicing-based reconstitution of split green fluorescent protein for monitoring protein-protein interactions in bacteria: Improved sensitivity and reduced screening time.*Anal. Chem.* 73:5866-5874.

Ozawa, T., Kaihara, A., Sato, M., Tachihara, K., and Umezawa, Y. 2001b. Split luciferase as an optical probe for detecting protein-protein interactions in mammalian cells based on protein splicing. *Anal. Chem.* 73:2516-2521.

Paulmurugan, R., Umezawa, Y., and Gambhir, S.S. 2002. Noninvasive imaging of protein-protein interactions in living subjects by using reporter protein complementation and reconstitution strategies. *Proc. Natl. Acad. Sci. U.S.A.* 99:15608-15613.

Pelletier, J.N., Campbell-Valois, F.X., and Michnick, S.W. 1998. Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. *Proc. Natl. Acad. Sci. U.S.A.* 95:12141-12146.

Rackham, O., and Brown, C.M. 2004. Visualization of RNA-protein interactions in living cells: FMRP and IMP1 interact on mRNAs. *EMBO J.* 23:3346-3355.

Remy, I. and Michnick, S.W. 1999. Clonal selection and in vivo quantitation of protein interactions with protein-fragment complementation assays. *Proc. Natl. Acad. Sci. U.S.A.* 96:5394-5399.

Remy, I. and Michnick, S.W. 2001. Visualization of biochemical networks in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 98:7678-7683.

Remy, I. and Michnick, S.W. 2004. A cDNA library functional screening strategy based on fluorescent protein complementation assays to identify novel components of signaling pathways. *Methods* 32:381-388.

Remy, I. and Michnick, S.W. 2006. A highly sensitive protein-protein interaction assay based on *Gaussia* luciferase. *Nat. Methods* 3:977-979.

Remy, I. and Michnick, S.W. 2007. Application of protein-fragment complementation assays in cell biology. *Biotechniques* 42:137-145.

Remy, I., Montmarquette, A., and Michnick, S.W. 2004. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat. Cell Biol.* 6:358-365.

Santangelo, P.J., Nix, B., Tsourkas, A. and Bao, G. 2004. Dual FRET molecular beacons for mRNA detection in living cells. *Nucleic Acids Res.* 32-e57.

Schmidt, U., Richter, K., Berger, A.B., Lichter, P. 2006. In vivo BiFC analysis of Y14 and NXF1 mRNA export complexes: Preferential localization within and around SC35 domains. *J. Cell Biol.* 172:373-381.

Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. 1997. Introduction of plasmid DNA into cells. *Curr. Protoc. Mol. Biol.* 37:1.8.1-1.8.10.

Silverman, A.P. and Kool, E.T. 2005. Quenched autoligation probes allow discrimination of live bacterial species by single nucleotide differences in rRNA. *Nucleic Acids Res.* 33:4978-4986.

Stains, C.I., Furman, J.L, Segal, D.J., and Ghosh, I. 2006. Site-specific detection of DNA methylation utilizing mCpG-SEER. *J. Am. Chem. Soc.* 128:9761-9765.

Takizawa, P.A. and Vale, R.D. 2000. The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p. *Proc. Natl. Acad. Sci. U.S.A.* 97:5273-5278.

Tuerk, C. and Gold, L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249:505-510.

Valencia-Burton, M., McCullough, R., Cantor, C.R., and Broude, N.E. 2007. RNA visualization in live bacterial cells using fluorescent protein complementation. *Nature Meth.* DOI 4 :421-427.

Vasl, J., Panter, G., Bencina, M., and Jerala, R. 2004. Preparation of chimeric genes without subcloning. *Biotechniques* 37:726-730.

Voytas, D. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

Wilson, K. 1997. Preparation of genomic DNA from bacteria. *Curr. Protoc. Mol. Biol.* 27:2.4.1-2.4.5.

Zhang, J., Campbell, R.E., Ting, A.Y., and Tsien, R.Y. 2002. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol.* 3:906-918.

CHAPTER 18
Cellular Aging and Death

INTRODUCTION

This chapter covers two distinct processes, cellular aging and cellular death. It is essential for multicellular organisms that the attainment of a permanent non-dividing state (replicative senescence) and patterned cell death (apoptosis) occur in a highly regulated manner. While the aging and death of cells are not strictly linked mechanistically, they are juxtaposed within this chapter since both processes occur at the end of the cellular life span and since the failure of both processes is intimately linked to tumor formation and cancer.

Somatic cells do not divide indefinitely under normal circumstances, although they may acquire the ability to do so if they, for instance, express viral gene products that allow them to escape the controls of senescence. Senescence is marked by phenotypic changes such as irreversible growth arrest and altered differentiated functions, as well as an increased resistance to apoptotic stimuli.

One useful marker for this transition is the accumulation of senescence-associated β-galactosidase (SA-β-gal; *UNIT 18.9*). Some level of lysosomal β-galactosidase can be detected in most cells at an optimum pH of around 4.0. However, the grossly exaggerated expression of β-galactosidase after the onset of senescence allows the differential detection of β-galactosidase activity in the senescent cells under suboptimal conditions (pH 6.0). This assay is straightforward and comparatively robust, allowing the detection of senescent cells in culture and tissue samples.

Senescence is also closely associated with changes in telomeric structures. Telomeres are structures at the ends of linear chromosomes that are composed of short, repeated DNA sequences and specialized proteins associated with these repeats. These structures are essential for the stability of linear chromosomes, but each round of DNA replication results in shortening of the telomeric repeats because the replication machinery inevitably leaves an unreplicated 3′ overhang. It has been proposed that senescence is triggered when telomeres shorten below some critical length, and considerable evidence is consistent with this hypothesis. Experimentally, telomere length can be measured by a number of different methods, including Southern blotting of chromosome terminal restriction fragments (*UNIT 18.6*), Q-FISH (*UNIT 18.4*) or Flow FISH. The elegant work of Blackburn and colleagues has demonstrated that the enzyme telomerase is capable of de novo addition of telomeric repeats to the ends of chromosomes. However, most somatic cells do not express telomerase, although immortal cells do. Some tumor cell lines express telomerase, but it does not appear that telomerase expression is strictly necessary or sufficient to escape senescence. Experimentally, telomerase activity can be measured by a sensitive PCR-based protocol known as telomere repeat amplification or TRAP (*UNIT 18.6*).

As discussed in *UNIT 18.1*, cell death can occur in either a programmed (apoptotic) or more accidental (necrotic) manner. Apoptosis is generally a preferred mode of cell death in multicellular organisms, since it is a more orderly dismantling of cellular structures and degradation of the cell's macromolecular contents and is therefore less likely to cause damage to surrounding cells. Apoptosis occurs in response to a number of stimuli,

Current Protocols in Cell Biology 18.0.1-18.0.2, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1800s47

including developmental signals and the engagement of receptors for cell death stimuli, DNA damage, or large amounts of unfolded protein within the endoplasmic reticulum. Apoptosis and necrosis can be distinguished by a number of criteria including cellular morphology and the patterns of DNA fragmentation (*UNIT 18.3*).

Mitochondria play a central role in the regulation and execution of apoptosis. *UNIT 18.5* describes protocols for the assessment of mitochondrial status during apoptosis. This unit includes protocols for the preparation of mitochondria, for the detection of mitochondrial release of cytochrome *c* and other intermembrane-space proteins, and for assaying apoptosis-associated transitions in mitochondrial membrane permeability and calcium release.

Apoptotic pathways promote the activation of cysteinyl aspartases (caspases; *UNIT 18.2*), a family of related proteases that act in a proteolytic cascade to bring about the fragmentation of the cellular contents and cell death. Activation of poly(ADP-ribose)polymerase 1 (PARP1) is an early event after DNA damage (*UNIT 18.7*). Once active, poly(ADP-ribosyl)ation by this enzyme is likely to promote chromatin remodeling in order to allow DNA repair and changes in gene expression. Caspase-7 and Caspase-3 efficiently cleave PARP1 after the activation of apoptotic pathways, allowing the use of its breakdown intermediates and enzymatic products to be used as a sensitive indicator of cell physiology during apoptosis.

The activation of DNA fragmentation programs during apoptotic cell death is most readily detected using TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assays (*UNIT 18.3*). TUNEL methods can be adapted for use in flow cytometric assays (*UNIT 18.8*). Phospholipids are asymmetrically distributed between the inner and outer leaflets of the plasma membrane, with phosphatidylserine located on the inner surface. This asymmetry is lost during apoptosis, exposing phosphatidylserine on the outside surface of the plasma membrane. This fact has been exploited for the staining of apoptotic cells using the fluorochrome-conjugated annexin V, which binds with high affinity to phosphatidylserine. Like DNA fragmentation, phosphatidylserine exposure can be effectively monitored in flow cytometric assays, as can caspase activation, PARP1 cleavage, collapse of the mitochondrial transmembrane potential, and other apoptotic changes in cell physiology (*UNIT 18.8*). Moreover, these methods may be likewise adapted for high throughput live cell imaging (*UNIT 18.10*), to facilitate the identification of pro-apoptotic compounds or the discovery of apoptosis regulators through RNAi-based screens.

Finally, it should also be noted that many of the processes of apoptosis have been reconstituted within cell-free mammalian and *Xenopus* systems (*UNIT 11.12*), and these systems should be increasingly helpful in understanding the molecular events surrounding programmed cell death.

Mary Dasso

Current Concepts in Cell Death

This unit attempts to define cell toxicity that leads to cellular demise, with a strong emphasis on cell death via both apoptosis and necrosis, by summarizing some of the more recent developments in cellular, molecular, and biochemical studies of the events that govern the induction and execution of cell death.

CELL DEATH

Cell death can occur via two processes, which are fundamentally different in their nature and biological significance (Kerr et al., 1972). These are termed necrosis and apoptosis and their features are summarized in Table 18.1.1.

Necrosis, or "accidental" cell death, was the classic model and was thought to be the universal mode of cell death until apoptosis was identified in 1972 as a separate mode of cell death. In general, necrosis is considered to be a passive process (Trump and Ginn, 1969) that is usually caused by extreme trauma or injury to the cell (Kerr et al., 1972). In tissues, necrosis is often seen as a lesion or patch consisting of many disintegrated cells. Typically, necrosis involves irreversible changes within the nuclei (such as karyolysis), loss of cytoplasmic structure, dysfunction in various organelles (especially mitochondria), and finally, cytolysis as a result of high-amplitude swelling. The release of the dying cell's contents into the extracellular space can cause further injury or even death of neighboring cells, and may result in inflammation or infiltration of proinflammatory cells into the lesion, leading to further tissue damage (Haslett, 1992).

The first references to a different type of cell death came from developmental studies. Glucksmann (1951) emphasized the existence of this phenomenon for the first time. This was followed by a surge of interest in the 1960s (for review see Lockshin, 1981). In the 1970s, histochemical studies of lysosomal changes in hepatic ischemia provided direct evidence for the existence of two distinct types of cell death (Kerr, 1971; Kerr et al., 1972). It was observed that rounded masses that developed in dead hepatic tissue contained cells that were morphologically different from those found in necrosis. These differences initially caused some confusion—i.e., the process was mistaken for a variation of necrosis and was called "shrinkage necrosis" (Kerr, 1971). The following year, Kerr, Wyllie, and Currie proposed the term "apoptosis" to describe the distinct morphological changes associated with this form of cell death (Kerr et al., 1972).

Apoptosis has been described as a form of cellular suicide, since death appears to result from induction of active processes within the cell itself. Typically, apoptosis involves margination and condensation of nuclear chromatin (pyknosis), cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, and finally, formation of apoptotic bodies.

A cell that is dying, whether by apoptosis or necrosis, undergoes rapid changes, which are reflected in its structure, morphology, and biochemistry. These are generally a result of various enzymes activated through elaborate signaling pathways. Since the first description of apoptosis in 1972, there has been a surge of interest in the study of apoptosis, and very little attention has been given to necrosis. This lack of parallel study of the two processes is also reflected in the degree of detail provided here describing apoptosis and necrosis.

STRUCTURAL CHANGES DURING CELL DEATH

Considerable biochemical changes occur within the apoptotic cell, which facilitate neat packaging and removal of apoptotic bodies by neighboring cells; however, as described above, necrosis is accompanied by high-amplitude cell swelling. Modifications in the cytoskeleton and cytoplasmic membranes are required for both shrinkage and swelling to occur. During apoptosis, this results in the loss of cell-cell contact, untethering of the plasma membrane, and rapid blebbing or zeiosis (Saunderson, 1982). Similarly, necrotic cells undergo membrane blebbing, but in contrast to apoptosis, the tracts of contiguous dying necrotic cells collapse without loss of contact with neighboring cells. This results in clumps of necrotic cells rather than single cells.

Tissue transglutaminase, a Ca^{2+}-dependent protein-glutamine γ-glutamyltransferase, is induced and activated in liver hyperplasia and in glucocorticoid-treated thymocytes (Fesus et al., 1987). This enzyme cross-links cytokeratin, a component of the cytoskeleton, through ε-(γ-glutamyl) lysine bonds. During apoptosis there is a significant increase in transglutaminase mRNA, as well as in the protein itself, its

Contributed by Boris Zhivotovsky and Sten Orrenius

Current Protocols in Cell Biology (2001) 18.1.1-18.1.18

Table 18.1.1 Features of Apoptosis and Necrosis

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological or pathological	Pathological (injury)
Occurrence	Single cells	Groups of cells
Reversibility	Limited	Limited
Cellular level		
Cell shape	Shrinkage and formation of apoptotic bodies	Swelling and later disintegration
Adhesion between cells	Lost (early)	Lost (late)
Phagocytosis by other cells	Present	Absent
Exudative inflammation	Absent	Present
Cellular organelles		
Membranes	Blebbing	Blebbing proir to lysis
Cytoplasm	Late-stage swelling	Very early swelling
Mitochondrial permeability transition	Present	Present
Nucleus	Convolution of nuclear outline and breakdown (karyorrexis)	Disappearance (karyolysis)
Biochemical level		
Gene activation	Present[a]	Absent[a]
Requirement for protein synthesis	Present[a]	Absent[a]
Lysosomal enzyme release	Present	Present
Activation of nonlysosomal enzymes	Present	Present
Activation of caspases	Present	Absent
Cleavage of specific proteins	Present	—[a]
Changes in cytoskeleton	Present	Present
Level of ATP required	High	Low
Bcl-2 protection	Present	Present
Nuclear chromatin	Compaction in uniformly dense masses	Clumping not sharply defined
DNA breakdown	HMW and internucleosomal	Randomized
RNA degradation	Present	—[a]
Phosphatidylserine exposure	Present	Absent

[a]This feature is not a universal event or there are conflicting reports.

enzyme activity, and protein-bound (γ-glutamyl) lysine (Fesus et al., 1987). It is thought that transglutaminase activity may stabilize apoptotic cells and prevent membrane leakage during the early stages of the process by forming a shell around the cell. In contrast, transglutaminase activity does not appear to change during necrosis. For example, it was recently reported that ethanol-induced cytotoxicity in astroglial cells was due to necrotic cell death, and that it occurred in the absence of any change in transglutaminase activity (Holownia et al., 1997).

A critical part of apoptosis is the efficient recognition and removal of the apoptotic cells by phagocytes (Savill and Fadok, 2000). This involves the rearrangement and biochemical alteration of the plasma membrane in the dying cell. There are a number of changes that occur in the plasma membrane of apoptotic cells. One such change results in the alteration of carbohydrates on the plasma membrane, which helps preferential binding of macrophages to apoptotic cells (Duvall et al., 1985). Recognition of apoptotic cells by macrophages can also be

mediated via the vitronectin receptor (CD36; Savill and Fadok, 2000). The loss of phospholipid asymmetry in the plasma membrane of cells undergoing apoptosis is yet another significant change leading to the externalization of phosphatidylserine at the surface of the cell, thereby enabling cell recognition by macrophages (Fadok et al., 2000). Development of fluorescently labeled annexin V, which binds specifically to phosphatidylserine residues, enables detection of this externalization in apoptotic cells (Koopman et al., 1994); however, under in vitro culture conditions, where phagocytic cells are absent, apoptotic cells and their fragments lyse in a process very similar to that observed in necrosis. This phenomenon is termed secondary necrosis or post-apoptotic necrosis. Necrotic cells do not undergo phagocytosis to a similar extent, and the spillage of their cellular contents through plasma membrane disruption into the extracellular milieu induces cellular inflammatory responses.

Cell-volume changes are another feature of cell death. Interestingly, the cell volume changes during apoptosis and necrosis go in different directions. Apoptotic cell shrinkage, which leads to a loss of 30% to 50% of the cell volume (Saunderson, 1982), has been proposed to be due to budding of the endoplasmic reticulum. Vesicles thus generated migrate and fuse to the plasma membrane, releasing their contents into the extracellular region. This process requires energy (ATP), since water is moved against the osmotic gradient (Saunderson, 1982). Mitochondria, which are postulated to remain structurally and functionally intact during the early phase of apoptosis, provide the necessary energy. In contrast, during necrosis the cell loses control of ion flux, resulting in a reduction in concentration gradients of Na^+, K^+, Ca^{2+}, and Mg^{2+}, as well as changes in osmotic pressure. This, in turn, leads to uptake of water, giving rise to high-amplitude swelling of the cell and its organelles. This process is energy-independent and does not require a large amount of ATP.

MACROMOLECULAR DEGRADATION DURING CELL DEATH

Cell death is associated with activation of lipases, nucleases, and different classes of proteases. In apoptosis there is an efficient and neat packaging of the cell contents into apoptotic bodies. This requires the rearrangement and breakdown of lipids, proteins, and nucleic acids. Another possible reason for efficient degradation of macromolecules in apoptotic cells prior to their phagocytosis is that contents from dead cells are thus prevented from entering the phagocyte or neighboring cells to cause injury. In necrosis, the enzymes activated appear to be different, with very little overlap with the apoptotic enzymes. The necrotic enzymes are largely lysosomal acidic hydrolases with less substrate specificity than the apoptotic enzymes. These enzymes are thought to be activated also in late apoptosis and in the absence of phagocytosis, when the cells undergo secondary necrosis. Thus, there is a similarity between the features of necrotic cells and those of apoptotic cells undergoing secondary necrosis. The major macromolecules and the mode of their degradation during apoptosis and necrosis are discussed below.

DNA Degradation

Three patterns of DNA degradation are already known to occur during apoptosis. One or more of these may occur during the progression of apoptosis in a single cell (for review see Robertson et al., 2000). These are: single-strand nicks (Gorczyca et al., 1992), chromatin cleavage with the formation of large (i.e., 50- to 300-kbp) fragments (Brown et al., 1993; Zhivotovsky et al., 1994), and finally formation of nucleosome-size fragments of 180 to 200 bp, which produce the ladder pattern that has long been accepted as a biochemical hallmark of apoptosis (Wyllie, 1980).

One or more nuclear endonucleases have been suggested as being responsible for these patterns of DNA fragmentation, since isolated nuclei can be induced to produce the same pattern of fragmentation. A number of endonucleases have been identified in different cell systems, with different ion requirements for their activity (for review see Robertson et al., 2000). For example, the endonuclease in thymocytes is Ca^{2+}/Mg^{2+}-dependent, whereas the endonuclease operating in HL-60 cells appears to function independently of these ions.

Among the different Ca^{2+}/Mg^{2+}-dependent endonucleases, DNase I is of particular interest. Several years ago, it was suggested that DNase I is involved in radiation-induced lymphoid cell death (for review see Robertson et al., 2000). DNase I is a secreted digestive enzyme that produces DNA strand breaks containing 3′-OH end groups—the same end groups found in DNA fragmentation products during apoptosis. This enzyme is localized within the rough endoplasmic reticulum, the Golgi complex, and in small (secretory) vesicles. In apoptotic cells,

DNase I is also found within the perinuclear space, which led to the speculation that during apoptosis damage to endoplasmic reticular and nuclear membranes permits entry of this enzyme into the nucleus where it is involved in DNA fragmentation. It is important to note that DNase I activity is inhibited by G actin gelsolin segment 1, suggesting that cytoskeletal interaction might play a role in the control of this enzyme. A potential problem in linking DNase I activity with apoptosis is the fact that this enzyme is believed to produce mainly single-strand breaks in DNA with 10-bp periodicity and only in the presence of Mn^{2+} can it introduce double-strand breaks; therefore, additional experiments are required to further clarify the role of DNase I in effecting DNA fragmentation during apoptotic cell death.

Endonuclease activities that are completely or partially independent of Ca^{2+} and Mg^{2+} have also been described. Specifically, endonuclease activity that is independent of Ca^{2+} and dependent on Mg^{2+} was implicated in the formation of high-molecular-weight DNA fragments, whereas Ca^{2+}/Mg^{2+}-independent and Mn^{2+}-dependent endonuclease activity was linked to the formation of DNA ladders in several experimental models (for review see Robertson et al., 2000). Moreover, some cell types appear to lack any constitutive Ca^{2+}-dependent endonuclease activity.

Little is known about the ionic requirement for the DNA fragmentation factor-40/caspase-activated DNase (DFF-40/CAD) and, hence, it is difficult to associate this enzyme with any of the nuclease classifications described above. Under normal or unstressed conditions, DFF-40/CAD is bound in an inactive form to DNA fragmentation factor-45/inhibitor of CAD (DFF-45/ICAD), which is an inhibitory component of DFF-40/CAD (for review see Nagata, 2000). In the presence of proapoptotic stimuli, DFF-45/ICAD is cleaved by specific proteases (caspases, see below) and liberates DFF-40/CAD resulting in the activation of nuclease activity, nuclear condensation, and DNA fragmentation. ICAD knock-out mice are refractory to DNA fragmentation and nuclear condensation in response to different apoptotic stimuli, indicating that the inhibitory molecule to CAD is required for normal function of this enzyme (for review see Nagata, 2000). Lastly, a recent study by Liu et al. (1999) indicated that DFF activation is dependent upon the association of DFF-40 with histone H1 and high-mobility group 2 protein, which grants DNA-binding ability and nuclease activity to this molecule. DFF-40/CAD cleaves DNA at internucleosomal spaces.

Endonuclease activity in cells undergoing apoptosis can be regulated by protooncogenes and tumor-suppressor genes, such as *c-myc*, Ha-*ras*, *bcl-2*, and *p53*, as shown by analysis of genetic control of susceptibility to apoptosis (Arends et al., 1993). The apoptotic endonuclease or endonucleases cleave DNA at the exposed linker regions, and it has been suggested that these nucleases are topologically constrained rather than sequence constrained. Results obtained from cloning and sequencing DNA fragments from apoptotic cells show that the nuclease has no preference for specific DNA sequences or for the type of DNA to cleave. This randomness may be functionally important, and reflects the known properties of the endonucleases. The finding that apoptotic endonucleases are constitutive enzymes and that their activity can be modulated by different signals in cells undergoing apoptosis underlines the important role of nucleases in both physiological and pathophysiological processes. Although the role of intracellular endonucleases is not yet understood, one may speculate that a major function is to maintain genomic stability. Unfortunately, both purpose and mechanisms of DNA fragmentation in apoptotic cells are still unclear. Such fragmentation could serve to destroy the genetic information of unwanted cells and thus act as an irreversible step in the process. On the other hand, it may be simply a mid-to-late event that reflects ion redistribution and subsequent chromatin hypersensitivity to endonuclease(s) present in apoptotic cells. Alternatively, it may facilitate cleavage of DNA before uptake of apoptotic bodies during phagocytosis. Whatever the reason, it is still important to understand the mechanisms of this intriguing step of apoptosis.

As compared to apoptosis, at least two patterns of DNA degradation are recognized as occurring during necrosis. The first is the formation of both single- and double-strand DNA breaks. The second is randomized chromatin fragmentation. It is unclear if there is a link between these two steps of chromatin disintegration, although the probability for that is rather high. DNA fragments isolated from necrotic cells contain 5′-OH and 3′-PO_4 end groups. Among different endonucleases isolated and characterized up to now, only one is able to produce such DNA fragments—i.e., DNase II, which is localized in lysosomes. This makes sense, since the activation of lysosomal enzymes has been observed in necrotic cells.

The search for enzymes responsible for cleavage of chromatin during necrosis is still in progress in many laboratories.

Several publications have implicated the involvement of DNase II in apoptosis as well, although its precise role in the apoptotic process remains unclear given the following considerations: (1) DNase II is a lysosomal enzyme and in order to cleave nuclear DNA in intact cells, this enzyme would need to be released from lysosomes and transported into the nucleus. While this scenario is possible, it seems unlikely since it has been demonstrated that lysosomes maintain membrane integrity during apoptosis. (2) DNase II–mediated cleavage of DNA produces 3′-phosphates, which have not been linked to cells undergoing apoptosis. Nevertheless, it cannot be ruled out that DNase II may contribute to DNA fragmentation during apoptosis when the intracellular pH may be more acidic. Recently, it was reported that intracellular acidification during apoptosis can occur even in the absence of a nucleus, suggesting that intracellular acidification and DNA fragmentation are independent consequences of caspase action during apoptosis (Wolf and Eastman, 1999). In this respect, it is likely that DNase II does not induce DNA fragmentation in whole cells, but digests chromatin within phagocytes, where pH is low and apoptotic remnants must be processed. Indeed, two recent publications have suggested that the DNA fragmentation that occurs during apoptosis not only can result from DFF-40/CAD or any other endonuclease activities within the apoptotic cell but can also be attributed to a lysosomal acid DNase after the apoptotic cells are engulfed (McIlroy et al., 2000; Wu et al., 2000).

In conclusion, it is still unclear how all of the nuclease activities described in this section correlate with each other and are involved in apoptosis. It may be that there are cell type and/or insult-dependent specificities for the activation of different enzymes, or it could be that different enzymes involved in DNA fragmentation are active at different stages in the overall process.

RNA Degradation

Changes in ribosomal counts occurring in response to cell injury have been known for a number of years. This phenomenon may be a result of the release of ribosomes from the endoplasmic reticulum at particular stages leading to necrosis (for review see Bowen, 1981). A detailed analysis of the role of ribosomes in the pathogenesis of liver-cell necrosis was conducted by Bernelli-Zazzera (1975). He showed that ischemia resulted in a decrease in the number of ribosomes, and that stripped rough endoplasmic reticulum from ischemic cells bound fewer added ribosomes than did the reticulum from normal cells. It was concluded that there is a "loosened relationship" between endoplasmic reticulum membranes and ribosomes in ischemic livers; however, it is unknown how specific these changes are for necrosis and what relationship they bear to this type of cell death.

There are also a number of reports that RNA, as well as DNA, is susceptible to cleavage during apoptosis. The changes in ribosomal RNA (rRNA) during apoptosis are best studied. Ribosomal RNA consists of conserved and divergent (or variable) domains numbered from D1 to D12 (for review see Houge and Doskeland, 1996). The cleavage of rRNA during apoptosis selectively affects the two largest divergent domains, D2 and D8, in the large 28S rRNA molecule of the 60S ribosomal subunit, while the 18S rRNA molecule in the 40S ribosomal subunit remains unaffected (Houge et al., 1993); however, the pattern of rRNA cleavage occurring during apoptosis cannot be reproduced in necrotic cells or when exposing cell lysates to random RNase activity, suggesting that it is specific to apoptotic cells.

Recently it was demonstrated that rRNA and DNA cleavages can occur independently in apoptotic cells (Samali et al., 1997). In other words, the previously observed correlation is likely to be coincidental. The absence of apoptotic rRNA cleavage in some cell types suggests that this phenomenon is tightly regulated and unrelated to DNA fragmentation; therefore it appears that the pattern of rRNA fragmentation seen during apoptosis is not part of a scheme for general macromolecular disintegration and degradation, but rather a trait present in only some cell types.

Protein Degradation

The prerequisite for proteolysis in apoptosis is well documented. Several proteolytic activities were implicated in both the induction and execution steps of cell killing (for review see Zhivotovsky et al., 1997). Early evidence of a role for proteases in apoptosis came from studies on granule proteases (granzymes/fragmentins). These investigations identified granzymes involved in the exocytosis pathway of lymphocyte-mediated cytotoxicity, which are responsible for the lethal damage inflicted by these cells upon target cells (Shi et al., 1992). More recently, a family of aspartic acid–spe-

cific cysteine proteases have been discovered in mammalian cells, which share homology with the *Caenorhabditis elegans* death gene, *ced-3* (Yuan et al., 1993). The newly adopted nomenclature for these enzymes, caspases, refers to the aspartic acid–specific cysteine protease activity specific to this family (Alnemri et al., 1996). At least fourteen of these proteases have now been purified and their genes cloned (for review see Budihardjo et al., 1999; Fadeel et al., 2000). The activation of the caspase family of proteases has been detected in numerous tissues and cell types and may function as a common pathway through which apoptotic mechanisms operate. Caspases are synthesized as zymogens, and an apoptotic signal converts the precursors into mature enzymes (Stennicke and Salvesen, 2000). Caspases differ in the length and sequence of their N-terminal prodomain and can be divided into two subgroups: procaspases-1, -2, -4, -5, -8, -9, and -10 with long prodomains, and procaspases-3, -6, -7, -11, and -13 with short prodomains. In the long prodomain–containing group of caspases, two distinct modules essential for protein-protein interaction have been identified. The first is referred to as the death effector domain (DED) and is present in both procaspases-8 and -10. The second is termed caspase recruitment domain (CARD) and is found in procaspases-1, -2, -4, and -9. The CARD domain is also present in Apaf-1, the mammalian homologue of the CED-4 protein. It seems that most, if not all, of the long prodomain–containing caspases are activated via oligomerization-induced autoproteolysis. For example, the oligomerization of procaspases-2 and -8 at the plasma membrane is sufficient for their autoactivation. The activation of procaspase-9 requires binding to Apaf-1 and the presence of at least two other components—i.e., dATP and cytochrome *c*. Apaf-1 first forms oligomers and then facilitates procaspase-9 autoactivation by oligomerizing its precursor molecules. On the other hand, the activation of short prodomain–containing caspases requires an initial first step cleavage (e.g., by active caspases or granzyme B) followed by a second autoproteolytic step. For full activation short prodomain–containing caspases also require cleavage in the interdomain linker. Thus, activated caspase-9 can initiate a caspase cascade involving the downstream executioners, caspases-3, -6, and -7.

All members of the caspase family share a number of amino acid residues crucial for substrate binding and catalysis. Despite their uniform requirement for an aspartate residue in the P_1 position of the substrate, caspases can be divided into three different groups according to their substrate preferences (Nicholson and Thornberry, 1997). The predicted caspase specificities correspond to the cleavage sites in the different intracellular target proteins. The effects of caspases in apoptosis appear to be accomplished by the cleavage of a large number of proteins located in the cell membranes, cytoplasm, and nucleus, although the significance of the particular cleavages for the cell death process is still unclear.

There have been a large number of reports on caspase involvement in apoptosis; however, there is very little evidence for the involvement of this family of proteases in necrosis. Recent work from our own group has shown that menadione-induced necrosis of hepatoma cells is independent of caspase activation (Samali et al., 1999a). Furthermore, Tomaselli and coworkers (Armstrong et al., 1997) observed the activation of caspase-3 in cerebellar granule neurons undergoing apoptosis but not necrosis. These results appear to be in line with the work carried out in our laboratory which has shown that after the acute, necrotic death of cerebellar granule neurons exposed to glutamate, the remaining neurons undergo a delayed, apoptotic death (Ankarcrona et al., 1995); however, we did not detect any caspase activation during the "necrotic" step of our experimental model (M. Ankarcrona, B. Zhivotovsky, S. Orrenius, and P. Nicotera, unpub. observ.).

Apart from the caspase family of proteases, a number of other proteolytic enzymes are also implicated in cell death. Studies from our laboratory have suggested the involvement of Ca^{2+}-dependent proteolytic activity in oxidant injury in the liver (Nicotera et al., 1986). Although the substrates for this protease activity during cell injury remain largely unidentified, it appears that cytoskeletal and membrane-integral proteins may be a major target for this proteolytic event during chemical toxicity (Mirabelli et al., 1989). Calpain, another Ca^{2+}-dependent neutral protease and member of the papain family of cysteine proteases, degrades a number of key cellular proteins including protooncogenes, steroid-hormone receptors, protein kinases, and cytoskeletal proteins (Croal and DeMartino, 1991; Vanags et al., 1996). Calpain, one of the degradative nonlysosomal proteases, is implicated in both apoptosis and necrosis (Arai et al., 1990).

In apoptotic cells calpain cleaves a number of substrates, including fodrin, which can also be cleaved by caspase-3. The cleavage sites for

both proteases are different, and it is still unclear why several enzymes should cleave the same protein during cell death. In vitro, calpain can cleave procaspase-3, although it is unclear if this cleavage activates or inactivates this very important effector caspase (for review see Johnson, 2000). Calpain also cleaves several apoptosis regulatory proteins, such as p53, Bax and procaspase-9; however, the impact of calpain-mediated cleavage of these substrates in vivo remains to be elucidated. Enhanced calpain activity has been observed in anoxic hepatocytes and neurons. Moreover, inhibition of calpain by acidosis, calpain protease inhibitors, and glycine delays anoxic injury. Precise mechanisms responsible for the stimulation of calpain activity during either necrosis or apoptosis are still unclear. More recently, it has been shown that hepatocellular carcinoma cells resist necrosis during anoxia by preventing phospholipase-mediated calpain activation (Arora et al., 1996); however, it is unknown whether sustained calpain activity promotes necrosis as a "deathase" by degrading key cytoskeletal proteins such as spectrin or by acting as a "signaling protease." Thus a better understanding of the protein substrates cleaved in necrosis will be required before the role of calpains in necrosis can be elucidated.

Another example of noncaspase proteases that may be involved in apoptosis is seen in the plasminogen activator system, which is a major proteolytic complex responsible for the breakdown of the extracellular matrix. Expression of the plasminogen activator inhibitor type 2 (PAI-2) in HeLa cells, which do not synthesize PAI-2, protects them from tumor necrosis factor–(TNF-) induced apoptosis (Dickinson et al., 1995). Interestingly PAI-2 has a high degree of structural similarity to crmA, which inhibits caspase-1-induced apoptosis (Dickinson et al., 1995). PAI-2 itself is not spared during apoptosis, and it loses its activity after cleavage by other proteases (Jensen et al., 1994). There are no reports about the involvement of this protease in necrosis.

An alternative proteolytic system, which is thought to play a role in cell death, is that of proteasomes. The contribution of proteasomal activity during apoptosis has primarily been evaluated by using inhibitors. There are several observations that show that inhibition of proteasomal activity by "specific" peptide inhibitors either fails to prevent receptor-mediated apoptosis (B. Zhivotovsky, D.H. Burgess, I. Ares-Pörn, and S. Orrenius, unpub. observ.) or drives cells into apoptosis (Shinohara et al., 1996); however, there are a few experimental models in which proteasome inhibitors antagonized apoptosis (for review see Orlowski, 1999). Currently there is no evidence that these enzymes directly activate caspases. Thus, a conclusive role for this enzymatic system in apoptosis remains to be elucidated.

The lytic nature of lysosomal enzymes and their release by various experimental procedures suggested long ago that they might be responsible for cell necrosis in damaged cells and tissues (for review see Bowen, 1981). The concept of the lysosome as a "suicide bag" was developed in the 1960s by de Duve (1963). This hypothesis states that particle-bound acid hydrolase can be released into the surrounding cytoplasm under appropriate conditions and bring about the cell's ultimate destruction. Such a response might occur during cellular injury. Much attention was paid to acid hydrolases in earlier publications. Hydrolases, however, were later shown to occur in association with the Golgi and/or Golgi–endoplasmic reticulum lysosomes, endoplasmic reticulum extracisternal spaces, and plasma membranes (for review see Bowen, 1981). In reference to inflammatory cells, it has been shown that acid hydrolases are directly transferred from the endoplasmic reticulum into foci of cytoplasmic degeneration (Van Lancker, 1975). It was later concluded that when cells die, areas of the cytoplasm become segregated for autolysis, and that these areas are supplied with hydrolases, either from primary lysosomes or directly from the endoplasmic reticulum. Furthermore, an accumulation of free acid phosphatases around ribosomes in the extracisternal space of the endoplasmic reticulum has been linked to this type of cell death (for review see Bowen, 1981). This early extracisternal buildup appears as a prelude to cell autolysis. On the other hand, histochemical studies showed that the liberation of acid phosphatases and esterases was not an early change (Kerr et al., 1972), and the authors of that study suggested that the release of lysosomal enzymes is not responsible for the initiation of necrotic cell death, but rather accompanies this type of death. There is no evidence for preferable degradation of any proteins by lysosomal proteases during necrosis. Thus, although the involvement of both lysosomal and nonlysosomal protease activities in necrosis was documented, the precise role of these protease activities in either induction or execution of necrosis, as well as the specific cleavage of cellular proteins, remain to be elucidated.

Recently some lysosomal proteases have entered the "apoptotic death scene" (Salvesen, 2001). It has been shown that in vitro cathepsins can readily cleave several caspases; however, it is unclear whether cathepsin-mediated cleavage of procaspases results in the activation of these enzymes. Moreover, it is unknown whether this cleavage occurs in whole cells (for review see Johnson, 2000). Given the lack of evidence for a direct role in caspase activation by lysosomal proteases in vivo, the authors hypothesized that an indirect mode of caspase activation may involve the Bcl-2 family member Bid (Stoka et al., 2001). Indeed, Bid is cleaved by lysosomal protease(s) and this cleavage may represent a mechanism by which proteases that leak from the lysosomes can participate in perpetuation of the apoptotic pathway. It seems that lysosomal rupture can trigger apoptosis in certain pathological conditions as well as during normal aging. Nevertheless additional work is required to understand the precise role of lysosomal enzymes in induction and execution of apoptosis.

CELLULAR SIGNALING DURING CELL DEATH

Signal transduction is thought to play a key role in the onset of both apoptosis and necrosis, and this may be mediated by an increase in intracellular Ca^{2+} levels, protein kinase C (PKC), cyclic adenosine monophosphate/protein kinase A (cAMP/PKA), or phosphatases. It has been demonstrated that glucocorticoid-induced cell death involves Ca^{2+} influx in lymphocytes and that this type of cell death can be mimicked using calcium ionophores (Kaiser and Edelman, 1978). Based on these observations, it appears that intracellular Ca^{2+} levels may initiate a death signal, probably through the activation of Ca^{2+}-dependent enzymes (proteases, phospholipases, and endonucleases) in this system. The evidence for a role of Ca^{2+} in necrotic cell killing is particularly strong in the central nervous system. Ca^{2+} appears to mediate the neurotoxicity of cyanide, heavy metals like lead and mercury, and organotin compounds (for review see Nicotera and Orrenius, 1998). Furthermore, intracellular Ca^{2+} overload, resulting from excessive stimulation of excitatory amino acid receptors and enhanced Ca^{2+} influx through membrane channels, appears to play an important role in ischemic brain damage. Much recent research has focused on glutamate-induced excitotoxicity and its contribution to brain damage in various diseases (Ankarcrona et al., 1995; Sims and Robinson, 1999 and references therein). The calcium ion plays a critical role in this process, and intracellular Ca^{2+} overload appears to mediate the lethal effect of *N*-methyl-D-aspartate (NMDA) receptor overactivation. This mechanism is responsible not only for the brain damage induced by certain neurotoxins, but also for excitotoxicity, which is strongly implicated in neuronal death following insults such as ischemia and trauma. Thus, NMDA receptor antagonists not only block Ca^{2+} influx and neuronal death elicited by glutamate or NMDA in vitro, but can also reduce the volume of infarction produced by focal ischemia in vivo. As discussed above, glutamate can trigger the onset of both apoptosis and necrosis in cerebellar granule cells (Ankarcrona et al., 1995). Radical scavengers and agents that inhibit the generation of nitric oxide by nitric oxide synthase were ineffective in preventing cell death in this system, whereas NMDA receptor/channel blockers prevented both necrosis and apoptosis in glutamate-treated cerebellar granule cells. These data suggest that Ca^{2+} overload, mediated by NMDA-receptor-operated channels, is sufficient to induce either necrosis or apoptosis in cerebellar granule neurons in vivo. It is clear that intracellular Ca^{2+} overload is an important factor in various in vitro models, and results from clinical studies appear to support this hypothesis. The Ca^{2+} overload seems to result from increased Ca^{2+} influx through both receptor-operated and L-type Ca^{2+} channels, as indicated by the neuroprotective effects of glutamate-receptor antagonists and L-type channel blockers. Various Ca^{2+}-dependent degradative processes have been found to contribute to Ca^{2+}-mediated necrotic cell killing in in vitro studies, and although their relative contribution is unclear, it appears that perturbation of cytoskeletal organization, impairment of mitochondrial function, and/or activation of certain proteases may be of particular importance. For example, it has been proposed that activation of phospholipase A2 by a sustained increase in cytosolic calcium plays an important role in necrotic cell killing. Support for this hypothesis comes from several observations that necrosis in the liver and heart caused by ischemia, as well as in hepatocellular carcinoma cells in anoxic condition, is prevented by inhibitors of this enzyme or by the inhibition of calpain activation (for review see Nicotera and Orrenius, 1998). Thus, it is likely that the calcium ion may play a determinant role in the necrotic process.

One of the classic signaling pathways is that of PKC, which is a multifunctional ser-

ine/threonine kinase that utilizes diacylglycerol as a second messenger (Nishizuka, 1984). Twelve different isoforms of PKC have been identified to date and are classified according to their calcium dependence and phorbol ester binding activity (for review see Lavin et al., 1996). There are some reports as to the involvement of PKC in apoptosis. For example, it has been reported that treatment of mouse thymocytes with phorbol esters, which activate PKC, induces apoptosis (Kizaki et al., 1989). Similarly, apoptosis induced by the calcium ionophore A23187 can be inhibited by the PKC inhibitor H7; however, most of the evidence to date indicates that PKC activation inhibits apoptosis (McConkey et al., 1996). This paradox may be explained by the large number of isoforms, which may be differentially regulated during apoptosis (Forbes et al., 1992).

Similarly, the role of protein phosphatases during apoptosis is not clear. There are some reports that a number of cell lines react to okadaic acid by undergoing apoptosis (Boe et al., 1991), while there are other reports that the inhibition of protein phosphatase activity blocks apoptosis in some other cell lines (Baxter and Lavin, 1992). From these reports it appears that protein activation/modification, through reversible protein phosphorylation by kinases and protein phosphatases rather than by de novo protein synthesis, may play a more central role in the regulation of apoptosis in some cells.

The anatomy of receptor-mediated death signaling machinery is well characterized. This pathway involves initiation of a cascade of signaling events leading to the activation of caspases. Members of this family, such as CD95 (Apo1/Fas), contain a death domain (DD) in their cytoplasmic tail, which is essential for transducing the apoptotic signal. Upon binding with its natural ligand, CD95-L, receptors undergo trimerization followed by very fast formation of death-inducing signaling complex (DISC). The first step in DISC formation involves binding of DD in CD95 with DD present in the adaptor protein Fas-associated death domain (FADD), which also carries a so-called death effector domain (DED). Next, DED in FADD by homologous interaction recruits the DED in another protein, FLICE, which contains on C-termini procaspase-8. During CD95-initiated cell death, small-accelerator for death signaling (SADS) enhances interaction of FADD and FLICE, and formation of DISC, thereby accelerating processing/activation of procaspase-8 (for review see Krammer, 2000). Active caspase-8 is either directly involved in cleavage and activation of procaspase-3, which results in completion of the cell death process, or it cleaves Bid, a Bcl-2 family member, which in turn activates the so-called, mitochondria-mediated pathway.

ROLE OF MITOCHONDRIA DURING CELL DEATH

Work from several laboratories has indicated that mitochondrial damage may be a common event in the development of cell injury caused by various agents (Nicotera and Orrenius, 1998). It was postulated that mitochondrial damage is initially manifested by a decrease in the mitochondrial membrane potential followed by ATP depletion (Zamzami et al., 1995). During necrosis, these changes occur irreversibly and usually lead to disruption of the mitochondrial structure. These changes set necrosis aside from apoptosis, during which mitochondrial structure remains morphologically intact. Mitochondria have nevertheless been implicated in apoptosis ever since the discovery that the Bcl-2 protein localizes to the outer mitochondrial membrane (Hockenbery et al., 1990). Moreover, by using different cell-free systems, it has been shown that nuclear apoptosis depends on the presence of ATP (Lazebnik et al., 1993; Newmeyer et al., 1994; Kass et al., 1996). It has been demonstrated that cytochrome *c* can induce cleavage and activation of procaspase-3 in cytochrome *c*–minus cytoplasmic extracts (Liu et al., 1996). It has also been shown that cytochrome *c* is released from the mitochondrial intermembrane space into the cytosol by cells undergoing apoptosis. In this case cytochrome *c* release is not preceded by changes in mitochondrial membrane potential (for review see Hengartner, 2000).

During apoptosis a number of proteins that are normally confined to the intermembrane space of the mitochondria are translocated to the cytosol, where they trigger reactions that give rise to the apoptotic process. The role of cytochrome *c* in activation of procaspases has already been described. Recently mitochondrial localization of at least three procaspases (2, 3, and 9) was described. It is still unclear whether any of these procaspases become active within the mitochondria or after their release into the cytosol. Mitochondrial procaspase-3 is present in a complex with HSP10 and 60, and upon induction of apoptosis, procaspase-3 is dissociated from the HSP complex simultaneously with the release of HSP from mitochondria and activation of caspases (Samali et al., 1999b). Another important regu-

lator of apoptosis, second mitochondria-derived activator of caspases (Smac) or direct IAP binding protein with low pH (DIABLO), is released from mitochondria into the cytosol, where it eliminates the inhibitory effect of many inhibitors of apoptosis proteins (IAPs) on the activation of several caspases (for review see Hengartner, 2000). Apoptosis-inducing factor (AIF) is also located in the intermembrane space of mitochondria and upon apoptosis induction redistributes to the nucleus where it is involved in nuclear chromatin condensation and chromatin fragmentation with formation of high-molecular-weight DNA fragments (Daugas et al., 2000). The role of other proteins released from mitochondria (i.e., adenylate kinase-2 and sulfite oxidase) in the apoptotic process is unknown.

Two groups have presented evidence that a high ATP level is required during the apoptotic process and suggested that the level of intracellular ATP determines whether a cell will die by apoptosis or necrosis (Nicotera and Leist, 1997; Tsujimoto, 1997). During exposure to glutamate, both mitochondrial membrane potential and ATP levels decline in many neurons (Ankarcrona et al., 1995). In neurons with irreversibly dissipated mitochondrial potentials, necrosis rapidly ensued. The surviving population recovered both mitochondrial membrane potential and energy levels and subsequently underwent delayed apoptosis. It was postulated that the loss of energy and the onset of rapid necrosis simply prevents the activation of the default apoptotic program. This postulate was supported by the observation that treatment of cerebellar granule cells with a combination of glutamate and the irreversible mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) resulted in necrosis for most of the neuronal population, rather than in the delayed apoptosis observed with glutamate alone.

There is abundant evidence for an ATP requirement in several apoptosis models, although the critical ATP-dependent steps in the process have not been characterized in detail. They are likely to be linked to both the signaling and the execution phases of apoptosis. It was suggested (Tsujimoto, 1997) that the caspase protease cascade is regulated by ATP-dependent reactions, and it has been shown (Yasuhara et al., 1997) that the active nuclear transport mechanism, which requires ATP hydrolysis, is involved in the apoptotic changes of the nuclei. It is likely that another ATP-dependent step or steps downstream from caspase activation is important for perpetuation of the apoptotic process. This fact could explain the previously published data on the ATP requirement for induction of nuclear apoptosis induced by different cytoplasmic extracts (Lazebnik et al., 1993; Newmeyer et al., 1994; Kass et al., 1996). Thus, it is not surprising that the depletion of cellular ATP blocks various events in apoptosis. The question that remains to be answered is whether and how ATP depletion redirects the death process towards necrosis when cells have been triggered with apoptotic stimuli, such as anti-CD95 antibodies. In addition to the possible requirement of ATP for caspase activation, redirection could also be explained by an unknown mechanism, which shortens the survival time of cells that would eventually die by necrosis resulting from energy deprivation.

The next important question that remains to be resolved is an apparent contradiction between, on the one hand, the induction of the mitochondrial permeability transition associated with a cessation of mitochondrial ATP synthesis, and on the other, a need for maintenance of the intracellular ATP level during development of apoptosis, which may take many hours or days. In addition, hyperproduction of reactive oxygen species (ROS) results from the loss of cytochrome *c* from the respiratory chain; yet the cell needs to maintain a reducing environment for optimal caspase activity. These controversies could be resolved if the proposed apoptosis-induced changes were restricted to a subset of mitochondria, or that the maintenance of the energy level required by cells to undergo apoptosis could occur by glycolysis. This would be consistent with the observation by Jacobson et al. (1993) that cells lacking mitochondrial DNA and a functional respiratory chain can still undergo apoptosis. It is very likely that the mitochondrial permeability transition associated with a cessation of mitochondrial ATP generation plays an important role in necrotic cell death, whereas leakage of cytochrome *c* from morphologically intact mitochondria via a yet unknown and more selective pathway than outer-membrane rupture following permeability transition–induced mitochondrial swelling plays an important role in apoptotic cell death.

GENETIC MODULATION OF APOPTOTIC CELL DEATH

In recent years, enough evidence has been gathered to suggest a role for the protein products of a number of oncogenes and tumor-suppressor genes, as well as stress proteins and acute phase proteins, in the modulation of cell

death. These can be grouped into genes whose products are positive or negative regulators of cell death. The genes with a key role in the modulation of cell death and their effects on both apoptosis and necrosis are reviewed below.

c-myc

The *c-myc* proto-oncogene is a short-lived nuclear protein and a member of a family of closely related genes that also include *v-myc*, *n-myc*, and *b-myc*. The *Myc* family plays a key role in the regulation of cell proliferation (for review see Ryan and Birnie, 1996). It has been demonstrated that *c-myc* is capable of driving apoptosis (Evan et al., 1992). For instance, when T cell hybridomas were stimulated through their CD3/T cell receptor, they rapidly underwent apoptosis that could be blocked using an antisense oligonucleotide to *c-myc* mRNA. Furthermore, in fibroblasts, *c-myc* overexpression can induce apoptosis. The decision to proliferate or die in cells overexpressing *c-myc* seems to depend on the presence or absence of extracellular survival factors (Evan and Littlewood, 1998).

p53

The p53 protein is the product of a tumor-suppressor gene, which is mutated in over half of all human tumors, thus implicating p53 inactivation in tumorigenesis. Nevertheless, the main functions of p53 appear to be in mediating the cellular response to DNA damage and maintaining genomic stability (Kastan et al., 1991). Many forms of genotoxic stress induce a rapid increase in p53 protein levels, through both stabilization of the protein (Kastan et al., 1991) and an increase in p53 mRNA levels (Sun et al., 1995). This protein induces growth suppression by regulating cell-cycle arrest and/or inducing apoptosis. Reintroduction and overexpression of wild-type p53 in cells that have lost endogenous p53 function may induce apoptosis (Yonish-Rouach et al., 1991). In this regard, p53 is shown to be required for both irradiation-induced G1 arrest and apoptosis in several cell systems (Lowe et al., 1993). Induction of growth arrest by p53 has been shown to be transcription activation–dependent and appears to involve induction of *p21* (*Waf1/Cip/sdi1*) and growth arrest and DNA damage-inducible gene 45 (*Gadd45*; for review see Somasundaram and El-Deiry, 2000).

Although transcriptional activation appears to be involved in the induction of growth arrest, little is known about the possible downstream elements in this apoptotic pathway. p53 has been shown to induce apoptosis in the presence of transcriptional activation, in which case p53 may induce expression of CD95. Moreover, p53 transcription activates several genes whose products can translocate to the mitochondrial outer membrane (e.g., the two proapoptotic Bcl-2 family members Bax and Noxa) or to an unknown submitochondrial compartment, presumably to the mitochondrial matrix (p53AIP1). In addition, direct death signal–induced translocation of p53 to mitochondria has been documented (for review see Ferri and Kroemer, 2001).

Recent identification of two homologues, p63 and p73, revealed that p53 is a member of a family of related transcription factors. Given that they share amino acid sequence identity, all three proteins should have redundant functions in the regulation of gene expression. Indeed, p73 can activate p53-regulated genes and suppress growth or induce apoptosis. Moreover, p73 and p53 are both induced by DNA damage, albeit through distinct mechanisms. Other evidence, however, suggests that p63 and p73 are important for regulation of normal development and in many p53-independent pathways (for review see Levrero et al., 2000; Yang and McKeon, 2000).

Bcl-2 family

This is a family of genes in which many members hold key positions in the apoptotic machinery. The proteins in this family share highly conserved regions, which have been referred to as *Bcl-2* homology domains (i.e., BH1 to BH4; for review see Gross et al., 1999). Family members that act as inhibitors of cell death harbor at least BH1, BH2, and BH4 domains, which are required for protein-protein interaction and the suppression of apoptosis, whereas BH3 serves as the minimal "death domain" in the proapoptotic members studied so far (for review see Fadeel et al., 1999).

Bcl-2 was the first member of this family to be identified in follicular B cell lymphomas (Bakhshi et al., 1985). Several other family members, which were discovered later, may function to inhibit apoptosis, including *Bcl-2, Bcl-X_L, Bcl-W, Bfl1/A1, Mcl-1, Bag-1, BRAG-1, NR-13, Boo/Diva,* and *BHRF-1,* while others function to accelerate the rate of apoptosis once it has been induced, including *Bax, Bcl-X_s, Bad, Bak, Bar, Bid, Bik/Nbk, Bok/Mtd, Bim/Bod, Blk, Hrk/DP5, Noxa, Bnip3, Nix, Bcl-G,* and *MAP-1* (for review see Fadeel et al., 1999).

Localization is an important theme in signal transduction and increased evidence has been

provided that the regulation of apoptotic signals by Bcl-2-like proteins is dependent on their specific intracellular movement.

Bcl-2 protein is localized to the mitochondria, endoplasmic reticulum, and nuclear membrane (Hockenbery et al., 1990). Bcl-2 appears to have antioxidant properties, as it can inhibit apoptosis induced by reactive oxygen species (Hockenbery et al., 1993); however, it has also been demonstrated that Bcl-2 can inhibit cell death under conditions that preclude the formation of reactive oxygen species (Jacobson et al., 1993). Thus, the ability of Bcl-2 to protect against the damaging effects of free radicals does not appear to account for the suppression of cell death by Bcl-2.

Bax is more ubiquitously expressed than Bcl-2 (Krajewski et al., 1994). The *bax* gene promoter contains four p53-binding sites, and it has been shown that the levels of its protein can be up-regulated by p53. The countervailing roles of pro- and antiapoptotic Bcl-2 family members can be regulated through protein-protein interaction and formation of homo- and/or heterodimers. Thus, Bcl-2 can heteromerize with Bax, Bid might interact with Bax and Bak, and Bax can also interact with Bcl-X_L, Mcl-1, and A1. Function of Bcl-2 like proteins in addition to homo- or heteromerization can be regulated via phosphorylation (i.e., Bcl-2, Bcl-X_L, Bim, and Bad), *N*-myristoylation (Bid), translocation (Bid, Bax, Bcl-X_L, and Bad), insertion (Bcl-2, Bcl-X_L, Bax, and Bid), conformational changes (Bak and Bax), and cleavage (Bcl-2, Bcl-X_L, and Bax). Many protein-protein interactions take place either at the level of mitochondria or induce the movement of proteins to mitochondria. Thus, after cleavage by caspase-8, the cleaved Bid (tBid) interacts in the cytosol with Bax. This interaction leads to conformational changes of Bax, its insertion into mitochondrial membrane and oligomerization. All these events result in cytochrome *c* release. Cleaved Bid can also translocate and insert into the outer mitochondrial membrane, where it might induce conformational changes of Bak constitutively anchored to this membrane. This again leads to oligomerization of Bak, its insertion into the membrane, and release of cytochrome *c*. The antiapoptotic proteins Bcl-2 and Bcl-X_L inhibit the conformational changes and/or oligomerization and insertion of Bax and Bak. Antiapoptotic proteins from the Bcl-2 family may counteract the effect of both caspase-dependent and caspase-independent models of cell death through manifold independent functions. Although much progress has been made toward understanding the mechanisms of pro- and antiapoptotic functions of Bcl-2 family proteins, numerous issues remain unresolved.

It has been shown that overexpression of Bcl-2 can also inhibit necrosis, at least in neuronal cells, when induced by such diverse stimuli as viruses, hypoxia, oxidative stress, or exposure to toxicants (Lezoulac'h et al., 1996; Shimizu et al., 1996). Whether this effect is cell-specific remains unclear. The mechanism of action of Bcl-2 in cells undergoing necrosis may simply involve antioxidant activity. The second possibility is that Bcl-2 prevents the consequences of mitochondrial dysfunction (i.e., collapse of the mitochondrial transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix calcium and glutathione, and release of soluble intermembrane proteins), all of which can lead to a bioenergetic catastrophe culminating in the disruption of plasma membrane integrity, which is the ultimate result of necrosis (Kroemer, 1997); however, more work should be done to justify this hypothesis and to understand the mechanism of Bcl-2-induced protection from necrosis.

Ras

Members of the Ras family of proteins play an essential role in the control of normal cell growth and may induce transformation. Certain evidence has led a number of groups to advocate that Ras, in addition to inducing transformation, can also prevent cell death as overexpression of the *ras* gene was shown to inhibit apoptosis in a number of cases (Wyllie et al., 1987; Arends et al., 1993). Ras appears to be a focal point for the convergence of many signaling pathways, especially during receptor/ligand interaction, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and nerve growth factor (NGF), T cell receptor–CD3 (TCR-CD3), and CD95. The question as to which protein downstream of the pathway is the key effector of the antiapoptotic signal through Ras is unclear, but it may involve the activation of MAP kinase or phosphotidylinositol 3 kinase or both.

Stress Proteins

The stress proteins are a set of evolutionarily conserved proteins that are synthesized or activated in response to stress (for review see Lindquist and Craig, 1988). The main function of these proteins is to afford protection to cells

in times of stress. When the stress element is removed, these cells continue to function normally and the levels drop back to normal. Heat-shock proteins (HSPs), a subgroup of stress proteins, were first demonstrated to be induced as a response to elevated temperatures.

There is strong evidence suggesting that the induction of HSPs coincides with the acquisition of tolerance to higher doses of stress, which may otherwise be lethal to the cell. In this regard, it was demonstrated that a mild heat shock induces the rapid synthesis of a number of HSPs (Jaattela et al., 1992; Mosser and Martin, 1992) and that heat-shocked or thermotolerant cells demonstrated a greater degree of resistance to environmental stress (Jaattela et al., 1992), probably by resisting apoptosis (Samali and Cotter, 1996). Indeed, several recent publications reveal an important role for heat-shock proteins in apoptosis (for review see Xanthoudakis and Nicholson, 2000). These proteins can positively or negatively regulate the apoptotic process. Thus, a direct interaction of caspase-3 with HSP60 ultimately results in stimulation of proenzyme maturation. Direct binding of HSP70 or HSP90 to Apaf-1 precludes the eventual recruitment of procaspase-9 to the apoptosome complex. HSP70 can inhibit apoptotic processes downstream of the release of cytochrome *c* and upstream of the activation of caspase-3 as well as exert a protective effect against apoptosis by targeting one or more steps downstream of caspase-3. Elevated levels of HSP70 prevent the morphological changes that are characteristic of dying cells as well as the death of tumor cells despite the presence of cytochrome *c* and active caspase-3 in the cytosol. In addition, HSP27 was shown to bind to cytochrome *c* upon its release from mitochondria, thus preventing its interaction with Apaf-1. HSP27 might also directly bind and inhibit processing of procaspase-3. Thus, it remains to be determined whether these observations reflect a difference in signaling between different cell types or whether the results can be reconciled on the basis of some experimental variable. It was demonstrated that thermotolerant or HSP-overexpressing cells are more resistant to cell death induced by hyperthermia or growth-factor withdrawal (Mailhos et al., 1993). This may suggest a possible role for HSPs in the resistance mechanism. Furthermore, HSPs are also implicated in drug resistance (McClean and Hill, 1992) and heat-shocked or HSP-overexpressing cells become more resistant to the cytotoxic effects of some anticancer drugs (Oesterreich et al., 1993) that are otherwise capable of inducing both apoptosis and necrosis (Martin et al., 1990).

CONCLUSIONS

Cell damage leading to cell death may occur via apoptosis or necrosis. The mode of cell death depends on the nature of the stimuli but in extreme situations the final outcome is cell disruption and disintegration. In other words, necrosis is the acute type of cell death or the cellular response to extreme injury. During necrosis, chromatin adopts a highly flocculated form, and the DNA from these cells is digested randomly to give a smear when analyzed by agarose gel electrophoresis. The main feature of necrosis is an increase in cell volume. The rapid increase in cell volume results in membrane rupture and cell lysis (for review see Trump and Berezesky, 1995).

After two decades of research and development in the field of cell death, it is now universally accepted that apoptosis is an essential strategy for maintaining the dynamic balance and equilibrium of living systems, and is observed to occur as a normal mechanism in development and homeostasis, as well as an altruistic mode of inducible cell death. Classification of cell death is now based on morphological and biochemical criteria, their circumstantial occurrence, or a combination of both. Although morphological characterization of apoptosis and the features distinguishing it from necrosis have been well documented, progress in our understanding of the mechanisms underlying the process has been quite slow.

There is now much evidence that classic apoptosis and necrosis represent only definitive endpoints for these two processes; however, both types of cell death can occur either simultaneously or sequentially in the same cell population or tissue depending on type and/or dose of toxic insult. This suggests that some early biochemical and morphological events might be similar or even identical in both types of cell death. If this is the case, different downstream events might be important in determining whether the cell will undergo apoptosis or necrosis. It could also be that after intensive treatment, the cellular apoptotic machinery becomes suppressed, after which necrosis would predominate. The events summarized in Table 18.1.1 can be observed at different levels of cellular organization. Thus, both physiological or pathological stimuli can induce apoptosis, but only pathological injury leads to necrosis. Apoptosis, in contrast to necrosis, occurs in individual cells in an asynchronous fashion

without damaging neighboring cells. Both processes can be protected by certain inhibitors before a point of no return is reached. Once the cascade of caspase proteolytic activities becomes active, apoptosis cannot be completely reversed. The point of no return during necrosis remains to be elucidated. Nevertheless, cells can be rescued from necrosis as well as from apoptosis.

Cellular shrinkage is a hallmark of apoptosis and is seen in many if not all cell types that undergo this type of death. Cell-volume regulation is a complex phenomenon, involving the homeostasis of intracellular ions such as chloride, sodium, and potassium. As the most abundant intracellular ion, K^+ has been implicated in several of the regulatory mechanisms governing cell volume, including regulatory volume decrease. It has been shown that intracellular K^+ content decreases in cells undergoing apoptosis induced by diverse stimuli (for review see Yu and Choi, 2000). Work in the authors' laboratory has shown a potential link between glutathione (GSH) efflux and apoptotic cell shrinkage (Nobel, 1997). Inhibitors of apoptotic GSH efflux were able to inhibit cell shrinkage in thymocytes induced to undergo apoptosis by etoposide without affecting DNA fragmentation. Moreover, the GSH efflux was accompanied by K^+ efflux in Jurkat cells treated with anti-CD95 antibody. The K^+ efflux was not blocked either by the inhibitor of GSH efflux or by any known inhibitors of K^+ channels and cotransporters tested. Concomitant with apoptotic K^+ efflux, significant inhibition of Na^+/K^+-ATPase activity was observed. Inhibition of this enzyme by ouabain caused a K^+ efflux of similar magnitude to that induced by anti-CD95 antibody, and it potentiated CD95-mediated cell shrinkage. This suggested that with Na^+/K^+-ATPase activation, K^+ and GSH effluxes facilitated apoptotic cell shrinkage, which was followed by the formation of apoptotic bodies. In contrast, necrotic cells showed extensive swelling, which resulted in subsequent disintegration of cells.

Apoptotic cells lose adhesion at the very early stages, while necrotic cells stay adherent until very late stages. As mentioned before, individual apoptotic cells can be efficiently recognized either by neighboring cells or professional macrophages and can be removed by phagocytosis. This step of the apoptotic process involves rearrangement of the plasma membrane. Removal of the dead-cell fragments before their plasma membranes are lysed has important implications for other cells within the tissues. It is assumed that apoptosis occurs without inflammation. Lysis of plasma membranes in necrotic cells seems to precede the removal of these remnants, and exudative inflammation is a common event during necrosis.

The significance of apoptosis is based on the fact that apoptotic cells tend to be "environmentally friendly" and package their contents into membrane-bound vesicles ready for ingestion by phagocytic cells, without releasing their contents into the intercellular matrix. Hence there is no inflammatory response. Apoptosis is also an altruistic mode of cell death, in that damaged or injured cells commit suicide to allow the neighboring cells to continue to proliferate without being affected by the death of the neighbor. In addition, sacrifice of individual abnormal, or potentially malignant, cells benefits the whole organism.

Most if not all mechanisms leading to cell death involve molecular damage that is potentially reversible by cellular repair mechanisms; therefore, if the repair mechanisms operate effectively, they may prevent cell death. It could be that in cells undergoing apoptosis, the cell-repair machinery is repressed by specific signals, which program cells to die. On the other hand, in cells undergoing necrosis this machinery might be directly suppressed by extremely strong insult (high doses of drugs or irradiation). Since apoptosis is a gene-directed process and many genes are involved in the regulation of this multistage action, the repression of repair processes can also be regulated on this level. There is no evidence for expression of any specific genes during necrosis, which is why it is difficult to discuss the possible requirement of gene regulation for necrotic cell death. Thus, it seems that classic necrosis is a passive process. An inhibition of the activity of a number of gene products involved in apoptosis can switch the death pathway from apoptosis to necrosis. Also rapid changes in the cellular ATP level, discussed above, and an imbalance in the ratio of nitric oxide and superoxide can switch the form of cell death. Recently, an additional form of cell death was described and named paraptosis by the authors (Sperandio et al., 2000). This internally programmed cell death is related to pathogenesis in the central nervous system and to development of some primitive eukaryotes. Although experiments indicate that this type of death depends on RNA and protein synthesis, the machinery required for apoptosis is inactive in paraptotic cells. Thus, morphologically these cells are characterized by swelling of endoplasmic reticulum and cytoplasmic vacuolization,

and do not display nuclear fragmentation and formation of apoptotic bodies. Paraptotic cells are TUNEL-negative and their DNA does not fragment. Moreover, paraptotic death is independent of well known sequential caspase activation, although expression of caspase-9 in the presence of pan-caspase inhibitors induced nonapoptotic cell death; therefore it is clear that there is "more than one way to go" (Wyllie and Golstein, 2001). The search at both molecular and biochemical levels for mechanisms of apoptosis, necrosis, and paraptosis, along with attempts to understand the differences/similarities between these types of cellular response to treatment, are currently subject to intense biological research.

LITERATURE CITED

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. 1996. Human ICE/CED-3 protease nomenclature. *Cell* 87:171.

Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A., and Nicotera, P. 1995. Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* 15:961-973.

Arai, A., Kessler, M., Lee, K., and Lynch, G. 1990. Calpain inhibitors improve the recovery of synaptic transmission from hypoxia in hippocampal slices. *Brain Res.* 532:63-68.

Arends, M.J., McGregor, A.H., Toft, N.J., Brown, E.J.H., and Wyllie, A.H. 1993. Susceptibility to apoptosis is differentially regulated by c-*myc* and Ha-*ras* oncogenes and is associated with endonuclease availability. *Br. J. Cancer* 68:1127-1133.

Armstrong, R.C., Aja, T.J., Hoang, K.D., Gaur, S., Bai, X., Alnemri, E., Litwack, G., Karanewsky, D.S., Fritz, L.C., and Tomaselli, K.J. 1997. Activation of the CED-3/ICE-related protease CPP32 in cerebellar granule neurons undergoing apoptosis, but not necrosis. *J. Neurosci.* 17:553-562.

Arora, A.S., de Groen, P.C., Croal, D.E., Emori, Y., and Gores, G.J. 1996. Hepatocellular carcinoma cells resist necrosis during anoxia by preventing phospholipase-mediated calpain activation. *J.Cell. Physiol.* 167:434-442.

Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., and Korsmeyer, S.J. 1985. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 41:899-906.

Baxter, G.D. and Lavin, M.F. 1992. Specific protein phosphorylation in apoptosis induced by ionizing radiation and heat shock in human lymphoid tumor lines. *J. Immunol.* 148:1949-1954.

Bernelli-Zazzera, A. 1975. Ribosomes in dying liver cells. *In* Pathogenesis and Mechanisms of Liver Cell Necrosis (D. Keppler, ed.) pp.103-111. MTP Press, Lancaster.

Boe, R., Gjertsen, B.T., Vintermyr, O.K., Houge, G., Lanotte, M., and Doskeland, S.O. 1991. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. *Exp. Cell Res.* 195:237-246.

Bowen, I.D. 1981. Techniques for demonstrating cell death. *In* Cell Death in Biology and Physiology (I.D. Bowen and R.A. Lockshin, eds.) pp. 379-444. Chapman & Hall, New York.

Brown, D.G., Sun, X.M., and Cohen, G.M. 1993. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. *J. Biol. Chem.* 268:3037-3039.

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. 1999. Biochemical pathways of caspase activation during apoptosis. *Ann. Rev. Cell Dev. Biol.* 15:269-290.

Croal, D.E. and DeMartino, G.N. 1991. Calcium-activated neutral protease (calpain) system: Structure, function and regulation. *Physiol. Rev.* 71:813-847.

Daugas, E., Susin, S.A., Zamzami, N., Ferri, K.F., Irinopoulou, T., Larochette, N., Prevost, M.C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. 2000. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J.* 14:729-739.

de Duve, C. 1963. The lysosome concept. *In* Lysosomes (A.V.S. deReuck and M.P. Cameron, eds.) pp.101-118. Churchill Livingston, London.

Dickinson, J.L., Bates, E.J., Ferrante, A., and Antalis, T.M. 1995. Plasminogen activator inhibitor type 2 inhibits tumor necrosis factor alpha–induced apoptosis. Evidence for an alternate biological function. *J. Biol. Chem.* 270:27894-27904.

Duvall, E., Wyllie, A.H., and Morris, R.G. 1985. Macrophage recognition of cells undergoing cell death (apoptosis). *Immunology* 56:351-358.

Evan, G. and Littlewood, T. 1998. A matter of life and cell death. *Science* 281:1317-1322.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Hartmut, L., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69:119-128.

Fadeel, B., Zhivotovsky, B., and Orrenius, S. 1999. All along the watchtower: On the regulation of apoptosis regulators. *FASEB J.* 13:1647-1657.

Fadeel, B., Orrenius, S., and Zhivotovsky, B. 2000. The most unkindest cut of all: On the multiple roles of mammalian caspases. *Leukemia* 14:1514-1525.

Fadok, V., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A., and Henson, P.M. 2000. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 405:85-90.

Ferri, K.F. and Kroemer, G. 2001. Mitochondria–the suicide organelles. *BioEssays* 23:111-115.

Fesus, L., Thomazy, V., and Falus, A. 1987. Induction and activation of tissue transglutaminase during programmed cell death. *FEBS Lett.* 224:104-108.

Forbes, I.J., Zalewski, P.D., Gianniakis, C., and Cowled, P.A. 1992. Induction of apoptosis in chronic lymphocytic leukaemia cells and its prevention by phorbol esters. *Exp. Cell Res.* 198:367-372.

Glucksmann, A. 1951. Cell death is normal vertebrate ontology. *Biol. Rev.* 26:59-86.

Gorczyca, W., Bruno, S., Darzynkiewicz, R.J., Gong, J., and Darzynkiewicz, Z. 1992. DNA strand breaks occurring during apoptosis: Their early detection by terminal deoxynucleotide transferase and nick translation assays and prevention by serine protease inhibitors. *Int. J. Oncol.* 1:639-648.

Gross, A., McDonel, J.M., and Korsmeyer, S.J. 1999. Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev.* 13:1899-1911.

Haslett, C. 1992. Resolution of acute inflammation and the role of apoptosis in tissue fate of granulocytes. *Clin. Sci.* 83:639-648.

Hengartner, M.O. 2000. The biochemistry of apoptosis. *Nature* 407:770-776.

Hockenbery, D.M., Nunez, G., Milliman, C., Schreiber, R.D., and Korsmeyer, S.J. 1990. Bcl-2 in inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334-336.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75:241-251.

Holownia, A., Ledig, M., and Menez, J.F. 1997. Ethanol-induced cell death in cultured rat astroglia. *Neurotoxicol. Teratol.* 19:141-146.

Houge, G. and Doskeland, S.O. 1996. Divergence towards a dead end? Cleavage of the divergent domain of ribosomal RNA in apoptosis. *Experientia* 52:963-967.

Houge, G., Doskeland, S.O., Boe, R., and Lanotte, M. 1993. Selective cleavage of 28S rRNA variable regions V3 and V13 in myeloid leukemia cell apoptosis. *FEBS Lett.* 315:16-20.

Jaattela, M., Wissing, D., Bauer, P., and Li, G.C. 1992. Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J.* 11:3507-3512.

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C., and Raff, M.C. 1993. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* 361:365-369.

Jensen, P.H., Cressey, L.I., Gjertsen, B.T., Madsen, P., Mellgren, G., Hokland, P., Gliemann, J., Doskeland, S.O., Lanotte, M., and Vintermyr, O.K. 1994. Cleaved intracellular plasminogen activator inhibitor 2 in human myeloleukaemia cells is a marker of apoptosis. *Br. J. Cancer* 70:834-840.

Johnson, D.E. 2000. Noncaspase proteases in apoptosis. *Leukemia* 14:1695-1703.

Kaiser, N. and Edelman, I.S. 1978. Further studies on the role of calcium in glutocorticoid-induced lympholysis. *Endocrinology* 103:936-942.

Kass, G.E.N., Eriksson, J.E., Weis, M., Orrenius, S., and Chow, S.C. 1996. Chromatin condensation during apoptosis requires ATP. *Biochem. J.* 318:749-752.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. 1991. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* 51:6304-6311.

Kerr, J.F.R. 1971. Shrinkage necrosis: A distinct mode of cellular death. *J. Pathol.* 105:13-20.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. 1972. Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* 26:239-257.

Kizaki, H., Tadakuma, T., Odaka, C., Maramatsu, J., and Isimura, Y. 1989. Activation of suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esters. *J. Immunol.* 143:1790-1794.

Koopman, G., Reutelingsperger, C.P.M., Kuijten, G.A.M., Keehnen, R.M.J., Pals, S.T., and van Oers, M.H.J. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 84:1415-1420.

Krajewski, S., Krajewska, M., Shabaik, A., and Reed, J.C. 1994. Immunohistochemical determination of in vivo distribution of bax, a dominant inhibitor of bcl-2. *Am. J. Pathol.* 145:1323-1336.

Krammer, P.H. 2000. CD95's deadly mission in the immune system. *Nature* 407:789-795.

Kroemer, G. 1997. Mitochondrial implication in apoptosis: Towards an endosymbiont hypothesis of apoptosis evolution. *Cell Death Differ.* 4:443-456.

Lavin, M.F., Watters, D., and Song, Q. 1996. Role of protein kinase activity in apoptosis. *Experientia* 52:979-994.

Lazebnik, Y.A., Cole, S., Cooke, C.A., Nelson, W.G., and Earnshaw, W.C. 1993. Nuclear events of apoptosis in vitro in cell-free mitotic extracts: A model system for analysis of the active phase of apoptosis. *J. Cell Biol.* 123:7-22.

Levrero, M., De Laurenzi, V., Costanzo, A., Sabatini, S., Gong, J., Wang, J.Y.J., and Melino, G. 2000. The p53/p63/p73 family of transcription factors: Overlapping and distinct functions. *J. Cell Sci.* 113:1661-1670.

Lezoulac'h, F., Rupprecht, R., Holsboer, F., and Behl, C. 1996. *Bcl-2* prevents hippocampal cell death induced by the neuroleptic drug haloperidol. *Brain Res.* 738:176-179.

Lindquist, S. and Craig, E.A. 1988. The heat shock proteins. *Annu. Rev. Genet.* 22:631-677.

Liu, X., Kim, N.C., Yang, J., Jemmerson, R., and Wang, X. 1996. Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome *c*. *Cell* 86:147-157.

Liu, X., Zou, H., Widlak, P., Garrard, W., and Wang, X. 1999. Activation of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease). Oligomerization with histone H1. *J. Biol. Chem*. 274:13836-13840.

Lockshin, R.A. 1981. Cell death in metamorphosis. *In* Cell Death in Biology and Physiology (I.D. Bowen and R.A. Lockshin, eds.) pp. 79-121. Chapman & Hall, New York.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362:847-849.

Mailhos C., Howard, M.K., and Latchman, D.S. 1993. Heat shock protects neuronal cells from programmed cell death by apoptosis. *Neuroscience* 55:621-627.

Martin, S.J., Lennon, S.V., Bonham, A.M., and Cotter, A.M. 1990. Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J. Immunol.* 145:1859-1867.

McClean, S. and Hill, B.T. 1992. An overview of membrane, cytosolic and nuclear proteins associated with the expression of resistance to multiple drugs in vitro. *Biochem. Biophys. Acta* 1114:107-127.

McConkey, D.J., Zhivotovsky, B., and Orrenius, S. 1996. Apoptosis: Molecular mechanisms and biomedical implications. *Mol. Aspects Med.* 17:1-110.

McIlroy, D., Tanaka, M., Sakahira, H., Fukuyama, H., Suzuki, M., Yamamura, K.-I., Uchiyama, Y., and Nagata, S. 2000. An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. *Genes Dev.* 14:549-558.

Mirabelli, F., Salis, A., Vairetti, M., Bellomo, G., Thor, H., and Orrenius, S. 1989. Cytoskeletal alterations in human platelets exposed to oxidative stress are mediated by oxidative and Ca^{2+}-dependent mechanisms. *Arch. Biochem. Biophys.* 270:478-488.

Mosser, D.D. and Martin, L.H. 1992. Induced thermotolerance to apoptosis in a human T lymphocyte cell line. *J. Cell. Physiol.* 151:561-570.

Nagata, S. 2000. Apoptotic DNA fragmentation. *Exp. Cell Res.* 256:12-18.

Newmeyer, D.D., Farston, D.M., and Reed, J.C. 1994. Cell-free apoptosis in Xenopus egg extracts: Inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 79:353-364.

Nicholson, D.W. and Thornberry, N.A. 1997. Caspases: Killer proteases. *Trends Biochem. Sci.* 22:299-306.

Nicotera, P. and Leist, M. 1997. Energy supply and the shape of cell death in neurones and lymphoid cells. *Cell Death Differ.* 4:435-442.

Nicotera, P. and Orrenius, S. 1998. The role of calcium in apoptosis. *Cell Calcium* 23:173-180.

Nicotera, P., Hartzell, P., Davis, G., and Orrenius, S. 1986. The formation of plasma membrane blebs in hepatocytes exposed to agents that increased cytosolic Ca^{2+} is mediated by the activation of a non-lysosomal proteolytic system. *FEBS Lett.* 209:139-144.

Nishizuka, Y. 1984. The role of PKC in cell surface signal transduction and tumour promotion. *Nature* 308:693-698.

Nobel, S. 1997. Thiol redox state in apoptosis: Physiological and toxicant modulation. Ph.D. Thesis, Karolinska Institutet, Stockholm.

Oesterreich, S., Weng, C., Qiu, M., Hilsenbeck, S.G., Osborne, C.K., and Fuqua, S.A.W. 1993. The small heat shock protein hsp27 is correlated with growth and drug resistance in human breast cancer cell lines. *Cancer Res.* 53:4443-4448.

Orlowski, R. Z. 1999. The role of the ubiquitin-proteasome pathway in apoptosis. *Cell Death Differ.* 6:303-313.

Robertson, J.D., Orrenius, S., and Zhivotovsky, B. 2000. Nuclear events in apoptosis. *J. Struct. Biol.* 129:346-358.

Ryan, K.M. and Birnie, G.D. 1996. *Myc* oncogenes: The enigmatic family. *Biochem. J.* 314:713-721.

Salvesen, G.S. 2001. A lysosomal protease enters the death scene. *J. Clin. Invest.* 107:21-22.

Samali, A. and Cotter, T.G. 1996. Heat shock proteins increase resistance to apoptosis. *Exp. Cell Res.* 223:163-170.

Samali, A., Gilje, B., Doskeland, S.O., Cotter, T.G., and Houge, G. 1997. The ability to cleave 28S rRNA during apoptosis is a cell type dependent trait unrelated to DNA fragmentation. *Cell Death Differ.* 4:289-293.

Samali, A., Nordgren, H., Zhivotovsky, B., Peterson E., and Orrenius, S. 1999a. A comparative study of apoptosis and necrosis in HepG2 cells. Oxidant-induced caspase activation leads to necrosis. *Biochem. Biophys. Res. Commun.* 256:6-11.

Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P., and Orrenius, S. 1999b. Presence of a pre-apoptotic complex of procaspase-3, Hsp60 and Hsp10 in the mitochondria fraction of Jurkat cells. *EMBO J.* 18:2040-2048.

Saunderson, C.J. 1982. Morphological aspects of lymphocyte mediated cytotoxicity. *In* Mechanisms of Cell Mediated Cytotoxicity (W.R. Clark and P. Golstein, eds.) pp. 3-21. Plenum, New York.

Savill, J. and Fadok, V. 2000. Corpse clearance defines the meaning of cell death. *Nature* 407:784-788.

Shi, L., Kam, C.M., Powers, J.C., Aebersold, R., and Greenberg, A. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interaction. *J. Exp. Med.* 176:1521-1529.

Shimizu, S., Eguchi, Y., Kamiike, W., Itoh, Y., Hasegawa, J., Yamabe, K., Otsuki, Y., Matsuda, H., and Tsujimoto, Y. 1996. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by *Bcl-2* and *Bcl-x$_L$*. *Cancer Res.* 56:2161-2166.

Shinohara, K., Tomioka, M., Nakano, H., Tone, S., Ito, H., and Kawashima, S. 1996. Apoptosis induction resulting from proteasome inhibition. *Biochem. J.* 317:385-388.

Sims, K.D. and Robinson, M.B. 1999. Expression patterns and regulation of glutamate transporters in the developing and adult nervous system. *Crit. Rev. Neurobiol.* 13:169-197.

Somasundaram, K. and El-Deiry, W.S. 2000. Tumor suppressor p53: Regulation and function. *Frontiers Biosci.* 5:D424-D437.

Sperandio, S., de Belle, I., and Bredesen, D.E. 2000. An alternative, non-apoptotic form of programmed cell death. *Proc. Natl. Acad. Sci. U.S.A.* 97:14376-14381.

Stennicke, H.R. and Salvesen, G.S. 2000. Caspases–controlling intracellular signals by protease zymogen activation. *Biochim. Biophys. Acta* 1477:299-306.

Stoka, V., Turk, B., Schendel, S.L., Kim, T.-H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M., Brömme, D., Krajewski, S., Reed, J.C., Yin, X.-M., Turk, V., and Salvesen, G.S. 2001. Lysosomal protease pathways to apoptosis. Cleavage of Bid, not procaspases, is the most likely route. *J. Biol. Chem.* 276:3149-3157.

Sun, X., Shimizu, H., and Yamamoto, K. 1995. Identification of a novel p53 promoter element involved in genotoxic stress-inducible p53 gene expression. *Mol. Cell. Biol.* 15:4489-4496.

Trump, B.F. and Berezesky, I.K. 1995. Calcium-mediated cell injury and cell death. *FASEB J.* 9:219-228.

Trump, B.F. and Ginn, F.L. 1969. The pathogenesis of subcellular reaction to lethal injury. *In* Methods and Achievements in Experimental Pathology, vol. 4 (E. Bajusz and G. Jasmin, eds.) p. 1. Karger, Basel.

Tsujimoto, Y. 1997. Apoptosis and necrosis: Intracellular ATP level as a determinant for cell death modes. *Cell Death Differ.* 4:429-434.

Vanags, D.M., Pörn-Ares, M.A., Coppola, S., Burgess, D.H., and Orrenius, S. 1996. Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J. Biol. Chem.* 271:31075-31085.

Van Lancker, J.L. 1975. Hydrolases and cellular death. *In* Pathogenesis and Mechanisms of Liver Cell Necrosis (D. Keppler, ed.) pp. 25-35. MTP Press, Lancaster.

Wolf, C.M. and Eastman, A. 1999. Intracellular acidification during apoptosis can occur in the absence of a nucleus. *Biochem. Biophys. Res. Commun.* 254:821-827.

Wu, Y.-C., Stanfiels, G.M., and Horvitz, H.R. 2000. Nuc-1, a *Caenorhabditis elegans* DNase II homolog, functions in an intermediate step of DNA degradation during apoptosis. *Genes Dev.* 14:536-548.

Wyllie, A.H. 1980. Glutocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284:555-556.

Wyllie, A. and Golstein, P. 2001. More than one way to go. *Proc. Natl. Acad. Sci. U.S.A.* 98:11-13.

Wyllie, A.H., Rose, K.A., Morris, R.G., Steel, C.M., Forster, E., and Spandidos, D.A. 1987. Rodent fibroblast tumors expressing human *myc* and *ras* genes: Growth, metastasis and endogenous oncogene expression. *Br. J. Cancer* 56:251-259.

Xanthoudakis, S. and Nicholson, D.W. 2000. Heat-shock proteins as death determinants. *Nature Cell Biol.* 2:E163-E165.

Yang, A. and McKeon, F. 2000. p63 and p73: p53 mimics, menaces and more. *Nature Rev. Mol. Cell Biol.* 1:199-207.

Yasuhara, N., Eguchi, Y.M., Tachibana, T., Imamoto, N., Yoneda, Y., and Tsujimoto, Y. 1997. Essential role of active nuclear transport in apoptosis. *Genes Cells* 2:55-64.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kim-Chi, A., and Oren, M. 1991. Wild-type *p53* induces apoptosis of myeloid leukaemic cells that is inhibited by interlukin-6. *Nature* 353:345-347.

Yu, S.P. and Choi, D.W. 2000. Ions, cell volume, and apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 97:9360-9362.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., and Horwitz, R.H. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to the mammalian interleukin-1β-converting enzyme. *Cell* 75:641-652.

Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.-A., Petit, P.X., Mignotte, B., and Kroemer, G. 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J. Exp. Med.* 182:367-377.

Zhivotovsky, B., Wade, D., Nicotera, P., and Orrenius, S. 1994. Role of nucleases in apoptosis. *Int. Arch. Allergy Immunol.* 105:333-338.

Zhivotovsky, B., Burgess, D.H., Vanags, D.M., and Orrenius, S. 1997. Involvement of cellular proteolytic machinery in apoptosis. *Biochem. Biophys. Res. Commun.* 230:481-488.

Contributed by Boris Zhivotovsky and Sten Orrenius
Karolinska Institutet
Stockholm, Sweden

Analysis of Caspase Activation During Apoptosis

This unit describes methods for the detection of caspase activation as cells undergo apoptosis. Caspases are intracellular cysteine proteases that are synthesized as zymogens and then proteolytically activated early in the course of apoptosis. This activation process can be detected by assays for cleavage of suitable fluorogenic or chromogenic substrates, by immunoblotting with monospecific antibodies, or by affinity labeling with reactive substrate analogs. Because the caspases are organized into distinct proteolytic cascades, identifying the caspases that are activated and their order of activation can provide insight into the biochemical pathways involved in that activation.

Three different approaches for assessing caspase activation are described. Enzymatic assays using suitable substrates are simple and relatively quantitative (see Basic Protocol 1). Because the various low-molecular-weight substrates available for these assays are not selective, however, the assays do not accurately distinguish between various caspases. Immunoblotting can be utilized to follow the activation of specific caspases (see Basic Protocol 2 and Alternate Protocol 1). When coupled with subcellular fractionation (Graham and Rickwood, 1997), this method can provide large amounts of temporal and spatial information about caspase activation. Like all immunological methods, however, immunoblotting is limited by the quality and availability of immunological reagents. Finally, affinity labeling can be utilized to detect active caspases in whole-cell lysates (see Basic Protocol 3 and Alternate Protocol 2) or subcellular fractions. With suitably designed affinity labels, this method can detect active caspases even when their identity and substrate specificity are unknown (e.g., when caspases are being explored in a new species before immunological reagents are available). Affinity labeling, however, is the most labor intensive of these techniques. In addition, because some procaspases label poorly with commercially available affinity labels, the lack of affinity labeling does not absolutely rule out the presence of active caspases.

ENZYMATIC ASSAYS FOR CASPASE ACTIVITY

BASIC PROTOCOL 1

In principle, the easiest method for determining whether caspases have been activated in apoptotic cells is to assay for their activity. This approach became feasible after the identification of sequences that are cleaved in various caspase substrates during apoptosis (reviewed in Nicholson and Thornberry, 1997; Earnshaw et al., 1999) and the subsequent synthesis of low-molecular-weight substrates containing the appropriate peptides coupled to fluorogenic or chromogenic leaving groups (Thornberry et al., 1992; Nicholson et al., 1995; Martins et al., 1997). It must be kept in mind, however, that the apoptotic caspases show varying degrees of preference rather than absolute specificity for the currently available caspase substrates. Accordingly, these enzymatic assays can indicate that caspases have been activated but will not necessarily provide an accurate indication of the individual caspases that have been activated.

The following protocol describes the preparation of cytosol and an assay for cleavage of the substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-DEVD-AFC). For this procedure, cells are initially subjected to conditions that are thought to induce apoptosis. These apoptosis-inducing treatments must be optimized for each cell line and inducing reagent before the protocol can be carried out. Because caspases are intracellular proteases, cells must then be lysed or fractionated. After the protein content in the lysates or subcellular fractions is measured, samples containing equal amounts of protein are incubated with the fluorogenic substrate. The change in

Contributed by Scott H. Kaufmann, Timothy J. Kottke, L. Miguel Martins, Alexander J. Henzing, and William C. Earnshaw

Current Protocols in Cell Biology (2001) 18.2.1-18.2.29

fluorescence is measured and compared to a standard curve constructed using known amounts of the fluorescent product.

Materials

Cells of interest and appropriate medium
Apoptosis-inducing stimulus
CMF-DPBS (*APPENDIX 2A*), ice cold
Lysis buffer (see recipe), 4°C
0.5 M EDTA, pH 7.4 (see recipe)
1 M dithiothreitol (DTT; *APPENDIX 2A*)
5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer, ice cold
20 mM (14.6 mg/ml) acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-DEVD-AFC; Biomol), or other fluorogenic or chromogenic caspase substrate, in dimethyl sulfoxide (DMSO)
HEPES/CHAPS buffer (see recipe), room temperature and ice cold
10 mM (4.6 mg/ml) 7-amino-4-trifluoromethylcoumarin (free AFC; Sigma), or appropriate standard for other substrate, in DMSO

Cell scraper (optional)
2-ml (total volume) tight-fitting Dounce homogenizers
Beckman TL100 ultracentrifuge and TL100.2 rotor, or equivalent, 4°C, and appropriate ultracentrifuge tubes
Fluorometer

Additional reagents and equipment for Ficoll-Hypaque density sedimentation (*UNIT 2.2*), cell trypsinization (for adherent cell lines only; *UNIT 1.1*), trypan blue staining to detect lysed cells (*UNIT 1.1*), and determination of protein concentration (*APPENDIX 3B*)

NOTE: Fluorogenic and chromogenic caspase substrates are available from a number of suppliers, including Bachem Bioscience, Biomol Research Laboratories, Calbiochem-Novabiochem, Molecular Probes, and Osaka Peptide Institute. Stock solutions for substrates and standards are prepared as 20 mM solutions in DMSO and stored in aliquots for up to 1 year at −20°C.

Induce apoptosis

1. Remove cells that have spontaneously become apoptotic before starting assay. For adherent cell lines, tap the flask containing cells of interest and change the medium to remove the dead cells. For nonadherent cell lines, remove dead cells by Ficoll-Hypaque density sedimentation (*UNIT 2.2*).

   This step reduces the background activity in this assay. For most adherent cell lines, apoptotic cells become nonadherent and can be simply washed away. Nonadherent cell lines such as leukemia and lymphoma cells require Ficoll-Hypaque density sedimentation to remove dead cells (Martins et al., 1997).

2. Treat cells with a suitable apoptosis-inducing stimulus or mock treatment. Use 1–3 $\times 10^8$ cells per time point for treatment and controls.

   The optimal treatment regimen (e.g., Kaufmann and Earnshaw, 2000) must be determined separately for each cell type and inducing agent. For reasons that are unclear, some cell lines (particularly leukemia cell lines) undergo apoptosis within 3 to 6 hr, whereas other cell lines (fibroblasts and solid tumor cell lines) take several days to die even though the same fraction of cells will ultimately die. These differences reflect varied lengths in the latent phase of apoptosis.

Harvest cells

3. Harvest cells for each time point. For adherent cells, use a cell scraper or trypsinization (*UNIT 1.1*) to release cells from their substratum.

   When working with an adherent cell line, cells that have become detached during the course of the experiment should also be collected. For many cell lines, one of the changes that occurs during apoptosis is detachment from the substratum. Discarding detached cells will cause an underestimation of the amount of apoptosis.

4. Transfer cells to a 50-ml conical centrifuge tube and centrifuge 10 min at 200 × *g*, room temperature.

5. Remove supernatant, resuspend cells in 10 ml ice-cold CMF-DPBS, and centrifuge 10 min at 200 × *g*, room temperature. Repeat once.

   All subsequent steps should be performed at 4°C unless otherwise indicated.

Isolate cell extracts

6. Remove supernatant and resuspend pellet in 1 ml ice-cold lysis buffer.

   This yields a cytosolic extract with a protein concentration ranging from 4 to 8 mg/ml.

7. Incubate sample 20 min on ice, then lyse cells in a 2-ml tight-fitting Dounce homogenizer.

   Glass-glass homogenizers work well for homogenizing cells under the conditions specified. Note that the power stroke with the homogenizer is the upstroke. Excessive force on the downstroke will cause a fountain effect. For leukemia cell lines, which have a sparse cytoskeleton, 20 to 30 strokes in a tight-fitting homogenizer are generally adequate. For epithelial cell lines, which have a dense cytoskeleton, many more strokes may be required. For some cell lines, alternative procedures such as freeze-thawing (see Basic Protocol 3) may also be employed. However, freeze-thawing disrupts membrane-enclosed compartments, making it difficult to assess subcellular distribution of activities.

8. Confirm cell lysis by mixing 3 to 5 µl homogenate with an equal volume of 0.4% (w/v) trypan blue in Hanks' balanced salt solution and examining with a hemacytometer (*UNIT 1.1*).

   If plasma membranes have been successfully disrupted, nuclei will stain blue. Continue homogenization until >95% of the nuclei stain blue.

9. Transfer homogenate to a suitable tube and centrifuge 10 min at 800 × *g* or 3 min at 16,000 × *g*, 4°C, to remove the nuclei.

10. Transfer the postnuclear supernatant to an ultracentrifuge tube. Add 0.01 vol of 0.5 M EDTA, pH 7.4.

    EDTA is added only after removal of nuclei to avoid the high viscosity that accompanies extraction of chromatin after chelation of divalent cations.

11. Centrifuge the postnuclear supernatant 60 min at 280,000 × g_{max}, 4°C, in a Beckman TL100 ultracentrifuge with a TL100.2 rotor.

12. Collect the supernatant (cytosol) and use an aliquot to determine its protein concentration (*APPENDIX 3B*).

    Caspase activity can also be examined in other fractions after suitable purification procedures (Martins et al., 1997).

    The authors generally use the bicinchoninic acid method (Smith et al., 1985) for protein concentration determination. Alternatives include the Lowry method (Cadman et al., 1979), the Coomassie blue method (Bradford, 1976), and the nitric acid method (Bible et al., 1999). No matter which method is utilized, protein measurements are required to adjust various samples to the same concentration.

13. To the remaining cytosol, add 1 M DTT to a final concentration of 1 mM.

    DTT is added to maintain sulfhydryl groups at caspase active sites in a reduced state. It could be added at earlier steps but would interfere with some protein assay methods.

14. Freeze the cytosol in 50-µl aliquots at −70°C.

    The authors generally avoid repeated freezing and thawing steps by freezing small aliquots. Control experiments from the authors' laboratory have indicated that activity capable of cleaving acetyl-DEVD-AFC is stable for ≥3 months at −70°C.

Measure enzymatic activity

15. Thaw a sample aliquot at 4°C and transfer a volume containing 50 µg cytosolic protein to a microcentrifuge tube. Dilute to 50 µl with ice-cold 5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer.

16. Add 50 µl ice-cold 5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer to a second microcentrifuge tube and treat in parallel as a negative control reaction.

    Alternatively, a reagent blank containing 50 µg boiled cytosolic protein or bovine serum albumin would also be acceptable.

17. Prepare 100 µM acetyl-DEVD-AFC in HEPES/CHAPS buffer and add 225 µl to each tube. Incubate 2 hr in a 37°C water bath.

    The 100 µM working substrate solution should be prepared immediately before use.

18. To stop the reaction, add 1.225 ml ice-cold HEPES/CHAPS buffer (final 1.5 ml) and place samples on ice.

    As indicated below, the authors have found the release of product to be a linear function of time for up to 4 hr when saturating substrate concentrations (five times the K_M) are utilized. If the reaction is run as an endpoint assay as described above, it is important to verify its linearity over time. Alternatively, continuous monitoring of fluorochrome release can be utilized to examine the kinetics of product release and/or the kinetics of enzyme inhibition if suitable equipment is available (Thornberry et al., 1992).

19. Use 10 mM free AFC in DMSO to set up a series of tubes containing a range of concentrations for a standard curve. Use 0 to 1500 pmol free AFC in 50 µl of 5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer. Add 1.45 ml HEPES/CHAPS buffer (final 1.5 ml at 0 to 1 µM AFC).

20. Measure the fluorescence of all samples in a fluorometer using an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

    Under the conditions specified, the reaction is fairly robust and can be monitored in a relatively inexpensive filter fluorometer. Alternatively, for continuous monitoring, fluorometric plate readers and fluorometers with thermostatically controlled cuvette holders are available.

    Some filter fluorometers do not have excitation and emission filters of the correct wavelength for AFC fluorescence. The authors have successfully used an excitation wavelength of 360 nm and an emission wavelength of 475 nm to measure AFC.

21. Prepare a standard curve from the fluorescence intensity of the known amounts of free AFC (step 19). Compare the fluorescence values in the cell extracts to determine the absolute amount of AFC liberated in each sample.

22. Subtract the amount of AFC liberated in the reagent blank (step 16) from each value to determine the amount of enzyme-mediated release.

23. Convert this to an activity measurement by expressing the data as product released per unit protein per unit time.

DETECTION OF CASPASE ACTIVATION BY IMMUNOBLOTTING

BASIC PROTOCOL 2

Immunoblotting provides an alternative method for detecting active caspases within cells. Caspases are synthesized as zymogens that undergo proteolytic cleavage during their activation (see Background Information, discussion of immunoblotting). Accordingly, the precursors and active caspase species can be distinguished by size differences on immunoblots using anti-caspase antibodies. The usual practice is to look for disappearance of zymogen and corresponding appearance of processed caspase subunit(s). Alternatively, it is possible to use antibodies that recognize neoepitopes (i.e., epitopes generated during caspase maturation, such as epitopes that contain the carboxylate moiety of aspartate at the C terminus of the large subunit or the free α-amino group at the N terminus of the small subunit). These anti-neoepitope antibodies have the advantage that they recognize species present only in cells containing active caspases. In using either type of antibody, the overall approach for detecting caspase activation is the same (also see *UNIT 6.2*). A second method for cell lysis is described (see Alternate Protocol 1).

Table 18.2.1 Selected Properties of Human Caspases[a]

New name	Old name(s)	Molecular weight (kDa)[b]			Preferred small substrates[c]		Antibody suppliers[d]
		Pr	Lg	Sm			
Caspase-1	ICE	45	24 20	14 10	YEVD/X	WEHD/X	BP, ORP, UB
Caspase-2	Ich-1_L, NEDD2	48	32 18	14 12	VDVAD/X	DEHD/X	BP, BTL, ORP, UB
Caspase-3	CPP32, YAMA, Apopain	32	20 17	12	DMQD/X	DEVD/X	BP, BTL, CI, ORP, UB
Caspase-4	Tx, Ich-2, ICE_{rel}II				LEVD/X	(W/L)EHD/X	BP, ORP
Caspase-5	ICE_{rel}III, Ty				Unknown	(W/L)EHD/X	CN
Caspase-6	Mch2	34	21 18	13 11	VEID/X	VEHD/X	BP, CI, CN, NEB, UB
Caspase-7	Mch3, CMH-1, ICE-LAP3	34	20	12	DEVD/X	DEVD/X	BP, BTL, NEB, ORP
Caspase-8	Mch5, FLICE, MACH	53 55	43 18	12 11	IETD/X	LETD/X	BP, CI, CN, NEB, ORP
Caspase-9	ICE-LAP6, Mch6	50	37	12	Unknown	LEDH/X	BP, CI, CN, NEB, UB
Caspase-10	Mch4, FLICE-2	55	43 17	12	IEAD/X	Unknown	CI, CN, NEB, UB
Caspase-13	ERICE				Unknown	Unknown	
Caspase-14	MICE	29	18	10	Unknown	Unknown	BTL, ORP

[a]Modified from Earnshaw et al. (1999).

[b]The appearance of multiple entries indicates partially processed and fully processed large (Lg) and small (Sm) subunits that result from sequential cleavage at the C-terminal end of the large subunit followed by removal of the linker peptide from the small subunit and the prodomain (Pr) from the large subunit. A blank in this column indicates that the molecular weight of the processed forms has not been reported.

[c]The left and right columns indicate the preferred low-molecular-weight substrate specificity reported by two groups: Talanian et al. (1997) and Thornberry et al. (1997), respectively. It is important to note, however, that additional factors also affect caspase cleavage of full-length polypeptides. Not all sites conforming to the indicated sequences are cleaved, perhaps due to limited accessibility. Conversely, polypeptides are sometimes cleaved at sites that would not be predicted based on analysis of the tetrapeptide preferences indicated in this table (Samejima et al., 1999).

[d]Abbreviations: BP, BD PharMingen; BTL, BD Transduction Laboratories; CI, Chemicon International; CN, Calbiochem-Novabiochem; NEB, New England Biolabs; ORP, Oncogene Research Products; UB, Upstate Biotechnology.

Materials

CMF-DPBS (*APPENDIX 2A*), ice cold
Serum-free tissue culture medium (appropriate for cells of interest), ice cold, optional
SDS sample buffer (see recipe)
Fast green dye solution: 0.1% (w/v) fast green FCF/20% (v/v) methanol/5% (v/v) acetic acid
Fast green destain: 20% (v/v) methanol/5% (v/v) acetic acid
Blocking buffer (see recipe)
Anti-caspase primary antibody (Table 18.2.1)
PBS-T (see recipe)
Appropriate secondary antibody conjugated to horseradish peroxidase (HRP), alkaline phosphatase (AP), or a radiolabel
3% (w/v) nonfat dry milk in CMF-DPBS
Enhanced chemiluminescence reagents (e.g., ECL from Amersham Pharmacia Biotech), for HRP-conjugated secondary antibodies
X-ray film

Cell scraper (optional)
Sonicator equipped with microprobe (e.g., Branson)
70° or 100°C water bath or heating block

Additional reagents and equipment for inducing apoptosis (see Basic Protocol 1), trypsinizing and counting cells (optional; *UNIT 1.1*), SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*), and electrophoretic transfer of polypeptides to a solid support (*UNIT 6.2*)

Induce apoptosis

1. Subject the desired number of cells to an apoptotic stimulus or mock treatment (see Basic Protocol 1, steps 1 and 2).

   It is convenient to load 2–5 × 10^5 cells per well for immunoblotting. To permit the running of multiple duplicate gels, each processed sample should contain 1–10 × 10^6 cells.

Harvest cells

2. Harvest cells at an appropriate time point. For adherent cells, use a cell scraper or trypsinization (*UNIT 1.1*) to release cells from their substratum.

   When working with an adherent cell line, cells that have become detached during the course of the experiment should also be collected. For many cell lines, one of the changes that occurs during apoptosis is detachment from the substratum. Discarding detached cells will cause an underestimation of the amount of apoptosis.

3. Transfer cells to a 15-ml conical centrifuge tube. Centrifuge 10 min at 200 to 400 × *g*, room temperature.

4. Remove supernatant and resuspend cells in 10 to 15 ml ice-cold CMF-DPBS or serum-free tissue culture medium.

   The purpose of this step is to remove serum proteins so that they do not interfere with subsequent protein determination, electrophoresis, or immunoblotting.

5. Count the cells in the sample (*UNIT 1.1*).

   If samples will be loaded based on protein concentration, cells do not need to be counted.

6. Centrifuge 10 min at 200 to 400 × *g*, room temperature. Remove supernatant without disturbing cell pellet.

Lyse cells

7. Add 20 µl SDS sample buffer per 2–5 × 10^5 cells to lyse cells. Vortex sample vigorously.

   If a cell count was not performed, a suitable sample volume is determined empirically by the inability to sonicate volumes that are too small and the inability to load volumes that are too large onto SDS-polyacrylamide gels. An alternative procedure for lysing cells in larger volumes and then concentrating them is given (see Alternate Protocol 1).

8. Use a sonicator equipped with a microprobe to deliver 40 pulses (0.33 sec each at 40% of the maximal output of the power supply) to the sample to shear the DNA.

9. Freeze sample indefinitely at −80°C or proceed to step 10 immediately.

   If samples are going to be loaded based on protein content, it is convenient to remove an aliquot for protein assays prior to freezing. Protein should be estimated using an assay that is not subject to interference by SDS, urea, or reducing agents, such as the nitric acid method (Bible et al., 1999) or the Lowry method after protein precipitation (APPENDIX 3B).

Electrophorese sample

10. Heat sample 20 min to 70°C or 3 min to 100°C in a water bath or heating block.

    This heat treatment is required to denature the protein and ensure that polypeptides are uniformly bound to SDS. If frozen samples are not heated prior to electrophoresis, there is a risk that SDS will not be uniformly bound to polypeptides and migration will be aberrant.

11. Immediately load a volume of sample containing 2–5 × 10^5 cells in the lane of an SDS-polyacrylamide gel with suitable separating capabilities.

    Alternatively, samples containing equal amounts of protein can be loaded.

    The authors prefer 20 × 20–cm gels for better resolution. Adequate results, however, can be obtained with minigels (6 × 8–cm).

    Because the subunits of active caspases are 10 to 12 kDa and 16 to 35 kDa, a gel that separates in this molecular-weight range is required. If the standard 30:0.8 acrylamide/bisacrylamide ratio (Laemmli, 1970) is utilized, a gel containing somewhere between 12% and 16% acrylamide is required to resolve in this molecular-weight range. Alternatively, a 5% to 15% or 5% to 20% gradient gel gives adequate separation of caspases and also permits analysis of their substrates by immunoblotting.

12. Electrophorese gel (*UNIT 6.1*) and electrophoretically transfer (*UNIT 6.2*) sample to a nitrocellulose or PVDF membrane.

    Because of their small sizes, caspases should readily transfer even from gels containing a high acrylamide content. If desired, transfer of higher-molecular-weight caspase substrates from these gels can be facilitated by including 0.02% to 0.1% (w/v) SDS in the transfer buffer. If this approach is taken, it is important to cool the transfer apparatus, as the SDS increases the current flow during transfer.

13. Stain the membrane for 1 min using fast green dye solution to confirm equal transfer of sample proteins. Remove unbound stain by rinsing briefly in fast green destain.

    Alternatively, blots can be stained in 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid (UNIT 6.2).

14. Rinse membrane several times in CMF-DPBS to remove residual acetic acid.

15. Incubate membrane on an orbital platform shaker ≥1 hr in blocking buffer at room temperature to block unoccupied protein-binding sites.

    This blocking step should be performed immediately after the transfer if PVDF membranes are used. Nitrocellulose membranes can be dried and blocked later.

Perform immunoblotting

16. Dilute an anti-caspase primary antibody in blocking buffer according to supplier's instructions.

    Most antisera or antibodies give good results when diluted in milk-containing buffer. Some, however, yield cleaner results when a different carrier protein, such as 1% to 3% (w/v) bovine serum albumin, is used.

17. Incubate membrane with diluted primary antibody on an orbital platform shaker overnight at room temperature.

    Some investigators prefer to incubate filters with antibody for a shorter period of time. The authors have obtained good signals after a 4 to 6 hr incubation with primary antibody. Others have reported good signals with incubation times as short as 1 hr, although the authors' experience has been that higher concentrations of antibody are required to detect a signal after a 1-hr incubation.

18. Remove the primary antibody and store it at 4°C for subsequent use.

    Aliquots of primary antibody can be used three to four times without detectable loss of signal. Additional penicillin, streptomycin, and sodium azide can cut down on microbial growth if the antibody is to be stored for several weeks.

19. Wash the membrane three times in 100 ml PBS-T (for a 20 × 20–cm membrane) for 15 min each.

    If there is extensive background on the film after an initial immunoblotting experiment, it may be helpful to use a wash buffer of increased stringency (e.g., PBS-T supplemented with 2 M urea or additional NaCl).

20. Wash the membrane twice with 100 ml CMF-DPBS for 5 min each.

21. Dilute an appropriate secondary antibody in 3% (w/v) nonfat dry milk in CMF-DPBS according to the supplier's instructions.

    If HRP-coupled secondary antibodies are used, it is important to avoid sodium azide from this point onward because azide inhibits peroxidase.

22. Add diluted secondary antibody to the membrane and incubate on an orbital platform shaker 1 hr at room temperature.

23. Discard the secondary antibody and wash filter with 100 ml PBS-T as follows:

    twice for 5 min each
    twice for 15 min each
    twice for 5 min each.

24. Follow manufacturer's instructions for enhanced chemiluminescence reagents for HRP- or AP-conjugated secondary antibodies.

    This step is omitted if radioconjugated secondary antibodies are used.

25. Expose the membrane to X-ray film for an appropriate length of time.

    When using ECL enhanced chemiluminescence reagents, the authors start with an initial 2-min exposure and then adjust the exposure time empirically.

    Primary and secondary antibodies can be removed from the membrane for reprobing (see Support Protocol 1).

CELL LYSIS WITH GUANIDINE HYDROCHLORIDE FOR IMMUNOBLOTTING

ALTERNATE PROTOCOL 1

It is sometimes inconvenient to lyse and sonicate cells in a small enough volume to permit loading on a gel. In addition, high concentrations of SDS and 2-mercaptoethanol (2-ME) interfere with many protein estimation methods. Finally, some of the authors' observations have raised the possibility that active caspase species bound to cleaved intermediate filaments (MacFarlane et al., 2000) might not be solubilized efficiently by SDS sample buffer (T.J.K. and S.H.K., unpubl. observ.). The following protocol provides an alternative that allows cells to be rapidly lysed in the strong denaturing agent guanidine hydrochloride, dialyzed into dilute SDS, and then concentrated to the desired volume prior to electrophoresis. This protocol is suitable for detecting a variety of polypeptides in addition to caspases (Kaufmann et al., 1997).

Additional Materials *(also see Basic Protocol 2)*

Guanidine hydrochloride lysis buffer (see recipe)
100 mM PMSF (*APPENDIX 2A*)
2-Mercaptoethanol (2-ME)
1.54 M (285 mg/ml) iodoacetamide in guanidine hydrochloride lysis buffer, prepared fresh
4 M urea (see recipe)/50 mM Tris·Cl, pH 7.4 at 4°C (*APPENDIX 2A*)
0.1% (w/v) SDS
1-cm dialysis tubing (MWCO 8000 to 10,000), double knotted at one end, and dialysis clips

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

CAUTION: 2-ME has a strong odor and its use is confined to the hood in some laboratories.

NOTE: All steps involving iodoacetamide should be performed under subdued light because of the light sensitivity of the carbon-iodine bond.

Prepare cell lysate

1. Subject cells of interest to apoptosis-inducing or mock treatment, harvest, and wash as described (see Basic Protocol 2, steps 1 to 6). Remove as much supernatant as possible after the second centrifugation.
2. To 1 ml guanidine hydrochloride lysis buffer add 10 µl of 100 mM PMSF and 10 µl of 2-ME. Immediately add this mixture to cell pellet and vortex vigorously.
3. Use a sonicator equipped with a microprobe to deliver 40 pulses (0.33 sec each at 40% of the maximal output of the power supply) to the sample to shear the DNA.

   At this point samples can be stored ≤10 weeks at room temperature without loss of reactivity as detected by a variety of antibodies.

4. Add 0.1 vol of 1.54 M iodoacetamide in guanidine hydrochloride lysis buffer to each sample. Incubate 1 hr at room temperature.

   This reaction blocks free sulfhydryl groups, which can otherwise reoxidize to form large disulfide-cross-linked polypeptide oligomers in the wells of an SDS-polyacrylamide gel.

5. Add 0.01 vol of 2-ME.

   This reducing agent reacts with any remaining iodoacetamide.

Dialyze lysate

6. Transfer sample to a length of knotted 1-cm dialysis tubing and close tubing with a dialysis clip.

7. Dialyze sample at 4°C against four or five changes (≥90 min each) of 10 to 100 vol ice-cold 4 M urea/50 mM Tris·Cl, pH 7.4.

   The purpose of dialyzing into urea initially is to avoid the simultaneous presence of guanidine and SDS, which form a poorly soluble salt. Because each change of dialysis buffer decreases the concentration of solutes by a multiplicative factor, the key to effective dialysis is multiple changes with moderate volumes rather than a few changes with large volumes. Sufficient time should be permitted for equilibration to occur before each buffer change.

8. Dialyze sample at 4°C against three changes (≥90 min each) of 10 to 100 vol of 0.1% SDS.

9. Transfer sample to a 2-ml microcentrifuge tube. Remove a small aliquot and determine its protein concentration (*APPENDIX 3B*).

   The authors prefer the bicinchoninic acid BCA method (Smith et al., 1985) or the folin (Lowry) method suitably modified for samples containing SDS (Cadman et al., 1979).

10. Measure and record the volume of the remaining sample and then lyophilize it until dry.

    Lyophilized sample can be stored up to 5 years at –20°C.

Electrophorese and immunoblot caspases

11. Reconstitute the lyophilized sample in SDS sample buffer at 5 mg protein/ml, transfer 10 to 15 µl to a microcentrifuge tube, and continue with analysis as described (see Basic Protocol 2, steps 10 to 25).

    The authors find it convenient to reconstitute samples at 5 mg protein/ml SDS sample buffer and then load 50 to 75 µg protein per well, which is about the loading limit of a 2-mm-wide × 0.75-mm-thick lane.

SUPPORT PROTOCOL 1

REMOVING (STRIPPING) PRIMARY AND SECONDARY ANTIBODIES FROM BLOTS

It is often useful to probe filters with more than one antibody solution. If the antigens of interest have sufficiently different molecular weights, it is possible to reprobe a blot without stripping it. Likewise, if the primary antisera were raised in different species and can therefore be distinguished by different secondary antibodies, it is not necessary to strip blots. However, sometimes it is useful to remove antibodies without removing the original proteins transferred to the blot. The following protocol serves this purpose.

Materials

Nitrocellulose or PVDF membrane with bound antibodies (see Basic Protocol 2 or Alternate Protocol 1)
Blot erasure buffer (see recipe)
CMF-DPBS (*APPENDIX 2A*)

Resealable plastic bags
65°C water bath

1. Place a nitrocellulose or PVDF membrane with bound antibodies in a resealable plastic bag.

2. Add 50 ml blot erasure buffer (for a 20 × 20–cm membrane). Seal the bag.
3. Incubate 20 to 30 min in a 65°C water bath with gentle agitation every 5 to 10 min.

   When blotting is performed after transfer of polypeptides to nitrocellulose, complete removal of some antibodies requires heating of blot erasure buffer to ≥50°C for 20 to 30 min (Kaufmann et al., 1987). On the other hand, after immobilization of polypeptides on PVDF, antibodies are efficiently dissociated by erasure buffer at room temperature.

4. Discard the erasure buffer. Wash the blot twice for 5 min each with 50 to 100 ml CMF-DPBS.
5. Reprobe the membrane as described (see Basic Protocol 2, steps 15 to 25).

   Although some antigens are destroyed by the stripping process, most can be readily detected even after blots have been stripped multiple times (Kaufmann et al., 1987).

   Be sure to incubate the membrane in blocking buffer after each stripping to ensure that all nonspecific binding sites are coated.

LABELING AND DETECTING ACTIVE CASPASES USING BIOTINYLATED SUBSTRATE ANALOGS

BASIC PROTOCOL 3

A protocol for detection of active caspases in cytosol preparations using *N*-(*N*$^{\alpha}$-benzyloxycarbonylglutamyl-*N*$^{\varepsilon}$-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone, or zEK(bio)D-aomk, is described below. Although not as sensitive as assays based on cleavage of substrate polypeptides in intact cells or fluorogenic substrates under cell-free conditions, this assay can nonetheless detect picogram levels of active caspases when combined with very sensitive chemiluminescent substrates currently available. This affinity-labeling approach is particularly useful in at least three situations. First, this assay can be utilized to unequivocally identify active caspase species in apoptotic cell extracts by comparing migration of species present in the extracts with migration of similarly labeled recombinant caspases (Martins et al., 1997). Second, by taking advantage of the affinity of streptavidin for the biotin moiety, it is possible to purify the affinity-labeled caspases for further study (Nicholson et al., 1995; Faleiro et al., 1997). Third, because only two inhibitor molecules bind to each caspase tetramer, the labeling procedure can be used to determine the molar concentration of labeled active caspases.

Materials

Incomplete KPM buffer (see recipe), 4°C
Complete KPM buffer (see recipe), 4°C
100 µM *N*-(*N*$^{\alpha}$-benzyloxycarbonylglutamyl-*N*$^{\varepsilon}$-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone [zEK(bio)D-aomk; Osaka Peptide Institute] in dimethyl sulfoxide (DMSO), stored in aliquots up to 2 years at −80°C
3× SDS sample buffer (see recipe)
5% (w/v) nonfat dry milk in PBS-T
PBS-T (see recipe)
Peroxidase-coupled streptavidin (e.g., Amersham Pharmacia Biotech)
Enhanced chemiluminescence reagents (e.g., ECL; Amersham Pharmacia Biotech)

Cell scraper (optional)
8 × 34–mm polycarbonate ultracentrifuge tubes (e.g., Beckman)
Beckman Optima TLX tabletop ultracentrifuge and TL100.1 rotor, or equivalent, 4°C

Additional reagents and equipment for inducing apoptosis (see Basic Protocol 1), cell trypsinization (optional; *UNIT 1.1*), protein determination (*APPENDIX 3B*), SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*), and electrophoretic transfer of polypeptides to a solid support (*UNIT 6.2*)

Induce apoptosis

1. Subject ~8×10^6 cells of interest at the desired density to suitable apoptosis-inducing conditions or mock treatment (see Basic Protocol 1, steps 1 and 2).

   It is important to include a sample that contains control (nonapoptotic cells) so that endogenous biotinylated proteins can be distinguished from species that label with zEK(bio)D-aomk. A second control for labeling specificity (see Support Protocol 2) can also be included.

   The authors estimate that a 10-cm tissue culture plate will yield ~8×10^6 cells or 400 μg cellular protein when prepared as described.

Harvest cells

2. At an appropriate time point, collect cells in a 15-ml tube, releasing cells from their substratum by trypsinization (*UNIT 1.1*) or scraping if they are adherent.

   All subsequent steps prior to incubation with the affinity labeling agent should be performed at 4°C.

3. Centrifuge cells 5 min at 300 × *g*, 4°C. Gently resuspend them in 5 to 10 ml incomplete KPM buffer and centrifuge again.

   In this and all following steps, incomplete and complete KHM buffer (see Alternate Protocol 2) can be substituted for incomplete and complete KPM buffer, respectively.

4. Gently resuspend cells in 1 ml complete KPM buffer and transfer them to a 1.5-ml microcentrifuge tube.

5. Centrifuge cells 3 min at 800 × *g*, 4°C. Remove supernatant and resuspend cell pellet in a volume of complete KPM buffer approximately equal to that of the pellet.

Prepare cytosolic lysates

6. Lyse cells using three cycles of freezing in liquid nitrogen (or on dry ice) and incubating in a 37°C water bath until sample thaws.

   It is not necessary for the cells to reach 37°C. The freeze-thaw lysis procedure ruptures mitochondria and lysosomes, providing cell lysates that contain soluble polypeptides from multiple subcellular organelles. As an alternative, it is possible to prepare various subcellular fractions and then affinity label them with zEK(bio)D-aomk (Martins et al., 1997).

7. Transfer lysed cells to an 8 × 34–mm polycarbonate ultracentrifuge tube and centrifuge 1 hr at 157,000 × g_{max}, 4°C, in a Beckman Optima TLX tabletop ultracentrifuge with a TL100.1 rotor.

8. Transfer cleared lysate to a 1.5-ml microcentrifuge tube.

9. Measure protein concentration (*APPENDIX 3B*).

   The authors prefer the folin (Cadman et al., 1979), BCA (Smith et al., 1985), or Coomassie blue methods (Bradford, 1976).

10. Dilute lysates to a final protein concentration of 5 mg/ml in complete KPM buffer. Freeze 200-μg aliquots up to 2 months at −80°C.

Affinity label lysate

11. Thaw an aliquot of lysate on ice.

12. To 40 μl cell lysate (200 μg) add 0.4 μl of 100 μM zEK(bio)D-aomk. Mix the sample thoroughly without forming bubbles and incubate 5 min at 37°C.

    When mixing the sample, it is important to avoid forming bubbles (e.g., by vortexing), as enzymes can be denatured at the resulting air-liquid interface.

13. Add 25 μl of 3× SDS sample buffer. Boil the sample 5 min.

Electrophorese labeled lysate

14. Apply samples (10 to 40 μg protein) to a 16% SDS-polyacrylamide gel (*UNIT 6.1*).

    Because the large subunits of active caspases have molecular weights of 17 to 35 kDa, a 16% gel gives good separation of various caspase species. Optimal resolution of the caspases is obtained using 0.75-mm-thick, 6 × 8–cm gels with separation at 200 V constant voltage for 1 hr. Alternatively, separation of charge variants of caspase species can be accomplished by two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis (Martins et al., 1997; also see UNIT 6.4). With this technique, 16% gels also give good separation in the second dimension.

    As an alternative to SDS-polyacrylamide gel electrophoresis and ligand blotting, the derivatized caspases can be affinity purified using immobilized avidin (Nicholson et al., 1995; Faleiro et al., 1997).

15. Transfer polypeptides electrophoretically to a nitrocellulose membrane (*UNIT 6.2*).

    For example, polypeptides can be transferred 30 min at 12 V, current limited to 5.5 mA/cm^2, in a Bio-Rad TransBlot SD SemiDry Transfer Cell.

Visualize labeled caspases

16. Incubate membranes in 5% (w/v) nonfat dry milk in PBS-T for 1 hr at room temperature to block unoccupied binding sites.

17. Wash membrane once for 15 min and two times for 5 min each with PBS-T.

18. Dilute peroxidase-coupled streptavidin in PBS-T and incubate blot 3.5 hr at room temperature in this solution.

    The authors use a 1:3000 dilution of peroxidase-coupled streptavidin supplied by Amersham Pharmacia Biotech. Suitable dilutions of similar reagents from other suppliers will need to be determined empirically.

19. Wash blot once for 15 min and four times for 5 min each with PBS-T to remove unbound peroxidase-coupled streptavidin.

20. Visualize the bound streptavidin using enhanced chemiluminescence reagents as described by the supplier.

    The membrane can be stripped and probed with a suitable antibody to confirm the activation of specific caspases (see Support Protocol 3).

IN VITRO ACTIVATION OF CASPASES IN NAIVE LYSATES FOLLOWED BY AFFINITY LABELING

ALTERNATE PROTOCOL 2

It is sometimes useful to assess the competence of lysates from nonapoptotic cells to activate caspase pathways. This can be accomplished by incubating lysates with reagents that are known to activate caspase cascades, such as active caspase-8 (Muzio et al., 1997) or cytochrome *c* plus dATP (Liu et al., 1996; Mesner et al., 1999). Detailed below is a protocol for the detection of active caspases following cytochrome *c* and dATP–dependent apoptosome formation or caspase-8 activation. For such experiments, lysates are prepared from nonapoptotic cells essentially as described (see Basic Protocol 3).

Additional Materials *(also see Basic Protocol 3)*

Incomplete KHM buffer (see recipe), 4°C
Complete KHM buffer (see recipe), 4°C
5 μg/ml active caspase-8 (BD PharMingen) in complete KHM buffer *or* 5 mg/ml cytochrome *c* (Sigma) in complete KHM buffer
10 mM dATP (Sigma) in complete KHM, pH 7.4, optional

100 µM *N*-(*N*$^{\alpha}$-benzyloxycarbonylglutamyl-*N*$^{\epsilon}$-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone [zEK(bio)D-aomk; Osaka Peptide Institute] in dimethyl sulfoxide (DMSO), stored in aliquots up to 2 years at −80°C

1. Prepare cell lysates from nonapoptotic cells (i.e., untreated cells; see Basic Protocol 3, steps 1 to 11), using incomplete and complete KHM buffer instead of incomplete and complete KPM buffer, respectively, throughout.
2. To 8 µl (40 µg) cell lysate add 10 µl of 5 µg/ml active caspase-8 in complete KHM buffer or 10 µl of 5 mg/ml cytochrome *c* in complete KHM buffer and 2 µl of 10 mM dATP (optional).

   Active caspase-8 can be utilized to trigger the caspase activation process without interfering with affinity labeling of endogenous caspases because caspase-8 does not label with zEK(bio)D-aomk. In contrast, caspase-9 does label with zEK(bio)D-aomk. To trigger caspase activation using endogenous caspase-9, cytochrome c can be added to cytosol (Li et al., 1997). It is important to keep in mind, however, that extracts from some cells contain sufficiently high levels of ATP to support this reaction, whereas other cell lysates might require addition of dATP or ATP (Liu et al., 1996; Mesner et al., 1999).

3. Mix the sample thoroughly without forming bubbles and incubate 1 hr at 37°C.

   It is important that mixing be performed without creating froth in the sample. Caspases, like other enzymes, can be denatured at air-water interfaces.

4. Add 0.2 µl of 100 µM zEK(bio)D-aomk in DMSO. Mix the sample and incubate 5 min at 37°C.
5. Add 10 µl of 3× SDS sample buffer. Boil the sample 5 min.
6. Electrophorese and detect the biotinylated species as described (see Basic Protocol 3, steps 14 to 20).

SUPPORT PROTOCOL 2

CONTROLS FOR SPECIFICITY OF AFFINITY-LABELED ACTIVE CASPASES

It is possible to competitively inhibit binding of the affinity label with caspase inhibitors. For example, preincubation of lysates with certain peptide chloromethyl ketones or fluoromethyl ketones completely abolishes subsequent labeling with zEK(bio)D-aomk (Martins et al., 1997). This type of competition experiment serves two purposes. First, cell lysates contain many polypeptides that are intrinsically biotinylated. These are unaffected by preincubation of the lysates with 100 µM acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (acetyl-YVAD-CMK), whereas labeling of caspases can be completely suppressed under the same conditions (Martins et al., 1997). Second, the same approach can sometimes be utilized to suggest the identity of the labeled caspase responsible for a particular biological function. For example, the SPI-2 serpin from rabbitpox virus and underivatized peptides corresponding to the lamin A cleavage site—two different types of reversible caspase inhibitors—were shown to inhibit lamin cleavage in vitro and simultaneously inhibit affinity labeling of one particular caspase (Takahashi et al., 1996a). In performing this latter type of experiment, it is important to optimize the labeling conditions so that the length of exposure to the affinity labeling reagent is minimized (e.g., 1 min). Because reversible inhibitors continually dissociate and rebind to the caspase active site (thereby providing an opportunity for binding to the affinity label), longer labeling times allow covalent modification of caspases even in the presence of a potent reversible inhibitor.

STRIPPING MEMBRANE IN THE PRESENCE OF d-BIOTIN FOR REPROBING WITH ANTIBODY

It is sometimes useful to confirm the activation of specific caspases detected by affinity labeling using suitable antibodies. In order to do so, it is necessary to dissociate the peroxidase-coupled streptavidin without removing the biotinylated caspases. To minimize removal of the membrane-bound caspases, the authors have devised a way to compete the peroxidase-coupled streptavidin from the membrane using biotin.

Additional Materials *(also see Basic Protocol 3)*

Membrane containing affinity-labeled caspases (see Basic Protocol 3)
2 mM d-biotin (Sigma) in PBS-T

1. Wash a membrane containing affinity-labeled caspases two times for 15 min each with PBS-T.

   It is convenient to use 50-ml of buffer for a 6 × 8–cm blot.

2. Incubate membrane 1 hr with 2 mM d-biotin in PBS-T at room temperature.

3. Wash membrane once for 15 min and two times for 5 min each with PBS-T.

   The membrane can be stored dry up to 6 months at room temperature.

4. Perform immunoblot procedure with an appropriate antibody as described (see Basic Protocol 2, steps 16 to 25).

REAGENTS AND SOLUTIONS

Use deionized or distilled water and ACS reagent–grade chemicals (or better) in all recipes and protocol steps. Filter sterilize buffers that are to be stored for long periods. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
150 mM NaCl
10% (w/v) nonfat dry milk
100 U/ml penicillin G
100 µg/ml streptomycin
1 mM sodium azide
Store up to 4 weeks at 4°C

CAUTION: *Sodium azide is poisonous. Follow appropriate precautions for handling, storage, and disposal.*

A 100× penicillin G/streptomycin solution is commercially available from Life Technologies.

Blot erasure buffer

62.5 mM Tris·Cl, pH 6.8 at 21°C
2% (w/v) SDS
Store up to 6 months at 4°C
Add 100 mM 2-mercaptoethanol (2-ME) immediately before use

CAUTION: *2-ME has a strong odor and its use is confined to the hood in some laboratories.*

CLAP, 1000×

Dissolve 5 mg chymostatin, 5 mg leupeptin, 5 mg antipain, and 5 mg pepstatin A (all from Sigma) in a total volume of 5 ml dimethyl sulfoxide (DMSO), for a final concentration of 1 mg/ml each. Store 50-µl aliquots up to 1 year at −20°C.

CMF-DPBS containing 0.05% Tween 20 (PBS-T)

To 900 ml H_2O add:
11.5 g Na_2HPO_4
2.96 g NaH_2PO_4
5.84 g NaCl
Add H_2O to 1 liter
Add 2.5 ml 20% (w/v) Tween 20 (e.g., Bio-Rad)

The pH of the CMF-DPBS should be 7.5 (before addition of Tween 20).

Complete KHM buffer

50 ml incomplete KHM buffer (see recipe)
50 µl 100 mM PMSF (*APPENDIX 2A*; 0.1 mM final)
50 µl 20 mM cytochalasin B (see recipe; 20 µM final)
500 µl 100 mM Na_3VO_4 (see recipe; 1 mM final)
50 µl 1000× CLAP (see recipe; 1× final)
Prepare on ice immediately before use

Complete KPM buffer

50 ml incomplete KPM buffer (see recipe)
50 µl 100 mM PMSF (*APPENDIX 2A*; 0.1 mM final)
50 µl 20 mM cytochalasin B (see recipe; 20 µM final)
500 µl 100 mM Na_3VO_4 (see recipe; 1 mM final)
50 µl 1000× CLAP (see recipe; 1× final)
Prepare on ice immediately before use

Cytochalasin B, 20 mM

Dissolve 10 mg cytochalasin B (Sigma) in 1 ml dimethyl sulfoxide (DMSO).
Store 50-µl aliquots up to 1 year at −20°C.

EDTA, 0.5 M, pH 7.4

Add 93.1 g $Na_2EDTA{\cdot}2H_2O$ to 400 ml H_2O. While stirring and continuously monitoring pH at room temperature, add two pellets solid NaOH every 5 to 10 min. Adjust pH to 7.4. Add H_2O to 500 ml. Store indefinitely at 4°C.

The white crystalline EDTA will go into solution as the pH rises toward 7.0.

EGTA, 0.5 M, pH 7.0 and pH 7.4

Add 19.02 g EGTA to 75 ml H_2O. Adjust pH to 7.0 with 10 M NaOH or to 7.4 with 5 M KOH, stirring vigorously. Add H_2O to 100 ml. Store indefinitely at 4°C.

The EGTA will not go into solution until the pH approaches 7.0. As the solution starts to clear, be careful not to overshoot the desired pH.

Guanidine hydrochloride lysis buffer

114.64 g guanidine hydrochloride (Sigma; 6 M final)
50 ml 1 M Tris·Cl, pH 8.5 at 21°C (*APPENDIX 2A*; 250 mM final)
4 ml 0.5 M EDTA, pH 7.4 (see recipe; 10 mM final)
Bring to 200 ml final volume
Store indefinitely at room temperature

HEPES, 1 M, pH 7.4

Dissolve 23.8 g HEPES in 50 ml H_2O. Adjust pH to 7.4 with 5 M KOH. Add H_2O to 100 ml. Store indefinitely at 4°C

HEPES/CHAPS buffer

To 450 ml H_2O add:
2.98 g HEPES (25 mM final)
Adjust pH to 7.5 with 10 M NaOH
Add 0.77 g dithiothreitol (DTT)
500 mg 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; 0.1% final)
H_2O to 500 ml
Store up to 2 months at 4°C
Immediately before use, for each 1 ml buffer add:
100 U aprotinin (Bayer)
10 µl 100 mM PMSF (*APPENDIX 2A*)

Incomplete KHM buffer

5 ml 1 M HEPES, pH 7.4 (see recipe; 50 mM final)
2.5 ml 2 M KCl (50 mM final)
2 ml 0.5 M EGTA, pH 7.4 (see recipe; 10 mM final)
100 µl 2 M $MgCl_2$ (2 mM final)
H_2O to 100 ml
Store indefinitely at 4°C

Incomplete KPM buffer

5 ml 1 M PIPES, pH 7.0 (see recipe; 50 mM final)
2.5 ml 2 M KCl (50 mM final)
2 ml 0.5 M EGTA, pH 7.0 (see recipe; 10 mM final)
100 µl 2 M $MgCl_2$ (2 mM final)
H_2O to 100 ml
Store indefinitely at 4°C

K-EDTA, 0.1 M, pH 7.4

3.72 g $Na_2EDTA{\cdot}2H_2O$
50 ml H_2O
Adjust pH to 7.4 with 1 M KOH
Add H_2O to 100 ml
Store indefinitely at room temperature

Lysis buffer

To 450 ml H_2O add:
2.98 g HEPES (25 mM final)
0.238 g $MgCl_2$ (5 mM final)
1 ml 0.5 M EGTA, pH 7.0 (see recipe; 1 mM final)
Adjust pH to 7.5 at 4°C with 10 M NaOH
Add H_2O to 500 ml
Store indefinitely at 4°C
Immediately before use, for each 1 ml buffer add:
10 µl 100 mM PMSF (*APPENDIX 2A*)
1 µl 10 µg/ml pepstatin A in ethanol
1 µl 10 µg/ml leupeptin in ethanol

Pepstatin A and leupeptin in ethanol can be stored indefinitely at −20°C.

Na_3VO_4, 100 mM

Add 1.84 g Na_3VO_4 (Sigma) to ~75 ml H_2O (pH ~14). Add concentrated HCl until the pH is 10. The solution will turn yellow at this point. Boil 3 min. The pH will then be ~9 and the solution will be almost white. Add 2.5 ml of 1 M NaOH and boil 3 min. Add H_2O to 100 ml. Store 1-ml aliquots indefinitely at −20°C.

PIPES, 1 M, pH 7.0

Dissolve 30.24 g PIPES in 50 ml H_2O. Adjust pH to 7.0 with 5 M KOH. Add H_2O to 100 ml. Store indefinitely at room temperature.

PIPES powder will not dissolve until the pH is adjusted to ≥6.7.

SDS sample buffer

To 200 ml H_2O add:
31.25 ml Tris base (62.5 mM final)
1 ml 0.5 M EDTA, pH 7.4 (1 mM final)
Adjust to pH 6.8 with concentrated HCl
Add 10 g SDS (2% final)
250 ml 8 M urea (see recipe; 4 M final)
Add H_2O to 500 ml
Store up to 3 months at 4°C
Immediately before use, add 50 µl of 2-mercaptoethanol (2-ME) per 950-µl aliquot

CAUTION: *SDS is a severe irritant. Use a dust mask to prevent inhaling SDS powder. 2-ME has a strong odor and its use is confined to the hood in some laboratories.*

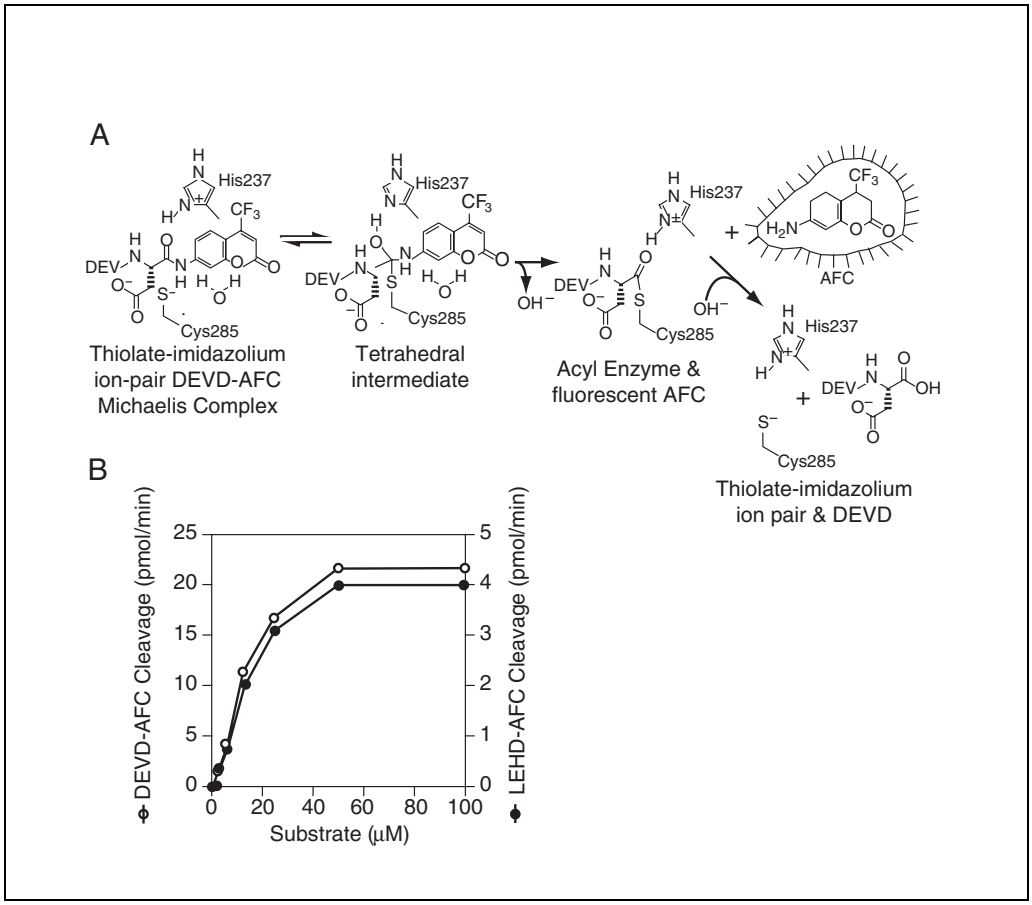


Figure 18.2.1 Measurement of caspase activity using a fluorogenic substrate. (**A**) Conversion of the substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-DEVD-AFC) to tetrapeptide and free 7-amino-4-trifluoromethylcoumarin (AFC). (**B**) Velocity versus substrate concentration curves for caspase-3. Purified recombinant human caspase-3 was incubated for 2 hr with increasing concentrations of acetyl-DEVD-AFC or acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-LEHD-AFC). From these data it is possible to determine that the maximal velocity is 5-fold lower for acetyl-LEHD-AFC than for acetyl-DEVD-AFC, whereas the concentration that results in half-maximal velocity (K_M) is the same (~12.5 µM) for each substrate.

SDS sample buffer, 3×

30 ml 0.5 M Tris·Cl, pH 6.8 at 20°C (150 mM final)
45 g sucrose (45% final)
6 ml 0.1 M K-EDTA, pH 7.4 (see recipe; 6 mM final)
30 ml 20% (w/v) SDS (9% final)
6 ml of 0.5% (w/v) bromphenol blue (0.03% final)
H_2O to 100 ml
Store 3-ml aliquots at −20°C
Just before use add 0.1 vol of 2-mercaptoethanol (2-ME; 10% final)

CAUTION: *SDS is a severe irritant. Use a dust mask to prevent inhaling SDS powder. 2-ME has a strong odor and its use is confined to the hood in some laboratories.*

Urea, 8 M

Gradually add 1920 g urea to 2 liters H_2O while stirring. Add H_2O to 4 liters. Filter through four layers of cheesecloth. Store 500-ml aliquots up to 2 months at room temperature. Prior to use, deionize aliquot with AG 501X-8 D mixed-bed resin (Bio-Rad).

Deionization removes cyanate breakdown products that can carbamylate polypeptides.

COMMENTARY

Background Information

Enzyme activity assays

Measurements of caspase enzymatic activity are performed to determine whether caspases have been activated after various treatments and to assess the timing of caspase activation relative to other events occurring as cells undergo programmed cell death. Similar assays can also be utilized to study the biochemistry of the caspase activation process under cell-free conditions and to purify active caspases.

The chemistry of the basic enzyme assay is illustrated in Figure 18.2.1A. Cell extracts (or recombinant proteases) are generally incubated with a substrate that generates a highly fluorescent or colored product when the amide bond on the C-terminal side of aspartate is cleaved. The substrates utilized in these reactions have been patterned after the cleavage sites in known caspase substrates (Sleath et al., 1990; Lazebnik et al., 1994; Takahashi et al., 1996b) or identified by studying relative rates of cleavage of libraries of derivatized peptides (Thornberry et al., 1992, 1997). The assays based on these substrates are not absolutely specific for caspases but are relatively specific because most mammalian proteases do not cleave very efficiently on the carboxyl terminal side of aspartate. Indeed, at the present time, the only other mammalian protease known to prefer aspartate in the P_1 position (i.e., immediately to the amino side of the cleavage site) is granzyme B, a serine protease that appears to be expressed exclusively in the granules of cytotoxic lymphocytes (Pham and Ley, 1997).

The principal advantages of assessing caspase activation using an enzymatic assay are its sensitivity and reliance on commonly available reagents and equipment. Because each caspase molecule can cleave multiple substrate molecules, there is an inherent amplification compared to techniques like affinity labeling (see Basic Protocol 3). Previous studies from the authors' laboratory have demonstrated low but detectable amounts of caspase activity in subcellular fractions whose content of active caspases is below the limit of detection by affinity labeling (Samejima et al., 1998). Moreover, the assay described above can also be performed with chromogenic substrates (e.g., commercially available peptidyl-*p*-nitroanilides), making it possible to assay for active caspases in any laboratory that has a spectrophotometer.

The principal disadvantage of this approach is its lack of specificity for any particular caspase. Currently available fluorogenic and chromogenic substrates were designed to mimic the sites that are cleaved in various substrate polypeptides (Earnshaw et al., 1999). Because most caspases have similar cleavage-site specificities (Table 18.2.1), these assays are not specific for individual caspases. Several examples illustrate this problem.

Analysis of tetrapeptide preferences has demonstrated that caspase-9 prefers acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl

coumarin (acetyl-LEHD-AFC) to other peptides tested (Thornberry et al., 1997). Contrary to the common interpretation of these results, this does not mean that acetyl-LEHD-AFC is a specific substrate for caspase-9. When caspase-3 is incubated with increasing concentrations of acetyl-DEVD-AFC or acetyl-LEHD-AFC, the enzyme displays the same K_M for these two substrates (Figure 18.2.1B). Although caspase-3 does display a lower turnover rate or maximum initial velocity (V_{max}) for acetyl-LEHD-AFC than acetyl-DEVD-AFC, it nonetheless cleaves both substrates quite well. Accordingly, cleavage of acetyl-LEHD-AFC in a cell lysate cannot be taken as evidence that caspase-9 has been activated. Indeed, if acetyl-LEHD-AFC is cleaved in a cellular extract, it is not immediately obvious how to determine the amount that has been cleaved by caspase-9 as opposed to the more abundant caspase-3.

This problem is not limited to acetyl-LEHD-AFC. Acetyl-DEVD-AFC, a substrate modeled after the cleavage site in poly(ADP-ribose) polymerase (PARP; Lazebnik et al., 1994), can be cleaved by caspases-1, -2, -3, -4, -6, -7, -8, -10, and -14 (Fernandes-Alnemri et al., 1995, 1996; Boldin et al., 1996; Srinivasula et al., 1996; Talanian et al., 1997; Hu et al., 1998; Faubion et al., 1999). Thus, when acetyl-DEVD-AFC cleavage activity is detected in a cell lysate, it

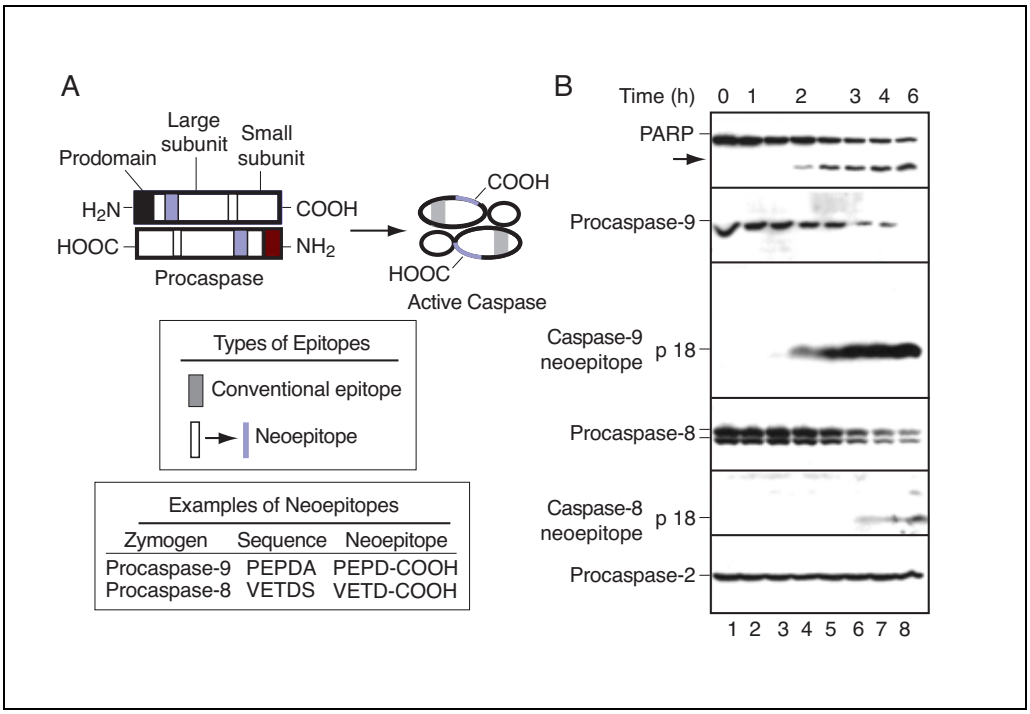


Figure 18.2.2 Assessment of caspase activation by immunoblotting. (**A**) Caspase activation. The prodomain, large subunit, and small subunit are indicated. Two types of epitopes, a conventional linear epitope in the large subunit and a neoepitope at the C terminus of the large subunit, are indicated. The lower box indicates the peptide sequences of procaspase-9 and procaspase-8 at the junction between the large and small subunits. When cleavage occurs on the C-terminal side of aspartate, new C-terminal epitopes (neoepitopes) are formed. (**B**) Results obtained when HL-60 human leukemia cells are treated with 68 μM etoposide, a topoisomerase II–directed agent that causes DNA damage, for 0, 1, 1.5, 2, 2.5, 3, and 4 or 6 hr. Adjacent lanes were loaded with 50 μg of total cellular protein harvested according to the guanidine hydrochloride cell lysis procedure (see Alternate Protocol 1). Blots were probed with monoclonal anti–poly(ADP-ribose) polymerase (PARP), a sensitive marker of caspase activation (Lazebnik et al., 1994); reagents that detect procaspases-2, -8, and -9; or anti-neoepitope sera that react with active caspase-9 (Mesner et al., 1999) or active caspase-8 species. Two major splice variants of procaspase-8 are detected in these cells. The arrow indicates the product resulting from PARP cleavage by caspases. The anti-neoepitope antisera were raised by immunizing rabbits with the neoepitope tetrapeptides indicated in (A). Note that the increased signal for active caspase-9 is at least as readily detected with the anti-caspase-9 neoepitope serum as it is with the anti-procaspase-9 serum. Also note that the signal for procaspase-2 does not change during the course of apoptosis in this cell line, illustrating the selective cleavage of some procaspases and not others during the course of apoptosis in situ (Martins et al., 1997).

is unclear whether the activity reflects caspase-3 activity or not. A more formal analysis of the K_M and k_{cat} (forward rate constant) of purified caspases acting on a variety of low-molecular-weight substrates (Talanian et al., 1997) revealed that caspases-3 and -7 preferred the *p*-nitroaniline (pNA)-coupled peptide acetyl-DEVD-pNA (K_M = 11 and 12 μM, respectively), caspase-1 preferred acetyl-YEVD-pNA (K_M = 7.3 μM), and caspase-6 preferred acetyl-VEID-pNA (K_M = 30 μM). Further analysis, however, revealed that these preferences were not absolute. Caspase-3 displayed a k_{cat}/K_M (a measure of catalytic efficiency) of 21.8×10^4 sec^{-1} for acetyl-DEVD-pNA, 6.1×10^4 sec^{-1} for acetyl-VEID-pNA, and 3.9×10^4 sec^{-1} for acetyl-YEVD-pNA, indicating that the cleavage efficiencies for the various substrates differed by only a factor of five. Aside from the inefficient cleavage of acetyl-DEVD-pNA by caspase-6, other caspases showed similar promiscuity. This lack of specificity of currently available substrates must be taken into account in interpreting results of these assays.

Given the ability of multiple caspases to cleave the same substrate, it is logical to ask whether other classes of proteases can also cleave these compounds. It has been reported that the proteasome can cleave acetyl-YVAD-AFC, albeit relatively inefficiently (Kobayashi et al., 1996). The possibility that other proteases might also cleave these substrates cannot absolutely be ruled out. Two approaches can be utilized to help ensure that cleavage of caspase substrates by other proteases is not misinterpreted as caspase activity. First, it is possible to perform the assay in the presence of a battery of protease inhibitors that inhibit many potentially confounding protease activities. The protease inhibitors used in Basic Protocol 1 will inhibit a wide range of serine, cysteine, and acid proteases and metalloproteinases. Second, it is usually important to bolster the conclusion derived from activity assays with some other measure of caspase activation (e.g., immunoblots showing cleavage of caspase zymogens or caspase substrates).

As an alternative to tetrapeptide or pentapeptide substrates, in vitro translated proteins have also been used as substrates to assay caspase activity in vitro (Nicholson et al., 1995; Liu et al., 1997; Margolin et al., 1997). Work of Andrade et al. (1998) has demonstrated the feasibility of measuring the K_M and k_{cat} using these more relevant macromolecular substrates. Recent data suggest that caspase cleavage preferences derived solely from the study of tetrapeptide-based substrates might not accurately predict cleavage sites in substrate polypeptides (Samejima et al., 1999). Additional experiments have shown that phosphatase treatment of extracts has no effect on cleavage of acetyl-DEVD-AFC but causes a significant increase in cleavage of PARP (Martins et al., 1998). Collectively, these studies suggest that macromolecular substrates might still have an important role in the study of caspases. On the other hand, disadvantages of this alternative approach include the need to perform both in vitro transcription and translation reactions to generate substrates and SDS-PAGE followed by fluorography to analyze results. In short, this labor-intensive approach is not suitable for casual inquiry.

A second disadvantage of using enzymatic assays to assess caspase activation is the potential to underestimate caspase activation in certain cell types. Previous studies have demonstrated that a number of caspase substrates are cleaved in MCF-7 human breast cancer cells. Nonetheless, activity that cleaves acetyl-DEVD-AFC is undetectable in cytosol prepared from this cell line (Janicke et al., 1998). Further analysis has revealed that caspase-3 and other active caspases can form tight complexes with caspase-generated fragments of intermediate filaments (MacFarlane et al., 2000) and are not solubilized under the conditions described. Whether this is a problem unique to the MCF-7 line, or whether substantial amounts of active caspases are also sequestered in an insoluble fraction in other cell types, remains to be established.

Immunoblotting

The ability to detect caspase activation by immunoblotting reflects the fact that these proteases are synthesized as zymogens that undergo proteolytic processing during the course of activation. The zymogens have the general organization shown in Figure 18.2.2A. An N-terminal prodomain is followed by sequences encoding a large subunit and a small subunit. In some procaspases, these two subunits are separated by a small spacer. Maturation of each zymogen appears to proceed by an ordered series of proteolytic cuts (Earnshaw et al., 1999). The first incision, which appears to increase the catalytic activity of the protease, occurs at the C terminus of the large subunit. If a spacer is present between the subunits, it is usually excised next. The final cut, which appears to be catalyzed by the caspase molecule

that is being matured, severs the prodomain from the rest of the molecule.

Three further aspects of caspase maturation require additional comment. First, mature caspases are $\alpha_2\beta_2$ tetramers consisting of two large subunits and two small subunits. Because the large subunit derived from one zymogen molecule associates with the small subunit derived from a second zymogen molecule in the dimeric precursor, caspase maturation actually involves cleavage of two zymogen molecules. Of course, the quaternary structure of the precursor and the mature caspase is lost under denaturing conditions. Second, the maturation of caspases appears to involve conformational changes as well as proteolytic cleavages. These conformational changes can be observed by immunofluorescence using certain conformation-sensitive anti-caspase antibodies (Srinivasan et al., 1998). Again, because SDS-PAGE and immunoblotting are performed after protein denaturation, these conformational changes are not detected by the present protocols. Finally, it is not clear that all caspases require cleavage for activation. In particular, recent results suggest that apoptotic protease activating factor-1 (Apaf-1) is capable of acting as an allosteric activator of procaspase-9 (Rodriguez and Lazebnik, 1999), even under conditions where procaspase-9 cannot be cleaved (Stennicke et al., 1999). Thus, when procaspase-9 is cleaved to its signature fragments (Table 18.2.1), this can be taken as presumptive evidence of procaspase-9 activation; but the lack of procaspase-9 cleavage cannot be taken as proof for a lack of caspase-9 activation.

Two types of reagents are available for detecting caspase cleavage by immunoblotting. First, antibodies raised against epitopes present in either the large or small subunits of the active enzymes are commercially available from a number of sources (Table 18.2.1). These antibodies should theoretically recognize the corresponding zymogen and active caspase with equal affinities. More recently, antibodies generated against neoepitopes (i.e., epitopes that are generated during the proteolytic activation of caspases) have been developed (Fig. 18.2.2). Such an antibody might recognize the C terminus of the large subunit, including the COOH group that is only present after zymogen cleavage at the proper site (i.e., activation). These reagents, which selectively detect active caspases on immunoblots, are becoming increasingly available.

The advantage of antibodies raised against epitopes present in the large or small subunit is the possibility that decreased zymogen levels can be directly correlated with increased amounts of processed caspases. Interestingly, a stoichiometric relationship between loss of caspase zymogen and appearance of processed caspase is rarely observed. Usually a decreased signal for caspase zymogen is accompanied by a much weaker increase in signal for the active caspase large or small subunit. One possible explanation for this phenomenon is a rapid degradation of caspase species once they are activated. Another possibility is that active caspases might leak from cells as they undergo secondary rupture late in the process of apoptosis. Whatever the explanation, the lack of a 1:1 relationship between loss of zymogen and appearance of active caspase species in cells undergoing apoptosis has practical implications: It makes it difficult to detect the decrease in zymogen levels and the appearance of active caspase species on the same blot with a single exposure of X-ray film. In many studies, a decrease of a procaspase signal is presented as the sole evidence of caspase activation. Whereas a decrease in zymogen level is consistent with caspase activation, it is also consistent with proteolytic degradation to fragments that lack caspase activity. Ideally, X-ray films exposed for different lengths of time are presented, one showing decreased zymogen levels and another showing the appearance of active subunits.

Anti-neoepitope antisera provide an alternative approach for detecting caspase activation (Fig. 18.2.2B). These sera are raised against epitopes that are present at the sites where caspases are cleaved during maturation. Accordingly, if these reagents are of high quality, they recognize only caspases that have been cleaved at the proper site to allow their activation to mature species. Cleavages at other sites will not liberate the epitopes and will not yield a signal. In other words, these sera provide information not only about the size of the caspase fragments that are generated during apoptosis, but also about the precise location of one of the cleavages giving rise to those fragments. The disadvantages of these anti-neoepitope antisera, however, are their current lack of widespread availability and the possibility that they might recognize the same two- to four-amino-acid epitope in other proteins that are cleaved during apoptosis. Nonetheless, these are potentially quite useful reagents.

Affinity labeling

The mapping of caspase cleavage sites in PARP, the lamins, and other substrates (Lazebnik et al., 1994; Takahashi et al., 1996a,b; Nicholson and Thornberry, 1997; Earnshaw et al., 1999) enabled the design of caspase-specific affinity labeling reagents. Each of these reagents consists of a four- or five-amino-acid peptide linked to a detector group (e.g., biotin) and a reactive group that irreversibly binds the caspase active site (e.g., chloromethyl, fluoromethyl, or acyloxymethyl ketone). In principle, the peptide binds to the substrate binding pocket of the caspase, the reactive group forms a covalent bond with the active site cysteine (Thornberry et al., 1994; Wilson et al., 1994), and the covalently modified subunit of the enzyme is then detected or purified using a reagent that reacts with the detector group (e.g., avidin, streptavidin).

This approach is feasible because caspases can tolerate the insertion of detector groups on substrate-like peptides. As was first shown for caspase-1 (Sleath et al., 1990), these enzymes

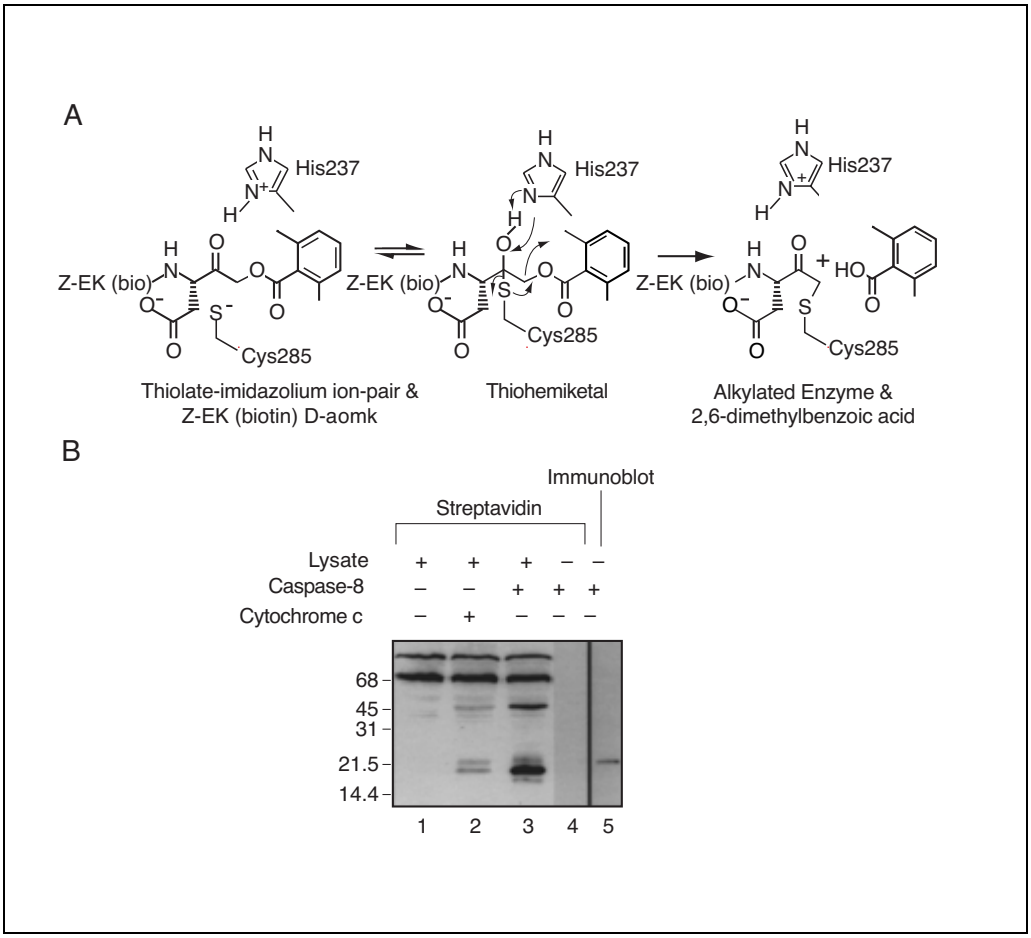


Figure 18.2.3 Labeling of active caspases by acyloxymethylketones. (**A**) The reaction mechanism of zEK(bio)D-aomk (Martins et al., 1997). The active site cysteine initially forms a reversible thiohemiketal. In a slower reaction, the methyl group of the substituted methylketone undergoes SN_2 attack by the same active site thiolate to form the thioether (alkylated enzyme) and liberate the leaving group 2,6-dimethylbenzoic acid. (**B**) Results obtained when *N*-(*N*$^{\alpha}$-benzyloxycarbonylglutamyl-*N*$^{\epsilon}$-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone (zEK[bio]D-aomk) is used to detect caspase activation. Cytosol from 1×10^6 cells (40 μg) was incubated for 1 hr at 37°C with diluent (lane 1), 100 ng cytochrome *c* (lane 2), or 50 ng active caspase-8 (lane 3). Samples were then covalently modified with zEK(bio)D-aomk as described in Basic Protocol 3. Lanes 4 and 5: 50 ng of active caspase-8 without cell lysate. Samples in lanes 1 to 4 were visualized using peroxidase-coupled streptavidin and enhanced chemiluminescence reagents. Lane 5 was probed with anti-caspase-8 antiserum followed by peroxidase-coupled secondary antibody. Collectively, these results demonstrate that endogenous biotinylated species are visualized by this technique (lane 1), that multiple zEK(bio)D-aomk-modified bands between 15 and 50 kDa are detected after caspase activation (lanes 2 and 3), and that active caspase-8 does not label with this reagent (lanes 4 and 5). Numbers at left, molecular weights of marker proteins in kilodaltons.

exhibit an absolute requirement for aspartate in the P_1 position. In contrast, because the sidechain of the P_2 residue is pointed away from the enzyme (Wilson et al., 1994), a wide range of unmodified or modified amino acids is permitted at this position (Thornberry et al., 1992). The observation that lysine is tolerated in the P_2 position (Thornberry et al., 1992) opened the door to a new family of labeling reagents that contain biotin on the ε-amino group of lysine. The first member of this family, acetyl-Tyr-Val-(ε-biotin)Lys-Asp-([2,6-dimethylbenzoyl]oxy)methylketone (YVK[bio]D-aomk), was shown to be a potent inhibitor of caspase-1 that could easily be detected with avidin-based reagents (Thornberry et al., 1994). The reagent utilized in Basic Protocol 3, *N*-(N^{α}-benzyloxycarbonylglutamyl-N^{ε}-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy) methylketone (zEK[bio]D-aomk; Martins et al., 1997), was synthesized based on the sequence of the caspase cleavage site in PARP (EVD↓G; Lazebnik et al., 1994) and lamin A (EID↓N; Takahashi et al., 1996a). This reagent has recently become commercially available.

The reaction leading to caspase derivatization by zEK(bio)D-aomk is illustrated in Figure 18.2.3A. Evaluation of the reaction of caspase-1 with a large number of aspartic ketones (Brady et al., 1999) has revealed that the inhibitory effects of these compounds could be classified as reversible, inactivating, or bimodal (i.e., reversible inhibition followed by slow inactivation). The acyloxymethyl ketone inhibitor zEK(bio)D-aomk can be categorized as a bimodal inhibitor, with a first-order rate of conversion of the reversible thiohemiketal complex to the inactive thioether. The thiolate-imidazolium ion pair of the active site Cys^{285} and His^{237} reversibly binds to the zEK(bio)D-aomk inhibitor to form a thiohemiketal complex. Here the imidazolium functions to polarize the carbonyl, enabling subsequent attack by the thiolate anion. The S-C-C_{α}-LG dihedral angle thereby adopts a 180∞ conformation necessary for SN_2 displacement. This conformation places the His^{237}Nd in a position that allows it to stabilize the charge that develops on the leaving group during the displacement reaction. In an irreversible SN_2 displacement reaction the thiolate attacks the $C_{\alpha'}$ carbon, thereby displacing the 2,6-dimethylbenzoic acid leaving group and simultaneously forming a thioether bond (and an alkylated enzyme). It is this alkylated enzyme that is detected upon subsequent blotting with streptavidin.

Critical Parameters

Enzyme activity assays

When setting up assays to measure caspase activity, there are a number of critical parameters to consider. Some of these relate to the experimental design, whereas others relate to the reagents utilized.

In terms of experimental design, it is important to remove cells that spontaneously become apoptotic before starting an experiment. For adherent cell lines, spontaneously apoptotic cells can be removed simply by changing the tissue culture medium at the start of the experiment. For nonadherent cells, these spontaneously apoptotic cells can be removed by Ficoll-Hypaque density gradient sedimentation (Martins et al., 1997). If this is not done, the spontaneously apoptotic cells contribute to a high level of caspase activity in the control sample.

At the time of the enzyme assay, it is important to use an appropriate substrate concentration. If the assay is being performed to quantitate relative amounts of enzyme present, as is usually the case, it is important to use saturating substrate concentrations (e.g., four to five times the K_M of the enzyme). The K_M can be estimated from data such as those presented in Figure 18.2.1B. If the substrate concentration is not saturating, changes in the amount of product released might be due to changes in either the number of active enzyme molecules (altered V_{max}) or the affinity for the substrate (altered K_M). Importantly, the common interpretation that changes in the amount of product released correspond to changes in the number of active enzyme molecules is only valid when the reaction is run under conditions where the substrate is saturating.

When the assay is run as an endpoint assay (see Basic Protocol 1), it is important to confirm that product release is a linear function of the length of incubation. If extracts are prepared from a variety of human cell lines after induction of apoptosis, the assay described above is linear for up to 4 hr under the specified conditions. If the assay is not linear, the most common causes of nonlinearity are substrate exhaustion and enzyme instability. Substrate exhaustion occurs when the enzyme uses most of the substrate, causing the actual substrate concentration to drop below saturating levels. This potential explanation for nonlinearity is suggested by the presence of a high concentration of product (i.e., approaching the nominal concentration of the substrate added). This problem

can be solved by decreasing the amount of enzyme (extract) present or by adding more substrate and determining whether linearity is restored.

If nonlinearity occurs in the absence of substrate exhaustion, the possibility of caspase instability needs to be considered. Based on the authors' experience, it does not appear that the caspases themselves are intrinsically labile at 37°C. However, nonlinearity can also arise if caspases are degraded by other components in the cellular extracts (e.g., proteases that are resistant to the cocktail of protease inhibitors). For certain tissues or cell types, it might be necessary to add additional protease inhibitors in order to preserve caspase stability in the apoptotic extracts.

Another critical parameter for these assays is the quality of the substrate utilized. The authors have found that acetyl-DEVD-AFC from some suppliers (not listed above) yields high levels of fluorescence when diluted in buffer without caspases, apparently as a consequence of contamination by free AFC.

Immunoblotting

When attempting to assess caspase activation by immunoblotting, there are at least two critical parameters. The first involves the method for preparing cell lysates. It has been reported that lysis of lymphoid cells in neutral detergents such as Triton X-100 can lead to artifactual caspase activation by granzyme B present in cytotoxic granules (Zapata et al., 1998). Other serine proteases can also activate certain caspases under cell-free conditions (Zhou and Salvesen, 1997), again raising the potential for artifactual caspase activation during preparation of cell lysates. To avoid this potential pitfall, it is important to solubilize cells under conditions that prevent procaspase cleavage and activation after cell lysis. The protocols described above involve lysis of cells under strongly denaturing conditions, such as in 2% (w/v) SDS containing 4 M urea and reducing agent (Martins et al., 1997; see Basic Protocol 2) or in 6 M guanidine hydrochloride containing reducing agent (Kaufmann et al., 1997; see Alternate Protocol 1). These conditions should avoid spurious caspase activation.

A second critical parameter is the quality of the anti-caspase antibody. Some currently available antibodies exhibit such a weak signal that the instructions accompanying them indicate that they can be used to detect caspase zymogens only in cells that have been transfected with cDNA encoding the procaspase under the control of a strong promoter. Other commercially available anti-caspase antibodies recognize purified recombinant protein but exhibit extensive cross-reactivity when applied to whole-cell lysates. Clearly, these reagents are not useful for the studies envisioned by most cell biologists. Because of the cost of obtaining commercially available antibodies from multiple sources (to say nothing of the frustration of failed experiments), it is important to assess the sensitivity and cross-reactivity of reagents whenever possible before purchasing them.

Affinity labeling

Several parameters are critical for successful detection of active caspases using the affinity labeling approach. First, it is essential to optimize detection conditions in order to achieve maximum signal and minimum background. High backgrounds can be a serious problem with this assay, particularly when caspase concentrations in the cell lysates are low. The most common cause for high backgrounds in the authors' experience is the choice of blocking agent. Although milk is traditionally discouraged when using avidin-biotin detection systems, the authors have found nonfat dry milk to be far superior to biotin-free blocking agents. However, they have also found that nonfat dry milk from different sources results in different levels of background. Accordingly, nonfat dry milk from several different sources might have to be compared to arrive at an optimal signal-to-noise ratio.

A second critical parameter is the chemistry of the reactive group on the affinity labeling reagent. The authors have found that affinity labeling reagents from some commercial sources give unacceptably high background labeling of polypeptides in nonapoptotic cells. Part of this problem might relate to the choice of reactive group. Chloromethyl ketones are highly reactive but relatively nonspecific reagents that covalently modify a wide range of sulfhydryl-containing enzymes (Shaw, 1990). Fluoromethyl ketones are also widely reactive with sulfhydryl-containing enzymes, as illustrated by the demonstration that zVAD-fmk and acetyl-DEVD-fmk can irreversibly inhibit cathepsins B and H (Faubion et al., 1999; Schotte et al., 1999) and by the observation that biotinylated zVAD-fmk can affinity label cathepsin B (Schotte et al., 1999). Accordingly, the authors recommend using peptidyl acyloxymethyl ketones in preference to the more readily available chloromethyl or fluoromethyl ketones.

A third critical factor is the quality of the affinity labeling reagent. The authors have found that the quality of commercially available acyloxymethyl ketones varies widely. These compounds are extremely difficult to synthesize cleanly. When the authors analyzed some commercially available reagents by high performance liquid chromatography, mass spectroscopy, and nuclear magnetic resonance, they noticed several problems, most notably cyclization and racemization of the aspartate. These alterations might reflect the reaction conditions utilized to synthesize these molecules, the quality of the purification step after the multistep synthesis, or the subsequent conditions used to store or ship these somewhat labile molecules. In any case, these impurities can contribute to lack of ability of the reagents to react with caspases and/or spurious reactions with other proteins.

Unfortunately, cost and quality of available reagents remains a consideration in deciding whether to apply this approach. Hopefully the presence of a potentially lucrative market will stimulate suppliers to improve the quality and availability of these reagents.

Anticipated Results

Enzyme activity assays

Despite all the caveats and limitations listed above, activity assays can be extremely useful when applied appropriately. They have been utilized as one means of successfully confirming that caspases are activated under a wide range of conditions. In addition, they have proven particularly useful in assessing the timing of caspase activation relative to other apoptotic events, in comparing the ability of cells to undergo apoptosis after different treatments, and in assessing the mechanism by which various antiapoptotic agents exert their effects. Compared to the immunoblotting and affinity labeling procedures, activity assays are easier and more quantitative. They must, however, be interpreted cautiously because they do not yield a definitive identification of the caspases that are activated.

Immunoblotting

Shown in Figure 18.2.2B are results obtained when HL-60 human leukemia cells were treated with the topoisomerase II poison etoposide, lysed in guanidine hydrochloride lysis buffer, and subjected to immunoblotting using several immunological reagents. Caspase activation, as reflected by the cleavage of the caspase substrate PARP (Lazebnik et al., 1994), is first detectable at 2 hr. Loss of caspase-9 zymogen, reflecting presumed proteolytic activation, and appearance of a species corresponding to the large subunit of active caspase-9 is also evident at 2 hr. Thus, immunoblotting with antibodies against either caspases or their substrates can be utilized to assess the timing of caspase activation. In addition, examination of other immunoblots in Figure 18.2.2B shows that some caspase zymogens are degraded to active species after caspase-9 activation (e.g., caspase-8) or are not activated at all (e.g., procaspase-2). Thus, by employing a series of reagents that recognize zymogens and active caspases, it is possible to confirm that specific caspases have been activated and to assess the relative timing of their activation in a population of cells undergoing somewhat synchronous apoptosis.

Affinity labeling

Results obtained in a typical experiment are shown in Figure 18.2.3B. In this example, cell lysate prepared from nonapoptotic cells has been treated with cytochrome *c* or active caspase-8 to activate endogenous caspases. After treatment with zEK(bio)D-aomk, the samples have been subjected to SDS-PAGE followed by detection with peroxidase-coupled streptavidin. As illustrated in lane 1, this technique detects several endogenous biotinylated polypeptides. Most of these have molecular weights above M_r ~50,000 and do not interfere with the detection of active caspase species. As shown in lanes 2 and 3, the most prominent polypeptides that label after caspase activation are species of M_r ~15,000 to 22,000. Two-dimensional gel electrophoresis has demonstrated that multiple discrete charged species are hidden in each band (Martins et al., 1997). Two different approaches have revealed that the most abundant species detected in apoptotic cell lysates are active forms of caspase-3 and caspase-6 (Faleiro et al., 1997; Martins et al., 1997). Further analysis has suggested that caspase-3 and caspase-6 each give rise to at least two size variants, a mature variant lacking the prodomain and a partially processed intermediate containing the prodomain. The charge variants appear to reflect, at least in part, differences in caspase phosphorylation (Martins et al., 1998).

As also illustrated in Figure 18.2.3B, zEK(bio)D-aomk does not react with active caspase-8. In contrast, biotinyl-DEVD-fmk has been reported to derivatize this enzyme (Muzio

et al., 1997). These observations illustrate the potential importance of testing several different affinity labeling reagents before concluding that active caspases cannot be detected in a particular biological system by affinity labeling.

Time Considerations

Enzyme activity assays

The entire enzyme activity assay procedure can be completed in 1 day. After the induction of apoptosis, the time required for preparing cell extracts is ~3 hr. These extracts can be stored for several months at −70°C without appreciable loss of caspase activity. Protein assays take 1 to 2 hr to complete. If the solutions are premixed, the assay itself can be set up in 30 min to 1 hr and then read 2 to 4 hr later.

Immunoblotting

It takes several days from start to finish to treat cells, prepare cell lysates, perform SDS-polyacrylamide gel electrophoresis, transfer polypeptides to nitrocellulose, and probe immunoblots. It should be obvious, however, that there are many stopping points in the procedure. The process can be speeded up by using minigels and a minitransfer apparatus, but the resolution of the smaller blots might be somewhat limited.

Affinity labeling

The entire affinity labeling procedure outlined above can be completed in 2 days. After the induction of apoptosis, the time required for preparing cell extracts is ~3 hr. These extracts can be stored for several months at −80°C without appreciable loss of reactivity. Protein assays take 1 to 2 hr to complete. The samples can then be subjected to electrophoresis (1 hr), transferred to nitrocellulose (30 min), blocked (overnight at 4°C), and incubated with streptavidin and visualized (4 to 5 hr).

Literature Cited

Andrade, F., Roy, S., Nicholson, D., Thornberry, N., Rosen, A., and Casciola-Rosen, L. 1998. Granzyme B directly and efficiently cleaves several downstream caspase substrates: Implications for CTL-induced apoptosis. *Immunity* 8:451-460.

Bible, K.C., Boerner, S.A., and Kaufmann, S.H. 1999. A one-step method for protein estimation in biological samples: Nitration of tyrosine in nitric acid. *Anal. Biochem.* 267:217-221.

Boldin, M.P., Goncharov, T.M., Goltsev, Y.V., and Wallach, D. 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. *Cell* 85:803-815.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Brady, K.D., Giegel, D.A., Grinnell, C., Lunney, E., Talanian, R.V., Wong, W., and Walker, N. 1999. A catalytic mechanism for caspase-1 and for bimodal inhibition of caspase-1 by activated aspartic ketones. *Bioorg. Med. Chem.* 7:621-631.

Cadman, E., Bostwick, J.R., and Eichberg, J. 1979. Determination of protein by a modified Lowry procedure in the presence of some commonly used detergents. *Anal. Biochem.* 96:21-23.

Earnshaw, W.C., Martins, L.M., and Kaufmann, S.H. 1999. Mammalian caspases: Structure, activation, substrates and functions during apoptosis. *Annu. Rev. Biochem.* 68:383-424.

Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. 1997. Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* 16:2271-2281.

Faubion, W.A., Guicciardi, M.E., Miyoshi, H., Bronk, S.F., Roberts, P.J., Svingen, P.A., Kaufmann, S.H., and Gores, G.J. 1999. Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. *J. Clin. Invest.* 103:137-145.

Fernandes-Alnemri, T., Litwack, G., and Alnemri, E.S. 1995. Mch2, a new member of the apoptotic Ced-3/ICE cysteine protease gene family. *Cancer Res.* 55:2737-2742.

Fernandes-Alnemri, T., Armstrong, R.C., Krebs, J., Srinivasula, S.M., Wang, L., Bullrich, F., Fritz, L.C., Trapani, J.A., Tomaselli, K.J., Litwack, G., and Alnemri, E.S. 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. U.S.A.* 93:7464-7469.

Graham, J. and Rickwood, D. (eds.) 1997. Subcellular Fractionation: A Practical Approach. IRL Press, Oxford University Press, New York.

Hu, S., Snipas, S.J., Vincenz, C., Salvesen, G., and Dixit, V.M. 1998. Caspase-14 is a novel developmentally regulated protease. *J. Biol. Chem.* 273:29648-29653.

Janicke, R.U., Ng, P., Sprengart, M.L., and Porter, A.G. 1998. Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J. Biol. Chem.* 273:15540-15545.

Kaufmann, S.H. and Earnshaw, W.C. 2000. Induction of apoptosis by cancer chemotherapy. *Exp. Cell Res.* 256:42-49.

Kaufmann, S.H., Ewing, C.M., and Shaper, J.H. 1987. The erasable western blot. *Anal. Biochem.* 161:89-95.

Kaufmann, S.H., Svingen, P.A., Gore, S.D., Armstrong, D.K., Cheng, Y.C., and Rowinsky, E.K. 1997. Altered formation of topotecan-stabilized

topoisomerase I-DNA adducts in human leukemia cells. *Blood* 89:2098-2104.

Kobayashi, T., Shinozaki, A., Momoi, T., Arahata, K., and Tsukahara, T. 1996. Identification of an interleukin-1β converting enzyme-like activity that increases upon treatment of P19 cells with retinoic acid as the proteasome. *J. Biochem.* 120:699-704.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., and Earnshaw, W.C. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371:346-347.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. 1997. Cytochrome c and dATP-dependent formation of apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. 1996. Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. *Cell* 86:147-157.

Liu, X., Zou, H., Slaughter, C., and Wang, X. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 89:175-184.

MacFarlane, M., Merrison, W., Dinsdale, D., and Cohen, G.M. 2000. Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. *J. Cell Biol.* 148:1239-1254.

Margolin, N., Raybuck, S.A., Wilson, K.P., Chen, W., Fox, T., Gu, Y., and Livingston, D.J. 1997. Substrate and inhibitor specificity of interleukin-1β-converting enzyme and related caspases. *J. Biol. Chem.* 272:7223-7228.

Martins, L.M., Kottke, T., Mesner, P.W., Basi, G.S., Sinha, S., Frigon, N. Jr., Tatar, E., Tung, J.S., Bryant, K., Takahashi, A., Svingen, P.A., Madden, B.J., McCormick, D.J., Earnshaw, W.C., and Kaufmann, S.H. 1997. Activation of multiple interleukin-1β converting enzyme homologues in cytosol and nuclei of HL-60 human leukemia cells lines during etoposide-induced apoptosis. *J. Biol. Chem.* 272:7421-7430.

Martins, L.M., Kottke, T.J., Kaufmann, S.H., and Earnshaw, W.C. 1998. Phosphorylated forms of activated caspases are present in cytosol from HL-60 cells during etoposide-induced apoptosis. *Blood* 92:3042-3049.

Mesner, P.W. Jr., Bible, K.C., Martins, L.M., Kottke, T.J., Srinivasula, S.M., Svingen, P.A., Chilcote, T.J., Basi, G.S., Tung, J.S., Krajewski, S., Reed, J.C., Alnemri, E.S., Earnshaw, W.C., and Kaufmann, S.H. 1999. Characterization of caspase processing and activation of HL-60 cell cytosol under cell-free conditions: Nucleotide requirement and inhibitor profile. *J. Biol. Chem.* 274:22635-22645.

Muzio, M., Salvesen, G.S., and Dixit, V.M. 1997. FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. *J. Biol. Chem.* 272:2952-2956.

Nicholson, D.W. and Thornberry, N.A. 1997. Caspases: Killer proteases. *Trends Biochem. Sci.* 22:299-306.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., and Lazebnik, Y.A. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376:37-43.

Pham, C.T. and Ley, T.J. 1997. The role of granzyme B cluster proteases in cell-mediated cytotoxicity. *Semin. Immunol.* 9:127-133.

Rodriguez, J. and Lazebnik, Y. 1999. Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev.* 13:3179-3184.

Samejima, K., Toné, S., Kottke, T.J., Enari, M., Sakahira, H., Cooke, C.A., Durrieu, F., Martins, L.M., Nagata, S., Kaufmann, S.H., and Earnshaw, W.C. 1998. Transition from caspase-dependent to caspase-independent mechanisms at the onset of apoptotic execution. *J. Cell Biol.* 143:225-239.

Samejima, K., Svingen, P.A., Basi, G.S., Kottke, T., Mesner, P.W. Jr., Stewart, L., Durrieu, F., Poirier, G.G., Alnemri, E.S., Champoux, J.J., Kaufmann, S.H., and Earnshaw, W.C. 1999. Caspase-mediated cleavage of DNA topoisomerase I at unconventional sites during apoptosis. *J. Biol. Chem.* 274:4335-4340.

Schotte, P., Declercq, W., Van Huffel, S., Vandenabeele, P., and Bayaevt, R. 1999. Non-specific effects of methyl ketone peptide inhibitors of caspases. *FEBS Lett.* 442:117-121.

Shaw, E. 1990. Cysteinyl proteinases and their selective inactivation. *Adv. Enzymol. Relat. Areas Mol. Biol.* 63:271-347.

Sleath, P.R., Hendrickson, R.C., Kronheim, S.R., March, C.J., and Black, R.A. 1990. Substrate specificity of the protease that processes human interleukin-1β. *J. Biol. Chem.* 265:14526-14528.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.

Srinivasan, A., Roth, K.A., Sayers, R.O., Shindler, K.S., Wong, A.M., Fritz, L.C., and Tomaselli, K.J. 1998. In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differ.* 5:1004-1016.

Srinivasula, S.M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R.C., Wang, L., Trapani, J.A., Tomaselli, K.J., Litwack, G., and Alnemri, E.S. 1996. The ced-3/interleukin-1 beta converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2 alpha are substrates for the apoptotic mediator CPP32. *J. Biol. Chem.* 271:27099-27106.

Stennicke, H.R., Deveraux, Q.L., Humke, E.W., Reed, J.C., Dixit, V.M., and Salvesen, G.S. 1999. Caspase-9 can be activated without proteolytic processing. *J. Biol. Chem.* 274:8359-8362.

Takahashi, A., Musy, P.-Y., Martins, L.M., Poirier, G.G., Moyer, R.W., and Earnshaw, W.C. 1996a. CrmA/SPI-2 inhibition of an endogenous ICE-related protease responsible for lamin A cleavage and apoptotic nuclear fragmentation. *J. Biol. Chem.* 271:32487-32490.

Takahashi, A., Alnemri, E.S., Lazebnik, Y.A., Fernandes-Alnemri, T., Litwack, G., Moir, R.D., Goldman, R.D., Poirier, G.G., Kaufmann, S.H., and Earnshaw, W.C. 1996b. Cleavage of lamin A by Mch2α but not CPP32: Multiple ICE-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 93:8395-8400.

Talanian, R.V., Quinlan, C., Trautz, S., Hackett, M.C., Mankovich, J.A., Banach, D., Ghayur, T., Brady, K.D., and Wong, W.W. 1997. Substrate specificities of caspase family proteases. *J. Biol. Chem.* 272:9677-9682.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J., et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356:768-774.

Thornberry, N.A., Peterson, E.P., Zhao, J.J., Howard, A.D., Griffin, P.R., and Chapman, K.T. 1994. Inactivation of interleukin-1 beta converting enzyme by peptide (acyloxy)methyl ketones. *Biochemistry* 33:3934-3940.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., and Nicholson, D.W. 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* 272:17907-17911.

Wilson, K.P., Black, J.A., Thomson, J.A., Kim, E.E., Griffith, J.P., Navia, M.A., Murcko, M.A., Chambers, S.P., Aldape, R.A., Raybuck, S.A., et al. 1994. Structure and mechanism of interleukin-1β converting enzyme. *Nature* 370:270-275.

Zapata, J.M., Takahashi, R., Salvesen, G.S., and Reed, J.C. 1998. Granzyme release and caspase activation in activated human T-lymphocytes. *J. Biol. Chem.* 273:6916-6920.

Zhou, Q. and Salvesen, G.S. 1997. Activation of pro-caspase-7 by serine proteases includes a non-canonical specificity. *Biochem. J.* 324:361-364.

Key References

Enari, M., Talanian, R.V., Wong, W.W., and Nagata, S. 1996. Sequential activation of ICE-like and CPP32-like proteases during fas-mediated apoptosis. *Nature* 380:723-726.

This paper illustrates the use of different tetrapeptide substrates to demonstrate the sequential activation of multiple caspases during apoptosis.

Schlegel, J., Peters, I., Orrenius, S., Miller, D.K., Thornberry, N.A., Yamin, T.T., and Nicholson, D.W. 1996. CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in fas-mediated apoptosis. *J. Biol. Chem.* 271:1841-1844.

This paper illustrates one of the first uses of immunoblotting to demonstrate caspase activation during apoptosis.

Takahashi et al., 1996b. See above.

This paper describes the first use of affinity labeling to detect active caspases in extracts from apoptotic cells.

Internet Resource

www.peptide.co.jp

The Web site of the Osaka Peptide Institute contains a variety of caspase substrates as well as acycloxymethyl ketones that can be used as affinity labels.

Contributed by Scott H. Kaufmann and Timothy J. Kottke
Mayo Clinic
Rochester, Minnesota

L. Miguel Martins
Imperial Cancer Research Fund
London, United Kingdom

Alexander J. Henzing and William C. Earnshaw
University of Edinburgh
Edinburgh, Scotland, United Kingdom

Assessment of Apoptosis and Necrosis by DNA Fragmentation and Morphological Criteria

Cell death may occur by two mechanisms: apoptosis, or programmed cell death, and necrosis, or cell death due to injury or trauma (see *UNIT 18.1*). Both types of cell death have their own specific and distinct morphological and biochemical hallmarks. Apoptotic cells share a number of common features, such as phosphatidylserine (PS) exposure, cell shrinkage, chromatin cleavage, nuclear condensation, and formation of pyknotic bodies of condensed chromatin. Necrotic cells exhibit nuclear swelling, chromatin flocculation, loss of nuclear basophilia, breakdown of cytoplasmic structure and organelle function, and cytolysis by swelling. Cell death can be induced by a wide variety of stimuli, such as growth factor withdrawal, heat shock, cold shock, radiation, heavy metals, genotoxic drugs, and a number of biological ligands such as Fas-L and tumor necrosis factor (TNF). Most if not all of these can induce both apoptosis and necrosis in a time- and dose-dependent manner.

A variety of techniques have been developed to assess cytotoxicity in untreated and/or treated cells, most of which are based on the loss of plasma membrane integrity. Membrane disruption can be detected based on the uptake of vital dyes (see Basic Protocol 1) However, during apoptosis, the cell membrane remains intact for a relatively long time, and therefore some of these assays cannot detect the early stages of apoptosis. Alternatively, assays that detect all types of cell death do not discriminate between apoptosis and necrosis. Therefore, more specific techniques have been developed to determine cell death, and the combination of several methods is required to distinguish between the two separate types. These techniques rely on specific morphological and molecular or biochemical changes associated with these two processes.

This unit describes some of the techniques most commonly used to detect cell death. Morphological assays include trypan blue exclusion (see Basic Protocol 1), differential staining (see Basic Protocol 2), and Hoechst staining (see Basic Protocol 3). Methods to detect chromatin cleavage include TUNEL assays for whole cells (see Basic Protocol 4) and paraffin sections (see Alternate Protocol 1), DNA fragmentation assays using whole cells (see Basic Protocol 5), assays of total genomic DNA (see Alternate Protocol 2), analysis of DNA fragmentation by agarose gel electrophoresis (see Alternate Protocol 3), phenol extraction of DNA for analysis of fragmentation (see Alternate Protocol 4), a quantitative assay for DNA fragmentation (see Basic Protocol 6), and detection of DNA fragmentation by pulsed-field gel electrophoresis (see Basic Protocol 7). A protocol is also provided for Cytospin preparations from cell suspensions (see Support Protocol 1). Table 18.3.1 describes how these assays are used to distinguish apoptotic cells from necrotic cells.

MORPHOLOGY ASSAYS

Loss of membrane integrity, which occurs late in apoptosis and relatively early in necrosis, can be detected by cellular uptake of the vital dye trypan blue (see Basic Protocol 1). Apoptosis and necrosis also can be identified morphologically based on the criteria described elsewhere (Bowen, 1980). These include cell shrinkage, nuclear condensation, and cleavage for apoptotic cells; and nuclear swelling, chromatin flocculation, and loss of nuclear basophilia for necrotic cells. The levels of both apoptosis and necrosis in a particular cell population can be estimated from Cytospin preparations (see Support Protocol 1) stained with RAPI-DIFF (see Basic Protocol 2; see Figure 18.3.1), Hoechst stain (see Basic Protocol 3), or propidium iodide.

Contributed by Boris Zhivotovsky and Sten Orrenius

Current Protocols in Cell Biology (2001) 18.3.1-18.3.23

Table 18.3.1 Methods for Determination of Apoptosis and Necrosis

Method	Protocol	Result for	
		Apoptosis	Necrosis
Morphological/cytological analysis			
Trypan blue staining	Basic Protocol 1	No staining (early apoptosis) Blue staining (late apoptosis)	Blue cells
Differential staining	Basic Protocol 2	Membrane blebbing, chromosome condensation and nuclear shrinkage, cytoplasmic constriction and loss of cell volume	Nuclear swelling, chromatin flocculation, membrane blebbing and disruption, appearance of cell "ghosts"
Hoechst staining	Basic Protocol 3	Increased blue fluorescence, fragmented or condensed nuclei	Blue nucleus with diffuse staining
Flow cytometry	—	Reduced forward and side scatter	Increased forward and side scatter
Annexin V binding and PI staining	—	Positive fluorescence response for annexin V binding; exclusion of PI	Positive fluorescence; uptake of PI
Assays for chromatin cleavage			
TUNEL assay	Basic Protocol 4 and Alternate Protocol 1	Detection of strand breaks	Detection of strand breaks
Whole-cell DNA fragmentation	Basic Protocol 5	DNA ladder	DNA smear
Total genomic DNA fragmentation	Alternate Protocol 2	DNA ladder	DNA smear
Simple protocol for DNA fragmentation	Alternate Protocol 3	DNA ladder	DNA smear
Phenol extraction for DNA fragmentation	Alternate Protocol 4	DNA ladder	DNA smear
Quantitative assay of DNA fragmentation	Basic Protocol 6	Increase in the percentage of fragmented DNA	Increase in the percentage of fragmented DNA
Pulsed-field detection of high-molecular-weight DNA	Basic Protocol 7	High-molecular-weight bands	DNA smear
Assays for caspase activation			
Specific substrate cleavage assay	*UNIT 18.2*	Positive detection of cleaved fluorescent substrate	No changes in fluorescence detected
Immunodetection of caspase activation	*UNIT 18.2*	Positive detection of cleavage products of procaspase	No cleavage products detected

BASIC PROTOCOL 1

Measurement of Cell Death by Trypan Blue Exclusion

This common cell viability assay is based on the ability of a cell with an intact membrane to exclude the dye trypan blue. Therefore, this assay allows one to distinguish between cells with intact and disrupted membranes. Since this method does not give an indication of the mode of cell death, it should be used only in conjuction with a more informative morphological method.

Materials

Cell suspension to be assessed
2× PBS tablets, pH 7.2 to 7.4 (Sigma) or 2× PBS (*APPENDIX 2A*)
0.2% (w/v) trypan blue (Sigma) in 2× PBS (store up to 1 to 2 months at 4°C)

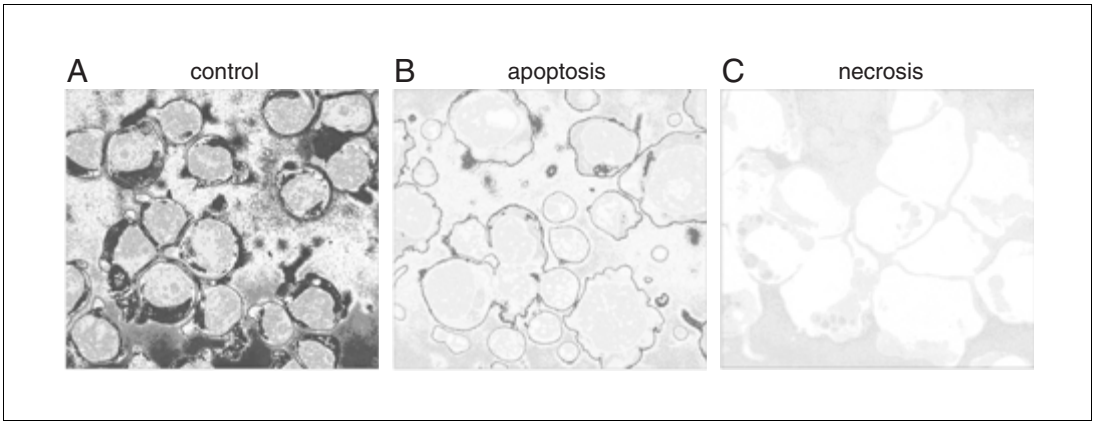


Figure 18.3.1 RAPI-DIFF staining of Cytospin preparation of HL-60 cells. Cells were treated with different concentrations of a cytotoxic drug to induce apoptosis or necrosis. (**A**) Untreated cells, (**B**) apoptotic cells, and (**C**) necrotic cells.

Hemacytometer: improved Neubauer type (Karl Hecht; Baxter) or equivalent
Coverslips (e.g., Chance Propper)
Light microscope

1. Remove from the cell suspension a sample containing ~5×10^4 cells.
2. Add an equal volume of 0.2% trypan blue, mix, and incubate 1 to 2 min at room temperature to permit dye uptake.
3. Load samples onto the hemacytometer.
4. Count the total number of cells and the number of unstained cells in five of the major sections of the hemacytometer (see Fig. 1.1.1). Calculate the average number of cells per section.
5. Calculate the number of cells/ml culture medium by multiplying the average number of cells per section by the dilution factor (2 in this case) and hemacytometer index (10^4). Determine the percentage viability according to the following formula:

$$\% \text{ viability} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

Control cells and cells in early stages of apoptosis exclude trypan blue; cells in the late stages of apoptosis and necrotic cells take up the dye and appear as blue cells.

Differential Staining of Cells

BASIC PROTOCOL 2

This staining procedure involves three steps: first cells are fixed in 100% methanol, then the nuclei are stained with an acid dye, and finally the cytoplasm is stained using a basic dye. Such three-step procedure allows differential staining and contrast between the cytoplasm and the nucleus. Apoptotic cells exhibit membrane blebbing, chromatin condensation and nuclear shrinkage, cytoplasmic constriction and loss of cell volume, and formation of apoptotic bodies. Necrotic cells undergo nuclear swelling, chromatin flocculation, cell membrane blebbing and disruption, and finally cell lysis resulting in the appearance of "ghost cells" (see Figure 18.3.1). This method can also be applied to cells growing attached to coverslips or chambered culture slides.

Materials

Cytospin preparations of cells (see Support Protocol 1)
100% methanol
Acid dye: 0.1% (w/v) eosin Y/0.1% (w/v) formaldehyde/0.4% (w/v) sodium phosphate dibasic/0.5% potassium phosphate monobasic
Basic dye: 0.4% (w/v) methylene blue–polychromed/0.4% (w/v) azure/0.4% (w/v) sodium phosphate dibasic/0.5% (w/v) monobasic potassium phosphate
DPX mountant (in solution; BDH)

Coverslips (e.g., Chance Propper)
Microscope slides (e.g., Menzel-Glaser)
Light microscope

1. Fix cells after Cytospin preparation (see Support Protocol 1) by immersing the slide ten times in 100% methanol.

   The three solutions required for this procedure are available as a kit, called RAPI-DIFF Stain kit, from Diagnostic Developments. The solutions can be stored 1 to 2 months at room temperature.

2. Stain the cell nuclei by dipping the slide in the acid dye ten times.
3. Stain the cell cytoplasm by dipping the slide in basic dye ten times.
4. Rinse the slide by dipping in distilled water, let it air dry, and mount it using DPX mountant.
5. Score the cells as normal, apoptotic, condensed and/or fragmented nuclei, or necrotic swollen cells, based on their morphological appearance under a light microscope (see *UNIT 18.1* and Fig. 18.3.1).

BASIC PROTOCOL 3

Hoechst Staining of Cells

Hoechst 33342, a bisbenzimide dye, is a cell-permeant, minor group–binding DNA stain that fluoresces bright blue upon binding to DNA. It is water soluble and relatively nontoxic. Hoechst 33342 can be excited with the UV lines of the argon-ion laser and most conventional fluorescence excitation sources, and it exhibits relatively large Stokes shifts (excitation and emission maxima ~350 and 460 nm), making it suitable for multicolor labeling experiments (Pollak and Ciancio, 1990). Cells are scored as apoptotic if they have fragmented nuclei. This method can also be applied to cells growing attached to coverslips or chambered culture slides.

Materials

Cell suspension
1× and 2× PBS, pH 7.2 to 7.4 (from Sigma 2× PBS tablets, or see *APPENDIX 2A*)
4% (w/v) paraformaldehyde (see recipe)
10 μg/ml Hoechst 33342 dye (Molecular Probes) in PBS (*APPENDIX 2A*)
50/50 (v/v) PBS/glycerol

Microscope slides (e.g., Menzel-Glaser)
Coverslips (e.g., Chance Propper)
Fluorescent microscope

Additional reagents and equipment for Cytospin preparations (see Support Protocol 1)

1. Place an aliquot of cell suspension containing $0.3–0.5 \times 10^6$ cells in a microcentrifuge tube, and centrifuge 5 min at $1000 \times g$, 4°C.

2. Remove and discard supernatant. Resuspend the dry pellet in 30 to 50 µl of 4% paraformaldehyde.
3. Spin the fixed cells onto a glass slide using a Shandon/Lipshaw Cytospin centrifuge (see Support Protocol 1). Alternatively, spread the cells onto a slide with a pipet tip, and air dry slide.
4. Stain slide with 10 µg/ml Hoechst dye.

   Hoechst 33342 dye can be diluted from 100× stock kept in the dark at 4°C.
5. Leave slide in the dark for 10 min at room temperature.
6. Rinse by dipping slide five times in distilled water, and let it air dry in the dark.
7. Mount slide with coverslip using PBS/glycerol. Monitor fluorescence with a blue filter in the fluorescence microscope, and score cells with fragmented nuclei as apoptotic.

   This method is useful for discrimination of apoptotic from nonapoptotic (untreated or necrotic) cells.

Cytospin Preparation of Cells for Analysis

SUPPORT PROTOCOL 1

Cytospin preparations can be used in many protocols for cell biology. Once slides are prepared they can be stained with variety of dyes and analyzed using light, fluorescent, and confocal microscopy, depending on the dye type. Before staining, slides can be stored up to 1 year at −20°C covered with aluminum foil. Fixed and stained slides can be stored indefinitely once mounted permanently.

Materials

Cell suspension
PBS (*APPENDIX 2A*)
Cytospin centrifuge and cups (Shandon/Lipshaw)

1. Place a sample of cell suspension containing ~0.5–1 × 10^5 cells in a microcentrifuge tube, and centrifuge 5 min at 1000 × *g*, 4°C.
2. Remove and discard supernatant. Resuspend pelleted cells in 100 µl PBS.
3. Add suspension to the Cytospin cup and slide setup, and centrifuge 2 min at 500 × *g*, 4°C.
4. Air dry the slide.

ASSAYS FOR CHROMATIN CLEAVAGE

Chromatin cleavage is a hallmark of apoptosis and involves the formation of high-molecular-weight (>50-kbp) and nucleosome-sized (200-bp) DNA fragments. The high-molecular-weight fragments can be separated by pulsed-field gel electrophoresis, and the nucleosome-sized fragments, when separated on a conventional agarose gel, demonstrate a ladder pattern. The DNA from necrotic cells, on the other hand, has a random and general cleavage pattern and produces a smear when electrophoresed on either a pulsed-field or conventional gel (see Fig. 18.3.2). Prior to the formation of the cleavage products, endogenous nucleases must generate a large number of DNA strand breaks. These strand breaks in DNA can be detected by attaching biotin-conjugated nucleotides to the 3′ hydroxyl termini in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT). A number of methods can be used to identify apoptotic or necrotic DNA. All of these methods are equally successful and require the same number of cells.

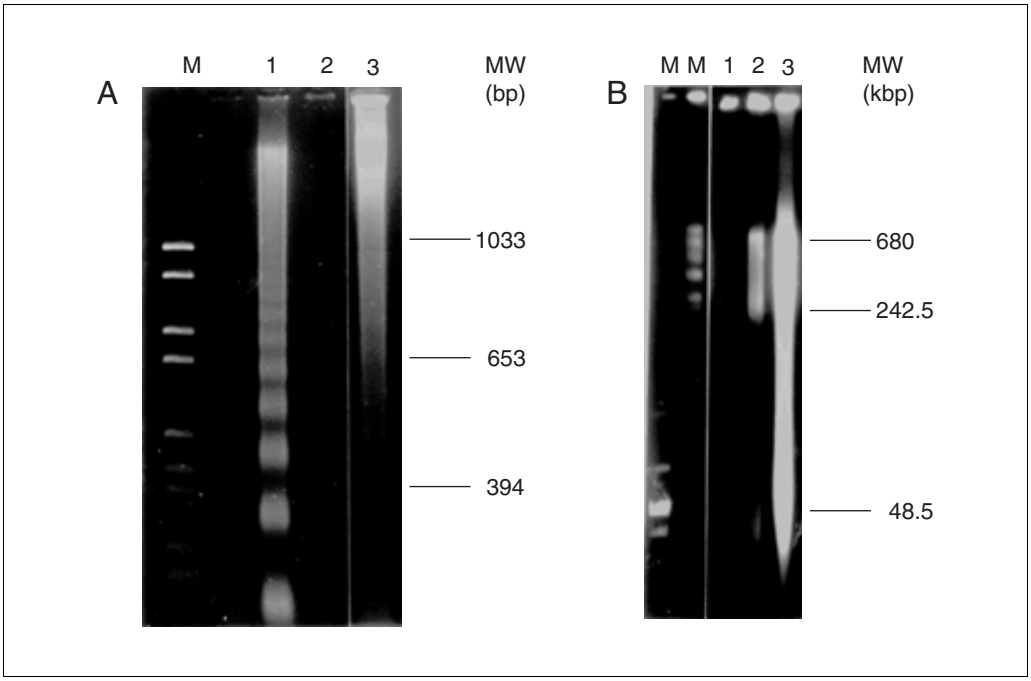


Figure 18.3.2 Gel electrophoresis of DNA from Jurkat cells treated with different concentrations of a cytotoxic drug. (**A**) Conventional gel: M, marker; lanes 1 to 3, DNA from apoptotic, untreated, and necrotic cells, respectively. The DNA ladder, when present, is a strong indicator of apoptosis. (**B**) Pulsed-field gel: MM, markers; lanes 1 to 3, DNA from untreated, apoptotic, and necrotic cells, respectively.

BASIC PROTOCOL 4

TUNEL Assay for DNA Fragmentation in Cells

Induction of apoptosis results in the generation of single-strand DNA breaks. These can be detected using the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method (Gavrieli et al., 1992). This method requires cell fixation with cross-linking agents such as formaldehyde, which, unlike some of the alcohols (such as ethanol), prevents the extraction of degraded DNA. In other words, this fixation step prevents the loss or reduction of the cellular DNA content after the extensive washings and staining involved in this protocol. Once cells are fixed with formaldehyde, treatment with alcohols does not affect the DNA content of cells.

Materials

Cell suspension
100% methanol, –20°C
PBS, pH 7.2 to 7.4 (*APPENDIX 2A*)
1% (v/v) formaldehyde
70% ethanol, ice cold
Terminal deoxyribonucleotidyltransferase (TdT; 25 U/ml) and 10× buffer (0.3 M Tris base/1.4 M sodium cacodylate, pH 7.2/1 mM DTT; e.g., Boehringer Mannheim)
25 mM $CoCl_2$
1 mM Bio-16-dUTP (e.g., Boehringer Mannheim)
Termination buffer: 300 mM NaCl/30 mM sodium citrate (e.g., Sigma or equivalent)
Staining buffer (e.g., Sigma; see recipe)
FACS fluid (e.g., Becton Dickinson; optional; for flow cytometry)
DPX mountant (BDH)
5 µg/ml propidium iodide in PBS (*APPENDIX 2A*)

Flow cytometer (e.g., Becton Dickinson) and tubes; *or* fluorescent microscope
Coverslips (e.g., Chance Propper)
Microscope slide (e.g., Objektträger, Menzel-Glaser)

1. Wash a sample of cell suspension containing ~10^6 cells by centrifuging 5 min at 1000 × *g*, 4°C, discarding supernatant, and resuspending in PBS. Transfer to microcentrifuge tube and centrifuge 5 min at 1000 × *g*, 4°C.
2. Remove and discard supernatant. Resuspend cells in 1% formaldehyde and fix for 15 min on ice.
3. Centrifuge 5 min at 1000 × *g*, 4°C. Remove and discard supernatant. Resuspend cell pellet in PBS, and repeat centrifugation. Remove and discard supernatant.
4. Resuspend cell pellet in 0.5 ml PBS and add to 5 ml ice-cold 70% ethanol.

   Cells can be stored in ethanol for several weeks at −20°C.
5. Centrifuge 5 min at 1000 × *g*, 4°C. Remove and discard ethanol. Resuspend cells in PBS, and repeat centrifugation.
6. Prepare elongation buffer (50 µl per sample):

   41.5 µl ultrapure water
   5 µl 10× TdT buffer
   2 µl 25 mM $CoCl_2$
   1 µl 1 mM Bio-16-dUTP
   0.5 µl 25 U/µl TdT enzyme.

   Add 50 µl elongation buffer to each cell pellet and resuspend. Incubate 30 min at 37°C.

   It is recommended that more than the required amount of elongation buffer be prepared; that is, multiply the total number of samples + 1 by 50 µl (the volume of the elongation buffer per sample) when calculating how much buffer to prepare. This buffer needs to be made fresh.
7. Add 5 ml PBS and centrifuge 5 min at 1000 × *g*, 4°C. Remove and discard supernatant.

   The reaction may first be stopped by incubating cells with termination buffer for 15 min at room temperature, though omission of this step does not appear to affect the results.
8. Resuspend cell pellet in 100 µl staining buffer, and incubate 30 min at room temperature.

   Again, it is recommended that extra buffer be prepared.
9. Add 2 ml PBS, resuspend cells, and centrifuge 5 min at 1000 × *g*, 4°C. Remove and discard supernatant. Repeat.
10. Add 1 ml of 5 µg/ml propidium iodide and incubate 30 min at room temperature in the dark.
11. Centrifuge 5 min at 1000 × *g*, 4°C, and remove supernatant.

12a. *For flow cytometry:* Resuspend cells in sufficient amount of PBS or FACS fluid and measure fluorescence (for FITC, 488 nm excitation, 520 ± 20 nm emission, FL-1 channel; for PI, 560 nm excitation, 640 nm emission, FL-2 or FL-3 channels).

12b. *For fluorescence microscopy:* Resuspend cells in 50 to 100 µl of PBS and spin them onto a slide as described under Cytospin preparations (see Support Protocol 1). Mount slides with DPX mountant and observe fluorescence with green and red filters under microscope.

Cells for fluorescence microscopy can be counterstained with hematoxylin for ≤30 sec, and then rinsed with water, before microscopy.

The number of single-strand breaks is increased during the early stages of apoptosis (increase in green fluorescence) without any change in the DNA content (measured by PI staining). With time the DNA content is decreased (red fluorescence) due to formation of apoptotic bodies, which also results in the reduction of TUNEL signal.

ALTERNATE PROTOCOL 1

TUNEL Assay in Paraffin-Embedded Sections

The TUNEL assay can also be performed on paraffin-embedded tissue sections.

Additional Materials *(also see Basic Protocol 4)*

Paraffin-embedded tissue sections on slides
4% (w/v) paraformaldehyde (see recipe) *or* 4% formaldehyde, in PBS (*APPENDIX 2A*)
96%, 90%, and 80% ethanol
Xylene
BSA (e.g., Sigma)
10 mM Tris·Cl, pH 8 (*APPENDIX 2A*)
20 µg/ml proteinase K in 10 mM Tris·Cl (*APPENDIX 2A* for Tris·Cl)
3% (v/v) methanol
2% (w/v) BSA in PBS (*APPENDIX 2A* for PBS)
ExtrAvidin-peroxidase (Sigma) diluted 1:50 in PBS/1% BSA/0.5% Tween 20
3-Amino-9-ethylcarbazole (AEC)

Fluorescent microscope
Coverslips (e.g., Chance Propper)
Microscope slide (e.g., Objektträger, Menzel-Glaser)

1. Fix section in 4% paraformaldehyde or formaldehyde, then immerse in PBS.
2. Remove paraffin from sections by incubating slide 10 min at 70°C or 30 min at 58° to 60°C.
3. Rehydrate sections by sequential incubation in xylene (twice, 5 min each), 96% ethanol (twice, 3 min each), 90% ethanol (3 min), and 80% ethanol (3 min), followed by 3 min in water.

   Because paraffin traces might interfere with the enzymatic reaction in tissue sections, use of fresh solvents is recommended.
4. Treat sections with 10 mM Tris·Cl, pH 8, for 5 min, then incubate 15 min in 20 µg/ml proteinase K at room temperature. Wash slide four times in ultrapure water, 2 min each time.
5. Incubate sections in 3% methanol for 30 min at room temperature, and rinse by dipping five times in ultrapure water.
6. Prepare 100 µl per slide of elongation buffer (see Basic Protocol 4, step 6; the buffer needs to be fresh). Incubate the sections 60 min at 37°C in humid atmosphere.
7. Rinse the sections by dipping three times in ultrapure water, and incubate in termination buffer for 15 min at room temperature.
8. Place sections sequentially in PBS (5 min), 2% BSA (10 min), and PBS (5 min), all at room temperature.

   Addition of BSA prevents nonspecific binding of ExtrAvidin-peroxide.
9. Incubate the sections in ExtrAvidin-peroxidase for 15 min at room temperature.
10. Incubate the sections in 2% BSA, and wash four times in PBS.
11. Stain the sections with AEC for 30 min at 37°C, then rinse by dipping three times in ultrapure water.
12. Mount slides and observe for fluorescence.

    After mounting the covered slides can stored for many months at 4°C.

    Tissue sections, like cells, can be counterstained with hematoxylin for ≤30 sec, followed by rinsing in water.

Detection of DNA Fragmentation in Whole Cells

In 1976, Skalka et al. reported that the DNA in chromatin of irradiated lymphoid tissues degrades in vivo into oligonucleosome-length fragments. This observation was first linked to endonuclease activation in 1980 (Wyllie, 1980). Since then several methods have been developed to measure internucleosomal DNA fragmentation. This method was adapted from the protocol first described by Sorenson et al. (1990) and does not require DNA purification. Appearance of a DNA ladder will correspond to apoptosis, whereas a DNA smear will indicate necrosis. This also applies to all the alternate protocols in this section.

Materials

1× and 5× TBE buffer (see recipe), pH ~8.0 at room temperature (do not adjust pH)
SeaKem GTG agarose (FMC Bioproducts)
Cell suspension
50 mg/ml RNase A (see recipe)
4× DNA loading buffer: 4× TBE buffer (see recipe) containing 40% (w/v) sucrose and 0.25% (w/v) bromphenol blue (e.g., Sigma; store up to 2 to 3 weeks at 4°C)
Ultrapure agarose (Life Technologies)
10% (w/v) SDS (see recipe)
20 mg/ml proteinase K in water (store in aliquots up to 1 year at −20°C)
1× DNA loading buffer: 1× TBE buffer (see recipe) containing 10% (w/v) sucrose and 0.25% (w/v) bromphenol blue (e.g., Sigma)
DNA marker VI (pBR328 DNA cleaved with *Bgl*I and *Hin*fI; Boehringer Mannheim), 1 µl in 20 µl of 1× DNA loading buffer
TE buffer, pH 8.0: 10 mM Tris·Cl/1 mM EDTA
10 mg/ml ethidium bromide (see recipe)

Boiling water bath or microwave oven
Gel electrophoresis apparatus: GNA-100 (Amersham Pharmacia Biotech), Buffer Puffer (Owl Scientific), or equivalent
Power supply (Power-Pac 300, Bio-Rad, or equivalent)
Shaker at 4°C
MacroVue UV Transilluminator (Hoefer Scientific Instruments) or equivalent
Photoman Polaroid gel documentation system (Hoefer Scientific Instruments) or equivalent

Prepare agarose gel for electrophoresis

1. Prepare sufficient volume of 1× TBE to cast the gel and to fill the electrophoresis tank.

   It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and in the gel. Differences in pH or ionic strength can greatly affect the DNA mobility.

2. Weigh 0.9 g SeaKem agarose in a flask, and add 50 ml of 1× TBE (for GNA-100 gel electrophoresis apparatus). Heat in a boiling water bath or microwave oven until dissolved, then allow the agarose to cool to 60°C for 10 to 15 min.

   This is a standard 1.8% agarose gel. The volume should not occupy >40% of the volume of the flask.

   For the Buffer Puffer gel electrophoresis apparatus, use 2.7 g agarose and 150 ml TBE.

3. Pour gel into mold. Remove unwanted air bubbles. Place comb in the gel and allow gel to set 1 hr.

Prepare sample

4. Place a sample of cell suspension containing 4–5 × 10^5 cells in a microcentrifuge tube, and centrifuge 3 min at 1000 × *g*, 4°C.

   If necessary, the pellet may be stored up to 2 to 3 weeks in microcentrifuge tubes at −20°C before proceeding.

5. Carefully remove supernatant and resuspend pellet in 16 µl ultrapure water.
6. Add 4 µl of 50 mg/ml RNase A (final concentration 10 mg/ml), mix, and leave 20 min at room temperature.
7. Add 5 µl of 4× DNA loading buffer.
8. Using a scalpel, cut out a piece of gel between the comb and the upper edge of the gel (do not remove the comb), leaving the gel above the first one or two wells intact.

Prepare digestion gel

9. Prepare 5 ml of digestion gel by weighing 40 mg Ultrapure agarose, adding 2.75 ml ultrapure water, and stirring on a hot plate to dissolve.

   Do not use the SeaKem agarose in this step because it causes SDS to come out of solution.

   For the Buffer Puffer gel electrophoresis apparatus, prepare 10 ml of digestion gel.

10. When steam starts to rise from the flask, add 1 ml of 5× TBE and 1 ml of 10% SDS. When agarose has boiled and dissolved, take flask off the heat and allow gel to cool to 50°C.
11. Add 250 µl of 20 mg/ml proteinase K, mix gently, and pour into the gap above the main gel. Allow to cool, and remove the comb.

Set up for electrophoresis

12. Mount the gel in the electrophoresis tank, and add just enough 1× TBE buffer to cover it to a depth of 1 mm.
13. Add 1 µl DNA marker VI to 30 µl of 1× DNA loading buffer and load the marker into the well that is separated from the digestion gel. Load all other samples into the wells connected with the digestion gel.

    For the Buffer Puffer apparatus, use 2 µl DNA marker VI dissolved in 30 µl of 1× DNA loading buffer.

14. Run the gel overnight (~14 hr) at 20 V to facilitate digestion of the sample.
15. Turn the voltage up to 90 V and run for an additional 1.5 hr.

    For the Buffer Puffer apparatus, run gel 2 hr at 100 V after the initial 20-V overnight run.

Develop gel

16. Remove the gel from the tank and rinse with water.
17. Add 100 ml TE buffer and 40 µl of 50 mg/ml RNase A. Place gel on shaker for 3 to 4 hr.
18. Rinse the gel with distilled water. Add 100 ml TE buffer and 5 µl of 10 mg/ml ethidium bromide. Place gel on the shaker for 40 min.
19. Wash several times with fresh TE to remove ethidium bromide, and photograph under UV light.

Detection of DNA Fragmentation in Total Genomic DNA

ALTERNATE PROTOCOL 2

This method is a modified version of the protocol described by McGahon et al. (1995). The presence of ethidium bromide in the gel avoids later staining and destaining of the gel. Although Basic Protocol 5 and this alternate protocol are equally sensitive, the latter is less time consuming. In addition, dry loading prevents the loss of material.

Additional Materials *(also see Basic Protocol 5)*

Lysis buffer: 2 mM EDTA/100 mM Tris·Cl, pH 8.0/0.8% (w/v) SDS (store at room temperature)

1. Place sample of cell suspension containing 4–6 × 10^5 cells in a 1.5-ml microcentrifuge tube. Centrifuge 5 min at 2000 × *g*, 4°C.
2. Remove and discard supernatant. Add 20 µl lysis buffer.
3. Add 2 µl of 50 mg/ml RNase A. Mix well by flicking the tip of the tube. Do not apply vigorous vortexing. Incubate at least 30 min at 37°C.
4. Add 10 µl of 20 mg/ml proteinase K, and incubate at least 1.5 hr at 50°C.
5. Add 8 µl of 4× DNA loading buffer.

   If necessary, after this step the samples can be stored for at least 1 week at 4°C.
6. Place 0.9 g SeaKem agarose (1.8%) in 50 ml of 1× TBE buffer in a flask (for GNA-100 gel electrophoresis apparatus). Heat in a boiling water bath or microwave oven until dissolved. Allow the agarose to cool to 60°C for 10 to 15 min. Add 3 µl of 10 mg/ml ethidium bromide.
7. Dry load the samples. Dissolve 1 µl DNA marker VI in 30 µl of 1 × DNA loading buffer and load into the gel. Apply low current (~35 mA) for ≥7 hr or higher current (~60 mA) for 4 hr.

   Samples are dry loaded and enough TBE buffer is added to the apparatus so it touches both sides of the gel but does not cover it. This is to ensure that the sample is not lost. After 10 min, when the samples have entered the gel, the current is stopped and more TBE is added so it covers the whole gel, and the current is then reapplied.
8. Photograph the gel under UV light.

Simple Protocol for Detection of DNA Fragments

ALTERNATE PROTOCOL 3

This simple protocol was developed in the authors' laboratory. It is particularly useful for detection of chromatin cleavage in lymphoid cells (Zhivotovsky et al., 1995) but less so for hepatocytes and epithelial cells. For these types of cells, see Alternate Protocol 4.

Additional Materials *(also see Basic Protocol 5)*

Lysis buffer, 4°C: 5 mM Tris·Cl, pH 8.0/20 mM EDTA/0.5% (v/v) Triton X-100
100% ethanol, −20°C
5 M NaCl (*APPENDIX 2A*)
RNase T1/A stock (see recipe)
Vacuum lyophilizer (e.g., Hetovac, Heto-Holten)

Prepare DNA sample

1. Place sample containing 1–2 × 10^6 cells in a microcentrifuge tube, and centrifuge 5 min at 2000 × *g*, 4°C. Resuspend pellet in 1 ml PBS and repeat centrifugation.
2. Resuspend pellet in 250 µl TE buffer, add 250 µl of 4°C lysis buffer, and vortex. Refrigerate 30 min.
3. Centrifuge sample 15 min at 15,000 × *g*, 4°C.

4. Transfer supernatant to a fresh microcentrifuge tube. Add 1 ml −20°C ethanol and 30 µl of 5 M NaCl. Mix and place in −20°C freezer overnight.
5. Centrifuge sample for 15 min at 15,000 × *g*, 4°C. Remove and discard supernatant.
6. Place precipitate in vacuum lyophilizer for 20 to 25 min.
7. Add 20 to 30 µl TE buffer and 1 µl RNase T1/A stock. Incubate 1 hr at 37°C.
8. Add 1 µl of 20 mg/ml proteinase K, and incubate an additional 1 hr at 37°C.
9. Add 8 µl of 4× DNA loading buffer.

Cast and run the gel

10. Dissolve 0.9 g SeaKem agarose in 50 ml TBE buffer (for GNA-100 gel electrophoresis apparatus) or 2.7 g SeaKem agarose in 150 ml TBE buffer (for Buffer Puffer gel electrophoresis apparatus). Heat in a boiling water bath or microwave oven until dissolved, then cool gel and pour into mold.
11. Mount the gel in the electrophoresis tank, and add just enough 1× TBE buffer to cover the gel to a depth of 1 mm.
12. Load DNA marker VI in the first well and samples in the rest of wells (see Basic Protocol 5, step 13).
13. Run the gel at 60 mA (for GNA-100) or 70 mA (for Buffer Puffer) until the bromphenol blue front is ~1 to 2 cm from the end of the gel.
14. Stain gel with ethidium bromide and photograph under UV light (see Basic Protocol 5, steps 18 to 19).

ALTERNATE PROTOCOL 4

Phenol Extraction of DNA Fragments for Agarose Gel Electrophoresis

This protocol is a modification of the classical method described by Wyllie (1980). This method requires DNA purification. Although it takes much more time than other techniques and needs many more cells for preparation, it yields very clean, protein-free DNA fragments.

Additional Materials *(also see Basic Protocol 5)*

Lysis buffer (see Alternate Protocol 3), 4°C
100% ethanol, −20°C
5 M NaCl (*APPENDIX 2A*)
RNase T1/A stock (see recipe)
Phenol, TE-saturated (see recipe)
24:1 (v/v) chloroform/isoamyl alcohol (store mixture at room temperature in a fume hood)
0.5% (w/v) SDS

Prepare DNA sample

1. Place sample containing 2–5 × 10^6 cells in microcentrifuge tube, and centrifuge 5 min at 2000 × *g*, 4°C.
2. Resuspend pellet in 250 µl TE buffer, add 250 µl of 4°C lysis buffer, vortex, and refrigerate 30 min at 4°C.
3. Centrifuge sample 15 min at 15,000 × *g*, 4°C.
4. Transfer supernatant to fresh microcentrifuge tube. Add 1 ml −20°C 100% ethanol and 30 µl of 5 M NaCl. Mix and place in −20°C freezer overnight.
5. Centrifuge sample 15 min at 15,000 × *g*, 4°C. Remove and discard supernatant.

6. Add 500 µl TE buffer and 5 µl RNase T1/A stock to pellet. Incubate 30 min at 37°C.
7. Add 250 µl TE-saturated phenol and 250 µl of 24:1 chloroform/isoamyl alcohol. Vortex, and centrifuge for 2 to 3 min at 5000 × *g*, 4°C. Transfer upper layer to fresh 2-ml microcentrifuge tube.
8. Add 500 µl of TE buffer to lower layer; repeat step 7. Transfer upper layer to 2-ml microcentrifuge tube and mix with upper layer from step 7.
9. Extract the mixed samples from steps 7 and 8 with 1000 µl of 24:1 chloroform/isoamyl alcohol and centrifuge again.
10. Transfer the upper layer to 1.5-ml microcentrifuge tubes, 500 µl per tube. Precipitate overnight with ethanol and NaCl as in step 4.
11. Repeat step 5.
12. Place precipitate in vacuum lyophilizer for 20 to 25 min.
13. Dissolve and mix precipitates from both microcentrifuge tubes in a total of 20 to 30 µl TE buffer/0.5% SDS. Add 5 µl of 4× DNA loading buffer.

Cast and run the gel

14. Mix a 1.8% agarose gel by dissolving 0.9 g agarose in 50 ml TBE buffer (for GNA-100 gel electrophoresis apparatus) or 2.7 agarose in 150 ml TBE buffer (for Buffer Puffer). Cool gel and pour into mold.
15. Mount the gel in the electrophoresis tank, and add just enough 1× TBE buffer to cover the gel to a depth of 1 mm.
16. Load DNA marker VI in the first well (see Basic Protocol 5, step 13) and samples in the rest of the wells.
17. Run the gel at 60 mA (for GNA-100) or 70 mA (for Buffer Puffer) until the bromphenol blue front is ~1 to 2 cm from the end of the gel.
18. Stain gel with ethidium bromide (see Basic Protocol 5, steps 18 to 19) and photograph under UV light.

Quantitative Assay of DNA Fragmentation

BASIC PROTOCOL 6

In addition to qualitative analysis by gel electrophoresis, DNA fragmentation can be quantitatively determined by using the diphenylamine reagent. This method, introduced by Burton (1956), cannot, however, discriminate between apoptotic and necrotic chromatin cleavage.

Materials

Cell suspension to be assessed
Lysis buffer (see Alternate Protocol 3), ice cold
10% (w/v) and 5% (w/v) trichloroacetic acid (TCA; keep at room temperature in dark flasks)
Diphenylamine reagent (see recipe)

10-ml conical glass tubes
Round-bottom glass tubes
Water bath, 100°C

1. Prepare cell suspension containing $1–10 \times 10^6$ cells in a 1-ml volume.
2. Transfer 0.8 ml of cell suspension to microcentrifuge tube. Add 0.7 ml ice-cold lysis buffer. Vortex, and allow lysis to proceed 15 to 30 min at 4°C.

3. Centrifuge 15 min at 15,000 × *g*, 4°C. Transfer supernatant to labeled conical glass tube.
4. Add 0.65 ml of 5% TCA to the pellet in microcentrifuge tube, and add 1.5 ml of 10% TCA to the sample in labeled glass tube.
5. Precipitate both samples overnight (≥4 hr) at 4°C.
6. Centrifuge conical tube 10 min at 2500 × *g*, room temperature.
7. Remove supernatant after centrifugation and add 0.65 ml of 5% TCA to the pellet. Prepare two blank tubes with 0.65 ml of 5% TCA to be treated the same for the remaining steps.
8. Make hole on the top of each microcentrifuge tube from step 5, and cover each conical tube with a marble.
9. Boil both sets of tubes for 15 min in 100°C water bath.
10. Cool to room temperature. Centrifuge samples 5 min at 2500 × *g*, room temperature.
11. Transfer 0.5 ml of each supernatant (from both glass and microcentrifuge tubes) to labeled, round-bottom glass tubes.
12. Add 1 ml diphenylamine reagent to each tube. Incubate ≥4 hr at 37°C with a marble covering each tube.
13. Read absorbance at 600 nm in spectrophotometer. Set zero with blanks from step 7. Express results as the percentage of DNA fragmented:

$$\% \text{ fragmented DNA} = \frac{\text{absorbance of the supernatant}}{\text{absorbance of supernatant + pellet}} \times 100$$

BASIC PROTOCOL 7

Detection of High-Molecular-Weight Chromatin Fragments by Pulsed-Field Agarose Gel Electrophoresis

Not all forms of apoptosis are accompanied by internucleosomal DNA fragmentation (Oberhammer et al., 1993a). However, the formation of high-molecular-weight apoptotic DNA fragments of 50 to 700 kbp (see Figure 18.3.2B), which was first observed by Walker et al. (1991), has been reported to occur in all the cell types studied to date (see Background Information in Commentary).

One of the forms of pulsed-field gel electrophoresis, field-inversion gel electrophoresis (FIGE), has been successfully used in several laboratories to identify high-molecular-weight DNA fragments in apoptotic cells and a smear in the same range of DNA size in necrotic cells. The authors have used both vertical and horizontal gel chambers; resolution of DNA fragments is the same. This protocol is an adaptation of an earlier method described by Anand and Southern (1990).

Materials

Cell suspension
Agarose buffer (for molds; see recipe)
SeaPlaque GTG low-melting-point agarose (FMC Bioproducts)
20 mg/ml proteinase K in water (store in aliquots up to 1 year at −20°C)
Proteinase buffer (for plugs; see recipe)
TE buffer, pH 8.0:10 mM Tris·Cl/1 mM EDTA
50 mM EDTA, pH 8.0 (*APPENDIX 2A*)
SeaKem GTG agarose (FMC Bioproducts)
5× TBE buffer (see recipe)

DNA size pulse markers: chromosomes from *Saccharomyces cerevisiae* (225 to 2200 kbp) and a mixture of λDNA *Hin*dIII fragments, λDNA, and λDNA concatemers (0.1 to 200 kbp; Sigma; supplied premade in syringe)

Gel leveling table

100-µl insert molds (Amersham Pharmacia Biotech), stored in 0.1 M HCl

12- or 24-well tissue culture plates

50°C incubator

100°C water bath or microwave oven

Pulsed-field gel electrophoresis system: vertical gel chamber with cooling elements (Protean II, Bio-Rad); horizontal gel chamber (HE 100B); power supply (PS 500 XT); and Switchback pulse controller (PC 500, Hoefer Scientific Instruments)

Thermostatic circulator Multi Temp III (Amersham Pharmacia Biotech)

Prepare sample plugs of agarose

1. Rinse insert molds extensively in distilled water and dry. Wrap Parafilm around the bottom of the molds and place them on ice to chill at least 10 min before pouring in the gel.

   Make sure to mark the sample wells with pencil or pen.

2. Place sample of cell suspension containing 1×10^6 cells in a microcentrifuge tube, and centrifuge 3 min at $2000 \times g$, 4°C. Remove and discard supernatant. Resuspend pellet in 100 µl of agarose buffer.

3. Prepare 1% low-melting-point agarose in agarose buffer, and place in a 60°C water bath until melted.

4. Add 100 µl of molten 1% agarose to the cell suspension, and mix with pipet. Immediately pipet mixture into two prechilled insert molds, 100 µl per mold.

5. Place the filled insert molds on ice for 10 to 15 min.

6. Remove Parafilm from the bottom of the insert molds, and dislodge each plug into separate well of a tissue culture plate.

   It is convenient to use 12- or 24-well tissue culture plates for this purpose.

7. Add 1 ml of proteinase buffer and 10 µl proteinase K (final concentration 0.2 mg/ml plug) to each well. Wrap Parafilm or clear tape around the plate, and incubate ≥24 hr on a shaker at 50°C.

8. Remove wrapping from plate, and remove buffer with pipet.

   If it is necessary to simultaneously analyze the low-molecular-weight DNA, it is possible to remove the DNA fragments that have leached out from the plugs during incubation with proteinase K by precipitating them from this buffer over 48 hr at −20°C. Add 0.2 volume of 10.5 M ammonium acetate and 2 volumes of cold absolute ethanol. This DNA can then be lyophilized, dissolved in 1× DNA loading buffer, and analyzed by conventional gel electrophoresis.

9. Wash each plug three times with 1 ml TE buffer, 2 hr per wash, on a shaker at 4°C.

10. Remove TE buffer, and add 1 ml of 50 mM EDTA.

    Plugs are now ready to put on a gel. To save samples for a long time, store the plugs in EDTA, in which DNA is stable for several months, at 4°C.

Prepare separating gel

11. Prepare 1% agarose (SeaKem GTG) gel in 0.5× TBE.

For horizontal gels

12a. Dissolve 2.5 g agarose in 250 ml 0.5× TBE in a boiling water bath or microwave oven. Allow the agarose to cool to 60°C for 10 to 15 min.

13a. Seal the edges of the gel platform with tape, and place platform on leveling table.

14a. Pour the gel into the mold, and remove unwanted air bubbles. Place the comb into the gel, and allow to set for 1 hr.

15a. Remove the comb very carefully so as not to break the gel. Remove tape.

For vertical gels

12b. Clamp two frosted glass plates together with 3-mm spacers in between. Seal the bottom with Parafilm to prevent leakage of warm agarose.

13b. Warm glass plates for 30 min in 50°C incubator.

14b. Melt agarose as in step 12a (1 g agarose in 100 ml 0.5× TBE), and pour into the prewarmed gel assembly to ~5 mm below top of plates. Insert comb into the gel, and allow to set for 1 hr.

15b. Remove the comb very carefully so as not to break the gel, and wash the wells with 0.5× TBE to remove partially polymerized agarose.

Load and run gel

16. Using a scalpel, cut out a 2-mm slab from one marker, and insert into first well of the gel with a spatula.

17. Repeat this procedure with the second marker.

    Make sure that the slab is flat on the bottom of the well. This applies to loading of samples as well.

18. To load sample, cut off 3 mm from sample plug, and insert it into individual well with a spatula. Repeat same procedure for all samples. Avoid introducing any air bubbles into the wells.

    Note and record the order of the samples!

19. Cement the slabs into wells with the remaining molten 1% agarose.

20. Transfer the loaded gel (either horizontal or vertical) into a precooled tank filled with 0.5× TBE.

    The buffer temperature should be 11° to 12°C at the beginning of the experiment.

21. Run the gel at a constant voltage with a suitable switcher program to achieve the desired resolution. The programs listed in Table 18.3.2 give good resolution of 50-, 300-, and 700-kbp fragments.

22. Remove gel carefully, and stain it in ethidium bromide for 1 hr (see Basic Protocol 5, step 18). Destain it in 0.5× TBE for 2 to 3 hr.

23. Visualize and photograph bands under UV light.

METHODS FOR ANALYSIS OF CASPASE PROTEOLYTIC ACTIVITY

Several proteolytic activities are implicated in apoptosis and necrosis (see *UNIT 18.1*). The activation of caspases, a family of apoptotic proteases, plays a key role in apoptosis induced by diverse stimuli. Caspases, which have been detected in numerous tissues and cell types, are synthesized as a precursor form, or proenzyme, and an apoptotic signal converts the precursor into mature enzyme. Moreover, until now there has been no

Table 18.3.2 Parameters for Running Pulsed-Field Agarose Gels

	Horizontal gel	Vertical gel
Running voltage[a]	170 V	200 V
Procedure	10 min: run the DNA into gel with continuous forward pulse	10 min: run the DNA into gel with continuous forward pulse
	6 hr: apply 20 sec forward time, 3:1 forward/back ratio, ramp factor 1.5[b]	2 hr: apply 12 sec forward time, 3:1 forward/back ratio, ramp factor 2[c]
	6 hr: apply 10 sec forward time, 3:1 forward/back ratio, ramp factor 2	3 hr: apply 2.4 sec forward time, 3:1 forward/back ratio, ramp factor 5.0
	12 hr: apply 0.8 sec forward time, 3:1 forward/back ratio, ramp factor 12.5	2.5 hr: apply 8.0 sec forward time, 3:1 forward/back ratio, ramp factor 3.0
Total running time	24 hr, 10 min	7 hr, 40 min

[a] Constant voltage

[b] This means that at the end of these 6 hr, the forward time should be 30 sec.

[c] This means that at the end of these 2 hr, the forward time should be 24 sec.

evidence for the involvement of caspase activities in necrosis. Therefore the detection of caspase activity in cells can be used as a discriminating criterion to distinguish apoptosis from necrosis.

All members of the caspase family of proteases share a number of amino acid residues crucial for substrate binding and catalysis. Despite their uniform requirement for an aspartate residue at the P_1 position of the substrate site, caspases can be divided into three different groups according to their substrate preferences. The first group (caspases 1, 4, and 5) has the optimal substrate cleavage sequence WEXD; the second (caspases 2, 3, and 7) preferentially cleaves targets at the DEXD motif; and the third (caspases 6, 8, and 9) has the optimal substrate cleavage sequence (L/V)EXD (Thornberry et al., 1997). The predicted caspase specificities correspond to the cleavage sites in the different intracellular caspase targets. Analysis of caspase activation is also based on the substrate specificity of the enzymes (see *UNIT 18.2*).

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified or other ultrapure water for all recipes and protocol steps. For common stock solutions, see **APPENDIX 2**; for suppliers, see **SUPPLIERS APPENDIX**.*

Agarose buffer *(for molds; used in Basic Protocol 7)*

Prepare 500 ml in H_2O:
0.15 M NaCl
2 mM KH_2PO_4/KOH, pH 6.8
1 mM EGTA
5 mM $MgCl_2$
Filter sterilize through 0.2-µm filter
Store at 4°C

Diphenylamine reagent

100 ml glacial acetic acid
1.5 g diphenylamine
1.5 ml concentrated sulfuric acid
0.5 ml 16 mg/ml acetaldehyde stock
Prepare just before use

Prepare acetaldehyde stock in ultrapure water; store in a dark flask up to 1 month at 4°C.

Ethidium bromide, 10 mg/ml

Stir 10 mg/ml ethidium bromide in water on magnetic stirrer for several hours. Store up to 2 months in a dark bottle at 4°C.

Paraformaldehyde, 4% (w/v)

Add 8 g paraformaldehyde powder (e.g., Sigma) to 100 ml water and heat to 60°C in a fume hood. If necessary, add NaOH dropwise (one or two drops each time) to help dissolve the powder. When the solids have completely dissolved, let the solution cool to room temperature. Then add 100 ml of 2× PBS (*APPENDIX 2A*). Store at 4°C.

Because this solution cannot be kept indefinitely, it is better to prepare small volumes every week.

Phenol, TE saturated

Allow phenol to warm to room temperature, and then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1%. Add a volume of TE buffer, pH 8.0 (10 mMTris·Cl/1 mM EDTA), equal to the volume of phenol, and vortex extensively. Allow the two phases to separate, and aspirate the upper aqueous phase. Repeat this saturation with TE buffer two to three times until volume of TE remains unchanged and the pH of the phenol phase has reached 8.0 (as measured with pH paper). Remove the aqueous phase, and add 0.1 vol fresh TE. Store up to 1 month in the dark at 4°C.

Hydroxyquinoline, a yellow antioxidant, provides a convenient way to identify the organic phase during extraction.

Proteinase buffer *(for treatment of plugs)*

Prepare 500 ml of buffer consisting of:
10 mM NaCl
10 mM Tris·Cl, pH 9.5
25 mM EDTA

Add *N*-lauroylsarcosine (Sarkosyl) to 1% final concentration and dissolve using magnetic stirrer. Check pH, and if necessary adjust to 9.5 with NaOH. Filter through 0.2-µm filter and store at 4°C.

RNase A, 50 mg/ml

Dissolve RNase A (e.g., Sigma) at a concentration of 50 mg/ml in 10 mM Tris·Cl, pH 7.5/15 mM NaCl. Heat 15 min at 50°C. Allow to cool at room temperature. Divide into aliquots and store up to several months at −20°C.

RNase T1/A stock

Mix 50 µl of 500,000 U/ml RNase T1 and 50 mg of RNase A in 5 ml of 10 mM Tris·Cl, pH 7.5/15 mM NaCl. Heat for 15 min at 50°C. Allow to cool at room temperature. Divide into aliquots and store up to several months at −20°C.

SDS, 10% (w/v)

Dissolve 10 g of sodium dodecyl sulfate (SDS) in 90 ml of water by mixing on magnetic stirrer for several hours or by heating to 65°C to assist dissolution. Adjust the volume to 100 ml with water. Store at room temperature for up to 1 month.

Staining buffer

54.2 µl water
25 µl 20× sodium cacodylate (SSC) stock solution
20 µl blotto (25% low-fat dried milk in PBS)
0.7 µl avidin-FITC (160×)
0.1 µl Triton X-100
Store up to several weeks at 4°C

TBE (Tris/borate/EDTA) buffer, 5×

445 mM Tris base
445 mM boric acid
10 mM trisodium EDTA
Store at room temperature

All components may be obtained from Sigma. Do not adjust pH, which should be about 8.0 at room temperature. When this solution is stored for a long time, a precipitate forms. To avoid problems, discard batches that develop a precipitate.

COMMENTARY

Background Information

A number of techniques have been developed to detect cytotoxicity and cell death in various cell types. The methods developed in the last two decades have been aimed at distinguishing between cell toxicity leading to apoptosis and that leading to necrosis. The choice of a particular method for analysis of cellular toxicity depends on the cell system; the toxin or toxicant; the expected mode of cell death; the type of information being sought; and, finally, technical limitations. There is no one single method that is satisfactory for obtaining all the information required. This is largely a result of technical and practical limitations. Therefore, to make the right interpretation of data and to draw the correct conclusions regarding the mode of cell death, a combination of some of the methods mentioned above should be used. It is recommended that a combination of at least three criteria of cell death be evaluated: cell morphology, DNA fragmentation (see Assays for Chromatin Cleavage), and/or caspase activation (see *UNIT 18.2*).

Although morphological changes are obviously secondary to biochemical alterations, many of the published reports on cellular toxicity are based mainly on histological, cytological, and electron microscopy studies (Kerr et al., 1972; Bowen, 1980). The interpretation of histological data is based on the changes in membrane permeability. However, as mentioned in the introduction to this unit, analysis of plasma membrane integrity fails to identify cells in the early stages of apoptosis, although it can be used to identify necrotic cells in a population of interest. Alternative morphological methods draw on a mixture of optical and electron microscopy techniques. Combination of these methods allows one to distinguish between apoptotic changes—cell shrinkage, high cytoplasm density, membrane blebbing, compaction of chromatin, and formation of apoptotic bodies—and those indicative of necrosis—chromatin clumping, gross swelling of organelles, early membrane breakdown, and cell disintegration. In addition, confocal microscopy allows the combination of morphological analysis with subcellular localization of biochemical changes within damaged cells.

Other changes that accompany cell death can be used as indicators of the mode of cell death. It has been shown that phagocytosis is a response to cell death and is particularly efficient in apoptosis. Apoptotic cells can be recognized by macrophages through a number of cell surface markers. The presence of PS on the outer plasma membrane is one such marker, and it can be detected efficiently by phagocytic cells, as blockage of PS renders phagocytosis less efficient. Therefore, PS exposure can be considered a biochemical feature of apoptosis with physiological importance (Koopman et al., 1994). Annexin V has recently been utilized as a probe to monitor changes in phospholipid distribution in the plasma membrane of cells undergoing apoptosis. In combination with propidium iodine, annexin V staining can reliably discriminate apoptosis from necrosis. Flow cytometry is a useful technique to examine not only changes in the plasma membrane, but also changes in cell size and granularity (Darzynkiewicz et al., 1994; Robinson et al., 1999). Apoptosis is accompanied by a dramatic

loss of water and consequent cell shrinkage, which can be distinguished from necrosis by a difference in light-scatter pattern. This is a rapid technique to identify apoptotic versus necrotic cells and is very widely used nowadays.

Several biochemical methods based on the detection of changes in chromatin structure have been developed. These vary from methods for detecting single-strand DNA nicks or breaks, to those for demonstrating high-molecular-weight DNA fragments, to those for showing the subsequent internucleosomal cleavage. DNA nicks can be detected using TUNEL (see Basic Protocol 4; Gavrieli et al., 1992). However, this method apparently recognizes not only nicks formed during apoptosis, but also those formed during necrosis. Thus this method should always be used in combination with other more discriminating methods.

Some other methods used for the analysis of DNA or chromatin damage appear to be better suited to discriminate between the two types of cell death. The different DNA gel electrophoresis techniques that are described here (see Basic Protocol 5; see Alternate Protocols 2, 3, and 4) can be utilized for a qualitative analysis (Wyllie, 1980; Sorenson, 1990). Although the quantitative analysis (see Basic Protocol 6) can provide limited information concerning the general cleavage of chromatin (Burton, 1956), the combination of different methods of gel electrophoresis provides more specific information concerning the type of chromatin cleavage (high-molecular-weight DNA fragments, DNA ladder, or smear). It has been shown that DNA laddering is not apparent in all apoptotic systems (Oberhammer et al., 1993a). In many types of cells, chromatin cleavage is restricted to the formation of high-molecular-weight DNA fragments (Walker et al., 1991; Oberhammer et al., 1993b). In such cases, again, the combination of two independent methods assists in drawing the correct conclusions in the study of cell death or toxicity. However, when DNA laddering occurs, it is highly indicative of apoptosis.

Activation of the caspase family of proteases has been detected in numerous cell systems and appears to function as a pathway through which apoptotic mechanisms operate (for review see Zhivotovsky et al., 1997). Upon apoptotic triggering, a hierarchy of caspases is believed to become activated in a process where more proximal caspases cleave and activate downstream caspases, giving rise to a proteolytic cascade that serves to amplify the death signal. However, not all of these caspases normally function in cell death. A recent classification divided caspases into two groups, one involved in cytokine processing (caspase 1–like proteases) and the other playing a role in cell death (Alnemri et al., 1996; Cryns and Yuan, 1998). This latter group can be further subdivided according to function as activators or executioners of cell death. Up to now there has been no evidence concerning the involvement of caspase activities in necrosis. Therefore the detection of caspase activity in cells can be used as a discriminating criterion to distinguish apoptosis from necrosis. Caspase activity is assessed by proteolytic cleavage of flurogenic substrates (see *UNIT 18.2*) or by immunodetection of protein bands corresponding to active caspases (see *UNIT 18.2*).

In summary, a combination of morphological and biochemical methods is recommended to discriminate between the mode of cell death, especially if the manifestations of cell death or the stimuli used have not been previously characterized in the cell type of interest.

Critical Parameters and Troubleshooting

Morphological assays

A characteristic feature of apoptosis is that membrane integrity of the dying cell is maintained long after the process has begun. However, a reduction in membrane integrity usually occurs during the late stages of apoptosis when the cells undergo a process commonly called "secondary necrosis" or "postapoptotic necrosis." Therefore, a disadvantage of the trypan blue exclusion assay (see Basic Protocol 1) is underestimation of the level of apoptosis in a cell population.

If the cytopsin preparations are understained after a full cycle of fixation and differential staining (see Basic Protocol 2), then the procedure can be repeated a second time until the desired staining is achieved. If overstaining is the problem, preparations can be destained by immersing them in 100% methanol for up to 5 sec, followed by rinsing with water. Because membrane blebbing is a feature common to both apoptosis and necrosis, one should take care not to use this as the sole criterion for determination of the mode of cell death.

If assessing the nuclear changes in apoptotic cells is problematic, then use other conventional methods of inducing apoptosis in cells as a positive control, stain cells treated both ways with Hoechst dye (see Basic Protocol 3), and observe nuclear changes under the UV micro-

scope. If no apoptotic changes are evident in cells, try to increase the time of incubation with apoptosis-inducing agents.

Chromatin cleavage assays

The TUNEL technique (see Basic Protocol 4) is used to detect DNA strand breaks. There are rare situations when apoptosis proceeds without DNA degradation. Conversely, extensive DNA degradation may accompany necrosis. Thus, one should always use another independent assay, along with the TUNEL method, to confirm whether cells have undergone apoptosis. Costaining cells with propidium iodide or Hoechst is a very useful method to discriminate between apoptosis and necrosis in samples that have stained positively with the TUNEL technique. TUNEL-positive cells that express condensed, fragmented nuclei after staining with PI or Hoechst are apoptotic, whereas those with diffuse staining of the nuclei with PI or Hoechst are necrotic.

If a high background is present in TUNEL-treated samples, make sure that there is no DNase contamination in the test tubes or buffers. It is also possible that the number of DNA strand breaks is so large that one cannot quantify the degree of cell labeling. To eliminate this problem, use fewer cells or decrease the incubation time.

For exogenous enzymes, such as the TdT enzyme used in TUNEL, to enter the cell, the plasma membrane has to be permeable. In many cases, the fixation procedure is sufficient to permeabilize cells; however, sometimes an additional short incubation on ice or short (1- to 2-min) treatment with proteinase is required. To avoid loss of low-molecular-weight DNA, the permeabilized cells have to be fixed with formaldehyde before permeabilization. This fixation cross-links DNA fragments to other cellular constituents and prevents their leakage during the permeabilization step.

The procedures for assessment of DNA fragmentation in whole cells (see Basic Protocol 5) may cause the samples to become viscous and difficult to load into gel sample wells. If this is the case, dilute samples with 1× DNA loading buffer. If no detectable DNA laddering is observed in a population of cells that are known to be apoptotic, increase the number of cells used for the assay. If it is difficult to observe distinctive bands because of high background, wash gel extensively with water. If an unusual band appears in the middle of the gel in all lanes, incubate gel longer with RNase (step 17).

For analysis of fragmentation in total genomic DNA (see Alternate Protocol 2), the reagents are optimized for the stated number of cells. If larger numbers of cells are used, RNA and protein digestion will not be complete, which may interfere with electrophoresis. In addition, the procedures cause the samples to be viscous. Therefore, for minimum sheering of DNA, it is better to use pipet tips that have the ends (3 to 4 mm) cut off with a razor blade to make wide-bore tips.

If no laddering is observed in DNA phenol extracted from a population of apoptotic cells (see Alternate Protocol 4), the DNA may have been lost in the organic phase during extraction. The aqueous phase should be removed carefully during extraction to prevent DNA loss. Another possible reason for failure to detect laddering may be degradation of DNA by exogenous nucleases. All solutions must be free of nuclease contamination. If, instead of a DNA ladder, a smear appears on the gel, this indicates random and general cleavage of chromatin, indicative of necrosis (see Figure 18.3.2A, lane 3).

For quantitative assays of DNA fragmentation (see Basic Protocol 6), if absorbance of DNA in supernatant samples is below the sensitivity of detection, increase the number of cells. If all absorbance is present in the supernatant and absent from the pellet, then the pellet was mistakenly transferred together with supernatant to the labeled conical glass tubes (step 4).

There could be several reasons for large fragments not to appear on the pulsed-field electrophoresis gel (see Basic Protocol 7). If the material is still in the wells, the pulse generator power supply may not have been working properly. If some of the material is in the wells, but no sharp bands are visible and the material moved in the wrong direction, the current may not have been applied in the correct direction. If there is no material in the wells, the chromatin may have been digested by exogenous exonucleases during the preparation. If very little material is in the well, a bigger slab may have to be cut from the sample plugs. If too much material is in the lane, a smaller slab may have to be cut from the sample plugs. If thin stripes appear in any lanes in the gel, it means that either a bubble was in the well or the slab was not placed flat. Sometimes there is a leakage of buffer from the top reservoir, which may also influence the results.

Anticipated Results

Morphological assays

In the trypan blue exclusion assay (see Basic Protocol 1), several thousand cells are sufficient

to quantify the number of trypan blue–positive cells and to count the percentage of cells with an intact plasma membrane. Necrotic cells with rapidly disrupted membrane take up trypan blue as well.

Differential staining (see Basic Protocol 2) is one of the fastest and easiest methods for identifying dead cells. Cells are viewed under a light microscope (40× magnification), and ~300 cells are counted from separate fields of view and scored as normal, apoptotic, or necrotic based on the following morphological characteristics: membrane blebbing, chromatin condensation and nuclear shrinkage, cytoplasmic constriction and loss of cell volume, and formation of apoptotic bodies for apoptotic cells; nuclear swelling, chromatin flocculation, cell membrane blebbing and disruption, and finally cell lysis resulting in the appearance of "ghost cells" for necrotic cells (see Figure 18.3.1).

Because chromatin condensation is one of the early events of apoptosis, apoptotic cells stained with Hoechst 33342 have increased blue fluorescence compared with live cells similarly stained (see Basic Protocol 3). The intensity of the blue fluorescence changes with the incubation time, so that an optimal incubation time should be found for each cell system for discrimination of apoptotic cells (usually 5 to 10 min). For quantification of apoptosis, a minimum of 150 cells, from 10 to 15 fields, should be evaluated for the changes described above.

Chromatin cleavage assays

In both TUNEL protocols (see Basic Protocol 4 and Alternate Protocol 1), very little material is required for quantitative measurement of TUNEL-positive cells. Proportions of apoptotic cells as low as 10% to 15% can be detected by this method. Although the enzymatic labeling methods are time consuming (due to multiple incubation and washing steps), they are very sensitive. The method can also be applied to cryostat sections. In this case, the proteolytic pretreatment (see Alternate Protocol 1, step 4) can be shorter or even be omitted.

The whole-cell DNA fragmentation method (see Basic Protocol 5) requires only $4–5 \times 10^5$ cells. It can be used for cells growing in suspension as well as attached cells. Usually, the resolution of DNA fragmentation using this protocol is distinct and high. This method does not require DNA purification and is relatively simple and fast. Although Basic Protocol 5 and Alternate Protocol 2 are equally sensitive, the latter is less time consuming. In addition, the dry loading prevents the loss of material. Alternate Protocol 3 is particularly useful for detecting chromatin cleavage in different lymphoid cells.

Phenol extraction (see Alternate Protocol 4) is the most commonly used technique for investigating internucleosomal chromatin cleavage. Although it takes much more time than other techniques (almost 2.5 working days) and needs many more cells for preparation ($2–5 \times 10^6$ cells), it yields very clean, protein-free DNA fragments.

The quantitative assay of DNA fragmentation described (see Basic Protocol 6) is only one quantitative method for measurement of DNA fragmentation. This method is very simple, and very reproducible results can usually be obtained.

Pulsed-field gel electrophoresis can be used to detect the presence of high-molecular-weight DNA fragments in apoptotic cells (see Basic Protocol 7). The method is very sensitive and requires 5×10^5 cells. Depending on which system is available, one can use either horizontal or vertical gel systems. If necessary, plugs can be kept for several months and run on another gel.

Time Considerations

Morphology assays

The time required for preparing solutions for trypan blue exclusion is 20 to 30 min; the solutions can then be kept up to 1 to 2 months at 4°C. Trypan blue staining takes 5 min, and calculation takes 10 min.

It takes 30 min to prepare the solutions for differential staining (which can be stored 1 to 2 months at room temperature); this time is not required if the commercial kit is used. 1 hr is required to prepare and analyze samples, and 30 min to evaluate results.

It takes about 30 to 40 min to prepare solutions for Hoechst staining and 1 hr to run the test. No more than 30 min are required to evaluate results.

Chromatin cleavage assays

Approximately 3 to 4 hr are required to run TUNEL assays. The evaluation of results requires an additional 1 to 2 hr, depending on the investigator's experience.

About 1 to 2 hr are required to prepare the buffers for assessment of fragmentation in whole cells, which then can be stored for at least 1 month. Usually, gel and sample preparation, which can be done simultaneously, take 2 hr, and gel electrophoresis takes 15 to 16 hr (over-

night is better). Incubation with RNase, staining, and destaining take another 6 to 7 hr. Thus, the whole experiment takes almost 1.5 days.

Analysis of fragmentation in total genomic DNA takes 1 working day.

Preparing solutions for quantitative analysis of DNA fragmentation takes 1.5 to 2 hr; however, the solutions can be stored for up to several weeks. The procedure takes 1.5 working days and 1 hr to evaluate results.

It takes about 1 week to perform the whole pulsed-field gel electrophoresis procedure. Vertical gels require a relatively shorter time (1 day less) compared with horizontal gels, with no difference in resolution.

Literature Cited

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. 1996. Human ICE/CED-3 protease nomenclature. *Cell* 87:171.

Anand, R. and Southern, E.M. 1990. Pulsed field gel electrophoresis. *In* Gel Electrophoresis of Nucleic Acid: A Practical Approach, 2nd ed. (D. Rickwood and B.D. Hames, eds.) pp. 101-123. IRL Press, Oxford.

Bowen, I.D. 1980. Techniques for demonstrating cell death. *In* Cell Death in Biology and Pathology (I.D. Bowen and R.A. Lockshin, eds.) pp. 379-444. Chapman & Hall, London–New York.

Burton, K. 1956. A study of the condition and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62:315-323.

Cryns, V., and Yuan, J. 1998. Proteases to die for. *Genes Devel.* 12:1551-1570.

Darzynkiewicz, Z., Robinson, J.P., and Crisstman, H.A. (eds.) 1994. *Methods Cell Biol.*, Vols. 41 and 42 (Flow Cytometry: Part A and Part B, 2nd ed.). Academic Press, San Diego.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. 1992. Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.* 119:493-501.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. 1972. Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* 26:239-257.

Koopman, G., Reutelingsperger, C.P.M., Kuijten, G.A.M., Keehnen, R.M.J., Pals, S.T., and van Oers, M.H.J. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B-cells undergoing apoptosis. *Blood* 84:1415-1420.

McGahon, A.J., Martin, S.J., Bissonnette, R.P., Mahboubi, A., Shi, Y., Mogil, R.J., Nishioka, W.K., and Green, D.R. 1995. The end of the (cell) line: Methods for the study of apoptosis in vitro. *Methods Cell Biol.* 46:153-185.

Oberhammer, F., Fritsch, G., Scmied, M., Pavelka, M., Printz, D., Purchio, T., Lassman, H., and Schulte-Hermann, R. 1993a. Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. *J. Cell Sci.* 104:317-326.

Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hichman, J.A., Wakeling, A.E., Walker, R.A., and Sikorska, M. 1993b. Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.* 12:3679-3684.

Pollak, A. and Ciancio, G. 1990. Cell cycle phase–specific analysis of cell viability using Hoechst 33342 and propidium iodide after ethanol preservation. *Methods Cell Biol.* 33:19-24.

Robinson, J.P., Darzynkiewicz, Z., Dean, P.N., Dressler, L.G., Orfao, A., Rabinovitch, P.S., Stewart, C.S., Tanke, H.J., and Wheeless, L.L. 1999. Current Protocols in Cytometry. John Wiley & Sons, New York.

Skalka, M., Matyasova, J., and Cejkova, M. 1976. DNA in chromatin of irradiated lymphoid tissues degrades in vivo into regular fragments. *FEBS Lett.* 72:271-275.

Sorenson, C.M., Barry, M.A., and Eastman, A. 1990. Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. *J. Natl. Cancer Inst.* 92:749-755.

Thornberry, N.A., Rano, T., Peterson, E., Rasper, D., Timkey, T., Garcia-Calvo, M., Houtzager, V., Nordstrom, P., Roy, S., Vaillancourt, J., Chapman, K., and Nicholson, D. 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B: Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* 272:17907-17911.

Walker, P.R., Smith, C., Youdale, T., Leblamc, J., Whitfield, J.F., and Sikorska, M. 1991. Topoisomerase II–reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.* 51:1078-1085.

Wyllie, A.H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284:555-556.

Zhivotovsky, B., Gahm, A., Ankarcrona, M., Nicotera, P., and Orrenius, S. 1995. Multiple proteases are involved in thymocyte apoptosis. *Exp. Cell Res.* 221:404-412.

Zhivotovsky, B., Burgess, D.H., Vanags, D.M., and Orrenius, S. 1997. Involvement of cellular proteolytic machinery in apoptosis. *Biochem. Biophys. Res. Commun.* 230:481-488.

Contributed by Boris Zhivotovsky and Sten Orrenius
Karolinska Institute
Stockholm, Sweden

Quantitative Fluorescence In Situ Hybridization (Q-FISH)

This unit describes a quantitative technique for measuring the lengths of telomere repeat sequences in individual chromosomes from single metaphase cells (see Basic Protocol). The technique is based on fluorescence in situ hybridization (FISH) adapted for use with peptide nucleic acid (PNA) probes. PNA is an example of novel synthetic oligonucleotide "mimetic" which has a higher affinity than regular oligonucleotide (RNA or DNA) probes for complementary single-strand (ss) DNA sequences. PNA oligonucleotides have excellent penetration properties due to their small size (typically 15 to 18–mers) and can be directly labeled with fluorochromes. These properties have been exploited to develop quantitative fluorescence in situ hybridization (Q-FISH) onto denatured single-stranded chromosomal DNA target sequences. The latter can be present in preparations of fixed metaphase cells on slides (Q-FISH) or in heat-treated (interphase) cells in suspension (flow-FISH).

Q-FISH requires fluorescence microscopy in combination with digital image acquisition to measure the length of telomere repeats in preparations of metaphase chromosomes. The basic Q-FISH techniques are also very suitable to obtain qualitative information about the presence of specific repeat sequences in the nuclei of cells. The bright fluorescence signals combined with the low background of directly labeled PNA probes offer unique possibilities for a variety of studies including studies of nuclear organization. Such studies are not discussed here but can be explored using the Basic Protocol provided.

The Q-FISH technique described here consists of four distinct steps: (1) sample preparation; (2) image capture; (3) image analysis; and (4) telomere length normalization and calibration. First, slides suitable for Q-FISH analysis are prepared. For this purpose, fixed metaphase cells are dropped onto slides and hybridized with directly labeled PNA probes using an optimized FISH technique (see Basic Protocol). Second, images of telomeres and chromosomes are captured using digital fluorescence microscopy (see Support Protocol 1). Third, the captured images are analyzed to extract telomere fluorescence and chromosome identification (karyotype) information (see Support Protocol 2). Finally, similar procedures incorporating all the three components are used to calibrate and validate Q-FISH for quantitative purposes and determine calibration values for telomere length measurements (see Support Protocol 3).

USING PNA PROBES TO VISUALIZE TELOMERES IN METAPHASE SPREADS

BASIC PROTOCOL

The following is a basic "generic" method to visualize telomeres and chromosomes in metaphase cells using fluorescence hybridization with directly labeled PNA probes. The slides that result from application will typically be of high quality and can be used for any type of qualitative analysis. However, in order to extract the quantitative information that is contained within these preparations, close attention to all steps described in subsequent protocols (see Support Protocols 1, 2, and 3) is essential.

The aim is to first prepare and deposit metaphase cells onto microscope slides. Standard cytogenetic techniques are used to prepare metaphase chromosomes from dividing cells. After fixation, metaphase cells are dropped onto slides and air-dried. A small volume of hybridization mixture containing Cy3- or FITC-labeled telomere (PNA) probe is placed onto coverslips and added to the chromosome preparations on the slides. The slides are subsequently heated at 80°C for 3 min using a thick metal (aluminum) plate preequili-

Contributed by Steven S.S. Poon and Peter M. Lansdorp
Current Protocols in Cell Biology (2001) 18.4.1-18.4.21

brated to 80°C in an oven. The heat and high (70%) formamide content of the hybridization mixture will result in complete denaturation of chromosomal DNA. Removal of the slides from the oven and subsequent incubation of the slides at room temperature result in conditions that allow hybridization of the PNA probes to chromosomal target sequences but not the renaturation of complementary DNA. Conventional FISH procedures (using RNA or DNA probes) must use hybridization conditions that allow renaturation of complementary DNA strands as well. In Q-FISH only the PNA probes can hybridize to the denatured telomere repeat target sequences in chromosomal DNA. As a result, the number of probes attached to target sites will correlate to the number of repeat sequences at that site. In this way, telomere probes provide information on the length of telomere repeats at individual telomeres. After hybridization, the slides are washed to remove unbound PNA, dried, and sealed using a coverslip and a small volume of an antifade solution containing DAPI (4′,6-diamidino-2-phenylindole,fs15 dihydrochloride) as a DNA counterstain. DAPI allows the visualization of cells and individual chromosomes and facilitates subsequent image acquisition, karyotyping, and telomere length analysis steps described in Support Protocols 1, 2, and 3.

Materials

Cells of interest
Cell-specific tissue culture medium supplemented with growth factors
10 μg/ml Colcemid (Life Technologies)
75 mM hypotonic KCl buffer (12.3 mM HEPES/0.53 mM EGTA/64.4 mM KCl; or commercially available from Stem Cell Technologies or Life Technologies), 37°C
3:1 (v/v) methanol/glacial acetic acid fixative (Fisher; BDH)
70%, 90%, 95%, and 100% ethanol
Phosphate buffered saline, pH 7.4 (PBS; without Ca^{2+} and Mg^{2+}, Stem Cell Technologies; also see recipe)
37% formaldehyde, reagent-grade (BDH)
Pepsin (Sigma-Aldrich)
1 N HCl, pH 2.0
Hybridization mixture (see recipe)
Wash solution I (see recipe)
Wash solution II (see recipe)
Mounting medium containing antifade and DAPI (see recipe)

25-cm^2 tissue culture flasks with vented blue plug seal caps (Becton Dickinson)
15- and 50-ml polypropylene conical tube (Becton Dickinson)
Precleaned microscope slides for hematology and histology (e.g., Micro World; *http://www.mwrn.com/)*
Lint-free paper towels
Phase-contrast microscope
100- and 150-ml glass slide staining jars and corresponding slide rack
Coverslips (22 × 60–mm; VWR)
Thick metal plate (~1-cm aluminum), e.g., 6 ×10–in.
80°C oven
Molded plastic slide box for 25 slides (Fisher)
Parafilm
Platform shaker (Barnstaed/Thermolyne)
Light-protected slide storage box

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic techniques should be used accordingly. Furthermore, it is prudent to consider cultured and primary human cells as biohazardous and adhere to appropriate biohazard

containment and disposal guidelines. Consult your institutional safety officer for specific instructions.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

CAUTION: To avoid inhalation of fumes from glacial acetic acid, methanol, formaldehyde, and formamide solutions, it is recommended that cell fixation and formamide washes be performed in a fume hood. Formamide is a harmful and irritant organic solution. Wear suitable protective clothing and gloves when handling; avoid inhalation of fumes and skin contact. Use an excess of water in case of skin contact. Refer to Material Safety Data Sheet and consult your institutional guidelines for appropriate disposal of chemical waste.

Prepare metaphase-arrested cells

1. Culture cells for several days in tissue culture medium supplemented with growth factors in 25-cm^2 tissue culture flasks.

   It is important to maximize the fraction of dividing cells in order to obtain sufficient mitotic cells and metaphase cells (chromosomes) in subsequent steps.

2. Add 10 µg/ml Colcemid to the culture medium (final 0.05 µg/ml) prior to harvesting mitotic cells.

   The duration of the Colcemid treatment varies with cell type (e.g., for lymphocytes, from 30 min to 2 hr; for fibroblasts, from 6 to 24 hr), the proportion of dividing cells, and the required quality of the metaphase chromosomes (the longer the Colcemid treatment, the more condense the chromosomes).

3. Harvest the cells by trypsinization and/or pipetting as required and transfer to 15-ml tubes.

4. Centrifuge cell suspension 8 to 10 min at 200 to 300 ×*g*, room temperature.

5. Remove supernatant, resuspend pellet in remaining supernatant (<0.5 ml) by vortexing the tube and adding 2 to 10 ml of prewarmed (37°C) 75 mM hypotonic KCl buffer depending on the size of the pellet.

6. Incubate 20 to 30 min in a 37°C water bath.

Fix cells

7. Centrifuge cells 8 to 10 min at 200 to 300 ×*g, room temperature, remove all but 0.5 ml of the hypotonic KCl solution, resuspend the cell pellet by hand tapping the tube, and add 1 to 5 ml of fresh 3:1 methanol/glacial acetic acid fixative with a Pasteur pipet. Let stand 15 min at room temperature.*

8. Centrifuge the tubes again as in step 7, discard supernatant, and suspend the cells in 1 to 5 ml fresh 3:1 methanol/glacial acetic acid fixative. Repeat this step one or two times until a suitable cell pellet is obtained.

   A suitable preparation will show nuclei devoid of cytoplasm as judged by microscopy after dropping the cells onto slides.

   After the last fixation, cells can be stored in fixative in a closed tube for at least 3 months at −20°C prior to the preparation of slides. However, typically, slides are prepared within 7 days.

Prepare slides

9. Clean glass microscope slides with 95% ethanol. Wipe slides with dry lint-free paper towels.

   Alternatively, precleaned slides can be used directly.

10. Resuspend the cells in a small amount of fresh 3:1 methanol/glacial acetic acid fixative until the suspension looks slightly turbid (~5×10^6 cells/ml). Drop 3 to 4 drops evenly on a slide covered with a thin film of distilled water. After the slide is completely dry, examine with a phase-contrast microscope to check the cell density and spread of metaphase chromosomes.

    If the cell density is too high, add a few more drops of fixative to the cell suspension. If the cell density is low, centrifuge the suspension and resuspend the pellet in a smaller amount of fixative.

11. With a Pasteur pipet, let two drops of cell suspension fall onto each cleaned, wet slide. Mark the slide with a diamond pen or a pencil. Air dry slide(s) overnight in the fume hood.

12. Pour 150 ml PBS without Ca^{2+} and Mg^{2+} in a 150-ml staining jar with a removable slide rack. Place slides in glass slide rack and immerse in PBS to rehydrate for 15 min at room temperature.

Fix slides in formaldehyde

13. In a fume hood, prepare 4% formaldehyde in PBS by adding 21.6 ml of 37% formaldehyde stock solution to 200 ml PBS.

14. In the fume hood, transfer the 4% formaldehyde solution into a staining jar. Transfer the slides in a slide rack into the fixative solution and fix for 2 min. Save fixative so it can be reused in step 18.

15. Wash slide(s) in three changes of fresh PBS for 5 min each change.

Treat slides with pepsin

16. Prepare pepsin solution by dissolving 200 mg pepsin in 200 ml of 0.01N HCl, pH 2.0. Pour the pepsin solution into a staining jar and equilibrate in a 37°C water bath. Prepare this solution during PBS washes (step 15).

17. Transfer slide(s) to pepsin solution and incubate 10 min at 37°C. Wash slides two times, 2 min each, in PBS.

18. Repeat the fix and wash steps (steps 14 and 15 above).

19. Dehydrate slide(s) in an ethanol series: 5 min each in 70%, 90%, and 100% ethanol.

Hybridize PNA probe

20. Leave slides in a vertical position to air dry or use air stream to dry.

21. Deposit two 10-µl drops hybridization mixture, with a micropipet, onto a clean 22 × 60–mm coverslip.

22. Gently place the slide(s) with fixed cells (upside down) on top of the coverslip (placed on a bench or glass plate). Turn slide so that coverslip faces up prior to complete spreading of the hybridization mixture between the slide and the coverslip.

    Avoid introducing air bubbles by holding the slide under a slight angle prior to contacting the drops of hybridization mixture on the coverslip.

Heat denature DNA

23. Denature chromosomal DNA by placing the slide(s), coverslip up, on a thick metal plate equilibrated at 80°C in a preheated oven for 3 min.
24. Place the slide(s) in a plastic slide box and place this box into a plastic beaker containing a damp paper towel.
25. Cover the beaker with Parafilm and let the sample hybridize for 1 to 2 hr at room temperature.

Wash slides

26. Prepare 500 ml of wash solution I and 500 ml of wash solution II.
27. In the fume hood, remove coverslips by soaking the slides in 100 ml of wash solution I. Use gloves and forceps to place slides back into a glass slide rack that fits into glass staining jars. Wash the slide(s) two times, 15 min each, in the remaining solution I.

    Do not force the coverslips off the slides, as this will damage the cells.

28. Wash the slide(s) three times, 5 min each, in wash solution II on a platform shaker.
29. Dehydrate the cells 5 min each in 70%, 90%, and 100% ethanol and allow the sample to air dry.
30. Place two 10-µl drops of mounting medium containing antifade and DAPI on a fresh coverslip.
31. Place the sample slide(s) on top of the coverslip (as in step 22).
32. Lift the slide (coverslip attached) and incubate in the dark, in a light-protected slide storage box, for at least 15 min to allow DAPI staining of DNA.

    The slides are now ready for analysis (see Support Protocols 1, 2, and 3) and can be stored in a light-protected storage box for several weeks at 4°C prior to fluorescence microscopy and Q-FISH analysis.

IMAGE CAPTURE

SUPPORT PROTOCOL 1

The following is a method for capturing images of telomeres and chromosomes prepared using the Basic Protocol. In order to capture images in a constant manner such that they can be used for quantitative analysis, special attention must be given to the steps described herein and in the selection of the appropriate microscope/image capture system. As there are a variety of microscope and image acquisition systems that are used in various laboratories, the method described here has been generalized to encompass the diversity of these systems. Thus, specific instructions of how to use a particular program or how to operate the microscope are not given. For specific details, the reader is referred to the manufacturers' manuals. For example, when instructions to adjust the focus are provided, the user could perform this task manually, using computer control, or via a separate focus controller, all of which would accomplish the same task.

In this protocol, reference images of fluorescent beads are first captured. The bead images indicate proper system set-up and operation. In addition, the fluorescence values extracted from the beads are used to calibrate and normalize subsequently captured telomere fluorescence data. A metaphase cell on the sample slide is then manually selected and placed into the field of view of the camera. Next, the best focus image of the telomeres is captured. The corresponding chromosome image is then captured and these images are stored as a pair for analysis (see Support Protocol 2). In a typical experiment, ~15 metaphase cells from a sample are required.

Materials

Immersion oil for fluorescence and general microscopy (NA = 1.5150 ±0.0002, Cargille Laboratories)

Sample slide of labeled telomeres and chromosomes (see the Basic Protocol)

Reference slide of fluorescent beads (see Support Protocol 3)

Fluorescence microscope (Zeiss Axioplan or equivalent) with the following components:

- Excitation and emission filters to match the probes used (DAPI/CY3 dichroic mirror/emission filter set, Chroma Technology)
- One high-quality objective lens (e.g., Plan Apochromat 63×/1.4, Zeiss or equivalent)
- Stable fluorescent light source (e.g., 200 W hybrid mercury/xenon lamp, OptiQuip)
- Fine focusing adjustment in ≤0.2-μm increments (e.g., piezo electric focus drive attached to the objective lens or fine focus control provided by the microscope)

Camera (e.g., MicroImager MI1400-12, Q-Imaging or equivalent) with the following characteristics:

- Variable exposure times with a maximum time of at least 10 sec to capture weak fluorescence signals
- At least 8 bits of photometric resolution (higher bit depth cameras generally are better as the upper 8 bits are less noisy and allow the user to select a sub-range of interest from the full range)
- Small pixel size (~7 × 7–μm such that better localization of the small telomere spots can be made)
- Large number of pixels (~1000 × 1000 such that the entire metaphase spread is captured within the camera's field of view)
- No automatic gain control or the ability to disable this function (e.g., manual gain control)

Image acquisition software with the following features:

- Visualization of the captured image on a monitor
- Capture and storage of images in the proprietary ".IMG" format or the Windows Bitmap 8 bit/pixel ".BMP" format
- Histogram option to display the intensity variation in the image (this feature will give an indication of the values of the minimum and maximum pixel intensity values that are present in the image)
- Feature to store the raw image data from the camera (such that unmodified images captured under controlled conditions can be consistently and objectively analyzed later)
- Feature to capture and display images in continuous mode (optional)
- Feature to switch into "intensity enhancement" mode where low light levels are scaled to appear much brighter and is useful for focusing (optional)
- Feature to switch into "binning" mode, an acquisition mode where images are captured at a faster rate (e.g., lower exposure times) but at a lower spatial resolution and is useful for focusing (optional)

CAUTION: Ensure that all automatic gain features are turned off when capturing data for image analysis. This includes the gain control setting on the camera. It also includes the "intensity enhancement", "binning" modes, and other similar modes that are available on various imaging systems.

NOTE: Ensure that the immersion oil on the slide connects evenly with the objective as this will reduce the optical distortion of the captured and viewed images.

Set focus for reference beads

1. Turn on the fluorescence light source and camera.

   These components need to warm up and become stabilized for ≥30 min prior to data acquisition. Other components in the system do not need a warm-up period and hence, these other components need only be powered on (if not already powered) just prior to performing the next step.

2. Place a reference slide of fluorescent beads (see Support Protocol 3) onto the fluorescence microscope and view this sample under the microscope with the 63× objective using the Cy3 excitation/emission filter set, and an ND3.0 neutral density filter (0.1% transmission) in the light path of the microscope.
3. Focus and capture an image of the beads.
4. Generate a histogram plot of the captured image and ensure that very few pixels, and preferably no pixels, are saturated (above the maximum intensity level of the camera).

   In cameras that have defective pixels, make allowances for saturated defective pixels that arise with long camera exposure times. The optimum image should have maximum signal intensities at ~230 out of 256 gray levels. For some images, the optimum values could not be achieved without going to higher exposure times that are beyond the capability of the camera. As a result, the lack of sensitivity in the system compromises the precision of the system. If saturation occurs, reduce the camera exposure time, re-capture the image, generate the histogram and verify that the image is not saturated.

5. If there is no continuous acquisition or live capture mode available, repeat the process manually, capturing an image after each time the focus has been changed. Select the focus position that produces the best focus.

   If the system supports continuous capture mode, adjust the focus of the object in small increments and observe the image on the monitor until the sharpest image is obtained. Repeat step 4 to ensure that the focused image has not crept into saturation as a result of better focus. If the system supports 'intensity enhancement' and 'binning' modes, use these options as this will speed up the acquisition process and thus will help to reduce the time to get the best focus image. However, remember to switch such modes off when the actual image is captured for analysis (to avoid creating erroneous images for analysis).

6. Capture and store the best focus image for later analysis. Record the exposure time used to capture the image and the mean fluorescence intensity of the captured image.

   These numbers should be very similar from experiment to experiment. Hence, they may be used as rough indicators of changes and problems with the overall set-up of the system.

7. Remove the bead slide and place the sample slide on the microscope.

Capture telomere images

8. Switch to the DAPI filter set and find a metaphase cell of interest in the sample.
9. Switch to the Cy3 filter set and perform steps 4 and 5 above to obtain the best focus image of the telomeres for telomere analysis.
10. Capture and store the best focus image of the telomeres for later analysis.

    If the software does not store the exposure time in the "IMG" file format, record the exposure time used and the file name of the stored image, as these values will be used later in the analysis program for normalization purposes. Alternatively, the exposure time can be fixed throughout the experiment and, thus, the exposure time only needs to be recorded once. The exposure time will differ between human and mouse telomeres and faint telomeres may require long exposure. As a result, the images acquired with a fixed exposure time may not be optimal. More importantly, there is a risk that some images may have saturated telomere values that will greatly compromise subsequent analysis (see Troubleshooting and Fig. 18.4.1).

11. Switch to the DAPI filter set and perform steps 4 and 5 above to obtain the best focus image of the metaphase chromosome image for karyotype analysis.

    The best focus is not as crucial for the chromosome images since these objects are larger and occupy a larger focal depth. As the microscope is adjusted for parfocality, the best focus level for the telomeres should be the same or at a fixed offset from the best focus level of the chromosomes.

12. Capture and store the image of the chromosomes for later analysis. Choose a file name that is similar to that for the telomere image as these files will be entered as pairs into the image analysis software (e.g., image 001c.bm for Cy3 stained telomeres and image 001d.bmp for DAPI stained chromosomes).

    Only grayscale/8 bit images are accepted. TIFF files can be opened in Adobe Photoshop and changed to gray scale. The best way is to copy the image to clipboard and paste into TFL-Telo and save as .IMG file (TIFF file formats are currently not supported by TFL-Telo but will be supported in later versions of this analysis software program)

    Some image acquisition software stores images in the TIFF or other proprietary file formats. In this instance, another utility program such as Adobe Photoshop will be needed to convert the combined TIFF file into individual telomere and chromosome BMP file formats.

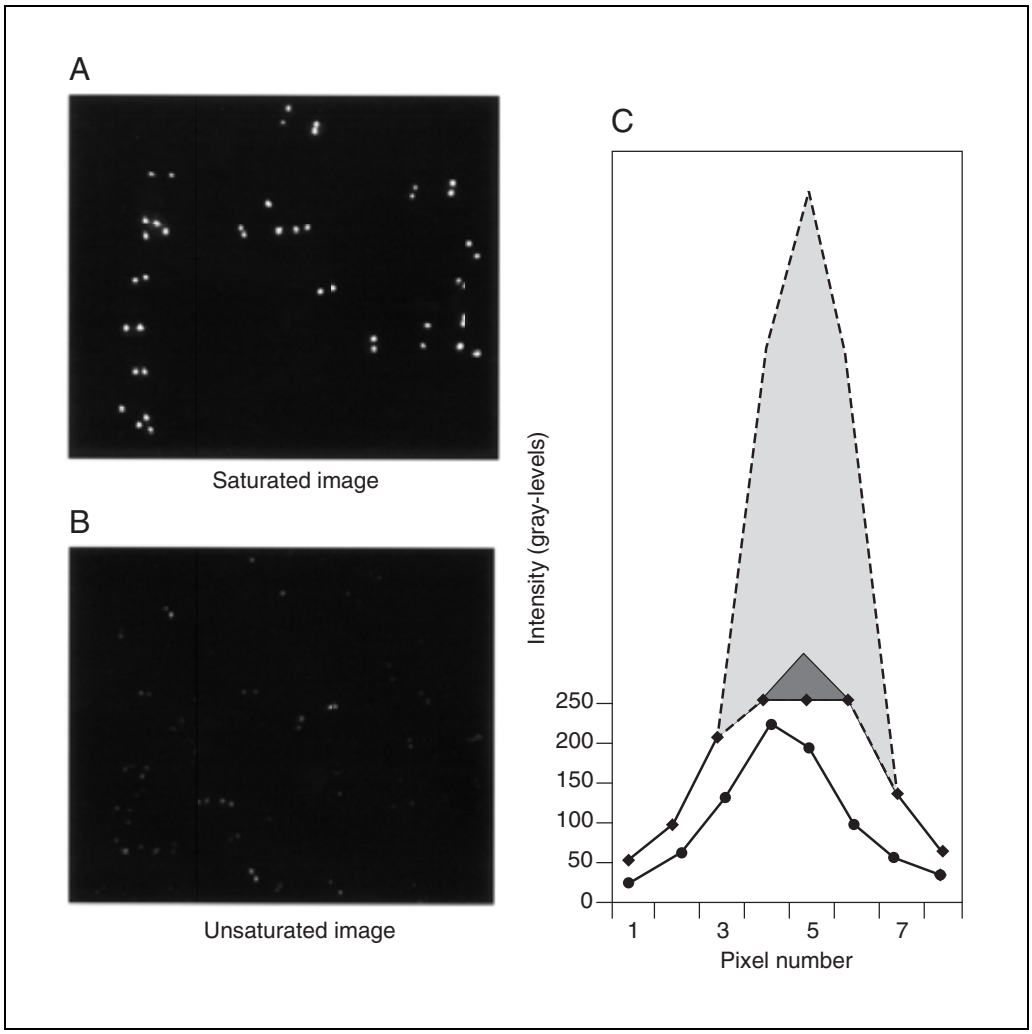


Figure 18.4.1 Intensity profile of saturated (**A**) and unsaturated (**B**) telomere signals. (**C**) There is no ambiguity in determining the total fluorescence of the unsaturated telomere signal (circles). However, there is uncertainty in the true fluorescence of the saturated signal (diamonds). Two possible estimates of the fluorescence signal (shaded regions) are shown.

13. Repeat steps 4 to 12 for ≥15 metaphase cells to obtain statistically relevant results for the sample.

    The number of metaphases for statistical relevance will vary from sample to sample. Variations in cell preparation (see Basic Protocol) and the quality of the microscope and imaging system components will also reduce the sensitivity of the system. Such variations can only be partially compensated by an increase in the number of metaphases that are analyzed.

14. To verify that the system has not drifted over the course of the experiment, repeat steps 2 to 5 using the reference bead slide.

    The fluorescence intensity of the beads should be similar to the values obtained earlier in the experiment and to the values from previous experiments.

IMAGE ANALYSIS

SUPPORT PROTOCOL 2

The following method uses the TFL-Telo image analysis software to measure the telomere values of individual chromosomes. A typical telomere/chromosome analysis session is shown in Figure 18.4.1. From the chromosome and telomere images, the program generates a combined image of the detected telomeres and chromosome boundaries superimposed onto the chromosome image. The user then interactively edits the image to correct for mistakes made in the segmentation (definition of the boundaries) of chromosomes, to karyotype the image, to assign and correct for the nomenclature of the telomeres on the p and q arms of the chromosome, and to split nearby telomeres on a specific arm of the chromosome into two. The results are saved into a formatted text file that can be imported into spreadsheets (e.g., Microsoft Excel) or statistical packages (e.g., Microsoft Origin) for analysis. Calibration/normalization of the results can then be performed using the reference bead image and the calibration parameters obtained from Support Protocol 3. To study the variation of the results and compare them to those of other metaphase cells, use a spreadsheet program to cut and paste the results into a combined results file.

Materials

Images of telomeres (see Support Protocol 1)
Corresponding images of chromosomes (see Support Protocol 1)
TFL-Telo (telomere image analysis software, this software is available upon request from the authors: plandor@bccancer.bc.ca)
Pentium-based personal computer with the following:
  Minimum of 8 Mb of RAM
  Graphics card with at least 256 colors and a display resolution of 640 × 480 pixels
  High resolution video monitor to match the video card
  Microsoft Windows 95, 98, or NT operating system

1. From the "Application Menu" in the TFL-Telo program, select the "Chromosome/Telomere" option.

2. First, enter the names of the files associated with the pair of chromosome (DAPI) and telomere images (Cy3) of the metaphase spread that is to be analyzed (i.e., the names assigned to the files stored from Support Protocol 1).

   If the files are not stored in the ".IMG" file format, enter the corresponding exposure time used for the telomere image. There is a threshold column value in this window with a default setting of 5. The threshold can be increased or deceased as required (e.g., when telomere signals are too faint or too bright on particular chromosomes).

3. Finally, enter the name of the file to store the results.

   The output file has to be in the ".IMG" format.

4. Double click on the "OK" button when all the fields for the file name have been entered. The program will then automatically determine the borders of the chromosomes and telomeres and calculate the total fluorescence value for each detected telomere in each chromosome.

   Once the program finishes its processing, a combined image of the telomere and chromosome results are displayed on the screen (see Fig. 18.4.1). A different color is assigned to the border of each of the telomeres in the chromosome for ease of user recognition (purple, red, blue, cyan, orange, and green are assigned to telomere number 1, 2, 3, 4, 5, and 6, respectively, or more). Similarly, a different color border is used to indicate the number of "telomere" objects in the chromosome (cyan, green, and yellow are assigned to ≤3, 4, and 5, respectively, or more objects). Each chromosome is also labeled with a unique number.

   Once the processing has been performed on the telomere/chromosome pair, the processed results can be reloaded into the program. To reload, select the "Open" option from the "File" menu and then enter the file name of the processed results. The combined telomere and chromosome image of the processed results will then be displayed.

5. From the "Options" menu, select the "Chromosome Statistics" option.

   A window will then appear showing a table with the following information: the label assigned to the chromosome number, karyotype number of the chromosome, up to five telomere values for the chromosome, and a number of other telomere and chromosome statistics.

6. Select the "Edit" option to open a submenu to edit the parameters for the selected chromosome such as the telomere belonging to the p or q arm of the chromosome and the karyotype number of the selected chromosome.

7. Select the "Save" option to save the changes made (changes are automatically saved in the latest version of the TFL-Telo software).

8. Find the mean fluorescence value of the beads. Perform system calibration by following Support Protocol 3, steps 6 to 8, for the fluorescent bead image and assign the mean value to $Bead_{exp}$.

9. To normalize the results, import the telomere fluorescence result from the output file with one of the following file extensions "*.LB2" or "*.LB3" (the last four columns are the telomere fluorescence values) into a spreadsheet program such as Microsoft Excel. Multiply the measured telomere fluorescence values using the following formula ($slope_{cal}$ and $bead_{cal}$ are obtained from Support Protocol 3):

$$\text{length (base pairs)} = \text{measured fluorescence} \times slope_{cal} \times bead_{exp}/bead_{cal}$$

SUPPORT PROTOCOL 3

SYSTEM CALIBRATION

The following method illustrates how data from different experiments can be normalized and how the telomere fluorescence measurements can be converted to telomere length estimates. While slides with fluorescent beads are used on a daily basis to calibrate the microscope, slides with hybridized plasmids are used occasionally to calibrate telomere fluorescence relative to telomere length. For the latter, plasmids (DNA with fixed numbers of telomeric repeat sequences) are used to verify and determine the linearity of the system using these protocols. In order to compensate for differences in illumination and other intensity variations from experiment to experiment, the total fluorescence measurements of fluorescent beads from each experiment is used for normalization purposes.

Materials

0.2-µm fluorospheres carboxylate-modified microspheres, orange fluorescent (540/560, Molecular Probes)
1:1 (v/v) PBS/FCS
Antifade reagent in glycerol/PBS (Slow Fade Antifade kit, component A, Molecular Probes)
PSXneo plasmids containing fixed numbers of telomere repeats (Hanish et al., 1994; Martens et al., 1998)

22 × 30–mm coverslips
Slide box
1.5-ml "sure lock" tubes (Rose Scientific)
Thermomixer (e.g., Eppendorf Model 5436) or 80°C water bath

Additional reagents and equipment for PNA probe hybridization (see Basic Protocol), image capture (see Support Protocol 1), and image analysis (see Support Protocol 2)

1. Prepare calibration slides with fluorescence microspheres. Dilute 0.2-µm fluorospheres carboxylate-modified microspheres 25× in a 1:1 mixture of PBS/FCS. Deposit 2 to 3 µl of microsphere suspension on to periphery of a 22 × 30–mm coverslip. With a regular slide, produce a thin homogenous smear over the coverslip (like a blood smear). Let the coverslip dry. Keep coverslips in slide box until use.

2. Prepare a calibration slide on the day of acquisition by depositing 5 µl of slow fade reagent on a slide and adding microsphere-coated coverslips.

   Note the different antifade reagent. Vectashield is not optimal for the preparation of calibration slides with fluorescence beads.

3. Prepare calibration slides with fluorescence plasmids. Add PSXneo plasmid DNA to hybridization mixture. For example, add 1 µl of plasmid DNA with 144 bp of telomere DNA at 0.1 µg/ml in 10 mM Tris·Cl to 99 µl of hybridization mixture in a 1.5-ml "sure lock" tube (see Basic Protocol).

4. Denature 10 min at 80°C in a thermomixer or water bath. Incubate 60 min at room temperature and put two 50-µl drops on a precleaned slide.

5. Incubate 30 min in the dark; some of the plasmids hybridized with fluorescent PNA will noncovalently attach to the glass surface. Proceed with wash steps, alcohol dehydration as described in Basic Protocol, steps 28 to 32.

Calibrate Q-FISH image acquisition and analysis system

6. Set up and prepare the microscope and image acquisition system for image capture (see Support Protocol 1, step 1).

7. Capture and store an image of fluorescence beads (see Support Protocol 1, steps 2 to 6).

8. Capture and store images of plasmids that contain 144 bp of telomere repeats (see Support Protocol 1, steps 9 and 10).

   Choose areas on the slide where the plasmids are spread out and not clumped. Adaptation to darkness is essential to see very faint spots of plasmids corresponding to a maximum of eight Cy3 molecules per plasmid. Ensure that a total of ≥50 to 100 plasmids are collected in one field. Too low a density of plasmids on the slides complicates acquistion and discrimination between plasmid and background fluorescence. Capture and store more images as required such that the desired minimum number of plasmids are acquired.

9. Repeat step 3 for the slides that contain plasmids with 400, 800, and 1600 bp of telomere repeats and capture the images.

Analyze the data

10. Execute the TFL-Telo program.
11. Select the "Open" option from the "File" menu to open a stored plasmid file.
12. Select the "Spot IOD" option from the "Application" menu.

    The program will then automatically find the telomere spots and then calculate the total fluorescence values of each detected spot.

    The calculated value needs to be normalized with the exposure time that was used to capture the image. If this value is entered with the image file, the program will make the calculation. Otherwise, divide the value obtained with the manually recorded exposure time for that image.

13. Select "Object Histogram" option from the "Option" menu to obtain the histogram of the fluorescence distribution of all detected objects. Obtain the mean fluorescence number of the telomeres by first rejecting the artifacts from the image as follows. First, point the mouse to the minimum fluorescence value of the objects in the histogram. Hold down the left mouse button and drag the mouse up the intensity scale of the histogram (i.e., towards the right). Release the button to select the maximum value of the intensity range. The selected range in the histogram will change color and the corresponding borders of the objects in the image will also change color. Repeat the range selection until the desired objects are highlighted (i.e., do not include debris or cluster objects). Record the mean fluorescence value for the selected range.
14. Repeat steps 11 to 13 for the other files containing images of plasmids that have different telomere insertion lengths.
15. Repeat steps 11 to 13 for the image of the fluorescent beads. Record the mean bead fluorescence value as $bead_{cal}$.

    This value will be used later for calibrating the results (see Support Protocol 2).

16. Plot the telomere fluorescence values as a function of the number of base pairs of telomere repeats in the plasmid.
17. Find the slope of the plot. Record this value as $slope_{cal}$ for use in image analysis (see Support Protocol 2).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

BSA stock solution, 10%

Dissolve 10 g BSA in 100 ml water by gently placing BSA powder on top of the water. Use a magnetic stirrer to dissolve. Do not stir at high speed, slow hydration works best. Filter through 0.4- and 0.2-μm filters to remove bacteria and debris. Store up to several years at −20°C in 1-ml aliquots.

Formamide, deionized

Add 5 g of resin beads to 100 ml of ultrapure formamide, pH 7.0 to 7.5 (Life Technologies). Stir for 2 hr in a fume hood. Filter through Whatmann no. 1 filter paper. Aliquot in 1-ml portions and store at −20°C.

Hybridization mixture

For 100-µl mix (5 slides):
70 µl deionized formamide (see recipe)
10 µl NEN blocking solution (see recipe)
5 µl 0.1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)
5 µl 10 µg/ml PNA Cy3-telomere probe (see recipe)
5 µl $MgCl_2$ buffer (82 mM NaH_2PO_4/9 mM citric acid/20 mM $MgCl_2$, pH 7.4)
5 µl distilled H_2O
Store up to a week at 4°C

Mounting medium

To 1 ml of Vectashield, add 1 µl of DAPI stock solution (0.2 mg/ml in water) to give final concentration of 200 ng DAPI/ml of antifade. Dilute with Vectashield if DAPI staining is too bright and is visible in Cy3 fluorescence filter combination. Store up to a week at 4°C.

NEN blocking solution

Dissolve (layer) 2.5 g of NEN blocking reagent (PE Biosystems or Life Sciences) in 100 ml distilled water in a glass beaker. Stir gently overnight. Filter through 0.4-µm filter and dispense into 3- to 4-ml aliquots. Store at −20°C.

PBS, 10×stock solution (pH 7.4)

Solution A:
16.02 g $Na_2HPO_4·2H_2O$
73.84 g NaCl
900 ml H_2O

Solution B:
2.76 g $NaH_2PO_4·H_2O$
16.56 g NaCl
200 ml H_2O

Adjust solution A to pH 7.4 with solution B. Store up to a year at room temperature.

Peptide nucleic acid probes

PNA probes can be synthesized manually or with dedicated equipment by chemists familiar with peptide and/or nucleic acid synthesis. Alternatively Cy3-labeled PNA can be ordered from DAKO as a kit (Telomere PNA FISH/Cy3) or from Boston Probes (*www.bostonprobes.com*) or Applied Biosystems as a custom order. The latter is the more economical because many hundreds of experiments can be done with just a few OD_{260} units of labeled PNA.

Good results will be obtained with FITC or Cy3-OO-CCCTAACCCTAACCCTAA-carboxy-terminus. More information on how to order and use PNA probes can be found at the website of Applied Biosystems (http://www.appliedbiosystems.com/ds/pna).

Wash solution I

For 500 ml:
5 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*, 10 mM final)
350 ml deionized formamide (see recipe, 70% v/v final)
5 ml 10% BSA stock solution (see recipe, 0.1% w/v final)
140 ml H_2O
Check pH and adjust to pH 7.4, if necessary, with 1 M NaOH or 1 M HCl
Store up to a week at 4°C

Wash solution II

For 500 ml:

50 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*, 0.1 M final)

50 ml 1.5 M NaCl (0.15 M final)

0.5 ml Tween 20 (0.1 % v/v final)

400 ml H_2O

Store up to a week at 4°C.

COMMENTARY

Background Information

PNA probe hybridization

Telomeres are specialized structures at the ends of chromosomes consisting of repetitive DNA and associated proteins (Blackburn, 1994; Zakian, 1995) that are maintained by the reverse transcriptase enzyme telomerase (Greider, 1995). In most somatic cells, telomere repeats are lost with each cell division and with age. Because telomere loss has been shown to limit the replicative life span of cells, the loss of telomere repeats is believed to be important in the aging of tissues with continuous cell turnover. Progressive telomere shortening has, furthermore, been shown to result in chromosome fusions and genetic instability (Blasco et al., 1997). This is increasingly recognized as an important mechanism contributing to malignant progression and the "mutator" phenotype of cancer cells (Wan et al., 1997; Artandi et al., 2000). Questions about the role of telomere shortening in aging and cancer have encouraged the development of novel techniques that can provide information about the length of telomere repeats present in individual chromosomes (Q-FISH; Lansdorp et al., 1996; Zijlmans et al., 1997; Martens et al., 1998) and in individual cells (flow FISH; Rufer et al., 1998, 1999). Such information cannot be obtained by the standard Southern blotting method to measure telomere length because it requires genomic DNA typically isolated from $>10^5$ cells (reviewed in de Lange, 1995).

Fluorescence in situ hybridization (FISH) with PNA probes has some distinct differences with the more established FISH procedures that employ DNA or RNA probes. The major differences are the inclusion of protein in the hybridization mixture to prevent nonspecific binding of the PNA probes and the use of very stringent hybridization conditions that are unsuitable for annealing of DNA or RNA probes to DNA target sequences. The denaturation of chromosomal DNA in the presence of the PNA probe simplifies the FISH procedure. Because hybridization of PNA probes to specific target sequences is fast and specific, the procedure is furthermore fast relative to conventional FISH procedures. The major advantage of Q-FISH with PNA probes over conventional FISH procedures with conventional telomeric DNA probes is that Q-FISH allows extraction of quantitative information. Such information is much more difficult to obtain using conventional FISH procedures because in conventional FISH procedures the hybridization of probes competes with the renaturation of complementary strands of the denatured genomic DNA. This competition inevitably introduces a stochastic component in conventional FISH experiments with the eventual probe signal depending on the concentration and size of the DNA or RNA probe, the efficiency of the denaturation of the genomic DNA, and the repetitive nature of DNA target sequences. The conditions that favor binding of small oligonucleotide probes to highly repetitive telomeric DNA, unfortunately, also result in efficient renaturation of genomic DNA. Even with multiple rounds of immunological amplification the net result of conventional hybridization for telomere targets is a highly variable signal on some but not all telomeres (Moyzis et al., 1988). Even to the inexperienced, the difference in telomere fluorescence obtained with indirect conventional labeling procedures using small RNA or DNA telomere probes and directly labeled PNA probes will be striking. The use of Q-FISH for quantitative purposes is supported by the overall good correlation between fluorescence from sister chromatids, the good correlation between telomere length data obtained by Southern blotting analysis, and Q-FISH analysis, and the analysis of plasmids with defined T_2AG_3 content (Lansdorp et al., 1996; Martens et al., 1998). While telomere repeats are ideal for use with PNA probes (little or no sequence divergence), other repeat sequences such as chromosome-specific alphoid

repeat sequences, can also be detected efficiently using selected 15- to 18-mer PNA probes (Taneja et al., 2000).

Image capture

Image acquisition and analysis systems have been around for a number of decades (Young and Roberts, 1951; Walton, 1952). However, it was not until the last decade that high-resolution, variable-exposure-time cameras were commercially available; these cameras can detect and measure weak fluorescent telomere signals (Poon and Hunter, 1994). The increased use of FISH analysis has also lead to a number of improvements to the fluorescence microscope, such as more efficient and wavelength-specific excitation and emission filters, better quality objective lenses for fluorescence detection, and better and more precise fine focus controls. While, in particular, the light source of standard fluorescence microscopes remains problematic, all these improvements have brought quantitative analysis from capture images of telomeres hybridized to directly labeled PNA probes within reach of dedicated experimentalists. It is expected that further improvements will make quantitative analysis a standard feature of future fluorescence microscope and imaging systems.

Image analysis

Interest in telomere length measurements has led to incorporation of a telomere analysis feature in the current version of a commercial chromosome analysis package (Metasystems V2.0). The automation of karyotyping by this software would greatly reduce the time required to identify chromosomes and assign telomere fluorescence values to specific chromosomes. Although, the accuracy of telomere analysis in this software needs to be validated, there is an option to export the karyotype results and link it with the telomere measurements obtained from the TFL-Telo program. A description of the algorithms used in the TFL-Telo program is presented elsewhere (Poon et al., 1999a,b).

Another new development for estimating the average telomere length in single cells in a sample is flow-FISH. This method uses the speed and power of the flow cytometer to quickly measure the total intensity of the fluorescent telomere probes in the cell. All these methods have specific advantages and disadvantages in studies of telomerase/telomere biology.

System calibration

Data normalization and calibration are the best means of ensuring that data from one experiment can be correlated to that of another. As the objective of the study is to measure telomere lengths, a good choice of test objects to use on the system would be those where the telomere lengths are known. Plasmids containing telomere inserts are good candidates as the telomere length inside the circular DNA sequence structure does not change as a function of cell division. Hence, these test objects can be duplicated for calibration purposes. As there can only be a limited number of telomeres that can be inserted to a plasmid, calibration points for long telomere lengths are extrapolated from the linear results of fluorescence intensities from different smaller-length telomeres. The smallest size insert also gives an indication of the telomere detection limit of the system. While the use of hybridized plasmids will allow conversion of arbitrary fluorescence units to telomere fluorescence units (TFU, with each unit corresponding to 1 kb of telomere repeats), comparisons of telomere length measurements by Southern blot analysis and Q-FISH are required to further validate the use of Q-FISH for telomere length measurements.

Critical Parameters

PNA probe hybridization

A number of issues in the Q-FISH protocol deserve careful consideration and attention. In general, it is recommended to include controls such as a slide with nicely fixed metaphase cells and a slide, done in a previous experiment, with good hybridization signals. These controls will help to identify common errors with metaphase preparation and/or microscope set-up. Sometimes it is difficult to obtain sufficient good quality metaphase spreads from cells. If this is the case, continuing with the hybridization procedure will not produce satisfactory results. Instead, it is probably a better idea to increase the yield of dividing cells and improve the metaphase preparation procedure. There are many tricks of the trade of a cytogeneticist, and there is undoubtedly an aspect of art to the preparation of good metaphase spreads. For example, for a discussion of the synchronization of cell cultures to increase the mitotic index or the climate-dependency of optimal results, the reader is referred to cytogenetic protocols for chromosome preparations. A visit to the local cytogenetic department may greatly help to resolve problems with metaphase prepara-

tions (one may even obtain some slides to do the first Q-FISH experiment).

The following issues are important if slides with satisfactory metaphase spreads have been obtained.

Prior to hybridization, slides with fixed cells should be dried extensively. Drying overnight in a fume hood produces satisfactory results. Shorter periods of drying (i.e., on a 65°C plate) may work, but insufficient dehydration (i.e., for 1 hr at room temperature) increases the nonspecific binding of the PNA probe.

The pepsin treatment can be omitted, but the signals at telomeres will be more variable.

The wash steps with formamide after hybridization can be replaced by washing for 5 min with wash solution II at 65°C. However, for optimal telomere signal and chromosome morphology, the authors recommend washes with wash solution I as described in the protocol.

Before adding antifade with DAPI, ensure that slides are completely dry again.

Image capture

The microscope set-up and sample preparation play a key role in the success of the experiment. It is important that the illumination over the field of interest (i.e., the metaphase spread) is constant. Otherwise, the fluorescence measurements taken from one area of the image will not be the same as those taken from another area. A slide of fluorescent beads or a plastic fluorescent slide could be used to check on the homogeneity of illumination over the field of view. Use objective features in the acquisition software (such as histograms) to measure the fluorescence intensity distribution over the

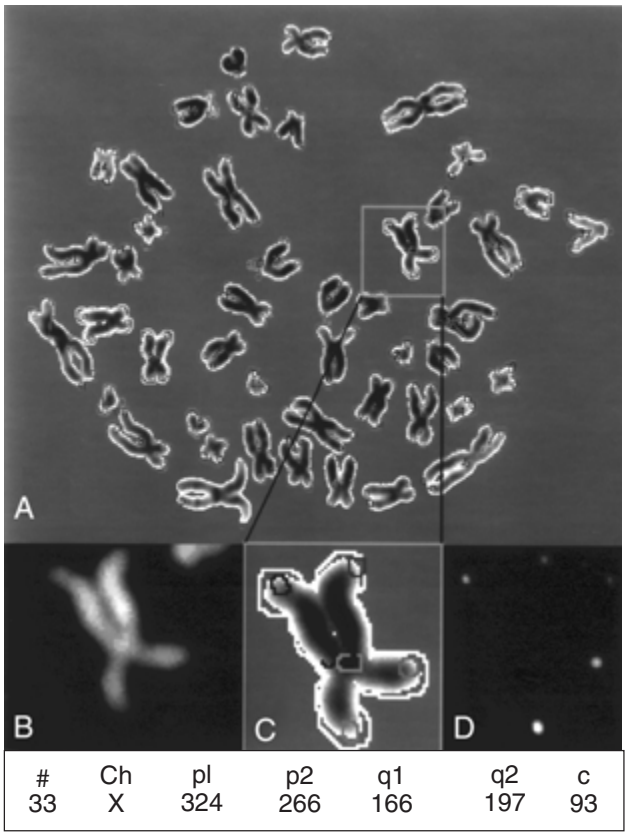


Figure 18.4.2 Output of TFL-Telo telomere length analysis software program. From imported image files the program generates an output image file shown in (**A**) in which chromosomes are given an arbitrary number. Telomere fluorescence values corresponding to that chromosome are calculated by the program and provided in a table. After editing and karyotyping by the user, the table provides information on the telomere fluorescence values for the two telomeres at the short (p) and long (q) arm of each selected chromosome (Ch X in the figure). The fluorescence intensity at the centromere is also calculated (c in the table). (**B**) A magnified view of a selected chromosome of the raw DAPI image file, (**C**) the output of the TFL-Telo program, and (**D**) the raw telomere fluorescence image are shown.

field of view rather than relying on subjective visual judgment. In instances where the light intensity varies significantly (e.g., >5%) between pixels, an appropriate correction of fluorescence values will increase the accuracy of results. Use flat-field correction in which all fluorescence pixel values are multiplied by a factor depending on the pixel location in the field of view that corrects for uneven illumination of the field.

The sensitivity of the acquisition system to detect the probes that are used has a major impact on the outcome of the experiment. Hence, it is worth the initial effort to experiment and choose the best components to use before starting experiments. For example, some objective lenses allow much more Cy3 fluorescence to pass through than other similar magnification lenses. The spectrum of the excitation and emission filters also plays a key role in the intensity of the observed fluorescence and the amount of interference or overlap of fluorescence from other wavelengths. The sensitivity of the camera and size of the camera pixels also affect the precision of the results obtained. Image fluorescence intensities, which span the 256-level intensity map of the camera, would give higher precision results than if the intensities were hovered around the lower levels. Similarly, smaller size pixels would give higher precision results than larger pixels as the area of the telomere can be defined better.

The focus of the telomeres also has an effect on the precision of the fluorescence measurements. Generally, calculations of total probe fluorescence can be made over a larger area that encompasses the probe, and hence focus variations do not play a major factor. However, telomeres can lie very close to each other, and as a result the signals will interfere with its neighbors' if they are out-of-focus. The TFL-Telo software has been written to identify the area occupied by an individual telomere and distinguish this from other nearby telomeres. A typical output of the TFL-Telo program is shown in Figure 18.4.2. As the algorithm used is focus-sensitive, the objects captured should be within 0.1-μm from best focus. Otherwise, the values obtained would be significantly lower than expected (see Fig. 18.4.3).

Photobleaching is another important aspect to consider in quantitative fluorescence microscopy. Antibleaching agents are often used to minimize the fading effects of probes used. If a sample shows significant photobleaching, a method will need to be incorporated to determine the amount of photobleaching so that the results generated can be correlated or compared with that of other cells. This will add to the complexity of the experiment since an estimate of how long the cells have been exposed to light will need to be determined, and extra care is needed to keep track of the locations on the slide that have been previously exposed to light. To avoid this complexity, it is better to experiment

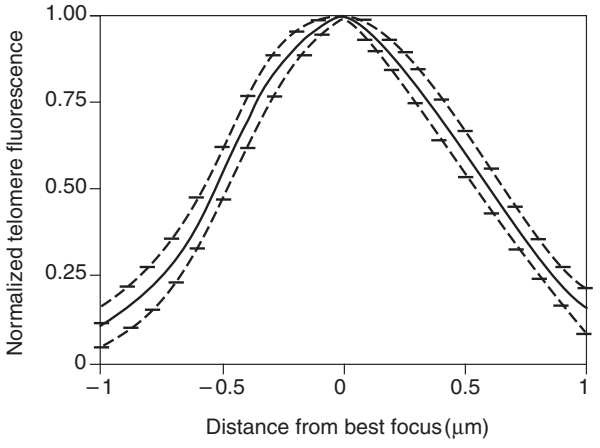


Figure 18.4.3 Variation of measured telomere fluorescence as a function of focus. In this experiment, the focus was varied at precise intervals using a piezzo electric drive connected to the objective lens. The telomere images were acquired and telomere fluorescence values calculated using the TFL-Telo program. Shown is the average (± s.d.) telomere fluorescence of all telomeres in the image (n = 184). Note that changing the focus by 0.5-μm results in a drop of calculated telomere fluorescence of up to 50%.

with the intensity of the light source and with different antifade agents. For example, the introduction of PNA probes tagged with different fluorescence dyes such as Cy5 that are specific for chromosome-specific alphoid repeats at centromeres may require a different antifade agent. Hence, experimentation with different antifade agents is needed when new sample preparation procedures are introduced. Reducing the intensity of the illumination (i.e., controlling the power applied to the lamp or placing a neutral density filter in the light path) is another method to reduce the effects of photobleaching. Although longer exposure times will be required, the results will typically be less dependent on the timing of the image capture. Closing the shutter between image captures is another good approach to reduce the effects of photobleaching.

The effects of photobleaching, light source stability, and system variability can be checked by repeatedly capturing images of the same metaphase (e.g., every 15 to 30 sec) over a 10-min interval. During this experiment, check and ensure that the focus of the metaphase does not drift over time. The images are then analyzed and the total fluorescence values from a number of selected telomeres are plotted as a function of time (Fig. 18.4.4). The slope of the graph gives an indication of the amount of photobleaching in the sample. The variability between adjacent measurements gives an indication of the amount of variability in the system. As a general rule, photobleaching (slope of plot) should be <5% of the total or average intensity over a 5-min working interval. In addition, the amount of variation in fluorescence intensity between consecutive captured images should be <10%. With experience, the acquisition time will typically decrease resulting in reduced photobleaching and improved data.

Troubleshooting

PNA probe hybridization

Poor morphology of chromosomes. The denaturation time and temperature are critical and may vary to some extent between cells from different tissues or between cell lines. Denaturation at too high a temperature or for >3 min will result in blurred chromosomes and poor morphology. Too low a temperature or too short a denaturation step will result in nice DAPI staining of chromosomes but poor telomere fluorescence signals. Consider reducing the amount of pepsin or the duration of the pepsin treatment for improved morphology. Do not skip fixation steps before or after pepsin treatment.

Poor signals. Include pepsin treatment of cells. Increase temperature and/or duration of denaturation step until morphology is compromised. Decrease salt concentration in hybridization mixture. Decrease time or temperature

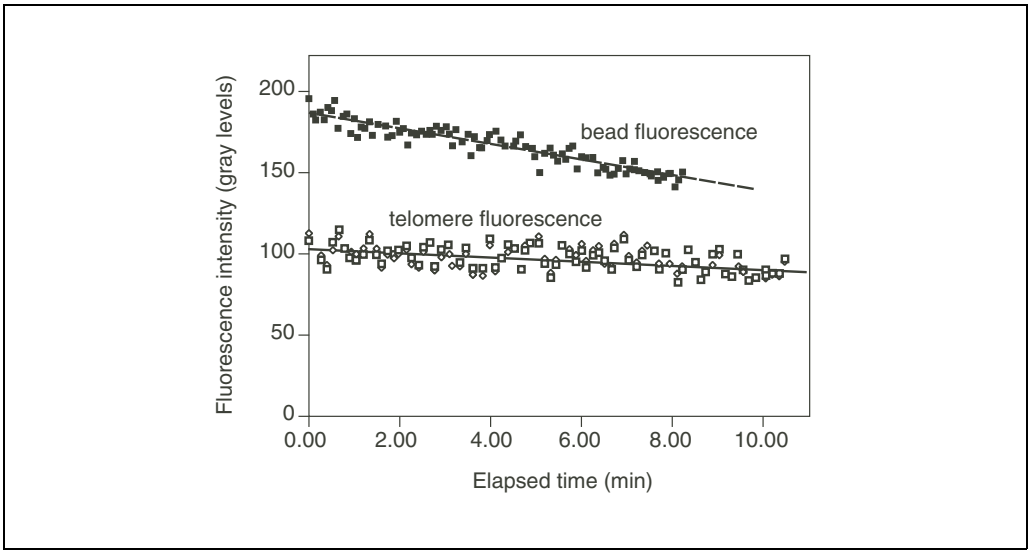


Figure 18.4.4 Photobleaching of fluorescent calibration beads and actual telomere fluorescence spots following PNA FISH and slide preparation as described in the text. Note that photobleaching of calibration beads (acquired at 0.1% of standard illumination) exceeds that of telomeres acquired at full light intensity and that photobleaching of telomere fluorescence over a 5-min period is <10%. The more pronounced variation in fluorescence values of beads and telomeres between sequentially acquired image files almost certainly reflects variation in the output of the light source.

of high-stringency wash with 70% formamide post hybridization.

High background PNA binding. If binding is localized to cytoplasm, increase number of fixation steps with methanol/acetic acid fixative when preparing fixed metaphase cells. If it is over the whole slide area, use different blocking protein or change concentrations of blocking protein in hybridization mixture. Insufficient drying of slides prior to denaturation will also increase background PNA fluorescence.

Image capture

As mentioned earlier, capturing an image of fluorescent beads at the beginning of every acquisition session helps to give an indication of the system setup. If the observed fluorescence intensity of the calibration beads differs significantly from the previous experiment (different intensity or similar intensity but at different exposure times), the change in the way the system is set up should be identified and corrected. If possible, confirm that the measured values with calibration beads correspond to the values obtained at the previous acquisition session.

If the telomere distribution plot of a sample appears to be skewed to the right of a normal distribution, check the raw image data for saturated pixels. Saturated images are excellent image candidates for the traditional spot counting as all telomeres can readily be seen (Fig. 18.4.5). However, telomere lengths are underestimated in saturated images giving rise to a large number of telomeres with fluorescence values clustered near the upper portion of the telomere length distribution. As illustrated in Figure 18.4.5, the underestimate in telomere length can be a few percent to a few hundred percent of the estimated fluorescence value.

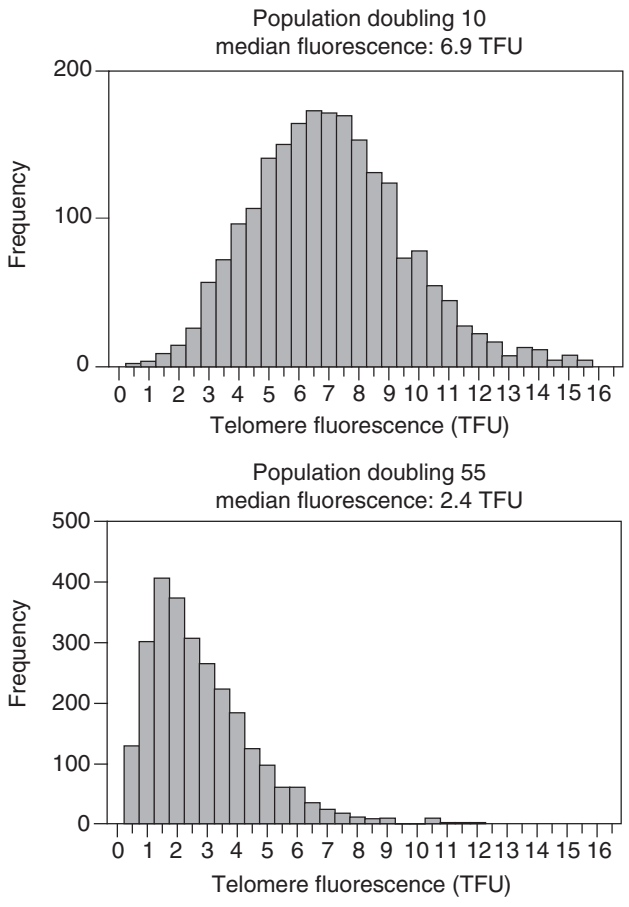


Figure 18.4.5 Accumulation of short telomeres in fibroblasts prior to replicative senescence. Note that the telomere fluorescence in early passage normal human fibroblasts shows a relatively normal distribution whereas at population doubling 55 (close to the Hayflick limit for these cells) the distribution is highly skewed towards the left. These observations indicate that a minimal number of telomere repeats need to be present on all chromosome ends in order for a cell to enter mitosis.

System calibration

The measured fluorescence values should be linearly related to the telomere length of the object. If the plot (see Support Protocol 3, step 12) does not generate a linear relationship, repeat the experiment to ensure that errors were not introduced in the sample preparation and image acquisition steps. For lower-sensitivity systems (microscope, camera), measurements of the smaller-size plasmids (e.g., 144 bp) may not be reliable, and hence this data point should be ignored from the determination of the slope used for calibration. Check also that the exposure time has been incorporated into the fluorescence measurement calculations.

Anticipated Results

PNA probe hybridization

All telomeres in normal human cells should easily be detectable. The signals from sister chromatids should be similar in intensity, and fluorescence of the telomere probe should be restricted to chromosome ends (sometimes a faint spot on human chromosome 2 identifies the area where two short primate chromosomes are fused to give rise to human chromosome 2 during evolution (Ijdo et al., 1991). Chromosomes should be easily identified using DAPI staining. No DAPI staining should be visible when analyzing telomere fluorescence.

Image capture

A typical experiment would give results that show the telomere fluorescence values of individual chromosomes. Figure 18.4.2 shows a raw DAPI image of chromosomes (B), a raw image of corresponding telomere spots (D), the image produced by the TFL-Telo program using the two raw image files (A,C), and the calculated telomere fluorescence of the p and q arms of the selected chromosomes in the metaphase as an example of the tabulated output of the TFL-Telo program.

Image analysis

Typical telomere length distributions of young and old cells are shown in Figure 18.5.5. These distributions resemble those obtained with the Southern blot technique (Oexle, 1998). Studies have been performed to show that telomeres on certain chromosomes are consistently shorter than others (Martens et al., 1998), and that there is a large variation in telomere lengths in mouse cells (Zijlmans et al., 1997). Other examples of telomere length studies using this protocol can be found in the literature (Blasco et al., 1997; Zhu et al., 1998; d'Adda di Fagagna et al., 1999; Hande et al., 1999a,b; Surralles et al., 1999; Wan et al., 1999; Martens et al., 2000; Niida et al., 2000).

Time Considerations

PNA probe hybridization

The time for the Colcemid treatment for different cell types can vary from 30 min to 24 hr. After the treatment, the preparation of metaphase chromosomes typically takes 2 to 3 hr. The Q-FISH protocol, on the next day, typically takes 5 to 6 hr to perform.

Image capture

The time to capture images also varies from user to user depending on the density of metaphase cells on the slide and one's experience in finding suitable metaphase spreads, focusing objects, as well as other aspects. Typically, after 30 min of initial setup, it takes ~1 hr to capture 15 metaphases on a slide with good quality metaphase spreads.

Image analysis

The time to analyze the data also varies from user to user as well as with the quality of the metaphases. The TFL-Telo program takes <1 min to extract the telomere length measurements (depending on the CPU speed and RAM of the computer). Editing the results can take up to 30 min per metaphase. If karyotyping is to be performed, then up to 1 hr can be added to the time for each metaphase.

System calibration

As mentioned earlier, telomere length measurements of plasmids need not be performed on a daily basis with each experiment. It takes several hours to prepare the plasmid slides, 1 to 2 hr to capture the images, and ~1 hr to extract the results.

Literature Cited

Artandi, S.E., Chang, S., Lee, S.L., Alson, S., Gottlieb, G.J., Chin, L., and DePinho, R.A. 2000. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* 406:641-645.

Blackburn, E.H. 1994. Telomeres: No end in sight. *Cell* 77:621-623.

Blasco, M.A., Lee, H.-W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A., and Greider, C.W. 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* 91:25-34.

d'Adda di Fagagna, F., Hande, M.P., Tong, W.-M., Lansdorp, P.M., Wang, Z.-Q., and Jackson, S.P.

1999. Functions of poly (ADP-ribose) polymerase in controlling telomere length and chromosomal stability (letter). *Nature Genet.* 23:76-80.

de Lange, T. 1995. Telomere dynamics and genome instability in human cancer. *In* Telomeres (E.H. Blackburn and C.W. Greider, eds.) pp. 265-293. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Greider, C.W. 1995. Telomerase biochemistry and regulation. *In* Telomeres (E.H. Blackburn and C.W. Greider, eds.) pp. 35-68. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Hande, M.P., Samper, E., Lansdorp, P., and Blasco, M.A. 1999a. Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. *J. Cell Biol.* 144:589-601.

Hande, P., Slijepcevic, P., Silver, A., Bouffler, S., van Buul, P., Bryant, P., and Lansdorp, P. 1999b. Elongated telomeres in scid mice. *Genomics* 56:221-223.

Hanish, J.P., Yanowitz, J.L., and de Lange, T. 1994. Stringent sequence requirements for the formation of human telomeres. *Proc. Natl. Acad. Sci. U.S.A.* 91:8861-8865.

Ijdo, J.W., Baldini, A., Ward, D.C., Reeders, S.T., and Wells, R.A. 1991. Origin of human chromosome 2: An ancestral telomere-telomere fusion. *Proc. Natl. Acad. Sci. U.S.A.* 88:9051-9055.

Lansdorp, P.M., Verwoerd, N.P., van de Rijke, F.M., Dragowska, V., Little, M.-T., Dirks, R.W., Raap, A.K., and Tanke, H.J. 1996. Heterogeneity in telomere length of human chromosomes. *Hum. Mol. Genet.* 5:685-691.

Martens, U.M., Zijlmans, J.M.J.M., Poon, S.S.S., Dragowska, W., Yui, J., Chavez, E.A., Ward, R.K., and Lansdorp, P.M. 1998. Short telomeres on human chromosome 17p. *Nature Genet.* 18:76-80.

Martens, U.M., Chavez, E.A., Poon, S.S.S., Schmoor, C., and Lansdorp, P.M. 2000. Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Exp. Cell Res.* 256:291-299.

Moyzis, R.K., Buckingham, J.M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, R.L., and Wu, J.-R. 1988. A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. U.S.A.* 85:6622-6626.

Niida, H., Shinkai, Y., Hande, M.P., Matsumoto, T., Takehara, S., Tachibana, M., Oshimura, M., Lansdorp, P.M., and Furuichi, Y. 2000. Telomere maintenance in telomerase deficient-mouse embryonic stem cells: Characterization of an amplified telomeric DNA. *Mol. Cell. Biol.* 20:4115-4127.

Oexle, K. 1998. Telomere length distribution and Southern blot analysis. *J. Theor. Biol.* 190:369-377.

Poon, S.S.S. and Hunter, D.B. 1994. Electronic cameras to meet the needs of microscopy specialists. *Adv. Imaging* 9:64-67.

Poon, S.S.S., Martens, U.M., Ward, R.K., and Lansdorp, P.M. 1999a. Telomere length measurements using digital fluorescence microscopy. *Cytometry* 36:267-278.

Poon, S.S.S., Ward, R.K., and Lansdorp, P.M. 1999b. Segmenting telomeres and chromosomes in cells. IEEE International Conference on Acoustics, Speech, and Signal Processing. IMDSP 8.4, Vol. 6, p. 3414-3416. IEEE, Washington, DC.

Rufer, N., Dragowska, W., Thornbury, G., Roosnek, E., and Lansdorp, P.M. 1998. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat. Biotechnol.* 16:743-747.

Rufer, N., Brummendorf, T.H., Kolvraa, S., Bischoff, C., Christensen, K., Wadsworth, L., Schultzer, M., and Lansdorp, P.M. 1999. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J. Exp. Med.* 190:157-167.

Surralles, J., Hande, M.P., Marcos, R., and Lansdorp, P.M. 1999. Accelerated telomere shortening in the human inactive X chromosome. *Am. J. Hum. Genet.* 65:1617-1622.

Taneja, K.L., Chavez, E.A., Coull, J., and Lansdorp, P.M. 2001. Multicolor fluorescence in situ hybridization with peptide nucleic acid probes for enumeration of specific chromosomes in human cells. *Genes Chrom. Cancer* 30:57-63.

Walton, W.H. 1952. Automatic counting of microscopic particles. *Nature* 169:321.

Wan, T.S.K., Chan, L.C., Ngan, H.Y.S., and Tsao, S.-W. 1997. High frequency of telomeric associations in human ovarian surface epithelial cells transformed by human papilloma viral oncogenes. *Cancer Genet. Cytogenet.* 95:166-172.

Wan, T.S.K., Martens, U.M., Poon, S.S.S., Tsao, S.-W., Chan, L.C., and Lansdorp, P.M. 1999. Absence or low number of telomere repeats at junctions of dicentric chromosomes. *Genes Chrom. Cancer* 24:83-86.

Young, J.Z. and Roberts, F. 1951. A flying spot microscope. *Nature* 167:231.

Zakian, V.A. 1995. Telomeres: Beginning to understand the end. *Science* 270:1601-1607.

Zhu, L., Hathcock, K.S., Hande, P., Lansdorp, P.M., Seldin, M.F., and Hodes, R.J. 1998. Telomere length regulation in mice is linked to a novel chromosome locus. *Proc. Natl. Acad. Sci. U.S.A.* 95:8648-8653.

Zijlmans, J.M.J.M., Martens, U.M., Poon, S.S.S., Raap, A.K., Tanke, H.J., Ward, R.K., and Lansdorp, P.M. 1997. Telomeres in the mouse have large inter-chromosomal variations in the number of T_2AG_3 repeats. *Proc. Natl. Acad. Sci. U.S.A.* 94:7423-7428.

Contributed by Steven S.S. Poon and Peter M. Lansdorp
BC Cancer Research Centre and University of British Columbia
Vancouver, Canada

Analysis of Mitochondrial Dysfunction During Cell Death

For many years mitochondria were considered only as a main energy supplier in the cell. In the middle of the 1990s it became clear that mitochondria actively participate in apoptosis, a form of cell death characterized by specific biochemical and morphological changes.

The following protocols represent basic tools widely used in estimating functional activity of mitochondria. The protocols are organized into three groups. The first group describes direct assessment of mitochondria in apoptotic cells—collection of proteins released from isolated mitochondria (see Basic Protocol 1), and from mitochondria within the cell (see Basic Protocol 2), immunoblot analysis of released proteins (see Basic Protocol 3), assessment of the mitochondrial membrane potential in apoptotic cells stained with TMRE (see Basic Protocol 4), and microscopy of apoptotic cells stained with MitoTracker Red (see Basic Protocol 5). The second group of protocols describes measurement of the mitochondrial permeability transition (MPT)—measurement of Ca^{2+} fluxes with an electrode (see Basic Protocol 6) or spectrophotometrically (see Alternate Protocol 1), measurement of the mitochondrial membrane potential with a TPP^{+} electrode (see Basic Protocol 7) or spectrophotometrically (see Alternate Protocol 2), estimation of mitochondrial swelling (see Basic Protocol 8), and measuring Ca^{2+} accumulation in permeabilized cells (see Basic Protocol 9). Support Protocols are included for the preparation of mitochondria from a number of sources—liver (see Support Protocol 1), brain (see Support Protocol 2), and cultured cells (see Support Protocol 3)—and for assessing the quality of the prepared mitochondria (see Support Protocol 4).

EVALUATION OF THE RELEASE OF PROTEINS FROM INTERMEMBRANE SPACE OF MITOCHONDRIA

The release of certain proteins from mitochondria in apoptotic cells is believed to be one of the key events in apoptosis. Therefore, estimation of the release of these proteins is important for understanding the role of mitochondria in apoptosis.

Collecting Samples of Proteins From Isolated Mitochondria

BASIC PROTOCOL 1

This method is based on the estimation of the amount of cytochrome *c* or any other protein released from the intermembrane space of mitochondria that remains in the incubation buffer after the mitochondria are removed by centrifugation. This protocol describes collecting samples of proteins released from isolated mitochondria incubated under different experimental conditions. Such experiments are very helpful in order to estimate a possible effect of certain pro-apoptotic compounds on mitochondria.

Materials

Isolated mitochondria (Support Protocols 1 to 3)
Buffer

1. Incubate mitochondria at a concentration of 1 mg protein/ml (time, conditions of incubation, and buffer to be used depend on the aim of the experiment).

   This buffer can be used for mitochondria isolated from different cources. The composition of the buffer is determined mainly by the aims of the experiment (see also Background Information section, MPT-dependent release of cytochrome c). For example, estimationof the release of cytochrome c is more suitable to perform in KCl-based ionic buffer, since the

Contributed by Vladimir Gogvadze, Sten Orrenius, and Boris Zhivotovsky
Current Protocols in Cell Biology (2003) 18.5.1-18.5.27

binding of cytochrome c to the inner mitochondrial membrane is significantly weaker as compared to mannitol-sucrose buffer. If the aim of the experiment is to assess MPT-independent release of cytochrome c, a calcium chelator EGTA, or cyclosporin A should be included.

2. Take a 200-µl aliquot and transfer to a microcentrifuge tube.
3. Microcentrifuge 5 min at 10,000 × *g*, 4°C.
4. Gently remove the supernatant without disturbing the pellet.
5. Resuspend the pellet in the original volume of the same buffer used for the incubation in step 1.
6. Freeze samples of supernatant (step 4) and pellet (step 5) and keep them at −20°C.

The samples are now ready for detection, e.g., of cytochrome c via immunoblotting (see Basic Protocol 3).

BASIC PROTOCOL 2

Evaluation of Cytochrome *c* Release from the Mitochondria of Apoptotic Cells

In order to analyze the release of certain proteins from mitochondria of apoptotic cells, the cellular plasma membrane should be disrupted and cytosolic fraction should be separated from membrane material. This can be achieved by preincubation of cells in a hypotonic solution that induces cell lysis.

Materials

Cells of interest (e.g., Jurkat cells, U 937, HeLa)
Apoptotic stimuli (e.g., etoposide, staurosporine)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
S100 buffer (see recipe), ice-cold
Refrigerated low-speed centrifuge and ultracentrifuge
Additional reagents and equipment for immunoblot analysis (*UNIT 6.2* and Basic Protocol 3 in this unit)

1. Incubate cells with apoptotic stimuli (type, concentration, and incubation time determined by cell type).
2. Wash cells twice, each time with 5 ml of ice-cold PBS.
3. Resuspend cells at 75×10^6 cells/ml in ice-cold S100 buffer.
4. Incubate on ice for 10 to 20 min.
5. Centrifuge cells 15 min at 10,000 × *g*, 4°C.
6. Transfer supernatant to ultracentrifuge tube and ultracentrifuge 1 hr at 100,000 × *g*, 4°C.
7. Use supernatant for immunoblot analysis (see Basic Protocol 3 and *UNIT 6.2*) with an antibody specific for cytochrome *c*.

BASIC PROTOCOL 3

Immunoblot Analysis of Proteins Released from the Mitochondria During Apoptosis

After separation as in Basic Protocol 1 or 2, the supernatant contains proteins released from mitochondria, while the pellet represents proteins associated with the organelles. The detection of these proteins is performed using electrophoresis with subsequent blotting and probing with specific antibodies.

Materials

Sample collected from apoptotic mitochondria (see Basic Protocol 1 or 2)
4× Laemmli's loading buffer (see recipe)
15% (w/v) SDS-PAGE gel (*UNIT 6.1*)
5% (w/v) nonfat milk in PBS (see *APPENDIX 2A* for PBS)
Antibody specific for apoptotic protein of interest (e.g., Becton Dickinson Biosciences)
PBS (*APPENDIX 2A*) containing 2.5% (w/v)
Nonfat dry milk
Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 1% (w/v) bovine serum albumin and 0.01% (w/v) azide (NaN_3)
PBS (*APPENDIX 2A*) containing 15% (v/v) Tween 20
Horseradish peroxidase–conjugated secondary antibody (e.g., Pierce)
ECL Western Blotting Detection Reagents kit (Amersham Biosciences)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and electroblotting (*UNIT 6.2*)

Perform electrophoresis

1. Mix 40 µl of sample with 12.5 µl of 4× Laemmli's loading buffer. Boil 5 min.
2. Separate the proteins on a 15% SDS-PAGE gel at 130 V (*UNIT 6.1*).

Perform immunoblotting and detect apoptotic proteins

3. Transfer proteins to nitrocellulose membranes by electroblotting (Basic Protocol 1 in *UNIT 6.2*) for 2 hr at 100 V.
4. Block membranes for 1 hr with 5% nonfat milk in PBS at room temperature.
5. Probe overnight in a cold room with an antibody (diluted 1:2500 in PBS containing bovine serum albumin and 0.01% NaN_3) specific for the apoptotic protein of interest.
6. Rinse membranes for 10 min in 30 to 40 ml of PBS.
7. Rinse membranes for 15 min in 30 to 40 ml of PBS with 0.15% Tween 20.
8. Rinse membranes for 10 min in 30 to 40 ml of PBS.
9. Incubate with a horseradish peroxidase–conjugated secondary antibody (diluted 1:10,000 in PBS containing 2.5% nonfat dry milk).

Visualize bound antibodies

10. Following incubation with the secondary antibody, rinse the membranes for 10 min in 30 to 40 ml of PBS.
11. Rinse membranes for 15 min in 30 to 40 ml of PBS with 0.15% Tween 20.
12. Rinse membranes for 10 min in 30 to 40 ml of PBS.
13. Detect bound antibodies using enhanced chemiluminescence (ECL kit) according to the manufacturer's instructions.

Assessment of the Mitochondrial Membrane Potential in Apoptotic Cells

BASIC PROTOCOL 4

The mitochondrial membrane potential ($\Delta\psi$), which drives oxidative phosphorylation (Mitchell and Moyle, 1967) and which also drives mitochondrial calcium uptake, is generated by an electron-transporting chain. When electron transport ceases, for example during ischemia, the inner-membrane potential is developed at the expense of ATP

hydrolysis by the mitochondrial ATP synthase. An early manifestation of apoptosis is often a decrease in the $\Delta\psi$, which is usually demonstrated using flow cytometry. The relationship between mitochondrial depolarization and apoptosis remains controversial. Some investigators consider a decrease in $\Delta\psi$ an early irreversible signal for apoptosis (Zamzami et al., 1996), while others describe it as a late event (Bossy-Wetzel et al., 1998).

Materials

Cells of interest (e.g., Jurkat cells, U 937, HeLa)
RPMI-1640 medium (Life Technologies) supplemented with 5% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml)
25 mM TMRE stock solution: dissolve 12.8 mg tetramethylrhodamine methyl ester (TMRE; Molecular Probes) in 1 ml ethanol; store per manufacturer's instructions
HEPES buffer (see recipe)
Flow cytometer (e.g., FACS; Becton Dickinson)

1. Prepare an aliquot of 10^6 cells in RPMI-1640 medium.
2. Dilute TMRE stock solution 1:1000 with HEPES buffer (for a concentration of 25 µM).
3. Add an aliquot of the diluted (25 µM) TMRE to cells for a final concentration of 25 nM.
4. Incubate cells with TMRE 20 min at 37°C.
5. Further dilute the 25 µM TMRE 1:1000 with HEPES buffer for a final concentration of 25 nM. Centrifuge cells 5 min at 200 × *g*, room temperature, and resuspend in fresh HEPES buffer containing 25 nM TMRE.
6. Analyze membrane potential by flow cytometry according to the manufacturer's instructions for the instrument used.

BASIC PROTOCOL 5

Microscopy of Apoptotic Cells Stained with MitoTracker

Fluorescent probes for mitochondria such as rhodamine 123 or tetramethylrosamine are readily sequestered by mitochondria. However, in experiments that require fixation of the organelles, these stains are easily washed out. In contrast, mitochondria-selective probes—e.g., MitoTracker Red (Molecular Probes)—are concentrated by active, energized mitochondria and retained during fixation. Once mitochondria are labeled, the cells can be fixed for further processing, for example, if cells are going to be subsequently labeled with antibody.

Materials

Cells of interest (e.g., Jurkat cells, U 937, HeLa) and appropriate culture medium
MitoTracker Red (Molecular Probes); store according the manufacturer's instructions
Dimethylsulfoxide (DMSO)
4% (w/v) paraformaldehyde in serum-free culture medium
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS (*APPENDIX 2A*) containing 0.2% (v/v) Triton X-100
PBS (*APPENDIX 2A*) containing 1% (v/v) fetal bovine serum (FBS)
Mounting medium: 50% (w/v) glycerol/PBS
Fluorescent or scanning confocal microscope (e.g., Bio-Rad), with 581 nm excitation filter and 644 nm emission filter
Additional reagents and equipment for cell culture (*UNIT 1.1*)

1. Grow cells on coverslips inside a petri dish filled with the appropriate culture medium to the optimal density.

   The optimal cell density depends on the cell type.

2. Open fresh vial of MitoTracker Red and add DMSO to create a 1 mM stock. Protect from light.

3. Dilute 1 mM MitoTracker Red to final working concentration in the culture medium used to grow the cells.

   Recommended Mito Tracker Red concentration is 100 to 500 nM. Diluted MitoTracker Red can be stored up to several months at –20°C

4. When cells have reached the desired confluence, remove the medium from the dish and add the prewarmed (37°) growth medium containing MitoTracker probe. Incubate for 30 min.

5. Replace the loading solution with fresh prewarmed medium.

Fix and permeabilize cells

6. Remove the growth medium covering the cells and replace it with fresh prewarmed medium containing 4% paraformaldehyde. Incubate 15 min at 37°C.

7. Rinse cells three times, each time for 5 min with room temperature PBS.

8. Incubate fixed cells in PBS containing 0.2% Triton X-100 at room temperature for 5 min.

9. Rinse cells three times, each time for 5 min with PBS containing 1% FBS.

10. Mount coverslips on glass slides in 50% glycerol/PBS and examine under fluorescent or scanning confocal microscope with excitation at 581 nm and emission at 644 nm.

ASSESSMENT OF THE MITOCHONDRIAL PERMEABILITY TRANSITION IN ISOLATED MITOCHONDRIA

The mitochondrial permeability transition (MPT) is one of the mechanisms that can lead to the release of cytochrome *c* from mitochondria during apoptosis. MPT is a consequence of Ca^{2+} overload. Accumulation of Ca^{2+} in mitochondria is driven by the mitochondrial membrane potential supported either by mitochondrial respiration or by ATP hydrolysis. Mitochondria release Ca^{2+} when the membrane potential is dropped either by inhibitors of mitochondrial respiratory chain (if the potential is supported by oxidation of a substrate) or by inhibitors of ATPase (if the source of potential is hydrolysis of ATP).

Retention of Ca^{2+} by mitochondria stimulates processes that result in mitochondrial deterioration. Mitochondria swell and become leaky, whereupon the membrane potential drops and Ca^{2+} is released. Although Ca^{2+} is obligatory for MPT induction, the sensitivity of mitochondria to permeability transition can be enhanced by different factors. Among these factors are elevated levels of phosphate, oxidative stress, and the depletion of adenine nucleotides. When phosphate or organic peroxide is added to Ca^{2+}-loaded mitochondria these organelles swell, membrane potential decays, and accumulated Ca^{2+} is released. All of these manifestations can be prevented by cyclosporin A (CsA), an inhibitor of pore opening. Therefore, MPT in isolated mitochondria can be assessed by monitoring either swelling, membrane potential, or the level of Ca^{2+} in the incubation buffer.

Accumulation of Ca^{2+} by mitochondria, as well as Ca^{2+} retention and release induced by different stimuli, can be monitored using a Ca^{2+}-sensitive electrode or spectrophotometrically with the Ca^{2+}-sensitive dye arsenazo III.

BASIC PROTOCOL 6

Monitoring of Ca^{2+} Fluxes Across the Inner Mitochondrial Membrane with a Ca^{2+}-Sensitive Electrode

One relatively easy method applicable to mitochondria and permeabilized cells employs a Ca^{2+}-sensitive electrode to follow the Ca^{2+}. Accumulation of Ca^{2+} by mitochondria can be followed by a decrease in the concentration of Ca^{2+} in the incubation buffer.

Materials

Incubation buffer for calcium-sensitive electrode (see recipe)
Isolated mitochondria (Support Protocol 1, 2, or 3)
2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at –20°C up to 1 month
10 mM $CaCl_2$: dissolve 1.47 mg $CaCl_2 \cdot 2H_2O$ in 1 ml H_2O; store frozen at –20°C up to 1 month
0.5 M KH_2PO_4: dissolve 136.1 mg KH_2PO_4 in 2 ml H_2O and adjust pH to 7.4 with KOH; store frozen at –20°C up to 1 month

Apparatus for membrane potential measurement consisting of:
Glass (or plastic) sample chamber large enough for incubation volume, which can be warmed by being connected to a water bath and can be constantly stirred
Ca^{2+}-sensitive electrode (e.g., Orion)
pH meter as source of current
Chart recorder with variable input and chart speed
Any commercially available reference electrode for pH measurements

1. Add 2 ml of incubation buffer for calcium-sensitive electrode to the sample chamber of the measurement apparatus and set to conditions of constant stirring. Add a volume of mitochondrial suspension (which will depend on the concentration of the mitochondrial preparation) containing 2 mg of protein to the sample chamber.
2. After 30 sec, add 2 µl of 2.5 mM rotenone, for a final concentration of 2.5 µM.

   When mitochondria are energized with the complex II substrate succinate, rotenone prevents formation of oxaloacetate, an inhibitor of succinate dehydrogenase that supresses mitochondrial respiration.

3. Turn on the chart recorder.
4. After 1 min (or when stabilization has been achieved as observed by recorder trace) add 50 to 60 nmol Ca^{2+} (from 10 mM $CaCl_2$ stock) per milligram of mitochondrial protein.

   The addition of Ca^{2+} to mitochondria leads to a rapid increase in the level of this cation in the reaction buffer, followed by a return to the initial level as mitochondria accumulate the excess Ca^{2+}.

5. After 1 min, add 20 µl of 0.5 M KH_2PO_4, for a final concentration of 5 mM.

   Inorganic phosphate is a trigger for permeability transition in mitochondria loaded with calcium.

6. Follow the retention of Ca^{2+} by its release as seen from the recorder trace.

Monitoring of Ca^{2+} Fluxes Across the Inner Mitochondrial Membrane with a Spectrophotometer

ALTERNATE PROTOCOL 1

Spectrophotometric estimation of Ca^{2+} fluxes across the mitochondrial membrane should be performed using a dual-wavelength spectrophotometer, in order to avoid interference from the turbidity of mitochondrial suspension.

Additional Materials *(also see Basic Protocol 6)*

25 mM arsenazo III: dissolve 19.4 mg arsenazo III (Sigma) in 1 ml H_2O; store frozen at –20°C up to 1 month

Dual-wavelength recording spectrophotometer set at 675 versus 685 nm, with appropriate cuvettes

1. Place 2 ml of incubation buffer for calcium-sensitive electrode in a spectrophotometric cuvette and set to conditions of constant stirring. Add 2 µl of 25 mM arsenazo III, for a final concentration of 25 µM.
2. Add an aliquot of mitochondrial suspension containing 2 mg of protein, for a final concentration of 1 mg/ml.

   The volume of the mitochondrial aliquot will depend on the concentration of the mitochondrial preparation.

3. After 30 sec add 2 µl of 2.5 mM rotenone, for a final concentration of 2.5 µM.
4. After 1 min add 50 to 60 nmol Ca^{2+} (from 10 mM $CaCl_2$ stock) per mg of mitochondrial protein.

   The addition of Ca^{2+} to mitochondria leads to a rapid increase in the level of this cation in the reaction buffer, followed by a return to the initial level as mitochondria accumulate the excess Ca^{2+}.

5. After 1 min, add 20 µl of 0.5 M KH_2PO_4, for a final concentration of 5 mM.

   The retention of Ca^{2+} is followed by its release upon induction of MPT as can be seen from the recorder trace.

DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL

Estimation of the mitochondrial membrane potential ($\Delta\psi$) can be performed using an electrode sensitive to the lipophilic cation tetraphenylphosphonium (TPP^+) or spectrophotometrically with a $\Delta\psi$-specific dye, safranin.

Measurements of Mitochondrial Membrane Potential with a TPP^+-Sensitive Electrode

BASIC PROTOCOL 7

Energized mitochondria rapidly accumulate TPP^+ from the incubation buffer and release this cation as $\Delta\psi$ decays. Accumulation of TPP^+ by mitochondria can be followed by a decrease in the concentration of TPP^+ in the incubation buffer.

Materials

Incubation buffer for TPP^+-sensitive electrode (see recipe)

TPP^+ stock solution: dissolve 3.75 mg tetraphenylphosphonium chloride (Aldrich) in 10 ml H_2O; store up to 1 month at room temperature

Isolated mitochondria (Support Protocol 1, 2, or 3)

2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at –20°C up to 1 month

10 mM $CaCl_2$: dissolve 1.47 mg $CaCl_2 \cdot 2H_2O$ in 1 ml H_2O; store frozen at –20°C up to 1 month

0.5 M KH_2PO_4: dissolve 136.1 mg KH_2PO_4 in 2 ml H_2O and adjust pH to 7.4 with KOH; store frozen at –20°C up to 1 month

Apparatus for membrane potential measurement consisting of:

Glass (or plastic) sample chamber that can accommodate 2 ml of incubation buffer, which can be warmed by being connected to a water bath and can be constantly stirred

TPP^+ electrode (purchase from Microelectrodes, Inc. or prepare in the laboratory, Kamo et al., 1979)

pH meter as source of current

Chart recorder with variable input and chart speed

Any commercially available reference electrode

1. Place 2 ml of incubation buffer for TPP^+-sensitive electrode in the sample chamber of the measurement apparatus and set conditions to constant stirring. Add 4 µl of TPP^+ stock, for a final concentration of 2 µM TPP^+, and turn on the chart recorder.

   The recorder trace shows the basal level of TPP^+ in incubation buffer.

2. Add mitochondrial suspension to a final concentration of 1 mg/ml to the incubation buffer under conditions of constant stirring.

   The volume of the mitochondrial aliquot will depend on the concentration of mitochondrial preparation.

   Mitochondria accumulate TPP^+ according to the membrane potential so that the level of TPP^+ in the incubation buffer decreases.

3. After 30 sec add 2 µl of 2.5 mM rotenone, for a final concentration of 2.5 µM.

4. After 1 min, add 50 to 60 nmol Ca^{2+} (from 10 mM $CaCl_2$ stock) per milligram of mitochondrial protein.

   Upon addition of Ca^{2+}, the mitochondrial membrane potential decreases, with subsequent restoration when Ca^{2+} is accumulated.

5. After restorationof the membrane potential, add 20 µl of 0.5 M KH_2PO_4 for a final concentration of 5 mM and monitor the release of TPP^+ from mitochondria, which is indicative of the drop of the membrane potential upon induction of MPT.

   If Ca^{2+} loading is not sufficient to induce permeability transition, mitochondria will maintain membrane potential. In this case Ca^{2+} loading should be increased.

ALTERNATE PROTOCOL 2

Measurement of Mitochondrial Membrane Potential Using a Spectrophotometer

Spectrophotometric measurement of the membrane potential can be performed using safranin, a cationic dye, because of its accumulation in mitochondria and a shift in absorption spectrum that occurs when membrane potential is high. In these experiments, a dual-wavelength spectrophotometer should be used in order to avoid a contribution by the turbidity of the mitochondrial suspension to light absorption.

Additional Materials *(also see Basic Protocol 7)*

Incubation buffer for calcium-sensitive electrode (see recipe)

10 mM safranin: dissolve 3.5 mg safranin in 1 ml H_2O; store frozen at –20°C up to 1 month

Dual-wavelength recording spectrophotometer set at 511 versus 533 nm, with appropriate cuvettes

1. Place 2 ml of incubation buffer for calcium-sensitive electrode in a spectrophotometric cuvette and set to conditions of constant stirring. Add 2 µl of 10 mM safranin, for a final concentration of 10 µM.
2. Add an aliquot of mitochondrial suspension containing 2 mg of protein, for a final concentration of 1 mg/ml.

   The volume of the mitochondrial aliquot will depend on the concentration of the mitochondrial preparation.
3. After 30 sec add 2 µl of 2.5 mM rotenone, for a final concentration of 2.5 µM.
4. After 1 min add 50 to 60 nmol Ca^{2+} (from 10 mM $CaCl_2$ stock) per milligram of mitochondrial protein.

   Upon addition of Ca^{2+}, the mitochondrial membrane potential decreases, with subsequent restoration when Ca^{2+} is accumulated.
5. After restoration of the membrane potential, add 20 µl of 0.5 M KH_2PO_4, for a final concentration of 5 mM, and monitor the decrease in the membrane potential upon induction of MPT.

Estimation of Mitochondrial Swelling

BASIC PROTOCOL 8

One of the most reliable manifestations of mitochondrial permeability transition is swelling of the organelles. Nephelometric or turbidometric techniques have generally been used to monitor mitochondrial swelling. Mitochondrial swelling can be monitored continuously as changes in OD_{540}.

Materials

Incubation buffer for swelling (see recipe)
Isolated mitochondria (Support Protocol 1, 2, or 3)
10 mM $CaCl_2$: dissolve 1.47 mg $CaCl_2 \cdot 2H_2O$ in 1 ml H_2O; store frozen at –20°C up to 1 month
2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at –20°C up to 1 month
0.5 M KH_2PO_4: dissolve 136.1 mg KH_2PO_4 in 2 ml H_2O and adjust pH to 7.4 with KOH; store frozen at –20°C up to 1 month

Spectrophotometer with 540-nm filter, or multiwavelength spectrophotometer
Chart recorder with variable input and chart speed

1. Place 2 ml of incubation buffer for swelling in a spectrophotometric cuvette and set to conditions of constant stirring. Add a volume of mitochondrial suspension (which will depend depends on the concentration of mitochondrial preparation) for a final concentration of 0.5 mg/ml.
2. After 30 sec, add 2 µl of 2.5 mM rotenone, for a final concentration of 2.5 µM.
3. After 1 min add 50 to 60 nmol Ca^{2+} (from 10 mM $CaCl_2$ stock) per milligram of mitochondrial protein.
4. After 1 min, add 20 µl of 0.5 M KH_2PO_4.
5. Monitor gradual decrease in OD_{540} nm due to mitochondrial swelling.

Estimation of Mitochondrial Ca^{2+} Accumulation in Digitonin-Permeabilized Cells

Permeabilization of the plasma membrane allows measurement of mitochondrial activity in situ, without isolation of these organelles and the accompanying potential risk of mitochondrial damage. For the permeabilization, a steroid glycoside, digitonin, is widely used. The affinity of digitonin for cholesterol allows a selective disruption of the plasma membrane, making mitochondria accessible to specific substrates and Ca^{2+}. The concentration of digitonin must be chosen carefully and usually should not exceed 0.005% (w/v), since higher concentrations might affect the outer mitochondrial membrane barrier functions.

Permeabilization of the plasma membrane allows estimation of the main functional parameters of mitochondria, such as respiration, membrane potential, and accumulation and release of Ca^{2+}. A description of the monitoring of Ca^{2+} fluxes is given below.

Induction of MPT in apoptotic cells is thought to be one of the reasons for the decrease in $\Delta\psi$. Pore opening during apoptosis (even in a subpopulation of mitochondria) will impair the ability of the total mitochondrial population to accumulate Ca^{2+}; therefore estimation of a threshold level of Ca^{2+} loading (the Ca^{2+} capacity of mitochondria) in cells might be a criterion for changes on the mitochondrial level.

Materials

Cells of interest (Jurkat cells, U 937, HeLa)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Incubation buffer for digitonin-permeabilized cells (see recipe)
1% (w/v) digitonin: dissolve 10 mg digitonin in 1 ml H_2O; shake vigorously before adding to cells; store up to 2 to 3 months at room temperature
2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at –20°C up to 1 month
10 mM $CaCl_2$: dissolve 1.47 mg $CaCl_2 \cdot 2H_2O$ in 1 ml H_2O; store frozen at –20°C up to 1 month

Apparatus for membrane potential measurement consisting of:
Glass (or plastic) sample chamber large enough for incubation volume, which can be warmed up by being connected to a water bath and can be constantly stirred
Ca^{2+}-sensitive electrode (e.g., Orion)
pH meter as source of current
Chart recorder with variable input and chart speed
Any commercially available reference electrode for pH measurements

1. Centrifuge $3–5 \times 10^6$ cells 5 min at $200 \times g$, room temperature. Remove supernatant, resuspend cells in PBS, and centrifuge again under the same conditions.
2. Resuspend cells in 50 µl of incubation buffer for digitonin-permeabilized cells, then add the resuspended cells to 450 µl of the same buffer in sample chamber with Ca^{2+} electrode at constant stirring. Turn on the chart recorder.
3. Following a 2-min stabilization period, add 1% digitonin to a final concentration of 0.005% and 2.5 mM rotenone to a final concentration of 5 µM.
4. Load mitochondria with pulses of Ca^{2+} (20 to 25 nmol each, added from 10 mM stock) to induce MPT, which can be observed by the release of accumulated Ca^{2+} as measured by the Ca^{2+}-sensitive electrode.

The calcium capacity is calculated as a sum of the amount of Ca^{2+} in each addition.

ISOLATION OF MITOCHONDRIA

Experiments with isolated mitochondria are a helpful supplement to experiments on a cellular level. The former type of experiments provide better understanding of the mechanisms responsible for the mitochondrial changes in apoptotic cells. All the methods of isolation of mitochondria are based on the disruption of tissue and subsequent differential centrifugation of homogenate.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Mitochondria should be kept on ice at all times.

Isolation of Rat Liver Mitochondria

SUPPORT PROTOCOL 1

Isolation of liver mitochondria represents a relatively "easy" procedure giving a high yield. The liver from a 150 to 200-g rat weighs 5 to 6 g. Liver can be easily disrupted and homogenized. The most important thing is keeping the temperature not higher than 4 to 6°C.

Materials

150- to 200-g rat
Buffer A for liver (see recipe)
Buffer B: Buffer A (for liver) without EDTA

Dissecting equipment
Motor-driven glass Dounce homogenizer and tight Teflon pestle
Refrigerated centrifuge and 50-ml centrifuge tubes

Additional reagents and equipment for protein assay (*APPENDIX 3H*) and determination of the respiratory control ratio for mitochondria (Support Protocol 4)

NOTE: All operations should be done on ice, using ice-cold buffers and instruments.

Harvest the liver

1. Let the rat starve overnight.

   This step is necessary to decrease the content of fat and glycogen in the liver.

2. Sacrifice rat in accordance with institutional guidelines and the aims of the experiment.

   Donovan and Brown (1995) provide a variety of protocols for rodent euthanasia.

3. Remove liver and immerse immediately in ice-cold buffer A for liver.

4. Cut liver into pieces with scissors and wash several times by replacing the ice-cold buffer A with fresh ice-cold buffer, in order to remove as much blood as possible.

Homogenize the liver

5. Homogenize the liver in 60 ml of buffer A with 5 to 6 up-and-down strokes of the tight Teflon pestle.

   Homogenization occurs as the piston of the homogenizer rotates (driven by an electrical motor) and the pestle is slowly moved up and down 5 to 6 times, so that there are no pieces of liver big enough to be seen by eye.

6. Transfer homogenate into two cold centrifuge tubes and balance them.

7. Centrifuge the homogenate 8 min at 600 × *g*, 4°C, to remove nuclei and unbroken cells.

Isolate mitochondria

8. Discard the pellet and centrifuge supernatant 15 min at 5500 × *g*, 4°C, to form a mitochondrial pellet.
9. Remove floating fat droplets with a paper towel or piece of tissue paper.
10. Detach the pellet from the bottom of the tube with the piston of the homegenizer, add 0.2 to 0.3 ml of buffer A for liver, resuspend the pellet using the pestle, and pass mitochondria gently 3 to 4 times through the tip of a 1-ml pipet tip using automatic pipettor.
11. Dilute the mitochondrial suspension with 30 ml of buffer B, then centrifuge again as in step 9.
12. Resuspend the final mitochondrial pellet to a volume of ~1 ml in buffer B by gentle passing through a 1-ml pipet tip, then transfer into a 10-ml tube and keep on ice.

Assess the preparation

13. Determine the protein concentration using Lowry's method (or any other method available; see *APPENDIX 3H*) with bovine serum albumin as a standard.

    The protein concentration of a final suspension usually is about 90 to 100 mg/ml.

14. As a control of the mitochondrial preparation, estimate the respiratory control ratio (see Support Protocol 4). Use within 4 hr.

    Measurements can be started directly after mitochondrial isolation.

SUPPORT PROTOCOL 2

Isolation of Brain Mitochondria

Another source of mitochondria frequently used in the investigation of mitochondrial functioning (e.g., during apoptosis) is the brain. The method for isolation of brain mitochondria is similar to that for liver mitochondria (see Support Protocol 1); however, in order to get a purer preparation, a purification of homogenate with a Ficoll gradient is involved.

Materials

150- to 200-g rat
SET buffer (see recipe), ice-cold
3% and 6% (w/v) Ficoll solutions (see recipe)
MSH buffer (see recipe)

Motor-driven glass Dounce homogenizer and tight Teflon pestle
Refrigerated centrifuge and 50-ml centrifuge tubes

Additional reagents and equipment for protein assay (*APPENDIX 3H*) and determination of the respiratory control ratio for mitochondria (Support Protocol 4)

Dissect brain

1. Sacrifice rat in accordance with institutional guidelines and the aims of the experiment.
2. Open the skull with scissors, gently remove the brain, place it in ice-cold SET buffer, and chop it into pieces with scissors.

3. Wash pieces of brain several times in ice-cold SET buffer in order to remove as much blood as possible.

Homogenize brain

4. Homogenize manually chopped brain in 30 ml of SET buffer at 4°C using a Dounce homogenizer with six up-and-down strokes of the tight pestle.
5. Dilute homogenate to a final volume of 80 ml with SET buffer.
6. Place the homogenate in two cold centrifuge tubes, balance them, and centrifuge 3 min at 2000 × *g*, 4°C.
7. Decant supernatant and centrifuge again for 3 min at 2000 × *g*, 4°C.

Isolate mitochondria

8. Collect the supernatant and centrifuge 15 min at 12,000 × *g*, 4°C, to obtain a crude mitochondrial fraction.
9. Resuspend the pellet in 6 ml of ice-cold 3% Ficoll solution and layer onto 25 ml of ice-cold 6% Ficoll solution.
10. Centrifuge 10 min at 11,500 × *g*, 4°C.
11. Resuspend the final pellet in MSH buffer to a final volume of ~1 ml and keep on ice.

Assess preparation

12. Determine the protein concentration (*APPENDIX 3H*).
13. As a control of the mitochondrial preparation estimate the respiratory control ratio (see Support Protocol 4). Use within 4 hr.

Measurements can be started directly after mitochondrial isolation.

Isolation of Mitochondria from Cultured Cells

SUPPORT PROTOCOL 3

Isolation of mitochondria from cultured cells is more problematic than isolation from tissue, since it is difficult to disrupt the cell membrane without affecting the mitochondrial membrane. In order to make the plasma membrane more vulnerable, different tactics are used. The plasma membrane can be sensitized to disruption by using a buffer that contains digitonin, a steroid glycoside with a high affinity for cholesterol that selectively disrupts the cholesterol-rich plasma membrane. Another approach is based on passing cells through a needle. Both methods are followed by a differential centrifugation commonly used for preparation of mitochondria.

Materials

Tissue culture cells (e.g., Jurkat cells, U 937, HeLa; *UNIT 1.1*)
Complete RPMI medium (*UNIT 1.2*) or appropriate medium for cells
Buffer A for cultured cells (see recipe), ice-cold
Buffer B: Buffer A (for cultured cells) without EGTA
1% (w/v) digitonin: dissolve 10 mg digitonin in 1 ml H_2O; shake vigorously before adding to cells; store up to 2 to 3 months at room temperature
Refrigerated centrifuge and tubes

1. Take an aliquot containing 5×10^7 cells growing in culture in complete RPMI medium and centrifuge 5 min at 200 × *g*, 4°C. Remove supernatant.
2. Resuspend cells in buffer A for cultured cells. Centrifuge 4 min at 200 × *g*, 4°C.

3. Add 1% digitonin to buffer A for a final concentration of 0.01%. Resuspend cells in 5 ml of the buffer A containing 0.01% digitonin and incubate 10 min on ice.
4. Add 5 ml of ice-cold buffer A and mix well. Sediment lysed cells by centrifuging 7 min at 500 × *g*, 4°C.
5. Discard the pellet and centrifuge supernatant 15 min at 5500 × *g*, 4°C, to form a mitochondrial pellet.
6. Detach the pellet from the bottom of the centrifuge tube with a pipet tip and resuspend the pellet in 0.1 ml of buffer B using an automatic pipettor.
7. Dilute the mitochondrial suspension with 2 ml of buffer B and centrifuge again for 15 min at 5500 × *g*, 4°C.
8. Resuspend the final mitochondrial pellet in buffer B by gently passing through a pipet tip, then transfer into an microcentrifuge tube and keep on ice.

Measurements can be started directly after mitochondrial isolation.

Unfortunately, the yield from this protocol is sufficient material for an experiment but not enough to assess protein concentration and respiratory control ratio as well.

SUPPORT PROTOCOL 4

Estimation of the Quality of Isolated Mitochondria: Measuring the Respiratory Control Ratio (RCR)

One of the keystones of the chemiosmotic theory of energy transduction is impermeability of the inner mitochondrial membrane to protons. Oxidation of substrates results in extrusion of protons from the mitochondrial matrix to generate the mitochondrial membrane potential. High membrane potential suppresses further extrusion of protons and therefore inhibits respiration. Under resting conditions, the rate of respiration of mitochondria is quite low and determined by a passive leakage of protons into the matrix space.

Phosphorylation of ADP requires translocation of protons into the matrix through mitochondrial ATP synthase. This results in a decrease in membrane potential that stimulates respiration. Comparison of the rates of respiration in the resting state and during phosphorylation of ADP is a useful measure of the efficiency of mitochondrial functioning. The most reliable and widely used criterion of the quality of a mitochondrial preparation is the respiratory control ratio (RCR). RCR is defined as the rate of respiration in the presence of ADP (phosphorylating respiration, state 3) divided by the rate obtained following the expenditure of ADP, state 4.

Materials

Mitochondria (Support Protocol 1, 2, or 3)
Incubation buffer for testing mitochondrial quality (see recipe)
2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at –20°C up to 1 month
0.5 M sodium succinate: dissolve 135 mg of sodium succinate in 1 ml of H_2O, store frozen at –20°C up to 1 month
50 mM ADP: dissolve 23 mg of ADP in 1 ml H_2O and adjust pH to 7.5 with KOH; store frozen at –20°C up to 1 month
1 mM CCCP: dissolve 0.2 mg CCCP in 1 ml ethanol and mix thoroughly; store frozen at –20°C up to 1 month
Biological oxygen monitor equipped with a Clark-type oxygen electrode (Yellow Spring Instrument Co.) or similar equipment (operate per manufacturer's instructions)
Chart recorder with variable input and chart speed
10- and 50-µl Hamilton syringes (e.g., Sigma)

1. Place 2 ml of incubation buffer for testing mitochondrial quality into the sample chamber of the oxygen monitor and set conditions to constant stirring. Add an aliquot of mitochondrial suspension (the volume of which will depend on the concentration of the mitochondrial preparation) for a final concentration of 2 mg/ml mitochondrial protein.
2. After 30 sec add 2 µl of 2.5 mM rotenone, for a final concentration of 2.5 µM.
3. Stop stirring and insert the electrode into the sample chamber. Expel all air through the slot in the plunger (slight twisting of the electrode helps to gather the bubbles at the slot) and set the chart speed.

   The desirable chart speed should be 10 to 15 mm/min.
4. After 1 min stabilization observed by recorder trace add 20 µl of 0.5 M sodium succinate, for a final concentration of 5 mM, through the slot on electrode's body with a Hamilton syringe.

   Mitochondria will now start to consume oxygen. The slope of the curve shows the rate of respiration.
5. After stabilization of respiration (~2 min) add 10 µl of 50 mM ADP.

   The rate of respiration will increase (state 3) with subsequent restoration of the initial rate of respiration (state 4) when all added ADP is phosphorylated.
6. Add 2 µl of 1 mM CCCP, for a final concentration of 1 µM.

   CCCP lowers the mitochondrial potential that stimulates oxygen consumption.
7. Calculate the rate of respiration from the recorder trace as the amount of oxygen consumed in 1 min.

   Assume that at room temperature and normal atmospheric pressure the concentration of oxygen in the incubation buffer is 250 µM.
8. Calculate the RCR by dividing the state 3 respiration rate by the state 4 respiration rate.

   Mitochondria with a respiratory control ratio above 4 are acceptable for use in the experiments.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A for cultured cells

38.26 g/liter mannitol (210 mM final)
23.96 g/liter sucrose (70 mM final)
0.2g/liter $MgCl_2 \cdot 6\ H_2O$ (1 mM final)
1.2 g/liter HEPES (5 mM final)
0.38 g/liter EGTA (1 mM final)
Adjust pH to 7.5 with KOH
Store up to 3 to 4 days at 4°C

Buffer A for liver

38.26 g/liter mannitol (210 mM final)
23.96 g/liter sucrose (70 mM final)
1.2 g/liter HEPES (5 mM final)
2 ml/liter 0.5 M EDTA (1 mM final)
Adjust pH to 7.5 with KOH
Store up to 3 to 4 days at 4°C

Ficoll dilution buffer

0.25 M mannitol
60 mM sucrose
100 μM potassium EGTA
10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Store up to 3 to 4 days at 4°C

Ficoll solutions, 3% and 6% (w/w)

Prepare 20% (w/v) Ficoll (Sigma) stock solution in Ficoll dilution buffer (see recipe). Store 3 to 4 days at 4°C. Before the experiement perpare 6% (v/v) and 3% (v/v) solutions by diluting 20% solution wiht Ficoll dilution buffer.

HEPES buffer

2.38 g/liter HEPES (10 mM final)
8.76 g/liter NaCl (150 mM final)
0.37 g/liter KCl (5 mM final)
0.2 g/liter $MgCl_2 \cdot 6H_2O$ (1 mM final)
Adjust pH to 7.4 with NaOH
Store up to 2 to 3 days at 4°C

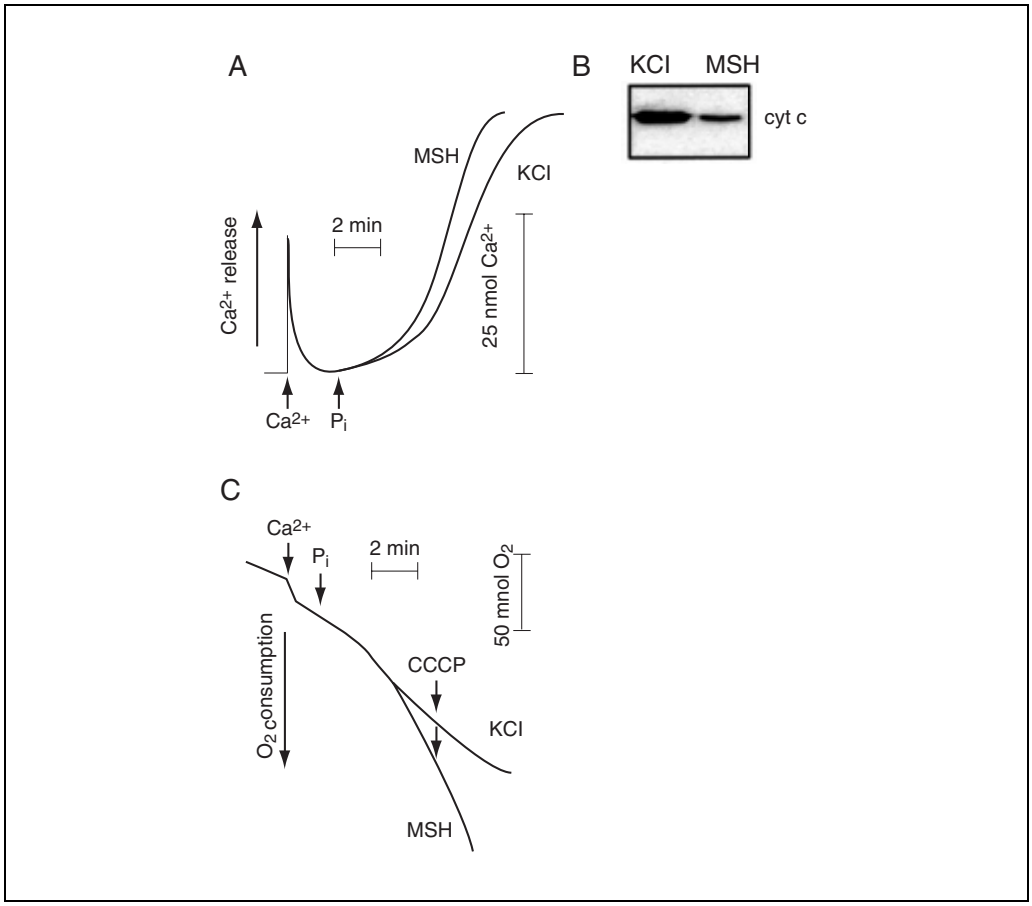


Figure 18.5.1 MPT-induced release of cytochrome *c* from mitochondria incubated in either MSH or KCl buffer. (**A**) Mitochondria were added to buffer at a concentration of 1 mg/ml. After a 2-min stabilization period, mitochondria were loaded with Ca^{2+} (50 nmol/mg protein), and MPT was induced by adding 5 mM inorganic phosphate (P_i). (**B**) Mitochondrial suspensions from (A) were centrifuged and the resulting supernatants were separated by SDS-PAGE and immunoblotted as described in Basic Protocol 3. (**C**) Mitochondrial respiration following MPT induction in either MSH or KCl buffer was analyzed as described in Support Protocol 4. The concentration of CCCP was 1 μM.

Incubation buffer for calcium-sensitive electrode

1.11 g/100 ml KCl (150 mM final)
13.1 mg/100 ml KH_2PO_4 (1 mM final)
60 mg/100 ml Tris (5 mM final)
135 mg/100 ml sodium succinate (5 mM final)
Adjust pH to 7.4 with HCl
Store up to 2 to 3 days at 4°C

Incubation buffer for digitonin-permeabilized cells

150 mM KCl
5 mM KH_2PO_4
1 mM $MgCl_2$
5 mM sodium succinate
5 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Store up to 2 to 3 days at 4°C

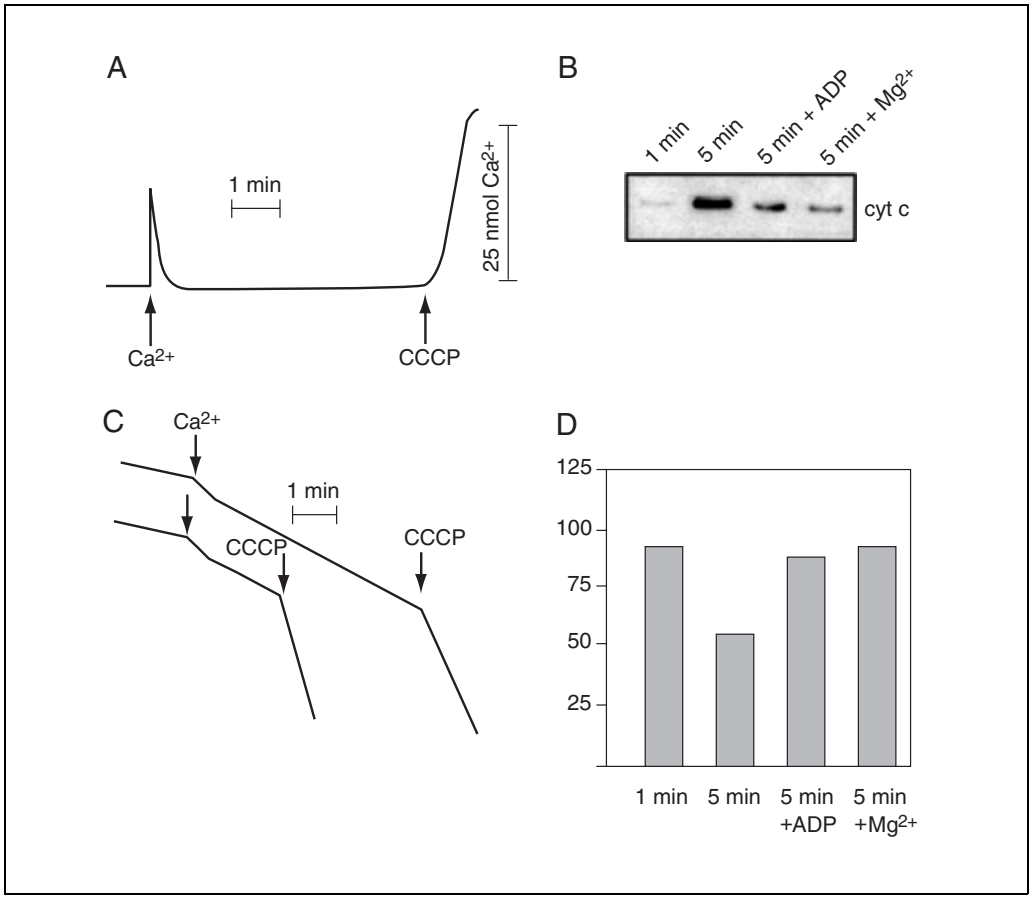


Figure 18.5.2 Ca^{2+}-induced release of cytochrome *c* from mitochondria in the absence of observable MPT. (**A**) Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 2-min stabilization period, mitochondria were loaded with Ca^{2+} (25 nmol/mg protein) prior to the addition of 1 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) at 5 min. (**B**) The amount of cytochrome *c* released from mitochondria after 1 and 5 min of Ca^{2+} retention in the presence and absence of 0.5 mM ADP or 1 mM Mg^{2+}. (**C**) Samples incubated under the same conditions as (A) were used to evaluate the rate of uncoupled respiration after 1 and 5 min. (**D**) Samples incubated under the same conditions as conditions as (B) were used to determine the effect of inhibitors of MPT on the rate of uncoupled respiration of Ca^{2+}-loaded mitochondria.

Incubation buffer for swelling

1.11 g/100 ml KCl (150 mM final)
6.8 mg/100 ml KH_2PO_4 (0.5 mM final)
60 mg/100 ml Tris (free base; 5 mM final)
135 mg/100 ml sodium succinate (5 mM final)
Adjust pH to 7.4 with HCl
Store up to 2 to 3 days at 4°C

Incubation buffer for testing mitochondrial quality

1.11 g/100 ml KCl (150 mM final)
13.1 mg/100 ml KH_2PO_4 (1 mM final)
60 mg/100 ml Tris (free base; 5 mM final)
Adjust pH to 7.4 with HCl
Store up to 3 to 4 days at 4°C

Incubation buffer for TPP^+-sensitive electrode

1.11 g/100 ml KCl (150 mM final)
27 mg/100 ml KH_2PO_4 (2 mM final)
60 mg/100 ml Tris (free base; 5 mM final)
135 mg/100 ml sodium succinate (5 mM final)
Adjust pH to 7.4 with HCl
Store up to 2 to 3 days at 4°C

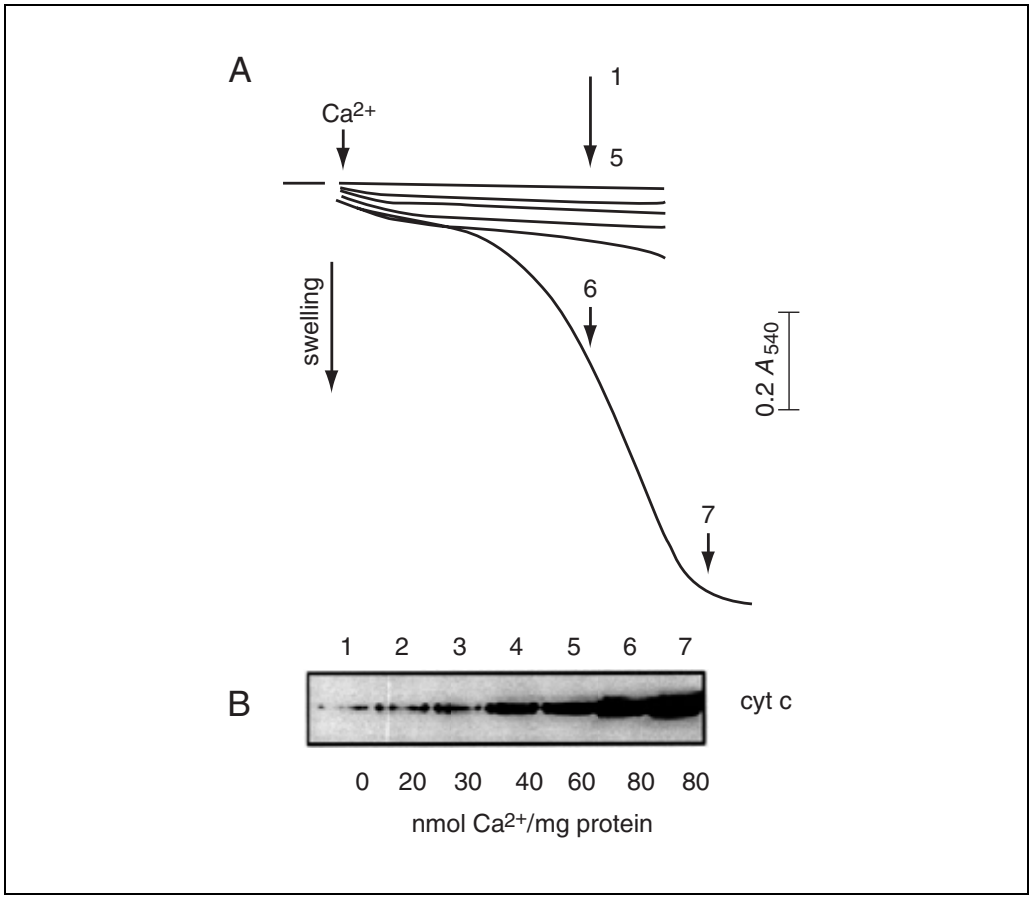


Figure 18.5.3 The effect of Ca^{2+} loading on mitochondrial swelling and the release of cytochrome *c*. Mitochondria (0.5 mg/ml) were incubated in 2 ml of KCl-based buffer. (**A**) Mitochondria were loaded sequentially with varied amounts of Ca^{2+} until MPT was induced. (**B**) Samples were taken after 5 min (lanes 1 to 6) or 8 min (lane 7) of incubation and the supernatants evaluated for cytochrome *c* content.

Laemmli's loading buffer, 4×

3.125 ml 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
4 ml glycerol
0.8 g sodium dodecyl sulfate
8 mg Bromphenol blue
0.8 ml 2-mercaptoethanol
H_2O to 10 ml
Store up to several weeks at 4°C

MSH buffer

210 mM mannitol
70 mM sucrose
5 mM HEPES
Adjust pH to 7.4 with KOH
Store up to 2 to 3 days at 4°C

S100 buffer

20 mM HEPES, pH 7.5
10 mM KCl
1.5 mM $MgCl_2$
1 mM EGTA
1 mM EDTA
0.1 mM PMSF
10 mg/ml leupeptin
5 mg/ml pepstatin A
2 mg/ml aprotinin
25 mg/ml calpain I inhibitor
1 mM DTT
Store at 4°C; prepare weekly

SET buffer

8.58 g/100 ml sucrose (0.25 M final)
0.5 ml of 100 mM potassium EGTA stock solution/100 ml (0.5 mM final)
120 mg/100 ml Tris (free base; 10 mM final)
Adjust pH to 7.4 with HCl

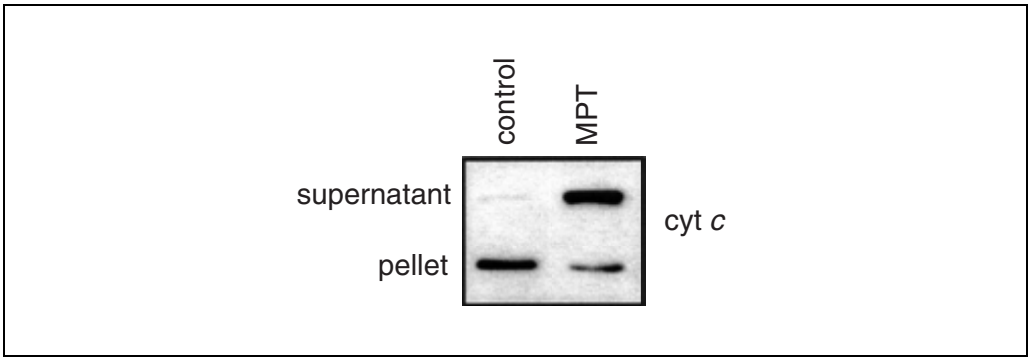


Figure 18.5.4 The release of cytochrome c from isolated rat liver mitochondria as a result of mitochondrial permeability transition (MPT). Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 2-min stabilization period, mitochondria were loaded with Ca^{2+}, and, when accumulation was complete, MPT was induced by 5 mM KH_2PO_4. A 200-µl aliquot of mitochondrial suspension was centrifuged and the resulting supernatant and pellet were separated by SDS-PAGE and immunoblotted as described under in Basic Protocol 3. Control mitochondria were incubated for the same time but without Ca^{2+} loading.

COMMENTARY

Background Information

Mitochondrial function in apoptotic cells

Attempts to identify a common, unifying step in the apoptotic program, in response to various cytotoxic stimuli, have focused on the role of mitochondrial participation in this form of cell death (Petit et al., 1997). Specifically, the release of several proteins, normally located in the intermembrane space of mitochondria, has been observed during the early stages of apoptotic cell death (Green and Reed, 1998). Among these proteins are cytochrome *c* and other intermembrane-space proteins, including AIF (apoptosis-inducing factor), HSPs (heat shock proteins), DIABLO/Smac (direct IAP-binding protein with low pI/Second mitochondria-derived activator of caspase), AK-2 (adenylate kinase-2), several pro-caspases (Mancini et al., 1998; Köhler et al., 1999; Samali et al., 1999a; Susin et al., 1999; Zhivotovsky et al., 1999; Du et al., 2000; Verhagen et al., 2000), endonuclease G, and sulfite oxidase. Although the role of many of these proteins in the apoptotic process is well characterized, the involvement of others (AK-2, sulfite oxidase) is still unclear. It is important to note that the function of the released proteins in the cytoplasm is different from their functions within mitochondria. The best-studied of these proteins is cytochrome *c*.

Cytochrome *c*, is a component of the electron transport chain that shuttles electrons between complexes III (bc1) and IV (cytochrome oxidase). Apo-cytochrome *c* is encoded by a nuclear gene, synthesized in the cytosol, and imported into the mitochondrial intermembrane space in an unfolded configuration where it receives its heme group. Covalent attachment of this heme group stimulates a conformational change, and holo-cytochrome *c* subsequently assumes its functional role as a component of the electron transport chain.

During the early phase of apoptosis, cytochrome *c* is released into the cytosol and interacts with Apaf-1 to form the apoptosome complex together with dATP and pro-caspase-9 (Li et al., 1997).

The mechanisms regulating cytochrome *c* release remain obscure. However, two distinct models for cytochrome *c* release have emerged, and these can be distinguished on the basis of whether Ca^{2+} is required for the event. In one instance, mitochondrial Ca^{2+} overload results in opening of a pore in the inner mitochondrial membrane, with subsequent swelling and rupture of the outer membrane followed by the release of cytochrome *c* and other intermembrane space proteins (Crompton, 1999). The Ca^{2+}-independent model asserts that a more selective protein release occurs without changes in mitochondrial volume. This mecha-

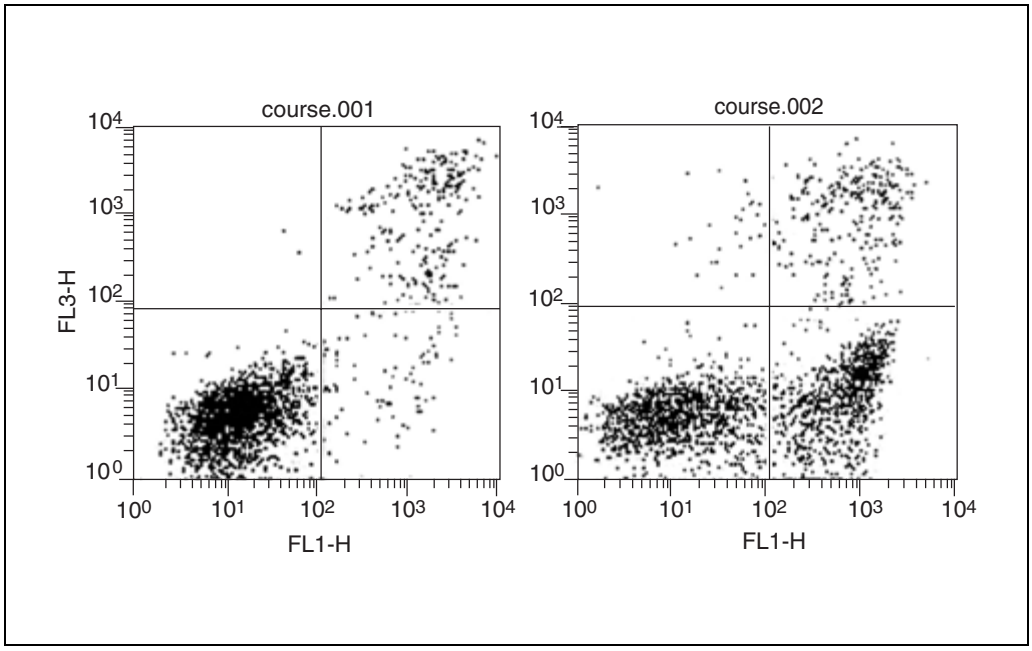


Figure 18.5.5 A typical image of FACS analysis of mitochondrial membrane potential in the cells undergoing apoptosis. Apoptosis was induced in Jurkat cells by 1 μM staurosporine and samples were analyzed 3 hr after initiation of apoptosis.

nism involves specific channels/pores in the outer mitochondrial membrane that may be opened and regulated by certain pro-apoptotic members of the Bcl-2 family of proteins, including Bax (Robertson and Orrenius, 2000). The precise manner in which Bax modulates cytochrome *c* release is controversial. In particular, the authors have reported recently that depending on the experimental conditions this protein can either act directly on mitochondria to stimulate the release of cytochrome *c* by forming a selective pore in the outer membrane, or it may facilitate opening of the permeability transition pore (Gogvadze et al., 2001). Recent evidence indicates that truncated Bid induces a conformational change in Bax that allows this protein to insert in the outer membrane, oligomerize, and stimulate cytochrome *c* release (Eskes et al., 2000).

Mitochondrial permeability transition

Energized mitochondria can take up and retain added Ca^{2+}. However this ability is not limitless. Mitochondria swell and become leaky; the membrane potential, which is the driving force for Ca^{2+} accumulation, drops, and accumulated Ca^{2+} is released. This process is called mitochondrial permeability transition (MPT). These changes are identified as a result of the opening of Ca^{2+}-dependent pore in the mitochondrial inner membrane.

MPT-dependent release of cytochrome c

Although the kinetics of MPT are unaffected by the composition of the incubation buffer (KCl versus MSH; Fig. 18.5.1A), the amount of cytochrome *c* released is much higher when mitochondria are incubated in the physiologically more relevant KCl buffer as compared to MSH buffer. Immunoblot analysis revealed that most cytochrome *c* was present in the supernatant fraction following MPT induction in KCl buffer, whereas only partial release of cytochrome *c* was observed in MSH buffer (Fig. 18.5.1B). Further evidence of this comes from the different effects that cytochrome *c* release has on mitochondrial respiration in the two buffers (Fig. 18.5.1C). Specifically, the induction of MPT in MSH buffer resulted in a time-dependent acceleration of oxygen consumption that persisted until all oxygen was consumed. In contrast, incubation of mitochondria in KCl buffer resulted in an initial acceleration of respiration that was followed by its suppression, and no additional stimulation of oxygen consumption was observed upon the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Taken together, these data indicate that while MPT-induced uncoupling of mitochondria occurs in both buffers, respiration is suppressed only in KCl buffer, an effect most likely due to a loss of cytochrome *c*.

It should be taken into account that mitochondrial preparations are heterogeneous. At a given concentration of Ca^{2+}, a certain subpopu-

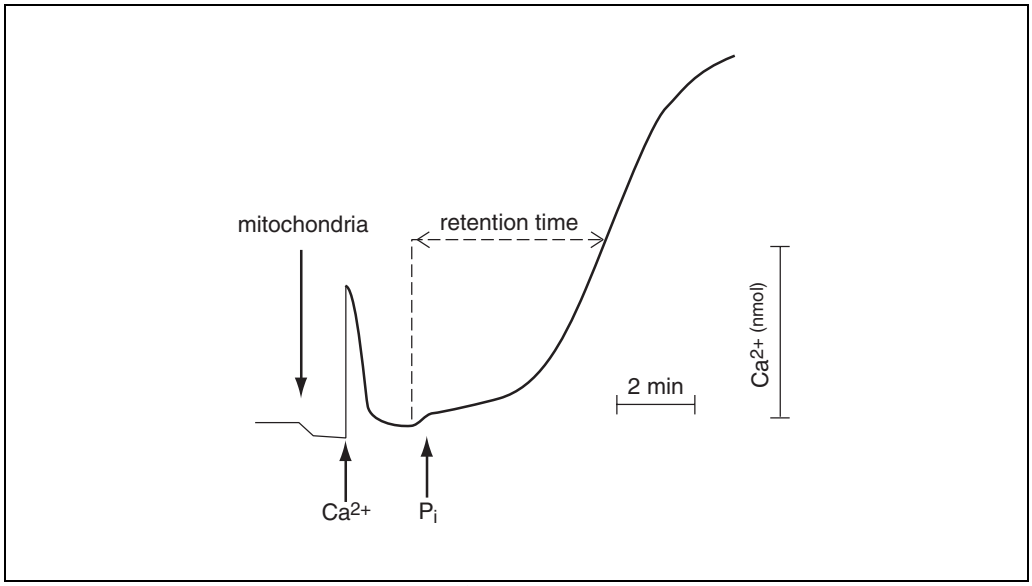


Figure 18.5.6 MPT monitored with a Ca^{2+}-sensitive electrode. Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 1-min stabilization period, mitochondria were loaded with Ca^{2+}, and, when accumulation was complete, MPT was induced by 5 mM KH_2PO_4.

lation (more susceptible) of mitochondria have undergone permeability transition at a given time, while the released Ca^{2+} is accumulated by more resistant mitochondria, which thereby increase their load until they also undergo permeabilization (Zoratti and Szabo, 1995).

In the aforementioned protocols, cytochrome *c* release was a consequence of MPT induction, accompanied by swelling of mitochondria and rupture of the outer membrane. When Ca^{2+} loading is insufficient to induce observable manifestations of MPT, the release of cytochrome *c* can still occur despite the absence of changes characteristic of MPT.

As seen in Figure 18.5.2A, moderate Ca^{2+} loading of mitochondria did not induce MPT, and this cation was retained by mitochondria unless the uncoupler CCCP was added. The accumulation of Ca^{2+} by mitochondria in the absence of observable MPT was nevertheless sufficient to stimulate a release of cytochrome *c*, an effect that was considerably more pronounced the longer mitochondria retained the accumulated Ca^{2+}. Inhibitors of MPT, such as Mg^{2+} and ADP, suppressed this release (Fig. 18.5.2B). In addition, Ca^{2+}-loaded mitochondria exhibited controlled respiration (Fig. 18.5.2C), although increasing the Ca^{2+} retention time from 1 to 5 min prominently diminished the rate of CCCP-directed uncoupled respiration (upper trace versus versus lower trace). Inhibitors of MPT, such as Mg^{2+} and ADP, suppressed this release (Fig. 18.5.2D) and restored the rate of uncoupled respiration.

Additional evidence of cytochrome *c* release in the absence of observable MPT is presented in Figure 18.5.3A, where the sequential addition of Ca^{2+} pulses to isolated mitochondria led to a step-wise decrease in the overall optical density without the large-amplitude swelling characteristic of MPT. Meanwhile, the amount of cytochrome *c* released from mitochondria was enhanced as Ca^{2+} loading increased (Fig. 18.5.3B). This release reflected MPT induction in a subpopulation of mitochondria, which was further supported by the fact that cotreatment of isolated mitochondria with 1 μM CsA (cyclosporin A) eliminated cytochrome *c* release induced by Ca^{2+} additions ranging between 20 to 80 nmol/mg protein (data not shown). Cytochrome *c* release ultimately reached a pinnacle at 80 nmol Ca^{2+}/mg protein (Fig. 18.5.3B, lanes 6 and 7) as all mitochondria underwent MPT (Fig. 18.5.3A).

MPT-independent release of cytochrome c

Although it was originally believed that MPT induction was the root mechanism responsible for cytochrome *c* release in response to different cytotoxic stimuli, more recently this notion has been challenged and the precise mechanisms regulating the release of this pro-

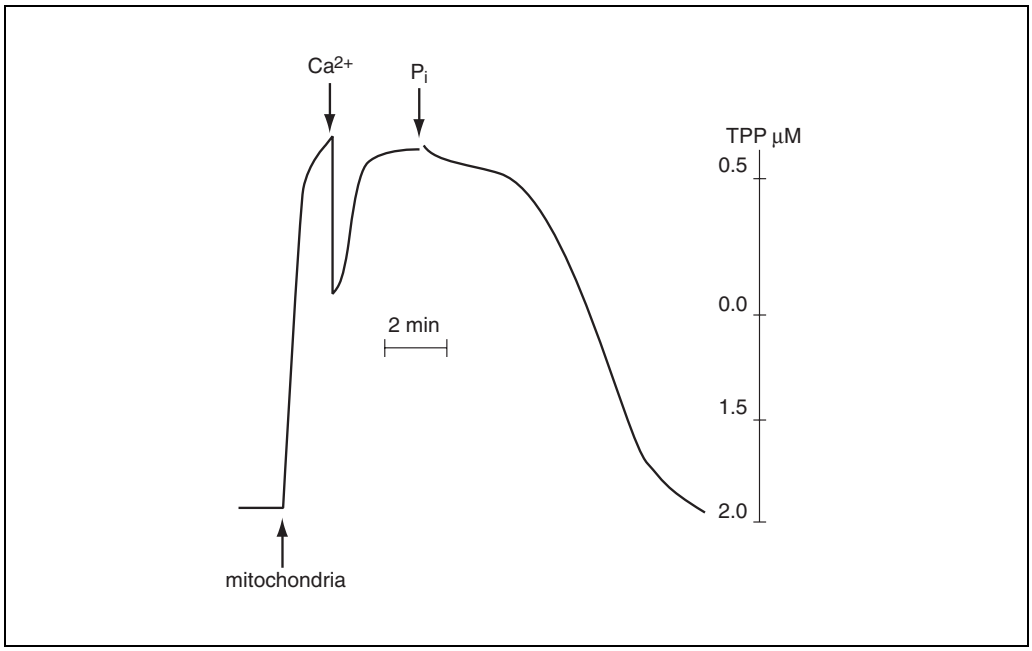


Figure 18.5.7 MPT monitored with a Δψ-sensitive electrode. Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 1-min stabilization period mitochondria were loaded with Ca^{2+}, and, when accumulation was complete and potential was restored, MPT was induced by 5 mM KH_2PO_4. The concentration of TPP^+ was 2 μM.

tein are unclear. In fact, many of the early results on mechanisms of cytochrome *c* release were generated using cell-free systems wherein isolated mitochondria and nuclei were treated with different MPT pore activators, which, in turn, led to mitochondrial swelling, the release of cytochrome *c* (and other proteins), and subsequent changes in nuclear morphology that were characteristic of apoptosis. However, ample evidence from more recent studies suggests that, while MPT is likely to be a mechanism responsible for cytochrome *c* release, it is no longer regarded as *the* mechanism.

In case of MPT induction, mitochondrial swelling leads to a rupture of the outer membrane; without MPT, permeabilization can be achieved by Bcl-2 family proteins Bax or Bid that form pores in the outer membrane, leaving the inner mitochondrial membrane intact.

Discrimination between MPT-dependent and MPT-independent release of cytochrome c

While analyzing the release of cytochrome *c* from mitochondria, one should keep in mind that even without added Ca^{2+}, MPT may occur in a subpopulation of mitochondria if the trace amount of Ca^{2+} in buffers is enough to induce MPT in "susceptible" mitochondria. Therefore the release of cytochrome *c* observed in the absence of added Ca^{2+} can be mistaken for MPT-independent release of cytochrome *c*. In order to distinguish between the MPT-dependent and -independent release of cytochrome *c*, experiments should be performed in the presence of the Ca^{2+} chelator EGTA, which sequesters Ca^{2+} and therefore prevents MPT induction. Similar results can be achieved with CsA.

Critical Parameters and Troubleshooting

Collecting released proteins (Basic Protocols 1 and 2)

Separation of the mitochondria from the supernatant should be done thoroughly. Aliquots of supernatant should be taken without disturbing the pellet.

Immunoblot analysis of released proteins (Basic Protocol 3)

For immunoblot analysis, too many bands on the membrane may indicate too high a concentration of primary and/or secondary antibodies. If the background of nonspecific binding of immunological probes is unacceptably high, increase the length of the time of washing with PBS. If there is no band on the film, increase the duration of incubation with primary antibodies. If a white spot on the film makes it difficult to detect the protein band of interest, a bubble may have been introduced during blotting and the whole procedure must be repeated. If a band on the film is too strong, decrease exposure time of X-ray film to the ECL-stained membrane. If the gel runs too fast

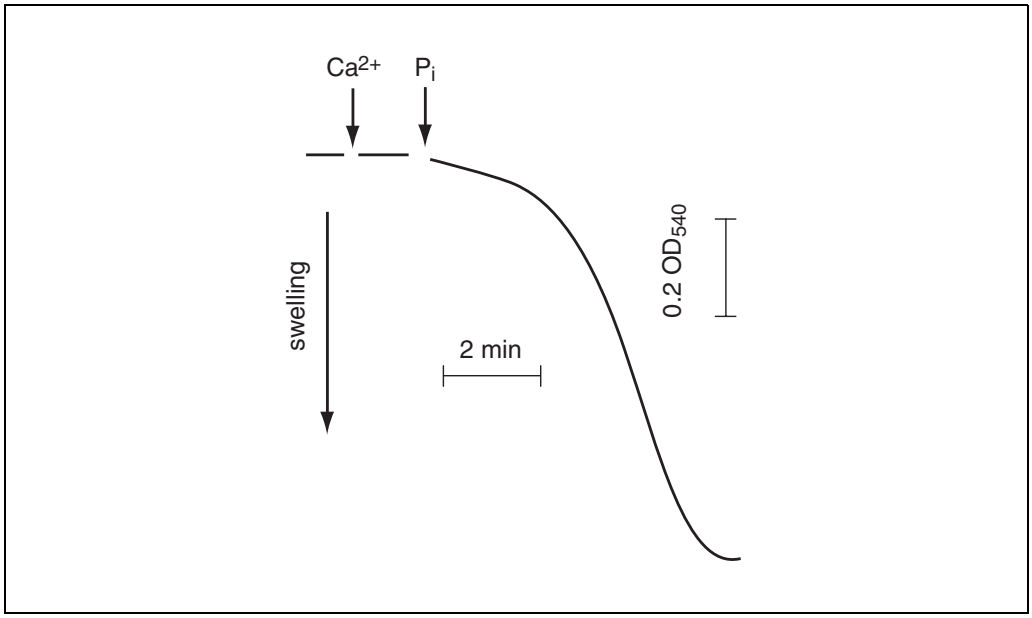


Figure 18.5.8 MPT assessed by mitochondrial swelling. Mitochondria (0.5 mg/ml) were incubated in 2 ml of KCl-based buffer. Mitochondria were loaded with Ca^{2+} and 5 mM phosphate was added. Swelling was monitored by a decrease of optical density at 540 nm.

or too slow and poor resolution of protein occurs, decrease or increase voltage, respectively, or check the buffer recipe. If no prestained markers appear on the nitrocellulose filter, check the transfer buffer recipe, or reposition the location of gel towards the cathode and anode.

FACS analysis of TMRE-stained cells (Basic Protocol 4)

FACS analysis needs an appropriate gating of the cells—a selection of the population of interest, especially if the initial population is a mixture of different types of cells (for example when blood samples are analyzed).

Another critical parameter is time. The analysis of the membrane potential should be done shortly after staining of the cells. TMRE is only accumulated by mitochondria with high membrane potential and any delay in analysis may negatively affect the functional state of the mitochondria and therefore cause leakage of the dye.

Microscopy of apoptotic cells stained with MitoTracker Red (Basic Protocol 5)

To reduce potential artifacts from overloading, the concentration of dye should be kept as low as possible. However if cells are not sufficiently stained, the concentration of dye or time of incubation should be increased.

Measurement of calcium release and membrane potential (Basic Protocols 6 and 7)

The release of Ca^{2+} from mitochondria may result not from opening of the pore, but simply because of a drop of the membrane potential. This may happen, for instance, if the mitochondria consume all available oxygen. However at a mitochondrial protein concentration of 1 mg/ml (or less), and with rapid stirring of the suspension, MPT usually occurs before the exhaustion of oxygen. If higher concentrations of mitochondrial suspension are used, possible anaerobiosis can be prevented by fine flow of oxygen blown onto the surface of the suspension.

Mitochondrial swelling (Basic Protocol 8)

Mitochondrial swelling assessed by a decrease in optical density of mitochondrial suspension represents one of the most reliable parameters of MPT induction in isolated mitochondria. However it should be kept in mind that anaerobiosis and subsequent release of accumulated Ca^{2+} due to the drop in membrane potential may prevent pore opening and terminate swelling. Anaerobiosis can be prevented by a fine flow of oxygen blown onto the surface of the suspension. On the other hand, a relatively low concentration of mitochondria (up to 1 mg/ml) should not lead to anaerobiosis.

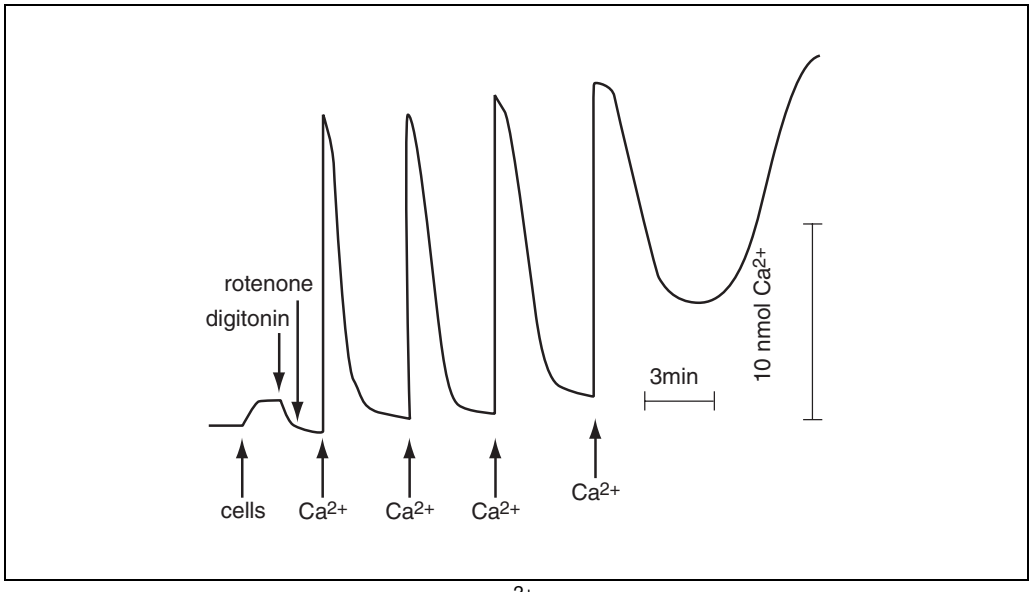


Figure 18.5.9 Estimation of mitochondrial Ca^{2+}accumulation in digitonin-permeabilized cells. Jurkat cells (2.5×10^6) were washed in PBS, resuspended in 500 µl of KCl-based buffer, and added to the incubation chamber. Following a 2-min stabilization period, cells were permeabilized with 0.005% digitonin, and 5 µM rotenone was added in order to maintain pyridine nucleotides in a reduced form. MPT was induced by sequential additions of $Ca^{2+}$and changes in the level of this cation were monitored using a Ca^{2+}-selective electrode.

Estimation of mitochondrial Ca^{2+} accumulation in digitonin-permeabilized cells (Basic Protocol 9)

Digitonin generally permeabilizes plasma membrane, which contains cholesterol; therefore, this detergent should be used with caution because relatively high concentrations of digitonin can affect also the outer mitochondrial membrane. The minimal concentration of digitonin that is necessary for the outer-membrane permeabilization can be detected with a Ca^{2+}-sensitive electrode. Cells do not accumulate added Ca^{2+}, since plasma membrane is permeable neither to Ca^{2+} nor to succinate, oxidation of which provide a driving force for Ca^{2+} accumulation. Upon addition of digitonin, the plasma membrane becomes permeable and the mitochondria start to accumulate Ca^{2+} from the incubation buffer.

Preparation of mitochondria (Support Protocols 1, 2, and 3)

The temperature during isolation should be kept around 4°C. Elevation of the temperature may result in activation of catabolic enzymes, in particular, mitochondrial phospholipase A_2, which leads to accumulation of free fatty acids in mitochondria. Free fatty acids are known as natural protonophores (agents that facilitate transport of protons through membrane); therefore, accumulation of fatty acids will decrease the mitochondrial membrane potential and destabilize mitochondria.

Anticipated Results

Analyzing functioning of isolated mitochondria and mitochondria in apoptotic cells: collection of released proteins and immunoblot analysis (Basic Protocols 1, 2, and 3)

Depending on the mechanisms involved in the release of cytochrome *c* or other proteins from mitochondria, the final result will be different. If the release was associated with mitochondrial swelling and the rupture of the outer membrane, most of the cytochrome *c* will be found in the supernatant after sedimentation of mitochondria. In such a situation, it would be informative also to present the data concerning changes in the content of cytochrome *c* in the pellet. Figure 18.5.4 represents a typical blot showing the release of cytochrome *c* from isolated liver mitochondria upon induction of MPT in KCl-based buffer.

FACS analysis of TMRE-stained cells (Basic Protocol 4)

A typical image of FACS analysis of mitochondria in the cells undergoing apoptosis is present in Figure 18.5.5. The lower left quadrant contains living cells, the lower right, apoptotic cells, and upper right, necrotic cells.

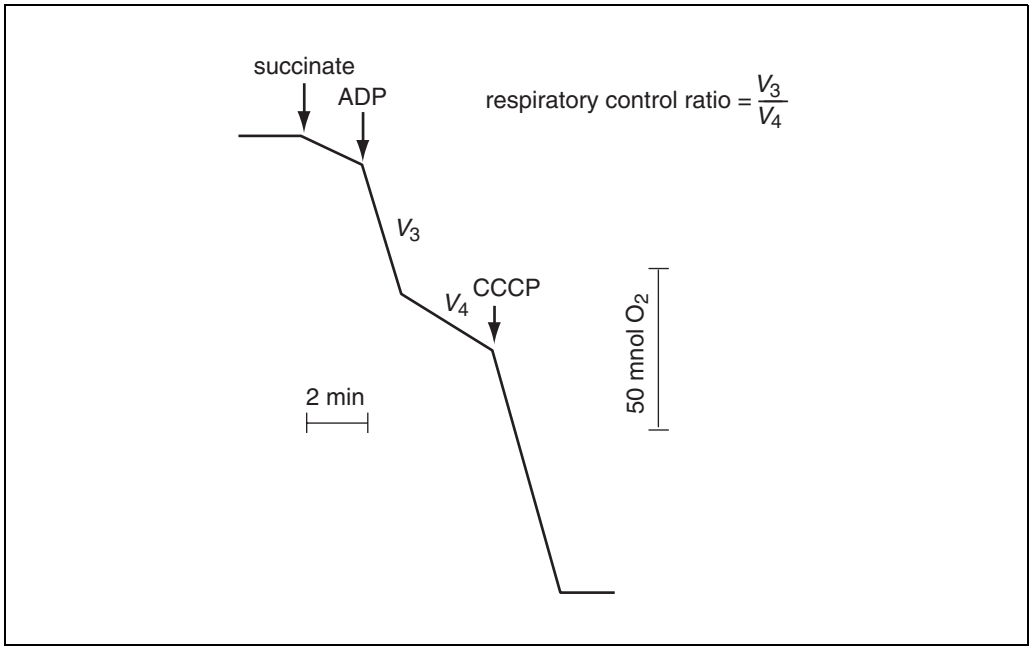


Figure 18.5.10 Estimation of mitochondrial respiration as described in Support Protocol 4.

Measurement of calcium release (Basic Protocol 6)

A typical response of mitochondria to Ca^{2+} is shown in Figure 18.5.6. Addition of Ca^{2+} to isolated mitochondria results in a quick rise in the basal level of Ca^{2+} in the incubation buffer, followed by restoration of the initial level due to accumulation of Ca^{2+} in mitochondria, whereupon a new steady state is reached. After addition of phosphate, mitochondria start releasing accumulated Ca^{2+}. The lag time before the onset of MPT induction, as well as the rate of release, depend on the Ca^{2+} load. If Ca^{2+} and other conditions are held constant, the effect of phosphate on MPT induction is dose-dependent. Therefore, the opening of the pore can be modulated either by varying the Ca^{2+} concentration while keeping the concentration of phosphate constant, or by varying the concentration of phosphate.

Both methods (spectrophotometric and ion-selective electrodes) are equally sensitive, and the choice is dictated by the availability of the equipment.

Measurement of membrane potential (Basic Protocol 7)

Energized mitochondria rapidly accumulate TPP^+ from the incubation buffer and release this cation as $\Delta\psi$ decays. A typical change in mitochondrial membrane potential upon MPT induction is shown on Figure 18.5.7. As can be seen in that figure, upon induction of MPT, two distinct phases of decrease in membrane potential occur. First, a relatively slow decrease starts when an MPT-inducing agent (P_i) is added, which is followed by a second, rapid phase, which reflects the induction of MPT in the whole population of mitochondria.

Measurement of mitochondrial swelling (Basic Protocol 8)

Figure 18.5.8 shows a typical example of results from measurement of mitochondrial swelling. When mitochondria are added to incubation buffer at a concentration of 0.5 mg/ml, the initial light absorption at 540 nm is about 1.3 to 1.4. Addition of Ca^{2+} to the mitochondria, followed by inorganic phosphate, results in a time-dependent decrease in the optical density (OD) of the mitochondrial suspension.

Estimation of mitochondrial Ca^{2+} accumulation in digitonin-permeabilized cells (Basic Protocol 9)

The addition of Ca^{2+} to a permeabilized cell suspension leads to a rapid increase in the level of this cation in the reaction buffer, followed by a return to the initial level as mitochondria accumulate the excess Ca^{2+} (Fig. 18.5.9); this is an effect that can be completely abrogated by 2 μg/ml antimycin, an inhibitor of mitochondrial respiratory chain (data not shown). Mitochondria accumulate sequential additions of Ca^{2+} until MPT is induced and the accumulated Ca^{2+} is released.

Preparation of mitochondria and assessing their quality (Support Protocols 1 to 4)

Isolation of mitochondria usually takes about 60 to 75 min. For liver the yield is about 20 to 25 mg protein of mitochondria per 1 g of tissue. High concentration of the final mitochondrial preparation is a prerequisite of mitochondrial stability.

A typical respiratory response of mitochondria and the method of calculating RCR are shown in Figure 18.5.10. Addition of CCCP when added ADP is phosphorylated and state 4 respiration is stabilized allows one to determine the rate of uncoupled respiration that represents the activity of the mitochondrial respiratory chain.

Time Considerations

The time of collecting samples and preparing for immunoblotting depends on the incubation conditions as well as the experience of a researcher. Basic Protocol 1 requires 45 to 60 min, Basic Protocol 2, 2 to 2.5 hr, and Basic Protocol 3, 2 days. TMRE (Basic Protocol 4) and MitoTracker Red (Basic Protocol 5) staining takes 1 hr and 1.5 hr, respectively. Measurement of the mitochondrial permeability transition (Basic Protocols 6, 7, 8, and 9 and Alternate Protocols 1 and 2) can be completed in 5 to 20 min.

Mitochondrial preparation (Support Protocols 1, 2, and 3) takes 60 to 80 min. Assessment of respiratory control ratio takes 20 to 35 min.

Literature Cited

Bossy-Wetzel E., Newmeyer, D.D., and Green, D.R. 1998. Mitochondrial cytochrome *c* release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J.* 17:37-49.

Crompton, M. 1999. The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* 341:233-249.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. 2000. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by elimination IAP inhibition. *Cell* 102:33-42.

Eskes, R., Desagher, S., Antonsson, B., and Martinou, J.C. 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol. Cell Biol.* 20:929-935.

Gogvadze, V., Robertson, J.D., Zhibotovsky, B., and Orrenius, S. 2001. Cytochrome c release occurs via Ca^{2+}-dependent and Ca^{2+}-independent mechanisms that are regulated by Bax. *J. Biol. Chem.* 276:19066-19071.

Green D.R. and Reed, J.C. 1998. Mitochondria and apoptosis. *Science* 281:1309-1312.

Kamo, N., Muratsugu, M., Hongoh, R., and Kobatake, Y. 1979. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J. Membr. Biol.* 49:105-121.

Köhler, C., Gahm, A., Noma, T., Nakazawa, A., Orrenius, S., and Zhivotovsky, B. 1999. Release of adenylate kinase 2 from the mitochondrial intermembrane space during apoptosis. *FEBS Lett.* 447:10-12.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489.

Mancini, M., Nicholson, D.W., Roy, S., Thornberry, N.A., Peterson, E.P., Casciola-Rosen, L.A., and Rosen, A. 1998. The caspase-3 precursor has a cytosolic and mitochondrial distribution: Implications for apoptotic signaling. *J. Cell Biol.* 140:1485-1495.

Mitchell, P. and Moyle, J. 1967. Chemiosmotic hypothesis of oxidative phosphorylation. *Nature* 213:137-139.

Petit, P.X., Zamzami, N., Vayssiere, J.L., Mignotte, B., Kroemer, G., and Castedo, M. 1997. Implication of mitochondria in apoptosis. *Mol. Cell. Biochem.* 174:185-188.

Robertson, J.D. and Orrenius, S. 2000. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. *Crit. Rev. Toxicol.* 30:609-627.

Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P., and Orrenius, S. 1999. Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. *EMBO J.* 19:2040-2048.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M.C., Alzari, P.M., and Kroemer, G. 1999. Mitochondrial release of caspase-2 and-9 during the apoptotic process. *J. Exp. Med.* 189:381-394.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J., and Vaux, D.L. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102:43-53.

Zamzami, N., Susin, S.A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. 1996. Mitochondrial control of nuclear apoptosis. *J. Exp. Med.* 183:1533-1544.

Zhivotovsky, B., Samali, A., Gahm, A., and Orrenius, S. 1999. Caspases: Their intracellular localization and translocation during apoptosis. *Cell Death Differ.* 6:644-651.

Zoratti M. and Szabo, I. 1995. The mitochondrial permeability transition. *Biochem. Biophys. Acta.* 121:139-176.

Contributed by Vladimir Gogvadze,
Sten Orrenius, and Boris Zhivotovsky
Institute of Environmental Medicine
Karolinska Institutet
Stockholm, Sweden

Analysis of Telomeres and Telomerase

This unit describes techniques to analyze telomeric length (Basic Protocol 1) and telomerase activity (Basic Protocol 2) in human cells. These assays can be used to study the in vitro cellular effects of aging and cancer treatments on telomere biology and telomerase activity.

Telomere length can be determined by a modification of Southern blotting in which the analysis of chromosome terminal restriction fragments (TRFs), as visualized with a radiolabeled telomeric repeat probe (Support Protocols 2 and 3), provides the average lengths of all telomeres in a cell population. A labeled molecular weight marker (Support Protocol 1) is used to determine telomere length.

Telomerase activity can be measured in vitro by a sensitive and efficient polymerase chain reaction (PCR)–based detection method, also known as telomeric repeat amplification protocol (TRAP). This method can use either radiolabeled or fluorescently labeled (Alternate Protocol) primers. Support Protocol 4 describes the preparation of tissue samples for TRAP; the preparation of other reagents that are needed for TRAP are described in Support Protocols 5 and 6.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiments and dispose of wastes in appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

TERMINAL RESTRICTION FRAGMENT SIZE DETERMINATION TO MEASURE AVERAGE TELOMERE LENGTHS

BASIC PROTOCOL 1

The simplified method for isolating DNA described below was developed in the authors' laboratory to efficiently process a large number of samples by eliminating phenol/chloroform extractions and avoiding alcohol precipitation. Because there are no steps where significant losses can occur, DNA concentrations can be calculated based on initial cell numbers, a more reproducible method than optical density measurements, which are frequently distorted by contaminating RNA. The measurement of telomere length relies on the absence of restriction enzyme recognition sites within the TTAGGG tandem repeat sequences. The length of telomeric repeats can be indirectly measured by analyzing the length of the terminal restriction fragment (TRF), the large DNA fragments remaining after the rest of the genomic DNA has been reduced to small sizes by digestion with frequent cutters (restriction enzymes with recognition sequences of four bases, such as *Hin*fI). There is also a region of mostly repetitive subtelomeric DNA, probably ranging from 2 to 5 kb in length in humans, that lacks functional restriction sites and contributes to the measured size of telomeres on gels. The DNA is probed with a labeled oligonucleotide containing TTAGGG repeats. The measured size of the TRF corresponds to the sum of the sizes of the subtelomeric DNA that lacks functional restriction sites and the length of the telomere repeats. Because small oligonucleotides easily penetrate dried agarose gels, it is more rapid and convenient to perform in-gel hybridization rather than Southern blot transfers.

Materials

Cell pellet from ≥300,000 cells and appropriate medium
Quick-prep lysis buffer (see recipe)
Triton X-100
20 mg/ml proteinase K

Contributed by Brittney-Shea Herbert, Jerry W. Shay, and Woodring E. Wright
Current Protocols in Cell Biology (2003) 18.6.1-18.6.20

TE buffer, pH 7.5 to 8.0 (*APPENDIX 2A*)

Restriction enzyme mix: equal volumes of *Hin*fI, *Rsa*I, *Msp*I, *Cfo*I, *Hae*III, and *Alu*I restriction enzymes (Boehringer Mannheim; 10 U/µl each before mixing, final 1.67 U/µl each)

10× TAE buffer (*APPENDIX 2A*)

10× TBE buffer (*APPENDIX 2A*)

10,000 cpm radiolabeled molecular weight markers mixed with ≥500 ng unlabeled, *Sty*I-digested λ DNA (or other appropriate unlabeled, digested DNA; see Support Protocol 1)

Denaturing solution: 0.5 M NaOH/1.5 M NaCl

Neutralizing solution: 1.5 M NaCl/0.5 M Tris·Cl, pH 8.0 (see *APPENDIX 2A* for Tris·Cl)

Hybridization solution (see recipe)

Radiolabeled telomeric repeat probe (see Support Protocols 2 and 3)

20× SSC (see recipe)

0.1× SSC/0.1% (w/v) SDS

2-ml polypropylene screw-cap tubes (Sarstedt) with an optional extra set of screw caps to be cut vertically with jigsaw to form open screw-cap ring (Fig. 18.6.1A)

37°, 55°, and 70°C water baths heating blocks, or PCR machine

Dialysis membrane sheets or tubing (e.g., Spectra/Por; Spectrum), molecular weight cutoff (MWCO) ≥6000 to 8000

Wide-bore pipet tips

Whatman 3MM filter paper

Hybridization oven, 42°C, and appropriate hybridization containers, or equivalent

PhosphorImager, including phosphor screens and ImageQuant software (Molecular Dynamics)

Additional reagents and equipment for agarose gel electrophoresis (*APPENDIX 3A*)

Lyse cells

1. Resuspend cell pellet from ≥300,000 trypsinized cells in medium and pellet cells in a 2-ml polypropylene screw-cap tube. Remove supernatant and freeze pellet at −80°C or proceed to next step.

   It is not necessary to wash cells with phosphate-buffered saline (PBS), as the small amount of medium in the cell pellet does not interfere with subsequent steps. At least 300,000 cells should be used; however, the collection of more cells is desirable (~3 million).

   Cell pellets can simply be placed at −80°C; it is not necessary to snap-freeze them.

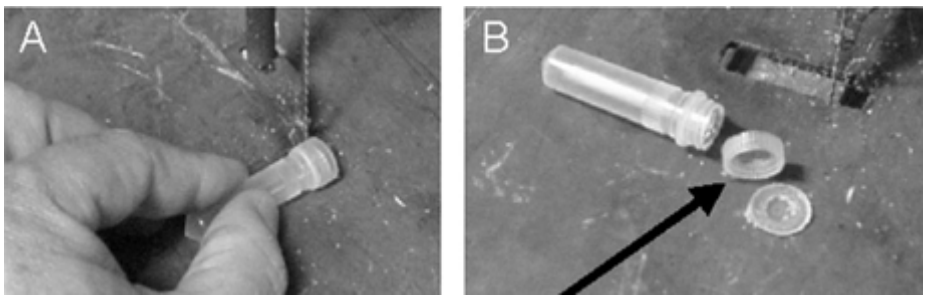


Figure 18.6.1 Construction of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1). Large numbers of these open-ended caps can easily be prepared as follows. (**A**) Screw the caps onto a tube so that the tube functions as a handle and cut off the end of the cap using a jigsaw. (**B**) The threads of the cap are attached as a ring on the tube (arrow). These rings are reusable as tube closures.

2. Thoroughly resuspend cell pellet (fresh or just after thawing from −80°C) in quick-prep lysis buffer, using 30 µl per million cells.

   100 mM EDTA is used in the lysis buffer to very rapidly inhibit nucleases in these concentrated cell suspensions. The lysis buffer should be added immediately to cells as they are thawing on ice to prevent nucleases from degrading the DNA.

3. Add Triton X-100 to 1% (v/v) final concentration and 20 mg/ml proteinase K to 2 mg/ml final concentration.

   Triton X-100 rather than SDS is used so that residual detergent present after dialysis does not inhibit digestion with restriction enzymes.

4. Incubate samples ≥2 hr at 55°C and inactivate proteinase K by heating 30 min at 70°C.

   Heat inactivation of proteinase K avoids the need to phenol/chloroform extract the DNA, and the residual protein present after dialysis does not inhibit enzyme digestion.

   After digestion with proteinase K, DNA should only be handled using wide-bore tips to prevent shearing of the DNA.

Dialyze sample

5a. *To dialyze sample in screw-cap tube:* Place a 1- to 2-cm^2 piece of dialysis membrane over top of tube and secure with an open screw-cap ring. Invert tube, making sure that sample (and not a bubble) is at the membrane, and place on floating rack in ≥200 vol TE buffer (Fig. 18.6.2). Dialyze 1 to 2 hr at 4°C with constant stirring.

   For larger volumes (>0.3 ml for >10 million cells), conventional dialysis (step 5b) can be used.

   Dialysis reduces salt and EDTA so that digests will not be inhibited later on.

5b. *To dialyze sample in dialysis tubing:* Wet dialysis tubing with distilled water and clip one end shut. With a wide-bore pipet tip, carefully collect sample and place in tubing without creating bubbles. Smooth any bubbles out and clip the other end shut, leaving room for buffer exchange and volume expansion. Place tubing in ≥200 vol TE buffer and dialyze 1 to 2 hr at 4°C with constant stirring.

   Dialysis reduces salt and EDTA so that digests will not be inhibited later on.

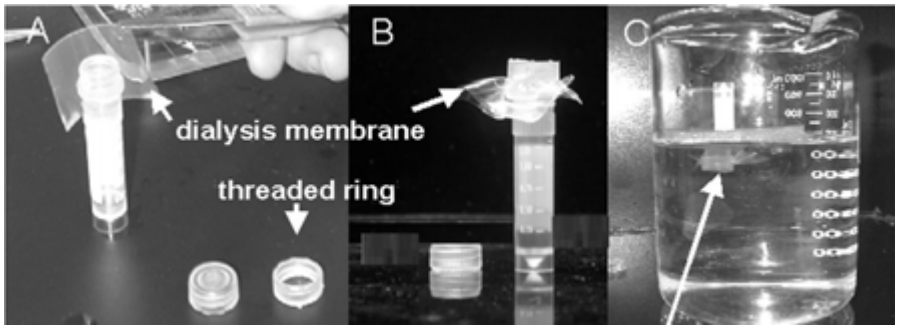


Figure 18.6.2 Assembly of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1). (**A**) A wet 1- to 2-cm^2 piece of dialysis membrane is placed on the tube containing the sample (save the original screw cap). (**B**) An open-end screw cap (threaded rings) is carefully threaded on the tube over the membrane without tearing. (**C**) The tube (arrow) is inverted and placed in a floating rack in 1× TE buffer. There should be no droplets remaining on the walls of the tube and no bubbles on the membrane when inverted, as this will prevent dialysis.

6. Change buffer to ≥200 vol fresh TE buffer and dialyze samples overnight at 4°C with constant stirring.

7. Discard dialysis membrane, replace threaded ring with a closed cap, and save threaded rings for re-use. Alternatively, remove sample from dialysis tubing and place in a fresh tube. Calculate the DNA concentration.

   DNA concentration is calculated at 6 μg/10^6 diploid cells or 10 μg/10^6 aneuploid cells divided by the final volume after dialysis. The samples should be viscous and they can now be stored for 1 year at 4°C (or for ≥1 year at −20°C) until they are digested.

Digest and electrophorese DNA

8. Digest 1 μg DNA to completion for 4 hr to overnight at 37°C with ≥1 U/μg restriction enzyme mix.

   The six enzymes ensure that genomic DNA, but not telomeric DNA, is digested. The authors generally use 1 μl enzyme mix/1 μg DNA (1.67 U each enzyme/μl) with 1× buffer A (supplied with the enzyme; Boehringer Mannheim).

9. Separate digested DNA on a 0.7% (w/v) agarose gel stained with ethidium bromide (0.5 μg/ml) for 16 hr at 70 V for a 25-cm-long gel in 1× TAE or 0.5× TBE buffer. Load 20,000 cpm radiolabeled molecular weight markers mixed with ≥250 ng unlabeled, *Sty*I-digested λ DNA on either side of the samples.

   0.5× TBE can be used to analyze telomeres less than ~8 kb, whereas 1× TAE buffer allows for better separation in the 8- to 20-kb range. The gel should be handled carefully as it may be fragile. Electrophoresis should be stopped temporarily before the <1-kb molecular weight markers migrate off the gel to document complete digestion of the DNA by photographing the gel. Actual time and voltage varies with each power supply and gel apparatus. Digested DNA will run as a smear below the 1-kb molecular weight marker. Anything above this size is incompletely digested. If the sample is not digested completely, it is usually sufficient to use an additional 1 μl enzyme mix for a longer amount of time. If the sample is completely undigested, the viscous sample may have floated up out of the well when the gel was loaded. Electrophoresis (at higher voltage) should be continued until the 1- to 1.5-kb marker is near the bottom of the gel.

   Radiolabeled molecular weight markers will allow the markers to be seen after hybridization with the telomere sequence–specific probe. The unlabeled, digested λ DNA is included so the markers can be visualized with ethidium bromide after electrophoresis.

Hybridize DNA

10. Denature gel 20 min in enough denaturing solution to cover the gel, rinse 10 min in distilled water, and dry gel upside down (so that the openings of the wells are against the paper) on two sheets of Whatman 3MM filter paper under vacuum for 1 hr at 50°C.

    Denaturing the DNA before rather than after drying the gel increases the signal intensity ~3- to 5-fold, presumably by permitting a much greater diffusion of the denatured DNA strands in the 0.7% agarose gel than in the very high percentage dried and rehydrated gel, and thus inhibiting the reannealing of the parental strands, which would compete with the probe.

    Rinsing with distilled water gets rid of the NaOH and substantially reduces sticking of the gel to the Whatman paper. Some loss of lower-molecular-weight DNA (particularly <400 bp) occurs during drying.

    Because most of the DNA samples do not fill the wells and are thus in the bottom half of the gel, the gels are flipped and dried with the upper surface against the filter paper support. This increases the distance between the DNA samples and the filter paper, and significantly reduces the loss of lower-molecular-weight DNA.

11. Neutralize gel 10 to 15 min in enough neutralizing solution to cover the gel, rinse 10 min in distilled water.

12. Prehybridize gel ≥10 min with 10 ml hybridization solution.

13. Hybridize gel overnight at 42°C in hybridization solution containing all of the radiolabeled telomeric repeat probe made in Support Protocol 2.

    The volume of the hybridization solution used depends on the gel size and hybridization setup. For a 20 × 25–cm gel rotating inside a glass cylinder in a hybridization oven, 10 ml is sufficient.

    Using DNA polymerase fill-in of uracil-containing templates (see Support Protocol 3) results in a probe with higher specific activity.

14. Wash gel 15 min at room temperature in 2× SSC and then one to three times for 10 min each in 0.1× SSC/0.1% SDS at room temperature. Expose gel ≥4 hr to a phosphor screen and then scan with a PhosphorImager and visualize using ImageQuant software.

    The number of washes can be adjusted as needed. Two 15-min washes with 0.1× SSC are generally sufficient.

15. Calculate mean TRF lengths from PhosphorImager scans of gels hybridized to the telomeric repeat probe. Position a grid of 30 boxes over each lane and determine the signal intensity and size (kb) corresponding to each box. Calculate the mean TRF length as the weighted mean using the following formula: $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the PhosphorImager output (signal intensity) and L_i is the length of the DNA at position *i*. Calculate sums over the range of the molecular weight marker used in the hybridized gel.

    TELORUN, a program that performs these calculations, is available (see Internet Resource).

MAKING A LABELED MOLECULAR WEIGHT MARKER FOR RUNNING ON AN AGAROSE GEL

SUPPORT PROTOCOL 1

Running a radiolabeled molecular weight marker on either side of the gel allows for quantitation of the telomere lengths.

Materials

~250 ng/µl DNA molecular weight marker (e.g., λ DNA digested with *Sty*I)
10× React 2 buffer (Roche)
100 µM dAGT mix: 100 µM each dATP, dGTP, dTTP in H_2O, stored up to 1 year at −20°C
[α-^{32}P]dCTP (3000 Ci/mmol)
2 U/µl Klenow fragment of *E. coli* DNA polymerase I
QIAquick nucleotide removal kit (Qiagen)

1. In a tube, mix the following:

   10 µl of ~250 ng/µl DNA molecular weight marker
   2 µl of 10× React 2 buffer
   5 µl of 100 µM dAGT mix
   5 µl [α-^{32}P]dCTP
   1 µl of 2 U/µl Klenow fragment

   Incubate 30 min at 37°C.

   The authors use Roche React 2 buffer because it is the same buffer used to digest the λ DNA with StyI. Any other buffer compatible with the Klenow fragment of DNA polymerase can be used.

2. Remove unincorporated label with QIAquick nucleotide removal kit.
3. Determine the cpm of the radiolabeled marker using a scintillation counter.

SUPPORT PROTOCOL 2

MAKING KINASED RADIOLABELED TELOMERIC REPEAT PROBE

Empirically, the G-rich probe tends to have higher signals, for unknown reasons, but both probes work reasonably well. G-rich probes generally give about 2-fold better signals than C-rich probes (probably due to possible G-quartet formation by the target G-strands which inhibits hybridization), so making a G-rich probe with 3000 Ci/mmol radioisotope is recommended if maximum sensitivity is needed.

Materials

$(T_2AG_3)_4$ or $(C_3TA_2)_4$ oligonucleotide (20 pmol/µl or 0.16 µg/µl)
[γ-^{32}P]ATP (3000 Ci/mmol)
5× forward or exchange reaction buffer (GIBCO/BRL)
10 U/µl T4 polynucleotide kinase (GIBCO/BRL)
QIAquick nucleotide removal kit (Qiagen)

1. In a tube, mix the following:

   2 µl $(T_2AG_3)_4$ or $(C_3TA_2)_4$ oligonucleotide
   24 µl [γ-^{32}P]ATP
   10 µl of 5× forward reaction buffer
   12 µl H_2O
   2 µl T4 polynucleotide kinase.

   Incubate 30 min at 37°C.

2. Remove unincorporated label with QIAquick nucleotide removal kit (Qiagen).
3. Determine the cpm of the radiolabeled marker using a scintillation counter (≥5000 cpm/µl). Store <2 weeks at −20°C.

SUPPORT PROTOCOL 3

SYNTHESIS OF A HIGH–SPECIFIC ACTIVITY TELOMERIC REPEAT PROBE

In this protocol, the template oligonucleotide contains uracils so that it can be degraded later with uracil deglycosylase, thus leaving a single-stranded probe. The template also contains a small mismatch at one end (GGGAAA rather than GGGTTA), so that the priming oligonucleotide will preferentially anneal in the correct spot rather than anywhere along the template. The template, GTU4, is an oligonucleotide of four G-rich telomeric repeats with uracil. The oligonucleotide T3C3+9 begins with TTTCCC followed by nine nucleotides of correct telomeric sequence. The annealed product of these two oligonucleotides is shown in Figure 18.6.3. Thus, six cytosines will be incorporated per synthesized product. If 50 µCi of [α-^{32}P]dCTP (3000 Ci/mmol) is used, then one has 16.7 pmol of [α-^{32}P]dCTP available. Assuming the target is to incorporate 10 pmol so that one gets mostly full-length products, then 1.7 pmol of annealed target is needed. In general, the efficiency of incorporation is so high that it should not be necessary to remove unincorporated dCTP. If desired, one can use plug dialysis on 20% (w/v) acrylamide. The authors currently use [α-^{32}P]dCTP as the radioisotope because they routinely have it on hand. G-rich probes generally give about 2-fold better signals than C-rich probes (probably because of possible G-quartet formation by the target G-strands, which inhibits hybridization), so making a G-rich probe with 3000 Ci/mmol radioisotope is recommended if maximum sensitivity is needed.

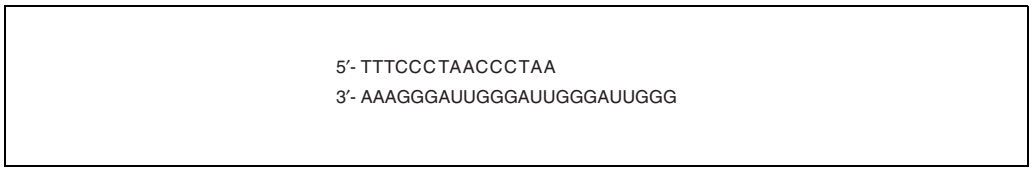


Figure 18.6.3 Annealed priming and template oligonucleotides for synthesis of a high–specific activity telomeric repeat probe. The template contains a small mismatch at its 3′ end (GGGAAA rather than GGGTTA) so the priming oligonucleotide will anneal in the correct spot.

Materials

10 pmol/µl GTU4 oligonucleotide dissolved in TE buffer:
5′-GGGUUAGGGUUAGGGUUAGGGAAA-3′
100 pmol/µl T3C3+9 oligonucleotide dissolved in TE buffer:
5′-TTTCCCTAACCCTAA-3′
1 M NaCl
10× buffer M: 10 mM Tris·Cl (pH 7.5)/10 mM $MgCl_2$/50 mM NaCl/1 mM dithioerythritol (DTE; Roche)
2 M Tris·Cl, pH 7.4 to 7.6 (*APPENDIX 2A*)
10 mg/ml BSA (e.g., Ambion)
1.25 mM dAdT: 1.25 mM each of dATP and dTTP
[α-^{32}P]dCTP (3000 Ci/mmol)
5 U/µl Klenow large fragment of *E. coli* DNA polymerase I
1 U/µl uracil deglycosylase (UDG)

37°, 95°, and 99°C water baths heating blocks, or PCR machines
PhosphorImager, including phosphor screens (Molecular Dynamics)

Additional reagents and equipment for DNA acrylamide gel electrophoresis (*APPENDIX 3A*)

1. To make 1.7 pmol/µl annealed template oligonucleotide stock, mix the following:

   3.4 µl 10 pmol/µl GTU4 oligonucleotide
   15.6 µl 100 pmol/µl T3C3+9 oligonucleotide
   1 µl 1M NaCl (50 mM final salt concentration).

   Incubate 1 min at 99°C, 15 min at 37°C, and 15 min at room temperature.

   The template oligonucleotide can be stored indefinitely at –20°C.

2. To make 8× buffer M, mix the following (total volume 625 µl):

   500 µl 10× buffer M
   100 µl 2 M Tris·Cl, pH 7.4 to 7.6
   25 µl 10 mg/ml BSA.

   Buffer M is the equivalent of Klenow buffer (50 mM Tris·Cl, pH 7.5; 10 mM $MgCl_2$; 1 mM DTT; 50 µg/ml BSA; 50 µM dNTP) without dNTP and with extra salt so that the oligonucleotides do not melt. It is very stable at –20°C.

3. To prepare the reaction mixture, mix together:

   3.125 µl of 8× buffer M (step 2)
   1 µl annealed template oligo (step 1)
   1 µl of 1.25 mM dAdT (final 50 µM)
   13.875 µl dH_2O
   5 µl [α-^{32}P]dCTP
   1 µl Klenow large fragment.

Incubate reaction mixture (25 µl total) 30 min at room temperature and then 5 min at 95°C.

The incubation at 95°C is important because if the Klenow is not inactivated, then the probe is degraded during UDG treatment.

4. Add 0.5 µl of 1 U/µl UDG and incubate 10 min at 37°C and then 10 min at 95°C.

   This step is to cleave deglycosylated uracils and release probe as a single-stranded molecule after denaturing the UDG.

5. To check probe, run 30 fmol labeled probe on a 20% (w/v) acrylamide minigel (170 V, 20 min), wrap gel in cellophane (while still attached to one glass plate), and expose 10 min to a phosphor screen. Visualize with a PhosphorImager to confirm that adequate signal is present. Store the probe <2 weeks at −20°C.

   The screen is saturated after a 1-hr exposure. A 10-bp ladder can be used for size markers, or a kinased probe or free dCTP can be used for reference, if desired.

   Approximately 40% of the [α-^{32}P]dCTP should be incorporated into the full-length probe.

   Either all or half the probe is used for the assay (>20,000 cpm/µl).

BASIC PROTOCOL 2

MEASUREMENT OF TELOMERASE ACTIVITY BY THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL

The telomeric repeat amplification protocol (TRAP) is a highly sensitive in vitro assay used for the detection of telomerase activity by the polymerase chain reaction (PCR) amplification of the products produced by the processive elongation of an oligonucleotide primer (Fig. 18.6.4). The current TRAP is a modification of the assay first described by Kim et al. (1994). This improved version allows for the elimination of the need for a wax barrier hot start, reduction of amplification artifacts, and better estimation of telomerase processivity by using a modified reverse primer sequence. Incorporation of an internal standard control allows for quantitation of telomerase activity. The TS primer is a nontelomeric sequence that telomerase can nonetheless recognize as a substrate. The reverse primer ACX contains several modifications (an anchor sequence at its 5′ end and two mismatches within the telomerase repeats) that both reduce PCR artifacts and more faithfully amplify the distribution of telomerase-elongated products than occurs with unmodified telomeric sequence primers.

Materials

100,000 cells grown in culture or tissue lysate containing 6 µg protein (see Support Protocol 4)
Liquid nitrogen
NP-40 lysis buffer (see recipe), ice cold
RNase
50× dNTP mix: 2.5 mM each dATP, dTTP, dGTP, and dCTP in RNase-free water
10× TRAP buffer (see recipe)
Radiolabeled TS primer (see Support Protocol 5)
Primer mix (see Support Protocol 6)
RNase-free H_2O (DEPC-treated)
50 mg/ml BSA, ultrapure (Ambion)
5 U/µl *Taq* DNA polymerase
Loading dye (see recipe)
0.5 M NaCl/50% (v/v) ethanol/40 mM sodium acetate (pH 4.2), optional

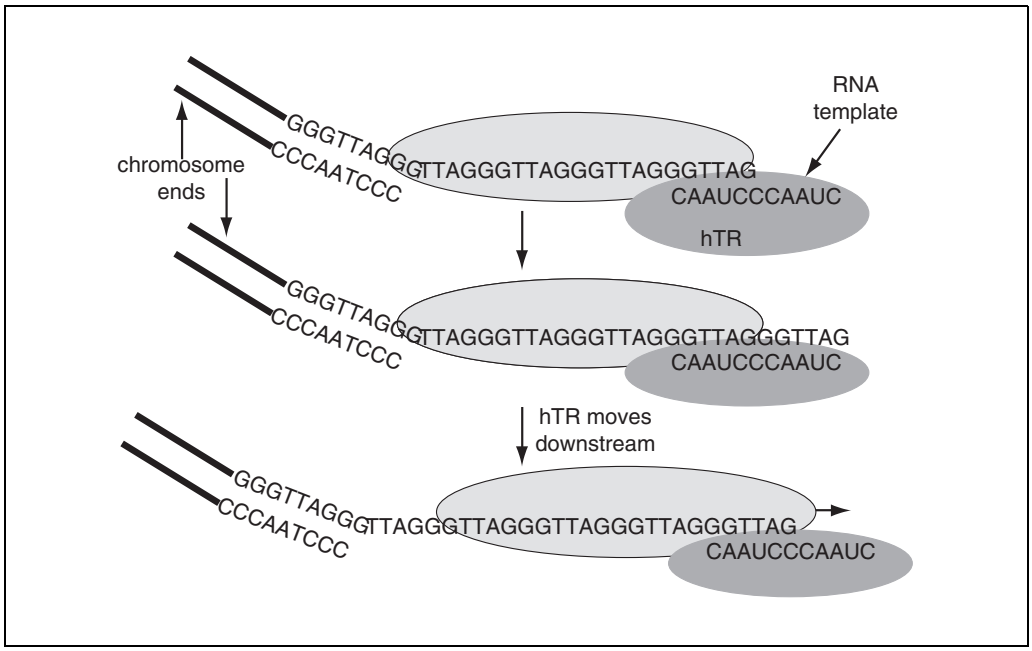


Figure 18.6.4 Telomere elongation by telomerase. Telomerase acts on the ends of chromosomes to add telomeric repeat sequences. Telomerase (hTERT) adds hexameric TTAGGG repeats by using its own RNA template (hTR) as a primer to add nucleotides. Telomerase then repositions its template RNA downstream for the addition of more telomeric repeats.

DNase-, RNase-free 0.5-ml microcentrifuge and PCR tubes
Tabletop centrifuge (e.g., 5415D; Eppendorf), room temperature and, optionally, 4°C
37°C water bath or 85°C heating block
Thermal cycler
PhosphorImager with phosphor screens and ImageQuant software (Molecular Dynamics)
Additional reagents and equipment for nondenaturing acrylamide gel electrophoresis (*UNIT 6.5*)

NOTE: Most of the reagents are included in the TRAPeze kit (Intergen).

Prepare sample

1. Place 100,000 cells in a DNase-, RNase-free 0.5 ml microcentrifuge tube. Pellet cells 6 min in a tabletop centrifuge at 6000 rpm (2940 × *g*), room temperature. Discard supernatant.

   Care must be taken not to contaminate any step in the TRAP assay with RNAses. RNA-Zap (Ambion) can be used to help eliminate RNAses.

   Much lower cell numbers can be used because one generally analyzes only 100 to 1000 cell equivalents.

   Washing the pellet is not necessary.

2. Snap freeze cell pellet in liquid nitrogen and store at −80°C until ready to lyse.

   Lead shot beads can be used as an alternative to snap freezing in liquid nitrogen. The beads are poured into a container and placed at −80°C. Samples added to the beads are snap-frozen in a couple of minutes.

   Alternatively, the cell pellet can be assayed immediately.

3. Resuspend sample (before it is fully thawed) in 100 µl ice-cold NP-40 lysis buffer and incubate 30 min on ice.

4. *Optional:* Centrifuge sample 20 min at 14,000 × *g*, 4°C, after lysis. Collect 80% of the supernatant in a fresh tube, making sure that no traces of cell debris from pellet are withdrawn.

   The whole cell lysate can be used instead of pelleting the insoluble material. The authors have found that recovery of telomerase activity is higher and more reproducible when the samples are not centrifuged and the entire lysate is analyzed. Centrifugation is usually needed only when tissues are used (see Support Protocol 4).

5. Snap-freeze lysate in liquid nitrogen and store at −80°C until analyzed by TRAP assay.

   Lead shot beads can be used as an alternative to snap freezing in liquid nitrogen. The beads are poured into a container and placed at −80°C. Samples added to the beads are snap-frozen in a couple of minutes.

   The TRAP assay can be performed using a radioactive or nonradioactive (see Alternate Protocol) primer.

   To prevent contamination with PCR-amplified products, the TRAP assay should be performed in a separate room from which the lysis took place.

Perform primer extension

6. As a negative control, incubate 5 µl lysate with 1 µg RNase for 20 min at 37°C or heat inactivate 10 min at 85°C prior to telomerase assay.

   A lysis-buffer-only control should also be included to check for presence of PCR contamination in the lysis buffer.

7. Place 1 to 2 µl lysate (equal to 100 to 1000 cells) in the bottom of an RNase-free PCR tube on ice and add the following:

   1 µl of 50× dNTP mix (50 µM final)
   5 µl of 10× TRAP Buffer
   2 µl radiolabeled TS primer
   1 µl primer mix
   38.6 µl RNase-free H_2O.

   Mix and add 0.4 µl of 50 mg/ml ultrapure BSA (20 µg final) and 0.4 µl of 5 U/µl *Taq* DNA polymerase (2 U final). Incubate 30 min at room temperature.

   This recipe is designed for one sample. A stock reaction mixture can be made just before assembling the reaction by combining the appropriate amount of dNTP mix, 10× TRAP buffer, BSA, primer mix, and water. The radiolabled TS primer and Taq DNA polymerase should be added immediately before the reaction mixture is added to each sample.

   To prevent contamination by PCR products, the preparation of samples and the assembly of the TRAP assay should take place in a separate room from that in which the PCR and electrophoresis are performed.

   During the initial 30-min incubation in the presence of the TS primer, varying numbers of hexameric repeats are added by telomerase. Subsequent amplification will yield a 6-bp incremental ladder.

8. Amplify extension products by polymerase chain reaction (PCR) in a thermal cycler as follows:

Initial step:	90 sec	94°C (to inactivate telomerase)
24 to 27 cycles:	30 sec	94°C
	30 sec	60°C.

Analyze extension products

9. Add 5 µl loading dye to 20 µl TRAP reaction mixture and run on a 10% (w/v) nondenaturing acrylamide gel in 0.5× TBE.

10. *Optional:* Fix gel 30 min in 0.5 M NaCl/50% ethanol/40 mM sodium acetate, pH 4.2.

    Fixing the gel to prevent diffusion of the bands may be performed when overnight exposure times are expected.

11. Wrap gel in plastic wrap and expose gel, without drying, to phosphor screens 30 min to overnight and visualize on a PhosphorImager using ImageQuant software.

12. Sum the intensities of the bands in the TRAP ladder and divide by the intensity of the internal standard control band to determine relative TRAP activity. Compare the activity of each sample with the negative and positive controls.

MEASUREMENT OF TELOMERASE ACTIVITY BY THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL USING FLUORESCENT PRIMERS

ALTERNATE PROTOCOL

Measurement of telomerase activity using fluorescent primers rather than radiolabeled primers can be used as an alternative to Basic Protocol 2 to reduce the exposure to radioactivity. This alternative procedure may be slightly less sensitive than the radioactive method.

Additional Materials *(also see Basic Protocol 2)*

100 ng/µl fluorescently labeled TS primer: 5′-Cy5-AATCCGTCGAGCAGAGTT (Integrated DNA Technologies), HPLC or PAGE purified

1. Prepare cells or tissue lysate as described (see Basic Protocol 2, steps 1 to 5).

2. Set up a negative control and TRAP reaction mixture as described (see Basic Protocol 2, steps 6 and 7), but use 100 ng/µl fluorescently labeled TS primer instead of radiolabeled TS primer in step 7.

3. Amplify extension products by polymerase chain reaction (PCR) in a thermal cycler as follows:

Initial step:	90 sec	94°C (to inactivate telomerase)
30 cycles:	30 sec	94°C
	30 sec	52°C
	45 sec	72°C.

4. Electrophorese the TRAP reaction mixture and fix the gel, if desired, as described (see Basic Protocol 2, steps 9 and 10).

   Fixing of the gel for nonradioactive TRAP allows for easier handling when the gel is placed on a PhosphorImager.

5. Wrap gel in plastic wrap and scan under Cy5 (Blue) fluorescence with a PhosphorImager using ImageQuant software (Molecular Dynamics). Analyze the TRAP ladder as described (see Basic Protocol 2, step 12).

SUPPORT PROTOCOL 4

LYSIS OF TISSUE SAMPLES FOR THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL

This procedure can be used to obtain lysates to analyze telomerase activity from tissue samples. Because the sample will be used in an RNase-sensitive procedure, standard precautions to avoid RNase contamination should be observed.

Additional Materials *(also see Basic Protocol 2)*

50 to 100 mg tissue sample, frozen at −80°C
Washing buffer (see recipe), ice cold
BCA protein assay kit (Pierce)

Kontes tubes and disposable pestles (VWR)
Hand-powered drill

1. *Optional:* Wash 50 to 100 mg frozen tissue sample in enough ice-cold washing buffer to cover the tissue. Centrifuge and remove buffer from tissue. Mince tissue with scalpel.
2. Homogenize tissue with 200 µl ice-cold NP-40 lysis buffer in a Kontes tube on ice using a disposable pestle rotating at 450 rpm with a hand-powered drill. Use short (<10- to 15-sec) pulses.

   Care must be taken not to overheat the samples and destroy the telomerase activity in the tissue.
3. Incubate sample 25 min on ice, centrifuge lysate 20 min at 16,000 × *g*, 4°C. Collect 80% of the supernatant in a fresh tube. Save an aliquot for protein assay. Rapidly freeze the supernatant in liquid nitrogen. Store up to ~1 year at −80°C.
4. Measure the concentration of protein in the lysate using a BCA protein assay kit (*APPENDIX 3H*).

   An aliquot of the extract containing 6 µg of protein will be used for each telomerase assay (see Basic Protocol 2, step 7).

SUPPORT PROTOCOL 5

MAKING RADIOLABELED TS PRIMER FOR TELOMERIC REPEAT AMPLIFICATION PROTOCOL

For the radioactive TRAP assay (see Basic Protocol 2), the TS primer (5′-AATC-CGTCGAGCAGAGTT-3′) is end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The following protocol makes enough primer for ten samples, and the amounts can be adjusted appropriately for varying number of samples.

Materials

100 ng/µl TS primer (5′-AATCCGTCGAGCAGAGTT-3′)
[γ-^{32}P]ATP (3000 Ci/mmol), sterile
10 U/µl T4 polynucleotide kinase and 5× forward kinase buffer (GIBCO/BRL)
Sterile, RNase-free H_2O

RNase-free 1.5-ml microcentrifuge tubes
37° and 85°C water baths or heating block, or PCR machine

1. Mix together the following in a RNase-free 1.5-ml microcentrifuge or PCR tube:

   10 µl of 100 ng/µl TS primer
   2.5 µl [γ-^{32}P]ATP
   4 µl of 5× forward kinase buffer
   3 µl sterile, RNase-free H_2O
   0.5 µl of 10 U/µl T4 polynucleotide kinase.

   Incubate 20 min at 37°C.

2. Heat inactivate enough kinase for one assay 5 min at 85°C and use immediately or freeze up to 2 weeks at −20°C. Do not freeze and thaw more than once.

MAKING THE PRIMER MIX FOR TELOMERIC REPEAT AMPLIFICATION PROTOCOL

SUPPORT PROTOCOL 6

The primer mix includes the reverse primer (ACX), the substrate for the 36-bp internal standard control (TSNT), and the reverse primer for the internal standard (NT). Preparation of the stock TSNT primer should be done in a separate room from the other TRAP steps and prepared with separate micropipettors. The TSNT primer should be purchased from a different company from the other two primers because it is possible to contaminate them at the production facilities with the TSNT oligonucleotide. This protocol makes enough primer mix for 100 sample reactions.

Materials

TSNT oligonucleotide:
5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′
RNase-free H_2O
1 µg/µl ACX primer: 5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′
1 µg/µl NT primer: 5′-ATCGCTTCTCGGCCTTTT-3′
10% (v/v) bleach
RNase-free 0.5-ml PCR tubes, including some that are siliconized

1. Dilute dry TSNT oligonucleotide in RNase-free water to 100 µM (1.0×10^{-10} mol/µl) in a siliconized, RNase-free PCR tube. Perform three dilutions (1:100, 1:1000, and 1:1000) in siliconized tubes so that the final concentration of the TSNT stock is 1.0×10^{-18} mol/µl.

   This step should be done in a separate room with separate micropipettors and tips from the other steps. Because extremely dilute solutions of the TSNT substrate are required, siliconized tubes should be used for all dilutions. The actual amount added to the primer mix below is empirically adjusted to compensate for experimenter error.

2. Mix 10 µl each of the 1 µg/µl ACX and NT primers (100 ng/µl final) and 79.0 µl RNase-free water together in an RNase-free tube.

3. Move to the area where the TSNT was prepared and add 1 ml dilute TSNT oligonucleotide (0.01 × 10-18 mol/ml final) to the mix. Clean the outside of the tube with 10% bleach.

4. Return to the nonTSNT work area and divide the primer mix into 25-µl aliquots. Use aliquots immediately or store up to 1 year at −20°C. Do not freeze and thaw more than once.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Denhardt solution, 100×

10 g Ficoll 400
10 g polyvinylpyrrolidone
10 g BSA (Pentax Fraction V; Miles Laboratories)
H_2O to 500 ml
Filter sterilize and store at −20°C in 25-ml aliquots

Hybridization solution

5× SSC buffer (see recipe)
5× Denhardt solution (see recipe)
10 mM Na_2HPO_4
1 mM $Na_2H_2P_2O_7$
Store up to 6 months at −20°C

Loading dye

0.25% (w/v) each of bromophenol blue and xylene cyanol in 50% (v/v) glycerol/50 mM EDTA. Store up to 1 year at 4°C.

For nonradioactive TRAPs, the xylene cyanol should be removed, as this shows up while imaging the gel.

NP-40 lysis buffer

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM $MgCl_2$
1 mM EDTA
1% (v/v) NP-40
0.25 mM sodium deoxycholate
10% (v/v) glycerol
150 mM NaCl (*APPENDIX 2A*)
5 mM 2-mercaptoethanol
0.1 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)
Store up to 6 months at −20°C

Quick-prep lysis buffer

100 mM NaCl
100 mM EDTA, pH 8.0 (*APPENDIX 2A*)
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Store up to 1 month at room temperature

SSC (sodium chloride/sodium citrate), 20×

3 M NaCl (175 g/liter)
0.3 M Na_3citrate·$2H_2O$ (88 g/liter)
Adjust pH to 7.0 with 1 M HCl (*APPENDIX 2A*)
Store up to 6 months at room temperature

TRAP buffer, 10×

200 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
15 mM $MgCl_2$
630 mM KCl
0.5% (v/v) Tween 20
10 mM EGTA
Store up to 6 months at −20°C

Washing buffer

10 mM HEPES (adjusted to pH 7.5 with KOH)
1.5 mM $MgCl_2$
10 mM KCl
1 mM DTT (*APPENDIX 2A*)
Store up to 6 months at 4°C

COMMENTARY

Background Information

Cells contain repeated TTAGGG DNA sequences, called telomeres, at the ends of chromosomes to provide genomic stability. Telomeres provide a source of expendable DNA, which alleviates the end-replication problem in which one DNA strand cannot complete copying all the way to its end during cell division (reviewed in Collins, 2000). An in-gel Southern hybridization analysis of the chromosome terminal (i.e., telomeres) restriction fragments (TRFs) allows for the determination of all telomere lengths in a cell population (Harley et al., 1990; Ouellette et al., 2000). Telomere length has been shown to decrease with time and with increasing age (Allsopp et al., 1992). Most normal human cells lack telomerase activity, and their telomeres shorten with each cell division until they enter replicative senescence, where cells cease to proliferate (Harley et al., 1990; Wright et al., 1996). Cells are mortal unless a rare survivor activates a mechanism to maintain its telomeres.

The mechanism predominately used to maintain human telomeres is the addition of telomeric sequences by telomerase. Human telomerase is an RNA-dependent DNA polymerase complex consisting of a reverse transcriptase catalytic subunit (hTERT), which uses the integral RNA component (hTR) of the complex as a template for adding TTAGGG repeats to the end of the chromosome (Fig. 18.6.4; reviewed in Greider and Blackburn, 1985; Cong et al., 2002). Telomerase is expressed in only a small number of normal proliferating cell types such as germline and somatic stem cells, but it is found in ~90% of human cancer cells. Once telomerase is activated, it stabilizes telomere length and permits continued cell division (Counter et al., 1992).

The development of a highly sensitive assay to detect telomerase activity in cells and tissues enhanced the evaluation of a wide variety of human tissue and tumor cells (Kim et al., 1994). The telomeric repeat amplification protocol (TRAP) measures in vitro telomerase activity by a primer extension assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers (Fig. 18.6.5; Kim et al., 1994). The products can then be amplified by the polymerase chain reaction (PCR) and run on an acrylamide gel. There are several publications describing modifications and alternative methods of extracting and detecting telomerase activity in human samples using the TRAP assay (Piatyszek et al., 1995; Wright et al., 1995; Holt et al., 1996; Norton et al., 1998; Gollahon and Holt, 2000). The protocol described in this unit incorporates the modifications suggested in these reports. Together with the TRF analysis, telomerase has been shown to be directly involved in telomere maintenance and has been linked with immortality (Kim et al., 1994).

Critical Parameters

Terminal restriction fragments

It is desirable to obtain ≥300,000 cells for the determination of telomere length so that 1 to 2 μg DNA can be assayed for each TRF analysis. The amount of DNA is estimated at 6 $\mu g/10^6$ diploid cells or 10 $\mu g/10^6$ aneuploid cells; therefore, 300,000 cells yields 1.8 μg DNA. Triton X-100 rather than SDS is used in the lysis so that the residual detergent present after dialysis does not inhibit subsequent digestion by restriction endonucleases. High concentrations of EDTA (100 mM) are present to inhibit nucleases very rapidly in these concentrated cell suspensions. Frozen cell pellets are first suspended in lysis buffer to quench nuclease activity and obtain a uniform distribution of cells before adding Triton X-100. Including Triton X-100 in the initial suspension buffer leads to a rapid clumping of cells. Lysis buffer should be added before cell pellets are fully thawed to prevent nuclease digestion. Heat inactivation of the proteinase K avoids the need to phenol/chloroform extract the DNA, and the residual salt, EDTA, and peptides present after dialysis do not inhibit enzyme digestion. After digestion with proteinase K, DNA should be handled using only wide-bore pipet tips to

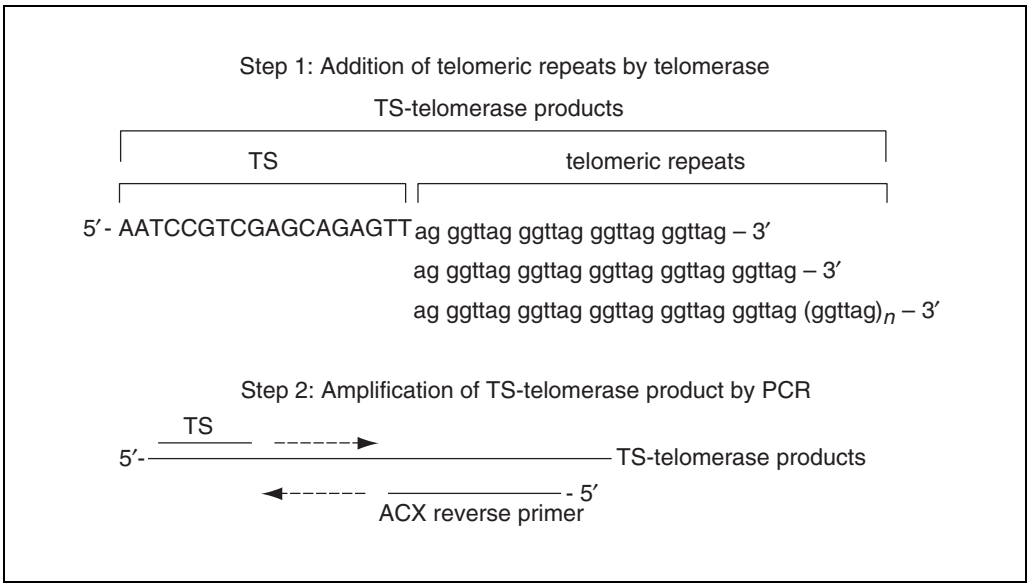


Figure 18.6.5 The telomeric repeat amplification protocol (TRAP; see Basic Protocol 2). In the first step, telomerase-mediated extension occurs on a nontelomeric oligonucleotide that serves as a substrate for telomerase (TS). In the second step, the TS-telomerase products are specifically amplified by the polymerase chain reaction (PCR) using the TS as an upstream primer and ACX as the reverse, downstream primer. The addition of telomeric repeats in the first step results in a TRAP ladder of 6-bp increments as seen on a 10% (w/v) acrylamide gel. Adapted from the manual for the TRAPeze Telomerase Detection Kit (Intergen).

prevent shearing of the DNA. The final DNA solution should be viscous. Lack of viscosity suggests nonspecific nuclease degradation of the DNA, which is easily tested by running 100 ng undigested DNA on a 0.7% (w/v) agarose gel. Degraded DNA is frequently seen the first time an investigator attempts this DNA purification procedure, but generally it is rarely present thereafter. If the DNA concentration is too low after dialysis to load a sufficient quantity on a gel, then concentration on a 30,000-molecular-weight spin-filter or ethanol precipitation of the DNA may be necessary. This is rarely necessary when starting with $\geq 1 \times 106$ cells.

Digested DNA should run as a smear below the 1-kb molecular weight marker. Anything above this size is incompletely digested. If the sample is not digested completely, an additional microliter of enzyme mix can be added to the sample and allowed to digest for a longer amount of time. It is essential to have the genomic DNA completely digested to have interpretable results after hybridization with the telomeric probe. If digestion problems persist, completion of the restriction digest can be checked ahead of time by electrophoresing 250 ng DNA on a small agarose gel stained with ethidium bromide.

TRF gels should be handled carefully as they are fragile until they are dried. The dried gel can be hybridized directly without the need to transfer the DNA to a membrane. Denaturing the DNA in the gel before rather than after drying the gel increases the signal intensity ~3- to 5-fold, presumably by permitting a much greater diffusion of the denatured DNA strands in the agarose gel than in the very-high-percentage dried and rehydrated gel, and thus inhibiting the reannealing of the parental strands, which would compete with the probe. Rinsing the denatured gel with distilled water gets rid of the NaOH used in the denaturing solution and substantially reduces sticking of the gel to the Whatman 3MM paper. Because most of the DNA samples do not fill the wells and are thus in the bottom half of the gel, the gels are flipped and dried with the upper surface against the filter paper support. This increases the distance between the DNA samples and the filter paper and significantly reduces the loss of lower-molecular-weight DNA. Some loss of lower-molecular-weight DNA (particularly <400 bp) occurs during drying, and this results in the lower limit of detection in the TRF analysis.

Telomeric repeat amplification protocol

Because the TRAP assay is a highly sensitive PCR-based assay, precautions must be taken to eliminate RNases and PCR product contamination. The following guidelines en-

Table 18.6.1 TRAP Troubleshooting Guide[a]

Problem	Possible cause	Solution
No visible products in any lane, including the internal standard control band	PCR not initiated	Check that all assay components were added, especially *Taq* DNA polymerase. Check thermal cycler for proper settings and operation.
Few visible TRAP ladder bands and weak internal control band	Cell or tissue extract contains *Taq* inhibitors	Dilute sample with lysis buffer.
No visible TRAP products in any lane but internal standard control band is present	Telomerase activity not initiated Possible presence of RNase contamination	Always use RNase-free tips, tubes, and solutions. Use fresh aliquots of solutions. Do not let pipet tips touch anything except the solutions. Keep samples on ice and reactions below 25°C. Add RNase inhibitor to lysis buffer.
Visible TRAP product in all lanes including lysis-buffer-only control	PCR carryover contamination	Use fresh aliquots of each component of the assay. Perform each step in a separate room with separate micropipettors and tips.
Several bands in the lysis-buffer-only control lane	Primer-dimer artifacts	Incorporate hot start into the PCR reaction: Assemble reaction mixture as described, except eliminate *Taq* polymerase, heat reaction 2 min at 94°C, add *Taq*, and continue PCR procedure as described.
Products in the heat-treated extracts	Insufficient heat inactivation, primer-dimer artifacts, or contamination of extract with TRAP products	Check temperature of heating block. Make and test new extract and check for PCR carryover contamination. Extreme care must be taken to prevent pipetting into multiple samples or solutions.
Extra bands between internal standard control band and TRAP ladder bands		
Weak intensity of internal standard control band	Telomerase activity too high	Dilute samples.

[a]Adapted from the manual for the TRAPeze Telomerase Detection Kit (Intergen).

sure that contamination is kept to a minimum. Gloves must be worn at all times and each step of the protocol should be done in separate rooms or at least in areas separated by barriers. DEPC-treated water must be used for all solutions, solutions should be kept from other solutions in the laboratory, and small aliquots of the solutions should be made and used only once. Separate micropipettors should be designated to be used only for the TRAP assay, and only aerosol-resistant pipet tips should be used with these micropipettors. Samples and solutions should also be kept on ice or snap-frozen, unless otherwise specified, to prevent degradation of telomerase.

Troubleshooting

The most common problem associated with the TRF assay is that the genomic DNA is not completely digested. This results in a smear or tight band >1 kb as visualized by ethidium bromide staining, and this interferes with the interpretation of telomere lengths after hybridizing the gel with a telomeric probe. A test gel using 200 ng of DNA digested with the enzyme mix can show whether the samples have been completely digested. If the DNA is not completely digested, the usual cause is that the sample contains too much EDTA, which interferes with the restriction enzymes. This usually results from dialyzing too many samples against an insufficient volume, or from a failure to ensure that all the liquid rests against the membrane, resulting in a few microliters of 100

mM EDTA failing to get dialyzed. Other occasional failures result from using too much DNA because of an inaccurate measurement of the viscous DNA. Another problem is that the sample may not contain any detectable DNA because of a tear in the dialysis membrane. Weak signals after scanning the hybridized gel may be due to too stringent washing or insufficiently labeled probe. Use a Geiger counter to ensure that the background signal of the gel is low but that the areas expected to have telomeres contain a higher signal before exposing the gel. Check to make sure that the probe is specific for telomeric sequences and that the radioisotope and other components are fresh.

Table 18.6.1 lists some common problems and solutions associated with the TRAP assay. The most common problems associated with the TRAP assay are RNase and PCR contamination or forgetting to add one of the components in the reaction, such as the *Taq* polymerase. Careful handling of the samples and reagents in the TRAP assay ensures successful detection of telomerase activity.

Anticipated Results

The results of an in-gel Southern hybridization of digested genomic DNA with a telomeric probe yields a smear that corresponds to the distribution of all telomere lengths within the population of cells collected (Fig. 18.6.6). Including a labeled molecular weight marker on the hybridized gel allows for calculation of the average telomere length as well as the longest and shortest telomere lengths.

Once the conditions for the TRAP assay have been optimized and RNase contamina-

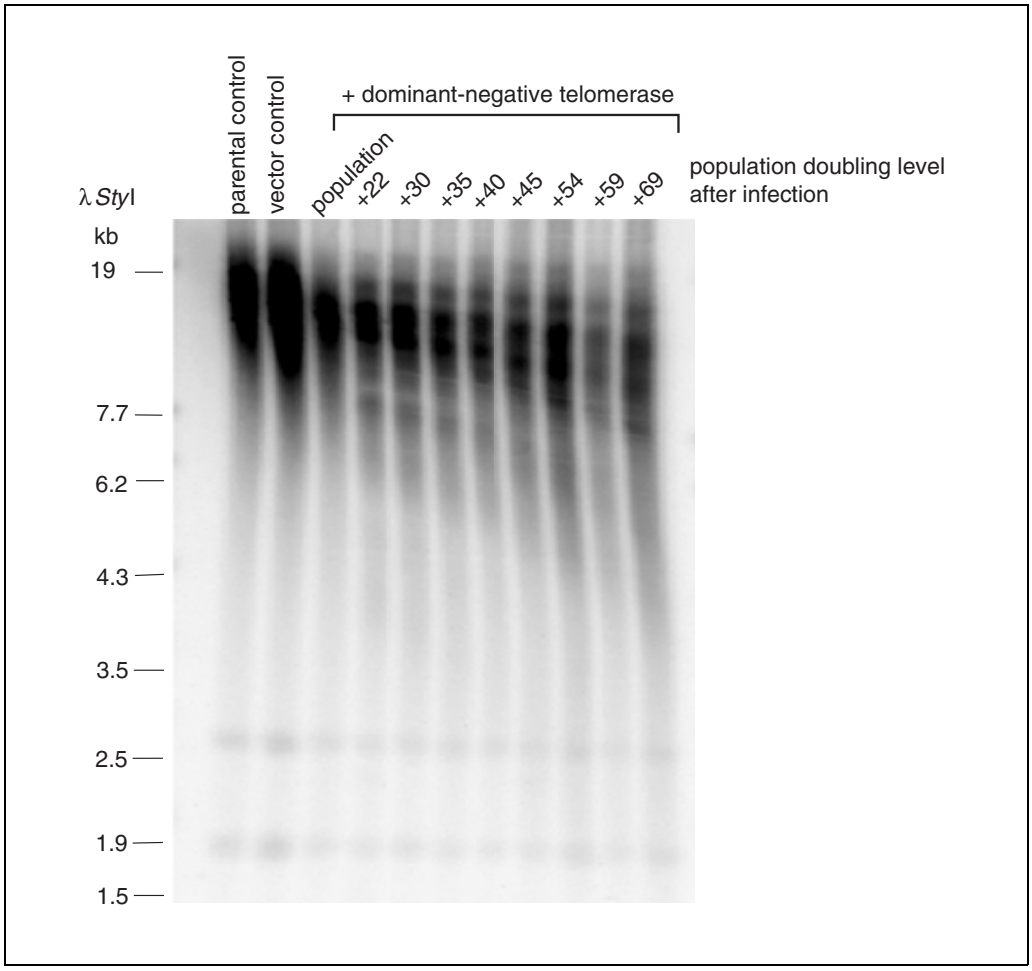


Figure 18.6.6 Determination of telomere length using the terminal restriction fragment (TRF) protocol in sequentially passaged (indicated by the population doubling level, or the number of doublings the population has accrued, after infection) H1299 lung carcinoma cells infected with a dominant-negative mutant of human telomerase to inhibit telomerase and induce telomere shortening. Telomere length was determined by digesting genomic DNA with a mixture of six restriction enzymes having four-base recognition sites (see Basic Protocol 1), and then analyzing the DNA on a 0.7% (w/v) agarose gel. λ DNA cut with *Sty*I was used as a molecular weight marker.

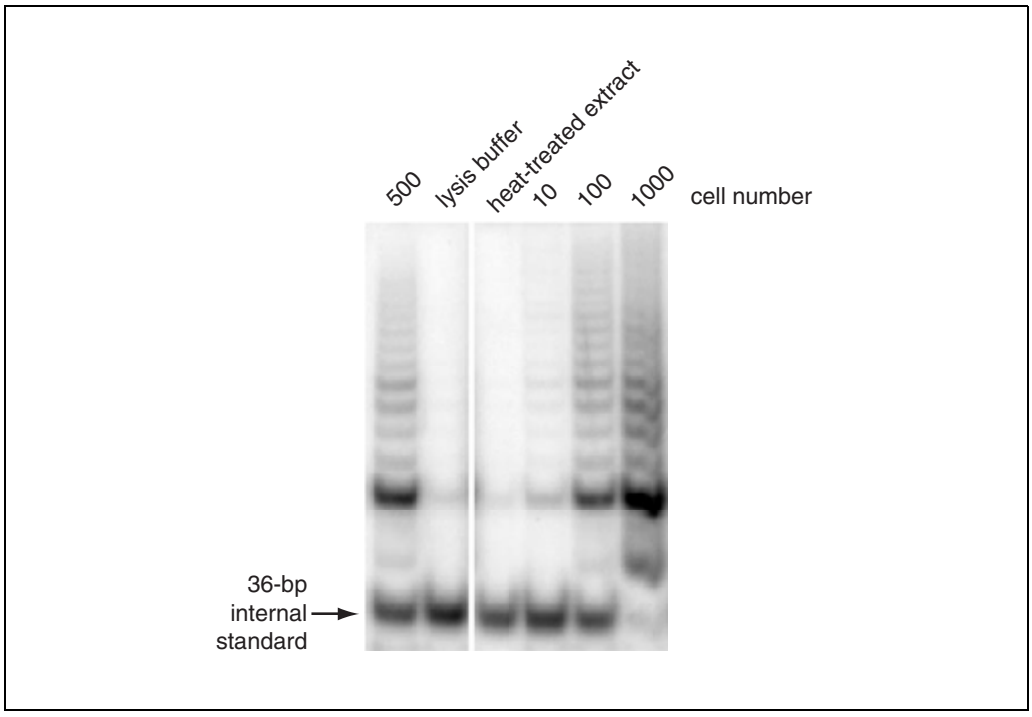


Figure 18.6.7 Analysis of telomerase activity using the telomeric repeat amplification protocol (TRAP; see Basic Protocol 2). Human cancer cell extracts (500 cell equivalents of H1299 lung carcinoma cells) are positive for telomerase activity as evidenced by the 6-bp incremental TRAP ladder. Treating the extract with heat inactivates the telomerase activity as evidenced by no TRAP ladder. Lysis buffer only serves as a negative control. Each TRAP reaction includes a 36-bp internal standard control. Ten-fold increases of H1299 cell equivalents (100 to 1000 cell equivalents) results in increased intensity of the TRAP ladder. The 1000 cell sample in this gel cannot be readily quantitated because of the faint signal in the internal standard (due to competition by excess telomerase products).

tioniseliminated,theamplifiedtelomerase-extended products can be seen as an incremental ladder (Fig. 18.6.7). Because human telomerase is processive, the 30-min incubation yields a TS primer extended by a variable number of hexameric repeats. When the resulting 10% (w/v) acrylamide gel is visualized by a PhosphorImager, a 6-bp DNA incremental ladder is seen as well as the internal standard control band. The lysis buffer only and the heat-treated sample lanes should contain only the internal standard control band. The intensities of each of the bands in the TRAP ladder can be summed together and divided by the internal standard control band to determine relative TRAP activity. Too much telomerase activity will result in intense ladders and a failure to amplify the internal standard. The actual amount of the TSNT primer used is empirically adjusted so that one gets an internal standard signal roughly equivalent to the most intense first band of the telomerase ladder using 100 to 1000 positive tumor cells as a control. The activity of each sample can then be compared with the negative and positive controls.

Time Considerations

The entire TRF procedure can be performed in 4 days (day 1: harvest and digest cells with proteinase K and dialyze overnight; day 2: restriction digest DNA and run gel overnight; day 3: dry gel and hybridize overnight; day 4: wash and expose gel). Alternatively, isolated genomic DNA can be stored at 4°C (or at −20°C for longer storage) until it is digested. The TRAP assay can be performed in 1 day or separated into a 3-day procedure: one day for lysing the samples, a second day for the PCR assay, and a third day for running and exposing the gel.

Literature Cited

Allsopp, R.C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E.V., Futcher, A.B., Greider, C.W., and Harley, C.B. 1992. Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 89:10114-10118.

Collins, K. 2000. Mammalian telomeres and telomerase. *Curr. Opin. Cell Biol.* 12:378-383.

Cong, Y.-S., Wright, W.E., and Shay, J.W. 2002. Human telomerase and its regulation. *Microbiol. Mol. Bio. Rev.* 66:407-425.

Counter, C.M., Avilion, A.A., LeFeuvre, C.E., Stewart, N.G., Greider, C.W., Harley, C.B., and Bacchetti, S. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* 11:1921-1929.

Gollahon, L.S. and Holt, S.E. 2000. Alternative methods of extracting telomerase activity from human tumor samples. *Cancer Lett.* 159:141-149.

Greider, C.W. and Blackburn, E.H. 1985. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* 43:405-413.

Harley, C.B., Futcher, A.B., and Greider, C.W. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature* 345:458-460.

Holt, S.E., Norton, J.C., Wright, W.E., and Shay, J.W. 1996. Comparison of the telomeric repeat amplification protocol (TRAP) to the new TRAP-eze telomerase detection kit. *Methods Cell Sci.* 18:237-248.

Kim, N.-W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* 266:2011-2015.

Norton, J.C., Holt, S.E., Wright, W.E., and Shay, J.W. 1998. Enhanced detection of human telomerase activity. *DNA Cell Biol.* 17:217-219.

Ouellette, M.M., Liao, M., Herbert, B.-S., Johnson, M., Holt, S.E., Liss, H.S., Shay, J.W., and Wright, W.E. 2000. Subsenescent telomere lengths in fibroblasts immortalized by limiting amounts of telomerase. *J. Biol. Chem.* 275:10072-10076.

Piatyszek, M.A., Kim, N.W., Weinrich, S.L., Hiyama, K., Hiyama, E., Wright, W.E., and Shay, J.W. 1995. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci.* 17:1-15.

Wright, W.E., Shay, J.W., and Piatyszek, M.A. 1995. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucl. Acids Res.* 23:3794-3795.

Wright, W.E., Brasiskyte, D., Piatyszek, M.A., and Shay, J.W. 1996. Experimental elongation of telomeres in immortal human cells extends the lifespan of immortal × normal cell hybrids. *EMBO J.* 15:1734-1741.

Internet Resource

http://www.swmed.edu/home_pages/cellbio/shay-wright/

Includes TELORUN for calculating mean TRF.

Contributed by Brittney-Shea Herbert, Jerry W. Shay, and Woodring E. Wright
The University of Texas Southwestern Medical Center
Dallas, Texas

Nonisotopic Methods for Determination of Poly(ADP-Ribose) Levels and Detection of Poly(ADP-Ribose) Polymerase

The techniques presented in this unit are designed to allow quantification and characterization of pADPr synthesized in vitro, in cultured cells, or in tissues from whole animals. Treatment of cells with DNA-damaging agents such as hydrogen peroxide or DNA-alkylating compounds stimulates PARP activity. After treatment, activated PARPs cleave nicotinamide adenine dinucleotide (NAD) into nicotinamide and ADP-ribose and synthesize pADPr polymers covalently attached to nuclear proteins. Following TCA precipitation of poly(ADP-ribosyl)ated proteins, the acid-insoluble fraction is used to purify pADPr in order to determine pADPr levels and its size distribution in intact cells (see Basic Protocol 1). This section presents novel applications for the immunodetection of pADPr in intact cells, such as immunodot blot analysis (see Basic Protocol 2), ELISA detection (see Alternate Protocol 1), size-distribution analysis by immunodetection (see Basic Protocol 3), detection of modified protein by immunoblot (see Basic Protocol 4), and cellular localization by immunocytochemistry (see Basic Protocol 5). In addition to determining the presence of pADPr, methods are also provided for detection of the PARPs themselves by immunoblot (see Basic Protocol 6), proteolytic cleavage during cell death (see Alternate Protocol 2), and nonisotopic–activity immunoblot (see Basic Protocol 7).

NONISOTOPIC METHODS FOR DETERMINATION OF POLY(ADP-RIBOSE) LEVELS IN INTACT CELLS

Poly(ADP-ribose) polymerase-1 (PARP-1) is the main enzyme responsible for poly(ADP-ribose) (pADPr) synthesis in response to DNA strand breaks caused by genotoxic agents. Analysis of pADPr is the easiest method that provides direct proof of PARP activation following genotoxic treatment in cells. Furthermore, monitoring changes in pADPr metabolism could serve as an important diagnostic tool to determine the impact of low-dose exposure to DNA-damaging agents.

The main challenge in pADPr research is its very low concentration in cells: ~2 to 10 pmol/mg DNA in untreated cells. To bypass this problem, some investigators have used isolated nuclei or permeabilized cells in order to study poly(ADP-ribosyl)ation of proteins following a DNA-damaging treatment; however, while this procedure provides higher levels of pADPr due to the loss of cellular integrity, the results are, by definition, nonphysiological. More recently, the availability of highly specific anti-poly(ADP-ribose) antibodies has become a powerful tool to investigate pADPr metabolism in cultured cells. The establishment of nonisotopic methods to study pADPr synthesis has provided safer and simpler laboratory procedures.

Poly(ADP-Ribose) Preparation from Cell Cultures

BASIC PROTOCOL 1

PADPr synthesis is a useful biological parameter for the investigation of cellular processes such as DNA repair and cell death. Cell treatments with DNA-damaging agents such as hydrogen peroxide or DNA alkylating compounds cause PARP-1 and -2 activation. It must be kept in mind that these pADPr-inducing treatments must be optimized for each cell line. The following protocol was first described by Aboul-Ela et al. (1988) and was subsequently modified in the authors' laboratory (Shah et al., 1995). These procedures are based on the principle that pADPr is covalently bound to proteins present in the TCA precipitate and released under alkaline conditions. Once released from proteins, the

Contributed by Jean François Haince, Guy G. Poirier, and Jim B. Kirkland
Current Protocols in Cell Biology (2003) 18.7.1-18.7.26

pADPr can be further purified by dihydroxyboryl Bio-Rex 70 (DHBB) chromatography (Alvarez-Gonzalez et al., 1983). This purification step is required to eliminate major contaminants such as DNA, RNA, and residual proteins present in the cell extract. In practice, it is useful to eliminate these contaminants to decrease background when using immunodetection techniques for pADPr analysis.

Materials

Cells of interest in appropriate medium
DNA-damaging agent
PBS, pH 7.4 (*APPENDIX 2A*), ice cold
20% (w/v) trichloroacetic acid (TCA), ice cold
100% ethanol, ice cold
1 M KOH/50 mM EDTA
AAGE9 buffer (see recipe)
HCl, concentrated
50% (w/v) dihydroxyboryl Bio-Rex 70 (DHBB) affinity resin (Alvarez-Gonzalez et al., 1983)
1 M ammonium acetate, pH 9.0
H_2O, 37°C

Rubber policeman
37° and 60°C water baths or heat blocks
10-ml Econocolumn (Bio-Rad)

Prepare acid-insoluble fraction from cultured cells

1. Treat cells of interest in medium with an appropriate DNA-damaging agent and incubate for sufficient time to allow for PARP activation.

   For example, treat confluent C3H10T½ mouse fibroblasts (ATTC# CCL-226) with 200 μM hydrogen peroxide or 100 μM N-methyl-N-nitro-N-nitrosoguanidine (MNNG) for 20 to 30 min at 37°C. This is a typical procedure for PARP-1 activation. An optimal incubation time must be determined by the investigator if using other DNA-damaging stimuli or other cell types. This time should be determined carefully, as pADPr can decrease rapidly at longer time points due to NAD depletion or repair processes.

2. Remove and discard the medium from the cell culture and quickly wash the monolayer with 5 ml ice-cold PBS, pH 7.4.

3. Remove the PBS, add 5 ml ice-cold 20% (w/v) TCA, and incubate cells 15 min on ice.

   This step is critical to prevent catabolism of polymer by poly(ADP-ribose) glycohydrolase and must be done as quickly as possible.

4. Scrape the TCA-insoluble material with a rubber policeman and transfer cells to a centrifuge tube. Pellet by centrifuging 10 min at 1800 × *g*, 4°C.

5. Wash the cell pellet once with ice-cold 20% TCA and twice with 5 vol ice-cold 100% ethanol.

6. Air dry the cell pellet.

   The pellet may be stored up to 6 months at −30°C or −80°C or directly processed as follows.

Isolate pADPr from cell pellet

7. Dissolve the TCA precipitate in 1 ml of 1 M potassium hydroxide/50 mM EDTA and mix by vortexing.

   In the case where TCA has not been thoroughly removed from the pellet, it may be necessary to utilize a glass homogenizer to disperse the pellet as much as possible.

8. Incubate the alkali-digest reaction mixture 1 hr at 60°C with occasional vortexing to promote separation of the pADPr from the proteins.

   Following this incubation, a small aliquot can be removed from each sample for determination of DNA content, if required.

Purify pADPr by DHBB chromatography

9. Add 9 ml AAGE9 buffer to the alkali-digested pADPr and adjust the pH to 9.0 ± 0.2 with concentrated hydrochloric acid.

   AAGE9 buffer contains 250 mM ammonium acetate, pH 9.0, 6 M guanidine·hydrochloride, and 10 mM EDTA.

10. Transfer 1 ml of 50% (w/v) DHBB suspension (0.5 ml packed affinity resin) into a 10-ml Econocolumn (Bio-Rad). Equilibrate with 5 ml AAGE9 buffer, 10 ml water, and 10 ml AAGE9 buffer, successively. Verify that the column pH is 9.0.

    The description and detailed preparation of DHBB resin is presented in Alvarez-Gonzalez et al. (1983).

11. Pass the alkali digest sample twice through the DHBB column. Wash with 25 ml AAGE9 buffer followed by 10 ml of 1 M ammonium acetate, pH 9.0.

12. Elute the pADPr with 0.5 ml water prewarmed to 37°C. Repeat four more times.

    The pADPr will be in fractions 2 and 3.

13. Lyophilize the eluted pADPr and keep frozen until further analysis (up to 1 year).

Quantification of pADPr by Immunodot Blot

BASIC PROTOCOL 2

The following protocol describes the utilization of an immunodot blot technique for the determination of DHBB-purified pADPr from cell extracts. This nonisotopic immunodetection method, developed in the authors' laboratory (Affar et al., 1999a), is based on the covalent binding of pADPr to a positively charged nylon membrane under alkaline conditions. The detection is carried out using an anti-poly(ADP-ribose) primary antibody, followed by an appropriate peroxidase-conjugated secondary antibody. Quantification is done with chemiluminescence reagents for HRP-conjugated secondary antibodies. This technique can be used routinely for rapid quantification of pADPr at femtomolar concentrations, so it provides great advantages over other procedures. In addition to a great sensitivity, the immunodot blot procedure is applicable to in vitro synthesized polymer as well as to cell culture isolated pADPr. More samples can be processed simultaneously by this method than is possible using HPLC methods.

Materials

DHBB-purified pADPr (see Basic Protocol 1)
0.4 M NaOH/10 mM EDTA
0.4 M NaOH
PBS-MT (see recipe)
Anti-pADPr primary antibody
Peroxidase-conjugated secondary antibody
PBS-T: PBS containing 0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*)
Chemiluminescence reagents for HRP-conjugated secondary antibodies

7.8 × 11.5–cm Hybond N^+ nylon membrane (Amersham Bioscience)
Dot blot manifold system (Life Technologies)
Orbital platform shaker

Cooled CCD camera with ChemiImager 4000 and Digital Imaging and Analysis System (Alpha Innotech) *or* X-ray film

Additional reagents and equipment for autoradiography and densitometry (*UNIT 6.3*; optional)

1. Dilute DHBB-purified pADPr in a vol of 0.4 M sodium hydroxide/10 mM EDTA.
2. Load samples onto a 7.8 × 11.5–cm Hybond N^+ nylon membrane using a dot blot manifold system.
3. Wash the membrane with 50 ml 0.4 M sodium hydroxide, remove from the manifold, and keep in water for further processing.
4. Incubate the membrane in 50 ml PBS-MT on a orbital platform shaker for at least 1 hr at room temperature.
5. Dilute anti-pADPr primary antibody in PBS-MT and incubate 50 ml with the membrane on a orbital platform shaker overnight at room temperature.

   Two antibodies, 10H and LP96-10, are commercially available for the detection of polymer ranged between 1 and 1000 fmol. Generally, experiments are performed with 10H antibody diluted 1:250 or LP96-10 at 1:5000. Under these specific conditions, both antibodies recognize pADPr in a dose-dependent manner, but LP96-10 polyclonal antibody shows a higher sensitivity (also see Critical Parameters).

6. Wash the membrane three times with 50 ml PBS-MT for 15 min each.
7. Dilute an appropriate peroxidase-conjugated secondary antibody in PBS-MT according to supplier's instructions.
8. Incubate the membrane 30 min with 50 ml secondary antibody on a orbital platform shaker at room temperature.
9. Discard the secondary antibody and wash the membrane as follows:

   Twice with 50 ml PBS-MT for 5 min each
   Three times with 50 ml PBS-T for 15 min each
   Twice with 50 ml PBS for 5 min each.

10. Illuminate the blot using chemiluminescence reagents for HRP-conjugated secondary antibodies according to instructions.

11a. *Quantify using digital imaging:* Quantify the amount of pADPr using a cooled CCD camera equipped with ChemiImager 4000. Analyze the data with the Digital Imaging and Analysis Systems.

   This is the technique used in the authors' laboratory. It removes the nonlinear steps involved in film exposure and developing, but traditional use of X-ray film and densitometry techniques (see below) are also effective, if the film is exposed correctly. With either technique, pADPr standards should be used to ensure that quantification is linear and accurate.

11b. *Quantify by densitometry:* Use standard X-ray densitometry techniques (*UNIT 6.3*) to quantify the signal from the secondary antibody.

Quantification of pADPr by Enzyme-Linked Immunosorbant Assay

The enzyme-linked immunosorbent assay (ELISA) is an interesting alternative method to the immunodot blot procedure (see Basic Protocol 2) for quantification of the pADPr synthesized following DNA-damaging or apoptosis-inducing treatments. This technique was developed to provide a simple procedure to investigate pADPr metabolism without radiolabeled compounds. The conditions of fixation and washing to detect low cellular levels of pADPr were optimized in the authors laboratory (Affar et al., 1999a). Briefly, pADPr isolated from cell cultures are alkali digested and fixed in a 96-well microplate. This cellular pADPr is incubated with a specific anti-poly(ADP-ribose) primary antibody. Following incubation with appropriate secondary antibodies, the 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) coloration is measured and compared to a standard curve made with known amounts of DHBB-purified pADPr synthesized in vitro.

Additional Materials *(also see Basic Protocol 2)*

1 μg/ml poly-L-lysine in PBS-T_2
PBS (*APPENDIX 2A*)
DHBB-purified pADPr samples (see Basic Protocol 1) in PBS-T_2
PBS-T_2: PBS (*APPENDIX 2A*)/0.05% (w/v) Tween 20
PBS-MT_2 (see recipe)
Anti-pADPr primary antibody diluted in PBS-MT_2
Peroxidase-conjugated secondary antibody
ABTS/H_2O_2 solution (see recipe)

96-well microtiter plate (Falcon 3912)
Microtiter plate reader

Prepare a microtiter plate

1. Coat a 96-well microplate with 200 μl per well of 1 μg/ml poly-L-lysine in PBS-T_2.
2. Incubate the plate overnight at 4°C.
3. Wash the plate twice with 200 μl/well PBS for 5 min each.

Add sample

4. Add each DHBB-purified pADPr sample prepared in PBS-T_2 (final volume 50 μl) to a well.
5. Incubate samples overnight at 37°C.
6. Wash the plate three times with 200 μl/well PBS-T_2 for 2 min each.

Block plate

7. Saturate the plate for 1 hr with PBS-MT_2 at 37°C.

   This blocking step should be done with 250 μl per well of PBS-MT_2 to efficiently saturate unoccupied binding sites.

8. Remove PBS-MT_2 from the plate and add 50 μl/well of anti-pADPr primary antibody diluted in PBS-MT_2.

   Generally, the authors use LP96-10 polyclonal antibody at a dilution of 1:5000. Other investigators have used monoclonal 10H antibody at a dilution of 1:250. The LP96-10 antibody is preferred because some studies have revealed that the 10H monoclonal antibody fails to recognize poly(ADP-ribose) smaller than 25 residues (also see Critical Parameters).

Incubate plate with antibody

9. Incubate pADPr samples 1 hr with the primary antibody at 37°C.
10. Wash the plate three times with 200 µl/well PBS-T_2 for 2 min each.
11. Dilute an appropriate peroxidase-conjugated secondary antibody 1:1000 in PBS-MT_2.
12. Incubate the plate 30 min with 50 µl/well secondary antibody at 37°C.
13. Discard the secondary antibody and wash the plate as follows:

    Three times with 200 µl/well PBS-T for 2 min each
    Twice with 200 µl/well PBS for 5 min each.

    At this stage, it is important to remove all traces of Tween 20 because it can interfere with the peroxidase assay.

Visualize bound antibody

14. Add 100 µl ABTS/H_2O_2 solution to each well and let the color develop 15 min at room temperature.
15. Read the absorbance at 405 nm using a microtiter plate reader.

BASIC PROTOCOL 3

High-Resolution Gel Electrophoresis and Immunodetection of pADPr

It has been observed that the size distribution of pADPr varies between unstimulated cells and cells subjected to DNA-damaging agents (Malanga and Althaus, 1994). With this information in mind, it can be useful to measure the levels of pADPr synthesized following DNA-damaging treatments. In order to characterize the size distribution of pADPr synthesized in treated cells, the authors have developed a novel immunodetection method involving the transfer of electrophoresed pADPr onto a positively charged membrane, followed by blotting with specific anti-pADPr antibodies to evaluate the size distribution of pADPr synthesized in vivo (Affar et al., 1999a). All pADPr larger than octamers were detected with this technique. Careful analysis of the blot has shown that short polymers (3 to 8 residues) have a weak binding capacity for the positively charged membrane. This class of pADPr is lost during washing in the immunodetection procedure.

Materials

20% (w/v) polyacrylamide gel (see recipe)
Electrode buffer: 54 mM Tris-borate buffer (pH 8.3)/1.2 mM EDTA
Alkali-digested pADPr sample isolated from cell culture (see Basic Protocol 1, step 8) or lyophilized DHBB-purified sample (see Basic Protocol 1)
Modified Laemmli sample buffer (see recipe)
Transfer buffer: 35 mM Tris-borate buffer (pH 8.3)/1 mM EDTA
PBS-MT (see recipe)
Anti-pADPr primary antibody
Peroxidase-conjugated secondary antibody
PBS-T: PBS containing 0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*)
Chemiluminescence reagents for HRP-conjugated secondary antibodies
Purified pADPr standard (see Basic Protocol 1)

Nitrocellulose Hybond N^+ membrane (Amersham Bioscience)
Hair dryer
312-nm UV source
X-Omat blue film (Kodak)

Additional reagents and equipment for preparing and running acrylamide gels (*UNIT 6.1*), immunoblot detection (see Basic Protocols 2, steps 5 to 10, and (*UNIT 6.2*), and autoradiography and densitometry (*UNIT 6.3*)

Prepare polyacrylamide gel

1. Prepare a $20 \times 20 \times 0.15$–cm 20% polyacrylamide gel.
2. Prerun the gel 1 to 2 hr in electrode buffer at 400 V.

Prepare pADPr

3. *Optional:* Air dry the alkali-digested pADPr isolated from cell culture.
4. Dilute the dried polymer (i.e., from alkali digested pADPr or lyophilized DHBB-purified sample) in modified Laemmli sample buffer.

   Do not heat the sample as pADPr can be degraded at higher temperatures. The use of bromophenol blue and xylene cyanol as standards is useful since they co-migrate with poly(ADP-ribose) molecules of ~8 and 20 residues, respectively.

5. Load a volume of sample containing 25 to 50 pmol pADPr per lane on the prerun polyacrylamide gel (step 2).

   pADPr is quantitated by immunodot blot (Basic Protocol 2) or ELISA (Alternate Protocol 1).

Electrophorese sample

6. Replace the electrophoresis buffer and electrophorese the sample at a constant voltage of 400 V for a period varying with the pADPr size of interest.

   The ratio of acrylamide/bisacrylamide (19:1) is not the same as that usually prepared for the SDS-PAGE. Consequently, use the recipes given in Reagents and Solutions.

   If the size is unknown, run the gel until the bromophenol blue reaches 5 cm from the end of the gel.

7. Electrotransfer the sample onto a nitrocellulose Hybond N^+ membrane 1.5 hr in transfer buffer at a constant current of 0.4 A at room temperature (*UNIT 6.2*).
8. Dry the membrane with a hair dryer. Cross-link the pADPr by exposing the membrane to a 312-nm UV source for 5 min.

Immunoblot and detect pADPr

9. Incubate the membrane in 50 ml PBS-MT on a orbital platform shaker for at least 1 hr at room temperature.
10. Perform immunoblot detection with an appropriate anti-pADPr antibody as described (see Basic Protocol 2, steps 5 to 10).

    This procedure was optimized to immunodetect quantities of 1 pmol pADPr isolated from cells when using the LP96-10 (1:5000) as anti-pADPr antibody (see Critical Parameters). The authors noticed that, with this technique, the 10H (1:250) anti-pADPr antibody failed to detect pADPr below 25 mers.

11. Expose the membrane to X-Omat blue film (Kodak; *UNIT 6.3*) for an appropriate length of time.

BASIC PROTOCOL 4

Detection of Poly(ADP-Ribosyl)ated Proteins by Immunoblotting

Poly(ADP-ribosyl)ation is a post-translational modification occurring on glutamate residues of nuclear acceptor proteins, including PARP-1 itself. A large number of acceptor proteins have been identified in a variety of cellular processes. The existence of pADPr in intact cells is a transient event, because poly(ADP-ribose) glycohydrolase (PARG) rapidly catabolizes pADPr by virtue of its exo- and endo-glycohydrolase activities. Under normal growth conditions, cultured cells display low basal levels of polymer, which can dramatically increase when cells are exposed to DNA-damaging agents. On immunoblots, basal protein-bound pADPr generally appears in concentrated smears that originates at ~116 and 89 kDa, likely representing automodification of PARP-1 and its apoptotic fragment (Boyonoski et al., 2002a,b); however, other molecular weights are observed, and the patterns vary between tissues. As a complementary approach to determine the presence of active PARPs, the following protocol provides a simple assay to monitor the accumulation of pADPr in a cellular or tissue extract. This assay uses a specific anti-pADPr primary antibody to quantify the accumulation of pADPr covalently bound to proteins. This technique provides an estimation of the total quantity of pADPr, chain lengths, and molecular weights of major acceptor proteins.

Materials

Cells in appropriate medium *or* pretreated anesthetized animal
DNA-damaging agents, oxidative stress, or apoptosis-inducing stimulus
HeBS (*APPENDIX 2A*), ice cold
HeBS containing 1 mM PMSF and Complete protease inhibitor cocktail (Roche), ice cold
Extraction buffer (see recipe)
2× SDS sample buffer (*APPENDIX 2A*)
Reducing sample buffer (see recipe)
Ponceau S dye (see recipe)
PBS-MT (see recipe)
Anti-pADPr primary antibody
Peroxidase-conjugated secondary antibody
PBS-T: PBS containing 0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*)
Chemiluminescence reagents for HRP-conjugated secondary antibodies
Prestained molecular weight standards (optional)

Cell scraper (optional)
15-ml conical centrifuge tube
Sonicator
65°C water bath
0.45-µm nitrocellulose membrane (Amersham Bioscieince)
Orbital platform shaker
X-Omat blue film (Kodak)

Additional regents and equipment for cell trypsinization (*UNIT 1.1*; optional), gel electrophoresis (*UNIT 6.1*), electrotransfer (*UNIT 6.2*), and immunoblotting (see Basic Protocol 2, steps 5 to 10)

Prepare samples

For cultured cell samples

1a. Subject cells of interest in appropriate medium to DNA-damaging agents, oxidative stress, or apoptosis-inducing treatments to stimulate pADPr synthesis.

2a. Remove and discard the medium from the cell culture and quickly wash the monolayer with 5 ml ice-cold HeBS.

3a. Harvest adherent cells in ice-cold 5 ml HeBS containing 1 mM PMSF and Complete protease inhibitor cocktail. Use a cell scraper or trypsinization (*UNIT 1.1*) to completely release cells from the dish.

The protease inhibitors may be excluded if direct immunoblotting is to be carried out.

Prepare tissue sample

1b. Remove tissues of interest as rapidly as possible from anesthetized pretreated animals. Flash freeze in liquid nitrogen and store up to 1 year at −80°C.

2b. Add chunks of frozen tissue to ice-cold extraction buffer. Homogenize on ice using a power unit.

The authors use ~0.25 g of tissue/ml buffer.

3b. Prepare ~100 to 200 µl tissue homogenate per milliliter of 2× SDS sample buffer and proceed immediately to step 9.

Lyse cells

4. Transfer harvested cultured cells to a 15-ml conical centrifuge tube and centrifuge 10 min at 600 × *g*, 4°C.

5. Wash the cell pellet twice with 5 ml of ice-cold HeBS, centrifuging as described (step 4).

6. Remove supernatant and lyse cells in reducing sample buffer at a concentration of 2×10^6 cells per milliliter.

The presence of urea in the sample buffer favors the dissociation of PARP-1 from DNA.

7. Sonicate resuspended cells on ice for three 20-sec bursts at 40% maximal output to shear the DNA.

8. Immediately freeze sample in liquid nitrogen and store up to 3 months at −80°C. Alternatively, proceed immediately to step 9.

This procedure was designed to obtain a cell extract inactivated as quickly as possible, because the pADPr is rapidly degraded by poly(ADP-ribose) glycohydrolase in whole cells.

Electrophorese sample

9. Incubate samples 15 min in a 65°C water bath.

10. Load a volume of sample equivalent to $1–2 \times 10^5$ cells or 20 µg protein/well onto a 6 × 8–cm 8% resolving SDS-polyacrylamide minigel (*UNIT 6.1*).

Do not forget to add appropriate molecular weight standards at this stage.

11. Electrophorese the gel (*UNIT 6.1*) and electrotransfer (*UNIT 6.2*) samples onto a 0.45-µm nitrocellulose membrane.

12. Stain the membrane with Ponceau S dye for 1 min and then wash extensively in deionized water to remove all residual dye.

This step is useful to identify molecular weight standards, confirm equal transfer of proteins, and visualize the protein profile of the samples. This step can be excluded if prestained molecular weight standards are used, although prestained standards are also less accurate if a close identification of molecular weight is required.

13. Incubate the membrane at least 1 hr in 50 ml PBS-MT on an orbital platform shaker at room temperature to block unoccupied protein-binding sites.

Immunoblot

14. Perform immunoblot detection with an appropriate anti-pADPr antibody as described (see Basic Protocol 2, steps 5 to 10) except incubate the membrane in anti-pADPr primary antibody at 4°C rather than room temperature (see Basic Protocol 2, step 5).

15. Expose the membrane to X-Omat blue film (Kodak; *UNIT 6.3*) for an appropriate length of time.

BASIC PROTOCOL 5

Immunocytochemical Detection of pADPr

Some investigators have proposed that the synthesis of pADPr could be a useful parameter to identify apoptotic cells (Donzelli et al., 1997; Scovassi and Poirier, 1999; Chang et al., 2002). The combination of pADPr synthesis with other apoptotic markers and the presence of PARP-1 cleavage (see Alternate Protocol 2) facilitates the determination of apoptosis in cell culture. PADPr synthesis is also a sensitive indicator of DNA damage from a variety of sources, such as alkylating agents and oxidative stress. The following procedure provides a sensitive and rapid method for identification of endogenous cellular pADPr synthesis, following DNA damage, by immunocytochemistry (see Fig. 18.7.1B). Alternatively, this procedure can be used to localize PARP family members in cultured cells.

Materials

Cells of interest in appropriate medium
DNA-damaging stimulus (e.g., 100 μM MNNG, 200 μM H_2O_2)
PBS (*APPENDIX 2A*), ice cold
70:30 (v/v) methanol/acetone, ice cold
PBS containing 1% (w/v) Triton X-100 and 10% (v/v) FBS
Anti-pADPr primary antibody
Fluorophore-conjugated secondary antibody
Fluoromount-*G* (Southern Biotech)

Coverslips and slides
Humid chamber (i.e., a petri dish lined with a wet paper towel)

Additional reagents and equipment for fluorescence microscopy (*UNIT 4.2*)

1. Grow cells of interest in appropriate medium on coverslips and expose them to DNA-damaging stimulus.

   Most DNA-damaging agents, oxidative stress, and apoptosis-inducing treatments can induce pADPr synthesis in different cell types. The authors prefer to treat mouse fibroblast C3H10T½ cells either with 100 μM MNNG for 15 min or with 200 μM hydrogen peroxide for 15 min. These two methods both give good results.

2. Wash the cells with 2 ml ice-cold PBS.

3. Fix cells by incubating them 15 min on ice in 2 ml ice-cold 70:30 methanol/acetone.

   A large number of fixation methods can be used at this stage. The authors have also obtained good results when cells were fixed for 10 min on ice with 10% ice-cold TCA (Lankenau et al., 1999); however, methanol/acetone fixation is preferred because traces of residual TCA may alter the integrity of the antibody used to detect pADPr.

4. Remove the methanol/acetone solution and wash four to five times with 2 ml ice-cold PBS.

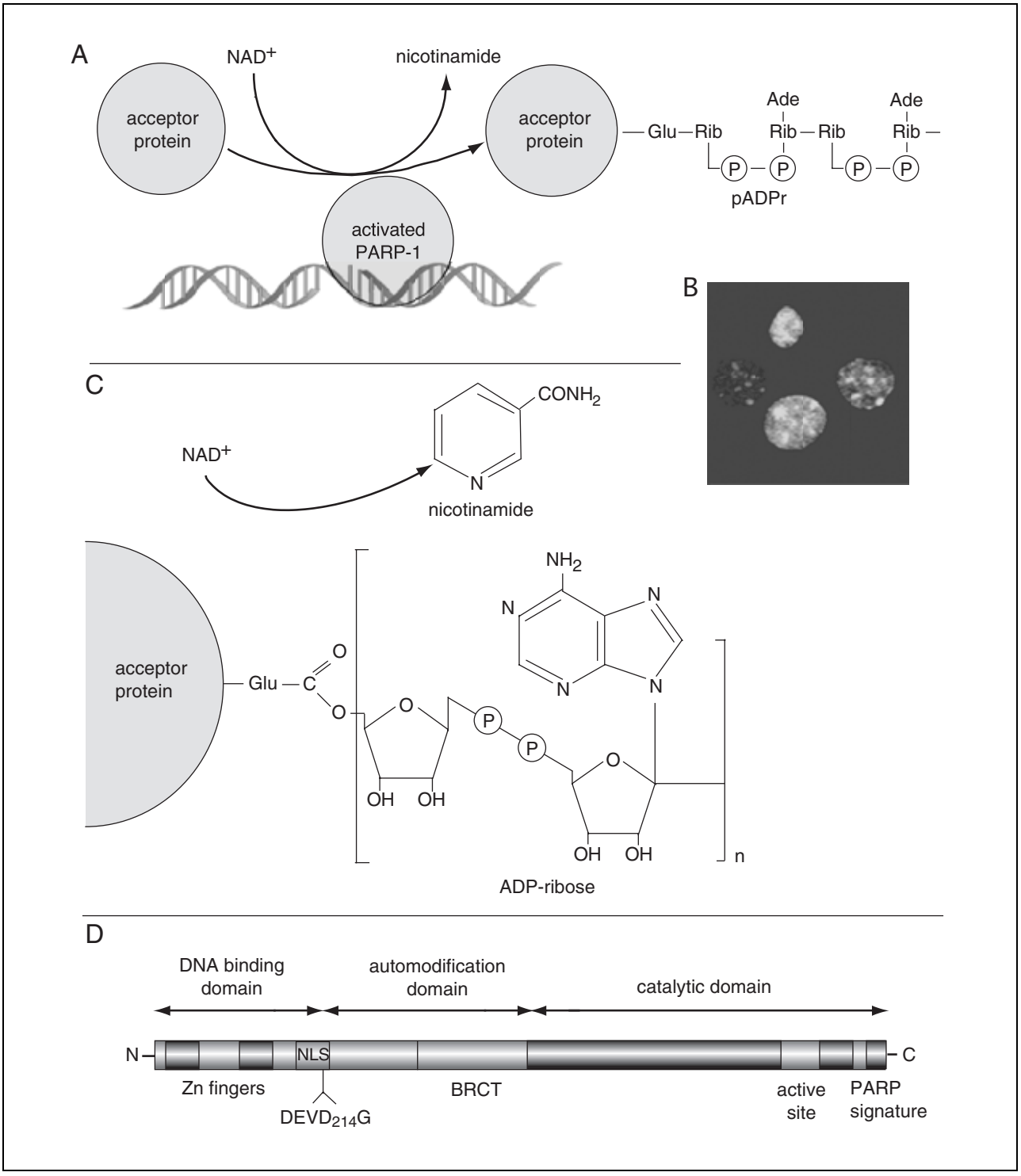


Figure 18.7.1 Poly(ADP-ribosyl)ation reaction and poly(ADP-ribose) polymerase-1 (PARP-1) structure. (**A**) PARP-1 is selectively activated by DNA strand breaks and transfers ADP-ribose from NAD^+ to a set of acceptor proteins. (**B**) Immunocytochemical detection of pADPr synthesized in mouse fibroblast C3H10T$_{1/2}$ cells exposed 15 min to 200 μM H_2O_2. Immunodetection was carried out with LP96-10 anti-pADPr polyclonal antibody (1:100) and revealed with 1:100 Texas Red–conjugated anti-rabbit second antibody (1:100). (**C**) The sequential addition of ADP-ribose units to a glutamic acid residue of an acceptor protein results in the formation of a long linear and branched chain of pADPr that is structurally similar to nucleic acids. (**D**) Schematic representation of PARP-1 domains. PARP-1 enzyme can be divided into three major domains (also see Fig. 18.7.2): a 46-kDa DNA binding domain, a central 22-kDa automodification region that contains the BRCT domain, and a 54-kDa C-terminal region that comprises the active site and the PARP signature. During caspase-dependent apoptosis, caspase-3 and -7 recognize a DEVD motif in the nuclear localization signal (NLS) and cleave PARP-1 into a p89 and a p24 fragment. (Adapted from the PARP link Web site: *http://parplink.u-strasbg.fr/index2.html*).

5. Permeabilize cells in 2 ml ml PBS containing 1% (w/v) Triton X-100 and 10% (v/v) FBS for 15 min at room temperature.

   FBS is used to block unspecific epitopes.

6. Remove permeabilizing solution and wash once with 2 ml ice-cold PBS.
7. Dilute the appropriate anti-pADPr primary antibody in PBS containing Triton X-100 and FBS according to supplier's instructions.
8. Incubate coverslips with 80 µl diluted primary antibody overnight at 4°C in a humid chamber.

   A petri dish lined with wet paper toweling is a suitable humid chamber.

9. Wash coverslips three to five times with 2 ml PBS.
10. Dilute an appropriate fluorophore-conjugated secondary antibody in PBS containing 1% Triton X-100 and 10% FBS according to supplier's instructions.
11. Incubate the coverslips 30 min with diluted secondary antibody at room temperature.

    This step should by done in darkness to avoid fluorochrome quenching.

12. Wash the coverslips several times with 2 ml PBS.
13. Mount coverslips on microscope slides with mounting medium (i.e., Fluoromount-*G*) according to manufacturer's instructions.

    Mounting slides with Fluoromount-G reduces fluorochrome quenching during analysis of slides by fluorescence microscopy. This mounting medium also provides a semipermanent seal for long-term storage of slides.

14. Allow mounted slides to air dry 5 min before analyzing the localization of cellular pADPr by fluorescence microscopy using an appropriate filter (*UNIT 4.2*).

DETECTION OF POLY(ADP-RIBOSE) POLYMERASE FAMILY MEMBERS IN CELLS

Poly(ADP-ribosyl)ation is a post-translational modification catalyzed mostly by the 116-kDa enzyme poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme that transfers an ADP-ribose moiety onto a limited number of nuclear proteins, including itself (D'Amours et al., 1999). When cells are exposed to environmental stresses such as alkylating agents or free radicals, there is up to a 500-fold increase in net pADPr synthesis in response to DNA strand breaks. The enzyme responsible for 80% to 90% of this stimulated pADPr synthesis is PARP-1, while other PARPs are responsible for the remaining 10% to 20%. The physiological meaning of these phenomena is not clear; however, it can be interpreted as a way of translating an event occurring on DNA to the nucleus by protein modification and finally to cytoplasm via NAD^+ depletion (Chiarugi, 2002). It has also been proposed that the presence of negatively charged pADPr at the site of DNA damage may play several roles in regulation of base excision repair, p53 functions, and apoptosis.

In the past few years, several additional proteins catalyzing poly(ADP-ribosyl)ation have been identified (Fig. 18.7.2). These new members of the PARP family (Shall, 2002) include PARP-2, PARP-3, vPARP (PARP-4), tankyrase-1 (PARP-5) and -2 (PARP-6), and finally Ti-PARP (PARP-7). However, recent characterization of PARP-2 demonstrated that it possesses a nuclear distribution similar to PARP-1. PARP-2 also possesses an N-terminal DNA-binding domain and is activated in response to DNA damage (Ame et al., 1999). The similarities between the activity and functions of PARP-1 and -2, and the fact that PARP-1 knockout cells still have the ability to undergo apoptosis, suggest that PARP-2

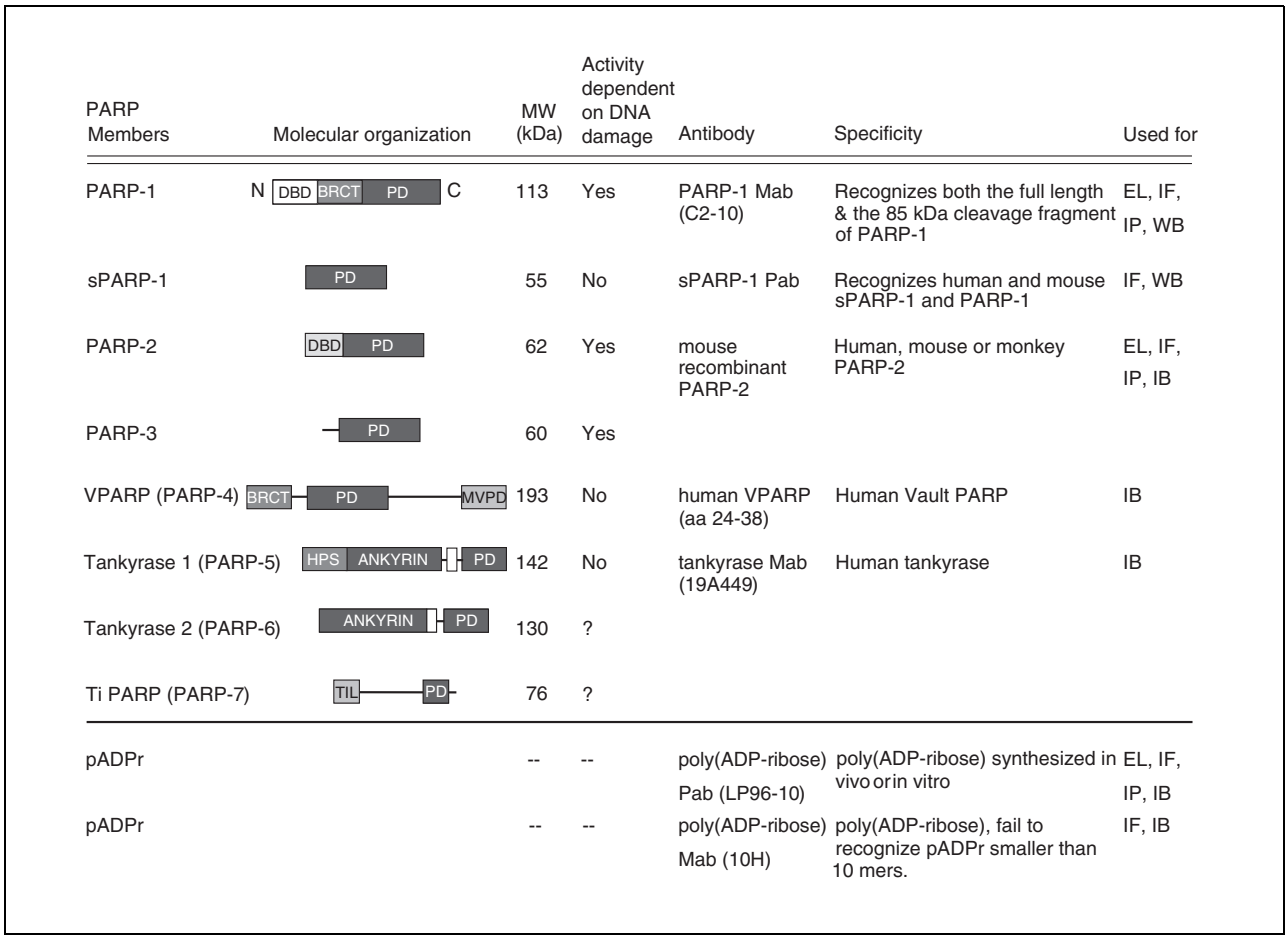


Figure 18.7.2 Poly(ADP-ribose) plolymerase family members and their antibodies. Abbreviations: EL, ELISA; IB, immunoblotting; IF, immunofluorescence; IP, immunoprecipitation.

may also play a role in apoptosis. Recent data suggest that PARP-3 is involved in centrosome duplication and cell cycle progression. The nucleoplasmic PARPs tankyrase-1 and -2 possess an N-terminal ankyrin domain and a C-terminal PARP domain and their PARP domains contain all of the amino acids necessary for catalysis (Cook et al., 2002). These PARPs have been described as being strand-break independent and are probably not responsive to DNA damage. A predominantly cytoplasmic PARP, vPARP, is a component of vault particles (Kickhoefer et al, 1999), but it is also observed in nuclear locations at some points of the cell cycle.

Detection of Poly(ADP-Ribose) Polymerase Enzymes by Immunoblot

BASIC PROTOCOL 6

Recently, identification of novel poly(ADP-risosyl)ating enzymes with distinct structures has led to a reconsideration of the role of pADPr in a large number of cellular process, such as DNA damage response, genomic stability, apoptosis, and chromatin modulation. PARP-1 has been shown to be stimulated directly or indirectly by free radicals or agents causing free radical release (Herceg and Wang, 2001). It is likely that PARP-1 is involved in the response to several different types of cell toxicity, because it can bind with very high affinity to double- or single-stranded breaks, and it consumes the NAD^+ pool in cells following genotoxic insults. Immunoblotting provides a useful method for the detection of various PARPs in cultured cells. Detailed below is a protocol for detection of PARP-1, but this procedure can also be applied to the detection of any PARP family member when an appropriate primary antibody is used (Fig. 18.7.2).

Materials

Tissue, fresh
Extraction buffer (see recipe), ice cold
2× SDS sample buffer (*APPENDIX 2A*)
HeBS (*APPENDIX 2A*), ice cold
Reducing sample buffer (see recipe)
Anti-PARP primary antibody
PBS-MT (see recipe) with and without 1 mM NaN_3
Horseradish peroxidase–conjugated secondary antibody
PBS-T: PBS/0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*)
Chemiluminescence reagents for HRP-conjugated secondary antibodies

Glass homogenizer with Teflon pestle
15-ml conical centrifuge tube
Sonicator
65°C water bath
0.45-µm nitrocellulose membrane
Orbital platform shaker
X-Omat blue film (Kodak)

Additional reagents and equipment for preparation of cell extracts (see Basic Protocol 4, steps 2 to 11), electrophoresis (*UNIT 6.1*), electrotransfer (*UNIT 6.2*), and autoradiography (*UNIT 6.3*)

Prepare tissue extracts

1. Homogenize fresh tissue in ice-cold extraction buffer using 10 to 20 strokes of a Teflon pestle in a glass homogenizer. Dilute the homogenized tissue to a final concentration of 2.5 to 5 mg/ml by adding enough 2× SDS sample buffer.

   A small aliquot should be kept after homogenization for subsequent protein determination.

   Alternatively, frozen tissue can be pulverized in liquid nitrogen using a mortar and pestle and then suspended in extraction buffer and diluted in 2× SDS sample buffer. Note that frozen tissues may display partial proteolysis of enzymes and give poor results.

Prepare cell extracts, electrophorese and transfer

2. Perform cell extraction, electrophoresis, and transfer as described above (see Basic Protocol 4, steps 2 to 11).

Immunoblot

3. Dilute an appropriate anti-PARP primary antibody according to the supplier's recommendation in PBS-MT supplemented with 1 mM sodium azide. Incubate along with the membrane on an orbital platform shaker overnight at room temperature.

   A large number of antibodies against PARP-1 are commercially available. The monoclonal antibodies F1-23 (BioMol Research) and the C2-10 (BioMol Research) recognize the N-terminal region of PARP-1, which is not present in other PARP. Thus, they are currently used for specific detection of PARP-1. In mouse tissues, where blood contamination is frequent, the authors recommend the use of LP96-72 (BioMol Research), polyclonal antibody raised against affinity-purified PARP-1; however, in all cases, it is often useful to test the conditions of immunoblotting to obtain optimal results.

   C-2-10 is a mouse monoclonal antibody generated by immunization of mice with purified calf thymus PARP-1 that recognizes full-length (116 kDa) PARP as well as the 85 to 90 kDa apoptosis-related cleavage fragment of PARP.

4. Wash the membrane three times with 50 ml PBS-MT for 15 min each.

5. Dilute an appropriate horseradish peroxidase-conjugated secondary antibody in PBS-MT according to supplier's recommendations.
6. Incubate the membrane with 50 ml secondary antibody on a orbital platform shaker for 30 min at room temperature.
7. Discard the secondary antibody and wash the membrane as follows:

   Three times with 50 ml PBS-MT for 10 min each
   Three times with 50 ml PBS-T for 15 min each
   Twice with 50 ml PBS for 5 min each.

8. Analyze the blot using chemiluminescence reagents for HRP-conjugated secondary antibodies according to the manufacturer's instructions.

   When extracts from limited quantities of cells are used for the immunoblotting procedure, the authors recommend using of the SuperSignal West Dura Extended Duration Substrate system (Pierce) for low signal detection.

9. Expose the membrane to X-Omat blue film (*UNIT 6.3*) for an appropriate length of time.

Detection of PARP-1 Cleavage During Cell Death

ALTERNATE PROTOCOL 2

It may be useful to monitor several apoptotic parameters in a unique sample. Recently, several authors have reported that poly(ADP-ribosyl)ation plays a critical role in the apoptotic process (Soldani and Scovassi, 2002). They suggested that proteolysis of PARP-1 is a sensitive marker for identifying apoptotic cells (Scovassi and Poirier, 1999). During apoptosis induced by a variety of chemotherapeutic agents, two caspases, caspase-3 and -7, cleave PARP-1 into its apoptotic 24- and 89-kDa fragments (Germain et al., 1999). PARP-1 was among the first caspase substrates to be identified. In particular, its cleavage by caspase-3 is well documented. The appearance of the 89-kDa proteolytic product is an early event in the apoptotic process (Kaufmann et al., 1993). It displays DNA-independent enzyme activity, and it may be poly(ADP-ribosyl)ated and thus recognized by an anti-polymer antibody in a variety of processes that lead to cell death. This protocol describes a method for the detection of PARP-1 cleavage that can also be used for the detection of cleavage of other caspase substrates.

Prepare apoptotic cell extracts

1. Treat cells of interest with an appropriate apoptosis-inducing agent and incubate for a sufficient length of time.

   For example, 1×10^6 HL-60 human promyelocytic leukemia cells may be treated with 68 μM etoposide for 0 to 12 hr. This procedure constitutes a positive control for immunoblotting of caspase cleavage targets including PARP-1 in etoposide-induced apoptosis. A variety of apoptosis-inducing agents can induce the cleavage of PARP-1, although with different kinetics; therefore, the incubation time producing maximal PARP-1 cleavage must be determined for each of these agents.

2. After drug treatment, remove and discard medium from cell culture. Quickly wash the monolayer with 5 ml ice-cold HeBS.
3. Harvest adherent cells and prepare apoptotic cell extracts as described (see Basic Protocol 4, steps 4 to 8).

   The apoptotic cell extract may be stored at –30°C or –80°C at this stage or directly processed on an SDS-PAGE gel (UNIT 6.1).

Electrophorese and immunoblot

4. Perform electrophoresis and immunoblotting of samples as described (see Basic Protocol 4, steps 9 to 16).

BASIC PROTOCOL 7

Nonisotopic-Activity Immunoblot Detection of Poly(ADP-Ribose) Polymerases

This technique involves automodification of nitrocellulose-immobilized poly(ADP-ribose) polymerases using nonisotopic NAD^+ as the substrate. Detection of pADPr synthesis is done using anti-pADPr antibodies to immunoblot. This technique may be useful to study the activity of apoptosis-associated fragments of PARPs that remain active upon cleavage by caspases. The use of anti-pADPr antibodies to detect polymer synthesis on nitrocellulose-immobilized PARPs enables the detection of these enzymes isolated from many species for which anti-polymerase antibodies are not available. In brief, this procedure is divided into four major steps: (1) reduction of oxidized cysteines in the gel, (2) renaturation of proteins resolved on nitrocellulose membrane, (3) synthesis of polymer, and (4) detection of pADPr by 10H or LP96-10 antibodies.

Materials

Tissue, fresh
Extraction buffer (see recipe), ice cold
2× SDS sample buffer (*APPENDIX 2A*)
HeBS (*APPENDIX 2A*), ice cold
Reducing sample buffer (see recipe)
6 × 8–cm 8% or 10% resolving SDS-polyacrylamide minigel (*UNIT 6.1*)
Prestained or biotinylated molecular weight standards
Running buffer (*UNIT 6.1*) containing freshly added 0.7 M 2-mercaptoethanol
Renaturation buffer (see recipe) with and without 100 μM NAD
Nonisotopic-activity immunoblot reducing buffer (see recipe)
PBS-MT (see recipe)
Anti-pADPr primary antibody
Horseradish peroxidase–conjugated secondary antibody
PBS-T: PBS/0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*)
Chemiluminescence reagents for HRP-conjugated secondary antibodies

Glass homogenizer with Teflon pestle
15-ml conical centrifuge tube
Sonicator
65°C water bath or heat block
Orbital platform shaker
X-Omat blue film (Kodak)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and autoradiography (*UNIT 6.3*)

Prepare cellular and tissue extracts

1. Homogenize fresh tissue in ice-cold extraction buffer using 10 to 20 strokes of a Teflon pestle in a glass homogenizer. Dilute the sample to a final concentration of 2.5 to 5 mg protein/ml in 2× SDS sample buffer.

   A small aliquot should be kept after homogenization for subsequent protein determination.

   Alternatively, frozen tissue can be pulverized in liquid nitrogen using a mortar and pestle then suspended in extraction buffer and diluted in 2× SDS sample buffer. Note that frozen tissues may display partial proteolysis of enzymes and give poor results.

Lyse cells

2. Transfer cells to a 15-ml conical centrifuge tube and centrifuge 10 min at 600 × *g*, 4°C.
3. Wash the cell pellet twice with 5 ml of ice-cold HeBS, centrifuging as described (step 2).
4. Remove supernatant and lyse cells in reducing sample buffer at a concentration of 2 × 10^6 cells per milliliter.

   The presence of urea in the sample buffer favors the dissociation of PARP-1 from DNA.

5. Sonicate resuspended cells on ice for three 20-sec bursts at 40% maximal output to shear the DNA.
6. Immediately freeze sample in liquid nitrogen and store up to 6 months at −80°C. Alternatively, proceed immediately to step 7.

Electrophorese and transfer

7. Incubate sample 15 min in a 65°C water bath or heat block.
8. Load a volume of sample equivalent to 75,000 cells or 20 µg protein in a well of a 6 × 8–cm 8% or 10% resolving SDS-polyacrylamide minigel. Also load prestained or biotinylated standards.

   Prestained or biotinylated molecular weight standards are used to avoid staining under acidic conditions, which may be detrimental to the polymerase activity (step 11).

9. Electrophorese gel as for a classical SDS-PAGE (*UNIT 6.1*).
10. After electrophoresis, soak gel 1 hr in running buffer containing freshly added 0.7 M 2-mercaptoethanol at 37°C.

    This treatment is necessary to avoid cross-linking between proteins and reduction of the cysteines present in poly(ADP-ribose) polymerases.

11. Transfer proteins to a nitrocellulose membrane as for a classical immunoblotting (*UNIT 6.2*).

    Do not stain the membrane with Ponceau S dye.

Renature proteins

12. Incubate the membrane on an orbital platform shaker 1 hr at room temperature in renaturation buffer without NAD.

    This step is important because it allows the restoration of the active PARP conformation.

13. Soak the nitrocellulose membrane on an orbital platform shaker for 1 hr at room temperature in 50 ml of renaturation buffer with 100 µM NAD.
14. Wash the membrane in 50 ml renaturation buffer without NAD four times for 15 min each at room temperature.
15. Wash the membrane in 50 ml nonisotopic-activity immunoblot reducing buffer to remove noncovalently bound pADPr.

Immunoblot

16. Wash the membrane three times with 50 ml PBS-MT for 15 min each.
17. Dilute an appropriate horseradish peroxidase–conjugated secondary antibody in PBS-MT.

18. Incubate the membrane with 50 ml of an appropriate horseradish peroxidase–conjugated secondary antibody on a orbital platform shaker for 30 min at room temperature.

19. Discard the secondary antibody and wash the membrane as follows:

    Three times with 50 ml PBS-MT for 10 min each
    Three times with 50 ml PBS-T for 15 min each
    Twice with 50 ml PBS for 5 min each.

20. Analyze the blot using chemiluminescence reagents for HRP-conjugated secondary antibodies according to the manufacturer's instructions.

    When extracts from limited quantities of cells are used for the immunoblotting procedure, the authors recommend using the SuperSignal West Dura Extended Duration Substrate system (Pierce) for low signal detection.

21. Expose the membrane to X-Omat blue film (*UNIT 6.3*) for an appropriate length of time.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AAGE9 buffer

250 mM ammonium acetate, pH 9.0
6 M guanidine hydrochloride
10 mM EDTA
Store 6 months at room temperature

ABTS/H_2O_2 solution

Just before use, prepare 100 ml citrate-phosphate buffer by dissolving 0.103 g anhydrous citric acid (MW 192.1) and 0.108 g anhydrous dibasic sodium phosphate in 100 ml water. Adjust to pH 4.6 with 1 N HCl. To this buffer, add 50 mg of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 50 µl of 30% (v/v) hydrogen peroxide.

Acrylamide/bisacrylamide (19:1), 30% (w/v)

28.5 g acrylamide
1.5 g bis(acrylamide)
100 ml H_2O
Store for a maximum of 4 weeks at 4°C in a brown bottle.

Extraction buffer

50 mM glucose
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
10 mM EDTA
1 mM PMSF
1× Complete protease inhibitor cocktail (Roche)
Prepare fresh

Modified Laemmli sample buffer

6 M urea
25 mM NaCl
4 mM EDTA
0.02% (w/v) xylene cyanol
0.02% (w/v) bromophenol blue
Store up to 6 months at room temperature

Nonisotopic-activity immunoblot reducing buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
100 mM NaCl
1 mM dithiothreitol (DTT)
2% (w/v) SDS
Prepare fresh

PBS-MT

PBS (*APPENDIX 2A*)
5 % (w/v) nonfat dried milk
0.1% (v/v) Tween 20
Prepare fresh

PBS-MT$_2$

PBS, pH 7.4 (*APPENDIX 2A*)
5% (w/v) nonfat dried milk
0.05% (v/v) Tween 20
Prepare fresh

Polyacrylamide gel, 20% (w/v)

100 ml 30% 19:1 (w/v) acrylamide/bisacrylamide (see recipe)
30 ml TBE electrophoresis buffer 5× (see recipe)
19 ml H_2O
Store up to 4 weeks at 4°C
Just before use add 0.75 ml 10% (w/v) ammonium persulfate (APS)
0.06 ml *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)

Ponceau S dye

2 g Ponceau S
30 g trichloroacetic acid (TCA)
30 g sulfosalicylic acid
100 ml H_2O
Store up to 1 year at room temperature

Reducing sample buffer

62.5 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
4 M urea
10% (v/v) glycerol
2% (w/v) SDS
0.00125% (w/v) bromophenol blue
Store up to 6 months at room temperature
Just before use, add 2-mercaptoethanol to 5% (w/v)

Renaturation buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
100 mM NaCl
1 mM dithiothreitol (DTT)
0.3% (v/v) Tween 20
2 mM $MgCl_2$
Prepare fresh

TBE electrophoresis buffer, 5×

54 g Tris base (890 mM)
27.5 g boric acid (890 mM)
980 ml H_2O
20 ml 0.5 M EDTA, pH 8.0 (20 mM final; see *APPENDIX 2A*)
Store up to 6 months at room temperature

TBE stands for Tris/borate/EDTA.

COMMENTARY

Background Information

PARP-1 structure and function

The enzyme poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) is a multifunctional enzyme that can be divided into three major domains (see Fig. 18.7.1D). A 41.5-kDa N-terminal region comprises the DNA binding domain (DBD), which contains two zinc-finger motifs and a nuclear localization signal (NLS). This region recognizes both double- and single-stranded DNA breaks in a nonsequence-dependent manner through the first and second zinc finger, respectively. A 16-kDa central automodification domain contains fifteen highly conserved glutamate residues thought be the targets of self-poly(ADP-ribosyl)ation, and a 55.4-kDa C-terminal region contains both the NAD binding site and the catalytic domain which synthesizes poly(ADP-ribose) (pADPr). The primary structure of the enzyme is highly conserved in eukaryotes (D'Amours et al., 1999; Pieper et al., 1999; Burkle, 2001; Herceg and Wang, 2001; Virag and Szabo, 2002).

Poly(ADP-ribosyl)ation is a post-translational modification of nuclear proteins, in which PARP-1 itself is the main poly(ADP-ribosyl)ated protein in vivo. PARP-1 is selectively activated by DNA strand breaks to catalyze the addition of pADPr from its substrate, nicotinamide adenine dinucleotide (NAD), to a set of acceptor proteins (see Fig. 18.7.1A). After initiation, PARP catalyzes elongation and branching reactions to synthesize a highly branched and complex structure of over 200 ADP-ribose residues into a large homopolymer that is structurally similar to nucleic acids (see Fig. 18.7.1C). The massive automodification of PARP-1 dramatically changes its physical properties. Thus, PARP-1 molecules carrying long, highly negatively charged, branched ADP-ribose polymers lose their affinity for DNA and are then inactivated. The automodification process represents a major regulatory mechanism leading to the down-regulation of the enzyme activity (reviewed in Lindahl et al., 1995).

Signaling function of pADPr

PADPr that is synthesized in response to massive DNA damage has a short half-life. It is rapidly hydrolyzed at ribose-ribose bonds and converted to free ADP-ribose units by the enzyme poly(ADP-ribose) glycohydrolase (PARG; Davidovic et al., 2001). The rapid response of PARG to pADPr synthesis indicates that pADPr degradation is also an important nuclear response to DNA damage. PARG constitutes an important component in the control of nuclear pADPr levels, which will in turn have direct effects on the activity of poly(ADP-ribosyl)ated proteins and on the levels of free pADPr. If there is a pADPr concentration threshold required to trigger apoptosis, the level at which PARG controls pADPr levels becomes critical for the decision point of apoptosis versus DNA repair and cell survival. The possible function of PARG in apoptosis and DNA repair is also supported by the fact that free pADPr generated by PARG may target many DNA damage checkpoints and DNA repair proteins. These cytoplasmic and nuclear proteins could interact strongly with free pADPr, by noncovalent interactions possibly mediated by a pADPr binding motif and alter their functions (Pleschke et al., 2000).

Poly(ADP-ribosyl)ation is involved in a variety of physiological events such as chromatin decondensation, DNA replication, DNA repair, apoptosis, and cellular differentiation (reviewed in Virag and Szabo, 2002). For example, poly(ADP-ribosyl)ation of histones decondenses chromatin structure, whereas subsequent degradation of the polymer restores chromatin to its condensed form. Relaxation of chromatin by poly(ADP-ribosyl)ation might facilitate the access of DNA regulatory and repair enzymes at sites of damage as well as at origins of replication and transcription initiation sites. In addition to modifying chromatin structure, PARP-1 has been shown to affect the function of several transcription factors, DNA replication factors and signaling molecules (i.e., NF-κB, AP-2, Oct-1, DNA-PK, p53, Topoi-

somerase-1, Lamin B) by noncovalent protein-protein interactions and by active poly(ADP-ribosyl)ation.

PARP-1 activation appears to be a sensitive marker of DNA damage at an early stage of the DNA damage response (see Fig. 18.7.3). Poly(ADP-ribosyl)ation is considered to be a major player in the cellular response to DNA damage because a large number of nuclear proteins are covalently modified with pADPr immediately following DNA breakage (Donzelli et al., 1997; Scovassi and Poirier, 1999; Duriez and Shah, 1997; Soldani and Scovassi, 2002). Moreover, the binding of PARP-1 to DNA strand breaks and the resulting synthesis of pADPr might constitute an emergency signal of damaged DNA to other compartments of cells, such as the mitochondrion (Chiarugi and Moskowitz, 2002).

PARP cleavage during cell death

When programmed cell death is activated, caspase-7 and -3 efficiently cleave and inactivate PARP-1 into a 24-kDa fragment containing the N-terminal DNA binding domain and a 89-kDa peptide comprising the central automodification domain, the C-terminal domain, and the catalytic domain (see Fig. 18.7.2). Proteolytic cleavage of PARP-1 into these two characteristic fragments was first recognized by Kaufmann and colleagues at an early stage of etoposide-induced apoptosis in treated human HL-60 cells (Kaufmann et al., 1993). The site of PARP-1 cleavage ($DEVD_{213}$ in human PARP-1) is located within the nuclear localization signal and is highly conserved. Separating the two zinc fingers from the catalytic domain renders the 89-kDa fragment insensitive to activation by additional DNA strand breaks, resulting in the inactivation of PARP-1 activity. However, several studies have shown that this 89-kDa apoptotic fragment can also serve as an early marker of apoptosis induced by a variety of chemotherapeutic agents.

The concept of PARP-mediated cell death following excessive DNA damage is supported by a number of studies that show prevention of cell death by specific PARP inhibitors and in mice deficient in PARP-1 (Shall and de Murcia, 2000; Burkle, 2001; Chiarugi, 2002; Virag and Szabo, 2002). For example, the PARP inhibitor, 3-aminobenzamide, has been shown to slow down the rejoining of DNA strand breaks and to increase the frequency of sister chromatid exchanges in MNNG-induced DNA damage (Oikawa et al., 1980). PARP inhibition also rendered cells more sensitive to cytotoxicity induced by DNA-damaging agents.

PARP-2

Studies from many laboratories have demonstrated the presence of additional proteins which are able to synthesize pADPr. This observation explains why PARP-1-deficient cells have some residual PARP activity. However, research on the biological role of these novel PARP enzymes is at an early stage. Moreover, some interesting differences in their structural domain, subcellular localization, and ability to be activated by DNA strand breaks have already been established (Fig. 18.7.2). PARP-1 is the best characterized of a growing family of enzymes; however, recent data have provided a model for the biological role of PARP-2 (Ame et al., 1999; Schreiber et al., 2002). This 62-kDa protein is capable of auto-poly(ADP-ribosyl)ation but could not poly(ADP-ribosyl)ate histones. Despite the fact that the BRCT domain is missing in PARP-2 and that the DNA binding domain (DBD) is very short, this enzyme also localizes to nuclei and is activated by DNA strand breaks. This led to the hypothesis that PARP-2 might be implicated in PARP-mediated cell death. Recently, PARP-2 has been demonstrated to be cleaved by caspase-8 in ischemia-induced neuronal apoptosis (Benchoua et al., 2002). Interestingly, cleavage of PARP-2 by caspase-8 dissociates the DNA binding domain (DBD) of PARP-2 from its catalytic domain, resulting in the inactivation of its DNA-dependent pADPr synthesis activity.

PARP-1 activation time course

Several investigators have examined the time course of PARP-1 activation and cleavage during apoptosis. They used immunofluorescence microscopy (see Basic Protocol 5 and Alternate Protocol 2) as well as immunoblot analysis with antibodies to PARP-1 and pADPr in intact cells or tissues (see Basic Protocols 4 and 6). The authors' laboratory has been actively involved in the establishment and improvement of several methods to study pADPr synthesis and catabolism (Shah et al., 1995; Affar et al., 1999a,b; Boucher et al., 2001). The development of standardized procedures to analyze pADPr synthesis and PARP-1 cleavage during cell death using in vitro models and in cultured cells yield specific tools to study many cell functions influenced by pADPr metabolism. One or more of the techniques presented

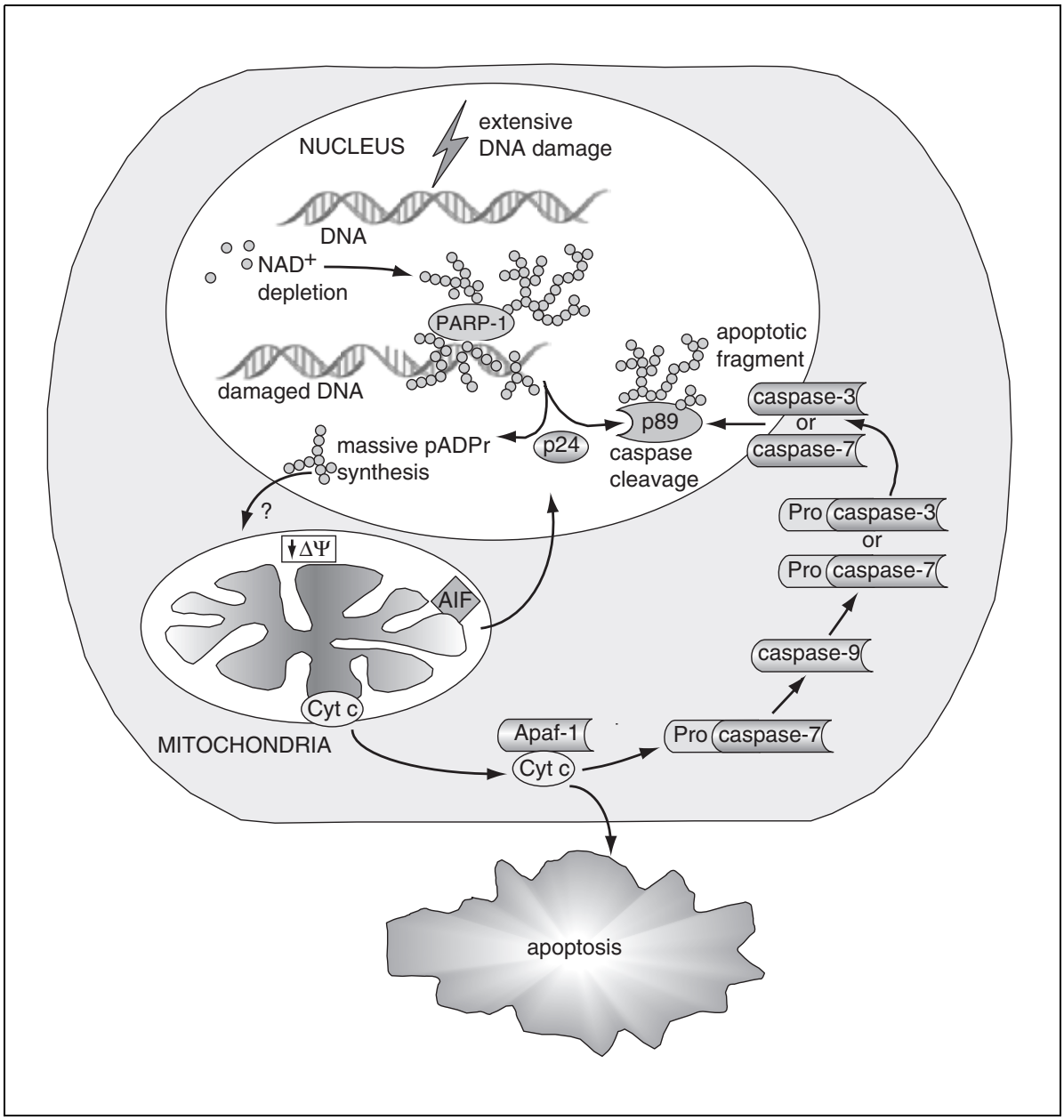


Figure 18.7.3 The concept of PARP-1-mediated cell death. Excessive DNA damage activates PARP-1 in the nucleus, leading to massive pADPr formation and resulting in a NAD^+ depletion. This high concentration of pADPr may trigger mitochondrial depolarization ($\downarrow\Delta\Psi$) and drive apoptosis through an unknown mechanism. Activated PARP-1 can mediate cell death by caspase-dependent and -delayed pathways. In the caspase-delayed pathway, the insults to DNA, produced by oxidative stress or DNA alkylating agents, induces the release of mitochondrial factors, such as AIF, that translocates to the nucleus and induces caspase-independent chromatin fragmentation into large 50-kDa fragments. However, in caspase-dependent apoptosis, caspase-3 and -7 cleave PARP-1 into two fragments: p89 and p24. Generation of 24-kDa apoptotic fragment contribute to PARP-1 inactivation. The early cleavage PARP-1 serves as preserving cellular ATP for the proper function of further energy-dependent stage of apoptosis.

in this unit has been used routinely for the detection of apoptotic events in numerous models of cell death. There are many reasons for using PARP-1 cleavage and pADPr synthesis as a sensitive assay for apoptosis. Although PARP-1 is not the only protein cleaved during apoptosis, it is a convenient tool to study caspase activity, and a wide range of detection reagents are currently available from various commercial suppliers. Nevertheless, most of the protocols presented in this unit can be used for studies of other PARPs in order to determine their possible role in cell death.

Critical Parameters

Immunodetection of pADPr

Several points should be considered when using the immunodot method to quantify the levels of pADPr in intact cells. To successfully quantify polymer levels in intact cells, a known concentration of pADPr sample should be used to make a standard curve. This will ensure a more accurate quantification. Quantities loaded should stay within the linear, or mildly curvilinear portion of the response curve. Measurement of polymer concentrations following DNA damage has been described in cultured cells (Affar et al., 1998), but quantitation in animal tissues may be more difficult (Boyonoski et al., 2002a,b). To efficiently quantify the concentration of pADPr in animal tissues, a known amount of protein-bound pADPr from rat liver samples, analyzed by HPLC methods, and/or automodified PARP-1 can be used as standards. These standards reflect chain length and branching similar to that found in other tissues from the whole animal. pADPr made in vitro and in many cultured cells appears to be larger and more complex than observed in vivo.

Although only a limited number of anti-pADPr antibodies are commercially available, monoclonal (10H) and polyclonal (LP96-10) anti-pADPr antibodies can serve for immunodetection of pADPr. The authors prefer LP96-10 antibody because some studies have revealed that the 10H monoclonal antibody fails to recognize pADPr smaller than 25 residues. The specificity of the anti-pADPr antibody LP96-10 or LP98-10 was confirmed in an in vitro competition study (Affar et al., 1999a), and it was shown that NAD, AMP, and ADP-ribose (10 nM) did not significantly interfere with antibody-pADPr binding. Cross-reactivity with nucleic acids is minimal with genomic and nicked DNA, transfer RNA, and total RNA, in contrast to the strong signal obtained with pADPr.

Detection of PARP-1 cleavage

The relationship between PARP-1 cleavage and apoptosis has been observed by a number of investigators. When immunoblotting detection methods are used for PARP-1 cleavage (see Alternate Protocol 2), one should always keep in mind the possibility of cross-reactivity between the anti-mouse IgG secondary antibodies and polypeptides present in cell lysates. Thus, the detection of PARP-1 cleavage should always be assessed along with morphological changes and DNA fragmentation observation, two other well defined markers of apoptosis. In cases where immunoblotting remains equivocal, it might even be necessary to use a PARP activity immunoblot (see Basic Protocol 7). When poly(ADP-ribose) polymerases are subjected to SDS-PAGE, transferred to nitrocellulose, renatured, and incubated with radioinert NAD, the synthesis of pADPr polymer can be detected using anti-pADPr antibodies. One advantage of this simple nonradioactive activity immunoblot technique is that it can detect any PARP activity in any sample.

Preparation of cell samples

Other critical parameters should be considered when preparing the cell extract. The pADPr synthesized following DNA damage has a very rapid turnover, due to its hydrolysis by PARG (see Basic Protocol 4). Consequently, work as quickly as possible during preparation of cell extracts to avoid the action of activated PARG. This can be achieved by retaining cell extracts on ice or using a specific inhibitor of the PARG enzyme. Note that the procedures described in this unit were optimized to have minimal pADPr degradation during processing of cell samples.

Anticipated Results

Quantitation of pADPr

Most cell lines have a basal level of pADPr in the range of 2 to 30 pmols/mg DNA or 10^8 cells (Affar et al., 1999b); however, in some cell lines, such as human keratinocytes, basal pADPr levels are ~50 to 65 pmols/mg DNA or 10^8 cells (Malanga and Althaus, 1994). Treatments with alkylating agents, such as MNNG, generally induce a 10- to 100-fold stimulation in pADPr synthesis following the formation of DNA strand breaks. The two quantitation methods described in this unit (see Basic Protocol 2

and Alternate Protocol 1), allow the detection of femtomolar concentrations of pADPr.

Immunodetection of pADPr

High-resolution gel electrophoresis, combined with immunodetection of pADPr (see Basic Protocol 3), provides a valuable method to quantify different sizes of pADPr in cultured cells. With this protocol, as little as 1 pmol pADPr may be detected. This high sensitivity can be used for monitoring changes in pADPr size distribution following DNA damage. Although Basic Protocol 2 and Alternate Protocol 1 could also serve to quantify the concentration of pADPr, these techniques do not provide much information about size distribution.

Immunocytochemical detection

The typical results obtained with immunocytochemical detection of pADPr are presented in Figure 18.7.1B. Generally, when mouse fibroblast C3H10T½ cells are grown on coverslips and stained for pADPr with pAb LP96-10, basal levels of polymer are not detected. However, the burst of pADPr synthesis, which occurs rapidly following DNA damage, peaks and can be visualized within 15 min (see Basic Protocol 5). The use of TCA as the method of fixation gives reliable and reproducible results, since pADPr is insensitive to TCA treatment. However, this method may be incompatible with colocalization experiments due to the possible loss of epitopic sites in some proteins. For pADPr colocalization experiments, methanol/acetone may be a better method of fixation.

Detection of PARP-1 Cleavage

It is generally accepted that PARP-1 proteolysis is a hallmark of caspase-dependent apoptosis. PARP-1 is cleaved by caspases 3 and 7 in the DEVD↑G_{214} during DNA damage–induced cell death. Both fragments can be easily recognized by immunoblot (see Alternate Protocol 2) with several antibodies. The visualization of PARP-1 and its apoptotic fragment is facilitated by the presence of urea in the sample buffer. However, due to the strong association of PARP-1 and DNA stands, it has been found that DNA-bound PARP-1 may be resistant to caspase-3 cleavage (D'Amours et al., 1998). The cleavage of PARP-1 remains a useful parameter to identify apoptotic cells when it is used in combination with other parameters like DNA fragmentation and morphological change.

Time Considerations

Preparation and quantification of pADPr

Several days (3 to 5) are necessary to perform accurate quantification of pADPr by ELISA or by immunodot blot. The entire quantification involves pADPr induction in cells, preparation of cell lysates, isolation and purification of pADPr from cell pellets, and quantification by immunological methods. Therefore, the process can be stopped at many specific steps and some steps can be done simultaneously. For example, during the induction of pADPr synthesis (~15 to 30 min) the DHBB resin could easily be equilibrated. The TCA precipitate cell pellets and lyophilized DHBB-purified samples can be stored for several months at −80°C. Also note that when the incubation time for the primary antibody is reduced to 2 hr in the immunodot blot procedure (see Basic Protocol 2, step 5) the signal is not sharp.

High-resolution gel electrophoresis

The entire electrophoresis and immunodetection procedure can be completed in 2 days.

After pre-electrophoresis of the 20% polyacrylamide gel, the time required to obtain a fine separation for each size class of pADPr is ~3 to 4 hr. The electrotransfer onto a nitrocellulose membrane takes 1.5 hr, blocking takes 1 hr, incubation with primary antibody is done overnight and finally, washes and incubation with secondary antibody takes ~2 hr. However, pADPr separated by high-resolution electrophoresis can be stained directly on the gel using GELCODE color silver stain kit (Pierce), a procedure less sensitive than the immunodetection method presented in Basic Protocol 3 but allowing the detection of polymer sizes up to 3 mers within the same day. This method has been improved by the group of Althaus for pADPr size quantification (Malanga et al., 1995).

Immunoblotting

This procedure usually takes more then a single working day to treat cells, prepare cell lysates, perform SDS-PAGE, transfer to nitrocellulose, and probe immunoblots. Therefore, samples could be prepared on the first day and the gels run on the second day. Here again, the incubation time for the primary antibody could be reduce to 2 hr. The process could also take advantage of minigels and a minitransfer apparatus, but note that the resolution of the smaller blots will be limited especially if the sample is highly complex.

Immunocytochemical detection of pADPr

The entire immunocytochemical detection of pADPr extends over 2 days. After cells are growing on coverslips, a 15-min DNA-damaging treatment will induce pADPr synthesis. The limiting step after treatment is the overnight incubation in primary antibody. Subsequently, second antibody incubation, washes, and microscopy analysis take no more than 1 hr.

Literature Cited

Aboul-Ela, N., Jacobson, E.L., and Jacobson, M.K. 1988. Labeling methods for the study of poly- and mono(ADP-ribose) metabolism in cultured cells. *Anal. Biochem.* 174:239-250.

Affar, E.B., Duriez, P.J., Shah, R.G., Sallmann, F.R., Bourassa, S., Kupper, J.H., Burkle, A., and Poirier, G.G. 1998. Immunodot blot method for the detection of poly(ADP-ribose) synthesized in vitro and in vivo. *Anal. Biochem.* 259:280-283.

Affar, E.B., Duriez, P.J., Shah, R.G., Winstall, E., Germain, M., Boucher, C., Bourassa, S., Kirkland, J.B., and Poirier, G.G. 1999a. Immunological determination and size characterization of poly(ADP-ribose) synthesized in vitro and in vivo. *Biochim. Biophys. Acta* 1428:137-146.

Affar, E.B., Shah, R.G., and Poirier, G.G. 1999b. Poly(ADP-ribose) turnover in quail myoblast cells: Relation between the polymer level and its catabolism by glycohydrolase. *Mol. Cell Biochem.* 193:127-135.

Alvarez-Gonzalez, R., Juarez-Salinas, H., Jacobson, E.L., and Jacobson, M.K. 1983. Evaluation of immobilized boronates for studies of adenine and pyridine nucleotide metabolism. *Anal. Biochem.* 135:69-77.

Ame, J.C., Rolli, V., Schreiber, V., Niedergang, C., Apiou, F., Decker, P., Muller, S., Hoger, T., Menissier-de Murcia, J., and de Murcia, G. 1999. PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. *J. Biol. Chem.* 274:17860-17868.

Benchoua, A., Couriaud, C., Guegan, C., Tartier, L., Couvert, P., Friocourt, G., Chelly, J., Menissier-de Murcia, J., and Onteniente, B. 2002. Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly(ADP-ribose) polymerase-2. *J. Biol. Chem.* 277:34217-34222.

Boucher, C., Gobeil, S., Samejima, K., Earnshaw, W.C., and Poirier, G.G. 2001. Identification and analysis of caspase substrates: Proteolytic cleavage of poly(ADP-ribose)polymerase and DNA fragmentation factor 45. *Methods Cell. Biol.* 66:289-306.

Boyonoski, A.C., Spronck, J.C., Gallacher, L.M., Jacobs, R.M., Shah, G.M., Poirier, G.G., and Kirkland, J.B. 2002a. Niacin deficiency decreases bone marrow poly(ADP-ribose) and the latency of ethylnitrosourea-induced carcinogenesis in rats. *J. Nutr.* 132:108-114.

Boyonoski, A.C., Spronck, J.C., Jacobs, R.M., Shah, G.M., Poirier, G.G., and Kirkland, J.B. 2002b. Pharmacological intakes of niacin increase bone marrow poly(ADP-ribose) and the latency of ethylnitrosourea-induced carcinogenesis in rats. *J. Nutr.* 132:115-120.

Burkle, A. 2001. Physiology and pathophysiology of poly(ADP-ribosyl)ation. *Bioessays* 23:795-806.

Chang, H., Sander, C.S., Muller, C.S., Elsner, P., and Thiele, J.J. 2002. Detection of poly(ADP-ribose) by immunocytochemistry: A sensitive new method for the early identification of UVB- and H_2O_2-induced apoptosis in keratinocytes. *Biol. Chem.* 383:703-708.

Chiarugi, A. 2002. Poly(ADP-ribose) polymerase: Killer or conspirator? The 'suicide hypothesis' revisited. *Trends Pharmacol. Sci.* 23:122-129.

Chiarugi, A. and Moskowitz, M.A. 2002. Cell biology. PARP-1—a perpetrator of apoptotic cell death? *Science* 297:200-201.

Cook, B.D., Dynek, J.N., Chang, W., Shostak, G., and Smith, S. 2002. Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres. *Mol. Cell Biol.* 22:332-342.

D'Amours, D., Germain, M., Orth, K., Dixit, V.M., and Poirier, G.G. 1998. Proteolysis of poly(ADP-ribose) polymerase by caspase 3: Kinetics of cleavage of mono(ADP-ribosyl)ated and DNA-bound substrates. *Radiat. Res.* 150:3-10.

D'Amours, D., Desnoyers, S., D'Silva, I., and Poirier, G.G. 1999. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem. J.* 342:2492-68.

Davidovic, L., Vodenicharov, M., Affar, E.B., and Poirier, G.G. 2001. Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism. *Exp. Cell Res.* 268:7-13.

Donzelli, M., Negri, C., Mandarino, A., Rossi, L., Prosperi, E., Frouin, I., Bernardi, R., Burkle, A., and Scovassi, A.I. 1997. Poly(ADP-ribose) synthesis: A useful parameter for identifying apoptotic cells. *Histochem. J.* 29:831-837.

Duriez, P.J. and Shah, G.M. 1997. Cleavage of poly(ADP-ribose) polymerase: A sensitive parameter to study cell death. *Biochem. Cell Biol.* 75:337-349.

Germain, M., Affar, E.B., D'Amours, D., Dixit, V.M., Salvesen, G.S., and Poirier, G.G. 1999. Cleavage of automodified poly(ADP-ribose) polymerase during apoptosis. Evidence for involvement of caspase-7. *J. Biol. Chem.* 274:28379-28384.

Herceg, Z. and Wang, Z.Q. 2001. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat. Res.* 477:97-110.

Kaufmann, S.H., Desnoyers, S., Ottaviano, Y., Davidson, N.E., and Poirier, G.G. 1993. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. *Cancer Res.* 53:3976-3985.

Kickhoefer, V.A., Siva, A.C., Kedersha, N.L., Inman, E.M., Ruland, C., Streuli, M., and Rome, L.H. 1999. The 193-kD vault protein, VPARP, is a novel poly(ADP-ribose) polymerase. *J. Cell. Biol.* 146:917-928.

Lankenau, S., Burkle, A., and Lankenau, D.H. 1999. Detection of poly(ADP-ribose) synthesis in Drosophila testes upon gamma-irradiation. *Chromosoma* 108:44-51.

Lindahl, T., Satoh, M.S., Poirier, G.G., and Klungland, A. 1995. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem. Sci.* 20:405-411.

Malanga, M. and Althaus, F.R. 1994. Poly(ADP-ribose) molecules formed during DNA repair in vivo. *J. Biol. Chem.* 269:17691-17696.

Malanga, M., Bachmann, S., Panzeter, S., Zweifel, B., and Althaus, F.R. 1995. Poly(ADP-ribose) quantification at the femtomole level in mammalian cells. *Anal. Biochem.* 228:245-251.

Oikawa, A., Tohda, H. Kanai, M., Miwa, M., and Sugimura, T. 1980. Inhibitors of poly(adenosine diphosphate ribose) polymerase induce sister chromatid exchanges. *Biochem. Biophys. Res. Commun.* 97:1311-1316.

Pieper, A.A., Verma, A., Zhang, J., and Snyder, S.H. 1999. Poly (ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol. Sci.* 20:171-181.

Pleschke, J.M., Kleczkowska, H.E., Strohm, M., and Althaus, F.R. 2000. Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *J. Biol. Chem.* 275:40974-40980.

Schreiber, V., Ame, J.C., Dolle, P., Schultz, I., Rinaldi, B., Fraulob, V., Menissier-de Murcia, J., and de Murcia, G. 2002. Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. *J. Biol. Chem.* 277:23028-23036.

Scovassi, A.I. and Poirier, G.G. 1999. Poly(ADP-ribosylation) and apoptosis. *Mol. Cell Biochem.* 199:125-137.

Shah, G.M., Poirier, D., Duchaine, C., Brochu, G., Desnoyers, S., Lagueux, J., Verreault, A., Hoflack, J.C., Kirkland, J.B., and Poirier, G.G. 1995. Methods for biochemical study of poly(ADP-ribose) metabolism in vitro and in vivo. *Anal. Biochem.* 227:1-13.

Shall, S. 2002. Poly (ADP-ribosylation)—a common control process? *Bioessays* 24:197-201.

Shall, S. and de Murcia, G. 2000. Poly(ADP-ribose) polymerase-1: What have we learned from the deficient mouse model? *Mutat. Res.* 460:1-15.

Soldani, C. and Scovassi, A.I. 2002. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: An update. *Apoptosis* 7:321-328.

Virag, L. and Szabo, C. 2002. The therapeutic potential of poly(ADP-Ribose) polymerase inhibitors. *Pharmacol. Rev.* 54:375-429.

Internet Resources

http://parplink.u-strasbg.fr/index2.html

The PARP Link Web Resource contains useful biological resources and pertinent information to the poly(ADP-ribose) polymerases (PARP) family of proteins and to their role in maintaining the integrity of the eukaryotic genome. This site offers instant access to the latest information and data on the PARP proteins and poly(ADP-ribosyl)ation and provides a valuable resource to the scientific community.

Contributed by Jean François Haince
and Guy G. Poirier
Laval University Medical Research Center
Ste.-Foy, Quebec, Canada

Jim B. Kirkland
University of Guelph
Guelph, Ontario, Canada

Figure 18.7.1 Poly(ADP-ribosyl)ation reaction and poly(ADP-ribose) polymerase-1 (PARP-1) structure. (**A**) PARP-1 is selectively activated by DNA strand breaks and transfers ADP-ribose from NAD^+ to a set of acceptor proteins. (**B**) Immunocytochemical detection of pADPr synthesized in mouse fibroblast C3H10T½ cells exposed 15 min to 200 μM H_2O_2. Immunodetection was carried out with LP96-10 anti-pADPr polyclonal antibody (1:100) and revealed with 1:100 Texas Red–conjugated anti-rabbit second antibody (1:100). (**C**) The sequential addition of ADP-ribose units to a glutamic acid residue of an acceptor protein results in the formation of a long linear and branched chain of pADPr that is structurally similar to nucleic acids. (**D**) Schematic representation of PARP-1 domains. PARP-1 enzyme can be divided into three major domains (also see Fig. 18.7.2): a 46-kDa DNA binding domain, a central 22-kDa automodification region that contains the BRCT domain, and a 54-kDa C-terminal region that comprises the active site and the PARP signature. During caspase-dependent apoptosis, caspase-3 and -7 recognize a DEVD motif in the nuclear localization signal (NLS) and cleave PARP-1 into a p89 and a p24 fragment. (Adapted from the PARP link web site: *http://parplink.u-strasbg.fr/index2.html*.

Figure 18.7.2 Poly(ADP-ribose) plolymerase family members and their antibodies. Abbreviations: EL, ELISA; IB, immunoblotting; IF, immunofluorescence; IP, immunoprecipitation.

Figure 18.7.3 The concept of PARP-1-mediated cell death. Excessive DNA damage activates PARP-1 in the nucleus, leading to massive pADPr formation and resulting in a NAD^+ depletion. This high concentration of pADPr may trigger mitochondrial depolarization ($\downarrow\Delta\Psi$) and drive apoptosis through an unknown mechanism. Activated PARP-1 can mediate cell death by caspase-dependent and -delayed pathways. In the caspase-delayed pathway, the insults to DNA, produced by oxidative stress or DNA alkylating agents, induces the release of mitochondrial factors, such as AIF, that translocates to the nucleus and induces caspase-independent chromatin fragmentation into large 50-kDa fragments. However, in caspase-dependent apoptosis, caspase-3 and -7 cleave PARP-1 into two fragments: p89 and p24. Generation of 24-kDa apoptotic fragment contribute to PARP-1 inactivation. The early cleavage PARP-1 serves as preserving cellular ATP for the proper function of further energy-dependent stage of apoptosis.

Flow Cytometry of Apoptosis

This unit describes the most common methods applicable to flow cytometry that make it possible to: (1) identify and quantify dead or dying cells, (2) reveal a mode of cell death (apoptosis or necrosis), and (3) study mechanisms involved in cell death. Gross changes in cell morphology and chromatin condensation, which occur during apoptosis, can be detected by analysis with laser light beam scattering. An early event of apoptosis, dissipation of the mitochondrial transmembrane potential, can be measured using a number of fluorochromes that are sensitive to the electrochemical potential within this organelle (see Basic Protocol 1). Another early event of apoptosis, caspase activation, can be measured either directly, by immunocytochemical detection of the epitope that characterizes activated caspase (see Basic Protocol 2), or indirectly by immunocytochemical detection of the caspase-3 cleavage product, the p85 fragment of poly(ADP-ribose) polymerase (see Basic Protocol 4). Exposure of phosphatidylserine on the exterior surface of the plasma membrane can be detected by the binding of fluoresceinated annexin V (annexin V–FITC); this assay is combined with analysis of the exclusion of the plasma membrane integrity probe propidium iodide (PI; see Basic Protocol 5). Also described are methods of analysis of DNA fragmentation based either on DNA content of cells with fractional (sub-G_1) DNA content (see Basic Protocol 6 and Alternate Protocol 1) or by DNA strand-break labeling (Terminal deoxynucleotidyltransferase–mediated dUTP Nick End Labeling, TUNEL; or In Situ End Labeling, ISEL; see Basic Protocol 7). Still another hallmark of apoptosis is the activation of tissue transglutaminase (TGase), the enzyme that cross-links protein and thereby makes them less immunogenic. Methods for analyzing TGase activation are presented in Basic Protocol 8 and Alternate Protocol 2.

STRATEGIC PLANNING

The choice of a particular method often depends on the cell type, the nature of the inducer of apoptosis, the desired information (e.g., specificity of apoptosis with respect to the cell cycle phase or DNA ploidy), and technical restrictions. For example, sample transportation or prolonged storage before the measurement requires prior cell fixation, thereby eliminating the use of "supravital" methods that rely on analysis of freshly collected live cells.

Positive identification of apoptotic cells is not always simple. Apoptosis was recently defined as a caspase-mediated cell death (Blagosklonny, 2000). Activation of caspases, therefore, appears to be the most specific marker of apoptosis (Shi, 2002). The detection of caspase activation, either directly (e.g., by antibody that is reactive with the activated enzyme; see Basic Protocol 2) or indirectly by the presence of poly(ADP-ribose) polymerase (PARP) cleavage product (PARP p85; see Basic Protocol 4), provides the most definitive evidence of apoptosis. Extensive DNA fragmentation is also considered as a specific marker of apoptosis. The number of DNA strand breaks in apoptotic cells is so large that intensity of their labeling in the TUNEL reaction (see Basic Protocol 7) ensures their positive identification and discriminates them from cells that have undergone primary necrosis (Gorczyca et al., 1992). However, in the instances of apoptosis when internucleosomal DNA degradation does not occur (Collins et al., 1992; Catchpoole and Stewart, 1993; Ormerod et al., 1994; Knapp et al., 1999), the number of DNA strand breaks may be inadequate to distinguish apoptotic cells by the TUNEL method. Likewise, in some instances of apoptosis, DNA fragmentation stops after the initial DNA cleavage to fragments of 50 to 300 kb (Collins et al., 1992, Oberhammer et al., 1993). The frequency of DNA strand breaks in nuclei of these cells is low, and therefore, they may not be easily detected by the TUNEL method.

Contributed by Piotr Pozarowski, Jerzy Grabarek, and Zbigniew Darzynkiewicz
Current Protocols in Cell Biology (2003) 18.8.1-18.8.33

The ability of cells to bind annexin V is still another marker considered to be specific to apoptosis. One should keep in mind, however, that use of the annexin V binding assay is hindered in some instances, e.g., when the plasma membrane is damaged during cell preparation or storage, leading to the loss of asymmetry in distribution of phosphatidylserine across the membrane. Furthermore, macrophages and other cells engulfing apoptotic bodies may also be positive in the annexin V assay (Marguet et al., 1999).

Apoptosis can be recognized with greater certainty when the cells are subjected to several assays probing different apoptotic attributes (Hotz et al., 1994). For example, the assay of plasma membrane integrity (exclusion of PI) and annexin V binding combined with analysis of PARP cleavage or DNA fragmentation may provide a more definitive assessment of the mode of cell death than can be determined by each of these methods used alone.

A plethora of kits designed to label DNA strand breaks and applicable to flow cytometry are available from different vendors. Most of these kits were designed by the authors (Gorczyca et al., 1992; Li and Darzynkiewicz, 1995). For example, Phoenix Flow Systems, BD PharMingen, and Alexis Biochemicals provide kits to identify apoptotic cells based on a single-step procedure utilizing either TdT and FITC-conjugated dUTP (APO-DIRECT; Li et al., 1995) or TdT and BrdUTP, as described in Basic Protocol 7 (APO-BRDU; Li and Darzynkiewicz, 1995). A description of the method, which is nearly identical to the one presented in this unit, is included with the kit. Another kit (ApopTag), based on a two-step DNA strand-break labeling with digoxygenin-16-dUTP by TdT, also designed by the authors (Gorczyca et al., 1992), was initially offered by ONCOR, later by Intergen, and most recently by Serologicals.

BASIC PROTOCOL 1

MITOCHONDRIAL TRANSMEMBRANE POTENTIAL ($\Delta\psi_m$) MEASURED BY RHODAMINE 123 OR DiOC$_6$(3) FLUORESCENCE

The critical role of mitochondria during apoptosis is associated with the release of two intermembrane proteins, cytochrome *c* and apoptosis-inducing factor (AIF), that are essential for sequential activation of pro-caspase 9 and pro-caspase 3 (Liu et al., 1996; Yang et al., 1997). AIF is also involved in proteolytic activation of apoptosis-associated endonuclease (Susin et al., 1997). Still another protein, Smac/Diablo, that interacts with the inhibitors of caspases, thereby promoting apoptosis, is released from mitochondria (Deng et al., 2002). Dissipation (collapse) of mitochondrial transmembrane potential ($\Delta\psi_m$), also called the permeability transition (PT), likewise occurs early during apoptosis (Cossarizza et al., 1994; Kroemer, 1998; Zamzani et al., 1998). However, a growing body of evidence suggests that this event may be transient when associated with the release of cytochrome *c* or AIF, and mitochondrial potential may be restored for some time in the cells with activated caspases (Finucane et al., 1999; Scorrano et al., 1999; Li et al., 2000).

The membrane-permeable lipophilic cationic fluorochromes such as rhodamine 123 (R123) or 3,3′-dihexyloxacarbocyanine iodide [DiOC$_6$(3)] can serve as probes of $\Delta\psi_m$ (Johnson et al., 1980; Darzynkiewicz et al., 1981, 1982). When live cells are incubated in their presence, the probes accumulate in mitochondria, and the extent of their uptake, as measured by intensity of cellular fluorescence, reflects $\Delta\psi_m$. A combination of R123 and PI discriminates among live cells that stain only with R123, early apoptotic cells that have lost the ability to accumulate R123, and late apoptotic/necrotic cells whose plasma membrane integrity is compromised and that stain only with PI (Darzynkiewicz et al., 1982; Darzynkiewicz and Gong, 1994). The specificity of R123 and DiOC$_6$(3) as $\Delta\psi_m$ probes is increased when they are used at low concentrations (<0.5 μg/ml). Still another probe of $\Delta\psi_m$ is the J-aggregate-forming lipophilic cationic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Its uptake by

charged mitochondria driven by the transmembrane potential is detected by the shift in color of fluorescence from green, which is characteristic of its monomeric form, to orange, which reflects its aggregation in mitochondria (Cossarizza and Salvioli, 2001). In light of the recent evidence that the collapse of $\Delta\psi_m$ may not be a prerequisite for release of cytochrome c, AIF, and other apoptotic events (Finucane et al., 1999; Scorrano et al., 1999; Li et al., 2000), one should be cautious in interpreting the lack of collapse of $\Delta\psi_m$ as a marker of non-apoptotic cells.

Materials

Cells of interest in appropriate complete culture medium
10 µM rhodamine 123 (R123; see recipe) *or* 10 µM $DiOC_6(3)$ (see recipe for 0.1 mM stock solution) *or* 0.2 mM JC-1 stock solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

12 × 75–mm tubes suitable for flow cytometer
Flow cytometer with 488-nm excitation and filters for collection of green, orange, and red fluorescence

Stain with R123 or $DiOC_6(3)$ and PI

1a. Add either 20 µl of 10 µM R123 (200 nM final) or 5 µl of 10 µM $DiOC_6(3)$ (50 nM final) to ~10^6 cells suspended in 1 ml complete tissue culture medium (with 10% serum), and incubate 20 min at 37°C in the dark.

2a. Centrifuge cells 5 min at 300 × *g*, room temperature. Resuspend cell pellet in 1 ml PBS.

3a. Add 10 µl PI solution and incubate 5 min at room temperature in the dark.

4a. Analyze cell fluorescence on the flow cytometer. Excite fluorescence with blue (488-nm) laser. Set the signal-triggering threshold on forward- and side-scatter signals. Collect green fluorescence [R123 or $DiOC_6(3)$] at 530 ± 20 nm and red fluorescence (PI) above 600 nm.

Stain with JC-1

1b. Suspend cell pellet (~10^6 cells) in 1 ml complete tissue culture medium with 10% serum.

2b. Add 10 µl of 0.2 mM JC-1 stock solution. Vortex cells intensely during addition and for the next 20 sec. Wash cells two times with PBS; centrifuge each time 5 min at 200 × *g*, room temperature.

Addition of JC-1 to the cell suspension without vortexing may lead to formation of precipitate. Vortexing too vigorously, on the other hand, may cause cell damage.

3b. Incubate cells 15 min at room temperature in the dark.

4b. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation. Collect green fluorescence at 530 ± 20 nm and orange fluorescence at 570 ± 20 nm with a band-pass filter or above 570 nm with a long-pass filter.

BASIC PROTOCOL 2

IMMUNOCYTOCHEMICAL DETECTION OF ACTIVATED CASPASES BY ZENON TECHNOLOGY

Caspases are activated by transcatalytic cleavage of their zymogen procaspase molecules into large and small subunits. The subunits then assemble to form the heterotetramer consisting of two small and two large subunits, which is the active caspase (Budihardjo et al., 1999; Earnshaw et al., 1999). Antibodies that are specific to activated caspase-3, caspase-8, and caspase-9 are now commercially available and one expects that antibodies reactive with other active caspases will soon be developed as well. It is possible, therefore, to detect caspase activation by immunocytochemical means. This protocol combines the use of activated caspase-specific antibody with staining of cellular DNA by propidium iodide (PI) to concurrently detect cells with activated caspases and relate caspase activation to the cell-cycle position.

The immunocytochemical detection of caspase-3 in this protocol makes use of Zenon technology (Haugland, 2002). Zenon technology consists of a labeling complex that is formed by a fluorochrome-labeled Fab fragment (Zenon Alexa Fluor 488) of an anti-IgG antibody that is directed against the Fc portion of a mouse (or rabbit) IgG_1 antibody. Mixing of the labeled Fab fragment with the primary antibody forms the labeling complex. Excess unbound labeled Fab fragments is removed by admixture of nonspecific mouse (or rabbit) IgG. The labeling complex is then used to stain cells in the same manner as a covalently labeled primary antibody (Haugland, 2002).

Materials

Cells of interest, both untreated (control) and induced to apoptosis (e.g., exponentially growing HL-60 cells incubated 2 to 4 hr with 0.15 μM camptothecin)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fixatives:
1% (v/v) methanol-free formaldehyde (Polysciences) in PBS, 0° to 5°C
4% (v/v) methanol-free formaldehyde (Polysciences) in PBS, room temperature
70% (v/v) ethanol, –20°C
Rinse solution (see recipe)
Primary antibody: cleaved (activated) caspase-3 antibody (Cell Signaling Technology, cat. no. 9661)
Zenon Alexa Fluor 488 rabbit IgG labeling kit (Molecular Probes, cat. no. Z-25302)
10% (v/v) Triton X-100 in PBS
DNA staining solution with PI (see recipe)

12 × 75–ml tubes suitable for use on flow cytometer
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend ~10^6 cells in 0.5 ml PBS.
2. Fix cells by transferring the above cell suspension into tubes containing 4.5 ml of 1% methanol-free formaldehyde in PBS at 0° to 5°C. Let stand 15 min at 0° to 5°C.
3. Centrifuge 5 min at 300 × *g*, room temperature. Decant supernatant.
4. Resuspend cell pellet in 3 ml of 70% ethanol at –20°C. Allow to sit at least 2 hr (cells can be stored several days in 70% ethanol at –20°C).
5. Bring the cell suspension in 70% ethanol to room temperature, add 2 ml PBS to this suspension, and let sit 5 min at room temperature.
6. Centrifuge 5 min at 300 × *g*, room temperature. Decant supernatant.

7. Resuspend cell pellet in 5 ml PBS and let sit 5 min at room temperature.
8. Centrifuge 5 min at 300 × *g*, room temperature. Decant supernatant.
9. Resuspend cell pellet in rinse solution. Let stand 30 min at room temperature.
10. Prepare the staining solution as follows.
    a. Mix 4 µl primary antibody (anti-caspase-3) with 16 µl rinse solution and with 5 µl solution A Zenon (from kit) in a 1.5-ml microcentrifuge tube.
    b. Keep 5 min in the dark at room temperature.
    c. Add 5 µl solution B Zenon (from kit).
    d. Keep 5 min in the dark at room temperature.
    e. Add 0.3 µl of 10% Triton X-100 in PBS.
11. Centrifuge the cell suspension (from step 9) 5 min at 300 × *g*, room temperature. Thoroughly drain the rinse solution by blotting on filter paper. Add 15 µl of the staining solution prepared in step 10, and 85 µl rinse solution, for a final volume of 100 µl. Resuspend the cell pellet.
12. Incubate cells with the staining solution 1 hr in the dark at room temperature.
13. Add 5 ml PBS, centrifuge 5 min at 300 × *g*, room temperature, and decant supernatant.
14. Resuspend cell pellet in 1 ml of 4% methanol-free formaldehyde in PBS and let stand 5 min at room temperature.
15. Centrifuge cells 5 min at 300 × *g*, room temperature. Decant supernatant.
16. Resuspend cell pellet in 1 ml DNA staining solution with PI.
17. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green Alexa 488 fluorescence at 530 ±20 nm and red PI fluorescence above 600 nm.

DETECTION OF APOPTOTIC CELLS USING FLUOROCHROME-LABELED INHIBITORS OF CASPASES (FLICAs)

BASIC PROTOCOL 3

Exposure of live cells to fluorochrome-labeled inhibitors of caspases (FLICAs) results in uptake of these reagents by apoptotic cells (Smolewski et al., 2001). Unbound FLICAs are removed from the nonapoptotic cells by rinsing the cells with wash buffer. The cells may also be fixed with formaldehyde; after fixation only apoptotic cells retain the label. Cells labeled with FLICAs can be examined by fluorescence microscopy, or their fluorescence can be measured by flow cytometry. FLICAs are convenient markers of apoptotic cells, and when used in combination with PI as described in the protocol below, they reveal three sequential stages of apoptosis. FAM-VAD-FMK, the inhibitor designed to react with all caspases, except perhaps caspase-2, is used in this protocol. It should be stressed, however, that FLICAs appear to react in apoptotic cells also with targets other than activated caspases. Cell labeling with FLICAs, therefore, although perhaps reflecting caspase activity and although reflecting caspase activation, cannot be interpreted as indicating reactivity with active enzyme centers of caspases only.

Materials

Cells of interest
Medium supplemented with 10% (v/v) serum or 1% (w/v) serum albumin
FLICA kit (Immunochemistry Technologies) containing:
  FAM-VAD-FMK reagent (see recipe)
  Fixative
  Hoechst stain
Rinse solution: 1% (w/v) BSA in PBS (*APPENDIX 2A*)
1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

12 × 75–ml tubes suitable for use on flow cytometer
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend ~10^6 cells in 0.3 ml medium containing 10% serum or 1% serum albumin.
2. Add 10 µl FAM-VAD-FMK working solution to this cell suspension (final concentration 10 µM). Mix gently and incubate 1 hr at 37°C.

   Sulforhodamine-labeled FLICA (SR-VAD-FMK) may be used instead of FAM-VAD-FMK to make apoptotic cells fluorescence in the red.

3. Add 2 ml rinse solution, mix gently, and centrifuge 5 min at 200 × *g*, room temperature.
4. Resuspend cell pellet in 2 ml rinse solution and centrifuge as in step 3.

   Cells may be fixed 15 min in 1% formaldehyde in PBS, then suspended in 70% ethanol and stored for several days. A fluorochrome of a different color than FLICA may be used to counterstain other cellular components (e.g., DNA) or other markers of apoptosis (e.g., DNA strand breaks).

5. Resuspend cell pellet in 1 ml rinse solution. Add 1.0 µl of 1 mg/ml PI stock solution. Keep 5 min at room temperature.

   Protect samples from light at all times.

   Staining with PI is optional. It allows one to distinguish the cells that have compromised plasma-membrane integrity (e.g., necrotic and late apoptotic cells, cells with mechanically damaged membranes, or isolated cell nuclei) to the extent that they cannot exclude PI.

6. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FAM-VAD-FMK fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

BASIC PROTOCOL 4

DETERMINATION OF POLY(ADP-RIBOSE) POLYMERASE (PARP) CLEAVAGE

PARP is a nuclear enzyme that is involved in DNA repair and activated in response to DNA damage (de Murcia and Menissier-de Murcia, 1994). Early in apoptosis, PARP is cleaved by caspases, primarily by caspase-3 (Kaufmann et al., 1993; Lazebnik et al., 1994; Alnemri et al., 1996). The specific cleavage of this protein results in distinct 85-kDa and 24-kDa fragments, usually detected electrophoretically, and this is considered to be a hallmark of the apoptotic mode of cell death.

The development of antibodies that recognize the cleaved PARP products prompted their use as immunocytochemical markers of apoptotic cells. The antibody that recognizes the 85-kDa fragment (PARP p85) was initially used to score the frequency of apoptosis in tissue sections (Sallman et al., 1997; Kockx et al., 1998). Recently, this antibody has been adapted to label apoptotic cells for detection by flow cytometry and laser scanning

cytometry (LSC; Li and Darzynkiewicz, 2000; Li et al., 2000). A good correlation was observed between the frequency of apoptosis detected cytometrically with PARP p85 Ab and that detected by the DNA strand-break (TUNEL) assay. However, at least in some cell systems, the cleavage of PARP occurs prior to the onset of DNA fragmentation (Li and Darzynkiewicz, 2000). In these instances, the correlation may not be apparent at early stages of apoptosis because the apoptotic index estimate based on PARP cleavage may exceed the estimate based on the TUNEL reaction. Cytometric analysis of cells differentially stained for PARP p85 and DNA, similar to the TUNEL assay, makes it possible not only to identify and score apoptotic cell populations but also to correlate apoptosis with the cell cycle position or DNA ploidy.

The classic immunocytochemical indirect (two-step) method to detect the 85-kDa PARP fragment is presented below. Alternatively, however, one can use the Zenon technology as described above (see Basic Protocol 2) for detection of activated caspases.

Materials

Cells of interest
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1% methanol-free formaldehyde (Polysciences) in PBS (*APPENDIX 2A*)
70% ethanol
0.25% (v/v) Triton X-100 (Sigma) in PBS (*APPENDIX 2A*); store at 4°C
PBS/BSA solution: 1% (w/v) bovine serum albumin (Sigma) in PBS; store at 4°C
Anti-PARP p85 antibody (Promega anti-PARP-85 fragment, rabbit polyclonal)
Fluorescein-conjugated anti-rabbit immunoglobulin antibody (Dako)
1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark
RNase stock solution (see recipe)

12 × 75–mm centrifuge tubes suitable for use on the flow cytometer
Pasteur pipets
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend ~10^6 cells in 0.5 ml PBS. Transfer this suspension to a 12 × 75–mm (5-ml) tube containing 4.5 ml of 1% methanol-free formaldehyde and fix cells 15 min on ice. Centrifuge the cells 5 min at 300 × *g*, 4°C, wash once with 5 ml PBS, centrifuge 5 min at 300 × *g*, and resuspend the cell pellet in 0.5 ml PBS. With a Pasteur pipet, transfer this cell suspension into a new 12 × 75–mm centrifuge tube containing 4.5 ml of ice-cold 70% ethanol.

   The cells may be stored several days in ethanol at –20°C.

2. Centrifuge cells 5 min at 200 × *g*, room temperature, and resuspend the cell pellet in 5 ml PBS; repeat centrifugation.

3. Resuspend cells in 5 ml 0.25% Triton X-100/PBS for 10 min.

4. Centrifuge cells 5 min at 300 × *g*, room temperature, and resuspend in 2 ml BSA/PBS solution for 10 min.

5. Centrifuge cells 5 min at 300 × *g*, room temperature, and resuspend in 100 µl BSA/PBS containing anti-PARP p85 Ab diluted 1:200. Incubate 2 hr at room temperature, or overnight at 4°C.

6. Add 5 ml BSA/PBS solution, let sit 5 min, and centrifuge 5 min at 300 × *g*, room temperature.

7. Resuspend cell pellet in 100 µl PBS/BSA containing fluorescein-conjugated secondary Ab [$F(ab')_2$ fragment, swine anti-rabbit immunoglobulin] diluted 1:30. Incubate 1 hr in the dark at room temperature.
8. Add 5 ml BSA/PBS, centrifuge 5 min at 200 × *g,* room temperature, and resuspend cell pellet in 1 ml PBS. Add 20 µl of 1 mg/ml PI and 50 µl RNase solution. Incubate 20 min in the dark at room temperature.
9. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-anti-PARP p85 fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

BASIC PROTOCOL 5

ANNEXIN V BINDING

Phospholipids of the plasma membrane are asymmetrically distributed between the inner and outer leaflets of the membrane. Phosphatidylcholine and sphingomyelin are exposed on the external leaflet of the lipid bilayer, while phosphatidylserine is located on the inner surface. During apoptosis, this asymmetry is disrupted and phosphatidylserine becomes exposed on the outside surface of the plasma membrane (Fadok et al., 1992; Koopman et al., 1994; van Engeland et al., 1998). Because the anticoagulant protein annexin V binds with high affinity to phosphatidylserine, fluorochrome-conjugated annexin V has found an application as a marker of apoptotic cells, in particular for their detection by flow cytometry (van Engeland et al., 1998). The cells become reactive with annexin V prior to their loss of plasma membrane ability to exclude cationic dyes such as PI. Therefore, by staining cells with a combination of annexin V–FITC and PI, it is possible to detect unaffected, non-apoptotic cells (annexin V–FITC negative/PI negative), early apoptotic cells (annexin V–FITC positive/PI negative), and late apoptotic ("necrotic stage" of apoptosis) as well as necrotic cells (PI positive).

Materials

Cells of interest
Fluorescein-conjugated annexin V (see recipe) in binding buffer (see recipe)
1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend 10^5 to 10^6 cells in 1 ml fluorescein-conjugated annexin V in binding buffer for 5 min at room temperature in the dark.
2. Prior to analysis, add an appropriate volume of 1 mg/ml PI solution to the cell suspension to have a final PI concentration of 1.0 µg/ml. Incubate 5 min at room temperature in the dark.
3. Analyze cells on the flow cytometer, using 488-nm excitation. Set gates based on light scatter. Collect green annexin V fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

DNA FRAGMENTATION: DETECTION OF CELLS WITH FRACTIONAL (SUB-G_1) DNA CONTENT USING PI

Endonucleases activated during apoptosis target internucleosomal DNA sections and cause extensive DNA fragmentation (Kerr et al., 1972; Arends et al., 1990; Nagata, 2000). The fragmented, low-molecular-weight DNA can be extracted from the cells following their fixation in precipitating fixatives such as ethanol. Conversely, fixation with cross-linking fixatives such as formaldehyde results in the retention of low-molecular-weight DNA in the cell and therefore should be avoided. Generally, the extraction occurs during the process of cell staining in aqueous solutions after transfer from the fixative. As a result, apoptotic cells often end up with deficient DNA content, and when stained with a DNA-specific fluorochrome, they can be recognized by cytometry as cells having less DNA than G_1 cells. On the DNA content frequency histograms, they form a characteristic sub-G_1 peak (Umansky et al., 1981; Nicoletti et al., 1991; Gong et al., 1994). It should be noted that loss of DNA may also occur as a result of the shedding of apoptotic bodies containing fragments of nuclear chromatin.

The degree of DNA degradation varies depending on the stage of apoptosis, cell type, and often the nature of the apoptosis-inducing agent. Hence, the extractability of DNA during the staining procedure also varies. A high-molarity phosphate-citrate buffer enhances extraction of the fragmented DNA (Gong et al., 1994). With some limitations, this approach can be used to extract DNA from apoptotic cells to the desired level in order to achieve their optimal separation by flow cytometry.

Materials

Cells of interest
PBS (*APPENDIX 2A*)
70% ethanol
DNA extraction buffer (see recipe)
DNA staining solution with PI (see recipe)

12 × 75–mm tubes suitable for use on the flow cytometer
Flow cytometer with 488-nm excitation and filter for collection of red fluorescence

1. Suspend 1–2 × 10^6 cells in 0.5 ml PBS and fix cells by adding suspension to 4.5 ml of 70% ethanol in a 12 × 75–mm tube on ice.

   Cells may be stored several weeks in fixative at −20°C.

2. Centrifuge cells 5 min at 200 × *g*, decant ethanol, suspend the cell pellet in 5 ml PBS, and centrifuge 5 min at 300 × *g*, room temperature.

3. Suspend cell pellet in 0.25 ml PBS. To facilitate extraction of low-molecular-weight DNA, add 0.2 to 1.0 ml DNA extraction buffer.

4. Incubate 5 min at room temperature, then centrifuge 5 min at 300 × *g*, room temperature.

5. Suspend cell pellet in 1 ml DNA staining solution with PI. Incubate cells 30 min at room temperature in the dark.

6. Analyze cells on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect forward light scatter and red fluorescence above 600 nm.

ALTERNATE PROTOCOL 1

DNA FRAGMENTATION: DETECTION OF CELLS WITH FRACTIONAL (SUB-G_1) DNA CONTENT USING DAPI

Cellular DNA may be stained with other fluorochromes instead of PI, and other cell constituents may be counterstained in addition to DNA. The following is the procedure used to stain DNA with DAPI. This protocol requires a UV laser.

Additional Materials *(also see Basic Protocol 6)*

DNA staining solution with DAPI (see recipe)
Flow cytometer equipped with UV excitation and filter for collection of blue fluorescence

1. Follow Basic Protocol 6, steps 1 to 4. Then, suspend the cell pellet in 1 ml DNA staining solution containing DAPI. Keep on ice 20 min.

2. Analyze cells on the flow cytometer, using UV excitation (e.g., 351-nm line from an argon-ion laser, or mercury lamp with a UG1 filter). Collect blue DAPI fluorescence in a band from 460 to 500 nm.

BASIC PROTOCOL 7

DNA FRAGMENTATION: DETECTION OF DNA STRAND BREAKS (TUNEL ASSAY)

DNA fragmentation during apoptosis, particularly when it progresses to internucleosomal regions (Arends et al., 1990; Oberhammer et al., 1993), generates a multitude of DNA strand breaks in the nucleus. The 3′-OH ends of the breaks can be detected by attaching a fluorochrome. This is generally done directly or indirectly (e.g., via biotin or digoxygenin) using fluorochrome-labeled deoxynucleotides in a reaction catalyzed preferably by exogenous terminal deoxynucleotidyltransferase (TdT; Gorczyca et al., 1992, 1993; Li and Darzynkiewicz, 1995; Li et al., 1995). The reaction is commonly known as TUNEL, from TdT-mediated dUTP-biotin nick-end labeling (Gavrieli et al., 1992). This acronym is actually a misnomer, since double-stranded DNA breaks are labeled, rather than single-stranded nicks. Of all the markers used to label DNA breaks, BrdUTP appears to be the most advantageous with respect to sensitivity, low cost, and simplicity of reaction (Li and Darzynkiewicz, 1995). When attached to DNA strand breaks in the form of poly-BrdU, this deoxynucleotide can be detected with a FITC-conjugated anti-BrdU Ab, the same Ab commonly used to detect BrdU incorporated during DNA replication. Poly-BrdU attached to DNA strand breaks by TdT, however, is accessible to the Ab without the need for DNA denaturation, which otherwise is required to detect the precursor incorporated during DNA replication.

It should be stressed that the detection of DNA strand breaks by this method requires pre-fixation of cells with a cross-linking agent such as formaldehyde. Unlike ethanol, formaldehyde prevents the extraction of small pieces of fragmented DNA. Thus, despite cell permeabilization and the subsequent cell washings required, the DNA content of early apoptotic cells (and the number of DNA strand breaks) is not markedly diminished through extraction. Labeling of DNA strand breaks in this procedure, which utilizes fluorescein-conjugated anti-BrdU Ab, can be combined with staining of DNA by a fluorochrome of another color (PI, red fluorescence). Cytometry of cells that are differentially stained for DNA strand breaks and for DNA allows one to distinguish apoptotic from non-apoptotic cell subpopulations and reveals the cell cycle distribution in these subpopulations (Gorczyca et al., 1992, 1993).

Materials

Cells of interest
1% (v/v) methanol-free formaldehyde (Polysciences) in PBS (*APPENDIX 2A*), pH 7.4 (primary fixative)
PBS (*APPENDIX 2A*)
70% ethanol (secondary fixative)
5× TdT reaction buffer (see recipe)
2 mM BrdUTP (Sigma) in 50 mM Tris·Cl, pH 7.5
TdT in storage buffer (both from Roche Diagnostics), 25 U in 1 µl
10 mM cobalt chloride ($CoCl_2$; Roche Diagnostics)
Rinsing buffer: PBS with 0.1% (v/v) Triton X-100 and 0.5% (w/v) BSA
FITC-conjugated anti-BrdU MAb (see recipe)
PI staining buffer: PBS with 5 µg/ml PI and 200 µg/ml DNase-free RNase
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

Fix cells

1. Fix 1–5 × 10^6 cells in suspension 15 min in 1% methanol-free formaldehyde in PBS, pH 7.4, on ice.
2. Centrifuge 5 min at 200 × *g*, 4°C, resuspend cell pellet (~2 × 10^6 cells) in 5 ml PBS, centrifuge 5 min at 200 × *g*, 4°C, and resuspend cells in 0.5 ml PBS.
3. Add the 0.5-ml cell suspension from step 2 to 5 ml ice-cold 70% ethanol.

   The cells can be stored several weeks in ethanol at −20°C.
4. Centrifuge 5 min at 200 × *g*, 4°C, remove ethanol, and resuspend cells in 5 ml PBS. Repeat centrifugation.

Stain cells

5. Resuspend the pellet (not more than 10^6 cells) in 50 µl of a solution that contains:

   10 µl 5× TdT reaction buffer
   2.0 µl 2 mM BrdUTP stock solution
   0.5 µl TdT in storage buffer (12.5 U final)
   5 µl 10 mM $CoCl_2$ solution
   32.5 µl dH_2O.
6. Incubate cells in this solution 40 min at 37°C.

   Alternatively, incubation can be carried out overnight at 22° to 24°C.
7. Add 1.5 ml rinsing buffer and centrifuge 5 min at 200 × *g*, room temperature.
8. Resuspend cells in 100 µl FITC-conjugated anti-BrdU MAb solution.
9. Incubate 1 hr at room temperature or overnight at 4°C. Add 2 ml rinsing buffer and centrifuge 5 min at 200 × *g*, room temperature.
10. Resuspend cell pellet in 1 ml PI staining solution containing RNase. Incubate 30 min at room temperature in the dark.
11. Measure cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-anti BrdU MAb fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

BASIC PROTOCOL 8

DETECTION OF TISSUE TRANSGLUTAMINASE ACTIVATION BY CELL RESISTANCE TO DETERGENTS

Extensive protein cross-linking takes place during apoptosis. The ubiquitous transglutaminase TGase 2 (also called "tissue transglutaminase"; tTGase) was identified as the enzyme responsible for this reaction (Fesus et al., 1987; Melino and Piacentini, 1998). It is presumed that activation of TGase 2 during apoptosis prevents release of soluble and immunogenic proteins from dying cells because protein cross-linking makes these proteins less soluble and thereby decreases a possibility of induction of autoimmune reaction. Furthermore, protein packaging into apoptotic bodies may be facilitated when proteins remain in solid state rather than in solution. The additional role of TGase 2 as one of the "executor enzymes" during apoptosis is still being debated.

This protocol is a simple and rapid approach to identify apoptotic cells with activated TGase 2. The method is based on the propensity of cross-linked protein to withstand treatment with detergents. The authors have noticed that when live, nonapoptotic cells are subjected to treatment with solutions of nonionic detergents, lysis of their plasma membrane and release of the content of cytoplasm is complete, resulting in preparation of isolated nuclei. In contrast, apoptotic cells resist the detergent treatment; their cytoplasmic protein remains insoluble and attached to the nucleus in the form of a shell-like cover (Grabarek et al., 2002). It is possible, therefore, by flow or laser scanning cytometry to distinguish apoptotic cells from the nuclei isolated from nonapoptotic cells, by means of the abundance of protein in the former. In addition, bivariate gating analysis of cellular DNA and protein content makes it possible to reveal the cell cycle distribution separately for the population of cells with cross-linked protein (activated TGase 2) and for the population of cells that did not show protein cross-linking (Grabarek et al., 2002).

Alternate Protocol 2 combines the detection of TGase 2 activity by binding of fluoresceinated cadaverine (F-CDV) with analysis of the cell cycle.

Materials

Cells of interest
DAPI/sulforhodamine 101/detergent solution (see recipe)
Flow cytometer equipped with UV excitation and filters for collection of blue and red fluorescence

1. Collect ~10^6 cells from the culture and centrifuge 5 min at $300 \times g$, room temperature.
2. Suspend the cell pellet in 1 ml DAPI/sulforhodamine 101/detergent solution and vortex 20 sec.
3. Analyze cells on the flow cytometer, using UV excitation (e.g., 351-nm line from an argon-ion laser, or mercury lamp with a UG1 filter). Collect blue DAPI fluorescence in a band from 460 to 500 nm and red fluorescence of sulforhodamine 101 above 600 nm.

ALTERNATE PROTOCOL 2

DETECTION OF TGase 2 ACTIVATION BY FLUORESCEINATED CADAVERINE (F-CDV) BINDING

This alternate protocol is based on the covalent attachment, by the activated TGase 2, of the fluorescein-tagged cadaverine to the respective protein substrates within the cell (Lajemi et al., 1998). This assay was adapted to flow cytometry and combined with concurrent analysis of cellular DNA content (Grabarek et al., 2002). Like the detergent-based assay, this method is simple and also offers good distinction between apoptotic cells with activated versus nonactivated TGase 2.

It should be noted that when the cost of the reagents for the procedure is taken into account, the detergent-based assay (see Basic Protocol 8) is less expensive by several orders of magnitude than this F-CDV assay.

Materials

Fluoresceinated cadaverine solution (F-CDV; see recipe)
Cells of interest
100% methanol
DNA staining solution with PI (see recipe)
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Add aliquots of F-CDV stock solution (1 part per 50) directly to cell cultures (10^6 to 10^7 cells) to obtain 50 µM final F-CDV concentration in the culture.
2. Incubate cells in the presence of F-CDV for the desired time interval during which activity of TGase 2 has to be detected (e.g., one to several hours).

   Because cross-linking is a cumulative process, intensity of cell labeling increases with time of incubation.

3. Harvest the culture by centrifuging 5 min at 200 × *g*, room temperature.
4. Suspend the cells in 0.5 ml PBS and fix in 5 ml of 100% methanol on ice. Keep in methanol ≥2 hr at 0° to 4°C; cells may be stored in the fixative for several days.
5. Centrifuge cells 5 min at 200 × *g*, room temperature, decant the fixative thoroughly, and suspend cell pellet in 2 ml DNA staining solution with PI.
6. Keep ≥30 min at room temperature.
7. Measure cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-CDV fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Binding buffer

10 mM HEPES-NaOH, pH 7.4
140 mM NaCl
5 mM $CaCl_2$
Store several weeks at 4°C

DAPI/sulforhodamine 101/detergent solution

Dissolve 100 µg DAPI, 2 mg sulforhodamine 101 (Molecular Probes), and 0.1 ml Triton X-100 in 100 ml PBS (or 0.1 ml Nonidet NP-40). Store several weeks at 0° to 4°C.

$DiOC_6$ (3) stock solution

Prepare a 0.1 mM solution of $DiOC_6$ (3) (Molecular Probes) by dissolving 5.7 mg dye in 10 ml dimethyl sulfoxide (DMSO). Store for weeks in small (e.g., 0.5- or 1-ml) aliquots protected from light at −20°C. Prior to use, dilute ten-fold with PBS to obtain 10 µM concentration.

DNA extraction buffer, pH 7.8

192 ml 0.2 M Na_2HPO_4
8 ml 0.1 M citric acid
Store for months at 4°C

DNA staining solution with DAPI

Dissolve 100 µg DAPI in 100 ml PBS. Store at 0° to 4°C for several weeks.

DNA staining solution with PI

200 µg propidium iodide (PI)
2 mg DNase-free RNase
10 ml PBS
Prepare fresh before use

FAM-VAD-FMK

Stock solution: following kit directions, dissolve FAM-VAD-FMK in dimethyl sulfoxide (DMSO) to obtain 150× solution. Store aliquots protected from light ≤3 months at −20°C.

Working solution: Just prior to use, prepare a 30× solution by diluting FAM-VAD-FMK stock solution 1:5 in PBS. Mix the vial until the solution becomes transparent and homogenous. Protect all FAM-VAD-FMK solutions from light. Discard unused portions. Do not store.

FITC-conjugated anti-BrdU MAb solution

100 µl PBS (*APPENDIX 2A*)
0.3 µg FITC-conjugated anti-BrdU MAb (Becton Dickinson)
0.3% (v/v) Triton X-100
1% (w/v) BSA
Prepare fresh before use

Fluoresceinated cadaverine solution

Dissolve 5-[(5-aminopentyl)thioureidyl]fluorescein dihydrobromide (F-CDV; Molecular Probes) in distilled water to obtain a 2.5 mM stock solution. Store aliquots (0.2 to 0.5 ml) of this solution several weeks at −20°C.

Fluorescein-conjugated annexin V

Dissolve FITC-conjugated annexin V (1:1 stoichiometric complex; e.g., from BRAND Applications) in binding buffer (see recipe) at a concentration of 1.0 µg/ml. This solution must be prepared fresh just prior to use.

JC-1 stock solution

Prepare a 0.2 mM solution of JC-1 (Molecular Probes) by dissolving 1.3 mg dye in 10 ml *N*,*N*-dimethylformamide (Sigma). Store for weeks in small (e.g., 0.5- or 1.0-ml) aliquots protected from light at −20°C. Use glass containers; *N*,*N*-dimethylformamide will dissolve plastics.

Rhodamine 123 (R123) stock solution

Prepare a 0.1 mM solution of R123 (Molecular Probes) by dissolving 0.38 mg dye in 10 ml methanol. Store for months in small aliquots protected from light at −20°C. Prior to use, dilute ten-fold with PBS to obtain 10 µM concentration.

Rinse solution

1 g BSA
0.1 ml Triton X-100
100 ml PBS (*APPENDIX 2A*)
Store up to 1 week at 4°C

RNase stock solution

Dissolve 2 mg RNase A (e.g., Sigma) in 1 ml distilled water. If the RNase is not DNase free, heat the solution 5 min at 100°C to inactivate any traces of DNase. Store ≤1 year at 4°C.

TdT reaction buffer, 5×

1 M potassium (or sodium) cacodylate
125 mM Tris·Cl, pH 6.6 (*APPENDIX 2A*)
0.125% bovine serum albumin (BSA)
Store for weeks at 4°C

COMMENTARY

Background Information

Applications of flow cytometry in cell necrobiology have two distinct goals (for reviews, see Darzynkiewicz et al., 1992, 1997; Ormerod, 1998; van Engeland et al., 1998; Vermes et al., 2000). One goal is to elucidate molecular and functional mechanisms associated with cell death. For this purpose, flow cytometry is used to measure cellular levels of components involved in the regulation and/or execution of apoptosis or cell necrosis. The most frequently studied are pro- and anti-apoptotic members of the Bcl-2 protein family, caspases, proto-oncogenes (e.g., *c-myc* or *ras)*, or tumor suppressor genes (e.g., p53 or pRB). Flow cytometry is also widely used to study functional attributes of the cell such as mitochondrial metabolism, oxidative stress, intracellular pH, or ionized calcium, all closely associated with mechanisms of apoptosis. The major advantage of flow cytometry in these applications is that it provides the possibility of multiparametric measurements of cell attributes. Multivariate analysis of such data reveals the correlation between the measured cell constituents. For example, when one of the measured attributes is cellular DNA content (the parameter that reports the cell cycle position or DNA ploidy), an expression of the other measured attribute can then be directly related to the cell cycle position (or cell ploidy) without a need for cell synchronization. Since individual cells are measured, intercellular variability can be assessed, cell subpopulations identified, and rare cells detected.

The second goal of cytometry applications is to identify and quantify dead cells and discriminate between apoptotic and necrotic modes of death. Dead-cell recognition is generally based on the presence of a particular biochemical or molecular marker that is characteristic for apoptosis, necrosis, or both. Numerous methods have been developed, especially for the identification of apoptotic cells. Apoptosis-associated changes in the gross physical attributes of cells, such as cell size and granularity, can be detected by analysis of laser light scattered by the cell in forward and side directions (Swat et al., 1981; Ormerod et al., 1995). Some methods rely on apoptosis-associated changes in the distribution of plasma membrane phospholipids (Fadok et al., 1992; Koopman et al., 1994). Others detect the loss of active transport function of the plasma membrane. Still other methods probe the mitochondrial transmembrane potential (Cossarizza et al., 1994; Kroemer, 1998; Zamzani et al., 1998). The detection of DNA fragmentation provides another convenient marker of apoptosis; apoptotic cells are then recognized either by their fractional (subdiploid, sub-G_1) DNA content due to extraction of low-molecular-weight DNA from the cell (Umansky et al., 1981; Nicoletti et al., 1991), or by the presence of DNA strand breaks, which can be detected by labeling their 3′-OH termini with fluorochrome-conjugated nucleotides in a reaction utilizing exogenous terminal deoxynucleotidyl transferase (TdT; Gorczyca et al., 1992, 1993; Li and Darzynkiewicz, 1995; Li et al., 1996). More recently, flow cytometric methods have been developed to detect activation of caspases and tissue transglutaminase (TGase 2; Grabarek et al., 2002). It should be noted, however, that the fluorochrome-labeled inhibitors of caspases (FLICAs), initially described as markers of caspase activation (Smolewski et al., 2001), or serine proteases, although convenient markers of apoptotic cells and most likely detecting activation of these proteases, do not have sufficient specificity to be used as specific probes of their active enzymatic centers (Pozarowski et al., 2003).

A variety of kits are commercially available to identify apoptotic cells using the methods presented in this unit. Since the reagents are

already prepackaged and the procedures are described in cookbook format, the kits offer the advantage of simplicity. Their cost, however, is many-fold higher than that of the individual reagents. Furthermore, the kits do not allow one the flexibility that is often required to optimize procedures for a particular cell system. In many situations, therefore, the preparation of samples for analysis by flow cytometry as described herein may be preferred.

Light-scattering changes during apoptosis

Intersection of cells with the laser light beam in a flow cytometer results in light scattering. Analysis of light scattered in different directions reveals information about cell size and structure. The intensity of the forward light-scatter signal correlates with cell size. Side scatter, on the other hand, yields information on light-refractive and light-reflective properties of the cell and reveals optical inhomogeneity of the cell structure resulting from condensation of cytoplasm or nucleus, granularity, and so on.

As a consequence of cell shrinkage, a decrease in forward light scatter is observed at a relatively early stage of apoptosis (Swat et al., 1981; Ormerod et al., 1995). Initially, there is little change in side scatter during apoptosis. In fact, in some cell systems, an increase in intensity of side-scatter signal may be seen, reflecting perhaps chromatin and cytoplasm condensation and nuclear fragmentation, the events that may lead to an increase in the light-refractive and light-reflective properties of the cell. When apoptosis is more advanced and the cells shrink in size, the intensity of side scatter also decreases (Fig. 18.8.1). Late apoptotic cells, therefore, are characterized by markedly diminished intensity of both forward- and side-scatter signals. In contrast to apoptosis, the early stages of cell necrosis are marked by cell swelling, which is detected by a transient increase in forward light scatter. Rupture of the plasma membrane and leakage of the cytosol during subsequent steps of necrosis correlate with a marked decrease in intensity of both forward- and side-scatter signals.

Analysis of light scatter is often combined with other assays, most frequently surface immunofluorescence (to identify the phenotype of the dying cell), or another marker of apoptosis. It should be stressed, however, that the change in light scatter alone is not a specific marker of apoptosis or necrosis. Mechanically broken cells, isolated nuclei, cell debris, and individual apoptotic bodies may also display diminished light-scatter properties. Therefore, the analysis of light scatter must be combined with measurements that can provide a more definite identification of apoptotic or necrotic cells.

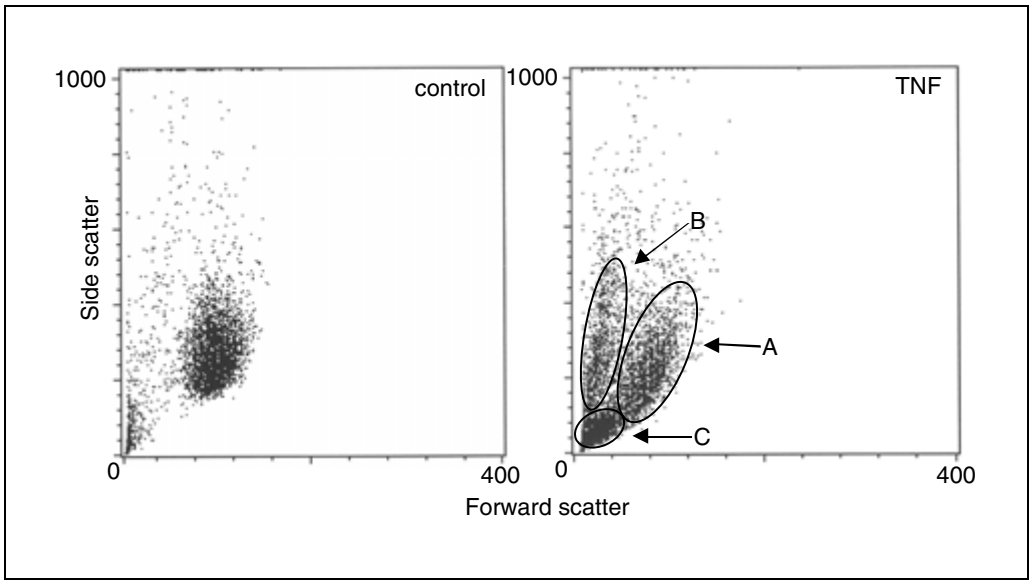


Figure 18.8.1 Changes in light scattering properties of cells undergoing apoptosis. HL-60 cells were untreated (left panel) or treated 3 hr with TNF-α and cycloheximide (CHX) to induce apoptosis (right panel). Cell population **A** in the treated culture (right panel) represents cells that have light scattering properties similar to those of untreated cells. Early apoptotic cells (**B**) have diminished forward scatter and are very heterogenous with respect to side scatter. Late apoptotic cells (**C**) have both forward and side scatter diminished.

Mitochondrial potential

A point to consider in measuring $\Delta\psi_m$ is that the mitochondrial potential probes lack absolute specificity and also accumulate in the cytosol. Probe distribution in mitochondria versus cytosol follows the Nernst equation, according to which the ratio of mitochondrial to cytosolic free cation concentration should be 100:1 at 120 mV mitochondrial transmembrane potential (Waggoner, 1979). However, the specificity of particular mitochondrial probes towards mitochondria is higher at low probe concentrations. It is advisable, therefore, to use these probes at the lowest possible concentration. The limit for the minimal dye concentration that still provides an adequate signal-to-noise ratio during the measurement is dictated by sensitivity of the instrument (laser power, optics, photomultiplier sensitivity) and by the mitochondrial mass per cell; the latter varies depending on the cell type or upon mitogenic stimulation (Darzynkiewicz et al., 1981).

A series of MitoTracker dyes (chloromethyltetramethylrosamine analogs) of different colors was introduced by Molecular Probes as new mitochondrial $\Delta\psi_m$-sensitive probes. Some of these dyes remain attached to mitochondria following cell fixation using cross-linking agents (Poot et al., 1997; Haugland, 2002). It should be noted, however, that because these dyes bind to thiol moieties within mitochondria, their retention after fixation may not be a reliable marker of the transmembrane potential (Ferlini et al., 1998; Gilmore and Wilson, 1999). Furthermore, they are potent inhibitors of respiratory chain I and may themselves induce dissipation of $\Delta\psi_m$ (Scorrano et al., 1999). Because it is likely that other $\Delta\psi_m$ probes may predispose the cells to the permeability transition, one has to be cautious in interpreting the data on their use in analysis of apoptosis.

It has been reported that other mitochondrial probes, 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green, are markers of mitochondrial mass and are not sensitive to $\Delta\psi_m$ (Ratinaud et al., 1988; Poot et al., 1997). It was proposed, therefore, to measure both $\Delta\psi_m$ and mitochondrial mass by using a combination of $\Delta\psi_m$-sensitive and $\Delta\psi_m$-insensitive probes (Petit et al., 1995). Recent observations, however, indicate that 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green are quite sensitive to changes in $\Delta\psi_m$ and therefore, either alone or in combination with $\Delta\psi_m$-sensitive probes, cannot be used as markers of mitochondrial mass (Keiji et al., 2000).

Caspases

Caspases (cysteine-aspartic acid specific proteases) are activated in response to different inducers of apoptosis (Kaufmann et al., 1993; Lazebnik et al., 1994; Alnemri et al., 1996). The process of their activation is considered to be the key event of apoptosis, a marker of cell commitment to disassemble the machinery that supports cell life (for reviews, see Budihardjo et al., 1999; Earnshaw et al., 1999; Shi, 2002). Caspases recognize a four-amino-acid sequence on their substrate proteins; within this sequence, the carboxyl end of aspartic acid is the target for cleavage. Detection of caspase activation is of primary interest in oncology as well as in other disciplines of medicine and biology, and several methods have been developed to accomplish this.

One approach that is potentially useful for cytometry utilizes fluorogenic caspase substrates. The peptide substrates are colorless or nonfluorescent, but upon caspase-induced cleavage, they generate colored or fluorescing products (Gorman et al., 1999; Hug et al., 1999; Liu et al., 1999; Komoriya et al., 2000; Telford et al., 2002). Many kits designed to measure activity of caspases using fluorometric or colorimetric assays are commercially available (e.g., from Biomol Research Laboratories or Calbiochem). Some kits can be used to detect activation of multiple caspases, while other are based on the substrates that are specific for caspase-1, caspase-3, or caspase-8.

The second approach in studies of caspase activation applicable to cytometry is based on immunocytochemical detection of the epitope of these enzymes that is characteristic of their active form. This epitope appears as a result of conformational changes that occur during activation of caspases, such as those associated with the transcatalytic cleavage of the zymogen pro-caspases (for reviews, see Budihardjo et al., 1999; Earnshaw et al., 1999). Antibodies developed to react only with the activated caspases have recently become commercially available (e.g., from Promega). These antibodies can be used in standard immunocytochemical assays. Basic Protocol 3 describes the methodology of detection of caspase-3 activation based on this principle, combined with the convenient immunocytochemical Zenon technology (Haugland, 2002). Another approach that was proposed to probe caspase activation utilizes fluorochrome-labeled inhibitors of caspases (FLICAs; e.g., FAM-VAD-FMK), which are reagents designed as affinity labels to the active enzyme center of these enzymes (Smolewski et al.,

2001). It was recently found, however, that these reagents lack the desired specificity with respect to the active enzyme center of caspases (Pozarowski et al., 2003). However, they are convenient and specific markers of apoptotic cells and can be used as such, particularly when there is a need to distinguish between apoptotic and necrotic modes of cell death. Basic Protocol 3 describes application of FAM-VAD-FMK to identify three sequential stages of apoptosis. Finally, caspase activation can be detected indirectly, by identifying specific protein fragments which are the products of cleavage by particular caspases. For example, immunocytochemical detection of the poly(ADP-ribose) polymerase 85-kDa fragment (PARP p85 fragment), which results from cleavage of the PARP substrate by caspase-3, is described in Basic Protocol 4. Concurrent analysis of cellular DNA content allows one to correlate activation of caspases with the cell cycle position or DNA ploidy. Using immunocytochemical methods, however, one has to remember that, often, antibodies that are applicable to immunoblotting or immunoprecipitation may not always be useful in immunocytochemical assays, and vice versa.

Critical Parameters and Troubleshooting

Positive and negative controls

There are instances when cells die by a process of atypical apoptosis that lacks one or more characteristic apoptotic features. Obviously, apoptosis cannot be detected if the feature serves as a marker. It is also possible that the assay (kit) used to identify apoptotic cells malfunctions for technical reasons. For example, the enzyme TdT used in the TUNEL assay may be inactive due to improper storage. A mistake might be made during the staining procedure. It is essential, therefore, to distinguish between the genuine lack of apoptosis and the inability to detect it due to technical causes. The distinction can be made using a positive control consisting of cells known to be apoptotic (confirmed by a standard method and inspection of cell morphology). Such control cells have to be processed in parallel with the investigated sample through all the prescribed protocol steps. Some vendors provide positive and negative control cells with their kits (e.g., APO-BRDU from Phoenix Flow Systems).

The positive control cells can be prepared in large quantity and stored in aliquots to be used during each experiment. Such a convenient control may be, to give an example, exponentially growing HL-60 or U-937 leukemic cells treated 3 to 6 hr in culture with 0.2 μM camptothecin (CPT) to induce apoptosis. The cells so treated consist of subpopulations of apoptotic (S-phase) and non-apoptotic (G_1-phase) cells, present in the same sample. However, to induce apoptosis of S-phase cells with CPT, it is critical to have the cultures in the exponential phase of growth, at relatively low cell density (<800,000 cells/ml); subconfluent cultures are quite resistant to CPT. A large batch of cells treated in such a manner can be appropriately fixed in 70% ethanol and then stored in aliquots at −20°C to be used as a positive and negative control for each assay that utilizes fixed cells. For the assays that require live cells, controls should be freshly made and must not be fixed. Cells from healthy, untreated cultures may also serve as negative controls.

False-positive apoptosis

The exposure of phosphatidylserine on cell surfaces that occurs during apoptosis (Fadok et al., 1992; Koopman et al., 1994) makes apoptotic cells and apoptotic bodies attractive to neighboring cells, which phagocytize them. The ability to engulf apoptotic bodies is not solely the property of professional phagocytes, but is shared by cells from fibroblast or epithelial lineages. It is frequently observed, especially in solid tumors, that the cytoplasm of both nontumor and tumor cells located in the neighborhood of apoptotic cells contains inclusions typical of apoptotic bodies. The remains of apoptotic cells engulfed by neighboring cells contain altered plasma membrane, fragmented DNA, and other constituents with attributes characteristic of apoptosis. Thus, if the distinction is based on any of these attributes, the live, nonapoptotic cells that phagocytized apoptotic bodies cannot be distinguished from genuine apoptotic cells by flow cytometry. For example, nonapoptotic cells that engulf apoptotic bodies become reactive with annexin V (Marguet et al., 1999). Most likely this is due to the fact that during engulfment, the plasma membrane of apoptotic bodies fuses with the plasma membrane of the phagocytizing cell. It has also been shown that nonapoptotic cells (primarily monocytes and macrophages) in bone marrow of patients undergoing chemotherapy have large quantities of apoptotic bodies in their cytoplasm and are strongly positive in the TUNEL reaction (Bedner et al., 1999).

Even after relative mild treatment such as trypsinization, mechanical agitation, detach-

ment with rubber policeman, or electroporation, the plasma membrane of live nonapoptotic cells may have phosphatidylserine, reactive with annexin V, exposed on the surface. Such cells may also be erroneously identified as apoptotic cells.

Distinction between apoptosis, necrosis, and the "necrotic stage" of apoptosis

There are several differences between typical apoptotic and necrotic cells (Kerr et al. 1972; Arends et al., 1990; Majno and Joris, 1995) that provided a basis for development of numerous markers and methods that can discriminate between these two modes of cell death (Darzynkiewicz et al., 1992, 1997). The major difference stems from the early loss of integrity of the plasma membrane during necrosis. This event results in a loss of the cell's ability to exclude charged fluorochromes such as trypan blue or PI. In contrast, the plasma membrane and membrane transport functions remain, to a large extent, preserved during the *early stages* of apoptosis. A cell's permeability to PI or its ability to retain some fluorescent probes, such as products of enzyme activity (e.g., fluorescein diacetate hydrolyzed by esterases), is the most common marker distinguishing apoptosis from necrosis (Darzynkiewicz et al., 1994). A combination of fluorochrome-conjugated annexin V with PI distinguishes live cells (unstainable with both dyes) from apoptotic cells (stainable with annexin V but unstainable with PI) from necrotic cells (stainable with both dyes; Koopman et al., 1994). The same holds true for a combination of PI with FLICA (Smolewski et al., 2001). However, while this approach works well in many instances, it has limitations and possible pitfalls.

(1) Late-stage apoptotic cells resemble necrotic cells to such an extent that the term "apoptotic necrosis" was proposed to define them (Majno and Joris, 1995). This is a consequence of the fact that the integrity of the plasma membrane of late apoptotic cells is compromised, which makes the membrane leaky and permeable to charged cationic dyes such as PI. Since the ability of such cells to exclude these dyes is lost, the discrimination between late apoptosis and necrosis cannot be accomplished by methods based on the use of dye exclusion (PI, trypan blue) or annexin V binding.

(2) The permeability and asymmetry of plasma membrane phospholipids (accessibility of phosphatidylserine) may change, as a result of prolonged treatment with proteolytic enzymes (trypsinization), mechanical damage (e.g., cell removal from flasks by rubber policeman, cell isolation from solid tumors, or even repeated centrifugations), electroporation, or treatment with some drugs.

(3) Many flow cytometric methods designed to quantify the frequency of apoptotic or necrotic cells are based on the differences between live, apoptotic, and necrotic cells in the permeability of plasma membrane to different fluorochromes such as PI, 7-AAD, or Hoechst dyes. It should be stressed, however, that plasma membrane permeability probed by dye accumulation in the cell may vary depending on the cell type and other factors unrelated to apoptosis or necrosis (e.g., very active efflux mechanism that rapidly pumps dye out of the cell). The assumptions, therefore, that live cells maximally exclude a particular dye, while early apoptotic cells are somewhat leaky and late apoptotic or necrotic cells are fully permeable to the dye, and that these differences are large enough to identify these cells, are not universally applicable.

It is particularly difficult to discriminate between apoptotic and necrotic cells in suspensions from solid tumors. Necrotic areas form in tumors as a result of massive local cell death due to poor accessibility to oxygen and growth factors when tumors grow in size and their local vascularization becomes inadequate. Needle-aspirated samples or cell suspensions from the resected tumors may contain many cells from the necrotic areas. Such cells are indistinguishable from late apoptotic cells by many markers. Because the AI in solid tumors, representing spontaneous or treatment-induced apoptosis, should not include cells from the necrotic areas, one has to eliminate such cells from analysis. Because incubation of cells with trypsin and DNase selectively digests all cells whose plasma membrane integrity is compromised, i.e., primarily necrotic cells (Darzynkiewicz et al., 1994), such a procedure may be used to remove necrotic cells from suspensions. It should be noted, however, that late apoptotic cells have partially leaky plasma membrane and are also expected to be sensitive to this treatment.

In conclusion, examination of cells by microscopy may be the only way to distinguish between apoptosis and necrosis, based on their characteristic differences in morphology.

Preferential loss of apoptotic cells during sample preparation

Cell detachment in culture. Early during apoptosis, cells detach from the surface of culture flasks and float in the medium. The standard procedure of discarding the medium, trypsinizing the attached cells or treating them with EDTA, and collecting the detached cells results in selective loss of those apoptotic cells that are discarded with the medium. Such loss may vary from flask to flask depending on how the culture is handled, e.g., the degree of mixing or shaking, efficiency in discarding the old medium, and so on. Surprisingly, some authors still occasionally report discarding the medium and trypsinizing the cells. Needless to say, such an approach cannot be used for quantitative analysis of apoptosis. To estimate the frequency of apoptotic cells in adherent cultures, it is essential to collect floating cells, pool them with the trypsinized ones, and measure them as a single sample. It should be stressed that trypsinization, especially if prolonged, results in digestion of cells with a compromised plasma membrane. Thus, collection of cells from cultures by trypsinization is expected to cause selective loss of late apoptotic and necrotic cells.

Density-gradient centrifugation. Similarly, density-gradient separation of cells (e.g., using Ficoll-hypaque or percoll solutions) may result in selective loss of dying and dead cells, because early during apoptosis the cells become dehydrated and have condensed nuclei and cytoplasm, and therefore have a higher density than nonapoptotic cells. Knowledge of any selective loss of dead cells in cell populations purified by such an approach is essential when one is studying apoptosis.

Centrifugations, mechanical agitations. Repeated centrifugations lead to cell loss by at least two mechanisms. One involves electrostatic cell attachment to the tubes and may be selective for a particular cell type. For example, preferential loss of monocytes and granulocytes is observed during repeated centrifugation of white blood cells, while lymphocytes remain in suspension (Bedner et al., 1997). Cell loss is of particular concern when hypocellular samples ($<5 \times 10^4$) are processed. In such a situation, carrier cells in excess (e.g., chick erythrocytes) may be added to preclude disappearance of the cells of interest through centrifugation. The second mechanism of cell loss involves preferential disintegration of fragile cells. Because apoptotic cells are very fragile, especially at late stages of apoptosis, they may selectively be lost from samples that require centrifugation or are repeatedly vortexed or pipetted. Addition of serum or bovine serum albumin to cell suspensions, shortened centrifugation time, and decreased gravity force all may have a protective effect against cell breakage by mechanical factors. Apoptotic cells may also preferentially disintegrate in biomass cultures that require constant cell mixing.

It should be noted that sensitivity of apoptotic cells to mechanical factors depends on activation of TGase 2. Cells with activated TGase 2 have their cytoplasmic protein crosslinked and are resistant to mechanical stress. They also resist treatment with detergents. In contrast, apoptotic cells that do not activate TGase 2 are overly sensitive and easily undergo disintegration. It was observed that cells may often undergo apoptosis without evidence of TGase 2 activation (Grabarek et al., 2002).

Abundance of extractable (fragmented) DNA is not a quantitative measure of apoptosis

A common misconception in analysis of apoptosis is that the amount of fragmented (low-molecular-weight, "extractable") DNA detected in cultures, tissue or cell extracts, or other samples reflects incidence of apoptosis. Many methods have been developed to estimate the abundance of fragmented DNA and numerous reagent kits are being sold for that purpose. They include direct quantitative colorimetric analysis of soluble DNA, densitometry of DNA ladders on gels, and immunochemical assessment of nucleosomes. These approaches and the related kits are advertised as quantitative, in that they provide information regarding the incidence of apoptosis in cell populations. Such claims are grossly incorrect for the reason that the amount of fragmented (low-molecular-weight) DNA that can be extracted from a *single* apoptotic cell varies over a wide range depending on the *stage* of apoptosis. Although at an early stage of apoptosis only a small fraction of DNA is fragmented and extractable, nearly all DNA can be extracted from the cell that is more advanced in apoptosis. Thus, the total content of low-molecular-weight DNA extracted from the cell population, or the ratio of low- to high-molecular-weight fraction, does not provide information about the *frequency* of apoptotic cells (AI), even in relative terms, e.g., for comparison of cell populations. For this simple reason, biochemical methods based on analysis of fragmented DNA can be used to *quantitatively* estimate the frequency of apop-

tosis only when comparing cell populations that have identical distribution of cells across the stages of apoptosis.

DNA laddering observed during electrophoresis provides evidence of internucleosomal DNA cleavage, which is considered one of the hallmarks of apoptosis (Arends et al., 1990). Analysis of DNA fragmentation by gel electrophoresis to detect such laddering is thus a valuable method to demonstrate the *apoptotic mode* of cell death; however, it should not be used as a means to quantify the *frequency* of apoptosis.

It should be noted that in some cell types, particularly of fibroblast and epithelial lineages, apoptosis may occur without internucleosomal DNA cleavage. The products of DNA fragmentation in these cells are large (50- to 300-kb) DNA sections that cannot be extracted from the cell (Oberhammer et al., 1993). Obviously, in these cases, apoptosis cannot be revealed by the presence of DNA laddering on gels or by analysis of low-molecular-weight products. These large DNA fragments, however, can be identified by pulse-field gel electrophoresis.

Changes in cell morphology, the "gold standard" for identification of apoptosis

Apoptosis was originally defined as a specific mode of cell death based on very characteristic changes in cell morphology (Kerr et al., 1972; Fig. 18.8.2). These changes are still considered the "gold standard" for identification of apoptotic cells. Although particular markers may be used in conjunction with flow cytometry for detection and quantitative assessment of apoptosis in cell populations, the mode of cell death should always be confirmed by inspection of cells by light or electron microscopy. If there is any ambiguity regarding the mechanism of cell death, the morphological changes should be the deciding factor in resolving the uncertainty.

The characteristic morphological features of apoptosis and necrosis are listed in Table 18.8.1. The most specific (and generic to apoptosis) of these changes is chromatin condensation; the chromatin of apoptotic cells is very "smooth" (structureless) in appearance, and the structural framework that otherwise characterizes the cell nucleus is entirely lost. Because of the condensation, chromatin shows strong hyperchromicity with any of the DNA-specific

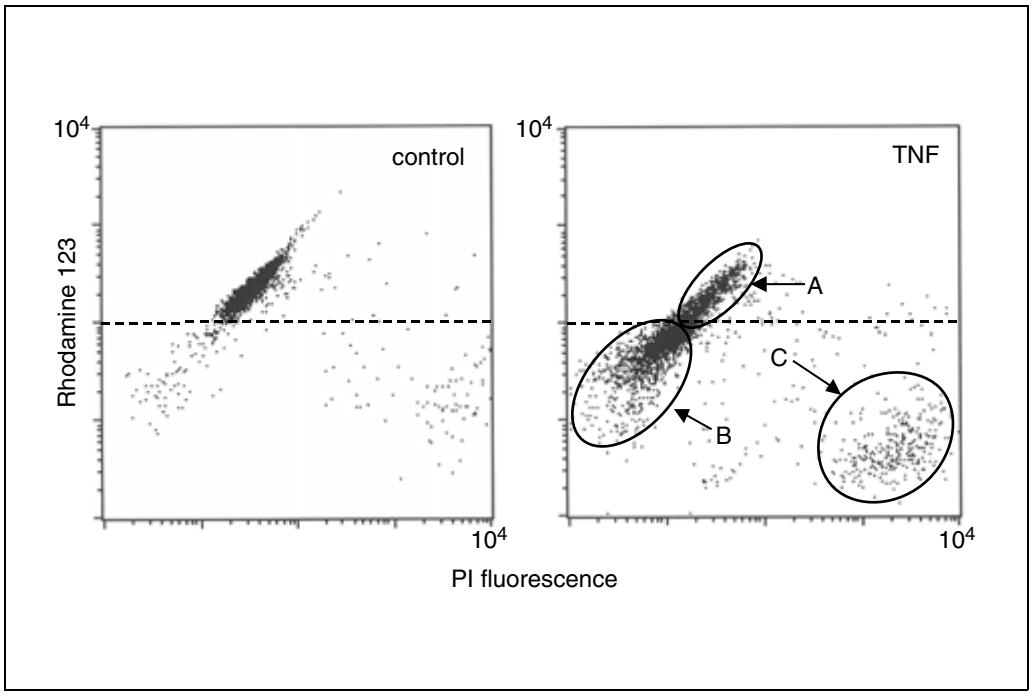


Figure 18.8.2 Detection of the collapse of mitochondrial electrochemical potential (ψ_m) by rhodamine 123 (R123). HL-60 cells were untreated (control; left panel) or treated 3 hr with TNF-α and CHX (right panel) to induce apoptosis. The cells were then incubated with R123 and PI according to Basic Protocol 1. The early apoptotic cells have diminished green fluorescence of R123 but exclude PI (cell population B). The late apoptotic (also necrotic) cells are stained strongly by PI (population C).

Table 18.8.1 Morphological Criteria for Identification of Apoptosis or Necrosis

Apoptosis	Necrosis
Reduced cell size, convoluted shape	Cell and nuclear swelling
Plasma membrane undulations ("blebbing," "budding")	Patchy chromatin condensation
Chromatin condensation (DNA hyperchromicity)	Swelling of mitochondria
Loss of the structural features of the nucleus (smooth, planate appearance)	Vacuolization in cytoplasm
Nuclear fragmentation (karyorrhexis)	Plasma membrane rupture ("ghost-like" appearance of lysed cells)
Presence of apoptotic bodies	Dissolution of nuclear chromatin (karyolysis)
Dilatation of the endoplasmic reticulum	Attraction of inflammatory cells
Relatively unchanged cell organelles	
Shedding of apoptotic bodies	
Phagocytosis of the cell remnants	
Cell detachment from tissue culture flasks	

dyes (Hotz et al., 1992). Apart from chromatin condensation, however, other changes are less generic to apoptosis and may not always be apparent. For example, nuclear fragmentation, although commonly observed during apoptosis of hematopoietic-lineage cells, may not occur during apoptosis of some epithelial- or fibroblast-lineage cells. Likewise, cell shrinkage, at least early during apoptosis, is not a universal marker of the apoptotic mode of cell death.

It should be stressed that optimal preparations for light microscopy require cytospinning of live cells followed by fixation and staining on slides. The cells become flat, facilitating assessment of their morphology. On the other hand, when cells are initially fixed and stained in suspension, transferred to slides, and analyzed under the microscope, their morphology is obscured by unfavorable geometry; the cells are spherical and thick, and require confocal microscopy to reveal details such as early signs of apoptotic chromatin condensation.

Differential staining of cellular DNA and protein of cells on slides with DAPI and sulforhodamine 101, respectively, is rapid and simple and provides very good morphological resolution of apoptosis and necrosis (Darzynkiewicz et al., 1997). A combined cell illumination with UV light (to excite the DAPI or other DNA fluorochrome) and light-transmission microscopy utilizing interference contrast (Nomarski illumination) is the authors' favorite method of cell visualization to identify apoptotic cells. Other DNA fluorochromes, such as PI, 7-aminoactinomycin D, or acridine orange, can be used as well.

In conclusion, regardless of which cytometric assay has been used to identify apoptosis, the mode of cell death should be confirmed by inspection of cells by light or electron microscopy. Morphological changes during apoptosis have a very characteristic pattern (Kerr et al., 1972; Majno and Joris, 1995) and should be the deciding factor in situations where any ambiguity arises regarding the mode of cell death. It should be noted, however, that the laser scanning cytometer (LSC), offering many attributes of flow cytometry and simultaneously providing the means to examine morphology of the measured cells (Darzynkiewicz et al., 1999; Kamentsky, 2001), appears to be the instrument of choice in analysis of apoptosis (Bedner et al., 1999).

Membrane potential

It should be stressed that $\Delta\psi_m$, like other functional markers, is sensitive to minor changes in cell environment. The samples to be compared, therefore, should be incubated and measured under identical conditions, taking into an account temperature, pH, time elapsed between the onset of incubation and actual fluorescence measurement, and other potential variables.

Annexin V

Interpretation of the results may be complicated by the presence of non-apoptotic cells with damaged membranes. Such cells may have phosphatidylserine exposed on the plasma membrane and therefore, like apoptotic cells, bind annexin V. Mechanical disaggregation of tissues to isolate individual cells, extensive use of proteolytic enzymes to disrupt cell aggregates, to remove adherent cells from cultures, or to isolate cells from tissue, mechanical removal of the cells from tissue-culture flasks (e.g., with a rubber policeman), or cell electroporation all affect the binding of annexin V. Such treatments, therefore, may introduce experimental bias in the subsequent analysis of apoptosis by this method.

Even intact and live cells take up PI upon prolonged incubation. Therefore, fluorescence measurement should be performed rather shortly after addition of the dye.

DNA fragmentation

It should be stressed that the degree of extraction of low-molecular-weight DNA and consequently the content of DNA remaining in apoptotic cells for flow cytometric analysis varies markedly depending on the extent of DNA degradation (duration of apoptosis), the number of cell washings, and the pH and molarity of the washing and staining buffers. DNA fragmentation is often so extensive that most DNA is removed during the post-fixation rinse with PBS and in the staining solution, and a DNA extraction step is therefore unnecessary. Conversely, when DNA degradation does not proceed to internucleosomal regions but stops after generating 50- to 300-kb fragments (Oberhammer et al., 1993), little DNA can be extracted, and this method may fail to detect such apoptotic cells. It also should be noted that if G_2, M, or even late S-phase cells undergo apoptosis, the loss of DNA from these cells may not be adequate to place them at the sub-G_1 peak, as they may end up with DNA content equivalent of that of G_1 or early S-phase cells and therefore be indistinguishable from the latter.

It is a common practice to use detergents or hypotonic solutions instead of fixation in the process of DNA staining for flow cytometry (Nicoletti et al., 1991). Such treatments cause lysis of plasma membrane and release of the nucleus. Although this approach is simple and yields excellent resolution for DNA-content analysis, when used to quantify apoptotic cells it introduces bias owing to the fact that nuclei of apoptotic cells are often fragmented. Lysis of cells with fragmented nuclei releases nuclear fragments rather than individual nuclei, and consequently several fragments can be released from a single cell. Likewise, lysis of mitotic cells that happen to be in the specimen releases individual chromosomes or chromosome aggregates. In the case of micronucleation (e.g., after cell irradiation), the micronuclei are released upon cell lysis. Each nuclear fragment, chromosome, or micronucleus is then recorded by the flow cytometer as an individual object with a sub-G_1 DNA content. Such objects are then erroneously classified as individual apoptotic cells. This bias is increased if DNA content is displayed on a logarithmic scale. Such a scale allows one to record objects with DNA content as little as 1% or even 0.1% of that of G_1 cells, which certainly cannot be individual apoptotic cells.

Activation of TGase 2

Although apoptotic cells with activated TGase 2 can be detected using either Basic Protocol 8 or Alternate Protocol 2, the differences between these assays should be underscored. The most distinct is the difference in the length of the time window that may be measured by the respective assay. Namely, the detergent-based assay (see Basic Protocol 8) detects cumulative protein cross-linking, reflecting the integrated cross-linking, from its onset to the time of cell harvesting. In contrast, the assay based on incorporation of F-CDV (see Alternate Protocol 2) detects cross-linking that occurs only during the time interval when this reagent is present in the culture. Thus, if F-CDV is included at time zero, i.e., when the inducer of apoptosis is added, its incorporation is a reflection of the cumulative protein cross-linking and thus is comparable with the detergent-based assay. However, if it is added during the final hour or two, it will reflect the cross-linking that took place only during this 1- or 2-hr time window. This difference between the assays should be kept in mind when comparing frequency of TGase 2–positive cells, which may vary between the assays, depending on the length of the respective time window.

Situations (e.g., following treatment with particular drugs) may occur in which cell proteins may become less soluble and more detergent resistant, not necessarily because of TGase 2 activation, but because of alteration by the drug. Some treatments unrelated to TGase 2 activity may also result in attachment of F-CDV to cellular proteins. Some cell types may be

more resistant to detergents. In all these cases, the assay may detect the "false-positive" TGase 2–positive cells. As with other markers of apoptosis, one has to be careful and additionally identify these cells by microscopy based on the characteristic changes in their morphology.

Apoptosis can be induced and may progress in some cells with no apparent TGase 2 activation (Grabarek et al., 2002). In general, the activation of TGase 2 is seen to occur in cells that show a high degree of chromatin and cytoplasm condensation leading to pronounced nuclear and cell shrinkage, and that either lack or have very limited nuclear fragmentation. In contrast, the apoptotic cells that appear larger and whose nuclei are excessively fragmented do not show activation of TGase 2. Apoptosis without activation of TGase 2 appears to occur more frequently when induced with higher drug doses, i.e., when cells enter the apoptotic process more rapidly following treatment. Thus, a note of caution should be added, that since TGase 2 activation may not be detected in some instances of apoptosis, the absence of its activation should not be considered a marker of nonapoptotic cells.

Anticipated Results

Light scatter. A decrease in forward light scatter characterizes early apoptotic cells (Fig. 18.8.1, cluster B). Late apoptotic cells and perhaps also larger apoptotic bodies show marked decrease in both forward and side light scatter (cluster C).

Mitochondrial potential. A combination of PI and R123 identifies nonapoptotic cells that stain only green, early apoptotic cells whose green fluorescence is diminished, and late apoptotic or necrotic cells that stain with PI and have red fluorescence only (Fig. 18.8.2). Likewise, a combination of $DiOC_6(3)$ and PI labels live nonapoptotic cells green, early apoptotic cells dim-green, and late apoptotic and necrotic cells red (not shown).

The change in binding of JC-1 is manifested by a loss of the orange fluorescence that represents the aggregate binding of this dye and that characterizes charged mitochondria (Fig. 18.8.3). JC-1 green fluorescence is expected to increase as a result of disaggregation of the complexed JC-1. However, either no change or a decrease in green fluorescence may be seen if JC-1 concentration within the cell is too high, which causes quenching of its fluorescence.

Caspases. Caspase-3 activation during apoptosis induced by topotecan (TPT), a camptothecin (CPT) analog, is reflected by the cells' ability to bind antibody that is reactive with the activated (cleaved) form of this enzyme (Fig. 18.8.4). Concurrent staining of cellular DNA with PI makes it possible to correlate caspase-3 activation with the cell cycle position. Note that activation of caspase-3 occurs preferentially in S-phase cells.

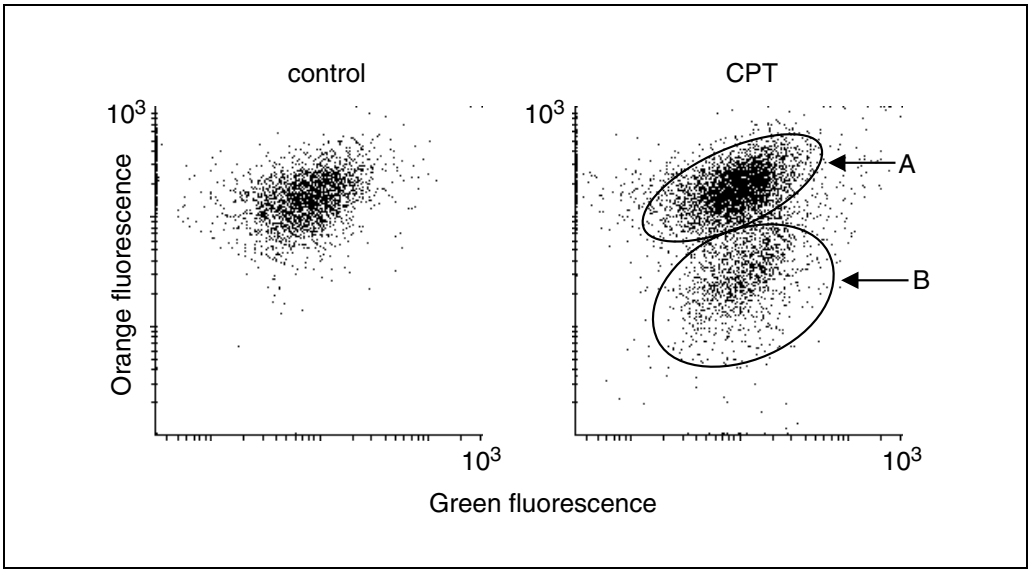


Figure 18.8.3 Detection of the collapse of mitochondrial electrochemical potential using the aggregate dye JC-1. HL-60 cells were untreated (control, left panel) or treated 3 hr with camptothecin (CPT, right panel) to induce apoptosis. Cells were then stained with JC-1 and their orange and green fluorescence was measured by cytometry, as described in Basic Protocol 2. Decreased intensity of orange fluorescence (subpopulation B) characterizes the cell with collapsed potential.

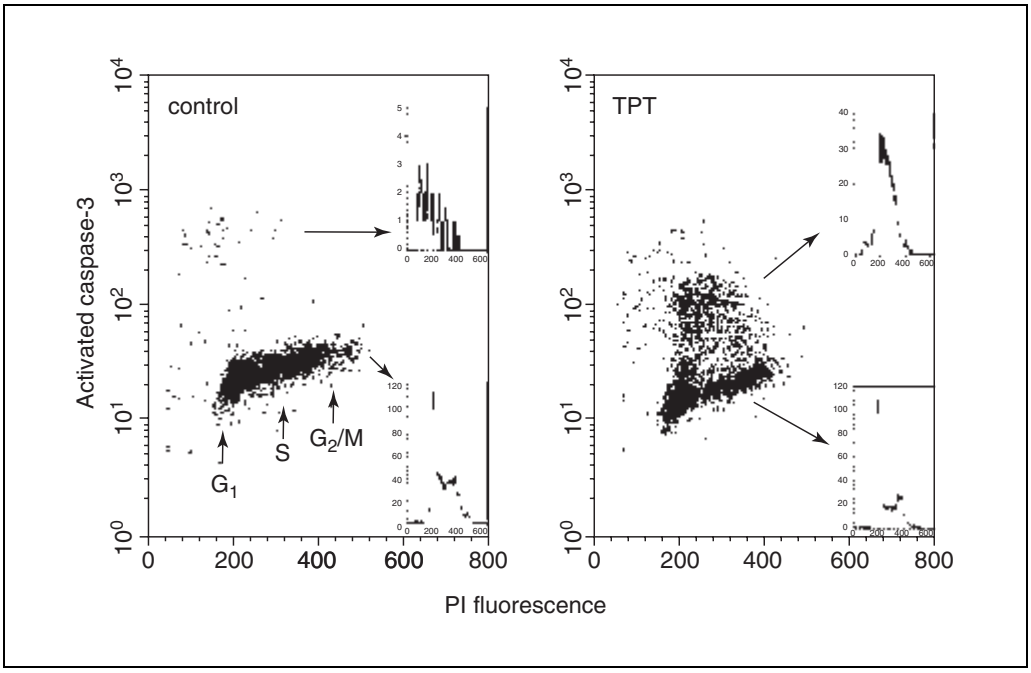


Figure 18.8.4 Immunocytochemical detection of caspase-3 activation using antibody reactive with the activated (cleaved) caspase-3. Apoptosis of HL-60 cells was induced by topotecan (TPT), an analog of CPT. Zenon technology (Haugland, 2002) was used to detect caspase-3 as described in Basic Protocol 2. Top and bottom insets in each panel show cellular DNA content frequency histograms of cells with activated and nonactivated caspase-3, respectively. Note that S-phase cells preferentially contain activated caspase-3 after induction of apoptosis by TPT.

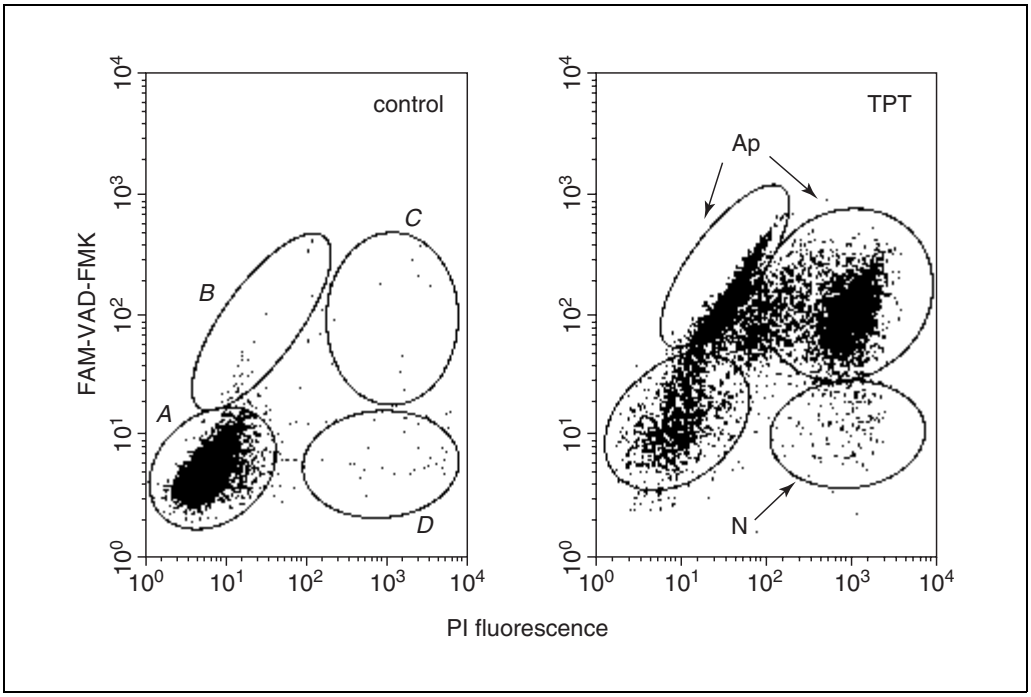


Figure 18.8.5 Binding of fluorochrome-labeled inhibitor of caspases (FLICA; FAM-VAD-FMK) and PI during apoptosis. Apoptosis of HL-60 cells was induced by TPT. The cells were stained according to Basic Protocol 3. Green (FAM-VAD-FMK) and red (PI) cellular fluorescence was measured by flow cytometry. Four cell subpopulations (A to D) can be identified, differing in their capability to bind FAM-VAD-FMK and PI. They represent sequential stages of apoptosis, starting with binding of FAM-VAD-FMK (B), loss of plasma membrane integrity to exclude PI (C), and loss of reactivity with FAM-VAD-FMK (D).

The bivariate distributions (scatterplots) of green and red fluorescence intensity representing cells supravitally stained with FAM-VAD-FMK (FLICA) and PI reveal the presence of four distinct subpopulations (Fig. 18.8.5). Nonapoptotic cells show neither FLICA nor PI fluorescence ($FLICA^-/PI^-$; subpopulation A). Early apoptotic cells bind FAM-VAD-FMK and still exclude PI ($FLICA^+/PI^-$; subpopulation B). More advanced in apoptosis are the cells that bind FAM-VAD-FMK but lose the ability to exclude PI ($FLICA^+/PI^+$; subpopulation C). The cells most advanced in apoptosis are FAM-VAD-FMK negative and are stained with PI ($FLICA^-/PI^+$; subpopulation D). Because the late phase of apoptosis during which the plasma membrane becomes permeable to cationic dyes such as PI or trypan blue has been defined as the "necrotic stage" of apoptosis (Majno and Joris, 1995; Darzynkiewicz et al., 1997), the $FLICA^+/PI^+$ and $FLICA^-/PI^+$ cells thus represent two consecutive phases of the "necrotic stage." It should be noted that genuine necrotic cells, i.e., cells that die by the mode of necrosis ("accidental" cell death), not having activated caspases and unable to exclude PI (Darzynkiewicz et al., 1997), have the same properties ($FLICA^-/PI^+$) as very late apoptotic cells.

As mentioned earlier, because of lack of specificity, the labeling of apoptotic cells with FAM-VAD-FMK, while likely the marker of caspase activation, is not in and of itself evidence of its binding to the active enzymatic center of caspases (Pozarowski et al., 2003).

PARP cleavage. Differences in intensity of PARP p85 immunofluorescence versus PI fluorescence (cellular DNA content) allow one to identify apoptotic cells and reveal the cell cycle distribution of both apoptotic (PARP p85–positive) and nonapoptotic (PARP p85–negative) cells (Fig. 18.8.6). It is quite evident that predominantly S-phase cells were undergoing apoptosis upon CPT treatment (Li and Darzynkiewicz, 2000).

Annexin V. Live nonapoptotic cells stained according to Basic Protocol 5 have minimal green (annexin V-FITC) fluorescence and also minimal red (PI) fluorescence (Fig. 18.8.7; subpopulation A). At early stages of apoptosis, cells stain green but still exclude PI and therefore continue to have no significant red fluorescence (subpopulation B). At late stages of apoptosis, cells show intense green and red fluorescence (subpopulation C). It should be noted that isolated nuclei, cells with severely damaged membranes, and very late apoptotic cells stain rapidly and strongly with PI and may not bind annexin V (subpopulation D).

DNA fragmentation. Apoptotic cells have decreased PI (or DAPI) fluorescence and diminished forward light scatter relative to cells in the main peak (G_1; Fig. 18.8.8). Optimally, the sub-G_1 peak representing apoptotic cells should be separated from the G_1 peak of the

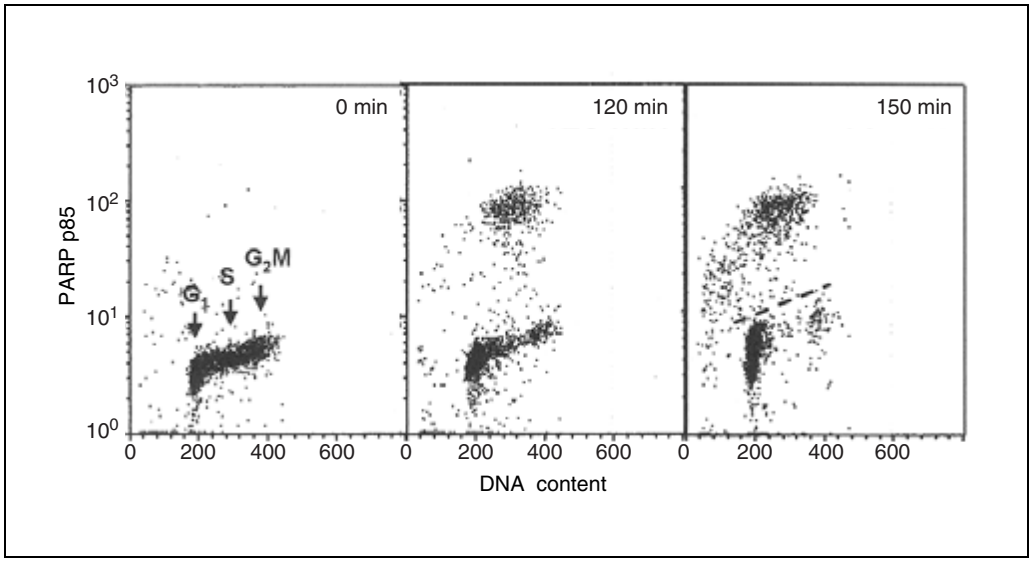


Figure 18.8.6 Identification of apoptotic cells by flow cytometry based on the immunocytochemical detection of the 85-kDa PARP cleavage fragment. To induce apoptosis, HL-60 cells were treated 60 min with TNF-α in the presence of CHX (Li and Darzynkiewicz, 2000). PARPp85 was detected immunocytochemically and DNA was counterstained with PI, as described in Basic Protocol 3.

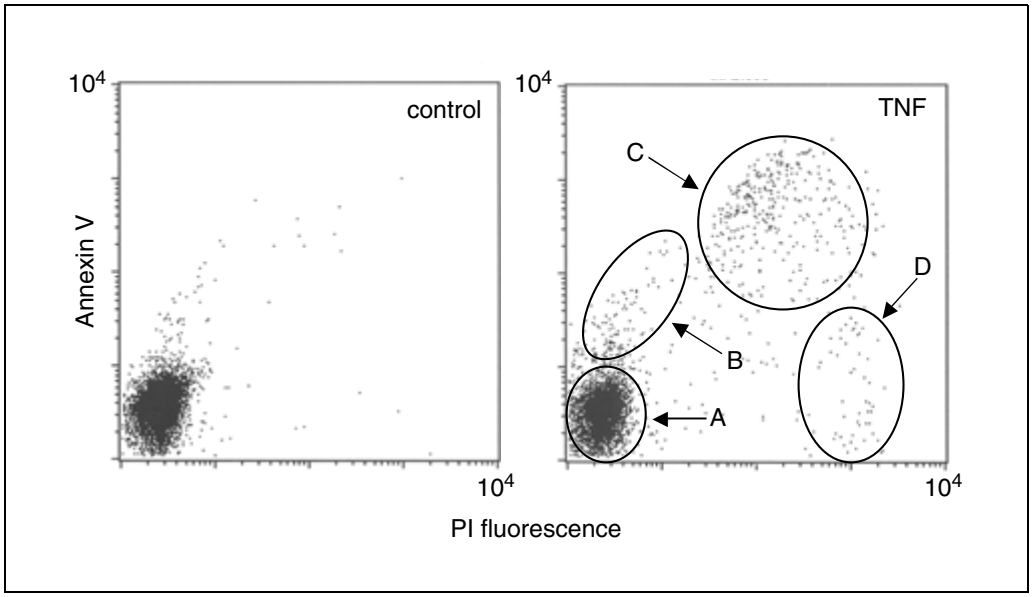


Figure 18.8.7 Detection of early and late apoptotic cells after staining with annexin V–FITC and PI. To induce apoptosis, HL-60 cells were treated 2 hr with TNF-α and CHX. Untreated (control; left panel) and TNF-α-treated (right panel) cells were then stained with annexin V-FITC and PI.

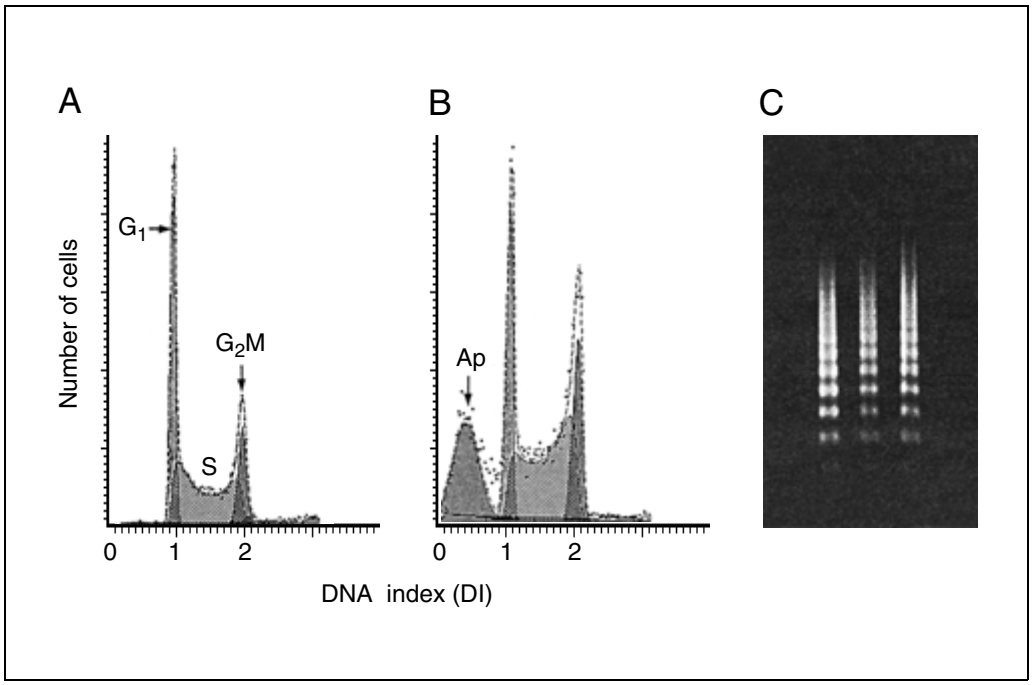


Figure 18.8.8 Detection of apoptotic cells by flow cytometry based on cellular DNA content analysis. (**A**) Normal cell plot. (**B**) To induce apoptosis, HL-60 cells were treated with the DNA topoisomerase II inhibitor fostriecin (Hotz et al., 1994). Cells were fixed in 70% ethanol, suspended in high-molarity phosphate buffer to extract fragmented DNA, and then stained with PI. A subpopulation of apoptotic cells (Ap) with fractional (sub-diploid) DNA content, i.e., with DNA index (DI) <1.0 (sub-G_1 cells), is apparent. Note also the increase in the proportion of S-phase cells in the nonapoptotic population. (**C**) The fragmented DNA extracted from the apoptotic cells by the buffer was subjected to gel elecrophoresis (Gong et al., 1994). Note “laddering" that reflects preferential DNA cleavage at internucleosomal sections, the characteristic feature of apoptosis (Arends et al., 1990).

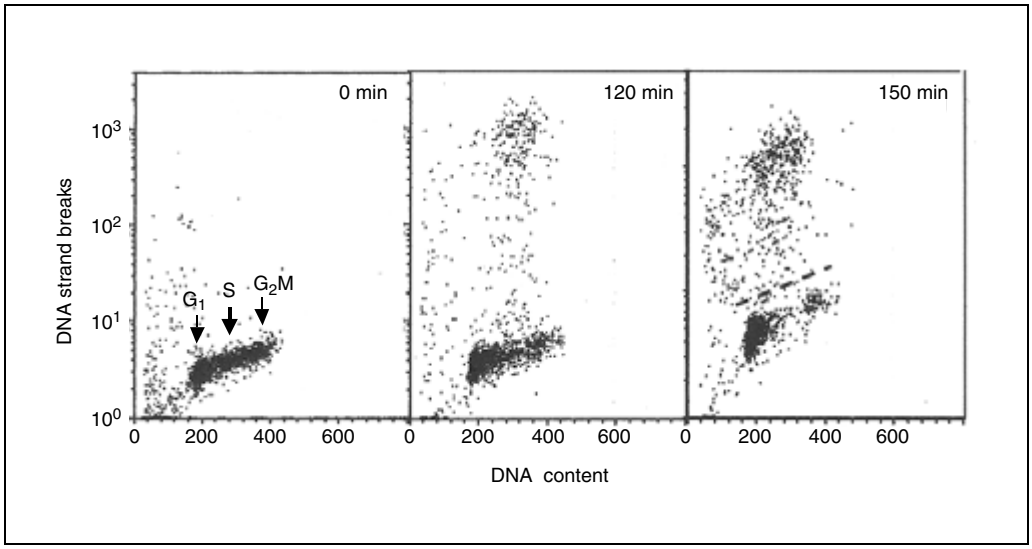


Figure 18.8.9 Detection of apoptotic cells by flow cytometry based on the presence of DNA strand breaks. To induce apoptosis, HL-60 cells were treated 120 or 150 min with CPT (Li and Darzynkiewicz, 2000). DNA strand breaks were labeled with BrdUTP using exogenous terminal deoxynucleotidyl transferase. The cell cycle distribution of both apoptotic and nonapoptotic cell subpopulations can be estimated based on the DNA content of individual cells. Note that in analogy to PARP cleavage (Fig. 18.8.6), preferentially S-phase cells undergo apoptosis following CPT treatment.

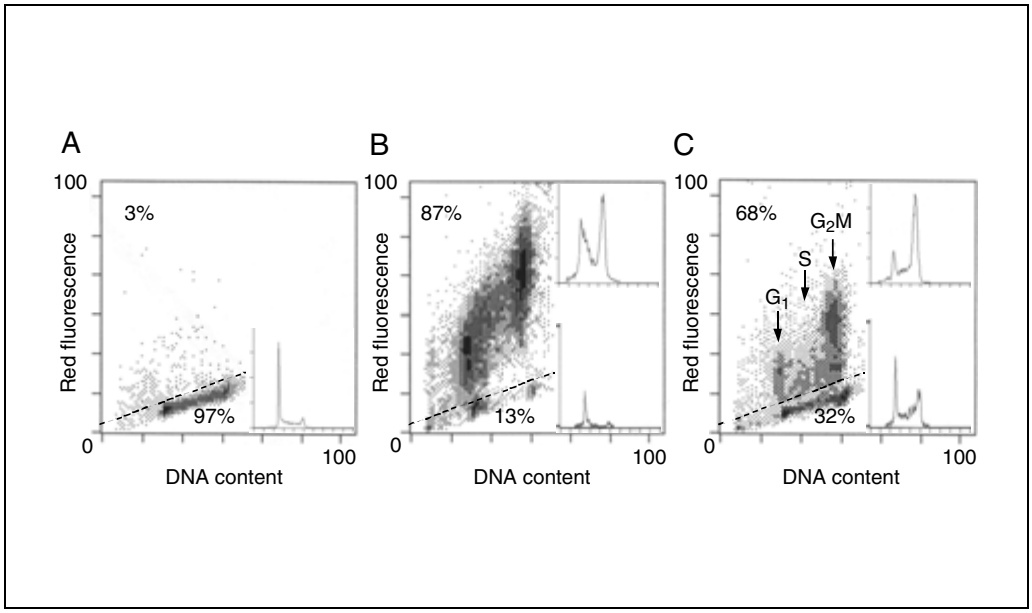


Figure 18.8.10 Detection of tissue transglutaminase (TGase 2) activation during apoptosis by the acquired resistance of the cytoplasmic proteins to detergent. Bivariate distributions illustrating red fluorescence of sulforhodamine 101 (protein content) versus blue fluorescence of DAPI (DNA content) of HL-60 cells, untreated (**A**) or exposed to hyperthermia (72 hr at 41.5°C) in the absence (**B**) and presence (**C**) of the cytotoxic RNase onconase (1.67 μM; Grabarek et al., 2002). Following cell lysis by Triton X-100 and staining with DAPI and sulforhodamine 101, the isolated nuclei of nonapoptotic cells from control culture (**A**) show low and uniform intensity of red fluorescence, reflecting low protein content. Subpopulations of apoptotic cells in **B** and **C** have their cytoplasmic protein crosslinked and therefore are resistant to detergent. They stain intensely with sulforhodamine 101. Note differences in DNA content (cell cycle) distribution of the cells with activated (top insets; cells gated above the dashed line) versus nonactivated TGase 2 (bottom insets; cells gated below the dashed line) in **B** and **C**. Percentage of cells with activated and non-activated TGase 2 in the respective cultures is indicated in each panel.

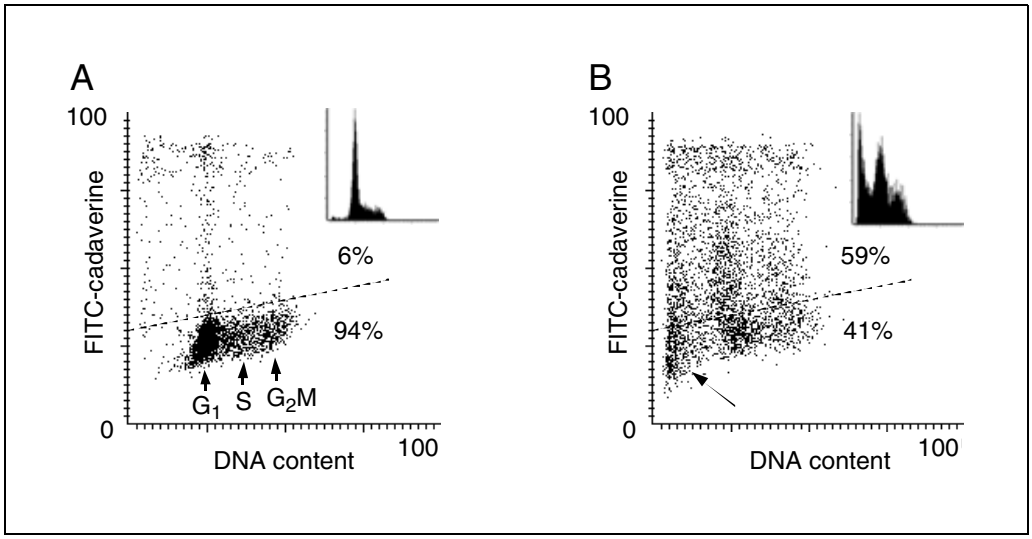


Figure 18.8.11 Detection of TGase 2 activity in HL-60 cells using FITC-conjugated cadaverine (F-CDV) as the enzyme substrate. Cultures of untreated (**A**) and hyperthermia (5 hr at 41.5°C)-treated (**B**) HL-60 cells were incubated 5 hr with 100 μM F-CDV. Cells were then fixed and their DNA was counterstained with PI in the presence of RNase. Note the presence in the untreated culture (**A**) of few cells that incorporated F-CDV (spontaneous apoptosis), and large numbers of F-CDV-labeled cells in the treated culture (**B**). Note also that some apoptotic cells with fractional DNA content (sub-G_1 subpopulation) in the treated culture do not show incorporation of F-CDV (arrow). Percentage of cells with activated and nonactivated TGase 2 in the respective cultures is indicated. The inset shows the cellular DNA content distribution histogram of all cells (Grabarek et al., 2002).

nonapoptotic cell population with little or no overlap between these two.

TUNEL. DNA strand breaks in apoptotic cells are strongly labeled with fluoresceinated anti-BrdU Ab that distinguishes them from the nonapoptotic cells (Fig. 18.8.9). Because of the high intensity of their green fluorescence, an exponential scale (logarithmic PMTs) often must be used for data acquisition and display. Simultaneous measurement of DNA content makes it possible to identify the cell cycle position of cells in apoptotic and nonapoptotic populations. It should be noted, however, that late apoptotic cells may have diminished DNA content because of prior shedding of apoptotic bodies (which may contain nuclear fragments), or due to such massive DNA fragmentation that small DNA fragments cannot be retained in the cell even after fixation with formaldehyde. Such late apoptotic cells may have sub-G_1 DNA content as shown in Figure 18.8.8.

Tissue transglutaminase (TGase 2). Detergent (Triton X-100) treatment of nonapoptotic cells as well as apoptotic cells without activated TGase 2 results in cell lysis and release of isolated nuclei or nuclear fragments that have minimal protein content. Such isolated nuclei or nuclear fragments have very low red fluorescence after staining with sulforhodamine 101 (Fig. 18.8.10, panel A). Apoptotic cells with activated TGase 2, on the other hand, have cross-linked proteins and resist lysis under these conditions. Intensity of their red fluorescence is several times higher than that of the isolated nuclei (subpopulations represented by the scatter plots above the dashed lines in panels B and C). Note the high heterogeneity among individual TGase 2–positive cells in intensity of their red fluorescence.

Because cellular DNA content (PI fluorescence) is measured concurrently with protein content, induction of protein cross-linking can be correlated with the cell cycle position. It is evident that the effects are cell cycle phase specific. Both hyperthermia and onconase lead to preferential protein cross-linking in G_2/M-phase cells.

Activation of TGase 2 in HL-60 cells, detected by cell labeling with F-CDV combined with cellular DNA content analysis, is shown in Fig. 18.8.11. As in the case of protein content (Fig. 18.8.10), populations of cells with activated TGase 2 either undergoing spontaneous apoptosis in control culture (Fig. 18.8.11A) or subjected to hyperthermia (Fig. 18.8.11B) are heterogeneous in terms of their TGase 2–related fluorescence. In the hyperthermia-treated culture, a large number of cells have fractional

DNA content, forming a characteristic sub-G_1 population typical of apoptotic cells. This population is heterogeneous in terms of intensity of F-CDV fluorescence, with many F-CDV-negative sub-G_1 cells (arrow). Thus, the degree of activation of TGase 2 is uneven, and many cells with apoptotic features (sub-G_1 DNA content) have undetectable level of TGase activity.

Time Considerations

Basic Protocol 1 takes ~15 min to prepare cells for incubation with mitochondrial probes followed by an additional 15- to 30-min incubation.

Basic Protocol 2 (activated caspase detection by Zenon techology) requires ~2 hr to complete (cell fixation not included).

In Basic Protocol 3, FLICA (FAM-VAD-FMK) is added directly to cultures at least 30 min before cell centrifugation. Optimal labeling is achieved after a 1- to 2-hr incubation with FLICA. Cell rinses and staining with PI require an additional ~15 min before cells are measured by flow cytometry.

Basic Protocol 4 requires ~4 hr to process cells from fixation through primary and secondary Ab incubations followed by staining with PI before they are analyzed by flow cytometry.

Basic Protocol 5 is a rapid procedure that can be completed in 15 min.

Basic Protocol 6 requires ~40 min to carry out cell rinsing and staining with PI following removal from fixative.

In Alternate Protocol 1, cell rinsing and staining can be completed in 25 to 30 min.

Basic Protocol 7 takes ~3 hr to carry out all steps of cell rinsing and incubations with the respective reagents after removal of cells from fixative.

Basic Protocol 8 is a rapid procedure. Cells can be analyzed by flow cytometry ~5 min after their removal from cultures.

In Alternate Protocol 2, the reagent F-CDV is added directly to cultures at different time intervals. It takes ~40 min following cell fixation to carry out cell rinsing and labeling with PI.

Literature Cited

Alnemri, E.S., Livingston, D.I., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. 1996. Human ICE/CED-4 protease nomenclature. *Cell* 87:171-173.

Arends, M.J., Morris, R.G., and Wyllie, A.H. 1990. Apoptosis: The role of endonuclease. *Am. J. Pathol.* 136:593-608.

Bedner, E., Burfeind, P., Gorczyca, W., Melamed, M.R., and Darzynkiewicz, Z. 1997. Laser scanning cytometry distinguishes lymphocytes, monocytes and granulocytes by differences in their chromatin structure. *Cytometry* 29:191-196.

Bedner, E., Li, X., Gorczyca, W., Melamed, M.R., and Darzynkiewicz, Z. 1999. Analysis of apoptosis by laser scanning cytometry. *Cytometry* 35:181-195.

Blagosklonny, M.V. 2000. Cell death beyond apoptosis. *Leukemia* 14:1502-1508.

Budihardjo, I., Oliver, H., Lutter, M., and Luo, X. 1999. Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol.* 15:269-290.

Catchpoole, D.R. and Stewart, B.W. 1993. Etoposide-induced cytotoxicity in two human T-cell leukemic lines. Delayed loss of membrane permeability rather than DNA fragmentation as an indicator of programmed cell death. *Cancer Res.* 53:4287-4296.

Collins, R.J., Harmon, B.V., Gobe, G.C., and Kerr, J.F.R. 1992. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int. J. Radiat. Biol.* 61:451-453.

Cossarizza, A. and Salvioli, S. 2001. Analysis of mitochondria during cell death. *Meth. Cell Biol.* 63:467-486.

Cossarizza, A., Kalashnikova, G., Grassilli, E., Chiappelli, F., Salvioli, S., Capri, M., Barbieri, D., Troiano, L., Monti, D., and Franceschi, C. 1994. Mitochondrial modifications during rat thymocyte apoptosis: A study at a single cell level. *Exp. Cell Res.* 214:323-330.

Darzynkiewicz, Z., Staiano-Coico, L., and Melamed, M.R. 1981. Increased mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. *Proc. Natl. Acad. Sci. U.S.A.* 78:2383-2387.

Darzynkiewicz, Z., Traganos, F., Staiano-Coico, L., Kapuscinski, J., and Melamed, M.R. 1982. Interactions of rhodamine 123 with living cells studied by flow cytometry. *Cancer Res.* 42:799-806.

Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M.A., Lassota, P., and Traganos, F. 1992. Features of apoptotic cells measured by flow cytometry. *Cytometry* 13:795-808.

Darzynkiewicz, Z., Li, X., and Gong, J. 1994. Assays of cell viability. Discrimination of cells dying by apoptosis. *Meth. Cell Biol.* 41:16-39.

Darzynkiewicz, Z., Juan, G., Li, X., Murakami, T., and Traganos, F. 1997. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 27:1-20.

Darzynkiewicz, Z., Bedner, E., Li, X., Gorczyca, W., and Melamed, M.R. 1999. Laser scanning cytometry. A new instrumentation with many applications. *Exp. Cell Res.* 249:1-12.

Deng, Y., Lin, Y., and Wu, X. 2002. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. *Genes Dev.* 16:33-45.

de Murcia, G. and Menissier-de Murcia, J.M. 1994. Poly(ADP-ribose) polymerase: A molecular nick sensor. *Trends Biochem. Sci.* 19:172-176.

Earnshaw, W.C., Martins, L.M., and Kaufmann, S.H. 1999. Mammalian caspases: Structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* 68:383-424.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148:22-29.

Ferlini, C., Scambia, G., and Fattorossi, A. 1998. Is chloromethyl-X-rosamine useful in measuring mitochondrial transmembrane potential? *Cytometry* 31:74-79.

Fesus, L., Thomazy, V., and Falus, A. 1987. Induction and activation of tissue transglutaminase during programmed cell death. *FEBS Lett.* 224:104-108.

Finucane, D.M., Waterhouse, N.J., Amaranto-Mendes, G.P., Cotter, T.G., and Green, D.R. 1999. Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL-60 cells. *Exp. Cell Res.* 251:166-174.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragementation. *J. Cell. Biol.* 119:493-501.

Gilmore, K. and Wilson, M. 1999. The use of chloromethyl-X-rosamine (Mitotracker Red) to measure loss of mitochondrial membrane potential in apoptotic cells is incompatible with cell fixation. *Cytometry* 36:355-358.

Gong, J., Traganos, F., and Darzynkiewicz, Z. 1994. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.*218:314-319.

Gorczyca, W., Bruno, S., Darzynkiewicz, R., Gong, J., and Darzynkiewicz, Z. 1992. DNA strand breaks occurring during apoptosis: Their early in situ detection by the terminal deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. *Int. J. Oncol.* 1:639-648.

Gorczyca, W., Bigman, K., Mittelman, A., Ahmed, T., Gong, J., Melamed, M.R., and Darzynkiewicz, Z. 1993. Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. *Leukemia* 7:659-670.

Gorman, A.M., Hirt, U.A., Zhivotovsky, B., Orrenius, S., and Ceccatelli, S. 1999. Application of a fluorometric assay to detect caspase activity in thymus tissue undergoing apoptosis in vivo. *J. Immunol. Methods* 226:43-48.

Grabarek, J., Ardelt, B., Kunicki, J., and Darzynkiewicz, Z. 2002. Detection of in situ activation of transglutaminase during apoptosis: Correlation with the cell cycle phase by multiparameter flow and laser scanning cytometry. *Cytometry* 49:83-89.

Haugland, R.P. 2002. Handbook of Fluorescent Probes and Research Chemicals. Ninth Edition. Molecular Probes, Eugene, OR.

Hotz, M.A., Traganos, F., and Darzynkiewicz, Z. 1992. Changes in nuclear chromatin related to apoptosis or necrosis induced by the DNA topoisomerase II inhibitor fostriecin in MOLT-4 and HL-60 cells are revealed by altered DNA sensitivity to denaturation. *Exp. Cell Res.* 201:184-191.

Hotz, M.A., Gong, J., Traganos, F., and Darzynkiewicz, Z. 1994. Flow cytometric detection of apoptosis: Comparison of the assays of in situ DNA degradation and chromatin changes. *Cytometry* 15:237-244.

Hug, H., Los, M., Hirt, W., and Debatin, K.M. 1999. Rhodamine 110-linked amino acids and peptides as substrates to measure caspase activity upon apoptosis induction in intact cells. *Biochemistry* 38:13906-13911.

Johnson, L.V., Walsh, M.L., and Chen, L.B. 1980. Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. U.S.A.* 77:990-994.

Kamentsky, L.A. 2001. Laser scanning cytometer. *Meth. Cell Biol.* 63:51-87.

Kaufmann, S.H., Desnoyers, S., Ottaviano, Y., Davidson, N.E., and Poirier, G.G. 1993. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. *Cancer Res.* 53:3976-3985.

Keiji, J.F., Bell-Prince, C., and Steinkamp, J.A. 2000. Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs. *Cytometry* 39:203-210.

Kerr, J.F.R., Wyllie, A.H., and Curie, A.R. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26:239-257.

Knapp, P.E., Bartlett, W.P., Williams, L.A., Yamada, M., Ikenaka, K., and Skoff, R.P. 1999. Programmed cell death without DNA fragmentation in the jimpy mouse: Secreted factors can enhance survival. *Cell Death Differ.* 6:136-145.

Kockx, M.M., De Meyer, G.R., Muhring, J., Jacob, W., Bult, H., and Herman, A.G. 1998. Apoptosis and related proteins in different stages of human atherosclerotic plaques. *Circulation* 97:2307-2315.

Komoriya, A., Packard, B.Z., Brown, M.J., Wu, M.L., and Henkart, P.A. 2000. Assessment of caspase activities in intact apoptotic thymocytes using cell-permeable caspase substrates. *J.Exp.Med.* 191:1819-1828.

Koopman, G., Reutelingsperger, C.P.M., Kuijten, G.A.M., Keehnen, R.M.J., Pals, S.T., and van Oers, M.H.J. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression of B cells undergoing apoptosis. *Blood* 84:1415-1420.

Kroemer, G. 1998. The mitochondrion as an integrator/coordinator of cell death pathways. *Cell Death Differ.* 5:547-548.

Lajemi, M., Demignot, S., and Adolphe, M. 1998. Detection and characterization, using fluoresceincadaverine, of amine acceptor substrates accessible to active transglutaminase expressed by rabbit articular chondrocytes. *Histochem. J.* 30:499-508.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., and Earnshaw, W.C. 1994. Cleavage of poly(ADP-ribose) polymerase by proteinase with properties like ICE. *Nature* 371:346-347.

Li, X., and Darzynkiewicz, Z. 1995. Labelling DNA strand breaks with BdrUTP. Detection of apoptosis and cell proliferation. *Cell Prolif.* 28:571-579.

Li, X., and Darzynkiewicz, Z. 2000. Cleavage of poly(ADP-ribose) polymerase measured *in situ* in individual cells: Relationship to DNA fragmentation and cell cycle position during apoptosis. *Exp. Cell Res.* 255:125-132.

Li, X., Traganos, F., Melamed, M.R., and Darzynkiewicz, Z. 1995. Single-step procedure for labeling DNA strand breaks with fluorescein- or BODIPY-conjugated deoxynucleotides: Detection of apoptosis and bromodeoxyuridine incorporation. *Cytometry* 20:172-180.

Li, X., Melamed, M.R., and Darzynkiewicz, Z. 1996. Detection of apoptosis and DNA replication by differential labeling of DNA strand breaks with fluorochromes of different color. *Exp. Cell Res.* 222:28-37.

Li, X., Du, L., and Darzynkiewicz, Z. 2000. During apoptosis of HL-60 and U-937 cells caspases are activated independently of dissipation of mitochondrial electrochemical potential. *Exp. Cell Res.* 257:290-297.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. 1996. Induction of apoptotic program in cell-free extracts: Requirements for dATP and cytochrome c. *Cell* 86:147-157.

Liu, J., Bhalgat, M., Zhang, C., Diwu, Z., Hoyland, B., and Klaubert, D.H. 1999. Fluorescent molecular probes V: A sensitive caspase-3 substrate for fluorometric assays. *Bioorg. Med. Chem. Lett.* 9:3231-3236.

Majno, G. and Joris, I. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* 146:3-16.

Marguet, D., Luciani, M.-F., Moynault, A., Williamson, P., and Chimini, G. 1999. Engulfment of apoptotic cells involves the redistribution of membrane phosphatidylserine on phagocyte and prey. *Nature Cell Biol.* 1:454-456.

Melino, E. and Piacentini, M. 1998. "Tissue" transglutaminase in cell death: A downstream or multifunctional upstream effector? *FEBS Lett.* 430:59-63.

Nagata, S. 2000. Apoptotic DNA fragmentation. *Exp. Cell Res.* 256:12-18.

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., and Riccardi, C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* 139:271-280.

Oberhammer, F., Wilson, J.M., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R., and Sikorska, M. 1993. Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.* 12:3679-3684.

Ormerod, M.G. 1998. The study of apoptotic cells by flow cytometry. *Leukemia* 12:1013-1025.

Ormerod, M.G., O'Neill, C.F., Robertson, D., and Harrap, K.R. 1994. Cisplatin induced apoptosis in a human ovarian carcinoma cell line without a concomitant internucleosomal degradation of DNA. *Exp. Cell Res.* 211:231-237.

Ormerod, M.G., Cheetham, F.P.M., and Sun, X.-M. 1995. Discrimination of apoptotic thymocytes by forward light scatter. *Cytometry* 21:300-304.

Petit, J.M., Ratinaud, M.H., Cordelli, E., Spano, M., and Julien, R. 1995. Mouse testis cell sorting according to DNA and mitochondrial changes during spermatogenesis. *Cytometry* 19:304-312.

Poot, M., Gibson, L.L., and Singer, V.L. 1997. Detection of apoptosis in live cells by MitoTracker Red CMXRos and SYTO dye flow cytometry. *Cytometry* 27:358-364.

Pozarowski, P., Huang, X., Halicka, D.H., Lee, B., Johnson, G., and Darzynkiewicz, Z. 2003. Interactions of fluorochrome-labeled caspase inhibitors with apoptotic cells. A caution in data interpretation. *Cytometry*. 55A:50-60.

Ratinaud, M.H., Leprat, P., and Julien, R. 1988. In situ cytometric analysis of nonyl acridine orange-stained mitochondria from splenocytes. *Cytometry* 9:206-212.

Sallman, F.R., Bourassa, S., Saint-Cyr, J., and Poirier, G.G. 1997. Characterization of antibodies specific for the caspase cleavage site on poly(ADP-ribose) polymerase: Specific detection of apoptotic fragments and mapping of the necrotic fragments of poly(ADP-ribose) polymerase. *Biochem. Cell Biol.* 75:451-458.

Scorrano, L., Petronilli, V., Colonna, R., Di Lisa, F., and Bernard, P. 1999. Chloromethyltetramethylrosamine (Mitotracker Orange) induces the mitochondrial permeability transition and inhibits respiratory complex I. Implications for the mechanism of cytochrome c release. *J. Biol. Chem.* 274:24657-24663.

Shi, Y. 2002. Mechanisms of caspase activation and inhibition during apoptosis. *Molec. Cell* 9:459-470.

Smolewski, P., Bedner, E., Du, L., Hsieh, T.-C., Wu, J.M., Phelps, D.J., and Darzynkiewicz, Z. 2001. Detection of caspases activation by fluorochrome-labeled inhibitors: Multiparameter analysis by laser scanning cytometry. *Cytometry* 44:73-82.

Susin, S.A., Zamzani, N., Larochette, N., Dallaporta, B., Marzo, I., Brenner, C., Hirsch, T., Petit, P.X., Geuskens, M., and Kroemer, G. 1997. A cytofluorometric assay of nuclear apoptosis induced in a cell-free system: Application to ceramide-induced apoptosis. *Exp. Cell Res.* 236:397-403.

Swat, W., Ignatowicz, L., and Kisielow, P. 1981. Detection of apoptosis of immature $CD4^+8^+$ thymocytes by flow cytometry. *J. Immunol. Methods* 137:79-87.

Telford, W.G., Komoriya, A., and Packard, B.Z. 2002. Detection of localized caspase activity in early apoptotic cells by laser scanning cytometry. *Cytometry* 47:81-88.

Umansky, S.R., Korol, B.A., and Nelipovich, P.A. 1981. In vivo DNA degradation in the thymocytes of gamma-irradiated or hydrocortisone-treated rats. *Biochim. Biophys. Acta* 655:9-17.

van Engeland, M., Nieland, L.J.W., Ramaekers, F.C.S., Schutte, B., and Reutelingsperger, P.M. 1998. Annexin V–affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 31:1-9.

Vermes, I., Haanen, C., and Reutelingsperger, C. 2000. Flow cytometry of apoptotic cell death. *J. Immunol. Methods* 243:167-190.

Waggoner, A.S. 1979. Dye indicators of membrane potential. *Annu. Rev. Biophys. Bioeng*. 8:47-68.

Yang, J., Liu, X., Bhalla, K., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., and Wang, X. 1997. Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. *Science* 275:1129-1132.

Zamzani, N., Brenner, C., Marzo, I., Susin, S.A., and Kroemer, G. 1998. Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene* 16:2265-2282.

Contributed by Piotr Pozarowski
School of Medicine
Lublin, Poland

Jerzy Grabarek
Pomeranian School of Medicine
Szczecin, Poland

Zbigniew Darzynkiewicz
New York Medical College
Valhalla, New York

Figure 18.8.1 Changes in light scattering properties of cells undergoing apoptosis. HL-60 cells were untreated (left panel) or treated 3 hr with TNF-α and cycloheximide (CHX) to induce apoptosis (right panel). Cell population **A** in the treated culture (right panel) represents cells that have light scattering properties similar to those of untreated cells. Early apoptotic cells (**B**) have diminished forward scatter and are very heterogenous with respect to side scatter. Late apoptotic cells (**C**) have both forward and side scatter diminished.

Figure 18.8.2 Detection of the collapse of mitochondrial electrochemical potential (ψ_m) by rhodamine 123 (R123). HL-60 cells were untreated (control; left panel) or treated 3 hr with TNF-α and CHX (right panel) to induce apoptosis. The cells were then incubated with R123 and PI according to Basic Protocol 1. The early apoptotic cells have diminished green fluorescence of R123 but exclude PI (cell population B). The late apoptotic (also necrotic) cells are stained strongly by PI (population C).

Figure 18.8.3 Detection of the collapse of mitochondrial electrochemical potential using the aggregate dye JC-1. HL-60 cells were untreated (control, left panel) or treated 3 hr with camptothecin (CPT, right panel) to indice apoptosis. Cells were then stained with JC-1 and their orange and green fluorescence was measured by cytometry, as described in Basic Protocol 2. Decreased intensity of orange fluorescence (subpopulation B) characterizes the cell with collapsed potential.

Figure 18.8.4 Immunocytochemical detection of caspase-3 activation using antibody reactive with the activated (cleaved) caspase-3. Apoptosis of HL-60 cells was induced by topotecan (TPT), an analog of CPT. Zenon technology (Haugland, 2002) was used to detect caspase-3 as described in Basic Protocol 2. Top and bottom insets in each panel show cellular DNA content frequency histograms of cells with activated and nonactivated caspase-3, respectively. Note that S-phase cells preferentially contain activated caspase-3 after induction of apoptosis by TPT.

Figure 18.8.5 Binding of fluorochrome-labeled inhibitor of caspases (FLICA; FAM-VAD-FMK) and PI during apoptosis. Apoptosis of HL-60 cells was induced by TPT. The cells were stained according to Basic Protocol 3. Green (FAM-VAD-FMK) and red (PI) cellular fluorescence was measured by flow cytometry. Four cell subpopulations (A to D) can be identified, differing in their capability to bind FAM-VAD-FMK and PI. They represent sequential stages of apoptosis, starting with binding of FAM-VAD-FMK (B), loss of plasma membrane integrity to exclude PI (C), and loss of reactivity with FAM-VAD-FMK (D).

Figure 18.8.6 Identification of apoptotic cells by flow cytometry based on the immunocytochemical detection of the 85-kDa PARP cleavage fragment. To induce apoptosis, HL-60 cells were treated 60 min with TNF-α in the presence of CHX (Li and Darzynkiewicz, 2000). PARPp85 was detected immunocytochemically and DNA was counterstained with PI.

Figure 18.8.7 Detection of early and late apoptotic cells after staining with annexin V–FITC and PI. To induce apoptosis, HL-60 cells were treated 2 hr with TNF-α and CHX. Untreated (control; left panel) and TNF-α-treated (right panel) cells were then stained with annexin V-FITC and PI.

Figure 18.8.8 Detection of apoptotic cells by flow cytometry based on cellular DNA content analysis. (**A**) Normal cell plot. (**B**) To induce apoptosis, HL-60 cells were treated with the DNA topoisomerase II inhibitor fostriecin (Hotz et al., 1994). Cells were fixed in 70% ethanol, suspended in high-molarity phosphate buffer to extract fragmented DNA, and then stained with PI. A subpopulation of apoptotic cells (Ap) with fractional (sub-diploid) DNA content, i.e., with DNA index (DI) <1.0 (sub-G_1 cells), is apparent. Note also the increase in the proportion of S-phase cells in the nonapoptotic population. (**C**) The fragmented DNA extracted from the apoptotic cells by the buffer was subjected to gel electrophoresis (Gong et al., 1994). Note laddering that reflects preferential DNA cleavage at internucleosomal sections, the characteristic feature of apoptosis (Arends et al., 1990).

Figure 18.8.9 Detection of apoptotic cells by flow cytometry based on the presence of DNA strand breaks. To induce apoptosis, HL-60 cells were treated 120 or 150 min with CPT (Li and Darzynkiewicz, 2000). DNA strand breaks were labeled with BrdUTP using exogenous terminal deoxynucleotidyl transferase. The cell cycle distribution of both apoptotic and nonapoptotic cell subpopulations can be estimated based on the DNA content of individual cells. Note that in analogy to PARP cleavage (Fig. 18.8.6), preferentially S-phase cells undergo apoptosis following CPT treatment.

Figure 18.8.10 Detection of tissue transglutaminase (TGase 2) activation during apoptosis by the acquired resistance of the cytoplasmic proteins to detergent. Bivariate distributions illustrating red fluorescence of sulforhodamine 101 (protein content) versus blue fluorescence of DAPI (DNA content) of HL-60 cells, untreated (**A**) or exposed to hyperthermia (72 hr at 41.5°C) in the absence (**B**) and presence (**C**) of the cytotoxic RNase onconase (1.67 μM; Grabarek et al., 2002). Following cell lysis by Triton X-100 and staining with DAPI and sulforhodamine 101, the isolated nuclei of nonapoptotic cells from control culture (**A**) show low and uniform intensity of red fluorescence, reflecting low protein content. Subpopulations of apoptotic cells in **B** and **C** have their cytoplasmic protein cross-linked and therefore are resistant to detergent. They stain intensely with sulforhodamine 101. Note differences in DNA content (cell cycle) distribution of the cells with activated (top insets; cells gated above the dashed line) versus nonactivated TGase 2 (bottom insets; cells gated below the dashed line) in **B** and **C**. Percentage of cells with activated and non-activated TGase 2 in the respective cultures is indicated in each panel.

Figure 18.8.11 Detection of TGase 2 activity in HL-60 cells using FITC-conjugated cadaverine (F-CDV) as the enzyme substrate. Cultures of untreated (**A**) and hyperthermia (5 hr at 41.5°C)-treated (**B**) HL-60 cells were incubated 5 hr with 100 μM F-CDV. Cells were then fixed and their DNA was counterstained with PI in the presence of RNase. Note the presence in the untreated culture (**A**) of few cells that incorporated F-CDV (spontaneous apoptosis), and large numbers of F-CDV-labeled cells in the treated culture (**B**). Note also that some apoptotic cells with fractional DNA content (sub-G_1 subpopulation) in the treated culture do not show incorporation of F-CDV (arrow). Percentage of cells with activated and nonactivated TGase 2 in the respective cultures is indicated. The inset shows the cellular DNA content distribution histogram of all cells (Grabarek et al., 2002).

Analysis of Cellular Senescence in Culture In Vivo: The Senescence-Associated β-Galactosidase Assay

The senescence-associated β-galactosidase (SA-β-gal) assay is based on a senescence-induced increase in levels of lysosomal β-galactosidase (Dimri et al., 1995). In nonsenescent cells, the lysosomal hydrolase β-galactosidase cleaves galactose from glycoproteins at an optimum pH of 4.0 to 4.5. Lysosomal β-galactosidase activity can be detected in most mammalian cells by performing a cytochemical assay at pH 4.0 in which cleavage of Xgal by β-galactosidase leads to the formation of a blue precipitate (Kurz et al., 2000). However, during senescence, there is an increase in lysosomal β-galactosidase protein levels and an overall increase in lysosomal size. The increase in β-galactosidase levels allows the detection of β-galactosidase activity at the suboptimal pH of 6.0 during senescence (Kurz et al., 2000).

ASSESSMENT OF CELLULAR SENESCENCE IN CULTURE USING THE SENESCENCE-ASSOCIATED β-GALACTOSIDASE ASSAY

BASIC PROTOCOL

In this protocol, cells are first fixed with a formaldehyde/glutaraldehyde mixture and then incubated together with the β-gal substrate in an acidic buffer. The development of a perinuclear blue color, which is indicative of senescent cells, can be followed using a standard light microscope (without phase; Fig. 18.9.1).

It is important to include quiescent cells, which are also growth arrested but do not express SA-β-gal, as negative controls in the assay.

Materials

Cells to be analyzed
1× PBS (*APPENDIX 2A*)
Fixing solution (see recipe)
Staining solution (see recipe)
Mounting medium
Colorless nail polish

Coverslips, sterile
10-cm-diameter petri dishes
Platform or orbital shaker
Light microscope
Glass microscope slides

1. Seed cells on coverslips (resting in petri dishes) at ∼50% confluence 24 hr before staining. Incubate 24 hr at 37°C.

   Seeding more cells may result in a confluent population and may produce nonspecific staining.

2. Wash cells three times, 2 min each, with 1× PBS at room temperature with gentle agitation on a platform or orbital shaker.

3. Aspirate the last wash solution and add enough room temperature fixing solution so that the cells are submerged.

   For 6-well plates, use 1.5 ml per well; for a 10-cm dish, use at least 6 ml fixing solution.

Contributed by Debdutta Bandyopadhyay, Catherine Gatza, Lawrence A. Donehower, and Estela E. Medrano
Current Protocols in Cell Biology (2005) 18.9.1-18.9.9

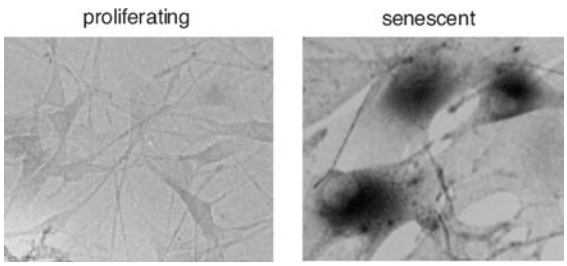


Figure 18.9.1 Senescence-associated β-galactosidase staining in normal human melanocytes (as performed in Basic Protocol). Senescent cells exhibit strong perinuclear activity. Note the enlarged, flat morphology of the senescent melanocytes compared with the much smaller, spindle-shaped morphology of the proliferating melanocytes. Similar morphological changes are also observed in other senescent cell types. This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.interscience.wiley.com/c_p/colorfigures.htm*

4. Incubate cells 5 min at room temperature with gentle agitation on a platform of orbital shaker.

   Incubation time should be determined uniquely for each cell type. Time course of fixation and integrity of cellular structure may be monitored using a microscope.

5. Aspirate fixing solution and immediately add room temperature 1× PBS.

   The volume of 1× PBS added should be at least two times that of the fixing solution.

6. Aspirate PBS, and then add just enough staining solution to cover cells completely.

   The same volume of staining solution may be used as for the fixing solution in step 3.

7. Incubate petri dishes containing coverslips in a 37°C incubator.

   The petri dishes should be placed in a 37°C incubator without carbon dioxide circulation. The presence of CO_2 will alter the pH of the staining solution, and nonspecific staining will result. Petri dishes should be incubated with lids closed to prevent evaporation. In addition, care should be taken to ensure that the plates are not tilted and the staining solution covers the whole area of the dish.

8. Using a light microscope, check for the development of a blue color in senescent cells. As soon as such coloration is detected, aspirate the staining solution and wash cells three times with distilled water.

   Time required for blue coloration to develop depends on cell type. Wait 2 to 4 hr after adding the staining solution before monitoring. Color development usually is complete after 16 hr. Some nonspecific staining, which is caused by loss of the buffering capacity of the staining solution, may be observed after a long staining incubation.

   Compare experimental sample with a negative control to detect color development. A proliferating cell population and/or a quiescent cell population may serve as good controls.

   After >8 hr of incubation, some salt crystals may be observed. These crystals can be removed by repeated washing with water.

9. Rinse coverslips briefly by dipping into distilled water and then mount upside down on glass microscope slides using any mounting solution. Aspirate excess mounting solution from the edge of the coverslip, and then seal coverslip to the slide with colorless nail polish.

   Coverslips protected in this way are stable for months at 4°C.

10. Visualize slides using light microscopy using direct light transmission.

ASSESSMENT OF CELLULAR SENESCENCE IN MURINE TISSUE USING THE SENESCENCE-ASSOCIATED β-GALACTOSIDASE ASSAY

ALTERNATE PROTOCOL

Historically, the identification of senescent cells in situ has been difficult, since many of the assays used to identify senescent cells in culture are not amenable to use in vivo. However, the discovery that senescent cells exhibit β-galactosidase activity at pH 6, whereas nonsenescent cells do not, has allowed the identification of senescent cells in human cell lines and tissues. Since this discovery, the same principle has been applied successfully in mouse and rat cell lines. Slight modifications in the protocol used to assay such cell lines also allow the successful identification of senescent cells in murine tissues.

The following protocol describes the identification of senescent cells in murine liver, spleen, and kidney tissue using the SA-β-gal assay. With some optimization of staining times, it is likely that this assay could be adapted for use in other tissue types. For use in this assay, tissue must be frozen in optimum cutting temperature (OCT) compound and sectioned using a cryotome (ThermoShandon). Immediately after cutting, sections are fixed and stained with a SA-β-gal staining solution and then counterstained with hematoxylin to identify the nucleus. The SA-β-gal-stained slide is then viewed under light microscopy, and the average number of senescent cells (i.e., those that exhibit perinuclear blue staining) is counted over a number of microscope fields. To determine the average number of total cells per field, a parallel slide is stained with hematoxylin and eosin and then viewed under light microscopy. The percentage of senescent cells is determined by dividing the average number of SA-β-gal-positive cells by the average number of total cells and multiplying the result by 100.

Additional Materials *(also see Basic Protocol)*

Tissue of interest (e.g., liver, spleen, kidney)
Optimum cutting temperature (OCT) compound
Liquid nitrogen
0.5% (v/v) glutaraldehyde (see recipe)
Senescence-associated β-galactosidase stain (see recipe)
Hematoxylin (Sigma) Mounting medium
Eosin (Sigma)

Cryomold
Standard cryotome (Thermo Electron Anatomical Pathology or equivalent)
Positively charged glass slides (e.g., VWR)
Humidity chamber: plastic box with lid
Pap pen
Coplin jars

Prepare tissue sections

1. Harvest tissue of interest from animal.
2. Cover the bottom of the cryomold with OCT compound. Place the tissue sample in the cryomold and cover completely with OCT compound. Allow OCT and tissue to freeze gradually in liquid nitrogen vapor. Store frozen tissue at −80°C until use.

   Gradual freezing of the tissue sample in OCT compound prevents tissue damage and the formation of air bubbles.

3. Using a cryotome, cut 4-μm (spleen and kidney) or 6-μm sections (liver), depending on tissue of interest. Mount sections on positively charged glass slides to increase tissue adhesion. Keep sections on dry ice until fixed.

   Cut multiple sections from each sample to ensure that good-quality sections, which are important for optimum staining, are obtained. Sections that are of poor quality (i.e., ripped, torn, or folded) can under- or overstain.

4. Immediately after cutting the sections, fix them by covering with 300 μl of 0.5% glutaraldehyde and incubating 15 min at room temperature. Rinse the sections with 1× PBS. To prevent sections from drying out, keep in 1× PBS until the staining solution is added.

   It is important to fix and stain the sections immediately after cutting. A loss of senescence-associated β-galactosidase staining activity can occur if sections are stored after cutting.

Stain for SA-β-gal-positive cells

5. Prepare a humidity chamber by placing wet paper towels in the bottom of a plastic box and closing the lid. Warm to 37°C.

6. Remove excess PBS from around the tissue section using a Kimwipe. Do not allow the tissue to dry out. Use a Pap pen to outline the tissue section, and cover with 300 μl fresh SA-β-gal stain. Place the slides in the humidity chamber and incubate at 37°C for up to 18 hr.

   For optimum staining, it is important to prepare the SA-β-gal stain immediately before use. The optimum staining time is 10 hr for liver tissue and 6 hr for spleen and kidney tissue. Optimum staining times can be determined for other tissue types.

7. After staining, rinse away the SA-β-gal stain with 1× PBS.

8. Counterstain the sections by immersing for 30 sec in a Coplin jar containing hematoxylin and then rinse with 1× PBS. Incubate the sections 2 min at room temperature in fresh 1× PBS in a Coplin jar. Use a Kimwipe to wipe away excess PBS and any remaining Pap pen markings.

9. Coverslip sections with mounting medium and dry slides overnight at room temperature in the dark.

10. Using a standard light microscope, count the SA-β-gal-positive cells in a number of different microscope fields, and use the results to calculate the average number of positive cells per field.

    This calculation will determine the average number of SA-β-gal-positive cells in a given tissue sample. Senescent cells will show strong perinuclear blue staining (see Fig. 18.9.2A), whereas nonsenescent cells will not.

Stain for total number of cells

11. Cut and fix sections for hematoxylin and eosin staining as previously described in steps 1 to 4.

12. Immerse the fixed sections in a Coplin jar containing hematoxylin for 30 sec at room temperature. Rinse with 1× PBS. Incubate in fresh 1× PBS for 2 min at room temperature.

13. Immerse sections in a Coplin jar containing eosin for 35 sec at room temperature. Rinse with distilled water. Remove excess distilled water and any remaining Pap pen markings with a Kimwipe. Do not allow sections to dry out.

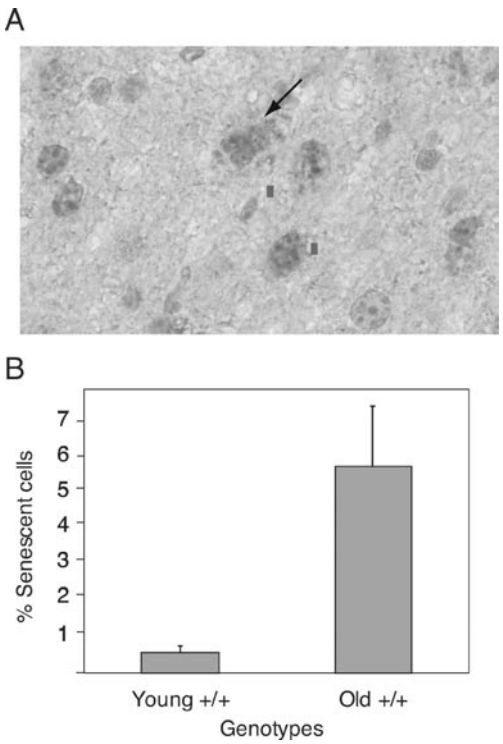


Figure 18.9.2 (**A**) Senescence-associated β-galactosidase staining in murine liver (performed as in Alternate Protocol). Senescent cells exhibit blue perinuclear staining (arrow) upon exposure to Xgal at pH 6.0, whereas nonsenescent cells do not. Nuclei are stained with hematoxylin. (**B**) Analysis of senescence in young (3-month-old) and old (21-month-old) wild-type murine liver tissue. 6-μm sections of young and old liver were treated with the senescence-associated β-galactosidase stain as described in the Alternate Protocol. Sections were observed under light microscopy to determine the percentage of senescent cells present. Levels of senescence were elevated in old wild-type liver compared with young wild-type liver ($n = 4$ for both sample sets). This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.interscience.wiley.com/c_p/colorfigures.htm*

14. Coverslip sections with mounting medium and dry slides overnight at room temperature.

    Optimal hematoxylin and eosin staining times vary according to tissue type and strength of reagents and can be determined empirically.

15. Using a standard light microscope, count all cells in a number of different microscope fields, and use the results to calculate the average number of cells per field.

    This calculation will determine the average total number of cells in a given tissue sample.

Calculate percentage of senescent cells

16. Divide the average number of SA-β-gal-positive cells per field by the average total number of cells per field and multiply the result by 100.

    This calculation yields the percentage of senescent cells in a given tissue sample. This value can be graphed and compared with values for other samples (see Fig. 18.9.2B).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Citric acid, 40 mM/sodium phosphate solution

Prepare 0.1 M citric acid by dissolving 2.1 g citric acid monohydrate in 100 ml water. Prepare 0.2 M dibasic sodium phosphate solution by dissolving 2.84 g Na_2HPO_4 in 100 ml water. Mix 36.85 ml of 0.1 M citric acid with 63.15 ml of 0.2 M dibasic sodium phosphate. Verify that pH is 6.0. Adjust pH to 6.0 with 0.1 M citric acid if necessary. Store at room temperature in the dark.

Fixing solution

1× PBS (APPENDIX 2A) containing:
2% (v/v) formaldehyde (commercially available formaldehyde is usually 37% v/v)
0.2% (v/v) glutaraldehyde (commercially available glutaraldehyde is usually 25% v/v)
Prepare fresh before each use

Glutaraldehyde, 0.5% (v/v)

200 µl 25% (v/v) glutaraldehyde
9.8 ml 1× PBS (*APPENDIX 2A*)
Prepare fresh before each use

Senescence-associated β-galactosidase (SA-β-gal) stain

40 mM citric acid/sodium phosphate, pH 6.0 (see recipe) containing:
1 mg/ml Xgal (add from 20 mg/ml stock in dimethylformamide [DMF]; store up to several months at −20°C in the dark)
5 mM potassium ferricyanide
5 mM potassium ferrocyanide
150 mM NaCl
2 mM $MgCl_2$
Prepare fresh before each use
Prepare long-term stocks of all of the above solutions (except for Xgal; see above) and store at room temperature in the dark
The pH of the citric acid/sodium phosphate solution in this stain is critical to the assay.

Staining solution

1 ml of 1 mg/ml Xgal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) in DMF
4 ml of citric acid/sodium phosphate solution (see recipe)
1 ml of 100 mM potassium ferrocyanide
1 ml of 100 mM potassium ferricyanide
0.6 ml of 5 M NaCl (*APPENDIX 2A*)
40 µl of 1 M $MgCl_2$ (*APPENDIX 2A*)
Make up to 20 ml with distilled H_2O
Prepare fresh before each use

COMMENTARY

Background Information

Replicative senescence, originally described by Leonard Hayflick almost four decades ago (Hayflick, 1965), is defined as the irreversible loss of the proliferative capacity of cells with maintenance of the metabolic functions necessary for cell survival. Cellular senescence can be induced by a variety of cellular stimuli, including shortened telomeres, DNA damage, oncogene expression, mitogenic signals, and chromatin disruption (Campisi, 2003). In response to these cellular stresses, the induction of cellular senescence may prevent neoplastic transformation by permanently arresting cell growth. Several tumor suppressors, including p53, $p16^{INK4A}$, p21, and Rb, have been shown to regulate the induction of cellular senescence (Bringold and Serrano, 2000).

Senescent cells permanently arrest in G_1 and do not enter S phase in response to mitogenic signals. Despite this permanent arrest, senescent cells remain metabolically active, and some cell types have been shown to be resistant to apoptosis during senescence. Due to their inability to proliferate and their resistance to apoptosis, it is thought that senescent cells can accumulate in vivo with age. An accumulation of senescent cells over time may contribute to aging phenotypes and pathologies such as loss of proliferative and tissue regenerative activity. The majority of evidence suggesting that senescent cells may accumulate in vivo comes from in vitro studies; however, the use of the senescence-associated β-galactosidase assay provides the investigator with a powerful tool for the examination of senescence levels in vivo.

Almost all cells express a lysosomal β-galactosidase that is optimally active at pH 4. Dimri et al. (1995) first reported that senescent cells express β-galactosidase, detectable by cytochemical staining with Xgal, at a pH of 6.0. Importantly, this senescence-associated β-galactosidase activity was not simply present in cultured human fibroblasts but was specific to senescent cells, as no staining was observed in quiescent or terminally differentiated cells. By examining human skin samples from donors of different ages, Dimri et al. (1995) were able to demonstrate an age-dependent increase in levels of SA-β-gal staining. (There are increases in lysosomal β-gal protein levels and lysosomal size that are thought to lead to the detection of β-gal at a suboptimum pH in senescent cells; to date, no other genes have been implicated in this phenomenon.) The results of this study suggested that SA-β-gal staining could serve as an in vivo marker of senescence.

More recently, it has been demonstrated that SA-β-gal activity can also be used to identify senescent cells in a variety of human, nonhuman primate, mouse, and chicken cell lines (Price et al., 2002; Paradis et al., 2001; Kim et al., 2002; Choi et al., 2000; Mishima et al., 1999). The SA-β-gal assay has also been used successfully to identify senescent cells in murine embryonic fibroblasts (MEFs) as well as whole murine embryos (Kim et al., 2002; Cao et al., 2003). In addition, a slight modification in the protocol has allowed the assay to be adapted for use in murine tissue. As a result, senescent cells can be identified in situ, and increased levels of senescence can be confirmed in mouse tissue sections without the creation of MEFs. This capability may prove to be important, as the standard method of culturing MEFs has recently been found to increase the accumulation of mutations (Busuttil et al., 2003).

Confirming increased levels of senescence in tissue can be difficult; most work on the identification of senescent markers has been performed in vitro. One potential approach to assaying senescence in tissue samples involves immunoblotting for various inducers of senescence. It is known that in vitro, levels of p53, $p21^{Waf-1}$, and $p16^{INK4a}$ are increased during senescence (Bringold and Serrano, 2000). By assessing tissue levels of these markers, one can also confirm an increase in the number of senescent cells within the tissue as a whole.

Critical Parameters and Troubleshooting

There are several critical parameters to consider when performing the SA-β-gal assay in tissue. Preparation of the tissue prior to the experiment is very important. Slowly freezing the tissue in OCT compound helps stabilize the tissue during cutting and also prevents cracking and bubble formation in samples. Good sections are critical for optimum staining in this assay. Sections that are ripped, torn, or folded will not stain properly. Preparation of good-quality sections can take practice, but the acquisition of several sections per sample will assure that some high-quality slides are available.

Once high-quality sections have been obtained, it is very important that sections be fixed and stained immediately after cutting.

Sections that are cut and then stored at −80°C for any length of time, even overnight, have been observed to exhibit decreased levels of SA-β-gal staining.

The SA-β-gal assay is based on the observation that senescent cells stain with Xgal at pH 6.0 and nonsenescent cells do not. Therefore, it is critical that the pH of the citric acid/sodium phosphate solution be 6.0. Nonsenescent cells stain with Xgal within a pH range of 4.0 to 4.5. Thus, alterations in the pH of the staining solution could lead to nonspecific staining. For optimum staining, it is also crucial to prepare fresh SA-β-gal staining solution before each use. In addition, for maximum activity, the Xgal used in the solution should be fairly new. All long-term stock solutions used to prepare the SA-β-gal staining solution should be stored away from light, as this appears to result in optimal activity.

By assessing staining at various time points, optimum staining times for each tissue type can be determined. Kidney, liver, and spleen tissues do not exhibit higher levels of staining with longer staining times. SA-β-gal staining is typically dark blue and localized to the perinuclear area. High levels of background staining are often observed in clusters around rips, tears, or folds in poor-quality sections. Background staining can appear as a diffuse, light-to-dark blue coloring throughout the section, and resectioning and restaining should be performed when high levels of such staining are encountered. Some tissues (particularly those obtained from young animals) show very low levels of senescence. Increasing the number of fields counted in these tissues allows quantification of the data and ensures accuracy.

Anticipated Results

The SA-β-gal staining assay is a good technique for analyzing levels of senescence in situ. Figures 18.9.1 and 18.9.2A show typical SA-β-gal staining in cultured melanocytes and liver sections, respectively. SA-β-gal staining is seen as a dark blue perinuclear coloring. Counterstaining cells with hematoxylin allows for easier detection of SA-β-gal staining by giving nuclei a contrasting purple color. Background levels of blue staining are expected to be relatively low, and senescence-associated staining should be fairly distinct. SA-β-gal staining is seen primarily in adult hepatocytes in liver and in both red and white pulp in spleen. Kidneys show staining primarily in cortical areas, with significantly lower levels observed in the medulla.

Typical results are presented in Figure 18.9.2B, in which percent senescence is compared between young and old wild-type liver tissues. Levels of senescence will vary with tissue type, but wild-type mice generally show low levels at a young age and higher levels with increasing age.

Time Considerations

The entire SA-β-gal staining assay can be completed in 2 days. Once tissue has been harvested and frozen in OCT compound, it can be stored at −80°C until use. Sections must be cut and stained on the same day, but tissue can be stained overnight (up to 18 hr) as an alternative stopping point. Typically, 45 min is required to take samples from the PBS wash to the point at which the stain is added. Staining times vary with tissue type. Spleen and kidney tissue require 6 hr to stain optimally, while liver requires 10 hr. Optimum staining times can be determined empirically for other tissue types. The time it takes to count positive cells will vary according to the level of senescence, according to the individual performing the counting, and according to the number of samples to be evaluated. However, it should be possible to easily complete counting on the second day of the experiment.

Literature Cited

Bringold, F. and Serrano, M. 2000. Tumor suppressors and oncogenes in cellular senescence. *Exp. Gerontol.* 35:317-329.

Busuttil, R.A., Rubio, M., Dolle, M.E., Campisi, J., and Vijg, J. 2003. Oxygen accelerates the accumulation of mutations during the senescence and immortalization of murine cells in culture. *Aging Cell* 2:287-294.

Campisi, J. 2003. Cellular senescence and apoptosis: How cellular responses might influence aging phenotypes. *Exp. Gerontol.* 38:5-11.

Cao, L., Li, W., Kim, S., Brodie, S.G., and Deng, C.X. 2003. Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. *Genes Dev.* 17:201-213.

Choi, J., Shendrik, I., Peacocke, M., Peehl, D., Buttyan, R., Ikeguchi, E.F., Katz, A.E., and Benson, M.C. 2000. Expression of senescence-associated beta-galactosidase in enlarged prostates from men with benign prostatic hyperplasia. *Urology* 56:160-166.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., and Pereira-Smith, O. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 92:9363-9367.

Hayflick, L. 1965. The limited in vitro lifespan of human diploid cell strains. *Exp. Cell Res.* 25:585-621.

Kim, H., You, S., Farris, J., Kong, B.W., Christman, S.A., Foster, L.K., and Foster, D.N. 2002. Expression profiles of p53-, p16(INK4a)-, and telomere-regulating genes in replicative senescent primary human, mouse, and chicken fibroblast cells. *Exp. Cell Res.* 272:199-208.

Kurz, D.J., Decary, S., Hong, Y., and Erusalimsky, J.D. 2000. Senescence-associated β-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell Sci.* 113:3613-3622.

Mishima, K., Handa, J.T., Aotaki-Keen, A., Lutty, G.A., Morse, L.S., and Hjelmeland, L.M. 1999. Senescence-associated β-galactosidase histochemistry for the primate eye. *Invest. Ophthalmol. Vis. Sci.* 40:1590-1593.

Paradis, V., Youssef, N., Dargere, D., Ba, N., Bonvoust, F., Deschatrette, J., and Bedossa, P. 2001. Replicative senescence in normal liver, chronic hepatitis C, and hepatocellular carcinomas. *Hum. Pathol.* 32:327-332.

Price, J.S., Waters, J.G., Darrah, C., Pennington, C., Edwards, D.R., Donell, S.T., and Clark, I.M. 2002. The role of chondrocyte senescence in osteoarthritis. *Aging Cell* 1:57-65.

Contributed by Debdutta Bandyopadhyay,
Catherine Gatza,
Lawrence A. Donehower, and
Estela E. Medrano
Baylor College of Medicine
Houston, Texas

High-Throughput Live Cell Imaging of Apoptosis

J.C. Puigvert,[1,2] Hans de Bont,[1] Bob van de Water,[1] and Erik H.J. Danen[1]

[1]Leiden University, Leiden, The Netherlands
[2]Netherlands Toxicogenomics Centre, Leiden, The Netherlands

ABSTRACT

Apoptosis is important for embryonic development, tissue homeostasis, and removal of cells with (potentially transforming) DNA lesions or other types of injuries. Functional genomics screens performed to unravel apoptotic signaling cascades in the context of toxicant-induced cell injury commonly use apoptosis as an end-point. Here, a method to detect the accumulation of apoptotic cells in real time that is well suited for high-throughput screens is described. The method uses automated microscopy in a 96-well format setting to visualize binding of fluorescent annexin V to the outer membrane leaflet of apoptotic cells. The automated image acquisition is followed by quantitative analysis using bioinformatics software. A protocol for each of the steps in this kinetic method is described, which includes the caspase-dependent apoptotic response to toxic compounds in multiple cell types and demonstrates that RNAi-based gene silencing of candidate apoptotic regulators affects the apoptosis kinetics as expected. This protocol will be useful for functional genomics as well as chemical (drug) screens. *Curr. Protoc. Cell Biol.* 47:18.10.1-18.10.13.

Keywords: apoptosis • image analysis • annexin V • cell death • microscopy

INTRODUCTION

Apoptosis is a complex and tightly regulated process also known as programmed cell death (Elmore, 2007). It is important for tissue formation during development and for tissue homeostasis in adults. It plays a key role in the removal of inflammatory cells, wound healing, and elimination of cells that contain DNA lesions that cannot be repaired and that could potentially lead to malignancies. Deregulation of signal transduction cascades in control of apoptosis can lead to pathologies, including developmental defects, autoimmune diseases, neurodegeneration, early aging, or cancer.

The method described here makes use of recombinant annexin V (Hanshaw and Smith, 2005) to detect phosphatidylserines that have translocated to the outer leaflet of the membrane in apoptotic cells. By coupling annexin V to a fluorescence dye, apoptosis can be detected in living cells using real-time fluorescence microscopy and quantified using image analysis software.

This unit describes a high-throughput method for detecting apoptosis in drug-treated cells (see Basic Protocol). Support protocols describe production of fluorescently labeled recombinant annexin V to use as a probe (see Support Protocol 1) and quantification of image analysis (see Support Protocol 2)

HIGH-THROUGHPUT ANALYSIS OF APOPTOSIS

BASIC PROTOCOL

This protocol is used to detect apoptosis in vitro in real time using high-throughput fluorescence microscopy. It makes use of recombinant fluorescently labeled annexin V, which binds to phosphatidylserines that accumulate in the outer leaflet of the plasma

Current Protocols in Cell Biology 18.10.1-18.10.13, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1810s47

membrane of apoptotic cells. The protocol describes how to prepare cultures of adherent cells in multi-well imaging plates, how to optimize annexin V concentration and exposure times, how to perform drug exposure studies, and how to perform real time imaging for kinetic analysis of the accumulation of apoptosis.

NOTE: All reagents and equipment coming into contact with living cells must be sterile and aseptic techniques should be used accordingly.

NOTE: All incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: For high-throughput purposes automated liquid handling robotics can be applied to these steps.

Materials

Cells
0.1% (w/v) gelatin in water
1 mg/ml bovine plasma fibronectin solution (Sigma)
Phosphate buffered saline (PBS; *APPENDIX 2A*)
0.05% (w/v) trypsin/EDTA
Pre-warmed culture medium
0.4% trypan blue in PBS
Labeled annexin V (0.5 mg/ml stock concentration; see Support Protocol 1)
Toxicant
Drugs to be tested
100 mM z-VAD-fmk stock solution (Bachem)

96- or 24-well plates, e.g., CELLSTAR SensoPlate or CELLSTAR μClear plates (Greiner)
Hemacytometer
Inverted fluorescence microscope system with automated stage, automated focus control, and temperature- and CO_2-controlled incubation system

Prepare cell cultures

1. Split cells 48 hr before the drug exposure (step 19) to reach a subconfluent culture.

   This protocol is applicable to a variety of cell lines as well as embryonic stem (ES) cells. Standard culture conditions for these cell types are used.

Coat multi-well plate(s)

2. Prepare a solution of 0.1% (w/v) gelatin in water or prepare a solution of 5 μg/ml bovine plasma fibronectin in PBS.

3. Add 100 μl protein solution per well in a 96-well plate or 350 μl/well in a 24-well plate.

   The multi-well plates used in this protocol must be suitable for cell culture as well as for high-quality image acquisition. For this purpose, CELLSTAR SensoPlate glass bottom or CELLSTAR μClear bottom plates (both from Greiner) can be used. Both types have optically clear bases bonded onto a black polystyrene frame that minimizes light cross-talk between wells.

   For glass-bottom plates and, to a lesser extent, for plastic-bottom plates, coating with adhesive substrates will optimize the formation of a healthy monolayer of cells.

4. Incubate for at least 1 hr at room temperature.

5. Remove the solution by aspiration 10 min before adding the cell suspensions (step 10).

6. Add PBS (100 µl per well in a 96-well plate or 500 µl/well in a 24-well plate) and remove by aspiration (plates are now ready to add the cell suspension). Take care not to let the plates dry out.

Prepare cells for drug exposure

7. Before (24 hr) the drug exposure, aspirate medium from the cell culture, wash once with PBS, and add 1 ml of (25-cm^2 flask) 0.05% trypsin/EDTA to detach cells.

8. Resuspend the cells gently in 4 ml (for 25-cm^2 flask) medium.

9. Remove a small sample, mix with trypan blue, and count cells on a hemacytometer (*UNIT 1.1*).

10. Plate cells in desired cell density in the coated multi-well plate(s).

    A good starting density is 1×10^4 cells per well in a 96-well plate and 1×10^5 cells per well in a 24-well plate.

    For ES cells, it is essential that this procedure is performed fast and gently and that cells are seeded at 75% confluency to maintain background cell death at a minimum.

    For high-throughput purposes, automated liquid handling robotics can be applied to these steps.

Titrate labeled annexin V and optimize exposure time for image acquisition

11. Seed cells for titration 24 hr prior to the experiment in the desired format as indicated above.

    At least 16 wells are needed for this titration experiment.

12. From the 0.5 mg/ml stock of annexin V, prepare a series of dilutions of labeled annexin V in culture medium, e.g., 1:10000, 1:5000, 1:1000, 1:500, 1:200, 1:100, and 1:50, and prepare a control tube without annexin V.

    A volume of 200 µl is needed for a 96-well plate and 700 µl is needed for a 24-well plate.

13. Divide each dilution into two tubes, and add a toxicant of choice to one of the tubes at a concentration that is expected to induce massive apoptosis in the cell type used.

    Some examples of toxicants are cisplatin (cis-diamminedichloroplatinum(II), CDDP), doxorubicin (hydroxydaunorubicin), vincristine (leurocristine), thapsigargin, hydrogen peroxide (H_2O_2), and tamoxifen.

14. Add the control (annexin V only) and toxicant-containing medium (100 µl for 96-well plates and 500 µl for 24-well plates) to the cells and follow the accumulation of fluorescence until the majority of cells exposed to toxicant have become positive.

15. Acquire images using a range of exposure times and choose the time that allows detection of fluorescent cells while maintaining a black background.

    Overexposure of the detectors would cause saturation and underestimation of the accumulation of apoptosis. The optimal exposure time must be determined in advance for a given microscope system and fluorophore. This is especially important given that fluorescence is typically expected to be absent at the start of any experiment. Optimization is done by exposing cells to a high concentration of toxicant that is expected to induce massive apoptosis in the presence of a high concentration of labeled annexin V (typically 0.05 mg/ml) to ensure a maximal signal.

16. Acquire fluorescence images every hour using the exposure time determined in step 15.

17. Determine the optimal annexin V concentration for the experimental conditions.

    The optimal annexin V concentration is that where fluorescence in the absence of toxicant is minimal while fluorescence in the presence of toxicant is maximal. For most cell types, efficient non-toxic labeling is reached using 5 μg/ml labeled annexin V.

Perform drug exposure studies

18. Generate a concentration range of the desired test drug in culture medium.

    Prepare sufficient amounts of the dilutions to test their effect in triplicate wells.

    For cisplatin, a typical range is 0.5 to 50 μM.

19. Based on the results obtained in the titrations, add the volume of labeled annexin V to achieve the optimal concentration to each tube.

20. Wash the multi-well plate with cells seeded 24 hr earlier, and that are ready to be exposed, once with warm PBS (100 μl/well for a 96-well plate; 500 μl/well for a 24-well plate).

    This will remove any floating, dead cells that would otherwise cause background signal.

21. Add 100 μl (96-well plate) or 500 μl (24-well plate) per well of fresh, warm medium containing the desired drug concentration and labeled annexin V.

    Cell death measured by this method may include cells dying through non-apoptotic pathways including necrosis. To exclude this, proper controls are needed. A recommended control consists of a duplicate for each test condition in which the general caspase-inhibitor z-VAD-fmk is added at a final concentration of 100 μM (see Fig. 18.10.1).

    For a combined detection of apoptosis and necrosis, incubation with propidium iodide can be incorporated into the procedure to detect necrotic (leaky) cells. However, this requires an additional exposure step, whereby enhancing the risk of phototoxicity.

22. Place the multi-well plate into the temperature- and CO_2-controlled imaging environment.

Acquire real-time image

23. Define at least four fields of interest for each well.

    When using a 10× objective, four fields allow a near complete coverage of a well in a 96-well plate.

24. Collect a fluorescence image (8-bit, gray-scale) for each field of interest, for each well, and for each time-point. Name the files "well01xy01time01C01.tif; well01xy02time01C01.tif; etc.", where C01 is the annexin V fluorescence channel. Use the exposure time determined by the concentration of annexin V, the fluorophore used, and the microscope system, as determined in the titration experiment (steps 15 and 16).

25. (Optional) Obtain a DIC (differential interference contrast; see Fig. 18.10.1) or phase contrast image (depending on the microscope system used) at the same time to correlate accumulation of apoptotic cells to total cell numbers. Name these files "well01xy01time01C02.tif; well01xy02time01C02.tif; etc.", where C02 is the DIC or the second fluorescence channel.

    As an alternative, cells may be engineered to express any variant of GFP that can be detected separately from the annexin V fluorophore. In this case, obtain a second, distinct fluorescence image at the same time to correlate accumulation of apoptotic cells to total cell numbers.

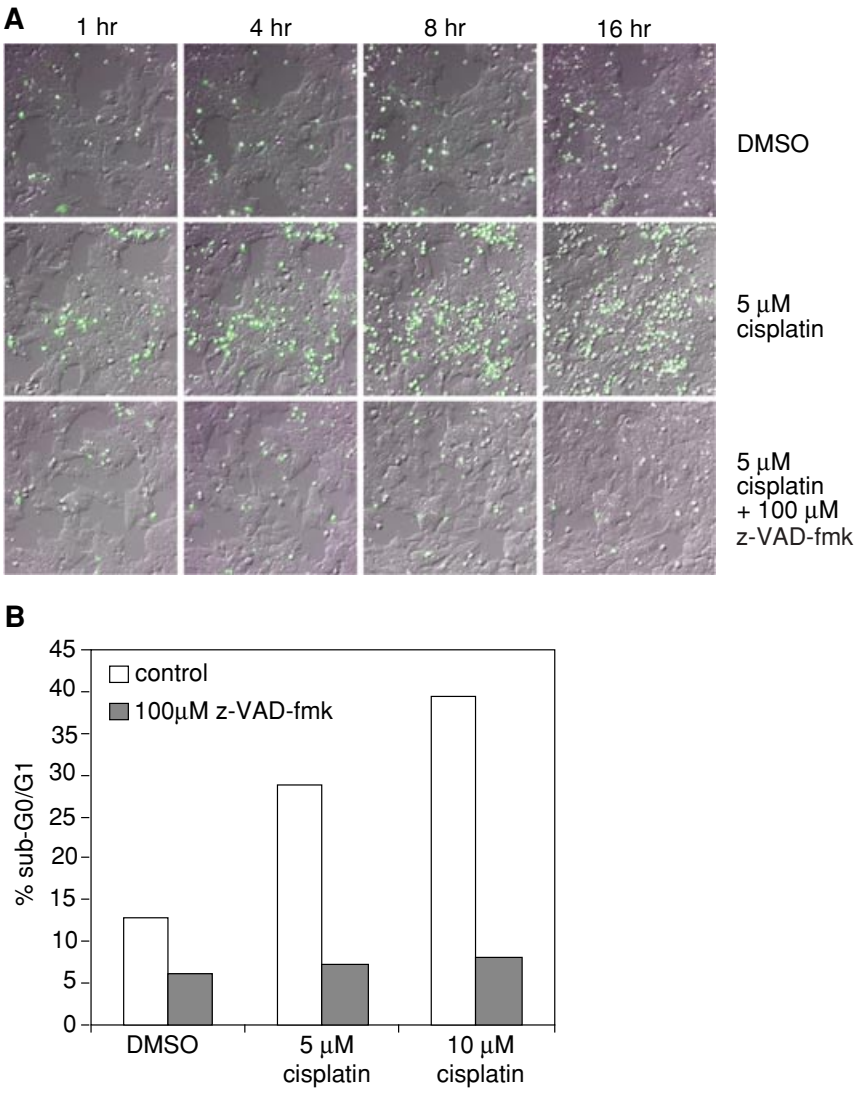


Figure 18.10.1 Accumulation of annexin V-FITC positive cells induced by cisplatin exposure. (**A**) Mouse ES cells were exposed to 5 μM cisplatin, annexin V-FITC. DIC and fluorescent images were taken during 16 hr. Over time, apoptotic cells accumulated in cisplatin, but not DMSO (control)-treated cells. In the presence of the pan-caspase inhibitor z-VAD-fmk signal accumulation was prevented, indicating that cells die by caspase-mediated apoptosis. (**B**) Comparison of this method with an alternative FACS-based assay gives similar results. Mouse ES cells were exposed to 5 μM and 10 μM of cisplatin in the presence or absence of the pan-caspase inhibitor z-VAD-fmk. Propidium iodide was added to determine the percent of sub-G0/G1 fraction by flow cytometry.

26. Acquire images of the fields of interest (step 23) every 30 min, for 16 to 48 hr depending on the cell type/toxicant combination.

    For instance, apoptosis develops in ES cells treated with 5 μM Cisplatin within 12 hr, whereas, U2OS cells treated with higher concentrations of Cisplatin require 24 to 48 hr (see Fig. 18.10.2).

    To perform automated analysis of apoptosis using this methodology, several robotic and microscopy units can be applied: (1) automated liquid handling for cell seeding and exposure to toxicants; (2) automated microscopy for real-time image acquisition; (3) combination of automated liquid handling and image acquisition (such as BD Pathway 855 from Becton Dickinson).

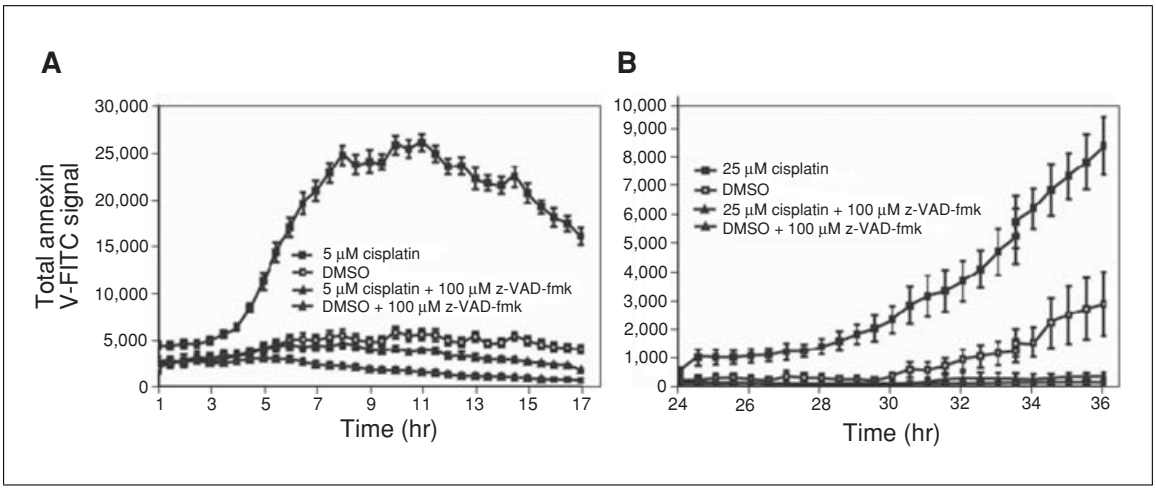


Figure 18.10.2 Increase of cisplatin-induced apoptosis in mouse ES cells and human U2OS cells detected by accumulation of annexin V-FITC positive cells. Mouse ES cells (**A**) and human osteosarcoma cells (U2OS; **B**) were exposed to the indicated concentrations of cisplatin in the presence or absence of z-VAD-fmk. Note that despite the much higher cisplatin concentration used for U2OS, the onset of apoptosis takes longer (time frame starts at 24 hr post-exposure for U2OS).

SUPPORT PROTOCOL 1

PREPARATION OF FLUORESCENTLY LABELED ANNEXIN V

Recombinant annexin V is isolated from a bacterial lysate and fluorescently labeled to use as a probe for apoptosis.

Materials

Bacteria: *E. coli* BL21-pLysS transformed with a pET/annexin V expression vector (available from Erik H.J. Danen; e.danen@lacdr.leidenuniv.nl)
LB medium containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin
IPTG
Spheroblast buffer (SBB; see recipe)
10 mg/ml lysozyme in SBB
Ultracentrifugation working solution (UCB; see recipe)
Bovine brain extract (Sigma)
1:2 (v/v) chloroform/methanol
Argon source
Diethyl ether
Liposome buffer (see recipe)
300 mM $CaCl_2$
Buffer 1: liposome buffer (see recipe) plus 5 mM $CaCl_2$
Buffer 2: liposome buffer (see recipe) plus 10 mM EDTA
Buffer 3: 20 mM bis-Tris, pH 6/0.02% (w/v) sodium azide
DEAE-Sepharose fast-flow column (~14 × 150–mm)
500 mM NaCl
Alexa fluor 488 protein labeling kit (Molecular Probes)

37°C incubator
250-ml Erlenmeyer flasks
Spectrophotometer
500-ml centrifuge tubes
Refrigerated centrifuge
50-ml plastic tubes
30-ml ultracentrifugation tubes
Refrigerated ultracentrifuge

250-ml glass beakers
10-kDa dialysis membranes

Additional reagents and equipment for SDS PAGE gel electrophoresis (*UNIT 6.1*) and Coomassie staining (*UNIT 6.6*)

Produce recombinant annexin V

1. Prepare an overnight culture of *E. coli* BL21-pLysS transformed with a pET/annexin V expression vector in 100 ml of LB medium containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin in a 250-ml Erlenmeyer flask at 37°C with shaking.
2. Dilute the culture 1:20 by adding 50 ml of overnight culture to 950 ml of fresh LB containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin.
3. Grow (~2 hr) at 37°C to $OD_{600} = 0.5$.
4. Add IPTG to 0.4 mM final concentration.
5. Incubate 4 hr at 37°C.

Produce crude extract containing recombinant annexin V

6. Split the culture in 500-ml centrifuge tubes and centrifuge 15 min at 5000 × g, 4°C.
7. Resuspend each bacterial pellet 25 ml SBB.
8. To each tube, add 2.5 ml of 10 mg/ml lysozyme stock in SBB (final concentration 1 mg/ml) and mix gently.
9. To each tube, add 192.5 ml of diluted SBB (1:1 in water) and mix gently.
10. Incubate 30 min on ice to allow spheroblast formation.
11. Centrifuge 30 min at 14,000 ×g, 4°C, to remove soluble components.
12. Resuspend and combine the four pellets in a total volume of 100 ml UCB working solution to open spheroblasts by osmotic shock.
13. Split into 25-ml aliquots in six 30-ml ultracentrifuge tubes.
14. Centrifuge overnight at 100,000 × g, 4°C, to remove large membrane parts.
15. Collect and combine the supernatants containing annexin V in a crude extract and store up to 2 years at −80°C.

Purify recombinant annexin V using bovine brain extract

16. Dissolve 500 mg of bovine brain extract in 6 ml of 2:1 chloroform/methanol in a 50-ml flask and dry under argon for 30 min.
17. Add 2.5 ml diethyl ether to the brain extract, mix, and dry under argon.
18. Dissolve in 20 ml liposome buffer.
19. Thaw the bacterial supernatant (which contains the annexin V in a crude extract) from step 16 slowly on ice.
20. In a 250-ml glass beaker on ice, mix the supernatant (100 ml) with the brain extract (20 ml) from step 20.
21. Add 300 mM $CaCl_2$ to a final concentration of 5 mM to bind the annexin V.
22. Stir for 30 min on ice.
23. Transfer to six 30-ml ultracentrifuge tubes and centrifuge 45 min at 40,000 × g, 4°C.
24. Resuspend the pellet in 40 ml of buffer 1.

25. Centrifuge 30 min at 50,000 × g, 4°C.
26. Resuspend the pellet in 20 ml of buffer 2.
27. Remove the liposomes by centrifuging 60 min at 50,000 × g, 4°C.
28. Collect the supernatant and dialyze overnight using a 10-kDa dialysis membrane at 4°C against 2 liters of buffer 3.

    Annexin V is ~30 kDa.

29. If a precipitate forms during dialysis, centrifuge 30 min at 20,000 × g, 4°C and collect the supernatant.

Column purify annexin V

30. Pre-rinse a DEAE-Sepharose fast-flow column (~14 × 150–mm) with 200 ml of buffer 3.
31. Add the annexin V-containing solution to the column.
32. Elute the annexin V protein with a linear NaCl gradient (0 to 500 mM; 1 ml/min).
33. Collect 4-ml samples.

    Annexin V elutes at 80 to 90 mM NaCl.

34. Run 4 µl of the 60 to 110 mM NaCl fractions on a 10% SDS PAGE gel (*UNIT 6.1*) to detect the 32-kDa annexin V protein by Coomassie staining (*UNIT 6.6*).
35. Combine fractions containing annexin V and store 1-ml aliquots up to 2 years at −80°C.

    The aliquots obtained will contain ~2 mg/ml of annexin V.

Fluorescently label purified annexin V

36. Fluorescently label annexin V (one aliquot at a time) with the Alexa fluor 488 protein labeling kit following the manufacturer's instructions.

    Protein labeling reagents are commercially available from multiple sources. The authors have successfully used the Alexa Fluor 488 kit from Molecular Probes but the choice of fluorophore is amendable. For use in combination with GFP for instance, Cy3 or Alexa 633 can be used. For labeling methodologies, refer to the methods included in such labeling kits.

    As an alternative, fluorescently labeled annexin V can be obtained from a number of suppliers (e.g., Immuno Tools, BD Biosciences). However, this protocol yields high amounts of pure fluorescently labeled annexin V at a cost that the authors have calculated to be ~20 times lower than that available from most commercial sources.

37. After labeling, adjust the concentration as desired (e.g., 0.5 mg/ml) and prepare small aliquots (e.g., 50-µl) store aliquots up to 2 years at −80°C.

    Subsequent protocols are based on a stock concentration of 0.5 mg/ml labeled annexin V.

SUPPORT PROTOCOL 2

QUANTITATIVE IMAGE ANALYSIS

The following steps describe the macro that is used to extract quantitative information on the accumulation of annexin V–positive cells over time. The macro has been designed by using Image Pro Plus from Media Cybernetics. This macro is available for download from *http://www.toxicologyleiden.nl*. This protocol describes an overview of the macro indicating the different steps that can be easily programmed into a comparable macro based on other image analysis software packages (see Fig. 18.10.3). In this particular macro, the DIC channel is used to determine cell numbers. If a second fluorescence marker is used for this purpose, then the macro can be adapted.

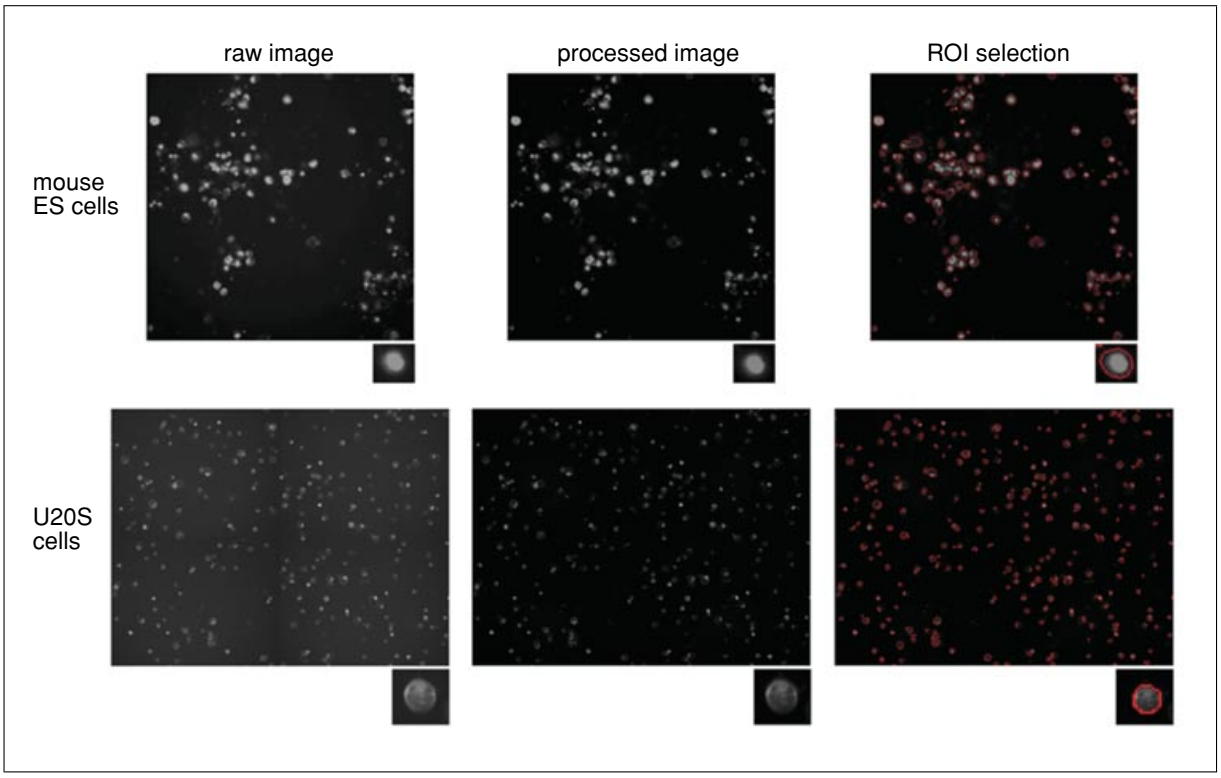


Figure 18.10.3 The accumulation of annexin V-FITC at the outer leaflet of the cell membrane is imaged and quantified. Three consecutive images are presented: a raw image corresponding to image directly acquired; a processed image that corresponds to the image after being adjusted to enhance the fluorescence label and reduce background noise; and finally the ROI selection image after the threshold is determined and the regions of interest are delineating the cells. The upper panel corresponds to mouse ES cells images acquired with a Nikon inverted fluorescent microscope, whereas the lower panel corresponds to U2OS cells images acquired with a BD Pathway 855 imager. Comparison of these data means that diverse types of image acquisition systems can be used to generate images that can be finally analyzed and quantified making use of the method presented in this unit. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb1810*.

Materials

Computer with tif images
Image Pro Plus software (Media Cybernetics; *http://www.toxicologyleiden.nl*)
Spreadsheet software (Excel)

Convert image from tif to avi

1. Convert the 8-bit, grayscale .tif image files created by the image acquisition software to uncompressed .avi files to generate time-lapse movies for each field of interest (using, for instance, Image J). Name these files "well01xy01C01.avi; well01xy01C02.avi; well01xy02C01.avi; well01xy02C02.avi; \ldots etc.", where C1 represents the annexin V fluorescence channel and C2 represents the DIC channel.

Start macro

2. Run macro in Image Pro Plus and open folder with .avi files.

   If, in addition to the annexin V fluorescence, DIC has been recorded to analyze total cell number, two .avi files are present for each field of interest for each time point. The macro sorts these file names alphabetically.

3. Select file "well01xy01C01.avi" and start Excel.

Define annexin V fluorescence intensity threshold

4. In the well01xy01C01.avi file, move to the image representing a time-point containing fluorescent cells.

5. Click Threshold button to open histogram model.
6. Move threshold ruler until fluorescent cells are red and background is black.

   Defining a threshold that is too low would result in overestimation of apoptosis while a threshold that is too high would cause underestimation of the number of apoptotic cells.

Define cell size in fluorescence and DIC channel

7. To exclude particles smaller than cells from the analysis, manually define the area of a cell in both channels. In well01xy01C01.avi and well01xy01C02.avi files, manually draw a region of interest (ROI) defining the area of a single cell (note that for the C01 file, a time-point is chosen where fluorescence has accumulated). To do this, click the button on the right side of "min" in the "area" window.

   If the area representing a single cell is already known from earlier experiments, this value can be introduced directly into the "area" window.

   It is advised to check the cell area also at the latter time-points as it may change during the experiment due to swelling or shrinking in response to toxicants. In such cases, draw a ROI representing the area of a single cell at later time-points and enter by clicking the button at the right side of "max" in the "area" window. The macro interpolates the values for the time-points in between the first and last.

   It is possible to obtain data without correcting for cell size. In this case, enter the value "1" in the "min" and "max" position.

Adjust DIC Edge filter settings for definition of cell-covered area

8. If using DIC to determine cell numbers, set background correction for DIC images.
9. In well01xy01C02.avi file, identify the total area covered with cells.

   The "Edge filter" extracts and enhances positive edges that are bright features on a dark background and is used here to optimize differentiation between plate surface and cells.

10. Select "high, medium, or low" settings for the [Width, Height, Strength, Passes] options of the "Edge filter" and run the macro followed by visual inspection.

    This must be done by trial and error and is determined by the DIC image acquisition settings. However, for a high-quality microscope system, once determined, the Edge filter settings can be applied to the entire experiment.

Run macro for fluorescence images

11. Click "calculate folder" to run the macro for all "well.xy.C01.avi" files (the annexin V fluorescence files). The macro will proceed with the following steps:

    a. Load file.
    b. Apply Edge Filter. Set values at: width: 4001; height: 4001; strength: 1; passes: 1.
    c. Select first frame.
    d. Set options to fill holes in segmentation.
    e. Segment picture with intensity threshold.
    f. Apply area range filter to avoid artifacts such as noise from particles smaller than the cells.
    g. Determine the sum of all segmented pixels (= total annexin V fluorescence).
    h. Divide total segmented pixels by the cell area (= number of positive cells).

       If the value "1" was entered in for the cell area, the total accumulation of annexin V signal is determined instead of the number of positive cells.

    i. Write result to Excel.
    j. Checks if 'Shift+S' key (signal to abort) is pressed. If 'Shift+S' is not pressed, the Macro will continue to the next .avi file.

Run macro for DIC images

12. If DIC files are present, run the macro for all "well.xy.C02.avi" files. The macro will go proceed with the following steps:

    a. Load file.
    b. Apply flatten background filter to reduce uneven illumination. Values will be set at: BrightOnDark: 1; ObjectSize: 20.
    c. Apply Edge Filter.
    d. Select first frame.
    e. Set options to NOT fill holes in segmentation.
    f. Segment picture with intensity threshold; this value will be automatically adjusted using the mean value of the Histogram Model.
    g. Apply area range filter, to avoid artifacts such as noise from particles smaller than the cells.
    h. Determine the sum of all segmented pixels (= total cell covered area).
    i. Divide total segmented pixels by the cell area (= number of cells; if the value 1 was entered in step h, number of cells is not determined).
    j. Write result to Excel.
    k. At this step the macro will check if 'Shift+S' key is pressed to stop the process. If 'Shift+S' is not pressed, the Macro will continue.

Calculate number of apoptotic cells

13. In Excel, divide the number of annexin V–positive cells obtained from the fluorescence files (step 11) by the total number of cells obtained from the DIC files (step 12) and multiply by 100 to obtain the percent of apoptotic cells.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Liposome buffer

20 mM Tris·Cl, pH 8 (*APPENDIX 2A*)
3 mM $MgCl_2$
100 mM NaCl
Store up to 3 days at 2° to 8°C

Spheroblast buffer (SBB)

0.5 mM EDTA
750 mM sucrose
200 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Store up to 3 days at 2° to 8°C

Ultracentrifugation buffer (UCB) stock solution

2 mM EDTA
5 mM $MgCl_2$
100 mM NaCl
20 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
0.1% (w/v) Triton X-100
Store up to 3 days at 2° to 8°C

continued

UCB working solution
To 100 ml UCB stock solution, add:
2 mM PMSF (2 ml of 100 mM stock in ethanol)
0.5 μg/ml pepstatin A (50 μl of 1 mg/ml stock in DMSO)
0.1 mg/ml RNaseA (1 ml of 10 mg/ml stock in TE)
10 U/ml benzonase (40 μl of 25 U/μl stock in TE)
Store up to 1 day at 2° to 8°C

COMMENTARY

Background Information

There are two main pathways that can drive apoptosis: the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway. An additional third pathway is named the perforin/granzyme route, and it involves T cell–mediated, perforin-granzyme-dependent, cytotoxicity. The three pathways converge in the same final execution pathway, which is initiated by the activation of executioner caspases such as caspase-3. Caspases are expressed in an inactive proenzyme form and, once activated, proteolytically cleave their substrates. These substrates also include other procaspases, thus initiating an irreversible amplification cascade.

Typical characteristics of apoptotic cells include extensive protein cross-linking through the expression and activation of tissue transglutaminase, DNA fragmentation by Ca^{2+}- and Mg^{2+}-dependent endonucleases and caspase-activated DNase, cell shrinkage, nuclear fragmentation, plasma membrane blebbing, and ultimately, cell fragmentation into apoptotic bodies, which are destined to be eliminated by phagocytosis.

A number of methods are currently available for the detection of apoptotic cells. These include detection of the typical profile of DNA laddering in cell lysates, TUNEL labeling, immunodetection of cleaved caspase-3, and flow cytometric analysis of DNA content to detect the sub-G0/G1 fraction using DAPI or propidium iodide in fixed cells. Since these methods require fixation or lysis of cells, they are by definition fixed endpoint methods. Although the response to apoptotic stimuli can be analyzed at several time-points, it would be preferable to detect the accumulation of apoptotic cells in real time to obtain kinetic information on the process of apoptosis. For this purpose, fluorescent probes are now commercially available for real-time imaging of caspase-3 activation.

In healthy cells, phosphatidylserines preferentially localize in the inner membrane leaflet due to the action of the aminophospholipid translocase that moves aminophospholipids from the outer to the inner leaflet. Endogenous annexin V proteins associate with the inner leaflet of the plasma membrane by binding to these phosphatidylserines (Gerke and Moss, 2002). In apoptotic cells, aminophospholipid translocase is inactivated while a scramblase, which catalyzes the randomization of phospholipids across the membrane bilayer, is activated. As a result, phosphatidylserines become exposed on the outer surface of apoptotic cells (Martin et al., 1995). Together with the expression of ligands for phagocytic cell receptors, this forms a signal for elimination by phagocytic cells. The method described in this unit makes use of recombinant, fluorescently labeled annexin V to detect apoptotic cells on the basis of accumulated phosphatidylserines on the outer surface of the plasma membrane.

Critical Parameters

The optimal concentration of labeled annexin V depends on the cell type used. For sensitive cell types, such as ES cells, excessive amounts of annexin V may be toxic. In addition, too low annexin V concentrations may require excessive exposure-times during image acquisition, which may cause phototoxicity and, hence, disturb the experiment. Therefore, a titration is required.

Overexposure of the detectors would cause saturation and underestimation of the accumulation of apoptosis. The optimal exposure time must be determined in advance for a given microscope system and fluorophore. This is especially important given that fluorescence is typically expected to be absent at the start of any experiment. Optimization is done by exposing cells to a high concentration of toxicant that is expected to induce massive apoptosis in the presence of a high concentration of labeled annexin V (typically 0.05 mg/ml) to ensure a maximal signal.

Anticipated Results

The authors' findings indicate that this method can be used for analysis of multiple

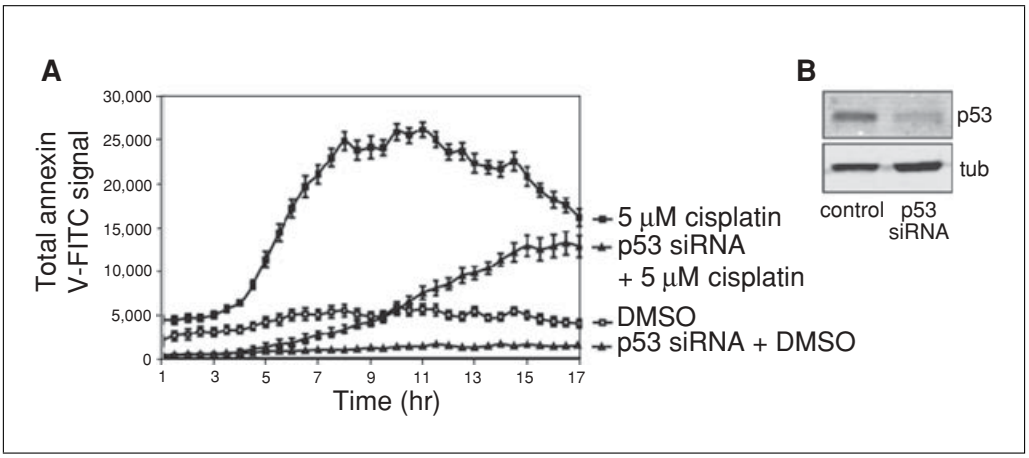


Figure 18.10.4 Cisplatin-induced apoptosis is inhibited by silencing p53. (**A**) Mouse ES cells were exposed to cisplatin in the presence or absence of a siRNA smartpool (Dharmacon; 50 nM) silencing the tumor suppressor gene p53. Silencing p53 in this cell type resulted in a strong delay of apoptosis. (**B**) p53 knock down efficiency is shown by western blot using p53 antibody or tubulin (tub) loading control antibody.

cell types, including ES cells and cancer cells, which undergo dose- and time-dependent apoptosis when exposed to a DNA-damaging drug such as cisplatin. Signal accumulation can be prevented using a pan-caspase inhibitor, z-VAD-fmk, indicating that it specifically depicts apoptosis. In addition, silencing the tumor suppressor gene p53 results in a delay of the apoptotic process as expected. Thus, the high-throughput live cell imaging method described in this unit provides kinetic information about the apoptotic process. Apoptosis is of special interest to toxicity studies. This particular analysis method can be performed for screening purposes to determine time- and dose-dependent responses to compounds and to investigate interactions between drugs, with special emphasis on the kinetics of the process. Moreover, RNA interference focused on understanding the apoptotic process can be very well approached with this methodology (see Fig. 18.10.4).

In vitro studies are often performed on different types of cell lines and using various compounds to induce apoptosis. It must be noted that due to the different characteristics of the chosen cell/drug combinations, responses are expected to display highly diverse kinetics. Obviously, exposure or pre-exposure periods to certain drugs in different cell types must be adjusted experimentally before moving toward larger scale screens (see Fig. 18.10.2).

Time Considerations

After the optimization phase (see Basic Protocol, steps 11 to 17), a complete single experiment will take ~24 hr. It should be mentioned here that automation is required for the multi-point imaging in multi-well plates (see Basic Protocol, steps 23 to 26). In addition to this, automation will greatly speed up the process of treating cells with concentration ranges of multiple drugs. Finally, for siRNA experiments, robotics will be required to efficiently move from analysis of small gene sets to larger screening efforts.

Acknowledgements

The authors are supported by the Dutch Cancer Society and by the Netherlands Genomics Initiative.

Literature Cited

Elmore, S. 2007. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* 35:495-516.

Gerke, V. and Moss, S.E. 2002. Annexins: From structure to function. *Physiol. Rev.* 82:331-371.

Hanshaw, R.G. and Smith, B.D. 2005. New reagents for phosphatidylserine recognition and detection of apoptosis. *Bioorg. Medicinal Chem.* 13:5035-5042.

Martin, S.J., Reutelingsperger, C.P.M., McGahon, A.J., Rader, J.A., van Schie, R.C.A.A., LaFace, D.M., and Green, D.R. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* 182:1545-1556.

CHAPTER 19
Whole Organism and Tissue Analysis

INTRODUCTION

The ultimate test of cell biological principles is in the intact organism. Ideally, concepts and conclusions based on in vitro biochemistry, molecular biology, or tissue culture studies should be validated in vivo in intact tissues and organisms. In addition, some studies can be performed only in living animals or plants. There are innumerable examples of conclusions from in vitro studies that have needed major qualification after analysis in vivo. One common class involves substantially altered conclusions about the necessity of a specific protein after testing whether it is essential by gene knockout analysis. Others include changing concepts about gene regulatory pathways in development and about the mechanisms and inhibitors of malignancy. An obvious reason is that organisms have complex microenvironmental, regulatory, and system-redundancy properties that are not mimicked adequately in vitro.

This chapter will provide detailed protocols for studies in whole organisms and tissues. Although experimental analyses in intact systems could theoretically span all of biology, the focus will be on current techniques particularly relevant to cell biology. The study of cancer metastasis shows the particular value, but also the many pitfalls, of analyzing the cell biology of this complex type of process in animals. In *UNIT 19.1*, members of a leading laboratory in the field (Paweletz, Charboneau, and Liotta) provide an insightful and thought-provoking overview of metastasis assays. The unit starts with a review of key cell biological principles and concludes with discussions of what constitutes a "good" animal model and some state-of-the-art approaches. As discussed in detail in the overview presented in *UNIT 19.1*, metastasis involves a plethora of cell biological steps. Relevant processes include angiogenesis, cell adhesion, cell motility and invasion, and local proteolysis, each of which will need understanding both in vitro and in the context of the living organism.

UNIT 19.2 by Elkin and Vlodavsky then provides practical details, focusing on the most frequently used approach, the analysis of metastasis by tail vein assay. This approach, often termed "experimental metastasis," involves injecting single-cell suspensions of cells into the tail vein of mice or rats. The cells lodge particularly effectively in lungs, where they form tumor colonies that can be quantitated. This procedure permits analyses of the steps of metastasis that occur during and after the extravasation of tumor cells from blood vessels into target organs. It has been used widely, and should continue to provide valuable insight into the mechanisms of metastasis and a system for testing candidate anti-metastatic drugs. An alternative to using adult rodents is the use of chick embryos for analyzing metastasis. Procedures for handling chick embryos are described in *UNIT 19.5*.

UNITS 19.3 & 19.4 provide complementary approaches to solving the difficult problem of how to obtain reliable quantitative determinations of gene expression with very small quantities of material. For example, tiny pieces of embryonic tissue or even laser-microdissected sites from histological sections are often the optimal sources of material for analyzing gene expression patterns in a specific part of a tissue from an intact organism. However, standard RT-PCR approaches for amplifying low quantities of nucleic acids from these sources can introduce distortions in gene expression data resulting from preferential

Current Protocols in Cell Biology 19.0.1-19.0.3, December 2009
Published online December 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1900s45

amplification of some transcripts and sequence errors introduced by *Taq* DNA polymerase. *UNIT 19.3* by Sakai, Larsen, and Yamada provides a procedure for analyzing ultra-micro amounts of sample, such as from laser-microdissected bits of tissue cut out of frozen sections, using high-fidelity T7-based RNA amplification. It also provides a detailed protocol for the Serial Analysis of Gene Expression (SAGE) procedure based on published protocols, which can provide digital quantification of gene expression. If larger (though still relatively tiny) amounts of sample are available, e.g., 1 to 2 μg of RNA, risks of amplification can be avoided by using the procedures described in *UNIT 19.4* by Cai, Ash, and Jabs, followed by SAGE analysis. In both cases, SAGE provides an approach that can identify novel genes that are not yet present in DNA microarrays and quantify expression levels for the full spectrum of genes expressed in each tissue.

Assays for angiogenesis in vivo are important for verifying predictions from in vitro angiogenesis models and for studying this complex process in the intact organism. The simplest in vivo angiogenesis model is the chick chorioallantoic membrane (CAM) assay. In *UNIT 19.5*, Ponce and Kleinman describe the advantages, caveats, and detailed protocols for this assay, which can be performed in virtually any laboratory without the need for an animal facility.

Although tumor metastasis is most frequently studied in mouse model systems, an attractive alternative is described in *UNIT 19.6* by Wilson and Chambers. The chick embryo provides an inexpensive and convenient system that has the added advantage of being immune-deficient and thus useful for analyzing tumor cells from any species including human without concerns about immunological compatibility. Besides methods for experimental metastasis assays using chick embryos, a protocol is provided for live-cell in vivo video microscopy. This procedure for real-time microscopy analysis of the thin, readily visualized chick embryo chorioallantoic membrane permits direct visualization of specific steps of metastasis as the injected tumor cells interact with host vasculature and invade tissues. New approaches now permit detailed characterizations of tumor cell invasion in vitro. In *UNIT 19.7*, Kedrin, Segall and colleagues provide detailed protocols for studying the real-time dynamics of migrating and invading cancer cells in rodent models. The authors describe their methods for dual-color imaging of invading tumor cells in living animals using GFP and CFP simultaneously, microscopy and image analysis, and additional supporting protocols.

The biogenesis of complex three-dimensional tissues, e.g., organogenesis and branching morphogenesis, can now be characterized and experimentally analyzed using serum-free organ culture. *UNIT 19.8* provides protocols for culturing several embryonic mouse organs. In that unit, Sakai and Onodera describe stepwise procedures and provide video demonstrations for dissecting mouse embryos to isolate specific organs. They then describe conditions for three-dimensional organ culture.

Experimental studies of the biology and pathology of human skin are now possible in vitro using three-dimensional models termed human skin equivalents. *UNIT 19.9* from Garlick and coauthors provides detailed protocols for establishing three-dimensional human skin models by combining epidermal cells with dermal fibroblasts grown in a collagen matrix. These models closely mimic normal human skin, and they can be used to study growth, differentiation, wound repair, and cancer of human skin.

Transgenic and knockout mouse technologies have revolutionized the study of normal in vivo physiology and biochemistry–as well as disease processes–by allowing genetic manipulation to over-express or ablate specific genes. A series of five units in this chapter from members of a facility specializing in gene targeting spans the spectrum of techniques for genetic modification of the mouse. *UNIT 19.10* by Haruyama, Cho, and Kulkarni provides a general overview of the process of producing appropriate DNA constructs

and engineering transgenic mice. It reviews principles and techniques for producing transgenic mice over-expressing a particular gene, as well as emerging techniques and typical problems that might be encountered. *UNIT 19.11* by Cho, Haruyama, and Kulkarni then provides highly detailed protocols for generating transgenic mice using pronuclear injection. A variety of methods and tips are provided that should provide the practical knowledge important for efficient production of transgenic mice.

The opposite approach to transgenic over-expression of a gene for determining function is to generate gene knockout mice using targeted homologous recombination. In *UNIT 19.12*, Hall, Limaye, and Kulkarni provide an overview of the principles of this process that uses electroporation of a custom DNA targeting construct into totipotent mouse embryonic stem cells to allow homologous recombination. *UNIT 19.13* by Limaye, Hall, and Kulkarni provides in-depth protocols for maintaining, transfecting, and selecting mutated pluripotent embryonic stem cells. Cells with successful gene knockout must then be injected into blastocyst embryos and implanted into pseudopregnant mice as described by Longenecker and Kulkarni in *UNIT 19.14*. The detailed techniques, practical advice, and troubleshooting information provided by these experts should facilitate the successful production of knockout mice.

Kenneth M. Yamada

Overview of Metastasis Assays

Cancer treatment and diagnosis usually begins at a late stage when most patients already have acquired occult or overt metastasis adding significantly to the diminishing success rates for localized surgery and cytotoxic approaches (Astrow, 1994). Consequently, metastatic dissemination is responsible for the majority of treatment failures and deaths.

During tumor progression, cells acquire genetic and proteomic changes as they transform from normal to hyperplastic, through dysplasia, to carcinoma in situ, and finally to invasive and metastatic. The time course of progression may extend as far back as 10 years prior to diagnosis (Fig. 19.1.1). Discerning the mechanism whereby tumor cells execute metastatic dissemination may provide the foundation necessary for successful treatment of the disease. For example, direct genetic evidence has linked in situ breast cancer to invasive carcinoma of the breast (Zhuang et al., 1995) supporting the generally accepted assumption that carcinoma in situ of the breast is a clonal expansion of hyperproliferating cells (Gallager and Martin, 1969; Spratt et al., 1986). This in turn may provide a more comprehensive and/or functionally directed target strategy for intervention and prevention of breast cancer.

Cancer metastasis is a highly complex process that involves the deregulation of interacting proteins and genes that are responsible for invasion, angiogenesis, circulation of tumor cells in blood vessels, colonization at secondary organ sites, and finally evasion of host defense systems (Fig. 19.1.2). Invasion can, furthermore, be subdivided into cellular adhesion, local proteolysis, and motility. While all cohorts mentioned above are necessary for successful metastasis, none alone is sufficient to cause metastasis.

Angiogenesis

The critical turning point in tumor progression is the initiation of local invasion and neovascularization leading to metastasis. Neovascularization, the formation of new blood vessels, is not only necessary for continuous tumor maintenance by providing adequate nutritional support but is also required for metastasis by providing a mechanism to allow tumor cells to

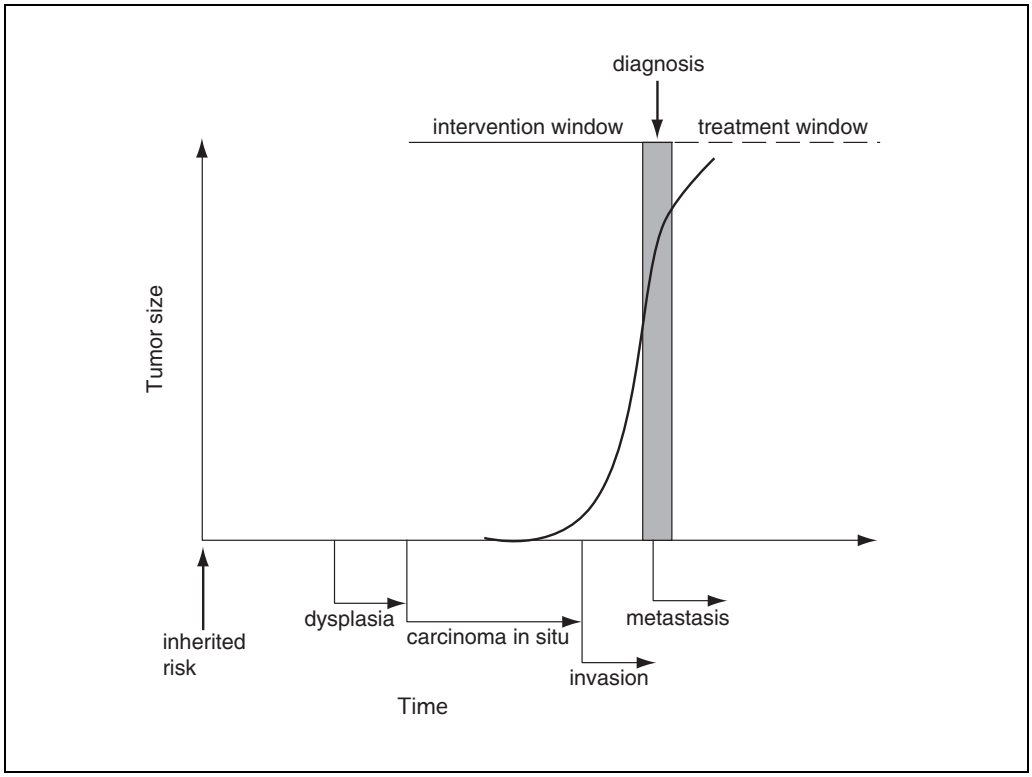


Figure 19.1.1 Theoretical improvement for intervention strategies as a function of time. Diagnosis of cancer usually is performed late into the disease, when most patients already present occult metastasis. However, progression of cancer from dysplasia to metastatic dissemination may extend as far back as 10 years providing a much larger window for intervention strategies before metastasis occurs.

Contributed by Cloud P. Paweletz, Lu Charboneau, and Lance A. Liotta
Current Protocols in Cell Biology (2001) 19.1.1-19.1.9

enter the blood circulation. A vascular entry port to dissemination is achieved by induced proliferation of highly permeable capillaries by tumor cells (Dvorak et al., 1995). These local capillaries mainly arise by angiogenic stimuli released by tumor or host cells. However, angiogenesis will cease when the local stimulus is removed. A variety of molecules have been found to mediate angiogenesis in vitro and in vivo. Among these are fibroblast growth factors (bFGF), angiogenin, vascular permeability factor (VPF), and transforming growth factor α and β (TGF-α, TGF-β; Folkman and Klagsbrun, 1987).

Neovascularization is not regulated by a single event but rather consists of tightly regulated molecular events that cause endothelial cells to degrade the basement membranes, proliferate, migrate, and initiate growth of capillaries (Fidler and Ellis, 1994; Folkman, 1995). Angiogenesis occurs in parallel with initiation of invasion. Rapidly growing tumors are capable of shedding up to millions of tumor cells into vascular circulation by angiogenesis and invasion alone (Liotta et al., 1974). Furthermore, experimental studies show that <0.05% of circulating tumor cells are successful in initiation of metastatic colonies making metastatic dissemination a highly inefficient process (Liotta et al., 1974; Nicolson, 1991). These studies are also further validated by the observation that circulating tumor cells are detected in patients who never form a metastasis.

Invasion

Invasion is the active translocation of neoplastic cells across tissue boundaries and through host cellular and extracellular matrix barriers. Cellular adhesion, local proteolysis, and motility characterize successful invasion. While all three cohorts are necessary for successful metastasis, none alone is sufficient enough to cause metastasis. Invasion is not due to growth pressure but involves additional genetic and proteomic deregulation over and above those molecular events that cause uncontrolled proliferation. Instead, cellular invasion is dependent on the coordinated activity of a series of interacting proteins extending from the inside of the cell to the cell surface and the imminent microenvironment.

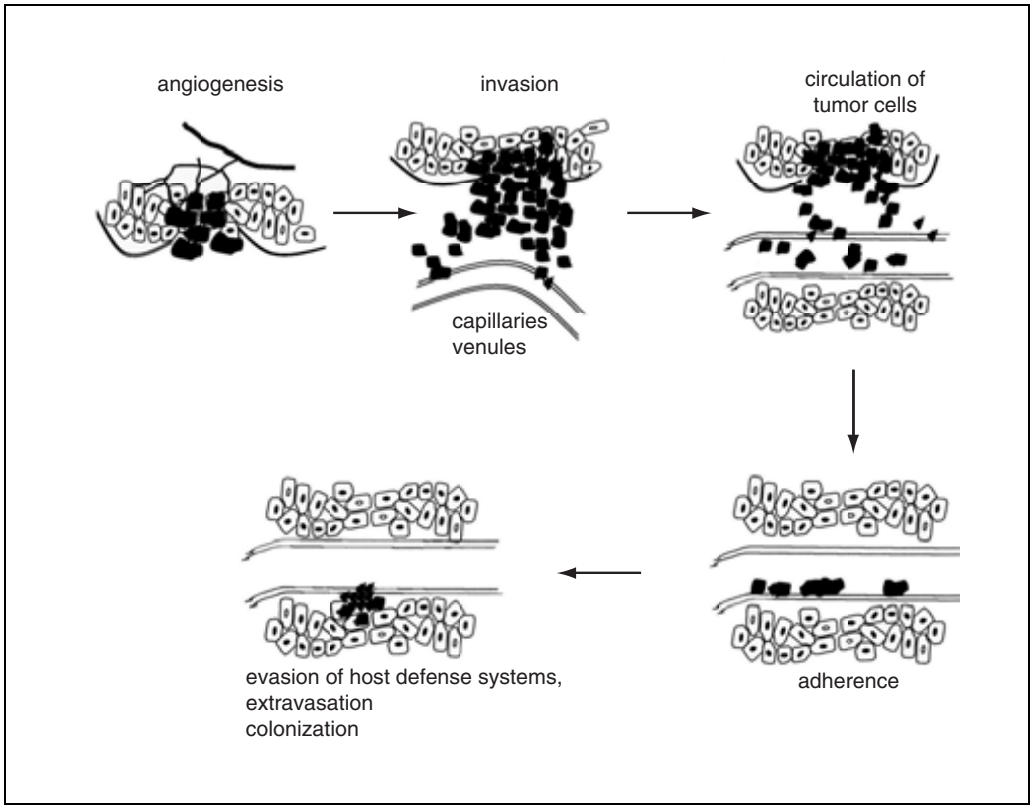


Figure 19.1.2 Pathogenesis of cancer. Cancer metastasis is a highly complex process that involves the deregulation of interacting proteins and genes that are responsible for angiogenesis, invasion, circulation of tumor cells in blood vessels, colonization at secondary organ sites, and finally evasion of host defense systems.

Cellular Adhesion

Maintaining cellular integrity with the extracellular matrix and/or basement membrane involves the rapid ability to respond to extracellular signals at receptors, which in turn affects corresponding intracellular signal transduction pathways that regulate proliferation, differentiation, and migration. These receptors can be membrane surface receptors that become activated by ligands (such as growth factors), or cytosolic/nuclear receptors that transmit hormone signals to regulate gene expression. Among the many types of cell surface receptors several classes have been shown to be predominant during tumorigenesis. These include integrins, cell adhesion molecules (CAMs), and cadherins.

The integrin family of cell surface extracellular matrix proteins consists of heterodimeric units, designated α (140 kDa) and β (95 kDa). An important aspect of this family is that integrins can either exist in an "on" or "off" state, thereby selectively changing its affinity for corresponding ligands, and changing molecular events in doing so (Hynes, 1992; Juliano and Haskill, 1993). Activation of integrins has been shown to be involved in cell migration, cell proliferation, and dissemination. However, loss of stimulus of integrins has been associated with apoptosis. This suggests a diverse switch-on/switch-off function for integrins. Increased expression of integrin receptors on cell surfaces has been associated with an invasive phenotype of melanoma and squamous carcinoma of the head and neck, whereas loss of expression status has been shown in cancers of breast, prostate, and colon (Chammas and Brentani, 1991; Natali et al., 1991).

The cadherins, on the other hand, comprise a family of transmembrane glycoproteins that mediate Ca^{2+}-dependent cell adhesion (Takeichi, 1991). Special intracellular proteins, the catenins, form zipper-like structures to form extracellular cell-cell bonds with the cell cytoplasm. These interactions are regulated by tyrosine phosphorylation as well as additional cell-to-cell communications. Down regulation of epithelial cadherin transcription, E-cadherin, has been shown to cause a more aggressive cancer cell phenotype (Frixen et al., 1991; Vleminckx et al., 1991). For example, transforming Madin-Darbey canine kidney cells (MDCK) by H-*ras* not only diminshed E-cadherin expression but also increased the invasive behavior of these cells. This effect could be reversed by transfecting E-cadherins back into the transformed cells (Behrens et al., 1989; Mareel et al., 1991; Vleminckx et al., 1991). Furthermore, overexpression of E-cadherin in highly invasive tumor types (i.e., bladder, breast, lung, and pancreas) showed loss of invasiveness in their respective transformed cell line.

Cellular adhesion receptors (CAMs) have also been studied in the context of cancer invasion. These include but are not limited to: intercellular adhesion molecules (ICAMs), L-, E-, and P-selectins, vascular cell adhesion molecules (VCAM), neural cell adhesion molecules (NCAM), and neuroglial cell adhesion molecules (NG-CAM). These adhesion receptors provide the necessary framework for the interaction of tumor cells with the microenvironment forming a vital part of cancer invasion and metastasis. Unlike other receptors that bind proteins, selectins bind carbohydrate ligands on endothelial cells. While adhesion alone may not be sufficient for metastasis, disregulation of any of the surface receptors above may alter signaling pathways responsible for positive and negative feedback controls and may therefore result in a more aggressive invasive phenotype.

Local Proteolysis

A positive correlation between tumor aggressiveness and protease levels has been documented (Liotta et al., 1980). Not only are proteases upregulated in tumor cells, and protease inhibitors down regulated, but zymogens are also activated into active protease enzymes. Local proteolysis by itself is not a sole metastatic phenomenon, and it is routinely observed in trophoblast implantation, embryo morphogenesis, wound healing, tissue remodeling, and angiogenesis. Local proteolysis is a tightly regulated process in normal tissue by appropriate protease inhibitors. Furthermore it has been indicated that deregulation between the equilibrium of protease to protease inhibitor regulates vascular morphogenesis and invasion (Ura et al., 1989). Metastasis cannot simply be a product of unbridled production of degradation enzymes as cell dissemination and invasion concomitantly are dependent on adhesion and motility. Deregulation of this tightly regulated process causes secretion of proteases by tumor cells that creates local access points across the basement membrane and/or extracellular matrix. This process takes place by coordinations of proteases including serine, aspartyl, cysteinyl, and metalloproteases (Nakajima et al., 1987; Ostrowski et al., 1988; Reich et al., 1988). Sufficient evidence exists that proteases

inside the cell may also be involved during invasion (Koblinski et al., 2000). Invasion is mostly hindered by collagens, which form with laminin, fibronectin, triple helical proteins, and vitronectin, important structural components of the basement membranes and extracellular matrices. Collagen I and III are located in stromal collagens, and collagen IV and V are predominant in the basement membrane. Among the many types of proteases involved in metastasis such as tissue-type plasminogen activator (tPA), plasmin, cathepsin -D, -B, -L, -G, the urokinase plasminogen activator (uPA), metalloproteinases, and the heparanases deserve special mentioning.

Urokinase plasminogen activator, a serine protease, has been shown to correlate with a metastatic phenotype of cells. Antibodies against uPA block human HEP-3 cell invasion and murine B16-F10 melanoma cell metastasis after tail vein injection (Ossowski and Reich, 1983; Esheicher et al., 1989). Alternatively, overexpression of uPA in H-*ras* transformed cell lines enhances lung metastases (Axelrod et al., 1989).

Enzymatic cleavage of collagens, necessary for invasion of tumor cells into the vascular vessel, is mostly carried out by metalloproteinases (MMP). Inhibition of metalloproteinases has been demonstrated to inhibit cell invasion (DeClerck et al., 1991). These inhibitors have also been shown to block metastasis. MMPs can be divided into three general classes: (1) interstitial collagenases; (2) stromelysins; and (3) gelatinases. Interstitial collagenase, degrade types I, II, III, and VII collagens. Stromelysins degrade types I, III, IV, V, IX collagens, laminin, fibronectin, and gelatin. The third group of the MMP family, the gelatinases (MMP-2 and MMP-9), are capable of degrading collagen types I, II, III, IV, V, VII, IX, and X, as well as fibronectin (Emonard and Grimaud, 1990). An important difference between the gelatinases and the other MMPs is that they are capable of interacting with endogeneous tissue inhibitor of metalloproteinases (TIMPs) of which there are two major subgroups, TIMP-1 and TIMP-2. Inhibition of MMP-2 by TIMP-1 has shown to reduce cellular invasion. Involvement of MMP-2 and MMP-9 in invasive phenotypes is abundant. Induction of H-*ras* oncogene enhances expression of MMP-2 and MMP-9. Invasive colonic, gastric, ovarian, and thyroid adenocarcinomas showed positive immunoreactivity for MMP-2, whereas normal colorectal, gastric mucosa, and benign ovarian cysts showed reduced or negative staining (Monteagudo et al., 1990; Levy et al., 1991). A fragile balance between TIMPs and MMPs may act as a postive and negative feedback control regulating vascular morphogenesis and invasion (Mignatti et al., 1989). Additional functions of MMPs besides degradation of tissue boundaries are also ascribed to its involvement in angiogenesis (Chambers and Matrisian, 1997).

Additional major components of tissue barriers to be degraded by proteases besides collagen, fibronectin, and laminin are heparan sulfate proteoglycans (HSPG). Involvement of heparanases, whose function involves the degradation of ubiquitous expressed heparan sulfate glycosaminoglycan side chains, with respect to metastasis has previously been demonstrated (Nakajima et al., 1988; Vlodavsky et al., 1995). Not only did heparin and similar polysaccharides inhibit metastasis (Parish et al., 1987), increased expression of heparanase genes and proteins were also found in tumor cells compared to normal cells (Vlodavsky et al., 1999). Discerning the fundamental mechanism whereby heparanases cause metastatic cell dissemination has become more achievable with the completion of the cloning of the heparanase gene (Hullet et al., 1999; Vlodavsky et al., 1999). In these studies, transfection of nonmetastatic murine T-lymphoma Eb cell lines with full-length human heparanase cDNA enhanced its metastatic phenotype, as shown by increased invasiveness of cells and a higher mortality rate of tumor-injected mice (Vlodavsky et al., 1999).

In the end, the coordination of families of proteases with adhesion molecules and locomotion is required for successful invasion. None alone can cause invasion, and it is likely that many other proteases will be found in the near future.

Motility

Translocation of individual cells across tissue membranes inevitably is a major part of invasion. However, as with local proteolysis and angiogenesis, cell migration is also part of normal cell development such as gastrulation and organogenesis. Diminishing cell adhesion single cell locomotion, and cohort cell translocations, have been reported to be involved in malignancy and metastasis (Liotta and Stetler-Stevenson, 1993; Nabeshima et al., 1999).

Early events in cell motility consist of extensions by pseudopods prior to translocation of the whole cell body (Stossel, 1993). These nuclear-free vessels would have all the neces-

sary requirements to sense and protrude within the three-dimensional structure of the tumor mass leading cell migration away from the primary organ (Condeelis, 1993; You et al., 1996). However, unregulated protease interactions alone cannot ad infinitum translocate the pseudopod, as the vessel would dig itself into a hole and thereby immobilize itself. Adhesion, interaction, and detachment with the ECM are essential parts of locomotion (Liotta et al., 1991; Damsky and Werb, 1992). Proteinases at the tip are vital for the disruption of the ECM, but as the matrix becomes more and more disrupted, the pseudopod must adhere to the matrix and pull the cell forward. At the rear of the pod, detachment from adjacent cells is necessary.

Movement across tissue boundaries is initiated by a chemotactic (directional) mechanism. A variety of chemoattractive compounds, among them certain cytokines (e.g., hepatocyte growth factor), collagen peptides, formyl peptides, or autocrine growth factors (e.g., autotaxin), have been identified to be important in metastasis locomotion (Anzano et al., 1983; Guirguis et al., 1987; Di Renzo et al., 1995; Nam et al., 2000). It needs to be realized, even if there exists a direct tumor host interaction, chemoattractant release to the migrating cell may only be sporadic at best and could not sustain metastatic dissemination. These factors are secreted by surrounding stroma, the tumor cells themselves, or by peptides affiliated with the extracellular matrix. For example, it has been demonstrated that the human melanoma cell line A2058 as well as human breast cell lines stimulate their own motility factors when treated with serum-free medium. Motility, like adhesion and local proteolysis, is necessary for metastasis but cannot alone cause metastasis.

In Vivo Models and Metastasis

Successful metastasis only occurs if positive and negative regulatory mechanisms that govern angiogenesis, proteolysis, motility, evading host defense systems, and cellular adhesion events are concomitantly deregulated. Metastasis is a multifactorial event that includes deregulations above and beyond those found during disturbances of cell proliferation and homeostasis. Ultimately, there exists a need to investigate proteins and genes involved in metastasis and invasion in vivo. While there exists a variety of assays to study any one of those specific characteristics in vitro, such as the modified Boyden chamber assay for the study of motility, transgenic, knock-out, and xenograft animal models are the only accurate tools available to investigate molecular mechanisms responsible for metastasis in an organism (Fidler and Hart, 1982; Guan et al., 2000; Zuber et al., 2000).

Organ trophism varies widely depending on the morphology and anatomic location of primary tumors. Clinical studies indicate that most metastases form micro-colonies in the first capillary bed found by circulating, invasive tumor cells. For example, lung cancer metastasizes predominately to the brain, or colorectal cancers to the liver. However, not all metastatic sites can be predicted by anatomical considerations alone. For example, clear-cell carcinoma of the kidney metastasizes to the bone and thyroid. Animal models that either show preferential organ metastases by purposeful selection or have not undergone purposeful selection have been designed to accompany and simulate these observations (see Table 19.1.1; Nicolson, 1988).

A "good" animal model is based on the following three basic axioms: (1) metastasis is not a random phenomenon; (2) neoplasms are heterogeneous in their cell make up and therefore observe different metastatic outcomes; and (3) the outcome of the metastasis is dependent on both the tumor cell and the host cell interactions (Fidler, 1991).

The human genome project in conjunction with other projects, such as the CGAP initiative of the NCI, is expected to provide a wealth of information of possible tumor (or metastasis) suppressor genes and oncogenes that are recessives and mutated dominants, respectively (Strausberg et al., 1997). However, it is necessary to establish whether these genes are actually involved in metastasis or tumorigenesis in vivo. A logical way is to transfect mutant forms of these candidate genes into a noninvasive cell line of the tumor to be studied, inject these modified cell lines intravenously, and determine the number of lung colonizations. Alternatively, the effects of possible oncogenes toward metastasis can be investigated by knock-out mice lacking certain candidate genes (Granovsky et al., 2000). The oncogene-deficient mice can then be compared to nondeficient mice by counting lung colonies of micro metastases and comparing tumor weight. Substantial decrease in colonies and tumor weight is expected if the gene that is "knocked out" is involved in metastasis.

An excellent example of how transfection of a gene provided valuable insights into metastasis was recently demonstrated by Vlo-

davsky et al. (1999). Initially, there existed sufficient evidence that heparanases may be involved in metastasis, as an increased level of heparanase was found in patient's urine and serum samples (Vlodavsky et al., 1999). However, the gene itself was (1) not cloned; and (2) not expressed in an animal model making accurate presumptions about its function difficult. After successful cloning of the gene, full-length human heparanase cDNA was transfected into nonmetastatic murine T-lymphoma Eb cell lines. Injection of transferred cell lines into DBA/2 mice not only increased the metastatic phenotype as shown by liver colonization but also decreased survival rates of injected mice. However, a word of caution is advised because transfection of genes and subsequent ectopic injections may cause "false-negatives," as it is possible to inject cells in such a way that they do not form metastases even if the cell possesses a real metastatic phenotype (Kerbel et al., 1991).

At present, there does not exist a completely accurate animal model that simulates a specific human histologic tumor type and its complete metastatic behavior. While the positive and negative attributes of animal models can be debated, their positive productive role in dissecting molecular mechanisms for metastasis and treatment cannot be disregarded.

Conclusion

Even though studies on metastasis have traditionally lagged behind other fields in cancer research, substantial progress has been made over the last 10 years. This is partly due to the separation of cancer metastasis into distinct fields such as angiogenesis, invasion, metastatic suppressor genes, and others. For each distinct field, novel technologies and methodologies have provided important insights. However, exciting and emerging technologies such as in vivo microscopy of cell motility (Farina et al., 1998) or proteomics combined with laser capture microdissection (Lawson et

Table 19.1.1 Organ Preferences of Metastasis in Some Human and Animal Selected Models[a,b]

Tumor system subline[c,d]	Lung	Liver	Brain	Ovary	Spleen	Lymph node
Murine B16 melanoma (i.v. or i.c.)						
B16-F1	+	±	±	±	−	±
B16-F10	++++	±	−	+	±	±
B16-B15b	+++	−	+++	+	±	±
B16-O13	++		−	+++	−	±
Murine RAW117 large cell lymphoma (i.v. or s.c.)						
Raw117-P	±	±	−	−	±	−
Raw117-H10	±	++++	−	−	++	−
Murine MT1 mammary carcinoma (i.v. or s.c.)						
TC3	+++	+	−	±		−
Chicken MD lymphoma (i.v.)						
AL-2	−	++++	−	±	−	−
AL-3	−	+	−	++++	−	−
Human A375 melanoma (i.v. in nude mice)						
A375-P	±	±	−	−	−	±
A375-SM	++	±	−	±	−	+
Human PC-3 prostatic carcinoma (i.v. in athymic mice)						
PC-3-125-IN	++++	−	−	−	−	−
PC-3-1-LN	++++	+	−	+	±	+++
Human MeWo melanoma (i.v. in nude mice)						
MeWo	+	−	−	−	−	−
MeWo-70-W	++	−	++	+	−	−

[a]Modified from Nicolson (1988).

[b]Metastesis: −, none; ±, sometimes; +, few; ++, moderate; +++, many; ++++, large numbers and heavy tumor burden.

[c]Abbreviations: i.v., intravenous; i.c., intracecum; s.c., subcutaneous.

[d]Common animal models used in the study of metastasis.

al., 1991; Emmert-Buck et al., 2000; see *UNIT 2.5*) will provide valuable insights into the field. For example, it is feasible to microdissect patient-matched normal, tumor, and metastatic tissue cells and analyze their corresponding DNA, mRNA, and protein levels, giving a true cellular snapshot. Information acquired through these studies will almost certainly target signal transduction pathways that may provide alternative intervention and prevention strategies much earlier than is possible today.

Literature Cited

Anzano, M.A., Roberts, A.B., Smith, J.M., Sporn, M.B., and De Larco, J.E. 1983. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. *Proc. Natl. Acad. Sci. U.S.A.* 80:6264-6268.

Astrow, A.B. 1994. Commentary: Rethinking cancer. *Lancet* 343:494-495.

Axelrod, J.H., Reich, R., and Miskin, R. 1989. Expression of human recombinant plasminogen activators enhance invasion and experimental metastasis of H-ras-transformed NIH 3T3 cells. *Mol. Cell. Biol.* 9:2133-2141.

Behrens, J., Mareel, M.M., van Roy, F.M., and Birchmeier, W. 1989. Dissecting tumor cell invasion: Epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* 108:2435-2447.

Chambers, A.F. and Matrisian, L.M. 1997. Changing views of the role of matrix metalloproteinases in metastasis *J. Natl. Cancer Inst.* 89:1260-1270.

Chammas, R. and Brentani, R. 1991. Integrins and metastases: An overview. *Tumour Biol.* 12:309-320.

Condeelis, J. 1993. Life at the leading edge: The formation of cell protrusion. *Annu. Rev. Physiol.* 9:411-444.

Damsky, C.H. and Werb, Z. 1992. Signal transaction by integrin receptors for extracellular matrix: Cooperation and processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772-781.

DeClerck, Y.A., Yean, T.D., Chan, D., Shimada, H., and Langley, K.E. 1991. Inhibition of tumor cell invasion of smooth muscle cell layers by recombinant human metalloproteinase inhibitor. *Cancer Res.* 51:2151-2157.

Di Renzo, M.F., Poulsom, R., Olivero, M., Comoglio, P.M., and Lemoine, N.R. 1995. Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. *Cancer Res.* 55:1129-1138.

Dvorak, H.F., Brown, L.F., Detmar, M., and Dvorak, A.M. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Path.* 146:1029-1039.

Emmert-Buck, M.R., Gillespie, J.W., Paweletz, C.P., Ornstein, D.K., Basrur, V., Appella, E., Wang, Q.-H., Huang, J., Hu, N., Taylor, P., and Petricoin, E.F. 2000. An approach to proteomic analysis of human tumors. *Mol. Carcinog.* 27:158-165.

Emonard, H. and Grimaud, J.A. 1990. Matrix metalloproteinases. A review. *Cell. Mol. Biol.* 36:131-153.

Esheicher, A., Wohlwend, A., Belin, D., and Vassalli, J.D. 1989. Characterization of the cellular binding site for the urokinase type plasminogen activator. *J. Biol. Chem.* 264:1180-1189.

Farina, K.L., Wyckoff, J.B., Rivera, J., Lee, H., Segall, J.E., Condeelis, J.S., and Jones, J.G. 1998. Cell motility of tumor cells visualized in living intact primary tumors using green fluorescent protein *Cancer Res.* 58:2528-2532.

Fidler, I.J. 1991. New developments in *in vivo* models of neoplasia. *Cancer Metastasis Rev.* 10:191-192.

Fidler, I.J. and Ellis, L.M. 1994. The implication of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79:185-188.

Fidler, I.J. and Hart, I.R. 1982. Biologic diversity in metastatic neoplasms–origins and implications. *Science* 217:998-1003.

Folkman, J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1:27-31.

Folkman, J. and Klagsbrun, M. 1987. Angiogenic factors. *Science* 235:442-447.

Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D., and Birchmeier, W. 1991. E-cadherin mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 113:173-185.

Gallager, H.S. and Martin, J.E. 1969. The study of mammary carcinoma by mammography and whole organ sectioning, early observation. *Cancer* 23:855-873.

Granovsky, M., Fata, J., Pawling, J., Muller, W.J., Khokha, R., and Dennis, J.W. 2000. Suppression of tumor growth and metastasis in Mgat5-deficient mice. *Nat. Med.* 6:306-312.

Guan, R.J., Ford, H.L., Fu, Y., Li, Y., Shaw, L.M., and Pardee, A.B. 2000. Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. *Cancer Res.* 60:749-755.

Guirguis, R., Margulies, I., Taraboletti, G., Schiffmann, E., and Liotta, L.A. 1987. Cytokine induced pseudopodial protrusion is coupled to tumor metastasis. *Nature* 329:261-263.

Hullet, M.D., Freeman, C., Hamdorf, B.J., Baker, R.T., Harris, M.J., and Parish, C.R. 1999. Cloning of mammalian heparinase, an important enzyme in tumor invasion and metastasis. *Nat. Med.* 5:735-736.

Hynes, R.O. 1992. Integrins: Versatility, modulation, and signalling in cell adhesion. *Cell* 69:11-25.

Juliano, R.L. and Haskill, S. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577-585.

Kerbel, R.S., Cornil, I., and Theodorescu, D. 1991. Importance of orthotopic procedures in assessing the effects of transfected genes on human tumor growth and metastasis. *Cancer Metastasis Rev.* 10:201-215.

Koblinski, E.J., Ahram, M., and Sloane, B.F. 2000. Unraveling the role of proteases in cancer. *Clin. Chim. Acta* 291:113-135.

Lawson, R.S., Latter, G., Miller, S.D., Goldstein, D., Naps, M., Burbeck, S., Teng, N.N.H., and Zuckerkandl, E. 1991. Quantitative protein changes in metastatic versus primary epithelial ovarian carcinoma. *Gynecol. Oncol.* 41:22-27.

Levy, A., Cioce, V., Sobel, M.E., Garbisa, S., Grigioni, W.F., Liotta, L.A., and Stetler-Stevenson, W.G. 1991. Increased expression of the 72 kDa type IV collagenase in human colonic adenocarcinoma. *Cancer Res.* 51:439-444.

Liotta, L.A. and Stetler-Stevenson, W.G. 1993. Principles of molecular cell biology of cancer: Cancer metastasis. *In* Cancer: Principles and Practices of Oncology, Fourth edition (V.T. deVita, Jr., S. Hellman, and S.A. Rosenberg, eds.) pp. 134-149. Lippincott, Philadelphia.

Liotta, L.A., Kleinerman, J., and Saidel, G. 1974. Quantitative relationships of intravascular tumor cells: Tumor vessels and pulmonary metastases following tumor implatation. *Cancer Res.* 34:997-1004.

Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M., and Shafie, S. 1980. Metastatic potential correlates with enzymatic degradation of basement membranes. *Nature* 284:67-68.

Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. 1991. Cancer metastasis and angiogenesis; an imbalance of positive and negative regulation. *Cell* 68:327-336.

Mareel, M.M., Behrens, J., Birchmeier, W., De Bruyne, G.K., Vleminckx, K., Hoogewijs, A., Fiers, W.C., and van Roy, F.M. 1991. Down regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cell tumors of nude mice. *Int. J. Cancer* 47:922-928.

Mignatti, P., Tsuboi, R., Robbins, E., and Rifkin, D. 1989. In vitro angiogenesis on the human amniotic membrane: Requirement for basic fibroblast growth factor-induced proteinases. *J. Cell Biol.* 108:671-682.

Monteagudo, C., Merino, M., San Juan, J., Liotta, L.A., and Stetler-Stevenson, W.G. 1990. Immunohistologic distribution of type IV collagenases in normal, benign, and malignant breast tissue. *Am. J. Path.* 136:585-592.

Nabeshima, K., Inoue, T., Shimao, Y., Kataoka, H., and Koono, M. 1999. Cohort migration of carcinoma cells: Differentiated colorectal cells move as coherent cell clusters or sheets. *Histol. Histopathol.* 14:1183-1197.

Nakajima, M., Welch, D., Belloni, P.N., and Nicolson, G.L. 1987. Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. *Cancer Res.* 47:4869-4876.

Nakajima, M., Irimura, T., and Nicolson, G.L. 1988. Heparanase and tumor metastasis. *J. Cell. Biochem.* 36:157-167.

Nam, S.W., Clair, T., Campo, C.K., Lee, H.Y., Liotta, L.A., and Stracke, M.L. 2000. Autotaxin (ATX), a potent tumor motogen, augments invasive and metastatic potential of ras-transformed cells. *Oncogene* 19:241-247.

Natali, P.G., Nictora, M.R., Cavaliere, R., Giannarelli, D., and Bigotti, A. 1991. Tumor progression in human malignant melanoma is associated with changes in α6/β1 laminin receptor. *Int. J. Cancer* 49:168-172.

Nicolson, G.L. 1988. Organ specificity of tumor metastasis: Role of preferential adhesion, invasion, and growth malignant cells at specific secondary sites. *Cancer Metastasis Rev.* 7:143-188.

Nicolson, G.L. 1991. Gene expression, cellular diversification and tumor progression to the metastatic phenotype. *BioEssays* 13:337-342.

Ossowski, L. and Reich, E. 1983. Antibodies to plasminogen activator inhibit human tumor metastasis. *Cell* 35:611-619.

Ostrowski, L.E., Rinch, J., King, P., and Matrisian, L. 1988. Expression pattern of a gene for a secreted metalloproteinase during late stages of tumor progression. *Mol. Carcinog.* 1:13-19.

Parish, C.R., Coombe, D.R., Jakobsen, K.B., and Underwood, P.A. 1987. Evidence that sulphated polysaccharides inhibit tumor metastasis by blocking tumor cell-derived heparanase. *Int. J. Cancer* 40:511-518.

Reich, R., Thompson, E., Iwamoto, Y., Martin, G.R., Deason, J.R., Fuller, G.C., and Miskin, R. 1988. Effects of inhibitors of plasminogen activator, serine proteases, and collagenase IV on the invasion of basement membranes by metastatic cells. *Cancer Res.* 48:3307-3312.

Spratt, J.S., Greenberg, R.A., and Heuser, L.S. 1986. Geometry, growth rates, and duration of cancer and carcinoma *in situ* of the breast before detection by screening. *Cancer Res.* 46:970-974.

Stossel, T.P. 1993. On the crawling of animal cells. *Science* 260:1086-1094.

Strausberg, R.L., Dahl, C.A., and Klausner, R.D. 1997. New opportunities for uncovering the molecular basis of cancer. *Nat. Genet.* 15:415-416.

Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251:1451-1455.

Ura, H., Bonfil, R.D., Reich, R., Reddel, R., Pfeifer, A., Harris, C.C., and Klein-Szanto, A.J. 1989. Expression of type IV collagenase and procollagen genes and its correlation with tumorigenic, invasive, and metastatic abilities of oncogene transformed human bronchial cells. *Cancer Res.* 49:4615-4621.

Vleminckx, K., Vakaet, L. Jr., Fiers, W., and Van Roy, F.M. 1991. Genetic manipulation of E-cad-

herin by epithelial tumor cells reveals an invasion suppressor role. *Cell* 66:107-119.

Vlodavsky, I., Mohsen, M., Lider, O., Svahn, C.M., Ekre, H.P., Vigoda, M., Ishai-Michaeli, R., and Peretz, T. 1995. Inhibition of tumor metastasis by heparanase inhibiting species of heparin. *Invasion Metastasis* 14:290-302.

Vlodavsky, I., Friedmann, Y., Elkin, M., Aingorn, H., Atzmon, R., Ishai-Michaeli, R., Bitan, M., Pappo, O., Peretz, T., Miichal, I., Spector, L., and Pecker, I. 1999. Mammalian heparanase: Gene cloning, expression and function in tumor progression and metastasis. *Nat. Med.* 5:793-802.

You, J., Aznavoorian, S., Liotta, L.A., and Dong, C. 1996. Responses of tumor cell pseudopod protrusion to changes in medium osmolality *J. Cell Physiol.* 167:156-163.

Zhuang, Z., Merino, M., Chuaqui, R., Liotta, L.A., and Emmert-Buck, M. 1995. Identical allelic loss on chromosome 11q13 in microdissected *in situ* and invasive human breast cancer. *Cancer Res.* 55:467-471.

Zuber, J., Tchernitsa, O.I., Hinzmann, B., Schmitz, A.C., Grips, M., Hellriegel, M., Sers, C., Rosenthal, A., and Schafer, R. 2000. A genome-wide survey of RAS transformation targets. *Nature Genet*. 24:144-152.

Contributed by Cloud P. Paweletz,
Lu Charboneau, and Lance A. Liotta
National Cancer Institute, NIH
Bethesda, Maryland

Tail Vein Assay of Cancer Metastasis

The most damaging change during cancer progression is the switch from a locally growing tumor to a metastatic killer. This switch involves numerous alterations that allow tumor cells to complete the complex series of events needed for metastasis (*UNIT 19.1*). In considering steps required for successful metastasis, extravasation from blood vessels in target organs is regarded as a critical process. Circulating tumor cells arrested in the capillary beds of different organs must invade the endothelial cell lining of blood vessels and degrade its underlying basement membrane in order to escape into the extravascular tissue where they establish metastasis. This unit describes the most common assay applied to evaluate the metastatic potential of blood-borne tumor cells. The protocol is often called "experimental metastasis", distinct from "spontaneous metastasis", where the tumor cells are first allowed to form a primary tumor in the site of injection and then escape into lymphatic or blood circulation. The Basic Protocol is to apply intravenous injection as a method for introducing tumor cells into circulation. For this purpose, cells maintained in tissue culture are dissociated into a single cell suspension, counted, and injected into the tail vein of mice or rats. After 12 to 20 days, the animals are euthanized and their lungs are removed, rinsed, and fixed. The number of pulmonary tumor colonies is then counted with the aid of a dissecting microscope. When an organ containing extensive secondary foci is seen, it is also removed and fixed for subsequent enumeration of tumor foci.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 7% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain a pH 7.4.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Cells of interest growing in tissue culture (e.g., B16 mouse melanoma, ATCC#CRL-1619; 3LL Lewis lung carcinoma; A375 human melanoma, ATCC#CRL-6322; 13762 MAT rat mammary adenocarcinoma)
Supplemented high-glucose DMEM-10 (*UNIT 10.4*)
Trypsin/EDTA solution (*UNIT 10.4*)
CMF-DPBS: divalent cation-free Dulbecco's PBS (CMF-DPBS; Life Technologies; *APPENDIX 2A*)
Mice for injection (or rats)
70% ethanol
3.8% (v/v) formaldehyde
Bouin's solution (see recipe; optional)

6-cm tissue culture dishes
15-ml polypropylene tubes
Sorvall RT6000D centrifuge and H-1000B rotor or equivalent
Beaker containing warm water (~50° to 60°C)
Lamp with 150-W light bulb (optional)
Restraining device (restrainer)
1-ml syringe, tuberculin type
27-G, ¾-in. needles
Dissecting microscope

Contributed by Michael Elkin and Israel Vlodavsky
Current Protocols in Cell Biology (2001) 19.2.1-19.2.7

Additional reagents and equipment for counting cells with hemacytometer (*UNIT 1.1*)

Prepare cells

1. Culture a sufficient number of cells in supplemented high-glucose DMEM-10 in 6-cm tissue culture dishes in a humidified 37°C, 7% CO_2 incubator.

   Other size dishes (i.e., 35-mm, 10-cm) can be used.

2. Aspirate medium from subconfluent cultures (0.5–1 $\times 10^5$ cells/cm^2), add 2 ml trypsin/EDTA solution per 6-cm tissue culture dish, and incubate 2 to 3 min at 37°C, or until the cells have rounded up, but are not detached.

3. Aspirate trypsin/EDTA solution, resuspend cells in 5 ml warm supplemented high-glucose DMEM-10, transfer into a 15-ml polypropylene tube, centrifuge 7 min at ~350 ×*g* (1000 rpm Sorvall RT6000D centrifuge, H-1000B rotor, or equivalent), room temperature, and resuspend in 5 ml warm CMF-DPBS.

   Trypsin/EDTA solution is commonly used to detach adherent cells. Its action is terminated by resuspending the cells in supplemented high-glucose DMEM-10. Prolonged (and unnecessary) trypsinization of tumor cells can alter their survival and metastatic behavior in vivo. Trypsinization >3 min may affect lung colony formation and should be avoided.

   Serum-free DMEM can be used instead of CMF-DPBS.

4. Count cells on a hemacytometer (*UNIT 1.1*), and prepare working cell suspension at 2.5 $\times 10^5$ to 1 $\times 10^6$ cells/ml, depending on the metastatic potential of the cells (e.g., 2.5 $\times 10^5$ B16F10 or A375SM cells/ml; 1 $\times 10^6$ A375 cells/ml).

   It is important to guard against clumping or aggregation of cells. The number of lung colonies is affected not only by the number of tumor cells injected, but also by the aggregation of cells and the tumor embolus size. To avoid clumping of cells, the cell preparations must be free of serum, and cells should be injected in a divalent cation- free balanced salt solution, which also serves to decrease clumping.

   Obviously, the trypsinization step is not applied when nonadherent cells (e.g., mouse Eb T-lymphoma) are tested. Cells growing in suspension are washed in CMF-DPBS, or serum-free medium, counted, and suspended at the desired cell density.

Intravenously inject tumor cells

5. Dilate the lateral tail veins by immersing the tail for 1 to 2 min in warm (50° to 60°C) water.

   The tail vein is dilated to facilitate the insertion of a 26- or 27-G needle. Alternatively, animals housed in a well-ventilated cage are placed for ~5 min under a lamp with a 150-W light bulb. The distance of the lamp from the cage can vary, but 4 to 8 in. is sufficient.

6. Place the mouse in a restrainer by grasping the mouse by the tail out of the cage and placing with tail protruding through the opening in the wall of a restraining device.

7. Fill 1-ml syringe (tuberculin type) with the cell suspension and remove air bubbles.

8. Hold the distal third part of the tail between thumb and middle finger, wipe clean with 70% ethanol, and rotate to position the lateral vein. Stabilize the vein with the index finger. Apply slight pressure to straighten the tail and further dilate the lateral vein.

9. Hold the 1-ml syringe with a 27-G, ¾-in. needle in the other hand. Direct the bevel of the needle upward. Force the needle gently through the skin (at a slight angle) and then immediately thread it almost parallel into the vein. When the vein is canulated, advance the needle into the lumen an additional 5 mm and slowly inject 0.2 to 0.3 ml of the cell suspension.

No significant pressure should be applied at this stage. If the needle is not in the vein, bleb formation and tissue resistance to the inoculum can easily be felt, indicating failure to canulate the vein. Perform the first injection at the distal third of the tail. In case of failure, additional attempts may be made proximally in the same or opposite lateral vein. Intravenous injection of anaesthetized mice, or of mice that struggle violently should be avoided.

Tail vein injection is delicate and requires pretraining, usually, 20 to 30 mice may suffice. Locating the lateral vein is relatively difficult with C57BL mice compared to Balb/c mice.

Enumerate pulmonary tumor colonies

10a. After 2 to 3 weeks post-injection, euthanize mice (or rats) by CO_2 asphyxiation or cervical dislocation (or other approved method), remove the lungs, rinse in tap water, fix in 3.8% formaldehyde, and count colonies with the aid of a dissecting microscope.

An illuminated colony–counting magnifying lens can be used instead of a dissecting microscope.

When an organ containing extensive secondary foci is observed, it is also removed, rinsed, and fixed for subsequent enumeration of tumor foci. Most pulmonary tumor colonies in mice are found near or on the surface of the lungs. Therefore, pigmented colonies that differ in color from the lung parenchyma (such as melanoma) can be counted when adequately magnified.

Tumor colonies that are not pigmented and do not differ in color from the pulmonary parenchyma are more difficult to enumerate. To induce contrast between tumor colonies and the lung parenchyma perform step 10b.

10b. *Optional:* Remove lungs (or other organs) and rinse in tap water. A few minutes later, place the lungs in a beaker containing Bouin's solution. After 24 hr, rinse the lungs in water to remove excess Bouin's solution and count the tumor colonies with the aid of a dissecting microscope.

The lung parenchyma will immediately turn yellow. After 24 hr, the white tumor colonies can readily be distinguished from the yellow lung parenchyma.

11. Specify the actual number of pulmonary colonies counted for each mouse, or show the range of counts by indicating the lowest and highest numbers of colonies observed within a given experimental group. Present the average number of nodules per mouse ± standard error or standard deviation, using the two-tailed student's *t*-test. Murine lungs with >300 colonies are reported as such.

Instead of actual counting of pulmonary colonies, one can remove and weigh the lungs of each mouse and express the results as average lung weight ± standard deviation. Normal lung weight is ~200 mg.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Bouin's solution

0.9% (v/v) picric acid
9% (v/v) formaldehyde
5% (v/v) acetic acid
Store up to 1 year at room temperature

COMMENTARY

Background Information

Metastasis is the foremost cause of death from cancer. Fortunately, metastasis is an extremely inefficient process, with few of the many cells shed from a primary tumor successfully forming secondary tumors (Hart and Fidler, 1980; Liotta, 1986; Fidler and Ellis, 1994; Ruoslahti, 1996). Sequential steps in the metastatic process are as follows: escape of cells from the primary tumor, entry of cells into lymphatic or blood circulation (intravasation), survival and transport in circulation, escape of cells from circulation (extravasation), and growth of cells to form secondary tumors in a new organ environment (Hart and Fidler, 1980; Liotta, 1986; Fidler and Ellis, 1994; Ruoslahti, 1996). The majority of cells that successfully escape from a primary tumor will not complete all of the steps necessary to give rise to metastatic tumors. Steps that have been considered to be major contributors to this inefficiency, and thus, rate limiting for metastasis, include cell survival in, and escape from, circulation (Hart and Fidler, 1980; Liotta, 1986; Fidler and Ellis, 1994; Ruoslahti, 1996). Once in circulation, the tumor cells often attach at or near junctions between adjacent endothelial cells, followed by retraction of the endothelial cells borders, and migration toward the exposed underlying basement membrane (BM). The invading tumor cells then degrade the subendothelial BM in order to migrate out of the vascular compartment. These steps are collectively addressed by the tail vein assay, commonly applied in vivo as a route for introducing cells into blood circulation (Fidler, 1973a, b).

Experimental metastasis, measured by the tail vein assay, is inhibited by molecules (i.e., RGD-containing peptides, laminin-derived peptides, sulfated polysaccharides) and antibodies which inhibit cell-cell and cell-matrix interactions (Humphries et al., 1986; Iwamoto et al., 1987). Because basement membranes underlying epithelial and endothelial cells appear to be clear barriers to metastasis, enzymes degrading BM proteins and glycosaminoglycans are regarded as promising targets for cancer therapy (Stetler-Stevenson et al., 1993; Finkel, 1999; Vlodavsky et al., 1999). Invasion through a reconstituted basement membrane (Matrigel; *UNIT 10.2*) is most often used to model extravasation (*UNIT 12.2*). Cell invasiveness in vitro correlates with the metastatic potential (i.e., lung colonization) of intravenously injected cells. Moreover, inhibitors of ECM degrading enzymes inhibit both BM invasion in vitro and experimental metastasis in vivo, further emphasizing the significance of the BM as a prominent physical barrier to metastasis (Parish et al., 1987; Stetler-Stevenson et al., 1993; Vlodavsky et al., 1994). Some of these molecules are being examined in cancer patients (Kohn and Liotta, 1995). Taking into account the complexity of the metastatic process, it is now understood that the mechanistic conclusions on the significance of a certain step and/or action of a given molecule cannot be drawn based on the endpoint outcome of the tail vein assay (i.e., number of metastases counted at the end of the experiment; Chambers and Matrisian, 1997). A procedure, intravital videomicroscopy (IVVM), for direct in vivo observation of early steps in metastasis, has recently provided evidence that some of the assumptions about mechanisms of metastasis need to be revised (Chambers and Matrisian, 1997; Al-Mehdi et al., 2000; Cameron et al., 2000). Unlike early studies, direct IVVM observation of intravenously injected cancer cells revealed that a large proportion of the injected cells not only survive injection and arrest in a target organ, but also succeed in extravasation (Chambers and Matrisian, 1997; Al-Mehdi et al., 2000; Cameron et al., 2000). It was suggested that, unlike previous assumptions, extravasation may be a relatively easy process and that metastatic inefficiency depends, to a large extent, on the inhibition of growth in a subset of extravasated cells (Cameron et al., 2000). Availability of a favorable microenvironment in the target organ is critical for seeding and proliferation of the disseminated cells ("seed and soil" theory; Killion et al., 1998).

Previous research has concentrated on the contribution of individual genes to metastasis. Initially, there was hope that a single metastasis-specific gene could be identified to be responsible for conversion to a metastatic phenotype. Although some cells could be converted to a metastatic phenotype by DNA transfection of certain genes, including those encoding ECM-degrading enzymes (Stetler-Stevenson et al., 1993; Vlodavsky et al., 1999), there is no single master "metastasis" gene that regulates cellular abilities necessary for cancer metastasis (Chambers and Matrisian, 1997). Gene-expression profiling, using high-density DNA microassays combined with the tail vein assay, led to the identification of several genes involved in ECM assembly and in regulation of the

actin-based cytoskeleton that are selectively upregulated in metastatic mouse and human melanoma cells (Clark et al., 2000). A particular significance was ascribed to RhoC, which may regulate metastasis by controlling cytoskeletal events essential for cell motility in response to extracellular factors (Clark et al., 2000).

Critical Parameters and Troubleshooting

General considerations for metastasis. The pathogenesis of metastasis begins with the invasion of tissues, blood vessels, and/or lymphatics by cells originating from a primary cancer. Following their release into circulation, most tumor emboli are initially arrested in the lung tissue, simply because the lung capillary bed is the first to be encountered (Fidler, 1973a; Hart and Fidler, 1980), although some cells recirculate and are trapped in other organs. It is because of this that the majority of intravenously injected cells extravasate and form metastatic colonies in the lungs, as in fact evaluated by the tail vein assay described in this unit. It should be noted that this assay, also known as "experimental metastasis", is restricted to evaluate the metastatic potential of circulating blood-borne cells. In contrast, the more complete cascade can be studied using the "spontaneous metastasis" assay in which the tumor cells are injected subcutaneously to first form a primary tumor. The tumor is either allowed to grow (Vlodavsky et al., 1999), or is resected when it reaches a certain size (Fitzer-Attas et al., 1997). The latter protocol better mimics the situation in patients who undergo resection of their primary tumor, a procedure which is often associated with stimulated vascularization and growth of already spread metastatic nodules (O'Reilly et al., 1994). Briefly, for measurements of spontaneous metastasis, mice are most often inoculated in the footpad with 2×10^5 cells per mouse. Local tumors are measured with calipers. When individual tumors reach a diameter of 8 mm, tumor-bearing legs are amputated (Fitzer-Attas et al., 1997). Two to 3 weeks post-amputation, the mice are euthanized and the number of pulmonary colonies or lung weights are determined as described for intravenously injected tumor cells (Fitzer-Attas et al., 1997).

Single cell suspension. A major problem likely to be encountered with the tail vein assay is the broad variation in the number of pulmonary colonies obtained among mice of the same group. In some experiments, these may vary between 10 and 100 nodules per mouse. This variation may primarily be due to inappropriate standardization of the cell suspension. Every effort should be made to obtain a single-cell suspension since cell clumping and aggregation often result in an increased number of lung colonies (Fidler, 1973b). Clearly, cell aggregation and clump (embolus) size influence the outcome of experimental metastasis assays. For example, mice injected with 10,000 clumps of 4 to 5 cells will develop more pulmonary foci than mice injected with 50,000 single tumor cells (Fidler, 1973b). The significance of cell aggregation is also demonstrated by the greatly increased number of tumor foci obtained when the tumor cells are injected simultaneously with lethally irradiated tumor cells, or with live syngeneic embryonic cells (Fidler, 1973b; Hart and Fidler, 1980). To avoid clumping, trypsinization and centrifugation of the cultured cells should be gentle and the dissociated cells should be suspended in serum-free, Ca^{2+}/Mg^{2+}-free balanced salt solution. Routine viability tests (e.g., trypan blue exclusion) and even-plating efficiency in vitro do not necessarily predict or correlate with the in vivo metastatic potential of over-trypsinized cells (Fidler, 1973b). Also, the 15-ml tube, as well as the syringe containing the cell suspension should be gently rotated from time to time and not be left standing for long periods of time (i.e., >30 min) to prevent the cells from settling and aggregating at the bottom of the tube or in the syringe. The cells should be pipetted gently prior to being taken up into the syringe for injection. Failure to obtain a single-cell suspension will result in a diverse and broad range of colony number among mice of the same experimental group, and in differences in colony size in the same mouse.

Cultures. An important parameter is the health of the cells. Cultures should be actively growing, but should be passaged at least 48 hr prior to the day of the tail vein assay. Also, the use of early passaged cultures (<20) is recommended. Relatively poor metastatic potential is often observed with late-passage cells. If the cells tend to loose their metastatic ability, it is advisable to go back and select for highly metastatic cells by means of a repeated isolation of pulmonary metastases and subsequent growth in culture, according to the scheme first described by Fidler (1973a) and applied by many other groups (Clark et al., 2000). Briefly, to select for highly metastatic tumor cells, pulmonary metastatic nodules are removed aseptically, minced, grown in vitro, and re-injected

intravenously into the tail vein of host mice. This procedure is repeated 2 to 5 times.

Anticipated Results

Using highly metastatic cell lines (e.g., B16-F10 or B16-BL6 mouse melanoma, A375SM human melanoma, 13762 MAT rat mammary carcinoma, D122 3LL Lewis lung carcinoma; Fidler, 1973a; Parish et al., 1987; Zhang et al., 1991; Vlodavsky et al., 1994; Fitzer-Attas et al., 1997; Clark et al., 2000), it is anticipated to obtain 200 to 300 pulmonary metastases per mouse (or rat) injected with 5×10^4 cells. The actual number of pulmonary colonies may range from ~80 to 350 in different mice of the same group. The lung weight of a C57BL mouse injected with 5×10^4 D122 3LL cells is expected to be ~800 ±140 mg versus a normal lung weight of ~200 mg (Fitzer-Attas et al., 1997). The effect of compounds that inhibit cell adhesion (mediated by selectins and integrin receptors, for example), or certain degradative enzymes (i.e., matrix metalloproteinases, plasminogen activator, heparanase) can be easily evaluated using the tail vein assay. The inhibitory molecule can be added to the cell suspension prior to the intravenous injection, or administered separately, subcutaneously or intraperitoneally. Generally, the test compound can be injected up to 12 hr prior to the cells, depending on its half-life time, but no later than 30 min following the tail vein injection of the cells. Effective compounds may decrease the number of visible pulmonary metastases by ~95% (Parish et al., 1987; Vlodavsky et al., 1994).

An example is presented in figure 19.2.1, showing a profound inhibition of experimental metastasis in mice treated with a chemically modified, non-anti-coagulant fragment of heparin (Vlodavsky et al., 1994). This effect is attributed primarily to efficient inhibition of heparanase activity (Vlodavsky et al., 1994). The heparanase enzyme has been shown to promote tumor angiogenesis and metastasis (Vlodavsky and Friedmann, 2001).

Time Considerations

Cell harvesting and tail vein injection require 2 to 4 hr, depending on the number of mice and treatment groups. The mice are generally euthanized 14 to 18 days after injection, unless injected with very low metastatic cells (i.e., A375 melanoma) where up to 8 weeks after injection may be required (Clark et al., 2000). The actual removal of lungs and enumeration of pulmonary colonies can be carried out in 1 day for a typical experiment of 4 to 5 groups, 6 to 8 mice each. In the case of tumor colonies that are not pigmented, it is recommended that lungs be first incubated in Bouin's solution for 24 hr.

Literature Cited

Al-Mehdi, A.B., Tozawa, K., Fisher, A.B., Shientag, L., Lee, A., and Muschel, R.J. 2000. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: A new model for metastasis. *Nat. Med.* 6:100-102.

Cameron, M.D., Schmidt, E.E., Kerkvliet, N., Nadkarni, K.V., Morris, V.L., Groom, A.C., Chambers, A.F., and MacDonald, I.C. 2000. Temporal progression of metastasis in lung: Cell survival, dormancy, and location dependence of metastatic inefficiency. *Cancer Res.* 60:2541-2546.

Chambers, A.F. and Matrisian, L.M. 1997. Changing views of the role of matrix metalloproteinases in metastasis. *J. Natl. Cancer Inst.* 89:1260-1270.

Clark, E.A., Golub, T.R., Lander, E.S., and Hynes, R.O. 2000. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* 406:532-535.

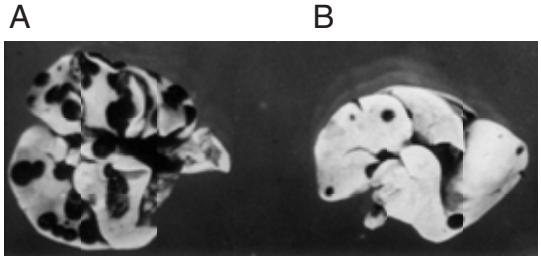


Figure 19.2.1 Lung colonization of B16-BL6 melanoma cells. B16-BL6 melanoma cells were injected into the tail vein of C57BL mice. The mice received a single subcutaneous injection of saline (**A**) or *N*-acetylated heparin fragment, 200 μg per mouse (**B**), 30 min prior to the cells. After 14 days, the mice were euthanized and evaluated for lung colonization (dark spots).

Fidler, I.J. 1973a. Selection of successive tumour lines for metastasis. *Nature New Biol.* 242:148-149.

Fidler, I.J. 1973b. The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. *Eur. J. Cancer* 9:223-227.

Fidler, I.J. and Ellis, L.M. 1994. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79:185-188.

Finkel, E. 1999. Potential target found for antimetastasis drugs. *Science* 285:33-34.

Fitzer-Attas, C.J., Do, M.S., Feigelson, S., Vadai, E., Feldman, M., and Eisenbach, L. 1997. Modification of PDGFalpha receptor expression or function alters the metastatic phenotype of 3LL cells. *Oncogene* 15:1545-1554.

Hart, I.R. and Fidler, I.J. 1980. Cancer invasion and metastasis. *Q. Rev. Biol.* 55:121-142.

Humphries, M.J., Olden, K., and Yamada, K.M. 1986. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science* 233:467-470.

Iwamoto, Y., Robey, F.A., Graf, J., Sasaki, M., Kleinman, H.K., Yamada, Y., and Martin, G.R. 1987. YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation. *Science* 238:1132-1134.

Killion, J.J., Radinsky, R., and Fidler, I.J. 1998. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Metastasis Rev.* 17:279-284.

Kohn, E.C. and Liotta, L.A. 1995. Molecular insights into cancer invasion: Strategies for prevention and intervention. *Cancer Res.* 55:1856-1862.

Liotta, L.A. 1986. Tumor invasion and metastases—role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res.* 46:1-7.

O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H., and Folkman, J. 1994. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79:315-328.

Parish, C.R., Coombe, D.R., Jakobsen, K.B., Bennett, F.A., and Underwood, P.A. 1987. Evidence that sulphated polysaccharides inhibit tumour metastasis by blocking tumour-cell-derived heparanases. *Int. J. Cancer* 40:511-518.

Ruoslahti, E. 1996. How cancer spreads. *Sci. Am.* 275:72-77.

Stetler-Stevenson, W.G., Liotta, L.A., and Kleiner, D.E., Jr. 1993. Extracellular matrix 6: Role of matrix metalloproteinases in tumor invasion and metastasis. *FASEB J.* 7:1434-1441.

Vlodavsky, I. and Friedmann, Y. 2001. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. *J. Clin. Invest.* 108:341-347.

Vlodavsky, I., Mohsen, M., Lider, O., Svahn, C.M., Ekre, H.P., Vigoda, M., Ishai-Michaeli, R., and Peretz, T. 1994. Inhibition of tumor metastasis by heparanase inhibiting species of heparin. *Invasion Metastasis* 14:290-302.

Vlodavsky, I., Friedmann, Y., Elkin, M., Aingorn, H., Atzmon, R., Ishai-Michaeli, R., Bitan, M., Pappo, O., Peretz, T., Michal, I., Spector, L., and Pecker, I. 1999. Mammalian heparanase: Gene cloning, expression and function in tumor progression and metastasis. *Nat. Med.* 5:793-802.

Zhang, R.D., Price, J.E., Schackert, G., Itoh, K., and Fidler, I.J. 1991. Malignant potential of cells isolated from lymph node or brain metastases of melanoma patients and implications for prognosis. *Cancer Res.* 51:2029-2035.

Contributed by Michael Elkin and
Israel Vlodavsky
Hadassah-Hebrew University Hospital
Jerusalem, Israel

Microanalysis of Gene Expression in Tissues Using T7-SAGE: Serial Analysis of Gene Expression After High-Fidelity T7-Based RNA Amplification

Comprehensive gene-expression data obtained from individual cell types in tissues and organs will provide novel insights into the fundamental molecular biology of embryonic development, disease, and other cell biological processes. There are presently two major types of gene expression profiling techniques, microarray analyses and serial analysis of gene expression (SAGE). SAGE has an advantage over microarray analysis in that it provides absolute transcript numbers in a digital format, and can readily identify previously unknown genes (Velculescu et al., 1995, 1997; Ishii et al., 2000). Microarrays have the advantages of being relatively easy to use and more suitable for high-throughput applications; however, mRNA quantitation is more accurate with SAGE than with microarrays (Polyak and Riggins, 2001).

A major disadvantage of SAGE is that it requires microgram quantities of starting poly(A)$^+$ mRNA, which prevents its use when mRNA is limited. Several techniques have been developed to overcome this limitation, but they mostly depend on amplification using the polymerase chain reaction (PCR). SAGE-Lite (Peters et al., 1999) and PCR-SAGE (Neilson et al., 2000) convert poly(A)$^+$ mRNA to cDNA, which is then amplified by PCR. Alternatively, MicroSAGE (Datson et al., 1999) and SAGE adaptation for downsized extracts (SADE; Virlon et al., 1999) depend on PCR amplification of SAGE ditags. Although the recently reported MicroSAGE protocol does not require a reamplification step (see *http://www.sagenet.org*), it is optimized for between 5×10^4 and 2×10^6 cells (St. Croix et al., 2000), and extra PCR amplifications would be necessary for fewer cells, such as when using laser-capture-microdissected tissue sections (Emmert-Buck et al., 1996).

Although PCR is a powerful method for amplifying rare DNA species from tissue samples, the widely used *Taq* DNA polymerase has relatively low fidelity, and it propagates errors through subsequent cycles of amplification (Van Gelder et al., 1990). The error rate of 1×10^{-3} for *Taq* polymerase (1 mismatch per 1000 bases synthesized) will result, on average, in several erroneous bases in most PCR-amplified cDNAs (Eberwine et al., 1992). Even though SAGE ditags are of approximately equal length for ditag amplification by PCR during SAGE, preferential amplification of some ditags cannot be completely prevented (Datson et al., 1999).

This unit begins with a procedure for T7-based RNA amplification (see Basic Protocol 1). In order to avoid extra PCR or other forms of amplification, the authors incorporate only two cycles of T7-based RNA amplification as the initial step in Basic Protocol 1. This T7-based amplification step has high accuracy, since a combined error rate of 2×10^{-4} has been calculated for avian myeloblastosis virus (AMV) reverse transcriptase and T7 RNA polymerase (Sooknanan et al., 1994). In addition, T7 RNA polymerase has high processivity (Muller et al., 1988) and functions effectively even when broad stretches of nucleotides are being amplified—unlike *Taq* polymerase, which works most efficiently for amplification of small regions of a few hundred nucleotides. Although no protocol that includes an amplification step can claim to permit determination of absolute transcript number, since slight changes in estimated transcript frequency are always possible, T7 procedures appear to be the safest to date (Wang et al., 2000; Bashiardes and Lovett,

Contributed by Takayoshi Sakai, Melinda Larsen, and Kenneth M. Yamada
Current Protocols in Cell Biology (2002) 19.3.1-19.3.30

2001). A procedure for T7-like amplification of tiny amounts of mRNA using the RiboAmp kit from Arcturus Engineering is also included (see Alternate Protocol).

In this unit, the authors describe a new technique, T7-SAGE (see Basic Protocol 2), in which a high-fidelity T7 amplification step is combined with SAGE analysis. This new T7-SAGE procedure should facilitate application of SAGE for gene-expression profiling using minimal quantities of starting material, such as from embryonic tissues and microdissected cells from histological sections of tissues.

Support Protocols are also provided for: preparation of total RNA (see Support Protocol 1); a phenol/chloroform procedure using Phase Lock Gel Light which is suitable for the numerous DNA extractions in T7-SAGE (see Support Protocol 2); kinasing to generate the linkers used in T7-SAGE (see Support Protocol 3); a gel-shift test (see Support Protocol 4) for completeness of biotinylation, used to evaluate the biotinylated oligo(dT) primer with which the biotinylated cDNA is synthesized in Basic Protocol 1; preparation of gels for the numerous PAGE procedures involved in T7-SAGE (see Support Protocol 5); and direct sequencing of PCR products from a SAGE library (see Support Protocol 6).

NOTE: Investigators should first become familiar with the underlying strategies of SAGE *(UNIT 19.4)* and then practice ahead of time (ideally several times) using larger amounts of starting material before performing the final T7-SAGE procedure with precious samples. Researchers are advised to access the SAGE home page on the internet at *http://www.sagenet.org/* organized by Kenneth Kinzler and co-workers at the Molecular Genetics Laboratory of the Johns Hopkins Oncology Center at Johns Hopkins University, which describes SAGE concepts, new SAGE applications, and recent publications.

NOTE: The quality of the data obtained from T7-SAGE libraries depends on the quality of the RNA and DNA used in its many steps. Consequently, DEPC-treated water should be used for all steps involving RNA. DNase-free water (e.g., obtained from a commercial source) is also recommended.

BASIC PROTOCOL 1

T7-BASED RNA AMPLIFICATION

This protocol describes how to perform high-fidelity T7-based RNA amplification, starting with only small amounts of total RNA, for use in SAGE procedure (Velculescu et al., 1995, 1997). This method can be used with tissue culture cell lines or with homogenates from animal tissues or organs. The protocol is a general method applicable to total RNA isolated in various ways in quantities as small as 4 ng. The Alternate Protocol is specifically designed for laser-microdissected histological samples, where quantities are even lower.

CAUTION: Investigators should wear gloves for all procedures involving RNA and take precautions in order to avoid cross-contamination of samples. See *APPENDIX 2A* for general guidelines when working with RNA. DEPC-treated water (*APPENDIX 2A*) must be used for steps 1 to 41.

Materials

4 to 5 ng purified total RNA from tissues or cells in a volume of 10.5 µl (see Support Protocol 1)

0.5 mg/ml T7-oligo(dT) primer:
5′-TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGT$_{21}$-3′
(custom-synthesized, e.g., by Integrated DNA Technologies or core facility)

Superscript Choice System for cDNA Synthesis kit (Invitrogen), including:
  5× first-strand reaction buffer
  0.1 M dithiothreitol (DTT)
  10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
  SuperScript II reverse transcriptase
  40 U/µl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen)
  5× second-strand reaction buffer
  10 U/µl DNA polymerase I
60 U/µl RNase H (PanVera)
60 U/µl DNA ligase (PanVera)
DEPC-treated H_2O (Quality Biological or other supplier; also see *APPENDIX 2A*)
8 U/µl T4 DNA polymerase (Promega)
QIAquick PCR Purification Kit (Qiagen)
Ampliscribe T7 High Yield Transcription Kit (Epicentre Technologies), including:
  Ampliscribe T7 reaction buffer
  100 mM ATP
  100 mM CTP
  100 mM GTP
  100 mM UTP
  0.1 M dithiothreitol (DTT)
  T7 RNA polymerase solution
10 U/µl DNase I (Boehringer Mannheim)
RNeasy Mini Kit (Qiagen)
1 mg/ml random primers (Invitrogen)
0.5 mg/ml biotinylated oligo(dT) primer: 5′- [biotin]T_{18} -3′ (custom-synthesized, e.g., by Integrated DNA Technologies or core facility), gel-purified; test for complete biotinylation before use by gel-shift test (see Support Protocol 4)

16°, 42°, 70°, and 95°C water baths
GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech) or any other spectrophotometer for determining UV absorbance of small samples (e.g., 20 to 50 µl)

Perform reverse transcription (RT) of RNA

1. Mix 4 to 5 ng purified RNA (in 10.5 µl; see Support Protocol 1) in a 1.5- to 1.7-ml microcentrifuge tube with 1 µl of 0.5 mg/ml T7-oligo(dT) primer to initiate first-strand synthesis.

   In general, small samples should be mixed by vortexing for 5 sec, then momentarily microcentrifuged at maximum speed (accelerate to maximal speed of 13,000 to 15,000 rpm, then immediately brake) to collect the sample at the bottom of the tube.

2. Incubate the primer and RNA at 70°C for 10 min, then incubate at 42°C for 5 min.

3. Add the following reagents to the microcentrifuge tube and mix:

   4 µl 5× first-strand reaction buffer
   2 µl 0.1 M DTT
   1 µl 10 mM dNTP mix
   0.5 µl 40 U/µl RNaseOUT RNase inhibitor
   1 µl SuperScript II reverse transcriptase.

4. Incubate at 42°C for 1 hr.

5. Add the following reagents to the tube and mix:

   30 µl 5× second-strand reaction buffer
   3 µl 10 mM dNTP mix
   1.5 µl 10 U/µl DNA polymerase I
   0.25 µl 60 U/µl RNase H
   0.25 µl 60 U/µl DNA ligase
   95 µl DEPC-treated H_2O.

6. Incubate at 16°C for 2 hr.

7. Add 2.5 µl of 8 U/µl T4 DNA polymerase and incubate for an additional 10 min at 16°C.

8. Purify the double-stranded (ds)-cDNA with a QIAquick PCR purification kit according to the manufacturer's instructions.

9. After collection from the column, vacuum dry the ds-cDNA to ~5 µl, then adjust the volume to 8 µl with DEPC-treated H_2O using a micropipettor, in preparation for in vitro transcription.

   Samples should not be evaporated to complete dryness, which reduces the efficiency of recovery. Instead, try to reduce the volume to ~5 µl, periodically checking the progress of volume reduction. Volumes can be determined using an adjustable 10-µl micropipettor: draw the sample up into a micropipet tip, then adjust the dial that sets the volume of the micropipettor until there is a precise match to the volume of the sample (i.e., after drawing up, no extra volume is left in the tube and no air is left at the bottom of the tip). Once the volume is reduced to <8 µl, the difference in volume to achieve the final 8 µl can be calculated and added using DEPC-treated water.

Amplify antisense RNA (aRNA) using T7 RNA polymerase

10. Mix the 8 µl ds-cDNA (from step 9) with the following reagents in the tube:

    2 µl 10× Ampliscribe T7 reaction buffer
    1.5 µl each of 100 mM ATP, CTP, GTP, and UTP
    2 µl 0.1 M DTT
    2 µl T7 RNA polymerase solution.

    All of the above reagents are included with the Ampliscribe T7 High-Yield Transcription kit from Epicentre Technologies.

11. Incubate at 42°C for 3 hr.

12. Add 1 µl of 10 U/µl DNase I, mix, and incubate at 37°C for 15 min.

13. Purify the resulting aRNA using an RNeasy Mini Kit according to the manufacturer's instructions.

14. Dry the aRNA down to ~5 to 10 µl using a vacuum concentrator, then adjust the volume to 10.5 µl using a micropipettor and DEPC-treated water.

Perform a second round of aRNA amplification

15. To the aRNA from the first-round amplification (step 14), add a 1-µl aliquot of 1 mg/ml random primers in a 1.5- to 1.7-ml microcentrifuge tube, and mix.

16. Incubate the mixture at 70°C for 10 min.

17. Chill the mixture on ice, and then allow it to equilibrate at room temperature for 10 min.

18. Add the following reagents (from the SuperScript kit) to the tube and mix:

    4 µl 5× first-strand reaction buffer
    2 µl 0.1 M DTT
    1 µl 10 mM dNTP mix
    0.5 µl 40 U/µl RNaseOUT RNase inhibitor
    1 µl SuperScript II reverse transcriptase.

19. Incubate at room temperature for 5 min and then at 37°C for 1 hr.

20. Add 0.5 µl of 60 U/µl RNase H, mix, and incubate at 37°C for 20 min.

21. Heat the reaction mixture to 95°C for 2 min and then chill it on ice.

22. For second-strand cDNA synthesis, add 1 µl of 0.5 mg/ml T7-oligo(dT) primer, mix, and incubate the mixture at 70°C for 5 min and then at 42°C for 10 min.

23. Add the following reagents to the tube and mix:

    30 µl 5× second-strand reaction buffer
    3 µl 10 mM dNTP mix
    1.5 µl 10 U/µl DNA polymerase I
    0.25 µl 60 U/µl RNase H
    0.25 µl 60 U/µl DNA ligase
    95 µl DEPC-treated H_2O.

24. Incubate the mixture at 16°C for 2 hr.

25. Add 2.5 µl of 8 U/µl T4 DNA polymerase, mix, and incubate at 16°C for 10 min.

26. Purify the ds cDNA with a QIAquick PCR purification kit according to the manufacturer's instructions.

27. Vacuum dry the cDNA to ~5 µl and adjust volume to 8 µl with DEPC-treated water.

    See annotation to step 9, above, for details of this procedure.

28. Using the same tube, add the following reagents to the 8 µl of ds cDNA (from step 27) and mix:

    2 µl 10× Ampliscribe T7 reaction buffer
    1.5 µl each of 100 mM ATP, CTP, GTP, and UTP
    2 µl 0.1 M DTT
    2 µl T7 RNA polymerase solution.

29. Incubate at 42°C for 3 hr.

30. Add 1 µl DNase I, mix, and incubate at 37°C for 15 min.

31. Purify the aRNA using an RNeasy Mini Kit according to the manufacturer's instructions.

32. Dry the aRNA down to 5 to 10 µl using vacuum concentrator, then adjust volume to 10.5 µl using a micropipettor and DEPC-treated water.

Synthesize biotinylated double-stranded cDNA (bds cDNA)

33. Mix the 10.5 µl aRNA from the second-round RNA amplification (step 32) with 1 µl of 1 mg/ml random primers.

34. Incubate the mixture at 70°C for 10 min, chill it on ice, and then allow it to equilibrate at room temperature for 10 min.

35. Add the following reagents to the tube and mix:

    4 µl 5× first-strand reaction buffer
    2 µl 0.1 M DTT
    1 µl 10 mM dNTP mix
    0.5 µl 40 U/µl RNaseOUT RNase inhibitor
    1 µl SuperScript II reverse transcriptase

36. Incubate at room temperature for 5 min and then at 37°C for 1 hr.

37. Add 0.5 µl of 60 U/µl RNase H, mix, and incubate the mixture at 37°C for 20 min.

38. Heat this reaction mixture to 95°C for 2 min, and then chill it on ice.

39. Add 1 µl of 0.5 mg/ml biotinylated oligo(dT) primer (5′- [biotin]T_{18} -3′) and mix.

    Before use, the biotinylated oligo(dT) primer should be tested for complete biotinylation by the gel-shift test (see Support Protocol 4).

40. Incubate the mixture at 70°C for 5 min and then at 42°C for 10 min.

41. Add the following reagents to the tube and mix:

    30 µl 5× second-strand reaction buffer
    3 µl 10 mM dNTP mix
    1.5 µl 10 U/µl polymerase I
    0.25 µl 60 U/µl DNA ligase
    0.25 µl 60 U/µl RNase H
    93.5 µl DEPC-treated H_2O.

42. Incubate the mixture at 16°C for 2 hr.

43. Purify the bds cDNA using a QIAquick PCR purification kit according to the manufacturer's instructions.

44. Elute with 60 µl of water.

45. Check the concentration of bds cDNA synthesized using a spectrophotometer appropriate for determining UV absorbance of small samples.

    A total of ~1 µg is needed to proceed to the SAGE protocol.

SUPPORT PROTOCOL 1

PREPARATION OF TOTAL RNA

Total RNA from tissues or cells can be prepared by any of the methods listed in Table 19.3.1, following the company's protocol if a commercial kit is used. A final yield of at least 4 ng total RNA in 10.5 µl water is needed. The column methods are generally recommended for isolating RNA from small samples, such as those obtained using laser microdissection of histological sections. DNase treatment, as described in the steps below, is needed to avoid any genomic DNA contamination. The integrity of RNA should be checked by gel electrophoresis, RT-PCR, or Northern blot hybridization.

Materials

Tissues or cells of interest
Kit or reagents for total RNA isolation (Table 19.3.1)
DEPC-treated H_2O (*APPENDIX 2A*; or supplied with isolation kit)
10× DNase I buffer (Ambion)
2 U/µl DNase I (RNase-free; Ambion)
40 U/µl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen)
RNeasy Mini Kit (Qiagen)

Table 19.3.1 Methods for Preparing Total RNA from Tissues or Cells

Method	Supplier or Reference
Standard solution-based methods	
Guanidium isothiocyanate method	Chomczynski and Sacchi (1987)
TRIzol reagenty	Invitrogen
RNAgents Total RNA Isolation System	Promega
Micro RNA Isolation Kit	Stratagene
Column methods	
RNeasy Mini Kit	Qiagen
Pinpoint Slide RNA Isolation System	Zymo Research
PicoPure RNA Isolation Kit	Arcturus
Micro-FastTrack 2.0 mRNA Isolation Kit	Invitrogen

CAUTION: Investigators should wear gloves for all procedures involving RNA and take precautions in order to avoid cross-contamination of samples. See *APPENDIX 2A* for general guidelines when working with RNA.

1. Isolate RNA from tissues or cells of interest using an appropriate method (Table 19.3.1).
2. Adjust the volume of the isolated RNA to 16 µl using DEPC-treated water.
3. Add the following reagents to the tube and mix:

   2 µl 10× DNase I buffer
   1 µl 2 U/µl DNase I
   1 µl 40 U/µl RNaseOUT RNase inhibitor.
4. Incubate at 37°C for 30 min.
5. Purify the treated RNA using an RNeasy Mini Kit according to manufacturer's instructions.

   Follow the RNeasy Mini Protocol for RNA Cleanup.
6. Vacuum dry the eluted RNA solution down to 10.5 µl.

   The product will be ready at this point for T7-based RNA amplification (see Basic Protocol 1).

SUBSTITUTION OF A COMMERCIAL KIT FOR AMPLIFYING TINY QUANTITIES OF RNA

ALTERNATE PROTOCOL

When samples are obtained by laser microdissection for isolation of cells from histological sections, or if investigators prefer to use a commercial kit for the RNA amplification, the RiboAmp kit from Arcturus is recommended for reliable T7-like amplification of tiny amounts of mRNA. This kit, designed to isolate and amplify extremely small amounts of material, can be substituted directly for Basic Protocol 1 through step 32. This kit generates antisense RNA that can be used for the synthesis of biotinylated double-stranded cDNA (bds cDNA).

Additional Materials *(also see Basic Protocol 1)*

RiboAmp RNA Amplification Kit (Arcturus Engineering)

1. Isolate RNA from samples and synthesize aRNA following the manufacturer's instructions for the RiboAmp RNA Amplification Kit.
2. Dry the aRNA down to 5 to 10 µl using a vacuum concentrator and then adjust the volume to 10.5 µl with DEPC-treated water.
3. Use the product in Basic Protocol 1 starting at step 33.
4. Check the concentration of bds cDNA using a spectrophotometer appropriate for determining UV absorbance of small samples.

BASIC PROTOCOL 2

T7-SAGE PROCEDURE

The following protocol is a modification of the SAGE technique developed by Kinzler and co-workers at Johns Hopkins University (Velculescu et al., 1995, 1997; see also SAGE Detailed Protocol version 1.0e at *http://www.sagenet.org*). Key alterations include the following changes:

a. The procedure is started with biotin-labeled double-stranded cDNA generated from antisense mRNA produced by T7 amplification.

b. Use of the commercial mRNA Capture Kit (Roche Molecular Biochemicals) is substituted for the first three steps involving binding of biotinylated double-stranded cDNA (bds cDNA) to streptavidin-magnetic beads, *Nla*III enzyme cleavage of cDNA and ligating of linkers, and release of cDNA tags using *Bsm*FI enzyme. This substitution permits completion of all three steps within two streptavidin-coated microcentrifuge tubes, which significantly reduces losses of material between successive steps.

c. Phase Lock Gel (PLG, Light; Eppendorf) for optimizing phase separation at several steps in the SAGE procedure for recovering DNA after phenol extraction; this technique significantly increases the recovery and purity of the DNA.

Materials

1 µg of bds cDNA generated by T7-based amplification (see Basic Protocol 1) in ~50 µl
mRNA Capture Kit (Roche Molecular Biochemicals) including:
  Streptavidin-coated PCR tubes
  Washing solution
10× NEBuffer 4 (New England Biolabs; supplied with corresponding restriction enzymes)
10 U/µl restriction endonuclease *Nla*III (New England Biolabs)
100× (10 µg/µl) BSA (New England Biolabs; supplied with corresponding restriction enzymes)
DNase-free H_2O
1 U/µl and 5 U/µl T4 DNA ligase and 10× ligase buffer (Invitrogen)
LoTE buffer (see recipe)
15 ng/µl annealed linker 1 (formed by annealing linkers 1A and 1B; see Support Protocol 3)
15 ng/µl annealed linker 2 (formed by annealing linkers 2A and 2B; see Support Protocol 3)
2 U/µl restriction endonuclease *Bsm*FI (New England Biolabs)
20 mg/ml glycogen (Boehringer Mannheim)
7.5 M ammonium acetate

70% and 100% ethanol
5× second-strand reaction buffer (Invitrogen; supplied with SuperScript kit, also see Basic Protocol 1)
10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP; Invitrogen; supplied with SuperScript kit; also see Basic Protocol 1)
5 U/ml Klenow fragment of DNA polymerase (Amersham Pharmacia Biotech or USB)
10× PCR buffer (see recipe)
Dimethyl sulfoxide (DMSO)
350 ng/µl primer 1: 5′-GGATTTGCTGGTGCAGTACA-3′ (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
350 ng/µl primer 2: 5′-CTGCTCGAATTCAAGCTTCT-3′ (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
5 U/µl Platinum *Taq* polymerase (Invitrogen)
12% and 8% polyacrylamide gels (see Support Protocol 5)
6× Loading Dye Solution (LDS; Fermentas)
10- and 25-bp DNA ladders (Invitrogen)
SYBR Green I (Molecular Probes)
1× TAE buffer (e.g., Advanced Technologies, or prepare as in *APPENDIX 2A*)
TE buffer, pH 7.4 (*APPENDIX 2A*), cold
Ready-to-Go 100-bp DNA ladder (Fermentas)
pZERO-1 plasmid (Invitrogen)
5 U/µl restriction endonuclease *Sph*I (New England Biolabs)
10× NEBuffer 2 (New England Biolabs; supplied with corresponding restriction enzymes)
1% and 3% agarose gels (see *APPENDIX 3A*)
One Shot TOP10 Electrocompetent *E. coli* cells (Invitrogen)
SOC medium (see recipe)
10-cm Zeocin-containing low-salt LB plates (see recipe)
350 ng/µl M13 forward primer: 5′-GTAAAACGACGGCCAGT-3′ (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
350 ng/µl M13 reverse primer: 5′-GGAAACAGCTATGACCATG-3′ (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
QIAquick 96 Multiwell PCR Purification Kit (Qiagen)
10 mg/ml ethidium bromide

16°, 50°, and 65°C water baths
2-ml PCR tubes
System for PCR using 96-well plates (e.g., GeneAmp PCR system 9600 or 9700, Applied Biosystems)
96-well PCR plates (Applied Biosystems)
Power Pac 200 power source (Bio-Rad)
18-G needle (1- to 1.5-in. length)
SpinX columns with collection tubes (Costar)
Gene Pulser II and Pulse Controller Plus (Bio-Rad) and 0.1-cm electroporation cuvettes
15-ml polypropylene snap-top tubes
Bacterial shaker
Micropipettors with sterile aerosol-barrier tips
SAGE 2000 software (freely available for noncommercial use; see *http://www.sagenet.org*) and Microsoft Access

Additional reagents and equipment for phenol/chloroform extraction using Phase Lock Gel Light (see Support Protocol 2), polymerase chain reaction (*APPENDIX 3F*), polyacrylamide gel electrophoresis (see *APPENDIX 3A* and Support Protocol 5), and agarose gel electrophoresis (see *APPENDIX 3A*)

Generation of Linked SAGE Tags

Capture the biotinylated cDNA

1. Add 25 µl of bds cDNA to each of two streptavidin-coated PCR tubes provided in the mRNA Capture Kit (~0.5 µg per tube).

   This step can be scaled up by using more bds cDNA and more pairs of tubes, keeping a ratio of approximately 0.5 µg bds cDNA per tube.

2. Incubate at 37°C for 5 min to allow the bds cDNA to attach to the walls of the tubes.

3. Aspirate the solution to remove non-bound bds cDNA and wash the tubes three times, each time with 50 µl of the washing solution provided in the mRNA Capture Kit.

Cleave the biotinylated cDNA with the anchoring enzyme NlaIII

4. After aspirating the washing solution, wash the streptavidin-coated PCR tubes (which contain the bds cDNA bound to the walls) three times, each time with 50 µl 1× NEBuffer 4.

5. Add the following reagents to each tube:

   2 µl 10 U/µl *Nla*III
   0.25 µl 100× (10 µg/µl) BSA
   2.5 µl 10× NEBuffer 4
   20 µl DNase-free H_2O.

6. Vortex the mixtures very gently (to avoid bubbles) for 10 sec, then momentarily centrifuge at maximum speed in a microcentrifuge.

7. Incubate for 3 hr at 37°C. Mix occasionally by flicking the tube with a finger or by slow vortexing.

8. Heat the tubes at 65°C for 20 min to inactivate the enzyme.

   NlaIII is easily inactivated at ambient or high temperature.When ordering NlaIII, request that it be shipped in dry ice. If dry ice shipping is not requested, this enzyme may be sent with an ice pack and arrive at room temperature, with a resulting loss of activity. Aliquots of NlaIII should be stored at –80°C, which helps to prolong its activity. Ensure that the enzyme is fresh, since it appears to have a half-life of only a few months even when stored at −80°C.

Ligate linkers to the bound cDNA

9. Aspirate the solutions, which will contain digested, unbound cDNA, from each of the streptavidin-coated PCR tubes. Keep the tubes and discard the solutions.

10. Wash the PCR tubes three times, each time with 50 µl washing solution (from the kit), then once with with 50 µl of 1× ligase buffer.

11. Add 5 µl 5× ligase buffer and 18 µl LoTE buffer to each tube.

12. Add 1 µl of 15 ng/µl annealed linker 1 to tube 1.

13. Add 1 µl of 15 ng/µl annealed linker 2 to tube 2.

14. Mix and heat the tubes for 2 min at 50°C and then let stand for 15 min at room temperature.

15. Add 1 µl of 5 U/µl T4 DNA ligase to each of the tubes and mix.

16. Allow the mixtures to ligate by incubating at 16°C for 2 hr.

    After ligation, the mixture can be stored at 4°C overnight.

Release cDNA tags using the tagging enzyme BsmFI

17. Remove the ligation solutions, and wash each of the PCR tubes three times, each time with 50 µl washing solution (from the mRNA Capture Kit).

18. Wash the PCR tubes once with 50 µl 1× NEBuffer 4.

19. Add the following reagents to each tube:

    2.5 µl 10× NEBuffer 4
    0.25 µl 100× (1 µg/µl) BSA
    21.25 µl LoTE buffer.

20. Add 1 µl of 2 U/µl *Bsm*FI to each tube and mix. Digest 1 hr at 65°C. Mix occasionally by flicking the tube with a finger or slow vortexing.

    BsmFI is the "tagging enzyme" that cuts cDNA 14 bp away from the enzyme site GGGAC to generate SAGE tags.

    Do not discard the supernatant, which contains the SAGE tags after BsmFI digestion.

Generation of 26-mer SAGE Ditags

Prepare the released, linked SAGE tags for blunt-ending

21. After the *Bsm*FI digestion, increase the volume of each reaction solution containing the released linked SAGE tags to 200 µl by adding LoTE buffer, then mix.

22. Transfer the reactions to 2-ml tubes numbered 1 and 2, each containing precentrifuged Phase Lock Gel Light (see Support Protocol 2). Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet 200 µl of the SAGE tags contained in the respective aqueous upper phases into fresh 1.5- to 1.7-ml microcentrifuge tubes numbered 1 and 2.

23. Add the following reagents to each of the tubes containing the 200 µl SAGE tag preparation.

    3 µl 20 mg/ml glycogen
    133 µl 7.5 M ammonium acetate
    700 µl 100% ethanol.

24. Mix well by vortexing and place the tube on dry ice for 10 to 20 min.

25. Microcentrifuge 30 min at maximum speed, 4°C.

26. Carefully remove the supernatants and discard them. Be careful not to disturb the pellets.

27. Wash the pellets twice, each time by gently adding 500 to 1000 µl of 70% ethanol solution down the wall of the tube using a micropipet, taking care to avoid disrupting the pellet, and decanting the solution gently.

28. After the final wash, microcentrifuge again briefly at maximum speed to collect any residual ethanol. Carefully remove all of the ethanol with a micropipet and air dry the pellet for 5 to 10 min.

29. Resuspend each pellet in 20 µl LoTE buffer.

Blunt-end the linked SAGE tags

30. Add the following reagents to the tubes 1 and 2 containing 20 µl of linked SAGE tags.

    6 µl 5× second-strand reaction buffer
    0.3 µl 100× (1 µg/µl) BSA
    1.25 µl 10 mM dNTP mix
    1.0 µl 5 U/µl Klenow fragment of DNA polymerase.

31. Mix well, microcentrifuge briefly to collect the solution at the bottom of the tube, and incubate at 37°C for 30 min.

32. Adjust the volume of each blunt-ending reaction to 200 µl by adding LoTE buffer. Mix, then transfer each mixture (no. 1 and no. 2) to a labeled 2.0-ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the SAGE tags contained in each aqueous upper phase into 1.5- to 1.7-ml microcentrifuge tubes numbered 1 and 2.

33. Precipitate the SAGE tags and wash and dry the pellets as in steps 23 to 28.

34. Resuspend each pellet in 4 µl LoTE buffer.

Ligate the linked SAGE tags to form linked ditags

35. Prepare the following reaction mix on ice in a sterile microcentrifuge tube.

    0.8 µl 5 U/µl T4 DNA ligase
    1.2 µl 5× ligase buffer
    2 µl blunt-ended sample 1 from tube 1 (see step 34)
    2 µl blunt-ended sample 2 from tube 2 (see step 34).

36. Prepare a parallel negative control reaction mix without ligase on ice in a sterile microcentrifuge tube.

    0.8 µl LOTE buffer
    1.2 µl 5× ligase buffer
    2 µl blunt-ended sample 1 from tube 1 (see step 34)
    2 µl blunt-ended sample 2 from tube 2 (see step 34).

37. Mix each of the reactions and incubate overnight at 16°C.

38. After the incubation, make up the volume in each (reaction and control) tube to 20 µl by addition of 14 µl LoTE buffer and mix.

Choose the optimal dilution for PCR amplification of the linked ditags

39. Dilute the following samples with sterile water as described below.

    Negative control (prepared in step 36; no ligase) to 1/5 (i.e., 1 vol of sample plus 4 vol of DNase-free H_2O).
    Template (prepared in step 35; ligated product) to 1/5, 1/10, 1/20, 1/40, and 1/80.

    Set up seven PCR reactions on ice using sterile PCR tubes labeled as:

    Tubes 1 to 5: Different dilutions of the template (1/5, 1/10, 1/20, 1/40, and 1/80)
    Tube 6: Negative control (no template, just H_2O)
    Tube 7: Negative control (no ligase, just H_2O).

To each tube, add the following:

5 µl 10× PCR buffer
3 µl DMSO
7.5 µl 10 mM dNTP mix
1 µl 350 ng/µl primer 1
µl 350 ng/µl primer 2
30.5 µl DEPC-treated H_2O
1 µl 5 U/µl Platinum *Taq* polymerase
1 µl ligation product or negative control at appropriate dilution.

Mix each of the reactions.

Ex Taq (PanVera) appears to produce the same results for SAGE ditag amplification as Platinum Taq, and the former source of Taq polymerase is at present slightly less expensive than the latter.

40. Transfer 50 µl of each reaction mixture into wells of a 96-well PCR plate. Amplify using the following cycling parameters:

1 cycle:	1 min	94°C	(denaturation)
26-30 cycles:	30 sec	94°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	70°C	(extension)
1 cycle:	5 min	70°C	(final extension).

41. Select the optimal dilution of linked ditag as follows:

    a. Mix 5 µl of each PCR product (the various amplified dilutions as well as the no-ligase control and no-template control) with 1 µl of 6× Loading Dye Solution (LDS, from Fermentas).

    b. Load the 6 µl of each sample mixture in a lane of a 12% PAGE gel. Load DNA ladder in additional lanes. Perform electrophoresis (see Support Protocol 5) at 150 V for 1.5 to 2 hr. Stop electrophoresis when the bromphenol blue marker is 1 cm from the bottom of the gel.

    c. Stain gels with a 1:10,000 dilution of SYBR Green I in 1× TAE buffer for 20 min.

    d. Wash gels with 1× TAE buffer for 5 min.

    e. Examine the gel with a UV transilluminator. Compare the lanes with the various dilutions and pick the lowest dilution (highest concentration of linked ditags) that gives a clear 102-bp band (an 80-bp band represents self-ligated linkers, and cannot be used).

    The dilution series and PAGE analysis are necessary to check the successful formation of SAGE ditags (102-bp band) and to identify the optimal concentration for the subsequent large-scale PCR amplification.

42. Perform multiwell large-scale amplification of linked ditag at optimal dilution (selected in step 41) as follows:

    a. Dilute the template (i.e., the remaining template solution from step 38) with water to the optimal dilution (determined in step 41).

    For example, 1/10 dilution of 19 µl will make 190 µl of diluted PCR product.

    b. Set up 198 to 288 individual PCR reactions in two to three 96-well PCR plates as in step 40, using 50 µl of the optimally diluted template per well.

    c. Amplify PCR reactions as in step 40.

    Experience has shown that, at least with presently available equipment, it is best to divide the reaction mixtures up into tiny (50-µl) aliquots and to perform PCR in a 96-well

thermocycler configuration to ensure high-efficiency amplification. Usually, 192 PCR reactions (using two 96-well PCR plates) are performed in parallel, although three to four 96-well plates may be needed if the SAGE ditags are diluted to a lower concentration.

Isolate linked ditags

43. Pool the PCR products from all of the wells of the 96-well plates from the large-scale amplification into one 50-ml tube containing precentrifuged Phase Lock Gel Light.

    There should be ~4.5 ml from each 96-well-plate.

44. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally transfer half of the aqueous phase (containing linked ditags) to each of two fresh 30-ml Oak Ridge centrifuge tubes.

45. To ~5 ml of the sample in each tube, add the following reagents:

    80 µl 20 mg/ml glycogen
    2 ml 7.5 M ammonium acetate
    15 ml 100% ethanol.

46. Mix well by vortexing and place the tubes on dry ice for 20 min. Microcentrifuge 30 min at maximum speed, 4°C.

47. Carefully remove and discard the supernatant. Be careful not to disturb the pellets.

48. Wash the pellets twice, each time with 25 ml of 70% ethanol.

49. Carefully remove the ethanol and air dry the pellets for 5 to 10 min.

50. Resuspend each pellet in 300 µl LoTE buffer.

51. Add 60 µl of 6× Loading Dye Solution (LDS) to the sample (total volume, 360 µl) and mix.

52. Load 10 µl of the sample mixture in each lane of a 10-well, 12% polyacrylamide gel, for a total of 36 lanes on four gels. Use a 10-bp DNA ladder as a marker on each gel. Perform gel electrophoresis at 150 V for 1.5 to 2 hr using a Power Pac 200 power source (or equivalent). Stop electrophoresis when bromophenol blue marker is 1 cm from the bottom of the gels.

53. Stain the gels with SYBR Green I at 1:10,000 dilution in 1× TAE for 20 min.

54. Wash gels with 1× TAE for 5 min.

55. Make a hole through the bottom of a 0.5-ml sterile microcentrifuge tube using an 18-G needle, and insert the tube into a 1.7-ml microcentrifuge tube.

    For safety, the hole should be punched from the inside toward the outside of the tube: insert the sharp end of a 1- to 1.5-in. 18-G needle into the microcentrifuge tube and slide it down to the center of the V-shaped bottom of the tube; push (away from one's hand) through the bottom of the tube to make the hole, then discard the needle in appropriate manner.

56. Visualize the bands in the gel using a UV transilluminator, taking care to keep all exposed areas of skin covered, e.g., with UV eye and face shielding, gloves, and laboratory coat. Using a single-edge razor blade, excise the 102-bp product from all 36 lanes of the gel by cutting out each band as a small rectangle.

57. Scoop up three excised gel pieces with a corner of the razor blade and place into a 0.5-ml sterile tube. Snap the lid of the 0.5-ml tube closed. Repeat this process for the remaining gel pieces. If there is a lid attached to the 1.7-ml microcentrifuge tube, cut through the plastic hinge with scissors or razor blade and discard the lid. Microcentrifuge 3 min at maximum speed, at room temperature. Discard the 0.5 ml tube, which should be empty, leaving behind fragmented gel in the 1.7-ml tube.

The purpose of this step is to fragment the gel by forcing it to pass through the needle hole by centrifugal force. The outer tube needs to be large enough to permit passage of all of the gel through the hole, so a 1.7-ml tube or even a 2.0-ml tube may be preferable to a 1.5-ml tube to permit collection of all gel fragments, depending on the shape of each of the microcentrifuge tubes.

58. Mix 5 vol of LoTE buffer with 1 vol of 7.5 M ammonium acetate. Add 300 µl of this mixture to the 1.7-ml microcentrifuge tube containing the fragmented gel. Make sure that all gel pieces are covered with buffer. Mix well by vortexing.
59. Incubate at 4°C overnight and then at 65°C for 30 min to elute the DNA from gel.
60. Transfer the contents of the tube to SpinX columns fitted to collection tubes.
61. Microcentrifuge 5 min at maximum speed, at room temperature.
62. Transfer the eluates (each ~300 µl) to new 1.5 to 1.7 ml microcentrifuge tubes.
63. Precipitate, wash, and air dry the DNA pellet as in steps 23 to 28, above.
64. Resuspend each pellet in 7 µl LoTE and then combine all samples (~126 µl).
65. Store the 102-bp ditags at −20°C for up to 1 month.

Purify the SAGE ditags

66. Aliquot the 102-bp product (126 µl) equally into two separate sterile microcentrifuge tubes (63 µl per tube). Add the following reagents to each of the two tubes:

    15 µl 10× NEBuffer 4
    2 µl 100× (1 µg/µl) BSA
    12 µl 10 U/µl *Nla*III (10 U/µl)
    58 µl DNase-free H_2O.

67. Mix the contents well and incubate for 2 hr at 37°C.
68. Adjust the volume in each tube to 200 µl by adding 50 µl LoTE buffer and mix.
69. Analyze 5 µl of the reaction by electrophoresis on a 12% gel to check the efficiency of *Nla*III digestion (see Support Protocol 5).

    Problems with incomplete NlaIII digestion may be due to contaminants, which may be resolved by further purifying the gel-purified 102-bp ditag preparation with a gel-filtration purification step (Angelastro et al., 2000).

70. Transfer the reaction to 200 µl with LoTE buffer and transfer to a 2-ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the SAGE tags contained in the aqueous upper phase into a fresh tube.
71. Precipitate the SAGE tags and wash and dry the pellets as in steps 23 to 28.
72. Combine the pellets and resuspend, on ice, in a total volume of 30 µl using cold TE buffer, pH 7.4.

    Note that standard TE buffer, pH 7.4, prepared as in APPENDIX 2A is used through step 76 instead of LoTE buffer. LoTE buffer is again used in step 81. The SAGE ditags are more stable in the presence of the higher-salt TE buffer (see Anticipated Results).

73. Mix with 6 µl of 6× LDS. Load 9 µl into each of 4 lanes of 10-well 1.5-mm-thick 12% polyacrylamide gel. Use a 10-bp DNA ladder to provide markers. Perform gel electrophoresis at 150 V for 1.5 to 2 hr. Stop electrophoresis when the bromophenol blue marker is 1 cm from the bottom of the gels.

74. Stain gels with SYBR Green I at 1:10,000 dilution in 1× TAE for 20 min. Wash the gels with water for 5 min.

75. Locate and excise each band containing the 26-bp product using the technique described in step 56, above. Referring to steps 55 to 63, above, combine two excised gel bands and fragment through each of two punctured microcentrifuge tubes, elute DNA (except use standard TE buffer in place of LoTE at step 58 and incubate at 37° instead of 65°C at step 59), then purify on SpinX columns and precipitate and wash DNA pellet according to steps 60 to 63.

    When eluting, be careful to incubate at 37°C instead of 65°C (see step 59); otherwise, the 26-bp cDNA SAGE tags may be degraded.

76. Resuspend the pellet in a total volume of 6.75 µl cold TE buffer on ice. Proceed immediately to the next step.

Ligate the SAGE ditags to form concatemers

77. Set up the following ligation reaction on ice and mix:

    6.75 µl SAGE ditag sample (step 76)
    2 µl 5× ligase buffer
    1.25 µl 5 U/µl T4 DNA ligase.

    Incubate 3 hr at 16°C.

78. After the ligation is complete, add 2 µl of 6× LDS, mix, and heat for 20 min at 65°C.

79. Load the 10-µl sample from step 78 in a single lane of a 10-well 1.5 mm-thick 8% polyacrylamide gel. Also load a 100-bp DNA ladder as a size marker. Perform electrophoresis at 100 V for 3 to 3.5 hr. Stop electrophoresis when bromophenol blue marker reaches a point 1 cm from the bottom of the gel.

80. Stain the gel with SYBR Green I at 1:10,000 dilution in 1× TAE for 20 min. Wash the gel with water for 5 min.

81. Locate and excise the product between 600 and 1200 bp from the sample lane of the gel using the technique described in step 56, above. Referring to steps 55 to 63, above, fragment the excised gel band through a punctured microcentrifuge tube, elute DNA, then purify on SpinX column and precipitate and wash DNA pellet according to steps 60 to 63.

    These procedures are performed exactly as in steps 55 to 63; use LoTE buffer and elute with incubation at 65°C.

    To be safe, save the gel region between 400 and 600 bp in case it is needed later; discard it if step 105 is successful.

82. Resuspend the pellet in a total volume of 6 µl LoTE buffer on ice. Proceed immediately to the next step.

Cloning and Sequencing of Concatemers (SAGE Tags)

Linearize pZero vector

83. Digest pZero-1 plasmid with *Sph*I by combining:

    1 µl 1 µg/µl pZero-1
    1 µl 5 U/µl *Sph*I
    0.2 µl 100× (10 µg/µl) BSA
    22 µl 10× NEBuffer 2
    15.8 µl sterile H_2O.

    Incubate at 37°C for 15 min.

84. Add 180 µl LoTE buffer to digestion mix, then transfer to a 2.0 ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the digest contained in the aqueous upper phase to a fresh tube.

85. Precipitate, wash, and air dry the plasmid DNA as in steps 23 to 28.

86. Resuspend the DNA in a total volume of 50 µl using LoTE buffer.

    The final concentration of the digested vector should be about 20 ng/µl.

87. Check the efficiency of *Sph*I digestion by agarose gel electrophoresis (*APPENDIX 3A*) using a 2-µl aliquot of the digestion mix and a 2-µl aliquot of 100 ng/µl undigested pZero-1 diluted with water on a 1% agarose gel. Use linearized pZero-1 vector immediately (step 88).

    The DNA can be stored for 1 to 2 weeks at −20°C but the cloning efficiency may decrease.

Ligate concatemers into SphI-digested vector

88. Set up the following 10-µl ligation reaction on ice and mix:

    6 µl concatemer sample (step 82)
    1 µl of ~20 ng/ml digested pZero-1 vector (step 87)
    2 µl 5× ligase buffer
    1 µl 5 U/µl T4 DNA ligase.

    Incubate for 3 hr at 16°C.

    Do not ligate at room temperature.

89. Adjust the volume of the above reaction mixture to 200 µl with LoTE buffer, and transfer to a 2-ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the concatemer ligated to digested contained in the aqueous upper phase, into a fresh 1.5- to 1.7-ml tube.

90. Precipitate, wash, and air dry the DNA pellet as in steps 23 to 28, above.

91. Resuspend the pellet in 12 µl water.

Transform DNA into E. coli

92. Add 1 µl of the resuspended DNA to 50 µl of One Shot TOP10 Electrocompetent *E. coli* suspension and mix gently.

93. Carefully transfer the cells and DNA to a chilled 0.1-cm electroporation cuvette on ice.

94. Electroporate the samples using Gene Pulser II and Pulse Controller with the following settings: voltage, 1.8 kV; capacitance, 25 µF; resistance, 200 Ω.

95. Add 950 µl of room temperature SOC medium to the cuvette, mix, and transfer the contents to a 15-ml polypropylene snap-top tube. Shake the tube in a bacterial shaker at 37°C for 1 hr at 200 to 225 rpm with the lid vented.

96. Plate 50 µl from each transformation on a test 10-cm low-salt Zeocin-containing LB plate.

97. Incubate each of the test plates at 37°C to grow the transformed bacteria for 12 to 16 hr and store the remaining transformation reactions at 4°C.

98. The next day, depending on the number of colonies observed from the previous test plating, plate out all of the transformation reaction or dilute the transformation reaction appropriately to ensure sufficient space between colonies, and plate all bacteria on 10-cm low-salt Zeocin-containing plates.

The ideal number of colonies is roughly 200 to 300 per 10-cm plate.

Identify insert sizes

99. Set up 96 PCR reactions on ice using sterile 0.2 ml PCR tubes containing the following components:

2.5 µl 10× PCR buffer
1.25 µl DMSO
1.25 µl 10 mM dNTP mix
0.5 µl 350 ng/µl M13 forward primer
0.5 µl 350 ng/µl M13 reverse primer (350 ng/µl)
19 µl H_2O
0.2 µl 5 U/µl Platinum *Taq* polymerase.

100. Add PCR components to wells of a 96-well PCR plate.

101. Use a micropipettor with a sterile 10-µl tip containing an aerosol barrier to collect each colony (about 2 µl of cell suspension). Transfer each colony to a separate well of PCR mix by pipetting the solution in and out several times.

102. Perform PCR using a 96-well thermal cycler using the following cycling parameters:

1 cycle:	2 min	95°C	(denaturation)
25-30 cycles:	30 min	95°C	(denaturation)
	1 min	56°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	5 min	70°C	(final extension).

103. Purify the resulting PCR products using the QIAquick 96 PCR purification kit system according to the manufacturer's instructions.

104. Perform gel electrophoresis using 2 µl of each sample plus 0.4 µl of 6× LDS on a 3% agarose gel (see *APPENDIX 3A*). Stain with ethidium bromide (use 1 µg/ml by mixing 5 µl 10 mg/ml ethidium bromide with 50 ml water; also see *APPENDIX 3A*).

105. Examine gel with a UV transilluminator. Select only wells showing PCR products over 600 bp, which should contain at least 25 tags, for sequence analysis.

To avoid low sequencing efficiency, an additional centrifugation step is recommended if the vacuum available for use with the QIAquick 96 PCR purification kit system is not sufficiently strong to remove all residual solution. Before eluting the PCR products with water, centrifuge the 96-column system at 3000 × g for 5 min using a 96-well plate holder in a low-speed centrifuge.

Sequence the SAGE library and analyze the SAGE tags

106. Sequence the PCR products in 96-well plates by sending them to a DNA sequencing facility or company that can directly sequence PCR products, or sequence in-house (see Support Protocol 6).

107. Extract SAGE tags from sequence files using the newest version of SAGE 2000 Software.

SAGE software is required in order to extract SAGE tag data and to generate quantitative information from the raw sequence data. A widely used software system for this purpose is

SAGE 2000, which is freely available for noncommercial use through a Material Transfer Agreement with Johns Hopkins University. Following the instructions for obtaining the software on the Web site http://www.sagenet.org. This software uses the Windows 2000 or Windows 98 operating system and requires the prior installation of Microsoft Access. An alternative SAGE software system is eSAGE, developed by Margulies and Innis (2000), which is also freely available for noncommercial use (the e-mail contact is ehm@umich.edu). This latter software uses the Windows 95/98 or NT version 4.0 operating system, and also requires the installation of Microsoft Access.

108. Identify transcripts using the NCBI SAGE tag database: *http://www.ncbi.nlm.nih.gov/SAGE.*

PHENOL-CHLOROFORM (PC8) EXTRACTION

SUPPORT PROTOCOL 2

The application of Phase Lock Gels as part of the SAGE procedure, for purification of the DNA after phenol extraction, significantly increases the purity and recovery of the DNA. If using a 50-ml tube, a centrifuge with a swinging-bucket rotor is recommended.

Materials

Phase Lock Gel Light (PLG; Eppendorf; purchased in 2- or 50-ml tubes)
DNA solution to be extracted
PC8 solution (see recipe)

1. Precentrifuge Phase Lock Gel Light tube without added sample immediately prior to use. If using a 2-ml tube, microcentrifuge 20 sec at maximum speed, room temperature; if using a 50-ml tube, centrifuge 1 min at 1500 × *g*, room temperature.
2. Add the sample and an equal volume of PC8 solution to the tube.
3. Shake to mix; e.g., by inverting the tube seven times.
4. If using 2-ml tubes, microcentrifuge 5 min at maximum speed, room temperature; if using 50-ml tubes, centrifuge 5 min at 1500 × *g*, room temperature, in a swinging-bucket rotor.
5. Transfer aqueous (top) layer to a new microcentrifuge tube using a pipet.

KINASING OF LINKERS

SUPPORT PROTOCOL 3

To ligate DNA with oligonucleotide linker, 5′ phosphorylation of the oligonucleotide linkers prior to ligation is necessary.

Materials

Linkers 1A, 1B, 2A, and 2B (see recipe)
LoTE buffer (see recipe)
10× kinase buffer (NEB)
10 mM ATP (NEB)
10 U/µl T4 polynucleotide kinase and 5× ligase buffer
6× Loading Dye Solution (Fermentas)
10-bp DNA ladder
12% polyacrylamide gel (see Support Protocol 5)
10 mg/ml ethidium bromide
1.5- to 1.7-ml RNAse/DNAse-free microcentrifuge tube (PGC Scientific or other supplier)
16°, 50°, and 65°C water baths or heating blocks

Additional reagents and equipment for polyacrylamide gel electrophoresis (see Support Protocol 5)

1. Dilute linkers 1A, 1B, 2A, and 2B to 350 ng/µl with water.
2. Prepare the following reaction mixes in two separate ~1.5-ml RNase/DNase-free microcentrifuge tubes:

   Tube 1:

   9 µl 350 ng/µl linker 1B
   6 µl LoTE buffer
   2 µl 10× kinase buffer
   2 µl 10 mM ATP
   1 µl 10 U/µl T4 polynucleotide kinase.

   Tube 2:

   9 µl 350 ng/µl linker 2B
   6 µl LoTE buffer
   2 µl 10× kinase buffer
   2 µl 10 mM ATP
   1 µl 10 U/µl T4 polynucleotide kinase.
3. Incubate at 37°C for 30 min.
4. Heat-inactivate at 65°C for 10 min.
5. Mix 9 µl of 350 ng/µl linker 1A with 20 µl of the kinased linker 1B (i.e., from the reaction mix in tube 1).

   The final concentration of both linker 1A and linker 1B should be 217.2 ng/µl.
6. Mix 9 µl of 350 ng/µl linker 2A with 20 µl of the kinased linker 2B (i.e., from the reaction mix in tube 2).

   The final concentration of both linker 2A and linker 2B should be 217.2 ng/µl.
7. Anneal linkers by heating as follows:

   95°C for 2 min
   65°C for 10 min
   37°C for 10 min
   Room temperature for 20 min.

   Store at –20°C.

   The result of the kinasing reaction will be "annealed linker 1" and "annealed linker 2," used in Basic Protocol 2.
8. Test effectiveness of the kinase reaction as follows.

   a. Prepare the following mixture in a sterile microcentrifuge tube on ice to self-ligate each annealed linker pair:

      1 µl 200 ng/µl linker 1 (annealed linker 1A and 1B, see step 5)
      1 µl 200 ng/µl linker 2 (annealed linker 2A and 2B, see step 6)
      5 µl LoTE buffer
      2 µl 5× ligase buffer.

   b. Complete the ligation reaction exactly as described in steps 14 to 16 of Basic Protocol 2.

c. Mix 5 µl of reaction with 1 µl of 6× Loading Dye Solution (LDS from Fermentas) and load on a 12% PAGE gel. Use a 10-bp DNA ladder to provide markers. Perform gel electrophoresis at 150 V for 1.5 to 2 hr (see Support Protocol 5). Stop electrophoresis when the bromphenol blue marker is 1 cm from the bottom of the gel.

d. Stain with ethidium bromide (use 1 µg/ml prepared by mixing 5 µl of 10 mg/ml ethidium bromide with 50 ml water).

e. Examine on a UV transilluminator for linker-linker dimers.

Kinased linkers should allow linker-linker dimers (80 to 100 bp) to form after ligation, while unkinased linkers will prevent self-ligation. Only linker pairs that self-ligate >70% should be used.

When linkers 1 and 2 are to be used at 15 ng/µl in steps 12 and 13 of Basic Protocol 2, 15 µl of each linker (200 ng/µl) are diluted with 185 µl of water.

GEL-SHIFT TEST FOR COMPLETE BIOTINYLATION OF BIOTIN-OLIGO dT

SUPPORT PROTOCOL 4

Biotinylated oligo(dT) primers synthesized by Integrated DNA Technology or by Invitrogen were used by the authors. Efficiency of binding to the streptavidin tube is an important factor for successfully constructing the T7-SAGE library and should be checked by investigators if they substitute another company's oligo(dT) primer.

Materials

Biotinylated oligo(dT) primer (custom-synthesized, e.g., by Integrated DNA Technologies or core facility; gel-purified)
1 µg/µl streptavidin
6× Loading Dye Solution (Fermentas)
100-bp DNA ladder
12% polyacrylamide gel (see Support Protocol 5)
10 mg/ml ethidium bromide

1. Prepare the following reaction mixes.

Tube 1:

2 µl 0.5 mg/ml biotinylated oligo(dT) primer
2 µl 1 µg/µl streptavidin
6 µl H_2O.

Tube 2 (negative control, no streptavidin):

2 µl 0.5 mg/ml biotinylated oligo(dT) primer
8 µl H_2O.

Tube 3 (negative control, no primer):

2 µl 1 µg/µl streptavidin
8 µl H_2O.

2. Incubate mixtures 15 min at room temperture.

3. Add 2 µl of 6× loading dye solution (LDS from Fermentas) to 5 µl of each reaction.

4. Load 6 µl of each reaction solution on a 12% PAGE gel. Use a 100-bp DNA ladder to provide markers. Perform gel electrophoresis at 150 V for 1.5 to 2 hr (see Support Protocol 5).

5. Stain with ethidium bromide (use 1 µg/ml prepared by mixing 5 µl of 10 mg/ml ethidium bromide with 50 ml of water). Examine on a UV transilluminator.

If the oligo dT is well-biotinylated, the entire band should be shifted to a single band of higher molecular weight, and this gel shift should occur only in the lane containing the streptavidin.

SUPPORT PROTOCOL 5

PREPARATION OF POLYACRYLAMIDE GELS USED IN SAGE ANALYSIS

Gels should be poured according to the following procedures before they are used in the respective protocols.

Materials

40% polyacrylamide (19:1 acrylamide:bis; Bio-Rad)
40% polyacrylamide (37.5:1 acrylamide:bis; Bio-Rad)
10× TAE buffer (*APPENDIX 2A*)
10% (w/v) ammonium persulfate (prepare fresh)
TEMED

XCELL II mini cell (Invitrogen)
Gel electrophoresis cassettes with 1.5 mm spacers (Invitrogen)
10-well combs (Invitrogen)
Power Pac 200 power source (Bio-Rad)

CAUTION: Refer to the manufacturer's instructions for electrophoresis conditions specific for the investigator's apparatus.

To prepare 12% PAGE gel (for purification of PCR products and ditags)

1a. Combine and gently mix (avoiding bubbles) the following reagents:

10.5 ml 40% polyacrylamide (19:1 acrylamide:bis)
20.7 ml H_2O
3.5 ml 10× TAE buffer
350 µl 10% ammonium persulfate
30 µl TEMED.

2a. Immediately pour into vertical gel apparatus (XCELL II mini cell) cassette with 1.5 mm spacers, or equivalent equipment. Add 10-well comb and allow gel to polymerize for at least 30 min.

To prepare 8% PAGE gel (for purification of concatemers)

1b. Combine and gently mix (avoiding bubbles) the following reagents:

7 ml 40% polyacrylamide (37.5:1 acrylamide:bis)
24.2 ml H_2O
3.5 ml 10× TAE buffer
350 µl 10% ammonium persulfate (make fresh)
30 µl TEMED.

2b. Immediately pour as described for 12% PAGE gel (step 2a).

DIRECT SEQUENCING USING A COMMERCIAL SEQUENCING KIT

If local arrangements can be made for direct sequencing of PCR products from the SAGE library after investigator preparation of samples for loading on an automated DNA sequencer, the following protocol using the ABI BigDye terminator sequencing system is recommended. This kit is suitable for performing fluorescence-based cycle sequencing reactions on PCR fragments using high-sensitivity dyes, e.g. 6-carboxyfluorescein (6-FAM).

Materials

ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems)
30 ng/µl PCR product to be sequenced (>600 bp; see Basic Protocol 2)
1 pmol/µl M13 forward primer

Low-speed centrifuge with 96-well plate holder
System for PCR using 96-well plates (e.g., GeneAmp PCR system 9700, Applied Biosystems)
Centri-Sep 96 plate (Princeton Separations)
Automated DNA sequencer (e.g., ABI Prism 3100 genetic analyzer system, Applied Biosystems)

1. For each reaction to be sequenced, combine the following reagents in individual wells of a 96-well PCR plate:

   8 µl Terminator ready reaction mix from kit
   4 µl 30 ng/µl PCR product
   4 µl 1 pmol/µl M13 forward primer (1 pmol/µl)
   4 µl H_2O.

2. Mix well and centrifuge 1 min at 3000 × *g*, 4°C. using a 96-well plate holder in a low-speed centrifuge.

3. Perform PCR using the following cycling parameters.

25 cycles:	10 sec	96°C	(denaturation)
	5 sec	50°C	(annealing)
	4 min	60°C	(extension).

4. Perform a rapid thermal ramp down to 4°C and hold at that temperature until ready to purify.

5. Microcentrifuge briefly at maximum speed to bring down the contents of the tubes.

6. Transfer extension products to a Centri-Sep 96 plate using a multichannel pipet. Purify according to the manufacturer's instructions for the Centri-Sep plate.

7. For the library, cycle-sequence the concatemer inserts (PCR products) using an ABI Prism 3100 genetic analyzer system according to the manufacturer's instructions.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Linkers

Linker 1A:
5′ TTT GGA TTT GCT GGT GCA GTA CAA CTA GGC TTA ATA GGG ACA TG 3′
Linker 1B:
5′ TCC CTA TTA AGC CTA GTT GTA CTG CAC CAG CAA ATC C [amino-modified C7] 3′
Linker 2A:
5′ TTT CTG CTC GAA TTC AAG CTT CTA ACG ATG TAC GGG GAC ATG 3′
Linker 2B:
5′ TCC CCG TAC ATC GTT AGA AGC TTG AAT TCG AGC AG [amino-modified C7] 3′

When purchasing the above oligonucleotides from a company that custom synthesizes oligonucleotides (the authors recommend Integrated DNA Technologies), or when submitting requests to a DNA synthesis core facility, the modifications described above must be communicated along with the sequence: i.e., for linkers 1B and 2B, a 3′ modification consisting of an amine moiety with a 7-carbon spacer. These oligonucleotides must be gel-purified; the investigator should request that this be done by the custom-synthesis company or core facility.

Amino-modified C7 is helpful to eliminate the native 3′-OH group from the oligo, which functionally blocks the oligo from participating as a primer in DNA synthesis, sequencing, or PCR (http://www.idtdna.com/program/techbulletins/amino.asp).

LoTE buffer

3 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.2 mM EDTA, pH 7.5 (*APPENDIX 2A*)
Store at 4°C

PC8 solution

Combine the following in the order indicated:
480 ml phenol, prewarmed to 65°C
320 ml 0.5 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
640 ml chloroform

Shake and place at 4°C. After 2 to 3 hr, shake again. After another 2 to 3 hr, aspirate aqueous (top) layer. Divide into aliquots and store at −20°C.

PCR buffer, 10×

166 mM $(NH_4)_2SO_4$
670 mM Tris·Cl, pH 8.8 (*APPENDIX 2A*)
67 mM $MgCl_2$
100 mM 2-mercaptoethanol
Store at −20°C

SOC medium

0.5% yeast extract
2% tryptone
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose
Store at room temperature (stable for years)

Zeocin-containing low-salt LB plates

For 1 liter:
10 g tryptone
5 g yeast extract
5 g NaCl

Adjust the pH to 7.5 and add 15 g Bacto agar. Autoclave solution. Allow to cool to 50°C, then add Zeocin to 50 μg/ml and pour into 10-cm plates.

COMMENTARY

Background Information

In order to acquire reliable gene expression data from nanogram quantities of total RNA, this set of protocols optimizes amplification of low-abundance RNA samples by combining T7-based antisense RNA (aRNA) amplification (Van Gelder et al., 1990) with serial analysis of gene expression (SAGE). The T7 procedure yields up to 10^6-fold linear amplification of high-fidelity aRNA from very small amounts of total RNA and is applicable regardless of whether total RNA or poly(A)$^+$ RNA is used. The effectiveness and accuracy of T7 amplification has been confirmed in microarrays (Wang et al., 2000). Recently, this method has been applied to microarray analyses with laser-capture microdissected samples (Luo et al., 1999), generation of full-length cDNA libraries from a single prostate cancer–cell (Ying et al., 1999) and analysis of gene expression of single live neurons (Eberwine et al., 1992).

For T7 amplification, an RNA polymerase promoter is incorporated into each cDNA molecule by priming cDNA synthesis with a synthetic oligonucleotide containing the T7 RNA polymerase promoter. After synthesis of double-stranded cDNA (ds-cDNA), T7 RNA polymerase is added, and aRNA is transcribed from the cDNA template. The repetitive synthesis of multiple RNA molecules from a single cDNA template results in amplified aRNA, which serves as the starting material for synthesizing first-strand cDNA with reverse transcriptase using random primers.

This T7-SAGE protocol includes some modifications to increase the efficiency of the procedure. Biotinylated ds-cDNA (bds-cDNA) containing the T7 promoter, rather than plain ds-cDNA, is synthesized using a biotinylated primer in place of the usual T7 primer after two cycles of T7 amplification. As a result, bds-cDNA is synthesized from amplified aRNA, instead of poly(A)$^+$ RNA, using a biotinylated primer. Consequently, the product can be added directly after silica-gel membrane column purification to a streptavidin-coated tube in order to immobilize it for SAGE. This tube-based modification reduces losses of valuable material.

This technique synthesizes a broad range of sizes (~100 to 3000 bp) of double-stranded cDNA (bds-cDNA) of high purity. However, the *Nla*III enzyme used for subsequently making ditags is sensitive to different conditions. It has been observed that *Nla*III often digests DNA templates with low efficiency because of its short half-life, improper storage, or the sensitivity of this enzyme to the digestion conditions (Angelastro et al., 2000). In order to maximize the effectiveness of *Nla*III, the bds-cDNA for this protocol is purified by a silica-gel membrane column. The resulting 102-bp bands were clearer and easier to excise from the gel than in published microSAGE (Datson et al., 1999) and SAGE-Lite (Peters et al., 1999) procedures. In order to isolate and amplify very small quantities of samples in this SAGE procedure, the authors used the mRNA Capture Kit (Boehringer Mannheim) to carry out in single tubes the multiple steps of enzyme cleavage of cDNA, ligation of linkers to bound cDNA, and release of cDNA tags (Datson et al., 1999).

The use of tiny quantities of starting material demands an amplification step. The authors found that only two cycles of T7 amplification were necessary for 4 ng of starting material. In the original SAGE procedure, 25 to 28 cycles of PCR are performed to amplify the pool of ditags. Although this PCR step should be relatively free of bias because all ditags are of approximately equal length, preferential amplification of some ditags will occur and will increase the possibility of duplicate ditags (Datson et al., 1999). Because the possibility of duplicate ditags occurring by chance is generally very low, their presence is thought to be the result of nonrepresentative overamplification (van Kampen et al., 2000). The SAGE software counts each exclusive ditag combination only once, thereby removing duplicate ditags. However, the frequency of duplicate ditags, and of ditags that are too long or short, are good parameters for judging the accuracy of a SAGE

library (van Kampen et al., 2000). Two-step ditag PCR amplifications were found to yield large numbers of duplicate ditags (Peters et al., 1999) and the artifactual ditag 5′-TCCCCGTA-CANNNTTAATAGGGA-3′ (Datson et al., 1999). In recent testing of the present protocol, the resultant T7-SAGE library contained only 0.8% repeated ditags (25 duplicated ditags out of 2986 tags), in contrast with a miniSAGE library containing 4% (160 duplicated ditags out of 3838 tags; Ye et al., 2000). The authors' T7-SAGE results also compared favorably with previously reported results using SAGE-Lite and conventional SAGE: 8.6% (994 duplicated ditags out of 11,495 tags; Peters et al., 1999) and 8.3% (5726 duplicated ditags out of 68,691 tags; Velculescu et al., 1997), respectively. The reason that the frequency of duplicated ditags is lower with T7-SAGE and miniSAGE is most likely due to the elimination of additional PCR amplification. Moreover, besides having a lower rate of duplicate ditags, the T7-SAGE

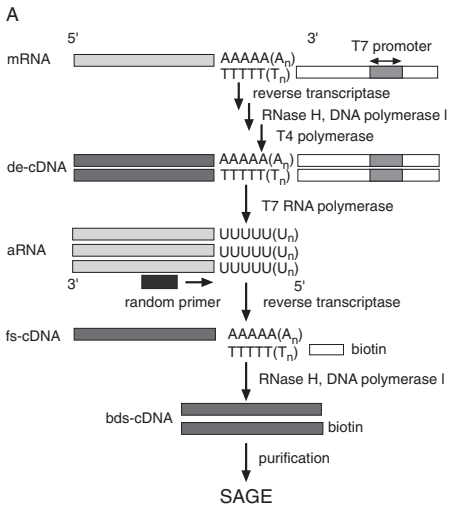


Figure 19.3.1 T7-based mRNA amplification and cDNA synthesis. (**A**) Schematic diagram of T7-based mRNA amplification. Modified from Van Gelder et al. (1990). Whole RNA is reverse-transcribed using a synthetic primer containing the T7 RNA polymerase binding site (sequences listed in Materials section of Basic Protocol 1). Second-strand cDNA synthesis to produce double-stranded (ds) cDNA is performed with *E. coli* DNA polymerase I and RNase H. After the cDNA is blunt-ended with T4 DNA polymerase, the cDNA is purified and transcribed with T7 RNA polymerase, yielding amplified antisense RNA (aRNA). These steps are repeated once to amplify the aRNA further. The amplified aRNA is reverse-transcribed using random primers to produce first-strand (fs) cDNA. Finally, biotinylated double-stranded (bds) cDNA is synthesized using a biotinylated oligo(dT) primer, *E. coli* DNA polymerase I, and RNase H, and then purified for SAGE. (**B**) Synthesis of biotinylated double-stranded (bds) cDNA from T7-amplified antisense RNA (aRNA). Input of total aRNA for the synthesis of bds cDNA is 4 nanograms, as indicated in Basic Protocol 1. This synthesis resulted in libraries of bds cDNA molecules that vary in length from ~100 to 3000 bp. Fragment sizes of the 100-bp DNA ladder are shown in bp.

library had no ditags that were too short, no ditags that were too long, and no other ditags indicative of artifacts.

Analysis of SAGE results is routinely performed using the NCBI SAGEtag database. Ongoing database additions continue to increase its value for the analysis of SAGE tags, and cDNA sequence data from RIKEN, NCI, and other sources should continue to promote SAGE research and to facilitate the identification of novel genes. Commercial databases using SAGE may also prove useful (e.g., Celera).

In conclusion, this T7-SAGE procedure, a new modification of SAGE, can be utilized in studies where mRNA is extremely limited. However, it is also effective for larger amounts of RNA, from which a 4- to 5-ng aliquot can be used. However, if large amounts of RNA are available, standard SAGE without amplification is recommended. Because studies of gene expression generally require confirmation of initial findings by independent methods such as RT-PCR and Northern blot analysis, any remaining RNA not used for this protocol can be saved for such additional analyses. Although

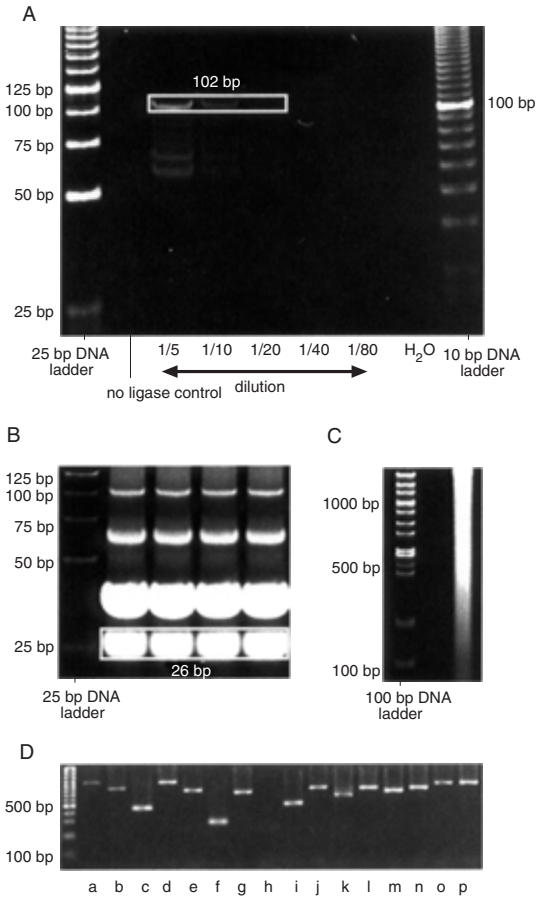


Figure 19.3.2 Selected steps of the SAGE procedure. (**A**) Linked SAGE ditags generated by T7-SAGE and separated on a 12% polyacrylamide gel. The 102-bp bands corresponding to amplified linked SAGE ditags are indicated by the rectangle. Shown after 28 PCR cycles are the no-ligase control, 1/5, 1/10, 1/20, 1/40 and 1/80 dilutions of the linked SAGE ditags, and the negative H_2O control (lanes 2-8), respectively. Lane 1, 25-bp DNA ladder. Lane 9, 10-bp DNA ladder. (**B**) SAGE ditags digested by *Nla*III and separated on a 12% polyacrylamide gel. Clear 26-bp bands (SAGE ditags), 40-bp bands (linkers) and 60- to 102-bp bands (products with incomplete *Nla*III digestion) should be observed. Fragment sizes of the 25-bp DNA ladder are shown in bp. (**C**) SAGE concatemer separated on an 8% polyacrylamide gel. A smear from ~100 bp to >1 kb should be observed. Fragment sizes of the 100-bp DNA ladder are shown in bp. (**D**) Example of sizes of inserts present in the T7-SAGE library. An insert of >300 bp (>500 bp band) will be contained in most of the clones (lanes a, b, d, e, g, and i to p). Lanes c and f contain small inserts. A 226-bp band indicates no insert (not shown). Fragment sizes of the 100-bp DNA ladder are shown in bp.

the authors have used T7-SAGE for global analysis of gene expression using only 4 ng of total RNA, they expect that the amount of starting material can be decreased further, perhaps down to a single cell. Microscale applications of SAGE such as this T7-SAGE method should help to broaden its future applications in studies of gene expression.

Critical Parameters and Troubleshooting

RNA preparation

It is essential to start with a clean preparation of RNA that is free of any salts or other substances that might inhibit reverse transcription. Successful RNA isolation from minute tissue samples depends on the complete elimination of ribonucleases. RNA and antisense RNA (aRNA) should be checked for intactness on a denaturing gel before cDNA synthesis if the investigator has sufficient RNA, or especially aRNA.

Purity of oligonucleotides

The quality of the primers is crucial to the success of the procedure. Where indicated (see materials lists of respective protocols), primers should be gel-purified, e.g., by the custom synthesis company from which they are purchased.

Production of 102-bp ditag-containing PCR products

Possible pitfalls in the synthesis of the 102-bp ditags include the following possible problems.

1. The cDNA was of poor quality or there were insufficient amounts. Check cDNA quality by gel electrophoresis and PCR. Check the extent of linker kinasing by self-ligation (see Support Protocol 3) and the extent of biotinylation of the oligo dT by a biotin gel shift test (see Support Protocol 4).

2. The protocols were not followed in exact detail. Because specific conditions in the protocol have been optimized, they should be followed as precisely as possible. For example, use the exact PCR sample sizes, buffers, and cycle temperatures described in the protocol.

3. Some reagent(s) were not of adequate quality. Obtain fresh reagents from the recommended companies.

4. The concentration of dNTPs was too high in the 102-bp PCR amplification. Although adding more dNTPs can increase amounts of PCR product, slightly exceeding a threshold can cause a paradoxical loss of the PCR product. Because of lot-to-lot variations in dNTP concentration, dNTPs should be titrated in a preliminary test before doing the large-scale preparation, e.g., starting with the lowest dNTP concentration of 0.5 mM.

NlaIII digestion of 102-bp ditag-containing PCR products

There are several reasons why the *Nla*III digestion may yield poor results.

1. The *Nla*III was not sufficiently active. *Nla*III should be stored in aliquots at −80°C, which helps to prolong its activity. Make sure the enzyme is fresh, since its half-life is only a few months even under optimal conditions. If the *Nla*III is less active and/or there are high yields of PCR product, more enzyme may be needed.

2. There was exonuclease contamination. The CATG cutting site of the PCR product may be missing because of exonuclease contamination or activity, e.g., from the DNA polymerase I. Phenol extractions and the use of Phase Lock Gel (PLG) is recommended to remove enzymes completely.

3. There were other interfering contaminants. The gel-purified 102-bp ditag preparation can be further purified by gel filtration before *Nla*III digestion as described by Angelastro et al. (2000).

Generation of large concatemers

Formation of sufficiently large concatemers depends on having sufficient amounts and purity of the ditags, as well as good ligation.

1. Problems can be caused by insufficient quantities of ditags present in the ligation reaction. The PCR protocol specified in the basic T7-SAGE protocol routinely uses 192 to 288 50-µl PCR reactions to ensure efficient production of 10 to 20 µg of 102-bp ditags. If a lower quantity of ditags is found, the safest course is to repeat the process to obtain at least 10 µg of ditags in order to ensure sufficient quantities for the *Nla*III digestion, so that enough 26-bp ditags are available for the ligation step to form concatemers.

2. The 26-bp ditags appear to degrade easily and should be kept at 4°C and in high-salt buffer at all times to prevent loss.

3. The 26-bp bands in gels are purified away from contaminating linkers by cutting them out with a razor blade. The cuts should be as close as possible to the bands to reduce contamination by materials of slightly different size, especially linkers, which interfere with the ligation reaction.

4. If linker contamination still appears to be causing poor concatemer formation, biotin-la-

beled primers can be substituted for primers 1 and 2 and then removed by binding to streptavidin beads (Powell, 1998).

Anticipated Results

Amplification of mRNA

The T7-based RNA amplification method (Fig. 19.3.1A) should be able to amplify the starting mRNA by ~1000-fold with one cycle (Luo et al., 1999). Theoretically, from 1 ng of total RNA containing roughly 1% mRNA (~10 pg), one mg of antisense RNA (aRNA) can be obtained after 2 cycles of T7 amplification. A clearly visible but smeared band (representing a range of RNA sizes) of amplified aRNA should be seen after running the sample on a gel.

Synthesis of biotinylated double-stranded (bds)-cDNA from aRNA

This synthesis should also result in a clearly visible but smeared zone of bds-cDNA molecules after running the sample on a gel; the bds-cDNA varies in length from ~100 to 3000 bp (Fig. 19.3.1B).

Synthesis of SAGE ditags (102 bp)

The102-bp bands corresponding to amplified linked SAGE ditags should be clearly visible for excision and purification away from several other background bands (Fig. 19.3.2A). In the large-scale PCR amplification step using 96-well plates, 192 to 288 50-µl PCR reactions are routinely performed. After pooling, this generates a total of 10 to 20 µg ditags (see Basic Protocol 2, step 65).

NlaIII digestion of SAGE ditags

The 26-bp band corresponding to SAGE ditags without linker should be visible for purification away from other background bands (Fig. 19.3.2B). If the 26-bp band cannot be seen clearly, the investigator should confirm the efficiency of the *Nla*III enzymatic reaction (see Troubleshooting above) and consider the possibility of degradation of ditags. The ditags occasionally melt in the presence of low salt, especially if the temperature rises above room temperature or if the DNA pellets are allowed to dry, although the ditags are stable in the presence of high salt. Therefore, subsequent procedures in Basic Protocol 2, such as, step 72, the overnight incubation in step 75, and then step 76, are performed under cold conditions, before the samples are placed in the high-salt TAE gel buffer. In addition, pellets are resuspended in TE instead of LoTE buffer.

Formation of SAGE concatemer

A clear high-molecular-weight smear above 400 bp should be visible in the gel (Fig. 19.3.2C). If the investigator cannot see evidence of these large concatemers, linker contamination or insufficient initial quantities of ditags are likely causes (see Critical Parameters and Troubleshooting).

The size of inserts present in the T7-SAGE library

An insert of >300 bp (>500-bp band) should be visible in most of the clones (Fig. 19.3.2D). A 226-bp band indicates the absence of any insert (negative control).

Time Considerations

In Basic Protocol 1, the entire procedure of T7-based RNA amplification, from steps 1 to 44, will require several days. In the Alternate Protocol, the procedure of T7-based RNA amplification using the RiboAmp kit from Arcturus will also require several days.

In Basic Protocol 2, the SAGE procedure from steps 1 to 105, can take several weeks, depending on the number of tags. Sequencing clones and analysis of sequence data, from steps 106 to 108, can require several weeks to months, depending on the number of tags and the efficiency of the sequencing facilities used.

In Support Protocol 1, RNA extraction takes about 1 hr, depending on the type of kit used. In Support Protocol 2, PC8 extraction requires about 30 min. In Support Protocol 3, the kinasing reaction for linkers takes about 4 hr. In Support Protocol 4, testing biotinylation of biotin-oligo dT requires about 3 hr. Support Protocol 5 takes 1 hr, and Support Protocol 6 takes 12 hr.

Literature Cited

Angelastro, J.M., Klimaschewski, L.P., and Vitolo, O.V. 2000. Improved *Nla*III digestion of PAGE-purified 102 bp ditags by addition of a single purification step in both the SAGE and microSAGE protocols. *Nucleic Acids Res*. 28:E62.

Bashiardes, S. and Lovett, M. 2001. cDNA detection and analysis. *Curr. Opin. Chem. Biol.* 5:15-20.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

Datson, N.A., van der Perk-de Jong, J., van den Berg, M.P., de Kloet, E.R., and Vreugdenhil, E. 1999. MicroSAGE: A modified procedure for serial analysis of gene expression in limited amounts of tissue. *Nucleic Acids Res*. 27:1300-1307.

Eberwine, J., Yeh, H., Miyashiro, K., Cao, Y., Nair, S., Finnell, R., Zettel, M., and Coleman, P. 1992. Analysis of gene expression in single live neurons. *Proc. Natl. Acad. Sci. U.S.A.* 89:3010-3014.

Emmert-Buck, M.R., Bonner, R.F., Smith, P.D., Chuaqui, R.F., Zhuang, Z., Goldstein, S.R., Weiss, R.A., and Liotta, L.A. 1996. Laser capture microdissection. *Science* 274:998-1001.

Ishii, M., Hashimoto, S., Tsutsumi, S., Wada, Y., Matsushima, K., Kodama, T., and Aburatani, H. 2000. Direct comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis. *Genomics* 68:136-143.

Luo, L., Salunga, R.C., Guo, H., Bittner, A., Joy, K.C., Galindo, J.E., Xiao, H., Rogers, K.E., Wan, J.S., Jackson, M.R., and Erlander, M.G. 1999. Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat. Med.* 5:117-122.

Muller, D.K., Martin, C.T., and Coleman, J.E. 1988. Processivity of proteolytically modified forms of T7 RNA polymerase. *Biochemistry* 27:5763-5771.

Neilson, L., Andalibi, A., Kang, D., Coutifaris, C., Strauss, J.F., 3rd, Stanton, J.A., and Green, D.P. 2000. Molecular phenotype of the human oocyte by PCR-SAGE. *Genomics* 63:13-24.

Peters, D.G., Kassam, A.B., Yonas, H., O'Hare, E.H., Ferrell, R.E., and Brufsky, A.M. 1999. Comprehensive transcript analysis in small quantities of mRNA by SAGE-lite. *Nucleic Acids Res.* 27:E39.

Polyak, K. and Riggins, G.J. 2001. Gene discovery using the serial analysis of gene expression technique: Implications for cancer research. *J. Clin. Oncol.* 19:2948-2958.

Powell, J. 1998. Enhanced concatemer cloning: A modification to the SAGE (Serial Analysis of Gene Expression) technique. *Nucleic Acids Res.* 26:3445-3446.

Sooknanan, R., Howes, M., Read, L., and Malek, L.T. 1994. Fidelity of nucleic acid amplification with avian myeloblastosis virus reverse transcriptase and T7 RNA polymerase. *Biotechniques* 17:1077-1080, 1083-1075.

St. Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K.E., Montgomery, E., Lal, A., Riggins, G.J., Lengauer, C., Vogelstein, B., and Kinzler, K.W. 2000. Genes expressed in human tumor endothelium. *Science* 289:1197-1202.

Van Gelder, R.N., von Zastrow, M.E., Yool, A., Dement, W.C., Barchas, J.D., and Eberwine, J.H. 1990. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl. Acad. Sci. U.S.A.* 87:1663-1667.

van Kampen, A.H., van Schaik, B.D., Pauws, E., Michiels, E.M., Ruijter, J.M., Caron, H.N., Versteeg, R., Heisterkamp, S.H., Leunissen, J.A., Baas, F., and van der Mee, M. 2000. USAGE: A web-based approach towards the analysis of SAGE data. Serial Analysis of Gene Expression. *Bioinformatics* 16:899-905.

Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. 1995. Serial Analysis Of Gene Expression. *Science* 270:484-487.

Velculescu, V.E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M.A., Bassett, D.E., Jr., Hieter, P., Vogelstein, B., and Kinzler, K.W. 1997. Characterization of the yeast transcriptome. *Cell* 88:243-251.

Virlon, B., Cheval, L., Buhler, J.M., Billon, E., Doucet, A., and Elalouf, J.M. 1999. Serial microanalysis of renal transcriptomes. *Proc. Natl. Acad. Sci. U.S.A.* 96:15286-15291.

Wang, E., Miller, L.D., Ohnmacht, G.A., Liu, E.T., and Marincola, F.M. 2000. High-fidelity mRNA amplification for gene profiling. *Nat. Biotechnol.* 18:457-459.

Ye, S.Q., Zhang, L.Q., Zheng, F., Virgil, D., and Kwiterovich, P.O. 2000. miniSAGE: Gene expression profiling using serial analysis of gene expression from 1 µg total RNA. *Anal. Biochem.* 287:144-152.

Ying, S.Y., Lui, H.M., Lin, S.L., and Chuong, C.M. 1999. Generation of full-length cDNA library from single human prostate cancer cells. *Biotechniques* 27:410-414.

Contributed by Takayoshi Sakai,
Melinda Larsen, and Kenneth M. Yamada
National Institute of Dental and Craniofacial Research
National Institutes of Health
Bethesda, Maryland

SAGE Analysis from 1 µg of Total RNA

Serial analysis of gene expression (SAGE; Velculescu et al., 1995; also see *UNIT 19.3*) is a powerful transcription-profiling method that allows simultaneous expression analysis of thousands of transcripts, provides absolute digital readout of the expression level, and identifies new genes. A disadvantage of SAGE is the relatively high amount of input RNA required. Consequently, several techniques have been developed to overcome this limitation, so that SAGE can be applied to very limited amounts of starting material, e.g., small biological samples such as tissue biopsies or microdissected materials. Here we describe a modified version of the original microSAGE protocol (Datson et al., 1999; also see *http://www.sagenet.org*), which requires only 1 to 2 µg total RNA. This method avoids PCR amplification of cDNA (Peters et al., 1999; Neilson et al., 2000) and reamplification of ditags (Datson et al., 1999; Virlon et al., 1999), procedures which potentially compromise the quantitative nature of this technique. Compared with conventional SAGE, this modified microSAGE protocol has several advantages: (1) reduction of material loss between steps with the use of a "single-tube" procedure for all steps from RNA isolation to tag release; (2) increase in the reaction efficiency with the use of Dynal magnetic beads instead of streptavidin-coated PCR tubes to ensure uniform surface contact; and (3) increase in the recovery and purity of the cDNA with the use of Phase Lock Gel for optimizing phase separation in the several steps that involve phenol extraction.

CONSTRUCTION OF SAGE LIBRARY FROM 1 µg OF TOTAL RNA

BASIC PROTOCOL

The protocol described here is suitable for creating a SAGE library from 1 to 2 µg of total RNA; for less starting material, refer to *UNIT 19.3*. After isolation of polyadenylated RNA using Dynabeads, the procedure includes the following operations: reverse transcription of mRNA into cDNA, digestion with *Nla*III to form one end of the tags, ligation with linkers 1 and 2, cleavage with *Bsm*FI, filling in 5′ overhangs, and PCR amplification with ditag primers 1 and 2. To complete construction of the library, release of 26-bp ditags, ligation of ditags to form concatemers, cloning into pZERO vector, and sequencing are then performed as in *UNIT 19.3*, Basic Protocol 2.

Materials

1 to 2 µg of total RNA from tissues or cells
DEPC-treated H_2O (*APPENDIX 2A*)
Dynabeads mRNA Purification Kit (Dynal) including:
  Oligo$(dT)_{25}$ beads
  Binding buffer
  Washing buffer B
1× first-strand/glycogen buffer (see recipe)
Superscript Choice System for cDNA Synthesis kit (Invitrogen), including:
  5× first-strand reaction buffer
  0.1 M dithiothreitol (DTT)
  10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
  SuperScript II reverse transcriptase
  40 U/µl RNaseOUT recombinant ribonuclease inhibitor
  5× second-strand reaction buffer
  10 U/µl *E. coli* DNA polymerase I
  10 U/µl *E. coli* DNA ligase
  2 U/µl *E. coli* RNase H
  1 U/µl T4 DNA polymerase
0.5 M EDTA, pH 7.5 (*APPENDIX 2A*)

Contributed by Jerry Cai, David Ash, and Ethylin Wang Jabs
Current Protocols in Cell Biology (2002) 19.4.1-19.4.10

1× B&W/SDS/glycogen buffer (see recipe)
1× B&W/BSA buffer (see recipe)
1× NEBuffer 4/BSA solution (see recipe)
LoTE buffer (*UNIT 19.3*)
100× (10 µg/µl) BSA (New England Biolabs; supplied with corresponding restriction enzymes)
10× NEBuffer 4 (New England Biolabs; supplied with corresponding restriction enzymes)
10 U/µl restriction endonuclease *Nla*III (New England Biolabs)
5 U/µl T4 DNA ligase and 10× ligase buffer (Invitrogen)
Annealed linker 1 and annealed linker 2 (see *UNIT 19.3*, Support Protocol 3)
7.5 M ammonium acetate
20 µg/µl glycogen
70% and 100% ethanol
10 U/µl Klenow fragment of DNA polymerase (Amersham Pharmacia Biotech or USB)
Platinum *Taq* DNA polymerase (Invitrogen) and 10× PCR buffer

16°, 50°, 65°, and 75°C water baths or heating blocks
Safe-Lock tubes (Eppendorf) or microcentrifuge tubes
Magnetic Particle Collector (MPC; Dynal, cat. no. 120.20)
Sample mixer (Dynal, cat. no. 947.01)
8-well PCR strips, sterile (USA Scientific)
Thermal cycler

Additional reagents and equipment for PCR (*APPENDIX 3F*) and constructing a SAGE library (see *UNIT 19.3*, Basic Protocol 2)

Isolate polyadenylated RNA

1. Suspend 1 to 2 µg total RNA in a final volume of 100 µl DEPC-treated water in a Safe-Lock or microcentrifuge tube.
2. Heat 2 min at 65°C to disrupt secondary structure. Place on ice.
3. Resuspend Dynabeads oligo$(dT)_{25}$ beads provided in the mRNA Purification Kit by vortexing.
4. Add 100 µl of the resuspended oligo$(dT)_{25}$ beads to a new Safe-Lock or microcentrifuge tube. Put on magnetic particle collector (MPC) for 30 sec, then remove the supernatant solution.
5. Resuspend the beads in 100 µl binding buffer from the Dynabeads kit. Put on MPC for 30 sec, then remove the solution.
6. Add 100 µl binding buffer and the RNA solution from step 2 to the beads, for a total volume of 200 µl in the tube.
7. Rotate on the sample mixer for 3 to 5 min at room temperature to allow hybridization of poly$(A)^+$ RNA to the oligo$(dT)_{25}$ which is immobilized on the beads. Put on MPC for 30 sec, then remove supernatant.
8. Gently wash beads twice, each time by adding 200 µl washing buffer B (provided with the kit) to the beads, then placing the tube on the MPC for 2 min and removing the solution.

Synthesize cDNA with SuperScript Choice kit

9. Wash beads four times with 100 µl 1× first-strand/glycogen buffer using the technique described in step 8.

10. Add the following reagents (provided in the SuperScript kit) to the tube:

    8 µl 5× first strand reaction buffer (directly from kit; no glycogen)
    4 µl 0.1 mM DTT
    2 µl 10 mM dNTP mix
    24 µl DEPC-treated H_2O.

11. Mix the contents by gently vortexing and collect the reaction at the bottom of the tube by microcentrifuging for 3 sec at maximum speed. Incubate 2 min at 37°C.

12. Add 2 µl SuperScript II reverse transcriptase. Incubate 1 hr at 37°C with gentle vortexing every 10 to 15 min, then place on ice to terminate the reaction.

13. Add the following reagents to the tubes:

    40 µl first-strand reaction buffer
    60 µl 5× second-strand reaction buffer
    182 µl DEPC-treated H_2O
    6 µl 10 mM dNTP mix
    2 µl 10 U/µl *E.coli* DNA ligase
    8 µl 10 U/µl *E.coli* DNA polymerase I
    2 µl 2 U/µl *E.coli* RNase H.

14. Mix the contents of the tube by gently vortexing, then collect the reaction at the bottom of the tube by microcentrifuging briefly at maximum speed. Incubate at 16°C for 2 hr, vortexing gently every 10 to 15 min.

15. Add 4 µl 1 U/µl T4 DNA polymerase. Incubate 5 min at 16°C.

16. Add 20 µl 0.5 M EDTA, pH 7.5, to terminate the reaction.

Wash the beads

17. Add 300 µl of 1× B&W/SDS/glycogen buffer, then heat at 75°C for 10 min.

18. Put on MPC for 30 sec, then remove the supernatant. Add 300 µl of 1× B&W/SDS/glycogen buffer, resuspend by vortexing, then put on MPC for 30 sec. Remove the supernatant.

19. Wash beads four times with 300 µl of 1× B&W/BSA buffer using the technique described in step 8.

Cleave the cDNA with anchoring enzyme NlaIII

20. Wash the beads with the bound ds cDNA in the Safe-Lock tube or microcentrifuge tube once with 200 µl of NEBuffer 4/BSA solution using the technique described in step 8.

21. Add the following reagents to the tube:

    173 µl LoTE buffer
    2 µl 100× (10 µg/µl) BSA
    20 µl 10× NEBuffer 4
    5 µl 10 U/µl *Nla*III.

22. Mix by vortexing gently, making sure to avoid bubbles. Collect the reaction in the bottom of the tube by microcentrifuging briefly at maximum speed, then incubate at 37°C for 1 hr, vortexing gently every 10 to 15 min.

23. Inactivate *Nla*III by washing the beads twice with 750 µl 1× B&W/SDS/glycogen buffer using the technique described in step 8. Wash an additional four times with 750 µl 1× B&W/BSA buffer in the same manner.

24. Add 150 µl of 1× T4 DNA ligase buffer and mix by vortexing. Put on MPC for 30 sec, then remove the supernatant.

25. Add 150 µl of 1× T4 DNA ligase buffer. Resuspend by vortexing. Split into two new Safe-Lock or microcentrifuge tubes, each of which should contain 75 µl solution with beads. Label these tube 1 and tube 2.

Ligate linkers to the bound cDNA

26. Put the two tubes prepared in step 25 on the MPC for 30 sec, then remove the supernatants.

27. Add the following to tube 1 and tube 2, respectively:

    Tube 1:
    13 µl LoTE buffer
    2 µl annealed linker 1
    4 µl 5× T4 DNA ligase buffer.

    Tube 2:
    13 µl LoTE buffer
    2 µl annealed linker 2
    4 µl 5× T4 DNA ligase buffer.

    Mix each tube, then heat 2 min at 50°C, and let stand 15 min at room temperature.

28. Add 1 µl of 5 U/µl T4 DNA ligase to each of the tubes, and mix. Incubate 2 hr at 16°C, vortexing gently every 10 to 15 min.

29. Wash beads five times, each time with 50 µl of 1× B&W/BSA buffer, using the technique described in step 8.

Release cDNA with the tagging enzyme BsmFI

30. Wash each tube twice, each time with 200 µl of 1× NEBuffer 4/BSA solution, using the technique described in step 8.

31. Add the following to each of the tubes:

    86 µl LoTE buffer
    10 µl 10× NEBuffer 4
    2 µl 100× (10 µg/µl) BSA
    2 µl 2 U/µl *Bsm*FI.

    Mix each of the reactions by vortexing gently and collect at the bottoms of the tubes by microcentrifuging briefly at maximum speed. Incubate at 65°C for 2 hr.

32. Put the tubes on the MPC for 2 min. Collect and retain the supernatants in separate tubes.

33. Wash beads in each tube twice with 50 µl LoTE buffer, using the technique described in step 8. Pool washings with the corresponding supernatants from step 32.

    Each tube should now contain 100 + (2 × 50) = 200 µl of solution.

34. Perform phenol/chloroform/isoamyl alcohol extraction on supernatants from each tube using Phase Lock Gel Heavy (see Support Protocol).

35. Ethanol precipitate by adding the following:

    133 µl 7.5 M ammonium acetate
    3 µl 20 µg/µl glycogen
    1000 µl 100% ethanol.

    Mix well and place on dry ice for 10 to 20 min. Microcentrifuge 30 min at maximum speed, 4°C, then carefully remove and discard the supernatants without disturbing the pellets.

36. Wash each pellet twice, each time by adding 200 µl of 70% ethanol down the wall of the tube with a micropipet, taking care to avoid disrupting the pellet, then decanting slowly. After the final wash, microcentrifuge briefly at maximum speed to collect residual ethanol. Remove all of the ethanol with a micropipet and air dry the pellets for 5 to 10 min. Resuspend each of the two pellets in 10 µl LoTE buffer.

Blunt-end the linked SAGE tags

37. Add the following reagents to tubes 1 and 2 containing 10 µl linked SAGE tags:

    10 µl 5× second-strand buffer (from SuperScript kit)
    1 µl 100× (10 µg/µl) BSA
    2.5 µl 10 mM dNTP mix
    23.5 µl H_2O
    3 µl 10 U/µl Klenow fragment of DNA polymerase.

    Incubate at 37°C for 30 min.

38. Adjust the volume in each tube to 200 µl by adding LoTE buffer, then combine the solutions in both tubes (for a total of 400 µl solution). Mix well and split into two new tubes labeled "+Ligase" and "–Ligase," each containing 200 µl solution.

39. Perform phenol/chloroform/isoamyl alcohol extraction on each of the solutions using 200 µl Phase Lock Gel Heavy (see Support Protocol). Ethanol precipitate the DNA from each of the tubes as in steps 35 to 36, then resuspend each pellet in 4 µl LoTE.

Ligate the linked SAGE tags to form linked ditags

40. Prepare the following two reaction mixes as follows:

    +Ligase:

    4 µl resuspended DNA from "+Ligase" sample (step 39)
    1.2 µl 5× T4 DNA ligase buffer
    0.8 µl 5 U/µl T4 DNA ligase.

    –Ligase:

    4 µl resuspended DNA from "–Ligase" sample (step 39)
    1.2 µl 5× T4 DNA ligase buffer
    0.8 µl H_2O.

    Mix each of the reactions and incubate at 16°C overnight.

41. After ligation, add 14 µl LoTE buffer to each of the reaction mixtures to raise the final volume to 20 µl.

PCR amplify ditags

42. Dilute the following samples (from step 41) with sterile water as follows:

    "–Ligase" (control) to 1/5 (1 µl sample + 4 µl H_2O).
    "+Ligase" (ligation product) to 1/5, 1/20, 1/50, 1/100, 1/200, 1/400.

43. Set up 8 PCR reactions using a sterile 8-well PCR strip as follows:

    Positions 1 to 6: Different dilutions of the template ("+ligase"; step 42)
    Position 7: No-ligase ("–ligase") control
    Position 8: H_2O control.

44. To each well add the following:

    5 µl 10× PCR Buffer (supplied with Platinum *Taq* polymerase)
    1 µl 10 mM dNTP mix
    1.5 µl 50 mM $MgCl_2$
    1 µl 350 ng/µl primer 1 (*UNIT 19.3*)
    1 µl 350 ng/µl primer 2 (*UNIT 19.3*)
    38.5 µl H_2O
    1 µl Platinum *Taq* DNA polymerase
    1 µl ligation product (at various dilutions) or control (see step 43).

45. Mix and amplify using the following cycle conditions:

1 cycle:	4 min	95°C	(denaturation)
26-30 cycles:	30 sec	95°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	4 min	72°C	(final extension).

46. Pick the lowest dilution of template that gives a clear 102-bp PCR product.

    The optimum PCR conditions are critical for isolating adequate DNA for SAGE. Using too few cycles or a substrate that is too diluted will result in a low yield, which may cause problems in the subsequent steps. Too many cycles or a substrate that is too concentrated will give erratic results. Therefore, it is very important to pick the right parameters to do large-scale PCR.

47. Using the PCR conditions in step 45 and the dilution identified in step 46, perform several hundred PCR reactions.

    Usually 384 reactions are performed in four 96-well plates to generate 10 to 20 µg DNA.

48. Complete the construction of the SAGE library continuing with step 43 of Basic Protocol 2 in *UNIT 19.3* ("Isolating Linked Ditags").

SUPPORT PROTOCOL

PCI EXTRACTION

Phase-Lock Gel will form a semisolid barrier, so one can extract the aqueous layer without contacting the organic phase. The advantage to using Phase-Lock Gel over the standard phenol/chloroform/isoamyl alcohol extraction is that it saves time while optimizing the recovery of DNA.

Materials

DNA solution to be extracted
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol solution (PCI; Invitrogen)
Phase-Lock Gel Heavy in 2-ml tubes (Eppendorf)

1. Microcentrifuge the 2-ml Phase-Lock Gel Heavy tube for 1 min at maximum speed without added sample.
2. Add the sample and an equal volume of PCI solution to the tube.
3. Shake to mix.
4. Microcentrifuge 5 min at maximum speed.
5. Transfer the top aqueous phase to a new microcentrifuge tube using a pipet.

SUBSTITUTING *Hsp*92II FOR *Nla*III AS THE ANCHORING ENZYME

ALTERNATE PROTOCOL

The anchoring enzyme *Nla*III is used, but it is very unstable, even at −80°C. *Hsp*92II has the same recognition and cut site, 5′-CATG-3′, as *Nla*III, so it can also be used as an anchoring enzyme.

Additional Materials *(also see Basic Protocol)*

10 U/µl restriction endonuclease *Hsp*92II (Promega)
100× BSA and 10× buffer K (provided with *Hsp*92II)

1. Isolate polyadenylated RNA and synthesize cDNA with SuperScript system (see Basic Protocol, steps 1 to 19).
2. Wash the beads with the bound ds cDNA in the Safe-Lock or microcentrifuge tube from step 19 of the Basic Protocol once with 200 µl of 1× NEBuffer 4/BSA solution, using the washing technique described in step 8 of the Basic Protocol.
3. Add the following reagents to the tube:

   173 µl LoTE buffer
   2 µl 100× BSA
   20 µl 10× Buffer K
   5 µl 10 U/µl *Hsp*92II.

4. Mix by vortexing gently, making sure to avoid bubbles. Collect the reaction in the bottom of the tube by microcentrifuging briefly at maximum speed, then incubate at 37°C for 1 hr, vortexing gently every 10 to 15 min.
5. Inactivate *Hsp*92II by washing twice with 750 µl 1× B&W/SDS/glycogen buffer using the washing technique described in step 8 of the Basic Protocol, then wash an additional four times with 750 µl 1× B&W/BSA buffer in the same manner.
6. Continue with the SAGE procedure (see Basic Protocol, steps 24 to 48).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

B&W/BSA buffer, 1×

To prepare 4 ml, mix the following:
2 ml 2× B&W buffer (see recipe)
40 µl 100× (10 µg/µl) BSA (New England Biolabs; provided with the restriction enzymes)
1.96 ml H_2O
Store up to 1 week at 4°C

B&W buffer, 2×

To prepare 10 ml, mix the following:
100 µl 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
20 µl 0.5 M EDTA, pH 7.5 (*APPENDIX 2A*)
4 ml 5 M NaCl (*APPENDIX 2A*)
5.88 ml H_2O
Store up to 1 week at room temperature

B&W/SDS/glycogen buffer, 1×

To prepare 3 ml, mix the following:
1.5 ml 2× B&W buffer (see recipe)
30 µl 1 µg/µl glycogen
300 µl 10% (w/v) SDS
1.17 ml H_2O
Prepare fresh just prior to use

First strand/glycogen buffer, 1×

To prepare 500 µl, mix the following:
100 µl 5× first-strand buffer (Invitrogen; provided with the SuperScript II kit)
5 µl 1 µg/µl glycogen (prepare with DEPC-treated H_2O; store at 4°C)
395 µl DEPC-treated H_2O (*APPENDIX 2A,* or provided with SuperScript II kit)
Store up to 1 week at 4°C

NEBuffer 4/BSA solution, 1×

To prepare 2 ml, mix the following:
200 µl 10× NEBuffer 4 (New England Biolabs)
20 µl 100× (10 µg/µl) BSA (New England Biolabs; provided with restriction enzymes)
1.78 ml H_2O
Store up to 1 week at 4°C

COMMENTARY

Background Information

The Basic Protocol in this unit optimizes the construction of a SAGE library from 1 µg of total RNA by combining Dynabeads mRNA isolation with SAGE. Preparation of high-quality and full-length mRNA is essential for gene-expression studies. Dynabeads Oligo$(dT)_{25}$ was chosen in the protocol to isolate poly$(A)^+$ RNA from total RNA. The isolation protocol relies on the base pairing between the poly(A) residues at the 3′ end of mRNA and the oligo (dT) residues covalently coupled to the surface of the Dynabeads. The unique paramagnetic properties of Dynabeads facilitate easy and efficient handling, while also reducing the requirement for centrifugation and precipitation steps, thereby significantly increasing yield. Using a solid phase enables all procedures to be conducted in a single tube and ensures that losses caused by multiple sample manipulations are minimized. This is particularly important in this protocol, since the starting total RNA is only 1 to 2 µg, and the detection of low-abundance transcripts is desired. The true spherical shape and the hydrophilic surface of the Dynabeads Oligo$(dT)_{25}$ also eliminates clumping and nonspecific binding associated with irregularly shaped particles, achieving uniformity of the reaction. The isolated mRNA can then be reverse transcribed to double-stranded cDNA and subjected to the standard SAGE procedure (also see *http://www.sage-net.org*).

It is well known that *Nla*III is very unstable even at −80°C. *Nla*III often digests DNA with low efficiency because it is very sensitive to digestion conditions. Recently the authors switched to *Hsp*92II, which is an isoschizomer of *Nla*III, but has a long half-life and is not very sensitive to digestion conditions. The resulting digestion (see Alternate Protocol) is improved and more consistent, giving a better yield of SAGE tags.

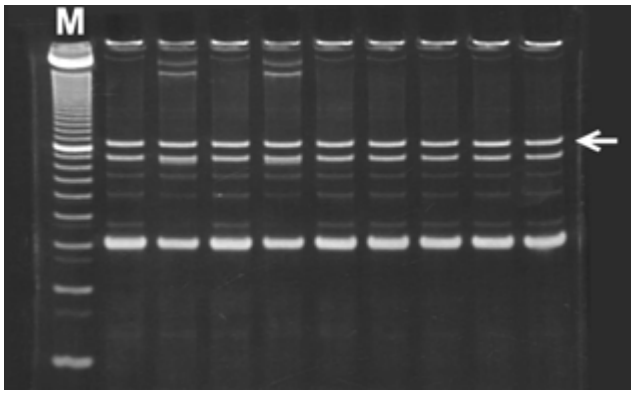


Figure 19.4.1 Linked SAGE ditags from large-scale PCR amplification are separated on a 12% polyacrylamide gel. The 102-bp bands corresponding to the linked SAGE ditags are indicated by an arrow. Lane M, 10-bp DNA ladder.

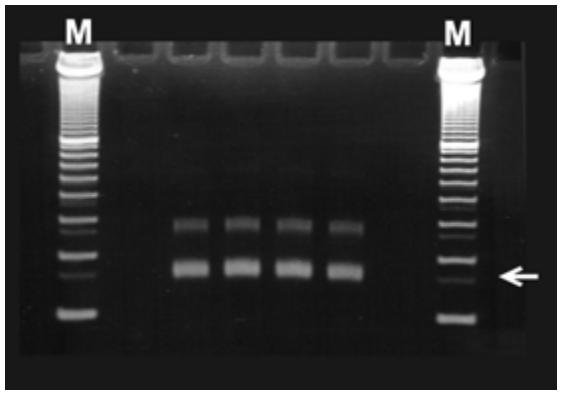


Figure 19.4.2 SAGE ditags digested by *Nla*III are separated on a 12% polyacrylamide gel. The 26-bp bands corresponding to the SAGE ditags without linkers are indicated by an arrow. The other bands (around 40-bp) are linkers. Lane M, 10-bp DNA ladder.

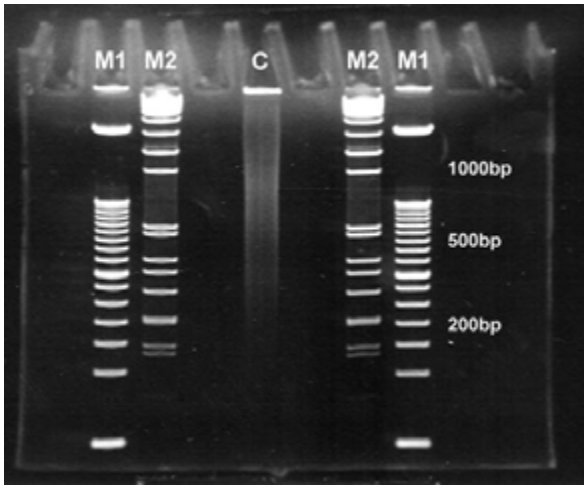


Figure 19.4.3 SAGE concatemers are separated on an 8% polyacrylamide gel. The concatemers appear as a smear in the lane indicated by C. Lane M1, 50-bp DNA ladder; lane M2, 1-kb DNA ladder.

Readers should also see the Commentary in *UNIT 19.3* to get more background information about SAGE.

Critical Parameters and Troubleshooting

RNA preparation

RNA quality is very critical to the success of the SAGE protocol. A high-quality RNA preparation without DNA contamination, salt, or other substances that might inhibit enzymatic activities is desired. If there is sufficient RNA, the integrity of the RNA should be checked on a denaturing RNA gel before the experiment.

mRNA isolation and cDNA synthesis

Isolation of mRNA is performed with the Dynabeads mRNA isolation kit. Since the beads tend to precipitate to the bottom, it is necessary to mix the solution from time to time during the incubation period by hand vortexing (flicking the bottom of the tube with one's finger) to ensure the uniformity of bead suspension.

Also see the Critical Parameters and Troubleshooting section in *UNIT 19.3* for more information.

Anticipated Results

Synthesis of 102-bp SAGE ditags

Figure 19.4.1 shows the typical result after large-scale PCR reaction. The 102-bp bands of linker SAGE ditags should be clearly visible for purification. This step usually generates 10- to 20-µg ditags.

NlaIII digestion of SAGE tags

Figure 19.4.2 shows a typical result after releasing 26-bp ditags by *Nla*III digestion. The 26-bp bands of SAGE ditags without linkers should be clearly visible from background bands. Since *Nla*III is unstable even at −80°C, *Hsp*92II may be used instead (see Alternate Protocol).

Formation of SAGE concatemers

Figure 19.4.3 shows a typical result of formation of SAGE concatemers. A smear should be clearly visible.

Sequencing clones and analysis of sequence data

From 1 to 2 µg total RNA, it is possible to generate 50,000 SAGE tags.

Time Considerations

In the Basic Protocol, steps 1 to 47 (including the large-scale PCR performed in step 47) take 1 week to complete. After large-scale PCR, step 48 in the Basic Protocol of this unit (which would comprise steps 43 to 106 in Basic Protocol 2 of *UNIT 19.3*) takes 2 weeks to complete.

The time requirement for sequencing clones and analysis of sequencing data depends on the number of tags and efficiency of the sequencing facilities used.

Literature Cited

Datson, N.A., van der Perk-de Jong, J., van den Berg, M.P., de Kloet, E.R., and Vregdenhil, E. 1999. MicroSAGE: A modified procedure for serial analysis of gene expression in limited amounts of tissue. *Nucleic Acid Res.* 27:1300-1307.

Neilson, L., Andalibi, A., Kang, D., Coutifaris, C., Strauss, J.F. 3rd, Santon, J.A., and Green, D.P. 2000. Molecular phenotype of the human oocyte by PCR-SAGE. *Genomics* 63:13-24.

Peters, D.G., Kassam, A.B., Yonas, H., O'Hare, E.H., Ferrell, R.E., and Brufsky, A.M. 1999. Comprehensive transcript analysis in small quantities of mRNA by SAGE-lite. *Nucleic Acid Res.* 27:E39.

Velculescu, V.E., Zhang, L., Vogelstein, B., and Kenzler, K.W. 1995. Serial analysis of gene expression. *Science* 270:484-487.

Virlon, B., Cheval, L., Buhler, J.M., Billon, E., Doucet, A., and Elalouf, J.M. 1999. Serial microanalysis of renal transcriptomes. *Proc. Natl. Acad. Sci. U.S.A.* 96:15286-15291.

Contributed by Jerry Cai, David Ash, and Ethylin Wang Jabs
Johns Hopkins University
Baltimore, Maryland

The Chick Chorioallantoic Membrane as an In Vivo Angiogenesis Model

BASIC PROTOCOL

This unit presents the basic steps involved in the chick chorioallantoic membrane (CAM) assay, a model originally developed to study the angiogenic activity of tumor samples (Ausprunk et al., 1974; Auerbach et al., 1974). One of the major advantages of this method is that it is the simplest of the in vivo angiogenic models. In contrast to other in vivo models, the CAM assay does not require any major training or highly technical surgical skills. In addition, there is no need for sophisticated equipment, and it does not require an animal protocol. The uninitiated can quickly learn this simple method. Although the CAM assay is a simple procedure, it requires considerable "hands-on" effort for the following two reasons. First, the initial number of eggs needed per data point (~20) is large due to embryo mortality during the assay, and second, the background of positive responses is ~20%. Other disadvantages to this assay include the fact that it takes 10 days to complete and that the number of samples that can be tested is limited by the number of embryos required. Therefore, the CAM assay is not recommended as a screening technique, but rather as a procedure that can be used to confirm results from other experiments suggesting that a substance has a potential to be active in vivo. Overall, the advantages well outweigh the disadvantages, since it is an in vivo assay that can be readily performed in any laboratory setting.

Materials

3-day-old embryonated chicken eggs (~20 eggs/condition), maintained in 37°C incubator (an egg incubator is not necessary)
70% ethanol
Test substance(s)
Positive control: bFGF (PeproTech) or another angiogenic stimulus
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Heavy cream

Sterile dissecting implements:
- Scalpel
- Curved tweezers
- Smooth-tipped (nonserrated) tweezers
- Scissors

6-ml syringes, sterile
20-G, 1.5-in. (3.5-cm) needles, sterile
1-in. (2.5-cm) wide clear adhesive tape
Single-edged razor blades
13-mm-diameter Thermanox coverslips (Nunc), sterile
Stereoscopic microscope of variable magnification, equipped with camera and light source

NOTE: As an additional, optional sterility precaution, this protocol may be performed in a sterile laminar-flow hood (also see *UNIT 1.3*).

Contributed by M. Lourdes Ponce and Hynda K. Kleinman
Current Protocols in Cell Biology (2003) 19.5.1-19.5.6

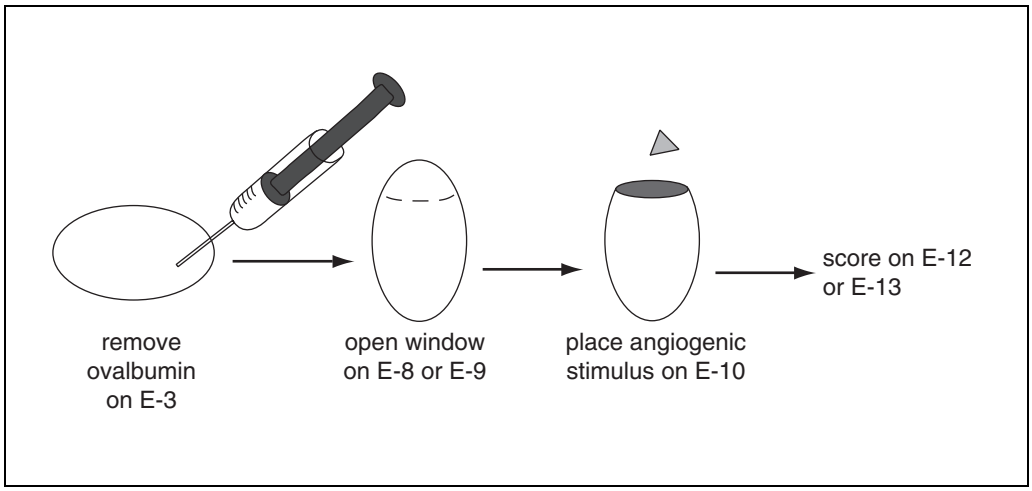


Figure 19.5.1 Schematic representation of the CAM procedure. Using a syringe and needle, the first step is to remove 3 to 4 ml of ovalbumin from 3 day-old embryonated eggs. The next step is to open a window on E-8 or E-9 after scoring the shell with a razor blade and pulling the egg shell out with a pair of tweezers. After the sample is dried out on the coverslip, the stimulus is placed on the CAM for 2 to 3 days. The eggs are then evaluated for angiogenesis under a stereoscopic microscope.

Prepare eggs

1. On embryonal day 3 (E-3), when the embryo is still small, prepare eggs to be used for the procedure by spraying them with 70% ethanol to minimize contamination. Make a small hole with a scalpel on the wide end of the egg, where the air sac is located (Fig. 19.5.1).

   IMPORTANT NOTE: Also spray eggs with 70% alcohol before each subsequent step in which they are to be opened.

2. Lay the egg sideways (Fig. 19.5.1). Insert the sterile needle with syringe into the hole at an angle of ~45°, without touching the far end of the egg shell, to a depth of ~1 to 1.25 in. (equivalent to 2.5 to 3.1 cm), and gently remove 3 to 4 ml of ovalbumin. Return the egg to its original position.

   If yolk is being removed, withdraw the needle and reposition it to withdraw ovalbumin.

   The ovalbumin ("egg white") is pale yellow in color. If any bright yellow-orange material is seen in the syringe, yolk is being aspirated and the viability of the embryo could be compromised. Ovalbumin is removed to lessen the volume of material in the egg so contents do not spill out when the window is opened.

   If only a small amount of yolk is removed, continue to incubate the egg as the embryo may survive.

3. Cover the hole with a small piece of tape, 0.5 to 0.75 in. (1.25 to 1.9 cm) long. Proceed in a similar manner with the rest of the eggs, making sure to return them to the incubator within 45 min to avoid decreased viability. Continue incubation.

4. On E-9, using a single-edged razor blade, score a circle (~1 in., or 2.5 cm, in diameter) or a square (of ~1 $in.^2$, or 6.25 cm^2) through the tape covering the egg (Fig. 19.5.1). Insert sterile curved tweezers through the needle hole created on E-3, puncturing the tape, and gently push up from inside the egg against the eggshell and all around the scored area until the shell comes out to produce an opening of the above dimensions in the eggshell. Remove any remaining membrane from the area immediately inside the window and close the opening with tape. Discard unviable embryos or unfertilized eggs.

If it is not possible to open the window on E-9, it can alternatively be done earlier on either E-7 or E-8, as necessary. Earlier times are not recommended since it increases the chances of contamination and decreases the number of viable chicks.

Unviable or dying embryos can be detected by observation. In these cases, vessels do not appear intact, the egg yolk looks discolored, and/or the embryo no longer moves.

Prepare the angiogenic stimuli

5. On E-10, examine embryos and determine the number of viable eggs; discard unviable or dying embryos (see annotation to step 4). Calculate the number of samples to be prepared for each condition.

   It is important that there be at least 12 eggs for each sample since some of the embryos will continue to die.

6. Using sterile conditions and instruments, cut the coverslips into quarters with scissors while holding with sterile smooth-tipped tweezers in such a way as to avoid scratches.

7. Dissolve the test substance in distilled water and place a 5-µl aliquot containing the desired amount to be tested in the center of one quarter of a coverslip. Prepare a minimum of 12 coverslips per sample (it is advisable to test at least two different concentrations of each test substance). Include controls that contain water only (negative control) or 50 ng of bFGF (positive control).

   Samples should be dissolved in water. The presence of buffers or salts might create crystal deposits that can yield false positive results. Variations of the assay that allow the presence of salts, buffers, media or cells have been developed using small collagen gels (see Gho et al., 2001).

8. Allow the coverslips to air dry for 10 to 20 min. Open the window by cutting off the tape from each egg, then place the air-dried coverslip containing the corresponding sample on top of the CAM with the sample in direct contact with the CAM. Cover the eggs with tape, and return them to the incubator.

   The area selected for placing the coverslip should be free of large vessels and away from any eggshell that might had fallen in on the CAM, since the shell can induce angiogenesis.

Score CAM for angiogenesis and photograph

Scoring of the CAM can take place on either day E-12 or E-13 depending on the degree of angiogenesis elicited by the test compound. Therefore, it is recommended that the CAM be examined on both days and that the best day for scoring be determined depending on the number of positive samples observed.

9. Remove the tape from the egg and using a stereoscopic microscope, look at the area where the coverslip was placed.

10. Score the CAMs by assessing the number of positive or negative responses or by assigning a score number varying from 0 to 3, in which 0 is no response and 3 is a strong response.

    A strong positive angiogenic stimulus will result in vessels that radiate from the center of the coverslip simulating spokes from a wheel (Fig. 19.5.2). When the effect is not as strong, smaller and fewer vessels will be observed. In some instances, a very weak response can be detected in the form of small capillaries. Another way in which a very weak response can be seen is by observing the orientation of the major vessels near the area of the stimulus; the vessels may appear to make a turn towards this area, which is evidence of a very weak stimulus. These very weak responses are scored as 1 (see Fig. 19.5.2).

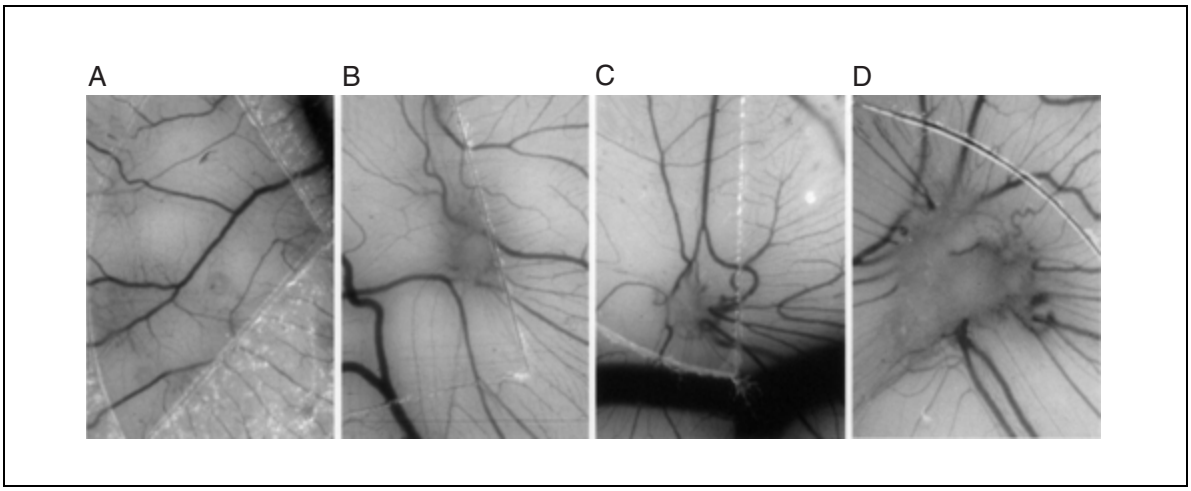


Figure 19.5.2 Positive responses and scoring. Examples of CAMs show different degrees of positive response. (**A**) A typical negative control showing normal CAM vessel levels but no induction of angiogenesis. Score = 0. (**B**) A slight response is observed in which some of the major vessels turn towards the stimuli. Smaller newly formed vessels are also observed. Score = 1. (**C**) A stronger angiogenic response is present. More, larger vessels are recruited to the area and a small "spoke wheel" begins to appear. Score = 2. (**D**) An intense angiogenic response is shown with large vessels and many smaller ones. The spoke wheel is present. Score = 3.

11a. *Photograph the CAM in situ:* Without dissecting, inject 3 to 4 ml of contrasting medium under the CAM consisting of a 1:1 mixture of PBS and heavy cream (or a similar substance with high buoyancy that would provide a contrasting background).

11b. *Photograph the CAM in dissected membrane:* Dissect out the area where the stimulus was placed using scissors and forceps, transfer it to a tissue culture dish, place the dish on a contrasting material, and photograph using the camera attached to the stereoscopic microscope.

COMMENTARY

Background Information

The formation of new blood vessels from pre-existing ones, i.e., angiogenesis, is important for physiological processes that occur during pregnancy, the menstrual cycle, and wound repair (Smith, 2001; Reynolds and Redmer, 2001; Sherer and Abulafia, 2001; Tonnesen et al., 2000). In addition, several pathological conditions have been associated with changes in the normal number of vessels present in various tissues. For instance, an increase in the level of the vascular supply has been linked to rheumatoid arthritis, psoriasis, corneal angiogenesis, and diabetic retinopathy, one of the leading causes of blindness in the U.S.A. (Arbiser, 1996; Yoshida et al., 1999; Campochiaro, 2000; Brenchley, 2001; Chang et al., 2001; Spanger and Pfeiffer, 2001). Vascular insufficiency, on the other hand, can also lead to serious conditions, such as myocardial ischemia, a form of heart disease, or ischemia of the limbs which can result, in extreme cases, in amputation (Majesky, 1996; Freedman and Isner, 2001; Vale et al., 2001). In recent years, angiogenesis has been the focus of numerous studies that utilize antiangiogenic agents to inhibit cancer. The vascularization of tumors is essential for their growth and, in advanced stages, for mestastasis. In essence, tumor growth and metastasis could be prevented by utilizing drugs that stop new vascularization. A number of laboratories have focused their efforts on the development of new antiangiogenic therapies that can affect tumor vessel formation. Several of these compounds are currently in clinical studies, including, but not limited to, endostatin, which is a collagen XVIII fragment, as well as thalidomide, interferon α, and antibodies to vascular endothelial growth factor and to integrin $\alpha v\beta 3$.

Angiogenesis is a complex multistep process that is usually initiated by a signal, an angiogenic stimulator, produced and/or released in the surrounding microenvironment. When the signal reaches the endothelium and binds to its receptor, the cell becomes activated

and the angiogenic cascade is initiated. This cascade includes degradation of the matrix, migration of endothelial cells, proliferation, establishment of a new matrix, and tubule formation and maturation. Due to the large number of conditions that are affected by angiogenesis and to the complexity of the process, it has been necessary to develop numerous methodologies for its study. Various in vitro assays have been used for this purpose and include migration, proliferation, formation of tubes on Matrigel or collagen gels, and aortic ring sprouting. In vivo studies include the rabbit corneal pocket assay, implanted sponges, subcutaneous Matrigel plugs, and the chick chorioallantoic membrane (CAM) assay, among others.

Angiogenesis in the chick CAM is a process that can be readily studied. During the development of the chick, the CAM undergoes rapid neovascularization—i.e., the formation of new vessels—which is complete by embryonal day 10 (E-10). When this neovascularization stops, the angiogenic process can be investigated. For this reason, the CAM assay is usually initiated on E-10, although some investigators have reported the application of the sample at earlier time points. It should also be noted that neovascularization can also be examined using the model described here, except that the sample is applied at an earlier stage (E-5 or E-6) when neovascularization is still taking place. In this case, the results are also observed 2 or 3 days later. In a similar manner, angiogenesis inhibitors can also be examined by mixing them together with an angiogenesis stimulator such as bFGF (Ponce et al., 2001).

Troubleshooting

As mentioned above, one of the caveats of the CAM assay is the high number of spontaneous positive responses obtained. In addition, false-positive responses can also be caused by the presence of salt crystals in the sample or pieces of eggshell that become lodged inside the egg. These responses are usually characterized by an inflammatory response that can cause indirect induction of angiogenesis.

Embryo viability, which can range between 30% and 70%, can also be seriously affected, in some cases, by the length of transportation between the farm and the laboratory, as well as the conditions of the trip, including bumpy rides and the time the eggs spend outside of the incubator. Although some of these issues can be addressed, often they are beyond the control of the investigator. Another serious problem that can reduce embryo viability and/or the number of samples is contamination. This problem can be reduced by following more stringent sterile techniques including the use of sterile equipment, careful swiping of the eggs with betadine or 70% alcohol before opening, working in a sterile hood in extreme cases, or maintaining a low incubator humidity.

At a minimum an experiment requires 12 embryos per treatment group, 12 embryos for a negative control, and 12 embryos for a positive control. If at the end of an experiment the number of embryos is too low and no statistical differences are observed between the negative and positive controls, the experiment should be discarded.

Anticipated Results

Many angiogenic and anti-angiogenic compounds have been tested using this assay including growth factors, such as bFGF, VEGF, and ICAM, antibodies (anti-bFGF, VEGF, and integrins), peptides and protein fragments (laminin and fibronectin, thrombospondin and collagen-derived), and small molecules (Auerbach and Auerbach, 1994; Ribatti et al., 2001). The angiogenic response will vary according to the strength of the compound being tested. For example, bFGF will usually give a strong response in which a well-formed spoke-wheel can be observed in as many as 80% of the eggs tested. Less angiogenic substances may not form a spoke-wheel, but the number of positive CAMs and strength of the response will vary depending on their angiogenicity and/or concentration. This will aslo be the case for anti-angiogenic molecules, in which the most potent ones will greatly reduce the angiogenicity of the inducing agent at lower amounts, whereas, weaker ones could require much larger amounts to observe a decreased response. All these factors will ultimately have to be experimentally determined by the investigator.

Time Considerations

The CAM assay takes 9 to 10 days to complete. The amount required for each one of the steps is as follows. Removal of ovalbumin on the first experimental day, E-3, requires ~1 to 1.5 hr per every 100 eggs. Opening of the windows on E-9 will take a minimum of 3.5 hr per 100 eggs depending on experience. On E-10, allow 2 to 3 hr for sample preparation and placing the coverslips on the CAM. On E-12, allow 0.5 to 1 hr for checking the angiogenic response. On the final day, E-12 or E-13, allow 2 to 3 hr to score and photograph the CAMs.

Literature Cited

Arbiser, J.L. 1996. Angiogenesis and the skin: A primer. *J. Am. Acad. Dermatol.* 4:486-497.

Auerbach, W. and Auerbach, R. 1994. ANgiogenesis inhibition: A review. *Pharmacol. Ther.* 63:265-311.

Auerbach, R., Kubai, L., Knighton, D., and Folkman, J. 1974. A simple procedure for the long-term cultivation of chicken embryos. *Dev. Biol.* 41:1391-394.

Ausprunk, D.H., Knighton, D.R., and Folkman, J. 1974. Differentiation of vascular endothelium in the chick chorioallantois: A structural and autoradiographic study. *Dev. Biol.* 38:237-248.

Brenchley, P.E. 2001. Antagonising angiogenesis in rheumatoid arthritis. *Ann. Rheum. Dis.* 60 Suppl 3:iii71-74.

Campochiaro, P.A. 2000. Retinal and choroidal neovascularization. *J. Cell Physiol.* 184:301-310.

Chang, J.H., Gabison, E.E., Kato, T., and Azar, D.T. 2001. Corneal neovascularization. *Curr. Opin. Ophthalmol.* 12:242-249.

Freedman, S.B. and Isner, J.M. 2001. Therapeutic angiogenesis for ischemic cardiovascular disease. *J. Mol. Cell Cardiol.* 33:379-393.

Gho, Y.S., Kim, P.N., Li, H.C., Elkin, M., and Kleinman, H.K. 2001. Stimulation of tumor growth by human soluble intercellular adhesion molecule-1. *Cancer Res.* 61:4253-4257.

Majesky, M.W. 1996. A little VEGF goes a long way: Therapeutic angiogenesis by direct injection of vascular endothelial growth factor-encoding plasmid DNA. *Circulation* 94:3062-3064.

Ponce, M.L., Nomizu, M., and Kleinman, H.K. 2001. An angiogenic laminin site and its antagonist bind through the alpha(v)beta3 and alpha5beta1 integrins. *FASEB J.* 15:1389-1397.

Reynolds, L.P. and Redmer, D.A. 2001. Angiogenesis in the placenta. *Biol. Reprod.* 64:1033-1040.

Ribatti, D., Nico, B., Vacca, A., Roncali, L., Burri, P.H., and Djonov, V. 2001. Chorioallantoic membrane capillary bed: A useful target for studying angiogenesis and anti-angiogenesis in vivo. *Anat. Rec.* 264:317-324.

Sherer, D.M., and Abulafia, O. 2001. Angiogenesis during implantation, and placental and early embryonic development. *Placenta* 22:1-13.

Smith, S.K. 2001. Regulation of angiogenesis in the endometrium. *Trends Endocrinol. Metab.* 12:147-151.

Spanger, J. and Pfeiffer, A.F. 2001. New concepts in pathogenesis and treatment of diabetic retinopathy. *Exp. Clin. Endocrinol. Diabetes* 109(Suppl):S438-S450.

Tonnesen, M.G., Feng, X., and Clark, R.A. 2000. Angiogenesis in wound healing. *J. Investig. Dermatol. Symp. Proc.* 5:40-46.

Vale, P.R., Isner, J.M., and Rosenfield, K. 2001. Therapeutic angiogenesis in critical limb and myocardial ischemia. *J. Interv. Cardiol.* 14:511-528.

Yoshida, A., Yoshida, S., Ishibashi, T., and Inomata, H. 1999. Intraocular neovascularization. *Histol. Histopathol.* 14:1287-1294.

Contributed by M. Lourdes Ponce and
Hynda K. Kleinman
National Institutes of Health
Bethesda, Maryland

Experimental Metastasis Assays in the Chick Embryo

Tumor metastasis is the spread of cancer from a primary tumor to form secondary tumors in distant organs. This process consists of a series of steps, starting with the release of cancer cells from a primary tumor into the blood or lymphatic circulation (intravasation), the transport of cells to distant organs via the circulation, arrest in capillaries in these sites, exit of the cancer cells from the circulation into the tissues of the new organ (extravasation), and the growth of some of these cells to form metastatic tumors in the new organ. Experimental metastasis assays model the latter half of this process by injection of cancer cells into the circulation of an experimental animal and the detection of the formation of metastatic tumors. This is in contrast to spontaneous metastasis assays, in which a primary tumor is created by injection of cancer cells into an appropriate tissue of an experimental animal and the presence of metastatic tumors in distant organs is assessed, which will not be discussed here.

Mice are most commonly used for experimental metastasis assays; however, for a variety of reasons (see Background Information), there are some distinct advantages to using alternate assays. For example, the chick embryo can provide a convenient and cost-effective animal model in which to study the process of tumor metastasis. It is a naturally immune-deficient host and can thus be used to assess the metastatic potential of cancer cells from a variety of species, including human and mouse. Also, housing requirements are minimal and accessible to most laboratories. Chicken embryos are readily available and experimental metastasis assays in this system are straightforward. Experimental metastasis assays can be combined with in vivo videomicroscopy on readily accessible thin tissues, such as the chick embryo chorioallantoic membrane (CAM), in order to learn about individual steps in the metastatic process. Methods for using chick embryos for experimental metastasis assays (see Basic Protocol 1) and in vivo videomicroscopy (see Basic Protocol 2) are provided in this unit, as are the details of routine maintenance of the embryos (see Support Protocol 1). Additional protocols for labeling cells with fluorescent nanospheres (see Support Protocol 2) and for assessing cells in tissues using fluorospheres (see Support Protocol 3) are also included.

NOTE: All protocols using live animals must be first reviewed and approved by the appropriate Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of these animals.

NOTE: All solutions and equipment coming into contact with cells and tissues must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Do not use a culture incubator for eggs.

EXPERIMENTAL METASTASIS ASSAY IN THE CHICK EMBRYO

BASIC PROTOCOL 1

Experimental metastasis assays measure the ability of cells injected intravenously into the circulation to form metastatic tumors in distant organs (see Welch 1997 for general review of techniques commonly used). In this assay using the chick embryo, which was described in 1982 by Chambers et al. (see Chambers and Tuck, 1988 for review), cells are injected intravenously (i.v.) into chorioallantoic membrane (CAM) veins. An illustration of this experimental approach is presented in Figure 19.6.1, which diagrams the injection of cells into a CAM vein of an 11-day-old chick embryo. Cells injected by this

Contributed by Sylvia M. Wilson and Ann F. Chambers
Current Protocols in Cell Biology (2003) 19.6.1-19.6.24

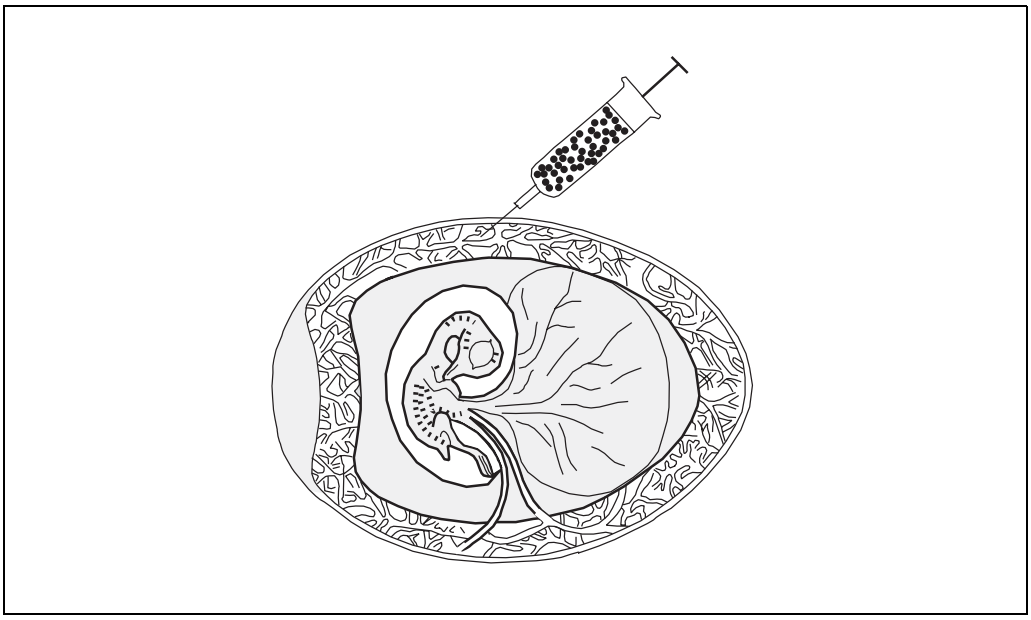


Figure 19.6.1 Diagram of experimental metastasis assay in chick embryos. Cancer cells are injected via a chorioallantoic membrane (CAM) vein, through a window opened in the shell of 11-day-old chick embryos. Cells injected via this route travel via the circulation first to the embryonic liver. After incubation for up to 7 days, metastatic cells in an embryonic organ such as liver are quantified using the ouabain plating assay, or (for rapidly growing cell lines) macroscopic metastases can be quantified. Republished with permission of Anticancer Research from Chambers and Tuck (1988).

route are taken by the circulation first to the embryonic liver. Some cell lines may grow rapidly in vivo in the chick, such that macroscopic metastatic tumors are visible in the liver and other internal organs at 7 days postinjection (Chambers and Ling, 1984; Chambers and Wilson, 1988); however, many cell lines, even highly metastatic ones, may not grow visible tumors in the time period available before embryo hatching. For these cell lines, the ouabain plating assay was developed (Chambers et al., l982). This assay permits the quantification of numbers of viable rodent cells in chick organs at various times after injection.

The ouabain plating procedure is based on natural interspecies differences in sensitivity to the cytotoxic effects of the drug ouabain: rodent cells are inherently considerably more resistant to ouabain than chick cells, while human cells are more sensitive than chick cells (Chambers et al., 1982; Chambers and Ling, 1984). Due to this latter sensitivity, this assay cannot be used for human cells in chick tissues. However, this approach can be adapted for use with human cells that carry a selectable drug resistance marker, such as neo/G418 (Chambers and Wilson, 1988). Because the assay requires adherent colonies to form, it is best suited for rodent cells that grow as adherent, monolayer cultures, rather than for cells that grow as suspension cultures.

A suspension of chick organ cells, in which there are unknown numbers of rodent cells, is plated in a concentration of ouabain sufficient to kill the chick cells while allowing the rodent cells to grow and form clonal colonies in vitro. At the end of the assay (the timing of which will depend on the growth rate of the cancer cells in vitro, in order to form clonal colonies of countable sizes), the rodent cell colonies that grow out from the chick liver suspension can be stained with methylene blue and counted to determine the number of viable (rodent) cells per chick embryo liver. Alternatively, viable cells can be recovered from some plates by standard trypsinization procedures to study the properties of cells that have been selected in vivo in the chick embryo (Chambers and Ling, 1984; Bondy et al., 1985; Chambers and Wilson, 1985; Hill et al., 1988).

Materials

Eggs containing 11-day-old chick embryos (see Support Protocol 1)
Adherent rodent tumor cells, optionally labeled with nanospheres (see Support Protocol 2)
Medium and serum used to grow tumor cells
Paraplast wax
Paraffin oil
70% ethanol
Sterile calcium- and magnesium-free PBS, 4°C (see recipe)
Hank's balanced salt solution (HBSS), 4°C (see recipe)
0.3% (w/v) collagenase/0.02% (w/v) trypsin (see recipe)
DNase I (see recipe)
2×10^{-3} M ouabain (see recipe)
Detergent or other decontaminating solution
Methylene blue stain (see recipe)

Egg candler (local farm supply store)
Pencil
Enclosed still hood with glass front, interior light, and electrical outlet
Dremel Moto tool with polishing wheel, $\frac{6}{8} \times \frac{1}{8}$–in. thick (available at local hobby shop)
38.5°C (99° to 100°F) automatic rotating egg incubator with 80% to 82% humidity (e.g., March Farms Roll-X incubators, Lyon Electric; available at farm supply stores)
Hemacytometer (*UNIT 1.1*) or Coulter counter
Adjustable gooseneck light source and fiber optic unit (e.g., Schott Glas Fiber Optics unit, Carl Zeiss unit) or focused intense light source
Egg support stand (i.e., three rubber corks glued on a plastic petri dish; Figs. 19.6.3 and 19.6.4)
1-cc syringes
30-G ½-in. needles
Cotton swab
Disposable underpad
1-liter plastic beaker lined with a 12-lb. plastic bag
Small plastic beaker
Dissecting instruments, autoclaved:
  Straight medium-point dissecting forceps, 115 mm in length
  Straight narrow-blades dissecting scissors with fine points, 110 mm in length
  Two straight fine-point dissecting forceps, 110 mm in length
  Two scalpel handles with no. 10 disposable blades
150 × 20–mm plastic dishes
100 × 15–mm nontissue and tissue culture dishes
24-well tissue culture plates
100-mm glass dishes (autoclave in a canister)
5-ml pipet
17 × 100–mm polystyrene test tubes
500-ml wash bottle
Funnel
Plastic wash basin with drainage holes drilled half-way up each end

Additional reagents and equipment for trypsinization (*UNIT 1.1*), adding accounting spheres (see Support Protocol 3), and inspecting for gross surface tumors (Chambers et al., 1982; Chambers and Wilson, 1988)

NOTE: During dissection, all instruments should be rinsed in 70% alcohol, wiped clean with a tissue, and flame sterilized between each step

Prepare egg windows

1. In a dark room, hold an egg candler directly against the shell on the air sac (blunt end) of an egg, which has been incubated for 11 days.

   The 11-day-old embryo and the CAM veins should be clearly visible under the shell. Dead or deformed embryos can be readily identified when candled, as they will have either no veins present or black, darkened areas. These eggs should be disposed of carefully without cracking, as they may have a strong sulfur odor.

2. Gently rotate the egg in hand to identify a large dark vein branched in a Y shape near the air sac. With a pencil (not pen or marker), mark a 10 × 10–mm square on the shell over this vein indicating the area to be drilled.

   Do not select veins that move freely when the egg is rotated with quick turns, as these cannot be injected. Drilling a square that is too small will make injection difficult, while if the square is too large, the window will cave in after drilling.

3. In an enclosed still hood with glass front, interior light, and electrical outlet, drill all four sides of the marked square using a Dremel Moto tool with a ⅝ × ⅛–in. polishing wheel using the following method (Fig. 19.6.2):

   a. Hold the egg with two fingers and the thumb of the left hand (assuming that the researcher is right-handed) so that the egg is at a slight angle, with the air sac end up, and the square marked for drilling facing up and toward the experimenter.

   b. Hold the Dremel drill in the right hand as if it were a large pencil.

   c. Bring the drill bit at right angles to the marked square on the shell. Use only gentle pressure to start cutting since the drill operates at high speed.

Figure 19.6.2 The drilling of the marked square or window on the egg over the selected vein area with a Dremel Moto tool is done in an enclosed still hood. To minimize breathing of eggshell dust, hang a cloth from the bottom of the glass front (or alternatively wear a mask and protective eyewear). Drill all four sides of the window, with the egg at a slight angle and the air sac end (blunt end) up and the marked window facing toward the person drilling. The bit of the Dremel tool is brought at right angles to the surface of the shell. Gentle pressure is sufficient for cutting due to the high speed of the drill. Only the shell is cut while avoiding the underlying smoother papery-white membrane or the underlying CAM.

d. Cut *only* the shell (which looks pebbly), and not the underlying papery-white shell membrane or the viable CAM tissue under this membrane. Do not remove the resulting window.

CAUTION: *For safety, minimize inhalation of egg shell dust by hanging a cloth from the bottom of the glass front on the enclosed still hood. Alternatively, or additionally, a mask and protective goggles can be worn.*

This step requires practice and a steady hand. If the CAM is nicked or bleeds, the egg should be discarded.

4. *Optional:* After drilling, return the eggs to a nonrotating egg incubator (i.e., with rotation turned off) for up to 1 hr before injection.

This step is useful when large numbers of eggs are to be injected in a single experiment; it helps to keep the embryos healthy.

Prepare tumor cells

5. Prepare a single-cell suspension of adherent rodent tumor cells from an 80% confluent culture, optionally with nanospheres, using standard laboratory procedures for trypsinization (*UNIT 1.1*). Count the cells using either a hemacytometer (*UNIT 1.1*) or Coulter counter.

6. Using the same medium and serum used to grow the tumor cells, adjust the volume to give the desired number of cells per embryo in a total injection volume of 0.1 ml per embryo. If desired, add accounting spheres (see Support Protocol 3).

The number of cells to inject into each chick depends on the metastatic properties of the cells. The number can range from 1–5 × 10^6 cells per embryo for poorly and nonmetastatic cells (Hill et al. 1988), down to 5 × 10^3 cells per embryo for highly metastatic cell lines (Chambers and Wilson, 1988).

The prepared cell suspension can be briefly (i.e., up to 2 hr) stored at 4°C until the eggs are ready for injection.

Set up injection equipment and area

7. In a small glass beaker melt Paraplast wax on a hot plate set at 60°C.

CAUTION: *Paraplast is flammable and should be carefully watched during this procedure; do not allow it to get hot enough to smoke.*

8. Set up the equipment needed for injection in the following manner:

a. Open the front of a tissue culture hood and place a fiber optic unit or focused intense light source near the back of the hood with the gooseneck light source extended to the front.

b. In front of this light source, position the egg support stand so that the egg will sit parallel to it.

c. Place empty 1-cc syringes, 30-G ½-in. needles, Kimwipes tissues, and a 1-cc syringe without needle half filled with paraffin oil into the hood.

d. When all the equipment is ready, place the eggs (windows cut but not removed, step 3 or 4) on their sides in an egg tray with the windows up and bring them into the tissue culture hood.

e. Once the Paraplast has melted, allow it to cool, but still remain liquid, and place it in the hood.

The arrangement of these components in preparation for egg injection is illustrated in Figure 19.6.3.

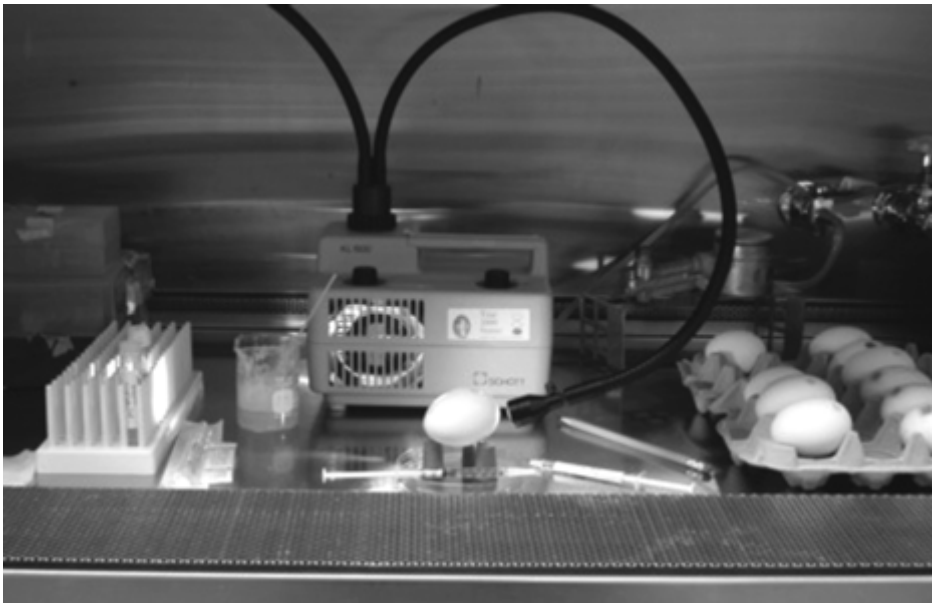


Figure 19.6.3 The arrangement of equipment inside the tissue culture hood just before injections proceed. The front of the hood is opened for easier access during the procedure with adequate sterility still being maintained. The gooseneck arm of the fiber optics light is illuminating the air sac end of an egg positioned on the egg stand (made by gluing three rubber corks onto a plastic petri dish) ready for injection. The remaining eggs have had the shell of the drilled window carefully removed over the vein area and are placed in a tray with the windows facing upwards awaiting injection. The cell suspension has been prepared at a predetermined concentration ready to be drawn up into the 1-cc syringe following gentle vortexing. The other 1-cc syringe contains the paraffin oil of which a drop will be placed on the exposed window area prior to injection. The melted Paraplast in the glass beaker (left) is cooling in preparation for sealing of the entire window area following injection.

Inject eggs

9. Gently remove the cut square of the shell from each egg with a pair of fine forceps. If the shell membrane or the underlying CAM become nicked or damaged, the egg cannot be used.

   This step also requires practice and a steady hand.

10. Set one of the eggs on the support stand with the air sac end facing the fiber optics so the veins inside the egg are illuminated. Add a drop of the paraffin oil from the 1-cc syringe to the surface of the shell membrane to further enhance visualization of the vein.

11. Gently vortex the suspension of cells to be injected (step 6). Draw the cell suspension up into a 1-cc syringe, attach the 30-G needle, and remove air plus any excess liquid with a Kimwipe.

    As a control, to determine the effect of passage of the cell suspension through a 30-G needle, it is recommended to perform an in vitro plating efficiency assay. For the cell lines used by the authors, passage of cells through a 30-G needle has not led to reduced plating efficiency of the cells.

12. Inject the CAM vein with tumor cells as follows (Fig. 19.6.4):

    a. With the beveled tip of the needle facing upwards and in line with the numbers on the barrel of the syringe (i.e., so both numbers and bevel face the experimenter), gently insert the needle into the CAM vein in the direction of blood flow.

    The paraffin oil enhances the visibility of the vein and thereby allows the quality of the injection to be monitored. When the CAM window is illuminated, vessels appear bright red and the egg contents appear yellow.

    b. Inject 0.1 ml quickly and evenly into the vein.

    c. Withdraw the needle carefully and smoothly from the vein to prevent bleeding.

    d. If any bleeding occurs or a clot forms at the injection site, discard the embryo.

    e. Return the injected egg to the egg tray in the hood, placing it on its side with the window facing upwards.

    To determine blood flow direction, follow the vein to where it branches; blood will generally travel along the two arms toward the single arm (Y). Areas of the vein near the branch seem to withstand injection the best. If a proper injection is done, the clear inoculum entering the vein will be visible as blood flow is temporarily blocked as will the pulsing of the blood flow beyond the end of the needle. See inset of Figure 19.6.4.

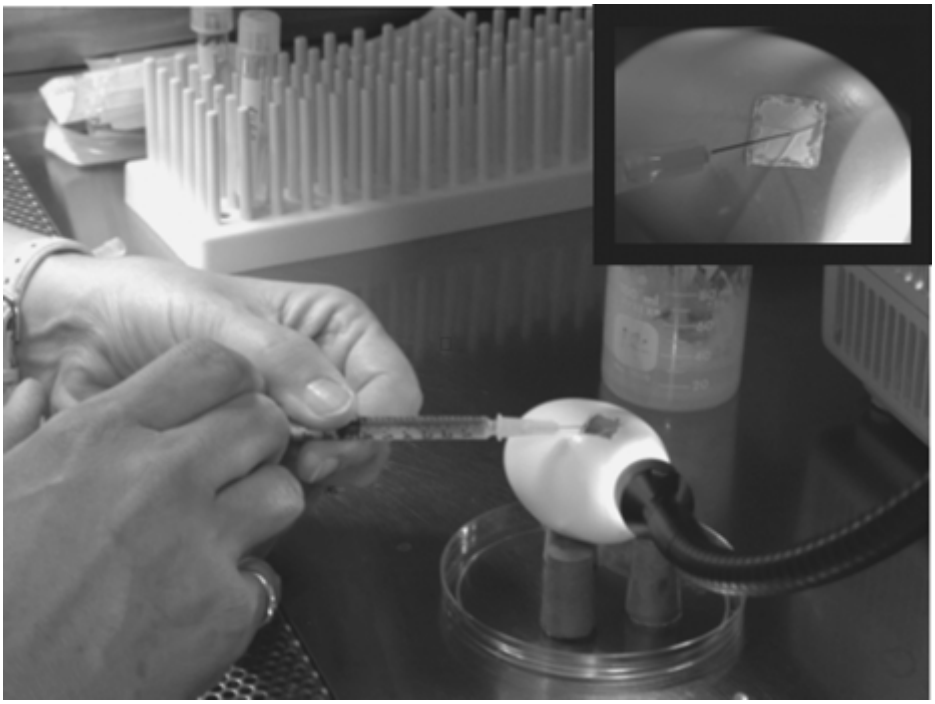


Figure 19.6.4 Injection of cells into a chick embryo CAM vein. Position the egg on the egg stand, with the fiber optics light illuminating the air sac end for visualization of the large Y shaped vein. The addition of a small drop of paraffin oil onto the injection site enhances the clarity of the vein. The beveled tip of the needle is facing upwards for gentle insertion into the branch area of the Y (see inset) just until the beveled end is completely into the vein. The needle should be at the angle seen in the picture above to prevent damage to the vein. If a proper injection is performed, the vein will temporarily become clear as the 0.1-ml inoculum is smoothly injected, and there will be no blood seeping from the injection point either during or after the injection.

13. Repeat steps 10 to 12 for each egg to be injected.

    When injecting several eggs from the same syringe of cells, be sure to agitate the syringe gently between injection, to avoid settling of the cells. This will ensure that all eggs receive the same number of cells.

14. Seal the exposed windows with cooled, but still melted Paraplast using a cotton swab. Cover the entire window area including at least 5 mm of shell on each side. After the Paraplast has hardened, apply a second coat to ensure a complete seal.

15. With a pencil, label the side of each egg with the date, cell line, and other important details.

Allow tumor cells to metastasize

16. Return the eggs, on their sides, to a nonrotating egg incubator (i.e., turn off rotator) for further incubation, up to a maximum of 7 days (hatching occurs on day 19 to 21). Use the same conditions of humidity and temperature as during the initial incubation (see Support Protocol 1)

    The incubation time depends on the type of assay, either having a single 7-day end point or, if determination of the growth kinetics of the cell is desired, a time course of intervals from 0 to 7 days from the time of injection.

    The temperature may be altered at this point if experiments are being performed to test the effects of temperature-sensitive mutants (Chambers and Wilson, 1985).

    Much information can be obtained from kinetics studies on the growth of injected cells in embryonic organs (Chambers et al., 1982; Chambers and Ling, 1984; Chambers and Wilson, 1985). Figure 19.6.5 shows an example of kinetic data, quantifying the in vivo increases in numbers of tumor cells (ouabain-resistant; i.e., rodent) in chick liver over time after injection. Figure 19.6.5B also illustrates how the chick embryo assay can be used to assess the in vivo effects of temperature-sensitive (ts) genes, by maintaining the embryos at different temperatures. In the example shown, the LA23-NRK cells carry a ts-src oncogene, which is active at 36° but inactive at 38°C, resulting in dramatic in vivo growth abilities of the cells shown in Figure 19.6.5B (Chambers and Wilson, 1985).

17. Check the viability of the embryos to be dissected by candling (step 1) in a darkened room.

    The embryo should be clearly visible and veins still present. If there are no veins or the eggs contain discolored interiors, the embryos have died and should be carefully discarded.

Set up dissection equipment and area

18. On a bench top near the tissue culture hood spread a disposable underpad. On the underpad, place a 1-liter plastic beaker lined with a 12-lb. plastic bag for disposal of the egg shells and other waste.

19. Place 115-mm, straight, medium-point dissecting forceps on the underpad outside of the hood. Place the remaining autoclave-sterilized dissecting instruments into a small plastic beaker containing 70% ethanol. Transfer the beaker with ethanol into the tissue culture hood.

Isolate chick embryo

20. Remove the embryo from the shell on the bench over the disposable underpad, as follows:

    a. Hold the egg with the air-sac end up. Break the shell at the air-sac end of the egg with a sharp tap from the handle of the 115-mm forceps.

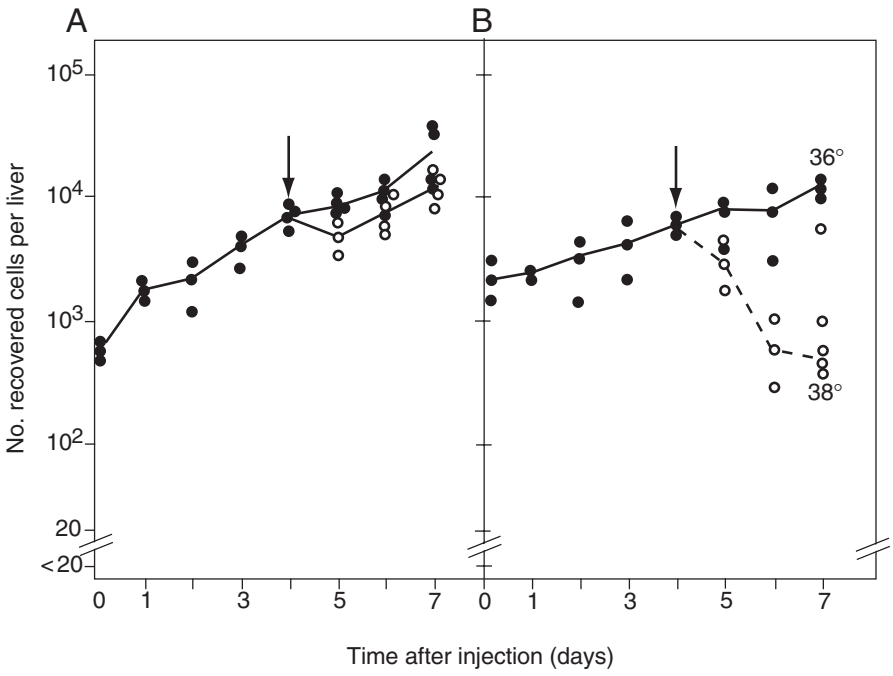


Figure 19.6.5 Example of in vivo growth kinetics results that can be obtained with the chick embryo experimental metastasis assay and the ouabain plating assay to recover rodent (and thus, ouabain-resistant) cells at various times after injecting cells via a CAM vein. (**A**) B77-NRK (normal rat kidney) cells transformed with the src oncogene, or (**B**) LA23-NRK cells (NRK cells carrying a temperature-sensitive src oncogene, which is active at 36° and inactive at 38°C were injected into CAM veins at 5×10^4 cells per embryo. Closed circles: embryos maintained at 36°C; Open circles: embryos transferred to 38°C at day 4. Each point represents the number of viable ouabain-resistant cells present in the liver of one embryo; lines connect median points. Republished with permission of American Society of Microbiologists from Chambers and Wilson (1985).

b. Over the 1-liter plastic-lined beaker, break through the air sac membrane towards the embryo.

c. Position the 115-mm forceps around the neck of the embryo and extract it from the shell.

Gloves should be worn for these steps.

21. Use the remaining shell to pull away any residual yolk or other tissue to completely free the embryo; discard the shell into the plastic-lined beaker.

The CAM lies directly under the shell membrane, and usually remains attached to the inside of the shell. It can be peeled from the inside of the shell and examined for tumor growth—e.g., if melanoma cells have been injected that produce easily visible tumors (Chambers et al., 1982)—or it can be used for histological assessment.

22. Use a 150 × 20–mm plastic dish to transfer the embryo to the tissue culture hood. Place each embryo into a 100 × 15–mm nontissue culture dish and decapitate the embryo with 110-mm, straight, narrow-blades, dissecting scissors with fine points. Cover with the lid.

The embryos can be left in the dishes inside the tissue culture hood until all the embryos have been extracted from the eggs.

Expose liver

23. Start dissecting by turning the embryo onto its back with the breast facing upwards. Just below the pectoral (breast) muscle, gently lift the skin with a pair of 110-mm, straight, fine-point dissecting forceps.

24. With sterile scissors, cut the skin above the breast all the way to the neck. Pull away the skin to the sides to expose the pectoral muscle, as well part of the liver (gold in color) and the gut.

25. Expose the entire liver and the heart as follows:

    a. Lift the area below the pectoral muscle at the point where a thin white membrane forms a V.

    b. Cut upwards through the middle of the pectoral muscle towards the neck, slightly lifting the muscle during cutting to avoid damaging the liver underneath.

    c. At the neck, make a transverse cut, then pull back the pectoral muscle to each side to expose the heart and entire liver.

Isolate liver

26. With sterile forceps, carefully detach the three areas of attachment of the liver to the other organs: two on the left side under one lobe and one under the right lobe.

27. Slip sterile opened forceps around the liver and move towards the heart which is the last point of attachment. Pinch the forceps together and lift out the liver.

28. In the tissue culture hood, transfer the liver to a well of a 24-well tissue culture plate containing 4°C sterile calcium- and magnesium-free PBS.

    The liver can remain up to 2 hr at 4°C.

    During this procedure (steps 25 to 27), do not puncture the gut while removing the areas of attachment under the liver. The gall bladder, situated directly under the liver, is usually dissected out at the same time as the liver. Avoid puncturing it if possible, but if this does occur, it will only discolor the PBS and not affect viability of cells present in the liver.

Dispose of waste and visually inspect livers for tumors

29. Incubate any undissected but viable eggs at 4°C for at least 1 hr prior to disposal to avoid hatching. Wrap the dissected embryos and shells in plastic bags and dispose by incineration.

30. Inspect the livers (and other organs) for gross surface tumors (Chambers et al., 1982; Chambers and Wilson, 1988) or abnormalities before dissociation, using proper sterile technique.

    Alternatively, organs may be fixed for histological examination and identification of internal micrometastases (Chambers and Ling, 1984).

Wash liver

31. Rinse each liver in a series of three sterile PBS washes in a 24-well plate by transferring the liver with sterile forceps from one well to the next.

    This allows six livers to be washed per 24-well dish.

32. Place the rinsed liver into a sterile 100-mm glass dish. Mince the liver into tiny pieces with crossed sterile scalpel blades.

    One scalpel can hold the tissue in place while the other cuts, then use both blades to finely mince the tissue. Avoid spreading the tissue over a large area to minimize drying.

Homogenize liver

33. Add 5 ml HBSS. Resuspend the minced liver tissue by pipetting up and down using a 5-ml pipet.

34. Dispense this suspension into a 17 × 100–mm polystyrene test tube (one tube per liver). Rinse the glass dish with another 5 ml HBSS to ensure all the tissue is removed from the petri dish. Pool with the 5 ml tissue suspension already in the test tube.

35. Centrifuge the liver suspension 10 min at 3000 × *g*, room temperature. Gently remove and discard the supernatant, leaving the liver pellet.

Prepare single-cell suspension

36. Add 3 ml freshly prepared 0.3% collagenase/0.02% trypsin and 0.5 ml DNase I into the tube and vortex.

37. Incubate ~1 hr in a 37°C heat block or water bath. Vortex occasionally during the incubation until a single-cell suspension is produced and there are no visible tissue fragments.

    The length of time necessary for complete dissociation of the liver tissue is dependent upon the age of the embryo—i.e., 18-day-old embryos require 1 hr, while 11-day-old embryos may require only 20 to 30 min. Other organs can also be dissociated; times will vary according to type of tissue and age of the embryo.

38. Centrifuge the single-cell suspension 10 min at 3000 × *g*, room temperature. Gently remove and discard the supernatant, leaving the liver cell pellet.

Select tumor cells with ouabain

39. Add 10 ml appropriate medium with serum for the cells injected with a final concentration of 2×10^{-5} M ouabain. Vortex the suspension to thoroughly mix.

40. Plate two dilutions of each liver suspension in 100 × 15–mm labeled tissue culture dishes containing 15 ml medium plus serum with 2×10^{-5} M ouabain. Use two to three plates per dilution.

    The total amount of liver cells plated should not exceed 1/20 of an 18-day old embryo liver per 100-mm dish, to avoid background problems due to large numbers of dying liver cells. All of the cells from younger (e.g., 11-day) livers can be plated in one to three dishes, without background problems. The fraction of the liver plated should reflect the expected number of rodent cells present in the liver and should be adjusted to give numbers of ouabain-resistant rodent colonies per dish that can be counted (optimal being 10 to 250 colonies per dish). Some suggestions of appropriate dilutions are 1/20 and 1/100 per 100-mm dish of a liver suspension for potentially low metastatic cell lines, and up to 1/100 and 1/2000 of liver suspension for a highly metastatic one.

41. Gently swirl each dish three times in both directions to ensure proper distribution of the cells over the bottom of the dish. Incubate the dishes undisturbed for 10 to 21 days, depending upon the doubling time of the injected cells.

    During the incubation of the diluted cell suspensions, the dishes should not be moved or small satellite colonies will be seeded that will make counting colonies difficult. Periodically, checking one plate during the incubation will assist in determining when to recover the cells from the colonies (following counting) for culturing or to stain for counting. Note which plate is used for these observations.

    Prior knowledge of the plating efficiency of the cell line injected will assist in determining the length of the incubation time.

Stain colonies and dry

42. Gently pour off the medium from the dishes into a beaker containing detergent or other decontaminating solution.

43. Add 5 ml methylene blue stain, from a 500-ml wash bottle, by allowing it to run down the side of the dish onto the bottom without disturbing the colonies. Make sure the bottom of the dish is completely covered with the stain. Allow to sit at least 15 min or until the colonies are a dark blue.

    The glutaraldehyde and alcohol in the stain will fix the cells to the dish.

44. Pour the stain off the colonies, collecting it into a 500-ml wash bottle using a funnel.

    This stain can be collected back into the 500-ml wash and reused.

45. Place a plastic wash basin with drainage holes drilled half-way up into a sink. Gently run tap water continously into the basin. Wash each dish individually by gently moving it slowly through the water. Do not allow direct water flow to dislodge colonies from the dish.

    The holes in the basin will allow the stained water to go down the sink while fresh water is poured in from the tap.

Determine number of rodent cells in each chick liver

46. Allow washed dishes to dry inverted, using the lids as a support to assist drying. Count colonies on the dried dishes any time, with the stained colonies being a permanent record.

47. Calculate the number of rodent cells present in each chick liver based on the fraction of liver plated in each plate.

    For example, calculate the mean of the total stained colonies counted per dish at a particular liver dilution multiplied by the dilution factor of the liver—e.g., (45 + 54 + 48)/3 × 20 (1/20 of liver plated per dish) = 980 cells per liver. For most dissociations, two separate liver dilutions are performed. In this case, do the above calculation for the second set of plates from the same liver—e.g., (9 + 11 + 10)/3 × 100 (1/100 of liver plated per dish) = 1000 cells per liver. To calculate the total number of cells per liver, take the two values obtained from each dilution and average: (980 + 1000)/2 = 990 cells per liver. The previously known plating efficiency in vitro of the cell line injected must be factored into this averaged value. If the cell line has a plating efficiency of 90% then calculate as follows: 990/0.9 =1100. Therefore, the mean final number of viable cells present in the chick livers would be 1100 cells. Standard deviations can be calculated from the variations in original cell counts per plate.

SUPPORT PROTOCOL 1

ROUTINE MAINTENANCE OF EGGS

Fertilized chick eggs (outbred White Leghorn) can be delivered fresh from a local hatchery on a regular schedule depending on experimental need (e.g., once a week). Larger eggs tend to be stronger and thus lead to more successful injections. During transport from the local hatchery, the eggs should not be subjected to extreme temperature changes and should be transported to the laboratory promptly. An insulated carrier may be necessary to keep them cool. When the eggs arrive in the laboratory, they should immediately be placed at 4°C, where they can be kept for up to one week before starting incubation. A schedule should be determined to provide embryos that have been incubated for exactly 11 days on the day on which the experiment is to be performed.

Materials

Fresh fertilized eggs (standard outbred White Leghorn), 4°C
Pencil
38.5°C (99° to 100°F) automatic rotating egg incubator with 80% to 82% humidity (e.g., March Farms Roll-X incubators, Lyon Electric; available at farm supply stores)
Hygrometer to routinely monitor egg incubator humidity

1. To start the incubation, remove fresh fertilized eggs from 4°C storage. On the blunt, rounded end of the egg, where the air sac is located, write the date when the incubation is begun in pencil.

   Do not use pen or marker, as these may be toxic to the embryos.

2. Place the eggs in a 38.5°C automatic rotating egg incubator with 80% to 82% humidity, with the air sac end up and the date clearly visible.

   The rotating incubator will prevent deformities from occurring in the embryos during incubation, by rotating the eggs at proper time intervals (New, 1966; Sturkie, 1976).

   Do not use a CO_2 incubator designed for tissue culture, as this will kill the embryos. The temperature is critical since a slightly lower incubator temperature will result in developmentally younger embryos that will not survive injection at day 11.

3. Continue incubating until eggs are the appropriate age. Record daily temperature readings to note fluctuations in temperature. Maintain the humidity by daily checking with a hygrometer and addition of either tap or distilled water into the interior egg incubator reservoir according to manufacturer's instructions.

   The incubators should be connected to an emergency back-up supply to prevent embryo death in case of a power failure.

INTRAVITAL VIDEOMICROSCOPY OF THE CHICK EMBRYO CAM

BASIC PROTOCOL 2

The technique of intravital videomicroscopy (IVVM) uses a videocamera attached to an inverted microscope to visualize, record, and quantify the movement of blood through microcirculatory pathways of intact organs in living animals. General information about this procedure can be found in MacDonald et al. (1992, 1998, 2002), and Chambers et al. (2002). Here the applications of this approach to studying the interactions of injected cancer cells with the chick embryo chorioallantoic membrane (CAM) will be discussed.

IVVM uses a fiber optic light source to supply oblique illumination of an organ of an experimental animal (e.g., chick embryo, mouse) placed on the stage of an inverted microscope. This approach yields high-resolution, three-dimensional images of the microcirculation. IVVM thus allows for the real-time observation of the hematogenous metastasis process involving the initial arrest of cancer cells, as well as their extravasation, post-extravasation migration, and replication to form tumors. This technique is able to visualize these events in thick tissues, such as mouse liver, to a depth of 50 to 100 μm. It is also very easy to visualize these events in thin tissues, such as mouse or rat mesentery, or chick CAM. Interactions between tumor cells and the host microvasculature and/or the surrounding tissues of the CAM of the chick embryo have been reported (e.g., Chambers et al., 1992; MacDonald et al., 1992; Koop et al., 1994, 1996; Morris et al., 1997).

The capillary network of the CAM closely resembles that of the lung (Sethi and Brookes, 1971), and at the embryonic stage it has a capillary plexus with a complete basement membrane, which is fully lined with endothelial cells (Rizzo and Defouw, 1993). This is an appropriate model for studying various aspects of metastasis, in that tumor cells in the

circulation are required to cross the endothelium and basement membrane barrier in order to form metastases. The CAM also provides an immune-deficient model easily accessible for observation without the need for surgery.

Labeled cells are injected intravascularly into the CAM vein of 11-day-old chick embryos (see Basic Protocol 1). Examination by IVVM of individual eggs can be done immediately (up to 4 hr) or at various times post-injection, up to 7 days. This provides a visual record of the interactions of the cancer cells with the CAM vasculature, which provides valuable data that can complement results from other procedures.

Materials

Eggs containing 11-day-old chick embryos (see Support Protocol 1)
Adherent tumor cells with or without nanospheres (see Support Protocol 2)
Medium and serum used to grow tumor cells
Citrate saline (see recipe)
Paraffin oil
Plasticine

Egg candler (local farm supply store)
Pencil
Enclosed still hood with glass front, interior light, and electrical outlet
Dremel Moto tool with polishing wheel, $\frac{6}{8} \times \frac{1}{8}$–in. thick (available at local hobby shop)
38.5°C (99° to 100°F) automatic rotating egg incubator with 80% to 82% humidity (e.g., March Farms Roll-X incubators, Lyon Electric; available at farm supply stores)
30-G ½-in. needle attached to a PE-10 cannula and 1-cc syringe
Hemacytometer (*UNIT 1.1*) or Coulter counter
Vinyl tape
No. 1 coverslip, 45 × 50–mm
180 × 130 × 3–mm acrylic viewing center with 40 × 40–mm hole cut in the center
Masking tape
Inverted microscope (e.g., Nikon Diaphot TMD) with 10× to 60× or 100× objectives and mercury arc lamp with B2-A filter block (570-nm dichroic mirror and 520-nm barrier filter; 450- to 490-nm excitation wavelength; Nikon)
Infrared heat lamp
Fiber optic light source with 150 W halogen bulb
Newvicon tube video camera with extended red sensitivity (Panasonic WV1550 or Hamamatsu C2400)

Additional reagents and equipment for preparing windows in eggs (see Basic Protocol 1, steps 1 to 6 and step 9) and injecting tumor cells into eggs (see Basic Protocol 1, steps 10 to 13 and steps 15 to 16)

Prepare eggs for injection

1. Prepare 11-day-old embryos for injection with tumor cells (see Basic Protocol 1, steps 1 to 6, and 9).

   Tumor cells can be labeled with fluorescent nanospheres (Support Protocol 2) to aid in their detection. Accounting spheres (Support Protocol 3) can be used to monitor blood flow and delivery of cells to the tissue.

2. Prepare to observe the initial interactions between injected cells and CAM microcirculation:

   a. Carefully place a 30-G ½-in. needle attached to a PE-10 cannula and 1-cc syringe containing warmed citrate saline rinse into the large CAM vein. Keep in place with vinyl tape.

   b. Inject 0.25 ml citrate saline, keeping the cannula filled with solution, to avoid vessel blockage by air bubbles.

Prepare window for IVVM

3. Remove the shell and outer shell membrane from the air sac region of the egg by gently using forceps to open an observation area ~3 cm in diameter and leaving the CAM at the same level as the border of the shell.

4. Apply paraffin oil to the inner shell membrane, covering the entire air sac area of the CAM to be observed so it becomes transparent.

5. Secure a no. 1 coverslip on the opening of the acrylic viewing platform, using masking tape, so that the CAM comes into direct contact, forming a window. Place the windowed egg on the platform, so that the CAM covered in paraffin oil comes in direct contact with the coverslip, thus creating a window to directly view the capillary plexus of the CAM.

Set up microscopic viewing system

6. Carefully place the acrylic platform and egg setup on the stage of an inverted microscope equipped with 10× to 60× or 100× objective, mercury arc lamp, and Nikon B2-A filter block.

   Refer to UNIT 4.2 for a discussion of fluorescence microscopy.

7. Keep the egg in place using vinyl tape on the shell and microscope stage and building a protective wall around the shell on the platform with Plasticine where the egg meets the acrylic platform, to provide stability and prevent cracking of the egg shell during the experiment. Maintain the temperature of the egg using an infrared heat lamp.

   The arrangement of the egg and CAM tissue on the stage of the inverted microscope is illustrated in Figure 19.6.6.

   With an inverted microscope, the thin CAM tissue rests on a stationary coverslip, minimizing the effects of embryonic and respiratory movement. By focusing up and down, the CAM tissue can be optically sliced throughout its depth.

8. Use the lateral window prepared for cell injection for oblique illumination using a fiber optic light source with 150 W halogen lamp. Direct the light at an angle of ~45° to the optical axis.

   This oblique angle will assist in obtaining high-contrast views of the microcirculation. This type of illumination refracts more light from one side of the cells than the other, producing a shadowy effect that gives a three-dimensional quality to the image. Fluorescent labeling of the cells aids in identifying them in vivo. To avoid quenching, however, the fluorescence illumination should only be used periodically (i.e., for positive identification of cells). Transillumination using the fiber optic light source should be used otherwise. The video camera with the extended red sensitivity avoids the problem of having a monochromatic red image due to the high blood volume in the light path.

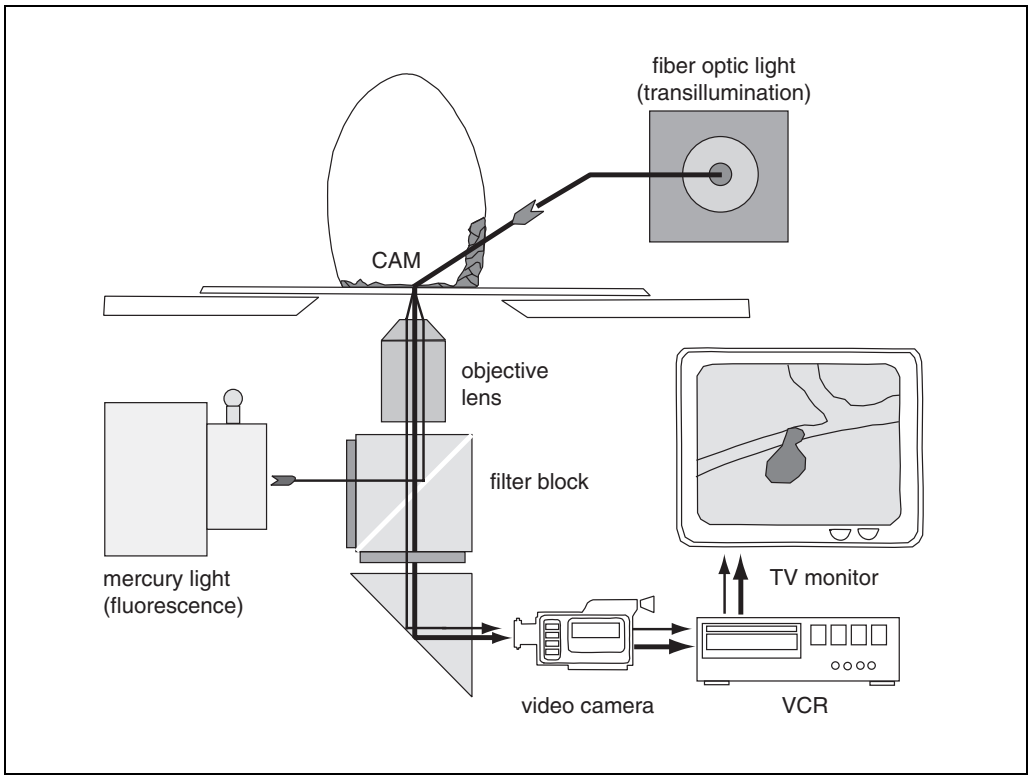


Figure 19.6.6 Schematic of the intravital videomicroscopy technique, as used for real-time observations of the interactions of injected cancer cells with the CAM microcirculation. The CAM is exposed and positioned on a plastic platform with a coverslip window above the objective lens of inverted microscope. For visualization of fluorescently labeled cancer cells, oblique transillumination is provided by the fiber optic light and epifluorescence through the objective aids in positive identification of the cancer cells. Images are collected through the video camera and saved to SVHS tape or digitally on a computer. Diagram by S. Koop and I.C. MacDonald, republished with permission of Plenum Publishers from Chambers et al. (1995).

Perform IVVM

9. Capture the microcirculation images using a Newvicon tube video camera with extended red sensitivity and appropriate capture device.

   These black-and-white images can be viewed directly on a video monitor, recorded on a SVHS videocassette recorder, or captured by computer programs such as Optimas Image Analysis (Media Cybernetics) or Northern Eclipse (Empix Imaging). A 35-mm camera can also be attached to the microscope for color photographs of the CAM. A character generator adds time, date, and stopwatch information to the video signal. A stage micrometer can be used to calibrate the magnification of the video image for each objective lens. Images can be obtained using either transillumination or epifluorescence alone, or a combination of both.

10. When the entire system is set up for viewing the CAM, inject 0.1 ml tumor cell suspension through the 30-G needle attached to the cannula (step 2).

11. View the cells at various magnifications starting with 10× to identify the cells. Carry out detailed examination of these cells and their immediate environment at higher magnification. Use video replay to measure cell and vessel dimensions, locations, and other parameters.

    Refer to Chambers et al. (1992), MacDonald et al. (1992, 1998), and Koop et al. (1994, 1995, 1996) for further discussion.

IVVM observations can be carried out for up to 4 hr post-injection without visible changes in blood flow or embryo movement. After the observations, the CAM tissue can be fixed in neutral-buffered formalin for later histopathological analysis.

The calibration scale on the fine-focus knob of the microscope permits the measurement of the cancer cell depth below the capillary plexus of the CAM. As a reference point for these measurements, the red blood cells within the plexus are clearly imaged, then the distance below this point, where the fluorescence image of the cancer cells are in sharpest focus, is determined using the fine-focus knob of the microscope.

Perform real-time in vivo kinetic analyses

12. Observe later stages of the interactions between tumor cells and CAM microcirculation by injecting as described (see Basic Protocol 1, steps 10 to 13 and 15 to 16). At specific time intervals (1 to 7 days postinjection) remove individual eggs from the incubator and observe as described above.

    Since the injection window is sealed, a new small window for observation with oblique illumination must be prepared (see Basic Protocol 1, step 3). The observation procedures are then carried out as described above.

LABELING CELLS WITH FLUORESCENT NANOSPHERES

SUPPORT PROTOCOL 2

In order to allow positive identification of cancer cells, human or rodent, within the CAM (or in mouse tissues), it is necessary that the cells be labeled in some way. Some lines, such as melanoma cells (e.g., B16F1), express melanin, which can be detected by eye (Chambers et al., 1982, 1992; Koop et al., 1994). Exogenous labels such as Calcein-AM (Chambers et al., 1992; Morris et al., 1994) or fluorescent nanospheres (~0.05-µm labeled polystyrene microspheres; Morris et al., 1994; Naumov et al., 2002) have been used to label the cytoplasm of cells to be injected without any effect on membrane integrity or growth potential of these cells. Alternatively, the gene for green fluorescence protein (GFP) can be transfected into cell lines, providing a heritable label that does not dilute with cell division (Naumov et al., 1999, 2002). Clones of GFP-transfected cells must be tested for brightness for use with IVVM as well as maintaining genotypic and phenotypic properties of the parental cell line (Chambers and Wilson, 1988; Naumov et al., 1999). Most cell lines readily take up the fluorescent nanospheres when incubated with them in vitro, prior to injection.

Materials

0.05-µm-diameter, fluorescent, carboxylated P(S/V-COOH), dragon-green (480/520 nm) nanospheres (Bangs Laboratories) for cell labeling; store at 4°C in the dark
OptiMEM serum-reduced medium (InVitrogen)
Cells to be labeled

Sonicator
50-ml conical polystyrene centrifuge tubes
0.2-µm syringe filter and 10-ml syringe
Aluminum foil
75-cm^2 tissue culture flask
150 × 15–mm tissue culture dishes
Tissue culture incubator, standard, 37°C, 5% CO_2

Additional reagents and solutions for culturing, trypsinizing, and counting cells (*UNIT 1.1*)

Prepare fluorescent nanospheres

1. Sonicate a 10-ml vial of 0.05-µm-diameter, fluorescent, carboxylated P(S/V-COOH), dragon-green nanospheres 15 min.
2. In a tissue culture hood, add 75 µl nanospheres to 10 ml OptiMEM serum-reduced medium or 375 µl nanospheres to 50 ml OptiMEM medium in a 50-ml conical polystyrene centrifuge tube.
3. Sterilize the nanosphere suspension by passing through a 0.2-µm syringe filter, attached to a 10-ml syringe, into a clean, sterile 50-ml conical centrifuge tube. Wrap the tube in aluminum foil and store undisturbed at 4°C overnight.

Prepare cells to be labeled

4. The next day, centrifuge the nanospheres 10 min at 3000 × *g*, room temperature. Carefully remove the upper 2.5 ml for a 10-ml suspension or 30 ml for a 50-ml suspension. Avoid disrupting the sediment at the bottom of the tube. Vortex the suspension.
5. Cells to be labeled to 80% confluency by standard laboratory methods (*UNIT 1.1*) in either a 75-cm^2 tissue culture flask or 150 × 15–mm tissue culture dish.
6. Wash the cells twice with 10 ml OptiMEM medium.

Allow cells to take up nanospheres

7. Completely remove the last wash and add either 7.5 ml filtered nanospheres for a 75-cm^2 tissue culture flask, or 20 ml for a 150 × 15–mm tissue culture dish.
8. Incubate 2 hr at 37°C in CO_2 incubator, swirling occasionally to distribute the nanospheres evenly.
9. Following the incubation, wash cells twice with 10 ml OptiMEM to remove excess nanospheres.

Prepare cells for injection

10. Trypsinize the cells by standard laboratory procedures (*UNIT 1.1*).
11. Perform a cell count with a hemacytometer to determine cell number (*UNIT 1.1*).
12. Using the medium and serum in which the cells are normally grown, resuspend and adjust the volume of the cell suspension to give the desired number of cells per 0.1 ml injection volume. Store the cells at 4°C for up to 2 hr prior to injection (see Basic Protocol 1, step 5).

    In general, fluorescent labeling of cells should be carried out using cytoplasmic rather than nuclear markers. Cells labeled with the nuclear stain acridine orange were shown to be prone to lysis, which markedly affects their metastatic properties in vivo (Morris et al., 1994). Alternatively, cells can be detected using stably transfected fluorescent markers such as green fluorescent protein (GFP; Naumov et al., 1999).

13. Determine if the uptake of the nanospheres affects the viability or growth of the cells by plating labeled and unlabelled cells in six 60-mm dishes at 100 cells per dish. Incubate undisturbed for 11 to 14 days. Stain colonies, count, and determine plating efficiencies:

    Plating efficiency = (no. colonies/no. cells plated) × 100%.

CELL ACCOUNTING IN TISSUES

This experimental procedure enables one to quantify the survival of cells injected into the CAM. To do this, the number of cells actually observed in a sampled volume of tissue at any time must be expressed relative to the number of cells that originally entered that volume. Based on the standard method of measuring blood flow, inert plastic microspheres (10 μm) are added to the cell suspension, prior to injection, at a known ratio (e.g., 1 microsphere:10 cells). These microspheres become trapped by size restriction in the blood capillaries where they remain indefinitely. Thus, the total number of microspheres in a particular volume of tissue provides a reference marker for the total number of cells that originally entered this area, and they can be assessed by either IVVM (see Basic Protocol 2) or histology on thick sections (30 to 50 μm). General information about this procedure can be found in Koop et al. (1995), Morris et al. (1997), and MacDonald et al. (1998).

Materials

Tumor cell suspension, with or without fluorescent nanospheres (see Support Protocol 2)
10-μm-diameter, yellow/green (505/515 nm) fluorescent, plastic microspheres (fluorospheres; Molecular Probes)
60-mm tissue culture dishes

Additional reagents and equipment for fluorescent microscopy (*UNIT 4.2*)

1. Prepare a tumor cell suspension labeled with fluorescent nanospheres if desired (see Support Protocol 2).

2. Calculate the final injection volume and adjust the tumor cell concentration in a smaller volume so the same number of cells are being injected, compensating for the additional volume of the predetermined amount of accounting spheres that will be needed to give the final volume of 0.1 ml per embryo.

   For example, the injection suspension is calculated as follows to compensate for the additional volume of the accounting sphere suspension: 1 ml (the final volume that will contain the predetermined number of cells and accounting spheres) minus the volume containing this number of accounting spheres (e.g., $^1/_{10}$ the number of tumor cells) = the volume of growth medium in which to resuspend the tumor cells.

3. Add the appropriate amount of 10-μm-diameter, yellow/green, fluorescent, plastic microspheres to the cell suspension.

   The fluorospheres, also known as accounting spheres, are at a known concentration and are already sterile. A recommended ratio is 1 accounting sphere:10 cells.

4. Confirm the intended calculated ratio by putting a drop of the suspension to be injected onto a microscope slide, and counting cells and beads in a given area using a standard light microscope (*UNIT 4.1*).

   The prepared suspension of cells with accounting spheres are now ready for injection and can be maintained at 4°C until injection into the CAM.

5. Inject the cell/microsphere suspension into the CAM.

6. Examine the embryonic tissue for the presence of accounting microspheres and fluorescently labeled cells (nanospheres). Use the numbers to calculate injected cell survival.

The ratio in the tissues at various times after injection, divided by the ratio in the syringe before injection, is referred to as the cell survival (CS) index (Koop et al., 1995; Morris et al.; 1997; MacDonald et al., 1998). Thus, a CS index of 1.0 (i.e., 10 cells observed in vivo for every microsphere in a given tissue volume) would imply 100% survival, whereas a decrease in CS index would imply <100% survival (i.e., if only five cells were observed for every microsphere in a given tissue volume at some time after injection, 50% of the cells could be accounted for, and 50% of the cells would have been lost, either by cell death or by passage through the organ). This assay allows for cell accounting relative to the total number of injected cells rather than the percentage of observed cells remaining in vivo at times after injection.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Citrate saline

4.4 g/liter sodium citrate
10.0 g/liter KCl
Sterilize by autoclaving 20 min
Store up to 3 months at 4°C

No pH adjustment is necessary.

Collagenase, 0.3%/trypsin, 0.02%

Prepare a trypsin stock by adding a 10-ml bottle of trypsin to 100 ml sterile HBSS (see recipe) using proper sterile technique. Store up to 2 weeks at 4°C.

Prepare a fresh collagenase stock by combining 300 mg collagenase IV with 80 ml HBSS. Stir 1 hr and filter through a 0.8-µm filter, then through 0.2-µm filter.

Prepare the collagenase/trypsin mix by adding 20 ml trypsin stock to 80 ml collagenase stock. Mix well and store up to 2 weeks at 4°C.

DNase I

Add 1 mg DNase I per milliliter HBSS (see recipe). Sterilize by passing through a 0.2-µm filter. Store up to 2 weeks at 4°C.

HBSS

Autoclave 450 ml water in a 500-ml bottle, then allow to cool. Under sterile conditions, add 50 ml of 10× HBSS solution (InVitrogen) and 1.5 ml of 7.5% sodium bicarbonate (InVitrogen), and adjust to pH 7.0. Store up to 3 months at 4°C.

Methylene blue stain

Prepare a 1% (w/v) solution of methylene blue in 70% ethanol. Stir for a few hours until the crystals are completely dissolved. Add glutaraldehyde to a final concentration of 1%. Store in a 500–ml plastic wash bottle up to 2 months at room temperature. Supplement with glutaraldehyde every third staining. If a precipitate forms, filter through chromatography paper.

Ouabain, 1×10^{-3} and 2×10^{-5} M

Prepare a 10^{-3} M stock by dissolving 72.86 mg ouabain in 100 ml tissue culture medium with serum for the cell line used. Filter sterilize by passing through a 0.2-µm filter. Store up to 2 weeks at 4°C.

Prepare the 2×10^{-5} M working solution by adding 10 ml of 10^{-3} M stock to a 500-ml bottle of tissue culture medium with serum. Store up to 2 weeks at 4°C.

PBS, calcium and magnesium free

8.0 g NaCl
0.2 g KCl
0.2 g KH_2PO_4
1.15 g Na_2HPO_4
Adjust to 1 liter with water
Autoclave for 20 min
Store up to 3 months at 4°C.

This buffer is calcium and magnesium free.

Trypsin, 2.5% (w/v), in citrate saline

Add one 10-ml vial of Difco Bacto-Trypsin to a 500-ml bottle of sterile citrate saline (see recipe). Store up to 3 months at 4°C. Warm in a 37°C water bath before trypsinization of cells.

COMMENTARY

Background Information

The experimental metastasis assay in the chick embryo chorioallantoic membrane (CAM) allows for two standard types of experiments. First, single time-point experiments are generally terminated 7 days after injection. These assays give a measure of metastatic growth ability of injected cancer cells in the chick liver, which is the first capillary bed reached by cells injected via CAM veins. These assays are useful for quickly comparing the metastatic properties of different cell lines or treatments. This end point would be analogous to single-time-point experiments in mice, in which the numbers and sizes of metastatic foci are determined at some time after i.v. injection. For quickly growing tumor cell lines, this end point (macroscopic tumors detected at the liver surface) can also be used in the chick embryo. In contrast, in the ouabain plating assay, the numbers of tumor (rodent) cells are counted. This number will be related to the number of metastases that grow, their growth rate, and the numbers of cells that remain without division.

Kinetic experiments can also easily be performed with the chick assay, by measuring the numbers of viable rodent cells in chick organs at various times after injection, as illustrated in Figure 19.6.5. See Chambers et al. (1982), Chambers and Ling (1984), and Chambers and Wilson (1985), for examples of the results that can be obtained with this assay. The fate of injected cells over time after arrival in the target organ determines the growth or death rates of cells in vivo. This information is generally not obtained from experimental metastasis assays in mice, because information on the cells is not obtained until metastases are large enough to be detected by the eye, and then only from a single end point. It should be noted that due to the short experimental time of this assay, genetically-manipulated cells which may have unstable genotypes should be considered for this assay rather than long-term tumor formation assays. The ability to easily perform growth kinetics, from immediately after injection to 7 days post-injection, is a strength of the chick embryo experimental metastasis assay.

The ability of the chick to develop over a range of temperatures can be a powerful genetic tool when applied to the study of metastatic properties of cancer cells having temperature-sensitive mutations. The experimental metastasis assay is also very sensitive to small changes in growth ability, to the point of being able to detect a response within a day of a change in growth conditions, as described for temperature-sensitive *src*-transformed cells shown in Figure 19.6.5B (see Chambers and Wilson, 1985).

The laboratory mouse is the most commonly used animal for in vivo metastasis assays. Many of these assays require the use of an immune-deficient host, such as athymic nude or SCID mice, which are expensive and require special care and housing. The authors have previously shown that the cost of assessing the metastatic properties of cell lines in the experimental metastatic assay using the chick embryo is approximately one-quarter the cost of testing the same cells in nude mice, and approximately one-fifth the cost of testing in nude mice and plating mouse livers to recover viable cells (Chambers et al., 1990). It should be noted that these calculations did not include the start-up cost for expensive facilities to house the immune-deficient mice. In contrast, the equipment for maintaining the chick embryo is inex-

pensive and readily available (Chambers et al., 1990). The experimental metastasis assay in the chick embryo is thus a cost-effective method that can be useful for prescreening cells, which can subsequently be tested in nude or SCID mice, using considerably smaller numbers of mice. The authors have shown that results from the two assays can give comparable results for individual cell lines (Chambers et al., 1990). In addition, in some institutions, use of chicken embryos may be subjected to less complex animal care and use regulations than those applied to the use of mice in tumor studies. Overall, the use of chick embryos to study metastasis provides a useful alternative to metastasis assays in mice and may offer advantages for many researchers.

Critical Parameters and Troubleshooting

Many of the critical parameters have been outlined above in the appropriate protocol sections. In general, several steps require a steady hand, such as the drilling of windows in the egg shells, lifting the shell to form the injection window, and injection of cells into CAM veins. These steps are not hard but do require practice. A good approach to developing injection skills is to carry out experiments in which a melanotic cell line, such as murine B16F1 or B16F10, are injected into CAM veins. Success of the injections can be determined 7 days later, as these cell lines form macroscopic tumors in the CAM tissue and liver; these can be readily detected upon dissection of the embryo and inspection by eye.

The fertility rate of chick embryos may vary seasonally and with geography and weather conditions; however, with experience, this rate can be predicted for the local situation. Extra eggs should be ordered until this fertility rate is known.

Anticipated Results

In Basic Protocol 1, some rapidly growing cancer cell lines (such as B16F1 and B16F10 melanoma) form metastases detectable by eye during a 7-day incubation period following injection into chick CAM veins (e.g. Chambers and Wilson, 1988, Koop et al., 1994). Detection of these may be aided by production of melanin by some cell types. Growth of many other cancer cell types can be detected using the ouabain recovery procedure, and these have been compared with results from experiments in mouse (Chambers et al., 1990). The in vivo growth kinetic analysis (Fig. 19.6.5). offers an especially powerful approach to characterizing in vivo behavior of cancer cell growth in metastatic sites and permits subtle changes in growth to be detected (e.g., due to a temperature shift, affecting a temperature-sensitive oncogene in the example shown; Chambers and Wilson, 1985).

Further information on the behavior of cancer cells in vivo can be obtained by direct observations, as permitted by the use of in vivo videomicroscopy (Basic Protocol 2). For example, use of this procedure permitted the in vivo effects of over-expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B16F10 cells to be determined (Koop et al., 1994). While it had been expected that TIMP-1 would inhibit escape of cancer cells from the microcirculation, that study found instead that the TIMP-1 overexpressing cells extravasated well, but had a defect in micrometastatic growth. Because the CAM is a thin structure, it is relatively easy to determine if cells are intravascular or extravascular, permitting kinetics and efficiency of extravasation to be easily quantified (e.g., Koop et al., 1995, 1996). These studies showed that the large majority of cells injected into the CAM circulation successfully extravasate, a finding also seen in mice (Chambers et al., 1995, 2002). The chick embryo thus can provide a low-cost, simple complement to more expensive and complex experiments in mice.

Time Considerations

The experimental metastasis assay (see Basic Protocol 1) involves injecting 11-day-old chick embryos and then collecting data at time intervals from time 0 to 7 days post-injection. Experiments must be planned ahead, in order to have embryos of the desired age on the appropriate day. The set-up time required for injection of the embryos is ~2 hr including the preparation of the cells for injection. The injection time is dependent on the number of embryos being injected. The dissection of the chick embryos, dissociation of the livers, and setting up of the ouabain plating assay usually require 3 hr, again depending on the number of embryos in the experiment. The in vitro part of this assay requires 10 to 14 days for formation of colonies, depending on the growth rate of the cells in vitro to form colonies.

IVVM of chick embryo CAMs (see Basic Protocol 2) can generate data in as short a time as 2 min post-injection and up to 4 hr. The embryos can be used from time 0 to 7 days post-injection. The set-up time for the embryo before visualization is ~2 hr including the cell

suspension preparation. IVVM, although a powerful observational tool, is generally a labor-intensive procedure, as information can be obtained in real-time for only one embryo at a time.

For Support Protocol 1, daily monitoring of the eggs in the incubator and addition of water takes ~15 min per day. For Support Protocol 2, labeling of cells with nanospheres, preparation of the nanospheres the day before the experiments requires 0.5 to 1 hr. Labeling of the cells and preparation of the cells on the day of the experiment takes ~4 hr. Addition of accounting spheres in Support Protocol 3 will require ~1 hr of preparation time.

Literature Cited

Bondy, G.P., Wilson, S., and Chambers, A.F. 1985. Experimental metastatic ability of H-ras-transformed NIH 3T3 cells. *Cancer Res.* 45:6005-6009.

Chambers, A.F. and Ling, V. 1984. Selection of experimental metastatic ability of heterologous tumor cells in the chick embryo after DNA-mediated transfer. *Cancer Res.* 44:3970-3975.

Chambers, A.F. and Tuck, A.B. 1988. Oncogene transformation and the metastatic phenotype. *Anticancer Res.* 8:861-872.

Chambers, A.F. and Wilson, S. 1985. Cells transformed with *ts* viral *src* mutant are temperature-sensitive for in vivo growth. *Mol. Cell. Biol.* 5:728-733.

Chambers, A.F. and Wilson, S. 1988. Use of Neo^R B16F1 murine melanoma cells to assess clonality of experimental metastases in the immune-deficient chick embryo. *Clin. Exper. Metast.* 6:171-182.

Chambers, A.F., Shafir, R., and Ling, V. 1982. A model system for studying metastasis using the embryonic chick. *Cancer Res.* 42:4018-4025.

Chambers. A.F., Wilson, S.M., Tuck, A.B., Denhardt, G.H., and Cairncross, J.G. 1990. Comparison of metastatic properties of a variety of mouse, rat, and human cells in assays in nude mice and chick embryos. *In Vivo* 4:215-219

Chambers, A.F., Schmidt, E.E., MacDonald, I.C., Morris, V.L., and Groom, A.C. 1992. Early steps in hematogenous metastasis of B16F1 melanoma cells in chick embryos studied by high-resolution intravital videomicroscopy. *J. Natl. Cancer Inst.* 84:797-803.

Chambers, A.F., MacDonald, I.C., Schmidt, E.E., Koop, S., Morris, V.L., Khokha, R., and Groom, A.C. 1995. Steps in tumor metastasis: New concepts from intravital videomicroscopy. *Cancer Metast. Rev.* 14:279-301.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer* 2:563-572.

Hill, S.A., Wilson, S., and Chambers, A.F. 1988. Clonal heterogeneity, experimental metastatic ability, and p21 expression in H-*ras*-transformed NIH 3T3 cells. *J. Natl. Cancer Inst.* 80:484-490.

Koop, S., Khokha, R., Schmidt, E.E., MacDonald, I.C., Morris, V.L., Chambers, A.F., and Groom, A.C. 1994. Overexpression of metalloproteinase inhibitor in B16F10 cells does not affect extravasation but reduces tumor growth. *Cancer Res.* 54:4791-4797.

Koop, S., MacDonald, I.C., Luzzi, K., Schmidt, E.E., Morris, V.L., Gratten, M., Khokha, R., Chambers, A.F., and Groom, A.C. 1995. Fate of melanoma cells entering the microcirculation: Over 80% survive and extravasate. *Cancer Res.* 55:2520-2523.

Koop, S., Schmidt, E.E., MacDonald, I.C., Morris, V.L., Khokha, R., Grattan, M., Leone, J., Chambers, A.F., and Groom, A.F. 1996. Independence of metastatic ability and extravasation: Metastatic *ras*-transformed and control fibroblasts extravasate equally well. *Proc. Natl. Acad. Sci. U.S.A.* 93:11080-11084.

MacDonald, I.C., Schmidt, E.E., Morris, V.L., Chambers, A.F., and Groom, A.C. 1992. Intravital videomicroscopy of the chorioallantoic microcirculation: A model system for studying metastasis. *Microvas. Res.* 44:185-199.

MacDonald, I.C., Schmidt, E.E., Morris, V.L., Groom, A.C., and Chambers, A.F. 1998. In vivo videomicroscopy of experimental hematogenous metastasis: Cancer cell arrest, extravasation, and migration. *In* Motion Analysis of Living Cells, Chapter 12 (D.R. Soll, and D. Wessels, eds.), pp. 263-285. John Wiley & Sons, New York.

MacDonald, I.C., Groom, A.C., and Chambers, A.F. 2002. Cancer spread and micrometastasis development: Quantitative approaches for in vivo models. *BioEssays* 24:885-893.

Morris, V.L., Koop, S., MacDonald, I.C., Schmidt, E.E., Grattan, M., Percy, D., Chambers, A.F., and Groom, A.C. 1994. Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. *Clin. Exp. Metast.* 12:357-367.

Morris, V.L., Schmidt, E.E., MacDonald, I.C., Groom, A.C., and Chambers, A.F. 1997. Sequential steps in hematogenous metastatasis of cancer cells studied by in vivo videomicroscopy. *Inv. Metast.* 17:281-296

Naumov, G.N., Wilson, S.M., MacDonald, I.C., Schmidt, E.E., Morris, V.L., Groom, A.C., Hoffman, R.M., and Chambers, R.M. 1999. Cellular expression of green fluorescent protein, coupled with high-resolution in vivo videomicroscopy, to monitor steps in tumor metastasis. *J. Cell Sci.* 112:1835-1842.

Naumov, G.N., MacDonald, I.C., Weinmeister, P.M., Kerkvliet, N., Nadkarni, K.V., Wilson, S.M., Morris, V.L., Groom, A.C., and Chambers, A.F. 2002. Persistence of solitary mammary carcinoma cells in a secondary site: A possible contributor to dormancy. *Cancer Res.* 62:2162-2168.

New, D.A.T. 1966. The Culture of Vertebrate Embryos. Chapter 3 (The Chick) pp. 47-98. Logos-Academic Press, London.

Rizzo, V. and Defouw, D.O. 1993. Macromolecular selectivity of chick chorioallantoic membrane microvessels during normal angiogenesis and endothelial cell differentiation. *Tissue Cell* 25:847-856.

Sethi, N. and Brookes, M. 1971. Ultrastructure of the blood vessels in the chick allantois and chorioallantois. *J. Anat.* 109:1-15.

Sturkie, P.D. 1976. Avian Physiology (Third Edition). Springer-Verlag, New York.

Welch, D.R. 1997. Technical considerations for studying cancer metastasis in vivo. *Clin. Exper. Metast.* 15:272-306.

Contributed by Sylvia M. Wilson and Ann F. Chambers
London Regional Cancer Centre
London, Ontario, Canada

Figure 19.6.1 Diagram of experimental metastasis assay in chick embryos. Cancer cells are injected via a chorioallantoic membrane (CAM) vein, through a window opened in the shell of 11-day-old chick embryos. Cells injected via this route travel via the circulation first to the embryonic liver. After incubation for up to 7 days, metastatic cells in an embryonic organ such as liver are quantified using the ouabain plating assay, or (for rapidly growing cell lines) macroscopic metastases can be quantified. Republished with permission of Anticancer Research from Chambers and Tuck (1988).

Figure 19.6.2 The drilling of the marked square or window on the egg over the selected vein area with a Dremel Moto tool is done in an enclosed still hood. To minimize breathing of eggshell dust, hang a cloth from the bottom of the glass front (or alternatively wear a mask and protective eyewear). Drill all four sides of the window, with the egg at a slight angle and the air sac end (blunt end) up and the marked window facing toward the person drilling. The bit of the Dremel tool is brought at right angles to the surface of the shell. Gentle pressure is sufficient for cutting due to the high speed of the drill. Only the shell is cut while avoiding the underlying smoother papery-white membrane or the underlying CAM.

Figure 19.6.3 The arrangement of equipment inside the tissue culture hood just before injections proceed. The front of the hood is opened for easier access during the procedure with adequate sterility still being maintained. The gooseneck arm of the fiber optics light is illuminating the air sac end of an egg positioned on the egg stand (made by gluing three rubber corks onto a plastic petri dish) ready for injection. The remaining eggs have had the shell of the drilled window carefully removed over the vein area and are placed in a tray with the windows facing upwards awaiting injection. The cell suspension has been prepared at a predetermined concentration ready to be drawn up into the 1-cc syringe following gentle vortexing. The other 1-cc syringe contains the paraffin oil of which a drop will be placed on the exposed window area prior to injection. The melted Paraplast in the glass beaker (left) is cooling in preparation for sealing of the entire window area following injection.

Figure 19.6.4 Injection of cells into a chick embryo CAM vein. Position the egg on the egg stand, with the fiber optics light illuminating the air sac end for visualization of the large Y shaped vein. The addition of a small drop of paraffin oil onto the injection site enhances the clarity of the vein. The beveled tip of the needle is facing upwards for gentle insertion into the branch area of the Y (see inset) just until the beveled end is completely into the vein. The needle should be at the angle seen in the picture above to prevent damage to the vein. If a proper injection is performed, the vein will temporarily become clear as the 0.1-ml inoculum is smoothly injected, and there will be no blood seeping from the injection point either during or after the injection.

Figure 19.6.5 Example of in vivo growth kinetics results that can be obtained with the chick embryo experimental metastasis assay and the ouabain plating assay to recover rodent (and thus, ouabain-resistant) cells at various times after injecting cells via a CAM vein. (**A**) B77-NRK (normal rat kidney) cells transformed with the src oncogene, or (**B**) LA23-NRK cells (NRK cells carrying a temperature-sensitive src oncogene, which is active at 36° and inactive at 38°C were injected into CAM veins at 5×10^4 cells per embryo. Closed circles: embryos maintained at 36°C; Open circles: embryos transferred to 38°C at day 4. Each point represents the number of viable ouabain-resistant cells present in the liver of one embryo; lines connect median points. Republished with permission of American Society of Microbiologists from Chambers and Wilson (1985).

Figure 19.6.6 Schematic of the intravital videomicroscopy technique, as used for real-time observations of the interactions of injected cancer cells with the CAM microcirculation. The CAM is exposed and positioned on a plastic platform with a coverslip window above the objective lens of inverted microscope. For visualization of fluorescently labeled cancer cells, oblique transillumination is provided by the fiber optic light and epifluorescence through the objective aids in positive identification of the cancer cells. Images are collected through the video camera and saved to SVHS tape or digitally on a computer. Diagram by S. Koop and I.C. MacDonald, republished with permission of Plenum Publishers from Chambers et al. (1995).

Imaging Tumor Cell Movement In Vivo

Understanding of the contribution of genetics to cancer has expanded greatly over the past several decades. However, studies of the functional significance of changes in oncogenic gene expression in vivo, as well as the effects of drugs on the oncogenic targets in vivo at the cellular level, have been limited by lack of technologies and associated techniques that would allow for dynamic high-resolution analysis. Moreover, the invasiveness of primary tumor cells makes a significant contribution to malignancy. Local invasion can enable tumor cells to spread from the primary tumor through neighboring connective tissues, and is responsible for poor patient prognosis in tumors that cannot easily be surgically removed, such and glioblastoma and head and neck cancer. Local invasion can also be important for the spread of tumor cells from the primary tumor into blood vessels or lymphatics in many other tumor types, including breast cancer. Although in vitro studies of cell motility and invasion are useful surrogates for beginning to understand the mechanisms that regulate invasiveness, the complexity of the in vivo microenvironment, with a wide variety of cell types, vessels, and extracellular matrix, is not yet possible to reproduce in vitro. Thus, it is important to evaluate tumor cell invasiveness using in vivo assays. In particular, the ability to image tumor cells as they are invading allows the direct evaluation of tumor cell motility and opens up experimental evaluation of specific mechanisms for tumor cell invasion.

This unit describes the methods that the authors of this unit have been developing for analyzing tumor cell motility in mouse and rat models of breast cancer metastasis. Rodents are commonly used in order to provide a mammalian system both for studying human tumor cells as xenografts in immunocompromised mice and for following the development of tumors from a specific tissue type in transgenic lines. Basic Protocol 1 describes the standard methods used for generation of mammary tumors and imaging them. Additional protocols for simultaneous CFP and GFP imaging (Alternate Protocol 1), microscope setup (Support Protocol 1), second harmonic imaging (Support Protocol 4), labeling macrophages (Support Protocol 2), blood vessel imaging (Support Protocol 3), and image analysis (Basic Protocol 2 and Alternate Protocol 2) are also included.

NOTE: All protocols using live animals must be reviewed and approved by the local Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

GENERATION AND IN VIVO IMAGING OF MAMMARY TUMORS

BASIC PROTOCOL 1

MTLn3, a highly metastatic rat mammary adenocarcinoma cell line, is often used by the authors' laboratory (Neri et al., 1982). The cells have been transfected with GFP (eGFP from pEGFP-N1 subcloned into PLXSN) so that they can be imaged. In general, cell lines used must stably express GFP, or any other fluorescent protein that can be imaged by the multiphoton microscope, because of the growth that occurs to form a tumor prior to the imaging step. The following protocol is specifically used for MTLn3 and its derivatives. A discussion of alternatives for other lines is provided in Wyckoff et al. (2005). Female 5- to 6-week-old BALB/c SCID mice or 7- to 8-week-old Fischer 344 rats are used. The animals are injected with 0.1 ml of cell suspension in the mammary fat pad, under the second nipple anterior from the tail, and tumors are allowed to grow for 3 to 4 weeks.

Materials

MTLn3 cells (available from authors; *segall@aecom.yu.edu*)
MTLn3 growth medium (see recipe)

Contributed by Dmitriy Kedrin, Jeffrey Wyckoff, Erik Sahai, John Condeelis, and Jeffrey E. Segall
Current Protocols in Cell Biology (2007) 19.7.1-19.7.17

2 mM EDTA (Invitrogen cat. no. 15575-038) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS; Invitrogen; also see *APPENDIX 2A*)

Dulbecco's phosphate-buffered saline (with calcium and magnesium; DPBS; Invitrogen; also see *APPENDIX 2A*)

Female 5- to 6-week-old BALB/c SCID/Ncr mice (National Cancer Institute), Fisher 344 rats (The Jackson Laboratory), or transgenic mouse tumor models (e.g., MMTV-pyMT; The Jackson Laboratory)

70% (v/v) ethanol

Isoflurane,USP (AERRANE, Baxter)

15-cm tissue culture dishes (Falcon)

15-ml conical polypropylene centrifuge tubes, sterile

Cell scrapers

Centrifuge (e.g., Sorvall GLC-1)

1-ml syringes with 25-G needles for cell injections

Oxygen/anesthesia apparatus (Forane Vaporizer, model 100, from SurgiVet)

100% compressed oxygen tanks to be used in combination with vaporizer

Surgical instruments

In vivo imaging microscope setup (Support Protocol 1)

Multiphoton apparatus setup (Support Protocol 4)

Surgical tape (optional)

Additional reagents and equipment for tissue culture techniques including counting cells (*UNIT 1.1*), housing of immunocompromised mice (Donovan and Brown, 1995), imaging blood vessels (Support Protocol 2 or 3; optional), inhalant anesthesia of rodents (Donovan and Brown, 1998), and image analysis and quantitation (Basic Protocol 2)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be carried out in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Grow and prepare cells for injection

1. Grow MTLn3 cells in MTLn3 growth medium to 50% to 70% confluence in a 15-cm tissue culture dish.

   UNIT 1.1 describes basic cell culture techniques.

2. Under sterile conditions, rinse the dishes three times, each time with 10 ml of 37°C sterile CMF-DPBS containing 2 mM EDTA.

   Since the cells are tightly attached to the dish, it is possible to aspirate all of the medium and gently add fresh medium to the side of the dish without losing many cells.

3. Add 12 ml of CMF-DPBS/2 mM EDTA and place at room temperature to detach cells.

   Depending on the thoroughness of the previous rinse steps, this can take from 10 to 30 min. Once 75% of the cells are detached after tapping the dish on a surface, the cells are ready to be collected.

   It may also be possible to use trypsin/EDTA without adverse affects on the cells. In this case, take care not to over-trypsinize, neutralize the trypsin with some serum, and then wash cells. Also see UNIT 1.1.

4. Add 6 ml DPBS containing Ca and Mg and detach the remaining cells by gently rinsing.

5. Collect the cells into two 15-ml sterile tubes, scraping the dish to collect the matrix and adding it to the tube as well. Take an aliquot and count cells using a hemacytometer (*UNIT 1.1*).

   Cell counting may be performed during the subsequent centrifugation step.

6. Centrifuge cells 5 min at 180 to 200 × *g*, room temperature.

7. On the basis of the cell count performed on the aliquot taken in step 5, determine the appropriate volume for resuspending the pellet to obtain a cell density of $\sim 1 \times 10^7$ cells/ml. Resuspend cells in sterile DPBS at 1×10^7 cells/ml, and recount (*UNIT 1.1*) to confirm actual density.

   Dilutions as low as 5×10^6 cells/ml can also be used, which produce tumors slightly more slowly.

8. Transfer cell suspension to a sterile 1.5-ml microcentrifuge tube and keep on ice until injection.

Inject cells for xenograft

9. With two people working together, one to hold the animal and another to inject the cells using a 1-ml syringe fitted with a 25-G, prepare for injection of cells.

   As one person prepares the mouse by picking it up by the back of the neck and holding the back leg with another hand, the second person takes the cell suspension off the ice and fills the syringe.

10. Spray the area at the second lowest nipple with 70% ethanol and insert the needle subcutaneously at a shallow angle in the mammary fat pad. Inject 100 µl of cell suspension and notice a slight bulge in the skin above the injected area.

    At this step it is important that the needle only penetrate the skin and stay within the mammary fat pad, so no cells spread into the peritoneum. To ensure this, keep the angle of injection very shallow.

    Injection of Fischer 344 rats is done in the same manner, but injection volume can be increased to 150 to 200 µl.

11. Keep the animals in the barrier-room facility (see Donovan and Brown, 1995) until the tumors are large enough to image (0.5 to 1 cm diameter for mice or up to 1.5 cm for rats, typically 3 to 4 weeks). Check tumor size on a regular basis to make sure it is growing.

    The tumor should be palpable after 1.5 weeks. After 3 to 4 weeks, the tumor should be about 5 to 7 mm in diameter (mice) or 1 cm in diameter (rats), at which time the animals are ready to be imaged.

    Smaller tumors are harder to image due to lack of good surfaces, because the tumor has not grown through the fat layers of the fat pad, which interferes with image collection. Larger tumors tend to become necrotic, thus destroying adequate imaging surfaces on the tumor, or the large tumor can invade the peritoneum, thus preventing skin-flap surgery from being adequate for imaging purposes.

    Alternatively, transgenic animals, such as those described by Wyckoff et al. (2004), can be used to image tumors. For this purpose, animals are grown until tumors reach a size adequate for imaging. Palpable tumors are large enough to image in transgenic animals, but due to staging of tumor progression, intravasion and metastasis do not occur generally until late-stage carcinoma. Therefore, each tumor model will be different in determining the best time for imaging.

Prepare animal for imaging

12. Place the animal under anesthesia with 5% isoflurane in O_2 using an oxygen/anesthesia apparatus.

    Isoflurane is to be administered via an oxygen mask to the animal prior to surgery, for the time of surgery, and while animal is used to collect imaging data.

13. *Optional:* Inject Texas red–dextran to image blood vessels (see Support Protocol 2 or 3).

14. Place the animal on the surgery stage and swab the tumor area with 70% ethanol. Perform a medial skin incision, exposing the tumor.

15. Clear the area around the tumor from fascia and fat while maintaining blood vessel supply as much as possible and keeping bleeding to a minimum.

    This must be done carefully and meticulously to avoid destroying the vasculature or cutting into the tumor itself.

16. Transfer the animal to a prewarmed imaging stage (30°C) with the exposed tumor placed on the coverslip (objective) for imaging. If necessary, use surgical tape to hold the tumor in place.

    The tumor should be isolated away from the animal as much as possible and secured to the microscope platform. Stages can be designed so as to make it possible to pin the skin around the tumor to cork; alternatively, the skin flap can be taped to the stage. This must be done carefully and without compressing the tumor too much. The tumor should be separated as far as possible from the mouse so as to minimize vibrations due to breathing.

    Fat cells work as lenses in the tumor and disrupt the multiphoton events. Fat should be removed as much as possible without disrupting the blood flow to the tumor, to allow for better image quality.

17. During imaging, sequentially lower isoflurane level in steps of 0.25% every 20 to 30 min to maintain a constant low breathing rate, defined as one or fewer distortions per image at 166 lines per second scan speed.

    Maintenance of breathing and minimization of drift and movement of the animal is key for successful time-lapse data collection. The best way to minimize breathing is by controlling the isoflurane level. The isoflurane should be monitored to control the amount of breathing during the imaging session, as described above. The ability to manipulate the breathing is why using isoflurane is a better choice than injectable anesthesias.

    If breathing is too rapid, distortions might make acquired data difficult to interpret, at which point the anesthesia levels should be adjusted or the position of the animal on the imaging platform should be adjusted to minimize drift and breathing artifact. With time, less anesthesia is required to maintain a low breathing rate; too much anesthesia will kill the animal. Typical final isoflurane levels are 2% to 3.5%.

Perform intravital microscopy

Each image is essentially in the *x-y* imaging plane. A *z*-series of individual *x-y* images is collected by stepping through the images. No specific detail on how to collect the *x-y* images is provided here; that will depend on the instrument used.

18. Focus in on fluorescence of the GFP tumor, looking for areas with flowing blood vessels by viewing through the eyepiece in epifluorescence mode. Upon finding an area that has bright fluorescence and flowing vessels, switch to imaging via the multiphoton mode.

    For one-color GFP imaging, the animal is placed under anesthesia as described above and imaged. On, average, each imaging sequence lasts 20 to 30 min, and the same animal can be maintained on the microscope for up to 4 hr.

19. While imaging in the multiphoton mode, move around the tumor until a good imaging area is obtained.

    If vessel imaging is desired, find a vessel to be imaged, or find the edge of the tumor, judging by fluorescence.

    Good imaging fields for cell invasion and intravasation are areas around flowing vessels with well defined cell boundaries and extracellular matrix. Flowing vessels can be visualized by labeling with a fluorescent dextran or by viewing vessels through the oculars of the microscope.

    The authors find that focusing at the outer tumor surface, where cells are just invading the matrix, and generating a z-series stepping into the tumor until the image degrades to a point where cell morphology is not well defined, gives the best images for analysis. Many systems now can vary the laser power as a function of z-position, allowing the use of more power to image greater depths without damaging or overexposing the tissue closer to the objective.

20. For the outermost plane of the *z*-series, focus in on the outside of the tumor by visualizing the collagen fiber network in the second harmonic channel (Fig. 19.7.1A).

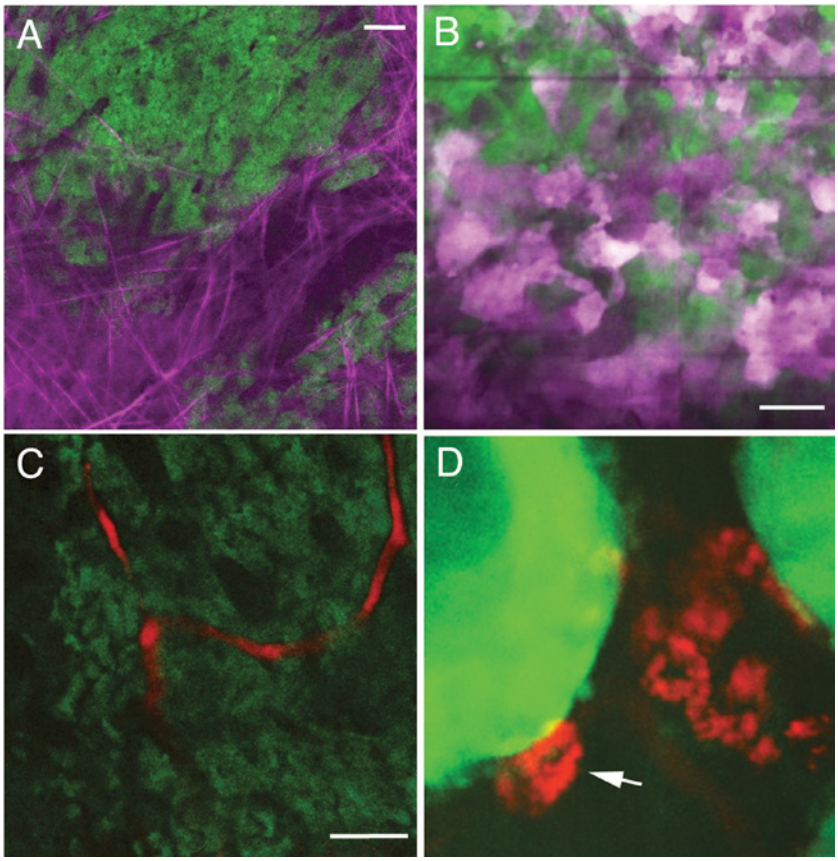


Figure 19.7.1 Multiphoton imaging of MTLn3 xenotransplant tumor in mouse. (**A**) GFP-expressing tumor cells are shown in the green channel and matrix second harmonic image is shown in both red and blue channels (for contrast). (**B**) Mix of GFP-expressing cells (green channel) with CFP-expressing cells (red and blue channels) in a single tumor. (**C**) Simultaneous imaging of GFP-expressing tumor cells (green) and blood vessels (red) following intravenous injection of Texas Red–dextran. (**D**) Imaging of macrophages labeled with Texas Red–dextran (red) in GFP-expressing MTLn3 tumor. Scale bars for A and C represent 50 μm, those for B and D represent 25 μm. For the color version of this figure go to *http://www.currentprotocols.com.*

Depending on the goal of the imaging session, the size of the z-series that is selected is constrained by: (1) the desired quality of each image, (2) the time-lapse interval desired between images at a particular plane, and (3) the z-axis resolution desired. The quality of each image is determined by the brightness of the fluorescence. For brightly labeled tumors, shorter imaging times per frame are needed, but for dimly labeled tumors, imaging time may become limiting. The authors typically use a scan rate of 166 lines per second for moderate-quality and 50 lines per second for high-quality images. The time-lapse interval required is determined by the type of structures that are being imaged. Subcellular shape changes using a high-magnification (40× or 60×) objective may require 10- to 30-sec time intervals. General tumor cell tracking using lower magnification objectives (20×) may be adequate at 1- to 3-min intervals.

21. Set up the time-lapse *z*-series (generally 10-μm steps for 100 μm and 1-min intervals for 30 min) and start collecting images.

    For time-lapse multiphoton imaging, as many z planes should be taken as is possible given the above constraints on image quality and time-lapse interval. Especially during the first 30 min of imaging, there is significant (typically >10 μm) drift. This z-series collection can compensate to some degree for this drift, but in the authors' experience, tracking a cell from one plane in a z-series to the neighboring plane always generates an artifact for the time interval during which the shift occurs. For detailed tracking of individual cells, 2.5- to 5-μm step sizes are best. For experiments in which one wishes to sample through as great a depth as possible to compare behavior at the periphery versus the interior of the tumor, 10-μm or larger steps may be required.

    This step provides information regarding the depth of tumor cells moving within the tumor.

22. Euthanized the animal at the end of data collection.

23. Perform image analysis (Basic Protocol 2).

SUPPORT PROTOCOL 1

IN VIVO IMAGING MICROSCOPE SETUP

A 10-W Tsunami Ti-Sapphire femtosecond pulsed laser from Spectra Physics is used in the authors' setup. This laser is used to run a Radiance 2000 multiphoton system (Bio-Rad). This system gives an output of 850 mW at 960 nm. Although the instrument from Bio-Rad is no longer sold, similar systems are available from Zeiss, Leica, and Prairie. This wavelength is optimal for imaging GFP fluorescence, but the authors have found that working between 860 and 880 nm is optimal for imaging GFP, CFP, matrix fibers, and Texas Red at the same time. The inverted Olympus IX70 microscope is ideal for placing an animal on the imaging platform.

It is important to have the animal in a 30°C environment; there is some tumor cell movement at lower temperatures but it is less, while at higher temperatures the animal begins to hyperventilate, presumably due to overheating. Also, other cell types are much more sensitive to temperature effects; T cells may be particularly affected. Thus, in the authors' laboratory there is a thermal chamber erected surrounding the imaging platform to maintain the animal's physiology while under anesthesia. The chamber takes air from a heating element and maintains a temperature of 30°C. An alternative is to cover the animal during imaging with a heating pad or small cloth, depending on the temperature of the room. Rooms that are highly air conditioned in order to keep the electronics from overheating may be too cool.

The objectives used are as follows: 20× Plan Apo 0.7NA (air); 40× LUMPlan/IR 0.8NA (water); and 60× LUMPlan/IR 0.9NA (water). The filters used are as follows (all from Chroma): 450/80 (blue), 480/30 (CFP), 515/30 (green) and 620/60 (red). The multiphoton instrumentation can be calibrated so that the images taken on separate days are of the same intensity by using fluorescent beads and doing a *z*-series at laser powers that give a range of intensity equal to a standard *z*-series performed when the laser has been

perfectly aligned. Laser power and gain settings are determined by the brightness of the chromophore in the tumor. An image should have a good dynamic range to be considered usable for data interpretation. Depending on the precise measurements and depth of image stack desired, it may be useful in some cases to be at saturation in cells in the uppermost slice to allow adequate signal from cells in deeper slices.

LABELING MACROPHAGES TO VISUALIZE BLOOD FLOW

SUPPORT PROTOCOL 2

To provide information about blood flow in and around the tumor, macrophages are labeled with Texas red–dextran by injecting the dye in the tail vein of a tumor-bearing animal.

Materials

Mouse with xenograft-induced tumor (Basic Protocol 1)
20 mg/ml Texas Red–dextran (70 kDa, Invitrogen) in DPBS (Invitrogen; also see *APPENDIX 2A*)
70% (v/v) ethanol

Mouse restrainer (Donovan and Brown, 2006)
1-ml syringe with 26-G needle
Superglue

Additional reagents and equipment for mouse restraint (Donovan and Brown, 2006)

1. Warm up the tail vein of the tumor-bearing animal by immersing the tail for 1 to 2 min in warm (50° to 60°C) water.

   This causes the tail vein to dilate so that a 26-G needle can be inserted. The animals can also be placed 4 to 8 in. (~10 to 20 cm) away from a lamp with 150-W light bulb for 5 min.

2. Place mouse into restrainer by holding it by the tail, and close the restrainer so the tail is easily accessible.

3. Wipe the tail with 70% ethanol and rotate it to position the lateral vein for injection. Stabilize the vein by holding the tail between thumb and middle finger and securing the vein with the index finger.

4. Prepare a 1-ml syringe with a 26-G needle containing 200 µl of 20 mg/ml Texas Red–dextran. Inject the mouse by orienting the bevel of the needle facing upward and forcing it gently through the skin, parallel to the vein, 1 to 1.5 in. (~2.5 to 4.25 cm) from the base of the tail.

   Successful insertions into the vein are indicated by easy sliding of the needle into the vein and easy injection of the solution without accumulation at the needle tip.

5. Inject slowly so that the 200 µl of the dextran solution enters the vein.

   If too much force is required for injecting the solution, that indicates the needle is not inserted into the vein. In this case, attempt additional injections, moving slightly closer to the base of the tail with each attempt.

6. Place the mouse back into the cage for later imaging.

   The animals are left for 1 to 2 hr. Because the ability to image macrophages depends on their ability to take up dextran molecules by phagocytosis, the authors have found that this time interval is adequate for labeling.

7. Image macrophages with red filter (Fig. 19.7.1D) while imaging the tumor with the green filter (515/30 filter).

   See Basic Protocol 1 for imaging steps.

SUPPORT PROTOCOL 3

BLOOD VESSEL IMAGING USING AN INDWELLING CATHETER

Either of two procedures can be used for blood vessel imaging: 200 μl of 20 mg/ml Texas Red–dextran (70-kDa Molecular Probes) is injected at a concentration of 20 mg/ml in Dulbecco's PBS via the tail vein (see Support Protocol 2) or via an indwelling intravenous catheter in the jugular vein of the rat or mouse as described in the protocol below. Tail-vein injection can be performed after anesthesia, but before surgery, whereas the indwelling catheter can be perfused while the animal is being imaged, if need be, when it is necessary to visualize vessels (Fig 19.7.1C). After introduction of dextran into the circulation, it can be visualized using the Texas Red channel of the Bio-Rad confocal microscope or by a comparable filter in the Bio-Rad Radiance 2000 multiphoton microscope.

This procedure requires some practice because catheter insertion is not trivial. It is also important to note that since multiphoton imaging requires another nonsurvival surgery, the incision for the catheter insertion should be minimized. Alternatively, direct catheterization of the tail vein can be performed.

Additional Materials *(also see Basic Protocol 1)*

Mouse with xenograft-induced tumor (Basic Protocol 1)
Heparinized PBS solution (Baxter, cat. no. FKB0953G)

Indwelling tail vein catheter (no. MTV-01, SAI Inc.; *http://www.sai-infusion.com*)
1-ml syringes
Waterproof marker (e.g., Sharpie)

Prepare catheter

1. Prepare indwelling catheter by pulling out the guide wire and attaching a 1-ml syringe filled with heparinized PBS.

   A catheter designed for the tail vein is used, but it will be inserted into the jugular vein for this protocol.

Insert the catheter

2. Insert the catheter into the guide needle so that 4 mm of catheter extends out the tip of the needle. Mark the catheter using a Sharpie where it inserts into the rear of the needle, as a guidepoint for the depth of insertion in to the vein.
3. With the animal anesthetized using 5% isoflurane (see Basic Protocol 1), spray the right thorax area with 70% ethanol.
4. Make a small incision to expose the left external jugular vein and the overlaying muscle layer.
5. Insert the guide needle through the muscle into the overlying jugular vein.
6. Push the catheter 4 mm into the vein and, while holding the catheter in the vein with a small forceps, pull out the guide needle over the catheter.
7. Place a drop of glue (e.g., Superglue) over the site of insertion of the catheter into the vein to hold it in place.

Test the catheter

8. Allow the glue to dry for several seconds and push a few microliters of PBS through the syringe to see if there is any leakage (an indicator of a bad insertion).
9. Try drawing blood to make sure that the catheter is patent.

   If correctly inserted, blood should be drawn into the catheter. However, in some cases, if the catheter tip is against the wall of the vessel, it may be difficult to draw blood.

Inject the dextran

10. Prepare another 1-ml syringe filled with 20 mg/ml Texas Red–dextran. Switch the catheter from the PBS syringe to the 20 mg/ml Texas Red–dextran syringe and prepare the animal for imaging (Basic Protocol 1).

11. After an area containing a flowing blood vessel has been discovered, inject the Texas Red–dextran into the vessel and observe the fluorescence by collecting a time-lapse image of the area (Basic Protocol 1).

SECOND HARMONIC FIBER IMAGING

SUPPORT PROTOCOL 4

A valuable benefit of multiphoton microscopy is the ability to use the second harmonic scattering effect (Zipfel et al., 2003; see *UNIT 4.15*) to generate images of extracellular matrix fibers (ECM) around the carcinoma cells of the tumors. The size, amount, and condition of the ECM fibers that are imaged can be determined by measuring the signal intensity and morphology of the images generated by the second harmonic (Ahmed et al., 2002). However, it is important to appreciate that such images label only a very restricted subset of matrix molecules that may be present. Typically only large, type I collagen fibers are likely to be imaged. With alternative wavelength comparisons, elastic fibers may also be identified (Zipfel et al., 2003), but other structures including small fibers, basement membrane, fibronectin fibers, and other cell types are not imaged. The authors are able to excite the second harmonic signal with wavelengths of 760 to 960 nm, and because the work is typically more concerned with imaging cells, the excitation wavelength is selected based on the cell to be imaged (860 to 880 nm for GFP), and the second harmonic image that is generated is then collected using a 450- to 480-nm-cutoff filter.

SIMULTANEOUS CFP AND GFP IMAGING

ALTERNATE PROTOCOL 1

The authors' group has previously shown that it is possible to image two separate populations of cancer cells in vivo using the multiphoton microscope (Sahai et al., 2005). To image two populations, it is important to choose the correct fluorescence pair, based on the excitation wavelength, so that both fluorophores can be excited at a single excitation wavelength (a common limitation of current multiphoton imaging is the difficulty in changing excitation wavelengths). In the authors' studies, CFP and GFP (Fig. 19.7.1B) are used due to the ease of excitation with the Ti-sapphire laser as well as the expression tolerance in most cell types. In the authors' experience, other fluorescent proteins such as YFP and RFP are not useful, due to the weak excitation at wavelengths attainable by the laser used. The optimal wavelength for imaging CFP and GFP was determined to be around 880 nm (Sahai et al., 2005). Because detection of the CFP signal in the system used by the authors overlaps with GFP, it was found that the gain for collection between the two signals needs to be set so that the CFP signal is equal in both fields. Also for ease of collection with a two-detector system, because the second harmonic image is captured with the same PMT as the CFP image, by making the gain equal between the CFP image and the GFP image the collagen fibers can also be identified as structures that are only present in the CFP channel and not in the GFP channel.

MULTIPHOTON TIME-LAPSE IMAGE ANALYSIS USING ImageJ AND CUSTOM MACROS

BASIC PROTOCOL 2

The authors of this unit have found ImageJ, a free image-processing program developed by Wayne Rasband at the NIH, to be a tremendously valuable image-manipulation program. ImageJ is written in Java, has the source code available, and is developing an ever larger user group. The authors have developed plugins for multiphoton time-lapse analysis

using ImageJ version 1.36b and running Java 1.5.0_06 under Windows XP; these are available at *http://asb.aecom.yu.edu/segall/ImageJ%20plugins/ImageJIntro.html*. People running an earlier version of either should try downloading the latest versions from *http://rsb.info.nih.gov/ij/download.html*. Download and install the latest version bundled with the JRE from that page.

It is useful to have a machine with lots of RAM for generating slice movies from *z*-series stacks (probably twice the total amount in the *z*-series files to be converted to movies, on the order of a gigabyte for current uses) because all the data are loaded into RAM in order to reshuffle them. Thus, an amount of RAM at least equal to the total file size must be allocated to ImageJ in the `.cfg` file before running it (i.e., the `-Xmx340m` command could be changed to `--xmx500m` to correspond to 500 Mb of RAM). If RAM is limited, it is possible to combine just a fraction of the total *z*-series, either by reducing the time resolution (i.e., taking every second or third *z*-series to make the movies), or else by making movie fragments that can be merged using the Concatenator plugin (downloaded from the ImageJ plugins download site).

The discussion below involves three typical procedures that are performed:

1. Generating time-lapse movies from time-lapse *z*-series.
2. Eliminating *x-y* drift in time-lapse movies.
3. Marking areas of interest in time-lapse movies.

Generating Time-Lapse Movies from Time-Lapse *z*-Series

The authors of this unit typically generate a sequence of *z*-series in which one *z*-series is accumulated every minute or so. In order to then image the motility that is occurring in a particular image plane in the stack, it is necessary to track particular planes. Currently, the authors have a set of plugins that are modifications of plugins available on the ImageJ Web site (in the Stacks to Movies folder). Place the entire folder in the plugins folder of ImageJ and start ImageJ. Next, in the plugins drop-down box, select the Stack to Movies option and click on StackstoMovies. The plugin begins by asking for a name that will be attached to each movie slice, and then asks whether it is a blue-green *z*-series (files ending in `01.pic` will be put in red and blue channels; files ending in `02.pic` will be in green channels), or red/green *z*-series (files ending in `01.pic` will be put in green channel; files ending in `02.pic` will be in red channel). These options can be modified by changing the `js1stepRGB_Stack_Merge.java` file. The program then loads the files, assembles color stacks, concatenates the stacks, rearranges them into movies of slices, and then checks where to save each one. The results can then be viewed using ImageJ and *x-y* drift can be compensated using the plugins described in the next section.

Items to put in Plugins folder

Stacks to Movies folder (*http://asb.aecom.yu.edu/segall/ImageJ%20plugins/ImageJIntro.html*).

Procedure

1. Start ImageJ.
2. Select StackstoMovies from the plugin drop-down menu.
3. Provide a name to be appended to each slice number.
4. Determine whether `...01.pic` files are to be red (option 1) or `...02.pic` files are to be red (option 2).

5. Select a place to store the completed slice movies and approve saving of each slice movie.

Possible memory issues for this step

It is necessary to have a machine with lots of RAM (probably twice the total amount in the *z*-series files that are to be converted to movies) because all the data are loaded into RAM in order to reshuffle them. In addition, an amount of RAM at least equal to the total file size must be allocated to ImageJ in the `.cfg` file before running it (i.e., the `-Xmx340m` command could be changed to `--xmx500m` to correspond to 500 Mb of RAM). If RAM is limited, it is possible to combine just a fraction of the total *z*-series, either reducing the time resolution or else making movie fragments which can be merged using various stack commands that can be downloaded from the ImageJ plugins download site.

Eliminating *x-y* Drift in Time-Lapse Movies

Philippe Thévenaz has written a valuable series of registration plugins that can reduce/eliminate *x-y* drift from color time-lapse sequences. This involves first downloading a plugin called TurboReg, and then downloading StackReg. In each case, the downloads are `.zip` files, which should be extracted to the plugins folder of Image J. The extractions will generate two folders in the plugins folder named `stackReg` and `TurboReg`. It is necessary to restart ImageJ, open the movie (only have one movie open at a time) from which drift is to be eliminated, and then go to the plugins drop-down menu in Image J, move to the StackReg line, and click on the StackReg box that appears. Various transformations can be chosen, but probably the default choice (rigid body) is best. More information is available at the download site *(http://bigwww.epfl.ch/thevenaz/stackreg/)*. Note that this plugin runs quite slowly: it can easily take a minute to step through a 30-frame movie. It registers each frame relative to the previous frame, and thus with time, black areas appear on the sides opposite the direction of drift of the movie. The program may not be able to accommodate rapid, large changes in position. In these cases, one can use TransformJ (again available from the plugins section of the ImageJ Web site) to shift sets of slices of the movie in the *x-y* plane in order to reduce the jumps enough for StackReg to track them. Changes in *z*-axis are extremely difficult to compensate for, requiring more extensive *z*-series collection (perhaps every 1 to 3 μm, depending on the scale of magnification) and then manually assembling images from the appropriate planes of each *z*-series.

Items to put in Plugins folder

StackReg folder: *http://bigwww.epfl.ch/thevanaz/pointpicker/*.

TurboReg folder: *http://bigwww.epfl.ch/thevanaz/pointpicker/*.

Procedure

1. Start ImageJ.
2. Open movie to be registered.
3. Select StackReg from the plugins drop-down menu.
4. Select Rigid body transformation.
5. Wait patiently until the progress bars in the ImageJ command bar stop appearing and the movie has stepped through all of its frames.

Marking Sites of Interest in Time-Lapse Movies

It can be useful to mark sites of interest in time-lapse movies; for example, the authors mark where tumor cells are moving as part of quantitating tumor cell motility, and they have written a simple series of macros/plugins that make use of another valuable plugin written by Philippe Thévenaz, called Pointpicker (*http://bigwww.epfl.ch/thevenaz/pointpicker/*). Pointpicker keeps a record of the *x*-*y* coordinates of the mouse when clicked and allows them to be saved or copied. The authors typically are just counting instances of tumor cell movement and thus wish to mark their locations and keep track of the total number. Usually, this is most accurately done viewing just the tumor cell channel in black and white; however, Pointpicker will work on either color or black-and-white movies.

For GFP-labeled cells, there is a simple plugin, Greentopointpicker, that selects the green channel and begins the Pointpicker plugin.

Items to put in Plugins folder

Greentopointpicker plugin: *http://asb.aecom.yu.edu/segall/ImageJ%20plugins/ImageJIntro.html*.

jsgetGreen plugin: *http://asb.aecom.yu.edu/segall/ImageJ%20plugins/ImageJIntro.html*.

Pointpicker: *http://bigwww.epfl.ch/thevanaz/pointpicker/* (folder with Pointpicker plugin and files in it).

Procedure

1. Start ImageJ.
2. Open time-lapse movie of slice to analyze (preferably already registered to remove drift, jitter, etc).
3. *Optional:* Select the color desired for the crosses by going to Edit-options-colors and change the selection color to the desired color, e.g., red.
4. *Optional:* Run the plugin Greentopointpicker: this extracts the green image (typically the cell image to be tracked) and then starts Pointpicker.
5. *Optional:* Convert crosses to just one color (double click on the pen+ [leftmost] button and choose monochrome).
6. Click on the "Apply to all slices" button (sixth from the left, has a sliced cube image on it) so that the crosses will be on all slices.
7. *Optional:* It can be useful to magnify the image 1× (click on magnifying glass, click on image, then click on the pen+ [leftmost] button to go back to marking mode).
8. *Optional:* It can be helpful to play the movie at high speeds, looping back and forth continuously to see motions (Stacks–animation options).
9. *Optional:* Expand the image to be tracked to the full screen (this is good for seeing small movements).
10. Start the movie running in a continuous loop.
11. Mark all the sites of interest (i.e., tumor cell motility) by clicking on the pen+ (leftmost) button and clicking with the mouse of the specific sites. A cross should appear at each marked site.

12. *Optional:* Errors can be removed by clicking on the pen– button (third from left) and clicking the mouse anywhere on the screen. However, this simply removes the marks in reverse order from that in which they were made; specific marks cannot be removed from this sequence.
13. Stop the movie and shrink the image back down to view the ImageJ tool bar.
14. Reveal the points list by clicking on the "document" button (eighth from left) and clicking on the "show" button from the drop-down menu.
15. The results box will appear with point, *x*, *y*, slice, and color columns. The information in each slice should be identical. Highlight the rows with the appropriate slice number corresponding to the slice currently being analyzed and paste them into an Excel file. For different analyses, the file can have different worksheets (i.e., tumor cell motility, host cell motility, movement on matrix, orientation towards blood vessel, etc). This then stores the number and coordinates of the locations of cell movement together with the slice number. Next, close the results box. If `jsDrawDot1` is to be used to mark the sites on a *z*-stack, copy and save the results with the number in the slice column being the slice number plus 1 (this provides the correct registration of the dots with the *z*-series using `jsdrawDot2`).
16. It is usually good to save the Excel file at this point. A file name indicating the date of the experiment and other distinguishing information is helpful for keeping the data organized.
17. *Optional:* To further analyze the same movie for a new category, remove the current markings by selecting the pen− button (third from the left) and click on the image until all the crosses are gone (and the pen+ button will automatically depress).
18. Repeat steps 10 through 16 for as many categories as desired.
19. Close the movie image.
20. Click the "return" button to return to the standard ImageJ menu, open the next file to be analyzed, and repeat the process.
21. *Optional:* save the Excel data file with the file name of the experiment in the same directory as the registered files.

Marking Specific Locations with Red Circles in a *z* Stack Using the Data Acquired from Pointpicker

Plugins used (put both just in the Plugins folder of ImageJ)

JsdrawDot1: *http://asb.aecom.yu.edu/segall/ImageJ%20plugins/ImageJIntro.html*.

IntLoader2: *http://asb.aecom.yu.edu/segall/ImageJ%20plugins/ImageJIntro.html*.

Procedure

1. To superimpose the dots on a *z*-series of the data, copy the columns (not the header column) and paste as unformatted text into a Word file, then save the Word file as plain text. This makes the data readable for `IntLoader2` (called by `jsdrawDot2`).
2. Open the *z*-series upon which you wish to superimpose the sites.
3. Run `jsdrawDot2`. It will remind the user what is required, and after clicking OK, it will ask for the location of the plain text file that has the data acquired from Pointpicker.

4. The program will note "All done" when finished.

The Manual Tracking plugin by Fabrice Cordelieres can be useful for following individual cells in two and three dimensions. Link to this plugin from ImageJ site: http://rsb.info.nih.gov/ij/plugins/track/track.html.

ALTERNATE PROTOCOL 2

IMAGE ANALYSIS AND QUANTIFICATION

Motility, invasion, and polarization

Because the data are collected as a time-lapse in *x,y* plane as well as a *z*-series, measurements of cell behavior, such as polarity, motility, and intravasation can be visualized.

1. Collect images for ~30 min at 1-min intervals.

   Each image is collected as an x-y image; the z-series (or z-stack) refers to a number of x-y images in a z plane.

2. Convert images into time-lapse movies using NIH ImageJ.

   This will depend on individual microscope image acquisition software. Macros can be written to shuffle the z-series slices into time-lapse movies in each image plane. Please see Basic Protocol 2 for image analysis using ImageJ.

3. Visually inspect the slices for moving cells and record their behavior patterns (e.g., movement on or off matrix fibers, interaction with host cells, movement towards blood vessels).

   Most MTLn3 cells will not be moving, but motile cells will move with speeds of up to 10 μm/min.

4. Examine each time-lapse *z*-series for the number of moving cells.

   This can be done by counting individual cells in each of the planes and adding them up. DIAS analysis can be used to determine such variables as speed, persistence, distance, and direction (Farina et al., 1998; Ahmed et al., 2002; Wang et al., 2003). For more detailed description of DIAS image analysis, see Cammer et al. (1997).

5. For quantification of cell orientation towards blood vessels, collect images as described above, with animals injected with Texas Red intravenously during imaging.

   Cells within two diameters of the vessels are defined as polarized if a cell's length is at least twice its width and directed towards the vessel.

6. Quantify motility along fibers and other cell-matrix interactions if the second harmonic images are collected as well.

7. Quantify attachment indirectly (by comparison only) by quantifying differences in cell-matrix colocalization, or by quantifying differences in cell-matrix locomotion (Ahmed et al., 2002).

Color (CFP-GFP) image analysis

8. Use the Image Calculator feature of ImageJ (available from *http://rsb.info.nih.gov/ij/*) to subtract the CFP fluorescence captured using either 450/80 or 480/30 filters from the mixture of GFP/CFP fluorescence passing through 515/30 filter.

   This procedure will yield the GFP fluorescence alone.

9. To normalize for differences in cell number in any given field, calculate the number of pixels for each cell type.

10. Monitor two separate populations of cells in the same tumor (Fig. 19.7.1B). By analyzing the time-lapse *z*-series of the tumors, compare the cells in terms of motility, morphology, protrusion, and polarization.

    Please refer to the Basic Protocol 2 for image analysis discussion.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

MTLn3 growth medium

Minimal Essential Medium, α modification (α-MEM; Invitrogen) containing:
5% (v/v) fetal bovine serum (Fisher cat. no. SH3008803IR)
1× penicillin/streptomycin (Invitrogen)

COMMENTARY

Background Information

In recent years, the use of multiphoton laser scanning microscopy, with its benefits of tissue penetration depth and high resolution, has shed light on many aspects of tumor microenvironment. Multiphoton fluorescence excitation uses a laser beam to focus photons of a wavelength two times longer than the excitation wavelength of the fluorophore at a single point (Wang et al., 2003; *UNIT 4.15*). This allows for greater penetration with less phototoxicity and bleaching during *z*-series collection.

Troubleshooting

Imaging tumors that are closer to the chest cavity is more difficult due to breathing possibly interfering with image collection. Animals with such tumors must be kept more sedated, and this may affect the length of the imaging session.

Data collection requires a large amount of hard drive space (500 Mb to 1 Gb/4D series), so make sure that the computer is up to date and able to handle large amounts of data for storage and transfer.

Anticipated Results

Using the metastatic MTLn3 rat mammary adenocarcinoma cell line transfected with GFP (Wyckoff et al., 2000), and comparing to the nonmetastatic MTC cell line, it is anticipated that ∼57% of blood vessels in tumors generated from the metastatic cell line have at least four cells oriented towards them, compared to ∼24% for the nonmetastatic cell line. Other parameters, determined as the number of fields with associated events that were found to be distinct, were protrusion (62% versus 47%), translocation (17% versus 10%), and cell fragmentation (6% versus 32%; Wyckoff et al., 2000).

Carcinoma cells in the metastatic MTLn3 cell line–derived tumors (Fig 19.7.1A) were found to be associated with and to locomote on collagen-containing fibers when compared to the nonmetastatic MTC cell line (Ahmed et al., 2002). Correlation of tumor cell behaviors with gene-expression analysis allows for a novel way of exploring cancer-related genes (Wang et al., 2005). From their experiments, the authors have found that the minimum motility machine genes are coordinately up-regulated in both metastatic tumors as compared to nonmetastatic tumors (Wang et al., 2004) and in the invasive population when compared to the metastatic tumor (Wang et al., 2004).

Using multicolor imaging (CFP and GFP cell populations with different metastatic potential in the same tumor), the authors were able to observe (Fig 19.7.1B) morphological differences, such as the movement speed between the two cell lines in the same microenvironment, and showed that motile cells often follow the same path (Sahai et al., 2005). Coinjection of parental MTLn3 cells with ErbB1-overexpressing MTLn3 cells showed that the ErbB1 overexpressor cells moved at a greater frequency than the parental cells and that more metastatic differences could be observed (Xue et al., 2006).

Host cells could be seen as shadows crawling over the background of the GFP fluorescence (Wyckoff et al., 2000). Some of these cells were determined to be macrophages by injecting Texas Red–dextran intravenously and allowing the macrophages to phagocytose the dextran (Fig. 19.7.1D). Imaging of tumor cell and macrophage cell interactions confirmed a paracrine loop involving EGF and CSF-1 and their corresponding receptors, which was discovered by using an in vivo invasion assay developed in the authors' laboratory (Wang et al., 2004). Tumor cells and macrophages could be imaged moving toward the bevel of a needle containing Matrigel and EGF and CSF-1, respectively (Wang et al., 2004).

Transgenic mice containing the PyMT antigen have been crossed with a *Cre-lox*-β–actin promoter-GFP to create mice with fluorescent mammary tumors (Ahmed et al., 2002). The

PyMT-generated tumors have been shown to mimic human disease (Lin et al., 2003). Many of the morphological characteristics seen in the orthotopically injected models have been seen, including cell motility, invasion, and intravasation. The authors have also crossed the PyMT mice with other GFP-containing transgenics to observe the role of host cells in tumors. These include Tie2-GFP mice with fluorescent endothelial cells (Motoike et al., 2000), lys-GFP mice (Faust et al., 2000), and C-fms-GFP mice (Sasmono et al., 2003) that label macrophages and granulocytes.

Time Considerations

Depending on the cell line used, it can take several months for animals to develop tumors (this is mainly true for human cell lines). In the authors' experience, MTLn3—a fast-growing cell line—is ideal in this respect, with tumors developing in ~3 weeks.

Each animal can stay on the microscope for up to 4 hr, under ideal conditions; thus, it is important to manage the breathing rate (by gradually reducing the isoflurane levels) to make sure that each animal is able to breathe steadily and evenly. Generally, time-lapse *z*-series images are collected for 30 min; macrophages are labeled 2 hr prior to imaging, and vessels are perfused with dextran immediately prior to imaging.

Literature Cited

Ahmed, F., Wyckoff, J., Lin, E.Y., Wang, W., Wang, Y., Hennighausen, L., Miyazaki, J., Jones, J., Pollard, J.W., Condeelis, J.S., and Segall J.E. 2002. GFP expression in the mammary gland for imaging of mammary tumor cells in transgenic mice. *Cancer Res.* 62:7166-7169.

Cammer, M., Wyckoff, J., and Segall, J.E. 1997. Computer-assisted analysis of single-cell behavior. *Methods Mol. Biol.* 75:459-481.

Donovan, J. and Brown, P. 1995. Managing immunocompromised animals. *Curr. Protoc. Immunol.* 14:1.2.1-1.2.6.

Donovan, J. and Brown, P. 1998. Anesthesia. *Curr. Protoc. Immunol.* 27:1.4.1-1.4.5.

Donovan, J. and Brown, P. 2006. Handling and restraint. *Curr. Protoc. Immunol.* 73:1.3.1-1.3.6.

Farina, K.L., Wyckoff, J.B., Rivera, J., Lee, H., Segall, J.E., Condeelis, J.S., and Jones, J.G. 1998. Cell motility of tumor cells visualized in living intact primary tumors using green fluorescent protein. *Cancer Res.* 58:2528-2532.

Faust, N., Varas, F., Kelly, L.M., Heck, S., and Graf, T. 2000. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood* 96:719-726.

Lin, E.Y., Jones, J.G., Li, P., Zhu, L., Whitney, K.D., Muller, W.J., and Pollard, J.W. 2003. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am. J. Pathol.* 163:2113-2126.

Motoike, T., Loughna, S., Perens, E., Roman, B.L., Liao, W., Chau, T.C., Richardson, C.D., Kaware, T., Kuno, J., Weinstein, B.M., Stainier, D.Y., and Sato, T.N. 2000. Universal GFP reporter for the study of vascular development. *Genesis* 28:75-81.

Neri, A., Welch, D., Kawaguchi, T., and Nicolson, G.L. 1982. Development and biologic properties of malignant cell sublines and clones of a spontaneously metastasizing rat mammary adenocarcinoma. *J. Natl. Cancer Inst.* 68:507-517.

Sahai, E., Wyckoff, J., Philippar, U., Segall, J.E., Gertler, F., and Condeelis, J. 2005. Simultaneous imaging of GFP, CFP and collagen in tumors in vivo using multiphoton microscopy. *BMC Biotechnol.* 5:14.

Sasmono, R.T., Oceandy, D., Pollard, J.W., Tong, W., Pavli, P., Wainwright, B.J., Ostrowski, M.C., Himes S.R., and Hume, D.A. 2003. A macrophage colony-stimulating factor receptor–green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* 101:1155-1163.

Wang, W., Wyckoff, J.B., Wang, Y., Bottinger, E.P., Segall, J.E., and Condeelis, J.S. 2003. Gene expression analysis on small numbers of invasive cells collected by chemotaxis from primary mammary tumors of the mouse. *BMC Biotechnol.* 3:13.

Wang, W., Goswami, S., Lapidus, K., Wells, A.L., Wyckoff, J.B., Sahai, E., Singer, R.H., Segall, J.E., and Condeelis, J.S. 2004. Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. *Cancer Res.* 64:8585-8594.

Wang, W., Goswami, S., Sahai, E., Wyckoff, J.B., Segall, J.E., and Condeelis, J.S. 2005. Tumor cells caught in the act of invading: Their strategy for enhanced cell motility. *Trends Cell Biol.* 15:138-145.

Wyckoff, J.B., Jones, J.G., Condeelis, J.S., and Segall, J.E. 2000. A critical step in metastasis: In vivo analysis of intravasation at the primary tumor. *Cancer Res.* 60:2504-2511.

Wyckoff, J., Wang, W., Lin, E.Y., Wang, Y., Pixley, F., Stanley, E.R., Graf, T., Pollard, J.W., Segall, J., and Condeelis, J. 2004. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res.* 64:7022-7029.

Wyckoff, J., Segall, J., and Condeelis, J. 2005 Single-cell imaging in animal tumors in vivo. *In* Live Cell Imaging, (R.D. Goldman and D.L. Spector, eds.) pp. 409-422. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Xue, C., Wyckoff, J., Liang, F., Sidani, M., Violini, S., Tsai, K.-L., Zhang, Z.-Y. Condeelis, J., and Segall, J.E. 2006. Epidermal growth factor

receptor overexpression results in increased tumor cell motility in vivo coordinately with enhanced intravasation and metastasis. *Cancer Res*. 66:192-197.

Zipfel, W.R., Williams, R.M., Christie, R., Nikitin, A.Y., Hyman, B.T., and Webb, W.W. 2003. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc. Natl. Acad. Sci. U.S.A.* 100:7075-7080.

Contributed by Dmitriy Kedrin, Jeffrey Wyckoff, John Condeelis, and Jeffrey E. Segall
Albert Einstein College of Medicine
Bronx, New York

Erik Sahai
London Research Institute
London, England

Embryonic Organ Culture

Takayoshi Sakai[1, 2] and Tomohiro Onodera[2]

[1]Department of Oral-Facial Disorders, Division of Functional Oral Neuroscience, Osaka University Graduate School of Dentistry, Osaka, Japan
[2]Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland

ABSTRACT

This unit provides detailed protocols for dissecting embryonic organs and performing organ culture to study questions in developmental biology. Procedures are described here for dissecting organs such as kidney, lung, and salivary gland. The unit also contains commentary including troubleshooting for embryonic organ culture. *Curr. Protoc. Cell Biol.* 41:19.8.1-19.8.8. © 2008 by John Wiley & Sons, Inc.

Keywords: embryonic organ culture • salivary gland • lung • kidney • branching morphogenesis

INTRODUCTION

Although cell culture model systems provide sensitive tools for the analysis of the molecular and cellular events of cytodifferentiation, they have only limited value when evaluating aspects of three-dimensional cell assembly during organogenesis and when exploring processes such as cell polarization and intercellular communication. Organogenesis involves many cellular processes, such as cell proliferation, cell adhesion, apoptosis, cell differentiation, changes in cell shape, and cell migration. Embryonic organ culture technology was developed for studying these processes in a three-dimensional environment.

Organ culture techniques, originally developed in the 1920s, make it possible to examine embryonic tissue fragments and whole organ rudiments under reproducible in vitro conditions. These techniques have also proven valuable in some toxicological studies. Studies undertaken using organ cultures provide advantages over cell cultures, since differentiation and certain physiological functions persist rather well (Saxen et al., 1983).

The transfilter technique created by Grobstein for analyzing kidney development has proven to be very effective, and it has been used for a great variety of other tissues as well (Grobstein., 1953, 1956, 1957). Grobstein's original setup consisted of a membrane filter as the substrate, supported by a Plexiglas ring fixed to a pair of glass rods. The original studies either used an embryonic organ on top of the filter or separated epithelial and mesenchymal tissues glued onto both sides of the filter membrane by a clot and cultivated in vitro. Various types of Millipore filters were used. These filters are made of cellulose ester and are available in different thicknesses and pore sizes. Millipore filters have a spongy structure and irregular pores, and they were later replaced by commercially available Nucleopore filters (Wartiovaara et al., 1974a,b). These latter filters are made of polycarbonate tape treated by exposure to charged particles in a nuclear reactor followed by chemical etching of the tracks. As a result of this process, the filters have a relatively uniform pore size and straight channels. The transfilter technique has simplified organ culture experiments, and culturing on filters is currently the standard approach.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

Current Protocols in Cell Biology 19.8.1-19.8.8, December 2008
Published online December 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1908s41

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH 7.4.

NOTE: When handling mice, investigators should wear gloves, laboratory coat, and surgical (dust or mist) mask to reduce exposure to mouse antigens. For dissections and culturing, investigators should wear gloves for all procedures and take precautions to avoid contamination. Gloves can be either presterilized surgical gloves or examination gloves that have been externally sprayed and rubbed with 70% ethanol after they have been put on.

BASIC PROTOCOL 1

MOUSE DISSECTION

Practicing dissections permits beginners to learn about the internal and external anatomical structures of animals (Rugh, 1968; Tuan, 2000). By taking a hands-on learning approach, beginners can obtain a practical sense of the relationships between embryonic organs and surrounding tissues. To dissect embryonic organs, the anatomical structure of pregnant mice and embryos must first be understood (see Background Information). Investigators should first become familiar with these dissections by practicing ahead of time (ideally several times) on, for example, E14 or E15 embryos using small amounts of starting material, before performing the dissection of embryonic organs in earlier stages such as E11 or E12.

Materials

70% ethanol
DMEM/F12 medium (Invitrogen), sterile
Pregnant ICR strain mice: embryonic day 12 (E12) to day 14 (E14)

Dissecting instruments including:
- Curved forceps, length 120 mm
- Two straight dissecting forceps, length 110 mm
- Straight scissors, length 140 mm
- Scalpel blade, size no. 11
- Glass dissection plate

100-mm and 30-mm culture dishes, sterile
Glass dissection plate, 10 to 15 cm, square or circular
Dissection microscope

Additional reagents and equipment for euthanasia of the mouse (Donovan and Brown, 2006)

Dissect uterus

1. Sterilize curved forceps and straight scissors by soaking in 70% ethanol for a minimum of 5 min. Keep instrument handles dry.
2. Prepare a 100-mm culture dish with ~10 ml cold DMEM/F12 on ice, then euthanize the pregnant mouse by CO_2 asphyxiation or cervical dislocation (Donovan and Brown, 2006).
3. Place the euthanized mouse in the supine position. Disinfect the abdomen of the mouse by thoroughly soaking with 70% ethanol using a squirt bottle.

   Washing the carcass with ethanol protects the tissues from artifacts caused by hair dragging through them.

   A video of this technique is available for CP Online subscribers.

4. Use straight scissors to make a ventral midline incision in the skin from the groin to the stomach.
5. Pull the skin back on the sides.
6. Dissect out the uterus of the mouse. Lift up the uterus with curved forceps and use straight scissors to cut the mesometrium away from the uterus. Do not squeeze embryos while grasping the mesometrium.
7. Place the uterus containing the embryos in the 100-mm culture dish containing DMEM/F12.

Isolate mouse embryos

8. Using two sterilized straight dissecting forceps, gently separate the embryo away from associated tissues (muscular wall of the uterus, Reichert's membrane, and the visceral yolk sac).
9. Remove the embryos from the uterus using two sets of sterilized straight dissecting forceps to open the uterine wall (embryos will usually "pop out" intact).

   A video of this technique is available for CP Online subscribers.

   When holding on to the animal during dissection, do not grab the near portion that includes the organ you want to culture (e.g., grasp the abdomen if you want to culture salivary glands or grasp the head if you want to culture lungs or kidneys).

10. Store embryos in the culture dish containing DMEM/F12 at 4°C or on ice before dissection.

Dissect organ of interest

All three of the sets of organs mentioned below can be harvested from the same animal if careful dissection technique is used.

For salivary submandibular glands

11a. Prepare a 35-mm culture dish with 3 ml DMEM/F12 and a sterile glass dissection plate.

12a. Place the embryo on its side in the lateral position onto the glass dissection plate.

   A video of this technique is available for CP Online subscribers.

13a. Stabilize the head using forceps and slice the head off with a scalpel blade, cutting between the neck and chin.

14a. Cut off the mandibles with a scalpel blade by cutting through the mouth above the tongue moving toward the back of the head.

   If it is hard to detect the mouth position, pushing gently below the nose with the non-cutting side of the scalpel blade should be helpful in finding the mouth opening.

15a. Place the mandibles into the 35-mm culture dish containing 3 ml DMEM/F12.

16a. Working under a dissection microscope, put the mandible onto a glass dissection plate caudal side up and cranial (tongue) side down. Turn the tip of the tongue to the 12 o'clock position (Fig. 19.8.1A, B).

   A video of this technique is available for CP Online subscribers.

17a. Using two sterile straight dissecting forceps, make a short longitudinal tear from the mandible tip (Fig. 19.8.1C). Scrape Meckel's cartilage from tongue base using sterile forceps, then pull off the tongue together with the two attached submandibular glands (Fig. 19.8.1D,E).

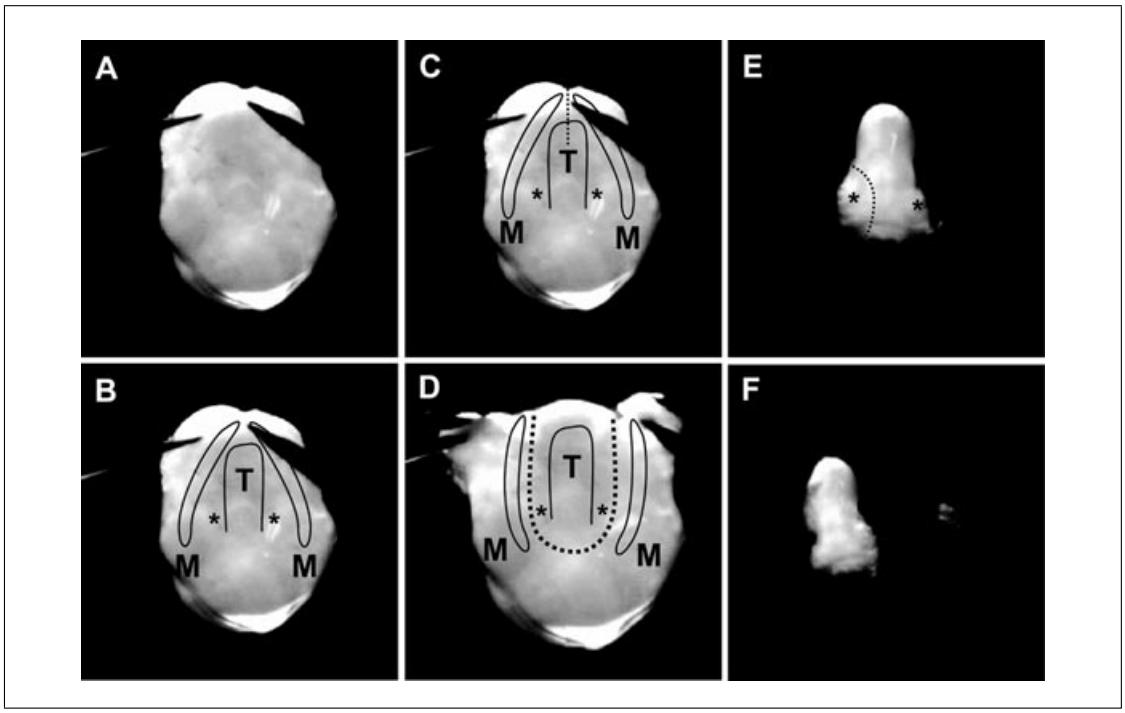


Figure 19.8.1 Salivary gland dissection. (**A, B**) Axial section of mandible tissue, oriented with the caudal side toward the objective and the front (anterior) of the embryo at the top. M, Meckel's cartilage; T, tongue; *, salivary gland. (**C**) The front of the mandible above the tongue is cut along the dashed line. (**D**) Meckel's cartilage is then discarded after cutting along the dashed line. (**E**) Isolated tongue with salivary glands. Cut along the dashed line to isolate the left salivary gland. (**F**) Separated tongue (left) and one of the salivary glands (right).

18a. Prick the center of the tongue with a pair of forceps to stabilize it, then cut off each submandibular gland from the tongue. Place the glands in a 35-mm culture dish containing 3 ml DMEM/F12 at 4°C, until ready for organ culture (Basic Protocol 2).

The organs may be stored up to several hours under these conditions.

For kidney

11b. Slice the head off by cutting at the neck, and place the embryo in the lateral position with the head end downward. Hold the posterior of the embryo with a set of forceps and cut out the liver, heart, and stomach using another pair of forceps.

A video of this technique is available for CP Online subscribers.

12b. Insert the forceps between the posterior body wall and the aorta/kidneys. Detach the aorta and kidneys carefully from the posterior wall (Fig. 19.8.2A-D) using closed forceps like a knife cutting parallel to the aorta. Next, with partially open forceps acting like a fork, lift the aorta and kidneys away from the body wall. Cut the aorta above the kidneys. Peel the aorta and kidneys away from the embryonic body wall using a single motion in the caudal direction toward the embryonic tail.

13b. Pick up the aorta above the kidneys and pull it away en bloc with both kidneys attached (Fig. 19.8.2E,F).

14b. Remove the remaining digestive organs, and cut off both kidneys using forceps. Place kidneys in a 35-mm culture dish containing 3 ml DMEM/F12 at 4°C, until ready for organ culture (Basic Protocol 2).

The organs may be stored up to several hours under these conditions.

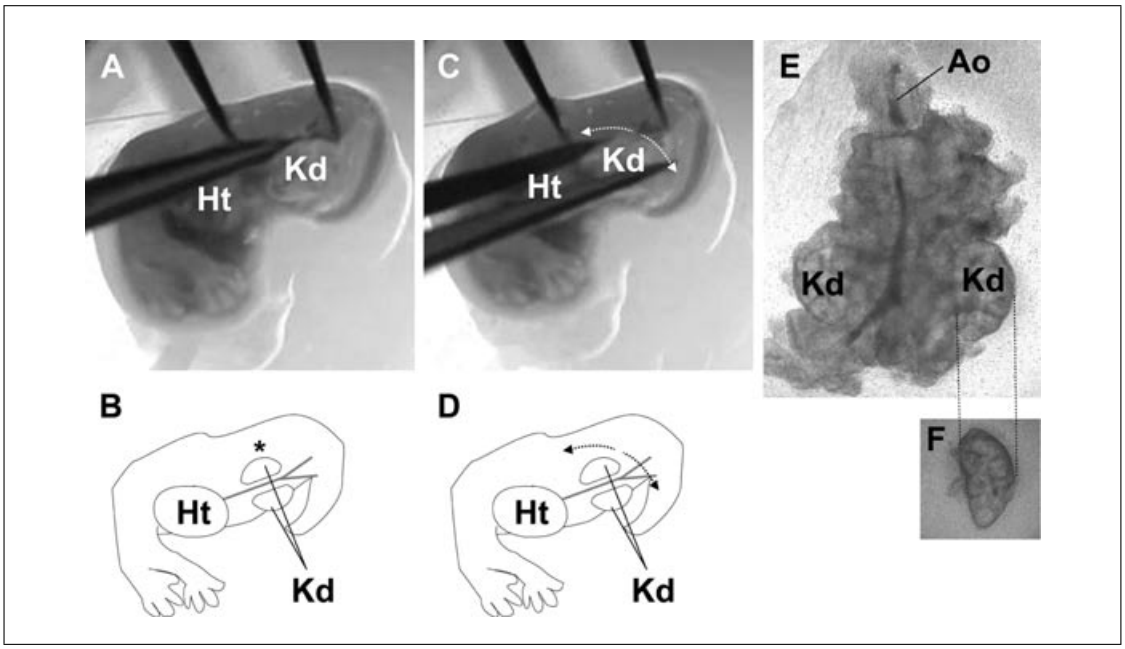


Figure 19.8.2 Kidney dissection. (**A**) Embryonic mouse in a lateral position with the front (anterior) facing down toward the bottom of the page. Ht, heart; Kd, kidney. (**B**) Proper insertion site for forceps. *, insertion site. (**C, D**) After insertion, open the forceps to move the aorta and kidneys in the direction of the dashed arrows. (**E**) Isolated aorta/kidneys. Ao, aorta; Kd, kidney. (**F**) Kidney isolated from aorta.

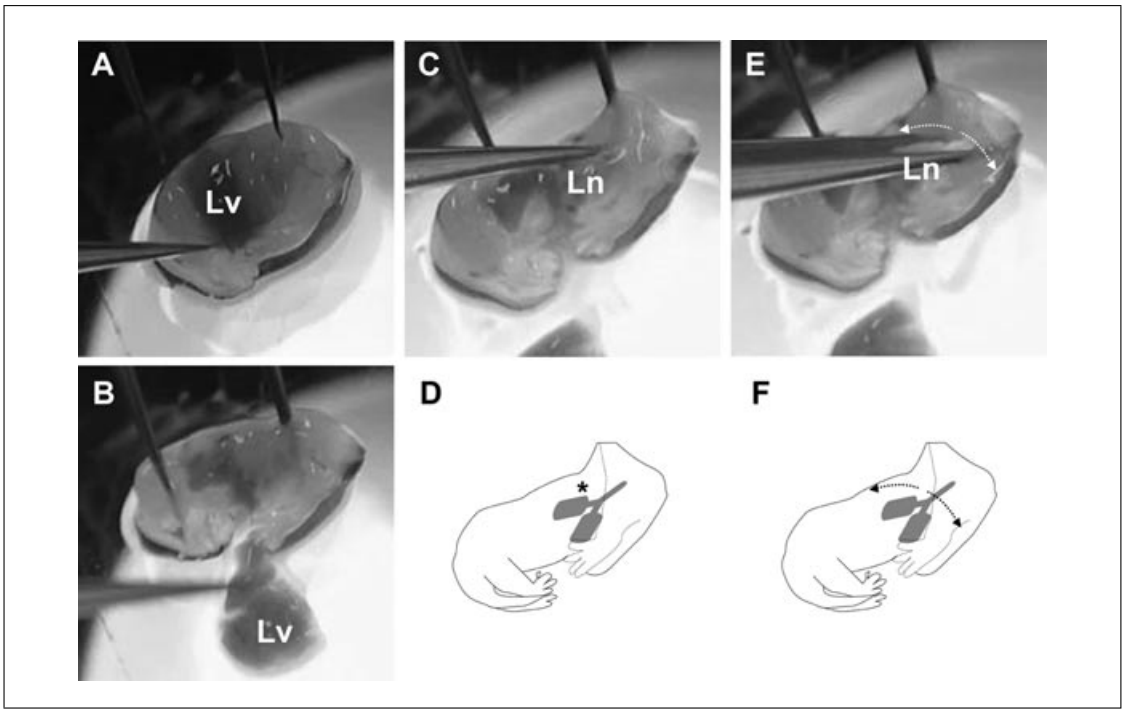


Figure 19.8.3 Lung dissection. (**A**) Embryonic mouse in lateral position with front (anterior) facing the bottom of the page. Lv, liver. (**B**) Scrape the liver toward the front using forceps. (**C**) Insert closed forceps tips between the posterior body wall and the trachea. (**D**) Diagram showing the proper insertion site of forceps. *, insertion site. (**E, F**) Open the forceps to separate the lungs away from the body as indicated by the dashed arrows.

For lungs

11c. Slice the head off by cutting at the neck, and place the embryo in the lateral position with the head end downward.

A video of this technique is available for CP Online subscribers.

12c. Holding the posterior of the embryo with a pair of forceps, remove the liver and heart with another pair of forceps (Fig. 19.8.3A,B).

13c. Detach the trachea carefully from the posterior body wall by piercing the tissue between the body wall and the trachea with a closed pair of forceps and opening the forceps to cut the tissue, freeing the trachea from the body wall (Fig. 19.8.3C-F).

14c. Carefully pull out the trachea and lungs. Lift the lungs gently away from the body wall with a pair of partially open forceps, to avoid injuring the lungs.

15c. Remove the remaining esophagus carefully if it is attached to the lungs. Place lungs in a 35-mm culture dish containing 3 ml DMEM/F12 at 4°C, until ready for organ culture (Basic Protocol 2).

The organs may be stored up to several hours under these conditions.

BASIC PROTOCOL 2

ORGAN CULTURE

This protocol describes how to perform the organ culture of embryonic salivary glands, kidneys, and lungs. Investigators should wear gloves for all procedures and take precautions in order to avoid cross-contamination of samples. Culture plate preparation should be done before organ dissection.

Materials

Culture medium (see recipe)
Mouse embryonic organs (Basic Protocol 1)

50-mm glass-bottom dish (MatTek Corporation)
Filter membrane (diameter 13 mm, polycarbonate, pore size 0.1 μm; Whatman)
Straight dissecting forceps, length 110 mm
Dissection microscope

1. Open the cover of a 50-mm glass-bottom dish.

   A video of this technique is available for CP Online subscribers.

2. Add 200 μl culture medium to the dish.

3. Using forceps, place the membrane on the surface of the culture medium so that the shiny side is on the top and the cloudy side is on the bottom.

4. Using forceps, gently place the organs of interest on the membrane.

   Be careful not to squeeze the organ; instead, scoop the organ up with the forceps. If the embryonic organ is in a drop of liquid, approach it with the open forceps' tips at a nearly horizontal angle and gradually close the tips to draw up the liquid and organ between the tips, then lift the organ in the drop of liquid.

5. Incubate in a 37°C, 5% CO_2 incubator for 3 days.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Culture medium

Purchase DMEM/F12 (in 500-ml bottle) from Invitrogen. Add penicillin to a final concentration of 100 U/ml and streptomycin to a final concentration of 100 μg/ml. Store at 4°C. Just before use, add vitamin C to 150 μg/ml and transferrin to 50 μg/ml.

COMMENTARY

Background Information

The great advantage of organ culture is that the overall three-dimensional structure of the tissue is retained. To avoid damaging vital organs, and to understand the procedures, an understanding of the anatomy is essential. *The Atlas of Mouse Development* (Kaufman, 1992) is recommended for beginners to study basic anatomy of the embryonic mouse.

Critical Parameters and Troubleshooting

Embryonic mouse organ dissection (salivary submandibular gland, kidney, lung)

It is essential to start with a fresh embryo so that it is easy for beginners to dissect organs; otherwise, embryos that are not fresh can make precise dissection difficult. Sharp forceps and

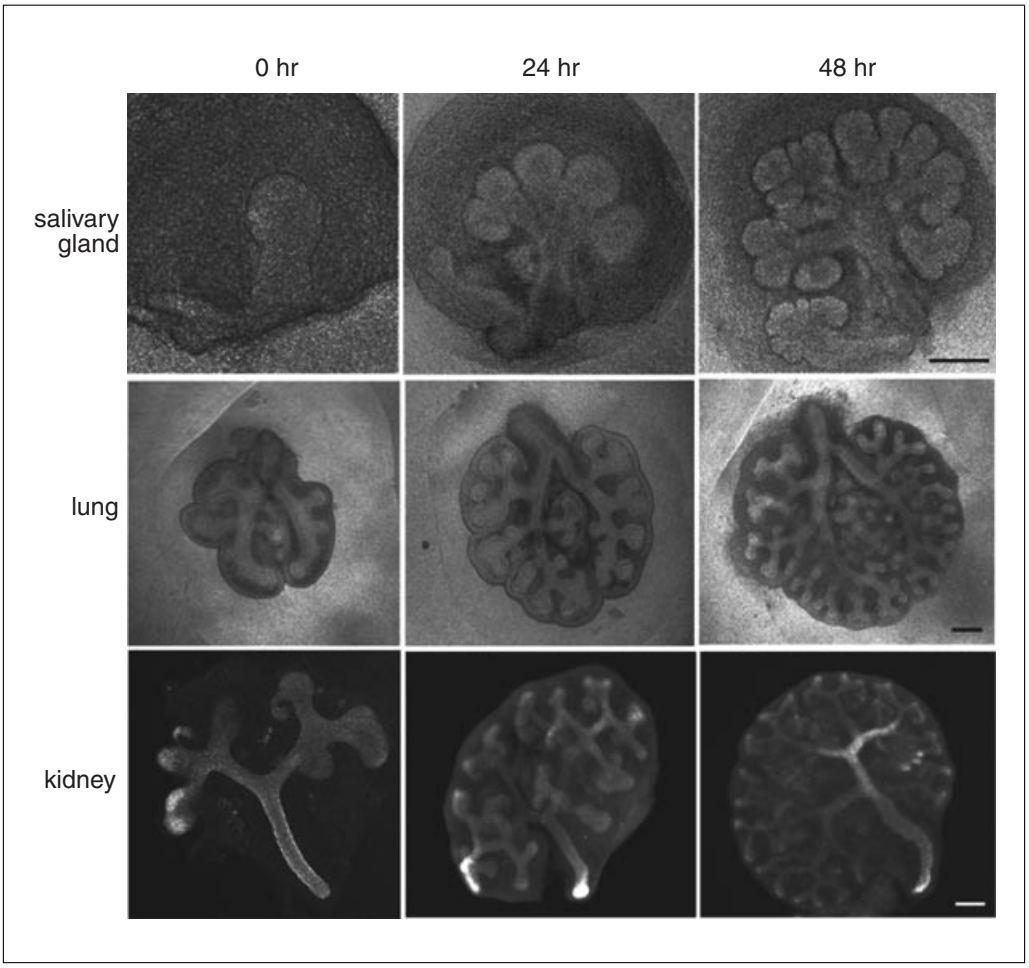


Figure 19.8.4 (**A** to **C**) The mouse submandibular salivary glands from E12 to E14 embryos were cultured for 72 hr based on these protocols. (**D** to **F**) Mouse lungs from E11.5 to E13.5 embryos. (**G** to **I**) Mouse kidneys from E11.5 to E13.5 embryos. The branching ureteric buds of all kidneys were visualized with anti-E-cadherin antibody as previously described (Sakai, 2003). Scale bar = 200 μm.

scalpel blades are recommended because embryonic tissues stick to scissors. Organ dissection from small tissue samples must be done quickly to ensure freshness and quality of tissues.

Anticipated Results

Each of the organs for which cultures are described in this unit have characteristic branching patterns. The organ culture method should produce growth of embryonic organs such as salivary glands, kidneys, and lungs (Fig. 19.8.4). Well-formed branching patterns should be visible in each organ.

Time Considerations

In Basic Protocol 1, the entire procedure for mouse dissection, mouse embryo isolation, and organ dissection will require a few hours for several pregnant mice. It is best to dissect as quickly as possible to ensure successful organ culture. In Basic Protocol 2, the procedure for setting up the organ culture technique can take several minutes, depending on the number of embryos. Cultures can be carried out for 48 to 72 hr with this method.

Literature Cited

Donovan, J. and Brown, P. 2006. Euthanasia. *Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Grobstein, C. 1953. Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter. *Nature* 172:869-870.

Grobstein, C. 1956. Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. *Exp. Cell Res.* 10:424-440.

Grobstein, C. 1957. Some transmission characteristics of the tubule-inducing influence on mouse metanephrogenic mesenchyme. *Exp. Cell Res.* 13:575-587.

Kaufman, M.H. 1992. The Atlas of Mouse Development. Academic Press, London.

Rugh, R. 1968. The Mouse: Its Reproduction and Development. Burgess Publishing Company, Minneapolis, Minn.

Sakai, T., Larsen, M., and Yamada, K.M. 2003. Fibronectin requirement in branching morphogenesis. *Nature* 423:876-881.

Saxen, L., Salonen, J., Ekblom, P., and Nordling, S. 1983. DNA synthesis and cell generation cycle during determination and differentiation of the metanephric mesenchyme. *Dev. Biol.* 98:130-138.

Tuan, R.S. and Lo, C.W. Developmental Biology Protocols. Humana Press, Totowa, N.J.

Wartiovaara, J., Linder, E., Ruoslahti, E., and Vaheri, A. 1974a. Distribution of fibroblast surface antigen: Association with fibrillar structures of normal cells and loss upon viral transformation. *J. Exp. Med.* 140:1522-1533.

Wartiovaara, J., Nordling, S., Lehtonen, E., and Saxen, L. 1974b. Transfilter induction of kidney tubules: Correlation with cytoplasmic penetration into nucleopore filters. *J. Embryol. Exp. Morphol.* 31:667-682.

Three-Dimensional Tissue Models of Normal and Diseased Skin

Mark W. Carlson,[1] Addy Alt-Holland,[1] Christophe Egles,[1] and Jonathan A. Garlick[1, 2, 3]

[1]School of Dental Medicine, Tufts University, Boston, Massachusetts
[2]School of Medicine, Tufts University, Boston, Massachusetts
[3]School of Engineering, Tufts University, Medford, Massachusetts

ABSTRACT

Over the last decade, the development of in vitro, human, three-dimensional (3D) tissue models, known as human skin equivalents (HSEs), has furthered understanding of epidermal cell biology and provided novel experimental systems. Signaling pathways that mediate the linkage between growth and differentiation function optimally when cells are spatially organized to display the architectural features seen in vivo, but are uncoupled and lost in two-dimensional culture systems. HSEs consist of a stratified squamous epithelium grown at an air-liquid interface on a collagen matrix populated with dermal fibroblasts. These 3D tissues demonstrate in vivo–like epithelial differentiation and morphology, and rates of cell division, similar to those found in human skin. This unit describes fabrication of HSEs, allowing the generation of human tissues that mimic the morphology, differentiation, and growth of human skin, as well as disease processes of cancer and wound re-epithelialization, providing powerful new tools for the study of diseases in humans. *Curr. Protoc. Cell Biol.* 41:19.9.1-19.9.17. © 2008 by John Wiley & Sons, Inc.

Keywords: organotypic culture • three-dimensional model • human skin • wound repair • intraepithelial neoplasia

INTRODUCTION

The development of human, three-dimensional (3D) models for advancing insights into the biology of skin keratinocytes requires the ability to construct tissues that mimic their in vivo counterparts. The linkage that exists in tissues between growth and differentiation is optimal only when cells are spatially organized to display the architectural features seen in vivo; it is lost in two-dimensional culture systems (Bissell and Radisky, 2001). Over the last decade, the development of tissue-engineered models that mimic human skin, known as human skin equivalents (HSEs) or organotypic cultures, have provided novel experimental systems for studying epidermal biology (Kolodka et al., 1998; Andriani et al., 2003). This unit describes methods for constructing 3D models of human epidermis that mimic the architectural features and behavior of normal human skin and the changes that occur during early skin cancer progression and wound re-epithelialization. This unit describes the construction of a multilayered epithelium that is composed of skin keratinocytes and grown upon a collagen substrate populated with dermal fibroblasts. When these protocols are used, keratinocytes differentiate upon exposure to an air-liquid interface to enable the tissue to recapitulate the morphologic and biochemical processes of human epidermis.

This unit describes the fabrication of normal skin and includes modifications to these protocols that will create novel environments that mimic disease states in cutaneous tissues. Basic Protocol 1 describes the fabrication of normal HSEs using primary keratinocytes, which allows the generation of tissues that mimic the morphology, differentiation, and

growth of human epidermis. An Alternate Protocol provides a tissue model that mimics the early stages of skin cancer progression; it incorporates keratinocytes from both a normal and a squamous cell carcinoma cell line. Basic Protocol 2 describes a skin-like tissue model for cutaneous re-epithelialization, in which 3D tissues are wounded and keratinocytes close the wound defect to re-establish epithelial integrity. A Support Protocol addresses alternative substrates for fabrication of HSEs.

While the methods described below are for the fabrication of HSEs using skin keratinocytes, they can be adapted to grow tissues from other stratified epithelia, e.g., the oral and cervical mucosa, esophageal lining, laryngeal epithelium, and conjunctiva. It is suggested that efforts be made to optimize the growth of these cells in monolayer culture before incorporating them into HSEs.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

BASIC PROTOCOL 1

CONSTRUCTION OF A THREE-DIMENSIONAL MODEL OF NORMAL HUMAN SKIN

The 3D tissue model of normal skin consists of a stratified epithelium harboring differentiated keratinocytes that are grown on a contracted collagen matrix populated with dermal fibroblasts. As seen in the schematic figure describing HSE assembly (Fig. 19.9.1), normal tissues are constructed through the following sequential steps: (1) A thin, acellular layer of collagen (acellular collagen layer) is constructed first and acts as an attachment substrate for the cellular collagen that is fabricated above it. This thin layer prevents the cellular collagen from contracting from the insert membrane and detaching from it. (2) A collagen matrix with human dermal fibroblasts (cellular collagen layer) is constructed and allowed to contract for 7 days while submerged in medium. Fibroblasts mixed within the collagen gel remodel the matrix upon production of extracellular matrix (ECM) proteins and cause contraction of the gel. (3) After contraction is complete and the matrix is stabilized, keratinocytes are added to the surface of the matrix and allowed to attach to this substrate to generate a confluent cellular monolayer that will initiate tissue stratification (epithelialization). (4) Tissues are raised to an air-liquid interface to enable complete stratification, as well as full morphological and biochemical differentiation (tissue stratification, organization, and differentiation).

Materials

Human foreskin fibroblasts
Fibroblast culture medium (see recipe)
Acellular and cellular collagen matrices (see recipe)
Feeder layer of mitotically inactivated mouse 3T3 fibroblasts (e.g., see Rheinwald and Green, 1975)
Human neonatal foreskin keratinocytes
Keratinocyte culture medium (see recipe)
Trypsin/EDTA (see recipe)
EDTA/PBS (see recipe)
PBS (phosphate-buffered saline; Invitrogen, cat. no. 14190; also see *APPENDIX 2A*)
Human skin equivalent (HSE) culture media (see Table 19.9.1) including:
  Epidermalization medium I
  Epidermalization medium II
  Cornification medium
10% (v/v) formalin
2 M sucrose

Embedding medium: Tissue-Tek optimal cutting temperature (OCT) medium (Ted Pella)
Liquid nitrogen

Pipets, chilled 15 min at −20°C before use
10-cm^2 cell culture plates
6-well tissue culture plate with 3-μm porous polycarbonate membrane inserts (Organogenesis, cat. no. MS-10-3-5)
15-ml centrifuge tubes
Centrifuge capable of 500-1000 × *g*
Scalpel
Tissue processing cassette
Aluminum foil
~2-cm bottle or cap (to use as a mold form)
Narrow-tip forceps
Metal rack (for embedded tissue)
Styrofoam box

Additional reagents and equipment for trypsinizing (*UNIT 1.1*) and counting (*UNIT 1.1*) cells

Fabricate cellularized collagen

1. Seed ~1×10^6 human foreskin fibroblasts (HFFs) in 10 ml fibroblast culture medium to a standard 10-cm^2 cell culture dish so that they are almost confluent 1 day before incorporation into the collagen. Change the medium every 2 days.

   After the fibroblasts have been passaged once from the frozen state, they will reach confluency in ~3 days.

2. The day before incorporation, passage fibroblasts at a 9:10 split ratio, i.e., resuspend the trypsinized cells (see *UNIT 1.1*) in 10 ml fibroblast culture medium, and add 9 ml of the cell suspension to a new plate.

   Passaging cells from a confluent plate ensures a higher fraction of actively dividing cells upon incorporation into collagen gels.

3. On the day of incorporation, prepare the acellular collagen mixture on ice. Use chilled pipets (15 min at −20°C) to prevent warming of collagen when it is mixed. Avoid creating air bubbles when mixing.

   Preparing the mixture on ice prevents premature gelation.

   Collagen should be a straw-yellow to light pink color to ensure optimal gelation. If the color is bright yellow, add a single drop of sodium bicarbonate and triturate until a straw-yellow color is seen.

4. Add 1 ml acellular collagen matrix to each 6-well tissue culture plate insert. Ensure that the gel coats the entire bottom surface of the insert and allow it to gel 20 min at room temperature. Do not move the plate while the mixture is undergoing gelation.

   The color will turn pink when the collagen has fully gelled.

5. Trypsinize (*UNIT 1.1*) with trypsin/EDTA, count viable cells in an aliquot (*UNIT 1.1*), and resuspend the fibroblasts to a final concentration of 3×10^5 cells/ml.

   A total of 5×10^5 fibroblasts will be used per 6-well plate.

6. Prepare the cellular collagen mixture on ice, adding the reagents in the order listed so that the cells (added last) will not be damaged by the acidic pH of the unneutralized collagen.

   It is important to neutralize the collagen so that the cells will not be damaged by the acid pH that exists before neutralization.

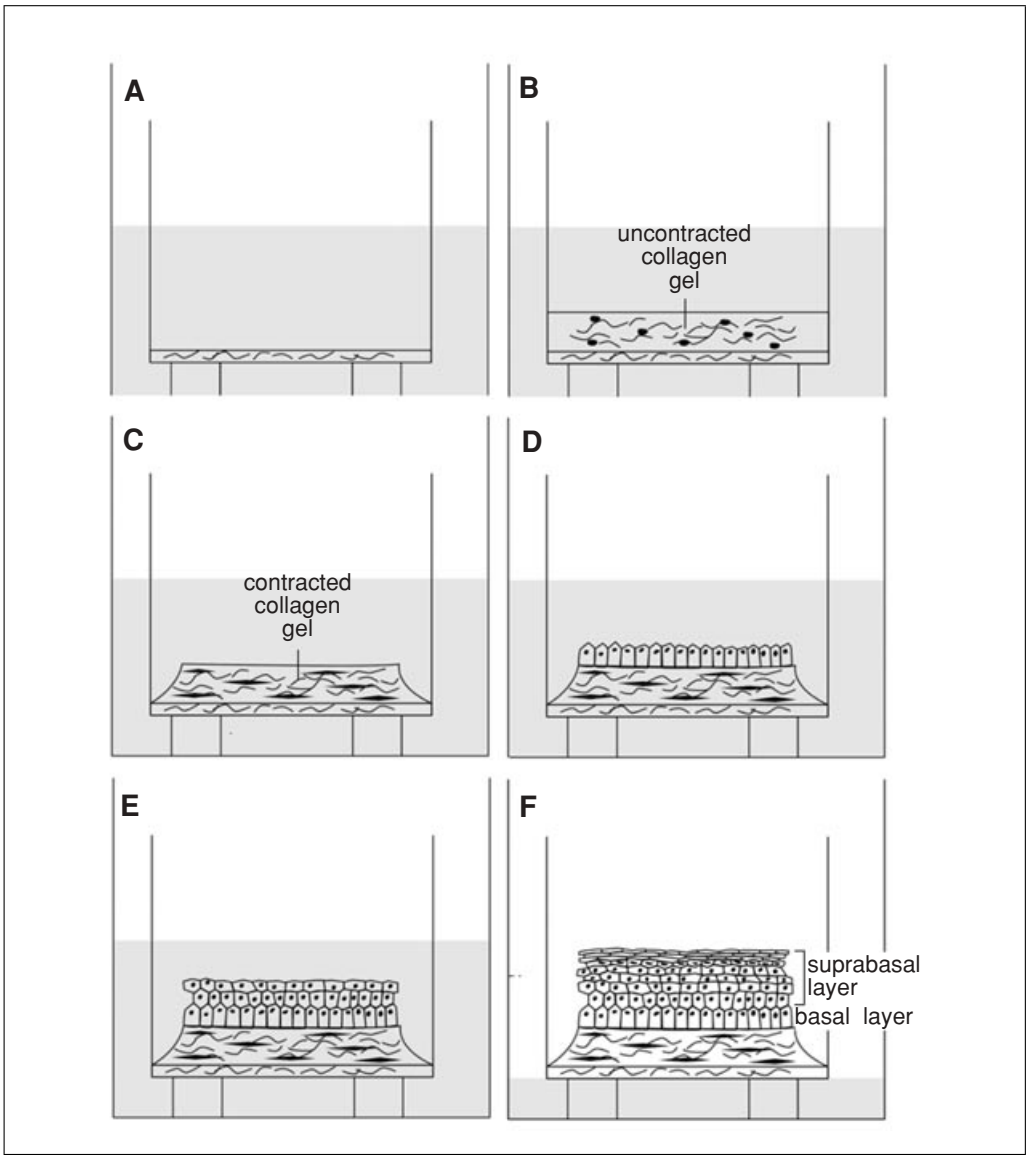


Figure 19.9.1 Schematic of three-dimensional tissue construction. (**A**) A thin, acellular layer of collagen is first constructed; it provides an attachment substrate for the cellular collagen. (**B**) A collagen gel embedded with human dermal fibroblasts is layered onto the acellular layer. (**C**) While submerged in medium for 7 days, dermal fibroblasts remodel the collagen matrix, causing it to contract away from the walls of the insert. The contracted collagen forms a plateau. (**D**) Keratinocytes are then added to the center of the plateau of contracted collagen and allowed to attach to the collagen (or intervening substrate such as AlloDerm) to create a monolayer that will form the basal layer of the tissue. (**E**) Tissues are raised to an air-liquid interface to initiate stratification. Keratinocytes stratify and differentiate and form a suprabasal layer that mimics in vivo skin both morphologically and biochemically. (**F**) Further exposure to the air-liquid interface and additional feedings with cornification medium results in an increase in the thickness of the spinous and cornified layers of the tissue.

7. Add the fibroblasts to the mixture last, gently triturate the cellular matrix to evenly incorporate fibroblasts into the collagen matrix, and add 3 ml into each insert on top of the gelled acellular collagen matrix. Gently transfer the mixture to the incubator for 30 min.

8. When the cellular matrix has turned pink and is completely gelled (usually less than 30 min), feed the gels with 12 ml of fibroblast culture medium by adding 10 ml of medium to the well around the insert and 2 ml of medium directly onto the insert.

Table 19.9.1 Supplements for HSE Organotypic Media[a]

Ingredient	Stock concentration	Final concentration	Epidermalization I (ml)	Epidermalization II (ml)	Cornification (ml)
O1O medium[b]			363	363	237
Ham's F12 supplements			120	120	237
L-glutamine	200 mM	4 mM	10	10	10
Adenine[b]	18 mM	40 μM	1	1	1
Hydrocortisone[b]	500×	1 μM	1	1	1
Triiodothyronine (T3)[b]	500×	20 nM	1	1	1
Transferrin[c]	5 mg/ml	10 μg/ml	1	1	1
Insulin[b]	5 mg/ml	10 μg/ml	1	1	1
Progesterone[b]	2 μM	2 nM	0.5	0.5	0
PES[c]	500×	1×	1	1	1
Calcium chloride	0.5 M	1.8 mM	0	1.8	1.8
FBS[d]			0.5	0.5	10

[a]Media construction for epidermalization I, epidermalization II, and cornification media, including stock concentrations, final concentration, and volume of each supplement for a 500 ml total volume.
[b]See recipe.
[c]Biosource.
[d]Fetal bovine serum (Hyclone, cat. no. 30071). The user is highly encouraged to test different FBS lots to find the optimal one. Lots used successfully by the authors include: ALG14153, AQC23532, AMA15402, and APB20546.

9. Incubate the matrix 5 to 7 days to allow complete gel contraction.

   During the first few days, the sides of the gel contract and will form a plateau in the center. Gels are stable from 5 to 10 days after initial construction.

Add keratinocytes

10. Aspirate off the medium from a feeder layer of mitotically inactivated mouse 3T3 fibroblasts and seed the feeder layer with 1×10^6 keratinocytes in 10 ml keratinocyte culture medium. Grow keratinocytes to no more than 50% confluence to minimize the number of differentiated cells seeded onto the collagen matrix.

    We typically expand 3T3 feeder cells (J2 line preferred in our lab) in ∼20 T-125 flasks, which yield ∼1×10^8 cells. It is important when expanding 3T3 cells to not let them lose their "stellate" morphology before irradiating; passage at ∼70% confluency during expansion is optimal.

    Trypsinized suspensions of the cells should be lethally irradiated. The irradiation level is determined by the user to provide cells that will still attach and survive for ∼1 week, but will not continue to divide.

    Frozen, irradiated cells last ∼3 months at −80°C, or indefinitely in liquid nitrogen. Each batch should be tested to verify support of normal human keratinocyte growth before large 3D experiments are started.

    After the keratinocytes have been passaged once from the frozen state, they will reach confluency in ∼3 days. One change of medium may be necessary.

    Alternatively, keratinocytes can be grown in monolayer culture in a low-calcium, serum-free medium (see Critical Parameters).

11. Remove the 3T3 feeder cells from the culture by incubating the dish in 5 ml EDTA/PBS for 5 min at 37°C. Displace the 3T3 cells by gentle pipetting so that keratinocytes will remain attached.

    It is important not to allow the cultures to incubate for an excessive time in EDTA/PBS, as the keratinocytes may detach from the dish as well.

12. As soon as the 3T3 cells have begun to detach, replace the EDTA/PBS with 5 ml PBS, gently rinse the plate three times with 5 ml PBS until all 3T3 cells have been completely removed, then remove the PBS, leaving only keratinocyte colonies attached to the dish.

13. Trypsinize (see *UNIT 1.1*) the keratinocytes with 2 ml trypsin/EDTA for 5 min at 37°C to obtain a single-cell suspension. Transfer the detached cells into a 15-ml tube containing 9 ml keratinocyte culture medium (to neutralize the trypsin) and count viable cells in an aliquot (*UNIT 1.1*).

14. Dispense the desired number of cells into a 15-ml tube. Centrifuge the cells 5 min at 1000 × *g*, room temperature, and resuspend in a volume of epidermalization I medium so that a total of 5×10^5 keratinocytes can be used per insert in 50 µl.

15. Remove all fibroblast culture medium from the plates with the contracted collagen 20 min before seeding keratinocytes so that keratinocytes can be seeded onto a moist collagen gel.

    Keratinocytes should be seeded directly onto the contracted collagen gels in an aliquot of 50 µl containing 5×10^5 cells. To modify the nature of the substrate on which keratinocytes are seeded, de-epidermalized dermis or coated polycarbonate inserts can be applied directly on top of the contracted collagen gels at this point (see the Support Protocol for specialized substrates).

16. Carefully add 50 µl of the cell suspension to the center of the contracted collagen gel (or onto the center of the intervening substrate placed on the collagen gel). Do not move the plate for 15 min, to allow the keratinocytes to attach. Then incubate 30 to 60 min at 37°C (without any additional medium) to allow the keratinocytes to fully adhere.

17. Add 12 ml of epidermalization I medium to each insert, adding 10 ml to the bottom of the well and 2 ml gently into the insert on top of the keratinocytes. Incubate at 37°C.

18. Feed each culture with medium every 2 days with appropriate HSE culture media as follows:

    a. Day 4: Add 12 ml epidermalization II medium.
    b. Day 6 until termination of the experiment: Add 7 ml cornification medium to the bottom of the well so that the insert just contacts the medium every two days. Aspirate medium from the inside of the insert so that tissues can be grown at the air-liquid interface.

    The morphological appearance of tissues at various stages of their development is shown in Figure 19.9.2.

Harvest tissues for analysis

19. Remove the medium from the inserts and gently rinse tissues twice in 5 ml PBS.

20. Cut away the insert membrane from the plastic insert at its base using a scalpel, and bisect the culture.

To fix the tissue

21a. Place one half of the culture in a tissue processing cassette and immediately immerse in 10% formalin for 1 hr.

    Tissues are very thin and thus only require a short fixation (1 hr).

22a. Proceed with tissue processing and paraffin embedding by sequential dehydration in graded ethanols and xylene, using standard techniques.

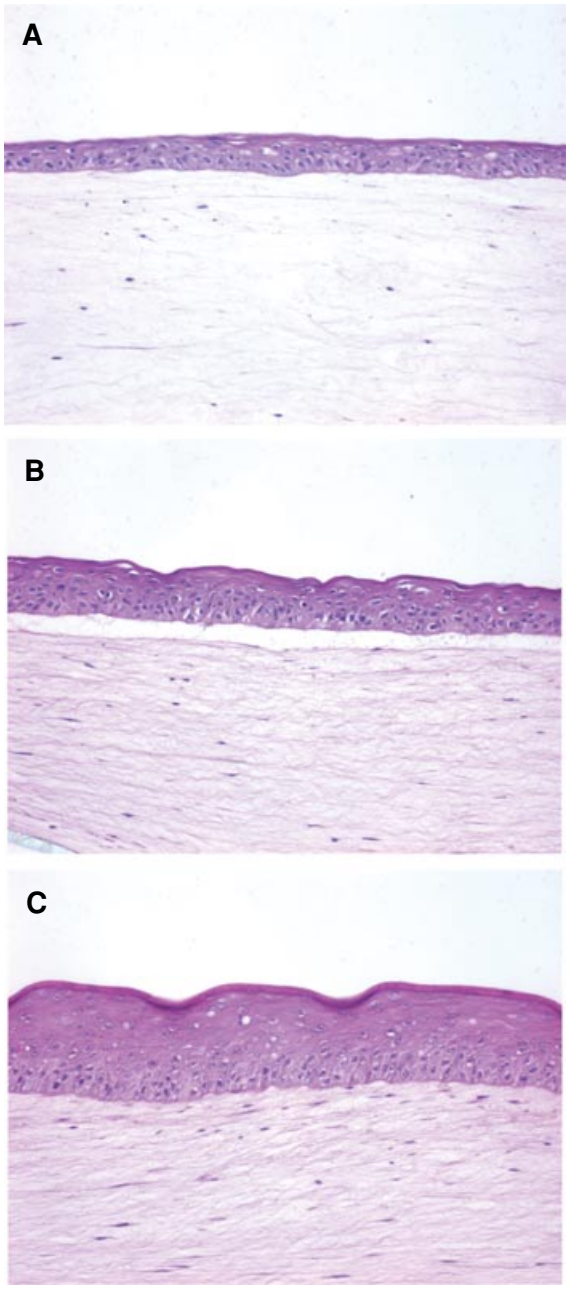


Figure 19.9.2 Morphologic development of human skin equivalents. Keratinocytes were grown directly on collagen gels for 4 days (**A**), 6 days (**B**), and 10 days (**C**). (A) Early epithelial development, evidenced by a thin epithelium, is seen while tissues are still submerged in epidermalization II medium. (B) The epithelium demonstrates a greater degree of tissue architecture and organization, demonstrated by the presence of cuboidal basal cells, after the tissue is exposed to the air-liquid interface for 2 days. (C) Full morphologic differentiation and stratification are seen after cells are exposed to the air-liquid interface for 6 days. Note that the clear space between the epidermis and dermis in panels A and B likely represents separation along the epithelium-stromal interface due to incomplete organization of the basement membrane at early time points of tissue development. A more mature tissue (C) has a more well developed basement membrane and is more resistant to separation during tissue processing.

To preserve the tissue by freezing

21b. Place the other half of the tissue in a 2 M sucrose solution. Soak the tissue in the sucrose solution at least 1 hr, but not more than 24 hr, at 4°C.

Sucrose replaces water in the hydrated collagen gel and protects against freezing damage during embedding and processing.

22b. Make a small mold with aluminum foil, using the cap or bottom of a small bottle (roughly 2 cm in diameter) as a form. Fill three-quarters of the mold with embedding medium.

23b. Gently remove the tissue from the sucrose using narrow-tip forceps, making sure to keep the tissue on the nylon insert membrane. Gently touch the membrane side of the tissue to a Kimwipe to remove excess sucrose solution.

24b. Place the tissue in the embedding medium and allow the tissue to soak 20 to 30 min at room temperature.

25b. Place a metal rack inside a Styrofoam box and fill it with liquid nitrogen to a height just under the top of the metal rack. Place the aluminum foil mold on top of the rack and stand the tissue inside to an upright position.

The liquid nitrogen vapors will freeze the embedding medium and tissue in about 5 min. The tissue can then be stored for decades at −80°C.

SUPPORT PROTOCOL

INCULDING SPECIALIZED SUBSTRATES FOR GROWTH OF HUMAN SKIN EQUIVALENTS ON BASEMENT MEMBRANE OR EXTRACELLULAR MATRIX PROTEINS

Specific applications may necessitate growing 3D tissues on basement membrane or defined extracellular matrix proteins using either de-epidermalized dermis or a coated polycarbonate membrane, respectively.

Materials

AlloDerm (de-epidermalized basement membrane; LifeCell, cat. no. 102-009)
PBS (phosphate-buffered saline; Invitrogen, cat. no. 14190; also see *APPENDIX 2A*)
6-well cell culture PET membrane inserts coated with the appropriate material:
  Collagen I (Becton-Dickinson, cat. no. 354442)
  Laminin I (Becton-Dickinson, cat. no. 354446)
  Fibrillar Collagen I (Becton-Dickinson, cat. no 354472)
  Fibronectin/collagen I (Becton-Dickinson, cat. no 354633)
  Fibronectin (Becton-Dickinson, cat. no 354440)
  Collagen IV (Becton-Dickinson, cat. no 354544)
Serum-free, low-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen)
1.4-cm stainless steel dermatological punch (Delasco, cat. no. KP-14)
Scalpel

To make de-epidermalized dermis substrate:

1a. Cut the de-epidermalized dermis (AlloDerm) with a dermatological punch to fit the diameter of the contracted collagen gel.

2a. Rehydrate the substrate in PBS 1 hr at 37°C before use.

To make coated insert substrates

1b. Cut the coated membrane from the inserts with a dematological punch.

2b. Rehydrate the coated membranes in serum-free DMEM for 1 hr at 37°C.

Add membrane or coated insert to collagen

3. Layer the de-epidermalized membrane or the coated membrane from the insert onto the contracted collagen gel 20 min before adding keratinocytes in step 15 of Basic Protocol 1.

FABRICATION OF THREE-DIMENSIONAL MODEL OF HUMAN SKIN CANCER

ALTERNATE PROTOCOL

The tissue microenvironment is defined by the complex network of intercellular interactions that are mediated by physical attachment (as in direct cell-cell or cell-extracellular matrix interactions) and by biochemical signals mediated by soluble molecules (Hagios et al., 1998). Cell adhesion is essential for the assembly of cells into 3D tissues and is required to maintain normalization of tissue architecture and homeostasis. In this way, cells receive adhesive cues that control their polarity, proliferation, differentiation, and survival. In recent years, evidence has accumulated that cancer is a disease of altered tissue architecture and that neoplastic progression is a consequence of abnormal interactions between tumor cells and their tissue microenvironment. As a result, it is essential to study the impact of the tissue microenvironment on cancer progression in human tissues that incorporate 3D tissue context so that they faithfully mimic their in vivo counterparts. Monolayer, 2D culture systems do not generate the spatially organized, 3D structures that are seen in vivo and have been of limited use in studying complex cellular responses. These inherent limitations have driven the construction of 3D tissue models that now provide novel experimental paradigms for studying cancer progression in biologically relevant tissues.

In one example of such tissue fabrication, it is now possible to construct 3D models of cutaneous, human squamous cell carcinoma that replicate multiple stages of its progression. To recapitulate the precancerous stages of progression of skin cancer, 3D tissue models must be constructed to reflect alterations in cell-cell interactions that could propel the tissue towards a neoplastic fate (Margulis et al., 2005). This has been accomplished by developing tissue models in which the fate of potentially malignant cells can be mapped by mixing normal keratinocytes with genetically-marked (with β-galactosidase) tumor cells at varying ratios to mimic precancerous conditions. We have previously found that interactions between genetically marked tumor cells and adjacent normal cells lead to tissue normalization and elimination of tumor cells when the two cell types are mixed in a ratio greater than 1:1 (normal cells/tumor cells) (Javaherian et al., 1998). Thus potentially malignant cells enter a quiescent state known as intraepithelial dormancy, in which the neoplastic potential of the tissue is conditionally suppressed and not realized. These studies have used 3D tissue models to directly implicate tissue architecture mediated by cell-cell interactions as a dominant regulator of the neoplastic phenotype.

Carcinoma cell lines can be grown as pure cultures at an air-liquid interface to simulate a carcinoma in situ, or they can be grown as mixtures with normal keratinocytes to mimic an earlier stage of epithelial dysplasia. Previous studies with different SCC cell lines indicate that tumor cells may either be maintained as individual cells within the tissue (Javaherian et al., 1998), or they may undergo intraepithelial expansion (Vaccariello et al., 1999). These tissues can be constructed by following the steps in Basic Protocol 1 for construction of normal skin equivalents and including the modifications presented below.

Additional Materials *(also see Basic Protocol 1)*

Cancer cell line of interest

1. Prepare cellularized collagen as described in Basic Protocol 1, steps 1 to 9.
2. Prepare a single cell suspension of normal keratinocytes as described in Basic Protocol 1, steps 10 to 13.

3. Culture the desired cancer cell line in a 10-cm^2 tissue culture dish in medium appropriate to the cell line.
4. Prepare a single cell suspension by incubating the cells with trypsin/EDTA for 5 min. at 37°C. Transfer the detached cells to a 15-ml tube and count viable cells in an aliquot (*UNIT 1.1*).
5. Dispense the desired number of normal keratinocytes and cancer cells into separate 15-ml tubes. Centrifuge the cells 5 min at 2000 × *g*, room temperature.
6. Resuspend each cell pellet in an appropriate volume of epidermalization I medium at 5×10^5 cells/50 μl.
7. Mix cancer cells with normal keratinocytes in the desired ratio, and seed a total of 5×10^5 combined cells/50 μl for each insert.
8. Proceed with Basic Protocol 1, starting at step 15, to culture tissues at the air-liquid interface and harvest for analysis.

BASIC PROTOCOL 2

FABRICATION OF THREE-DIMENSIONAL WOUND HEALING MODEL OF HUMAN SKIN

In vitro studies of wound re-epithelialization have often been limited by their inability to simulate wound repair as it occurs in humans. For example, wound models using skin explants or monolayer, submerged keratinocyte cultures demonstrate limited stratification, partial differentiation, and hyperproliferative growth culture systems have been helpful in studying keratinocyte migration in response to wounding. However, they have been of limited use in studying the complex nature of keratinocyte response during wound repair, as these cultures do not provide the proper tissue architecture to study the in vivo wound response.

We have previously found that HSEs adapted to study wound repair in human epithelium simulate the chronology of events that occur during re-epithelialization in human skin, and we have advanced the understanding of the healing of wounds in human skin and other stratified epithelia (Garlick and Taichman, 1994b; Garlick et al., 1996). This tissue model has allowed direct determination of the key response parameters of wounded epithelium including cell proliferation, migration, differentiation, growth-factor response, and protease expression. The application of tissue engineering technology demonstrates the utility of HSEs in studying phenotypic responses that are characteristic of the switch from a normal to a regenerative epithelial tissue during wound re-epithelialization.

This protocol describes a method for determining the response of HSEs that mimics re-epithelialization of wounded human skin, from the initiation of keratinocyte activation until restoration of epithelial integrity. Using these protocols, HSEs are fabricated as described in Basic Protocol 1. They are wounded 7 days after keratinocytes are seeded onto the contracted collagen gel. One week before wounding these tissues, an additional collagen gel, using both acellular and cellular collagen, is fabricated; this gel serves as a substrate to which the wounded tissue will be transferred. This method requires sequencing the experiment so that preparation of the additional collagen gel will be initiated one week in advance of wounding (see Fig. 19.9.3), i.e., at approximately the same time that the keratinocytes are seeded onto the first collagen matrix.

Materials

Human skin equivalent (HSE; see Basic Protocol 1, steps 1-18) with keratinocyte cultures at day 7
Contracted collagen gel (prepared 1 week in advance of the wounding; Basic Protocol 1 steps 1-9)
Cornification medium (see Table 19.9.1)

10-cm^2 sterile tissue culture dish
1.4-cm stainless steel dermatological punch (Delasco, cat. no. KP-14), optional
Forceps
Dental mirror
Scalpel with #22 blade, sterile

Wound the tissue

1. Aspirate all medium from the HSE culture after 7 to 10 days of culture.
2. Remove the insert from the well and place it upside down in a sterile dish.
3. Using a scalpel, cut away the insert membrane and place the culture in a 10-cm^2 sterile dish right-side up.
4. Trim the culture with the scalpel by cutting around the raised, mesa-like region to remove the part of the collagen gel not covered by keratinocytes.

   This will facilitate the removal and transfer of the wounded tissue from the membrane.

5. Wound the cultures with either an incisional or excisional wound.

   An incisional wound can be generated by incising tissues with a scalpel in a way that will allow the wound edges to be separated to generate an elliptical wound.

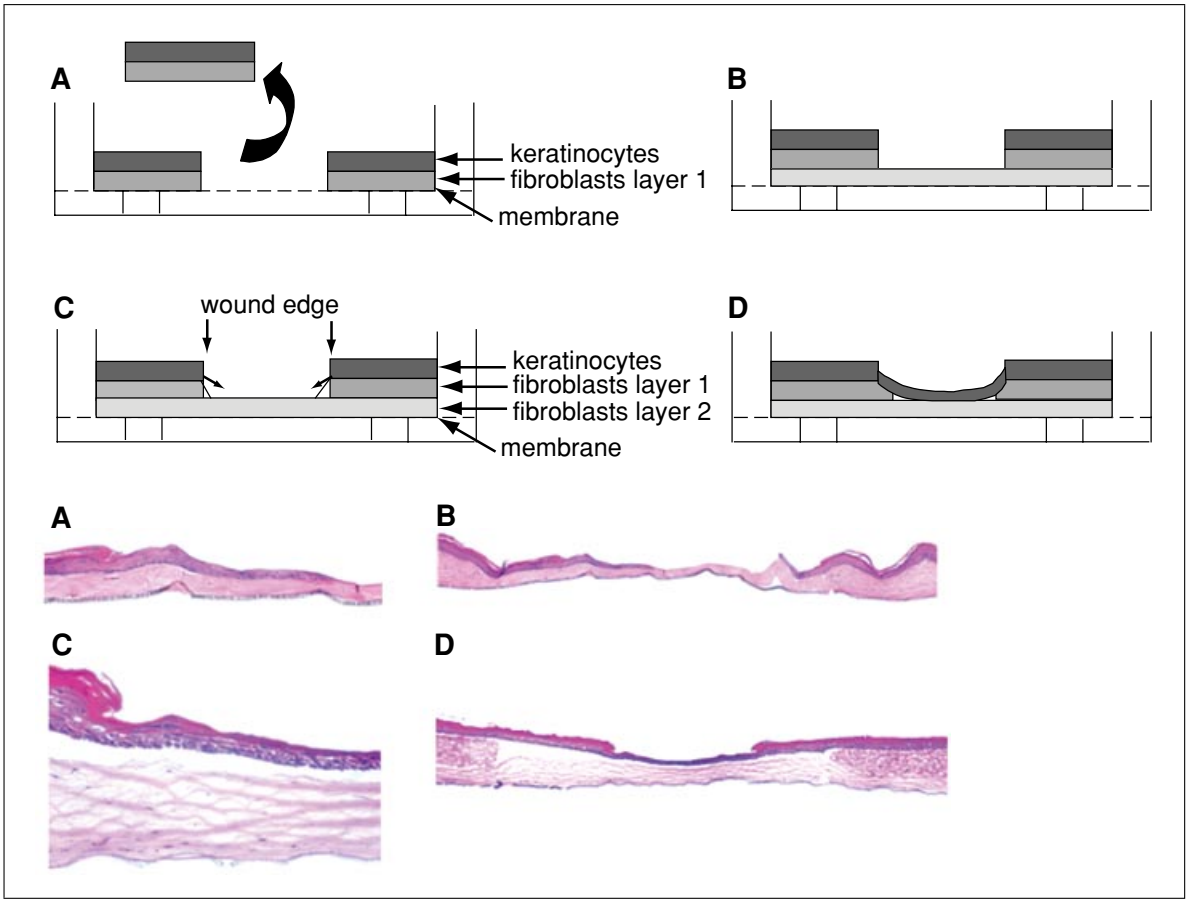


Figure 19.9.3 Three-dimensional tissue model of wound re-epithelialization: schematic (upper panels) and photomicrographs (lower panels; magnification: 5×). (**A**) A wound is generated through the full thickness of a human skin equivalent (HSE) and the excised tissue is removed. (**B**) The wounded tissue is placed on a second, contracted collagen gel. (**C**) Keratinocytes undergo migration to close the wound gap. (**D**) Keratinocytes have restored epithelial integrity, have closed the wound gap, and undergo stratification.

An excisional wound can be generated using an elliptical dermatological punch that completely penetrates the center of the tissue through the epidermis, collagen, and membrane. The excised tissue can be fixed and preserved for H&E staining in 10% formalin.

Transfer tissue to new gel

6. Use forceps to gently lift the edge of the wounded tissue by separating the collagen gel from the membrane. Drag the tissue onto a dental mirror while leaving the membrane behind.

   The transfer may be easier if the mirror is moistened with medium.

7. Unfold any wrinkles in the culture by gently moving the tissue back and forth on the mirror using the forceps. Once the culture is smooth, pull one side of the culture slightly over the edge of the mirror.

8. Move the mirror directly over the second contracted collagen matrix so that the edge of the mirror and wounded tissue are in contact with the matrix. Slide the tissue onto the second collagen gel by teasing it gently with a closed forceps as the mirror is slowly pulled away, leaving the culture on the contracted collagen gel.

9. Using the forceps, tease apart the tissue wounded by incision to create an elliptical space that should be 2 to 3 mm at its greatest width. Smooth the tissue with the forceps to ensure that it is completely free of any folds or wrinkles.

10. Maintain the tissue at an air/liquid interface by adding 8 ml of cornification medium beneath the insert, and change the medium every 2 days until the end of the experiment.

Histologically analyze wound repair

11. For histological analysis, process the tissue as described in Basic Protocol 1, steps 19 to 25, making sure to bisect the tissue perpendicular to the long axis of the wound (i.e., along the greatest width of the wound). Mount the tissue for sectioning en face so that the greatest width of the wound is sectioned first.

    It is essential to capture the first two sections as these will be the most informative sections of the wound.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acellular and cellular collagen matrices

10× MEM (minimum essential medium with Earle's salts; Cambrex, cat. no. 12-684F)
200 mM L-glutamine (Invitrogen, cat. 25030-081)
Fetal bovine serum (FBS; Hyclone, cat. SH30071)
187 µl 71.2 mg/ml $NaHCO_3$ (Cambrex, cat. no. 17-605E)
Bovine type I collagen (Organogenesis, cat. no. 200-055)
3×10^5 fibroblasts/ml (see Basic Protocol 1)

NOTE: Keep all components on ice and mix in the order listed to avoid alteration in pH that will result in inability of collagen to gel.

See Table 19.9.2 for volumes to use for acellular and cellular matrix construction. Prepare fresh and use immediately. Do not store the final matrix.

Table 19.9.2 Construction of Collagen Matrix

Component[a]	Acellular collagen matrix[b]	Cellular collagen matrix[b]
10× MEM	0.6 ml	1.8 ml
L-glutamine	54 µl	162.5 µl
FBS	0.68 ml	2.02 ml
Sodium bicarbonate	187 µl	0.56 ml
Collagen	5 ml	15 ml
Fibroblasts	–	1.65 ml

[a]See recipe for acellular and cellular collagen matrix components for more information about component solutions and the fibroblast suspension.
[b]Component volumes are listed for the construction of acellular collagen and cellular collagen matrices. Volumes are for six inserts. Use 1 ml per insert for acellular collagen matrix and 3 ml per insert for cellular collagen matrix.

Adenine, 18 mM

Dissolve 0.972 g adenine (MP Biomedicals, cat. no. 100190) in 2.4 ml 4 N NaOH. Bring to 400 ml with water. Store up to 1 year at −20°C.

Cholera toxin, 10^{-7} M

Dissolve cholera toxin (Sigma, cat. no. C-8052) at 9 ng/ml in water and store up to 1 year at −20°C.

EDTA/PBS

50 ml of 5 mM EDTA: dilute 5 ml of 0.5 M EDTA, pH 8.0 (Invitrogen, cat. no. 15575) to 500 ml with PBS (Invitrogen, cat. no. 14190; also see *APPENDIX 2A*); store up to 1 year at room temperature

EGF, 10 µg/ml

Dissolve human recombinant epidermal growth factor (EGF; Austral Biological) at 10 µg/ml in 0.1% (v/v) bovine serum albumin (BSA; tissue culture tested); store up to 1 year at −20°C.

Fibroblast culture medium

500 ml serum-free, low-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen)
55.6 ml fetal bovine serum (FBS; Hyclone, cat. no. SH30071; 10% final)
5.6 ml 800 mM HEPES (see recipe; 8 mM final)
3.4 ml 100× penicillin/streptomycin (Invitrogen)
Store up to 2 weeks at 4°C

HEPES, 800 mM

Dissolve 47.24 g HEPES (Sigma) in 250 ml H_2O. Store up to 1 year at −20°C.

Hydrocortisone, 0.25 mg/ml (500×)

Dissolve 0.0538 g hydrocortisone (Sigma) in 200 ml H_2O. Store up to 1 year at −20°C.

The concentration in this solution is 0.55 mM.

Insulin, 5 mg/ml

Dissolve 50 mg insulin (Sigma) in 10 ml of 0.005N HCl. Store up to 1 year at −20°C.

Keratinocyte culture medium

338 ml serum-free, low-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen)
112 ml F12 nutrient mixture (Ham), containing L-glutamine (Invitrogen)
25 ml fetal bovine serum (FBS; Hyclone, cat. no. SH30071; 5% final)
5 ml 18 mM adenine (see recipe; 0.18 mM final)
3.4 ml 100× penicillin/streptomycin (Invitrogen)
5 ml 800 mM HEPES (see recipe; 8 mM final)
1 ml 0.25 mg/ml hydrocortisone (see recipe; 0.5 μg/ml final)
0.5 ml 10^{-7} M cholera toxin (see recipe; 10^{-10} M final)
0.5 ml 10 μg/ml EGF (see recipe; 10 ng/ml final)
0.5 ml 5 mg/ml insulin (see recipe; 5 μg/ml final)
Store up to 2 weeks at 4°C

CAUTION: *Cholera toxin is very toxic. Use appropriate precautions when handling stock solutions.*

O1O Medium

43 g DME powder, containing no glucose and no $CaCl_2$ (JRH Biosciences, special order)
5 liters H_2O
0.5 g $MgSO_4$
18.5 g $NaHCO_3$
Store up to 6 months at 4°C

Progesterone, 2 μM

Dissolve 1 mg progesterone (Sigma, cat. no. P-8783) in 1 ml absolute ethanol and then add 14.7 ml H_2O. Dilute 1 ml of this solution in 100 ml serum-free, low glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen). Store up to 1 year at −20°C

Triiodothyronine (T3), 500×

1 ml 3,3′,5-triiodo-L-thyronine sodium salt (Sigma, T-5516)
99 ml H_2O
Store up to 6 months at 4°C

Trypsin/EDTA

50 ml of 0.1% (v/v) trypsin: prepared by diluting 50 ml 0.25% trypsin (Invitrogen, cat. no. 15050) with 75 ml PBS (Invitrogen, cat. no. 14190; also see *APPENDIX 2A*); store up to 1 year at −20°C
50 ml of 5 mM EDTA: prepared by diluting ultrapure 0.5 M EDTA, pH 8.0 (Invitrogen, cat. no. 15575) with PBS; store up to 6 months at room temperature
Store up to 1 year at −20°C

COMMENTARY

Background Information

A biologically relevant 3D human tissue model of epidermis must faithfully recreate the morphological and biochemical features of the in vivo tissue. Upon fabrication, HSEs become spatially organized as they undergo growth and differentiation, due to stromal-epidermal communication (see Fig. 19.9.2). This provides numerous advantages in the study of epidermal biology when compared to 2D monolayer culture systems (Carlson et al., 2007), as components of the model can be altered and tissue outcomes monitored in order to investigate morphological and molecular responses. The ability to manipulate either the cells or the tissue microenvironment provides opportunities to investigate cancer progression and injury response in human skin. For example, soluble

factors (e.g., growth factors) can be added to the medium to directly observe HSE response. A pulse of bromodeoyuridine (BrdU) can be added to the medium to enable analysis of the proliferation rates of the cells in 3D tissues.

The protocols described above enable tissue growth on a variety of ECM substrates. Substrates can be selected to answer specific questions about the impact of different environments and how they affect tissue development. De-epidermalized dermis (AlloDerm, LifeCell) provides an interface on which the assembly of basement membrane is optimized, while HSEs grown on a contracted collagen gel do not assemble structured basement membrane (Andriani et al., 2004). Tissues grown on polycarbonate membranes coated with specific ECM proteins can be used to study how specific ECM or basement membrane (BM) proteins affect tissue growth and architecture (Segal et al., 2008).

Observations from early studies performed in monolayer 2D culture systems revealed that cell-cell contact between normal cells and adjacent tumorigenic cells could suppress the transformed phenotype. However, the mechanistic basis for these observations remained unclear due to a lack of human tissue models that could more fully mimic the biologically meaningful pathways that couple the cell-cell adhesion and in vivo phenotype, and more accurately represent these early events in tumorigenesis as they occur. Three-dimensional tissue models have now been designed to reflect the incipient stages of spontaneous tumors in human epithelial tissues, where potentially neoplastic cells are found in the context of normal, cellular neighbors during the premalignant stage of epithelial cancer development.

Three-dimensional tissue models can be adapted to understand the intraepithelial dynamic that occurs in precancerous legions. The fate of potentially malignant cells can be monitored in 3D tissues by mixing normal keratinocytes with genetically marked (with β-glactosidase) tumor cells at varying ratios. These models can reflect the alterations in cell-cell interactions that could propel the tissue towards a neoplastic fate. Using this approach, we had previously found that interactions between genetically marked tumor cells and adjacent normal cells led to normalization and elimination of tumor cells. Thus, potentially malignant cells entered a quiescent state known as intraepithelial dormancy, in which the neoplastic potential of the tissue was conditionally suppressed and not realized. This dormant state can be overcome by altering tissue dynamics in response to the tumor promoter TPA (Karen et al., 1999), irradiating with UV light (Mudgil et al., 2003), decreasing adhesive interactions between tumor cells and adjacent epithelia, or enabling tumor cells to interact with basement membrane proteins (Andriani et al., 2004). Both TPA and UV have different effects on normal versus premalignant cells. For example, premalignant cells are resistant to UV-induced apoptosis and undergo selective expansion in the presence of TPA, leading to intraepithelial expansion of premalignant cells. Thus, the opportunities created through the use of 3D tissue models have directly implicated tissue architecture, mediated by cell-cell interactions, as a dominant regulator of the neoplastic phenotype since maintenance of normal tissue architecture can constrain cancer progression at a premalignant stage.

Critical Parameters

The proliferative potential of keratinocytes is a critical factor in the successful fabrication of HSEs with normalized tissue architecture. Most keratinocytes seeded onto HSE cultures will adhere to the connective tissue substrate, but only replicating cells will grow after seeding. Keratinocytes that have undergone terminal differentiation while in submerged culture will also attach to the substrate, but will not undergo further proliferation to become well stratified HSEs. In order to generate tissues with normalized tissue architecture, it is critical that keratinocytes seeded to generate HSEs have an elevated growth potential. Nearly all keratinocytes seeded in HSEs will adhere to their connective tissue substrate, but only replicating cells will grow.

It is, therefore, important to grow keratinocytes so that a high growth fraction is present in the monolayer cultures at the time of passage to HSEs. This can be accomplished by growing keratinocytes as small colonies at high clonal density in submerged cultures on 3T3 feeder layers so that terminal differentiation will be minimized and the fraction of replicating cells will be maximized. Alternatively, keratinocytes can be grown in low calcium medium before passage to HSEs, using defined medium conditions without 3T3 fibroblasts.

Keratinocyte strains can be tested in HSEs to determine those that will provide the best growth and morphologic differentiation. This may also be accomplished by testing the clonogenic growth of keratinocyte strains in monolayer cultures on feeder layers in order

to determine the growth potential of these cells. We have observed variations in growth and morphology of HSEs when different keratinocyte and fibroblast strains are incorporated. Therefore, keratinocyte strains should be screened by constructing HSEs to identify strains that can achieve optimal morphologic differentiation and tissue architecture.

An advantage of studying keratinocyte phenotype in HSEs lies in the ability to control and modify the cellular milieu in which these tissues are grown. For example, it is possible to add soluble factors directly to these cultures to determine the phenotypic response to such environmental conditions. In addition, growth conditions can be modified by culturing tissues in the absence of fibroblasts by eliminating them after contraction of the collagen gel (Andriani et al., 2003). Furthermore, control of the milieu facilitates analysis of these tissues, as it is possible to directly determine proliferation indices by adding a pulse of BrdU directly to the medium.

The protocols described in this unit provide techniques that allow growth of epithelial tissues on a variety of connective tissue substrates, each of which can be tailored to answer experimental questions. The interface of tissues grown on the de-epidermalized dermis promotes the rapid assembly of structured basement membrane and optimization of tissue morphology (Andriani et al., 2003). One source of such a dermal substrate is AlloDerm, a commercially available cadaveric dermis that is used in a variety of clinical applications to treat burns, periodontal disease, and surgical defects (LifeCell). Tissues grown directly on contracted collagen gels do not assemble intact basement membrane, but provide excellent support for keratinocyte growth and differentiation. Cultures grown on polycarbonate membranes coated with specific extracellular matrix proteins can be used to directly study the effect of these proteins on tissue architecture and phenotype of these tissues (Segal et al., 2008).

Troubleshooting

We have found some variability in the degree to which fibroblast strains support keratinocyte growth after their incorporation into collagen gels. It appears that fibroblast support of HSE organization and growth is directly related to the degree to which fibroblasts are able to contract the collagen gel. Fibroblast strains demonstrating more shrinkage of the collagen gel before adding keratinocytes are better able to support keratinocyte growth. This parameter can be used to screen fibroblast strains for optimal growth-support when planning initial experiments.

It is critical to preserve tissue architecture during processing of tissues after culture is complete. Since collagen gels are greatly hydrated, they can undergo significant artifactual damage during tissue processing. For this reason, tissues are soaked in 2 M sucrose for 2 hr at room temperature or overnight at 4°C in order to replace water in the tissue and prevent freezing damage. Furthermore, tissues need to be gradually frozen in liquid nitrogen vapor to prevent destruction of tissue architecture. Tissues should never be snap-frozen by immersing them in liquid nitrogen.

Fabrication of the collagen matrix requires that all components be kept on ice until the gel mixture is placed onto the insert. This will ensure that collagen will not prematurely precipitate from these solutions. Plastic pipets used for collagen should be chilled before use.

Anticipated Results

Several points regarding some subtleties of keratinocyte behavior in HSEs should be mentioned. The first concerns the length of time which cultures can be maintained at the air-liquid interface. In our experience, cultures can be kept at this interface for 7 to 14 days. After this time, the surface layers of the epithelium become very thick due to a failure to desquamate. As a result, lower layers of the epithelium become compressed and the longevity of cultures is limited. A second and related issue concerns the growth potential of keratinocytes in HSEs. While HSEs demonstrate a basal level of proliferation that is greater than human skin, it has been shown that these cultures have a tremendous potential for cell growth and are very responsive to external growth stimuli. For example, we have shown that disruption of HSEs upon wounding results in a 10-fold increase in basal cell proliferation (Garlick and Taichman, 1994a).

It should be kept in mind that although keratinocytes grown in HSEs share many morphologic and biochemical features with their in vivo skin and mucosal keratinocytes, there are differences in tissue phenotype. For example, integrin receptors (the αv integrins) not normally expressed in skin are constitutively expressed in keratinocytes grown in HSEs. In addition, HSEs are somewhat deficient in barrier function. Keratinocytes grown

in HSEs can be thought of as tissues undergoing de novo development (e.g., newly re-epithelialized, healed wounds in vivo) wherein morphologic differentiation is complete, but cells are still in a somewhat activated state.

Time Considerations

Construction of HSEs requires ~3 to 4 weeks from the time HFF are seeded in monolayer culture until 3D tissues are fully mature. HFF cells should be confluent two days before construction of the collagen gels. At that time, cells should be passaged at a 9:10 ratio, to provide cells with a growth stimulus before incorporation into collagen gels. Complete contraction of the collagen gels requires 7 to 10 days, during which time the normal human keratinocytes cell cultures are initiated and expanded. Growth and differentiation of keratinocytes on the contracted collagen gels requires, sequentially, 2 days in epidermalization I medium, 2 days in epidermalization II medium, and 7 to 10 days in cornification medium to allow full tissue organization and stratification. Histological analysis takes 2 to 3 days.

Literature Cited

Andriani, F., Margulis, A., Lin, N., Griffey, S., and Garlick, J.A. 2003. Analysis of microenvironmental factors contributing to basement membrane assembly and normalized epidermal phenotype. *J. Invest. Dermatol.* 120:923-931.

Andriani, F., Garfield, J., Fusenig, N.E., and Garlick, J.A. 2004. Basement membrane proteins promote progression of intraepithelial neoplasia in 3-dimensional models of human stratified epithelium. *Int. J. Cancer* 108:348-357.

Bissell, M.J. and Radisky, D. 2001 Putting tumours in context. *Nat. Rev. Cancer JID* 101124168 1:46-54.

Carlson, M.W., Iyer, V.R., and Marcotte, E.M. 2007. Quantitative gene expression assessment identifies appropriate cell line models for individual cervical cancer pathways. *BMC Genomics* 8:117.

Garlick, J.A. and Taichman, L.B. 1994a. Effect of TGF-beta 1 on re-epithelialization of human keratinocytes in vitro: An organotypic model. *J. Invest. Dermatol.* 103:554-559.

Garlick, J.A. and Taichman, L.B. 1994b. Fate of human keratinocytes during re-epithelialization in an organotypic culture model. *Lab. Invest.* 70:916-924.

Garlick, J.A., Parks, W.C., Welgus, H.G., and Taichman, L.B. 1996. Re-epithelialization of human oral keratinocytes in vitro. *J. Dent. Res.* 75:912-918.

Hagios, C., Lochter, A., and Bissell, M.J. 1998. Tissue architecture: The ultimate regulator of epithelial function? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 353:857-70.

Javaherian, A., Vaccariello, M., Fusenig, N.E., and Garlick, J.A. 1998. Normal keratinocytes suppress early stages of neoplastic progression in stratified epithelium. *Cancer Res.* 58:2200-2208.

Karen, J., Wang, Y., Javaherian, A., Vaccariello, M., Fusenig, N.E., and Garlick, J.A. 1999. 12-*O*-tetradecanoylphorbol-13-acetate induces clonal expansion of potentially malignant keratinocytes in a tissue model of early neoplastic progression. *Cancer Res.* 59:474-481.

Kolodka, T.M., Garlick, J.A., and Taichman, L.B. 1998. Evidence for keratinocyte stem cells in vitro: Long-term engraftment and persistence of transgene expression from retrovirus-transduced keratinocytes. *Proc. Natl. Acad. Sci. U.S.A.* 95:4356-4361.

Margulis, A., Zhang, W., Alt-Holland, A., Crawford, H.C., Fusenig, N.E., and Garlick, J.A. 2005. E-cadherin suppression accelerates squamous cell carcinoma progression in three-dimensional, human tissue constructs. *Cancer Res.* 65:1783-1791.

Mudgil, A.V., Segal, N., Andriani, F., Wang, Y., Fusenig, N.E., and Garlick, J.A. 2003. Ultraviolet B irradiation induces expansion of intraepithelial tumor cells in a tissue model of early cancer progression. *J. Invest. Dermatol.* 121:191-197.

Rheinwald, J.G. and Green, H. 1975. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell* 6:331-344.

Segal, N., Andriani, F., Pfeiffer, L., Kamath, P., Lin, N., Satyamurthy, K., Egles, C., and Garlick, J. 2008. The basement membrane microenvironment directs the normalization and survival of bioengineered human skin equivalents. *Matrix Biol.* 27:163-170.

Vaccariello, M., Javaherian, A., Wang, Y., Fusenig, N.E., and Garlick, J.A. 1999. Cell interactions control the fate of malignant keratinocytes in an organotypic model of early neoplasia *J. Invest. Dermatol.* 113:384-391.

Overview: Engineering Transgenic Constructs and Mice

Naoto Haruyama,[1] Andrew Cho,[1] and Ashok B. Kulkarni[1]

[1]National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland

ABSTRACT

Cell biology research encompasses everything from single cells to whole animals. Recent discoveries concerning particular gene functions can be applied to the whole animal for understanding genotype-phenotype relationships underlying disease mechanisms. For this reason, genetically manipulated mouse models are now considered essential to correctly understand disease processes in whole animals. This unit reviews the basic mouse technologies used to generate conventional transgenic mice, which represent a gain-of-function approach. First, an overview of transgenic construct design is presented. This unit then explains basic strategies for the identification and establishment of independent transgenic mouse lines, followed by comments on historical and emerging techniques. It then describes typical problems that are encountered when researchers start to generate transgenic mice. *Curr. Protoc. Cell Biol.* 42:19.10.1-19.10.9. © 2009 by John Wiley & Sons, Inc.

Keywords: transgenic mice • plasmid vector • constructs • gene expression • reporter gene

INTRODUCTION

Genetically manipulated mouse models are now considered essential for studying gene functions in whole animals. Gene knockout mice represent a loss-of-function strategy, whereas the transgenic mice discussed in this unit represent a gain-of-function approach to define molecular and cellular functions of a gene of interest. This approach can also be used to analyze tissue-specific or developmental stage–specific gene expression by introducing reporter genes, such as β-galactosidase (*lacZ)* or green fluorescent protein (GFP), under the control of a specific gene promoter. If the functional domain of a gene of interest is well characterized, it is possible to create transgenic mice with gene ablation by introducing the dominant negative forms of the gene into the mouse genome. Typically, transgenic mice are generated by microinjecting the transgenic construct into a fertilized egg (oocyte or zygote). An alternative way to effectively introduce a transgene into an egg is the use of a retroviral vector. However, the retroviral method has limitations: high titers must be used to obtain the vector integration, and mosaic mice may be generated that do not carry the transgene in each and every cell. Another method to generate transgenic mice is to transfect a transgenic construct into mouse embryonic stem (ES) cells and then inject these cells into mouse blastocysts. This method is particularly useful to obtain a low copy number of the transgene in the mice, especially when embryonic lethality is expected in the resultant transgenic mice. Chimeric mice generated by the ES cell transfer method may be able to transmit the transgene to embryos, allowing analysis of transgenic embryos in the dams that have mated with the chimera.

This unit presents the most widely used strategies for generating plasmid-based transgenic constructs to be injected into fertilized mouse eggs, identifying potential founders by genotyping, and establishing separate founder lines. Frequent problems that can be encountered when researchers start to generate transgenic mice will be discussed in the Commentary sections.

DESIGN OF THE TRANSGENIC CONSTRUCT

Like other plasmid constructs used to express proteins in a cell culture system, transgenic constructs must have all the critical elements for gene expression to occur, such as promoters, introns, the protein coding sequence of interest (cDNA or genomic DNA) and a poly(A) site (Fig. 19.10.1A). A cloning strategy should be clearly planned with a good understanding of each functional element so as to obtain the expected expression

Current Protocols in Cell Biology 19.10.1-19.10.9, March 2009
Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1910s42

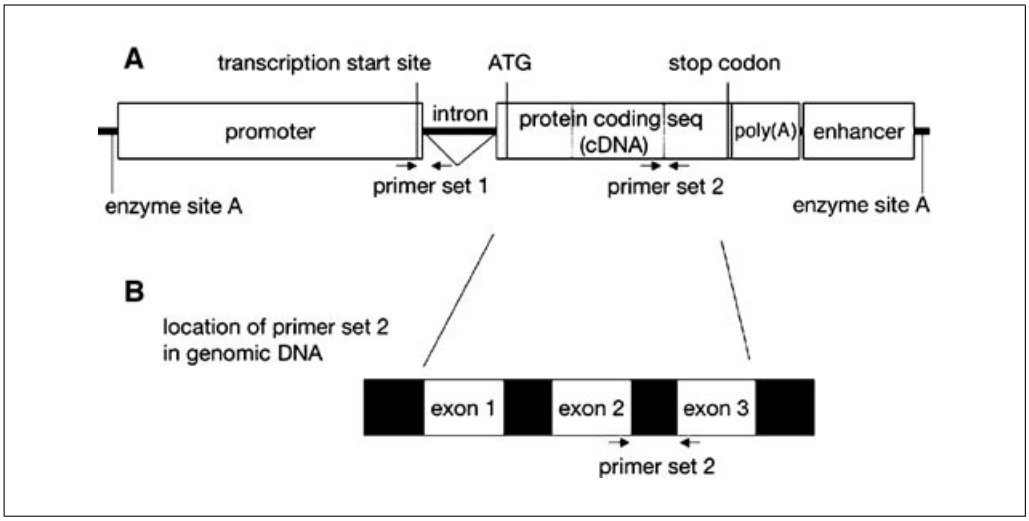


Figure 19.10.1 Representative transgenic construct and primer designs for genotyping. (**A**) Typical transgene construct is depicted, consisting of promoter, intron, protein-coding sequence (cDNA) from a gene of interest, stop codon, poly(A) sequence, and enhancer sequences. The promoter sequence normally contains the transcriptional start site, although there are variations. The inclusion of an intron leads to a greater percentage of active transgene expression in mouse lines. The protein coding sequence (cDNA) needs to have a translation start site, typically consisting of a Kozak consensus sequence in addition to an ATG start codon. The entire fragment can be cut out from the vector backbone by a single restriction enzyme (site A) prior to zygote injection. A restriction enzyme site that can be cut only once inside of the fragment should be considered at the construct-design stage for future genotyping by Southern blotting. Primer set 1, designed at the junction of 2 different elements, can amplify a unique sequence. (**B**) Alternative strategy of primer design. The primers for genotyping can also be designed in cDNA to bridge between two or more different exons so that the endogenous gene would be amplified with a larger size at low efficiency. ATG, translation start codon; stop, translation stop codon; seq, sequence.

of a transgene. Well defined expression in a cell line is certainly a good indicator that a transgenic construct was properly designed, although this will not guarantee transgene expression in vivo. A transgene must be excised out of the vector backbone prior to injection into mouse eggs (see *UNIT 19.11*). Unnecessary sequences from a plasmid vector (derived from prokaryotic sequences) should be removed as much as possible to increase the probability of transgene expression in mice, although the expression seems unaffected by up to 100 extra bases. Inclusion of extra sequences between each element of a transgene does not usually affect the transgene's function as long as some regulatory elements are not inadvertently created. To release a transgenic cassette from a vector, the construct must be designed with unique restriction enzyme sites flanking the transgenic cassette (Fig. 19.10.1A). The size of a transgene does not affect efficiency of generating transgenic mice.

Promoter

A promoter is a critical sequence required for regulating the spatial and temporal expression pattern of a transgene. Promoter sequences are isolated from upstream regions of endogenous mammalian genes. A promoter sequence normally includes a transcriptional start site as well as transcription regulatory sequences. Many promoters have been reported that can successfully achieve tissue-specific and developmental stage–specific expression of transgenes. A database search such as PubMed or the International Mouse Strain Resource (see Internet resources with annotations) is the preferable way to find promoters for a tissue or cell type of interest. Promoters should be tested to determine if they contain the appropriate transcriptional regulatory elements. For example, transgenic mice harboring 0.9 kb of the type I collagen (*Col1a1*) promoter expressed the transgene at relatively low levels, almost exclusively in the skin. On the other hand, mice with a sequence containing 2.3 kb of this proximal promoter expressed the transgene at high levels in mineralized tissues, but not in other type I collagen–producing cells. Transgenic mice harboring 3.2 kb of the proximal promoter showed an additional high-level expression of the transgene in tendon and fascia fibroblasts (Rossert et al., 1995).

The best way to choose a promoter to generate transgenic mice is to review original papers that examined its endogenous expression patterns. To prevent any significant difference from the original work, investigators should ask the group that published the work to provide the vector with detailed information about the promoter sequence.

Inducible expression of a transgene in mice is possible by utilizing inducible promoters. For example, the metallothionein-1 promoter is induced by heavy metals like zinc (Mayo et al., 1982; Sumarsono et al., 1996), and interferon-inducible gene expression has been reported for Cre (P1 bacteriophage cyclization recombinase) in transgenic mice (Kühn et al., 1995). Tetracycline, used for tightly regulated expression of a transgene, is the most frequently used promoter and activator of binary systems (Gossen and Bujard, 1992; see *UNIT 20.8*). In this system, the expression of a fusion protein, consisting of the viral transactivation domain and the prokaryotic tetracycline repressor (*tetR*) domain, is controlled by a tissue- specific promoter. Because the fusion protein binds to both tetracycline and the operator sequences (*tetO*) of the tet operon in the target transgene, the target transgene can be controlled by a minimal promoter that contains only a TATA box, depending on the tetracycline concentrations in tissues. There are two options in this system: in the first one, the tetracycline-controlled transactivator (tTA) cannot bind to DNA when the tetracycline is present (tet-off), whereas in the second one, the reverse tTA (rtTA) binds to DNA only when tetracycline is present (tet-on).

Protein-Coding Sequence and Transcription Start Site

A protein-coding sequence is usually a full-length cDNA derived from the RNA of a gene of interest. This sequence normally contains a translational start codon (ATG), a translational stop codon, and a Kozak sequence upstream of the start codon so that the ribosome can scan and recognize the proper translation start and stop sites on mRNA. An authentic 10- to 15-nucleotide sequence found at the 5′ end of the start codon in a gene of interest can be assumed to have appropriate translational start signals. Alternatively, a Kozak consensus sequence such as GCCGCC (G/A) NN ATG G can be incorporated as a translational start site (Kozak, 1987). The sequence between the transcriptional start site and the translational start site should be checked for inadvertent ATG start codons, stop codons, or other potential regulatory elements created during construction of a transgene.

Adding protein tag sequences such as VSV, 6×His, HA, V5, or Myc to the transgene may be useful to detect transgene expression because reliable antibodies to these epitopes are available. Additionally, a tag sequence provides a way to differentiate between endogenous gene expression and transgene expression. It is important to avoid disruption of transgene function when adding a tag sequence. Introducing an internal ribosomal entry site (IRES) followed by a reporter gene such as *lacZ* or GFP is also useful for checking the expression pattern and for genotyping, although the efficiency of IRES-mediated bicistronic expression may require additional studies.

If cDNA cloning is difficult because of highly repetitive sequences, and if a genomic clone containing all of the required exons and introns is available, then the promoter can be ligated with the genomic sequence (Tanaka et al., 2001).

Roles of Intron, Stop, Poly(A) Addition, and Enhancer Sequences

Variation in transgene expression levels is influenced by a number of factors. However, inclusion of an intron in a transgene construct leads to significantly greater transgene expression (Brinster et al., 1988); Fig. 19.10.1). The intron seems to have important effects on mRNA stabilization and the efficient translocation from nucleus to cytoplasm (Huang and Gorman, 1990). Examples of introns commonly used are the rabbit β-globin intron or the simian virus 40 (SV40) intron. The first authentic intron located upstream of exon 2, which often contains a translational start site, can also be used in experiments where tissue-specific expression is important. Each transgene must also contain a transcriptional stop signal to match the transcriptional start signal. Eukaryotic transcriptional stop signals include a poly(A)-addition sequence (AAUAAA), as well as hundreds of downstream nucleotides whose function is important but not clearly understood. The transcriptional stop signal including poly(A) addition sequence (designated poly(A) as shown in Figure 19.10.1) is added at the 3′ end of the protein coding sequence. Examples of transcriptional stop sequences are those from SV40, bovine growth hormone (BGH), and human growth hormone (hGH; Sheets et al. 1987; Goodwin and

Rottman 1992). If an enhancer sequence for the gene of interest is identified, it can be included at either end of all the fragments mentioned above.

IDENTIFICATION OF POTENTIAL FOUNDERS AND GENOTYPING STRATEGY

About 3 weeks after the zygote injection, potential founders (F0) should be born. Genomic DNA for genotyping should be isolated from either ear-punch or snipped tail according to the approved animal study protocol. All the founders should be screened for the presence of the transgene to establish separate transgenic lines. The founders will have variable copy numbers and unique transgene insertion sites because of the random integration of the transgene.

Polymerase chain reaction (PCR) and Southern blotting are the most commonly used methods to detect the presence of the transgene. Southern blotting is highly recommended to identify founder animals because of the low false-positive rate and because more information can be obtained from the blot. If investigators obtain too many potential founders to be genotyped by Southern blotting, a PCR assay can be used to screen for positive mice first, and Southern blotting can be used with the reduced sample size. PCR is probably the best method for genotyping the offspring of the founders, but the result of the PCR assay should be validated with Southern blotting at least once. In either case, the validation protocol must ensure that the methods have enough sensitivity to detect one copy of the transgene and that established lines can be maintained without loss in the future.

PCR Method to Identify the Transgene

Because of the high sensitivity of PCR, cross-contamination between samples and low primer sensitivity should be eliminated prior to genotyping. It is, therefore, always preferable to use wild-type mouse genomic DNA as a negative control and a positive control of wild-type mouse genomic DNA spiked with a transgene fragment or a plasmid at 1 and 10 copies per diploid genome. Neither the pure transgene fragment nor the plasmid should be used for the positive control.

The input DNA for PCR should be 20 to 200 ng of genomic DNA for a 25-µl reaction. If the PCR products smear in the electrophoresed gel, the amount of input DNA should be decreased. PCR primers should consist of around 30 nucleotides and produce a short amplicon of between 100 and 500 nucleotides for robust and specific amplification. The best primer pair can be designed in flanking sequences of the junction between two elements uniquely combined in the transgene (primer set 1 in Fig. 19.10.1A). This primer pair distinguishes the transgene from the endogenous gene. Alternatively, if a transgene construct has a foreign sequence from other species, such as a GFP, the primers designed in the sequence can amplify the unique product. If a cDNA is used as a coding sequence, the primers can be designed to bridge between two or more different exons so that the endogenous gene will be amplified as a larger product (primer set 2 in Fig. 19.10.1A and B).

Southern Blotting Method to Identify the Founders

A restriction enzyme that can cut only once in the transgene fragment is frequently used to digest genomic DNA for the genotyping of transgenic mice by Southern blotting. Since the majority of cytosines in the context of the CpG dinucleotide are methylated in normal adult somatic tissues, a restriction enzyme that is CpG methylation sensitive should not be used. Because the transgenic construct integrates as multiple head-to-tail tandem copies in the mouse genome in most cases, digestion with a restriction enzyme that cuts at one site in the transgene will result in a fragment that is the same size as the original transgene (enzyme site A in Fig. 19.10.2A). These fragments will appear as the most intense band by using either a transgene-specific probe or a probe made from the entire injected fragment. The transgene flanking fragments, which consist of the genomic sequence and a part of the transgene, can also be detected as two obvious bands with different sizes depending on the integration sites. These bands can be used as single-copy standards to calculate the absolute copy numbers of the transgene. If more than three different flanking sequences appear, the founder mouse may have multichromosomal integration (Fig. 19.10.2B, Lane E).

We recommend using the entire injected fragment as a probe (Fig. 19.10.2A). Because the promoter sequence in the probe fragment binds to the endogenous promoter region, the band derived from the endogenous promoter region can be used as a control to check whether equal amounts of DNA were used for each of the samples. In addition, the band from the endogenous promoter could be used as a

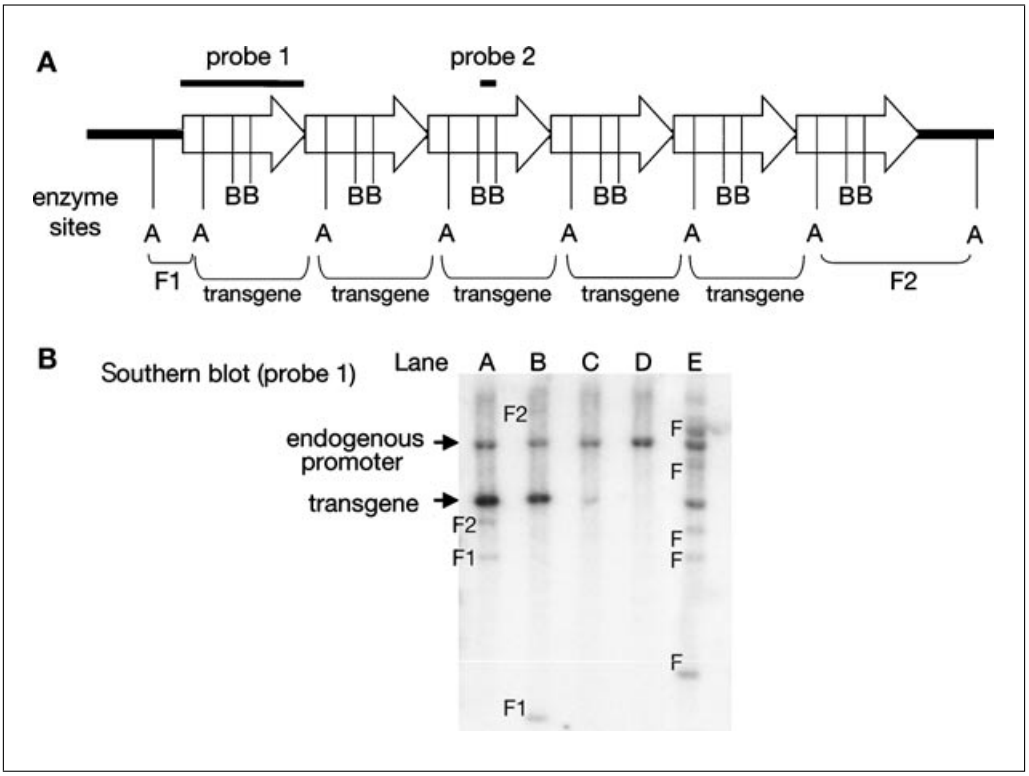


Figure 19.10.2 Diagrams of transgenes integrated in the mouse genome and the strategy for genotyping by Southern blotting. (**A**) The scheme assumes there are six copies of a transgene integrated head-to-tail. (**B**) Example for genotyping of transgenic mouse by Southern blotting. Enzyme sites A and B in panel (A) represent hypothetical locations of the restriction enzyme sites in integrated locus. If the genomic DNA containing the transgenes is digested with enzyme A, five copies of transgenes with the size of the injection fragment (transgene) and two flanking sequences (F1 and F2) will be released from the genomic DNA. The five copies of transgene fragments will be detected as the most intense band by using a probe made from the entire injected fragment, probe 1 (transgene in panel B, lane A). The flanking fragments of the transgene, which consist of the genomic sequence and a part of the transgene, can also be detected as two obvious bands with unique sizes depending on the integration sites (bands F1 and F2 in panel B, lane A). These bands can be used as single copy standards to calculate the absolute copy numbers of the transgene. Because the transgene is randomly integrated in the mouse genome, different lines show different sizes of flanking sequences (bands F1 and F2 in panel B, lane B). The band from an endogenous promoter may also be detected in all the samples (endogenous promoter in panel B). Lane C in panel B shows a transgene band with less intensity (~1 copy), suggesting that this line may be a mosaic mouse. If more than three different flanking sequences appear, the founder mouse may have multichromosomal integration (bands F in panel B, lane E). Lane D represents the wild-type mouse control. If the transgene fragment is too long to resolve using standard Southern blotting (>15 to 20 kb), a restriction enzyme that cuts two or more times in the injected fragment can be used (enzyme site B in panel A). In this case, probe 2 detects six copies of transgene fragments generated by enzyme B with no flanking sequences.

single-copy standard in case only one endogenous promoter with a unique sequence exists in the mouse genome. For example, if the transgene band has an apparent low intensity compared to the promoter band, the founder may be mosaic (Fig. 19.10.2B, Lane C).

In some cases, it may not be possible to use a restriction enzyme that can cut only once in the transgene. Especially if the transgene is longer than 15 to 20 kb, the fragment will be too long to resolve using standard Southern blotting (pulsed-field gel electrophoresis might be used). If that is the case, a restriction enzyme that cuts two or more times in the injected fragment can be used (enzyme site B in Fig. 19.10.2A). A probe designed in one of the internal fragments can hybridize and detect the intense band provided by the transgene. A series of wild-type genomic DNA samples spiked with 1 to 100 copies of the transgene can be used to generate a standard curve for calculating the copy numbers.

Calculation of Transgene Mass to Spike One Copy per Diploid Genome

Assume the mouse genome has around 6.0×10^9 bp/cell and the injected transgenic fragment has 6.0 kb (6.0×10^3 bp).

The ratio (the size difference) between the foreign DNA fragment and the mouse genome is:

$$(6.0 \times 10^3 \text{ bp})/(6.0 \times 10^9 \text{ bp}) = 1.0 \times 10^{-6}$$

To obtain a single copy of the fragment, the size difference or the ratio between the fragment and the total genome should be translated into the concentration of the DNA.

For example, if 10 μg of genomic DNA is to be analyzed by Southern blotting, multiply the difference to obtain a single-copy amount of transgene, i.e., 10 μg of genomic DNA $\times$ $1.0 \times 10^{-6} = 1.0 \times 10^{-5} = 10$ pg.

Therefore, 10 pg of the 6.0-kb transgene fragment is equal to 1 copy.

If 200 ng of genomic DNA is used for the PCR reaction, the single-copy amount for spiking is 0.20 pg. Note that the copy number of transgene usually has a strong correlation with the transgene expression level, although it will not guarantee the expression level of protein (see Transgene silencing and leaky expression, below).

ESTABLISHMENT OF INDEPENDENT MOUSE LINES FOR EXPERIMENTAL USE

Once the founders reach sexual maturity, they can be crossed with wild-type mice to establish separate transgenic lines. However, the wild-type mouse strain should be considered before the founders are mated. Because the phenotype may vary depending upon the strain background, generating littermates with a variety of genetic background ratios should be avoided. If the founders are generated from inbred strains such as C57BL6/J or FVB, the same background should be maintained by crossing with the same strain so that no mixed backgrounds will result. After verifying transmission from founder mice (F0) to offspring (F1), transgene expression within each line of the F1 or F2 generation has to be examined in most of the organs to prove that the line has proper temporal and spatial gene expression patterns and no leaky expression. Undesirable leaky (ectopic) expression in organs or tissues may result in unexpected phenotypes. In case the transgene is integrated into multiple chromosomes, further crossing with wild-type mice is necessary for the transgene to be segregated into different chromosomes to establish separate mouse lines.

Reverse transcriptase–polymerase chain reaction (RT-PCR) and/or northern blotting can be used for the screening of expression patterns. It is advisable to perform northern blots to minimize the effect of trace amounts of genomic DNA contaminations in RNA, especially when cDNA is used as a transgene. Immunoblots should also be performed to verify that the desired proteins were translated from transgenes.

At least two or three different lines generated from the same construct should be established and analyzed. Because each line should have different transgene insertion sites, copy numbers, and protein expression levels, an analysis of multiple transgenic lines may provide dose-dependent phenotypes. Multiple-line analysis also rules out the possibility of analyzing phenotypes from an unknown gene mutation or ectopic expression of the transgene.

GENERAL CONSIDERATIONS

Background Information

Generation of the first transgenic mouse was reported by Gordon et al. (1980). They produced transgenic mice by pronuclear injection of a pBR322-based plasmid containing HSV (herpes simplex virus) and the SV40 DNA sequence. After this report, the technique spread immediately and was well pursued. It is now an indispensable tool when gene functions need to be analyzed at the whole-body level, such as in developmental biology, immunology, and neuroscience. A large number of promoters have been characterized that direct a wide variety of transgene expression patterns. Varieties of transgenic mice that express Cre (P1 bacteriophage cyclization recombinase) in a tissue-specific manner are now available for creating conditional knockout mice utilizing the Cre-*loxP* system (Sauer and Henderson 1988; Gu et al. 1994).

Historically, the majority of transgenic mice were generated by injecting plasmid-based recombinant DNA, which limited the insert size to less than 20 kb because of the cloning capability of bacteria, but investigators could still expect faithful amplification, easy manipulation, and a good yield of recombinants. Relatively shorter inserts excised from plasmids can integrate as tandem-repeat concatemers, resulting in high copy number in most cases. To a certain extent, transgene silencing is inevitable from position effects

caused by the use of shorter transgenes (see Transgene silencing and leaky expression).

To overcome the problems related to size-restricted plasmid vectors, larger constructs can be made in YAC (yeast artificial chromosome), BAC (bacterial artificial chromosome), or PAC (P1-derived artificial chromosome). A premade BAC library can especially facilitate the cloning and identification of the gene of interest. The advantages of using a large clone such as YAC are reported in a number of papers (e.g., Giraldo and Montoliu, 2001). Transgenic mice created from large genomic clones showed position-independent, copy number–dependent expression of the transgene, suggesting that most of the regulatory sequences are needed to stably maintain the expression. Recently, Lois et al. (2002) reported that an alternative method of introducing a transgene by lentiviral vectors was successfully used to generate transgenic mice. This virus can be introduced either by microinjection in the perivitelline space or by coincubation with a denuded zygote. Furthermore, this method could be adapted with tightly regulated tet-on/off RNA interference (RNAi) technology to achieve conditional gene expression and knock-down in mice (Szulc et al. 2006). These emerging technologies will provide further insights for analyzing gene functions and developing therapeutics.

Troubleshooting

No founders born

If no founder mice are obtained, it may be the result of technical problems with microinjection. Alternatively, if a transgene carries fatal mutations or integrates at very high copy number, it may become toxic to embryos. Another possibility is that the transgene functions may be critical for the development of mouse embryos or for survival before the weaning stage. Investigators may need to reconsider these issues prior to repeating microinjections. There is also a possibility that the potential founder may be a false positive because of poor specificity for PCR primer sets or probes (see Identification Of Potential Founders and Genotyping Strategy). Lethal phenotypes can be analyzed in the founder mice during the embryonic development.

Germline transmission

The offspring (F1) from founder mice (F0) should inherit the transgene at almost 50% according to the Mendelian ratio, if the transgene is integrated into a single chromosome.

If investigators encounter no germline transmission, the genotype of the founder should be verified again. If F1 offspring inherit the transgene at a rate substantially less than 50%, the founder is most likely a mosaic mouse, which means that not all of the cells carry the transgene because of late integration. The mosaic founder may never transmit the transgene to offspring, or it may transmit it at very low efficiency, depending upon how many germ cells carry the transgene. If the transgene is integrated into multiple chromosomes, more than 50% of the offspring will inherit the transgene. Separate lines may be obtained if the transgenes can be successfully segregated to different chromosomes in the F1 generation. If the transgene integrates into the X chromosome of male founders, the female offspring will inherit the transgene at 100%, but the male offspring will inherit none.

Transgene silencing and leaky expression

Transgene silencing occurs when a transgene is inserted into a transcriptionally inactive region in the mouse genome. Even though the transgene is present in the genome, the transcript (mRNA) cannot be made because of positional effects. Thus, the copy numbers of an integrated transgene will not necessarily correlate with the transgene expression level. Transcriptionally inactive regions may result from repressor sites near the inserted promoter, insertion of the transgene into a gene with a repressed promoter, DNA methylation, X-inactivation, or genome imprinting. The use of an insulator sequence, which can insulate or buffer the activity of transgene from the effects of the activity of *cis*-acting regulatory elements, such as enhancer or silencer regions of the DNA, may decrease the probability of transgene silencing caused by the positional effects (Rivella et al., 2000). On the other hand, leaky (ectopic) expression of a transgene in unexpected tissues, or unrestricted temporal expression, can result from insertion into the vicinity of an endogenous enhancer or promoter. The transgene expression level may decrease after several generations and may eventually stop in some cases. This epigenetic silencing happens when the animals with high copy number become older. To reduce this risk, creating two or more lines of transgenic mice is preferable.

Table 19.10.1 Time Line for Identifying Founders and Establishing Lines

Week	Action
0	Microinjection
3	Birth of potential founders (F0)
4∼6	Tag (tattooing), tail biopsy, genotyping for founder identification
6	Wean
9	Set up founder mating
12	Birth of first founder offspring (F1)
13∼15	Tag (tattooing), tail biopsy, genotyping for transgenic mouse identification

Unknown gene mutations as a result of random integration

Because the transgene integrates randomly into mouse genomes, there is a possibility of disrupting a normal gene sequence. Such insertion may lead to an unexpected phenotype even in heterozygous mice because of haploinsufficiency. Since the phenotype caused by most of the mutations should be recessive, the phenotype may not appear until homozygotes are generated by crossing heterozygotes. Homozygotes may also have higher transgene expression compared to hemizygotes. To rule out the possibility of unknown gene knock-down phenotypes, more than two independent lines should be examined, because the independent lines should not have the same insertion site.

Mixing different lines

Transgenic mice created from the same construct may have similar but different phenotypes. It is always important to maintain independent lines without mixing them. The results of PCR genotyping will produce the same amplicon from different lines with same transgene, so regular PCR genotyping cannot distinguish lines accidentally mixed. In this case, Southern blotting can distinguish such lines by comparing the different sizes of flanking fragments.

Time Considerations

Table 19.10.1 outlines the major events in creating a transgenic line. Potential founders are born 3 weeks after zygote injection. Pups are typically genotyped before weaning. After founders are identified, mating can be set up once the founders reach sexual maturity around 9 weeks of age. The founders' offspring (F1) can be used for the initial phenotypic analysis. Germline transmission and transgene copy numbers should also be verified in this generation. If all F1 pups from a founder have the same copy numbers and flanking sequences, any positive mouse among the F1 littermates could be used as a single established line.

ACKNOWLEDGEMENT

This work was supported by the Division of Intramural Research of the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Md.

LITERATURE CITED

Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E., and Palmiter, R.D. 1988. Introns increase transcriptional efficiency in transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 85:836-840.

Giraldo, P. and Montoliu, L. 2001. Size matters: Use of YACs, BACs and PACs in transgenic animals. *Transgenic Res.* 10:83-103.

Goodwin, E.C. and Rottman, F.M. 1992. The 3′-flanking sequence of the bovine growth hormone gene contains novel elements required for efficient and accurate polyadenylation. *J. Biol. Chem.* 267:16330-16334.

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A., and Ruddle, F.H. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. U.S.A.* 77:7380-7384.

Gossen, M. and Bujard, H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* 89:5547-5551.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., and Rajewsky, K. 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* 265:103-106.

Huang, M.T. and Gorman, C.M. 1990. Intervening sequences increase efficiency of RNA 3′ processing and accumulation of cytoplasmic RNA. *Nucleic Acids Res.* 18:937-947.

Kozak, M. 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 15:8125-8148.

Kühn, R., Schwenk, F., Aguet, M., and Rajewsky, K. 1995. Inducible gene targeting in mice. *Science* 269:1427-1429.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868-872.

Mayo, K.E., Warren, R., and Palmiter, R.D. 1982. The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. *Cell* 29:99-108.

Rivella, S., Callegari, J.A., May, C., Tan, C.W., and Sadelain, M. 2000. The cHS4 insulator increases the probability of retroviral expression at random chromosomal integration sites. *J. Virol.* 74:4679-4687.

Rossert, J., Eberspaecher, H., and de Crombrugghe, B. 1995. Separate *cis*-acting DNA elements of the mouse pro-alpha 1(I) collagen promoter direct expression of reporter genes to different type I collagen-producing cells in transgenic mice. *J. Cell Biol.* 129:1421-1432.

Sauer, B. and Henderson, N. 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. U.S.A.* 85:5166-5170.

Sheets, M.D., Stephenson, P., and Wickens, M.P. 1987. Products of in vitro cleavage and polyadenylation of simian virus 40 late pre-mRNAs. *Mol. Cell Biol.* 7:1518-1529.

Sumarsono, S.H., Wilson, T.J., Tymms, M.J., Venter, D.J., Corrick, C.M., Kola, R., Lahoud, M.H., Papas, T.S., Seth, A., and Kola, I. 1996. Down's syndrome-like skeletal abnormalities in Ets2 transgenic mice. *Nature* 379:534-537.

Szulc, J., Wiznerowicz, M., Sauvain, M.O., Trono, D., and Aebischer, P. 2006. A versatile tool for conditional gene expression and knockdown. *Nat. Methods* 3:109-116.

Tanaka, T., Veeranna, O., Ohshima, T., Rajan, P., Amin, N.D., Cho, A., Sreenath, T., Pant, H.C., Brady, R.O., and Kulkarni, A.B. 2001. Neuronal cyclin-dependent kinase 5 activity is critical for survival. *J. Neurosci.* 21:550-558.

KEY REFERENCES

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. 2003. Production of transgenic mouse. *In* Manipulating the Mouse Embryo: A Laboratory Manual, 3rd ed. pp. 289-358. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

This book is one of the most read laboratory manuals for the production of transgenic mice.

Voncken, J.W. 2003. Genetic modification of the mouse: General technology; pronuclear and blastocyst injection. *In* Transgenic Mouse: Methods and Protocols (M.H. Hofker and J. van Deursen, eds.) pp. 9-34. Humana Press, Totowa, N. J.

General mouse technologies including basic husbandry, and microinjections are very well described.

INTERNET RESOURCES

http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed

PubMed is the world's largest literature database. Search using keywords "transgenic," "mouse," and the tissue or cell type of interest to find out the preferable promoters.

http://www.mshri.on.ca/nagy/

Nagy Lab Cre and Flox mouse database.

http://www.informatics.jax.org/imsr/index.jsp

International Mouse Strain Resource.

http://www.informatics.jax.org/

Mouse genome informatics.

http://www.jax.org/

These Jackson Laboratory website provide links to a variety of mouse-related information, such as mutant resources and literature pertaining to mouse genetics.

Generation of Transgenic Mice

Andrew Cho,[1] Naoto Haruyama,[1] and Ashok B. Kulkarni[1]

[1]National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland

ABSTRACT

This unit describes detailed step-by-step protocols, reagents, and equipment required for successful generation of transgenic mice using pronuclear injection. The experimental methods and practical tips given here will help guide beginners in understanding what is required and what to avoid in these standard protocols for efficiently generating transgenic mice. *Curr. Protoc. Cell Biol.* 42:19.11.1-19.11.22. © 2009 by John Wiley & Sons, Inc.

Keywords: DNA purification • genotyping • embryo harvesting • embryo implantation • transgenic mice • zygote

INTRODUCTION

The technology for gene transfer into the early developing embryos has enabled researchers to make animal models to study human development, diseases, and disorders. We can now generate transgenic, knockout, conditional-knockout, or knock-in mice to elucidate the precise functions of candidate genes implicated in development, disease, and disorders. Transgenic mouse models are now considered ideal tools to delineate the molecular mechanisms of the gene products and their interactions with one another that influence all cellular processes that form the basis of physiological systems. The technique for generating the genetically altered animal models has been perfected over the years, and it is now used widely in many laboratories.

This unit describes the pronuclear-microinjection technique in detail to successfully generate transgenic mice, which represents a gain-of-function approach. In short, generating transgenic mice involves five basic steps: purification of transgenic construct (Basic Protocol 1), harvesting donor zygotes (Basic Protocol 2), microinjection of transgenic construct (Basic Protocol 3), implantation of microinjected zygotes into the pseudopregnant recipient mice (Basic Protocol 4), and genotyping and analysis of transgene expression in founder mice (Basic Protocol 5). The timeline for generating transgenic mice is shown in Table 19.10.1.

NOTE: Mice to be used for generating transgenic mice should be purchased from approved animal vendors that provide healthy and pathogen-free mice suitable for research. The number of mice and methods for housing, breeding, and manipulating the mice for the purpose of generating transgenic mice must be approved by the appropriate Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations.

PURIFICATION OF TRANSGENIC CONSTRUCT USING A SUCROSE GRADIENT

BASIC PROTOCOL 1

To generate transgenic mice successfully, every step is critical, from designing the transgenic construct to embryo transfer. Preparing a clean DNA sample is a vital step because it affects the health of the embryo and the DNA integration efficiency. Only a DNA fragment without any trace of vector sequence should be microinjected. The vector sequence, as well as any chemical residues remaining in the final DNA solution are generally toxic to mouse zygotes and will result in death of the embryo or poor efficiency in generating

Current Protocols in Cell Biology 19.11.1-19.11.22, March 2009
Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1911s42

transgenic mice. The final purified DNA should be dissolved in a microinjection buffer and should be centrifuged to remove any impure particles in the solution just before performing microinjection. In the case of a larger DNA construct such as bacterial artificial chromosomes (BAC) DNA, it should be dissolved in a specialized buffer. We routinely use a sucrose gradient or a gel-purification method (Alternate Protocol) to purify the DNA.

Materials

Plasmid DNA of interest, digested to remove plasmid backbone
TE buffer, pH 7.4 (KD medical)
10% (w/v) and 40% (w/v) sucrose solutions (see recipes)
1.2% agarose gel
Microinjection buffer *or* BAC DNA buffer (see recipes)

Sucrose gradient DNA purification apparatus (Fig. 19.11.1) containing:
  Custom-made sucrose mixer
  Stirrer/hot plate
  Glass microcapillary (Kimble Glass)
  Rubber tubing
  Magnetic bar
  14 × 95–mm centrifuge tubes (Beckman)
Ultracentrifuge (e.g., Beckman Coulter, model L-70)
Swinging-bucket rotors (e.g., Beckman Coulter, model SW 40 Ti)
1.7-ml microcentrifuge tubes
Microcapillary pipet (Kimble Glass, 20-μl)
Dialysis cassette (Pierce, 10,000 MWCO)

Additional reagents and equipment for checking the DNA concentration using a spectrophotometer (*APPENDIX 3D*)

Prepare the gradients

1. Start out with the plasmid digest containing the transgene fragment and the vector backbone at a concentration of ~50 μg in 500 μl TE buffer.
2. Prepare 10% (w/v) and 40% (w/v) sucrose solutions. Dispense 6.5 ml of 10% sucrose into chamber 1 (containing a small magnetic stirring bar) and 6.5 ml of 40% sucrose into chamber 2 of the custom-made sucrose gradient mixer shown in Figure 19.11.1.
3. Prepare a 10% to 40% sucrose gradient from top to bottom of the 14 × 95–mm ultracentrifuge tube by gently mixing the two sucrose concentrations by opening two pigots, and gently spinning a small magnetic bar in chamber 1 using a stirring plate.

Separate transgene and vector

4. After the ultracentrifuge tube is filled up to the top with the gradient, slowly discard 500 μl of the sucrose gradient from the top, and load 250 μl of the digested plasmid DNA to the top of the gradient.
5. Separate the transgene fragment from the vector backbone by ultracentrifuging at 16 hr at ~160,000 × *g* (30,000 rpm in a Beckman Coulter, SW 40 Ti rotor), 4°C in the swinging-bucket rotor.

Figure 19.11.1 *(at right)* Purification of a transgenic construct using the sucrose gradient method. (**A**) Sucrose mixer with dimensions of 3-in. (W) × 2-in. (H) × 1 7/8-in. (D) is made by making two chambers of 5/8-in. (D) × 2-in. (H), which are connected together on the bottom with an outlet and two spigots that open and close controlling the sucrose flow into the ultracentrifuge tube. (**B, C**). A picture and schematic drawing showing how gradients are made from 10% to 40% sucrose.

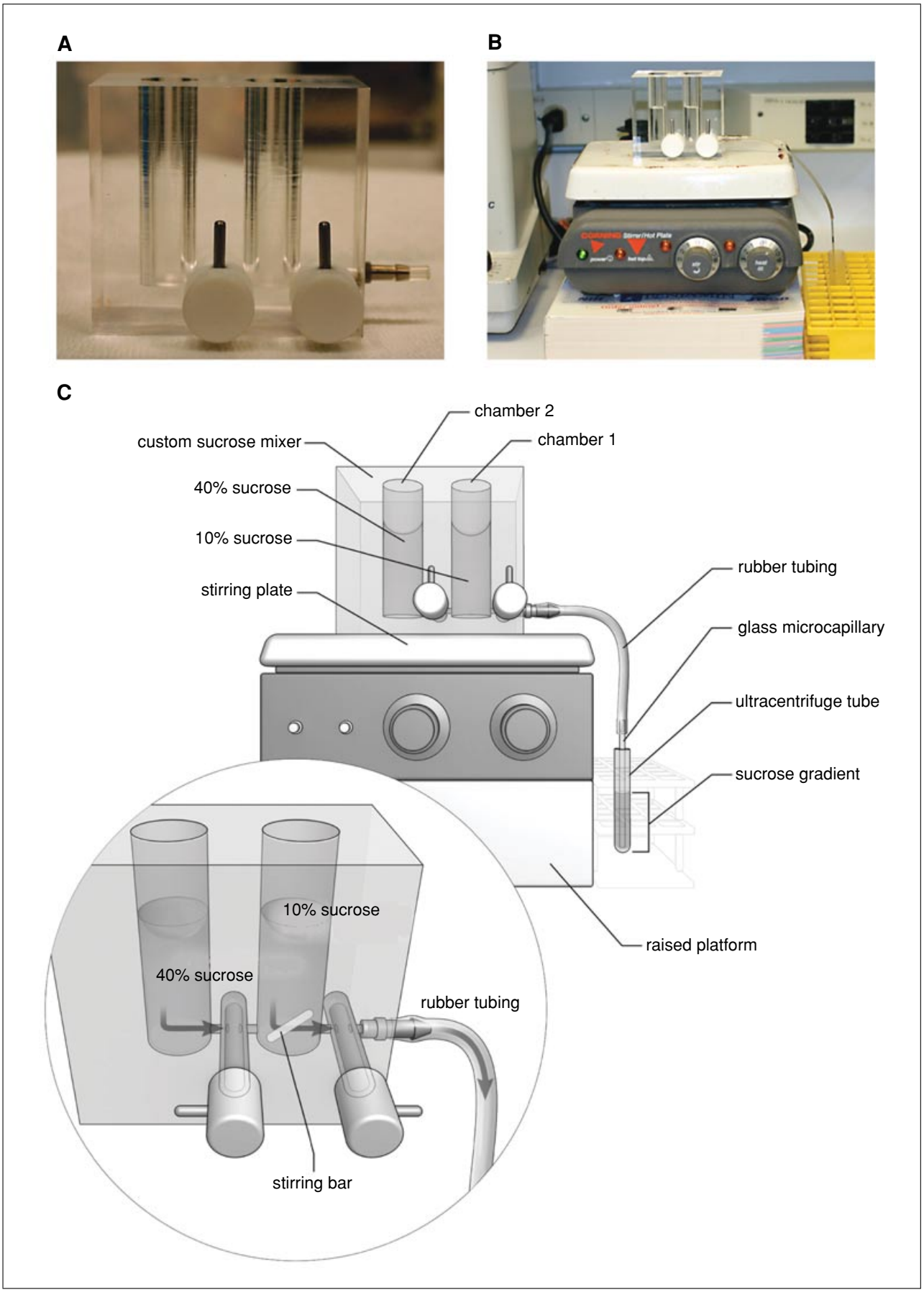


Figure 19.11.1 *(legend at left)*

6. Serially elute the gradient by collecting ~200 µl fractions in 1.7-ml microcentrifuge tubes through a hole in the bottom of the centrifuge tube.

7. Load 20 µl of the eluted fractions onto a 1.2% agarose gel in 1× TE buffer to determine which fractions contain only the transgene fragment (and not the vector DNA).

   The size of the transgene should be sufficiently different from the vector for better separation by ultracentrifugation and subsequent check by gel electrophoresis. Pooled fractions to be used for the next step should contain only the transgene DNA fragment.

Dialyze the transgene DNA

8. Pool the transgene-containing fractions from the microcentrifuge tubes containing the transgene fragments and dialyze against the microinjection buffer according to the manufacturer's instruction. Dialyze three more times against the fresh microinjection buffer to purify the DNA.

9. Centrifuge the dialyzed solution 2 min at 16,000 × *g*, room temperature, to remove any particulate impurities. Check the DNA concentration using a spectrophotometer (*APPENDIX 3D*).

10. Based on the accurate DNA concentration, carefully dilute the DNA to 2 ng/µl with the microinjection buffer.

    The dialyzed DNA can be stored up to 1 week at 4°C or in −20°C freezer for a longer period.

ALTERNATE PROTOCOL

PURIFICATION OF TRANSGENIC CONSTRUCT USING A GEL-BASED DNA PURIFICATION METHOD

We routinely use either of two methods to purify DNA (sucrose gradient or a gel-purification method). Both methods have merits of their own in that the sucrose gradient method yields the purest DNA for microinjection, whereas the gel purification method is a quick and easy method to yield adequately clean DNA for microinjection.

***Additional Materials** (also see Basic Protocol 1)*

Transgene DNA of interest, digested to remove plasmid backbone (see Basic Protocol 1)
QIAEX II Gel Extraction Kit (QIAGEN, cat. no. 20051) including the following components:
Buffer QX1
QIAEX II DNA binding particles
Buffer PE

Razor blade
50°C water bath
Vortex

Gel-purify the construct

1. Run the digested plasmid DNA transgenic construct (see Basic Protocol 1) on a 1.2% agarose gel in 1× TE buffer.

2. Excise the transgene DNA band from the agarose gel with a clean razor blade.

   You can visualize the DNA bands under low-intensity UV light to minimize the damage to the DNA.

Extract the DNA

3. Weigh the gel slice and add 3 vol of buffer QX1 to 1 vol of gel for a DNA fragment between 100-bp and 4-kb in size. If the DNA fragment is >4 kb add 3 vol of buffer QX1 plus 2 vol of dH_2O to 1 vol of gel.

Purify the DNA

4. Resuspend the QIAEX II DNA binding particles, add the binding particles to the sample in step 3, and mix gently. If the amount of DNA is <2 μg add 10 μl of QIAEX II or add 30 μl if the DNA amount is between 2 and 10 μg. Add an additional 30 μl for each additional 10 μg of DNA.

5. Incubate 10 min at 50°C in a water bath to solubilize the agarose gel and allow QIAEX II particles to bind to the DNA. Mix the tubes by vortexing every 2 min to maintain QIAEX II in suspension.

6. Centrifuge the sample 30 sec at 16,000 × *g*, room temperature and carefully discard the supernatant.

Wash the construct-containing pellet

7. Wash the pellet by adding 500 μl of Buffer QX1, vortexing, centrifuging 30 sec at 16,000 × *g*, room temperature and discarding the supernatant.

8. Wash the pellet twice, each time by adding 500 μl of Buffer PE, vortexing, centrifuging 30 sec at 16,000 × *g*, room temperature, and discarding the supernatant.

9. Air dry the pellet for 10 to 15 min.

Elute the DNA

10. Elute the DNA from the pellet by adding 20 μl of microinjection buffer followed by vortexing. Incubate the mixture 5 min at room temperature for a DNA amount of <4 kb size, or incubate 5 min at 50°C for DNA size between 4 and 10 kb, or 10 min at 50°C if >10 kb.

11. Centrifuge the mixture 30 sec at 16,000 × *g*, room temperature and carefully pipet out the supernatant containing the purified DNA into a 1.7-ml clean tube.

12. With the remaining material, repeat steps 10 and 11 to increase the DNA yield and combine the eluted fractions.

13. Dissolve the DNA pellet in 100 μl of microinjection buffer.

14. Centrifuge the purified DNA solution 2 min at 16,000 × *g*, room temperature to remove all the traces of impurities that might still be present in the final DNA solution. Carefully pipet out the supernatant into another clean tube.

Prepare construct DNA for microinjection

15. Measure the DNA concentration (*APPENDIX 3D*) and dilute the DNA to 2 ng/μl in microinjection buffer or to 1 ng/μl in a BAC buffer if transgene is BAC DNA.

    The methods for preparing BAC DNA suitable for microinjection are detailed in Conner (2004).

HARVESTING DONOR ZYGOTES (FERTILIZED EGGS) FOR MICROINJECTION

BASIC PROTOCOL 2

To obtain the maximum number of donor zygotes to use for microinjection to generate a large number of transgenic founders with minimal effort and cost, all egg-donor female mice need to be superovulated by injecting PMSG (pregnant mare's serum gonadotropin) and HCG (human chorionic gonadotropin) prior to mating them with the stud mice. We routinely harvest ~250 zygotes from 15 superovulated and plugged female mice. The donor mouse strain we typically use is FVB/N, which is one of the most widely used inbred mouse strains because its embryos have a relatively large pronucleus and are robust enough to withstand the microinjection procedure.

Materials

PMSG (pregnant mare's serum gonadotropin; Sigma Chemical Inc., cat. no. G4877-2000IU)
20 donor female mice (4- to 5-week-old FVB/N mice)
HCG (human chorionic gonadotropin; Sigma Chemical Inc., cat. no. CG10-1VL)
20 stud male mice (sexually mature male, 2- to 12-month-old, FVB/N mice)
M16 medium (Sigma Chemical, cat. no. M7292)
M2 medium (Sigma Chemical, cat. no. M7167)
Hyaluronidase (Sigma Chemical, cat. no. H3884-1G)

35 × 10–mm tissue culture dish (Falcon, cat. no. 353001)
60 × 10–mm tissue culture dish (Falcon, cat. no. 353002)
Dissecting microscope for handling zygotes (Zeiss, Stemi SV II Apo)
Light source for dissecting microscope (Zeiss, KL 1500 LCD)
Surgical scissors (Roboz, RS-5840)
Microdissection forceps (Roboz, RS-5055)
Mouth-controlled pipet assembly (Fig. 19.11.2) including the following items:
Plastic mouth piece
Portion of a 1-ml pipet that includes cotton piece
Rubber tubing, 1/8-in. i.d., 1/32-in. wall thickness (Thomas Scientific, cat. no. 9521R67)
Glass tubing to make embryo-handling pipets (Drummond Scientific, cat. no. 9-000-1181)
Glass tubing with a rubber cap (Drummond Scientific, cat. no. 1-000-0300)
Bunsen burner
CO_2 incubator (Thermo Electron Corporation, Model 370)

Additional reagents and equipment for peritoneal injections (Donovan and Brown, 2006a) and euthanasia of mice (Donovan and Brown, 2006b)

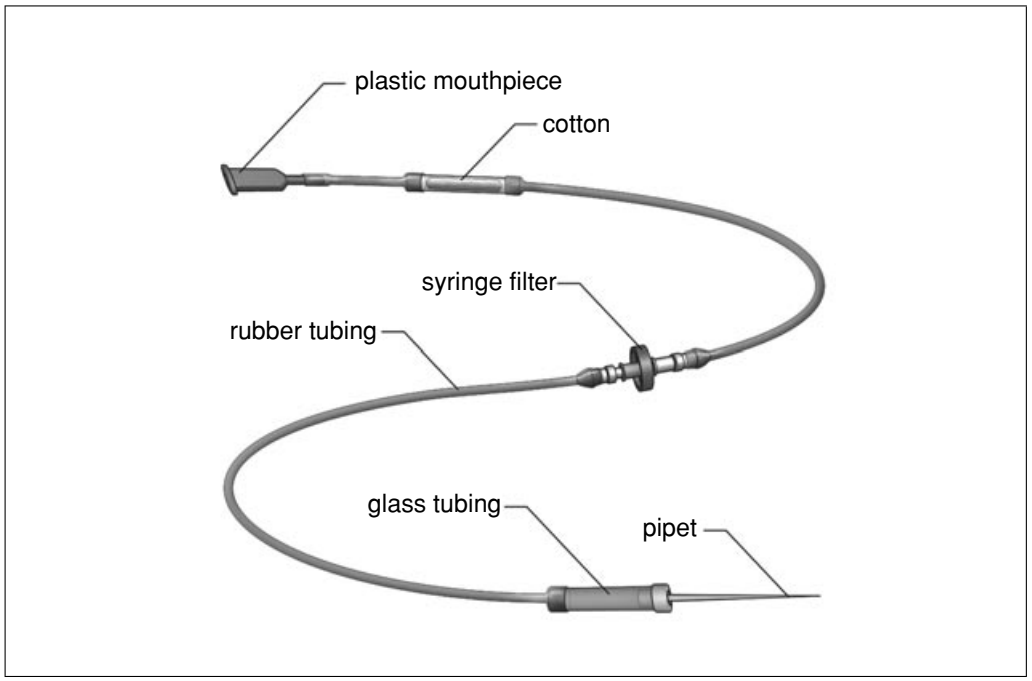


Figure 19.11.2 Schematic diagram of the components necessary for preparing a mouth-controlled pipet device for handling zygotes. Rubber tubing with 1/8-in. i.d. and 1/32-in. wall is cut in three pieces, and the mouth piece, 0.2-μm syringe filter unit, 1-ml pipet cotton piece, and glass tubing are attached to each end of rubber tubing.

Prepare donor females

1. To obtain the maximum number of zygotes for microinjection, superovulate the donor mice by administering an intraperitoneal (i.p.) injection (Donovan and Brown, 2006a) of 5 U of PMSG per mouse at 2:00 PM 2 days prior to HCG injections and mice matings.
2. Inject each mouse intraperitoneally (Donovan and Brown, 2006a) with 5 U of HCG at 1:00 PM 2 days after PMSG injections.
3. Following the HCG injection, set up mating pairs consisting of one superovulated female and one stud male per cage.

   Mating one female to one male mouse will enhance the plugging performance.

   Set up mating at late afternoon so that the mice will mate during the dark cycle.
4. Check the donor female mice to see if mice have mated overnight by checking for the presence of vaginal plugs the next morning, preferably before 9 AM.

   To get fresh fertilized eggs, harvest them from the plugged female mice before noon as described below.

Isolate the ovary and oviduct

5. Prepare two 35 × 10–mm dishes of M16 medium, by transferring 200 µl to the center of the dish and cover the medium with 5 ml of mineral oil. Then, prepare three 35 × 10–mm dishes with 5 ml of M2 medium and one 35 × 10–mm dish of M2 medium containing 1.5 mg of hyaluronidase in 5 ml of M2 medium, as shown in Figure 19.11.3A.

   Carry two M16 dishes in a 60 × 10–mm dish for easy transportation and incubate both dishes containing M16 medium at 37°C in the 5% CO_2 incubator.
6. Kill the plugged female mouse by cervical dislocation (Donovan and Brown, 2006b), put it on its back, and using a pair of surgical scissors and microdissection forceps open the body cavity (as shown in Fig. 19.11.3B).
7. Locate and harvest the entire ovary and the oviduct by cutting between the upper part of the uterus and the fat body. Transfer the oviduct and ovary to one of the 35 × 10–mm dishes containing M2 medium, as shown in Figure 19.11.3 panels C and D.

Free and wash the zygotes

8. To release the zygotes from each oviduct, transfer one dissected tissue at a time to the dish containing M2 medium with hyaluronidase. Locate the ampulla (swollen area of the oviduct) and carefully tear open the corner of the ampulla using microdissection forceps to release the eggs into the medium, as shown in Figure 19.11.3 panels E and F.
9. Let the eggs separate from the surrounding cumulus cells in the M2/hyaluronidase solution, as shown in Figure 19.11.3G.

   By the time all ampullas are torn open, the zygotes will be separated from the cumulous cells and should be ready for transfer to the next petri dish.
10. Pick up each zygote that is separated from cumulus cells using the mouth-controlled pipet assembly and transfer it to fresh M2 medium for washing.

    To make good embryo handling pipets, flame the middle part of the glass tubing over the Bunsen burner to melt it, and when it gets red hot, pull from each end to make a pipet with a long, thin shaft. Then, cut the stretched middle part with a diamond pencil to make two embryo handling pipets with blunt ends.
11. Wash embryos at least four times by picking them up and transferring them to different drops of M2 medium. At this stage, remove any unfertilized or abnormal eggs.

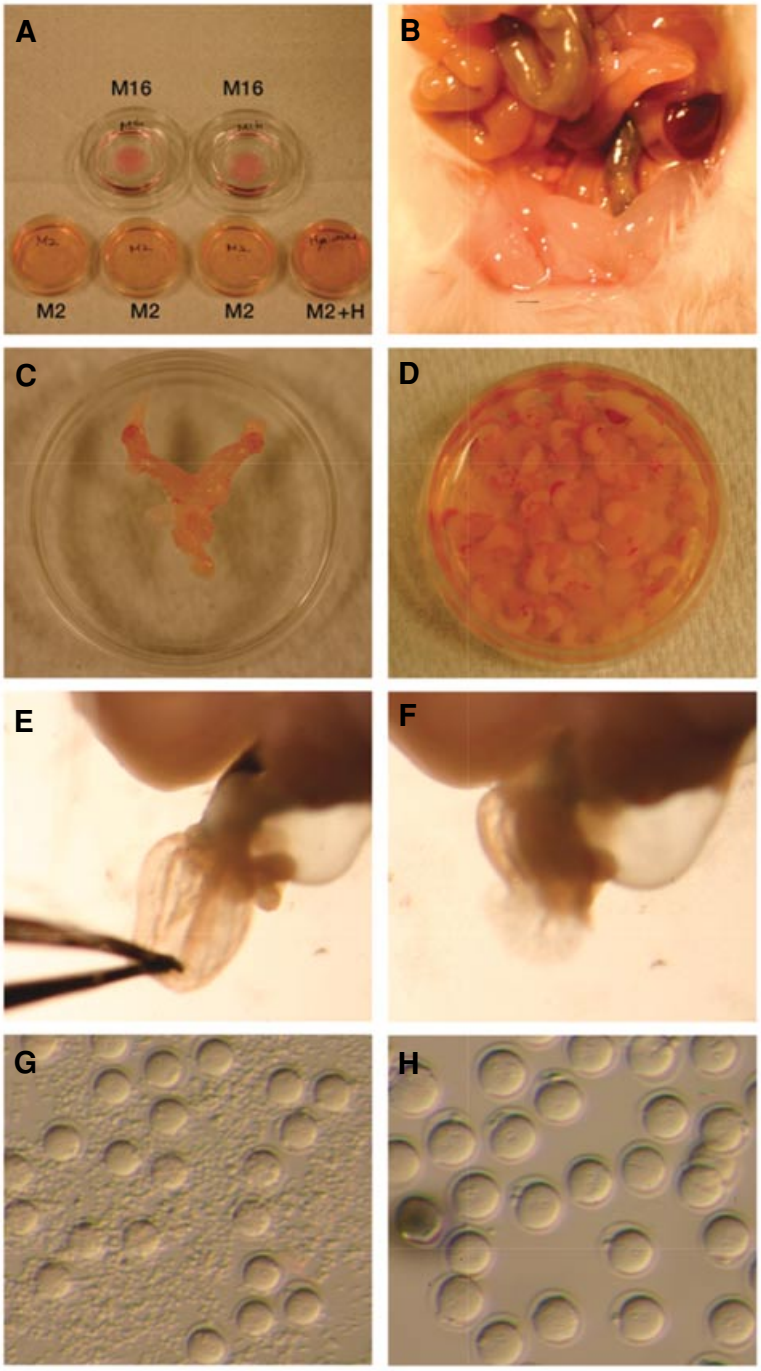


Figure 19.11.3 Sequence of embryo harvesting: (**A**) Two M16, three M2, and one M2 medium with hyaluronidase plates are prepared for handling or culturing embryos throughout the whole process of transgenic mice generation. (**B, C**) The reproductive organs: uterus, ovary, and oviduct are removed from the donor mice. (**D**) All the oviducts are collected in a single M2 dish. (**E**) The ampulla is located and torn apart with forceps under the microscope. (**F**) The zygotes and the cumulus cells are spilled into the M2 medium with hyaluronidase. (**G**) The zygotes are slowly separated from the surrounding cumulus cells. (**H**) Thoroughly washed zygotes are ready for microinjection.

12. Transfer washed eggs into one of the two M16 culture medium dishes that have been pre-incubated at 37°C in the 5% CO_2 incubator. Put the dish back into the incubator until ready for the microinjection procedure.

The second M16 culture medium dish will be used later for eggs after they are microinjected with DNA.

MICROINJECTION OF THE TRANSGENE DNA CONSTRUCT INTO MOUSE ZYGOTE

BASIC PROTOCOL 3

To ensure a successful transfer of foreign DNA into mouse zygotes, and to increase the chance of integration into the host genome, a number of parameters have to be carefully evaluated and applied when performing microinjection. First, the purified DNA should be diluted to obtain an optimal DNA concentration. We use a DNA concentration of 2 ng/μl for the microinjection. A DNA concentration that is too high may cause zygotes to lyse, and a DNA concentration that is too low may yield poor or no integration of DNA. In our experience, a DNA concentration of 2 ng/μl gives the best transgenic efficiency. Second, the injection pressure with which the DNA solution is injected into the pronucleus should be low enough not to damage the DNA. We use a constant flow rate of 50 hPa (hectopascal) to deliver the DNA using an automated microinjector. There are many advantages in using the constant-flow function. It ensures that every zygote gets the same amount of DNA solution as long as the injection time is equal for each zygote. It also helps the operator because there is no need to push the injecting button every single time for each zygote. Third, the manner in which the injection is performed is very important. Gentle yet swift injections of DNA minimize the trauma to the zygotes. This is done first by carefully breaking the tip of the needle against the holding pipet in order to create ideal DNA flow out of the injection needle. Puncture the zygote and pronucleus membranes as quickly as possible to avoid injecting DNA into the cytoplasm. Lastly, the timing of the microinjection should be strictly followed since the optimal window for DNA integration into the mouse genome is narrow and easy to miss if you start the procedure late. Our injection time window is between 11:00 AM and 2:00 PM (about 11 to 13 hr post-conception) to give the pronuclei of microinjected zygotes plenty of time to prepare to fuse together to initiate the first round of cell division. Injections done later than 2:00 PM may yield no integration, or may result in the generation of mosaic mice, in which only certain cell populations will carry the transgene.

Materials

M2 medium (Sigma Chemical, cat. no. M7167)
Mineral oil (Sigma Chemical, cat. no. M3516-1L)
Washed, ready-to-inject zygotes (Basic Protocol 2)
M16 medium (Sigma Chemical, cat. no. M7292)

Microforge (Technical Products International Corporation, MF-1)
Microinjection system including the following components (Fig. 19.11.6 A):
  Inverted microscope (Zeiss, Axiovert 135M)
  Anti-vibration table (Kinetic systems)
  Automated DNA injector (Eppendorf, Femptojet)
  Capillary holder for holding pipet (Mitutoyo)
  Micromanipulator (Narishige, model MO-202U)
  Compressed Nitrogen gas cylinder
Needle/pipet puller (Kopf Instruments, model 720)
Glass tubing for microinjection needles (Sutter Instrument, cat. no. BF100-78-10)
Microloaders (Eppendorf, cat. no. 930001007)
One-chambered plastic slide to be used for the microinjection platform (Nalge Nunc International, cat. no. 177372)
Mouth-controlled pipet assembly for handling zygotes (Fig.19.11.2)
CO_2 incubator (Thermo Electron Corporation, model 370)

Prepare for microinjection

1. Make a holding pipet using the microforge as shown and described in Figure 19.11.4. Insert a holding pipet into the capillary holder on the left side of the microscope. Connect this holder to the oil-based hydraulic system.

   To make good holding pipets, bring the hand-pulled pipet close to the heated glass ball until you get a diameter on the open end that is ~1/3 of the diameter of an embryo. The opening should have a smooth edge.

2. Make injection needles using a vertical pipet puller (as shown in Figure 19.11.5) at a setting of 16 (heating filament intensity) × 2.5 (force of pulling down), and insert it into the capillary holder on the right side of the microscope. Connect this holder to the automatic microinjector.

   Making successful injection needles can be achieved using the pipet puller. The setting mentioned above gives us good sharp needles with long shafts to minimize the damage to the embryo during the microinjection process.

3. Load the DNA solution in the back of the injection needle using a microloader pipet.

   To ensure a clean DNA solution free of debris for microinjection, first microcentrifuge the tube containing the DNA solution 2 min at maximum speed, room temperature to settle any suspended debris that might clog the injection needle. After that, check the DNA concentration (APPENDIX 3D) and dilute it to 2 ng/μl for the plasmid DNA constructs or 1 ng/μl for BAC DNA constructs.

   When handling BAC DNA, do not vortex at any time for it may shear the DNA.

Prepare the microinjection platform

4. Prepare the microinjection platform by putting a few drops of M2 medium in the middle of the one-chambered slide followed by overlaying the medium with mineral oil, as shown in Figure 19.11.6B.

5. Transfer forty zygotes to the upper portion of the M2 medium to be prepared for microinjection.

6. Transfer the slide carefully onto the microscope stage.

Microinject zygotes

7. Test the holding needle by holding and releasing one zygote.

   Make sure that not too much oil is flowing out of the holding pipet onto M2 medium. When lowering the holding pipet onto the glass slide, first go to the mineral oil field and control the oil flow, then go into M2 medium. Once the holding pipet is inside the M2 medium, draw up a little bit of the medium into the pipet before attempting to hold the zygote.

8. Set the automatic microinjector to continuous flow mode at a pressure of 50 hPa, and lower the injection needle into the M2 medium on the slide. Inject the DNA into the male pronuclei inside the zygote, as shown in Figures 19.11.7 and 19.11.8.

   Prior to microinjection, carefully break the tip of the injection needle against the holding pipet to obtain a good DNA flow. Inject the first egg and watch how fast the pronucleus expands to determine the condition of the injection needle. The pronucleus should expand noticeably if the needle is good. If the pronucleus swells too fast and bursts open, the injection needle is too big and it should be changed to a new one immediately. Also, when microinjecting, be sure to puncture the pronucleus as quickly as possible to avoid injecting DNA into the cytoplasm.

   Inject one batch of eggs (~40 eggs) at a time to avoid keeping zygotes outside the incubator for too long.

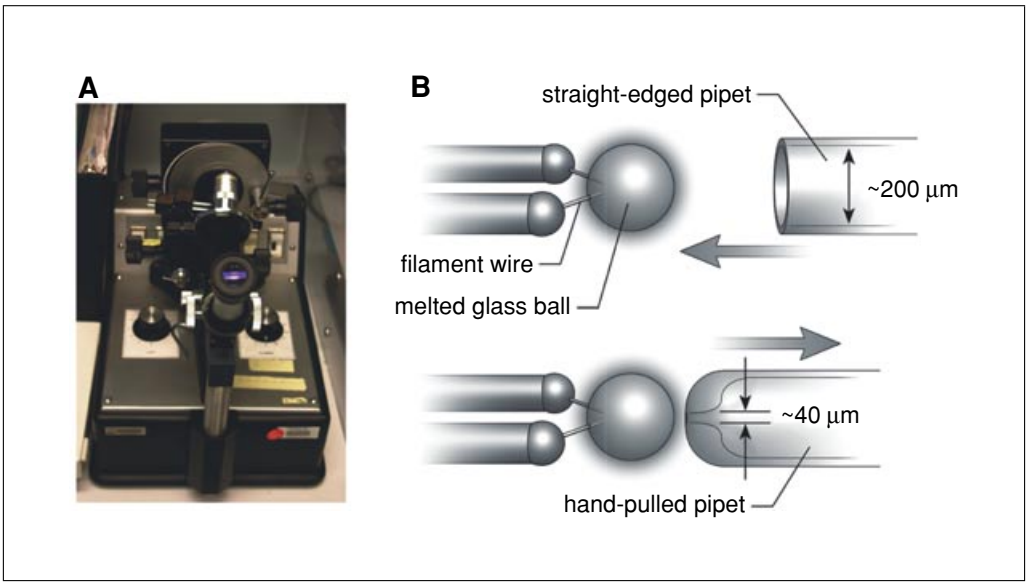


Figure 19.11.4 Making of a holding pipet. (**A**) Microforge. (**B**) The schematic diagram of preparing holding pipets. The hand-pulled and diamond pencil-cut straight-edged pipet is selected and is brought closer to the heated glass ball to melt evenly to give a polished holding pipet with a small opening of ~20 μm.

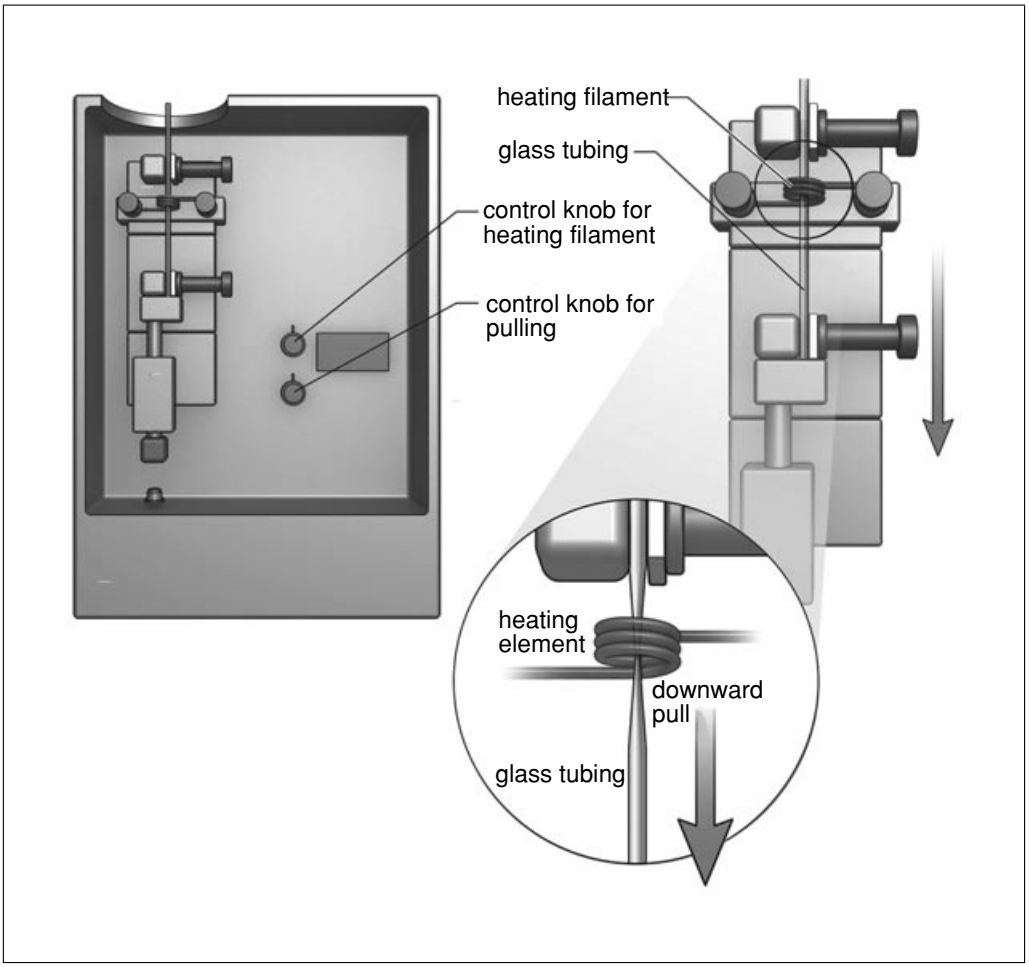


Figure 19.11.5 A schematic diagram for preparing injection needles. A glass tubing of 1.0-mm o.d. × 0.78-mm i.d. is inserted between two rubber pads and is pulled downward with the setting of 2.5 (downward force) × 16 (heat intensity) to prepare excellent and reproducible injection needles.

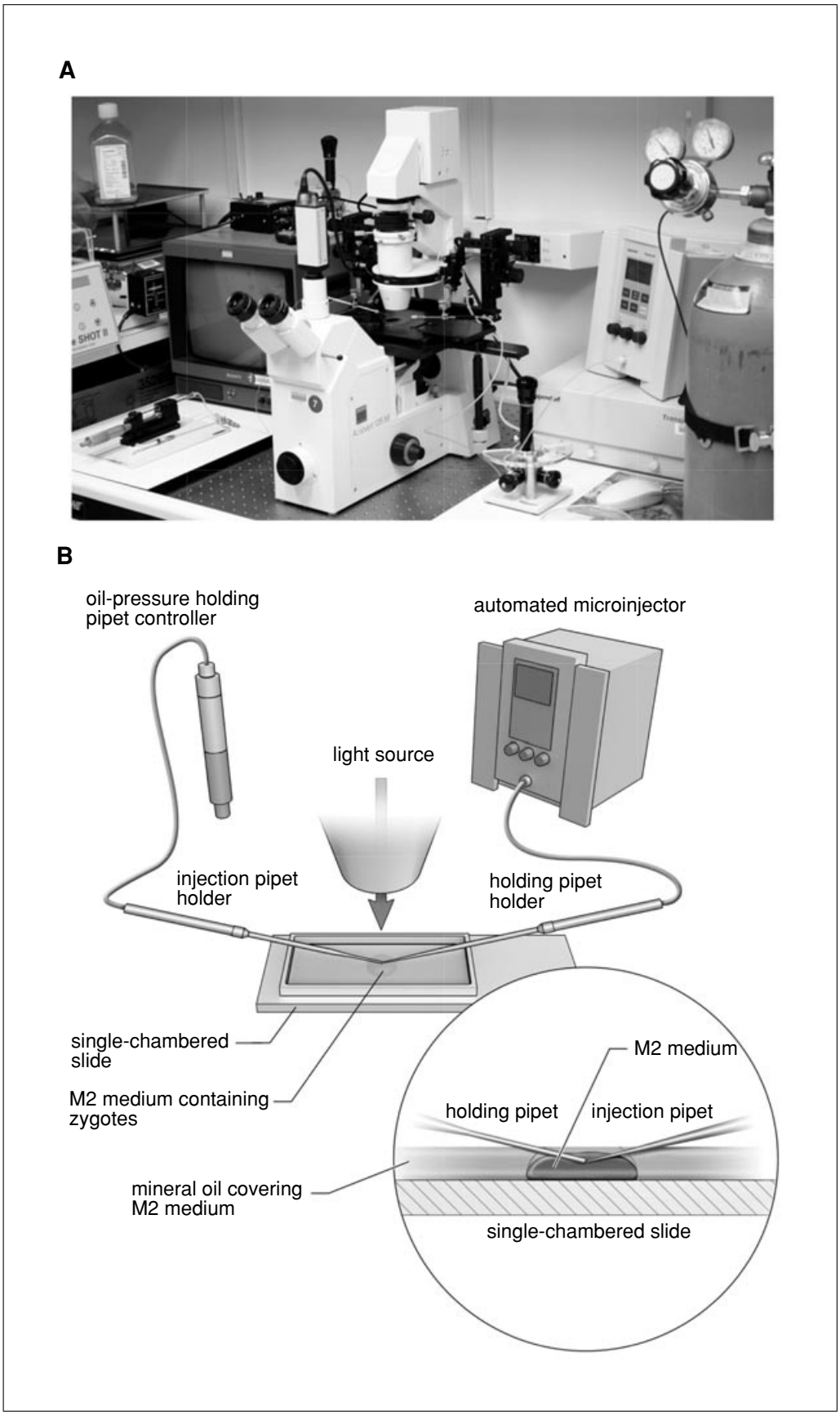


Figure 19.11.6 Microinjection setup. (**A**) A microinjection system. (**B**) Schematic diagram of the microinjection setup. A holding pipet is attached to the oil-pressured controller on the left side of the microscope whereas the injection pipet (needle) is attached to the automatic microinjector on the right side.

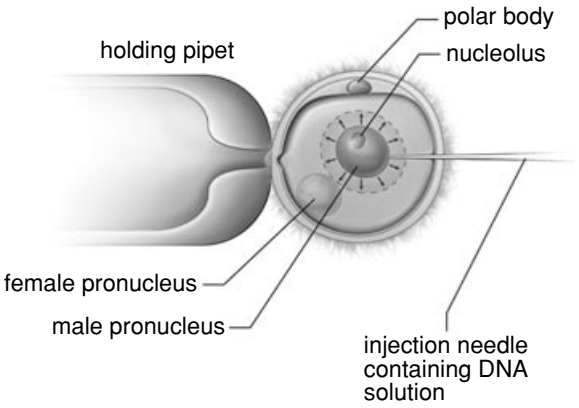


Figure 19.11.7 A schematic diagram depicting microinjection of transgenic DNA into the pronucleus. The size of the microinjected male pronucleus expands as the DNA is injected. A quick horizontal injection maneuver while minimizing damage to the zygote is critical for successful gene delivery.

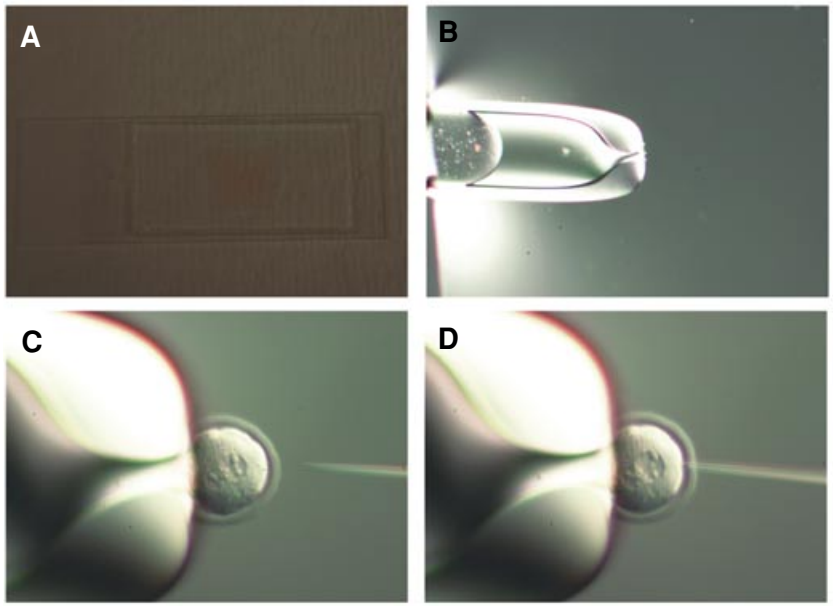


Figure 19.11.8 A sequence of events for the microinjection technique. (**A**) Injection platform for microinjection. Note the rectangular M2 medium in the center that is overlaid with mineral oil. (**B**) A polished holding pipet is lowered onto the injection platform and a small volume of M2 medium is sucked into the pipet. (**C**) A zygote is held in place by the holding pipet and the injection needle is lowered and the tip is focused. (**D**) DNA solution is injected into the male pronucleus. Note the swelling of the pronucleus, which confirms the successful transfer of the transgene.

9. To differentiate microinjected zygotes from uninjected ones during microinjection, after injecting each egg, move the microscope stage down and release the zygote on the bottom of the M2 medium.

10. Transfer all the microinjected zygotes into the one remaining, pre-incubated, fresh M16 culture medium dish. (See Basic Protocol 2, step 12.) Maintain the zygotes at 37°C in a 5% CO_2 incubator until ready for implantation into recipient mice.

IMPLANTATION OF MICROINJECTED ZYGOTES INTO PSEUDO-PREGNANT RECIPIENT MICE

The surgical steps required for embryo transfer need to be mastered first if one wants to be proficient in generating transgenic mice. The technique requires much patience and practice to become a skillful and efficient generator of the transgenic mice. Locating the infundibulum, the opening of an oviduct into which the microinjected zygotes need to be transferred, is hard for beginners, and inexperienced hands can cause massive bleeding problems around the surgical site. Steady hands are critical to avoid rupturing blood vessels and for successful embryo transfers. The embryo transfer can be performed on the same day that the embryos are harvested and injected or the next day after culturing zygotes to the two-cell stage overnight. In our experience, we found no difference between either the timing of the embryo transfer on the development of embryos or on the litter size.

Materials

Forty wild-type female mice (CB6F1, 6- to 8-weeks-old) to be mated with vasectomized mice to generate pseudo-pregnant recipient mice
Twenty vasectomized BALB/c mice (housed in separate cages)
M16 dish containing microinjected zygotes (see Basic Protocol 3)
70% ethanol
0.25% (v/v) Avertin for anesthesia
Alcohol swabs
Betadine antibiotic swabs
0.5% (v/v) Marcaine for analgesia

Animal cages
CO_2 incubator (Thermo Electron Corporation, model 370)
Glass bead sterilizer for the surgical instruments (Fine Science Tools, type 250)
Dissecting microscope for transferring zygotes (Zeiss, Zeiss Stemi SV II Apo)
Electric clippers
Surgical instruments (Sigma Surgical Instrument) including:
- Forceps
- Scissors
- Clamp skin closer with wound clips
- Sutures
- Curved forceps

Mouth-controlled pipet assembly (Fig. 19.11.2)
Inverted microscope to perform surgery (Zeiss, Stemi 2000)
Light source to illuminate the surgical area (Zeiss, KL 1500 LCD)
Slide warmer preheated to 37°C (Lab-line Instruments, model 26005)

Additional reagents and equipment for anesthetizing the mouse (Donovan and Brown, 2006c) and intraperitoneal injections (Donovan and Brown, 2006a)

Prepare pseudo-pregnant recipient mice

1. Set up twenty mating cages by putting two wild-type female mice to one vasectomized male mouse per cage 1 day before embryo surgery is to be performed.
2. On the day of the embryo transfer, check each female mouse for the presence of a vaginal plug.
3. Pool all the vaginal-plugged female mice to be used as pseudo-pregnant mice for embryo transfer surgery.

Collect materials for surgery

4. Transport the dish containing the microinjected zygotes (see Basic Protocol 3) to the embryo transfer room, if applicable.
5. Place the dish containing microinjected zygotes at 37°C, in a 5% CO_2 incubator of the embryo transfer room.
6. Sterilize the surgical area with 70% ethanol and sterilize all surgical tools using a glass bead sterilizer.
7. Anesthetize a recipient mouse (Donovan and Brown, 2006c) with a dose of 0.25% (v/v) Avertin (0.015 to 0.017 ml/g) into the peritoneal cavity (Donovan and Brown, 2006a; e.g., 0.3 ml for a 20-g mouse).

Load embryos

8. Take out the dish containing the microinjected eggs from the CO_2 incubator, and load the transfer needle with the following items using the sequence as shown in Figure 19.11.9: small amount of M16 medium, small air bubble, medium, air bubble, medium, air bubble, medium, air bubble, fifteen to twenty tightly packed zygotes (transferred using the mouth-controlled pipet). Place the mouth-controlled pipet around the neck of the microscope until ready to transfer.

   Transferring fifteen to twenty microinjected zygotes on each side of the mouse will allow for the optimum number of pups to be born.

Prepare the recipient mouse

9. Put the anesthetized recipient female mouse on its belly, and shave the mouse back from the hump to just above the legs on both sides with an electric clipper as shown in Figure 19.11.10A.
10. Clean the surgical sites of the mouse with alcohol and Betadine antiseptic swabs three times alternately.
11. Make a small incision of ~0.5 cm on the right side of the mouse skin above where the ovary is located, as shown in Figure 19.11.10B.
12. Locate the ovary beneath the transparent muscle membrane, make a small incision of ~0.2 cm on the muscle membrane, and pull out the ovary by grabbing the fat pad using a small surgical metal clip, as shown in Figure 19.11.10C.

    Make sure not to cut the big blood vessel that runs across the muscle membrane when making an incision.

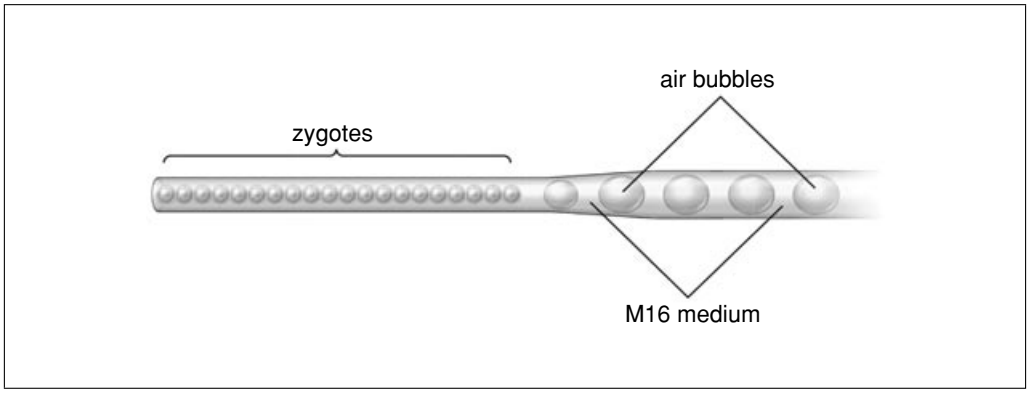


Figure 19.11.9 A schematic diagram of preparing transfer pipets for zygote transfers. A hand-pulled and diamond pencil-cut straight-edged pipet with ~100-μm opening size is first loaded with the five alternate sequence of M16 medium and small bubbles and followed by fifteen to twenty tightly packed zygotes.

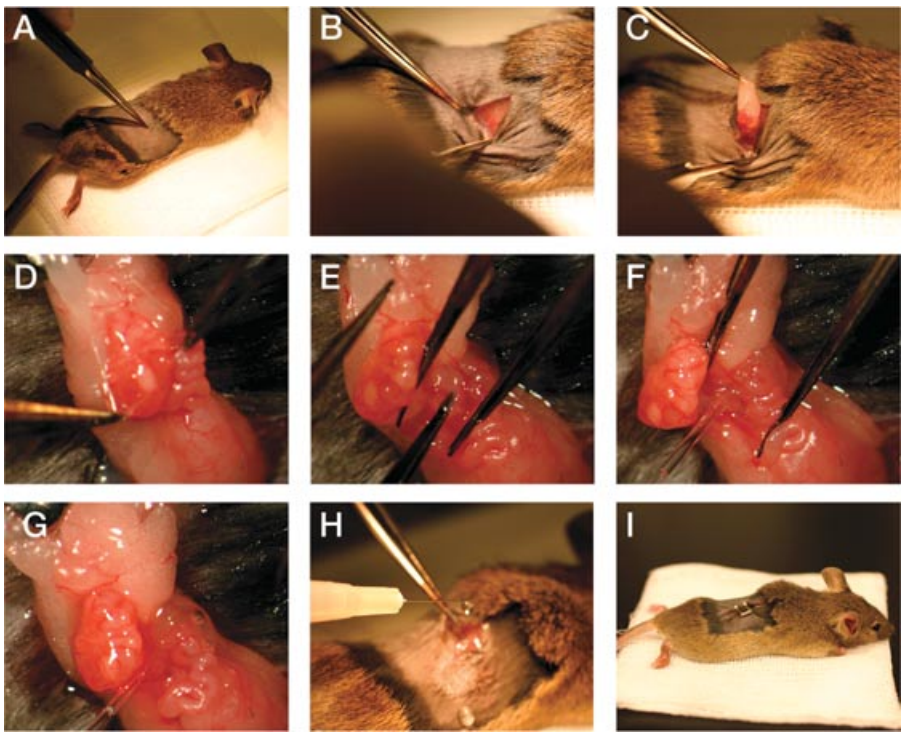


Figure 19.11.10 A sequence of events for zygote transfer into oviduct. (**A**) A surgical site is located and shaved. (**B**) A small skin incision is made and the ovary and fat pad are located before the muscle membrane incision is made. (**C**) The ovary and an oviduct are pulled out by the fat pad with the small metal clip. (**D**) The bursa is carefully peeled off and tucked under the ovary with a pair of forceps. (**E**) The space between the ovary and oviduct is opened with a pair of forceps while the tip of another forceps is inserted into the opening of the oviduct called the infundibulum for locating and testing prior to transferring zygotes with transfer pipet. (**F, G**) The transfer pipet is inserted into the infundibulum and the whole embryo content is blown slowly into the oviduct. (**H**) The muscle membrane is sutured and one drop of analgesia is administered to relieve pain during recovery. (**I**) the recipient mouse is placed on the slide warmer at 37°C.

13. Carefully tear open the thin, transparent membrane called bursa covering the ovary with fine forceps, and tuck it behind the ovary as, shown in Figure 19.11.10D.

    Again, when tearing open the membrane, avoid tearing the blood vessels.

14. Carefully open the space between the oviduct and the ovary using forceps, and locate the opening of the oviduct (infundibulum), as shown in Figure 19.11.10E.

    After locating the infundibulum, check it with the tip of the forceps to make sure it is the opening before inserting the transfer needles to transfer the zygotes.

Deposit the embryos

15. Using a mouth-controlled pipet, slowly blow in the fifteen to twenty microinjected zygotes into the infundibulum along with a few air bubbles to make sure the eggs are blown into the swollen area of the oviduct (ampulla), as shown in Figures 19.11.11 and 19.11.10F.

    After blowing in the zygotes, push two air bubbles trapped in the oviduct to the ampulla using the forceps to ensure all the eggs are pushed into the ampulla (as shown in Fig. 19.11.10G).

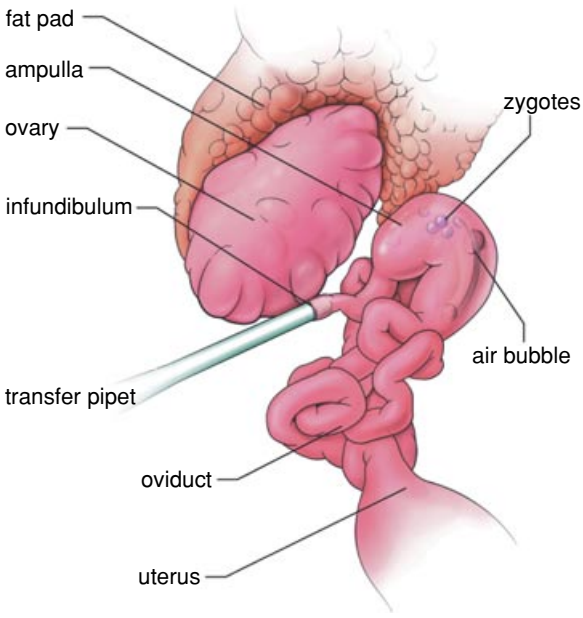


Figure 19.11.11 A schematic diagram depicting zygote transfer into the infundibulum, which is often hidden within coils of oviduct. The infundibulum can be made visible by peeling off the bursa membrane that covers the ovary and by separating the space between the ovary and the oviduct using fine forceps.

Complete the surgery

16. After the transfer is complete, carefully tuck the oviduct and ovary back into the body cavity.
17. Suture the muscle membrane and administer one drop of 0.5% (v/v) Marcaine on the sutured surgical site to relieve pain during recovery (as shown in Fig. 19.11.10H).
18. Staple the skin together using a wound clip.
19. Repeat the procedure for the other side (steps 11 to 18).
20. Place the animal on a slide warmer that has been preheated to 37°C for recovery, and wait for the mouse to move, which takes ~1 hr, before putting the mice into the new cage, as shown in Figure 19.11.10I.
21. Repeat this procedure for other recipient mice until you finish transferring all zygotes.
22. Put the cages into the mice rack in the animal room.

PREPARATION OF TISSUE SAMPLES FOR GENOTYPING FOUNDER MICE

BASIC PROTOCOL 5

After microinjection and embryo transfers, most recipient mice should be pregnant and deliver the pups after 20 days. Before pups are born, sometime around embryonic day 17, each pregnant mouse should be housed separately to avoid having multiple litters in the same cage. After delivery of pups, the pups should be monitored every day to see if they develop normally. Any pups that have retarded or abnormal growth should be noted. After 10 days from birth, a tissue biopsy, either a small piece of tail or a piece of ear, should be obtained for the purpose of genotyping to determine the transgenic founders. We snip ~0.50 cm from the tail to isolate DNA as described below, and we tattoo the mouse finger for identification by piercing it with a 30-G needle stained with tattoo paste.

Materials

Potential founder mice
Tail lysis buffer (see recipe)
Proteinase K
Phenol
Chloroform
100% and 70% ethanol
1× TE buffer

Microtattoo system (Fine Science Tools, cat. no. 24201-00)
Green tattoo paste (Fine Science Tools, cat. no. 24201-01)
30-G needle (Becton Dickinson, cat. no. 305106)
Labeled microcentrifuge tubes
Hybridization oven (Boekel, Little Shot II, Model 230501)

Additional reagents and equipment for tail DNA isolation for Southern blotting (Brown, 1993) and PCR (*APPENDIX 3F*)

Prepare the DNA

1. Snip from the potential founder mouse tail 0.5-cm length, tattoo the mouse finger for identification by piercing it with a 30-G needle stained with tattoo paste, and transfer it to labeled microcentrifuge tubes corresponding to the tattooed mouse finger number.
2. Add 0.5 ml of tail lysis buffer (excluding proteinase K) to each tube.
3. Add 10 μl of 10 mg/ml proteinase K to each tube.

Extract the DNA

4. Rotate the tubes in a hybridization oven overnight at 55°C.
5. Add 500 μl of phenol and 500 μl of chloroform to each tube.
6. Rotate the tubes in the rotating rack for 1 hr at room temperature.
7. Centrifuge the tubes 5 min at 16,000 × *g*, in the microcentrifuge at room temperature.
8. Carefully take out the supernatant and transfer it to the newly labeled tubes.

   Take out ~250 μl of the supernatant to avoid taking cell debris.

9. Add 700 μl of chloroform to each tube.
10. Rotate the tubes in the rotating rack for 1 hr at room temperature.
11. Centrifuge the tubes 5 min at 16,000 × *g*, in the microcentrifuge at room temperature.
12. Carefully take out the supernatant and transfer it to the newly labeled tubes.
13. Add 100% ethanol at 2.2× the amount of the supernatant in step 12 and mix tubes gently by hand to precipitate the DNA.
14. Centrifuge the tubes 30 sec at 16,000 × *g*, in the microcentrifuge at room temperature to pellet the DNA.

Wash the DNA

15. Add 600 ml 70% ethanol to wash the DNA.
16. Centrifuge the tubes 30 sec at 16,000 × *g*, in the microcentrifuge at room temperature to pellet the DNA.
17. Decant the 70% ethanol and air dry the DNA pellet for 1 hr.

18. Add 200 μl of 1× TE buffer to the DNA pellet to dissolve the DNA.

19. Check the DNA concentration for genotyping the mice by PCR (*APPENDIX 3F*) or Southern blot analysis (Brown, 1993).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BAC DNA buffer

10 mM Tris·Cl, pH 7.4
0.1 mM EDTA, pH 8.0 (*APPENDIX 2A*)
100 mM NaCl
30 μM spermine
70 μM spermidine

For preparing the buffer, use ultrapure water.

Microinjection buffer

5 mM Tris·Cl, pH 7.4
0.1 mM EDTA, pH 8.0 (*APPENDIX 2A*)

For preparing the buffer, use ultrapure water.

Sucrose solutions, 10% (w/v) and 40% (w/v)

10% (w/v) sucrose solution
For 100 ml:
10 g sucrose
5 ml 1M Tris·Cl, pH 7.4
20 ml 5M NaCl
2 ml 0.5M EDTA, pH 8.0 (*APPENDIX 2A*)
Adjust volume to 100 ml using 1× phosphate-buffered saline (PBS)

40% (w/v) sucrose solution
For 100 ml:
40 g sucrose
5 ml 1M Tris·Cl, pH 7.4
20 ml 5M NaCl
2 ml 0.5M EDTA, pH 8.0 (*APPENDIX 2A*)
Adjust volume to 100 ml using 1× phosphate-buffered saline (PBS)

Tail lysis buffer

100 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
5 mM disodium EDTA pH 8.0
0.2% (v/v) SDS
200 mM NaCl
100 μg/ml proteinase K (due to differing storage conditions, add separately after the other buffer components have been added)
Store up to 6 months at room temperature (6 months at 4°C for proteinase K)

COMMENTARY

Background Information

In 1981, Gordon and colleagues reported the generation of the first transgenic mouse using the pronuclear injection technique with a foreign gene sequence of a pBR322-based plasmid containing the herpes simplex virus and simian virus 40 DNA sequence that could be integrated into the mouse genome. Shortly thereafter, several other groups reported similar success (Brinster et al., 1981; Wagner

et al., 1981). Since then, thousands of transgenic mice have been generated to unravel the functions of many genes implicated in human development, diseases, and disorders. There are other methods to deliver transgenes into the genome of the mouse. The use of the retrovirus carrying the transgene to infect the pre-implanted embryos and its successful germline transmission has been reported (Jahner et al., 1985; Van der Putten et al., 1985; Lois et al., 2002). However, this method did not gain much popularity because of cloning limitations of the retroviral vectors.

Recently, the discovery of new vectors such as YACs, PACs, and BACs have enabled the cloning of larger transgenes, which are more advantageous in transgene expression than plasmid vector–based cloning technology. (Sternberg et al., 1992; Schedl et al., 1993; Giraldo et al., 2001). Sometimes, minitransgenes (10 kb or less) that are introduced into the genome can cause mouse lethality by complicating normal mouse development. This might be due to the positional effect of the genes surrounding the transgene integration site or the true effect of the transgene. BACs, however, do not suffer from such positional effect because of its representation of the full gene with all the regulatory elements of the endogenous genes. Therefore, BACs play an important tool in functional genomics due to their large cloning capacity (around 150 kb), and due to their greater convenience in handling over other large cloning vectors like YACs and PACs (Testa et al., 2004). Moreover, the availability of high-quality, arrayed BAC libraries encompassing the mouse or human genome makes identification and isolation of specific clones relatively trivial (Conner, 2004). The newly developed Red/ET recombination method that allows BAC-based cloning is relatively easy (Zhang et al., 1998, 2000; Muyrers et al., 1999). There are variations of the method that can be used to make a large transgene for microinjection into oocytes from the BACs (Testa et al., 2004) that make pronuclear injections more attractive than ever in functional genomics.

Critical Parameters

Every step involved in creating transgenic mice requires tremendous focus and attention. To generate transgenic mice at the expected efficiency on a regular basis, all the parameters have to be examined and evaluated. For example, the stud males and the vasectomized male mice have to be replaced every 10 months, the hormone stock should be fresh at all times, and techniques to handle the embryos and the mice throughout the transgenic mice production have to be consistent. Other factors that cannot be controlled such as variations in the mouse responses to the hormone treatment, anesthetic agent, and environment have to be identified immediately and should be addressed as soon as possible. All the procedures should be evaluated from time to time to ensure that they are being performed at an optimal level and that there is no deviation from the best practices. The learn this technology, one should master the implantation surgery skill before learning injections and zygote harvesting. An experienced transgenic generator should focus her/his efforts on making transgenic mice before any other duties to maintain the highest efficiency. Good animal husbandry practices are a must to maintain mice at their best, including mating performance, response to hormone or anesthetics, and recovery after the surgery.

Troubleshooting

No transgenic founders and/or very low transgenic efficiency

Possible solutions: (1) Check the transgene to make sure it is the right transgenic fragment. (2) Make sure the DNA is ultraclean with no trace of vector DNA. (3) Check to make sure the DNA concentration is 2 ng/μl. (4) Be sure to set the injection pressure to 50 hPa and the injection mode to continuous flow. (5) Inject the mice as early as possible and finish the injections before 2:30 PM at the latest on the injection day. (6) If all of the above are followed and there are still no positives, check to see if the transgene is causing embryonic lethality.

Having a hard time injecting the DNA

Suggestions: (1) DNA contains impure particles. Centrifuge the DNA solution for 2 min at maximum speed in the microcentrifuge, take the DNA from the upper portion of the solution and check the DNA concentration, and inject. (2) Injection needle is too thin. Break the tip of the injection needle to increase the diameter slightly and inject. (3) Injection needle is clogged with cell debris from the zygotes. Change the injection needle immediately.

The founder mice are mosaic

Recommendation: Start injecting early and finish long before the 2 pronuclei get close to fusing together, with our strain, inject between 11 AM and 2 PM.

Getting very small litter size

Possible explanations: (1) The mother does not take care of the pups or kills the pups. (2) The transgene causes embryonic lethality, especially if the pups are delivered but die soon after birth.

Anticipated Results

Mating of 15 to 20 mice should be set up to provide at least 200 zygotes to be microinjected. With good DNA and injection technique, at least 95% of the zygotes should survive the injection procedure and be ready for implantation. The injected zygotes should be implanted into at least 4 recipient mice per each transfer day. From 30 to 40 implanted zygotes per mouse (15 to 20 zygotes in each side of the mouse), at least 10 pups (25%) should be born. Out of the total pups born, 10% to 20% should be the founder mice. Most founder mice are expected to transmit the transgene to their offspring.

Time Considerations

To generate several transgenic founders for each DNA construct, a total of 4 days are needed from the injection of hormone to the implantation surgery. Although much attention has been placed on aspects of the technique, animal care and preparation are also important. The donor mice are received and housed every week, hormone has to be prepared and administered, and mice must be mated and checked for plugs, all of which takes substantial amounts of time. After the implantation, the mice need to be separated and housed individually to avoid having multiple litters; a mouse tissue sample, either a piece of tail or a piece of ear, must be obtained, and the mouse has to be tagged for identification (Table 19.10.1). The harvesting of zygotes from mice takes ~2 hr, microinjection takes ~2 hr, and surgery takes ~3 hr.

Literature Cited

Brinster, R.L., Chen, H.Y., Trumbauer, M., Senear, A.W., Warren, R., and Palmiter, R.D. 1981. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* 27:223-231.

Brown, T. 1993. Southern Blotting. *Curr. Protoc. Molec. Biol.* 21:2.9.1-2.9.20.

Conner, D.A. 2004. Transgenic mouse production by zygote injection. *Curr Protoc. Molec. Biol.* 68:23.9.1-23.9.23.

Donovan, J. and Brown, P. 2006a. Parenteral injections. *Curr. Protoc. Immunol.* 73:1.6.1-1.6.10.

Donovan, J. and Brown, P. 2006b. Euthanasia. *Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Donovan, J. and Brown, P. 1998. Anesthesia. *Curr. Protoc. Immunol.* 27:1.4.1-1.4.5.

Giraldo, P. and Montoliu, L. 2001. Size matters: Use of YACs, BACs and PACs in transgenic animals. *Transgenic Res.* 10:83-103.

Jahner, D., Haase, K., Mulligan, R., and Jaenisch, R. 1985. Insertion of the bacterial *gpt* gene into the germ line of mice by retroviral infection. *Proc. Natl. Acad. Sci. U.S.A.* 82:6927-6931.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868-872.

Muyrers, J.P., Zhang, Y., Testa, G., and Stewart, A.F. 1999. Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res.* 27:1555-1557.

Schedl, A., Larin, Z., Mountoliu, L., Thies, E., Kelsey, G., Lerach, H., and Schutz, G. 1993. A method for the generation of YAC transgenic mice by pronuclear microinjection. *Nucleic Acids Res.* 21:4783-4787.

Sternberg, N.L. 1992. Cloning high molecular weight DNA fragments by the bacteriophage P1 system. *Trends Genet.* 8:11-16.

Testa, G., Vintersten, K., Zhang, Y., Benes, V., Muyrers, J.P., and Stewart, A.F. 2004. BAC engineering for the generation of ES cell-targeting constructs and mouse transgenes. *Methods Mol. Biol.* 256:123-139.

van der Putten, H., Botteri, F.M., Miller, A.D., Rosenfeld, M.G., Fan, H., Evans, R.M., and Verma, I.M. 1985. Efficient insertion of genes into the mouse germ line via retroviral vectors. *Proc. Natl. Acad. Sci. U.S.A.* 82:6148-6152.

Wagner, T. E., Hoppe, P.C., Jollick, J.D., Scholl, D.R., Hodinka, R.L., and Gault, J.B. 1981. Microinjection of a rabbit beta-globin gene in zygotes and its subsequent expression in adult mice and their offspring. *Proc. Natl. Acad. Sci. U.S.A.* 78:6376-6380.

Zhang, Y., Buchholz, F., Muyrers, J.P., and Stewart, A.F. 1998. A new logic for DNA engineering using recombination in *Escherichia coli. Nat. Genet.* 20:123-128.

Zhang, Y., Muyrers, J.P., Testa, G., and Stewart, A.F. 2000. DNA cloning by homologous recombination in *Escherichia coli*. *Nat. Biotechnol.* 18:1314-1317.

Key References

Brown, G.A.J. and Corbin, T.J. 2002. Transgenesis in the Mouse: Oocyte injection. *In* Transgenesis Techniques. *Methods in Molecular Biology*, Vol. 180 (A.R. Clarke, Ed.) pp. 39-70. Humana Press, Totowa, New Jersey.

This chapter contains very useful information for the production of transgenic mice, especially for setting up a new transgenic facility.

Conner, 2004. See above.

The most current information and methodologies for the generation of transgenic mice are found in this protocol.

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. 2003. Manipulating the Mouse Embryo: A laboratory Manual, 3rd Edition, Cold Spring Laboratory Press, Cold Spring Harbor, New York.

A comprehensive guide for generating genetically altered mice along with excellent diagrams and illustrations.

Overbeek, P.A. 1994. Factors Affecting Transgenic Animal Production *In* Transgenic Animal Technology: A Laboratory Handbook (Pinkert, C.A. Ed.) pp. 72-109. Academic Press, San Diego.

A detailed description of different strains of mice, excellent husbandry practices, and transgenic phenomenology are discussed in this chapter.

Pinkert, C.A. 1994. Introduction to Transgenic Animals *In* Transgenic Animal Technology: A Laboratory Handbook (Pinkert, C.A. Ed.) pp. 3-11. Academic Press, San Diego.

A thorough and complete history of the development of transgenic technology, as well as a compendium for practical applications of transgenesis via pronuclear injections.

Pinkert, C.A. and Polites, H.G 1994. Transgenic Animal Production Focusing on the Mouse Model *In* Transgenic Animal Technology: A Laboratory Handbook (Pinkert, C.A. Ed.) pp. 15-65. Academic Press, San Diego.

Another excellent resource for the production of transgenic animals via pronuclear microinjections.

Testa et al., 2004. See above.

An excellent guide for designing specific BAC transgenic constructs.

Overview: Generation of Gene Knockout Mice

Bradford Hall,[1] Advait Limaye,[1] and Ashok B. Kulkarni[1]

[1]National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland

ABSTRACT

The technique of gene targeting allows for the introduction of engineered genetic mutations into a mouse at a determined genomic locus. The process of generating mouse models with targeted mutations was developed through both the discovery of homologous recombination and the isolation of murine embryonic stem cells (ES cells). Homologous recombination is a DNA repair mechanism that is employed in gene targeting to insert a designed mutation into the homologous genetic locus. Targeted homologous recombination can be performed in murine ES cells through electroporation of a targeting construct. These ES cells are totipotent and, when injected into a mouse blastocyst, they can differentiate into all cell types of a chimeric mouse. A chimeric mouse harboring cells derived from the targeted ES cell clone can then generate a whole mouse containing the desired targeted mutation. The initial step for the generation of a mouse with a targeted mutation is the construction of an efficient targeting vector that will be introduced into the ES cells. *Curr. Protoc. Cell Biol.* 44:19.12.1-19.12.17. © 2009 by John Wiley & Sons, Inc.

Keywords: gene targeting • homologous recombination • gene knockout

INTRODUCTION

Two discoveries have been instrumental in creating the ability to generate knockout mice—the isolation of stem cells and the discovery of homologous recombination. The significance of these findings was demonstrated when Mario Capecchi, Oliver Smithies, and Martin Evans were awarded the 2007 Nobel Prize in Physiology or Medicine for their work in establishing the knockout mouse model (Vogel, 2007). The key step needed for making knockout mice was the isolation of embryonic stem cells (ES cells). The ES cells were isolated from the inner cell mass of a 3.5-day post-coitum mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981). These stem cells could progressively grow in tissue culture and were pluripotent (also see *UNIT 19.1*). By injecting the stem cells into a mouse blastocyst, Bradley et al. (1984) were able to generate chimeric mice. The production of chimeric mice proved that the stem cells were able to differentiate into multiple cell lineages and were able to contribute to the development of the mouse embryo. Germline transmission was also achieved by using the pluripotent ES cells. After breeding the chimeric mice, the resulting offspring were clearly derived from the ES cells, as seen by the transmission of coat color. Therefore, the introduced stem cells could become established into the germline of the chimeric mice. The isolation of stem cells basically meant that an ES cell clone that was genetically modified in culture could eventually be used to derive a mouse.

The second important step in the establishment of knockout mice was the discovery of homologous recombination within mammalian cells (also see Mortensen et al., 2006, for basic principles of this technique). Lin et al. (1984) were first able to demonstrate that homologous recombination was feasible by reconstructing a functional thymidine kinase gene in mouse L cells. Later, Smithies et al. (1985) were able to modify the β-globin locus with the insertion of the neomycin-resistance gene. This work provided a means for specifically targeting the DNA insert to a planned genetic locus, in contrast to the random integration of a construct that is used with transgenic technology.

The convergence of these two technologies provided the means to generate knockout mice. The first ideal genetic locus to test gene targeting was for the enzyme hypoxanthine-guanosine phosphoribosyl transferase (*HPRT*). Loss of the *HPRT* gene could be tested with treatment using

Current Protocols in Cell Biology 19.12.1-19.12.17, September 2009
Published online September 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1912s44

6-thioguanine, while restoration of the gene could be selected in *HPRT*-null cells with addition of hypoxanthine/aminopterin/thymidine (HAT) medium. In addition, the *HPRT* gene is X-linked so that, with male stem cells, only one allele needed to be targeted for drug selection. Doetschman et al. (1987) proved that a mutant *HPRT* gene could be corrected through homologous recombination in ES cells, while Thomas and Capecchi (1987) demonstrated targeted gene disruption. These experiments led the way to targeting of nonselectable genes, starting with *int-2* and *c-abl* knockout mice (Mansour et al., 1988; Schwartzberg et al., 1989).

Since the first knockouts, there has been an explosive growth in the number of animal models derived through the technique of gene targeting. While mouse stocks with spontaneous or radiation-induced mutations existed in the past, the mutated alleles did not correspond to the majority of biologically important cloned genes (Mak, 1998). Through homologous recombination, a cloned gene could be directly utilized to disrupt the target allele. Currently, a targeting strategy is often planned shortly after the mouse gene is originally cloned. Therefore, targeted recombination has become well established as an important tool to inactivate a gene in order to study its function in vivo.

The process of gene targeting provides a means to alter a specified gene in order to better discern its biological role. Through homologous recombination, an engineered mutation can be directed to a designated genetic locus. In this manner, a potentially important genomic clone can directly be utilized to create a mutation in a selected gene. Even within the 2.5 Gb of the mouse genome, the cellular DNA repair mechanisms are able to align a targeting vector with its corresponding region of homology and cause recombination into the chromosome.

While the goal of transgenic technology is to overexpress a gene to study its biological role in vivo, homologous recombination is typically employed to create a loss-of-function mutation. The most common application of gene targeting is to produce knockout mice, where a drug-resistance marker replaces an essential coding region in a genetic locus. In the majority of cases, the importance of a gene cannot be determined by simply recognizing amino acid motifs in the protein (Iredale, 1999). Additionally, the role of a gene often cannot be completely revealed by examining closely related family members. Therefore, gene inactivation is the best way to delineate the biological role of a protein, and gene targeting is a direct means to disrupt a gene's open reading frame and block its expression in a mouse. Not surprisingly, during the twenty years that gene-targeting techniques have been available, thousands of genes have been knocked out. To date, about 11,000 genes have been knocked out in mice, which accounts for roughly half of the mouse genome (Sikorski and Peters, 1997; Vogel, 2007). Through a combination of gene targeting and gene trapping, a global effort is underway to make a knockout mouse for each of the 25,000 mouse genes (Grimm, 2006).

The knockout mouse has been a valuable tool for geneticists to discern the role of a gene in embryonic development and in normal physiological homeostasis. Mice act as a good analog for most human biological processes, since humans and mice share ~99% of the same genes (Capecchi, 1994). Additionally, mice are useful experimental animals because they are small, have relatively short life spans, and are prolific. So, for geneticists, the targeted deletion of a gene in a mouse provides an important means to determine the biological role of a genetic allele.

While useful for studying in vivo gene function, some knockout mice have also served as valuable animal models for human genetic diseases. When a human mutation is found to disable a protein, the corresponding knockout mouse can be an important resource to study the underlying pathophysiology and to develop therapies to treat a genetic disease (Majzoub and Muglia, 1996). Additionally, pharmaceutical companies obtain clues about inhibiting a protein by first looking at the phenotype of a knockout mouse (Zambrowicz and Sands, 2003). Thus, knockout mice can provide insight into a gene's physiological role in humans.

Rather than just inactivating a gene, however, some genetic diseases result in the expression of a mutated protein. Point mutations, microdeletions, or insertions are responsible for many human genetic diseases. These subtle mutations can also be mimicked in a mouse model using gene targeting. Instead of disrupting a gene, as in most knockout mice, homologous recombination is employed to swap the normal copy of an exon with a mutated version. As long as a similar mutation can be reproduced in the mouse protein, then the corresponding amino acid substitution, deletion, or insertion can be targeted into a gene of interest to replicate the human disease. The effects

of the altered protein can then be studied in the animal model.

With some knockout mouse models, the severity of the phenotype can preclude analysis of a gene's role in the organogenesis of a particular tissue. For example, about 15% of all knockout mice have mutations that result in developmental lethality (*http://www.genome.gov/12514551*). To circumvent this problem, Cre/loxP technology has been employed to create conditional knockout mice. Derived from the P1 bacteriophage, the Cre recombinase will excise any region of DNA placed between two *loxP* sites (locus of crossing over; Sternberg and Hamilton, 1981; Sauer and Henderson, 1998). The *loxP* site is a 34-bp nucleotide sequence that can be genetically targeted around an essential exon in a gene (Gu et al., 1994). The resulting mice contain the floxed (flanked by *loxP* sites) allele in all tissues, but are phenotypically wild-type. These floxed mice are then bred to Cre-expressing transgenic animals, wherein the promoter used to drive Cre expression will determine the site of the gene deletion. For spatial inactivation of a gene in a mouse, a cell-type-specific promoter is used to limit Cre expression to a particular tissue.

Although less commonly used than Cre/loxP technology, the Flp recombinase also provides a similar means to rearrange a genetic locus. Flp (flippase) was isolated from *Saccharomyces cerevisiae* and, like Cre, the recombinase will also excise DNA flanked between 34-bp sequences known, in this case, as FRT sites (Dymecki, 1996). So, through the use of either the Cre/loxP or the Flp/FRT systems, gene expression can be disrupted in a spatial and temporal manner, and the lethality of a knockout mouse phenotype can be overcome. With this versatility, mice utilizing Cre/loxP or the Flp/FRT systems are often shared among research laboratories studying differing physiological systems.

In addition to spatial excision of a floxed allele, temporal control of Cre-mediated recombination is also possible in a conditional knockout mouse. The timing of recombination can be regulated by use of a tamoxifen-inducible Cre (Feil et al., 1997; Hayashi and McMahon, 2002). In this strategy, Cre is ligated to a mutated ligand-binding domain of the estrogen receptor that restricts transcription until tamoxifen is present. Cre is, therefore, not expressed until tamoxifen is applied either topically or through injection. Another inducible Cre system takes advantage of the reverse tetracycline-controlled transactivator (rtTA; Utomo et al., 1999). Doxycycline is administered to activate the rtTA which, in turn, will induce transcription of Cre. Lastly, Cre can also be delivered through injection of a viral vector (Anton and Graham, 1995). When and where the Cre is expressed is controlled by the timing and site of injection for the virus. The amount of DNA rearrangement can be adjusted by varying the titer of the virus. The numbers of conditional knockout mice have dramatically increased since Cre/loxP and Flp/FRT technologies were introduced into gene targeting. This proliferation of conditional animal models attests to the value of the recombinases as a molecular switch.

Another interesting application of gene targeting is knock-in technology, in which any gene of interest can be placed under the cis-acting regulatory elements of another gene (Cohen-Tannoudji and Babinet, 1998). Originally, knock-in mice were derived as a means to visualize a gene's expression due to targeted recombination of the *lacZ* reporter gene into a genetic locus (LeMouellic et al., 1990). The initial coding sequence of a gene would be replaced with the *lacZ* marker, which is inserted under the direction of the gene's promoter. The strategy of using homologous recombination to knock in a reporter gene, like *lacZ*, allows not only for the creation of homozygous null mice for a gene, but also provides a technique to study the targeted gene's expression in the heterozygous mice, which are often phenotypically normal. The knock-in idea was later elaborated to include the replacement of a gene by the sequence coding for a similar isoform of the protein (Hanks et al., 1995). Similar protein isoforms could thus be tested for redundancy. Through knock-in mice, genetically similar proteins can be examined to determine if two isoforms are truly biologically distinct or if the proteins are basically functionally equivalent. with differing patterns of expression being the only key divergence.

VECTOR DESIGN

Homologous recombination takes advantage of a cell's own DNA-repair machinery to replace a targeted genetic locus with homologous genomic sequence. The targeted event probably occurs through strand transfer by the recombination RAD52 epistasis group (Rijkers et al., 1998; Vasquez et al., 2001). For recombination to occur at all in a cell, around 2 kb of sequence homology is required (Melton, 2002). However, 6 to 14 kb of homology is typical for targeting constructs. In

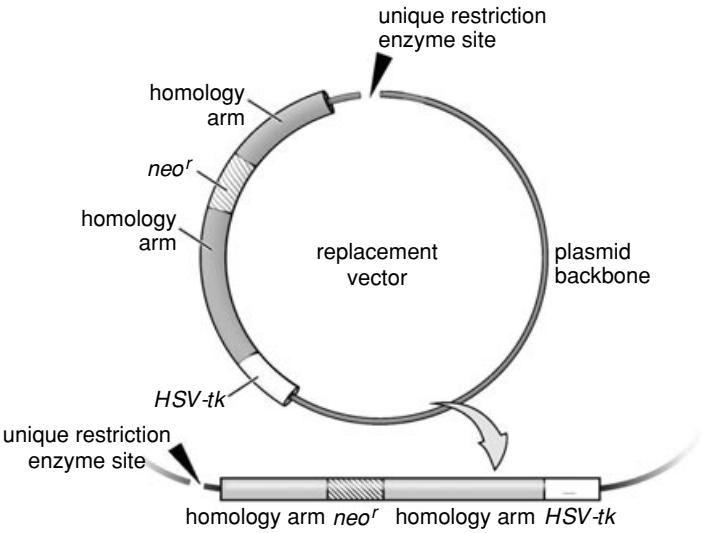


Figure 19.12.1 A schematic of a replacement vector. Two homology arms flank a positive drug-selection marker (*neo*^{r}). A negative-selection marker (*HSV-tk*) is placed adjacent to one of the targeting arms. A unique restriction-enzyme site is located between the vector backbone and the homology arm. When linearized for gene targeting, the vector backbone will then protect the *HSV-tk* from nucleases.

addition, linear DNA was found to be the preferred substrate for the recombination proteins (Hasty et al., 1992). The actual targeted event takes place in only a small percentage of cells, as homologous recombination occurs at a rate ~1000-fold lower than random insertions (Sargent and Wilson, 1998). During a stem cell experiment, only about 10^{-2} to 10^{-3} of the DNA integrations are homologous recombination events (Melton, 2002). Therefore, a thorough screening process employing Southern blotting or PCR is necessary to identify cells with the targeted event. Electroporation became the preferred way to deliver the replacement vectors into a large number of stem cells. While microinjection has a better targeting ratio (1:15 targeted recombinants to random integrants), a mass delivery system was needed to introduce the targeting constructs into cells, and electroporation provided the most efficient technique (1:2400 targeting ratio; Vasquez et al., 2001). However, since the transformation efficiency is low (10^{-3}), a positive selection marker is needed to enrich clones that have inserted the targeting vector into their genome (Ledermann, 2000). The likelihood of recombination peaks when cells are in early to mid-S phase, and the process occurs rapidly, in ~30 min after the construct is taken into the nucleus (Wong and Capecchi, 1987; Cappecchi, 1989). The rate of homologous recombination is not dependent on the input concentration of vector used in the targeting experiment (Thomas et al., 1986; Capecchi, 2005). So, the capacity to locate homologous sequence within the recipient cell genome is not a rate-limiting step, since this would be influenced by the input concentration of DNA. Rather, the cellular machinery performing the homologous recombination sets the reaction rate for gene targeting.

Primarily, two classes of vectors have been used to generate targeted mutations. Most gene-targeting experiments employ replacement vectors (Fig. 19.12.1), which have been particularly instrumental for efficiently generating knockout mice. In the design of a replacement vector, the open reading frame of a genomic clone is disrupted by the placement of an intervening drug-selection marker. Two homologous recombination events function to insert the targeting construct containing the drug-resistance gene into a homologous genetic locus (Fig. 19.12.2). The drug-resistance gene works for the positive selection of cells that have integrated the targeting vector into their chromosome. Neomycin is the most common drug used for positive selection. Integration of the neomycin phosphotransferase (*neo*^{r}) gene allows for resistance to neomycin, an aminoglycoside that interferes with protein synthesis in eukaryotic cells. Other drug-selection markers include resistance genes for puromycin and hygromycin.

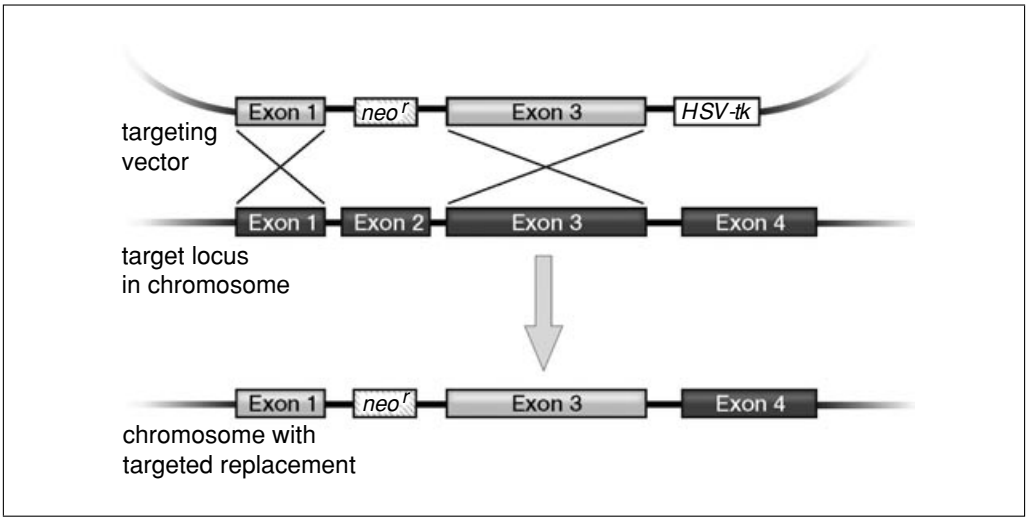


Figure 19.12.2 Gene inactivation through a replacement vector. Homologous recombination with a replacement vector requires a positive-selection marker (*neo*^{r}), a negative-selection marker (*HSV-tk*), and two targeting arms (homologous sequence is depicted with exons in light gray). A representative target gene (with exons in dark gray) is aligned with the targeting vector. For this example, the targeting vector is designed so that Exon 2 is substituted by the *neo*^{r} gene. The replacement of Exon 2 by the *neo*^{r} gene is then recapitulated in the target locus as homologous recombination exchanges genomic sequence for the homologous sequence of the targeting vector. Two homologous recombination events (depicted through the crosses) occur via the long and short targeting arms to introduce nonhomologous sequence (i.e., *neo*^{r}) into the designated gene. Insertion of the *neo*^{r} gene is selected for by treatment of cells with neomycin sulfate (G418) in tissue culture. The negative-selection marker (*HSV-tk*) is not recombined into the chromosome and is lost during gene targeting. If the targeting construct is randomly integrated anywhere in the genome, the *HSV-tk* gene would be intact. Random integrants can be selected against by either gancyclovir or FIAU treatment.

Mansour et al. (1988) established positive and negative selection for gene targeting by the additional placement of the HSV thymidine kinase (*HSV-tk*) gene adjacent to one of the vector arms containing targeted homology to a genetic locus. Random integrants will usually contain an intact form of the *HSV-tk* gene when inserted into the genome. Cells with random integrants are killed during negative selection via treatment with gancylovir or FIAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil], compounds that require phosphorylation by HSV-tk to inhibit DNA synthesis. An ~2000-fold enrichment of targeted clones can occur with this type of vector. Even with positive and negative selection, a substantial number of ES cell clones that arise in culture are false positive, since *HSV-tk* can be inactivated before recombination by events like partial deletions. The random integrants are, therefore, insensitive to negative drug selection. To avoid subjecting the ES cells to drugs like gancyclovir and FIAU, some targeting vectors omit using *HSV-tk* in favor of a negative-selection marker like the diphtheria toxin gene (Yagi et al., 1990). In a gene-targeting experiment, a replacement vector directs the disruption of an essential coding exon (or exons) of a gene through the insertion of a positive drug-resistance marker. This replacement occurs via recombination at the two flanking homology arms. Any important coding region that is essential for a gene's function can be thereby targeted for deletion.

When designing a targeting construct, a few factors should be considered that could result in an incomplete knockout. A gene may be residually expressed if there exist alternative or cryptic promoters that are not disrupted in the targeted allele (Müller, 1999). Differential splicing could also generate RNA species where the selection marker is skipped. Read-through transcription of the drug-resistance gene is another way mutant mRNA that has some coding sequence from the targeted allele can appear. In gene targeting via a replacement vector, a strong promoter is typically used to drive the *neo*^{r} marker in the stem cells, but the poly(A)-addition site can sometimes be skipped during transcription of the drug-resistance gene. Therefore, as the *neo*^{r} gene is transcribed, downstream exons could possibly be spliced into the mRNA. The *neo*^{r} gene is often oriented in the opposite direction with

respect to gene transcription for the targeted allele, to avoid any potential transcription of downstream exons. In addition, this orientation also ensures that the strong promoter on the *neo*^{r} gene does not influence any downstream genes. Therefore, the size and location of the targeted deletion should be carefully considered when designing a replacement vector, to help avoid an incomplete knockout of the gene function.

Double replacement vectors are a variation of the knockout vector design that is primarily used to target subtle mutations into a selected genetic allele (Askew et al., 1993; Stacey et al., 1994). Also known as tag and exchange, this strategy requires two rounds of homologous recombination to create the desired mutation. In contrast to a replacement vector, the *HSV-tk* gene is placed adjacent to *neo*^{r}, rather than at the end of the construct. Both the positive and negative selection markers are inserted into the targeted genomic site, with the homology arms flanking both genes. In the first tag step, a targeted stem cell clone is isolated with only neomycin treatment. Once isolated, the ES cells then undergo a second round of electroporation with a targeting vector that will exchange both drug-selection markers for a mutated exon. This second targeting vector is basically a genomic clone containing the desired point mutation, deletion, or insertion. During this second round of gene targeting, gancyclovir is added to isolate cells that have lost the *HSV-tk* gene from homologous recombination with the second vector. The *HPRT* gene can be used to replace both the *neo*^{r} and *HSV-tk* genes in the construct design. The *HPRT* gene is part of a purine salvage pathway that can be adapted as a drug marker for both positive and negative selection (Melton, 2002). HAT (hypoxanthine/aminopterin/thymidine) medium is used for positive selection, since it forces a cell to depend on the salvage pathway that needs an intact copy of the *HPRT* gene. Negative selection involves the purine analog 6-thioguanine, which is phosphorylated by the *HPRT* gene to generate a toxic compound for the cell. ES cells with a mutated copy of their endogenous *HPRT* gene, however, are needed for this type of strategy. So, with a double replacement vector, two rounds of targeted recombinations are required to obtain the final ES cell clones with the expected subtle mutation. Several mutations can readily be placed into the modified gene once targeted ES cell clones from the first electroporation are isolated.

Subtle mutations can also be introduced with vectors that take advantage of Cre/loxP technology (Fig. 19.12.3; Ferradini et al., 1996; Cohen-Tannoudji and Babinet, 1998). Similar to a replacement vector, the targeting construct is designed with a drug-selection marker flanked by two homology arms. Both positive and negative selection are required to isolate cells with the properly recombined mutation. Instead of replacing an entire exon with a drug-selection marker, the goal, here, is to exchange normal coding sequence in a targeted allele for a mutated version. One homology arm will carry the planned point mutation, micro-deletion, or insertion to be introduced into the targeted gene. However, in this strategy, the positive drug-selection marker needs to be removed because it will interfere with transcription of the mutated allele. Using a *neo*^{r} gene that is floxed allows a way to eventually remove the drug-selection marker by using Cre recombinase. In the construct design, the *neo*^{r} gene is inserted into a noncoding region. Upon recombination, only a 34-bp *loxP* site remains in the intron, creating a clean mutation (without a drug-selection marker) in the targeted genetic locus. Without disruption by a drug-selection marker, the modified allele should be transcribed to produce the expected mutant protein, and the remaining *loxP* site within an intron should not interfere with gene expression. Like double replacement vectors, this strategy is also a two-step process, except that Cre is used to remove the drug-selection marker rather than a second round of homologous recombination.

Making a targeting construct for a conditional knockout is essentially like making a clean mutation, but, with this strategy, the inserted mutation results in the addition of *loxP* sites into a targeted genetic locus (Fig. 19.12.4). In the final targeted allele, two *loxP* sites will flank an essential exon (or exons) to be excised by Cre in a conditional manner (Gu et al., 1994; Cohen-Tannoudji and Babinet, 1998). As with the clean mutation, a floxed *neo*^{r} gene is needed for positive selection and will eventually be removed using Cre. This process of Cre excision will leave one of the necessary *loxP* sites required to flox a targeted exon(s). One of the homology arms from the replacement vector is then used to insert the other flanking *loxP* on the opposite side of this exon(s). Both *loxP* sites must be positioned in the introns of the targeted gene, and should not interfere with the either the coding sequences or the promoter of the gene,

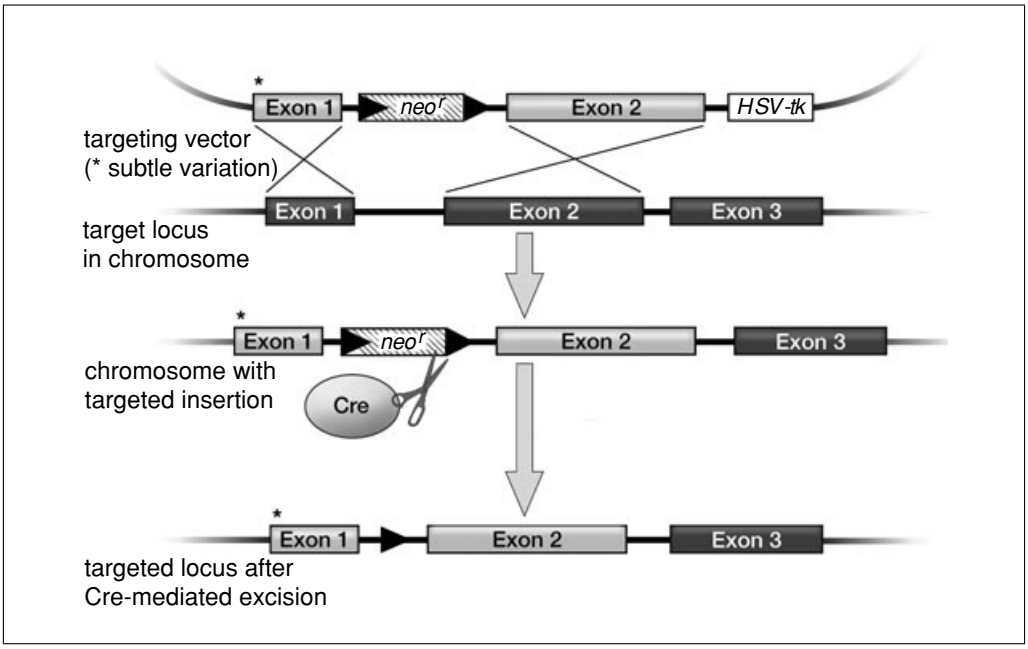


Figure 19.12.3 Gene targeting to insert a subtle mutation. In the targeting vector, a subtle mutation (depicted by the asterisk) is designed into one of the homology arms. Subtle mutations can consist of point mutations, microdeletions, or insertions. When sequence in the target gene is exchanged with homologous sequence from the targeting vector, the subtle mutation is introduced into the chromosome. Homologous recombination occurs through both the long and short homology arms. A positive drug-selection marker (*neo*^{r}) is needed in this strategy to select for clones that have undergone recombination. With a clean mutation, the *neo*^{r} gene is inserted into the targeted locus, but no exons are lost as a result of recombination. The *neo*^{r} gene needs to be removed so that it does not interfere with transcription of the recombined allele. The *loxP* sites (depicted as black triangles) are used to flank the *neo*^{r} gene in order to facilitate its removal. These 34-bp *loxP* sites are recognized by the Cre recombinase, which can be introduced into targeted stem cells by transient transfection. If the *loxP* sites are in the same direction, the Cre recombinase will circularize out any intervening sequence. With Cre-mediated recombination, only a single *loxP* site will remain. The *loxP* site in the recombined allele is situated within intron sequence so that it does not interfere with transcription of the mutant protein.

nor with any neighboring genes. After homologous recombination, the mutated genetic locus will initially have three *loxP* sites consisting of the floxed exon(s) followed by a floxed *neo*^{r} gene. Once the targeted clones are identified, the targeted ES cells are then transiently transfected with a Cre expression vector to remove the *neo*^{r} gene. Three scenarios arise when Cre is used to recombine the targeted allele. In one situation, the targeted allele is properly recombined and Cre only excises the *neo*^{r} gene. However, recombination can also result in the exclusion of the floxed exon(s) or both the exon(s) and the *neo*^{r} gene. A second round of genomic screening by Southern blot or PCR is needed to find the ES cell clone that is missing the *neo*^{r} gene, but which has the floxed exon intact. Transcription of the targeted gene should not be impacted when the final floxed allele is generated. The mutated mice are then bred to a Cre-expressing transgenic line to allow for conditional disruption of the gene in the tissue of interest.

Knock-in strategies basically adapt replacement vectors to usurp the endogenous promoter of a gene to transcribe an exogenous cDNA of interest (Fig. 19.12.5; LeMouellic et al., 1990; Hanks et al., 1995; Cohen-Tannoudji and Babinet, 1998). During recombination, the protein start site of the targeted gene is disrupted when a selected cDNA is knocked into the promoter, essentially resulting in a targeted transgenic mouse. For this vector design, both the cDNA to be knocked in and the *neo*^{r} gene are brought into the genome by two flanking homology arms. The 5′ arm, in this case, will typically contain homologous genomic sequence that is upstream from the protein start site. Similar to a transgenic construct, this regulatory sequence is followed by a cDNA and a poly(A)-addition signal. For positive selection, a drug-resistance gene like

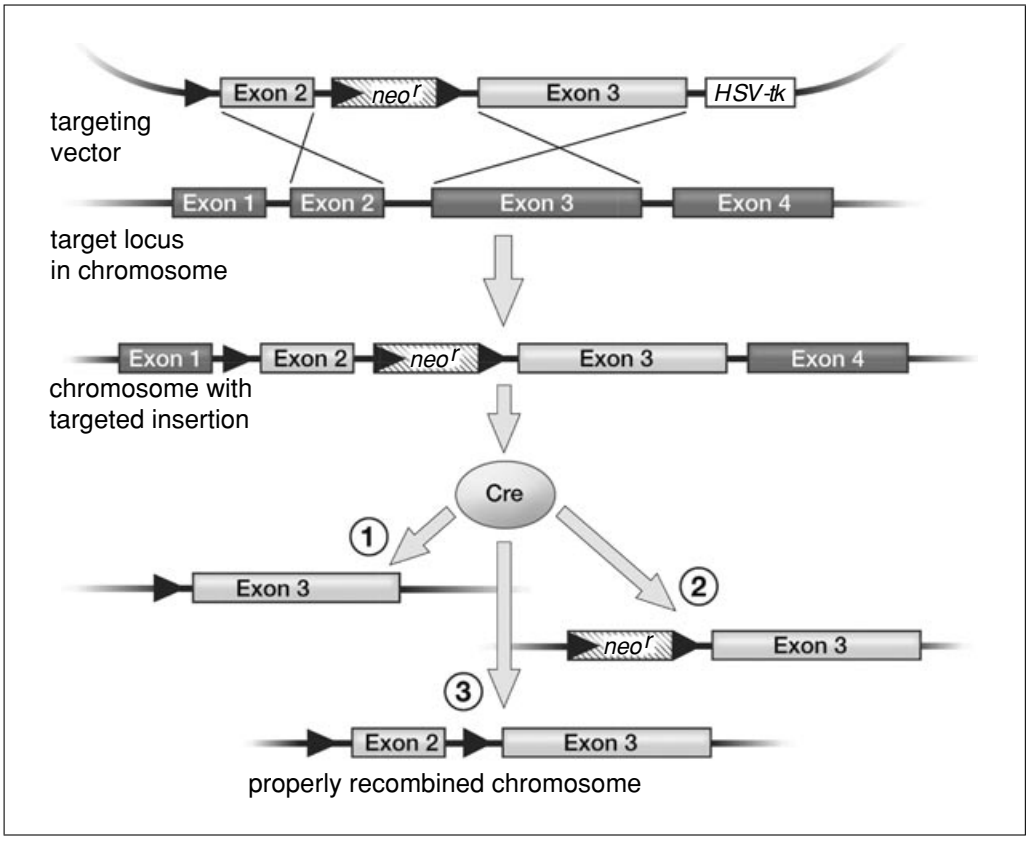


Figure 19.12.4 Gene targeting to create a conditional knockout. In this strategy, the targeting construct is designed so that a *loxP* site (indicated with a black triangle) is located next to an essential exon within the homology arm. A floxed *neo*^{r} gene is then positioned on the opposite side of this exon. Both the *loxP* site and the floxed *neo*^{r} gene are introduced into the target locus through homologous recombination. Unlike a conventional knockout experiment, the targeting vector is assembled so that no exons are lost as a result of homologous recombination. After the stem-cell clone with the properly targeted insertion is identified, the Cre recombinase is then introduced into the cells through a transient transfection. The Cre recombinase can produce three types of recombinations. In the first example (shown as a circled "1"), both Exon 2 and the *neo*^{r} gene are excised from the chromosome, leaving only one *loxP* site. In the second scenario, (shown as "2"), only Exon 2 is removed while the floxed *neo*^{r} gene remains in the targeted chromosome. In the third case ("3"), just the *neo*^{r} gene is excised while two *loxP* sites remain to flank Exon 2, creating a floxed allele. Only the stem-cell clones with this specific recombination are injected into blastocysts to generate floxed mice. Conditional deletion of Exon 2 in vivo is then accomplished typically by breeding the floxed mice with a Cre-expressing transgenic line. After the removal of *neo*^{r} in tissue culture and subsequent conditional deletion of Exon 2 in vivo, the final allele has only one *loxP* site remaining within the intron sequence of the targeted gene (as depicted in "1").

neo^r is placed downstream of the cDNA. A second 3′ homology arm then follows after the *neo*^{r} gene. Like a replacement vector, two homologous recombination events function to replace the coding sequence of the gene with the cDNA and *neo*^{r} drug marker in the construct. If positioned correctly, the knocked-in cDNA should then be in frame and expressed by the recombined gene. Through this design, a gene can be knocked out as the cDNA of closely related isoform is simultaneously knocked in. Protein domains can be switched by homologous recombination as well. In addition, marker genes, like *lacZ*, can be knocked in not only to produce a null mouse, but also to help track the targeted gene's expression pattern through the mutated gene. Even though knock-in mice are more time consuming to generate than transgenic mice, the knocking in of a cDNA into a targeted genetic locus can be useful to overcome problems of unreliable transgene expression that sometimes occur from the randomness of the site of integration and the variability in transgene copy number (Jasin et al., 1996; Ledermann, 2000). If the knocked-in cDNA is correctly in frame, expression of the knocked-in transgene should more faithfully mirror the transcription pattern

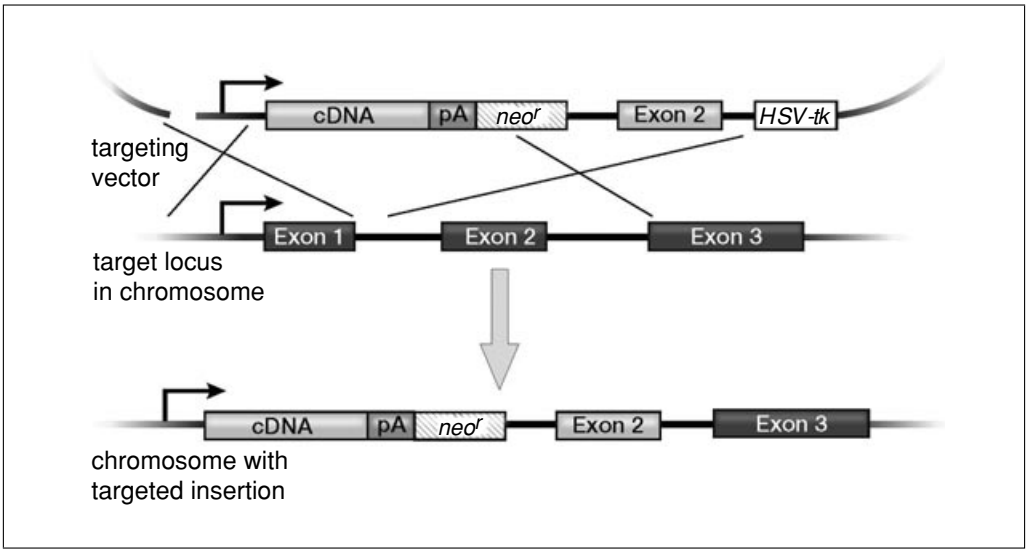


Figure 19.12.5 Knock-in of a cDNA through the use of gene targeting. Knock-in constructs are similar in design to conventional knockout gene-targeting vectors, except that additional sequence (i.e., a cDNA or protein domain of interest) is inserted into the target gene. Through homologous recombination, this foreign sequence is introduced in frame into the target locus to be expressed by its promoter. Essential coding sequence in the target locus is simultaneously lost during recombination with the targeting construct. In this example, homologous recombination places a cDNA under the control of the promoter of the target gene. Concurrently, the translational start sequence in Exon 1 of the target gene is also replaced by the cDNA. The cDNA, in this example, has a poly(A)-addition signal (pA) which will stop any further transcription downstream of the targeted insertion. For a knock-in targeting vector, one of the homology arms must consist of genomic sequence upstream of the planned insertion site for the cDNA. To knock in a cDNA, as shown, a targeting vector must use promoter sequence for one of its homology arms (as depicted with the directional arrow). A positive drug-selection marker (*neo*^{r}) is still needed to select for clones that have inserted the designated cDNA into the target gene. Two homologous recombination events serve to insert the cDNA and *neo*^{r} gene into the target location while knocking out Exon 1.

for the targeted promoter. In one such example, Cre was targeted to the Cd19 genetic locus in order to maintain all of the regulatory elements for transcription in B cells (Rickert et al., 1997).

Lastly, gene targeting with a replacement vector can be applied to generate a single-copy transgenic mouse. The use of *HPRT*-null ES cells allows for only a single copy of a transgene to be integrated at a known insertion site (Bronson et al., 1996). With this replacement-vector design, homologous recombination is needed to correct the *HPRT* gene. The homology arms of the targeting construct restore two missing exons needed for a functional copy of the *HPRT* gene during recombination in the stem cells. During homologous recombination, a single copy of a transgene is carried into the restored *HPRT* genetic locus. The transgene flanked by the targeting arms has its own promoter, selected cDNA, and poly(A)-addition site. With a reconstituted *HPRT* gene available as a drug-selection marker, clones with the proper homologous recombination can then be isolated with HAT medium.

Another less commonly used gene-targeting strategy employs insertion vectors to disrupt a genetic locus (Fig. 19.12.6). These insertion vectors are designed using just one arm of homologous sequence, and a single recombination event is all that is required to insert a drug-selection gene like *neo*^{r} into the targeted gene (Hasty et al., 1991). A restriction-enzyme site located within the homology arm is used to linearize the construct, so that homologous recombination can occur. Instead of replacing sequence, the whole insertion vector is integrated into the genome. In this case, the homology arm serves merely to direct the site of integration to the targeted gene. Insertion vectors result in gene duplication during homologous recombination because the entire targeting construct is inserted where the homology arm was linearized. In most cases, the *neo*^{r} gene will interfere with transcription in the targeted gene to create a loss-of-function mutation. However, since homologous recombination with an insertion vector produces gene duplication, mutant RNA species may still be expressed through altered splicing.

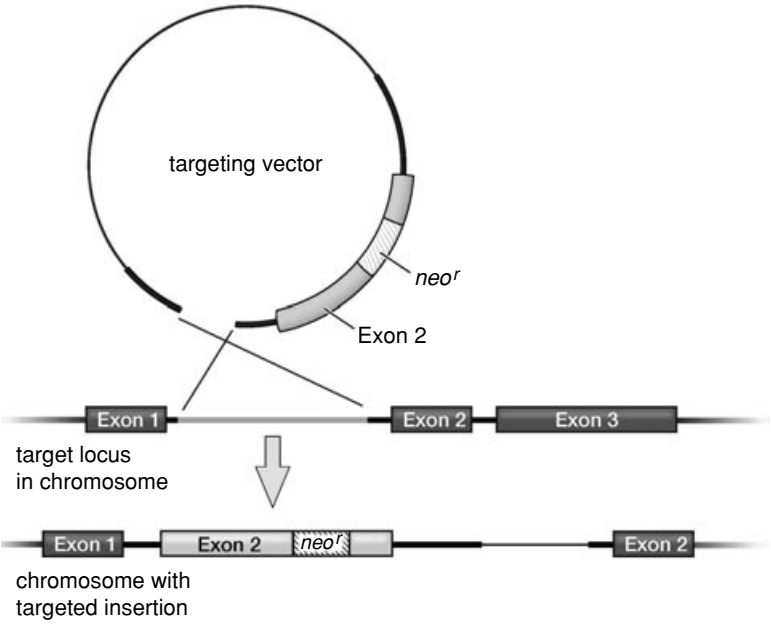


Figure 19.12.6 Gene inactivation with an insertion vector. For an insertion vector, only one homologous recombination event is needed for targeted insertion of DNA into a designated gene. In this case, recombination occurs around a double-strand break that is located in the homology arm of the targeting construct. The entire insertion vector is then incorporated into the gene, including the plasmid backbone (represented by the thin line). A drug-selection marker like the *neo*^{r} gene is still needed for positive selection, but this marker can be positioned either in the targeting arm or in the plasmid backbone of the insertion vector. In this example, the positive drug-selection marker is designed into the homology arm in order to replace essential coding sequence of the target gene (as shown with the disruption of Exon 2 by the *neo*^{r} gene). For insertion vectors, a knockout allele is essentially generated because the target gene is disrupted with insertion of the *neo*^{r} gene and by duplication of exonic sequence.

In addition, intrachromosomal recombination can also lead to regeneration of the wild-type allele. Compared with replacement vectors, though, constructs of this type have a higher frequency of recombination (Hasty et al., 1991). A variation of the insertion-vector strategy is to create a subtle mutation through a hit-and-run or in-out strategy (Vanlancius and Smithies, 1991). With this technique, the homology arm contains a desired mutation to be inserted into the targeted gene. Through homologous recombination, both the *neo*^{r} and *HSV-tk* genes are inserted into the targeted gene. After initial positive selection with *neo*^{r}, the isolated cells then undergo a second round of drug selection with gancyclovir. During this next step, the duplicated gene should align in such a way as to undergo intrachromosomal recombination. Both drug-selection markers will then be lost in the process. The remaining recombined allele will retain the subtle mutation, and the mutated protein will be expressed.

STRATEGIC PLANNING FOR DESIGNING A KNOCKOUT TARGETING CONSTRUCT

While creating a knockout mouse is costly and labor intensive (>$12,000 and ~1 year from electroporation of the targeting vector in ES cells to the generation of homozygous null mice; *http://www.med.umich.edu/tamc*), the understanding of gene functions in mammalian physiology and development have made the efforts worthwhile. To design a vector for gene targeting, the following steps are suggested:

1. After selecting a gene to be disrupted, the genomic structure of the target allele should be researched. Exon/intron sequence information should be gathered. The size of the gene and the chromosomal location of the allele to be targeted should also be known. Lastly, the location of most major restriction enzyme sites should be identified to aid with subcloning. A good restriction-enzyme map of the genomic locus will be useful in

the construction of the replacement vector. Once an allele is selected for targeted deletion, the flanking genomic sequence should be examined to ensure that any possible neighboring genes are not disrupted during recombination (Olson et al., 1996). Check for potential open reading frames that could be disturbed when a targeted mutation is generated. Most genomic sequence information can be gathered through the Mouse Genome Sequencing Consortium (MGSC). The mouse genome sequence is freely available in public databases (GenBank accession number CAAA01000000) and is accessible through various genome browsers (*http://www.ensembl.org/Mus_musculus/*; *http://genome.ucsc.edu/*, and *http://www.ncbi.nlm.nih.gov/genome/guide/mouse/*; Mouse Genome Sequencing Consortium, 2002). Lastly, perform a thorough search to ensure that your gene of interest has not been knocked out already. About 700 knockouts have been made three or more times (Grimm, 2006).

2. A mouse genomic fragment containing a large portion of the gene to be targeted needs to be isolated to create the homology arms in the targeting construct. A 129/Sv genomic clone is most commonly used for constructing targeting vectors, since most stem cells were derived from this mouse strain. Using a genomic clone of a mouse strain different from the ES cell strain can reduce the frequency of homologous recombination. In general, homologous recombination is four to five times more efficient with targeting vectors using isogenic DNA (Deng and Capecchi, 1992). For any given genetic locus, however, the rate of recombination will depend on the amount of nonhomologous sequence between the mouse strains, as a 20-fold decreased efficiency has been reported with the use of nonisogenic DNA in the targeting vector (te Riele et al., 1992). Searching a BAC (Bacterial Artificial Chromosome) library is one means to get the genomic sequence needed to make a targeting vector. Due to sequencing of the mouse genome, BLAST can be used to readily find a BAC clone that contains the selected gene for targeted disruption. Genomic DNA from a female 129S6/SvEvTac (Taconic) mouse, for example, was used to create the RPCI-23 BAC library. A BAC clone will generally contain from 100 to 300 kb of genomic sequence. Another means to obtain the homology arms for the targeting vector is through PCR. PCR can be used to amplify the homology arms, if a source of purified 129/Sv genomic DNA is available. A high-fidelity *Taq* polymerase is needed that can amplify long stretches of genomic DNA. Even with a high-fidelity *Taq* polymerase, however, the PCR products must be carefully sequenced to ensure that no coding errors were generated during the reaction. Lastly, a mouse 129/Sv Lambda phage genomic library is another option to isolate the sequence for the gene to be targeted. A probe containing some homology to the chosen allele is used to screen the phage library. Once a genomic clone is isolated, most of the common restriction enzyme sites should be verified.

The total amount of homology needed in most successful gene-targeting experiments is about 5 to 10 kb in length. The efficiency of homologous recombination increases when increasing the length of homology in the targeting construct, but this peaks after 14 kb of sequence (Deng and Capecchi, 1992). For a typical replacement vector, the total length of homologous sequence will consist of roughly a 1- to 2-kb span of DNA for a short homology arm and a separate, larger 4- to 6-kb genomic fragment as a long homology arm. The short arm can be as small as 0.5 kb, however, without affecting targeting efficiency (Hasty et al., 1991). In the case of the long arm, though, increasing the length of homology from 8 up to 110 kb does not enhance the frequency of homologous recombination (Lu et al., 2003). In the construct, the short and long arms will share homology with genomic sequences that flank the designated coding region to be deleted from the gene to be targeted.

3. Both positive and negative selection genes need be chosen to construct the targeting vector. The most common positive-selection marker is the neomycin phosphotransferase (*neo*^{r}) gene. The drug-resistance genes for puromycin and hygromycin have also been used as positive-selection markers. When designing a targeting vector, the sequence of the *neo*^{r} gene is often inserted in the opposite orientation to transcription in the target allele. With negative selection, the HSV thymidine kinase (*HSV-tk*) gene is often used to select against random integrants. If the targeting construct is randomly integrated, the cells will mostly contain an intact copy of the *HSV-tk* gene that will phosphorylate gancyclovir into a cytotoxic drug (Mansour et al., 1988). The diphtheria toxin gene has also been used for negative selection against random integrants (Yagi et al., 1990). A frequently used plasmid for making replacement vectors is pPNT (Tybulewicz et al., 1991). This plasmid contains both the *neo*^{r} and *HSV-tk* genes. All that

is required is to ligate the two homology arms into the unique restriction enzyme sites that flank the *neo*^{r} gene.

With the acquisition of the drug markers and a genomic clone, one can proceed with piecing a targeting construct together.

DESIGNING A KNOCKOUT TARGETING CONSTRUCT (REPLACEMENT VECTOR)

1. Once a gene is chosen for targeted inactivation, determine the best region of genomic sequence to disrupt through homologous recombination. With a replacement vector, two homologous recombination events will occur at the targeted locus via the long and short homology arms. The positive drug-resistance gene is inserted into a gene while coding sequence is simultaneously deleted. The length of genomic sequence replaced by the drug-selection marker is governed by the location of these homology arms. So, any intervening sequence situated between the two directed sites of recombination is omitted during the process of homologous recombination. The length of sequence that can be replaced by the positive-selection marker does not seem to influence the efficiency of homologous recombination. Up to 15 kb of sequence, for example, was deleted with a replacement vector to generate the T cell–receptor knockout mouse (Mombaerts et al., 1991). With most targeting constructs, however, one to a few critical exons are generally disrupted in gene-targeting experiments. Small deletions of 0.5 to 1 kb in length are typical for most gene-targeting experiments, and are usually sufficient to inactivate a gene (DeChiara, 2001). Genetic coding sequence essential for protein function should be selected for targeted deletion because there is the possibility that the inserted drug marker can be bypassed during transcription of the targeted allele. Any resulting truncated protein generated should, therefore, not be functional and would hopefully be rapidly degraded so as not to interfere with the cell's normal physiology. Generally, deleting 5′ exons provides a better chance to totally disrupt the formation of protein than a strategy that targets downstream exons at the C-terminus (Hasty et al., 1993). With genes smaller than 20 kb in length, some investigators prefer to design replacement vectors that disrupt all the coding exons in order to absolutely ensure gene inactivation (Cheah and Behringer, 2001). For any gene-targeting strategy, though, be sure that no neighboring genes are affected during homologous recombination.

2. After designating a location for targeted deletion, identify neighboring restriction-enzyme sites that are convenient for generating a short homology arm (~1 to 2 kb in length) as well as the long homology arm (~4 to 6 kb). A total amount of 5 to 8 kb of homology is typical in most targeting vectors (Hasty et al., 1993). Both the short and long homology arms may need to be subcloned into a vector such a pBluescript (Stratagene) in order to facilitate the assembly of the final targeting construct. Often, DNA-modifying enzymes are needed with subcloning, since convenient restriction enzyme sites may not be readily available in the genomic clone. The following DNA modifications may need to be applied for subcloning the homology arms:

a. Incompatible overhangs produced from a restriction enzyme digest can be blunted to help facilitate the subcloning of a homology arm. Blunt ligatable ends can be generated with enzymes like mung bean nuclease (to remove a 3′ overhang) or Klenow polymerase (to fill in a 3′ recessed end). However, sticky end modification can result in reduced ligation efficiency.

b. Adaptors can be purchased (from New England Biolabs, Stratagene, etc.) to insert a desired restriction enzyme site onto blunt-ended DNA. Alternatively, oligonucleotide adaptors can be generated to insert a restriction-enzyme site into cleaved DNA with sticky ends. Oligos must be designed so that, upon annealing, sticky ends are generated that are compatible to the cleaved DNA. The synthesized oligos are diluted to a concentration of 100 ng/μl in a buffer of 10 mM Tris·Cl/50 mM NaCl, pH 8.0. This solution is then heated at 95°C for 15 min and cooled to room temperature. The annealed oligos are ligated with the cleaved DNA at a 500 to 1000 molar excess. This high concentration helps to facilitate ligation of the oligo and inhibits mere re-ligation of the vector.

c. For ligation, the vector may need to be modified to prevent re-ligation, particularly if only one restriction enzyme is being used to cleave the DNA. In this case, the vector needs to be dephosphorylated to prevent re-ligation. Dephosphorylation of the DNA can be achieved by incubating the cleaved vector with an alkaline phosphatase (typically either calf intestinal phosphatase or shrimp alkaline phosphatase).

As mentioned above, PCR provides another means to clone out the homology arms, but careful attention should be made to ensure that no coding errors were generated during the reaction.

3. The targeting construct can be assembled after both homology arms and the drug-selection markers have been gathered. The targeting construct should be ligated so that the long and short homology arms flank the positive-selection marker. A negative-selection marker is subcloned outside of this grouping. Try to ligate the vector so that, upon recombination, the positive-selection marker is transcribed in the opposite orientation with respect to the targeted gene. A unique restriction enzyme site must be maintained to linearize the targeting construct to improve the efficiency of homologous recombination (Hasty et al., 1992). This restriction enzyme site should be located outside the regions of homology, typically between the plasmid backbone and a targeting arm. With linearization of the targeting construct, the plasmid DNA will protect the negative-selection marker (e.g., *HSV-tk* gene) from nucleases (Cheah and Behringer, 2001). The placement of either the long or the short arm between the two drug markers has varied among targeting vectors. While some investigators prefer to locate the *HSV-tk* adjacent to the long targeting arm (Torres and Kühn, 1997), other vectors place the *HSV-tk* gene at the end of the short targeting arm because this design may help improve the efficiency of negative selection (Melton, 2002).

Plasmids such as pPNT (Tybulewicz et al., 1991) or the pKO Scrambler Series (Lexicon Genetics; *http://www.lexgen.com*) can be useful in designing targeting constructs, since these vectors contain both *neo*^{r} and thymidine kinase genes. In addition, common restriction enzyme sites are positioned in locations that facilitate the subcloning of the homology arms. With pPNT, for example, one homology arm can be subcloned into the restriction enzyme sites (*Xba*I, *Bam*HI, *Kpn*I, and *Eco*RI) located between the *neo*^{r} and *HSV-tk* genes. Both of these drug-selection marker genes in this vector are driven by the 3-phosphoglycerate kinase promoter (PGK; McBurney et al., 1991), with the PGK pA-addition site. PGK is a housekeeping enzyme and the promoter is useful to drive high expression of these drug markers. The second homology arm can then be placed adjacent to the *neo*^{r} gene with *Not*I and *Xho*I restriction enzyme sites. Since *Not*I is a rare 8-base-pair cutter, this site is useful for linearizing targeting constructs. An example of a targeting construct is illustrated in Figure 19.12.1.

4. While the replacement vector is being constructed, a strategy to identify the targeted clones can be planned using all the collected sequence information about the genomic clone. Typically, to be certain of proper recombination, a Southern blot test should be designed to identify the targeted stem cell clone. With a Southern blot, an appreciable fragment-size difference must appear between the wild-type and the mutated allele. An ideal restriction enzyme should be identified that will best show a band difference from the digested genomic DNA. Southern blot analysis usually tests either the insertion or deletion of a restriction enzyme site due to homologous recombination. A fragment of the genomic clone is generally retained to make a Southern probe that contains sequence flanking the homology arms. This ensures that the Southern probe will not bind with random integrants. Therefore, the only bands to be detected with the Southern probe will be from either the recombined allele or the wild-type gene. PCR can also be used to find properly targeted clones. PCR can provide a fast, high-throughput means to test for targeted clones. With PCR, one primer will typically be designed within the *neo*^{r} gene and the other primer will be located outside the short arm of the replacement vector. Screening by PCR, however, can be tricky since long PCR products need to be amplified. In addition, there is always the risk of either false positives or negatives when using PCR to find a clone. Once a positive clone is identified, though, PCR becomes the preferred strategy to genotype the resulting mutant mice.

DESIGNING A KNOCK-IN TARGETING CONSTRUCT

Designing a knock-in construct follows the same basic rules as a knockout replacement vector. One homology arm, however, must consist of genomic sequence upstream of the protein initiation site. In this case, the 5′ and 3′ homology arms will flank both the knocked in cDNA and a positive drug-selection marker. Be sure to include a pA-addition signal for the knocked in cDNA. Upon recombination, the promoter in the targeted gene will drive expression of the inserted cDNA (Fig. 19.12.5). For efficient homologous recombination, the length of nonhomologous DNA (i.e., the cDNA and *neo*^{r} gene) inserted into a target locus should not exceed

the length of total homology (the long and short arms of the targeting construct).

DESIGNING A CONDITIONAL KNOCKOUT TARGETING CONSTRUCT

With a conventional knockout vector, an essential coding region in the targeted gene is replaced with a drug-selection marker during homologous recombination. For a conditional knockout mouse, however, the final targeted allele needs to be functionally intact. In this case, the end result of gene targeting is the placement of *loxP* sites around an essential coding region to create a floxed allele. Therefore, no sequence should be omitted by gene targeting. Basically, a continuous length of homologous genomic sequence is ideal in making a vector for a conditional knockout. A fragment of the genomic clone is merely cut in two with a restriction enzyme to derive a long and short homology arm. This design contrasts with a conventional knockout, where two separate lengths of homologous genomic sequence are needed to make the targeting vector. One of the homology arms is then modified to position a *loxP* site next to an essential exon for conditional deletion.

While a positive drug-selection marker (i.e., *neo*^{r} gene) is needed for initial enrichment of targeted clones, it must be floxed so that it will not interfere with the final mutated gene. Once the targeted clones are identified, this drug marker is removed by transient transfection to express Cre recombinase. If the correct recombination event occurs, then only a *loxP* site will remain after Cre excises the *neo*^{r} gene. In combination with the *loxP* site in the targeting arm, the exon(s) designated for conditional deletion should basically be floxed upon Cre-mediated rearrangement. However, Cre recombination can also result in the exclusion of the floxed exon(s) or both the exon(s) and the *neo*^{r} gene, so stem-cell clones must be screened to determine the correct rearrangement (Fig. 19.12.4).

1. A homologous 129/Sv genomic fragment continuous with the gene of interest needs to be isolated and characterized. The length of genomic DNA needed for a targeting vector follows the same rules listed for a conventional knockout vector, with roughly 5 to 10 kb of homologous sequence required for efficient recombination. Exon/intron boundaries and restriction-enzyme sites need to be mapped. Afterwards, the coding region to be floxed should be determined. The final *loxP* sites should be located within intronic sequence so that gene expression is not affected. In addition, a *loxP* site should not be designed into any promoter regulatory sequence, as this may interfere with transcription. In the floxed allele, all the coding sequence should be in frame. Again, check for any neighboring open reading frames that could be disrupted after Cre recombination.

2. A plasmid with a floxed *neo*^{r} gene should be acquired to create the targeting construct. A negative-selection marker should also be obtained, such as the *HSV-tk* gene.

3. Restriction enzyme sites in the genomic clone should be identified to aid in the placement of the *loxP* sites. It is best if two unique restriction enzyme sites can be found that flank a vital coding region for placement for the floxed *neo*^{r} gene, as well as a *loxP* site. As mentioned, the *loxP* sites should only be placed in intronic sequences. If available, the floxed *neo*^{r} gene can be subcloned into one of the unique restriction enzyme sites. In the second unique restriction enzyme site, a synthetic *loxP* site can then be ligated into the vector. Oligonucleotides with the *loxP* site and restriction-enzyme site overhangs can be synthesized, annealed, and ligated into the vector. The annealed synthetic oligo should be as shown in Figure 19.12.7.

A *loxP* site consists of two 13-bp inverted repeats that flank an asymmetric 8-bp core (underlined in Fig. 19.12.7). The asymmetric core determines the directionality of the *loxP* site. All *loxP* sites in the targeting construct must be in the same orientation. Otherwise, if the *loxP* sites are in opposite orientation, the floxed sequence will be inverted rather than deleted from the gene. To anneal the oligos, dissolve the DNA to form a 100 ng/μl solution in 10 mM Tris·Cl/50 mM NaCl, pH 8.0. Combine

```
5′-RE Site—ATAACTTCGTATAGCATACATTATACGAAGTTAT—RE Site-3′

3′-RE Site-TATTGAAGCATATCGTATGTAATATGCTTCAATA-RE Site-5′
```

Figure 19.12.7 Annealed synthetic oligos with *loxP* site and restriction-enzyme site overhangs. See text for additional detail.

sense and antisense strands in a microcentrifuge tube, heat at 95°C for 15 min, then let slowly cool to room temperature for proper annealing. If needed, the vector pBS246 can be obtained to procure the *loxP* sites. This plasmid has two *loxP* sites that flank a multiple cloning site (Sauer, 1993). Sequence can then be ligated around the *loxP* sites to get the desired floxed allele.

4. After proper placement of the floxed *neo*^{r} gene and a *loxP* site within the homologous genomic fragment, this cluster should then be ligated together with a negative-selection marker like *HSV-tk*. Again, a unique restriction enzyme site must be located adjacent to the homology arm so that the targeting vector can be linearized.

5. Eventually, after electroporation and isolation of targeted clones, the floxed *neo*^{r} gene should be removed by transient expression of Cre. Therefore, a Cre expression vector must be acquired for generation of the final mutated allele. One such plasmid is pBS185 (Sauer and Henderson, 1990). This vector will express Cre under the CMV promoter.

6. A PCR protocol should be designed to identify the floxed allele. With the sequence information from the genomic clone, primers can be designed to detect wild-type, floxed, and Cre-recombined alleles. If possible, cell culture work should be performed to test Cre excision and PCR analysis before the actual electroporation of the targeting construct into the stem cells.

LITERATURE CITED

Anton, M. and Graham, F.L. 1995. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: A molecular switch for control of gene expression. *J. Virol.* 69:4600-4606.

Askew, G.R., Doetschman, T., and Lingrel, J.B. 1993. Site-directed point mutations in embryonic stem cells: A gene-targeting tag-and-exchange strategy. *Mol. Cell. Biol.* 13:4115-4124.

Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 309:255-256

Bronson, S.K., Plaehn, E.G., Kluckman, K.D., Hagaman, J.R., Maeda, N., and Smithies, O. 1996. Single-copy transgenic mice with chosen-site integration. *Proc. Natl. Acad. Sci. U.S.A.* 93:9067-9072.

Capecchi, M.R. 1989. The new mouse genetics: Altering the genome by gene targeting. *Trends Genet.* 5:70-76.

Capecchi, M.R. 1994. Targeted gene replacement. *Sci. Am.* 270:52-59.

Capecchi, M.R. 2005. Gene targeting in mice: Functional analysis of the mammalian genome for the twenty-first century. *Nat. Rev.* 6:507-512.

Cheah, S.S. and Behringer, R.R. 2001. Contemporary gene targeting strategies for the novice. *Mol. Biotechnol.* 19:297-304.

Cohen-Tannoudji, M. and Babinet, C. 1998. Beyond knock-out mice: New perspectives for the programmed modification of the mammalian genome. *Mol. Hum. Reprod.* 4:929-938.

DeChiara, T.M. 2001. Gene targeting in ES cells. *Methods Mol. Biol.* 158:19-45.

Deng, C. and Capecchi, M.R. 1992. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol. Cell Biol.* 12:3365-3371.

Doetschman, T., Gregg, R.G., Maeda, N., Hooper, M.L., Melton, D.W., Thompson, S., and Smithies, O. 1987. Targeted correction of a mutant *HPRT* gene in mouse embryonic stem cells. *Nature* 330:576-578.

Dymecki, S.M. 1996. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 93:6191-6196.

Evans, M.J. and Kaufman, M.H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Feil, R., Wagner, J., Metzger, D., and Chambon, P. 1997. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem. Biophys. Res. Commun.* 237:752-757.

Ferradini, L., Gu, H., De Smet, A., Rajewsky, K., Reynaud, C.A., and Weill, J.C. 1996. Rearrangement-enhancing element upstream of the mouse immunoglobulin kappa chain J cluster. *Science* 271:1416-1420.

Grimm, D. 2006. A mouse for every gene. *Science* 312:1862-1866.

Gu, H., Marth, J.D., Orban, P.C., Mossman, H., and Rajewsky, K. 1994. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* 265:103-106.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A.B., and Joyner, A.L. 1995. Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. *Science* 269:679-682.

Hasty, P., Rivera-Pérez, J., and Bradley, A. 1991. The length of homology required for gene targeting in embryonic stem cells. *Mol. Cell. Biol.* 11:5586-5591.

Hasty, P., Rivera-Pérez, J., and Bradley, A. 1992. The role and fate of DNA ends for homologous recombination in embryonic stem cell. *Mol. Cell. Biol.* 12:2464-2474.

Hasty, P., Abuin, A., and Bradley, A. 1993. Gene targeting, principles, and practice in mammalian cells. *In* Gene Targeting: A Practical Approach (A.L. Joyner, ed.) pp. 1-35. Oxford University Press, New York.

Hayashi, S. and McMahon, A.P. 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: A tool for temporally regulated gene activation/inactivation in the mouse. *Dev. Biol.* 244:305-318.

Iredale, J.P. 1999. Gene knockouts. *Mol. Pathol.* 52:111-116.

Jasin, M., Moynahan, M.E., and Richardson, C. 1996. Targeted transgenesis. *Proc. Natl. Acad. Sci. U.S.A.* 93:8804-8808.

Ledermann, B. 2000. Embryonic stem cells and gene targeting. *Exp. Physiol.* 85.6:603-613.

LeMouellic, H., Lallemand, Y., and Brûlet, P. 1990. Targeted replacement of the homeobox gene Hox-3.1 by the *Escherichia coli lacZ* in mouse chimeric embryos, *Proc. Natl. Acad. Sci. U.S.A.* 87:4712-4716.

Lin, F.L., Sperle, K., and Sternber, N. 1984. Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences. *Proc. Natl. Acad. Sci. U.S.A.* 82:1391-1395.

Lu, Z.H., Brooks, J.T., Kaufman, R.M., and Ley, T.J. 2003. Long targeting arms do no increase the efficiency of homologous recombination in the β-globin locus of murine embryonic stem cells *Blood* B:1531-1533.

Mak, T.W. (ed.) 1998. The Gene Knockout Factsbook. Academic Press, New York.

Majzoub, J.A. and Muglia, L.J. 1996. Knockout mice. *New Engl. J. Med.* 334:904-907.

Mansour, S.L., Thomas, K.R., and Capecchi, M.R. 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: A general strategy for targeting mutations to non-selectable genes *Nature* 336:348-352.

Martin, G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

McBurney, M.W., Sutherland, L.C., Adra, C.N., Leclair, B., Rudnicki, M.A., and Jardine, K. 1991. The mouse Pgk-1 gene promoter contains an upstream activator sequence *Nucleic Acids Res.* 19:5755-5761.

Melton, D.W. 2002. Gene-targeting strategies *Methods Mol. Biol.* 180:151-173.

Mombaerts, P., Clarke, A.R., Hooper, M.L., and Tonegawa, S. 1991. Creation of a large genomic deletion at the T-cell antigen receptor β-subunit locus in mouse embryonic stem cells by gene targeting. *Proc. Natl. Acad. Sci. U.S.A.* 88:3084-3087.

Mortensen, D. 2006. Overview of gene targeting by homologous recombination *Curr. Protoc. Mol. Biol.* 76:23.1.1-23.5.12.

Mouse Genome Sequencing Consortium. 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature* 420:520-562.

Müller, U. 1999. Ten years of gene targeting: Targeted mouse mutants, from vector design to phenotype analysis *Mech. Dev.* 82:3-21.

Olson, E.N., Arnold, H.H., Rigby, P.W., and Wold, B.J. 1996. Know your neighbors: Three phenotypes in null mutants of the myogenic bHLH gene MRF4 *Cell* 85:1-4.

Rickert, R.C., Roes, J., and Rajewsky, K. 1997. B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res.* 25:1317-1318.

Rijkers, T., Van Den Ouweland, J., Morolli, B., Rolink, A.G., Baarends, W.M., Van Sloun, P.P.H., Lohman, P.H.M., and Pastink, A. 1998. Targeted inactivation of mouse RAD52 reduces homologous recombination but not resistance to ionizing radiation *Mol. Cell Biol.* 18:6423-6429.

Sargent, R.G. and Wilson, J.H. 1998. Recombination and gene targeting in mammalian cells *Curr. Res. Mol. Ther.* 1:584-592.

Sauer, B. 1993. Manipulation of transgenes by site-specific recombination: Use of Cre recombinase. *Meth. Enzymol.* 225:890-900.

Sauer, B. and Henderson, N. 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol.* 2:441-449.

Sauer, B. and Henderson, N. 1998. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. U.S.A.* 85:5166-5170.

Schwartzberg, P.L., Goff, S.P., and Robertson, E.J. 1989. Germ-line transmission of a *c-abl* mutation produced by targeted gene disruption in ES cells. *Science* 246:799-803.

Sikorski, R. and Peters, R. 1997. Transgenics on the internet. *Nat. Biotech.* 15:289.

Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A., and Kucherlapati, R.S. 1985. Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. *Nature* 317:230-234.

Stacey, A., Schnieke, A., McWhir, J., Cooper, J., Colman, A., and Melton, D.W. 1994. Use of double-replacement gene targeting to replace the murine α-lactalbumin gene with its human counterpart in embryonic stem cells and mice. *Mol. Cell Biol.* 14:1009-1016.

Sternberg, N. and Hamilton, D. 1981. Bacteriophage P1 site-specific recombination. *J. Mol. Biol.* 150:467-486.

te Riele, H., Maandag, E.R., and Berns, A. 1992. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc. Natl. Acad. Sci. U.S.A.* 89:5128-5132.

Thomas, K.R. and Capecchi, M.R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51:503-512.

Thomas, K.R., Folger, K.R., and Capecchi, M.R. 1986. High frequency targeting of genes to specific sites in the mammalian genome. *Cell* 44:419-428.

Torres, R.M. and Kühn, R. 1997. Laboratory Protocols for Conditional Gene Targeting. Oxford University Press, New York.

Tybulewicz, V.L.J., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C. 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-able proto-oncogene. *Cell* 65:1153-1163.

Utomo, A.R.H., Nikitin, A.Y., and Lee, W.H. 1999. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat. Biotechnol.* 17:1091-1096.

Vanlancius, V. and Smithies, O. 1991. Testing an "in-out" targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. *Mol. Cell Biol.* 11:1402-1408.

Vasquez, K.M., Marburger, K., Intody, Z., and Wilson, J.H. 2001. Manipulating the mammalian genome by homologous recombination. *Proc. Natl. Acad. Sci. U.S.A.* 98:8403-8410.

Vogel, G. 2007. A knockout award in medicine. *Science* 318:178-179.

Wong, E.A. and Capecchi, M.R. 1987. Homologous recombination between coinjected DNA sequences peaks in early to mid-S phase. *Mol. Cell Biol.* 7:2294-2295.

Yagi, T., Ikawa, Y., Yoshida, K., Shigetani, Y., Takeda, N., Mabuchi, I., Yamamoto, T., and Aizawa, S. 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A–fragment gene in negative selection. *Proc. Natl. Acad. Sci. U.S.A.* 87:9918-9992.

Zambrowicz, B.P. and Sands, A.T. 2003. Knockouts model the 100 best-selling drugs—will they model the next 100? *Nat. Rev.* 2:38-51.

Key References

Kmiec, E.B. (ed.) 2000. Gene Targeting Protocols. Humana Press, Totowa, N.J.

Gene knockout methodologies discussed in this book include different strategies for gene transfer and techniques for gene targeting.

Internet Resources

http://www.cellmigration.org/resource/komouse/komouse_resources_targeting4beginners.shtml

This Web site was established throuhg the Cell Migration Consortium to provide strategies for gene targeting as well as important links useful for acquiring genomic sequence information.

Manipulation of Mouse Embryonic Stem Cells for Knockout Mouse Production

Advait Limaye,[1] Bradford Hall,[1] and Ashok B. Kulkarni[1]

[1]National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland

ABSTRACT

The establishment of mouse embryonic stem (ES) cell lines has allowed for the generation of the knockout mouse. ES cells that are genetically altered in culture can then be manipulated to derive a whole mouse containing the desired mutation. To successfully generate a knockout mouse, however, the ES cells must be carefully cultivated in a pluripotent state throughout the gene-targeting experiment. This unit describes detailed step-by-step protocols, reagents, equipment, and strategies needed for the successful generation of gene knockout embryonic stem cells using homologous recombination technologies. *Curr. Protoc. Cell Biol.* 44:19.13.1-19.13.24. © 2009 by John Wiley & Sons, Inc.

Keywords: mouse embryonic stem cells • homologous recombination

INTRODUCTION

The isolation of murine embryonic stem cells (ES) has been an important discovery necessary for the development of mice with designer mutations (*UNIT 19.12*). Stem cells are capable of self-renewal in culture and can be maintained in an undifferentiated state under certain growth conditions (Support Protocol 2). Stem cells are also totipotent, and, when injected into a host blastocyst (*UNIT 19.14*), can contribute to the somatic and germ-cell lineages of the resulting chimeric mouse. If germline transmission occurs, an offspring of the chimeric mouse can be produced that was derived from the injected ES cell clone. The ability to pass on germline transmission means that a mouse can be generated from ES cells that are genetically manipulated in culture.

With the discovery of homologous recombination, stem cells were seen as an ideal tool that could be used to make genetically altered mice. Homologous recombination in stem cells was first applied for the development of knockout mice through targeted gene inactivation. Eventually, these techniques were adapted for creating conditional knockout mice, knock-in mice, and mice with subtle mutations, such as genetic point mutations, deletions, and insertions. With gene targeting in mice, scientists have been able to create animal models that mimic human genetic diseases. Additionally, these animal models have also provided an important tool for delineating the function of a gene in vivo.

For knockout mouse production, the mouse ES cells must be transformed with a plasmid bearing the target gene construct (*UNIT 19.12*). Electroporation is the method of choice for transforming the cells. Once electroporated, the ES cells are subjected to drug selection, cloned, frozen, and characterized (Basic Protocol 2).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator unless otherwise indicated.

Current Protocols in Cell Biology 19.13.1-19.13.24, September 2009
Published online September 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1913s44

BASIC PROTOCOL 1

INTRODUCTION OF PLASMID DNA INTO PLURIPOTENT MOUSE EMBRYONIC STEM CELLS

With the discovery of homologous recombination, scientists quickly realized that ES cells would be useful for gene-targeting experiments. A targeting construct is delivered into a stem cell where homology arms in the vector align with a targeted genetic locus to introduce a gene modification. While not exactly the most efficient technique for producing homologous recombination events, electroporation (Basic Protocol 1) became the preferred and easiest way for mass delivery of targeting constructs into a large number of stem cells (Vasquez et al., 2001). Targeting constructs usually contain a positive drug-selection marker to enrich for clones that have successfully taken up the vector. The neomycin phosphotransferase (*neo*^{r}) gene is a well known positive-selection marker that confers resistance to the drug neomycin sulfate (G418) in tissue culture. In addition, gene-targeting vectors also generally include a negative drug-selection marker outside of the homology arms to select against random integrants (Mansour et al., 1988). For example, the HSV thymidine kinase (*HSV-tk*) gene is a widely used negative selection marker that phosphorylates drugs like gancyclovir or FIAU (1-[2′-deoxy-2′-fluoro-β-D-arabinofuranosyl]-5-iodouracil) into cytotoxic compounds. Clones with random integration of the complete vector are thereby excluded upon drug treatment. Thus, after electroporation of the targeting construct, the stem cells are plated onto culture dishes and treated for about 10 days under drug selection to select for clones that have undergone a true homologous recombination event. Once a stem cell with the designated genetic alteration is identified, the ES cell clone is then usually expanded and injected into a blastocyst to derive a chimeric mouse containing cells with the targeted mutation (*UNIT 19.14*). The mice produced from mating chimeric mice with the wild-type mice will be either wild-type or heterozygous for the targeted mutation, and these heterozygous mice must be bred to homozygosity.

Materials

Naïve (non-electroporated) ES cells growing in culture (Support Protocol 2) on mitotically arrested feeder layers (Support Protocol 1) in 6-cm^2 dishes
Embryonic stem cell medium (ESM; see recipe)
Targeting vector (*UNIT 19.12*)
Electroporation buffer (EB; see recipe)
Calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS; Cellgro)
0.25% (w/v) trypsin/EDTA (Invitrogen)

15- and 50-ml sterile disposable centrifuge tubes
Refrigerated centrifuge
Electroporation cuvette 0.4-mm gap (BioRad)
Gene Pulser Xcell electroporation system with capacitance extender (BioRad)
250-ml disposable plastic Erlenmeyer flask, sterile (Corning)
6-cm^2 tissue culture plates of PMEF cells (MMC-treated; Support Protocol 1)

Additional reagents and equipment for restriction enzyme digestion (Bloch and Grossman, 1995), confirmation of linearized targeting vector (e.g., agarose gel electrophoresis; Voytas, 2000), ethanol precipitation of DNA (Moore and Dowhan, 2002), and counting cells (*UNIT 1.1*)

Prepare ES cells and vector

1. Refeed the ES cell culture dishes with ESM to encourage cell growth before electroporation. Grow cells to 70% to 80% confluency.

   Cells should be ready for electroporation (step 10) in 2 to 3 days, depending upon the plating density.

2. Linearize the targeting vector with the appropriate restriction enzyme (Bloch and Grossman, 1995). Confirm that the targeting vector is linearized by running a small aliquot of the digested vector on an agarose gel (Voytas, 2000). Dry the vector, generally through ethanol precipitation (Moore and Dowhan, 2002).

   The plasmid should be free of any contaminants such as ethanol and phenol.

3. Dissolve 25 μg of targeting vector in 25 μl of EB to make a final concentration of 1 μg/μl DNA.

   In other protocols, the targeting vector is dissolved in sterile CMF-DPBS.

Collect ES cells

4. Aspirate the medium from the ES cell dishes, and wash with 2 ml of CMF-DPBS per dish.

5. Aspirate the CMF-DPBS and then cover the cells with 1 ml of 0.25% trypsin/EDTA per dish. Incubate for 3 min at 37°C.

6. Inactivate the trypsin by adding 4 ml ESM per dish. Pipet the cells vigorously up and down to dissociate the stem cell colonies into a single-cell suspension.

7. Pool the cell/medium mixture from all the dishes and collect in a 50-ml centrifuge tube. Wash any residual cells remaining on the culture dishes with an additional 5 ml of ESM and add to the pooled cell suspension.

8. Centrifuge the ES cells 5 min at 693 × *g*, 4°C.

9. Aspirate the supernatant and resuspend the pellet in 5 to 10 ml ESM.

   10 ml is a convenient volume for calculation of total number of cells.

Prepare cells for electroporation

10. Count a small aliquot of the ES cells using either a hemacytometer (*UNIT 1.1*) or an automated cell counting device.

    If using the hemacytometer, the ES cells will appear rounder and smaller than the arrested feeder cells. Some investigators use trypan blue to differentiate between dead and live cells. The dead (blue) cells will not be able to remove the trypan blue and should be excluded from the count if using a hemacytometer.

11. Select a volume of cell suspension that will ensure that at least 1×10^7 cells are available for the electroporation and transfer to a 1.5-ml microcentrifuge tube.

12. Centrifuge the ES cells 5 min at 693 × *g*, 4°C. Aspirate the supernatant and resuspend the cell pellet in 0.8 ml of EB.

    Some investigators have used sterile phosphate-buffered saline on ice as the buffer for electroporation.

13. Transfer the cell suspension to a sterile 1.5-ml microcentrifuge tube.

Electroporate the cells

14. Add the targeting construct (from step 2) to the tube containing the ES cells, and gently mix the DNA with the ES cells.

    At this stage, it is important to have a single-cell suspension.

15. Transfer the mixture to a 0.4-mm electroporation cuvette (try to avoid forming air bubbles).

16. Place the cuvette into the shock pod of the electroporator so that the metal plates on the cuvette are in contact with the metal plates of the shock pod.

17. Electroporate the cell suspension at the appropriate setting.

For most cells, e.g., R1, use settings of 230 V, 500 μF, and an electrode gap of 0.4 cm with an exponential decay. With these settings, the time constant should be between 5.6 to 8.0 msec, depending upon the solutes in the medium and the cell concentration. Alternatively, settings of 400 V and 25 μF have been used. In this case, the time constant should be between 0.4 and 0.5 msec.

After the shock, bubbles or foam at the top of the cuvette may be apparent, signaling that current has passed through the solution.

Optimal settings will vary depending upon ES cell type and must be determined experimentally.

Plate the electroporated cells

18. After electroporation of the cells, leave the cuvette containing the electroporated cells at room temperature for 10 min.

For mammalian cells, it is not critical to chill the cells before or after electroporation.

19. Transfer the entire contents of the electroporation cuvette to 200 ml of ESM in a sterile disposable 250-ml Erlenmeyer flask, resulting in a concentration of 5×10^4 cells per ml to be plated onto feeder dishes. Wash the cuvette with about 0.8 ml of the ESM and transfer to the flask to retain as many of the electroporated cells as possible.

As a result of electrophoretic shock, many of the cells will die off and will be seen floating in the medium. With the initial cell density of 1×10^7 cells, enough cells should survive to undergo drug selection.

20. Plate 4 ml electroporated cells per 6-cm^2 plate with mitomycin C–treated PMEF feeder cells. Incubate at 37°C.

BASIC PROTOCOL 2

SELECTING, FREEZING, AND CHARACTERIZING DRUG-RESISTANT ES CELLS

Successfully electroporated ES cells are exposed to a selecting drug based on the plasmid structure. These cells will survive the selection process and can be used for creating chimeric mice.

Materials

Electroporated ES cells (Basic Protocol 1) growing in 6-cm^2 dishes
Embryonic stem cell medium (ESM; see recipe)
Positive selection agent (G418; see recipe)
Negative selection agent (FIAU; see recipe)
Naïve (non-electroporated) ES cells
Calcium- and magnesium-free Dulbecco's PBS (CMF-DPBS; Cellgro)
0.25% (w/v) trypsin/EDTA (Invitrogen)
2× ES cell freezing medium (see recipe)
0.2% gelatin (from porcine skin, type A; Sigma) in tissue culture-grade water, sterilized by autoclaving
Liquid nitrogen

Pipettor from 20 μl to 200 μl to mix cell suspensions, plus sterile tips (Rainin)
Pipettor from 200 μl to 1000 μl to mix cell suspensions, plus sterile tips (Rainin)
Repeat pipetting device from 100 μl to 1000 μl (useful but optional; Eppendorf)
Light-Touch Pipetting System (Rainin; optional) requires less hand pressure and reduces fatigue
24-well tissue culture plates (Fisher Scientific)
Forceps

Flame sterilizer (Fireboy Plus, Integra Biosciences)
Glass cloning cylinders in petri dish (see recipe)
2-ml liquid nitrogen cryovials (Nalgene/Nunc)
12-well tissue culture plates (Fisher Scientific)
Mr. Frosty slow-cool chamber (Nalgene) containing isopropanol (Fisher Scientific)
Liquid nitrogen freezer (Thermo-Forma)

Additional reagents and equipment for preparation of PMEF feeder plates (Support Protocol 1), purification of DNA by isopropanol precipitation (Laird et al., 1991), Southern blotting (Brown, 1993), and PCR (*APPENDIX 3F*)

Treat electroporated ES cells with selective drugs

1. Begin drug selection for resistant clones (as described in the steps below) within 20 hr of electroporation of targeting vector DNA into ES cells (see Basic Protocol 1).

   Drug selection helps reduce the population of nontargeted ES cells.

2. Treat the majority of dishes with ESM supplemented with positive drug-selection agent (G418) and negative selection agent (FIAU). Treat one or two dishes with ESM and G418 only (i.e., only positive drug selection) to check for the degree of random targeting vector insertion (targeting vector inserted randomly in the genome should include the *HSV-tk* cassette, and this would be toxic to the ES cell colony).

   If diphtheria toxin A (DTA) is the negative selection cassette, no negative selection agent is necessary.

   The ideal G418 concentration must be determined experimentally for each ES cell strain by performing a kill curve. Briefly, plate 1 × 10^6 naïve (non-electroporated) ES cells. Start with a G418 concentration of 150 μg/ml and increase in 50- to 100-μg increments to a maximal concentration of 350 μg/ml. Grow the cells 3 to 4 days, feeding daily. On the third or fourth day, assess the number of viable cells (UNIT 1.1) per concentration. Select the lowest concentration that kills all of the naïve ES cells within 3 to 4 days of selection pressure to use for drug selection.

   The FIAU is used at an 0.2 μM concentration. To obtain this concentration in the ESM plus G418 plus FIAU medium, add 0.444 ml of the 270 μM negative selection agent (FIAU) stock solution (see recipe in Reagents and Solutions) to 600 ml of the ESM plus G418 solution (G418 concentration determined as described above), which will result in 0.2 μM FIAU.

3. Replace the medium daily with ESM plus G418 plus FIAU for 4 to 5 days, followed by ESM plus G418 only for 3 to 4 days.

   The total period for drug selection should be about 8 to 10 days.

4. After this period, assess the dishes for pinpoints of circular, white, drug-resistant colonies that are visible without a microscope. Lift each dish and circle the underside of the dish to mark candidate colonies for expansion.

   Colonies that are starting to differentiate will sometimes appear more flat, with a diffuse haze around them. Avoid picking these colonies.

Prepare PMEF plates for receiving colonies

5. The day before picking colonies, prepare 24-well MMC-treated PMEF feeder plates to receive the targeted clones for expansion as described in Support Protocol 1, at a concentration of 0.8 to 1.0 × 10^5 cells/ml. Dispense 0.5 ml of the feeder suspension per well on gelatinized 24-well plates. Prepare enough plates and wells to receive at least 200 colonies.

   The gelatinization of the plates is also described in Support Protocol 1.

6. The next day, replace the 0.5-ml volume per well in the 24-well plates with 1 ml of ESM, before starting to pick the stem cell colonies from the 6-cm^2 dishes.

Pick colonies

7. Aspirate the medium from one 6-cm^2 dish of drug-treated ES cells and replace with 1 to 2 ml of CMF-DPBS.

   The CMF-DPBS washes away the serum from the ESM, which would inhibit the action of trypsin in subsequent steps.

8. Aspirate the CMF-DPBS from the dish of cells. Sterilize a pair of forceps with a flame sterilizer and use the forceps to vertically lift the cloning cylinders swiftly off the glass dish.

   The grease should be evenly distributed along the bottom of the cylinder.

9. Place a greased cloning cylinder around a marked suitable colony and gently press onto the culture dish. Ensure that the cylinder is positioned around only a single colony so that no adjacent colonies are harvested in the process.

   Whenever possible, try to avoid picking two colonies in close proximity to each other. In such cases, one colony may pass on neomycin resistance to the nearby colony, and as both colonies would fit under the same cloning cylinder, they would be picked together. This is called the "bystander effect."

10. Repeat these steps (flaming the forceps each time) until all good colonies on the dish have a cloning cylinder above them.

11. Using the repeat pipettor, dispense 200 µl of 0.25% trypsin/EDTA into each cloning cylinder.

    The vacuum grease will hold the liquid inside the cylinder, acting as a miniwell.

    Usually it is best to limit the number of dishes to one or two at a time, to prevent over-trypsinizing the colonies.

12. Incubate the dish in the tissue culture incubator for 3 min at 37°C.

13. After 3 min, use a 200-µl pipet and a sterile tip to pipet the trypsin up and down in the cloning cylinder.

    Pipetting is needed to disperse the colony clumps. If dissociated correctly, a single-cell suspension should be achieved through trypsinization.

14. Take the entire contents of each miniwell on the dish and dispense into one well of the 24-well plate (prepared in steps 5 to 6) containing MMC-treated PMEFs in 1 ml of ESM supplemented with G418 (see step 2 annotation for determining appropriate concentration).

15. Repeat steps 13 and 14, using new pipet tips each time, until all the colonies have been transferred to the 24-well plates of feeders.

16. Replace the medium in the 24-well feeder plates every day with 1.0 ml/well of ESM supplemented with G418 until the ES cells have grown sufficiently. Watch the stem cells daily. Be prepared to freeze the cells whenever the growing colonies in the well appear to be getting confluent or close to touching.

    The stem cells should be ready for harvesting within a few days after the colonies are isolated with the cloning cylinders.

    While an ample number of cells need to be grown for storage and freezing, ensure that the stem cell culture does not become overgrown and start to differentiate. The ES cell colonies should have very smooth, sharp borders (see Figure 19.13.3) and should not appear flat.

If the medium indicator appears orange to yellow, then the colonies are probably over-confluent and may be ready to differentiate. These wells should be avoided whenever possible.

17. Prepare the 2× ES cell freezing medium at this stage (see Reagents and Solutions). Dispense 0.5 ml of 2× ES cell freezing medium into each 2-ml cryovial and store at 4°C.

18. Prepare gelatinized 12-well plates by adding 1 ml/well of 0.2% gelatin and then incubating ~30 min at room temperature in a sterile laminar flow hood. Discard gelatin solution and allow dishes to dry to provide a surface for cells to attach.

    These plates will be needed for DNA testing of the potentially targeted clones.

19. Examine each well daily to determine which colonies need to be frozen down. Mark the appropriately confluent wells on the 24-well plates to prepare for harvesting, leaving the subconfluent wells unmarked until they become confluent.

    The growth rate of the stem cell colonies will vary among the wells on the tissue culture plate. Often, these differences can be due to the number of cells recovered when picking the colonies.

Freeze clones

20. Aspirate the medium from the marked wells and replace with 1 ml/well of CMF-DPBS.

21. Replace the CMF-DPBS with 200 µl of 0.25% trypsin/EDTA and transfer to the incubator for 3 min at 37°C.

22. Neutralize the trypsin with 800 µl of ESM.

    The total volume in each well of the marked 24-well plates should be 1 ml.

23. Using a 1-ml pipettor with a sterile tip, pipet up and down to make a single-cell suspension.

    It is often convenient to use a pipettor that requires less force to pipet up and down, particularly when many clones are to be frozen down. We have used the Light-Touch Pipetting System pipettors and pipet tips (Rainin) for this purpose.

24. Remove 500 µl of the cell suspension and transfer it to a prefilled freezer vial (see step 17) to make the total volume 1 ml. Place the freezer vials in a slow-cool chamber and place the chamber in a –80°C freezer.

25. Add 500 µl ESM to each gelatin-coated well of a gelatinized 12-well plate (see step 18). Transfer the remainder of the cell suspension (~500 µl) to an ESM-containing well of the plate. Transfer the 12-well plates to the incubator.

    Over several days, the ES cells in the 12-well plates should differentiate and overgrow, resulting in the color of the indicator in the medium turning yellow. It is not necessary to maintain pluripotency in the 12-well plates, since the cells will only be used to purify genomic DNA.

26. After collecting the stem cell freezer vials, transfer the vials in the slow-cool chamber into a −80°C freezer (the temperature in the chamber should drop about 1°C/min).

27. Once the freezer vials are frozen to −80°C, transfer the vials to a liquid nitrogen freezer for longer-term storage.

    Generally, these tubes should be stored in the vapor phase of the freezer, which minimizes the chance of the plastic cracking, which would result in liquid nitrogen seeping into the freezer vials and cross-contaminating the samples.

Verify that the clones contain the desired sequence

28. Purify genomic DNA from the clones on the gelatinized plates with medium that has turned yellow, indicating overgrown cells (Laird et al., 1991).

    Briefly the cells are lysed with proteinase K solution and DNA is precipitated using an equal volume of isopropanol. The DNA clump is picked out of solution, briefly dried, then transferred to water or 1× TE buffer.

29. Use the purified DNA to determine whether a targeted event has taken place, either by Southern blot hybridization (Brown, 1993) or by PCR (*APPENDIX 3F*).

SUPPORT PROTOCOL 1

CULTURE OF PRIMARY MOUSE EMBRYONIC FIBROBLASTS AND PREPARATION OF MOUSE EMBRYONIC STEM CELL FEEDERS

For a gene-targeting experiment, mouse embryonic stem cells in culture need to be maintained in a pluripotent stage until they are injected into mouse blastocysts. Usually, to maintain these conditions, a feeder cell layer is used to provide an environment that helps to prevent differentiation. The feeder cells secrete unknown growth factors that help preserve pluripotency. Feeder cells are produced from either STO embryonic fibroblasts or primary mouse embryonic fibroblasts (PMEFs). PMEFs are typically employed in gene-targeting experiments and are derived from embryonic day 14.5 embryos. The embryos are minced, and the dissociated cells are placed into culture. The adherent fibroblasts are then usually passaged a few times to obtain an adequate number of cells to be frozen in aliquots. PMEFs can also be purchased from commercial sources, which can save time and cage space. One important point is that the PMEFs should be resistant to whichever positive selection cassette is used within the gene-targeting construct (e.g., neomycin). Otherwise, this feeder layer will not support ES cell growth once the drug selection is introduced. PMEFs can be generated from the embryos of an established knockout mouse that bears the same drug-resistance cassette (see Support Protocol 1 materials list). Fibroblasts derived from the knockout mice should have the necessary resistance cassette integrated into their genome. Feeder cells need to be growth inhibited before coculture with ES cells. Typically, the cells are mitotically inactivated either by treatment with the drug mitomycin C or through γ irradiation. If using mitomycin C treatment, be sure to wash the cells thoroughly since this drug can cause differentiation of the stem cells. To enhance the growth conditions needed to maintain pluripotency, the culturing of ES cells on PMEFs is usually supported by the use of gelatin-coated plates and the addition of LIF (leukemia inhibitory factor) to the stem cell medium. LIF is a cytokine that helps to prevent differentiation of the cells. While ES cells can be maintained with LIF in the absence of PMEFs, an increased tendency towards differentiation will develop with long-term culture of these cells (Nichols et al., 1990). This seems to be the case especially with ES cells that have undergone many passages. A brief illustration depicting the critical steps in the generation of targeted embryonic stem cells is shown in Figure 19.13.1.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male mice harboring a positive-resistance cassette on both alleles (e.g. homozygous knockout mice such as α-galactosidase A knockout mice obtained from The Jackson Laboratory, Accession no. 003535); and homozygous knockout female mouse

Calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS; Cellgro)

70% ethanol
0.05% (w/v) trypsin/EDTA (Invitrogen)
Embryonic feeder medium (EFM; see recipe)
2× PMEF cell freezing medium (see recipe)
0.2% gelatin (from porcine skin, type A; Sigma) in tissue culture-grade water, sterilized by autoclaving
Embryonic feeder medium (EFM) containing 10 μg/ml mitomycin C (see recipe)
10-cm^2 and 6-cm^2 tissue culture-grade dishes (non-gelatin-coated; Fisher Scientific)
Curved iris scissors (Roboz Surgical Instruments)
Straight fine scissors (Roboz Surgical Instruments)
Forceps
Sterilizer pouches to autoclave instruments (Fine Science Tools)
Single-edged razor blades
50- and 15-ml conical centrifuge tubes
Dedicated centrifuge able to achieve 4°C (e.g., Beckman-Coulter Allegra X-22R)
2-ml cryovials (Nalge/Nunc)
Mr. Frosty slow-cool chamber (Nalgene) containing isopropanol (Fisher Scientific)
250-ml disposable plastic Erlenmeyer flasks, sterile (Corning)
Additional reagents and equipment for euthanasia of the mouse (Donovan and Brown, 2006) and counting cells (*UNIT 1.1*)

Collect embryos

1. Mate homozygous knockout male mice harboring a positive resistance cassette to a similar homozygous knockout female mouse.
2. The next morning, observe the copulation plug (embryonic day 0.5) and wait until the embryos are embryonic day 13.5 to 14.5.
3. Prepare three 10-cm^2 tissue culture dishes each containing 15-ml sterile CMF-DPBS; also prepare sterile, autoclaved scissors and forceps.
4. Euthanize the mouse by CO_2 asphyxiation, cervical dislocation, or whichever method has been approved by your Institutional Animal Care and Use Committee.

   Donovan and Brown (2006) provides protocols for all common methods of mouse euthanasia.

5. Wipe the fur surrounding the abdominal cavity with 70% ethanol to clean it and to prevent fur and dander from entering into the abdominal cavity.
6. Make a vertical incision into the abdominal cavity and locate the uterus.

   The uterus should be swollen with embryos, resembling a sac with many bulges.

7. Detach the uterus at both ends with the straight scissors and remove from the abdominal cavity with fine-tipped scissors. Place the uterus into one of the 10-cm^2 dishes filled with 15 ml sterile CMF-DPBS that were prepared in step 3.
8. Move the uterus around in the dish to wash off some of the blood.
9. Carefully cut open the uterus, releasing the embryos into the CMF-DPBS.

Dissect embryos

10. Remove the placenta and membranes. Transfer the embryos to a new 10-cm^2 dish of CMF-DPBS (prepared in step 3).
11. Hold each embryo with forceps, scoop out the liver, and decapitate the embryos with a single-edged razor blade.

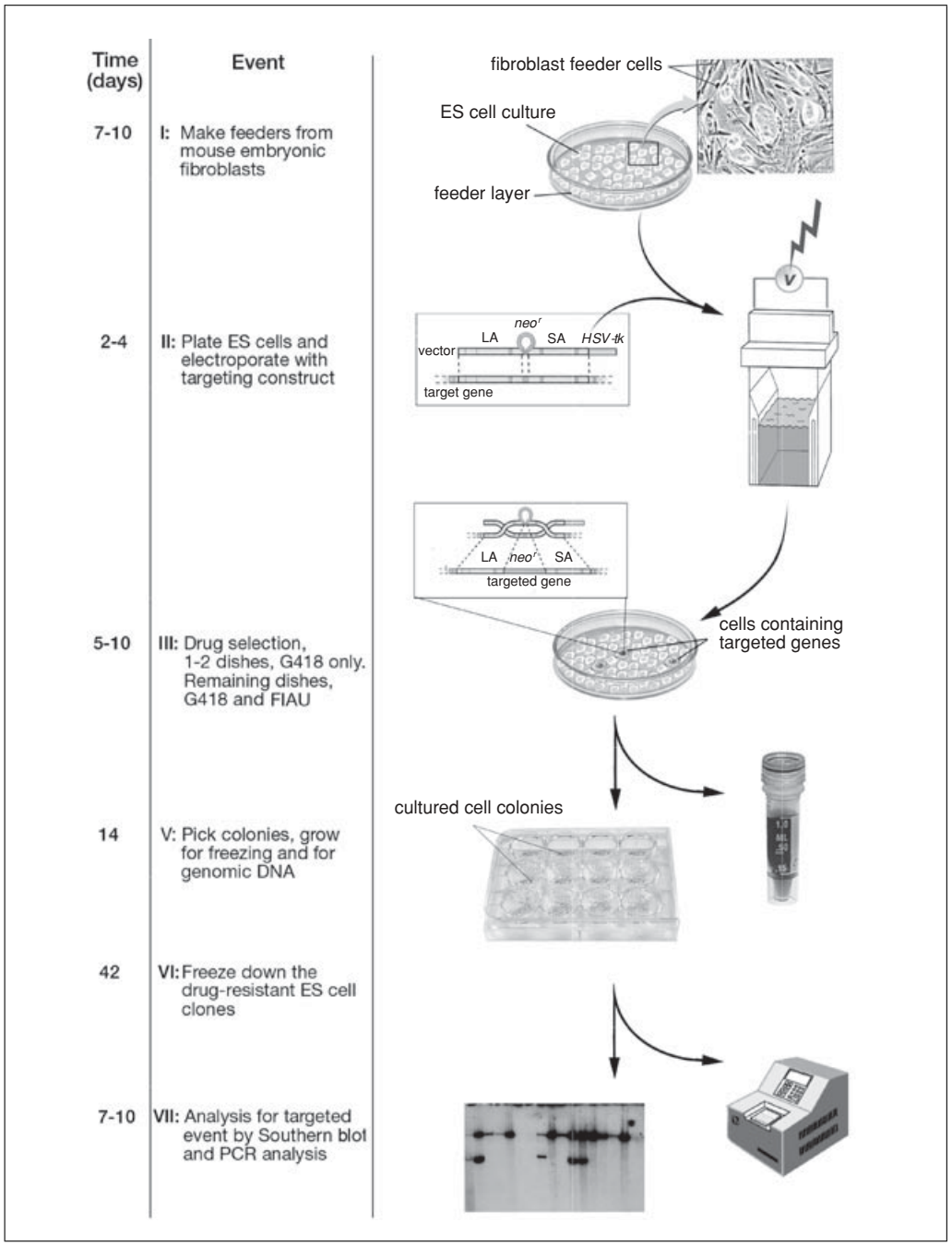


Figure 19.13.1 Overview of electroporation and selection. The left column shows the approximate number of days at each critical stage in the preparation of targeted embryonic stem cells. The middle column describes key procedural events. The right column is an illustration of each critical event. Note that in stages II through V, embryonic stem cells should remain as close to pluripotent as possible. Once the cultured ES cells have been plated to the tissue culture plate depicted in stage V, they may become differentiated. These cells will used for DNA screening as depicted in stage VII. In stage VII, the left panel represents an autoradiographic film image of a Southern blot. Southern blotting is the definitive test for detection of a targeted event. Many investigators first screen all clone DNA samples by PCR followed by confirmation with Southern blot. A strategy for differentiating between the knockout allele and the wild-type allele should be worked out in advance of initiating a targeting experiment.

12. Rinse the embryo carcasses and transfer to the third new 10-cm^2 dish of CMF-DPBS that was prepared in step 3. Try to get rid of as many red blood cells as possible.

Digest embryos

13. Prepare one 10-cm^2 tissue culture dish with 15 ml of 0.05% trypsin/EDTA. Place this dish inside a clean laminar flow hood. Place the decapitated embryos into the trypsin dish and quickly mince the tissue with the forceps and curved scissors until the tissue can be sucked up into a 10-ml serological pipet. Pipet minced tissue up and down into the same dish six to seven times.

14. Add another 5 ml of 0.05% trypsin/EDTA, and continue to disperse the tissue with a narrower-bore 5-ml pipet, using the technique described in step 13.

15. Place this dish in the incubator for 10 min.

Collect cells

16. Transfer the contents of the dish to a sterile 50-ml conical tube and allow the undigested tissue to settle at the bottom of the tube.

    This should take about 2 min.

17. Decant the supernatant to a new, empty sterile 50-ml tube and bring the volume up to 50 ml with EFM.

18. Centrifuge this tube 5 min at 193 × *g*, 10°C. Discard the supernatant.

Culture the cells

19. Resuspend the cell pellet in 50 ml fresh EFM. Transfer 10 ml of suspension to a sterile 10-cm^2 tissue culture dish.

20. Allow the fibroblasts to attach overnight in the incubator. The next day replace the medium from each dish with 10 ml EFM. Allow these fibroblasts to grow until the plates appear confluent.

    At this point the primary mouse embryonic fibroblasts (PMEF) will be passage 0.

    Often, passage 0 fibroblasts are "split" into several more dishes to permit preparation of a bigger batch of frozen PMEF.

Expand PMEFs for frozen stock

21. To split the PMEF, aspirate the EFM from each plate. Add ~5 ml CMF-DPBS to each dish, and then aspirate the CMF-DPBS off of the dish.

    This wash reduces the number of dead cells and removes any serum remaining from the medium, which can inhibit the action of trypsin.

22. Add 2 ml 0.05% trypsin/EDTA to the dish and place in the tissue culture incubator at 37°C for 3 min.

23. Gently tap the side of the tissue culture dish and check that the PMEF have detached from the bottom of the dish.

24. Add 3 ml EFM to the dish to stop the action of trypsin. Pool all the dishes that were trypsinized in a 50-ml sterile tube and bring the volume up to the amount necessary for 10 ml of cells per 10-cm^2 dish. Replate cells at 10 ml/10-cm^2 dish and return to incubator.

    For example a splitting ratio of 1:3, would require 10 ml cell suspension to 20 ml EFM.

    The passage number of the newly split cells would now be passage 1 (P1). Depending upon the density of the PMEF, the dishes will be confluent in about 4 or 5 days.

Freeze aliquots of PMEFs

25. Prepare cell suspensions as described in steps 21 to 24.

    We generally freeze down aliquots of PMEF at passage 3.

26. Count a small aliquot of the cell suspension with a hemacytometer (*UNIT 1.1*) or automated counting device.

    Using a hemacytometer, 10 μl of cell suspension is added to the chamber and will flow by capillary action to the counting grid. Count the number of PMEF in each outer corner square. Divide this total number by 4 for the average number per square. Multiply this value by 1×10^4 (the cubic volume) to get the number of cells per ml.

    The number of dishes prepared for freezing should be sufficient for 2×10^7 cells per 0.5 ml volume.

27. Combine 0.5 ml of the cell suspension with 0.5 ml of 2× PMEF cell freezing medium per 2-ml cryovial. Freeze a large batch of vials by placing the vials into a slow cool chamber. Transfer the chamber to a –80°C freezer overnight. Then, transfer the vials to a liquid nitrogen freezer for longer term storage.

    The final concentration will be 1×10^7 cells per vial.

28. Test two or three representative vials for mycoplasma and other potential mouse pathogens before a stem cell experiment is initiated.

    Many commercial services perform this mouse pathogen testing (e.g., GlobalStem, Rockville, Maryland; http://www.globalstem.com).

Expand PMEFs for production of feeders

29. Prewarm 9 ml of EFM in a sterile 15-ml tube to 37°C.

30. Thaw a vial of PMEF in the 37°C water bath until the cells are half thawed.

31. Clean the exterior surface of the vial with 70% ethanol.

32. Take one-half of the vial's total volume (generally a total of 1 ml) and transfer to the centrifuge tube with the prewarmed EFM (prepared in step 29), then transfer a portion of the prewarmed medium back into the cryovial to thaw the remaining PMEF stock.

    This ensures the rapid thawing of the PMEF and the quick dilution of the freezing medium containing DMSO.

33. Aspirate the entire contents of the cryovial back into the centrifuge tube.

34. Centrifuge the cells 5 min at $693 \times g$, 4°C, and aspirate the supernatant.

35. Resuspend the cell pellet in 20 ml EFM, and then dispense the medium and cells at 10 ml per dish to give two 10-cm^2 dishes.

36. Transfer the dishes to a tissue culture incubator at 37°C with 5% CO_2.

    After 3 to 4 days, depending upon the density of cells, the PMEFs should be confluent. It is not necessary to re-feed the confluent PMEFs prior to splitting.

37. Once confluent, aspirate the medium from each dish and wash the cells by adding 2 ml/dish CMF-DPBS (to cover the cells).

38. Aspirate the CMF-DPBS, and then cover the cells with cold 0.05% trypsin/EDTA (2 ml per dish).

39. Incubate for 3 min at 37°C until the PMEFs to detach from the dish.

40. Neutralize the trypsin by adding EFM (3 ml per dish). Pipet up and down several times to detach the cells and make a single-cell suspension.

41. Pool the medium from both dishes into a 50-ml centrifuge tube. Wash off any remaining cells on the culture dishes with an additional 5 ml EFM and add to the pooled medium.

42. Centrifuge 5 min at 693 × *g*, 4°C.

43. Aspirate the supernatant, resuspend the cells, and dilute in a sterile 250-ml Erlenmeyer flask with EFM to a volume of 150 to 200 ml total.

44. Add 10 ml of the cell suspension per 10-cm^2 dish to make 15 to 20 dishes of PMEFs.

Treat PMEFs with mitomycin C (MMC)

45. Gelatinize about forty 6-cm^2 dishes by adding 4 ml/dish of 0.2% gelatin and incubating for ~30 min at room temperature in a sterile laminar flow hood. Discard the gelatin, and allow the dishes to dry to provide a surface for the PMEF to attach.

46. Replace the EFM medium in 15 dishes (~5 dishes at a time) of PMEF with EFM containing 10 μg/ml mitomycin C. Reserve the remaining 5 dishes of PMEF for MMC treatment later in this protocol.

    CAUTION: *MMC is a potent toxin, so care must be taken to handle it carefully and to dispose of it as chemical waste.*

47. Place the MMC-treated PMEF in the incubator for 3 hr to permit mitotic arrest of the PMEF.

48. Aspirate the EFM with the MMC into the appropriate chemical waste container. Wash each dish three times, each time with 5 ml CMF-DPBS, to ensure total removal of the MMC. Treat the washes as hazardous chemical waste.

49. Add 2 ml/dish of 0.05% trypsin/EDTA to cover the fibroblasts in each dish and incubate 3 min at 37°C.

50. Neutralize the action of trypsin by adding 3 ml/dish of EFM, and pool the volume (5 ml) from each dish into a 50-ml centrifuge tube. Wash each stack of 5 dishes with an additional 5 ml of EFM and add to the pooled cell suspension.

51. Centrifuge 5 min at 693 × *g*, 4°C.

52. Aspirate the medium, then resuspend the pooled cell pellets in a convenient concentrated volume (e.g., one-tenth of the original volume, ~15 ml).

53. Count a small aliquot of the cell suspension using either a hemacytometer (*UNIT 1.1*) or an automated cell counting device. Dilute the cells in EFM to a concentration of 0.8–1.0 × 10^5 cells per ml.

    This concentration is ideal to form a monolayer for the ES cells to grow.

54. Transfer this cell suspension to at least 25 gelatin-treated 6-cm^2 dishes that were prepared in step 45.

    Within the next day or two, the feeders will assume a cobblestone-like appearance (see Figure 19.13.2). These feeders will secrete factors into the medium, which will provide an environment along with LIF (leukemia inhibitory factor) for the mouse ES cells to grow.

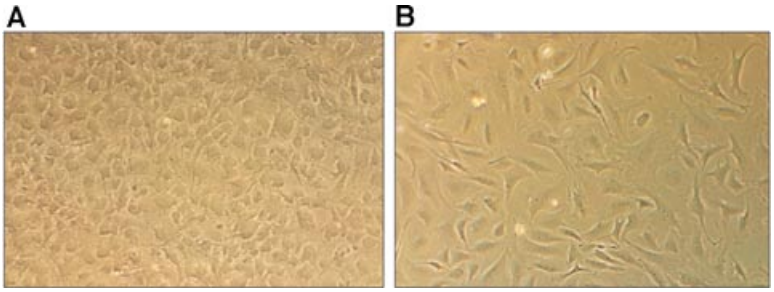


Figure 19.13.2 Mitotically inactivated ES cell feeders and PMEF. (**A**) Primary mouse embryonic fibroblasts that have been treated with mitomycin C. Note that occasionally the mitomycin–C treated PMEFs will not appear as dramatically different as shown in panel A when compared with panel B. The treatment arrests the cell division and the cells produce cytokines, which inhibit differentiation of ES cells. Note that the cell bodies appear swollen and suggest a cobblestone-like appearance. (**B**) PMEFs that have not been treated with mitomycin C. These fibroblasts have a elongated and sickle-shaped appearance. For color version of figure go to *http://www.currentprotocols.com/protocols/cb1913*.

SUPPORT PROTOCOL 2

CULTURING MOUSE EMBRYONIC STEM CELLS

Embryonic stem cells must be cultured under carefully controlled conditions to maintain the pluripotent state and prevent differentiation.

Materials

Embryonic stem cell medium (ESM; see recipe)
Pluripotent mouse embryonic stem cells (as frozen stocks; lower passage preferred, usually no more than 20 passages) e.g.:
R1 (129 strain–based; ATCC, cat. no. SCRC-1011)
W4129/S6 (129 strain–based; Taconic, cat. no. ES_W412956)
Bruce4 (C57BL/6 strain–based; Millipore, cat. no. SF-CTMI-2)
Pluristem B6 albino (C57BL/6 strain–based; Millipore, cat. no. SCR011; note these cells require IMDM media and 7% CO_2 humidified incubation)
Mouse embryonic feeder in 6-cm^2 tissue culture dishes (Support Protocol 1)
0.25% (w/v) trypsin/EDTA (Invitrogen)
15-ml centrifuge tubes, sterile

1. Prewarm 9 ml of filter-sterilized ESM in a sterile 15-ml centrifuge tube to 37°C.
2. Thaw one vial of ES cells (1 ml) by warming until half-thawed at 37°C. Transfer half the contents to the medium-containing tube (from step 1) and mix. Transfer 0.5 ml of the medium back to the cryovial, thaw the remaining cells, then transfer to the medium-containing 15-ml tube and mix.

   This dilution is done in order to dilute out the DMSO cryoprotectant.

   Generally, the ES cryovial should contain about 1×10^6 cells. If the vial contains too few cells, then growth may be inhibited. If the vial contains too many cells, then differentiation may occur, as the cells will be too close to each other.

3. Centrifuge the ES cells 5 min at $693 \times g$, 4°C.
4. Gently resuspend the cells in 5 ml ESM then transfer to a 50 ml tube and add another 10 to 15 ml ESM at 37°C.
5. Aspirate the EFM from three to four feeder dishes created in Support Protocol 1, and replace with 4 ml of the ES cell suspension (from step 4) per dish.

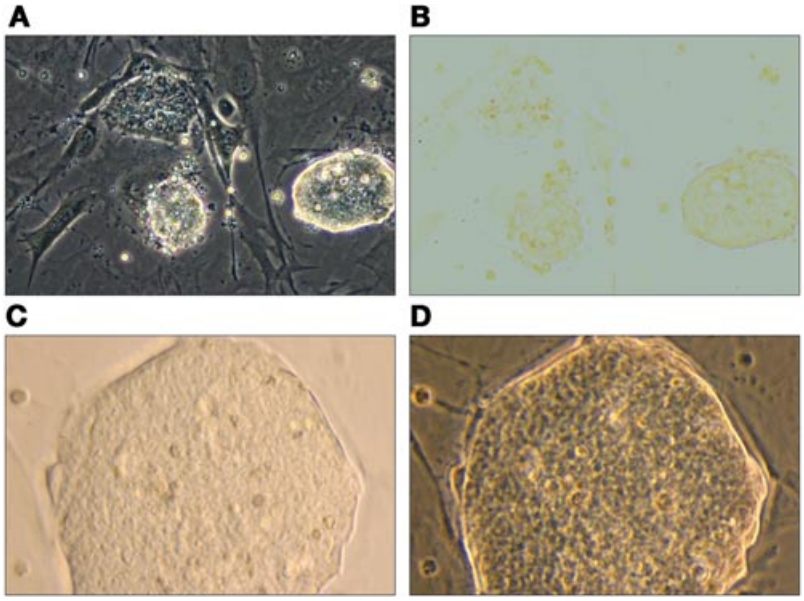


Figure 19.13.3 (**A, D**) Images of embryonic stem cells taken with phase-contrast filter. (**B, C**) Bright field images of the same cells in (A, D). The advantage of the phase-contrast filter is in highlighting the sharp borders of the ES cell colonies as well as the underlying feeder cells. (**A**) Embryonic stem cells under drug-selection pressure. The far-right colony appears to be surviving drug selection (note the sharp, bright border). The other two colonies (rough border) probably would not survive drug selection. (**D**) A high magnification of a good embryonic stem cell colony. The border appears very sharp and the embryonic stem cells within the mass appear indistinguishable from each other. For color version of figure go to *http://www.currentprotocols.com/protocols/cb1913.*

6. Once ES cells have been plated onto feeders, feed daily with ESM (which contains LIF to help maintain pluripotency).

   Depending upon the freezing density of the ES cells, the plate should be ready for harvesting in 2 to 3 days.

   Under suitable conditions, the ES-cell colonies should have very smooth, sharp borders with respect to the underlying feeders and have a dome-like, almost shiny appearance (see Figure 19.13.3 panel A and D).

7. Split stem cells maintained in culture 1:3 or 1:4 every 2 to 4 days to prevent differentiation. Use 0.25% trypsin to dissociate the ES cells and form a single-cell suspension; stop the action of trypsin by adding ESM (see Basic Protocol 1).

   Differentiation can occur if the stem cells are plated at either too low or too high a density. The stem cell colonies should not be allowed to become large and overgrown, and the ES cells within these cultured colonies should appear indistinguishable. Differentiation is generally seen when stem cell colonies become large and flattened with jagged borders.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Electroporation buffer (EB)

10% (v/v) Hanks' balanced salt solution (HBSS; Invitrogen)
20 mM HEPES buffer solution (add from 1 M stock; Invitrogen)
1 mM sodium hydroxide (NaOH) in water
110 μM 2-mercaptoethanol, tissue culture grade (Invitrogen)
Store for the duration of the electroporation experiment at 4°C, then discard

Embryonic feeder medium (EFM)

Dulbecco's modified Eagle medium (DMEM) with high glucose and phenol red (purchase in 500-ml bottle from Invitrogen) supplemented with:
50 U/ml (1×) penicillin/streptomycin (add from 1000× stock; Invitrogen)
6 mM (1×) L-glutamine solution (add from 100× stock; Invitrogen)
10% (v/v) fetal bovine serum (FBS, Gemini BioProducts)
Store for the duration of the experiment at 4°C, then discard

Embryonic feeder medium (EFM) containing 10 μg/ml mitomycin C

Dissolve one 2-mg vial of mitomycin C (MMC; Sigma, cat. no. M-0503) in 1 ml of calcium and magnesium-free Dulbecco's PBS (CMF-DPBS; Cellgro). Transfer all of this solution into 199 ml of EFM (see recipe) for a 10 μg/ml final concentration. Store at 4°C for the duration of the experiment, then discard into a chemical waste container.

Embryonic stem cell medium (ESM)

Dulbecco's modified Eagle medium (DMEM) with high glucose and phenol red (purchase in 500-ml bottle from Invitrogen; for certain cells as directed in Support Protocol 2 substitute Iscove's Modified Dulbecco Medium, IMDM, also from Invitrogen) supplemented with:
15% fetal bovine serum (FBS, embryonic stem cell qualified; Gemini BioProducts)
50 U/ml (1×) penicillin/streptomycin (add from 1000× stock; Invitrogen)
6 mM (1×) L-glutamine (add from 100× stock; Invitrogen)
90 μM 2-mercaptoethanol, tissue culture grade (Invitrogen)
100 μM nonessential amino acids (add from 100× stock; Invitrogen)
1000 U/ml leukemia inhibitory factor (LIF; Chemicon-Millipore)
Store up to 4 weeks at 4°C

ESM should be used in less than 4 weeks before glutamine begins to degrade.

Lots of FBS should be screened to ensure that the serum is able to maintain stem cell cultures in an undifferentiated state. An alkaline phosphatase antibody staining kit is available from Millipore to test the quality of the FBS. Stem cell cultures which are undifferentiated will stain pink to red, in contrast to the differentiated ES cell cultures.

Since FBS can contain growth factors that may encourage differentiation, a serum-free formulation, KnockoutSR, can be substituted. It is available from Invitrogen.

ES cell freezing medium, 2×

90 ml ESM (see recipe)
30 ml fetal bovine serum (FBS, ES cell–qualified; Gemini BioProducts)
Filter sterilize using a 0.22-μm filter (Millipore)
Add 30 ml dimethyl sulfoxide (DMSO; Sigma-Aldrich)
Dispense 0.5-ml aliquots into 2-ml cryovials (Nalgene/Nunc)
Store up to 4 weeks at 4°C

Addition of the DMSO to the filter unit sometimes clogs the membrane; therefore DMSO is added after filtering.

Glass cloning cylinders

Purchase 8 × 8–mm cloning cylinders (Bellco Glass). Lightly and evenly apply high-vacuum grease (Fisher Scientific) to the bottom of a 150 × 20–mm KMX glass petri dish (PGC Scientific). Fill the dish by placing the flat bottoms of the cloning cylinders into the grease. Cover and autoclave. Store indefinitely at room temperature after autoclaving.

Negative selection agent (FIAU)

Dissolve the entire contents of a 1-mg vial of FIAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil; Moravek Biochemicals] in 1 ml 50% ethanol/50% water (the FIAU will dissolve slowly over 1 to 2 hours; vortex periodically). Ensure that no precipitate is present at the bottom of the vial. The resulting solution is 2.7 mM. Transfer the entire vial contents to 9 ml of ethanol/water mix (for a stock concentration of 270 μM). Aliquot into several tubes and store at –20°C for ~6 months (the manufacturer states that the decay rate is 0.2% per month).

To use, add 444 μl of this stock to 600 ml of ESM plus G418 (see annotation to step 2 of Basic Protocol 2) for a final concentration of 0.2 μM. Filter-sterilize through a 0.2-μm filter. Store at 4°C for the duration of the experiment, then discard.

PMEF cell freezing medium, 2×

2 ml fetal bovine serum (FBS, Gemini BioProducts)
6 ml embryonic feeder medium (EFM; see recipe)
Filter sterilize using a 0.22-μm filter
Add 2 ml dimethyl sulfoxide (DMSO; Sigma)

For freezing large batches of PMEFs, the volume of each ingredient must be increased. The ratio ingredients is kept the same. Store at 4°C until the ready for freezing, then discard.

Positive selection agent (G418 sulfate)

Add 1 g G418 sulfate powder (743 μg/mg activity; Invitrogen) to 10 ml tissue culture-grade water to make a stock solution. Refrigerate at 4°C up to 1 to 2 weeks.

To use, supplement a 600-ml bottle of ESM (see recipe) with 2.1 ml of the G418 solution described above for a final G418 concentration of 350 μg/ml (adjust volume of G418 stock solution to add depending upon the optimal concentration for the particular ES cell line, depending upon the "kill curve"; see Basic Protocol 2, step 2). Filter-sterilize ESM plus G418 medium before use. Store at 4°C for the duration of the experiment, then discard.

G418 is an analog of neomycin.

COMMENTARY

Background Information

The earliest attempts to culture mouse embryos were basically unsuccessful. In general, when a mouse blastocyst was placed in culture, the trophectoderm layer would grow into giant cells while the inner cell mass (ICM) would fail to proliferate (Cole and Paul, 1965). Long-term cultures were eventually developed from mouse blastocysts, but over time, these would primarily consist of either epithelioid or fibroblastic cells (Sherman, 1975).

Because of the initial problems in culturing blastocysts, many researchers turned to embryonal carcinoma (EC) cells to study embryonic development. Researchers discovered that when embryonic day 1- to embryonic day 7.5 mouse embryos were transplanted into extrauterine sites in a host mouse, a teratocarcinoma would eventually develop (Solter et al., 1970; Stevens, 1970). EC cell cultures were then derived from these teratocarcinomas. Most of the EC cell lines were obtained through experiments with 129 mice, a mouse strain that was known to develop spontaneous testicular teratomas (Stevens and Little, 1954). These cultured EC cells behaved like cells seen in an early embryo. In addition, the pluripotent EC cells could differentiate into all three primary germ layers and could be directed to form embryoid bodies in culture (Martin and Evans, 1975). Unlike normal embryonic cells, however, the EC cells had an abnormal karyotype.

Investigations into embryonic development were further augmented by the ability to generate chimeric mice. The cell-cell interactions that occur in a developing embryo could be studied by the injection of foreign cells into a host mouse blastocyst. R.L. Gardner (Gardner, 1968) was the first investigator able

to show how the injection of cells into a blastocyst could produce mouse chimeras. This technique was then applied to show that EC cells could differentiate into various cell lineages to populate the developing chimeric mouse embryo (Brinster, 1974; Papaioannou et al., 1975). The EC cells, however, were not transmitted through the germ line to produce progeny of the chimeric mouse.

The lessons learned from culturing EC cells led to the isolation of embryonic stem (ES) cells. The optimal stage of embryonic development was determined by comparing cell-surface antigens and patterns of protein synthesis between EC cells and mouse embryonic cells at various stages (Evans and Kaufman, 1981). In addition, the growth conditions used in maintaining EC cells were instrumental for the eventual isolation of ES cells. The transformed feeder cells used in some EC cell cultures, for example, were helpful in preventing differentiation of the embryonic cells. Evans and Kaufman (1981) were the first to isolate ES cells by explanting whole blastocysts. Independently, G.R. Martin (Martin, 1981) was able to obtain ES cells by isolating the inner cell mass using immunosurgery. The isolated stem cells could propagate in culture and were able to differentiate into various cell types under specified growth conditions. Unlike EC cells, the stem cell cultures were karyotypically normal. Bradley et al. (1984) showed that, when injected into a blastocyst, these stem cells could populate a chimeric mouse, further proving the pluripotency of the ES cells. Of major significance, though, was the ability to achieve germ-line transmission with the newly isolated cell cultures, a finding that could be confirmed through transmission of coat color. The cytokine leukemia inhibitory factor (LIF) was later added to cultures to suppress differentiation. Before being cloned and characterized, LIF was initially added to EC cultures under the name DIA (differentiation inhibitory activity). With improvements in culturing conditions, ES cells from mouse strains other than 129 could be isolated.

The properties of ES cells include pluripotency and self-renewal in tissue culture. Embryonic stem cells were originally derived from the inner cell mass of an embryonic day 3.5 mouse embryo and were grown successfully in vitro (Evans and Kaufman, 1981; Martin, 1981). These isolated stem cells remain karyotypically normal in culture and do not transform into teratocarcinomas. Under specified growth conditions, ES cells can also be induced to form multiple cell types and even embryoid bodies. This pluripotent property allows scientists to test which growth factors are involved in turning an undifferentiated cell into specified cell types. With the capability to differentiate into various cell lineages, stem cells are also being tested for efficacy in regenerative medicine. An additional characteristic of the pluripotent stem cells is the ability to incorporate into a developing mouse embryo when injected into a blastocyst (Bradley et al., 1984; *UNIT 19.14*). The stem cells participate in the development of the host embryo to produce chimerism in the resulting mouse. The production of mouse chimeras, an important step required to generate genetically modified mice, can alternatively be achieved by aggregating ES cells together with an embryonic day 2.5 morula-stage embryo (Nagy and Rossant, 2000; Eakin and Hadjantonakis, 2006). If truly undifferentiated, the cells derived from an ES cell clone will be established in the germ line of a chimeric mouse. Characteristics of the ES cell, such as coat color, can then be transmitted from the germ line into the progeny of the chimeric mice. Thus, an ES cell clone that has been mutated in tissue culture can then be used to eventually derive a mouse containing this genetic modification.

Culture conditions

Important clues about ES cell culture were originally derived by observations of teratocarcinomas. Grafting of early embryos ectopically into a histocompatible host leads to experimentally derived teratocarcinomas from which embryonic carcinoma (EC) cell lines could be cultured (Solter et al., 1970; Stevens, 1970). EC cells can differentiate into all three types of precursor tissues necessary for the development of a mouse, namely the ectoderm, mesoderm, and endoderm. Maintaining the EC lines on feeder cells of transformed mouse fibroblasts, however, helped to prevent differentiation (Martin and Evans, 1974, 1975). EC cells were found to share similar morphology and appearance with preimplantation-stage embryos (Evans and Kaufman, 1981). The knowledge gained by studying EC cells, including the use of feeders to prevent differentiation, allowed for the eventual isolation of stem cells from the mouse embryo. Initial isolation of ES cells was performed by either explanting whole blastocysts (Evans and Kaufman, 1981) or by isolating the inner cell mass by immunosurgery (Martin, 1981). In the two decades since the initial isolation of these stem cells, more the 1200 scientific papers have been published with mouse ES cells

as a research emphasis (Downing and Battey, 2004).

One very important factor that aided in the culture of undifferentiated ES cells was myeloid leukemia inhibitory factor (LIF). This cytokine was initially given the name DIA for differentiation inhibitory activity and was isolated from various sources as a soluble factor capable of inhibiting differentiation in both ES and EC cells. Eventually DIA was cloned and characterized as the molecule LIF (Williams et al., 1988). LIF is a multifunctional cytokine that acts as a hemopoietic regulator. In addition, LIF is expressed by the trophectoderm of a developing embryo. Embryonic stem cell and embryonic carcinoma cell lines were shown to display high-affinity receptors for LIF. When highly purified recombinant LIF was added to cultures, more than 95% of the resultant colonies displayed the stem cell phenotype of compact colonies. In contrast, cells cultured in normal culture medium (without LIF) eventually differentiated to form colonies of large, flat differentiated cells. LIF mediates cell signaling, particularly through the activation of STAT3, and plays a key role in promoting ES cell propagation while blocking cellular differentiation (Burdon, et al., 1999). Other refinements to the ES cell culture medium have included the addition of β-mercaptoethanol and nonessential amino acids, which are useful to maintain the viability of the cultured stem cells (Robertson, 1987).

Characteristics of ES cells

Recently, key transcription factors have been identified that may be responsible for maintaining pluripotency in stem cells. The combination of four transcription factors—Oct-3/4, Sox2, c-Myc, and Klf4—was shown to be able to reprogram mouse embryonic fibroblasts (MEFs) into a stem cell–like state (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Oct-4, in particular, has been seen as a key regulator of pluripotency, and high expression of this transcription factor is characteristic of undifferentiated stem cells (Niwa et al., 2000). Upon retroviral infection of these transcription factors, MEFs are transformed into induced pluripotent stem (iPS) cells that behave similarly to ES cells. After proper selection, these iPS cells could populate a chimeric mouse and be transmitted through the germ line. The expression of the transcription factor Nanog, a downstream target of Oct3/4 and Sox2, is also critical for the maintenance of pluripotency in stem cells. Nanog deficiency in mice results in the inability of the inner cell mass to form an epiblast (Mitsui et al., 2003). Therefore, expression of Nanog is also related to maintenance of pluripotency.

Changes in the expression of cell-surface molecules can additionally be studied to determine if the stem cell cultures are undergoing differentiation. For example, changes in the expression level of stage-specific embryonic antigens (SSEA) are useful in determining if stem cells are undifferentiated. Differentiation of murine ES cells results in the loss of SSEA-1 expression and an increase in both SSEA-3 and SSEA-4 (Solter et al., 1979). This shift, however, is exactly opposite to what is seen with human ES cells. Lastly, a high level of alkaline phosphatase is also a good indicator that the stem cells are in an undifferentiated state (Pease et al., 1990).

Mouse strains

Historically, the most common ES cell lines were derived from the 129sv inbred mouse strain. The 129 mouse was often used to develop stem cells because this strain was commonly used to study embryonic development and was the favored strain in the derivation of EC cells. ES cells obtained from the 129 strain were unusually adept in colonizing and competing with cells from the inner cell mass of blastocysts. Commonly used 129-derived ES cell lines include ES-D3 (Doetschman et al., 1985), J1 (Li et al., 1992), and R1 (Nagy et al., 1993). Initially, only 129 strain mice were conducive or permissive for establishing ES cells in vitro. As cell culture conditions were improved to favor proliferation and reduce differentiation, stem cell lines could be maintained in culture for more passages and ES cells could be isolated from previously nonpermissive strains, such as from C57 mice (Ledermann and Bürki, 1991; Köntgen and Stewart, 1993) and BALB/c mice (Noben-Trauth et al., 1996; Baharvand and Matthaei, 2004).

Gene targeting

In addition to their use in gene targeting, ES cells have been used as a vehicle for transgenesis. ES cells can be genetically modified in culture through transfection of a transgenic construct. This ES cell route to making a transgenic mouse provides researchers with a means to screen for the desired genetic alteration before producing an animal model (Gossler et al., 1986). Along with electroporation (*UNIT 20.5*) or calcium phosphate transfection (*UNIT 20.3*) of transgenic

constructs, viruses have also been a commonly employed for gene transfer into ES cells. One of the first experiments in genetic manipulation of ES cells involved the delivery of exogenous DNA through a retroviral vector (Robertson et al., 1986). The modified ES cells were then used to create genetically altered mice. Transduction of ES cells has been performed with adenoviral and lentiviral vectors as well (Smith-Arica et al., 2003; Kosaka et al., 2004). Gene transfer through viral vectors has sometimes provided better transduction efficiency and less cytotoxicity than electroporation.

Transgenesis using gene-trap vectors is an alternative means of making knockout mice, although via indiscriminant gene inactivation (Gossler et al., 1989; Stanford et al., 2001). Unlike gene targeting, gene-trap vectors are not site directed through homology arms, and are therefore only a random means to disrupt a gene. Gene-trap vectors generally consist of a promoterless reporter gene like *lacZ* (β-galactosidase), whose expression is dependent on random integration into a functioning gene. A splice acceptor is also commonly placed preceding the *lacZ* gene to ensure expression when integrated within a gene intron. Additionally, a gene trap vector usually will contain a neo^r gene for positive drug selection.

Homozygous null ES cells can also be derived in tissue culture by treating a properly targeted clone to increased positive drug-selection pressure. For example, clones with a targeted insertion of the neo^r gene can be treated with a high concentration of G418 to force the replacement of the wild-type gene for the mutated copy (Mortensen et al., 1992). The high concentration of G418 seems to force another homologous recombination event to accommodate for the increased drug-selection pressure. Null stem cells can then be characterized in culture to determine if gene inactivation affects the differentiation into various cell lineages. The homozygous null ES cells can also be injected into a mouse blastocyst to make a chimeric mouse. In the chimeric mouse, the development of the null cells can then be followed to determine if the effects of gene inactivation are cell-autonomous.

As techniques were perfected in applying homologous recombination for gene targeting, researchers quickly realized that murine stem cells could be used as a means by which to make genetically altered mice. With the convergence of these technologies, a targeting construct is first delivered into cultured ES cells to introduce a desired mutation into a selected gene. The designated mutation is inserted into the targeted gene via recombination at sequences of genetic homology. The stem cell clone with the desired recombination event is then used to derive a mouse with the targeted mutation through injection into a blastocyst. The combination of homologous recombination and ES cells was first applied in gene targeting of hypoxanthine-guanosine phosphoribosyl transferase gene (*HPRT*). The *HPRT* gene was an ideal candidate to test gene targeting, since both correction (Doetschman et al., 1987) and inactivation (Thomas and Capecchi, 1987) of this gene could be easily tested in tissue culture. Later refinements allowed for the production of knockout mice via targeted inactivation of nonselectable genes, such as *int-2* and *c-abl* (Mansour et al., 1988; Schwartzberg et al., 1989). These refinements included positive and negative drug selection (Mansour et al., 1988). Soon, the methods used to make knockout mice were modified to create other gene-targeted mutations. In this manner, conditional knockout mice, knock-in mice, and mice with subtle genetic mutations were generated. The significance of gene targeting was seen when Mario Capecchi, Oliver Smithies, and Martin Evans won the 2007 Nobel Prize in Physiology or Medicine. Additionally, the explosive growth in the number of animal models derived through gene targeting attests to the usefulness of these discoveries. With targeted gene inactivation in mice, the precise role of a gene can be discerned by analyzing its absence in vivo. Through gene targeting to disrupt genes or create subtle mutations in mice, human genetic disease can be mimicked in an animal model. Therefore, using stem cells in combination with gene targeting has been a valuable means to gain insights into mammalian biology.

Alternate method

In general, one can expect a targeting efficiency (number of clones possessing the recombined allele divided by the total number of clones) of about 1 to 3 percent (te Riele et al., 1992). In many cases, it is advantageous to have more than one targeted clone to increase the chances of obtaining good chimeric mice. The protocol described here can be very labor intensive, particularly at the steps where clones are picked and individually frozen. It is not uncommon to freeze 200 to 300 clones per project. Other investigators have developed a less laborious method that uses 96-well tissue culture plates and a multichannel pipettor to reduce fatigue

(Ramirez-Solis et al., 1993). In this method, feeders are plated onto 96-well tissue culture plates instead of 24-well plates. After drug selection, ES cell clones are scraped around the colony and plucked from the dish using a pipet tip, and are then trypsinized and transferred to corresponding 96-well feeder plates. When the cells are confluent and ready to freeze, a multichannel pipettor is used to wash, add trypsin and medium, and resuspend the cells. This method can generate many more clones and reduce the time involved and the resulting fatigue. Many investigators have used this method, particularly if they use only positive selection or when there is concern regarding TK-induced sterility of transgenic males (e.g., conditional knockout mice where the exon has flanking *loxP* sites and an incorporated *HSV-tk* cassette; Salomon et al., 1995) or when it is not feasible to include a negative-selection cassette due to a lack of appropriate restriction enzyme sites. There are, of course, disadvantages using this high-throughput method. The chief disadvantage is that there is less cell lysate from the 96-well as compared to the 24-well method. Usually, there is only enough lysate to permit one Southern blot. Secondly, an entire plate must be harvested when the cells are ready to freeze if a multichannel pipettor is used, and the number of cells per well will vary.

Feeder-free system

The methods detailed above use mouse embryonic fibroblast cells that have been arrested; these feeders provide not only the cytokines necessary to maintain undifferentiated embryonic stem cells, but also a support for the stem cell colony to grow on. In some cases where the population of dispersed embryonic stem cells is sparse relative to the number of feeder cells, it is difficult to differentiate between the wild-type genomic DNA from the feeder cells and the genomic DNA from the potentially null stem cells. In addition, it may be necessary to culture the PMEF for 1 or 2 weeks, adding time to any project. These problems led to identifying the particular growth factors in feeder cells that sustained self-renewal in embryonic stem cells. This possibility was especially useful in the propagation of primate stem cells where generation of feeders would be impractical. For a feeder-free system, LIF and the bone morphogenetic proteins (BMP-4) seem to be the only growth factors necessary for mouse ES cells to remain undifferentiated (Ying et al., 2003) without the presence of feeder cells. Millipore has developed a feeder-free kit for murine embryonic stem cell culture, and information for this kit can be obtained from their Web site (*http://www.millipore.com*).

Critical Parameters and Troubleshooting

Embryonic stem cell passage number

Occasionally, after targeted clones have been injected, chimeric mice are not produced, or chimeric mice are bred but the targeted ES cells do not contribute to the germline. One reason for this appears to be the amount of time and passages spent in vitro. The steps necessary to generate and test new stem cell lines can be very time consuming and require considerable skill. As a result, many facilities purchase stocks of these stem cell lines then freeze down large batches. If the passage number becomes very high, then the ability of these clones to contribute to the germ line diminishes. One such study found that previously euploid (40 chromosome count in mice) ES cell clones, when passaged in vitro for more than 20 passages, rapidly became aneuploid (higher chromosome counts), and the capacity to contribute to the germ line dropped to zero (Longo et al., 1997). Some facilities routinely screen new stem cell line batches by karyotyping chromosomal spreads, and some facilities offer this screening as a service. Karyotyping or G-banding naïve ES cell batches may help when troubleshooting problems with germline transmission.

Mouse strain

When planning to make genetically altered mice, careful consideration should be made in choosing the mouse strain from which to derive ES cells to use for experimentation. Most stem cells are derived from the 129 strain of mice because, historically, these mice were commonly used for the derivation of EC cell lines. However, extensive genetic variation has been reported between the 129 mouse substrains and the ES cells derived from these mice (Simpson et al., 1997). Additionally, the 129 strain of mice tend to have reduced fertility as compared with other mouse strains. Lastly, some reports indicated that this background displays some abnormalities in anatomy, immunology, and behavior (Seong et al., 2004). Still, 129 stem cells are easier to propagate in vitro when compared with stem cells from other mouse strains. In addition, stem cells from other mouse strains do not populate a blastocyst as efficiently and are less capable of leading to germ-line transmission. This is

particularly seen with higher-passage-number stem cells (Schoonjans et al., 2003). Therefore, of the thousands of targeted mice described in the literature, most were created using the 129 mouse strain background. For example, only 38 targeted mice have been developed with a C57BL/6 background even though two C57BL/6-derived ES cell lines have been available for more than a decade (Seong et al., 2004). To get a pure breeding background, though, requires at least another year of backcrosses. Therefore, trying a stem cell from strains other than 129 can sometimes be a worthwhile option. For example, stem cells from the BALB/c mouse were derived because this strain is commonly used for experiments in immunology (Dinkel et al., 1999). The C57 strain of mice is also favored for studying various phenotypes, since this strain has been well characterized. Therefore, C57BL/6 stem cell lines have been developed, and more genomic DNA libraries have recently been constructed from C57 strain mice to help enable investigators to develop knockout mice with this background. With this strategy, C57-derived ES cells (e.g., LK1 or Bruce4) are microinjected into C57 donor blastocysts and foster mothers. Coat color cannot be used as a marker for chimerism since the pups would have a black coat color regardless of recombined ES cells or native (from the donor blastocyst) ES cells. Recently, C57 ES cells have been developed with a tyrosinase mutation that imparts a white color (albino), allowing coat-color assessment of chimerism. Although the C57-derived ES cells require more fastidious care than the 129-derived cell lines, the targeting frequency between these two strains is roughly similar (Seong et al., 2004). In addition, while the 129-derived ES cells are better able to proliferate in a host blastocyst, the capacity to transmit the targeted event through the germ line is not very different between the two strains. Regardless of which strain of ES cell lines is used, care must be taken to ensure that these ES cells remain pluripotent until they are transferred to a host blastocyst and foster mouse.

Anticipated Results

The transformation efficiency of electroporation of ES cells is between 1% and 3%, so that an electroporation of 1×10^7 ES cells should produce $1–3 \times 10^5$ transformed cells. Of these, 200 to 300 survive and form colonies. Of the colonies formed, ~2 to 3 contain the desired mutant gene structure.

Time Considerations

It takes ~2 weeks to prepare PMEFs for use as feeder layers. PMEFs should not be used beyond passage 6.

Electroporation requires ~3 hr. Positive/negative drug selection takes at least 5 to 10 days. Picking colonies takes 3 to 4 hr, depending on the number of colonies. Expansion of the colonies before freezing requires ~2 days. Screening the colonies by Southern blotting requires 2 to 3 weeks, and by PCR takes 1 to 2 days.

Literature Cited

Baharvand, H. and Matthaei, K.I. 2004. Culture condition difference for establishment of new embryonic stem cell lines from the C57BL/6 and BALB/c mouse strains. *In Vitro Cell Dev. Biol. Anim.* 40:76-81.

Bloch, K.D. and Grossman, B. 1995. Digestion of DNA with restriction enzymes. *Curr. Protoc. Mol. Biol.* 31:3.1.1-3.1.21.

Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines *Nature* 309:255-256.

Brinster, R.L. 1974. The effect of cells transferred into the mouse blastocyst on subsequent development. *J. Exp. Med.* 14:1049-1056.

Brown, T. 1993. Southern blotting. *Curr. Protoc. Mol. Biol.* 22:2.9A.1-2.9A.15.

Burdon, T., Stracey, C., Chambers, I., Nichols, J., and Smith, A. 1999. Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Dev. Biol.* 210:30-43.

Cole, R.J. and Paul, J. 1965. Properties of cultured mammalian ova. *In* Ciba Foundation Symposium, Preimplantation Stages of Pregnancy (G.E.W. Wolstenholme and M. O'Connor, eds.) pp. 95-98. Little, Brown and Company, Boston.

Dinkel, A., Aicher, W.K., Warnatz, K., Bürki, K., Eibel, H., and Ledermann, B. 1999. Efficient generation of transgenic BALB/c mice using BALB/c embryonic stem cells. *J. Immunol. Methods* 223:255-260.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27-45.

Doetschman, T., Gregg, R.G., Maeda, N., Hooper, M.L., Melton, D.W., Thompson, S., and Smithies, O. 1987. Targetted correction of a mutant *HPRT* gene in mouse embryonic stem cells. *Nature* 330:576-578.

Donovan, J. and Brown, P. 2006. Euthanasia. *Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Downing, G.J. and Battey, J.F. 2004. Technical assessment of the first 20 years of research using

mouse embryonic stem cell lines. *Stem Cells* 22:1168-1180.

Eakin, G.S. and Hadjantonakis, A.K. 2006. Production of chimeras by aggregation of embryonic stem cells with diploid or tetraploid mouse embryos. *Nat. Protoc.* 1:1145-1153.

Evans, M.J. and Kaufman, M.H. 1981. Establishment in culture of pluripotential cells from mouse embryos *Nature* 292:154-156.

Gardner, R.L. 1968. Mouse chimeras obtained by the injection of cells into the blastocyst. *Nature* 220:596-597.

Gossler, A., Doetschman, T., Korn, R., Serfling, E., and Kemler, R. 1986. Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proc. Natl. Acad. Sci. U.S.A.* 83:9065-9069.

Gossler, A., Joyner, A.L., Rossant, J., and Skarnes, W.C. 1989. Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science* 244:463-465.

Köntgen, F. and Stewart, C.L. 1993. Simple screening procedure to detect gene targeting events in embryonic stem cells. *Methods Enzymol.* 225:878-890.

Kosaka, Y., Kobayashi, N., Fukazawa, T., Totsugawa, T., Maruyama, M., Yong, C., Arata, T., Ikeda, H., Kobayashi, K., Ueda, T., Kurabayashi, Y., and Tanaka, N. 2004. Lentivirus-based gene delivery in mouse embryonic stem cells. *Artif. Organs* 28:271-277.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R., and Berns, A. 1991. Simplified mammalian DNA isolation procedure. *Nucl. Acids.Res.* 19:4293.

Ledermann, B. and Bürki, K. 1991. Establishment of a germ-line competent C57BL/6 embryonic stem cell line. *Exp. Cell Res.* 197:254-258.

Li, E., Bestor, T.H., and Jaenisch, R. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69:915-926.

Longo, L., Bygrave, A., Grosveld, F.G., and Pandolfi, P.P. 1997. The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. *Transgenic Res.* 6:321-328

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K., and Hochedlinger, K. 2007. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* 1:55-70.

Mansour, S.L., Thomas, K.R., and Capecchi, M.R. 1988. Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: A general strategy for targeting mutations to nonselectable genes. *Nature* 336:348-352.

Martin, G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

Martin, G.R. and Evans, M.J. 1974. The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture. *Cell* 2:163-172.

Martin, G.R. and Evans, M.J. 1975. Differentiation of clonal lines of teratocarcinoma cells: Formation of embryoid bodies in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 72:1441-1445.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113:631-642.

Moore, D. and Dowhan, D. 2002. Purification and concentration of DNA from aqueous solutions. *Curr. Protoc. Mol. Biol.* 59:2.1A.1-2.1A.10.

Mortensen, R.M., Conner, D.A., Chao, S., Geister-Lowrance, A.A., and Seidman, J.G. 1992. Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell Biol.* 12:2391-2395.

Nagy, A. and Rossant, J. 2000. Production and analysis of ES cell aggregation chimeras. *In* Gene Targeting: A Practical Approach, 2nd edition. (A.L. Joyner, ed.) pp. 177-206. Oxford University Press, New York.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 90:8424-8428.

Nichols, J., Evans, E.P., and Smith, A.G. 1990. Establishment of germ-line competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development* 110:1341-1348.

Niwa, H., Miyazaki, J., and Smith, A.G. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* 24:372-376.

Noben-Trauth, N., Köhler, G., Bürki, K., and Ledermann, B. 1996. Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line. *Transgenic Res.* 5:487-491.

Okita, K., Ichisaka, T., and Yamanaka, S. 2007. Generation of germ line-competent induced pluripotent stem cells. *Nature* 448:313-317.

Papaioannou, V.E., McBurney, M.W., Gardner, R.L., and Evans, M.J. 1975. Fate of teratocarcinoma cells injected into early mouse embryos. *Nature* 258:70-73.

Pease, S., Braghetta, P., Gearing, D., Grail, D., and Williams, R.L. 1990. Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). *Dev. Biol.* 141:344-352.

Ramïrez-Solis, R., Davis, A.C., and Bradley, A. 1993. Gene targeting in embryonic stem cells. *Methods Enzymol.* 225:855-878.

Robertson, E.J. 1987. Embryo-derived stem cells. *In* Teratocarcinomas and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.) pp. 71-112. IRL Press, Oxford.

Robertson, E., Bradley, A., Kuehn, M., and Evans, M. 1986. Germ-line transmission of genes

introduced into cultured pluripotential cells by retroviral vector. *Nature* 323:445-448.

Salomon, B., Maury, S., Loubière, L., Caruso, M., Onclercq, R., and Klatzmann, D. 1995. A truncated herpes simplex virus thymidine kinase phosphorylates thymidine and nucleoside analogs and does not cause sterility in transgenic mice. *Mol. Cell Biol.* 15:5322-5328.

Schoonjans, L., Kreemers, V., Danloy, S., Moreadith, R.W., Laroche, Y., and Collen, D. 2003. Improved generation of germ line-competent embryonic stem cell lines from inbred mouse strains. *Stem Cells* 21:90-97.

Schwartzberg, P.L., Goff, S.P., and Robertson, E.J. 1989. Germ-line transmission of a *c-abl* mutation produced by targeted gene disruption in ES cells. *Science* 246:799-803.

Seong, E., Saunders, T.L., Stewart, C.L., and Burmeister, M. 2004. To knockout in 129 or in C57BL/6: That is the question. *Trends Genet.* 20:59-62.

Sherman, M.I. 1975. The culture of cells derived from mouse blastocysts. *Cell* 5:343-349.

Simpson, E.M., Linder, C.C., Sargent, E.E., Davisson, M.T., Mobraaten, L.E., and Sharp, J.J. 1997. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nat. Genet.* 16:19-27.

Smith-Arica, J.R., Thomson, A.J., Ansell, R., Chiorini, J., Davidson, B., and McWhir, J. 2003. Infection efficiency of human and mouse embryonic stem cells using adenoviral and adeno-associated viral vectors. *Cloning Stem Cells* 5:51-62.

Solter, D., Skreb, N., and Damjanov, I. 1970. Extrauterine growth of mouse egg-cylinders results in malignant teratoma. *Nature* 227:503-504.

Solter, D., Shevinsky, L., Knowles, B.B., and Strickland, S. 1979. The induction of antigenic changes in a teratocarcinoma stem cell line (F9) by retinoic acid. *Dev Biol* 70:515-521.

Stanford, W.L., Cohn, J.B., and Cordes, S.P. 2001. Gene-trap mutagenesis: Past, present and beyond. *Nat. Rev. Genet.* 2:756-768.

Stevens, L.C. 1970. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev. Biol.* 21:364-382.

Stevens, L.C. and Little, C.C. 1954. Spontaneous testicular teratomas in an inbred strain of mice. *Proc. Natl. Acad. Sci .U.S.A.* 40:1080-1087.

te Riele, H., Maandag, E.R., and Berns, A. 1992. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc. Natl. Acad. Sci. U.S.A.* 89:5128-5132.

Thomas, K.R. and Capecchi, M.R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51:503-512.

Vasquez, K.M., Marburger, K., Intody, Z., and Wilson, J.H. 2001. Manipulating the mammalian genome by homologous recombination *Proc. Natl. Acad. Sci. U.S.A.* 98:8403-8410.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448:318-324.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. 1988. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336:684-687.

Voytas, D. 2000. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat. Biotechnol.* 21:183-186.

Generation of Gene Knockout Mice by ES Cell Microinjection

Glenn Longenecker[1] and Ashok B. Kulkarni[1]

[1]National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland

ABSTRACT

This unit describes protocols used in the production of chimeric mice that are then used for the generation of gene knockout mice. These protocols include the collection of blastocyst embryos, ES cell injection, and uterine transfer of injected blastocysts. Support protocols for the superovulation of blastocyst donor mice, generation of pseudopregnant recipients, fabrication of glass pipets for embryo and cell manipulations, and generation of germline mice are also included. Practical tips and solutions are mentioned to help troubleshoot problems that may occur. *Curr. Protoc. Cell Biol.* 44:19.14.1-19.14.36.

Keywords: embryonic stem cells • blastocyst • blastocyst injection • uterine transfer • chimeric mice • knockout mice

INTRODUCTION

The generation of knockout mice through the process of creating chimeric mice via embryonic stem (ES) cell injection is a powerful tool for understanding in vivo functions of a particular gene of interest. Knockout mouse models may be used to elucidate the molecular mechanisms underlying human diseases, and could aid in the creation of new therapies to treat these diseases.

ES cell injection is the most common method used to generate these chimeras, but morula aggregation is also used. There is a very narrow time window in embryonic development for the ES cell injection procedure to be successful in the generation of chimeric mice. For this reason, all components of the procedure for generating chimeric mice—harvesting (Basic Protocol 1), injection (Basic Protocol 2), and surgical transfer of the mouse blastocysts (Basic Protocol 3)—are usually done the same day and require good planning and coordination.

A timeline for the generation of knockout mice by the methods described in this unit is shown in Table 19.14.1.

Learning all of the procedures involved requires time and practice. It also requires skills not everyone might possess. How quickly and easily these techniques are learned will depend on previous experience, natural abilities, and the desire to learn them.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator unless otherwise indicated.

NOTE: All animal work performed must be approved by the Institutional Animal Care and Use Committee of the institution where the work will be taking place and must conform to governmental regulations for animal care and use. The animal facility where the mice are held should ideally be pathogen free.

Current Protocols in Cell Biology 19.14.1-19.14.36, September 2009
Published online September 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb1914s44

Table 19.14.1 Timeline for the Generation of Knockout mice[a]

Event	Timing
Injection of targeted ES cells into C57BL6 host blastocyst	Day 1
Transfer of injected blastocyst into foster mother	Day 1
Chimeric pups born	~3 weeks
Chimerism seen in chimeric mice	~1 month
Mating of chimeric mice to C57BL6 mice	~1.5 months
Birth of potential F1 heterozygote founders	~2 months, 1 week
Determination of germline transmission by coat color	~2.5 months
Mating of F1 heterozygous pair	~3 months, 3 weeks
F2 knockouts born	~4.5 months

[a]This is a suggested timeline and may vary due to animal reproduction.

BASIC PROTOCOL 1

COLLECTION OF BLASTOCYSTS

Embryonic day (E) 3.5 blastocysts can be found free-floating in the uterine horn. Note that all embryonic day designations in this unit refer to days post-coitum (p.c.) from the time of the appearance of the copulation plug. To collect these embryos, the uterine horns are generally flushed out with medium. Although this is considered a straightforward technique, it does require a certain amount of manual dexterity. Another important component is the ability to use a mouth pipet in the collection and washing of embryos. Without this ability it would be hard, if not impossible, to collect the mouse embryos. Even though, in theory, only blastocysts should be obtained from this procedure, it is not uncommon to obtain earlier-stage embryos as well. Of these earlier-stage embryos, morulas should be kept in culture along with blastocysts. Depending on the stage of development, the morulae may become blastocysts in culture and can be used for ES cell injections if needed. When flushing out the blastocysts, a zoom setting of 1.2 is suggested for the dissecting microscope, since it will allow a viewing of the uterine horn all the way to the opening of the cervix. For washing blastocysts, a zoom setting of 3.2 is recommended, because this setting offers enough magnification to discern the blastocyst well, but still allows for a relatively large viewing area to see the embryos in the dish.

Materials

C57BL/6NCr 3.5-day post-coitum (p.c.) superovulated female mice (see Support Protocol 1; 10 are needed)
Injection medium (see recipe)
70% ethanol
Microdrop cultures (see recipe)

Bench paper (absorbent material with plastic backing)
35 × 10–mm tissue culture dishes (Falcon 3001 or equivalent)
3-cc tuberculin syringes
30-G, 0.5-in. needles (Becton Dickson, cat. no. 305106 or equivalent)
70% ethanol pads
Surgical instruments (cleaned and sterilized with alcohol pads):
  Dissection scissors (Roboz RS-5880 or equivalent)
  No. 55 forceps (Roboz RS-5063 or equivalent)
Dissecting microscope
Transfer pipet (see Support Protocol 2)
Mouth pipet assembly (see Fig. 19.11.2) including:
  Mouthpiece (Fisher Scientific no. 13-647-10 or equivalent)

Saliva trap (cotton plug from a 1-mm pipet; BD Falcon, cat. no. 7521, or equivalent)
Syringe filter, 0.22-μm (Gelman no. 4602 or equivalent)
Pipet insert and reservoir (Drummond microcaps no. 1-000-0300 or equivalent); a modified 1000-μl pipet tip can be used as a reservoir.
Approximately 23 in. of natural latex tubing (1/8-in. i.d.; 1/32-in. wall)
Male luer, 5/32-in. (Ark-Plas Products no. 10-12ML016N or equivalent) to connect female end of syringe filter to rubber tubing
Female luer, 5/32-in. (Ark-Plas Products no. 10-15FL016N or equivalent) to connect male end of syringe filter to rubber tubing

Additional reagents and equipment for euthanasia of the mouse (Donovan and Brown, 2006a)

Harvest uterine horns

1. Take out injection medium from the refrigerator and allow it to warm to room temperature.
2. Place bench paper on the area where the mice will be euthanized.
3. Pour injection medium into a 35 × 10–mm tissue culture dish, and also load injection medium into a 3-cc syringe and attach a 30-G needle onto it. Tap the syringe to allow bubbles to travel to the plunger end.
4. Swab a pair of no. 55 forceps and dissection scissors with 70% ethanol pads and allow to dry.
5. Euthanize a mouse by cervical dislocation (Donovan and Brown, 2006a).

   It is suggested that all ten blastocyst donor females be used even if a plug is not seen in some of them. These females could potentially be plugged even though a copulation plug was not seen. Failure to use all of the mice could potentially decrease the embryo yield.

6. Place each mouse on its back (Fig. 19.14.1), and orient it such that the head and tail are in the 3 o'clock and 9 o'clock positions, respectively. With a squeeze bottle filled with 70% ethanol, spray the abdomen of each mouse.

   Figure 19.14.1 illustrates many of the manipulations described in the following steps.

7. Gently, with the fur placed between the index finger and thumb of each hand, pull the skin apart toward the head and tail of the mouse.
8. With a pair of no. 55 forceps and dissection scissors, grasp the peritoneal abdominal muscle wall and make a vertical incision. Move the intestines to the anterior side, away from the ovaries.
9. Next, using the no. 55 forceps, grasp the white fat pad of the ovary and snip the ligamentum latum attached to the uterus and the kidney. Carefully cut away the fat and blood vessel structure (mesometrium) running along the uterine horn, and cut at the cervix. Avoid cutting the side of the uterine horn.
10. Repeat step 9 on the other horn, cut at the oviduct, and place the uterus into the dish containing the injection medium.
11. Repeat steps 5 through 9 for the remaining mice until all uteri have been harvested and placed in the same 35 × 10–mm tissue culture dish.

Flush uterine horns

12. Take the uteri out of the dish one at a time and place in another 35 × 10–mm dish that has no injection medium in it.
13. Under a dissecting microscope, straddle the uterine horn, without the ovary attached, with forceps, holding the outer edges.

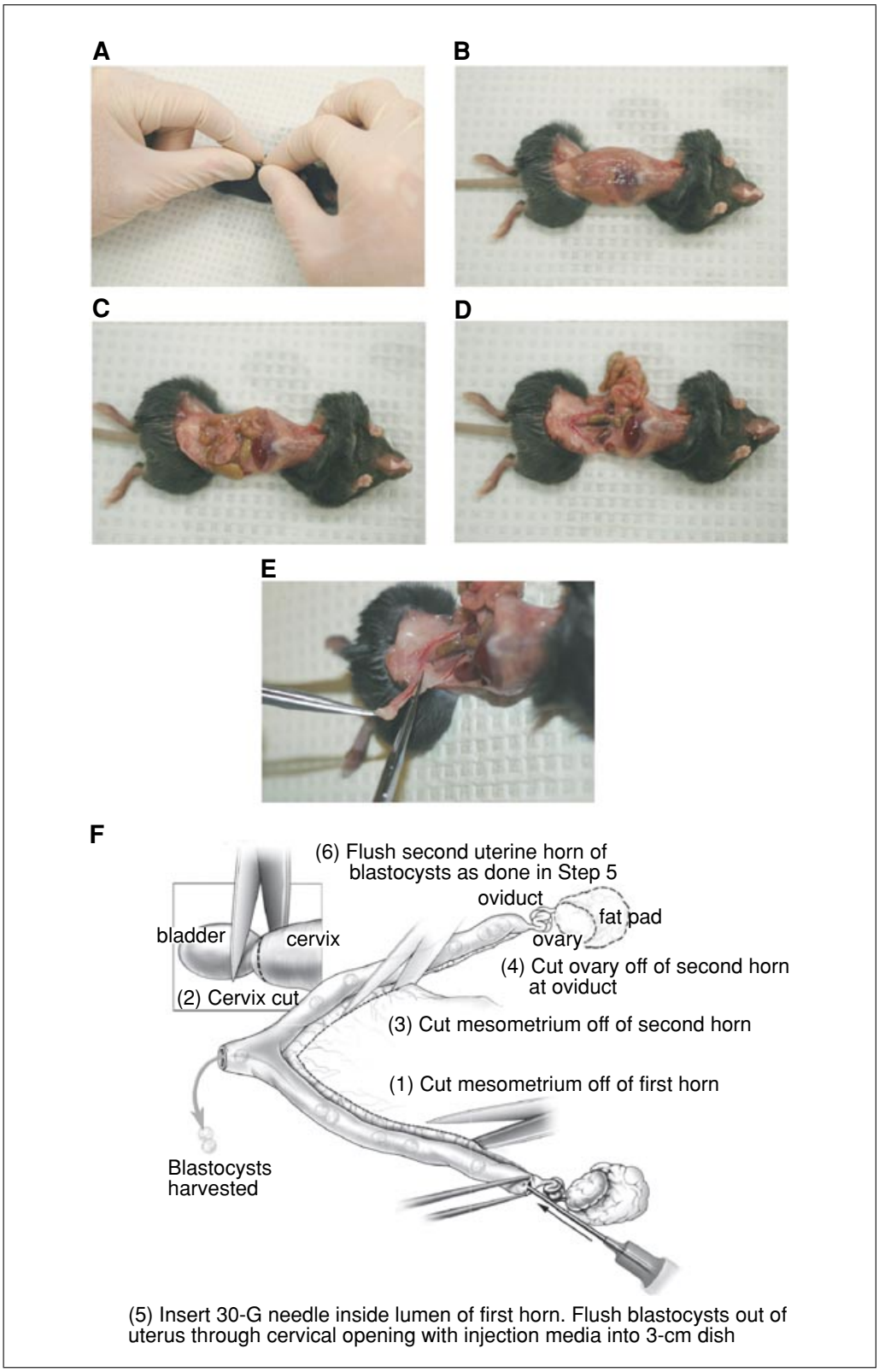


Figure 19.14.1 Collection of blastocyst embryos. (**A**) Holding of skin and fur prior to removal. (**B**) Removal of skin and fur. (**C**) Removal of peritoneal membrane. (**D**) Uterine horns exposed. (**E**) Removal of mesometrium tissue from the uterine horns. (**F**) Drawing showing the harvesting and flushing of uterine horns.

14. Insert the 30-G needle (attached to the 3-cc syringe with injection medium; see step 3) into the tip of the uterine horn inside the lumen. Inject medium through the cervix while holding the uterine horn down onto the dish until it is flushed well with medium.

    If the needle does not move freely, and the uterine wall expands, the needle is not inside the lumen. A good sign that the uterine horn is being flushed out properly is that the medium can be seen coming out of the cervix and the uterine horn expands, showing the rib-like structures of the uterine wall.

    When flushing out through the cervix, orient the cervix opening so that it is to the side and slightly up. This allows one to see the medium being flushed out and helps prevent medium from squirting out of the dish. It should take one or two quick and forceful presses on the syringe to fully flush out each uterine horn. The amount of medium required would be about 0.25 to 0.5 ml per uterine horn.

15. Repeat step 14 on the other uterine horn with the ovary still attached.

16. Repeat steps 13 to 15 for all mice in one or two tissue culture dishes if more medium is required to flush out the uterine horns.

Wash and collect blastocysts

17. When all flushing is complete, working under a dissection microscope with a polished transfer pipet (prepared as in Support Protocol 2) attached to a mouth pipet (with a cotton plug from a 1-mm pipet as a saliva trap), collect the embryos on the dish while avoiding the collection of any debris in the process.

    Transfer pipets used for washing the embryos should be a little larger than the embryo diameter to allow for microcapillary action, but not so large that there is no control in the mouth pipetting of embryos.

    It would be expected that on average 60 unhatched blastocysts will be recovered. The authors only use injection medium and no oil, due to the fact that the debris may stick to the oil, possibly making the transfer pipet unusable.

18. Transfer embryos into a second 35 × 10–mm dish containing injection medium until all the embryos are mostly free of any debris or other tissue.

19. Repeat step 18 again on a third 35 × 10–mm dish containing injection medium, to continue the wash.

20. Transfer the washed embryos with the transfer pipet into a microdrop culture (30-µl drop of injection medium under light mineral oil on a 35 × 10–mm plate; see Reagents and Solutions), and place this in a 37°C incubator until ready to inject.

    Prepare additional plates of medium/ with oil overlays for blastocysts to be injected.

SUPEROVULATION OF EMBRYO DONOR FEMALES

SUPPORT PROTOCOL 1

C57BL/6 females at 3 weeks of age are considered to be the best choice for superovulation in the production of blastocysts. If not available, then 4-week-old females would be the next best choice. Mice that are 5 to 6 weeks of age or older are not considered the best choice for superovulation because of the fact that the females would be producing the comparable hormones themselves. If using C57BL/6 females at 5 to 6 weeks of age, you should not use hormones for superovulation; rather, let the mice naturally ovulate instead. An advantage of superovulation is that fewer mice are needed to produce the number of blastocysts required for ES cell injections. Four times more females would be needed for each injection day for naturally ovulating females versus superovulated females (Hogan et al. 1994). The drawback with superovulation is the tendency to produce abnormal embryos. However, once at the blastocyst stage, most abnormal or defective embryos should have died or stopped at an earlier developmental time point. C57BL/6 blastocysts are generally considered the standard in blastocyst injection to generate chimeric mice.

However, other groups have used different strains for this purpose. The strain of mice chosen will depend on the ES cell line being used.

One hormone that we use in our lab is pregnant mare serum gonadotropin (PMSG), which is a follicle-stimulating hormone (FSH) involved in the formation of the egg follicles. Another hormone we use is human chorionic gonadotropin (HCG), which is a luteinizing hormone (LH) that releases the formed eggs and also acts as an attractant to the stud male (Hogan et al., 1994). Ten C57BL/6 females are used for each injection day, and one female is mated to each stud male housed in a separate cage. C57BL/6 males are only used once per week to ensure plugging and fertility. A light/dark cycle of 14 hr of light/10 hr of dark is a good choice for the rooms where the animals will be held, as this is thought to be more conducive to mating.

Materials

C57Bl/6NCr females or equivalent strain (3 to 4 weeks of age; ten are needed per injection day
50 IU/ml pregnant mare's serum gonadotrophin (PMSG; see recipe)
50 IU/ml human chorionic gonadotrophin (HCG; see recipe)
10 C57Bl/6NCr males (7 weeks to 10 months of age)

1-cc tuberculin syringes
30-G, 0.5-in. needles (Becton Dickson no. 305106 or equivalent)

Additional reagents and equipment for injection of mice (Donovan and Brown, 2006b)

1. At a time point 6 days before the planned day of blastocyst injections, intraperitoneally inject 0.1 ml of 50 IU/ml PMSG at 1300 hr into each C57BL/6NCRr female mouse.

   Intraperitoneal injection is described in Donovan and Brown (2006b). For this injection and that in the following step, use a 1-cc tuberculin syringe and a 30-G, 1/2-in. needle.

2. At a time point 2 days (47 hr) later, intraperitoneally inject 0.1 ml of 50 IU/ml HCG at 1200 hr into each C57BL/6NCRr female mouse.

3. Mate C57BL/6NCRr females 1:1 (one female mated with each male) with C57BL/6NCr males right after all females are injected with HCG.

4. The next morning, before 0900 hr, check females for copulation plugs.

   Actual times may vary depending on your animal facility and other variables. The plugging by C57BL/6 males becomes variable after 10 months of age, with a reduced number of blastocysts and a greater number of earlier-stage embryos. At about 11 months to 12 months of age, mice that are superovulated may produce only zygote-stage embryos at E3.5 p.c. At one year of age the C57BL/6 males will be become sterile. The C57BL/6 stud males are replaced at 9 to 10 months of age.

SUPPORT PROTOCOL 2

FABRICATION OF GLASS PIPETS

Some of the pipets used in the making of chimeric mice require much more skill and practice than others to prepare. The pipet that is the most difficult to make is the injection pipet, followed by the transfer pipet, and then the holding pipet.

The injection pipet has a similar inner diameter to that of the holding pipet, but has a much more gradual taper. This aids in the control of loading and expelling ES cells during blastocyst injections. The holding pipet has a more dramatic taper, which aids in holding of the blastocyst onto the holding pipet during ES cell injections. Control in this instance is not as important as it is for the injection pipet. The transfer pipet, in general, has a much larger opening to allow blastocysts to enter inside for washing after harvesting,

transferring between the oil overlay and injection dishes, and transferring of blastocysts during surgical uterine transfer.

Three variables that will affect the quality of pipets produced are the melting of the glass tubing, the length of time the glass tubing is pulled, and how much force is applied to the glass tubing during the pull. When first learning to pull the transfer pipet under a microflame, these variables are harder to control. Experience is required to control these variables and pull a good transfer pipet. For the pipet puller that the authors use in the laboratory, time and force of the pull on the glass tubing are controlled by the solenoid, and temperature is set by the heater control knob. How fast the ends of the pipets are melted by the glass bead on the microforge will depend upon how close the pipet is to the glass bead, the heat level setting of the filament, and the size of the glass bead.

Both injection and holding pipets can be purchased from vendors, but these premade pipets can be costly if many of them will be used. After the initial expenditure of buying the equipment, such as a microforge and pipet puller, the real expense is that of the glass tubing. See Figure 19.14.2 for a summary of these procedures.

Materials

Plasticene (Fisher Scientific, cat. no. P148-1LB or equivalent)
1.25% Tween 80 solution (see recipe)
Glass tubing (Drummond Scientific no. N-51A: I.D. 0.8 mm × O.D. 1.0 mm × length 150 mm or equivalent)
Microflame assembly (for pulling transfer pipets and polishing ends of pipets, consisting of:
  Rectangular support stand plus rod (PGC Scientific, or equivalent)
  Alumaloy tri-grip utility buret clamps with micro clamp holder (PGC Scientific, or equivalent)
  9-in. Pasteur pipet shortened to 7 in. (clamped vertically to the support stand, using the buret clamps, to emit a small flame)
  Tygon tubing, formulation R-3603 (I.D. 1/4 in. × O.D. 3/8 in. × wall 1/16 in.) for conveying gas to the Pasteur pipet
Diamond pencil
Microforge (Defonbrune style, with 10× eye pieces and reticle, 4× and 10× objectives or equivalent; Technical Products International, *http://www.nuhsbaum.com*); glass bead added to platinum wire filament in the laboratory with pulled injection or holding pipet
Instrument holder to hold pipets (Leica, cat. no. 520145 or equivalent)
C-flex tubing: I.D. 1/32 in. × O.D. 3/32 in. × wall 1/32 in. × length ~4 mm long (Cole Parmer, cat. no.6424-60 or equivalent) to hold pipets inside instrument holder
150 × 25–mm tissue culture dish (Falcon 3025 tissue culture plate or equivalent)
Pipet puller (Kopf 1720 vertical pipet puller with nichrome heater coil or equivalent)
Top of 100 × 20–mm tissue culture dish (Falcon 3010 tissue culture plate or equivalent)
Dissecting microscope (10×/23 eye pieces, 0.6-6.6× zoom, with base illumination)
1-cc tuberculin syringe
30-G, 0.5 in. needle (Becton Dickson, cat. no. 305106 or equivalent)
35 × 10–mm tissue culture dishes (BD Falcon, cat. no. 3001, or equivalent)
Syringe pipet assembly (to coat injection pipets with 1.25% Tween 80 solution) including:
  12-cc tuberculin syringe
  Female luer 1/8 in. (Ark-Plas Products no. 10-15FL012N or equivalent) to connect syringe with tubing

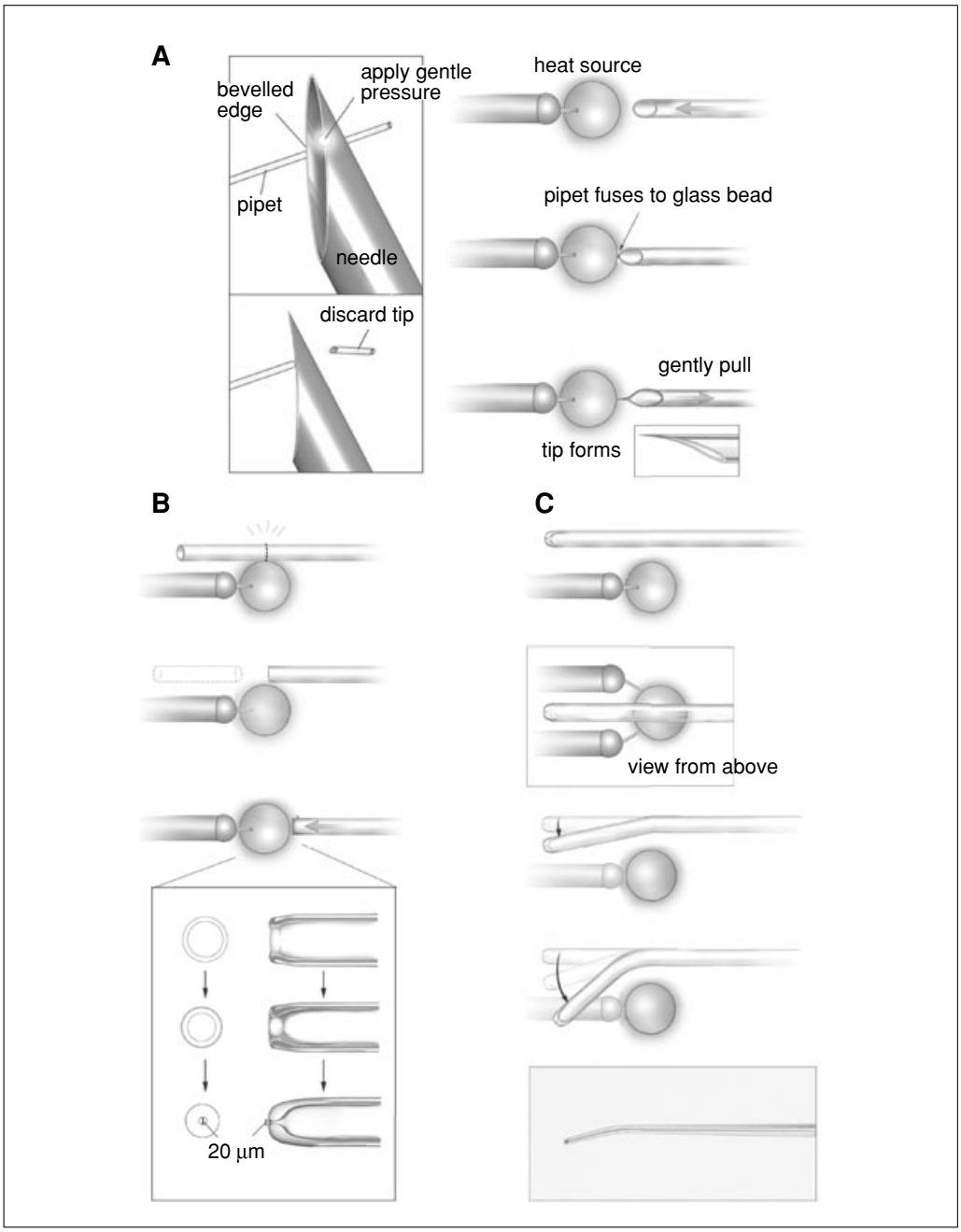


Figure 19.14.2 Fabrication of pipets with a microforge. (**A**) Breaking pulled injection pipet with a 30-G needle and sharpening tip with the microforge. A broken injection pipet is moved to the glass bead so that the tip is touching the glass bead. The injection pipet is allowed to fuse and is pulled slightly. The power is turned off and the glass allowed to contract, providing a sharp-tipped pipet with an inner diameter of 20 μm. (**B**) Breaking and polishing a holding pipet using the glass bead of the microforge. A pulled holding pipet is moved to the glass bead so that the edge of the tubing is touching the top and allowed to fuse. The power is turned off and the tubing broken by contraction. The inner diameter is melted to ∼20 μm. (**C**) Bending then holding pipet at 30° using glass bead. The holding pipet is brought near the glass bead of the microforge and allowed to bend at ∼30°. A transfer pipet would be made the same way in panel B, except the edges of the pipet are only polished and are not bent at a 30° angle.

Pipet insert and reservoir (Drummond microcaps no. 1-000-0300 or equivalent); a modified 1000-μl pipet tip may also be used as a reservoir.
Natural latex tubing (I.D. 1/8 in. × wall 1/32 in. × ~8 in. long)

Prepare transfer pipets

1. While holding both ends of a piece of glass tubing, one in each hand, place the tubing into a microflame.
2. Leave the glass tubing in the upper part of the flame until it becomes soft and can be easily moved up and down.
3. Quickly, take the glass tubing out of the flame and pull it apart in one continuous motion.
4. After the pipets have been pulled and allowed to cool for a few seconds, position the narrow end of each of the two tapering pieces of glass tubing between the thumbnail and forefinger and break apart.

   The pipets can be broken right after the pipets have been pulled and allowed to cool for a few seconds. A diamond pencil can be used to score the glass first before breaking it.

5. Look at the pulled pipet to see if it is the right size.

   Viewing the pulled transfer pipet takes practice. The only way to gauge the size of the transfer needle is to use a microforge that has a reticle in the eyepiece.

6. Insert a previously pulled transfer pipet into a pipet holder on a microforge.
7. Orient the transfer pipet so that the one can see it in the microforge optics.
8. Under 40× magnification, adjust the focusing knob so that the outer diameter of the transfer pipet is in focus.
9. Move the filament so that the outer diameter of the glass bead is also in focus, especially the area that will touch the transfer pipet.
10. Turn on the power for the filament that will heat the glass bead, and touch an area near the tip of the pipet.

    The authors use a power filament setting of 3. A different setting may work better, depending on the microforge unit you are using and the amount of glass that is in the glass bead.

    Avoid any areas that are chipped, broken, or cracked.

11. When the transfer pipet has fused with the glass bead on the platinum wire filament, turn off the power to the filament and allow the glass bead to contract, breaking the transfer pipet.

    When the transfer pipet fuses with the glass bead, a small indentation will form on the pipet.

12. Repeat steps 10 and 11 if the pipet becomes distorted and there is not a clean break.
13. Turn on the power to the filament to reheat the glass bead, and bring the glass bead next to the opening of the transfer pipet. Polish the opening, but not so much that the polishing actually narrows the opening of the transfer pipet.

    The authors use a power setting of 6 on the microforge to polish the edges of the transfer pipet. A different setting may work better depending on the microforge unit you are using and the amount of glass that is in the glass bead.

14. If needed, shorten the nontip side of the transfer needle with a diamond pencil by scoring the glass tubing and breaking at the score.

15. Store in a 150 × 25–mm tissue culture dish containing two strips of Plasticene to hold the pipets. Store all pipets facing in one direction.

    IMPORTANT NOTE: *The inner diameter of the transfer pipet should be large enough so that the fully expanded blastocysts are not constricted, which could possibly damage them. A pipet that is too small is more susceptible to blockage by blood clotting. The pipet should also not be too large that the blastocysts are bunched up together side-by-side instead of being lined up in a row. A general range for the inner diameter would be approximately 8 to 10 units at 40× magnification. A washing pipet would require a larger diameter due to the fact that some of the blastocysts may be fused together, and because of the debris that can be encountered. If using a reticle to make different types of pipets for ES cell injection, the measurement scale for the reticle at 40× magnification is 1 unit = 2.5 μm, and at 100×, 1 unit = 10 μm.*

Prepare holding pipets

16. Insert glass tubing into a pipet puller, adjust settings, and pull pipet.

    The authors use a heat setting of 12.0 units and a solenoid setting of 1.0 A for pulling holding pipets in the pipet puller.

17. Insert a previously pulled holding pipet into a pipet holder on a microforge.

18. Looking through the optics, move the pipet until it is the correct outer diameter on the reticle scale.

    At 40× magnification, this is ∼5 units and at 100× magnification this would be ∼12 to 13 units.

19. Make sure that the holding pipet is in focus, and then orient the glass bead on the filament so that it is also in focus, especially the edge of the glass bead that will touch the holding pipet.

20. With the edge of the holding pipet and the edge of the glass in the same plane, turn on the power of the filament that will heat the glass bead and gently touch the holding pipet. Allow the edge of the holding pipet to fuse with the glass bead.

    When the holding pipet fuses with the glass bead, a small indentation will form on the pipet.

21. Turn off the power to the filament and glass bead and allow the holding pipet to break.

    The authors use a power filament setting of 3. A different setting may work better depending on the microforge unit you are using and the amount of glass that is in the glass bead.

22. Repeat steps 20 and 21 if the pipet becomes distorted and there is no clean break.

23. Turn on the power to the filament and glass bead, and bring the glass bead up next to the opening of the holding pipet.

24. Melt the inner diameter of the holding pipet to 20 μm in size.

    At 100× magnification, this would be 2 units on the reticle scale.

    For the 20-μm inner diameter of the holding pipet, it is necessary to use a higher magnification than 40×. Magnification of at least 100× will work well for this purpose. If 40× magnification is used, the inner diameter will be inconsistent, and most likely larger than 20 μm. Inconsistency in size will lead to control problems with the holding pipet. This is especially important if the blastocyst is held by the trophoblasts and not the inner cell mass during injections. This can make injections very difficult, and the blastocyst could be suctioned into the holding pipet.

The authors use a power setting of 6 to melt the inner diameter of the holding pipet. A different setting may work better depending on the microforge unit that you are using and the amount of glass that is in the glass bead.

25. Turn on the power to the filament and glass bead, and, at some distance from the end of the holding pipet, bring the glass bead next to the holding pipet and make a 30° bend in the holding needle.

    The authors use a power setting of 6 to make this bend of the holding pipet. A different setting may work better depending on the microforge unit that you are using and the amount of glass that is in the glass bead.

26. Polish the back end of the holding pipet with a microflame by briefly putting the end into the flame.

    If the pipet is allowed to stay too long in the flame, it will be sealed closed.

27. Store in a 150 × 25–mm tissue culture dish containing two strips of Plasticene to hold the pipets. Store all pipets facing in one direction.

Prepare injection pipets

28. Insert glass tubing into a pipet puller, adjust settings, and pull pipet.

29. Place a previously pulled injection pipet on top of a lid from a 100 × 20–mm plastic tissue culture dish under a dissection microscope.

30. With a 30-G needle attached to a 1-cc syringe, gently tap the glass pipet at a perpendicular angle with the edge of the needle to break the glass (see Fig. 19.14.2A).

    IMPORTANT NOTE: *When viewing the bevel from the side, it is easier to see the edge of the 30-G needle (see Fig. 19.14.2A).*

31. Under a dissecting microscope, check the tip of the broken injection pipet. If the tip of the injection pipet needle appears to be in good shape overall, with no barbs, breaks, or jagged edges, save the pipet.

    With the settings that authors use for pulling injection pipet, the pipet can be broken a few times to get a proper broken pipet. After several attempts, it is best to use another pulled pipet.

32. Place previously broken injection pipet into a pipet holder on a microforge.

33. Orient the injection pipet so that it can be seen in the microforge optics.

34. Under 100× magnification, check again if the broken injection pipet appears to be free of defects and is of good size and shape. If defects are seen, discard the pipet.

    For the inner diameter of the injection pipet, we prefer a size of ∼20 μm.

35. If the pipet appears to still be of good quality, orient the pipet such that the bevel opening is either facing directly toward or away from you under the optics.

36. Focus the tip of the injection pipet and then orient the glass bead on the filament so that it is also in focus. Orient the tip of the injection pipet so that it is either facing toward or away from you.

37. Turn on the power to the filament that will heat the glass bead, and gently touch the tip of the pipet to the glass bead and allow it to melt just a little.

    The temperature setting should not be too high, or the inner diameter will become smaller. The authors use a power setting of 4.75 to melt the tip of injection pipet. A different setting may work better depending on the microforge unit that you are using and the amount of glass that is in the glass bead. The temperature of the glass bead should be high enough

to actually melt the pipet tip. Melting and pulling the pipet tip too much will create a bevel that is too long, which would make ES cell injections more difficult. If the tip is too long or there is a filament-like structure on the tip, the tip can be gently broken again and the melting of the tip can be repeated. If the tip is still is too short after the first melting, it can be repeated. If the inner diameter becomes too narrow or has a bevel too long, it is best to start over with another broken injection pipet. The authors prefer an injection pipet with an inner diameter of ~20 μm, which can be successfully obtained by using a heat setting of 14.2 units and a solenoid setting of 4.2 A when pulling injection pipets.

38. Pull away the glass bead only very slightly, and turn off the power to the filament, allowing the tip of the needle to contract from the glass bead.
39. Take the sharpened injection pipet out of the holder and set it aside.
40. Pour 1.25% Tween 80 solution into a sterile 35 × 10–mm dish.
41. Insert the injection pipet inside the rubber insert of the glass reservoir of the syringe pipet assembly.
42. Withdraw the Tween 80 solution inside the injection pipet to the hub and expel it back into the dish.
43. Lift out the injection pipet and continue expelling the Tween 80 solution out of the injection pipet to remove any excess.
44. Repeat steps 14 and 15 if necessary to ensure that the inside of the injection pipet is coated.

    Coating the inside of the injection pipet will help minimize debris and oil sticking to the inside of the pipet (Wells, 1993; Hogan et al., 1994).

45. Polish the back end of the injection pipet with a microflame by briefly putting the blunt end into the flame.

    If the pipet is allowed to stay too long in the flame, it will be sealed closed.

46. Store in a 150 × 25–mm tissue culture dish containing two strips of Plasticene to hold the pipets. Store all pipets facing in one direction.

BASIC PROTOCOL 2

INJECTION OF BLASTOCYSTS WITH ES CELLS

When injecting ES cells into blastocysts, the optimal type of blastocyst to inject would be between the middle and late stages, with a good-sized blastocoel cavity. Larger, more developed blastocysts tend to re-expand more quickly after injection, but are more susceptible to collapse prior to injection. Trying to inject any hatched blastocysts (see Fig. 19.14.3) can be difficult because they lack rigidity from the loss of the zona and have a very sticky outer surface, so they should be avoided. The morphology of ES cells to inject should be round and smooth and cells should appear opaque in nature. It is not uncommon to have small, medium, and large cell sizes. Medium-sized cells would be the preferred choice in that they will likely provide a higher chance for germline transmission, and they are less likely to be differentiated. Avoid the largest cells because they are mouse embryonic fibroblasts (MEFs; Stewart, 1993; see Fig. 19.14.3). Morula injections could be used instead of blastocyst injections to increase the success of generating chimeras, and have higher germline efficiency. A circulating pump may be used during the cooling stage in place of the dish to cool the ES cells and blastocysts. A suggested microinjection setup is listed in the materials list below (also see Fig. 19.14.4).

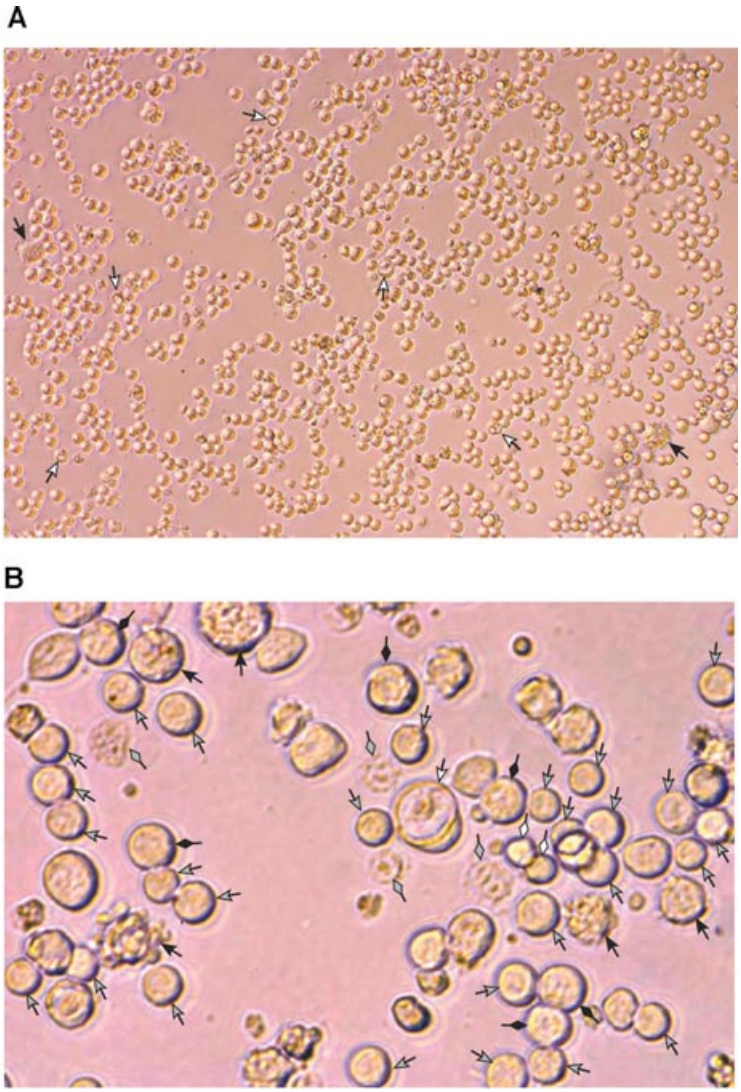


Figure 19.14.3 ES cell morphology. ES and MEF cell suspension and the different types of cells and morphology seen. (**A**) 100× magnification. Larger MEF cells are denoted by black arrows and differentiated ES cells by white arrows. (**B**) 400× magnification. Various types of cells seen in suspension. MEF cells are marked by black arrows. Differentiated ES cell is marked by a white arrow. Good morphology, medium-sized ES cells are marked by a gray arrows. Good-morphology, large, undifferentiated ES cells are marked by a black diamond. Small ES cells are marked by a white diamond. Translucent ES cells are marked by a gray diamond. It is suggested that medium-sized, good-morphology ES are the cells to inject into the blastocysts for optimal results.

Materials

Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Mediatech, cat. no. 21-031-CV, or equivalent)
Liquid soap (e.g., VioNex; any liquid soap will probably work)
Injection medium (see recipe)
ES cell suspension (1×10^6/ml) transformed with desired transgene (*UNIT 19.13*)
Filtered light white mineral oil (Sigma M-3516 embryo-tested or equivalent)
Blastocysts (see Basic Protocol 1)

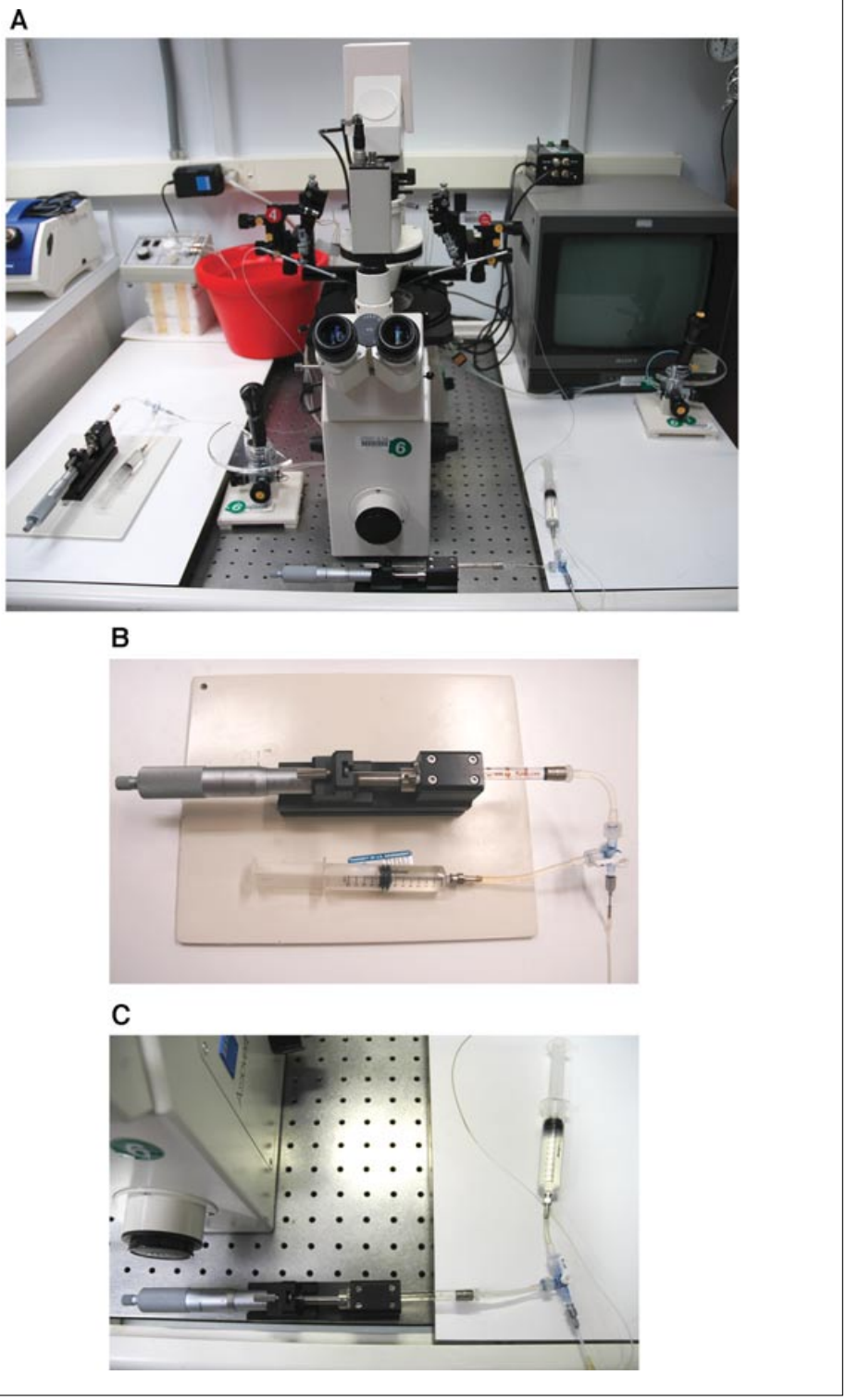


Figure 19.14.4 ES microinjection setup. (**A**) Picture of microinjection setup. (**B**) Picture of holding system detailing micrometer syringe assembly, syringe reservoir, stopcock, tubing, and fittings. (**C**) Picture of injection system detailing micrometer syringe assembly, syringe reservoir, stopcock, tubing, and fittings.

60 × 15–mm tissue culture plate (Falcon 3002 or equivalent)
70% ethanol pads
Injection chamber with cooling system (Fig. 19.14.5)
  Water circulating pumps (Baxter, cat. no. 8329 or equivalent)
  Siliconized tubing, I.D. 3/16-in., with plastic connections
  Male luers with 1/16 in. barb (Cole Parmer, cat. no. 31507-62 or equivalent) to connect plastic connections of silicone tubing to inflow and outflow Tygon tubing
  Injection dish (see Fig. 19.14.5)
  Tygon tubing for inflow and outflow cooling lines, I.D. 3/32 in. × O.D. 5/32 in. × wall 1/32 in.
  60 cc tuberculin syringe with Tygon tubing to load water jacket of injection dish
  Female luer with 3/16 in. to 1/4 in. barb (Cole Parmer, cat. no. 31507-62 or equivalent) connected to inflow tubing as a weight in ice water
Transfer pipet, cleaned and polished (Support Protocol 2)
Mouth pipet assembly (see Fig. 19.11.2 and Basic Protocol 1)
Dissecting microscope (10×/23 eye pieces, 1.0× objective, 0.6 to 6.6× zoom, with base illumination)
Injection pipet (see Support Protocol 1)
Holding pipet (see Support Protocol 1)
Microinjection setup (see recipe and see Fig. 19.14.4) consisting of:
  Injection apparatus:
  Inverted microscope (Zeiss 135M or equivalent)
  Coarse micromanipulators (microscope-mounted; Narishige MMN-1 or equivalent)
  Fine micromanipulators (Joy-stick micromanipulator; Narishige MO-202U or equivalent)
  Mounting bracket for mounting Narishige course manipulators
  Video camera (Dage-MTI CCD-72 or equivalent)
  Video monitor (Sony PVM-137 or equivalent)
  Injection line:
  Instrument holder to hold injection pipet (Leica, cat no.520145 or equivalent)
  Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × ∼4-mm long tubing insert for instrument holder to connect injection pipet to PE 60 tubing
  Polyethylene tubing for injection line (Becton Dickinson PE 60, no. 427415, I.D. 0.030 in. × O.D. 0.048 in. × length ∼2 ft. or equivalent)
  Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × ∼8 mm long or equivalent to connect between Tygon tubing AAC00002 and PE 60 tubing
  Tygon tubing AAC00002: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × ∼1 cm long or equivalent to connect Tygon AAC00001 tubing to PE 200 tubing
  Polyethylene tubing for injection line (Becton Dickson PE 200; Clay Adams no. 427440, I.D. 0.055 in. × O.D. 0.075 in. or equivalent), ∼3.5 cm. in length
  Female luer flare type adapter (Popper no. 6193 or equivalent) to connect PE 200 tubing to 3-way stopcock
  3-way stopcock (Baxter no. K177A or equivalent)
  Tygon tubing (Cole Parmer no. 6408-63, I.D. 3/32 in. × O.D. 5/32 in. × wall 1/32 in. or equivalent) for injection line between the stopcock and to either the syringe reservoir or the Hamilton syringe
  Female luer with barb (BioRad, cat. no. 7318223 or equivalent) to connect Hamilton syringes to Tygon tubing
  Male luer with barb (BioRad, cat. no. 7318226 or equivalent) to connect to luer lock connection of stopcock to Tygon tubing

Micrometer syringe (Minnetonka Instruments or equivalent) with Mitutoyo syringe, 0 to 50 mm, with 50-μl gas-tight syringe (Hamilton no. 80920 or equivalent for injection line)

Female luer connection with barb (Cole Parmer no. 31507-65 or equivalent) to connect Cole Parmer 6408-63 to tuberculin syringe reservoir

Tuberculin syringe (12 cc) containing Dow Corning silicone oil 200 for reservoir

Holding line:

Instrument holder to hold holding pipet (Leica, cat. no. 520145 or equivalent)

Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × length ~4 mm or equivalent tubing insert for instrument holder to connect holding pipet to PE 60 tubing

Polyethylene tubing for holding line (Becton Dickinson PE 100; Clay Adams no. 427425, I.D. 0.034 in. × O.D. 0.06 in. × approximate length 2 ft., or equivalent)

Tygon tubing AAC00001: I.D. 1/16 in. × O.D. 1/8 in. × wall 1/32 in. × length ~1.8 cm, or equivalent, to connect between PE 100 tubing and blunted 19-G needle

Blunted 19-G needle to connect male luer end of stopcock to Tygon AAC00001 tubing

3-way stopcock (Baxter, cat. no. K177A or equivalent)

Tygon tubing (Cole Parmer no. 6408-63: I.D. 3/32 in. × O.D. 5/32 in. × wall 1/32 in., or equivalent) for injection line from the stopcock to either the syringe reservoir or the Hamilton syringe

Female luer with barb (BioRad, cat. no. 7318223 or equivalent) to connect Hamilton syringe to Tygon tubing

Male luer with barb (BioRad, cat. no. 7318226 or equivalent) to connect luer lock connection of stopcock to Tygon tubing

Micrometer syringe (Minnetonka instruments or equivalent) with Mitutoyo syringe, 0 to 50 mm with 250 μl gas-tight syringe (Hamilton, cat. no. 81120 for holding line)

Female luer connection with barb (Cole Parmer, cat. no.31507-65 or equivalent) to connect Cole Parmer 6408-63 to tuberculin syringe reservoir

Tuberculin syringe (12 cc) containing silicone oil 200 for reservoir

Ice bucket containing ice and water

Air suspension platform setup:

Air suspension table (Kinetic Systems 9101-02-46 or equivalent)

Gas regulator (Curtis Mathis 1H-580 or equivalent)

Nitrogen gas cylinder (Roberts Oxygen no. R-31 or equivalent)

Check microinjection apparatus

The microinjection system should be functioning properly before proceeding with injection of ES cells into blastocysts. Address all problems before starting the actual microinjection procedure. Both injection and holding systems should be in good working order before attempting ES cell injections. See Critical Parameters and Troubleshooting for details on problems encountered when injecting blastocysts.

1. Fill a 60 × 15–mm tissue culture dish with DPBS, and check the condition of both the injection and holding pipets by using them to take up the DPBS.

   The pipets should be in good condition and not clogged or broken, with no debris sticking to the inside. The uptake of DPBS into both pipets should be a smooth and fluid motion.

2. If using a silicone- or mineral oil-based system, check to see that there are no bubbles or leaks present in either the injection or holding line systems.

   If bubbles or leaks are present, severe control problems can occur.

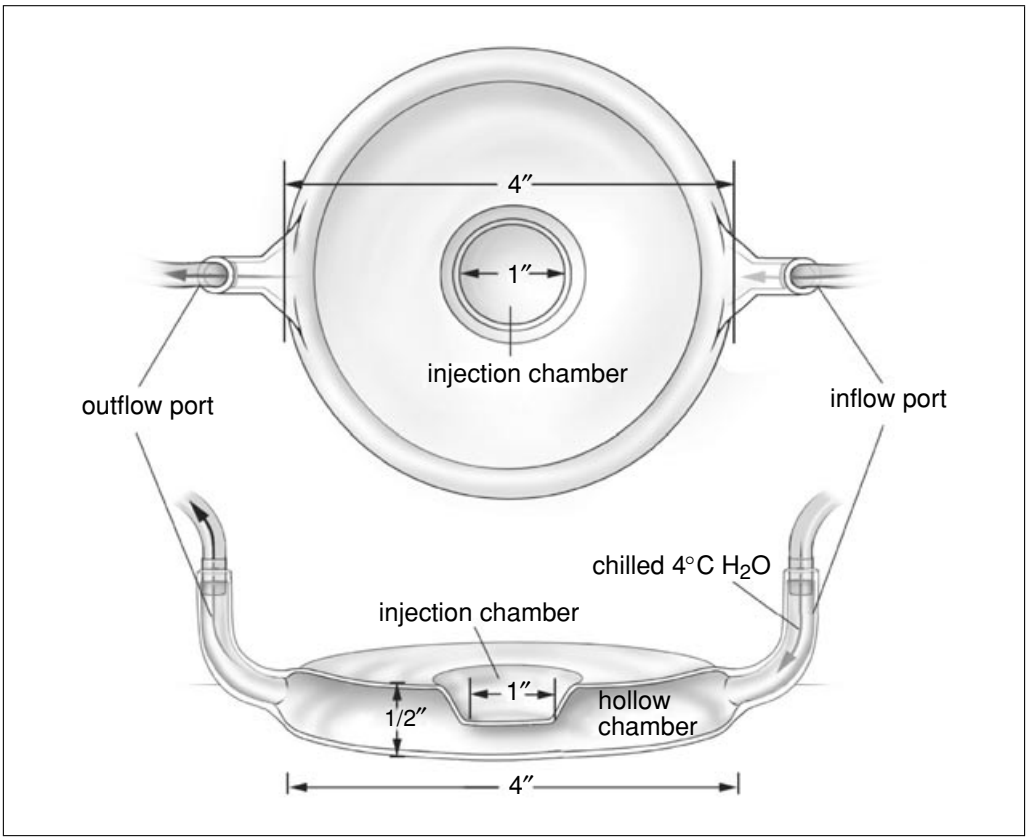


Figure 19.14.5 Diagram of injection dish. The dish has an outer diameter of 4 in., an inner diameter of ~1 in., and a height of 0.5 in. The dimensions of the inflow and outflow ports are ~1.5 in. long with an inner diameter of 3/8 in.

Prepare injection dish

For a cooling system, we use a glass injection chamber with an internal hollow opening around the injection chamber to allow for chilled (4°C) water to circulate (see Fig. 19.14.5). There are two reasons to use some sort of cooling apparatus. The first is to delay the re-formation of surface proteins after trypsinizing the cells so they do not become sticky. Secondly, cooling is conducive to the rigidity of the blastocysts. The injection dish should be free of alcohol, as this can cause differentiation of the ES cells. For the following steps, refer to Figure 19.14.5.

3. Swab the injection chamber area thoroughly with a 70% ethanol pad, then wash liberally with sterile DPBS.
4. Add a very light film of liquid soap under the bottom of the injection dish to prevent water droplets from forming during the cooling process.
5. Attach inflow tubing from the water circulating pump to the input opening of the injection dish.
6. Next, insert outflow tubing into the output opening of the injection dish, which has been placed in a chilled water reservoir, and then start the circulating pump.
7. Pipet 1.5 ml injection medium into the well of the injection dish and add 30 μl of the 1×10^6 cells/ml ES cell suspension.

   The authors recommend a cell suspension of 1×10^6 per ml. This is more than enough cells for injection purposes. If too many cells are added to the injection dish, the cells tend to become sticky. A high ratio of ES cells to feeder cells is best for injections. Having

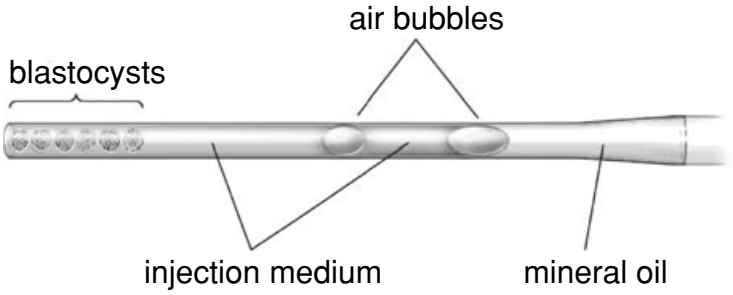


Figure 19.14.6 Diagram of transfer pipet loaded with blastocysts. The air bubbles are omitted for blastocyst injection (Basic Protocol 2), but are used when loading a transfer pipet for uterine implantation (Basic Protocol 3). Mineral oil is first loaded inside the pipet. This is followed by the first bubble, injection medium, another bubble, and then the blastocysts.

some feeder cells can be a benefit in that they can be used to clear debris from inside the injection pipet. This is done by loading a feeder cell inside the injection pipet and moving it back and forth to dislodge any debris inside the pipet.

8. Overlay with light mineral oil.

Transfer embryos to microinjection dish

Blastocysts are more tolerant to pH and temperature changes than earlier-stage embryos such as zygotes. Lowering the temperature will delay the onset of blastocyst hatching. Keeping blastocysts in the cold injection chamber too long might be detrimental to their vitality.

9. Take the dish containing the blastocysts with mineral oil and injection medium out of the incubator (see Basic Protocol 1, step 20).

10. Take a clean and polished transfer pipet, and mouth pipet light mineral oil to the hub of the pipet (Fig. 19.14.6).

11. Load unhatched blastocysts inside the transfer pipet from the dish containing the blastocysts (see Figure 19.14.6).

    The air bubbles shown in Figure 19.14.6 are omitted for this procedure. The authors use only oil and media, and no bubbles, to transfer the blastocysts from the medium and oil overlay dish to the injection dish and back.

12. Take the transfer pipet and gently expel the embryos into the chamber of the injection dish, while clearing an area for injections in the cell suspension. Only transfer as many blastocysts to the injection dish as can be injected within 30 min.

    Any investigator with a reasonable skill level should be able to inject 10 to 20 blastocysts, depending on injection conditions.

Inject ES cells

When first learning ES cell injections, 200× magnification should be used. Focus on the outer ring of trophoblast cells and then the tip of the injection pipet before attempting to inject inside the blastocoel cavity. This ensures that the injection pipet is in the correct plane of the z axis, being approximately in the center of the blastocoel cavity. After becoming proficient in ES cell injections, a tapping technique, which looks at indentations on the blastocyst, can be employed to make sure the injection pipet is in the right plane. 100× magnification will work well with this procedure and has the advantage of a broad field of view to collect the ES cells, and changing the objective is not required. The general range for the number of ES cells that can be injected inside a blastocyst

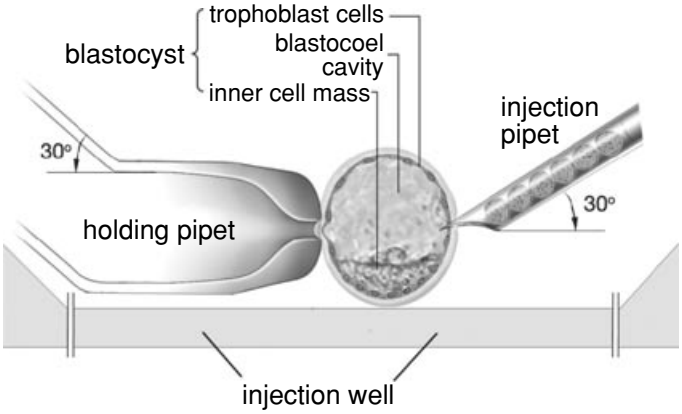


Figure 19.14.7 Diagram of blastocyst injection chamber. The blastocyst is held by the holding pipet and is touching the bottom of the injection well for stability. The injection pipet is unbent and is inside the blastocoel cavity at a 30° angle.

is considered to be between 10 and 20 (Papaioannou and Johnson, 2002). The authors' preference is to inject 15 ES cells, which balances the chance of germline transmission versus normal homeostasis in embryonic development. It is advisable not to inject more than 20 ES cells (Wells, 1993), as this is thought to be detrimental to the developing mouse embryo.

13. After blastocysts have been transferred to the bottom of the inner chamber on the injection dish, lower the holding and injection pipets (Fig. 19.14.7).

    All blastocysts should be injected within 30 min of putting them on the injection dish.

14. With the injection micrometer syringe, take up a small amount of injection medium inside the injection pipet.

    This will allow a buffer area between the cells and the oil and help prevent injection of oil inside the blastocoel cavity.

15. Load 15 ES cells inside the injection pipet with as little injection medium as possible.

    The ES cells do not have to be touching each other when inside the injection pipet. If the cells are touching, there is a possibility that they may stick to each other and, consequently, many of the cells may come out of the blastocyst when withdrawing the injection pipet.

16. Orient the blastocyst so that the inner cell mass (ICM) is in the 6 o'clock position with the injection pipet. Make sure the bevel of the injection needle is pointed down (Fig. 19.14.8).

    The blastocyst can be oriented with respect to the injection pipet by moving the joystick in either a clockwise or counterclockwise motion. Using the holding pipet to orient the blastocyst will disperse the cells away from the injection area and also other blastocysts.

17. With the holding micrometer syringe, take up injection medium inside until the blastocyst is held securely to the holding pipet. Lower the holding pipet until the blastocyst is just touching the bottom of the injection dish.

18. Focus on the zona and outer trophoblast layers of the blastocyst, and then move the injection pipet so it is also in focus.

    This puts the injection pipet in the correct plane of the z axis.

    It is better to inject at the junction between the trophoblast cells, reducing damage to the embryo. Sometimes this junction may be hard to see.

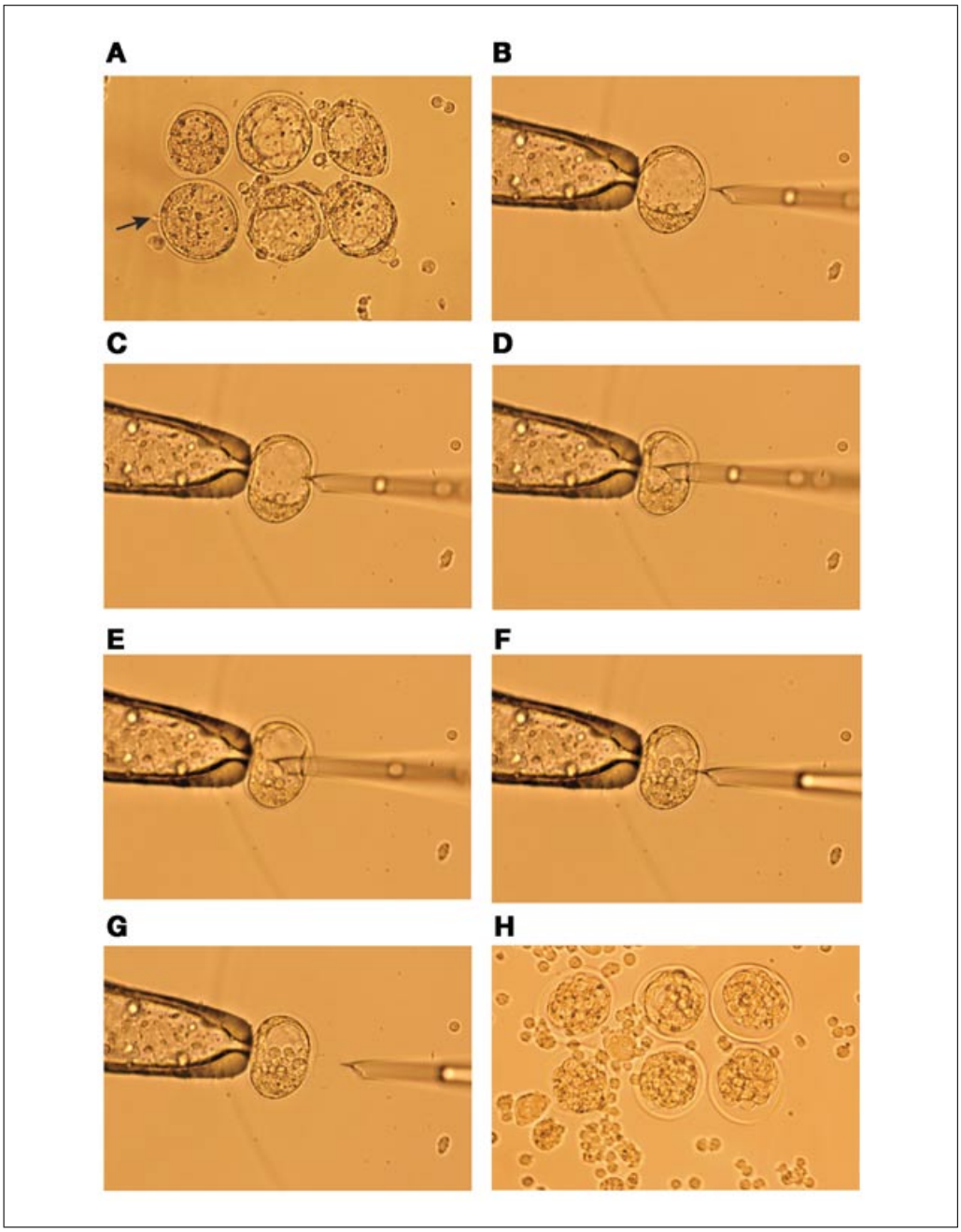


Figure 19.14.8 Blastocysts and sequential ES injection. (**A**) Blastocysts at various stages of development: top left, early stage blastocyst; top middle, middle-to-late stage blastocyst; top right, oval-shaped blastocyst; bottom left, blastocyst in the process of hatching (arrow) showing early bud structure; bottom middle, blastocyst on the verge of hatching with pronounced trophoblast morphology; and bottom right, hatched blastocyst. (**B-G**) Panels showing sequential steps in blastocyst injection. (**H**) Injected blastocysts in various stages of collapse.

19. With a quick, short popping and jabbing motion, insert the injection pipet inside the blastocoel cavity, being careful not to touch the ICM. Pull back the injection pipet slightly after inserting into the blastocoel cavity.

    Touching the ICM is considered harmful to the blastocyst. If the injection pipet is inserted too slowly, the blastocyst will likely collapse, making injection difficult. If the pipet is only partially inside the blastocoel cavity and the blastocyst collapses, medium can be injected to re-expand the blastocyst, and another attempt to inject the blastocyst can be made. Otherwise, move the blastocyst to the side and perform injection on another.

20. Gently expel the ES cells inside the blastocoel cavity onto the ICM, being careful not to inject oil into the blastocoel cavity.

21. Withdraw the injection pipet slowly out of the blastocoel cavity, avoiding the expulsion of the ES cells outside of the blastocyst. Allow the injection site to close before fully withdrawing the injection pipet.

    Letting some medium escape will enhance the settling of the injected ES cells onto the ICM.

    When injecting, consider the pressure inside the blastocoel cavity. If too much medium is injected inside the blastocyst or too much holding force is applied by the holding pipet, the injected ES cells may be forced out of the blastocoel cavity.

22. After injection of the blastocyst, move it to an area away from the uninjected ones on the injection dish with the injection pipet using a technique similar to that described in step 16.

23. When all blastocysts on the injection dish have been injected, use a transfer pipet to place the injected blastocysts into a new microdrop culture (see recipe and see Basic Protocol 1, step 20) and put back into the incubator.

24. Allow the blastocysts to re-expand in culture.

    Re-expansion of the blastocoel cavity of the blastocyst is a possible sign of its vitality.

    The ES cell clones should be injected (see Basic Protocol 3) within 2 hr after putting them on the injection dish.

    If both the holding and injection pipets are flushed out with oil, they have the potential to be reused for another injection day.

UTERINE TRANSFER OF INJECTED BLASTOCYSTS

BASIC PROTOCOL 3

Surgical transfers have to be performed on the same day as the ES cell injections, and E2.5-day pseudopregnant recipient females are generally used to allow the blastocyst to catch up developmentally prior to implantation at E4.5 to E5.0 days. If E2.5 recipients are unavailable, the authors have had success with E1.5 and E2.0 recipients with this procedure. Pups are generally born 18 to 21 days p.c. of the pseudopregnant recipient.

The authors recommend a zoom setting of 3.2 for the surgical microscope, which will allow a large enough field of view for the uterine horn and still make it possible to see the hole in the uterine horn wall.

Materials

Injection medium (see recipe)
Filtered light white mineral oil (Sigma M-3516 embryo tested or equivalent)
Injected blastocysts in microdrop cultures (see Basic Protocol 2)
2.5-day p.c. pseudopregnant B6D2F1 female mice or equivalent (see Support Protocol 3)
2.5% Avertin working solution (see recipe)
0.5% Marcaine (Hospira, cat. no. NDC 0409-1610-50; *http://www.hospira.com/*) or 0.5% Naropin analgesic (AstraZeneca, cat. no. NDC0186-0863-61)

Transfer pipets (see Support Protocol 2)
Mouth pipet assembly (see Fig. 19.11.2 and Basic Protocol 1)
35 × 10–mm tissue culture dishes (BD Falcon, cat. no. 3001)

100 × 20-mm tissue culture dish (BD Falcon, cat. no. 3010) containing two Plasticene rolled strips (Fisher Scientific, cat. no. P148-1LB or equivalent); do not sterilize prior to surgery

Dissecting microscope (10×/23 eye pieces, 1.0× objective, 0.6 to 6.6× zoom, with base illumination)

Mouse fur clippers (Oster finisher trimmer no. 76059-030 or equivalent)

70% ethanol pads

Betadine swabs

Surgical stand (6-1/8 in. × 7-1/2 in. Nalgene container top with vinyl bumpers attached to bottom, or equivalent)

Surgical microscope (16×/16 eye pieces, 0.63× objective, 0.8 to 6.6× zoom) with Diagnostics SMS6B sliding zoom stand or equivalent)

Fiber-optic light source (Zeiss KL-1500 or equivalent)

Surgical instruments, cleaned and sterilized by placing in glass bead sterilizer (Fine Science Tools FTS 250 or equivalent) for 10 sec prior to use in surgery:

Dissection scissors (Roboz RS-5880 or equivalent)

No. 7 curved forceps (Roboz RS-5064 or equivalent)

Foerster forceps, straight tips (Roboz RS-5065 or equivalent)

Dieffenbach clamp (Roboz RS 7422 or equivalent)

Wound clip–removing forceps (Roboz RS-9268 or equivalent)

Wound clip applier, Reflex 9 (Roboz RS-9260 or equivalent)

9-mm wound clips (Roboz 9262 or equivalent)

Autoclaved sterilization pouch 5-1/4 in. × 10 in. (Fisher Scientific, cat. no. 01-812-54 or equivalent) to serve as sterile field for instruments

Sterile gauze, 2 × 2-in. and 4 × 4-in.

25-G, 5/8-in. needles

1-cc tuberculin syringes

Sterile cotton swabs

37°C slide warmer

Additional reagents and equipment for injection of the mouse (Donovan and Brown, 2006b)

Load transfer pipets with injected blastocysts

1. Take an already-made transfer pipet and load mineral oil to the hub of the transfer pipet by mouth pipetting (see Fig. 19.14.6).
2. On the inside of the top section of a 35 × 10–mm tissue culture dish, place a small amount (one drop) of injection medium.
3. Under low-power magnification, (a zoom control knob setting of 1.2 with 10× eye pieces and 1× objective), place the transfer pipet into the drop of medium and then onto the area that does not contain medium, and then gently take up the medium, forming an air gap.
4. Immediately place the transfer pipet into the drop of medium, while keeping the air gap, to form a bubble.
5. Repeat steps 3 and 4 to form a second air bubble.
6. Under high power (a zoom control knob setting of 6.6 with 10× eye pieces and 1× objective), take the plate containing medium with injected blastocysts under oil and place the transfer pipet into the medium drop. Take up the medium until the last bubble is just out of the field of view.

If an oil droplet forms on the outside of the transfer pipet upon placing it into the drop of medium, gently shake the transfer pipet back and forth until the drop of oil comes off.

7. Next, take up the injected blastocysts slowly one at a time, allowing as little medium as possible to be drawn up along with them, until all embryos for a given recipient mouse are loaded into the transfer pipet.

8. Make sure the blastocysts are stabilized inside the transfer pipet so that the embryos do not move. If they do move up the transfer pipet, repeat steps 1 through 7.

9. Store transfer pipets with loaded embryos in a tissue culture dish containing two strips of Plasticene prior to surgery.

   The tissue culture dish with Plasticene is not sterilized prior to surgery. Loaded and unloaded transfer pipets are oriented in the same direction on this dish. Only load the transfer pipets that will be used on the next surgical transfer. Since the blastocysts are loaded in the transfer piper just prior to surgery, they are not kept in the incubator while in the transfer pipet. Make up two transfer pipets if surgery is to be performed on both uterine horns.

Surgically transfer embryos

10. Swab the abdomen of an E2.5-day pseudopregnant mouse with an ethanol pad and anesthetize the mouse by intraperitoneal injection (Donovan and Brown, 2006b) of 0.015 to 0.017 ml/g body weight of 2.5% Avertin.

11. When the mouse is fully anesthetized, prepare the animal by shaving the surgical area using clippers.

12. With a 70% ethanol swab, wipe away all loose fur, and then swab the skin with a Betadine swab. Repeat again with another ethanol and Betadine swab in the same order. Place the surgically prepared mouse on a surgical stand and illuminate the surgical area with a fiber-optic light source.

13. Next make a 5-mm incision in the skin ~1 cm away from the thigh area of the hind limb on the back of the mouse, holding the skin with a no. 7 curved forceps (Fig. 19.14.9).

14. Once an incision has been made through the skin, grasp the underlying muscle with a pair of Foerster forceps, and cut through until reaching the peritoneal cavity.

15. Under the surgical microscope, look for the fat pad and the ovary and, using Foerster forceps, gently pull out the ovary by the fat pad.

    The ovary and fat pad may be seen through the dorsal muscle if there is a low amount of fat on the mouse. The ovary will have a bright red color due to the formation of the corpus luteum. Do not use mice that lack a corpus luteum on the ovary. This would indicate that the mouse is not pseudopregnant. The ovarian fat pad will appear bright white in color with a smooth texture. Avoid the rough-looking, beige tissue that is attached to the spleen, which is most likely the pancreas.

16. Grab the fat pad with a Dieffenbach clamp and gently lay it over the other side of the back onto 2 × 2–in. sterile gauze.

    The mesometrium structure should be on the bottom of the field of view.

17. Orient the mouse in such a way that the ovary and fat pad are in the 9 o'clock position. Gently grasp the uterus with Foerster forceps, and, with a 25-G needle attached to a 1-cc syringe, make a hole in the uterus under the forceps into the lumen.

    The hole for the transfer pipet should be made near the tip of the uterine horn just as the taper from utero-tubal junction ends and becomes full size. This will allow for a higher rate of implantation than transferring near the cervix. This offers the added advantage of being easier than transferring at the very tip of the uterine horn, especially since there is a much bigger lumen to put the 25-G needle and transfer pipet into.

    If the needle is inside the lumen, it should move freely with no resistance.

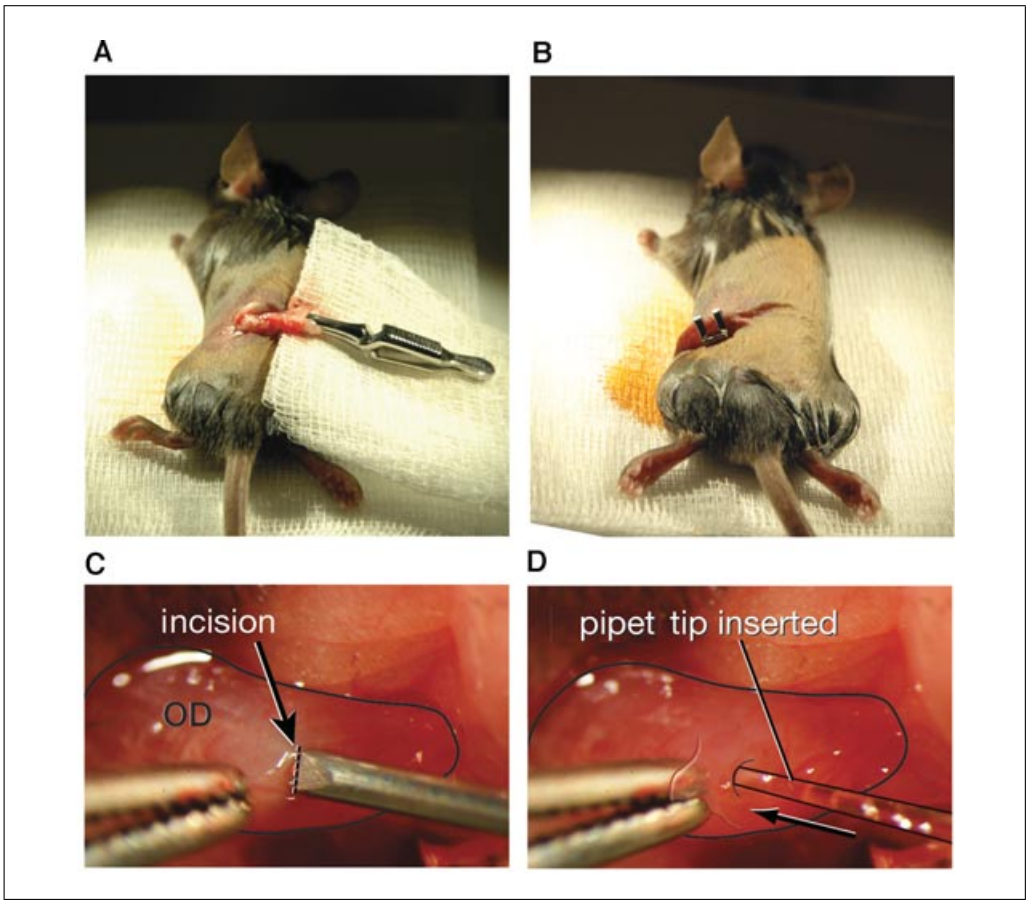


Figure 19.14.9 Diagram of uterine transfer of blastocysts. (**A**) The uterine horn has been extracted from the peritoneal cavity. (**B**) The surgical incision closed by wound suture. (**C**) Hole made in the uterus by 25-G needle into the lumen. (**D**) Transfer pipet placed inside the hole of the uterus into the lumen, showing air bubbles present inside the pipet.

18. Insert the transfer pipet, loaded with injected blastocysts, just barely inside the hole in the uterus. Gently blow the embryos inside the uterus by mouth pipetting, using the bubbles as a marker to see if the blastocysts are inside the lumen.

    If the bubbles inside the transfer pipet do not move when blowing them into the lumen of the uterus, the transfer pipet may be touching the inside uterine wall. Slightly pull back the transfer pipet and try again. If this does not work, take out the transfer pipet and place in a dish containing a small amount of injection medium under the dissecting microscope. If a blood clot is seen, dislodge it and reload the transfer pipet again with the injected blastocysts. Repeat step 18.

19. Under a dissecting microscope, check to see if there are any embryos left in the transfer pipet. Repeat step 18 if blastocysts are left inside the transfer pipet.

20. Release the Dieffenbach clamp and hold the fat pad with the Foerster forceps. Place the uterus and ovary back inside the peritoneal cavity. Do this while holding the incision open by the skin with the curved no. 7 forceps.

21. Gently swab the lateral dorsal muscle incision with a sterile cotton swab saturated with 0.5% Marcaine or 0.5% Naropin analgesic.

22. Hold together the skin incision with a no. 7 curved forceps, and close the wound with a 9-mm wound clip.

23. Repeat steps 13 to 22 if transferring blastocysts to the other uterine horn.

24. Place the mouse on a 37°C slide warmer that has gauze placed on top of it. Allow the mouse to stay on the slide warmer until it wakes up.

25. Place the mouse in a cage for further recovery. When the mouse appears fully recovered from the Avertin, transfer it back into the animal room.

Enough embryos should be transferred to allow the pups to be born healthy. If too few embryos are born, the pups will tend to be large in size, causing trauma to the pups and mother, as well as decreased lactation. If too many embryos are transferred, the pups could be severely runted and die. Based on the authors' experience using B6D2F1 and B6CBAF1 recipient mice, only six mice will be implanted per uterine horn; none of the blastocysts will migrate to the other uterine horn. Five to ten embryos should be transferred into each uterine horn, with a maximum of twelve embryos transferred if transferring into both horns (Hogan et al., 1994). If new to this surgical procedure, allow for a greater number of embryos when transferring, to allow for enough embryos to implant.

GENERATION OF PSEUDOPREGNANT RECIPIENT FEMALES

SUPPORT PROTOCOL 3

Various strains of mice can be used for the purpose of serving as pseudopregnant foster mothers. Outbred mice tend to be the best mothers, and two notable examples are the Swiss Webster and CD1 strains. C57BL/6 hybrid strains are another alternative, as well as B6D2 and B6CBA. The use of inbred strains is generally not recommended, but if it is unavoidable, the FVB/N strain is the best choice. The strain of mouse chosen should be proven to have good mothers that take care of their offspring.

Vasectomized males of just about any strain could be used as long as they are known for their ability to plug females. Balb/c males seem to be the most commonly used for this purpose, though other strains of males could be used.

Even if the vasectomized mice are from a reliable supplier, the males should be tested for sterility. Before the vasectomized males become too old and lose their plugging ability, new ones should be ordered, and these should be tested prior to use. The new vasectomized males could be housed with one or two females up to a maximum of 2 months to test for sterility. If time is a factor, there is the option of superovulating the females with PMSG and HCG (Support Protocol 1) and then housing them with the vasectomized males to see if the latter are truly sterile.

Table 19.14.2 shows a schedule for embryo collection and transfer into recipient females.

Materials

B6D2F1 (C57Bl/6NCr x DBA/2NCr) female mice, or equivalent strain (10 to 12 weeks of age)

Balb/cAnNCr vasectomized male mice, or equivalent strain (at least 6 to 7 weeks to 18 months of age)

1. Mate one female to one vasectomized male 4 days prior to the harvest date for collection and injection of blastocysts.

2. The next morning, before 0900 hr, check the mated females for copulation plugs. Remove the plugged females into another cage and mark with the date they were plugged.

The authors initially mate 25 females 4 days before the start of ES cell injections. An additional 25 females are used to replace any plugged females. Unplugged females are kept with the males until 2 days prior to the last injection day for the week. Any unused females will then be separated out from the males and then can be reused for future matings. A period of at least 5 days should elapse before the mice are mated again. This will allow the mice to reestablish the estrus cycle.

Table 19.14.2 Embryo Collection and Recipient Schedule[a,b,c]

Day	Collect embryos	PMSG (1:00 p.m.)	HCG (12:00 p.m.)	Separate embryo donor females	Pseudopregnant recipient females
Monday	C		B		Separate, and mate again
Tuesday		C		B	Separate, and mate again
Wednesday	A				Separate
Thursday		A	C		No matings, rest day
Friday	B			C	Mate (25 B6D2 F1)
Saturday		B	A		Separate, and mate again
Sunday				A	Separate, and mate again

[a]Each letter denotes a group of (ten) C57B16 females used for a certain injection day.
[b]Recipient females that are plugged are separated and each is replaced with another B6D2 female, for a total of 50 B6D2 females for the week of injections.
[c]Group A females could be separated on Monday instead of Sunday if it is not necessary to know the number of plugged donor females.

The time window from when the female is plugged by the male to when the plug can no longer be seen is thought to be 12 hr. If necessary, females can be mated with the males in the morning to generate plugged pseudopregnant recipients in the afternoon. The plugs should look white or beige in color. If the females are checked for plugs soon after being impregnated by the male, the plugs will look very large, and after a longer period they will look smaller.

BASIC PROTOCOL 4

GENERATION OF GERMLINE KNOCKOUT MICE

Cells from various strains can, in theory, be used as the host blastocyst and as the ES cell line. Generally the most common strain of mouse used for the host blastocyst is C57BL/6 mice and, for the ES cells, the 129 strain. It is best to use strains of mice that are different in coat color for the blastocyst and the ES cell line. The mating scheme to generate germline knockout mice will be determined by what strains of mice are used in the generation of chimeric mice.

If one is using a C57BL/6 donor strain for the blastocyst, the chimeras could be mated with either a C57BL/6 or a Black Swiss to generate germline mice. Each of these mice has disadvantages and advantages. The Black Swiss mice are better breeders, and the mothers take better care of their offspring and, unlike C57BL/6 females, are not disturbed easily by noise and vibration (Doetschman, 1999). However, if C57BL/6 mice are used, the genetic background is conserved, and if Black Swiss mice are used, this might be lost since they are outbred mice. This could have possible implications for the phenotype of the knockout mouse created. Also, if using either the C57BL/6 or Black Swiss strains of mice to mate with the chimeric mice, the germline mice will be on a mixed strain background, and in order to achieve an isocongenic C57BL/6, FVB/N, or other strain background, the mice will have to be back-crossed at least ten times (Silver, 1995). It is possible to use speed congenics and PCR methodology to limit the number of back-crosses needed for a deleted gene of interest on a particular isogenic strain background (Wong, 2002). This would be done by obtaining tail snips from the offspring of parental founder transgenic or gene knockout mice that have been mated to a certain isocongenic strain of interest. DNA is then extracted from these samples and microsatellite markers amplified by PCR to determine the amount of isocongenic purity of these offspring. The offspring with the highest isocongenic purity is then mated back to the same isocongenic strain.

Another option is the mating of the C57BL/6 × 129 chimera with an isogenic strain of 129 mice that has been used to generate an ES cell line. The 129 isogenic strain can be mated to the chimera to obtain F1 founder mice on the isogenic strain of the ES cell line in only one back-cross generation. One drawback to this scheme is that some strains of 129 mice have low fecundity, and this may result in breeding problems in the analysis of the mouse knockout phenotype. If ES cells have contamination from other strains in the genetic background (Simpson et al., 1997), then the mice will still have to be back-crossed in order to obtain isocongenic purity.

If using a coat-color marker to determine the chimeric composition of the ES cells, it is best to choose the chimeras with the highest contribution possible. They should be robust and preferably male, and they should not be dwarfs. Female chimeras could be used if needed, but are not preferred in that ES cells are generally male. Any female chimera would likely have lost the Y chromosome, becoming XO, and this is thought to be more unstable (Papaioannou and Johnson, 2002).

Female chimeras have been known to generate germline mice even with the loss of the Y chromosome. Another advantage of using male chimeras over females is that a large number of offspring can be generated in a short period of time, ensuring the transfer of the knockout allele to the F1-generation offspring.

When mating the chimeras, the most efficient mating scheme would be the mating of two females to one male chimera, Another approach is the so-called "shotgun" approach, the mating of four females to one male chimera. One drawback to this approach is that there could be a delay in the generation of offspring because of the fact that the males may become distracted by so many females and delay plugging. Furthermore, there could be a lot of mice generated at one time, which would take up a lot of cage space.

The mating scheme chosen to generate germline mice must conform to the IACUC regulations of the institution where the animal work is taking place.

REAGENTS AND SOLUTIONS

Use tissue culture-grade water and reagents when working with cells and embryos. For common stock solutions see ***APPENDIX 2B****; for suppliers see* ***SUPPLIERS APPENDIX****.*

Avertin, 2.5% (w/v)

To make 100% Avertin stock solution: Add 50 ml of tertiary amyl alcohol (Fisher Scientific, cat. no. A730-1) to 50 g of 2,2,2-tribromoethanol (Fluka, cat. no. 90710). Add the smallest stirring bar possible to the mixture and place on a stirring plate. Not all 50 ml of the tertiary amyl alcohol may fit inside the 2,2,2-tribromoethanol bottle due to the stirring bar inside. Allow the mixture to stir at room temperature until the stirring bar is heard. Keep stirring the mixture overnight at 4°C to make sure the 2,2,2-tribromoethanol is dissolved. The next day, using a 1-ml pipet, check to see if the 2,2,2-tribromoethanol is completely dissolved. If there are no solid crystals in solution, the stock solution is ready. The color of the Avertin stock solution should have a clear to very pale yellow color. If the solution has a dark brown color, discard.

continued

For the 2.5% working solution: Allow sterile Dulbecco's phosphate-buffered saline (DPBS without calcium or magnesium; Mediatech cat. no. 21-031-CV or equivalent) to warm up to room temperature. To 10 ml of DPBS, add 250 μl of the 100% Avertin stock solution, then shake and vortex until fully dissolved. Filter this solution through a 0.22-μm, nonpolystyrene filter unit. Store in a polypropylene tube covered with foil.

There will be some variation in each batch of stock Avertin, so it is essential that it be tested on mice. prior to using in surgery, for efficacy and toxicity. Both solutions should be stored for a minimal period of time at room temperature and shielded from light, which will cause the breakdown of Avertin into toxic products. If a glass container is unavailable, use plastic containers such as polypropylene. Avoid using polystyrene, as it will chemically react with the Avertin stock solution and become toxic to the mouse, possibly leading to peritonitis.

The recommended dosage of Avertin is between 0.015 and 0.017 ml/g body weight. If the working solution is being reused, it is suggested that the solution be refiltered soon after use to reduce the risk of possible bacterial contamination. If it is thought that the working solution is contaminated, discard it.

Human chorionic gonadotropin (HCG), 50 IU/ml

Under sterile conditions, add 10 ml DPBS (without calcium or magnesium, Mediatech, cat. no. 21-031-CV or equivalent) to a vial containing 10,000 IU of chorionic gonadotropin (Sigma, cat. no. CG10). Take 2 ml of this solution and dilute into 40 ml of PBS. Aliquot this solution into tubes and store up to 6 months in a –30°C freezer.

Injection medium

81 ml Dulbecco's Modified Eagle's Medium (DMEM) containing 4500 mg/liter D-glucose, L-glutamine, and HEPES (Invitrogen, cat. no. 12430054)
8 ml of fetal bovine serum (FBS; preferably already ES-cell tested)
0.5 ml penicillin/streptomycin solution containing 10,000 units per ml penicillin and 10,000 μg/ml streptomycin (Invitrogen, cat. no. 15140-122)

Filter the prepared medium with a 0.22-μm filter unit. Divide the injection medium into three 50-ml tubes. Store up to 1 month at 4°C. Use one tube for each injection day.

Microdrop cultures

Place 30 μl of injection medium (see recipe) on a 35 × 10–mm tissue culture plate. Overlay the drop of medium with prefiltered, embryo-tested mineral oil (Sigma, cat. no. M-3516 or equivalent).

Microinjection setup

An inverted microscope is generally used, and it should have 10× and 20× objectives, with the 40× objective being optional. The 20× objective is more useful for those who are first learning ES cell injections, but the 10× objective is used more often by more experienced researchers. There are three optical systems most commonly used for microinjections. Hoffman generally works best for viewing items through plastic, Nomarski or differential interference contrast (DIC) is better suited for looking through glass, and phase contrast can be used for either plastic or glass (see *UNIT 4.1* for the principles of these optical systems). In the authors' laboratory, DIC optics are used in the microinjection microscope. General injection

continued

and holding line systems would consist of a syringe-type unit, line tubing, and pipet holder, and may or may not have a stopcock attached to an oil-filled syringe. Luer-locking or other types of connection systems should be used whenever possible to minimize or eliminate possible leaks that might occur. For the injection and holding line systems, a syringe that is filled with oil, rather than air, will tend to give better control. Silicone oil would be a better choice than mineral oil in that mineral oil will tend to shrink, causing air bubbles that affect control. An injection line system with a stopcock and a tuberculin-type syringe with an oil-filled reservoir will allow the flushing out of debris and air bubbles from the injection and holding lines and both pipets. A 12-cc oil-filled syringe reservoir will allow the microsyringe units to be aligned back to the midpoint, as opposed to a system that does not use an oil-filled syringe.

For a microsyringe controlling unit, the authors prefer a device made by Minnetonka Instruments, which has an internal Hamilton syringe and an external Mitutoyo syringe. The injection line tubing and Hamilton syringe should be a smaller volume than the holding system because the injection pipet is much smaller than the holding one. As for micromanipulators, the authors have had successful experience with Narishige and Leica manual manipulators. There are multiple options available, and users should decide which model will work best for their use. It should include fine as well as coarse movement. A manual model is preferred for the actual injection of the blastocyst, because it allows for more precise movement and control, unlike some powered systems. To avoid any vibration problems that could affect microinjections, an air suspension table using N_2 or CO_2 gas cylinders should be considered.

Also see Basic Protocol 2 and Figure 19.14.4 for the makeup of the microinjection system.

Pregnant mare serum gonadotropin (PMSG) 50 IU/ml

Under sterile conditions, add 3 ml of sterile PBS to a vial of 2000 units of PMSG (Sigma, cat. no. G 4527). Add this solution to a tube containing 40 ml of DPBS (without calcium or magnesium, Mediatech, cat. no. 21-031-CV or equivalent) aliquot it into tubes, and store the tubes up to 6 months in a –30°C freezer.

Tween 80 solution, 1.25% (v/v)

Add 1.25 ml of Tween 80 solution (Fluka, cat. no. 93780) to 98.75 ml of sterile water. Filter through a 0.22-μm filter unit. Store up to 1 year at 4°C.

COMMENTARY

Background Information

Early cancer studies in mice initially done by Stevens (1970, 1973) led to the generation of the strain of mice known as 129. Subsequent developmental studies in these mice made possible the creation of two major mouse cell lines, the embryonal carcinoma (EC) cells and the embryonal stem (ES) cells. EC cells were originally derived from teratomas that came from Stevens' mice (Kahan and Ephrussi, 1970; Rosenthal et al., 1970). ES cells were initially derived from the inner cell mass (ICM) of mouse blastocyst-stage embryos from 129 mice (Evans and Kaufman, 1981; Martin, 1981).

With the development of the cell injection technique into blastocyst mouse embryos (Gardner, 1968), the true potential of these cell lines to be able to contribute to the developing mouse embryo could then be assessed. The EC cells initially showed promise with pluripotency in the formation of chimeric mice and the contribution to various tissues (Brinster, 1974). However, EC cells had problems with developmental abnormalities, tumors, and a lower germline transmission efficiency compared to ES cells (Papaioannou and Rossant, 1983). ES cells, on the other hand, showed this pluripotency in chimeric formation, as well as totipotency in germline

transmission to the offspring, without the problems associated with EC cells (Bradley et al., 1984).

Upon understanding the mechanisms of homologous recombination, generating knockout mice became possible. Folger et al. (1982) were the first to demonstrate that, through homologous recombination, nonreplicating DNA could be transferred into a mammalian cell. This would later lead to gene targeting of a plasmid into the β-globin gene of mammalian cells (Smithies et al., 1985). The first gene to be targeted in ES cells was the hypoxanthine phosphoribosyltransferase (*HPRT*) gene (Thomas and Capecchi, 1987; Doetschman, et al. 1988). This had the benefit of being X-linked, so recombination of only one copy was needed to show deletion in male ES cells. Later, ES cells with the *HPRT* mutation would be shown to possess the ability for germline transmission (Hooper et al., 1987; Kuehn et al., 1987; Koller et al., 1989; Thompson et al., 1989). While the generation of a mutant mouse with this gene showed initial promise as a model for Lesch-Nyhan syndrome, the phenotype did not correlate with the human disease (Samuel et al., 1993). The *HPRT* knockout mouse phenotype highlights what may happen when one attempts to delete a human gene in the mouse.

An alternative approach for generating chimeric mice is the ES embryo aggregation technique. This technique can be further divided into two basic methods. One of them is the diploid aggregation technique, which involves ES cells cultured with morula-stage embryos. This can further be divided into two techniques. One technique developed by Wood et al. (1993) involves culturing the morula on a layer of ES cells. The other technique, devised by Khillan and Bao (1997), also uses morulas and ES cells, but with a defined microwell and a given number of cells. A second morula aggregation method, in contrast, uses a tetraploid developed by Nagy et al. (1993)—2-cell embryos are electrically fused together and cultured until the 4-cell stage. These 4-cell tetraploid embryos, which are developmentally compromised, are then used to sandwich the ES cells, allowing integration (fusion) to occur. This is done in a well on a tissue culture plate. All three methods have the advantage of not requiring injection skills or the expensive equipment needed to perform the ES microinjection procedure. Ultimately the success rate of all these methods mentioned, as well as blastocyst injection, is determined by parental line used and targeted clones obtained from this line.

The last key component for generating chimeric mice and subsequent knockout mice is the uterine surgical transfer technique. This technique was initially developed by McLaren and Michie (1956). They determined that a critical parameter for success in the uterine transfer technique is the surgical transfer of E3.5 blastocyst embryos into E2.5-aged recipient females. Based on this work, McLaren and Biggers (1958) cultured morula-stage embryos in vitro to the blastocyst stage and surgically transferred them by the uterine technique to successfully generate live offspring.

A recent development suggests the possibility of using other types of stem cells instead of ES cells to generate chimeric and knockout mice. Guan et al. (2006) isolated spermatogonia stem cells (SSC) and maintained them under ES cell growth conditions. As a result, these cells retain characteristics of both SSC and ES cells, and they were subsequently named multipotent adult germline stem cells (maGSCs). This technique may be another method to derive cells that are ES cell capable, without having to obtain them from the inner cell mass of a blastocyst. This may make it easier to acquire ES-like cells from a strain of mouse where currently none is available.

Critical Parameters and Troubleshooting

There are three main areas where problems may be encountered while creating chimeric mice: harvesting blastocyst embryos, injecting ES cells into the blastocyst embryo, and surgically reimplanting embryos into pseudopregnant female mice. The ES injection technique can largely be controlled by conditions in the laboratory, which can make problems in this area easier to solve. The other two areas—harvesting the blastocyst embryos and surgically reimplanting them—depend on conditions in the lab, the in vivo variability of the mice themselves, and conditions in the animal facility. Thus, solving problems in these last two areas can be quite complex. It is probably best to look at laboratory conditions first, which are more easily controlled, then proceed to looking at the mice themselves and the animal-facility conditions. It is important that detailed notes be taken, which can greatly help to pinpoint problems. Aspects of all related procedures should be assessed periodically so that potential problems can be addressed early.

Low number of blastocysts collected

The number of blastocysts obtained through the process of superovulation and harvesting of blastocyst embryos can depend on several factors. The age of the mice and the conditions in the animal facility are two areas affecting blastocyst yield. Females that are 3 weeks old are preferable because they appear to be more affected by the PMSG and HCG hormones. If 3-week-old females are not available, 4-week-old females are the next best choice. Female mice at 6 weeks or older should be producing their own reproductive hormones, which could interfere with any hormone injected intraperitoneally. For the breeding stud males, a range of 7 weeks to ~10 months of age has worked well in our experience. Around 10 months of age, plugging by the stud males becomes variable. Another sign that the stud males are too old is that only very early-stage embryos are recovered. Replacing the stud males will solve both of these problems. In the animal room where the mice are being held, the most important factor is the timing of the light and dark cycle. A light and dark cycle of 14 hr light and 10 hr dark is considered to be conducive to mating by the mice. The light cycle should be checked if there is any doubt that it is working correctly.

Hormone-related issues can also influence blastocyst yield. These would include the source where the hormones came from, a possible bad lot, or how the hormones were prepared. A reputable source that others have had success with should be the first choice. Another lot number of the same hormones should be tried to see if similar results are obtained. If the hormones have not been diluted to the proper concentration, they should be discarded. Freezing and thawing of hormones is to be avoided, as this will decrease the activity and effectiveness. The proper dosage of hormone being injected intraperitoneally and the timing of the hormone injections are also important.

What day and time the blastocysts are to be harvested will determine the timing of the hormone injections. If, at the desired time of day, only morula- or earlier-stage embryos are obtained, both of the hormone injections should be done at earlier times. If only hatched blastocysts are collected, hormone injections should be done at later times.

When new to the technique of harvesting and collecting blastocysts, it is not uncommon to obtain a lower embryo yield than that of someone who is experienced. Blastocysts can be lost while harvesting the uterine horns. Since the blastocysts are free-floating inside the uterine horns, care should be taken to not cut holes in the uterine wall when cutting off the mesometrium structure. Letting the uterine horns snap like a rubber band should also be avoided when extracting out the uterine horn, as this can also contribute to losing blastocysts. After all of the uterine horns have been taken out of the original holding dish, check to see if there are any blastocysts left on the dish. If there are a lot of them on the dish, then there is likely a problem with the harvesting technique. Practice is needed until the technique is mastered. The way the uterine horns are flushed out can also affect blastocyst yield. The opening of the cervix should be oriented slightly up and to the side. This will allow one to see the injection medium being flushed out of the cervix while keeping the flushed medium inside the 35 × 10–mm dish. In our experience, a 30-G needle works best for flushing the blastocysts out of the uterine horns. A good amount of pressure can be applied to the syringe with less chance of medium coming out of the dish, unlike that which occurs with larger-gauge needles. It should be noted that, even under optimal conditions, the process of superovulation and harvesting the blastocysts does not always work well all of the time. There will be days when very few embryos are recovered.

Blastocyst injection procedure

Blastocyst-stage embryos are very hardy and can withstand injections quite well, unlike embryos at earlier developmental stages, such as zygotes. There are three main areas where problems might arise during ES cell injections of blastocysts: the injection and holding line systems, the injection pipet, and the holding pipet (Bradley, 1993).

In the injection and holding line systems, problems that may commonly occur are that the oil does not move inside the pipet, there is a lack of control or the movement is very slow, or the cells are continuously taken up by the holding and injection pipet when the line is open. These problems could be due to air in the system, a leaky or worn-out stopcock, or a leaky joint or connection. If air is found in the system, a 19-G needle and syringe filled with oil (or the oil-filled syringe reservoir) is a good way to purge this out. A leaky stopcock due to wear must be replaced with a new one. Reconnect the loose joint or connection and replace any fittings or pieces of tubing if necessary.

Another area where problems might arise is the injection pipet. Debris or oil can become

stuck to the inside and outside of the injection pipet; there may be blockage or air bubbles that form inside the injection pipet; or cells may lyse inside the injection pipet. The injection pipet may not penetrate inside the blastocyst, and may cause it to collapse before penetrating the blastocoel cavity. Debris, silicone oil, air bubbles, or other types of blockage may be flushed out with the syringe reservoir filled with silicone oil. If this method does not remove the debris or oil attached to the inside of the pipet, a smaller MEF cell may be used much like a pipe-cleaning device. Debris on the outside of the injection pipet may be removed by moving up the injection pipet through the medium/oil and oil/air interfaces. The holding pipet could be used to pull debris off the injection needle, but care should be taken not to block the inside of the holding pipet. The reason for lysis of ES cells inside the injection pipet could be the fact that some of the ES cells inside the pipet are too large, are of poor quality, or have come in contact with oil. ES cells should be well trypsinized to remove cell-surface proteins, and kept cold to inhibit them from reforming. If not, this can cause stickiness inside and outside the injection pipet, impeding injections. If the inner diameter of the injection pipet is too small, then the cells could lyse. Failure to inject into the blastocoel cavity can be the result of a dull or badly damaged injection pipet. It is best to inject in the junction between the ICM and the trophoblast cells, which will make injections easier. The injection pipet should be at approximately the midpoint of the blastocyst height, so that it will be in the center of the blastocoel cavity. If it is on the bottom of the blastocoel cavity, it will be harder to inject. The injection pipet should be raised up to avoid this. It is very important to have extremely good injection pipets when new to the injection procedure, as this will make injections much easier and can help avoid much of the frustration that is often encountered when learning the injection technique. If all other attempts fail to correct problems encountered in injections of the blastocyst, the injection pipet needs to be replaced.

The third and last area where problems can occur is the holding pipet. It could become blocked with debris or have air bubbles forming inside. Erratic holding control may be experienced, or there may be no holding suction at all. If bubbles form or the inside of the holding pipet becomes blocked, the holding pipet may be flushed out with the syringe reservoir filled with silicone oil. Usually, this method will not work with a large blockage. If this happens, the holding pipet will need to be replaced. In our experience, a holding pipet with an inner diameter of ~20 μm works best for holding control. A smaller diameter may not have enough holding power, while a larger diameter tends to give more erratic control and the possibility of the blastocyst ending up inside the holding pipet. If the blastocyst moves when attempting to inject, this could be due to the fact that the holding pipet is too low with respect to the surface of the well of the injection dish and there is not enough room between the holding pipet and the surface of the well. It also may mean that the holding pipet is not properly aligned or is too close to the shoulder of the injection dish. Touching the blastocyst to the bottom of the well adds much-needed stability for holding.

Low birth and survival rate

There could be a number of reasons for a small litter size or for pseudopregnant females not taking care of the pups. The best place to start is to do in utero analysis of uninjected blastocysts by extracting out the uterine horns of pseudopregnant recipient females and looking at E9.5 embryos. If there is a low percentage of embryos being implanted, this could most likely be due to factors related to the surgical technique.

Surgical technique can be influenced by several factors. Only perform the uterine surgical transfer procedure on mice that have the corpus luteum (red spots) present on the ovary. Absence of the corpus luteum suggests the female was not plugged or was not in estrus when plugged by the vasectomized male. Avoid touching the uterine horns and ovary when possible, except to hold the uterus to make the hole into the lumen and insert the transfer pipet. When making the surgical incision in the dorsal muscle wall, avoid cutting the blood vessels, which can cause excessive bleeding, making it hard to find the ovary and fat pad. Like the surgical incision, avoid the blood vessels in the uterine wall when inserting the 25-G needle in the uterine wall to make the hole in the lumen. If a blood vessel is damaged during this procedure, bleeding might clog the inside of the transfer pipet, and mouth pipetting the embryos will be difficult if not impossible. Reload the transfer pipet, and make another attempt to transfer the blastocysts. Both the 25-G needle and transfer pipet should pass freely inside the hole in the uterine wall. This indicates that both have entered inside the lumen. The attempt to transfer the

blastocysts will not be successful if there is any obstruction. The procedure of mouth pipetting, the quality of the transfer pipet, and how the transfer pipet is loaded can influence the outcome of the surgical transfer procedure. As the investigator becomes skilled in using the mouth pipet, this factor can largely be ruled out. If the inner diameter of the transfer pipet is too small, there is a tendency for the transfer pipet to become easily clogged by bleeding which can happen during the procedure, and if the inner diameter is too big, this may affect the control in that it will be too sensitive and the blastocysts will migrate up the transfer pipet. Also, a transfer pipet that is too small in diameter may make it difficult to blow the blastocysts inside the lumen of the uterus. The way a transfer pipet is loaded with blastocysts can make a difference in the transfer results. There is a lot of variation in the way a transfer pipet can be loaded with blastocysts. Air bubbles and oil are factors that can help to stabilize the embryos inside the transfer pipet, but they will move as the embryos are blown out with the mouth pipet. After the transfer of the blastocysts has taken place, examine the transfer pipet under a dissecting microscope to see if there are any embryos left inside. If any blastocysts are left, they should be transferred until they are no longer in the transfer pipet.

If the rate of implantation of E9.5 embryos is high, then there may be factors other than the surgical technique that affect survival and litter size; one of these is related to the culture conditions of the ES cells and blastocyst embryos. Reagents that are used to grow the ES cells or that may come into contact with the blastocysts could be the culprit. Serum, DMEM medium, and oil should be tested on the parental cell line first before they are used on targeted ES cell clones. Reagents used more specifically with the in vitro blastocyst should be tested before the blastocysts are to be injected with ES cells. If these reagents have been tested before, they are most likely not the cause. However, if a new lot is now being used, it is worthwhile to investigate. Another lot should be tried to see if similar results are obtained. If a new lot of a particular reagent has been determined as the cause of survival failure, it should be discarded and replaced as soon as possible.

Haploid insufficiency of the targeted gene, or the way the construct is designed, may influence embryo lethality and pup death; if other targeted ES cell clones are generally doing well, then this could be the cause.

The other area that may affect the gestation of the pseudopregnant recipient litters concerns the conditions in the animal room where the mice are being housed. Care should be exercised not to stress the recipient mother, because that could cause physiological conditions leading to reabsorption of the mouse embryos in utero or the death of the newborn pups. Probably the key embryonic developmental time points where this stress would likely be the most detrimental are at E4.5 to E5, when the embryos implant, and then shortly before birth. The first week of life is the most critical time for the newborn pups. If they survive this week, there generally should be no complications. Another stress factor is the Bruce effect, whereby the odor of strange males can induce abortion of the surgical pregnancy. Other factors to consider are ammonia levels, noise, vibration, animal handling, animal food nutrition, ventilation, and the light/dark cycle. These conditions in the room should be monitored to determine which of them might be the cause. Infectious agents such as viruses (Brayton et al., 2004), which may be present as a latent infection in the animal colonies, can affect knockout mouse production. Ideally the mice should be housed in specific-pathogen-free facility to avoid pathogen exposure.

Finding the exact cause of embryo lethality can be difficult and time-consuming, as numerous factors affecting pregnancy need to be ruled out. Even the simplest of factors might be the cause of the problem and should not be overlooked. For example, we determined that a defective wheel on one of the animal racks was the possible cause for the pups being reabsorbed in utero and the pseudopregnant mothers not taking care of the pups after birth. When the rack was replaced, these problems went away. Lastly, one factor that is often overlooked is the effect of lighting on embryos (Takenaka et al., 2007), whether it is in the lab or surgical room. This could cause apoptosis to occur in the ICM of the blastocyst embryo, which would have detrimental effects on injected embryos and the potential for live pups being born.

Anticipated Results

With practice, this surgical transfer technique should result in at least 70% to 80% of the surgical recipients giving birth. Even uninjected blastocysts, when surgically transferred and implanted, may end up being reabsorbed during gestation. This can happen as early as E9.5. The number of pups born will be

influenced by the strain of female mouse used for the pseudopregnant recipient. In our experience, the B6D2 and B6CBA strains of mice have given birth to no more than six pups per uterine horn, regardless of how many were surgically transferred. If more than six embryos are surgically transferred, these extra embryos most likely will not implant.

Blastocyst-stage embryos are very hardy, especially when compared to earlier stages. They tolerate changes in temperature, pH, and the technique of ES injection. Unless there is a severe trauma to the embryos, like damage to the ICM, the embryos should not be negatively impacted. In the hands of a skilled injectionist, the ES cell injection procedure should not adversely affect the birth rate of pups being born.

There are numerous ES cell lines available for the purpose of making chimeras to generate knockout mice. The parental ES cell lines the investigator plans to use should first be evaluated for any possible ill effects from these cells. Their efficiency in generating chimeric mice needs to be evaluated before carrying out any gene targeting–related experiments on them. Some markers to look at in evaluating the effectiveness of the parental cell include the percentage of chimerism, the ratio of the number of male chimeras born to females, and the ability to generate germline offspring. In a good parental ES cell line, the chimerism should be high, the male-to-female ratio high, and the number of pups needed to see germline transmission low. It is possible to see chimerism or germline transmission at as early as 5 days, when a small patch of fur becomes visible. This is much easier to see if an albino ES cell line is used, in that these white areas would appear nude before the fur starts to grow in. Germline transmission is detected on other types of 129 ES cell lines used with a C57BL/6 host blastocyst as either a medium brown or agouti (golden brown) appearance.

Even if good parental lines are used, there can be great variability in a targeted clone's ability to create a high percentage of chimeras, and in the germline potential of these chimeric mice. This is most likely due to trauma that the ES cells have to undergo in the electroporation of the gene-targeting construct and selection process to determine a correctly targeted clone. In theory, it should take only two clones to generate two different germline chimeric lines, leading to the establishment of two independent knockout mouse lines. This would confirm that the phenotype is due to the gene-targeting construct and not to the cells themselves. Several targeted clones should be available to ensure that there is germline transmission from two independent clones, should some of the clones give low chimerism and not be germline.

A situation may arise where lethality is most likely related to the clones being injected or the construct that is electroporated inside the ES cells. This can result in no chimeric pups being born at all, or only a few surviving to birth and then dying shortly thereafter. If everything is generally going well with other constructs and ES cell clones, then other factors affecting gestation can be ruled out. If the ES clones in question have been injected additional times and large numbers of blastocysts have been injected with similar results, then this further suggests a construct-related problem. Further injections of ES cells with the suspect construct will most likely be of little use.

There is also the possibility of embryonic and perinatal lethality occurring in the germline F1 mice. As previously mentioned with chimeras, this could be due to targeting construct–related issues. The construct should be re-evaluated and any problems corrected. Additional clones can be generated and injections performed. Lastly, lethality could be due to the function of the gene deleted and its essential role in embryonic development. This haploid insufficiency may be the reason for embryonic and pup lethality. If this occurs, the only kinds of phenotypic analyses that might be carried out would be in utero analysis of the developing embryos or in vitro tissue culture analysis.

Time Considerations

Plan to start the initial intraperitoneal PMSG hormone injections 1 week before the day when the blastocysts will be needed. The entire process of harvesting the blastocysts, injecting them, and surgically transferring them back to a pseudopregnant recipient female is generally performed on the same day. Even for the experienced injectionist, this can make for a long day, especially when problems with the injection system and bad cell morphology of the ES cell clones occur on the same day. The surgical procedure should be practiced before attempting the injection procedure, since it generally takes longer to master. Having a video display for all the procedures aids greatly in the visualization of these techniques and can shorten the time required to

learn them, which is expected to take up to 6 months. How long it takes will ultimately depend on previous experience, the person teaching the procedures, and the desire of the investigator to learn the procedures.

Literature Cited

Bradley, A. 1993. Production and analysis of chimeric mice. *In* Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. (E.J. Robertson, ed.) pp. 113-151. Oxford University Press, New York.

Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 309:255-256.

Brayton, C., Mähler, M., and Nicklas, W. 2004. Viral infections. *In* The Laboratory Mouse. (H. Hedrich, ed.) pp. 357-391. Elsevier Academic Press, New York.

Brinster, R.L. 1974. The effects of cells transferred into the mouse blastocyst on subsequent development. *J. Exp. Med.* 140:1049-1056.

Doetschman, T. 1999. Interpretation of phenotype in genetically engineered mice. *Lab. Anim. Sci.* 49:137-143.

Doetschman, T., Maeda, N., and Smithies, O. 1988. Targeted mutation of the Hprt gene in mouse embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 85:8583-8587.

Donovan, J. and Brown, P. 2006a. Euthanasia. *Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Donovan, J. and Brown, P. 2006b. Parenteral injections. *Curr. Protoc. Immunol.* 73:1.6.1-1.6.10.

Evans, M.J. and Kaufman, M.H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Folger, K.R., Wong, E.A., Wahl, G., and Capecchi, M.R. 1982. Patterns of integration of DNA microinjection into cultured mammalian cells: Evidence for homologous recombination between injected plasmid DNA molecules. *Mol. Cell. Biol.* 2:1372-1387.

Gardner, R.L. 1968. Mouse chimeras obtained by the injection of cells into the blastocyst. *Nature* 220:596-597.

Guan, K., Nayernia, K., Maier, L.S., Wagner, S., Dressel, R., Lee, J.H., Nolte, J., Wolf, F., Li, M., Engel, W., and Hasenfuss, G. 2006. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 440:1199-1203.

Hogan, B., Beddington, R., Constantini, F., and Lacy, E. 1994. Manipulating the Mouse Embryo: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. 1987. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germ line colonization by cultured cells. *Nature* 326:292-295.

Kahan, B.W. and Ephrussi, B. 1970. Developmental potentialities of clonal in vitro cultures of mouse testicular teratoma. *J. Natl. Cancer Inst.* 44:1015-1036.

Khillan, J.S. and Bao, Y. 1997. Preparation of animals with a high degree of chimerism by one-step coculture of embryonic stem cells and preimplanation embryos. *Biotechniques* 22:544-549.

Koller, B.H., Hagemann, L.J., Doetschman, T., Hagaman, J.R., Huang, S., Williams, P.J., First, N.L., Maeda, N., and Smithies, O. 1989. Germline transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 86:8927-8931.

Kuehn, M.R., Bradley, A., Robertson, E.J., and Evans, M.J. 1987. A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* 326:295-298.

Martin, G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

McLaren, A. and Michie, D. 1956. Studies on the transfer of fertilized mouse eggs to uterine foster-mothers: I. Factors affecting the implantation and survival of native and transferred eggs. *J. Exp. Biol.* 33:394-416.

McLaren, A. and Biggers, J.D. 1958. Successful development and birth of mice cultivated in vitro as early embryos. *Nature* 182:877-878.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 90:8424-8428.

Papaioannou, V.E. and Rossant, J. 1983. Effects of the embryonic environment on proliferation and differentiation of embryonal carcinoma cells. *Cancer Surveys* 2:165-183.

Papaioannou, V. and Johnson, R. 2002. Production of chimeras by blastocyst and morula injection of targeted ES cells. *In* Gene Targeting: A Practical Approach, 2nd ed. (A.L. Joyner, ed.) pp. 133-175. Oxford University Press, New York.

Rosenthal, M.D., Wishnow, R.M., and Sato, G.H. 1970. In vitro differentiation of clonal populations of multipotent mouse cells derived form a transplantable testicular teratocarcinoma. *J. Natl. Cancer Inst.* 44:1001-1014.

Samuel, K., Clarke, A.R., Ansell, J.D., and Hooper, M.L. 1993. Age dependent selection against phosphoribosyl transferase–deficient cells in mouse haematopoiesis. *Development* 118:859-863.

Silver, L.M. 1995. Mouse Genetics: Concepts and Application. Oxford University Press, New York.

Simpson, E.M., Linder, C.C., Sargent, E.E., Davisson, M.T., Mobraaten, L.E., and Sharp, J.J. 1997. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nat. Genet.* 16:19-27.

Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A., and Kucherlapati, R.S. 1985. Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. *Nature* 317:230-234.

Stevens, L.C. 1970. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev. Biol.* 21:364-382.

Stevens, L.C. 1973. A new inbred subline of mice (129/terSv) with a high incidence of spontaneous congenital testicular teratomas. *J. Natl. Cancer Inst.* 50:235-242.

Stewart, C.S. 1993. Production of chimeras between embryonic stem cells and embryos. *In* Guide to Techniques in Mouse Development. Methods in Enzymology, Vol. 225 (P.M. Wassarman and M.L. DePamphilis, eds.) pp. 823-854. Academic Press, New York.

Takenaka, M., Horiuchi, T., and Yanagimachi, R. 2007. Effects of light on development of mammalian zygotes. *Proc. Natl. Acad. Sci. U.S.A.* 104:14289-14293.

Thomas, K.R. and Capecchi, M.R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51:503-512.

Thompson, S., Clarke, A.R., Pow, A.M., Hooper, M.L., and Melton, D.W. 1989. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. *Cell* 56:313-321.

Wells, D. 1993. Production of chimeras derived from murine embryonic stem cells. *Methods Mol. Biol.* 18:217-237.

Wood, S.A., Pascoe, W.S., Schmidt, C., Kemler, R., Evans, M.J., and Allen, N.D. 1993. Simple and efficient production of embryonic stem cell–embryo chimers by coculture. *Proc. Natl. Acad. Sci. U.S.A.* 90:4582-4585.

Wong, G.T. 2002. Speed congenics: Applications for transgenic and knock-out mouse strains. *Neuropeptides* 36:230-236.

Key References

Hogan et al., 1994. See above.

Good overall reference on mouse surgeries and injections.

Robertson, E.J. 1993. Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Oxford University Press, New York.

This reference source is especially useful for working with ES cells and solving troubleshooting problems encountered during ES cell injections. Bradley et al. (1984) in this compilation is particularly relevant to the subject matter of this unit.

Joyner, A.L. 2000. Gene Targeting; A Practical Approach, 2nd ed. Oxford University Press, New York.

Another good reference for the procedures related to the production of chimeric mice. Especially useful for information in construct design. Papaioannou and Johnson (2002) in this compilation is particularly relevant to the subject matter in this unit.

Wassarman, P.M. and DePamphilis, M.L. 1993. Guide to techniques in mouse development. *Methods Enzymol.* vol. 225.

This reference is good in characterizing the best morphology of ES cells to inject. It also describes multiple versions of the same procedures. Stewart (1993) in this compilation is particularly relevant to the subject matter of this unit.

Internet Resources

http://www.biosupplynet.com/

This is a useful Web site for finding products and reagents required for procedures in the generation of chimeric mice and related procedures.

http://www.microinjectionworkshop.net

This site is useful for links to other transgenic-related Internet sites. There are also transgenic-related protocols as well as images of these techniques.

http://oacu.od.nih.gov/additional/survivalrodent.html

Ordering page for Training in Survival Rodent Surgery CD-ROM (2001), developed by a subcommittee of the NIH Animal Research Advisory Committee.

CHAPTER 20
Expression and Introduction of Macromolecules into Cells

INTRODUCTION

Cells are fundamentally insular, isolated and protected from their surroundings by their plasma membranes. However, it is frequently necessary for cell biologist to overcome the isolation of the cell's interior in order to probe its workings at a molecular level. As a result, many experimental strategies employed by cell biologists rely upon the introduction or expression of particular macromolecules within cells of interest. The goals of such techniques include the expression or introduction of fluorescently-tagged proteins, allowing the examination of the subcellular localization and behavior of the protein within living cells. Similarly, these techniques can be used to perturb the function of particular proteins through the addition of antibodies, inhibitors or mutant forms of the protein or through inappropriate expression with altered abundance or timing of protein accumulation. Additionally, techniques for introduction of macromolecules into cells are frequently utilized to load cells with impermeant dyes that can be used to monitor various aspects of cellular physiology, including changes in ion concentrations. This chapter will cover methods that have been developed for such experimental strategies. The first two units of this chapter discuss simple and inexpensive methods for the introduction of a variety of macromolecules (*UNIT 20.1*) or specific fusion proteins (*UNIT 20.2*) into cells. *UNITS 20.3*, *20.4*, *20.5*, *20.6 & 20.7* discuss the introduction of nucleic acids into cells for the purpose of altering gene expression.

UNIT 20.1 provides a variety of flexible protocols that can be inexpensively used to load a broad range of macromolecules into cells. Methods covered in *UNIT 20.1* include scrape loading, scratch loading, bead loading and syringe loading. These techniques all rely upon the physical disruption of the plasma membrane to promote the uptake of target molecules, and upon the capacity of cell to re-seal the plasma membrane after damage. The techniques discussed in *UNIT 20.1* have a number of advantages, including the fact that their implementation does not require specialized equipment and that they can be utilized to simultaneously load a large number of cells. They are also useful because a large range of macromolecules can be introduced in this manner. These techniques would not be preferred under conditions where the macromolecule being loaded is particularly precious, since each of the techniques require a relatively large amount of such material.

UNIT 20.2 describes the introduction of proteins into cells using an eleven amino acid sequence from the HIV-TAT transduction domain. This amino acid sequence has the remarkable property of allowing the HIV-TAT protein to pass through the intact plasma membrane and enter the cytoplasm of cells. It has also been shown to confer this property to a number of other proteins when expressed as a fusion moiety. Thus, investigators can express this sequence as a tag to their proteins of interest in bacteria, purify the resultant fusion peptide using standard biochemical means, and introduce the fusion protein into cells by simply introducing the fusion protein to the cell culture medium. Like the methods discussed in *UNIT 20.1*, these techniques have considerable advantages because of their simplicity and because of the fact that they are economical and have minimal requirements for specialized equipment.

Contributed by Mary Dasso
Current Protocols in Cell Biology (2005) 20.0.1-20.0.2

While *UNITS 20.1 & 20.2* offer the possibility of direct introduction of proteins into cells, expression of cloned genes from transfected plasmids is more commonly used to probe protein function. *UNITS 20.3*, *20.4*, *20.5*, *20.6 & 20.7* discuss transfection protocols to introduce DNA into cells for this purpose. These protocols include calcium phosphate transfection (*UNIT 20.3*), DEAE dextran transfection (*UNIT 20.4*), electroporation (*UNIT 20.5*) and lipid-mediated transfection (*UNIT 20.6*). Calcium phosphate (*UNIT 20.3*) and DEAE dextran (*UNIT 20.4*) chemically induce the association of plasmid DNA to the cell surface, resulting in its uptake through endocytosis. Cationic lipids spontaneously associate with nucleic acids through charge interactions. In the case of plasmids, the resulting DNA-lipid structures are also capable of transducing genes into cells through endocytosis (*UNIT 20.6*). In all cases, the pathway through which DNA eventually enters the nucleus after endocytosis is poorly defined. By contrast, electroporation uses an electric field to open pores in the cell to allow the entry of DNA through diffusion. Electroporation is less dependent upon special characteristics of the cell than other transfection techniques and can therefore be used for introduction of plasmid DNA into a very broad spectrum of cell types. Calcium phosphate, DEAE dextran, and lipid-mediated transfection are typically employed in the transfection of adherent cell lines. Lipid-mediated transfection and electroporation are typically used for non-adherent cells. Since the successful use of all transfection methods depends heavily on the cell line under study and conditions of the particular experiment, *UNIT 20.7* discusses strategies for optimization of transfection efficiency using reporter systems.

It is frequently desirable to control the expression of mRNA from plasmids after their introduction into cells, particularly those that encode toxic protein products. *UNIT 20.8* describes the use of tetracycline-regulated gene expression systems for this purpose. This system is based upon an artificial transcriptional transactivator (tTA), derived from the fusion of the tetracycline repressor of *E. coli* to the transcriptional activation of the herpes simplex VP16 protein. In the absence of tetracycline, this fusion protein binds to and activates transcription of plasmids bearing tetracycline-resistance operator elements from the Tn10 operator. *UNIT 20.8* further discusses selection of stable cell lines bearing such plasmids, which provides a mechanism for quick and synchronous expression of target proteins in a population of cultured cells.

Together, these techniques comprise some of the most basic tools available to cell biologists. In each case, they are designed to overcome the challenges posed by the insular nature of cells and to help us to manipulate and understand the machinery of the intact cell.

Mary Dasso

Direct Introduction of Molecules into Cells

Techniques for introducing normally impermeant macromolecules into the living cell—referred to as "cell-loading techniques"—open up many possibilities for the cell biologist. Examples of the investigations that can be carried out using such methods include measuring cytosolic ion concentrations accurately by means of a large, highly membrane-impermeant fluorescent probe; defining the native location of a protein (fluorescently tagged) dynamically in time and space; perturbing the normal functioning of a cellular protein (for example, by introducing antibodies or other specific inhibitors); or altering the genome (by introducing antisense or expression-vector nucleotide sequences). Microinjection is probably the most commonly used technique for introducing fluorescent probes, fluorescently tagged proteins, and antibodies into living cells for short-term studies of cell physiology and protein location and function. It is, however, not the only technique available, nor the easiest or least expensive to implement. Among the alternatives are several closely related techniques that, like microinjection, rely on the cell's ability to reseal a mechanically induced plasma membrane disruption (see McNeil, 2002, for a review) created in order to gain temporary access to cell cytosol. Four such techniques are described here: scrape loading (see Basic Protocol), scratch loading (see Alternate Protocol 1), bead loading (see Alternate Protocol 2), and syringe loading (see Alternate Protocol 3). Although these techniques may not be competitive with microinjection in terms of economy of use of the macromolecule to be loaded, or as efficient at loading very large macromolecules (>100,000 mol. wt.), their implementation does not require the acquisition of a specialized skill or expensive equipment. Additionally, unlike microinjection, they allow one to rapidly load (in a matter of minutes) thousands or even many millions of many types of mammalian cells with normally impermeant molecules, and so to facilitate quantitative analyses of the effect of loading (Doberstein et al., 1993).

STRATEGIC PLANNING

An excellent probe for testing the effectiveness of each of the loading techniques below, and for working out the critical parameter of imposed mechanical load (see Critical Parameters), is fluorescein-labeled dextran (available from Sigma or Molecular Probes). It is inexpensive and can be purchased in a range of molecular weights (from ~3×10^3 to 5×10^6), allowing one to approximately match it in terms of size to the protein or other probe ultimately to be loaded. Fluorescein-labeled dextran is available in a fixable form (conjugated with lysine residues), allowing it to be used initially with fixed rather than living cell specimens.

Because the loading techniques described in this unit, like microinjection, damage cells, it is important to compare control populations of loaded cells, such as those loaded with fluorescein dextran only, with experimental cells loaded with both fluorescein dextran and the molecule of interest, before concluding that an effect of loading is specific to the molecule loaded. Mixing the molecule of interest with fluorescein dextran creates a "loading solution" that will provide this experimental population. It is important also to compare the relevant behavior of the fluorescein dextran–loaded "control" population with that of undisturbed (nonfluorescent) cells, which are always present in the populations generated by these techniques. In this way, those effects on cell behavior that are caused by the loading procedure alone can be detected. In this regard, the authors of this unit have noticed that cells suffering plasma membrane disruptions often contain more intracellular vesicles than undisturbed neighbors (this is probably related to the membrane-membrane fusion process that mediates resealing; Terasaki et al., 1997), but, in the cell types that have been studied in the authors' laboratory, there has been no evidence

Contributed by Paul L. McNeil

Current Protocols in Cell Biology (2003) 20.1.1-20.1.7

that apoptosis is induced among loaded cells. Indeed, one can observe fibroblasts that have been scratch-loaded with fluorescein dextran locomoting into adjacent denuded zones of the coverslip within 60 min post-wounding, and then undergoing cell division there 12 to 24 hr later (Swanson and McNeil, 1987). Cytosolic levels of Ca^{2+} are rapidly (within seconds to minutes) restored to normal levels after wounding (by microinjection or other, more radical means of making cell-surface disruptions)—another indication of the remarkable, and biologically essential, capacity of cells to rapidly reseal and hence survive plasma membrane disruptions (McNeil, 2002).

One or more of these techniques should be applicable to any type of mammalian cell, and also to other eukaryotic cells that lack an external cell wall. For cells that must be loaded or are most conveniently loaded as a suspension, syringe loading is the technique of choice. For adherent cells, any of the additional techniques described below could be used.

A final question is what quantity of one's, often precious molecule is needed? The answer to this question will depend, of course, on the experimenter's goal. If it is simply to produce a fluorescent signal readily visualized or measured microscopically or via a flow cytofluorometer, then initial tests with a fluorescent dextran of appropriate size and concentration (e.g., the size and concentration of the molecule of interest) should provide a useful preliminary answer. The authors find that 0.5 to 1.0 mg/ml solutions of fluorescein dextran (mol. wt., 10 to 70 kDa) result in a readily assayed or measured fluorescent signal from cells loaded by any of these techniques. Sample volume is another important issue. Some of these techniques require that only a very small volume of loading solution be employed. For example, using syringe loading, an ultramicropipettor (1- to 10-µl range), and ultramicro pipet tips, no more than 1 µl is needed. In general, the minimum usable volume is that which prevents the cells from being damaged by drying during the ~1 to 2 min required to execute the crucial plasma membrane–disrupting step of each of these techniques.

BASIC PROTOCOL

SCRAPE LOADING

Transient, survivable plasma membrane disruptions are produced in the presence of the molecule to be loaded by tearing the cells off of their culture substratum (McNeil et al., 1984).

Materials

Adherent cells of interest, growing in tissue culture (also see *UNIT 1.1*)
Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*) or equivalent physiological saline containing 1 to 1.5 mM $CaCl_2$ at physiological temperature (37°C for mammalian cells)
Loading solution: DPBS (with 1 to 1.5 mM calcium) containing molecule to be loaded, at physiological temperature (37°C for mammalian cells)

Rubber policeman
Circular tissue culture dishes

Additional reagents and equipment for cell culture (*UNIT 1.1*)

1. Culture cells on a substratum to which they adhere strongly and which allows an unobstructed approach with a rubber policeman (see step 4).

   Circular-profile tissue-culture-grade dishes are usually a good choice for mammalian cells, as unimpeded access is available after the lid has been removed.

   Basic techniques for culturing mammalian cells are presented in UNIT 1.1.

2. Wash the cells twice with 37°C DPBS (or other physiological saline compatible with the macromolecule to be loaded and with cell viability).

   This and all saline solutions used in subsequent steps should be maintained as closely as possible to 37°C, the optimum resealing temperature.

3. Remove the second DPBS wash. Add the loading solution and swirl to mix it thoroughly with any plain saline that might still be present on top of the cell layer.

   The minimal volume required is that which will prevent cell-drying damage during the minute or two of the scraping procedure.

4. Scrape the cells off of their substratum using a rubber policeman. Leave the cells 5 to 10 min in the loading solution before proceeding with the next step. Check for completeness of cell removal by examining with a phase-contrast microscope.

5. Mix the now suspended and loaded cell population with a 10-fold or larger volume of plain DPBS, and wash the cells several times by centrifuging for 10 to 20 min at 5000 × *g*, at a temperature appropriate for the cell type, removing the supernatant, resuspending the cells in ≥10 volumes of DPBS, and repeating the centrifugation.

6. Replate the cells and return them to normal culturing conditions if the goal is to study adherent, loaded cells, e.g., for microscopic analysis, or use them immediately after washing if the goal is to study suspended, loaded cells, e.g., for flow cytofluorometric analysis.

SCRATCH LOADING

ALTERNATE PROTOCOL 1

Partial, rather than total, cell removal (as in Basic Protocol), as well as severing of cell processes, is used to create plasma disruptions (Swanson and McNeil, 1987).

Additional Materials *(also see Basic Protocol)*

30-G syringe needle or similar sharp implement (e.g., Fisher)
Glass coverslips

1. Culture cells on any substratum to which they adhere strongly and which allows approach with a syringe needle tip (see step 4).

   A glass coverslip is a good choice, especially if the goal is to observe loaded cells under the microscope. Basic techniques for culturing mammalian cells are presented in UNIT 1.1.

2. Wash the cells twice with DPBS (or other physiological saline compatible with the macromolecule to be loaded and with cell viablility).

   If using a coverslip, the washings are easily accomplished by grasping the coverslip with forceps and then transferring it from one beaker containing saline wash to another.

   This and all saline solutions used in subsequent steps should be maintained as closely as possible to 37°C, the optimum resealing temperature.

3. Cover the monolayer with loading solution and mix well to ensure that the loading solution combines thoroughly with any plain saline still present on top of the layer.

   If a coverslip is being used, pipetting the loading solution onto and off of the cells several timers will accomplish this.

4. Scratch the monolayer surface one or more times with a 30-G syringe needle.

   This will denude small (two- to four-cell-wide) strips of the monolayer. Loaded cells will be present along these denuded zones but not elsewhere in the culture. Increasing the number of scratches will increase the proportion of loaded cells.

5. Wash the monolayer three to four times thoroughly with plain DPBS or other appropriate physiological saline.

6. Examine or experiment with the cells.

   The cells are ready for immediate microscopic analysis. Many of the successfully loaded cells along the denudation sites will have morphologies quite normal in appearance; others will be slightly rounded. Within ~30 min, loaded cells will display obvious signs of viability—locomotion into the denuded zone and division there ~12 to 24 hr later.

ALTERNATE PROTOCOL 2

BEAD LOADING

Survivable plasma membrane disruptions are produced in the presence of the molecule to be loaded by the impact of glass beads falling onto and rolling across cells, which remain adherent to the culturing substratum (McNeil and Warder, 1987).

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Glass beads, 50- to 500-μM diameter (Sigma)
Glass coverslips

1. Culture cells on any substratum to which they adhere strongly and which will allow glass beads to be added and removed (see steps 3 and 4).

   A glass coverslip is convenient for this purpose. Basic techniques for culturing mammalian cells are presented in UNIT 1.1.

2. Wash cells twice with DPBS and immerse them in loading solution (see Alternate Protocol 1, steps 2 and 3). Mix the loading solution well with any residual DPBS.

3. Sprinkle glass beads (50- to 500-μm diameter) onto the cell monolayer evenly, until ~75% of the monolayer surface appears, by eye, to be covered by them.

   To increase the frequency of loading, cause the beads to roll around on the monolayer by rocking it to and fro several times. The glass beads can be used "off the shelf," or sterilized by autoclaving if desired in experiments where sterility must be maintained.

4. Wash monolayer thoroughly with plain DPBS to remove beads and loading solution (see Alternate Protocol 1 for technique).

5. Examine or experiment with cells.

   As with Alternate Protocol 1, many cells will have apparently normal morphologies immediately after this loading procedure.

ALTERNATE PROTOCOL 3

SYRINGE LOADING

Survivable plasma membrane disruptions are created in the presence of the molecule to be loaded by shear forces generated by aspirating and expelling cells repeatedly through a narrow orifice, such as that of a syringe needle or micropipettor tip (Clarke and McNeil, 1992).

Additional Materials *(also see Basic Protocol)*

Adherent *or* suspension cells of interest, growing in tissue culture (*UNIT 1.1*)
30-G syringe needle and 1-ml syringe *or* micro- or ultramicropipettor (1- to 10-μl range) and appropriate pipet tips (e.g., Fisher)

1. Trypsinize or otherwise harvest cells (*UNIT 1.1*), and wash twice, each time by centrifuging for 10 to 20 min at 5000 × *g*, at a temperature appropriate for the cells, removing the supernatant, resuspending the cells in ≥10 volumes of PBS, and repeating the centrifugation.

   Basic techniques for mammalian cell culture, including trypsinization, are presented in UNIT 1.1.

2. Resuspend the pellet from the final wash in the loading solution.

3. Draw the cell suspension up into a 1-ml syringe fitted with a 30-G needle, or into a micro- or ultramicropipet tip and then expel it (five to ten times is a good starting point). Repeat this maneuver as many times as necessary in order to achieve the desired compromise of loading level in comparison to cell viability (see Critical Parameters).

4. Wash the cells (see Basic Protocol, step 5).
5. Replate the cells and return them to normal culturing conditions if the goal is to study adherent, loaded cells, e.g., for microscopic analysis, or use them immediately after washing if the goal is to study suspended, loaded cells, e.g., for flow cytofluorometric analysis.

COMMENTARY

Background Information

The capacity to seal a plasma membrane disruption is critical to the survival of many cells (McNeil, 2002). This is because many normal, mechanically active cell environments, including many tissues of the body, promote membrane disruption injury. Resealing, in other words, is not merely Nature's gift to the experimental biologist. Knowledge of this capacity did, however, lead to the development of each of the techniques described in this unit. Conditions that promote resealingphysiological temperature and calcium levelsmust be maintained during each procedure.

How does one choose among the various methods presented—scrape loading, scratch loading, bead loading, and syringe loading? If analysis post-loading is best done on a suspension of cells, e.g., flow cytofluometry, then scrape or syringe loading should be used; if it is best done on adherent cells, e.g., microscopy, then scratch or bead loading should be used. However, scrape, scratch, and bead loading require adherent cells as starting material.

Microinjection is the most obvious alternative method. In cases where there is a minimal, limited amount of reagent for loading, microinjection is unrivaled in its economy of reagent: one needs only enough of the reagent to load a microneedle, e.g., less than a microliter. However, mictoinjection requires special equipment and is labor intensive. The loading techniques described in this unit are by comparison, far less costly to implement and easier to learn. Moreover, they can produce virtually unlimited numbers of loaded cells in minutes.

Critical Parameters

The common feature of all of these techniques is that they bring mechanical force to bear on cells in order to create transient plasma membrane disruptions. Normally impermeant molecules can then enter into the cytosol, until resealing prevents further access. Therefore, to increase loading efficiency by these mechanically based techniques, one simply increases the force applied and hence the number and size of the disruptions. On the other hand, as the extent of plasma membrane disruption increases, viability decreases. Hence, when first attempting to load a particular cell by these techniques, it will usually be advisable to vary the mechanical force imposed over a wide range, and then to select the loading conditions that provide an acceptable level of both loading and viability.

The following provides some guidelines for manipulating the level of mechanical force imposed by each technique.

In scrape loading (see Basic Protocol), the extent of plasma membrane disruption is mainly a function of the strength of cell-substratum attachment. Agents such as poly-L-lysine can, for many cells, be used to increase adhesion and therefore loading efficiency. Conversely, a wash or two with Ca^{2+}-free saline decreases adhesion for many cultured cells, and therefore decreases the level of loading. These same considerations apply also to the closely related technique of scratch loading (Alternative Protocol 1).

In bead loading (see Alternate Protocol 2), the extent of plasma membrane disruption depends on the size of the beads employed (the authors have used beads ranging from 50 to 500 μm in diameter), the number of beads sprinkled onto the cells, and the degree to which the beads are caused to roll around on the monolayer.

In syringe loading (see Alternate Protocol 3), the size of the orifice and probably other hydrodynamic factors related to barrel length and shape are important. Certainly, smaller orifices, which produce greater shear forces, result in increased loading. Additionally, higher ejection pressures increase loading efficiency. The authors of this unit have described an automated device that allows one to precisely control pressure, but this is not necessary unless one desires a high level of reproducibility between one loading procedure and the next (Clarke and McNeil, 1994). Any narrow-bore orifice can be used, and the authors often find it convenient in minimizing loading solution volume to employ a micropipettor and pipet tips instead of a syringe and needle.

Resealing does not occur in the cold or in the absence of Ca^{2+}, so these conditions must be avoided during the step when plasma mem-

brane disruptions are being created, and for ~1 min thereafter (McNeil and Steinhardt, 1997).

Troubleshooting

Successful loading by the techniques described in this unit requires: (1) that sufficient mechanical force be brought to bear on the cell plasma membrane for tearing or disrupting it; and (2) that the cell then be able to reseal the disruption thus created. Poor efficiency in loading can be explained by a problem in either or both of these two areas.

One or more, though not necessarily all, of the techniques described above will result in the imposition of sufficient force for the loading of most mammalian cells. For example, cells smaller than a typical cultured mammalian cell kept in suspension (~10 μm diameter or greater) may not be amenable to syringe loading with a 30-G needle; a needle of this bore may not impose sufficient shear stress, and another technique must be utilized. As a case in point, the authors have found that mammalian red blood cells (~5 μm diameter) are not susceptible to wounding by syringe loading (P. McNeil, unpub. observ.). Red blood cells can, however, be wounded by scraping after they have been stuck to a plastic substratum coated with poly-L-lysine.

On the other hand, too much force can create disruptions too large or too numerous to be resealed. Low (<50%) recovery of viable cells is a key indicator of this problem, which can be solved by reducing the amount of mechanical force applied. For example, this can be accomplished by using smaller beads in the bead-loading technique, by using a larger-gauge needle or fewer intake and expulsion strokes in the syringe-loading technique, or by treatments that loosen cell substratum adherence (such as prescraping rinses with low-calcium medium for mammalian cells) in the scrape-loading technique. Moreover, it is essential for cell viability when applying these membrane-disrupting techniques that resealing occur. For this to happen, cells need physiological levels of extracellular Ca^{2+} and a near-physiological temperature. Thus, if loading fails, with a heavy loss of cell viability, one should check to ensure that these two requirements have been met—both during the loading procedure, when disruptions are being created, and for the 1- to 5-min period after membrane disruption is initiated, when resealing is taking place.

A few cell types, notably echinoderm eggs, reseal extremely rapidly (even with disruptions >1000 μm^2 in extent), and so provide the experimenter much less temporal access to cytosol. Indeed, it is the authors' experience that these cells are difficult to load by the techniques described, but easy to microinject since they are rarely killed by this membrane-disrupting technique. In theory, this rapid resealing capacity could be countered by reducing extracellular Ca^{2+} below physiological levels or by chilling the cells that rapidly reseal.

Anticipated Results

The authors and others have successfully loaded fibroblasts, endothelial cells, smooth muscle cells, epithelial cell lines, neurons, and free-living amebas with these techniques. It is expected that they will work on almost any cell type lacking a cell wall.

The extent of loading is a direct function of the concentration of the macromolecule and an inverse exponential function of its molecular weight. Both of these observations are similar to what would be predicted for a process dependent on diffusion down a concentration gradient through a hole in an otherwise impermeable barrier. Therefore smaller molecules are more effectively loaded than larger ones, and the highest possible concentration of the molecule to be loaded should be employed in the loading solution. One can expect from all of these techniques that the extent of loading will vary over a large range (three-log scale as assessed by flow cytofluorometry). This can be of advantage if one wishes to conduct, for example, a dose-response type of experiment. If, on the other hand, a homogeneous population of, say, heavily loaded cells is desired, then some selection process must be employed, such as flow sorting or microscopic discrimination, based on whole-cell fluorescence derived from the macromolecule loaded.

Time Considerations

These techniques are very rapid, generally taking <30 min.

Literature Cited

Clarke, M.S.F. and McNeil, P.L. 1992. Syringe loading introduces macromolecules into living mammalian cell cytosol. *J. Cell Sci.* 102:535-541.

Clarke, M.S.F. and McNeil, P.L. 1994. Syringe loading: A method for inserting macromolecules into cells in suspension. *In* Cell Biology: A Laboratory Handbook, vol. 3. (J.E. Celis, ed.) pp. 30-36. Academic Press, San Diego, Calif.

Doberstein, S.K., Baines, I.C., Wiegand, G., Korn, E.D., and Pollard, T.D. 1993. Inhibition of contractile vacuole function in vivo by antibodies

against myosin-I [see comments]. *Nature*. 365:841-843.

McNeil, P.L. 2002. Repairing a torn cell surface: Make way, lysosomes to the rescue. *J. Cell Sci.* 115:873-879.

McNeil, P.L. and Steinhardt, R.A. 1997. Loss, restoration and maintenance of plasma membrane integrity. *J. Cell Biol.* 137:1-4.

McNeil, P.L. and Warder, E. 1987. Glass beads load macromolecules into living cells. *J. Cell Sci.* 88:669-678.

McNeil, P.L., Murphy, R.F., Lanni, F., and Taylor, D.L. 1984. A method for incorporating macromolecules into adherent cells. *J. Cell Biol*. 98:1556-1564.

Swanson, J.A. and McNeil, P.L. 1987. Nuclear reassembly excludes large macromolecules. *Science* 238:548-550.

Terasaki, M., Miyake, K., and McNeil, P.L. 1997. Large plasma membrane disruptions are rapidly resealed by Ca^{2+}-dependent vesicle-vesicle fusion events. *J. Cell Biol.* 139:63-74.

Contributed by Paul L. McNeil
Medical College of Georgia
Augusta, Georgia

Protein Transduction: Generation of Full-Length Transducible Proteins Using the TAT System

Described here is the technology that allows an investigator to transduce full-length proteins by utilizing a minimal, eleven–amino acid, HIV-TAT transduction domain that can be fused to a protein of choice using the pTAT or pTAT-HA protein expression plasmids. Bacterial expression (see Basic Protocol 1), followed by solubilization of protein aggregates with a denaturing agent, affords high yields of transducible fusion protein. The fusion protein, once added to the culture medium, can cross the cell membrane and then be degraded or refolded by the cellular machinery. Correct targeting and function of the fusion protein can be easily examined by fluorescent microscopy or immunohistochemistry.

This strategy was established and improved to its current state by the purification and transduction of a multitude of fusion proteins. Because the pool of fusion proteins span many different functions including sequestering proteins (i.e., p16, p27, and CDK2DN), proenzymes (caspase-3), viral proteins (HPV E6, E7, and E1A), enzymes (HIV protease, β-galactosidase), GTPases (rac, rho and cdc-42), and transcriptional regulators (E2F-1-5, pRb), the protocols cover a wide variety of commonly used protein isolation and characterization methods. Table 20.2.1 lists a few examples of some of TAT fusions and details the size, optimal isolation method, dose required to yield a phenotypic result, biological result obtained, and time in which the result was observed.

No special equipment is necessary to generate or transduce fusion proteins, although Basic Protocol 2 does recommend the use of fast protein liquid chromatography (FPLC) to reproducibly bind and elute denatured fusion proteins. FPLC, although recommended, is not required. Bulk ion-exchange resins are available and have been successfully used in place of the Mono Q/Mono S resins that Basic Protocol 2 describes. Another frequently used column is the PD-10 column (Amersham Pharmacia Biotech). This is a disposable column, packed with a gel-filtration resin, which is ideal for the removal of small molecules such as salt, urea, or unconjugated fluorescent molecules.

The unit illustrates the steps of the basic procedure with various fusions, to give the investigator a broader base of information upon which to begin specific isolations.

CAUTION: TAT-protein fusions have been shown to cross most lipid bilayers, including all tissues in a mouse. Therefore, when designing and using TAT-fusion proteins, precautions regarding safe handling and disposal are necessary. It is very important to analyze the health effects of each fusion protein individually and to observe appropriate biosafety procedures for disposal and decontamination. The authors suggest using a 0.1% (w/v) trypsin solution to decontaminate any large spills of fusion proteins. NIH Biosafety Level 2/3 containment should be used at all times. Also, refer to Backus et al. (2001), which provides further guidelines for the safe handling of TAT-transducing proteins.

STRATEGIC PLANNING

This unit is broken down into two sections: (1) isolation, optimization, and large-scale production of the fusion protein (see Basic Protocols 1 and 2 and Alternate Protocols 1 to 4) and (2) analysis of the transduction of the fusion proteins into target cells (see Basic Protocol 3 and Alternate Protocol 5). This unit contains a compilation of different

Contributed by Michelle Becker-Hapak and Steven F. Dowdy
Current Protocols in Cell Biology (2003) 20.2.1-20.2.25

Table 20.2.1 Detailed Description of Selected TAT-Fusions

Fusion protein	Apparent size (kDa)	Isolation method	In vitro dose (nM)	Biological effect	Time biological effect was examined	References
TAT-p16 WT	24	Denaturing/ PD-10	200-1000	Inhibitor of CDK4/6, and induces G1 arrest	30 hr after addition, to G1-arrested, synchronized cells	Ezhevsky et al., 1997
TAT-HA-p27 WT	35	Denaturing/ Mono S ion-exchange	100-200	Inhibitor of CDK2/4/6 complexes, and induces cell scattering	30 min	Nagahara et al., 1998
TAT-E1A WT	60	Denaturing/ rapid dialysis	100	Sequesters pRb	15 min	Unpub. observ.
TAT-HA-E7 Wt	20	Denaturing/ PD-10	100	Sequesters pRb	3 hr	Lissy et al., 1998
TAT-HA-CDK2 DN	40	Soluble/ PD-10	200	Inactivates cyclin E:CDK2 complexes, resulting in G1 arrest	30 hr after addition to synchronized cells	Nagahara et al., 1998
TAT-HA-Caspase 3 WT	39	Denaturing/ Mono Q ion-exchange	100	Processed form induces apoptosis	1-6 hr	Vocero-Akbani et al., 1999
TAT-HA-HIV protease	20	Denaturing/ Mono S ion-exchange	100	Cleaves HIV protease Recognition sequence	1-6 hr	Vocero-Akbani et al., 1999
TAT-HA-β-galactosidase	120	Soluble/ PD-10	100	Cleaves ONPG and Xgal	30 min	Schwarze et al., 1999
TAT-HA-cdc42	21	Denaturing/ PD-10	25	Filopodia formation	5 min	Becker-Hapak et al., 2001

techniques that have been used to successfully isolate many different fusion proteins. One rule of thumb that must be remembered is that every TAT fusion protein is unique, and, while the method described in this unit can cover many fusions, every fusion is different. Therefore the following describes a starting point to begin one's isolations. Figure 20.2.1, outlines the overall strategy.

Isolation and Purification of Fusion Protein

In general, first identify bacterial clones expressing the cDNA of interest using the pTAT or pTAT-HA expression vector (Fig. 20.2.2). The plasmid containing the appropriate insert is then transformed into an *E. coli* strain that is specifically designed for the expression of recombinant proteins. Then, clones expressing the desired fusion may need to be boosted with inducer molecules such as IPTG to yield sufficient quantities of fusion protein for the desired study. The pTAT/TAT-HA vector utilizes a 6-His domain for the convenient isolation of recombinant protein by Ni-NTA chromatography resin. The 6-His domain can lead to fusions that are generally insoluble and compartmentalized into inclusion bodies within *E. coli*. Therefore, buffered urea is routinely used as a denaturing agent to obtain large quantities of unfolded recombinant protein that can bind to the nickel affinity resin. Once the protein is bound and the resin washed, imidazole is used as a competitor to elute the fusion protein from the nickel resin.

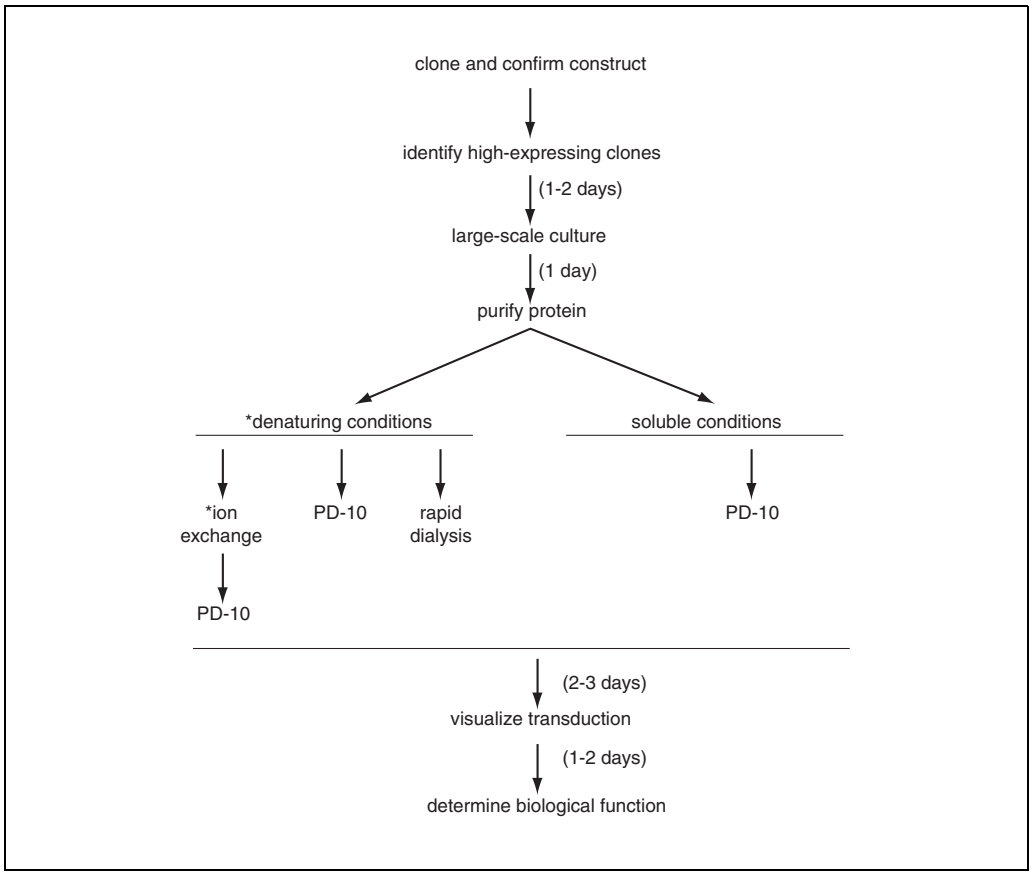


Figure 20.2.1 Flow diagram outlining the overall strategy and time frame required to perform protein transduction using the TAT system. Asterisks (*) denote the preferred methods that consistently lead to fusions that are biologically active.

To remove the urea from the peak nickel affinity protein fraction, one of three different approaches can be used: ion-exchange, gel filtration, or rapid dialysis. The most reliable method for producing transducible recombinant proteins from inclusion bodies is through the use of strong ion-exchange resins to capture the unfolded protein by its ionic charge on an anion (Mono Q) or cation (Mono S) exchange resin using an FPLC platform. Once the unfolded protein is captured, the environment in the column is immediately changed to an aqueous one. The protein is quickly released from the resin using a salt bump, which theoretically leads to a pool of correctly folded and misfolded proteins. The pool of proteins is then desalted and ready for use or storage. The routine method of urea removal from bacterially expressed proteins involves the removal of the denaturant by slow dialysis. While this method works when preparing small quantities of soluble, properly folded proteins, dialysis of high concentrations of the TAT-fusion proteins usually leads to dramatic protein precipitation. Another method of urea removal utilizes a disposable gel-filtration column (PD-10) to exchange the buffer environment around the protein. This procedure has afforded somewhat better success than dialysis, but it is not routinely advisable. Note that the PD-10 column is used in this unit for more than one purpose. While it is not recommended to routinely remove urea from the nickel chromatography purification portion of the procedure, it is ideal for buffer exchange and removal of small molecules such as unconjugated FITC.

In some cases, the 6-His TAT-fusion proteins are maintained in the bacterium in a soluble conformation. In these rarer cases, the fusion protein can be isolated after simply suspending the bacteria in an aqueous buffer, sonicating then clarifying the suspension, and finally performing nickel affinity chromatography.

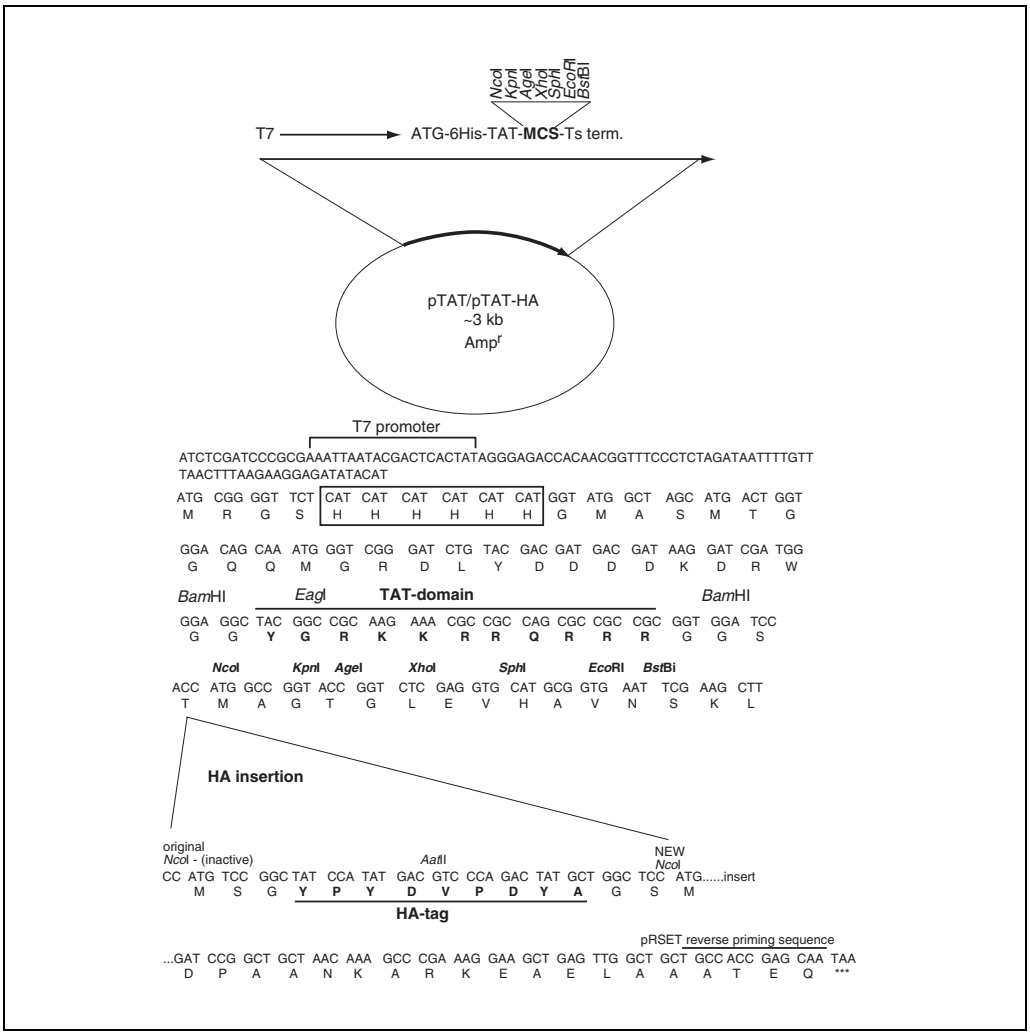


Figure 20.2.2 Vector map of pTAT/pTAT-HA. The functional domains are in boldface. Convenient forward and reverse priming sequences are noted, as well as the peptide sequence of the TAT-HA leader. The pTAT vector does not contain the HA insert. Insertion of desired cDNA using any of the restriction enzymes noted in the multiple cloning site (MCS) will yield an in-frame fusion.

Tranduction and Detection

Two general methods are given for monitoring of full-length TAT-fusion transduction into target cells. The first method detects the intracellular location of the fusion protein, by fluorescently labeling the protein using fluorescein isothiocyanate (FITC), adding the labeled protein to the target cells, washing, fixing, and then observing the resultant transduction by fluorescence microscopy. This standard method for protein labeling covalently attaches the fluorescein molecule to basic residues such as lysine and arginine. Eight out of the eleven amino acids in the TAT-transduction domain are comprised of these basic residues and over-labeling in this functional domain can lead to artificial inhibition of transduction.

The second method given to detect protein transduction is indirect immunofluorescence. This method uses commercially available, fluorescent antibodies to detect transduced protein within cells that have been subjected to the fusion protein over various amounts of time, washed, and then fixed. It is the method of choice when detecting transduction on adherent cells.

Both of these methods focus solely on detection of the fusion protein inside the cell and provide no evidence of fusion protein function. Phenotypic results of fusion-targeted

events are clearly the ultimate detection of protein transduction however, these methods are fusion specific and will not be covered in this unit.

Controls

Depending on the goal of the study, one may choose to use one of two negative controls. The first control to consider is the preparation of a 6-His-fusion of the protein of interest without the transduction domain. This control would be advantageous in cases where proof of the transduction is required. If this is necessary, simply digest the pTAT/pTAT-HA vector containing the cDNA of interest with *Bam*HI, purify, and re-ligate. This will "pop out" the TAT domain of the protein while maintaining the expression of the 6-His-HA tagged protein of interest. Removal of the TAT transducing domain can be verified by digestion of the re-ligated plasmid with the restriction enzyme *Eag*I (Fig. 20.2.2). If the transducing domain has been removed, it will no longer be possible to linearize the plasmid with this restriction enzyme. The purification of this species is generally the same as for the TAT-fused protein.

The second control to consider is the creation of a site-specific mutant within the protein of interest. These fusions are highly recommended when doing in vitro studies in order to prove the specificity of the TAT-fusion protein. Generally, no major deviations from the already optimized protocol for the wild-type fusion will be necessary.

EXPRESSION, VERIFICATION, AND YIELD OPTIMIZATION OF TAT-FUSION PROTEINS

BASIC PROTOCOL 1

This protocol assumes that the investigator has already confirmed the insertion of the cDNA of interest into the TAT expression vector, as well as the identity of the cDNA. It is important that the DNA sequence be confirmed and that the possibility of frame shifts or point mutations be eliminated. The verified plasmid should be stored as a glycerol stock in *E. coli*, DHSα. Storage of the plasmid in bacteria used for protein expression is not generally recommended because of possible plasmid instability. If the investigator needs further background into creating fusion cDNA, see *APPENDIX 3A*, which provides references for procedures that are not described in detail in this unit.

Materials

Pure pTAT/pTAT-HA expression vectors (Nagahara et al., 1998) with and without the gene of interest inserted (available from Dr. S. Dowdy, sdowdy@ucsd.edu)

E. coli strains BL-2 (DE3) pLysS (Novagen) and DH5α (Invitrogen, Life Technologies)

LB medium and plates both containing 50 μg/ml ampicillin (see recipe)

2× SDS-PAGE sample buffer (*APPENDIX 2A*)

Antibody specific for target protein *or* anti-HA mAb (Berkeley Antibody Company) if using pTAT-HA vector

Glycerol, ultrapure, 50% (v/v), sterile filtered

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), Coomassie blue staining (*UNIT 6.6*), and basic molecular biology procedures (including transformation of bacteria and IPTG induction; see *APPENDIX 3A*)

1. Transform the verified, pure plasmid, into competent *E. coli* strain BL-21(DE3)pLysS (*APPENDIX 3A*). Select transformants on LB plates containing 50 μg/ml ampicillin. Also transform the original vector (without the gene of interest) in the same *E. coli* strain for later use as a whole bacterial protein control (empty-vector control).

   This bacteria will not produce any detectable 6-His fusion protein if using the pTAT vector, or will express the hemagglutinin antigen (HA-tag) if using the pTAT-HA vector (6 to 9 kDa).

2. Incubate the agar plate overnight. Pick 6 to 10 colonies from the fusion-positive plate and grow in 3 ml of liquid LB medium containing 50 μg/ml ampicillin, overnight at 37°C with shaking at 220 rpm. Pick one colony from the empty-vector control plate and grow in the same fashion.

3. Vortex the overnight cultures. Remove 50 μl of each culture and place into an individual microcentrifuge tube containing 50 μl of 2× SDS-PAGE sample buffer.

4. Vortex briefly and heat samples 5 min in a boiling water bath.

5. Microcentrifuge the lysate 3 min at maximum speed, room temperature, to bring down any particulates.

6. Load 20 μl of each supernatant from step 5 (including that from the negative control) on two separate SDS-PAGE gels of the appropriate percentage (*UNIT 6.1*).

   One gel will be stained with Coomassie Brilliant Blue (UNIT 6.6) and the other will be transferred to nitrocellulose for immunoblotting (UNIT 6.2).

7. Place one of the two gels in Coomassie Brilliant Blue staining solution for 1 hr with gentle agitation, then remove staining solution and replace with destain solution. Change the destaining solution every 15 min until desired background is achieved.

   The procedures and solutions used here are described in UNIT 6.6.

8. In the lanes containing the clones of interest, determine if there is an overexpressed band at the appropriate size when compared to the lane loaded with lysed bacteria transformed with pTAT or pTAT-HA alone (the empty-vector control). If no clear differences are evident, rely on the immunoblot for confirmation of the expression of the fusion protein.

9. Perform immunoblotting and detection procedures (*UNIT 6.2*). Use the anti-HA mAb at a 1:5000 dilution for the primary antibody if using the HA tag as a marker to follow fusion protein expression. For an antibody against a specific protein, use the dilution recommended by the manufacturer. Use the secondary antibody (*UNIT 6.2*) at a concentration of 1:1000.

   The authors typically use extended-life chemiluminescent development reagents such as SuperSignal (Pierce).

10. Using the results from the immunoblot, determine the size of the fusion protein.

    The TAT/TAT-HA fusion proteins run 6 to 9 kDa larger than the untagged gene of interest.

11. *Optional:* If a sufficient level of overexpression of the desired fusion is not observed by immunoblotting, induce the system using IPTG (*APPENDIX 3A*).

    See Troubleshooting for other possible solutions to poor fusion expression.

12. Determine the clone or clones that express the protein of interest at the highest levels. Prepare glycerol stocks by adding 700 μl of overnight culture into 300 μl of sterile 50% glycerol, and freeze at −80°C.

    As mentioned earlier, not all fusions are stable in BL-21(DE3)pLysS bacteria. If this is discovered, transform into the expression bacteria, let the cells recover for 1 hr without antibiotic selection, and then prepare a 100-ml overnight inoculum in LB medium containing 5 μg/ml ampicillin. Use this culture to inoculate a large-scale culture as described in the next section.

    Also, for permanent storage of the unstable plasmid, transform the plasmid into DH5α and store as glycerol stock at −80°C as mentioned earlier.

LARGE-SCALE ISOLATION OF THE TAT-FUSION PROTEIN

This protocol describes the large-scale isolation of the TAT-fusion protein from the high-expressing clones identified in the previous steps. See Key References for more information on these approaches. To perform ion exchange, it is necessary first to determine the isoelectric point (pI) of the purified protein. This can be done easily on various molecular biology Web sites (see Internet Resources for one of these). It is then necessary to determine which ion-exchange resin will best suit the protein. The pI of the fusion protein will, in large measure, determine whether to use a Mono Q column (for basic proteins) or a Mono S column (for acidic proteins). Although the TAT leader is a basic entity (8 of 11 residues are basic), it has been the experience of the authors that ~50% of all TAT fusion proteins will bind to the Mono S resin regardless of pI predictions. Following the successful elution of the protein, pool the appropriate fractions and exchange the buffer using a PD-10 column. The columns are provided as prepacked disposable columns of 30 per box and should be stored at 4°C.

In general, when isolating proteins from crude extracts, the preparation should be kept in the cold or on ice. However, in this procedure, it is not necessary to keep the preparation on ice until the urea has been removed from the sample, except when sonicating the bacteria. In fact, if the Ni-NTA purified fractions are kept on ice after elution from the column, crystallization of the eluate will be observed. Therefore, during this phase, avoid cold conditions unless it is necessary to freeze the partially purified protein for storage and later purification.

Materials

LB medium containing 50 µg/ml ampicillin (see recipe)
Glycerol stock of clone with high expression of TAT fusion protein (see Basic Protocol 1)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Buffer Z (see recipe) containing 1× protease inhibitors (see recipe)
5 M (340 g/liter) imidazole (store in foil-wrapped bottle at 4°C)
50% (w/v) stock suspension of Ni-NTA agarose (Qiagen)
100 mM, 250 mM, 500 mM, and 1 M imidazole in buffer Z (see recipe for buffer Z), prepared fresh daily
PBS (*APPENDIX 2A*) containing 0.1% (w/v) sodium azide
20 mM HEPES, pH 8 (for Mono Q resin) or pH 6.5 (for Mono S resin)
Buffer A (binding): 20 mM HEPES, pH 8.0, for Mono Q; pH 6.5 for Mono S
Buffer B (elution): 20 mM HEPES/1 M NaCl, pH 8.0, for Mono Q; pH 6.5 for Mono S
PBS (*APPENDIX 2A*) containing 1× protease inhibitors (see recipe)
Bovine serum albumin (BSA)
Glycerol (ultrapure), 50% (v/v) sterile filtered

Sorvall refrigerated centrifuge with GSA rotor, or equivalent
Sonicator with microprobe (Branson)
Disposable 50-ml Econo columns (Bio-Rad)
Mono Q or Mono S 5/5 or 10/10 ion-exchange FPLC columns *or* bulk resin (Resource Q or S), all products of Amersham Pharmacia Biotech
FPLC apparatus
PD-10 gel filtration columns (Amersham Pharmacia Biotech)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and Coomassie blue staining (*UNIT 6.6*)

Prepare fusion-protein-containing lysate

1. Inoculate 200 ml of LB medium containing 50 µg/ml ampicillin with a sterile loop or scraping of the high-expressing clone glycerol stock (see Basic Protocol 1). Incubate overnight at 37°C with shaking at 220 rpm.

2. Pour the entire contents of the overnight culture into 1 liter of LB medium containing 50 µg/ml ampicillin. Incubate 5 to 6 hr at 37°C with shaking at 220 rpm.

   Protein production usually decreases after stationary phase has begun in E. coli.

   If it has been determined that IPTG is required to obtain large quantities of the protein, be sure to add it at this step.

3. Centrifuge the cell suspension 5 min at 5000 × *g*, 4°C, in a Sorvall GSA or equivalent rotor. Discard supernatant.

4. Resuspend pellet in PBS. Centrifuge the suspension again as in step 3.

   The washed pellet can be stored at −20°C for one month if necessary.

5. Decant supernatant and add 10 ml buffer Z with protease inhibitors to the pellet. Make a homogenous suspension by pipetting up and down using a wide-bore pipet, or by vortexing.

   A homogenous suspension is critical for efficient lysis of the bacteria by sonication in the next step.

6. Sonicate the suspension using four 15-sec on/off cycles at 60% (microtip limit), at 4°C or on ice.

   The cold temperatures are required to keep the proteins from being irreversibly denatured by the heat generated during the sonication process.

7. Centrifuge the suspension 10 min at 12,000 × *g*, 4°C. Carefully decant the supernatant into a clean tube and measure its volume, then add sufficient 5 M imidazole to a final concentration of 20 mM imidazole.

   The concentration of imidazole to add at this point must be determined experimentally. In most cases the fusion protein binds specifically at 20 mM imidazole; however, some proteins will require lower concentrations (from 5 to 15 mM). At lower imidazole concentrations, the background (non-6-His labeled proteins) can also bind to the nickel resin. Usually, this is not a problem because the desired protein is in vast excess with respect to the contaminating proteins. Additionally, the contaminating bacterial proteins do not contain the transduction domain and therefore will not transduce.

Purify fusion protein on Ni-NTA agarose

8. Prepare a 5-ml bed volume Ni-NTA affinity column by adding 10 ml of the 50% stock suspension of Ni-NTA agarose to a 50-ml Bio-Rad Econo-Column with the Luer lock in place to control the flow of buffers or extract to be added.

9. Wash the resin twice, each time with 10 bed volumes (50 ml) of Milli-Q water, to remove the resin storage buffer.

10. Equilibrate the resin with 10 bed volumes of buffer Z containing 20 mM imidazole (or whatever concentration of imidazole was added as in step 7).

    Remember to save a small portion of each purification step so that it will be possible to follow the protein purification by SDS-PAGE.

11. Apply the clarified sonicated bacterial lysate (from step 7) to the resin.

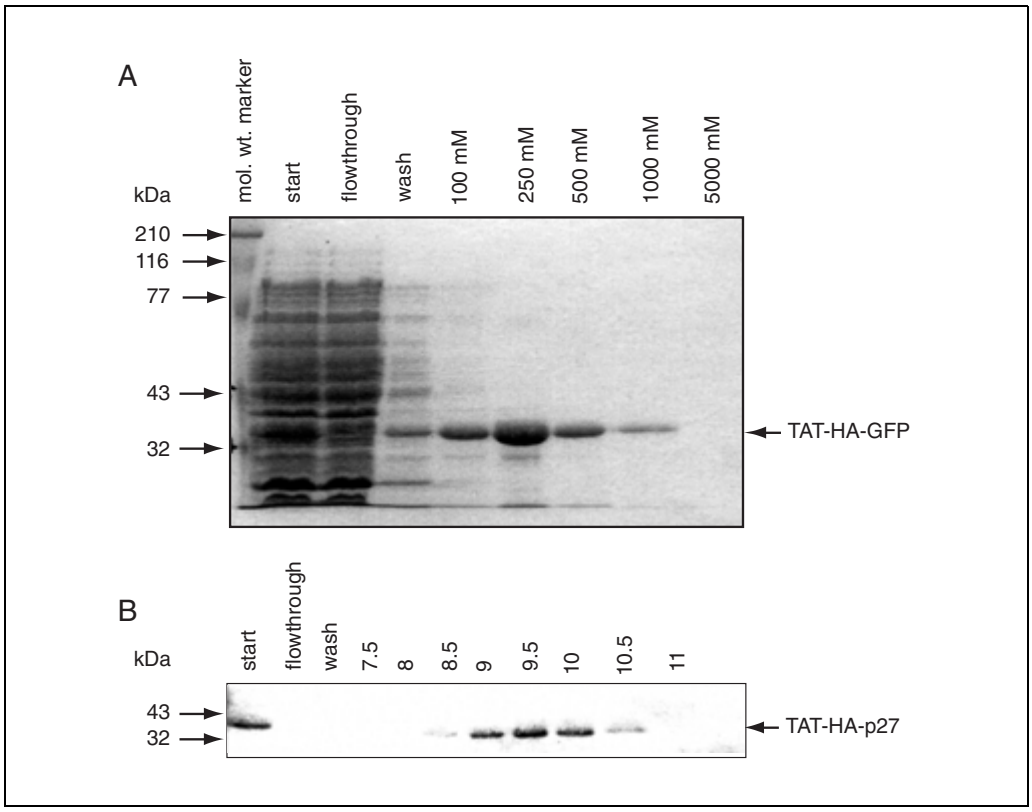


Figure 20.2.3 (**A**) Typical elution profile of TAT-fusion protein (TAT-HA-GFP) from a Ni-NTA agarose resin. BL-21(DE3)pLysS bacteria were transformed with pTAT-HA-GFP plasmid and cultured in 200 ml of LB medium containing 50 μg/ml ampicillin, overnight. This inoculum was then added to 1 liter of LB-ampicillin and cultured for another 5 hr with shaking at 220 rpm, 37°C. The bacteria were lysed in buffer Z with protease inhibitor cocktail (see Reagents and Solutions), clarified, and the imidazole concentration was brought up to 20 mM. The crude lysate was applied to a 5-ml-bed-volume Ni-NTA column and washed with 50 ml of buffer Z containing 20 mM imidazole. The fusion protein was then eluted with 5 ml aliquots of buffer Z containing 100, 250, 500, 1000, and 5000 mM imidazole. The 12.5% SDS-PAGE gel was loaded with 5 μl of each fraction noted. The gel was stained for 60 min with Coomassie Blue and destained as necessary. (**B**) Ion-exchange profile of TAT-p27 WT on a Mono S (5/5) column, equilibrated in buffer A and loaded with a 1:1 dilution of a Ni-NTA elution fraction. The column was washed with 40 ml of buffer A. TAT-HA-p27 WT fusion protein consistently eluted after 8 through 11 ml of buffer B. 10 μl of each elution fraction was separated on a 15% SDS-PAGE gel and stained with Coomassie Blue for 1 hr, then destained as necessary. The numbers above each band denote the volume of elution buffer (buffer B) that had passed through the Mono S (5/5) column.

Some lysates can be very viscous. If this is observed, dilute the lysate with more buffer Z or apply the lysate directly and use slight pressure on the column to gently force the lysate through the resin. If the lysate is not very viscous and clears the resin too quickly, reduce the flow rate and apply the lysate over the resin again.

Remember to maintain the imidazole concentration (20 mM or other concentration added at step 7) throughout the column application and wash steps.

12. Wash the resin with 10 ml of buffer Z containing 20 mM imidazole, then with an additional 40 ml of buffer Z containing 20 mM imidazole (ten bed volumes total).

    The first 10 ml of wash will contain flow through proteins. The subsequent 40 ml is the wash that removes weakly bound proteins.

13. Elute the protein stepwise by sequential addition of 5 ml each of 100 mM, 250 mM, 500 mM, and 1 M imidazole in buffer Z, and collect fractions. Finally, strip the resin with 5 ml of 5 M imidazole.

14. Wash the resin with 20 bed volumes of PBS. Store the column at 4°C in PBS containing 0.1% sodium azide.

15. Load 10 µl of each purification fraction (from step 13) on an appropriate-percentage SDS-PAGE gel (*UNIT 6.1*). Perform electrophoresis, then stain the gel with Coomassie Brilliant Blue for 1 hr and destain appropriately (*UNIT 6.6*).

    Note that purified protein can be readily observed as early as 2 min after placing the gel in the staining solution.

16. Determine the fraction(s) that contain the desired protein.

    See Fig. 20.2.3A for an example of a typical urea elution profile.

    The procedure can be suspended at this step if necessary. The imidazole-eluted fractions may be stored at −20°C until the SDS-PAGE is completed and one is ready to do the urea-removal steps (no more than one month).

Purify by ion-exchange chromatography

17. Pool the Ni-NTA fractions containing the peak protein concentration determined by SDS-PAGE.

18. Dilute the pooled fractions 1:1 with 20 mM HEPES, pH 8.0 (for Mono Q), or pH 6.5 (for Mono S).

    This dilutes the protein pool to a low-enough salt and denaturant concentration to allow binding to the ion-exchange resin.

19. Inject the sample into a 10/10 (preferably) Mono Q or S column attached to an FPLC apparatus and equilibrated in buffer A.

    A gravity column may be used in place of FPLC (see Alternate Protocol 1).

20. Wash with ~50 ml buffer A and elute with 10 ml buffer B, collecting 0.5-ml fractions.

    Switching from the non-urea-containing buffer A to buffer B results in elution via a single 1 M NaCl step.

21. Analyze the fractions by SDS-PAGE (*UNIT 6.1*) and stain with Coomassie Brilliant Blue (*UNIT 6.6*).

    Figure 20.2.3B shows an example of an elution profile from ion-exchange chromatography. The sample is ready at this point for desalting on a PD-10 column equilibrated in PBS, followed by collection and final analysis by SDS-PAGE (steps 22 to 26). The only decision that must be made by the investigator at this point is the equilibration buffer to be used. The authors recommend desalting into PBS and adding sterile glycerol to a minimum concentration of 10% (v/v). For further discussion on the use of this column see Becker-Hapak (2001).

    IMPORTANT NOTE: *If the protein fails to bind to the predicted resin, or weak binding of protein is observed, try the other column type (Mono Q or Mono S) regardless of predicted pI. If strong binding is observed with weak elution, decrease (for Q resin) or increase (for S resin) the pH of buffer A by steps of 0.5 pH units, until a small amount of protein is detected in the flowthrough fraction. Also see Troubleshooting for problems associated with the ion-exchange step.*

Perform buffer exchange using PD-10 column

22. Drain the storage buffer from the PD-10 column and equilibrate the column with 25 ml of PBS with 1× protease inhibitors.

    Culture medium such as RPMI-1640 (e.g., Life Technologies), without serum and antibiotics, but containing 1× protease inhibitors, can be used in place of the PBS.

23. Apply the pooled protein fraction to the column (do not exceed 2.4 ml), and allow the solution to absorb into the resin. Apply 4 ml PBS with 1× protease inhibitors to the column and collect fourteen 0.5-ml fractions.

    The protein will begin eluting in the sixth or seventh fraction. If 2.4 ml of protein was applied to the resin, then the protein will stop eluting in the thirteenth or fourteenth fraction.

    The column can be reused once by applying another 25 ml of buffer with protease inhibitors onto the column; however, it is not wise to reuse the column if working with different fusion proteins, because it is always possible to carry over contaminants from a previous preparation.

24. Analyze the fractions by SDS-PAGE (*UNIT 6.1*) using BSA as a standard. Load 0.1 to 2 µg protein standard per lane and a known volume of the purified fusion protein on the same gel.

25. Stain the gel with Coomassie Blue and destain as desired (*UNIT 6.6*).

26. Pool the appropriate fractions and add glycerol to a final concentration of 10% (v/v). Divide into 0.25-ml aliquots and flash freeze on dry ice. Store fractions at −80°C.

    Fusions have been stored for over 2 years in this manner and still maintained activity.

USE OF ION-EXCHANGE GRAVITY COLUMNS INSTEAD OF FPLC

ALTERNATE PROTOCOL 1

If an FPLC system is not available, bulk ion-exchange resin can be used to pack a gravity-flow column.

Additional Materials *(also see Basic Protocol 2)*

30-µm Resource Q or S ion-exchange resin (Amersham Pharmacia Biotech; see Basic Protocol 2 for choice of resin)
50-ml Econo columns (Bio-Rad)

NOTE: Perform all steps at 4°C and add 1× protease inhibitors (see recipe) to all solutions.

Replace steps 19 to 21 of Basic Protocol 2 with the following:

1. Pack a 10-ml bed-volume ion exchange column using 30-µm Resource Q or S resin.
2. Wash the resin with 20 bed volumes of Milli-Q water to remove the storage buffer and rehydrate the resin.
3. Wash the column with 10 bed volumes of buffer A.
4. Apply the diluted sample (see Basic Protocol 2, step 18) onto the resin and allow the protein to enter the gel bed slowly.
5. When the protein has completely entered the gel, wash with 10 bed volumes of buffer A.
6. Elute with two bed volumes of ml buffer B, collecting 0.5-ml fractions. Analyze the fractions by SDS-PAGE (*UNIT 6.1*) and pool the fractions containing the protein of interest.

    IMPORTANT NOTE: *At this point the fusion protein is very pure. It is absolutely necessary to maintain the protein on ice from this point on. Failure to do so will yield degraded protein.*

7. Perform buffer exchange and analyze the final fractions (see Basic Protocol 2, steps 22 to 26).

ALTERNATE PROTOCOL 2

DIRECT BUFFER EXCHANGE OF UREA-DENATURED PROTEIN

In theory, rapid desalting of a denatured protein from 8 M urea through an aqueous interface of PBS or culture medium without serum forces the protein to rapidly hide its hydrophobic residues and become soluble in an aqueous environment. The PD-10 desalting column has a 1:1.4 dilution factor; therefore denatured proteins are rapidly separated from each other, helping to avoid aggregation of the proteins and subsequent precipitation on the column. In this procedure, 1 to 1.5 ml of the Ni-NTA affinity-purified TAT fusion protein in 8 M urea (i.e., in buffer Z) is applied to the PD-10 column equilibrated in PBS/HEPES buffer or serum-free culture medium. Column fractions of 0.5 ml are isolated and analyzed by SDS-PAGE as in Basic Protocol 2. Reasonable success has been achieved by this rapid and inexpensive procedure, however, use of this method routinely leads to dramatic protein precipitation. While some soluble protein can be obtained using this protocol, more soluble (and therefore, transducible) protein can be obtained by working out the ion-exchange conditions.

Additional Materials *(also see Basic Protocol 2)*

Serum-free culture medium (e.g., RPMI-1640) without antibiotics, containing 1× protease inhibitor cocktail (see recipe) *or* PBS plus 1× protease inhibitor cocktail

Replace steps 17 to 26 of Basic Protocol 2 with the following:

1. Equilibrate PD-10 column with 25 ml of culture medium without serum or antibiotics but with 1× protease inhibitors.

   Alternatively, the fusion can be buffer exchanged into PBS plus 1× protease inhibitor cocktail.

2. Load a maximum of 1.5 ml of the peak protein fraction from the Ni-NTA affinity column (see Basic Protocol 2, steps 16 and 17) on to the gel bed.

3. After the sample has completely entered the gel bed, apply more of the culture medium with protease inhibitors to the column and collect 0.5-ml fractions.

   Protein can precipitate as it is eluted from this column. Microcentrifuging the fractions immediately for 5 min at maximum speed, 4°C, can minimize this precipitation. Transfer the supernatant fractions in separate microcentrifuge tubes.

4. Keeping the fractions on ice at all times, determine which fractions contain the desired protein by SDS-PAGE (*UNIT 6.1*).

5. Pool the fractions containing the protein of interest and and add glycerol to a final concentration of 10% (v/v). Store the protein in 0.2-ml aliquots at −80°C.

   Some proteins may require the additional 0.1% (w/v) BSA to stabilize the pure protein. This must be determined experimentally. In general, storing the protein in the 10% glycerol will be sufficient. Also, keep in mind that if the fusion protein is an enzyme, it may not be active if frozen at any point.

ALTERNATE PROTOCOL 3

DIALYSIS OF THE UREA-DENATURED PROTEIN

One may choose to rapidly dialyze the Ni-NTA affinity-purified protein into the desired buffer, replacing the ion-exchange and gel-filtration steps (Basic Protocol 2, steps 17 to 23). However, 6-His fusion proteins are highly susceptible to precipitation when using this method to remove urea. Also, some proteins are not biologically active after dialysis, whereas ion-exchange chromatography will routinely yield proteins with higher biological specific activity. For example, TAT-p27 WT, if prepared by rapid dialysis, will not

cause G1 arrest, whereas if it is subjected to ion-exchange chromatography, it does (Nagahara et al., 1998). Conversely, other proteins such as TAT-E1A and TAT-E7 are biologically active when desalted by rapid dialysis (Beker-Hapak, unpub. observ.).

Additional Materials *(also see Basic Protocol 2)*

Slide-A-Lyzer dialysis cassettes (Pierce) with membrane of MWCO appropriate for protein of interest

Replace steps 17 to 23 of Basic Protocol 2 with the following:

1. If the protein in the purified fraction is > 5 μg/μl, dilute at least 1:1 in buffer Z.
2. Apply the sample to the dialysis cassette. Be sure to remove all air bubbles.

   In the authors' laboratory, use of the Slide-A-Lyzer cassettes with large surface-to-volume ratio provided by Pierce has proven to yield more soluble protein than regular dialysis tubing.

3. Dialyze the protein at 4°C against 4 liters of pre-chilled buffer appropriate for the downstream application. Change the buffer after 1 hr, then again 2 hr later, and let stir overnight. After the overnight incubation period, remove the protein from the dialysis cassette and remove any solids by centrifuging 10 min at 5000 × *g* , 4°C, prior to use or storage.

   For example, if using the protein for orthophosophate labeling, use 20 mM HEPES/137 mM NaCl, pH 7.2, but if simply adding the protein to tissue culture cells, use 1× PBS at 4°C.

4. Analyze the protein concentration with BSA standards on a SDS-PAGE gel and store the protein in aliquots (see Basic Protocol 2, steps 24 to 26).

ISOLATION OF SOLUBLE TAT-FUSION PROTEINS

ALTERNATE PROTOCOL 4

Some proteins can or must be isolated under soluble (nondenaturing) conditions. For example TAT-β-galactosidase is not active if purified in the presence of any urea. Use of nondenaturing conditions can reduce yield as well as transduction efficiency; therefore, use of this protocol is not generally recommended. However, several reports successfully utilized the isolation of TAT fusions under nondenaturing conditions. According to the authors' experience, the yield can be much lower than proteins prepared under denaturing conditions and the transduction efficiency may be lower. Exact effects must be determined experimentally.

For materials, see Basic Protocol 2.

1. Prepare pellet of cells from 5- to 6-hr culture (see Basic Protocol 2, steps 1 to 4).
2. Resuspend the pellet fraction in 10 ml of PBS containing 1× protease inhibitors. Perform Ni-NTA purification (see Basic Protocol 2, steps 5 to 14), replacing the buffer Z with PBS with protease inhibitors. Identify the fractions containing the protein of interest by SDS-PAGE (see Basic Protocol 2, steps 15 and 16).

   Sonication of cells in PBS is more difficult than when done in buffer Z. One may need to modify the sonication procedure to optimally lyse the bacteria without damaging the fusion protein.

   The ion-exchange steps (Basic Protocol 2, steps 17 to 21) are not performed.

3. After Ni-NTA chromatography, remove the imidazole and exchange the buffer using PD-10 column, and store the protein in 10% (v/v) glycerol at −80°C (see Basic Protocol 2, steps 22 to 26).

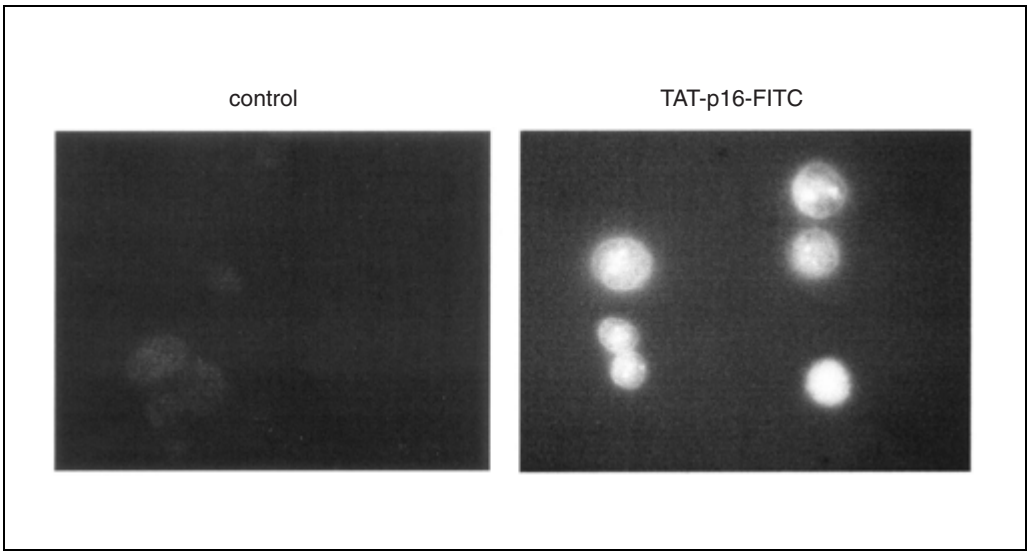


Figure 20.2.4 Confocal microscopy analysis of Jurkat T cells transduced with p16 WT-FITC, control (left panel) or TAT-p16WT-FITC protein (right panel). Jurkat T cells were transduced for 1 hr with the FITC-labeled pure protein. Cells were washed in PBS and fixed with 4% paraformaldehyde, washed again, and then mounted on slides with antifade mounting medium. Note the generalized fluorescence of TAT-p16WT-FITC protein in the cell. Higher-intensity staining can be observed in the nucleoli, typical of nuclear targeted TAT-fusions. The cells treated with non-TAT-fused FITC-labeled p16 show little to no fluorescence.

BASIC PROTOCOL 3

TRANSDUCTION AND DETECTION WITH FLUOROPHORE-LABELED FUSION PROTEIN

TAT-mediated protein transduction occurs without the use of specialty reagents or instrumentation. A TAT-fusion protein can be simply added to cultured cells along with the culture medium. The process is concentration dependent but seemingly temperature independent (see Commentary for a detailed discussion of all of the parameters affecting transduction). This unit will not detail a regimen for transduction because the procedure will be different for every fusion, cell type, and cell culture system. To optimize, the researcher should consider trying several different doses of the transducing protein (10 to 200 nM) in culture medium, varying incubation times with the target cell population to achieve the lowest concentration of protein in shortest time frame required to achieve the phenotypic effect. Suspension (e.g., Jurkat T-cell) and adherent (e.g., NIH 3T3) cell lines, are used in this section to illustrate two different and routinely used methods for visualizing transduced proteins in tissue culture cells. This protocol describes transduction of a fusion protein labeled with fluorescein. Alternatively, other fluorescent molecules such as Alexa (Molecular Probes) can be used to label the fusion. These molecules are reported to have a higher half-life when compared to FITC. The authors recommend using the manufacturer's labeling protocol whenever using an alternative fluorophore.

Materials

Fluorescein isothiocyanate (FITC; Molecular Probes)
DMSO
Purified fusion protein (see Basic Protocol 2 and Alternate Protocols 1 to 4)
10× FITC conjugation buffer (see recipe)
PBS (*APPENDIX 2A*) containing 1× protease inhibitors (see recipe)
Glycerol, ultrapure
Cell line of interest for transduction or Jurkat T cell culture
Paraformaldehyde fix solution (see recipe)
Antifade mounting medium (Molecular Probes)

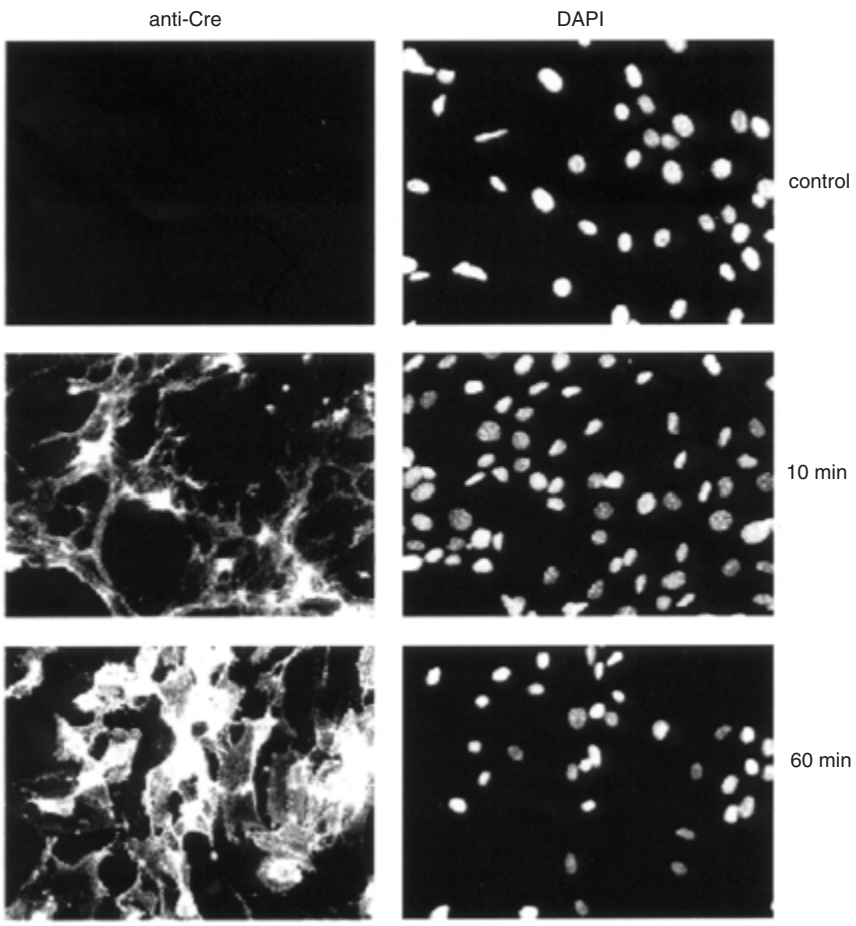


Figure 20.2.5 Detection of TAT-HA-Cre transduced into NIH-3T3 cells containing a phloxed β-galactosidase gene. Cells were transduced for 0 (no TAT-HA-Cre), 10, and 60 min in 8-chamber Lab-Tek slides. The cells were washed and fixed as described in Alternate Protocol 5. Rabbit anti-Cre polyclonal antibody was used at a 1:3000 dilution and a TRITC-labeled goat anti-rabbit secondary antibody was used at a 1:1000 dilution. TAT-HA-Cre is detected as early as 10 min after introduction of the protein into the cells. The panel showing the cells treated for 10 min clearly shows that many of the cells have detectable protein in the cytoplasm. Accumulation of the fusion protein in most cell nuclei is evident at 60 min.

Clear nail polish

PD-10 gel-filtration columns (Amersham Pharmacia Biotech)
Microscope slides and coverslips

Label TAT fusion protein

1. Prepare a FITC stock solution by dissolving 1 mg FITC per 0.5 ml DMSO. Keep in the dark.

   The FITC stock solution should be prepared fresh daily.

2. Prepare the labeling reaction by combining:

   540 µl (0.1 to 0.5 µg) purified fusion protein
   60 µl 10× conjugation buffer
   1 µl FITC stock solution (see step 1).

It is wise to set up at least three separate labeling reactions of high, medium, and low protein concentration to be conjugated to FITC. Overlabeling can cause inhibition of the transduction, presumably due to blockage of the basic groups in the transduction domain. Therefore, it is a good idea to label and purify the reactions prepared at all three protein concentrations; one of them will provide the best visualization of the transduction.

3. Incubate at room temperature in the dark for 2 hr.

Purify the fluorophore-labeled fusion protein

4. Equilibrate a PD-10 column with 25 ml of PBS with 1× protease inhibitors.
5. Apply the entire labeling reaction from step 3 to the column.
6. After the volume enters the gel bed, apply more PBS with protease inhibitors and collect twelve 0.5-ml fractions.

   The unconjugated FITC will remain in the gel bead because of its small size. Do not reuse the column.

7. Pool fractions 6 to 8 containing the labeled protein (which will be slightly yellow), add glycerol to 10% (v/v), and store at −80°C.

Transduce labeled fusion protein

8. Incubate various volumes of FITC-labeled TAT fusion protein with 5×10^5 suspension cells (e.g., Jurkat T cells) in 200 µl culture medium (i.e., RPMI/10% FBS) for 30 min at 37°C.

   Equilibration is reached in as little as 5 to 15 min.

9. Microcentrifuge 5 min at 5000 rpm, 4°C. Remove the supernatant, add 0.5 ml of ice-cold PBS and immediately microcentrifuge again. Remove the supernatant to complete the wash.

Fix and visualize cells

10. Resuspend the pellet in 500 µl paraformaldehyde fix solution and incubate cells for 15 min at room temperature on an end-over-end rotator.
11. Gently pellet the cells by microcentrifuging 5 min at 5000 × *g*. Resuspend the cell pellet in 200 µl of PBS and mount onto slides (using antifade mounting medium if slides are to be stored overnight prior to examination). Seal the coverslips with clear nail polish.
12. Examine slides by fluorescence microscopy using excitation and emission wavelengths appropriate to the fluorophore (*APPENDIX 1E*).

    See Figure 20.2.4 for an example of FITC-labeled TAT-p16 WT transduced into Jurkat T cells.

ALTERNATE PROTOCOL 5

TRANSDUCTION AND DETECTION BY INDIRECT IMMUNOFLUORESCENCE

When the necessity of colocalization of transduced protein to cellular organelles or substructure is required, use of indirect immunofluorescence is highly desirable. The method described below will allow the researcher to make direct observations of where the TAT fusion protein is located within an individual cell. Use of the Lab-Tek 8-chamber slides makes it easy to examine and manipulate many conditions (i.e., antibody concentration) at the same time. Be sure to include a secondary antibody control to ensure low nonspecific binding of the fluorescent secondary antibody. An example of immunofluorescence completed on NIH 3T3 cells transduced with TAT-Cre is shown in Figure 20.2.5.

Materials

Adherent cells of interest for transduction, e.g. NIH 3T3 cells
Culture medium for NIH 3T3 cells (i.e., DMEM/10% FBS)
Purified fusion protein (see Basic Protocol 2 and Alternate Protocols 1 to 4)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold and room temperature
Paraformaldehyde fix solution (see recipe)
100% ethanol, ice-cold
1% and 0.1% (w/v) bovine serum albumin (BSA) in PBS (prepare from 10% w/v BSA stock)
Primary antibody: antibody of choice to fusion protein or mAb to the HA epitope (Berkeley Antibody Company)
TRITC- or PE-labeled secondary antibody
0.2 µg/ml DAPI (prepare fresh from 1 mg/ml DAPI stock; store stock in dark at 4°C)
Slowfade mounting medium (Molecular Probes)
Clear nail polish

Lab-Tek 8-chamber glass slides with lids (Nalge Nunc International)
40°C heat block
50 × 24–mm coverslips (Fisher)

Prepare cells in chamber slides

1. Culture 1000 NIH 3T3 cells/chamber on a Lab-Tek 8-chamber slide overnight in a minimum volume of 0.5 ml of culture medium.
2. Wash the cells by flooding the chambers with fresh medium followed by removal of the medium by gentle aspiration.

   It is a good idea to aspirate from the same position in the well for each wash to minimize loss of cells during the wash steps.

Transduce fusion protein

3. Transduce fusion protein into cells as desired for specific time points.

   The useful concentration of the protein will vary from 10 to 200 nM and the times for transduction will also vary depending on the investigator. The minimum volume to cover the cells is 200 µl per chamber. Transduction can be detected in as early as 5 min and usually maximizes between 2 and 6 hr after protein addition (this is fusion dependent).

4. Remove medium and wash once with ice-cold PBS using the technique described in step 2.
5. Fix cells by adding 400 µl of ice-cold paraformaldehyde fix solution per chamber and incubating on ice for 15 min.

   It is critical that the fixation buffer be prepared fresh daily; its pH must be verified prior to use.

Expose transduced cells to antibody reagents

6. Wash three times with ice-cold PBS using the technique described in step 2.
7. Permeabilize the cells by adding 400 µl of ice-cold 100% ethanol and incubating on ice for 10 min.
8. Wash three times with ice-cold with PBS using the technique described in step 2.
9. Block with 1% BSA and incubate 10 min on top of a 40°C heat block.
10. Wash five times with PBS at room temperature using the technique described in step 2.

11. Dilute the primary antibody in PBS containing 0.1% BSA according to the manufacturer's recommendation. Add 400 µl of the diluted antibody to each chamber and incubate 15 min at 40°C.

    For anti-HA, a 1000- to 3000-fold dilution is optimal.

12. Wash five times with with PBS at room temperature.

13. Dilute TRITC- or PE-labeled secondary antibody 1000- to 5000-fold in PBS containing 0.1% BSA. Add 400 µl of the diluted antibody to each chamber and incubate 15 min at 40°C.

    If the background (i.e., cells stained with secondary antibody alone) is high, increase the dilution of this antibody.

14. Wash five times with PBS at room temperature.

Counterstain cells with DAPI

15. Counterstain with DAPI by adding 200 µl of 0.2 µg/ml DAPI to each chamber and incubating 15 min at room temperature.

16. Rinse chambers with water, remove the attached chambers and seal, then dry in the dark.

Visualize the results

17. After drying is complete, add 5 drops of Slowfade to mount, add 50 × 24–mm coverslip then seal coverslip with nail polish. Keep in dark until ready to view.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer Z

480.0 g urea
8.0 g NaCl
4.8 g HEPES
Adjust the pH to 8.0 with NaOH
Add H_2O to 1 liter
Store up to 1 month at room temperature

FITC conjugation buffer, 10×

Dissolve 0.84 g $NaHCO_3$ in 9 ml of H_2O. Adjust to pH 9.0 with NaOH, then add H_2O to a final volume of 10 ml. Store at room temperature; prepare fresh solution every week.

LB medium and plates

LB liquid medium: Add 25.0 g LB broth mix (Sigma) per liter of water. Autoclave for 30 min on liquid cycle. Let cool to 50°C and add ampicillin to final concentration of 50 µg/ml.
LB plates: Prepare as above, but include 5 g/liter agar and pour plates.

Paraformaldehyde fix solution 4% (w/v)

4 g paraformaldehyde
100 ml of PBS (without calcium or magnesium; *APPENDIX 2A*)
Heat gently with constant stirring (do not boil).
Adjust pH to exactly 7.2 after solution has cooled to room temperature
Chill on ice prior to use
Store up to one week at 4°C

Protease inhibitor stock solutions

Prepare stocks:

1 mg/ml aprotinin in H_2O (1000× stock; store up to 1 year at 4°C)

1 mg/ml leupeptin in H_2O (1000× stock; store up to 1 year at 4°C)

10 mg/ml phenylmethylsufonyl fluoride (PMSF) in 100% ethanol (200× stock; store up to 1 year at −20°C)

Add each inhibitor to desired solution for a final concentration of 1×

COMMENTARY

Background Information

There are many methods that can be used to introduce biologically interesting molecules into live cells. Most of these methods such as electroporation, microinjection, and lipofection can be inefficient or laborious. Other methods, such as the use of small peptides, which can essentially diffuse into a live cell, are not desirable because of the high concentrations of the peptides required to achieve the biological effect. Therefore, a method which can deliver full-length, target-specific, concentration-dependent, and biologically active molecules into a live cell in the absence of harsh conditions or mechanical disruption would be highly advantageous.

Domains that have the ability to transport (transduce) heterologous molecules have been identified in many different biological systems (Wadia and Dowdy, 2002). Briefly, four different biological systems have been exploited as tools for the exogenous delivery of heterologous proteins: the Antennapedia protein from *Drosophila* (Derossi et al., 1998),VP22 protein from HSV (Elliot and O'Hare, 1997), MPG domain (synthetic fusion peptide of HIV-1 gp41and the NLS of SV40 large T-antigen; Morris et al., 1997), and TAT from HIV (Fawell et al., 1994). The focus of the method described here, is the transduction domain that originated from the 87 amino acid HIV-Tat protein. The transduction phenomenon was first identified in 1988 when full-length, exogenously added HIV-Tat protein was shown to trans-activate a reporter gene when added to cultured cells at concentrations as low as 1 nM. Fawell et al. (1994) expanded on this observation by demonstrating that proteins chemically cross-linked to a 36 amino acid peptide (residues 37-72) of HIV-Tat were able to transduce into cells. Vives et al. (1997) using a FITC-labeled Tat-peptide showed that residues 37-60 were important for transduction ability. This region was shown to adopt an α-helical structure with amphipathic characteristics from residues 38-49 but a cluster of basic amino acids from residues 49-58 appeared to be unstructured due to charge repulsions (Loret et al., 1991). The eleven amino acids used in the pTAT/pTAT-HA vectors encompass residues 47-57 of the HIV-Tat molecule. Residues 53-57 (QRRR) seem to be essential for the transduction since FITC-labeled peptides devoid of this region failed to transduce into HeLa cells (Vives et al., 1997). Various mutations in this minimal domain have been completed and certain substitutions have been shown to enhance transduction of peptides (Ho et al., 2001). Translation of these transduction domains (PTDs) into expression vectors is the subject of ongoing investigation, and use of these domains could provide even greater transduction potential (S.F. Dowdy, unpub. observ.).

To date, the exact mechanism of HIV Tat-mediated transduction across cellular membranes remains unclear. An attempt at defining this mechanism was reported in 1997 (Vives et al., 1997) using various versions of residues 37-72. They determined that internalization of TAT peptides did not involve endocytosis (temperature sensitive), potocytosis (caveolae or non-coated plasmalemmal vesical–mediated), or membrane destabilization. Another proposed mechanism is analogous to the Penitratin-1 transduction system. In this case, internalization could be caused by local reorganization of the lipid bilayer, resulting in inverted micelles and eventual delivery of the protein in the cytoplasm (Vives et al., 1997). Alternatively, the Tat transduction domain could utilize heparan sulfate proteoglycans as a means of gaining entry into the cell (Tyangi et al., 2001). Clearly differences in mechnisms of transduction between peptides using protein transduction domains (PTDs) and full-length fusions could vary, and this may explain the differences observed in these studies. Although the mechanism by which full-length TAT-fusion proteins gain entry into cells is not concrete, phenotypic effects of these proteins are being observed in many biological systems (Table 20.2.2), and

Table 20.2.2 Selected TAT-Fusions and the Various Cell Types Transduced

TAT fusion	Function	Cell type/line transduced	Reference
Filamin A	Actin binding Scaffolding protein of caveoli MAPK signaling cascade	HEK-293 Parathyroid cells	Hjalm et al., 2001
Ovalbumin	Used to sensitize dendritic cells (DC) in a solid tumor model	EL-4 thymoma cells Mouse dendritic cells	Shibagaki and Udey, 2002
HPC-1/syntaxin 1A	Binds to SNAP-25 or VAMP2 forming stable SNARE complexes	PC-12 neuronal Cells	Fujiwara et al., 2002
Rac/Rho	GTPases	Eosinophils Leukocytes	Alblas et al., 2001
Pur alpha[a]	Multifunctional DNA and RNA binding protein	T98G (human glioblastoma) U-87 MG (human astrocytoma) J3671 and Daoy (human medulloblastoma)	Darbinian et al., 2001
Merlin/NF2	Neurofibromatosis Type 2 tumor suppressor	Schwannoma tumors Normal Schwann cells	Bashour et al., 2002
ApolipoproteinB	Lipid metabolism	Rat primary hepatocytes McArdle RH7777	Yang et al., 2001
Bcl X_L PEA-15	Apoptosis inhibitors	BTC-3 (insulinoma cells) Rat islet cells	Embury et al., 2001
eGFP	Fluorescent protein	NIH 3T3 Myoblasts	Caron et al., 2001
CRIB	Inhibitor of Cdc42-GTP	Peripheral blood mononuclear cells	Haddad et al., 2001
C3 (exoenzyme of *C. botulinum*)	Inhibitor of Rho	Rat vascular smooth muscle cells	Sauzeau et al., 2001

[a]A C-terminal TAT-fusion molecule.

new reports of novel applications of this technique are rapidly growing.

A TAT fusion protein can be easily constructed using the pTAT/pTAT-HA vectors. The pTAT-HA vector has several features that make it ideal for use as an expression vector. The HA tag can be used to follow the fusion protein throughout purification and transduction in a specific system. A 6-His domain in the N-terminus of the fusion protein affords a simple method of affinity purifying the protein on a nickel affinity resin pre- or post-transduction. The vector contains a multiple cloning region that yields an in-frame fusion protein. A 3′ transcriptional terminator is also present if truncations of the fusion proteins are desired. Other fusion vectors have recently been described in which the HIV-Tat transduction domain has been used for C-terminal fusions (Darbiniah et al., 2001) instead of N-terminal fusions that pTAT/pTAT-HA would yield (see Table 20.2.2).

Many bacterially expressed recombinant proteins are stored as insoluble inclusion bodies because the bacteria lack the machinery to correctly fold the eukaryotic protein they are forced to produce. The bodies are insoluble aggregates that cannot be resolublized without the use of detergents or denaturing agents such as guanidine·HCl or urea. The system described in this unit makes use of 8 M urea to denature the aggregates, allowing binding to an ion-exchange resin, and immediate exchange of the protein into an aqueous environment. This yields protein that is stable enough to be added to tissue culture media where it can be correctly refolded or degraded once inside the cell. Fluorescently labeled fusion proteins have been shown to distribute uniformly in the nucleus and cytoplasm of all cells tested, including: peripheral blood lymphocytes (PBLs), diploid human fibroblasts, keratinocytes, bone marrow stem cells, osteoclasts, fibrosarcoma cells, leukemic T cells, osteosarcoma, glioma, hepatocellular carcinoma, renal carcinoma, NIH 3T3 cells, and all cells present in whole blood, in a concentration-dependent manner (Nagahara et al., 1998). A TAT-β-galactosidase fusion has been shown to penetrate every cell type in an intact live mouse and remain enzymati-

cally active after traversing all of the cellular layers in various organs, including the brain (Schwarze et al., 1999). Fusion proteins of up to 120 kDa have been successfully transduced and shown to be biologically active; therefore the size of the fusion protein does not appear to retard transduction or function, unlike other transduction domains (Wadia et al., 2002). The concentration of the transduced protein required to achieve a biological function is very low when compared to peptide mimetics. This feature of TAT transduction technology makes it ideal when targeting therapeutic strategies.

The method has already been applied to many biological questions. For example, fusions of TAT-p16 were used to define the phosphorylation events of the retinoblastoma protein (pRb) in cell cycle progression (Ezhevsky et al., 1997); TAT-E7 has been shown to sequestor pRb and rescue T-cells from T-cell Receptor-Activated Induced Cell Death (TCR-AID; Lissy et al., 1998); and the role of p27 in inducing hepatocellular carcinoma cell scattering was elucidated using TAT-p27 (Nagahara et al., 1998). This technology has been exploited to "trick" an HIV-infected cell into committing suicide by inducing caspase-3 cleavage (Vocero-Akbani et al., 1999). The implications of this idea are broadly applicable to any disease that depends upon protease-specific cleavage of pro-molecules. Many more applications have been and continue to be reported (Table 20.2.2).

The limitations for the use of this strategy seem to be three-fold. First, mammalian systems have evolved to defend against foreign proteins. Clearly, proteolytic degradation of exogenously added fusion proteins found in serum reduces the effective half-life of the protein in vitro and can be amplified in vivo. Similarly, inhibitors within serum can interfere with function of transduced molecules. For example, TAT–thymidine kinase prepared by the soluble method described in this unit (see Alternate Protocol 4) is fully functional and rapidly transduces into cells; however, the fusion protein showed no enzymatic activity after lysis of the transduced cells (S.R. Schwarze, unpub. observ.) because of a serum-derived enzymatic inhibitor. Culture systems can minimize the serum effects by using protease inhibitors or by increasing the dosage of the fusion proteins. Additionally, one can use agents such as TNF/cyclohexamide in vitro (Embary et al., 2001) to slow the cellular machinery and allow the cell to refold the transduced proteins more rapidly. The second limitation to this technology may be easily addressed. Since some eukaryotic proteins are modified post-translationally (i.e., phosphorylation and glycosylation), and bacteria do not have the ability to make such modifications, a eukaryotic fusion protein expression system must be designed in order to achieve fully functional protein. Lastly, localization of membrane fusion proteins is probably impossible. Studies with fluorescinated TAT-PTEN (which contains a C2 domain that is known to bind to phospholipid membranes; Lee et al., 1999) showed aggregation of the fusion protein at LnCaP cell membranes (S.R. Schwarze, unpub. observ.).

Critical Parameters

Maintaining protein solublility

Since many TAT-fusion proteins will be isolated in 8 M buffered urea and then quickly exchanged into an aqueous buffer, it is important to determine if the protein maintains stability after freeze/thaws and addition into the culture medium. Centrifuging the proteins at $5000 \times g$ for 10 min at 4°C will ensure that only soluble protein will be added to the cells. If a pellet is evident after centrifugation, another SDS-PAGE gel comparing protein concentrations should be completed.

If precipitation of the fusion protein is noticed on the cells during the transduction procedure, the buffer in which the fusion is stored should be changed. Use of the PD-10 column to exchange the buffer for cell-specific culture media without serum or any other additives usually solves the problem. If the protein is stored in the culture medium, the authors suggest using 0.1% (w/v) BSA to stabilize the protein. Also, when freezing the protein, always add at least 10% (v/v) glycerol, prior to flash freezing and storage at –80°C.

When thawing the proteins, quick thawing at 37°C is recommended to decrease the chances of precipitation and shearing from ice crystals. Immediately after the quick thaw, keep the proteins on ice until they are added to the medium. The thawed protein can be kept on ice at 4°C for up to 1 week with no significant loss of transducibility. Continual storage of the protein at 4°C will eventually result in protein precipitation, and is therefore not recommended for long-term storage.

Addition of the fusion proteins to tissue culture system

Because of the efficiency of this transduction system, it may be important to add the fusion protein to the culture medium prior to

addition of the medium to the cells. Therefore, after plating the cells at the appropriate density for the experimental procedure, wash the cells once in fresh medium. Add the appropriate volume of fusion protein to the required volume of tissue culture medium—i.e., 0.2 ml to 1.8 ml of DMEM containing 10% fetal bovine serum and 1× penicillin/streptomycin per well of 6-well dish—and then add this volume to the cells. It is important that the volume of fusion protein not exceed 10% of the tissue culture volume. Toxic effects and slowing of the cell cycle have been observed in some cases when greater volumes were used.

Dose and toxicity of the fusion proteins

It is virtually impossible to predict possible toxicity of the fusion protein in certain cell culture systems. If toxicity is noticed, several options are available. First, one can decrease the molar concentration of the fusion protein to the cells. In general, concentrations of >200 nM fusion protein are not necessary. The working range of these proteins vary from 50 to 200 nM. In a rare case, 1 μM was necessary (Ezhevsky et al., 1997). Therefore, the range in which a protein can be used must be decided on a fusion-to-fusion basis. Second, changing the buffer in which the protein is stored may be necessary (see Alternate Protocols 2 and 3).

The final concentration of cells used in a transduction experiment is critical. The concentration of transducing protein that is required to observe an effect is dependent upon the starting cell population. The smaller the target cell population, the greater the intracellular concentration will be. Studies using FITC-labeled TAT-p16 in normal diploid fibroblasts have showed greater fluoresecence intensity of the fusion when fewer cells were plated (M. Becker-Hapak, unpub. observ.). Therefore, in suspension cultures or adherent cells, it is generally recommended that the lowest possible concentration of cells be used when fusion protein concentrations are limited.

Half-life of fusion proteins

The authors have observed dramatic differences in the half-lives of fusion proteins. Degradation of the fusion protein can be determined by immunoblotting extracts of the trypsinized and washed cells. Generally, if the transducing protein is to be incubated for longer than 24 hr, addition of supplemental protein is suggested. The necessity for supplementary additions of any protein of interest must also be experimentally determined.

Troubleshooting

Established clone is no longer expressing fusion protein

In some cases, the bacteria may stop expressing the fusion protein. If this occurs, simply go to another one of the backup clones or retransform the plasmid into fresh BL-21 (DE3)pLysS and rescreen for high-expressing clones.

Poor expression of the fusion protein

It may be necessary to induce the fusion protein of interest. Bl-21 cells, carry an IPTG-inducible, T7 polymerase (DE3) and a T7 polymerase specific protease (pLysS) under a chloramphenicol resistance marker. The pLysS keeps the T7 polymerase at a negligible concentration until the organism goes into log phase (during IPTG induction). If the expression of the specific fusion protein is low, induce the T7 polymerase by adding 2 μg/ml IPTG when the 200 ml overnight culture is added to 1 liter of LB-ampicillin medium. Culture as described in the protocols above.

Another factor affecting the protein expression levels is the choice of supplier for the LB medium. In the authors' experience, LB medium purchased from certain vendors have produced more recombinant protein than others, so it is recommended that various vendors and lots be tested for maximal yield of any protein of interest. One may, at the same time, culture the fusion in a richer medium such as Terrific Broth (TB). However, the routine use of this medium is not recommended because some fusions have proven to express more robustly in LB than TB.

Many T7 promotor–driven fusions can be expressed in a multitude of BL-21(DE3) derivatives. The authors have found a single source of many of these hosts. Novagen provides a reasonably priced set of competent DE3 derivatives (cat. no. 71032-3) which are very helpful in expression of various eukaryotic proteins that cannot be efficiently expressed in BL-21 (DE3) pLysS. Transformation of the pure plasmid into a panel of these hosts and side-by-side detection via immunoblotting can show dramatic differences in host-to-host differences in fusion expression.

Ion-exchange chromatography

Difficulties encountered during any standard ion-exchange procedure are also observed with TAT-fusion proteins. The authors highly recommend the batch test tube procedure outlined in Amersham Pharmacia Biotech's ion-exchange

handbook (Amersham Biosciences, 2002) to rapidly identify binding and elution protocols.

Protein seems to be degrading during the purification procedure

Be sure to use the protease inhibitor cocktail described in Reagents and Solutions. This cocktail should be added at every step of the purification when a new buffer is used. If the protein was sonicated in PBS maintain the sonicate at 4°C at all times and perform the chromatographic steps in the cold.

Protein precipitates after dialysis

The concentration of the protein may be too high. Dilute the protein in buffer Z to a concentration of <1 mg/ml before applying it to the dialysis cassette.

Try using the PD-10 column to remove the urea. However, the authors have noticed that if the protein precipitates during dialysis, it is likely to precipitate during the PD-10 step as well. Therefore, the only way to ensure soluble protein is to use the ion-exchange method of achieving soluble proteins.

No biological effect with wild-type protein

The transduced protein, may require a concentration higher than 200 nM (working concentrations with in cell culture varies from 50 to 200 nM, but can require upwards of 1 μM) to obtain the specific effect. For example, the authors have observed that in wild-type cells (i.e., SiFTs) that have an already high concentration of p16 (later passage), less TAT-p16 wt is required to achieve a G1 arrest, than in those expressing low concentrations of the protein.

Another factor that can affect function of the TAT-fusion protein is the position of the TAT-leader. The authors have observed that the TAT-leader inhibits the ability of p16 to bind to TAT-CDK4, but the leader has no effect on TAT-CDK2. In such cases, Cé terminal fusions should help.

Check if the protein is active after freeze-thaw. Some proteins cannot be frozen at any step of the purification from bacterial harvesting to storage. If this is the case, store the protein on ice at 4°C. BSA may also be required to help keep the protein from precipitating or degrading.

Unknown serum factors can inhibit, inactivate, or degrade exogenously added fusion proteins causing negative results. To examine if this is occurring with the fusion, simply dilute the pure protein into cell-free culture medium (usually in the presence of 10% v/v fetal bovine serum) at 37°C for varying amounts of time, then immunoblot for the pure protein. Nonspecific proteolytic degradation can be minimized by the addition of specific protease inhibitors. However, solutions to these types of problems are most likely found by the preparation of point-specific mutants.

Choice of tags

While the pTAT-HA vector utilizes the hemagglutinin tag to allow one to detect the fusion, other popular tags used concomitantly can inhibit transduction of the purified protein. It is presumed that the very acidic nature of tags such as FLAG can associate with the TAT domain and very effectively negate the transducibility of the fusion (M. Becker-Hapak, unpub. observ.).

Anticipated Results

Protein yield

The quantity of protein yielded by a 1-liter LB-ampicillin culture will vary depending upon the construct. For example, TAT-β-galactosidase will yield 4 to 6 mg of total protein, whereas TAT-caspase 3 only yields 500 μg. In general non-cytotoxic constructs, such as TAT-p27, yield 1 to 2 mg of protein per liter of LB-ampicillin medium.

Fluorescein-labeled fusion proteins

The efficiency of fluorescein labeling will vary from day to day and protein to protein. One can quantitate the fluorescence intensity using a fluorometer prior to addition of the label to the cell culture. When comparing fluorescently labeled TAT-fusion versus the nontransducing pure protein by confocal microscopy, TAT-fusion proteins will show an even fluorescence intensity throughout the cell, with some punctate staining in the nucleoli (Fig. 20.2.4). The nontransducing protein will show a lower intensity "rim" fluorescence due to nonspecific sticking of the labeled protein to the cell membrane. If examining transduction by immunohistochemistry, typical results show detection of the transducing protein after as little as 5 min and peaking between 4 and 6 hr after transduction, when incubated at 37°C. Early time points will show the protein nonspecifically distributed throughout the whole cell, while the later time points will show a more specific staining pattern. The putative nuclear localization sequence will not prevent the fusion from going in and out of the nucleus. For example, TAT-p27 WT shows a ubiquitous distribution when transduced into HepG2 cells in the first 12 hr, then concentrates in the nucleus 24 hr after introduction of the fusion protein. However, if HepG2 cells are

cultured in the presence of HGF, TAT-p27 is shuttled out of the nucleus and into the cytoplasm over a 24 hr period (S.S. McAllister and S.F. Dowdy, unpub. observ.).

Biological activities of transduced TAT-fusion proteins

The activities expected of the TAT-fusion proteins vary with application. Consequently, phenotypic readouts vary as well. In general, the time frame in which the phenotype can be recorded can vary from 5 min (enzymatic assays) to days (morphological changes). Table 20.2.1 shows some results obtained from various classes of TAT-fusion molecules. The table details the dose of fusion that was required, biological effect observed, and time frame in which the result was measured. To expand on examples of the utilization of TAT-full length fusion technology, examples of selected fusions are shown in Table 20.2.2. The table not only lists the TAT-fusion created and the function, but it also summarizes some other cell types that were not used in transduction experiments listed in Table 20.2.1.

Time Considerations

Total time required

Identification of protein and determining necessity of IPTG induction requires 2 days

Isolation of protein requires 1 to 2 days.

Detection of protein transduction requires 1 to 2 days.

For biological readouts the time required is pathway/protein–dependent.

Stopping points

In general, one should consider any protein left at room temperature as being susceptible to degredation. Therefore, if one wishes to stop at any point during the protein purification, one should consider freezing the preparation if the duration will be longer than overnight. Alternatively, store the protein at 4°C on ice overnight, if the protein of interest is sensitive to freeze/thaw. For example; TAT-β-gal, whose enzymatic activity is abolished if frozen at any point but it retains transducibility, must be stored at 4°C in the presence of 0.1% BSA to remain active for up to one month on ice at 4°C.

Literature Cited

Alblas, J., Ulfman, L., Hordijk, P., and Koenderman, L. 2001. Activation of PhoA and ROCK are essential for detachment of migrating leukocytes. *Molec. Biol. Cell.* 12:2137-2145.

Amersham Biosciences. 2002. Ion Exchange Chromatography, Principles and Methods. Amersham Biosciences, Piscataway, N.J.

Backus, B.D., Dowdy, S., Boschert, K., Richards, T., and Becker-Hapak, M. 2001. Safety guidance for laboratory personnel working with trans-activating transduction (TAT) protein transduction domains. *Am. Chem. Soc. J. Chem. Health Safety* 8:March/April 2001.

Bashour, A.M., Meng, J.J., Ip, W., MacCollin, M., and Ratner, N. 2002. The neurofibromatosis type 2 gene product, merlin, reverses the F-actin cytoskeletal defects in primary human Schwannoma cells. *Mol. Cell Biol.* 22:1150-1157.

Becker-Hapak, M., McAllister, S.S., and Dowdy, S.F. 2001. TAT-mediated protein transduction into mammalian cells. *Methods* 24:247-256.

Caron, N.J., Torrente, Y., Camirand, G., Bujold, M., Chapdelain P., Leriche, K., Bresolin, N., and Tremblay, J.P. 2001. Intracellular delivery of a Tat-eGFP fusion protein into muscle cells. *Mol. Ther*. 3:310-318.

Darbinian, N., Gallia, G.L., King, J., DelValle, L., Johnson, E.M., and Khalili, K., 2001. Growth inhibition of glioblastoma cells by human Pur alpha. *J. Cellular Physiol.* 189:334-340.

Derossi, D., Chyassaing, G., and Prochiantz, A. 1998. Trojan peptides: The Penetratin system for intracellular delivery. *Trends Cell Biol.* 8:84-87.

Elliot, G. and O'Hare, P. 1997. Intracellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* 88:223-233.

Embury, J., Klein, D., Pileggi, A., Ribeiro, M., Jayaraman, S., Molano, R.D., Fraker, C., Kenyon, N., Ricordia, C., Inveradrdi, L., and Pastori, R.L. 2001. Proteins linked to a protein transduction domain efficiently transduce pancreatic islets. *Diabetes* 50:1706-1713.

Ezhevsky, S.A., Nagahara, H., Vocero-Akbani, A.M., Gius, D.R., Wei, M.C. and Dowdy, S.F. 1997. Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D: Cdk4/6 complexes results in active pRb. *Proc. Natl. Acad. Sci. U.S.A.* 94:10699-10704.

Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L.L., Pepinsky, B., and Barsoum, J. 1994. Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci.U.S.A. 91:664-668.*

Fujiwara, T., Yamamori, T., and Akagawa, K. 2001. Suppression of transmitter release by Tat HPC-1syntaxin 1A fusion protein. *Biochim. Biophys. Acta.* 1539:225-232.

Haddad, E., Zugaza, J.L., Louache, F., Debili, N., Crouin, C., Schwarz, K., Fischer, A., Vainchenker, W., and Bertoglio, J. 2001. The interaction between Cdc42 and WASP is required for SDF-1-induced T-lymphocyte chemotaxis. *Blood* 97:33-38.

Hall, D.J., Cui, J., Bates, M.E., Stout, B.A., Koenderman, L., Coffer, P.J., and Bertics, P.J. 2001. Transduction of a dominant-negative H-Ras into human eosinophils attenuates extracellular sig-

nal-regulated kinase activation and interleukin-5-mediated cell viability. *Blood* 98:2014-2021.

Hjalm, F., MacLeod, J.R. Kifor, O., Chattopadhyay, N., and Brown, E.M. 2001. Filamin-A binds to the carboxyl-terminal tail of the calcium-sensing receptor, an interaction that participates in CaR-mediated activation of mitogen-activated protein kinase. *J. Biol. Chem.* 276:34880-34887.

Ho, A., Schwarze, S.R., Mermelstein, S.J., Waksman, G., and Dowdy, S.F. 2001. Synthetic protein transduction domains: Enhanced transduction potential in vitro and in vivo. *Cancer Res.* 61:474-477.

Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P., and Pavletich, N.P. 1999. Crystal structure of the PTEN tumor suppressor: Implications for its phosphoinositide phosphatase activity and membrane association. *Cell* 99:323-334.

Lissy, N.A., Van Dyk, L.F., Becker-Hapak, M., Vocero-Akbani, A., Mendler, J.H., and Dowdy, S.F. 1998. TCR antigen-induced cell death occurs from a late G1 phase cell cycle check point. *Immunity* 8:57-65.

Loret, E.P., Vives, E., Ho, P.S., Rochat, H., Van Rietschoten, J., and Johnson, W.C., Jr. 1991. Activating region of HIV-1 Tat protein: Vacuum UV circular dichroism and energy minimization. *Biochemistry* 30:6013-6023.

Morris, M.C., Vidal, P., Chaloin, L., Heitz, F., and Divita G. 1997. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res.* 25:2730-2736.

Nagahara, H., Vocero-Akbani, A.M., Snyder, E.L., Ho, A., Latham, D.G., Lissy, N.A., Becker-Hapak, M., Ezhevsky, S.A., and Dowdy, S.F. 1998. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27^{kip1} induces cell migration. *Naure Med.* 4:1449-1452.

Sauzeau, V., Le Mellionnec, E., Bertoglio, J, Scalbert, E., Acaud, P., and Lorirand, G. 2001. Human urtensin II–induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-Kinase. *Circ. Res.* 88:1102-1104.

Schwarze, S.R., Ho, A., Vocero-Akbani, A., and Dowdy, S.F. 1999. In vivo protein transduction: Delivery of a biologically active protein into the mouse. *Science* 285:1569-1572.

Shibagaki, N. and Udey, M.C. 2002. Dendritic cells transduced with protein antigens induce cytotoxic lymphocytes and elicit antitumor immunity. *J. Immunol.* 168:2393-2401.

Tyagi, M., Rusnati, M., Presta, M., and Giacca, M. 2001. Internalization of HIV-1 TAT requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 276:3254-3261.

Vives, E., Brodin, P., and Lebleu, B. 1997. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 272:16010-16017.

Vocero-Akbani, A., Vander Heyden, N., Lissy, N.A., Ratner, L., and Dowdy, S.F. 1999. Killing HIV-infected cells by transduction with an HIV-protease-activated Caspase-3 protein. *Nature Med.* 5:29-33.

Wadia, J.S. and Dowdy, S.F. 2002. Protein transduction technology. *Cur. Opin. Biotech.* 13:52-56.

Yang, Y., Ballatori, N., and Smith H.C., 2001. Apolipoprotein B mRNA editing and the reduction in synthesis and secretion of he athrerogenic risk factor, apolipoprotein B100 can be effectively targeted through TAT-Mediated protein transduction. *Molec. Pharmacol.* 61:269-276.

Key References

Amersham Biosciences, 2002. See above.

Excellent resource for the theory of ion-exchange chromatography and troubleshooting measures for difficulties associated with using strong ion-exchange resins.

Backus et al., 2001. See above.

Addresses all safety issues regarding the safe handling procedures for individuals working with TAT fusion proteins.

Nagahara et al., 1998. See above.

First description of the utility of TAT-fused full-length fusion proteins.

Schwarze et al., 1999. See above.

First demonstration in vivo delivery of a biologically active TAT-fusion protein and showed that TAT-fusions as large as 120 kDa could be delivered to virtually every cell type of a live mouse.

Qiagen. 1997. The QIAexpressionist: A Handbook for high level expression of 6× His-tagged proteins. Qiagen, Chatsworth, Calif.

Contains general procedures and considerations for the expression and purification of b-His-tagged proteins.

Wadia and Dowdy, 2002. See above.

Recent review on protein transduction methodologies.

Internet Resources

http://www.expasy.ch/tools/pi_tool.html

Tools for rapid calculation of pI of a protein.

Contributed by Michelle Becker-Hapak
Washington University School of Medicine
Saint Louis, Missouri

Steven F. Dowdy
University of California, San Diego
La Jolla, California

Calcium Phosphate Transfection

This unit presents two methods of calcium phosphate–based eukaryotic cell transfection that can be used for both transient and stable transfections. In these protocols, plasmid DNA is introduced to monolayer cell cultures via a precipitate that adheres to the cell surface. A HEPES-buffered solution is used to form a calcium phosphate precipitate that is directly layered onto the cells (see Basic Protocol). For some cells, shocking the cells with glycerol or DMSO (see Support Protocol) improves transfection efficiency. In the alternate high-efficiency method, a BES-buffered system is used that allows the precipitate to form gradually in the medium and then drop onto the cells (see Alternate Protocol). The alternate method is particularly efficient for stable transformation of cells with circular plasmid DNA. For transformation with linear plasmid or genomic DNA, or for transient expression, however, the Alternate Protocol is comparable to the Basic Protocol. Both methods of transfection require very high-quality plasmid DNA. Factors that can be optimized for calcium phosphate transfections are presented in *UNIT 20.7*. Additional details of mammalian cell culture are given in *UNIT 1.1*.

TRANSFECTION USING CALCIUM PHOSPHATE–DNA PRECIPITATE FORMED IN HEPES

BASIC PROTOCOL

A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES-buffered saline solution with a solution containing calcium chloride and DNA. This precipitate adheres to the surface of cells and should be visible in the phase contrast microscope the day after transfection. Depending on the cell type, up to 10% of the cells on a dish will take up the DNA precipitate through an as yet undetermined mechanism. Glycerol or dimethyl sulfoxide shock increases the amount of DNA absorbed in some cell types (see Support Protocol).

Materials

Exponentially growing eukaryotic cells (e.g., HeLa, BALB/c 3T3, NIH 3T3, CHO, or rat embryo fibroblasts)
Complete medium (depending on cell line used)
CsCl-purified plasmid DNA (10 to 50 µg per transfection)
2.5 M $CaCl_2$ (see recipe)
2× HEPES-buffered saline (HeBS; see recipe)
PBS (*APPENDIX 2A*)

10-cm tissue culture dishes
15-ml conical tube

Additional reagents and equipment for ethanol precipitation (*APPENDIX 3A*) and mammalian cell tissue culture (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. Split exponentially growing eukaryotic cells into 10-cm tissue culture dishes the day before transfection. Feed cells with 9.0 ml complete medium 2 to 4 hr prior to precipitation.

   When transfecting adherent cells that double every 18 to 24 hr, a 1:15 split from a confluent dish generally works well. On the day of the transfection, it is important that cells are thoroughly separated on the dish, as the ability to take up DNA is related to the surface

Contributed by Robert E. Kingston, Claudia A. Chen, and Hiroto Okayama
Current Protocols in Cell Biology (2003) 20.3.1-20.3.8

area of the cell exposed to the medium. Cells should be split in a manner that accomplishes this.

The desired density of cells on dishes to be transfected will vary with cell type and the reason for doing the transfection. The optimal density is that which produces a near confluent dish when the cells are harvested or split into selective medium.

2. Ethanol precipitate the DNA to be transfected and air dry the pellet by inverting the microcentrifuge tube on a fresh Kimwipe inside a tissue culture hood. Resuspend the pellet in 450 µl sterile water and add 50 µl of 2.5 M $CaCl_2$.

   The amount of DNA that is optimal for transfection varies from 10 to 50 µg per 10-cm plate, depending on the cell line to be transfected.

   DNA to be transfected should be purified twice by CsCl gradient centrifugation. DNA can also be prepared using column procedures. Some column procedures produce DNA that does not transfect well, so column-purified DNA should be tested and compared to CsCl-purified DNA for transfection efficiency. Supercoiled DNA works well in transfections. Impurities in the DNA preparation can be deleterious to transfection efficiency. A description of how to optimize the amount of DNA to transfect and other parameters of calcium phosphate–mediated transfection is provided in the discussion of optimization of transfection (UNIT 20.7).

   Ethanol precipitation sterilizes the DNA to be transfected. For transfections that will be harvested within 3 to 4 days (transient analysis), this is not necessary. For transient experiments, many researchers make a 450-µl aqueous solution containing the DNA directly, without ethanol precipitation. If this is done, care should be taken to keep the amount of Tris in the solution to a minimum, as Tris may alter the pH of the precipitate and therefore reduce transfection efficiency.

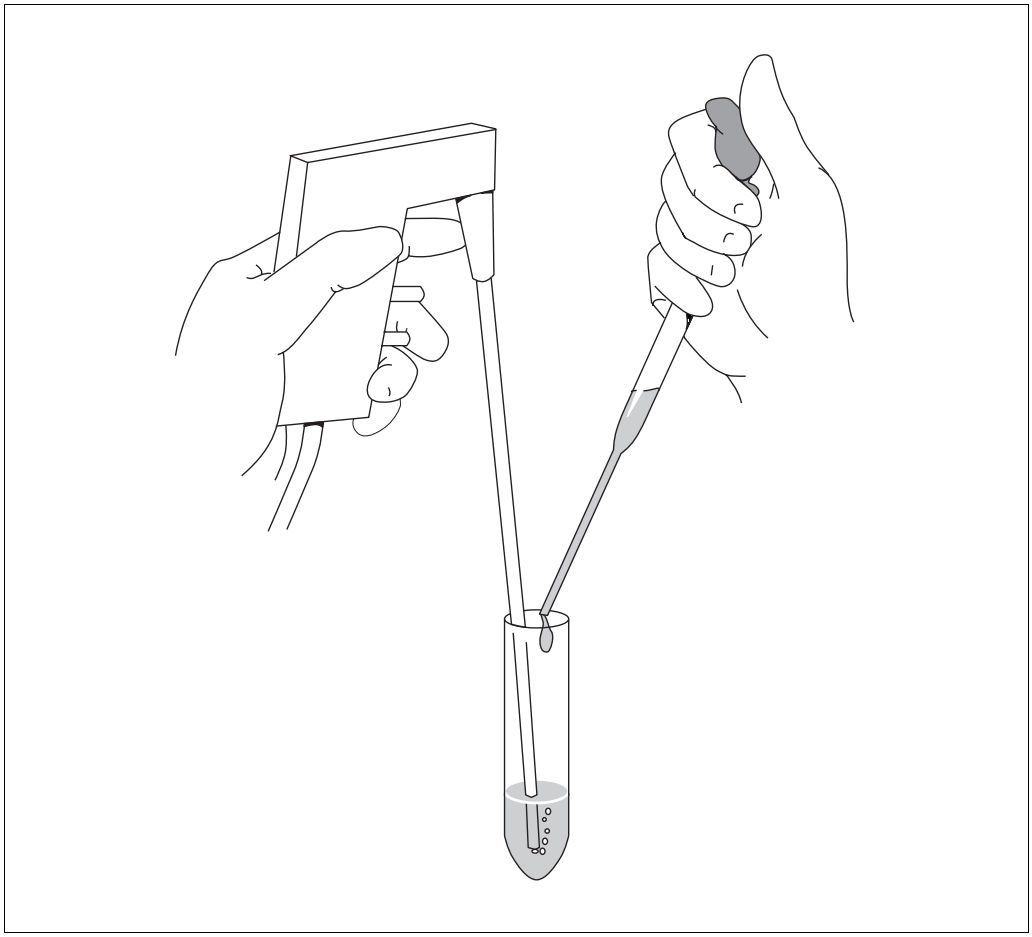


Figure 20.3.1 Formation of calcium phosphate precipitate.

3. Place 500 µl of 2× HeBS in a sterile 15-ml conical tube. Use a mechanical pipettor attached to a plugged 1- or 2-ml pipet to bubble the 2× HeBS and add the DNA/$CaCl_2$ solution dropwise with a Pasteur pipet (see Fig. 20.3.1). Immediately vortex the solution for 5 sec.

   If no mechanical pipettor is available, the solution can be bubbled by blowing through rubber tubing that is attached to a pipet via a filter. The filter is necessary to maintain sterility. This does not give as reproducible results as the mechanical pipettor.

4. Allow precipitate to sit 20 min at room temperature.

5. Use a Pasteur pipet to distribute the precipitate evenly over a 10-cm plate of cells and gently agitate to mix precipitate and medium.

6. Incubate the cells 4 to 16 hr under standard growth conditions. Remove the medium. Wash cells twice with 5 ml PBS and feed cells with 10 ml complete medium.

   The amount of time that the precipitate should be left on the cells will vary with cell type. For hardy cells such as HeLa, NIH 3T3, and BALB/c 3T3, the precipitate can be left on for 16 hr. Other cell types will not survive this amount of exposure to the precipitate. See discussion of optimization of transfection (UNIT 20.7) for optimization of this step as well as for a discussion of how to determine whether glycerol shock is useful.

7. For transient analysis, harvest the cells at the desired time point. For stable transformation, allow the cells to double twice before plating in selective medium.

GLYCEROL/DMSO SHOCK OF MAMMALIAN CELLS

SUPPORT PROTOCOL

The Basic Protocol works well for cell lines such as HeLa, BALB/c 3T3, NIH 3T3, and rat embryo fibroblasts. Transfection efficiency in some cell lines, such as CHO DUKX, is dramatically increased by "shocking" the cells with either glycerol or DMSO. Precipitates are left on the cell for only 4 to 6 hr, and the cells are shocked immediately after removal of the precipitate.

Additional Materials *(also see Basic Protocol)*

10% (v/v) glycerol solution or DMSO in complete medium, sterile
PBS (*APPENDIX 2A*), sterile

Replace step 6 of the Basic Protocol with the following:

6a. Incubate the cells 4 to 6 hr and remove the medium. Add 2.0 ml of a sterile 10% glycerol solution. Let the cells sit 3 min at room temperature.

   Alternatively, 10% or 20% DMSO can be used. DMSO tends to be somewhat less harmful to the cells, but also may not work as well.

6b. Add 5 ml of PBS to the glycerol solution on the cells, agitate to mix, and remove the solution. Wash twice with 5 ml of PBS. Feed the cells with complete medium.

   It is important to dilute the glycerol solution on the cells with PBS before removing the glycerol solution so the cells do not stay in glycerol too long. Excessive exposure to glycerol will kill cells.

ALTERNATE PROTOCOL

HIGH-EFFICIENCY TRANSFECTION USING CALCIUM PHOSPHATE–DNA PRECIPITATE FORMED IN BES

A solution of calcium chloride, plasmid DNA, and *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer, pH 6.95, is added to a plate of cells containing culture medium. The plates are incubated overnight while a calcium phosphate–DNA complex forms gradually in the medium under an atmosphere of 3% CO_2. With this method, 10% to 50% of the cells on a plate stably integrate and express the transfected DNA. Transient expression under these conditions is comparable to that obtained with the Basic Protocol. Glycerol or DMSO shock does not increase the number of cells transformed.

Materials

Exponentially growing mammalian cells (see Critical Parameters)
Complete medium: Dulbecco modified Eagle medium containing 10% (v/v) fetal bovine serum (FBS)
CsCl-purified plasmid DNA
TE buffer, pH 7.4 (*APPENDIX 2A*)
2.5 M $CaCl_2$ (see recipe)
2× BES-buffered solution (BBS; see recipe)
PBS (*APPENDIX 2A*)
Selection medium (*APPENDIX 3A*; optional)

10-cm tissue culture dishes
35°C, 3% CO_2 humidified incubator
35° to 37°C, 5% CO_2 humidified incubator
Fyrite gas analyzer (optional; Fisher Scientific or Curtin Matheson)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

1. Seed exponentially growing mammalian cells at 5×10^5 cells/10-cm tissue culture dish in 10 ml complete medium the day prior to transfection.

   There should be <10^6 cells/dish just prior to infection. Enough surface area should remain on the plate for at least two more doublings.

2. Dilute CsCl-purified plasmid DNA with TE buffer to 1 µg/µl. Store the DNA solution at 4°C.

   Purity of the plasmid DNA is critical.

   The optimum amount of plasmid to use can be determined by transfecting three dishes of cells with 10, 20, and 30 µg of plasmid DNA and incubating overnight. The dishes should then be examined with a microscope at 100×. A coarse, clumpy precipitate will form at DNA concentrations that are too low, a fine (almost invisible) precipitate will form at concentrations that are higher than optimal, and an even, granular precipitate will form with optimal DNA concentrations.

3. Prepare 0.25 M $CaCl_2$ from 2.5 M stock. Mix 20 to 30 µg plasmid DNA with 500 µl of 0.25 M $CaCl_2$. Add 500 µl of 2× BBS, mix well, and incubate 10 to 20 min at room temperature.

4. Add the calcium phosphate–DNA solution dropwise onto the medium-containing plate while swirling the plate. Incubate 15 to 24 hr in a 35°C, 3% CO_2 incubator.

   Level of carbon dioxide is critical. Use a Fyrite gas analyzer to measure percent CO_2 prior to incubation.

5. Wash the cells twice with 5 ml PBS, and add 10 ml complete medium. For stable transformation, incubate overnight in a 35° to 37°C, 5% CO_2 incubator. For studies involving transient expression, incubate the cells for 48 to 72 hr after adding the DNA.
6. Split the cells 1:10 to 1:30, depending on the growth rate of the host cell, before beginning to select stable transformants. Incubate overnight in a 35° to 37°C, 5% CO_2 incubator.
7. Start selection by changing the medium to selection medium or by incubating cells under appropriate selection conditions.

REAGENTS AND SOLUTIONS

*Use deionized, distilled water or equivalent for all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

BES-buffered solution (BBS), 2×

50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; Calbiochem-Novabiochem)
280 mM NaCl
1.5 mM Na_2HPO_4, pH 6.95
800 ml H_2O
Adjust to pH 6.95 with 1 N NaOH, room temperature
Add H_2O to 1 liter
Filter sterilize through a 0.45-µm nitrocellulose filter (Nalgene)
Store in aliquots at −20°C

As discussed in the Commentary, the pH of this solution is critical (pH 6.95 to 6.98). When a new batch of 2× BES buffer is prepared, its pH should be checked against a reference stock prepared (and tested) earlier.

This solution can be frozen and thawed repeatedly.

$CaCl_2$, 2.5 M

183.7 g $CaCl_2 \cdot 2H_2O$ (Sigma; tissue culture grade)
H_2O to 500 ml
Filter sterilize through a 0.45-µm nitrocellulose filter (Nalgene)
Store at −20°C in 10-ml aliquots

This solution can be frozen and thawed repeatedly.

HEPES-buffered saline (HeBS) solution, 2×

16.4 g NaCl (0.28 M final)
11.9 g HEPES (N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 0.05 M final)
0.21 g Na_2HPO_4 (1.5 mM final)
800 ml H_2O
Titrate to pH 7.05 with 5 N NaOH
Add H_2O to 1 liter
Filter sterilize through a 0.45-µm nitrocellulose filter
Test for transfection efficiency
Store at −20°C in 50-ml aliquots

An exact pH is extremely important for efficient transfection. The optimal pH range is 7.05 to 7.12.

There can be wide variability in the efficiency of transfection obtained between batches of 2× HeBS. Efficiency should be checked with each new batch. The 2× HeBS solution can be rapidly tested by mixing 0.5 ml of 2× HeBS with 0.5 ml of 250 mM $CaCl_2$ and vortexing. A fine precipitate should develop that is readily visible in the microscope. Transfection efficiency must still be confirmed, but if the solution does not form a precipitate in this test, there is something wrong.

COMMENTARY

Background Information

Calcium phosphate transfection was first used to introduce adenovirus DNA into mammalian cells by Graham and van der Eb (1973). It was later found to be possible to integrate exogenous DNA into mammalian chromosomes using this technique (Wigler et al., 1978). The protocol described here has evolved from these methods; in particular, the order of addition of the HEPES-buffered saline solutions (HeBS) to calcium chloride is different. In addition, many investigators have found that it is not necessary to add chromosomal DNA as carrier when doing a transfection. Transfections work well if only plasmid DNA, such as pUC vectors, is used.

This HEPES-based approach has been used to analyze replication and promoter function using "transient" protocols, in which cells are harvested 48 to 60 hr after the transfection is started. It also is presently the most widely used technique for producing cell lines in which transfected DNA is stably integrated into the chromosome, although the BES-based technique (see Alternate Protocol) is gaining widespread use for production of stable transformants. Calcium phosphate–mediated transfection tends to work much better than DEAE-dextran-mediated transfection (*UNIT 20.4*) in formation of stable cell lines. It is believed that this is because cells in a calcium phosphate transfection pick up more DNA than DEAE-dextran-transfected cells. Electroporation (*UNIT 20.5*) and liposome-mediated transfection (*UNIT 20.6*) can also be successfully used to produce stable cell lines.

Another major strength of calcium phosphate transfection is that transfected cells generally contain a representative sampling of the various plasmids in the precipitate. Hence, one can prepare a 10:1 ratio of two plasmids and expect that the plasmids will be present in that ratio in the transfected cells.

The BES-based high-efficiency calcium phosphate transfection (see Alternate Protocol) is a modification of the standard calcium phosphate method that employs a buffer system originally developed for phage particle–mediated gene transfer (Ishiura et al., 1982). With this buffer system the calcium phosphate–DNA precipitate forms gradually in the culture medium, dropping gently onto the cells over a 15- to 24-hr period. This method can stably transform most common mammalian fibroblast and epithelial cell lines 10- to 100-fold more efficiently than other methods. For transient expression, it is no better than the standard method.

Critical Parameters and Troubleshooting

Calcium phosphate transfections are finicky—they are not difficult to do, they just do not always work—even in the hands of people who routinely do them. In the Basic Protocol, the most common reason for failure is a 2× HeBS solution that is no longer at the appropriate pH. The optimum pH range for transfection is extremely narrow (between 7.05 and 7.12; Graham and van der Eb, 1973). The pH of the solution can change during storage and an old 2× HeBS solution may not work well. Some investigators also have noticed that the 2.5 M $CaCl_2$ solution can go bad over time. Both solutions should be made fresh if transfections have stopped working well.

A second problem is that the pH of the medium can turn acidic while the transfection is in progress. This results in an extremely heavy precipitate (making the medium resemble orange juice) and generally results in cell death. Care should be taken to maintain a pH of 7.2 to 7.4 and CO_2 concentrations in the incubator as listed in the protocols. Incubator and medium conditions that are fine for routinely growing cells may not suffice for calcium phosphate transfection.

Many factors can influence the efficiency of HEPES-buffered calcium phosphate–mediated transfection. A description of experiments that can be done to optimize transfection efficiency using both this procedure and the BES procedure can be found in the discussion of optimization of transfection (*UNIT 20.7*).

Several parameters are crucial to achieve high efficiency with the Alternate Protocol: pH of the 2× BES-buffered saline (BBS), percentage of carbon dioxide in the incubator during formation of precipitate, and form and amount of DNA used.

A pH curve of the 2× BBS buffer should be made because minor variations in pH can have substantial effects on transfection efficiency. Perform pilot experiments with buffers of varying pH. The optimal pH is within a very narrow range (6.95 to 6.98). Once the optimal buffer is found, use it as a reference to prepare buffer stocks. If no precipitate forms, the concentration of calcium chloride or 2× BES solution (BBS) may be wrong. Be sure to mix reagents

thoroughly before adding them to the DNA. Crystal formation upon the addition of calcium chloride indicates incorrect calcium chloride concentration, and the transfection must be repeated.

The first overnight incubation should be at 3% CO_2, but a variation of 0.5% may be acceptable. After overnight incubation at 3% CO_2 the culture medium should be alkaline (pH 7.6). Measure the CO_2 levels of the incubator before using. A Fyrite device is recommended for this.

Only plasmid DNA gives high efficiencies of gene transfer with the Alternate Protocol. Efficiency is also dependent on the purity and concentration of the DNA. Toxicity that often occurs with common fibroblasts and epithelial cell lines is usually caused by impure DNA, not calcium phosphate. DNA prepared by cesium chloride gradient centrifugation is rarely toxic to cells. The optimal DNA concentration varies among plasmid preparations as well as with different cells and media. Each new plasmid preparation and each new cell line being transfected should be tested for optimum DNA concentration.

If it is suspected that a particular plasmid preparation is toxic, use a control plasmid—one known not to be toxic to these cells—to test for toxicity. If the plasmid DNA is toxic, prepare new DNA.

Cotransfection efficiency is 10- to 20-fold better with the BES method (see Alternate Protocol) than with the Basic Protocol, although efficiencies vary with the plasmid and marker gene used. A 1:10 ratio of selectable marker to nonselected gene is recommended, but the efficiency of transfection will depend on optimum DNA dose. Glycerol shock or DMSO treatment will not increase the number of cells transfected with this method.

The BES protocol (see Alternate Protocol) has been optimized for use with cells that grow in Dulbecco modified Eagle medium containing 10% (v/v) fetal bovine serum (FBS). RPMI and minimal essential mediumα (MEMα) have also been demonstrated to give good results under the stated conditions. The FBS must be tested before use by examining the growth, plating efficiency, and transformation efficiency of at least two cell lines. (Serum is tested without heat inactivation.) Because FBS is very costly, 10% (v/v) newborn calf serum and 5% (v/v) FBS can be added to the medium (a total of 75 ml serum/500 ml medium) instead of 10% FBS. This gives equivalent efficiencies of stable transformation. The lots of newborn calf serum also must be tested for growth and plating efficiencies, as these have been found to vary. If horse or other serum or medium is required, optimum conditions may need to be rechecked.

The Alternate Protocol works well for most established cell lines that grow as monolayers, including mouse, rat, hamster, monkey, and human. It does not seem to work well for neuronal lines. This may be due to the deleterious effect of the calcium on these cells. Cells that grow in suspension are transfected rather poorly by this method, but their stable transformation frequencies seem to be better than those obtained by the Basic Protocol and almost comparable to those obtained by electroporation.

Anticipated Results

The efficiency that can be obtained with calcium phosphate–mediated transfection varies with cell type and other parameters as described. Methods for optimizing these parameters are presented in the discussion of optimization of transfection (*UNIT 20.7*). Up to 10^3 colonies can be obtained by transfecting 1 μg of a plasmid containing a dominant selectable marker into 10^6 cells by the HEPES-based protocol. Efficiencies for stable transformants are generally >10- to 100-fold higher when the BES-based protocol is used, with 10% to 50% of the cells on a plate stably transformed (Chen and Okayama, 1987). Transient expression is comparable in the Basic and Alternate Protocols (Chen and Okayama, 1988).

Time Considerations

For the Basic Protocol, preparation of twelve DNA precipitates and addition of the precipitates to the cells takes 1 to 2 hr. Without the ethanol precipitation step, the procedure can be done in 1 hr. With practice, the actual mixing of the $CaCl_2$ and 2× HeBS solutions will take ~1 min. This means that up to eighteen precipitates can be made before the first precipitate is ready to apply to the cells.

For the Alternate Protocol, no ethanol precipitation step has been necessary for either transient or stable transfections. It takes slightly less time than the Basic Protocol because the 2× BBS does not need to be added dropwise to the calcium chloride–DNA solution.

Literature Cited

Chen, C. and Okayama, H. 1987. High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745-2752.

Chen, C. and Okayama, H. 1988. Calcium phosphate–mediated gene transfer: A highly efficient system for stably transforming cells with plasmid DNA. *BioTechniques* 6:632-638.

Graham, F.L. and van der Eb, A.J. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456.

Ishiura, M., Hirose, S., Uchida, T., Hamada, Y., Suzuki, Y., and Okada, Y. 1982. Phage particle–mediated gene transfer to cultured mammalian cells. *Mol. Cell. Biol.* 2:607-616.

Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* 14:725.

Key References

Chen and Okayama, 1987. See above.

Ishiura et al., 1982. See above.

Provides the basis for BES-mediated transfection.

Contributed by Robert E. Kingston
(HEPES method)
Massachusetts General Hospital
and Harvard Medical School
Boston, Massachusetts

Claudia A. Chen (BES method)
National Institute of Mental Health
Bethesda, Maryland

Hiroto Okayama (BES method)
Osaka University
Osaka, Japan

Transfection Using DEAE-Dextran

Transfection of cultured mammalian cells using diethylaminoethyl (DEAE)-dextran/DNA can be an attractive alternative to other transfection methods in many circumstances. The major advantages of the technique are its relative simplicity and speed, limited expense, and remarkably reproducible interexperimental and intraexperimental transfection efficiency. Disadvantages include inhibition of cell growth and induction of heterogeneous morphological changes in cells. Furthermore, the concentration of serum in the culture medium must be transiently reduced during the transfection. Any of these factors may adversely affect or be incompatible with some bioassays or experimental goals. In addition, for nonstandard cell types there may be a requirement for extensive preliminary investigation of optimal transfection conditions. Together, these factors influence the suitability of this technique to specific purposes. In general, DEAE-dextran DNA transfection is ideal for transient transfections with promoter/reporter plasmids in analyses of promoter and enhancer functions, and is suitable for overexpression of recombinant protein in transient transfections or for generation of stable cell lines using vectors designed to exist in the cell as episomes. The procedure may also be used for expression cloning (Aruffo and Seed, 1987; Kluxen and Lubbert, 1993; Levesque et al., 1991), although electroporation is usually preferred for this purpose (Puchalski and Fahl, 1992).

This unit presents a general description of DEAE-dextran transfection (see Basic Protocol) as well as two more specific protocols for typical experimental applications (see Alternate Protocols 1 and 2).

The Basic Protocol is suitable for transfection of anchorage-dependent (attached) cells. For cells that grow in suspension, electroporation (*UNIT 20.5*) or lipofection (*UNIT 20.6*) is usually preferred, although DEAE-dextran-mediated transfection can be used (Fregeau and Bleackley, 1991). For suspension cells, the transfection step should be performed on collected cells that have been resuspended at 10^7 cells/ml in transfection medium, using reagents and conditions that are otherwise similar to those of the Basic Protocol.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be followed accordingly.

NOTE: All culture incubations are performed in a 37°C, 5% CO_2 incubator unless otherwise specified.

GENERAL PROCEDURE FOR DEAE-DEXTRAN TRANSFECTION

BASIC PROTOCOL

Cultured cells are incubated in medium containing plasmid DNA and DEAE-dextran, which form complexes that are taken up by cells via endocytosis. Chloroquine can be included to inhibit degradation of plasmid DNA. Cells are exposed transiently to DMSO or another permeabilizing agent to increase DNA uptake (DMSO "shock"). Important variables include the concentration of DEAE-dextran, the ratio of DNA to DEAE-dextran, the duration of transfection, and the presence and timing of chloroquine exposure (see Critical Parameters). This protocol is suitable for transfection of COS and CV1 cells; Alternate Protocols 1 and 2 describe two examples of transfection experiments.

Materials

Cells to be transfected and appropriate culture medium (e.g., complete DMEM; *UNIT 1.1*) with and without 10% FBS

100 mM (1000×) chloroquine diphosphate in PBS, filter-sterilized (store at 4°C)

Plasmid DNA(s), prepared by CsCl density-gradient centrifugation or affinity chromatography

TE buffer (*APPENDIX 2A*)

Contributed by Tod Gulick

Current Protocols in Cell Biology (2003) 20.4.1-20.4.10

10 mg/ml DEAE-dextran stock solution (see recipe)
10% (v/v) dimethyl sulfoxide (DMSO) in PBS, filter-sterilized (store up to 1 month at room temperature)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Appropriate-sized tissue culture vessels (Table 20.4.1)
Inverted microscope

Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

1. Plate cells at a density that will achieve 50% to 75% confluence on the target day for transfection. For COS or CV1 cells, perform a 1:10 split 2 days prior to transfection.

   The surface area of various cell culture vessels given in Table 20.4.1 can be used to determine how to split cells to the desired density.

   Some cell types including many primary cells show particular sensitivity to the toxicity of DEAE-dextran. These cells should be plated at higher density or transfected after reaching near-confluence.

2. Determine the total volume of medium to be used in the transfection based on the number of culture vessels containing cells to be transfected and the volume per vessel shown in Table 20.4.1. Make up this amount of medium (plus some excess) to contain 2.5% FBS by combining 1 part medium containing 10% FBS with 3 parts serum-free medium.

   DEAE-dextran can precipitate in the presence of high medium protein, necessitating use of a low FCS concentration. Alternatively, NuSerum (Collaborative Research), which contains only ~30% serum, can be used at a final concentration of 10%.

3. Add 100 mM (1000×) chloroquine diphosphate stock solution to the 2.5%-FBS-containing transfection medium prepared in step 2 to achieve a final concentration of 100 µM. Warm transfection medium to 37°C.

   Chloroquine is toxic to all cells, so exposure time should be limited to <4 hr. If longer transfection times are required for optimal transfection of a particular cell type, chloroquine should be added during the final hours of the transfection.

Table 20.4.1 Surface Areas of Commonly Used Tissue Culture Vessels and Corresponding Appropriate DEAE-Dextran Transfection Medium Volumes

Vessel	Area (cm^2)	Appropriate vol. DEAE-dextran medium[a] (ml)
T175 flask	175	
T150 flask	150	
T75 flask	75	
T25 flask	25	
150-mm dish	148[b]	10
100-mm dish	55[b]	4
60-mm dish	21[b]	2
35-mm dish	8[b]	1
6-well plate (35-mm wells)	9.4[b]	1
12-well plate (22-mm wells)	3.8[b]	0.5
24-well plate (15.5-mm wells)	1.9[b]	0.25

[a]These volumes are roughly a linear function of vessel surface area. To ensure that cells are completely covered by medium during the transfection, small wells require proportionately larger volumes due to annular sequestration of medium because of surface tension at the periphery.
[b]Costar; other manufacturer products may deviate slightly.

4. Dilute plasmid DNA in TE buffer or distilled water to between 1.0 and 0.1 µg/µl, depending on the quantity to be transfected. Add the DNA solution directly to the warmed transfection medium to a final concentration of 1.0 µg/ml.

   DNA solution(s) should comprise <1% of the total volume of transfection medium, so that the concentration of medium components is not significantly altered. Optimal DNA concentration in the transfection medium may have to be determined experimentally.

   Maintaining dilute stock DNA solutions for dedicated use in transfections reduces interexperimental variation as well as the time required to set up transfection experiments.

5. Warm the 10 mg/ml DEAE-dextran stock solution to 37°C and mix thoroughly by inversion. Add to the DNA-supplemented transfection medium to a final concentration of 100 µg/ml DEAE-dextran and mix by inversion.

   The order of addition to the transfection medium is critical. Adding plasmid DNA to medium that has already been supplemented with DEAE-dextran can result in precipitation, seen as a ropy white glob. Optimal DEAE-dextran concentration in the transfection may have to be determined experimentally.

6. Aspirate medium from the 50% to 70% confluent cell cultures (see step 1) and replace with the appropriate volume of 37°C DEAE-dextran/DNA-supplemented transfection medium (see Table 20.4.1). Incubate 4 hr.

   Uniformity of transfection efficiency may be improved by placing culture vessels on a rocker platform within the incubator during the transfection to ensure even exposure of cells to DEAE-dextran/DNA in the medium and to avoid dessication of cells in the center of the vessel. Optimal transfection time may have to be determined experimentally.

7. Examine cells with an inverted microscope.

   Cells may appear granular, some cell nuclei may appear pyknotic, and some cell borders may be somewhat ragged. An efficient DEAE-dextran transfection is usually associated with 25% to 75% cell death.

8. Warm the 10% DMSO/PBS to 37°C. Aspirate the transfection medium, note the volume, and replace with 2 to 3 volumes of 37°C DMSO/PBS. Incubate at room temperature for >2 but <10 min. Aspirate the DMSO/PBS and wash the cell layer with a volume of PBS equal to the amount of DMSO/PBS removed. Aspirate and replace with a standard amount of complete medium containing 10% FBS.

   Loss of firm cellular anchorage to the culture vessel may occur. Medium exchange and cell washing should therefore involve careful aspiration and pipetting, perhaps by holding the tip of the pipet against a wall of the culture dish or well. It is sometimes advisable to omit the PBS wash (as in the experiment described in Alternate Protocol 1, step 8) and simply add the complete medium, then change the medium a second time several hours after the DMSO shock when cells have recovered and are more firmly adherent.

9. Continue incubating the cells and analyze at times appropriate to the bioassay or intended purpose of the experiment.

   The onset and duration of expression of the transfected gene varies from one cell type to another, and especially with the expression vector used. It is advisable to perform a parallel transfection with a readily assayable reporter gene in the identical vector to assess the temporal features of expression. A reporter that is secreted by the cell into the culture medium, such as human growth hormone or secreted alkaline phosphatase, is ideal for this purpose, since aliquots of medium from a single transfection sample can be collected at serial time points. This parallel transfection can also be used in preliminary experiments to optimize transfection conditions.

ALTERNATE PROTOCOL 1

SAMPLE EXPERIMENT: TRANSFECTION TO TEST PROMOTER FUNCTION

In this protocol for a typical application of DEAE-dextran transfection, a thyroid hormone–response element in a hormone-responsive gene promoter will be mapped. Thyroid hormone (T_3) modulates transcription by activating the thyroid hormone receptor (TR), a transcription factor that binds to specific response elements in target-gene promoters as a heterodimer with the retinoid X receptor (RXR). Expression of TR, but not RXR, is minimal and limiting in fibroblasts, such that overexpression of TR using an expression vector significantly increases transcriptional response to T_3 in these cells. In this example, four promoter/reporter constructs will be tested, representing a promoter 5′ deletion series. The hormones, retinoids, and fatty acids in fetal bovine serum (FBS) can interfere with or cause high background in transcription assays in transfected cells. These moieties can be removed from FBS by charcoal treatment (see Support Protocol).

This protocol corresponds step-for-step with Basic Protocol; variations from the original procedure and reagents specific to this particular experiment are noted.

Additional Materials *(also see Basic Protocol)*

CV-1 cells (ATCC #CCL 70) growing in 100-mm dish
Complete DMEM medium (*UNIT 1.2*) with and without 10% FBS
Complete DMEM medium (*UNIT 1.2*) with 10% charcoal-treated FBS (see Support Protocol)
Plasmid DNAs:
  Control reporter plasmid (e.g., β-galactosidase, secreted alkaline phosphatase, or growth hormone, driven by a viral promoter)
  Four test promoter constructs (promoter/CAT or promoter/luciferase)
  Expression plasmid with TR gene insert (pTR)
  No-insert expression plasmid (p[–])
Complete DMEM medium (*UNIT 1.2*) with 10% charcoal-treated FBS (see Support Protocol), supplemented with 10 nm thyroid hormone (T_3)

12-well tissue culture plates
100-ml tissue culture dishes

Additional reagents and equipment for trypsinizing and subculturing monolayer cells (*UNIT 1.1*) and analyzing reporter gene activity (*APPENDIX 3A*)

1. Two days prior to the transfection, trypsinize and suspend CV1 cells from a confluent 100-mm dish in 36 ml complete DMEM medium/10% FBS. Place 1 ml complete DMEM/10% FBS in each well of four 12-well plates, then add 250 µl of the cell suspension to each of these wells. Plate the residual cells in 100-mm dishes for later use, or discard. 12 to 24 hr before the transfection, change medium to complete DMEM/10% charcoal-treated FBS.

   The sensitivity of most reporter assays permits use of many fewer cells/transfection conditions than are generally used, with consequent cost savings. CV1 cells in a well of a 12-well plate will provide sufficient reporter-gene activity for most promoter/reporters. In this protocol, triplicate wells for each condition will be analyzed. Four promoter/CAT or promoter/luciferase reporters will be tested. Cells will include or exclude pTR cotransfection. Transfected cells will be incubated in the presence or absence of T_3. Thus, four 12-well plates are required for the experiment—i.e., 3 wells (triplicates) × 4 wells (four reporters) × 2 wells (with and without pTR cotransfection) × 2 wells (with and without T_3) = 48 wells. These 48 wells include ~180 cm^2 (see Table 20.4.1) such that approximately one-third of the cells on one 100-mm (55 cm^2) dish will be used in order to achieve a 1:10 split.

Addition of suspended cells to empty tissue culture plates or wells results in an uneven distribution of adherent cells, which can introduce undesirable intersample variability; hence medium is added to the wells first.

Preincubation in medium supplemented with charcoal-stripped FBS (see Support Protocol) ensures that any hormones of interest are absent from medium bathing the control cells during subsequent experiments.

2. Add 7 ml DMEM/10% charcoal-treated FBS to 21 ml serum-free DMEM to make 28 ml of DMEM/2.5 % FBS.

   In this experiment, the FBS in the DMEM/10 has been stripped of low molecular weight hydrophobic moieties, including T_3, using charcoal.

   The amount here was calculated as 48 wells multiplied by 0.5 ml/well, and a small excess was added. In experiments in which the transfection medium will be divided into multiple aliquots carrying different plasmids or plasmid combinations, it is useful to carry a volume excess throughout the preparation of the separate transfection media to adjust for pipet-calibration errors.

3. Add 28 µl 100 mM chloroquine diphosphate to the medium and place the tube in a 37°C water bath.

4. Dilute the reporter plasmids (control and test promoter constructs) in TE buffer to 1 µg/µl; dilute the TR (pTR) and no-insert (p[–]) expression plasmids in TE buffer to 0.2 µg/µl. Add 14 µl of the diluted reporter plasmid to the transfection medium (final concentration, 0.5 µg/ml). Divide this medium into four equal 6.6-ml aliquots and add 6.6 µl of a test promoter construct to each separate tube (final concentration, 1 µg/ml). Divide each of these into two tubes, each containing 3.2 ml. Add 8 µl pTR to one set of four tubes and 8 µl p[–] to the other set of four tubes (final concentration, 0.5 µg/µl). Use each of these eight transfection medium samples to transfect cells within six wells in step 6, below.

   All cells will be transfected with a control reporter—e.g., β-galactosidase, secreted alkaline phosphatase, or growth hormone—driven by a viral promoter. Each of the four test promoter/reporters will be transfected into cells within 12 wells. A TR expression vector will be transfected into 6 of each of these sets of 12 wells. Cells in triplicate wells in each of these conditions will be cultured in medium supplemented with T_3, while those in the other triplicate wells will be cultured in medium devoid of T_3.

   Plasmids that are included in more than one transfection condition are added prior to division of medium into separate aliquots to ensure that these samples receive equivalent amounts of plasmid DNA. The final concentration of DNA in each transfection medium sample should be equivalent. For this and other reasons, an "empty vector" should be used as described for p[–] above.

5. Warm the stock DEAE-dextran to 37°C, mix by inversion, and add 32 µl to each of the eight tubes containing DNA-supplemented transfection medium (final DEAE-dextran concentration, 100 µg/ml). Mix by gentle inversion.

6. Aspirate medium from six wells on one plate, and replace with one of the eight transfection-medium samples prepared in step 4 at 500 µl per well. Repeat for each of the different transfection-medium samples. Incubate 4 hr.

7. Examine cells with inverted microscope.

8. Aspirate medium from wells in one plate and add 1 ml DMSO/PBS per well. Repeat for each plate. Return to the first plate, aspirate the DMSO/PBS and replace with 1 ml/well complete DMEM/10% charcoal-treated FBS. Incubate cells for 4 to 12 hr. Aspirate medium from wells and replace the medium from one set of triplicate wells for each transfection condition with 1 ml/well T_3-supplemented DMEM/10% char-

coal-treated FBS and the other set of triplicate wells for each transfection condition with (T_3-unsupplemented) DMEM/10% charcoal-treated FBS.

9. Incubate cells 24 to 48 hr. Aspirate 500 µl of medium from each separate well and save for control reporter activity determinations in order to normalize test reporter activities. Wash wells with PBS and harvest cells for CAT or luciferase activity measurements.

   Reporter activity is used to confirm and quantitate promoter T_3 responsiveness ($+T_3$ /$-T_3$), to verify a direct transcriptional response mediated by the TR (augmentation of response to T_3 in pTR-cotransfected cells), and to map the T_3-responsive region of the promoter (region present in a T_3-responsive promoter/reporter and absent in an unresponsive one).

   The use of a control reporter permits normalization for transfection efficiency and for nonspecific-stimulus effects on gene expression. In many cases, when using this transfection technique, adequate and informative preliminary experiments can be conducted without this control (since transfection efficiency is so uniform), thereby saving time and expense. This is seldom possible when using other transient-transfection techniques, especially calcium phosphate coprecipitation (UNIT 20.3), where transfection efficiency varies markedly within an experiment. Of course, initial experiments should be performed to exclude nonspecific stimulus effects.

ALTERNATE PROTOCOL 2

SAMPLE EXPERIMENT: TRANSFECTION TO TEST ENZYME STRUCTURE/ACTIVITY RELATIONSHIPS

In this experimental application of DEAE-dextran transfection, an enzyme and several enzyme mutants are overexpressed in COS cells to provide material for kinetic analyses in a structure/activity analysis. A vector designed for high-level expression that replicates in SV40-transformed cells (e.g., CDM8) will be used.

This protocol corresponds step-for-step with Basic Protocol; variations from the original procedure and reagents specific to this particular experiment are noted.

Additional Materials *(also see Basic Protocol)*

COS cells (ATCC #1650) growing in 100-mm dish
Complete DMEM medium (*UNIT 1.2*) with and without 10% FBS
Control plasmid containing reporter gene (e.g., luciferase, CAT, or secreted alkaline phosphatase)
CDM8 vectors containing gene for wild-type enzyme and genes for four mutant enzymes
100-ml tissue culture dishes
Additional reagents and equipment for analyzing reporter gene activity (*APPENDIX 3A*) and analysis of recombinant proteins (*APPENDIX 3A*)

1. Two days prior to the transfection, split five 100-mm dishes of confluent COS cells into fifty 100-mm dishes.

   Ten 100-mm dishes of COS cells will provide sufficient recombinant enzyme activity for kinetic analyses. Recombinant wild-type enzyme and four mutant enzymes will be overexpressed. Thus, fifty dishes are required for this experiment.

2. Add 50 ml DMEM/10% FBS to 150 ml serum-free DMEM to make 200 ml DMEM/2.5% FBS.

   This was calculated as fifty dishes at 4 ml/100-mm dish (see Table 20.4.1).

3. Warm the transfection medium in a 37°C water bath. Do not add chloroquine diphosphate.

Chloroquine treatment increases DEAE-dextran transfection efficiency, but may reduce the amount of recombinant protein produced by the transfected cells. It is advisable to test this in early pilot experiments.

4. Dilute the reporter gene plasmid and each of the CDM8/enzyme expression vectors to 1 µg/µl in TE buffer. Add 20 µl reporter plasmid to the transfection medium and mix (final concentration, 0.1 µg/ml). Divide the medium into five aliquots of 40 ml. Add 160 µl of one CDM8/enzyme expression plasmid to one aliquot (final concentration, 4 µg/ml) and repeat this for each of the CDM8/enzyme expression plasmids.

   All cells will be transfected with a control plasmid containing a reporter gene such as luciferase, CAT, or secreted alkaline phosphatase to evaluate transfection efficiency. Each of the five enzyme expression vectors will be used to transfect cells in ten dishes.

   If the COS cells have an endogenous activity identical or similar to the activity of the recombinant protein to be overexpressed, it may be prudent to include cells that are transfected with "empty vector" in experiments of this type to permit parallel assays of endogenous COS cell activities for "background" subtraction.

   Plasmids may compete for replication and/or transcription factors. The control reporter plasmid can be included at low concentration because the reporter has a very sensitive assay.

5. Warm the stock DEAE-dextran to 37°C, mix by inversion, and add 800 µl to each of the tubes containing DNA-supplemented transfection medium (final DEAE-dextran concentration, 200 µg/ml). Mix by gentle inversion.

6. Aspirate medium from ten dishes and replace with 4 ml of the appropriate DEAE-dextran/DNA-supplemented transfection medium. Repeat for each set of ten dishes. Incubate 3 hr.

7. Examine cells with inverted microscope. Continue the transfection until some cells appear slightly granular.

8. Aspirate transfection medium from one of the sets of ten dishes and replace with 10 ml/dish DMSO/PBS. After 2 min, aspirate the DMSO/PBS and wash gently with 10 ml PBS. Aspirate PBS and add 10 ml DMEM/10 FBS. Repeat for each set of ten dishes.

9. Incubate cells 48 to 96 hr. Harvest cells as appropriate for recombinant protein assays. Save aliquots of culture medium (for secreted control reporter) or cell extract (intracellular reporter) to determined transfection efficiency.

   Normalization for transfection efficiency may not be necessary, particularly in circumstances where an independent assay for recombinant protein production is available, as might be provided by a specific antibody.

CHARCOAL STRIPPING OF FETAL BOVINE SERUM

SUPPORT PROTOCOL

Activated charcoal is used to remove low-molecular-weight lipophilic compounds from serum including hormones, retinoids, and fatty acid ligands of nuclear receptor transcription factors.

Materials

Fetal bovine serum (FBS), heat-inactivated (*UNIT 1.1*)
Activated charcoal, acid-washed (Sigma)
Ultracentrifuge with Beckman SW 28, JA-20.1, or equivalent swinging-bucket rotor

1. Add 2 g activated charcoal per 100 ml heat-inactivated FBS. Add a stir-bar and place on a stir plate in a 4°C cold room or refrigerator. Stir for 2 hr.

   Although it is difficult to maintain aseptic conditions during charcoal stripping, care should be taken to avoid flagrant contamination and to keep the serum at 4°C.

2. Collect serum in centrifuge tubes and centrifuge 30 min at 72,000 × *g* (20,000 rpm in SW 28 rotor) *or* 60 min at 51,500 × *g* (20,000 rpm in JA-20.1), 4°C.

3. Gently pour off serum from each centrifuge tube into a sterile beaker, divide into aliquots in sterile conical tubes, and store frozen at −20°C.

   Some residual charcoal may be present but the serum should be only lightly peppered with charcoal after centrifugation.

4. Prior to use, thaw a tube of charcoal-stripped medium and use immediately to supplement complete medium. Filter sterilize the medium using a 0.22-µm bottletop filter and collect in a sterile bottle. Store at 4°C.

   The serum is filtered after it is added to the medium because the undiluted serum is too viscous to filter readily.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DEAE-dextran stock solution, 10 mg/ml (100×)

Make a 10 mg/ml stock solution of diethylaminoethyl (DEAE)-dextran (mol. wt. ~500,000 Da; Sigma) in tissue culture–quality PBS (*APPENDIX 2A*). Mix well, filter sterilize using an 0.22-µm filter, mix again, divide into aliquots, and store up to 3 months at 4°C.

Warm to 37°C and mix well by inversion immediately before each use.

COMMENTARY

Background Information

The mechanism by which cells take up DNA in DEAE-dextran-mediated transfection appears to involve endocytosis after adsorption of DNA/DEAE-dextran complexes onto cells. The advantages of the technique relate largely to the relative simplicity, limited expense, and lack of interexperiment and intraexperiment variability in transfection efficiency.

Critical Parameters

There are several crucial parameters in the transfection procedure, and the weight of importance of each differs for different cell types. Furthermore, maximizing efficiency of a transfection (percent of cells transfected) does not necessarily correspond with optimizing the desired goal of the transfection, such as production of recombinant protein (Kluxen and Lubbert, 1993) or maintenance of colony-forming potential of cells containing transfected DNA (Puchalski and Fahl, 1992). Systematic analyses of variables in DEAE-dextran/DNA transfection for particular cell types have been reported (Fregeau and Bleackley, 1991; Puchalski and Fahl, 1992; Kluxen and Lubbert, 1993; Yang and Yang, 1997), and these studies provide useful information. However, the existence of numerous variables and their mutual codependence makes interpolation, extrapolation, or guessing transfection conditions toward any specific end for any particular cell type difficult to extract from literature reports. Thus, the investigator should carry out a modest set of pilot experiments to optimize conditions.

The major variables that influence DEAE-dextran/DNA transfection include: (1) DEAE-dextran concentration; (2) DNA concentration and the ratio of DNA concentration to DEAE-dextran concentration; (3) duration of transfection; (4) use of chloroquine; (5) use of permeabilizing agents; and (6) serum concentration. The influence of each of these factors on the various goals of DNA transfection will be addressed.

The concentration of DEAE-dextran used in transfections varies from 50 to 500 μg/ml. There is an inverse relationship between the concentration used and the duration of exposure prior to onset of cytotoxicity for all cell types. In initial experiments, it may be best to use a low concentration (100 or 200 μg/ml) to provide an adequate temporal window to evaluate cytotoxicity microscopically. Lethal cytotoxicity can precede microscopic evidence of the same, such that transfections with high concentrations of DEAE-dextran can result in near-complete cell loss even when most cells appear healthy at the end of the transfection period.

The DNA concentration used depends in part on the vector, cell type, and the purpose of the transfection. For maximal cell adsorption of complexes and maximal transfection efficiency, the ratio of transfection-medium DEAE-dextran concentration to DNA concentration should be 40:1 to 50:1, perhaps because of the existance of an optimal electrostatic cell membrane/DEAE-dextran/DNA-complex interaction (Yang and Yang, 1997). Thus, at a DEAE-dextran concentration of 200 μg/ml, plasmid DNA should be included at 4 to 5 μg/ml. Since this ratio influences transfection efficiency, "empty vector" should be included in controls at the same concentration as test vectors. This ideal [DEAE-dextran]/[DNA] ratio may depend on transfection-medium serum concentration.

The amounts of DNA to be used in cotransfection experiments again depend on the intended purpose of each component transfection. When a control reporter plasmid is used to simply normalize for transfection efficiency, it is often possible and desirable to include a low concentration of the plasmid, since reporter enzyme assays are generally exquisitely sensitive. In experiments in which simultaneous cotransfection of single cells with two plasmids is a goal, it is appropriate to add the plasmids at the same or similar concentrations. Furthermore, transfection efficiency is of paramount importance in this circumstance, since the percentage of cells that take up both plasmids is a fraction of those that take up each alone. This is particularly important in a case like that presented in Alternate Protocol 1, where coexistence of the transcription-factor expression vector and the promoter/reporter plasmid in a single cell is important.

DEAE-dextran-mediated cytotoxicity is a function of exposure time. In general, efficient transfections can be achieved with a 4-hr incubation if other parameters are adjusted appropriately. This is a convenient transfection duration since it corresponds to the maximal allowable period of cell exposure to chloroquine. Shorter transfection times may be appropriate for some cell types.

The utility of chloroquine in increasing the efficiency of DEAE-dextran transfection is well documented. However, there may be an attenuation in the amount of transfected gene expression (Kluxen and Lubbert, 1993) and increased cell loss and disruption of cellular morphology (Puchalski and Fahl, 1992) when this reagent is included. Thus, use of chloroquine is appropriate for purposes where transfection efficiency is a dominant priority. In other cases, preliminary experiments should evaluate the impact of this agent on the bioactivity desired. While some investigators add chloroquine in the final stage of (or after) a longer transfection, it is generally easier to increase the DEAE-dextran concentration in this circumstance to permit a single 4-hr transfection in medium containing all components.

Using a final cell "shock" with a permeabilizing agent markedly increases DEAE-dextran transfection efficiency without additional cytotoxicity (Lopata et al., 1984) and should be used universally, unless this manipulation somehow interferes with the desired bioactivity. DMSO at a concentration of 10% is generally used, although 15% glycerol may be more effective for some cell types. Enhancement of transfection efficiency increases as a function of permeabilizing-agent exposure time up to 2 min, after which there is no additional impact (Sussman and Milman, 1984). Since longer exposure, up to 5 to 10 min, produces no negative effect, the "shock" should be for >2 min, but need not be rigorously timed or reproduced, even between transfected samples within an experiment.

Use of transfection medium supplemented with 10% FBS results in formation of macroscopic protein/DEAE-dextran/DNA complexes that are not compatible with efficient transfection. Serum-free medium can be used during the transfection, as can medium supplemented with either 2.5% FBS or 10% NuSerum. The concentration of serum in the transfection medium affects the extent of DEAE-dextran-mediated and chloroquine-mediated cytotoxicity, with a protective effect provided by the serum. FBS concentration may also influence the optimal ratio of [DEAE-dextran]/[DNA].

Anticipated Results

The efficiency of DEAE-dextran-mediated transfection varies considerably among cell types. One can expect to achieve 20% to 60% transfection efficiency with many cells using this procedure if proper attention is paid to optimization of transfection conditions. Following transfection using this technique, there are generally significant morphological changes in cells, and some of these may weigh against the use of this technique for certain purposes.

Time Considerations

Splitting cells into the required number of plates several days prior to the transfection may take ~30 min. Depending on the complexity of the transfection, preparing transfection media may take 5 min to 1 hr. If stock solutions of DNA, DEAE-dextran, chloroquine, and DMSO/PBS are prepared in advance, 30 min should be adequate for this phase, even in a moderately complex transfection experiment like that presented in Alternate Protocol 1. During the subsequent transfection, cells should be monitored using a microscope periodically after 3 hr. The DMSO or glycerol shock, cell wash, and medium replacement requires only ~10 min. Thus, with minimal but careful planning of experimental design, a transfection can readily be completed in 5 hr.

Literature Cited

Aruffo, A. and Seed, B. 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. U.S.A.* 84:8573-8577.

Fregeau, C.J. and Bleackley, R.C. 1991. Factors influencing transient expression in cytotoxic T cells following DEAE-dextran-mediated gene transfer. *Somatic Cell Mol. Genet.* 17:239-257.

Kluxen, F.-W. and Lubbert, H. 1993. Maximal expression of recombinant cDNAs in COS cells for use in expression cloning. *Anal. Biochem.* 208:352-356.

Levesque, J.P., Sansilvestri, P., Hatzfeld, A., and Hatzfeld, J. 1991. DNA transfection in COS cells: A low-cost serum-free method compared to lipofection. *Biotechniques* 11:313-318.

Lopata, M.A., Cleveland, D.W., and Sollner-Webb, B. 1984. High level transient expression of a chloramphenical acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment *Nucl. Acids Res.* 12:5707-5717.

Puchalski, R.B. and Fahl, W.E. 1992. Gene transfer by electroporation, lipofection, and DEAE-dextran transfection: Compatibility with cell-sorting by flow cytometry.*Cytometry* 13:23-30.

Sussman, D.J. and Milman, G. 1984. Short-term, high-efficiency expression of transfected DNA. *Mol. Cell. Biol.* 4:1641-1643.

Yang, Y.-W. and Yang, J.-C. 1997. Studies of DEAE-dextran-mediated gene transfer *Biotechnol. Appl. Biochem.* 25:47-51.

Contributed by Tod Gulick
Massachusetts General Hospital
Charlestown, Massachusetts

Transfection by Electroporation

Electroporation—the use of high-voltage electric shocks to introduce DNA into cells—is a procedure that is gaining in popularity. It can be used with most cell types, yields a high frequency of both stable transformation and transient gene expression, and, because it requires fewer steps, can be easier than alternate techniques (*UNITS 20.3, 20.4 & 20.6).*

The Basic Protocol describes the electroporation of mammalian cells. The alternate protocol outlines modifications for preparation and transfection of plant protoplasts.

ELECTROPORATION INTO MAMMALIAN CELLS

BASIC PROTOCOL

Electroporation can be used for both transient and stable transfection of mammalian cells. Cells are placed in suspension in an appropriate electroporation buffer and put into an electroporation cuvette. DNA is added, the cuvette is connected to a power supply, and the cells are subjected to a high-voltage electrical pulse of defined magnitude and length. The cells are then allowed to recover briefly before they are placed in normal growth medium. Factors that can be varied to optimize electroporation effectiveness are discussed in *UNIT 20.7*.

Materials

Mammalian cells to be transfected
Complete medium (*UNIT 1.2*) without and with appropriate selective agents (*APPENDIX 3A*)
Electroporation buffer, ice-cold (see recipe)
Linear or supercoiled, purified DNA preparation (see step 7)

Beckman JS-4.2 rotor or equivalent
Electroporation cuvettes (Bio-Rad #165-2088) and power source

Additional reagents and equipment for stable transformation in selective medium and for harvesting transfected cells (*APPENDIX 3A*)

Prepare the cells for electroporation

1. Grow cells to be transfected to late-log phase in complete medium.

   Each permanent transfection will usually require 5×10^6 cells to yield a reasonable number of transfectants. Each transient expression may require $1–4 \times 10^7$ cells, depending on the promoter.

2. Harvest cells by centrifuging 5 min at $640 \times g$ (1500 rpm in a JS-4.2 rotor), 4°C.

   Adherent cells are first trypsinized (UNIT 1.1) and the trypsin inactivated with serum.

3. Resuspend cell pellet in half its original volume of ice-cold electroporation buffer.

   The choice of electroporation buffer may depend on the cell line used. See Critical Parameters for a complete discussion.

4. Harvest cells by centrifuging 5 min as in step 2.

5. Resuspend cells at 1×10^7/ml in electroporation buffer at 0°C for permanent transfection. Higher concentrations of cells (up to 8×10^7) may be used for transient expression.

6. Transfer 0.5-ml aliquots of the cell suspension into desired number of electroporation cuvettes set on ice.

Contributed by Huntington Potter
Current Protocols in Cell Biology (2003) 20.5.1-20.5.6

Add DNA and electroporate the cells

7. Add DNA to cell suspension in the cuvettes on ice.

   For stable transformation, DNA should be linearized by cleavage with a restriction enzyme that cuts in a nonessential region and purified by phenol extraction and ethanol precipitation. For transient expression, the DNA may be left supercoiled. In either case, the DNA should have been purified through two preparative CsCl/ethidium bromide equilibrium gradients followed by phenol extraction and ethanol precipitation. The DNA stock may be sterilized by one ether extraction; the (top) ether phase is removed and the DNA solution allowed to dry for a few minutes to evaporate any remaining ether. See APPENDIX 3A for cross-references to these procedures.

   For transient expression, 10 to 40 μg is optimal. For stable transformation, 1 to 10 μg is sufficient. Cotransfection, although not recommended because of the work required to select and test transformants, can be done with 1 μg of a selectable marker containing DNA and 10 μg of the DNA containing the gene of interest.

8. Mix DNA/cell suspension by holding the cuvette on the two "window sides" and flicking the bottom. Incubate 5 min on ice.

9. Place cuvette in the holder in the electroporation apparatus (at room temperature) and shock one or more times at the desired voltage and capacitance settings.

   The number of shocks and the voltage and capacitance settings will vary depending on the cell type and should be optimized (Critical Parameters; see also UNIT 20.7).

10. After electroporation, return cuvette containing cells and DNA to ice for 10 min.

Culture and harvest the transfected cells

11. Dilute transfected cells 20-fold in nonselective complete medium and rinse cuvette with this same medium to remove all transfected cells.

12a. *For stable transformation:* Grow cells 48 hr (about two generations) in nonselective medium, then transfer to antibiotic-containing medium.

   Selection conditions will vary with cell type. For example, neo selection generally requires ~400 μg/ml G418 in the medium. XGPRT selection requires 1 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine in the medium.

   It is often convenient to plate adherent cells at limiting dilution immediately following the shock, or suspension cells at the time of antibiotic addition.

12b. *For transient expression:* Incubate cells 50 to 60 hr, then harvest cells for transient expression assays.

   Transfected cells can be visualized by standard transient expression assays.

ALTERNATE PROTOCOL

ELECTROPORATION INTO PLANT PROTOPLASTS

This is a modification of the Basic Protocol that is intended for use with plant cells. Plant cells are stripped of their cell walls and DNA is introduced into the resulting protoplasts.

Additional Materials *(also see Basic Protocol)*

5-mm strips (1 g dry weight) sterile plant material
Protoplast solution (see recipe)
Plant electroporation buffer (see recipe)

80-μm-mesh nylon screen
Sterile 15-ml conical centrifuge tube

Additional reagents and equipment for plant RNA preparation (*APPENDIX 3A*)

1. Obtain protoplasts from carefully sliced 5-mm strips of sterile plant material by incubating in 8 ml protoplast solution for 3 to 6 hr at 30°C on a rotary shaker.
2. Remove debris by filtration through an 80-µm-mesh nylon screen.
3. Rinse screen with 4 ml plant electroporation buffer. Combine protoplasts in a sterile 15-ml conical centrifuge tube.
4. Centrifuge 5 min at 300 × *g* (1000 rpm in a JS-4.2 rotor). Discard supernatant, add 5 ml plant electroporation buffer, and repeat wash step. Resuspend in plant electroporation buffer at $1.5–2 \times 10^6$ protoplasts/ml.

   Protoplasts can be counted with a hemacytometer (UNIT 1.1).

5. Carry out electroporation as described for mammalian cells (steps 6 to 11 of the Basic Protocol). Use one or several shocks at 1 to 2 kV with a 3- to 25-µF capacitance as a starting point for optimizing the system.

   Alternatively, use 200 to 300 V with 500 to 1000 µF capacitance if the phosphate in the electroporation buffer is reduced to 10 mM final.

6. Harvest cells after 48 hr growth and isolate RNA, assay for transient gene expression, or select for stable transformants.

   Protoplasts can also be selected and grown into full transgenic plants (Rhodes et al., 1988).

REAGENTS AND SOLUTIONS

Use deionized distilled water for all recipes and protocol steps. For common solutions, see ***APPENDIX 2A****; for suppliers see* ***SUPPLIERS APPENDIX****.*

Electroporation buffers

Choice of electroporation buffer depends on the cells being used in the experiment (see Critical Parameters). The following buffers (stored at 4°C) can be used:

1. PBS (*APPENDIX 2A*) *without* Ca^{2+} or Mg^{2+}
2. HEPES-buffered saline (HeBS; *UNIT 20.3*)
3. Tissue culture medium *without* FBS (*UNIT 1.1*)
4. Phosphate-buffered sucrose: 272 mM sucrose/7 mM K_2HPO_4 (adjusted to pH 7.4 with phosphoric acid)/1 mM $MgCl_2$

Plant electroporation buffer

Prepare in PBS (APPENDIX 2A):
0.4 M mannitol
5 mM $CaCl_2$
Store at 4°C

Protoplast solution

2% (w/v) cellulase (Yakult Honsha)
1% (w/v) macerozyme (Yakult Honsha)
0.01% (w/v) pectylase
0.4 M mannitol
40 mM $CaCl_2$
10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 5.5
Prepare fresh before use

COMMENTARY

Background Information

DNA transfection by electroporation is a technique that is applicable to perhaps all cell types. It yields a high frequency of stable transformants and has a high efficiency of transient gene expression. Electroporation makes use of the fact that the cell membrane acts as an electrical capacitor that is unable to pass current (except through ion channels). Subjecting membranes to a high-voltage electric field results in their temporary breakdown and the formation of pores that are large enough to allow macromolecules (as well as smaller molecules such as ATP) to enter or leave the cell. The reclosing of the membrane pores is a natural decay process that is delayed at 0°C.

During the time that the pores are open, nucleic acid can enter the cell and ultimately the nucleus. Linear DNA with free ends is more recombinogenic and more likely to be integrated into the host chromosome to yield stable transformants. Supercoiled DNA is more easily packaged into chromatin and is generally more effective for transient gene expression.

The use of high-voltage electric shocks to introduce DNA into cells was first performed by Wong and Neumann using fibroblasts (Neumann et al., 1982; Wong and Neumann, 1982). The technique was then generalized (Potter et al., 1984) to all cell types—even those such as lymphocytes that, unlike fibroblasts, cannot be transfected with other procedures (e.g., calcium phosphate or DEAE-dextran DNA coprecipitates). Although whole plants or leaf tissue have been reported to be transfectable by electroporation, plant cells must generally be made into protoplasts before DNA can be easily introduced into them (Alternate Protocol; Fromm et al., 1985; Ou-Lee et al., 1986). Like mammalian cells, plant protoplasts may be electroporated under a variety of electrical conditions (Critical Parameters). Both high voltage with low capacitance (short pulse duration) or low voltage with high capacitance (long pulse duration) have been used to achieve successful gene transfer (Chu et al., 1987).

The wide use of electroporation has been made possible in large part by the availability of commercial apparatuses that are safe and easy to use and that give extremely reproducible results. Designs of these machines vary substantially, but fall into two basic categories that use different means of controlling pulse duration and voltage (the two electrical parameters that govern pore formation). One kind uses a capacitor discharge system to generate an exponentially decaying current pulse, and the other generates a true square wave (or an approximation thereof). The capacitor discharge instruments charge their internal capacitor to a certain voltage and then discharge it through the cell-DNA suspension. Both the size of the capacitor and the voltage can be varied. Because the current pulse is an exponentially decaying function of (1) the initial voltage, (2) the capacitance setting of the instrument, and (3) the resistance of the circuit (including the sample), changing the capacitor size to allow more (or less) charge to be stored at the voltage will result in longer (or shorter) decay times and hence a different effective pulse duration. In contrast, square wave generators control both the voltage and pulse duration with solid-state switching devices. They also can produce rapidly repeating pulses.

Most of our electroporation experiments have used the Bio-Rad Gene Pulser, a capacitor discharge device, but are directly applicable to other capacitor discharge devices, and with some adjustment to square wave generators. Capacitor discharge devices are also available from Life Technologies, BTX, Hoeffer Scientific, and International Biotechnologies (see *SUPPLIERS APPENDIX* for suppliers' addresses). These machines, either in a single unit or through add-on components, can deliver a variety of electroporation conditions suitable for most applications. Square wave generators are available from BTX or Baekon and offer great control over pulse width, allow multiple, rapid pulses, and can be more effective for cells that are very sensitive or otherwise difficult to transfect. These machines are generally more expensive. It has become apparent that alternating current pulses at ~100 kHz may be the most effective wave form for electroporation and possibly electrofusion (Chang, 1989). However, dedicated electroporation devices utilizing such waves are not yet commercially available and must be constructed from components. For a complete discussion of electroporation instruments, see Potter (1988).

Electroporation can be easier to carry out than alternative techniques, which is why it is becoming popular. Its drawback for use with transient analysis is that almost five-fold more cells and DNA are needed than with either calcium phosphate– or DEAE-dextran-mediated transfection (*UNITS 20.3 & 20.4*). The main difference between electroporation and cal-

cium phosphate coprecipitation procedures is the state of the integrated DNA after selection in appropriate antibiotic media. In the case of calcium phosphate, the amount of DNA taken up and integrated into the genome of each transfected cell is in the range of 3×10^6 bp. As a result, the transfected DNA often integrates as large tandem arrays containing many copies of the transfected DNA. This would be an advantage when transfection of genomic DNA into recipient cells and selection for some phenotypic change such as malignant transformation is desired; here a large amount of DNA integrated per recipient cell is essential. In contrast, electroporation can be adjusted to result in one to many copies of inserted DNA per recipient cell. This would be an advantage for gene expression studies, as the identity of the particular copy responsible for the gene expression can be controlled.

Critical Parameters

As discussed above, the two parameters that are critical for successful electroporation are the maximum voltage of the shock and the duration of the current pulse (see also *UNIT 20.7*). The voltage and capacitance settings must be optimized for each cell type, with the resistance of the electroporation buffer being critical for choosing the initial instrument settings. The guidelines presented in this unit are meant to be adapted according to the manufacturers' instructions and the individual investigator's needs. Optimal stable and transient transformations occur at about the same instrument settings, so transient expression can be used to optimize conditions for a new cell type.

For low-resistance (high-salt) buffers such as PBS, HeBS, or tissue culture medium, start with a capacitor setting of 25 μF and a voltage of 1200 V for 0.4-cm cuvettes, then increase or decrease the voltage until optimal transfection is obtained (usually at ~40% to 70% cell viability as detected by trypan blue exclusion; *UNIT 1.1*). For many cell types, the choice between PBS, HeBS, and tissue culture medium is arbitrary. However, some cells (especially primary cells) are very easily killed and thus electroporate poorly at the high voltages needed for PBS or HeBS electroporation buffers. Particularly sensitive cells seem to prefer tissue culture medium, though it has been shown that the calcium and magnesium ions in the medium lower the electroporation efficiency (Neumann et al., 1982). Phosphate-buffered sucrose has the advantage that it can be optimized at voltages several hundred volts below those used with PBS or HeBS. Alternatively, Chu et al. (1987) found many sensitive cells were electroporated more effectively in HeBS with a low voltage/high capacitance setting that resulted in at least 10-fold longer pulse duration. For these conditions, start at 250 V/960 μF and change the voltage up to 350 V or down to 100 V in steps to determine optimal settings.

Keeping cells on ice (at 0°C) often improves cell viability and thus results in higher effective transfection frequency, especially at high power which can lead to heating (Potter et al., 1984). However, Chu et al. (1987) found that under low voltage/high capacitance conditions, some cell lines electroporate with higher efficiency at room temperature. Therefore, steps 6 to 10 of the Basic Protocol should be carried out separately at both temperatures to determine the optimum conditions for a new cell line.

Another factor contributing to cell death appears to be the pH change that results from electrolysis at the electrodes. This problem can be reduced by replacing some of the ionic strength of the PBS with extra buffer (e.g., 20 mM HEPES, pH 7.5).

Optimal parameters for plant electroporation differ depending on whether tissue culture cells or various parts of the whole plant are used as a source of protoplasts. In particular, the high salt in PBS can be damaging to protoplasts freshly produced from plant tissue. Replacing the NaCl in PBS with 135 mM LiCl may increase CAT transient gene expression in electroporated plant protoplasts 4- to 70-fold (Saunders et al., 1989). Alternatively, an electroporation buffer of 0.6 M mannitol/25 mM KCl for leaf cells, or 0.7 M mannitol/40 mM KCl/4 mM MES (pH 5.7)/1 mM 2-ME added for root and stem cells, is recommended (Sheen, 1990). In addition, 0.1% BSA/15 mM 2-ME/1 mM $MgCl_2$ can be added to either protoplast isolation buffer and the $CaCl_2$ reduced to 1 mM final. A low salt concentration in the electroporation buffer reduces the optimal capacitance setting to 200 μF.

Anticipated Results

The efficiency of transfection by electroporation is dependent upon cell type. For fibroblasts, which are easily transfected by calcium phosphate or DEAE-dextran coprecipitation (*UNITS 20.3 & 20.4*), electroporation gives a stable transformation frequency of 1 in ~10^3 to 10^4 live cells—approximately that obtainable by the above traditional procedures. For cells refractory to traditional methods, electropora-

tion gives a stable transformation frequency between 1 in 10^4 to 10^5 for most cell types. Occasionally a cell line (e.g., some T lymphocytes) will transfect poorly under our standard conditions (1 in 10^6), and even this frequency is sufficient to obtain significant numbers of transfectants. In general, cells that transfect efficiently for stable transformants also do so for transient gene expression. Increasing the number of cells and the amount of DNA used in the electroporation for studying transient gene expression can circumvent problems of low transfection efficiency and low promoter/enhancer efficiency.

For plant protoplast electroporation, the frequency of stable transformants is between 1 in 10^2 and 1 in 10^3 dividing cells.

Time Considerations

The entire process of electroporation of mammalian cells will take <1 hr. Electroporation of plant cells requires ≤6 hr to prepare the protoplasts and <1 hr for the actual electroporation process. As with other transfection procedures, the experiment should be planned to allow for harvest or splitting of the cells 1 to 2 days after transfection.

Literature Cited

Chang, D.C. 1989. Cell poration and cell fusion using an oscillating electric field. *Biophys. J.* 56:641-652.

Chu, G., Hayakawa, H., and Berg, P. 1987. Electroporation for the efficient transfection of mammalian cells with DNA. *Nucl. Acids Res.* 15:1311-1326.

Fromm, M., Taylor, L.P., and Walbot, V. 1985. Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proc. Natl. Acad. Sci. U.S.A.* 82:5824-5828.

Neumann, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, P.H. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* 1:841-845.

Ou-Lee, T.M., Turgeon, R., and Wu, R. 1986. Uptake and expression of a foreign gene linked to either a plant virus or *Drosophila* promoter in protoplasts of rice, wheat and sorghum. *Proc. Natl. Acad. Sci. U.S.A.* 83:6815-6819.

Potter, H. 1988. Electroporation in biology: Methods, applications, and instrumentation. *Anal. Biochem.* 174:361-373.

Potter, H., Weir, L., and Leder, P. 1984. Enhancer-dependent expression of human κ immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc. Natl. Acad. Sci. U.S.A.* 81:7161-7165.

Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D., and Detmar, J.J. 1988. Genetically transformed maize plants from protoplasts. *Science* 240:204-207.

Saunders, J.A., Matthews, B.F., and Miller, P.D. 1989. Plant gene transfer using electrofusion and electroporation. *In* Electroporation and Electrofusion in Cell Biology (E. Neumann, A.E. Sowers, and C.A. Jordan, eds.) p. 343-354. Plenum, New York.

Wong, T.K. and Neumann, E. 1982. Electric field mediated gene transfer. *Biochem. Biophys. Res. Commun.* 107:584-587.

Key Reference

Potter et al., 1984. See above.

The original paper from which the Basic Protocol is adapted.

Contributed by Huntington Potter
Harvard Medical School
Boston, Massachusetts

Transfection of Cultured Eukaryotic Cells Using Cationic Lipid Reagents

The development of high-efficiency methods for the introduction of functional genetic material into eukaryotic cells using cationic lipid–based transfection reagents has accelerated biology research in the studies of gene expression, control of cell growth, and cell lineage. Cationic lipid–mediated transfection techniques are commonly used in industrial protein production as well as in some clinical gene therapy protocols.

Most natural lipids are either neutral or negatively charged (anionic). Positively charged (cationic) lipids were first introduced in 1987 (Felgner et al., 1987). The cationic lipids function by spontaneous electrostatic interaction of their positive charges with the negative charges in the backbone of DNA, RNA, or oligonucleotides, condensing the extended macromolecules to a compact structure. The cationic charges and the lipophilic nature of the cationic lipids then allow the condensed aggregates to interact with and cross the negatively charged and hydrophobic cell membrane and enter the target cells. It is also possible to deliver some proteins into cells using cationic lipids (Sells et al., 1995). Most (but not all) cationic lipid reagents used for transfection consist of mixtures of cationic and neutral lipids (e.g., DOPE, cholesterol) that are formulated in water to yield noncovalent structures called liposomes—hollow spheres with aqueous cores and a diameter of 100 to 400 nm (Felgner et al., 1987). Some are dissolved in ethanol and form micelles (Behr et al., 1989). There are many cationic lipid reagents available (see Background Information and Table 20.6.1). They perform with various efficiencies in different applications and target cells.

This unit describes cationic lipid–mediated transfection of a variety of cell types. DNA transfection is presented for adherent mammalian cells (cell lines as well as primary cultures; see Basic Protocol 1), along with a modified protocol for enhanced transfection (see Alternate Protocol). DNA transfection of suspension mammalian cells (lymphoid, myeloid, and leukemic-derived cells) is also presented (see Basic Protocol 2). For adherent mammalian cells, RNA transfection is also covered (see Basic Protocol 3). Finally, DNA transfection is presented for insect cells (see Basic Protocol 4). To determine

Table 20.6.1 Partial Listing of Commercially Available Cationic Lipid Transfection Reagents

Supplier	Reagent	Uses
Invitrogen	PerFect Lipid Transfection Kit (8 lipids)	Test for different cell types
Life Technologies	LipofectAmine 2000	General, rapid
	LipofectAmine Plus	Difficult-to-transfect adherent cells
	LipofectAmine	General
	Lipofectin	Liver and endothelial cells, oligonucleotides
	DMRIE-C	Suspension cells, RNA
	CellFectin	Insect cells
Promega	Transfectam	General
	Tfx Transfection Trio	Test for different cell types
Qiagen	Effectene	General
Roche/BMB	DOTAP	General
	DOSPER	General
	FuGENE 6	General

Contributed by Pamela Hawley-Nelson and Valentina Ciccarone
Current Protocols in Cell Biology (2003) 20.6.1-20.6.17

the best transfection conditions for a particular reagent, use the optimization/fine-tuning procedure (see Support Protocol).

NOTE: There are minor differences among the recommended protocols for the various commercially available cationic lipid reagents. The appropriate product profile sheets should be consulted before beginning an experiment.

NOTE: All culture incubations for mammalian cells should be performed in a humidified 37°C, 5% CO_2 incubator. Some media may require altered levels of CO_2 (e.g., for growth of CHO cells in suspension, 8% CO_2 is preferable) to maintain pH 7.4. Insect cells are cultured at 27°C.

BASIC PROTOCOL 1

CATIONIC LIPID–MEDIATED TRANSFECTION OF ADHERENT MAMMALIAN CELLS WITH DNA

This protocol describes the procedure for transfection of DNA into most adherent mammalian cell lines or cultures of primary cells grown attached to culture vessels (Fig. 20.6.1). DNA and lipid reagent are diluted into separate aliquots of serum-free medium, and are then mixed together and allowed to form complexes. Complexes and transfection medium (which may contain serum) are added to the cells in one of two ways. Either the complexes are diluted with transfection medium and this mixture is added to cells that have been rinsed and aspirated (as shown in Fig. 20.6.1), or complexes are added directly to transfection medium that has already been added to the cells (with or without washing). The cells and complexes are incubated together for several hours. After transfection, the volume of the medium is increased if necessary to prevent drying, serum is added if the transfection was serum free, and cells are incubated an additional day or two to allow expression of the transgene.

It is advisable to optimize the conditions for transfection using the Support Protocol. Suggested starting ranges for optimizing the various components of these mixtures are given in Table 20.6.2 for six different popular culture vessel sizes. Transfections can be scaled up to other vessel sizes by increasing the amounts of lipid, DNA, and medium in

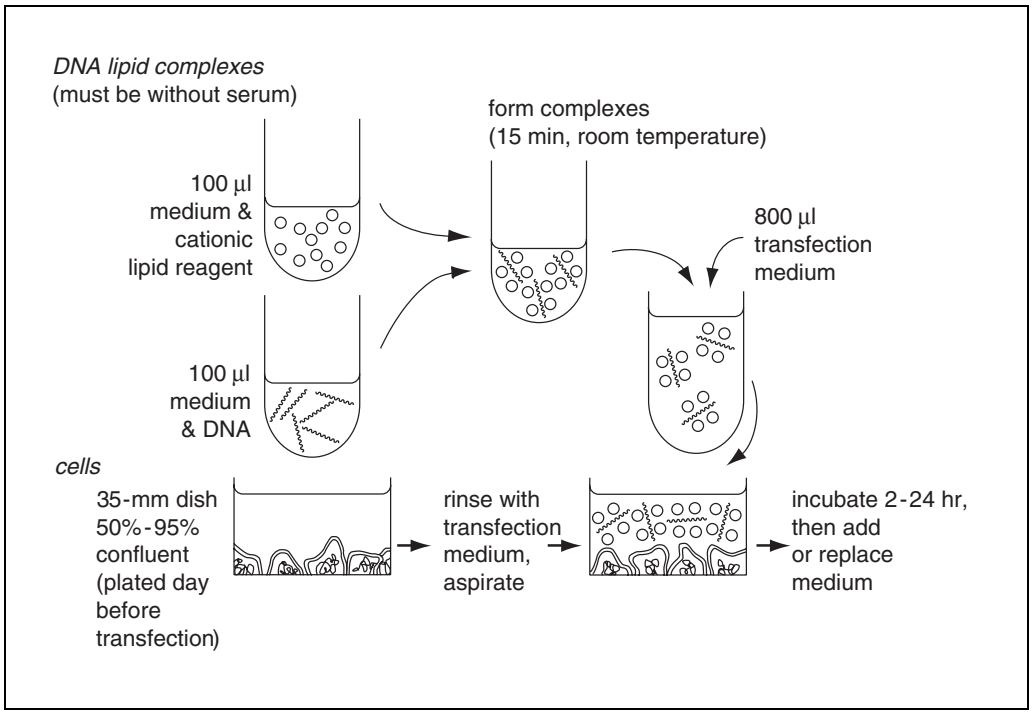


Figure 20.6.1 Diagram of cationic lipid–mediated transfection procedure.

proportion to the difference in surface area. Table 20.6.3 shows the surface areas of several popular culture vessels.

Materials

Adherent cells
Cell culture medium with serum (e.g., complete DMEM, *UNIT 1.2*)
Dilution medium: serum-free cell culture medium or specialized medium for lipid-mediated transfection (e.g., Opti-MEM I, Life Technologies)
Plasmid DNA, purified by anion-exchange chromatography (e.g., Concert High Purity columns, Life Technologies; or see Goldsborough et al., 1998), cesium chloride density gradient, or alkaline lysis (*UNIT 1.6*)
Cationic lipid reagent (see Table 20.6.1)
Polystyrene or polypropylene tubes

Additional reagents and equipment for trypsinization and counting of cells (*UNIT 1.1*), Xgal staining, and selection of stable transformants (*APPENDIX 3A*)

1. The day before transfection, trypsinize and count adherent cells (*UNIT 1.1*). Plate cells in cell culture medium with serum so that they are 50% to 95% confluent on the day of transfection. Avoid antibiotics at the time of plating and during transfection.

   The single most important factor in reproducible, high-efficiency transfection is a consistent number of healthy, proliferating cells. Transfection is most efficient when the cells are maintained in mid-log growth. Because transfection efficiency is sensitive to culture

Table 20.6.2 Suggested Starting Ranges of Reagents for Lipid-Mediated Transfection[a]

Culture vessel	DNA (µg)	Dilution medium (µl)	Cationic lipid reagent (µl)	Transfection medium (ml)	Transfection volume (ml)
	Step 2	Steps 2 & 3	Step 3	Step 5	Step 6
96-well	0.05-0.4	10-25	0.075-1.5	0.08-0.1	0.1-0.15
24-well	0.2-1.6	25-50	0.5-10	0.2-0.5	0.25-0.6
12-well	0.4-3.2	50-100	1-20	0.4-1	0.5-1.2
6-well	1-8	100-250	2.5-50	0.8-2.5	1-3
60-mm	2-16	250-500	5-100	2-5	2.5-6
100-mm	6-48	750-1500	15-300	5-15	6.5-18

[a]Step numbers are indicated from Basic Protocol 1. Volumes have been optimized for LipofectAmine 2000. See Support Protocol for additional optimization strategies.

Table 20.6.3 Surface Areas of Commonly Used Culture Vessels

Culture vessel	Surface area (cm^2)
96-well	0.3
24-well	2
12-well	4
6-well	10
35-mm	8
60-mm	20
100-mm	60
150-mm	140
T25	25
T75	75
T150	150

confluency, cultures should be maintained carefully and passaged frequently, and a standard seeding protocol should be followed from one experiment to the next. Antibiotics may cause some toxicity if present during transfection. For some transfection reagents, the higher cell density is recommended (90% to 95%).

Multiwell cell culture dishes are the easiest to use. Some cells are weakly adherent. If necessary, increase adherence by plating cells on polylysine-coated wells (0.1 mg/ml poly-L-lysine, Sigma; poly-D-lysine-precoated plates, Becton Dickinson).

This protocol is written without specific amounts of reagents. Table 20.6.2 gives the recommended starting amounts for several popular vessels. See Support Protocol for fine tuning the reagent volumes for highest transfection efficiency.

2. On the day of transfection, dilute plasmid DNA into dilution medium in a polystyrene or polypropylene tube and mix. Prepare in bulk for multiple transfections.

   Although DNA prepared by anion-exchange chromatography or CsCl gradients yield the best results, DNA prepared by alkaline lysis (miniprep) will work with lower efficiency.

   Commercial medium that is specialized for lipid-mediated transfection (e.g., Opti-MEM I) yields the highest efficiency, but other serum-free media may be used.

   Polystyrene or polypropylene tubes work well for dilutions. For small-scale transfections, round-bottom 96-well plates with covers are suitable.

3. Dilute cationic lipid reagent into dilution medium in a second tube and mix. Prepare in bulk for multiple transfections.

   See Table 20.6.1 for a partial listing of commercially available reagents. A preparation of the cationic lipid DDAB with the neutral lipid DOPE can be made in the laboratory (Rose et al., 1991), although efficiency may be lower than with some commercial preparations.

   If using Lipofectin, dilute into Opti-MEM I and allow to incubate at room temperature for 30 to 45 min (Ciccarone and Hawley-Nelson, 1995). If using LipofectAmine 2000, dilute into Opti-MEM I and allow to incubate no more than 30 min (Ciccarone et al., 1999).

4. Combine diluted DNA and diluted cationic lipid reagent, mix, and incubate for 15 min at room temperature.

   Incubation times >15 min (up to 6 hr for some reagents) work just as well.

5. While complexes are forming, replace medium on the cells with the appropriate volume of fresh transfection medium.

   This step is not necessary if the complexes are diluted with transfection medium or if using LipofectAmine 2000 (see step 6).

   This medium can be the same as the dilution medium. It is possible to use serum in the transfection medium at this step.

6. Add DNA-lipid complexes to each well containing cells. Mix complexes into the medium gently, holding the plate at a slant. Incubate 5 hr at 37°C in 5% CO_2.

   Adding transfection medium directly to the cells (as described in the steps) helps prevent cells in multiwell plates from drying out, as transfection medium can be added to multiple wells rapidly. Alternatively, complexes can be diluted with the appropriate volume of fresh transfection medium and added to cells from which the cell culture medium has been aspirated (with or without washing, see Fig. 20.6.1). If using LipofectAmine 2000, fresh transfection medium is not required. Undiluted complexes can be added directly to the cell culture medium.

   The exposure time may be >5 hr (up to overnight). Be sure that there is sufficient medium to prevent the cells from drying out (it is not necessary to increase other components if this is done). If necessary to maximize cell growth, replace the medium containing complexes with fresh complete medium after 5 hr incubation.

7. After 5 hr incubation, add cell culture medium to reach normal volume and add serum to bring the final concentration to that of normal cell culture medium.

   This step is omitted in some protocols, especially when serum is present during transfection and volumes are adequate to prevent drying.

8a. *For transient expression analysis:* Assay cell extracts or stain cells in situ 24 to 48 hr after the start of transfection, depending on cell type and promoter activity.

8b. *For stable expression analysis*: Passage cells into fresh culture medium 1 day after the start of transfection. At 2 days posttransfection, add the appropriate antibiotic to select for expression of the transfected antibiotic-resistance gene.

   Several days or weeks of selection are required for stable expression.

ENHANCED CATIONIC LIPID–MEDIATED TRANSFECTION OF ADHERENT MAMMALIAN CELLS WITH DNA

ALTERNATE PROTOCOL

This is an efficient and reproducible protocol for transfection of DNA into most adherent mammalian cell lines or cultures of primary cells grown attached to culture vessels (Dube, 1997; Shih et al., 1997; Fig. 20.6.2). The procedure is essentially as described in Basic Protocol 1, except that DNA is diluted into serum-free medium along with a proprietary enhancing reagent and incubated for 15 min, allowing the formation of precomplexed DNA before addition to the diluted cationic lipid reagent.

The protocol that follows was developed for LipofectAmine Plus, which is composed of two reagents: LipofectAmine (the cationic lipid reagent) and Plus reagent (the enhancer). One of the main advantages of the Plus enhancer is a high plateau of transfection activity

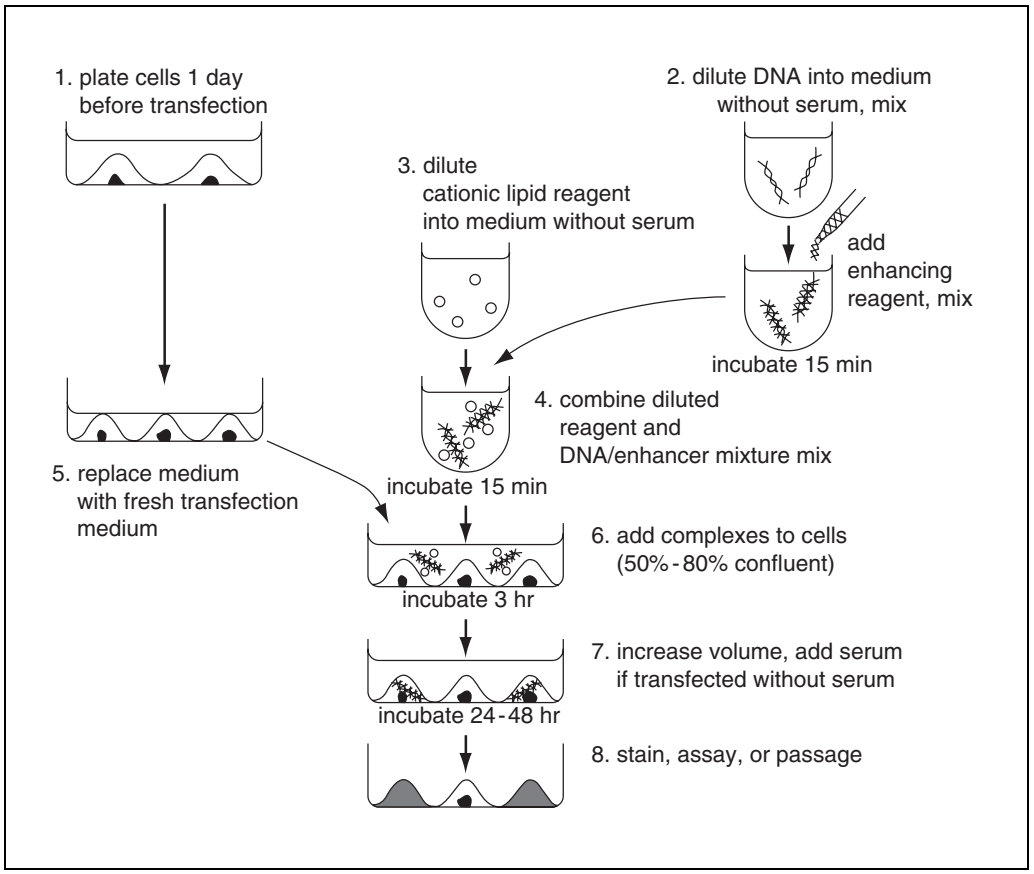


Figure 20.6.2 Diagram of enhanced cationic lipid–mediated transfection procedure.

Table 20.6.4 Suggested Starting Amounts of Reagents for Transfection with LipofectAmine Plus[a]

Culture vessel	DNA (µg)	Plus reagent (µl)	Dilution medium (µl)	LipofectAmine (µl)	Transfection medium (ml)	Transfection volume (ml)
	Step 2	Step 2	Steps 2 & 3	Step 3	Step 5	Step 6
96-well	0.1	1	10	0.5	0.08	0.1
24-well	0.4	4	25	1	0.2	0.250
12-well	0.7	5	50	2	0.4	0.5
6-well	1	6	100	4	0.8	1.0
60-mm	2	8	250	12	2	2.5
100-mm	4	20	750	30	5	6.5

[a]Step numbers indicated from Alternate Protocol. It is possible to fine tune transfections by testing a range of lipid and DNA concentrations to obtain optimal efficiency; however, the peak activity is usually a broad plateau with this reagent.

across a broad range of lipid and DNA concentrations. This decreases the necessity for optimization and allows the recommendation of specific starting conditions (Table 20.6.4). Transfections can be scaled up to other vessel sizes by increasing the amounts of lipid, DNA, enhancer, and medium in proportion to the difference in surface area (see Table 20.6.3).

The other commercially available enhanced cationic lipid reagent is Effectene (Qiagen, see Table 20.6.1). If using this product, consult the appropriate product profile sheets before beginning the experiment.

NOTE: Consult annotations of the standard transfection procedure (see Basic Protocol 1) for additional details, which also apply to this protocol.

Additional Materials *(also see Basic Protocol 1)*

Cationic lipid reagent and enhancer (e.g., LipofectAmine Plus, including LipofectAmine and Plus reagents; Life Technologies)

1. The day before transfection, trypsinize and count adherent cells (*UNIT 1.1*). Plate cells in cell culture medium with serum so that they are 50% to 80% confluent on the day of transfection. Avoid antibiotics at the time of plating and during transfection.

   For this protocol, refer to Table 20.6.4 for recommended amounts of reagents in different vessels.

2. On the day of transfection, dilute plasmid DNA into dilution medium in a polystyrene or polypropylene tube and mix well. Add enhancer, mix, and incubate 15 min at room temperature.

   DMEM is preferred over Opti-MEM I in this protocol. It is important to add the DNA first and mix well before adding the Plus reagent to avoid precipitation of the DNA. Incubation times >15 min (up to an hour) work just as well.

3. Dilute cationic lipid reagent into dilution medium in a second tube and mix.

4. Combine precomplexed DNA and diluted cationic lipid reagent, mix, and incubate for 15 min at room temperature.

   Incubation times >15 min (up to an hour) work just as well when LipofectAmine is the cationic lipid and Plus reagent is the enhancer.

5. While complexes are forming, replace medium on the cells with the appropriate volume of fresh transfection medium.

   The medium can be the same as the dilution medium. It is possible to use serum in the transfection medium at this step. In some cells (e.g., HeLa and NIH 3T3), transfection in medium containing serum is as efficient or more efficient than in medium without serum.

6. Add DNA-enhancer-lipid complexes to each well containing cells. Mix complexes into the medium gently, holding the plate at a slant. Incubate at 37°C in 5% CO_2 for several hours.

   The exposure time with LipofectAmine Plus may be as short as 3 hr or up to overnight. Be sure there is sufficient medium to prevent the cells from drying out (it is not necessary to increase other components if this is done).

7. After incubation, add cell culture medium to reach normal volume and add serum to bring the final concentration to that of normal cell culture medium.

8. Perform transient or stable expression analysis (see Basic Protocol 1, steps 8a and 8b).

CATIONIC LIPID–MEDIATED TRANSFECTION OF SUSPENSION CELLS WITH DNA

BASIC PROTOCOL 2

This protocol is essentially the same as for adherent cells (see Basic Protocol 1) in that lipid and DNA are diluted separately into dilution medium, mixed, and allowed to form complexes before exposing to cells. However, complexes are formed in the wells of multiwell culture plates, and cells are then distributed into the wells containing complexes and allowed to transfect.

Materials

Dilution medium: cell culture medium without serum or specialized medium for transfection (e.g., Opti-MEM I, Life Technologies)
Cationic liposome reagent (e.g., DMRIE-C or LipofectAmine 2000, Life Technologies; also see Table 20.6.1)
Plasmid DNA, purified by anion-exchange chromatography or Goldsborough et al., 1998), cesium chloride density gradient, or alkaline lysis
Cell suspension: 1×10^7 cells/ml in normal cell culture medium without serum or antibiotics
Cell culture medium (e.g., complete DMEM; *UNIT 1.2*)
Serum
6-well tissue culture plates

1. To each well of a 6-well tissue culture plate add 0.5 ml dilution medium.

   Commercial medium that is specialized for lipid-mediated transfection (e.g., Opti-MEM I), without serum or antibiotics, gives the best results. However, other serum-free media may also be used.

   When transfecting in different-sized culture plates, change the amounts of DNA, cationic lipid reagent, and medium in proportion to the difference in surface area (see Table 20.6.3).

2. Add 0, 2, 4, 6, 8, or 12 µl cationic lipid reagent to each well and mix gently by swirling the plate.

   DMRIE-C was found to give high efficiency transfection of DNA in Jurkat (human T cell lymphoma), K562 and KG-1 (human myeologenous leukemia), and MOLT-4 (human lymphoblastic leukemia) cell lines. It is a lipid suspension that may settle with time. To ensure that a homogenous sample is taken, mix thoroughly by inverting the tube 5 to 10 times before removing a sample for transfection.

3. Add 0.5 ml dilution medium containing 4 µg plasmid DNA to each well. Mix by swirling plate.

   The amount of DNA should be optimized for each cell line.

4. Incubate at room temperature for 15 to 45 min to allow formation of lipid-DNA complexes.

5. Add 0.2 ml cell suspension (2×10^6 cells) to each well and mix gently.

   The single most important factor in reproducible, high-efficiency transfection is a consistent number of healthy, proliferating cells. Transfection is most efficient when the cells are maintained in mid-log growth.

6. Incubate several hours at 37°C in a 5% CO_2 incubator.

   A 4-hr incubation is adequate for DMRIE-C transfections.

7. To each well add 2 ml cell culture medium containing 1.5× the usual amount of serum.

   For Jurkat and MOLT-4 cells, addition of 1 μg/ml phytohemagglutinin (PHA) and 50 ng/ml phorbol myristate acetate (PMA) enhances promoter activity and gene expression. For K562 and KG-1 cells, PMA alone enhances promoter activity.

8. Assay the cells at 24 or 48 hr post-transfection for transient or stable expression (see Basic Protocol 1, step 8a or 8b).

BASIC PROTOCOL 3

CATIONIC LIPID–MEDIATED TRANSFECTION OF ADHERENT CELLS WITH RNA

In this protocol, lipid is first diluted into dilution medium and mixed. RNA is then mixed directly into the diluted lipid and immediately added to cells (which have been rinsed with serum-free medium), and cells are incubated for transfection.

Materials

Adherent cells
Cell culture medium with serum (e.g., complete DMEM; *UNIT 1.2)*
Dilution medium: serum-free cell culture medium or specialized medium for transfection (e.g., Opti-MEM I, Life Technologies)
Cationic lipid reagent (e.g., DMRIE-C, Life Technologies; also see Table 20.6.1)
mRNA (see *APPENDIX 3A*)

6-well or 35-mm tissue culture plate
12 × 75–mm polystyrene tubes

Additional reagents and equipment for trypsinizing, counting, and plating cells (*UNIT 1.1*)

1. The day before transfection, trypsinize and count adherent cells (*UNIT 1.1*). In each well of a 6-well tissue culture plate, or in six 35-mm tissue culture plates, seed ~2–3 $\times 10^5$ cells in 2 ml of the appropriate cell culture medium supplemented with serum.

   Transfection is most efficient when the cells are growing rapidly. Cultures should be maintained carefully and passaged frequently. As transfection efficiency may be sensitive to culture confluency, it is important to maintain a standard seeding protocol from experiment to experiment.

2. Incubate at 37°C in a 5% CO_2 incubator until the cells are ~80% confluent.

   This will usually take 18 to 24 hr, but the time will vary among cell types.

3. On the day of transfection, wash the cells in each well with 2 ml dilution medium at room temperature.

   Commercial medium that is specialized for lipid-mediated transfection (e.g., Opti-MEM I), without serum or antibiotics, gives the best results. However, other serum-free media may also be used.

4. Add 1.0 ml dilution medium to each of six 12 × 75–mm polystyrene tubes.

5. Add 0, 2, 4, 6, 8, or 12 µl cationic lipid reagent to each tube and mix or vortex briefly.

   DMRIE-C was found to give high-efficiency transfection of RNA in adherent cell lines (Ciccarone et al., 1995). It is a lipid suspension that may settle with time. To ensure that a homogenous sample is taken, mix thoroughly by inverting the tube 5 to 10 times before removing a sample for transfection.

6. Add 2.5 to 5.0 µg RNA to each tube and vortex briefly.

   mRNA that is capped and polyadenylated is translated more efficiently and is more stable within the cell.

7. Immediately add lipid-RNA complexes to washed cells and incubate 4 hr at 37°C in a 5% CO_2 incubator.

   The time of exposure of cells to lipid-RNA complexes, as well as the amount of RNA added to the cells, should be adjusted for each cell type.

8. Replace transfection medium with cell culture medium containing serum.

9. Allow cells to express the RNA for 16 to 24 hr and analyze them for expression of the transfected RNA as appropriate for the transgene used.

CATIONIC LIPID–MEDIATED TRANSFECTION OF ADHERENT *Sf*9 AND *Sf*21 INSECT CELLS WITH BACULOVIRUS DNA

BASIC PROTOCOL 4

As for transfecting mammalian cells (see Basic Protocol 1), cationic lipid reagent and nucleic acid are diluted separately into serum-free medium and then mixed and allowed to form complexes. Complexes are then diluted with fresh transfection medium and added to the cells for transfection. After the cells are fed and incubated, budded virus can be isolated from the medium.

Materials

Insect cells: *Sf*9 or *Sf*21 cells
Insect medium (e.g., Sf-900 II SFM, Life Technologies) with and without serum and antibiotics
Baculovirus DNA: purified DNA or bacmid DNA miniprep (Anderson et al., 1995)
Cationic lipid reagent (Table 20.6.1)

6-well tissue culture plate
27°C incubator
12 × 75–mm polystyrene tubes, sterile

Additional reagents and equipment for culturing insect cells and harvesting baculovirus from cell supernatants (*APPENDIX 3A*)

1. In each well of a 6-well tissue culture plate, seed ~9×10^5 insect cells in 2 ml insect medium without serum or antibiotics.

   Insect cells must be plated when they are in mid-log growth phase. Cells that have reached stationary phase transfect and infect at very low efficiency. Therefore, it is advisable to maintain a standard cell passage protocol that keeps the cells in log growth. For Sf9 or Sf21 cells adapted in Sf-900 II SFM, cells are passaged twice weekly to a density of 3×10^5 *cells/ml in suspension, and plated for transfection on the third day postseeding, when they are in mid-log phase. For other cell culture media and growth conditions, adjust conditions to maintain similar growth characteristics.*

   For culture of insect cells, use 50 units/ml penicillin and 50 µg/ml streptomycin (half the usual final concentration). For transfection, it is preferable to omit antibiotics from the medium to avoid toxicity.

2. Allow cells to attach at 27°C for ≥1 hr.
3. For each transfection, dilute 1 to 2 µg baculovirus DNA into 100 µl insect medium without serum or antibiotics in a 12 × 75–mm polystyrene tube.
4. For each transfection, dilute 1.5 to 9 µl cationic lipid reagent into 100 µl insect medium without serum or antibiotics in a separate 12 × 75–mm polystyrene tube.

   The suggested amount is 6 µl, but this should be optimized for each system.

   CellFectin gives high-efficiency transfection of DNA in insect cell lines (Anderson et al., 1995). It is a lipid suspension that may settle with time. To ensure that a homogenous sample is taken, mix thoroughly by inverting the tube 5 to 10 times before removing a sample for transfection.
5. Combine the two solutions, mix gently, and incubate at room temperature for 15 to 45 min to form lipid-DNA complexes.
6. For each transfection, add 0.8 ml insect medium without serum or antibiotics to each tube containing lipid-DNA complexes and mix gently.
7. Aspirate medium from cells and overlay diluted lipid-DNA complexes onto the washed cells.

   Alternatively, the medium on the cells can be replaced with 0.8 ml fresh insect medium and the undiluted complexes can be added directly to the fresh medium on the cells.
8. Incubate cells for 5 hr in a 27°C incubator. Protect plates from evaporation by putting them in a humidified container.
9. Remove transfection mixture and add 2 ml insect medium containing antibiotics and serum, if desired. Incubate cells in a 27°C incubator for 72 hr.
10. Harvest baculovirus from cell supernatants.

    Gene expression may also be evaluated in the cells after removal of virus-containing medium.

SUPPORT PROTOCOL

FINE TUNING OR OPTIMIZING CONDITIONS FOR CATIONIC LIPID REAGENT TRANSFECTIONS

This protocol provides an example of a simple one-step procedure for determining conditions conducive to high-efficiency transfections using cationic lipid reagents in any target cell type. A matrix of DNA and lipid reagent concentrations is used on transfections performed in a multiwell plate (Fig. 20.6.3). Once the best conditions have been determined, the transfections may be scaled up to larger vessels using the relative surface area (see Table 20.6.3) to increase the amounts of all reagents proportionately. This protocol can be modified for use with any transfection protocol.

Additional Materials *(also see Basic Protocol 1 and Alternate Protocol)*

24-well tissue culture plates
96-well round-bottom plates (sterile, with lid)

1. The day before transfection, trypsinize and count cells (*UNIT 1.1*). Plate cells in each well of a 24-well tissue culture plate using normal cell culture medium with serum, so that they are 50% to 95% confluent on the day of transfection. Avoid antibiotics at the time of plating and during transfection.

   The single most important factor in high-efficiency transfection is healthy, proliferating cell cultures. Antibiotics may cause some toxicity if present during transfection.

   In a 24-well plate, seeding 4×10^4 to 2×10^5 cells per well will usually give good plating density. Any type of plate may be used by scaling the reagent and cell amounts in proportion to the relative surface area (see Table 20.6.3).

2. Dilute DNA into dilution medium (appropriate for the lipid being optimized) without serum or antibiotics in four microcentrifuge tubes. Use a range of DNA concentrations, and use a volume of dilution medium that is 7× the appropriate protocol volume (see Basic Protocol 1, step 2, and Table 20.6.1). Mix gently after each addition.

   This makes enough DNA per tube for seven wells on a 24-well plate. Good ranges include 0.2 to 1.6 μg DNA per well.

   If the Plus enhancer is being used, include it in the diluted DNA tubes, using 10 μl Plus reagent per μg DNA. Add the Plus reagent to the diluted DNA after mixing well. If the Plus reagent is added first, precipitation may occur.

3. Dilute cationic lipid reagent into dilution medium without serum or antibiotics in six microcentrifuge tubes. Use a range of DNA concentrations, and use a volume of dilution medium that is 5× the appropriate protocol volume (see Basic Protocol 1, step 3, and Table 20.6.1). Mix gently after each addition.

   Be sure to observe timing that works best for the cationic lipid reagent being used.

   This makes enough diluted lipid per tube for five wells on a 24-well plate. Good ranges include 0.5 to 5 μl lipid reagent per well.

4. Pipet equal per-well volumes of diluted DNA and diluted cationic lipid reagent into the wells of a 96-well plate in a matrix corresponding to the wells on the 24-well plate (Fig. 20.6.3). Mix the complexes with the pipet tip by triturating. Cover the plate and incubate for 15 min at room temperature.

   Incubation times >15 min (up to an hour) work just as well, but be sure the complexes do not dry by covering them well.

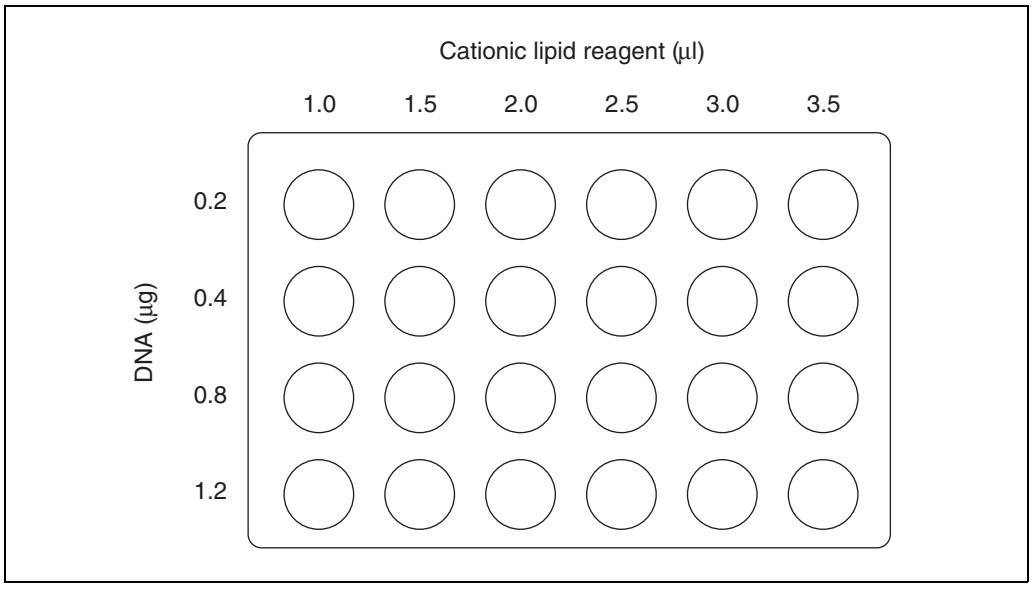


Figure 20.6.3 A sample matrix for fine tuning (optimizing) transfection reagent efficiencies using cationic lipid reagents.

5. While complexes are forming, replace medium on the cells with fresh transfection medium.

   See Basic Protocol 1, steps 5 and 6, for alternate procedures for combining complexes, medium, and cells. The medium can be the same as the serum-free dilution medium. It is possible to use serum in the transfection medium at this step. It is also possible to omit this step when using LipofectAmine 2000.

6. Add aliquots of DNA-lipid complexes (total volume from wells in step 4) to each well containing cells with fresh transfection medium. Mix complexes into the medium gently, holding the plate at a slant. Incubate at 37°C in 5% CO_2 for 5 hr.

   The exposure time may be >5 hr (up to overnight). Be sure there is sufficient medium to prevent the cells from drying out (it is not necessary to increase other components if this is done). If using the Plus enhancer, a 3-hr exposure is sufficient.

7. After 5 hr incubation, add cell culture medium to reach normal volume and add serum to bring the final concentration to that of normal cell culture medium.

   If necessary to maximize cell growth, replace the medium containing the complexes with fresh complete medium after 5 hr incubation. This step may be omitted entirely for some protocols.

8. Check expression as described (see Basic Protocol 1, steps 8a and 8b).

   If peak activity is found to occur on the edge of the matrix of concentrations tested, adjust the concentrations to include the observed peak at the center of a new matrix and repeat the transfection.

COMMENTARY

Background Information

There are currently at least eight companies that market cationic lipid–based transfection reagents. A partial listing of companies and products may be seen in Table 20.6.1. Many companies offer more than one type of reagent. Among the more popular ones are LipofectAmine 2000 and LipofectAmine Plus (Life Technologies), DOTAP and FuGENE 6 (Roche), and Effectene (Qiagen). Some of the structures are proprietary. The structures that are published can be classified into two general categories based on the number of positive charges in the lipid headgroup. The first cationic lipid (DOTMA) has a single positive charge per molecule and is used in Lipofectin (Life Technologies; Felgner et al., 1987). Several other cationic lipid–based transfection reagents such as DOTAP liposomal transfection reagent (Roche) and DMRIE-C (Life Technologies) also make use of singly charged cationic lipid molecules. Increasing the number of positive charges per cationic lipid molecule to as many as five improved transfection efficiency dramatically in most cell types. This can be seen in the examples of DOGS, the cationic lipid in Transfectam (Promega; Behr et al., 1989); DOSPA in LipofectAmine (Life Technologies; Hawley-Nelson et al., 1993); and TMTPS in CellFectin (Life Technologies; Anderson et al., 1995). Further increase in transfection efficiency can sometimes be achieved by precomplexing DNA with a proprietary enhancer. Two commercially available transfection kits with enhancers are LipofectAmine Plus (Life Technologies; Shih et al., 1997) and Effectene (Qiagen).

Life Technologies has designed cationic lipid reagents with specialized applications such as high-efficiency transfection of insect cells (see Basic Protocol 4) or delivery of RNA (see Basic Protocol 3). Lipofectin has high activity for endothelial cell transfection (Tilkins et al., 1994).

Basic Protocol 1 and the Alternate Protocol described in this unit are the procedures with the highest potential for efficient DNA transfection of adherent mammalian cells (Shih et al., 1997; Ciccarone et al., 1999). LipofectAmine 2000 has a simple protocol that yields the highest transfection efficiencies in many cell types. Using the enhancer reagent results in more reproducible transfections without extensive optimization because of the overall high activity. Prior to the availability of enhanced cationic liposome transfections (e.g., using

LipofectAmine 2000 and Effectene), the most effective procedure for transfection of adherent mammalian cells with DNA was with other polycationic reagents (e.g., LipofectAmine; Hawley-Nelson et al., 1993) following Basic Protocol 1. In order to achieve high-efficiency transfections with Basic Protocol 1, it is necessary to optimize lipid and DNA concentrations with the target cells at the desired plating density using a procedure similar to that described in the Support Protocol. Many cationic lipid reagents, as well as transfection reagents based on other chemistries, are available that can be used in Basic Protocol 1 for adherent mammalian cell DNA transfection, but they may yield lower efficiencies than the Alternate Protocol with the enhancer. Optimization using the Support Protocol is highly recommended when not using the enhancer, and the protocol can be modified for use with any cationic lipid reagent.

Critical Parameters

The most critical parameter for successful transfection is cell health. Cells should be proliferating as rapidly as possible at the time they are plated for transfection. On the day of transfection, mitoses should be abundant in healthy cultures. Fresh cultures with a finite life span should be used at the earliest possible passage.

For reproducible transfection results, it is critical to plate the same number of healthy cells for each transfection. Cells should always be counted, preferably using a hemacytometer and trypan blue (*UNIT 1.1*).

Although optimization is not required for high-efficiency transfection when using an enhancer (see Alternate Protocol), it is essential for success without the enhancer, and may improve efficiency even with the enhanced method.

The medium used to dilute and form complexes between the cationic liposomes and the DNA must not contain serum. Serum contains sulfated proteoglycans and other proteins, which compete with the DNA for binding to the cationic lipids. The medium should also not contain antibiotics. There is toxicity to the cells when cationic lipid reagents are used in the presence of antibiotics.

The dilution medium/plating medium for the cells may have some influence on transfection efficiency. Some proprietary serum-free media contain components that inhibit transfection and should be replaced with Opti-MEM I, DMEM, or other media without serum during transfection (Hawley-Nelson and Ciccarone, 1996).

Serum may be present in the medium on the cells during transfection. For most cationic lipid reagents, on most cell types, transfection activity is not inhibited in the presence of serum provided the complexes were formed in serum-free medium (Brunette et al., 1992; Ciccarone et al., 1993, 1999; Shih et al., 1997).

The specific serum-free medium used to dilute the lipid and DNA can have a slight effect on the efficiency of transfection. For the enhanced protocol (Alternate Protocol), normal culture medium such as DMEM is recommended. For the standard procedure (Basic Protocols 1, 2, and 3), Opti-MEM I medium works best. The improvements resulting from using the recommended media are less than two fold. When using Lipofectin, dilution in Opti-MEM I followed by a 30- to 45-min incubation is recommended (Ciccarone and Hawley-Nelson, 1995). With LipofectAmine 2000, the reverse is true: extended incubation (>30 min) of LipofectAmine 2000 in Opti-MEM prior to addition of DNA results in lower transfection activity (Ciccarone et al., 1999).

High-purity DNA will increase transfection efficiency. Miniprep DNA does work, however, when efficiency is not critical. A wide range of sizes of polynucleotides may be transfected, from 18-mer single-stranded oligonucleotides (Chiang et al., 1991; Bennett et al., 1992; Yeoman et al., 1992; Wagner et al., 1993) to 400-kb YAC DNAs (Lamb et al., 1993). Excess vortexing of complexes or DNA solutions may result in some shearing, especially with larger molecules. The concentration of EDTA in the diluted DNA should not exceed 0.3 mM.

Transgene expression may be increased in some cell types by inducing the promoter. This is observed in Jurkat cells when phytohemagglutinin and phorbol myristate acetate are added following transfection to activate the cytomegalovirus promoter (Schifferli and Ciccarone, 1996).

Troubleshooting

The most common complaints surrounding transfections include decreased transfection efficiency and low cell yield. Decreases in efficiency often result from changes in the target cell line. Cultured cell lines are usually aneuploid and often consist of a mixture of genotypes and phenotypes that can be subject to selection in the laboratory environment. Primary cultures, although usually genotypically uniform, often consist of a mixture of phenotypes from different tissues and can change their population characteristics in response to

their environment. Whenever a decrease in transfection efficiency is observed, the first thing to try is to work with a freshly thawed culture or isolate (Hawley-Nelson and Shih, 1995). Be sure the same number of cells is plated in each experiment, since plating density affects efficiency and peak position (Hawley-Nelson et al., 1993).

Low cell yield often results from the use of too much DNA or cationic lipid reagent. Use lower concentrations of these two components and examine the results for transfection efficiency as well as cell yield. Acceptable efficiency can usually be obtained with higher cell yield by using lower concentrations of lipid and DNA (Hawley-Nelson et al., 1993; Life Technologies, 1999).

Cell yield can also be improved in several other ways. (1) Increasing the plating density. This usually requires adjustment of lipid and DNA amounts, but often the transfection efficiency as well as the cell yield increases with higher plating input (Life Technologies, 1999). (2) Decreasing time of exposure of the cells to cationic lipid–DNA complexes. This can be done by increasing volume and adding back serum at earlier times or by removing the complexes from the cells at the end of transfection. (3) Performing the transfection in the presence of serum. Most cationic lipid reagents work

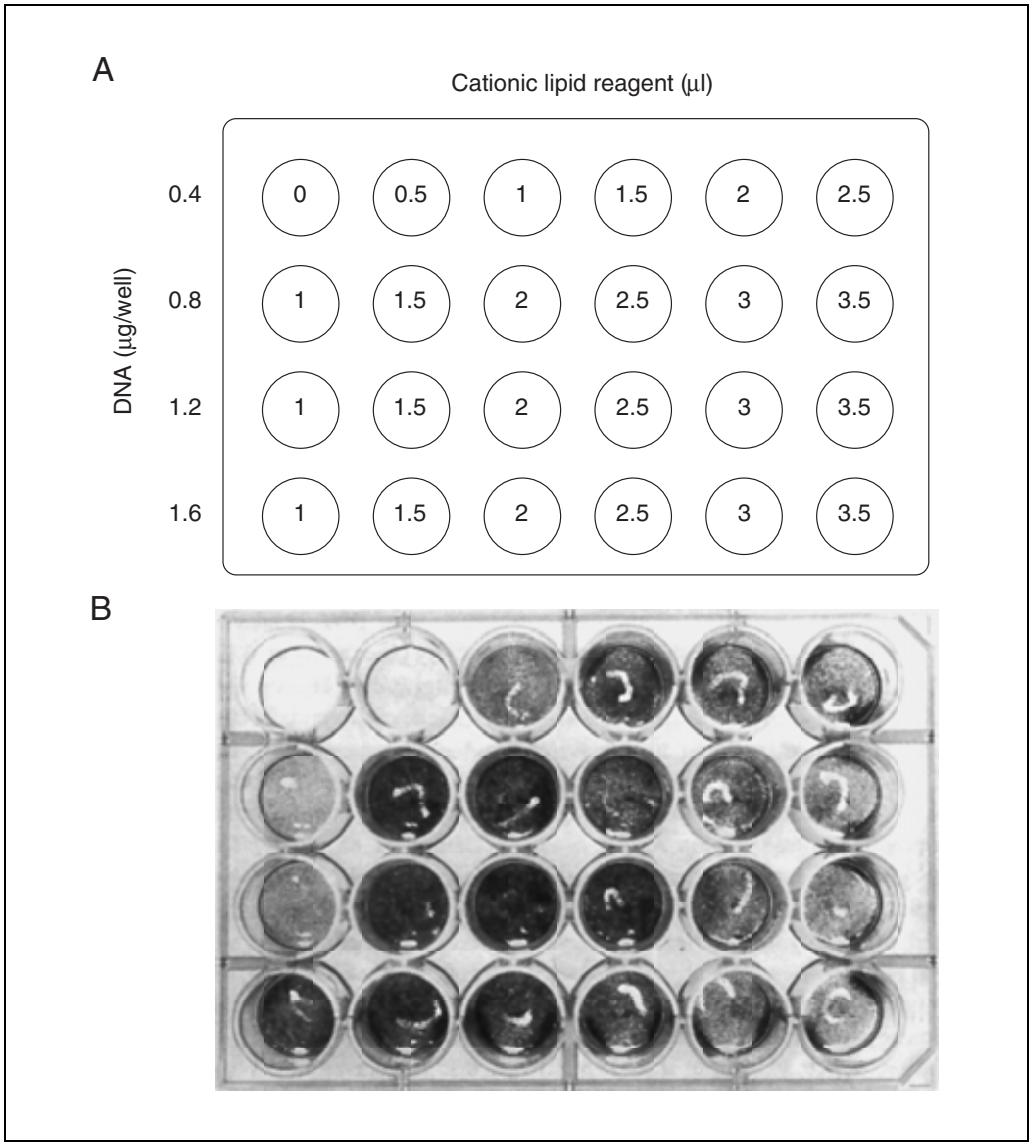


Figure 20.6.4 Results of fine-tuning or optimizing conditions for transfection. Before transfection, 293 H cells were plated at 2×10^5/well in a 24-well plate precoated with poly-D-lysine. The following day, cells were transfected with pCMV·SPORTβgal DNA using LipofectAmine 2000 as described (see Support Protocol). One day posttransfection, cells were fixed and stained with Xgal. (**A**) Amounts of DNA and lipid reagent used. (**B**) Results of Xgal staining.

Table 20.6.5 Activity for a Scaled-up Transfection Using LipofectAmine Plus in BHK-21 Cells

Plate	Surface area ratio to 24-well plate	Cells/well ($\times 10^4$)	DNA/well (μg)	Plus reagent (μl)	LipofectAmine reagent (μl)	β-gal (ng/cm^2)[a]
24-well	1	4	0.4	2	2	188 ± 5
12-well	2	8	0.8	4	4	193 ± 12
6-well	5	20	2	10	10	179 ± 27
60-mm	10	40	4	20	20	171 ± 16
100-mm	28	112	11.2	560	56	157

[a]Results are the mean of three transfections ± the standard deviation.

well in transfection medium containing serum (Brunette et al., 1992; Ciccarone et al., 1993, 1999; Shih et al., 1997). One exception is LipofectAmine without the Plus enhancer.

Some cationic lipid solutions are naturally cloudy. Sometimes cloudiness is observed when complexes are made with DNA. Usually this does not interfere with transfection efficiency. Most cationic liposome solutions (especially DMRIE-C and CellFectin) should be mixed gently by inversion just before use to produce a uniform suspension. With Plus reagent, it is possible to precipitate the DNA when the Plus reagent is diluted first into the DMEM and DNA is added second. Always dilute the DNA into DMEM and mix well before adding Plus reagent.

Anticipated Results

Transfection should be observed for most adherent mammalian cells transfected with DNA using Basic Protocol 1. Efficiencies vary with cell type. For example, 293, COS-7, and CHO-K1 cells yield 95% or more blue cells following Xgal staining of cells transfected with pCMVSPORTβgal plasmid DNA using LipofectAmine 2000. The authors have noted efficiencies of other cell types as high as 49% for SK-BR3 breast cancer cell lines, 77% for BE(2)C human neuroblastoma cells, and 43% for MDCK canine kidney cells (Ciccarone et al., 1999). Efficiencies also vary for suspension cells. The authors note that while LipofectAmine Plus is relatively inefficient for transfecting Jurkat cells, DMRIE-C can yield up to 85% blue cells following pCMV·SPORTβgal plasmid DNA transfection, gene activation

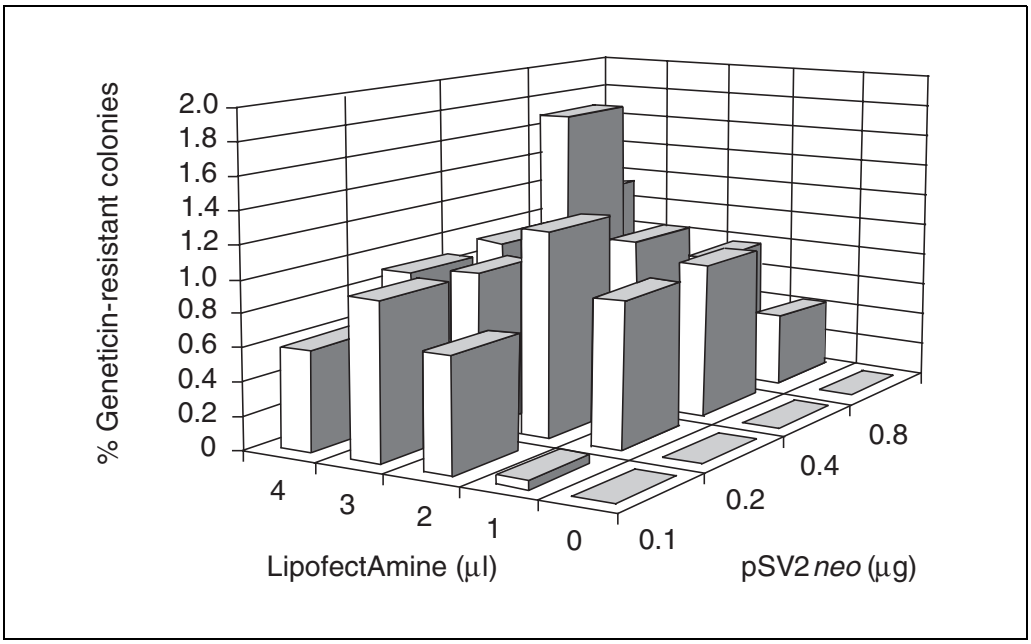


Figure 20.6.5 Stable transfection of NIH 3T3 cells. Cells were plated at 6×10^4 cells/well in 24-well plates. The day after plating, cells were transfected with LipofectAmine Plus complexed with pSV2*neo* DNA. The following day, cells were passaged at a total dilution of 1/150. Cells were exposed to 0.6 mg/ml Geneticin antibiotic from day 3 to day 13, and were then washed once with PBS and stained with 0.2% toluidine blue in PBS with 10% formalin.

with phytohemagglutinin and phorbol myristate acetate, and Xgal staining (Ciccarone et al., 1995, Schifferli and Ciccarone, 1996).

The result of a typical fine-tuning/optimization protocol is shown in Figure 20.6.4. The transfection reagent was LipofectAmine 2000, the DNA was pCMV·SPORTβgal, the cells were 293 H. Cells were stained the day following transfection and were allowed to stain overnight at 37°C. A selection of transfection conditions can be made.

Conditions found to be advantageous for transfection in small wells may be scaled up, the results of a typical scale-up are given in Table 20.6.5.

The results of a stable transfection of NIH 3T3 cells with pSV2*neo* DNA using an enhancer reagent (LipofectAmine Plus) are shown in Figure 20.6.5. The transfection was done on 24-well plates, the cells were passaged onto 6-well plates the following day, and selection with geneticin was done for 10 days. The figure shows optimization of conditions and the generally high efficiency that can be achieved using this method.

Time Considerations

Counting and plating the cells should be done the day before transfection and will usually require <1 hour. Transfection is usually done in the morning. Depending on the number of conditions being evaluated, it may require all morning plus a short period in the afternoon to increase medium volume or feed the cells. The total time required is <1 day.

Literature Cited

Anderson, D., Harris, R., Polayes, D., Ciccarone, V., Donahue, R., Gerard, G., Jessee, J., and Luckow, V. 1995. Rapid generation of recombinant baculovirus and expression of foreign genes using the Bac-to-Bac baculovirus expression system. *Focus* 17.2:53-58.

Behr, J.-P., Demeneix, B., Loeffler, J.-P., and Perez-Nutul, J. 1989. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. U.S.A.* 86:6982-6986.

Bennett, C.F., Chiang, M.-Y., Chan, H., Shoemaker, J.E.E., and Mirabelli, K. 1992. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* 41:1023.

Brunette, E., Stribling, R., and Debs, R. 1992. Lipofection does not require the removal of serum. *Nucl. Acids Res.* 20:1151.

Chiang, M.-Y., Chan, H., Zounes, M.A., Freier, S.M., Lima, W.F., and Bennett, C.F. 1991. Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J. Biol. Chem.* 266:18162-18171.

Ciccarone, C. and Hawley-Nelson, P. 1995. Lipofectin transfection activity increased by protocol improvement. *Focus* 17:103.

Ciccarone, V., Hawley-Nelson, P., and Jessee, J. 1993. Cationic liposome-mediated transfection: Effect of serum on expression and efficiency. *Focus* 15:80-83.

Ciccarone, V., Anderson, D., Lan, J., Schifferli, K., and Jessee, J. 1995. DMRIE-C reagent for transfection of suspension cells and for RNA transfections. *Focus* 17.3:84-87.

Ciccarone, V., Chu, Y., Schifferli, K., Pichet., J.-P., Hawley-Nelson, P., Evans, K., Roy, L., and Bennett, S. 1999. LipofectAmine 2000 Reagent for rapid, efficient transfection of eukaryotic cells. *Focus* 21.2:54-55.

Dube, S. 1997. Transfection using LipofectAmine Plus Reagent. *Focus* 19.3:57.

Felgner, P.L., Gadek, T.R., Holm, M. Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. 1987. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417.

Goldsborough, M.D., Evans, K., Xu, L., and Young, A. 1998. High purity plasmid DNA from anion exchange chromatography. *Focus* 20:68-69.

Hawley-Nelson, P. and Shih, P.-J. 1995. Sensitivity of transfection efficiency to culture age. *Focus* 17:62.

Hawley-Nelson, P. and Ciccarone, V. 1996. Transient transfection efficiency of human keratinocytes in two serum-free media. *Focus* 18.2:43-44.

Hawley-Nelson, P., Ciccarone, V., Gebeyehu, G., Jessee, J. and Felgner, P. 1993. LipofectAmine reagent: A new, higher efficiency polycationic liposome transfection reagent. *Focus* 15.3:73-79.

Lamb, B.T., Sisodia, W.W., Lawler, A.M., Slunt, H.H., Kitt, C.A., Kearns, W.G., Pearson, P.L., Price, D.L., and Gearhart, J.D. 1993. Introduction of the 400 kilobase precursor amyloid protein gene in transgenic mice. *Nature Genet.* 5:22-30.

Life Technologies. 1999. Guide to eukaryotic transfections with cationic lipid reagents, 2nd ed. Life Technologies, Inc., Rockville, Md.

Rose, J.K., Buonocore, L., and Whitt, M. 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *BioTechniques* 10:520-525.

Schifferli, K.P. and Ciccarone, V. 1996. Optimization of cationic lipid reagent-mediated transfection for suspension cell lines. *Focus* 18:45-47.

Sells, M.A., Li., J., and Chernoff, J. 1995. Delivery of proteins into cells using cationic liposomes. *BioTechniques* 19:72-78.

Shih, P.J., Evans, K., Schifferli, K., Ciccarone, V., Lichaa, F., Masoud, M., Lan, J., and Hawley-

Nelson, P. 1997. High efficiency transfection with minimal optimization using the Lipofect-Amine Plus Reagent. *Focus* 19.3:52-56.

Tilkins, M.L., Hawley-Nelson, P., and Battista, P. 1994. Transient transfection of endothelial cells. *Focus* 16.4:117-119.

Wagner, R.W., Matteucci, M.D., Lewis, J.G., Gutierrez, A.J., Moulds, C., and Froehler, B.C. 1993. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science* 260:1510.

Yeoman, L.C., Danels, Y.J., and Lynch, M.J. 1992. Lipofec'tin enhances cellular uptake of antisense DNA while inhibiting tumor cell growth. *Antisense Res. Dev.* 2:51.

Key References

Kriegler, M. 1990. Gene Transfer and Expression: A Laboratory Manual. Stockton Press, New York.

Good general review on gene expression.

Life Technologies, 1999. See above.

Describes history and gives protocols and considerations for cationic lipid reagent transfections.

Tilkins, M.L., Hawley-Nelson, P., and Ciccarone, V. 1998. Transfection of mammalian and invertebrate cells using cationic lipids. *In* Cell Biology: A Laboratory Handbook, Vol. 4, 2nd ed. (J.E. Celis, ed.) pp. 145-154. Academic Press, New York.

Review of cationic lipid transfection procedures.

Felgner et al., 1987. See above.

The original description of cationic lipid transfection.

Contributed by Pamela Hawley-Nelson
and Valentina Ciccarone
Life Technologies, Inc.
Rockville, Maryland

Optimization of Transfection

When embarking upon any transfection procedure, a critical first step is to optimize conditions. Every mammalian cell type has a characteristic set of requirements for optimal introduction of foreign DNA; there is a tremendous degree of variability in the transfection conditions that work, even among cell types that are very similar to one another. Often, an experimenter must screen a wide variety of cell types for a desired regulatory trait, such as an appropriate response to a particular effector molecule. It is thus helpful to have a straightforward, systematic approach to optimizing transfection efficiency. Transient assay systems are particularly useful for this purpose. A fusion gene that is known to function in mammalian cells can be transfected into cells under a variety of conditions, and transfection efficiency can be easily monitored by assaying for the fusion gene product. The human growth hormone (hGH) assay system is particularly useful for this purpose because both harvest and assay take very little time. However, any reporter system can be used to optimize transfection efficiency.

The single most important factor in optimizing transfection efficiency is selecting the proper transfection protocol. This usually comes down to a choice among calcium phosphate–mediated gene transfer (*UNIT 20.3*), DEAE-dextran-mediated gene transfer (*UNIT 20.4*), electroporation (*UNIT 20.5*), and liposome-mediated transfection (*UNIT 20.6*). Fusion techniques such as protoplast fusion and microinjection may also be considered. Cells are variable with respect to which transfection protocol is most efficient. It is recommended that any adherent cell line under investigation be tested for transfection ability with DEAE-dextran, calcium phosphate, and liposome-mediated transfection. Nonadherent cell lines can be transfected by electroporation and liposome-mediated transfection. Generally, if a cell can be grown in culture, it can be transfected.

CALCIUM PHOSPHATE TRANSFECTION

The primary factors that influence efficiency of calcium phosphate transfection (*UNIT 20.3*) are the amount of DNA in the precipitate, the length of time the precipitate is left on the cell, and the use and duration of glycerol or DMSO shock. A calcium phosphate optimization is shown in Table 20.7.1. Generally, higher concentrations of DNA (10 to 50 μg) are used in calcium phosphate transfection. Total DNA concentration in the precipitate can have a dramatic effect on efficiency of uptake of DNA with calcium phosphate–mediated transfection. With some cell lines, more than 10 to 15 μg of DNA

Table 20.7.1 Optimization of Calcium Phosphate Transfection

Dish (10-cm)	pXGH5 (μg)	pUC13 (μg)	Exposure to precipitate (hr)	Glycerol shock (min)
1	5	5	6	—
2	5	15	6	—
3	5	35	6	—
4	5	5	16	—
5	5	15	16	—
6	5	35	16	—
7	5	5	6	3
8	5	15	6	3
9	5	35	6	3
10	5	5	16	3
11	5	15	16	3
12	5	35	16	3

Contributed by John K. Rose

Current Protocols in Cell Biology (2003) 20.7.1-20.7.4

added to a 10-cm dish results in excessive cell death and very little uptake of DNA. With other cell types, such as primary cells, a high concentration of DNA in the precipitate is necessary to get any DNA at all into the cell on a routine basis. For example, with human foreskin fibroblasts, transfection of 5 μg of a reporter plasmid with 5 μg of carrier DNA (e.g., pUC13) gives significantly less expression than does transfection of 5 μg of reporter plasmid with 35 μg of carrier DNA. Presumably, this is because the amount of DNA affects the nature of the precipitate and thus alters the fraction of the applied DNA that is taken up into cells.

The optimal length of time that the precipitate is left on cells varies with cell type. Some cell types, such as HeLa or BALB/c 3T3, are efficiently transfected by leaving the precipitate on for 16 hr. Other cell types cannot survive this length of exposure to the precipitate. Transfection efficiency of some cell types, such as CHO DUKX BII, is dramatically increased by glycerol or DMSO shock (*UNIT 20.3*). The pilot experiment listed will indicate whether the cell type is tolerant to long exposure to a calcium phosphate precipitate and whether glycerol shock should be used. Once the results of this experiment are in hand, finer experiments can be done to further optimize conditions. For example, if shocking with 10% glycerol for 3 min enhances transfection efficiency, an experiment varying the time of glycerol shock or also trying 10% and 20% DMSO shock might be done.

Once optimal conditions for transfection are found, extensive DNA curves varying the amount of reporter plasmid should be prepared. The total amount of DNA should be kept constant at the optimal level determined in the first experiment. The amount of reporter plasmid DNA (e.g., pXGH5) should be varied, and carrier DNA (e.g., pUC13) should be used to make up the difference. This is to ensure that transfections are performed under conditions where the amount of reporter plasmid in the cell is not saturating the cellular transcription and translation machinery.

DEAE-DEXTRAN TRANSFECTION

There are several factors that can be varied in DEAE-dextran transfection (*UNIT 20.4*). The number of cells, concentration of DNA, and concentration of DEAE-dextran added to the dish are the most important to optimize. To a first approximation, most cell types that can be transfected using DEAE-dextran will have a preference for 1 to 10 μg DNA/10-cm dish and for 100 to 400 μg DEAE-dextran/ml of medium. Table 20.7.2 shows how the dishes in an optimization might be chosen. The 20-dish experiment consists of two sets of 10 dishes; one set is plated at 5×10^5 cells/dish, the other is plated at 2×10^6 cells/dish. Each set contains dishes that will be transfected with 1 to 10 μg of a reporter plasmid and

Table 20.7.2 Optimization of DEAE-Dextran Transfection

5×10^5 cells/10-cm dish:			2×10^6 cells/10-cm dish:		
Dish	pXGH5 (μg)	DEAE-dextran (μg/ml)	Dish	pXGH5 (μg)	DEAE-dextran (μg/ml)
1	1	400	11	1	400
2	1	200	12	1	200
3	1	100	13	1	100
4	4	400	14	4	400
5	4	200	15	4	200
6	4	100	16	4	100
7	10	400	17	10	400
8	10	200	18	10	200
9	10	100	19	10	100
10	0	200	20	0	200

100 to 400 μg/ml DEAE-dextran. If an hGH expression vector such as pXGH5 is used, a time course of expression under each condition can be determined by removing 100-μl aliquots of the medium 2, 4, and 7 days posttransfection (with a medium change after the day 4 aliquot is removed).

With the results of this pilot experiment in hand, a second experiment using a narrower range of DEAE-dextran concentrations and a wider range of DNA doses should be undertaken. For example, if the cells appear to express more hGH at 100 μg/ml DEAE-dextran than at higher concentrations in the pilot experiment, the second experiment should cover from 25 to 150 μg/ml DEAE-dextran. Because DEAE-dextran is toxic to some cells, a brief exposure to small concentrations may be optimal. The wide range of added DNA in this experiment is crucial in two respects. First, it is valuable to know the smallest amount of the transfected reporter gene that can give a readily detectable signal. Second, the linearity of the dose of DNA with the amount of reporter gene expression generally decays for large amounts of input DNA. When excessive (i.e., nonlinear) amounts of DNA are used in transfection experiments, it is possible that the effects observed are dose-response effects rather than the phenomenon intended for study. This serious and common problem can be eliminated by doing a careful DNA dose–response curve as above.

ELECTROPORATION

Perhaps because it is not a chemically based protocol, electroporation (*UNIT 20.5*) tends to be less affected by DNA concentration than either DEAE-dextran- or calcium phosphate–mediated gene transfer. Generally, DNA amounts in the range of 10 to 40 μg/10^7 cells work well, and there is a good linear correlation between the amount of DNA present and the amount taken up. The parameter that can be varied to optimize electroporation is the amplitude and length of the electric pulse, the latter being determined by the capacitance of the power source. The extent to which this can be varied is determined by the electronics of the power supply used to supply the pulse. The objective is to find a pulse that kills between 20% and 60% of the cells. This generally is in the range of 1.5 kV at 25 μF. If excessive cell death occurs, the length of the pulse can be lowered by lowering the capacitance. Settings between 3 and 25 μF can be tried.

LIPOSOME-MEDIATED TRANSFECTION

Three primary parameters—the concentrations of lipid and DNA and incubation time of the liposome-DNA complex—affect the success of DNA transfection by cationic liposomes (*UNIT 20.6*). These should be systematically examined to obtain optimal transfection frequencies.

Concentration of Lipid

In general, increasing the concentrations of lipid improves transfection of four cell lines examined (CV-1 and COS-7 with Lipofectin, and HeLa and BHK-21 with TransfectACE; see *UNIT 20.6*). However, at high levels (>100 μg), the lipid can be toxic. For each particular liposome mixture tested, it is important to vary the amount as indicated in Table 20.7.3.

Concentration of DNA

In many of the cell types tested, relatively small amounts of DNA are effectively taken up and expressed. In fact, higher levels of DNA can be inhibitory in some cell types with certain liposome preparations. In the optimization protocol outlined in Table 20.7.3, the standard reporter vector pSV2CAT is used; however, any plasmid DNA whose expression can be easily monitored would be suitable.

Table 20.7.3 Optimization of Liposome-Mediated Transfection

Dish (35-mm)	pSV2CAT (μg)	Liposomes (μl)	Dish (35-mm)	pSV2CAT (μg)	Liposomes (μl)
1	0.1	1	11	5	5
2	0.1	2	12	5	10
3	0.1	4	13	5	15
4	0.1	8	14	5	20
5	0.1	12	15	5	30
6	0.5	1	16	10	5
7	0.5	2	17	10	10
8	0.5	4	18	10	15
9	0.5	8	19	10	20
10	0.5	12	20	10	30

Time of Incubation

When the optimal amounts of lipid and DNA have been established, it is desirable to determine the length of time required for exposure of the liposome-DNA complex to the cells. In general, transfection efficiency increases with time of exposure to the liposome-DNA complex, although after 8 hr, toxic conditions can develop. HeLa or BHK-21 cells typically require ~3 hr incubation with the liposome-DNA complex for optimal tranfection, while CV-1 and COS-7 cells require 5 hr of exposure.

Contributed by John K. Rose
Yale University School of Medicine
New Haven, Connecticut

Inducible Gene Expression Using an Autoregulatory, Tetracycline-Controlled System

Tetracycline-regulated gene expression systems have been developed to overcome some of the obstacles encountered using other strategies for inducible gene expression in mammalian cells. These difficulties include pleiotropic, nonspecific effects or toxicity of inducing agents or treatments, and high uninduced background levels of expression. This unit describes protocols for using a modified tetracycline-regulated system in which a transcriptional transactivator drives expression of itself and a target gene in cultured cells and, to some extent, in transgenic mice. This transactivator (tTA) is a fusion protein consisting of the tetracycline-repressor of *E. coli* and the transcriptional activation domain of the VP16 protein of herpes simplex virus. In the absence of tetracycline, tTA binds to and activates genes preceded by a heptamerized version of the tetracycline-resistance operator of Tn*10* plus a minimal CMV promoter (here collectively referred to as Tet P). Binding of tTA to Tet P and subsequent gene activation are blocked in the presence of tetracycline. The plasmid pTet-Splice (Fig. 20.8.1A) contains Tet P upstream, and SV40 splice and polyadenylation signals downstream, of a multiple cloning site into which sequences encoding the open reading frame (ORF) of a target gene of choice is easily inserted. Autoregulatory tTA expression is driven from the plasmid pTet-tTAk (Fig. 20.8.1B), in which the tTA ORF (including an optimal sequence for initiation of translation according to Kozak) has been inserted into pTet-Splice.

The protocols in this unit describe the transfection of adherent cells and the testing of resultant clones for inducible transactivator or target gene protein expression. Stably transfected fibroblast cell lines expressing transactivator and target gene(s) can be derived by first cotransfecting pTet-tTAk and a plasmid encoding a selectable marker and obtaining stable lines with inducible transactivator expression (see Basic Protocol). These lines are subsequently stably cotransfected with plasmids encoding the target gene(s) and a second selectable marker. The procedure may also be used to cotransfect pTet-tTAk with the target gene–encoding plasmid(s) and a single selectable marker plasmid. The choice of method depends upon the feasibility of screening for the protein products of the target genes. While the consecutive method is more systematic, cotransfection may be faster given a relatively straightforward screening method for expression of the target gene (see Critical Parameters).

A Support Protocol also describes methods to test stably transfected cell lines for inducible gene expression, for transient transfection and induction of tet-regulated plasmids, and for detection of the tTAk gene in cells (or transgenic mice).

CALCIUM PHOSPHATE-MEDIATED STABLE TRANSFECTION OF NIH3T3 CELLS WITH pTET-tTAk AND TETRACYLINE-REGULATED TARGET PLASMIDS

BASIC PROTOCOL

This protocol describes the stable transfection of adherent cells with pTet-tTAk for the derivation of cell lines expressing inducible tTA. In the first round of transfection stable cell lines expressing inducible tTA alone are produced. The single transfection procedure may also be used for stable cotransfection of pTet-tTAk and plasmids expressing the target gene(s). In the second round of transfection tTA-expressing lines are transfected with plasmids expressing the target gene(s).

Contributed by Penny Shockett and David Schatz
Current Protocols in Cell Biology (2005) 20.8.1-20.8.10

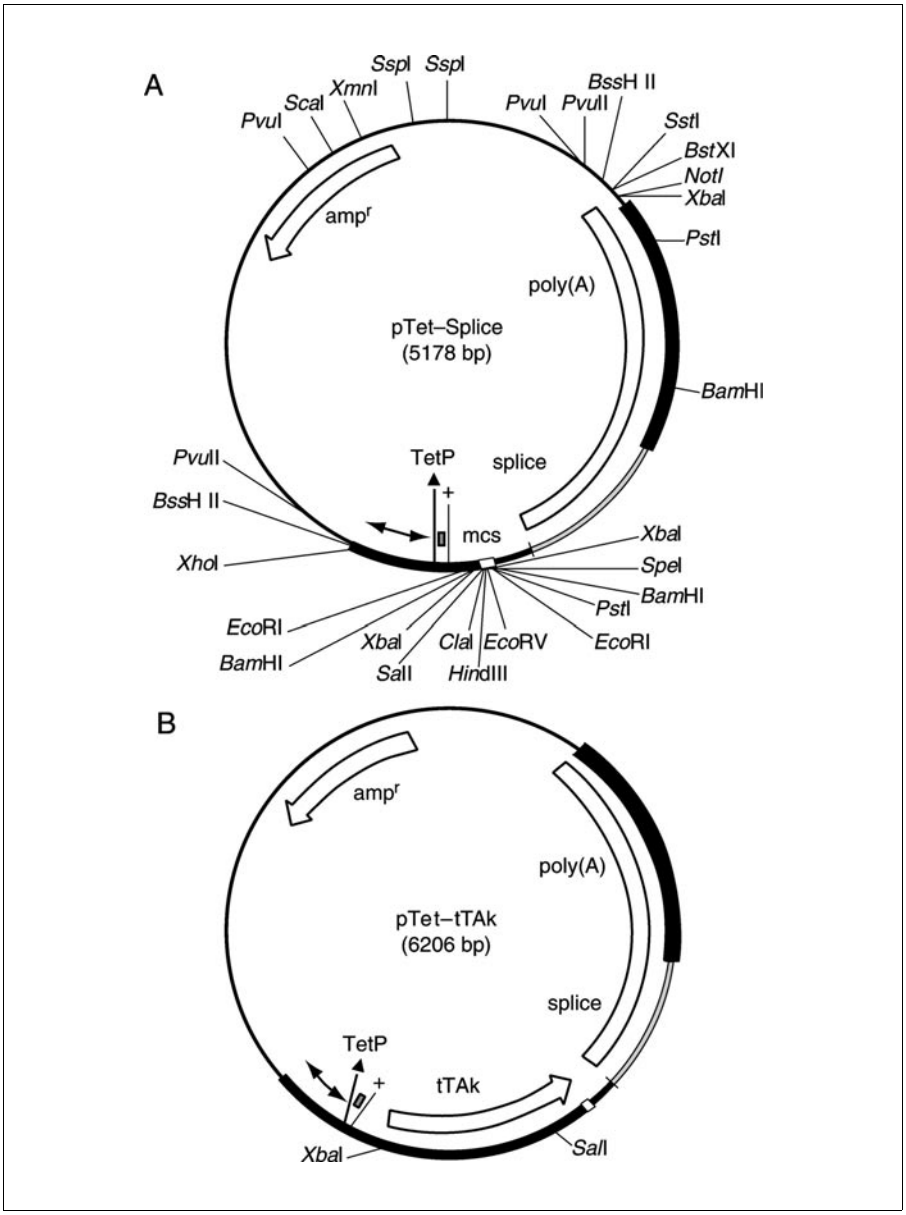


Figure 20.8.1 (**A**) The plasmid pTet-Splice (Shockett et al., 1995) is designed to drive tetracycline-regulated expression of a target gene inserted into the multiple cloning site (mcs). The tetracycline-regulated promoter (TetP) consists of a heptamerized tetracycline operator (double-ended arrow) upstream of a minimal human CMV promoter that includes bases −53 (triangle) to +75. The transcriptional start site (+) and TATAA box (small rectangle) are also indicated. This TetP fragment is an *Xho*I-*Sal*I fragment derived from pUHC13-3 (Gossen and Bujard, 1992). SV40-derived sequences downstream of the MCS drive mRNA splicing and polyadenylation. The backbone of the plasmid is from Bluescript II KS+ (Stratagene) and carries the ampicillin resistance gene (*amp*^{r}). (**B**) pTet-tTAk (Shockett et al., 1995) consists of the tTAk open reading frame inserted into the *Hin*dIII-*Eco*RV sites of pTet-Splice.

Materials

NIH3T3 cells
Complete DMEM-10 medium (see recipe)
Complete DMEM/tet: complete DMEM-10 medium (see recipe) containing 0.5 μg/ml tetracycline hydrochloride (Sigma; dilute 10 mg/ml stock in 70% ethanol and store protected from light at −20°C)
Selection medium (see recipe) containing 125 μM, 250 μM, or 500 μM L-histidinol
Plasmids for first-round or cotransfection procedure: pTet-tTAk (Life Technologies) and plasmids containing target gene ORF(s) cloned into pTet-Splice (Life Technologies), pSV2-His, or another selectable marker plasmid; purified by CsCl banding or anion-exchange chromatography
Plasmids for second round transfection procedure: plasmids containing target gene ORF(s) cloned into pTet-Splice, pPGKPuro, or another selectable marker plasmid; purified by CsCl banding or anion-exchange chromatography
2 M $CaCl_2$
HEPES-buffered saline (HeBS; see recipe)
10 mg/ml chloroquine (19 mM; optional; Sigma); dilute in water and store at −20°C
85% (v/v) HeBS/15% (v/v) glycerol, prewarmed to 37°C
3 mg/ml puromycin (Sigma) diluted in PBS (*APPENDIX 2A*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1× trypsin/EDTA (Invitrogen)
10-cm and 6-cm tissue culture plates
4-ml polystyrene tubes (Falcon)
24-well and 6-well tissue culture plates

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO_2 incubator.

Grow the cells

1a. *First round only:* Grow cells in complete DMEM-10 medium. The day before transfection split cells into 10-cm tissue culture plates in complete DMEM/tet to achieve one-third confluence on the day of the transfection.

From this point on cells are kept in the presence of 0.5 μg/ml tet.

One plate per transfection is needed at this stage. A typical experiment might include one plate for tTA only, one for tTA plus target gene, and one to serve as the untransfected control plate.

1b. *Second round only:* Grow stable cell lines that inducibly express autoregulatory tTA in selection medium/500 μM L-histidinol. The day before transfection split into 10-cm plates in this same medium to achieve one-third confluence on the day of transfection.

Transfect the cells

2. Linearize plasmids prior to transfection and adjust concentration to ≥0.5 mg/ml.

See Damke et al. (1995) for discussion of other selectable markers. All plasmids should be purified by CsCl banding (APPENDIX 3A) or on a Qiagen column.

3a. *First round only:* Mix 10 to 20 μg of pTet-tTAk (in the presence or absence of an equimolar amount of target gene plasmids) plus 1 to 2 μg pSV2-His (a molar ratio of ~10:1 of each tet plasmid to selectable marker plasmid) with 500 μl HeBS in a clear 4-ml polystyrene tube.

A control mock transfection should be performed with no DNA added to the transfection. All of these cells should die in the selection medium/125 μM L-histidinol introduced in step 14.

3b. *Second round only:* Mix 10 to 20 μg each of target gene plasmid(s) plus 1 to 2 μg pPGKPuro (a molar ratio of ~10:1 of each tet plasmid to selectable marker plasmid) with 500 μl HeBS in a clear 4-ml polystyrene tube.

A control mock transfection should be performed with no DNA added to the transfection. All of these cells should die in the presence of the puromycin introduced in step 14. The optimal killing concentration for puromycin (lowest dose between 0.1 μg/ml to 10 μg/ml that kills all untransfected cells within a few days) should be determined empirically prior to the transfection and varies with the cell type.

4. Add 32.5 μl of 2 M $CaCl_2$ to plasmid DNA and mix immediately by gentle vortexing. With occasional gentle mixing, allow precipitate to form for 15 to 30 min at room temperature or until solution is visibly cloudy when compared to a tube containing water.

5. Aspirate all of the medium from cells, doing one plate at a time. Mix precipitate a few times by pipetting with a Pasteur pipet, and apply dropwise and evenly over cells.

6. Incubate 30 min, gently rocking the plate after 15 min to ensure even coverage over entire plate.

7a. *First round only:* Add 10 ml complete DMEM/tet, with or without 25 μM chloroquine (final), to each plate.

Although the use of chloroquine may further reduce cell integrity during the glycerol shock (step 9), it can improve transfection efficiency.

7b. *Second round only:* Add 10 ml selection medium/500 μM L-histidinol, with or without 25 μM chloroquine (final), to each plate.

8. Incubate 4 to 5 hr.

The optimal length of incubation may vary for different cell types.

9. Gently aspirate medium from cells with minimal disruption of the precipitate that has settled onto the cells. Shock cells by adding dropwise 2.5 ml of prewarmed 85% HeBS/15% glycerol.

It is normal for the cells to look somewhat ragged before and especially after glycerol shock. Two to four plates may be shocked at one time, depending on the speed of the researcher.

10. Aspirate HeBS/glycerol after exactly 2.5 min. Work quickly, as glycerol can be very toxic to the cells.

The length of time cells are exposed to glycerol solution can be varied and increased up to 4 to 5 min to optimize transfection efficiency for different cell types. Cells should be shocked the maximal length of time which results in the least cell death.

11a. *First round only:* Immediately, gently, and quickly wash cells twice by adding 10 ml complete DMEM/tet and immediately aspirating.

Because cells tend to come loose from the plate after glycerol addition, add all medium to a single spot on the plate.

11b. *Second round only:* Immediately, gently, and quickly wash cells twice by adding 10 ml selection medium/500 μM L-histidinol and immediately aspirating.

Again, add medium to a single spot on the plate to avoid loosening the cells.

12a. *First round only:* Add 10 ml complete DMEM/tet. Incubate cells overnight.

12b. *Second round only:* Add 10 ml selection medium/500 μM L-histidinol. Incubate cells overnight.

13a. *First round only:* The morning after the transfection, aspirate the medium and replace with 10 ml complete DMEM/tet. Continue incubation.

13b. *Second round only:* The morning after the transfection, aspirate the medium and replace with 10 ml selection medium/500 μM L-histidinol. Continue incubation.

Select and clone transfected cells

14a. *First round only:* At 48 hr posttransfection, split cells into selection medium/125 μM L-histidinol at several dilutions ranging from 3×10^4 to 1×10^6 cells per 10-cm plate. Make more than one plate in the mid-range that corresponds to an approximate split from one confluent plate of 1:16 to 1:32.

14b. *Second round only:* At 48 hr posttransfection, split cells as above, using selection medium/500 μM L-histidinol containing 3 μg/ml puromycin (final).

The optimal killing concentration for puromycin (lowest dose between 0.1 μg/ml to 10 μg/ml that kills all untransfected cells within a few days) should be determined empirically prior to the transfection and varies with the cell type. The concentration of 3 μg/ml puromycin is sufficient for selection of transfected NIH3T3 cells.

15a. *First round only:* Refeed cells 4 days later with selection medium/125 μM L-histidinol. When colonies have formed, increase the concentration of L-histidinol in the selection medium to 250 μM.

L-histidinol is normally toxic to cells. The concentration of L-histidinol in the selection medium is therefore kept low initially and is raised as the number of cells expressing pSV_2-His at high levels reaches a critical mass.

15b. *Second round only:* Refeed cells 4 days later with selection medium/500 μM L-histidinol/puromycin.

16. When colonies are well established (at about day 12 to 14 of selection), circle their borders with a marker. Aspirate medium from plate and place a plastic cloning ring (autoclaved upright in vacuum grease) on the plate to surround an individual clone. Wash clones quickly with ~100 μl PBS and add 2 drops of trypsin (~100 μl) for 30 sec to 1 min.

Pick cells from plates on which individual colonies are moderately spaced and can easily be distinguished.

17a. *First round only:* Loosen cells by pipetting up and down with a Pasteur pipet and transfer colonies to wells of a 24-well plate into 1 ml selection medium/250 μM L-histidinol.

17b. *Second round only:* Loosen cells as for first round, transferring them into 1 ml selection medium/500 μM L-histidinol/puromycin.

18a. *First round only:* When cells are heavy in wells, split into 6-cm dishes in selection medium/500 μM L-histidinol.

18b. *Second-round only:* When cells are heavy in wells, split into 6-cm dishes in selection medium/500 μM L-histidinol/puromycin.

All trypsinization is performed by standard methods (UNIT 1.1), involving a quick PBS wash, a 1 to 3 min trypsin/EDTA incubation (2 ml per confluent 10-cm plate), and using 3rd selection medium/500 μM L-histidinol (± puromycin) and containing 10% calf serum to dilute and stop the trypsin.

19a. *First round only:* Expand cells for testing in selection medium/500 μM L-histidinol. Freeze aliquots of cells for storage in liquid nitrogen and grow in selection medium/500 μM L-histidinol from this point on. Test for tTA or target gene expression (if applicable; see Support Protocol for methods that may be used). Or, if applicable, repeat transfection procedure with target gene plasmid(s), following steps 1 to 18 and using the options listed for second-round transfection.

19b. *Second round only:* Test for target gene expression by northern or immunoblotting (*UNIT 6.2*) after induction (see Support Protocol). Freeze aliquots for storage in liquid nitrogen and grow in selection medium/500 μM L-histidinol/puromycin from this point on.

SUPPORT PROTOCOL

ANALYSIS OF TARGET GENE PROTEIN EXPRESSION

This protocol outlines methods for the analysis of target gene expression and inducibility. Instructions for inducing stable cell lines, for examining transient target gene expression with and without induction, and for PCR amplification of the tTA gene are included, with references to detection procedures such as Southern, northern, and immunoblotting (*UNIT 6.2*) techniques.

Induction of Stable Cell Lines

Stable cell lines can be tested for tTA or target gene expression by comparing induced to uninduced cells for tTA mRNA or target gene mRNA (see Detection of tTA Transgene in Cellular or Tail DNA by Southern Blotting), or protein expression or protein activity. Multiple lines may be screened at a time.

The night before induction, the cells are plated in selection medium/500 μM L-histidinol (see recipe) containing 3 μg/ml puromycin at an appropriate density such that cells will be subconfluent to confluent at the time of harvest. Cells are washed three times with PBS (*APPENDIX 2A*), with gentle swirling. Immediately, the medium is replaced with selection medium *without* 0.5 μg/ml tetracycline hydrochloride (tet). (For tet^+ controls, simply aspirate medium and replace with fresh selection medium containing tet.) Cells are incubated 6 to 48 hr in a humidified 37°C, 5% CO_2 incubator, then trypsinized (*UNIT 1.1*) and harvested at 4°C, and an aliquot of 0.15–0.4 $\times$ 10^6 cells is analyzed by immunoblotting (see *UNIT 6.2*).

Alternatively, cells may be grown in selection medium in the presence of tet, transferred to tubes [with a quick wash with cold PBS followed by trypsinization (*UNIT 1.1*) and stopping of the trypsin by addition of selection medium containing tet], washed three times with PBS (or just pelleted, for tet^+ controls), and replated into selection medium with and without tet at an appropriate density such that the cells will be subconfluent to confluent at the time of harvest.

Induction of Gene Expression in Transiently Transfected Cells

Transient transfection of tet-regulated plasmids is useful in several situations, including the initial testing of the autoregulatory system in a given cell line, screening stable tTA expressors for inducible expression, and biological applications where transient expression is specifically desired.

The night before the transfection, cells are split into medium containing 0.5 μg/ml tetracycline hydrochloride; the following day they are then transfected by methods appropriate for the cells being used (*UNITS 20.3–20.6*). Cells are induced by washing them three times in medium without tet. For $CaPO_4$ transfection, washes are incorporated into those normally performed after glycerol shock (see Basic Protocol, step 11). Uninduced cell controls are

washed with medium containing tet. Medium with and without tet is added to the appropriate plates, then the cells are incubated for 12 to 48 hr in a humidified 37°C, 5% CO_2 incubator. The cells are harvested at 4°C and, if trypsinized (*UNIT 1.1*), cold medium containing 10% FBS (with and without tet, as appropriate) is used to stop the action of the trypsin. Cells are pelleted for freezing or lysis, and tTA or target gene (experimental or reporter) expression can be analyzed by northern blotting, immunoblotting (*UNIT 6.2*), or by an appropriate activity assay (see Commentary).

Detection of tTA Transgene in Cellular or Tail DNA by PCR

PCR is routinely used to detect the Tet-tTAk transgene in candidate transgenic mouse tail DNA. The forward primer derives from the minimal human CMV promoter, CMV-F1:

5′-TGACCTCCATAGAAGACACC-3′

The reverse primer, TTA-REV1, is specific for the tTA ORF:

5′-ATCTCAATGGCTAAGGCGTC-3′

Hot-start PCR (*APPENDIX 3F*) is performed on 150 ng of each tail DNA to be analyzed in a reaction mix containing 1.5 mM $MgCl_2$, 0.5 μM each primer, and 0.2 mM each dNTP. PCR cycling conditions are as follows:

1 cycle:	3 min	94°C	
		80°C	(pause) add *Taq* polymerase
30 cycles:	45 sec	94°C	(denaturation)
	45 sec	58°C	(annealing)
	90 sec	72°C	(extension)
1 cycle:	10 min	72°C	(extension)
		80°C	(end).

Products are analyzed on a 1% to 1.3% agarose gel; the main product of interest is visible as a 290-bp band after ethidium bromide staining.

Detection of tTA Transgene in Cellular or Tail DNA by Southern Blotting

The tTA transgene may also be detected by Southern blot analysis (*APPENDIX 3A*). Tail DNA is digested with *Eco*RI and blots are probed with a 761-bp *Xba*I-*Sal*I tTA insert from pTet-tTA. This fragment detects a 1094-bp tTA fragment of the transgene. This probe may also be used to detect tTA mRNA by northern blotting (*APPENDIX 3A*).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete DMEM-10

Dulbecco's minimal essential medium containing:
10% (v/v) donor bovine calf serum (JRH Biosciences)
100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen)
2 mM glutamine (Invitrogen)

All DMEM complete medium used in this unit (with or without selection reagents or 0.5 μg/ml tetracycline hydrochloride) may be stored protected from light ~1 month at 4°C).

Fetal bovine serum (FBS) may also be used in place of donor bovine calf serum, but the latter is less expensive.

HEPES-buffered saline (HBS)

6 mM dextrose
137 mM NaCl
5 mM KCl
0.7 mM $Na_2HPO_4 \cdot 7H_2O$
21 mM HEPES (free acid)
Adjust final pH to 7.05 with NaOH
Filter sterilize and store in aliquots at −20°C

Selection medium

Complete histidine-free DMEM (Irvine Scientific, purchased without glutamine), containing:
10% (v/v) donor bovine calf serum (JRH Biosciences)
100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen)
2 mM glutamine (Invitrogen)
0.5 μg/ml tetracycline·HCl (Sigma; dilute 10 mg/ml stock in 70% ethanol and store protected from light at −20°C)
125 μM, 250 μM, or 500 μM L-histidinol (Sigma, dilute in water as a 125 mM stock and store at −20°C)

COMMENTARY

Background Information

Inducible, tetracycline-regulated gene expression systems were initially developed to allow the controlled expression in eukaryotic cells of foreign genes not tolerated constitutively in cultured cells or during the development of transgenic animals. The general features of tetracycline-regulated gene expression strategies and their improvements over previous inducible expression systems have been addressed in current review articles (Gossen et al., 1993; Barinaga, 1994; Damke et al., 1995; Shockett and Schatz, 1996). The autoregulatory tTA system used in this protocol derives directly from a constitutive tTA system described by Gossen and Bujard (1992). Although tight regulatory control and high inducibility was achieved with the original system in HeLa cells, the inability to detect clones expressing moderate to high levels of tTA by immunoblotting suggested that the tTA was toxic when expressed constitutively. The autoregulatory tTA system was designed to overcome possible toxic effects of constitutive tTA expression by making tTA expression itself tetracycline regulated. Autoregulated tTA expression theoretically allows for the selection of clones expressing higher levels of tTA via an autoregulatory feed-forward mechanism that is activated only in the absence of tetracycline. In the presence of tetracycline, low-level tTA and target gene expression are driven from the minimal human CMV promoter. However, any tTA produced is unable to bind to tet operators upstream of the tTA or target gene. Conversely, when tetracycline is removed from the system, the small amounts of tTA protein expressed from the minimal promoter can bind the *tet* operators upstream of the tTA gene, driving higher levels of tTA (for controlled periods of time) and, subsequently, target gene expression.

The theoretical benefits of the autoregulatory tTA system have been confirmed by experiments in stably transfected NIH3T3 cell lines (Shockett et al., 1995). In these experiments, expression of the recombination activating genes *RAG-1* and *RAG-2*, and subsequent DNA recombination activated by these proteins, was higher and more frequently detected among stable transfectants expressing autoregulatory tTA than in constitutive tTA expressors. In transgenic mice expressing a luciferase reporter target transgene, the levels of expression appear to be 1 to 2 orders of magnitude greater with the autoregulatory system, although the uninduced levels also appear to be higher. Several studies have successfully used the autoregulatory tTA system for regulated gene expression in cell lines or transgenic animals (for examples, see Sheehy and Schlissel, 1999; Sikes et al., 1999; Chen et al., 2002; Shockett et al., 2004).

Since the description of the early tTA systems, several laboratories have created modified vectors, including streamlined versions containing both tTA and the target gene, viral vectors, and vectors in which

expression of two different target genes may be differentially or co-regulated. Some of these systems and their applications have recently been reviewed (Shockett and Schatz, 1996; Blau and Rossi, 1999).

Critical Parameters and Troubleshooting

Cell lines stably expressing both autoregulatory tTA and target genes have been derived at fairly high efficiencies by simultaneous transfection of all plasmids. This method may be faster, but it may require the screening of more clones than if stable lines with low basal and high induced levels of tTA are first derived and subsequently transfected with plasmids encoding the target genes. For the derivation of these clones, any selectable marker combination should theoretically work for consecutive cotransfection. Additionally, although the Basic Protocol describes calcium phosphate–mediated transfection of adherent fibroblast cell lines, the procedure can be adapted for other cell types using their optimal methods of transfection and selection. The protocol can also be scaled down to require fewer cells by using smaller dishes or wells and reducing all components proportionately.

Using the autoregulatory tTA system, tTA mRNA induction appears to be a good indicator of induced tTA expression (see Support Protocol). Alternatively, the vector pUHC13-3 (Life Technologies) encoding luciferase under tet control may be transiently transfected into putative stable tTA expressors as previously described (see Support Protocol and Damke et al., 1995). Cells are then cultured for 12 to 48 hr in the presence and absence of tetracycline. Luciferase activity is easily measured in cell lysates using a kit (Luciferase Assay System and Dual-Luciferase Reporter Assay System; Promega) in which luciferase activity in cell lysates is normalized either to total protein determined using a Bradford protein assay (*APPENDIX 3H*), or to a transfection control, respectively. Although basal expression of target plasmids tends to be higher when transiently transfected and luciferase detection is extremely sensitive, this method can be useful for the initial testing of the system in a given cell type (Damke et al., 1995).

It is imperative after stable transfection with pTet-tTAk that cells be maintained in medium containing 0.5 μg/ml tetracycline to prevent any toxic effects of tTA expression and subsequent selection against clones expressing high levels of tTA.

Anticipated Results

In the authors' experience with stably transfected NIH3T3 cells, expression of induced tTA and target gene has been observed by 6 hr and peaks at ~12 hr after induction. In cells that stably express tTA, transient target gene expression has been observed by 12 hr. In cells transiently expressing tTA and a tet-sensitive luciferase reporter (pUHC13-3), luciferase activity induced by 2 orders of magnitude has been observed by 20 hr.

Time Considerations

Starting with the plasmid vectors and following the transfection protocols above, stable clones expressing tTA (or tTA + target gene(s) if cotransfecting) are obtained in ~12 to 14 days. Approximately 2 additional weeks are required for expansion and testing of candidate clones. Subsequent transfection of a stable inducible tTA clone with vectors expressing target genes will require the same amount of time. Transient transfection and inducible gene expression may be achieved within 48 hr.

Literature Cited

Barinaga, M. 1994. Researchers devise a master gene control switch. *Science* 265:26-28.

Blau, H.M. and Rossi, F.M.V. 1999. Tet B or not tet B: Advances in tetracycline-inducible gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 96: 797-799.

Chen, J., Kelz, M.B., Zeng, G., Steffen, C., Shockett, P.E., Terwilliger, G., Schatz, D.G., and Nestler, E.J. 2002. Inducible, reversible hair loss in transgenic mice. *Transgenic Res.* 11: 241–247.

Damke, H., Gossen, M., Freundlieb, S., Bujard, H., and Schmid, S.L. 1995. Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Methods Enzymol.* 257: 209-220.

Gossen, M. and Bujard, H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* 89:5547-5551.

Gossen, M., Bonin, A.L., and Bujard, H. 1993. Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem. Sci.* 18:471-475.

Sheehy, A.M. and Schlissel, M.S. 1999. Overexpression of RelA causes G_1 arrest and apoptosis in a Pro-B cell line. *J. Biol. Chem.* 274:8708–8716.

Shockett, P.E. and Schatz, D.G. 1996. Commentary: Diverse strategies for tetracycline-regulated inducible gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 93:5173-5176.

Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. 1995. A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 92:6522-6526.

Shockett, P.E., Zhou, S., Hong, X., and Schatz, D.G. 2004. Partial reconstitution of V(D)J rearrangement and lymphocyte development in RAG-deficient mice expressing inducible, tetracycline-regulated RAG transgenes. *Mol. Immunol.* 40:813–829.

Sikes, M.L., Suarez, C.C., and Oltz, E.M. 1999. Regulation of V(D)J recombination by transcriptional promoters. *Mol. Cell Biol.* 19:2773–2781.

Contributed by Penny Shockett
Southeastern Louisiana University
Hammond, Louisiana

David Schatz
Howard Hughes Medical Institute and
Yale University School of Medicine
New Haven, Connecticut

CHAPTER 21
Fluorescent Protein Technology

INTRODUCTION

The development of fluorescent proteins as molecular tags over the past decade has provided an essential new tool for understanding biological systems by allowing proteins to be visualized in living cells. The fluorescent proteins can be fused to a protein of interest and then expressed within cells to visualize, track, and quantify the behavior of a protein, providing fundamentally new insights into protein function and cellular processes in the complex environment of the cell. The fluorescent protein that has been most widely used in this regard is green fluoresent protein (GFP) from the jellyfish *Aequorea victoria*. GFP is a β-barrel-shaped protein of ~27 kDa that contains an amino acid triplet (Ser-Tyr-Gly) located in its center that functions as a fluorophore. Molecular engineering of the GFP coding sequence has resulted in optimized expression of GFP in different cell types, as well as the generation of GFP variants with optimal spectral properties, including increased brightness, relative resistance to the effects of pH, and photostability. When expressed within cells, GFP fusion proteins are bright and they are resistant at low illumination to photobleaching, which is the photo-induced alteration of a fluorophore that abolishes its fluorescence. These characteristics allow GFP chimeras expressed within cells to be imaged with low illumination levels over many hours, providing a simple method for visualizing the dynamics and behavior of the tagged protein.

Time-lapse observations of fluorescently tagged proteins can provide information about the steady-state distribution of the protein over time, but they usually do not reveal a protein's kinetic properties. These properties include whether a protein undergoes free or restricted diffusional motion, how rapidly it binds to and releases from other molecules, and whether it undergoes directed movement between compartments. One way to obtain this type of information with fluorescently tagged proteins is to utilize the technique of photobleaching. In this approach, a selected pool of fluorescent proteins is photobleached with a high-intensity laser pulse and the movement of unbleached molecules from neighboring areas into the bleached area is recorded by time-lapse microscopy. Analysis of the observed recovery kinetics can then be used to determine the diffusion coefficient of the fluorescent protein, its binding properties, or its transport routes. *UNIT 21.1* describes the use of fluorescent proteins in photobleaching techniques. Several specific applications of photobleaching are detailed, including fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), and selective photobleaching. The unit also describes how to extract kinetic parameters from the photobleaching data, including a protein's diffusion coefficient and its $t_{1/2}$ and mobile fraction. Use of these techniques provides a powerful method for characterizing the movement of proteins and their interactions with cellular components in the environment of the living cell.

UNIT 21.2 describes the technique known as fluorescence localization after photobleaching (FLAP), which allows localized photolabeling of proteins and subsequent tracking of these molecules in living cells. This technique, like FRAP and FLIP described in *UNIT 21.1*, overcomes the limitations of conventional fluorescence localization by revealing the relocation of molecules during dynamic, steady-state processes such as diffusion and polymerization/depolymerization. The principle underlying FLAP is simple and relies

Current Protocols in Cell Biology 21.0.1-21.0.2, March 2008
Published online March 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2100s38

on photobleaching to label the targeted molecules. This is accomplished by labeling the molecular species of interest with two different fluorophores that can be imaged independently but simultaneously by fluorescence microscopy. Then one of the fluorophores is rapidly photobleached at a chosen location. Because the unbleached (reference) fluorophore remains co-localized with the target fluorophore, the subsequent fate of the photobleached molecules can be revealed by processing simultaneously acquired digital images of the two fluorophores. The FLAP method thus overcomes one drawback of FRAP and FLIP by enabling the bleached as well as unbleached molecules to be tracked in an experiment.

UNIT 21.3 describes the technique of bimolecular fluorescence complementation analysis (BiFC), which can be used to visualize protein interactions in living cells. It is based on complementation between non-fluorescent fragments of the yellow fluorescent protein (YFP) when they are brought together by an interaction between proteins fused to different fragments. This produces a fluorescent protein that allows the direct visualization of protein interactions within living cells and the site of this interaction. The unit also describes a multicolor BIFC assay that allows competition between interactive partners to be tested in cells. In this approach, fragments of different fluorescent proteins are attached to different proteins capable of interacting. The molecules are then allowed to compete with each other for binding with the read-out being a fluorescent signal. In this way, the multicolor BiFC assay allows comparison of the subcellular locations of different protein interactions in the same cell, as well as analysis of the competition among mutually exclusive interaction partners for complex formation with a common partner.

The advent of green fluorescent protein technology—in which proteins can be tagged with naturally fluorescent proteins derived from marine organisms and visualized in cells—is revolutionizing the field of cell biology. *UNIT 21.4* describes how to design fluorescent fusion proteins as probes of cell structure and function. Strategies for the selection of a fluorescent protein and for attaching it to a protein of interest using molecular biology techniques are described. Finally, assays are presented for testing whether the fusion protein maintains its function after being expressed in a living cell. *UNIT 21.5* extends the topic of green fluorescent protein technology to the color palette that fluorescent proteins offer as reporter molecules. Starting with an historical background of GFP development, the unit then delves into the details of the physical and chemical characteristics of GFP's unique chromophore, discussing its brightness, maturation, photostability, and oligomeric properties. The unit then discusses the broad range of fluorescent protein genetic variants that have been developed, including blue, cyan, green, yellow, orange, and red fluorescent proteins. Also discussed in *UNIT 21.5* are optical highlighter fluorescent proteins. These molecules have the advantage of being able to be "switched on" in response to a specific wavelength of light. The unit concludes with a discussion of future developments of fluorescent proteins aimed at making variants optimized for use in different imaging techniques, such as resonance energy transfer and multiphoton excitation.

UNIT 21.6 discusses the recent advance of photoactivation microscopy involving the use of fluorescent proteins that have the capacity to be "switched on" by photoactivation. These fluorescent proteins are invisible at the imaging wavelength until activated by a different wavelength of light. This unit discusses technical aspects regarding how to use the photoactivatable fluorescent proteins, including which laser lines should be used for photoactivation versus imaging, how to avoid photobleaching, and how to identify particular objects prior to photoactivation. Finally, specific applications of phtoactivation as they relate to cell imaging are discussed.

Jennifer Lippincott-Schwartz

Measuring Protein Mobility by Photobleaching GFP Chimeras in Living Cells

This unit describes photobleaching methods used in combination with green fluorescent protein (GFP) chimeras to analyze protein and organelle dynamics in living cells. Photobleaching is the photoinduced alteration of a fluorophore that abolishes the fluorophore's fluorescence signal. The diffusive characteristics of fluorescently tagged proteins or organelles in the cell can be studied by photobleaching a selected region of a fluorescently labeled cell with intense light and then quantitating the movement of nonbleached fluorescent molecules into the photobleached area using an attenuated light source (Lippincott-Schwartz et al., 1999, 2001; Meyvis et al., 1999). Other processes that lead to fluorescesce recovery into the bleached area (including vesicle transport or flow-based movement) also may be quantitated and studied by this method. Insights into a variety of aspects of protein and organelle dynamics thus can be addressed using photobleaching. These include: (1) estimation of the diffusion coefficient, *D*, of a fluorescent protein, (2) determination of the fraction of fluorescent molecules able to move under different cellular conditions, (3) assessment of continuity or discontinuity of an organelle, and (4) characterization of protein rates to or from an organelle (Ellenberg et al., 1997; Hirschberg et al., 1998; Partikian et al., 1998 Dayel et al., 1999; Marguet et al., 1999; Nehls et al., 2000). Photobleaching can also be used to reduce fluorescence from background noise to reveal faint populations of fluorescent proteins.

The usefulness of photobleaching methodologies depends on the availability of fluorescently labeled molecules. Until recently, these were limited to fluorescent dyes and to fluorescently labeled antibodies, which only permitted labeling of the plasma membrane, unless microinjected into cells. With the advent of GFP, a naturally fluorescent protein from the jellyfish *A. victoria* (Tsien, 1998), this has changed. Virtually any protein of interest can be tagged with GFP. When expressed within cells, these proteins usually retain their parent protein-targeting behavior. An important property of GFP is that it can be bleached without detectable damage to the surrounding environment. This is presumably because the GFP's cage-like structure, which surrounds a small cyclic peptide fluorophore (Prendergast, 1999), shields the external environment from any damaging effects caused by reactive photobleaching intermediates. These characteristics of GFP chimeras make them ideal reagents in photobleaching experiments.

In this unit, two photobleaching techniques for confocal laser scanning microscopes (CLSM) are described: FRAP (fluorescence recovery after photobleaching; see Basic Protocol 1 and Alternate Protocol 1) and FLIP (fluorescence loss in photobleaching; see Basic Protocol 2). In FRAP, a distinct region of interest in a cell expressing a GFP chimera is briefly photobleached with a high-intensity laser, and the movement of unbleached fluorescent molecules into the bleached region is followed with low-intensity laser light. FRAP is useful for determining a protein's diffusion coefficient (*D*), which measures the random movement or Brownian motion of a molecule. Knowing a protein's *D* can be used, in turn, to obtain information about the viscosity of a protein's environment and whether the protein is part a of much larger complex. FRAP also can be used to determine the fraction of molecules capable of recovering into a photobleached area, referred to as the mobile fraction (M_f). FLIP differs from FRAP in that photobleaching is repeated several times, alternating each photobleach with a low-laser-intensity image of the whole cell. FLIP reveals the connectedness of cellular compartments and whether immobile pools of proteins are spatially segregated.

Contributed by Erik L. Snapp, Nihal Altan, and Jennifer Lippincott-Schwartz
Current Protocols in Cell Biology (2003) 21.1-21.1.24

The protocols are described for mammalian cells, and the concepts can apply to other systems, including plant cells, yeast cells, and bacteria. Basic Protocols 1 and 2 (FRAP and FLIP, respectively) and Alternate Protocol 1 (FRAP with older confocal laser scanning microscopes) describe photobleaching techniques that can be used to visualize and quantitate protein mobility and organelle dynamics. Alternate Protocol 2 is a selective photobleaching protocol, which can be applied to the study of protein trafficking and protein flux through organelles. Support Protocol 1 discusses some practical issues of expressing and imaging GFP chimeras in living cells. In that protocol, preparation of transfected mammalian cells for imaging on a CLSM and setup of the CLSM is described. Data processing for FRAP is described in Support Protocol 2. Support Protocol 3 describes a simulation of inhomogeneous diffusion that can be used to obtain the diffusion coefficient *D*.

BASIC PROTOCOL 1

QUANTITATIVE FRAP BY STRIP PHOTOBLEACHING USING A LASER SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE PHOTOBLEACHING

Fluorescence recovery after photobleaching (FRAP) can be performed by irreversibly photobleaching a fluorescent marker in a region of a cell with a high-intensity laser beam and then following diffusion of unbleached fluorescent proteins into the bleached region by imaging with nonbleaching attenuated laser light (Fig. 21.1.1A). Several forms of FRAP or FPR (fluorescence photobleaching recovery) have been described. Popular methods include spot bleaching, pattern photobleaching, polarized photobleaching, and strip bleaching. The protocols in this unit will focus on the strip photobleach method for confocal laser scanning microscopes. Strip photobleaching permits imaging of the whole cell while monitoring the fluorescence recovery into the photobleached region of interest (ROI). It allows the investigator to readily determine information concerning the spatial distribution of fluorescence. It also can reveal whether the cell, stage, or focus have moved during imaging, events that make FRAP analysis difficult.

There are two types of strip FRAP protocols: quantitative and qualitative. Using the quantitative FRAP protocol, it is possible to obtain an effective diffusion coefficient, *D*, and to calculate the mobile fraction, M_f, of a fluorescent protein. Images are acquired rapidly and the data can be used to plot a recovery curve from which *D* can be determined (Fig. 21.1.1B). The qualitative protocol is used to obtain high-quality images for visualizing the diffusion process in a single cell. Because the images are obtained by line averaging and slow scan speeds, they take longer to acquire and therefore are usually unsuitable for calculation of *D*, which requires time points immediately after bleaching.

This FRAP protocol has been designed for the Zeiss LSM 510 microscope (using the physiology software package) and for other laser scanning confocal microscopes capable of selective photobleaching (e.g., from Leica and Bio-Rad). FRAP can also be performed using older laser scanning confocal microscopes without the selective photobleaching capacity (see Alternate Protocol 1). Selective photobleaching means that the investigator can bleach any shape or size of ROI. Specific settings will vary for each microscope. The user must determine conditions for photobleaching and postbleach recovery imaging that do not result in significant photobleaching of the cell (steps 1 to 6). The actual FRAP experiment is described in steps 7 to 9, and data analysis is described in the FRAP data-processing section (see Support Protocol 2).

Materials

Cell samples expressing GFP chimeric protein of interest (Support Protocol 1)
Confocal laser scanning microscope capable of selective photobleaching (e.g., Zeiss LSM 510)

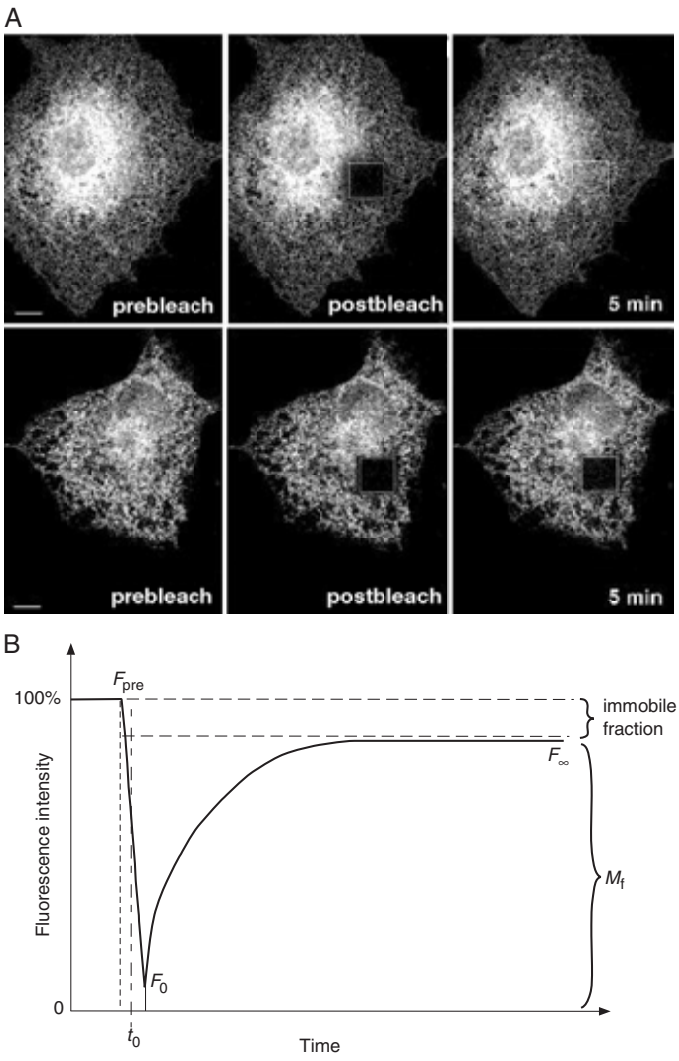


Figure 21.1.1 Photobleaching techniques. (**A**) Example of FRAP. Cells expressing VSVG-GFP were incubated at 40°C to retain VSVG-GFP in the ER under control conditions (untreated, upper row) or in the presence of tunicamycin (lower row). FRAP revealed VSVG-GFP was highly mobile in ER membranes at 40°C but was immobilized in the presence of tunicamycin. (**B**) Plot of fluorescence intensity in a ROI versus time after photobleaching a fluorescent protein. The prebleach (F_{pre}) is compared with the asymptote of the recovery (F_∞) to calculate the mobile and immobile fractions. Information from the recovery curve (from F_0 to F_∞) can be used to determine D of the fluorescent protein.

Additional reagents and equipment for preparing imaging chambers and microscope for photobleaching experiments (Support Protocol 1)

Establish FRAP conditions

1. Set up the microscope and prepare cells in imaging chamber with imaging medium as described in Support Protocol 1. Prewarm the imaging stage to 37°C or other desired temperature and warm up the microscope lasers for at least 5 min to avoid power fluctuations during imaging.

2. Identify the cell of interest on the confocal microscope. Bring it to the desired focus. Scan an image of the whole cell at the desired excitation light intensity, line averaging,

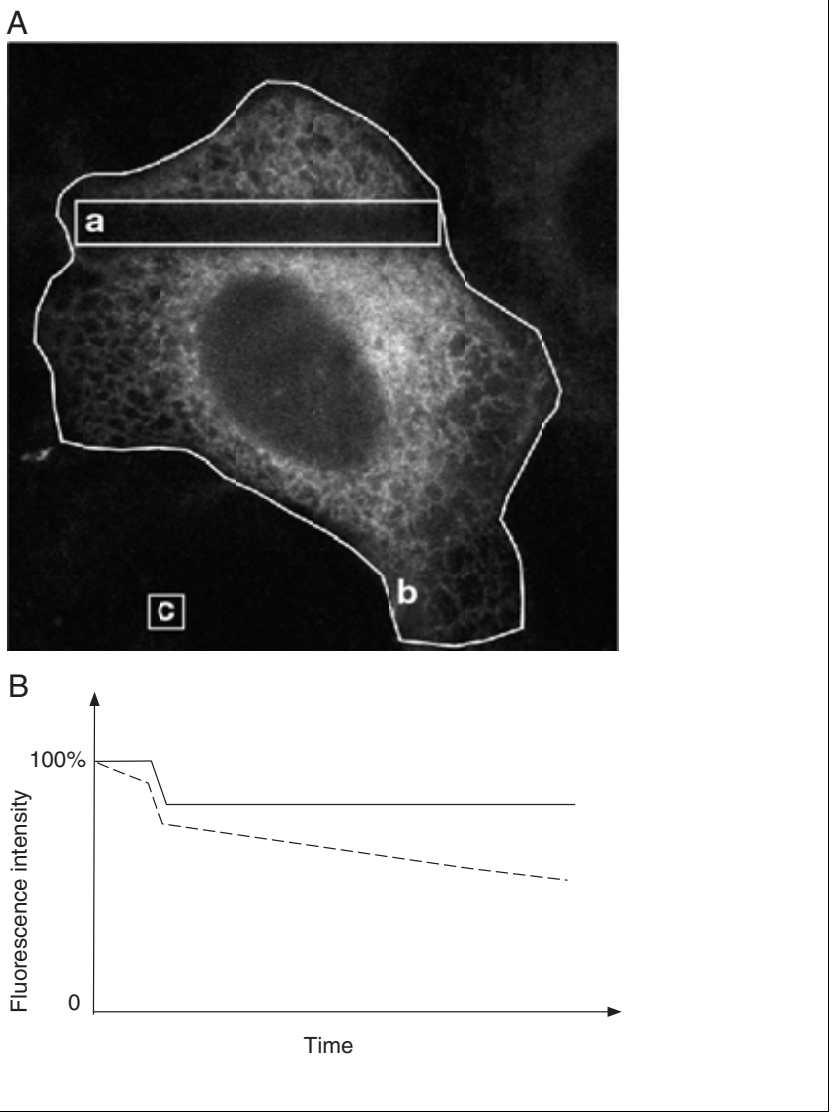


Figure 21.1.2 Defining ROIs and illustrating bleaching during imaging. (**A**) Example of ROIs, where *a* is the bleach ROI, *b* is the whole cell ROI, and *c* is the background ROI. (**B**) Plot of whole cell fluorescence (ROI *b*) versus time. The solid line represents an ideal plot in which the prebleach intensity of the cell does not change with time and where after the photobleach there is a drop in mean cellular fluorescence. There is no further loss of fluorescence. The dashed line represents a problematic plot of ROI *b*. The prebleach intensity steadily decreases with time, suggesting that the cell is being imaged at too high a laser power, resulting in photobleaching of the whole cell. The fluorescence loss continues after the photobleach of ROI *a*. The investigator must establish new imaging conditions.

zoom, and other parameters. Modify pinhole and detector gain for maximal fluorescence signal with no pixel saturation (pixel intensities that exceed the detector scale, i.e., >255 for an 8-bit image).

Saturated pixels only register as the maximum detector value, 255, so the true intensity of the pixel cannot be calculated under these conditions. Detector gain and offset will vary depending on the concentration of the fluorophore, the laser power, and the width and thickness of the fluorescently labeled organelle or region. The imaging parameters used for this image should be used for the fluorescence recovery time series. It may be useful to

record the detector gain settings to compare recoveries in cells expressing high versus low amounts of fluorescent protein.

3. Define a region-of-interest (ROI) for the photobleach (see Fig 21.1.2A), usually a 2- to 4-μm strip across the width of the cell.

   For soluble proteins that diffuse rapidly, the photobleach ROI may need to be relatively large (i.e., one-third to one-half of the cell). When performing FRAP on a discrete organelle, such as the nucleus or the Golgi complex, the photobleach ROI does not need to extend across the entire cell. It is sufficient for the edges of the strip to extend beyond the edges of the structure being analyzed. Note that larger photobleach ROIs will require longer recovery times.

4. Empirically determine photobleaching conditions (i.e., scan speed, zoom, laser power, microscope objective, and the minimal number of laser iterations required for photobleaching) so that after photobleaching, the fluorescent signal of the photobleach ROI decreases to within background intensity levels. Use imaging software to quantitate fluorescence intensity in the photobleach ROI and the whole cell prior to bleaching (Fig. 21.1.2A)

   It is necessary to know the fluorescence intensities in the whole cell and ROI prior to bleaching in order to determine the extent to which the photobleach ROI and the whole cell undergo bleaching during the course of imaging (Fig. 21.1.2B).

   It is useful to establish photobleaching conditions on fixed samples, as rapidly diffusing species may be impossible to bleach to background levels. Fix the plated GFP-expressing cells (either on a coverslip or in a Lab-Tek chamber) for 15 min in PBS (APPENDIX 2A) containing 4% formaldehyde at room temperature. Wash twice with PBS and then either place the coverslip on an imaging chamber with imaging medium or fill the Lab-Tek chamber with imaging medium. Under no circumstances must an antifade reagent (such as Fluoromount-G or phenylenediamine) be used, because this will significantly alter the photobleaching properties of the fluorophore. Image the fixed cells in imaging medium.

   Typical bleaching conditions require a 100 to 1000-fold increase in laser power (decrease in attenuation) for 1 to 5 bleach iterations (roughly 0.01 to 0.5 sec) for many organelles. If an ROI requires >20 sec to photobleach, a more powerful laser is necessary (see below).

   To ensure that bleaching laser power does not damage the cell, it is useful to repeat FRAP on the same ROI for the same cell. The diffusion coefficient (see discussion of data processing under Support Protocol 2) now should not change, but the mobile fraction (see Support Protocol 2) should be close to 100%. This is because any immobile fluorophores in the first FRAP will have been bleached and therefore will not contribute to the percent recovery observed in the second photobleach.

5. For acquisition of fluorescence recovery time points, empirically determine imaging conditions that do not significantly photobleach the cell outside of the bleach ROI (Fig. 21.1.2B)

   Recommended conditions for photobleaching with a 40-mW 488/514 nm argon or 25-mW argon laser are 45% to 60% power with 100% transmission. For acquisition of recovery time points, use the same power with 0.1% to 1.0% transmission. For quantitative FRAP, the whole cell is usually scanned at scan speed 8 to 10 (0.798 to 3 sec per 512 × 512 frame) with either two-line averaging or no-line averaging. In qualitative FRAP experiments, where the goal is to obtain high-quality images, the intervals at which images are collected during recovery need not be rapid (3- to 10-sec intervals, for membrane proteins). Soluble and lumenal proteins tend to diffuse rapidly and both quantitative and qualitative FRAP may require the bleaching of a large ROI and very rapid imaging conditions.

Collect FRAP data

6. To perform data analysis of a FRAP experiment, collect a prebleach image of the cell, a series of postbleach images of the whole cell that extend from an immediate

postbleach image to several images after the bleach ROI fluorescence intensity reaches a plateau, and a table of fluorescence intensity values including the photobleach ROI, the whole-cell ROI, and a background ROI. Note the corresponding time for each image and the pixel size in microns.

For the Zeiss 510 microscope, the physiology software contains several windows which permit the user to determine the number of images to collect, laser intensities for bleaching and imaging, number of bleach iterations, how many images to collect before bleaching, and drawing of the photobleach ROI. Other microscopes may possess their own software packages or may require writing macros for the photobleach time series.

7. Collect multiple prebleach images to establish the prebleach fluorescence intensity and to confirm that the prebleach fluorescence intensities of the cell and the bleach ROI do not fluctuate significantly.

8. Using the conditions determined in step 4, photobleach the ROI with intense laser illumination. Continue to image the whole cell at low laser illumination (the same conditions as the prebleach images) until the recovery process has reached a steady state.

   This must be determined quantitatively, as the human eye is incapable of distinguishing small differences in intensity. Typically, a 4-μm wide bleach ROI strip will recover within 90 to 120 sec for most freely mobile soluble lumenal proteins and within 350 sec for membrane proteins in the plasma membrane, endoplasmic reticulum, or Golgi. Cytoplasmic proteins may recover as rapidly as 5 to 20 sec. In this way, the investigator can establish conditions to obtain data sufficient for estimation of $t_{1/2}$ and M_f (see Support Protocol 2). The investigator should not attempt to refocus a cell during a FRAP experiment, because the fluorescence intensities will shift and the recovery curves will no longer be smooth. Time series that contain a shift in focal plane should be discarded.

   If a protein has a high protein diffusion coefficient (e.g., 5 μm^2/sec), the investigator may not observe a defined bleach region following the photobleach. Rapidly diffusing fluorescent proteins may appear homogeneously distributed immediately following a photobleach. The investigator must either: (1) increase the image collection rate, by changing the scan speed or reducing the size of the total frame to be collected, (2) increase the bleach ROI size, (3) increase the laser power, or employ a combination of these suggestions.

   To ensure that FRAP did not damage the area that was being scanned, repeat FRAP on the same area and compare the diffusion coefficients obtained from both experiments. D should remain the same but the mobile fraction should now be close to 100%. This is because the immobile fraction was bleached in the previous experiment.

9. Collect at least 10 to 20 data sets for each fluorescently labeled protein and treatment for statistical analysis.

   A fraction of the data sets are usually discarded because of problems that potentially bias imaging results (e.g., recovery was not complete, the focal plane shifted, or the recovery curve-fitting method failed).

   In Lippincott-Schwartz et al. (1999), a method for imaging the bleach ROI alone for rapid collection of a large number of data points is described. The caveat of the method is that the cell may shift in focus or position during the course of imaging. Imaging the whole cell offers the advantage of visualizing any movement of the cell or focal plane, thus allowing the researcher to reject unusable data upon visual inspection.

FRAP WITH CONFOCAL LASER SCANNING MICROSCOPES WITHOUT THE CAPACITY FOR SELECTIVE PHOTOBLEACHING

ALTERNATE PROTOCOL 1

FRAP (see Basic Protocol 1) can also be performed with older confocal laser scanning microscopes. Photobleaching is accomplished by zooming in on a small region of the cell and scanning with full laser power. The procedure for photobleaching is described below. The actual FRAP protocol is otherwise identical to Basic Protocol 1. For quantitative FRAP experiments for determining D, images should be acquired rapidly after the bleach, and this is best done if fluorescence is collected from the ROI only. If the investigator acquires a prebleach and a postrecovery image of the whole cell, it will be possible to calculate the M_f and D.

Additional Materials *(also see Basic Protocol 1)*

Confocal laser scanning microscope without capacity for selective photobleaching (e.g., Zeiss LSM 410)

Photobleach by zooming

1. Define a region of interest (ROI) for the photobleach. Define the ROI to be bleached at the highest zoom possible (usually zoom 8).

   At high zooms the laser will dwell longer on an ROI per line scan and thus will deliver more bleaching radiation.

2. To photobleach the ROI, set laser power at maximum and remove all neutral density filters from the path of the laser beam. Scan (photobleach) the ROI.

3. Bleach a desired ROI with high-intensity light and then rapidly and reliably switch to scanning the ROI with low levels of light and collect emission.

   A photobleaching macro can be written for the confocal system to perform bleaching and recovery imaging automatically.

CELL TRANSFECTION AND IMAGING SETUP

SUPPORT PROTOCOL 1

Cells first must be transfected with a construct in which DNA for GFP is linked to the protein of interest. Then the cells must be prepared for imaging. Transiently transfected adherent cells can be grown and imaged in a LabTek chambered slide or on a coverslip. Transfected suspension cells must be attached to a coated coverslip for imaging.

Materials

Eukaryotic cells of interest
Vector for expression of GFP chimeric protein: most laboratories today use the enhanced GFP (EGFP) variant (Clontech); although spectral variants of GFP are available, EGFP is best suited for photobleaching experiments; see Commentary for details
Cell culture medium (e.g., DMEM with serum) appropriate for cells of interest
Imaging medium (see recipe)
Silicon grease (optional)
5 to 10 mg/ml poly-L-lysine (Sigma) in PBS (see *APPENDIX 2A* for PBS)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Confocal laser scanning microscope equipped with a filter set for GFP and a 488-nm excitation laser that is at least 10 mW and preferably ≥25 mW
Imaging chamber: chambered coverglass system (LabTek from Nalgene) *or* glass coverslips and silicon rubber gasket for constructing silicon rubber chamber (see recipe in *UNIT 4.4*; also see Fig. 4.4.1)
Immersion oil (for oil-immersion microscope objectives)

Stage heater (e.g., Model ASI 400 Air Stream Stage Incubator, Nevtek; for cells that grow at 37°C)

Temperature probe: e.g., Thermolyne pyrometer (Cole-Parmer)

Computer system capable of processing large image files: 350 MHz or faster processor, multiple gigabyte hard drive, and at least 128 MB RAM

Image processing software (i.e., NIH Image or Metamorph)

Additional reagents and equipment for transfection of eukaryotic cells by electroporation (*UNIT 20.5*) or use of lipid transfection reagents (*UNIT 20.6*)

Transfect cells

1. To enhance the probability of having multiple cells to choose from, transfect cells with GFP chimeric protein vector using a high-efficiency transfection method, such as electroporation (*UNIT 20.5*) or a lipid transfection reagent—e.g., FuGENE6 (Roche) or LipofectAMINE 2000 (Invitrogen)—as described in *UNIT 20.6*.

   The cell density for imaging depends on cell type, cell size, and experimental design.

   Before performing a photobleaching protocol, the investigator must ensure that there is sufficient GFP fluorescence in the expressing cell to maintain a significant fluorescent signal relative to background noise after photobleaching. Most standard transfection protocols are sufficient to provide bright specimens. Stable transfectants express lower levels of protein. Transient transfectants usually express higher levels of proteins; this sometimes results in overexpression artifacts, such as protein aggregation or saturation of protein targeting machinery, which lead to inappropriate localization. Whichever transfection method is selected, the investigator needs several usable cells for each experiment.

   Adherent cells should be transiently transfected 16 to 36 hr prior to the experiment. The commercially available GFP expression vectors are under the control of a very strong promoter, the CMV promoter, so cell toxicity or mislocalization of GFP chimeras to other regions of the cell should always be a concern.

2. Determine the optimal level of expression and timing of imaging empirically for each sample and condition.

Set up imaging system

3. Set up the confocal laser scanning microscope and its associated hardware.

   It is assumed that the investigator is familiar with the basic operation of a confocal microscope. The investigator should understand both the concept and the operation of a pinhole, as well as the considerations related to scan speed, zoom, detector gain, laser power, photobleach, and collection of a time series.

Set up the imaging chamber

To image adherent cells on chambered slides

4a. Plate adherent cells on LabTek chambered coverglass.

   The cell density for imaging depends on cell type, cell size, and experimental design.

   These chambers consist of wells with a cover glass bottom, which permits the use of high-numerical-aperture oil objectives for viewing.

5a. Fill the wells to the rim with imaging medium and place on the stage of an inverted microscope.

   This ensures that cells have sufficient nutrients during the imaging session. For experiments that will last longer than 1 hr, the top cover of the chamber should be sealed onto the chamber using petroleum jelly or silicon grease. These steps prevent rapid evaporation of the medium above the cells and decrease the alkalinization of the medium by preventing room air from entering the chamber.

To image adherent cells in silicon rubber chambers

4b. Plate cells on glass coverslips.

The cell density for imaging depends on cell type, cell size, and experimental design.

5b. Invert the cell-containing coverslips on rubber gaskets that have a hole punched in them to form silicon rubber chambers (see *UNIT 4.4* and Fig. 4.4.1).

These gaskets are mounted on glass slides with petroleum jelly or silicon grease. The hole is filled with imaging medium and the coverslips are inverted and pressed onto the hole allowing the cells to face the medium. The coverslip adheres to the gasket by capillary action. Excess liquid from the top of the coverslip is wicked away with absorbant tissue. See UNIT 4.4 for full details.

To image suspension cells

4c. Precoat LabTek chambers or clean coverslips 5 to 10 mg/ml poly-L-lysine in PBS. Incubate for 15 min. Wash twice with PBS or distilled water.

The coating is usually good for up to 1 week.

5c. Wash suspension cells three times in PBS, then place them on the poly-L-lysine-coated surface and incubate 2 to 5 min. Remove nonadhering cells by washing twice with PBS. Finally, immerse the cells in imaging medium.

The cell density for imaging depends on cell type, cell size, and experimental design.

6. Keep cells warm on the microscope stage using a stage heater, and use a temperature probe to confirm that the proper temperature is maintained at the coverslip/chamber.

In addition to the chambers described above, Bioptechs offers a completely closed chamber with built-in perfusion. This type of setup permits the maintenance of cells on the microscope stage for extended periods of time (>24 hours) with minimal loss in viability. In addition, the investigator can perfuse drugs, dyes, and other reagents with controlled precision throughout the experiment. Thermal collars for objectives are another option for heating/maintaining the temperature of the chamber/coverslip. Thermal collars are available from Bioptechs.

Collect data

7. Collect FRAP data as described in Basic Protocols 1 or 2 or Alternate Protocols 1 or 2.

Many laser scanning confocal microscopes provide the investigator with a number of options for data collection, including but not limited to image size (e.g., 512 × 512 or 1024 × 1024, pixels), range of data collection (8-bit or 12-bit), and file formats. It is worth determining the requirements for image-analysis software in advance. For example, some image-analysis programs (see, e.g., Support Protocol 3) cannot process 12-bit images or only process PGM (portable graymap) image files, not TIFF (tagged-image file format) files.

See Support Protocol 2 for processing of FRAP data.

FRAP DATA PROCESSING

SUPPORT PROTOCOL 2

After collecting FRAP data using either Basic Protocol 1 or Alternate Protocol 1, the data need to be processed for quantitation. The three parameters that can be determined are the mobile fraction (M_f) of the fluorescent fusion protein, its diffusion coefficient (D), and the $t_{1/2}$ of the fluorescence recovery. When first performing FRAP experiments, it is an excellent idea to perform calibration experiments with a fluorescent protein whose D and M_f have been previously characterized (see Table 21.1.1).

Table 21.1.1 Diffusion Rates of GFP and GFP Chimeras Using FRAP[a]

Molecule	D_{eff} (μm²/sec)	References
GFP in water	87	Swaminathan et al. (1997)
GFP in cytoplasm	25	Swaminathan et al. (1997)
GFP in the ER lumen	5-10	Dayel et al. (1999)
Elastase-GFP in the ER lumen	0.5	Subramanian and Meyer (1997)
GFP in the mitochondrial matrix	20-30	Partikian et al. (1998)
Nucleoplasm		
GFP-HMG-17	0.45	Phair and Misteli (2000)
GFP-SF2/ASF	0.24	Phair and Misteli (2000)
GFP-fibrillarin	0.53	Phair and Misteli (2000)
Endoplasmic reticulum membrane		
Lamin B receptor–GFP (in ER)	0.35	Ellenberg et al. (1997)
GFP–MHC class I (murine)	0.46	Marguet et al. (1999)
TAP1-GFP (murine)	0.12	Marguet et al. (1999)
Galactosyltransferase-GFP (in ER)	0.48	Nehls et al. (2000)
Signal recognition particle β subunit–GFP	0.26	Nehls et al. (2000)
KDEL receptor–GFP (+BFA)	0.43	Cole et al. (1996)
VSVG-GFP ts045 (in ER+BFA, 32°C)	0.49	Nehls et al. (2000)
VSVG-GFP ts045 (in ER, 40°C)	0.45	Nehls et al. (2000)
Cytochrome P450-GFP	0.03-0.06	Szczesna-Skorupa et al. (1998)
Golgi apparatus membrane		
Galactosyltransferase-GFP (in Golgi)	0.54	Cole et al. (1996)
Mannosidase II–GFP	0.32	Cole et al. (1996)
KDEL receptor–GFP	0.46	Cole et al. (1996)
Plasma membrane		
E-cadherin-GFP	0.03-0.04	Adams et al. (1998)
GFP-Ki-Ras (12V)	0.19	Niv et al. (1999)
GFP-aquaporin (1 and 2)	0.009	Umenishi et al. (2000)
Lutenizing hormone receptor–GFP	0.16	Horvat et al. (1999)
Endosomal membrane		
GFP-rab5	0.1	Roberts et al. (1999)
E. coli cytoplasm		
GFP	7.7	Elowitz et al. (1999)
Maltose D–GFP	2.5	Elowitz et al. (1999)

[a]Abbreviations: BFA, brefeldin A; ER, endoplasmic reticulum; GFP, green fluorescent protein; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing; VSVG, vesicular stomatitis virus G protein.

Mobile fraction (M_f)

M_f refers to the percentage of fluorescent proteins capable of diffusing into a bleached ROI during the time course of the experiment (see Fig. 21.1.1B). M_f and D are distinct parameters and must be understood as such. D is a characteristic of the mobile pool of fluorescent proteins. The proteins in the immobile fraction do not diffuse.

An approximation of M_f can be calculated using the following equation, based on Feder et al. (1996), with the inclusion of a photobleaching correction (Lippincott-Schwartz et al., 1999):

$$M_f = 100 \times \frac{F_{\text{precell}} - F_{\text{background}}}{F_{\infty\text{cell}} - F_{\text{background}}} \times \frac{(F_{\infty\text{cell}} - F_{\text{background}}) - (F_0 - F_{\text{background}})}{(F_{\text{pre}} - F_{\text{background}}) - (F_0 - F_{\text{background}})}$$

Equation 21.1.1

where F_{precell} is the whole cell prebleach intensity, F_{pre} is the bleach ROI prebleach intensity, $F_{\infty\text{cell}}$ is the asymptote of fluorescence recovery of the whole cell, $F_{\text{background}}$ is the mean background intensity, F_∞ is the bleach ROI asymptote, and F_0 is the bleach ROI immediate postbleach intensity.

In the above equation, the bleach ROI and whole cell ROIs are background-subtracted. Next, the bleach ROI data are transformed such that the prebleach fluorescence intensity is defined as 100% fluorescence intensity. The equation includes a correction for the loss of total cellular fluorescence (due to the photobleach of the ROI and bleaching of the whole cell during imaging). The correction is calculated by determining the prebleach fluorescence intensity of the whole cell ROI (F_{precell}) and dividing it by the whole cell ROI intensity at time t. Without this bleach correction, the bleach ROI intensity can never recover to 100% of the prebleach fluorescence because the photobleach depletes 5% to 20% of the total cellular fluorescence. Finally, to convert the fraction to a percentage, it is multiplied by 100.

The F_0 value is an approximation for the fluorescence intensity immediately following the photobleach. For conditions in which the ROI is bleached to near background levels, F_0 will be relatively accurate. However, for rapidly diffusing proteins or for narrow bleach ROIs, the immediate postbleach ROI intensity may be closer to 30% to 40% of the prebleach fluorescence intensity. This can lead to the appearance of an artificial immobile fraction. The true F_0 may be much lower and can potentially be derived by rapidly collecting images of the bleach ROI alone, or by attempting to determine F_0 by nonlinear regression analysis.

Diffusion Analysis

The diffusion coefficient, D, reflects the mean-square displacement (usually μm^2/sec or cm^2/sec) that a protein explores through a random walk (i.e., Brownian motion) over time. It is important to recognize that D is not a simple linear rate (see Fig. 21.1.3). That is, the time required to cover increasing distances will not increase in a linear fashion. Time increases as the square of the distance covered divided by the D. The primary constraints on protein diffusion are the viscosity of the environment, whether the protein is soluble or integrated into a membrane, and, to a lesser extent, the molecular radius of the protein. Protein-protein interactions and collisions with other molecules also hinder free diffusion. By measuring D, the investigator can obtain information related to a protein's environment and in some cases, whether the protein is interacting with large complexes.

Several groups have derived a number of equations and computer simulations to determine D. The majority of available equations have been written for spot photobleaching. Related equations and simulations are available for strip photobleaching, including those described in Ellenberg et al. (1997) and Wey and Cone (1981). These equations, however, make several assumptions about the experimental system that may or may not be met. For example, the equation from Ellenberg et al. (1997) assumes that the bleach is complete, that there is no immobile fraction, that the cell is a uniform rectangle, and that the width of the bleach ROI is much less than the distance to either end of the cell. D values calculated with this equation can differ by 50% or more from an assumed D. Given these concerns, investigators are cautioned in the use this equation.

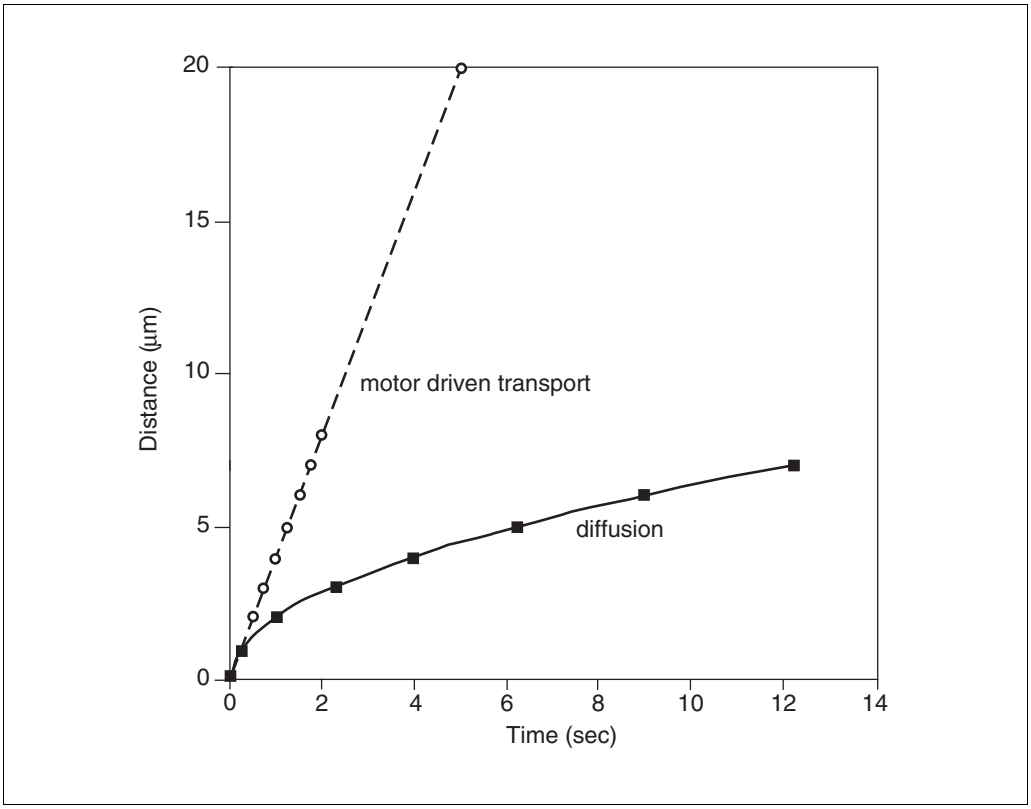


Figure 21.1.3 Diffusion versus linear movement. Plot of distance versus time for a protein, such as a motor protein, that moves directionally at a linear rate of 4 μm/sec (open circles, dashed line) and a protein that diffuses with a diffusion coefficient of 4 μm^2/sec (filled squares, solid line). Note that a protein travels 4 μm in 1 sec for the linear rate, but statistically takes 4 secs to travel the same distance by diffusion.

The best method the authors have found for obtaining *D* is through simulation of diffusive recovery into a strip bleach (Siggia et al., 2000). The Siggia simulation models inhomogeneous diffusion of nonbleached proteins in the cell into the photobleach ROI. The simulation then compares the simulated recovery to the actual data to determine *D* (Siggia et al., 2000). The program has been used for ER membrane proteins (Ellenberg et al., 1997; Zaal et al., 1999) and has been used to calculate *D* values comparable to values observed by other laboratories using different methods. Like other methods for determining *D*, simulation encounters difficulties when a significant fraction of the fluorescent protein is immobilized (Siggia et al., 2000). In such cases, the simulation either fails to fit the simulation data to the experimental recovery data or the simulation cannot calculate *D*. The Siggia simulation can be obtained by contacting Dr. Eric Siggia at *siggia@eds1.rockefeller.edu*. The simulation has been written for Unix systems and has been compiled for Macintosh computers. After signing the licensing agreement with Dr. Siggia, the Macintosh-compiled simulation can be obtained from the authors of this unit. Support Protocol 3 is a simple user's guide to the simulation.

After running any simulation or calculating a least-squares fit to determine *D*, it is essential to compare the simulation or fit data to the experimental data by plotting the data sets in a spreadsheet program. Even if a program or equation produces *D*, a poor fit means that *D* is questionable at best. "Goodness of fit" describes how well the simulation or equation data overlap the experimental data. The majority of the experimental data must overlap the simulation or equation plot. If the simulation misses the experimental data, the calculated *D* is questionable, at best. If there are any doubts about "goodness of fit," the user should consider another method of calculating the *D*.

A number of potential errors may occur in the course of data fitting. These errors are not unique to D calculations. They are part of the general problem of nonlinear regression analysis. Individuals that anticipate performing diffusion analysis are encouraged to become familiar with nonlinear regression analysis. An excellent introduction to the theory and problems of nonlinear regression analysis has been written by Dr. Harvey Motulsky and is available as a free download at *http://www.graphpad.com/www/nonlin.pdf*.

$t_{1/2}$

The mobilities of some proteins depend on complex behaviors, such as binding and release or populations with multiple D values. Such conditions complicate the analysis of D and may prevent fitting of data by traditional diffusion analysis. When a diffusion equation or simulation is unable to fit fluorescence recoveries, the $t_{1/2}$ measure can be used to compare relative recovery rates between samples. The $t_{1/2}$ is the time required for the fluorescence intensity in the bleach ROI to recover to 50% of the asymptote or plateau fluorescence intensity. This measure is independent of the prebleach ROI fluorescence intensity. In addition, it should be emphasized that while the $t_{1/2}$ can be a useful tool, it is only relevant for the user's system and conditions. It cannot be used to relate to $t_{1/2}$ values obtained from other experiments, since the $t_{1/2}$ value is relative to a particular experimental setup. The $t_{1/2}$ value requires two data manipulations: (1) conversion of time with the half-time of the bleach as time zero (t_0), and (2) conversion of bleach ROI recovery data into fractional fluorescence. Note that fluorescence recovery into a photobleach ROI must plateau or $t_{1/2}$ analysis cannot be performed. The $t_{1/2}$ can be determined visually or by solving the following equation (Feder et al., 1996):

$$F(t) = 100 \times [F_0 + F_\infty (t / t_{1/2})] / [1 + (t / t_{1/2})]$$

Equation 21.1.2

where t is the time for each ROI intensity value, usually in seconds or milliseconds, $t_{1/2}$ is the time required for the bleach ROI to recover to 50% of the asymptote (see discussion of $t_{1/2}$, below), and the remaining variables are defined as for Equation 21.1.1.

Fractional Fluorescence

To directly visualize and determine the $t_{1/2}$, transform the fluorescence intensity [$F(t)$] data to a 0% to 100% scale (see Fig. 21.1.4). The measurement is independent of the prebleach intensity and is not bleach corrected, as the relevant data occur after the photobleach. It is critical that the bleach ROI fluorescence recovery reaches a true plateau for accurate measurements. It is also important to have a large signal difference between the first postbleach intensity and the plateau. If the signal difference is too small, the plotted recovery will tend to be very broad and difficult to interpret. The following equation will convert the bleach ROI fluorescence recovery into fractional fluorescence data:

$$F_2(t) = 100 \times [F(t) - F_0] / [F_\infty - F_0]$$

Equation 21.1.3

where $F(t)$ is the bleach ROI fluorescence intensity at time t and the remaining variables are defined as for Equation 21.1.1. Time t must be rescaled relative to t_0, as described above. The $F_2(t)$ data are then plotted versus time (in seconds) to determine $t_{1/2}$.

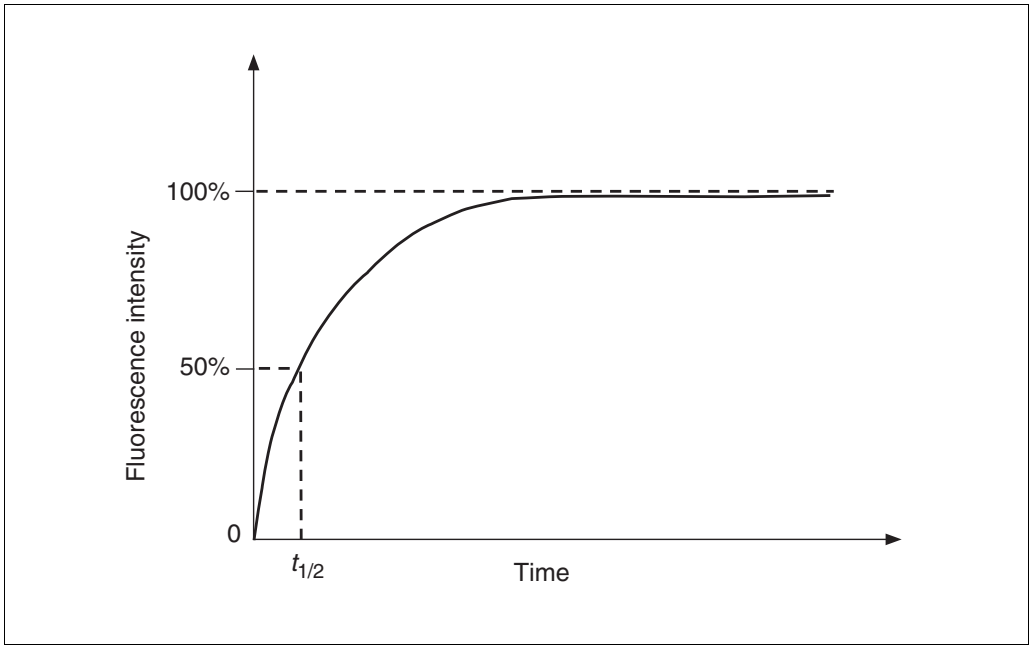


Figure 21.1.4 Fractional fluorescence. A plot of fractional fluorescence can be used to determine $t_{1/2}$, the time at which the fluorescence has recovered to 50% of the asymptote. The recovery data points (from F_0 to F_∞ in Fig. 21.1.1B) have been transformed to a scale of 0% to 100%.

The measurement is sensitive to the initial time (or t_0) of the bleach. When varying the size of the bleach ROI, the time to bleach the ROI will change. Practically, this means that the time for collection of the first postbleach image will vary between samples with different-sized bleach ROIs, which will alter the start time for data collection for each sample. Different groups define t_0 slightly differently. For this unit, t_0 is defined as the half-time of the bleach. To modify the time, subtract t_0 from each time value, where t_0 is defined by the following equation:

$$t_0 = \{[(t_{post} - t_{pre}) - (\text{time per image frame})]/2\} + t_{pre}$$

Equation 21.1.4

where t_{post} is the time of first image after photobleach and t_{pre} is the time of image immediately prior to photobleach.

ALTERNATE PROTOCOL 2

SELECTIVE PHOTOBLEACHING TO ENHANCE DIM STRUCTURES WHILE IMAGING OR TO VISUALIZE AND MEASURE NONDIFFUSIVE TRANSPORT INTO AN ORGANELLE

Photobleaching has additional uses besides those defined in Basic Protocols 1 and 2 and Alternate Protocol 1. For example, a GFP chimera may localize to two organelles in close proximity. One organelle may accumulate a substantial amount of the protein, while the adjacent organelle is dim. The bright organelle can be photobleached to permit imaging of the dim structure. Another application is to use photobleaching to visualize trafficking or flux through an organelle. For example, the Golgi complex (Nichols et al., 2001) or lysosomes can be photobleached, and then fluorescence recovery—either of fluorescence into the photobleached organelle or fluorescence trafficking out of the unbleached organelle into the photobleached area surrounding the organelle—can be imaged for both qualitative and quantitative analysis. Selective photobleaching essentially follows the FRAP protocol (see Basic Protocol 1). Instead of a strip, the investigator defines an ROI

appropriate to the organelle or structure of interest. Imaging conditions must be determined to photobleach either the structure of interest or around the structure of interest and to avoid bleaching of the overall cell during the course of imaging. When bleaching a bright structure to visualize dimmer structures, the conditions need to be set such that the dim structure or structures are sufficiently bright, but not saturating. The bright structure can be saturated, as it will be photobleached.

SIMULATION OF INHOMOGENOUS DIFFUSION

SUPPORT PROTOCOL 3

This simulation (Siggia et al., 2000) is a method for obtaining the diffusion coefficient *D* through diffusive recovery into a strip bleach (also see Support Protocol 2, discussion of Diffusion Analysis). The Siggia simulation can be obtained by contacting Dr. Eric Siggia at *siggia@eds1.rockefeller.edu*. The simulation has been written for Unix systems and has been compiled for Macintosh computers. After signing the licensing agreement with Dr. Siggia, the Macintosh compiled simulation can be obtained from the authors of this unit. Before running this simulation on a Macintosh computer (note the authors collect all of their data on a PC system with the confocal microscope and process diffusion data using a Macintosh), a substantial amount of RAM (50 to 100 Mb) should be allotted to the program. When running the simulation, other programs will run very slowly; therefore it is suggested that other programs be turned off. Before running the simulation, determine the time interval between frames and the size of each pixel.

1. To run the simulation, convert the TIFF (tagged-image file format) files to PGM (portable graymap), a format recognized by the simulation program.

   TIFF files can be converted to PGM files with the Macintosh program Graphic Converter (http://www.lemkesoft.de/us_gcabout.html). The authors recently discovered a bug in version 4.04 of the program, available online. The program will create PGM files; however, the simulation program does not recognize these files. The authors have used version 4.01 without incident. To run the simulation, the simulation program must be in the same folder as the PGM files. All files generated by the simulation will appear in this folder.

2. Open the first postbleach image and move the cursor to determine the coordinates of the smallest region that contains the whole cell. Write down the coordinate numbers (*x*, *y*), which should be in the range between 0 and 511, for the upper left corner of the box and the lower right corner of the box. Repeat for the photobleach ROI.

3. Start the simulation program.

   The program is designed to be user-friendly. The first few steps are self explanatory.

4. Enter the name of prebleach file (include full name, e.g., b009.pgm). If any colored saturation pixels are present in the images, the program will request that the user choose a channel (r, g, or b). Assuming the user has used GFP, enter g.

5. When asked Is this is a FRAP? and Are you supplying a series of postbleach images?, answer y for each question.

6. When asked Do you want to supply a background value?, answer n .

   Usually the simulation determines an accurate number, though the user is free to change the value.

7. When asked to enter the *x*,*y* coordinates of the ROI (note that the program is referring to the whole cell ROI, not the photobleach ROI), enter each coordinate for the upper left-hand corner of the box defined in step 2 (first *x* and then *y*) separated by a space (no parentheses or commas). Press the Return key and then enter the lower right-hand coordinates, each separated by a space.

8. When asked if one wants to monitor diffusion in individual rectangles, if one desires to use this option, open the postbleach_grid.pnm file and select grid rectangles of interest.

   The coordinates for each rectangle are determined by numbering the rectangles (0 to 9) across and down from the upper left corner of the grid. Each rectangle will correspond to x y, entered with a single space and no punctuation. The user may opt for this method to measure diffusion differences in different regions of the cell. However, the rectangle grid generated by the program will not necessarily align with the photobleach ROI. In addition, D values will be different for different regions of the cell. This information can be useful, but the time required for equilibration of a region of the cell some distance from the photobleach ROI is often much longer than the equilibration time in the photobleach ROI.

9. To obtain *D* in the bleach box ROI, type n and hit the Return key. Next, enter the coordinates (x y) of the bleach box ROI that were determined using graphic converter or NIH Image. Enter a single space between coordinates with no punctuation.

10. For time, unless one has used different times between frames, type y (without punctuation) if the time intervals between postbleach frames are the same. Next, enter the time between frames in seconds.

    Decimals (e.g., 0.798) are permitted.

11. Hit the Return key to run the simulation.

    The program takes 3 to 10 min to run depending on computer speed, the size of the cell ROI, and the number of images to be processed. The program becomes especially slow after the words "Starting inhomogeneous diffusion" appear. Finally, the program will state the effective diffusion constant for the photobleach ROI or the rectangles selected.

    IMPORTANT NOTE: *At the end of the program, the user is asked, "Do you wish to plot the simulation data with a different D_eff (yes or no)? The user must answer y or "n, and hit the Return key or the program will not create the experimental data file. Without this file, the user cannot compare the experimental recovery relative to the simulated recovery.*

12. Compare the sim001.dat versus the exp001.dat data files by simultaneously plotting both in a spreadsheet program.

    It is essential to plot the simulation data to determine whether the diffusion recovery has been accurately modeled. If the simulation misses the experimental data, the calculated diffusion rate is questionable, at best. The majority of the experimental data must overlap the simulation plot. A simulation that only overlaps with the initial steep rise or only with the later more gradual rise is not correct, and these D values should not be used. It is important to use all of the recovery data because removing parts of the recovery can significantly alter the calculated D. However, if the photobleach ROI fluorescence intensity has reached a genuine plateau and then rises or drops due to a focal shift or photobleaching, then the images after the plateau may be excluded from the analysis by simply removing them from the folder.

13. If the simulation quits before stating that *D* cannot be determined, recheck to see if incorrect coordinates have been entered (e.g., by placing the photobleach ROI outside of the whole cell ROI). Repeat the simulation, with the correct coordinates (be sure to remove any files generated by the previous run of the simulation).

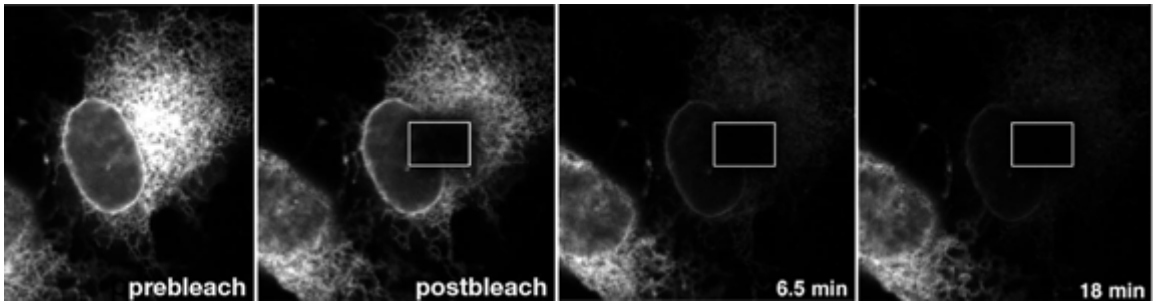


Figure 21.1.5 Example of FLIP. Protein fluorescence in a small area of the cell (box) is bleached repetitively. Loss of fluorescence in areas outside the box indicates that the fluorescent protein diffuses between the bleached and unbleached areas. Repetitive photobleaching of an ER GFP-tagged membrane protein reveals the continuity of the ER in a COS-7 cell. Image times are indicated in the lower right corners. The postbleach image was obtained immediately after the first photobleach. The cell was repeatedly photobleached in the same box every 40 sec. After 18 min, the entire ER fluorescence was depleted, indicating that all of the GFP-tagged protein was highly mobile and that the entire ER was continuous with the region in the bleach ROI.

FLIP USING A LASER SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE PHOTOBLEACHING

BASIC PROTOCOL 2

FLIP (fluorescence loss in photobleaching) is similar to FRAP (see Basic Protocol 1) in that an ROI is photobleached with a high-power laser. However, in FLIP, the ROI is repeatedly bleached over time to examine the behavior of the entire fluorescent pool. If the fluorescent molecules are completely mobile and have access to the photobleaching ROI, the entire fluorescent pool will be depleted (see Fig. 21.1.5). Results from FLIP experiments therefore provide information about the connectedness of structures containing GFP chimeras.

For FLIP analysis, the user must obtain a prebleach image, an immediate postbleach image following the first photobleach, and images following successive photobleaches (often 2 to 3 images following each photobleach) until the entire cell is depleted of fluorescence, or until several successive photobleaches do not further deplete cell fluorescence. The time for each image, pixel size, and fluorescence intensity values for the whole cell and the background intensity must also be determined.

Materials

Cell samples expressing GFP chimeric protein of interest (Support Protocol 1)
Confocal laser scanning microscope capable of selective photobleaching (e.g., Zeiss LSM 510)

Additional reagents and equipment for preparing imaging chambers and microscope for photobleaching experiments (Support Protocol 1)

1. Set up the microscope and prepare cells in imaging chamber with imaging medium as described in Support Protocol 1. Prewarm the imaging stage to 37°C or other desired temperature and warm up the microscope lasers for at least 5 min to avoid power fluctuations during imaging.

2. Identify the cell of interest on the confocal microscope.

   The ideal situation is to have two adjacent cells of similar fluorescence intensity in the imaging field. The cell that is not bleached provides a control to ensure that the imaging conditions do not cause nonspecific photobleaching of all fluorescence in the field of view. It is essential that imaging conditions do not significantly photobleach any adjacent cells during the experiment.

3. Bring the desired cell into focus. Scan an image of the whole cell at the desired excitation light intensity, averaging, and other parameters. Modify pinhole and detector gain for maximal fluorescence signal and minimal pixel saturation.

   Detector gain and offset will vary depending on the concentration of the fluorophore, the laser power, and the width and thickness of the fluorescently labeled organelle or region. The imaging parameters used for this image should be used for the fluorescence recovery time series.

4. Define a region-of-interest (ROI) for the photobleach, which should be between 5% and 20% of the structure of interest.

   The bleach ROI does not need to be a strip. A square is equally appropriate.

5. Determine imaging and photobleaching conditions (i.e., scan speed, zoom, laser power, number of laser iterations required for photobleaching, and microscope objective) that photobleach 90% or more of the fluorescent signal in the ROI, without causing significant photobleaching of the cell outside the photobleach ROI.

   A 40-mW 488/514 nm argon laser can be used at 45% to 60% power with 100% transmission for photobleaching and 1% transmission for imaging. Usually, cells are scanned at 0.8 to 3 sec per image with two- to eight-line averaging.

6. Collect three to five prebleach images to establish the prebleach fluorescence intensity and to confirm that the prebleach fluorescence intensity does not significantly fluctuate.

7. Collect two to five prebleach images and then photobleach the bleach ROI. Collect two to four images of the whole cell and photobleach the ROI again. Repeat the process until the fluorescent structure intensity is similar to background.

   The pauses allow unbleached molecules to diffuse into the photobleached ROI. In addition, collecting images between bleaches permits monitoring of progress and confirms that the cell remains in focus and has not moved. As this protocol requires longer time periods, the focal plane may shift. It is acceptable to refocus the cell during the experiment. However, if the cell or focus moves such that the region being photobleached also moves laterally, then the experiment should be aborted. Successful FLIP requires that the bleach ROI position within the cell is stable. If the cell migrates during the course of the experiment, then the data will be unusable.

8. Execute a quantitative FLIP.

   This procedure can be automated with a macro to program the bleaching and imaging timing. A FLIP macro for the Zeiss 510 laser scanning microscope (the advanced time-series macro in combination with the physiology software package) is available from Zeiss. It is also possible to perform FLIP manually. To do this, photobleach the bleach ROI in cells for several (i.e., 20 to 50) bleach iterations. Next, collect three images of the cell. Repeat the bleach and image collection many times, up to 15 to 20 min (or longer). If the proteins are mobile and the fluorescent structure is continuous, the total structure fluorescence will be depleted. If the protein diffuses relatively slowly, the investigator can modify the routine to include a time delay between images, to reduce overall photobleaching due to laser scanning of the cell.

9. Collect at least three to five data sets for each fluorescently labeled protein and treatment.

   A fraction of data sets may be unusable because the focal plane shifted, the cell moved, or other reasons.

   For CLSMs that are unable to perform selective photobleaching of an ROI, it is possible to perform FLIP using essentially the same protocol as that described above. The primary differences are the need to photobleach the cell by zooming (see Alternate Protocol 1) and

the requirement for a macro to automate the FLIP experiment. Essentially, the protocol must alternate between photobleaching the ROI and scanning images of the whole cell between photobleaching. The photobleaching and imaging scans must be performed at regular time intervals such that it will be possible to directly compare the rates of fluorescence depletion between two cells. For a more extensive discussion on writing this macro, see the review by Lippincott-Schwartz et al. (1999). Some FLIP experiments may not need to be quantitative and it is possible to manually perform FLIP.

REAGENTS AND SOLUTIONS

Use deionized , distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Imaging medium

Phenol-red free medium (e.g., RPMI or DMEM)
10% (v/v) fetal bovine serum (FBS)
2 mM glutamine
25 mM HEPES, pH 7.4
Prepare fresh

COMMENTARY

Background Information

Diffusion is defined as the rate of movement or flux of particles due to random or Brownian motion. An example of diffusion is the mixing of a drop of dye in a liquid solution. Random movements of proteins in a lipid bilayer (lateral diffusion), in the cytosol, or within an organelle are all examples of diffusion in a cell. Diffusion primarily depends on the viscosity of the environment and the size of the molecule. For soluble spherical molecules, the diffusion coefficient, *D*, is inversely proportional to the cube root of the radius of the molecule (Stokes-Einstein equation). This means that a molecule must increase 8- to 10-fold in size to decrease *D* by one-half. The size dependence of *D* for membrane proteins relates primarily to the radius of the transmembrane segments, rather than aqueous domains. This is because the lipid bilayer is more viscous than cytoplasm or organelle lumena. Other factors, such as protein-protein interactions or binding to a matrix can also profoundly affect the *D* of a protein.

Since Frye and Edidin (1970) demonstrated that proteins on the plasma membrane of cells move by diffusion, investigators have sought to directly measure diffusion coefficients of cellular proteins in their cellular context. Poo and Cone (1974) and Liebman and Entine (1974) performed some of the first FRAP experiments by photobleaching retinal rod outer segments; these experiments successfully measured the *D* value of the transmembrane protein rhodopsin. The analytical equations and methodology that form the basis of FRAP were described originally by Axelrod et al. (1976). Labeling of proteins that are exposed to the extracellular face of the plasma membrane of living cells, using fluorescent antibodies, has been the primary labeling method for FRAP studies for 20 years. However, with that method, studies were restricted primarily to proteins on the cell surface, leaving all of the vesicles, organelles, and cytoskeletal components of cells unexplored. Some researchers have exploited microinjection techniques to place fluorescent antibodies or fluorescently labeled proteins in the cytoplasm. However, standard microinjection does not deliver proteins to the lumena of organelles, leaving these important cell environments unprobed. An additional barrier to investigating protein mobility was that traditional FRAP methodology required a dedicated custom microscope (Wolf, 1989).

Today, the combination of advances in fluorescence imaging methods, user-friendly commercially available laser scanning microscopes, and powerful cost-effective computing resources has made it possible for nonspecialists to exploit GFP chimeras and to probe their mobilities and interactions. For examples and more information, the reader is referred to several reviews of FRAP theory and techniques (Edidin, 1992; Ellenberg et al., 1997; Lippincott-Schwartz et al., 1999, 2001; Meyvis et al., 1999; White and Stelzer, 1999; Nichols et al., 2001).

Critical Parameters and Troubleshooting

The critical parameters for data collection are described in the protocols. There are several considerations for data analysis and interpretation that require additional emphasis. The major concerns are discussed below.

EGFP is better suited than other spectral variants for photobleaching experiments

Photoconversion and reversible photobleaching are phenomena that can seriously complicate photobleaching analysis by generating artificially high *D* values. Photoconversion is the process by which a fluorophore is excited and becomes transiently or permanently altered in its fluorescence excitation and emission spectra. Reversible photobleaching occurs when a fluorophore's excitation state is changed by intense illumination, which appears to an observer as destruction of the fluorophore. However, the fluorophore reverts to its native excitation and emission spectra and becomes fluorescent again. While many fluorophores exhibit varying degrees of both phenomena, photoconversion and reversible photobleaching are minimal for enhanced green fluorescent protein (EGFP). In contrast, wild-type GFP readily undergoes photoconversion. Thus, FRAP experiments using wild-type GFP chimeras should be avoided.

Correct for photobleaching due to bleaching of the ROI and repeated scanning of the cell

One method to measure the amount of photobleaching is to directly measure the change in the fluorescence of the whole cell used in an experiment. A decrease in fluorescence can be used for data correction and can also help the investigator modify imaging conditions to achieve minimal sample bleaching. In addition, this method offers an additional advantage in that the investigator can monitor fluorescence changes in every frame and can be alerted to cell movement and focal drift, which can change the apparent fluorescence recovery. Alternatively, the investigator can fix the sample (using 4% formaldehyde in PBS for 15 min) and acquire the time-lapse images of the fixed sample using exactly the same imaging parameters (e.g., excitation light intensity, duration, zoom, and number of images) as in living cells. Loss of fluorescence under these conditions will be due only to photobleaching and not to biological degradation processes. Once the photobleaching rate has been determined, the experimental values obtained from living cells can be corrected.

Photobleaching experiments must produce recoveries with a true plateau or asymptote

M_f and $t_{1/2}$ calculations cannot be performed reliably when the asymptote has not been reached. Often, in the course of data processing, what appears to be a flat plateau with raw data may become an incomplete recovery following data processing. This result emphasizes the importance of determining the optimal imaging time to obtain a true plateau.

D and M_f must be interpreted in the context of the cell

Data analysis requires a commitment on the part of the investigator not to blindly accept numbers generated by the equations without attempting to determine whether the results are biologically and physically reasonable. For example, a *D* of 2 μm^2/sec for an integral membrane protein exceeds the highest recorded diffusion coefficient (0.4 to 0.5 μm^2/sec) for a membrane protein in a cell by four-fold. Such a number could indicate that a protein is no longer membrane-associated or that the GFP chimera is incorrectly processed or targeted. Another less likely possibility is that processes besides diffusion are occurring (e.g., flow; Sciaky et al., 1997) that do not exhibit diffusive behavior.

Interpretation of D and M_f can be confounded by the connectedness of fluorescent structures

The problem concerning compartment connectedness is not always readily apparent. That is, an organelle or compartment may appear connected at the light level, but not actually be connected. This situation will result in a low apparent mobile fraction since fluorescent molecules in nearby but unconnected areas will be unable to diffuse into the recovery box. One way to clarify whether a structure is continuous or disconnected is to perform FLIP and check to see whether fluorescence of an otherwise mobile protein is depleted from all areas of the compartment (indicating connectivity) or only specific areas (indicating a lack of connectedness). If the molecule being studied resides within a discontinuous compartment, it will be difficult to estimate its *D*, since the equations and simulation programs used for calculating *D* assume free diffusion throughout a compartment.

Table 21.1.2 Troubleshooting Guide for Photobleaching

Problem	Possible cause and solution
Autofluorescence noise	Lysosomes are notorious for autofluorescence when excited with light between 400 and 488 nm. To decrease autofluorescence, lower the intensity of the excitation beam and use narrower-bandpass emission filters. Also, avoid phenol red and high serum concentrations (>20%) in the medium.
Fluorophore bleaches too rapidly during acquisition	Decrease the excitation light intensity using either neutral density filters or by lowering the voltage to the Acousto-Optical Modulator and increase the gain on the detector side to collect light more efficiently. If this does not work try adding Oxyrase[a], an oxygen scavenger, to the medium.
Cells round up during imaging	This could be due to gross photodamage to the cells from long periods of exposure to an intense excitation beam. To correct, decrease the intensity of excitation light. Alternatively, the cells may have depleted some nutrient in the medium due to remaining in coverslip chambers for too long. Replenishing the imaging medium should help.
Cells are excited with a constant light intensity, but the fluorescence intensity varies over time	If this is not due to a biologically relevant process such as recruitment/degradation of fluorophore, then either the focus is shifting during acquisition or the laser power output is unstable. Maintain the focus either manually or by using autofocus software, which is available for some CLSMs. If laser output is a concern, then check it by exciting fluorescent beads and quantifying the emission over time. Fluctuating laser output may be due to the laser being operated at low power output. Increasing the power output to 50% may help. If this fails to correct the problem, then contact your confocal service representative.
Focus drifts	Try using relatively flat cells (i.e., COS-7) which may alleviate the problem. Try imaging with the pinhole partly or entirely open and use lower-NA objectives. Be sure to pre-equilibrate the sample to the temperature of the objective, as this will prevent expansion/contraction of the coverslip/chamber during imaging. Make sure that the stage insert is mounted securely and that the sample is seated properly in the holder.
D values of two subsequent experiments of the same ROI in the same cell do not match	This suggests the area that has been photobleached has been damaged. Try using a different GFP variant that requires a lower (and less damaging) wavelength of laser light, such as YFP. Note that YFP may reversibly photobleach.
Slow recovery or lack of recovery after bleaching	Either the fluorophore is relatively immobile or there are discontinuities within the structure where the fluorophore is located. This is relevant for isolated membrane structures with little connection between membranes, such as structures of the endocytic system (e.g lysosomes and endosomes).
Recovery is significantly faster than expected	A faster than expected *D* for a membrane protein may indicate that the protein is no longer correctly assembled or inserted in the membrane. This can be tested by performing immunofluorescence colocalization with a marker for the organelle of interest or performing immunoblotting to determine whether the GFP chimera has become partially degraded. Another condition that may affect fluorescence recovery is loss of cell integrity or viability.

continued

Table 21.1.2 Troubleshooting Guide for Photobleaching, *continued*

Problem	Possible cause and solution
Nondiffusive behavior	A failure to fit recovery-curve data by a simulation or to a least squares fit equation may suggest that the protein is not moving by simple diffusion. There are conditions that cannot be described by equations for simple diffusion, e.g., in the case of anomalous diffusion[b] and when there are multiple populations diffusing at different rates.[c]

[a]Product of Oxyrase Inc.; see *SUPPLIERS APPENDIX*.

[b]Potential mechanisms of anomalous diffusion include binding and collisional interactions with mobile and immobile obstacles (Periasamy and Verkman, 1998) and recovery due to vesicular transport.

[c]The labeled protein in the FRAP experiments may alternate between multiple states, i.e., different-sized oligomers or bound and unbound to a membrane or complex. These conditions pose significant problems to diffusion analysis. Gordon et al. (1995) describe the mathematical difficulties of separating multiple *D* coefficients. The main problem is that the investigator does not usually know the fraction of each population or the actual number of populations. It is worth noting that at least one group has successfully teased out multiple diffusing populations (Marguet et al., 1999). In this example, the group independently determined the *D* coefficients for the two populations and biochemically determined the relative percentages of each population.

Troubleshooting

A number of common problems that may arise in photobleaching experiments and potential solutions for them are presented in Table 21.1.2.

Anticipated Results

The methods described in this unit should permit the investigator to obtain and interpret fluorescence intensity recovery data following photobleaching of GFP chimeras with a CLSM. The investigator should be able to calculate the M_f and either the D or $t_{1/2}$ of fluorescence recovery of a protein in a cell. The investigator should also be able to perform a FLIP experiment to determine the connectivity of GFP-labeled cellular compartments and organelles. Finally, the investigator may take advantage of selective photobleaching either to reveal dimmer fluorescent structures obscured by bright structures or to visualize dynamic processes under steady-state conditions, such as vesicular trafficking.

Time Considerations

Creation of GFP chimeras involves standard cloning procedures and biochemical or genetic assays to confirm that the properties of the chimera are similar to those of the parent protein. Preparation for photobleaching experiments consists of transferring cells to coverslips or imaging chambers and transfecting the cells with a GFP construct 16 to 48 hr prior to imaging. The initial setup for photobleaching experiments requires a time investment of a few hours to determine conditions for each protein to be bleached. The actual FRAP experiments can be very rapid, ranging from 90 sec for the recovery of a soluble lumenal ER protein into a 4-μm bleach ROI, to 6 min for a membrane protein. FLIP experiments often take 10 to 20 min. FRAP experiments require at least 5 (and preferably 10) data sets for useful statistics to compare *D* values. Data processing depends on the method used and the power of the computer. The Siggia simulation will process a 250-image data series in 2 to 3 min on a Macintosh G4 450 MHz computer. Due to the large numbers of data sets to be processed, data analysis can be time consuming.

Literature Cited

Adams, C.L., Chen, Y., Smith, S.J., and Nelson, W.J. 1998. Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J. Cell Biol.* 142:1105-1119.

Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E., and Webb, W.W. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055-1069.

Cole, N.B., Smith, C.L., Sicaky, N., Terasaki, M., Edidin, M., and Lippincott-Schwartz, J. 1996. Diffusional mobility of Golgi proteins in membranes of living cells. *Science* 273:797-801.

Dayel, M.J., Hom, E.F.Y., and Verkman, A.S. 1999. Diffusion of green fluorescent protein in the aqueous-phase lumen of endoplasmic reticulum. *Biophys. J.* 76:2843-2851.

Edidin, M. 1992. Translational diffusion of membrane proteins. *In* The Structure of Biological Membranes (P. Yeagle, ed.) pp. 539-572. CRC Press, Boca Raton, Fla.

Ellenberg, J., Siggia, E.D., Moreira, J.E., Smith, C.F., Presley, J.F., Worman, H.J., and Lippincott-Schwartz, J. 1997. Nuclear membrane dynamics and reassembly in living cells: Targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell Biol.* 138:1193-1206.

Elowitz, M.B., Surette, M.G., Wolf, P., Stock, J.B., and Leibler, S. 1999. Protein mobility in the cytoplasm of *Escherichia coli*. *J. Bacteriol.* 181:197-203.

Feder, T.J., Brust-Mascher, I., Slattery, J.P., Baird, B., and Webb, W.W. 1996. Constrained diffusion or immobile fraction on cell surfaces: A new interpretation. *Biophys. J.* 70:2767-2773.

Frye, L.D. and Edidin, M. 1970. The rapid intermixing of cell surface antigens after formation of mouse human heterokaryons. *J. Cell Sci.* 7:319-335.

Gordon, G.W., Chazotte, B.F., Wang, X.F., and Herman, B. 1995. Analysis of simulated and experimental fluorescence recovery after photobleaching: Data for two diffusing components. *Biophys. J.* 68:766-778.

Hirschberg, K., Miller, C.M., Ellenberg, J., Presley, J.F., Siggia, E.D., Phair, R.D., and Lippincott-Schwartz, J. 1998. Kinetic analysis of secretory protein traffic and characterization of Golgi to plasma membrane transport intermediates in living cells. *J. Cell Biol.* 143:1485-1503.

Horvat, R.D., Nelson, S., Clay, C.M., Barisas, B.G., and Roess, D.A. 1999. Intrinsically fluorescent luteinizing hormone receptor demonstrates hormone-driven aggregation. *Biochem. Biophys. Res. Commun.* 255:382-385.

Liebman, P.A. and Entine, G. 1974. Lateral diffusion of pigment in photoreceptor disk membranes. *Science* 185:457-459.

Lippincott-Schwartz, J., Presley, J., Zaal, K., Hirschberg, K., Miller, C., and Ellenberg, J. 1999. Monitoring the dynamics and mobility of membrane proteins tagged with green fluorescent protein. *In* Green Fluorescent Proteins, vol. 58 (K.F. Sullivan and S.A. Kay, eds.) pp. 261-291. Academic Press, San Diego.

Lippincott-Schwartz, J., Snapp, E.L., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* 2:444-456.

Marguet, D., Spiliotis, E.T., Pentcheva, T., Lebowitz, M., Schneck, J. and Edidin, M. 1999. Lateral diffusion of GFP-tagged H2Ld molecules and of GFP-TAP1 reports on the assembly and retention of these molecules in the endoplasmic reticulum. *Immunity* 11:231-240.

Meyvis, T.K.L., De Smedt, S.C., Oostveldt, P.V., and Demeester, J. 1999. Fluorescence recovery after photobleaching: A versatile tool for mobility and interaction measurments in pharmaceutical research. *Pharm. Res.* 16:1153-1162.

Nehls, S., Snapp, E., Cole, N., Zaal, K.J.M., Kenworthy, A., Roberts, T.H., Ellenberg, J., Presley, J., Siggia, E., and Lippincott-Schwartz, J. 2000. Dynamics and retention of misfolded proteins in native ER membranes. *Nature Cell Biol.* 2:288-295.

Nichols, B.J., Kenworthy, A., Polishchuk, R.S., Lodge, R., Roberts, T.H., Hirschberg, K., Phair, R.D., and Lippincott-Schwartz, J. 2001. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* 153:529-542.

Niv, H., Gutman, O., Henis, Y.I., and Kloog, Y. 1999. Membrane interactions of a constitutively active GFP-Ki-Ras 4B and their role in signaling. *J. Biol. Chem.* 274:1606-1613.

Partikian, A., Olveczky, B., Swaminathan, R., Li, Y., and Verkman, A.S. 1998. Rapid diffusion of green fluorescent protein in the mitochondrial matrix. *J. Cell Biol.* 140:821-829.

Periasamy, N. and Verkman, A.S. 1998. Analysis of fluorophore diffusion by continous distributions of diffusion coefficients: Application to photobleaching measurements of multicomponent and anomalous diffusion. *Biophys. J.* 75:557-567.

Phair, R.D. and Misteli, T. 2000. High mobility of proteins in the mammalian cell nucleus. *Nature* 404:604-609.

Poo, M.M. and Cone, R.A. 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature* 247:438-441.

Prendergast, F.G. 1999. Biophysics of the green fluorescent protein. *Methods Cell Biol*. 58:1-18.

Roberts, R.L., Barbieri, M.A., Pryse, K.M., Chua, M., Morisaki, J.H., and Stahl, P.D. 1999. Endosome fusion in living cells overexpressing GFP-rab5. *J. Cell Sci.* 112:3667-3675.

Sciaky, N., Presley, J., Smith, C., Zaal, K.J.N., Cole, N., Moreira, J.E., Terasaki, M., Siggia, E., and Lippincott-Schwartz, J. 1997. Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J. Cell Biol.* 139:1137-1155.

Siggia, E.D., Lippincott-Schwartz, J., and Bekiranov, S. 2000. Diffusion in a inhomogeneous media: Theory and simulations applied to a whole cell photobleach recovery. *Biophys. J.* 79:1761-1770.

Subramanian, K. and Meyer, T. 1997. Calcium-induced restructuring of nuclear envelope and endoplasmic reticulum calcium stores. *Cell* 89:963-971.

Swaminathan, R., Hoang, C.P., and Verkman, A.S. 1997. Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cells: Cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion. *Biophys. J.* 72:1900-1907.

Szczesna-Skorupa, E., Chen, C., Rogers, S., and Kemper, B. 1998. Mobility of cytochrome P450 in the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. U.S.A*. 95:14793-14798.

Tsien, R.Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 67:509-544.

Umenishi, F., Verbavatz, J.M., and Verkman, A.S. 2000. cAMP regulated membrane diffusion of a green fluorescent protein-aquaporin 2 chimera. *Biophys. J.* 78:1024-1035.

Wey, C. and Cone, R.A. 1981. Lateral diffusion of rhodopsin in photoreceptor cells measured by fluorescence photobleaching and recovery. *Biophys. J.* 1981:225-232.

White, J. and Stelzer, E. 1999. Photobleaching GFP reveals protein dynamics inside living cells. *Trends Cell Biol.* 9:61-65.

Wolf, D.E. 1989. Designing, building, and using a fluorescence recovery after photobleaching instrument. *Methods Cell Biol.* 30:271-306.

Zaal, K.J.M., Smith, C.L., Polishchuk, R.S., Altan, N., Cole, N.B., Ellenberg, J., Hirschberg, K., Presley, J.F., Roberts, T.H., Siggia, E., Phair, R.D., and Lippincott-Schwartz, J. 1999. Golgi membranes are absorbed into and reemerge from the ER during mitosis. *Cell* 99:589-601.

Contributed by Erik L. Snapp, Nihal Altan, and Jennifer Lippincott-Schwartz
Cell Biology and Metabolism Branch
NICHD
National Institutes of Health
Bethesda, Maryland

Fluorescence Localization After Photobleaching (FLAP)

Fluorescence localization after photobleaching (FLAP) is a new method for localized photolabeling and subsequent tracking of specific molecules within living cells. The molecular species to be located carries two different fluorophores that can be imaged independently but simultaneously by fluorescence microscopy. For the method to work, these two fluorophores should be accurately colocalized throughout the cell so that their images are closely matched. One of the fluorophores (the target fluorophore) is then rapidly photobleached at a chosen location. The unbleached (reference) fluorophore remains colocalized with the target fluorophore; thus the subsequent fate of the photobleached molecules can be revealed by processing simultaneously acquired digital images of the two fluorophores. For example, an absolute FLAP image, which shows the location of the photobleached molecules, is calculated simply by subtracting the target intensity from the reference intensity at each pixel. This image is effectively the same as images obtained directly by photoactivation methods, so absolute FLAP can be considered to be a method of virtual photoactivation. In addition, however, a relative FLAP image can be calculated to show the photobleached fraction of molecules within each pixel. This useful information is not available with other methods.

The Basic Protocol in this unit demonstrates the simplicity and effectiveness of the FLAP method in revealing both fast and slow molecular dynamics in living cells. As an example, cDNA fusion constructs of β-actin with yellow and cyan fluorescent proteins (YFP and CFP) are microinjected into the nuclei of transformed rat fibroblasts. Using a Zeiss LSM 510 laser-scanning microscope, the authors show that it is possible to follow simultaneously the fast relocation dynamics of monomeric (globular) G-actin and the much slower dynamics of polymeric (filamentous) F-actin in expressing cells.

In order to give a step-by-step protocol for the FLAP method, it is necessary to focus on a particular configuration of fluorophores and microscopy methods. CFP-actin and YFP-actin fusion proteins have been chosen as the fluorescent molecules, since YFP can be efficiently and rapidly photobleached. Moreover, the predominance of native actin in the cell means that noise-free images can be obtained at low relative expression levels of the tagged molecules. Using wide-field fluorescence microscopy, it is possible to separate the emission spectra of these two fluorophores and thus obtain simultaneous images. For this, both fluorophores are excited simultaneously, and a commercially available arrangement of fixed dichroic mirrors and filters (Cairn Research Ltd.) enables the two images to be projected side-by-side onto a single CCD chip. However, in view of the large overlap of emission spectra of CFP and YFP, laser scanning microscopy, which permits alternately exciting the two fluorophores line-by-line and yields images that are effectively simultaneous, has been chosen for the Basic Protocol. Some laser scanning microscopes, such as the Zeiss LSM 510 used here, have the further advantages of versatile choice of size, shape and location of the bleach region, and rapid resumption of image scanning after bleaching. Time-lapse recording using no intervals between scanned frames will reveal rapid diffusion dynamics, whereas intervals of several seconds or longer may be required to reveal the movements of polymerized or bound molecules without excessive fading of the fluors.

Contributed by Graham A. Dunn, Mark R. Holt, Daniel Y. H. Soong, Colin Gray, and Daniel Zicha
Current Protocols in Cell Biology (2004) 21.2.1-21.2.16

FLAP OF ACTIN IN LIVING CELLS

This protocol describes the FLAP method using transformed rat fibroblasts of the lines K2 and T15 (Pavel Vesely, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nam. 2, 166 37 Prague 6 Dejvice, Czech Republic; e-mail, *pvy@zeus.img.cas.cz*). Many other cell types that the authors have tried work equally well. A notable exception is the Swiss 3T3 line, which shows significant autofluorescence on the YFP channel. Extensive details on preparation of constructs, cell culture, and microinjection methods will not be given, as these are standard procedures. Also a basic familiarity with the software and operation of the Zeiss LSM 510 upright microscope will be assumed. Once the microscope and software have been prepared (Support Protocol 1), there are two main sequential stages to the Basic Protocol: (1) preparation of cells and setting up the microscope (steps 1 to 4) and (2) microscopy (steps 5 to 17). Post-processing and analysis of images (Support Protocol 2) can be performed later.

Materials

Rat fibroblast cell line K2 or T15
Hanks' Minimal Essential Medium (MEM; Cancer Research UK; *daniel.zicha@cancer.org.uk*) containing 10% bovine serum and no antibiotics
cDNA constructs of eCFP-β-actin and eYFP-β-actin (see recipe)
Experimental reagents of interest (e.g., myosin light chain kinase inhibitor, ML-7)
Hot wax mixture (see recipe)
Non-toxic immersion oil optimized for 37°C, refractive index 1.515 (Cargille Labs)
18 × 18–mm glass coverslips
35-mm plastic petri dishes (Costar)
Microinjection system (also see *UNITS 4.10 & 17.1*) including:
  5171 micromanipulator (Eppendorf)
  5246 transjector (Eppendorf)
Zeiss Axiovert 35 microscope
Microneedles (GC120TF-10, Harvard Apparatus)
P97 Flaming/Brown micropipette puller (Sutter)
Optical chambers (see recipe)
Zeiss upright LSM 510 microscope (see Support Protocol 1 for full configuration) contained within a 37°C environmental control incubator (e.g., Microscope Temperature Control System, Life Imaging Services) *or* a similar apparatus assembled in house (Fig. 21.2.1)
Software:
  Zeiss LSM 510 operating software for image acquisition
  Zeiss LSM Reader for image review (free download; see Internet Resources)
Additional reagents and equipment for cell culture (*UNIT 1.1*), microinjection (see *UNITS 4.10 & 17.1*), and use of LSM 510 operating software (see Support Protocol 1)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH 7.4.

Prepare cells and set up microscope

1. Seed cells at the required density onto the 18 × 18–mm coverslips in 35-mm plastic petri dishes and flood with Hanks' Minimal Essential Medium/10% bovine serum (see *UNIT 1.1* for basic cell culture techniques). Incubate 72 hr.

Figure 21.2.1 Zeiss upright LSM 510 microscope contained within a 37°C environmental control incubator. In the authors' laboratory, the enviromental control chamber is a specially built Plexiglass box with access hatches (Cancer Research UK workshop). The essential components for the heater and control system are a centrifugal fan (part no. 40BTFL from Air Flow Developments), and a temperature controller (208-2739), thermocouple type T (219-4680), box (584-615), mini-3-pole plug (449-269), mini-3-pole socket (449-275), fuse holder (418-603), optical relay (394-535), type T panel socket (219-4860), type T line plug (219-4876), enclosed heater (224-565), and thermocouple connector (219-4876), all from RS Components. A similar commercially available system (Microscope Temperature Control System) may be obtained from Life Imaging Services (see Internet Resources).

MEM has been optimized by the authors at Cancer Research UK for the cell lines used here. However, it might be possible to obtain satisfactory results with the MEM formulation supplied by Sigma.

In the authors' laboratory, experiments usually require that the cell density still be well below confluence (~50%) after this incubation.

2. Microinject the two cDNA constructs at concentrations of 50 ng/μl (YFP-actin) and 75 ng/μl (CFP-actin) into the nuclei of 20 to 30 cells located near the center of each coverslip. Return to incubator for 2 to 3 hr.

   Expression and folding times may vary widely with other fluorescent protein constructs.

3. During the incubation, switch environmental heating and argon laser on the LSM 510 to on and open a database with previous software settings (see Support Protocol 1).

4. Assemble a coverslip culture (from step 2) onto an optical chamber filled with medium and any experimental reagents. Seal chamber with hot wax mixture, wash outside of chamber with clean distilled water, and blow dry before placing on the stage of the LSM 510.

Perform microscopy

5. Restore settings for tile scanning by selecting the previous tile scan from the saved database and reusing the settings (Support Protocol 1). Check that scan rate is now at fastest setting and laser power settings are correct.

Tile scanning provides a montage of adjacent fields acquired sequentially in order to overview a large area for identification of expressing cells.

6. Use the stage control joystick to locate the center of the chamber on the microscope axis, switch to direct viewing using a low-power phase objective (5× to 25×), change phase setting of condenser as necessary, and focus on cells manually.

7. Start Tile scan in Stage controls.

   The authors generally use a 4 × 4 tile array with 256 × 256 pixel images.

8. Mark the expressing cells. Create an image database and use Save As to store the tile scan.

9. Restore software settings for FLAP imaging by selecting a FLAP sequence from the saved database and reusing the settings (Support Protocol 1). Use the stage-control joystick to move the chamber away from objective, apply immersion oil, and recentralize chamber by moving to the first marked cell. Switch to high-aperture oil-immersion objective (63× Plan apochromat Ph3) and change phase setting of condenser as necessary.

   CAUTION: *Do not attempt to switch objectives automatically unless it has been ascertained that they are accurately parfocal (Support Protocol 1) and that the lower surface of the upper coverslip is in focus. The high-power objective may have a very short working distance and any error could cause a disastrous crash between the objective and the specimen.*

10. Start a fast scan while displaying split channels and manually focus on a cell.

    The interference reflection microscopy (IRM) image will brighten suddenly when one is focused on the lower surface of the upper coverslip. A small distance below this surface (turn top of fine focus knob away from you) will usually be the optimal setting with maximal brightness of the fluorescence images.

11. If necessary, centralize the cell in image field using the X/Y buttons and the nudge wheel on the joystick control. Cease fast scanning as soon as possible.

    Note that the IRM image will often be out of focus at the optimal setting for fluorescence imaging; a compromise is necessary if good IRM images are required.

12. Select orientation and zoom factor (the authors usually keep a standard zoom of 2) and do a single scan.

    When it is critical to record fast molecular dynamics immediately after bleaching, note that the top of the reoriented image will be scanned first.

13. Click the use ROI button to set up region of interest (ROI) if required in order to reduce scan times.

    It is sometimes advantageous to image the whole cell for accurate estimates of fluorophore fade during imaging (Support Protocols 2). Note that the top one or two scan lines will sometimes show a defective match between channels and should be avoided (see Troubleshooting).

14. Do a single scan while displaying split channels. Switch to the custom Hall palette (Support Protocol 1). Using a minimal number of further scans, adjust laser gains and offsets so that the two fluorescence channels are accurately matched with no saturation and with a low but finite background intensity. Adjust settings for the other two channels if necessary. Return to the no palette display.

    If matched, the fifth combined image should show the fluorescent regions as pure white (Fig. 21.2.2). The phase-contrast and IRM channels should show good contrast.

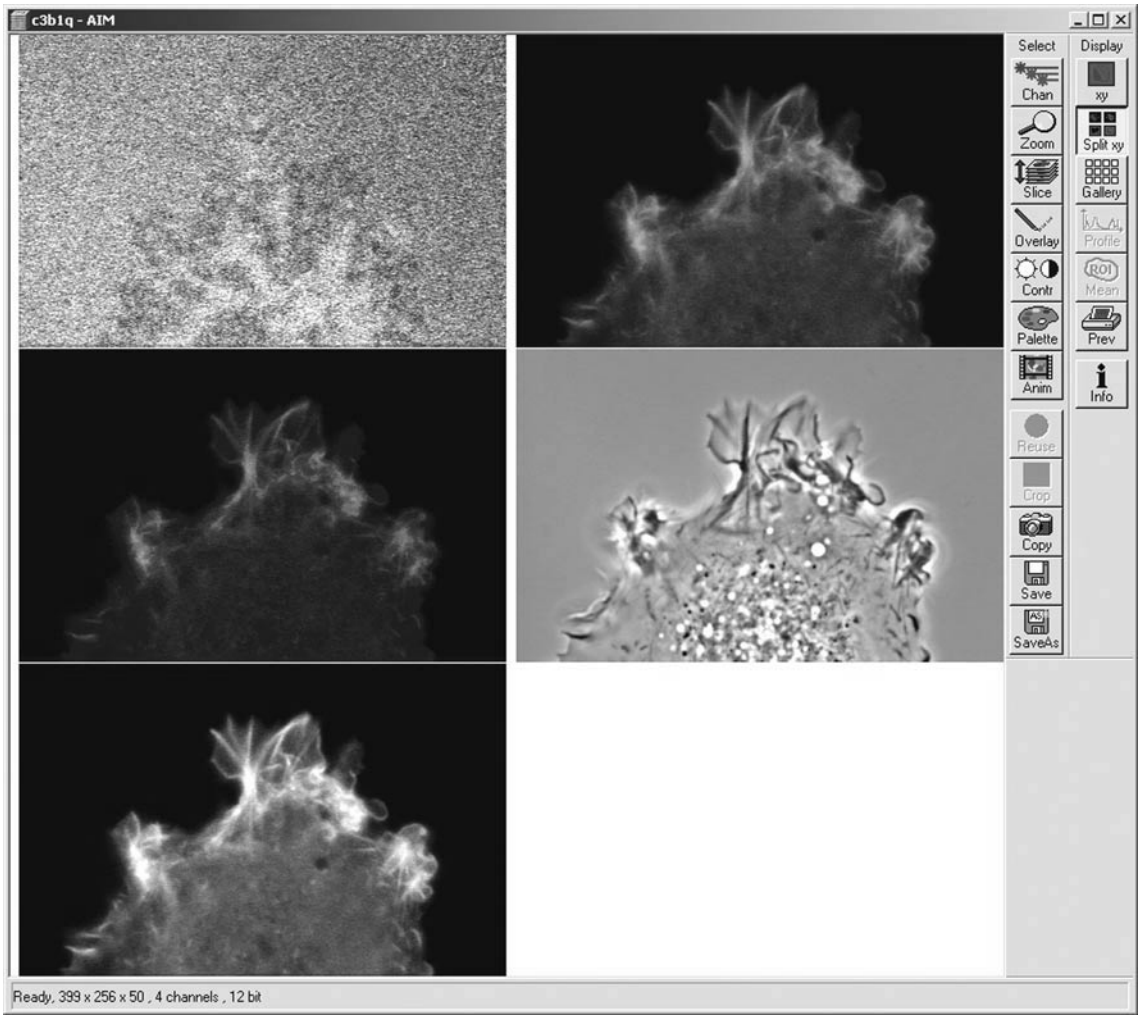


Figure 21.2.2 Window from Zeiss LSM software showing four channels and fifth combined channel after setting gains and offsets and laser powers. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss UK. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

15. Select the bleach panel, choose a bleach region, and change the bleach parameters if necessary.

    The authors use 50 bleach scans at the full-power laser setting. Note that bleach times depend on the height of the bleach region (regardless of its shape) but not on its width, so a horizontal narrow strip will bleach a large area rapidly.

16. Select the time series panel and change the time-lapse parameters if necessary. Do one single scan to check that everything is still OK (i.e., that the current setup still provides a satisfactory image of the selected cell). Choose the number of scans to average and start the recording.

    The authors normally average four scans per frame and record two frames before bleaching and 50 afterwards with no time-lapse interval. Recording of the FLAP signal is best monitored in a large window showing the combined image. After bleaching, the bleach-labeled molecules will then show as a bluish color against a white background (Fig. 21.2.3). Avoid transmitting any vibrations to the microscope during recording.

17. Save the recorded sequence if satisfactory, export the bleach region image for later reference (showing the bleach region and with the zoom aspect set to 1:1) as a

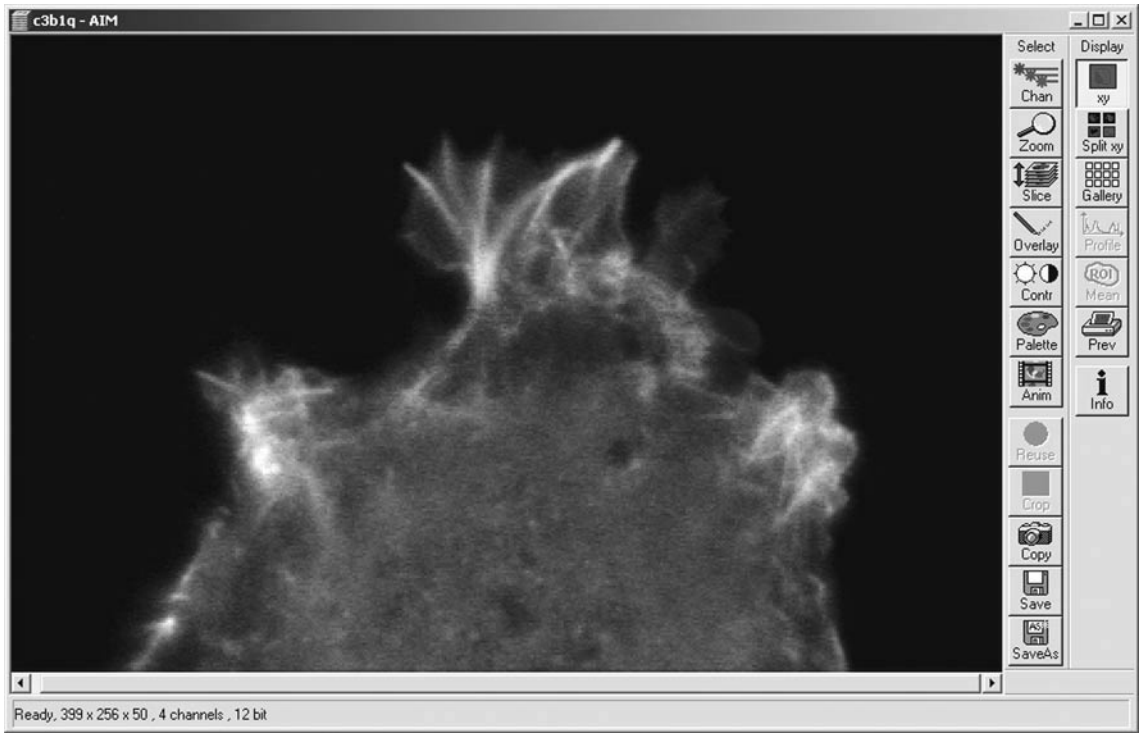


Figure 21.2.3 Window from Zeiss LSM software showing only the combined channel after bleaching during data recording. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss U K. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

single-image TIFF file, reset the scan averaging to 1, move to the next marked cell using the stage-control panel, and repeat steps 10 through 16 for all marked cells. Return to step 5 to start a new culture chamber.

SUPPORT PROTOCOL 1

SETTING UP THE LSM 510 AND ITS SOFTWARE

It is important that the microscope be configured optimally—e.g., pinhole alignment should be set correctly. Optimization of settings for tile scans and FLAP imaging will require a trial run through the Basic Protocol.

Materials

Zeiss upright LSM 510 microscope and software (see Basic Protocol 1)
Small beads (e.g., TetraSpeck microspheres, 0.2 μm; Molecular Probes)

1. Make sure that the objectives to be used are accurately parfocal using small beads and Parfocal Settings from the `CLM32.EXE` program.
2. Define the pseudocolor lookup table (LUT) for the custom Hall palette (Fig. 21.2.4) to be used for gain and offset adjustments and for viewing fully processed FLAP images.

   Other preferred LUTs may be used, but it is critical that minimum and maximum gray levels be easily distinguishable from the rest.

3. For tile scans, use three channels (CFP fluorescence, YFP fluorescence, and phase contrast), a low number of pixels (256 × 256), and fast scanning.

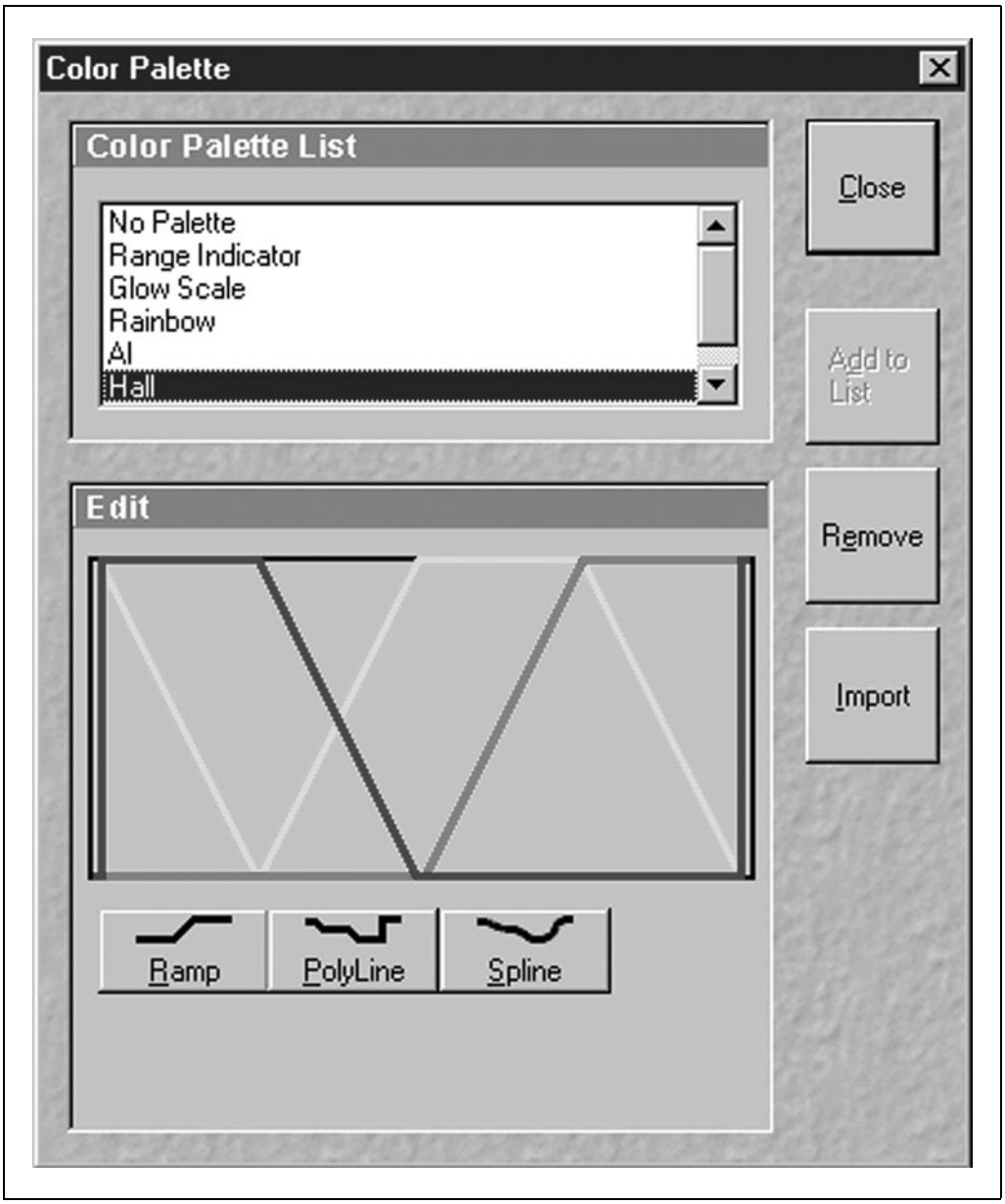


Figure 21.2.4 Window from Zeiss LSM software showing custom Hall pseudocolor palette. Note that the lowest intensity level is coded as black and the highest as white. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss UK.

4. For FLAP imaging, set the microscope to use 4 channels: CFP fluorescence, YFP fluorescence, phase contrast, and IRM (Fig. 21.2.5) with 512 × 512 pixels.

   Tile scan is used to identify expressing cells for FLAP imaging.

5. Set (typically) laser line 514 nm power to 1% and 548 nm power to 21%; pinhole diameters Ch1 to 408 μm; Ch2 to 409 μm; and Ch3 to 414 μm.

   These pinhole sizes give optical sections of 3 μm in all channels.

6. Set dichroic mirror HT to HTF 458/514; set dichoic mirror NT1 to NFT 635 VIS; set dichroic mirror NT2 to NFT 545; set dichroic mirror NT3 to Plate; set filter Ch1 to None for IRM; set filter Ch2 to BP 475-525 for CFP fluorescence; set filter Ch3 to LP 530 for YFP fluorescence; and set filter ChD for phase-contrast microscopy (Fig. 21.2.5).

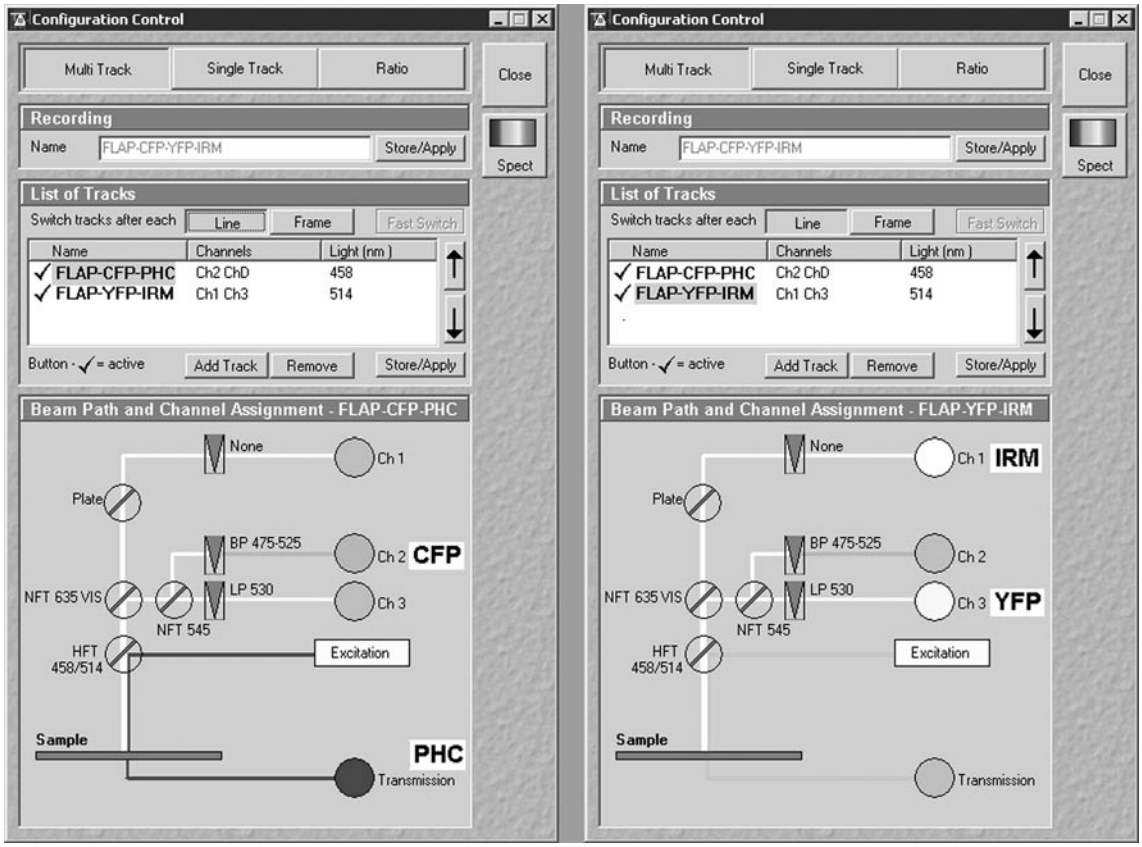


Figure 21.2.5 Window from Zeiss LSM software showing both tracks on the configuration panel. Courtesy˜of Carl Zeiss, Germany; reprinted with permission of Zeiss UK.

7. Set pixel size to 0.29 μm × 0.29 μm and pixel dwell time to 1.6 μsec.
8. Use laser line 514 nm under the bleach setup at maximum power measured as 1.32 mW.
9. Set palette of the CFP fluorescence channel to RGB = (0, 128, 255) and YFP fluorescence channel to RGB = (255, 128, 0) so that the cyan and yellow channels are easily identified and equal intensities combine to a white image when the phase contrast and IRM are switched off from the overlay panel.
10. Save settings by saving the trial run as a database for future Reuse. Do not check Re-use Objective in the Save Settings menu, to avoid unexpected movements of the objective revolver.

Further runs will be required to refine gain and laser power settings for fade matching of channels (Support Protocol 2).

SUPPORT PROTOCOL 2

IMAGE PROCESSING AND ANALYSIS

The authors perform the image processing in Mathematica (Wolfram Research). This has the advantage of immense flexibility over dedicated image-processing software and can be programmed to read the information in the `*.lsm` Zeiss file headers. A commercial image acquisition and processing program from Kinetic Imaging (*http://www.kineticimaging.com*) can also read these file headers as, of course, can the LSM Reader software distributed as a free download by Zeiss. The LSM Reader will also export the individual images of the four channels as 12-bit TIFF format files. The required

image processing is fairly basic, and it will be assumed that a package is available that can read and operate on the raw 12-bit TIFF files exported from LSM Reader.

Materials

Mathematica v 4.2 or 5 (Wolfram Research) or a dedicated image-processing package capable of processing 12-bit TIFF images

1. Subtract a constant from the intensity values of all the CFP and YFP images (it may be different for each image) so that the background regions outside the cell(s) have a mean intensity of zero. Set all negative intensity values to zero.

   The intensity values should now be proportional to the number of fluorescent molecules at each location if care was taken to avoid saturation in step 14 of the Basic Protocol.

2. Multiply the intensity values of the last pre-bleach YFP image by a factor so that the total image intensity matches that of the last pre-bleach CFP image.

   The two images should now look identical.

3. Multiply all the remaining YFP images by the factor obtained in step 2.

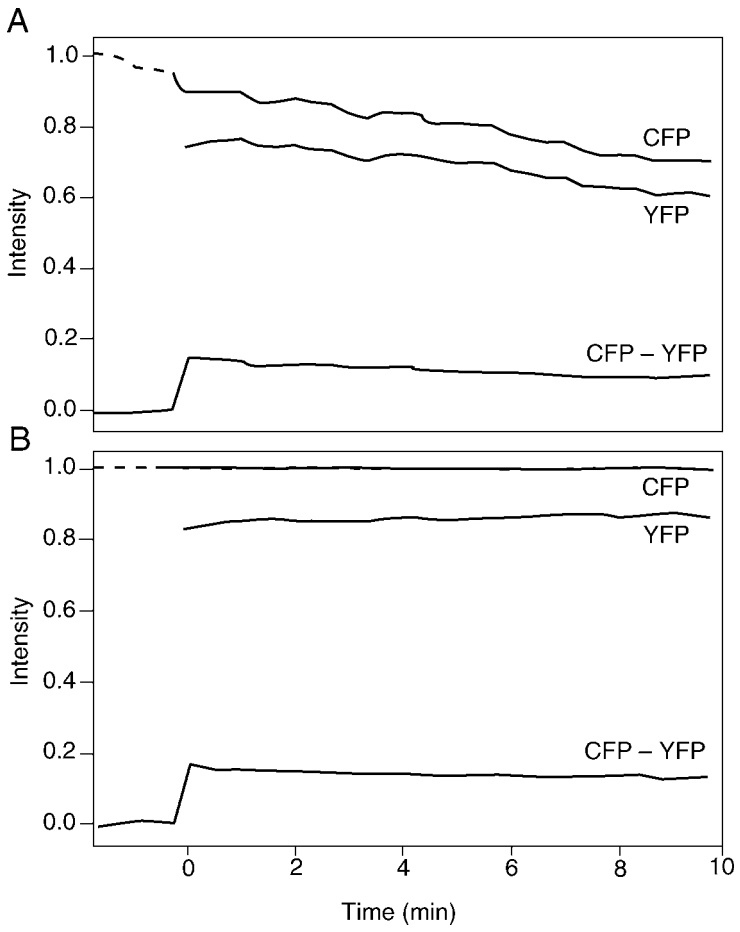


Figure 21.2.6 (**A**) The summed intensity values for a whole cell during a 10-min time series after step 3 in Support Protocol 2. These values have been normalized so that the total intensity of the first CFP image is 1. (**B**) The same intensity values after fade compensation as in step 4 of Support Protocol 2. This consists of dividing each CFP image by a factor so that the total intensity is 1 and then dividing the corresponding YFP image by the same factor. Note that fluctuations due to cyclical focus drift have also been compensated.

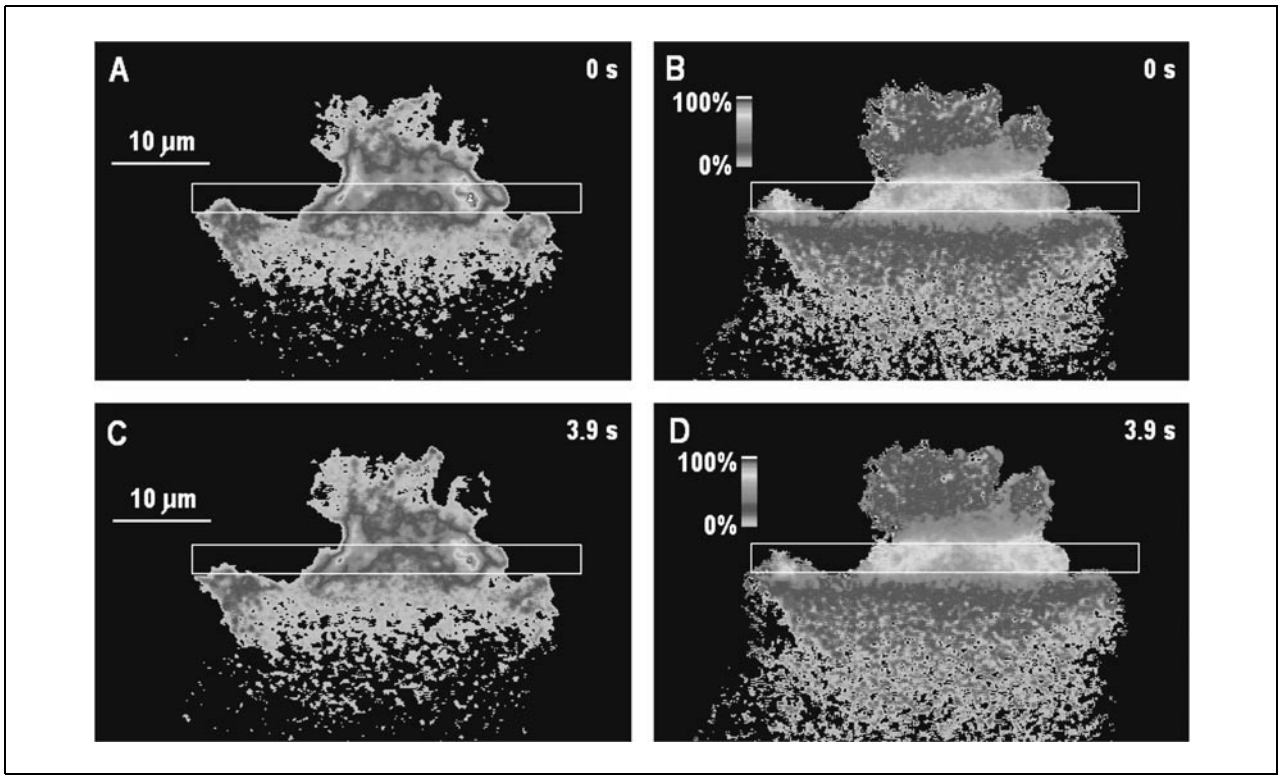


Figure 21.2.7 Absolute FLAP (**A, C**) and relative FLAP (**B, D**) images of the cell featured in Figures 21.2.2 and 21.2.3 recorded immediately after bleaching (**A, B**) and 3.9 sec later (**C, D**). Bleach box is shown as white rectangle. Hall palette. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

If the YFP and CFP channels do not fade at the same rate (see Support Protocol 1), it will be necessary to change this factor for each YFP image. This is best calculated if a whole cell is contained within the image. Plots of the total image intensity of each channel against time will then reveal the fade rates and any compensation that is necessary (Fig. 21.2.6A).

4. If planning to calculate absolute FLAP images, multiply all images after the first pair by a factor to compensate for fade.

   The fade rate is best calculated on whole cells as in step 3. After compensation, plots of the total image intensity of each channel against time should be horizontal lines with a step fall at bleaching in the YFP channel (Fig. 21.2.6B).

5. For each pair of simultaneous images, subtract the YFP image from the CFP image. Set all negative intensity values to zero.

   If fade compensation has been performed as in step 4, the resulting images are now absolute FLAP images.

6. Divide each image obtained in step 5 by the corresponding CFP image to obtain the relative FLAP images.

   Note that this operation has an infinite or undefined result for pixels in which the CFP intensity is zero and an unreliable result where intensity values are low. A threshold level of CFP intensity should be chosen (by trial and error) below which the result of this operation is set to zero.

7. View the final FLAP images, if desired, using a pseudocolor lookup table or palette.

   The authors often use the Hall palette (Support Protocols 1). The relative FLAP images (Fig. 21.2.7B) have intensity values in the range 0 to 1, and a scale bar showing the corresponding pseudocolors can be calibrated from 0% to 100%, showing the fraction

of bleached YFP molecules in each pixel. Absolute FLAP images (Fig. 21.2.7A) could in theory be calibrated for numbers of molecules, but this would require knowing numbers of expressed and native molecules for the whole cell. If the images are noisy and the noise levels in the raw images cannot be improved further (see Troubleshooting), there are two helpful image-processing procedures. A 3 × 3 or higher-order smoothing filter can be applied to the raw images before or after step 1, and/or a threshold can be used below which values are displayed as zero when viewing the FLAP images. Note that smoothing filters other than a block mean may change the intensity value total.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

cDNA constructs of eCFP-β-actin and eYFP-β-actin

Use a pEGFP-β-actin construct (Dunn et al., 2002; *daniel.zicha@cancer.org.uk*) as the basic starting vector. Use enhanced yellow and cyan fluorescent protein coding regions from pEYFP-C1 and pECFP-C1 (Clontech), respectively, to substitute the eGFP coding region using conserved restriction enzyme sites (James Monypenny and Daniel Zicha, Cancer Research UK; *daniel.zicha@cancer.org.uk*). Maintain the 45-bp linker (TCC GGA CTC AGA TCT CGA GCT CAA GCT TGC GGC CGC GCC GCG GCC) between eGFP and β-actin in the pEGFP-β-actin vector in the new fusions. Microinject the DNA in distilled water. The amino acid translation (SGLRSRAQACGRAAA) has net positive charge and 40% hydrophobic residues, ensuring linker flexibility in a cytosolic environment.

Hot wax mixture

Prepare a 1:1:1 (w/w/w) mixture of beeswax, soft yellow paraffin, and paraffin (melting point 46°C) maintained at 54°C in a wax bath.

All of the above materials are available from Fisher.

Optical chambers

Fabricate optical chambers from 76 × 26–mm (as cut) glass microscope slides (Chance Propper), No. 3, 76 × 26–mm glass coverslips, and No. $1\frac{1}{2}$, 18 × 18–mm glass coverslips (Chance Propper). Drill a ~10 mm hole centrally through the glass slide using a diamond-tipped drill (Proxon) under water. Fix a 76 × 26–mm coverslip to one face using Sylgard 184 silicone elastomer (Dow Corning) and allow it to set overnight at room temperature.

While in use, the other face of the medium-filled chamber is sealed with an 18 × 18–mm coverslip carrying the cell culture (see Basic Protocol).

COMMENTARY

Background Information

Fluorescence microscopy has long been the most important tool for revealing the changing distribution of specific proteins within living cells, but it is only recently that fluorescence methods have enabled the study of other aspects of molecular dynamics (see reviews by Lippincott-Schwartz et al., 2001; Holt et al., 2004). One serious limitation of conventional fluorescence localization is that it reveals little of the relocation of molecules during cyclical, steady-state dynamics. Thus critical activities such as the constant depolymerization, diffusive transport, repolymerization, and treadmilling of structural molecules of the cytoskeleton cannot be visualized by this means.

Two methods developed more recently for studying the relocation of molecules, fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), can have a sufficiently high time resolution to enable diffusion rates of proteins

within the cytoplasm to be estimated (see review by Lippincott-Schwartz et al., 2001). Their chief disadvantage is that the bleached molecules themselves cannot be tracked. On the other hand, fluorescent speckle microscopy (FSM) permits the study of slow molecular movements such as treadmilling and translocation within polymerized structures (*UNIT 4.10*; Waterman-Storer and Salmon, 1997). It relies on a low percentage of tagged molecules (<1%) to produce a discontinuous pattern that can then be used to determine whether the labeled polymer is stationary or translocating. It cannot detect the much more rapid movements of freely diffusing molecules, but this is considered an advantage because it identifies which molecules are polymerized. A refinement of this technique detects the signals from single fluorophores and permits the sites of polymerization and depolymerization of the tagged molecule to be located and their rates to be estimated (Watanabe and Mitchison, 2002). Although complementary to some extent, FRAP, FLIP, and FSM cannot easily provide a full picture of the dynamics within a cyclical polymerization/depolymerization system because the depolymerized molecules cannot be tracked directly.

In contrast, photoactivation of fluorescence (PAF) allows specific molecules to be "flash" labeled at a chosen site and then tracked directly, regardless of whether or not they are polymerized. Earlier PAF methods relied on caged fluorophores and, although they yielded much significant information in the hands of skilled investigators (see review by Mitchison et al., 1998), results were generally disappointing. Uncaging the fluorophore requires exposure to intense UV light, and radiation damage cannot be ruled out. Moreover, molecules tagged with caged fluorophores have to be microinjected or otherwise introduced directly into the cell, and often clump or fail to distribute to their natural locations within the cell. This latter shortcoming led to the development of fluorescent proteins that could be expressed by the cells from cDNA constructs and photoactivated directly. Again, early results were disappointing, and it is only very recently that efficiently photoactivatable fluorescent proteins have been developed (Patterson and Lippincott-Schwartz, 2002; Chudakov et al., 2003). These promise to contribute a great deal to the knowledge of cellular dynamics in the future.

The FLAP method is an alternative approach to the problem of direct and localized photolabeling of a molecular species (Dunn et al., 2002). There is no need to use specialized fluorophores. The principle is simple and relies on photobleaching instead of photoactivation to label the targeted molecules. Suppose that, instead of a single fluorophore, each molecule carries two fluorophores that have differing spectral properties so that they can be imaged separately. If only one of the fluorophores is photobleached, then the resulting molecules are unambiguously labeled: they are the only molecules carrying just one active fluorophore. Although the bleached molecules cannot be imaged directly, one fluorescence image shows where all the molecules are, while the other shows only where the unbleached molecules are. If image intensities have been matched, simply subtracting the second image from the first generates an absolute FLAP image that shows where the bleach-labeled molecules are. Thus FLAP combines the best features of FRAP and PAF. In FRAP, the molecules can no longer be imaged after they have been targeted, whereas in PAF they cannot usually be imaged before they have been targeted. With FLAP, full information is retained on the distributions of both targeted and nontargeted molecules. It is therefore possible to construct other useful virtual images such as relative FLAP, which shows the percentage of bleach-labeled molecules at any given site (Zicha et al., 2003).

It is not an absolute requirement of the FLAP method that a single molecule carry both fluorophores. Indeed, there are some advantages when they are carried by different individual molecules of the same species. In this case, if each colocalizes with the corresponding native molecules as expected, they will still colocalize with each other. However, colocalization now depends on the statistical properties of molecular distribution and, in order to obtain noise-free FLAP images, it is important that both tagged molecules be present in large numbers. This is usually not a problem if the corresponding native molecules are abundant in the cell. It is hard to specify how many molecules need to be carrying fluorophores compared to the number of native molecules, since this depends on many other factors such as detection efficiency, pixel size, and post-processing. A general guideline is that results comparable to PAF or better will be obtained if the two fluorophores give well matched, noise-free images prior to photobleaching. The effect of having the fluorophores on different molecules is, roughly, that the error noise in the final FLAP image is doubled (see Critical Parameters).

The chief competitors to FLAP for flash labeling molecules at specific sites are PAF techniques. Each method has advantages and shortcomings. Although first in the field, the earliest PAF methods using caged fluorophores are probably now the ones with the most disadvantages. First, there are difficulties in conjugating and purifying the protein in sufficient quantity without impairing its activity, as well as in introducing it into cells so that it colocalizes with native protein. A serious limitation is that caged compounds cannot yet be incorporated into cDNA constructs and expressed by the cells. The photoactivatable fluorescent proteins are much more promising in this respect, and recent developments suggest that some will soon be able to be activated and deactivated repeatedly and efficiently (Chudakov et al., 2003), which will be a decided advantage over current FLAP methods.

On the other hand, an interesting feature of the FLAP method as described here is that there is much less need to worry about photodamage occuring to the photolabeled molecules. The method calculates where the virtual bleached molecules are located, i.e., where the bleached molecules would be assuming that they were not damaged, and it is not of much concern what happens to the real bleached molecules. While it is still possible that reactive intermediates generated by photobleaching might cause problems, the compact barrel-like structure of GFP and its variants is thought to shield the external environment from these (Lippincott-Schwartz et al., 2001). Coupled with this, FLAP has the advantages of not requiring specialized fluorophores, and the bleaching process is rapid and efficient using the 514-nm line of a standard argon laser on the LSM 510, whereas photoactivation may require an additional blue diode laser (the alternative two-photon photoactivation is very slow; *UNIT 4.11*). The main advantage of FLAP, however, is that all the tagged molecules are available for imaging throughout the experiment, whether or not they have been photobleached. In the authors' experiments (see Anticipated Results) it has been possible to see throughout where all the actin is, as well as where it has been FLAP-labeled. It may turn out to be possible to do this in conjunction with PAF, but it will require either a second reference fluorophore, as with FLAP, or a second way of exciting the molecules to fluoresce before photoactivating them.

A potential shortcoming of the FLAP method is that colocalization of the two fluorophores is not perfect, but relies on the statistical properties of large numbers of individual molecules. In practice, the authors have not found this to be a problem and have obtained results that compare very favorably with photoactivation methods (but see Critical Parameters). For the future, however, one way around this problem would be to achieve almost perfect colocalization by fusing both fluorophores to the same molecule. This has not been done in this unit because, besides the potential problems of creating a bulkier molecule that may no longer colocalize with corresponding native molecules, it would no longer be possible to ignore any photodamage that might occur to the bleached molecules. Moreover, there is an additional complication that the two fluorophores used in this unit are capable of fluorescence resonance energy transfer (FRET; *UNIT 17.1*) when very close together on the same molecule. Even so, there are early indications that the FRET complication may not be a serious disadvantage and may actually enhance the FLAP signal; however, there are still worries that the FRET efficiency may alter during configuration changes such as polymerization.

Critical Parameters

Expression levels are critical. At high expression levels, there is a danger that FRET may occur in regions of high fluorophore density even when the two fluorophores are carried on separate molecules as described in this unit (see Troubleshooting). On the other hand, the FLAP images would be unusably noisy if the density of fluorescent molecules were very low, as is required by the FSM method. This is especially true when the fluorophores are carried on separate molecules. The binomial theorem can be used to predict these statistical errors in the FLAP signals. Consider first the case in which there happen to be 1000 molecules of CFP-YFP-actin in a pixel and half of them are expected to have a photobleached YFP. Theory predicts that, in repeated experiments, the number of molecules found to be bleached by absolute FLAP would be 500 ± 16 (mean $\pm$ SEM). In the case of relative FLAP, the bleached fraction would be 0.5 ± 0.016. Now, consider the case in which the two fluorophores are not on the same molecule but there still happen to be 2000 fluorophores in the pixel: half are expected to be CFP-actin and half YFP-actin. Half of the latter are expected to have been photobleached. Theory now predicts that the number of molecules found to be bleached by absolute FLAP would be 500 ± 37, and the fraction of YFP-actin molecules

found to be bleached by relative FLAP would be 0.5 ± 0.027. Thus, one can conclude that having the fluorophores on different molecules approximately doubles the error of the FLAP estimates. This also holds true if there are only 200 fluorophores in the pixel, in which case the figures are: 50 ± 5; 0.5 ± 0.05; 50 ± 12; and 0.5 ± 0.09, respectively. The relative errors are proportional to the square of the number of molecules, so it is necessary to express four times as many fluorescent molecules in order to halve the relative error.

A further requirement, as with most fluorescence microscopy, is that the tagged molecules accurately colocalize with native molecules of the same species. There is evidence that GFP variants, fused to actin in the configuration used by the authors, do this well (Choidas et al., 1998). Ensuring that the two different variants are incorporated into otherwise identical cDNA constructs will give the best colocalization for FLAP even when colocalization with the native species is slightly impaired.

Even the best apochromatic objectives show a significant lateral and axial chromatic aberration. This means that the CFP and YFP images may not exactly coincide both laterally and vertically, and it is necessary for good results that the cell be close to the optical axis of the objective (see Troubleshooting) and that there be no drift in focus. Temperature stabilization is essential for maintaining a fixed focal plane. In the past, the authors have obtained satisfactory results using a fan and temperature-controlled heater within a spacious microscope dust cover placed over the entire microscope. However, a properly constructed air-flow chamber (Fig. 21.2.1) is essential for consistently good results. The thermocouple is placed very close to, but not touching, the optical chamber (see Troubleshooting). The temperature controller is

Table 21.2.1 Troubleshooting Guide to FLAP

Problem	Possible cause	Solution
Regions of cell edge show in FLAP images even before bleaching	Chromatic aberration	Minimize by using fluorescent beads to find best region of objective.
Rhythmic noise or parallel lines	Vibration from heater, thermocouple, table, or computer	Eliminate fan/computer vibration; make sure that table is properly isolated.
Focus drift	Temperature fluctuation	Check heater stability and presence of drafts.
Artifacts on top 1-2 lines of first image scanned after a frame interval	Software bug in acousto-optic driver	Avoid frame intervals if possible. Avoid placing critical part of cell near top of image.
Difference signal is not zero everywhere before bleach	Images not matched	See Basic Protocol, step 14 or match images during post-processing.
	Fluorophores not colocalized	Choose new fluorophore(s).
Striped or fluctuating phase image	Polarizing filter in the light path	Remove polarizing filter from the light path.
Lower (negative) FLAP signal in areas of dense expression before bleach	FRET	If unacceptable, use shorter expression times, compensate during post-processing, or choose new fluorophore(s).
Noisy fluorescence images on one or both channels.	Low expression	Wait longer for expression. Adjust cDNA injection ratio.
	Too much gain in photodetector amplifier	Increase laser power and reduce gain (but keep fade rates matched if possible).
	Too much magnification (pixels too small)	Use lower zoom factor or average more frames.

trained to maintain a temperature of 37°C, and should do so, ideally, within a range of ± 0.1°C.

Troubleshooting

See Table 21.2.1 for a troubleshooting guide.

Anticipated Results

FLAP has been used to estimate the diffusion rate of G-actin in cytoplasm (Zicha et al., 2003), and the result agrees well with previous estimates using FRAP (McGrath et al., 1998). Relative FLAP has proven particularly useful for detecting the rapid transport of actin to the cell's leading edge from regions up to 12 μm behind the edge (Zicha et al., 2003). Four FLAP images showing some of the results obtained from the cell featured in Figures 21.2.2 and 21.2.3 are shown in Figure 21.2.7. Note that the absolute FLAP images (Fig. 21.2.7A and C) show the highest intensity in parts of the bleach region where the density of actin is greatest (see Fig. 21.2.3), whereas relative FLAP (Figure 21.2.7B and D) gives a more uniform signal throughout the bleach region. In the later relative FLAP image which started 3.9 sec after bleaching (Figure 21.2.7D) highly labeled actin (∼60%) can be seen at small regions of the cell's leading edge. This phenomenon is not noticeable in the absolute FLAP image (Figure 21.2.7C) because these thin marginal regions have a relatively low density of actin. When interpreting the FLAP images, bear in mind that the top of the image has been scanned first and that there can be a time difference of several seconds between the top and bottom of the image. It is especially important to compensate for the scanning time when fitting mathematical models of fast molecular processes such as diffusion of monomer (see Internet Resources).

Time Considerations

Cell culture

Plan ahead by at least 72 hr to allow for sufficient cell density, depending on growth rate of cell type.

Microinjection

This should take no more than 15 min if injecting cells at room temperature without CO_2.

Expression of constructs

Allow at least $2\frac{1}{2}$ hr for expression and maturation of GFP and variants.

Filming chamber preparation

Chambers should be prepared well in advance (usually left overnight) and kept sterile in a sealed container until needed.

Literature Cited

Choidas, A., Jungbluth, A., Sechi, A., Murphy, J., Ullrich, A., and Marriott, G. 1998. The suitability and application of a GFP-actin fusion protein for long-term imaging of the organization and dynamics of the cytoskeleton in mammalian cells. *Eur. J. Cell Biol.* 77:81-90.

Chudakov, D.M., Belousov, V.V., Zaraisky, A.G., Novoselov, V.V., Staroverov, D.B., Zorov, D.B., Lukyanov, S., and Lukyanov, K.A. 2003. Kindling fluorescent proteins for precise in vivo photolabeling. *Nat. Biotechnol.* 2:191-194.

Dunn, G.A., Dobbie, I.M., Monypenny J, Holt, MR., and Zicha, D. 2002. Fluorescence Localization After Photobleaching (FLAP): A new method for studying protein dynamics in living cells. *J. Micros.* 205:109-112.

Holt, M.R., Soong, D.Y.H., Monypenny J., Dobbie, I.M., Zicha, D., and Dunn, G.A. 2004. Using bioprobes to follow protein dynamics in living cells. *In* Cell Motility: From Molecules to Organisms, Chapter 7 (A. Ridley, P. Clark, and M. Peckham, eds.) John Wiley and Sons, Hoboken, N.J..

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* 2:444-456.

McGrath, J.L., Tardy, Y., Dewey, C.F., Jr, Meister, J.J., and Hartwig, J.H. 1998. Simultaneous measurements of actin filament turnover, filament fraction, and monomer diffusion in endothelial cells. *Biophys. J.* 75:2070-2078.

Mitchison, T.J., Sawin, K.E., Theriot, J.A., Gee, K., and Mallavarapu, A. 1998. Caged fluorescent probes. *Methods Enzymol.* 291:63-78.

Patterson G.H. and Lippincott-Schwartz, J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297:1873-1877.

Watanabe, N. and Mitchison, T.J. 2002. Single-molecule speckle analysis of actin filament turnover in lamellipodia. *Science* 295:1083-1086.

Waterman-Storer, C.M. and Salmon, E.D. 1997. Actomyosin-based retrograde flow of microtubules in the lamella of migrating epithelial cells influences microtubule dynamic instability and turnover and is associated with microtubule breakage and treadmilling. *J. Cell Biol.* 139:417-434.

Zicha, D., Dobbie, I.M., Holt, MR., Monypenny J., Soong, D.Y.H., Gray, C., and Dunn, G.A. 2003. Rapid actin transport during cell protrusion. *Science* 300:142-145.

Key References

Dunn et al., 2002. See above.

First description of the technique.

Zicha et al., 2003. See above.

Describes an application of the technique.

Internet Resources

http://www.lis.ch

Web site of Life Imaging Services, which supplies microscope temperature control systems.

http://www.zeiss.com/us/micro/home.nsf/Contents-FrameDHTML/286BA4D22B14DEE985256B4A007C3686

Zeiss Web site from which LSM Reader can be downloaded.

http://www.sciencemag.org/cgi/content/full/300/5616/142/DC1

Supplementary online material for Zicha et al. (2003). Describes diffusion modeling.

Contributed by Graham A. Dunn, Mark R. Holt, and Daniel Y. H. Soong
The Randall Division
King's College London
London, United Kingdom

Colin Gray and Daniel Zicha
Cancer Research UK
London Research Institute
Lincoln's Inn Fields Laboratories
London, United Kingdom

Visualization of Protein Interactions in Living Cells Using Bimolecular Fluorescence Complementation (BiFC) Analysis

Protein interactions control many cellular functions. The functional characteristics of many proteins are determined by the proteins that they interact with in each cell. Interactions with different partners and in response to different extracellular signals can occur in different subcellular locations. Determination of the locations of protein complexes and their regulation can provide insight into the functional roles of the protein interactions.

This unit describes the bimolecular fluorescence complementation (BiFC) assay for the visualization of protein interactions in living cells (Hu et al., 2002). This approach is based on complementation between nonfluorescent fragments of the yellow fluorescent protein (YFP) when they are brought together by an interaction between proteins fused to each fragment (Fig. 21.3.1). The BiFC assay allows direct visualization of the subcellular locations of protein interactions in living cells (see Basic Protocol). This approach can be used for the analysis of many protein interactions and does not require information about the structures of the interaction partners.

Most proteins have a large number of alternative interaction partners in each cell. Many of these interactions are mutually exclusive, resulting in competition for shared interaction partners in the cell. To study the competition for interactions between alternative interaction partners in living cells, the authors of this unit have developed a multicolor BiFC assay by fusing fragments of different fluorescent proteins to the interaction partners (see Alternate Protocol 1). The multicolor BiFC assay is based on the use of fragments of fluorescent proteins with distinct spectral characteristics. Bimolecular complexes formed between these fragments can be visualized using different excitation and emission wavelengths, enabling parallel visualization of multiple interactions in the same cell. The

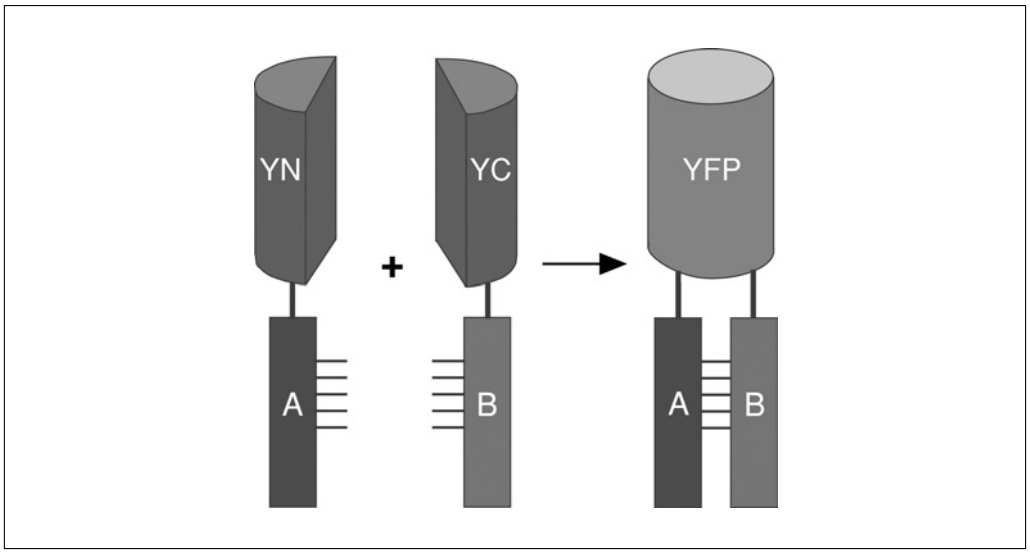


Figure 21.3.1 Schematic representation of the principle of the BiFC assay Two fragments (YN and YC) of the yellow fluorescent protein (YFP) are fused to putative interaction partners (A and B). The association of the interaction partners allows formation of a bimolecular fluorescent complex. For the color version of this figure, go to *http://www.currentprotocols.com.*

Contributed by Chang-Deng Hu, Asya V. Grinberg, and Tom K. Kerppola
Current Protocols in Cell Biology (2005) 21.3.1-21.3.21

multicolor BiFC assay allows comparison of the subcellular locations of different protein interactions in the same cell, as well as analysis of the competition among mutually exclusive interaction partners for complex formation with a common partner (Alternate Protocol 2).

NOTE: Cells are cultured in a humidified 37°C, 5% CO_2 incubator. Cells can be incubated in a 30°C, 5% CO_2 incubator to promote maturation of the fluorophore and thereby increase the signal (see Commentary). Cells are maintained under conditions required for each line (*UNIT 1.1*).

BASIC PROTOCOL

VISUALIZATION OF PROTEIN INTERACTIONS IN LIVING CELLS

The BiFC assay allows direct visualization of protein interactions in living cells based on complementation between nonfluorescent fragments of YFP or other fluorescent proteins.

Materials

Plasmid vectors for expression of proteins of interest (also see Critical Parameters): e.g., pFlag-CMV2 (Sigma) or pHA-CMV (Clontech)
DNA encoding amino acid residues 1 to 154 of yellow fluorescent protein (YN fragment; also see Critical Parameters and Table 21.3.1)
DNA encoding amino acid residues 155 to 238 of yellow fluorescent protein (YC fragment; also see Critical Parameters and Table 21.3.1)
Linkers (see Critical Parameters)
DNA encoding proteins (interaction partners, wild-type) of interest
DNA encoding mutated, single amino acid–substitution variants of the protein of interest that do not interact with each other (controls)
Cells that can be transfected using plasmid DNA (preferably adherent, monolayer cell line)
Appropriate culture medium (*UNIT 1.2*)
FuGENE 6 (Roche Diagnostics) or other transfection reagent
Tissue culture vessels appropriate for experiment: e.g., cluster plates, slide chambers, or glass coverslips
Inverted fluorescence microscope (*UNIT 4.2*) equipped with:
  Sensitive CCD camera
  20× to 100× objectives
  Filters for visualization of YFP (excitation 500 ± 10 nm; emission 535 ± 15 nm)
  Software for instrument control and data analysis
Additional reagents and equipment for expressing proteins in mammalian cells (see *APPENDIX 3A* for cross-reference), mammalian cell culture (*UNIT 1.1*), and immunoblotting (see *UNIT 6.2*)

Table 21.3.1 Selection of Fluorescent Protein Fragments for BiFC and Multicolor BiFC Analyses

Fluorescent protein fragments	Applications	Filters
YN155+YC155	BiFC analysis	YFP
YN173+YC173	BiFC analysis	YFP
CN155+CC155	BiFC analysis	CFP
YN155+CN155+CC155	Multicolor BiFC analysis	CFP YFP

Construct plasmid vectors

1. Using the appropriate plasmid vectors, construct mammalian expression vectors by fusing the sequences encoding amino acid residues 1 to 154 (YN) and residues 155 to 238 (YC) of the yellow fluorescent protein to sequences encoding the proteins of interest. Whenever possible, include fusions to both the N- and C-terminal ends of the proteins to be investigated (Fig. 21.3.2). As controls, construct plasmids encoding mutated variants of the proteins that do not interact with each other.

   For additional information about the design of fusion constructs, see Critical Parameters; for cross-references to molecular biology techniques, see APPENDIX 3A. *The chimeric coding regions can be expressed in mammalian cells using vectors such as pFlag-CMV2 (Sigma) or pHA-CMV (Clontech).*

   As an alternative, fragments of the cyan fluorescent protein (CFP) may be used (see Table 21.3.1)

Prepare cells

2. Seed cells the day before transfection at a density that will allow for cell proliferation over the course of the experiment and, at the same time, allow for the effects of cell growth and density on the interaction under investigation.

   The BiFC assay can be used for the analysis of protein interactions in many mammalian cell lines. The authors have studied protein interactions using the BiFC assay in COS-1, HEK293, HeLa, Hep3B, αTN4, and NIH3T3 cells (Hu et al., 2002; Grinberg et al., 2004; Rajaram and Kerppola, 2004).

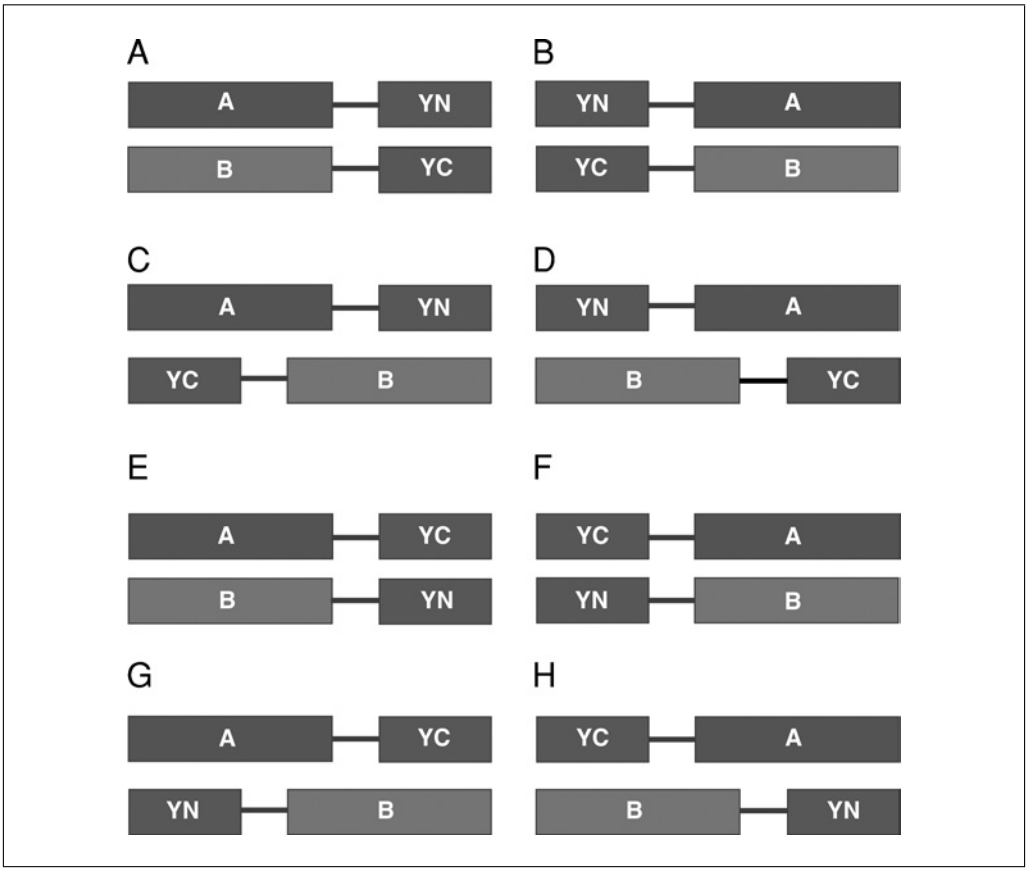


Figure 21.3.2 Multiple combinations of fusion proteins should be tested for bimolecular fluorescence complementation. Amino- and carboxyl-terminal fusions can be used to test eight distinct combinations in cells that are healthy and that have a morphology similar to nontransfected cells. For the color version of this figure, go to *http://www.currentprotocols.com*.

Cluster plates are convenient for processing multiple transfections in parallel. If short-working-distance objectives will be used to visualize the interaction, the cells should be grown in slide chambers or on glass coverslips.

UNIT 1.1 describes basic mammalian cell culture techniques.

3. When cells reach appropriate confluence (e.g., ~50%), transfect cells with appropriate amounts (e.g., 0.25 μg) of the BiFC plasmid (from step 1) encoding the fusion proteins. As controls, transfect cells in parallel with the plasmids encoding mutated variants of the proteins that do not interact with each other.

   FuGENE 6 from Roche Diagnostics is used according to the manufacturer's instructions to introduce plasmid DNA into cells, as it has low background fluorescence and requires minimal manipulation of the cells. Users need to follow specific protocols and optimize the conditions of transfection if other transfection reagents are used (UNITS 20.3-20.7).

4. Grow cells until fluorescence is detected (12 to 36 hr). If necessary, incubate the cells at 30°C with a 5% CO_2 atmosphere to promote maturation of the fluorophore and to increase the signal (however, interpret results obtained under such conditions with care, as incubation at a lower temperature could alter protein interactions). Compare the levels of fusion protein expression with those of the endogenous proteins by immunoblot analysis (see *UNIT 6.2*).

Examine cells

5. Wash the cells with PBS to remove dead cells and debris, then add fresh medium.

6. Image the cells using an inverted fluorescent microscope with a 20× long-working-distance objective and excitation at 500 ± 10 nm with emission detection at 535 ± 15 nm. Confirm that fluorescent cells are alive and exhibit normal morphology compared to nontransfected cells.

   Higher-numerical-aperture objectives may be necessary to visualize weak signals and higher magnification may be helpful to determine the subcellular localization.

   Cells grown on coverslips can be fixed and individual proteins can be visualized by indirect immunofluorescence analysis.

   If CFP is used as the reporter, the excitation wavelength is 436 ± 5 nm and the emission wavelength is 470 ± 15 nm.

Assess results

7. Compare results obtained with cells transfected with the wild-type interaction proteins and those transfected with the control, mutant constructs with single amino acid substitutions that prevent interactions. Compare the number of fluorescent cells observed in cultures expressing fluorescent protein fragment fusions with that observed in cultures expressing intact fluorescent proteins.

   Fluorescence in a small subpopulation of cells is difficult to interpret, since it may represent complementation due to nonspecific interactions, or it may reflect a regulated interaction that occurs only in response to signaling in a subset of cells.

   The following conclusions may be drawn:

   Specific interaction: If fluorescence is detected when wild-type proteins are expressed, and this signal is eliminated or significantly reduced by single amino acid substitutions that prevent the interaction, it is likely that the bimolecular fluorescence complementation represents a specific interaction between the proteins fused to the fragments of fluorescent proteins.

Nonspecific interaction: If mutations that are known to eliminate the interaction do not eliminate the fluorescence, then the bimolecular complementation is due to nonspecific interactions between the fusion proteins. If this is the case, the BiFC assay may not be an appropriate assay for the study of the two proteins. Alternatively, a different combination of fusion proteins or linkers should be tested.

No fluorescence complementation detected: The lack of fluorescence complementation in BiFC assay does not prove the absence of an interaction, even if coexpression of the same fusion proteins with other interaction partners results in bimolecular fluorescence complementation. Factors that may contribute to this are discussed in the Commentary. Different combinations of BiFC constructs or alternative approaches should be employed.

QUANTIFICATION OF FLUORESCENCE COMPLEMENTATION EFFICIENCY

SUPPORT PROTOCOL 1

Quantification of the efficiency of fluorescence complementation is useful for comparison of interactions involving closely related proteins. It is particularly important for determining whether fluorescence complementation represents a specific interaction by comparison of wild-type and mutated proteins. The relative efficiencies of bimolecular fluorescence complementation by structurally unrelated proteins cannot be used to determine the efficiencies of complex formation, since many factors unrelated to the efficiency of complex formation influence the efficiency of bimolecular fluorescence complementation.

Additional Materials *(also see Basic Protocol)*

Plasmid encoding full-length CFP (assuming YFP fragments are used in the Basic Protocol) or other spectrally distinguishable fluorescent protein

Filters for visualization of YFP (excitation 500 ± 10 nm; emission 535 ± 15 nm) and CFP (excitation 436 ± 5 nm; emission 470 ± 15 nm) fluorescence

1. Follow steps 1 to 5 of the Basic Protocol, but add 50 ng of a plasmid encoding full-length CFP or another spectrally distinguishable fluorescent protein into each transfection.
2. Image cells with a fluorescence microscope using filters optimized for separation of CFP and YFP signals. Collect images of at least 100 cells that show detectable fluorescence using both filter sets.
3. Quantify the fluorescence intensities using both YFP and CFP filters integrated over the area of each cell. Subtract the background in an area of the field containing no cells.
4. Calculate the ratio of YFP/CFP fluorescence for each cell.

   This represents the complementation efficiency for the combination of proteins expressed in individual cells.

5. Plot the data in a histogram by binning cells with similar ratios as shown in Fig. 21.3.3.
6. Assess result.

   If a mutation significantly reduces the YFP/CFP ratio compared to the wild-type protein (but the levels of expression and localization of the proteins are not altered), the observed fluorescence is likely to represent a specific interaction between the proteins. If the ratios for wild-type and mutated proteins are similar, the observed fluorescence signal may represent nonspecific fluorescence complementation, or the mutation may not affect the interaction between the proteins.

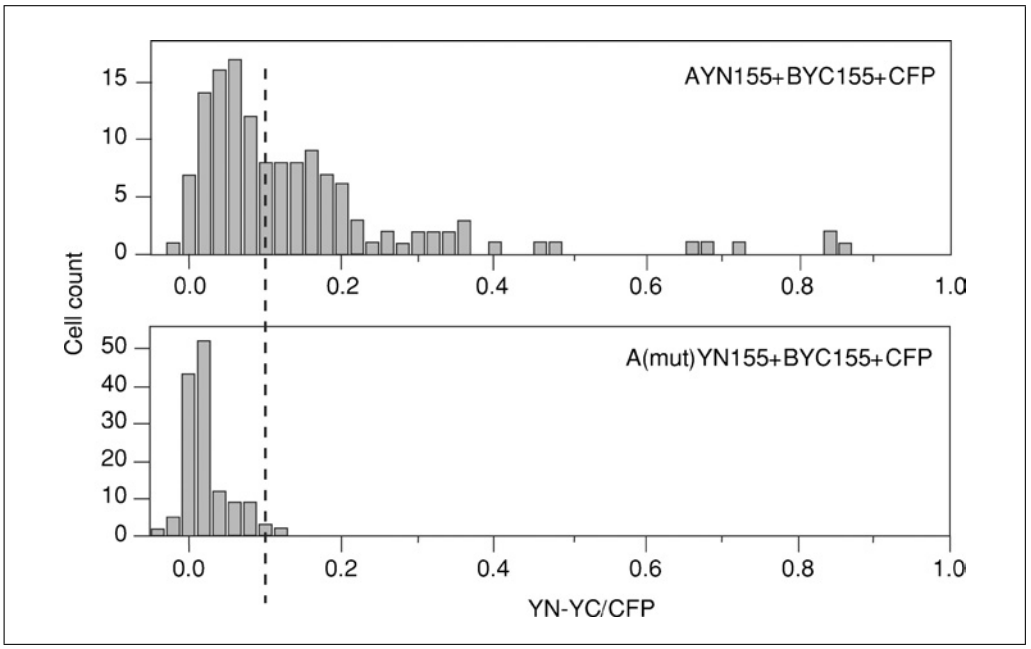


Figure 21.3.3 The effects of mutations that eliminate the interaction interface should be tested on the efficiency of bimolecular fluorescence complementation (data adapted from Hu et al., 2002). Plasmids encoding either wild-type (upper panel) or mutated (lower panel) interaction partners were transfected into cells together with an internal reference (CFP). The fluorescence intensities produced by bimolecular fluorescence complementation (YN-YC) and the internal reference (CFP) were measured in individual cells. The distribution of ratios between the fluorescence intensities in individual cells is plotted in each histogram. The dashed line indicates the maximal ratio produced by nonspecific interactions.

ALTERNATE PROTOCOL 1

SIMULTANEOUS VISUALIZATION OF MULTIPLE PROTEIN INTERACTIONS IN THE SAME CELL

Comparison of the subcellular localization of different protein-protein complexes can provide information about similarities and differences in their functions based on colocalization or nonoverlapping distributions of the complexes. The locations of different complexes can be compared in different cells by using markers for various cellular structures to define the localization of each complex separately. However, it is often difficult to find markers that precisely match the localization of a particular complex. It is therefore preferable to compare the locations of protein complexes in the same cell. This protocol describes a multicolor BiFC approach that enables simultaneous visualization of several interactions in the same cell (Hu and Kerppola, 2003; Grinberg et al., 2004).

The multicolor BiFC assay is based on complementation between fragments of different fluorescent proteins that produce bimolecular fluorescent complexes with distinct spectra. These fragments are fused to alternative interaction partners such that complexes formed with different partners can be visualized independently in the same cell using different excitation and emission wavelengths. This approach provides a unique method for comparison of the distributions of different protein complexes in the same cell and for analysis of the relative efficiencies of interactions with mutually exclusive interaction partners (Alternate Protocol 2).

Additional Materials *(also see Basic Protocol)*

DNA encoding amino acid residues 155 to 238 of cyan fluorescent protein (also see Critical Parameters and Table 21.3.1)
DNA encoding proteins of several alternative interaction partners

DNA encoding amino acid residues 1 to 154 of the cyan and yellow fluorescent proteins (also see Critical Parameters and Table 21.3.1)

Interference filters for fluorescence microscope designed to minimize cross-talk between the fluorescence signals from bimolecular fluorescent complexes formed by fragments of different fluorescent proteins (see below)

Construct plasmid vectors

1. Using the appropriate plasmid vectors, construct mammalian expression vectors by fusing the sequences encoding amino acid residues 155 to 238 of the cyan fluorescent protein (CFP) to the protein whose interactions are to be investigated (this protein is designated Z-CC155), and fuse sequences encoding amino acid residues 1 to 154 of YFP and residues 1 to 154 of CFP to two alternative interaction partners (these proteins are designated A-YN155 and B-CN155, respectively). As controls, construct plasmids encoding these fluorescent protein fragments fused to the same interaction partners.

   For additional information about the design of fusion constructs, see Critical Parameters; for cross-references to molecular biology techniques, see APPENDIX 3A.

   Other fluorescent proteins whose fragments produce bimolecular fluorescent complexes with distinct spectra may be used (Hu and Kerppola, 2003; also see Critical Parameters).

Prepare cells

2. Seed cells the day before transfection at a density that will allow for cell proliferation over the course of the experiment and, at the same time, allow for the effects of cell growth and density on the interaction under investigation.

   If short-working-distance objectives will be used to visualize the interaction, the cells should be grown in slide chambers or on glass coverslips.

3. When cells reach appropriate confluence (e.g., ~50%), transfect cells with an appropriate amount (e.g., 0.25 μg) of the plasmids encoding A-YN155, B-CN155, and Z-CC155. As a control, transfect each pair of expression vectors (A-YN155 and Z-CC155 as well as B-CN155 and Z-CC155) into separate cells.

4. Grow cells until fluorescence from both complexes (i.e., A-YN155—Z-CC155 and B-CN155—Z-CC155) is detected using filters optimized for the detection of YFP and CFP fluorescence, respectively. If necessary, incubate the cells at 30°C with 5% CO_2 to promote maturation of the fluorophores and to increase the signal (however, interpret results obtained under such conditions with care, as incubation at a lower temperature could alter protein interactions).

5. Compare the levels of expression of the proteins by western analysis (*UNIT 6.2*) and adjust the amounts of plasmids transfected if necessary.

Examine cells

6. Wash the cells with PBS to remove dead cells and debris, then add fresh medium.

7. Image the cells using an inverted fluorescence microscope with a 20× long-working-distance objective.

   To image the fluorescence emissions of A-YN155—Z-CC155 and B-CN155—Z-CC155 complexes use two filter sets with excitation at 500 ± 10 nm and emission at 535 ± 15 nm (to detect YN—CC complexes), and excitation at 436 ± 5 nm and emission at 470 ± 15 nm (to detect CN—CC complexes). The emission and excitation wavelengths are separated using a dichroic mirror with transmission windows at 450 to 490 nm and 520 to 590 nm.

   The fluorescence emissions of YN155—CC155 and CN155—CC155 bimolecular fluorescent complexes can be resolved with less than 2% cross-talk between the signals using the filters optimized for YFP and CFP detection.

ALTERNATE PROTOCOL 2

ANALYSIS OF THE COMPETITION BETWEEN ALTERNATIVE INTERACTION PARTNERS IN LIVING CELLS

The selectivity of protein interactions is determined by the relative binding affinities of alternative interaction partners and by the local concentrations of each protein. It is difficult to predict the selectivity of protein interaction in cells based on in vitro studies, since many factors, including covalent modifications, differences in subcellular distributions, interactions with additional proteins, and binding affinity, can influence the selectivity of interactions. The following protocol describes a quantitative multicolor BiFC assay that can provide information about the relative efficiencies of complex formation by mutually exclusive interaction partners in living cells (Hu and Kerppola, 2003; Grinberg et al., 2004).

Principles of competition experiment

To investigate the competition between two alternative interaction partners (i.e., A and B) for a shared partner (i.e., Z), the proteins are fused to fragments of different fluorescent proteins that can form spectrally distinct bimolecular fluorescent complexes (i.e., A-YN155, B-CN155, and Z-CC155). Equal concentrations of the two competing interaction partners (i.e., A-YN155 and B-CN155) are expressed with a limiting concentration of the shared partner (i.e., Z-CC155). The fluorescence intensities of bimolecular fluorescent complexes formed by the alternative interaction partners (i.e., A-YN155–Z-CC155 and B-CN155–Z-CC155) are measured in the same cells. For purposes of this protocol, the phrase "fluorescence intensities of complexes" and variations thereof will be used to refer to the fluorescence intensities of individual cells with specific spectral characteristics.

Additional Materials *(also see Basic Protocol)*

Constructs prepared in Alternate Protocol 1, step 1

Prepare cells

1. Seed cells the day before transfection at a density that will allow for cell proliferation over the course of the experiment and, at the same time, allow for the effects of cell growth and density on the interaction under investigation.
2. When cells reach appropriate confluence (e.g., ~50%), transfect cells with an appropriate amount (e.g., 0.05 μg) of the plasmid encoding Z-CC155 to produce a limiting concentration of the protein (established by immunoblot analysis) and appropriate amounts (i.e., 0.25 μg) of the plasmids encoding A-YN155 and B-CN155 to produce equal amounts of these proteins that are in molar excess relative to Z-CC155. For quantitation using absolute fluorescence intensities (see Support Protocol 2), also transfect each pair of expression vectors (A-YN155 and Z-CC155; as well as B-CN155 and Z-CC155) in separate cells. For quantitation using relative fluorescence intensities (see Support Protocol 3), also transfect expression vectors encoding fusions with the interaction partners fused to different combinations of fluorescent protein fragments (i.e. A-YN155 + A-CN155 + Z-CC155; as well as B-YN155 + A-CN155 + Z-CC155) into separate cells.
3. Grow cells until fluorescence from both complexes (i.e., A-YN155–Z-CC155 and B-CN155–Z-CC155) is detected. If necessary, incubate the cells at 30°C with a 5% CO_2 atmosphere to promote maturation of the fluorophore and to increase the signal (however, interpret results obtained under such conditions with care, as incubation at a lower temperature could alter protein interactions).
4. Compare the levels of expression of the proteins and adjust the amounts of plasmids transfected if necessary.

Examine cells

5. Wash the cells with PBS to remove dead cells and debris and add fresh medium.
6. Image the cells using an inverted fluorescence microscope with a 20× long working distance objective. Identify cells that are healthy and that have a morphology similar to nontransfected cells.

   Interactions between competing partners will result in the formation of two BiFC complexes, A-YN155–Z-CC155 and B-CN155–Z-CC155, with distinct spectral characteristics. To image the fluorescence emissions of A-YN155–Z-CC155 and B-CN155–Z-CC155 complexes, use filters with excitation at 500 ± 10 nm and emission at 535 ± 15 nm to detect YN–CC complexes and filters with excitation at 436 ± 5 nm and emission at 470 ± 15 nm to detect CN–CC complexes. The emission and excitation light are separated using a dichroic mirror with transmission windows at 450 to 490 nm and 520 to 590 nm.

QUANTITATION OF MULTICOLOR BiFC ANALYSIS

The authors of this unit have developed two methods for quantification of data from the multicolor BiFC assay that can provide information about the relative efficiencies of complex formation by mutually exclusive interaction partners in living cells (Hu and Kerppola, 2003; Grinberg et al., 2004). The first method, designated absolute competition (Support Protocol 2), is based on comparison of the absolute fluorescence intensities of bimolecular fluorescent complexes produced when a pair of interaction partners is expressed in the presence and absence of an alternative interaction partner in separate cells. The second method, designated relative competition (Support Protocol 3), is based on comparison of the relative fluorescence intensities of bimolecular fluorescent complexes produced when a protein is expressed together with two alternative interaction partners in the same cells. It is best to use both methods in combination to eliminate potential caveats inherent in each approach.

Comparison of the Absolute Fluorescence Intensities of Bimolecular Fluorescent Complexes Produced When a Pair of Interaction Partners Is Expressed in the Presence and Absence of a Competitor in Separate Cells

SUPPORT PROTOCOL 2

The absolute fluorescence intensities of bimolecular fluorescent complexes are compared when two fusion proteins are expressed alone (i.e. A-YN155+Z-CC155) and when they are expressed in the presence of a competitor (i.e. A-YN155+B-CN155+Z-CC155) in separate cells. This approach is designated absolute competition. This method is well suited for analysis of large differences in the efficiencies of complex formation between alternative interaction partners. Since it involves comparison of different cell populations, it is subject to error due to variations between the populations. Since the absolute fluorescence intensities vary between individual cells, this requires comparison of the fluorescence intensities of the populations or representative subsets of the populations. Using this approach, it is generally possible to detect only large differences in the efficiencies of complex formation. This approach compares the fluorescence intensities of bimolecular fluorescent complexes formed by the same interacting pair, in the absence and presence of competitor. This circumvents the effects of differences in the steric arrangement of the fluorescent protein fragments between different interaction partners that prevent comparison of the efficiencies of complex formation based on the absolute fluorescence intensities of different bimolecular fluorescent complexes.

Carry out Alternate Protocol 2, steps 1 to 6, then continue with the following steps.

1. Measure the fluorescence intensities of YN-CC complexes in 100 to 300 cells expressing A-YN155 + Z-CC155.
2. Measure the fluorescence intensities of YN-CC complexes in 100 to 300 cells expressing A-YN155 + B-CN155 + Z-CC155.
3. Measure the fluorescence intensities of CN-CC complexes in 100 to 300 cells expressing B-CN155 + Z-CC155.
4. Measure the fluorescence intensities of CN-CC complexes in 100 to 300 cells expressing A-YN155 + B-CN155 + Z-CC155.
5. Compare the fluorescence intensities of YN–CC complexes measured in steps 1 and 2 as well as the CN–CC complexes measured in steps 3 and 4.
6. Assess results.

   Case i: If the fluorescence intensities of the two bimolecular fluorescent complexes (i.e., A-YN155–Z-CC155 and B-CN155–Z-CC155) expressed in the same cells are ∼50% of the fluorescence intensities of the complexes when expressed separately, then the two complexes form with comparable efficiencies.

   Case ii: If the fluorescence intensity of one complex is similar whether it is expressed separately or in combination, whereas the fluorescence intensity of the other complex is reduced by >50% when expressed in combination, then the former complex forms with higher efficiency than the latter.

   Case iii: If the fluorescence intensities of both complexes expressed together are equal or higher than those observed when they are expressed separately, then the interaction partners do not compete for complex formation. The shared interaction partner may not be present at limiting concentration, and the relative efficiencies of complex formation cannot be determined based on the data.

   Case iv: If the fluorescence intensities of both complexes are reduced by significantly more than 50% when they are expressed together, or if the changes in fluorescence intensities are not reciprocal, then the interaction partners may affect the expression or functions of each other, and the relative efficiencies of complex formation cannot be determined based on the data.

SUPPORT PROTOCOL 3

Comparison of the Relative Fluorescence Intensities of Bimolecular Fluorescent Complexes Produced When a Protein Is Expressed with Two Alternative Interaction Partners in the Same Cells

The relative fluorescence intensities of bimolecular fluorescent complexes are compared when a fusion protein is expressed with two alternative interaction partners fused to fragments of different fluorescent proteins (i.e., A-YN155 + B-CN155 + Z-CC155). This approach is designated relative competition. This approach is suitable for detection of relatively modest differences in the efficiencies of complex formation between structurally related alternative interaction partners. Since this approach compares complexes in the same cells, it is less sensitive to variations between cells. However, since this approach compares the relative fluorescence intensitites of bimolecular fluorescent complexes produced by different interaction partners, it is subject to variations in the efficiency of association of the fluorescent protein fragments when they are fused to different interaction partners. Therefore, this approach can be used only when there are no large differences in the steric constraints to association of the fluorescent protein fragments between different complexes.

Carry out Alternate Protocol 2, steps 1 to 6, then continue with the following steps.

1. Measure the fluorescence intensities of 100 to 300 cells expressing A-YN155 + A-CN155 + Z-CC155 to use as a reference.
2. Measure the fluorescence intensities of 100 to 300 cells expressing A-YN155 + B-CN155 + Z-CC155.
3. Measure the fluorescence intensities of 100 to 300 cells expressing B-YN155 + A-CN155 + Z-CC155.
4. Plot the fluorescence intensities of YN–CC versus CN–CC complexes for each individual cell as a scatter plot as shown in Figure 21.3.4 (middle row).
5. Compare the slopes produced by different combinations of interaction partners. The relative slopes of these plots provide information about the relative efficiencies of complex formation between alternative interaction partners. Alternatively, calculate the ratio YN-CC/(YN-CC + CN-CC) and plot the data as a histogram as shown in Figure 21.3.4 (bottom row). The distribution of ratios in the population can provide information about the relative efficiencies of complex formation.
6. Assess results.

   Case i: If the relative fluorescence intensities of all combinations of complexes display a linear relationship, and the slopes of the relationships are not significantly different, then the two interaction partners compete with indistinguishable efficiencies for the shared partner.

   Case ii: If the fluorescence intensities of all combinations of complexes display a linear relationship, and the slopes of the relationships are significantly different, then the two interaction partners compete with different efficiencies for the shared partner.

   Case iii: If the fluorescence intensities of one or all combinations of complexes do not display a linear relationship, or if the slopes cannot be determined with sufficient confidence, then the relative efficiencies of competition cannot be determined based on the data.

Comparison of Results from the Absolute and Relative Competition Approaches

The relative competition approach (Support Protocol 3) is generally more accurate for complexes with similar structures. However, this approach can only be used when there are no differences in the steric constraints to association of the fluorescent protein fragments.

To confirm that the identities of the fluorescent protein fragments fused to the alternative interaction partners do not influence the relative efficiencies of complex formation, it is necessary to exchange the fragments between the interaction partners (i.e., compare A-YN155 + B-CN155 + Z-CC155 with B-YN155 + A-CN155 + Z-CC155). In the absolute competition approach, exchange of the fragments should not affect the difference in fluorescence intensity in the presence and absence of the competitor. In the relative competition approach, the slope of the plot of YN-CC versus CN-CC intensities should shift in the opposite direction relative to the slope observed for the reference complexes (A-YN155 + A-CN155 + Z-CC155 compare slopes of the plots on the right and the left of the middle row of Fig. 21.3.4 with that of the plot in the center). If exchange of the fluorescent protein fragments between the interaction partners does not produce the predicted results, it is possible that the fluorescent protein fragments influence the specificity of the interaction. In this case, the relative efficiencies of complex formation cannot be determined using multicolor bimolecular fluorescence complementation analysis.

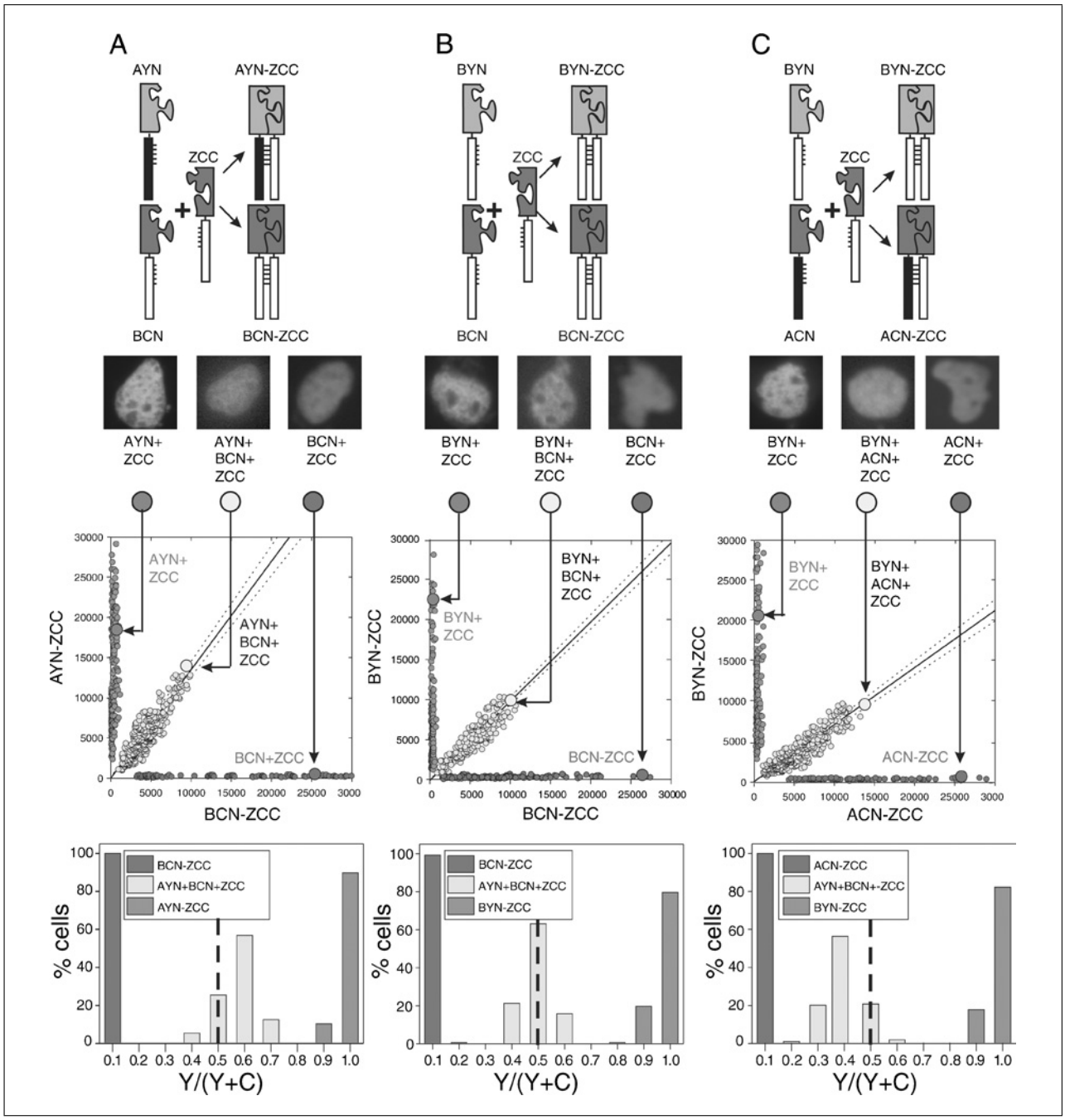


Figure 21.3.4 Legend at right.

Limitations of the Method

The multicolor BiFC assay for measurement of the efficiencies of protein interactions in cells enables determination of the relative efficiencies of competition between alternative interaction partners for a shared partner, but this does not necessarily reflect their relative binding affinities, since the complexes are not at equilibrium for the entire time of the experiment. The association of the fluorescent protein fragments is relatively slow ($t_{\frac{1}{2}} \approx 60$ sec) and essentially irreversible in vitro (Hu et al., 2002). The relative efficiencies of complex formation reflect the competition between alternative interaction partners during the time before association of the fluorescent protein fragments. This is predicted to give a valid estimate of the relative efficiencies of complex formation by proteins with rapid exchange rates. However, interaction partners with very slow rates of association may not compete efficiently for complex formation in the assay.

The multicolor BiFC approach is based on comparison of the relative amounts of bimolecular fluorescent complexes with different spectral characteristics. Differences between the rates of degradation of proteins in different complexes could alter the steady-state ratio between the complexes and thereby affect the apparent efficiencies of complex formation. The relative fluorescence intensities should therefore be measured at several time pointss, and the steady-state levels of proteins should be compared by western analysis. The rates of degradation of the complexes can be compared by monitoring the decrease in fluorescence in cells treated with protein-synthesis inhibitors (e.g. cycloheximide). The rates of degradation of the fusion proteins can also be measured directly using pulse-chase analysis.

Example of the Application of Quantitative Multicolor BiFC Analysis for Determination of the Relative Efficiencies of Complex Formation

To examine the relative efficiencies of complex formation by protein Z with two alternative interaction partners, A and B, the authors of this unit performed an experiment in which the proteins were fused to fluorescent protein fragments (A-YN, B-CN, and Z-CC) and were expressed separately and in combination (Grinberg et al., 2004). The fluorescence intensities of the alternative complexes (i.e., A-YN–Z-CC and B-CN–Z-CC) were measured when the complexes were expressed in separate cells and when they

Figure 21.3.4 *(at Left)* Multicolor fluorescence complementation analysis of the competition for dimerization between alternative interaction partners. The relative efficiencies of complex formation between protein Z and two alternative interaction partners, A and B, can be quantified by fusing them to fragments of different fluorescent proteins and expressing them in different combinations. (**A**) BiFC analysis of A-YN + Z-CC (green), B-CN + Z-CC (red) and A-YN + B-CN + Z-CC (yellow). (**B**) BiFC analysis of B-YN + Z-CC (green), B-CN + Z-CC (red) and B-YN + B-CN + Z-CC (yellow). (**C**) BiFC analysis of B-YN + Z-CC (green), A-CN + Z-CC (red) and B-YN + A-CN + Z-CC (yellow). The diagrams at the top indicate the combinations of proteins expressed either pairwise or in three-way competition in each experiment. The images show cells expressing the proteins indicated below each image. The cells were imaged using filters (described in the instrumentation section) that distinguish the fluorescence emissions of YN–CC (green) and CN–CC (red) complexes, and the images were superimposed. The fluorescence intensities of YN–CC complexes were plotted as a function of the fluorescence intensities of CN–CC complexes in individual cells (middle row of graphs). The fluorescence intensities are shown in green and red for cells that express the interaction partners pairwise, and in yellow for cells that express two alternative interaction partners in direct competition. The best fit of a linear function to the data from cells coexpressing three proteins is shown. The slope of this function reflects the relative efficiencies of complex formation between the alternative interaction partners. The ratio between the fluorescence emissions from each complex [Y/(Y + C) where Y represents YN–CC and C represents CN–CC] was determined in individual cells in each population, and was plotted in histograms (lower row of graphs) using the same colors as in the middle row. For the color version of this figure, go to *http://www.currentprotocols.com.*

were expressed together in the same cell (Fig. 21.3.4A). As a reference, the fluorescent proteins were fused to the same interaction partners (B-YN, B-CN and Z-CC), and their fluorescence intensities were analyzed in parallel (Fig. 21.3.4B). The fluorescence intensities of the complexes were plotted in scatter plots (middle panels) and histograms (lower panels) when the complexes were expressed separately (red and green) and when they were expressed together in the same cells (yellow). The ratio between the fluorescence intensities of the complexes reflects the relative efficiencies of complex formation between the alternative interaction partners.

When A-YN and B-CN were coexpressed with Z-CC, the fluorescence intensities produced by A-YN–Z-CC and B-CN–Z-CC exhibited a linear relationship with a slope of 1.3 and a 95% confidence interval of 0.08 (Fig. 21.3.4A, middle row). In comparison, when B-YN and B-CN were coexpressed with Z-CC, the fluorescence intensities produced by B-YN–Z-CC and B-CN–Z-CC exhibited a linear relationship with a slope of 0.98 and a 95% confidence interval of 0.05 (Fig. 21.3.4B, middle row). Likewise, the distribution of ratios between the fluorescence intensities [Y/(Y+C)] was shifted toward higher values for complexes formed by A-YN and B-CN with Z-CC compared to complexes formed by B-YN and B-CN with Z-CC (compare lower row of graphs for Fig. 21.3.4, panels A and B). These results are consistent with the interpretation that Z favors complex formation with A over complex formation with B. The absolute fluorescence intensities of A-YN–Z-CC and B-YN–Z-CC were comparable when expressed separately, suggesting that the efficiencies of association of the fluorescent protein fragments were comparable under conditions where no competitors for complex formation were present.

To confirm that the fluorescent protein fragments fused to the alternative interaction partners did not influence the relative efficiencies of complex formation, the authors exchanged the fragments between the interaction partners (i.e., B-YN, A-CN and Z-CC) and compared the fluorescence intensities of bimolecular fluorescent complexes formed by these proteins (Fig. 21.3.4C). The fluorescence intensities of these complexes exhibited a linear relationship with a slope of 0.7 and a 95% confidence interval of 0.04 (Fig. 21.3.4C, middle row). Likewise, the distribution of fluorescence intensity ratios was shifted in the opposite directions compared to the proteins in which the fragments were fused to the opposite interaction partners (see lower row of graphs for Fig. 21.3.4; compare panel C with panels A and B). These data are consistent with the interpretation that the difference between the efficiencies of bimolecular fluorescent complex formation by A and B fusions is not due to a difference between the fluorescent protein fragments fused to these proteins, but is caused by a difference between the efficiencies of complex formation between A and Z versus B and Z (Grinberg et al., 2004).

Controls for Quantitative Multicolor BiFC Analysis

The use of both absolute and relative competition methods for the quantification of relative efficiencies of complex formation is designed to correct for many of the factors that influence the fluorescence intensities of bimolecular fluorescent complexes formed by fragments of different fluorescent proteins. However, there are additional factors that need to be considered and examined as follows.

1. The relative levels of A-YN155 and B-CN155 expression affect the relative amounts of complexes formed. The alternative interaction partners must therefore be expressed at equal concentrations and the shared interaction partner must be expressed at a lower, limiting concentration. All protein concentrations should preferably be within the physiological range to observe the competition under normal cellular conditions. The levels of protein expression can be measured by immunoblot analysis using antibodies directed against the same epitope tag on the alternative interaction partners.

2. The efficiencies of association of different fluorescent protein fragments as well as the fluorescence intensities of the resulting bimolecular complexes can differ. It is therefore essential to determine the relative fluorescence intensities of the bimolecular fluorescent complexes when they are brought together by the same interaction partners (i.e., A-YN155, A-CN155, and Z-CC155). The relative fluorescence intensities of the two bimolecular fluorescent complexes (i.e., A-YN155–Z-CC155 and A-CN155–Z-CC155) reflect both differences between the efficiencies of association of the fluorescent protein fragments and differences between their fluorescence intensities when they are brought together by the same interaction partners.

3. The relative efficiencies of bimolecular fluorescence complementation can be affected by steric constraints imposed by the interaction partners on the association of the fluorescent protein fragments. To investigate this possibility, the fluorescent protein fragments can be fused to the interaction partners using several different extended linkers. If fusion proteins containing different linkers produce identical results, then the efficiencies of bimolecular complex formation are unlikely to be affected by steric constraints.

4. Different fluorescent protein fragments could have differential effects on the efficiencies of competition for the shared interaction partner. It is therefore essential to exchange the fluorescent protein fragments between the interaction partners (i.e., B-YN155, A-CN155, and Z-CC155) to determine if differences between the fluorescent protein fragments affect the relative efficiencies of competition. This can also be verified in vitro by measuring the relative amounts of bimolecular fluorescent complexes formed when different ratios of the fluorescent protein fragments fused to the same interaction partners are mixed (Hu and Kerppola, 2003). If the amounts of complexes formed are proportional to the relative concentrations of fusion proteins in the reaction, then differences between the fluorescent protein fragments do not influence the relative efficiencies of complex formation.

COMMENTARY

Background Information

The biological functions of proteins are determined in large part by their interactions with other proteins in the physiological environment. Numerous protein interactions have been identified using genetic screens and in vitro binding assays. It is important to develop experimental approaches that allow determination of the subcellular locations of these interactions.

The BiFC assay has been used to visualize interactions between many structurally unrelated proteins (Hu et al., 2002; Atmakuri et al., 2003; Deppmann et al., 2003; von der Lehr et al., 2003; Yu et al., 2003; Bracha-Drori et al., 2004; de Virgilio et al., 2004; Farina et al., 2004; Grinberg et al., 2004; Hynes et al., 2004a,b; Kanno et al., 2004; Rackham and Brown, 2004; Rajaram and Kerppola, 2004; Remy and Michnick 2004a,b; Remy et al., 2004; Tsuchisaka and Theologis, 2004a,b; Tzfira et al., 2004; Walter et al., 2004; Wei et al., 2004; Zal and Gascoigne, 2004; Zhang et al., 2004; Zhu et al., 2004). The authors of this unit and other investigators have determined the localization of complexes formed by Fos, Jun, and ATF-2 (Hu et al., 2002); c-Maf and Sox (Rajaram and Kerppola, 2004); BATF and Jun (Deppmann et al., 2003); p65, p50, and IkB (Hu et al., 2002); Myc, Max, Mxi1, Mad3, and Mad4 (Grinberg et al., 2004); Skp2 and c-Myc (von der Lehr et al., 2003); and Brd2 and histone H4 (Kanno et al., 2004).

Protein interactions are crucial for the control of most cellular functions. Several methods have been developed to study protein interactions in living cells. One of the most commonly employed methods is FRET (*UNIT 17.1*). The FRET assay is based on the use of two fluorophores either chemically linked or genetically fused to two proteins whose interaction is to be examined. Compared to the BiFC assay, FRET analysis generally requires higher levels of protein expression to detect energy transfer. Also, structural information or a great deal of luck is required to place the two fluorophores within 100 Å of each other, which is the maximal distance over which any significant energy transfer between fluorescent proteins can be detected. The fraction of proteins

that forms complexes must also be high to produce a sufficient change in the donor and acceptor fluorescence intensities. To exclude alternative interpretations of the results, numerous controls must be performed and the fluorescence intensities must be measured with high quantitative accuracy. Despite these limitations, FRET has been successfully used for the analysis of many protein interactions in living cells (Sorkin et al., 2000; Li et al., 2001; Majoul et al., 2002; Hink et al., 2003; Larson et al., 2003; Miyawaki, 2003; Tsien, 2003). A great advantage of FRET over BiFC analysis is the reversibility of complex formation, which potentially allows analysis of the interaction under equilibrium conditions. FRET is therefore superior for studies of the kinetics of protein association and dissociation.

Bimolecular complementation between fragments of a variety of proteins has been used to investigate protein interactions in cells (Johnsson and Varshavsky, 1994; Rossi et al., 1997; Pelletier et al., 1998; Rossi et al., 1998; Ghosh, 2000; Galarneau et al., 2002; Wehrman et al., 2002; Paulmurugan and Gambhir, 2003). The basic principle is to fuse fragments of a reporter protein to the interaction partners. Selected fragments of many proteins are able to associate with each other to produce a functional complex. This complex can be detected by the use of chromogenic or fluorogenic substrates or ligands. Reporter proteins that have been used to date include ubiquitin (Johnsson and Varshavsky, 1994), β-glactosidase (Rossi et al., 1997), dihydrofolate reductase (Pelletier et al., 1998), luciferase (Paulmurugan and Gambhir, 2003), and β-lactamase (Galarneau et al., 2002; Wehrman et al., 2002). BiFC employs the classical principle of complementation between protein fragments (Ullmann et al., 1967, 1968). The advantage of the BiFC approach over other complementation approaches is that the reconstituted fluorescent protein has strong intrinsic fluorescence that allows direct visualization of the protein complex. The complex can therefore be visualized with minimal perturbation of the cells. Using the BiFC approach, living cells can be observed over long periods, and the possibility of experimental manipulations altering the result is minimized. Potential problems with unequal distributions of the chromogenic or fluorogenic substrates or ligands are also avoided. Moreover, as described in Alternate Protocols 1 and 2, multiple protein interactions can be visualized in parallel using spectrally distinct bimolecular fluorescent complexes.

One limitation of the BiFC approach is the time required for fluorophore maturation. This prevents real-time detection of rapid changes in interactions using the BiFC assay. In addition, bimolecular fluorescent complex formation is irreversible in vitro. These characteristics prevent the use of the BiFC assay for studies of the dissociation and subunit exchange of protein complexes in cells.

Characteristics of the BiFC assay

The BiFC assay has several features that make it particularly useful for the study of protein interactions. First, it enables direct visualization of protein interactions and does not rely on their secondary effects. Second, the interactions can be visualized in living cells, eliminating potential artifacts associated with cell lysis or fixation. Third, the proteins are expressed in a relevant biological context, ideally at levels comparable to their endogenous counterparts. This increases the likelihood that they will reflect the properties of native proteins, including any post-translational modifications. Fourth, the BiFC assay does not require stoichiometric complex formation but can detect interactions between subpopulations of each protein. Fifth, multicolor BiFC analysis allows simultaneous visualization of multiple protein complexes in the same cell and enables analysis of the competition between alternative interaction partners for complex formation. Finally, BiFC does not require specialized equipment, apart from an inverted fluorescence microscope equipped with objectives that allow imaging of fluorescence in cells. The simple detection of bimolecular complex formation requires no post-acquisition image processing for interpretation of the data.

Critical Parameters

Design of BiFC fusion constructs

The fusion proteins for BiFC analysis must be designed with the specific proteins to be investigated as well as the purpose of the experiment in mind. Schematic diagrams of the different permutations of fusion proteins that can be used for BiFC analysis are shown in Figure 21.3.2. Some general guidelines for the design of fusion proteins for BiFC analysis are discussed below.

Fragments of fluorescent proteins

Several combinations of fluorescent protein fragments support bimolecular fluorescence complementation (Hu and Kerppola, 2003). The combinations of fluorescent protein fragments recommended for BiFC analysis are

listed in Table 21.3.1. For most purposes, fragments of YFP truncated at residue 155 (designated YN155, containing N-terminal residues 1 to 154, and YC155, containing of C-terminal residues 155 to 238) are recommended, as they exhibit a relatively high complementation efficiency when fused to many interaction partners, yet produce low fluorescence when fused to proteins that do not interact with each other. Fragments of YFP truncated at residue 173 (designated YN173 containing residues 1 to 172, and YC173, containing residues 172 to 238) can also be used. Other combinations of fluorescent protein fragments can be also used (Hu and Kerppola, 2003) as described in the multicolor BiFC assay (see Alternate Protocols 1 and 2).

Positions of fusions

The positions of the fusions must be determined empirically to fulfill three essential criteria:

1. The fusions must allow the fragments of the fluorescent proteins to associate with each other if the fusion proteins interact. Information about the structure and location of the interaction interface may be useful for this purpose. However, this information is not essential since BiFC vectors can be designed by screening multiple combinations of fusion proteins for fluorescence complementation. A simple strategy for the identification of fusion proteins that allow bimolecular fluorescence complementation is to fuse each of the fluorescent protein fragments to the N- and C-terminal end of each interaction partner, and to test the fusion proteins for complementation in all eight combinations that contain both fragments of the fluorescent protein (Fig. 21.3.2).

2. The fusions must not affect the localization or the stabilities of the proteins. This should be confirmed by comparing the localization and the level of expression of the fusion proteins with those of wild-type proteins lacking the fusions using indirect immunofluorescence (*UNIT 4.3*) and immunoblot (*UNIT 6.2*) analyses.

3. The fusions must not affect the functions of the proteins under investigation. Ideally, the protein functions should be validated using assays that evaluate all of the known functions of the endogenous proteins, and they should be performed under the conditions used to visualize the protein interactions.

Linkers

The linkers connect the fragments of the fluorescent proteins to the proteins of interest in the fusion proteins. The linkers must provide flexibility for independent motion of the fluorescent protein fragments and the interaction partners, allowing the fragments to associate when the proteins interact. The authors have used the RSIAT and RPACKIPNDLKQKVMNH linker sequences in many fusion constructs used for BiFC analysis (Hu et al., 2002; Hu and Kerppola, 2003). These linkers have been used for the visualization of interactions between many structurally unrelated proteins. The sequence AAANSSIDLISVPVDSR encoded by the multiple cloning sites of the pCMV-FLAG vector (Sigma) has also been successfully used as a linker in many BiFC experiments. Although these linker sequences worked well in the proteins examined, it is possible that the length or the sequence of the linker affects the complementation efficiency between other proteins.

Design of constructs for multicolor BiFC analysis

The basic principles for the design of BiFC constructs also apply to the design of multicolor BiFC constructs. The main difference is the use of fluorescent protein fragments that provide maximal spectral separation of the fluorescence signals from different bimolecular complexes. There are several combinations of fragments that can be used for multicolor BiFC analysis (Table 21.3.1). The use of CC155 (C-terminal fragment of CFP) paired with YN155 versus CN155 (N-terminal fragment of CFP) provides good spectral separation and high complementation efficiency. These combinations are therefore appropriate for the simultaneous analysis of two protein interactions. For the simultaneous analysis of more than two interactions, more selective interference filters and more complex spectral separation algorithms are required.

Expression system

The selection of an expression system should be based on the purpose of the experiment. To determine whether a pair of proteins can interact in cells and to determine the subcellular location of the complex, a transient overexpression system can be used. However, overexpression of proteins in cells can result in mislocalization of the proteins and formation of non-native complexes. To ensure that the observed fluorescence signal reflects native interactions, the fusion proteins should be expressed at levels comparable to the endogenous proteins. This can be done by the use

of plasmids with weak promoters and plasmid vectors that do not replicate in mammalian cells. Also, cells can be transfected using small amounts of plasmid DNA, and they can be observed soon after transfection.

More consistent results can be obtained by using inducible expression vectors integrated into the genomes of stable cell lines (e.g., pIND). This allows for the control of protein expression at relatively uniform levels in the entire cell population.

Controls

In order to determine if any fluorescence observed reflects a specific protein interaction, it is essential to include negative controls in each experiment. This is especially important because the fluorescent protein fragments are able to form fluorescent complexes with a low efficiency even in the absence of a specific interaction. This spontaneous complementation is generally reduced when the fragments are fused to proteins that do not interact with each other. The validity of bimolecular fluorescence complementation results must therefore be confirmed using fusions to proteins in which the interaction interface has been mutated (Hu et al., 2002; Grinberg et al., 2004). The mutant proteins must be fused to the fluorescent protein fragments in a manner identical to the wild-type protein. The mutation must not affect the stability or the subcellular location of the fusion protein. The level of expression and the localization of the mutant protein should be compared with the wild-type fusion protein by immunoblot (*UNIT 6.2*) and indirect immunofluorescence analyses (*UNIT 4.3*). The efficiencies of fluorescence complementation by the wild-type and mutant proteins should be quantified and compared. If the interaction interface has not been previously characterized, it is possible to screen for mutations that alter the efficiency of bimolecular fluorescence complementation, and thereby to determine if the complementation reflects a specific interaction. The BiFC assay can therefore be used to determine whether two proteins interact in cells without prior knowledge of the location or the structural nature of the interaction interface.

Protein concentrations

The levels of expression of fusion proteins used for BiFC analysis can have a profound influence on the experimental results and on interpretation of the data. A high level of expression may produce non-native protein interactions, and may result in bimolecular complementation independent of specific protein interactions. It may also alter the subcellular distributions of the fusion proteins and alter cell functions. These may lead to the misinterpretation of the observed interactions. It is therefore important to control the levels of expression of the fusion proteins and to compare them with those of the endogenous proteins. This can be accomplished by using regulated expression vectors and by making stably transfected cell lines. Differences between the levels of expression of the fusion proteins and their endogenous counterparts should also be considered when interpreting the results from competition studies using the multicolor BiFC assay.

Time and temperature

The time required for the detection of fluorescence varies depending on the complementation efficiency. For most productive interactions, transiently transfected cells exhibit fluorescence at 12 to 36 hr after transfection. In the case of some interactions, fluorescence can be detected as early as 8 hr after transfection (Hu et al., 2002; Grinberg et al., 2004). Long incubation times should be avoided since this may result in higher expression of fusion proteins and complementation due to nonspecific interaction. Since fluorophore maturation is sensitive to high temperature, incubation at 30°C for 30 min to several hours can increase the signal. Keep in mind that results obtained at 30°C should be carefully interpreted, as low-temperature incubation may alter protein localization and/or interactions.

Interpretation of lack of fluorescence complementation

The lack of fluorescence complementation does not prove the absence of an interaction because fusion of nonfluorescent fragments to the proteins of interest can alter their structures, which could interfere with an interaction between the proteins. Moreover, the spatial arrangement of the fragments of fluorescent proteins may not allow bimolecular complex formation. Only in cases where fluorescence complementation can be induced by an extracellular signal can the lack of fluorescence complementation in the absence of the signal be tentatively interpreted to reflect the absence of an interaction or a change in complex architecture. If there is strong evidence to indicate that the proteins interact in cells, fluorescence complementation could be examined in different cell types or in the presence of different extracellular stimuli. Alternatively, additional

fusion proteins containing different linker sequences could be tested.

Quantification of the efficiency of bimolecular fluorescence complementation

The efficiency of fluorescence complementation is defined as the fluorescence intensity produced by bimolecular fluorescent complex formation relative to the levels of fusion proteins present in the cell. The efficiencies of bimolecular fluorescence complementation by structurally unrelated proteins cannot be used to determine their efficiencies of complex formation, since many factors unrelated to the efficiency of complex formation influence the efficiency of bimolecular fluorescence complementation. However, in situations where all of these factors are expected to be identical, differences in the efficiency of bimolecular fluorescence complementation can provide information about the relative efficiencies of complex formation. Thus, the effects of single amino acid substitutions that do not alter the level of protein expression or its localization can be examined by quantifying the efficiencies of fluorescence complementation by the wild-type and mutated proteins (Hu et al., 2002; Hu and Kerppola, 2003). To quantify the efficiency of fluorescence complementation, it is necessary to include an internal control in the experiment to normalize for differences in the efficiencies of transfection and protein expression in individual cells. For this purpose, cells are cotransfected with plasmids encoding the two fusion proteins together with a plasmid encoding a full-length fluorescent protein with distinct spectral characteristics (e.g., CFP). The fluorescence intensities derived from both bimolecular fluorescence complementation (i.e., YFP) and the internal control (i.e., CFP) are measured in individual cells. The ratio of YFP to CFP emission is calculated after subtraction of background signal (Fig. 21.3.3). The ratio of YFP to CFP fluorescence is a measure of the efficiency of bimolecular fluorescence complementation. For structurally related proteins under carefully controlled conditions, the efficiency of complementation can provide information about the efficiency of the protein interaction.

Anticipated Results

If the fusion proteins under investigation support fluorescence complementation within 12 to 36 hr after transfection, it is likely that the complementation reflects a specific interaction. This possibility must be verified by the analysis of mutated proteins. For proteins that display complementation after longer times, it is increasingly probable that the complementation results from a nonspecific association. If eight different combinations of amino- and carboxy-terminal fusions are tested and no complementation is observed, it is unlikely that additional combinations will produce fluorescence complementation, although this does not exclude the possibility that the proteins may interact.

Time Considerations

The construction of plasmid vectors for BiFC and multicolor BiFC analysis can be accomplished in 1 to 2 weeks. For transfection, preparation of the cells takes 1 day and transfection takes 1 hr using FuGENE 6. Next, 1 to 2 days incubation at 37°C is required. To promote chromophore maturation, incubation at 30°C for 30 min to several hours or overnight may be necessary. Imaging of the cells may take a few hours depending on how the data will be analyzed. Quantitative analysis requires about half a day to 1 day for each experiment. If everything goes smoothly, 3 to 4 weeks should be enough to obtain preliminary results. However, it takes much longer to validate the results by performing all the necessary controls. Confirmation of the interaction using cells stably transfected with inducible expression vectors also takes longer, since it requires the establishment of stable cell lines expressing the fusion proteins.

Literature Cited

Atmakuri, K., Ding, Z., and Christie, P.J. 2003. VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of *Agrobacterium tumefaciens*. *Mol. Microbiol.* 49:1699-1713.

Bracha-Drori, K., Shichrur, K., Katz, A., Oliva, M., Angelovici, R., Yalovsky, S., and Ohad, N. 2004. Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. *Plant J.* 40:419-427.

Deppmann, C.D., Thornton, T.M., Utama, F.E., and Taparowsky, E.J. 2003. Phosphorylation of BATF regulates DNA binding: A novel mechanism for AP-1 (activator protein-1) regulation. *Biochem. J.* 374:423-431.

de Virgilio, M., Kiosses, W.B., and Shattil, S.J. 2004. Proximal, selective, and dynamic interactions between integrin alpha II beta 3 and protein tyrosine kinases in living cells. *J. Cell Biol.* 165:305-311.

Farina, A., Hattori, M., Qin, J., Nakatani, Y., Minato, N., and Ozato, K. 2004. Bromodomain protein Brd4 binds to GTPase-activating SPA-1, modulating its activity and subcellular localization. *Mol. Cell. Biol.* 24:9059-9069.

Galarneau, A., Primeau, M., Trudeau, L.E., and Michnick, S.W. 2002. Beta-lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein protein interactions. *Nat. Biotechnol.* 20:619-622.

Ghosh, I., Hamilton, A.D., and Regan, L. 2000. Antiparallel leucine zipper-directed protein reassembly: Application to the green fluorescent protein. *J. Am. Chem. Soc.* 122:5658-5659.

Grinberg, A.V., Hu, C.D., and Kerppola, T.K. 2004. Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells. *Mol. Cell. Biol.* 24:4294-4308.

Hink, M.A., Borst, J.W., and Visser, A.J. 2003. Fluorescence correlation spectroscopy of GFP fusion proteins in living plant cells. *Methods Enzymol.* 361:93-112.

Hu, C.D. and Kerppola, T.K. 2003. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat. Biotechnol.* 21:539-545.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. 2002. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* 9:789-798.

Hynes, T.R., Mervine, S.M., Yost, E.A., Sabo, J.L., and Berlot, C.H. 2004a. Live cell imaging of G(s) and the beta(2)-adrenergic receptor demonstrates that both alpha(s) and beta(1)gamma(7) internalize upon stimulation and exhibit similar trafficking patterns that differ from that of the beta(2)-adrenergic receptor. *J. Biol. Chem.* 279:44101-44112.

Hynes, T.R., Tang, L.N., Mervine, S.M., Sabo, J.L., Yost, E.A., Devreotes, P.N., and Berlot, C.H. 2004b. Visualization of G protein beta gamma dimers using bimolecular fluorescence complementation demonstrates roles for both beta and gamma in subcellular targeting. *J. Biol. Chem.* 279:30279-30286.

Johnsson, N. and Varshavsky, A. 1994. Split ubiquitin as a sensor of protein interactions in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 91:10340-10344.

Kanno, T., Kanno, Y., Siegel, R.M., Jang, M.K., Lenardo, M.J., and Ozato, K. 2004. Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. *Mol. Cell* 13:33-43.

Larson, D.R., Ma, Y.M., Vogt, V.M., and Webb, W.W. 2003. Direct measurement of Gag-Gag interaction during retrovirus assembly with FRET and fluorescence correlation spectroscopy. *J. Cell Biol.* 162:1233-1244.

Li, H.Y., Ng, E.K., Lee, S.M., Kotaka, M., Tsui, S.K., Lee, C.Y., Fung, K.P., and Waye, M.M. 2001. Protein-protein interaction of FHL3 with FHL2 and visualization of their interaction by green fluorescent proteins (GFP) two-fusion fluorescence resonance energy transfer (FRET). *J. Cell. Biochem.* 80:293-303.

Majoul, I., Straub, M., Duden, R., Hell, S.W., and Soling, H.D. 2002. Fluorescence resonance energy transfer analysis of protein-protein interactions in single living cells by multifocal multiphoton microscopy. *J. Biotechnol.* 82:267-277.

Miyawaki, A. 2003. Visualization of the spatial and temporal dynamics of intracellular signaling. *Dev. Cell* 4:295-305.

Nagai, T., Sawano, A., Park, E.S., and Miyawaki, A. 2001. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *Proc. Natl. Acad. Sci. U.S.A.* 98:3197-3202.

Paulmurugan, R. and Gambhir, S.S. 2003. Monitoring protein-protein interactions using split synthetic renilla luciferase protein-fragment-assisted complementation. *Anal. Chem.* 75:1584-1589.

Pelletier, J.N., Campbell-Valois, F.X., and Michnick, S.W. 1998. Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. *Proc. Natl. Acad. Sci. U.S.A.* 95:12141-12146.

Rackham, O. and Brown, C.M. 2004. Visualization of RNA-protein interactions in living cells: FMRP and IMP1 interact on mRNAs. *EMBO J.* 23:3346-3355.

Rajaram, N. and Kerppola, T. 2004. Transcription activation by Maf and Sox, and their subnuclear localization are disrupted by a mutation in Maf that causes cataract. *Mol. Cell. Biol.* 24:5694-5709.

Remy, I. and Michnick, S.W. 2004a. A cDNA library functional screening strategy based on fluorescent protein complementation assays to identify novel components of signaling pathways. *Methods* 32:381-388.

Remy, I. and Michnick, S.W. 2004b. Regulation of apoptosis by the Ft1 protein, a new modulator of protein kinase B/Akt. *Mol. Cell. Biol.* 24:1493-1504.

Remy, I., Montmarquette, A., and Michnick, S.W. 2004. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat. Cell Biol.* 6:358-365.

Rossi, F., Charlton, C.A., and Blau, H.M. 1997. Monitoring protein-protein interactions in intact eukaryotic cells by beta-galactosidase complementation. *Proc. Natl. Acad. Sci. U.S.A.* 94:8405-8410.

Rossi, F.M., Guicherit, O.M., Spicher, A., Kringstein, A.M., Fatyol, K., Blakely, B.T., and Blau, H.M. 1998. Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16. *Nat. Genet.* 20:389-393.

Sorkin, A., McClure, M., Huang, F., and Carter, R. 2000. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr. Biol.* 10:1395-1398.

Tsien, R.Y. 2003. Imagining imaging's future. *Nat. Rev. Mol. Cell. Biol.* Suppl. SS16-21.

Tsuchisaka, A. and Theologis, A. 2004a. Heterodimeric interactions among the 1-amino-cyclopropane-1-carboxylate synthase polypeptides encoded by the *Arabidopsis* gene family. *Proc. Natl. Acad. Sci. U.S.A.* 101:2275-2280.

Tsuchisaka, A. and Theologis, A. 2004b. Unique and overlapping expression patterns among the arabidopsis 1-amino-cyclopropane-1-carboxylate synthase gene family members. *Plant Physiol.* 136:2982-3000.

Tzfira, T., Vaidya, M., and Citovsky, V. 2004. Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. *Nature* 431:87-92.

Ullmann, A., Jacob, F., and Monod, J. 1967. Characterization by in vitro complementation of a peptide corresponding to an operator-proximal segment of the beta-galactosidase structural gene of *Escherichia coli*. *J. Mol. Biol.* 24:339-343.

Ullmann, A., Jacob, F., and Monod, J. 1968. On the subunit structure of wild-type versus complemented beta-galactosidase of *Escherichia coli*. *J. Mol. Biol.* 32:1-13.

von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K.I., Soderberg, O., Kerppola, T.K., and Larsson, L.G. 2003. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. *Mol. Cell* 11:1189-1200.

Walter, M., Chaban, C., Schutze, K., Batistic, O., Weckermann, K., Nake, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K., and Kudla, J. 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* 40:428-438.

Wehrman, T., Kleaveland, B., Her, J.H., Balint, R.F., and Blau, H.M. 2002. Protein-protein interactions monitored in mammalian cells via complementation of beta-lactamase enzyme fragments. *Proc. Natl. Acad. Sci. U.S.A.* 99:3469-3474.

Wei, G.H., Liu, D.P., and Liang, C.C. 2004. Charting gene regulatory networks: Strategies, challenges and perspectives. *Biochem. J.* 381:1-12.

Yu, H., West, M., Keon, B.H., Bilter, G.K., Owens, S., Lamerdin, J., and Westwick, J.K. 2003. Measuring drug action in the cellular context using protein-fragment complementation assays. *Assay Drug Dev. Technol.* 1:811-822.

Zal, T. and Gascoigne, N.R. 2004. Using live FRET imaging to reveal early protein-protein interactions during T cell activation. *Curr. Opin. Immunol.* 16:418-427.

Zhang, S.F., Ma, C., and Chalfie, M. 2004. Combinatorial marking of cells and organelles with reconstituted fluorescent proteins. *Cell* 119:137-144.

Contributed by Chang-Deng Hu,
Asya V. Grinberg, and
Tom K. Kerppola
Howard Hughes Medical Institute and
University of Michigan Medical School
Ann Arbor, Michigan

Design and Use of Fluorescent Fusion Proteins in Cell Biology

The discovery that green fluorescent protein (GFP) variants and coral fluorescent proteins can be functionally expressed in heterogeneous systems has revolutionized cell biology (Lippincott-Schwartz et al., 2001; Miyawaki et al., 2003). Unmodified fluorescent proteins (FPs) can be visualized by fluorescence microscopy and can serve as probes of environments within living cells. The addition of targeting and retention sequences to FPs can be exploited to highlight specific cellular organelles and to follow their dynamics. The ability of FPs to fold, even when fused to cellular proteins, has made it possible to directly study the biology of proteins in vivo. A protein of interest can be monitored in cells or even in whole animals without having to purify, label, and deliver the protein into cells. Thus, it is now possible to label and observe proteins in previously inaccessible environments, such as organelle lumena. Fusion of FPs to proteins of interest can reveal a wealth of data, including information on a protein's steady-state distribution, dynamics, history, and association with other proteins (Lippincott-Schwartz et al., 2001; 2003). In this unit, strategies and background for designing and creating fluorescent fusion proteins (FFPs) are described.

To design an FFP, the investigator must consider what the FFP's intended use is, which fluorescent tag to add, whether the FP has complicating issues related to the protein of interest's environment (e.g., pH sensitivity, enhanced aggregation), and where to insert the FP. The actual construction of FFPs can be readily accomplished using standard molecular biology techniques and will only be described in general terms in the Basic Protocol. Investigators seeking specific advice on cloning techniques are referred to *Current Protocols in Molecular Biology* (Ausubel et al., 2005) or *Molecular Cloning: A Laboratory Manual* (Sambrook and Russell, 2001).

DESIGN OF A FLUORESCENT FUSION PROTEIN

BASIC PROTOCOL

Most fluorescent protein (FP) cDNAs are commercially available from BD Biosciences Clontech or from the laboratories that first described the FP. Often, FP coding regions are positioned adjacent to a multicloning site in the plasmid for ease of subcloning. The cDNA for the protein of interest can sometimes be directly cloned into the FP vector. However, in many cases, construction of a fluorescent fusion protein (FFP) will necessitate modification of the protein of interest and/or the FP. As described below, placement of an FP can affect the localization and functionality of the protein of interest. Because the required modifications will vary on a case-by-case basis, two different general cloning strategies are described below:

Strategy 1: When a protein's functional and targeting domains are unknown

Even if only minimal information on a protein is available, it is still possible to study the protein's environment and behavior in cells. However, the investigator should first assess the steady-state distribution of the unmodified protein of interest (e.g., by immunolocalization; *UNIT 4.3*). This is necessary to ensure that the corresponding FFPs localize properly. It is also useful to compare the distribution of the untagged protein in different cell types, as some proteins localize very differently in different types of cells. For example, procollagen only folds properly and exits from the ER in cell types expressing specific chaperones (Nagai et al., 2000).

Contributed by Erik Snapp
Current Protocols in Cell Biology (2005) 21.4.1-21.4.13

To maximize the likelihood of creating a functional and properly targeted FFP, the investigator should design two constructs. One construct should contain the FP at the NH_2-terminus of the protein of interest, and the other should have the FP at the COOH-terminus, as many proteins fold with their NH_2- and COOH-termini exposed on the protein surface, rather than buried in the protein core (Hovmöller and Zhou, 2004). It is often useful to include a small linker of two to ten amino acids, such as glycine interspersed with serine residues (to enhance the solubility of the linker), to provide flexibility between the FP and the protein of interest (Miyawaki et al., 2003). The linker can help promote proper folding and functioning of both the FP and the protein of interest. Note that the necessary size of a linker can only be determined empirically. The position of the FP can also affect the need for a linker. For example, the COOH-terminus of enhanced green fluorescent protein (EGFP) is floppy and rarely requires a linker when fused to the NH_2-terminus of the protein of interest (Miyawaki et al., 2003). A linker can be added by PCR to the cDNA encoding the protein of interest or to the FP cDNA.

The final FFP construct will contain an in-frame fusion between the FP and the protein of interest, an unambiguous initiating methionine within the appropriate Kozak sequence (for eukaryotes, this is 5′-ACCATGG-3′, where the internal ATG is the initiating methionine; Kozak, 1992), a linker between the FP and the protein of interest (if necessary), and appropriate regulatory elements (e.g., promoter, 5′ and 3′ untranslated regions). Appropriate regulatory elements are often included in commercially available FP subcloning vectors.

Once a cloning strategy and PCR primers have been designed, the investigator can generate the FFP construct. The final step of a cloning strategy is to place the FFP in an appropriate expression vector. For example, the cytomegalovirus promoter–containing EGFP-C1 vector (Clontech) can be used to express the FFP in mammalian cells, but not in bacteria or yeast. Insertion of the construct into an appropriate vector can be accomplished by ligation into a multicloning site.

In preparation for future experiments, it is often useful to make several color variants of the desired FFP. Because the GFP variant sequences are identical to each other at the NH_2- and COOH-termini, it is easy to use the same PCR primers to amplify multiple variants simultaneously.

Strategy 2: When a protein's function can be assayed and targeting domains have been identified

In this situation, the investigator can exploit the full potential of FFPs. The FP can be inserted at an optimal position (i.e., one at which the FP will not interfere with targeting domains or protein folding), and the resulting FFP can be assayed for function. As in strategy 1, it is important to be able to assess the steady-state distribution of the wild-type protein of interest. This will help the investigator distinguish whether the FP affects the spatial distribution of the protein being studied.

The example of a lumenal endoplasmic reticulum (ER) protein illustrates some of the considerations affecting FP placement (Fig. 21.4.1). A typical lumenal ER protein contains two critical sequences with targeting information—a signal sequence at the NH_2-terminus, and an ER-retention sequence at the COOH-terminus. The signal sequence is essential for the targeting and translocation of the nascent peptide into the lumen of the ER (Martogolio and Dobberstein, 1998). Thus, the FP must be placed after the signal sequence, but where?

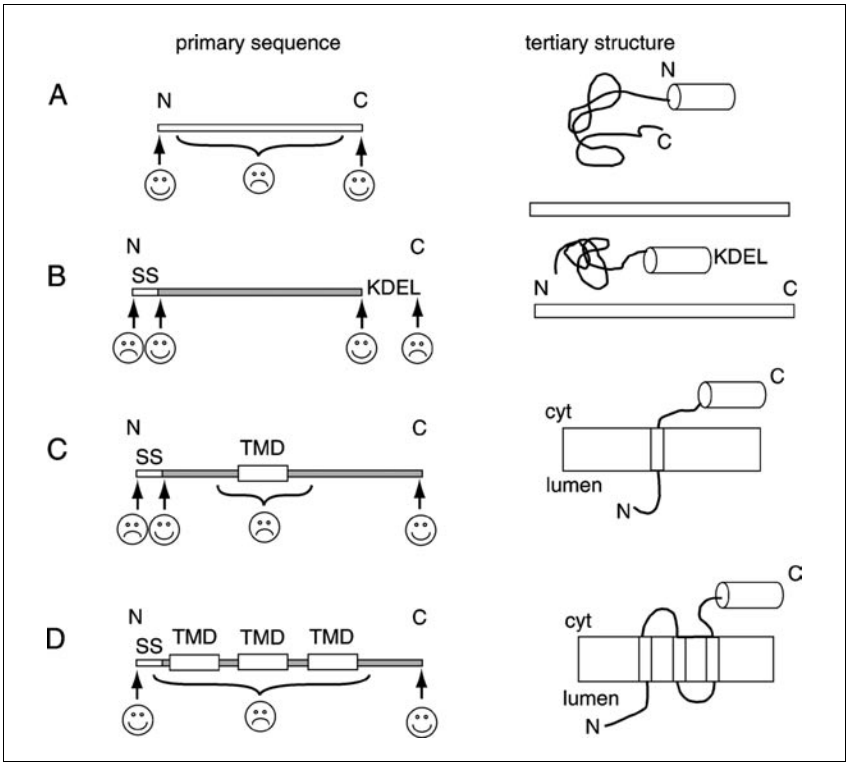


Figure 21.4.1 Appropriate positioning of a fluorescent protein (FP) in a fluorescent fusion protein construct. Preferred sites of FP fusion in the primary sequence of the protein of interest are indicated by happy face icons, and domains to be avoided are indicated by sad face icons. Each tertiary structure shows the folding of the sample construct with the FP (represented as a cylinder) fused at an optimal site. (**A**). A hypothetical globular protein expressed in the cytoplasm can have the FP fused at either the NH_2- or the COOH-terminus. Typically, one end of the protein of interest will contain a functional domain that may be sterically hindered by an FP, and so it is useful to make both of the possible constructs. (**B**) A hypothetical lumenal protein contains an NH_2-terminal signal sequence (SS), a mature domain, and a COOH-retention sequence (KDEL). An FP placed immediately after the SS or immediately before the retention sequence is less likely to interfere with the functioning of either sequence. (**C**) A single membrane–spanning protein has the additional constraint that the FP cannot be placed within or near the transmembrane domain (TMD), as this will disrupt the domain and cause problems with membrane integration. (**D**) A membrane multispanning protein has the same constraint as the example in panel C, but in multiple locations. The loops between the transmembrane domains are also poor choices, because the exact spacing between transmembrane domains is often important for protein folding, and because these loops often contain functional domains. Abbreviation: cyt, cytoplasm.

If the functional domain of the protein is near the COOH-terminus, then placing the FP after the signal sequence is a reasonable strategy. It is advantageous to place the FP two to ten amino acids downstream of the predicted signal-sequence cleavage site (R.S. Hegde, unpub. observ.). This will enhance the efficiency of signal-sequence cleavage and help promote the efficiency of translocation of the FFP into the ER.

If the functional domain of the protein is toward the NH_2-terminus, then the FP should be placed toward the COOH-terminus, but not necessarily at the absolute terminus. For example, in animal cells, the KDEL motif must account for the final four amino acids of the protein, as this motif functions as a lumenal ER retention sequence (Pelham, 1990). In such a case, the FP should be inserted just before the start of the KDEL motif.

Additional examples of appropriate and inappropriate FP placement sites are illustrated in Figure 21.4.1. Once the FP has been inserted, as in strategy 1, the last step is to place the FFP in an appropriate expression vector.

Finally, in both strategies, the investigator must confirm that the FFP construct has the correct DNA sequence and assess whether the expressed FFP is fluorescent, localizes in a pattern similar to that of the wild-type protein within the cell (e.g., by performing immunofluorescence and colocalization studies; *UNIT 4.3*), and retains the functionality of the native protein (as determined by the investigator's own assays). In the absence of functional characterization, the investigator should be vigilantly skeptical of inferring too much from studies of FFPs.

COMMENTARY

Background Information

Considerations for the design and application of FFPs

The various fluorescent proteins (FPs) have different advantages and limitations. Before designing and generating an FFP, the investigator needs to identify what questions the FFP will be used to address. It is worth spending some time asking whether the planned construct will be able to fulfill the stated purpose. Additional concerns include whether the FFP will be sufficiently bright, whether it will be appropriate for the time scale of the planned experiment, and whether the necessary equipment is available for the application.

The simplest fluorescent fusion proteins (FFPs) are FPs fused to targeting sequences (e.g., a nuclear localization sequence or a signal sequence) for the highlighting of an organelle or a cellular domain of interest. Such FFPs permit the investigator to colocalize a protein of interest with a specific organelle or to follow the dynamics of an organelle in a living cell (Lippincott-Schwartz et al., 2001; Miyawaki et al., 2003). In addition, these FFPs can be coupled with photobleaching methods (*UNIT 21.1*) to probe the viscosity or crowdedness of a cellular environment (Dayel et al., 2000; Nehls et al., 2000; Lippincott-Schwartz et al., 2001). FFPs containing a full-length protein fused to an FP are potentially valuable tools that can be exploited to illuminate a protein's function or behavior in its native environment.

How much of a fluorescent protein is enough?

The first issue to carefully consider is the normal expression levels of the protein of interest. Ranges of expression levels for a variety of proteins are provided in Table 21.4.1. Compare these values with the concentrations of FP that are required for visualization over background fluorescence in cells. For example, to achieve a twofold increase in fluorescence over background fluorescence, enhanced green fluorescent protein (EGFP) must be expressed at 200 nM (Patterson et al., 1997). Thus, to visualize a homogeneously distributed cytoplasmic FFP, the FFP must be expressed at levels two orders of magnitude higher than those for a kinase such as MAPKKK (Table 21.4.1). However, if all of the FFP will be concentrated in discrete compartments or domains, then low expression levels may be sufficient to visualize the protein or organelle of interest; in contrast, if a protein normally binds to a receptor present at low levels, the excess fluorescence of the unbound FFP could obscure the physiologically relevant population. More importantly, the potential biological consequences of the overexpressed protein should not be underestimated. Therefore, knowing the normal expression levels and localization of one's protein of interest can greatly assist in the planning and interpretation of experiments. To determine a protein's relative expression level, the investigator can compare immunoblots from lysates of cells that natively express the protein of interest and lysates of cells stably transfected with the FFP, using the same antibody for the native protein and the FFP (*UNIT 6.2*).

Rate of fluorophore maturation

FFPs are generally useful as markers for and probes of protein and organelle dynamics. However, the temporal aspects of FP maturation may place constraints on the utility of FFPs in certain experiments. For example, current FFPs are unlikely to be useful for following the behavior of nascent proteins within the first few minutes of translation. This is because EGFP takes up to 30 min to fold and fluoresce in solution (Heim et al., 1994). However, EGFP may become fluorescent more rapidly in cells (Prendergast, 1999).

The *Discosoma* red fluorescent protein (DsRed) is even slower, taking up to 48 hr

Table 21.4.1 Typical Protein Concentrations in Cells

Protein	Concentration[a,b] (μM)	Reference
Enzymes		
Ornithine decarboxylase	15 (rabbit liver)	Albe et al., 1990
Hexokinase	0.5 (rabbit muscle)	Albe et al., 1990
Aldolase	15 (rat liver); 809 (rabbit muscle)	Albe et al., 1990
Signaling molecules		
MAPKKK (Mos)	0.006 to 0.015	Huang and Ferrell, 1996
ras p21	0.25	Hand et al., 1987
MAPK phosphatase	0.024 to 0.6	Huang and Ferrell, 1996
MAPKinase (p42)	0.24 to 6	Huang and Ferrell, 1996
MAPKK (Mek1)	0.24 to 6	Huang and Ferrell, 1996
cAMP-dependent kinase	2	Francis and Corbin, 1994
Calmodulin	30	Manalan and Klee, 1984
Cytoskeleton		
Actin	95	Luby-Phelps, 2000
Vimentin	3	Luby-Phelps, 2000

[a] According to Niswender et al. (1995) and Patterson et al. (1997), a concentration of at least 200 nM enhanced green fluorescent protein (EGFP) is necessary to visualize a fluorescence increase over background autofluorescence in HeLa cells. Enhanced yellow fluorescent protein (EYFP) is 1.5× brighter than EGFP (Table 21.4.2), and so only 133 nM EYFP is necessary for a detectable fluorescence increase. See Table 21.4.2 for relative brightness data on other fluorophores.

[b] The volume of a BHK cell (3900 μm^3; Griffiths et al., 1984) was used to calculate concentration when only an absolute protein amount was provided in the literature.

to make 90% of all newly synthesized proteins fluoresce (Baird et al., 2000). However, new red FP variants are available that fold in <1 hr (Bevis and Glick, 2002; Campbell et al., 2002). The slow folding of one DsRed variant, DsRed-E5, has been exploited to visually distinguish between proteins younger and older than 9 hr (Terskikh et al., 2000).

Size matters

Unlike an epitope tag, FP fusions are not inherently small modifications to proteins (Fig. 21.4.2). An FP represents a significant addition to a protein and thus may have steric consequences for protein folding, function, or targeting. GFP variants and the coral FPs are 27 kDa in size. The crystal structures of all of the FPs show β-barrels that are 3 nm in diameter and 4 nm in length (Yang et al., 1996). In addition, many of the coral FPs exist as obligate tetramers of these β-barrels (Baird et al., 2000), which not only increases their size but also tends to induce formation of aggregates. For these reasons, investigators need to be alert to FP-related effects on a protein's behavior. Alternatives to FPs are briefly discussed in the following section.

Selecting a fluorescent protein

Once the investigator has determined that an FFP will meet the requirements of the experiment, he or she must choose an FP. In the past few years, the variety of available FPs has increased dramatically. In addition to GFP and its spectral variants, coral FPs that span the visible spectrum and FPs with new functionalities have become available (Miyawaki et al., 2003). Many of the currently available FPs are listed in Table 21.4.2. Generally, the investigator will want to select the brightest possible FPs to increase the signal-to-noise ratio. The relative brightness values of various FPs are indicated in Table 21.4.2. It is also important to confirm that the necessary equipment (e.g., filter sets, excitation lasers) for using the FP of interest is available. BD Biosciences provides a useful Web resource that profiles the excitation and emission spectra of several fluorescent dyes and proteins (*http://www.bdbiosciences.com/spectra/*).

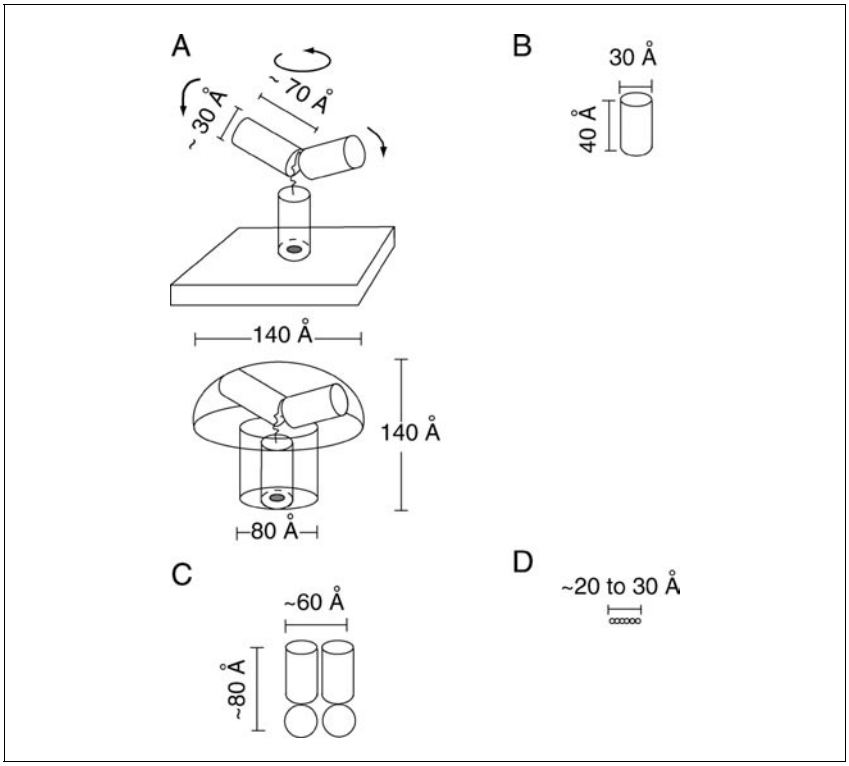


Figure 21.4.2 Relative sizes of (**A**) immunoglobulin G (IgG; reference for comparison with panels B to D), (**B**) green fluorescent protein (GFP), (**C**) the *Discosoma* red fluorescent protein (DsRed) tetramer, and (**D**) biarsenical tetracysteine.

Families of FPs that are currently available are described below. It is likely that improved variants will continue to become available, and so the investigator should regularly search the literature for the latest FPs.

Note that many of the FP cDNAs are either native sequences or have been codon-optimized for the intended host cell type. Codon-optimized variants are available for mammalian, fungal, and plant cells (Yang et al., 1996; Davis and Vierstra, 1998; Sheff and Thorn, 2004). If the FP expresses poorly in organisms such as bacteria or fungi, codon bias or Kozak sequence–related issues should be considered.

GFP and its spectral variants

The first FP to be cloned and characterized was green fluorescent protein (GFP) from the jellyfish species *Aequorea victoria* (Prasher et al., 1992). GFP folds to form a β-barrel bearing a triplet of internal amino acids (SYG) that autocatalytically form a fluorophore within the barrel (Heim et al., 1994). Wild-type GFP does not fold efficiently, is sensitive to pH, and can undergo reversible photobleaching (Patterson et al., 1997), which is a problem in FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) experiments (*UNIT 21.1*). To improve the utility of GFP, the codon bias was modified to make the protein more suitable for expression in mammalian cells, and two amino acids were mutated to enhance brightness and protein folding (S65T and P64L). The resulting variant is termed EGFP and is available from Clontech (Yang et al., 1996).

Note that EGFP and its variants have a modified amino acid numbering system. When EGFP was first created, amino acid 2, a valine, was deleted. However, publications generally use the original wild-type GFP amino acid numbering scheme. Thus, the fourth amino acid (starting from the NH_2-terminus) in EGFP would be numbered as amino acid 5 in most publications. This can be important when sequencing or mutagenizing the EGFP variants.

Point mutations can alter the spectral properties of EGFP to create blue (ECFP) and yellow (EYFP) variants, also available from Clontech. These variants differ in other ways besides spectral excitation and emission.

Table 21.4.2 Properties of Commonly Used Fluorescent Proteins[a]

Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)	Relative brightness[b]	Reference
wtGFP	397	508	20,448 (0.6×)	Patterson and Lippincott-Schwartz, 2002
EGFP	490	509	33,000 (1×)	Rizzo et al., 2004
PAGFP (preactivation)	504	515	2691 (0.08×)	Patterson and Lippincott-Schwartz, 2002
PAGFP (postactivation)	400	517	13,746 (0.4×)	Patterson and Lippincott-Schwartz, 2002
EYFP	514	527	51,240 (1.5×)	Rizzo et al., 2004
Venus	515	528	52,554 (1.6×)	Nagai et al., 2002
Citrine	516	529	58,520 (1.8×)	Griesbeck et al., 2001
ECFP	433	476	10,730 (0.3×)	Rizzo et al., 2004
Cerulean Blue	433	475	26,660 (0.8×)	Rizzo et al., 2004
DsRed	558	583	45,030 (1.4×)	Campbell et al., 2002
T1	554	586	12,642 (0.4×)	Bevis and Glick, 2002
mRFP	584	607	11,000 (0.3×)	Campbell et al., 2002
Kaede (preactivation)	508	518	78,400 (2.4×)	Ando et al., 2002
Kaede (postactivation)	572	582	19,932 (0.6×)	Ando et al., 2002
HcRed	592	645	na	Gurskaya et al., 2001
KFP-1 (preactivation)	na	600	<123 (0.004×)	Chudakov et al., 2003
KFP-1 (postactivation)	580	600	4130 (0.1×)	Chudakov et al., 2003
mAzami-Green	492	505	33,858 (1×)	Karasawa et al., 2003

[a]Abbreviation: na, not available.
[b]Quantum yield multiplied by extinction coefficient. Number in parentheses is the brightness of the fluorescent protein relative to EGFP.

For example, ECFP is substantially dimmer than EGFP, while EYFP is slightly brighter (Table 21.4.2). To calculate the brightness of an FP, multiply the protein's extinction coefficient (ε, the efficiency of photon absorption) by its quantum efficiency (ϕ, the ratio of photons emitted to photons absorbed; Table 21.4.2).

Recently, EGFP and its variants have been further improved. All EGFP variants can undergo weak transient dimerization in living cells (Zacharias et al., 2002). This dimerization can produce false-positive FRET results and lead to the reorganization of membrane structures when an enhanced FP is fused to a membrane protein (Fig. 21.4.3A; Zacharias et al., 2002; Snapp et al., 2003). Fortunately, dimerization can be disrupted by any one of three different point mutations (A206K, L221K, or F223R) without changing the fluorescent properties of EGFP (Zacharias et al., 2002). Such monomerized variants can be generated by mutagenesis or can be obtained from the author of this unit.

Another advance in EGFP variants has been the generation of brighter versions that fold more efficiently. Two YFP variants (called Venus and Citrine; Nagai et al., 2002; Griesbeck et al., 2001) that are more resistant to the effects of chloride ions and pH, as well as a CFP variant (called Cerulean Blue; Rizzo et al., 2004) that has improved fluorescence properties, have become available, and the reader is encouraged to use these improved versions.

Other groups have modified GFP to create environmental sensors. For example, the sensitivity of wild-type GFP to pH has been exploited by Miesenbock et al. (1998) to create mutants that can function as pH sensors.

Coral fluorescent proteins

GFP and its variants cover the visible spectrum from 400 to ~540 nm. Until recently, FPs were not generally available at the red

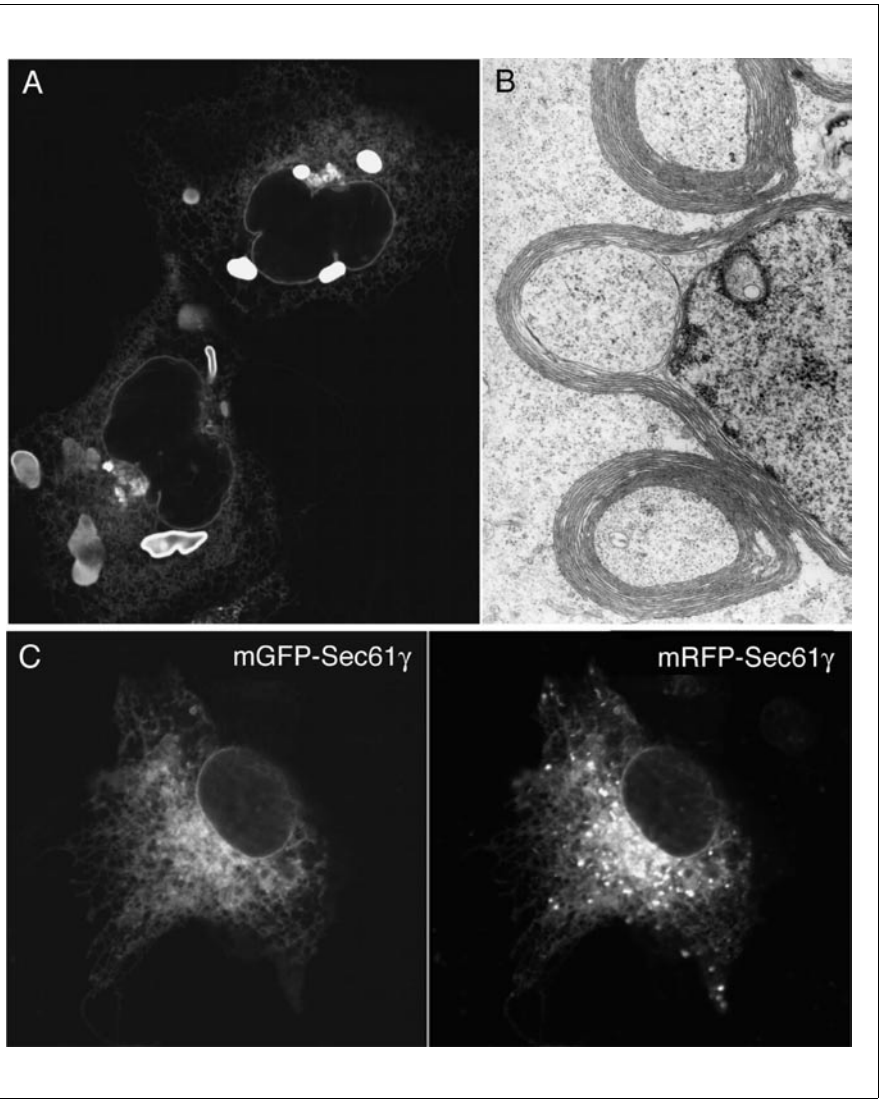


Figure 21.4.3 The fusion of a fluorescent protein (FP) to a native protein may change the protein's normal localization pattern or may lead to the formation of aggregates or oligomers. (**A**) The fusion of nonmonomerized enhanced green fluorescent protein (EGFP) to a resident endoplasmic reticulum (ER) membrane protein induces the formation of an organized smooth ER structure. (**B**) The fusion of monomerized EGFP to the same protein does not grossly alter the structure of the ER. (**C**) A Cos-7 cell expressing two fluorescent fusion proteins (FFPs), one containing monomerized green fluorescent protein (mGFP; left-hand image) and the other containing monomerized red fluorescent protein (mRFP; right-hand image). The mGFP-containing FFP localizes to the ER network, and similarly, the mRFP-containing FFP colocalizes to the ER membranes. The image yielded by the mRFP-tagged protein shows bright puncta, which are probably mRFP aggregates.

end of the spectrum. FPs that emit at longer wavelengths are highly desirable, because autofluorescence is reduced at these wavelengths (Miyawaki et al., 2003). The discovery of a series of red FPs and chromophores in coral species remedied this deficit. The first of the coral FPs, DsRed, has a spectral emission in the range of 583 nm (Table 21.4.2). This property offers the investigator an additional marker for imaging in the same cell with CFP and GFP/YFP fusion proteins. However, wild-type DsRed, like wild-type GFP, has significant drawbacks. It is an obligate tetramer, it matures slowly (48 hr), and its excitation and

emission profiles are in the same spectral range as EGFP during the first 6 hr of the maturation phase (Baird et al., 2000). Two enhanced versions of DsRed have become available—DsRed.T4 (Bevis and Glick, 2002) and mRFP (Campbell et al., 2002). DsRed.T4 matures much more quickly than DsRed (<1 hr) but is still a tetramer. The spectral properties of mRFP, a monomerized variant of DsRed created by Campbell et al. (2002), make it substantially dimmer than DsRed (Table 21.4.2). Yet, the rate of folding for this protein is ten times faster, which effectively results in similar levels of brightness compared with wild-type DsRed in cells (Campbell et al., 2002).

It should be noted that while mRFP is generally less prone to aggregation than previous variants of DsRed, researchers in the Lippincott-Schwartz laboratory have encountered difficulties with the fusion of mRFP to integral membrane proteins. Often, bright red puncta can be observed in cells expressing these FFPs (Fig. 21.4.3C), while GFP-based FFPs do not form these puncta. The most likely explanation is that attachment of mRFP to a membrane protein reduces that protein's rotational diffusion, increases its effective concentration (in the two dimensions of the membrane), and results in a tendency to form aggregates. In general, when using a new FP to make an FFP, it is useful to compare the FFP distribution with the distribution of the native protein fused to an EGFP variant or the immunofluorescence distribution of the native protein alone.

Additional coral FPs include a far red–emitting FP, HcRed (Gurskaya et al., 2001), as well as a green-emitting FP (Karasawa et al., 2003), among others (Table 21.4.2). Some of these proteins are also obligate oligomers, and the investigator should be familiar with the properties of these FPs before deciding to use them.

Photoactivatable proteins and timer proteins

Not all FPs are restricted to a single color. As noted in the preceding section, wild-type DsRed undergoes a slow maturation involving a phase as a green-emitting FP. A variant, DsRed-E5, has a more rapid transition from green to red (within 5 hr of protein synthesis) and permits investigators to follow both the steady-state distribution and the relative age of an FFP (Terskikh et al., 2000). The ratio of more mature (red) proteins to immature (green) proteins can be used to obtain information about the lifetime of the protein and whether proteins of different ages have different distributions.

To mark discrete populations of proteins, three FP options are currently available—PAGFP (Patterson and Lippincott-Schwartz, 2002), Kaede (Ando et al., 2002), and KFP-1 (Chudakov et al., 2003), which are all photoactivatable FPs. Because a particular population of protein molecules tagged with one of these FPs can be photoactivated (i.e., the fluorescent properties of the FP tag can be "turned on") at a discrete time, the new synthesis of fluorescent proteins during the time course of an experiment is eliminated as a potential problem. PAGFP is derived from GFP and is monomerized. Initially, it is excited by UV light (Patterson and Lippincott-Schwartz, 2002) and barely emits in the green range (505 to 530 nm). Upon brief stimulation with intense UV light (similar to a photobleaching experiment), however, it can be excited under the same conditions as EGFP and will fluoresce 100 times more brightly in the green range compared with the unstimulated protein. (Note that PAGFP is fluorescent in the blue range both before and after photoactivation.) UV photoactivation requires a 405- or 413-nm excitation laser or a mercury arc lamp with the proper filter set. For a more detailed description of working with this FP, see Patterson and Lippincott-Schwartz (2004). Once photoactivated, PAGFP can also be used for photobleaching applications, such as FRAP.

Kaede, a variant of DsRed, can be photoactivated with 350- to 415-nm excitation to increase its fluorescence intensity up to 1000-fold over background. Absorption shifts from 508 nm to 572 nm upon activation, and emission shifts from 518 nm to 582 nm (Ando et al., 2002). In contrast to PAGFP, Kaede undergoes a complete conversion in both its absorption and emission spectra, and this conversion readily permits the simultaneous spectral separation of activated and unactivated proteins. However, Kaede is an obligate tetramer and requires UV light for photoactivation. To avoid exposing cells to intense UV light, investigators can use the coral FP KFP-1, which can be excited with a less phototoxic, longer-wavelength laser (532 nm) to increase fluorescence 30-fold over background (Chudakov et al., 2003). In addition, KFP-1 differs from the other two photoactivatable FPs in that it is inherently nonfluorescent in its unactivated form. However, like Kaede, KFP-1 is an obligate tetramer.

In addition to having pulse-labeling applications, photoactivatable proteins can be used to mark organelles or whole cells and may be useful for following cell lineages in development experiments.

Alternatives

As noted above, none of the FPs are small, and some proteins do not fold or are nonfunctional following the attachment of a bulky FP (Andresen et al., 2004). If this is the case, a few alternative options are available.

Fluorescent dyes

Prior to the cloning of GFP, investigators studying proteins or compartments in living cells often used organelle-specific dyes (e.g., MitoTracker; Invitrogen) or microinjected purified proteins conjugated to dyes such as fluorescein or rhodamine into cells. These methods are still used and can be quite powerful by themselves or in combination with FFPs. Dye labeled proteins offer certain advantages over natural FPs, including (1) tight control over the amount of fluorescently labeled protein in the cell; (2) the elimination of concerns about new fluorescent protein synthesis; (3) the ability to acutely introduce otherwise cytotoxic proteins into the cell; (4) the wide variety of available dyes, compared with the relatively small selection of FPs; and (5) the much smaller size of dye molecules, making them less likely to cause steric hindrance. The primary concerns with dye-labeled proteins are that (1) dyes may render a protein nonfunctional; (2) many readily undergo photobleaching, which can cause significant photodamage to cells (although some of the newer dyes, such as Invitrogen's Alexa dyes, are more resistant to photobleaching); (3) a microinjector is often required to deliver dye-labeled proteins to the cytoplasm; and (4) the lumena of many organelles cannot be accessed or labeled.

Short fluorophore-binding epitopes

Some of the smallest available options for fluorescently labeling proteins include fluorescent biarsenical-linked dyes (namely, FlAsH and ReAsH). The addition of a tetracysteine amino acid sequence (CCPGCC) to a flexible exposed domain of a protein of interest (typically the COOH-terminus) makes the protein competent to bind these fluorescein- (FlAsH) or rhodamine-derived (ReAsH) arsenicals (Griffin et al., 1998; Gaietta et al., 2002). These modified dyes are membrane-permeable and relatively small. Thus, it is possible to fluorescently label both cytoplasmic and lumenal proteins in cells without resorting to microinjection or other protein delivery methods. An important advantage is the comparatively small size of the tetracysteine tag and the bound dyes. Andresen et al. (2004) recently demonstrated that specific yeast tubulin isoforms were functional when one or two tetracysteine motifs were added, whereas complementation in mutant cells was not possible when three tetracysteine motifs or a single GFP was added.

Another useful property of these two dyes is that they can be added sequentially to differentially label newly synthesized proteins, resulting in a kind of biochemical photoactivation (Gaietta et al., 2002). Furthermore, ReAsH can photoconvert diaminobenzidine (DAB) to produce an electron-dense reaction product, permitting tetracysteine-tagged proteins to be imaged in the same cells by fluorescence, bright-field, and electron microscopy (Gaietta et al., 2002).

The tetracysteine-binding dyes do have at least one limitation associated with their use. Stroffekova et al. (2001) observed a significant fluorescence background even in FlAsH-treated cells not expressing a tetracysteine-tagged protein, as mammalian cells were found to contain proteins that naturally bind FlAsH. Those authors concluded that FlAsH labeling was best suited to proteins that are expressed at especially high levels (Stroffekova et al., 2001). However, proteins that concentrate in discrete cellular domains, such as gap junctions (Gaietta et al., 2002), probably would not require substantial overexpression to be visualized over background. The biarsenical dyes are comparatively new and may have other limitations. Nonetheless, FlAsH and ReAsH represent viable alternatives to the more commonly used FPs and are worth considering.

Troubleshooting

See Table 21.4.3 for a guide to troubleshooting in FFP design and use.

Anticipated Results

The methods described in this unit should permit the investigator to generate a fluorescently labeled protein that is expressed at levels comparable to those for the native unlabeled protein, that targets to the correct compartment in the cell, and that exhibits behavior similar to that of the native protein (in terms of half-life, dynamics, and protein-protein interactions). The investigator may exploit the FFP in live cells, developing animals, or in solution.

Table 21.4.3 Troubleshooting Guide for the Design and Use of Fluorescent Fusion Proteins[a]

Problem	Cause	Solution
FFP is not fluorescent	FFP's environment may suppress fluorescence (e.g., pH is too low)	Try an FP that is more tolerant of different environments
	FFP may not be folded	Place a short linker (2 to 10 amino acids) between the protein of interest and the FP; this may help to ensure proper folding of both the protein and the FP
	FFP is not synthesized or is highly unstable	Determine whether the FFP is expressed by performing immunoblot analysis (*UNIT 6.2*) or pulse-labeling/immunoprecipitation analysis (*UNITS 7.1 & 7.2*) of transfected cells[b]
FFP does not localize correctly	FP interferes with native protein's targeting sequence	Construct the FFP with the FP at the opposite end of the protein of interest Place a short linker (2 to 10 amino acids) between the protein of interest and the FP; this may help to ensure proper folding of both the protein and the FP Targeting machinery may be saturated; determine whether mistargeting is seen specifically in cells with higher expression of the FFP; if so, limit observations to dimmer cells[c]
	FP oligomerizes	Use a monomeric FP
FFP is nonfunctional	FP may sterically hinder folding or obstruct functional domains	Try constructing the FFP with the FP placed elsewhere in the protein of interest Place a short linker (2 to 10 amino acids) between the protein of interest and the FP; this may help to ensure proper folding of both the protein and the FP Try using a fluorescent biarsenical-linked dye (FlAsH or ReAsH) in place of the FP Try using a dye-labeled protein instead of an FFP

[a] Abbreviations: FFP, fluorescent fusion protein; FP, fluorescent protein.

[b] If the protein is expressed but unstable, the FP must be placed in a different position. Try fusing the FP gene near the other terminus of the cDNA encoding the protein of interest. Alternatively, the FP may prevent incorporation of the FFP into a complex, thereby reducing FFP stability. If this is suspected, consider FlAsH or other smaller fluorescent labels. If the FFP is not expressed, check the sequence of the construct again. If the sequence is correct and contains an appropriate Kozak sequence, then the flanking sequences of the FFP gene may be interfering with transcription or translation. If this is the case, the FFP coding sequence (with Kozak sequence) should be excised and inserted in the multicloning site of an appropriate expression vector.

[c] Overall expression can be reduced by stably transfecting cells and/or by placing the FFP construct in a vector with an inducible promoter and modulating FFP gene expression.

Time Considerations

The time required for creation of an FFP will depend on the number of cloning steps required to generate the construct. If convenient restriction sites are present in the DNA sequence encoding the protein of interest and there are no concerns with targeting sequences, an FFP can be created in the time it takes to perform digestion, DNA purification, and ligation, followed by transformation and screening for the resulting DNA construct (1 to 3 days). More complex cloning strategies may require one or more PCR steps, the design of oligonucleotides, and more screening steps; in such cases, FFP creation can take up to 1 to 2 weeks. Characterization of the new construct with regard to proper localization will require the time necessary to transfect cells and perform immunocolocalization experiments (typically 4 hr to label cells and <1 hr to image them), and the time needed for functional assays will be application dependent.

Literature Cited

Albe, K.R., Butler, M.H., and Wright, B.E. 1990. Cellular concentrations of enzymes and their substrates. *J. Theor. Biol.* 143:163-195.

Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. 2002. An optical marker based on the uv-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99:12651-12656.

Andresen, M., Schmitz-Salue, R., and Jakobs, S. 2004. Short tetracysteine tags to β-tubulin demonstrate the significance of small labels for live cell imaging. *Mol. Biol. Cell* 15:5616-5622.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 2005. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Baird, G.S., Zacharias, D.A., and Tsien, R.Y. 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci. U.S.A.* 97: 11984-11989.

Bevis, B.J. and Glick, B.S. 2002. Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). *Nat. Biotechnol.* 20:83-87.

Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., Zacharias, D.A., and Tsien, R.Y. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99: 7877-7882.

Chudakov, D.M., Belousov, V.V., Zaraisky, A.G., Novoselov, V.V., Staroverov, D.B., Zorov, D.B., Lukyanov, S., and Lukyanov, K.A. 2003. Kindling fluorescent proteins for precise in vivo photolabeling. *Nat. Biotechnol.* 21:191-194.

Davis, S.J. and Vierstra, R.D. 1998. Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol. Biol.* 36:521-528.

Dayel, M.J., Hom, E.F., and Verkman, A.S. 1999. Diffusion of green fluorescent protein in the aqueous-phase lumen of endoplasmic reticulum. *Biophys. J.* 76:2843-2851.

Francis, S.H. and Corbin, J.D. 1994. Structure and function of cyclic nucleotide-dependent protein kinases. *Annu. Rev. Physiol.* 56:237-272.

Gaietta, G., Deerinck, T.J., Adams, S.R., Bouwer, J., Tour, O., Laird, D.W., Sosinsky, G.E., and Tsien, R.Y. 2002. Multicolor and electron microscopic imaging of connexin trafficking. *Science* 296:503-507.

Griesbeck, O., Baird, G.S., Campbell, R.E., Zacharias, D.A., and Tsien, R.Y. 2001. Reducing the environmental sensitivity of yellow fluorescent protein: Mechanism and applications. *J. Biol. Chem.* 276:29188-29194.

Griffin, B.A., Adams, S.R., and Tsien, R.Y. 1998. Specific covalent labeling of recombinant protein molecules inside living cells. *Science* 281:269-272.

Griffiths, G., Warren, G., Quinn, P., Mathieu-Costello, O., and Hoppeler, H. 1984. Density of newly synthesized plasma membrane proteins in intracellular membranes: I: Stereological studies. *J. Cell Biol.* 98:2133-2141.

Gurskaya, N.G., Fradkov, A.F., Terskikh, A., Matz, M.V., Labas, Y.A., Marynov, V.I., Yanushevich, Y.G., Lukyanov, K.A., and Lukyanov, S.A. 2001. GFP-like chromoproteins as a source of far-red fluorescent proteins. *FEBS Lett.* 507:16-20.

Hand, P.H., Vilasi, V., Caruso, A., and Schlom, J. 1987. Absolute values of *ras* p21 defined by direct binding liquid competition radioimmunoassays. *Biochim. Biophys. Acta* 908:131-142

Heim, R., Prasher, D.C., and Tsien, R.Y. 1994. Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 91:12501-12504.

Hovmöller, S. and Zhou, T. 2004. Why are both ends of the polypeptide chain on the outside of proteins. *Proteins* 55:219-222.

Huang, C.Y. and Ferrell, J.E. 1996. Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.* 93:10078-10083.

Karasawa, S., Araki, T., Yamamoto-Hino, M., and Miyawaki, A. 2003. A green-emitting fluorescent protein from *Galaxeidae* coral and its monomeric version for use in fluorescent labeling. *J. Biol. Chem.* 278:34167-34171.

Kozak, M. 1992. Regulation of translation in eukaryotic systems. *Annu. Rev. Cell Biol.* 8:197-225.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* 2:444-456.

Lippincott-Schwartz, J., Altan-Bonnet, N., and Patterson, G.H. 2003. Photobleaching and photoactivation: Following protein dynamics in living cells. *Nat. Cell Biol.* 5 (Suppl.):S7-S14.

Luby-Phelps, K. 2000. Cytoarchitecture and physical properties of cytoplasm: Volume, viscosity, diffusion, intracellular surface area. *Int. Rev. Cytol.* 192:189-221.

Manalan, A.S. and Klee, C.B. 1984. Calmodulin. *In* Advances in Cyclic Nucleotide Protein Phosphorylation Research (P. Greengard and G.A. Robison, eds.) pp. 227-278. Raven Press, New York.

Martogolio, B. and Dobberstein, B. 1998. Signal sequences: More than just greasy peptides. *Trends Cell Biol.* 8:410-415.

Miesenbock, G., DeAngelis, D.A., and Rothman, J.E. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394:192-195.

Miyawaki, A., Sawano, A., and Kogure, T. 2003. Lighting up cells: Labeling proteins with fluorophores. *Nat. Cell Biol.* 5 (Suppl.):S1-S7.

Nagai, N., Hosokawa, M., Itohara, S., Adachi, E., Matsushita, T., Hosokawa, N., and Nagata, K. 2000. Embryonic lethality of molecular chaperon hsp47 knockout mice is associated with defects in collagen biosynthesis. *J. Cell Biol.* 150:1499-1506.

Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., and Miyawaki, A. 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* 20: 87-90.

Nehls, S., Snapp, E.L., Cole, N.B., Zaal, K.J., Kenworthy, A.K., Roberts, T.H., Ellenberg, J., Presley, J.F., Siggia, E. and Lippincott-Schwartz, J. 2000. Dynamics and retention of misfolded proteins in native ER membranes. *Nat. Cell Biol.* 2:288-295.

Niswender, K.D., Blackman, S.M., Rohde, L., Magnuson, M.A., and Piston, D.W. 1995. Quantitative imaging of green fluorescent protein in cultured cells: Comparison of microscopic techniques, use in fusion proteins and detection limits. *J. Microsc.* 180:109-116.

Patterson, G.H. and Lippincott-Schwartz, J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297:1873-1877.

Patterson, G.H. and Lippincott-Schwartz, J. 2004. Selective photolabeling of proteins using photoactivatable GFP. *Methods* 32:445-450.

Patterson, G.H., Knobel, S.M., Sharif, W.D., Kain, S.R., and Piston, D.W. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73:2782-2790.

Pelham, H.R. 1990. The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* 15:483-486.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J. 1992. Primary structure of the *Aqueorea victoria* green-fluorescent protein. *Gene* 111:229-233.

Prendergast, F.G. 1999. Biophysics of the green fluorescent protein. *Methods Cell Biol.* 58:1-18.

Rizzo, M.A., Springer, G.H., Granada, B., and Piston, D.W. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* 22:445-449.

Sambrook, J. and Russell, D.W. (eds.) 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sheff, M.A. and Thorn, K.S. 2004. Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast* 21:661-670.

Snapp, E., Hegde, R., Francolini, M., Lombardo, F., Colombo, S., Pedrazzini, E., Borgese, N., and Lippincott-Schwartz, J. 2003. Formation of stacked cisternae by low affinity protein interactions. *J. Cell Biol.* 163:257-269.

Stroffekova, K., Proenza, C., and Beam, K.G. 2001. The protein-labeling reagent FLASH-EDT2 binds not only to CCXXCC motifs but also nonspecifically to endogenous cysteine-rich proteins. *Pflugers Arch.* 442: 859-866.

Terskikh, A., Fradkov, A., Ermakoua, G., Zaraisky, A., Tan, P., Kajava, A.V., Zhao, X., Lukyanou, S., Matz, M., Kim, S., Weissman, I., and Siebert, P. 2000. "Fluorescent timer": Protein that changes color with time. *Science* 290:1585-1588.

Tsien, R.Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 67:509-544.

Yang, T., Cheng, L., and Kain, S.R. 1996. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res.* 24:4592-4593.

Zacharias, D.A., Violin, J.D., Newton, A.C., and Tsien, R.Y. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science*. 296:913-916

Contributed by Erik Snapp
Albert Einstein College of Medicine
Bronx, New York

The Fluorescent Protein Color Palette

INTRODUCTION

Although the first report of fluorescence emission in the bioluminescent hydrozoan jellyfish species *Aequorea victoria* was recorded in the mid-twentieth century (Davenport and Nichol, 1955), and a protein extract was independently demonstrated by two investigators to be responsible for this "green" fluorescence in the 1960s and 1970s (Shimomura et al., 1962; Morin and Hastings, 1971a), it took another 20 years and a number of significant advances in the technology of molecular and cellular biology to witness the elucidation of the primary amino acid structure (Prasher et al., 1992). Following shortly thereafter was the astonishing demonstration that the jellyfish green fluorescent protein (GFP) could be readily employed as a useful marker for gene expression (Chalfie et al., 1994) in cells evolutionarily far removed from the jellyfish. During the next several years, a number of "enhanced" genetic variants having fluorescence emission spectral profiles in the blue (Yang et al., 1998), cyan (Heim and Tsien, 1996), green (Heim et al., 1995), and yellow (Wachter et al., 1998) regions of the visible spectrum were developed by engineering specific mutations of the original GFP nucleotide sequence. One of the most significant advances following the initial cloning and early mutagenesis efforts on the *Aequorea victoria* green fluorescent protein was the discovery of similar proteins in nonbioluminescent reef corals and sea anemones (Matz et al., 1999; Shagin et al., 2004) that not only provided a large spectrum of new emission colors, but also demonstrated that these protein motifs occur in a wide range of organisms.

Over the past decade, fluorescent proteins have launched a new and unprecedented era in cell biology by enabling investigators to apply routine molecular cloning methods, fusing these optical probes to a wide variety of protein and enzyme targets, in order to monitor cellular processes in living systems using fluorescence microscopy and related methodology (for recent reviews, see: Chudakov et al., 2005; Miyawaki et al., 2005; Shaner et al., 2005; Dixit et al., 2006; Prescott et al., 2006). The spectrum of applications for fluorescent proteins ranges from reporters of transcriptional regulation and targeted markers for organelles and other subcellular structures to fusion proteins designed to monitor motility and dynamics. These fascinating probes have also opened the door to creating biosensors for numerous intracellular phenomena, including pH and ion concentration fluctuations, protein kinase activity, apoptosis, voltage, and cyclic nucleotide signaling (Zhang et al., 2002; Meyer and Teruel, 2003; Miyawaki, 2003; Zaccolo, 2004). By applying selected promoters and targeting signals, fluorescent protein biosensors can be introduced into an intact organism and directed to a host of specific tissues, cell types, as well as subcellular compartments, to enable an unprecedented focus on monitoring a variety of physiological processes.

When coupled to recent technical advances in widefield fluorescence and confocal microscopy, including ultra-fast low-light-level digital CCD cameras, spinning-disk and swept-field instruments, as well as multitracking laser control systems with acousto-optic tunable filter (AOTF) control, the green fluorescent protein and its color-shifted genetic derivatives have demonstrated invaluable service in many thousands of live-cell imaging experiments. Compared to many traditional synthetic fluorophores, which are often toxic or photoreactive, the use of fluorescent proteins is minimally invasive for living cells, enabling visualization and recording of time-lapse image sequences for extended periods of time (Lippincott-Schwartz and Patterson, 2003; Day, 2005). Furthermore, continued advances in genetically fine-tuning the properties of fluorescent protein variants have led to increased brightness levels, improved photostability, and significantly better expression in mammalian cells (Verkhusha and Lukyanov, 2004; Shaner et al., 2005). These functional enhancements have stimulated a wide variety of investigations into protein dynamics and function using fluorescent protein chimeras imaged at low-light intensities for many hours to extract valuable information about changes in the steady-state distribution.

Among the most noteworthy attributes of the original green fluorescent protein derived from the *Aequorea victoria* jellyfish, as well as the more recently developed palette of color-shifted genetic variants, is that a stable and highly-defined cylindrical polypeptide

Contributed by Scott G. Olenych, Nathan S. Claxton, Gregory K. Ottenberg, and Michael W. Davidson

Current Protocols in Cell Biology (2007) 21.5.1-21.5.34

structure is essential for the development and maintenance of fluorescence in this very remarkable family of proteins (Rizzo and Piston, 2005a). The principle fluorophore (often termed a chromophore) in GFP is a tripeptide consisting of the residues serine, tyrosine, and glycine at positions 65 to 67 in the sequence. Although this simple amino acid motif is commonly found throughout nature, it does not generally result in fluorescence. The chromophore forms spontaneously after translation without the requirement for cofactors or external enzyme components (other than molecular oxygen), through a self-catalyzed intramolecular rearrangement of the tripeptide sequence to produce the fluorescent species (as illustrated in Figure 21.5.1 for the enhanced GFP variant). The first step in maturation is thought to be a series of torsional peptide and side-chain bond adjustments that relocate the carboxyl carbon of the serine amino acid at position 65 (Ser65) in close proximity to the amino nitrogen atom of Gly67. Nucleophilic attack by this carbon atom on the amide nitrogen of glycine, followed by dehydration, results in the formation of an imidazolin-5-one heterocyclic ring system (Cody et al., 1993). Fluorescence occurs when oxidation of the tyrosine (Tyr66) α-β carbon bond by molecular oxygen extends electron conjugation of the imidazoline ring system to include the tyrosine phenyl ring and its para-oxygen substituent (Heim et al., 1994). The result is a highly conjugated π-electron resonance system that largely accounts for the spectroscopic properties of the protein. Extensive

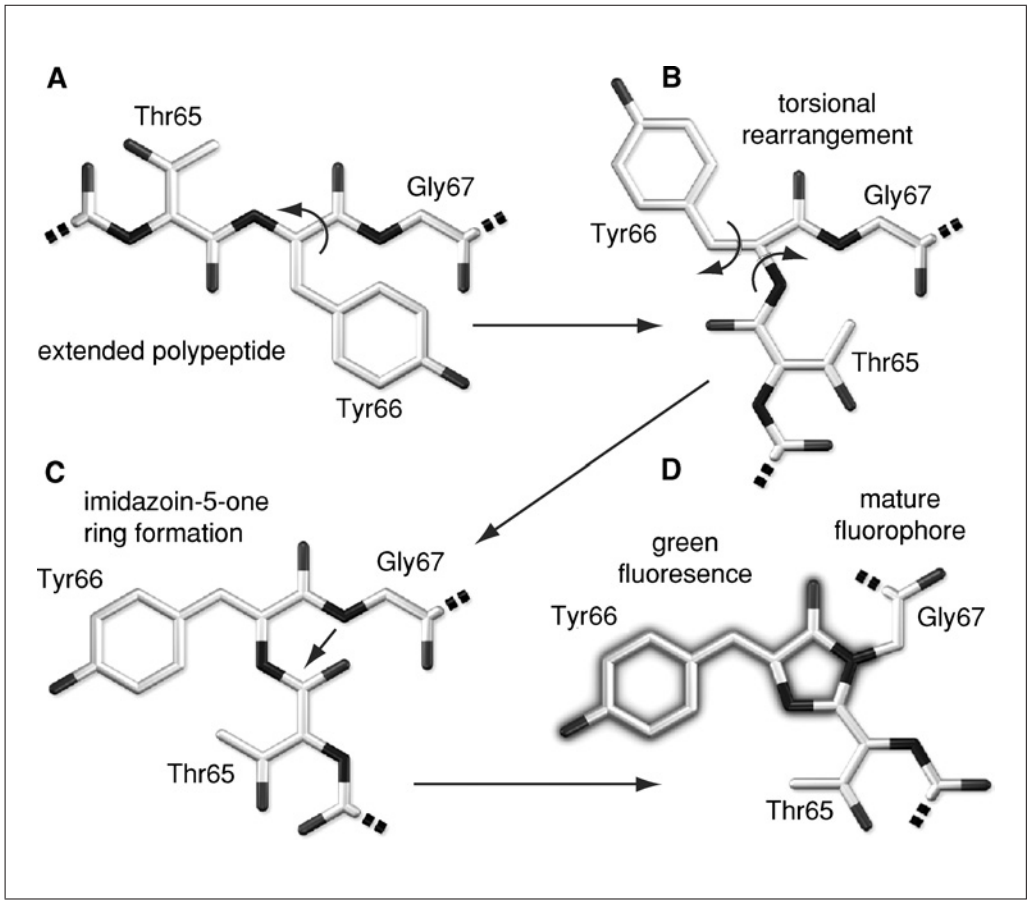


Figure 21.5.1 Schematic diagram of the chromophore formation in maturing enhanced green fluorescent protein (EGFP). (**A**) The prematuration EGFP fluorophore tripeptide amino acid sequence (Thr65-Tyr66-Gly67) stretched into a linear configuration so that the threonine residue is positioned in the upper left-hand corner of the diagram. The first step in maturation is a series of torsional adjustments (**B**) and (**C**) that relocate the carboxyl carbon of Thr65 so that it is in close proximity to the amino nitrogen of Gly67. Nucleophilic attack by this carbon atom on the amide nitrogen of glycine, followed by dehydration, results in formation of an imidazolin-5-one heterocyclic ring system. (**D**) Fluorescence occurs when oxidation of the tyrosine α-β carbon bond by molecular oxygen extends electron conjugation of the imidazoline ring system to include the tyrosine phenyl ring and its para-oxygen substituent.

mutagenesis studies suggest that the glycine residue at position 67 is critical in formation of the chromophore (Cubitt et al., 1995; Delagrave et al., 1995), and indeed, no fluorescent proteins have yet been discovered that lack this essential element.

Several fundamental physical and chemical aspects of the fluorescent protein chromophore have profound implications for the application of these biomolecules as intracellular probes. A striking aspect of the fluorescent protein chromophores is that although their presence to date has only been detected in sea creatures, they are able to form functional units in a wide variety of organisms ranging from bacteria to plants and mammals. The native *Aequorea victoria* GFP is composed of a single-domain protein with 238 amino acids and a molecular weight of ~27 kDa. Even though the photophysics of GFP are rather complex (Chattoraj et al., 1996; Jung et al., 2005), the molecular structure of the protein is quite robust and can accommodate a high degree of modification (executed through the amino acid sequence) without destroying the ability to emit fluorescence. A wide variety of amino acid substitutions to the original configuration have been successful in fine-tuning the fluorescence of native GFP to provide a broad range of derivative fluorophores that emit colors traversing the blue to the yellow regions of the visible spectrum (Tsien, 1998; Shaner et al., 2005). Site-directed and random mutagenesis investigations (Heim and Tsien, 1995; Tsien, 1998; Zacharias and Tsien, 2006) have revealed that fluorescence is very dependent on the three-dimensional structure of amino acid residues surrounding the chromophore. Denaturation of the protein, as might be expected, destroys fluorescence (Alkaabi et al., 2005; Ward, 2006), and mutations in residues immediately adjacent to the chromophore can significantly alter the fluorescent properties. Surprisingly, amino acid substitutions in regions of the polypeptide far removed from the chromophore can also affect the spectral characteristics of the protein (Zacharias and Tsien, 2006).

The remarkably well-conserved cylindrical geometry of all the fluorescent proteins discovered thus far appears to be ideally suited to the primary function of protecting the chromophore. Formed in the shape of a cylinder (Ormö et al., 1996) having dimensions of ~25 × 40 Å, the polypeptide backbone is wound into 11 strands of an extensively hydrogen bonded β sheet that surround a central α helix containing the chromophore with short helical segments protruding from the ends of the cylinder. Dubbed a "β can" because of the geometrical symmetry (Yang et al., 1998), the tight packing of amino acid residues bestows a high level of stability to the protein (Phillips, 2006), which often results in relatively large fluorescent quantum yields for GFP and its derivatives (up to 80%). A lack of clefts and gaps for access of small ligands (such as ions and oxygen), combined with the fact that the chromophore is located near the exact center of the protein, partially explains the extraordinary photostability and high quantum yields that are observed. In addition, both the N- and C- termini are "conveniently" exposed on the surface of the β can to readily participate in protein fusions that do not affect the structural integrity. This consolidated protein organizational motif also enhances resistance to changes in pH, temperature, fixation with paraformaldehyde, and the disruptive action of many common denaturing agents, such as urea and guanidine hydrochloride. Mutations in the amino acid sequence that affect the fluorescence profile generally also produce negative effects on overall stability, usually resulting in a reduction of quantum yield, increased rates of photobleaching, and enhanced environmental sensitivity.

FLUORESCENT PROTEIN BRIGHTNESS AND MATURATION

When designing experiments using fluorescent protein reporters, investigators should ensure that the highest possible signal levels are achieved, especially in cases where the targeted species is rapidly turned over or exhibits low overall population abundance. The apparent brightness of a fluorescent protein, a seemingly qualitative concept, is determined by a number of factors that include the efficiency and rate of maturation, the level of protein expression, the molar extinction coefficient values within the excitation wavelength range, and the quantum yield (Tsien, 1998; Shaner et al., 2005; Ward, 2006). In addition, the fluorescence filter combination, spectral distribution of the illumination source, and the digital camera parameters utilized for imaging of fluorescent proteins are of paramount importance in determining whether adequate contrast and signal-to-noise ratios can be achieved.

The intrinsic "brightness" of a fluorescent protein is determined by the product of the molar extinction coefficient at the peak of the absorption band and the integrated emission quantum yield (Rizzo and Piston, 2005a;

Shaner et al., 2005). As an example, if two proteins have identical quantum yields but one has twice the extinction coefficient at the wavelength of excitation, then the protein with the largest extinction coefficient would have double the brightness of the protein with the smaller extinction coefficient. Likewise, in fluorescent proteins with similar molar extinction coefficients, the one with the highest quantum yield will be the brightest. Quantitative assessment of extinction coefficients and quantum yields is a tedious process that requires a highly purified and correctly folded protein with >95% of the molecules having an active fluorescent chromophore (Ward, 2006). In addition, the total protein concentration must be accurately determined and the measurements of absorption and fluorescence emission performed in a reliable, calibrated spectrophotometer and fluorimeter. Quantum yield assessment requires the comparison of emission spectra between the unknown fluorescent protein and a reference standard having a similar wavelength profile. Investigators should be skeptical of purely qualitative fluorescent protein brightness evaluations (often made by commercial distributors) that lack quantitative information pertaining to the extinction coefficient and quantum yield. It is difficult, if not impossible, to accurately perform brightness comparisons between fluorescent proteins without knowledge of these critical parameters.

Fluorescent protein maturation efficiency is also determined by a number of variables, including the origin of the protein and the abundance of species-specific optimum codons in the organism chosen for expression. In general, fluorescent proteins that have been codon-optimized for expression in mammalian cells will be expressed with high efficiency and mature rapidly at 37°C, although a small percentage of the product may not fold correctly, resulting in poor targeting and high background fluorescence. Many of the protein variants derived from the original *Aequorea* jellyfish green fluorescent protein have been optimized (with regards to the wild-type GFP) through mutagenesis for expression in mammalian systems (Tsien, 1998; Nagai et al., 2002; Zapata-Hommer et al., 2003; Shaner et al., 2005) at high efficiency. Fluorescent proteins from reef corals and sea anemones generally express well at 37°C, presumably because the native species from which the proteins are obtained have evolved in warmer habitats (Salih et al., 2000). The presence of molecular oxygen is also a critical factor in fluorescent protein chromophore development during the maturation process. During the formation of chromophores in *Aequorea* protein variants, at least one oxygen molecule is required for dehydrogenation, while reef coral proteins that emit in the orange-red spectral regions usually require two molecules (Tsien, 1998; Gross et al., 2000; Zimmer, 2002). In mammalian cell cultures, fluorescent protein expression is rarely hampered by a lack of oxygen, but anoxia could become a limiting factor in other systems.

Regardless of the intrinsic brightness displayed by a particular fluorescent protein, the ability to achieve high signal levels is primarily determined by the configuration of the imaging equipment. The laser system or arc-discharge lamp coupled to fluorescence filters used to excite the chromophore should strongly overlap the chromophore absorption profile and the emission filters must have the widest possible bandpass region coinciding with the emission spectrum. In addition, the camera system must be capable of recording images with high quantum efficiency in the fluorescence emission region of interest (Patterson et al., 1997), and the optical system of the microscope should have high throughput in the wavelength regions necessary for producing excitation and gathering emission. Even with research-level instrumentation, it is often quite difficult to achieve the maximum potential fluorescent protein brightness levels in each spectral class unless the fluorescence filter sets are completely optimized for imaging the proteins. Many core imaging facilities have limited inventories of filter sets that are typically designed for traditional synthetic fluorophores rather than fluorescent proteins. For example, the standard DAPI, FITC, TRITC, and Texas Red fluorescence filter combinations, which are often marketed by default with widefield arc-discharge microscopes, are not suitable for many fluorescent proteins and are less than optimal for others.

The hazards of using incorrect filter sets for imaging fluorescent proteins are illustrated in Figure 21.5.2 for several of the more useful fluorescent proteins with standard widefield microscopy filter sets. The cyan fluorescent protein, Cerulean, is depicted in Figure 21.5.2A and 21.5.2B with the absorption and emission spectral profiles superimposed over a DAPI filter set (Fig. 21.5.2A), as well as a set designed specifically for cyan fluorescent proteins (Fig. 21.5.2B). Note the poor overlap of the DAPI filters with the Cerulean absorption and emission bands, which yields low signal when this combination is employed. Narrowing the

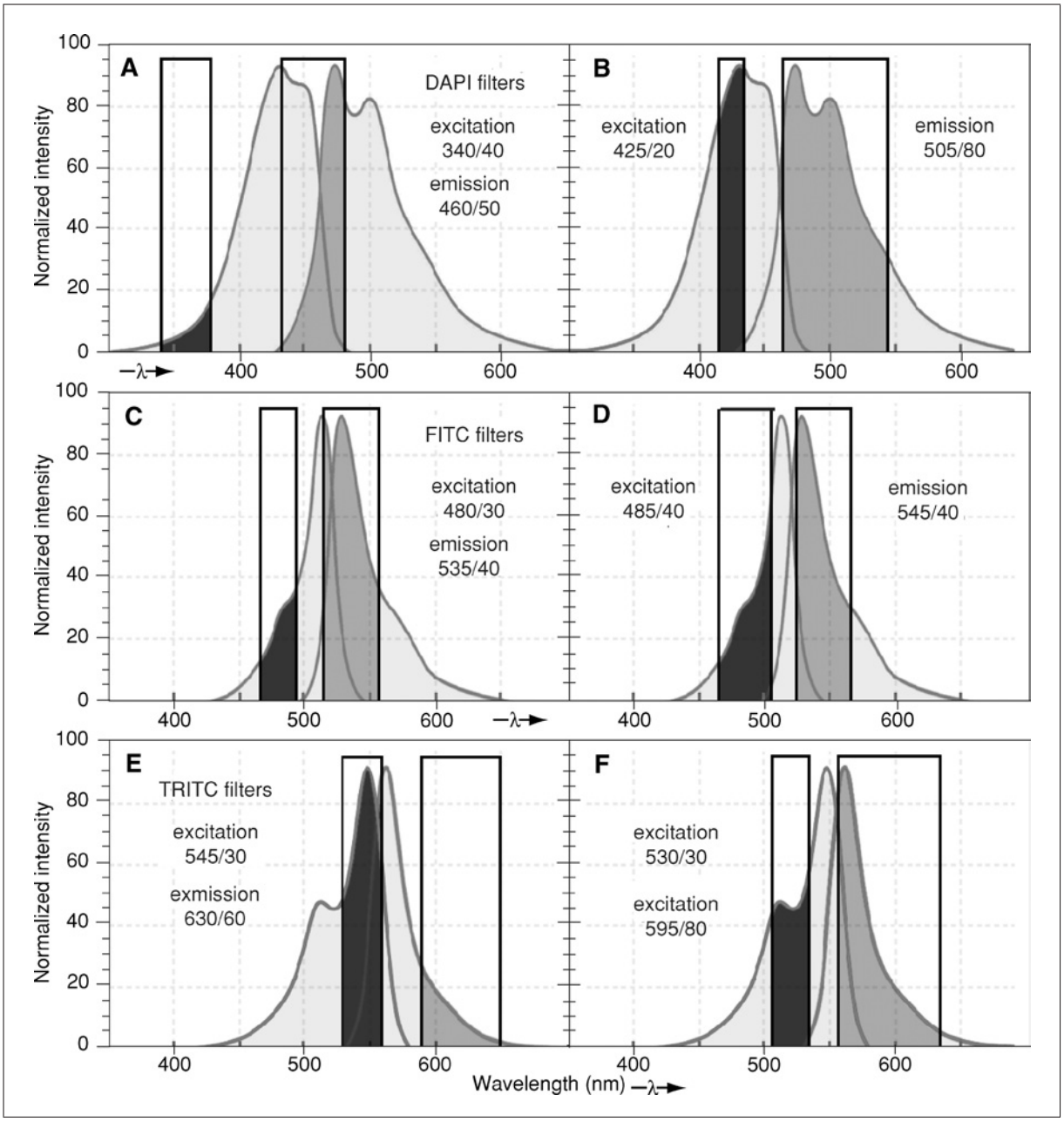


Figure 21.5.2 Spectral profiles of three common fluorescent proteins, Cerulean (**A**) and (**B**), Venus (**C**) and (**D**), and mKusabira Orange (**E**) and (**F**) superimposed over standard ultraviolet, blue, and green excitation filter combinations (**A**), (**C**), (**E**) and custom sets optimized to image the fluorescent proteins (**B**), (**D**), and (**F**). Filters are drawn as rectangles identifying the bandpass region and are described in terms of center wavelength and spectral bandpass.

excitation band to 20 nm and moving the center wavelength 85 nm to longer wavelengths significantly improves the excitation of Cerulean (Fig. 21.5.2B). Likewise, the emission filter effectively captures a majority of the fluorescence emission with a wideband (80-nm) filter having a center wavelength of 505 nm. In general, cyan fluorescent proteins are best imaged with custom filter sets specifically designed for their blue-violet excitation range. The standard FITC filter set is a much closer match for the absorption profile of the yellow fluorescent protein, Venus (Fig. 21.5.2C), but a better combination increases the excitation filter bandwidth to 40 nm and moves the center wavelength to 485 nm (red-shifted by 5 nm). The optimum emission filter bandwidth for Venus is similar to the FITC version with an increase of 10 nm for the center wavelength. Most of the green and yellow fluorescent proteins can be satisfactorily imaged with the FITC filter set, but quantitative imaging benefits from optimizing the central wavelengths and bandwidths for each protein. Orange fluorescent proteins have spectral profiles that fall between the FITC and TRITC filter

wavelength regions for optimal excitation and gathering of emission. Imaging mKusabira Orange with a TRITC filter combination (Fig. 21.5.2E,F) is a good example of the mismatch. The TRITC excitation filter covers an acceptable cross-section of the absorption spectrum, but it is shifted slightly too far towards the emission wavelengths (Fig. 21.5.2E). In addition, the emission filter for this set captures only a small portion of the fluorescent protein emission spectral tail, leading to a loss of signal. A more effective filter set for mKusabira Orange is illustrated in Figure 21.5.2F. Fortunately, a majority of the red fluorescent proteins (DsRed, mCherry, JRed, mPlum, etc.) can be imaged with good results using either the TRITC or Texas Red filter combinations.

PHOTOTOXICITY AND PHOTOSTABILITY

Aside from the toxicity that occurs due to excessive concentrations of synthetic fluorophores and the overexpression or aggregation of poorly localized fluorescent proteins, the health and longevity of optimally labeled mammalian cells in microscope imaging chambers can also suffer from a number of other deleterious factors. Foremost among these is the light-induced damage (phototoxicity) that occurs upon repeated exposure of fluorescently labeled cells to illumination from lasers and high-intensity arc-discharge lamps. In their excited state, fluorescent molecules tend to react with molecular oxygen to produce free radicals that can damage subcellular components and compromise the entire cell. In addition, several reports have suggested that particular constituents of standard culture media, including the vitamin riboflavin and the amino acid tryptophan, may also contribute to adverse light-induced effects on cultured cells (Spierenburg et al., 1984; Silva et al., 1991; Edwards et al., 1994; Lucius et al., 1998). Fluorescent proteins, due to the fact that their fluorophores are buried deep within a protective polypeptide envelope, are generally not phototoxic to cells (Shaner et al., 2005). In contrast, many of the common synthetic fluorophores, such as the MitoTracker and most nuclear stains (including Hoechst, SYTO cyanine dyes, and DRAQ5), can be highly toxic to cells when illuminated for even relatively short periods of time (Durand and Olive, 1982; Minamikawa et al., 1999). In designing experiments, synthetic fluorophores as well as fluorescent proteins that exhibit the longest excitation wavelengths possible should be chosen in order to minimize damage to cells by short wavelength illumination. Thus, rather than creating fusion products with blue or cyan fluorescent proteins (excited by ultraviolet and blue illumination, respectively), variants that emit in the yellow, orange, and red regions of the spectrum should, when possible, be used instead.

Investigators should take care to perform the necessary control experiments when using new fluorescent protein variants, chimeric fusion protein vectors, and cell lines to ensure that cytotoxicity and phototoxicity artifacts do not obscure important biological phenomena. In some cases, lipophilic reagents induce deleterious effects that may be confused with fluorescent protein toxicity during imaging in cell lines following transient transfections. Oligomeric fluorescent proteins (discussed below) from reef corals have a far greater tendency to form aggregates (combined with poor subcellular localization) than do the monomeric jellyfish proteins, but improperly folded fusion products can occur with any variant. Recently, a fluorescent protein capable of generating reactive oxygen species (ROS) upon illumination with green light has been reported (Bulina et al., 2006) as an effective agent for inactivation of specific proteins by chromophore-assisted light inactivation (CALI) and photodynamic therapy. Appropriately named KillerRed, this genetically encoded photosensitizer is capable of killing both bacteria and eukaryotic cells upon illumination in the microscope. Previous studies on GFP phototoxicity indicate that even though the chromophore is capable of generating singlet oxygen (Greenbaum et al., 2000), the probe is inefficient as a photosensitizer (Rajfur et al., 2002; Tour et al., 2003). However, prolonged illumination of cells expressing GFP and its variants can result in physiological alterations and eventual cell death (Dixit and Cyr, 2003), a definite signal of the potential for phototoxicity in long-term imaging experiments.

In live-cell experiments, fluorescent proteins are highly advantageous for extended imaging due to their reduced rate of photobleaching when compared to synthetic fluorophores (Zumbusch and Jung, 2000; Zhang et al., 2002; Rizzo and Piston, 2005a). Although there is a high degree of uncorrelated variability between fluorescent proteins in terms of photostability, most variants are useful for short-term imaging (from 1 to 25 captures), while several of the more photoresistant proteins can be employed in time-lapse sequences that span periods of 24 hr or longer

(in which hundreds to thousands of images are gathered). The long-term stability of any particular protein, however, must be investigated for every illumination scenario (e.g., widefield, confocal, multiphoton, swept-field) because differences in photostability are often observed with the same protein when illumination is produced by an arc-discharge lamp versus a laser system. Thus, in terms of photostability, the selection of fluorescent proteins is dictated by numerous parameters, including the illumination conditions, the expression system, and the effectiveness of the imaging setup.

OLIGOMERIZATION

All of the fluorescent proteins discovered to date display at least a limited degree of quaternary structure, exemplified by the weak tendency of native *Aequorea victoria* green fluorescent protein and its derivatives to dimerize when immobilized at high concentrations (Campbell et al., 2002; Zacharias et al., 2002). This tenant is also verified by the obligate dimerization necessary for solubility in fluorescent proteins isolated from *Renilla* sea pansies (Ward, 2006) and the strict tetramerization motif of the native yellow, orange, and red fluorescent proteins isolated in reef corals and anemones (Baird et al., 2000; Gross et al., 2000; Yarbrough et al., 2001; Verkhusha and Lukyanov, 2004). Oligomerization can be a significant problem for many applications in cell biology, particularly in cases where the fluorescent protein is fused to a host protein that is targeted at a specific subcellular location. Once expressed, the formation of dimers and higher order oligomers induced by the fluorescent protein portion of the chimera can produce atypical localization, disrupt normal function, interfere with signaling cascades, or restrict the fusion product to aggregation within a specific organelle or the cytoplasm. This effect is particularly marked when the fluorescent protein is fused to partners such as actin, tubulin, or histones, which themselves participate in natural oligomer formation. Fusion products with proteins that form only weak dimers (i.e., most *Aequorea victoria* variants) may not exhibit aggregation or improper targeting, provided the localized concentration remains low (see Fig. 21.5.3). However, when dimeric fluorescent proteins are targeted to specific cellular compartments, such as the plasma membrane (Zacharias et al., 2002), the localized protein concentration can, in some circumstances, become high enough to permit dimerization. This is a particular concern when conducting FRET experiments, which can yield complex data sets that are easily compromised by dimerization artifacts.

The basic strategy for overcoming oligomerization artifacts is to modify the fluorescent protein amino acid sequence to include residues that disrupt intermolecular binding, a procedure that varies in complexity depending upon the nature and origin of the protein. For many of the *Aequorea* protein variants, dimerization can be either significantly reduced or eliminated completely (Zhang et al., 2002) by replacing the hydrophobic amino acid side chains in the dimer interface with positively charged residues at several key sequence positions. The three most successful mutations, in decreasing order of effectiveness, are F223R, L221K, and A206K, where the nonpolar amino acids phenylalanine, leucine, and alanine are replaced by their hydrophilic relatives, arginine or lysine. In cases where close molecular associations are suspected involving a fusion protein and where quantitative FRET interactions are being investigated, it is highly recommended that *Aequorea* variants (CFP, GFP, YFP) should be converted into monomers using one of these point mutations (preferably A206K; Shaner et al., 2005; Zacharias and Tsien, 2006).

Creating fluorescent protein monomers from the tetrameric coral reef and sea anemone proteins is usually far more difficult. For example, even at exceedingly low concentrations, the DsRed fluorescent protein is an obligate tetramer (Baird et al., 2000; Vrzheshch et al., 2000; Wiehler et al., 2001) that cannot be dissociated without irreversible denaturation of the polypeptides (Mizuno et al., 2001). In the tetrameric unit, each DsRed protein interacts with two adjacent neighbors, one through a hydrophobic interface and the other through a hydrophilic interface resulting in a complex assembly (Baird et al., 2000; Campbell et al., 2002; Verkhusha and Lukyanov, 2004). Other Anthozoan proteins, such as the *Zoanthus* variants and eqFP611 (Zacharias, 2002; Wiedenmann et al., 2005), have simpler interfaces that may prove easier to disrupt into monomers. The most successful approaches (Campbell et al., 2002; Zacharias and Tsien, 2006) utilized so far to generate fluorescent protein monomers with Anthozoan species have involved repeated site-directed mutagenesis to disrupt the tetrameric interfaces, usually by substitution of hydrophilic or charged amino acids for hydrophobic and neutral moieties. Because a significant decrease in fluorescence emission quantum yield usually accompanies

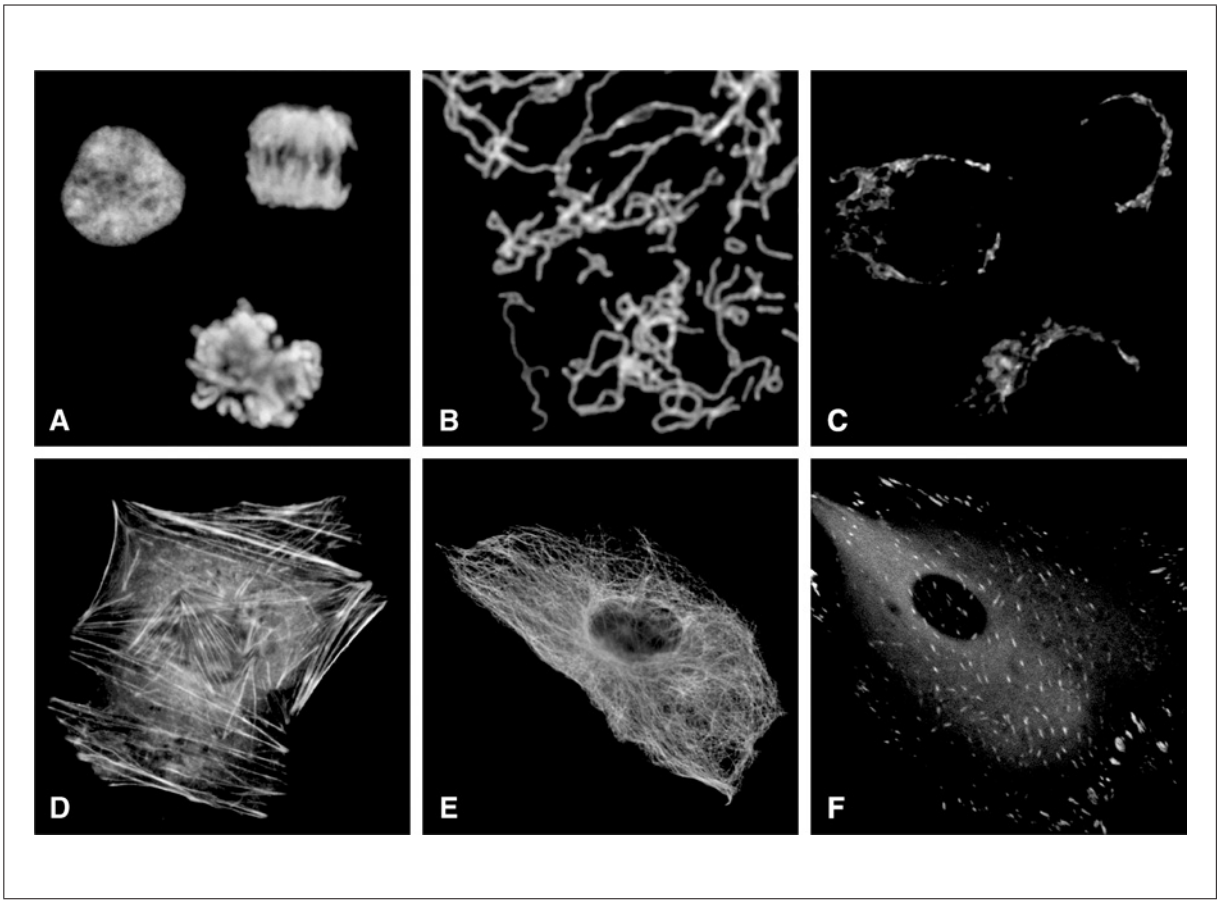


Figure 21.5.3 Localization of fluorescent protein fusion tags to specific subcellular compartments. (**A**) The chimera of mPlum and histone H2B localizes to the nucleus and can be utilized to observe chromosomes during cell division. (**B**) The monomeric red fluorescent protein mCherry highlights mitochondria when fused to the targeting sequence from subunit VIII of human cytochrome c oxidase. (**C**) A fusion between EYFP and the N-terminal 81 amino acids of human β-1,4-galactosyltransferase localizes the yellow fluorescent marker to the Golgi apparatus. (**D**) The actin cytoskeleton becomes fluorescent when Cerulean fluorescent protein is fused to the gene for human β-actin. (**E**) A EGFP-tubulin fusion vector marks the microtubule network in COS-7 cells. (**F**) Focal adhesions become fluorescent with a chimera of mKusabira Orange fluorescent protein and vinculin.

these genetic modifications, a second round of random mutagenesis is often necessary to rescue fluorescence (as discussed below). Another technique involves creating vectors containing two sequential coding regions separated by a short linker of nonspecific amino acids, known as tandem dimers, from proteins that exist naturally as dimeric and tetrameric complexes. Upon expression, the fused fluorescent proteins preferentially bind to each other to form an intramolecular dimeric unit that performs as a monomer, although at twice the molecular weight (and size). This method was successfully applied with HcRed by fusing two copies of the DNA sequence, separated by a short linker of four amino acids, to several subcellular localization proteins (Fradkov et al., 2002). Tandem dimer constructs have also been developed with DsRed (Campbell et al., 2002) and a photoconversion fluorescent protein known as Eos (Ivanchenko et al., 2005). Other mechanisms for reducing fluorescent protein oligomerization and aggregation effects include removing several basic residues from the N terminus (Yanushevich et al., 2002) and simultaneous co-expression of fluorescently tagged proteins with an excess of either fusion partner alone or with a nonfluorescent mutant of the marker protein (Lauf et al., 2001; Bulina et al., 2003). Regardless of the specific mechanism employed to overcome fluorescent protein oligomerization, the most important point is that experimental results are not compromised by artifacts induced by the existence of quaternary structures.

THE FLUORESCENT PROTEIN COLOR PALETTE

A broad range of fluorescent protein genetic variants have been developed (Verkhusha and Lukyanov, 2004; Chudakov et al., 2005; Shaner et al., 2005) over the past several years; these feature fluorescence emission spectral profiles spanning almost the entire visible light spectrum (see Table 21.5.1). Mutagenesis efforts in the original jellyfish green

Table 21.5.1 Properties of Popular and Useful Fluorescent Proteins[a]

Protein (Acronym)	Ex (nm)[a]	Em (nm)[a]	EC × 10^{-3}	QY	In vivo structure	Relative brightness (% of EGFP)[b]	References
Blue Fluorescent Proteins							
EBFP	383	445	29.0	0.31	Monomer[c]	27	Patterson et al., 1997
Sapphire	399	511	29.0	0.64	Monomer[c]	55	Zapata-Hommer et al., 2003
T-Sapphire	399	511	44.0	0.60	Monomer[c]	79	Zapata-Hommer et al., 2003
Cyan Fluorescent Proteins							
ECFP	439	476	32.5	0.40	Monomer[c]	39	Cubitt et al., 1995
mCFP	433	475	32.5	0.40	Monomer	39	Zacharias et al., 2002
Cerulean	433	475	43.0	0.62	Monomer[c]	79	Rizzo et al., 2004
CyPet	435	477	35.0	0.51	Monomer[c]	53	Nguyen and Daugherty, 2005
AmCyan1	458	489	44.0	0.24	Tetramer	31	Matz et al., 1999
Midoriishi Cyan	472	495	27.3	0.90	Dimer	73	Karasawa et al., 2004
mTFP1	462	492	64.0	0.85	Monomer	162	Ai et al., 2006
Green Fluorescent Proteins							
EGFP	484	507	56.0	0.60	Monomer[c]	100	Heim et al., 1995
aceGFP	480	505	50.0	0.55	Monomer[c]	82	Gurskaya et al., 2003
TurboGFP	482	502	70.0	0.53	Monomer[c]	110	Shagin et al., 2004
Emerald	487	509	57.5	0.68	Monomer[c]	116	Cubitt et al., 1999
Azami Green	492	505	55.0	0.74	Monomer	121	Karasawa et al., 2003
ZsGreen	493	505	43.0	0.91	Tetramer	117	Matz et al., 1999
Yellow Fluorescent Proteins							
EYFP	514	527	83.4	0.61	Monomer[c]	151	Miyawaki et al., 1999
Topaz	514	527	94.5	0.60	Monomer[c]	169	Tsien, 1998
Venus	515	528	92.2	0.57	Monomer[c]	156	Nagai et al., 2002
mCitrine	516	529	77.0	0.76	Monomer	174	Griesbeck et al., 2001

continued

Table 21.5.1 Properties of Popular and Useful Fluorescent Proteins[a], *continued*

Protein (Acronym)	Ex (nm)[a]	Em (nm)[a]	EC × 10^{-3}	QY	In vivo structure	Relative brightness (% of EGFP)[b]	References
Yellow Fluorescent Proteins, continued							
YPet	517	530	104	0.77	Monomer[c]	238	Nguyen and Daugherty, 2005
PhiYFP	525	537	124	0.39	Monomer[c]	144	Shagin et al., 2004
ZsYellow1	529	539	20.2	0.42	Tetramer	25	Matz et al., 1999
mBanana	540	553	6.00	0.70	Monomer	13	Shaner et al., 2004
Orange and Red Fluorescent Proteins							
Kusabira Orange	548	559	51.6	0.60	Monomer	92	Karasawa et al., 2004
mOrange	548	562	71.0	0.69	Monomer	146	Shaner et al., 2004
dTomato	554	581	69.0	0.69	Dimer	142	Shaner et al., 2004
dTomato-Tandem	554	581	138	0.69	Monomer	283	Shaner et al., 2004
DsRed	558	583	75.0	0.79	Tetramer	176	Matz et al., 1999
DsRed2	563	582	43.8	0.55	Tetramer	72	Bevis and Glick, 2002
DsRed-Express (T1)	555	584	38.0	0.51	Tetramer	58	Bevis and Glick, 2002
DsRed-Monomer	556	586	35.0	0.10	Monomer	10	Clontech, 2005
tangerine	568	585	38.0	0.30	Monomer	34	Shaner et al., 2004
mStrawberry	574	596	90.0	0.29	Monomer	78	Shaner et al., 2004
AsRed2	576	592	56.2	0.05	Tetramer	8	Matz et al., 1999
mRFP1	584	607	50.0	0.25	Monomer	37	Campbell et al., 2002
JRed	584	610	44.0	0.20	Dimer	26	Shagin et al., 2004
mCherry	587	610	72.0	0.22	Monomer	47	Shaner et al., 2004
HcRed1	588	618	20.0	0.015	Dimer	1	Gurskaya et al., 2001a
mRaspberry	598	625	86.0	0.15	Monomer	38	Wang et al., 2004
HcRed-Tandem	590	637	160	0.04	Monomer	19	Fradkov et al., 2002
mPlum	590	649	41.0	0.10	Monomer	12	Wang et al., 2004
AQ143	595	655	90.0	0.04	Tetramer	11	Shkrob et al., 2005

[a]The excitation and emission peak values listed may vary in published reports due to the broad spectral profiles. In actual fluorescence microscopy investigations, spectral profiles and wavelength maxima are influenced by environmental effects, such as pH, ionic concentration, and solvent polarity, as well as fluctuations in localized probe concentration. Therefore, the listed extinction coefficients and quantum yields may differ from those actually observed under experimental conditions.

[b]The computed brightness values were derived from the product of the molar extinction coefficient and quantum yield, divided by the value for EGFP. This listing was created from scientific and commercial literature resources and is not intended to be comprehensive, but instead represents fluorescent protein derivatives that have received considerable attention in the literature and may prove valuable in research efforts.

[c]The protein actually exists in solution as a weak dimer, which can be converted to a true monomer with the A206K modification.

fluorescent protein have resulted in new fluorescent probes that range in color from blue to yellow (Tsien, 1998) and are some of the most widely used in vivo reporter molecules in biological research. Longer wavelength fluorescent proteins, emitting in the orange and red spectral regions, have been developed from the marine anemone *Discosoma striata* and reef corals belonging to the class Anthozoa (Matz et al., 1999). Still other species have been mined to produce similar proteins having cyan, green, yellow, orange, red, and far-red fluorescence emission (Chudakov et al., 2005; Shaner et al., 2005). Developmental research efforts are ongoing to improve the brightness and stability of fluorescent proteins, thus improving their overall usefulness.

Essential to the understanding of spectral diversity in the wide range of fluorescent proteins discovered thus far are structural investigations of the stereochemical nature of the fluorophore and the effects of its surrounding environment on fluorescent properties. Aside from the jellyfish proteins, there appears to be a high degree of variation in the fluorophores of red-shifted fluorescent proteins (Shkrob et al., 2005). Even though the DsRed fluorophore configuration, termed planar *cis*, appears to be the predominant structure in most proteins that emit in the orange and red regions, there are at least two additional motifs, planar *trans* and nonplanar *trans*, which have been elucidated through x-ray diffraction studies. A planar *trans* motif is found in the red fluorescent protein eqFP611, isolated from the sea anemone *Entacmaea quadricolor* (Peterson et al., 2003), which displays one of the largest Stokes shifts and red-shifted emission wavelength profiles of any naturally occurring Anthozoan fluorescent protein. In contrast, the nonplanar *trans* conformation is characteristic of the nonfluorescent chromoprotein Rtms5 from *Montipora efflorescens* (Beddoe et al., 2003). Several guiding motifs have emerged in recent years regarding the origins and manipulation of fluorescent protein emission color (Remington, 2006). The most important consideration appears to be the physical extent of π-orbital conjugation contained within the chromophore, which largely determines the general spectral class (i.e., cyan, green, yellow, or red). In addition, local environmental variables, such as the position of charged amino acid residues, hydrogen bonding network, and hydrophobic interactions within the protein matrix, are capable of producing either blue or red spectral shifts in the absorption and emission maxima by as much as 20 nm (Tsien, 1998; Remington, 2006; Shu et al., 2006). As further studies into the complex characteristics of fluorescent protein chromophores yield clues about the structure-function relationship with the polypeptide backbone, the task of genetically engineering more finely-tuned color variants and broadening the spectral range of useful proteins will undoubtedly become easier.

Blue Fluorescent Proteins

Fluorescent proteins emitting in the blue region of the visible light spectrum (~440 to 470 nm) were first obtained from site-directed mutagenesis efforts targeted at the tyrosine amino acid residue at position 66 in the GFP chromophore. Conversion of this residue to histidine (Y66H) produces a blue fluorescent protein (BFP) that exhibits a broad absorption band in the ultraviolet centered close to 380 nm and an emission maximum at 448 nm (Heim et al., 1994; Cubitt et al., 1999). The original protein exhibited only ~15% to 20% of the parent GFP brightness value due to a low quantum yield and required additional secondary mutations to increase its folding efficiency and expression levels. Subsequent investigations and several additional mutations led to an enhanced BFP version that is still only 25% as bright as the enhanced green variant (Patterson et al., 1997; Yang et al., 1998; Patterson et al., 2001;) and displays limited photostability compared to many other fluorescent proteins.

The primary motivation for developing blue fluorescent proteins in the mid-to-late 1990s was the keen interest in creating matched pairs for fluorescence resonance energy transfer (FRET) experiments and multicolor labeling (Tsien, 1998). Because the spectral characteristics (fluorescence emission profile) of BFP are readily distinguishable from EGFP, this protein combination was one of the first utilized for multicolor imaging (Heim and Tsien, 1996; Rizzuto et al., 1996). Blue fluorescent protein also has the distinction of being incorporated into the first genetically encoded biosensor along with an enhanced GFP variant to demonstrate FRET through linkage of the two fluorescent proteins via an intervening protease-sensitive spacer (Heim and Tsien, 1996; Mitra et al., 1996). The broad emission peak of BFP overlaps to a significant extent with the excitation spectrum of red-shifted GFP variants to yield a Förster distance of 4.1 (Patterson et al., 2000), a reasonable value for measuring FRET. Blue fluorescent protein has also been coupled with several GFP

derivatives into biosensors designed to monitor transcription factor dimerization (Periasamy and Day, 1998), calcium (Miyawaki et al., 1997; Romoser et al., 1997), and apoptosis (Rehm et al., 2002).

Aside from the limited brightness levels and rapid photobleaching, blue fluorescent proteins also suffer from the fact that they must be excited with ultraviolet light, which is phototoxic to mammalian cells, even in limited doses (Potter, 1996; Stephens and Allan, 2003; Khodjakov and Rieder, 2006). Furthermore, working in this spectral region is often hampered by autofluorescence and high absorption levels by cells and tissues, as well as light scattering. Microscopes operating in the ultraviolet also require specialized light sources, optics, and filter combinations that further complicate imaging (Rizzo and Piston, 2005a). For all of the reasons listed above, the quest for more efficient blue fluorescent proteins has only been pursued by a few research groups. Investigations of mutagenesis using nonnatural amino acids positioned in and around the chromophore have led to several blue-shifted "artificial" fluorescent protein variants that may find utility in several biological and photophysical applications (Kajihara et al., 2005).

Cyan Fluorescent Proteins

The first fluorescent protein emitting in the bluish-green cyan spectral region (CFP; ranging from ~470 to 500 nm) was discovered simultaneously with BFP during mutagenesis studies (Heim et al., 1994) that converted the tyrosine residue in the GFP chromophore to tryptophan (Y66W). This single mutation yielded a chromophore that displays an absorption maximum at 436 nm with a very broad fluorescence emission spectral profile centered at 485 nm. Subsequent refinements, including the F64L maturation improvement and S65T (discussed in Green Fluorescent Proteins), resulted in the production of an enhanced version (ECFP) with greater brightness and photostability (Cubitt et al., 1995; Patterson et al., 2001). Even these modifications failed, however, to increase the brightness level of ECFP beyond 40% of that shown by EGFP. Other than providing an additional hue for multicolor imaging, initially the most promising aspect of ECFP was the potential for utility as a biosensor FRET partner with yellow fluorescent proteins in widefield and confocal microscopy using blue-violet filter sets or the 457-nm spectral line of an argon-ion laser (Miyawaki et al., 1997; Tsien, 1998). Greater photostability compared to EBFP also positions ECFP as a more useful protein for time-lapse imaging in localization and dynamics studies.

By far the most successful applications using ECFP have taken advantage of the ability of this variant to be coupled with yellow fluorescent protein (YFP) and derivatives to generate FRET biosensors capable of monitoring a wide spectrum of intracellular processes (reviewed in Zhang et al., 2002; Miyawaki, 2003; Meyer and Teruel, 2003; Zaccolo, 2004). Although a host of FRET investigations have been performed using the ECFP-EYFP pair, the experimental results are often problematic due to limitations in the fluorophore properties that restrict measurements to a small dynamic range (Rizzo and Piston, 2005a). A majority of these biosensors exhibit a typical overall ratio change of 10% to 30% during FRET analysis, with a few exceptions (Ting et al., 2001; Zhang et al., 2001; Rizzo et al., 2002). Such a low level of contrast presents difficulties with modern digital microscopy due to a noise level that can approach 10% of the signal at low imaging intensities (Swedlow et al., 2002). These biosensors are further complicated by the potential to form dimers when constrained with tight two-dimensional spatial restrictions such as those that exist in membranes (Ward, 2006). To overcome the artifacts associated with dimerization, hydrophobic residues at the dimer interface were replaced with positively charged amino acids in both ECFP and EYFP to produce true monomeric variants (mCFP and mYFP), which achieve a far higher level of efficiency in FRET experiments (Zacharias et al., 2002). The critical mutation is the substitution of lysine for alanine at position 206 (A206K, as discussed above), which can be applied to virtually any *Aequorea victoria* variant in order to generate a true monomer capable of superior performance in applications such as localization, FRET, and protein dynamics.

Despite the host of improvements in CFP and the large database of experiments performed with this variant, continued investigation has produced additional useful fluorescent proteins in the cyan spectral class. Among the improved cyan fluorescent proteins that have recently been introduced, CyPet and an enhanced cyan variant termed Cerulean show the most promise (Rizzo et al., 2004; Nguyen and Daugherty, 2005) as fusion tags, FRET biosensors, and for multicolor imaging (see Figs. 21.5.3 and 21.5.4). The Cerulean fluorescent probe (named for the sky-blue color) was rationally engineered by site-directed mutagenesis

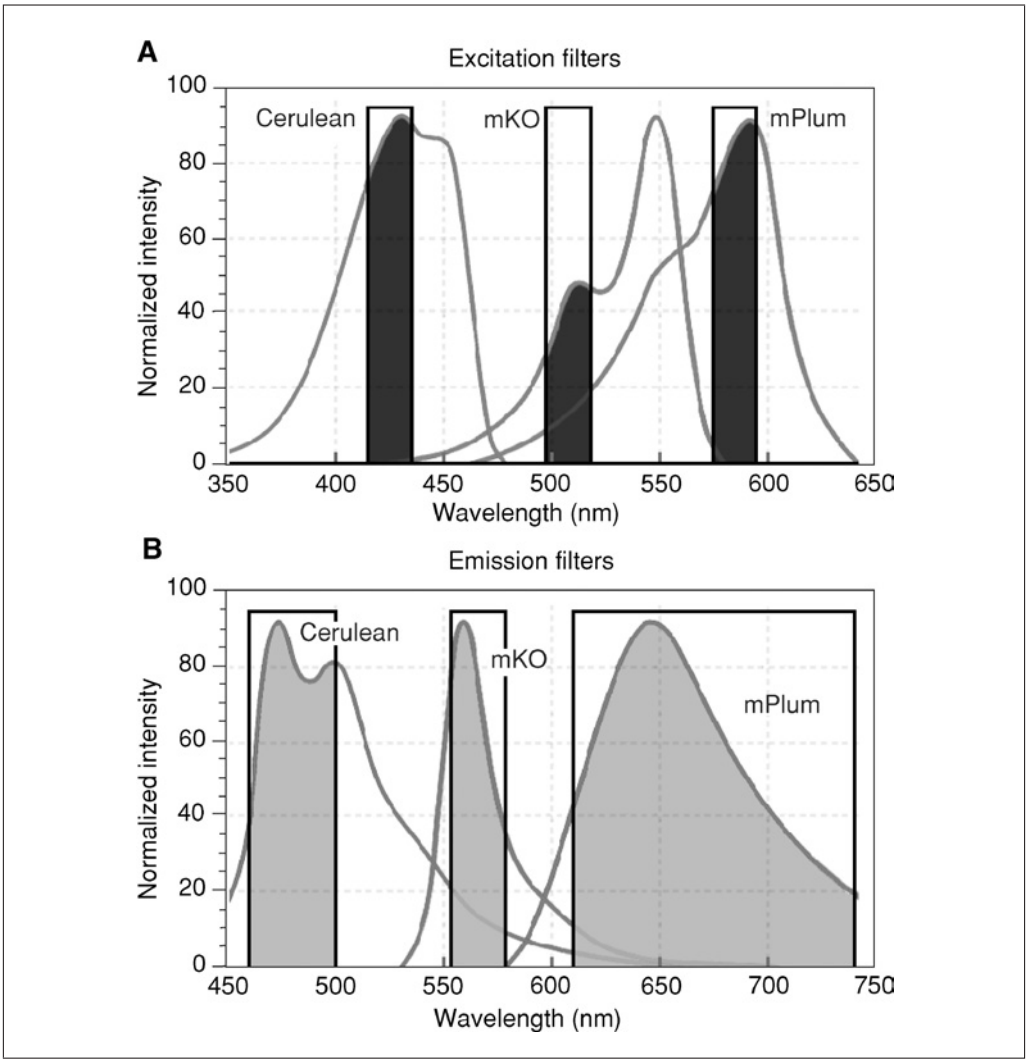


Figure 21.5.4 Optimized fluorescence filter combinations for multicolor imaging of three fluorescent proteins spanning the cyan through far-red wavelength regions. (**A**) Excitation filters optimized for Cerulean, mKO, and mPlum fluorescent proteins having center wavelengths of 425, 508, and 585 nm, respectively. The bandwidth of all excitation filters is 20 nm. (**B**) Emission filters optimized for the same probes having center wavelengths of 480, 564, and 675 nm with bandwidths of 40, 28, and 130 nm, respectively. The mKO excitation filter is designed to work off-peak to minimize simultaneous excitation of mPlum. Bleed-through of Cerulean fluorescence emission into the mKO filter is reduced by the fact that mKO is four times brighter at equimolar concentrations and requires much shorter exposure intervals for image capture.

of enhanced cyan fluorescent protein to yield a higher extinction coefficient, improved quantum yield, and a fluorescence lifetime decay having a single exponential component (useful in lifetime decay measurements of FRET; Wallrabe and Periasamy, 2005). Cerulean is at least 2-fold brighter than enhanced cyan fluorescent protein and has been demonstrated to significantly increase contrast, as well as the signal-to-noise ratio when coupled with yellow-emitting fluorescent proteins, such as Venus (see below), in FRET investigations. In addition to site-directed mutations designed to improve folding and brightness (Rizzo et al., 2004), random mutagenesis was conducted on preCerulean variants to further increase the molar extinction coefficient, and the optimized protein has been "monomerized" to enhance its utility in fusions (Rizzo and Piston, 2005b). The abundance of advantageous features afforded by Cerulean render this protein the most useful all-purpose cyan derivative.

The cyan fluorescent protein variant named CyPet (from the acronym: Cyan fluorescent Protein for energy transfer) was derived through a quantitative and unique strategy utilizing fluorescence-activated cell sorting (FACS; Boeck, 2001) to optimize the cyan and yellow pairing for FRET (Nguyen and Daugherty, 2005). Libraries were screened for

FRET efficiency and the best clones were subjected to several evolutionary cycles consisting of random mutagenesis and synthetic DNA shuffling. A total of seven mutations resulted in the production of the CyPet protein, which features absorption and emission maxima positioned at 435 and 477 nm, respectively. CyPet is about half as bright as EGFP and two-thirds as bright as Cerulean, but expresses relatively poorly at 37°C (Shaner et al., 2005). However, when paired with its FACS-optimized partner, YPet, in FRET biosensors, this cyan variant exhibits a dynamic range that is more than six times higher than CFP-YFP (Nguyen and Daugherty, 2005), dramatically improving contrast and potentially leading to far more sensitive detection of subtle intracellular processes. CyPet has a more blue-shifted and narrower fluorescence emission peak than mCFP, which greatly increases its utility in multicolor imaging, and is the most photostable cyan fluorescent protein of the weakly dimeric and monomeric versions currently available.

Several potentially useful cyan proteins have been isolated in Anthozoan species. Derived from the reef coral *Anemonia majano*, the AmCyan1 fluorescent protein (Matz et al., 1999), which is now commercially available (Clontech), has been optimized with human codons for enhanced expression in mammalian cell systems (Richards et al., 2002). Originally named amFP486 (am, *Anemonia majano*; FP, fluorescent protein; 486 emission maximum) in accordance with a nomenclature scheme (Matz et al., 1999) devised to simplify the discussion of myriad Anthozoan proteins, this variant exhibits a similar brightness level, but a significantly better resistance to photobleaching than CFP. The absorption maximum of AmCyan1 occurs at 458 nm while the fluorescence emission peak resides at 489 nm. Note that both peaks are shifted to longer wavelengths by 19 and 13 nm, respectively, compared to ECFP. On the downside, similar to most of the other reef coral proteins, the probe has a tendency to form tetramers, which will significantly complicate attempts to employ this protein as a fusion tag or a FRET biosensor.

First isolated by Miyawaki and associates from an *Acropara* stony coral species (Karasawa et al., 2004), the cyan-emitting Midoriishi-Cyan (abbreviated MiCy) probe was originally designed as the donor in a new FRET combination with the monomeric Kusabira Orange fluorescent protein to generate a biosensor probe with high spectral overlap (Förster distance of 5.3; mKO is discussed in the section on Orange Fluorescent Proteins). This protein features the longest absorption and emission wavelength profiles (472 and 495 nm, respectively) reported for any probe in the cyan spectral class. The high molar extinction coefficient and quantum yield exhibited by MiCy render the protein of equal brightness to Cerulean, although the spectra are far more sensitive to pH. Also similar to Cerulean, MiCy features a single exponential lifetime decay component with a time constant of 3.4 ns, which should be useful for measurements of FRET in combination with fluorescence lifetime imaging microscopy (FLIM). An unusual feature of MiCy is that it forms a homodimeric complex similar to the GFP variant isolated from the bioluminescent sea pansy, *Renilla reniformis* (Ward and Cormier, 1979), rather than the obligate tetramer observed in most coral reef species. Although the dimerization motif may be a problem in some fusion proteins, it should be far easier than a tetramer to mutate MiCy into a true monomer.

Recently, a new monomeric cyan fluorescent protein having excellent brightness, pH insensitivity, and photostability has been introduced for live-cell imaging of fusion partners, and as a FRET donor for yellow and orange acceptor fluorescent proteins in biosensors (Ai et al., 2006). Termed mTFP1 (monomeric teal fluorescent protein 1), the variant was produced from a synthetic gene library built around the tetrameric cyan protein, cFP484, originating from a *Clavularia* soft coral. Displaying red-shifted spectral profiles (excitation and emission maxima at 462 and 492 nm, respectively) when compared to other cyan members of this spectral class, mTFP1 has a total of 31 amino acid substitutions relative to the wild-type tetramer. The red-shifted spectra were considered when classifying the new color as teal rather than cyan. Unlike other members of the cyan fluorescent protein group, which generally feature the aromatic amino acid tryptophan at position 66 in the chromophore, mTFP1 contains the classical tyrosine residue at this location, characteristic of many GFP derivatives. Substituting tyrosine for tryptophan reduces the broad fluorescence emission spectral width from ~60 nm to a narrower and more manageable 30 nm, a factor that is useful for reducing bleed-through in multi-color experiments. The high quantum yield (0.85) of mTFP1 provides an excellent alternative to the cyan derivatives, mCFP and mCerulean, as a FRET donor having a Förster distance exceeding 5.0 when

combined with either yellow or orange fluorescent proteins.

Green Fluorescent Proteins

The original green fluorescent protein isolated from *Aequorea victoria* (termed wild-type) has been the principal subject of numerous investigations (Tsien, 1998), but it is not useful in a majority of the practical applications involving fluorescent proteins due to the bimodal absorption band (395 and 475 nm peaks), which is hampered by relatively low extinction coefficients and an absorption maximum in the ultraviolet part of the spectrum. Shortly after GFP was demonstrated to be a useful marker for gene expression (Chalfie et al., 1994), a point mutation altering the serine residue at position 65 in the chromophore to threonine (S65T) produced a new version of the protein having a well-defined absorption profile with a single peak at 484 nm (Heim et al., 1995). This mutation is featured in the most popular variant of green fluorescent protein, termed enhanced green fluorescent protein (EGFP), which can be imaged using commonly available filter sets designed for fluorescein (FITC); it is among the brightest and most photostable of the *Aequorea victoria* fluorescent proteins (Shaner et al., 2005). These features have rendered enhanced green fluorescent protein one of the most popular probes and the best choice for most single-label fluorescent protein experiments. As previously discussed, EGFP was coupled with blue fluorescent proteins as a FRET acceptor in early biosensor investigations, but it has subsequently been largely replaced by yellow, orange, and red variants. The only drawbacks to the use of EGFP as a fusion tag are a slight sensitivity to pH and a weak tendency to dimerize.

In addition to EGFP, several other green-emitting variants (in the range of ~500 to 525 nm) are currently being utilized in live-cell imaging (Rizzo and Piston, 2005a). Among the best of these in terms of photostability and brightness may be the Emerald variant (Cubitt et al., 1999), but lack of a commercial source (until recently) has limited its use. Emerald contains the S65T mutation featured in EGFP, but also has four additional point mutations that improve folding, expression at 37°C, and brightness. Although Emerald is far more efficient than EGFP in folding and developing fluorescence in mammalian cells, it has a fast photobleaching component that might affect quantitative imaging in some environments. One of the most interesting derivatives of wtGFP is Sapphire (Zapata-Hommer and Griesbeck, 2003), which contains a critical mutation of isoleucine for threonine at position 203 (T203I), abolishing the absorption peak at 475 nm. The result is a protein having an absorption maximum at 399 nm with emission in the green spectral region (511 nm) to yield a surprisingly large Stokes shift of over 100 nm. Several Sapphire variants have been developed to improve folding, including a probe known as T-Sapphire (for Turbo), and derivatives featuring circular permutations are available that might be useful for altering the orientation geometry with fusion partners. Due to the significant separation between absorption and emission peaks, the greatest potential for the Sapphire proteins is their pairing with orange and red derivatives as FRET donors.

A wide variety of additional fluorescent proteins emitting in the green spectral region have been isolated from other sources, including different *Aequorea* species, copepods, and coral reefs. One of the most promising of these probes, derived by random mutagenesis of a colorless protein isolated from *Aequorea coerulescens*, is known as aceGFP (Gurskaya et al., 2003). The conversion of glutamic acid into glycine at position 222 (E222G) transformed the wild-type protein into a highly fluorescent species with a relatively symmetrical pair of spectral profiles having an absorption maximum at 480 nm and an emission peak at 505 nm. The high molar extinction coefficient and quantum yield of aceGFP combine to produce a brightness level similar to that displayed by EGFP. Demonstrated to exist as a monomer by electrophoresis and gel filtration, this protein is commercially available from several sources (Clontech and Evrogen, as AcGFP1 and AceGFP) with human-optimized codon replacements. Proper localization of fusion products targeted at specific subcellular components and organelles (such as filamentous actin, the Golgi, nucleus, mitochondria, etc.; see Fig. 21.5.3) indicates that aceGFP is quite useful as a marker and could have potential for partnership with red-emitting proteins in a novel FRET combination. However, the photostability characteristics of aceGFP remain unknown, and there are no clear advantages to the use of this protein over the more common EGFP and Emerald variants.

Several closely related GFP-like proteins have been isolated from an assortment of copepod aquatic crustacean species. The brightest of these probes, originally referred to as ppluGFP2 (Shagin et al., 2004), have been

made commercially available (Evrogen) under the names CopGFP and TurboGFP (an enhanced variant). CopGFP is efficiently excited using an argon-ion laser or FITC filter set (absorption maximum at 482 nm) and produces green fluorescence at 502 nm with a brightness value ~30% higher than EGFP and a much greater resistance to changes in pH. Reported to be a monomer in dilute solution, CopGFP matures significantly faster than EGFP and is ideal for applications as a fusion partner targeted at expression in subcellular regions of low pH. Limitations of this probe include the inability to isolate stable cell lines and the formation of aggregates in long-term cultures. An improved version, TurboGFP, derived from site-directed and random mutagenesis, retains the fast maturation kinetics of the parent protein with a slight loss in brightness and substantially lower resistance to acidic environments. Despite the improved folding kinetics and excellent optical properties of these proteins, however, photostability data have not been reported and no compelling evidence exists to demonstrate a significant benefit over the application of the extensively studied original GFP derivatives.

Green fluorescent proteins have also been mined from reef corals and several of these are commercially available. A brightly fluorescent protein termed Azami Green (Karasawa et al., 2003), bearing only a surprisingly scant (<6%) sequence homology to EGFP, was isolated from the stony coral *Galaxeidae* and has been demonstrated to mature rapidly during expression in mammalian cell lines. Likewise, one of the original Anthozoan coral reef proteins from *Zoanthus* reported by Matz and coworkers (1999) has also been transformed into a commercial product (Clontech) under the trade name ZsGreen. The probes have absorption maxima at 492 and 496 nm and emission peaks at 505 and 506 nm, respectively, readily allowing visualization and imaging with standard lasers and filter combinations in confocal and widefield microscopy. However, similar to most of the other proteins isolated in corals, Azami Green and ZsGreen both exist as tetramers in the natural state, which significantly interferes with their use as fusion partners and as a FRET donor or acceptor in biosensors. To overcome the oligomerization problem, site-directed and random mutagenesis efforts were successful in creating a monomeric version of Azami Green, but this type of effort has not been reported for ZsGreen although the protein has been re-engineered with human codons to optimize expression (resulting in a variant termed ZsGreen1). Because reliable photostability data is lacking, it is unclear whether either of these proteins will outperform EGFP in long-term imaging experiments.

The sea pansy, an Anthozoan soft coral, is the source of several green fluorescent proteins that have been characterized in detail and are now commercially available (Cormier and Eckroade, 1962; Morin and Hastings, 1971b Miyawaki, 2002). A protein isolated from *Renilla reniformis* that exhibits properties similar to EGFP is the best characterized of the probes in this class. Having absorption and emission maxima at 485 and 508 nm, respectively, in addition to a similar sensitivity to pH, the *Renilla* protein would be an excellent substitute for EGFP were it not for the fact that it is an obligate dimer (Ward, 2006). Aside from the oligomerization problem, *Renilla* GFPs may be useful in many applications and have been expressed in a wide variety of organisms, including bacteria, fungi, and mammalian cells. Versions with human codon sequences are available from the manufacturers, as are derivatives optimized for expression in other species. There is a general lack of reliable data concerning extinction coefficients, quantum yields, and photostability for the commercial *Renilla* proteins, so valid comparisons to EGFP in terms of brightness and photobleaching are not possible.

Yellow Fluorescent Proteins

Yellow fluorescent proteins, as a spectral class, are among the most versatile genetically-encoded probes yet developed. Ranging in emission wavelength maxima from ~525 to 555 nm, those proteins residing in the shorter wavelength region actually appear green, rather than yellow, when viewed in a widefield fluorescence microscope. The first member in what has become a rather large family of probes was rationally engineered after the high-resolution crystal structure of green fluorescent protein revealed that threonine residue 203 (Thr203) was positioned near the chromophore and potentially able to alter the spectral characteristics upon substitution (Ormö et al., 1996; Rizzo and Piston, 2005a). Mutations of this aliphatic amino acid to several aromatic moieties were introduced in order to induce π-orbital stacking and attempt stabilization of the excited state dipole moment of the chromophore. The most successful mutant

proved to be tyrosine (T203Y; termed mutant 10C, the original YFP), which resulted in almost a 20-nm shift to longer wavelengths for both the excitation and emission spectra (Ormö et al., 1996; Tsien, 1998; Wachter et al., 1998). Several YFP variants were initially constructed in attempts to maximize brightness as well as to increase the speed of maturation and optimize expression at 37°C (Tsien, 1998; Cubitt et al., 1999). One of these variants, named Topaz, has been of service in fusion tag localization, intracellular signaling, and FRET investigations (Tavaré et al., 2001; Cornea and Conn, 2002; Sturman et al., 2006).

In an effort to improve the performance of FRET biosensors, further sequence refinements led to the development of the enhanced yellow fluorescent protein (EYFP), which is one of the brightest and most widely utilized fluorescent proteins (Miyawaki et al., 1999; Miyawaki, 2005; Shaner et al., 2005). EYFP was constructed from the original yellow variant by introduction of lysine to position 69 in place of glutamine (Q69K). The high brightness level and fluorescence emission spectrum wavelength profile of EYFP combine to make this probe an excellent candidate for multicolor imaging experiments in fluorescence microscopy, although maturation is slower, especially in organelles. Enhanced yellow fluorescent protein has also been widely employed as an acceptor for energy transfer experiments when paired with enhanced cyan fluorescent protein. However, all of the original yellow fluorescent protein variants present some problems in that they are very sensitive to acidic pH and lose ~50% of their fluorescence at pH 6.5. In addition, most of the *Aequorea*-derived proteins in this class have also been demonstrated to have significant sensitivity to chloride ions, undergo weak dimerization, exhibit relatively poor expression at 37°C, and photobleach much more readily than many of the green fluorescent proteins. Several investigations have taken advantage of the environmental sensitivity of YFP to measure cytosolic pH (Llopis et al., 1998), chloride ion concentrations (Jayaraman et al., 2000; Kuner and Augustine, 2000), and FRET efficiency (Llopis et al., 2000). The A206K mutation, substituting the charged amino acid lysine for alanine at the hydrophobic dimer interface, has been applied to YFP (Zacharias et al., 2002) in order to generate a true monomer.

Continued genetic development of the YFP family (Griesbeck et al., 2001) led to the discovery that a single point mutation near the chromophore, the substitution of methionine for glutamine at position 69 (Q69M), dramatically increases the acid stability of the protein and reduces the chloride sensitivity. Named Citrine in recognition of the yellow color and acid resistance, this variant also expresses at a much higher level in mammalian cell culture (especially when targeted to acidic organelles) and demonstrates almost twice the photostability of many previous yellow fluorescent proteins. Citrine features absorption and fluorescence emission maxima at 516 and 529 nm, respectively, and is 75% brighter than EGFP, although much less photostable. FRET biosensors constructed with Citrine demonstrate better performance than sister analogs containing EYFP, and the probe is a better choice for multicolor experiments in general. Although Citrine exists as a weak dimer in solution, the protein can be converted into a monomer with the A206K mutation (Shaner et al., 2005).

Introduction of yet another novel point mutation into *Aequorea*-derived YFP, the substitution of leucine for phenylalanine at position 46 (F46L), produced a variant that was named Venus in honor of the brightest star in the night sky (Nagai et al., 2002). This mutation, which was discovered during random mutagenesis experiments with circularly permuted GFP derivatives, dramatically accelerates oxidation of the chromophore, the rate-limiting step in fluorescent protein maturation. Additional mutations were also introduced in order to increase the tolerance of Venus to acidic environments and to reduce the sensitivity to chloride. The absorption and emission spectral peaks (515 and 528 nm, respectively) of Venus are shifted to longer wavelengths by a single nanometer compared to EYFP, but the brightness level is retained. Unfortunately, the photostability of Venus is only ~25% that of EYFP, a significant problem for long-term imaging experiments. Venus has been demonstrated to perform well in intracellular organelle fusion protein pH assays (Tsuboi and Rutter, 2003), for localization studies in yeast (Sheff and Thorn, 2004), in bimolecular fluorescence complementation analysis (Shyu et al., 2006), and holds significant potential as an acceptor in FRET biosensors (Nagai and Miyawaki, 2004; Sheridan and Hughes, 2004). Genetic conversion of the weakly dimeric Venus into a monomer using the A206K mutation will further expand the use of this probe in biological investigations (Rizzo and Piston, 2005b).

During the same fluorescence-activated cell sorting investigation that led to the generation

of the cyan fluorescent protein CyPet (Nguyen and Daugherty, 2005), the evolutionary optimized complementary FRET acceptor, termed YPet (YFP for energy transfer), was also obtained from Venus and YFP variants. Named after its proficiency in FRET, YPet is the brightest yellow fluorescent protein variant yet developed and demonstrates very good photostability. The resistance to acidic environments afforded by YPet is superior to Venus and other YFP derivatives, which will enhance the utility of this probe in biosensor combinations targeted at acidic organelles. However, although the optimized CyPet-YPet combination should be the preferred starting point in the development of new FRET biosensors, the utility of this pair has not yet been tested in widespread practice (Shaner et al., 2005). Likewise, the suitability of CyPet and YPet in fusion tags for localization experiments, bimolecular complementation analysis, and other routine fluorescent protein assays has yet to be established. Both proteins exist in solution as weak dimers, but presumably can be converted to true monomers using the A206K mutation that has worked so well with other *Aequorea* variants.

Although the potential for new discoveries of yellow and green fluorescent proteins in Hydrozoan species other than *Aequorea victoria* is significant, only one candidate has surfaced so far. Isolated from the *Phialidium* jellyfish, a protein termed phiYFP (Shagin et al., 2004) is reported to demonstrate very bright yellow fluorescence (absorption and emission at 525 and 537 nm, respectively) and to be useful for N-terminal fusion tags. An extraordinary feature of phiYFP is that the naturally occurring protein contains the same mutation at position 64 (leucine) that was introduced by Venus to increase the folding efficiency (Nagai et al., 2002). The probe also naturally contains tyrosine at position 203 (Ormö et al., 1996), another site-directed modification of the native GFP that resulted in yellow fluorescence. This remarkable discovery of a natural similarity between the structure of phiYFP and genetically modified *Aequorea* proteins is a testament to the efficacy of protein engineering efforts directed at GFP to adjust the spectral properties. The phiYFP protein has been optimized by random mutagenesis to produce a monomeric version without compromising the spectral properties.

Site-directed and random mutagenesis of a monomeric Anthozoan fluorescent protein (mRFP1) from *Discosoma* have resulted in the creation of two monomeric coral reef derivatives with spectral properties falling in the range of *Aequorea* yellow fluorescent proteins (Shaner et al., 2004). Named after similarly colored fruits, mHoneydew and mBanana both emit fluorescence in the yellow spectral region. The broad absorption band of mHoneydew (peaks at 487 and 504 nm) enables effective excitation with an argon-ion laser or standard FITC filter combination. However, the similarly broad emission spectrum (peaks at 537 and 562 nm) tails into the near-infrared, hampering the use of this protein in multicolor experiments. In addition, a low extinction coefficient and quantum yield render mHoneydew the dimmest member of the monomeric yellow fluorescent protein cadre. The mBanana variant is only twice as bright as mHoneydew, but features much narrower excitation and emission spectra (peaks at 540 and 553 nm, respectively). Because both proteins exhibit relatively poor photostability, and mBanana is highly pH-sensitive, neither will probably find great utility in imaging experiments. Perhaps the most promising aspect of these probes is that the mere existence of mHoneydew (a cyan-type Y67W mutant) demonstrates that the tryptophan-based chromophore of CFP can undergo a further maturation into a longer-wavelength emitting species (Shaner et al., 2004).

ZsYellow (originally referred to as zFP538) is a yellow fluorescent protein that was discovered in the Anthozoan button polyp *Zoanthus* during a search in reef corals for naturally occurring GFP analogs emitting fluorescence in longer wavelength regions (Matz et al., 1999; Gurskaya et al., 2001b Yanushevich et al., 2002). One of the most unique features of the ZsYellow fluorescence emission spectrum is that the peak (538 nm) occurs almost midway between those of EGFP (508 nm) and DsRed (583 nm), presenting an opportunity to investigate proteins emitting fluorescence in the yellow portion of the visible light spectrum. The ZsYellow fluorescent protein chromophore features a novel three-ring system and peptide backbone cleavage due to the substitution of lysine for serine as the first amino acid residue in the chromophore tripeptide sequence (Zagranichny et al., 2004; Remington et al., 2005;). As a result of the unique chromophore motif, the degree of conjugation observed in ZsYellow is intermediate between that observed with EGFP and DsRed (one double bond more than EGFP, and one less than DsRed), which accounts for the positioning

of emission wavelengths in the yellow region. ZsYellow exhibits a marked tendency to form tetramers when expressed in vivo, hampering the use of this protein as a fusion partner for localization investigations. Furthermore, the reduced brightness level of ZsYellow when compared to enhanced green fluorescent protein (25% of EGFP) also limits the utility of this reporter in fluorescence microscopy (the human codon-optimized version is commercially available as ZsYellow1). The unique emission spectral profile of ZsYellow, however, should encourage the search for genetic modifications that alleviate the tendency to form tetramers while simultaneously increasing the quantum yield and extinction coefficient, an effort that could ultimately yield a high-performance monomeric yellow fluorescent protein.

Orange Fluorescent Proteins

In contrast to the relatively large number of fluorescent proteins engineered in the cyan, green, and yellow spectral classes, only three probes have been developed so far in the orange portion of the spectrum (ranging from ~555 to 580 nm). Even so, all three of the existing orange fluorescent proteins, which were isolated from coral reef species, have the potential to be useful in a variety of imaging scenarios. Perhaps the most versatile of these is monomeric Kusabira Orange (Karasawa et al., 2004), a protein originally derived as a tetramer from the mushroom coral *Fungia concinna* (known in Japanese as Kusabira-Ishi). Kusabira Orange was engineered by site-specific mutagenesis from a cDNA clone of the coral by adding ten amino acids to the N terminus. The resulting protein has an absorption maximum at 548 nm (ideal for excitation with a 543-nm laser) and emits bright orange fluorescence at 561 nm. A monomeric version of Kusabira Orange (abbreviated mKO) was created using a strategy similar to that employed for DsRed to create mRFP1 (discussed in the section on red fluorescent proteins) by introducing over 20 mutations through site-directed and random mutagenesis. The monomer exhibits similar spectral properties to the tetramer and has a brightness value similar to EGFP, but is slightly more sensitive to acidic environments. The photostability of this probe, however, is the best of any fluorescent protein in all of the spectral classes, making mKO an excellent choice for long-term imaging experiments. Furthermore, the emission spectral profile is sufficiently well separated from cyan fluorescent proteins to increase the FRET efficiency in biosensors incorporating mKO, and the probe is useful in multicolor investigations (see Figure 21.5.4) with a combination of cyan, green, yellow, and red fluorescent proteins.

The mRFP1 derivative, mOrange (Shaner et al., 2004), was derived after four rounds of directed evolution to yield a probe absorbing at 548 nm and emitting orange fluorescence at 562 nm. The mOrange variant is slightly brighter than mKusabira Orange, but has <10% the photostability, thus severely compromising its application for experiments that require repeated imaging. However, mOrange remains the brightest protein in the orange spectral class and is still an excellent choice where intensity is more critical than long-term photostability. In addition, combined with the green-emitting T-Sapphire, mOrange is a suitable alternative to CFP-YFP proteins as a FRET pair to generate longer wavelength biosensors, and can be coupled with fluorescent proteins in other spectral regions for multicolor investigations. A novel orange fluorescent protein isolated from the *Cerianthus* tube anemone (Ip et al., 2004) is commercially available (trade name cOFP; Stratagene) and has spectral properties that are similar to mOrange and mKusabira Orange, but like the other anemone proteins isolated to date, exists in solution as a tetramer. The brightness and photostability of cOFP have not been reported so this protein cannot be directly compared to other orange fluorescent proteins, and its utility will be further limited until it can be converted into a monomer.

Red Fluorescent Proteins

The quest for a well-behaved red-emitting fluorescent protein has long been the holy grail of live-cell imaging. This is primarily due to the requirement for probes in this spectral region in multicolor imaging experiments, as well as the fact that longer excitation wavelengths generate less phototoxicity and can probe deeper into biological tissue. As an added convenience, most of the proteins in the red region of the visible spectrum can be imaged with the common TRITC and Texas Red widefield fluorescence filter sets, as well as cheap helium-neon (543, 561, 594, and 633 nm) lasers in confocal microscopy. After five years of unsuccessful mutagenesis efforts in the *Aequorea* GFP-derived proteins (Tsien, 1998), the first real breakthrough occurred with the discovery of potentially fluorescent chromoproteins in nonbioluminescent Anthozoa coral species (Matz et al., 1999). To

date, a wide spectrum of potentially useful red fluorescent proteins has been reported (spanning the emission wavelength range of 580 to 630 nm), many of which still suffer from some degree of the obligatory quaternary structure bestowed by their species of origin (Verkhusha and Lukyanov, 2004; Chudakov et al., 2005; Miyawaki, 2005; Shaner et al., 2005). Unlike the jellyfish proteins, most of the native and genetically engineered variants of coral reef proteins mature very efficiently at 37°C, presumably due to differing water temperatures of their respective habitats (Chudakov et al., 2005; Miyawaki et al., 2005).

The first Anthozoa-derived fluorescent protein to be extensively investigated was derived from the sea anemone *Discosoma striata* and originally referred to as drFP583, but is now commonly known as DsRed (Matz et al., 1999). Once the protein has fully matured, the fluorescence emission spectrum of DsRed features a peak at 583 nm, whereas the excitation spectrum has a major peak at 558 nm and a minor peak around 500 nm. Several problems are associated with DsRed in practice. Maturation of DsRed fluorescence occurs slowly and proceeds through an intermediate chromophore stage where a majority of the fluorescence emission is seen in the green region (Baird et al., 2000). Termed the "green state," this artifact has proven challenging for multiple labeling experiments in combination with other green fluorescent proteins because of the spectral overlap. In addition, DsRed is an obligate tetramer, an undesirable characteristic that interferes in fusion protein constructs, often leading to poor localization, and increases the tendency to form large protein aggregates in living cells. Although these side effects are not important when the probe is utilized simply as a reporter for gene expression, the utility of DsRed as an epitope tag is severely compromised (Rizzo and Piston, 2005a). In contrast to the large *Aequorea* family of proteins that have been employed to successfully tag hundreds of fusion proteins, DsRed fusion proteins have proven far less successful and often exhibit toxic effects.

Several of the major problems with DsRed fluorescent protein have been overcome through site-directed and random mutagenesis efforts. The second-generation version of DsRed, known as DsRed2, contains a series of silent nucleotide substitutions corresponding to human codon preferences and several internal sequence mutations that increase the maturation rate (Bevis and Glick, 2002). In addition, the elimination of basic amino acid residues (changed to acidic or neutral moieties) at the peptide amino terminus in DsRed2 reduces the tendency of the protein to form aggregates in fusion constructs (Yanushevich et al., 2002). The DsRed2 protein still forms a tetramer in solution, but it is more compatible with green fluorescent proteins in multiple labeling experiments due to the increased maturation rate (Rizzo and Piston 2005a). Further reductions in maturation time have been realized with the third generation of DsRed mutants, which also display an increased brightness level in terms of peak cellular fluorescence. Red fluorescence emission from DsRed-Express (a commercial vector from Clontech) can be observed within an hour after expression, as compared to ~6 hr for DsRed2 and 11 hr for DsRed (Bevis and Glick, 2002; Rizzo and Piston, 2005a). The presence of a green state in DsRed-Express is not apparent, rendering this fluorescent protein the best choice of tetrameric DsRed variants for multiple labeling experiments. Because these probes remain obligate tetramers, however, they have largely been replaced with dimeric and monomeric versions and are now only of historical interest. A commercially available monomeric version of DsRed (Clontech) has recently been introduced, but the intrinsic brightness and photostability of this probe are reported to be poor (Shaner et al., 2005).

Several additional red fluorescent proteins showing a considerable amount of promise have been isolated from the reef coral organisms. One of the first to be adapted for mammalian cell applications is HcRed1 (Gurskaya et al., 2001a), which was isolated from the anemone *Heteractis crispa* and is now commercially available (Clontech and Evrogen). HcRed1 was originally derived from a nonfluorescent chromoprotein that absorbs orange light through site-directed and random mutagenesis. A total of six amino acid substitutions were necessary to create a red fluorescent species that matured rapidly and efficiently at 37°C (absorption and emission at 588 and 618 nm, respectively). However, similar to many other reef coral proteins, the resulting red fluorescent HcRed displays a tendency to form obligate tetramers when expressed in bacteria. Additional mutagenesis efforts resulted in a brighter dimeric variant (Gurskaya et al., 2001a), but a monomeric version of the protein has not yet been discovered. In order to generate a species of the protein that is useful in creating fusion products for localization

studies, a tandem dimer expression vector of HcRed (two head-to-tail identical copies of the protein) has been constructed (Fradkov et al., 2002). When fused to a gene product that itself forms biopolymers (such as actin or tubulin), the HcRed tandem dimer forms an intramolecular self-association complex (mimicking a monomeric tag) that apparently does not interfere with the biological activity of the resulting chimera. However, because the overall brightness and photostability of this twinned protein combination has not yet been improved, it remains a secondary choice for routine applications in live-cell microscopy.

A far-red fluorescent protein, termed eqFP611, was isolated from the sea anemone *Entacmaea quadricolor* and displays one of the largest Stokes shifts and red-shifted fluorescence emission wavelength profiles (excitation and emission maxima at 559 and 611 nm, respectively) of any naturally occurring Anthozoan fluorescent protein (Wiedenmann et al., 2002). The quantum yield and extinction coefficient of eqFP611 combine to yield a probe approximately as bright as EGFP. During the in vivo fluorophore maturation process, which occurs in ~12 hr, the protein passes through a green intermediate state. After maturation, however, only a small fraction of this green species (<1 percent) can be detected. In contrast to other Anthozoan fluorescent proteins, eqFP611 has a reduced tendency to oligomerize at lower concentrations as evidenced through electrophoresis and single-molecule experiments (Schenk et al., 2004), although at high concentrations the protein does form tetramers. Site-directed mutagenesis efforts have yielded functional dimeric variants of eqFP611 (Wiedenmann et al., 2005), and continued efforts may finally lead to a monomeric far-red fluorescent protein from this species.

Two additional reef coral red fluorescent proteins, AsRed2 and JRed, are commercially available (Clontech and Evrogen), but these probes form tetrameric and dimeric complexes, respectively, and are less useful than the monomeric proteins described below. AsRed2 was originally isolated as a chromoprotein from *Anemonia sulcata* (Matz et al., 1999) and modified through mutagenesis to yield a protein having an absorption maximum at 576 nm and an emission peak at 595 nm (Lukyanov et al., 2000) with a very modest quantum yield (0.05). Although the protein has been optimized with human codons for expression in mammalian cell lines, it exhibits only ~10% the brightness level of EGFP and the photostability has not been reported. The dimeric protein, JRed, was derived through extensive mutagenesis of a jellyfish chromoprotein (Shagin et al., 2004) to produce a novel red fluorescent marker with peak absorption and emission wavelengths of 584 and 610 nm, respectively. The probe has been demonstrated to produce useful fusion tags, but is unsuitable for expression in prokaryotes due to folding problems. JRed is ~25% as bright as EGFP and exhibits limited photostability when illuminated in the 560 to 580 nm region, but it can be successfully employed for long-term imaging experiments when excited with a 543-nm laser.

The construction of true monomeric DsRed variants, as well as monomers from proteins in other Anthozoan species, has proven to be a difficult task (Rizzo and Piston, 2005a). A total of 33 amino acid alterations to the DsRed sequence were required for the creation of the first-generation monomeric red fluorescent protein (termed RFP1; Campbell et al., 2002). However, this derivative exhibits significantly reduced fluorescence emission compared to the native protein and photobleaches very quickly, rendering it much less useful than analogous monomeric green and yellow fluorescent proteins. Extensive mutagenesis research efforts (Shaner et al., 2004), including novel techniques such as iterative somatic hypermutation (Wang et al., 2004), have successfully been applied in the search for yellow, orange, red, and far-red fluorescent protein variants that further reduce the tendency of these potentially efficacious biological probes to self-associate while simultaneously pushing emission maxima towards longer wavelengths. The result has been improved monomeric fluorescent proteins that feature increased extinction coefficients, quantum yields, and photostability, although no single variant has yet been optimized by all criteria. In addition, the expression problems with obligate tetrameric red fluorescent proteins are being overcome by the efforts to generate true monomeric variants, which have yielded derivatives that are more compatible with biological function.

Perhaps the most spectacular development on this front has been the introduction of a new harvest of fluorescent proteins (Patterson, 2004) derived from monomeric red fluorescent protein (mRFP1) through directed mutagenesis targeting the Q66 and Y67 chromophore residues (Shaner et al., 2004), which have been

demonstrated to play key roles in determining the spectral characteristics in *Aequorea* proteins. The resulting cadre of monomeric fluorescent proteins exhibit maxima at wavelengths ranging from 560 to 610 nm and have been named in honor of common fruits that bear colors similar to their respective fluorescence emission spectral profiles. Among the potentially efficacious members in the fruit series of fluorescent proteins are mStrawberry, mCherry, and tdTomato (a tandem dimer), all of which have fluorescence emission profiles in the orange and red regions of the spectrum. Of these probes, tdTomato, which has an orange-red emission maximum at 581 nm, is the brightest and most photostable under widefield illumination (Shaner et al., 2005), although the dimer is twice the molecular weight of the monomers. The red proteins, mCherry and mStrawberry (emission peaks at 610 and 596 nm, respectively), have brightness levels of ~50% and 75% of EGFP, but mCherry is far more photostable than mStrawberry and is the best probe choice to replace mRFP1 for long-term imaging experiments. These new proteins essentially fill the gap between the most red-shifted jellyfish fluorescent proteins (such as Venus) and the multitude of oligomeric coral reef red fluorescent proteins that have been reported and are commercially available. Although several of these new fluorescent monomeric proteins lack the brightness and photostability necessary for many imaging experiments (Shaner et al., 2005; Tsien, 2005), their existence is encouraging as it suggests the eventuality of bright, stable, monomeric fluorescent proteins across the entire visible spectrum.

Far-Red Fluorescent Proteins

Further extension of the fruit protein spectral class through iterative somatic hypermutation (Wang et al., 2004) has yielded two new fluorescent proteins with emission wavelength maxima of 625 and 649 nm, representing the first true far-red (ranging from 630 to 700 nm) genetically engineered probes. The most potentially useful probe in this pair was named mPlum, which has a rather limited brightness value (10% of EGFP), but excellent photostability. This monomeric probe should be useful in combination with fluorescent proteins emitting in the cyan, green, yellow, and orange regions for multicolor imaging experiments (see Figure 21.5.4) and as a biosensor FRET partner with green and yellow proteins, such as mEmerald (Förster distance, 4.4) and mCitrine (Förster distance, 5.0). Another far-red fluorescent protein, termed AQ143, has been derived from mutagenesis efforts on a chromoprotein isolated from the anemone *Actinia equine* (Shkrob et al., 2005). The excitation and emission maxima of AQ143 are 595 and 655 nm, respectively, and the brightness is comparable to mPlum. On the downside, the photostability of this protein has not been reported and it forms an obligate tetramer.

OPTICAL HIGHLIGHTER FLUORESCENT PROTEINS

Protein chromophores that can be activated to initiate fluorescence emission from a quiescent state (a process known as photoactivation), or are capable of being optically converted from one fluorescence emission bandwidth to another (photoconversion), represent perhaps the most promising approach to the in vivo investigation of protein lifetimes, transport, and turnover rates. Appropriately termed molecular or optical highlighters, photoactivated fluorescent proteins generally display little or no initial fluorescence under excitation at the imaging wavelength, but dramatically increase their fluorescence intensity after activation by irradiation at a different (usually lower) wavelength (for reviews see: Lippincott-Schwartz et al., 2003; Miyawaki, 2004; Lukyanov et al., 2005). Photoconversion optical highlighters, on the other hand, undergo a change in the fluorescence emission bandwidth profile upon optically-induced changes to the chromophore. These effects result in the direct and controlled highlighting of distinct molecular pools within the cell (see Fig. 21.5.5). Because only the limited population of photoactivated molecules exhibit noticeable fluorescence, their lifetime and behavior can be followed independently of other proteins that are newly synthesized. In a similar manner, fluorescent proteins that are capable of being optically modulated through photoconversion to reversibly alter emission intensity and/or color are also potentially very useful for examining dynamic processes in living cells.

The ideal optical highlighter proteins should be readily photoconvertible (through the process of fluorescence activation and/or emission wavelength shifts) to produce a high level of contrast, as well as being monomeric for optimum expression in the target system. These probes will be especially useful in experiments paralleling results obtained with photobleaching techniques, such as recovery (FRAP) and loss (FLIP) of photobleaching,

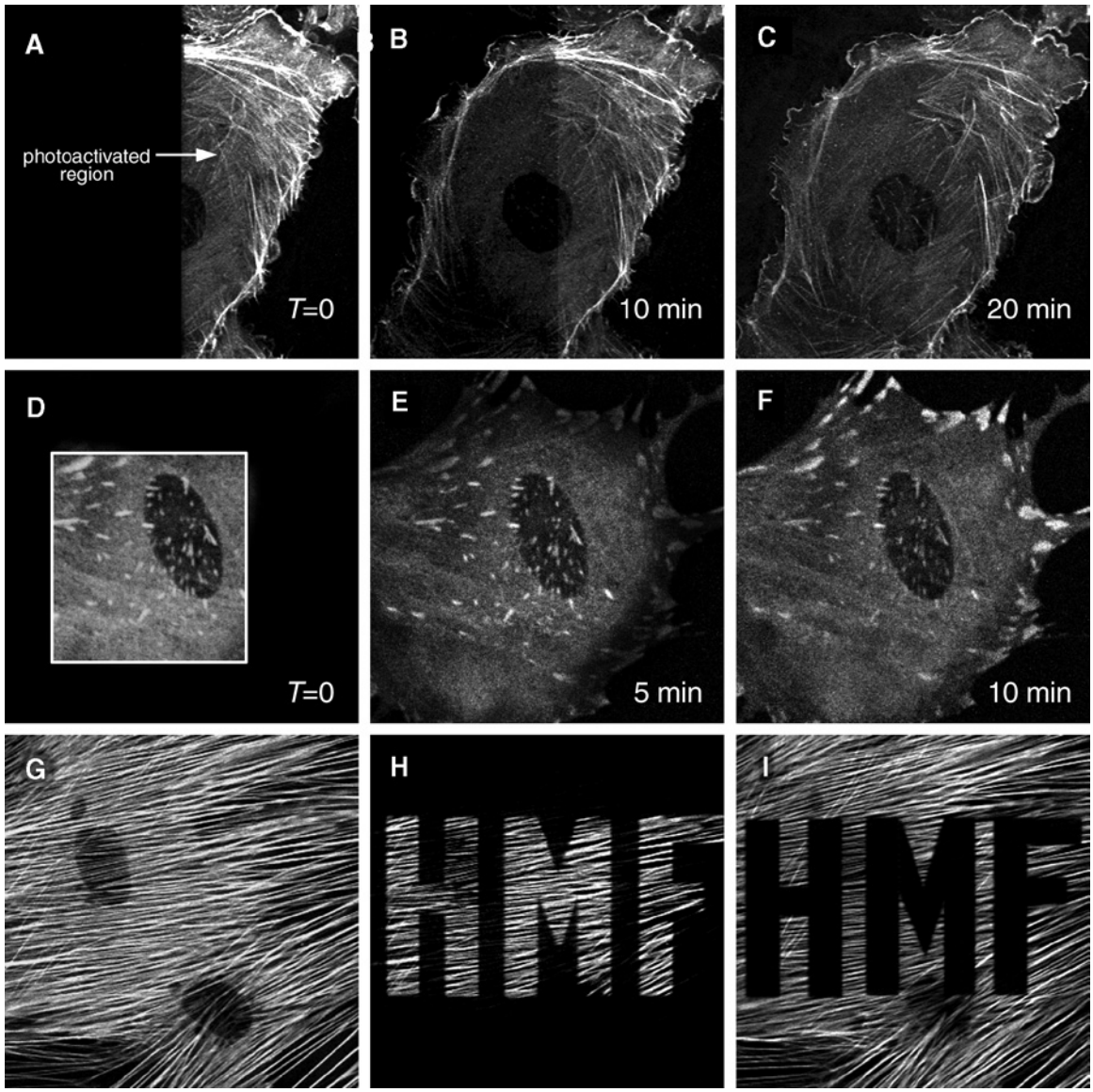


Figure 21.5.5 Photoactivation, photoconversion, and photoswitching with optical highlighter fluorescent proteins. (**A**) to (**C**) PA-GFP fused to human β-actin expressed in an epithelial cell is illuminated at high intensity with a 405-nm diode laser in the right-hand portion of (**A**) to photoactivate the protein, which is slowly distributed across the filamentous network (**B**) and (**C**) over a period of 20 min. (**D**) to (**F**) A fusion tag containing EosFP and vinculin is localized to the focal adhesion regions in a fibroblast cell. After photoconversion of fluorescence from green to red in a selected region (**D**); only the red channel is illustrated, the converted highlighter diffuses through the cytoplasmic pool slowly making its way to the focal adhesion areas over a period of 10 min (**E**) and (**F**). (**G**) to (**I**) Photoswitching of Dronpa fused to human β-actin in a fibroblast. (**G**) Image of the actin cytoskeletal network with a 488-nm laser. (**H**) An AOTF was used to spell the letters "HMF" and the labeled molecules within the letters were photoswitched to the "on" state using a 405-nm diode laser after photoswitching molecules outside to the "off" state with a 488-nm laser. (**I**) Molecules inside the letters were photobleached with the 405-nm laser while imaging exterior molecules with the 488-nm laser.

because they have the advantage that measurements are not influenced by newly synthesized or non-converted proteins, which either remain invisible or continue to emit the original wavelengths (Lippincott-Schwartz et al., 2003). Also, by repeated excitation in the region of interest, optical highlighters can be continuously photoconverted at a specific intracellular location. This technique is more efficient than FLIP because the translocation of activated proteins can be directly imaged. In addition, the time required for photoactivation (a few seconds) is often much less than the time required to completely photobleach a similar region. Investigations involving extremely rapid cellular processes will clearly benefit from such improvements in temporal resolution. Presented in Table 21.5.2 is a compilation of important data for optical highlighter fluorescent proteins that display significant potential in applications as in vivo probes targeting cellular structure and function. Included are the excitation (or absorption) and fluorescence emission maximum wavelengths for both the activated and nonactivated species, as well as the molar extinction coefficients, quantum yields, molecular structure (monomer, dimer, tetramer, etc.), and relative brightness level compared to enhanced green fluorescent protein (EGFP). Also listed are references to the original literature sources and review articles. This table should serve as a convenient guide for comparing the properties of optical highlighters.

The first useful optical highlighter designed specifically for photoactivation studies is a variant of the jellyfish (*Aequorea victoria*) green fluorescent protein, termed PA-GFP (an acronym for Photo Activatable Green Fluorescent Protein). This photoactivatable version of GFP was developed by improving on the photoconversion efficiency of the natural wild-type protein chromophore from a predominately neutral form to a species that is anionic in character (Patterson and Lippincott-Schwartz, 2002a,b; Lippincott-Schwartz and Patterson, 2003). Substitution of histidine for threonine at position 203 produced a variant with negligible absorbance in the region between 450 and 550 nm, thus dramatically enhancing the contrast between the nonactivated and activated species. After photoactivation with violet light, the absorption maximum at 504 nm in PA-GFP increases ~100-fold. This event evokes very high contrast differences between the converted and unconverted pools of PA-GFP, and is useful for tracking the dynamics of molecular subpopulations within a cell. Note that after photoactivation, both PA-GFP and the wild-type GFP exhibit comparable levels of fluorescence when illuminated with a 488-nm laser. On the downside, intracellular targets expressing PA-GFP are not readily distinguishable before photoconversion without the use of a low-intensity violet illumination source (such as a 405-nanometer blue diode laser), making it difficult to identify the proper regions to be selectively targeted in photoactivation experiments using microscopes having only blue and green lasers.

Several new photoactivatable proteins have been produced using site-directed mutagenesis of monomeric red-shifted reef coral fluorescent proteins. In particular, the monomeric derivative of DsRed fluorescent protein, mRFP1, has been converted into probes that are photoactivated by either green or violet irradiation (Verkhusha and Sorkin, 2005). These have been collectively termed PA-mRFP1s, the brightest of which exhibits a 70-fold increase of fluorescence intensity upon activation by wavelengths between 380 and 400 nm. The critical molecular determinant residues for producing photoactivatable proteins with coral reef variants appear to be the amino acids at positions 146, 161, and 197 (DsRed numbering scheme). These residues, which lay in close proximity to the chromophore, serve to stabilize the tyrosine residue at position 66 in either a *cis* (fluorescent) or *trans* (nonfluorescent) configuration to form a fluorescent protein (*cis*) or a chromoprotein (*trans*) that absorbs light but does not emit fluorescence. The monomeric red fluorescent protein optical highlighters have been demonstrated to be useful in some cases as a fusion tag in mammalian systems for investigation of intracellular dynamics. However, the relatively low level of fluorescence of PA-mRFP1s in the photoactivated form (3% of EGFP), along with a tendency to form nonspecific aggregates when fused to naturally oligomeric proteins (for example, actin and tubulin), render these probes less useful than PA-GFP for live-cell investigations (Verkhusha and Sorkin, 2005). Improved monomeric yellow, orange, and red fluorescent proteins (such as mCherry, mStrawberry, and mPlum) hold the potential to produce efficient photoactivatable optical highlighters with emission wavelengths extending into the deep red portion of the visible light spectrum.

A novel photoconversion optical highlighter, termed photoswitchable cyan fluorescent protein (PS-CFP), derived from the *Aequorea coerulescens* green fluorescent protein

Table 21.5.2 Fluorescent Proteins that Have Potential as In Vivo Probes[a]

Protein(Acronym)	Ex (nm)	Em (nm)	EC × 10^{-3}	QY	in vivo Structure	Relative Brightness (% of EGFP)	References
PA-GFP (G/NA)	400	515	20.7	0.13	Monomer	8	Patterson and Lippincott-Schwartz, 2002a
PA-GFP (G)	504	517	17.4	0.79	Monomer	41	Patterson and Lippincott-Schwartz, 2002a
PS-CFP (C)	402	468	34.0	0.16	Monomer	16	Chudakov et al., 2004
PS-CFP (G)	490	511	27.0	0.19	Monomer	15	Chudakov et al., 2004
PS-CFP2 (C)	400	468	43.0	0.20	Monomer	26	Chudakov et al., 2004
PS-CFP2 (G)	490	511	47.0	0.23	Monomer	32	Chudakov et al., 2004
PA-mRFP1 (R)	578	605	10.0	0.08	Monomer	3	Verkhusha et al., 2005
Kaede (G)	508	518	98.8	0.88	Tetramer	259	Ando et al., 2002
Kaede (R)	572	580	60.4	0.33	Tetramer	59	Ando et al., 2002
Kikume (KikGR; G)	507	517	53.7	0.70	Tetramer	112	Tsutsui et al., 2005
Kikume (KikGR; R)	583	593	35.1	0.65	Tetramer	68	Tsutsui et al., 2005
mEosFP (G)	505	516	67.2	0.64	Monomer	128	Wiedenmann et al., 2004
mEosFP (R)	569	581	37.0	0.62	Monomer	68	Wiedenmann et al., 2004
Dendra2 (G)	490	507	45.0	0.50	Monomer	67	Gurskaya et al., 2006
Dendra2 (R)	553	573	35.0	0.55	Monomer	57	Gurskaya et al., 2006
Kindling (R)	580	600	59.0	0.07	Tetramer	12	Labas et al., 2002
Dronpa (G)	503	518	95.0	0.85	Monomer	240	Ando et al., 2004

[a]A compilation of important data for fluorescent proteins that display significant potential in applications as in vivo probes targeting cellular structure and function.

variant, aceGFP, has been observed to transition from cyan (468 nm) to green fluorescence (511 nm) upon illumination at 405 nm. The PS-CFP highlighter was generated by site-directed mutagenesis of aceGFP (Chudakov et al., 2004). Expressed as a monomer in vivo, this probe should be useful in photobleaching, photoconversion, and photoactivation investigations because it can potentially be fused to a wide variety of proteins without altering their behavior. Another advantage of PS-CFP is the significant level of cyan fluorescence that is present before photoconversion, a factor that allows investigators to track and selectively illuminate specific intracellular regions or entire cells for study. However, the fluorescence emission intensity from PS-CFP is ~2.5-fold dimmer than PA-GFP, and the probe is inferior

to other highlighters in terms of photoconversion efficiency (the 40-nm wavelength shift in fluorescence emission upon photoconversion is less than that observed with similar optical highlighters). A high-contrast, brighter variant of PS-CFP, designated PS-CFP2, is commercially available (Evrogen) as a cloning vector.

Several interesting and potentially useful optical highlighters have been developed in fluorescent proteins cloned from reef coral and sea anemone species. One of the first and most important examples, a tetrameric fluorescent protein isolated from the stony Open Brain coral, *Trachyphyllia geoffroyi*, has been found to photoconvert from green to red fluorescence emission in the presence of ultraviolet light (Ando et al., 2002). It is interesting to note that the discovery of this highlighter occurred when researchers accidentally left a test tube containing the protein on a laboratory bench near a window, and then astutely observed the shift from green to red. The unusual color transition prompted investigators to name the protein Kaede, after the leaves of the Japanese maple tree, which turn from green to red in the fall months. Irradiation of the commercially available (MBL) Kaede optical highlighter with light between 380 and 400 nm results in a rapid spectral shift from principal maxima at 508 nm (absorption) and 518 nm (emission) to longer wavelength peaks at 572 and 582 nm, respectively. Upon photoconversion, Kaede exhibits a dramatic increase in the red-to-green fluorescence ratio (~2000-fold, considering both the decrease in green and the increase in red emission). The conversion is stable and irreversible under aerobic conditions. Neither exposure to dark for extended periods nor strong illumination at 570 nm is able to restore green fluorescence to the chromophore. The red fluorescent state of the Kaede chromophore is comparable to the green in terms of brightness and stability, and because the unconverted protein emits very little fluorescence above 550 nm, the appearance of strong red signal provides excellent contrast.

Similar proteins capable of being photoconverted by violet and ultraviolet illumination have been discovered in the Great Star coral (mcavRFP; derived from *Montastraea cavernosa*; Shagin et al., 2004), soft corals (DendFP; derived from members of the genus *Dendronephthya*; Pakhomov et al., 2004), and the mushroom coral (rfloRFP; derived from *Ricordea florida*; Shagin et al., 2004). All of these highlighters (including Kaede, Eos, and KikGR, discussed below) contain a chromophore derived from the tripeptide His-Tyr-Gly that initially emits green fluorescence until driven into a red state by light rather than chemical oxidation, as is the case with many red fluorescent proteins derived from reef corals. These amino acids form the imidazolinone chromophore (similar to the *Aequorea* proteins) that is transformed from green to red fluorescence emission under illumination at shorter visible and longer ultraviolet wavelengths. Irradiation induces cleavage between the amide nitrogen and α-carbon atoms in the histidine residue with subsequent formation of a highly conjugated dual imidazole ring system, a process requiring catalysis by the intact protein and resulting in the dramatic shift of fluorescence emission to red wavelengths (Mizuno et al., 2003; Nienhaus et al., 2005). The unconventional chemistry involved in this chromophore transition should serve fluorescent protein engineers with an excellent foundation upon which to develop more advanced highlighters.

The stony coral *Favia favus* has yielded a promising tetrameric derivative that exhibits efficient photoconversion from green to red fluorescence emission wavelengths (similar to Kaede) upon irradiation with near-ultraviolet or violet light (Tsutsui et al., 2005). Genetic engineering efforts based on structural analysis of this protein produced a variant, termed KikGR (named after Kikume-ishi, the Japanese term for *Favia*), which is several-fold brighter than Kaede in both the green and red states when expressed in mammalian cells. Commercially available (MBL) under the name Kikume Green-Red, the highlighter has been demonstrated to be successfully photoconverted using multiphoton excitation at 760 nm, which can be used for specific labeling of cells with high spatial resolution in thick tissues. Furthermore, the Kikume highlighter features a wider separation of green and red emission maxima (75 nm vs. 54 nm) than Kaede.

Another tetrameric stony coral (*Lobophyllia hemprichii*) fluorescent protein similar to Kaede, termed EosFP (named after the goddess of dawn in Greek mythology), emits bright green fluorescence at 516 nm that shifts to orange-red (581 nm) when illuminated with light at wavelengths in the near-ultraviolet region of 390 to 405 nm (Wiedenmann et al., 2004). Two single point mutations have been employed to split the wild-type tetramer into dimeric subunits, each of which is capable of being incorporated into functional fusion constructs with a variety of proteins that retain

normal biological activity. By introducing a combination of both single point mutations into the wild-type EosFP tetramer, a true monomeric protein has been created that is referred to as mEosFP. Similar to the dimer subunits, the monomer is able to be incorporated into functional biological chimeras to serve as a marker in live-cell imaging (Ivanchenko et al., 2005), although the monomer fusions are only efficiently expressed at temperatures below 30°C (limiting their applications in mammalian systems). Fluorescence spectroscopy of Eos indicates a pH dependence on photoconversion, suggesting that the protonated form of the chromophore is necessary for activation, which is consistent with an excited-state proton transfer mechanism.

Recently, an additional highlighter that undergoes green-to-red photoconversion has been isolated from the soft coral *Dendronephthya* and named Dendra (an acronym derived from the first four letters of the genus name, *Dend*, and *r*ed *a*ctivatable). Originally isolated as a tetramer, Dendra was converted into a true monomer through site-directed mutagenesis to yield a green fluorescent probe that can be photoconverted into a red species using either ultraviolet or blue light (Gurskaya et al., 2006). Upon photoconversion, Dendra exhibits up to a 4000-fold increase in red fluorescence and demonstrates remarkable photostability. Unlike many of the *Aequorea* protein variants, maturation of the Dendra green chromophore is very efficient over a wide temperature range, from 20°C to 37°C. A commercial version, Dendra 2 (see Table 21.5.2), contains the substitution of a single amino acid, valine for alanine, at position 224 (A224V), which leads to more complete maturation and brighter fluorescence both before and after photoconversion. The ability to photoconvert Dendra using a 488-nm laser at high intensity is distinctive in that other green-to-red optical highlighters require more phototoxic ultraviolet or violet (405-nm) light. However, caution should be used when adjusting the laser for imaging Dendra at 488 nm to avoid unwanted photoconversion.

The unique green-to-red optical highlighters discovered thus far in reef coral proteins certainly warrant an aggressive effort focused on solving the problems associated with aggregation, as well as fine-tuning photoactivation requirements and imaging spectral profiles. By engineering a variant that matures rapidly at 37°C, the monomeric Eos protein, which features optical properties very similar to Kaede and KikGR, should be far more useful as an epitope tag due to the dramatically reduced tendency to form intermolecular associations between fusion proteins in vivo. In the future, by engineering optical highlighter proteins that shift photoconversion illumination wavelengths to the blue and green spectral regions, which are significantly less toxic to living cells, coupled with shifts of fluorescence emission to the yellow through far-red wavelength bands, the potential applications for Kaede, KikGR, and Eos derivative probes can be greatly expanded.

A useful non-*Aequorea* optical highlighter, the Kindling fluorescent protein (KFP1) has been developed from a nonfluorescent chromoprotein isolated in *Anemonia sulcata* (Labas et al., 2002; Chudakov et al., 2003), and is now commercially available (Evrogen). Kindling fluorescent protein does not exhibit emission until illuminated with green or yellow light in the region between 525 and 580 nm. Low-intensity light results in transient red fluorescence (kindling) with excitation and emission maxima at 580 and 600 nm, respectively, which slowly decays upon cessation of illumination as the protein relaxes back to its initial non-fluorescent state (exhibiting a half-life of ~50 to 60 sec). Irradiation with intense blue light quenches the kindled fluorescence immediately and completely, allowing tight control over fluorescent labeling. Note that both kindling with low-intensity green light and quenching of fluorescence by blue light are reversible processes for the wild-type protein. In contrast, high-intensity illumination or continued irradiation at moderate levels results in irreversible kindling with a fluorescence intensity ~30-fold greater than that of the nonactivated protein. Irreversibly kindled molecules do not lose their fluorescence and are not quenched by illumination with blue light. This feature allows for stable long-term highlighting of cells, tissues, and organelles similar to PA-GFP and other highlighter proteins. The major drawback of kindling protein is its tendency to aggregate into tetramers, which seriously affects the potential for use as a protein fusion tag without some degree of disturbance to normal biological processes. However, the kindling protein is an excellent candidate for bulk photolabeling and tracking of individual organelles and cells within a large population.

A new generation of specialized optical highlighters with reversible on-off switching capabilities was heralded by the introduction of Dronpa, a monomeric fluorescent protein derived from Pectiniidae, a family of stony reef

corals featuring several members that naturally emit faint fluorescence upon irradiation with ultraviolet light (Ando et al., 2004). Named after a fusion of the ninja term for vanishing (dron) and photoactivation (pa), Dronpa exhibits unusual photochromic behavior due to its ability to toggle fluorescence on and off by illumination with two different excitation wavelengths. Dronpa was engineered using both directed and random mutagenesis to generate a monomeric version of the wild-type oligomeric fluorescent protein having a major absorption maximum at 503 nm and a minor peak at 390 nm at neutral pH. The absorption peak at 503 nm is due to the deprotonated species of the protein, while the smaller peak at 390 nm arises from the protonated form. When irradiated at 488 nm, the fluorescence emission spectral profile of the deprotonated species has a maximum at 518 nm with a relatively high quantum yield of 0.85. In contrast, the protonated form of the protein is almost nonfluorescent. Photoswitching of Dronpa occurs by interconversion between the deprotonated and protonated forms of the optical highlighter (Habuchi et al., 2005). Upon intense irradiation at 488 nm, the protein population is very efficiently driven to the protonated species with a commitment decrease in fluorescence to produce a dim (off) state in which the 390-nm absorption peak predominates. The dim state is readily converted back to the original fluorescent (on) deprotonated state with minimal illumination at 405 nm.

Although photochromism (the ability to switch fluorescence on and off) has been observed in the wild-type and several yellow fluorescent protein variants of *Aequorea victoria* GFP at the single molecule level (Cinelli et al., 2001; McAnaney et al., 2005; Tinnefeld and Sauer, 2005), none have demonstrated this phenomenon when measured in bulk. In these studies, during several minutes of illumination at 488 nm, the molecules produced fluorescence for several seconds, followed by an equally short time interval without emission, followed again by resumption of emission. Termed blinking behavior (Dickson et al., 1997), this on-and-off switching sequence can be repeated a number of times before each green fluorescent protein molecule ultimately reaches a final long-lived nonfluorescent state upon the emission of $\sim 10^6$ photons. The bright state can be recovered by irradiation with violet light (405 nm) for a period of 5 min before re-initiating the sequence by illumination with a 488-nm laser. In this manner, many native green fluorescent protein derivatives display an optically induced switching effect in which each molecule is individually addressable. However, the ability of large Dronpa-tagged molecular ensembles (including organelles and entire cells) to collectively act as an information storage medium with the ability to write, erase, and read information nondestructively, and with high efficiency, is truly unique in the field of fluorescent proteins.

THE FUTURE OF FLUORESCENT PROTEINS

The current thrust of fluorescent protein development is centered on fine-tuning the current palette of blue to yellow fluorescent proteins derived from the *Aequorea victoria* jellyfish, while simultaneously developing monomeric fluorescent proteins emitting in the orange to far-red regions of the visible light spectrum. Progress toward these goals has been substantial, and it is not inconceivable that near-infrared emitting fluorescent proteins loom on the horizon. The latest efforts in jellyfish variants have resulted in new and improved monomeric probes for the cyan, green, and yellow region, while the search for a bright monomeric and fast-maturing red fluorescent protein has yielded a host of excellent candidates spanning the longer wavelengths. Continuing protein engineering of the existing fluorescent proteins coupled with new technologies, such as the application of unnatural amino acids and circular permutation, should further expand the color palette.

The complex interplay of biological roles for an ever-increasing number of fluorescent proteins derived from a wide variety of marine species is only beginning to be understood. Light-induced changes to the autocatalytic chromophores, including photoactivation and photoconversion, may serve as a highly evolved photo-protection mechanism to assist these organisms in the useful dissipation of high-energy sunlight, especially the damaging shorter wavelengths, via the absorption and subsequent fluorescence re-emission of longer and safer wavelengths. In many cases, these remarkable fluorescent proteins display very high photostability and dynamic photo-induced transformation properties, including spectral fine-tuning of donor-acceptor pairs (and even cascades) for resonance energy transfer. The large number of spectral variants already discovered, featuring emission profiles covering the entire visible spectrum, suggests

that the diverse optical and biochemical properties of these proteins will generate a host of new candidates as probes for biological investigations and ensure the continued development of unique genetically engineered fluorescent proteins.

As the development of optical highlighters continues, fluorescent proteins useful for optical marking should evolve towards brighter, monomeric derivatives with high contrast that can be easily photoconverted and display a wide spectrum of emission colors. For example, proteins capable of reversible photoactivation, red-to-green photoconversion, improved expression at elevated temperatures, and derivatives emitting in the far-red or near-infrared regions of the spectrum would be especially useful. Coupled with these advances, microscopes equipped to smoothly transition between illumination modes for fluorescence observation and regional marking will become commonplace in most cell biology laboratories. The combination of photoactivation and photoconversion with advanced fluorescence techniques, such as resonance energy transfer and multiphoton excitation, will enable the study of protein dynamics with greater precision and spatial resolution. Ultimately, these innovations have the potential to achieve significant advancements in the understanding of spatial and temporal dynamics in signal transduction systems.

LITERATURE CITED

Ai, H.-W., Henderson, J.N., Remington, S.J., and Campbell, R.E. 2006. Directed evolution of a monomeric, bright, and photostable version of *Clavularia* cyan fluorescent protein: structural characterization and applications in fluorescent imaging. *Biochem. J.* (in press) Doi: 10.1042/BJ20060874

Alkaabi, K.M., Yafea, A., and Ashraf, S.S. 2005. Effect of pH on thermal- and chemical-induced denaturation of GFP. *Appl. Biochem. Biotechnol.* 126:149-156.

Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. 2002. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99:12651-12656.

Ando, R., Mizuno, H., and Miyawaki, A. 2004. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science* 306:1370-1373.

Baird, G.S., Zacharias, D.A., and Tsien, R.Y. 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci. U. S.A.* 97:11984-11989.

Beddoe, T., Ling, M., Dove, S., Hoegh-Guldberg, O., Devenish, R.J., Prescott, M., and Rossjohn, J. 2003. The production, purification and crystallization of a pocilloporin pigment from a reef-forming coral. *Acta Crystallog. D: Biol. Crystallog.* 59:597-599.

Bevis, B.J. and Glick, B.S. 2002. Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). *Nat. Biotechnol.* 20:83-87.

Boeck, G. 2001. Current status of flow cytometry in cell and molecular biology. *Internatl. Rev. Cytol.* 204:239-298.

Bulina, M.E., Verkhusha, V.V., Staroverov, D.B., Chudakov, D.M., and Lukyanov, K.A. 2003. Heterooligomeric tagging diminishes nonspecific aggregation of target proteins fused with Anthozoa fluorescent proteins. *Biochem. J.* 371:109-114.

Bulina, M.E., Chudakov, D.M., Britanova, O.V., Yanushevich, Y.G., Staroverov, D.B., Chepurnykh, T.V., Merzlyak, E.M., Shkrob, M.A., Lukyanov, S., and Lukyanov, K.A. 2006. A genetically encoded photosensitizer. *Nat. Biotechnol.* 24:95-99.

Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., Zacharias, D.A., and Tsien, R.Y. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99:7877-7882.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802-805.

Chattoraj, M., King, B.A., Bublitz, G.U., and Boxer, S.G. 1996. Ultra-fast excited state dynamics in green fluorescent protein: Multiple states and proton transfer. *Proc. Natl. Acad. Sci. U.S.A.* 93:8362-8367.

Chudakov, D.M., Belousov, V.V., Zaraisky, A.G., Novoselov, V.V., Staroverov, D.B., Zorov, D.B., Lukyanov, S., and Lukyanov, K.A. 2003. Kindling fluorescent proteins for precise *in vivo* photolabeling. *Nat. Biotechnol.* 21:191-194.

Chudakov, D.M., Verkhusha, V.V., Staroverov, D.B., Souslova, E.A., Lukyanov, S., and Lukyanov, K.A. 2004. Photoswitchable cyan fluorescent protein for protein tracking. *Nat. Biotechnol.* 22:1435-1439.

Chudakov, D.M., Lukyanov, S., and Lukyanov, K.A. 2005. Fluorescent proteins as a toolkit for *in vivo* imaging. *Trends Biotechnol.* 23:605-613.

Cinelli, R.A.G., Pellegrini, V., Ferrari, A., Faraci, P., Nifosi, R., Tyagi, M., Giacca, M., and Beltram, F. 2001. Green fluorescent proteins as optically controllable elements in bioelectronics. *Appl. Phys. Lett.* 79:3353-3355.

Clontech, 2005. Living colors fluorescent protein vectors. *Clontechniques* 20:18-20.

Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G., and Ward, W.W. 1993. Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. *Biochemistry* 32:1212-1218.

Cormier, M.J. and Eckroade, C.B. 1962. Studies on the bioluminescence of *Renilla reniformis*. III. Some biochemical comparisons to other *Renilla* species and determinations of the spectral energy distributions. *Biochim. Biophys. Acta* 64:340-344.

Cornea, A. and Conn, P.M. 2002. Measurement of changes in fluorescence resonance energy transfer between gonadotropin-releasing hormone receptors in response to agonists. *Methods* 27:333-339.

Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A., and Tsien, R.Y. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:448-455.

Cubitt, A.H., Wollenweber, L.A., and Hein, R. 1999. Understanding structure-function relationships in the *Aequorea victoria* green fluorescent protein. *Methods Cell Biol.* 58:19-30.

Davenport, D. and Nichol, J.A.C. 1955. Luminescence in hydromedusae. *Proc. Royal Soc. London, Ser. B* 144:399-411.

Day, R.N. 2005. Imaging protein behavior inside the living cell. *Molec. Cell. Endocrin.* 230:1-6.

Delagrave, S., Hawtin, R., Silva, C., Yang, M., and Youvan, D. 1995. Red-shifted excitation mutants of the green fluorescent protein. *Nat. Biotechnol.* 13:151-154.

Dickson, R.M., Cubitt, A.B., Tsien, R.Y., and Moerner, W.E. 1997. On/off blinking and switching behavior of single molecules of green fluorescent protein. *Nature* 388:355-358.

Dixit, R. and Cyr, R. 2003. Cell damage and reactive oxygen species production induced by fluorescence microscopy: Effect on mitosis and guidelines for non-invasive fluorescence microscopy. *The Plant J.* 36:280-290.

Dixit, R., Cyr, R., and Gilroy, S. 2006. Using intrinsically fluorescent proteins for plant cell imaging. *The Plant J.* 45:599-615.

Durand, R.E. and Olive, P.L. 1982. Cytotoxicity, mutagenicity, and DNA damage by Hoechst 33342. *J. Histochem. Cytochem.* 30:111-116.

Edwards, A.M., Silva, E., Jofre, B., Becker, M.I., and De Ioannes, A.E. 1994. Visible light effects on tumoral cells in a culture medium enriched with tryptophan and riboflavin. *J. Photochem. Photobiol. B* 24:179-186.

Fradkov, A.F., Verkhusha, V.V., Staroverov, D.B., Bulina, M.E., Yanushevich, Y.G., Martynov, V.I., Lukyanov, S., and Lukyanov, K.A. 2002. Far-red fluorescent tag for protein labeling. *Biochem. J.* 368:17-21.

Greenbaum, L., Rothmann, C., Lavie, R., and Malik, Z. 2000. Green fluorescent protein photobleaching: A model for protein damage by endogenous and exogenous singlet oxygen. *Biol. Chem.* 381:1251-1258.

Griesbeck, O., Baird, G.S., Campbell, R.E., Zacharias, D.A., and Tsien, R.Y. 2001. Reducing the environmental sensitivity of yellow fluorescent protein: Mechanism and applications *J. Biol. Chem.* 276:29188-29194.

Gross, L.A., Baird, G.S., Hoffman, R.C., Baldridge, K.K., and Tsien, R.Y. 2000. The structure of the chromophore within DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci. U.S.A.* 97:11990-11995.

Gurskaya, N.G., Fradkov, A.F., Terskikh, A., Matz, M.V., Labas, Y.A., Martynov, V.I., Yanushevich, Y.G., Lukyanov, K.A., and Lukyanov, S.A. 2001a. GFP-like chromoproteins as a source of far-red fluorescent proteins. *FEBS Lett.* 507:16-20.

Gurskaya, N.G., Savitsky, A.P., Yanushevich, Y.G., Lukyanov, S.A., and Lukyanov, K.A. 2001b. Color transitions in coral's fluorescent proteins by site-directed mutagenesis. *BMC Biochem.*, 2:6-12.

Gurskaya, N.G., Fradkov, A.F., Pounkova, N.I., Staroverov, D.B., Bulina, M.E., Yanushevich, Y.G., Labas, Y.A., Lukyanov, S., and Lukyanov, K.A. 2003. A colorless green fluorescent protein homologue from the non-fluorescent hydromedusa *Aequorea coerulescens* and its fluorescent mutants. *Biochem. J.* 373:403-408.

Gurskaya, N.G., Verkhusha, V.V., Shcheglov, A.S., Staroverov, D.B., Chepurnykh, T.V., Fradkov, A.F., Lukyanov, S., and Lukyanov, K.A. 2006. Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. *Nat. Biotechnol.* 24:461-465.

Habuchi, S., Ando, R., Dedecker, P., Verheijen, W., Mizuno, H., Miyawaki, A., and Hofkens, J. 2005. Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa. *Proc. Natl. Acad. Sci. U.S.A.* 102:9511-9516.

Heim, R. and Tsien, R.Y. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* 6:178-182.

Heim, R., Prasher, D.C., and Tsien, R.Y. 1994. Wavelength mutations and post-translational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 91:12501-12504.

Heim, R., Cubitt, A.B., and Tsien, R.Y. 1995. Improved green fluorescence. *Nature* 373:663-664.

Ip., D.T.M., Chan, S.H., Allen, M.D., Bycroft, M., Wan, D.C.C., and Wong, K.B. 2004. Crystallization and preliminary crystallographic analysis of a novel orange fluorescent protein from the *Cnidaria* tube anemone *Cerianthus* sp. *Acta Crystallog. D: Biol. Crystallog.* D60:340-341.

Ivanchenko, S., Roecker, C., Oswald, F., Wiedenmann, J., and Nienhaus, G.U. 2005. Targeted green-red photoconversion of EosFP, a fluorescent marker protein. *J. Biol. Phys.* 31:249-259.

Jayaraman, S., Haggie, P., Wachter, R.M., Remington, S.J., and Verkman, A.S. 2000. Mechanism and cellular applications of green fluorescent protein-based halide sensor. *J. Biol. Chem.* 275:6047-6050.

Jung, G., Wiehler, J., and Zumbusch, A. 2005. The photophysics of green fluorescent protein: Influence of the key amino acids at positions 65, 203, and 222. *Biophys. J.* 88:1932-1947.

Kajihara, D., Hohsaka, T., and Sisido, M. 2005. Synthesis and sequence optimization of GFP mutants containing aromatic non-natural amino acids at the Tyr66 position. *Prot. Engineer. Design Select.* 18:273-278.

Karasawa, S., Araki, T., Yamamoto-Hino, M., and Miyawaki, A. 2003. A green-emitting fluorescent protein from *Galaxeidae* coral and its monomeric version for use in fluorescent labeling. *J. Biol. Chem.* 278:34167-34171.

Karasawa, S., Araki, T., Nagi, T., Mizuno, H., and Miyawaki, A. 2004. Cyan-emitting and orange-emitting fluorescent proteins as a donor/acceptor pair for fluorescence resonance energy transfer. *Biochem. J.* 381:307-312.

Khodjakov, A. and Rieder, C.L. 2006. Imaging the division process in living tissue culture cells. *Methods* 38:2-16.

Kuner, T. and Augustine, G.J. 2000. A genetically encoded ratiometric neurotechnique indicator for chloride: Capturing chloride transients in cultured hippocampal neurons. *Neuron* 27:447-459.

Labas, Y.A., Gurskaya, N.G., Yanushevich, Y.G., Fradkov, A.F., Lukyanov, K.A., Lukyanov, S.A., and Matz, M.V. 2002. Diversity and evolution of the green fluorescent protein family. *Proc. Natl. Acad. Sci. U.S.A.* 99:4256-4261.

Lauf, U., Lopez, P., and Falk, M.M. 2001. Expression of fluorescently tagged connexins: A novel approach to rescue function of oligomeric DsRed-tagged proteins. *FEBS Lett.* 498:11-15.

Lippincott-Schwartz, J. and Patterson, G.H. 2003. Development and use of fluorescent markers in living cells. *Science* 300:87-91.

Lippincott-Schwartz, J., Altan-Bonnet, N., and Patterson, G.H. 2003. Photobleaching and photoactivation: Following protein dynamics in living cells. *Nat. Cell Biol.* 5:S7-S14.

Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G., and Tsien, R.Y. 1998. Measurement of cytosolic, mitochondria, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. U.S.A.* 95:6803-6808.

Llopis, J., Westin, S., Ricote, M., Wang, J., Cho, C.Y., Kurokwa, R., Mullen, T.M., Rose, D.W., Rosenfeld, M.G., Tsien, R.Y., and Glass, C.K. 2000. Ligand-dependent interactions of coactivators steroid receptor coactivator-1 and peroxisome proliferator-activated receptor binding protein with nuclear hormone receptors can be imaged in live cells and are required for transcription. *Proc. Natl. Acad. Sci. U.S.A.* 97:4363-4368.

Lucius, R., Mentlein, R., and Sievers, J. 1998. Riboflavin-mediated axonal degeneration of postnatal retinal ganglion cells *in vitro* is related to the formation of free radicals. *Free Rad. Biol. Med.* 24:798-808.

Lukyanov, K.A., Fradkov, A.F., Gurskaya, N.G., Matz, M.V., Labas, Y.A., Savitsky, A.P., Markelov, M.L., Zaraisky, A.G., Zhao, X., Fang, Y., Tan, W., and Lukyanov, S. A. 2000. Natural animal coloration can be determined by a non-fluorescent green fluorescent protein homolog. *J. Biol. Chem.* 275:25879-25882.

Lukyanov, K.A., Chudakov, D.M., Lukyanov, S., and Verkhusha, V.V. 2005. Innovation: Photoactivatable fluorescent proteins. *Nat. Rev. Molec. Cell Biol.* 6:885-891.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., and Lukyanov, S.A. 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* 17:969-973.

McAnaney, T. B., Zeng, W., Doe, C.F.E., Bhanji, N., Wakelin, S., Pearson, D.S., Abbyad, P., Shi, X., Boxer, S.G., and Bagshaw, C.R. 2005. Protonation, photobleaching, and photoactivation of yellow fluorescent protein (YFP 10C): A unifying mechanism. *Biochemistry* 44:5510-5524.

Meyer, T. and Teruel, M.N. 2003. Fluorescence imaging of signaling networks. *Trends Cell Biol.* 13:101-106.

Minamikawa, T., Sriratana, A., Williams, D.A., Bowser, D.N., Hill, J.S., and Nagley, P. 1999. Chloromethyl-X-rosamine (MitoTracker Red) photosensitizes mitochondria and induces apoptosis in intact human cells. *J. Cell Sci.* 112:2419-2430.

Mitra, R.D., Silva, C.M., and Youvan, D.C. 1996. Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein. *Gene* 173:13-17.

Miyawaki, A. 2002. Green fluorescent protein-like proteins in reef Anthozoa animals. *Cell Struct. Funct.* 27:343-347.

Miyawaki, A. 2003. Fluorescence imaging of physiological activity in complex systems using GFP-based probes. *Curr. Opin. Neurobiol.* 13:591-596.

Miyawaki, A. 2004. Fluorescent proteins in a new light *Nat. Biotechnol.* 22:1374-1376.

Miyawaki, A. 2005. Innovations in the imaging of brain functions using fluorescent proteins. *Neuron* 48:189-199.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. 1997. Fluorescent indicators for calcium based on green fluorescent proteins and calmodulin. *Nature* 388:882-887.

Miyawaki, A., Griesbeck, O., Heim, R., and Tsien, R.Y. 1999. Dyanmic and quantitative calcium measurements using improved cameleons. *Proc. Natl. Acad. Sci. U.S.A.* 96:2135-2140.

Miyawaki, A., Nagai, T., and Mizuno, H. 2005. Engineering fluorescent proteins. *Adv. Biochem. Engineer. Biotechnol.* 95:1-15.

Mizuno, H., Swano, A., Eli, P., Hama, H., and Miyawaki, A. 2001. Red fluorescent protein from *Discosoma* as a fusion tag and a partner for fluorescence resonance energy transfer. *Biochemistry* 40:2502-2510.

Mizuno, H., Mal, T.K., Tong, K.I., Ando, R., Furuta, T., Ikura, M., and Miyawaki, A. 2003.

Photo-induced peptide cleavage in the green-to-red conversion of a fluorescent protein. *Molec. Cell* 12:1051-1058.

Morin, J.G. and Hastings, J.W. 1971a. Energy transfer in a bioluminescent system. *J. Cell. Physiol.* 77:313-318.

Morin, J.G. and Hastings, J.W. 1971b. Biochemistry of the bioluminescence of colonial hydroids and other coelenterates. *J. Cell. Physiol.* 77:305-312.

Nagai, T. and Miyawaki, A. 2004. A high-throughput method for development of FRET-based indicators for proteolysis. *Biochem. Biophys. Res. Commun.* 319:72-77.

Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., and Miyawaki, A. 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* 20:87-90.

Nguyen, A.W. and Daugherty, P.S. 2005. Evolutionary optimization of fluorescent proteins for intracellular FRET. *Nat. Biotechnol.* 23:355-360.

Nienhaus, K., Nienhaus, G.U., Wiedenmann, J., and Nar, H. 2005. Structural basis for photo-induced protein cleavage and green to red conversion of fluorescent protein EosFP. *Proc. Natl. Acad. Sci. U.S.A.* 102:9156-9159.

Ormö, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y., and Remington, S.J. 1996. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* 273:1392-1395.

Pakhomov, A.A., Martynova, N.Y., Gurskaya, N.G., Balashova, T.A., and Martynov, V.I. 2004. Photoconversion of the chromophore of a fluorescent protein from *Dendronephthya* sp. *Biochemistry (Moscow)* 69:901-908.

Patterson, G.H. 2004. A new harvest of fluorescent proteins. *Nat. Biotechnol.* 22:1524-1525.

Patterson, G.H. and Lippincott-Schwartz, J. 2002a. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297:1873-1877.

Patterson, G.H. and Lippincott-Schwartz, J. 2002b. Selective photolabeling of proteins using photoactivatable GFP. *Methods* 32:445-450.

Patterson, G.H., Knobel, S.M., Sharif, W.D., Kain, S.R., and Piston, D.W. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73:2782-2790.

Patterson, G.H., Piston, D.W., and Barisas, B.G. 2000. Förster distances between green fluorescent protein pairs. *Anal. Biochem.* 284:438-440.

Patterson, G.H., Day, R.N., and Piston, D.W. 2001. Fluorescent protein spectra. *J. Cell Sci.* 114:837-838.

Periasamy, A. and Day, R.N. 1998. FRET imaging of Pit-1 protein interactions in living cells. *J. Biomed. Optics* 3:154-160.

Peterson, J., Wilmann, P.G., Beddoe, T., Oakley, A.J., Devenish, R.J., Prescott, M., and Rossjohn, J. 2003. The 2.0-~crystal structure of eqFP611, a far red fluorescent protein from the sea anemone *Entacmaea quadricolor*. *J. Biol. Chem.* 278:44626-44631.

Phillips, G.N. 2006. The three-dimensional structure of green fluorescent protein and its implications for function and design. *In* Green Fluorescent Protein: Properties, Applications, and Protocols, 2nd ed. (M. Chalfie and S.R. Kain, eds.) pp. 67-82. John Wiley & Sons, Hoboken, N.J.

Potter, S.M. 1996. Vital imaging: Two photons are better than one. *Curr. Biol.* 6:1595-1598.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111:229-233.

Prescott, M., Battad, J.M., Wilmann, P.G., Rossjohn, J., and Devenish, R.J. 2006. Recent advances in all-protein chromophore technology. *Biotechnol. Annu. Rev.* 12:31-66.

Rajfur, Z., Roy, P., Otey, C., Romer, L., and Jacobson, K. 2002. Dissecting the link between stress fibers and focal adhesions by CALI with EGFP fusion proteins. *Nat. Cell Biol.* 4:286-293.

Rehm, M., Duessmann, H., Jaenicke, R.U., Tavare, J. M., Koegel, D., and Prehn, J.H.M. 2002. Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. *J. Biol. Chem.* 277:24506-24514.

Remington, S.J. 2006. Fluorescent proteins: maturation, photochemistry, and photophysics. *Curr. Opin. Struct. Biol.* 16 (in press) doi: 10.1016/j.sbi.2006.10.001

Remington, S.J., Wachter, R.M., Yarbrough, D.K., Branchaud, B., Anderson, D.C., Kallio, K., and Lukyanov, K.A. 2005. zFP538, a yellow-fluorescent protein from *Zoanthus*, contains a novel three-ring chromophore. *Biochemistry* 44:202-212.

Richards, B., Zharkikh, L., Hsu, F., Dunn, C., Kamb, A., and Teng, D.H. 2002. Stable expression of Anthozoa fluorescent proteins in mammalian cells. *Cytometry* 48:106-112.

Rizzo, M.A. and Piston, D.W. 2005a. Fluorescent protein tracking and detection. *In* Live Cell Imaging: A Laboratory Manual (R.D. Goldman andD.L. Spector, eds.) pp. 3-23. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Rizzo, M.A. and Piston, D.W. 2005b. High-contrast imaging of fluorescent protein FRET by fluorescence polarization microscopy. *Biophys. J.* 88:L14-L16.

Rizzo, M.A., Magnuson, M.A., Drain, P.F., and Piston, D.W. 2002. A functional link between glucokinase binding to insulin granules and conformational alterations in response to glucose and insulin. *J. Biol. Chem.* 277:34168-34175.

Rizzo, M.A., Springer, G.H., Granada, B., and Piston, D.W. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol.* 22:445-449.

Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Hein, R., Tsien, R.Y., and Pozzan, T. 1996. Double labeling of subcellular structures with organelle-targeted GFP mutants in vivo. *Curr. Biol.* 6:183-188.

Romoser, V.A., Hinkle, P.M., and Persechini, A. 1997. Detection in living cells of calcium-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. *J. Biol. Chem.* 272:13270-13274.

Salih, A., Larkum, A., Cox, G., Kuhl, M., and Hoegh-Buldberg, O. 2000. Fluorescent pigments in corals are photoprotective. *Nature* 408:850-853.

Schenk, A., Ivanchenko, S., Roecker, C., Wiedenmann, J., and Nienhaus, G.U. 2004. Photodynamics of red fluorescent proteins studied by fluorescence correlation spectroscopy. *Biophys. J.* 86:384-394.

Shagin, D.A., Barsova, E.V., Yanushevich, Y.G., Fradkov, A.F., Lukyanov, K.A., Labas, Y.A., Semenova, T.N., Ugalde, J.A., Meyers, A., Nunez, J.M., Widder, E.A., Lukyanov, S.A., and Matz, M.V. 2004. GFP-like proteins as ubiquitous metazoan superfamily: Evolution of functional features and structural complexity. *Molec. Biol. Evol.* 21:841-850.

Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N.G., Palmer, A.E., and Tsien, R.Y. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* 22:1567-1572.

Shaner, N.C., Steinbach, P.A., and Tsien, R.Y. 2005. A guide to choosing fluorescent proteins. *Nat. Methods.* 2:905-909.

Sheff, M.A. and Thorn, K.S. 2004. Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast* 21:661-670.

Sheridan, D.L. and Hughes, T.E. 2004. A faster way to make GFP-based biosensors: Two new transposons for creating multicolored libraries of fluorescent fusion proteins. *BMC Biotechnol.* 4:1-17.

Shimomura, O., Johnson, F.H., and Saiga, Y. 1962. Extraction, purification, and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J. Cell. Comp. Physiol.* 59:223-239.

Shkrob, M.A., Yanushevich, Y.G., Chudakov, D.M., Gurskaya, N.G., Labas, Y.A., Poponov, S.Y., Mudrik, N.N., Lukyanov, S., and Lukyanov, K.A. 2005. Far-red fluorescent proteins evolved from a blue chromoprotein from *Actinia equine*. *Biochem. J.* 392:649-654.

Shu, X., Shaner, N.C., Yarbrough, C.A., Tsien, R.Y., and Remington, S.J. 2006. Novel chromophores and buried charges control color in mFruits. *Biochemistry* 45:9639–9647.

Shyu, Y.J., Liu, H., Deng, X., and Hu, C.D. 2006. Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions. *BioTechniques* 40:61-66.

Silva, E., Salim-Hanna, M., Edwards, A.M., Becker, M.I., and De Ioannes, AE. 1991. A light-induced tryptophan-riboflavin binding: Biological implications. *Adv. Exp. Med. Biol.* 289:33-48.

Spierenburg, G.T., Oerlemans, F.T., van Laarhoven, J.P., and de Bruyn, C.H. 1984. Phototoxicity of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-buffered culture media for human leukemic cell lines. *Cancer Res.* 44:2253-2254.

Stephens, D.J. and Allan, V.J. 2003. Light microscopy techniques for live cell imaging. *Science* 300:82-86.

Sturman, D.A., Shakiryanova, D., Hewes, R., Deitcher, D., and Levitan, E.S. 2006. Nearly neutral secretory vesicles in *Drosophila* nerve terminals. *Biophys. J.* 90:L45-L47.

Swedlow, J.R., Hu, K., Andrews, P.K., Roos, D.S., and Murray, J.M. 2002. Measuring tubulin content in Toxoplasma gondii: A comparison of laser-scanning confocal and wide-field fluorescence microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 99:2014-2019.

Tavare, J.M., Fletcher, L.M., and Welsh, G.I. 2001. Using green fluorescent protein to study intracellular signalling. *J. Endocrinol.* 70:297-306.

Ting, A.Y., Kain, K.H., Klemke, R.L., and Tsien, R.Y. 2001. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 98:15003-15008.

Tinnefeld, P. and Sauer, M. 2005. Branching out of single-molecule fluorescence spectroscopy: Challenges for chemistry and influence on biology. *Angewandte Chemie* 44:2642-2671.

Tour, O., Meijer, R.M., Zacharias, D.A., Adams, S.R., and Tsien, R.Y. 2003. Genetically targeted chromophore-assisted light inactivation. *Nat. Biotechnol.* 21:1505-1508.

Tsien, R.Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem.* 67:509-544.

Tsien, R.Y. 2005. Building and breeding molecules to spy on cells and tumors *FEBS Lett.* 579:927-932.

Tsuboi, T. and Rutter, G.A. 2003. Insulin secretion by "kiss-and-run" exocytosis in clonal pancreatic islet *beta*-cells. *Biochem. Soc. Trans.* 31:833-836.

Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N., and Miyawaki, A. 2005. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Reports* 6:233-238.

Verkhusha, V.V. and Lukyanov, K.A. 2004. The molecular properties and applications of Anthozoa fluorescent proteins and chromoproteins. *Nat. Biotechnol.* 22:289-296.

Verkhusha, V.V. and Sorkin, A. 2005. Conversion of the monomeric red fluorescent protein into a photoactivatable probe. *Chem. Biol.* 12:279-285.

Vrzheshch, P.V., Akovbian, N.A., Varfolomeyev, S.D., and Verkhusha, V.V. 2000. Denaturation and partial renaturation of a tightly tetramerized DsRed protein under mildly acidic conditions. *FEBS Lett*. 487:203-208.

Wachter, R.M., Elsliger, M.A., Kallio, K., Hanson, G.T., and Remington, S.J. 1998. Structural basis of spectral shifts in the yellow-emission variants of green fluorescent protein. *Structure* 6:1267-1277.

Wallrabe, H. and Periasamy, A. 2005. Imaging protein molecules using FRET and FLIM microscopy. *Curr. Opin. Biotechnol.* 16:19-27.

Wang, L., Jackson, W.C., Steinbach, P.A., and Tsien, R.Y. 2004. Evolution of new nonantibody proteins via iterative somatic hypermutation. *Proc. Natl. Acad. Sci. U.S.A.* 101:16745-16749.

Ward, W.W. 2006. Biochemical and physical properties of green fluorescent protein. *In* Green Fluorescent Protein: Properties, Applications, and Protocols, 2nd ed. (M. Chalfie and S.R. Kain, eds.) pp. 39-65. John Wiley & Sons, Hoboken, N.J..

Ward, W.W. and Cormier, M.J. 1979. An energy transfer protein in coelenterate bioluminescence: Characterization of the *Renilla* green-fluorescent protein (GFP), *J. Biol. Chem.* 254:781-788.

Wiedenmann, J., Schenk, A., Roecker, C., Girod, A., Spindler, K.D., and Nienhaus, G.U. 2002. A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from *Entacmaea quadricolor* (Anthozoa, Actinaria). *Proc. Natl. Acad. Sci. U.S.A.* 99:11646-11651.

Wiedenmann, J., Ivanchenko, S., Oswald, F., Schmitt, F., Rocker, C., Salih, A., Spindler, K., and Nienhaus, G.U. 2004. EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proc. Natl. Acad. Sci. U.S.A.* 101:15905-15910.

Wiedenmann, J., Vallone, B., Renzi, F., Nienhaus, K., Ivanchenko, S., Roecker, C., and Nienhaus, G.U. 2005. Red fluorescent protein eqFP611 and its genetically engineered dimeric variants. *J. Biomed. Optics* 10:1-7.

Wiehler, J., von Hummel, J., and Steipe, B. 2001. Mutants of *Discosoma* red fluorescent protein with a GFP-like chromophore. *FEBS Lett.* 487:384-389.

Yang, T.T., Sinai, P., Green, G., Kitts, P.A., Chen, Y.T., Lybarger, L., Cervenak, R., Patterson, G.H., Piston, D.W., and Kain, S.R. 1998. Improved fluorescence and dual color detection with enhanced blue and green variants of the green fluorescent protein. *J. Biol. Chem.* 273:8212-8216.

Yanushevich, Y.G., Staroverov, D.B., Savitsky, A.P., Fradkov, A.F., Gurskaya, N.G., Bulina, M.E., Lukyanov, K.A., and Lukyanov, S.A. 2002. A strategy for the generation of non-aggregating mutants of Anthozoa fluorescent proteins. *FEBS Lett.* 511:11-14.

Yarbrough, D., Wachter, R.M., Kallio, K., Matz, M.V., and Remington, S.J. 2001. Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0 ~resolution. *Proc. Natl. Acad. Sci. U.S.A.* 98:462-467.

Zaccolo, M. 2004. Use of chimeric fluorescent proteins and fluorescence resonance energy transfer to monitor cellular responses. *Circ. Res.* 94:866-873.

Zacharias, D.A. 2002. Sticky caveats in an otherwise glowing report: Oligomerizing fluorescent proteins and their use in cell biology. *Science STKE* 131:pe23.

Zacharias, D.A. and Tsien, R.Y. 2006. Molecular biology and mutation of green fluorescent protein. *In* Green Fluorescent Protein: Properties, Applications, and Protocols, 2nd ed. (M. Chalfie and S. R. Kain, eds.) pp. 83-120. John Wiley & Sons, Hoboken, N.J.

Zacharias, D.A., Violin, J.D., Newton, A.C., and Tsien, R.Y. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* 296:913-916.

Zagranichny, V.E., Rudenko, N.V., Gorokhovatsky, A.Y., Zakharov, M.V., Shenkarev, Z.O., Balashova, T.A., and Arseniev, A.S. 2004. zFP538, a yellow fluorescent protein from coral, belongs to the DsRed subfamily of GFP-like proteins but possesses the unexpected site of fragmentation. *Biochemistry* 43:4764-4772.

Zapata-Hommer, O. and Griesbeck, O. 2003. Efficiently folding and circular permuted variants of the Sapphire mutant of GFP. *BMC Biotechnol.* 3:5-11.

Zhang, J., Ma, Y., Taylor, S.S., and Tsien, R.Y. 2001. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc. Natl. Acad. Sci. U.S.A.* 98:14997-15002.

Zhang, J., Campbell, R.E., Ting, A.Y., and Tsien, R.Y. 2002. Creating new fluorescent probes for cell biology. *Nat. Rev. Molec. Cell Biol.* 3:906-918.

Zimmer, M. 2002. Green fluorescent protein (GFP): Applications, structure, and related photophysical behavior. *Chem. Rev.* 102:759-781.

Zumbusch, A. and Jung, G. 2000. Single molecule spectroscopy of the green fluorescent protein: A critical assessment. *Single Molecules* 1:261-270.

Contributed by Scott G. Olenych, Nathan S. Claxton, Gregory K. Ottenberg, and Michael W. Davidson
Florida State University
Tallahassee, Florida

Photoactivation and Imaging of Photoactivatable Fluorescent Proteins

George H. Patterson[1]

[1]Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

ABSTRACT

A major advance in the microscopic study of cells and tissues is the introduction of photoactivatable fluorescent proteins, which can specifically mark proteins of interest within a living cell. Fluorescent proteins are now available that allow a pool of molecules to be "turned on" by photoactivation. This unit discusses technical aspects for the general use of photoactivatable fluorescent proteins and introduces some specific applications in the concluding remarks. *Curr. Protoc. Cell Biol.* 38:21.6.1-21.6.10. © 2008 by John Wiley & Sons, Inc.

Keywords: photoactivatable • fluorescent protein • microscopy

INTRODUCTION

Cell biologists rely on the many advances in microscopy methods to study cells and tissues. A major advance is the introduction of photoactivatable fluorescent proteins (PA-FPs), which can specifically mark proteins of interest within a living cell. Fluorescent proteins are now available which allow a pool of molecules to be "turned on" by photoactivation. This unit discusses technical aspects for the general use of photoactivatable fluorescent proteins and introduces some specific applications in the concluding remarks.

BACKGROUND

Photoactivatable fluorescent proteins differ from normal fluorescent proteins in that they exhibit little or no fluorescence at certain excitation and emission wavelengths prior to photoactivation. However, they do retain many of the usual advantages associated with fluorescent proteins. The term "certain wavelengths" is used to avoid confusion about some PA-FPs that are highly fluorescent in their native inactivated states; this fluorescence arises from a different spectral region than that of the protein in its photoactivated state. Proteins from several marine species have been reported, along with several variants and approaches for their use as photoactivatable fluorescent proteins (Lukyanov et al., 2005; Table 21.6.1). For the purpose of a photoactivation protocol, these molecules are categorized in this unit on the basis of their spectral characteristics. Descriptions of their other characteristics that might be of interest for other types of experiments, as well as evidence for the mechanism for photoconversion, are discussed elsewhere (Lukyanov et al., 2005).

Photoactivatable Green Fluorescent Proteins

One of the first PA-FPs resulted from a photo-induced conversion (photoconversion) within the wild-type (wt) green fluorescent protein (GFP; Yokoe and Meyer, 1996). The wt GFP chromophore population exists as neutral phenols when Y66 within the chromophore is protonated and as anionic phenolates when Y66 is deprotonated, and these different populations produce a major absorbance peak at ~397 nm and a minor absorbance peak at 475 nm, respectively. After irradiation at ~400 nm, the absorbance at 475 nm increases, and this leads to an increase in the fluorescence upon excitation at 475 nm. This phenomenon was utilized in developing a GFP mutant with decreased absorbance at 488 nm (compared to wt GEP) that could also undergo photoconversion when irradiated at ~400 nm (Patterson and Lippincott-Schwartz, 2002). The first of these mutants was T203I, which has a mostly neutral phenol chromophore population giving a major absorbance peak at ~400 nm and little absorbance at the common 488-nm laser line used for excitation (Heim et al., 1994; Ehrig et al., 1995). Substitution of the threonine at position 203 on the protein lead to the T203H mutant, finally named PA-GFP (Fig. 21.6.1A), which gave the

Current Protocols in Cell Biology 21.6.1-21.6.10, March 2008
Published online March 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2106s38

Table 21.6.1 Selected Photoactivatable Fluorescent Proteins

Protein	Activation[a] wavelength (nm)	State[b]	Excitation wavelength (nm)	Emission wavelength (nm)	Reference
PA-GFP	405	Pre Post	400 504	515 517	Patterson and Lippincott-Schwartz, 2002
PS-CFP	405	Pre Post	402 490	468 511	Chudakov et al., 2004
PS-CFP2	405	Pre Post	400 490	470 511	See supplier's Web site
Dronpa[c]	490, 405	Pre	503	518	Ando et al., 2004
Dendra	405 or 488	Pre Post	486 558	505 575	Gurskaya et al., 2006
Dendra2	405 or 488	Pre Post	486 558	505 575	See supplier's Web site
Kaede	405	Pre Post	508 572	518 582	Ando et al., 2002
Kikume Green-Red (KikGR)	405	Pre Post	507 583	517 593	Tsutsui et al., 2005
EosFP	405	Pre Post	506 571	516 581	Wiedenmann et al., 2004
d1EosFP	405	Pre Post	505 571	516 581	Wiedenmann et al., 2004
d2EosFP	405	Pre Post	506 569	516 581	Wiedenmann et al., 2004
mEosFP	405	Pre Post	505 569	516 581	Wiedenmann et al., 2004
KFP1	532	Post	580	600	Chudakov et al., 2003
PAmRFP1-1	405	Post	578	605	Verkhusha and Sorkin, 2005
PAmRFP1-2	405	Post	578	605	Verkhusha and Sorkin, 2005
PAmRFP1-3	405	Post	578	605	Verkhusha and Sorkin, 2005

[a]The wavelength range for activation can be broad for each protein. The wavelengths listed here are suggestions based on commonly available laser lines.

[b]The wavelengths to the right of Pre represent the major peaks prior to photoactivation; The wavelengths to the right of Post represent the major peaks after photoactivation; absence of Pre wavelengths indicates that the protein is not initially fluorescent (values not published).

[c]Dronpa displays green fluorescence with excitation at 503 nm and emission at 518 nm. Excitation at 490 nm results in loss of absorbance at 503 nm. Upon irradiation at 400 nm, green fluorescence is restored.

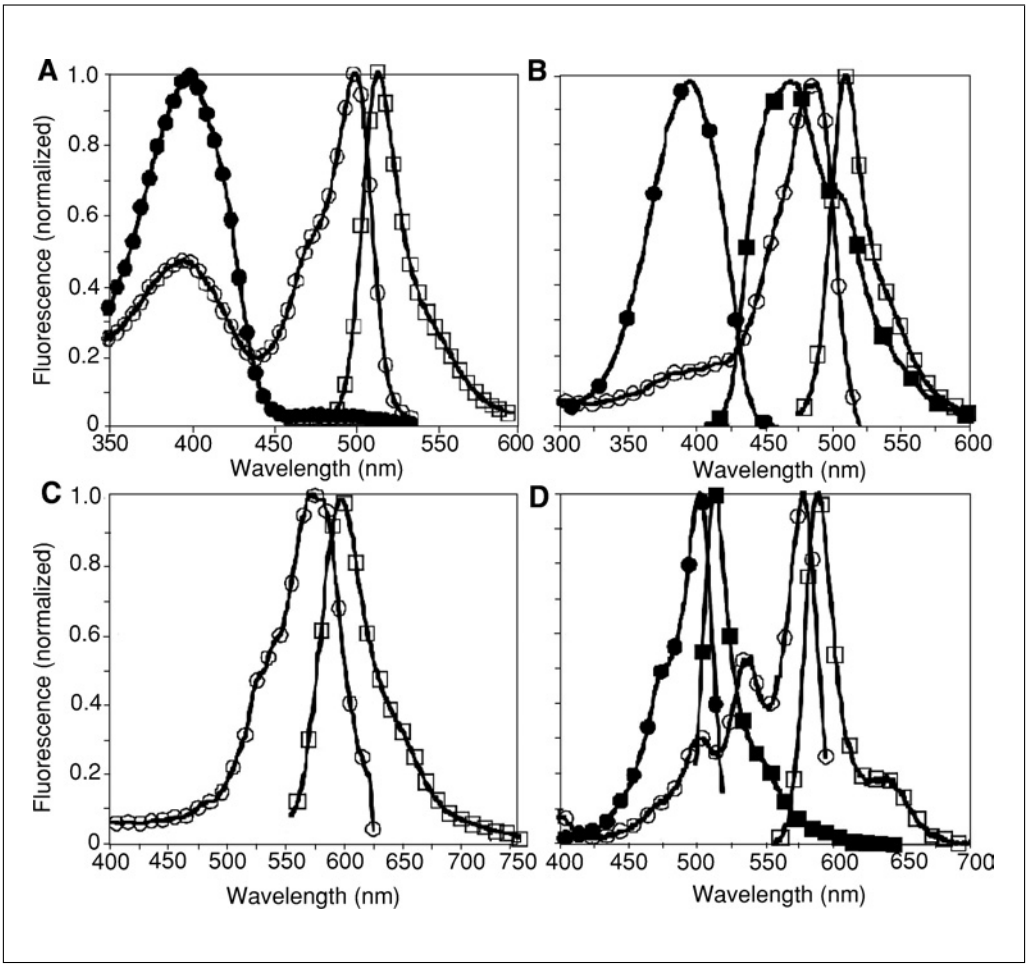


Figure 21.6.1 Fluorescence excitation and emission spectra of selected photoactivatable fluorescent proteins show the diversity that is now available from this class of fluorescent proteins. (**A**) The excitation spectra for PA-GFP are shown prior to (closed circles) and after (open circles) photoactivation. The emission spectrum after photoactivation is shown in open squares. (**B**) The excitation spectra for PS-CFP2 are shown prior to (closed circles) and after (open circles) photoactivation. The emission spectra for PS-CFP2 are shown prior (closed squares) to photoactivation and after (open squares) photoactivation. (**C**) The excitation spectra (open circles) and emission spectrum (open squares) are shown for photoactivated KFP1. (**D**) The excitation spectra for KikGR are shown prior to (closed circles) and after (open circles) photoactivation. The emission spectra for KikGR are shown prior to (closed squares) and after (open squares) photoactivation.

highest fluorescence emission contrast after photoactivation compared to nonphotoactivated protein (Patterson and Lippincott-Schwartz, 2002).

Dronpa is another green PA-FP; it is derived from *Pectimiidae* (Ando et al., 2004). Its spectra resemble those of PA-GFP (Fig. 21.6.1A), except that it has a much higher extinction coefficient and the photoactivation is reversible. Initially, Dronpa displays green fluorescence with excitation at 503 nm and emission at 518 nm. Excitation at 490 nm (0.4 W/cm^2) results in loss of the absorbance at 503 nm and an increase at ~390 nm. However, after irradiation at 400 nm (0.14 W/cm^2), 503-nm absorbance increases and the green fluorescence is restored.

Photoswitchable Green Fluorescent Proteins: Cyan-to-Green

Contrary to the previous examples, photoswitchable-cyan fluorescent protein (PS-CFP) displays a shift of both absorbance and emission from cyan to green fluorescent protein (Chudakov et al., 2004; Fig. 21.6.1B). It exhibits initial excitation at 402 nm and emission at 468 nm (Chudakov et al., 2004) which shift to 490 nm and 511 nm, respectively, and give ~1500-fold increase in the green-to-cyan

fluorescence ratio upon photoactivation at ~400 nm.

Photoactivatable Red Fluorescent Proteins

The monomeric red fluorescent protein, mRFP1 (Campbell et al., 2002), was converted into a series of photoactivatable fluorescent proteins, PAmRFP1-1, PAmRFP1-2, and PAmRFP1-3 by Verkhusha and Sorkin (2005). The brightest, PAmRFP1-1, has little fluorescence before activation and gives ~70-fold increase in red fluorescence upon ~400-nm irradiation (Verkhusha and Sorkin, 2005).

Less well known is asFP595 (emission λ_{max} = 595 nm), a protein isolated from the sea anemone, *Anemonia sulcata*. Its red fluorescence can be enhanced by exposure to green light and quenched by exposure to blue light (Lukyanov et al., 2000). Kindling fluorescent protein (KFP1) is an improved variant of asFP595 (Chudakov et al., 2003). Activation with 532-nm laser light increases its red fluorescence ~30-fold, and it can revert to the inactive state or be stabilized in the photoactivated state, depending on the intensity of the photoactivation light (see Fig. 21.6.1C).

Photoactivatable Green-to-Red Fluorescent Proteins

Many of the naturally occurring and engineered photoactivatable fluorescent proteins exhibit a spectral shift from a green fluorescent protein into a red fluorescent protein (see Fig. 21.6.1D). The first of these to be discovered is Kaede, from a stony coral, *Trachyphyllia geoffroyi* (Ando et al., 2002). Kaede absorbs maximally at 508 nm and emits at 518 nm, is photoactivated by irradiation at ~400 nm, and then exhibits absorbance at 572 nm and emission at 582 nm afterward. Since both the excitation and emission peaks are shifted, ratio imaging results in a >2000 fold increase in the red-to-green ratio. KiKGR is another fluorescent protein from coral that was engineered to undergo green-to-red photoactivation (Tsutsui et al., 2005). Its spectra are shown in Figure 21.6.1D as an example of this group of PA-FPs. EosFP, from another stony coral, *Lobophyllia hemprichii*, also exhibits a green-to-red fluorescence photoconversion after exposure to ultraviolet or near ultraviolet light (Wiedenmann et al., 2004). EosFP has a preactivated excitation maximum at 506 nm with emission at 516 nm, which shift to 571 nm and 581 nm, respectively, after photoactivation. The latest addition to this category is Dendra from *Dendronephthya sp*., which gives up to a 4500-fold increase in the red-to-green ratio after photoactivation (Gurskaya et al., 2006). Uniquely, Dendra can be activated with potentially less phototoxic wavelengths (~488 nm) in addition to the ~400-nm light required by the other green-to-red PA-FPs.

REQUIREMENTS FOR PHOTOACTIVATING FLUORESCENT PROTEINS

These general procedures can be applied to most photoactivatable fluorescent proteins, but the varied characteristics of each protein must be considered when addressing many of the points listed below.

Locating Positive Cells and Structures

Molecules that have little fluorescence prior to photoactivation produce the first obstacle, which is locating a cell and a specific region of a cell to activate. This is straightforward for the green-to red and cyan-to-green PA-FPs because the unactivated green or cyan emission wavelength can be used for targeting. Initially, the Dronpa protein has a 503-nm excitation peak and 518-nm fluorescence, but after irradiation with light in the 470- to 510-nm wavelength range, it is rendered nonfluorescent and develops absorbance at 390 nm. The PA-GFP emits green fluorescence when excited with low levels of ~400-nm light, and this may be sufficient to locate positive cells and structures. Alternatively, cotransfection with a second red or cyan fluorescent protein can usually be used to locate transfected cells.

Photoactivating the Fluorescent Protein

Photoactivation generally requires a separate excitation source (or at least a different filter set) than that used for imaging. For most of the molecules listed in Table 21.6.1, ~400-nm irradiation is required. Notable exceptions are the KFP1, which uses green (~532 nm) light and Dendra, which can be activated with ~400-nm light and ~490-nm light.

Imaging the Photoactivated Fluorescent Protein

Imaging of these molecules is similar to imaging any other molecule, with a few differences that can be advantageous or disadvantageous, depending on the experiment. Unlike conventional fluorescent proteins in which the starting level of fluorescence is usually the highest level that will be observed, the level of

fluorescence after photoactivation must be estimated to avoid detector saturation at a given imaging excitation power and detector gain. A simple procedure for estimation of post-photoactivation fluorescence involves taking a prephotoactivation image, measuring the mean fluorescence within the region to be photoactivated, background subtracting that mean and multiplying that number by the *x*-fold increase in fluorescence emission reported for the protein being used. This will give a rough estimation of the pixel values after photoactivation, but a little trial and error with various detector gain settings (contrast or voltage settings) will help the experimenter gain an appreciation of the levels of fluorescence to be expected with each experiment.

Optimizing Photoactivation

Regardless of the excitation source, some effort must be expended to obtain optimal photoactivation. Photoactivation is dependent on the duration of excitation power per unit area. In addition to photobleaching during the imaging after the photoactivation, photobleaching can also be encountered during the photoactivation event. Therefore, to maximize the contrast between pre- and post-photoactivation, power and duration of the photoactivation must be optimized such that the highest level of photoactivation is obtained with the least amount of photobleaching. The power and duration dependence are most easily demonstrated and optimized with cells expressing the PA-FP. A straightforward approach to optimization is to image a cell or field of cells between brief exposures to the activation light. This procedure is analogous to a fluorescence loss in photobleaching (FLIP; e.g., see *UNIT 21.1*) experiment, with the major difference being that fluorescence is increased instead of lost. As long as similar parameters (objective, zoom factor, photoactivation wavelength) are used, this optimization routine should suffice. However, fluctuations in laser power and alignment or changes in the parameters listed above will require additional optimizations. For a more detailed procedure, see below.

OPTIMIZATION PROCEDURES

1. Set the instrument light path configurations for imaging both the photoactivated PA-FP and inactive PA-FP, if desired. This will require proper mirrors and emission filters to detect the green and/or red fluorescence depending on the PA-FP in use. It is important to remember that a dichroic mirror capable of reflecting the photoactivation light source to the sample is also required. For most of the PA-FPs, this will be light of ~400 nm for one-photon excitation, with the notable exceptions of KFP1 and Dendra, discussed earlier. If two-photon excitation is to be used, be aware that the dichroic mirror should reflect the near-infrared wavelengths being used.

2. Set the imaging parameters (magnification or zoom factor, pixel dwell time or scan time, imaging excitation power, detector gain) required for the experiment. Some of these parameters may change for different experiments, but if the light path (mirrors, alignment etc.), magnification, and pixel dwell time remain similar, optimized photoactivation conditions should be similar for each experiment.

3. Locate a cell or cells expressing the PA-FP. As discussed earlier, this will depend on the PA-FP being used.

4. Set the photoactivation light power level to be used for the experiment. This parameter will have to be optimized depending on the requirements of the experiment and the light sensitivity of the cell type. Photoactivation light levels can affect both cell viability and the function of the protein of interest, but this depends on the protein as well as the cell type. It is the investigator's responsibility to test both possibilities. Regardless of the light level required, the procedure outlined here will be the same. To begin, use the maximum power available. This will lead to conditions for the most rapid photoactivation.

5. Set the photoactivation time. Depending on the instrument used, this may depend on the pixel dwell time or scan time used for imaging, or it may be set separately. Set this to a period of photoactivation as short as possible.

6. Set up an experiment with the following steps: (a) Image the PA-FP in the photoactivated spectrum before photoactivation, perform one photoactivation using the desired photoactivation power level, and image the PA-FP in the photoactivated spectrum again. (b) Repeat the photoactivation and imaging steps in sequence 10 to 100 times. Over the course of the experiment, the fluorescence should increase to a maximum and perhaps begin to decrease gradually. If the fluorescence is quantified within the photoactivated cells, the maximum level represents the optimal contrast that is obtainable over the inactivated PA-FPs. The number of photoactivation cycles required

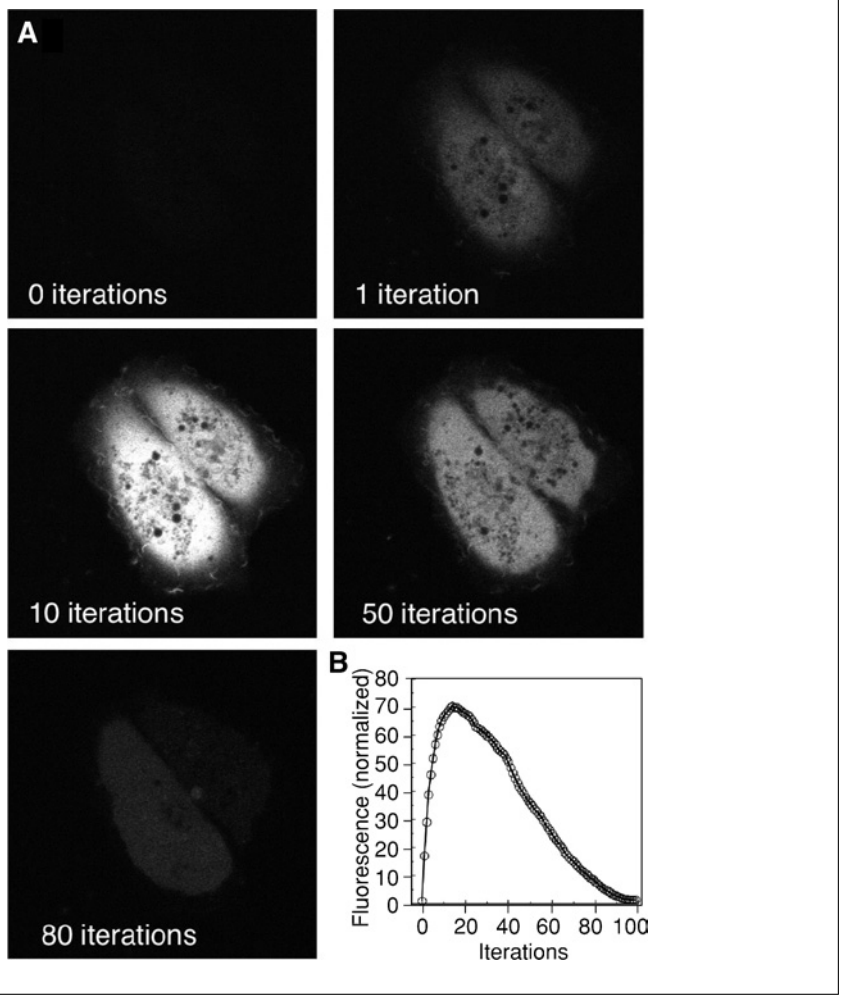


Figure 21.6.2 Photoactivation of PA-GFP. (**A**) COS-7 cells expressing PA-GFP were repeatedly imaged using 488-nm excitation. Between images, the entire field was briefly irradiated by two-photon excitation using 800-nm laser light. The total number of two-photon irradiations is shown in the lower left corner of the image. (**B**) The 488-nm excited fluorescence within the photoactivated region was normalized to the initial value and displayed as a function of the accumulated irradiations.

to reach the maximum fluorescent signal determines the optimal photoactivation time required for this photoactivation power level, magnification, and light path. An example of such an optimization is shown in Figure 21.6.2 using two-photon excitation for the photoactivation. A note of caution here is that if the photoactivation power level is too high for use with the PA-FP of choice, it may be photoactivated and photobleached during the same iteration. Thus, it may appear that no photoactivation is observed during the optimization. Performing the same protocol with lower photoactivation power levels will test for this possibility and help the experimenter find optimal photoactivation conditions.

GENERAL PHOTOACTIVATION EXPERIMENT

An example of a photoactivation experiment using the following procedure is shown in Figure 21.6.3. This protocol is written for PA-GFP using a laser scanning confocal microscope, but it should work for most of the PA-FPs in Table 21.6.1 and on other types of microscopes with appropriate alterations. Most commercial confocal systems have a software module devoted to performing photobleaching experiments. These can often be used for photoactivation experiments as well, as long as the laser wavelength used for the photoactivation event is close to ~400 nm rather than the normal imaging laser line.

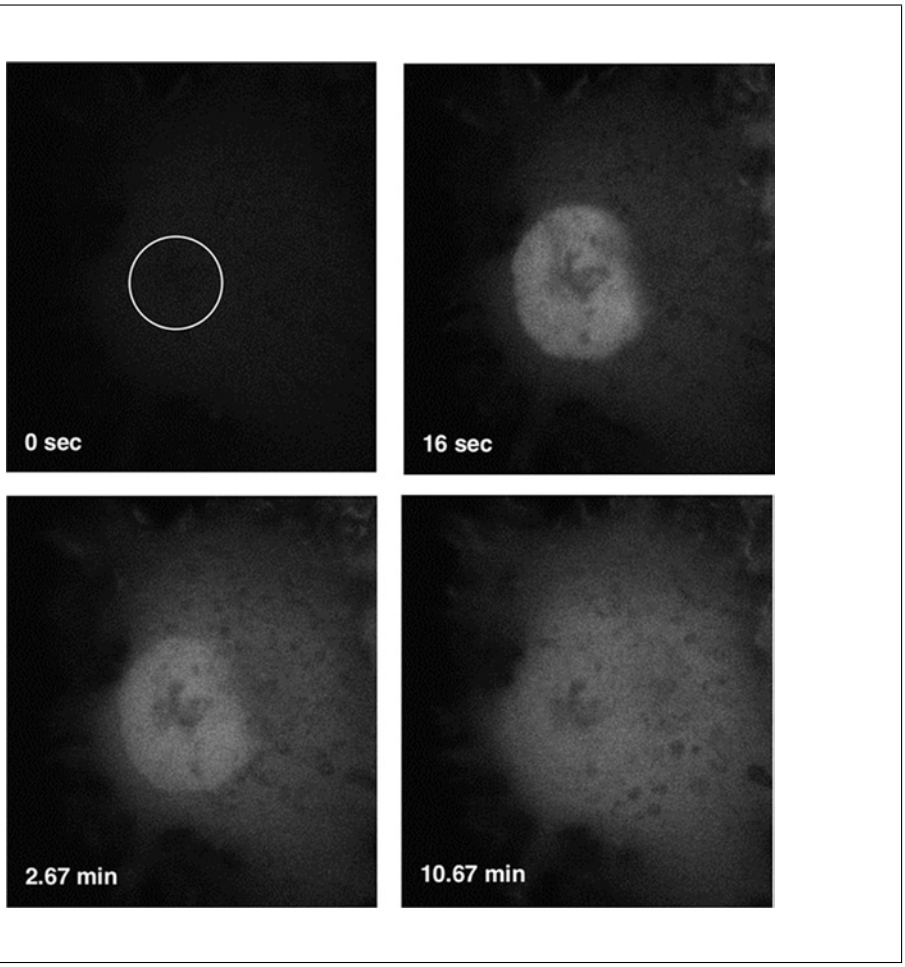


Figure 21.6.3 Selective photoactivation of PA-GFP. A COS-7 cell expressing PA-GFP was imaged using low levels of 488-nm light before and after photoactivation of the nuclear region (indicated by the white circle) with ~1 sec of 413-nm light. The images were acquired at 15.75-sec intervals.

1. Locate an expressing cell and the region of interest. The photobleaching or perhaps photoactivation software module generally has a feature for selecting the region to be photobleached or photoactivated. Select the region using the appropriate function.

2. Adjust the 488-nm laser power and detector gain for the expected fluorescence increase. This may require some of the trial and error indicated earlier.

3. Using the time series software module, program a time-lapse image experiment that will encompass the entirety of the process under study. This should collect images at the desired intervals, which will depend on the time scale of the process of interest.

4. Begin the time-lapse experiment by initially imaging the cell for one to ten frames with 488 nm to establish the baseline fluorescence.

5. Switch the irradiation wavelength to ~400 nm. For one photon, the increasingly common 405-nm diode lasers work well. A mercury lamp with a diaphragm can be used, but it is less efficient and has more limited temporal resolution. For two-photon excitation, published reports indicate 800 to 820 nm works well (Post et al., 2005; Schneider et al., 2005).

6. Irradiate the region of interest using the activation power levels and activation times determined in the optimization above.

7. Switch excitation back to ~488 nm and image the cell at the desired time intervals until the end of the experiment.

USES OF PHOTOACTIVATABLE FLUORESCENT PROTEINS

Some examples making use of PA-FPs are introduced here to convey the diverse applications of PA-FPs. With the exception of photoactivated localization microscopy (PALM; see below), where generally only a few molecules are activated at a time, the

procedures discussed earlier should assist or at least serve as an appropriate starting point for optimization of many PA-FP experiments.

Protein Dynamics

One of the most common uses of PA-FPs is complementary to the photobleaching technique, fluorescence photobleaching recovery (FPR) or fluorescence recovery after photobleaching (FRAP; *UNIT 21.1*). However, a distinct difference between FRAP and photoactivation is that in photoactivation, rather than monitoring the recovery of fluorescence within a region, a decrease in fluorescence can monitored when molecules are activated in one region and move to another. When monitoring rapid movement, this potentially provides two helpful characteristics compared to photobleaching. First, photoactivation can often rapidly and efficiently produce high contrast between highlighted and nonhighlighted molecules. Second, the highest signal-to-background ratio is found in the earliest points of the photoactivation experiment. Thus, the movement of proteins by diffusion or transport within a cell or organelle, movement of organelles within a cell, or even movement of a cell within an organism, can be readily monitored.

One disadvantage in photoactivation is that often the signal generated in one region dissipates as it moves throughout surrounding regions. To compensate for this signal dilution, it is possible to repeatedly photoactivate the same region, which is a variation similar to the photobleaching technique, fluorescence loss in photobleaching (FLIP; e.g., see *UNIT 21.1*). It relies on repeatedly photoactivating within the same region while monitoring the fluorescence increases in another region (Kim et al., 2006).

Fluorescence Pulse Labeling

New protein synthesis is often a concern when monitoring dynamics of fluorescent protein–tagged molecules of interest. This can often lead to artifacts in experiments unless synthesis is inhibited. However, photoactivation overcomes this problem because it affects only the protein that is already synthesized and properly folded at the time of photoactivation. Proteins synthesized before photoactivation fluoresce at the photoactivated wavelengths, while those that are synthesized after photoactivation do not. This characteristic of PA-FPs has been used to monitor the fate of cells during embryogenesis with KFP1 (Chudakov et al., 2003), the movements of chromatin loci in *Drosophila* embryos (Post et al., 2005), and the formation of new peroxisomes in cell culture (Kim et al., 2006).

This fluorescence pulse labeling ability also introduces an approach to monitoring protein turnover. A brief pulse with the activation wavelength of the tagged proteins of interest labels a population of molecules, and protein degradation is monitored by imaging the loss of fluorescence. It must be realized that the population of fluorescent molecules labeled under these conditions includes only those PA-FPs that are fully synthesized and properly folded, and the PA-FP must be degraded along with the protein of interest for proper read-out. Biochemical pulse-labeling thus should be carried out in parallel experiments to verify results. The temporal resolution of fluorescence pulse-labeling is essentially limited by the instrument parameters (usually milliseconds-seconds are required for activation), has subcellular spatial resolution (dependent on the optics used for imaging), and allows study of protein turnover in a single living cell.

Photoquenching Fluorescence Resonance Energy Transfer (PQ-FRET)

Fluorescent proteins have contributed to the study of protein-protein interactions within living cells by Förster resonance energy transfer (FRET; Day et al., 2001; *UNIT 17.1*). Since FRET requires that the distance between the donor and acceptor fluorophores be <10 nm, the energy transfer can be interpreted as an interaction of the tagged proteins of interest. An interesting approach to the study of protein-protein interactions in cells has been termed photoquenching FRET (PQ-FRET) and relies on the use of photoactivated PA-GFP as an acceptor to quench the fluorescence of a donor fluorophore, cyan fluorescent protein (CFP; Demarco et al., 2006). In the inactive state, the absorbance spectrum of PA-GFP has little overlap with the emission spectrum of CFP, but after photoactivation of PA-GFP it exhibits spectral overlap that makes FRET possible. Using PQ-FRET, the authors were able to monitor both the movement of the proteins and the interactions of the heterochromatin protein (HP1α) and the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα) within distinct domains of the nuclei of living cells (Demarco et al., 2006). Thus, PQ-FRET offers the capability of monitoring the dynamic interactions of molecules

at steady state within different regions of the cell. Although the use of other PA-FPs in PQ-FRET has not been reported at the time of this writing, this technique should be expandable by using them in combination with the plethora of fluorescent proteins that are now available.

Photoactivated Localization Microscopy (PALM)

Unlike the applications mentioned above, the high-resolution imaging technique, photoactivated localization microscopy (PALM), does not benefit from optimizing PA-FP photoactivation and actually requires only a few molecules to be activated with each activation cycle. Fluorescent proteins tagged to a protein of interest offer spatial information that is accurate within a few nanometers. Yet, the limit of resolution for conventional optical techniques is $\sim$100$\times$ more than the size of the fluorescent proteins. PALM provides near molecular resolution of proteins (Betzig et al., 2006). PALM involves imaging of molecules individually and then localizing them to high precision by determining their centers of fluorescence emission. This is achieved by performing a statistical fit of their measured photon distributions with ideal point spread functions. When the background noise is negligible compared to the molecular signal, the error in the fitted position is $\sigma_{xy} \approx s/\sqrt{N}$, where s is the standard deviation of a Gaussian distribution approximating the point spread function of the objective, and N is the total number of detected photons (Cheezum et al., 2001; Thompson et al., 2002).

Determination of protein localization using PALM is usually limited to molecules that are separated by the distance required of conventional optics ($\sim$250 nm). However, within most fluorescent specimen, hundreds or thousands of molecules may be present within one point spread function. PALM approaches this problem using both spectral and temporal means to isolate individual molecules. It relies on the serial photoactivation (using low levels of photoactivation light) of a small population of PA-FPs and their subsequent imaging until photodestruction. The photoactivation of small populations ensures that the density of imaged molecules remains much less than one molecule per normal resolution limit, and their subsequent photodestruction allows a new subset of molecules to be imaged. By fitting the fluorescent signal from each molecule to a two-dimensional Gaussian distribution, the coordinates for the location of the molecule and its uncertainty are determined. Each molecule is then rendered in a new image as a two-dimensional Gaussian distribution of standard deviation based on its uncertainty centered at the determined coordinates.

Reversible Saturable Optical Fluorescence Transitions (RESOLFT)

Reversible saturable optical fluorescence transitions (RESOLFT; Hofmann et al., 2005), relies on the switching behavior of the reversible PA-FPs such as asFP495 (Lukyanov et al., 2000) or Dronpa (Ando et al., 2004) to increase obtainable resolutions to the 50- to 100-nm range. This technique relies on the photoactivation of the reversible PA-FP within the normal optical diffraction–limited spot in conjunction with photoswitching off of the PA-FP in the same diffraction limited spot with a zero node inactivation. This produces a region of inactivation that leaves a region of photoactivated PA-FP smaller than the diffraction limited spot.

FUTURE DIRECTIONS OF PA-FPS

A long-term goal in optical imaging is to monitor the behavior of individual molecules of interest within the living cells of an organism, and the developments using PA-FPs such the ones discussed above have certainly advanced this goal (see Hofmann et al., 2005; Betzig et al., 2006). If the trend in fluorescent protein development continues, molecules with more defined absorbance cross sections, narrower emission spectra, higher fluorescence quantum efficiencies, more photostability, and less blinking and/or flickering are likely to be introduced in the near future. These will undoubtedly serve to advance the techniques mentioned above and lead to new and clever optical approaches in studies of cell biology.

LITERATURE CITED

Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. 2002. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99:12651-12656.

Ando, R., Mizuno, H., and Miyawaki, A. 2004. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science* 306:1370-1373.

Betzig, E., Patterson, G.H., Sougrat, R., Lindwasser, O.W., Olenych, S., Bonifacino, J.S., Davidson, M.W., Lippincott-Schwartz, J., and Hess, H.F. 2006. Imaging intracellular

fluorescent proteins at nanometer resolution. *Science* 313:1642-1645.

Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., Zacharias, D.A., and Tsien, R.Y. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99:7877-7882.

Cheezum, M.K., Walker, W.F., and Guilford, W.H. 2001. Quantitative comparison of algorithms for tracking single fluorescent particles. *Biophys. J.* 81:2378-2388.

Chudakov, D.M., Belousov, V.V., Zaraisky, A.G., Novoselov, V.V., Staroverov, D.B., Zorov, D.B., Lukyanov, S., and Lukyanov, K.A. 2003. Kindling fluorescent proteins for precise in vivo photolabeling. *Nat. Biotechnol.* 21:191-194.

Chudakov, D.M., Verkhusha, V.V., Staroverov, D.B., Souslova, E.A., Lukyanov, S., and Lukyanov, K.A. 2004. Photoswitchable cyan fluorescent protein for protein tracking. *Nat. Biotechnol.* 22:1435-1439.

Day, R.N., Periasamy, A., and Schaufele, F. 2001. Fluorescence resonance energy transfer microscopy of localized protein interactions in the living cell nucleus. *Methods* 25:4-18.

Demarco, I.A., Periasamy, A., Booker, C.F., and Day, R.N. 2006. Monitoring dynamic protein interactions with photoquenching FRET. *Nat. Methods* 3:519-524.

Ehrig, T., O'Kane, D.J., and Prendergast, F.G. 1995. Green-fluorescent protein mutants with altered fluorescence excitation spectra. *FEBS Lett.* 367:163-166.

Gurskaya, N.G., Verkhusha,V.V., Shcheglov, A.S., Staroverov, D.B., Chepurnykh, T.V., Fradkov, A.F., Lukyanov, S., and Lukyanov, K.A. 2006. Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. *Nat. Biotechnol.* 24:461-465.

Heim, R., Prasher, D.C., and Tsien, R.Y. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 91:12501-12504.

Hofmann, M., Eggeling, C., Jakobs, S., and Hell, S.W. 2005. Breaking the diffraction barrier in fluorescence microscopy at low light intensities by using reversibly photoswitchable proteins. *Proc. Natl. Acad. Sci. U.S.A.* 102:17565-17569.

Kim, P.K., Mullen, R.T., Schumann, U., and Lippincott-Schwartz, J. 2006. The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *J. Cell Biol.* 173:521-532.

Lukyanov, K.A., Fradkov, A.F., Gurskaya, N.G., Matz, M.V., Labas, Y.A., Savitsky, A.P., Markelov, M.L., Zaraisky, A.G., Zhao, X., Fang, Y., Tan, W., and Lukyanov, S.A. 2000. Natural animal coloration can be determined by a nonfluorescent green fluorescent protein homolog. *J. Biol. Chem.* 275:25879-25882.

Lukyanov, K.A., Chudakov, D.M., Lukyanov, S., and Verkhusha, V.V. 2005. Innovation: Photoactivatable fluorescent proteins. *Nat. Rev. Mol. Cell Biol.* 6:885-891.

Patterson, G.H. and Lippincott-Schwartz, J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297:1873-1877.

Post, J.N., Lidke, K.A., Rieger, B., and Arndt-Jovin, D.J. 2005. One- and two-photon photoactivation of a paGFP-fusion protein in live *Drosophila* embryos. *FEBS Lett.* 579:325-330.

Schneider, M., Barozzi, S., Testa, I., Faretta, M., and Diaspro, A. 2005. Two-photon activation and excitation properties of PA-GFP in the 720-920-nm region. *Biophys. J.* 89:1346-1352.

Thompson, R.E., Larson, D.R., and Webb, W.W. 2002. Precise nanometer localization analysis for individual fluorescent probes. *Biophys. J.* 82:2775-2783.

Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N., and Miyawaki, A. 2005. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep.* 6:233-238.

Verkhusha, V.V. and Sorkin, A. 2005. Conversion of the monomeric red fluorescent protein into a photoactivatable probe. *Chem. Biol.* 12:279-285.

Wiedenmann, J., Ivanchenko, S., Oswald, F., Schmitt, F., Rocker, C., Salih, A., Spindler, K.D., and Nienhaus, G.U. 2004. EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proc. Natl. Acad. Sci. U.S A.* 101:15905-15910.

Yokoe, H. and Meyer, T. 1996. Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nat. Biotechnol.* 14:1252-1256.

CHAPTER 22
Cell Biology of Chromosomes and Nuclei

INTRODUCTION

Organizing and expressing the information within the genome is an enormous challenge faced by all living organisms. Notably, the isolation of chromosomal DNA within the nucleus is one of the defining characteristics of eukaryotic cells. This isolation has important consequences for the organization of the genome, as well as for expression, trafficking, and interpretation of genetic information. This chapter is concerned with methods that address the expression, structure, and maintenance of chromosomes.

Chromosomes are highly dynamic structures, and the chromosomal DNA must undergo a complete, single round of duplication and packaging to allow accurate distribution of the genome to daughter cells. DNA replication is initiated at multiple sites along the chromosome (replication origins). There is considerable evidence that the timing of replication for different chromosomal sites is highly regulated with respect to expression patterns of genes within these domains and with respect to their distribution along the chromosome. Replication origins are organized into "replication factories" that can be visualized as punctate foci when sites of DNA synthesis are pulse-labeled within interphase mammalian nuclei. *UNIT 22.10* discusses the use of pulse-labeling strategies to characterize the organization of DNA replication within mammalian cells. Specifically, this unit includes bromodeoxyuridine (BrdU) labeling protocols for analysis of replication foci assembly, as well as for investigating their distribution with respect to chromosomal domains or subnuclear regions. This unit also discusses dual replication pulse labeling with chloro- and iododeoxyuridine, to examine the dynamics of DNA synthesis, and replication labeling of DNA fibers, to observe the distribution of replication sites on individual DNA fibers rather than on intertwined DNA strands within the nucleus. Units from other chapters that deal with DNA replication include *UNIT 11.5*, which describes systems for studying mammalian cell replication using the large T antigen-dependent replication of plasmids within mammalian cell extracts, and *UNIT 11.10*, which describes the assembly and analysis of replication-competent nuclei in *Xenopus laevis* egg extracts. *Xenopus* egg extracts provide a highly manipulable in vitro system (see *UNITS 11.10*, *11.11*, *& 11.13*), allowing the selective removal or addition of individual proteins.

UNITS 22.1 to *22.7* concern the cytogenetic analysis of vertebrate chromosomes. *UNIT 22.1* provides an overview of cytogenetic analysis techniques. These techniques are particularly important in analyzing subtle changes in chromosome structure that are associated with genomic instability in many contexts. *UNIT 22.2* describes culture methods and the preparation of mitotic and interphase samples for this analysis. *UNIT 22.3* describes traditional banding methods that permit the identification of chromosomes by number and allow the identifications of abnormalities. *UNIT 22.7* discusses the analysis of sister chromatid exchange using mitotic chromosomes from cells that have been labeled during DNA replication with 5′-bromodeoxyuridine (BrdU). Domains of exchange can be recognized through differential staining of the labeled strand by Hoechst dye and Giemsa, allowing direct monitoring of template switching during strand synthesis. Increased rates of sister chromatid exchange are diagnostic of a number of inherited diseases, and they are also observed in response to ionizing radiation or chemical insults.

Current Protocols in Cell Biology 22.0.1-22.0.3, March 2009
Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2200s42

UNIT 22.4 describes the use of fluorescence in situ hybridization (FISH) for the analysis of chromosomal structure. FISH analysis of mitotic chromosomes has provided a wealth of information about genetic changes in cancers and in genetic syndromes, such as chromosomal translocations and inversions. These methods have been extended with multicolor FISH applications to identify aberrations in genomic structure (*UNIT 22.5*). These techniques include M-FISH, wherein individual chromosomal paints are distinguished from one another during acquisition using distinct excitation filters. Alternatively, individual chromosomal paints within a mixture can be distinguished through spectral karyotyping (SKY) methods, which rely on Fourier transform methods for their analysis and separation. Moreover, fluorescence in situ hybridization analysis through comparative genomic hybridization (CGH; *UNIT 22.6*) provides sensitive determination of the net genomic gains and losses in test samples in comparison to normal reference genomic DNAs, giving a detailed picture of how genomes change during the progression of human disease.

Chromosomal instability is frequently associated with aberrations in spindle structure, and *UNIT 22.8* provides guidance for the examination of non-chromosomal spindle components by immunofluorescence, particularly microtubules and centrosomal proteins. *UNIT 22.9* provides protocols for assembling mitotic chromosomes in *Xenopus laevis* egg extracts and for the physical analysis of their structure. These methods should therefore be highly valuable in studying the construction and properties of mitotic chromosomes.

Among the most important aspects of nuclear biology is the regulation of messenger, transfer, and ribosomal RNA (mRNA, tRNA, and rRNA) synthesis. Closely related problems are involved in the post-transcriptional modification of RNAs, their assembly into ribonucleoprotein complexes (RNPs), and their trafficking between the nucleus and the cytoplasm. The expression of the RNA is controlled through a number of mechanisms, including modification of both chromatin components and DNA. One aspect of this modification is discussed in *UNIT 22.12*, which presents protocols for the analysis of DNA methyltransfereases at a single-cell level; these protocols utilize irreversible trapping of enzymatically active MTases to genomic DNA substituted with the nucleoside analog 5-aza-2-deoxycytidine (5-aza-dC).

Systems for analyzing transcription, processing, and export of mRNAs are described in a number of units: *UNIT 11.6* contains protocols for the preparation and use of mammalian and *Drosophila* extracts that accurately initiate basal and activated polymerase II transcription. *UNIT 11.17* describes protocols for in vitro analysis of post-transcriptional mRNA splicing, and *UNIT 11.14* describes protocols for RNA export assays, using microinjected *Xenopus* oocytes. Protocols for the analysis of mRNA export in both yeast cells and mammalian cells using oligo(dT) FISH are described in *UNIT 22.13*, along with methods to analyze mRNA export in live cells through microinjection of fluorescently labeled pre-mRNA within the nucleus. *UNIT 22.11* describes techniques for analysis of ribosomal (rRNA) maturation and ribosome assembly, using metabolic labeling and biochemical separation of preribosomal particles. Protocols for the analysis of nuclear trafficking in digitonin-treated cells are described in *UNIT 11.7*.

Other experimental approaches to nuclear processes are provided in Chapters 13, 17, and 18. *UNIT 13.5* describes the application of photobleaching techniques to proteins within the nucleus, providing temporally and spatially resolved data regarding the dynamics of nuclear structures. The measurement of protein-DNA interactions in the context of chromatin, through chromatin immunoprecipitation (ChIP) assays, is described in *UNIT 17.7*. Analysis of nuclear functions during apoptosis is provided in Chapter 18, particularly in *UNIT 18.4*, which discusses monitoring of telomeres through quantitative in situ hybridization, and *UNIT 18.6* which discussed other assays for telomere status and telomerase activity.

Together, these methods provide experimental avenues to address questions about the organization, maintenance, and expression of the genome—some of the most complex and fascinating problems in modern biology.

Mary Dasso

Overview of Cytogenetic Chromosome Analysis

This set of units describes the common techniques used in the preparation and analysis of mammalian chromosomes. Although the protocols mainly describe analysis of human chromosomes, the techniques are often applicable, with minor modifications, to the study of cytogenetics in other species. The amount of cytogenetic information that can be generated from a given piece of tissue or from a cell line is vast. Thus, the specific application of the techniques described depends on the type of question being asked by the researcher.

The first visualization of chromosomes in the late 1950s opened up the field of cytogenetics. Successful culturing of primary tissues or cell lines (*UNIT 22.2*) is paramount for obtaining a sufficient number of high-quality metaphase preparations for chromosome analysis. Once a culture has been established from a tissue or cell line, chromosome spreads need to be prepared (*UNIT 22.2*).

Banding techniques such as Giemsa banding (G-banding) have permitted the identification of chromosomes by number as well as the detection of chromosomal abnormalities (*UNIT 22.3*). For the past 40 years, these banding techniques have enabled the identification of chromosomal abnormalities in hematological malignancies, sarcomas, carcinomas, and genetic syndromes.

The 1990s brought about a second golden age for the field of cytogenetics with the advent of fluorescence in situ hybridization (FISH), where labeled DNA probes (*UNIT 22.4*), and, more recently, protein nucleic acid (PNA) probes, could now be hybridized to the chromosome target, detected, and analyzed using fluorescence microscopy (*UNIT 22.4*). This provided an important tool for gene mapping and for the identification of specific chromosomal aberrations associated with disease. Commercial diagnostic FISH tests could now routinely be included as an adjunct to classical banding analysis in the clinical hospital cytogenetics laboratory. In addition, with FISH, the drawback of having few metaphase spreads in specimens could be overcome by using gene/locus-specific probes and examining interphase nuclei. Tissues embedded in paraffin can also be examined by interphase FISH.

Multicolor FISH analysis of all human and mouse chromosomes can be carried out in one experiment. This recent development is a technological milestone and brings the field of cytogenetics full circle. Now, very subtle chromosomal aberrations such as small translocations or inversions can be readily identified without having to rely solely on the subjective interpretation of classical banding patterns. Chromosomes that were once dubbed as marker chromosomes, indicating that they provide insufficient banding information for chromosomal identification, can now easily be identified using advanced cytogenetics techniques. In conjunction with standard FISH experiments, small structural changes such as inversions, microdeletions, and amplifications can easily be assessed using locus-specific probes. Improvements in microscopy and fluorochromes make it possible to carry out many tests on one specimen.

Another FISH-based technology now routinely used is comparative genomic hybridization (CGH). This technique permits the determination of net genomic gains and losses in a given DNA sample. The test/tumor DNA is compared to a normal reference DNA sample and hybridized to normal metaphase slides. A two-color FISH detection experiment is performed, and image-analysis software calculates the change in the green/red ratio along the length of the chromosomes, corresponding to the tumor (green) DNA and normal reference (red) DNA that has hybridized, competitively, to the normal metaphase target. Increase in green/red ratios indicates a gain of genomic material at that chromosomal locus in the test/tumor, while a decrease in green/red ratio indicates a loss of genomic material at that chromosomal locus. CGH can provide important information from specimens where short-term culture has proven unsuccessful or impossible. DNA for CGH can readily be extracted from fresh or frozen tissue, paraffin-embedded tissue, or microdissected cells. More recently, CGH methods are being used to identify chromosomal imbalances using microarray target slides.

Sister chromatid exchange (SCE) refers to the interchange of DNA between replication

Contributed by Jane Bayani and Jeremy A. Squire

Current Protocols in Cell Biology (2004) 22.1.1-22.1.3

products. Each exchange represents a point of DNA template exchange during strand synthesis. SCEs are visualized as asymmetric chromatid staining or as harlequin chromosomes, because BrdU incorporation during strand synthesis causes differential staining with Hoechst dye and Giemsa. SCEs can be used to identify chromosomal anomalies and errors in the mitotic machinery.

Finally, improvements in antibody systems and identification of new proteins permit the study of the events that occur during DNA replication and the components involved in the mitotic machinery.

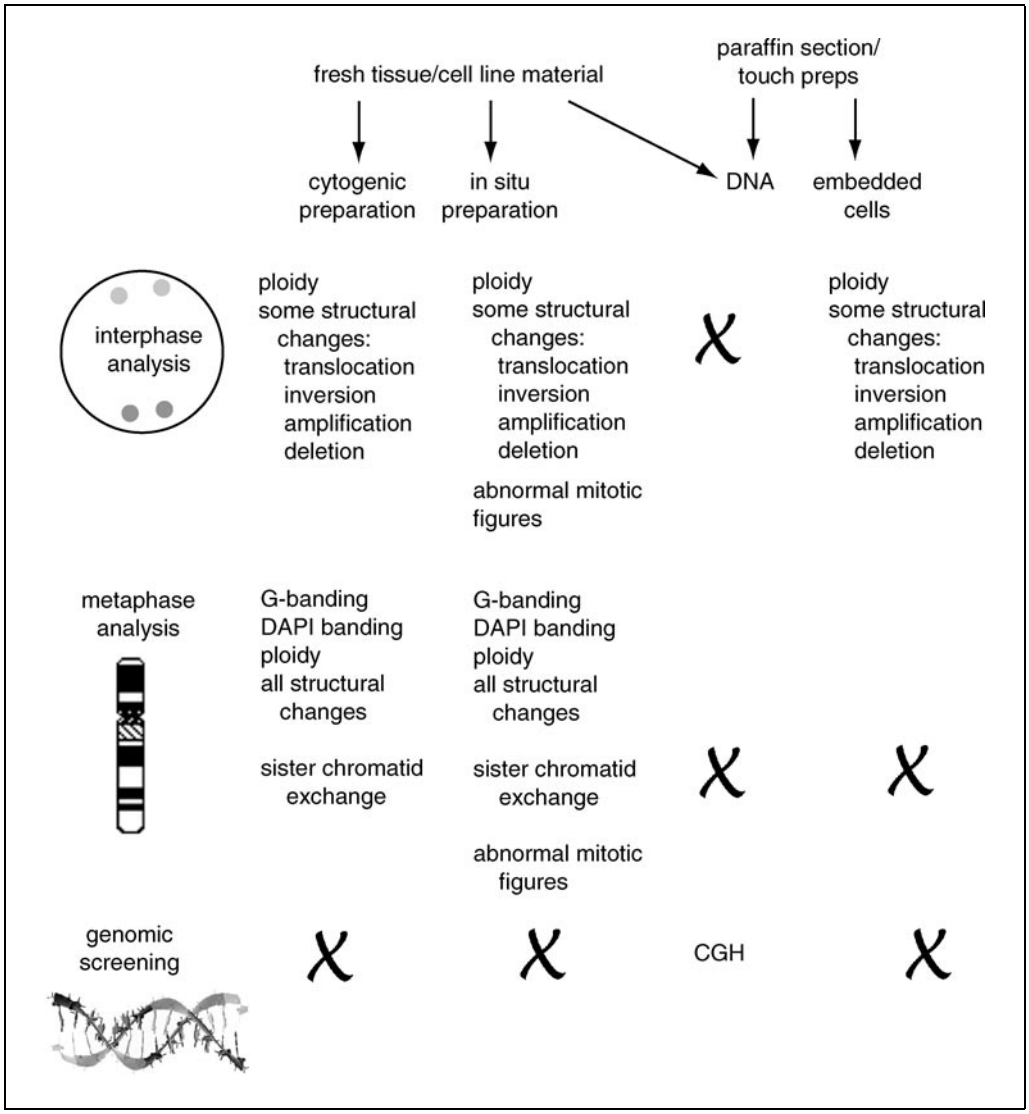


Figure 22.1.1 Overview of techniques for chromosomal analysis. Information regarding the status of whole chromosomes or specific chromosomal loci can be obtained from fresh material, such as fresh tissues and cultured cell lines, as well as from archival material including paraffin-embedded tissues, using FISH analysis. However, only cultured cells make it possible to perform specific chromosomal analysis using metaphase spreads as well as interphase cells. Tissues embedded in paraffin material can only provide information derived from interphase nuclei. Metaphase analysis provides information regarding ploidy, as well as the presence of structural changes such as balanced and unbalanced translocations, inversions, deletions, and additions. Complex extrachromosomal structures such as double-minute chromosomes (dmins), homogeneously staining regions (HSRs), ring chromosomes (r), and multicentric or acentric chromosomes can also be revealed by metaphase analysis; these features can only be indirectly inferred by interphase analysis. Both fresh and archival material will yield DNA that can be used for comparative genomic hybridization (CGH), a genome-screening technique. Other techniques that reveal chromosomal abnormalities and errors in the mitotic machinery include analysis of sister chromatid exchange and in situ hybridization using antibodies against proteins involved in the mitotic machinery, such as centrosomes.

Figure 22.1.1 describes the types of chromosomal analysis that can be performed on a given specimen and the information that can be generated from such analysis. Since each assay provides different information, it is important to determine which assay is most applicable for addressing a specific research question. Furthermore, the type, amount, and status of the tissue will greatly influence the type of analysis that can be carried out.

Contributed by Jane Bayani and
Jeremy A. Squire
University of Toronto
Ontario, Canada

Preparation of Cytogenetic Specimens from Tissue Samples

This unit describes basic tissue culture techniques that enable the investigator to prepare metaphase or interphase cells suitable for cytogenetic analysis from short-term cultured cells derived from tissue or body fluids. Established cell lines from sources such as the American Type Cell Collection (ATCC) are well adapted to in vitro growth and can also be used. A comprehensive discussion of tissue culture techniques for long-term cell lines can be found in *UNIT 1.1* (also see Barch, 1991; Freshney, 1993). This unit will describe the general parameters for establishing a short-term culture from primary tissue for cytogenetic studies (see Basic Protocol 1 and Alternate Protocols 1 to 3).

Success in obtaining chromosomes for analysis depends on achieving a mitotically active cell population. Different tissues and cell types, depending on the nature of the experiment, may require specific growth factors and medium supplements (*UNIT 1.2*). In addition, some cell types may sustain mitotic activity for a few days in culture because of residual in vivo growth-promoting effects. It is suggested that the investigator refer to current literature sources for the most effective growth conditions and media required for specific tissue types to ensure successful in vitro mitotic activity. These factors may be essential when maintaining primary or cell line cultures for several days. Moreover, abnormal cells (e.g., tumors, xenografts, or transgenic tissues) will have different growth kinetics than normal cells, and optimal conditions to favor growth of the cell type of interest should be selected. The culture techniques described here are applicable to tissues acquired from human as well as murine and other rodent sources.

After the cultures are established, the viable cells must be harvested and fixed to make a cytogenetic suspension (see Basic Protocol 2 and Alternate Protocol 4). Growing cultures are treated with a microtubule stabilizer called Colcemid that arrests cells in metaphase. Following exposure to Colcemid, the cells are hypotonically treated to enhance chromosome morphology and to increase chromatin accessibility for appropriate fixation using methanol and acetic acid. Once fixed, the cytogenetic preparation can be stored as cell pellets, under fixative, at −20°C for several months or even longer. Metaphase slides are prepared by resuspending the cells and applying them to glass slides. Obtaining adequate-quality chromosome spreads is multifactorial, and is discussed in detail by Henegariu et al. (2001) and Barch (1991). Oftentimes there is insufficient material to establish a culture, and it may be necessary to harvest any remaining viable cells before the culture dies. Sometimes the cell type of interest will only grow adherently, so short-term cultures may have to be grown on a glass slide or coverslip and treated in situ. The techniques for obtaining metaphase spreads for these types of cultures are also described here and in the cited literature.

CULTURING CELLS DERIVED FROM SOLID TISSUES FOR CHROMOSOMAL ANALYSIS

BASIC PROTOCOL 1

This protocol describes the preparation and culture of cells from solid tissues; these cells may be fully or partially adherent. The tissue is subjected to enzymatic disaggregation (if required) of the solid tissue, and the cells are plated and maintained in vitro.

Materials

Solid tissue specimen maintained in transfer medium
Appropriate culture medium (*UNIT 1.2*)
10× collagenase IV stock (see recipe)

Contributed by Jane Bayani and Jeremy A. Squire
Current Protocols in Cell Biology (2004) 22.2.1-22.2.15

Sterile medium-sized petri dishes (e.g., 60-mm)
Tabletop centrifuge
Scalpel and scalpel blades
Vented 25-cm^2 tissue culture flasks or 10-cm diameter petri dishes
15-ml conical centrifuge tube

Additional reagents and equipment for cell culture (Chapter 1)

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO_2 incubator. Some media, e.g., DMEM, require increased levels of CO_2 to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

Dissociate solid specimen

1. Using aseptic techniques (see Chapter 1), remove solid tissue specimen from transfer medium/container and place in a sterile petri dish.

   If required, transfer transport medium and remaining cells to a sterile centrifuge tube, centrifuge 5 min at 200 to 300 × g in a tabletop centrifuge, room temperature, and transfer the cell pellet to a sterile tissue culture flask as in step 5. Transfer medium typically contains the same basal medium, growth factors, serum, and other ingredients as the medium for culture; it may contain higher concentrations of antibiotics or fungicide.

2. Mince tissue, using scalpels into a fine pulp.

3. Add a few milliliters of tissue culture medium to the minced tissue and gently draw into a 5-ml pipet. Transfer to a vented 25-cm^2 tissue culture flask (or 10-cm diameter petri dish) containing sufficient medium (~10 to 15 ml) to cover the surface of the flask.

   The culture should be densely seeded to promote growth.

4. Continue mincing the large tissue pieces (remaining in the original petri dish) until they are a fine pulp, and transfer to the flask.

5. Depending on the nature of the tissue, add sufficient 10× collagenase IV stock to the tissue culture flask containing the medium and minced tissue such that the final collagenase concentration is 1×.

   Tissues that are fibrous or contain bone may benefit from collagenase treatment. Other, softer tissues, such as brain, may not require collagenase treatment. Additionally, some tissues may be sensitive to collagenase treatment and die. Thus, if there is sufficient tissue, one flask should be prepared with collagenase IV and one without collagenase IV. For the culture without collagenase IV, proceed to step 12.

6. Cap the flask securely and place it on its side in the 37°C CO_2 incubator. Incubate overnight.

7. The following day, use a sterile pipet to gently pipet the treated tissue up and down to break up remaining solid masses and obtain a single-cell suspension.

8. Examine the culture using phase-contrast microscopy to assess the extent of digestion. Also assess the extent of cell adhesion and colony formation. Transfer the single-cell suspension to a 15-ml conical centrifuge tube. Add fresh medium to flask with the adherent cells

   Any adherent cells in the original flask should remain untouched.

9. Centrifuge the single-cell suspension 5 min at 200 to 300 × *g*, room temperature, in a tabletop centrifuge. Pour off the supernatant and resusupend the pellet in 5 ml of fresh medium.

   There may still be large fragments of tissue remaining. These tissues can be treated again with collagenase IV in a separate culture.

10. Add fresh medium and transfer the resuspended pellet back to the flask.

    If there is a very high density of cells, one or more other cultures can be established.

Establish culture

11. Place the cultures back into the incubator and leave undisturbed overnight.
12. Check cultures daily to obtain a general estimate of doubling time, and feed cultures as required (*UNIT 1.1*; also see Critical Parameters).
13. Once culture is ~80% confluent, proceed to Basic Protocol 2.

    If required, subculture cells as described in UNIT 1.1.

CULTURING TISSUES IN SITU

ALTERNATE PROTOCOL 1

This protocol is especially useful when a very small amount of tissue is available for analysis. The procedure is essentially the same as that in Basic Protocol 1.

Additional Materials *(also see Basic Protocol 1)*

Poly-L-lysine-coated glass coverslips *or* slides (see Support Protocol) *or* chambered glass tissue culture slides (Nunc)

Phase-contrast microscope

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO_2 incubator. Some media, e.g., DMEM, require increased levels of CO_2 to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

1. Proceed with transfer and mincing of the solid specimen (see Basic Protocol 1, steps 1 to 2), except transfer the minced tissue to a 15-ml conical centrifuge tube.
2. Mince remaining large tissue pieces again and transfer to the 15-ml conical centrifuge tube.
3. Depending on the nature of the tissue, add sufficient 10× collagenase IV to the 15-ml conical centrifuge tube for a final collagenase concentration of 1×.

   Tissues that are fibrous or contain bone may benefit from collagenase treatment. Other softer tissues, such as brain may not require collagenase treatment. Furthermore, some tissues may be sensitive to collagenase treatment and die. Thus, if there is sufficient tissue, prepare one culture with collagenase IV and one without collagenase IV. For the culture without collagenase IV, proceed to step 6.

4. Cap the tube loosely and place in the 37°C CO_2 incubator. Incubate overnight.
5. The following day, gently pipet the treated tissues up and down to further disaggregate to a single-cell suspension. Centrifuge 5 min at 200 to 300 × *g*, room temperature, in a tabletop centrifuge. Pour off the supernatant and resuspend the pellet in 3 to 4 ml fresh medium.
6. If using coverslips or nonchambered glass slides, carefully place each in a sterile petri dish and add a few drops of cell suspension, followed by a few drops of fresh

medium. If using chambered slides, add sufficient medium to cover the chambered area. Using a sterile plastic transfer pipet, add a few drops (~500 µl total) of cell suspension. View using a phase-contrast microscope and add more cell suspension if the cell density appears low.

Depending on preference, use glass coverslips or glass slides directly for in situ culturing or coat with an appropriate matrix (see Support Protocol). Commercially available chambered, coated tissue culture slides can also be used. The investigator should refer to the current literature to determine the ideal matrix for promoting attachment of the specific cell type.

Do not use plastic coverslips if subsequent fluorescent-based work will be carried out because plastics produce autofluorescence.

7. Place the slides or coverslips with the cells in the 37°C CO_2 incubator. Incubate overnight, undisturbed.
8. The following day, carefully examine the slides/coverslips with a phase-contrast microscope to determine the extent of adherence. Add fresh medium as required by carefully pipetting fluid from the slide or corverslip and replacing with new medium. Monitor growth daily until the cultures are 80% confluent, then proceed to Basic Protocol 2.

SUPPORT PROTOCOL

PREPARE GLASS COVERSLIPS OR GLASS SLIDES FOR IN SITU CULTURING

In many cases, cells will adhere to uncoated glass or plastic surfaces; however, the adherence process can be facilitated by the pretreatment of regular glass coverslips and slides. This protocol describes the steps to coat and store glass slides and coverslips for in situ culturing as in Alternate Protocol 1. Poly-L-lysine-coated slides can also be purchased commercially from many companies.

Materials

Glass coverslips *or* slides, autoclaved
1 mg/ml poly-L-lysine stock solution: mix 100 mg poly-L-lysine (Sigma) with 100 ml sterile H_2O
Sterile petri dishes or Coplin jar

1. Place autoclaved glass coverslips or slides in a petri dish or Coplin jar containing the 1 mg/ml poly-L-lysine stock solution. Incubate at least 1 hr at room temperature.
2. Carefully remove the coverslips and/or slides and allow to dry.
3. Store in a dry cool place up to 1 month at room temperature, in a sterile petri dish sealed with Parafilm.

ALTERNATE PROTOCOL 2

CULTURING NONADHERENT OR FLUID TISSUES FOR CHROMOSOMAL ANALYSIS

This protocol describes the culturing of fluid tissue sources and nonadherent cells for cytogenetic preparations.

Additional Materials (also see Basic Protocol 1)

Fluid specimen maintained in transfer medium

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO_2 incubator. Some media, e.g., DMEM, require increased levels of CO_2 to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

1. Using aseptic techniques, transfer fluid tissue-containing specimen to a sterile 15-ml conical centrifuge tube and centrifuge 10 min at 200 to 300 × *g*, room temperature, in a tabletop centrifuge.
2. Pour off supernatant and resuspend pellet in 5 ml fresh culture medium.
3. Transfer cells to a sterile 25-cm^2 vented tissue culture flask containing 10 ml of medium.

   Alternatively, if there is abundant material, the investigator may wish to establish a larger culture in a 75-cm^2 vented tissue culture flask. Keep in mind that high cell density tends to promote growth.

4. Place in a 37°C CO_2 incubator and incubate overnight.
5. Monitor growth daily by phase-contrast microscopy and pH changes as revealed by changes in medium color.
6. If required, subculture the cells (*UNIT 1.2*).

CULTURE OF PERIPHERAL BLOOD CELLS

ALTERNATE PROTOCOL 3

This is a standard protocol for obtaining metaphase spreads from blood. Depending on the nature of the specimen (e.g., specimens from hematological malignancies), most blood specimens will be processed in the same fashion. The blood is collected in heparin and processed for culturing as soon as possible. Although whole blood can simply be cultured with the medium, this protocol describes the separation of the white blood cells using a Ficoll-Hypaque density gradient and centrifugation. The isolated white blood cells are then cultured for 72 hr in medium containing phytohemagglutanin, a cell synchronizer. At a time point ~24 hr prior to harvesting, the cultures may be half-fed (see Critical Parameters).

Materials

Blood collected in heparin
Phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile
Ficoll-Hypaque (density 1.077; Amersham Biosciences; store at 4°C protected from direct light)
Supplemented RPMI 1640 medium (see recipe)

50-ml conical tubes (e.g., Falcon)
15-ml conical centrifuge tubes (e.g., Falcon)
Refrigerated centrifuge
Sterile disposable plastic transfer pipets (Fisher)
25-cm^2 tissue culture flasks

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO_2 incubator. Some media, e.g., DMEM, require increased levels of CO_2 to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

Isolate white blood cells by Ficoll-Hypaque gradient

1. Transfer 12 ml of blood collected in heparin to a sterile 50-ml tube and add 20 ml sterile PBS. Cap tube and mix by inversion.

2. To each of four 15-ml conical centrifuge tubes, add 3 ml Ficoll-Hypaque.
3. Layer 8 ml of the blood/PBS mixture onto the top surface of the Ficoll-Hypaque.

   Add the blood/PBS mixture slowly, in such a way that it does not mix with the Ficoll.

4. Cap the tubes and carefully place them into a 4°C centrifuge. Centrifuge 20 min at 300 × *g*, 4°C, without the brake.

   If a refrigerated centrifuge is not available, a room temperature centrifuge can be used, but the investigator is cautioned to work quickly, as Ficoll can be toxic to cells.

5. At end of centrifugation, carefully remove the tubes from the centrifuge without disturbing the separated phases.
6. Using a sterile disposable plastic transfer pipet carefully remove the band of white blood cells located at the interface above the packed red cell pellet, and transfer into a new 15-ml conical tube.

   Cells from all four tubes may be combined into the same conical tube.

7. Fill the conical tube almost to the top with supplemented RPMI 1640 medium. Cap and mix by inversion. Centrifuge 5 min at 200 to 300 × *g*, room temperature.
8. Remove the supernatant and resuspend the pellet by gently tapping the tube. Add ~4 ml of fresh medium.

Establish cultures

9. Add 9 ml of supplemented RPMI 1640 medium to each of four 25-cm^2 tissue culture flasks. Add 1 ml of the resuspended cells to each flask for a total volume of 10 ml.
10. Place flasks on their sides in a 37°C CO_2 incubator and incubate 48 hr. At end of incubation, add 5 ml of medium to the flask and gently pipet the cells up and down to break up any clumps of cells. Return flasks to incubator.
11. After another 24 hr of incubation (total culture time of 72 hr), proceed to Basic Protocol 2.

    A normal peripheral blood culture usually requires a 1-hr Colcemid treatment at the standard concentration (see Basic Protocol 2).

BASIC PROTOCOL 2

PREPARATION OF METAPHASE SPREADS

The following protocol describes the steps required to obtain metaphase spreads from both adherent and nonadherent cultures as well as those from cells grown in situ. Briefly, the cultures are treated with Colcemid, pelleted by centrifugation, and hypotonically swollen. Finally the cells are fixed to maintain cellular architecture and to ensure that cellular proteins do not interfere with subsequent detection steps. The hypotonically swollen and fixed cytogenetic suspension is then applied to glass slides and air dried under controlled humidity. The slides are then ready for various analyses. The exposure time and concentration of Colcemid varies and depends on factors including tissue type and overall growth characteristics. The incubation times and concentrations listed below are typical starting points that will require modification. Barch (1991) provides a detailed summary of harvesting conditions for many of the hematopoietic malignancies and clinical samples such as amniocytes and chorionic villus cells. Harvesting solid tumors is far less predictable and requires experience borne from trial and error. Human, murine, and rodent cells are treated in the same fashion; however just as different cultures may respond differently to harvesting techniques within the same tissue type, there may also be some variability between species. An empirical approach to optimizing harvesting procedures is critical.

Materials

Adherent or nonadherent cell culture (Basic Protocol 1 or Alternate Protocol 2 or 3), 80% confluent
10 μg/ml Colcemid (Invitrogen) in H_2O (store up to 2 months at 4°C)
Appropriate tissue culture medium with serum
1× trypsin/citrate saline (see recipe)
0.075 M KCl (see recipe), prewarmed to 37°C in oven or water bath
3:1 (v/v) methanol/acetic acid fixative (see recipe)

15-ml conical centrifuge tubes
Tabletop centrifuge
Sterile disposable plastic transfer pipets (Fisher)
Coplin jars
Glass slides
Slide box

Additional reagents and equipment for cell culture (Chapter 1), including trypsinization (*UNIT 1.1*)

Treat cells with Colcemid

1. To 80% confluent adherent or nonadherent culture, add sufficient 10 μg/ml Colcemid stock to give a final concentration of 0.1 μg/ml.

2. Return the cultures to the 37°C CO_2 incubator and continue incubating for 1 hr.

See Troubleshooting for discussion of Colcemid sensitivity and incubation time.

Prepare single-cell suspension

3a. *For adherent and partially adherent cells*: At end of incubation, transfer the medium to a 15-ml conical centrifuge tube and centrifuge 5 min at 200 to 300 × *g* in a tabletop centrifuge, room temperature. Discard the supernatant. Trypsinize attached cells in the flask using 1× trypsin/citrate saline (also see *UNIT 1.1*) and transfer to the 15-ml tube containing the pellet of cells from the medium. When the cells have detached, add fresh medium, collect the cells, add to the tube, and centrifuge 5 min at 200 to 300 × *g*, room temperature. Discard supernatant.

3b. *For nonadherent cells*: Transfer cell suspension to a 15-ml conical centrifuge tube and centrifuge 5 min at 200 to 300 × *g*, room temperature. Discard supernatant.

4. Resuspend the cell pellet in the remaining solution by gently tapping the bottom of the conical tube. Resuspend as much of the pellet as possible into a uniform cell suspension.

Swell and fix cells

5. To the resuspended cells, add the first milliliter of prewarmed (37°C) 0.075 M KCl (hypotonic solution) in a dropwise fashion using a plastic disposable transfer pipet, gently tapping the bottom of the tube between drops. Once the first milliliter has been added, slowly fill the tube with the hypotonic solution. Gently mix by inversion and incubate 10 min at 37°C.

6. Again using a plastic disposable transfer pipet, add 5 to 10 drops of 3:1 methanol/acetic acid fixative. Cap tube and gently mix by inversion.

7. Centrifuge 5 min at 200 to 300× *g*, room temperature, in a tabletop centrifuge.

The pellet should look white and should have doubled in size.

8. Discard supernatant and resuspend the pellet thoroughly by flicking the bottom of the tube.
9. Using a plastic disposable transfer pipet, add 3:1 methanol/acetic acid fixative in a dropwise fashion for the first milliliter with gentle flicking between drops. Slowly fill the tube with 3:1 fixative after the addition of the first millliliter.
10. Centrifuge again as in step 7. Discard supernatant and resuspend pellet by gentle flicking of the bottom of the tube.
11. Repeat fixation by simply filling the conical tube with fixative, capping, and mixing by inversion.
12. Repeat steps 10 and 11 once more. If necessary, store the cytogenetic suspension as a pellet under fixative at −20°C.

Prepare cytogenetic slides

13. Discard fixative and resuspend pellet by gentle flicking of the bottom of the tube. Add sufficient fixative such that the suspension appears opaque.

    After preparing the first slide, the cell density may be adjusted as needed with fixative.

14. To a clean Coplin jar containing distilled water, add a clean glass slide.

    Different techniques for slide making exist. A comprehensive discussion can be found in Henegariu et al. (2001) and Barch, (1991).

15. Remove one slide and hold roughly at a 45° angle (Fig. 22.2.1).

    A thin layer of water will still remain.

16. Using a 1-ml plastic disposable transfer pipet, add 1 to 2 drops of the cytogenetic suspension. Allow the slide to dry.

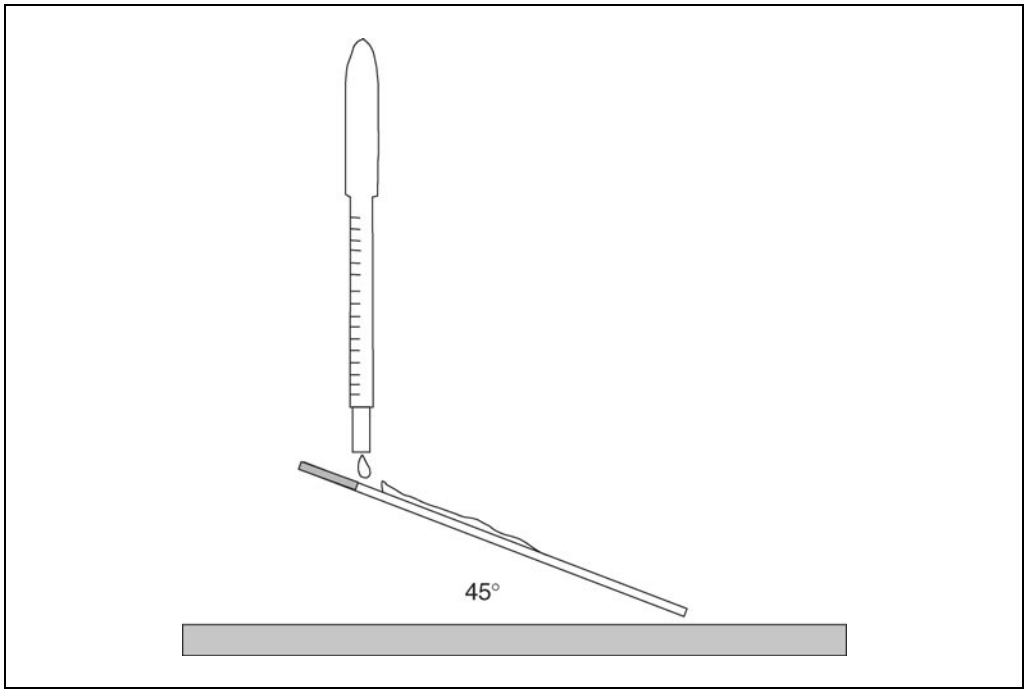


Figure 22.2.1 Schematic representation of "drop-method" of slide making. Shown in the figure is the general method for preparing slides for molecular cytogenetic analysis from a fixed suspension. Using a plastic disposable transfer pipet, add 1 or 2 drops (or 200 μl) to a clean slide held at ~45° angle. The slide may be pretreated with water or fixative. Allow the suspension to run down the slide. Expose the slide to humidity or heat as required to achieve the proper drying conditions.

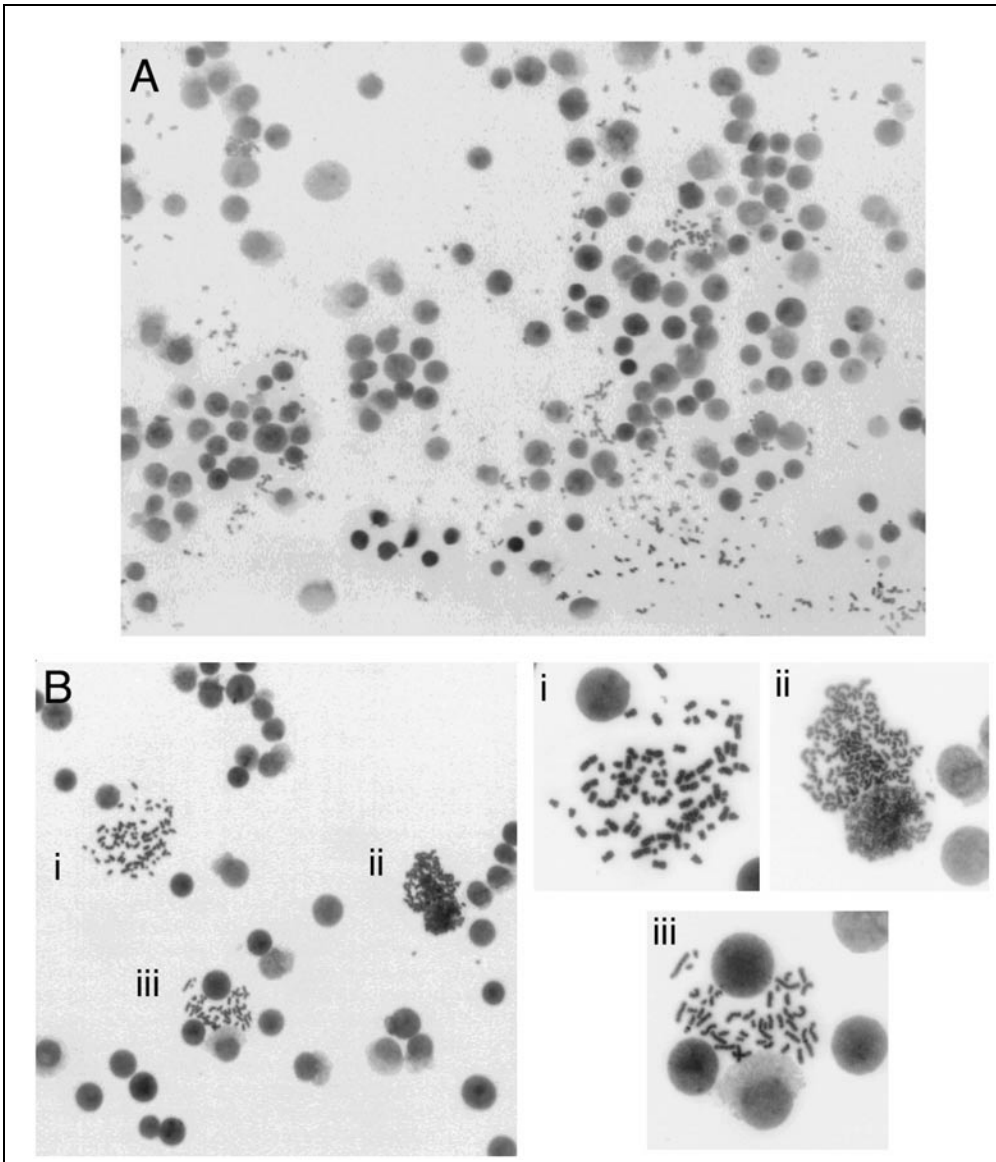


Figure 22.2.2 Phase-contrast images of slides that are appropriate and not appropriate for molecular cytogenetic analysis. (**A**) This panel is a 40× view of a prepared cytogenetic slide where the cell density is too high; thus, less suspension should be applied to the slide, or the main suspension should be diluted with fixative. This slide would be appropriate for interphase analysis, since the nuclei are intact and flattened, and chromosome analysis would not be accurate on this slide, since the metaphase spread is not intact. There is the appearance of "chromosome soup," making it difficult to discern the chromosomal content of one cell. The bursting of the cells may have occurred from rapid fixation following hypotonic treatment, or from excessive hypotonic treatment. If the entire slide has this appearance, a change in dropping technique could be used to prevent overspreading of chromosomes; however it is suggested that another suspension be made. (**B**) This panel shows a cell density that is manageable, with many intact metaphase spreads acceptable for analysis. Each metaphase spread in this field has been magnified. Metaphase "i" shows a well spread metaphase with few overlapping chromosomes that are at a reasonable length for banding analysis. Metaphase "ii" shows a tight spread with longer chromosomes. This metaphase is not appropriate for banding analysis. Metaphase "iii" shows a spread with good-length chromosomes and few overlaps. Its location between nuclei may make access to the spread difficult if there is residual cytoplasmic debris; however this is an analyzable metaphase.

17. Examine by phase-contrast microscopy. Change slide-making strategy as required based on visual assessment of slides (see Troubleshooting and Fig. 22.2.2).

18. Store slides in a slide box in a dry area until ready for use (see Troubleshooting).

ALTERNATE PROTOCOL 4

CYTOGENETIC PREPARATIONS FROM IN SITU CULTURES

This protocol describes the technique used for cells grown on slides or coverslips (Alternate Protocol 1). The principles of hypotonic treatment and fixation are similar to those of Basic Protocol 2.

Additional Materials *(also see Basic Protocol 2)*

In situ cultures (Alternate Protocol 1), 80% confluent
Cytoseal 60 mounting medium (Richard-Allan Scientific)

1. To 80% confluent cultures, add sufficient 10 μg/ml Colcemid stock to give a final concentration of 0.1 μg/ml.

   The amount of Colcemid used will depend on the amount of medium present (i.e., after adding fresh medium to the initial drops of cell suspension).

2. Return the cultures to the 37°C CO_2 incubator and continue incubating for 1 hr.

   See Troubleshooting for discussion of Colcemid sensitivity and incubation time.

3. Carefully remove medium and add fresh medium to wash the Colcemid from the cells.

4. Remove medium and carefully add prewarmed (37°C) 0.075 M KCl. Place in a 37°C incubator or dry oven for 10 min.

5. Add 5 to 10 drops of 3:1 methanol/acetic acid fixative and gently mix by rocking the petri dish or chambered slide.

6. Using a plastic disposable transfer pipet, remove solution. Slowly add fresh 3:1 methanol/acetic acid fixative and let stand at room temperature for 5 min.

7. Remove fixative and repeat fixation twice. Air dry slides

8. Mount coverslip on slide using mounting medium. Examine slides using phase-contrast microscopy. Change slide-making strategy as required based on visual assessment of slides (see Troubleshooting and Fig. 22.2.2).

9. Store slides in a slide box in a dry area until ready for use (see Troubleshooting).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Citrate-saline, 10×

0.44 g sodium citrate (0.44% w/v)
1.0 g potassium chloride (1% w/v)
100 ml H_2O
Dilute to 1× using H_2O

Collagenase IV, 10×stock

153 mg of 260 U/mg Collagenase IV (Sigma)
20.0 ml sterile culture medium or H_2O
Filter sterilize
Store in 500-µl single-use aliquots indefinitely at −20°C

KCl, 0.075M

2.8 g KCl (Sigma; 0.075 M final)
500.0 ml H_2O
Store at room temperature or 37°C up to 1 month

Methanol/acetic acid fixative, 3:1

90.0 ml methanol (molecular biology grade)
30.0 ml glacial acetic acid (molecular biology grade)

Fixative should be made fresh, however it may be stored at −20°C for several days.

Supplemented RPMI 1640

415.0 ml RPMI 1640 medium with antibiotics (e.g., Life Technologies)
75.0 ml fetal bovine serum (15% v/v final)
5 ml 100× L-glutamine (Invitrogen; 1× final)
5 ml phytohemagglutinin (M Form, Invitrogen; reconsititute lyophilized powder per manufacturer's instructions)
Store up to 1 month at 4°C

Trypsin/citrate saline, 1×

10 ml 10× trypsin/EDTA (Invitrogen) (1× final)
90 ml 1× citrate-saline (see recipe)
Store indefinitely at 4°C
Place at room temperature or 37°C before use

COMMENTARY

Background Information

Cell cultures

Growing tissues in culture has given investigators the ability to analyze and control cellular events. However, it has become increasingly important to characterize the gross chromosomal changes and correlate them to abnormal cellular processes and phenotypes identified. A comprehensive discussion on tissue culture techniques can be found in Chapter 1 of this manual, as well as in Freshney (1993) and Barch (1991). The brief discussion in this unit covers basic tissue culture issues relevant for preparing cytogenetic preparations.

Slide preparation

The first karyotypes were made from peripheral lymphocytes by Hungerford et al. (1959), using a squash technique that is now considered primative. Later, Moorehead et al. (1960) published the method for air drying peripheral blood chromosomes, a modification of the method of Rothfels and Siminovitch (1958) using methanol/acetic acid. Prior to this, plant studies had revealed that mitotic-spindle disruptors such as colchicine made it possible to discern the beginning and end of distinct chromosomes, since the chromatin material was condensed and arrested at metaphase; this technique was later applied to mammalian cells. In the procedure used here, the hypotonic swelling of the cells causes a concentration gradient and stretches the cell membrane. Fixation helps to maintain this state until the cell is forced to flatten out when applied to the slide. It is evident from the many cytogenetic publications since those early reports that identifying the chromosomal content of a cell has significance in diagnosing, monitoring, and treating disease and genetic abnormalities. The success of fluorescence in situ hybridization (FISH)–based technologies lies largely in the meticulous principles of harvesting and slide making established by early cytogeneticists.

Finally, obtaining optimal chromosome spreads is an art in and of itself, and techniques

vary from laboratory to laboratory as well as between individuals in the same laboratory. It can be compared to a group of people who are given the same recipe and same ingredients and asked to prepare the same meal. Invariably some will prepare a better-tasting meal than others, based on their careful observations and years of culinary experience. This analogy is worth considering when preparing slides. The sections below (see Critical Parameters and see Troubleshooting) will discuss some of the more critical aspects of slide making, but the onus is on the individual to determine the best method of slide making from trial and error.

Critical Parameters

Cell cultures

Aceptic techniques and proper biohazard safety practices should always be implemented when handling tissue, especially human tissue. These precautions serve not only to protect the investigator and the laboratory but also to ensure that unwanted microbes or fungi in the cultures are minimized.

As mentioned, a search of the current literature will provide the requirements necessary for optimal short-term culture of the tissue of interest. Since not all tissues will have the same growth rate, the investigator should monitor the progress of the cultures daily to ensure that they are not becoming contaminated with normal cells and to estimate the likelihood that sufficient cells will grow. Investigators are urged to consult the current literature concerning the cells being used to determine the best way of limiting the growth of unlimited cell populations. Feeding should be carried out just as the medium starts to become more acidic, based on the change in color of the indicator present in most media. Cultures may be heterogeneous with respect to the types of cells present in the culture, ranging from fully adherent or nonadherent to partially adherent with floating viable cells. When feeding cultures, it is suggested that the cultures be half-fed. To do this, remove half of the tissue culture medium and centrifuge to bring down any floating cells. Discard the supernatant. Add fresh medium to the pellet, resuspend the pellet, and return the cells back to the culture. This ensures that the culture obtains fresh nutrients and at the same time retains critical growth factors or cytokines present in the original medium, which may have been produced by the cells themselves. Finally, some tissues may fail to thrive in culture after enzymatic treatment.

In this unit, collagenase IV is featured as the main enzyme for tissue disaggregation; however, there are several types of collagenase as well as other enzymes that may be used for tissue disaggregation. Caution should be exercised when treating with an enzyme, because some cells do not recover well from enzymatic treatment.

Preparing slides

Cell division may be stimulated by subculturing the cells 1 or 2 days before harvesting for cytogenetic suspension (Basic Protocol 2 or Alternate Protocol 4). Cultures do not necessarily need to be split into new flasks or dishes, but they can be passaged into a new flask simply to induce cell division by disaggregation while keeping cell density at a level just below confluence to avoid contact inhibition of proliferation.

The cultures to be harvested should be actively dividing. When using established cell lines, it is usually easy to identify and count rounded-up mitotic pairs of daughter cells at anaphase/telophase. However, cultures from primary tissues often do not have a uniform morphology, and it can be difficult to distinguish between proliferating and apoptotic or necrotic cells. Oftentimes there is not sufficient material to stain cells with trypan blue; thus the investigator should monitor primary cultures daily. Some tissues fail to thrive in vitro and may need to be harvested within 72 hr. Cellular heterogenetity with respect to cell cycle will also influence the mitotic index of the final cytogenetic suspension. Treatment with cell synchronizers is discussed in Barch (1991) and may help to increase the mitotic index.

Treatment with the drug Colcemid will also influence the mitotic index of a cytogenetic suspension. Since Colcemid arrests cells by disrupting the spindle, increasing treatment times will improve the mitotic index by accumulating cells arrested at metaphase. However, prolonged exposure or use of higher concentrations of the drug will cause increasing chromosome contraction, resulting in shorter chromosomes. As the chromatin within chromosomes becomes condensed, the ability to identify banding patterns continues to decrease.

Hypotonic swelling is generally carried out using KCl. However some have used a mixture of KCl with culture medium or water (Squire, 1983; Barch, 1991; Henegariu et al., 2001). These variations help to prevent the sudden rush of water into the cells. Should the hypotonic treatment occur too quickly, the cell

membranes may burst, yielding "chromosome soup" (Fig. 22.2.2A). Too little swelling will prevent the membranes from adequately releasing the chromosomes from the confines of the cell membrane, preventing them from spreading. Hypotonic treatment is typically carried out slowly, with gentle mixing. Vortexing is usually too harsh, although some individuals have used this method with success. A successful hypotonic treatment will yield a pellet that has doubled in size and that often looks translucent due to increased water content within cells.

The first fixation, termed the pre-fix, occurs after the hypotonic treatment. It involves the addition of a few drops of fixative to the swollen cells. This pre-fix serves to harden cell membranes to maintain cellular architecture and the swollen cell state. The first full fixation is carried out gradually. Once the first full fixation is complete, the subsequent fixations can be carried out in normal fashion. Repeated fixations help to remove cytoplasmic and other cellular debris as well as to preserve the cells.

Fixed cells can be stored for several years, provided that they are stored as pellets under fixative in tightly capped tubes at −20°C. The reasons why cytogenetic suspensions degrade over time are that the cells were not pelleted, they were stored at room temperature or 4°C, or that the methanol has evaporated, leaving an increased concentration of acetic acid, which will damage the cells.

The consensus of many experienced cytogeneticists is that the relative humidity in the room where slides are made is the most critical parameter for slide making. It is not uncommon to find laboratories hoping for a rainy or humid day so that their slide preparation will be optimal. In the authors' experience, a relative humidity of at least 30% to 35% is optimal for preparing well spread chromosome preparations. This can be achieved by having a boiling kettle, beaker of water, or water bath nearby, over which to pass the freshly prepared slide. Humidity in excess of 50% will require aided drying, usually with a warm-to-the-touch hot plate. Determining the best conditions for slide making requires frequent changes in test conditions and trips to a phase-contrast microscope to check the results. Fast drying can be controlled by applying the suspension to slides that are wet (with water). In more humid conditions, suspensions may be applied to dry slides. Others choose to wet their slides with fixative, but this may speed up the drying process. In many state-of-the-art cytogenetics laboratories, a humidity- and temperature-controlled unit (about the size of a fume hood) called a Thermotron (*http://www.thermotron.com/cryogen.html*) has been used to establish the correct humidity and temperature for slide making.

Storing of cytogenetic slides is also subjective. Some laboratories simply store slides at room temperature, while others store slides at −20°C (in or free of ethanol), and still others store in dessicators. Each laboratory will need to determine the best storage conditions. However, if there is left-over cytogenetic suspension, fresh slides can always be made as needed.

Troubleshooting

Cell cultures

Depending on the likelihood that tissues were handled aseptically before reaching the cell culture facility (i.e., from an operating room or pathology laboratory), it may be necessary to include antifungal agents in addition to the routine antibiotics supplementing both the culture and transport media. Once again, caution should be exercised because antifungal agents are often toxic to cells.

If bacterial or fungal contamination does occur, particularly in adherent cultures, and there are no backup cultures, the contaminants may be removed from the culture, the cultures washed with sterile PBS, and fresh medium added. The cultures must be harvested (Basic Protocol 2) within 24 hr so that the remaining contaminants do not have sufficient time to overgrow. The investigator should be warned that the presence of contaminating bacteria or fungi will cause subsequent background problems for banding analysis and FISH analysis because they contain DNA and antigens that bind the antibodies conjugated to a fluorochrome.

Slide preparation

See Table 22.2.1 for a troubleshooting guide to slide preparation for cytogenetic analysis.

Anticipated Results

Establishing short-term cultures from primary tissues requires careful and systematic monitoring to determine the optimal time to initiate harvesting. Longer-term cell lines will generally yield better quality cytogenetic preparations, as they have undergone adaptation to in vitro growth and usually exhibit high mitotic activity. The investigator should make

Table 22.2.1 Troubleshooting Guide for Cell Harvesting and Slide Preparation for Cytogenetics Analysis[a]

Problem	Possible cause	Solution
No metaphase spreads	Slowly dividing cell culture	No immediate solution to increasing cell growth; however growth can be stimulated by providing a half-feed of the culture the day before harvesting
	Insufficient Colcemid incubation	Treatment time with the standard Colcemid concentration of 0.1 μg/ml may need to be increased in increments from 60 to 90 min
	Insufficient Colecmid concentration	If the culture appears to be dividing (empirically determined), then increase the Colcemid concentration, but either maintain the same incubation time, or decrease it
Chromosomes too long	Short Colcemid incubation	Every cell type reacts differently to Colcemid treatment; if chromosomes are too long, this suggests that they can tolerate longer incubations or higher concentrations of Colcemid
	Insufficient Colecmid concentration	As described above, Colcemid incubation time and/or concentration can be increased to achieve shorter chromosomes
Chromosomes too short	Cells sensitive to Colcemid treatment	This is more of a problem than the situation above. Decrease amount of Colcemid but maintain the same incubation time, or maintain the same concentration of Colcemid, but decrease the incubation time.
	Colcemid incubation too long	Decrease the amount of Colcemid but maintain the same incubation time, or maintain the same concentration of Colcemid, but decrease the incubation time.
Metaphase spreads too tight	High cell density	If the concentration of cells in the working suspension is too high, it will not permit the chromosomes to spread. Either add less cell suspension to the slide, or dilute the suspension further while maintaining the amount applied to the slide.
	Insufficient hypotonic swelling	The chromosomes must burst out of the plasma membrane and spill onto the slide. If the hypotonic treatment is insufficient to build the necessary tension, then the chromosomes will fail to spread or burst from the plasma membrane. Increase the hypotonic treatment to by 5 to 10 min. However, too much swelling will cause the cells to be too fragile.
Presence of cytoplasm	High cell density	If the concentration of cells in the working suspension is too high, it will not permit the chromosomes to spread. Either add less cell suspension to the slide, or dilute the suspension further while maintaining the amount applied to the slide.
	Insufficient hypotonic swelling	The chromosomes must burst out of the plasma membrane and spill onto the slide. If the hypotonic treatment is insufficient to build the necessary tension, then the chromosomes will fail to spread or burst from the plasma membrane. Increase the hypotonic treatment to by 5 to 10 min. However, too much swelling will cause the cells to be too fragile
Refractile-looking chromosomes	Slides drying too quickly or too slowly	This is the "art" of slide making. Ambient temperatures in the lab and the time of year will greatly affect slide making and their appearance. During the dry months of the year, keep a beaker or kettle of water boiling, or have a water bath on in the lab to increase the relative humidity.
		For the humid days of the year, keep a "warm-to-the-touch" hotplate on to facilitate drying.

[a] A comprehensive discussion can be found in Barch (1991).

note of increasing debris from dead cells and the number of cells that appear to be inactive or senescent, and thus decide the best time to harvest the cells for optimal cytogenetic preparations.

Every harvest will be different, even when harvesting multiple cultures of the same cells. However, with experience, the investigator will learn when to change Colcemid concentration, alter hypotonic conditions, and vary slide-making techniques.

Under the perfect conditions, a slide will have a relatively high mitotic index, such that at least three metaphases can be seen under low power (Figure 22.2.2). Chromosomes should not be too long or too short. Cytoplasmic debris and other cellular debris should be minimal with nuclei and chromosomes appearing gray to dark gray.

Time Considerations

It takes several days to establish a primary culture from tissue and for the cells to reach 80% confluence, depending on the growth characteristics of the cells in question.

It takes 2 to 3 hr to prepare slides for cytogenetic analysis depending on the skill of the investigator and the number of slides to be prepared.

Literature Cited

Barch, M.J. 1991. The ACT Cytogenetics Laboratory Manual, 2nd Edition. Raven Press, New York.

Freshney, R.R. 1993. Culture of Animal Cells: A Manual of Basic Techniuqes, 3rd Ed. Wiley-Liss, New York.

Henegariu, O., Heerema, N.A., Wright, L.L., Bray-Ward, P., Ward, D.C., and Vance, G.H. 2001. Improvements in cytogenetic slide preparation: Controlled chromosome spreading, chemical aging and gradual denaturing. *Cytometry.* 43:101-109.

Hungerford, D., Donnely, A., Nowel, A., and Beck, S. 1959. The chromosome constitution of a human phenotypic intersex. *Am. J. Hum. Genet.* 11:215.

Moorhead, P., Nowell, P., Mellman, W., Battips, D., and Hungerford, D. 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp.l Cell Res.* 20:613.

Rothfels, K. and Siminovitch, L. 1958. An air-drying technique for flattening mammalian cells grown in vitro. *Stain. Technol.* 33:73.

Squire, J. 1983. Dilution of culture medium of trypsinized cells as an alernative to conventional KCl hypotonic treatment. *Karyogram.* 9:27

Internet Resources

http://www.thermotron.com/cryogen.html

Thermotron Web site for equipment specifications and description.

Contributed by Jane Bayani and
Jeremy A. Squire
University of Toronto
Ontario, Canada

Traditional Banding of Chromosomes for Cytogenetic Analysis

This unit describes the two most commonly used methods for banding chromosomes, Giemsa banding (G-banding; see Basic Protocol) and DAPI banding (see Alternate Protocol). G-banding is the conventional means of staining chromosomes to reveal their banding patterns. It is a nonfluorescent technique requiring only a standard light microscope for visualization. DAPI banding utilizes a DNA stain that fluoresces. With appropriate pretreatment (if required), DAPI banding identifies the same major banding patterns revealed by G-banding. Imaging tools can convert the DAPI image into a black-and-white image, which can then be converted into a G-banding-like image, more familiar to cytogeneticists. Both techniques permit the identification of chromosomes and detection of the presence of grossly abnormal chromosomes and abnormal structures. G-banding requires sufficient chromosome aging, by enzymatic digestion, to remove cytoplasmic debris and allow access of the Giemsa stain to the DNA. The incubation times for each step may be variable depending on the type and quality of specimen. DAPI staining, on the other hand, is not as finicky; slides can be used as is and can normally be stained directly with minimal or no pretreatment.

GIEMSA BANDING (G-BANDING) OF METAPHASE CHROMOSOMES

BASIC PROTOCOL

This protocol describes the basic steps for G-banding metaphase spreads. The enzymatic treatment of the slides requires optimization, either with respect to duration or trypsin concentration. Similarly, the time for staining requires adjustments to suit the investigator's preference and the slide quality. The investigator must be able to recognize the morphology of overtrypsinized or undertrypsinized chromosomal banding patterns. It is suggested that a few practice slides be used first to determine the optimal conditions for digestion and staining. Practice slides should ideally be slides made from the same suspension at the same time and under the same aging conditions as the test slides.

Materials

Metaphase slide preparation made 1 to 2 days before banding (*UNIT 22.2*)
Trypsin working solution (see recipe)
1% (v/v) fetal bovine serum (FBS): mix 1 ml FBS with 99 ml H_2O
Gurr's buffer, pH 6.8: dissolve 1 Gurr's buffer table (Bio/medical Specialties) in 1 liter sterile H_2O
Giemsa stain working solution (see recipe)

55° to 60°C drying oven
Forceps
Coplin jars
Phase-contrast microscope

1. Age the air-dried slide overnight in a drying oven at 55° to 60°C. Remove and bring to room temperature just prior to banding.
2. Grasp the slide with forceps and immerse it in a Coplin jar containing the 0.025% trypsin working solution for 8 to 10 sec, moving the slide back and forth.

   The exposure to trypsin will need to be adjusted depending on the quality of the cytogenetic specimen and the resulting banding (see Troubleshooting and Fig. 22.3.1).

Contributed by Jane Bayani and Jeremy A. Squire
Current Protocols in Cell Biology (2004) 22.3.1-22.3.7

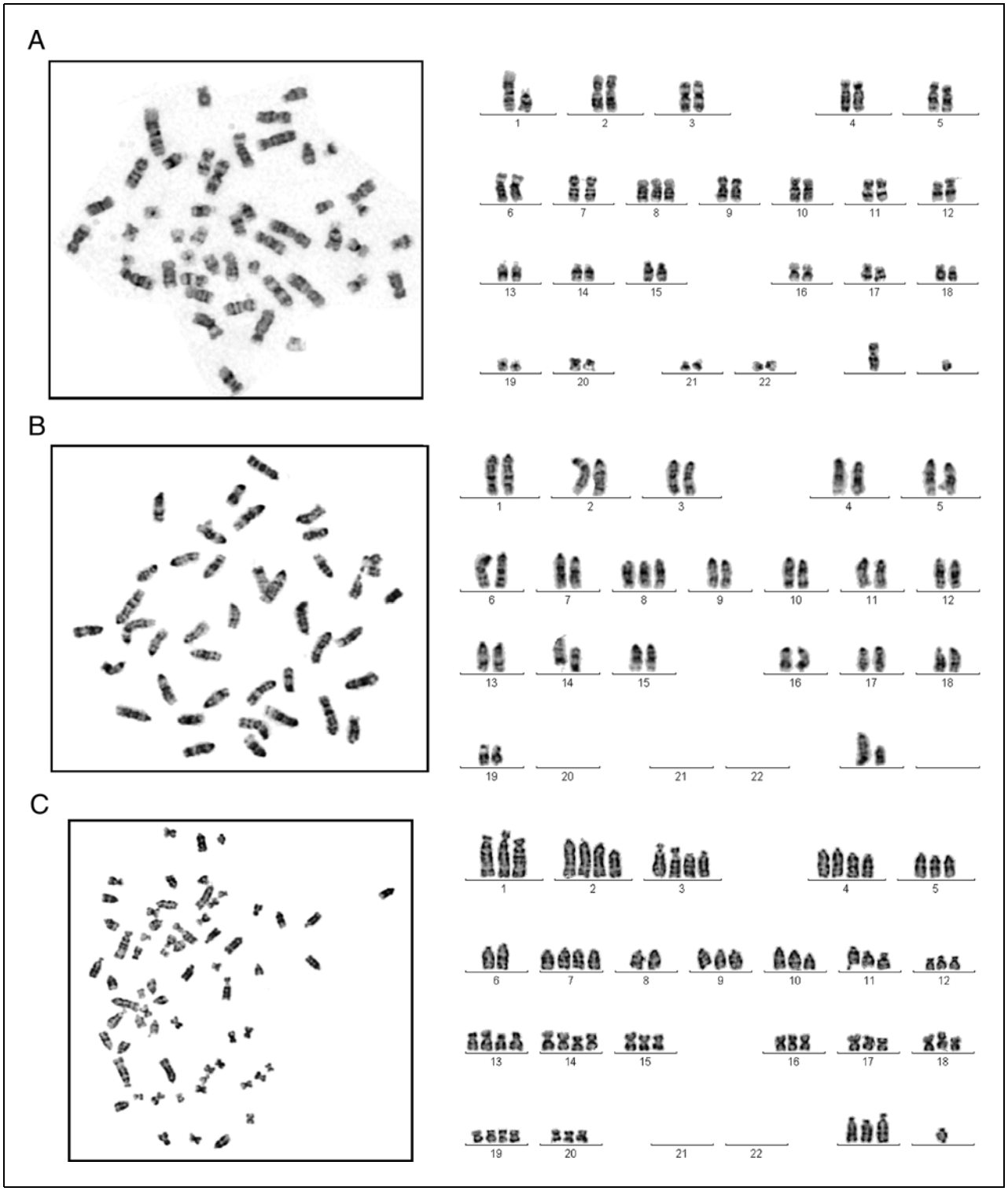


Figure 22.3.1 Images of G-banded metaphase spreads and karyotypes from human, mouse and rat tumors. For each panel, a G-banded metaphase spread and its karyotype has been shown for tumors derived from (**A**) human, (**B**) mouse, and (**C**) rat. All spreads show banding patterns that are well defined with few or no overlapping chromosomes.

3. Briefly rinse the slide in a Coplin jar containing 1% FBS to inactivate the trypsin.
4. Pre-rinse the slide by dipping in a Coplin jar containing Gurr's buffer, using the same agitation technique as in step 2.
5. Place slide in a Coplin jar containing Giemsa staining solution for 8 to 10 min.

The degree of staining will also require adjustment following visualization by phase-contrast microscopy.

6. Rinse slide in autoclaved distilled water until the stain no longer discolors the water, using the same agitation technique as in step 2.

   CAUTION: *Long exposure to water will result in destaining of the slide.*

7. Allow slide to air dry. Examine by light microscopy using a phase-contrast microscope to determine the quality of banding. Adjust trypsin exposure or duration of staining as required.

8. Once the optimal banding quality has been achieved, analyze the slides. Store the slides in a slide box free of dust and dirt at room temperature.

4′-6-DIAMIDINO-2-PHENYLINDOLE (DAPI) STAINING OF METAPHASE CHROMOSOMES

ALTERNATE PROTOCOL

In this protocol, cytogenetic specimens are stained with DAPI, which is typically mixed with an antifade mounting medium. Antifade mounting medium can be made in-house or bought commercially (e.g., Vectashield from Vector Laboratories). Unlike G-banding, DAPI staining requires no slide pretreatment and is fluorescently based.

Materials

Metaphase slide preparation (*UNIT 22.2*)
DAPI/antifade medium (see recipe)

Glass coverslips
Fluorescent microscope with DAPI filters

1. Add 50 μl of the DAPI/antifade medium to the metaphase slide preparation. Add a coverslip.

2. Visualize the chromosome banding pattern using fluorescence microscopy (Fig. 22.3.2). Store the slide at −20°C.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DAPI/antifade medium

DAPI stock solution: Dilute an entire container of DAPI (4′,6-diamidino-2-phenylindole, Sigma) powder with sufficient water to prepare a 100 μg/ml stock solution. Store in small aliquots up to several months at −20°C.

DAPI/antifade medium:

Combine in the following order:
5 ml PBS (*APPENDIX 2A*)
500.0 μl of 100 μg/ml DAPI stock (see above; 1 μg/ml final)
0.5 g *p*-phenylenediamine (Sigma; 10 mg/ml final)
Dissolve well, then add:
45 ml glycerol (90% final)
Total volume, 50.0 ml

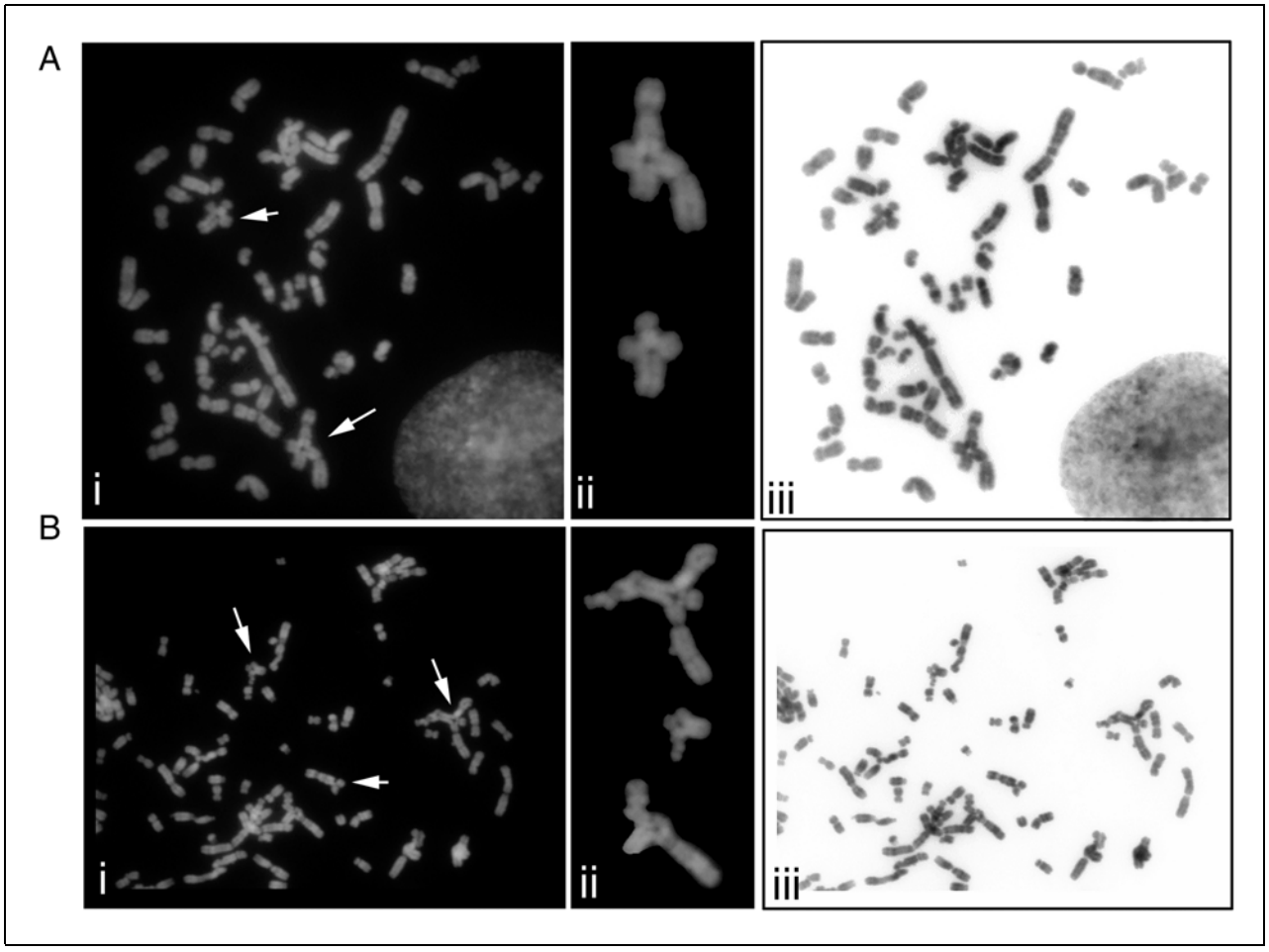


Figure 22.3.2 DAPI staining of a prostate cell line subjected to a chemotherapeutic agent and 4 Gy radiation. Shown in panel **A**, labeled "i," is a DAPI image of a prostate cell line subjected to mitomycin C treatment. Structural aberrations, e.g., radial structures, are labeled "ii.". Also in panel A is the inverted DAPI image (labeled "iii") demonstrating that DAPI banding provides similar banding patterns as G-banding, but at a lower resolution. Panel **B** illustrates the same prostate cell line subjected to 4 Gy of ionizing radiation, showing complex structural aberrations. Other aberrations, including DNA fragmentation, chromatid breaks, and dicentrics, can be identified. Arrows indicate multiradial chromosomal structures as shown enlarged in (ii).

The resulting medium is very viscous. Transfer to a 50-ml conical polypropylene centrifuge tube (e.g., Falcon), wrap tube in foil (product is light-sensitive), and place on an end-over-end rotator to ensure proper mixing (30 min). Store, protected from light, in 1-ml aliquots for several months at −20°C.

Giemsa stain working solution

1 ml Giemsa stain (Azure Blend, Harleco 620G/75, from EM Science; 2% v/v final)
50 ml Gurr's buffer solution, pH 6.8: dissolve 1 Gurr's buffer table (Bio/medical Specialties) in 1 liter sterile H_2O
Store up to 2 months at room temperature

Trypsin working solution 0.025%

0.5 g trypsin (Difco, 0.025% w/v final)
200.0 ml Earle's balanced salt solution (e.g., Life Technologies)
Store up to 1 day at room temperature

COMMENTARY

Background Information

Since chromosomes were first visualized in 1959, there has been an obvious need to determine ways of identifying individual chromosomes. Although size, shape, and the position of the centromere is clearly a means of identifying chromosomes, it became readily apparent that there were many chromosomes that were very similar in size and shape. Solid staining and chromosome measurement were used in the early 1960s; however, this still did not provide an adequate method for unequivocal chromosome identification. The Paris Conference of 1971 first systematically described chromosome bands that could be distinguished from adjacent segments on the basis of darker or lighter appearance by one or more techniques. Banded chromosomes were seen as appearing with a consistent series of dark and light bands. Human banding patterns and their nomenclature can be found in the International System of Cytogenetic Nomenclature (1995). Briefly, convention states that the short arm of a normal chromosome is called the p arm and the long arm is referred to as the q arm. A centromere is identified as a constriction along the length of the chromosome that defines the transition from the p to the q arm. Individual bands along each arm are designated numerically, with subdivisions of bands denoted as fractions of the main band. The resolution of bands is influenced by the length of the chromosome; hence, longer chromosomes produce more bands than shorter chromosomes.

There are several banding techniques that are described in Barch (1991). Today, G-banding is considered the gold standard for cytogenetics. Introduced in 1971 by Sumner et al. (1971), G-banding permits stable and effective banding of chromosomes without the need for fluorescence, which was used in an earlier technique called Q-banding (quinicrine banding). Through investigations by Sumner and colleagues, and others (reviewed in Barch, 1991), it was determined that the most effective means of revealing the banding pattern of human chromosomes was through digestion with a protease. Trypsin is used as the protease because it was found to effectively hydrolyze the proteins surrounding the chromatin. The differential protein composition along the length of the chromosome then reveals the variation of chromatin compaction associated with euchromatin and heterochromatin. These differences in chromatin condensation are easily revealed through the staining procedure.

Giemsa is a complex mixture of dyes including azure A, azure B, azure C, thionin, methylene blue, and eosin. Each dye component binds different protein moieties along the length of the chromosome, resulting the in typical banding patterns seen in Figure 22.3.1.

4′-6-diamidino-2phenylindole (DAPI) is a fluorescent dye that has an emission of 450 nm. It has AT specificity, which enables the elucidation of constitutive heterochromatin–containing regions. DAPI staining is typically used to detect centromeric regions, and it is useful in identifying pericentromeric breakpoints in chromosomal rearrangements. DAPI is also a good method for determining the quality of DNA on the slide and for identifying chromosomes when performing FISH. If the DNA fails to stain, it is often indicative of damaged DNA or slides that are too old to use.

Typical applications

Banding and staining of chromosomes permit the visualization of gross chromosomal abnormalities, including gains and losses of whole or partial chromosomes, the presence of extrachromosomal material, double-minute chromosomes, ring chromosomes, homogeneously staining regions (HSRs), chromosome fragments, and chromatid breaks (see Fig. 22.3.2). Banding and staining can also identify large structural abnormalities such as additions, deletions, inversions, and translocations. The technique has usefulness in xenograft studies, where the investigator can identify contaminating cells from the host (i.e., murine) and estimate the purity of the tissue. It is also useful for determining the extent of in vitro chromosomal (genetic) change that a given culture may have developed. Recently, investigations of genomic instability have used these simple methods for determining the rate of change of normal, tumor, or transfected cells using drug or radiation treatment (Gebhart et al., 1981; Ellis et al., 1999).

Critical Parameters

The most critical parameter in successful banding is a well spread metaphase with few overlapping chromosomes that are of good length. Generally there are spreads of varying chromosome lengths; thus the investigator is able to carry out effective analysis. Sometimes, particularly in abnormal cells, metaphase spreads will typically be too long or too short (see *UNIT 22.2*), a result of physiological responses to Colcemid treatment.

Table 22.3.1 Troubleshooting Guide to Banding Chromosomes

Problem	Cause	Solution
G-banding		
Solid staining	Insufficient trypsin treatment	Increase the trypsin incubation time in increments of 2-3 sec
	Excessive Giemsa staining time	Decrease the time in Giemsa. This will help to determine whether the trypsin time is sufficient.
No stain uptake	Excessive trypsin treatment	Decrease trypsin incubation time. Puffy chromosomes are also an indication of overtrypsinization.
	Insufficient Giemsa staining time	If the chromosomes do not appear overtrypsinized, increase the staining time in Giemsa. If the stain has been used for many specimens, the dye may be more dilute than before. If this is the case, prepare a fresh dye mixture.
Puffy chromosomes	Excessive trypsin treatment	Decrease trypsin incubation time. If the slides were not sufficiently aged prior to treatment, try more aggressive aging.
DAPI banding		
Solid staining	Insufficiently aged slides	Heat-treat the slides prior to DAPI staining or pass through an ethanol dehydration series.
	DAPI concentration too high	If the concentration of DAPI is too high (typically the case of in-house formulations), the bright signal intensity may obscure banding patterns. Remove coverslip and wash in 2 × SSC for 10 min at room temperature. Mount in a diluted DAPI/antifade medium (i.e., add more glycerol and PBS) and visualize again.
Puffy chromosomes	Insufficiently aged slides	Although no enzymatic treatment is required, very fresh chromosome preparations, and preparations made during high humidity (i.e., summer time), will produce puffy chromosomes with poor banding. Age slides artificially.

By phase-contrast microscopy, chromosomes should appear flat and range from pale gray to dark gray. The artificial aging of slides helps to sharpen the chromosome contours and increase band resolution. For G-banding, slides not aged sufficiently appear fuzzy after banding; this is due to the protease treatment. Very fat or puffy chromosomes, with little banding or stain uptake, are an indication of excessive trypsin treatment. Trypsin treatment (duration and concentration) must be optimized with each slide and for each batch of trypsin solution made. Duration adjustments will often be on the order of seconds. The brand of typsin and the supplier also influences trypsin digests markedly. Fetal bovine serum is used to inactivate the action of trysin; however, other rinses can be used such as 0.85% saline. Time spent in the Giemsa stain will also vary depending on the extent of trypsinization and the investigator's preference.

The criteria for DAPI banding are similar to those described above. Aged slides are preferred for DAPI banding, as discussed for Giemsa staining.

Careful consideration should be given to solution preparation and quality control. The trypsin solution may be used for the course of the day, although it loses its enzymatic activity as a function of use during this time period.

Be sure to take this into consideration, since a freshly prepared solution will digest more quickly than a solution that has been sitting for a few hours at room temperature or that has already been used to prepare several slides. Similarly, the concentration of Giemsa stain will become progressively more dilute, and the stain will start to precipitate as specimens are passed through it. As the day progresses, the stain requires an increase in incubation time to produce the same results. The stain is known for producing a slight film along the stain surface in the container. This can be removed by skimming the surface of the stain with some filter paper or a paper towel to prevent deposition of purple scum or precipitate on the slide as it is pulled out of the Coplin jar.

Troubleshooting

See Table 22.3.1 for troubleshooting chromosome banding techniques. The techniques are applicable to human and animal chromosomes.

Anticipated Results

Under the proper conditions, the resulting banding patterns should permit identification of the chromosomes and detection of gross anomalies or abnormal chromosomal DNA structures if present.

Time Considerations

Although the bench time is minimal, the most time-consuming portion of chromosomal banding is determining the correct trypsinization and staining times. Each specimen may require some minor adjustments.

Acknowledgements

The authors wish to thank Jana Karaskova, Department of Cellular and Molecular Biology, University Health Network, Ontario Cancer Insitute.

Literature Cited

Barch, M.J. 1991. The ACT Cytogenetics Laboratory Manual, 2nd Ed. Raven Press, New York.

Ellis, N.A., Proytcheva, M., Sanz, M.M., Ye, T.Z., and German, J. 1999. Transfection of BLM into cultured bloom syndrome cells reduces the sister-chromatid exchange rate toward normal. *Am. J. Hum. Genet.* 65:1368-1374.

Gebhart, E. 1981. Sister chromatid exchange (SCE) and structural chromosome aberration in mutagenicity testing. *Hum. Genet.* 58:235-254.

Mitelman, F. 1995. ISCN. 1995 An International System for Human Cytogenetic Nomenclature 1995. S. Karger, Basel, Switzerland.

Sumner, A.T., Evans, H.J., and Buckland, R.A. 1971. New technique for distinguishing between human chromosomes. *Nat. New Biol.* 7:232:31-32.

Contributed by Jane Bayani and
Jeremy A. Squire
University of Toronto
Ontario, Canada

Fluorescence In Situ Hybridization (FISH)

As early as 1988 (Lichter et al., 1988), FISH was used to visualize labeled DNA probes hybridized to chromosome and interphase nuclei preparations. The improvement of cloned DNA sources, antibodies, fluorochromes, microscopy and imaging equipment, and software has permitted a variety of scientific investigations.

This unit is divided into five parts—probe preparation, slide preparation, hybridization, post-hybridization washes, and interpretation. Probes are prepared by nick translation (see Basic Protocol 1) or degenerative oligonucleotide primer PCR (DOP-PCR; see Alternate Protocol 1) using hapten- or fluorochrome-labeled nucleotide. The amount of hapten label incorporated is quantified by dot blotting (see Support Protocol 1). Cytogenetic slide preparations are suitable for FISH (see Basic Protocol 2) if they are appropriately aged (see Support Protocol 2); these slides may be used even if they have previously been G-banded (see Alternate Protocol 2). In addition, slides made from paraffin-embedded tissues are suitable specimens for hybridization with DNA (see Alternate Protocol 3) or protein nucleic acid (PNA) probes (see Alternate Protocol 4). Hybridization conditions must be appropriate for both the sample and probe materials: cytogenetic slides can be hybridized with DNA (see Basic Protocol 3) and PNA (see Alternate Protocol 5) probes, as can paraffin sections (see Alternate Protocols 6 and 7). Post hybridization wash and detection conditions vary depending on the probe—indirectly labeled DNA probes (see Basic Protocol 4), directly labeled DNA probes (see Alternate Protocol 8 or 9), and peptide nucleic acid (PNA) probes (see Alternate Protocol 10).

NOTE: Coplin jars containing solutions at temperatures other than room temperature should be prewarmed and maintained in a water bath at the specified temperature.

LABELING DNA PROBES FOR FISH

This section describes methods for labeling probes for fluorescence in situ hybridization (FISH) analysis. The first protocol (see Basic Protocol 1) describes DNA labeling by nick translation either indirectly with a hapten (biotin or digoxigenin) or directly with a fluorochrome (*APPENDIX 1E*) conjugated to dUTP. This method is appropriate for most DNAs listed in Table 22.4.1; however, smaller DNAs, such as cDNAs, may require labeling by PCR methods, which are also described (see Alternate Protocol 1). The choice of label, whether direct or indirect, is based on the type of DNA probe, its size, and the application. Small probe inserts (such as cDNAs) should be labeled with a hapten, enabling the option of signal amplification. Larger inserts can be both directly or indirectly labeled (Table 22.4.1). The probe's signal strength can vary depending on the application and sequence specificity (Table 22.4.2). Furthermore, the choice of label or fluorochrome will also depend on the availability of conjugated antibodies, the nature of the experiment, and the availability of filters for the microscope utilized for visualization. A list of fluorochrome properties can be found in Table 22.4.3. For both labeling methods discussed here, assessment of probe labeling is determined by size, gel electrophoresis, and for indirectly labeled probes, dot blot analysis of incorporation (see Support Protocol 1). The labeled DNA is ethanol precipitated in the presence of excess unlabeled DNAs, which serve as carriers (sonicated salmon sperm DNA) and as suppressors of repetitive sequences (Cot-1 DNA). The final product is resuspended in a hybridization buffer and is then ready for use in FISH experiments (see Hybridization). The labeling procedures outlined can be applied to all species of DNA.

Contributed by Jane Bayani and Jeremy A. Squire
Current Protocols in Cell Biology (2004) 22.4.1-22.4.52

Table 22.4.1 Sources of DNA Probes for FISH Analysis[a]

Type	Application	Amplification of signal required	Labeling methods applicable	Indirect or direct labeling
Plasmids (10–20 kb insert)	Locus specific	Yes	Nick translation and PCR	Indirect
Cosmids (15–30 kb insert)	Locus specific	Yes	Nick translation and PCR	Indirect
PACs (~100 kb insert)	Locus specific	No	Nick translation and PCR	Both
BACs (~200 kb insert)	Locus specific	No	Nick translation and PCR	Both
YACs (300–1.5 Mb insert)	Locus specific	No	Nick translation and PCR	Direct
Genomic DNA from flow sorted chromosomes	Chromosome paints	No	PCR labeling	Both
Genomic DNA from microdissected DNA	Locus specific	Yes	PCR labeling	Both

[a]Shown are the most common types of DNAs used as probes for FISH analysis. Many of these DNAs can be labeled by nick translation (see Basic Protocol 1) or PCR (see Alternate Protocol 1). For probe inserts <2.0 kb, labeling by PCR is recommended.

Table 22.4.2 Different Classes of Commonly Used Probes for FISH Analysis[a]

Type	Fluorescence intensity	Typical applications
Centromere probes	Strong	Ideal probe for the beginner to learn basic FISH techniques. Commonly used to enumerate chromosomal monosomies and trisomies, and for sex determination in transplantation studies.
Subtelomere specific probes	Moderate/weak	Used for determining whether small terminal rearrangements near telomeres have taken place.
Chromosome paints	Strong/moderate	Useful for identifying small marker chromosomal aberrations where a specific chromosome is suspected. Also useful in confirming SKY or MFISH findings.
Translocation junction unique-sequence probes	Moderate	Used for detecting the presence of specific translocations in interphase cells or in metaphase spreads.
Microdeletion unique-sequence probes	Moderate	Identification of small submicroscopic deletions using metaphase preparations.
Probes detecting gene amplification	Strong	For detecting oncogene copy number increases (gene amplification) in interphase and metaphase cells

[a]Shown are the most common types of commercially available probes used in FISH experiments and their typical applications. Signal strength will vary depending on the size and application of the probe as well as the target DNA specimen.

Another choice for FISH analysis is a peptide nucleic acid (PNA) probe. These probes also bind DNA in a sequence-specific manner but are created by oligonucleotide synthesis. The fabrication of PNA probes is not easily accomplished in a typical molecular cytogenetic laboratory, so they are currently only available commercially (see Internet Resources).

BASIC PROTOCOL 1

Labeling FISH Probes by Nick Translation

This protocol, adapted from Beatty et al. (2002), outlines the basic steps for either directly or indirectly labeling DNA probes for FISH analysis. Once labeled probes are ethanol precipitated, they are ready for final preparation prior to hybridization to the target specimen.

Materials

DNA probe (i.e., cosmid, plasmid, PAC, BAC, or YAC; Table 22.4.1)
10× nick translation buffer (see recipe)

Table 22.4.3 Fluorescent Properties of Labels and DNA Stains Used for FISH[a]

Label	Excitation (nm)	Emission (nm)
Haptens[b]		
Digoxigenin	NA	NA
Biotin	NA	NA
Fluorochromes		
Alexa 488[c]	490	520
Alexa 532[c]	525	550
Alexa 546[c]	555	570
Alexa 594[c]	590	615
Amino-methyl coumarin	399	445
Cascade Blue	400	420
Cyanine 2[d]	489	506
Cyanine 3[d]	550	570
Cyanine 5[d]	649	670
Cyanine 7[d]	743	767
Fluorescein isothiocyanate (FITC)	495	523
Rhodamine B	560	580
Spectrum Aqua[e]	433	480
Spectrum FRed[e]	655	675
Spectrum Green[e]	497	524
Spectrum Gold[e]	530	555
Spectrum Orange[e]	559	588
Spectrum Red[e]	587	612
Tetramethylrhodamine isothiocyanate (TRITC)	550	570
Texas Red	595	610
DNA stains		
Chromomycin A3	430	570
4′,6-Diamindine-2-phenylindole (DAPI)	538	461
Ethidium bromide	518	615
Hoechst 33258 (bis-benzimide)	352	461
Propidium bromide	535	617

[a]Shown are the commonly used fluorochromes and DNA stains used in FISH analysis. Of these, DAPI is the most widely used.
[b]Haptens, such as digoxigenin and biotin are detected with antibodies conjugated to one of the fluorochromes listed.
[c]The Alexa family of fluorochromes can be purchased from Molecular Probes.
[d]The Cyanine family of fluorochromes can be purchased from Amersham Biosciences.
[e]The Spectrum family of fluorochromes can be purchased from Vysis.

DNase I dilution buffer (see recipe)
1 mM dNTP mixture (see recipe)
Fluorochrome/dTTP or hapten/dTTP mixture (see recipes)
3 mg/ml DNase I (see recipe)
10 U/μl *E. coli* DNA polymerase I (Roche)
5 × loading dye (see recipe)

2% (w/v) agarose gel (see recipe)
100-bp DNA ladder
Sonicated salmon sperm DNA standards (i.e., 12.5, 25.0, and 500 ng/μl; see recipe)
1× TBE buffer (see *APPENDIX 2A*)
300 mM EDTA (*APPENDIX 2A*)
10 mg/ml sonicated salmon sperm DNA (Invitrogen)
1 μg/μl human or mouse Cot-1 DNA (Invitrogen)
3 M sodium acetate (*APPENDIX 2A*)
100% ethanol
70% ethanol, cold
Hybridization buffer (see recipe)

Water bath or PCR machine
0.5-ml PCR tube (optional)

Additional reagents and equipment for agarose gel electrophoresis (*APPENDIX 3A*) and determination of hapten incorporation by dot blot analysis (see Support Protocol 1)

Prepare equipment and reagents

1. Set water bath or PCR machine to 15°C.
2. Put DNA probe and all labeling reagents (i.e., 10× nick translation buffer, DNase I dilution buffer, 1 mM dNTP mixture, and fluorochrome/dTTP or hapten/dTTP mixture) on ice.
3. Prepare 10× DNase I solution (1.0 ml total) by combining 1.0 μl of 3 mg/ml DNase I and 999.0 μl DNase I dilution buffer. Place on ice.

Perform labeling reaction

4. For each DNA to be labeled, prepare the following mixture in a 1.5-ml microcentrifuge or 0.5-ml PCR tube, adding the 10× DNase I solution and *E. coli* polymerase I last:

   2 μg DNA probe
   10.0 μl 10× nick translation buffer
   5.0 μl 1 mM dNTP mixture (i.e., dATP, dCTP, dGTP)
   4.0 μl fluorochrome/dTTP or hapten/dTTP mixture
   10.0 μl 10× DNase I solution
   2.5 μl *E. coli* DNA polymerase.
   Adjust the volume to 100 μl with water.

   The amount of DNase I solution added will vary (see Commentary).

   A probe prepared using hapten/dTTP is indirectly labeled, while one prepared with fluorochrome/dTTP is directly labeled.

5. Incubate 90 to 120 min at 15°C (see Table 22.4.22 and Critical Parameters).
6. After incubation, place tubes on ice.

Determine size of products from labeling reaction

7. Transfer 10 μl labeling reaction to a fresh tube and mix with 4 μl of 5× loading dye. Load onto a 2% agarose gel (*APPENDIX 3A*) along with a 100-bp DNA marker and 10 μl of each salmon sperm concentration standard.

   The salmon sperm standards are used to help determine the amount of labeled DNA probe, as well as to act as a molecular-size marker.

8. Electrophorese at 100 V for ~20 min or until the dye front is two thirds of the way through the gel.

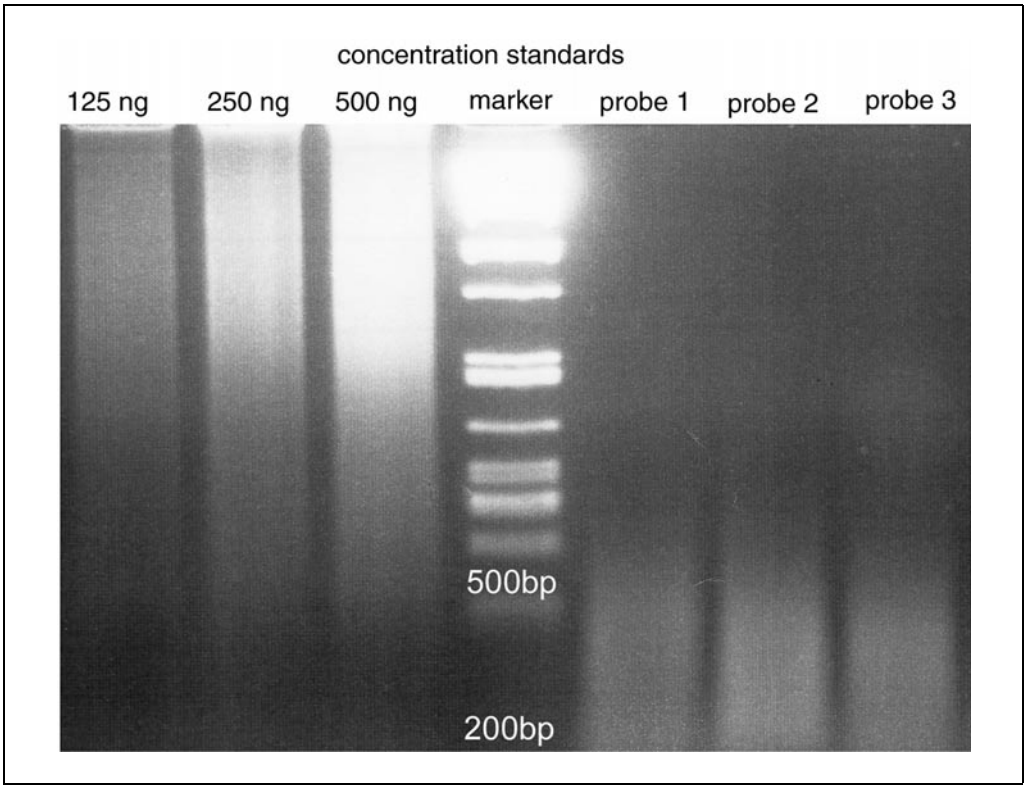


Figure 22.4.1 Nick translated DNA fragments electrophoresed on a 2% agarose gel. A volume of 10.0 μl labeled product was loaded. The fragment sizes range from 200 to 500 bp as determined from the molecular marker. The estimated concentration of the probe is 200 ng in 10 μl, yielding ~20 ng/μl.

The final fragment sizes should be between 200 and 500 bp, with ~200 ng labeled DNA present in the gel, assuming the original amount of labeled probe was 2 μg. This can be ascertained using the sonicated salmon sperm concentration standards (Figure 22.4.1).

9. If the fragment sizes are too large, return the labeling reaction to 15°C for 20 to 30 min (or time as required) and reassess the size of the fragments (i.e., repeat steps 5 to 8).

It may be necessary to spike the labeling reaction with additional DNase I solution and E. coli DNA polymerase I. If the fragments are too small, labeling (step 4) will need to be repeated with an adjustment in the amount of DNase I added to the labeling reaction. If there is no significant change in fragment size following further digestion, the investigator should start again and review Table 22.4.22 for possible reasons of insufficient digestion.

Determine amount of labeled probe

10. Once the proper labeling size has been achieved, add 10 μl of 300 mM EDTA to the labeling reaction to stop the action of the enzymes.

11. Estimate the amount of labeled probe on the gel using the salmon sperm standards as a guide. Calculate the amount of labeled DNA remaining in the tube.

Be sure to consider the volumes removed from the starting volume for loading onto the gel(s) when calculating the amount of labeled DNA left in the tube.

Based on the concentration standards, the labeled probe in Figure 22.4.1 appears to be somewhere between 125 ng/10 μl (1.25 ng/μl) and 250 ng/10 μl (25.0 ng/μl). A conservative estimate is 200 ng/10 μl (20 ng/μl). Thus, from an original 100 μl starting volume, 10 μl was removed for a gel, leaving 90 μl. If the estimated concentration is 20 ng/μl, the remaining labeled probe is 1800 ng (20 ng/μl × 90 μl = 1800 ng).

Add carrier and supressor of repetitive sequences

12. For each 1 μg labeled DNA in the tube, add 5 μl of 10 mg/ml sonicated salmon sperm DNA (50 μg).

    For example, for 1.8 μg DNA, add ~100 μg sonicated salmon sperm DNA.

13. For each microgram labeled DNA in the tube, add 5 to 10 μg human or mouse Cot-1 DNA.

    For the example given above (1.8 μg labeled DNA), ~20 μg Cot-1 DNA is required

    The amount of Cot-1 used is dependent on the number and extent of repeat elements in the DNA insert used as the probe. Generally, cDNA probes will have fewer repeats than genomic probes, so they will require relatively less Cot-1 suppression. BAC and YAC probes may need more suppression.

Determine incorporation

14. If the DNA probe has been labeled with hapten, check incorporation by dot blot analysis (see Support Protocol 1).

Ethanol precipitate DNA

15. Add 0.1 vol. of 3 M sodium acetate followed by 2.5 vol of 100% ethanol.

16. Incubate overnight at −20°C or 2 to 3 hr at −80°C.

    The recovery of DNA during the precipitation process can be increased by lengthening the time at −20°C or −80°C.

17. Microcentrifuge 20 min at 13,000 rpm, 4°C.

Resuspend DNA

18. Carefully remove the supernatant and wash the pellet with cold 70% ethanol.

19. Microcentrifuge 20 min at 13,000 rpm, 4°C.

20. Remove the ethanol and allow the pellet to air dry.

21. Resuspend the pellet in hybridization buffer to a final concentration of 10 ng/μl. Store labeled probe at −20°C up to several months (directly labeled probe) or several years (indirectly labeled probe).

    The final concentration should be based on the labeled DNA content, not the total DNA content, which includes the unlabeled Cot-1 and salmon sperm DNA. Thus, for a labeled DNA amount of 1.8 μg, 180 μl hybridization buffer should be added to reach a final concentration of 10 ng/μl.

    The labeled probe can now be used for FISH experiments.

    The amount of labeled probe used will depend on the area of the slide to be hybridized. For example, 10 μl is sufficient to cover a 22 × 22–mm coverslip, 20 μl is sufficient to cover a 22 × 30–mm coverslip, and 30 μl is sufficient to cover a 22 × 50–mm coverslip.

ALTERNATE PROTOCOL 1

Labeling Probes for FISH by Degenerative Oligonucleotide Primer Polymerase Chain Reaction (DOP-PCR)

This protocol describes direct and indirect labeling procedures using PCR labeling. The user has various choices in the primer sets. These may be specific primers for a region, specific primers for vector sequences, universal primers, or DOP primers.

Additional Materials *(also see Basic Protocol 1)*

DNA template (Table 22.4.1)
10× PCR buffer: 100 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)/500 mM KCl (store up to several months at −20°C)

2 mM dNTP mixture (i.e., dATP, dCTP, dGTP; see recipe)
5 μM each primers 1 and 2 (*APPENDIX 3F*)
50 mM $MgCl_2$
5 to 10 U/μl *Taq* DNA polymerase
Water, sterile
Mineral oil, sterile

1. For each labeling reaction, mix the following:

   1 to ng DNA template
   5.0 μl 10× PCR Buffer
   4.0 μl 2 mM dNTP
   2.0 μl 1 mM fluorochrome/dTTP or hapten/dTTP
   1.5 μl 5 μM primer 1
   1.5 μl 5 μM primer 2
   1.5 μl 50 mM $MgCl_2$
   0.5 μl 5–10 U/μl *Taq* DNA polymerase.

   Adjust final volume to 50.0 μl with sterile water. Overlay the mixture with sterile mineral oil and put on ice until ready to place in the thermocycler.

   The 10× PCR buffer is often supplied with the enzyme.

2. Place in a thermocycler and program the following steps:

Initial step:	5 min	93°C	(denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	90 sec	72°C	(extension)
Final step:	5 min	72°C	(extension)
Hold:	indefinitely	4°C.	

3. Mix 5 μl labeled reaction with 1 μl of 5× loading dye and run on a 2% (w/v) agarose gel with molecular weight markers and sonicated salmon sperm DNA concentration standards as described (see Basic Protocol 1, steps 7 and 8).

   The final PCR product should fall between 200 and 500 bp.

4. Calculate the amount of labeled DNA remaining in the tube, add carrier and supressor DNA, precipitate, and resuspend in hybridization buffer as described (see Basic Protocol 1, steps 11 to 20).

5. If the DNA probe has been labeled with hapten, check incorporation by dot blot analysis (see Support Protocol 1).

Determination of Hapten Incorporation by Dot Blot Analysis

SUPPORT PROTOCOL 1

Gel electrophoresis of the labeled DNA FISH probe allows the investigator to determine whether the DNA has been nicked (see Basic Protocol 1) or amplified (see Alternate Protocol 1) to the appropriate sizes. It does not, however, allow determination of whether the hapten has been incorporated into the DNA. Using a dot blot assay, the degree of hapten incorporation can be assessed to determine the efficiency of the labeling protocol and the activity of the DNA polymerase I or *Taq* DNA polymerase. This method is useful in determining whether the lack of FISH signal is due to a poorly labeled probe or to hybridization or slide preparation factors. This method involves spotting labeled product onto a nylon filter along with a control. The labeled DNA is detected by immunolabeling and colorimetric visualization.

Materials

100 mM Tris·Cl (pH 7.5)/15 mM NaCl (*APPENDIX 2A*)
Indirectly labeled probe (i.e., with biotin or digoxigenin; Table 22.4.3)
Control DNA labeled with biotin or digoxigenin (i.e., labeled DNA probe known to produce good signal strength)
0.5% (w/v) BSA in 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)/15 mM NaCl (store up to 6 months at 4°C)
AP-labeled antibody mixture (see recipe)
100 mM Tris·Cl (pH 9.5)/100 mM NaCl/50 mM $MgCl_2$ (*APPENDIX 2A*)
NBT/BCIP (see recipe)

Charged nylon membrane (~5 × 5 cm per sample)
Filter paper
37°C dry oven
Rotating platform

Prepare and load nylon membrane

1. Cut a charged nylon membrane to a size appropriate for the number of samples.
2. Soak the membrane 5 min in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl, room temperature. Blot the membrane with filter paper.
3. Pipet aliquots of indirectly labeled probe and control DNA labeled with biotin or digoxigenin in a dilution series (e.g., 200, 100, 50 ng) onto the membrane, leaving ample space between spots. Incubate 5 to 10 min, room temperature.

Block and label membrane

4. Incubate the membrane 1 min in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl, making sure it is saturated.
5. Incubate the membrane 30 min in 0.5% (w/v) BSA in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl at room temperature.
6. Transfer the membrane to an alkaline phosphatase–labeled antibody mixture in a plastic container and incubate 30 min in a 37°C dry oven on a rotating platform, making sure the membrane is completely saturated.

Develop dot blot

7. Remove the membrane and wash 15 min with 100 mM Tris·Cl (pH 7.5)/15 mM NaCl.
8. Remove the membrane and wash 2 min with 100 mM Tris·Cl (pH 9.5)/100 mM NaCl/50 mM $MgCl_2$, room temperature.
9. Remove the membrane and incubate 5 to 10 min in NBT/BCIP solution under dimmed lighting until the blot develops.
10. Wash the membrane with water and air dry.

The membrane can be stored at room temperature. Over time the intensity of the chemical stain will fade. Typical results are shown in Figure 22.4.2.

TARGET SLIDE PREPARATION

Slide quality can greatly influence the success of FISH assays. When slide preparations are less than optimal, treatment with a protease can decrease the risk of high background and increase access of the probe to the DNA target by removing protein barriers to allow efficient hybridization. FISH carried out on paraffin sections requires more aggressive

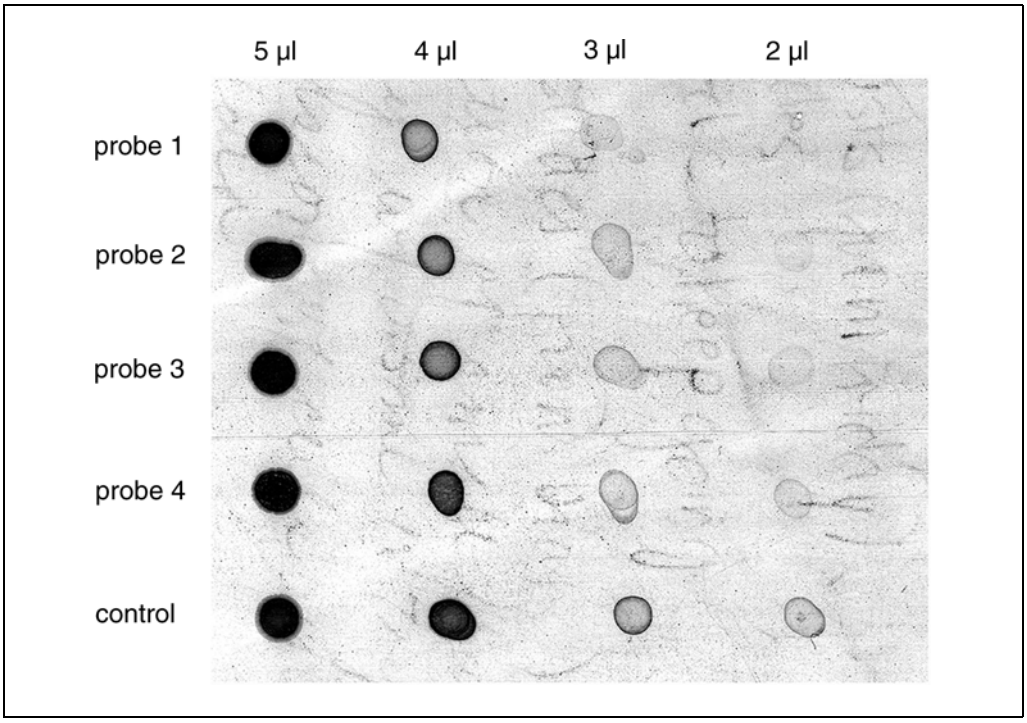


Figure 22.4.2 Dot-blot analysis of biotin incorporation in probes shown in Figure 21.4.1. Compared to control labeled DNA, the labeled probes appear to have incorporated biotin well, with probe 4 showing the greatest incorporation compared to probe 1. The concentration of each probe was estimated at 20 ng/µl; however, it is evident that the labeling efficiency was greater in some probes over others.

protease treatment and assessment. Following optimal slide pretreatment, the specimen is subjected to denaturation, causing double-stranded DNA to become single stranded. This will permit the efficient hybridization of the denatured probe to the DNA target on the slide.

Preparation of Cytogenetic Specimens for FISH

BASIC PROTOCOL 2

The following protocol describes the pretreatment of cytogenetic slides (*UNIT 22.2*) for FISH analysis with DNA probes (in-house or commercial) or commercially obtained PNA probes. In this protocol, pepsin is recommended for use as the protease; however, proteinase K is also often used. The concentration and conditions for proteinase K treatment are also noted in the protocol. The treatment with pepsin will vary depending on the extent of digestion required on the slide. In some cases, pepsin treatment is not needed.

Materials

Cytogenetic slide preparation (*UNIT 22.2*): age naturally at least 2 days at room temperature or artificially (see Support Protocol 2)

10% (w/v) pepsin—100 mg pepsin powder (Sigma) in 1.0 ml H_2O; store in 20-µl aliquots up to several months at −20°C—and 0.01 M HCl, 37°C *or*

14 mg/ml proteinase K (Roche Diagnostics) and proteinase K buffer—i.e., 20 mM Tris·Cl, pH 7 (*APPENDIX 2A*)/0.2 mM CaCl; store up to several months at room temperature

1× PBS (*APPENDIX 2A*)

70% formamide/2× SSC (pH 7.0), 72°C (see *UNIT 18.6* for SSC): prepare fresh

70% ethanol, ice-cold (for DNA probes)

Table 22.4.4 Coplin Jars Needed for Preparation of Cytogenic Specimens for FISH[a]

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	70% ethanol[b]	4°C
1 or 2[b]	80% ethanol	Room temperature
1 or 2[b]	100% ethanol	Room temperature
1	70% formamide in 2× SSC[b]	72°C
1	1× PBS	Room temperature
1	Pepsin/0.01 HCl *or* proteinase K/buffer	37°C or room temperature (respectively)

[a]See Basic Protocol 2.
[b]For DNA probes only.

Phase contrast microscope (*UNIT 4.1*)
Coplin jars

Additional reagents and equipment for hybridization (see Basic Protocol 3 and Alternate Protocol 5)

CAUTION: Formamide is a carcinogen and should be handled with care. Discard according to biohazard rules of the institution.

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.4.

Detect and remove cytoplasmic contamination by protease treatment

1. Using a phase-contrast microscope, determine the extent of cytoplasmic residue on the cytogenetic slide preparation. If there is no cytoplasm, proceed to step 6.

2a. *For pepsin digestion:* Add 10 to 15 µl of 10% (w/v) pepsin to 50 ml warm 0.01 M HCl in a Coplin jar. Incubate the slides in the protease 5 min at 37°C.

2b. *For proteinase K digestion:* Dilute 14 mg/ml proteinase K to a final concentration of 0.1 µg/ml in proteinase K buffer in a Coplin jar. Incubate the slides in the protease 6.5 min at room temperature.

Remove protease and determine efficacy

3. Wash the slide 5 min at room temperature in a Coplin jar containing 1× PBS.

4. Pass the slide through a dehydrating series of 70%, 80%, and 100% ethanol in Coplin jars for 5 min each. Allow to air dry after the final ethanol treatment (~5 min).

5. View the slide using phase-contrast microscopy to determine the extent of protein digestion. If necessary, repeat the protease step.

Prepare slides for hybridization

6a. *For PNA probes:* Proceed to hybridization (see Alternate Protocol 5).

6b. *For DNA probes:* Denature the slide for the recommended time (see Table 22.4.5) in 70% formamide/2× SSC (pH 7.0), 72°C.

The time will vary according to the age and quality of the slide.

7b. Promptly place the slide into a Coplin jar containing ice-cold 70% ethanol for 5 min. Pass the slides through a dehydration series of 80% and 100% ethanol for 5 min each.

Table 22.4.5 Formamide Denaturation Guide for Cytogenetic Specimens[a]

Slide criteria	Suggested denaturation time[b]
Fresh slide stored 1–2 days at room temperature	60–90 sec
Fresh slide artificially aged 1–2 days	60–90 sec
Aged slide stored 1–2 weeks at room temperature	1.5–2 min
Aged slide stored 2–4 weeks at room temperature	2 min
Aged slides stored 1–2 months	2–3 min
Previously G-banded slides >1-week old	30–40 sec
Previously G-banded slides <1-week old	20–30 sec

[a]Shown are general guidelines for denaturation of slides of varying ages and conditions. The investigator should pay attention to the slide quality and monitor the changes in denaturation conditions as the slides age or are processed. Each laboratory will possess different environmental conditions as well as slide making procedures, which will affect the denaturation procedures significantly.
[b]Denaturation conditions are 72°C in 70% formamide/2× SSC.

8b. Air-dry the slide after the final ethanol treatment and proceed to hybridization (see Basic Protocol 3).

Artificial Aging of Cytogenetic Slide Preparations for FISH

SUPPORT PROTOCOL 2

The chromosomes on freshly prepared slides are often too fragile for immediate use—i.e., the high temperatures used during the slide denaturation procedure can damage the DNA, making it less optimal for hybridization. Such slides require aging, which can be achieved by allowing them to naturally age a few days at ambient temperature; however, an experiment must occasionally be performed immediately. The protocol below outlines a method for artificially aging freshly prepared slides so that FISH results are available within 12 to 24 hr after preparation from the cytogenetic suspension. This method may also be used when the relative humidity in the laboratory is high due to local weather conditions.

To age, incubate a freshly prepared cytogenetic slide (*UNIT 22.2*) at least 1 hr (up to 3 hr) in a Coplin jar containing 2× SSC (*UNIT 18.6*), 37°C. Remove the slide and pass through a dehydration series of 70%, 80%, and 100% ethanol for 5 min each, and air dry (5 to 10 min). Proceed with enzyme digestion if required.

Note that a list of all Coplin jars used in this protocol is given in Table 22.4.6.

Preparation of Previously G-Banded Cytogenetic Specimens for FISH

ALTERNATE PROTOCOL 2

The following method outlines the steps involved in pretreating a previously Giemsa (G)-banded cytogenetic slide (*UNIT 22.3*) for subsequent use in FISH analysis. The slides must be completely free of any oils and must also be destained. Since the slide has already been treated with a protease, it will require no additional protease digestion; however, stripping the proteins, which are protective to the DNA, will cause it to become much more sensitive to degradation during the denaturation process. The ability to re-use a previously banded slide for FISH will be dependent on the age of the slide, the degree of trypsin digestion during the banding, and whether residual immersion oils or mounting buffers have degraded the DNA.

Materials

Banded cytogenetic slide specimen (*UNIT 22.3*)
Xylene

Table 22.4.6 Coplin Jars Needed for Artificial Aging of Cytogenetic Slide Preparations for FISH[a]

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	80% ethanol	Room temperature
1	100% ethanol	Room temperature
1	2× SSC	37°C

[a]See Support Protocol 2.

Table 22.4.7 Coplin Jars Needed for Preparation of Previously G-Banded Cytogenetic Specimens for FISH[a]

No. Coplin jars	Contents	Temperature
1	70% ethanol	4°C
1	70% ethanol	Room temperature
2	80% ethanol	Room temperature
2	100% ethanol	Room temperature
1	70% formamide/2× SSC, pH 7.0	72°C
1	Methanol	Room temperature
1	Xylene[b]	Room temperature

[a]See Alternate Protocol 2.
[b]*CAUTION:* Maintain in a fume hood.

Methanol
70%, 80%, and 100% ethanol
70% formamide in 2× SSC (pH 7.0; *UNIT 18.6*), 72°C: prepare fresh
70% ethanol, ice-cold
Coplin jars

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.7.

1. Remove residual oils from previously banded cytogenic slide specimen by incubating 5 min in a Coplin jar containing xylene under a vented chemical hood.
2. Destain by incubating the slide ~5 to 10 min in a Coplin jar containing room-temperature methanol.
3. Pass the slide through a dehydrating series of 70%, 80%, and 100% ethanol for 5 min each. Allow the slide to air dry (5 to 10 min) after the final ethanol treatment.
4. Denature slide 20 to 30 sec in 70% formamide/2× SSC (pH 7.0), 72°C.

   The time will vary according to the age and quality of the slide (Table 22.4.5).

5. Promptly place the slide into a Coplin jar containing ice-cold 70% ethanol for 5 min following denaturation.
6. Proceed through a dehydration series of 80% and 100% ethanol for 5 min each. Air-dry the slide after the final ethanol treatment and proceed with hybridization (see Hybridization).

Preparation of Paraffin-Embedded Specimens for Hybridization with DNA Probes

FISH analysis on paraffin sections is becoming increasingly important in both diagnostic and research laboratories. This protocol outlines the steps involved in pretreating paraffin sections for FISH. While the preparation of paraffin-embedded specimens can be modified, the most critical component is the quality of the starting section (see Commentary). Different histopathology laboratories will have various methods of tissue fixation which should be taken into consideration when assessing the success of an experiment.

Materials

5- to 10-μM paraffin sections on silanized slides
Xylene
100% ethanol
0.5% (w/v) pepsin in 0.85% (w/v) NaCl (pH 1.5), 45°C (see recipe)
2× SSC, pH 7.0 *(UNIT 18.6)*
Propidium iodide (PI) or DAPI in antifade (see recipes)
70%, 80% and 100% ethanol

Coplin jars
45°C water bath
Fluorescence microscope (*UNIT 4.2*) with a FITC, and PI or DAPI filter set

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.8.

Remove wax

1. Add 5- to 10-μM paraffin sections on silanized slide to a Coplin jar containing xylene. Gently agitate 5 min at room temperature. Transfer slides to a second Coplin jar containing fresh xylene for an additional 5 min with gentle agitation.
2. Transfer the slide to a Coplin jar containing 100% ethanol. Soak 5 min, then transfer slides to fresh 100% ethanol and soak another 5 min. Agitate 2 to 3 times during each 5-min period.
3. Remove the slide from ethanol and allow to air dry (5 to 10 min).

Perform protease digestion and counterstain

4. Place slide in a Coplin jar containing 0.5% (w/v) pepsin in 0.85% NaCl, 45°C. Incubate 15 to 20 min in a 45°C water bath (see Table 22.4.5).
5. Rinse the slide 20 to 30 sec in a Coplin jar containing 2× SSC, pH 7.0.

Table 22.4.8 Coplin Jars Needed for Preparation of Paraffin-Embedded Specimens for Hybridization with DNA Probes[a]

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	80% ethanol	Room temperature
3	100% ethanol	Room temperature
1	0.5% pepsin in 0.85% NaCl	45°C
4	2× SSC, pH 7.0	Room temperature
2	Xylene[b]	Room temperature

[a] See Alternate Protocol 3.
[b] *CAUTION:* Maintain in a fume hood.

Table 22.4.9 Troubleshooting Guide for Pretreatment of Paraffin-Embedded Sections

Problem	Cause	Solution
Green autofluorescence upon inspection/or poor uptake of counterstain	Under-digestion with pepsin solution	Remove coverslip and wash with 2× SSC as described (see Alternate Protocol 3, step 5). Return the slide to pepsin digestion buffer (see Alternate Protocol 3, step 4). The time required for subsequent protease treatment will be dependent on the existing degree of digestion.
		Should repeated attempts at digestion fail, consider another enzyme such as proteinase K (use at 10 mg/ml in 2× SSC for 20 min at 37°C) in place of the pepsin buffer (see Alternate Protocol 3, step 4) or in combination with the pepsin buffer.
Lifting of tissue from slide	Loosening by protease treatment	Occasionally the protease treatment will loosen the tissue from the slide. Try to work carefully such that the tissue is relatively undisturbed.
	Glass slides not coated with silane	Associated with this is the use of charged or silanated slides. Many pathology labs will use coated slides. Check if the sections were mounted onto such slides. If not, try to obtain sections on these slides.
Holes in tissue or "ghosty" appearance	Over-digestion	Over-digestion will be evident from the degradation of the tissue. Start again and decrease the tissue treatment.

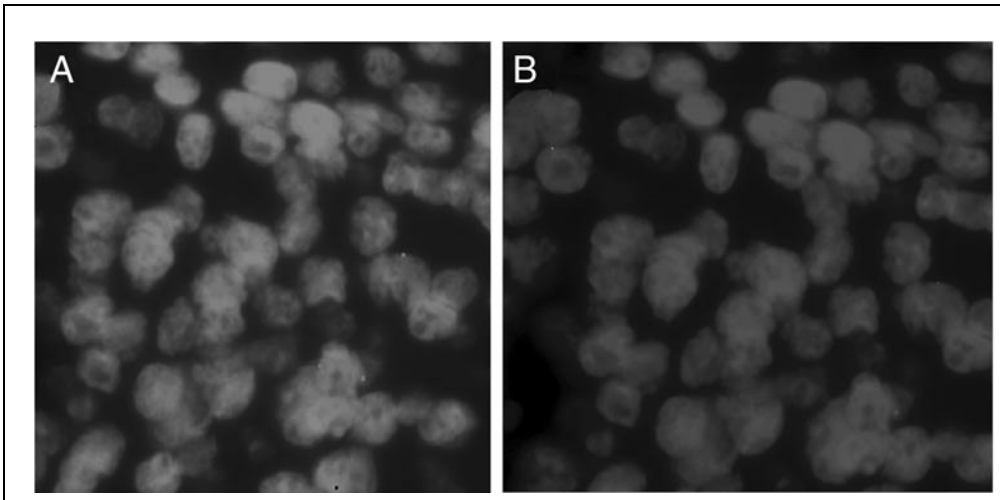


Figure 22.4.3 Digestion of paraffin-embedded specimen for FISH. (**A**) Under-digestion is indicated by weak uptake of the DAPI stain. (**B**) Increased digestion permits better access to the DNA and increase uptake of DAPI. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure, see color plates.*

6. Apply 10 μl PI or DAPI in antifade to counter stain and coverslip.

   The choice between PI or DAPI depends on the label color of the probe being analyzed.

7. View the slide using a fluorescence microscope with FITC, and PI or DAPI filter set. Evaluate the tissue sections for under-digestion, appropriate digestion, or over-digestion using the guidelines in Table 22.4.9 and Figure 22.4.3.

For certain types of tissue additional troubleshooting steps may also be necessary (see Troubleshooting).

8. Carefully remove the coverslip and rinse slide three times in separate Coplin jars containing 2× SSC, pH 7.0, for 5 min each, agitating 5 to 10 sec in each rinse.
9. Dehydrate slide in a series of 70%, 80%, and 100% ethanol washes for 1 min each.
10. Allow the slide to air dry. Proceed to hybridization with labeled DNA probes.

Preparation of Paraffin-Embedded Specimens for Hybridization with PNA Probes

ALTERNATE PROTOCOL 4

PNA probes are also applicable to paraffin sections. Like pretreatment for hybridization with DNA probes (see Alternate Protocol 3), pretreatment for hybridization with PNA probes requires some minor modifications as described below.

Materials

5- to 10-μM paraffin sections on silanized slides
Xylene
100% ethanol
100 μg/ml RNase I (see recipe)
2× SSC (*UNIT 18.6*)
1 M sodium thiocyanate, 80°C
5 mg/ml pepsin in 0.85% (w/v) NaCl, 45°C (see recipe)
0.1 M triethanolamine, pH 8.0
Acetic anhydride (Sigma)
1× PBS (*APPENDIX 2A*)
70%, 80%, and 100% ethanol
45°C hot plate or slide warmer
Coplin jars
37°C oven

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.10.

Remove wax

1. Preheat 5- to 10-μm paraffin sections on silanized slide on a 45°C slide warmer or hot plate for ~30 min (i.e., until the wax melts).

Table 22.4.10 Coplin Jars Needed for Preparation of Paraffin-Embedded Specimens for Hybridization with PNA Probes[a]

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	80% ethanol	Room temperature
4	100% ethanol	Room temperature
2	1× PBS	Room temperature
1	5 mg/ml pepsin in 0.85% NaCl	45°C
1	1 M sodium thiocyanate	80°C
5	2× SSC	Room temperature
1	Triethanolamine, pH 8.0	Room temperature
2	Water	Room temperature
3	Xylene[b]	Room temperature

[a] See Alternate Protocol 4.
[b] *CAUTION:* Maintain in a fume hood.

2. Dewax by transferring the slide to a Coplin jar containing 50 ml xylene and incubating 5 to 10 min at room temperature. Repeat twice with fresh xylene.

   Time for dewaxing is dependent on many factors (e.g., tissue section thickness, surface area, type of tissue).

3. Remove xylene by soaking slide 5 to 10 min in 100% ethanol. Repeat twice with fresh ethanol.

4. Allow slides to air dry (5 to 10 min).

   The tissue should turn white at this point.

Treat with RNase, SSC, and sodium thiocyanate

5. Add 40 µl of 100 µg/ml RNase I to the slide. Cover with a coverslip and incubate 1 hr at 37°C.

6. Wash twice in Coplin jars containing fresh 2× SSC for 5 min each time.

7. Incubate slide 8 min in a Coplin jar containing 1 M sodium thiocyanate, 80°C.

8. Rinse twice in Coplin jars containing fresh distilled water for 1 min each.

Digest with protease

9. Incubate 7 to 9 min at 45°C in a Coplin jar containing 5 mg/ml pepsin in 0.85% NaCl, pH 1.5.

10. Wash twice for 1 min each in Coplin jars containing fresh 2× SSC.

Acetylate

11. Start acetylation by placing slide in a Coplin jar containing 0.1 M triethanolamine, pH 8.0.

12. While stirring gently, slowly add 125 µl acetic anhydride to give a final concentration of 0.25% (v/v). Incubate 10 min at room temperature.

    The acetylation procedure is carried out to reduce the electrostatic binding of probe to positive charges on the tissue, thereby reducing the background.

Wash and dehydrate

13. Rinse 5 min in a Coplin jar containing 1× PBS. Repeat the rinse using a fresh Coplin jar of PBS.

    Washes from this point forward can be reduced in duration if adherence of tissue to the slide is poor.

14. Rinse 5 min in a fresh Coplin jar containing 2× SSC.

15. Dehydrate through a series of 70%, 80%, and 100% ethanol and allow to air dry.

16. Proceed to hybridization with PNA probes (see Alternate Protocol 7).

HYBRIDIZATION

In the following section, hybridization of the probe to the slide is described. The prepared DNA probe is denatured to a single-stranded state in the presence of denaturing buffer. Depending on the probe type, there may be a preannealing step. The denatured probe is applied to the denatured cytogenetic specimen and allowed to hybridize overnight. Additionally, this section also describes the hybridization conditions for paraffin sections, where the slide and probe are simultaneously denatured. If commercial DNA probes are being used, the manufacturer's instructions for probe denaturation are followed and the hybridization conditions are adjusted accordingly. If a PNA probe is utilized, the probe

is applied to the slide, and together the probe and target are denatured and permitted to hybridize for at least 1 hr.

Hybridization of Labeled DNA Probes to Cytogenetic Specimens

BASIC PROTOCOL 3

Hybridization of a cyotgenetic specimen with the probe requires heat-denaturation of the DNA probe in the hybridization buffer, and a pretreated and denatured slide specimen; together they are permitted to hybridize at 37°C. This protocol can be applied to cytogenetic specimens that are unstained or were previously G-banded (see Alternate Protocol 2).

Materials

DNA probe (see Basic Protocol 1 and Alternate Protocol 1)
Pretreated and denatured cytogenetic slide specimen (see Basic Protocol 2 and Support Protocol 2)
Rubber cement

75°C water bath or PCR machine
37°C dry oven
Glass coverslips
Hybridization box, slightly dampened (e.g., black plastic video tape box lined with slightly moistened gauze or paper towel)
Hybridization container (i.e., any plastic container with lid or a black video cassette box)

1. Heat-denature the required amount of labeled DNA probe 5 min in a 75°C water bath or PCR machine. Preanneal 1 hr in a 37°C dry oven (see Critical Parameters).
2. Add the appropriate amount of DNA probe to the pretreated and denatured cytogenetic slide specimen using guide given in Table 22.4.11. Coverslip and ring the perimeter with rubber cement to seal in place.

Table 22.4.11 Guide to Appropriate Probe Volume for a Given Coverslip Size

Probe volume	Coverslip size (mm)
10 µl	22 × 22
20 µl	22 × 30
30 µl	22 × 50

Table 22.4.12 Examples of Minimal Hybridization Times Required for Representative DNA Probes

Probe type (300–500 bp)	Minimal hybridization time (37°C)
Centromere Probes[a]	4 hr
Subtelomere chromosome-specific probes[a]	24 hr
Chromosome paints	4 hr
Translocation junction unique-sequence probes	24 hr
Microdeletion unique-sequence probes	24 hr
Probes detecting gene amplification	24 hr

[a]Protein nucleic acid (PNA) probes, which are commercially available, require a minimum of 60 min for hybridization.

3. Place slide in a slightly dampened hybridization box.
4. Transfer slide to a hybridization container and incubate overnight in a 37°C dry oven (see Table 22.4.12).
5. Proceed to post-hybridization washes and immunofluorescent detection of indirectly (see Basic Protocol 4) or directly labeled DNA probes (see Alternate Protocol 8)

ALTERNATE PROTOCOL 5

Hybridization of PNA Probes to Cytogenetic Specimens

PNA probes, as discussed below (see Critical Parameters), can be commercially obtained. Use of PNA probes requires minor modifications in the hybridization to both cytogenetic specimens and paraffin-embedded sections, as described below.

Additional Materials *(also see Basic Protocol 3)*

Labeled PNA probe (Applied Biosystems)
Pretreated cytogenetic specimen, not denatured (Basic Protocol 2)
80°C oven, hot plate, or HYBrite (Vysis)

1. Add the required amount of labeled PNA probe to the pretreated, undenatured, cytogenetic specimen.
2. Coverslip and seal with rubber cement. Allow the rubber cement to set and dry.
3. Place the slide in an 80°C oven or HYBrite, or on an 80°C hot plate for 90 sec.
4. Remove the slide and place it into a hybridization box. Hybridize at least 1 hr at room temperature.
5. Proceed to post-hybridization washes for specimens hybridized with PNA probes (see Alternate Protocol 10).

ALTERNATE PROTOCOL 6

Hybridization of Labeled DNA Probes to Sections from Paraffin-Embedded Material

Unlike cytogenetic specimens, tissue sections from paraffin-embedded material require higher denaturation temperatures and longer denaturation times. Hybridization to these sections requires co-denaturation of the probe and tissue section simultaneously. All experiments using previously paraffin-embedded material and a DNA probe should include a minimal 24-hr hybridization.

Additional Materials *(also see Basic Protocol 3)*

Deparaffinized and enzyme-digested specimen (see Alternate Protocol 3)
90°C oven, hot plate, or HYBrite (Vysis)

1. Apply DNA probe to the deparaffinized and enzyme-digested specimen using the guide given in Table 22.4.11. Add a glass coverslip and apply rubber cement along the perimeter of the coverslip. Allow the rubber cement to dry.
2. Denature the probe and target DNA simultaneously by placing the slide in a 90°C oven or HYBrite, or on a 90°C hot plate for 12 min.
3. *For slide denatured in an oven or on a hot plate:* Transfer the slide to a hybridization container lined with a wet paper towel or damp gauze, and incubate overnight in a 37°C dry oven.
4. *For slide denatured in a HYBrite:* Program the unit to hold at 37°C. Incubate overnight.

5. Proceed to post-hybridization washes and immunofluorescent detection of indirectly or directly labeled DNA probes (see Basic Protocol 4 or Alternate Protocols 8 or 9).

Hybridization of PNA Probes to Sections from Paraffin-Embedded Material

ALTERNATE PROTOCOL 7

The following protocol describes hybridization of commercially available PNA probes to pretreated sections from paraffin-embedded samples. Unlike DNA probes, PNA probes require a minimum of 60 min for hybridization.

Additional Materials *(also see Basic Protocol 3)*

PNA probes (Applied Biosystems)
Deparaffinized and enzyme-digested specimen (see Alternate Protocol 4)
80°C oven, hot plate or HYBrite (Vysis)

1. Apply the appropriate amount of PNA probe (as suggested by the manufacturer) to the deparaffinized and enzyme-digested paraffin section using the guidelines provided in Table 22.4.11. Coverslip and seal with rubber cement. Allow the rubber cement to dry.
2. Place in an 80°C oven or HYBrite, or on an 80°C hot plate for 3 min.
3. Place in a hybridization container and allow to hybridize at least 1 hr at room temperature.
4. Proceed to post-hybridization wash for slides hybridized with PNA probes (see Alternate Protocol 10).

POST-HYBRIDIZATION WASHES AND DETECTION

Following hybridization, unbound probes, whether DNA or PNA, must be removed from the specimen. This is accomplished through washes of appropriate stringency, using formamide and SSC in varying amounts. After immunodetection, final washes contain detergents to remove unbound antibodies. The slides are counterstained and mounted in an antifade medium for visualization. Variations on washing procedures reflect the nature of the probe, whether directly or indirectly labeled.

Post-Hybridization Washes and Immunofluorescent Detection of Indirectly Labeled DNA Probes

BASIC PROTOCOL 4

This protocol describes post-hybridization washes and detection of indirectly labeled DNA probes. Following overnight hybridization, the coverslip is removed and the slide is immersed in a solution of formamide to remove any unbound probe. The slide is then washed in stringent SSC washes and blocked with a solution of BSA. Depending on the type of DNA probe used, amplification of the signal may be required. This is achieved by the addition of primary and secondary antibodies that may or may not be conjugated with a fluorochrome. Detergent washes are carried out after each antibody incubation; it is critical that the slide not be permitted to dry out at any point of the assay. The slide is then counterstained and ready for visualization. In multicolor FISH experiments, the user may have both indirectly and directly labeled probes on the same specimen. If this is the case, it is important to keep the ambient light dim to prevent quenching of the signal; Coplin jars with lids are useful for this purpose. If using multiple indirectly labeled probes, be sure that the primary and secondary antibodies are raised in different animals such that there is no cross-reaction. This protocol is applicable to cytogenetic slides or sections from paraffin-embedded samples; however, more stringent or additional washes may be required for paraffin experiments.

Table 22.4.13 Coplin Jars Needed for Post-Hybridization Washes and Immunofluorescent Detection of Indirectly Labeled DNA Probes[a]

No. Coplin jars	Contents	Temperature
3	50% formamide in 2× SSC	45°C
3	1× SSC	45°C
9	0.1% Tween 20 in 4× SSC	45°C

[a]See Basic Protocol 4.

Materials

Hybridized slides in a hybridization box (see Basic Protocol 3 and Alternate Protocol 6)
50% formamide in 2× SSC, 45°C (prepare fresh)
1× SCC, 45°C (*UNIT 18.6*)
Blocking solution: 1% BSA (w/v)/0.1% (v/v) Tween 20 in 4× SSC (store indefinitely at −20°C or up to several months at 4°C)
Primary, secondary, and tertiary antibodies (see recipe for antibodies)
0.1% (v/v) Tween 20 in 4× SSC, 45°C
DAPI in antifade (see recipe)
Clear nail polish

Coplin jars
22 × 50–mm glass coverslips
37°C oven
Fluorescent microscope and appropriate filter sets (*UNIT 4.2*)

CAUTION: Formamide is a carcinogen and should be handled with care. Discard according to biohazard rules of the institution.

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.13.

Remove coverslip

1. After 24 hr, remove hybridized slide from the hybridization box.
2. Carefully peel the rubber cement from the hybridized slide and immerse in a Coplin jar containing 50% formamide in 2× SSC, 45°C.
3. Allow the coverslip to fall off and let stand 5 min in solution with gentle agitation.

Wash and block

4. Remove slide and transfer to second Coplin jar containing formamide/SSC, 45°C, for 5 min. Agitate gently. Repeat with a third Coplin jar containing formamide/SSC, 45°C.
5. Wash the slide for 5 min each in three consecutive Coplin jars containing 1× SSC, 45°C, agitating gently between washes.
6. Drain excess solution but do not allow the slides to dry. Add 80 µl blocking solution, coverslip with 22 × 50–mm glass, and place back in hybridization box. Incubate 40 min at 37°C.

Label with primary antibody

7. Remove coverslip and add 80 µl primary antibody. Coverslip and place back into the hybridization box. Incubate 40 min at 37°C.

Table 22.4.14 Common Primary and Secondary Antibody Systems and Final Concentrations[a]

Hapten	Primary antibody	Secondary antibody	Tertiary antibody	Color	Probe Type
Biotin (no signal amplification)	5 μg/ml FITC-avidin	—	—	green	Centromere chromosome paints
Biotin	5 μg/ml FITC-avidin	5 μg/ml biotinylated goat anti-avidin	5 μg/ml FITC-avidin	Green	Locus-specific cDNAs
Biotin	5 μg/ml rhodamine-avidin	5 μg/ml biotinylated goat anti-avidin	5 μg/ml rhodamine-avidin	Red	Locus-specific cDNAs
Digoxigenin (no signal amplification)	2 μg/ml rhodamine anti-Dig	—	—	Red	Centromere chromosome paints
Digoxigenin (no signal amplification)	2 μg/ml FITC anti-Dig	—	—	Green	Centromere chromosome paints
Digoxigenin	0.5 μg/ml mouse anti-digoxigenin	2 μg/ml digoxigenin anti-mouse	2 μg/ml rhodamine anti-Dig	Red	Locus-specific cDNAs
Digoxigenin	0.5 μg/ml mouse anti-digoxigenin	2 μg/ml digoxigenin anti-mouse	2 μg/ml FITC anti-Dig	Green	Locus-specific cDNAs

[a]Shown in this table are the most common antibody systems for the detection of biotinylated and digoxigenin (Dig)-labeled DNA. Final concentrations for antibodies are also stated but may require adjustment. A variety of antibody systems are available from Molecular Probes and Roche Diagnostics. If a two–color FISH approach is being used, be sure that respective primary, secondary, and tertiary antibodies do not cross-react. This may require sequential hapten detection rather than concomitant hapten detection. The scheme above uses antibodies raised against mouse and goat, thus no cross-reaction will occur. Antibodies raised in rabbits will also offer more variety in hapten detection.

This antibody may or may not have a fluorescent moiety conjugated to it, depending on the nature of the probe (see Critical Parameters). Common primary antibody and secondary antibody systems are outlined in Table 22.4.14.

8. Remove coverslip and wash slide for 5 min each in three consecutive Coplin jars containing 0.1% Tween 20/4× SSC, 45°C, with gentle agitation. If proceeding with signal amplification (see Table 22.4.15) continue to step 9. Otherwise, proceed to step 14.

9. Drain excess wash solution, but do not allow slide to dry. Add 80 μl blocking solution, coverslip, and place back into the hybridization box. Incubate 10 min at 37°C.

Label with secondary antibody

10. Remove coverslip and add 80 μl secondary antibody (Table 22.4.14). Coverslip and place back into the hybridization box. Incubate 30 min at 37°C.

11. Remove coverslip and wash slide 5 min each in three consecutive Coplin jars containing 0.1% Tween 20 in 4× SSC, 45°C, with gentle agitation.

Label with tertiary antibody

12. Remove coverslip and add 80 μl tertiary antibody (conjugated to fluorochrome). Coverslip and place back into the hybridization box. Incubate 30 min at 37°C.

13. Remove coverslip and wash slide 5 min each in three consecutive Coplin jars containing 0.1% Tween 20 in 4× SSC, 45°C, with gentle agitation.

Counterstain and seal

14. Drain excess wash solution, but do not allow the slides to dry. Add 40 μl DAPI in antifade counterstain. Coverslip and seal with clear nail polish.

The slides are now ready for visualization. When not in use, store slides up to several weeks or months at −20°C, depending on the frequency of visualization.

15. Examine using a fluorescent microscope and appropriate filter sets.

Table 22.4.15 Interpretation of Parallel Positive Control Experiments

Problem	Slide hybridized with new probe	Slide hybridized with good known probe	Analysis
Background	Present	Not present	New probe may still contain unincorporated dNTPs or many small labeled fragments are present and did not hybridize. Slide quality is not the problem. Washing conditions also do not appear to be the cause.
Background	Present	Present	Problems likely related to washing conditions or slide quality rather than issues with the probe.
Weak Signal (indirect-labeled probe)	Yes	No	If using antibody detection system for indirectly labeled probes, this scenario indicates that the antibodies are in good working order. Look to problems with incorporation of the hapten into the DNA.
Weak signal (direct-labeled probe)	Yes	No	This indicates a problem with the labeling of the probe. Check that all enzymes and fluorochromes are within their shelf life. This also indicates that washing conditions are correct.
Weak signal (indirect labeled probe)	Yes	Yes	This indicates a problem with the antibody detection system. Ensure that the concentrations are correct and that the antibodies are fresh. It may also indicate a problem with hybridization, insufficient denaturation of the target DNA on the slide, or overall quality of the DNA specimen.
Weak signal (direct-labeled probe)	Yes	Yes	The problem may be in the post-hybridization washes: too stringent or temperatures too high.
Poor chromosome morphology with weak signal	Yes	Yes	Over denaturation if the chromosomes appear puffy.
Good chromosome morphology but no signal	Yes	Yes	Slides are likely too old and resistant to denaturation. Change to a more fresh preparation. Otherwise the experiment can be repeated but the denaturation of the slide should be increased.

ALTERNATE PROTOCOL 8

Post-Hybridization Washes and Detection of Directly Labeled DNA Probes

The protocol below outlines steps for washing slides hybridized with directly labeled probes. If using commercially produced probes, follow the manufacturer's instructions. This protocol is applicable to hybridized cytogenetic slides or paraffin sections. See Basic Protocol 4 for materials.

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.16.

1. After 24 hr hybridization, carefully peel off rubber cement from slide and immerse in a Coplin jar containing 50% formamide in 2× SSC, 45°C. Allow the coverslip to fall off and let stand 5 min.
2. Remove slide and transfer to a fresh Coplin jar containing formamide/SSC, 45°C. Incubate 5 min. Repeat with a third Coplin jar containing formamide/SSC, 45°C.

Table 22.4.16 Coplin Jars Needed for Post-Hybridization Washes and Detection of Directly Labeled DNA Probes[a]

No. Coplin jars	Contents	Temperature
3	50% formamide in 2× SSC	45°C
3	1× SSC	45°C
3	0.1% Tween in 4× SSC	45°C

[a]See Alternate Protocol 8.

Table 22.4.17 Coplin Jars Needed for Rapid Wash of Directly Labeled Probes[a]

No. Coplin jars	Contents	Temperature
1	0.3% (v/v) NP-40 in 2× SSC	72°C
1	0.3% (v/v) NP-40 in 2× SSC	Room temperature
1	2× SSC	Room temperature

[a]See Alternate Protocol 9.

3. Wash slide 5 min each in three consecutive Coplin jars containing 1× SSC, 45°C.
4. Wash slide 5 min each in three consecutive Coplin jars containing 0.1% Tween 20 in 4× SSC, 45°C, with gentle agitation.
5. Drain excess wash solution, but do not allow the slide to dry. Add 40 µl DAPI in antifade counterstain. Coverslip with 22 × 50–mm glass and seal with clear nail polish. Store slide up to several months at −20°C.

   Fluorescence fading will depend upon the frequency of viewing.
6. Examine using a fluorescent microscope equipped with the appropriate filters.

ALTERNATE PROTOCOL 9

Rapid Wash of Directly Labeled Probes

This protocol describes the use of high temperatures and stringent SSC washes for the removal of unbound probe from target DNA. This protocol is generally effective for directly labeled DNA, particularly from commercial sources. It is important to consider the differing stringency requirements of each probe being used when planning this protocol since excessive temperature can strip bound probe from the target. This protocol is applicable to cytogenetic slides or paraffin sections.

Additional Materials *(also see Basic Protocol 4)*

2× SSC (*UNIT 18.6*)
0.3% (v/v) NP-40 in 2× SSC, room temperature and 72°C

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.17.

1. Carefully remove rubber cement from the hybridized slide. Place the slide and coverslip in a Coplin jar containing 2× SSC to gently remove coverslip.
2. Once the coverslip has fallen off, incubate the slide 1 to 2 min in a Coplin jar containing 0.3% NP-40 in 2× SSC, 72°C.
3. Remove slide and wash 1 min in a Coplin jar containing 0.3% NP-40/2× SSC, room temperature.
4. Drain solution from slide and add DAPI in antifade counterstain. Add a 22 × 50–mm glass coverslip and seal with clear nail polish.
5. Examine the slide with a fluorescent microscope equipped with the appropriate filters.

ALTERNATE PROTOCOL 10

Post-Hybridization Washes for Specimens Hybridized with PNA Probes

This protocol describes a procedure for removing unbound PNA probe from cytogenetic specimens or from paraffin sections.

Materials

Slides hybridized with PNA probes (see Alternate Protocol 5 or 7)
70% (v/v) formamide/10 mM Tris·Cl (pH 7.0 to 7.5)/0.1% (w/v) BSA (see recipe)
0.1 M Tris·Cl (pH 7.0 to 7.5)/0.15 M NaCl/0.08% (v/v) Tween 20 (store up to several weeks at room temperature)
70%, 90%, and 100% ethanol
DAPI in antifade solution (see recipe)
Fluorescent microscope and appropriate filters

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.18.

1. Remove coverslip from slide hybridized with PNA probes and wash 15 min in a Coplin jar containing 70% formamide/10 mM Tris·Cl (pH 7.0 to 7.5)/0.1% BSA. Repeat once.
2. Wash 5 min each in three consecutive Coplin jars containing 0.1 M Tris·Cl (pH 7.0 to 7.5)/0.15 M NaCl/0.08% Tween 20.
3. Dehydrate slide by incubating 5 min each in Coplin jars containing 70%, 90%, and 100% ethanol. Air dry (5 to 10 min).
4. Apply DAPI in antifade and coverslip.
5. Examine the slide using a fluorescent microscope and appropriate filters.
6. Store slides up to several months at −20°C

INTERPRETATION OF FISH FINDINGS

This section discusses interpretation of FISH experiments and relevant troubleshooting measures. Each laboratory may adopt a different means of assessing positive and negative results as is applicable to the experiment and the question being asked. The guidelines below can generally be applied to both interphase and metaphase analysis.

Evaluating FISH

FISH encompasses four main components: the slide and its preparation (see Target Slide Preparation), the DNA probe and its preparation (see Labeling DNA Probes for FISH),

Table 22.4.18 Coplin Jars Needed for Post-Hybridization Washes for Specimens Hybridized with PNA Probes[a]

No. Coplin jars	Contents	Temperature
2	70% formamide/10 mM Tris·Cl (pH 7.0–7.5)/0.1% BSA	Room temperature
3	0.1 M Tris·Cl (pH 7.0–7.5)/0.15 M NaCl/0.08% Tween 20	Room temperature
1	70% ethanol	Room temperature
1	90% ethanol	Room temperature
1	100% ethanol	Room temperature

[a]See Alternate Protocol 10.

denaturation and hybridization of the probe to the slide (see Hybridization), and finally, post-hybridization washes and detection (see Post-Hybridization Washes and Detection). Each of these components is, in itself, multistepped. The discussion below outlines many of the problems encountered in FISH experiments, including factors influencing signal strength, background, and preserving optimal chromosome morphology. Other sources for FISH optimization and parameters can be found in Beatty et al. (2002), Henegariu et al. (2001), Schwarzacher and Heslop-Harrison (2000), and van de Rijke et al. (1996).

Signal strength

Slide age. In the author's experience, optimal results have been obtained from slides not older than 2 months. As the slides age further (3 to 6 months), they become harder to denature. Conversely, very old preparations (>1 year) often have partially degraded DNA that may reduce or preclude effective FISH experiments. In such situations, preparations tend to become very sensitive to denaturation. Previously G-banded slides (*UNIT 22.3*) have an even shorter life span and should be processed within 2 weeks (refer to Table 22.4.5).

Cytoplasmic debris. The presence of cytoplasm on the slide may inhibit binding and contribute to overall background. A more aggressive protease pretreatment may be required to reduce cytoplasmic protreinacious noise. This can be accomplished by increasing the time of digestion or the amount of protease added (maintain the same digestion time). One must also consider whether the slide is made from a dropped suspension or FISHed to a slide made from an in situ culture, since the latter slides tend to possess more cytoplasmic and cellular debris. A high background obscures true signals. If there is minimal background but the signal is weak in different parts of the slide, then there may be a gradient or patchiness of cellular protein in areas of the slide with a higher density of fixed cells.

Excessive slide pretreatment. Excessive enzymatic treatment may damage the target DNA, making it less efficient for hybridization with the probe. This is usually indicated by weak uptake of the counterstain and the presence of bright centromeres.

Denaturation time. As the slide ages (see above), the chromosomes become harder to separate into single strands. Slides used within 1 to 2 weeks of preparation should be denatured for 1.5 to 2 min. Slides that are older may require times that range from 2 to 3 min. Under-denaturaton results in insufficient strand separation of the target DNA, decreasing the effective hybridization efficiency. Over-denaturation of the target DNA causes DNA damage and reduces the amount of target DNA that is able to hybridize with the probe; it also results in reduced hybridization efficiency. Over-denaturation usually also results in poor uptake and banding using the DNA counterstain and very bright centromeres.

Sealing of coverslip. It is critical that the coverslip be adequately sealed with rubber cement during incubations to prevent any moisture from entering the hybridized area, thus diluting the probe. Do not use contact cement or other ultra-adhesive glues.

Proper temperature for washes and incubations. Check that the temperature in the oven, hot plate unit, or water bath is correct for hybridization and other incubations. High temperatures during post-hybridization washes may remove bound probe with weak sequence homology (see Critical Parameters), thus decreasing the amount of DNA available for signal detection or antibody binding. Avoid taking shortcuts during incubation times with blocking reagents or antibody detection reagents. Also be sure that the reagents do not dry up on the slide; this makes washing more difficult.

Detergent type and concentration. Increasing the detergent content or choice of detergent may influence signal strength. Tween 20 is generally less harsh than NP-40. Increasing

the detergent concentration in combination with increasing temperature will remove more probe and/or bound antibodies. Be sure that the concentration and temperatures are correct.

SSC concentration. Decreasing SSC concentration increases stringency. Thus 0.1× SSC destabilizes double-stranded DNA more readily than 2× SSC. Double-stranded DNA stability is also affected by the extent of sequence homology between the probe and target DNAs. Ensure that the proper SSC concentrations are used during washes.

Proper filter sets. For fluorescence, make sure that the proper filter sets are used for image acquisition. The use of filters with incorrect spectral characteristics for the fluorochrome being used can severely impair the ability to detect the correct signals or they may increase the amount of autofluorescence. Also refer to *UNIT 4.2* and *APPENDIX 1E*.

Amplification of signal. When using an indirectly labeled DNA probe, signal amplification may be required. If the signal is amplified but still weak, check the concentrations and shelf lives of antibodies used. If the antibodies are in good order, relabel the probe and check the labeling efficiency (see Critical Parameters). For directly labeled probes, consider the labeling efficiency of the probe.

Probe characteristics

In-house probes. If probes are labeled in-house (see Labeling DNA Probes for FISH), strict controls must be undertaken to ensure that proper hapten or fluorochrome incorporation has been obtained to produce a high-quality DNA probe. Haptens may be assessed using the dot blot method of incorporation. Directly labeled probes can be assessed by removing a small aliquot of probe, placing it on a slide, and viewing it by fluorescence microscopy.

Cot-1 suppression. Excessive Cot-1 suppression may be the cause of reduced signal. Smaller DNA probes or cDNAs contain fewer repeat elements compared to larger inserts. Normally, 5 to 10 μg Cot-1 is sufficient per 100 ng labeled DNA; adjust as required.

Commercial probes. Usually commercial suppliers properly process the product with the necessary quality controls. Check that the probes were properly stored and used before the expiration date. For some experiments it is possible to optimize procedures using concentrations of commercial probes at levels slightly below those recommended by the supplier. This can prove very cost effective if a specific commercial probe is going to be used routinely.

Probe concentration. The probe is usually used in excess of the target DNA; however, make sure that sufficient probe volume has been added to adequately cover the area of interest on the slide (see Table 22.4.11).

Preannealing. Preannealing at 37°C is usually carried out for 1 hr following denaturation. This step is performed to permit the annealing of repetitive sequences in the DNA probe with unlabeled Cot-1 DNA to prevent cross-hybridization. If there are few repetitive elements in the probe, or the probe is a cDNA or small insert, this step may be omitted. The probe is simply denatured and applied to the denatured slide.

Background

Cytoplasmic debris. This is the most common cause of background. Increase the incubation time during protein digestion or maintain the same time but change the concentration of the enzyme.

Bacterial/yeast contamination. Microorganisms that contaminate cultures used to make the slide, or reagents used to prepare the slide or for hybridization are deposited onto the slide. If the contamination is minor, then it is only necessary to analyze the slide in areas that contain fewer deposits of fungi or bacteria. If the contamination is heavy, the slide or reagent must be prepared again.

Coverslips. Coverslips should be clean and dust free. If cells are to be grown in situ, use only glass coverslips as plastic coverslips autofluoresce.

Residual oils. Slides that have been previously visualized using immersion oil (e.g., G-banded slides) should be cleaned with xylene. Residual oils will prevent hybridization and cause background problems.

Dust and other particles. Particles may be deposited on the slide during various transfers into solutions. Be sure that solutions are well mixed and filtered as needed. Some solutions may form precipitates that may bind to the slide.

In-house probes. Ensure that nonincorporated conjugated nucleotides are removed from the final probe preparation.

Commercial probes. These probes are usually not the cause of background, especially when the same probe on another slide preparation has not produced background; however, note the lot number for future reference. It is also very helpful to introduce a new batch of a commercial probe into routine use before the previous lot is exhausted in case the supplier has had production difficulties

Unlabeled DNA: carrier and Cot-1. An excess of unlabeled DNA is added to the probe mixture. Although it is unlabeled, it can contribute to background by trapping antibodies. To reduce this problem, ensure that the resuspended probe pellet is fully dissolved in sufficient hybridization buffer. If the pellet does not completely dissolve, add more buffer. This will not significantly alter the reaction.

Denaturation time. The probe is usually heat denatured a minimum of 5 min. This should be sufficient time to denature the probe and dissolve any remaining DNA. The denaturation time may be increased to 10 min without any damage to the DNA probe.

Post-hybridization washes. Make sure that the correct temperature has been maintained for washes and incubations. Agitation during the washes can help to remove unbound probe and antibodies. Increasing the stringency of the washes by either increasing the temperature or altering the amount of SSC in the washes can also help considerably if background is encountered. Avoid drying the slide with any residual blocking or detection reagents.

Detergent concentration. Increasing the detergent concentration in post-hybridization washes in combination with increasing temperature removes more probe and/or bound antibodies. Be sure that the concentration and temperatures are correct.

Fading signals

In-house probes. Ensure that stocks of directly conjugated dNTPs are properly stored in the dark and used well before the expiration date. Unlike indirectly labeled probes, directly labeled DNAs have a much shorter shelf life.

Commercial probes. Although these probes are supposed to be quality controlled, these too are subject to the same concerns as in-house probes (see above). During denaturation

and hybridization, keep lights dimmed and hybridize in a light-proof container for directly labeled probes.

Directly-labeled DNA probes. When directly labeled probes are used, slides should be washed with the lights dimmed to prevent unnecessary exposure to extraneous light.

Mounting medium. Check that the DAPI/antifade mounting solution is used before its expiration date. Normally, the medium is clear with a slight pink tinge; medium that is degrading will turn increasingly amber. Expired antifade medium causes rapid signal degradation and displays a red glow when viewed under the microscope.

Image acquisition. Extended exposure of the hybridized slide to UV sources leads to signal quenching. When not in use, store slides at −20°C. During visualization, keep the light source off when not in use.

Poor chromosome morphology

Slide age. See Signal Strength (above) for information concerning this parameter.

Cytoplasmic debris. See Signal Strength (above) for information concerning this parameter.

Excessive slide pretreatment. Excessive enzymatic treatment may damage the target DNA, making it less efficient for hybridization with the probe

Previously G-banded slides. G-banding (*UNIT 22.3*) causes DNA to become sensitive to subsequent denaturation. For this reason, denaturation times are greatly reduced. This also has an effect on the quality of hybridization and signal strength.

Over-denaturation. The cause of poor chromosome morphology is usually over-denaturation, which causes the DNA to be destroyed. This can be sample specific and/or slide-age related.

Denaturation temperature. Check that the temperature for denaturation is accurate. The final internal temperature of the Coplin jar should be 72°C. Add 1°C for each slide that is denatured in the jar. Avoid denaturing more than five slides in one jar at one time. Note that plastic Coplin jars have a greater differential temperature between bath and internal temperatures as compared to glass.

Probe cross-hybridization

Clone identity. When using in-house FISH probes for research applications, ensure that the clone identity and the insert are correct for the experiment. Some genes have highly homologous sequences elsewhere in the genome and may cross-hybridize to other family members.

DNA contamination. Consider whether stock DNA was contaminated with DNA from another clone. Increase the hybridization temperature if contamination is suspected. Typically, the hybridization temperature is 37°C. If cross-hybridization occurs, try increasing the hybridization temperature to 42°C.

Stringency of washes. Some probes may cross-hybridize, particularly centromere probes. Usually cross-hybridization signals are significantly weaker than true signals. More stringent washes can be undertaken by decreasing the SSC concentration, increasing wash temperature, or adding additional washes.

Evaluation of a New DNA Probe

The most critical factor in optimizing a new FISH probe which was labeled in-house is first determining whether the probe of interest maps to the correct location. Many vectors (e.g., plasmids, cosmids, BACs) have traveled from laboratory to laboratory and have changed hands many times. These DNAs, as well as cloned DNA obtained from reputable repositories, can be mislabeled or misidentified. After a cloned DNA has been extracted and labeled, the first FISH experiment should be carried out on normal lymphocyte spreads and should address the following questions.

Is a signal visible on a pair of chromosomes?

If there is a weak signal, refer to the section above.

Does the DNA probe map to the right chromosomal location? Does it map to only one location?

It helps to have someone who can identify the chromosomes by DAPI banding. Most people are not as proficient as trained cytogeneticists, thus it may be useful to carry out a two-color experiment using a commercially available centromere probe for the chromosome of interest. Refer to *UNIT 22.3* for general conventions for identification of chromosomes according to ISCN nomenclature. Some DNA sequences/genes may belong to families with similar sequences. It is possible that hybridization to these similar regions may occur. Usually the true signals are stronger than the cross-hybridized ones. If this is the case, wash conditions can be adjusted or another clone should be obtained.

Does a positive control probe work properly?

It is useful to perform parallel experiments with a probe known to give good signals in the same experiment. This will help to identify any problems that are not related to the newly labeled probe (see Table 22.4.15 for interpretation of such control experiments).

For example, in panel iii of Figure 22.4.4A, two mouse BAC probes were labeled (one green and the other red) and assessed. A separate slide with a previously labeled BAC known to give good signal and no background was prepared as a control at the same time (not shown). The newly labeled BAC probes shown in panel iii of Figure 22.4.4A mapped to the proper location with weak signal intensity and high background. Analysis of this experiment is as follows.

The BAC probes map to the correct location. Because the signal intensity was weak consider (1) starting DNA quality and quantity, (2) labeling efficiency, (3) fidelity of DNA polymerase I and DNase I, (4) labeling time and temperature, (5) effective removal of nonincorporated nucleotides, and (6) insufficient Cot-1 suppression. Because the control probe displayed no background problems, one can assume that the hybridization and wash conditions were sufficient.

Evaluation of Hybridization Efficiency

The minimum number of cells or metaphase spreads required to obtain a given result reflects the clinical context of the finding, the limitations of the patient material available for study, and the question being asked. With tissues or cells that are hard to obtain, a single abnormal metaphase may be significant. For example, in some situations, limited FISH data may be supported by results obtained using PCR and/or Southern blot analysis.

Prior to enumerating or analyzing FISH results on a test sample, it is important to carefully assess the overall quality, uniformity, and effectiveness of hybridization as discussed

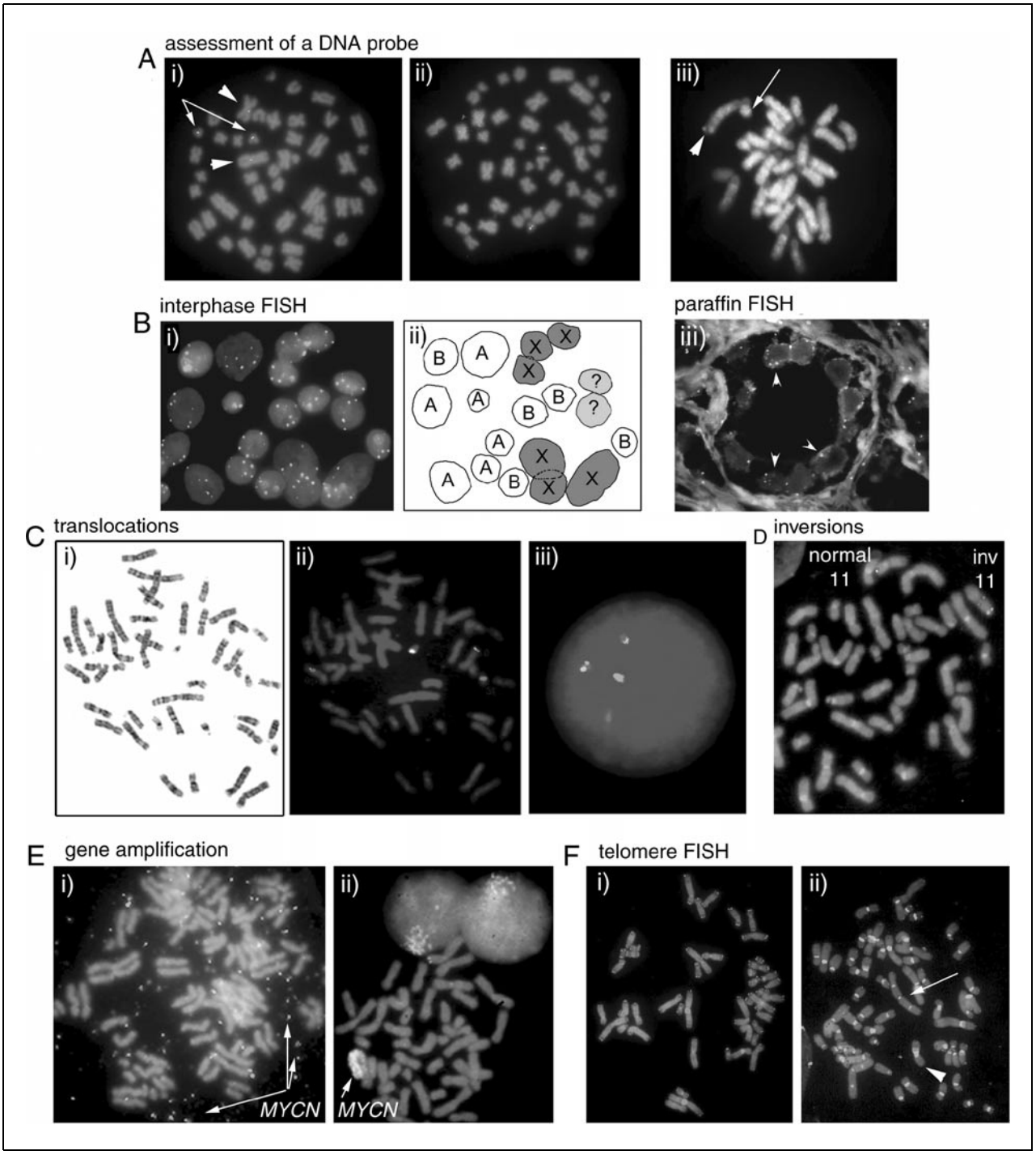


Figure 22.4.4 Legend at right.

above. Each hybridized slide should be evaluated for the specificity of the hybridization, the probe signal intensity, and the signal-to-background noise, to determine if the hybridization was optimum for the given analyses. There should be minimal background or nuclear fluorescent noise. At least 85% of all nuclei in the target area should be easily innumerable. For some applications (such as the detection of mosaicism or minimal residual disease), more rigorous analytical sensitivities and hybridization efficiencies are required.

1. Perform the FISH protocol appropriate for the type of patient and control slides in the experiment.
2. Prior to determining the hybridization efficiency, quickly scan the whole slide, noting the general signal-to-noise levels in different regions of the slide and any areas with high background or unusually weak signals. It may be useful to mark the underside surface of the slide in these areas with a diamond pen. If the signal intensity far exceeds background levels, proceed with estimating the hybridization efficiency.
3. Pick several representative areas of the slide and score at least 200 nuclei from the areas selected, following the selection criteria described below in Selecting Cells for FISH Microscopy. Keep a running log of the number of cells scored and the observed signal counts for the patient and control slides.
4. For all slides, add up the number of cells with no signal. In general, hybridization is considered to be adequate if $>85\%$ of the cells scored have one or more signals. Lower hybridization efficiencies may be encountered with smaller probes (generated

Figure 22.4.4 *(at Left)* Composite figure illustrating FISH hybridization. (**A**) Assessment of a DNA probe. (i) Cross-hybridization of probe (arrowhead) to chromosome 1. True signals are located on chromosome 22. (ii) Changes in stringency washes and post-hybridization removes cross-hybridization. (iii) High background associated with a insufficient repetitive sequence (Cot-1) suppression in this mouse metaphase spread. Weak signals (arrows) indicate poor labeling efficiency. (**B**) Interphase FISH analysis. (i) A cytogenetic specimen from a short-term ovarian primary culture hybridized with PNA probes for centromeres 7 (green) and 8 (red). When scoring interphase nuclei, it is especially important to focus through the cells since signals may be present at different *z* planes. (ii) Schematic indicating the nuclei that are acceptable for scoring: A, cell acceptable for scoring; B, cell is likely acceptable for scoring, but requires careful attention; ?, cell has qualities that make it questionable for scoring; X, cell is not acceptable for scoring. (iii) Interphase analysis using PNA probes specific for centromeres 7(green) and 8 (red) on a paraffin section from a prostate carcinoma. Arrowheads indicate cells containing changes in ploidy. (**C**) Analysis of translocation probes. (i) and (ii) Results of consecutive hybridization of a G-banded metaphase spread with the Vysis *BCR/ABL* translocation probe. Two fusion signals (yellow) are produced from the hybridization of *BCR/ABL* fusion on the Philadelphia chromosomes (Ph) on chromosome 22 and the reciprocal *ABL/BCR* on the derivative chromosome 9. Separate green and red signals from the normal chromosomes 9 and 22 are also seen. (iii) Interphase pattern from this specimen. (**D**) Example of a chromosomal inversion on chromosome 11. An inversion was identified involving the terminal portion of chromosome 11 by gross cytogenetic analysis. Clones 200 kb apart and in the 11p15.5 region, containing the *IGF2* gene (green) and *H19* (red) were hybridized to the patient specimen. The normal chromosome 11 shows the red and green signal hybridizing on top of each other at 11p15.5. The inverted 11 shows the clear spit of signal along the abnormal chromosome 11 indicating the breakpoint lies within the 200 kb between *IGF2* and *H19.* (**E**) Gene amplification of *MYCN* in neuroblastoma specimens. (i) Double minute chromosomes (dmns) containing hundreds of copies of the *MYCN* gene. This is in contrast to amplification of *MYCN* in (ii) as a large block of signal called a homogeneously staining region (HSR). Interphase nuclei nearby show the typical hybridization pattern for an HSR. (**F**) Use of subtelomeric and pan-centromeric PNA probes. (i) Hybridization of a prostate cell line with PNA subtelomeric probes. (ii) Hybridization of another prostate cell line with subtelomeric and pan-centromeric PNA probes. Loss of telomeric sequences are indicated by the arrow while the presence of multicentric chromosomes are indicated by arrow heads. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure, see color plates.*

for example in a research laboratory). Extreme caution must be exercised when using probes with lower hybridization efficiencies or elevated background signal to provide clinical information.

Selecting Cells for FISH Microscopy

Generally, look at all areas of the slide and analyze regions with uniformity in signal strength. Compare the intensity of the background signals to the intensity of the signals in the nuclei or metaphases of interest. The FISH signal intensity should consistently be greater than the background intensity in the regions of the slide chosen for analysis. If the background signals are equivalent to signals in the nuclei, then the counts will be skewed and the results biased. The following provides a guide to selecting targets for analysis.

1. Ideally analyze cells from all areas of the slide. Systematically select representative areas from different regions of the slide. Any regions that have unacceptable background or weak signals identified in prescreening evaluations should be excluded.
2. Select nuclei or metaphase cells that do not touch or overlap (Fig. 22.4.4B).
3. Select intact nuclei that have smooth well-rounded borders. Partially ruptured nuclear membranes may have lost informative chromatin. Similarly select metaphase spreads that have no evidence of preparation artifacts or breakage.
4. Select cells that are not surrounded by cytoplasmic material and that are without evidence of potential drying artifacts such as rings or clumped cells.
5. Do not evaluate interphase nuclei with signals located on the extreme periphery of the nucleus.
6. Do not score regions of the slide containing nuclei that have no signals. Absence of signals may represent uneven or patchy hybridization, resulting in some areas of the slide having very weak or absent signals.

Analysis of Interphase Nuclei

Interpretation of interphase FISH is very much dependent on statistical analyses and has inherent technical challenges. For instance, the presence of signal is dependent upon the probe and its fluorescent label successfully entering the cell and hybridizing to the target DNA. Detection of the correct number of signals can be complicated by signals overlapping or splitting. Any background hybridization whatsoever leads to major complications in interpretation. It is not uncommon to find monosomy or trisomy in nuclei that reflect technical artifact or false positive background signals. Therefore, the accuracy of interphase FISH analysis is dependent upon recognizing these technical issues, correcting for them, and standardizing the scoring criteria accordingly. Interphase analysis is typically used for enumeration of chromosomes using centromere probes, detection of gene amplification or deletion (see below), and detection of the presence of translocations, so that many cells should be scored. Metaphase FISH analysis, although more informative, is more difficult, especially when there are few metaphase spreads present.

1. Select nuclei in which signals generally have the same intensity.
2. Focus up and down in the *z* axis to accommodate spatial configurations of probe signals within the nucleus (Fig. 22.4.4B).
3. Signals that are more intense in some nuclei than the specific signal indicate the presence of regional background. Care must be taken when analyzing any sample with this type of background noise.

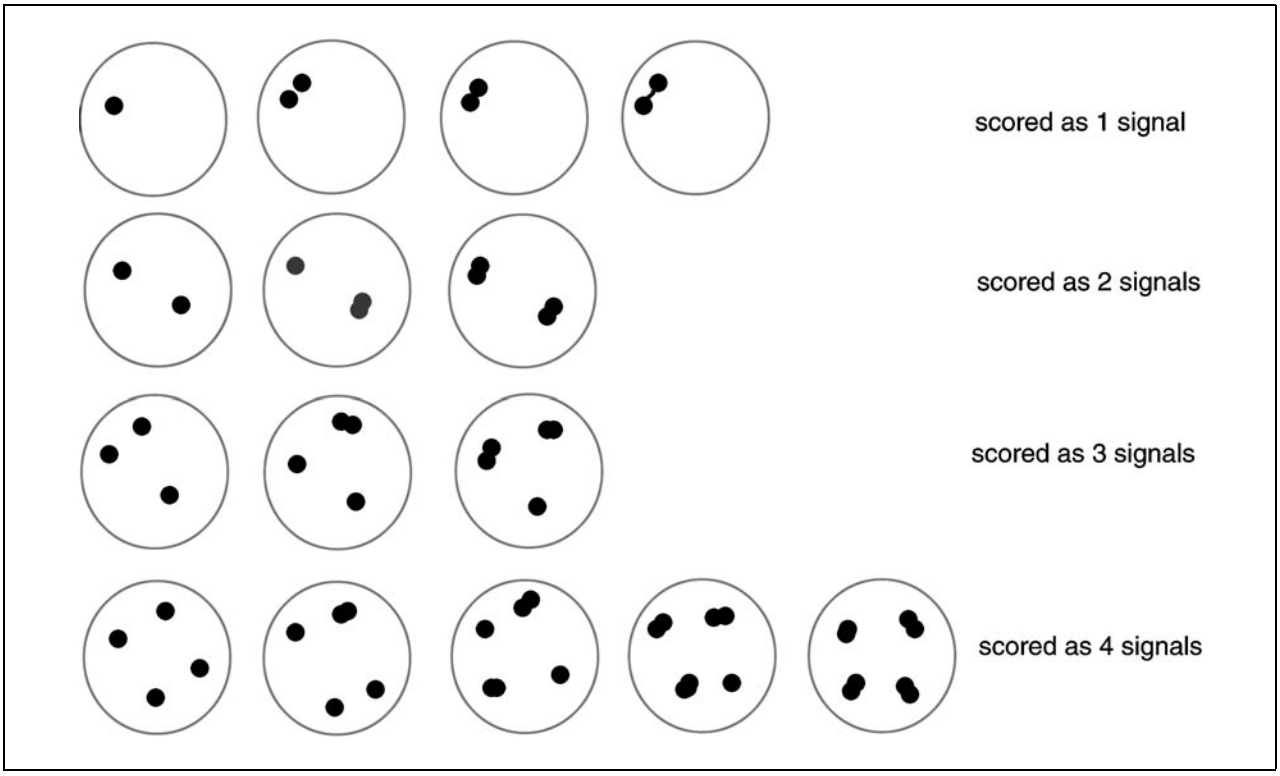


Figure 22.4.5 Schematic representation of scoring criteria. Shown in the illustration are typical hybridization signal configurations. These images pertain to a single probe, such as a centromere- or locus-specific probe. Signals must be more than one signal width apart to be considered one signal. Signals joined by a string of hybridization are also considered as one signal.

4. Do not count lower level nonspecific hybridization signals. These signals can usually be recognized by their lower intensity and different shape.
5. Some nuclei will have passed through the S phase of the cell cycle and may be present as G2-paired signals (i.e., two smaller signals in very close proximity). These paired signals represent a single chromosome already divided into chromatids and should be counted as one signal (Fig. 22.4.5).
6. Count two signals connected by a strand of fluorescence as one signal. Sometimes centromere or long genomic probes will generate signals that are not spherical. Typically, FISH signals appear as separate fluorescent dots on each chromatid of a metaphase chromosome when the target size is 100 to 250 kb. Similarly, in interphase nuclei, such probes will also generate discrete easy-to-interpret signals. Larger probes can appear as fused signals straddling both chromatids, and in interphase nuclei, these probes can generate signals that present more diffuse or dispersed hybridization spots in the chromatin of interphase nuclei. Knowledge of the probe size and anticipated configuration in both metaphase spreads, and interphase nuclei is essential. As long as the signal is continuous it should be scored as one signal.
7. Count only nuclei in which a definite enumeration can be made. Do not analyze or enumerate inconclusive cells.
8. Use two people to score 200 consecutive nuclei from each sample such that each person scoring will analyze ~100 nuclei from a given sample. The slides should be coded and scored independently by two analysts. Any discrepancies may mean the established scoring criteria for the FISH assay are not being adhered to rigidly.

Analytical sensitivity of interphase FISH assays

Once a particular probe set is made available as a routine FISH test for a clinical service, it is important that the laboratory perform assay validation and create a database establishing reportable range and general laboratory experience with each probe. The analytical sensitivity assay measures the success of a given FISH test in a particular laboratory environment and on a given tissue type. Since there are known differences in the cell populations and tissue types, it is important to use the appropriate positive control tissue for the assessment of analytical sensitivity and for the establishment of the database. Analytical sensitivity analyses are performed by scoring 200 interphase nuclei representing at least five normal, preferably male, individuals. (Pooling samples on one slide is acceptable.) The nuclei are scored for the percentage of nuclei exhibiting the appropriate number of distinct signals.

For constitutional studies using FISH, the recommended analytical sensitivity for probes intended for nonmosaic detection is 90%, while for probes intended to detect mosaicism, it is 95%. Similarly, for detection of minimal residual disease, sensitivities $\geq$95% are helpful. Databases for each probe should be established so that it will then possible to determine the mean and standard deviation of results from a series of normal samples processed and analyzed in the same manner as clinical samples. False positive rates can then be calculated and used for final scoring reports. More discussion on this issue is available from the following sources: VYSIS guidelines for single (*http://www.vysis.com/tech_sup_fishproto_quality_single.asp*) and dual probes (*http://www.vysis.com/tech_sup_fishproto_quality_dual.asp*), and scoring criteria for preimplantation genetic diagnosis of numerical abnormalities for chromosomes X, Y, 13, 16, 18, and 21 (Munne et al., 1998).

Statistical considerations concerning interphase FISH analysis of paraffin sections

Due to truncation of the nuclei during sectioning, loss of signal from areas of the nucleus excluded from the target slide will be encountered when enumerating signals after FISH has been performed on paraffin sections. The criteria for determining the significance of loss or gain of signals in interphase nuclei will depend on a number of parameters (e.g., nuclear diameter, age of patient, type of tissue). Readers are referred to some of the scientific literature where suggested cutoff values are adopted from the available literature (Qian et al., 1996). In the authors' experience with FISH analysis of prostate cancer, chromosomal gains can been identified when more than ~10% of the nuclei exhibit more than two signals. Panel iii of Figure 22.4.4B, shows an example of a prostate section hybridized with centromere probes for centromeres 7 (green) and 8 (red). Chromosomal losses have been identified when more than 50% of the nuclei exhibit a reduction of signal number, and tetraploidy has been assumed when all chromosomes investigated show signal gains up to four. For some classes of tumor, extreme polyploidy together with complex patterns of chromosomal rearrangement means that it is not realistic to select a suitable control chromosomal region in which two signals are expected. In such situations, it may be helpful to perform flow cytometric analysis of DNA content in parallel with interphase FISH analysis.

Analysis of Translocation and Inversion Probes

If the probes based on green and red fluorescence used for FISH are close to specific translocation breakpoints on different chromosomes, they will appear joined as a result of the translocation, generating a yellow color fusion signal. Commercial probes are now available for many of the common translocations in cancers (Table 22.4.19). One such probe from Vysis detects the Philadelphia chromosome (Ph) resulting from the

Table 22.4.19 Commonly Used Commercial Probes for Detection of Translocations in Sarcomas and Hematological Malignancies

Neoplasm	Chromosomal location	Probe	Scoring method
CML/pediatric ALL	9q34/22q	*BCR/ABL*	Color fusion observed in metaphase and interphase
Various leukemias	11q23	*MLL*	Split signal, metaphase
Various leukemias	5q31	*EGFR1*	Loss of signal, metaphase/interphase
Various leukemias	7q31	*DSS486*	Loss of signal, metaphase/interphase
AML M4 EO	Inv(16)	*CBFB*	Split signal, metaphase
Various leukemias	20q13.2	*ZNF217*, *D20S183*	Loss of signal, metaphase/interphase
Various hematologic malignancies	8q24 /14q32	*MYCC/IgH*	Color fusion observed in metaphase and interphase
AML-M1	12p13/21q22; 8q22/21q22	*TEL/AML1*; *AML1/ETO*	Color fusion observed in metaphase and interphase
AML-M3	15q22/17q21.1	*PML/RARA*	Color fusion observed in metaphase and interphase
Ewings sarcoma	t(11;22) (q24;q12)	*FLI1/EWS*	Color fusion observed in metaphase and interphase
Rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX/FKR*	Color fusion observed in metaphase and interphase

translocation between ABL on chromosome 9 and BCR on chromosome 22. Shown in Figure 22.4.4C is an example of combined G-banding and FISH analysis using the Vysis *BCR/ABL* probe set. Panel i of Figure 22.4.4C shows the G-banded metaphase spread to which the *BCR/ABL* probe was subsequently hybridized. In panel iii of Figure 22.4.4C, detection of a Ph chromosome in interphase nuclei of leukemia cells is achieved by the presence of two double-fusion (D-FISH) signals. All nuclei positive for the translocation contain one red signal (*BCR* gene), one green signal (*ABL* gene), and two intermediate fusion yellow signals because the 9;22 chromosome translocation generates two fusions, one on the 9q+ and a second on the 22q−. The following general guidelines may be helpful for performing this type of assay.

1. Green and red signals that are juxtaposed but not overlapping should be scored as ambiguous.
2. Do not score nuclei that are missing a green or red signal. This assay is looking for the presence or absence of a fusion signal, not the absence of a green or red signal.
3. Atypical signal patterns have been reported and are now considered to be clinically important (Kolomietz et al., 2001).

Table 22.4.19 lists some of the commonly used FISH assays in hematological cancers as well as sarcomas. In addition to the scientific literature, readers are referred to the suppliers web sites (see Internet Resources and *SUPPLIERS APPENDIX*), which will provide the most up-to-date listing of currently available probes and the preferred scoring method.

Inversions are related to translocations such that a break and rejoining occurs within the resident chromosome. Probes are available to detect common inversions present in AMLs

(see Table 22.4.19). Mapping of breakpoints is common in many research cytogenetic laboratories and involves chromosome walking. To identify the locus containing the breakpoint, rough cytogenetic analysis locates the band region. Clones are then obtained spanning the putative breakpoint. These clones are then FISHed to the specimen of interest. Such is the case in Figure 22.4.4D. An inversion was identified involving the 11p15.5 region. Clones 200 kb apart, containing the *IGF2* gene (green) and *H19* (red) were hybridized to the patient specimen. The normal chromosome 11 shows the red and green signal hybridizing on top of each other at 11p15.5. The inverted 11 shows the clear split of signal along the abnormal chromosome 11 indicating the breakpoint lies within the 200-kb distance between *1GF2* and *H19.*

Use of FISH Probes in Assessing Solid Tumors and Gene Amplification

Gene amplification is one of the mechanisms by which cancer cells achieve over expression of some classes of oncogenes, which involves an increase in the relative number of copies of a gene per cell. This can range from one or two additional copies per cell to extreme examples where over a thousand copies per cell have been reported. Gene amplification can occur in association with the over-expression of oncogenes, thus conferring a selective growth advantage or mechanism of acquired resistance to chemotherapeutic agents leading to poor prognosis. Gene amplification is highly suited to FISH analytical approaches that have the added benefit of excellent sensitivity and the ability to address cellular heterogeneity.

Neuroblastoma is characterized by the frequent occurrence of a highly amplified oncogene, *MYCN*. It has been known for many years that the presence of this aberration is strongly associated with poor outcome. More aggressive management is usually required when *MYCN* is found to be amplified. Similarly, breast cancer can be accompanied by an amplified oncogene *HER2/Neu*, and presence or absence of this aberration may determine which of different treatment regimens are followed. Examples of metaphase and interphase FISH assays for gene amplification are shown in Figure 22.4.4E. In this figure, a DNA probe containing the *MYCN* gene was hybridized to a cytogenetic specimen from a neuroblastoma patient. Panel i of Figure 22.4.4E illustrates double minute (dmns) chromosomes containing hundreds of copies of the *MYCN* gene as extrachromosomal bodies. This is in contrast to another patient where amplification of the *MYCN* gene occurs as a homogeneously staining region (HSRs) inserted in a chromosome other than the resident site of *MYCN* (normally at 2p24). Interphase nuclei in this image show a large patch of hybridization signal characteristic of HSRs. Some of the commonly detected aberrations observed in solid tumors which are amenable to FISH analysis are presented in Table 22.4.20.

Analysis of Telomere Probes

Telomeres are located at the ends of chromosomes and are characterized as (T_2AG_3) repeat sequences and their associated proteins (Poon et al., 1999). Maintained by the ribonucleoprotein complex, telomerase, they function to protect chromosomes from end-to-end fusions. In most normal tissues, telomerase is expressed at very low levels. As such, each round of DNA replication results in the gradual shortening of the telomeres. The shortening of telomeres is associated with replicative cell senescence. The up-regulation of telomerase extends the proliferative lifespan of a normal cell. In abnormal cells, expression of telomerase is associated with the maintenance of telomere length or telomere lengthening. Conventional Southern blot analysis gives average telomere length but fails to yield information on individual chromosome ends. It also underestimates the size and number of short telomeres. Although DNA probes for these sequences are available commercially, the use of commercial PNA probes for such sequences, as well as for centromeric and pancentromeric sequences, has enabled researchers to determine the overall

Table 22.4.20 FISH Probes Used For Assessing Solid Tumors and Gene Amplification

Neoplasm	Chromosomal location	Probe	Scoring method
Neuroblastoma	2p24; 2p23-24	*MYCN*	Interphase, metaphase; amplification
Ewings sarcoma	t(11;22) (q24;q12)	*FLI1/EWS*	Color fusion observed in metaphase and interphase
Breast cancer	17q11.2-q12	*HER2/neu*	Interphase amplification
Glioblastoma	7p10-p21	*EGF-R*	Interphase amplification
Bladder cancer	Centromere regions of chromosomes 3, 7,17 and 9p21 region of chromosome 9	Centromere 7, 13, 9	Interphase enumeration

telomere lengths of individual cells (interphase nuclei) and chromosomes (metaphases) using quantitative digital imaging, as described by Poon (1999), with greater specificity and accuracy. Both fixed cytogenetic cells (Poon et al., 1999) and paraffin-embedded samples (Vukovic et al., 2003) have been used for telomere analysis. Panel i of Figure 22.4.4F illustrates subtelomeric PNA probes hybridized to a prostate cell line metaphase spread. Digital imaging and signal intensity ratios determine the relative telomere length. In some cases, loss of telomere signals can also be identified as in panel ii of Figure 22.4.4F (arrow). In this figure, a prostate cell line was hybridized with subtelomeric PNA probe as well as a PNA pan-centromeric probe. The pancentromeric probe confirmed the presence of multicentric chromosomal structures indicative of chromosomal instability.

For those who wish to engage in telomere studies, access to digital imaging and analysis software capable of determining telomere length is suggested. Like all other FISH experiments, the background should be minimal. Telomere signals are relatively small and located at the ends of chromosomes. Background, such as antibody speckling can greatly affect the sensitivity of the analysis. The proper controls must also be established. Since telomere length is dependent on the number of cell divisions, age- and sex-matched controls should be included in all experiments.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose gel, 2% (w/v)

Dissolve 2 g molecular biology–grade agarose in 1× TBE buffer (*APPENDIX 2A*) by either microwaving or placing on a hot plate, and allow to cool slightly. Add 5 μl of 10 mg/ml ethidium bromide in a well vented chemical hood. Pour into casting trays (*APPENDIX 3A*) and allow to solidify. Store up to 1 week at 4°C covered with foil.

AP-labeled antibody mixture

Dilute anti-biotin or anti-digoxigenin conjugated to alkaline phosphatase (AP; Invitrogen) to a final concentration of 0.75 U/ml in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl (*APPENDIX 2A*). Prepare fresh for each experiment.

Antibodies

Prepare antibodies stocks according to the manufacturer's instructions. Tertiary antibodies are conjugated to fluorchromes. Store up to several months as frozen aliquots. Prepare working solutions of primary and secondary antibodies according to the manufacturer's instructions using the guidelines for final antibody listed in Table 22.4.14. Diluted antibodies should be kept at 4°C in light-proof containers for 2 to 3 months.

Minor adjustments in concentration will need to be made to account for background and signal intensity.

DAPI, 100 μg/ml

Dilute all of the powder from a 1-mg bottle of 4′,6-diamidino-2-phenylindole (DAPI; Sigma) in 10 ml buffer to produce a 100 μg/ml stock solution. Store in small aliquots up to 1 year at −20°C.

CAUTION: *DAPI is a potential carcinogen and should be handed with care.*

DAPI in antifade

Combine the following in order:
5.0 ml 1× PBS (*APPENDIX 2A*)
500.0 μl 100 μg/ml DAPI stock (see recipe; 1 μg/ml final)
0.5 g *p*-phenylenediaminie (Sigma; 10 mg/ml final; dissolve well)
45.0 ml glycerol (90% v/v final)

Transfer to a 50.0-ml conical tube, wrap with aluminum foil (product is light sensitive), and place on a rotator 30 min to ensure proper mixing. Store in 1-ml aliquots up to 1 year at −20°C.

The resulting solution is very viscous

This reagent is available commercially as Vectashield (Vector Laboratories).

DNase I, 3 mg/ml

3.0 mg DNase I powder
500.0 μl glycerol (50% v/v final)
50.0 μl 1 M Tris·Cl, pH 7.5 (50 mM final; *APPENDIX 2A*)
5.0 μl 1 M $MgCl_2$ (5 mM final)
1.0 μl 1 M 2-mercaptoethanol (1 mM final)
1.0 μl 10 mg/ml BSA (10 μg/ml final)
Adjust volume to 1.0 ml with H_2O
Store in 50-μl aliquots up to 1 year at −20°C.

DNase I dilution buffer

250.0 μl 1 M Tris·Cl, pH 7.0 (50 mM final; *APPENDIX 2A*)
25.0 μl 1 M $MgCl_2$ (5 mM final)
5.0 μl 1 M 2-mercaptoethanol (1 mM final)
20.0 μl 10 mg/ml BSA (4 μg/ml final)
4.7 ml H_2O
Store in 1-ml aliquots up to 6 months at −20°C *or*
In 1 ml aliquots up to 1 month at 4°C.

d*NTP mixture, 1 and 2 mM*

For a 1 mM mixture:
1.0 μl 100 mM dATP
1.0 μl 100 mM dCTP
1.0 μl 100 mM dGTP
97.0 μl H_2O, sterile
Store in 100-μl aliquots up to several months at −20°C

For a 2 mM mixture: Double the volume of each 100 mM dNTP added (i.e., use 2 μl of 100 mM dNTP) and reduce the amount of water to 94 μl. Store up to several months.

The total volume of either solution is 100 μl.

Fluorochrome/dTTP mixture

The amount of fluor-dUTP will vary. Thus, the recipes below are guidelines. Refer to the manufacturer's suggested final concentrations. All labeled nucleotides should be stored in 20 to 30-μl aliquots up to 6 months at −20°C in light-proof containers.

FITC-dUTP/dTTP:
3.5 μl 1 mM dTTP (0.6 mM final)
1.75μl 1 mM fluorescein (FITC)-dUTP (0.3 mM final; Roche)

The total volume, 5.25 μl, is appropriate for one labeling reaction and can be scaled up as needed.

Rhodamine-dUPT/dTTP mixture:
3.5 μl 1 mM dTTP (0.6 mM final)
1.75 μl 1 mM rhodamine-dUTP (0.3 mM final; Roche)

The total volume, 5.25 μl, is appropriate for one labeling reaction and can be scaled up as needed.

Texas Red-dUPT/dTTP mixture:
3.5 μl 1 mM dTTP (0.7 mM final)
1.0 μl 1 mM Texas Red-dUTP (0.2 mM final; Roche)

The total volume, 4.5 μl, is appropriate for one labeling reaction and can be scaled up as needed.

Cy3-dUPT/dTTP mixture:
3.5 μl 1 mM dTTP (0.7 mM final)
1.25 μl 1 mM Cy3-dUTP (0.2 mM final; Amersham Biosciences)

The total volume, 4.75 μl, is appropriate for one labeling reaction and can be scaled up as needed.

Cy5-dUPT/dTTP mixture:
3.5 μl 1 mM dTTP (0.7 mM final)
1.25 μl 1 mM Cy5-dUTP (0.2 mM final; Amersham Biosciences)

The total volume, 4.75 μl, is appropriate for one labeling reaction and can be scaled up as needed.

Formamide, 70%/Tris·Cl (pH 7.0 to 7.5), 10 mM/BSA, 0.1% (w/v)

Dissolve 100 mg BSA in 29 ml water. To this solution, add 70 ml formamide (Invitrogen) and 1 ml of 1 M Tris·Cl, pH 7.0 to 7.5 (*APPENDIX 2A*). Store up to several weeks.

CAUTION: *Formamide is a carcinogen and should be handled with care. Discard according to institution's biohazard rules*.

Total volume is 100.0 ml; however, the solution can be made in larger quantities and stored up to several weeks at 4°C.

Hapten/dTTP mixture

The amount of hapten-dUPT will vary. Thus, the recipes below are guidelines. Refer to the manufacturer's suggested final concentrations. Store up to several months or a year at −20°C.

Biotin-dTTP/dUTP mixture:
3.5 µl 1 mM dTTP (0.6 mM final)
1.75 µl 1 mM biotin-16dUTP (0.3 mM final; Invitrogen)

The total volume, 25 µl, is appropriate for one labeling reaction and can be scaled up as needed.

Digoxigenin-dTTP/dUTP mixture:
3.5 µl 1 mM dTTP (0.6 mM final)
1.75µl 1 mM dig-11dUTP (0.3 mM final; Roche)

The total volume, 5.25 µl, is appropriate for one labeling reaction and can be scaled up as needed.

Hybridization buffer

500.0 µl high grade formamide (50% v/v final; Invitrogen)
100.0 µl 20× SSC (2× final; *UNIT 18.6*)
100.0 µl dextran sulfate (10% final)
300.0 µl H_2O
Store in 100-µl aliquots up to several months at 4°C

Total volume is 1.0 ml.

Hybridization buffer can also be purchased from DAKO.

Loading dye, 5×

0.125 g bromphenol blue (0.25% final)
15.0 ml glycerol (30% final)
35.0 ml H_2O
Store in 1.0-ml aliquots up to several months at 4°C

Total volume is 50.0 ml.

NBT/BCIP

22.5 µl 75 mg/ml NBT (Invitrogen)
17.5µl 50 mg/ml BCIP (Invitrogen)
4.96 ml 100 mM Tris·Cl (pH 9.5; *APPENDIX 2A*)/100 mM NaCl/50 mM $MgCl_2$
Prepare fresh for each experiment.

Volumes can be scaled up or down as required.

Nick translation buffer, 10×

1.0 µl 10 mg/ml BSA (0.1 µg/µl final)
10.0 µl 1 M 2-mercaptoethanol (0.1 M final)
50.0 µl 1 M Tris·Cl (0.5 M final)
5.0 µl 1 M $MgCl_2$ (50 mM final)
34.0 µl H_2O
Store in 100-µl aliquots up to several months at 4°C

Total volume is 100.0 µl.

PBS, 1×/$MgCl_2$, 0.05 M

950.0 ml 1× PBS
50.0 ml 1 M $MgCl_2$
Store at room temperature until ready for use.

The total volume is 1 liter.

Pepsin, 0.5% (w/v) in NaCl, 0.85%

Dissolve 500 mg pepsin in 100.0 ml of 0.85% (w/v) sodium chloride. Adjust pH to 1.5 with 12 N HCl. Prepare fresh for each experiment.

PI, 100 µg/ml

Dissolve the powder from an entire 10-mg bottle of propidium iodide (PI; Sigma) in 10 ml water to a final concentration of 100 µg/ml. Store in small aliquots indefinitely at −20°C.

PI in antifade

Combine in the following order:
5.0 ml 1(PBS; *APPENDIX 2A*)
150.0 µl 100 µg/ml PI (0.3 µg/ml final; see recipe)
0.5 g *p*-phenylenediaminie (10 mg/ml final; Sigma; dissolve well)
45.0 ml glycerol (90% final)

Transfer to a 50.0-ml conical tube, wrap in aluminum foil (the product is light sensitive), and place on a rotator 30 min to ensure proper mixing. Store in 1-ml aliquots up to several months at −20°C.

The resulting solution (50 ml total) is very viscous.

This solution is available commercially available from Vectashield (Vector Laboratories).

RNase I, 100 µg/ml

Prepare a final 100 µg/ml solution of RNase I in 2× SSC (*UNIT 18.6*) fresh for each experiment using any molecular-grade RNase enzyme.

Sonicated salmon sperm DNA standards

Prepare 50.0 ng/µl salmon sperm DNA standard by combining 5.0 µl of a 10 µg/µl stock (Invitrogen), 300 µl of 5× loading dye (e.g., *UNIT 18.6*), and 695 µl water. Prepare a 25.0 ng/µl standard by combining 500 µl of the 50 ng/µl standard, 100 µl of 5× loading dye, and 400 µl water. Prepare a 12 ng/µl standard by combining 500 µl of the 25.0 ng/µl standard, 100 µl of 5× loading dye, and 400 µl water. Store all standards up to several months at 4°C or indefinitely at −20°C.

The average size of the commercial salmon sperm is ∼500 to 2.0 kb

Triethanolamine (pH 8.0), 0.1 M

660.0 μl triethanolamine (final 0.1 M; Sigma)
50.0 ml H_2O
Prepare fresh for each experiment.

Total volume is 50.0 ml.

COMMENTARY

Background Information

Applications of FISH

As early as 1988 (Lichter et al., 1988), FISH was used to visualize labeled DNA probes hybridized to chromosome and interphase nuclei preparations. The improvement of cloned DNA sources, antibodies, fluorochromes, microscopy and imaging equipment, and software has permitted a variety of scientific investigations. FISH analysis is routinely used in clinical cytogenetic laboratories for detection of syndromes in prenatal assessments, including Prader-Willi/Angelman Syndrome, DiGeorge Syndrome, and Cri-du-chat. Cancer cytogenetics laboratories use FISH to confirm and monitor hematological malignancies such as CML and AML (Table 22.4.19 and Table 22.4.20). Gene amplification studies for neuroblastoma (MYCN amplification), and breast cancer (HER2/NEU amplification) are also routinely carried out. Mapping of newly identified genes have relied heavily on FISH (Squire et al., 1993; Lichter et al., 1988) both in humans and other species (Boyle et al., 1990, Giguere et al., 1995). Metaphase spreads may not always be easy to obtain, thus interphase cells offer a means of obtaining information, albeit via an indirect method. This has been useful for gauging chromosome instability (Speicher et al., 1995; Ghadimi et al., 1999; Al-Romaih et al., 2003; Vukovic et al., 2003) and determining normal versus abnormal content, as well as gene amplification or deletion. FISH applied to paraffin-embedded sections appeared in the early 1990s (Thompson et al., 1994) and was applied to both thick (>5 μm) and thin (5 μm) paraffin sections. Application of FISH to paraffin-embedded sections provides a tremendous opportunity to correlate the histopathological classification to the genomic changes detected. Furthermore, it enables the investigator to study concepts of cellular heterogeneity, tumor focality, and metastasis (Squire et al., 1996). Paraffin FISH analysis requires patient and careful technical expertise. Proper controls must be used when interpreting final results. Depending on the type of information sought, paraffin FISH is an acceptable form of data collection when fresh tissues for cytogenetic suspensions are not available; however, paraffin FISH will not provide chromosome-based information.

Preparation of probes

The development of reliable cloning strategies in the 1980s facilitated the genomic analysis and sequencing of specific DNA fragments. Mapping of these genes to their chromosomal locations was previously laborious and infrequently reliable. The emergence of FISH in the early 1990s paved the way for an effective and direct means of mapping specific DNA fragments to their chromosomal locations.

Types of probes. The creation of comprehensive genomic libraries as a result of the human genome project provides renewable resources for FISH probes. At present, bacterial artificial chromosomes (BACs) are the most popular cloned forms of genomic DNA used for FISH probes. Although yields are generally considered low in comparison to plasmids and cosmids (predecessors of BACs), BAC inserts are larger (200 kb) and can produce a stronger FISH signal compared to cosmids and plasmids, which are considerably smaller in insert size (i.e., ranging from 2 to 30 kb). P1 artificial chromosomes (PACs) and yeast artificial chromosomes (YACs) have also been used in the past and are still used in many research laboratories. Other probes, such as RNA and oligonucleotide probes are options and are reviewed in Speel (1999), and Schwarzacher and Heslop-Harrison (2000). More recently, peptide nucleic acid (PNA) probes (Lansdorp, 1996), have been used for FISH experiments with great success (Martens et al., 1998; Vukovic et al., 2003.). Using the same nucleotide bases as DNA probes, PNA probes do not have a phosphate or deoxyribose sugar backbone. Specific fluorochromes, haptens, or enzymes can be chemically attached to the bases and used in the same fashion as DNA probes. PNA probes are sequence specific. Thus, sequence information must accompany a PNA custom probe

order to companies such as Applied Biosystems (*http://www.appliedbiosystems.com*). There are several advantages of PNA probes. For instance, PNA has a neutral backbone, which provides stronger binding and greater specificity of interaction. Also, the uncharged PNA structure creates stronger binding independent of salt concentration, allowing more robust hybridization applications. Finally, the fact that PNA oligomers have resistance to nucleases and proteases, permitting more robust in situ experiments, is also an advantage. The drawback of PNA probes are in the cost of their fabrication. The typical molecular (cytogenetic) laboratory cannot generate these probes in-house. The cost-effectiveness of PNA probes, therefore, can only be appreciated if the laboratory is carrying out a particular hybridization frequently, as in the case of a clinical cytogenetic laboratory.

Methods for preparing probes. By far the most commonly used strategy for labeling DNA probes is by nick translation. Nick translation involves the simultaneous actions of two enzymes: DNase I and DNA polymerase I. DNase I randomly nicks the DNA fragment in each strand of the double-stranded DNA molecule. DNA polymerase I (derived from *E. coli*), with its three activities—i.e., exonuclease function (removes bases in the 5′ to 3′ direction), polymerase function (adds nucleotides from the 3′ nick site), and the 3′ to 5′ proof reading function—incorporates the label, whether indirectly using a hapten (i.e., biotin or digoxigenin) or directly (i.e., fluorescein or rhodamine). Nick translation can be applied to all cloned DNA sources without the need to remove vector sequences. Polymerase chain reaction (PCR) is another commonly used method of labeling DNA probes from cloned sources, as well as from microdissected genomic DNA or flow-sorted chromosomes. PCR employs multiple rounds of template denaturation, primer annealing, and template replication, facilitated by *Taq* DNA polymerase. Primers are variable and may include sequence-specific primers to amplify those targeted fragments, vector sequence primers to amplify and label cloned DNA, and/or universal primers. Compared to nick translation, which requires micrograms of starting DNA, PCR requires only nanograms of template. Furthermore, nick translation is most efficient when the starting DNA is of high molecular weight with little or no degraded material. PCR labeling is a little more forgiving of DNA quality; thus, suboptimal DNA may still be used when not acceptable for nick translation. Depending on the template, several rounds of amplification may be required before labeling, as in the case of in-house production of chromosome painting probes. Other labeling strategies, including random priming and end-labeling, are also options and are described in Schwarzacher and Heslop-Harrison (2000).

For both nick translation and PCR labeling, commercial kits and enzyme mixes are readily available and optimized. Usually, commercial kits combine DNase I and DNA polymerase I as an enzyme mix. The drawback of combining them is the fact that one enzyme (usually the DNA polymerase I) will lose its enzymatic activity before the other. Commercial kits can also be costly. Making an in-house kit allows the investigator to alter enzyme concentrations as necessary to better optimize the reaction. This alleviates the combined-enzyme problem with commercial kits. In-house kits are also expensive and they do require rigorous internal quality control and troubleshooting from time to time.

See Critical Parameters for discussion of slide preparation, hybridization, post-hybridization washes and detection, and interpretation of FISH findings.

Critical Parameters

Preparation of probes

DNA quality. Ensure that the starting DNA to be labeled has a high molecular weight with no degraded DNA fragments or protein. Contaminating RNA will contribute slightly to the spectrophotometric quantification of the DNA and cause a miscalculation of the actual starting amount of DNA. DNA should be assessed using an accurate spectrophotometer and by gel electrophoresis. Compare the concentration of DNA as determined using a spectrophotometer and gel with a DNA of known concentration. The DNA used as a standard for concentration can be another undigested clone or genomic DNA.

The starting DNA should be dissolved in water rather than Tris/EDTA (TE) buffer. TE is normally used to inhibit any DNases that may be present in the water. By that reasoning, TE buffer has been known to inhibit the enzymatic activity of the DNase during the nick translation reaction. Furthermore, it may inhibit the polymerase activity for nick translation and PCR labeling.

Reagents. Ensure that reagents are used prior to their expiration dates. The most critical reagents in the labeling reactions are:

1. DNA polymerase I/*Taq* DNA polymerase. Failure of polymerase activity in the reaction will lead to inefficient incorporation of the labeled nucleotide into the DNA, resulting in a very weak FISH signal.

2. Conjugated nucleotides. Nucleotides conjugated to a fluorochrome are light sensitive. Repeated and prolonged exposure to direct light will quench the fluorescent signal. Store these reagents in small aliquots at −20°C in light-proof containers. Restrict exposure to light during the labeling procedure and store labeled DNA at −20°C in light-proof containers. Unlike nonfluorescently labeled probes, these directly-labeled DNAs will have a shorter shelf life. The concentration of nucleotides can vary from as much as 1 mM to as low as 0.2 mM in different nick translation protocols. The labeled nucleotide should always be in at least a 2- fold excess of the corresponding unlabeled nucleotide. Furthermore, the labeled nucleotide can be interchangeable between hapten-dUTP or hapten-dATP. If the investigator chooses to use Biotin 14-dATP rather than the dUPT form, the nucleotide mixture should be adjusted accordingly.

3. 2-Mercaptoethanol. Buffers containing 2-mercaptoethanol should be replaced every 3 to 4 months, particularly if not stored at −20°C.

4. DNase I. Although the activity of DNase I is usually potent enough that it may be used for up to a year when stored at −20°C, the specific activity may decrease over time. This is evident when the fragment sizes are larger than expected when the standard DNase I concentration and incubation times are used. Simply increase the amount of DNase I used in the reaction and keep the same period of incubation, or keep the same amount of DNase I used in the reaction and increase the time of incubation.

5. PCR factors. Treat PCR labeling like any other PCR experiment and consider the following factors: amount of template, pH, magnesium ion concentration, and primer sequence.

Equipment. Small volumes are used for both labeling strategies. Pipets should be calibrated and serviced often. Use an accurate spectrophotometer and water bath that can effectively maintain a constant temperature, if a thermocycler is not available for nick translation.

Preparation of slides

FISH involves the hybridization of the labeled and denatured DNA probe to its denatured DNA target located on the slide. Each component requires care to obtain the most effective results. Pretreatment of the slide with a protease before denaturation permits the removal of any cellular and cytoplasmic debris. In the case of paraffin-embedded sections, more aggressive treatment is required to remove residual wax as well as to allow sufficient protease treatment to permit access of the probe to the intended target DNA.

Protease treatment. Treatment with pepsin has been featured in this protocol because its action is relatively mild as compared to other proteases such as proteinase K; however, many laboratories use proteinase K alone or in combination with pepsin, particularly for the digestion of paraffin-embedded sections, to obtain the proper digestion conditions for FISH (see Table 22.4.9). The incubation times of any protease treatment will vary according to the type of tissue (e.g., paraffin-embedded), quality of the slide (i.e., quantity of cytoplasmic debris/cellular debris), as well as the lot or batch of pepsin used. It is suggested that incubation times be increased rather than the concentration of pepsin used in the digest. The temperature at which the incubations occur is also critical. Higher temperatures facilitate faster digestions as opposed to those at room temperature or below. While variations in the pretreatment protocol can be made, the starting quality of the fixed tissue is the most critical factor. Ideally, the tissue should be fixed shortly after removal from the host.

Paraffin-embedded samples. In the case of paraffin-embedded tissues, formalin is the fixative of choice for routine use in most clinical laboratories; however, superior results for most research needs may be obtained if tissue sections are fixed using ethanol or paraformaldehyde prior to embedding. Buffered formalin solutions have also been implemented to provide better recovery of DNA, RNA, and protein for future studies. The time spent in fixation is equally as important as the type of fixative. The longer the time spent in fixative, the more difficult it may be to denature the DNA to a single-stranded state. This is due to the cross-linking action of the fixative to proteins that enables the cells to maintain their morphology. Sections for FISH should be cut fresh from the original block, preferably a few sections from the exposed face. This will help to ensure that the DNA has not been exposed to DNases, or oxidative damage from the handling of the block or from the local environment. Several sections should be taken so that different pretreatment conditions can be tested. It is suggested that the investigator take the time to contact the

histology laboratory preparing the specimens to document the fixation procedure for troubleshooting later.

Denaturation. Denaturation is also a critical parameter for successful FISH. Too much denaturation of the target DNA causes excessive DNA damage, yielding poor banding and/or hybridization. Too little denaturation results in ineffective hybridization due to limited access to the target DNA. The extent of denaturation will depend on several factors.

1. Quality of the slide. Namely, the presence of cytoplasmic and/or cellular debris.
2. Age of the slide. Whether using a fresh slide, a fresh slide with artificial aging, a slide that is 1 week or 1 month old, and so forth.
3. Previous assays. Namely if banding has previously been performed on the slide.
4. Tissue type. Whether fibrous, bony, or muscle tissue, or the like, is being used.
5. Age and method of fixation. See above.
6. Section thickness. Normally 5-μm-thick sections are used.

Hybridization

Optimal hybridization is influenced by many factors that will affect the target DNA and the probe itself. These factors are sources for inefficiencies and should be carefully monitored and documented such that effective troubleshooting can be carried out.

Stringency. The hybridization of a DNA probe to its DNA target is dependent on the extent of sequence homology, the complexity of the DNA sequence, and the chemical factors that influence the success of maximal binding efficiency. Stringency refers to the percentage of matches and mismatches between the probe and target such that the hybridized double helix remains stable. FISH experiments are typically run at 70% to 90% stringency, with 70% representing a lower relative stringency than 90%. This is critical when determining the feasibility of carrying out cross-species FISH experiments (i.e., human probes on mouse targets or vice versa), where sequence homology may be considerably lower in parts of the probe. The reagents present in hybridization buffers, as well as the temperature significantly influence stringency.

Temperature. DNA strand melting and annealing are influenced by temperature. Since reagents such as formamide are present in the hybridization buffer, high temperatures (i.e., 80° to 90°C) are not required for DNA denaturation. This is the reason denaturation of the DNA can be accomplished at 72°C in the presence of formamide. Increasing temperatures also increases stringency, such that a 1°C alteration increases the stringency by 1%. Thus, increasing the hybridization temperature from 37° to 42°C will increase the stringency of the hybridization step, requiring the probe to possess higher sequence homology with its target. This may be particularly useful if there is a chance of cross-hybridization, which is typically the case with locus-specific probes of genes that belong to a class of genes/sequences that are very similar. Pseudogenes may also cross-hybridize. Lowering the hybridization temperature under 37°C will result in lower stringency conditions permitting the hybridization of the probe to targets with greater mismatch. This also has advantages and disadvantages. At low stringency, mismatched spurious hybridization events will be more common and contribute to more experimental noise at the detection step.

Probe size. The rate of hybridization is also influenced by probe size. Generally, longer fragments (>500 bp) will require a longer time to hybridize than shorter ones. Once again, the hybridization is affected by the extent of sequence homology.

PNA probes. In the case of PNA probes, the specificity of the probe, as a required function of their fabrication through oligosynthesis, eliminates some of the complexities experienced by DNA probes. Since the PNA probe has an uncharged backbone, there is no charge repulsion when the probe hybridizes to the DNA, thus creating a stronger bond. Furthermore, PNA/DNA bonds will melt 15°C above DNA/DNA bonds, almost independently of salt concentration. The specificity of the PNA/DNA bond lies in the increased destabilization of the PNA/DNA bond when a mismatch occurs.

Post-hybridization washes and detection

Stringency. The factors that affect hybridization also influence the conditions involved in post-hybridization washes and antibody incubations. Incubation at 37°C under a range of 50% to 60% formamide in 2× SSC sets a stringency of ~70% to 80%. This permits hybridization of sequences with a 20% to 30% mismatch and maintains duplex stabilization. Increasing hybridization temperatures raises the stringency, requiring greater sequence homology for effective hybridization. Conversely, decreasing temperatures permits hybridization with greater mismatch. Decreasing salt ion strength (i.e., SSC) also increases

Table 22.4.21 Stringency of DNA/DNA Hybridization and Post-Hybridization Washes[a]

SSC concentration	Temperature[b]	Stringency as a function of % formamide						
		60	55	50	45	40	35	30
5×	37°C	76	73	70	67	64	61	58
2×	37°C	83	80	77	74	71	68	65
1×	37°C	88	85	82	79	76	73	70
0.75×	37°C	90	87	84	81	78	75	72
0.5×	37°C	93	90	87	84	81	78	75
0.2×	37°C	100	96	93	90	87	84	81
0.1×	37°C	105	101	98	95	92	89	86
5×	42°C	81	78	75	72	69	66	63
2×	42°C	88	85	82	79	76	73	70
1×	42°C	93	90	87	84	81	78	75
0.75×	42°C	95	92	89	86	83	80	77
0.5×	42°C	98	95	92	89	86	83	80
0.2×	42°C	105	101	98	95	92	89	86
0.1×	42°C	110	106	103	100	97	94	91

[a]This Table describes the changes in stringency for probes with 43% GC content at 300 bp (modified from Schwarzacher and Heslop-Harrison, 2000). Increasing the formamide content or temperature, or decreasing the SSC strength will increase stringency. A combination of these three factors can be used to modulate the stringency.

[b]Hybridization of probe to DNA typically occurs under 50% formamide in 2× SSC at 37°C, giving a 77% stringency. Washing of unbound probe following hybridization typically occurs under 50% formamide in 2× SSC at 42°C, giving an 82% stringency.

stringency. The combination of temperature and salt concentration can affect hybridization kinetics considerably. The same holds true for removal of unbound probe.

Typically, post-hybridization washes are carried out at 42° to 45°C. This temperature, coupled with 50% formamide and 2× SSC, permits 90% stringency for probes with a 43% GC content, at a probe size of 300 bp (Schwarzacher and Heslop-Harrison, 2000). Table 22.4.21 describes the stringency conditions based on these parameters.

Detergents. Some suppliers of commercial probes may suggest higher temperature washes of 72°C in a low ion (high stringency) SSC wash containing a detergent. This too is based on high sequence homology and the relative GC sequence content of the probe. This rapid wash method eliminates the need for formamide. Caution should be exercised when using high temperatures, since there is the risk of partial denaturation of the hybridized probe and target. This will result in loss of signal. A rapid wash should only be used with probes that possess high sequence homology and high labeling efficiency. Commercial probes tend to fit these criteria.

The subsequent SSC washes and washes with detergents serve to remove any remaining unbound probe and antibodies. As before, decreasing the SSC content will create a more stringent wash. The choice of detergent is subjective, largely based on the preference and experience of the investigator. The most commonly used detergents include Tween 20, NP-40, IPEGAL, SDS, and Triton X-100 (reviewed in Speel, 1999). In general, the actions of these detergents are comparable when used at the standard 0.1% in 4× SSC; however, SDS, Triton X-100, and NP-40 are generally harsher than Tween 20. If high background persists, the number of washes with the detergent can be increased or the percentage of detergent and SSC strength can be altered.

Antibodies. The choice of antibody systems is also fairly standard. Many companies provide antibodies raised in different animals and conjugated to different fluorochromes so that a variety of probes may be used and detected at one time. Some antibodies may require a higher concentration; thus, it is important that the signal strength be monitored carefully for background and the antibody concentration adjusted as required. As discussed

above, caution should be exercised when using various antibody combinations for detecting more than one probe: ensure that there is no cross-reactivity between antibodies raised in different species.

Counterstains. Finally the use of a counterstain helps to visualize the cells or spreads. DAPI stains DNA blue upon fluorescence and is most commonly used. DAPI, however, may obscure a green signal if using a dual filter (i.e., FITC/DAPI), so a red counterstain, such as propidium iodide (PI), may be more appropriate. If the color of probe detected is red, do not use PI as a counterstain. The signals are protected from rapid quenching through the reagents used in the mounting medium. These counterstains in mounting (antifade) medium can be purchased commercially. If the counterstain is prepared in the laboratory, be sure to use high quality glycerol, since some lower quality glycerols can cause autofluorescence. The concentration of counterstain may require adjustment. Higher counterstain concentrations may obscure FISH signals. If this is the case, dilute the counterstain in antifade by adding more glycerol and PBS in amounts that will be consistent with the final concentrations.

Storage. All slides should be stored at −20°C for long-term storage (i.e., up to 1 year) and at 4°C for short-term storage (i.e., up to 6 months).

Troubleshooting

Preparation of probes

See Table 22.4.22 for a troubleshooting guide to labeling.

Preparation of slides

Cytogenetic specimens. The influence of pretreatment and denaturation cannot be readily assessed until the entire FISH protocol has been carried out and the slides are visualized by fluorescence microscopy (Henegariu et al., 2001). The slides, however, may be visualized by phase-contrast microscopy for cytogenetic preparations after the dehydration step following pepsin treatment to determine the extent of digestion. The slides may be returned for digestion if the cytoplasmic debris does not appear to have been removed. Similarly, for treatment of paraffin sections, following visualization for autofluorescence, the slides may be returned for digestion and reassessed.

If there are no cells remaining on the slide following pepsin treatment, then the treatment was too harsh. If many cells have lifted off, the treatment was too harsh. Adjust the time or pepsin concentration as required.

Paraffin sections. The diameter of nuclei in various tissue types will vary between 4 and 8 μm. The estimated diameter for the tissue of interest should be ascertained from the literature and sections of the same thickness or 2 to 4 μm greater should be cut, so that it is likely that the entire nucleas is included in the section.

Pepsin digestion works for most tissue types. Occasionally, the tissue will exhibit persistent green autofluorescence of nuclei and no signal (see Table 22.4.19). Variation in histology fixation procedures and quality control performed by the hospital pathology laboratory necessitates careful assessment of treatment times and/or concentration of pretreatment (also see Table 22.4.9). Certain tissue sections are more resistant to effects of protein digestion than others. This may be due to the tissue type or amount of protein cross-linking elicited by excessive formalin fixation. If surplus fresh tissue can be obtained, then ethanol or paraformaldehyde fixation will cause less cross-linking and provide better results for most research applications. It should also be noted that normal tissues are often more refractory to protein digestion than tumor material derived from the same tissue type. If there are a limited number of slides remaining from the patient sample of interest it is sometimes helpful to use a superfluous slide from another sample of the same age and cell type to derive the optimal digestion parameters with respect to aging and storage. More comprehensive guidelines for improved paraffin results are summarized by Hyytinen et al. (1994).

Anticipated Results

Preparation of probes

Successful labeling for FISH will yield fragment sizes between 200 and 500 bp. Fragments up to 1 kb are acceptable, but larger fragments will contribute to background. Investigators should expect that the actual amount of DNA aliquoted onto the gel for visualization should be fairly close to the expected amount loaded onto the gel. The labeling reaction is fairly tolerant to variations in concentration from half the actual amount to two-times greater than the actual amount.

Once the labeling conditions have been established and tested in control FISH experiments, the investigator can be confident that subsequent labeling experiments will proceed without incident. Minor adjustments must

Table 22.4.22 Troubleshooting Guide to FISH Probe Labeling[a]

Problem	Cause	Solution
DNA failed to digest	DNase I not present	Ensure that DNase I was added to the labeling reaction.
	Loss of DNase I activity	If DNase I was added, check the expiration date or change to a fresh aliquot.
	TE buffer used to dissolve DNA (EDTA inactivates DNase I)	If TE buffer was used to resuspend the DNA, reprecipitate the DNA, resuspend in water, quantify by spectrophotometry, and relabel.
Fragments too small	Too much DNase I added	Pipetting errors are the leading cause of many experimental problems. Use accurate pipettors.
	DNA starting concentration inaccurate	Refer back to spectrophotometer readings and gel images of undigested DNA to determine whether there were any discrepancies. Contaminating RNA and proteins may contribute to an inaccurate reading, such that the actual amount of starting DNA was less than expected, thus changing the labeling (digestion) kinetics for a specified amount of DNA over a specific time at a specific DNase I concentration. Relabel and increase the amount of starting DNA as required
	Starting DNA was degraded	If the starting DNA already contains degraded fragments, they will simply continue to degrade into relatively smaller fragments. Refer back to the gel of the undigested DNA and determine the percentage of degraded fragments present. Compensate for the fragments that will be digested away and increase the amount of DNA required to relabel. Consider PCR labeling strategies or obtain a better DNA sample.
	Labeling time too long	Be sure that the correct incubation time was used.
	Unstable water bath temperature	The labeling reaction has been optimized for a specific temperature. Increasing the temperature will facilitate faster nicking, while decreasing the temperature will slow it.
Fragments too long	Insufficient DNase I	Use accurate pipettors (see above).
	DNA starting concentration inaccurate	As described above, check the spectrophotometer reading and gel image. If too much DNA was added to the labeling reaction, the same amount of DNase I used to nick a given amount of DNA in a specific time will increase when there is more DNA present. The labeling of this DNA can be salvaged by continuing to incubate for 20 to 30 min (or as required). Be sure to spike the reaction with fresh enzymes.

continued

Table 22.4.22 Troubleshooting Guide to FISH Probe Labeling[a] , *continued*

Problem	Cause	Solution
	Starting DNA was dissolved in TE buffer	Check whether the starting DNA was dissolved in TE as this will inhibit the nicking of DNA. The labeling of this DNA can be salvaged by continuing to incubate as required. Be sure to spike the reaction with fresh enzyme.
	Decreasing activity of DNase I	Over time, the specific activity of DNase I will decrease. If this is the case, increase the amount of DNase I used in the reaction, while keeping the same incubation period. Otherwise, keep the same DNase I concentration and increase the labeling time.
No fragments present	Absence of DNA added to reaction	Make sure that DNA was added to the reaction
	Insufficient DNA added	As discussed above, check to see that the concentration of DNA is accurate and pipettors are accurate.
	Starting DNA degraded	If a significant portion of the starting DNA was degraded to begin with, as shown by gel electrophoresis, this will cause an increase in fragmentation speed, with the smaller fragments running off the gel. A new DNA specimen should be used, or the investigator should consider PCR methods.
	Too much DNase I added	Check that the proper DNase I concentration and/or volume was used in the labeling reaction.
Expected amount of DNA too low/too high (but fragmented to the right size)	DNA starting concentration inaccurate	Assess using a concentration standard. If the actual amount of DNA is less than expected, as determined by gel electrophoresis, then the starting amount of DNA was insufficient or caused by some of the factors described above. If the investigator chooses to relabel, increase the amount of starting DNA as required. If the actual amount of DNA is greater than expected, again as determined by gel electrophoresis, then the starting amount of DNA was incorrect. The investigator may choose to proceed and use this probe for FISH, but the overall incorporation of label into the DNA will be lower since the labeling reaction contains sufficient dNTPs for a specified DNA quantity. If the investigator chooses to relabel, decrease the amount of starting DNA material as required.
PCR failed	Amount of template, pH, or $MgCl_2$ concentration incorrect, or quality of template poor	Refer to *APPENDIX 3F* and *APPENDIX 2A*.

[a]The guide assumes that the labeling conditions have generally been optimized but occasionally anomalies occur.

be made when using new reagents. Once a probe has been FISHed, the signal should be clearly visible with little or no background or cross-hybridization. The investigator should be aware that other factors, such as hybridization efficiency, slide quality, antibody quality, stringency of post-hybridization washes can also affect the signal strength.

Preparation of slides

Optimal pretreatment of cytogenetic or paraffin-embedded slides should produce slides that are relatively free of cytoplasm/cellular debris, free of paraffin, and have adequately denatured DNA for FISH. The samples should not be autofluorescent.

Hybridization

Due to the multistepped nature of FISH, it is difficult to determine the success of hybridization until the entire assay is completed. Successful hybridization will depend on adequate denaturation of the target DNA on the slide, as well as adequate denaturation of the probe. Proper suppression of repetitive sequence elements with Cot-1 DNA can increase the specificity of hybridization. The specificity of hybridization is also regulated by stringency factors described above (see Background information; also see Critical Parameters). Successful hybridization will allow the identification of chromosomal location of the probe sequence.

Post-hybridization washes and detection

The protocols described provide a basic framework for adjusting post-hybridization washes and detections. Under ideal conditions, the stringency of the post-hybridization washes is sufficient to adequately remove any unbound/nonhybridized probe from the target DNA. The specificity of the probe and the suppression of repetitive elements during preannealing will also decrease the number of cross-hybridization signals. The concentration of detergent used in the washes is also optimized to remove any unbound antibodies, although minor modifications, as required, should be made when using a different brand, lot, or type of antibody. The result, upon visualization with a microscope, is a clean preparation showing minimal background and an easily visualized signal(s) on the DNA target.

Time Considerations

Preparation of probes

The most time consuming portion of labeling for FISH is in the optimization of the labeling procedure if using an in-house protocol. As discussed previously, commercial kits for labeling are available and give relatively consistent results.

Preparation of slides

The preparatory work for preparing cytogenetic slides requires minimal time and the protocol is fairly robust. Optimization for paraffin-embedded sections requires more careful time and trial-and-error. The preparation of cytogenic slides for FISH can be accomplished in ~1 hr. Once optimized conditions have been obtained for paraffin-embedded tissues, it too can be accomplished in 1 to 2 hr. However, initial optimization may require several experimental attempts.

Hybridization

The hybridization step of FISH typically occurs overnight (i.e.,. DNA probes), ~18 to 24 hr at 37°C. The time may be lengthened to 48 or 72 hr (as in the case with weekends), with no adverse affects. Hybridization with PNA probes require less time, at least 1 hr at room temperature. Caution, however should be taken that the slides are adequately sealed such that the probe in hybridization solution does not evaporate. Maintaining a slightly dampened slide container will help alleviate this problem.

Post-hybridization washes and detection

The time allotted for post-hybridization washes and detection will vary depending on the type of DNA probe used in the experiment. Directly labeled probes (DNA or PNA probes) require very little bench time since no antibody detection is required; typically they take up to 30 minutes. Probes are simply washed using high stringency washes as described in the methods. Indirectly labeled probes require incubations with antibodies (primary, secondary and/or tertiary), with the final incubation with a fluorochrome-conjugated antibody, as well as detergent washes and/or blocking steps between each antibody incubation. If the signal requires amplification, the bench time increases as each antibody incubation should last at least 30 minutes. A wash protocol using an indirectly labeled probe with signal amplification will range from 3.5 to 4 hours.

Acknowledgments

The authors would like to thank Elena Kolomeitz, Jana Karaskova, Paula Marrano, Ajay Pandita, Bisera Vukovic, and Zong Mei Zhang from the Department of Cellular and Molecular Biology, University Health

Network, Ontario Cancer Institute in Toronto, Ontario, Canada.

Literature Cited

Al-Romaih, K., Bayani, J., Vorobyova, J., Karaskova, J., Park, P.C., Zielenska, M., and Squire, J.A. 2003. Chromosomal instability in osteosarcoma and its association with centrosome abnormalities. *Cancer Genet. Cytogenet*. 144:91-99.

Beatty, B., Mai, S., and Squire, J.A. 2002. FISH: A Practical Approach. Oxford University Press. New York.

Boyle, A.L., Ballard, S.G., and Ward, D.C. 1990. Differential distribution of long and short interspersed element sequences in the mouse genome: Chromosome karyotyping by fluorescence in situ hybridization. *Proc. Natl. Acad. Sci. U.S.A*. 87:7757-7761.

Ghadimi, B.M., Heselmeyer-Haddad, K., Auer, G., and Ried, T. 1999. Interphase cytogenetics: At the interface of genetics and morphology. *Anal. Cell Pathol*. 19:3-6.

Giguere, V., Beatty, B., Squire, J., Copeland, N.G., and Jenkins, N.A. 1995. The orphan nuclear receptor ROR alpha (RORA) maps to a conserved region of homology on human chromosome 15q21–q22 and mouse chromosome 9. *Genomics*. 10:28:596-598.

Henegariu, O., Heerema, N.A., Wright, L.L., Bray-Ward, P., Ward, D.C., and Vance, G.H. 2001. Improvements in cytogenetic slide preparation: Controlled chromosome spreading, chemical aging and gradual denaturing. *Cytometry* 43:101-109.

Hyytinen, E., Visakorpi, T., Kallioniemi, A., Kallioniemi, O.P., and Isola, J.J. 1994. Improved technique for analysis of formalin-fixed, paraffin-embedded tumors by fluorescence in situ hybridization. *Cytometry* 1;16:93-99.

Kolomietz, E., Al-Maghrabi, J., Brennan, S., Karaskova, J., Minkin, S., Lipton, J., and Squire, J.A. 2001. Primary chromosomal rearrangements of leukemia are frequently accompanied by extensive submicroscopic deletions and may lead to altered prognosis. *Blood* 97:3581-8.

Lansdorp, P.M. 1996. Close encounters of the PNA kind. *Nat. Biotechnol*. 14:1653.

Lichter, P., Cremer, T., Borden, J., Manuelidis, L., and Ward., D.C. 1988. Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. *Hum. Genet*. 80:224-234.

Munne, S., Marquez, C., Magli, C., Morton, P., and Morrison, L. 1998. Scoring criteria for preimplantation genetic diagnosis of numerical abnormalities for chromosomes X, Y, 13, 16, 18 and 21. *Mol. Hum. Reprod.* 4:863-870.

Martens, U.M., Zijlmans, J.M., Poon, S.S., Dragowska, W., Yui, J., Chavez, E.A., Ward, R.K., and Lansdorp., P.M. 1998. Short telomeres on human chromosome 17p. *Nat. Genet*. 18:76-80.

Poon, S.S., Martens, U.M., Ward, R.K., Lansdorp, P.M. 1999. Telomere length measurements using digital fluorescence microscopy. *Cytometry* 36:267-278.

Qian, J., Bostwick, D.G., Takahashi, S., Borell, T.J., Brown, J.A., Lieber, M.M., and Jenkins, R.B. 1996. Comparison of fluorescence in situ hybridization analysis of isolated nuclei and routine histological sections from paraffin-embedded prostatic adenocarcinoma specimens. *Am. J. Pathol*. 149:1193-1199.

Schwarzacher, T. and Heslop-Harrison, P. 2000. Practical In Situ Hybridization. Springer-Verlag New York.

Speel, E.J.M. 1999. Detection and amplification systems for sensitive, multiple-target DNA and RNA in situ hybridization: Looking inside cells with a spectrum of colors. *Histochem. Cell Bio.* 112:98-113.

Speicher, M.R., Jauch, A., Walt, H., du Manoir, S., Ried, T., Jochum, W., Sulser, T., and Cremer, T. 1995. Correlation of microscopic phenotype with genotype in a formalin-fixed, paraffin-embedded testicular germ cell tumor with universal DNA amplification, comparative genomic hybridization, and interphase cytogenetics. *Am. J. Pathol*. 146:1332-1340.

Squire, J., Meurs, E.F., Chong, K.L., McMillan, N.A., Hovanessian, A.G., and Williams, B.R. 1993. Localization of the human interferon-induced, ds-RNA activated p68 kinase gene (PRKR) to chromosome 2p21–p22. *Genomics* 16:768-770.

Squire, J.A., Thorner, P., Marrano, P., Parkinson, D., Ng, Y.K., Gerrie, B., Chilton-Macneill, S., and Zielenska, M. 1996. Identification of MYCN copy number heterogeneity by direct FISH analysis of neuroblastoma preparations. *Mol. Diagn*. 1:281-289.

Thompson, C.T., LeBoit, P.E., Nederlof, P.M., and Gray, J.W. 1994. Thick-section fluorescence in situ hybridization on formalin-fixed, paraffin-embedded archival tissue provides a histogenetic profile. *Am. J. Pathol*. 144:237-243.

van de Rijke, F.M., Vrolijk, H., Sloos, W., Tanke, H.J., and Raap, A.K. 1996. Sample preparation and in situ hybridization techniques for automated molecular cytogenetic analysis of white blood cells. *Cytometry* 24:151-157.

Vukovic, B., Park, P.C., Al-Maghrabi, J., Beheshti, B., Sweet, J., Evans, A., Trachtenberg, J., and Squire, J. 2003. Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. *Oncogene* 22:1978-1987.

Internet Resources

http://www.appliedbiosystems.com

The Applied Biosystems website. This company supplies PNA probes and custom orders PNA probes.

http://www.cytocell.co.uk

The Cytocell website. This is a commercial supplier of DNA FISH probes

http://www.biochem.roche.com

The Roche Molecular Biochemicals website. This company is a supplier of antibody and labeling reagents.

http://www.vysis.com

The Vysis website. This company is a supplier of commercial DNA FISH probes.

Contributed by Jane Bayani and
Jeremy A. Squire
University of Toronto
Ontario, Canada

Multi-Color FISH Techniques

Small structural chromosomal aberrations are often difficult to determine with certainty using conventional cytogenetic banding methods alone (*UNIT 22.3*). The problems that can typically arise in both clinical and cancer cytogenetics are the presence of structural chromosome aberrations with unidentifiable chromosomal regions, or very complex chromosomes (sometimes called marker chromosomes) in which no recognizable region appears to be present. Confirmation of the cytogenetic origins of such chromosomal aberrations can sometimes be obtained by the judicious application of locus-specific FISH analysis (*UNIT 22.4*) if the investigator has some general impression regarding a possible identity. However, such an approach is very subjective and risky and requires some knowledge of the specific loci and available probes likely to be involved in the aberration. A more systematic approach is to use whole-chromosomal paints in succession, until the marker chromosome and its constituents can be identified. While this strategy will eventually identify each chromosomal region involved, it is both costly and time-consuming and may lead to the depletion of valuable patient samples. Recently, some generalized screening FISH techniques utilizing sensitive and differentially labeled chromosome-specific paints (also see *UNIT 22.4*) have been developed, and these allow the full chromosome complement to be analyzed to identify unknown aberrations. In this unit, the commonly available methods will be described with suggested protocols and approaches to troubleshooting.

There are currently two to three slightly different imaging systems available that utilize the mechanical rotation of fluorescence excitation filters to distinguish the distinct fluorescence of a mixture of chromosomal paints during image acquisition. Such filter-based systems are generically termed multicolor FISH (M-FISH; Speicher et al., 1996; each supplier has their own modified acronym for this technique). The second more frequently used system, called spectral karyotyping (SKY), utilizes image analysis based on Fourier transformation to spectrally analyze the differential fluorescence of each chromosomal paint (Schrock et al., 1996). Both the SKY and M-FISH methods require the use of human whole-chromosomal paints that are differentially labeled, so that each chromosome emits a unique combination of colors following hybridization, this color combination is used for identification purposes. Each method can be performed as a laboratory procedure and is capable of identifying the cytogenetic origins of all chromosomes in the complement in one image acquisition step (as in SKY) or through sequential imaging with specific filters (M-FISH).

The protocols in this unit employ the basic FISH techniques already discussed in *UNIT 22.4*. However, since multi-chromosome full-painting probe cocktails are different for specific assays, it is prudent to discuss the labeling strategy and denaturation, hybridization, and post-hybridization steps in detail. The probe labeling protocol is similar to those discussed in *UNIT 22.4*, where normally, genomic DNA from cloned sources serves as the template. However, the technique described for SKY and M-FISH requires multiple rounds of amplification. The in situ hybridization steps, whether for SKY or M-FISH, are very similar, differing only in the post-hybridization washes and specific reagents. The critical difference between the two assays is the method of image acquisition and analysis. All of the critical parameters of slide making, probe labeling, and washing stringency apply to SKY and M-FISH (see *UNIT 22.4*).

This unit includes protocols for labeling SKY and M-FISH probes (see Basic Protocol 1) and for multi-color FISH (see Basic Protocol 2 and Alternate Protocol 1), including

Contributed by Jane Bayani and Jeremy Squire
Current Protocols in Cell Biology (2004) 22.5.1-22.5.25

multi-color FISH of previously G-banded slides (see Alternate Protocol 2) and using locus-specific probes with previously SKY or M-FISH hybridized slides (see Alternate Protocol 3).

Multi-color FISH assays can be easily completed if the reagents are purchased commercially. If the investigator plans to produce the probes and antibody mixtures in-house, many more experiments and quality-control experiments will be required.

BASIC PROTOCOL 1

LABELING WHOLE-CHROMOSOME DNA PROBES FOR MULTI-COLOR FISH ASSAYS

This labeling protocol has been kindly provided by the laboratory of Dr. Thomas Ried; it outlines the general labeling strategy for in-house production of whole-chromosome paints to be used in SKY and M-FISH experiments. Some modifications may be required for optimization. The labeling protocol is divided into three parts: (1) primary amplification of flow-sorted chromosomes, (2) secondary amplification of the degenerate oligonucleotide primed (DOP) PCR-amplified primary product, and (3) labeling of the secondary amplification product with fluorochromes or haptens. Each labeling experiment involves the use of DOP primers as described by Telenius et al. (1992). The labeling protocol below includes tables of the human fluorochrome/hapten combinations currently used by Applied Spectral Imaging for use with the SKY system, the labeling combinations used by Vysis for use with the SpectraView System, and the combinations used by Applied Spectral Imaging for a Mouse SKYPaint (see Tables 22.5.3, 22.5.5, and 22.5.6). These labeling combinations can be used to custom produce probes for use with any standard FISH imaging system, provided that the proper filters are available on the microscope and there is a means of identifying and analyzing custom multi-color FISH experiments. Custom experiments may involve all the chromosomes, a subset of them, or specific bands along one chromosome (MBand). MBand, which differentially labels major chromosomal bands along the length of a chromosome is offered by Metasystems but is not covered in this unit.

Materials

Template DNA (flow-sorted chromosomes in a volume of 32.0 μl; Gray et al., 1986)
5× buffer D (accompanying 10 mM dNTP from Invitrogen)
10 mM dNTP nucleotide mix (see recipe)
5 μM 5′-CCGACTCGAGNNNNNNATGTGG-3′ primer
15 U/μl Super *Taq* polymerase (CPG)
5× loading buffer (see recipe)
2% (w/v) agarose gel with ethidium bromide (see recipe)
5 U/μl Ampli*Taq* polymerase (Perkin Elmer)
10× PCR buffer
25 mM $MgCl_2$
2 mM dNTP for secondary amplification (see recipe)
2 mM dNTP for labeling (see recipe)
100 μM Rhodamine 110 (Perkin Elmer)
1 mM FITC
1 mM Texas Red (see recipe for *x*-dUTPs)
50 mM biotin (Boehringer Mannheim)
50 mM digoxygenin (Boehringer Mannheim)
1 mM Spectrum green (see recipe for *x*-dUTPs)
1 mM Spectrum orange (see recipe for *x*-dUTPs)
1 mM Spectrum aqua (see recipe for *x*-dUTPs)
1 mM Spectrum gold (see recipe for *x*-dUTPs)

1 mM Spectrum far red (see recipe for *x*-dUTPs)
1 mg/ml human Cot-1 DNA (GIBCO)
10.0 mg/ml sonnicated salmon sperm DNA (Invitrogen)
3 M sodium acetate (*APPENDIX 2A*)
100% and 70% ethanol
Hybridization buffer (see recipe)

PCR tubes
PCR thermal cycler
Gel system and power source
0.5- and 2-ml microcentrifuge tubes
Refrigerated centrifuge

NOTE: Use aseptic technique throughout. Be sure to change pipet tips for each reagent and before addition to each tube.

DOP-PCR amplify flow-sorted chromosomes

1. Vortex flow-sorted chromosomes in PCR tubes well in order to detach chromosomes from the inner walls. Microcentrifuge for ≥3 min (~32.0 µl volume) at maximum speed, or 200 to 300 × *g*, room temperature.

   This will be the tube to which the PCR mix will be added. The total volume will be 50 µl.

2. Combine the following PCR mixture for each sample (make sure to change tips for each reagent):

   10.0 µl 5× buffer D
   4.0 µl 10 mM dNTP
   1.5 µl 5 µM 5′-CCGACTCGAGNNNNNNATGTGG-3′ primer
   2.0 µl ddH_2O
   0.25 µl 15 U/µl Super *Taq* polymerase.

3. Lightly vortex each tube and microcentrifuge 30 sec at maximum speed.

4. Immediately transfer tubes to PCR thermal cycler and run PCR with the following parameters:

1 cycle:	10 min	93°C
4 cycles:	1 min	94°C
	1.5 min	30°C
	3 min	30°–70°C
	3 min	72°C
34 cycles:	1 min	94°C
	1 min	62°C
	3 min, add 1 sec/cycle	72°C
1 cycle:	10 min	72°C (final extension)
		4°C (hold).

5. When the program is complete, mix a 2-µl aliquot with 1 µl 5× loading buffer from each reaction and run on a 2% agarose gel to determine the efficiency of the initial amplification reaction.

   The resulting smear migrates at ~500 bp. The efficiency of the amplification can be assessed by comparing the intensity of the resulting smear with concentration standards by gel electrophoresis or by standard spectrophotometry. The amplified product may be stored indefinitely at −20°C.

Table 22.5.1 Master Mix for Secondary Amplification

Reagent	Volume per reaction (μl)	Total for 13 reactions (μl)	Total for 12 reactions (μl)
Sterile ddH_2O	65	845	780
10× PCR buffer	10	130	120
25 mM $MgCl_2$	8	104	96
2 mM dNTP	10	130	120
5 μM primer (for 13 reactions)	4	52	48
Ampli*Taq* DNA polymerase	1	13	12

Perform secondary amplification of product

6. Label twenty-five 0.5-ml tubes with chromosome number and date (1 to Y and a control tube without DNA).

   Secondary amplification using DOP-PCR is used to amplify the primary product above. Future labeling experiments will start from this step.

7. Thaw template DNA at 37°C if previously frozen, vortex, and centrifuge briefly in a picofuge prior to dispensing the DNA into aliquots.

8. Pipet 2 μl of the amplified flow-sorted chromosomal DNA into each PCR tube and place closed PCR tubes temporarily at 4°C.

9. Since there are a total of 25 reactions (22 autosomes, 2 sex chromosomes, and a negative control), prepare the master mix in two 2-ml microcentrifuge tubes according to Table 22.5.1. Vortex the two tubes, centrifuge, and place on ice.

   One tube will contain enough master mix for 13 reactions and the other will contain enough for 12 reactions.

10. Take out the Ampli*Taq* polymerase, mix carefully (tap with finger), centrifuge, and then add an appropriate amount to each master mix. Vortex the master mix tubes, centrifuge, and put back on ice.

11. Place PCR tubes containing DNA in order and open each tube by handling only the outside of the tube. Pipet 96 μl of master mix into each tube (change tips with each tube) and place on ice.

12. Vortex each tube, centrifuge briefly, and place each into the thermal cycler. Amplify using the following amplification program:

29 cycles:	1 min	94°C
	1 min	56°C
	3 min, add 1 sec/cycle	72°C
1 cycle:	10 min	72°C (final extension)
		4°C (hold).

13. After completion, remove the tubes from the PCR machine, vortex, centrifuge, and place on ice.

14. Run a 2-μl aliquot with 1 μl 5× loading buffer from each reaction on a 2% agarose gel.

    The resulting smear migrates at ∼500 bp. The efficiency of the amplification can be assessed by comparing the intensity of the resulting smear with concentration standards by gel electrophoresis or by standard spectrophotometry. The amplified product may be stored indefinitely at −20°C.

Label amplified products

15. Label PCR tubes according to labeling scheme corresponding to the software/hardware system used (i.e., ASI or Vysis). Remember to include a zero control for each color (master mix with no DNA sample).

    Commercial probes for SKY and M-FISH are available from ASI and Vysis, however, some laboratories may prefer to produce their own probes to use with their system of choice. This labeling protocol can also be used to produce mutli-color custom probe cocktails, whether they be whole-chromosome paints, or band-, locus-, or centromere-specific probes, the investigator simply substitutes the template DNA as required.

    The critical difference between the two probe cocktails lies in the fact that SKY probes are both directly and indirectly labeled, thus requiring antibody detection, whereas the M-FISH probe cocktail is entirely directly labeled and does not require antibody detection.

    Contact ASI for any changes in probe cocktail combinations as this will affect the reference library, which has been integrated into the SKY software. Furthermore, should the investigator choose to alter the probe combination, the investigator should make sure that the reference library is concomitantly altered to reflect the changes made in the probe. Contact ASI or refer to the user manual for instructions on how to change the combinatorial table (referred to as a .ctb file).

16. Thaw template DNA at 37°C, vortex, and centrifuge briefly. Dispense a 4-μl aliquot of the appropriate DNA into each PCR tube, seal lid, and temporarily place at 4°C.

17a. *For SKY probes:* Label five 2-ml microcentrifuge tubes A through E and make the master mix for each fluorochrome according to Table 22.5.2 and in the following order: ddH_20, 10× PCR buffer, 25 mM $MgCl_2$, 2 mM dNTP, and 100 μM primer.

    Table 22.5.3 lists the fluorochrome combinations for specific chromosomes.

17b. *For M-FISH probes:* Label five 2-ml microcentrifuge tubes A through E and make the master mix for each fluorochrome according to Table 22.5.4 and in the following order: ddH_20, 10× PCR buffer, 25 mM $MgCl_2$, 2 mM dNTP, and 100 μM primer.

    Table 22.5.5 lists the fluorochrome combinations for specific chromosomes.

18. Dilute the Texas Red and spectrum-dUTPs 1:5 in sterile water immediately before use (1 mM final). Vortex the labeled fluor-dUTPs, centrifuge briefly, add each to the appropriate master mix, and place back on ice.

Table 22.5.2 SKY Master Mix

Reagent	Rhodamine (A)	Texas Red (B)	Biotin/Cy5 (C)	FITC (D)	Dig(Cy5.5) (E)
10× PCR buffer	120 μl	110 μl	130 μl	140 μl	120 μl
25 mM $MgCl_2$	96 μl	88 μl	104 μl	112 μl	96 μl
2 mM dNTPs	60 μl	55 μl	65 μl	70 μl	60 μl
Sterile ddH_2O	756 μl	693 μl	819 μl	872 μl	756 μl
100 mM primer	58 μl	44 μl	52 μl	56 μl	58 μl
Ampli*Taq* polymerase	12 μl	11 μl	13 μl	14 μl	12 μl
x-dUPT	60 μl	65 μl (of 1:5 dilution)	65 μl	80 μl (of 1:5 dilution)	60 μl
Total reactions	12	11	13	14	12

Table 22.5.3 Human SKY Labeling Scheme

Chromosome	Labeling scheme[a]
1	BCD
2	E
3	ACDE
4	CD
5	ABDE
6	BCDE
7	BC
8	D
9	ADE
10	CE
11	ACD
12	BE
13	AD
14	B
15	ABC
16	BD
17	C
18	ABD
19	AC
20	A
21	DE
22	ABCE
X	AE
Y	CDE

[a]A, rhodamine; B, Texas Red; C, biotin/Cy5; D, FITC; E, digoxigenin/Cy5.5.

Table 22.5.4 M-FISH Master Mix

Reagent	Spectrum FarRed (A)	Spectrum Aqua (B)	Spectrum Green (C)	Spectrum Gold (D)	Spectrum Red (E)
10× PCR buffer	110 µl	100 µl	110 µl	110 µl	120 µl
25 mM $MgCl_2$	88 µl	80 µl	88 µl	88 µl	96 µl
2 mM dNTPs	55 µl	50 µl	55 µl	55 µl	60 µl
Sterile ddH_2O	693 µl	630 µl	693 µl	693 µl	751 µl
100 µM primer	44 µl	40 µl	44 µl	44 µl	48 µl
Ampli*Taq* polymerase	11 µl	10 µl	11 µl	11 µl	12 µl
x-dUPT (1:5 dilution)	65 µl	60 µl	65 µl	65 µl	70 µl
Total reactions	11	10	11	11	12

Table 22.5.5 Human M-FISH Labeling Scheme[a]

Chromosome	Labeling scheme[b]
1	D
2	E
3	B
4	CE
5	AD
6	C
7	A
8	AE
9	DE
10	ABD
11	BE
12	CD
13	BC
14	CDE
15	BDE
16	AC
17	ACE
18	ADE
19	ACD
20	ABE
21	BCD
22	BCE
X	AB
Y	BD

[a]Like the SKY probes, contact Vysis for the latest probe combinations. Ensure that in-house probe matches combinatorial libraries established by the SpectraView software.
[b]A, Spectrum Far Red; B, Spectrum Aqua; C, Spectrum Green; D, Spectrum Gold; E, Spectrum Red.

19. Mix the Ampli*Taq* enzyme carefully (tap with finger), centrifuge briefly, then add appropriate amounts to each master mix. Vortex the master mix tubes, centrifuge, and put back on ice.

20. Put labeled PCR tubes with aliquoted DNA in fluorochrome order (all As in one row, all Bs in the next, and so on).

21. Carefully open each tube in row A by handling only the outside of the tube. Vortex the master mix for spectrum-dUTPs, pipet 96 µl into each tube (change tips with each tube) and put on ice. Repeat the same procedure for each remaining *x*-dUTP. Vortex each tube, centrifuge briefly, and put on ice again.

Table 22.5.6 Mouse SKY Labeling Scheme

Chromosome	Labeling scheme[a]
1	ACD
2	CE
3	AE
4	ABD
5	AC
6	BE
7	BD
8	AB
9	E
10	C
11	BC
12	CDE
13	D
14	ACE
15	AD
16	A
17	BCD
18	B
19	DE
X	ABC
Y	ADE

[a]A, rhodamine; B, Texas Red; C, biotin/Cy5 antibodies (see recipe); D, FITC; E, digoxigenin/Cy5.5.

22. Start the thermal cycler, arrange the tubes in the PCR machine, close lid, and start the program using the following parameters:

29 cycles:	1 min	94°C
	1 min	56°C
	3 min, add 1 sec/cycle	72°C
1 cycle:	10 min	72°C (final extension)
	hold	4°C.

Analyze labeling results

23. After completion, remove the tubes from the thermal cycler, vortex, centrifuge, and place on ice.

24. Analyze a 2-µl aliquot from each reaction on a 2% agarose gel.

The resulting smear migrates at ~500 bp.

At this point, the custom probe cocktail is generated according to the labeling scheme. The chromosome paint for each chromosome will be added together for each of the 22 autosomes and 2 sex chromosomes according to Table 22.5.3 or 22.5.5, or according to Table 22.5.6 for mouse chromosome probes.

The total number of differently labeled DNAs for the ASI labeling scheme is 57, while the total number of labeled DNAs for the Vysis labeling scheme is 52.

Purify probe

25. To produce the SKY cocktail, add 3 μl of labeled, amplified product according to the labeling scheme into a 1.5-ml microcentrifuge tube. Add 20 μl of 1 mg/ml Cot-1 DNA and 1 μl of 10.0 mg/ml salmon sperm DNA.

    This precipitation protocol outlines the amount of probe required for one slide to be hybridized. It is suggested that sufficient probe for five or ten assays should be precipitated together and stored as aliquots.

26. Add 1/10 vol of 3 M sodium acetate, 2.5 vol of 100% ethanol, mix well, and store overnight at −20°C or 1 hr at −80°C.

27. Centrifuge 20 min at 200–300 × *g* (13,000 to 14,000 rpm), 4°C. Wash the pellet with cold 70% ethanol and repeat centrifugation. Dry the pellet either by air drying or vacuum.

28. Dissolve pellet in 15 to 20 μl hybridization buffer at room temperature.

    If the probe does not readily dissolve back into solution, add more hybridization buffer.

29. Store the probe in aliquots sufficient for five to ten slide hybridizations at −20°C for long-term storage (>2 months), or at 4°C for short-term use (1 to 2 months).

IN SITU HYBRIDIZATION FOR SPECTRAL KARYOTYPING (SKY)

BASIC PROTOCOL 2

The following outlines the SKY protocol, which is simply FISH (*UNIT 22.4*) with different multi-color probes. The metaphase preparation is pre-treated with pepsin, formalin-fixed, and denatured. The prepared probe is denatured, allowed to pre-anneal and added to the denatured slide. After 48 hr, the slides are washed, detected with primary and secondary antibodies, counterstained, and visualized.

Materials

Metaphase slides (*UNIT 22.2*)
0.01 M HCl (see recipe), prewarmed to 37°C
10% (w/v) pepsin (see recipe)
1× PBS
1% formaldehyde/1× PBS/50 mM $MgCl_2$ (see recipe)
70% formamide/2× SSC (see recipe), prewarmed to 72°C
70%, 80%, and 100% ethanol
SKY kit (Applied Spectral Imaging) containing probe cocktail, block reagents, detection reagents, and counterstain *or* in-house custom-made probes in hybridization solution (see Basic Protocol 1)
Rubber cement
50% formamide/2× SSC, 45°C (3 Coplin jars)
1× SSC (see recipe), 45°C (3 Coplin jars)
0.1%Tween-20/4× SSC (see recipe), 45°C (7 Coplin jars)
Blocking solution (see recipe)
Avidin-Cy5 antibodies (see recipe for antibodies)
Mouse anti-digoxigenin (see recipe for antibodies)
Cy5.5 anti-mouse antibodies (see recipe for antibodies)
DAPI/antifade counterstain (see recipe)
Clear nail polish (do not use if planning to re-probe slide)

Phase-contrast microscope
37°C oven
Fume hood
22 × 22–mm coverslips

Hybridization container
Fluorescent microscope and associated Applied Spectral Imaging Acquisition hardware and software (Applied Spectral Imaging)

Prepare slide

1. Using a phase-contrast microscope, determine the extent of cytoplasmic residue on the metaphase slide preparation (*UNIT 22.2*).
2. To 50 ml of prewarmed 0.01 M HCl, add 10 to 15 μl of 10% pepsin. Allow the slide to incubate for 5 to 10 min at 37°C.
3. Wash the slide in 1× PBS for 5 min at room temperature. Then, wash in 1× PBS/50 mM $MgCl_2$ for 5 min at room temperature.
4. Incubate the slide in 1% formaldehyde/1× PBS/50 mM $MgCl_2$ for 10 min at room temperature in a well-ventilated area or fume hood.

Prepare the probe

5a. *For commercial probes:* From the commercially available SKY kit, pipet 10 μl of probe cocktail (vial 1) for each slide to be hybridized. Heat denature the probe for 10 min at 72°C, then place 1 hr at 37°C.

This is sufficient probe to cover a 22 × 22–mm area.

5b. *For in-house probes:* Heat denature prepared probe (labeled probe in hybridization buffer, see Basic Protocol 1) for 10 min at 72°C, then place 1 hr at 37°C.

6. Wash the slide in 1× PBS for 5 min at room temperature.
7. Pass slide through an ethanol dehydration series: 70%, 80%, and 100% ethanol for 5 min each. Allow slide to air dry after the final ethanol wash.
8. Denature the slide in 70% formamide/2× SSC for 1.5 to 2 min at 72°C.

The time required for denaturing will vary according to the age and quality of slide.

9. Promptly place the slide into 70% ethanol following denaturation and proceed through the dehydration series as in step 7.

Hybridize and wash slides

10. Add 10 μl of the preannealed probe to the denatured slide. Apply a 22 × 22–mm coverslip and seal with rubber cement.
11. Transfer the slides to a hybridization container that has been slightly dampened with a wet paper towel or gauze and place at 37°C.
12. After 48 hr, carefully peel rubber cement from slide and immerse in the first Coplin jar of 50% formamide/2× SSC at 45°C. Allow the coverslip to fall off and let stand for 5 min.
13. Remove slide and transfer to second Coplin jar of 50% formamide/2× SSC for 5 min. Repeat with third Coplin jar of 50% formamide/2× SSC.
14. Wash the slide three times in three separate Coplin jars of 1× SSC, 5 min each wash, at 45°C.
15. Briefly wash the slide in 0.1%Tween-20/4× SSC at 45°C.

Block and detect slides

16a. *For commercial probes:* Drain excess solution, but do not allow slide to dry. Add 80 μl of blocking reagent (vial 2 from the SKY kit) to the slide. Apply coverslip and place back in hybridization container for 40 min at 37°C.

17a. Remove coverslip and add 80 µl of detection reagent (vial 3 from SKY kit) to the slide. Apply coverslip and place back in hybridization container for 40 min at 37°C.

16b. *For in-house probes:* Drain excess solution, but do not allow slide to dry. Add 80 µl of blocking solution, apply coverslip, and place back in hybridization container for 40 min at 37°C.

17b. Add 40 µl each of Avidin-Cy5 antibodies and mouse anti-digoxigenin antibodies. Apply coverslip and place back in hybridization container for 40 min at 37°C.

18. Remove coverslip and wash slide in three times in three separate Coplin jars of 0.1% Tween-20/4× SSC, 5 min each wash, at 45°C with gentle agitation.

19a. *For commercial probes:* Drain excess solution, but do not allow slide to dry. Add 80 µl detection reagent (vial 4 from the SKY kit) to the slides. Apply coverslip and place back in hybridization container for 40 min at 37°C.

19b. *For in-house probes:* Drain excess solution, but do not allow slide to dry. Add 80 µl of diluted Cy5.5 anti-mouse antibodies. Apply coverslip and place back in hybridization container for 40 min at 37°C.

20. Remove coverslip and wash slide three times in three separate Coplin jars of 0.1% Tween-20/4× SSC, 5 min each wash, at 45°C with gentle agitation.

Counterstain and visualize

21. Drain excess solution, but do not allow slide to dry. Add 40 µl of DAPI/antifade counterstain (vial 5 from SKY kit or from another source). Apply coverslip and seal with clear nail polish. However, do not seal with nail polish if there are plans to re-probe the slide (see Alternate Protocols 1, 2, and 3).

    Slides may be stored at −20°C and visualized for up to 6 months with sustained signal strength.

22. Acquire and analyze metaphase preparation using SKY labeling scheme with the hardware and software provided by Applied Spectral Imaging (*http://www.spectral-imaging.com/*).

    See Figure 22.5.1 for a diagram of signal acquisition and Figure 22.5.2 for typical results.

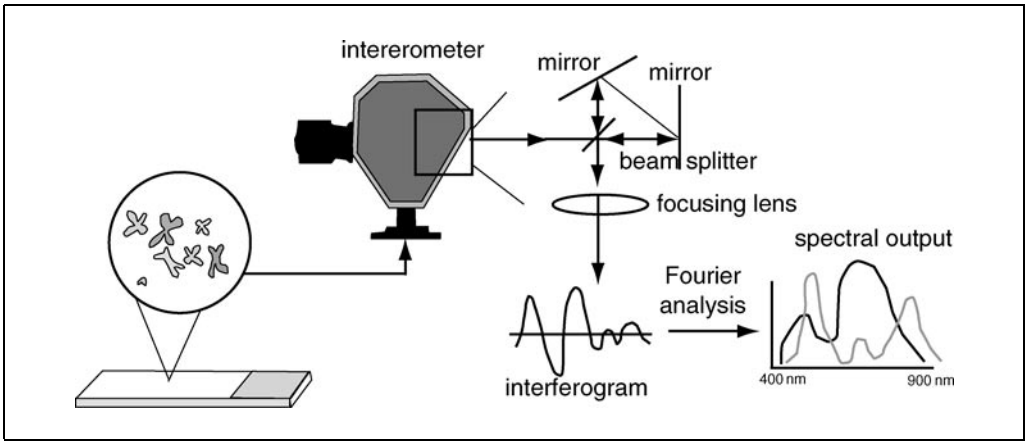


Figure 22.5.1 Schematic representation of spectral karyotyping image acquisition and image analysis. The metaphase, following hybridization with the SKYPaints is visualized by fluorescence microscopy. The light passes into the optical head containing the interferometer where 100 different images are taken. This information is Fourier transformed and processed using the SKYView software for analysis. (Adapted from the ASI manual.)

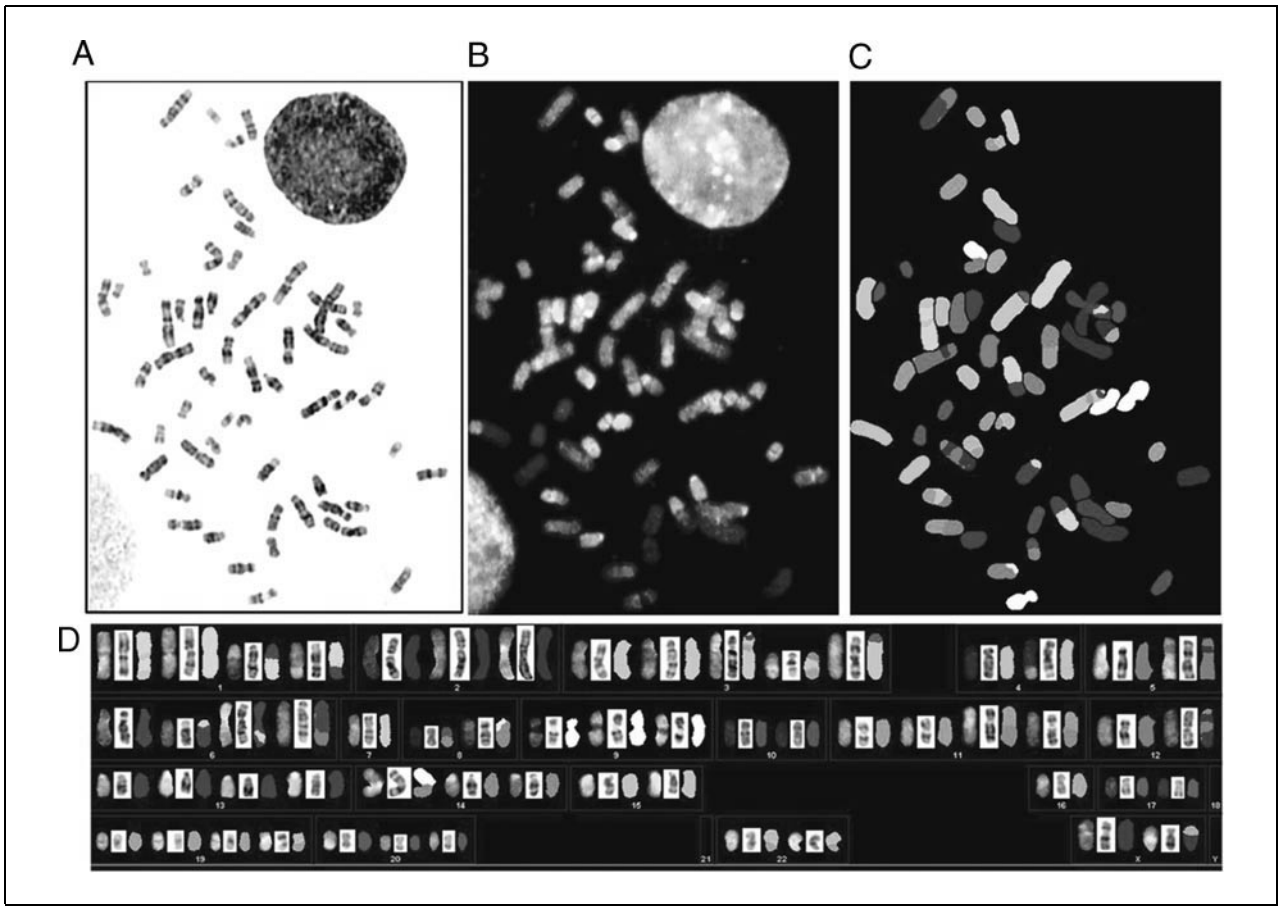


Figure 22.5.2 SKY analysis of a primary ovarian carcinoma. (**A**) Typical representations of SKY analysis. An inverted DAPI image reveals banding patterns similar to G-banding. (**B**) Red-Green-Blue (RGB) image of the hybridized spread as it would appear with the probe colors. (**C**) Classified or pseudo-colored image to help identify chromosomal aberrations not readily evident in the RGB image. Each chromosome is identified by a specific pseudo-color. A change in color along the length of a chromosome indicates a different chromosome. (**D**) SKY karyotype showing each representation of the chromosomes: RGB, inverted DAPI, and classified images. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

ALTERNATE PROTOCOL 1

IN SITU HYBRIDIZATION FOR M-FISH KARYOTYPING

The M-FISH protocol is very similar to the SKY protocol with one critical difference. Like SKY, the metaphase preparation is pretreated with pepsin, formalin-fixed, and denatured. The prepared probe is denatured, allowed to pre-anneal, and added to the denatured slide. After 48 hr, the slides are washed, counterstained, and ready for visualization. Because M-FISH probes are labeled using directly labeled dUTPs, there is no need for antibody detection.

Materials

Chromosome spreads (*UNIT 22.2*)
70%, 80%, and 100% ethanol
RNase A working solution (see recipe)
2× SSC (see recipe)
10% pepsin stock (see recipe)
0.01 M HCl (see recipe), 37°C
1× PBS (2 coplin jars)
1% formaldehyde/1× PBS/50 mM $MgCl_2$ (see recipe)
70% formamide/2× SSC (see recipe), 72°C
Commercially available M-FISH probes (Vysis) *or* in-house custom probes

0.4× SSC/0.3% NP-40 (see recipe), 73°C
2× SSC/0.1% NP-40 (see recipe)
DAPI/antifade counterstain (DAPI III, Vysis or see recipe)
50% formamide/2× SSC (see recipe), 45°C
1× SSC (see recipe), 45°C
0.1% Tween-20/4× SSC (see recipe), 45°C
Clear nail polish (do not use if planning to re-probe slide)

Phase-contrast microscope
Glass coverslips
37°C dry oven or incubator
Fume hood

Prepare slide

1. Prepare chromosome spreads as outlined in *UNIT 22.2*.
2. Under phase-contrast microscopy, determine the extent of cytoplasmic residue on the slide preparation.
3. Dehydrate the slide in a 70%, 80%, 100% ethanol series, 5 min each, and allow to air dry.
4. Apply 20 µl of the RNase A working solution to slide and coverslip. Incubate 30 min at 37°C.
5. Remove the coverslip and wash slide in 2× SSC for 5 min.

   This and the following incubations should use solutions in Coplin jars.

6. Add 10 to 15 µl of 10% pepsin to 50 ml prewarmed 0.01 M HCl and treat slide for 5 min. Then wash the slide in 1× PBS for 5 min.
7. Incubate the slide in 1% formaldehyde/1× PBS/50 mM $MgCl_2$ for 10 min at room temperature in a well-ventilated area or fume hood.
8. Wash the slide in 1× PBS for 5 min at room temperature.
9. Pass the slide through a 70%, 80%, and 100% ethanol series, 5 min each, and allow the slide to air dry.

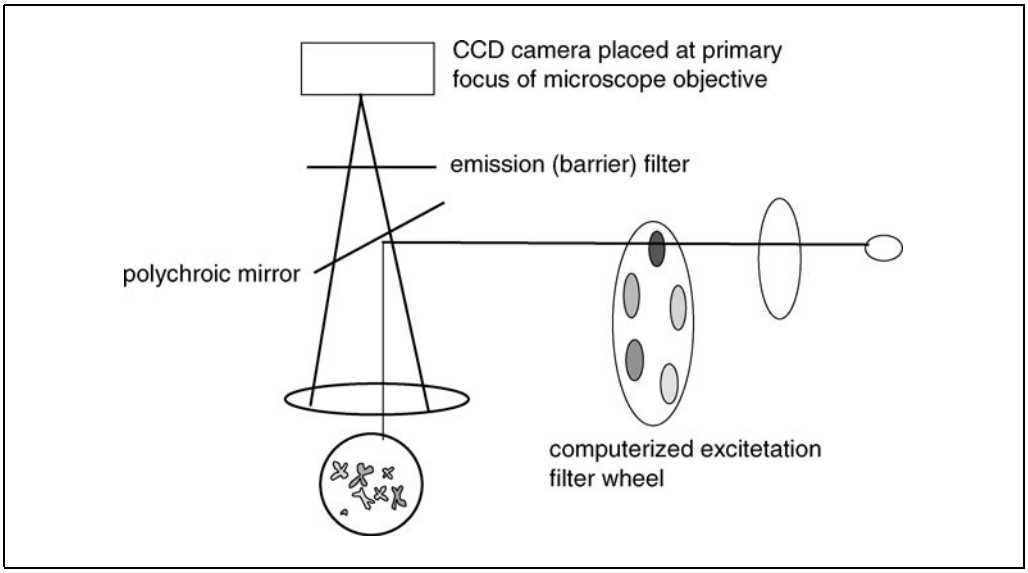


Figure 22.5.3 Schematic representation of M-FISH image acquisition and image analysis. M-FISH experiments involve the imaging of each dye or fluorochrome using a specific filter. The images are then stacked to produce the final merged image. (Adapted from the Vysis manual.)

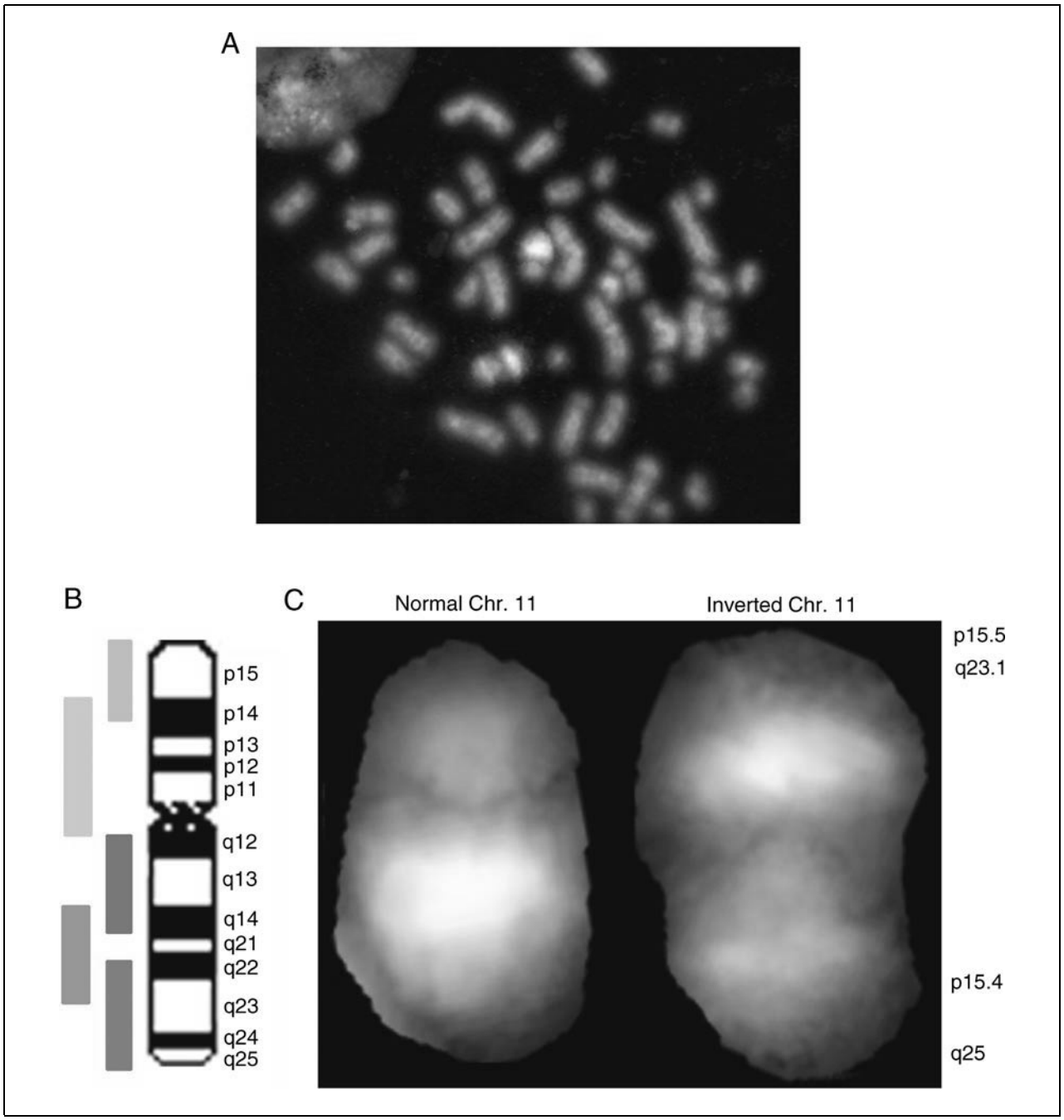


Figure 22.5.4 MBand using the 11Cyte probe and imaging system from Metasystems. (**A**) Metaphase spread from a patient possessing an inversion on chromosome 11, hybridized with the 11Cyte chromosome paint. Five different dyes are used to create the painting probe along the length of the chromosome in a specific order. (**B**) Ideogram of chromosome 11 showing the color order of the dyes along the length of the chromosome and the band regions they span. (**C**) The normal and inverted chromosome 11. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

10. Denature the slide for 1.5 to 2 min in 70% formamide/2× SSC at 72°C.
11. Promptly place the slide into 70% ethanol following denaturation and proceed through the dehydration series as in step 9.
12. Remove the slide from the ethanol, add the recommended amount of denatured and pre-annealed commercial probe or 15 to 20 µl of in-house probe and hybridize for 48 hr at 37°C.

Vysis rapid wash

13a. Carefully remove the coverslip from the slide and immerse in 0.4× SSC/0.3% NP-40 for 2 min at 73°C.

14a. Wash the slide in 2× SSC/0.1% NP-40 for 1 min at room temperature.

15a. Wash the slide in 1× PBS for 2 min at room temperature.

16a. Air dry the slide and stain with DAPI/antifade counterstain. Store slides at −20°C.

Slides can be stored for several months at −20°C.

17a. Visualize slide using a fluorescent microscope and the associated hardware and software provided by Vysis.

Traditional wash protocol (for in-house probes)

13b. After 48 hr, carefully peel off rubber cement from slide and immerse in the first Coplin jar of 50% formamide/2× SSC at 45°C. Allow the coverslip to fall off and let stand for 5 min. Remove slide and transfer to second Coplin jar for 5 min. Repeat with third solution.

14b. Wash the slide three times in three separate Coplin jars of 1× SSC, 5 min each wash, at 45°C.

15b. Remove coverslip and wash slide in three washes of 0.1% Tween-20/4× SSC, 5 min each wash, at 45°C with gentle agitation.

16b. Drain excess solution, but do not allow slide to dry and add 40 µl of DAPI antifade counterstain. Apply coverslip and seal with clear nail polish. However, do not seal with nail polish if there are plans to re-probe the slide (see Alternate Protocols 2, and 3).

The slides are now ready for visualization. When not in use, store slides at −20°C.

17b. Analyze the slide using fluorescence microscopy with the appropriate filters and imaging system capable of integrating multiple fluorochromes.

See Figure 22.5.3 for a diagram of signal acquisition and Figure 22.5.4 for typical results.

PRETREATMENT OF PREVIOUSLY G-BANDED SLIDES FOR SKY OR M-FISH

ALTERNATE PROTOCOL 2

This procedure can be used when a previously G-banded slide is needed for subsequent SKY or M-FISH analysis. The G-banded slide must be cleaned of residual oils using xylene. It is then destained using methanol. See Table 22.5.7 for guidelines for handling G-banded slides.

Materials

G-banded metaphase slides (*UNIT 22.3*)
Xylene
100% methanol
70%, 80%, and 100% ethanol
1× PBS
1× PBS/50 mM $MgCl_2$
1%formaldehyde/1× PBS/50 mM $MgCl_2$ (see recipe)
70% formamide/2× SSC (see recipe), 72°C
Fume hood

Table 22.5.7 General Guide for Processing Cytogenetic Preparations for G-Banding Analysis Followed by SKY or M-FISH

Banding steps	Slide 3 days to 1 month	Slide >1 month	In situ slide preparation 3 days to 1 month	In situ slide preparation >1 month
Trypsin (working solution)	10–20 sec with agitation	20–30 sec with agitation	40–60 sec with agitation	60–90 sec with agitation
Stain	50 sec	1+ min	50 sec	1+ min
Water	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Water	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Denaturing time	20–30 sec	45 sec–1 min	30–45 sec	1 min-?

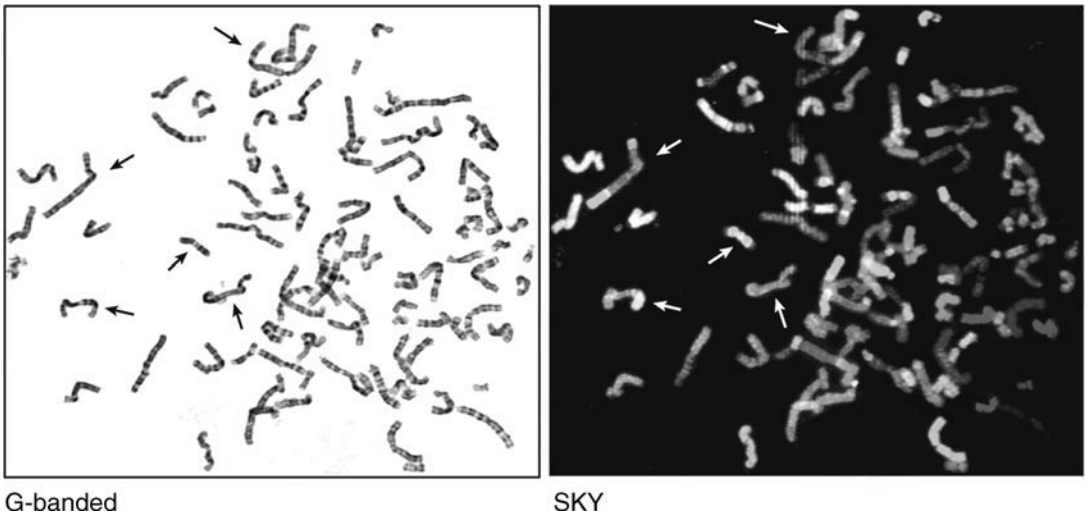


Figure 22.5.5 The results of sequential G-banding (**A**) and SKY (**B**) analysis of a rhabdomyoscaroma cell line. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

Destain slide

1. For previously G-banded slide, wash in xylene for 5 min.
2. Destain with 100% methanol for 10 min.
3. Pass the slide through a 70%, 80%, and 100% ethanol series, 5 min each wash, and allow the slide to air dry after the final ethanol wash.
4. Rehydrate in 1× PBS for 5 min.

Denature slide

5. Incubate the slide in 1% formaldehyde/1× PBS/50 mM $MgCl_2$ for 10 min at room temperature in a well-ventilated area or fume hood.
6. Wash the slide in 1× PBS for 5 min at room temperature.
7. Pass the slide through a 70%, 80%, 100% ethanol dehydration series, 5 min each wash, and allow the slide to air dry after the final ethanol wash.

8. Denature the slide in 70% formamide/2× SSC for 20 to 30 sec at 72°C.
9. Promptly place the slide into 70% ethanol following denaturation and proceed through the dehydration series as in step 6.

Hybridize slide

10. Proceed with probe denaturation and pre-annealing as outlined in the SKY (see Basic Protocol 2, step 6) and hybridization to slide (see Basic Protocol 2, step 11) or M-FISH protocols described above (see Alternate Protocol 1, step 12).
11. Proceed with the respective protocol (SKY or M-FISH) following the appropriate post-hybridization detection and washing procedures (see Basic Protocol 2 or Alternate Protocol 1).

See Figure 22.5.5 for an example of SKY hybridization of previously G-banded chromosomes.

FISH ANALYSIS USING LOCUS-SPECIFIC OR CHROMOSOME PAINTING PROBES FOLLOWING SKY/M-FISH HYBRIDIZATION

ALTERNATE PROTOCOL 3

This protocol can be used in conjunction with previously G-banded slides as well as SKY/M-FISH-hybridized slides. In some cases, investigators may wish to know the status of a specific DNA probe within the context of the SKY or M-FISH analysis that has been ascertained, thus it is particularly useful to be able to sequentially use locus-specific or chromosome paint probes on the same metaphase spread that was analyzed using SKY or M-FISH. The hybridized slides are washed free of mounting medium and dehydrated. The slides are redenatured and hybridized with the locus-specific or chromosome paint probe of interest. After hybridization, the slides are washed, the probe is detected with antibody (if needed), counterstained, and visualized.

Materials

Hybridized SKY or M-FISH slides (see Basic Protocol 2 or Alternate Protocol 1, respectively)
2× SSC (see recipe)
0.1% Tween-20/4× SSC (see recipe)
70%, 90%, and 100% ethanol
70% formamide/2× SSC (see recipe), 75°C
Labeled DNA probe (as prepared in *UNIT 22.4* or from a commercial supplier)
Rubber cement

Coverslips
Hybridization box
37°C dry oven or incubator

1. For SKY or M-FISH hybridized slide, record the microscope coordinates of the metaphase spreads of interest.
2. Carefully remove the coverslip and wash slide in 2× SSC for 5 to 10 min at room temperature. Gently agitate.
3. Transfer slide to 0.1% Tween-20/4× SSC and wash for 5 min at room temperature with gentle agitation.
4. Dehydrate the slide in a 70%, 90%, and 100% ethanol dehydration series, 5 min each wash. After the final ethanol (100%) wash, allow the slide to air dry.
5. Denature the probe at 75°C and pre-anneal as needed or follow the manufacturer's instructions.

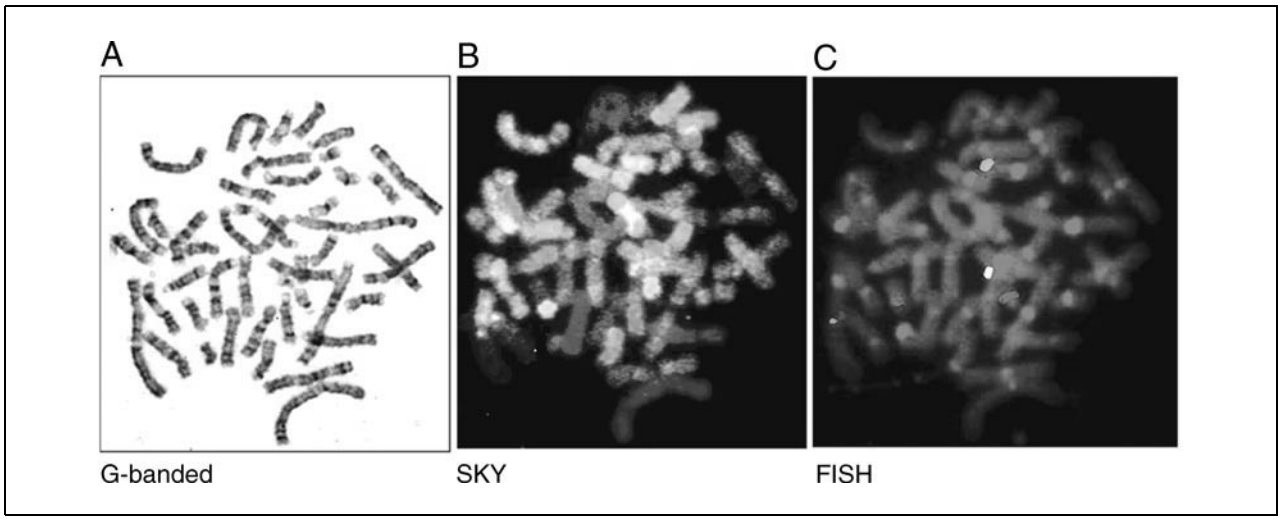


Figure 22.5.6 The results of sequential G-banding (**A**), SKY (**B**), and locus-specific FISH (**C**) for the BCR-ABL translocation in a CML sample. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/ colorfigures.*

6. Denature the slide in 70% formamide/2× SSC for 2 min at 75°C (for previously G-banded/SKY or M-FISH slides, denature for 1 min).
7. Immediately place the slide in 70% ethanol and continue with the dehydration series as in step 4.
8. Add the appropriate amount of the denatured probe to the denatured slide. Apply coverslip and ring with rubber cement. Hybridize at 37°C overnight.
9. Follow the necessary post-hybridization washes and detections as required (see *UNIT 22.4*)

See Figure 22.5.6 for typical results.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protcol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose gel, 2% (w/v)

Dissolve 2 g of agarose into 100 ml of 1× TBE buffer (*APPENDIX 2A*) by warming the solution, e.g., in a microwave oven. After the agarose has been dissolved, add 5 ml of ethidium bromide. Pour into electrophoresis tray with combs and allow to solidify at room temperature.

Antibodies

Avidin-Cy5 antibodies (Amersham Bioscience): Reconstitute antibodies using water according to the manufacturer's instructions. Dilute 1:200 in blocking solution (see recipe).

Mouse anti-digoxigenin (Sigma): Reconstitute antibodies using water according to the manufacturer's instructions. Dilute 1:500 in blocking solution (see recipe).

Cy5.5 anti-mouse antibodies (Amersham Bioscience): Reconstitute antibodies using water according to the manufacturer's instructions. Dilute 1:200 in blocking solution (see recipe).

Store antibodies in 100-μl aliquots up to 1 year at −20°C. Aliquots of working solutions may be stored for 2 to 3 months at 4°C protected from the light.

Blocking solution

1.0 g BSA (Sigma; final 1% w/v)
100.0 µl Tween-20 (Sigma; final 0.1% v/v)
20.0 µl 20× SSC (final 4×)
80.0 µl H_2O
Store indefinitely at −20°C

DAPI/antifade medium

Dissolve the entire powder of DAPI (4′,6-diamidino-2-phenylindole; Sigma) in water to a 100 µg/ml stock solution. Store in 500-µl aliquots up to 1 year at −20°C.

To combine DAPI in antifade medium, combine the following in order:
5.0 ml PBS
500.0 µl 100 µg/ml DAPI stock (final 1 µg/ml)
0.5 g *p*-phenylenediamine (Sigma; final 10 mg/ml)
Dissolve well then add
45.0 ml glycerol (final 90% v/v)

The resulting medium is very viscous. Transfer to a 50-ml Falcon tube and place on a rotator to ensure proper mixing (30 min). This product is light sensitive, so wrap the tube with foil. Store in 1-ml aliquots 1 year at −20°C. New medium must be made when an increasing amber tint appears.

This is also commercially available from Vectashield (Vector Laboratories).

CAUTION: *DAPI is a potential carcinogen and should be handled with caution.*

dNTP (dATP, dTTP, dGTP, dCTP) for secondary amplification, 2 mM

10.0 µl 100 mM dATP (final 2 mM)
10.0 µl 100 mM dCTP (final 2 mM)
10.0 µl 100 mM dTTP (final 2 mM)
10.0 µl 100 mM dGTP (final 2 mM)
460.0 µl sterile H_2O (final 2 mM)
Store up to 1 year at −20°C.

dNTP (dATP, dGTP, dCTP, 1.5 mM dTTP) for labeling, 2 mM

10.0 µl 100 mM dATP (final 2 mM)
10.0 µl 100 mM dCTP (final 2 mM)
10.0 µl 100 mM dGTP (final 2 mM)
7.5 µl 100 mM dTTP (final 1.5 mM)
460.0 µl sterile H_2O (final 2 mM)
Store up to 1 year at −20°C.

dNTP (dATP, dTTP, dGTP, dCTP), 10 mM

10.0 µl 100 mM dATP (final 10 mM)
10.0 µl 100 mM dCTP (final 10 mM)
10.0 µl 100 mM dTTP (final 10 mM)
10.0 µl 100 mM dGTP (final 10 mM)
60.0 µl sterile H_2O (final 10 mM)
Store up to 1 year at −20°C.

x-dUTPs

Dilute 1 mM Texas Red 1:5 in sterile H_2O (13 ml Texas Red + 52 ml H_2O)
Dilute 1 mM spectrum red 1:5 in sterile H_2O (14 ml spectrum red + 56 ml H_2O)
Dilute 1 mM spectrum far red 1:5 in sterile H_2O (14 ml spectrum far red + 56 ml H_2O)
Dilute 1 mM spectrum aqua 1:5 in sterile H_2O (14 ml spectrum aqua + 56 ml H_2O)
Dilute 1 mM spectrum green 1:5 in sterile H_2O (14 ml spectrum green + 56 ml H_2O)
Dilute 1 mM spectrum gold 1:5 in sterile H_2O (14 ml spectrum green + 56 ml H_2O)
Store up to 1 year at −20°C

1% formalin/PBS/$MgCl_2$

2.7 ml 37% formaldehyde (Sigma; final 1% v/v)
100.0 ml 1× PBS/$MgCl_2$ (see recipe)
Make fresh

Discard in accordance with the regulations of the institution.

70% formamide/2× SSC

35.0 ml formamide (Invitrogen; final 70% v/v)
5.0 ml 20× SSC (final 2×)
10.0 ml H_2O
Make fresh

CAUTION: *Formamide is a carcinogen and should be handled with caution. It should be discarded according to biohazard rules of the institution.*

HCl, 0.01 M

0.5 ml 1 M HCl (final 0.01 M)
49.5 ml H_2O
Store at room temperature until ready for use

Hybridization buffer

500.0 µl high-grade formamide (final 50% v/v)
100.0 µl 20× SSC (final 2×)
100.0 µl dextran sulfate (final 10% v/v)
300.0 µl H_2O

Alternatively, this solution may be purchased from DAKO.

Loading dye, 5×

0.125 g bromophenol blue (final 0.25% w/v)
15.0 ml glycerol (final 30% v/v)
35.0 ml H_2O
Store for several months to 1 year at room temperature or 4°C

1× PBS/$MgCl_2$

950.0 ml 1× PBS (*APPENDIX 2A*)
50.0 ml 1M $MgCl_2$ (final 50 mM)
Store at room temperature until ready for use

Pepsin stock, 10%

100 mg pepsin powder (Sigma; final 10% w/v)
1.0 ml H_2O
Store in 20-µl aliquots up to 1 year at −20°C

RNase A stock solution

10.0 mg RNase A (DNase-free; final 10 mg/ml)
1.0 ml 2× SSC
Dispense into 10-µl aliquots and store at −20°C
To make an RNase A working solution:
10.0 µl 10 mg/ml RNase A stock solution
990.0 µl 2× SSC
Vortex to mix. Do not freeze-thaw. Discard after use.

20× SSC

175.3 g NaCl
88.2 g sodium citrate
Adjust pH with 10 M NaOH to pH 7
Adjust volume to 1 liter with H_2O
Store for several months at room temperature

0.4× SSC/0.3% NP-40 solution

20.0 ml 20× SSC (final 0.4×)
950.0 ml H_2O
3.0 ml NP-40 (final 0.3% v/v)
Add up to 1 liter with H_2O
Store up to 6 months at room temperature

Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

2× SSC/0.1% NP-40 solution

100.0 ml 20× SSC (final 2×)
850.0 ml H_2O
1.0 ml NP-40 (final 0.1% v/v)
Add up to 1 liter with H_2O
Store up to 6 months at room temperature

Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

0.1%Tween-20/4× SSC

1.0 ml Tween-20 (Sigma; final 0.1% v/v)
200.0 ml 20× SSC (final 4×)
799.0 ml H_2O
Store at room temperature until ready for use

COMMENTARY

Background Information

Probe labeling

See Commentary on probe labeling in *UNIT 22.4*.

SKY

SKY is a combination of optical microscopy, high-resolution imaging, and the measurement of spectral emissions by Fourier spectroscopy (Malik et al., 1996; Fig. 22.5.1). It is an interferometer-based method of image acquisition and analysis and has specific requirements for its correct operation. The ability for SKY to properly identify chromosomal segments relies on the proper calibration of the spectral reference library. Upon installation, the optical head is carefully aligned with the digital camera. Slides hybridized with each pure dye are then imaged to create the spectral reference library for that particular system

and microscope. Since SKY analysis is based on the recognition of distinct spectral signatures, a less than optimal signal intensity can still generate a coherent chromosome classification, provided that there are no shifts in the spectra. Furthermore, should shifts in the spectra occur, it is possible to create a new spectral library based on the spectral signatures of hybridized slides. The advantage of SKY acquisition is the use of only the SKYCube to detect all the dyes and a short exposure using the DAPI filter for inverted banding images, rather than the lengthier exposure of the slide to five different filters as with M-FISH. The SKY software provides the capability to manually identify the spectral properties of each pixel along the length of a chromosomal region and compare this to the reference library. Such analyses can be very helpful in determining the identity of chromosomes where identity is inconclusive.

The commercial SKY probes are derived from flow-sorted chromosomes that are amplified and labeled using DOP-PCR (Telenus et al., 1992). A 24-chromosome probe cocktail is generated by the combinations of five pure dyes—rhodamine, FITC, Cy5, Cy5.5, and Texas Red. This allows 2^{n-1} or 31 combinations. Thus, each chromosome has a unique spectral signature generated by the specific combination of the five pure dyes. The generation of a spectral image is achieved by acquiring ~100 frames of the same image that differ from each other only by their optical path differences. Once a spectral image is acquired, the SKYVIEW software compares the acquired spectral image against the combinatorial library containing the fluorochrome combinations for each chromosome to generate a classified image. The classified image pseudocolors the chromosomes to aid in the delineation of specific structural aberrations where the RGB (red-green-blue) display images (Figure 22.5.2), which displays the fluorescent colors of the chromosomes, may appear quite similar. For every chromosomal region, identity is determined by measuring the spectral emission at that point. Regions where sites for rearrangement or translocation between different chromosomes occur, are visualized by a change in the display color at the point of transition.

M-FISH

All other M-FISH methods rely on the use of fluorochrome-specific filters moved sequentially during the image capturing process (Speicher et al., 1996; Fig. 22.5.3). The identity of each chromosome or chromosome region is based on which combination of the five fluorochromes hybridizes together in one location. As with SKY, the unique identification dye combinations are stored in a reference library within the software to which the test specimen is compared and aberrations are delineated in the same manner as described for SKY. Filter-based systems are more sensitive to the influences of high background and weak signal strength. Weak signal intensity or high background can misclassify a chromosome identity since the system may not consider the weak signal as true, thus choosing the next best chromosome filter pattern for identification. SKY, on the other hand, overcomes this obstacle since the fluorochrome spectral signature is the method of determining chromosome identity. Although a signal may be weak, its spectral output will remain the same. Nevertheless, both systems are widely used. Research laboratories are inclined to use SKY analysis, while clinical cytogenetic laboratories rely on M-FISH, since they already tend to have the filters and cytogenetic/FISH systems.

M-FISH probes are generated in the same manner as the SKY probes, differing only by their dye combinations. Commercial M-FISH probes can be purchased from Vysis as SpectraVision probes. The fluorochromes used for this probe set includes Spectrum Far Red, Spectrum Red, Spectrum Gold, Spectrum Green, and Spectrum Aqua used in a combinatorial fashion. Metasystems also provides M-FISH probes in their 24Xcyte kit. Metasystems has generated band-specific probes (M BAND) along the length of a given chromosome. Since each chromosome has a specific five-color FISH assay, each chromosome analysis must be performed individually (Fig. 22.5.4). Therefore, this assay is particularly useful when a specific rearrangement has been identified by banding, SKY, or MFISH, and the investigator wishes to further examine the rearranged region in detail to delineate the breakpoint or determine whether a more complex inversion or deletion is also present.

Locus- or band-specific M-FISH

Clearly the use of M-FISH-based techniques has increased the amount of cytogenetic data that can be derived from a given specimen. The integration of locus-specific or band-specific FISH permits further analysis regarding specific breakpoint locations and the status of specific genes or chromosomal loci. The ability to sequentially carry out traditional

banding analysis, SKY, or M-FISH, and locus-specific FISH on the same metaphase spread eliminates the question of clonality by identifying aberrations with increasing specificity, thereby identifying unique aberrations of common aberrations.

Critical Parameters and Troubleshooting

The investigator is referred to *UNIT 22.4* for general troubleshooting issues associated with FISH experiments. However, the investigator should also consider the following factors.

The use of commercially available probes significantly reduces the preparatory work and problems associated with quality control. If the laboratory chooses to produce custom probes, careful planning and control experiments should be carried out to ensure that each chromosome probe has been efficiently labeled.

Both SKY and M-FISH involve the hybridization of whole chromosome paints, thus the risk of background is high. Slide age and pre-treatment are critical to minimizing background. Similarly, if antibodies are being used, they should be diluted to the proper concentration and maintained according to the manufacturer's instructions. The microscope and imaging software should be well maintained, and the user should be fully trained. Although SKY and M-FISH reveal the same information, the methods by which these data are generated are quite different.

SKY

Criteria for successful image acquisition and analysis should be provided by the companies supplying the microscope, imaging equipment, and analytical software. As mentioned previously, all parameters that influence basic FISH analysis will apply to multi-color FISH assays, regardless of the analysis format.

The overall signal intensity along the length of the chromosome should be high with minimal background. The acquisition times, which are usually automatically set by the software, should be relatively short, as longer exposure times will amplify signals arising from background and increase the contribution of electronic noise associated with all forms of digital imaging. Similarly, bright spots that arise as a result of insufficient antibody or probe washing can skew automatic exposure times. This results in a low exposure of the metaphase of interest and increases the risk of ambiguous or erroneous chromosomal assignments.

Interpretation of the analysis relies both on the familiarity of the investigator with the basic technicalities of FISH assays, chromosomal identification by banding methods, and an understanding of fluorescence microscopy. One of the greatest concerns in the analysis is distinguishing between true translocation events and some of the preparation artifacts that may be misinterpreted during M-FISH and SKY. Regions of repetitive sequences such as centromeric areas and telomeric chromosomal ends can often give false classifications due to the differential suppression of those regions either with unlabeled Cot-1 DNA or by the labeled probe. Through careful analysis of normal metaphase spreads and confirmation by locus-specific FISH, real translocations can be distinguished from artifacts. Another phenomenon that contributes to misclassification is the sandwich effect where the junction between a true chromosomal translocation produces an intermediate color. This intermediate color could be identified by the software as another chromosomal segment giving the false impression of a tiny insertion at the site of translocation. Since 24-color painting analysis relies on the combination of up to five different fluorochromes, it is implicit that the fluorescence of two different chromosomal junction regions may in some circumstances be misclassified as a third chromosome undergoing insertion and bearing the spectra or dye combinations of the two partner chromosomes that are actually undergoing a simple rearrangement. This phenomenon can also be seen in chromosomes that are overlapping. The region of overlap will produce a color pattern contributed by both chromosomes, and the software will identify the fluorescence at the region of overlap as that which most closely matches the reference library. In trying to determine whether a translocation is real or not, it is advisable to look for longer chromosomes for analysis in another metaphase. If the insertion is no longer present, then the misclassification is likely to be due to suboptimal hybridization and/or the sandwich effect. Obviously, it is necessary to confirm any uncertain rearrangement of interest using single whole chromosome paint or locus-specific FISH assays on another slide from the same preparation or by re-hybridizing the slide after elution of chromosomal paints.

M-FISH

As discussed for SKY, criteria for successful image acquisition and analysis should be provided by the companies supplying

the microscope, imaging equipment, and analytical software. However, some general points should be considered regardless of which method of image acquisition and analysis is used. The overall signal intensity along the length of the chromosome should be high with minimal background. This is more critical for filter-based systems because the analysis is based on the presence or absence of that fluorochrome. Any background or bleed-over into another filter can influence the outcome of the analysis. The acquisition times, which are usually automatically set by the software, should be relatively short, as longer exposure times will amplify signals arising from background and increase the contribution of electronic noise associated with all forms of digital imaging. Similarly, bright spots that arise as a result of insufficient antibody or probe washing can skew automatic exposure times. This results in a low exposure of the metaphase of interest and increases the risk of ambiguous or erroneous chromosomal assignments.

M-FISH is also subject to the sandwich affect. Caution should be taken when analyzing small insertions. Be sure that the sandwiched chromosome is not simply the mixture of the flanking chromosomal fluorescence. Analyzing longer chromosomes will help to alleviate this problem.

M-FISH imaging relies on the sequential acquisition of images through specific filters and detecting the combination and the presence/absence of the reference dyes hybridized to the metaphase spreads. This entails the acquisition of five different images that are then merged to form the final image. One of the most critical factors is the use of the proper filter sets designed specifically for the M-FISH probe cocktail being used. Careful consideration must be taken with respect to pixel shifts from one image to the next. Careful attention must also be paid to the order in which the images are taken because it is known that some fluorochromes/dyes emit a different wavelength upon degradation or exposure to UV. Such interaction between two fluorochromes could cause misclassification should, for example, a red fluorescing dye that degrades to a yellow emission after exposure to UV, be imaged before the yellow probe is exposed. Because the slides are exposed to different filters, there will be varying exposure times for each dye, and this may also differentially quench the signals from the other fluorochromes/dyes later in the exposure sequence. Investigators are urged to refer to the manufacturer's instructions for both reagents and hardware/software for optimal results.

Like the SKY software, most M-FISH software packages allow the user to identify the fluorochromes that are present along the chromosome of interest and compare it to the combinatorial library. In the event of weak signals or small inconclusive regions of rearrangement, this function is very convenient.

Locus- or band-specific M-FISH

The use of SKY or M-FISH in combination with other cytogenetic or FISH methods provides more detailed information but also increases the damage to the DNA target. As with any FISH experiment, particularly with previously banded slides, the quality of the metaphase spreads, the age and pretreatment (for banding and SKY/M-FISH) influence the outcome for locus-specific FISH following these two assays. In many cases, the overall signal intensity of the locus-specific FISH probe will be weaker following banding and SKY/M-FISH. This is due to the fact that the target DNA has undergone a series of digestions and denaturations, thus compromising the quality of the DNA for hybridization. The overall background intensity will also increase since the slide was previously probed with a whole genome–painting probe. The denaturation of the slide prior to hybridization with the locus-specific probe should remove any bound probe and antibodies from the chromosomes, however, this is never complete. This is certainly the case if there is residual cytoplasm and a high density of cells on the slide. An indication that the DNA has been severely damaged is if it fails to take up the counterstain, such that the chromosomes appear a very faint blue and the centromeres are very bright.

Anticipated Results

In the best conditions, a given metaphase spread will classify perfectly.

If the DNA is not damaged, the resulting FISH should provide signals that can be clearly visualized by microscopy. These signals can be enhanced using imaging tools. For examples, see Figures 22.5.2, 22.5.4, 22.5.5, and 22.5.6.

Time Considerations

The time required to complete analysis depends on the complexity of the karyotype.

Acknowledgements

The authors wish to thank Thomas Ried of the Genetics Branch of the NIH, and Paula Marrano, Jana Karaskova, Bisera Vukovic,

Ajay Pandita, and Gloria Lim, of the University of Toronto for their help with these methods.

Literature Cited

Gray, J.W., Lukas, J., Peters, D., Pinkel, D., Trask, B., Van den Engh, G., and Van Dilla, M. 1986. Flow karyotyping and sorting of human chromosomes. *In* Proceedings of Cold Spring Harbor Symposium in Quantitative Biology 51 Pt. 1:141-149. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Malik, Z., Dishi, M., and Garini, Y. 1996. Fourier transform multipixel spectroscopy and spectral imaging of protoporphyrin in single melanoma cells. *Photochem. Photobiol.* 63:608-614.

Schrock, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M.A., Ning, Y., Ledbetter, D.H., Bar-Am, I., Soenksen, D., Garini, Y., and Ried, T. 1996. Multicolor spectral karyotyping of human chromosomes. *Science* 273:494-497.

Speicher, M.R., Gwyn Ballard, S., and Ward, D.C. 1996. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat. Genet.* 12:368-375.

Telenius, H., Pelmear, A.H., Tunnacliffe, A., Carter, N.P., Behmel, A., Ferguson-Smith, M.A., Nordenskjold, M., Pfragner, R., and Ponder, B.A. 1992. Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes Chromosomes Cancer.* 4:257-263.

Contributed by Jane Bayani and Jeremy Squire
Ontario Cancer Institute
Toronto, Canada

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH; Kallioniemi et al., 1992) is a FISH-based technique that is conceptually different from the FISH experiments already discussed in this chapter (*UNITS 22.4 & 22.5*). The critical difference between CGH and standard FISH assays is that the target DNA for CGH, located on the slide, consists of metaphase chromosome preparations derived from cells of a karyotypically normal individual (Fig. 22.6.1). In standard FISH studies, the target cellular DNA is hybridized with a purified DNA probe, such as a specific gene or chromosomal locus or a chromosomal paint. In CGH, the labeled probe consists of differentially labeled genomic DNA. An equal amount of labeled normal reference DNA is mixed with a labeled tumor/test DNA. Together, these probes are allowed to hybridize to the normal metaphase slide and each labeled DNA is detected in a dual-color experiment. Many of the details of the steps required to perform CGH have already been reviewed in the previous units of Chapter 22, with minor changes. The investigator will be referred to these sections throughout the discussion. In this assay, DNA must be extracted from the test sample; this can be DNA extracted from cell lines or fresh/frozen bulk tissue (see Support Protocol 1 for extraction; see Support Protocol 3 for labeling) or DNA extracted from paraffin-embedded tissues (see Support Protocol 2; see Support Protocol 4 for labeling). DNA must also be extracted from any normal source, such as blood, tonsils, or placenta (see Support Protocol 5 and *UNIT 22.2*). If the experiment is based on DNA extracted from paraffin-embedded tissue, the normal control should also be extracted from paraffin-embedded material. Finally, the normal reference DNA should be derived from a male, as should the normal metaphase slide (see Critical Parameters). Typically, as described in the Basic Protocol (indirect labeling), the normal reference DNA is labeled with digoxigenin and the test/tumor DNA is labeled with biotin. The differentially labeled DNA samples are denatured and allowed to hybridize to the denatured normal metaphase spreads. Following a 72-hr hybridization, the slides are washed to remove any unbound probe and detected with FITC/avidin and rhodamine-conjugated to anti-digoxigenin, such that the tumor DNA is detected by a green signal and the normal DNA is detected by a red signal. Alternatively, as also described in the Basic Protocol, the two probes may be directly labeled with green and red fluorochromes, respectively; in this case, no antibody detection procedure is required. Imaging and CGH analysis software calculates the green/red ratio along the length of each chromosome and determines the net changes between the tumor and normal reference. CGH is useful when no sample is available for short-term culture or for archival material either frozen or embedded in paraffin.

STRATEGIC PLANNING

CGH is a lengthy process, requiring attention to each step both at the bench and at the microscope. A time-saving hint is to set up the hybridization just before a weekend and allow it to occur over the weekend, and then to carry out the post-hybridization washes and microscopy the following week.

An overview of the assay is as follows:

Preparatory work

1. Tissue/Cell acquisition.
2. DNA extraction.
3. DNA labeling (*UNIT 22.4*).
4. Metaphase slide preparation (culturing and slide making; *UNIT 22.2*).

Contributed by Jane Bayani and Jeremy A. Squire
Current Protocols in Cell Biology (2004) 22.6.1-22.6.15

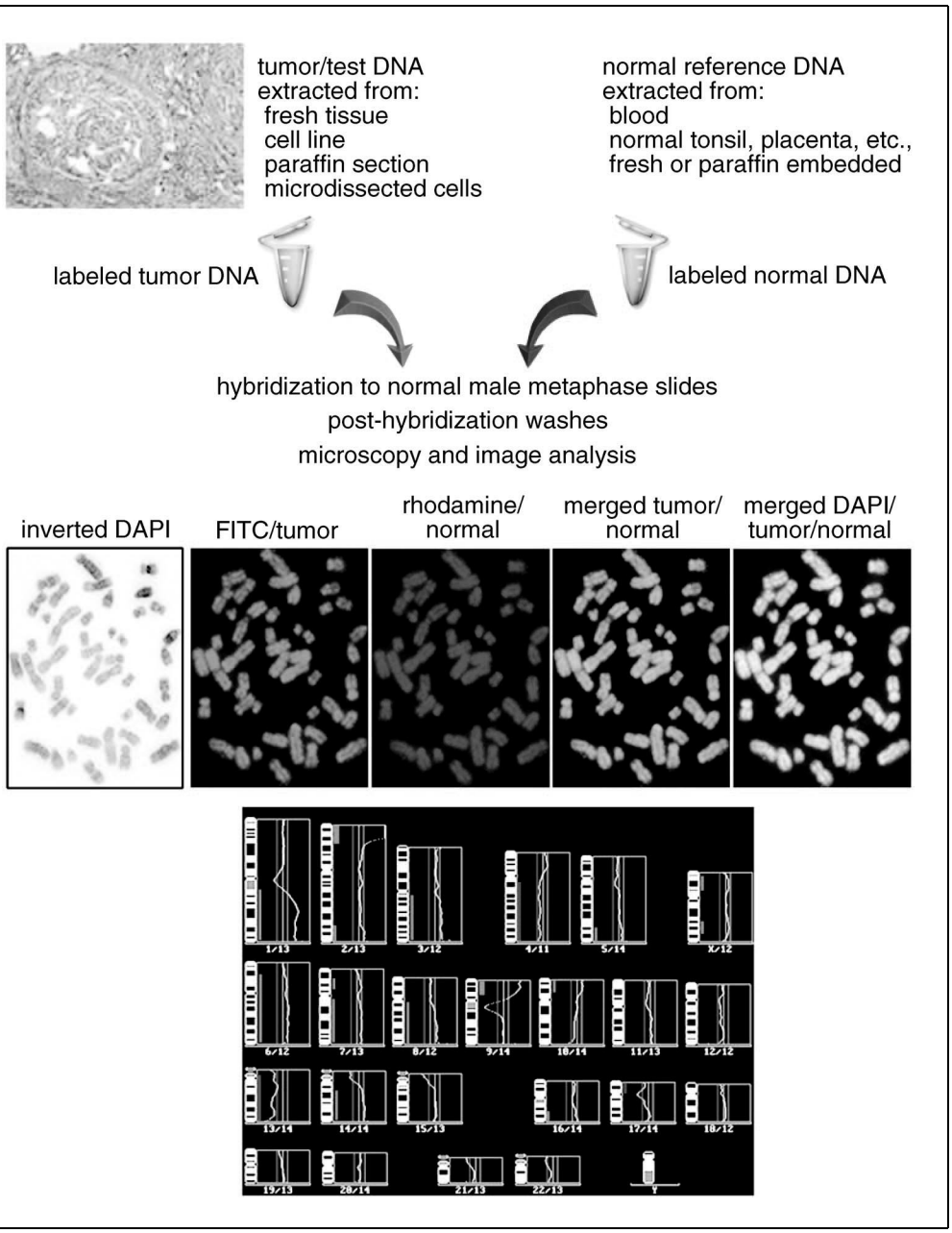


Figure 22.6.1 Schematic representation of CGH analysis. CGH analysis involves the labeling of normal reference DNA and the tumor/test DNA such that they can be identified by either green or red fluorescence (tumor and normal reference, respectively). Equal amounts of these labeled DNAs are hybridized and detected by standard in situ methods to normal metaphase spreads. The metaphase cells are then imaged and karyotyped. CGH analysis determines the change in green/red ratio along the length of each chromosome, corresponding to the net genomic change of DNA at a specific chromosomal locus in the tumor in comparison to the normal reference. The metaphase is imaged using three filters: a DAPI filter to identify chromosomes for karyotyping purposes (the blue image is converted into a pseudo G-banded image), a green filter (FITC/Spectrum Green) to detect the tumor DNA, and a red filter (rhodamine/Spectrum Orange) to detect the normal DNA hybridized to the normal chromosomes. These images can be overlayed as shown. Areas of genomic gain or amplification are seen as fluorescing green, while areas of loss are seen as fluorescing red. Regions of no genomic change are seen as yellow/orange. The three-color merged image (DAPI/FITC/rhodamine) is also displayed. Analysis of 7 to 10 metaphase spreads results in a CGH karyotype as shown at the bottom of the figure. The CGH karyotype of this tumor is as follows: +1q, amp 2p24-qter, +3q,−4q,+5q33-qter, +6p22-qter,+7p13-qter, +8q,amp9p,+10p,−13,+14q12-qter,+16q23-qter,−17p,+Xp11-14, +Xq221-22. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.interscience.wiley.com/c_p/colorfigures.htm.*

FISH (UNIT 22.4)

1. Slide pretreatment and denaturation.
2. Probe denaturation and preannealing.
3. Hybridization for 72 hr.
4. Post-hybridization washes.

Microscopy and analysis

1. Acquisition of images.
2. Karyotyping and CGH analysis.

COMPARATIVE GENOMIC HYBRIDIZATION

BASIC PROTOCOL

Slide pretreatment and denaturation should be carried out according to *UNIT 22.4*. There should be a high mitotic index, with chromosomes that are neither too short nor too long (preventing frequent overlapping chromosomes). Meanwhile, equal amounts of labeled normal DNA and tumor/test DNA should be mixed, denatured, and allowed to preaneal for 1 hr at 37°C. Next, the slides and probe are added together, coverslipped, sealed, and allowed to hybridize for at least 72 hr at 37°C. The slides are then washed of any unbound probes using the methods described in *UNIT 22.4*. Haptens are detected in a single round of antibody incubation; thus, the antibodies should be conjugated to their respective fluorochrome. In the case of directly labeled probes, no antibody detection is required.

Materials

10 ng/μl tumor DNA: indirectly labeled with biotin or directly labeled with Spectrum Green, FITC, or other green-fluorescing dye (see *UNIT 22.4* and Support Protocols in this unit) in hybridization buffer (see *UNIT 22.4* for buffer)

10 ng/μl normal reference DNA: indirectly labeled with digoxigenin or directly labeled with Spectrum Orange, rhodamine, or other red-fluorescing dye (see *UNIT 22.4* and Support Protocols in this unit) in hybridization buffer (see *UNIT 22.4* for buffer)

Pretreated and denatured male metaphase slides (*UNIT 22.4*)

Rubber cement

50% (v/v) formamide/2× SSC (see *UNIT 18.6* for 20× SSC), 45°C

2× SSC (*UNIT 18.6*), 45°C

Blocking solution: 1% BSA (w/v)/0.1% (v/v) Tween 20 in 4× SSC (store indefinitely at –20°C or up to several months at 4°C)

FITC-labeled avidin *and* rhodamine-labeled anti-digoxigenin antibody (*UNIT 22.4*; for detection of indirectly labeled probes only)

0.1% (v/v) Tween-20/4× SSC (see *UNIT 18.6* for 20× SSC), 45°C

DAPI in antifade (*UNIT 22.4*)

75°C water bath

37°C dry incubator or oven

Coverslips

Hybridization box: e.g., black videocassette box containing slightly dampened moist paper towel or gauze

Coplin jars

Fluorescent microscope with appropriate filter sets (*UNITS 4.2 & 22.4*)

Image acquisition software (*UNIT 4.2*)

CGH analysis software (e.g., Leica, Applied Imaging, Metasystems)

Additional reagents and equipment for FISH (*UNIT 22.4*)

Preanneal probe

1. In a 1.5-ml microcentrifuge tube, combine 200 ng of labeled normal DNA and 200 ng of labeled tumor/test DNA (as respective 10 ng/μl solutions). Mix well.

   Some protocols have used up to 1 μg each of labeled DNAs for one experiment. Since the DNAs are in great excess, there is no need to use such large amounts. In the event that the experiment fails (see Troubleshooting) due to factors other than the labeling, using the above amounts, the investigator will still have sufficient material to repeat the experiment without having to repeat the labeling procedure.

2. Denature the combined probes for 10 min at 75°C, then place in a 37°C oven for 1 hr.

Hybridize probe to metaphase slides

3. Add the preannealed probe to pretreated and denatured normal metaphase slides (also see *UNIT 22.4*).

4. Place a coverslip on top of the area of the slide containing the preparation, being careful to avoid bubbles. Seal with rubber cement and place in the dampened hybridization box at 37°C for 72 hr.

5. Carefully remove coverslip by peeling off the rubber cement and placing the slide in a Coplin jar containing 50% formamide/2× SSC at 45°C for 5 min; the coverslip may fall off unaided, but it might be necessary to gently ease it off of the slide surface. Repeat this procedure for a total of three washes, agitating gently between washes.

6. Wash slide three additional times as in step 5, except use 2× SSC at 45°C. For indirectly labeled probes proceed to step 7; for directly labeled probes, proceed to step 9.

Use antibody to detect indirectly labeled probes

7. Remove the slide from the last wash and allow excess solution to drain, but do not allow the slides to dry. Add 60 μl blocking solution to the slide. Add a coverslip and place in the hybridization box at 37°C for 30 min.

8. Remove coverslip and add FITC-labeled avidin and rhodamine-labeled anti-digoxigenin antibody in a total volume of 60 μl (also see *UNIT 22.4*). Apply a coverslip and place in the hybridization box at 37°C for 30 to 40 min.

9. Remove coverslip and wash slide three times with 0.1% Tween 20/4× SSC at 45°C using the technique described in step 5.

Perform imaging

10. Remove the slide from the last wash and allow the excess solution to drain, but do not let the slide to dry. Add 50 μl of DAPI in antifade (mounting medium) to the slide. Apply coverslip.

    The slides are now ready for visualization. When not in use, slides should be kept at −20°C.

11. Acquire the image using a fluorescent microscope equipped with the appropriate filters.

    CGH mapping is based on the average green/red ratio along the length of properly identified chromosomes. Approximately 7 to 10 well-hybridized spreads (see Troubleshooting) should be karyotyped, and the average green/red ratio from all the karyotyped chromosomes should be calculated, giving the final results. Metaphase spreads should have few to no overlapping chromosomes, since these will be excluded from analysis, and they should have strong signal strength across the metaphase spread with minimal to no background. It is crucial that the chromosomes be properly karyotyped; thus, if the DAPI banding is poor, so that identification of the chromosomes cannot be accomplished, the experiment should be repeated with changes in slide conditions.

    To ensure accurate calculation of ratios, there should be no pixel shifts when changing to different filters during image acquisition.

12. Analyze the image using CGH software.

Currently, there are several commercial CGH systems available from Leica (http://www.leica-microsystems.com), Applied Imaging (http://www.aicorp.com) and Metasystems (http://www.metasystems.de). As each system is different, consult with the company representative for the specification and training related to the system.

Standard metaphase CGH imaging systems have a mapping resolution of ~10 Mb. However Applied Imaging has recently introduced metaphase CGH imaging algorithms with improved mapping resolution (stated to be ~3 Mb) and improved sensitivity for dealing with normal-cell contamination (see Critical Parameters). Such improved resolution may be helpful for the detection and mapping of smaller deletions or focal amplifications.

EXTRACTION OF DNA FROM FRESH/FROZEN TISSUES OR CELL LINES

SUPPORT PROTOCOL 1

DNA extraction from fresh/frozen tissues or cell lines uses standard proteinase K treatment and phenol:chloroform extraction. Commercially available DNA extraction kits are available if the investigator prefers. Irrespective of the extraction method, the final DNA sample should be of high molecular weight with little or no degraded fragments and should be resuspended in water.

Materials

Tissue sample or cell pellet
TE buffer, pH 7.5 (*APPENDIX 2A*)
10% (w/v) SDS
14 mg/ml proteinase K
Molecular-biology-grade phenol, TE-saturated (*UNIT 18.3*)
1:1 (v/v) phenol/chloroform (see recipe)
Chloroform
3 M sodium acetate (*APPENDIX 2A*)
100% and 70% ethanol, cold
DNase-free H_2O

Scalpels and scalpel blades
15-ml polypropylene conical centrifuge tubes
Centrifuge
Platform rotator

Digest sample

1a. *For tissues:* Using sterile techniques, mince tissue into small pieces with a scalpel and place into 15-ml conical tube.

1b. *For cultured cells:* Place cells in a 15-ml tube and centrifuge 5 min at 200 × *g*, room temperature or 4°C. Discard supernatant and retain pellet.

2. Add 5 ml of TE buffer, 500 µl of 10% SDS (1% final), and 10 µl of 14 mg/ml proteinase K to the minced tissue or cell pellet. Cap tightly and place on a platform rotator at 37°C overnight.

If there is too much tissue or the resulting digest is too thick, add more TE buffer and SDS to the tube.

3. The following day, check digest. If there are still tissue pieces remaining, add more proteinase K and continue incubation at 37°C.

Extract DNA with phenol

4. When the tissue has completely digested, add an equal volume of TE-saturated phenol. Cap tightly and place on a platform rotator at room temperature for 10 min, or mix by manual inversion.
5. Centrifuge 10 min at 3000 × *g*, room temperature. Carefully remove the tube without disrupting the phases.
6. Using a sterile 1-ml plastic bulb pipet, carefully transfer the aqueous (upper) layer to a clean tube. Do not remove any solid matter that may be at the interface.
7. To the extracted aqueous layer, add an equal volume of TE-saturated phenol and repeat the extraction and centrifugation for a total of three extractions.

Extract DNA with phenol/chloroform

8. To the extracted aqueous layer removed from the third phenol extraction, add an equal volume of 1:1 (v/v) phenol/chloroform. Cap tightly and place on a platform rotator at room temperature for 10 min, or mix by inversion.
9. Centrifuge 10 min at 3000 × *g*, room temperature. Carefully remove the tube without disrupting the phases.
10. Using a sterile 1-ml plastic bulb pipet, carefully transfer the aqueous (upper) layer to a clean tube. Do not remove any solid matter that may be at the interface.
11. To the extracted aqueous layer, add an equal volume of 1:1 (v/v) phenol/chloroform and repeat the phenol/chloroform extraction and centrifugation for a total of three extractions.

Extract DNA with chloroform

12. To the extracted aqueous layer removed from the third 1:1 (v/v) phenol/chloroform extraction, add an equal volume of chloroform. Cap tightly and place on a platform rotator at room temperature for 10 min, or mix by inversion.
13. Centrifuge 10 min at 3000 × *g*, room temperature. Carefully remove the tube without disrupting the phases.
14. Using a sterile 1-ml plastic bulb pipet, carefully transfer the aqueous (upper) layer to a clean tube. Do not remove any solid matter that may be at the interface.
15. To the extracted aqueous layer, add an equal volume of chloroform and repeat the chloroform extraction and centrifugation for a total of three extractions.

Precipitate and resuspend DNA

16. To the extracted aqueous layer from the third chloroform extraction, add 1/10 volume of 3 M sodium acetate and 2.5 vol of cold 100% ethanol. Invert gently.

    The DNA will spool into a clump of thread-like wisps.

17. Collect the spooled DNA into a clean 1.5-ml tube using a pipet tip and rinse with 70% ethanol.
18. Microcentrifuge 5 min at maximum speed, room temperature, and remove the ethanol supernatant.
19. Resuspend the DNA in 100 µl DNase-free water. Tap or flick the tube to dissolve the DNA back into solution.

    If the solution is too thick, add more water.

20. Store the DNA at −20°C until ready for use.

The DNA is best stored at −20°C and can be kept for several years. DNA concentration can now be assessed by spectrophotometry (APPENDIX 3D) and DNA purity can be assessed by gel electrophoresis (see APPENDIX 3A for cross-references).

DNA EXTRACTION FROM PARAFFIN SECTION

SUPPORT PROTOCOL 2

DNA can also be extracted from paraffin-embedded tissue. The paraffin is removed and the tissue is digested under more harsh conditions than those described for fresh tissue in Support Protocol 1. The digest is then phenol/chloroform extracted as previously outlined in Support Protocol 1, steps 4 to 15. This method is typically used for the bulk extraction of large tissue sections. The DNA from small numbers of microdissected cells would be lost in this extraction method, so it is simply added to the labeling reaction following brief digestion (see Support Protocol 4).

Materials

Paraffin sections
Xylene
Methanol
1 M sodium thiocyanate, 55°C (prepare fresh)
DNA extraction buffer (see recipe), 55°C (prepare fresh)
Proteinase K

Microtome
Polypropylene tubes
Oven maintained at 55°C

1. Using a microtome, cut 30 to 50 slices from paraffin block, each 6 μm thick, and place the sections in a polypropylene tube.
2. Add 1 ml xylene and incubate 10 min. Remove xylene and repeat with 1 ml fresh xylene. Remove xylene, add 1 ml methanol, and incubate 10 min. Remove methanol.
3. Incubate overnight in 1 ml of 1 mM sodium thiocyanate at 55°C.
4. Digest for 3 days in 1 ml of extraction buffer at 55°C. Add 0.2 mg of proteinase K each day.
5. Perform extractions and precipitation of DNA (see Support Protocol 1, steps 4 to 20).

LABELING DNA EXTRACTED FROM FRESH TISSUES FOR CGH

SUPPORT PROTOCOL 3

UNIT 22.4 describes the basic method for labeling DNA for FISH analysis using nick translation. For CGH, the test/tumor DNA is typically labeled with biotin and detected with FITC/avidin; the normal reference DNA is labeled with digoxigenin and detected with rhodamine conjugated to anti-digoxigenin antibody. Direct labeling of each DNA sample can also be carried out using Spectrum Green or fluorescein for the tumor DNA and Spectrum Orange or rhodamine for the normal control DNA . Unlike conventional FISH experiments described previously, the final fragment sizes should be between 500 bp and 2 kbp. The final probe concentration should be determined by gel electrophoresis. The probes should be precipitated in a similar fashion using salmon sperm carrier and Cot-1 DNA and resuspended in hybridization buffer to a final concentration of 10 ng/μl, as outlined for FISH probes. It is always suggested that a normal control be carried out, particularly if the investigator is not familiar with the assay. In this case, normal DNA should be labeled with biotin (or directly labeled with a green-fluorescing fluorochrome), as well as labeled with digoxigenin (or directly labeled with a red-fluorescing fluorochrome), and included as another component of the experiment.

SUPPORT PROTOCOL 4

LABELING DNA EXTRACTED FROM PARAFFIN-EMBEDDED TISSUES FOR CGH

DNA extracted from paraffin-embedded tissues can range in both quantity and quality. If the DNA yield from bulk extracted tissue is sufficient and is of high molecular weight, labeling by nick translation can be employed. However, if the yield is significantly lower and it is not possible to obtain more material, then amplification by degenerate oligonucleotide primer (DOP)–PCR may be required, as described below. This amplification protocol is also applicable to microdissected cells. The DOP-amplified products may then be labeled by nick translation as described in *UNIT 22.4*, or may require labeling by DOP-PCR methods, also described in *UNIT 22.4*. As in the case of labeling for fresh tissue the test/tumor DNA is typically labeled with biotin and detected with FITC/avidin, while the normal reference DNA is labeled with digoxigenin and detected with rhodamine-conjugated anti-digoxigenin antibody. Direct labeling of the DNA can also be carried out using Spectrum Green or fluorescein for the tumor DNA and Spectrum Orange or rhodamine for the normal control DNA. In the case of DOP-PCR labeling strategies, the fragments tend to be generally smaller than those obtained with nick translation. The final probe concentration should be determined by gel electrophoresis. The probes should be precipitated in a similar fashion using the salmon sperm carrier and Cot-1 DNA, and resuspended in hybridization buffer to a final concentration of 10 ng/μl. Before proceeding with any method for genome amplification, the investigator should carry out the necessary control experiments (see Critical Parameters) to ensure that there is representative amplification across the entire genome, rather than any bias leading to over- or under-representation of the amplified product. As before, normal DNA should be labeled with biotin (or directly labeled with a green-fluorescing fluorochrome), as well as digoxigenin (or directly labeled with a red-fluorescing fluorochrome), and included as another component of the experiment. To optimize the PCR amplification and labeling, it is strongly suggested that the investigator label both normal DNA from the paraffin source and from fresh/frozen DNA (e.g., if using a paraffin-extracted test sample, the normal DNA should come from normal tissue embedded in paraffin). The protocol below is based on the techniques used by Stefan Joos, Carsten Schwänen, and Peter Lichter (Beatty et al., 2002), although other protocols can be performed (Speicher et al., 1993), including a recent method developed for whole-genome amplification termed "SCOMP" (single cell comparative genomic hybridization; Stoecklein et al., 2002). SCOMP has been assessed on archival tissues of different ages, and the method has been shown to be very well suited for formalin-fixed paraffin-embedded samples obtained by nuclei extraction or laser microdissection.

Materials

Isolated (microdissected) cells (20 to 200) or DNA extracted from paraffin section
1× DOP-PCR buffer (see recipe) containing 0.5 μg/μl proteinase K, freshly added
10× DOP-PCR buffer (see recipe)
2 mM dNTP mix (see recipe)
10× DOP primer (see recipe)
Taq DNA polymerase (Perkin-Elmer)
TBE buffer (*APPENDIX 2A*)

500 μl PCR reaction tube
50° and 95°C ovens
Thermal cycler

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 6.7*) and labeling probes by nick translation (*UNIT 22.4*)

1. Collect 20 to 200 isolated cells in a 500-µl reaction tube containing 20 µl of 1× DOP-PCR buffer with 0.5 µg/µl freshly added proteinase K. Incubate at 50°C for at least 2 hr.

   If using extracted DNA, skip steps 1 and 2 and proceed directly to step 3.

2. Inactivate proteinse K by incubation at 95°C for 20 min.

3. Add the following reagents to the tube:

   Reaction from step 2 *or* 1 to 100 ng template DNA in 20-µl volume
   8.0 µl 10× DOP-PCR buffer
   10.0 µl 2 mM dNTP mix
   10.0 µl 10× DOP primer
   5 U *Taq* DNA polymerase
   H_2O to 100 µl.

   As a negative control combine the same solutions, but add water instead of any DNA.

4. For universal amplification of the genomic DNA, use the following program:

1 cycle:	10 min	94°C	(denaturation)
5 cycles:	1 min	94°C	
	1.5 min	30°C	
	3 min	30°-72°C transition	
	3 min	72°C	
35 cycles:	1 min	94°C	
	1 min	62°C	
	3 min (add 1 sec/cycle)	72°C	
1 cycle:	10 min	72°C	
1 cycle	indefinite	4°C	(hold).

5. Run 5 µl of the PCR reaction on a 1% agarose gel in TBE buffer.

   A smear of DNA ranging in size from about 200 to 2000 bp should be visible.

6. Compare reaction product to controls and concentration standards (*UNIT 22.4*) to calculate total amplified product.

7. Label the amplified DNA by nick translation as described in *UNIT 22.4*.

PREPARATION OF NORMAL METAPHASE SLIDES: PRETREATMENT AND DENATURATION

SUPPORT PROTOCOL 5

Cell culture, harvesting, and slide making are described in *UNIT 22.2*. The most commonly used source for normal metaphase spreads is a peripheral lymphocyte culture, ideally from a male. Once normal metaphase slides have been made, the slides can be pretreated with pepsin and denatured (*UNIT 22.4*). The criteria for metaphase slides for CGH analysis are discussed in the Commentary of this unit.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DNA extraction buffer

25.0 μl 1 M EDTA (25 mM final)
75.0 μl 1 M NaCl (75 mM final)
0.5 μl Tween 20 (0.5% v/v final)
28.0 μl 14.4 mg/ml proteinase K (0.4 mg/ml final)
H_2O to 1.0 ml
Prepare fresh for each experiment

dNTP mix (dATP, dCTP, dGTP), 2 mM

2 μl 100 mM dATP (2 mM final)
2 μl 100 mM dCTP (2 mM final)
2 μl 100 mM dGTP (2 mM final)
94 μl sterile water (total volume 100.0 μl)
Store up to several months at −20°C

DOP-PCR buffer, 10×

2.0 μl 1 M $MgCl_2$ (2 mM final)
50.0 μl 1 M KCl (50 mM final)
10 μl 1 M Tris hydrochloride (10 mM final)
0.1 mg gelatin
Sterile H_2O to 1 ml
Prepare using sterile technique and store in 100.0-μl aliquots up to several months at −20°C

DOP primer, 10×

Using sterile technique, prepare a 20 μM solution in sterile water of the following oligonucleotide:
5′-CCG ACT CGA GNN NNN NAT GTG G-3′, (N = A, C, G, or T).
Store in 100.0-μl aliquots up to several months at −20°C.

Phenol:chloroform, 1:1 (v/v)

50 ml molecular-biology-grade phenol, TE-saturated (*UNIT 18.3*)
50 ml chloroform
Total volume, 100.0 ml
Store up to 1 month at 4°C

Use appropriate eye and skin protection when using these chemicals and use in well ventilated areas or designated areas.

COMMENTARY

Background Information

Comparative genomic hybridization (CGH) was first introduced in 1992 (Kallioniemi et al., 1992). It is described as a FISH-based method by which information regarding the net gain or loss of genomic material in a given DNA can be assessed without the need for fresh material, as required for short-term culture and chromosome analysis. As shown in Figure 22.6.1, normal reference DNA and the tumor/test DNA are differentially labeled and hybridized to normal metaphase spreads. The normal DNA and tumor/test DNA are detected using different fluorochromes (green and red) and visualized by fluorescence microscopy. Metaphase spreads are karyotyped by inverted DAPI banding and the average green/red (tumor/test:normal) ratio is calculated. Regions of genomic gain in the tumor are expressed as an increase in green/red ratio,

whereas regions that are lost in the tumor are expressed as a decrease in green/red ratio. Regions that are normal maintain a 1:1 ratio. High-level amplifications can be readily seen as a spike at a specific chromosomal locus and are generally values that exceed 1.5. The major advantage of CGH as compared to other cytogenetic methods—chromosome banding (*UNIT 22.3*) and FISH (*UNIT 22.4*)—is the fact that, instead of metaphase chromosomes or interphase nuclei, only a few micrograms of genomic DNA are required from the cells or tissue to be studied. The best CGH results are obtained from DNA that is prepared from fresh or frozen tissue samples. However, it is also possible to analyze paraffin-embedded tissue samples, small populations of cells, or single tumor cells. Small samples require physical enrichment of tumor cells, e.g., by cell sorting or micromanipulation, in combination with a representative amplification of genomic DNA by universal PCR. However, CGH *only* provides the net genomic changes in a given DNA sample. Information regarding the specific karyotype of those cells that result in those genomic changes is lost; thus, it is not possible to determine whether gains of genomic regions are the result of duplications of whole or partial chromosomes, unbalanced translocations, or more complex cytogenetic changes such as the formation of double-minute chromosomes or homogeneously staining regions. Similarly, loss of genomic regions can result from whole or partial chromosome losses, from deletions that in turn result from translocations or inversions, or from small interstitial deletions. Furthermore, the determination as to karyotypic clonality is lost, since CGH will only provide a gross average of genomic imbalance present in the sample. Aberrations such as balanced translocations will not be detected, since in such cases there is no net genomic change, but rather a change of structure. Nevertheless, CGH has been an important tool for identifying genomic profiles for various human tumors (Zielenska et al., 2001; Bayani et al., 2002; Knutsen et al., 2003) as well as murine systems (Crabtree et al., 2003), and enabling investigators to identify novel regions of genomic loss and amplification that may contain putative tumor-suppressor genes and oncogenes.

Critical Parameters

Since CGH is a FISH-based strategy, those parameters discussed in *UNIT 22.4* are also applicable. However there are some additional critical factors, specific to CGH, that should be taken into consideration. An excellent guide for CGH parameters and troubleshooting can be found in the January 1995 issue of *Cytometry*.

Controls for CGH experiments

For an investigator just starting CGH, it helps to optimize the labeling, hybridization, washes, and analysis using a DNA that has been cytogenetically characterized. In these situations, an established cell line with full-chromosome gains and losses are useful, not only for standard nick-translation methods, but also as a positive copy-number control for universally amplified products and PCR labeling. The control cell line can also be pelleted and embedded in paraffin blocks, serving as a useful qualitative control for optimization of DNA extraction from paraffin-embedded cells. In addition, it is helpful for ensuring that amplification and labeling procedures are efficient. The control karyotype should be relatively simple and clonal—i.e., consistent from cell to cell. The presence of numerous structural aberrations and marker chromosomes can make analysis more difficult. By using a well characterized cell line, the investigator knows what to expect from the CGH analysis. Another method for determining accuracy is to use the sex chromosomes as a guide (Kirchhoff et al., 1998). It is for this reason that the target slides and reference DNA come from a male. Changes in the intensity of the X and Y chromosome can help to determine the success of an experiment. One caveat to this method is that it is quite common for established cell lines and tumors to gain or lose whole copies of the sex chromosomes. Thus, the investigator may know that the tumor/test DNA comes from a female or male, but due to tumor progression it loses a sex chromosome, and the CGH analysis may not reflect this. However, if the researcher is investigating breast or ovarian cancer and uses a male reference, one can be assured that no Y chromosome should be in excess or lost. Similarly, the normal reference DNA can be female if the investigator is studying prostate cancer, and this will allow determination of whether loss or gain of the Y (or X) has occurred.

Interpreting CGH results

Having established the technique and level of sensitivity on controls, the challenge of CGH is in determining the accuracy on a given DNA sample where no previous cytogenetic information is available. Generally, it is useful to include the labeled cell line control and normal reference pair (discussed above) with every experiment. Since the investigator already knows that the labeling produced good results,

it will help to determine whether any technical problems that arise are due to the slide, hybridization, or post-hybridization portions of the assay, and whether the new DNA/normal pair to be tested has labeling efficiency problems. Often CGH is performed because little or no cytogenetic analysis could be carried out, leaving only bulk frozen tissue, paraffin section, or extracted DNA as the source for analysis. When CGH analysis is not concordant with previous cytogenetic analysis, one must consider the following situations. First, the cytogenetic analysis only reflects the clonal changes, which is described by ISCN nomenclature as a case where a gain is seen in at least three metaphases, a loss is seen in at least two metaphases, or structural aberration is seen in at least two metaphases. Moreover, cytogenetic analysis only applies to the relatively few cells actively proliferating and producing metaphase spreads. The over-representation of cytogenetic information in resting interphase cells may contribute disproportionately to copy-number changes detected by CGH, either masking aberrations or leading to inconsistencies with metaphase findings. Nonclonal changes within the tumor can contribute to the relative normalization of copy number gains and losses. This leads to the complex issue of cytogenetic heterogeneity (or chromosomal instability) that can make CGH interpretation of tumors problematic. It is not uncommon for a given tissue to have different levels of chromosomal instability, i.e., gains of a chromosome in one cell population can be offset by an equivalent level of loss in other cells. Finally, the investigator should consider the level of normal contamination. Theoretically, there should be at least a 50% abnormal cell population to be detectable. The most recent Applied Imaging CGH system claims to detect copy number changes when 70% of the cell population consists of contaminating normal cells. In this context, the word "abnormal" refers to a cell that is not diploid. If the ploidy status of the abnormal cell is upwards of triploid or tetraploid, then the amount of normal contamination that the assay can withstand may be slightly higher. Confounding this issue is the fact that visual inspection of a tissue, whether this is simply gross inspection or carried out by histopathological means, may not be accurate in its estimation of the relative contribution of normal or tumor tissue. Some tumors tend to maintain a diploid status, (e.g., the hematological malignancies), whereas others show high levels of aneuploidy (e.g., ovarian and breast tumors and sarcomas). The possibility also arises that cells characterized as tumor cells may possess normal karyotypes. Nevertheless, the investigator can gain additional confidence in the CGH results by repeating the experiment with the labels for the normal and tumor reversed. The normal-versus-normal control and control cell line–versus–normal control should reveal the expected CGH profiles.

DNA quality

For any labeling reaction, the starting material, in this case total genomic DNA, should be of high molecular weight, with little degraded material. Depending on the sample, this may be difficult to achieve, particularly if the specimen contains necrotic or apoptotic tissue, as may be the case in many fresh tumor samples. DNA extracted from bony or cartilaginous tissues, as well as from tissues composed primarily of fat, will also produce low DNA yields of varying quality. The starting DNA should be assessed by gel electrophoresis. For DNA extracted from paraffin-embedded tissues, the criteria that influence paraffin FISH (see *UNIT 22.4*) will also influence DNA extraction. The age of the tissue block, its storage conditions, and quality of the fresh tissue before embedding will influence the subsequent quality of DNA extracted.

Labeling efficiency

Since the fluorescent intensities along the length of each chromosome will be calculated and averaged, it is important that the labeling efficiency be equal between the two samples (tumor and normal). It is suggested that the two be labeled as pairs during the labeling procedure, and that they also be labeled with reversed labels. Therefore, the normal DNA is detected with green fluorescence and the tumor detected with red fluorescence. If the profiles are consistent between both experiments, then the investigator can be sure that the results are accurate. All reagents should be used before their expiration dates.

Target metaphase slides

Since CGH is also dependent on identification of chromosomes, the banding quality of the metaphase spreads following hybridization is crucial. If the identification of a chromosome cannot be made based on the banding or by the cytogenetic skill of the investigator, there is a good chance that misclassification will occur giving inaccurate results. While a slide may produce good hybridization signals, failure to identify the chromosomes renders the experiment just as useless as if no signals were present.

Karyotyping

As already discussed above, the investigator will need to employ the help of a cytogeneticist or spend a good amount of time learning to karyotype chromosomes. With enough practice, the investigator should be able to easily identify and karyotype a metaphase spread.

Adequate Cot-1 suppression

Unlike FISH experiments, more Cot-1 suppression is needed, since the probes being used are derived from total genomic DNA. Hybridization is also targeted to the entire genome. Hybridization experiments can be severely impaired by insufficient suppression of repetitive sequences. This might manifest itself by a strong staining of the heterochromatin blocks on human chromosomes 1, 9, and 16. An increase in the amount of the Cot-1 DNA fraction and longer preannealing times (the latter being less effective) can be used to achieve adequate suppression of signals in these regions of the genome.

Low quality of equipment

The optical instrumentation, the camera system, and the image analysis software used can also contribute to insufficiencies. These are discussed elsewhere (du Manoir et al., 1995a,b; Lundsteen et al., 1995; Piper et al., 1995). Microscope lamps must be properly centered and the proper filter sets should be used. Image capture using a good-quality 63× fluorescence objective tends to produce the best results. See *UNIT 4.2* for general considerations in fluorescence microscopy.

Metaphase spreads for CGH analysis

CGH analysis can be carried out on 7 to 10 well hybridized metaphase spreads. The signal strengths should be high, with low background levels. Metaphase spreads should have no overlapping chromosomes; otherwise these must be excluded from analysis. Choose spreads where the chromosomes are long enough to produce a recognizable banding pattern. There should also be consistency with respect to chromosome length when choosing metaphases for analysis. If a chromosome cannot be readily identified, exclude it from the analysis. It is far better to exclude a questionable chromosome from the analysis than to karyotype it incorrectly. Analysis must be derived from one slide. Do not "mix-and-match" experiments. If a particular DNA is repeated, analyze it as its own experiment. Variables such as slide age, batch, and hybridization conditions can influence the quality of the hybridization and hence the results (see Troubleshooting below).

Troubleshooting

CGH, like other FISH assays is multifaceted, involving the preparation of many different components and bringing them together to achieve the final results. *UNIT 22.4* covers many of the typical problems encountered when carrying out FISH and FISH-based assays. The following discussion deals with common CGH difficulties and the strategies to pinpoint the cause of such difficulties.

Weak signal intensity of both normal and tumor DNAs

This can be caused by a number of factors at different points of the assay. Ensure that the labeling reaction was efficient. Check that all labeling reagents were maintained at the right storage conditions and used prior to their expiration dates, particularly the enzymes. If a dot-blot test was used to check the label incorporation (see *UNIT 22.4*) and the results were satisfactory, then the problem lies elsewhere. The quality of the DNA should also be investigated, making sure that it is resuspended in water rather than TE buffer and that it is of high molecular weight. If the labeling reaction can be ruled out, then consider the target slide. If the metaphases are puffy with poor banding, overdenaturation, which destroys the DNA, may be the problem. On the other hand, if the morphology of the chromosomes is very good, with sharp, crisp banding, there may have been insufficient denaturation as the result of over-aging of the slides or insufficient time for denaturation. If another CGH experiment using the same slides during the same hybridization experiments produces good results, it is likely not a slide problem. Weak signal intensity can also be a result of poor hybridization caused by the dilution of the probe and associated buffers if the coverslip was not adequately sealed. This will occur if there is so much moisture in the hybridization chamber that the moisture leaches under the poorly sealed coverslip, diluting the probe and/or causing the probe to leak out. The temperature for hybridization should be at 37°C with a minimum of 72 hr for hybridization. As discussed previously, longer fragments require more time to hybridize to their targets. Factors affecting signal intensity during the post-hybridization washes and detection are discussed in *UNIT 22.4*; these include stringency of washes, temperature, and quenching of the fluorescence of conjugated antibodies.

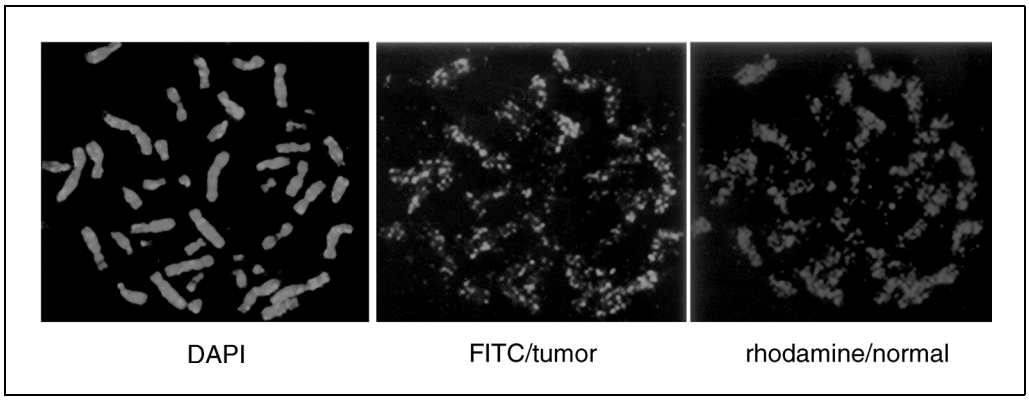


Figure 22.6.2 Examples of poor hybridization resulting in a speckled or patchy appearance of fluorescence across the metaphase. From left to right: DAPI-stained metaphase, FITC-detected tumor DNA, and rhodamine-detected normal reference DNA. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.interscience.wiley.com/c_p/colorfigures.htm.*

Weak signal intensity of one of the two DNAs

This is usually an issue with the labeling efficiency of the problem DNA. Check the quality of the starting DNA by examining gel photos of the undigested DNA. Contaminating proteins can inhibit labeling as can resuspension in TE buffer. Furthermore, check the antibody used to detect the labeled DNA, as this is the other factor causing weak signal intensity.

Granularity of hybridization

A good CGH experiment will have even hybridization across the metaphase spread with good signal intensity, as shown in Figure 22.6.1. Under these conditions, gross aberrations, e.g., gains or losses of a whole chromosome or distinct high-copy-number amplifications, can be detected visually without any equipment for digitized image acquisition or analysis, as seen in the three-color image (red-green-blue) in Figure 22.6.1. If the hybridization signals show a patchy or speckled appearance, this indicates that improper hybridization has occurred (Fig. 22.6.2). This can be caused by insufficient protease treatment, resulting in impaired access of the probe to the DNA target. Insufficient or overdenaturation of the metaphase slide can also cause this appearance, as can labeled DNA that is too long. High granularity is also indicated by large confidence intervals that indicate the variability of averaged ratio values when the analysis has been completed. The investigator should look to all these factors when analyzing a slide.

Poor DAPI banding

The ability to visualize the chromosomes and identify them using DAPI banding (which is normally converted into a pseudo-G-banding image by all CGH softwares) is critical to successful CGH. *UNIT 22.4* describes the possible causes for poor banding and methods for improvement.

Failure of control CGH experiments

The value of performing control experiments using a DNA with known gains and losses lies in its ability to establish the level of sensitivity. Having obtained good signal strength and banding in an experiment that has reached the analysis stage, the third critical aspect is whether the CGH assay has been able to detect the expected genomic changes.

None of the changes detected: If none of the expected changes were detected, the investigator must go back to the beginning of the procedure (i.e., starting from the DNA extraction and labeling through to hybridization). One of the biggest concerns would be the inequality of normal and tumor/test DNA used for the experiment. The investigator should review the labeling procedure, paying special attention to the quantity of DNA used in the initial labeling procedure and the quantitation using the gel images and standards. Hybridization problems can also contribute to lack of CGH findings (discussed above). Finally, poor karyotyping skills will significantly affect the CGH results.

Some of the changes detected: If some of the expected changes are detected, while others are not, this suggests either that karyotyping of those chromosomes may be suspect or that the hybridization of that particular experiment was not optimal; thus, some changes were detected while others were not. The investigator can analyze a few more metaphase spreads and increase the number of analyzed cells to the final profile. The drawback is that a poorly hybridized experiment will accentuate

false results, aggravated primarily by amplified background signals. It is suggested that the experiment be repeated with adjustments in slide pretreatment and/or denaturation, making sure that all hybridization and washing conditions are correct.

CGH results skewed in favor of gains or losses: Having carried out the analysis, if one finds that all the chromosomes have a tendency for gain or loss, this suggests that there was an unequal amount of one labeled DNA over the other in the experiment.

Anticipated Results

A good CGH experiment will have bright signal strength along the length of each chromosome and across the metaphase spread. The chromosome banding should be crisp and distinct. There should also be minimal background.

Time Considerations

The preparatory work may take several days, depending on the method of DNA extraction and the source of DNA being extracted. Hybridization is carried out for a minimum of 72 hr. Post-hybridization washes and detection will require ~2 hr. Image acquisition and image analysis will take several hours to several days, depending on the karyotyping skills of the investigator.

Acknowledgments

The authors wish to acknowledge the assistance of Paula Marrano, Ontario Cancer Institute, and Ajay Pandita, Hospital for Sick Children, Toronto, Ontario, Canada.

Literature Cited

Bayani, J., Brenton, J.D., Macgregor, P.F., Beheshti, B., Albert, M., Nallainathan, D., Karaskova, J., Rosen, B., Murphy, J., Laframboise, S., Zanke, B., and Squire, J.A. 2002. Parallel analysis of sporadic primary ovarian carcinomas by spectral karyotyping, comparative genomic hybridization, and expression microarrays. *Cancer Res.* 15:62:3466-3476.

Beatty, B., Mai, S., and Squire, J.A. 2002. FISH: A Practical Approach. Oxford University Press, New York.

Crabtree, J.S., Scacheri, P.C., Ward, J.M., McNally, S.R., Swain, G.P., Montagna, C., Hager, J.H., Hanahan, D., Edlund, H., Magnuson, M.A., Garrett-Beal, L., Burns, A.L., Ried, T., Chandrasekharappa, S.C., Marx, S.J., Spiegel, A.M., and Collins, F.S. 2003. Of mice and MEN1: Insulinomas in a conditional mouse knockout. *Mol. Cell. Biol.* 23:6075-6085.

du Manoir, S., Schrock, E., Bentz, M., Speicher, M.R., Joos, S., Ried, T., Lichter, P., and Cremer, T. 1995a. Quantitative analysis of comparative genomic hybridization. *Cytometry.* 19:27-41.

du Manoir, S., Kallioniemi, O.P., Lichter, P., Piper, J., Benedetti, P.A., Carothers, A.D., Fantes, J.A., Garcia-Sagredo, J.M., Gerdes, T., and Giollant, M. 1995b. Hardware and software requirements for quantitative analysis of comparative genomic hybridization. *Cytometry* 19:4-9.

Kallioniemi, A., Kallioniemi, O.P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F., and Pinkel, D. 1992. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 258:818-821.

Kirchhoff, M., Gerdes, T., Rose, H., Maahr, J., Ottesen, A.M., and Lundsteen, C. 1998. Detection of chromosomal gains and losses in comparative genomic hybridization analysis based on standard reference intervals. *Cytometry* 31:163-173.

Knutsen, T., Pack, S., Petropavlovskaja, M., Padilla-Nash, H., Knight, C., Mickley, L.A., Ried, T., Elwood, P.C., and Roberts, S.J. 2003. Cytogenetic, spectral karyotyping, fluorescence in situ hybridization, and comparative genomic hybridization characterization of two new secondary leukemia cell lines with 5q deletions, and MYC and MLL amplification. *Genes Chromosomes Cancer* 37:270-281.

Lundsteen, C., Maahr, J., Christensen, B., Bryndorf, T., Bentz, M., Lichter, P., and Gerdes, T. 1995. Image analysis in comparative genomic hybridization. *Cytometry* 19:42-50.

Piper, J., Rutovitz, D., Sudar, D., Kallioniemi, A., Kallioniemi, O.P., Waldman, F.M., Gray, J.W., and Pinkel, D. 1995. Computer image analysis of comparative genomic hybridization. *Cytometry* 19:10-26.

Speicher, M.R., du Manoir, S., Schrock, E., Holtgreve-Grez, H., Schoell, B., Lengauer, C., Cremer, T., and Ried, T. 1993. Molecular cytogenetic analysis of formalin-fixed, paraffin-embedded solid tumors by comparative genomic hybridization after universal DNA-amplification. *Hum. Mol. Genet.* 2:1907-1914.

Stoecklein, N.H., Erbersdobler, A., Schmidt-Kittler, O., Diebold, J., Schardt, J.A., Izbicki, J.R., and Klein, C.A. 2002. SCOMP is superior to degenerated oligonucleotide primed-polymerase chain reaction for global amplification of minute amounts of DNA from microdissected archival tissue samples. *Am. J. Pathol.* 161:43-51.

Zielenska, M., Bayani, J., Pandita, A., Toledo, S., Marrano, P., Andrade, J., Petrilli, A., Thorner, P., Sorensen, P., and Squire, J.A. 2001. Comparative genomic hybridization analysis identifies gains of 1p35 approximately p36 and chromosome 19 in osteosarcoma. *Cancer Genet. Cytogenet.* 130:14-21.

Contributed by Jane Bayani and Jeremy A. Squire
Princess Margaret Hospital and The Ontario Cancer Institute
University of Toronto
Toronto, Ontario, Canada

Sister Chromatid Exchange

Sister chromatid exchange (SCE) refers to the interchange of DNA between replication products. The technique takes advantage of the semiconservative nature of DNA synthesis. 5′-bromodeoxyuridine (BrdU) is incorporated into the newly synthesized DNA. Colcemid is added to the culture, and conventional cytogenetic preparations are made. Differential staining with Hoechst dye and Giemsa allows the newly synthesized DNA within a chromatid to be recognized because BrdU incorporation leads to much weaker staining. SCEs represent a point of DNA template exchange during strand synthesis, visualized as asymmetric chromatid staining or "harlequin" chromosomes. There are a number of minor variations in the basic SCE protocol, which are discussed in Barch (1991). The protocol below uses a nonfluorescent method of microscopy.

STRATEGIC PLANNING

To carry out SCE experiments, the investigator should be familiar with the growth kinetics of the cells being analyzed. Since SCE involves DNA replication, the cells should be actively dividing. Depending on the nature of the experiment, drug or radiation treatment should be carefully modulated. The researcher should refer to the current literature regarding growth medium and other relevant information. A quick guide to SCE analysis is as follows:

Cell culture

1. Growth of cells (culture medium should not contain thymidine).
2. Culture treatment.
3. BrdU treatment.
4. Cell recovery.

Cell harvesting

1. Colcemid treatment.
2. Preparation of cytogenetic suspensions and slide making.

Cell staining

Microscopy

HOECHST-GIEMSA STAINING FOR SCE AND REPLICATION BANDING

BASIC PROTOCOL

This technique is also known as the fluorescence plus Giemsa (FPG) technique for detection of SCE (Barch, 1991). The following protocol employs the basic technique of cell harvesting outlined in *UNIT 22.2*. BrdU is added to the culture and the cells are allowed to undergo replication. Colcemid is added to arrest the cells in metaphase, and the cells are harvested to make cytogenetic suspensions. Metaphase slides are prepared as described previously (*UNIT 22.2*), then stained with Giemsa.

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Contributed by Jane Bayani and Jeremy A. Squire
Current Protocols in Cell Biology (2004) 22.7.1-22.7.4

Materials

Subconfluent cell culture
5′-bromodeoxyuridine (BrdU; Sigma): follow manufacturer's instructions and keep away from direct light
10 μg/ml Colcemid (Invitrogen) in H_2O
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Hoechst 33258 working solution (see recipe)
4% (v/v) Giemsa staining solution
2× SSC (see *UNIT 18.6* for 20×)
15-ml conical centrifuge tubes
Coplin jars
Large petri dish
UV light source, e.g., the UV light in a tissue culture hood
Additional reagents and equipment for harvesting cells and preparing metaphase spreads (*UNIT 22.2*)

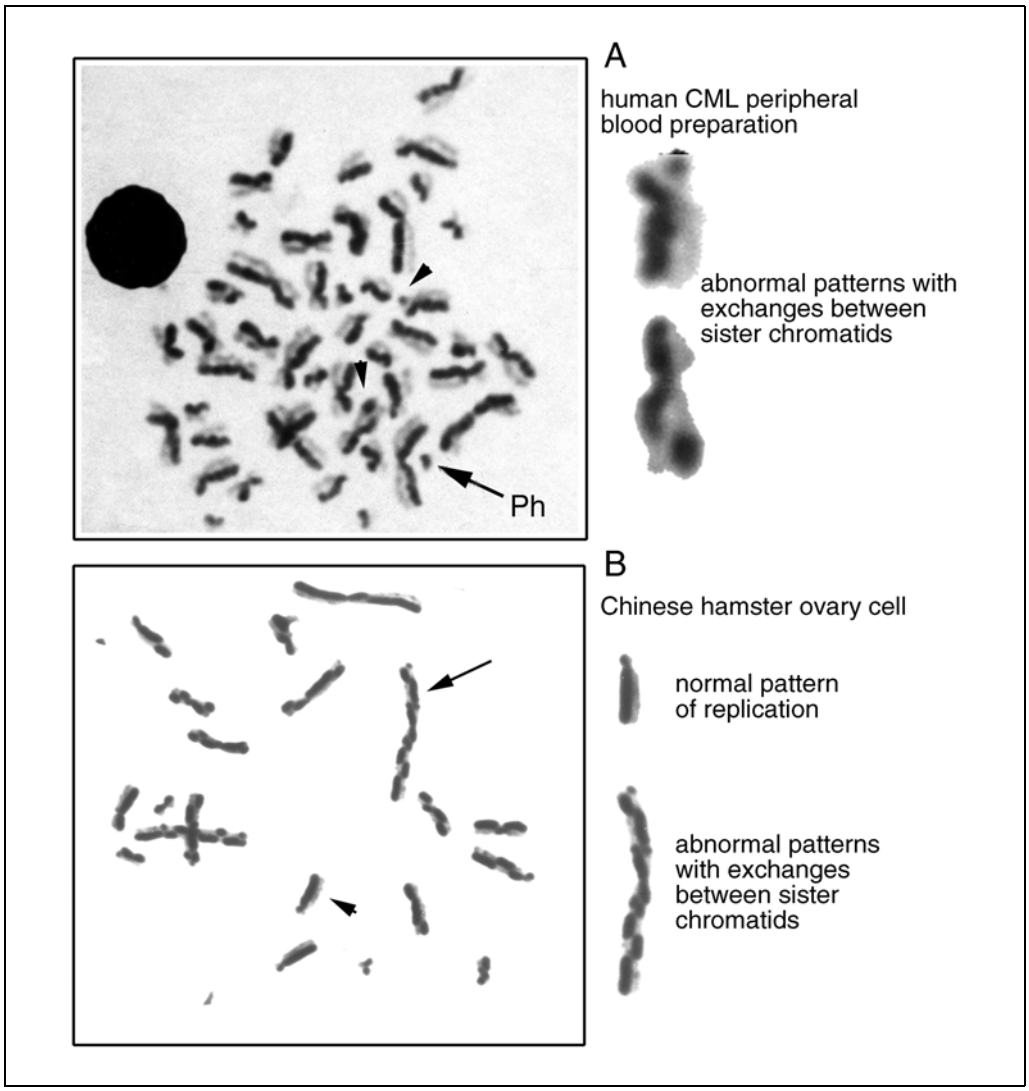


Figure 22.7.1 Sister chromatid exchange in a human CML spread and Chinese hamster ovary cells. Examples of sister chromatid exchange in a human CML spread (**A**) and in Chinese hamster ovary cells (**B**). Images kindly provided by Turid Knutsen, Hesed Padilla-Nash, and Thomas Ried, Genetics Branch, National Institute of Health, Bethesda, Maryland. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.interscience.wiley.com/c_p/colorfigures.htm.*

1. To a subconfluent cell culture, add BrdU to a final concentration of 5 μg/ml. Keep the cultures in the dark by wrapping flasks in foil. Return the flask to the incubator for 24 to 48 hr. Allow the cells to recover.

   Depending on the nature of the experiment, a period of cell recovery in medium without BrdU may be required. This period may range from one to several hours.

2. Add sufficient 10 μg/ml Colcemid stock to the culture to give a final concentration of 0.1 μg/ml. Return the flask to the incubator and continue incubating 1 hr.

3. Harvest the cells and prepare cytogenetic suspensions. Make metaphase spreads on clean glass slides (all techniques described in *UNIT 22.2*).

4. Incubate slides in Coplin jars containing PBS for 5 min at room temperature.

5. Incubate slides in Hoechst 33258 working solution for 5 min at room temperature, then rinse briefly in PBS.

6. In a large uncovered petri dish, place the slides face up in 2× SSC and expose to UV light for 10 min, then rinse in fresh 2× SSC.

7. Stain slides by immersing them in a Coplin jar containing 4% Giemsa stain for 3 to 5 min at room temperature.

8. Rinse slides in water, then visualize by light microscopy.

   The sister chromatid with BrdU incorporation will shown decreased staining with Giemsa.

9. Score the metaphases for sister chromatid exchange (see Fig. 22.7.1).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Giemsa stain, 4% (v/v)

2 ml Giemsa stain (Azure Blend, Harleco 620G/75, from EM Science; 2% v/v final)
48 ml Gurr's buffer solution, pH 6.8: dissolve 1 Gurr's buffer table (Bio/medical Specialties) in 1 liter sterile H_2O
Store up to 2 months at room temperature

Hoechst 33258 working solution

Stock solution (75 μg/ml): Dissolve 7.5 mg Hoechst 33258 (e.g., Molecular Probes) in 100 ml water. Prepare fresh each time or store at room temperature up to 1 month. Protect from direct light.
Working solution: Combine 50.0 ml Hoechst stock solution with 25.0 ml PBS (*APPENDIX 2A*). Prepare fresh.

COMMENTARY

Background Information

Sister chromatid exchange (SCE) refers to the interchange of DNA between replication products. The mechanism of chromatid exchange is still not fully understood; however what occurs is the interchange of DNA at the same location in each chromatid. This requires fidelity in DNA repair. SCEs were first observed by Taylor et al. (1957), using autoradiography on cells that had undergone one cycle of tritiated thymidine incorporation followed by a replication cycle in non-radioactive medium (also see Barch, 1991). BrdU is now used in place of radiolabeled thymidine. SCEs have typically been studied for the detection of DNA damage (Gebhart et al., 1981). Exposure of cells to carcinogens, mutagens, or ionizing radiation has

been known to induce chromosomal aberrations. SCE has been used to diagnose inherited diseases such as Bloom's syndrome, Fanconi's anemia, and ataxia-telangiectasia (Ellis et al., 1999).

Critical Parameters

The use of BrdU is a quicker and much easier method than the use of radioactive thymidine for labeling newly synthesized DNA. BrdU is incorporated into DNA in the place of thymidine, but the BrdU concentration should be closely monitored because subtoxic levels may induce exchanges. To avoid these issues, it is necessary to have the appropriate controls. These controls will include establishing thresholds of toxicity by adjusting either the amount of BrdU or the time of BrdU exposure. Furthermore, cells treated with BrdU are light-sensitive; thus the cultures should be maintained in the dark.

BrdU incorporation complements the use of Hoechst 33258, since the Hoechst dye will fluoresce less efficiently when bound to poly (dA-dBrdU) than when bound to poly (dA-dT). Detecting exchanges relies on the semiconservative nature of DNA synthesis. 5′-bromodeoxyuridine (BrdU) is incorporated into the newly synthesized DNA. Differential staining with Hoechst dye and Giemsa allows the newly synthesized DNA within a chromatid to be recognized, since BrdU incorporation in only one DNA stand leads to much weaker staining than in the strand without any incorporation. Thus, SCEs represent a point of DNA template exchange during strand synthesis, visualized as asymmetric chromatid staining or "harlequin" chromosomes (see Fig. 22.7.1).

The modification of the basic SCE assay described in this unit employs Giemsa staining. The FPG technique is based on the theory that light energy absorbed, but not emitted, by the fluorescent dyes can promote selective degradation of BrdU-substituted DNA.

As with any assay involving chromosomes, the success of this assay requires mitotically active cells. The resulting cytogenetic suspension should have a high mitotic activity and good chromosome length, such that the exchanges can be easily identified. Refer to *UNIT 22.2* for culturing and harvesting conditions.

Troubleshooting

The detection of exchanges requires that the investigator be able to see subtle differences in staining intensities between the newly replicated strands. If the staining is too strong, it will obscure the subtle differences; in such cases, one should reduce the stain time or stain concentration. The investigator should also consider whether the concentration of BrdU was sufficient to label the new DNA. As mentioned above, caution should be taken when establishing BrdU quantities, since it can induce exchanges.

Anticipated Results

If sister chromatid exchanges are present they should be detectable at 40 × magnification. Exchanges should be reciprocal. The anticipated results will depend on the nature of the experiment. The frequency of exchanges can be determined and can be used in a correlative fashion (e.g., varying exposure to different drugs or to radiation).

Time Considerations

A SCE experiment should take 2 to 3 days depending on the duration of culture in BrdU.

Acknowledgments

The authors wish to acknowledge the assistance of Paula Marrano and Jana Karaskova, Ontario Cancer Institute, and Thomas Ried, Turid Knutsen, and Hesed Padilla-Nash, National Institutes of Health, Bethesda, Maryland.

Literature Cited

Barch, M.J. 1991. The ACT Cytogenetics Laboratory Manual, 2nd Edition. Raven Press, New York.

Ellis, N.A., Proytcheva, M., Sanz, M.M., Ye, T.Z., and German, J. 1999. Transfection of BLM into cultured Bloom syndrome cells reduces the sister-chromatid exchange rate toward normal. *Am. J. Hum. Genet.* 65:1368-1374.

Gebhart, E. 1981. Sister chromatid exchange (SCE) and structural chromosome aberration in mutagenicity testing. *Hum. Genet.* 58:235-254.

Taylor, J.H., Woods, P.S., and Hughes, W.L. 1957. The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labeled thymidine. *Proc. Natl. Acad. Sci. U.S.A.* 43:122-128.

Contributed by Jane Bayani and Jeremy A. Squire
Princess Margaret Hospital and The Ontario Cancer Institute
Toronto, Ontario, Canada

Detection of Mitotic Figures and Components of the Mitotic Machinery

BASIC PROTOCOL

This unit describes the detection of proteins associated with the mitotic machinery. Mitotic figures can easily be identified by the condensation of chromatin into chromosomes as the cells undergo cell division. Staining of DNA and of the proteins associated with mitosis (e.g., tubulins and centromere-associated binding proteins) help to identify mitotic figures. Many different appropriate primary antibodies are available for this purpose, and they can be used with secondary antibodies conjugated to fluorochromes (indirect immunofluorescence, *UNIT 4.3*). Each assay requires a series of antibody incubations with intervening washes. The investigator should refer to the current literature to determine whether an antibody is available for the protein complex under study. The suppliers of each antibody will provide guidelines regarding optimal blocking and concentrations for the fixative that have been used. In the following protocol, γ-tubulin is used as a model. Table 22.8.1 outlines the other antibodies that can be used to detect mitotic figures and components of the mitotic machinery.

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Cells growing on coated glass slides, coverslips, or chambered tissue culture slides (*UNIT 22.2*), 75% confluent
Phosphate-buffered saline (PBS; *APPENDIX 2A*), room temperature and cold
4% (w/v) paraformaldehyde in PBS (see recipe), cold
70% and 100% ethanol, cold
Permeabilizing solution (see recipe)
Blocking solution (see recipe)
Primary antibody: mouse anti γ-tubulin antibody (Sigma) at working concentration recommended by manufacturer
0.1% (v/v) NP-40 in PBS (prepare fresh)
Secondary antibody: rhodamine-conjugated anti-mouse IgG antibody (Sigma) at working concentration recommended by manufacturer
DAPI in antifade (see recipe in *UNIT 22.4* or purchase from Vector Laboratories)
Nail polish
Petri dishes
Coplin jars
37°C dry incubator or oven
Hybridization box: e.g., black videocassette box containing slightly dampened moist paper towel or gauze
Fluorescence microscope and appropriate filters (*UNIT 4.2* and *APPENDIX 1E*)

Grow and fix cells

1. Grow cells to 75% confluency on coated glass slides, coverslips, or chambered tissue culture slides (*UNIT 22.2*).

   If cells were grown on chambered slides, the chambers may be removed. If cells were grown on coverslips, petri dishes may be used for the incubations. Be sure to keep track of which side of the slide or coverslip the cells are were grown on.

Contributed by Jane Bayani and Jeremy A. Squire
Current Protocols in Cell Biology (2004) 22.8.1-22.8.6

Table 22.8.1 Components of the Mitotic Machinery Commonly Used for Analysis

Antibody	Structure(s) detected	Supplier(s)[a]
Aurora	Centrosomes	IHC World, Bethyl Laboratories, Abcam
CENPA	Kineticore, centromere	Abcam
CENPB	Kineticore, centromere	Bethyl Laboratories
Centrin	Centrosomes, spindle apparatus	Sigma
α-Tubulin	Centrosomes, spindle apparatus	Sigma
β-Tubulin	Centrosomes, spindle apparatus	Sigma
γ-Tubulin	Centrosomes, spindle apparatus	Sigma

[a]See Internet Resources.

2. Remove the medium and wash once with cold PBS, either by floating coverslips in a petri dish or by using a Coplin jar for slides.
3. Fix the cells using cold 4% paraformaldehyde in PBS for 10 min, either by floating coverslips in a petri dish or by using a Coplin jar for slides. Wash the cells with 1× PBS for 2 min using the same technique.
4. Fix with 100% ethanol for 10 min. Store slide in 70% ethanol at 4°C, or process immediately as in the following steps.
5. Hydrate the cells in PBS for 5 min at room temperature.

Prepare cells for staining

6. Permeabilize cells by immersing in permeabilizing solution for 15 min at room temperature.
7. Drain solution, but do not allow the slide or coverslip to dry. Apply blocking solution to the area with the cells. Place coverslip over this area and incubate at least 30 min at 37°C in a hybridization box.

Treat with primary antibody

8. Remove coverslip. Drain blocking solution, but do not allow slide or coverslip to dry. Add primary antibody to the area with the cells. Place coverslip over this area and incubate at least 1 hr 37°C.
9. Remove coverslip. Wash three times with 0.1% NP-40 in PBS, each time for 5 min at room temperature, either by floating coverslips in a petri dish or by using a Coplin jar for slides.
10. Drain solution, but do not allow the slide to dry. Apply blocking solution to the area with the cells. Place coverslip over this area and incubate at least 30 min at 37°C.

Treat with fluorochrome-conjugated secondary antibody

11. Remove coverslip. Drain blocking solution, but do not allow slide or coverslip to dry. Add rhodamine-conjugated secondary antibody to the area with the cells. Place coverslip over this area and incubate at least 1 hr 37°C.
12. Remove coverslip. Wash three times with 0.1% NP-40 in PBS, each time for 5 min at room temperature, using the washing technique described in the previous steps. Briefly rinse with PBS at room temperature.

Mount and visualize slides

13. Drain excess liquid but do not allow cells to dry. If cells were grown on coverslips, add 50 μl of DAPI in antifade solution (counterstain) to a clean glass slide and place the coverslip over it cell-side-down. If cells were grown on a slide, add the 50 μl of DAPI in antifade to the area on the slide with the cells, then apply the coverslip. Seal the coverslip to the slide using nail polish.

14. Visualize the slide preparation under a fluorescence microscope (*UNIT 4.2*). When slides are not in use, store at −20°C.

See Figure 22.8.1 for examples of mitotic figures.

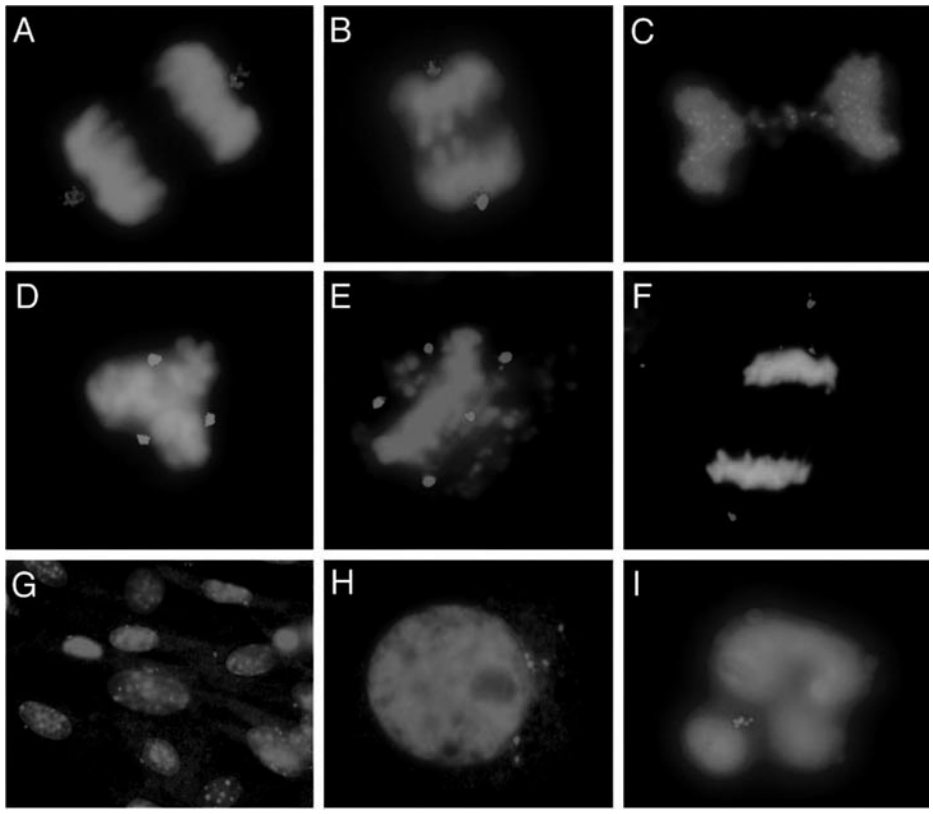


Figure 22.8.1 Examples of aberrant mitotic figures. Shown are examples of mitotic cells stained with γ-tubulin to detect centrosomes (red) and the spindle apparatus. Counterstaining with DAPI (blue) was used to visualize DNA. (**A**) and (**B**) show cells undergoing mitosis, where duplicated chromosomes are being pulled from the metaphase plate to their respective poles. (**C**) In this image, the cells were stained with antibodies against centromere/kinetichore proteins and illustrate the formation of an anaphase bridge. Abnormal mitotic figures are shown in (**D**), (**E**) and (**F**), where centrosome amplification has occurred, resulting in multipolar mitotic figures (D and E) and functional centrosomes. In (F), centrosome amplification has occurred, but the extra centrosome is apparently nonfunctional, and the cell maintains normal division. Amplification of centrosomes are also seen in interphase cells. (**G**) Mouse metastatic osteosarcoma cells derived from the lung show amplified centrosomes. (**H**) Amplified centrosomes are present in a clear cell ovarian cancer cell. (**I**) A multi-nucleated ovarian cancer cell shows amplified centrosomes. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.interscience.wiley.com/c_p/colorfigures.htm.*

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking solution

3 g bovine serum albumin (BSA; 3% w/v final)
100 ml PBS (*APPENDIX 2A*)
Warm slightly to dissolve if necessary
Store up to 6 months at 4°C in small aliguots or up to 1 year at −20°C

Paraformaldehyde in PBS, 4% (v/v)

10.0 ml 16% paraformaldehyde (e.g., Electron Microscopy Sciences; 4% v/v final)
30.0 ml PBS (*APPENDIX 2A*)
Wrap bottle to protect from light
Store up to 1 month at 4°C

Permeabilizing solution

0.5 ml NP-40 (0.5% v/v final)
99.5 ml PBS (*APPENDIX 2A*)
Store at room temperature

COMMENTARY

Background Information

Chromosomal abnormalities are closely linked to errors in the mitotic machinery. Chromosomal instability (CIN) is thought to arise as a result of aberrations in mitotic checkpoints (Cahill et al., 1998) and pathways responsible for maintaining genomic integrity (Difilippantonio et al., 2000). There is controversy over whether aberrations in mitotic checkpoints are a prerequisite for chromosomal changes. Tetraploidy is a common feature in many carcinomas and sarcomas; it is believed to be an initial stage in the development of aneuploidy arising either through disruption of chromosome segregation during mitosis and/or through failure of cytokinesis (Andreassen et al., 2001). The failure to arrest in G1 following tetraploidization may be exacerbated by the abnormal function of genes including *p53*, *BRCA1/2*, *Bub1*, *BubR1*, and *Mad2* (Skoufias et al., 2001), such that the cell may not be able to stably maintain the arrest of the tetraploid in G1. When tetraploids fail to arrest in G1, the cell then re-enters G1 with a tetraploid content and double the number of centrosomes. Centrosomes maintain genomic stability through the establishment of bipolar spindles during cell division, ensuring equal segregation of replicated chromosomes into two daughter cells (Nigg, 2002). Centrosome duplication normally occurs during S phase through a cdk2-dependent mechanism and is under a system of constraint that ensures there is only one duplication event during interphase (Doxsey et al., 2001; Nigg, 2002; Pihan et al., 2003). As a result of fidelity in duplication, a nontransformed cell has two centrosomes at mitosis, which dictate the formation of two spindle poles. If more than one duplication event occurs in interphase, a multipolar spindle could result, in which case the genome would segregate in an aneuploid manner. The value of these experiments is in the identification of cell populations that possess characteristics of aberrant cell cycle function. They have been observed in breast carcinomas (Pihan et al., 2003), colorectal cancer (Ghadimi et al., 2000), prostate cancer (Pihan et al., 2003), and osteosarcomas (Al-Romaih et al., 2003).

Critical Parameters

Cell growth and division are the most critical factors when investigating errors in the mitotic machinery. Cells should be monitored daily such that contact inhibition does not occur. Careful preparation of fixatives such as the 4% paraformaldehyde should be monitored. The reagents are best used when freshly prepared. If the cells are not used immediately, they may be stored at 4°C in 70% ethanol. If the slides are kept in a petri dish under ethanol, be sure that the lid is well sealed to prevent evaporation. The slides may also be stored in plastic or glass Coplin jars. The alcohol will evaporate over time, leaving a higher water content, so it is strongly suggested that the fixed cells

be checked every few days to be sure there is enough ethanol.

When assessing the extent of mitotic errors, it is essential to select well matched normal, positive, and negative control cells. Subtle perturbations in the mitotic machinery may have significant implications for processes such as chromosomal instability.

Troubleshooting

The most common problem with immunostaining assays is the possibility of high background from the fluorochrome. If a high background is present and the signals are visible, it is possible to rewash the slide in the detergent solution. Increasing the percentage to 0.2% NP-40 may also help to remove background signals. If this does not decrease the background signal, the experiment should be repeated paying close attention to the following points.

1. Make sure the antibody used has high specificity for the protein of interest.

2. Ensure the cells are adequately fixed in the paraformaldehyde solution. Increase the fixation time by 5 min.

3. Ensure that the BSA powder used to make the blocking solution is adequately dissolved. A high percentage of BSA is used for this assay (3%), but some protocols use higher BSA content (4% to 6%). The solution should be stored at 4°C to discourage any unwanted microbial growth. The solution may be warmed in a water bath or incubator to make sure the BSA does not precipitate out.

4. Adjust antibody concentrations. Check the manufacturer's suggested working concentration. Be sure that the antibodies are thoroughly mixed. If the antibody concentration is suspected as being the culprit for background, decrease the concentration while maintaining the incubation time.

5. Washes using detergent should also include gentle agitation to facilitate the removal of any unbound antibody. The detergent concentration may also be increased.

Lack of signal is the next most common problem and is typically caused by the following.

1. Lack of antibody specificity or insufficient incubation time with the antibody.

2. Insufficient membrane permeation. Increase the incubation time using the permeablizing solution. The amount of detergent may also be increased.

3. Incorrect secondary antibody (conjugated to fluorochrome) concentration or incubation. The fluorochrome may also have quenched due to expired reagents.

4. Wash conditions are too stringent.

Anticipated Results

A successful experiment will yield signals specific to the structure/protein against which the antibody is directed, with low to no background.

Time Considerations

The greatest amount of time is expended in cell culture. Great care and time must be invested in determining the growth characteristics of the cells under study, so that the maximum number of cells at fixation will be in mitosis. The entire staining procedure can be accomplished during the course of a day.

Acknowledgments

The authors wish to acknowledge the assistance of Bisera Vukovic, Khaldoun Al-Romaih, and Paul C. Park of the University of Toronto.

Literature Cited

Al-Romaih, K., Bayani, J., Vorobyova, J., Karaskova, J., Park, P.C., Zielenska, M., and Squire, J.A. 2003. Chromosomal instability in osteosarcoma and its association with centrosome abnormalities. *Cancer Genet. Cytogenet.* 144:91-99.

Andreassen, P.R., Lacroix, F.B., Lohez, O.D., and Margolis, R.L. 2001. Neither p21WAF1 nor 14-3-3σ prevents G2 progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21WAF1 induces stable G1 arrest in resulting tetraploid cells. *Cancer Res.* 61:7660-7668.

Cahill, D.P., Lengauer, C., Yu, J., Riggins, G.J., Willson, J.K., Markowitz, S.D., Kinzler, K.W., and Vogelstein, B. 1998. Mutations of mitotic checkpoint genes in human cancers. *Nature* 392:300-303.

Difilippantonio, M.J., Zhu, J., Chen, H.T., Meffre, E., Nussenzweig, M.C., Max, E.E., Ried, T., and Nussenzweig, A. 2000. DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature* 404:510-514.

Doxsey, S.J. 2001. Centrosomes as command centres for cellular control. *Nat. Cell Biol.* 3:E105-E108.

Ghadimi, B.M., Sackett, D.L., Difilippantonio, M.J., Schrock, E., Neumann, T., Jauho, A., Auer, G., and Ried, T. 2000. Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations. *Genes Chromosomes Cancer* 27:183-190.

Nigg, E.A. 2002. Centrosome aberrations: Cause or consequence of cancer progression? *Nat. Rev. Cancer.* 2:815-825.

Pihan, G.A., Wallace, J., Zhou, Y., and Doxsey, S.J. 2003. Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res.* 63:1398-1404.

Skoufias, D.A., Andreassen, P.R., Lacroix, F.B., Wilson, L., and Margolis, R.L. 2001. Mammalian mad2 and bub1/bubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints. *Proc. Natl. Acad. Sci. U.S.A.* 98:4492-4497.

Internet Resources

http://www.abcam.com/

Abcam Web site.

http://bethyl.com/

Bethyl Laboratories Web site.

http://www.ihcworld.com/

IHC World Web site.

Contributed by Jane Bayani and Jeremy A. Squire
Princess Margaret Hospital and The Ontario Cancer Institute
Toronto, Ontario, Canada

Assembly and Micromanipulation of *Xenopus* In Vitro–Assembled Mitotic Chromosomes

During mitosis, chromatin undergoes dramatic changes resulting in the assembly of mitotic chromosomes. The mitotic chromosomes of a typical vertebrate cell exhibit a very high level of DNA compaction (~10,000 times). How this high level of compaction is achieved is largely unknown. Several generations of biologists have been involved in studying the structure of mitotic chromosomes, but these studies have not resulted in a generally accepted model for the structural organization of these objects. Numerous structures, including loop domains attached to a central protein scaffold (Paulson and Laemmli, 1977), hierarchical folding of chromatin (Manuelidis, 1990), or spaghetti-like disorder (DuPraw, 1966) were proposed. These models were derived mainly from microscopy studies. The use of microscopy techniques suffers, however, from several limitations. For example, the resolution of light microscopy is within the range of a few hundred nanometers and cannot be used for detailed characterization of the mitotic chromosome organization, while electron microscopy uses harsh treatment that may destroy chromosome structure.

Recently, a novel approach for studying the organization of mitotic chromosomes was developed that measures the elastic response of mitotic chromosomes to mechanical stress. Elasticity reflects the interactions that hold objects together and the capacity of the objects to reorganize their structure upon external stress, and these are strongly dependent on the structural organization of the object. Knowledge of the elastic properties is essential for building a proper model of an object, and, in the case of chromosomes, allows comparison with the different chromosome structures reported in the literature.

In vitro–assembled *Xenopus* chromosomes are very suitable for elasticity measurements since they can be easily manipulated (Houchmandzadeh and Dimitrov, 1999). Hard data for these in vitro–assembled chromosomes have been obtained, indicating that the structure of these chromosomes must be formed by one or a few thin rigid elastic axes surrounded by a soft chromatin envelope (Houchmandzadeh and Dimitrov, 1999). The diameter of these elastic axes is estimated from elasticity calculations to be <20 nm (Houchmandzadeh and Dimitrov, 1999). What are the components of these axes? Are the axes organized by proteins alone or by proteins along with and DNA and/or RNA? What is the structure of the axes? How is chromatin attached to the axes? These and many other questions remain unanswered and elasticity measurements should undoubtedly help achieve deeper insight into them.

A video illustrating the experimental approach for chromosome elasticity measurements may be viewed by going to *http://www.interscience.wiley.com/c_p/videos.htm*. Briefly, the chromosome is grabbed by its two ends and stretched, and its elastic response is measured. The procedure consists of the following steps. First, the chromosomes are assembled in mitotic extract isolated from *Xenopus* eggs. Next, one end of the chromosome is aspirated and fixed to a micropipet. The same operation is repeated with another micropipet and the other end of the chromosome. One of the micropipets is very flexible and immobilized, while the other one is relatively rigid and mobile. The chromosome is the material link between these two micropipets; any action of one micropipet on the chromosome will be felt by the other micropipet via the chromosome, and, consequently, when the rigid pipet moves, it exerts tension on the chromosome. The flexible, immobile micropipet

Contributed by Sébastien Almagro and Stefan Dimitrov
Current Protocols in Cell Biology (2005) 22.9.1-22.9.23

feels the tension applied on the chromosome, and a deflection of this micropipet is induced. By measuring this deflection, one can easily calculate the force applied to the chromosome. From the force applied to the chromosome, determined as a function of its relative extension, the stretch modulus, i.e., the force necessary to elongate the chromosome to twice its initial length, is measured. The stretch modulus is an important elasticity parameter that allows modeling of the chromosome structure (Poirier et al., 2000; Almagro et al., 2003a,b).

This unit describes in detail the procedures for measuring the elastic properties of mitotic chromosomes (see Basic Protocol). Support protocols describe preparing sperm nuclei (Support Protocol 1), assembling mitotic chromosomes (Support Protocol 2), assembling and setting up the equipment for elasticity measurements (Support Protocol 3), designing, producing, and testing micropipets (Support Protocol 4), calibrating pixel size (see Support Protocol 5), measuring the spring constant of micropipets (see Support Protocol 6), and preparing the force calibration reference (see Support Protocol 7).

BASIC PROTOCOL

STRETCHING A MITOTIC *XENOPUS* CHROMOSOME

The mitotic chromosome could be viewed as a spring: it is possible to stretch it, to measure the force applied on it, and to return it reversibly to its initial state, and thereby to determine its elasticity (Poirier, et al., 2000; Almagro, et al., 2003a,b). *Xenopus* in vitro–assembled mitotic chromosomes are about 10 μm long and 0.8 μm wide. They are relatively flexible and fluctuate at room temperature (Almagro, et al., 2003a,b). Since these chromosomes are assembled in the test tube and are free in solution, their elastic characteristics can be precisely measured. Below is a detailed protocol for stretching a *Xenopus* in vitro–assembled mitotic chromosome. The protocol is not complicated, but it requires some experience.

The analysis of stretch data comprises the determination of the force applied to the chromosome as a function of its relative extension at the moment where the force is applied. The analysis consists of the following steps. First, the video record is digitized to transform it into a stack of images. Second, the positions of the two pipets are determined for all images. Finally, since the spring constant of the flexible pipet (see Support Protocol 6) and the size of the pixels (see Support Protocol 5) are known, the force applied on the chromosome and its extension are calculated as a function of time.

Materials

Xenopus chromosome suspension (see Support Protocol 2)
Stain buffer: buffer to be used in experiment (usually EB buffer; see recipe), containing 1×10^{-5} Hoechst 33258
EB buffer (see recipe)
Experimental equipment setup, isolated from vibration (also see Support Protocol 3, Fig. 22.9.1, and Fig. 22.9.2) consisting of:
- 3 to 4 reservoirs (see Support Protocol 3)
- Inverted microscope equipped for phase-contrast and fluorescence microscopy using Hoechst 33258 excitation and emission filters (see Support Protocol 3 for greater detail)
- Objectives: Olympus LCPlanFl 20× (NA = 0.40; air; phase 1) and Olympus long-working-distance LCplanFl 40× (NA = 0.60; air; phase 2)
- Micromanipulators (see Support Protocol 3 for greater detail)
- Motor-driven injector, syringe pump, nitrogen source, connectors, and other necessary hardware (see Support Protocol 3 for greater detail)
- CCD camera (see Support Protocol 3 for greater detail)
- S-VHS videocasette recorder (AG-TL-700, Panasonic) with S-VHS tape and TV monitor

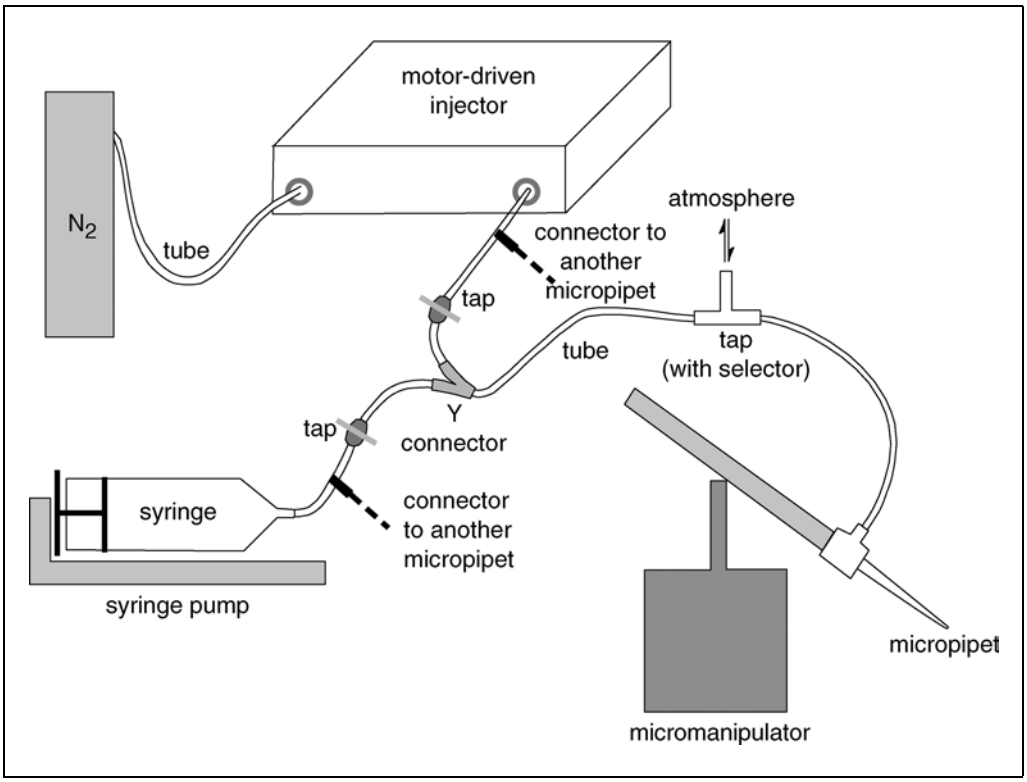


Figure 22.9.1 Schematic presentation of the pressurization/depressurization system. The pressurization system comprises an N_2 gas bottle linked to a motor-driven microinjector. A tap is present to isolate the pressurization system from the rest of the system. The depressurization system comprises a syringe mounted on a syringe pump along with another isolation tap. The two systems are linked together with a "Y" connector, and feed into a third tap (a three-way valve) that gives the user the ability either to isolate the global system from the atmosphere (under-pressure position, i.e., system closed, with the micropipet linked to the microinjector/syringe pump) or to vent it to the atmosphere (no pressure position, i.e., the system is opened to the outside and is not linked to the microinjector/syringe pump) This latter tap is the so-called pipet tap.

Computer (see Support Protocol 3 for greater detail) with PCI bus frame grabber (DT-3155, Datatranslation, Inc.)

UTHSCSA ImageTool image analysis software with plug-ins installed (see Internet Resources)

20 to 30 rigid micropipets and the same number of flexible micropipets (see Support Protocol 4)

Micropipet filling needles (MicroFil MF34G, World Precision Instruments)

10-ml syringes

24-mm × 24-mm × 170-μm glass coverslips

Additional reagents and equipment for pixel calibration (see Support Protocol 5) and measuring the spring constant of a micropipet (see Support Protocol 6)

Prepare the reservoir with the chromosomes

1. When the *Xenopus* chromosomes are completely formed (see Support Protocol 2, step 4), deposit a drop containing 10 μl of the chromosome suspension in a new reservoir. Immediately, add 7.5 μl of stain buffer and, to preserve from dust contamination, cover the reservoir with a 24-mm × 24-mm × 170-μm coverslip.

   Do not use fix/stain buffer for chromosome stretching; use stain buffer instead.

2. Wait 5 min. During this time, make sure that the 20× objective is attached to the microscope and positioned properly. Adjust the mode of the microscope to phase contrast.

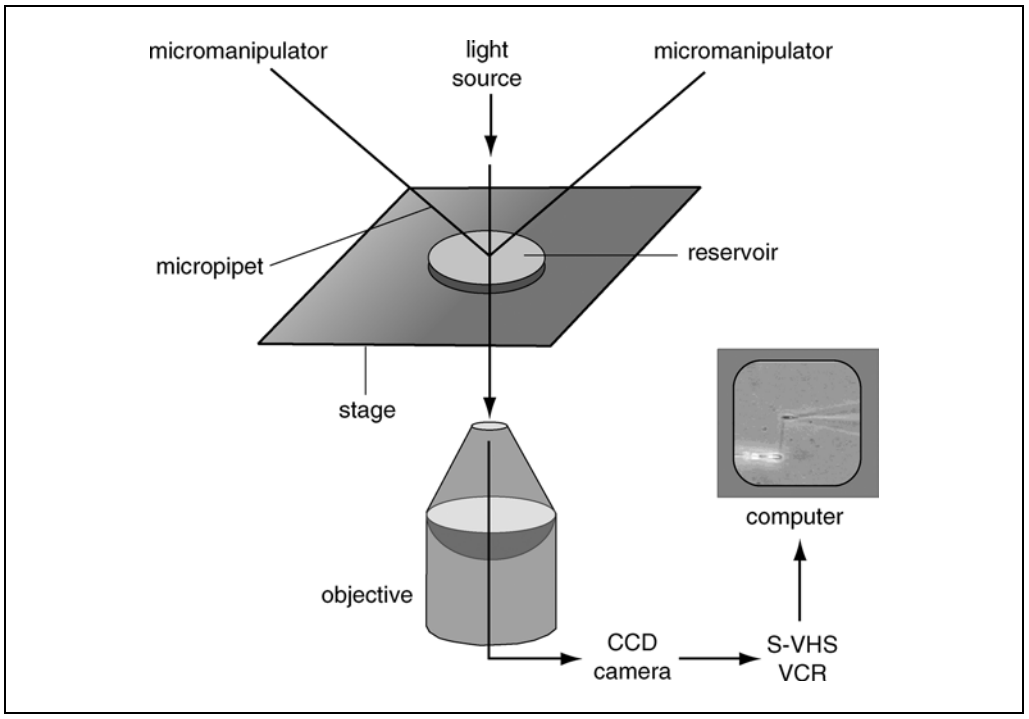


Figure 22.9.2 General scheme of the experimental set-up used for chromosome elasticity measurements. A reservoir containing the chromosome solution is placed on the motorized stage of an inverted microscope. The two micromanipulators holding the micropipets are located on each side of the microscope. The experiment is filmed with the help of a CCD camera and the video sequence is stored using an S-VHS VCR. A computer is linked to the VCR in order to analyze the results.

3. Remove the coverslip and add 300 μl of the buffer (usually EB buffer), then place the filled reservoir on the stage of the inverted microscope.

Prepare the micropipets

4. Using a micropipet-filling needle and syringe, fill either one of the two micropipets with EB buffer. Adjust the hold mode of the motor-driven injector on 10 psi and the clear mode on 25 psi (time = 10 sec). Mount the micropipet quickly on its respective micromanipulator, and activate the superpressure (hold mode) inside the pipet.

   Until the micropipet is out of the reservoir, it must be manipulated quickly (within 1 min) in order to prevent dust from sticking to the tip of the micropipet.

   To activate the superpressure mode, the motor-driven injector tap must be opened, the syringe tap must be closed, and the pipet tap must be in the under-pressure position (also see Fig. 22.9.1).

5. Center the micropipet quickly with the help of the 20× objective.

6. Activate the clear mode of the motor-driven injector by pressing the ”clear” button, and, within 10 sec of doing this, lower and place the micropipet in the reservoir liquid with the help of the micromanipulator. Check to make sure that the micropipet conducts fluid by applying a pressure on it and watching the end of the pipet; the particles neighboring the end of the pipet tip should move away from it.

   The tip of the micropipet must be situated approximately at at equal distances from the surface and the bottom of the reservoir. Do not touch the bottom of the reservoir.

7. Repeat steps 4 to 6 with the other micropipet on the other micromanipulator.

   If the first micropipet was the flexible one, the second will be the rigid one.

Stretch the chromosomes and record the video

8. Mount the 40× objective and change to fluorescence mode (adjust the excitation/emission filter wheel to see the chromosomes labeled with Hoechst). Find a chromosome.

   Because the chromosomes are all moving, concentrate on the ones that are at the bottom of the reservoir since they are less mobile. The atmosphere of the room agitates the surface of the reservoir and thus agitates the chromosomes that are close to the surface.

9. Change the microscope mode to phase contrast. Cut the pressure of one micropipet by turning the three-way valve to the "atmosphere" position (see Fig. 22.9.1). Lower this micropipet until it is near the chromosome, then turn the three-way valve to the "under-pressure" position.

10. With one end of the chromosome 2 to 5 μm from the tip of the micropipet, activate the syringe pump, using very low speed to create a very small suction.

11. Once a small part of one end of the chromosome has penetrated the pipet, cut off the suction by adjusting the tap to "atmosphere."

12. Repeat steps 9 to 11 with the other micropipet and the opposite end of the same chromosome such that the chromosome becomes attached between the two micropipets.

13. Coalign the tips of the micropipets on the long axis of the chromosome (see Fig. 22.9.3) without applying tension on the chromosome.

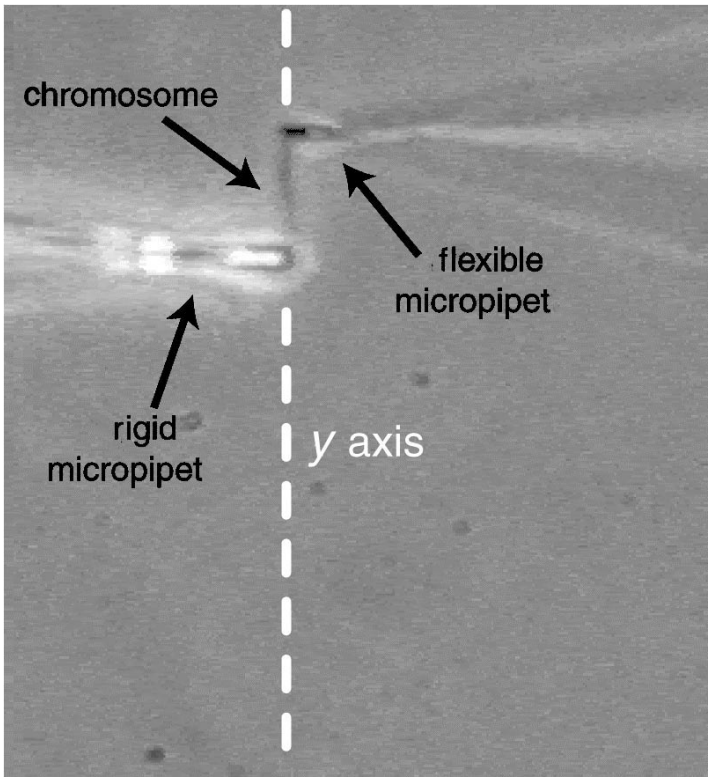


Figure 22.9.3 Positions of the two micropipets and the chromosome during a stretching experiment. The two micropipets are positioned upon on the *y*-axis, and each one has caught one end of the chromosome.

14. Program the MP–285 micromanipulator and activate the program to stretch the chromosome**.** Simultaneously, activate the CCD camera and VCR and record the experiment.

    The program for stretching consists of only two parameters: the distance at which the rigid pipet will be moved and the velocity at which the movement will take place. These parameters are entered directly in the keypad of the controller. To enter these two parameters in the program, first measure the length of the chromosome that is held between the two micropipets, then deduce the amplitude of movement to be applied to the chromosome and choose a velocity. Type the program on the keypad of the controller of the micromanipulator. For example, if the object is to stretch a chromosome of 10 μm length to twice its initial length at a velocity of 5% of its length per sec, type: "Distance = 10 μm; Velocity = 0.5 μm/sec."

    The chromosome stretching is stopped if the chromosome breaks or when the stretching program is over (indicated by a light switching off).

    Use the S–VHS VCR with S–VHS tape in PAL mode to obtain a resolution of 768 × 576 pixels. The recorded sequence will be later used for the computer analysis of the experiment.

15. Once the stretching is completed, prepare a new reservoir with some new chromosomes as in step 1 and repeat the experiment.

    The chromosomes can be used up to 8 hr after beginning the assembly reaction (see Support Protocol 2).

    Repeat the procedure a sufficient number of times to get statistically significant values (see Almagro, et al., 2003a,b for comparison values).

Digitize the video

16. Insert the videotape of the experiment in the VCR and note the time (t_0) at the beginning of the measurement and the time (t_1) corresponding to the end of the measurement. Calculate the length of the sequence (L_m) by subtracting the initial time from the final time ($L_m = t_1 - t_0$).

17. Choose a rate of images per sec (R_i). Calculate the total number of images (N_i) by multiplying the length of the sequence by the rate of image ($N_i = L_m \times R_i$).

    In most cases a collection of 100 to 2000 images is sufficient for the analysis. Acquiring large numbers of images will greatly increase the time of analysis and the amount of hard disk space consumed.

18. Open the video acquisition window in ImageTool and choose PCI frame grabber as a source. Acquire a stack of images.

    A typical picture stack is ~60 Mb and usually comprises from 100 to a few thousand images.

    ImageTool plug-ins must be installed.

19. Enter the delay between images (R_i) and the total number of images (N_i) to be taken.

20. Rewind the videotape to several seconds before t_0. Play the videotape and, when t_0 is reached, start the video acquisition process.

21. Save the image stack in a suitable image format. Store the image stack in a folder specially reserved for these images.

    Do not forget that some image formats decrease the resolution due to their lossy compression algorithms. This problem could be avoided by choosing, for example, a TIFF format without compression.

Extract the results from the images

22. With ImageTool running, open the image stack previously acquired.

23. Zoom in on the first image to enlarge it and place the mouse cursor on the extremity of each micropipet that is in contact with the chromosome (refer to Fig. 22.9.3), noting the *x*- and *y*-axis coordinates of the mouse cursor at each of these two points.

    The position of the mouse at the image appears in the bottom left part of the Image Tool window. Increase the size of the image when measuring the micropipet position.

24. Repeat the previous step for each image of the stack. Always refer to the same point on each micropipet all through the image stack.

25. Calculate the length (in pixels) of the chromosome on each image by subtracting the *y* coordinates of the two micropipets (the flexible and the rigid one).

26. Measure the deflection in pixels of the flexible micropipet relative to its initial position before stretching.

27. Calibrate pixel size (see Support Protocol 5). Convert all distances that are in pixels to meters by multiplying the value in pixels by the value obtained for the size calibration of the pixel.

28. Multiply the deflection values obtained in step 26 by the spring constant of the soft micropipet (see Support Protocol 6) in order to obtain the value of the force applied on the chromosome for each image recorded during the chromosome stretching experiment.

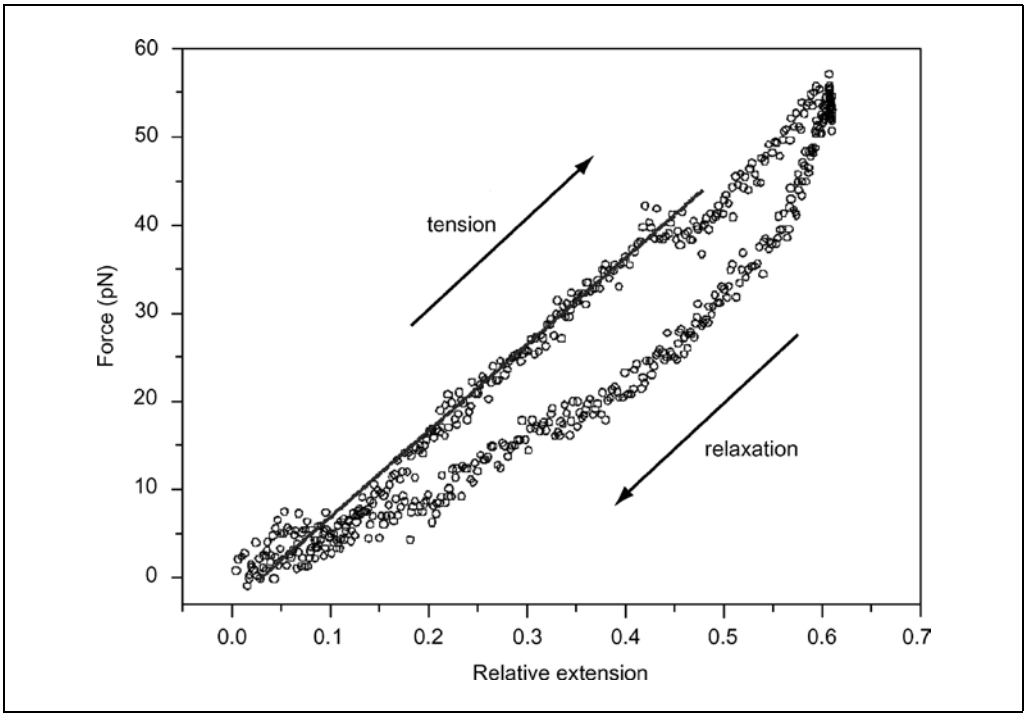


Figure 22.9.4 Graphic representation of the results of a chromosome-stretching experiment. The force applied on the chromosome during the stretching experiment is plotted as a function of its relative extension. Data points are represented as circles. The line represents the linear fit of the experimental data and its slope is the stretch modulus.

29. Calculate the relative extension (R_e) of the chromosome as $R_e = (L - L_0)/L_0$ where L_0 is the initial length of the chromosome and L the chromosome length on image number n.

    Note that the relative extension is dimensionless.

    Because the chromosome length varies from one chromosome to another, it is important to measure it each time in order to normalize the applied force.

30. Plot the measured force as a function of the relative extension [$F = f(R_e)$] (see Fig. 22.9.4, circles).

    The slope of the curve (Fig. 22.9.4, line) is the stretch modulus, i.e., the force needed to double the length of the chromosome.

    Figure 22.9.4 shows the two phases of the stretching experiment: the chromosome is first tensed by the moving the rigid pipet and then it is relaxed by returning the rigid pipet to its initial position.

    The stretch modulus may easily be calculated using a computer, but, for this, dedicated home-made software must be designed, since such software is not commercially available.

SUPPORT PROTOCOL 1

PREPARING *XENOPUS* SPERM NUCLEI

Incubation of sperm nuclei with the mitotic *Xenopus* egg extract allows the assembly of mitotic chromosomes. The use of sperm nuclei (not nuclei from somatic cells) is mandatory for the assembly of well-structured chromosomes. The assembly procedure is simple, straightforward, and reproducible. The assembled chromosomes are relatively large, and thus are easy to manipulate. They consist of one chromatid only and recapitulate the essential structural properties of somatic chromosomes (Housmandzadeh and Dimitrov, 1999; Almagro et al. 2003a,b).

In order to successfully carry out chromosome assembly, it is important to prepare sperm nuclei that are well demembranated and not aggregated. The following protocol for isolation of sperm nuclei, based on Lohka and Masui (1983), Smythe and Newport (1991), and de la Barre et al. (1999), satisfies these requirements.

Materials

Two or three male *Xenopus* frogs
Buffer T (see recipe), 4°C
Buffer S (see recipe), 4°C
Fix/stain solution (see recipe)
Buffer R (see recipe), 4°C

Instruments necessary for frog dissection: forceps, scalpel, scissors
Petri dishes
20-ml glass beaker
15-ml conical centrifuge tubes (e.g., Falcon)
Tabletop centrifuge (e.g., IEC Clinical)
Fluorescence microscope with excitation and emission filters for Hoechst 33258 fluorescent dye and oil-immersion objective (NA ~1.3) with 60×/100× magnification

Additional reagents and equipment for counting cells using a hemacytometer (*UNIT 1.1*)

1. Anesthetize two to three frogs by immersing them 10 min in a mix of ice and water.

   Better nuclear preparations are obtained when more than one animal is used.

2. Sacrifice a frog by decapitation and open its peritoneal cavity with a scalpel and scissors.

   A video showing the dissection of Xenopus is available (see Internet Resources).

3. Push the intestines carefully to one side of the frog in order to gain access to the testes.

   The testes are white, oval, hard to the touch, and ~1 cm long.

4. With scissors and forceps, remove the testes and deposit them in a petri dish containing buffer T.

5. Rinse the testes in the petri dish several times with buffer T.

6. Place the testes on a piece of absorbent paper and roll them gently several times in order to remove contaminating intestinal debris and blood.

7. Remove the fat body surrounding each testis with scissors, and place the testes in a 20-ml glass beaker containing 2 ml of buffer T.

8. With forceps, crush the testes forcefully. Place the crushed testes in a 15-ml conical centrifuge tube.

9. Centrifuge 10 to 20 sec at at 150 × *g*, room temperature, and, using a 1-ml pipet, transfer the supernatant, to a new 15-ml conical centrifuge tube.

10. Place the pellet (which now contains cell debris) back into the 20-ml beaker, add 2 ml of buffer T, and repeat step 9. Add the new supernatant to the original one in the tube from step 9.

11. Centrifuge 5 min at 1500 × *g*, room temperature, and discard the supernatant.

    The pellet consists of two parts: the upper one (white) is the sperm and the lower one (red) contains red blood cells.

12. Cut a 200-µl pipet tip so the internal diameter of the tip after cutting is ~2 or 3 mm. Using this tip carefully remove the upper part of the pellet containing the sperm and place it in an another 15-ml tube. Resuspend the sperm in 4 ml of buffer T.

    It is critical to avoid contamination of the sperm by red blood cells.

13. Repeat step 11 and step 12 once more, then repeat step 11.

14. Resuspend the sperm in 100 µl buffer T. Add 300 µl buffer S and let stand 5 min at room temperature.

15. Place the sperm suspension at 4°C and remove a 1-µl aliquot. Add 1 µl of fix/stain buffer to this aliquot and examinet with a fluorescence microscope.

    If the sperm nuclei are well demembranated, they should be uniformly labeled. If nonuniform labeling is observed, the demembranization has not gone to completion and an additional 5-min incubationof the sperm suspension at room temperature is necessary. After this additional incubation, check the sperm labeling again by fluorescence microscopy. Repeat all these manipulations until complete demembranation is achieved.

16. Block the reaction by adding 1.2 ml buffer R.

17. Centrifuge 5 min at 500 × *g*, room temperature, and resuspend the pellet in 1.5 ml of buffer R. Centrifuge again for 5 min at 500 × *g*, room temperature. Discard the supernatant and resuspend the pellet in 50 to 100 µl of buffer T.

18. Measure the sperm nuclei concentration with a hemacytometer (*UNIT 1.1*).

    A correct sperm concentration for in vitro Xenopus mitotic chromosome assembly in high-speed extract is between 0.8 to 1.2 μg/μl (about 4000 nuclei/μl). The sperm concentration is an important parameter for chromosome assembly (see Critical Parameters and Troubleshooting).

19. Divide the suspension of sperm nuclei into 5-μl aliquots and store up to several years at −80°C.

SUPPORT PROTOCOL 2

ASSEMBLING *XENOPUS* CHROMOSOMES

The assembly of mitotic chromosomes depends strongly on the quality of both the egg extract and the sperm nuclei. Different protocols for the preparation of the egg extract are available and some of them are described in this book (*UNIT 11.10*). The authors have been successfully using the the procedure of de la Barre, et al. (1999) for the isolation of a *Xenopus* mitotic egg extract.

Materials

Mitotic egg extract (de la Barre et al., 1999; *UNIT 11.10*)
EB buffer (see recipe)
10× ATP regenerating system (see recipe)

Coverslips
Fluorescence microscope with excitation and emission filters for Hoechst 33259 fluorescent dye

1. Dilute 20 μl mitotic egg extract with 20 μl of EB buffer.
2. Add 4 μl of 10× ATP regenerating system and mix gently.
3. Add 1 μl of sperm nuclei suspension to the extract, mix gently again, and incubate at a temperature between 20° and 24°C.

   Lower temperatures result in very slow chromosome assembly and higher temperatures lead to the formation of broken chromosomes.

4. Take 5-μl aliquots of the reaction mixture at different time points (e.g., 0, 10, 45, 90, 120, 150, and 180 min after sperm addition). Deposit each aliquot immediately on a coverslip and add an equal amount of fix/stain buffer. Follow the steps of chromosome assembly using a fluorescence microscope.

   Once the incubation time necessary for chromosome assembly is determined, in any further stretching experiments, simply check with a 2-μl aliquot that chromosomes are well formed at this time of incubation. If this is the case, use them directly for stretching. There is no need to make a time course before each stretching experiment. Frog chromosomes assembled in the egg extract are of different lengths and behave identically upon mechanical stress. The authors have stretched >100 chromosomes with lengths ranging from 5 to 20 μm, and no differences have been observed in their elasticity; no broken chromosomes were detected in these studies.

SUPPORT PROTOCOL 3

BUILDING THE EXPERIMENTAL SETUP FOR MEASUREMENT OF CHROMOSOME ELASTICITY

The experimental setup necessary for chromosome stretching is composed of relatively simple items; the quality of the technique used for assembling them is, however, critical. This protocol begins with design and construction of the reservoir used for single-chromosome stretching experiments and continues with a general description of the experimental setup and instructions on how to isolate it from mechanical vibrations.

Materials

50-mm × 26-mm × 170-μm glass coverslips
O-rings (rubber; internal diameter = 13 mm, external diameter = 19 mm)
Metal cylinders (height = 10 cm, diameter = 19 mm; weight = ~250 g; custom-prepared in machine shop)
Dow Corning 732 adhesive/sealant
24 × 24-mm × 170-μm glass coverslips
Table with pneumatic vibration isolators (TLC, Integrated Dynamics Engineering)
Inverted microscope (IX70, Olympus) with left-side connector tube for camera and 1.25× lens adapted to the connector tube, motorized stage (Newport, cat. no. 860–C2) and two filters for the Hoechst dye (excitation, 365 nm; emission, 465 nm)
Two micromanipulators: one (MP-285, Sutter Instrument Company) with a displacement precision of about 40 nm and a second (DC3 XYZ, World Precision Instrument) with a lower precision, and corresponding controllers
Motor–driven injector (IM–300, Narishige) connected to a N_2 gas bottle.
Syringe pump (World Precision Instruments, model no. SP100i), equipped with 50-ml syringe
Taps: two 2-way valves and one 3-way valve (available in aquarium supply stores)
Several meters (length depending on distance between components of setup) of plastic tubing (internal diameter, ~1 mm)
CCD camera (Micam VHR–2000 from Digital Vision Technologies, 512 × 512 pixels, 8 bits)
S–VHS VCR (AG–TL700, Panasonic)
Personal computer (PC), with Microsoft Windows 2000 or XP installed, sufficient RAM and hard drive space to work with stacks of pictures, and a free PCI bus
PCI bus frame grabber (DT3155 from Data Translation Inc.)
Spirit level
Strong bungee cords

Construct the reservoirs

The reservoir (see Fig. 22.9.5) must be prepared 4 to 6 hr before the experiment. It is recommended that several reservoirs be prepared because each reservoir is used only once (the O-rings may be reused, but only after incubation in 70% ethanol for several days).

1. Place a 50 × 26-mm × 170-μm glass coverslip on a clean piece of paper. Place an O-ring at the center of the coverslip.
2. Press the O-ring on the coverslip by placing the metal cylinder over it.
3. Using a thin object such as a micropipet tip or scalpel, apply Dow Corning 732 adhesive/sealant all around the O-ring in order to avoid leakage of liquid from the reservoir. Wait ≥3 hr for the glue to dry.

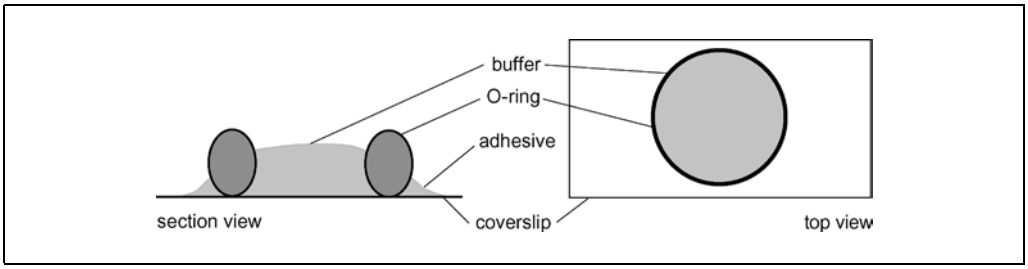


Figure 22.9.5 Diagram of the reservoir used for chromosome stretching experiments. An O-ring is attached to a coverslip with adhesive and the chromosome suspension is poured into the cavity formed by the O-ring.

The adhesive contains a solvent that can destroy the chromosomes and it is imperative to wait several hours for it to dry before addition of the chromosome suspension.

4. Remove the metal cylinder carefully and cover the reservoir with a 24 × 24-mm × 170-μm coverslip in order to prevent dust from entering in the reservoir.

   Sometimes the adhesive sticks the cylinder to the joint; if this happens, carefully remove the adhesive with a scalpel.

Set up the system and isolate it from vibrations

5. Locate a table equipped with pneumatic vibration isolators in an appropriate part of the laboratory and mount a wooden shelf ∼1 meter above the table to serve as a support for the power supply and the controllers of the micromanipulators and motor-driven injector. Drill a sufficient number of holes in the shelf to allow passage of all cables.

   The shelf will thus hold components of the system that do not need to be on the vibration-isolated table itself, thereby avoiding overloading of the table while still keeping the peripheral components close to the operator. Overloading of the table may lead to experimental problems.

6. Assemble the microscope, micromanipulators, motor-driven injector, and syringe pump on the pneumatic table, connecting these items with the appropriate taps and lengths of plastic tubing (see Fig. 22.9.1). Place a nitrogen tank with attached pressure reducer on the floor next to the table in proximity to the motor-driven injector (making sure to observe the safety precautions detailed below) and attach it to the corresponding port of the injector using plastic tubing. Connect the micromanipulators and motor-driven injector to their respective controllers on the wooden shelf with the appropriate cables according to the manufacturer's instructions. Situate the VCR and the computer on a separate table or bench near the rest of the apparatus.

   CAUTION: *Secure the nitrogen tank to avoid having it fall over. Bear in mind that pressurized nitrogen can be dangerous. Contact a qualified person to mount the pressure reducer, secure the bottle, ascertain that room ventilation is adequate, and inform laboratory personnel of the safety rules when using pressurized gas. Close the bottle when the experiment is finished; excessive nitrogen concentration in air can lead to asphyxiation.*

   The experimental setup (see Fig. 22.9.1) is very similar to the one used by Houchmandzadeh and Dimitrov (1999). The stage of the inverted microscope is motorized. The reservoir containing the chromosome suspension is placed on the stage with two micromanipulators positioned one on each side of the reservoir. A micropipet (Support Protocol 4) is attached to each one of the micromanipulators. The micropipet is connected with capillary tubes to a positive pressure system (a motor-driven injector), and a negative pressure system (syringe pump).

   The image obtained by the inverted microscope is redirected to the CCD camera (bottom of Fig. 22.9.2) attached to the microscope's left side port and recorded on the S-VHS VCR. A 1.25× lens adaptor is placed between the CCD camera and the left side port in order to obtain an optimal image size with the camera. The recorded sequences are then digitized using a PCI frame grabber.

7. Regulate the air pressure in each of the four pneumatic elements of the table such that it is high enough to raise the table and low enough to cut the vibrations.

   Vibrations are ubiquitous; the building where the experiment will be conducted vibrates continuously even if no one feels it, and the air in the laboratory continuously moves along with the hands of the laboratory personnel. These vibrations may lead to numerous problems and it is very important to isolate the microscope from them.

   With the model of pneumatic table recommended above, a distance of 5 mm between the base of the pneumatic element and the table seems to be the best compromise.

8. Test the isolation by mounting a micropipet with the smallest spring constant that will be used on the micromanipulator, then jumping on the floor near the table. If the micropipet has an amplitude of vibration >5 μm, reduce the pressure in the pneumatic elements. Acertain that the table is horizontal using a spirit level.

   The table must be placed as horizontally as possible (i.e., the slope of the table must not exceed 0.1%).

9. Firmly fix all the cables to the table with strong bungee cords.

   It is recommended that the frame of the table be fixed to the floor.

DESIGNING THE MICROPIPETS

SUPPORT PROTOCOL 4

Micropipets are the key elements of the experiment; they are used to catch the chromosome and to measure the force applied to it. Preparation of the micropipets is a delicate operation and requires both time and patience.

The micropipets must have a defined tip diameter, an ability to conduct fluids, and a specific spring constant falling within the range of forces used in the chromosome stretching experiments. Particular attention has to be paid to avoiding dust contamination during the preparation of the pipets and the manipulations (a 1-μm particle of dust can block the ability to conduct fluid). The micropipets and the capillaries are manipulated only with gloves.

Materials

Hellmanex II detergent (Helma; store in dust-free location)
Ultrapure H_2O (conductivity ~12 MΩ), filtered with a 0.22-μm filter immediately before use
EB buffer (see recipe)
Capillaries (GC100–T10; Harvard Apparatus Ltd; internal diameter= 0.78 mm; external diameter = 1 mm)
60°C water bath
Micropipet filling needles (MicroFil MF34G, World Precision Instruments)
10-ml syringes
Micropipet puller (P–97) with through filament (FT320B), both available from Sutter Instrument Company
Experimental setup for measuring chromosome elasticity (see Support Protocol 3; also see Basic Protocol)
UTHSCSA ImageTool image analysis software with plug-ins installed (see Internet Resources)
Additional reagents and equipment for calibrating pixel size (see Support Protocol 5) and measuring the spring constant of micropipets (see Support Protocol 6)

Clean the capillaries

1. Prepare 400 ml of 4% (v/v) solution of Hellmanex II in filtered ultrapure water in a 500-ml dust-free beaker.

   Hellmanex II is a detergent that is used to clean the capillaries and to make filling easier.

2. Place 20 to 30 capillaries in the beaker, then cover with Parafilm and shake vigorously.

   Two micropipets can be prepared from a single capillary.

3. Remove the Parafilm and cover the beaker with a sheet of aluminum foil. Incubate 45 min at 60°C, then let the beaker stand on the benchtop for 15 min.

4. Rinse capillaries vigorously 10 to 15 times, each time by swirling them in a beaker containing ~400 ml filtered ultrapure water. Cover the beaker with a new sheet of aluminum foil.

5. Attach a micropipet filling needle to a 10-ml syringe. Fill the syringe with filtered ultrapure water.

6. Place an absorbent paper on the bench. Rinse the interior of each capillary with the syringe, then completely remove the water from the capillary after the rinsing by placing one of its ends on the absorbent paper.

   Be careful to prevent dust from entering the capillaries. For this reason, cover them with a sheet of aluminium foil immediately after cleaning.

7. Place the clean capillaries in a beaker covered with a sheet of aluminium foil and incubate 45 min in a 120°C dry incubator. After incubation, let the capillaries cool down for about 20 min.

   Cleaned capillaries can be stored up to 36 hr in a covered beaker.

Pull the micropipets

As explained in the introduction to this protocol, the micropipets must have several specific properties. To obtain micropipets with these properties, the micropipets must be pulled with a specially designed pulling program. Unfortunately, it is impossible to create a general program for micropipet pulling, and the investigator must determine the pulling parameters for the micropipet puller being used.

8. Check to be sure that the crystals in the air-drying system of the micropipet puller have the right color (see the instrument manual).

   If the cartridge contains Drierite, crystals must be blue. Crystals of Drierite can be regenerated by heating 2 to 3 hr at 225°C.

9. Measure the ramp value of the micropipet puller according to the manufacturer's instructions.

10. Check to be sure that the filament of the micropipet puller has good geometry (see Fig. 22.9.6 and the instrument manual). If not, readjust it with tweezers, then measure the ramp value again. If it is different from that obtained in the previous step, note the new value.

    The geometry of the filament is extremely important for the reproducibility of the micropipet preparation and the fluid conduction.

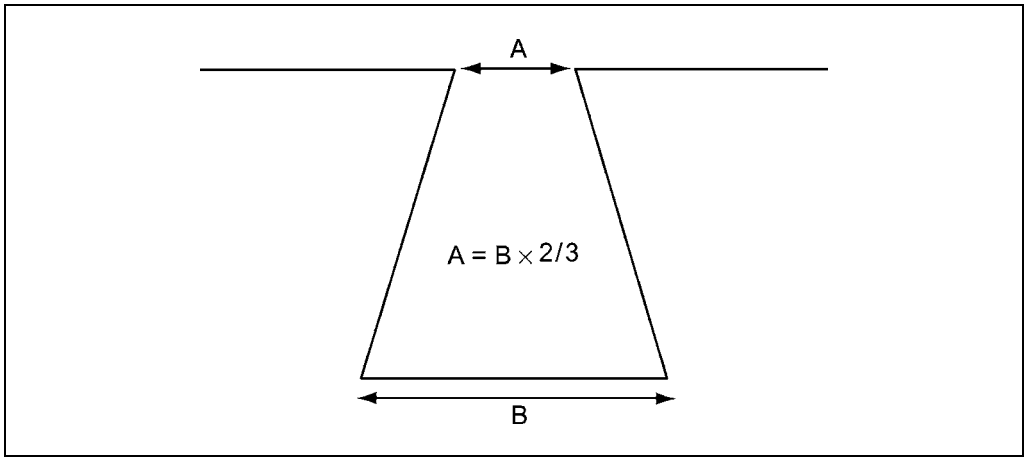


Figure 22.9.6 Correct geometry of the filament of the micropipet puller. The filament must have a trapezoidal shape with the length of the smallest base being 2/3 of the length of the largest base.

11. Check to make sure that the mounted capillary passes through the center of the micropipet puller filament. If not, center the filament, then measure the ramp value again. If it is different from that obtained in the previous step, note the new value.

12. Pull the capillary using, e.g., one of the following programs as a startup base for setting up the micropipet puller (ramp value = 225).

    To obtain a rigid micropipet with a spring constant (K) of ~2 nN/μm and an internal diameter of 1 μm:
    Pressure = 500
    Heat = 205
    Pull = 120
    Velocity = 90
    Time = 45.
    To obtain a flexible micropipet with spring constant (K) of ~100 pN/μm and an internal diameter of 1 μm:
    Pressure = 500
    Heat = 215
    Pull = 140
    Velocity = 150
    Time = 45.

    The filament is fragile and it should be touched as little as possible. The pulled micropipets must be stored in a covered container, e.g., a beaker with cover. They may only be used once.

Test the micropipets

Before conducting the real experiment, test the quality of micropipets as follows.

13. Place a reservoir on the stage of the microscope.

14. Using a syringe with a micropipet filling needle, fill a micropipet with EB buffer. Mount the filled micropipet on a micromanipulator, center it on the computer image, lower it into the reservoir, and measure its internal diameter using ImageTool software (see Support Protocol 5 for calibrating the size of the pixel.

    As explained in the Basic Protocol, two types of micropipets are used for chromosome stretching: flexible (K = 20 to 100 pN/μm) and rigid (K > 1 nN/μm). Both micropipets have internal diameters of ~1 μm and must conduct fluid.

15. Apply pressure inside the micropipet and observe if the micropipet is able to conduct fluid.

    Some 2-μm microbeads may be added to the reservoir to make it easier to visualize fluid conduction.

16. If the micropipet is able to conduct fluid, measure the spring constant of the micropipet (see Support Protocol 6).

17. If the desired spring constant has been obtained, repeat steps 14 to 16 for 10 different micropipets.

    Usually the variability in the measured micropipet spring constant value is from ~50 % to ~100 % (the older the filament, the higher the variability). If about half of the pipets exhibit the wanted spring constant, the pulling program used may be considered as appropriate.

    Note that using a micropipet puller with a laser instead of a filament reduces the variability of the prepared micropipets considerably. Such puller is, however, more expensive.

Prepare the remaining micropipets

18. Continue pulling micropipets. Each time use the same program parameters for the preparation of the micropipets

    See Critical Parameters and Troubleshooting for problems that may arise in micropipet pulling, and possible solutions.

SUPPORT PROTOCOL 5

CALIBRATING PIXEL SIZE

The following procedure is used to determine the effective size of the pixel. It is extremely important to know the size of the pixel, because without this knowledge it is impossible to analyze the results.

Additional Materials *(also see Basic Protocol and Support Protocol 3)*

Stage micrometer (graduated slide) with 100 divisions, 10 μm per division (Graticules Ltd., model PS 8)

1. Adjust the microscope at the magnification to be used for calibration.
2. Turn on the video system and the computer.
3. Place the stage micrometer on the stage and take an image of the stage micrometer.
4. Measure the number of pixels between the graduations of the stage micrometer on the image acquired with the computer (see Fig. 22.9.7), then calculate the size of the pixels in micrometers according to the following formula: number of pixels/distance = n pixels/μm.
5. Rotate the stage micrometer 90° and repeat step 4.

    It is necessary to calibrate on both the x and the y axes because some CCD cameras have different resolution on the x and y axes.

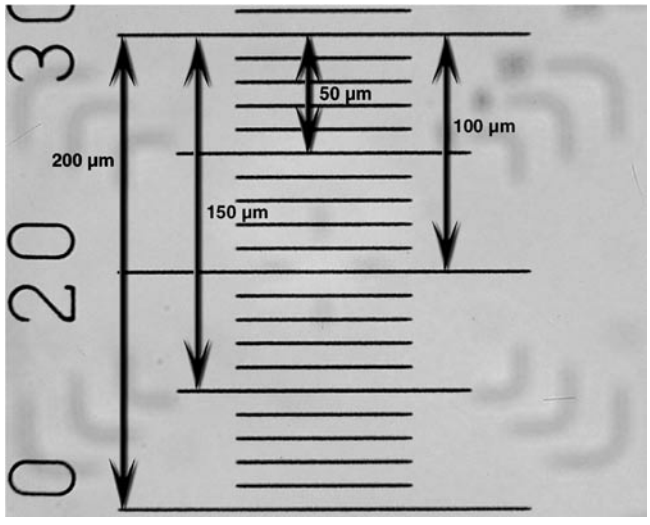


Figure 22.9.7 Size calibration of the pixel. The graduations of the stage micrometer are 10 μm apart. In order to calibrate the size of the pixel on the image it is necessary to measure the number of pixels between graduations.

MEASURING THE SPRING CONSTANT OF THE MICROPIPET

This method of pipet calibration requires that the ratio of spring constant between the two objects (the needle and the micropipet) be within a factor of 10. The protocol is illustrated in a video that may be viewed at *http://www.interscience.wiley.com/c_p/videos.htm* (a needle instead of a micropipet is used in the video).

Additional Materials *(also see Basic Protocol and Support Protocol 3)*

Experimental buffer (usually EB, see recipe) containing 50 to 100 mM NaCl
Calibrated needle (see Support Protocol 7)

1. Fill a reservoir with the experimental buffer (EB buffer, in general).

   Electrostatic interactions can sometimes provoke repulsion of the pipet from the needle; therefore, it is recommended that the buffer contain 50 to 100 mM NaCl to minimize such interactions.

2. Fix the previously calibrated needle (Support Protocol 7) on the lower-precision micromanipulator and adjust its position in order to visualize it on the computer screen.

3. Mount the micropipet on the higher-precision micromanipulator and adjust its position so that it is in the vicinity of the needle.

4. Use the micropipet to lift the needle up, moving the pipet on the *y* axis. Take a first image using ImageTool. Measure the respective *y*-axis positions (y_0) of the pipet ($y_{0\text{pipet}}$) and the needle ($y_{0\text{needle}}$) at their extremities on the image.

   Both the pipet and needle are deflected by the force they are applying to each other.

5. Quickly move the pipet along the *x* axis away from the needle without changing its *y* position, so that both the pipet and the needle reach a new position. Take another image with ImageTool. Measure the respective *y*-axis positions (y_1) of the pipet ($y_{1\text{pipet}}$) and the needle ($y_{1\text{needle}}$) at their extremities on the image (see video at *http://www.interscience.wiley.com/c_p/videos.htm*).

   Since there is no more contact between the pipet and the needle, they are no longer deflected.

6. Calculate the deflection of the pipet as $D_{\text{pipet}} = |y_{1\text{pipet}} - y_{0\text{pipet}}|$. Calculate the deflection of the needle as $D_{\text{needle}} = |y_{1\text{needle}} - y_{0\text{needle}})$.

7. Calculate the spring constant of the pipet (K_{pipet}) using the following equation.

   $K_{\text{pipet}} = (K_{\text{needle}} \times D_{\text{needle}})/D_{\text{pipet}}$

8. Repeat steps 4 to 7 several (n) times and measure $K_{1\rightarrow n}$. Determine the correct (average) value of the spring constant as:

$$K_{final} = \frac{K_1 + K_2 + \ldots + K_n}{n}$$

   Calculate the error on each measurement (E_i) as:

$$E_i = \frac{\left|K_i - K_{final}\right|}{K_{final}} \times 100$$

Calculate the final error (Err) on the spring constant as the average of all errors:

$$Err = \frac{E_1 + E_2 + \ldots + E_n}{n}$$

The error is typically within the range of 0.5% to 4%.

To calibrate micropipets with a much lower (e.g., 100×) spring constant than that of the needle, it is necessary to calibrate an intermediate micropipet. First, calibrate a micropipet against the needle. This intermediate pipet must be about 10× more flexible than the needle. Finally, use this intermediate micropipet to calibrate the pipet to be used in the stretching experiments.

SUPPORT PROTOCOL 7

PREPARATION OF THE FORCE CALIBRATION REFERENCE

The preparation of the force calibration reference is a key point of the experiment, so it has to be carried out very carefully. In this protocol, the investigator prepares a needle as the calibration reference for all the experiments, which is used to calibrate the spring constant of all the micropipets that will in turn be used to stretch the chromosomes. Therefore, an error at this point could lead to a systematic error in all force measurements.

Materials

Thin copper electric wire, 50 cm
Balance capable of precision within 1 mg
Mirror (at least 10 cm wide)
Transparent plastic ruler (graduated in millimeters)
Needle (MicroFil MF34G, World Precision Instruments)
Binocular microscope, preferably with zoom from 5× to 20×
Tweezers

1. Cut different lengths of thin copper electric wire and bend each of them into a U form.
2. Weigh them individually on high-precision balance (i.e., one capable of weighing <1 mg).

   Typically, fragments of copper wire must have a weight of 1 to 10 mg.

3. Using the mirror, needle, plastic ruler, and binocular microscope, construct the experimental setup shown in Figure 22.9.8.
4. Adjust the binocular microscope, the mirror, the needle, and the ruler to view the needle superimposed upon the ruler.

   A video illustrating the following two steps may be viewed at http://www.interscience.wiley.com/c_p/videos.htm.

5. Measure the initial position (x_0) of the needle on the ruler.
6. Using tweezers, put one copper wire fragment (with weight m_1) at the free extremity of the needle and note the new position (x_1) of the needle on the ruler. Calculate its deflection (D_1) using the equation $D_1 = |x_1 - x_0|$.
7. Repeat step 6 *n* times with at least five different copper wire fragments and calculate the deflection values.

   A reasonable value for n is when the investigator obtains an error on the calibration of <3 % with at least five independent measurements. If after 10 to 15 measurements this error range is not obtained, the investigator must take another needle and weigh all the thin copper wire filaments once again.

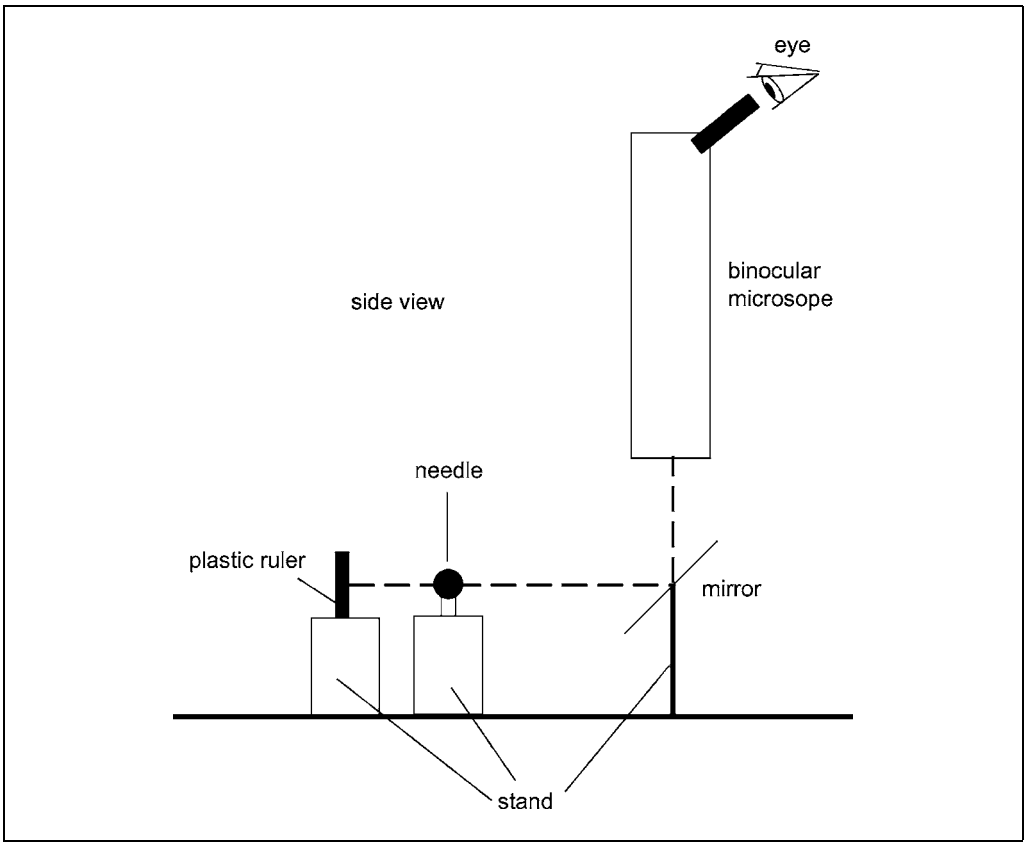


Figure 22.9.8 Schematic of the needle calibration apparatus. Three stands are aligned and a mirror, a needle, and a transparent plastic ruler are aligned on top of them. A binocular microscope is placed vertically above the mirror and adjusted to allow observation of both the needle and the plastic ruler.

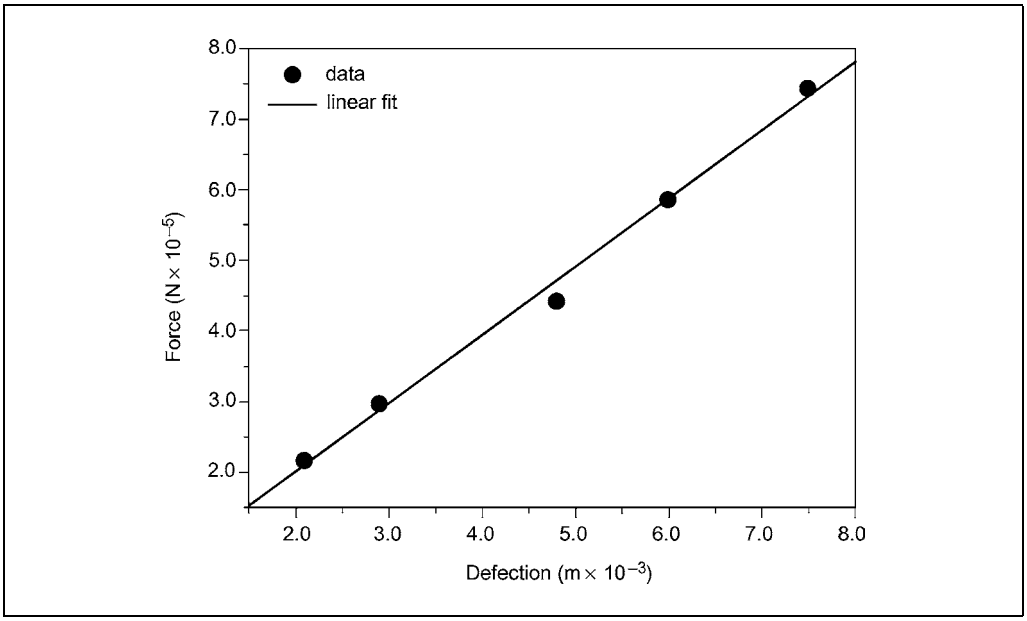


Figure 22.9.9 Graphic depiction of the spring constant of the needle. The force applied to the needle is plotted as a function of its deflection. The data points (circles) are fitted (gray line) to a line through zero. The slope of the gray line is the spring constant of the needle.

8. Calculate the force applied by the different copper wires (each one having a weight m) on the needle by the relation $F = mg$ where F is the force in newtons (N), m the weight in kg and g is the acceleration due to gravity (~ 9.8 meters/sec^2).

9. Plot the measured deflections (in meters) as a function of the force (in N) applied on the needle.

   For an example of graph, see Figure 22.9.9.

   The slope of the curve is the spring constant of the needle (K_{needle}).

10. Save the needle.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ATP regenerating system, 10×

Prepare the following 10× solutions fresh just before use:
200 mM phosphocreatine in 10 mM potassium phosphate buffer, pH 7.0 (*APPENDIX 2A*)
20 mM ATP, pH 7.0
50 μg/ml creatine kinase in 10 mM potassium HEPES, pH 7.5/50% (v/v) glycerol
Add each solution individually to reaction at final concentration of 1×

Buffer R

15 mM PIPES, pH 7.4
15 mM NaCl
7 mM $MgCl_2$
80 mM KCl
0.2 M sucrose
3% (w/v) bovine serum albumin
Prepare fresh and cool to 4°C before use

Buffer S

15 mM PIPES, pH 7.4
15 mM NaCl
7 mM $MgCl_2$
80 mM KCl
0.2 M sucrose
20 mM maltose
0.05% (w/v) lysolecithin
Prepare fresh and cool to 4°C before use

Buffer T

15 mM PIPES, pH 7.4
15 mM NaCl
7 mM $MgCl_2$
80 mM KCl
0.2 M sucrose
Prepare fresh and cool to 4°C before use

EB buffer

80 mM β-glycerophosphate (Sigma)
20 mM EGTA, pH 7.3
15 mM $MgCl_2$
1 mM DTT, pH 7.3
Store up to several months at −20°C

Fix/stain buffer

Prepare the following in filtered ultrapure H_2O:
1×10^{-5} M Hoechst 33258
200 mM sucrose
7.4% (w/v) formaldehyde
10 mM HEPES pH 7.5
Store up to 1 month at 4°C

This buffer must only be used for chromosome observation, not chromosome manipulation (because of the presence of formaldehyde).

COMMENTARY

Background Information

The first experiment where chromosomes were stretched (Nicklas, 1983) was performed using grasshopper somatic chromosomes. The goal of that experiment was to measure the force applied by the mitotic spindle on the chromosomes during anaphase. The first report on the elasticity of mitotic chromosomes assembled in *Xenopus* egg extract was published in 1999 (Houchmandzadeh and Dimitrov, 1999). In this work, an original combination of the measurements of both the bending rigidity and the longitudinal deformability of individual chromosomes was applied to the study of mitotic chromosome organization. Hard physical data were obtained indicating that the chromosome is formed by a few rigid elastic axes surrounded by a soft chromatin envelope. It was demonstrated that these axes are implicated in the maintenance of the overall chromosome structure. Recently, by coupling stretching experiments with microinjection of DNase I and proteases on an individual chromosome, Almagro et al. (2003a,b) showed that the intactness of DNA and proteins is important for the maintenance of chromosome integrity. Therefore, both the DNA and some specific proteins are involved in the organization of the chromosome axes. Almagro et al. (2003a,b) have shown that the axes exhibit a modular structure where the structural maintainance of chromosome (SMC) proteins are major players.

Critical Parameters and Troubleshooting

See Tables 22.9.1 and 22.9.2 for problems and possible solutions.

Anticipated Results

Sperm nuclei preparation

The demembranated sperm nuclei show a wormlike shape, and, if demembranation is complete, they are uniformly labeled by

Table 22.9.1 Troubleshooting Guide to Preparation of Sperm Nuclei

Problem	Possible cause	Solution
Few sperm nuclei visible when observed under the fluorescence microscope	Suspension of sperm nuclei is not concentrated enough	Repeat step 17 and step 18 to concentrate the sperm suspension
Sperm nuclei are aggregated	Sperm nuclei concentration is too high	Resuspend pellet with more buffer T during step 17
	Sperm nuclei are sensitive to repeated cycles of freezing/unfreezing	Use each aliquot only once and thaw just before use

Table 22.9.2 Troubleshooting Guide to Preparation of Micropipets

Problem	Possible cause	Solution
Micropipets are too rigid	Heat is too low	Increase the heat (be careful not to melt the filament).
	Velocity is too low	Increase the velocity (too high a value leads to bad reproducibility).
Micropipets are too flexible	Heat is too high	Decrease the heat.
	Velocity is too high	Decrease the velocity.
Micropipets are not reproducible	Heat is too high and/or Pressure is too low[a]	Adjust Heat and/or Pressure[a].
	Filament does not have the correct geometry	Reform the filament with the correct geometry (Fig. 22.9.6).
Some particles seem to be blocking the fluid in the micropipet.	Dust has penetrated into the micropipet	Be careful to not contaminate the capillaries with dust. Pull the micropipet just before use.
	Bad geometry of the filament	Reform the filament with the correct geometry (Fig. 22.9.6).

[a] See Support Protocol 3, step 12.

staining with Hoechst 33258. The expected yield per testis is the equivalent for 30 to 50 chromosome assemblies.

Chromosome assembly

If the chromosome assembly has gone to completion, the chromosomes are well separated from each other. This is a requirement for carrying out successful stretching experiments. It is difficult to quantify the chromosome concentration, but there are ~10,000 to 15,000 per μl of the chromosome assembly mix.

Chromosome elasticity

The in vitro–assembled mitotic chromosomes have a mean stretch modulus around 100 to 110 pN (standard deviation, ~60 pN and standard error, ~20 pN, see Almagro et al. 2003a,b, for details).

Time Considerations

Sperm nuclei preparation

Preparation of sperm nuclei can be completed in 1 day.

Chromosome assembly

The chromosome should be assembled in no more than 5 hr. If the assembly takes longer than that, use another mitotic extract.

Micropipet preparation

The preparation of the micropipets takes 2 days. During the first day the capillaries are cleaned and dried, and on the second day the micropipets are pulled.

Chromosome stretching experiment

The whole experiment must be completed within the same day. In the morning, start the assembly of the chromosome. During the assembly, prepare the reservoirs and verify that all parts of the apparatus work correctly. In the afternoon, stretch the chromosomes. The authors have found that chromosomes are stable for a period of 8 hr after the start of chromosome assembly.

Analyzing the results

The time required for analyzing the results of one chromosome stretching is variable. It depends on the duration of the chromosome stretching experiment, the number of images that were taken, and the availability of a homemade software that is able to automatically detect the position of the micropipets on the images. Thus, the time needed to extract the results from one chromosome stretching experiment varies from <1 hr to 1 day.

Literature Cited

Almagro, S., Riveline, D., Hirano, T., Houchmandzadeh, B., and Dimitrov, S. 2003a. The mitotic chromosome: An assembly of rigid elastic axes, organized by SMC proteins and surrounded by a soft chromatin envelope. *J. Biol. Chem.* 279:5118-5126.

Almagro, S., Dimitrov, S., Hirano, T., Vallade, M., and Riveline, D. 2003b. Individual chromosomes as viscoelastic copolymers. *Europhys. Lett.* 63:908-914.

de la Barre, A.E., Robert-Nicoud, M., and Dimitrov, S. 1999. Assembly of mitotic chromosomes in *Xenopus* egg extract. *Methods Mol. Biol.* 119:219-229

DuPraw, E.J. 1966. Evidence for a "folded-fibre" organization in human chromosomes. *Nature* 209:577-581

Earnshaw, W.C., Halligan, B., Cooke, C.A., Heck, M.M., and Liu, L.F. 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell. Biol.* 100:1706-1715

Houchmandzadeh, B. and Dimitrov, S. 1999. Elasticity measurements show the existence of thin rigid cores inside mitotic chromosomes. *J. Cell. Biol.* 145:215-223.

Lohka. M.J. and Masui, Y. 1983. Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science* 220:719-721.

Manuelidis, L. 1990. A view of interphase chromosomes. *Science* 250:1533-1540.

Paulson, J.R. and Laemmli, U.K. 1977. The structure of histone-depleted metaphase chromosomes. *Cell* 12:817-828.

Poirier, M., Eroglu, S., Chatenay, D., and Marko, J.F. 2000. Reversible and irreversible unfolding of mitotic newt chromosomes by applied force. *Mol. Biol. Cell.* 11:269-276.

Smythe, C. and Newport, J.W. 1991. Systems for the study of nuclear assembly, DNA replication, and nuclear breakdown in *Xenopus laevis* egg extracts. *Methods Cell. Biol.* 35:449-468

Internet Resources

http://www.curry.edschool.virginia.edu/go/frog/

Detailed video, audio and pictures explaining dissection of Xenopus.

http://www.ddsdx.uthscsa.edu/dig/itdesc.html

Web site where ImageTool version 3 and its plug-ins can be downloaded.

Contributed by Sébastien Almagro and Stefan Dimitrov
Institut Albert Bonniot
Grenoble, France

Replication Labeling with Halogenated Thymidine Analogs

DNA replication is essential for cell proliferation. Genetic information contained in the genome must be duplicated before each cell division (Gilbert, 2002; Goren and Cedar, 2003). Because the genome of eukaryotes consists of long, linear chromosomes, DNA replication must initiate at many positions along the length of the entire chromosome in order to complete replication in a timely fashion. DNA replication proceeds via the synchronous firing of clusters of about six replication origins that together form coordinately replicated domains of several hundred kilobases (Berezney et al., 2000). These coordinately replicated "replication domains" appear as punctuate foci when sites of DNA synthesis are pulse-labeled. Despite the fact that replication domains derive from many different chromosomal sites, the same cohort of replication foci replicate synchronously from cell cycle to cell cycle, implying a tight coordination of the temporal order of replication domains throughout S-phase (Ma et al., 1998; Dimitrova and Gilbert, 1999; Zink et al., 2004). Although the biological function of this temporal order is not understood, it has been shown that domains that replicate at particular times are localized to distinct regions of the nucleus, suggesting a relationship between replication domains and nuclear organization (McNairn and Gilbert, 2003). Mounting evidence suggests that replication domains form structural and functional units of chromosome organization (Bornfleth et al., 1999; Edelmann et al., 2001; Sadoni et al., 2004). Because replication is regulated at the level of large chromosomal domains, studies relating DNA replication to nuclear structure and function can be performed with the light microscope.

In higher eukaryotes, the molecular factors that dictate where and when replication will initiate are poorly understood. Replication origins do not appear to consist of any particular DNA sequence (Gilbert, 2001, 2004). Furthermore, replication origin activity is regulated both during development and in response to DNA damage or the metabolic state of cells (Anglana et al., 2003; Gilbert, 2005; Norio et al., 2005). Hence, studies of replication origins must be performed without any bias regarding where they may appear under different conditions. The distribution and spacing of origins throughout the genome, as well as the rate of elongation of replication forks, can reveal important information regarding the physiological state of cells without knowledge of the specific initiation sites. In addition, observing the positions of replication origins along the length of specific DNA fibers can reveal the positions of initiation sites to within a few kilobases.

In this unit, several conventional protocols to visualize replication foci in mammalian cells are described. Basic Protocol 1 describes visualizing DNA replication sites with bromodeoxyuridine (BrdU); this is the most basic procedure to visualize replication foci by identifying the sites of DNA synthesis. Newly synthesized DNA can be labeled with halogenated thymidine analogs such as BrdU. After immunostaining, replication foci containing BrdU-labeled replication forks can be observed using fluorescence microscopy. Basic Protocol 2, a combination of replication labeling and fluorescence in situ hybridization, is a typical application to examine the replication and subnuclear location of specific sequences, revealing the positional relationship between DNA synthesis and specific DNA regions. Basic Protocol 3, dual replication labeling with chloro- and iododeoxyuridine, provides an opportunity to examine the dynamics of DNA synthesis without the need for cumbersome cell-synchronization methods. This protocol distinguishes two different stages of S-phase by separating two different pulse labels with increasing chase times. Finally, Basic Protocol 4, replication labeling of DNA fibers, is

Contributed by Tomoki Yokochi and David M. Gilbert
Current Protocols in Cell Biology (2007) 22.10.1-22.10.14

a convenient method to observe replication sites on stretched DNA fibers, rather than on tangled DNA strands in the nucleus. Methods to examine the positional relationship between DNA synthesis and specific proteins or histone modifications have been described elsewhere (Wu et al., 2005b).

BASIC PROTOCOL 1

VISUALIZING DNA REPLICATION SITES WITH BROMODEOXYURIDINE (REPLICATION LABELING)

This protocol describes replication labeling. BrdU is added to culture medium to label replication sites (BrdU incorporation). Cells are treated with fixatives and detergents as necessary (fixation and permeabilization). BrdU incorporated in the genome is recognized by incubation with an appropriate antibody (immunostaining). Fluorophore-conjugated antibodies are observed by fluorescence microscopy (visualization). Anticipated images are shown in Figure 22.10.1.

Materials

Adherent tissue culture cells and appropriate medium
10 mg/ml bromodeoxyuridine (BrdU) stock in sterile tissue culture–grade water (store indefinitely at −20°C)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
2% (w/v) paraformaldehyde in PBS
0.4% (v/v) Triton X-100
1.5 N HCl
5% (w/v) BSA in PBST (0.5% v/v Tween 20 in PBS)
Primary antibody: monoclonal mouse anti-BrdU antibody (Becton Dickinson)

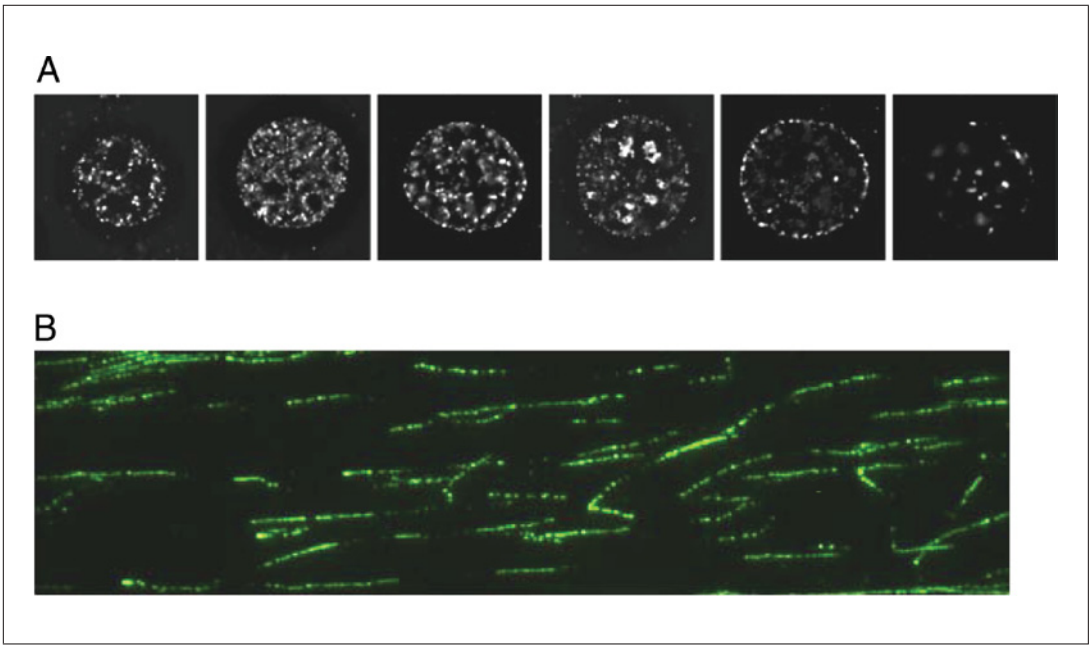


Figure 22.10.1 (**A**) Replication patterns visualized by BrdU incorporation during S-phase. Images are shown by courtesy of The American Society For Cell Biology. (Wu et al., 2005a). (**B**) DNA fibers visualized by CldU incorporation (F. Li and D.M. Gilbert, unpub. observ.). For the color version of this figure go to *http://www.currentprotocols.com*.

Secondary antibody: Alexa Fluor 495–conjugated goat anti-mouse antibody (Molecular Probes)
PBST: 0.5% (v/v) Tween 20 in PBS
1 μg/ml DAPI in Vectashield (Vector Labs) mounting medium

100-mm tissue culture dishes for plating cells on coverslips
12-mm-diameter round glass coverslips, sterilized by flaming after dipping in 70% ethanol or by autoclaving
24-well tissue culture plate
Glass microscope slides
Clear nail polish
Fluorescence microscope (*UNIT 4.2*) with appropriate color filters for fluorophores used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas Red, Alexa Fluor 594)

Additional reagents and equipment for cell culture (*UNIT 1.1*) and fluorescence microscopy (*UNIT 4.2*)

Prepare cells labeled with BrdU

1. Plate 5×10^6 adherent cells in a 100-mm tissue culture dish containing several round coverslips. Incubate overnight to allow cells to attach to coverslips.

   The authors use mammalian cells in their laboratory but believe that any tissue culture cells can be used in this protocol. The size of the dish and number of cells plated may vary depending upon the density desired. If cell density is too low, the yield in terms of cell number will be compromised; if density is too high, BrdU incorporation is decreased. Typically use 0.5–2 × 10^6 cells/ml.

   Basic cell culture techniques are described in UNIT 1.1.

2. Add sufficient 10 mg/ml BrdU stock solution for a final concentration of 10 μg/ml. Incubate the dish 30 min at 37°C.

3. Transfer the coverslips into a new container (24-well tissue culture plate recommended) containing prewarmed growth medium. Rinse the cells on the coverslip twice, each time with 1 ml PBS.

   Use of a 24-well culture dish as a container is convenient for handling many coverslips. Coverslips can be transferred to the 24-well dish one at a time prior to the BrdU labeling step to allow multiple time points with the same cell population.

4. Aspirate PBS. Add 0.5 ml/well of 2% paraformaldehyde solution and incubate for 10 min at room temperature. Wash twice with 1 ml/well PBS and remove the medium. Add 0.5 ml/well of 0.4% Triton X-100 and incubate for 10 minutes to permeabilize the cells. Wash twice with 1 ml/well PBS.

   If 3-D structure of the nucleus is not important, simply add 70% ethanol to fix the cells and incubate for 10 min at room temperature. The fixed cells on the coverslip can be stored in the 70% ethanol solution at 4°C for 1 week.

5. Aspirate PBS. Add 0.5 ml/well of 1.5 N HCl and incubate for 30 min to denature DNA.

6. Wash twice with 1 ml/well PBS and remove the medium.

7. Add 5 ml/well of 5% BSA and incubate for 10 min at room temperature. Remove the medium.

Immunostain cells

The antibody dilution factors below are those typically used in the authors' laboratory, but these should be determined empirically, and they depend on the cells and culture conditions used.

8. Dilute primary antibody (i.e., anti-BrdU mouse monoclonal antibody) 1:10 to 1:100 (depending on affinity of secondary antibody) in PBST. Add 30 µl of the diluted antibody to the cells on each coverslip. Incubate 30 min at room temperature.
9. Wash three times with 1 ml/well PBST. Aspirate PBST.
10. Dilute fluorophore-conjugated secondary antibody (i.e., Alexa Fluor 495–conjugated goat anti-mouse antibody) 1:100 in PBST. Add 30 µl of the diluted secondary antibody to the cells on each coverslip. Incubate 30 min at room temperature.
11. Wash three times with 1 ml/well PBST. Aspirate PBST.

Mount cells for imaging

12. Pipet 5 µl Vectashield containing 1 µg/ml DAPI on a glass microscope slide. Place the round coverslip with the labeled cells, cell-side-down, over the drop and seal with clear nail polish.

    Avoid air bubbles. If trapped air bubbles are a problem, Vectashield can be pipetted directly onto the coverslip first; the coverslip is then placed on the glass slide with the cell side facing down.

13. Observe the cells using fluorescence microscopy (*UNIT 4.2*) with appropriate filters for the fluorophore.

BASIC PROTOCOL 2

COMBINATION OF REPLICATION LABELING AND FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

This protocol describes how to simultaneously visualize replication sites and specific sequences in the genome by fluorescence in situ hybridization (FISH). Cells are swollen in hypotonic buffer before being fixed using methanol/acetic acid (3:1). The DNA region of interest—gene locus, promotor region, or functional domain—is visualized by in situ hybridization with a specific DNA probe, while replication domains are visualized by BrdU labeling. Up to eight glass slides can be processed in one Coplin jar using this procedure.

Materials

Adherent tissue culture cells and appropriate medium
10 mg/ml bromodeoxyuridine (BrdU) stock (store indefinitely at −20°C)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Trypsin solution (see recipe)
Fixative: 3:1 (v/v) methanol/acetic acid (prepare fresh and store on ice)
75 mM KCl
0.0625 µg/µl probe DNA sequence in TE buffer (see *APPENDIX 2A* for TE buffer)
Roche Nick Translation Mix (for digoxigenin or biotin, depending on desired labeling
0.5 mM disodium EDTA, pH 8.0
Hybridization mix (see recipe)
2× SSC (*APPENDIX 2A*) containing 0.1 mg/ml RNaseA
2× and 0.1× SSC (*APPENDIX 2A*)
70%, 90%, and 100% (v/v) ethanol
Clear nail polish
2× SSC (*APPENDIX 2A*)/70% (v/v) formamide
2× SSC (*APPENDIX 2A*)/50% (v/v) formamide
SSCT: 4× SSC (*APPENDIX 2A*)/0.1% (v/v) Tween 20
Blocking solution: SSCT (see above) containing 5% (w/v) nonfat dry milk

Primary antibody solution: cocktail in blocking solution of sheep anti-digoxigenin, FITC-conjugated (Molecular Probes) and mouse anti-BrdU (Becton Dickinson), each at a dilution of 1:10

Secondary antibody solution: cocktail in blocking solution of rabbit anti–sheep IgG, FITC-conjugated (Vector Labs), diluted 1:10, and goat anti–mouse IgG, Alexa Fluor 594–conjugated (Molecular Probes), diluted 1:500

Vectashield mounting medium (Vector Labs)

100-mm tissue culture dishes

15-ml conical centrifuge tubes

Centrifuge

Glass microscope slides

15°C water bath (kept in cold room)

45°, 65°, and 80°C water bath

Coplin jars

Vacuum desiccator with desiccant (e.g., silica gel)

18 × 18–mm square glass coverslips, sterile

Slide warmer (Precision Scientific)

Humidified chamber (e.g., covered Tupperware container with moistened paper towels on the bottom)

22 × 40–mm glass coverslips

Fluorescence microscope (*UNIT 4.2*) with appropriate color filters for fluorophores used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas Red, Alexa Fluor 594)

Additional reagents and equipment for cell culture technique including counting cells (*UNIT 1.1*) and fluorescence microscopy (*UNIT 4.2*)

Grow cells and treat with BrdU

1. Grow the cells in a 100-mm tissue culture dish to the appropriate cell density (*UNIT 1.1*).

   For overnight incubation seed 5 × 10^6 cells.

   If cell density is too low, the yield in terms of cell number will be compromised; if density is too high, BrdU incorporation is decreased. Typically, use 0.5–1 × 10^6 cells/ml.

2. Add 10 mg/ml BrdU to a final concentration of 10 μg/ml. Incubate the dish for 30 min at 37°C.

3. Rinse the cells twice with 10 ml PBS.

Permeabilize cells by hypotonic shock

4. Dissociate the cells into a single-cell suspension using 2 ml trypsin solution (also see *UNIT 1.1*), add 6 ml of serum-containing growth medium to stop the reaction, and collect them in a 15-ml conical tube. Determine cell number (*UNIT 1.1*).

5. Centrifuge cells 1 min at 200 × *g*, room temperature. Remove the supernatant. Resuspend the cells gently with 10 ml of 75 mM KCl at 1 × 10^6 cells/ml (total 1 × 10^7 cells) and incubate for 15 min at room temperature to swell the cell bodies.

   Cell concentration is critical for swelling.

   Prepare fresh fixative solution (3:1 methanol/acetic acid) during this time and keep it on ice.

Fix cells

6. Add 300 μl methanol/acetic acid fixative per 10 ml of KCl solution, dropwise, while gently vortexing the cell suspension. Leave the mixture 5 to 10 min at room temperature.

   During this incubation period, do not agitate the cell suspension, as this causes cells to aggregate.

7. Centrifuge the cells 1 min at 200 × *g*, room temperature. Remove the supernatant.
8. Add sufficient methanol/acetic acid fixative to adjust the cell density to 2×10^6 cells/ml. Incubate cells at −20°C for at least 1 hr or keep this cell suspension overnight in −20°C freezer.
9. Transfer 1×10^7 cells into a new 15-ml conical tube. Centrifuge the cells and resuspend the pellet with 5 ml methanol/acetic acid fixative.

   Note that the final cell density should be ~ 2×10^6 cells/ml.

   This step is essential to remove unnecessary salt that may interfere with clear imaging on the microscope. The cell suspension after this step can be stored at −20°C for up to 3 months.

Prepare mitotic spreads on glass slides

10. Dilute the fixed cell suspension 1/2 to 1/20 in methanol/acetic acid fixative.
11. Place or drop 10 µl of the diluted cell suspension on the glass microscope slide. Dry the specimen at room temperature for 10 min.

    At this stage, slides can be stored in the dark at room temperature indefinitely.

Prepare DNA probe labeled with digoxigenin or biotin and prepare probe mix

12. Mix 16 µl of 0.0625 µg/µl probe DNA solution (total amount 1 µg DNA) and 4 µl of Roche Nick Translation Mix (for digoxigenin or biotin, depending on desired labeling). Incubate for 90 min at 15°C in a water bath kept in a cold room.
13. Add 1 µl of 0.5 mM disodium EDTA pH 8.0, to stop the reaction. Heat the mixture at 65°C for 10 min. Combine 1 µl of this labeled probe DNA with 12 µl hybridization mix to prepare the probe mix. Mix well and denature 5 min at 95°C, then immediately place on ice and let stand 5 min.

Perform in situ hybridization

14. In Coplin jars, prewarm 40 ml 2× SSC containing 0.1 mg/ml RNase A at 37°C and 40 ml 2× SSC/70% formamide at 80°C.
15. Incubate the slides in 2× SSC containing 0.1 mg/ml RNase A in the first Coplin jar at 37°C for 30 min.
16. Transfer the slides to a second Coplin jar containing 2× SSC and wash briefly.
17. Dehydrate slides in Coplin jars containing 40 ml of each of the following ethanol solutions at room temperature for the times indicated:

    70% ethanol for 4 min
    90% ethanol for 4 min
    100% ethanol for 4 min.

18. Desiccate the slides under low pressure in a vacuum desiccator containing desiccant (e.g., silica gel) for at least 10 min.
19. Denature the slides in 40 ml 2× SSC/70% formamide at 80°C for 2 min.
20. Immediately dehydrate slides in Coplin jars containing 40 ml of each of the following ethanol solutions at room temperature for the times indicated:

    70% ethanol for 2 min
    90% ethanol for 2 min
    100% ethanol for 2 min.

21. Desiccate the slides in a vacuum desiccator for at least 10 min.

22. Warm the slides, probe mix (from step 13), and 18 × 18–mm coverslips on a slide warmer at 37°C. Place a humidified chamber in a 37°C incubator.

23. Pipet 12 μl probe mix on a prewarmed coverslip, then put it upside down on the slide. Seal with clear nail polish and place in the prewarmed humidified chamber. Incubate in 37°C incubator for 6 to 12 hr.

    Placing probe mix solutions on coverslips first before placing on the glass slides helps avoid trapping air bubbles under the coverslip.

24. Prewarm at least 120 ml of 2× SSC/50% formamide and at least 120 ml of 2× SSC at 45°C. Prewarm at least 120 ml of 0.1× SSC at 45°C to 80°C (optimal temperature determined empirically, see annotation to step 25).

25. Remove the nail polish from around the coverslip and wash the slides in Coplin jars for 5 min with 40 ml each of the washing solutions shown below:

    2× SSC/50% formamide at 45°C, three washes
    2× SSC at 45°C, three washes
    0.1× SSC at 45° to 80°C (optimized empirically), three washes.

    Every probe has unique hybridization properties. To achieve better signal-to-noise ratio, conditions for the final washing step (with 0.1× SSC) should be determined empirically. If background proves too high in a pilot experiment, use a higher temperature of 0.1 × SSC (i.e., more stringent washing conditions; 80°C); if background is low and signal is low, use a lower temperature of 0.1 × SSC (more relaxed washing conditions; 45°C).

26. Wash briefly in 40 ml SSCT in a Coplin jar at room temperature. Incubate the slides in a Coplin jar with 40 ml blocking solution at room temperature for >30 min.

Immunostain cells

27. Pipet 30 μl primary antibody solution onto the slide and cover with a 22 × 40–mm coverslip. Incubate the slide for 40 min at room temperature.

    It should be noted that the primary antibodies used here to detect the BrdU and FISH (digoxigenin) signals must be produced in different animal species in order to prevent cross-reaction of the secondary antibodies.

    Note that although the primary and secondary antibodies in this protocol are applied as a cocktail, in some cases this is not possible owing to the incompatibility of antibodies. For instance, if one of the secondary antibodies (e.g., rabbit anti–sheep IgG-FITC) were mouse anti-sheep IgG-FITC, it would not be possible to use the cocktail method because another secondary antibody (goat anti-mouse IgG-Alexa 594) could have a cross-reaction. In that case, the secondary antibodies would have to be applied sequentially, e.g., goat anti–mouse IgG–Alexa 594 first, then mouse anti–sheep IgG-FITC second.

    If biotin was used to label the probe (see step 12) only an anti-BrdU primary antibody is used, and the FISH signal is detected by fluorochrome-labeled avidin.

28. Wash the slides in Coplin jars three times, each time with 40 ml SSCT for 2 min.

29. Repeat steps 27 and 28 for the secondary antibodies.

    Signal intensity can be increased by sequential "sandwich" method if necessary (e.g., use avidin-Texas red as a first layer. Then use goat anti-avidin antibody conjugated to biotin as the second layer. Repeat this cycle to increase the signal).

Mount cells for imaging

30. Add 5 μl Vectashield mounting medium per slide and cover with 18 × 18–mm coverslip. Seal with clear nail polish and observe using a fluorescence microscope with appropriate filters for the fluorophores used (*UNIT 4.2*).

BASIC PROTOCOL 3

DUAL REPLICATION LABELING WITH CHLORO- AND IODODEOXYURIDINE

This section describes the procedure for visualizing replication sites using two halogenated thymidine analogs such as chlorodeoxyuridine (CldU) and iododeoxyuridine (IdU). It should be noted that the choice of primary antibodies is the key in this protocol. CldU is recognized by rat anti-BrdU antibody (Accurate Chemical), while IdU is recognized by mouse anti-BrdU antibody (Becton Dickinson). Anti-rat and anti-mouse IgG antibodies are used as secondary antibodies, respectively.

Materials

Cell culture on 12-mm round coverslips (Basic Protocol 1) in culture dish
10 mg/ml chlorodeoxyuridine (CldU) stock solution (store indefinitely at −20°C)
10 mg/ml iododeoxyuridine (IdU) stock solution (store indefinitely at −20°C)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
70% (v/v) ethanol
100% methanol
1.5 N HCl
PBST (0.5% v/v Tween 20 in PBS) containing 5% (w/v) BSA
PBST: 0.5% (v/v) Tween 20 in PBS
Primary antibody for CldU: rat anti-BrdU (Accurate Chemical)
Secondary antibody for CldU: FITC-conjugated goat anti-rat Ig (Molecular Probes)
Primary antibody for IdU: mouse anti-BrdU (Becton Dickinson)
Secondary antibody for IdU: Texas Red–conjugated donkey anti–mouse Ig (Jackson ImmunoResearch)
High-salt buffer (see recipe)
Vectashield mounting medium (Vector Labs)
Clear nail polish

12-mm-diameter round glass coverslips, sterilized by flaming after dipping in 70% ethanol or by autoclaving
100-mm petri dishes and 24-well plates
Humidified chamber (e.g., covered Tupperware container with moistened paper towels on the bottom)
Glass microscope slides
Fluorescence microscope (*UNIT 4.2*) with appropriate color filters for fluorophores used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas Red, Alexa Fluor 594)

Additional reagents and equipment for fluorescence microscopy (*UNIT 4.2*)

Pulse-chase-pulse label with CldU and IdU

1. Culture the cells on 12-mm round coverslips as in Basic Protocol 1.
2. Add the first halogenated thymidine analog (e.g., CldU) to the culture from 10 mg/ml stock solution for a final concentration of 10 μg/ml, to incorporate CldU in the genome. Incubate 30 min at 37°C.

   This is the first pulse step.
3. Change the medium to remove the analog and incubate the cells for the desired length of chase time.

   This is the chase step. The chase time depends on the objective of dual labeling and the properties of the cells (length of S-phase). To visualize the transition of replicating foci, the chase time can be omitted. To visualize the early and late replicating foci through the S-phase, the chase time should be 6 to 10 hr, depending on the length of the entire S-phase.

4. Add the second halogenated thymidine analog (e.g., IdU) to the culture from 10 mg/ml stock solution for a final concentration of 10 μg/ml. Incubate the dish for 30 min at 37°C.

   This is the second pulse step.

5. Rinse the cells on the coverslip twice with 10 ml PBS.

Prepare fixed cells on coverslips after double labeling

6. Immerse the coverslip in 10 ml of 70% ethanol in a 100-mm petri dish for 5 min at room temperature.

   Alternatively, transfer coverslips individually into wells of a 24-well plate at this step. In that case, use 1 ml/well of following reagents: 100% methanol (step 7), PBS (step 8), and 1.5 N HCl (step 9) per coverslip directly in the wells.

   The specimens can be stored indefinitely until use.

7. Incubate with 10 ml of 100% methanol for 5 min at room temperature.
8. Wash with 10 ml PBS twice.
9. Incubate with 10 ml 1.5 N HCl for 30 min at room temperature.
10. Wash with 10 ml PBS twice.

Stain with antibodies against CldU and IdU

Perform CldU staining

11. Block by incubating with 10 ml of 5% BSA in PBST for 20 min at room temperature. Transfer the coverslips to wells of a 24-well plate or a humidified chamber. Aspisate blocking solution.

    If the glass coverslips are kept inside a 24-well plate, the humidified chamber is not necessary; otherwise all staining steps should be done inside the humidified chamber.

12. Pipet 30 μl primary antibody (rat anti-BrdU antibody; diluted 1:10 in 5% BSA/PBST) onto the coverslip. Incubate 1 hr at room temperature.
13. Wash the coverslip three times, each time with 1 ml PBST for 5 min.
14. Pipet 30 μl secondary antibody (goat anti–rat IgG conjugated to FITC; diluted 1:10 in 5% BSA/PBST) onto the coverslip and incubate 1 hr at room temperature.
15. Wash three times with 1 ml PBST, each time for 5 min.

Perform IdU staining

16. Block by incubating with 10 ml of 5% BSA in PBST in a 100-mm petri dish for 20 min at room temperature. Transfer the coverslips to wells of a 24-well plate or a humidified chamber.

    If the glass coverslips are kept inside a 24-well plate, the humidified chamber is not necessary; otherwise all staining steps should be done inside the humidified chamber.

    When CldU and IdU staining are performed sequentially, this blocking step can be omitted, but be sure to prepare the antibody dilutions in blocking solution.

17. Pipet 30 μl primary antibody solution (mouse anti-BrdU antibody; diluted 1:10 in 5% BSA/PBST) onto the coverslip. Incubate for 1 hr at room temperature.
18. Wash the coverslip with 1 ml PBST three times, each time for 5 min at room temperature, then wash with 1 ml high-salt buffer for 15 min at room temperature.

    The wash with high-salt buffer is essential to avoid cross-reactivity of antibodies.

19. Pipet 30 µl secondary antibody (donkey anti–mouse IgG conjugated to Texas Red; diluted 1:10 in 5% BSA/PBST) onto the coverslip and incubate for 1 hr at room temperature.

20. Wash three times, each time with 1 ml PBST for 5 min.

Mount coverslips for imaging

21. Pipet 5 µl Vectashield mounting medium on a glass microscope slide. Place the coverslip with the labeled cells, cell-side-down, over the drop and seal with clear nail polish. Observe by fluorescence microscopy (*UNIT 4.2*) with appropriate filters for the fluorophores.

BASIC PROTOCOL 4

REPLICATION LABELING OF DNA FIBERS

This section describes the procedure to visualize replication sites on DNA fibers. BrdU-labeled cells are lysed in detergent-containing buffer on a glass slide. Tilting the glass slide extends DNA fibers along the surface of the slide. The fibers are fixed with methanol/acetic acid and are then immunostained.

Materials

Adherent tissue culture cells and appropriate medium
10 mg/ml bromodeoxyuridine (BrdU) stock (store indefinitely at −20°C)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Lysis buffer (see recipe)
Fixative: 3:1 (v/v) methanol/acetic acid (prepare fresh and store on ice)
Blocking buffer: 1% (w/v) BSA in PBST (0.5% v/v Tween 20 in PBS)
PBST: 0.5% (v/v) Tween 20 in PBS
Primary antibody solution: mouse monoclonal anti-BrdU (Becton Dickinson), diluted 1:10 in blocking buffer (see above)
Secondary antibody solution: Alexa Fluor 594–conjugated goat anti–mouse IgG (Molecular Probes) diluted 1:500 in blocking buffer (see above)
Vectashield mounting medium (Vector Labs)

100-mm culture dishes
15-ml conical centrifuge tubes
Centrifuge
Glass microscope slides
Diamond pen (optional)
Coplin jars
Humidified chamber (e.g., covered Tupperware container with moistened paper towels on the bottom)
Fluorescence microscope (*UNIT 4.2*) with appropriate color filters for fluorophores used, e.g., blue (DAPI), green (FITC, Alexa Fluor 488), and red (e.g., Texas Red, Alexa Fluor 594)

Additional reagents and equipment for cell culture (including trypsinization and cell counting; *UNIT 1.1*) and fluorescence microscopy (*UNIT 4.2*).

Grow and label cells

1. Grow the cells in a 100-mm culture dish to the appropriate cell density (*UNIT 1.1*).

   For overnight incubation seed 5 × 10^6 cells.

   If cell density is too low, the yield in terms of cell number will be compromised; if density is too high, BrdU incorporation is decreased. Typically, use 0.5–1 × 10^6 cells/ml.

2. Add 10 mg/mlBrdU stock for a final concentration of 10 μg/ml. Incubate the dish 30 min at 37°C. Prepare cells without BrdU treatment under the same conditions at the same time.

   Alternatively, CldU and/or IdU can be used.

3. Trypsinize the cells (*UNIT 1.1*) and collect them in a 15-ml conical tube.

4. Centrifuge 1 min at 200 × *g*, room temperature, and remove supernatant. Add 10 ml PBS to the pellet, centrifuge again as before, and remove the supernatant.

5. Count the cells (*UNIT 1.1*). Prepare a single-cell suspension at 2×10^6 cells/ml in PBS.

   Mixing 20 μl labeled cells and 80 μl unlabeled cells can help to reduce the density of BrdU-incorporated DNA fibers on the slide, yielding clearer images. Unlabeled cells are also used as a negative control for immunostaining.

Prepare fibers

6. Place 2 μl of the cell suspension onto the edge of a glass slide.

7. Add 10 to 50 μl lysis buffer and mix briefly on the slide with a pipet tip. Incubate the slide for no more than 10 min at room temperature.

   The smaller the volume of lysis buffer, the better the result. However, it might be a bit difficult to handle a 10-μl volume of lysis buffer; therefore, beginners should try 20 to 50 μl.

8. Tilt the slide gradually to a 15° to 30° angle (e.g., by laying the slide on the edge of a test tube rack so that the one side of the slide is raised ∼2 cm).

   The cell lysate deposits DNA fibers as the cell suspension flows down the surface of the glass slide.

   It is useful to mark the extent of flow of the cell lysate on the opposite side of the slide with a diamond pen.

9. Pipet 400 μl freshly prepared fixation solution directly onto the slide. Incubate for 2 min, then remove the fixative and allow the slide to air dry.

   At this step, the glass slide can be stored in the dark at room temperature indefinitely.

Immunostain DNA fibers

10. Add sufficient blocking buffer (1% BSA in PBST) to cover the glass slide (∼1 ml) and incubate 20 min at room temperature.

    Large numbers of glass slides can be processed at the same time by using Coplin jars. Otherwise, pipet the solutions directly onto the glass slide.

11. Pipet 30 μl primary antibody solution onto the slide. Cover the slide with an 18 × 18–mm glass coverslip. Incubate the slide for 1 hr at room temperature in a humidified chamber.

12. Wash slides three times by immersion in 40 ml PBST in Coplin jars, each time for 3 min.

13. Add 30 μl secondary antibody and incubate for 1 hr at room temperature in a humidified chamber.

14. Wash slides three times by immersion in 40 ml PBST in Coplin jars, each time for 3 min.

Mount cells for imaging

15. Add 5 μl Vectashield mounting medium per slide and cover with 24 × 50–mm coverslip. Seal with clear nail polish and observe with a fluorescence microscope (*UNIT 4.2*) using the appropriate filters for the fluorochromes.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

High-salt buffer

Phosphate-buffered saline (PBS; APPENDIX 2A) containing:
0.5 % (v/v) Tween 20
0.4 M NaCl
0.2% (v/v) Nonidet P-40 (NP-40)
Prepare fresh

Hybridization mix

2 × SSC (APPENDIX 2A) containing:
50% (v/v) formamide
1% (v/v) Tween 20
10% (w/v) dextran sulfate
Prepare fresh

Lysis buffer

0.5% (w/v) sodium dodecyl sulfate (SDS; see *APPENDIX 2A* for 20% stock)
50 mM disodium EDTA
200 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Prepare fresh

Trypsin solution

Hanks' balanced salt solution (HBSS: APPENDIX 2A) without Ca, Mg, or bicarbonate, containing:
0.01% (w/v) trypsin
0.1 mM disodium EDTA

COMMENTARY

Background Information

Since the late 1960s, a variety of cytochemical methods have been developed to understand DNA metabolism in the nucleus. These techniques include autoradiography using tritium as well as dye staining using, e.g., Giemsa. Considering the sensitivity required to measure small amounts of newly synthesized DNA, the identification of proliferating cells has usually been accomplished by demonstrating [^{3}H]thymidine incorporation into DNA by autoradiography (Cleaver, 1967; Baserga and Malamud, 1969). The problem with tritium incorporation is that autoradiographic studies are time consuming. In addition, the radiation hazard posed by [^{3}H]thymidine has been a major barrier to its application in the field of clinical diagnostics and treatment, including cell kinetics and histopathology of human patients. Thus, investigators have been seeking a faster and less hazardous technique.

In the early 1970s, fluorescence quenching of DNA-specific fluorochromes by incorporated bromodeoxyuridine was developed (Latt, 1973; Stubblefield, 1975). Fluorescence quenching techniques using BrdU were first applied to flow cytometric studies of DNA replication (Dutrillaux et al., 1973). In the mid-1970s, antibodies raised against BrdU made possible an immunochemical method for detection of BrdU incorporated into DNA (Gratzner et al., 1975, 1976, 1978). In particular, the development of monoclonal anti-BrdU antibody significantly advanced microscopic methods, including image analysis, in the study of DNA synthesis (Gratzner, 1982). In combination with the use of fluorophore-conjugated secondary antibodies, immunochemical staining of incorporated BrdU and other halogenated thymidine analogs has been applied both to single cells and to tumor tissues, to estimate the labeling index of individual specimens (Raza et al., 1984; Nagashima and Hoshino, 1985).

Critical Parameters and Troubleshooting

Obviously, the most critical parameters common to all protocols described in this unit

are the concentration and incubation time for labeling with halogenated thymidine analog. Low concentrations result in lower signal-to-noise ratio, while high concentrations may affect cell viability because the thymidine analogs are toxic to cells. Short incubation periods yield low signal, whereas long incubation times result in larger regions being labeled, and, hence, lower resolution. In mammals, each replication focus takes 45 to 60 min to complete synthesis (Ma et al., 1998; Dimitrova and Gilbert, 1999), so periods longer than that will label several cohorts of foci. While the final concentration of 10 μg/ml and 30-min incubation time typically gives good results with most mammalian cells, the dose and period should be determined empirically. Different cell types may differ in their permeability or in the levels of endogenous thymidine pools, which affect the final intracellular concentration of labeled nucleoside. Thus, optimization may need to be performed if unexpected images are obtained.

It is feasible to employ a combination of the protocols in this unit to further analyze DNA replication. For example, it should be noted that the replication foci visualized by BrdU incorporation can be either replication origins or replication forks. To measure the density of active origins, IdU and CldU double labeling (Basic Protocol 3) should be employed in the DNA-fiber method (Basic Protocol 4). Cells are briefly labeled with IdU, followed by a longer CldU chase to allow forks to progress and to label the remainder of the long DNA fiber. DNA is then stretched on glass slides and stained with differentially labeled antibodies to highlight the sites of IdU and CldU incorporation. Short IdU stretches flanked on either side by long CldU stretches identify replication origins. The distances between these short IdU stretches can be measured under each experimental condition to evaluate the effects on the density of active replication origins (Pasero et al., 2002; Anglana et al., 2003; Norio et al., 2005).

Anticipated Results

Typical replication patterns visualized by BrdU incorporation are shown in Figure 22.10.1A. Cells were labeled with BrdU followed by anti-BrdU antibody immunofluorescence staining. Sites of BrdU incorporation (replication pattern) were shown in the order of appearance from early-S to late-S (left to right; Wu et al., 2005a). The results of dual replication labeling have been published (Dimitrova and Gilbert, 1999; Dimitrova et al., 1999; Li et al., 2003). DNA fibers labeled with CldU and visualized as per Basic Protocol 4 are shown in Figure 22.10.1B (F. Li and D.M. Gilbert, unpub. observ.). It should be noted that DNA fibers are not necessarily a single stretch of double-stranded DNA.

Time Considerations

Typically, replication labeling with one halogenated thymidine analog takes 30 min. After the labeling, an additional 1 hr is required to process the specimen. For immunostaining, typically 1 to 2 hr is required. Since FISH involves a hybridization step of 6 to 12 hr, 2 working days may be required. Preparation of DNA fibers usually takes <1 hr.

Literature Cited

Anglana, M., Apiou, F., Bensimon, A., and Debatisse, M. 2003. Dynamics of DNA replication in mammalian somatic cells: Nucleotide pool modulates origin choice and interorigin spacing. *Cell* 114:385-394.

Baserga, R. and Malamud, D. 1969. Autoradiography: Technique and Application. Harper & Row, New York.

Berezney, R., Dubey, D.D., and Huberman, J.A. 2000. Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci. *Chromosoma* 108:471-484.

Bornfleth, H., Edelmann, P., Zink, D., Cremer, T., and Cremer, C. 1999. Quantitative motion analysis of subchromosomal foci in living cells using four-dimensional microscopy. *Biophys. J.* 77:2871-2886.

Cleaver, J. 1967. Thymidine Metabolism and Cell Kinetics. North Holland, Amsterdam.

Dimitrova, D.S. and Gilbert, D.M. 1999. The spatial position and replication timing of chromosomal domains are both established in early G1 phase. *Mol. Cell.* 4:983-993.

Dimitrova, D.S., Todorov, I.T., Melendy, T., and Gilbert, D.M. 1999. Mcm2, but not RPA, is a component of the mammalian early G1-phase prereplication complex. *J. Cell Biol.* 146:709-722.

Dutrillaux, B., Laurent, C., Couturier, J., and Lejeune, J. 1973. Staining of human chromosomes with acridine orange after treatment with 5-bromodeoxyuridine. *C.R. Acad. Sci. Hebd. Seances Acad. Sci. D.* 276:3179-3181.

Edelmann, P., Bornfleth, H., Zink, D., Cremer, T., and Cremer, C. 2001. Morphology and dynamics of chromosome territories in living cells. *Biochim. Biophys. Acta.* 1551:M29-M39.

Gilbert, D.M. 2001. Making sense of eukaryotic DNA replication origins. *Science* 294:96-100.

Gilbert, D.M. 2002. Replication timing and transcriptional control: Beyond cause and effect. *Curr. Opin. Cell Biol.* 14:377-383.

Gilbert, D.M. 2004. In search of the holy replicator. *Nat. Rev. Mol. Cell Biol.* 5:848-855.

Gilbert, D.M. 2005. Origins go plastic. *Mol. Cell* 20:657-658.

Goren, A. and Cedar, H. 2003. Replicating by the clock. *Nat. Rev. Mol. Cell Biol.* 4:25-32.

Gratzner, H.G. 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 218:474-475.

Gratzner, H.G., Leif, R.C., Ingram, D.J., and Castro, A. 1975. The use of antibody specific for bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells and chromosomes. *Exp. Cell Res.* 95:88-94.

Gratzner, H.G., Pollack, A., Ingram, D.J., and Leif, R.C. 1976. Deoxyribonucleic acid replication in single cells and chromosomes by immunologic techniques. *J. Histochem. Cytochem.* 24:34-39.

Gratzner, H.G., Ettinger, N., Ingram, D., and Castro, A. 1978. Immunochemical studies of 5-bromodeoxyuridine. *Res. Commun. Chem. Pathol. Pharmacol.* 20:539-548.

Latt, S.A. 1973. Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. *Proc. Natl. Acad. Sci. U.S.A.* 70:3395-3399.

Li, F., Chen, J., Solessio, E., and Gilbert, D.M. 2003. Spatial distribution and specification of mammalian replication origins during G1 phase. *J. Cell Biol.* 161:257-266.

Ma, H., Samarabandu, J., Devdhar, R.S., Acharya, R., Cheng, P.C., Meng, C., and Berezney, R. 1998. Spatial and temporal dynamics of DNA replication sites in mammalian cells. *J. Cell Biol.* 143:1415-1425.

McNairn, A.J. and Gilbert, D.M. 2003. Epigenomic replication: Linking epigenetics to DNA replication. *Bioessays* 25:647-656.

Nagashima, T. and Hoshino, T. 1985. Rapid detection of S-phase cells by anti-bromodeoxyuridine monoclonal antibody in 9L brain tumor cells in vitro and in situ. *Acta Neuropathol. (Berl.)* 66:12-17.

Norio, P., Kosiyatrakul, S., Yang, Q., Guan, Z., Brown, N.M., Thomas, S., Riblet, R., and Schildkraut, C.L. 2005. Progressive activation of DNA replication initiation in large domains of the immunoglobulin heavy chain locus during B cell development. *Mol. Cell* 20:575-587.

Pasero, P., Bensimon, A., and Schwob, E. 2002. Single-molecule analysis reveals clustering and epigenetic regulation of replication origins at the yeast rDNA locus. *Genes Dev.* 16:2479-2484.

Raza, A., Kuliczkowski, K., Weidong, G., and Preisler, H.D. 1984. Comparison of in vitro assays for detecting subpopulations of P388 leukemic cells resistant to AraC. *Med. Oncol. Tumor Pharmacother.* 1:163-168.

Sadoni, N., Cardoso, M.C., Stelzer, E.H., Leonhardt, H., and Zink, D. 2004. Stable chromosomal units determine the spatial and temporal organization of DNA replication. *J. Cell Sci.* 117:5353-5365.

Stubblefield, E. 1975. Analysis of the replication pattern of Chinese hamster chromosomes using 5-bromodeoxyuridine suppression of 33258 Hoechst fluorescence. *Chromosoma* 53:209-221.

Wu, R., Terry, A.V., Singh, P.B., and Gilbert, D.M. 2005a. Differential subnuclear localization and replication timing of histone H3 lysine 9 methylation states. *Mol. Biol. Cell* 16:2872-2881.

Wu, R., Terry, A.V., and Gilbert, D.M. 2005b. Observing S-phase dynamics of histone modifications with fluorescently labeled antibodies. *In* Nuclear Reprogramming: Methods and Protocols (S. Pells, ed.) pp. 139-148. Humana Press, Totawa, N.J.

Zink, D., Amaral, M.D., Englmann, A., Lang, S., Clarke, L.A., Rudolph, C., Alt, F., Luther, K., Braz, C., Sadoni, N., Rosenecker, J., and Schindelhauer, D. 2004. Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei. *J. Cell Biol.* 166:815-825.

Contributed by Tomoki Yokochi and David M. Gilbert
Department of Biological Science
Florida State University
Tallahassee, Florida

Assays for Ribosomal RNA Processing and Ribosome Assembly

Dimitri G. Pestov,[1] Yevgeniya R. Lapik,[2] and Lester F. Lau[2]

[1]University of Medicine and Dentistry of New Jersey, School of Osteopathic Medicine, Department of Cell Biology, Stratford, New Jersey
[2]University of Illinois at Chicago College of Medicine, Department of Biochemistry and Molecular Genetics, Chicago, Illinois

ABSTRACT

The synthesis of ribosomes is a major metabolic activity critical for cell growth and homeostasis. Understanding the mechanisms of ribosome biogenesis has important implications for studying both protein synthesis and cell cycle control. This unit describes several techniques for the analysis of rRNA maturation and ribosome assembly adapted for mammalian cells. Metabolic labeling of rRNA and hybridization analysis of precursors can be used to assess changes in rRNA processing that occur under experimental conditions of interest. Separation of preribosomal particles by sucrose gradient centrifugation is suitable for the analysis of proteins associated with preribosomes during their assembly and maturation in the cell nucleus. *Curr. Protoc. Cell Biol.* 39:22.11.1-22.11.16.

Keywords: ribosome biogenesis • rRNA • preribosome • pre-rRNA • nucleolus • ribonucleoprotein • RNA processing • mammalian cells

INTRODUCTION

During periods of rapid growth and high biosynthetic activity, transcription of ribosomal RNAs (rRNAs) accounts for the majority of RNA synthesis in the cell. One way to follow rRNA transcription and processing in cultured mammalian cells is to incubate the cells with a radioactive RNA precursor and analyze labeled RNA by gel electrophoresis. Basic Protocol 1 describes labeling of rRNA by using [5,6-^{3}H]uridine. Labeling with ^{32}P-orthophosphate ($^{32}P_i$; see Alternate Protocol 1) produces stronger signals and is preferred for the analysis of small rRNAs. Alternate Protocol 2 describes pulse-chase labeling of rRNA with the precursor of methyl groups L-[*methyl*-^{3}H]methionine to examine the kinetics of rRNA processing. The Support Protocol describes fluorographic detection of ^{3}H-labeled rRNA blotted on membranes.

A detailed picture of the steady-state levels of various pre-rRNA intermediates can be obtained by hybridization of cellular RNA with oligonucleotide probes specific to various regions of the rRNA or rRNA precursors (pre-rRNAs). This approach is detailed in Basic Protocol 2.

Processing of the rRNA transcript occurs in nuclear ribonucleoprotein complexes (preribosomes) that contain both ribosomal proteins and auxiliary factors necessary for the assembly and maturation of the ribosomal subunits. Basic Protocol 3 describes sucrose gradient centrifugation of preribosomal particles isolated from cell nuclei for the analysis of associated proteins.

CAUTION: Observe proper techniques for working with radioactivity and disposal of radioactive waste (*APPENDIX 1D*). To prevent accidental spillage of radioactive media inside a tissue culture incubator, place a plastic tray lined with an absorbent pad inside the incubator prior to adding the labeling plates.

Current Protocols in Cell Biology 22.11.1-22.11.16, June 2008
Published online June 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2211s39

NOTE: All culture incubations should be performed in a humidified 37°C incubator with the concentration of CO_2 appropriate for the cell culture medium used.

BASIC PROTOCOL 1

METABOLIC LABELING OF rRNA WITH [^{3}H]URIDINE

Labeling of the newly synthesized rRNA with tritiated uridine is a simple and inexpensive technique suitable for analysis of large rRNA precursors. In the following protocol, [5,6-^{3}H]uridine is added to a monolayer cell culture grown in a tissue culture plate. After a 2-hr incubation, cells are rinsed of excess label and total RNA is isolated using a phenol/guanidine thiocyanate reagent. The labeled RNA obtained in this way can then be analyzed by standard gel electrophoresis techniques, followed by nylon membrane blotting and fluorography.

The volumes in this protocol are for a 6-well plate. The procedure can be scaled in proportion to the surface area of the tissue culture vessel used.

Materials

Adherent cell cultures (e.g., NIH 3T3 cells)
Complete cell culture medium (e.g., DMEM supplemented with 10% FBS)
1 mCi/ml [5,6-^{3}H]uridine (specific activity ≥35 Ci/mmol)
Phenol/guanidine thiocyanate reagent (e.g., TRI Reagent, MRC or TRIzol, Invitrogen)
Deionized formamide
Scintillation fluid

6-well tissue culture plates
37°C, CO_2 humidified cell culture incubator
Flask for radioactive waste
Microcentrifuge tubes with screw-caps equipped with a rubber O-ring (tubes must withstand centrifugation forces of at least 12,000 × *g*)
55° to 65°C water bath
Liquid scintillation vials and counter

Additional reagents and equipment for RNA isolation (see manufacturer's protocol for the isolation reagent used) and RNA gel electrophoresis and nylon membrane transfer (Brown et al., 2004)

1. Set up cultures of adherent cells in a 6-well culture plate 1 to 2 days prior to labeling so that the cultures are subconfluent on the day of the experiment.

   For NIH 3T3 cells, plate ~2.5 × 10^5 cells per well of a 6-well plate in 2 ml DMEM containing 10% FBS 1 day before labeling. Confirm that cells look healthy prior to the experiment. rRNA synthesis will be significantly reduced if cell growth is inhibited by suboptimal conditions or contact inhibition.

2. Add [5,6-^{3}H]uridine to medium in each well to a final concentration of 3 μCi/ml. Immediately return cells to the 37°C, CO_2 humidified cell culture incubator and incubate for 2 hr.

3. Aspirate radioactive medium from each well into a flask for safe disposal. Rinse cell monolayers two times, each time adding 2 ml complete culture medium to a well and aspirating the medium into the same disposal flask.

4. Add 1 ml phenol/guanidine thiocyanate reagent directly to cells and lyse them by gentle mixing. Transfer lysates into screw-cap microcentrifuge tubes. Isolate total RNA following manufacturer's instructions for the RNA isolation reagent used.

   It is important to use screw-cap tubes that provide a tight seal to prevent leakage of radioactive samples during RNA purification.

5. Following isolation, dissolve RNA pellets in 15 µl deionized formamide. To ensure that RNA is completely dissolved, incubate 30 min on ice with occasional mixing followed by 10 min at 55° to 65°C.

^{3}H-labeled RNA dissolved in formamide can be stored for 1 to 2 months at −20°C or at least 1 year at −70°C.

Formamide protects RNA from degradation and simplifies handling of samples.

6. Measure [^{3}H] incorporation by liquid scintillation counting of 1 µl.

Incorporation observed in actively growing NIH 3T3 cells is typically 5–10 × 10^3 cpm/µg RNA, but may vary significantly depending on the cell line and growth conditions.

Optionally, determine RNA concentration either by measuring absorbance at 260 nm or fluorescent quantitation (APPENDIX 3D).

7. Separate RNA by agarose-formaldehyde gel electrophoresis and transfer to a nylon membrane.

Follow standard protocols for electrophoresis and blotting of RNA found in common molecular biology manuals (Brown et al., 2004). Detection of ^{3}H-labeled RNA blotted on nylon membranes by fluorography is described in the Support Protocol (also see UNIT 6.3). See Figure 22.11.1A for an example of results.

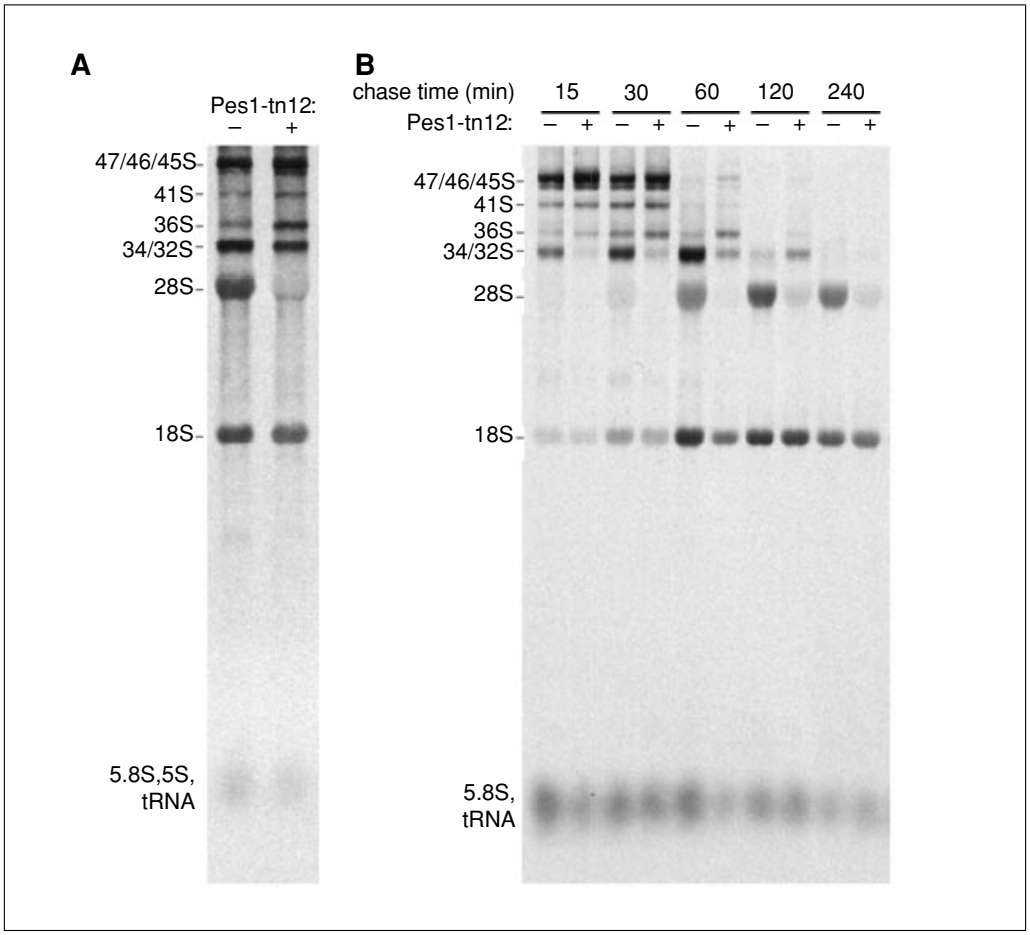


Figure 22.11.1 Gel analysis of labeled rRNA precursors from mouse cells. (**A**) An example of metabolic labeling with [^{3}H]uridine. (**B**) An example of pulse-chase labeling with L-[*methyl*-^{3}H]methionine. In the experiments shown, maturation of pre-rRNA in normal cells (– lanes) is compared with cells inducibly expressing Pes1-tn12 (+ lanes), a dominant-negative mutant of the processing factor Pes1 (Lapik et al., 2004). The positions of the major labeled RNA species are indicated on the left of each panel.

ALTERNATE PROTOCOL 1

METABOLIC LABELING OF rRNA WITH $^{32}P_i$

To improve detection of low-molecular-weight rRNA species (e.g., 5S, 5.8S) and their immediate precursors, it is more practical to use ^{32}P labeling, which significantly reduces exposure times necessary for detection. Cells are first pre-incubated in phosphate-free medium, followed by addition of $^{32}P_i$. After a 1-hr incubation, cells are briefly washed to remove the excess label and incubated for an additional 1.5 hr in complete medium. Total RNA is isolated using a phenol/guanidine thiocyanate reagent. ^{32}P-Labeled RNA separated on a gel can be detected using autoradiography or phosphorimaging (*UNIT 6.3*).

The volumes in this protocol are for a 6-well plate. The procedure can be scaled in proportion to the surface area of the tissue culture vessel used.

Additional Materials *(also see Basic Protocol 1)*

Phosphate-free cell culture medium (e.g., phosphate-free DMEM) supplemented with serum dialyzed against phosphate-free saline, prewarmed to 37°C
^{32}P-Labeled orthophosphate, carrier-free (10 mCi/ml $^{32}P_i$)
Aerosol-resistant tips
Plexiglas shields and other protective equipment for safe work with ^{32}P (*APPENDIX 1D*)

1. Grow cells as described in Basic Protocol 1, step 1.
2. One hr before labeling, replace cell growth medium with 1 ml prewarmed phosphate-free medium.
3. Add $^{32}P_i$ to the medium in wells containing cells to a final concentration of 20 μCi/ml.
4. Incubate cells 1 hr in a 37°C, CO_2 humidified cell culture incubator.
5. Discard $^{32}P_i$-containing medium into a radioactive waste container, and add 3 ml prewarmed complete growth medium to cells. Incubate for an additional 1.5 hr in a 37°C, CO_2 humidified cell culture incubator.

   It is recommended to use a pipettor equipped with aerosol-resistant tips to remove media containing high amounts of ^{32}P. Using a vacuum aspirator will spread radioactivity over large volumes, increasing exposure to the energetic radiation from ^{32}P.

6. Follow Basic Protocol 1, steps 3 to 6, to isolate RNA from cells.

   ^{32}P-Labeled RNA must be analyzed within a few days of labeling due to the short half-life of ^{32}P.

7. Carry out gel electrophoresis using either a denaturing polyacrylamide gel for analysis of small RNA species or an agarose-formaldehyde gel for large precursors (Brown et al., 2004).

   Follow standard procedures for electrophoresis of RNA found in common molecular biology manuals (see APPENDIX 3A). Since ^{32}P is a strong β-emitter, no transfer of RNA to membrane after electrophoresis is required; gels can be dried and analyzed directly by autoradiography or phosphorimaging (see UNIT 6.3).

ALTERNATE PROTOCOL 2

PULSE-CHASE LABELING OF rRNA WITH L-[*METHYL*-^{3}H]METHIONINE

L-[*methyl*-^{3}H]Methionine is a precursor for methyl groups that are abundant modifications in mature rRNAs. Because of the rapid turnover of the cellular methionine pool, this reagent can be used for pulse-chase labeling to study the kinetics of pre-rRNA processing. In addition, the overall background in this labeling is much lower compared to uridine or phosphate labeling because only methylated RNA is detected. However, any changes in rRNA methylation can affect labeling of precursors in this approach.

In this protocol, cells are briefly preincubated in methionine-free medium and then pulse-labeled for 30 min in medium containing L-[*methyl*-^{3}H]methionine. The chase is performed by incubating cells in medium containing an excess of methionine for various amounts of time. Total RNA is isolated using a phenol/guanidine thiocyanate reagent. The labeled RNA obtained this way can be analyzed by standard gel electrophoresis techniques, followed by nylon membrane blotting and fluorography (see Support Protocol).

The volumes in this protocol are for a 6-well plate (alternatively, 35-mm dishes can be used). The protocol can be scaled in proportion to the surface area of the tissue culture vessels used.

***Additional Materials** (also see Basic Protocol)*

Methionine-free cell culture medium (e.g., methionine-free DMEM) supplemented with dialyzed serum
1 mCi/ml L-[*methyl*-^{3}H]methionine (specific activity 70 to 85 Ci/mmol)
10× methionine medium: add methionine to culture medium at a final concentration of 0.3 mg/ml, prewarmed to 37°C

NOTE: L-[*methyl*-^{3}H]Methionine is prone to rapid decomposition if not properly stored. For storage conditions and shelf life, refer to the manufacturer's recommendations.

1. Grow cells as described in Basic Protocol 1, step 1.

   Prior to growing cells, determine the number of time points in the chase. Separate cell culture plates should be prepared for each time point.

2. For each plate, aspirate medium from wells and rinse cell monolayers once with 1 ml prewarmed methionine-free cell culture medium. Add 1 ml methionine-free cell culture medium and incubate 15 min in a 37°C, CO_2 humidified cell culture incubator.

3. Add L-[*methyl*-^{3}H]methionine to wells containing cells to a final concentration of 50 μCi/ml.

4. Incubate cells 30 min in a 37°C, CO_2 humidified cell culture incubator to label the RNA.

5. Aspirate L-[*methyl*-^{3}H]methionine-containing medium into a flask for safe disposal. Rinse wells with 2 ml prewarmed 10× methionine medium, aspirating it into the same disposal flask.

   Work fast to avoid temperature fluctuations.

6. Add 3 ml prewarmed 10× methionine medium to cells and return plate to the 37°C, CO_2 humidified cell culture incubator (note the time—this is the "zero" time point for the chase).

7. At appropriate times, remove corresponding cell culture plates from the incubator, isolate total RNA, and proceed with analysis as described in Basic Protocol 1, steps 3 to 7.

   Useful time points are 0, 15 min, 30 min, 1 hr, and 2 hr of chase. Depending on the cell type, a 4-hr time point may also be informative. Labeling efficiencies with methionine are usually lower than those obtained by using other techniques described in this unit, with specific activities in the range of 800 to 3500 cpm/μg RNA typically observed. See Figure 22.11.1B for an example of results using this method.

SUPPORT PROTOCOL

FLUOROGRAPHIC DETECTION OF ^{3}H-LABELED RNA ON NYLON MEMBRANES

Tritium (^{3}H) used for rRNA labeling in Basic Protocol 1 and Alternate Protocol 2 is a weak β-emitter that can be detected by coating RNA blots with a thin layer of a scintillant followed by exposure to a high-sensitivity photographic film. In this protocol, membranes containing blotted RNA are first sprayed with EN^{3}HANCE and then soaked in carbon tetrachloride, which acts as a solvent for the fluor and facilitates even coating of the membrane.

CAUTION: Work in a fume hood. Avoid breathing vapors.

NOTE: Nylon membranes used in the protocol are unaffected by carbon tetrachloride. Some plastic containers may dissolve in the fluor or carbon tetrachloride; if using other types than those recommended, check them first for solvent resistance.

Materials

Nylon membrane with transferred ^{3}H-labeled RNA (see Basic Protocol 1 or Alternate Protocol 2)
EN^{3}HANCE Spray (PerkinElmer Life)
Carbon tetrachloride (Sigma)

Glass or polypropylene tray (a size to fit the membrane)
Filter paper
High-sensitivity radiography film (e.g., Kodak BioMax MS or equivalent)

1. Before starting the procedure, make sure the RNA blot is completely dry.
2. Place the membrane in a suitable glass or polypropylene tray with the RNA side up.
3. Working in a fume hood, spray the membrane lightly with EN^{3}HANCE. Immediately add a small volume of carbon tetrachloride to completely cover the blot surface. Gently agitate the tray for several minutes to distribute the solution over the surface of the membrane.

   Carbon tetrachloride evaporates very rapidly creating a progressively more concentrated solution of the fluor, which will form a thin coating on the membrane once dried. Do not allow the solution to become too viscous, as it may lead to uneven coating.

4. Remove membrane when solvent has partially evaporated, but there is still enough free-flowing liquid to cover entire surface of membrane. Air dry membrane for 5 min on a piece of filter paper.
5. Expose membrane with direct contact to a radiography film in a light-proof cassette at −80°C. Develop film after a predetermined amount of time.

   Exposure length must be determined empirically. A good starting point is 1 to 2 days if labeling was performed with uridine and 2 to 5 days if labeled with methyl-methionine. Membranes can be repeatedly treated with carbon tetrachloride (with or without respraying, depending on the signal strength from the previous coating) and re-exposed until a satisfactory film is obtained.

BASIC PROTOCOL 2

HYBRIDIZATION OF rRNA PRECURSORS WITH OLIGONUCLEOTIDE PROBES

This protocol is designed to identify pre-rRNA species on a northern blot and examine changes in their steady-state levels. Oligonucleotide probes complementary to specific regions of the primary rRNA transcript are labeled at the 5′ termini with T4 polynucleotide kinase and [γ-^{32}P]ATP. The radiolabeled oligonucleotide probes are hybridized with RNA blotted onto a membrane.

Materials

Oligonucleotides (see Critical Parameters for details)
10× T4 polynucleotide kinase buffer (supplied with enzyme)
[γ-^{32}P]ATP (10 mCi/ml, specific activity ≥3000 Ci/mmol)
T4 polynucleotide kinase
0.5 M EDTA (*APPENDIX 2A*)
TE buffer (*APPENDIX 2A*)
Liquid scintillation fluid, optional
Nylon membranes containing total cellular RNA
2× and 5× SSC (see recipe)
Hybridization solution (see recipe)
2× SSC/0.1% SDS, room temperature and 70°C
0.1× SSC/0.1% SDS

1.5-ml microcentrifuge tubes
37°C and 60° to 75°C water baths or hybridization ovens
Spin columns (e.g., Micro Bio-Spin P-6 columns, BioRad) or equivalent
Liquid scintillation counter and vials, optional
Plastic boxes with tight-fitting lids or hybridization tubes suitable for the hybridization equipment used

Additional reagents and equipment for autoradiography or phosphorimaging (*UNIT 6.3*)

Radiolabel probes

1. Set up the labeling reaction by mixing the following components in a 1.5-ml microcentrifuge tube:

   10 pmol oligonucleotide
   2.5 μl 10× T4 polynucleotide kinase buffer
   16.5 pmol [γ-^{32}P]ATP (e.g., 5 μl of the 3000 Ci/mmol, 10 mCi/ml label)
   15 U T4 polynucleotide kinase
   water to 25 μl

2. Incubate 30 to 60 min in a 37°C water bath.
3. Stop the reaction by adding 0.5 μl of 0.5 M EDTA (final concentration of 10 mM) or by heating the reaction 10 min at 70°C.
4. Add 25 μl TE buffer to the labeling reaction and remove unincorporated [γ-^{32}P]ATP by centrifugation through a spin column according to the manufacturer's instructions.
5. Measure [γ-^{32}P]ATP incorporation by Cerenkov or scintillation counting.

   The typical specific activity of a probe determined by scintillation counting is 2–6 × 10^6 cpm/pmol. Alternatively, use a Geiger counter to quickly estimate the amount of radioactivity in the column eluate and the unincorporated label left in the column. Incorporation of the label should not be less than 50%.

   Probes can be stored at −20°C but they should be used within 2 to 3 days to avoid their degradation due to radiolysis.

Hybridize membrane

6. Prewet membrane to be hybridized in 5× SSC. Drain excess liquid.
7. Prehybridize membranes in hybridization solution (~0.2 ml/cm^2 membrane) 2 to 5 hr at 60°C.

8. Add the radiolabeled oligonucleotide directly to the hybridization solution in the container with the membrane to a final concentration of 100 to 200 pM.

   If using a probe directed against mature rRNAs, reduce oligonucleotide concentration to 20 pM to avoid an overly strong signal.

9. Hybridize overnight (12 to 18 hr) at 60°C.

10. Discard hybridization mixture and rinse membrane briefly in 2× SSC/0.1% SDS. Incubate with agitation in fresh changes of 2× SSC/0.1% SDS (0.6 to 0.7 ml/cm^2 membrane) as follows:

    a. 5 min at room temperature
    b. 10 min at room temperature
    c. 10 min at 70°C
    d. 5 min at room temperature.

    CAUTION: *Hybridization solution and all wash solutions should be treated as radioactive waste and disposed of appropriately.*

    If initial experiments show an excessive background or smear due to probe cross-hybridization, increase stringency of the high-temperature wash by 5° to 10°C.

11. Rinse the membrane in 2× SSC and wrap it securely in plastic wrap to prevent drying out. Use either autoradiography or phosphorimaging to detect hybridization signal.

    Keeping filters moist during exposure allows their subsequent reprobing after stripping. To strip filters, bring 0.1× SSC/0.1% SDS (0.6 to 0.7 ml/cm^2 membrane) to a boil and pour over the membrane. Incubate with agitation at room temperature until the solution cools down to 30° to 40°C. Repeat the procedure once, rinse the membrane with 2× SSC and check for the reduction of signal intensity by autoradiography or phosphorimaging.

BASIC PROTOCOL 3

SUCROSE GRADIENT ANALYSIS OF PRE-RIBOSOMAL RIBONUCLEOPROTEIN COMPLEXES

Preribosomes can be isolated from mammalian cell nuclei as heterogeneous particles with sedimentation coefficients ranging from ~50S to >100S. The first steps in the protocol describe isolation of nuclei and a sonication procedure for the extraction of preribosomes. The remaining steps describe separation of preribosomal particles by sucrose gradient centrifugation. A detailed discussion of the theory and methods for preparing and running sucrose density gradients can be found in *UNIT 5.3*. Proteins associated with preribosomal particles are recovered from gradient fractions by precipitation with TCA and can be further analyzed by SDS-PAGE and western blotting *(UNITS 6.1 & 6.2)*.

Materials

10% and 30% (w/w) sucrose solutions (see recipe)
Three 150-mm plates of subconfluent NIH 3T3 cells (~2 × 10^7 cells)
Phosphate buffered saline (PBS; *APPENDIX 2A*), ice cold
LSB (see recipe)
LSB$^+$ (see recipe)
10% (w/v) Igepal CA-630 (Sigma)
10% (w/v) sodium deoxycholate
Sonication buffer (see recipe)
0.1% (w/v) BSA (*APPENDIX 2A*)
100% TCA, 4°C
100% ethanol
Urea sample buffer (see recipe)
Polyallomer ultracentrifuge tubes for the SW41Ti rotor (9/16 × 3^1/$_2$– in., Beckman)

Plastic cell scraper
15-ml conical-bottom plastic centrifuge tubes
Refrigerated centrifuge with a swinging bucket rotor accommodating 15-ml conical-bottom tubes
1.5-ml microcentrifuge tubes
Ultrasonic processor equipped with a microtip
Refrigerated microcentrifuge
1-ml syringes and 20-G needles
Ultracentrifuge and SW41Ti rotor (Beckman)
50° and 95° to 100°C heating blocks

Additional reagents and equipment for gradient formation and fractionation (*UNIT 5.3*) and SDS-PAGE and immunoblotting (*UNITS 6.1 & 6.2*)

NOTE: This protocol was developed for NIH 3T3 cells. For other cell types, the amounts of detergents in steps 11 to 12 may require adjustment to isolate nuclei.

NOTE: Unless otherwise indicated, all steps in the protocol are performed at 4°C.

Prepare gradients

1. Prepare 10% to 30% (w/w) sucrose gradients in ultracentrifuge tubes (total volume 11.0 to 11.2 ml per tube) and store them at 4°C before use.

   Linear gradients can be prepared using any of the gradient makers available commercially (UNIT 5.3). Alternatively, a simple diffusion-based method can be used. Pipet 5.5 ml of 30% sucrose into a centrifuge tube, carefully overlay it with 5.5 ml of 10% sucrose solution, securely cap the tube with a silicone rubber stopper, and slowly lower the tube to a horizontal position. Keep the tube on its side for 3 hr at room temperature without moving, then carefully set it upright, and store overnight at 4°C. The resulting gradient will have an S-shaped rather than strictly linear concentration profile, but it is suitable for most types of work.

Isolate nuclei

2. Culture cells in three 150-mm tissue culture plates to obtain actively growing, sub-confluent cultures on the day of the experiment.
3. Discard growth medium from one plate and place plate on ice.
4. Rinse cell monolayer once with 10 ml ice-cold PBS.
5. Add 4 ml ice-cold PBS and scrape cells into the buffer. Pipet cell suspension into a 15-ml conical-bottom tube and store on ice.
6. Repeat with the remaining two plates combining cell suspensions into the same tube.
7. Pellet cells by centrifuging 5 min at 400–500 × *g*, 4°C, in a swinging bucket rotor.
8. Resuspend cell pellets in 2 ml ice-cold LSB by gently vortexing and incubate 10 min on ice.
9. Centrifuge as in step 7 to pellet swollen cells.
10. Resuspend cells in 2 ml LSB$^+$ by vortexing.
11. Add 66 µl of 10% Igepal CA-630 and briefly vortex tube to mix.
12. Add 44 µl of 10% sodium deoxycholate and vigorously vortex for 30 sec.

    The addition of detergents lyses the plasma membranes of swollen cells and facilitates removal of cytoplasmic material.

13. Centrifuge 5 min at 1000 × *g*, 4°C, to sediment crude nuclei.
14. Resuspend pellet in 2 ml LSB$^+$ by pipetting up and down with a 1-ml tip.

15. Repeat centrifugation as in step 13.

Phase-contrast microscopic examination of the pellet should show undamaged nuclei that may still have portions of the cytoplasmic membrane attached.

Sonicate nuclei and set up gradients

16. Resuspend nuclear pellets in 500 µl sonication buffer by gently pipetting up and down, and transfer to 1.5-ml microcentrifuge tubes.

17. Sonicate samples on ice using an ultrasonic processor equipped with a microtip to disrupt the nuclei.

When the procedure is performed for the first time, nuclear disruption should be monitored by phase-contrast microscopy. The intensity and duration of sonication should be adjusted so as to just break down nuclei in the sample; avoid oversonication. As a starting point, try ten 1-sec pulses at a low-to-medium output; briefly spin down samples in a microcentrifuge and repeat sonication once. Make sure to immerse the sonicator tip deep enough to avoid foaming. If working with several samples, keep tubes on ice between sonication cycles.

18. Incubate samples 5 min on ice with occasional mixing.

19. Microcentrifuge 15 min at 15,000 × *g*, 4°C. Transfer supernatant to a new microcentrifuge tube.

20. Remove and store a small (25-µl) aliquot at −80°C as a gradient 'input' sample.

21. Layer the nuclear extract (~450 µl total) onto a 10% to 30% (w/w) sucrose gradient (prepared in step 1) using a 1-ml syringe with a 20-G needle attached.

22. Carefully slide the tubes into pre-chilled buckets of the ultracentrifuge rotor, taking care not to disturb the gradients.

23. Ultracentrifuge gradients 3 hr at 160,000 × *g* (36,000 rpm), 5°C.

Fractionate gradients

24. Remove tubes from the rotor in a cold room or chromatography cabinet where the fractionation equipment is set up.

25. Fractionate gradients at 4°C and collect twelve 1-ml fractions. Monitor A_{254} to A_{260} of the gradient by using a flow cell connected between the tube and fraction collector (Fig. 22.11.2).

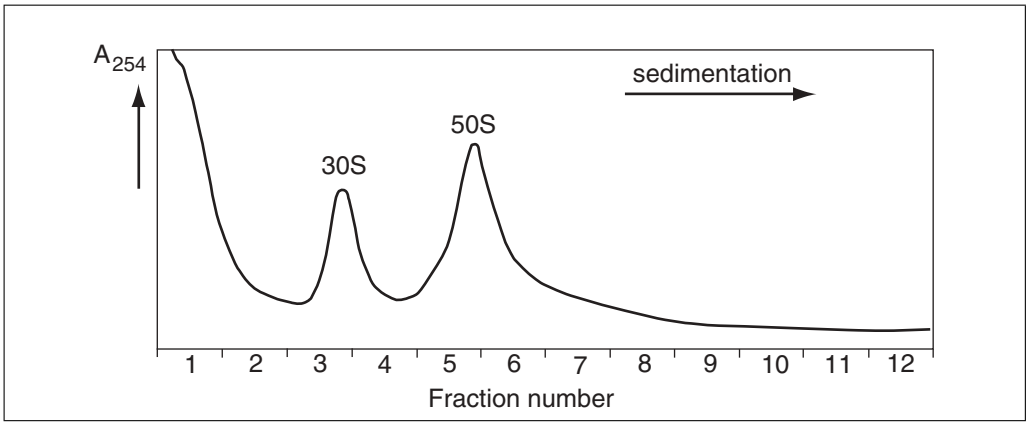


Figure 22.11.2 A typical profile of UV absorbance obtained upon fractionation of nuclear extracts on a sucrose gradient. The two major peaks sedimenting at ~30S and 50S contain small and large ribosomal subunits, respectively, and their precursors. Earlier preribosomal particles are found in fractions sedimenting ahead of 50S (fractions 6 through 9).

If possible, use upward displacement to fractionate gradients: pump 55% (w/w) sucrose through a needle inserted into the bottom of the tube at ~1 ml/min and collect fractions from the top using the funnel cap supplied by the separation system.

Analyze gradient fractions

26. To precipitate proteins, add 10 µl of 0.1% BSA and 100 µl cold 100% TCA to each 1-ml fraction, thoroughly mixing samples after each addition. Incubate 1 hr on ice.

    Because of the high viscosity of sucrose-containing samples, a combination of inverting the tube and vortexing may be necessary to achieve complete mixing. BSA serves as a carrier to ensure uniform protein recovery from different fractions.

27. Microcentrifuge tubes 15 min at ~12,000 × *g*, 4°C.

28. Wash protein pellets two times with 100% ethanol to remove residual TCA. Each time, incubate pellets at least 30 min at room temperature with occasional mixing and then microcentrifuge for 5 min at a maximum speed, 4°C.

    Samples can also be stored under ethanol overnight at −20°C—this is a convenient breakpoint in the procedure.

29. Air dry pellets for 5 to 10 min and dissolve in 50 µl freshly prepared urea sample buffer by incubating 5 min at 50°C followed by thorough mixing.

30. Briefly spin the samples in a centrifuge and store at −80°C.

    Urea in the sample buffer helps solubilize proteins.

31. Transfer an aliquot of each sample to be loaded onto a gel into a separate tube, heat 2 to 3 min at 95° to 100°C, and microcentrifuge 3 min at maximum speed, room temperature.

    A 15-µl aliquot is usually sufficient for one lane of a minigel. Avoid repeated heating of samples as it may lead to protein degradation.

32. Analyze samples by SDS-PAGE (*UNIT 6.1*) followed by immunoblotting *(UNIT 6.2)*.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Denhardt's solution, 50×

1% (w/v) Ficoll (type 400)
1% (w/v) polyvinylpyrrolidone
1% (w/v) BSA (fraction V; Sigma)
Filter sterilize through a 0.45-µm filter
Store in 25- to 50-ml aliquots up to 1 year at −20°C

Hybridization solution

5× SSC (see recipe)
5× Denhardt's solution (see recipe)
0.5% (w/v) SDS
25 µg/ml denatured salmon sperm DNA
Prepare fresh from stock solutions just before use

Low salt buffer (LSB)

10 mM HEPES-NaOH, pH 7.5
10 mM NaCl
2 mM $MgCl_2$

continued

1 mM EGTA
Filter sterilize through a 0.22-μm filter
Store up to 6 months at 4°C

LSB+

To LSB (see recipe), add 5 μl/ml protease inhibitor cocktail (Sigma) immediately before use.

Sonication buffer

25 mM Tris·Cl (*APPENDIX 2A*), pH 7.5, 25°C
100 mM KCl
1 mM NaF
2 mM EDTA
0.05% (v/v) Igepal CA-630 (Sigma)
Filter through a high protein-binding filter (nylon or nitrocellulose) to sterilize and remove any contaminating RNase traces
Store up to 6 months at 4°C
Add before use:
1 mM DTT (from 1 M stock solution stored at −20°C)
10 μl/ml protease inhibitor cocktail (Sigma)
50 to 100 U/ml RNase inhibitor (e.g., Promega or Fermentas)

SSC, 20×

3 M NaCl
0.3 M sodium citrate
Adjust pH to 7.0 with HCl
Sterilize by autoclaving
Store at room temperature

Sucrose solutions

To prepare 100-ml gradient solutions, start by weighing out 10.38 g sucrose for the 10% (w/w) solution, or 33.81 g sucrose for the 30% (w/w) solution. Add water and appropriate KCl and EDTA stock solutions to bring the final volume to 97.5 ml and 100 mM KCl and 2 mM EDTA final concentrations.

Filter the solution through a 0.22-μm filter and transfer to a glass bottle with a tight cap.

Add diethylpyrocarbonate (DEPC) to 0.05% (v/v), close cap securely, shake vigorously to mix, and incubate overnight at 60°C to decompose DEPC. Add 2.5 ml of 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*), at 25°C. Store up to 6 months at 4°C.

CAUTION: *Wear gloves and work in a fume hood when using DEPC, as it is toxic and a suspected carcinogen.*

Before making a gradient, add DTT to a final concentration of 1 mM to the working portion of solution.

Note that the percentage of sucrose used for making gradients is expressed as w/w. Use high-quality sucrose that is certified to have low A_{260}. DEPC treatment inactivates RNases that often contaminate commercial sucrose. Do not autoclave sucrose solutions because this will lead to caramelization and an increase of A_{260}.

Urea sample buffer

62.5 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
3 M urea

continued

2% (w/v) SDS
0.01% (w/v) bromophenol blue
0.1 M DTT
Prepare fresh

Urea-containing solutions should be freshly prepared to minimize adverse effects of urea hydrolysis products.

COMMENTARY

Background Information

Ribosome biogenesis in eukaryotes occurs largely in the nucleolus, with final stages of subunit maturation taking place in the nucleoplasm and cytoplasm. In mammalian cells, the large primary 47S pre-rRNA transcript is synthesized by RNA polymerase I and then processed through a series of endo- and exonucleolytic processing steps and covalent nucleotide modifications to mature 18S, 5.8S, and 28S rRNAs (Bowman et al., 1981; Eichler and Craig 1994; Fig. 22.11.3). The small 5S rRNA is synthesized by RNA polymerase III.

rRNAs and their precursors (pre-rRNAs) are among the most abundant RNA species in the cell, and therefore can be detected simply by running metabolically labeled total RNA on a gel. Metabolic labeling of the newly synthesized rRNA can be accomplished with ^{3}H- or ^{32}P-labeled precursors. The use of ^{3}H for labeling offers a number of advantages including minimal exposure of the investigator to radiation and the excellent stability of radiolabeled samples. Coating membranes containing ^{3}H-labeled RNA with a thin layer of fluor is a sensitive method for detection of ^{3}H. Commercial intensifying screens for ^{3}H have been developed; however, they tend to produce artificially low signals from bands containing very large amounts of RNA (e.g., 28S), apparently due to inability of the weak β-emission to reach the screen in such areas. Labeling with $^{32}P_i$ results in stronger signals that can

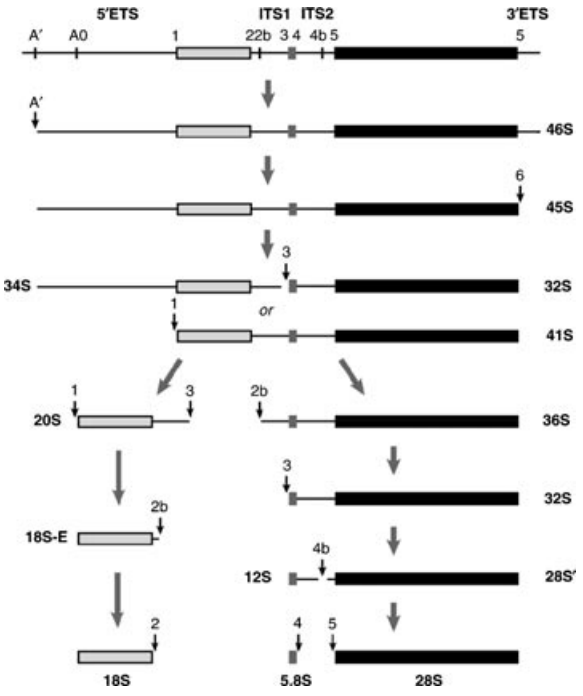


Figure 22.11.3 Structure of the primary transcript (47S) and major rRNA processing intermediates in mammals. The 47S pre-rRNA contains the sequences of mature 18S, 5.8S, and 28S rRNAs separated by the internal transcribed spacers (ITS1 and 2) and flanked by the external transcribed sequences (5'ETS, 3'ETS). Processing sites involved in the formation of each precursor are indicated. Adapted from Bowman et al. (1981) and Eichler and Craig (1994), with modifications.

be detected directly with autoradiography or using a phosphorimager.

[^{3}H]uridine labeling is the simplest type of rRNA labeling that does not require any special media or dialyzed serum, both of which are necessary for labeling with $^{32}P_i$ and [^{3}H]methyl-methionine (specialty media and sera are commercially available but could also be prepared in the laboratory). Pulse-chase labeling with [^{3}H]methyl-methionine is a highly sensitive technique that can often reveal more information about the temporal order and efficiency of different pre-rRNA processing events than other types of labeling.

For the isolation of radiolabeled RNA, commercial monophasic reagents containing phenol and guanidine thiocyanate are recommended because they require fewer manipulations than other methods, thus decreasing chances of accidental equipment contamination. Protocols for running RNA gels and northern blotting can be found in manuals such as *Current Protocols in Molecular Biology* (Brown et al., 2004).

Hybridization analysis of pre-rRNA allows unequivocal identification of precursor bands and is suitable for quantitative analysis of changes in steady-state levels of different processing intermediates. A readily detectable signal with rRNA precursors can be obtained on standard northern blots containing total cellular RNA using oligonucleotide probes ^{32}P-labeled at the 5′ termini with T4 polynucleotide kinase. A comprehensive analysis of pre-rRNA processing in a cell requires a series of hybridizations using probes that match different parts of spacer regions (5′ETS, 3′ETS, ITS1, ITS2) and mature rRNA sequences.

Processing of pre-rRNAs occurs within large ribonucleoprotein complexes that can be separated by centrifugation through sucrose gradients. The first studies that introduced this methodology date back to the 1960s (Warner and Soeiro, 1967). In addition to preribosomes, the gradients obtained from nuclear lysates contain other large complexes, including the abundant nuclear ribosomes, and may also contain contaminating ribosomal subunits from the cytoplasm. When gradient fractions are monitored for A_{254} to A_{260}, two prominent peaks of ~30S and ~50S are observed that are largely due to nuclear ribosomal subunits, whereas different preribosomal complexes are generally found in the faster sedimenting (55S to 100S) portion of the gradient.

The initial step in the isolation of preribosomal particles described in this unit involves preparation of nuclei by cell lysis using low concentrations of detergents in a hypotonic buffer. This method yields a relatively crude nuclear preparation, but is reliable and easy to perform. More complicated protocols may be needed if contamination with cytoplasmic material is of concern (*UNIT 3.10*). A number of techniques have been described to lyse the nuclei and release preribosomal particles. Many ribosome synthesis factors remain stably associated with preribosomal complexes under different conditions of isolation, but it is obvious that no single technique can preserve all interactions. The protocol in this unit uses sonication to disrupt nuclei in the presence of EDTA (Strezoska et al., 2000). A different approach in which nuclei are lysed in high salt buffer offers better protection of pre-rRNAs from endogenous ribonucleases but strips more proteins from preribosomes (Lapik et al., 2004).

Methods for preparing and running sucrose gradients and a discussion on the various types of equipment used for gradient fractionation can be found in *UNIT 5.3*. A typical setup for the analysis of preribosomes by upward displacement consists of a pump, an assembly for piercing the tube, a tube cap, a UV-monitoring cell, and a fraction collector. Fractionation of gradients by puncturing the tube and collecting drops from the bottom is not recommended as it leads to a considerable loss of resolution.

Critical Parameters and Troubleshooting

Metabolic labeling of rRNA

The rate of ribosome synthesis in mammalian cells strongly depends on the growth state of culture. A number of adverse conditions, such as contact inhibition, lack of growth factors or nutrients, or various chemical inhibitors including some transfection reagents, can inhibit ribosome biogenesis and greatly reduce incorporation of radioactive precursors into rRNA. Even under optimal conditions, kinetics of pre-rRNA processing may differ between cell lines. In particularly slow cell types, increasing the time of labeling to 2.5 to 3 hr may improve the signal detectable in mature rRNAs (especially 28S).

Gel analysis of large pre-rRNAs

Standard northern blotting procedures (Brown et al., 2004) apply to the analysis of rRNA and their precursors. Problems arising in the analysis of pre-rRNAs, such as smeared, poorly resolved bands, often result from gel overloading during electrophoresis. If samples are loaded based on their total cpm (e.g., to obtain similar intensities across the gel), it may

be useful to process one unlabeled RNA sample from an identical cell culture to estimate the total RNA amount obtained in the protocol. Loading 2 to 3 μg total RNA per lane is usually sufficient for a typical small- to medium-sized agarose gel. One should also keep in mind that rRNA precursors can be very large (the primary transcript in mammalian cells is >13 kb). Therefore, gels should be run for a sufficiently long time to ensure good separation.

Fluorography of ^{3}H-labeled rRNA

Exposure times necessary for detection of a weak ^{3}H signal after fluorography depend on multiple parameters, including type of labeling, total amount of radioactivity loaded, and the amount and uniformity of fluor coating. To obtain a high-quality picture, it is better to remove membranes from a relatively dilute fluor solution rather than try to evaporate the solvent almost to dryness. Excessive amounts of fluor residue left on the membrane may result in a spotty or blotchy signal after exposure to film. This can be corrected by repeating the solvent soaking procedure without additional spraying. Before re-exposing blots that have been already exposed for several days, recoating is recommended, as the fluor itself is volatile and tends to gradually disappear from a membrane starting from its edges. The sensitivity of film used for detection can also dramatically affect the exposure time.

Hybridization with pre-rRNA

Oligonucleotide probes used for hybridization must be reverse complementary to the regions of interest in the (pre)-rRNA. For optimal results with the protocol described in this unit, the oligonucleotide probes should be ~30 nucleotides in length and have a 50% to 70% GC content. Sequences that significantly deviate from these parameters may require an adjustment of the hybridization and/or wash temperatures. Sequences of probes designed for mouse pre-rRNA can be found in Strezoska et al. (2002) and Lapik et al. (2004). Synthetic oligonucleotides obtained from commercial sources can usually be used without additional purification. To allow for repeated reprobing of the same blot, it is recommended to use nylon membranes for blotting of the RNA. When a series of hybridizations is planned, one may wish to start with probes designed to detect those parts of pre-rRNA that are normally quickly degraded in the cell, such as 5′ETS or 3′ETS, because strong signals produced by mature rRNAs or highly abundant pre-rRNAs (e.g., 32S) can be difficult to eliminate completely by stripping the membrane.

Sucrose gradients

The efficient release of preribosomes from the sonicated nuclear material requires chelation of metal ions with EDTA and the presence of non-ionic detergents. Sonication conditions should be optimized for a particular instrument used. Insufficient sonication can result in inadequate nuclear breakage and reduced yield, while oversonication could lead to partial preribosome fragmentation. Although preribosomal complexes are relatively stable in sucrose gradient solutions, some degradation of pre-rRNAs due to endogenous RNase activity is commonly observed. To avoid introducing extraneous RNases, using RNase-free plasticware and reagents is strongly recommended. If a sonication probe is also used for processing bacterial cultures, it should be thoroughly cleaned before use.

Anticipated Results

Metabolic labeling of RNA in mammalian cells should yield a pattern of discrete bands corresponding to radiolabeled mature rRNAs and their precursors upon analysis by gel electrophoresis. Changes in pre-rRNA biosynthesis can manifest as (1) under- or over-accumulation of normal rRNA precursors, and/or (2) appearance of improperly processed (aberrant) rRNA intermediates. A series of hybridizations with oligonucleotide probes should provide the clues as to which processing steps are affected. Separation of nuclear extracts on sucrose gradients should yield two distinct major peaks corresponding to nuclear ribosomal subunits when the gradient is monitored for UV absorbance during fractionation. Many preribosome-associated proteins will co-sediment with large-molecular-weight complexes after separation; however, some factors may be detected in the top fractions of a gradient if they are loosely associated or require divalent cations for binding.

Time Considerations

Cells for rRNA analysis are typically plated 1 to 2 days in advance. Labeling with radioactive precursors and RNA isolation can be completed in 1 day. Detection of [^{3}H]-labeled RNA blotted onto a membrane by fluorography may take anywhere from 1 day to 1 to 2 weeks, depending on labeling efficiency, fluor coating, and film sensitivity. Hybridization of an RNA blot with one oligonucleotide probe requires

~3 days to complete if phosphorimaging is used for detection. Analysis of preribosomes by gradient centrifugation usually requires 3 to 5 days.

Literature Cited

Bowman, L.H., Rabin, B., and Schlessinger, D. 1981. Multiple ribosomal RNA cleavage pathways in mammalian cells. *Nucleic Acids Res.* 9:4951-4966.

Brown, T., Mackey, K., and Du, T. 2004. Analysis of RNA by northern and slot blot hybridization. *Curr. Protoc. Mol. Biol.* 67:4.9.1-4.9.19.

Eichler, D.C. and Craig, N. 1994. Processing of eukaryotic ribosomal RNA. *Prog. Nucl. Acid Res. Mol. Biol.* 49:197-239.

Lapik, Y.R., Fernandes, C.J., Lau, L.F., and Pestov, D.G. 2004. Physical and functional interaction between Pes1 and Bop1 in mammalian ribosome biogenesis. *Mol. Cell* 15:17-29.

Strezoska, Z., Pestov, D.G., and Lau, L.F. 2000. Bop1 is a mouse WD40 repeat nucleolar protein involved in 28S and 5 8S rRNA processing and 60S ribosome biogenesis. *Mol. Cell Biol.* 20:5516-5528.

Strezoska, Z., Pestov, D.G., and Lau, L.F. 2002. Functional inactivation of the mouse nucleolar protein Bop1 inhibits multiple steps in pre-rRNA processing and blocks cell cycle progression. *J. Biol. Chem.* 277:29617-29625.

Warner, J.R. and Soeiro, R. 1967. Nascent ribosomes from HeLa cells. *Proc. Natl. Acad. Sci. U.S.A.* 58:1984-1990.

Visualization and Measurement of DNA Methyltransferase Activity in Living Cells

Lothar Schermelleh,[1] Fabio Spada,[1] and Heinrich Leonhardt[1]

[1]Ludwig Maximilians University Munich (LMU), Department of Biology II, Martinsried, Germany

ABSTRACT

In this unit, a live-cell assay to measure DNA (cytosine-5) methyltransferase (MTase) activity at the single-cell level is described. This method takes advantage of the irreversible binding of enzymatically active MTases to genomic DNA substituted with the mechanism-based inhibitor 5-aza-2′-deoxycytidine (5-aza-dC). The procedure comprises incorporation of this nucleoside analog into DNA during replication and quantification of the time-dependent MTase immobilization by fluorescence recovery after photobleaching (FRAP). This trapping assay monitors kinetic properties and activity-dependent immobilization of MTases in their native environment and enables direct comparison of mutations and inhibitors that affect MTase regulation and catalytic activity in single living cells. In addition, a simplified protocol to obtain qualitative information on the activity of either endogenously or exogenously expressed MTases is provided. *Curr. Protoc. Cell Biol.* 39:22.12.1-22.12.16. © 2008 by John Wiley & Sons, Inc.

Keywords: DNA methylation • DNA (cytosine-5) methyltransferase • Dnmt1 • trapping assay • fluorescence recovery after photobleaching • 5-aza-2′-deoxycytidine • mechanism-based inhibitor

INTRODUCTION

In this unit, a method to assay enzymatic activity of DNA (cytosine-5) methyltransferases (MTases) in living cells, which also provides information on protein kinetics and subcellular localization, is described. The method relies on incorporation of 5-aza-2′-deoxycytidine (5-aza-dC, also known as decitabine), an analog of 2′-deoxycytidine (dC) that acts as a mechanism-based inhibitor for all MTases. 5-aza-dC is added to the growth medium and is incorporated into the DNA during replication in place of dC. Under normal conditions, the catalytic mechanism of MTases involves the formation of a transient covalent bond with the base ring in dC that is released after methyl group transfer (Fig. 22.12.1). When an MTase is engaged in methylation of 5-aza-dC, this covalent bond cannot be resolved and the enzyme becomes irreversibly trapped at the site of action. As more 5-aza-dC gets incorporated in replicating cells, the enzyme becomes progressively immobilized at postreplicative CpG sites. Thus, the MTase immobilization rate directly reflects the efficiency of the methylation process. The enzyme activity can be quantified by expressing fluorescent MTase fusions and measuring their mobility by fluorescence recovery after photobleaching (FRAP; Schermelleh et al., 2005). Alternatively, both endogenous MTases and exogenously expressed MTase constructs can be assayed by fixing the cells at different time points after addition of 5-aza-dC and subsequent immunostaining (see Alternate Protocol).

STRATEGIC PLANNING

The trapping assay (see Basic Protocol) requires tissue culture experience (*UNIT 1.1*), knowledge in handling of cells for live-cell microscopy, and experience in advanced confocal live-cell microscopy techniques, in particular photokinetic applications and image analysis (*UNITS 13.5 & 21.1*). Generation of MTase constructs (fluorescent fusions and/or

Current Protocols in Cell Biology 22.12.1-22.12.16, June 2008
Published online June 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2212s39

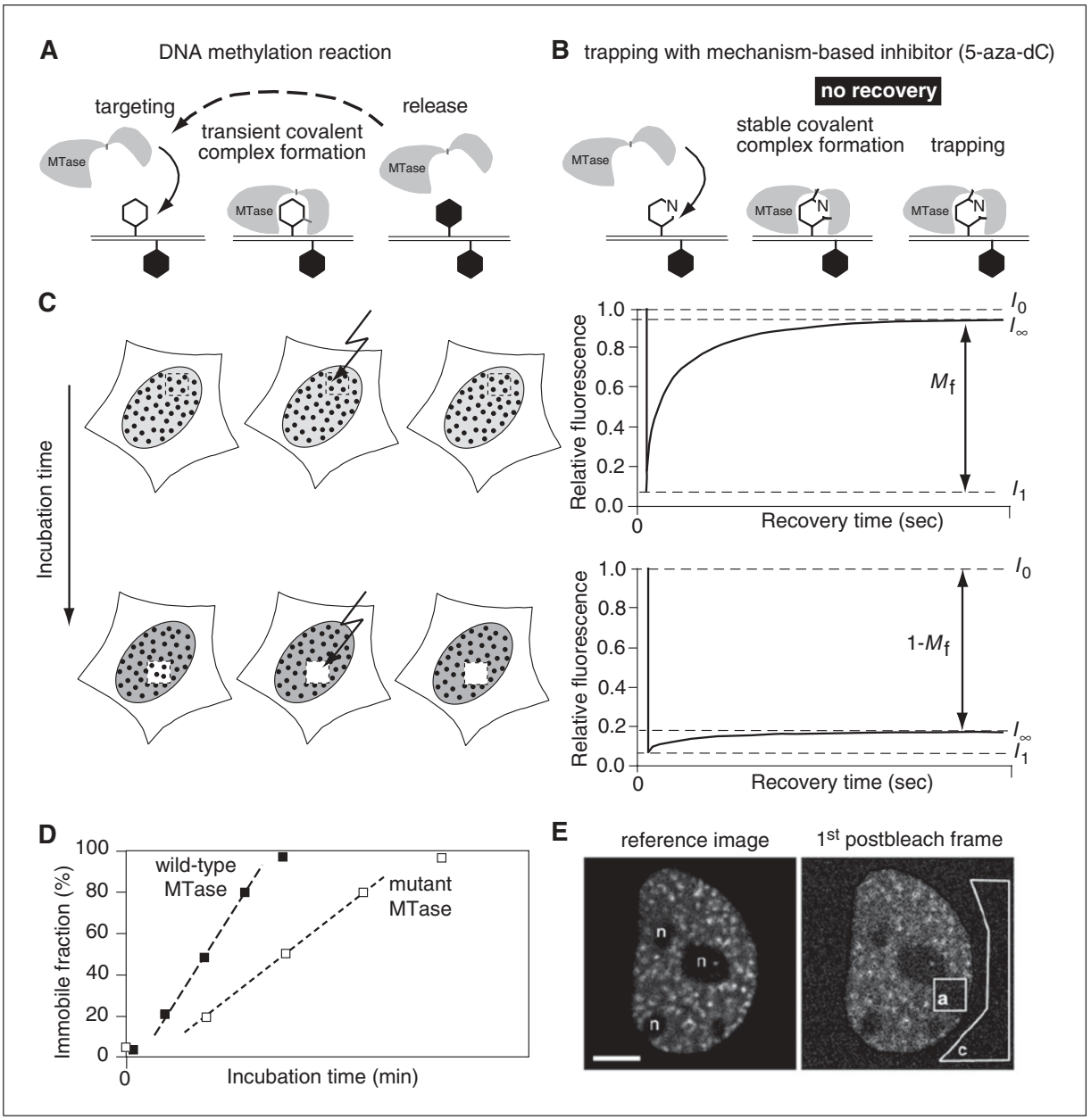


Figure 22.12.1 Principle of the trapping assay (shown here for the maintenance MTase Dnmt1 with hemimethylated CpG substrate sites). (**A**) Methylated and non-methylated cytosines are depicted as black and white hexagons, respectively. MTase binds to (hemimethylated) CpG sites produced during DNA replication and forms a transient covalent complex with the cytosine residue (gray line). After methyl group transfer, the enzyme is released and becomes available for another round of methylation (dotted arrow). (**B**) Mechanism-based inhibitors such as 5-aza-dC (hexagons with red N at position 5) are incorporated into DNA during S-phase. The MTase forms a stable covalent complex with 5-aza-dC (black line) and becomes trapped. (**C**) Dynamic exchange of MTases is visualized as fluorescence recovery after photobleaching of GFP-MTase in the outlined region (top row). Immobilization of GFP-MTase is detected as reduced recovery after photobleaching and decrease of mobile fraction (M_f) and increase of the immobile fraction, respectively (bottom row). Dots indicate MTase accumulation at replication foci (RF). Covalent trapping leads to an increase of the RF-associated fraction and a decrease of diffuse fraction. (**D**) To compare, e.g., specific mutant MTases with wild-type MTase, the immobile fractions (1 – M_f) are plotted as a function of incubation time. Such a comparison may also be performed simultaneously in single living cells when wild-type and mutant MTase tagged with spectrally distinct fluorescent proteins are co-expressed. (**E**) Example of ROI selection for quantitative FRAP evaluation. The left panel shows a high S/N reference image of an early S-phase mouse myoblast cell expressing GFP-Dnmt1 before bleaching (n, nucleoli). The right panel shows the corresponding first postbleach frame of the FRAP series. Bleach ROI (a), total ROI (b), and background ROI (c) are indicated. Bar = 5 μm. For color version of this figure, go to *http://www.currentprotocols.com*

specific mutants) and transfection in appropriate cell lines may be necessary (*UNITS 20.6, 20.7 & 21.4*). Establishment of stable cell lines may be considered as alternative to transient transfections (see Critical Parameters). Co-expression with a red fluorescent S-phase cell cycle marker (e.g., RFP-PCNA, RFP-DNA ligase I) is obligatory if the green fluorescent MTase fusion construct is not known to localize at replication sites during S-phase. The method described in the Alternate Protocol employs standard immunofluorescence detection techniques (*UNIT 4.3*) to yield qualitative data. It is technically less demanding and especially suited if only a standard epifluorescence microscope without FRAP capability is available.

FRAP-BASED QUANTITATIVE MEASUREMENT OF MTase ACTIVITY IN LIVING CELLS

BASIC PROTOCOL

The following protocol is based on routine procedures for the quantitative analysis of fluorescent Dnmt1 fusion constructs in mammalian cells and may require adaptation for different MTases, cell lines, and organisms.

The FRAP technique has been reviewed, e.g., in Lippincott-Schwartz et al. (2001). A detailed description of the FRAP method is given in *UNITS 13.5 & 21.1*.

Materials

Cultured cells
Expression vectors encoding MTase fusions with fluorescent proteins suitable for FRAP analysis (e.g., eGFP and RFP)
Transfection reagent (e.g., Transfectin, BioRad) or stably expressing cell line
5-Aza-2′-deoxycytidine (5-aza-dC; see recipe)

Live-cell chamber slides (e.g., Lab-Tek, Nunc or μ-Slides, Ibidi)
Confocal laser scanning microscope (CLSM) system (e.g., Leica TCS SP2/5 AOBS) suited for live-cell microscopy (inverted set up) equipped with the following:
High numerical aperture (1.2 to 1.4 NA) oil, glycerol, or water objectives (60×, 63×, or 100×)
High-power blue laser to provide a 488-nm line for bleaching and GFP excitation
Green laser (543-nm/561-nm) to excite red fluorescent protein
Temperature-controlled incubator box (recommended)
Motorized *xy*-stage (optional)

Prepare cells and carry out transient transfection

1. Seed cells in standard growth medium on live-cell chamber slides.

   In principle, any adherently growing cell line can be used. A normal to high proliferation rate and ease of transfection are advantageous.

   The seeding density depends on the cell line and should be chosen such that the culture reaches ~50% confluency at the desired time of transfection.

   Alternatively, perfusable live-cell chambers (e.g., FCS2 chamber, Bioptechs; POC-Chamber, LaCon) can be used.

2. Incubate cells until they have adhered on the slide surface and have reached ~50% confluence (from a few hours to overnight depending on the cell line).

3. Transiently transfect cells with expression vector (plasmid DNA) encoding a GFP-MTase fusion protein of choice.

   Optionally, co-transfect with RFP-PCNA or RFP-DNA ligase I if a reference marker of replication sites in S-phase cells is desired.

   The transfection method of choice must be determined and optimized for each cell line (see UNITS 20.6 & 20.7). Ideally, select transfection conditions that combine high transfection rates with moderate expression levels (see Critical Parameters).

4. Incubate cells for at least 16 hr, preferably 24 hr, to allow optimal expression of the fusion construct(s).

   Some transfection protocols require a medium exchange 2 to 4 hr after addition of the transfection mix. Depending on the following steps of the protocol, a change to the live-cell observation medium (without phenol red, with HEPES, see Critical Parameters) can be made at this stage.

Measure fluorescence recovery after photobleaching (FRAP)

5. For the confocal laser scanning microscope set up, set temperature of the incubator box (if available) to 37°C and allow the system to equilibrate for an appropriate time (~1 hr) to avoid thermal shifts during data acquisition. If an adjustable argon-ion laser is used to excite and bleach GFP, set it to maximum power.

6. Mount live cell chamber slide with transfected cells onto the microscope stage of the confocal laser scanning microscope.

   Alternatively, widefield or spinning disk confocal microscope systems equipped with a laser-based FRAP module may be employed.

7. Set CLSM parameters for FRAP measurements.

   For the Leica TCS SP2 AOBS (63×/1.4 oil objective, 75 mW argon-ion laser) and measurement of GFP-tagged Dnmt1 variants in mouse or human cells, the following settings are recommended (adaptation for other microscope systems and samples may be necessary; also see Critical Parameters). Scan size: 256 × 256 pixel; Zoom factor: 9.3 (resulting in a pixel size of 100 nm); Pinhole diameter: 3 Airy units; PMT settings should be kept constant with gain setting not exceeding 680 V; AOTF (transmission) setting of the 488-nm line may be varied from 2% to 5% depending on the brightness of the sample. Time intervals (minimum frame rate): 150 msec (using 1400-Hz scan speed and bidirectional scan) or 208 msec (1000-Hz, bidirectional scan); 10 to 20 prebleach frames, 1 to 2 bleach frames, 400 to 600 postbleach frames; for bleaching (using the FRAP-Wizard of the Leica software), select a 3 × 3–μm square ROI, set the 48-nm laser line to 100% transmission and activate "zoom in" option. Keep parameter settings constant for subsequent measurements.

   If the microscope is equipped with a motorized xy-stage, it can be useful to first search the sample for suitable S-phase cells and to store their positions before performing FRAP measurements. One cell may be FRAPed at several time points. Be aware, however, that photodamage induced by extensive bleaching and acquisition may slow down or even stall DNA replication. Consequently, 5-aza-dC incorporation and thus trapping can be negatively affected.

8. (Optional) Acquire a high-quality, high signal-to-noise ratio (S/N) reference image of all channels (GFP, RFP, transmission) using multiple averaging before recording the FRAP series.

9. Record reference FRAP series before 5-aza-dC treatment.

10. Add 5-aza-dC to the medium or, if a perfusable live-cell chamber is used, exchange with CO_2-adapted medium supplemented with the desired concentration of 5-aza-dC.

    Typically, a final concentration of 10 to 30 μM produces optimal results (see Critical Parameters).

    Note that 5-aza-dC is unstable in aqueous solution, thus effectiveness decreases when stored for more than a few days at 4°C. The 10 mM stock solution of 5-aza-dC should be dispensed into 0.2-ml aliquots and stored at least 1 year at −20°C.

11. Record FRAP series of ideally 10 or more cells during 5-aza-dC incubation.

    Take note of the exact incubation time after addition of 5-aza-dC for each series (see Critical Parameters).

Quantitatively evaluate data

FRAP may be evaluated using proprietary microscope software. Here, a procedure using the ImageJ image processing freeware (*http://rsb.info.nih.gov/ij/*) and a conventional spreadsheet program (e.g., Excel) is described.

12. Import FRAP data as image series into ImageJ (data may be converted to 8-bit grayscale).

13. Define the square evaluation region-of-interest (ROI) for the bleached region (bleach ROI) using the rectangular selection tool and measure mean gray values at all time points (Image → Stacks → Plot z-axis Profile) (Fig. 22.12.1E).

14. Define a correction ROI covering the total nucleus (total ROI) using the polygon selection tool and measure mean gray values.

    The nucleus must not shift laterally with respect to the ROI over the time course. Rather downsize the ROI so that it does not contain extra-nuclear (dark) pixels at any time point. Excessive lateral cell movement may be corrected with an image registration tool (e.g., StackReg Plug-in for ImageJ).

    Datasets of cells showing excessive z-drift should not be considered for evaluation.

15. Define background ROI covering a variable region outside of the cell. Measure mean gray values.

16. Transfer the numerical values to a spreadsheet program (e.g., Excel).

17. Within the spread sheet, create a column with the respective time stamp information (e.g., from the bleach series log-file). Set the first postbleach time point to 0 plus the time delay required to record the first frame.

18. For each time point subtract background value from the bleach ROI (B) and total ROI (T) values, respectively. Normalize postbleach ROI values by the mean of the last five prebleach values (B_0 and T_0) to compensate for gray level fluctuations.

    The first prebleach values of a FRAP series should not be considered, as the fluorescence typically drops and reaches a prebleach equilibrium after acquiring two to five image frames.

19. To correct for total loss of nuclear fluorescence over the time course (bleaching by image acquisition) and in some cases also for the gain of nuclear fluorescence due to import from the cytoplasm, multiply by the reciprocal of the total ROI values. The relative fluorescence intensity (normalized and corrected) at a given postbleach time point (I_t) is given by

$$I_t = \frac{B_t}{B_0} \times \frac{T_0}{T_t}$$

    where B_t and T_t represent the background subtracted values from the bleach ROI and the total ROI, respectively, at postbleach time point (t); B_0 and T_0 represent the mean of the last five prebleach values after background subtraction.

20. Determine the value I_∞, where the recovery curve reached its plateau (mean of the last values). Typically, the mobile fraction (M_f) is given by

$$M_f = \frac{I_\infty - I_1}{1 - I_1}$$

    where I_1 represents the first relative intensity value after bleaching (see Fig. 22.12.1C and Critical Parameters).

21. Plot the immobile fraction $1 - M_f$ of each FRAP series as a function of the 5-aza-dC incubation time.

Apply linear regression to the linear part of the plot (excluding initial ~5 min lag-phase and saturation effect near full immobilization) to compare different enzymes or mutants.

ALTERNATE PROTOCOL 1

QUALITATIVE MTase ACTIVITY ASSAY ON FIXED CELLS USING BrdU REPLICATION LABELING

For qualitative evaluation or if no FRAP equipment is available, cells co-expressing a GFP-MTase fusion and RFP-PCNA can be fixed at several time points after addition of 5-aza-dC. Alternatively, endogenous MTases can be detected by immunofluorescence in combination with BrdU replication labeling or PCNA immunostaining or combinations thereof (Fig. 22.12.2). The thymidine analog BrdU is incorporated into replicating DNA of S-phase cells. Thus, both PCNA and BrdU (when applied 15 to 30 min before fixation) mark sites of active DNA replication. Detection of incorporated BrdU requires denaturation of the genomic DNA. When combining BrdU labeling and visualization of GFP-tagged proteins, denaturation using DNase I is mandatory, as other protocols using HCl or NaOH destroy GFP fluorescence (also see *UNIT 4.3* for standard immunofluorescence staining and *UNIT 8.3* for DNA replication labeling with BrdU).

NOTE: All incubation steps and washes are carried out at room temperature unless stated otherwise.

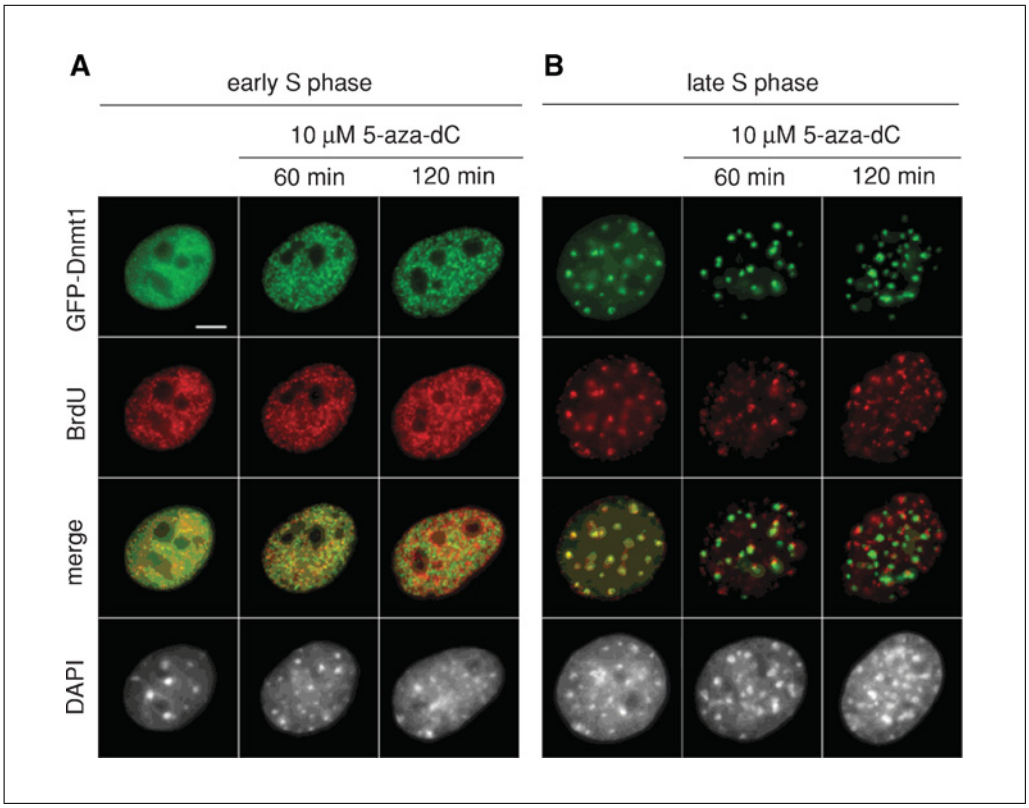


Figure 22.12.2 Fixed cell assay to visualize Dnmt1 immobilization after 5-aza-dC treatment. Typical early S-phase (**A**) and late S-phase nuclei (**B**) of mouse myoblast cells are shown. Cells were BrdU labeled and fixed without or after a 60- and 120-min treatment with 5-aza-dC. Replication foci were marked by BrdU immunostaining of nascent DNA. Note the stronger association of Dnmt1 at or near replication sites after 5-aza-dC treatment. At 120 min, a clear loss of co-localization is observed as the pool of initially mobile Dnmt1 is fully trapped at earlier replicated genomic DNA. The effect seems more pronounced in late S-phase, likely reflecting the higher density of methylated CpG sites in late replicating constitutive heterochromatin. Bar = 5 µm. For color version of this figure, go to *http://www.currentprotocols.com*

Materials

Cultured cells grown on coverslips of appropriate size (e.g., 15 × 15–mm) and thickness (e.g., 0.17 ± 0.01 mm) in small plastic petri dishes
5-Aza-2′-deoxycytidine (5-aza-dC; see recipe)
5-Bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich)
Phosphate-buffered saline (PBS, *APPENDIX 2A*)
Fixation solution (see recipe)
PBST (see recipe)
10% (w/v) Triton X-100 (*APPENDIX 2A*)
Blocking solution (see recipe)
1 M $MgCl_2$ (*APPENDIX 2A*)
1:1000 mouse anti-BrdU antibodies (e.g., MAb IU-4, CALTAG Labs)
DNase I (see recipe)
PBSTE (see recipe)
Green and/or red fluorescent secondary antibodies (e.g., Alexa Fluor 488 and 555 conjugates, Invitrogen)
4′,6-diamidino-2-phenylindole (DAPI, see recipe)
Anti-fade mounting medium (e.g., Vectashield, Vector Laboratories)
Transparent nail polish (base coat)

6-well plates or 35-mm culture dishes
Fine-tip forceps
37°C incubator
Glass microscope slides
CLSM or epifluorescence microscope system appropriately equipped with CCD camera, fluorescence filter sets, and high-NA objectives

Label cells

1. Grow cells to ≥50% confluence on coverslips (cleaned in 80% ethanol and placed individually in suitably sized cell culture dishes (6-well plates or 35-mm culture dishes).

   If required, transfect cells with any desired MTase construct the day before or use a stable cell line.

2. Add 5-aza-dC to the cell culture medium to a final concentration of 10 to 30 μM and incubate for different time periods (e.g., 30 min, 1 hr, 2 hr) until fixation. Leave one sample untreated as control.

3. Add BrdU to a final concentration of 10 μM 15 min before fixation.

Fix cells

4. Transfer the coverslips briefly to PBS and then rapidly to fixation solution. Incubate for 10 min.

   Fixation and subsequent washing steps may be performed in 6-well plates or 35-mm culture dishes.

   Drying of cells must be avoided at any step of the protocol. Make sure that coverslips always remain covered by a film of liquid when changing solutions. The mild detergent Tween 20 in the washing solution (PBST) acts as a surfactant to lower the surface tension and thus helps to avoid drying artifacts.

   For transferring coverslips, use fine-tip forceps.

5. Exchange fixation solution with PBST.

   To avoid fixation artifacts, such as wrinkled nuclear morphology, first remove only ∼2/3 of the fixation solution and fill up with PBST before complete solution exchange with PBST.

   At this stage, cells may be stored overnight at 4°C.

Permeabilize cells and expose to antibodies

6. Permeabilize cells with 0.5% Triton X-100 in PBS for 5 min.
7. Incubate in blocking solution for 10 min.
8. Incubate with mouse anti-BrdU primary antibody solution (appropriate dilution in blocking solution containing 5 mM $MgCl_2$ and 10 U/ml DNase I) for 30 min at 37°C.

   There may be batch-to-batch variations of DNase I activity and the optimal concentration may vary according to the cell type/tissue. Optimal DNase I concentrations usually range from 5 to 20 U/ml and should be determined by titration in advance.

   Place drops of the antibody solution (typically 100 μl for 15 × 15–mm coverslips) onto a piece of Parafilm (flattened on a smooth plastic surface) and then place the coverslips cell-side down on the drops. Incubation should be performed in a dark humid chamber to prevent drying and fluorescence fading.
9. Wash three times, 5 min each time, with PBSTE.
10. Incubate with 1 μg/ml of secondary antibodies (typically 1:500 to 1:2000) in blocking solution for 1 hr at room temperature.
11. Wash thoroughly with PBST with several exchanges of the washing buffer.

    Extensive washing reduces background fluorescence of the sample and increases the signal-to-noise ratio.

Counterstain and mount

12. Counterstain nuclei with 50 ng/ml DAPI in PBST for 5 min and rinse briefly in PBST and then finally in water to remove salts.
13. Mount cells by adding a drop of antifade medium onto a glass microscope slide and quickly placing coverslip cell-side down onto the drop. Gently remove excess solution with soft tissue paper before sealing edges of coverslip with transparent nail polish.

ALTERNATE PROTOCOL 2

IMMUNODETECT PCNA

PCNA immunodetection may be performed as an alternative to BrdU labeling. Note, however, that with most if not all commercially available PCNA antibodies (e.g., MAb PC-10), a methanol treatment is required for epitope binding. In this case, replace Alternate Protocol 1, step 6, with a 5-min incubation with 100% ice-cold methanol followed by an exchange with PBST. In Alternate Protocol 1, step 8, prepare antibody solution without DNase I and $MgCl_2$ and incubate 1 hr at room temperature.

ALTERNATE PROTOCOL 3

IMMUNODETECT ENDOGENOUS MTases

Endogenous MTases may be detected by immunostaining as an alternative to exogenous expression of GFP fusion constructs. In this case, add a specific MTase antibody at an appropriate concentration to the primary antibody solution (see Alternate Protocol 1, step 8) and a matching green fluorescent secondary antibody to the secondary antibody solution (see Alternate Protocol 1, step 9). For this two-color immunostaining, care must be taken that the secondary antibodies do not show any cross-species reactivity. To avoid potential problems, use highly cross-adsorbed antibodies and/or perform cross-reactivity controls before using a specific antibody combination for the first time.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

5-aza-dC, 100 mM

10 mg 5-Aza-2′-deoxycytidine (5-aza-dC, dry chemical; Sigma-Aldrich)
438 μl DMSO

For 10 mM 5-aza-dC
100 mM stock solution diluted 1:10 in PBS

Store in 0.2-ml aliquots at least 1 year at −20°C

Blocking solution

2% (w/v) bovine serum albumin (BSA fraction V Sigma) in PBST (see recipe)
Store up to 1 month at 4°C

DAPI stock solution

5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma) in water

Store indefinitely at 4°C
To make up DAPI working solution:
50 ng/ml DAPI stock solution in PBST (see recipe)

Store up to 1 month at 4°C

DNase I stock solution

10,000 to 20,000 U/ml DNase I (code D; Worthington)
5 mM sodium acetate, adjusted to pH 4.5
1 mM $CaCl_2$
50% (v/v) glycerol
Mix gently, do not vortex
Store in 1-ml aliquots at least 1 year at −20°C

Fixation solution

37% reagent-grade formaldehyde solution 1:10 diluted in PBS
Prepare fresh
Alternatively, freshly prepare 4% para-formaldehyde (Sigma) in PBS.

PBST

0.05% Tween 20 in PBS
Store up to 6 months at room temperature

PBSTE

1 mM EDTA in PBST (see recipe)
Store up to 6 months at room temperature

COMMENTARY

Background Information

DNA methylation is essential for establishment and/or maintenance of cell lineage–specific expression patterns as part of an intricate network of epigenetic mechanisms. Thus, by affecting the transcriptional potential of genes, DNA methylation plays an important role in many fundamental processes such as cell differentiation, embryonic development, genome stability (silencing of transposable elements), neoplastic transformation, imprinting, and X chromosome inactivation (Bird, 2002). In metazoans, DNA (cytosine-5) methyltransferase (MTase) activity is the only detectable DNA methyltransferase activity and nearly all methylated cytosines occur as CpG dinucleotides. Four catalytically active DNA methyltransferases (Dnmts) have been

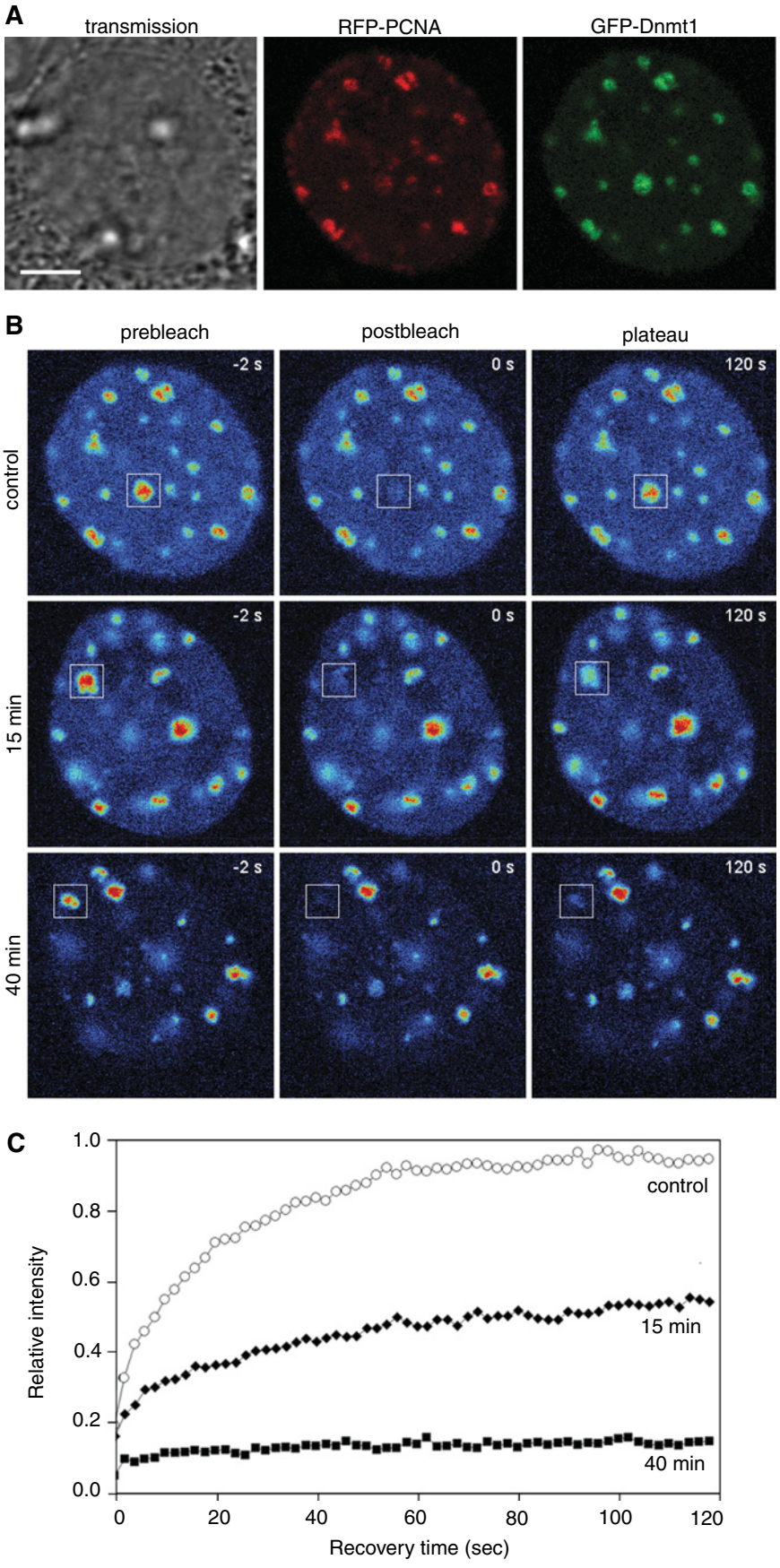


Figure 22.12.3 Legend at right.

described. Dnmt1 is the most abundant and ubiquitous enzyme and it is mainly responsible for maintaining genomic methylation patterns through successive DNA replication cycles. Dnmt2 shows very weak DNA methyltransferase activity, may be responsible for cytosine methylation in sequences other than CpG, and is involved in methylation of cytoplasmic $tRNA^{Asp}$. Dnmt3a and b are mainly responsible for de novo establishment of methylation patterns during embryonic development and maturation of germ cells (Hermann et al., 2004; Goll and Bestor, 2005).

Classically, MTase function is studied by knock-out experiments (Lei et al., 1996; Okano et al., 1998, 1999; Jackson-Grusby et al., 2001; Chen et al., 2007). Exogenous expression of the MTase and variants thereof in the respective knock-out cells (rescue/complementation) can also be used to gain further insight in the regulation of enzyme activity (Schermelleh et al., 2007). These studies are laborious and require extensive experimental set up for generation of knock-out and complemented cell lines or mouse models and analysis of genomic methylation patterns. In addition, these studies do not provide kinetic data. Enzyme kinetics is usually assayed in vitro under highly artificial conditions that cannot reproduce the complexity of the interactions occurring in vivo between MTases and their cofactors or their native chromatin substrate. The method presented here allows analysis of MTase activity in the native cellular environment and at the same time provides information on protein kinetics and subcellular localization of the enzyme with an experimental schedule of ~2 days (see Time Considerations).

The catalytic mechanism of MTases involves the formation of a transient covalent bond with position 6 of the cytosine ring, which is released by β-elimination after methyl group transfer. When an MTase is engaged in methylation of 5-aza-dC, the nitrogen atom at position 5 of the base lacks the hydrogen atom necessary for β-elimination and the enzyme is covalently trapped at the site of action. As more 5-aza-dC is incorporated into replicating cells, the initially free pool of enzyme becomes progressively immobilized at postreplicative CpG sites (Fig. 22.12.1).

Routinely, the procedure described in the Basic Protocol is used to assay postreplicative methyltransferase activity of wild-type and mutant DNA methyltransferase 1 (Dnmt1) constructs fused to GFP (GFP−Dnmt1), which are expressed either transiently or stably in different cultured cell lines (Schermelleh et al., 2005, 2007; Spada et al., 2007; Fig. 22.12.3). Co-expression of RFP-PCNA may serve as a marker of replication sites. Proliferating cell nuclear antigen (PCNA) is a core component of the replication machinery forming a trimeric ring around the DNA at the replication fork, which serves as a loading platform for various replication factors, such as DNA ligase I, pol δ, and others (Maga and Hubscher, 2003; Sporbert et al., 2005).

However, this method may be used to assay any MTase provided that is relatively mobile, as enzymes with very slow protein kinetics would not allow reliable measurement of trapping rates. This experimental strategy allows direct comparison of enzyme kinetics and subcellular localization before and after addition of 5-aza-dC within the same cell. Alternate Protocol 1 provides qualitative assessment of MTase activity and subcellular localization without the use of expensive and sophisticated equipment for imaging and photobleaching (Fig. 22.12.3).

The MTase trapping assay described here is set to be a useful tool not only for detailed studies on the properties of MTases but also for screening potential DNA methyltransferase inhibitors. When a functional MTase inhibitor (e.g., a small molecule inhibitor that blocks the catalytic center) is added to the cells along with 5-aza-dC it is expected to decrease or prevent

Figure 22.12.3 FRAP analysis of the Dnmt1 mobility before and during 5-aza-dC treatment in a single living cell. (**A**) Reference image (confocal mid section) of a mouse myoblast cell in late S-phase co-transfected with RFP-PCNA and GFP-Dnmt1 (wild-type). PCNA and Dnmt1 largely co-localize at replication foci. Bar = 5 μm. (**B**) FRAP of GFP-Dnmt1 (shown in false color) localized at a late replication focus is shown before treatment (control), as well as after a 15- and 40-min incubation of the same nucleus with 30 μM 5-aza-dC. The last prebleach, the postbleach and the final frame of each series is shown. Boxes indicate the bleach ROIs. FRAP series were recorded at different *z*-levels. Note the continuous loss of the diffuse fraction with longer incubation time. (**C**) Quantitative evaluation of the FRAP series shown in B demonstrating the decrease of the mobile fraction with 5-aza-dC incubation. For color version of this figure, go to *http://www.currentprotocol.com*

trapping of the enzyme at 5-aza-substituted CpG sites. As aberrant methylation patterns have been involved in the generation and/or progression of different tumor types, screening of potential DNA methyltransferase inhibitors would be helpful for drug development and evaluation as therapeutic agents.

Critical Parameters and Troubleshooting

Expression of fluorescent fusion proteins

For expression in mammalian cells, constructs where the cDNA coding for the MTase of interest is cloned into the pEGFP−C1 vector (Clontech) are routinely used. A clean plasmid DNA preparation is essential for good transfection rates. Transient transfection protocols are usually selected and optimized for a given cell line to provide high transfection rates, decent expression levels, and low toxicity. Unlike in biochemical assays, the optimal expression levels of GFP-fusion proteins for live-cell microscopy are rather moderate (ideally in the range of the respective endogenous proteins). Overexpressing cells showing typical artifacts like additional cytoplasmic signal or atypically large replication foci should not be considered for kinetic measurements. As the assay requires actively replicating cells, a high number of S-phase cells is desirable. Also, the relative number of transfected cells in S-phase should be in the range of untreated cells as an indication of their well-being. The use of stable cell lines should be considered to avoid problems arising from low or variable transfection rates and heterogeneous expression. Although the establishment of stable cell lines requires considerable time (2 to 4 weeks), it offers the advantage of nearly 100% expressing cells and the possibility of selecting for defined ratios of endogenous to exogenous protein levels.

Live cell imaging conditions

In principle, any standard culture medium can be used during live cell observation. However, using a medium without phenol red, to reduce autofluorescence, is recommended. If an "open" live-cell chamber system is used, medium containing 25 mM HEPES may be advantageous to avoid an increase in pH when imaging for extended time periods without a CO_2 supply (>2 hr). Experiments with mammalian cell lines should ideally be performed at a physiological temperature of 37°C; however, most cell lines will proliferate and replicate at room temperature, although at a reduced rate, which will likewise affect trapping rates. Thus, for direct comparability of experimental results, the temperature stability is maybe more important than the absolute temperature. This is important if no temperature-controlled incubator box or live-cell chamber is available.

Phototoxicity

Extensive illumination with an HBO mercury light source should be avoided (e.g., when searching for suitable cells using the epifluorescence light path of the confocal microscope). Epifluorescence filters are usually leaky in the range of the intense UV excitation peak of the HBO lamp, which likely induces unwanted photodamage even before recording of FRAP series. Thus, keeping the exposure with HBO light as short as possible or using the laser scanning mode of the microscope to select for suitable cells is recommended. Alternative light sources (e.g., standard halogen lamp) in combination with an additional UV filter(s) may also be considered (Chuang et al., 2006).

CLSM settings for FRAP experiments

Scan region size, scan speed, laser power, and gain settings should be selected to strike an optimal balance between resolution, frame rate, bleaching by image acquisition, and signal-to-noise (S/N) ratio. To increase frame rate, the image size should be reduced (e.g., 256 × 256 pixel). In this case, the zoom factor should be set to obtain a pixel size of 100 nm providing a scan region that usually covers typically sized mammalian cell nuclei while keeping the image resolution relatively high. To increase signal (at the cost of reduced *z*-sectioning), the pinhole may be opened to some extent (e.g., 3 Airy units instead of 1 as typical for standard imaging).

Generally, PMT gain and offset settings should be set so that the dynamic range (usually 8-bit, i.e., 0 to 255 gray values) is fully used, avoiding pixel saturation (use false color LUT to display pixels with values beyond the dynamic range). The PMT setting should be a reasonable compromise between signal strength and electronic noise and should be kept constant between individual measurements. To accommodate differences in sample brightness, the AOTF (transmission) setting of the 488-nm laser line should be varied rather than PMT gain setting. Here, the limit is set by the bleaching caused by image acquisition over the time course. As a rule of thumb, this "bleaching-by-acquisition" should not exceed 30%.

At least ten prebleach time points should be recorded to measure the prebleach equilibrium before disturbance by the actual bleaching pulse. Time intervals between postbleach time points are typically set between 100 and 500 msec, depending on the mobility of the MTase and limits of the imaging system. The number of postbleach time points should be chosen to allow the fluorescence to reach its plateau of recovery with the selected intervals/frame rates. When measuring fast kinetics, the time interval between the last bleach frame and the first postbleach frame should be as short as possible since fast diffusing molecules invading the bleached region during this short time period may, in extreme cases, affect the calculated mobile fraction. Intervals between subsequent postbleach time points may be extended if this option is available.

The bleach region of interest (ROI) should be set to a suitable size (e.g., 3 × 3 μm) covering a representative number of replication foci or binding sites (also see below). For bleaching, set the 488-nm laser line to 100% transmission (additional laser lines below 500 nm may also be set to 100% if available) and choose the "zoom in" option (Leica TCS SP2/5) or similar options, if available, to provide maximum bleaching efficiency. Bleaching efficiency and number of bleaching iterations should be determined in advance on a fixed sample (do not mount this sample in antifade medium). The first postbleach (mean gray) value of the bleach ROI should be as close as possible to the background level (i.e., I_1 should be near 0; see Fig. 22.12.1C) using the shortest and most intense bleach pulse possible (i.e., with a minimum number of iterations and total bleaching time not exceeding 1/10 of the half recovery time). This critically depends on the technical features of the CLSM system, in particular, maximal laser power to provide fast bleaching and scan speed to provide minimum delay between bleaching and first postbleach acquisition.

Factors affecting trapping rate

Time-dependent immobilization of a given MTase fusion construct measured with the trapping assay is related to the enzyme activity/efficiency, but also to the amount of (hemimethylated) 5-aza-substituted CpG sites produced during replication (Fig. 22.12.4). The number of "traps" set within a given time is itself a function of various parameters that include: (1) DNA-synthesis rate, which might be influenced by environmental conditions, the state of the culture, and of selected cells. (2) Density of (methylated) CpG sites that may vary considerably from early replicating euchromatin to late replicating constitutive heterochromatin and among cell types or species. The selection of the bleach-ROI indirectly also relates to this parameter, as the "traps" may not be homogeneously distributed, but locally concentrated at replication foci (RF) and hardly present in nucleoli. Thus, bleach-ROIs in different FRAP-measurements should contain a similar ratio of RF associated to diffuse enzyme. (3) The dose and incorporation efficiency of the mechanism-based inhibitor used (and possibly also the affinity of the MTase for the inhibitor). The 5-aza-dC inhibitor is likely the most potent mechanism-based inhibitor of MTases. Unlike the related (and widely used) inhibitor 5-aza-C, this precursor does not require reduction of the ribose and is also not incorporated into RNA explaining the approximately five-fold higher effectiveness of 5-aza-dC over 5-aza-C (Jones and Taylor, 1980; Schermelleh et al., 2005). Typically, when used as a demethylating agent, a much lower 5-aza-dC concentration (in the range of 0.1 to 0.3 μM) is applied and its effects are assayed after longer periods of time. For the trapping assay, typically, an ~100-fold higher concentration (10 to 30 μM) is applied to approach a maximum substitution rate in the newly synthesized DNA strand. Higher 5-aza-dC concentrations do not lead to a further (linear) increase in trapping rates, indicating a saturation effect (L. Schermelleh, F. Spada, and H. Leonhardt, unpub. observ.). This relatively high concentration ensures that an initially free pool of fluorescently labeled, enzymatically active MTase molecules becomes fully immobilized within a fraction of the S-phase. Nevertheless, one has to be aware that prolonged incubation with 5-aza-dC may result in cytotoxic effects likely due to the formation of bulky DNA-MTase adducts (Juttermann et al., 1994; L. Schermelleh, F. Spada, and H. Leonhardt, unpub. observ.). Therefore, experiments with cells that have been incubated in the presence of 5-aza-dC at high concentrations for several hours should be considered with caution.

Less clear is the effect of differences in expression levels and absolute numbers of exogenously and endogenously expressed MTase molecules. In the authors' experience, the assay is rather robust against variation of GFP-Dnmt1 fusion protein levels, which might be explained by an excess of traps over the absolute amount of MTase molecules.

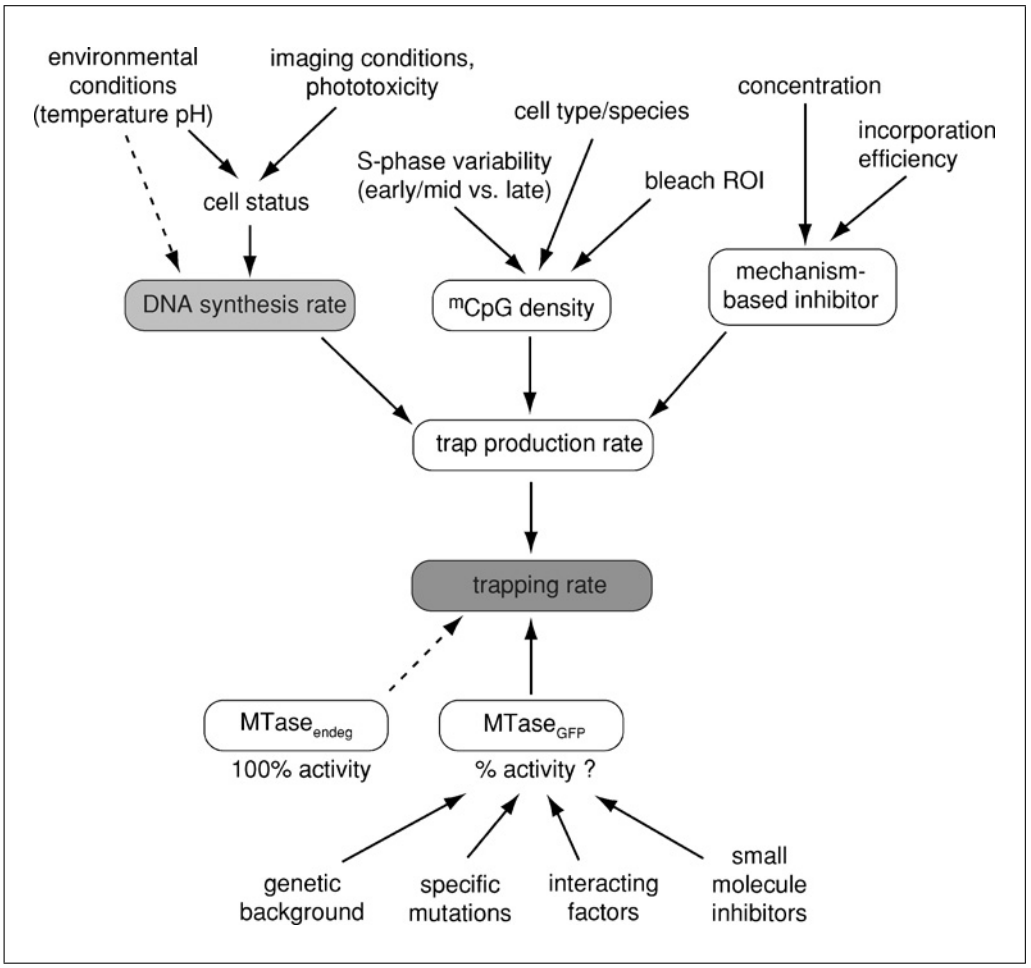


Figure 22.12.4 Summary of factors affecting the trapping rate of GFP-MTase fusion proteins. For comparability of results, experimental conditions that potentially affect the trap production rate should be kept constant. Two enzymes may also be directly compared side-by-side in single living cells using, e.g., red and green fluorescent fusions (see text for details).

When assaying MTase activity variations (e.g., as consequence of a specific mutation, catalytic inhibition by small molecules, or specific gene knock outs/downs), all other possible variables, such as cell type, cell cycle stage, bleach ROI, drug concentration, etc., should be kept as constant as possible to obtain meaningful and comparable results. On the other hand, the method also offers the possibility to assay other variables by using the same GFP construct, thus broadening the potential applicability of the trapping assay (Fig. 22.12.4).

Protocol variations and optimizations

The trapping assay also offers the possibility to test wild-type and mutant MTase simultaneously if one is labeled with GFP and the other with RFP. As an alternative to the classical FRAP approach, the trapping assay may be performed using a photoactivation approach with photoactivatible GFP (paGFP) fusions and a microscope system additionally equipped with a near-UV laser (405-nm diode laser; Schermelleh et al., 2005). Here, co-transfection with a red fluorescent cell cycle marker (e.g., RFP-PCNA) is mandatory for identifying S-phase cells and defining the photoactivation ROI.

The use of a motorized and programmable *xy*-stage, multi-well chamber slides (e.g., 6-well μ-slides, Ibidi), sophisticated software/macro programming (for semi-automation of different steps, such as target cell recognition, data acquisition and evaluation) are possible and should help raising the throughput, e.g., for screening of potential MTase inhibitors.

Anticipated Results

With regard to an anticipated outcome of a single FRAP experiment, most fluorescence MTase constructs that have been tested and that have shown reasonable mobility, reach the recovery plateau within <2 min. Plotting the

immobile fractions from all acquired FRAP curves against incubation time typically shows a short lag-phase of <5 min after adding the drug, until its incorporation into DNA. Also, a small saturation effect sometimes becomes obvious at late time points when the depletion of the mobile fraction is nearly completed.

Maintenance DNA methyltransferase 1 (Dnmt1) is likely the most efficient MTase on postreplicative DNA. Thus, trapping of GFP-tagged wild-type Dnmt1 (GFP-Dnmt1) can be considered a positive control. It has been previously shown that in various mouse and human cell lines (C2C12, SH-EP, HeLa), full immobilization of GFP-Dnmt1 is reached between 30 and 45 min when incubated with 30 μM of 5-aza-dC (Schermelleh et al., 2005, 2007; Spada et al., 2007). In contrast, a mutant Dnmt1 construct lacking the PCNA-binding domain (PBD), which targets the enzyme to the replication machinery, requires about a two times longer incubation for complete immobilization, reflecting the reduced methylation efficiency of these mutants in vivo (Schermelleh et al., 2007; Spada et al., 2007).

Time Considerations

The FRAP-based trapping requires seeding of cells on live-cell chamber slides, which is typically done the day before transient transfection (2 days before the actual trapping assay). Transient transfection typically requires ~1 hr hands-on work and an optional medium exchange 2 to 4 hr after adding the plasmid DNA-transfection reagent mix onto cells. The FRAP experiments should ideally be performed 24 hr after transient transfection. Several hours (depending on the number of experiments) should be scheduled for recording a FRAP series. A preparation lead time required to let the temperature of the microscope system stabilize, find the optimal CLSM settings, and (visually) screen the sample for suitable cells, should be taken into account. These steps may require up to 2 hr before recording the FRAP series of untreated cells. The length of a FRAP measurement depends on the mobility of the measured construct, i.e., how fast postbleach equilibration of fluorescence is reached. In the authors' experiments with human or mouse Dnmt1 fusion constructs, 2 min are usually sufficient. Progressive immobilization of MTase may be assayed over 0.5 to 2 hr depending on the construct, cell type, and cell cycle stage (early or late S).

Literature Cited

Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev.* 16:6-21.

Chen, T., Hevi, S., Gay, F., Tsujimoto, N., He, T., Zhang, B., Ueda, Y., and Li, E. 2007. Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. *Nat. Genet.* 39:391.

Chuang, C.H., Carpenter, A.E., Fuchsova, B., Johnson, T., de Lanerolle, P., and Belmont, A.S. 2006. Long-range directional movement of an interphase chromosome site. *Curr. Biol.* 16:825-831.

Goll, M.G. and Bestor, T.H. 2005. Eukaryotic cytosine methyltransferases. *Annu. Rev. Biochem.* 74:481-514.

Hermann, A., Gowher, H., and Jeltsch, A. 2004. Biochemistry and biology of mammalian DNA methyltransferases. *Cell Mol. Life Sci.* 61:2571-2587.

Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E., and Jaenisch, R. 2001. Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. *Nat. Genet.* 27:31-39.

Jones, P.A. and Taylor, S.M. 1980. Cellular differentiation, cytidine analogs and DNA methylation. *Cell.* 20:85-93.

Juttermann, R., Li, E., and Jaenisch, R. 1994. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl. Acad. Sci. U.S.A.* 91:11797-11801.

Lei, H., Oh, S., Okano, M., Juttermann, R., Goss, K., Jaenisch, R., and Li, E. 1996. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development.* 122:3195-3205.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. 2001. Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* 2:444-456.

Maga, G. and Hubscher, U. 2003. Proliferating cell nuclear antigen (PCNA): A dancer with many partners. *J. Cell Sci.* 116:3051-3060.

Okano, M., Xie, S., and Li, E. 1998. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res.* 26:2536-2540.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell.* 99:247-257.

Schermelleh, L., Spada, F., Easwaran, H.P., Zolghadr, K., Margot, J.B., Cardoso, M.C., and Leonhardt, H. 2005. Trapped in action: Direct visualization of DNA methyltransferase activity in living cells. *Nat. Methods* 2:751-756.

Schermelleh, L., Haemmer, A., Spada, F., Rösing, N., Meilinger, D., Rothbauer, U., Cardoso, M.C., and Leonhardt, H. 2007. Dynamics of Dnmt1 interaction with the replication machinery and its role in postreplicative maintenance of DNA methylation. *Nucleic Acids Res.* 35:4301-4312.

Spada, F., Haemmer, A., Kuch, D., Rothbauer, U., Schermelleh, L., Kremmer, E., Carell, T., Langst, G., and Leonhardt, H. 2007. Dnmt1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells. *J. Cell Biol.* 176:565-571.

Sporbert, A., Domaing, P., Leonhardt, H., and Cardoso, M.C. 2005. PCNA acts as a stationary loading platform for transiently interacting Okazaki fragment maturation proteins. *Nucleic Acids Res.* 33:3521-3528.

Monitoring mRNA Export

Kazuaki Tokunaga[1] and Tokio Tani[1]

[1]Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Kurokami, Kumamoto, Japan

ABSTRACT

Transport of mRNA from the nucleus to the cytoplasm is an essential process for gene expression in eukaryotic cells. In this unit, methods for monitoring nuclear mRNA export are described. Visualization of cellular mRNAs by fluorescence in situ hybridization with oligo(dT) probes is effectively applied to monitoring mRNA export from the nucleus in yeast and mammalian cells. In addition to the protocols for fluorescence in situ hybridization, this unit includes an alternate method that the authors have been developing for visual analysis of nuclear mRNA export in living mammalian cells by microinjection of fluorescently labeled pre-mRNA into the nuclei. *Curr. Protoc. Cell Biol.* 41:22.13.1-22.13.20. © 2008 by John Wiley & Sons, Inc.

Keywords: mRNA • transport • in situ hybridization • microinjection • yeast • mammalian cells

INTRODUCTION

This unit contains protocols for monitoring transport of mRNA from the nucleus to the cytoplasm in yeast and mammalian cells. The first three protocols describe commonly used methods for monitoring mRNA export by fluorescence in situ hybridization with oligo(dT) probes that bind to the poly(A) tail of mRNA in the fission yeast *Schizosaccharomyces pombe* (Basic Protocol 1), the budding yeast *Saccharomyces cerevisiae* (Alternate Protocol 1), and mammalian cells (Alternate Protocol 2). The fourth protocol describes an alternate method for visual monitoring of mRNA export in a living mammalian cell by nuclear microinjection of fluorescently labeled pre-mRNAs that are synthesized in vitro (Basic Protocol 2).

NOTE: All steps in the following protocols must be performed using RNase-free, DEPC-treated water and all solutions must be prepared with RNase-free DEPC-treated water (see recipe in *APPENDIX 2A*). Also see *APPENDIX 2A* for general considerations when working with RNA.

MONITORING NUCLEAR mRNA EXPORT BY FLUORESCENCE IN SITU HYBRIDIZATION IN FISSION YEAST

BASIC PROTOCOL 1

Inhibition of mRNA export from the nucleus to the cytoplasm results in accumulation of mRNAs in the nucleus and concomitant decrease in cytoplasmic mRNAs. Based on such changes in cellular mRNA distribution, we can monitor nuclear mRNA export in eukaryotic cells. This protocol describes a procedure for fluorescence in situ hybridization using a biotin-labeled oligo(dT) probe to visualize the distribution of mRNAs in the fission yeast *Schizosaccharomyces pombe.* The oligo(dT) used as a probe hybridizes specifically to the poly(A) tail of mRNA.

The experiment can be divided into three stages. First, cells to be analyzed are fixed with paraformaldehyde and their cell walls are lysed with enzymes. The cells are then adhered onto a slide, followed by permeabilization with ethanol dehydration. In the second stage, after a short incubation with a prehybridization solution, cells are incubated with a hybridization solution, which allows the biotin-labeled oligo(dT) probe to bind to the

Current Protocols in Cell Biology 22.13.1-22.13.20, December 2008
Published online December 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2213s41

poly(A) tails of mRNAs in the fixed cells. In the final stage of the experiment, the cells are washed with a series of sodium chloride/sodium citrate (SSC) solutions to remove unbound probe, then treated with avidin-FITC to visualize cellular mRNAs hybridized with the biotin-labeled probe.

Materials

S. pombe cells to be analyzed (e.g., wild-type strain *972* or temperature-sensitive export mutant strain *ptr8*; both strains available from Dr. Tokio Tani, Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Japan)
YE medium (see recipe)
4% paraformaldehyde solution (see recipe; freshly prepared)
0.1 M sodium phosphate buffer, pH 6.0 (*APPENDIX 2A*)
PEMS (see recipe), ice cold
Novozym (*http://www.novozymes.com*) or lysing enzyme from *Trichoderma harzanum* (Sigma, cat. no. L1412)
Zymolyase 100T (ICN Biomedicals)
70%, 90%, and 100% ethanol
Prehybridization solution for yeast (see recipe)
Hybridization solution for yeast: 1 ng/μl biotin-labeled oligo(dT) probe (Support Protocol 1) in prehybridization solution for yeast (see recipe); store at –20°C
4× SSC (see recipe for 20×)
2 μg/ml fluorescein isothiocyanate (FITC)–conjugated avidin (Roche, cat. no. 1975595) in 4× SSC/1% (w/v) RNase-free BSA (prepare fresh)
4× SSC/0.1% (v/v) Triton X-100 (prepare from 0.5% Triton stock, see recipe)
0.1 μg/ml DAPI solution (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Mounting medium (see recipe)
Clear nail polish

30° (for wild-type yeast) or 26°C (for temperature-sensitive mutants) shaking incubator
Refrigerated microcentrifuge with swinging-bucket rotor (e.g., Kubota model MF-2036)
Phase-contrast microscope
Coplin jars
Multi-well slide coated with poly-L-lysine (see recipe)
Glass coverslips
Moist chamber: place several sheets of filter paper soaked with distilled H_2O in a petri dish and put two toothpicks on the paper; slide on the toothpicks and seal the dish with tape or Parafilm
42°C air incubator
Epifluorescence microscope with FITC or DAPI filter set
Digital image acquisition system with a cooled CCD camera

Grow S. pombe cells

1. At a time point 2 days before the experiment, inoculate 5 ml of YE medium with a single colony of the *S. pombe* strain to be analyzed. Grow this preculture overnight with shaking to saturation at a temperature suitable for growth of the strain (in general, 30°C for the wild-type strain and 26°C for temperature-sensitive strains).

   When growing liquid cultures for in situ hybridization, gentle shaking is recommended to maintain uniform growth conditions.

2. The day before the experiment, inoculate 5 ml of YE medium with the appropriate amount of the saturated preculture. Grow overnight at the temperature used in step 1 to a mid-log phase—OD_{600} of 0.3 to 0.5 depending on the strain (1×10^7 cells/ml).

   Growth phase and cell density are very important for reproducible results of in situ hybridization. Saturated cultures cannot be used for the experiment. It is advisable to inoculate three independent culture tubes with varying amounts of the preculture (e.g., add 0.1, 0.2, or 0.5 ml preculture to 5 ml YE medium), then measure the OD_{600} of each culture the next day.

3. If the yeast strain to be analyzed is a temperature-sensitive mutant and its nuclear mRNA export at the nonpermissive temperature is being assessed, split the culture into two culture tubes and incubate one tube at a nonpermissive temperature (for example, at 37°C) using a water bath. Maintain the other tube at the permissive temperature during the temperature shift.

Harvest cells

4. Transfer 1.5 ml of each culture to a microcentrifuge tube. Harvest cells by centrifuging 2 min at 2000 × *g* (5000 rpm in Kubota microcentrifuge with swinging-bucket rotor MF-2036), 4°C. Discard supernatant by aspiration.

   We highly recommend a swinging-bucket rotor in centrifugation for collecting cells in this protocol. Centrifugation in a fixed-angle rotor causes loss of most cells during removal of supernatants, especially after digestion of cell walls, as spheroplast cells stick to the side walls of microcentrifuge tubes.

Fix cells

5. Resuspend the cells in 1 ml of 4% paraformaldehyde solution.

   The paraformaldehyde solution should be freshly prepared before the experiment.

6. Incubate at room temperature for 60 min to fix the cells.

7. Collect the cells by centrifugation for 2 min at 2000 × *g* (5000 rpm in Kubota MF-2036 swinging-bucket rotor), 4°C. Discard supernatant by aspiration.

8. Wash the cells three times, each time by resuspending them in 500 µl of 0.1 M sodium phosphate buffer, pH 6.0, and then collecting them by centrifuging 2 min at 2000 × *g* (5000 rpm in Kubota MF-2036 swinging-bucket rotor), 4°C, and discarding the supernatant by aspiration.

Digest cell walls

9. Resuspend the cells in 500 µl of PEMS containing 1 mg/ml Novozym (or 1mg/ml lysing enzyme from *Trichoderma harzianum*) and 1 mg/ml Zymolyase 100T.

10. Incubate at 37°C in a water bath for 15 to 20 min, then withdraw a drop of the cell suspension from the tube and check cells using a phase-contrast microscope.

    When cell walls are digested, cells look dark. If the percentage of dark cells is low, then continue incubation until the percentage of dark cells reaches 70% to 80%; do NOT continue incubation until the percentage of dark cells reaches 100%. Overdigestion of cells at this step will result in poor preservation of cell morphology and high background hybridization of the probe, and sometimes gives false positive signals in the nuclei.

11. Wash the cells three times with ice-cold PEMS (no enzymes), using the volumes and centrifugation conditions described in step 8.

    It is very important to use ice-cold PEMS for washing to prevent overdigestion of the cells during centrifugation and resuspension.

12. After washing, resuspend the cells in 300 µl ice-cold PEMS.

13. Put 50 μl of the cell suspension on a well of a multiwell slide coated with poly-L-lysine.
14. Leave the slide at room temperature for 30 min to allow the cells to settle to the surface of the well.
15. Aspirate the solution.

    An aspirator with a trapping jar is very convenient for quick removal of solutions from the wells of a slide. We connect a Pasteur pipet to the aspirator and attach a disposable pipet tip to the pipet when aspirating solutions.

Prepare cells for hybridization

16. Add 50 μl of 70% ethanol to each well.
17. Aspirate the ethanol solution to remove unattached cells.
18. Dehydrate the cells by incubating successively in 70%, 90%, and 100% ethanol in Coplin jars for 5 min each.
19. Air dry the slide at room temperature for 20 min.

    If desired, the dried slides can be stored in a plastic bag at −80°C after wrapping them with aluminum foil.

Prehybridize slides

20. Add 50 μl of prehybridization solution to each well.
21. Incubate the slide at room temperature for 30 min.
22. Discard the prehybridization solution by aspiration.

    Wells should not be allowed to dry during this and subsequent steps.

Hybridize slides

23. Add 10 μl of hybridization solution [prehybridization solution containing 1 ng/μl biotin-labeled oligo(dT) probe] to each well.
24. Put a glass coverslip on the multiwell slide to prevent evaporation of the hybridization solution.

    Usually, it is not necessary to seal the coverslip with rubber cement if the slide is incubated in a moist chamber.

25. Incubate the slide at 42°C overnight in a moist chamber.

Wash the slides

26. After incubation, remove the cover glass and wash the wells with 4× SSC (at 42°C) four times, each time for 10 min.

    To wash the wells, place the slide in 20 ml of 4× SSC in a petri dish or a small plastic container and change the 4× SSC every 10 min. After washing, wipe off the bottom of the multiwell slide carefully with paper towels to remove excess 4× SSC; otherwise deposition of salts on the glass surface will hinder microscopic observation.

27. Add 50 μl of 4× SSC/0.1 % Triton X-100 (at room temperature) to each well and aspirate the solution after 10-sec incubation at room temperature.
28. Add 50 μl 2 μg/ml FITC-avidin freshly diluted in 4× SSC/1% BSA to each well.
29. Incubate at room temperature for 30 min.

    Keep the slide in a dark box (or equivalent) to avoid fading of fluorescence.

Visualize hybridized probe

30. Aspirate the FITC-avidin and wash wells twice, each time for 10 min with 80 μl/well of 4× SSC.
31. Wash wells twice, each time for 10 min with 80 μl/well of 4× SSC/0.1% Triton X-100.
32. Wash each well twice, each time with 80 μl of 4× SSC.
33. Aspirate the 4× SSC, then add 50 μl of 0.1 μg/ml DAPI solution to each well and incubate for 2 min at room temperature.
34. Wash each well once with 50 μl PBS.
35. Apply 10 μl of mounting medium to each well, put a glass coverslip on the slide, and seal it with clear nail polish.

    Make sure not to trap air bubbles in the wells when placing the coverslip on the slide.

36. Inspect cells under an epifluorescence microscope equipped with a FITC or DAPI filter set and record images with a digital image acquisition system.

    If nuclear mRNA export takes place normally, fluorescent signals for mRNAs will be distributed throughout the cell, including the nucleus and the cytoplasm. If export of mRNA from the nucleus is blocked, strong nuclear signals will be observed, due to the accumulation of transcribed mRNAs in the nucleus, and the cytoplasmic signals will be concomitantly decreased.

MONITORING NUCLEAR mRNA EXPORT BY FLUORESCENCE IN SITU HYBRIDIZATION IN BUDDING YEAST

ALTERNATE PROTOCOL 1

These procedures are for fluorescence in situ hybridization in the budding yeast *Saccharomyces cerevisiae*. Reagents and steps for cell wall digestion are modified for *Saccharomyces cerevisiae*, as cell walls of *S. cerevisiae* are easily digested with Zymolyase 100T.

Additional Materials *(also see Basic Protocol 1)*

S. cerevisiae cells to be analyzed
YPD medium (see recipe)
Sorbitol buffer (see recipe), ice cold
2 mg/ml Zymolyase 100T
2-Mercaptoethanol

1. Perform steps 1 to 8 of Basic Protocol 1, except cultivate *S. cerevisiae* cells in YPD medium instead of YE medium.
2. Suspend fixed cells in 500 μl of sorbitol buffer. Add 2 μl of 2-mercaptoethanol and 20 μl of 2 mg/ml Zymolyase 100T dissolved in sorbitol buffer.
3. Incubate at 37°C in a water bath for 15 to 20 min.

   After incubation for 10 to 15 min at 37°C, withdraw a drop of the cell suspension from the tube and check cells using a phase-contrast microscope. If cell walls are digested, cells look dark under a phase-contrast microscope. Incubate until 70% to 80% of cells are digested.

4. Wash the cells three times, each time by resuspending them in 500 μl of sorbitol buffer, and then collecting them by centrifuging 2 min at 2000 × *g* (5000 rpm in Kubota MF-2036 swinging-bucket rotor), 4°C, and discarding the supernatant.
5. Resuspend the resulting spheroplast cells in 300 μl of sorbitol buffer and perform steps 13 to 36 of Basic Protocol 1 to label and visualize mRNA.

ALTERNATE PROTOCOL 2

MONITORING NUCLEAR mRNA EXPORT IN MAMMALIAN CELLS BY FLUORESCENCE IN SITU HYBRIDIZATION

Fluorescence in situ hybridization with an oligo(dT) probe can also be applied to the monitoring of nuclear mRNA export in mammalian cells. In the case of the fluorescence in situ hybridization of mammalian cells, an oligo(dT) probe labeled with digoxigenin is preferable to a biotin-labeled probe, as the biotin-labeled probe sometimes shows false-positive signals caused by an endogenous avidin-binding activity of mammalian cells. Detection of the digoxigenin-labeled probe hybridized to mRNAs is carried out by treatment with an anti-digoxin antibody followed by treatment with a fluorochrome-conjugated secondary antibody. The anti-digoxin antibody (Sigma) can recognize the digoxigenin specifically and yields low background staining.

Materials

HeLa cells (ATCC no. CCL-2) grown in a 100-mm tissue culture dish
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Trypsin/EDTA (see recipe)
DMEM with 10% FBS (see recipe)
3% paraformaldehyde (see recipe; freshly prepared)
0.5% (v/v) Triton X-100 (see recipe)
1× and 2× SSC (*APPENDIX 2A*)
Hybridization solution for mammalian cells (see recipe)
Rubber cement
PBS-BAG (see recipe)
Primary antibody: mouse anti-digoxin antibody (Sigma, cat. no. D8156)
Secondary antibody: anti–mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC)
0.1 μg/μl DAPI solution (see recipe)
Mounting medium (see recipe)
Clear nail polish

Hemacytometer (*UNIT 1.1*)
12-mm glass coverslips, round or square
35-mm tissue culture dishes
Glass microscope slides
Moist chamber: place several sheets of filter paper soaked with distilled H_2O in a petri dish and put two toothpicks on the paper; slide on the toothpicks and seal the dish with tape or Parafilm
Forceps
Epifluorescence microscope with FITC or DAPI filter set
Digital image acquisition system with a cooled CCD camera

Additional reagents and equipment for counting cells using a hemacytometer (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Subculture cells for in situ hybridization

1. Aspirate medium from a confluent monolayer of HeLa cells in a tissue culture dish.
2. Add PBS (0.1 to 0.2 ml/cm^2 tissue culture surface), rinse the cells, and discard rinse.

   This step is necessary to remove traces of serum, which could inhibit the activity of trypsin.

3. Add trypsin/EDTA (0.1 ml/cm^2 tissue culture surface) to the dish.
4. Leave the trypsin/EDTA on the cells for 30 sec to 40 sec and aspirate the solution.
5. Incubate the dish at 37°C until the cells become round.
6. Add DMEM with 10% FBS to the dish (0.2 ml/cm^2 tissue culture surface) and disperse cells by repeated pipetting over the surface of the dish.

   Overtreatment of cells with trypsin/EDTA will reduce cell viability.

7. Count cells in an aliquot using a hemacytometer (*UNIT 1.1*) and dilute the cell suspension to 10^4 cells/ml with DMEM containing 10% FBS.
8. Put up to four sterilized 12-mm glass coverslips in a 35-mm tissue culture dish and then seed the dish with the diluted cell suspension (0.2 ml/cm^2).
9. Incubate the dish at 37°C in 5% CO_2 for 2 days.

Fix cells

10. Remove the medium and rinse cells once with PBS (0.1 to 0.2 ml/cm^2 tissue culture surface).
11. Add 3% paraformaldehyde (0.1 ml/cm^2 tissue culture surface) and incubate for 15 min at room temperature to fix the cells.

    The paraformaldehyde solution should be freshly made on the day of the experiment.

12. Wash the cells with PBS (0.2 ml/cm^2 tissue culture surface) three times, each time for 10 min.

Permeabilize cells

13. Add 0.5% Triton X-100 prepared in PBS (0.1 ml/cm^2 tissue culture surface) and incubate the dish for 5 min on ice to permeabilize the cells.
14. Rinse the cells with PBS (0.2 ml/cm^2) three times, each time for 10 min.
15. Rinse the cells with 2× SSC (0.2 ml/cm^2 tissue culture surface), each time for 10 min.
16. Remove coverslips from the dish using forceps and blot excess moisture on the coverslip with a paper towel.

Hybridize mRNA

17. Place 30 µl hybridization solution for mammalian cells onto the coverslip.
18. Invert the coverslip onto a glass slide and seal with rubber cement.
19. Place the slide/coverslip in a moist chamber and incubate overnight at 37°C.

    A moist chamber should be used to protect the coverslip from drying.

20. Peel rubber cement from the coverslip with forceps.

    Do not allow the coverslip to dry from this step on.

21. Place the coverslip with the cell culture side up in a 35-mm dish and wash cells three times, each time with 2 ml of 2× SSC for 10 min at 37°C.
22. Wash cells for 10 min with 2 ml of 1× SSC for 10 min at 37°C.
23. Add 2 ml of PBS-BAG and incubate for 30 min at room temperature.

Detect hybrids

24. Remove the coverslip from the dish. Place 30 μl of anti-digoxin primary antibody diluted 1:200 in PBS-BAG onto the coverslip and invert it onto a glass slide. Incubate the slide/coverslip in a moist chamber at room temperature for 60 min.

25. Wash the coverslip three times, each time with 2 ml PBS for 10 min in a 35-mm dish at room temperature.

26. After washing, place 30 μl of anti–mouse IgG secondary antibody conjugated with FITC, diluted 1:200 in PBS-BAG onto the coverslip and then invert it on a glass slide. Incubate the slide/coverslip in a moist chamber at room temperature for 60 min.

    Keep the coverslip in the dark during this and subsequent steps.

27. Wash the coverslip three times, each time with 2 ml PBS in a 35-mm dish for 10 min at room temperature.

28. Stain the cells on the coverslip with 100 μl of 0.1 μg/μl DAPI solution.

29. Wash the coverslip once for 10 min with 2 ml PBS in a 35-mm dish at room temperature.

Mount the coverslip

30. Remove the coverslip from the dish using forceps and blot excess moisture from the coverslip using a paper towel.

31. Apply 10 μl mounting medium to the coverslip and invert it onto the glass slide. Seal the coverslip with clear nail polish.

    Do not trap air bubbles in the mounting medium when placing the coverslip on the slide.

32. Observe cells under an epifluorescence microscope equipped with a FITC or DAPI filter set and record images with a digital image acquisition system.

    If nuclear mRNA transport is not impaired, fluorescent signals for mRNAs will be observed as a speckled pattern in the nucleus and a uniform distribution in the cytoplasm. If nuclear mRNA transport is inhibited, fluorescent signals in the nuclear speckles will be increased.

BASIC PROTOCOL 2

MONITORING NUCLEAR mRNA EXPORT IN A LIVING MAMMALIAN CELL BY MICROINJECTION OF FLUORESCENTLY LABELED PRE-mRNA INTO THE NUCLEUS

This protocol is designed to monitor mRNA export in a living mammalian cell by visualization of mRNAs injected into the nucleus. Pre-mRNAs transcribed in vitro are labeled with a fluorescent dye and microinjected into the nuclei. The injected pre-mRNAs have been shown to be spliced in the nucleus and exported to the cytoplasm via an energy-dependent process (Tokunaga et al., 2006). The process of mRNA transport from the nucleus to the cytoplasm in living mammalian cells can be analyzed visually under an epifluorescence microscope equipped with a cooled CCD camera.

Materials

HeLa cells (ATCC no. CCL-2)
DMEM supplemented with 10% FBS, penicillin, and streptomycin (see *APPENDIX 2A*)
Phenol red–free DMEM with 10% FBS (see recipe for DMEM with 10% FBS, but use phenol red–free DMEM)
3 M sodium acetate, pH 5.3 (*APPENDIX 2A*)
100% ethanol

Microinjection buffer (see recipe)
1.5 mg/ml FITC-dextran (see recipe)
Cy3-labeled *fushi tarazu* pre-mRNA (Support Protocol 2)
3% paraformaldehyde solution (see recipe)
Mounting medium (see recipe)
Clear nail polish

35-mm glass-bottom dishes
Humidified 5% CO_2 incubator
Refrigerated centrifuge with Kubota AF-2536A rotor (or equivalent)
Microloader pipet tip (Eppendorf or equivalent)
Inverted epifluorescence microscope
Digital image acquisition system with a cooled CCD camera
Microscope stage incubator, 37°C, 5% CO_2 (optional)
Microinjection needle (Eppendorf Femtotips II or equivalent)
Microinjector and manipulator (Eppendorf 5170 microinjector or equivalent)

Additional reagents and equipment for trypsinizing HeLa cells and preparing cell suspension (Alternate Protocol 2, steps 1 to 7)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Subculture cells for microinjection

1. At a time point 2 days before mRNA injection, trypsinize HeLa cells and make a cell suspension of 10^4 cells/ml with DMEM supplemented with 10% FBS, penicillin, and streptomycin as described in steps 1 to 7 in Alternate Protocol 2.
2. Seed a 35-mm glass bottom dish with the cell suspension.
3. Incubate the dish at 37°C in a 5% CO_2 incubator.

   The cells should be ~80% confluent on the day of microinjection.

Prepare reagents for microinjection

4. Change the medium to fresh phenol red–free DMEM supplemented with 10% FBS.

   Phenol red in the medium generates red fluorescence that competes with the Cy3 fluorescence of the labeled mRNAs.

5. Ethanol-precipitate the labeled pre-mRNA by adding 1/10 vol of 3 M sodium acetate, pH 5.3, and 2.5 vol of cold 100% ethanol. After keeping the mixture at –80°C for 30 min, centrifuge 15 min at 20,000 × *g* (14,500 rpm in Kubota AF-2536A rotor), 4°C, and discard the supernatant. Dry the RNA pellet under vacuum and resuspend the RNA pellet in microinjection buffer to make a 1.5 μM labeled pre-mRNA working solution.
6. Mix equal volumes of 1.5 μM labeled pre-mRNA solution and 1.5 mg/ml FITC-dextran. To avoid clogging the injection needle, centrifuge the mixture 5 min at 2400 × *g* (5000 rpm in Kubota AF-2536A rotor), 4°C, and use the supernatant for microinjection.

   FITC-dextran (70 kDa), which cannot move to the cytoplasm through nuclear pores by passive diffusion, is used as an injection marker and coinjected into the nuclei to confirm the nuclear injection of the fluorescent pre-mRNA.

7. Load a microinjection needle with ~0.5 µl of the pre-mRNA/FITC-dextran mixture using a microloader pipet tip.

   Standard femtotip microinjection needles work well for the Eppendorf microinjection system and are convenient for obtaining reproducible results.

Microinject fluorescently labeled pre-mRNA into the nuclei

8. Place the dish on the stage of the inverted epifluorescence microscope and locate the cells to be injected using a 10× objective lens.
9. Attach the microinjection needle loaded with the pre-mRNA/FITC-dextran mixture to the manipulator.
10. Move the microinjection needle close to a cell and change the objective lens to 40×.
11. Microinject fluorescently labeled pre-mRNA into the nuclei of cells in the center of the glass-bottom dish. Inject at least 50 cells per experimental condition.

    In the case of the Eppendorf 5170 microinjector, we usually set the injection pressure to 50 hPa, the compensation pressure to 45 hPa, and the injection duration to 0.5 sec.

    It is necessary to maintain a constant flow from the needle to keep it from clogging. Injection of pre-mRNAs into cells in one dish should be finished within 20 min.

12. Place the dish in a 37°C, 5% CO_2 incubator. After 15, 30, and 60 min of incubation, inspect cells with an inverted epifluorescence microscope equipped with a cooled CCD camera.

Observe the cells

13. If a microscope stage incubator is available, set the dish in it after injection and observe migration of fluorescent mRNAs from the nucleus to the cytoplasm under the inverted epifluorescence microscope.
14. To obtain clear images of the injected mRNAs, fix the cells with 2 ml freshly prepared 3% paraformaldehyde for 15 min at room temperature.
15. Rinse the cells three times, each time with 2 ml PBS 10 min, then add the mounting medium to the glass-bottom dish and seal with a coverslip and clear nail polish.
16. Observe cells with an inverted epifluorescence microscope and record images with a digital image acquisition system.

SUPPORT PROTOCOL 1

PREPARATION OF A DIGOXIGENIN (OR BIOTIN)–LABELED OLIGO(dT) HYBRIDIZATION PROBE

In this protocol, a 50-mer oligo(dT) is enzymatically labeled with digoxigenin, biotin, or fluorochrome such as FITC or Texas red, at its 3′-end with terminal transferase. Digoxigenin is a steroid that occurs naturally only in digitalis plants.

Materials

DIG oligonucleotide 3′-end labeling kit (Roche Applied Science)
1 mM biotin-16-ddUTP (for preparation of biotin-labeled probe)
STOP solution (see recipe)
4 M LiCl
100% ethanol, cold
70% ethanol
TE buffer (*APPENDIX 2A*)

Microcentrifuge with, Kubota AF-2536A rotor (or equivalent), 4°C
Vacuum desiccator

1. Mix the following reagents from the Roche DIG oligonucleotide 3′-end labeling kit with oligo dT (50-mer) in a microcentrifuge tube:

   8 µl 5× reaction buffer (vial 1)
   8 µl $CoCl_2$ solution (vial 2)
   2 µl 1 mM DIG-11-ddUTP solution (vial 3)
   2 µl terminal transferase (vial 4)
   2 µl oligo(dT) (50-mer, 100 pmol/µl)
   18 µl distilled H_2O.

   For preparation of the biotin-labeled probe, add 2 µl of 1 mM biotin-16-ddUTP for the reaction in place of the digoxigenin-labeled dUTP.

2. Incubate at 37°C for 20 min in a water bath.
3. Add 4 µl of STOP solution, 5 µl of 4 M LiCl, and 150 µl of cold 100% ethanol.
4. Mix by vortexing and incubate at −80°C for 30 min.
5. Microcentrifuge 15 min at 20,000 × *g* (14,500 rpm in Kubota AF-2536A rotor), 4°C. Remove supernatant.
6. Add 300 µl of 70% ethanol and microcentrifuge 10 min at 20,000 × *g* (14,500 rpm in Kubota AF-2536A rotor), 4°C.
7. Remove supernatant carefully and dry pellet for 10 min under vacuum.
8. Resuspend the pellet in 50 µl of TE buffer. Store at −20°C.

PREPARATION OF FLUORESCENTLY LABELED PRE-mRNA

SUPPORT PROTOCOL 2

Materials

pSP64 (poly A)-ftz plasmid (available from Dr. Tokio Tani, Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, Japan)
Restriction enzyme *Eco*RI and high-salt restriction buffer
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol
3 M sodium acetate, pH 7.0
3 M sodium acetate, pH 5.2 (*APPENDIX 2A*)
100% ethanol, cold, and 70% ethanol
TE buffer (*APPENDIX 2A*)
5× transcription buffer (see recipe)
100 mM DTT
10 mM ATP, UTP, GTP, and CTP
m^7G cap analog (Promega, cat. no. P171B)
15 U/µl SP6 RNA polymerase
1 U/µl DNase I (RNase-free grade)
RNase-free H_2O (DEPC-treated; *APPENDIX 2A*)
RNA dye solution (see recipe)
8% polyacrylamide/7 M urea gel (also see Brown et al., 2004)
1 µg/ml ethidium bromide
10× labeling buffer (PanVera Corp.)
Label IT reagent (PanVera Corp.)
Microcentrifuge with Kubota AF2536A rotor (or equivalent), 4°C
Vacuum desiccator
MicroSpin G-50 spin columns (GE Healthcare)
Spectrophotometer capable of reading at 260 nm
90°C water bath or heat block

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000), extraction of DNA from agarose gels (Moore et al., 2002), and denaturing polyacrylamide electrophoresis of RNA (Brown et al., 2004)

Linearize plasmid

1. To prepare a linearized substrate for in vitro transcription, digest 25 μg of the plasmid DNA in a 100-μl solution containing 50 U of *Eco*RI and 1× high-salt restriction buffer (supplied by the manufacturer of the restriction enzyme) at 37°C for at least 6 hr.

   pSP64 (poly A)-ftz was constructed as follows. pGEM-pre ftz (Rio, 1988) was digested with HindIII and SacI, and a fragment containing a part of the Drosophila fushi tarazu (ftz) genomic gene was inserted between HindIII and SacI sites of the pSP64-poly A vector (Promega), which contains SP6 promoter and a stretch of dA:dT residues for generation of a poly(A) tail.

2. Extract digested DNA with 100 μl 25:24:1 phenol/chloroform/isoamyl alcohol by vortexing for 1 min and centrifuging 10 min at 20,000 × *g* (14,500 rpm in Kubota AF2536A rotor), room temperature.

3. Take the upper phase and add 1/10 vol of 3 M sodium acetate, pH 7.0, and 2.5 vol of cold 100% ethanol. Mix and incubate at −80°C for at least 30 min.

4. Centrifuge 10 min at 20,000 × *g*, 4°C, and remove supernatant.

5. Add 500 μl of 70% ethanol and centrifuge 10 min at 20,000 × *g*, 4°C.

6. Remove supernatant and dry the pellet for 10 min under vacuum. Suspend the DNA pellet in TE buffer at a concentration of 0.5 μg/μl. Check the digested plasmid DNA by electrophoresis on an agarose gel (Voytas, 2000). If undigested plasmid DNA remains, purify the cleaved DNA fragment by electrophoresis on an agarose gel followed by extraction of DNA from a gel slice (Moore et al., 2002).

Transcribe to pre-mRNA

7. Mix the following and incubate at 37°C for 60 min:

   10 μl 5 μg/μl template DNA (from step 6)
   20 μl 5× transcription buffer
   10 μl 100 mM DTT
   10 μl 10 mM ATP
   10 μl 10 mM CTP
   10 μl 10 mM UTP
   2 μl 10 mM GTP
   8 μl 10 mM m^7G cap analog
   3 μl 15 U/μl SP6 RNA polymerase
   7 μl distilled H_2O.

   Transcription of pSP64 (poly A)-ftz linearized with EcoRI generates pre-ftz mRNA (497 nt) containing one intron (151 nt) and a poly(A) sequence (30 nt).

8. Add 5 μl of DNase I (1 U/μl, RNase-free grade) and incubate at 37°C for 60 min to digest the template DNA.

9. Add 100 μl of 25:24:1 phenol/chloroform/isoamyl alcohol and mix by vortexing for 1 min.

10. Centrifuge 10 min at 20,000 × *g*, room temperature.

11. Transfer the upper phase to a new microcentrifuge tube and add 1/10 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of cold 100% ethanol. Incubate for 30 min at −80°C.

12. Centrifuge 15 min at 20,000 × *g*, 4°C, and remove supernatant.
13. Add 500 µl of 70% ethanol and centrifuge 10 min at 20,000 × *g*, 4°C.
14. Remove supernatant and dry the RNA pellet under vacuum.

Purify pre-mRNA

15. Resuspend the RNA pellet in 50 µl of RNase-free (DEPC-treated) water.
16. Remove unincorporated ribonucleotides from the RNA sample using a MicroSpin G-50 column according to the manufacturer's instructions.
17. Measure the concentration of RNA using a spectrophotometer with absorbance at 260 nm.
18. To check integrity of the synthesized pre-mRNA, mix 1 µg of the RNA sample and 19 µl of RNA dye solution. After heating at 90°C for 3 min, electrophorese the mixture on an 8% polyacrylamide/7 M urea gel (also see Brown et al., 2004) followed by staining with 1 µg/ml of ethidium bromide solution for 10 min.

    It is also possible to run the RNA in the gel system with the ethidium bromide contained in the gel and running buffer, but the authors have found that post-staining results in more uniform staining of the gel, although it takes a little bit longer to perform the experiments.

Cy3-label pre-mRNA

19. To label RNA with a Cy3 fluorescent dye, set up the following reaction mixture in a final volume of 50 µl:

    10 µg pre-mRNA transcribed in vitro as described in the previous steps
    5 µl 10× labeling buffer
    5 µl Label IT reagent.

20. Incubate at 37°C for 1 hr.
21. Add 5 µl of 3 M sodium acetate, pH 5.2, and 125 µl of cold 100% ethanol. Mix by vortexing and incubate for 30 min at −80°C.
22. Centrifuge 15 min at 20,000 × *g*, 4°C.
23. Discard supernatant and rinse the RNA pellet with 70% cold ethanol as described in step 13.
24. Dry the RNA pellet under vacuum for 10 min.
25. Suspend the pellet in 50 µl of RNase-free water.

Purify the fluorescently labeled pre-mRNA

26. Purify the labeled pre-mRNA from unincorporated fluorescent dye using the MicroSpin G-50 column according to the manufacturer's instructions.

    The amount of Cy3 coupled with pre-mRNA can be measured spectrofluorometrically at an excitation wavelength of 550 nm. On the average, the pre-mRNA should be labeled with 18 molecules of Cy3 per molecule.

REAGENTS AND SOLUTIONS

Use RNase-free DEPC-treated (APPENDIX 2A) deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Carbonate-bicarbonate buffer

Mix 2 ml of 0.2 M anhydrous sodium carbonate (1.06 g/50 ml), 2 ml of 0.2 M sodium bicarbonate (1.84 g/50 ml), and 96 ml of distilled water. Store up to several months at 4°C.

DAPI solution

0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI)
1× PBS (*APPENDIX 2A*)
Store up to several months at −20°C

Denhardt solution, 100×

10 g Ficoll 400
10 g polyvinylpyrrolidone
10 g bovine serum albumin (Pentax Fraction V)
H_2O to 500 ml
Filter sterilize
Store up to several months at –20°C in 25-ml aliquots

DMEM with 10% FBS

Dulbecco's Modified Eagle Medium (formulation containing L-glutamine; use phenol-red free formulation where specified in protocol) supplemented with:
10% fetal bovine serum (FBS)
100 U/ml penicillin
100 μg/ml streptomycin

FITC-Dextran

Prepare 3 mg/ml FITC-dextran (70 kDa) in microinjection buffer. Filter the solution with a 0.2-μm filter and store up to several weeks at 4°C protected from the light.

Hybridization solution for mammalian cells

20 μl formamide
10 μl 10 mg/ml tRNA
10 μl 20× SSC (see recipe)
20 μl 50% (w/v) dextran sulfate
3 μl oligo(dT) probe labeled with digoxigenin (Support Protocol 1)
37 μl distilled H_2O
Prepare fresh

Microinjection buffer

10 mM sodium phosphate buffer, pH 7.2 (*APPENDIX 2A*)
70 mM KCl
Store up to several months at 4°C

Mounting medium

8.5 ml glycerol (nonfluorescent grade)
750 μl 1× PBS (*APPENDIX 2A*)
10 mg *p*-phenylenediamine

Mix the above reagents in a small beaker wrapped with aluminum foil and stir ½ day to overnight to dissolve *p*-phenylenediamine, which is a chemical antibleaching agent that greatly retards the photobleaching of fluorophores. Then, add ∼20 drops of carbonate-bicarbonate buffer (see recipe) and check pH using pH paper. The pH of the solution should be 8.0. Store the mounting medium at −80°C in 200-μl aliquots for long-term storage. Working mounting medium can be stored up to several weeks at −20°C in the dark, although it will become dark brown with time, whereupon it should be discarded.

Multiwell slides, poly-L-lysine-coated

Place 50 µl of 1 mg/ml poly-L-lysine (mol. wt., 150,000 to 300,000) into each well of a multiwell slide (immunofluorescence slide from Polysciences, or equivalent). Leave at room temperature for 15 min, and then aspirate the solution, rinse each well once with 50 µl distilled water, and allow to air dry completely. Store slides up to several days at room temperature.

Paraformaldehyde solution, 3% or 4%

After suspending 0.4 g (for 4% solution) or 0.3 g (for 3%) of paraformaldehyde in 5 ml of distilled water, add 2 to 3 drops of 5 N NaOH, heat at 50° to 60°C in a water bath to dissolve paraformaldehyde completely, and then mix with 5 ml of 0.2 M phosphate buffer, pH 6.0 (see recipe). Prepare fresh.

CAUTION: *All operations involving paraformaldehyde should be performed in a fume hood, as formaldehyde (generated from the paraformaldehyde) is a carcinogen and may cause irritation to skin and mucous membranes.*

PBS-BAG

1× PBS (*APPENDIX 2A*)
1% (w/v) bovine serum albumin
0.1% (w/v) NaN_3
0.5% (w/v) cold water fish skin gelatin (Sigma)
Filter with a 0.45-µm filter.
Store up to several months at 4°C

PEMS

100 mM PIPES, pH 6.9
0.1 mM $MgCl_2$
1 mM EGTA
1.2 M sorbitol
Store up to several months at 4°C

Prehybridization solution for yeast

4× SSC (see recipe)
5× Denhardt solution (see recipe)
1 mg/ml baker's yeast tRNA (Sigma)
Store up to several months at −20°C

RNA dye solution

90% (v/v) formamide
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EDTA
0.1% (w/v) xylene cyanol
0.1% (w/v) bromphenol blue
Store up to several months at room temperature

Sodium phosphate buffer, pH 6.0, 0.2 M

Solution A: 13.9 g $NaH_2PO_4 \cdot H_2O$; make up to 500 ml with distilled H_2O
Solution B: 35.85 g $Na_2HPO_4 \cdot 12H_2O$; make up to 500 ml with distilled H_2O

Mix 87.7 ml of solution A and 12.3 ml of solution B to make a 0.2 M phosphate buffer (pH 6.0). Store up to several months at 4°C.

Sorbitol buffer

1.2 M sorbitol
0.1 M sodium phosphate buffer, pH 6.0 (see recipe)
Store up to several months at 4°C

SSC (sodium chloride/sodium citrate), 20×

3 M NaCl (175 g/liter)
0.3 M Na_3citrate·$2H_2O$ (88 g/liter)
Adjust pH to 7.0 with 1 M HCl
Store up to several months at room temperature

STOP solution

0.2 M EDTA, pH 8.0 (*APPENDIX 2A*)
100 μg/ml glycogen
Store up to several months at −20°C

Transcription buffer, 5×

250 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
30 mM $MgCl_2$
10 mM spermidine
500 μg/ml bovine serum albumin (RNase free)
Store up to several months at −20°C

Triton X-100, 0.5% (v/v)

1× PBS (*APPENDIX 2A*)
0.5% (v/v) Triton X-100
Store up to several months at 4°C

Trypsin/EDTA

0.25% (w/v) trypsin
1 mM EDTA (*APPENDIX 2A*)
1× PBS (*APPENDIX 2A*)
Store up to several weeks at −20°C

YE medium

0.5% (w/v) yeast extract
3% (w/v) glucose
Store up to several weeks at room temperature

YPD medium

1% (w/v) yeast extract
2% (w/v) Bacto-peptone
2% (w/v) glucose
Store up to several weeks at room temperature

COMMENTARY

Background Information

In eukaryotic cells, domains for transcription and translation are spatially separated by the nuclear membrane. Therefore, transport of mRNAs from the nucleus to the cytoplasm is an essential step for gene expression in eukaryotic cells. Understanding of the mechanisms of mRNA transport from the nucleus to the cytoplasm has increased through the identification of genes involved in the export process. Genetic studies in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* have been an effective approach for identifying genes involved in nuclear mRNA export (for a

review see Strässer and Hurt, 1999). Dozens of yeast temperature-sensitive mutants defective in mRNA export were isolated by screening with fluorescence in situ hybridization using an oligo(dT) as a probe, and the genes responsible for the mutations have been cloned (e.g., Heath et al., 1995; Azad et al., 1997; Mizuki et al., 2007).

In yeast, the accumulation of mRNA in the nucleus by inhibition of mRNA export is easily visualized in the in situ hybridization assay, as most yeast mRNAs in the cytoplasm have relatively short half-lives and the accumulated nuclear mRNAs are considerably more stable. Average half-lives ranging from 16 to 23 min have been measured for mRNAs in the yeast *S. cerevisiae* (Herrick et al., 1990). In addition, yeast mRNAs have been shown to exit the nucleus within 2 min after synthesis under normal growth conditions (Groner and Phillips, 1975). Therefore, blockage of the mRNA transport pathway in yeast cells results in a drastic change in the steady-state distribution of mRNAs, which can be easily detected by fluorescence in situ hybridization with an oligo(dT) probe that binds to the poly(A) tail of mRNAs.

Fluorescence in situ hybridization with the oligo(dT) probe has been also used for monitoring nuclear mRNA export in mammalian cells (e.g., Shibata et al., 2002). However, the generally long half-lives of mammalian mRNAs and the presence of stable poly(A)$^+$ RNAs in specific nuclear compartments termed "speckles" or "splicing factor compartments" (Lamond and Spector, 2003) sometimes make it difficult to detect the inhibition of nuclear mRNA export using fluorescence in situ hybridization.

A *Xenopus* oocyte system has also been used for analyses of nuclear mRNA export (e.g., Jarmolowski et al., 1994). In that system, ^{32}P-labeled mRNAs are microinjected into the nuclei of *Xenopus* oocytes, followed by incubation for an appropriate time. The nucleus and the cytoplasm are then dissected, and RNAs are extracted from each fraction to be analyzed by electrophoresis on a gel with subsequent autoradiography. Compared to the *Xenopus* oocyte system, the microinjection assay for fluorescent pre-mRNA described in this unit has the advantage of analysis of mRNA export in a living mammalian cell in real time. It has been demonstrated that, like endogenous mRNAs, the injected fluorescent pre-mRNAs are exported to the cytoplasm after splicing by an energy-dependent process (Tokunaga et al., 2006).

Critical Parameters

In situ hybridization assay

To obtain strong hybridization signals with low background, it is important to use oligo(dT) probes efficiently labeled with biotin or digoxigenin. Labeling of the oligo(dT) with biotin or digoxigenin can be checked by spotting of 1 μl of the probe onto a nylon membrane and treating the membrane with avidin or anti-digoxigenin antibody conjugated with alkaline phosphatase or horseradish peroxidase, followed by appropriate detection reagents. Temperatures for hybridization and subsequent washing are also important. Hybridization at low temperatures (for example, at 37°C) sometimes causes high background hybridization.

One possible cause of weak or no hybridization signals in yeast cells is incomplete digestion of cell walls. Although removal of cell walls is essential for penetration of a probe into cells, overdigestion of cells damages cell morphology and gives background hybridization in the nuclei. As efficiencies of cell wall digestion by Novozym or Zymolyase 100T vary depending on the growth phase of the cells, inspection with a phase-contrast microscope at the digestion step is important for stopping the digestion reaction at the correct time for in situ hybridization (~80% of cells are spheroplasted). Washing cells with ice-cold PEMS or sorbitol buffer after treatment with the enzymes is also critical to prevent overdigestion of cells during the washing steps. Oligo(dT) labeled directly with fluorochrome, such as FITC, can be used as a probe for fluorescence in situ hybridization in yeast and mammalian cells. The signal intensity of the fluorochrome-labeled oligo(dT) probe, however, is generally weak compared to that of a biotin- or digoxigenin-labeled probe, which can amplify the signal intensities.

Microinjection assay

Labeling efficiency of pre-mRNAs with the Cy3 fluorescent dye is important for visualization of the export process in living mammalian cells. Too much labeling with the Cy3 fluorescent dye might inhibit the mRNA export process. The amount of Cy3 coupled with pre-mRNA can be measured spectrofluorometrically at an excitation wavelength of 550 nm. Pre-mRNAs labeled with ~18 molecules of Cy3 per molecule on average are suitable for the microinjection assay.

Table 22.13.1 Troubleshooting Guide for Fluorescence In Situ Hybridization for Monitoring mRNA Export

Problem	Possible cause	Solution
High background signal	Washing of the probe is insufficient	Increase washing steps and time. Increase volume of the washing buffer.
	Cells dried up during hybridization and subsequent washing steps	Make sure not to leave the sample without solution for a long time. Use an aspirator to quickly exchange solutions when removing supernatants.
	The probe is too concentrated	Decrease the amount of the oligo(dT) probe in the hybridization solution
	Antibodies or FITC-avidin are too concentrated	Dilute antibodies or FITC-avidin further
	Temperature of hybridization is low	Perform hybridization at 42°C or above
	Quality of ethanol used for the dehydration step is not suitable for in situ hybridization (for yeast cells only)	Some manufacturers' ethanols generate high fluorescent background. Change ethanol to an other manufacturer's absolute ethanol.
No or weak signal	Labeling efficiency of oligo(dT) with digoxigenin or biotin is low	Extend the incubation time for the labeling reaction and check the labeled probe by spotting on the nylon membrane as described in the protocol
	Cell walls are not sufficiently digested (for yeast cells only)	Extend the incubation time with the cell wall digestion enzymes. Make sure that the cells to be analyzed are in the mid-log phase. Cell walls of stationary-phase cells are hard to digest.
False hybridization in the nuclei (for yeast cells only)	Cells are overdigested by cell wall digestion enzymes	Shorten the incubation time with the enzymes. Cells should be checked frequently with a phase-contrast microscope to determine the best incubation time for cell wall digestion.
Poor morphological preservation (for yeast cells only)	Fixation of cells is not sufficient	Prepare fresh 4% paraformaldehyde on the day of the experiment
	Spheroplasted cells are centrifuged at inadequate speed	Make sure that centrifugation of spheroplasted cells in carried out at low speed (e.g., 3000 to 5000 rpm in a microcentrifuge)
	Cells are over-digested by cell wall digestion enzymes	Shorten the incubation time with the enzymes
Loss of cells during washing steps (for yeast cells only)	Poor packing of cells after centrifugation	Use a swinging-bucket rotor to collect cells

Table 22.13.2 Troubleshooting Guide for the Microinjection Assay

Problem	Possible cause	Solution
Microinjection needle clogs often	Pressure for constant flow from the needle is weak	Change the injection pressure, compensation pressure and injection time of the microinjector
	Insoluble particles are present in the microinjection mixture	Centrifuge and filter the microinjection mixture with a 0.2-μm filter
RNA samples are degraded	RNase contamination	Prepare all reagents with DEPC-treated RNase-free water

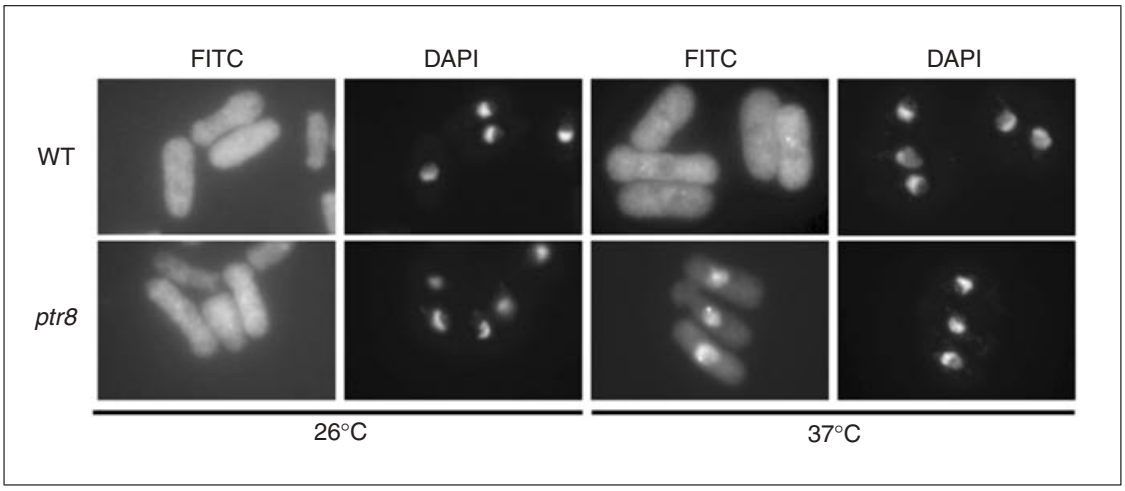


Figure 22.13.1 Fluorescence in situ hybridization with oligo(dT) probe in *S. pombe*. The wild-type strain *972* ("WT," upper panels) and the mRNA export mutant *ptr8* (lower panels, Mizuki et al., 2007) were grown at 26°C and either maintained at 26°C or shifted to 37°C for 60 min. The cells were fixed and analyzed by fluorescence in situ hybridization with the biotin-labeled oligo(dT)$_{50}$ probe. Hybridized signals were detected by FITC-conjugated avidin. The FITC columns show the distribution of mRNA in the respective cells. The DAPI columns show staining of DNA of the cells in the same field.

To monitor the mRNA export, fluorescent pre-mRNAs should be accurately injected into the nuclei. The injected pre-mRNAs are localized to the speckles in the nucleus, spliced, and then exported to the cytoplasm (Tokunaga et al., 2006). Intron-less mRNAs transcribed from cDNAs localize uniformly in the nucleus after injection and are not distributed to the speckles even after long incubation periods (Tokunaga et al., 2006). It is usually difficult to detect the nuclear export of intron-less mRNAs in the microinjection assay, as they degrade more rapidly than pre-mRNAs in cells. Pre-mRNA splicing might facilitate formation of export-competent RNP complexes and stabilize the injected mRNAs. We, therefore, recommend pre-mRNAs with high splicing efficiency, such as *fushi tarazu* pre-mRNA, as an injection substrate.

Troubleshooting

Tables 22.13.1 and 22.13.2 describe problems commonly encountered when performing the fluorescence in situ hybridization and the microinjection assays. Possible causes of these problems and how to solve them are also given.

Anticipated Results

In situ hybridization assay

In wild-type yeast cells, hybridization signals should be observed throughout the cells at all temperatures tested (Fig. 22.13.1, upper panels). In contrast, due to defects in mRNA export, temperature-sensitive mRNA export mutants will give strong nuclear signals and weak cytoplasmic signals in cells cultured at nonpermissive temperatures in the in situ hybridization assay with the oligo(dT) probe (Fig. 22.13.1, lower panels; Mizuki et al., 2007).

In the case of in situ hybridization of mammalian cells, hybridization signals will be detected as a speckle pattern in the nucleus and a uniform distribution in the cytoplasm under normal conditions. When nuclear mRNA export is inhibited in mammalian cells, intensities of hybridized signals in the speckles increase significantly (e.g., Shibata et al., 2002).

Microinjection assay

The fluorescent pre-mRNAs injected into the nuclei of HeLa cells are first distributed uniformly in the nucleus and then localized to the speckles within ∼15 min. After that, the cytoplasmic fluorescent signals will increase with incubation time, with concomitant decreases in nuclear fluorescence, suggesting that the injected mRNAs are exported to the cytoplasm from the nucleus.

Time Considerations

After culturing cells, the entire procedure for fluorescence in situ hybridization in yeast and mammalian cells will take 2 days, depending on the length of hybridization. If hybridization with the oligo(dT) probe is carried out within several hours, the procedures can be done in a single day. In the case of in situ hybridization in yeast cells, however, overnight hybridization is recommended

for reproducible results. After dehydration of yeast cells with ethanol (step 18 of Basic Protocol 1), the protocol can be interrupted. The slides can be stored at −80°C for more than several months.

For the microinjection assay, preparation of fluorescently labeled pre-mRNAs will require 1 day. Injection of the fluorescent pre-mRNAs into the nuclei of ∼50 cells will take 15 to 20 min, depending on the experimenter's skills for microinjection.

Literature Cited

Azad, A.K., Tani, T., Shiki, N., Tsuneyoshi, S., Urushiyama, S., and Ohshima, Y. 1997. Isolation and molecular characterization of mRNA transport mutants in *Schizosaccharomyces pombe*. *Mol. Biol. Cell* 8:825-841.

Brown, T., Mackey, K., and Du, T. 2004. Analysis of RNA by northern and slot-blot hybridization. *Curr. Protoc. Mol. Biol.* 67:4.9.1-4.9.19.

Groner, B. and Phillips, S.L. 1975. Polyadenylate metabolism in the nuclei and cytoplasm of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 250:5640-5646.

Heath, C.V., Copeland, C.S., Amberg, D.C., Del Priore, V., Snyder, M., and Cole, C.N. 1995. Nuclear pore complex clustering and nuclear accumulation of poly $(A)^+$ RNA associated with mutation of the *Saccharomyces cerevisiae* RAT2/NUP120 gene. *J. Cell Biol.* 131:1677-1697.

Herrick, D., Parker, R., and Jacobson, A. 1990. Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10:2269-2284.

Jarmolowski, A., Boelens, W.C., Izaurralde, E., and Mattaj, I.W. 1994. Nuclear export of different class of RNA is mediated by specific factors. *J. Cell Biol.* 124:627-635.

Lamond, A. and Spector, D.L. 2003. Nuclear speckles: A model for nuclear organelles. *Nat. Rev. Mol. Cell Biol.* 4:605-635.

Mizuki, F., Namiki, T., Sato, H., Furukawa, H., Matsusaka, T., Ohshima, Y., Ishibashi, R., Andoh, T., and Tani, T. 2007. Participation of XPB/Ptr8p, a component of TFIIH, in nucleocytoplasmic transport of mRNA in fission yeast. *Gene Cells* 12:35-47.

Moore, D., Dowhan, D., Chory, J., and Ribaudo, R.K. 2002. Isolation and purification of large DNA restriction fragments from agarose gels. *Curr. Protoc. Mol. Biol.* 59:2.6.1-2.6.12.

Rio, D.C. 1988. Accurate and efficient pre-mRNA splicing in *Drosophila* cell-free extracts. *Proc. Natl. Acad. Sci. U.S.A.* 85:2904-2908.

Shibata, T., Matsuoka, Y., and Yoneda, Y. 2002. Nucleocytoplasmic transport of proteins and poly $(A)^+$ RNA in reconstituted Tpr-less nuclei in living mammalian cells. *Genes Cells* 7:421-434.

Strässer, K. and Hurt, E. 1999. Nuclear RNA export in yeast. *FEBS Lett.* 452:77-81.

Tokunaga, K., Shibuya, T., Ishihama, Y., Tadakuma, H., Ide, M., Yoshida, M., Funatsu, T., Ohshima, Y., and Tani, T. 2006. Nucleocytoplasmic transport of fluorescent mRNA in living mammalian cells: Nuclear mRNA export is coupled to ongoing gene transcription. *Genes Cells* 11:305-317.

Voytas, D. 2000. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

Analysis of DNA Replication in *Saccharomyces cerevisiae* by Two-Dimensional and Pulsed-Field Gel Electrophoresis

Satoru Ide[1] and Takehiko Kobayashi[1,2]

[1]National Institute of Genetics, Shizuoka, Japan
[2]The Graduate University for Advanced Studies, Sokendai, Mishima, Japan

ABSTRACT

Two methods to analyze DNA replication in budding yeast are described here. One is two-dimensional (2D) gel electrophoresis, which has been used to analyze replication intermediates in yeast. The other method is pulsed-field gel electrophoresis (PFGE) that monitors the replication status of a chromosome. These are convenient and easy, and they do not require specialized equipment. *Curr. Protoc. Cell Biol.* 49:22.14.1-22.14.12.

Keywords: two-dimensional gel electrophoresis • pulsed-field gel electrophoresis • DNA replication • agarose plug • replicative intermediate

INTRODUCTION

Two methods to analyze DNA replication in budding yeast are described in this unit. One is two-dimensional (2D) gel electrophoresis, which has been used to analyze replication intermediates in yeast. This method was developed by Brewer and Fangman (1987) to characterize the activity of autonomously replicating sequence elements (ARS) in yeast. In addition to mapping the replication origin, this method makes it possible to analyze the dynamics of replication forks, including blocking of replication forks (or slowing formation) and termination. Electron microscopy (EM) can also detect replication intermediates, but sample preparation is time-consuming and EM cannot recognize regions through which the detected replication fork has passed. The protocol described herein for two-dimensional gel electrophoresis is more convenient and does not require specialized equipment. Moreover, two-dimensional gel analysis can detect replication intermediates in a target locus using a specific probe and can follow temporal changes of replication intermediates.

The other method introduced here is pulsed-field gel electrophoresis (PFGE) that monitors replication status of a chromosome. PFGE was developed by Schwartz and Canter (1984) as a means of resolving very large DNA molecules (>20,000 bp). In the case of budding yeast, all of its linear chromosomes are resolvable by PFGE. However, replicating chromosomes cannot enter the gel and remain in the well because such chromosomes have a higher-order structure consisting of replication intermediates. Therefore, PFGE analysis can distinguish replication status by determining whether a chromosome can migrate in the gel or not. As a method to analyze the cell cycle, fluorescence-activated cell sorting (FACS) is known to be a powerful and convenient tool. For this application, FACS is used to measure cellular DNA content. While FACS can monitor the whole genome, it cannot determine the replication status of each chromosome. In contrast, PFGE can monitor replication status of each chromosome if chromosome-specific probes are used. In addition, when replication stalls, the DNA degradation products are detectable as a smear by Southern blotting.

Current Protocols in Cell Biology 22.14.1-22.14.12, December 2010
Published online December 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb2214s49

Two-dimensional gel analysis and PFGE share the same DNA preparation method. In both assays, DNA is eluted from yeast cells after being embedded in an agarose plug. This method of immobilizing the DNA prior to electrophoresis is crucial as it prevents unstable replication intermediates from forming secondary structures and minimizes random cleavage due to physical stress.

BASIC PROTOCOL 1

TWO-DIMENSIONAL AGAROSE GEL ELECTROPHORESIS TO ANALYZE DNA STRUCTURES

Two-dimensional gel electrophoresis distinguishes branched DNA structures from linear structures. In the first dimension of this analysis, digested DNA in an agarose plug is electrophoresed under conditions that separate DNA molecules on the basis of molecular mass (in a low-concentration agarose gel and relatively weak electric field). In the subsequent second dimension, electrophoresis is run under conditions that maximize the contribution of DNA shape to migration rate (in a higher concentration agarose gel and a stronger electric field, at lower temperature, and with the intercalating agent ethidium bromide to stiffen the DNA). Nonlinear DNA molecules (including replication intermediates) are more affected and migrate slowly; in comparison, linear DNA molecules migrate faster in a higher-concentration agarose gel. It is more difficult for nonlinear DNA to move through denser gel matrices because of their complicated structures (Bell and Byers, 1983).

Materials

Agarose solution no. 1 for the first dimensional (1D) gel electrophoresis (see recipe)
Agarose solution no. 2 for the second dimensional (2D) gel electrophoresis (see recipe)
Restriction enzyme–digested DNA in agarose plug (Support Protocol 2)
1× TBE (*APPENDIX 2A*)
Ethidium bromide
0.2 N HCl
Denaturation solution: 1.5 M NaCl/0.5 N NaOH
Neutralization buffer: 1 M Tris·Cl (pH 7.4)/1.5 M NaCl
20× SSC (*APPENDIX 2A*)
Phosphate-SDS hybridization buffer (see recipe)
Radiolabeled DNA probe made with Rediprime II DNA Labeling System (GE)
2% (w/v) SDS (*APPENDIX 2A*)

12 × 14–cm gel casting tray
Well combs
Electrophoresis apparatus
Power supply
Hybond N+ nylon membrane (GE)
UV light
Stratalinker (Stratagene)
200-ml roller bottle
65° and 68°C incubator
Plastic wrap
Imaging plate (Fuji film)

Prepare the agarose solutions

1. Melt the agarose solution by heating it in a microwave with frequent mixing (should take ~3 min at a 750-W microwave setting). Cool until it is comfortable to the touch (~45°C).

   For the second agarose solution, add the ethidium bromide after cooling.

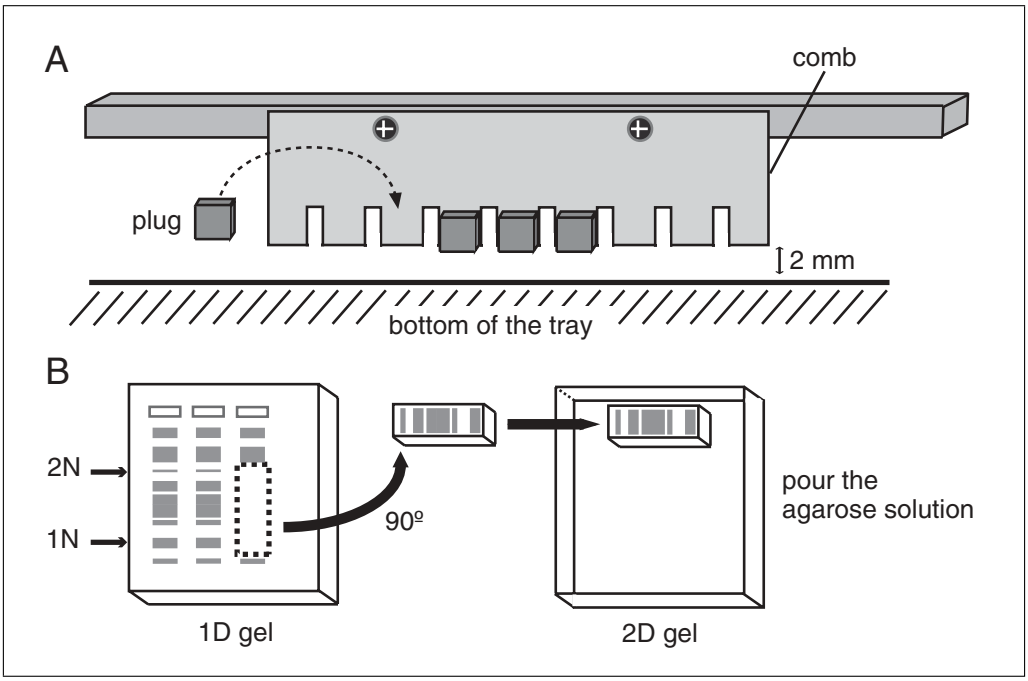


Figure 22.14.1 Tips for two-dimensional gel electrophoresis. (**A**) Embedding agarose plugs in agarose gel. (**B**) How to transfer a gel run in the first dimension for electrophoresis in the second dimension. Excise the portion of the gel containing DNA molecules ranging from 1N to 2N. Turn the excised gel piece 90° and re-position it at the top of the second gel. The agarose solution for the second dimension gel has been poured in the tray.

The second agarose solution should be prepared while the first dimension gel is stained with ethidium bromide.

Prepare and run the first dimension gel

2. Place the agarose plugs (tweleve plugs on one gel) from the restriction solution on the comb teeth (corresponding to wells), and remove buffer around the plug by blotting with tissue paper (Fig. 22.14.1A).

3. Place the comb with the agarose plugs into the casting tray leaving a 2-mm gap at the bottom (Fig. 22.14.1A).

4. Pour 100 ml agarose solution no. 1 (0.4% agarose) and allow to harden at 4°C.

5. Carefully remove the comb leaving the plugs in the gel.

6. Perform electrophoresis at 1.32 V/cm at room temperature for 14 hr.

Stain the gel

7. Stain the gel in 300 ml of 1× TBE containing 0.3 μg/ml ethidium bromide for 30 min at room temperature, and check the electrophoresis pattern.

   DNA fragments undergoing replication should be distributed between the non-replicating fragments of unit mass (1N) and ~2N (Fig. 22.14.1, one-dimensional gel). In the case of the yeast ribosomal gene that we are using, the fragment digested with BglII is 4.5 kb (1N, non-replicating fragment). Therefore, DNA fragments undergoing replication are distributed between 4.5 kb (1N) and 9.0 kb (2N), as assessed by the molecular size maker.

8. Excise the portion of the gel containing fragments ranging from 1N to 2N, turn it 90°, and re-position at the top of the second gel tray. Pour agarose solution no. 2 and allow it to harden at room temperature (Fig. 22.14.1B).

   It takes ~30 min for the agarose solution to harden.

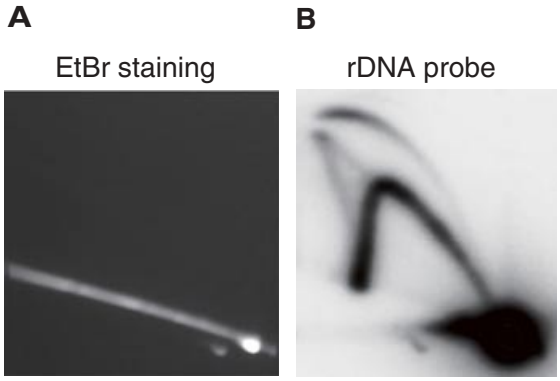


Figure 22.14.2 Migration pattern of replication intermediates separated by two-dimensional gel analysis. DNA is eluted from exponentially growing yeast cells, digested with *Nhe*I, and subjected to two-dimensional gel electrophoresis. (**A**) The gel was stained with ethidium bromide and photographed under UV illumination. Only linear DNA can be detected by ethidium bromide staining. (**B**) Hybridization pattern with an rRNA gene–specific probe is shown. Simple Y-shaped, replication bubble, and X-shaped molecules are observed.

9. Perform the electrophoresis in 750 ml of 1× TBE buffer containing 0.3 μg/ml ethidium bromide at 6.0 V/cm for 6 hr at 4°C.

   A representative example is shown in Figure 22.14.2A.

   This protocol is optimized for separation of ~4.5-kb fragments (1N). Separation of larger or smaller fragments will require adjustment of gel strength (percentage of agarose) and strength of the electric field.

Transfer the DNA to the membrane

10. Incubate the gel in 0.2 N HCl for 20 min at room temperature with agitation for depurination of DNA.
11. After depurination, denature the gel in denaturation solution (completely cover the gel) for 30 min at room temperature, with gentle agitation.
12. Following denaturation, neutralize the gel in neutralization buffer for 30 min at room temperature, with gentle agitation.
13. Proceed with standard capillary transfer using 20× SSC as a transfer buffer to transfer the DNA to a positively charged nylon membrane (Hybond N+), which is prewet with 6× SSC before blotting. Perform the transfer of DNA for 16 to 24 hr.
14. After the capillary transfer, rinse the membrane briefly with 6× SSC to remove any agarose and cross-link the DNA to the membrane with UV light at 254 nm (using a Stratalinker).

Hybridize the membrane-bound DNA

15. Place the membrane into a 200-ml roller bottle together with 40 ml of phosphate-SDS hybridization buffer.
16. Prehybridize 5 min at 65°C.
17. Add a radiolabeled probe (e.g., 3,000Ci/mmol dCTP with 32P for labeling) and incubate it overnight at 65°C.
18. Remove hybridization buffer and rinse the membrane once with 50 ml 2× SSC, 2% SDS at 68°C.
19. Wash the membrane once with 50 ml 2× SSC, 2% SDS at 68°C for 30 min.

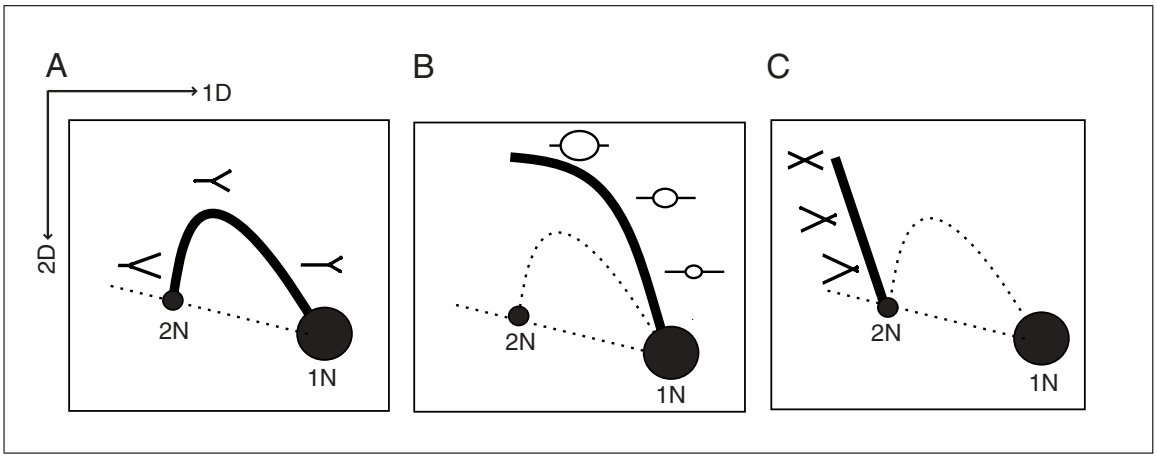


Figure 22.14.3 Behavior of replication intermediates and recombination intermediates in two-dimensional gel electrophoresis. The first dimension from left to right separates molecules in proportion to mass. The second dimension (top to bottom) mostly separates molecules as a function of their three-dimensional shape. The dashed lines mark the location of linear molecules of various sizes. Replication intermediates of the detected fragments vary in mass from 1N (large filled circle) to 2N linear fragments (small filled circle). (**A**) Fragments replicated passively from an outside origin correspond to Y-shaped simple replication fork signals. These consist of two branched daughter strands and one non-replicated stem strand. As a replication fork progresses, the branched strands increase in length and the stem strand shrinks. The migration pattern of a Y fork is determined by the ratio of branched strands to the stem strand. (**B**) Fragments containing a replication origin correspond to the bubble-shaped signal. The diameter of the bubble determines the mobility along a gently curved line. (**C**) Fragments containing a replication termination site corresponds to the X-shaped signal. Recombination intermediates undergoing repair due to broken replication forks also form X-shaped molecules. Fragments that have different termination sites or that undergo crossing-over migrate differentially along a linear line.

20. Wash once with 50 ml 0.2× SSC 0.2% SDS at 68°C for 30 min.
21. Rinse the membrane briefly five times, each time with 50 ml 0.2× SSC 0.2% SDS at room temperature.
22. Wrap the damp blot in plastic wrap, and expose it to the imaging plate (Fuji Film).

An example obtained with a probe specific for the rRNA gene cluster is shown in Figure 22.14.2B using bio-imaging analyzer system BAS2500 (Fuji Film). The fragment contains a replication origin. Figure 22.14.3 explains the behavior of various kinds of replication intermediates.

DNA PREPARATION IN LOW-MELTING-POINT AGAROSE

SUPPORT PROTOCOL 1

This protocol describes how to prepare DNA from yeast in low-melting-point agarose. Cells are treated with Zymolyase to hydrolyze the cell walls. The spheroplasts formed are subsequently embedded in a low-melting-point agarose plug, and are then incubated in detergent containing proteinase K to disrupt the plasma membrane and to hydrolyze proteins associated with DNA, respectively.

Materials

Cultures of yeast cells
10% (w/v) sodium azide solution
Sorbitol solution (see recipe)
Zymolyase solution (see recipe)
Agarose solution no. 3 (see recipe)
ES solution: 0.5 M EDTA (pH 9.0)/1% (v/v) sodium *N*-lauroyl sarcosinate
Proteinase K (Merck)
TE buffer, pH 9.0 (*APPENDIX 2A*)

50-ml tubes
37° and 50°C incubators
Mold for making agarose plugs, 10-mm (height) × 5-mm (width) × 1.5-mm (thickness; Bio-Rad)

Collect the cells

1. Harvest exponentially growing yeast cells (~8.0×10^7) in a 50-ml tubes, add 1/100 volume 10% azide solution, and mix gently.
2. Pellet the cells by centrifuging 5 min at 3000× *g*, 4°C, and wash twice with 1 ml cold sorbitol solution.
3. Resuspend the cell pellets in 47 µl sorbitol solution on ice.
4. Add 3 µl Zymolyase solution, mix gently by pipetting, and incubate at 37°C for 30 to 60 min until the solution becomes viscous.

Embed the cells

5. Add an equal volume of agarose solution no. 3 and mix gently by pipetting. Immediately, pour the mixture into molds for making plugs.
6. Hold the filled mold at 4°C for 20 min to allow the agarose to harden.
7. Transfer the agarose plugs from the mold into 1 ml ES solution and incubate for 10 min at room temperature.
8. Replace the ES solution with 1 ml fresh ES solution containing 1 mg/ml proteinase K.
9. Incubate at 50°C for 8 to 24 hr, until the color of plug become transparent from white.
10. Wash the agarose plug five times in 1 ml TE, pH 9.0, for 10 min.
11. Store the washed plugs in TE buffer at 4°C.

SUPPORT PROTOCOL 2

DIGESTION OF DNA IN LOW-MELTING AGAROSE

This protocol describes how to digest DNA embedded in agarose. It should be noted that the choice of appropriate restriction enzyme is critical for effective digestion. Some restriction enzymes are very sensitive to agarose. The agarose-sensitivity of restriction enzymes is generally described by the supplier.

Materials

Agarose plug (Support Protocol 1)
Restriction enzyme buffer (H buffer; Takara)
Restriction enzyme (e.g., *Bgl*II 30 U/µl to 60 U/µl; Takara)

NOTE: Restriction enzymes that digest the target region into small fragments (<10 kb) should be selected. Because the DNA fragment (<10kb) undergoing replication is shorter than 20 kb, it can be separated by the gel electrophoresis.

1. Cut the agarose plug in half (final dimensions of 5 mm × 5 mm × 1.5 mm).
2. Place half of the plug into 1 ml of restriction enzyme buffer.
3. Incubate for 30 min at room temperature.
4. Replace the solution with fresh 1 ml restriction enzyme buffer and incubate for an additional 30 min.

5. Remove the buffer and add 250 μl enzyme buffer containing 250 units of restriction enzyme.

6. Incubate the agarose plug–embedded DNA at an appropriate temperature for ~8 hr.

   To prevent DNA fragments from dispersing out of the plug, put the plugs on the comb right after the digestion with the restriction enzyme.

CONTOUR-CLAMPED HOMOGENEOUS ELECTRIC FIELD PULSED-FIELD GEL ELECTROPHORESIS TO MONITOR CHROMOSOME REPLICATION STATUS

BASIC PROTOCOL 2

Pulsed-field gel electrophoresis (PFGE) was developed as a means of separating large DNA molecules by periodically switching the direction and duration of the electric field. DNA molecules ranging from ~10 kbp to 10 Mbp are amenable to separation by PFGE, which can be used for a variety of purposes (e.g., direct visualization of chromosomes, RFLP analysis of large restriction fragments, and isolation of YACS and BACS). Here, we demonstrate an application for monitoring replication status of chromosomes isolated from the yeast *Saccharomyces cerevisiae*.

Contour-clamped homogeneous electric field (CHEF) electrophoresis, a popular form of PFGE, produces sharp reproducible DNA bands in a straight lane. Electrodes are set along a hexagonal array to make a homogeneous electric field using computer-controlled digital/analog converters at each electrode. This system can generate an unlimited variety of field strengths and angles.

Materials

Yeast DNA in agarose plugs (Support Protocol 3)
Agarose solution for CHEF (200 ml): 1.0% (w/v) pulsed-field certified agarose (Bio-Rad)/0.5× TBE
0.5× TBE (*APPENDIX 2A*)
Ethidium bromide

43°C water bath
Gel casting tray
Well combs
Electrophoresis apparatus
CHEF mapper (Bio-Rad)

Prepare the gel

1. Melt a fresh agarose solution for CHEF (1% w/v agarose in 0.5× TBE) by heating in a microwave with frequent mixing.

2. Cool the solution to 43°C in a water bath, but do not allow the agarose to gel.

3. Assemble casting tray to make the gel.

4. Place the agarose plug on the comb teeth (corresponding to wells), and remove buffer around the plug by blotting with tissue paper.

   The maximum number of plugs per gel is 44.

5. Place the comb with the agarose plug into the casting tray leaving a 2-mm gap at the bottom.

6. Pour the agarose solution (43°C) into the tray and allow the gel to harden completely for 20 min at 4°C.

7. Carefully remove the comb leaving the plugs in the gel, and seal each well with the remaining agarose solution (43°C) allowing the seals to harden for 5 min.

Sealing of the wells is required to prevent the plugs from breaking loose of the gel during the process of capillary transfer. The replication intermediates remain in the plugs.

Run the gel

8. Set the parameters for switching time, voltage, and duration of run, to separate the chromosomes. A typical program follows:

Initial switch time:	0.22 sec
Final switch time:	266 sec
Run time:	15.16 hr
Voltage:	6.0 V/cm
Included Angle:	120°.

9. Run the CHEF apparatus at 14°C by circulating 2.2 liters of 0.5× TBE buffer in the running tank.

Visualize the chromosomes bands

10. After the electrophoresis is complete, place the gel into 300 ml of 0.5 μg/ml ethidium bromide solution and incubate for 30 min at room temperature with gentle agitation.

11. Following the ethidium bromide staining, replace the solution with 400 ml deionized water and incubate for at least 20 min at room temperature. Check the migration pattern of each chromosome under UV illumination (Fig. 22.14.4A, left panel).

If necessary, destain the gel overnight in deionized water to further reduce background staining.

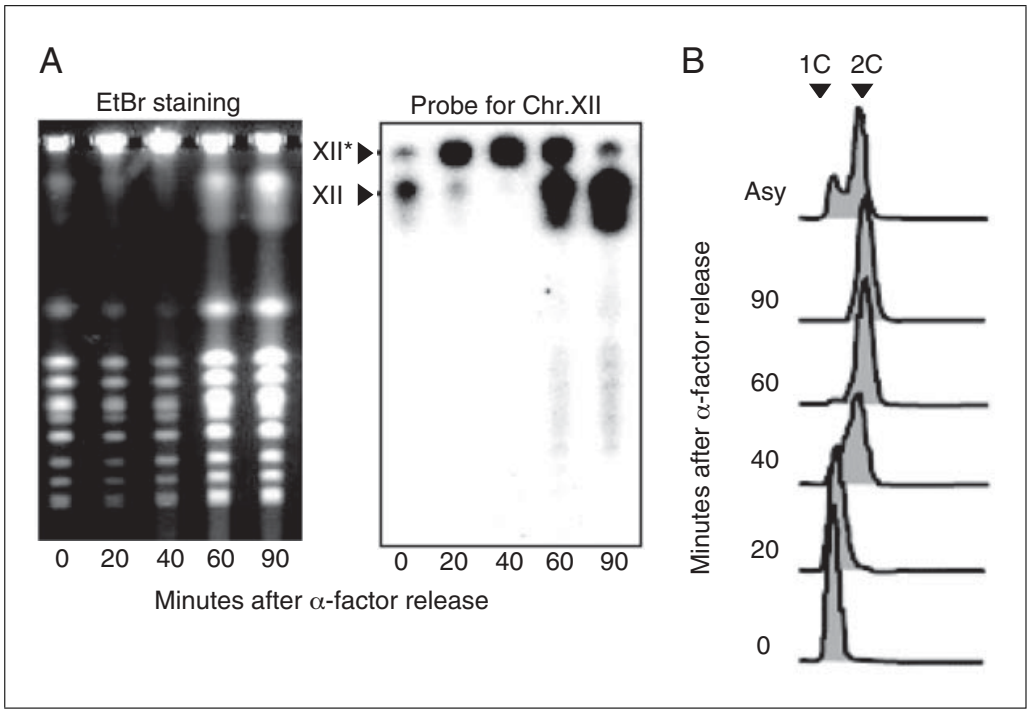


Figure 22.14.4 Migration of chromosomes from synchronously grown yeast. (**A**) Cells were released from G1 arrest by α-factor into YPD medium (see recipe) at 23°C. At the indicated times, DNA was prepared in agarose gel plugs and analyzed by CHEF. Chromosome patterns were detected by ethidium bromide staining (left) and by Southern blot hybridization using a chromosome XII specific probe (right). Triangles show the positions of chromosome XII in the well (with asterisk) and gel. (**B**) Flow cytometry analysis for cell cycle progression after release from G1-arrest by a-factor.

12. Because of the large size of the DNA fragments, it is necessary to nick them by acid treatment to achieve efficient transfer.

    A typical transfer protocol including acid treatment is shown in Basic Protocol 1 (steps 10 to 13).

    A typical migration pattern is shown in Figure 22.14.4A (right panel). At G1 (time 0 after released from the YPD medium containing α-factor), chromosomes enter the gel because they are linear. In S phase (time 20 to 40 min), most chromosomes get stuck in the well and fail to migrate because of the presence of replication intermediates. After completion of replication (60 to 90 min), chromosomes again are able to migrate into the gel. At the same time, the change in total DNA content can be followed by FACS analysis (Fig. 22.14.4B). FACS analysis confirms that total DNA content gradually increases with progression of replication.

DNA PREPARATION IN LOW-MELTING-POINT AGAROSE GEL FOR PFGE

SUPPORT PROTOCOL 3

This section describes an alternate protocol for preparing DNA from yeast in a low-melting-point agarose gel. While the DNA preparation protocol for two-dimensional gel analysis can be used for PFGE, it is not recommended, as it leads to chromosome degradation during Zymolyase treatment because spheroplasts are extremely fragile. Therefore, we highly recommend that yeast cells be embedded in low-melting-point agarose prior to Zymolyase treatment (even if this prolongs sample processing by a day), because this prevents chromosomes from being sheared, even if the spheroplast is burst.

***Additional Materials** (also see Basic Protocol 1)*

Agarose solution: 1.5% Sea plaque GTG agarose, 0.25 M EDTA (pH 8.0)
Synchronously grown yeast cells harvested from YPD medium (pH 4.0) containing 3.0 μM α-factor.
50 mM EDTA
LET buffer (see recipe)
ESP buffer (see recipe)
TE buffer, pH 9.0 (*APPENDIX 2A*)

Add cells to agarose

1. Heat agarose solution in a microwave until the agarose is melted and then equilibrate to 50°C.
2. Harvest ~8.0×10^7 cells, transfer the cells into a 50-ml tube, and pellet the cells by centrifuging 5 min at 3000 × *g*, 4°C.
3. Resuspend the cells with 1 ml ice-cold 50 mM EDTA and then centrifuge again.
4. Resuspend the cell pellets in 47 μl of 50 mM EDTA.
5. Warm the cells to 50°C.
6. Add 3 μl Zymolyase solution and 50 μl of agarose solution, and mix gently by pipetting.

Prepare the mold

7. Immediately pour the mixture into mold to form plugs.
8. Place the filled mold at 4°C for 20 min to allow the agarose to gel.
9. Transfer the agarose plug into 1 ml of LET buffer and incubate overnight at 37°C.

    LET buffer promotes degradation of the cell wall by Zymolyase

10. Replace the LET solution with 1 ml of ESP buffer containing 1 mg/ml proteinase K and incubate overnight at 50°C.

11. Wash the agarose plug with 1 ml TE buffer, repeat the wash at least five times, and store at 4°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose solution no. 1, 100 ml

0.4% (w/v) Sea Kem agarose
1× TBE (*APPENDIX 2A*)

Agarose solution no. 2, 100 ml

1.2% (w/v) Sea Kem agarose
1× TBE (*APPENDIX 2A*)
0.3 μg/ml ethidium bromide

Agarose solution no. 3

1% (w/v) Sea plaque GTG agarose
0.1 M EDTA, pH 7.5 (*APPENDIX 2A*)
Heat in a microwave oven until the agarose melts and then equilibrate to 45°C

ESP buffer

100 mM Tris·Cl, pH 9.0 (*APPENDIX 2A*)
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
1% (v/v) *N*-Lauroylsarcosine sodium
1 mg/ml proteinase K (Merck)
Prepare fresh

LET buffer

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
1 M 2-mercaptoethanol
Prepare fresh

Phosphate-SDS hybridization buffer

0.5 M Na_2HPO_4, pH 7.2
7% (w/v) SDS (*APPENDIX 2A*)
1% (w/v) bovine serum albumin (BSA)
1 mM EDTA (*APPENDIX 2A*)
Prepare fresh

Sorbitol solution

1 M sorbitol
0.1 M EDTA, pH 7.5
0.1% (w/v) sodium azide
Store up to 6 months at 4°C

YPD medium

Final concentration:
1% (w/v) Bacto yeast extract
2% (w/v) Bacto polypeptone
2% (w/v) glucose

Zymolyase solution

10 mg/ml Zymolyase (100T, Seikagaku BC)
20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
50% (v/v) glycerol
Store up to 3 months at −20°C

COMMENTARY

Background Information

Since the late 1980s, two-dimensional gel electrophoresis has been used as a sensitive physical mapping technique to identify chromosomal DNA replication origins. Two groups independently reported use of two-dimensional gel electrophoresis to localize replication origins and termination sites in *S. cerevisiae*. One used a neutral/neutral type of two-dimensional gel electrophoresis (Brewer and Fangman, 1987), while the other used a neutral/alkaline type of two-dimensional gel electrophoresis (Nawotka and Huberman, 1988). In the neutral/neutral type, it is possible to observe DNA molecules by electron microscopy, following excision of the gel band. This technique has also been applied to higher eukaryotic cells (Dijkwel and Hamlin, 1997). In the case of higher eukaryotic cells, enrichment for replicate intermediates is required. Enrichment involves: (1) synchronization of cell populations in the S phase, and (2) removal of nonreplicating DNA by chromatography using benzoylated naphthoylated DEAE (BND)-cellulose (Dijkwel and Hamlin, 1997). These enrichment techniques can also be applied to yeast as needed.

The CHEF pulsed-field gel system was first used to analyze DNA replication as an assay of chromosome damage in replication-deficient yeast mutants (Hennessy et al., 1991). It has since been shown that CHEF can monitor replication status in synchronized cells. Moreover, CHEF distinguishes chromosomes undergoing repair as the recombination intermediates formed during repair are unable to enter the gel because of their higher order structure (Ide et al., 2007; Ganley et al., 2009).

Critical Parameters and Troubleshooting

Condition of cells

The ratio of replication intermediates to nonreplicating DNA is the most critical parameter in two-dimensional gel electrophoresis. As described above, synchronization of the cell cycle is an effective means of increasing replication intermediates. In case of yeast, an exponentially growing population contains enough S phase cells for adequate detection. We recommend harvesting exponentially growing cells at a density of $<3.0 \times 10^6$ cells/ml.

Killing cells by azide solution

The first step in preparing the DNA is to kill yeast cells by exposure to azide solution. Treatment with azide stops DNA replication immediately and completely.

Efficiency of DNA digestion

Incomplete DNA digestion by restriction enzymes sometimes occurs because the DNA is embedded in an agarose plug. If another restriction enzyme cannot be used, we have two recommendations. One is extension of the reaction time from hours to overnight, for example. The other is to decrease cell density in the plug.

Detection of replication intermediates in single-copy loci

Two-dimensional gel electrophoresis can also be applied to single-copy loci. Although signals from single-copy loci are weaker than from repetitive sequences, e.g., ribosomal RNA genes, the protocol described here can detect a variety of replication intermediates. If the signal density of replication intermediates is found to be weak, we recommend using more cells.

Temperature of agarose solution

One critical parameter of CHEF electrophoresis to monitor the replication status is the temperature of the agarose solution when it is poured into the tray. Agarose solution at much higher temperatures may denature linear DNA in the plugs attached on the comb. As a result, the majority of the chromosome in the plugs stacks in the well and the migration pattern does not reflect the replication status. To avoid such artificial conformational change of DNA in the plugs, the temperature of the agarose solution should be lower than 45°C.

Anticipated Results

Typical DNA migration patterns resulting from two-dimensional gel analysis and

CHEF analysis are shown in Figure 22.14.2B and Figure 22.14.4A. In the case of two-dimensional gel analysis of rDNA, three kinds of nonlinear DNA, simple Y-shaped, replication bubble, and X-shaped molecules, are observed. They are detected by hybridization with the radiolabeled rDNA probes. Generally, as the signal intensity of nonlinear DNA is weaker than that of linear DNA, the blot is exposed to the imaging plate for longer time (~1 week). In CHEF analysis, linear chromosomes are separated from replicating chromosomes. Linear chromosomes are detected as bands in the gel and replicating chromosomes are detected as stacked bands in the well.

Time Considerations

For two-dimensional gel analysis, preparation of DNA in the plug takes 2 days. After preparation of DNA, first and second gel electrophoreses take 14 hr (overnight) and 6 hr, respectively. Capillary transfer and the subsequent hybridization require 2 days to complete, and the time to expose the blot to the imaging plate is 1 to 7 days, depending on the signal intensity of target DNA. In CHEF, analysis requires almost the same amount of time as two-dimensional gel analysis does, except the electrophoresis requires ~16 hr.

Literature Cited

Bell, L. and Byers, B. 1983. Separation of branched from linear DNA by two-dimensional gel electrophoresis. *Anal. Biochem.* 130:527-535.

Brewer, B.J. and Fangman, W.L. 1987. The localization of replication origins on ARS plasmids in *S. cerevisiae*. *Cell* 51:463-471.

Dijkwel, P.A. and Hamlin, J.L. 1997. Mapping replication origins by neutral/neutral two-dimensional gel electrophoresis. *Methods* 13:235-245.

Ganley, A.R.D., Ide, S., Saka, K., and Kobayashi, T. 2009. The effect of replication initiation on gene amplification in the rDNA and its relationship to aging. *Mol. Cell* 35:1-11

Hennessy, K.M., Lee, A., Chen, E., and Botstein, D. 1991. A group of interacting yeast DNA replication genes. *Genes Dev.* 5:958-969.

Ide, S., Watanabe, K., Watanabe, H., Shirahige, K., Kobayashi, T., and Maki, H. 2007. Abnormality in initiation program of DNA replication is monitored by the highly repetitive rDNA array on chromosome XII in budding yeast. *Mol. Cell. Biol.* 27:568-578.

Nawotka, K.A. and Huberman, J.A., 1988 Two-dimensional gel electrophoretic method for mapping DNA replicons. *Mol. Cell. Biol.* 8:1408-1413.

Schwartz, D.C. and Canter, C.R. 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 37:67-75.

CHAPTER 23
Stem Cells

INTRODUCTION

Few issues in cell biology have garnered more public attention in recent years than stem cells. Research into stem cells holds the promise for novel therapeutic interventions that could revolutionize the treatment of a range of human diseases. On the other hand, objections to the use of human embryonic stem cells for research have been raised on religious, moral, and ethical grounds. While keeping in mind these two important aspects of the ongoing debate, scientists have steadily learned to exploit the potential of stem cells for unraveling the molecular mechanisms of cell differentiation and organismal development. This chapter presents a comprehensive collection of protocols describing the use of stem cells to study cell differentiation.

Stem cells have the dual ability to undergo self-renewal and to generate lineages of more differentiated or mature cells. *UNIT 23.1* is an overview of the current knowledge on stem cells and their pathways of differentiation. The overview describes the properties of stem cells, defines the meaning of totipotent, multipotent, pluripotent, and unipotent cells, and discusses the different cell lineages that can be derived from various stem cell types. The discussion deals with both embryonic and adult stem cells and gives examples of their use for tissue repair. The overview ends with two sections dealing with the genetic manipulation and potential therapeutic applications of stem cells.

UNIT 23.2 comprises a series of protocols for the production and handling of embryonic stem cells. The initial protocols describe procedures for the derivation, culture, and preservation of mouse embryonic stem cells. This is followed by a description of methods for the differentiation of mouse embryonic stem cells into "embryoid bodies," which are spherical masses of cells with regions of lineage-specific differentiation. This unit also includes protocols for the propagation of human stem cells and their differentiation into embryoid bodies, though not for their initial derivation.

UNIT 23.3 begins a series of units devoted to the differentiation of embryonic stem cells into various lineages. This unit deals specifically with the maintenance and differentiation of mouse embryonic stem cells to generate blood vessels. These vessels form early during embryonic development by two processes: vasculogenesis and angiogenesis. In vasculogenesis, mesodermally derived angioblasts differentiate to form primitive blood vessels. This is followed by angiogenesis, in which endothelial cells derived from the angioblasts proliferate and migrate, leading to vessel expansion and sprouting. Both of these processes can be studied by differentiation of embryoid bodies over a period of 8 days. Blood vessels can be identified by staining for various vascular markers (e.g., PECAM-1) or by performing the β-galactosidase reaction on differentiated cultures generated from mouse embryonic stem cells in which the *lacZ* gene is driven from a vascular-specific promoter.

UNIT 23.4 deals with the differentiation of mouse embryonic stem cells and human adult stem cells into adipocytes. Adipocytic differentiation of mouse embryonic stem cells also starts with the formation of embryoid bodies, which are first committed by the addition of retinoic acid and then terminally differentiated by the addition of adipogenic

Current Protocols in Cell Biology 23.0.1-23.0.2, September 2007
Published online September 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2300s36

factors (e.g., insulin, triiodothyronine, rosiglitazone). Adipocytes can also be derived from adult human multipotent adipose-derived stem (hMADS) cells or human mesenchymal stem (hMS) cells. For both of these adult stem cell types, differentiation does not require retinoic acid and is achieved in monolayer culture. hMADS are driven to adipogenic differentiation by addition of isobutylmethylxanthine (IBMX) and dexamethasone, whereas adipogenic differentiation of hMS occurs simply in the presence of fetal bovine serum. The resulting adipocytes can be easily identified through visualization of their lipid droplets by bright-field microscopy of unstained cells or of cells stained with the triglyceride-specific stain, Oil Red O. Adipocytic differentiation can also be monitored by northern analysis of the expression of adipose-specific genes.

UNIT 23.5 presents protocols for the differentiation of mouse embryonic stem cells to chondrocytes and osteocytes. The process also starts from embryoid bodies, which spontaneously differentiate into various cell types, including chondrogenic and osteogenic precursors. Mesenchymal condensations containing chondrogenic precursors and, at later stages of differentiation, cartilage nodules with mature chondrocytes and osteocytes, are isolated using either a microdissector or a microscalpel. Differentiated cells can be studied either in the aggregates or upon dissociation with collagenase followed by monolayer culture. Samples can be analyzed by histochemical staining, immunofluorescent staining, in situ hybridization, or RT-PCR, to test for the expression of various markers for cartilage or bone. The characteristic features of ES cell-derived mesenchymal condensations and cartilage nodules can also be visualized by light microscopy and further examined at the ultrastructural level by electron microscopy.

UNIT 23.6 features protocols to study the differentiation of human embryonic stem cells into hematopoietic progenitors and endothelial cells for coculture with bone marrow stromal cell lines. Cells belonging to both lineages can be derived simultaneously and characterized by immunostaining and flow cytometry. A colony-forming assay can be used to examine the potential of hematopoietic progenitors to differentiate into erythroid, granulocytic, macrophage, and megakaryocyte cells. The various cell lineages produced by differentiation of human embryonic stem cells can be isolated, as well as separated from the bone marrow stromal cells, by magnetically-activated cell sorting and preparative flow cytofluormetry.

UNIT 23.7 describes protocols for neural differentiation of human embryonic stem cells. Pure populations of neural precursors can be derived from human embryonic stem cells for differentiation in chemically defined serum-free culture medium containing noggin and fibroblast growth factor. Neural precursors derived in this way can be cultured for long periods or further induced to differentiate into mature neurons and glial cells. Other protocols describe methods for characterizing neural precursors and their derivatives by immunostaining for specific marker antigens followed by fluorescence microscopy or flow cytometric analysis of stained cells.

Juan S. Bonifacino

Stem Cells: An Overview

Stem cells are specialized cells that possess a capacity to undergo self-renewal while at the same time having the ability to give rise to at least one or more differentiated or mature cell type. They therefore represent a fundamental cornerstone during the life of all vertebrates, playing central roles in the production of new and replacement cells for tissues during development and homeostasis, including repair following disease or injury. Although all tissues during vertebrate development appear to possess cells with stem cell properties, whether this is the case in the adult remains to be clearly demonstrated. Certainly, the skin, muscle, intestine, hematopoietic system, and liver can be regenerated following acute trauma, but it is only now becoming accepted that similar properties may also be attributable to tissues of the brain, heart, and pancreas (Dor and Melton, 2004).

A diverse range of stem cells have been described according to their long-term ability to maintain stem cell–like properties in vitro and in vivo and the number and type of derivatives that they give rise to. The first category is the unipotential stem cell, which undergoes self-renewal and gives rise to only one mature cell type. One example is the keratinocyte stem cell of the dermis that divides to give rise to a population of closely related keratinocytes. The second category is the multipotent stem cell, characterized by an ability to undergo self-renewal and the capacity to yield at least two or more differentiated fetal or adult cell types. Examples of multipotential stem cells include a range of stem cells in the developing fetus and many of the adult stem cells so far described, e.g., neural stem cells (NSCs), neural crest stem cells (NCSCs) of the nervous system, and hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) of the bone marrow. A third category of stem cell is the pluripotent stem cell, which is also capable of self-renewal and gives rise to a vast array of mature cell types, but not every cell type characteristic of that species. Examples of pluripotent stem cells are the in vitro–generated embryonic germ (EG) cells of the gonads, embryonal carcinoma (EC) cells, which are derivatives of tumorigenic germinal tissue, and embryonic stem (ES) cells. ES cells are pluripotent in vitro derivatives of the blastocoel inner cell mass or epiblast (Mollard et al., 2002). They have a wide developmental potential and have been demonstrated to give rise to every cell type of the adult organism. However, they give rise only to a restricted subset of their respective extraembryonic lineages represented during development. A fourth type of stem cell is the totipotent stem cell, which includes the fertilized oocyte and premorula blastomeres. Totipotent stem cells replicate and generate all adult and extraembryonic tissues of their species, but undergo either no or only limited self-renewal. A relatively new fifth category is the cancer stem cell. These cells replicate and undergo self-renewal, yet their compromised self-renewal pathways result in neoplasia with accompanying undifferentiated, partially differentiated, and/or differentiated cell types.

Much attention has been focused on stem cells because of their wide potential for therapeutic applications and their potential for unraveling molecular mechanisms of development. They possess the potential to provide replacement tissue or a means to understand disease mechanisms, thus providing cures for numerous diseases and injuries, such as Parkinson's disease, spinal injury, cardiac failure, and diabetes. Before this is possible, however, and in addition to developing methods to determine how to differentiate stem cells into exactly the right lineage desired at a homogenous or near-homogenous frequency, a number of important factors need to be addressed. Firstly, the ethics of using ES cells need to be discussed widely in both the scientific and the general community. Secondly, issues related to the immune rejection of foreign stem cells need to be overcome. Thirdly, the issue of accessibility of stem cells to appropriate regions of the body must be determined.

THE STEM CELL NICHE

Maintenance of the stem cell phenotype in vivo is critically dependent upon its surrounding microenvironment or niche. This microenvironment is composed of a basement membrane, extracellular matrix, cell-cell interactions, cytokines, and other effector or maintenance proteins. Together, such factors regulate the stem cell's balance between self-renewal and differentiation. Functional genomic attempts to determine the genetic factors driving the balance between self-renewal

Contributed by Mark Denham, Brock Conley, Fredrik Olsson, Timothy J. Cole, and Richard Mollard

Current Protocols in Cell Biology (2005) 23.1.1-23.1.18

and differentiation in the stem cell niche have been recently undertaken (Hackney et al., 2002). The fetal liver, for example, provides the early microenvironment for expansion of its stem cell pool required for the liver in adult life. The cellular constituents of this hepatic stem cell niche were examined by cloning fetal stromal cell lines that support primitive hematopoietic cells in vitro (Hackney et al., 2002). Elucidating the genetic mechanisms that maintain stem cell populations in their stem cell state will aid in the production of large quantities of stem cells for therapeutic applications.

Stem Cell Division

The stem cell niche provides an environment that maintains cells in an undifferentiated stem cell state. Local injury in the surrounding tissue releases morphogenic signals to invoke proliferative and differentiation events within the niche, allowing tissue repair. This alters the balance within the stem cell niche, and through intrinsic mechanisms within the stem cell, a switch occurs from self-renewal (symmetric division) to a proliferative differentiation event (asymmetric division), leading to the production of more mature cellular derivatives.

Asymmetrical cell division produces both another stem cell and a more mature cell type known as a progenitor cell (in hematopoiesis) or transit-amplifying cell (in other tissues). Progenitor or transit-amplifying cells often undergo a more rapid expansion, producing fully differentiated cell types with specific characteristics appropriate to their function in the particular tissue. Stem cells generally display a slower rate of cell cycle progression. For example, stem cells of the epidermis cycle slowly, displaying a long-term retention of the nuclear label bromodeoxyuridine (Cotsarelis et al., 1990). Asymmetrical cell divisions thus maintain the local stem cell pool while contributing mature differentiated cells for tissue growth, turnover, or repair. Symmetrical cell division produces either two identical stem cells or two progenitor or transit-amplifying cells. Symmetrical cell divisions therefore maintain, increase, or deplete the stem cell pool.

IMMORTALITY: TELOMERASE AND STEM CELLS

The terminal ends, or telomeres, of eukaryotic chromosomes are protected from degradation, incomplete replication, or fusion by enzymes known as telomerases (Fig. 23.1.1). In the absence of telomerase activity, cells become subject to age-dependent mortality, with shortening of the chromosomes until a threshold senescence limit is reached. At this point, cells have a reduced replicative potential and therefore can no longer divide, an event characteristic of differentiated cell types. In order for stem cells to maintain their stem cell pool throughout the life of an organism and not reach replicative senescence, a higher telomerase activity is utilized by many stem cell populations (Morrison et al., 1996). Germ cells, cancer cells, and some stem cells, such as HSCs, express high levels of telomerase (Morrison et al., 1996). However, the amount of telomerase expressed in adult stem cells is not sufficient to keep the telomeres of chromosomes at the same length. Instead, enough is expressed to enable adult stem cells to divide and provide sufficient cells for an organism's life. ES cells, on the other hand, are theoretically immortal, and thus presumably produce enough telomerase such that their telomeres never shorten. However, depending upon the specific culture conditions used, it appears that karyotypic abnormalities can occur (Buzzard et al., 2004; Draper et al., 2004).

THE EMBRYONIC STEM CELL AS A PLURIPOTENT STEM CELL

ES cells can be maintained indefinitely in an undifferentiated pluripotent state in vitro. Their capacity to contribute to functional derivatives of all cells of the body is best exemplified by tetraploid embryo complementation studies in the mouse (Nagy et al., 1990, 1993). Mouse ES cells (mESCs) represent the best characterized ES cell lines, and in addition to their pluripotent properties described in vivo, have also been induced to differentiate into diverse cell lineages such as cardiac muscle, neural crest cells, neural stem cells, and hematopoietic cell lineages in vitro (Fig. 23.1.2). Human ES cells (hESCs), although less well characterized, are also known to possess similar properties to mESCs, but they also display some different characteristics. Inoculation of hESCs beneath the testicular capsule of severe combined immunodeficient (SCID) mice has produced solid teratomas containing representative cells from all three embryonic germ cell layers, including glandular and squamous epithelia, muscle, bone, and neuroectoderm. This represents a test for pluripotency in the human comparable to blastocyst injection of mESCs in mice (Reubinoff et al., 2000). In

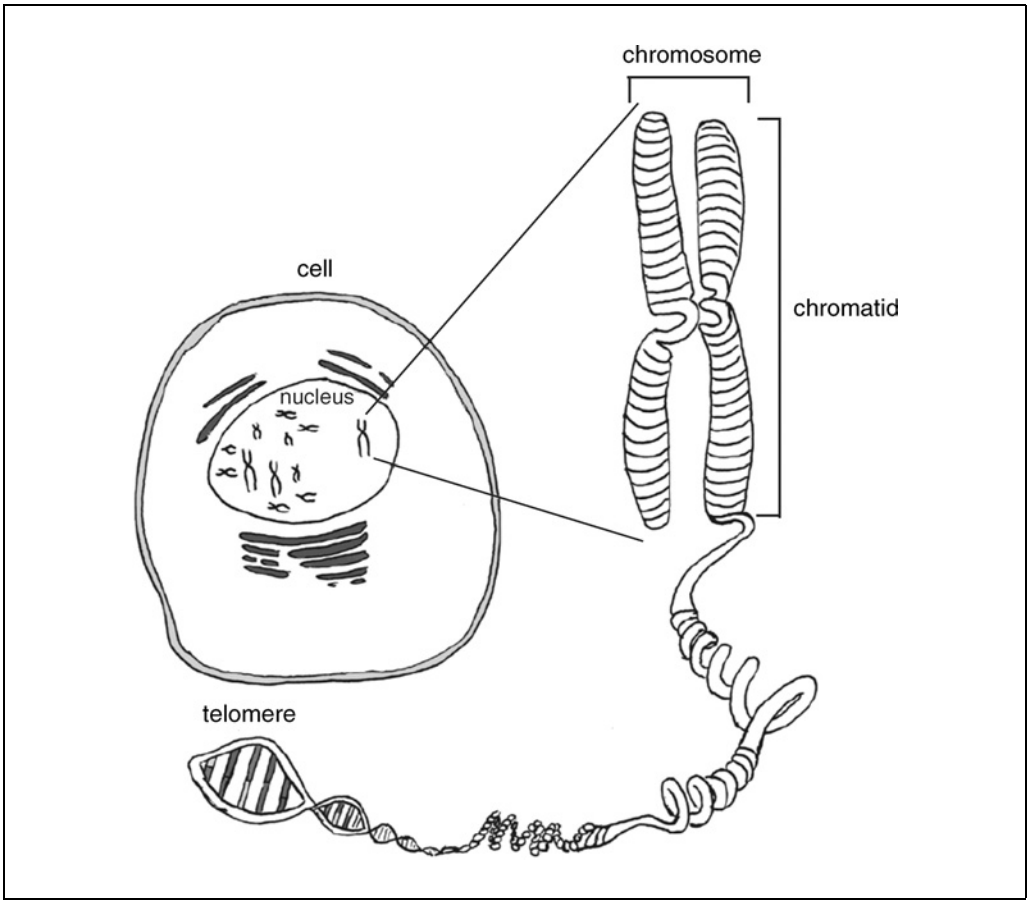


Figure 23.1.1 Eukaryotic cells express telomerase. Telomeres are located at the end of each chromosome in eukaryotic cells. Telomeres shorten with each cell division. Stem cells express telomerase, an enzyme that maintains telomere length during division. Maintenance of telomere length is crucial to the prevention of cell senescence.

addition to the blastocyst, isolation of hESCs has also been reported recently from the inner cell mass of human morula (Strelchenko et al., 2004). With the demonstrated pluripotency of mESCs in vivo and their amenability to manipulation in vitro, it is believed that hESCs may be induced in vitro to differentiate into any desired cell of the body for potential use therapeutically.

A common method for differentiating hESCs is embryoid body (EB) formation, which can be achieved through several different techniques such as suspension culture or culture in methylcellulose-containing media (Itskovitz-Eldor et al., 2000; Conley et al., 2004). ES cells cultured in these conditions form spherical structures which are called embryoid bodies (EBs), which refers to the appearance of lineage-specific regions of cell differentiation and a gene-expression profile similar to that found in the early embryo. These EBs contain cells representative of all three embryonic germ cell layers (ectoderm, mesoderm, and endoderm), and, under specific culture conditions, give rise to a number of distinct differentiated cell types. The isolation of relatively pure populations of several of these desired cell types has been subsequently achieved by culture with growth factors to enhance certain cell populations, by culture in selection media, or by fluorescence-activated cell sorting (FACS) for lineage-specific markers or lineage-specific reporter gene expression. In addition to EB formation, directed differentiation of ES cells into desired lineages has been achieved by coculture with selected tissues. For example, differentiation of hESCs into immature cardiomyocyte lineages has been reported following culture on a cell layer of a spontaneously differentiating visceral endoderm-like cell line derived from the P19 embryonal carcinoma cell line (Mummery et al., 2002). Similarly, culture of hESCs on a layer of γ-irradiated S17 murine bone marrow stromal cell line or the C166 yolk sac endothelial cell line has been reported to induce formation of hematopoietic colonies that give rise to erythroid, myeloid, and

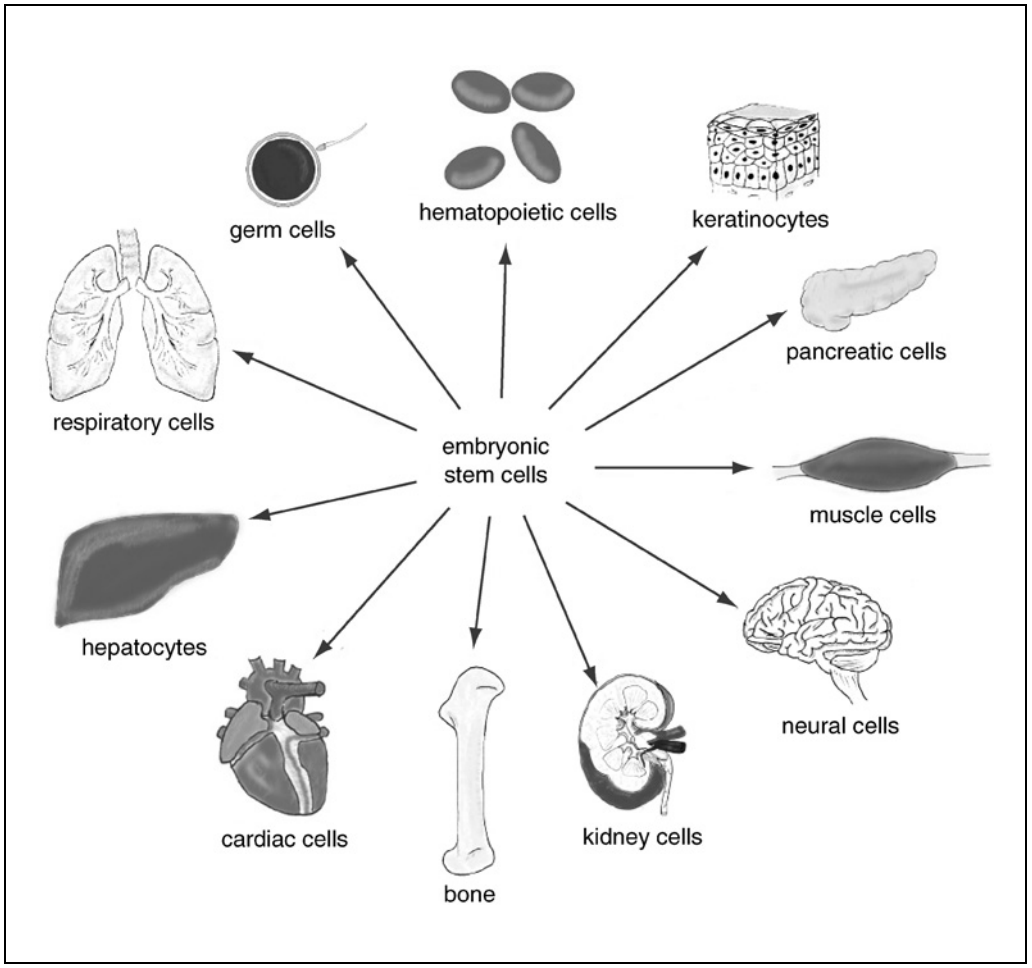


Figure 23.1.2 Pluripotency of embryonic stem cells. Embryonic stem cells have been reported to differentiate into a vast array of cells types in vitro. Differentiated cell types include: germ cells, respiratory cells, hepatocytes, cardiomyocytes, osteoblasts of the bone, neural cells, skeletal and smooth muscle, islet cells of the pancreas, keratinocytes, and hematopoietic cells.

megakaryocyte colonies following transfer to semisolid media in the presence of hematopoietic growth factors (Kaufman et al., 2001). Furthermore, prolonged cultivation of hESCs to a higher cell density has been shown to induce the formation of (1) contracting cells that stained positively for muscle-specific forms of actin and (2) cells possessing elongated processes that stain positively for neurofilament proteins and N-CAM, a neural cell adhesion molecule (Schuldiner et al., 2001). The differentiation of ES cells into motor neurons has been achieved by first producing EBs, then culturing them in the presence of retinoic acid (RA) to produce caudal neural cells, and finally adding an Shh signaling antagonist (Hh-Ag1.3) to induce the formation of ventral motor neurons. This procedure mimics the molecular signals known to promote normal development of motor neurons (Wichterle et al., 2002). Following implantation into the chick spinal cord, these cells were demonstrated to specifically innervate muscle. In another recent example, mESCs were treated with the phosphoinositide-3-kinase (PI3-K) inhibitor (LY294002) to induce differentiation of ES cells into islet-like cells, which could be transplanted into the pancreas of mice, thereby rescuing several effects associated with induced diabetes mellitus (Lumelsky et al., 2001; Hori et al., 2002).

MULTIPOTENT STEM CELLS

A potential source of cells for replacement therapeutics, apart from ES cells, is that of multipotent stem cells, in particular the hematopoietic and mesenchymal stem cells (HSCs and MSCs, respectively) of the bone marrow.

Hematopoietic Stem Cells

In terms of molecular properties, ease of isolation, developmental potential, and

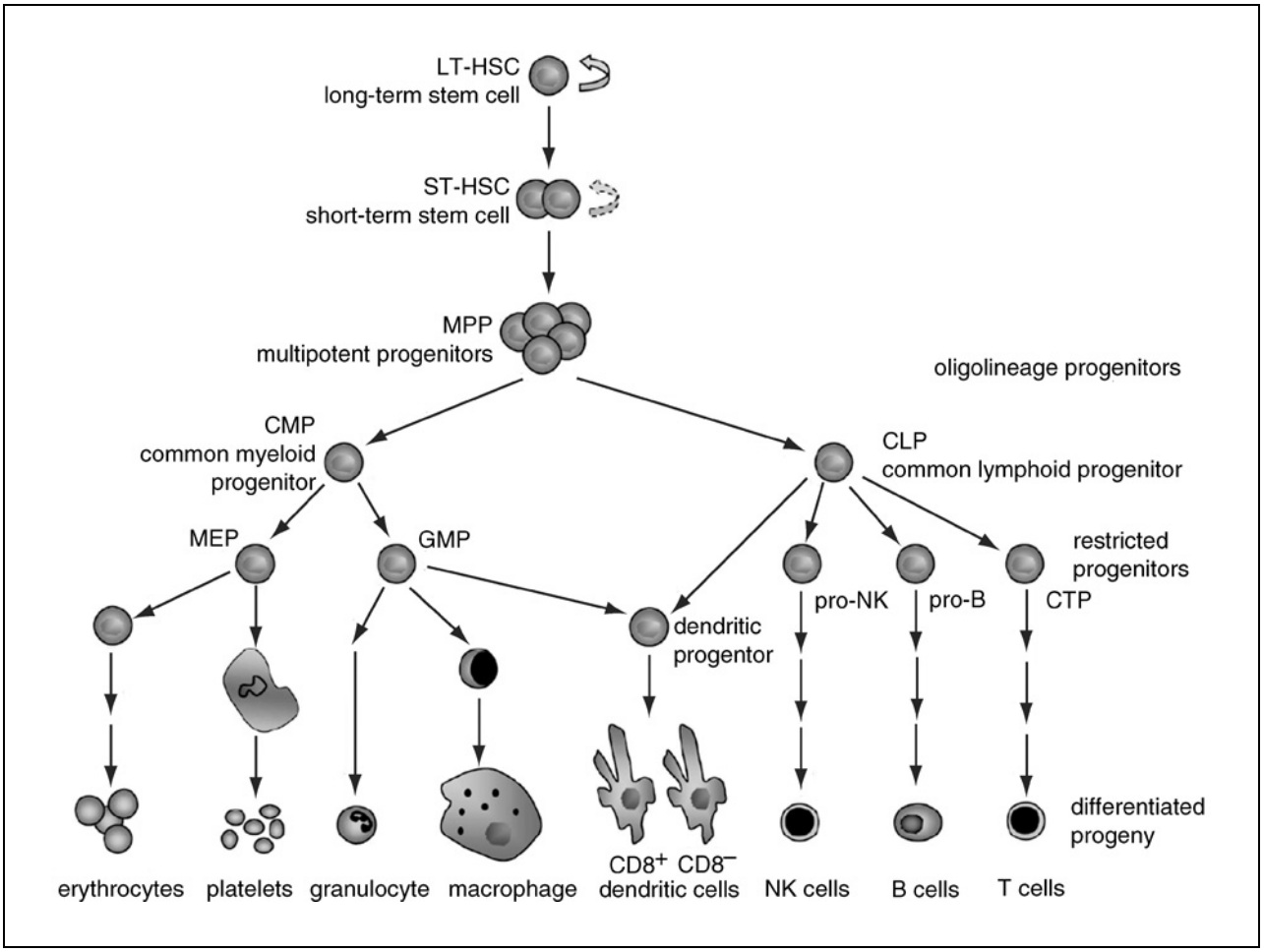


Figure 23.1.3 The hematopoietic hierarchy. Long-term hematopoietic stem cells (LT-HSCs) are located at the top of the hierarchy and give rise to short-term hematopoietic stem cells (ST-HSCs). In turn, ST-HSCs give rise to multipotent progenitors (MPPS) that generate the two distinct common myeloid and lymphoid progenitor populations (CMPs and CLPs, respectively). CMPs give rise to erythrocytes, platelets, granulocytes, and macrophages, whereas CLPs give rise to natural killer (NK), B cells, and T cells.

thrapeutic applications, HSCs represent the best characterized and understood of all stem cells (Orkin, 2000). HSCs found within adult bone marrow can give rise to all lymphoid and myeloid blood cell types and can be divided into two subtypes: long-term HSCs (LT-HSCs), which undergo extensive self-renewal, and their progeny, short-term repopulating HSCs (ST-HSCs), which undergo more limited self-renewal prior to becoming multipotential lymphoid or myeloid progenitors. At a single-cell level, LT-HSCs can be defined as being able to reconstitute all blood types of lethally irradiated mice (Osawa et al., 1996; Morrison et al., 1997). Although numerous definitions exist, LT-HSCs have also been defined within both the $CD34^+/lin^-$ population and Hoechst 33342/rhodamine 1223 effluxing ($Sca\text{-}1^+c\text{-}kit^+CD43^+CD45{+}lin^-$) side population (SP) of the bone marrow (Krause et al., 1994; Goodell et al., 1996, 1997; Christensen and Weissman, 2001; Scharenberg et al., 2002). Multipotential progenitors derived from ST-HSCs do not undergo self-renewal, but instead they provide both myeloid and lymphoid precursors that rapidly divide and give rise to all blood cell types (Kondo et al., 1997; Akashi et al., 1999a,b; Fig. 23.1.3). Although both LT-HSC and ST-HSC equivalents have been identified in both mouse and human, a number of cell surface markers have been shown to differ (Okuno et al., 2002).

With respect to therapies, both syngeneic (i.e., usually from twins) and allogeneic HSC transplantations can be used following systemic chemotherapy and chemoradiotherapy to replace the depleted hematopoietic systems and to induce immune tolerance of patients with severe aplastic anemias (80% of cases have neutropenia and megakaryocytic anemia), fatal leukemias and other

hematological malignancies (lymphomas and myelomas), clinically severe autoimmune disease, and thalassemias. Although success rates vary widely (between 15% and 20% for patients with acute leukemia and ~80% for patients with chronic myeloid leukemia) more than 20,000 transplants were performed worldwide in 2004 alone (see American Cancer Society, Cancer Facts and Figures, 2005; *http://www.cancer.org*).

Mesenchymal Stem Cells

MSCs were first identified as a mixed population of fibroblast-like plastic-adherent cells that separated from the nonadherent hematopoietic bone marrow fraction in culture (Friedenstein et al., 1966). Reports demonstrate that residual pre-B progenitor cells and granulocytic cells can best be removed on the basis of CD34/CD45/CD11b immunodepletion (Ortiz et al., 2002). MSCs provide a supportive role to HSCs during hematopoiesis, secreting growth factors and effecting direct cell-cell interactions, which play a role in HSC proliferation and differentiation. MSCs additionally possess the capacity to differentiate into a range of mature connective tissue cell types including chondrocytes, osteocytes, and adipose and smooth muscle cells. MSCs are also called bone marrow stromal cells, as they are most often isolated from the tibia, fibula, iliac crest, or the thoracic and lumbar spine. However, MSCs that possess the same multipotency have similarly been isolated from skeletal muscle, adipose tissue, deciduous teeth, and the synovium (Poulsom et al., 2002; Barry and Murphy, 2004). MSCs have therefore also been named mesenchymal progenitor cells because of the ability, for example, of MSCs isolated from adipose tissue to produce adipocytes and chondrocytes. The capacity to direct differentiation of MSCs preferentially into bone, cartilage, adipose tissue, or muscle has been demonstrated to be dependent upon the source tissue for extracting MSCs. Furthermore, different culture conditions can also prejudice the percentage or preference of differentiation into a specific lineage. For example, osteoblastic differentiation can be achieved by supplementing the medium with fetal bovine serum, dexamethasone, ascorbic acid, and β-glycerol phosphate in combination (Rodriguez et al., 2004). Preferential differentiation into chondroblastic lineages can be achieved by the addition of TGF-β, growth in a 3-D ultrastructure, and the removal of serum from the medium. However, differentiation of MSC into chondrocytes does not require all of these methods to be combined; chondrocyte differentiation can be achieved with the addition of TGF-β or by growth in 3-D culture, but if TGF-β is added to the 3-D culture, increased chondrocyte differentiation will result (Johnstone et al., 1998; Li et al., 2005). Differentiation of MSCs into adipocytes has been reported to be induced by monolayer culture of MSCs in the presence of isobutylmethylxanthine (Suzawa et al., 2003). Differentiation of MSCs into skeletal muscle induced by culture in the presence of amphotericin B has also been reported (Phinney et al., 1999). Therapeutically, MSCs have been reported to contribute to the repair of many tissues; this will be discussed below.

Neural Stem Cells

NSCs comprise the bulk of early fetal brain cells, and despite some conjecture, they have been reported to arise in the adult brain in the hippocampal dentate gyrus subgranular zone and the subventricular/subependymal zone of the lateral ventricles (Lois and Alvarez-Buylla, 1993; Doetsch et al., 1999; Johansson et al., 1999; Gage, 2000; Seaberg and van der Kooy, 2002). Linear differentiation of multipotent NSCs through more restricted progenitor populations provides all differentiated cells of the fetal neural system during development, as well as neurons and glia (oligodendrocytes and astrocytes) as required for learning and memory and following functional loss of established systems in the adult (Reynolds and Weiss, 1996; Shors et al., 2001; Carlen et al., 2002; Nakatomi et al., 2002). The regulation of NSCs and their progenitors into neuronal, astrocytic, or oligodendrocytic fates occurs according to the specificities of developmentally patterned transcriptional programming and the neural-specific microenvironmental niche (Song et al., 2002; Doetsch, 2003; Shen and Zhang, 2004). In addition to contributing to daily cellular turnover, neural cells possess a limited replacement potential following injury, but they are unable to restore function after damage.

Fetal neuronal transplantation has demonstrated some success in restoring motor function, memory, and spatial learning in rats following cortical grafting in focal ischemia–induced injury within the forebrain (Hodges et al., 1996). Furthermore, although some

patients display graft rejection and others develop severe dyskinesis, striatal grafting of fetal mesencephalic precursors or dopaminergic neurons can restore dopamine release to near-normal levels in patients with Parkinson's disease (Freed et al., 2001; Lindvall and Bjorklund, 2004). It is unlikely, however, that fetal neural tissue will become a routine source of material for treating neural disease, due to lack of availability and difficulties in standardizing functional outcome (Lindvall and Bjorklund, 2004). Several workers are therefore investigating the use of transplantable NSCs and their derivatives as a means to repair damage associated with diseases such as Parkinson's disease and multiple sclerosis.

NSCs isolated from fetal and adult brains of mice or humans can be expanded in culture from single cells in the presence of epidermal growth factor (EGF), fibroblast growth factor (FGF), or both EGF and FGF together, to form neurospheres (Reynolds and Weiss, 1992; Richards et al., 1992; Carpenter et al., 1999; Tropepe et al., 1999; Piper et al., 2001). Although some differences exist between NSCs isolated from humans and mice, expanded neurospheres from both species maintain a small percentage of multipotent NSCs and can be plated to give rise to neurons, oligodendrocytes, and astrocytes, or dissociated into single cells for serial neurosphere propagation. NSCs derived from fetal brain provide stable lines after extensive culture, and they maintain the capacity to engraft as neurons and glia following transplantation to the 6-hydroxydopamine-lesioned striata of cyclosporin-treated rats (Vescovi et al., 1999). Furthermore, multipotential cells isolated from the mouse optic nerve, hippocampus, and adult subventricular zone demonstrate remyelination of damaged axons, repopulation of the hippocampus, and rostral migration to the olfactory bulb where they produce two different types of olfactory neurons, respectively (Crang et al., 1992; Lois and Alvarez-Buylla, 1994; Ray and Rodrigues, 1995; Yandava et al., 1999). Perinatal intracallosal xenograft of more committed oligodendrocyte progenitor cells from both the late-gestation and adult human ventricular zone into myelin basic protein–deficient shiverer mice (*shi*$^{-/-}$), a model of perinatal leukodystrophy, generated myelinating oligodendrocytes (Windrem et al., 2004). Thus, several strategies aimed at developing transplantation therapies for correcting neurological disease and genetic deficits are underway.

ADULT STEM CELLS DISPLAYING UNEXPECTED MULTIPOTENTIALITY

In addition to the ability of NSCs to undergo linear differentiation into the three neural derivatives (neuron, astrocyte, and oligodendrocyte) and the ability of HSCs to differentiate into all known hematopoietic lineages, as well as the capacity of MSCs to give rise to a restricted subset of mesodermal-like derivatives, evidence has appeared in the literature suggesting an unexpected increased plasticity of these cell types (Bjornson et al., 1999; Clarke et al., 2000; Lagasse et al., 2000; Willenbring and Grompe, 2004). These reports indicate that both bone marrow (BM)–derived stem cells and NSCs may in fact also be capable of giving rise to a wider array of fully differentiated cell types, not previously expected.

Repair of Liver Tissue by Adult Stem Cells

Following liver damage, a subpopulation of hepatocytes with regenerative potential and oval cells recruited from the biliary duct epithelium were traditionally believed to potentiate hepatic regeneration and restore vital liver function (Overturf et al., 1997; Alison et al., 1998). With reports that BM-derived cells displayed greater multipotency than previously recognized, the contribution of specific bone marrow populations to liver regeneration was investigated. Informative studies have taken advantage of fumarylacetoacetate hydrolase (FAH)–deficient (*Fah*$^{-/-}$) mice, which normally display a lethal tyrosinemia manifested as progressive liver failure and chronic renal tubular damage unless maintained on 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione (NTBC) in their drinking water (Grompe et al., 1995). Lethally irradiated *Fah*$^{-/-}$ mice reconstituted intravenously with whole bone marrow or HSCs purified for c-kit^{high} Thy^{low} Lin^{-} Sca-1^{+} (Lin, lineage markers CD2, CD3, CD4, CD5, CD8, NK1.1, B220, Ter119, GR-1, and Mac-1) could survive after being withdrawn from NTBC (Lagasse et al., 2000).

Subsequent studies by the same group and others, aimed at understanding the underlying mechanisms of the apparent transdifferentiation events, demonstrated that appearance of hepatic phenotypes from the BM-derived cells could be best explained in terms of cell-cell

fusion events. Serial transplantation of HSCs along with karyotypic analysis and expression of donor versus host alleles in regenerating liver nodules confirmed that the apparent change in the HSC derivative phenotype and reinstated liver function was achieved by a mechanism associated with cell-cell fusion (Vassilopoulos et al., 2003; Wang et al., 2003).

Dissection of the HSC derivatives participating in the cell-cell fusion demonstrated that fusogenic macrophages are sufficient for the appearance of *Fah*$^{+/+}$ hepatocytes in the recipient mice. It was further demonstrated that mature hepatocytes did not fuse (Willenbring and Grompe, 2004). Conjecture, however, remains over the precise cell type and the extent of the role that a specific injury may play in the induction of fusion between donor HSC-derived cells and host liver. Two-dimensional homed Fr25lin$^-$ marrow cell lines, selected as both functionally and phenotypically representing HSCs, were reported to convert to a hepatocyte lineage in the absence of fusion following engraftment in carbon tetrachloride–induced hepatocyte injury in vivo (Jang et al., 2004). Similarly an independent study using Z/EG Cre-reporter BM donors to reconstitute lethally irradiated β-actin-Cre recipients reported transdifferentiation of BM-derived cells to hepatocytes in the absence of any apparent cell-cell fusion (Harris et al., 2004).

Repair of Cardiac Tissue by Adult Stem Cells

Myocardial infarction (MI) is an ischemic insult resulting in cardiomyocyte apoptosis and fibrous replacement. MI and its associated process of scarring is a leading cause of congestive heart failure (CHF) and morbidity in Western societies (Tang and Francis, 2003). Although cardiomyocytes or resident lin$^-$c-kit$^+$CD45$^-$ cells may regenerate the heart and improve function to some minor extent following injury, this process is largely insufficient. Therefore, heart transplantation, left-ventricular implantation devices, and pharmaceutical intervention remain as current clinical treatments. Novel strategies aimed at reducing heart failure after MI are warranted and greatly needed.

Investigations into the possibility that circulating myogenic precursors may contribute to cardiomyocyte regeneration demonstrated that cardiac and skeletal muscle of *mdx* mutant mice (a mouse model approximating Duchenne's muscular dystrophy) could be engrafted following total-body irradiation and hematopoietic reconstitution with donor whole BM (Bittner et al., 1999). BM engraftment of infarcted cardiac tissue was supported by several left coronary artery (LCA) ligation studies, including the intravenous injection of SP BM–derived cells (CD31$^{-/low}$/c-kit$^+$,Sca-1$^+$) following lethal irradiation and hematopoietic reconstitution, and the direct peri-infarct injection of lin$^-$c-kit$^+$ BM–derived cells (Jackson et al., 2001; Orlic et al., 2001). In both studies, transdifferentiation of BM-derived cells into cardiomyocytes and vascular endothelium was reported. In the latter, engraftment was reported to improve cardiac hemodynamics. In addition to providing a means of ameliorating the symptoms of heart disease, the delivery of BM cells to cardiac tissue was suggested as a viable means of delivering dystrophin to patients with Duchenne's muscular dystrophy.

Subsequent and more detailed studies of the mechanisms underlying engraftment using conditional transgenic mice revealed that BM–derived cells and cardiomyocytes can undergo cell-cell fusion (Alvarez-Dolado et al., 2003). Furthermore, c-kit$^+$, c-kit$^+$lin$^-$ and c$^-$kit$^+$lin$^-$Thy-1^{lo}Sca-1$^+$ BM–derived cells injected directly into ischemic hearts following LCA ligation, into c-kit$^+$Lin$^-$ BM-derived cells following freeze injury, or into α-myosin-GFP c-kit$^+$lin$^-$ BM–derived cells following LCA ligation or cauterization, although transiently present in the graft, did not express markers characteristic of cardiomyocyte differentiation (Murry et al., 2004; Nygren et al., 2004). Some benefit in limiting ventricular dilation and dysfunction in the long-term was observed; however, 30-day survival and infarct size did not differ significantly between control and treated groups, suggesting that recruitment of resident cardiomyocyte progenitors was minimal (Murry et al., 2004). Similarly, lethal irradiation followed by reconstitution with β-actin-GFP or α-actin-GFP c-kit$^+$lin$^-$Sca-1$^+$CD45$^+$ BM–derived cells, then LCA ligation and mobilization of stem cells with cytokines or reconstitution with α-myosin-GFP c-kit$^+$lin$^-$ BM–derived cells, followed by LCA ligation, did not show evidence for engraftment of donor cells displaying cardiac phenotype within the infarct (Murry et al., 2004; Nygren et al., 2004). Following lethal irradiation and reconstitution, however, a small number of donor cells were observed to take on characteristics of cardiomyocytes through a process of cell fusion with endogenous

cardiomyocytes within the peri-infarct zone. What effect these fused cells have on cardiac function is not known; however, it was suggested that the previously reported benefit in function is most likely attributable to left-ventricular remodeling and/or the potentiation of endogenous endothelial vascularization.

Despite this conjecture in the literature with animal models, several clinical trials have already been conducted, and mixed success in terms of functional benefit has been reported. Injection of BM-derived cells or cytokines has been reported to be safe and beneficial, yet it has also been associated with occlusion of microcoronary circulation, induction of myocardial infarction, increases in cardiac enzymes, and increased rates of restenosis (Lee et al., 2004).

Repair of Skeletal Muscle by Adult Stem Cells

Self-renewal, growth, and regeneration of skeletal muscle is effected by slowly dividing, self-renewing $CD45^-Sca1^-/CD34^+Sca1^-$ mononuclear satellite cells that lie between the muscle fiber plasmalemma and associated basement membrane (Campion et al., 1984; Zammit and Beauchamp, 2001; Asakura et al., 2002; Sherwood et al., 2004). Following injury, or in patients suffering degenerative myopathies, satellite cells divide and fuse to form multinucleated replacement myotubes. Satellite cells display a reduced self-renewal capacity with age, resulting in their gradual loss. In combination with chronic or severe injury, satellite cells are eventually depleted such that absent muscle tissue can be replaced only with scar tissue.

In addition to satellite cells, unfractionated, nonadherent and adherent BM-derived cells were reported to engraft cardiotoxin (CTX)–injured skeletal muscle fibers and SP cells were reported to engraft *mdx* null skeletal muscle fibers (Ferrari et al., 1998; Bittner et al., 1999; Gussoni et al., 1999; Ferrari et al., 2001). The production of mononuclear myoblasts and subsequent skeletal muscle engraftment by unfractionated whole BM following irradiation and/or exercise-induced injury were reported to occur after passage through a muscle satellite cell intermediary phenotype (LaBarge and Blau, 2002). Both $Sca\text{-}1^+CD45^+$ and $Sca\text{-}1^+CD45^-$ resident skeletal muscle cells were reported to harbor myogenic activity (Asakura et al., 2002; Polesskaya et al., 2003). With respect to $Sca\text{-}1^+CD45^+$ resident cells, myogenic recruitment into CTX-induced skeletal muscle injury was coupled to activation of Wnt signaling (Polesskaya et al., 2003). Although the precise identity of any single BM-derived cell type harboring myogenic activity is unknown, Camargo et al. (2003) showed that 0.03% to 0.08% of all skeletal muscle cells in the CTX-injured tibialis anterior muscle were derived from a single SP $Sca1^+$ $CD45^+$ HSC cell previously transplanted following irradiation and long-term hematopoietic chimerism. Furthermore, $CD45^+$ BM cells derived from SP $Sca1^+CD45^{+-}$ reconstituted mice, injected into lethally irradiated *mdx* mice, demonstrated a 0.13% TA myofibril contribution. No donor-derived cells were found in uninjured muscle or desmin-null muscle, and transplantation of $CD45^-$ BM-derived cells did not result in TA engraftment following injury. The presence of $\beta\text{-gal}^+$ cells in CTX-injured TA of bitransgenic *Lysozyme M-Cre recombinase* (*LysM-Cre*) × $ROSA^{flox/STOP}$ mice indicated that engraftment by BM derivatives was attributable to circulating myelomonocytic precursors, macrophages, and/or granulocytes. Furthermore, engraftment was attributable to fusion with myoblasts produced from resident satellite cells during muscle fiber regeneration and not transdifferentiation. Thus, it still remains unclear whether BM-derived stem cells may be exploited for myogenic therapeutics. However, it appears that fractionated cell types with myogenic potential (with or without fusion) can be isolated from both bone marrow and adult skeletal muscle, and can, to varying degrees, contribute to repair in an injury-specific fashion. Therefore, it remains uncertain whether the incidence of BM contribution will be high enough to offer therapy for muscle dystrophies and whether both subsets may eventually play equal roles in future therapeutics.

Neural Stem Cell Plasticity

In a similar fashion to BM-derived stem cells, mouse NSC differentiation pathways have been reported to be permissive to reprogramming, transdifferentiation, or transdetermination in response to ectopic stimuli (Frisen, 2002). Adult and fetal forebrain NSC derivatives were reported to reconstitute the hematopoietic system following intraportal injection of sublethally irradiated mice. Adult NSCs were reported to give rise to representatives of all three embryonic germ cell layers following injection into the amniotic cavity of chick embryos, and intrablastocoel injection and transfer to pseudopregnant mice (Bjornson et al., 1999; Clarke et al., 2000). Neurospheres derived from a human fetal brain

were injected into irradiated *scid-hu* mice and similarly displayed hematopoietic reconstituting activity (Shih et al., 2001). Coculture of adult mouse paraventricular NSCs and human embryonic NSCs with myoblasts and direct injection into CTX-injured tibialis anterior muscle were reported to result in transdifferentiation of NSCs into skeletal muscle cells (Galli et al., 2000). NSCs were thus suggested as an alternative source of cells for therapeutic strategies where caveats associated with therapeutic cloning or immune rejection of MHC-disparate donor cells limit the application of ES cells.

Theories concerning stem cell plasticity have not been unequivocally accepted (Anderson, 2001; Morshead et al., 2002). The ability of the blastocyst environment to alter NSC fate was considered an abnormal situation and repeat experiments using NSC/morula aggregation failed to demonstrate embryonic integration of NSC derivatives (Tropepe et al., 2001). Experiments aimed at repeating hematopoietic reconstitution of irradiated mice using NSCs similarly failed. It was suggested that this reported property was more likely due to a rare in vitro genetic or epigenetic alteration in long-term cultured NSCs or the presence of contaminating HSCs in the original NSC culture (Morshead et al., 2002; Magrassi et al., 2003). More recently, culture of mouse NSCs with human endothelium induced a type of transdifferentiation into endothelial lineages (Wurmser et al., 2004). No fusion was observed in vitro and injection of NSCs into the telencephalon of embryonic day 14 (E14) mice resulted in expression of endothelial markers by a fraction of the cells by E16. Although it is not known whether NSC conversion to endothelial cell phenotype represents a normal physiological process, the appearance of a small percentage of cells (1.6%) from all input cells opens up this possibility.

STEM CELLS STIMULATE ENDOGENOUS REPAIR

Despite debates over transdifferentiation and the capacity of transplanted adult stem cells to replace damaged tissue in a functionally relevant context, some studies suggest adult stem cell transplantation may stimulate endogenous repair mechanisms. These studies suggest direct consequences for treating diseases such as type I diabetes, multiple sclerosis, and idiopathic pulmonary fibrosis. For example, transplantation of c-kit$^+$ adult BM-derived stem cells was reported to reduce hyperglycemia in streptozotocin-induced damage of NOD-SCID mouse pancreas by contributing to neovasculogenesis, engrafting the pancreas, and stimulating endogenous islet cell proliferation and insulin secretion (Hess et al., 2003). Intraventricular or systemic injection of adult periventricular neural progenitors into myelin oligodendrocyte glycoprotein induced autoimmune encephalomyelitis, which resulted in decreases in astroglyosis, demyelination, axonal loss, and associated functional impairment (Pluchino et al., 2003). Neural progenitors were reported to home to the site of injury, remyelinate neurons, stimulate endogenous oligodendroglia, and attenuate reactive astrogliosis. Roughly a quarter of all mice in each group were reported to recover completely from the disease, whereas mice that were either sham-transplanted or transplanted with other cell types showed no recovery. Similarly, systemically administered plastic adherent and CD11bCD34CD45 immunodepleted whole BM MSCs were reported to home to bleomycin-induced respiratory injury. Notwithstanding issues of cell-cell fusion, MSCs adopted an epithelial-like morphology and were reported to graft as type II epithelial cells. Furthermore, MSC administration immediately following bleomycin-induced respiratory injury reduced inflammation and fibrosis with an associated decrease in collagen deposition and increase in metalloproteinase gene expression (Ortiz et al., 2003).

GENETIC MANIPULATION OF EMBRYONIC STEM CELLS

The ability to manipulate mESCs has proven to be a valuable tool in developing understanding of processes of differentiation, development, and disease. The generation of genetically altered cell lines is standard in many laboratories. The capacity to similarly genetically manipulate hESCs is fast becoming a reality. Several strategies have been developed that allow the successful transfection of hESCs. These have resulted in the generation of ES cell lines with ubiquitous or specific promoter-driven reporter genes for lineage tracing and gene-targeting events such as knock-in/up/out. The first major success came from liposome-based ExGen500 (Fermentas) technology, which produces a high transfection efficiency when compared to both standard electroporation conditions and other liposome-mediated systems (Eiges et al., 2001). This technique was used for the generation of stable hESCs with an EGFP reporter

gene driven by the Rex-1 promoter (Eiges et al., 2001). An alternative approach has been the use of both adenoviral and lentiviral vectors. Adenoviral vectors Ad5 containing the β-galactosidase reporter gene have proven the most successful, generating transient transfection efficiencies of 11.2% with no apparent effect on differentiation status (Smith-Arica et al., 2003). Self-inactivating lentiviral vectors have also been particularly successful in generating stable transgenic hESCs (Gropp et al., 2003; Ma et al., 2003). These viral vectors have been reported to generate stable transduction efficiencies of between 20% and 80% (Gropp et al., 2003; Ma et al., 2003). More importantly, electroporation of hESCs have resulted in both the *HPRT1* and *POU5F1* loci to be successfully targeted using homologous recombination (Zwaka and Thomson, 2003). Most recently, a novel transfection method has been reported that combines electroporation with a chemical-based technology. This method is termed nucleofection, as it enhances nuclear targeting of exogenous DNA to the nucleus, thus enhancing expression and genomic integration (Lakshmipathy et al., 2004).

The generation of defined lineage-restricted populations has also been explored through the use of genetic manipulation. The directed differentiation of mESCs has been successfully achieved through the manipulation of lineage-specific transcription factors. Ectopic expression of Nurr1 in transgenic mESCs directs their differentiation into a midbrain-type dopaminergic neuronal phenotype (Chung et al., 2002). Similarly, forced constitutive expression of GATA-6 and GATA-4 induces mESC differentiation into extraembryonic endoderm (Fujikura et al., 2002). The production of a more tightly regulated induction has recently been reported, whereby insulin-producing cells have been generated from mESCs via tetracycline-off regulated exogenous *Pdx-1* integrated into the *ROSA26* locus (Miyazaki et al., 2004). Alternatively, episomal constructs that are active as extrachromosomal vectors have also been shown to be effective in directing lineage-specific differentiation. In this study, the Wnt antagonist Sfrp2 was used to stimulate the production of neural progenitors (Aubert et al., 2002).

The development of new technologies such as RNA interference (RNAi)–based protocols may also efficiently result in lineage restriction of ES cells by modifying gene expression within the cell. RNAi technology has been applied to mESCs to suppress Oct4 expression, resulting in trophectodermal differentiation (Velkey and O'Shea, 2003). In the human context, Vallier et al. (2004) have shown that hairpin RNAi constructs can efficiently induce gene-specific knockdown of reporter gene expression in hESCs.

Advances in methods for high-efficiency transfection of hESCs, production of novel expression systems, and advances in the ability to direct specific activation and inactivation of gene expression provide powerful technologies for the future of human embryonic stem cell research. These, coupled with growing databases of genes implicated in pluripotency and lineage-specific differentiation, greatly enhance the ability to specifically regulate ES cell differentiation and thus fully harness the potential of ES cells.

THERAPEUTIC APPLICATIONS OF STEM CELLS

Mouse ES cells were first described some 17 years before hESCs, which were first isolated in 1998 (Martin, 1981; Thomson et al., 1998). Research using mESCs is currently more advanced, with several in vivo examples reporting appropriate physiological function of the transplanted cell types. Reports of mESC derivatives improving heart function (Min et al., 2002; Hodgson et al., 2004), curing blood disease (Rideout et al., 2002), normalizing weight, longevity, and insulin levels in diabetic mice (Hori et al., 2002), reducing symptoms of Parkinson's disease (Bjorklund et al., 2002; Kim et al., 2002), and partially repairing damaged spinal cords (McDonald et al., 1999; Liu et al., 2000) have already appeared in the literature. More sophisticated studies demonstrating similar physiological function of hESC derivatives in vivo, until now, have been lacking. Kehat et al. (2004) recently described the production of cardiac-like cells from hESCs, and, for the first time, meaningful in vivo physiological function of hESC derivatives. They successfully treated pigs with an atrioventricular block and restored a heart rhythm compatible with the survival of approximately half the animals tested. As acknowledged by the authors, this work was limited in part by the failure to test for cell fusion (between the transplanted cells and the cells already existing in the pig heart) and the inability to discriminate between direct electrical effects of the cells versus indirect instructive effects upon neighboring cells. This research provides important evidence to support the suggestion that hESCs

may represent suitable candidates for relieving heart conditions and disorders, and that hESCs may one day play an important role in cell-transplantation therapeutics.

The therapeutic application of ES cells is not only challenged by the ability to generate specific cell types capable of repairing various tissues, but it is also confronted with the additional hurdle of immune rejection. Currently, histocompatible donors and immune-suppression drugs are used to reduce these effects in organ-transplantation patients. However, alternatives in overcoming immune rejection will broaden the availability and success of organ transplantation and ES cell therapy. One approach is that of therapeutic cloning, whereby patient tissue is cloned into an oocyte for the generation of ES cells, which can then be differentiated into the desired cell type for tissue regeneration (Fig. 23.1.4). These cells, being genetically identical to the patient, will circumvent an immunological response. An alternative to therapeutic cloning may be in the form of ES cell banks. It is envisaged that, by generating large numbers of ES cells from a range of genetic backgrounds, a histocompatible hESC line will be available for any patient (Baharvand et al., 2004; Heins et al., 2004). Alternatively, reprogramming adult cells into the phenotype of an ES cell without the use of oocytes is also currently being investigated. ES cell extracts may be capable of de-differentiating/reprogramming adult patient cells to take on an ES-like phenotype, whereupon subsequent differentiation strategies can then be utilized (Tada et al., 2001).

A fourth approach has been that of manipulating immunological tolerance. Such methods include second-signal blockade (Rifle and Mousson, 2002), manipulation of recipient

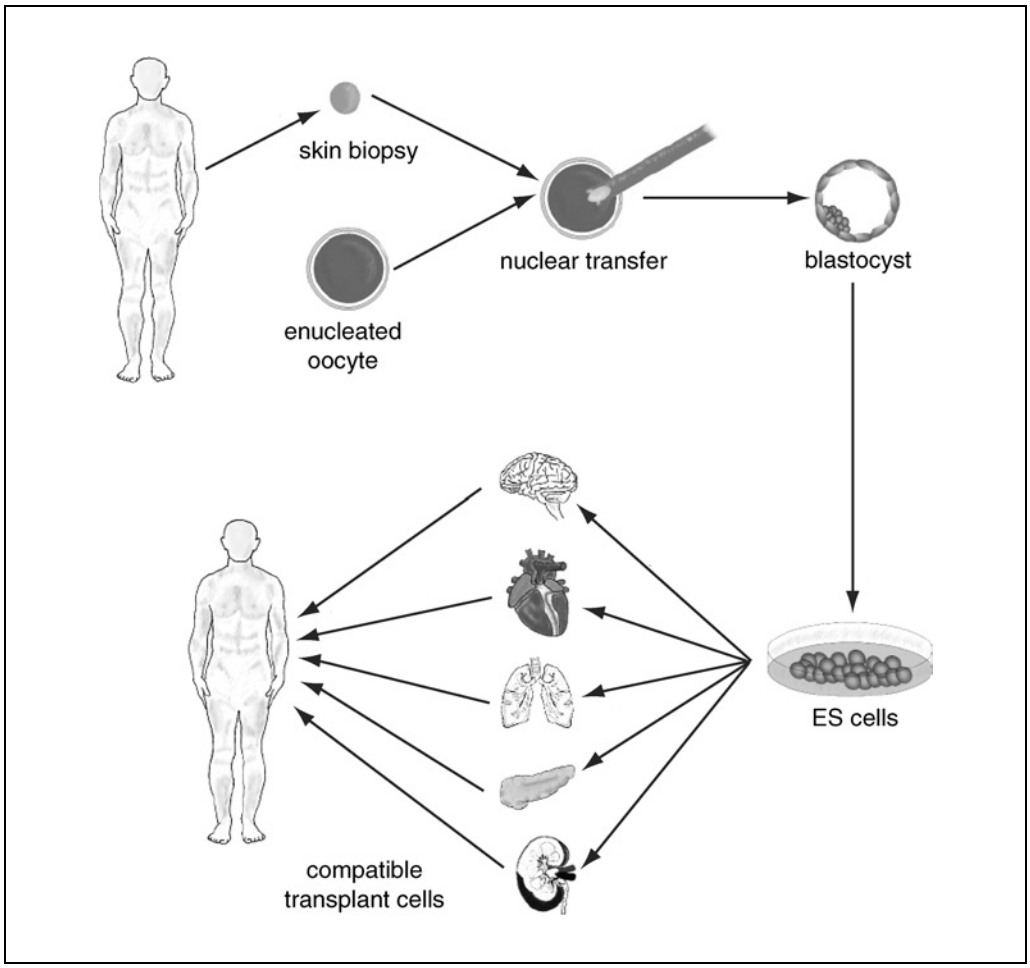


Figure 23.1.4 Therapeutic cloning. Therapeutic cloning involves the production of custom-designed embryonic stem (ES) cells by the transfer of a person's own DNA (nuclear transfer) into a donor oocyte. Following nuclear transfer, the oocyte is incubated to develop into a blastocyst, from which ES cells are derived. ES cells are then coaxed to become a desired cell type (e.g., neural, cardiac, respiratory, pancreatic, or renal) and made available for transplantation. Because the resulting tissue for transplant contains the same DNA as the donor, it should not be rejected.

antigen-presenting cells, and chimerism of the immune system. Chimerism involves the injection of donor bone marrow into the recipient in order to improve organ allograft survival (Monaco and Wood, 1970; Deng et al., 2004). It has been suggested that a combination of donor myeloid (immature) dendritic cells (Rifle and Mousson, 2002) and donor T cells aids in the induction of transplantation tolerance (Tian et al., 2004). This technique, combined with ES cell therapy, could aid in long-term survival for donor cells in patients and in overcoming the requirements for ES cell banks and therapeutic cloning.

CONCLUSION

Current research on the culture and differentiation of both adult and embryonic stem cells has allowed advances in unraveling the molecular mechanisms behind specific cellular differentiation events. In vitro expansion of stem cell populations, combined with differentiation protocols and genetic manipulation of ES cells, has enabled the tracing of various cell lineage differentiation events and the development of an understanding of several of the molecular pathways involved. Ethical issues surrounding the use of ES cells may be resolved by current research into adult stem cell plasticity, which offers a potential source of cells for replacement therapies in certain diseases and the ability to overcome immune-rejection issues. Overall, it is hoped that homogenous differentiation of stem cells into a desired precursor, or specific differentiated cell lineage, will facilitate the development of effective clinical methods for treating a wide range of diseases, and also increase understanding of the mechanisms leading to these diseases.

Literature Cited

Akashi, K., Kondo, M., Cheshier, S., Shizuru, J., Gandy, K., Domen, J., Mebius, R., Traver, D., and Weissman, I.L. 1999a. Lymphoid development from stem cells and the common lymphocyte progenitors. *Cold Spring Harb. Symp. Quant. Biol.* 64:1-12.

Akashi, K., Traver, D., Kondo, M., and Weissman, I.L. 1999b. Lymphoid development from hematopoietic stem cells. *Int. J. Hematol.* 69:217-226.

Alison, M., Golding, M., Lalani el, N., and Sarraf, C. 1998. Wound healing in the liver with particular reference to stem cells. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 353:877-894.

Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J.M., Fike, J.R., Lee, H.O., Pfeffer, K., Lois, C., Morrison, S.J., and Alvarez-Buylla, A. 2003. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425:968-973.

Anderson, D.J. 2001. Stem cells and pattern formation in the nervous system: The possible versus the actual. *Neuron* 30:19-35.

Asakura, A., Seale, P., Girgis-Gabardo, A., and Rudnicki, M.A. 2002. Myogenic specification of side population cells in skeletal muscle. *J. Cell Biol.* 159:123-34.

Aubert, J., Dunstan, H., Chambers, I., and Smith, A. 2002. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. *Nat. Biotechnol.* 20:1240-1245.

Baharvand, H., Ashtiani, S.K., Valojerdi, M.R., Shahverdi, A., Taee, A., and Sabour, D. 2004. Establishment and in vitro differentiation of a new embryonic stem cell line from human blastocyst. *Differentiation* 72:224-229.

Barry, F.P. and Murphy, J.M. 2004. Mesenchymal stem cells: Clinical applications and biological characterization. *Int. J. Biochem. Cell Biol.* 36:568-584.

Bittner, R.E., Schofer, C., Weipoltshammer, K., Ivanova, S., Streubel, B., Hauser, E., Freilinger, M., Hoger, H., Elbe-Burger, A., and Wachtler, F. 1999. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. *Anat. Embryol. (Berl.)* 199:391-396.

Bjorklund, L.M., Sanchez-Pernaute, R., Chung, S., Andersson, T., Chen, I.Y., McNaught, K.S., Brownell, A.L., Jenkins, B.G., Wahlestedt, C., Kim, K.S., and Isacson, O. 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc. Natl. Acad. Sci. U.S.A.* 99:2344-2349.

Bjornson, C.R., Rietze, R.L., Reynolds, B.A., Magli, M.C., and Vescovi, A.L. 1999. Turning brain into blood: A hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 283:534-537.

Buzzard, J.J., Gough, N.M., Crook, J.M., and Colman, A. 2004. Karyotype of human ES cells during extended culture. *Nat. Biotechnol.* 22:381-382.

Camargo, F.D., Green, R., Capetanaki, Y., Jackson, K.A., and Goodell, M.A. 2003. Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. *Nat. Med.* 9:1520-1527.

Campion, D.R., Purchas, R.W., Merkel, R.A., and Romsos, D.R. 1984. Genetic obesity and the muscle satellite cell. *Proc. Soc. Exp. Biol. Med.* 176:143-147.

Carlen, M., Cassidy, R.M., Brismar, H., Smith, G.A., Enquist, L.W., and Frisen, J. 2002. Functional integration of adult-born neurons. *Curr. Biol.* 12:606-608.

Carpenter, M.K., Cui, X., Hu, Z.Y., Jackson, J., Sherman, S., Seiger, A., and Wahlberg, L.U. 1999. In vitro expansion of a multipotent

population of human neural progenitor cells. *Exp. Neurol.* 158:265-278.

Christensen, J.L. and Weissman, I.L. 2001. Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 98:14541-14546.

Chung, S., Sonntag, K.C., Andersson, T., Bjorklund, L.M., Park, J.J., Kim, D.W., Kang, U.J., Isacson, O., and Kim, K.S. 2002. Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. *Eur. J. Neurosci.* 16:1829-1838.

Clarke, D.L., Johansson, C.B., Wilbertz, J., Veress, B., Nilsson, E., Karlstrom, H., Lendahl, U., and Frisen, J. 2000. Generalized potential of adult neural stem cells. *Science* 288:1660-1663.

Conley, B.J., Trounson, A.O., and Mollard, R. 2004. Human embryonic stem cells form embryoid bodies containing visceral endoderm-like derivatives. *Fetal Diagn. Ther.* 19:218-223.

Cotsarelis, G., Sun, T.T., and Lavker, R.M. 1990. Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 61:1329-1337.

Crang, A.J., Franklin, R.J., Blakemore, W.F., Noble, M., Barnett, S.C., Groves, A., Trotter, J., and Schachner, M. 1992. The differentiation of glial cell progenitor populations following transplantation into non-repairing central nervous system glial lesions in adult animals. *J. Neuroimmunol.* 40:243-253.

Deng, W., Han, Q., Liao, L., Li, C., Ge, W., Zhao, Z., You, S., Deng, H., and Zhao, R.C. 2004. Allogeneic bone marrow-derived flk-1^{+}Sca-1^{-} mesenchymal stem cells leads to stable mixed chimerism and donor-specific tolerance. *Exp. Hematol.* 32:861-867.

Doetsch, F. 2003. A niche for adult neural stem cells. *Curr. Opin. Genet. Dev.* 13:543-550.

Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97:703-716.

Dor, Y. and Melton, D.A. 2004. How important are adult stem cells for tissue maintenance? *Cell Cycle* 3:1104-1106.

Draper, J.S., Moore, H.D., Ruban, L.N., Gokhale, P.J., and Andrews, P.W. 2004. Culture and characterization of human embryonic stem cells. *Stem Cells Dev.* 13:325-336.

Eiges, R., Schuldiner, M., Drukker, M., Yanuka, O., Itskovitz-Eldor, J., and Benvenisty, N. 2001. Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. *Curr. Biol.* 11:514-518.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279:1528-1530.

Ferrari, G., Stornaiuolo, A., and Mavilio, F. 2001. Failure to correct murine muscular dystrophy. *Nature* 411:1014-1015.

Freed, C.R., Greene, P.E., Breeze, R.E., Tsai, W.Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, H., Culver, S., Trojanowski, J.Q., Eidelberg, D., and Fahn, S. 2001. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *New Engl. J. Med.* 344:710-719.

Friedenstein, A.J., Piatetzky, S. II, and Petrakova, K.V. 1966. Osteogenesis in transplants of bone marrow cells. *J. Embryol. Exp. Morphol.* 16:381-390.

Frisen, J. 2002. Stem cell plasticity? *Neuron* 35:415-418.

Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki Ji, J., and Niwa, H. 2002. Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev.* 16:784-789.

Gage, F.H. 2000. Mammalian neural stem cells. *Science* 287:1433-1438.

Galli, R., Borello, U., Gritti, A., Minasi, M.G., Bjornson, C., Coletta, M., Mora, M., De Angelis, M.G., Fiocco, R., Cossu, G., and Vescovi, A.L. 2000. Skeletal myogenic potential of human and mouse neural stem cells. *Nat. Neurosci.* 3:986-991.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* 183:1797-1806.

Goodell, M.A., Rosenzweig, M., Kim, H., Marks, D.F., DeMaria, M., Paradis, G., Grupp, S.A., Sieff, C.A., Mulligan, R.C., and Johnson, R.P. 1997. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat. Med.* 3:1337-1345.

Grompe, M., Lindstedt, S., al-Dhalimy, M., Kennaway, N.G., Papaconstantinou, J., Torres-Ramos, C.A., Ou, C.N., and Finegold, M. 1995. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. *Nat. Genet.* 10:453-460.

Gropp, M., Itsykson, P., Singer, O., Ben-Hur, T., Reinhartz, E., Galun, E., and Reubinoff, B.E. 2003. Stable genetic modification of human embryonic stem cells by lentiviral vectors. *Mol. Ther.* 7:281-287.

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M., and Mulligan, R.C. 1999. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401:390-394.

Hackney, J.A., Charbord, P., Brunk, B.P., Stoeckert, C.J., Lemischka, I.R., and Moore, K.A. 2002. A molecular profile of a hematopoietic stem cell niche. *Proc. Natl. Acad. Sci. U.S.A.* 99:13061-13066.

Harris, R.G., Herzog, E.L., Bruscia, E.M., Grove, J.E., Van Arnam, J.S., and Krause, D.S. 2004.

Lack of a fusion requirement for development of bone marrow-derived epithelia. *Science* 305:90-93.

Heins, N., Englund, M.C., Sjoblom, C., Dahl, U., Tonning, A., Bergh, C., Lindahl, A., Hanson, C., and Semb, H. 2004. Derivation, characterization, and differentiation of human embryonic stem cells. *Stem Cells* 22:367-376.

Hess, D., Li, L., Martin, M., Sakano, S., Hill, D., Strutt, B., Thyssen, S., Gray, D.A., and Bhatia, M. 2003. Bone marrow-derived stem cells initiate pancreatic regeneration. *Nat. Biotechnol.* 21:763-70.

Hodges, H., Sowinski, P., Fleming, P., Kershaw, T.R., Sinden, J.D., Meldrum, B.S., and Gray, J.A. 1996. Contrasting effects of fetal CA1 and CA3 hippocampal grafts on deficits in spatial learning and working memory induced by global cerebral ischaemia in rats. *Neuroscience* 72:959-988.

Hodgson, D.M., Behfar, A., Zingman, L.V., Kane, G.C., Perez-Terzic, C., Alekseev, A.E., Puceat, M., and Terzic, A. 2004. Stable benefit of embryonic stem cell therapy in myocardial infarction. *Am. J. Physiol. Heart Circ. Physiol.* 287:H471-H479.

Hori, Y., Rulifson, I.C., Tsai, B.C., Heit, J.J., Cahoy, J.D., and Kim, S.K. 2002. Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:16105-16110.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N. 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol. Med.* 6:88-95.

Jackson, K.A., Majka, S.M., Wang, H., Pocius, J., Hartley, C.J., Majesky, M.W., Entman, M.L., Michael, L.H., Hirschi, K.K., and Goodell, M.A. 2001. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J. Clin. Invest.* 107:1395-2402.

Jang, Y.Y., Collector, M.I., Baylin, S.B., Diehl, A.M., and Sharkis, S.J. 2004. Hematopoietic stem cells convert into liver cells within days without fusion. *Nat. Cell Biol.* 6:532-539.

Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U., and Frisen, J. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 96:25-34.

Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., and Yoo, J.U. 1998. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* 238:265-272.

Kaufman, D.S., Hanson, E.T., Lewis, R.L., Auerbach, R., and Thomson, J.A. 2001. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 98:10716-10721.

Kehat, I., Khimovich, L., Caspi, O., Gepstein, A., Shofti, R., Arbel, G., Huber, I., Satin, J., Itskovitz-Eldor, J., and Gepstein, L. 2004. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat. Biotechnol.* 22:1282-1289.

Kim, J.H., Auerbach, J.M., Rodriguez-Gomez, J.A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S.H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., and McKay, R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418:50-56.

Kondo, M., Weissman, I.L., and Akashi, K. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91:661-672.

Krause, D.S., Ito, T., Fackler, M.J., Smith, O.M., Collector, M.I., Sharkis, S.J., and May, W.S. 1994. Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. *Blood* 84:691-701.

LaBarge, M.A. and Blau, H.M. 2002. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* 111:589-601.

Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I.L., and Grompe, M. 2000. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat. Med.* 6:1229-1234.

Lakshmipathy, U., Pelacho, B., Sudo, K., Linehan, J.L., Coucouvanis, E., Kaufman, D.S., and Verfaillie, C.M. 2004. Efficient transfection of embryonic and adult stem cells. *Stem Cells* 22:531-543.

Lee, S., Bick-Forrester, J., Makkar, R.R., and Forrester, J.S. 2004. Stem-cell repair of infarcted myocardium: Ready for clinical application? *Am. Heart Hosp. J.* 2:100-106.

Li, W.J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K.G., Hall, D.J., and Tuan, R.S. 2005. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials* 26:599-609.

Lindvall, O. and Bjorklund, A. 2004. Cell therapy in Parkinson's disease. *Neurorx* 1:382-393.

Liu, S., Qu, Y., Stewart, T.J., Howard, M.J., Chakrabortty, S., Holekamp, T.F., and McDonald, J.W. 2000. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc. Natl. Acad. Sci. U.S.A.* 97:6126-6131.

Lois, C. and Alvarez-Buylla, A. 1993. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc. Natl. Acad. Sci. U.S.A.* 90:2074-2077.

Lois, C. and Alvarez-Buylla, A. 1994. Long-distance neuronal migration in the adult mammalian brain. *Science* 264:1145-1148.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R., and McKay, R. 2001. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292:1389-1394.

Ma, Y., Ramezani, A., Lewis, R., Hawley, R.G., and Thomson, J.A. 2003. High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. *Stem Cells* 21:111-117.

Magrassi, L., Castello, S., Ciardelli, L., Podesta, M., Gasparoni, A., Conti, L., Pezzotta, S., Frassoni, F., and Cattaneo, E. 2003. Freshly dissociated fetal neural stem/progenitor cells do not turn into blood. *Mol. Cell Neurosci.* 22:179-187.

Martin, G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

McDonald, J.W., Liu, X.Z., Qu, Y., Liu, S., Mickey, S.K., Turetsky, D., Gottlieb, D.I., and Choi, D.W. 1999. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat. Med.* 5:1410-1412.

Min, J.Y., Yang, Y., Converso, K.L., Liu, L., Huang, Q., Morgan, J.P., and Xiao, Y.F. 2002. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J. Appl. Physiol.* 92:288-296.

Miyazaki, S., Yamato, E., and Miyazaki, J. 2004. Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells. *Diabetes* 53:1030-1037.

Mollard, R., Denham, M., and Trounson, A. 2002. Technical advances and pitfalls on the way to human cloning. *Differentiation* 70:1-9.

Monaco, A.P. and Wood, M.L. 1970. Studies on heterologous antilymphocyte serum in mice. VII. Optimal cellular antigen for induction of immunologic tolerance with antilymphocyte serum. *Transplant. Proc.* 2:489-496.

Morrison, S.J., Prowse, K.R., Ho, P., and Weissman, I.L. 1996. Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity* 5:207-216.

Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E., and Weissman, I.L. 1997. Identification of a lineage of multipotent hematopoietic progenitors. *Development* 124:1929-1939.

Morshead, C.M., Benveniste, P., Iscove, N.N., and van der Kooy, D. 2002. Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. *Nat. Med.* 8:268-273.

Mummery, C., Ward, D., van den Brink, C.E., Bird, S.D., Doevendans, P.A., Opthof, T., Brutel de la Riviere, A., Tertoolen, L., van der Heyden, M., and Pera, M. 2002. Cardiomyocyte differentiation of mouse and human embryonic stem cells. *J. Anat.* 200:233-242.

Murry, C.E., Soonpaa, M.H., Reinecke, H., Nakajima, H., Nakajima, H.O., Rubart, M., Pasumarthi, K.B., Virag, J.I., Bartelmez, S.H., Poppa, V., Bradford, G., Dowell, J.D., Williams, D.A., and Field, L.J. 2004. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 428:664-668.

Nagy, A., Gocza, E., Diaz, E.M., Prideaux, V.R., Ivanyi, E., Markkula, M., and Rossant, J. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110:815-821.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 90:8424-8428.

Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S., Hatano, O., Kawahara, N., Tamura, A., Kirino, T., and Nakafuku, M. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110:429-441.

Nygren, J.M., Jovinge, S., Breitbach, M., Sawen, P., Roll, W., Hescheler, J., Taneera, J., Fleischmann, B.K., and Jacobsen, S.E. 2004. Bone marrow–derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nat. Med.* 10:494-501.

Okuno, Y., Iwasaki, H., Huettner, C.S., Radomska, H.S., Gonzalez, D.A., Tenen, D.G., and Akashi, K. 2002. Differential regulation of the human and murine CD34 genes in hematopoietic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:6246-6251.

Orkin, S.H. 2000. Diversification of haematopoietic stem cells to specific lineages. *Nat. Rev. Genet.* 1:57-64.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S.M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D.M., Leri, A., and Anversa, P. 2001. Bone marrow cells regenerate infarcted myocardium. *Nature* 410:701-705.

Ortiz, B., Porras, F., Jimenez-Martinez, M.C., Montano, L.F., Martinez-Cairo, S., Lascurain, R., and Zenteno, E. 2002. Differential expression of a 70 kDa O-glycoprotein on T cells: A possible marker for naive and early activated murine T cells. *Cell Immunol.* 218:34-45.

Ortiz, L.A., Gambelli, F., McBride, C., Gaupp, D., Baddoo, M., Kaminski, N., and Phinney, D.G. 2003. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc. Natl. Acad. Sci. U.S.A.* 100:8407-8411.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 273:242-245.

Overturf, K., al-Dhalimy, M., Ou, C.N., Finegold, M., Tanguay, R., Lieber, A., Kay, M., and Grompe, M. 1997. Adenovirus-mediated gene therapy in a mouse model of hereditary tyrosinemia type I. *Hum. Gene Ther.* 8:513-521.

Phinney, D.G., Kopen, G., Isaacson, R.L., and Prockop, D.J. 1999. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: Variations in yield, growth, and differentiation. *J. Cell Biochem.* 72:570-585.

Piper, D.R., Mujtaba, T., Keyoung, H., Roy, N.S., Goldman, S.A., Rao, M.S., and Lucero, M.T. 2001. Identification and characterization of neuronal precursors and their progeny from human fetal tissue. *J. Neurosci. Res.* 66:356-368.

Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A., Furlan, R., Comi, G., Vescovi, A.L., and Martino, G. 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422:688-694.

Polesskaya, A., Seale, P., and Rudnicki, M.A. 2003. Wnt signaling induces the myogenic specification of resident $CD45^+$ adult stem cells during muscle regeneration. *Cell* 113:841-852.

Poulsom, R., Alison, M.R., Forbes, S.J., and Wright, N.A. 2002. Adult stem cell plasticity. *J. Pathol.* 197:441-456.

Ray, K. and Rodrigues, V. 1995. Cellular events during development of the olfactory sense organs in *Drosophila melanogaster*. *Dev. Biol.* 167:426-438.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. 2000. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. *Nat. Biotechnol.* 18:399-404.

Reynolds, B.A. and Weiss, S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255:1707-1710.

Reynolds, B.A. and Weiss, S. 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* 175:1-13.

Richards, L.J., Kilpatrick, T.J., Bartlett, P.F., and Murphy, M. 1992. Leukemia inhibitory factor promotes the neuronal development of spinal cord precursors from the neural tube. *J. Neurosci. Res.* 33:476-484.

Rideout, W.M. 3rd, Hochedlinger, K., Kyba, M., Daley, G.Q., and Jaenisch, R. 2002. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* 109:17-27.

Rifle, G. and Mousson, C. 2002. Dendritic cells and second signal blockade: A step toward allograft tolerance? *Transplantation* 73:S1-S2.

Rodriguez, J.P., Gonzalez, M., Rios, S., and Cambiazo, V. 2004. Cytoskeletal organization of human mesenchymal stem cells (MSC) changes during their osteogenic differentiation. *J. Cell. Biochem.* 93:721-731.

Scharenberg, C.W., Harkey, M.A., and Torok-Storb, B. 2002. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99:507-512.

Schuldiner, M., Eiges, R., Eden, A., Yanuka, O., Itskovitz-Eldor, J., Goldstein, R.S., and Benvenisty, N. 2001. Induced neuronal differentiation of human embryonic stem cells. *Brain Res.* 913:201-205.

Seaberg, R.M., and van der Kooy, D. 2002. Adult rodent neurogenic regions: The ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J. Neurosci.* 22:1784-1793.

Shen, L.H. and Zhang, J.T. 2004. Ginsenoside Rg1 promotes proliferation of hippocampal progenitor cells. *Neurol. Res.* 26:422-428.

Sherwood, R.I., Christensen, J.L., Weissman, I.L., and Wagers, A.J. 2004. Determinants of skeletal muscle contributions from circulating cells, bone marrow cells, and hematopoietic stem cells. *Stem Cells* 22:1292-1304.

Shih, C.C., Weng, Y., Mamelak, A., LeBon, T., Hu, M.C., and Forman, S.J. 2001. Identification of a candidate human neurohematopoietic stem-cell population. *Blood* 98:2412-2422.

Shors, T.J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T., and Gould, E. 2001. Neurogenesis in the adult is involved in the formation of trace memories. *Nature* 410:372-376.

Smith-Arica, J.R., Thomson, A.J., Ansell, R., Chiorini, J., Davidson, B., and McWhir, J. 2003. Infection efficiency of human and mouse embryonic stem cells using adenoviral and adeno-associated viral vectors. *Cloning Stem Cells* 5:51-62.

Song, H., Stevens, C.F., and Gage, F.H. 2002. Astroglia induce neurogenesis from adult neural stem cells. *Nature* 417:39-44.

Strelchenko, N., Verlinsky, O., Kukharenko, V., and Verlinsky, Y. 2004. Morula-derived human embryonic stem cells. *Reprod. Biomed. Online* 9:623-629.

Suzawa, M., Takada, I., Yanagisawa, J., Ohtake, F., Ogawa, S., Yamauchi, T., Kadowaki, T., Takeuchi, Y., Shibuya, H., Gotoh, Y., Matsumoto, K., and Kato, S. 2003. Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. *Nat. Cell Biol.* 5:224-230.

Tada, M., Takahama, Y., Abe, K., Nakatsuji, N., and Tada, T. 2001. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr. Biol.* 11:1553-1558.

Tang, W.H. and Francis, G.S. 2003. Trends and treatment of heart failure developing after acute myocardial infarction. *Am. Heart Hosp. J.* 1:216-218.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.

Tian, C., Bagley, J., Forman, D., and Iacomini, J. 2004. Induction of central tolerance by mature T cells. *J. Immunol.* 173:7217-7222.

Tropepe, V., Sibilia, M., Ciruna, B.G., Rossant, J., Wagner, E.F., and van der Kooy, D. 1999. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev. Biol.* 208:166-188.

Tropepe, V., Hitoshi, S., Sirard, C., Mak, T.W., Rossant, J., and van der Kooy, D. 2001. Direct neural fate specification from embryonic stem cells: A primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 30:65-78.

Vallier, L., Rugg-Gunn, P.J., Bouhon, I.A., Andersson, F.K., Sadler, A.J., and Pedersen, R.A. 2004. Enhancing and diminishing gene function in human embryonic stem cells. *Stem Cells* 22:2-11.

Vassilopoulos, G., Wang, P.R., and Russell, D.W. 2003. Transplanted bone marrow regenerates liver by cell fusion. *Nature* 422:901-904.

Velkey, J.M. and O'Shea, K.S. 2003. Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells. *Genesis* 37:18-24.

Vescovi, A.L., Parati, E.A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frolichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A., and Galli, R. 1999. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp. Neurol.* 156:71-83.

Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M. 2003. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 422:897-901.

Wichterle, H., Lieberam, I., Porter, J.A., and Jessell, T.M. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385-397.

Willenbring, H. and Grompe, M. 2004. Delineating the hepatocyte's hematopoietic fusion partner. *Cell Cycle* 3:1489-1491.

Windrem, M.S., Nunes, M.C., Rashbaum, W.K., Schwartz, T.H., Goodman, R.A., McKhann, G. II, Roy, N.S., and Goldman, S.A. 2004. Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. *Nat. Med.* 10:93-97.

Wurmser, A.E., Nakashima, K., Summers, R.G., Toni, N., D'Amour, K.A., Lie, D.C., and Gage, F.H. 2004. Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. *Nature* 430:350-356.

Yandava, B.D., Billinghurst, L.L., and Snyder, E.Y. 1999. "Global" cell replacement is feasible via neural stem cell transplantation: Evidence from the dysmyelinated shiverer mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* 96:7029-7034.

Zammit, P. and Beauchamp, J. 2001. The skeletal muscle satellite cell: Stem cell or son of stem cell? *Differentiation* 68:193-204.

Zwaka, T.P. and Thomson, J.A. 2003. Homologous recombination in human embryonic stem cells. *Nat. Biotechnol.* 21:319-321.

Contributed by Mark Denham,
Brock Conley, Fredrik Olsson,
Timothy J. Cole, and Richard Mollard
Monash University
Melbourne, Australia

Mouse Embryonic Stem Cell Derivation, and Mouse and Human Embryonic Stem Cell Culture and Differentiation as Embryoid Bodies

Embryonic stem (ES) cells are pluripotent cells derived from developing mouse blastocysts in vitro that maintain long-term self renewal and the capacity to give rise to all cell types in the adult body (including some extraembryonic cell types) when subjected to the appropriate conditions. It is envisaged that the development of methods enabling controlled differentiation of mouse ES cell counterparts from human blastocysts would enable the provision of an unlimited supply of tissue for cell and tissue transplantation therapies for the repair and replacement of diseased, injured, and senescent tissue. Furthermore, derivation of mouse ES cells has allowed for the generation of thousands of gene-targeted mouse mutants. Culture of mouse ES cells as embryoid bodies (EBs) has provided a convenient system for studying early mouse developmental processes, including several aspects of extraembryonic lineage and axis formation associated with the pre- and peri-gastrulating mouse embryo. Relatively little is known regarding the corresponding development of the early human embryo due to limitations associated with the acquisition of relevant tissue material for study. The transfer of methods such as EB formation to human systems should, by association, facilitate a more advanced understanding of similar processes associated with early human development. This unit describes protocols for isolating mouse embryonic stem cells and methods for propagating, freezing, and producing EBs from both mouse and human embryonic stem cells.

The protocols in this unit are designed to provide a basis for the derivation of mouse ES cells (see Basic Protocol 1) and the successful propagation of both mouse (see Basic Protocol 1 and Alternate Protocol 1) and human ES cells (see Basic Protocol 3 and Alternate Protocol 2) in culture. In addition, protocols for the production of embryoid bodies (EBs) from both mouse (see Basic Protocol 2) and human (see Basic Protocol 4 and Alternate Protocol 4) ES cells are described. Descriptions of mouse embryonic fibroblast (MEF) feeder layer preparation (see Support Protocol 1), inactivation by mitomycin C (see Support Protocol 2) or γ-irradiation (see Support Protocol 3), mouse and human ES cell media preparation, methods for propagating ES cells on MEFs (see Basic Protocols 1 and 3), gelatin, and Matrigel (see Alternate Protocol 3), methods for passaging human ES cells by mechanical dissection (see Basic Protocol 4) and enzymatic digestion (see Alternate Protocol 3), conditioned medium preparation and storage (see Alternate Protocol 2), mouse blastocyst derivation, handling and cultivation techniques, vitrification, freezing and thawing protocols for human ES cells (see Basic Protocol 5), mouse ES cells and MEFs (see Support Protocol 4), and mouse (see Basic Protocol 2) and human EB (see Basic Protocol 3) formation using hanging drop and suspension culture are presented.

NOTE: For all procedures described in this unit, tissue culture, reagent preparation, and wash-up and sterilizing facilities are required. Experiments should be performed under sterile conditions in either Class II Biological Hazard Flow Hoods or laminar flow horizontal draft hoods. When working with human embryonic stem cells, Class II Biological Hazard Flow Hoods are recommended.

NOTE: Ethics approval for the described protocols is usually required from the appropriate institutional research office.

Contributed by Brock J. Conley, Mark Denham, Lerna Gulluyan, Fredrik Olsson, Timothy J. Cole, and Richard Mollard
Current Protocols in Cell Biology (2005) 23.2.1-23.2.22

NOTE: All incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

BASIC PROTOCOL 1

DERIVING, CULTURING, AND FREEZING MOUSE EMBRYONIC STEM CELLS

Mouse embryonic stem cells (mES) are currently the best defined ES or ES-like cells. Due to the relative ease of working with mouse ES cells when compared to their human counterparts, it may be convenient for researchers working for the first time in this discipline to set up both systems in the laboratory. Basic culture techniques for working with mouse ES cells are outlined below. For further information, see Key References.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

Materials

3.5 days post coitum (dpc) pregnant strain 129sv mouse
M2 medium (Sigma), sterile
M16 medium (Sigma), sterile
Cell culture–grade distilled water (JRH Biosciences), sterile
Organ culture dishes treated with 0.1% (w/v) gelatin and coated with mitotically inactivated MEFs (see Support Protocol 2 or 3)
mES cell medium (see recipe), sterile
PBS, calcium- and magnesium-free (CMF-PBS; Invitrogen), sterile
0.025% trypsin/0.04% EDTA (see recipe), sterile
Cell culture–grade distilled water (JRH Biosciences), sterile
1-ml syringe and 26-G needle
Petri dishes
Organ culture dish (Becton Dickinson)
Finely drawn glass capillary pipets, 1 mm i.d.
Microscope
25-cm^2 tissue culture flask (Becton Dickinson), optional

Collect embryos

1. Sacrifice a 3.5-days dpc pregnant mouse by cervical dislocation. Fill a 1-ml syringe with M2 medium, attach a 26-G needle, then insert the needle into the oviduct of the mouse and gently flush out the embryos into a petri dish containing M2 medium.
2. Transfer embryos to 1 ml M16 medium in the central well of an organ culture dish and incubate at a 37°C until the embryos become expanded blastocysts. Add 5 ml cell culture–grade sterile distilled water to the outer compartment of organ culture dish to help maintain humidity during culture.

   Some freshly isolated embryos may already be expanded blastocysts, ~1 day in culture should be sufficient for all blastocysts to expand.

Isolate ICMs

3. Place four embryos in an organ culture dish treated with 0.1% (w/v) gelatin and mitotically inactivated MEFs in 1 ml mES cell medium (see Support Protocol 2 or 3).

   Embryos should attach within 2 days. The trophectoderm cells will be seen to migrate out onto the feeder cells and the inner cell mass (ICM) will be seen to expand.

4. Pick off each ICM with a finely drawn glass capillary and place in a microdrop (10 to 20 μl) of 0.025% trypsin/EDTA at room temperature.

5. Disaggregate the ICM with a finely drawn glass capillary into single cells, then replate onto a new organ culture dish coated with MEFs in 1 ml mES cell medium. Culture one dissociated ICM per MEF-coated organ culture dish.

   The ICM cells should proliferate in clumps as ES cells. Some non-ES cells may persist for a few days.

Grow ES cells

6. Change mES medium daily, removing and replacing 1 ml per central well.

7. After an additional 2 to 3 days, remove the medium from the plate, wash by adding 1 ml CMF-PBS and then removing the buffer, then add 200 μl of 0.025% trypsin/EDTA. Incubate 5 to 10 min at 37°C.

8. Observe cells detaching under a microscope and, prior to their disaggregation to single cells, add 600 μl mES cell medium while gently pipetting up and down with a finely drawn glass pipet.

9. Transfer cells to a fresh MEF-coated organ culture dish, incubate at 37°C, and change mES cell medium the following day.

10. Two days later, treat cells with 0.025% trypsin/EDTA as per previous steps 7 and 8.

11. After transferring the collected cells to a new MEF-coated organ culture dish, add 500 μl of 0.025% trypsin/EDTA to the old plate and incubate for an additional 5 to 10 min at 37°C.

12. Add 1 ml mES cell medium to stop reaction, transfer remaining cells to a second new MEF-coated organ culture dish and incubate overnight at 37°C.

13. Change medium the following day on both dishes and analyze under a microscope.

    The first plate should contain ES cell colonies, the second plate should not. If the second plate contains ES cell colonies, repeat previous steps 7 to 9.

Passage ES cells

14. When colonies have expanded (2 to 3 days), treat with 0.025% trypsin/EDTA as described in steps 7 and 8, and transfer to a fresh MEF-coated organ culture dish or a 25-cm^2 tissue culture flask coated with MEFs.

15. Trypsinize cells and transfer to a new dish or flask no longer than every 3 days for the first few passages. Observe cells each day and transfer when 80% to 90% confluent.

    For purposes of tracking passage number, begin counting passages the first time single cells are plated, i.e., step 5.

    To scale up the ES culture, cells can be split into two or more flasks or into larger flasks.

16. Freeze cells at regular intervals to prevent loss of cell line (see Support Protocol 4).

    It is recommended to freeze ES cells following the second or third passage at step 10.

17. Monitor ES cells at regular intervals via karyotypic analysis (G-banding; *UNIT 22.3*) and undifferentiated marker analysis.

    See Conley (2004a) for discussion of markers.

    The morphology of a typical mouse ES cell colony grown on MEFs can be seen in Figure 23.2.1C.

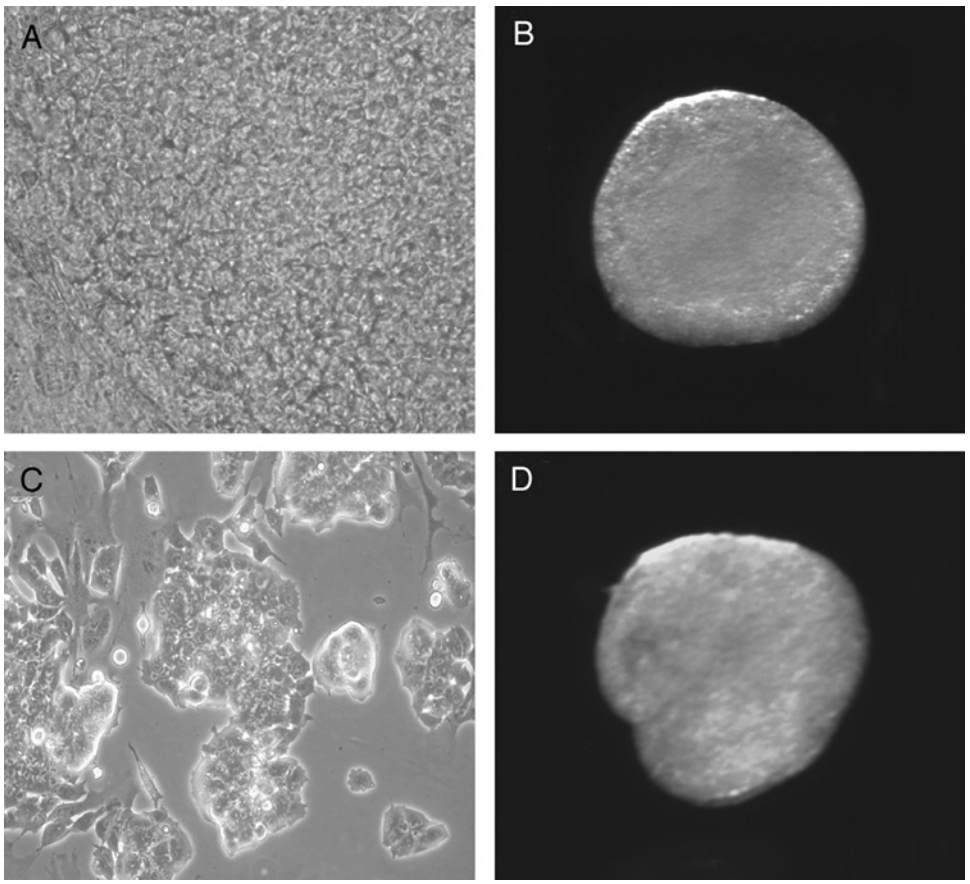


Figure 23.2.1 Photomicrographs of human ES cell and mouse ES cell propagation and growth as EBs. (**A**) Human ES cells (HES-2) propagated on MEFs (40× magnification). (**B**) An EB formed from human ES cells following mechanical dissection and culture in suspension for 7 days (2.5× magnification). (**C**) Zin40 mouse ES cells (see Munsie et al., 1998) propagated on gelatin in the presence of LIF (40× magnification). (**D**) An EB formed from Zin40 ES cells following 7 days of culture in a hanging drop (2.5× magnification).

ALTERNATE PROTOCOL 1

MOUSE ES CELL CULTURE IN THE ABSENCE OF FEEDER CELLS

Subsequent to their derivation, mES cells can be maintained in the absence of an MEF feeder layer in mES cell medium supplemented with 2000 U/ml leukemia inhibitory factor (LIF). Cells are grown in flasks and passaged when 75% to 85% confluent. Medium should be changed every 1 to 2 days.

SUPPORT PROTOCOL 1

PREPARATION OF MOUSE EMBRYONIC FIBROBLASTS

Mouse embryonic fibroblasts (MEFs) are used as a feeder support layer for the derivation of mouse ES cells and the propagation of both mouse and human ES cells. This protocol requires the investigator to possess basic animal handling, dissection, and tissue culture skills.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

Materials

12.5 to 13.5 days post coitum (dpc) pregnant strain 129sv females
PBS, calcium- and magnesium-free (CMF-PBS; Invitrogen), sterile
0.025% (w/v) trypsin/EDTA (see recipe), sterile
MEF medium (see recipe), sterile

90-mm bacteriological petri dish
Scalpel, sterile
20-G needle (Becton Dickinson)
75-cm^2 tissue culture flasks (Becton Dickinson/Falcon)
Platform shaker
15-ml tubes

Dissect and process embryos

1. Dissect the pregnant females at 12.5 to 13.5 days post coitus and remove the fetuses from the uterine horns into 90-mm bacteriological petri dishes containing sterile CMF-PBS.

2. Decapitate and eviscerate the fetuses to isolate the carcasses. Slice the carcasses into small, ~0.1-cm^2 fragments with a sterile scalpel.

3. Digest the carcass slices in 0.025% trypsin/EDTA for ~5 min at 37°C.

   In this and the following step, enough trypsin solution should be used to immerse the tissue.

4. Dissociate the tissue further by passing through a 20-G needle and then incubate the tissue in 0.025% trypsin/EDTA for an additional 10 min at 37°C.

5. Plate all of the cells from one carcass in a 75-cm^2 tissue culture flask containing 20 ml of MEF medium. Incubate at 37°C.

Passage MEFs

6. When the cells reach 80% to 90% confluency (~1 to 2 days), aspirate and discard the MEF medium, rinse with 2 ml CMF-PBS, replace with 1.5 ml of 0.025% trypsin/EDTA, and incubate 1 to 2 min at room temperature with agitation.

7. Inactivate trypsin by adding 1 ml MEF culture medium down the growing surface to dislodge all cells. Transfer cells to a 15-ml tube.

8. Centrifuge cells 2 min at ~700 × *g*, room temperature, to pellet. Remove and discard supernatant and resuspend the cells in 8 ml MEF medium.

9. Dispense cell suspension into 2-ml aliquots and add each aliquot to 18 ml of prewarmed MEF medium in 75-cm^2 tissue culture flasks. Incubate at 37°C (1:4 split).

10. Allow cells to reach 80% to 90% confluency (1 to 3 days) and passage again according to steps 6 to 9.

11. Passage cells four to five times to ensure minimal non-fibroblast contamination and to create a stockpile of genetically equivalent MEFs.

    For the purpose of counting passage number, begin counting following step 9.

    MEFs can be utilized following three or four passages, but for maximum efficiency, utilize them at passages five and six. After about seven passages, MEFs begin to lose their ability to support ES cells.

    MEFs can be frozen for storage at each passage number according to the freezing protocol (see Support Protocol 4).

Other strains of mice can be used to derive MEFs; however, these must be batch tested with ES cells to assess compatibility. Mouse ES cell derivation efficiency is severely reduced when not using the inbred 129sv mice. Mouse ES cells and MEFs need not be strain-matched. MEFs used for human ES cell culture provides a potential means for the xenobiotic transfer of pathogens from the mouse to human tissue, thus making these cells unsuitable for human therapeutic use. Human fetal fibroblasts have been utilized for propagation of human ES cells; however, currently, the use of MEFs in human ES cell culture is the most accessible and effective means for their propagation (Richards et al., 2002).

SUPPORT PROTOCOL 2

PASSAGING AND MITOTIC INACTIVATION OF MEFs BY MITOMYCIN C

Before MEFs can be used as a feeder layer for ES cells, they must be mitotically inactivated to prevent overgrowth or contamination of ES cells. They can be mitotically inactivated by treatment with mitomycin C or γ-irradiation (see Support Protocol 3). Mitomycin C treatment is an efficient and effective way to inactivate MEFs. Following treatment, ~90% to 95% of cells are seen to be inactivated.

Materials

MEF cultures in 75-cm^2 culture flasks (see Support Protocol 1)
MEF medium (see recipe)
Mitomycin C (see recipe)
0.1% (w/v) gelatin (see recipe)
PBS, calcium- and magnesium-free (CMF-PBS; Invitrogen)
0.025% (w/v) trypsin/EDTA (see recipe)
Cell culture–grade water (JRH Biosciences), sterile

Organ culture dishes
15-ml centrifuge tubes

Additional reagents and equipment for counting cells (*UNIT 1.1*)

Inactivate MEFs

1. Incubate all MEFs from one 75-cm^2 culture flask at passage 4 or 5 with 20 ml of MEF medium containing 10 μg/ml mitomycin C for a minimum of 2.5 hr (maximum 3 hr) at 37°C.
2. During incubation, pretreat the central compartment of organ culture dishes with 1 ml of 0.1% gelatin for 30 min at room temperature. After 30 min, aspirate the gelatin and allow the plates to dry at room temperature.
3. Aspirate MEF medium containing mitomycin C from MEFs and wash cells one time with 20 ml of prewarmed MEF medium at 37°C.

Collect cells

4. Wash three times each time by adding and then removing 20 ml of CMF-PBS at room temperature.
5. Detach cells from the flasks by incubating with 1.5 ml of 0.025% trypsin/EDTA for 1 to 2 min at room temperature with agitation.
6. Inactivate the trypsin by adding 6 ml of pre-warmed MEF medium at 37°C. Pipet up and down the growing surface of the flask to ensure all cells are dislodged.
7. Transfer cells to a 15-ml centrifuge tube and centrifuge 2 min at ~700 × *g*, room temperature, to pellet cells.

Set up organ culture dishes

8. Resuspend the cells in 8 ml of MEF or human ES culture medium. Pipet a 10-µl aliquot and count total cell number using a hemacytometer (*UNIT 1.1*).
9. Plate MEFs onto the pretreated, gelatin-coated central portions of organ culture dishes (from step 2) at a concentration of 1.75–1.8 × 10^5 cells/ml per dish.
10. Pipet 5 ml of tissue culture–grade distilled water into the outer section of the organ culture dish to maintain humidity. Place dishes at 37°C.

ES cells may be added 24 hr after the MEFs are plated (i.e., after the MEFs have attached).

PASSAGING AND MITOTIC INACTIVATION OF MEFs BY γ-IRRADIATION

SUPPORT PROTOCOL 3

γ-Irradiation can be used to mitotically inactivate MEFs. This method is as effective as mitomycin C inactivation provided that an appropriate level of radiation is utilized. A disadvantage to this method is that it requires additional equipment to irradiate MEFs, whereas mitomycin C is a reagent that can be directly applied in almost any laboratory.

Materials

MEF cultures (for fresh MEF, see Support Protocol 1 or for frozen MEF, see Support Protocol 4)
0.025% (w/v) trypsin/EDTA (see recipe)
MEF medium (see recipe)
Human ES (hES) cell culture medium (see recipe)
75-cm^2 culture flasks
50-ml centrifuge tube
γ irradiator (Gammacell 1000, Nordion)
150- and 100-mm gelatin-coated tissue culture dishes (optional)
Organ culture dishes

1. Grow MEFs at passage 4 to 5 or a thawed frozen vial of primary MEFs (from an earlier preparation, see Support Protocol 4) to confluence in 75-cm^2 culture flasks or on 150-mm gelatin-coated tissue culture dishes.
2. Detach cells using 0.025% trypsin/EDTA as described in Support Protocol 2, steps 4 to 6, and collect in a 50-ml centrifuge tube.
3. Centrifuge cells 2 min at ~700 × *g*, room temperature, to pellet and resuspend pellet in 25 ml MEF medium.
4. Irradiate each tube of cells with 3000 to 10,000 rads of γ-irradiation.

This varies with the cell line used, therefore, a titration should be performed to determine the effective dose.

5. Plate irradiated cells onto gelatin-coated plates at 3–4 × 10^6 cells per 100-mm gelatin-coated dish, or 1.8 × 10^5 cells per organ culture dish, in MEF or hES culture medium.

FREEZING MEFS

SUPPORT PROTOCOL 4

Early passaged MEFs can be frozen to provide cells for use at a later point.

Materials

Freezing solution (see recipe)
MEF cultures in 75-cm^2 tissue culture flasks (see Support Protocol 1)

PBS, calcium- and magnesium-free (CMF-PBS; Invitrogen)
0.05% (w/v) trypsin/EDTA (Invitrogen)
MEF medium (see recipe)
Liquid nitrogen
1-ml cryovial

1. Prior to freezing cells, store the freshly prepared freezing solution on ice. Label 1.0-ml cryovials with passage number and cell line and chill on ice (use two vials per 75-cm^2 tissue culture flask).
2. Aspirate medium from 80% to 90% confluent MEFs cultured in 75-cm^2 tissue culture flasks and wash gently with 2 ml CMF-PBS to remove all traces of medium.

   Freeze aliquots of MEFs from passage 1 to 5 for subsequent thawing and inactivation.
3. Add 1.5 ml of 0.05% trypsin/EDTA to cells and incubate for 1 to 2 min at room temperature. Tap flask/dish to gently dislodge the cells.
4. Add 6 ml MEF medium to inactivate the trypsin. Pellet cells by centrifuging 2 min at ~700 × *g*, room temperature.
5. Aspirate supernatant from the cell pellet and resuspend cells in 2 ml freezing solution.
6. Transfer 1 ml freezing solution and cells to each 1.0-ml cryovial and place at −80°C. After 24 hr, transfer the cryovials to liquid nitrogen.

   MEFs can be stored indefinitely in liquid nitrogen. Upon thawing, cells may take 1 to 3 days to recover to 80% to 90% confluency for subsequent use.

BASIC PROTOCOL 2

MOUSE EMBRYOID BODY FORMATION (HANGING DROP CULTURE)

Mouse embryoid body (EB) formation is routinely utilized as an initial differentiation method as it initiates differentiation into a variety of cell types that can be subsequently purified and analyzed. This method is for use with mES cells propagated in the absence of a feeder layer. It is important that the mES cell medium used in EB formation lacks LIF. A typical mouse EB can be seen in Figure 23.2.1D.

Materials

mES cell culture in the absence of a feeder layer (see Alternate Protocol 1) in a 25-cm^2 tissue culture flask
PBS, calcium- and magnesium-free (CMF-PBS; Invitrogen)
0.025% (w/v) trypsin/EDTA (see recipe)
mES medium (see recipe) without LIF
Cell culture–grade distilled water (JRH Biosciences), sterile
15-ml centrifuge tubes
90-mm bacterial culture plate
Additional reagents and equipment for counting cells (*UNIT 1.1*)

Collect cells

1. Aspirate the medium from a 25-cm^2 tissue culture flask of undifferentiated mES cells.
2. Rinse the growing surface with 2 ml CMF-PBS at room temperature.
3. Apply 1 ml of 0.025% trypsin/EDTA and incubate 3 to 7 min at room temperature or until cells have detached.
4. Inactivate trypsin by adding 6 ml mES medium without LIF down the growing surface to collect all cells.

5. Transfer cells and medium to a 15-ml centrifuge tube and centrifuge 2 min at ~700 × *g*, room temperature.

6. Aspirate supernatant and resuspend pellet in 3 ml mES medium without LIF. Count cells using a hemacytometer (*UNIT 1.1*).

7. Dilute cells in mES medium without LIF to obtain a concentration of 1×10^4 cells/ml.

Set up cultures for EB formation

8a. *For hanging drop cultures:* Apply 30- to 50-µl droplets (300 to 500 cells/droplet) onto the lid of a 90-mm bacterial culture plate (ensuring sufficient space is left between the droplets to avoid fusion of neighboring drops on lid—between 30 to 35 individual drops per lid) and flip lid in a smooth, steady manner to invert. Place the lid onto the bacterial dish, which has been half filled with cell culture–grade distilled water to maintain humidity.

8b. *For solution culture:* Alternatively, produce EBs by transfering dissociated ES cells to a bacteriological culture dish at a concentration of 2×10^6 cells in 10 ml mES cell medium without LIF.

EB formation is slower with this method and smaller spheres are observed after 2 to 3 days in culture.

9. Incubate at 37°C for desired time.

After 1 to 2 days of incubation, spheres can be observed; these increase in size.

Cell types observed in EBs may vary between cell lines used and between methods used.

HUMAN EMBRYONIC STEM CELL PROPAGATION

BASIC PROTOCOL 3

Human ES cell handling techniques are a little more challenging than mouse ES cell handling techniques. The following protocols are modifications of those described in Reubinoff et al. (2000) and those found at *http://www.geron.com/PDF/scprotocols.pdf*. The morphology of a typical human ES cell colony grown on MEFs can be seen in Figure 23.2.1A. The mechanical human ES cell propagation procedure is illustrated in Figure 23.2.2.

Materials

Mitomycin C–treated MEFs in organ culture dish (see Support Protocol 2)
Human ES cell medium (see recipe)
Cultures of human ES cells
Finely drawn glass capillaries, 1.0-mm o.d. (Clark Electromagnetic Industries)

1. On the day prior to human ES cell transfer, equilibrate mitomycin C–treated MEFs by incubating in human ES cell medium at 37°C.

2. At ~7 days of culture, mechanically dissect the human ES cell colonies into ~0.1-cm^2 morphologically designated undifferentiated fragments using a glass capillary finely drawn over a blue flame of a Bunsen burner. Gently lift the fragment from the plate with the end of a 20-µl micropipettor tip.

Morphologically undifferentiated fragments, visualized through a dissection microscope, appear as a uniform and consistent white color with distinct edges, whereas differentiated regions are more heterogenous and irregular in appearance often with cystic (fluid-filled) regions. Differentiated regions have indistinct edges and often display processes extending away from the colony. Often colonies of hES cells will contain a densely packed region in the center, from which the colony has initially arisen. This region also contains differentiated cells and thus is not transferred to a new dish.

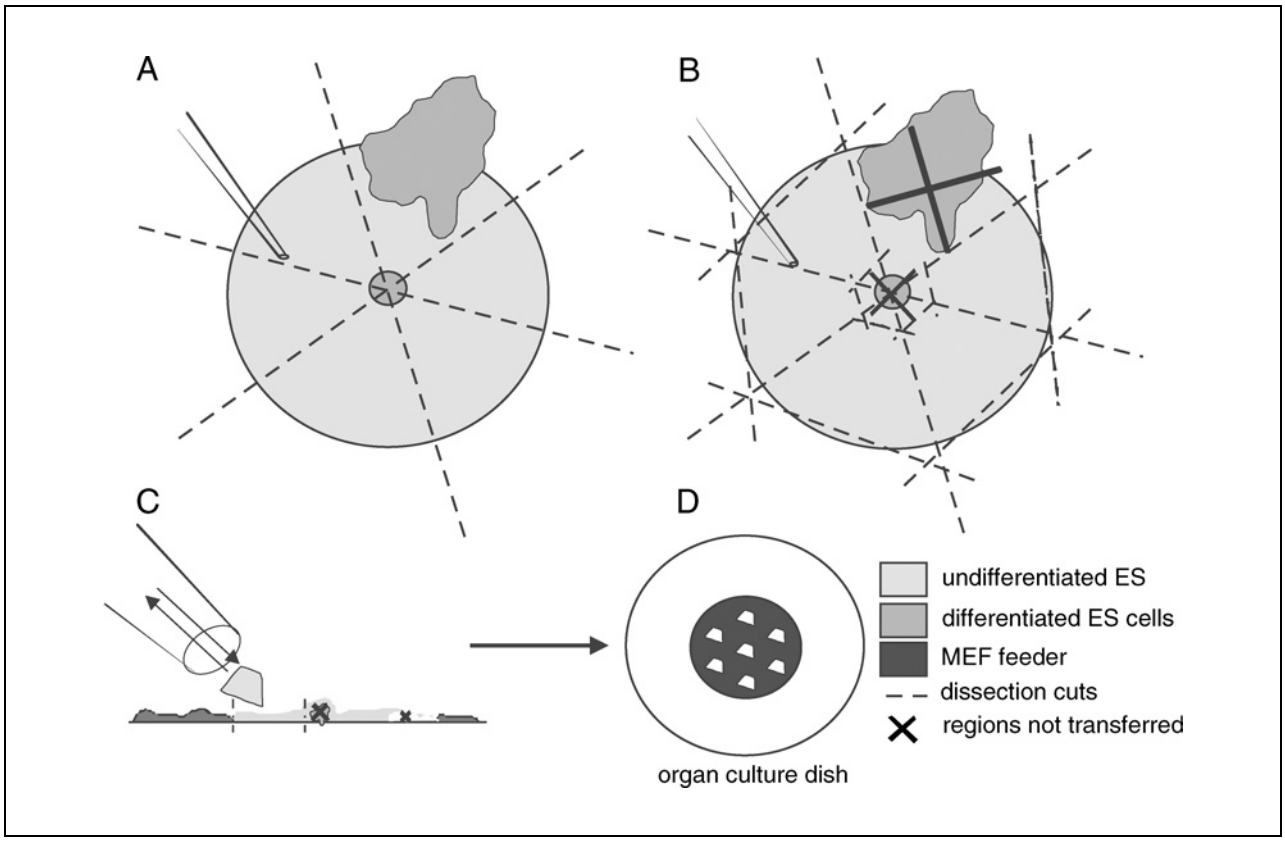


Figure 23.2.2 Schematic representation of the human ES cell mechanical dissociation culture procedure. (**A, B**, and **C**) Human ES cell colonies are dissected with a drawn glass micropipet according to the dotted lines. Isolated undifferentiated regions are gently lifted with a micropipettor to separate them from any differentiated cells. The undifferentiated regions are then transferred to wash plates. (**D**) Approximately six to nine regions of undifferentiated cells from the original colony are transferred to a new MEF layer for growth.

3. Transfer the undifferentiated fragments to a fresh organ culture dish containing 1 ml room temperature hES medium, then transfer to a second organ culture dish containing 1 ml medium for a second wash.
4. Transfer six to nine fragments to an equilibrated MEF-coated organ culture dish.
5. Change the medium daily (1 ml per dish) until the following transfer at ~7 days.

   Depending on the number of fragments seeded, one organ culture dish after 7 days should yield 25 to 35 colonies.

ALTERNATE PROTOCOL 2

PREPARATION OF CONDITIONED MEDIUM FEEDER-LAYER-FREE CULTURE OF HUMAN ES CELLS

Human ES (hES) cells can be cultured in the absence of a feeder layer using MEF cell conditioned medium supplemented with FGF-2 on an extracellular matrix (see Alternate Protocol 3). This method is efficient for larger cultures; however, prolonged culture in this manner (>23 passages) has been shown to produce chromosomal abnormalities (Mitalipova et al., 2005).

Materials

Mitotically inactivated MEFs (see Support Protocol 2 or 3)
MEF medium (see recipe)
Serum-free medium (see recipe)

0.1 mg/ml human recombinant basic fibroblast growth factor stock (hbFGF; Invitrogen): reconstitute 10 μg in 100 μl of 10 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*), store at −20°C
hES cells
25-cm^2 tissue culture flasks
0.22-μm filter

1. Plate mitomycin C–treated or γ-irradiated MEFs at 5.6×10^4 cells/cm^2 in 5 ml MEF medium in 25-cm^2 tissue culture flasks. Incubate overnight at 37°C.
2. Replace the MEF medium the following day with 5 ml of serum-free medium. Incubate overnight at 37°C.
3. Collect medium from the flask, filter through a 0.22-μm filter, and add 8 ng/ml hbFGF. Transfer the new conditioned medium (CM) either to human ES cells (see Basic Protocol 3) or freeze for storage.

   CM can be stored for 1 month at −20°C.

4. Add 5 ml of fresh serum-free medium to the flask and incubate cells overnight at 37°C.
5. Repeat steps 3 and 4 for up to 7 days.

ENZYMATIC PASSAGE OF HUMAN ES CELLS FOR CULTURE ON FEEDER LAYERS AND ON MATRIGEL

ALTERNATE PROTOCOL 3

Human ES cells have also been propagated more recently using enzymatic methods as well as growth in the absence of feeders on the extracellular matrix substrate Matrigel. Some controversy remains with respect to the reliability of this culture method, however, especially in regard to human ES cell karyotype integrity following prolonged culture (see Mitalipova et al., 2005). Nevertheless, these protocols provide a more time-efficient method for regular maintenance of human ES cells in short-term cultures.

Materials

hES cell cultures in Matrigel-coated 4-well plates (see recipe for plates)
1 mg/ml collagenase IV (see recipe)
PBS, calcium- and magnesium-free (CMF-PBS; Invitrogen)
Serum-free medium (see recipe)
Conditioned medium (CM, see Alternate Protocol 2)
Matrigel-coated 4-well plates (see recipe)
15-ml centrifuge tubes

1. Replace the medium on human ES cells with 0.5 ml of 200 U/ml collagenase IV per well of a Matrigel-coated 4-well plate.
2. Incubate for 5 to 10 min at 37°C or until colonies just begin to detach from plate.
3. Gently remove collagenase IV and wash gently with 1 ml CMF-PBS.

4a. *For passage onto MEFs:* Replace CMF-PBS with 1 ml serum-free medium equilibrated at 37°C and proceed to Basic Protocol 3, step 4.

4b. *For passage onto Matrigel:* Remove CMF-PBS and add 1 ml CM to each well.

5. Gently pipet cells up and down, scraping with the pipet to dislodge cells, and transfer to a 15-ml centrifuge tube.

6. Dissociate cells with gentle pipetting to small clusters (50 to 400 cells), then transfer between 1/3 and 1/6 of the suspension to a Matrigel-coated well containing 1 ml of CM medium.

   Colonies should appear within days and should continue to expand.

7. Passage when human ES cells comprise 80% of the surface area of the well.

BASIC PROTOCOL 4

PRODUCING HUMAN ES CELL EMBRYOID BODIES BY MECHANICAL DISSOCIATION

Mechanical dissection is more laborious but more selective with the ability to select for morphologically undifferentiated regions whereby enzymatic dissociation obtains both differentiated and undifferentiated cells together. This results in a difference in cell potential and thus a difference in EBs produced.

Materials

Cultures of human ES cell colonies in organ culture dishes on MEF feeder layer, 6 to 9 colonies/dish
Human ES cell medium (see recipe)
Finely drawn glass capillary (1.0-mm o.d.)
90-mm bacteriological petri dish

1. Dissociate human ES cells colonies with a finely drawn glass capillary to obtain small undifferentiated regions of between 100 and 300 cells.
2. Transfer as many of these aggregates as required to 90-mm bacteriological petri dishes containing 10 ml human ES cell medium and culture as a suspension (see Conley et al., 2004b).
3. Incubate at 37°C.

   A typical 7-day-old EB formed from human ES cells can be seen in Figure 23.2.1B.

ALTERNATE PROTOCOL 4

PRODUCING HUMAN ES CELL EMBRYOID BODIES BY ENZYMATIC DIGESTION

Human EBs can also be produced following enzymatic digestion. EBs are produced this way from smaller digested fragments of ES cells and thus are generally smaller. Digestion additionally leads to more variability in ES cell fragment size as well as resultant EB size.

Materials

Human ES cell colonies
Serum-free medium (see recipe)
1 mg/ml collagenase/dispase stock (see recipe)
Certified fetal bovine serum (Invitrogen)
Human ES cell medium (see recipe)
Finely drawn glass capillary (1.0-mm o.d.)
15-ml centrifuge tube
90-mm bacteriological petri dish

1. Mechanically dissect each colony with a finely drawn glass capillary to obtain undifferentiated areas (see Basic Protocol 3).
2. Transfer fragments to a 15-ml centrifuge tube and centrifuge 2 min at ~700 × *g*, room temperature.

3. Aspirate as much medium as possible and resuspend pellet in 500 µl serum-free medium.
4. Add 500 µl of 1 mg/ml collagenase IV/dispase stock (final concentration 0.5 mg/ml for each enzyme) and mix gently with a pipet.
5. Incubate 3 min at 37°C and then agitate with a pipet ten times.
6. Add 5 ml certified fetal bovine serum and invert gently three to five times to mix.

   FBS should be batch tested for compatibility with ES cells.
7. Add 7 ml human ES cell medium, mix, and centrifuge 3 min at ~700 × *g*, room temperature.
8. Resuspend the pellet in 5 ml human ES cell medium and transfer to a 90-mm bacteriological petri dish containing 3 to 5 ml medium to allow aggregation as EBs in suspension.

   EBs can be seen to form after ~2 days.

VITRIFICATION/THAWING OF HUMAN ES CELLS

BASIC PROTOCOL 5

The vitrification protocol described is the one that can be found in Reubinoff et al. (2001). It is used to maintain frozen stocks of human ES cell lines.

Materials

Bench medium (BM; see recipe)
Vitrification solution 1 (VS1; see recipe)
Vitrification solution 2 (VS2; see recipe)
Liquid nitrogen
hES cell culture
Warming solution 1 (WS1; see recipe)
Warming solution 2 (WS2; see recipe)
Organ culture dish seeded with mitotically inactivated MEFs (see Support Protocol 2 or 3)
Human ES cell medium (see recipe)
4-well tissue culture plates (NUNC)
5-ml cryovials (NUNC)
18-G needle
Canes for liquid nitrogen tank
20-µl micropipettor
Vitrification straws (LEC Instruments)
Forceps

Prepare equipment for vitrification

1. Prior to freezing human ES cell fragments, prepare a vitrification plate. Pipet 1 ml of each of the solutions BM, VS1, and VS2, into separate wells of a 4-well tissue culture plate and pre-warm at 37°C.
2. Label a 5-ml cryovial with all details pertaining to cells being frozen. Using a flame-heated 18-G needle, punch holes in the upper section, base, and lid of the cryovial and attach the cryovial to a labeled cane. Submerge in a bucket of liquid nitrogen.

Prepare cells for freezing

3. Prepare human ES cell pieces for freezing by cutting fragments slightly larger than that required for usual transfer (0.1 cm^2). Transfer about eight fragments to the first well of the 4-well tissue culture plate containing BM using a 20-µl micropipettor.

4. With a fresh tip, transfer all fragments to the second well containing VS1 for 1 min, ensuring all pieces settle to the bottom of the viscous solution. During this minute, pipet a 20-µl drop of VS2 onto the inside of the 4-well tissue culture plate lid. Ensure a fresh drop is prepared for each straw to be frozen.
5. Transfer the human ES cell fragments to the last well containing VS2 for 25 sec and again ensure that all pieces settle to the bottom.
6. Transfer the fragments in the least possible volume to the 20-µl VS2 drop on the 4-well tissue culture plate lid.
7. Using a second pipet set at 3 µl, pick up the fragments and make a small, high droplet on the lid.

Collect cells in vitrification straws and freeze

8. Immediately after making the droplet, touch the narrow end of a vitrification straw to the side of the droplet at an angle of 30° to 45° to the plane of the lid and draw up the droplet and fragments into the straw by capillary action.
9. Plunge the straw into the bucket of liquid nitrogen at an angle of 45°. Without removing the straw from the liquid nitrogen, carefully transfer into the prepared cryovial. Repeat until all fragments have been collected (three to five straws per cryovial). Store the cryovial in liquid nitrogen.

Thaw and process ES cells

10. Prior to thawing the cryovial containing the human ES cell straws, prepare a 4-well tissue culture plate containing 1 ml of each of solutions WS2, WS1, and 2 wells of BM in separate wells and prewarm at 37°C.
11. Collect the cryovial containing the straws to be thawed in a bucket of liquid nitrogen. Remove a straw using forceps and hold it between the thumb and middle finger with the index finger blocking the larger end of the straw which is devoid of cells.
12. Immediately submerge the narrow end of the straw containing the fragments into the first well containing WS2 where, as the gas expands, the human ES cell fragments will expel from the straw and into the well.
13. After 1 min transfer the fragments to the next well containing WS1 for 5 min.
14. Transfer the fragments into the third well containing BM for an additional 5 min.
15. Transfer fragments to the last well containing BM for an additional 5 min.
16. Transfer the fragments onto a previously prepared organ culture dish containing mitotically inactivated MEFs (see Support Protocol 2 or 3) and 1 ml of human ES cell medium, and culture at 37°C.

REAGENTS AND SOLUTIONS

Use deionized or distilled tissue culture grade water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Bench medium (BM)

Add 800 µl DMEM/F12 (Invitrogen)
200 µl FBS (20% v/v; JRH Biosciences)
Store up to 2 weeks at 4°C

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been

hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

Collagenase IV, 1 mg/ml

Dilute 100 mg collagenase type IV (Invitrogen) in 100 ml knockout DMEM (Invitrogen). Dispense into aliquots and store up to 6 months at −20°C.

Collagenase IV/dispase stock, 1 mg/ml

Add 100 mg collagenase type IV (Invitrogen) and 100 mg dispase (Invitrogen) to 100 ml of filter-sterilized serum-free medium (see recipe). Store in aliquots at −20°C.

Gelatin, 0.1% (w/v)

Dilute 5 ml of 1% (w/v) gelatin stock (see recipe) in 50 ml distilled water. Store up to 4 weeks at 4°C.

Gelatin, 1% (w/v)

Dissolve 0.4 g of gelatin powder (Sigma) in 40 ml distilled water, autoclave, store in 30- to 50-ml aliquots up to 2 months at −20°C.

Freezing solution

Prepare a mixture of 90% (v/v) FBS and 10% (v/v) DMSO. Prepare fresh and store on ice.

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

hbFGF stock, 0.1 mg/ml

Reconstitute 10 μg human basic FGF (Invitrogen) in 100 μl of 10 mM Tris·Cl, pH 7.6. Store up to 6 months in 20- to 50-μl aliquots at −20°C.

Human ES cell medium

High-glucose DMEM (Invitrogen) supplemented with:
20% (v/v) certified fetal bovine serum (FBS; Invitrogen)
1% (v/v) nonessential amino acids (Invitrogen)
2 mM L-glutamine (Invitrogen)
50 U/ml penicillin/50 mg/ml streptomycin (Invitrogen)
1× insulin-transferrin-selenium supplement (Invitrogen)
0.1 mM β-mercaptoethanol (Sigma)
Store up to 2 weeks at 4°C

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

Matrigel-coated plates

Thaw an aliquot of Matrigel stock (see recipe) at 4°C. Dilute stock 1:10 in cold knockout DMEM (Invitrogen). Apply 500 μl of diluted Matrigel to each well of a 4-well tissue culture plate. Incubate 1 hr at 37°C and aspirate liquid immediately prior to use.

Matrigel stock

Thaw 10-ml bottle of Matrigel (Becton Dickinson) overnight at 4°C, add 10 ml cold knockout DMEM (Invitrogen), and mix well. Store in 10-ml aliquots up to 6 months at −20°C.

mES cell medium

High-glucose DMEM (Invitrogen) supplemented with:
10% (v/v) fetal bovine serum (FBS; JRH Biosciences)
2 mM L-glutamine (Invitrogen)
50 U/ml penicillin/50 mg/ml streptomycin (Invitrogen)
1% (v/v) nonessential amino acids (Invitrogen)
0.1 mM β-mercaptoethanol (Sigma)
1000 U/ml recombinant mouse leukemia inhibitory factor (LIF; Chemicon)
Store up to 2 weeks at 4°C

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

MEF medium

High-glucose DMEM; (Invitrogen) supplemented with:
10% (v/v) fetal bovine serum (FBS; JRH Biosciences)
2 mM L-glutamine (Invitrogen)
50 U/ml penicillin/50 mg/ml streptomycin (Invitrogen)
Store up to 2 weeks at 4°C

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

Mitomycin C

Reconstitute a 2-mg ampule of mitomycin C powder (Sigma) with 1 ml sterile PBS using a 21-G needle, with a 26-G needle in place to vent the ampule. Remove solution and filter through a 0.22-μm syringe filter. Store solution up to 2 weeks at 4°C protected from light.

CAUTION: *Mitomycin C is a toxic substance, refer to the product data sheet from the manufacturer for handling instructions.*

Serum-free medium (SFM)

High-glucose DMEM (Invitrogen) supplemented with:
1% (w/v) nonessential amino acids (Invitrogen)
2 mM L-glutamine (Invitrogen)
50 U/ml penicillin/50 mg/ml streptomycin (Invitrogen)
1× insulin-transferrin-selenium supplement (Invitrogen)
mM β-mercaptoethanol (Sigma)
Store up to 2 weeks at 4°C

Serum replacement medium

80% (v/v) knockout DMEM (Invitrogen)
20% (v/v) knockout serum replacement (Invitrogen)
1% (v/v) nonessential amino acids (Invitrogen)
2 mM L-glutamine (Invitrogen)
50 U/ml penicillin/50 mg/ml streptomycin (Invitrogen)
1× insulin-transferrin-selenium supplement (Invitrogen)
0.1 mM β-mercaptoethanol (Sigma)
Store up to 2 weeks at 4°C

Sucrose solution, 2 M

Dissolve 6.846 g sucrose (Sigma) in ~5 ml DMEM/F12 (Invitrogen). Add DMEM/F12 to a final volume of 10 ml. Prepare fresh.

Trypsin/EDTA, 0.025% (w/v)

Dilute 20 ml of 0.05% (w/v) trypsin/EDTA (Invitrogen) in 20 ml calcium- and magnesium-free PBS (CMF-PBS; e.g., Invitrogen) for a final concentration of 0.025%. Store in aliquots up to 2 weeks at 4°C or up to 6 months at −20°C.

Vitrification solution 1 (VS1)

600 µl DMEM/F12 (Invitrogen)
200 µl FBS (Invitrogen; 20% v/v final)
100 µl DMSO (10% v/v final)
100 µl ethylene glycol (Sigma; 10% v/v final)
Prepare fresh

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

Vitrification solution 2 (VS2)

250 µl sucrose solution (0.5 M final; see recipe for 2 M sucrose)
150 µl DMEM/F12 (Invitrogen)
200 µl FBS (Invitrogen; 20% v/v final)
200 µl DMSO (20% v/v final)
200 µl ethylene glycol (20% v/v final)
Prepare fresh

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

Warming solution 1 (WS1)

100 µl sucrose solution (0.2 M final; see recipe for 2 M sucrose)
700 µl DMEM/F12 (Invitrogen)
200 µl FBS (Invitrogen; 20% v/v final)
Prepare fresh

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

Warming solution 2 (WS2)

50 µl sucrose solution (0.1 M final; see recipe for 2 M sucrose)
750 µl DMEM/F12 (Invitrogen)
200 µl FBS (Invitrogen; 20% v/v final)
Prepare fresh

FBS purchased from JRH Biosciences has been traditionally used in the authors' laboratory for mouse ES cell and MEF cultures, while it has been observed that certain batches of FBS purchased from Invitrogen maintained human ES cells more effectively. It has been hypothesized that may be due to a low endotoxin level in some of the batches from Invitrogen (however, this has not been proven definitively). Therefore, batch testing of all serum is imperative.

COMMENTARY

Background Information

Mouse embryonic stem (ES) cells are an in vitro–derived cell type isolated from the epiblast/inner cell mass (ICM) of mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). mES cells are said to possess properties consistent with an immortal phenotype: demonstrating continual self-renewal and maintaining a normal chromosomal content over extended periods in culture. mES cells further retain several properties of their in vivo predecessors, including a primitive pluripotent phenotype enabling differentiation into all functional cell types of the adult and some extraembryonic tissues given the appropriate stimuli (see Nagy et al., 1990, 1993).

The isolation of ES cells, or ES-like cells, has more recently been reported from other species including human and nonhuman primates (see Thomson et al., 1995, 1998). Functional studies, however, remain more advanced for mouse ES cell systems because of the relatively long time since their first description some 24 years ago and, when compared to human ES cell work, the relatively relaxed ethical regulations associated with their research (for a review, see Fischbach and Fischbach, 2004). For example, mouse ES cell protocols for culture and genetic manipulation at the single cell level, tetraploid complementation and blastocyst injection, and the transfer of mouse ES cell derivative structures to pseudopregnant females for full-term pregnancy are routine (but technically challenging) procedures in many laboratories (see Thomas and Capecchi, 1987; Zimmer and Gruss 1989; Nagy et al., 1993). Advances in these disciplines have led to a relatively sophisticated understanding of mouse ES cell definition, developmental molecular genetics and, in association, several techniques for controlling mouse ES cell fate reliably into predicted and functionally relevant phenotypes in vitro and in vivo (see Klug et al., 1996; Li et al., 1998; McDonald et al., 1999; Kim et al., 2002; Rideout et al., 2002; Wichterle et al., 2002). Failing such technological advances and due to the limitations that result from currently applied ethical regulations, the true pluripotential identity of human ES cell lines derived to date remains obscure and many researchers still refer to human ES cells as human ES-like cells (see Buehr and Smith, 2003).

The ability to manipulate mouse ES cell fate in vitro and in vivo, however, has driven the belief that ES-like cells derived from human blastocysts (human ES cells) may one day be similarly manipulated and thus used to produce sufficient tissue for replacement therapeutics aimed at treating a variety of human diseases and injuries. Technological advances supporting this notion include the development of strategies describing human ES cell differentiation into several desired lineages (for a review, see Pera and Trounson, 2004), clonal (albeit inefficient) derivation (Amit et al., 2000), cryopreservation (Reubinoff et al., 2001), knock-ins and knockouts using homologous recombination (Zwaka and Thomson, 2003), and biological interference using RNAi (Vallier et al., 2004a). With respect to reports describing differentiation into desired lineages, evidence has been restricted mostly to morphological and biochemical characterization of cells found, for example, within teratomas created form human embryonic stem cells following injection under the kidney capsule of NOD-SCID mice, or following the application of differentiation protocols designed to favor differentiation of particular cell types from an in vitro cultured cell colony (see Thomson et al., 1998; Reubinoff et al., 2000; Kaufman et al., 2001; Levenberg et al., 2002; Mummery et al., 2003; Rambhatla et al., 2003; Conley et al., 2004a). In the absence of relevant functional studies and in

agreement with arguments outlined above, absolute identification as the desired cell type is difficult to argue. Only recently has a study of human ES cell derived functionality appeared in the literature. According to this study, transplantation of cardiac-like cells derived from human ES cells to the heart of a pig model of atrioventriclular block reinstated heart rhythm in a manner compatible with survival in ~50% of the subject animals (Kehat et al., 2004).

In addition to the promise of providing tissue for therapeutic replacement strategies, ES cell culture facilitates a means to study several early developmental processes (see Doetschman et al., 1985). Reports in the literature suggest that human ES cells possess a capacity to produce representatives of both the trophoblast and the visceral endoderm in vitro when cultured in the presence of growth factors or as structures known as embryoid bodies (EBs; Xu et al., 2002; Conley et al., 2004b; Gerami-Naini et al., 2004; Vallier et al., 2004b). EB formation has additionally provided an integral first step in many in vitro differentiation protocols (see Zhang et al., 2001; Levenberg et al., 2002). It is believed that research on the differentiation of human ES cells into trophectoderm and visceral endoderm derivatives may provide important information regarding nutrient and gas exchange and patterning of the early human embryo.

Critical Parameters

All tissue culture is to be performed in Class 2 biohazard hoods and/or laminar flow hoods using sterile technique. All reagents and media must be sterilized either by autoclaving or filtering through a 0.22-μm filter. (Do not autoclave solutions containing proteins, such as media, FBS, amino acids.) Bottles used to store media should be washed thoroughly to avoid contamination by soaps or detergents, then placed for 4 hr in a 140°C sterilization oven prior to use.

Dissection of human ES cell colonies is to be performed using a dissecting microscope in a laminar flow hood. A microscope with a dark field function is useful in determining undifferentiated regions.

For human ES cell culture, the batch of FBS utilized is essential for maintenance of the undifferentiated state. FBS containing a low endotoxin level (<1.5 EU/ml) is proposed to be efficient for human ES cell culture. Batch testing is essential prior to culture with human ES cells. FBS should be aliquoted and stored at −20°C and thawed only once to make medium as repeated freeze/thaw cycles severely compromises its cell culture efficiency. Media should be discarded after 2 weeks as this can influence the differentiation of ES cells.

MEFs are an important and essential part of the human ES cell culture protocol; it is essential that the MEFs are not used beyond six passages, that they grow well, and that they look healthy. The strain of mouse used for MEF derivation can also influence the efficiency of human ES cell culture. It is advisable to utilize an inbred strain of mouse (e.g., 129sv) and to test each new batch of MEFs for their ability to support human ES cell culture. When testing MEFs, it is important to propagate human ES cells on the new MEFs for a minimum of three passages to determine their efficiency. Following the first week of culture on new MEFs, ES cell colonies may contain a proportion of differentiated cells; however, ES cells may subsequently equilibrate on the new MEFs following repeated transfer and propagate normally thereafter.

Human ES cell mechanical transfer must be delicately performed in the shortest possible time due to reduced cell viability and increased differentiation rates with increased dissection times. Additionally, for enzymatic digestion of human ES cells, it is important to stop the reaction prior to dissociation to single cells as cell viability is severely compromised otherwise.

For mouse ES cell derivation, efficiency can be dependent on the strain of mouse used.

Once derived, mouse and human ES cells are theoretically immortal and can be passaged extensively without loss of differentiation potential, although some karyotypic abnormalities may arise over time. It is therefore advisable to regularly karyotype ES cells and test them for appropriate pluripotent markers such as oct-4, nonog, stage specific embryonic antigen (SSEA) -3 and -4, Tra-1-60, Tra-1-81 and GCTM-2 for hES cells, and oct-4, nanog and SSEA-1 for mES cells. Freezing stocks of cells at regular passage numbers (e.g., passages 3, 5, 10, 15, 30, 50) is essential to prevent loss of the cell line. If proper culture conditions are maintained, the cells should not accumulate karyotypic changes; cells cultured over 150 passages in the authors' laboratory still maintain normal karyotype.

Troubleshooting

If human ES cells begin to spontaneously differentiate in culture, it is advisable to immediately transfer them regardless of the day following the last transfer. New medium should

be prepared and sterilized by filtration through a 0.22-μm filter. A new aliquot of MEFs should then be thawed and prepared for transfer of human ES cells.

Anticipated Results

MEF derivation is highly efficient and should be successful every time. Each batch of MEFs, however, may differ in their ability to support human ES cell culture and therefore must be tested for a minimum of three passages of human ES cells. Mouse ES cells are generally more tolerant of MEFs, especially when medium containing LIF is used.

Mouse ES cell derivation has an efficiency of 10% to 15% depending on the mouse strain used. Although inbred strains other than 129sv have been used, success rates for mouse ES cell derivation were significantly reduced. Mouse ES cell cultures tend to contain 5% to 10% differentiated cells, this increases upon confluency, thus passaging prior to confluence is important. When mouse ES cells are transferred to hanging drop cultures, ~90% to 95% of drops are seen to form EBs. At the meniscus of the drop, cells will aggregate and by 1 to 2 days will be seen to form spheres that continue to grow in the droplet until the desired time point. When mouse ES cells are transferred to suspension, EB formation is somewhat slower, with cells forming smaller spheres both with their progeny (clonal) and by aggregating with other cells. By 2 to 3 days, spheres should be present, and these spheres should continue to expand.

Human ES cell culture is efficient, provided that competent MEFs and sterile medium are used and care is taken when transferring them. Upon transfer of clumps of human ES cells, they attach forming colonies that expand in a regular manner. When freezing/thawing human ES cells, expect ~10% to 30% cell death. With human EB formation, ~70% to 80% of ES cell aggregates placed in suspension will form EBs, with some clumps attaching to the dish. Clumps should become spherical balls after 2 to 3 days in culture and continue to grow until ~8 to 12 days when fluid-filled cysts become obvious and EBs may begin to form attachments with the dish.

Time Considerations

MEFs

Dissection of fetuses will depend on the number of fetuses and competence of the operator. Approximately 1 to 1.5 hr should be spent dissecting and dissolving tissue into single cells. Subsequent to derivation, passaging of MEFs requires 20 to 40 min depending on number of flasks. Mitomycin C inactivation of MEFs requires ~3.5 hr (including a 2.5-hr incubation). Freezing of MEFs or mouse ES cells requires ~20 to 30 min. Thawing cells takes 20 to 30 min.

Media

Approximately 1 to 1.5 hr should be allowed for media preparation 2 weeks prior to culture.

Mouse ES cells

Mouse ES cell derivation is laborious and great care is required to ensure their subsequent growth, due primarily to the low efficiency of derivation. Maintenance of mouse ES cells is relatively time efficient when using LIF alone, with simple tissue culture passaging required; however, time requirements significantly increase when growing the cells on MEFs because of the time-consuming process of cell preparation and mitotic inactivation.

Mouse EB formation

The setup for EB formation requires 20 to 30 min depending on the number of drops needed for hanging drops; it takes 10 to 15 min to set up a suspension culture.

Human ES cells

Following MEF preparation, human ES cell mechanical dissection should take ~10 min or less per organ culture dish. Medium must be changed daily until the following transfer. With prewarmed medium, this can take 5 to 15 min depending on the number of plates. Medium must be made and MEFs prepared fresh each week for transfers or used to collect conditioned medium, this is quite a laborious undertaking and can take up to a full day.

Human EB formation

Human EB formation takes approximately the same time as required for transfers, with clumps cut smaller than those for transfers and then placed in suspension.

Literature Cited

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P., Waknitz, M.A., Itskovitz-Eldor, J., and Thomson, J.A. 2000. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* 227:271-278.

Buehr, M. and Smith, A. 2003. Genesis of embryonic stem cells. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358:1397-1402.

Conley, B.J., Young, J.C., Trounson, A.O., and Mollard, R. 2004a. Derivation, propagation and differentiation of human embryonic stem cells. *Int. J. Biochem. Cell. Biol.* 36:555-567.

Conley, B.J., Trounson, A., and Mollard, R. 2004b. Human embryonic stem cells form embryoid bodies containing visceral endoderm-like derivatives. *Fetal Diagn. Ther.* 193:218-224.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27-45.

Evans, M.J. and Kaufman, M.H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Fischbach, G.D. and Fischbach, R.L. 2004. Stem cells: Science, policy, and ethics. *J. Clin. Invest.* 114:1364-1370.

Gerami-Naini, B., Dovzhenko, O.V., Durning, M., Wegner, F.H., Thomson, J.A., and Golos, T.G. 2004. Trophoblast differentiation in embryoid bodies derived from human embryonic stem cells. *Endocrinology* 145:1517-1524.

Kaufman, D.S., Hanson, E.T., Lewis, R.L., Auerbach, R., and Thomson, J.A. 2001. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 98:10716-10721.

Kehat, I., Khimovich, L., Caspi, O., Gepstein, A., Shofti, R., Arbel, G., Huber, I., Satin, J., Itskovitz-Eldor, J., and Gepstein, L. 2004. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat. Biotechnol.* 22:1282-1289.

Kim, J.H., Auerbach, J.M., Rodriguez-Gomez, J.A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S.H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., and McKay, R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418:50-56.

Klug, M.G., Soonpaa, M.H., Koh, G.Y., and Field, L.J. 1996. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *J. Clin. Invest.* 98:216-224.

Levenberg, S., Golub, J.S., Amit, M., Itskovitz-Eldor, J., and Langer, R. 2002. Endothelial cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:4391-4396.

Li, M., Pevny, L., Lovell-Badge, R., and Smith, A. 1998. Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr. Biol.* 8:971-974.

Martin, G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

Mitalipova, M.M., Rao, R.R., Hoyer, D.M., Johnson, J.A., Meisner, L.F., Jones, K.L., Dalton, S., and Stice, S.L. 2005. Preserving the genetic integrity of human embryonic stem cells. *Nat. Biotechnol.* 23:19-20.

McDonald, J.W., Liu, X.Z., Qu, Y., Liu, S., Mickey, S.K., Turetsky, D., Gottlieb, D.I., and Choi, D.W. 1999. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat. Med.* 5:1410-1412.

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., de la Riviere, A.B., Passier, R., and Tertoolen, L. 2003. Differentiation of human embryonic stem cells to cardiomyocytes: Role of coculture with visceral endoderm-like cells. *Circulation* 107:2733-2740.

Munsie, M., Peura, T., Michalska, A., Trounson, A., and Mountford, P. 1998. Novel method for demonstrating nuclear contribution in mouse nuclear transfer. *Reprod. Fertil. Dev.* 10:633-637.

Nagy, A., Gocza, E., Diaz, E.M., Prideaux, V.R., Ivanyi, E., Markkula, M., and Rossant, J. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110:815-821.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 90:8424-8428.

Pera, M.F. and Trounson, A.O. 2004. Human embryonic stem cells: Prospects for development. *Development* 131:5515-5525.

Rambhatla, L., Chiu, C.P., Kundu, P., Peng, Y., and Carpenter, M.K. 2003. Generation of hepatocyte-like cells from human embryonic stem cells. *Cell Transplant.* 12:1-11.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. 2000. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. *Nat. Biotechnol.* 18:399-404.

Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A., and Ben-Hur, T. 2001. Neural progenitors from human embryonic stem cells. *Nat. Biotechnol.* 19:1134-1140.

Richards, M., Fong, C.Y., Chan, W.K., Wong, P.C., and Bongso, A. 2002. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat. Biotechnol.* 20:933–936.

Rideout, W.M. 3rd, Hochedlinger, K., Kyba, M., Daley, G.Q., and Jaenisch, R. 2002. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* 109:17-27.

Thomas, K.R. and Capecchi, M.R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51:503-512.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. 1995. Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U.S.A.* 92:7844-7848.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.

Vallier, L., Rugg-Gunn, P.J., Bouhon, I.A., Andersson, F.K., Sadler, A.J., and Pedersen, R.A. 2004a. Enhancing and diminishing gene function in human embryonic stem cells. *Stem Cells* 22:2-11.

Vallier, L., Reynolds, D., and Pedersen, R.A. 2004b. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev. Biol.* 275:403-421.

Wichterle, H., Lieberam, I., Porter, J.A., and Jessell, T.M. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385-397.

Xu, R.H., Chen, X., Li, D.S., Li, R., Addicks, G.C., Glennon, C., Zwaka, T.P., and Thomson, J.A. 2002. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat. Biotechnol.* 20:1261-1264.

Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., and Thomson, J.A. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* 19:1129-1133.

Zimmer, A. and Gruss, P. 1989. Production of chimaeric mice containing embryonic stem ES cells carrying a homoeobox Hox 1.1 allele mutated by homologous recombination. *Nature* 338:150-153.

Zwaka, T.P. and Thomson, J.A. 2003. Homologous recombination in human embryonic stem cells. *Nat. Biotechnol.* 21:319-321.

Key References

Joyner, A.L. ed. 1993. Gene Targeting: A Practical Approach. (D. Rickwood and B.D. Hames, eds.), IRL Press, Oxford, UK.

Protocols for gene targeting.

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. 2002. Manipulating the Mouse Embryo: A Laboratory Manual. 3rd Ed. Cold Spring Harbor Laboratory Press, New York.

Important work describing manipulation of mouse embryo at various stages, and culture conditions required.

Tymms, M.J. and Kola, I. 2001. Gene Knockout Protocols. Methods in Molecular Biology, Vol. 158. Humana Press, Totowa, NJ, USA.

Protocol for gene knockout.

Contributed by Brock J. Conley, Mark Denham, Lerna Gulluyan, Fredrik Olsson, Timothy J. Cole, and Richard Mollard
Monash University
Melbourne, Australia

Maintenance and In Vitro Differentiation of Mouse Embryonic Stem Cells to Form Blood Vessels

Embryonic stem (ES) cells are pluripotent cells that have been derived from the inner cell mass of blastocyst-stage embryos. Early studies of the ES differentiation model demonstrated that vasculogenesis and hematopoiesis were among the earliest developmental processes to occur (Doetschman et al., 1988; Schmitt et al., 1991; Wiles and Keller, 1991; Wang et al., 1992). Furthermore, the endothelial cells that differentiate during this model coalesce to form primitive blood vessels that are analogous to the first vessels that form in the developing embryo and yolk sac (Wang et al., 1992). Vascular development during mouse ES cell differentiation in vitro occurs by two processes; vasculogenesis and angiogenesis, similar to embryonic vascular development in vivo (Poole and Coffin, 1989; Risau, 1997). In vasculogenesis, mesodermally derived precursor cells, known as angioblasts, differentiate and coalesce to form a primitive blood vessel. In angiogenesis, endothelial cells from preexisting vessels coordinate cell proliferation and sprouting migration as a basis for expansion of vessels. Primitive erythrocytes and embryonic macrophages mature alongside vascular endothelial cells during ES cell differentiation (Wiles and Keller, 1991; Inamdar et al., 1997; Kearney and Bautch, 2003). Thus this model is ideal for studying molecular and cellular aspects of early vascular and hematopoietic development. This unit describes protocols for maintaining and differentiating mouse embryonic stem cells and methods of analyzing the vasculature via immunohistochemistry and β-galactosidase staining.

A protocol for the formation of embryoid bodies (EBs) and subsequent in vitro differentiation of mouse ES cells is described (see Basic Protocol). In combination with the in vitro differentiation protocol, an explanation of how to test different lots of fetal bovine serum (FBS) for maintaining differentiated cultures has been described (see Support Protocol 8). Methods for analysis of differentiation are included: testing for optimum ES cell differentiation via benzidine staining (see Support Protocol 1) and PECAM/Mac-1 double immunostaining (see Support Protocol 2), antibody immunolocalization (see Support Protocol 3), and *lacZ* staining of in vitro differentiated ES cells (see Support Protocol 4). Other protocols in this unit are designed to provide a method for maintaining mouse ES cells (see Support Protocol 5). A protocol for harvesting 5637 cell–conditioned medium, which contains the LIF that prevents differentiation, is explained (see Support Protocol 6). In addition, a protocol used for testing FBS lots from different manufacturers for ES Medium for maintenance is described (see Support Protocol 7).

NOTE: The protocols in this unit should be conducted under aseptic conditions using sterile reagents and equipment. Experiments should be performed in a Class II Biological Flow Hood.

NOTE: All incubations should be performed in a humidified 37°C, 5% CO_2 incubator, unless otherwise indicated.

PROGRAMMED IN VITRO DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

BASIC PROTOCOL

Mouse embryonic stem cells have the distinctive ability to differentiate into numerous cell types. In vitro ES cell differentiation complements their ability to contribute many tissue types in vivo, thus providing a unique model system for aspects of early mammalian

Contributed by Nicholas C. Kappas and Victoria L. Bautch
Current Protocols in Cell Biology (2007) 23.3.1-23.3.20

development. The conditions that are used initiate a programmed differentiation that leads to the formation of many of the cell types typically found in the mouse embryo and yolk sac. The following utilizes an unmanipulated differentiation protocol that involves the removal of differentiation inhibitory factors, thus allowing the ES cells to undergo a programmed differentiation to form cells that will in turn provide developmental signals to each other. In this case, cultures are examined for vasculogenesis and angiogenesis.

Materials

5- to 6-day-old ES cell culture (Support Protocol 5)
1× CMF-PBS (Sigma)
2.4 U/ml dispase, grade II stock (Boehringer-Mannheim; see recipe)
Differentiation medium (see recipe)

50-ml centrifuge tubes (Sarstedt), sterile
10-cm bacteriological petri dishes (do not use TC-treated plates)
24-well tissue culture plates (Costar)
Medidroppers (Fisher Scientific), autoclaved

NOTE: All volumes are given assuming that one 6-cm dish of ES cell colonies is being used. Double all volumes if using 10-cm dishes.

Collect ES cells

1. Choose the dish that has the best ES cell clumps for differentiation.

   ES cell clumps should be round and differentiated on the very edge and tight, shiny, and undifferentiated in the middle (Figure 23.3.1). ES cell clumps are collected from dishes after incubation for 5 to 6 days at 37°C without feeding after normal passage.

2. Aspirate medium from the ES cell dish. Wash two times, each time with 5 ml of cold 1× CMF-PBS. Aspirate PBS.

3. Add 1 ml of cold 1.2 U/ml dispase (diluted 1:1 with cold CMF-PBS just before use), and let dish sit at room temperature for 1 to 2 min. Check to see if ES cell clumps have detached from dish bottom by shaking dish.

   If the majority of cells clumps have not detached, let the solution sit longer. Dispase produces small clumps of cells that are used to start the differentiation.

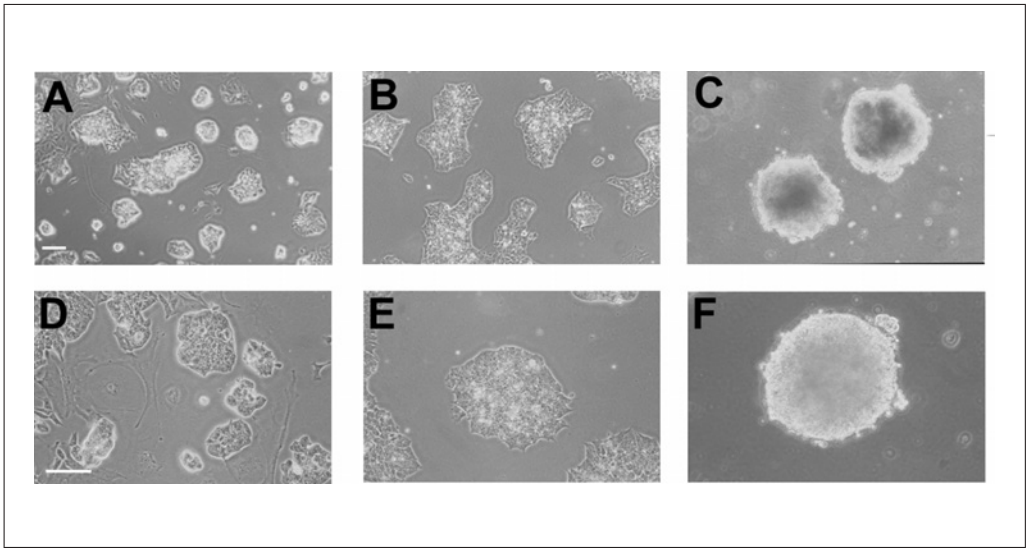


Figure 23.3.1 ES cells are typically processed for passage after 3 days of growth, when they are observed in tight, shiny clumps (**A, D**). ES cells undergo enzymatic disruption treatment for in vitro differentiation on day 5, when the ES clumps are large, but appear differentiated on the edges (**B, E**). Day 3 EBs (**C, F**) prior to plating (Bar = 100 μm).

4. When a majority of the cell clumps have detached from the dish, use a 5-ml pipet to gently transfer the cells into a 50-ml tube containing 35 ml of room temperature 1× CMF-PBS. Rinse the dish with 5 ml of 1× CMF-PBS and add the rinse to the 50-ml tube. Cap tube, and invert the tube once gently to mix.

5. Let the tube sit until the cell clumps have settled to the bottom of the tube.

Wash and plate the clumps

6. Aspirate all but 4 to 5 ml of CMF-PBS, carefully avoiding the ES cell clumps.

7. Add another 35 ml room temperature 1× CMF-PBS gently down the side of the 50-ml tube. Gently swirl the tube to redistribute the cell clumps. Cap tube, and invert gently to mix.

8. Let the tube sit until the cell clumps have settled to the bottom of the tube.

9. Aspirate the CMF-PBS (leaving 2 to 3 ml CMF-PBS/ES cell clump suspension at the bottom), and gently add 5 ml prewarmed (37°C) differentiation medium down the side of the tube.

10. Pipet 10 ml of prewarmed differentiation medium into a labeled 10-cm bacteriological petri dish. Using a 25-ml pipet, transfer the contents of the 50-ml tube (cell clumps/CMF-PBS/medium) to the 10-cm dish.

11. Check the density of cell clumps in each dish (~100 clumps). Incubate at 37°C in a humidified incubator with 5% CO_2.

    The authors attempt to achieve ~100 clumps/dish. Fewer clumps is not an efficient use of medium, while more clumps may allow for individual clumps to aggregate together.

Allow formation of embryoid bodies

12. Change the medium at least every other day after the dispase treatment (day 0). To feed the EBs, remove dish from incubator, place under hood, and gently swirl the dish in a circular manner so that the EBs migrate to the center of the dish.

13. Aspirate spent medium from the dish.

    This is easily done by aspirating along the inside edge of the dish (away from the EBs).

14. Add 10 ml of fresh prewarmed differentiation medium to the dish. Return dish to incubator.

Differentiate embryoid bodies

15. Set up reattachment cultures on day 3 after the dispase treatment (Fig. 23.3.1). Add 1.5 ml of prewarmed differentiation medium to each well of a 24-well tissue culture plate that is to be seeded with EBs.

16. Use a sterile medidropper to transfer EBs from the dish to the wells of a 24-well plate. Generally, dispense between 10 and 20 EBs per well.

    Holding the dish up and looking at it from underneath is helpful when determining the number of EBs in a well.

17. Ensure that the EBs are spread evenly in the well by gently shaking/moving the plate, or if necessary, use a pipettor with a sterile tip to gently pipet medium up and down in the well.

18. Place the plate in the incubator. Keep plate level when placed in incubator to ensure that the EBs do not settle to one side of the well. Incubate at 37°C in a humidified incubator with 5% CO_2.

    Attachment generally occurs within a few hours.

19. Feed the attached cultures every other day. To feed, aspirate medium, then slowly add 1.5 to 2 ml fresh prewarmed differentiation medium down the side wall of the well so as not to disturb the attached cultures.

20. Monitor the cultures for hematopoietic and vascular development (see Support Protocols 1 to 4).

    Using this protocol, the authors typically monitor vascular development in cultures that have been differentiated for 8 days (day of dispase treatment is day 0). Angioblast formation is generally observed at day 4 to 6, while vessel formation occurs at day 6 to 8.

    All volumes given are for staining cultures from a well of a 24-well plate. Gently add/remove all solutions from culture dishes, as harsh removal and addition of solutions may result in detachment of cultures.

SUPPORT PROTOCOL 1

BENZIDINE STAINING

Benzidine staining is performed on day-8 differentiated cultures to visualize the hemoglobin of red blood cells.

Materials

8-day differentiated ES cell cultures (Basic Protocol)
Benzidine solution (see recipe), 4°C
Differentiation medium (see recipe)
1× CMF-PBS (Sigma)

1. Remove 8-day differentiated cultures from incubator and add 0.2 ml of cold (4°C) benzidine solution per 2 ml (per well) of differentiation medium. Incubate at room temperature.

   Benzidine solution is added directly to the cultured cells while still in differentiation medium (i.e., do not rinse cells in CMF-PBS at this time).

   The benzidine reaction will occur immediately: upon addition of benzidine solution, the medium will turn a bluish/brown color, while positive cells will turn blue.

2. Observe/photograph cells within 1 hr, as the stain is not very stable.

   It is recommended that wells be rinsed once with 1× CMF-PBS before taking photographs of stained wells.

SUPPORT PROTOCOL 2

DOUBLE STAINING WITH PECAM AND Mac-1

PECAM/Mac-1 double staining is performed on day 10 differentiated cultures to visualize both blood vessels (PECAM) and embryonic macrophages (Mac-1).

Materials

Day-10 differentiated cultures (from Basic Protocol)
1× CMF-PBS (Sigma)
4% (w/v) paraformaldehyde fixative (see recipe)
1:500 trypsin solution (see recipe)
100% heat-inactivated fetal bovine serum
Staining medium (see recipe)
Primary and secondary antibodies (see recipe);
  Rat anti-mouse CD31 (PECAM-1; Mec 13.3; BD Pharmingen)
  FITC-AffinPure F(ab′)$_2$ fragment of donkey anti-rat IgG (Jackson Immunoresearch)
  Mac-1-BNHS (biotin coupled to Mac-1 antibody); clone M1/70; Rat anti-mouse Mac-1 (BD Pharmingen)
  Streptavidin-RPE (Southern Biotechnology Associates)
Rat serum staining medium (see recipe)

Treat cultures with trypsin

1. Remove day-10 differentiated cultures from incubator and bring plate to room temperature. Remove medium, wash cells twice with 1× CMF-PBS at room temperature, and fix cells in 4% paraformaldehyde (PFA). Use 1 ml of cold 4% PFA/well for 5 to 10 min at room temperature.
2. Aspirate PFA and add 2 ml room temperature 1× CMF-PBS/well. Allow dish to sit for 2 min before aspirating CMF-PBS. Repeat wash 1 more time. Either store at 4°C in CMF-PBS until ready to stain, or proceed with the staining protocol.
3. Add 0.2 ml room temperature 1:500 trypsin solution/well of a 24-well plate. Let 1:500 trypsin solution sit for no more than 60 to 75 sec at room temperature.

   Incubation times longer than 60 to 75 sec may result in complete detachment of differentiated cultures. This step slightly loosens cells and enhances Mac-1 staining.
4. Stop the trypsin reaction by adding 0.5 ml of room temperature 100% heat-inactivated FBS to the well. Immediately remove the solution from the well with gentle aspiration or pipetting.
5. Proceed with immunostaining or store in staining medium at 4°C.

Stain with PECAM

6. Gently add 1.5 to 2 ml (per well of a 24-well plate) of staining medium. Incubate for 1 hr at 37°C to block nonspecific sites.
7. Remove plate from incubator, aspirate staining medium, and add fresh staining medium containing Mec13.3 (PECAM antibody) at 1:1000 dilution. Incubate for 1 hr at 37°C.

   PECAM is expressed by mouse endothelial cells.

   Typically, 250 μl of staining medium/antibody is used per well of a 24-well plate.
8. Remove plate from incubator and aspirate PECAM antibody. Wash wells twice, each time with 2 ml room temperature staining medium.
9. Add fresh staining medium containing FITC-AffinPure F(ab')$_2$ donkey anti-rat antibody at 1:100 dilution. Incubate for 1 hr at 37°C in the dark.

   Typically, 250 μl of staining medium/secondary antibody is used per well of a 24-well plate.

Stain with Mac-1

10. Remove plate from incubator and aspirate FITC reagent. Wash wells twice, each time with 2 ml staining medium at room temperature.

    If desired, the plate can be stored at 4°C, in the dark, for later processing. Be sure to leave staining medium in wells.
11. Add 2 ml/well of rat serum staining medium. Incubate for 30 min at 37°C in the dark.

    If desired, the plate can be stored at 4°C, in the dark, for later processing. Be sure to leave rat serum staining medium in wells.
12. Remove rat serum staining medium. Add fresh rat serum staining medium containing Mac-1-BNHS at 1:800. Incubate for 1 hr at 37°C, in the dark.

    Mac-1 is expressed by embryonic macrophages.

    Typically, 250 μl of rat serum staining medium/ antibody is used per well of a 24-well plate.

13. Remove Mac-1-BNHS and wash well(s) twice with 2 ml staining medium at room temperature. Add fresh staining medium containing streptavidin-R-PE at 1:800. Incubate for 1 hr at 37°C in the dark.

    Typically, 250 μl of staining medium/secondary antibody is used per well of a 24-well plate.

14. Remove streptavidin-RPE and wash well(s) once with 2 ml staining medium at room temperature, then aspirate. Next, wash well(s) twice with 2 ml 1× CMF-PBS. Add 2 ml CMF-PBS and store plate at 4°C in the dark.

15. Examine the plate using fluorescence microscopy with the appropriate filter sets.

SUPPORT PROTOCOL 3

IMMUNOLOCALIZATION

An important aspect of a model system is that it recapitulates the expression of molecular markers seen in vivo. Much of the vascular development that is observed in this in vitro ES differentiation system is analogous to blood island and vascular development that is observed in the mouse embryo and yolk sac. Fortunately, it is with general ease that one can observe primitive ES cell–derived mouse blood vessels using several markers and protocols. There are a number of identified molecular markers for the early mouse vasculature. Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD-31), VEGF (vascular endothelial growth factor) receptor 2 (Flk-1), VE-cadherin, and intercellular adhesion molecule 2 (ICAM-2) have proven particularly useful in visualizing primitive vessels. It has been shown that PECAM-1 and Flk-1 are expressed on mouse angioblasts and endothelial cells (Vittet et al., 1996; Redick and Bautch, 1999). Other vascular markers such as ICAM-2 and VE-cadherin are detectable later, when endothelial cells coalesce to form vessels. This is a protocol for visualizing expressed genes in the in vitro ES cell differentiation model system that utilizes antibodies specific for several vascular markers.

Materials

Differentiated ES cell culture (from Basic Protocol)
1× CMF-PBS (Sigma)
Methanol/actetone fixative (see recipe): 1:1 (v/v) methanol (Fisher Scientific)/acetone (Mallinckrodt) *or* 4% (w/v) paraformaldehyde (PFA; Polysciences; see recipe)
Staining medium (see recipe)
Primary and secondary antibodies (see recipe)
Fluorescent microscope, inverted (equipped with epifluorescence and camera)

NOTE: All volumes given are for staining cultures from a 24-well plate. Gently add and remove all solutions from culture plates, as harsh removal and addition of solutions may result in detachment of cultures.

Fix the cells

1. Remove differentiated cultures from incubator, and allow to slightly cool. Aspirate medium and wash twice with 2 ml/well of 1× CMF-PBS at room temperature.

2. Add 1 ml of cold methanol/acetone fixative/well and incubate for 5 min at room temperature.

    PECAM-1 and ICAM-2 antibodies work in both PFA and methanol/acetone fixatives. Both fixatives are listed here because some applications (not those described here) favor one fixative over another. The authors generally use methanol/acetone fixative because it is less harsh on the attached cultures.

3. Aspirate fixative and add 2 ml of room temperature 1× CMF-PBS/well. Allow plate to sit for 2 min before aspirating CMF-PBS. Repeat wash two more times. Either store at 4°C in CMF-PBS until ready to stain, or proceed with the staining protocol.

Stain the cells

4. If the plate has been stored at 4°C after fixation, allow the plate to come to room temperature.
5. Aspirate 1× CMF-PBS, and gently add 1.5 to 2 ml of staining medium. Incubate for 45 min to 1 hr at 37°C.
6. Remove plate from incubator, aspirate staining medium, and add fresh staining medium containing the properly diluted primary antibody. Incubate for 1 to 2 hr at 37°C.

   Typically, 250 μl of staining medium/primary antibody is used per well of a 24-well plate.

7. Remove plate from incubator, aspirate, and wash well(s) two times (2 to 3 min/wash) with 1.5 to 2 ml staining medium. Add fresh staining medium containing the properly diluted secondary antibody. Incubate for 1 hr at 37°C.

   Typically, 250 μl of staining medium/secondary antibody is used per well of a 24-well plate.

8. Remove plate from incubator, aspirate, and wash well(s) one time for 2 to 3 min with 1.5 to 2 ml staining medium. Aspirate wash, then wash one to two times (2 min/wash) using 1× CMF-PBS.
9. Aspirate last wash and add 1.5 to 2 ml of 1× CMF-PBS to each well, store in CMF-PBS at 4°C in the dark.
10. To visualize the vasculature, set up an inverted microscope equipped with epifluorescence and a camera.

β-GALACTOSIDASE STAINING

SUPPORT PROTOCOL 4

Another commonly used technique to visualize blood vessels in the in vitro ES cell differentiation model system relies on the β-galactosidase reporter system. The authors have mouse ES cells in which expression of the *lacZ* gene is under the control of a vascular promoter (Flk-1, Flt-1). The β-galactosidase protein is the gene product of *lacZ* and is easily assayed. β-galactosidase catalyzes the hydrolysis of the colorless substrate Xgal, to form a blue precipitate in β-galactosidase-expressing cells. This protocol describes the steps involved in β-galactosidase staining of ES cell–derived blood vessels.

Materials

Day-8 differentiated cultures in 24-well plate (Basic Protocol)
0.1 M phosphate buffer, pH 7.3 to 7.5 (see recipe)
Glutaraldehyde fixative solution (see recipe)
Xgal stain (see recipe)
Wash buffer (see recipe)

NOTE: All volumes given are for staining cultures from a 24-well plate. Gently add and remove all solutions from culture dishes, as harsh removal and addition of solutions may result in detachment of cultures.

1. Remove differentiated cultures from incubator, and allow to slightly cool. Aspirate medium and wash well(s) once with 1.5- to 2 ml 0.1 M phosphate buffer (pH 7.3 to 7.5) at room temperature.

2. Aspirate phosphate buffer and add 1 ml glutaraldehyde fixative solution to each well and fix for 5 min at room temperature.
3. Aspirate fixative. Wash cells three times (5 min/wash), each time with 1.5 to 2 ml 0.1 M phosphate buffer at room temperature.
4. Aspirate phosphate buffer. Add 200 µl Xgal stain to each well. Stain cells for 4 hr to overnight at 37°C depending on level of *lacZ* activity.
5. After staining, remove Xgal stain, add 1 ml wash buffer to each well, aspirate, then replace with 1 ml wash buffer to each well.
6. Examine plates for blue-staining structures indicating blood vessels (see Fig. 23.3.2C).

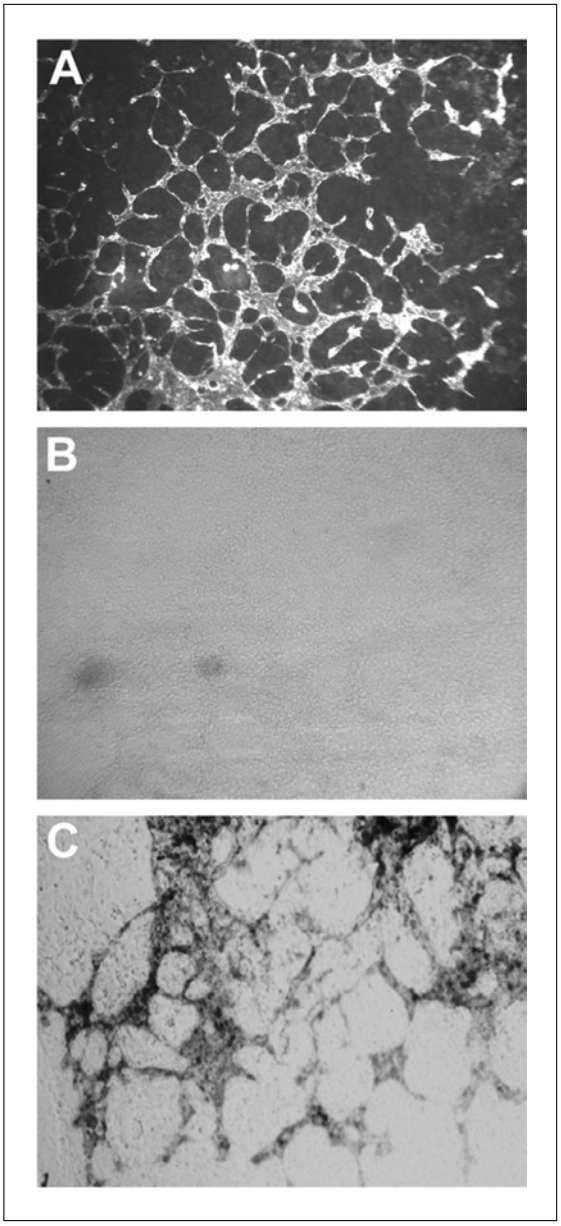


Figure 23.3.2 The vasculature of differentiated ES cell attached cultures can be visualized by both immunolocalization (PECAM stain) and enzymatic reaction (β-galactosidase stain). (**A, B**) ES cell culture differentiated to day 8, fixed and stained using the PECAM antibody, depicting a typical vascular pattern by immunofluorescence (**A**), and the corresponding phase image (**B**). (**C**) ES cell culture differentiated to day 8, fixed and stained for β-galactosidase, showing a vascular pattern (**C**) in phase.

CULTURING MOUSE EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER CELLS

There are numerous published protocols describing mouse embryonic stem cell (ES) maintenance (Bautch, 2002; Kearney and Bautch, 2003). Here the authors describe the maintenance of mouse ES cells for in vitro differentiation. Leukemia Inhibitory Factor (LIF) is a leading component that contributes to stem cell maintenance and prevention of cell differentiation. Traditionally, ES cells are maintained on a layer of feeder cells (usually mouse embryo fibroblasts or STO cells), which provide LIF to the surrounding ES cells. Here the authors describe the maintenance of ES cells using medium that has been conditioned by the 5637 human bladder cancer cell line. This cell line produces LIF that is subsequently used for ES cell maintenance. The authors prefer the 5637 cell–conditioned medium to commercial LIF because they feel that it best preserves the undifferentiated morphology of the ES cells grown off of feeder layers.

Materials

3- to 4-day-old ES cell dishes (*UNIT 23.2*), without feeder layer, passage 3 to 45
1× CMF-PBS (Sigma)
0.25× trypsin/EDTA (see recipe)
Trypsin stop solution (see recipe)
ES cell culture medium (see recipe)

Gelatin-coated dishes (see recipe)
6-cm tissue culture (TC)–treated culture dishes (Corning)

NOTE: All volumes are given assuming that one 6-cm dish of ES cell colonies is being used. Double all volumes if using 10-cm dishes. Prewarm all solutions to 37°C prior to use.

1. Remove 3- to 4-day-old ES cell dish(es) from 37°C incubator. Aspirate medium. Wash two times, each time with 5 ml 1× CMF-PBS at room temperature and then remove.

   ES cells must be passed frequently to prevent differentiation. The authors typically pass ES cells every 3 to 4 days. On the day of passage, ES colonies are typically found in tight, shiny clumps (Fig. 23.3.1). If the colonies are large and flat, the optimal time for passage has passed.

2. Add 1 ml 0.25× trypsin/EDTA solution to dish. Place the dish in 37°C incubator until a majority of ES cell clumps disassociate upon gentle agitation (1 to 3 min).

   This 1-ml volume should just cover the bottom of the dish.

3. Stop trypsinization reaction by adding 4 ml trypsin stop solution to dish. Gently draw the ES cells/trypsin stop solution up and down with a pipet a few times to break up the cell clumps.

4. Remove a gelatin-coated 6-cm dish from incubator. Aspirate gelatin solution, and add 5 ml pre-warmed ES cell culture medium. Add 2 to 3 drops of the cell suspension into the dish. Observe the sizes of ES cell clumps under a microscope.

   ES cells should be in a single-cell suspension, or in clumps of no more than 4 to 6 cells/clump. If cell clumps are significantly larger, pipet solution to further break apart cell clumps.

5. Place dish in 37°C incubator with 5% CO_2 and gently move dish in a back-and-forth motion in order to evenly disperse ES cells throughout the dish.

SUPPORT PROTOCOL 6

PREPARATION OF 5637 CELL–CONDITIONED MEDIUM (CM)

The conditioned medium of confluent 5637 cells contains LIF (leukemia inhibitory factor/differentiation inhibiting activity), which is used as a medium component for the maintenance of ES cell cultures.

Materials

5637 human bladder carcinoma cell line (ATCC #HTB9)
5637 cell growth medium (recipe)
Collection medium (see recipe)

6- and 15-cm tissue culture (TC)–treated culture dishes (Corning)
15- and 50-ml centrifuge tubes (Sarstedt)
200-ml centrifuge tubes, if desired (NUNC)
1-, 3-, 10, and 25-ml disposable pipets
0.22-μm cellulose acetate filter units with bottle (Corning) *or* Nalgene SFCA 0.2-μm bottle top filter with 33-mm neck to fit standard 500-ml glass bottles
500-ml bottles, autoclaved

NOTE: The following protocol describes the collection of 5637 medium from one 15-cm plate, however, more plates are typically processed for collection. Adjust volumes as needed if more plates are to be processed for 5637 collection.

1. Thaw and grow 5637 cells in 5637 cell growth medium to 80% to 90% confluence (usually 2 to 3 days after thaw) in 15-cm tissue-culture dishes. Grow cells in 37°C incubator.

   The authors typically use 25- to 30 ml 5637 cell growth medium/15-cm dish.

2. At cell confluence (2 to 3 days), remove dishes from incubator, aspirate 5637 cell growth medium and feed cells using collection medium. Use 25 to 30 ml collection medium/15-cm dish.

3. After 48 hr, remove the dishes from the incubator and carefully transfer the 5637–conditioned medium from the 15-cm dishes of cells to 50-ml or 200-ml centrifuge tubes using a 25-ml pipet. Avoid scraping the monolayer of cells. Add 25 to 30 ml of fresh collection medium and return plate to incubator.

   The addition of fresh medium to the dish allows for subsequent collection of additional 5637–conditioned medium.

4. Balance the volumes in the 50-ml tubes and centrifuge 10 min at 770 × *g*, 4°C.

5. In the hood, remove the supernatant from each tube and sterile filter through a 0.22-μm cellulose acetate filter unit.

   The supernatant will take a long while to filter.

6. After filtration is complete, place 3 ml of the filtered 5637–conditioned medium in a 6-cm dish into the incubator and check for contamination after 24 hr.

7. Collect conditioned medium every 2 to 3 days for 5 or 6 collections (30 ml/collection/plate). Combine/pool the filtered collected media. Store 5637 cell–conditioned medium up to 1 month at 4°C. Freeze medium at −20°C for use at a later time (up to 6 months). Use the conditioned medium in ES cell culture medium.

   Collect conditioned medium until the cells look sub-optimal (usually after 5th collection). Suboptimal cells will typically detach from the dish or have a disrupted cell monolayer.

ES cell culture medium containing individual pooled collections of conditioned medium is subsequently used for maintenance of ES cell cultures. It is important for 5637 cell– conditioned medium to maintain ES cells in an undifferentiated state. Overly differentiated ES cells, as evidenced by large, flat cells in colonies and no shiny center, are an indication of sub-optimal 5637 cell–conditioned medium.

LOT TESTING OF FBS FOR PASSAGE OF ES CELLS

SUPPORT PROTOCOL 7

A number of different lots of FBS (in parallel with the current lot) are tested in ES medium to ensure the best maintenance and growth of undifferentiated ES cells. To test different lots of FBS, ES cells are maintained in ES medium containing one lot of FBS (one lot of FBS/batch ES cell culture medium), for three passages. During these three passages, ES cells are analyzed for their proper morphology (shiny, tight clumps of cells) and growth. After this point, ES cells are differentiated to day 8, then analyzed for a healthy and normal vasculature pattern via PECAM antibody staining. In this way, ES cells maintained in medium containing different FBS lots during cell passage can be compared. Typically, three to four lots of FBS are tested independently in batches of ES cell culture medium.

Materials

ES cell culture medium (see recipe), prepared with different lots of FBS
3- to 4-day-old ES cell culture dish (Support Protocol 5)
6-cm tissue culture–treated culture dishes (Corning)

Additional reagents and equipment on in vitro differentiation of ES cells (Basic Protocol), passaging of ES cells (Support Protocol 6), and fixing and staining differentiated cultures (Support Protocol 3)

1. Prepare different batches of ES cell culture medium, with each batch containing a different lot of FBS including the currently used lot. To test FBS lots, use the same ES clone to test all lots.
2. Remove 3- to 4-day-old ES cell culture dish from 37°C incubator. Passage ES cells (Support Protocol 6) setting up one to two 6-cm plates of ES cells per lot of FBS to be tested.
3. Passage ES cells for three passages using the same ES cell culture medium and FBS lot to be tested to maintain the same group of ES cells for the duration of the three passages.

   One parameter used to assess whether an FBS lot is of good quality is if ES cells maintain an undifferentiated morphology of tight, shiny clumps for the duration of the three passages.

4. After completion of the third passage, in vitro differentiate the groups of ES cells (Basic Protocol).
5. On day 8 of in vitro differentiation, remove cells from incubator. Fix and stain differentiated cultures with anti-mouse PECAM antibody (Support Protocol 3).

   By maintaining/differentiating ES cells and assessing the vasculature (via PECAM antibody staining) of ES cells maintained in different lots of FBS, it is possible to evaluate the ability of each lot of FBS to properly maintain ES cells to form blood vessels.

SUPPORT PROTOCOL 8

LOT TESTING OF FETAL BOVINE SERUM FOR IN VITRO DIFFERENTIATION OF MOUSE ES CELLS

A number of different lots of FBS are tested in differentiation medium to ensure the best in vitro differentiation of ES cells into blood vessels. To test different lots of FBS, differentiated ES cells are maintained in differentiation medium containing a unique test lot of FBS (one lot of FBS/batch of differentiation medium), until day 8 of differentiation. Day 8 differentiated cultures are then fixed and stained for vasculature (PECAM stain; Support Protocol 2), red blood cell differentiation (benzidine stain; Support Protocol 1), and embryonic macrophages (Mac1 stain; Support Protocol 2). This way, differentiated cells maintained in medium containing different FBS lots can be compared by the expression of markers that are observed in differentiated ES cultures.

Typically, three to four lots of FBS, including the lot currently in use, are tested independently in batches of differentiation medium.

Materials

Differentiation medium (see recipe), prepared with different lots of FBS
5-day-old to 6-day-old ES cell culture (Support Protocol 5)

Additional reagents and equipment for in vitro differentiation of ES cells (Basic Protocol)

1. Make up different batches of differentiation medium, with each batch containing a different lot of FBS. To test FBS lots, use the same differentiated clone for all test media.
2. Remove 5-day-old ES cell dishes from incubator. Ensure that there is one 6-cm dish per batch of differentiation medium (containing a unique FBS lot) to be processed for in vitro differentiation.
3. In vitro differentiate ES cells (see Basic Protocol).
4. For each group of differentiated ES cells (corresponding to each FBS lot to be tested), set up attached cultures as described in Basic Protocol (steps 15 to 20). Attach each group of differentiated cells in four wells in each of two 24-well plates.

   The four wells of one 24-well plate will be used for benzidine staining, while the four wells of the other 24-well plate will be used for double immunostaining with PECAM and Mac-1.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antibodies, primary and secondary (and streptavidin-R-PE)

Dilute all antibodies in staining medium (see recipe).

Primary antibodies: Dilute rat anti-mouse PECAM-1 (CD31; Pharmingen) to 1:1000, rat anti-mouse ICAM-2 (3C4; Pharmingen) to 1:500, rat anti-mouse Mac-1-BNHS (Pharmingen, no. 01711D) to 1:800. Prepare just prior to use.

Secondary antibodies: Dilute reconstituted secondary antibody goat anti-rat Alexa Fluor 488 (Molecular Probes) to 1:250 (for detecting PECAM-1 and ICAM-2), FITC-AffinPure F(ab')$_2$ donkey anti-rat to 1:100 (Jackson Immunoresearch, no. 712-096.150), and streptavidin-R-PE (Southern Biotechnology, no. 7100-09) to 1:800. Store at 4°C after reconstitution (according to the manufacturer) for up to 6 months.

Do not freeze reconstituted antibodies.

Benzidine solution

Stock solution: Prepare a benzidine base stock solution that is 3% (w/v) benzidine base (Sigma) in 90% (v/v) acetic acid (Fisher Scientific)/10% water. Store up to 6 months at 4°C in the dark.

Working solution: To make benzidine solution, add 1 part benzidine base stock, 1 part 30% hydrogen peroxide, and 5 parts water. Prepare fresh for each use.

Cell growth medium, 5637

Heat inactivate FBS (lot tested for ES cell maintenance) at 55°C for 1 hr. Store up to 6 months at −20°C for future use.

Prepare growth medium:
DMEM-H containing:
10% (v/v) heat-inactivated FBS
50 μg/ml gentamicin (final concentration)
1× (75 μM) monothioglycerol (MTG; Sigma)
Freeze up to 6 months at −20°C. Avoid freeze thaws.
Once thawed, store at 4°C and use within 1 month.

Collection medium

Dulbecco's modified Eagle medium (DMEM-H; Invitrogen)
5% (v/v) heat-inactivated FBS
50 μg/ml gentamicin (final concentration)
1× (75 μM) monothioglycerol (MTG; Sigma)
Freeze up to 3 months at −20°C. Avoid freeze thaws.
Once thawed, store at 4°C and use within 1 month

Differentiation medium

Add gentamicin (Invitrogen) to a final concentration of 50 μg/ml per 500-ml bottle of DMEM-H (Invitrogen). Add lot-selected FBS (Support Protocol 8) to 20% (v/v) and MTG (Sigma) to 2× (150 μM final concentration). Store at 4°C. Use within 2 weeks.

Dispase

Dilute dispase, grade II stock (2.4 U/ml) 1:1 in 1× CMF-PBS (1.2 U/ml final). Use cold. Store at 4°C for up to 1 month.

ES cell culture medium

Stock solutions

DMEM-H/gentamicin: To a 500-ml bottle of DMEM-H add 0.5 ml of 50 mg/ml gentamicin (50 μg/ml final) and invert to mix.

100× MTG: Prepare a 7.5 mM solution of MTG in CMF-PBS. Store at 4°C.

FBS: Heat inactivate 100% lot-tested (Support Protocol 7) FBS at 55°C for 1 hr.

5637 cell–conditioned medium (Support Protocol 6)

Working solution:
100 ml 5637–conditioned medium (66% v/v final)
26 ml heat-inactivated, lot-tested FBS (17% v/v final)
1.5 ml 100× MTG [1× (75μm) final]
24 ml DMEM-H/gentamicin (16% v/v final)
Store at 4°C. Use within 2 weeks.

Gelatin-coated dishes

Add 2 ml of 0.1% (w/v) gelatin (Type A gelatin, porcine, Bloom Factor 200; Difco) in CMF-PBS (Sigma) per 6-cm dish or 4-ml per 10-cm dish. Ensure that bottom of dish is gelatin-coated by tilting dish back and forth. Incubate dish for at least 1 hr at 37°C.

Dishes can be prepared up to 1 week in advance, however, to ensure that there is no bacterial or fungal contamination, check the dish under a microscope.

Glutaraldehyde fixative solution

For 50 ml:
0.4 ml 25% (v/v) glutaraldehyde (Polysciences)
2.5 ml 100 mM EGTA, pH 7.3 (Sigma)
0.1 ml 1 M magnesium chloride (Mallinckrodt)
47 ml 0.1 M phosphate buffer (see recipe)
Prepare fresh for each use

Methanol/acetone fixative

Prepare a 1:1 (v/v) mixture of methanol/acetone fresh on the day of use.

Methanol and acetone can be stored separately at −20°C.

Monothioglycerol (MTG)

Prepare 100× MTG stock by adding 32.5 µl of monothioglycerol stock (Sigma no. M6145) to 50 ml CMF-PBS (7.5 mM final). Store at 4°C and use within 2 weeks.

Paraformaldehyde (PFA) fixative, 4% (w/v)

Add 2 g PFA (Polysciences) powder to 50 ml 1× CMF-PBS in a 50-ml conical centrifuge tube. Heat solution to 60°C, shaking occasionally until PFA is in solution. Cool to room temperature, then filter through a 0.45-µm filter fitted to a syringe. Divide into 3-ml aliquots and freeze up to 6 months at −20°C. Alternatively, store at 4°C in dark and use within 2 weeks.

Phosphate buffer, 0.1 M (pH 7.3 to 7.5)

Prepare 500 ml 0.1 M phosphate buffer solution by combining 115 ml 0.1 M sodium phosphate monobasic (Mallinckrodt) and 385 ml 0.1 M sodium phosphate dibasic (Mallinckrodt). The mixture should give a pH between 7.3 and 7.5. Store at room temperature and use until solution becomes cloudy.

Rat serum staining medium

5% (v/v) rat serum
3% (v/v) FBS, heat inactivated 1 hr at 55°C
0.1% (w/v) NaN_3 (Fisher Scientific)
1× CMF-PBS
Store up to 6 months at 4°C

Staining medium

3% (v/v) FBS, heat inactivated 1 hr at 55°C
0.1% (w/v) NaN_3 (Fisher Scientific)
1× CMF-PBS
Store up to 6 months at 4°C

Trypsin/EDTA, 0.25×

Add 1 ml 1× trypsin/EDTA stock solution (0.2% (w/v) trypsin/0.53 mM EDTA; Invitrogen) to 3 ml CMF-PBS. Store at 4°C. Use within 2 weeks.

Trypsin solution, 1:500

Dilute 2.5% (w/v) trypsin (no EDTA; Invitrogen) 1:500 using 1× CMF-PBS. Prepare fresh each time.

Trypsin stop solution

35% (v/v) FBS, heat inactivated 1 hr at 55°C
1× CMF-PBS
Store up to 1 month at 4°C

Wash buffer

For 200 ml:
0.4 ml 1 M magnesium chloride (Mallinckrodt)
2 ml 2% (v/v) Nonidet P-40 (Sigma)
197.6 ml 0.1 M phosphate buffer (see recipe)
Filter sterilize
Store at room temperature until solution becomes cloudy

Xgal stain

For 50 ml:
2 ml of 25 mg/ml Xgal stock (Bethesda Research Labs) in dimethyl formamide (Fisher Scientific)
0.106 g potassium ferrocyanide (Sigma)
0.082 g potassium ferricyanide (Sigma)
48 ml wash buffer (see recipe)
Store at 37°C for 2 weeks or until cloudy

COMMENTARY

Background Information

Embryonic stem (ES) cells are pluripotent cells that have been derived from the inner cell mass of developing blastocysts (Evans and Kaufman, 1981). ES cells maintained on embryonic fibroblasts in culture can repopulate all different cell lineages, including the germ line, after being injected into host blastocysts (Bradley et al., 1984). Furthermore, in vitro studies over the past twenty years have shown that ES cells can differentiate into a variety of cell lineages under appropriate conditions in culture (Keller, 2005). Many of the cell types that differentiate under theses conditions are those that are found in the developing embryo and yolk sac, such as hematopoietic cells, endoderm, and endothelial cells (Wiles and Keller, 1991; Schmitt et al., 1991; Wang et al., 1992; Keller et al., 1993). The vascular endothelial cells that arise during ES cell differentiation have the potential to form primitive blood vessels, comparable to the vessels that first form in the developing embryo and yolk sac.

An extensive discussion of nonmouse ES cell differentiation into endothelial cells and vascular structures is beyond the scope of this unit, but several excellent reviews have recently highlighted similarities and differences between mouse and human or nonhuman primate ES cells (reviews: Smith, 2001; Pera and Trounson, 2004; Hematti et al., 2005). Although human ES cells (hES) differ from mouse ES cells in requirements to maintain their undifferentiated status, hES can be induced to differentiate and form embryoid bodies similar to their mouse counterparts. Several reports have characterized endothelial progenitors, endothelial cells, and vascular structures derived from hES cells, although the development of hematopoietic cells and vascular structures takes longer than with mouse ES cell differentiation (Levenberg et al., 2002; Gerecht-Nir et al., 2003; Wang et al., 2004).

These recent findings suggest that the basic cell-cell interactions and differentiation programs are shared among the different species, but that the protocols must be modified when changing the species source of ES cells.

Mouse ES cells were initially cultured on embryonic fibroblast feeder cells (Evans and Kaufman, 1981; Martin, 1981), or with leukemia inhibitory factor (LIF) once it was identified as a factor to prevent differentiation (Smith et al., 1988; Williams et al., 1988). It was subsequently shown that LIF activates the transcription factor STAT3, and this activation is critical to ES cells (Matsuda et al., 1999). Sources of LIF include: recombinant LIF that is commercially available, transient transfection of COS cells with LIF-expressing plasmids, and harvesting medium conditioned by the 5637 human bladder cancer cell line (Kearney and Bautch, 2003). The authors prefer the 5637–conditioned medium because they feel it best preserves the undifferentiated ES cell morphology, perhaps because other factors in the medium co-operate with LIF to efficiently maintain undifferentiated ES cells. There are several methods used for initiating ES cell differentiation. ES cells are removed from medium containing LIF and cultured in differentiation medium in suspension where they aggregate to form a colony of partially differentiated cells known as an embryoid body (EB; Doetschman et al., 1985; Wang et al., 1992; review: Keller, 1995). Alternatively, ES cells are cultured directly onto stromal cells to provide an environment conducive to hematopoietic differentiation (Nakano et al., 1994). A commonly used stromal cell line for such differentiation is the OP9 cell line, isolated from CSF-1 deficient op/op mice (Yoshida et al., 1990). A third method utilizes the culture of ES cells in a monolayer on extracellular matrix proteins (Nishikawa et al., 1998). Also, EBs can be disaggregated into single ES cells and plated in methyl cellulose, and subsequent analysis of the colonies that form shows that EBs contain many of the hematopoietic progenitors found in bone marrow (Schmitt et al., 1991). More recent studies have shown that single cells disassociated from EBs and plated in methyl cellulose generate blast-cell colonies that indicate the existence of a common progenitor for hematopoietic and endothelial cells (Kennedy et al., 1997; Choi et al., 1998).

While this protocol discusses the formation of EBs by enzymatic disruption of ES cell clumps, an alternative method of creating EBs is by culturing ES cells in hanging drops for several days (Wobus et al., 1991; *UNIT 23.2*). Whether forming EBs by enzymatic disruption or by hanging drops, differentiation of the EBs can be performed in suspension rather than as attached cultures, as described here. EBs, which contain an inner and outer layer, can be expanded to form a large lumen and are called cystic embryoid bodies (CEBs; Wang et al., 1992). CEBs have hemoglobinized areas that are indicative of hematopoietic development, primitive blood vessels, and areas of beating that indicate differentiation of cardiac muscle (Doetschman, et al., 1985; Risau et al., 1988; Wang et al., 1992).

All methods of ES cell differentiation have both advantages and disadvantages. The formation of EBs (either enzymatic disruption or hanging drop) has the advantage of providing for a cellular structure that enhances cell-cell interactions. Cells within an EB provide critical developmental cues involved in differentiation of other cell types (review: Keller, 2005). However, EBs have the potential to generate unknown factors that can complicate analysis of developmental programs. The advantage of coculturing ES cells with stromal cells is that stromal cells can provide a beneficial growth environment for hematopoietic development. However, undefined factors produced by stromal cells may influence the differentiation of ES cells to one cell type over another (review: Keller, 2005). Separation of differentiated ES cells from the stromal cell layer has also been found to be a disadvantage of this method. The formation of EBs is sometimes preferred over the formation of CEBs, because reattachment of EBs lends itself to enhanced experimental manipulations such as in situ localization and image analysis (Kearney and Bautch, 2003).

The protocol of ES cell differentiation from EBs that is described here provides a tool for further understanding the process of vascular differentiation (vasculogenesis) and vascular expansion (angiogenesis) in an in vitro setting that is analogous to the in vivo setting. A number of markers for the early vasculature have been identified and are particularly useful, and protocols for immunolocalization and β-galactosidase staining described here are beneficial tools for analyzing vascular marker expression.

Critical Parameters

All tissue culture should be performed under sterile conditions in a class II biological flow hood. All reagents and media should be sterile. Pipet tips and medidroppers should be

sterilized by autoclaving. All bottles should be thoroughly washed and autoclaved before use. All incubations should be performed in a humidified 37°C, 5% CO_2 incubator, unless otherwise indicated.

ES cells should be passed on day 3 or 4 after the last passage. As the source of LIF is depleted over time, ES cells will become more differentiated, thus passage of ES cells on day 3 is preferred over day 4. Enzymatic dissociation of ES cells using trypsin should be carried out for no longer than 2 to 3 min as described. Longer incubations have the potential to cleave cell surface proteins important for cell viability. Once the trypsin reaction is stopped with trypsin stop solution, it is important to gently draw cells up and down with a pipet for further ES cell dissociation, as harsh handling of the ES cells at this step can result in decreased cell viability. The authors typically culture ES cells for 50 passages, after which, maintenance of cell viability declines and undifferentiated ES cells are not preserved.

For ES cell culture, 5637 cell–conditioned medium is a critical component for ensuring that ES cells are maintained in their undifferentiated state. Testing of pooled collections of 5637 cell–conditioned medium on ES cells for proper ES cell maintenance is essential. The lot of FBS that is utilized for proper maintenance of mouse ES cells is also a critical factor in ES cell maintenance. Thus, lot testing of FBS as described in Support Protocols 7 and 8 is essential. FBS and 5637 cell–conditioned medium aliquots should be stored at −20°C and repeated freeze-thaws should be avoided.

When choosing 5- to 6-day-old ES cells for in vitro differentiation, it is important to choose the dish containing ES clumps that are undifferentiated in the middle (tight and shiny appearance), but differentiated along the edges (Figure 23.3.1). This morphology indicates a partial differentiation that promotes further differentiation. After treating the ES cells with dispase to generate small clumps of cells, it is imperative to then transfer the cell clumps to bacteriological dishes to prevent them from sticking to the dish bottom. This will ensure that the cell clumps will form EBs in suspension culture. Some cell clumps will invariably attach to the dish, but are discarded upon EB transfer to attachment dishes. The authors have found that timely feeding of attached cultures with fresh differentiation medium is important for good differentiation. The pH of medium can be monitored with phenol red, and if the medium on the cultures is light orange or yellow after 24 hr, it should be changed every day. Daily feedings are sometimes necessary for densely seeded wells or for later days of a time course (past day 8) when there are a large number of cells in each well. The lot of FBS that is utilized for proper maintenance and differentiation of ES cells is also a critical parameter and it should be emphasized that often different lots are optimal for maintenance versus differentiation. Thus, lot testing of FBS as described in Support Protocol 8 is essential. When performing immunolocalization experiments, it is important to set up control conditions for the experiment. Set up attached cultures that do not receive primary antibody (staining medium alone), but do receive secondary antibody. This step should help determine if the secondary antibody exhibits nonspecific binding.

Troubleshooting

Maintaining undifferentiated ES cells during ES cell passage is a commonly encountered problem. There are a few potential causes for overly differentiated ES cells: (1) The 5637 cell–conditioned medium does not contain enough LIF. Thus, it is important to test the different pools of combined 5637 collections on ES cells to determine which pool is optimal at keeping ES cells undifferentiated. (2) The FBS lot is not efficient in maintaining ES cells. Testing lots of FBS from different manufacturers is critical for proper ES cell maintenance. (3) Cell passage number is too high. The authors typically stop maintaining ES cells once they reach passage 50.

If the ES cells begin to differentiate in culture after 1 or 2 days since passage, it is advisable to immediately feed cells with fresh ES medium. If ES cells do not adhere to the gelatin-coated dish, make sure that the gelatin-coated dishes are used within 1 week after preparation.

When feeding EBs on day 2 after enzymatic disruption of ES clumps for in vitro differentiation, many of the EBs stick to the dish bottom. If this occurs, it may be necessary to transfer the EBs to a fresh 10-cm bacteriological dish using a medidropper.

When setting up attached cultures for in vitro differentiation, it is best to select medium-sized EBs for attachment (Figure 23.3.1). Larger EBs do not adhere as well, and while smaller EBs do attach to the dish, they often do not spread out and differentiate as well. Another factor that can influence ES cell differentiation is the quality of FBS. Thus, testing lots of FBS from different manufacturers is critical for optimal ES cell differentiation.

A problem that is commonly encountered when fixing and staining differentiated ES cultures is detachment of cells from the dish. As mentioned in Support Protocol 4, the authors prefer to use methanol/acetone fixative over PFA when applicable, because methanol/acetone fixative is less harsh on the attached cultures. However, it is recommended that attention be given to treating the cultures as gently as possible during the fixing and staining procedures.

If background signal problems occur after β-galactosidase staining, try increasing the pH of the phosphate buffer. The β-galactosidase staining procedure works at a pH of 8.5.

Anticipated Results

Although maintenance and passage of mouse ES cells with 5637 cell–conditioned medium should result in cultures containing a population of only undifferentiated ES cells, the authors typically do not observe this. In general, they notice that ~80% of cultured cells appear to be undifferentiated ES cells on day 3. This percentage goes down significantly if the batch of 5637 cell–conditioned medium is sub-optimal.

Attached cultures of EBs are set up on day 3 following the dispase treatment. By day 4 of differentiation (1 day after attachment of EBs), most EBs have attached to the bottom of the plastic dishes, and the rest are removed by aspiration. This attachment is strengthened as the EBs spread out further on the dish bottom over time. Angioblasts are first observed between days 4 and 6 of differentiation. Vessels are routinely visualized between days 6 and 8, with some expansion of the vasculature after day 8. A typical time course to observe and analyze vascular development would begin on day 5 and conclude on day 8. While cultures are viable past day 8, they begin to deteriorate around day 10 of culture under differentiation conditions.

Time Considerations

Passaging mouse ES cells

Approximately 15 min should be allowed to prepare all solutions. Maintaining mouse ES cells is fairly time efficient. After allowing solutions to warm to appropriate temperatures (5 to 15 min depending on volumes), a beginner can anticipate about 20 to 30 min to process 1 to 2 6-cm dishes of ES cells.

Preparation of 5637 cell–conditioned medium (CM)

After 5637 cells are thawed and passed, cells are typically grown for 48 to 72 hr (or until 80% to 90% confluency in a 15-cm plate. At this point growth medium is replaced with collection medium, and the cells are cultured for a further 48 hr. Approximately 45 min should be allowed to process one 15-cm plate for 5637 cell–conditioned medium. More time should be added if more than one 15-cm plate is processed (typically about 5 min more for every plate added).

Enzymatic disruption of ES cells (dispase treatment)

Approximately 15 min should be allowed to prepare all solutions, and 25 to 30 min should be allowed to process one 6-cm dish of ES cells. More time should be added if more than one 6-cm dish is processed (typically about 2 to 3 min for every added dish).

In vitro differentiation

Medium must be changed on day 2 after the dispase treatment. After warming differentiation medium to 37°C, ~3 to 4 min should be allowed to aspirate and feed one dish of EBs. The time required to set up attached cultures varies depending on the number of samples that are to be processed and the number of wells to be plated. It typically takes only a couple of minutes to plate one sample into four wells of a 24-well plate. Differentiated samples must be fed every other day after plating. Generally, it takes 5 min or less to feed the wells of a 24-well plate.

Benzidine staining

Approximately 10 to 15 min should be allowed to prepare all of the solutions for the benzidine staining protocol. Including incubation times, ~5 min to 1 hr should be allowed for completion of protocol (benzidine stain does not typically last longer than 1 hr).

Double immunostaining with PECAM and Mac-1

Approximately 20 to 30 min should be allowed to prepare all of the solutions for this protocol. Including incubation times, ~7 to 7.5 hr should be allowed to double immunostain 24 wells of a 24-well plate.

Immunolocalization

Approximately 10 to 15 min should be allowed to prepare all of the solutions for the

immunolocalization protocol. Including incubation times, ~4 to 4.5 hr should be allowed to stain all 24 wells of a 24-well plate with antibody.

β-galactosidase staining

Approximately 30 to 40 min should be allowed to prepare all of the solutions for β-galactosidase staining protocol, and ~30 to 40 min should be allowed to complete all protocol steps leading up to the addition of Xgal stain. Cells are incubated with Xgal stain for 4 hr to overnight depending on the level of β-galactosidase activity.

Literature Cited

Bautch, V.L. 2002. Embryonic stem cell differentiation and the vascular lineage. *In* Methods in Molecular Biology, Vol. 185: Embryonic Stem Cells: Methods and Protocols (K. Turksen, ed.) pp. 117-125. Humana Press. Totawa, N.J..

Bradley, A., Evans, M., Kaufman, M., and Robertson, E. 1984. Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* 309:255-256.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J., and Keller, G. 1998. A common precursor for hematopoietic and endothelial cells. *Development* 125:725-732.

Doetschman, T., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines; formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27-45.

Doetschman, T., Williams, P., and Maeda, N. 1988. Establishment of hamster blastocyst-derived embryoid stem (ES) cells. *Dev. Biol.* 127:224-227.

Evans, M. and Kaufman, M. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Gerecht-Nir, S., Ziskind, A., Cohen, S., and Itskovitz-Eldor, J. 2003. Human embryonic stem cells as an in vitro model for human vascular development and the induction of vascular differentiation. *Lab. Invest.* 83:1811-1820.

Hematti, P., Obrtlikova, P., and Kaufman, D. 2005. Nonhuman primate embryonic stem cells as a preclinical model for hemtopoietic and vascular repair. *Exp. Hematol.* 33:980-986.

Inamdar, M., Koch, T., Rapoport, R., Dixon, J.T., Probolus, J.A., Cram, E., and Bautch, V.L. 1997. Yolk-sac-derived murine macrophage cell line has a counterpart during ES cell differentiation. *Dev. Dyn.* 210:487-497.

Kearney, J. and Bautch, V.L. 2003. In vitro differentiation of mouse ES cells: Hematopoietic and vascular differentiation. *Methods Enzymol.* 365:83-98.

Keller, G. 1995. In vitro differentiation of embryonic stem cells. *Curr. Opin. Cell Biol.* 7:862-869.

Keller, G. 2005. Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. *Genes Dev.* 19:1129-1155.

Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M. 1993. Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell Biol.* 13:473-486.

Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N., and Keller, G. 1997. A common precursor for primitive erythropoiesis and definitive hematopoiesis. *Nature* 386:488-493.

Levenberg, S., Golub, J., Amit, M., Itskovitz-Eldor, J, and Langer, R. 2002. Endothelial cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:4391-4396.

Martin, G. 1981. Isolation of a pluripotent cell line from the early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. 1999. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J.* 18:4261-4269.

Nakano, T., Kodama, H., and Honjo, T. 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 265:1098-1101.

Nishikawa, S.I, Nishikawa, S., Hirashima, M., Matsuyoshi, N., and Kodama, H. 1998. Progressive lineage analysis by cell sorting and culture identifies FLK+VE-cadherin cells at a diverging point of endothelial and hematopoietic lineages. *Development* 125:1747-1757.

Pera, M. and Trounson, A. 2004. Human embryonic stem cells: Prospects for development. *Development* 131:5515-5525.

Poole, T. and Coffin, J. 1989. Vasculogenesis and angiogenesis: Two distinct morphogenetic mechanisms establish embryonic vascular pattern. *J. Exp. Zool.* 251:224-231.

Redick, S. and Bautch, V.L. 1999. Developmental platelet endothelial cell adhesion molecule expression suggests multiple roles for a vascular adhesion molecule. *Am. J. Pathol.* 154:1137-1147.

Risau, W. 1997. Mechanisms of angiogenesis. *Nature* 386:671-674.

Risau, W., Sariola, H., Zerwes, H-G., Sasse, J., Ekblom, P., Kemler, R., and Doetschman, T. 1988. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development* 102:471-478.

Schmitt, R., Bruyns, E., and Snodgrass, H. 1991. Hematopoietic development of embryonic stem cells in vitro: Cytokine and receptor gene expression. *Genes Dev.* 5:728-740.

Smith, A. 2001. Embryo-derived stem cells: Of mice and men. *Annu. Rev. Cell Dev. Biol.* 17:435-462.

Smith, A., Heath, J., Donaldson, D., Wong, G., Moreau, J., Stahl, M., and Rogers, D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336:688-690.

Vittet, D., Prandini, M.-H., Berthier, R., Schweitzer, A., Martin-Sisteron, H., Uzan, G., and Dejana, E. 1996. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* 88:3424-3431.

Wang, L., Li, L., Shojaei, F., Lavec, K., Cerdan, C., Menendez, P., Martin, T., Rouleau, A., and Bhatia, M. 2004. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity* 21:31-41.

Wang, R., Clark, R., and Bautch, V.L. 1992. Embryonic stem cell-derived cystic embryoid bodies form vascular channels: An in vitro model of blood vessel development. *Development* 114:303-316.

Wiles, M. and Keller, G. 1991. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* 111:259-267.

Williams, R., Hilton, D., Pease, S., Willson, T., Stewart, C., Gearing, D., Wagner, E., Metcalf, D., Nicola, N., and Gough, N. 1988. Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336:684-687.

Wobus, A., Wallukat, G., and Hescheler, J. 1991. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. *Differentiation* 48:173-182.

Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., and Shultz, L.D. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 345:442-444.

Contributed by Nicholas C. Kappas and Victoria L. Bautch
The University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

Differentiation of Mouse Embryonic Stem Cells and of Human Adult Stem Cells into Adipocytes

Severe obesity is the result of increases in fat cell size and increased fat cell number. Mature adipocytes do not undergo cell division, and new fat cells arise from a pre-existing pool of adipose stem cells that are present irrespective of age. The development of established preadipose cell lines has facilitated the study of different steps leading to terminal differentiation of preadipocytes into adipocytes. The key events have been characterized by the identification of transcription factors that play a regulatory role in the differentiation process. The best characterized transcription factors shown to be important in the development of mature adipocytes are members of the CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferative-activated receptors (PPARs) families (Tontonoz et al., 1995; Mandrup and Lane, 1997). However, established preadipose cell lines are limited for studying early events of differentiation as they represent cells that are already committed to the adipogenic lineage. Master genes that commit multipotent stem cells toward adipocytes remain to be identified. In vitro differentiation of multipotent stem cells to the adipogenic lineage provides an alternative source of adipocytes for study in tissue culture and offers the possibility to investigate regulation of the first steps of differentiation and to test effects of drugs on adipose cell development.

This unit includes a protocol for culture conditions in which mouse embryonic stem (mES) cells can be maintained at an undifferentiated state (Support Protocol 1) or committed to undergo adipocyte differentiation at a high rate and in a highly reproducible fashion (Basic Protocol 1). There are also protocols for maintaining (Support Protocol 2) and differentiating human adult stem cells, isolated form adipose tissue (hMADS cells) and from bone marrow (hMS cells), into adipocytes (Basic Protocol 2). These culture systems provide a powerful means for studying the first step of adipose cell development and the effects of drugs on the biology of adipocytes. There are also protocols for visualization of adipocytes (Support Protocol 3) and analysis of adipocyte gene expression (Support Protocol 4).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

DIFFERENTIATION OF MOUSE ES CELLS TO ADIPOCYTES

BASIC PROTOCOL 1

Adipocyte differentiation of mES cells is initiated by aggregation of ES cells to form embryoid bodies (EBs). The authors routinely use the hanging drop method for the formation of EBs. Two phases can be distinguished in adipogenesis from ES cells. The first phase, between day 2 and 5 after EB formation, corresponds to the permissive period for the commitment of ES cells; this phase requires retinoic acid (RA). The second phase corresponds to the permissive period for terminal differentiation and is influenced by adipogenic factors (e.g., insulin, triiodothyronine, and rosiglitazone) as previously shown for the differentiation of cells from preadipose clonal lines. The treatment leads to 60% to 80% of outgrowths containing adipose cells compared to 2% to 5% in the absence of RA treatment.

Contributed by Brigitte Wdziekonski, Phi Villageois, and Christian Dani
Current Protocols in Cell Biology (2007) 23.4.1-23.4.14

Materials

ES cells
Growth medium for mES cells (see recipe)
Leukemia inhibitory factor (LIF)
CMF-PBS (phosphate-buffered saline, calcium and magnesium free; Cambrex)
Trypsin solution (see recipe)
Fetal bovine serum (FBS, see recipe)
Retinoic acid (RA, see recipe)
Differentiation medium for mES cells (see recipe)

60- and 100-mm bacteriological grade petri dishes (Greiner)
20-ml conical sterile tube
100-mm gelatinized-tissue culture dishes (see recipe)

Additional reagents and equipment for cell counting (*UNIT 1.1*)

Collect ES cells

ES cells are originally plated at 10^6 cells per 25-cm^2 flask and are grown for 2 or 3 days until they reach a confluency of 80%. At this point, cells are still maintained from the original plating in the presence of LIF, in order to keep ES cells in the undifferentiated state. ES cell cultures may contain a proportion of "differentiated" cells which have lost their pluripotency. It is crucial to minimize this proportion of differentiated cells. This is achieved by the addition of LIF in a high-quality culture medium, i.e., an adequate batch of serum. Identification of pluripotent stem cells is difficult unless one is familiar with the appropriate cellular morphology. Pluripotent stem cells: (1) are small, (2) have a large nucleus containing prominent nucleoli structures, and (3) have minimal cytoplasm. Pluripotent stem cells, in contrast to differentiated cells, grow rapidly.

1. Change medium on ES cells with complete growth medium supplemented with 1000 U/ml LIF 2 hr before subculture.
2. Aspirate medium and wash twice, each time with 5 ml of CMF-PBS.
3. Aspirate the CMF-PBS and add 1 ml of trypsin solution. Incubate 2 to 3 min.
4. Add 5 ml of growth medium to stop trypsinization and suspend the cells by vigorous pipetting. Transfer the cells to a sterile 20-ml tube and centrifuge 5 min at 250 × *g*, room temperature.
5. Aspirate the medium and resuspend the cell pellet in 10 ml of growth medium without LIF to prepare a single-cell suspension. Count the cells (*UNIT 1.1*). After cell counting, adjust the suspension to a concentration of 5×10^4 cells/ml.

Induce EB formation

6. Place aliquots of 20 µl of this suspension onto the lids of 10-cm bacteriological grade dishes.

   This is defined as day 0 of EB formation.

   Bacterial grade petri dishes are used to prevent cell attachment to the substrate. EBs have a tendency to attach to the bottom of the plastic dish. EBs that are firmly attached to the dish should be eliminated as these EBs seem to have no adipogenic capacity.

7. Invert the lid and place it over the bottom of a bacteriological petri dish filled with 8 ml CMF-PBS containing a few drops of FBS to decrease the surface tension of the liquid.

   It is essential to cover the bottom of the dish with the liquid to prevent the evaporation of the hanging drops. When the lid is inverted, each drop hangs and the cells fall to the bottom of the drop where they aggregate into a single clump (EB).

Attachment of EBs on petri dishes can be eliminated by coating the dish with poly (2-hydroxyethylmethylacrylate) supplied as Cellform polymer (ICN Biomedicals). Dishes are filled with a film of Cellform working solution (0.5 g of Cellform dissolved in 42 ml of 100% ethanol) and left in a culture hood, with the cover of the dish removed, to evaporate ethanol. Dishes are rinsed with CMF-PBS before use.

8. At a time point 2 days later, remove the lid, invert it and collect drops containing EBs in a 20-ml conical sterile tube. Let stand for 5 min at room temperature to allow the aggregates to sediment.

9. Aspirate the supernatant and resuspend the pellet in 4 ml of growth medium supplemented with 10^{-7} M retinoic acid (RA). Transfer the suspension to 60-mm bacteriological grade petri dishes.

   RA is light-sensitive.

10. Incubate for 3 days in the presence of RA changing the growth medium every day.

    Plate EBs on gelatin and differentiate.

Induce differentiation

11. At day 5 after EB formation, change the medium to growth medium without the addition of RA and plate 2 to 4 EBs per cm^2 in 100-mm gelatinized-tissue culture dishes.

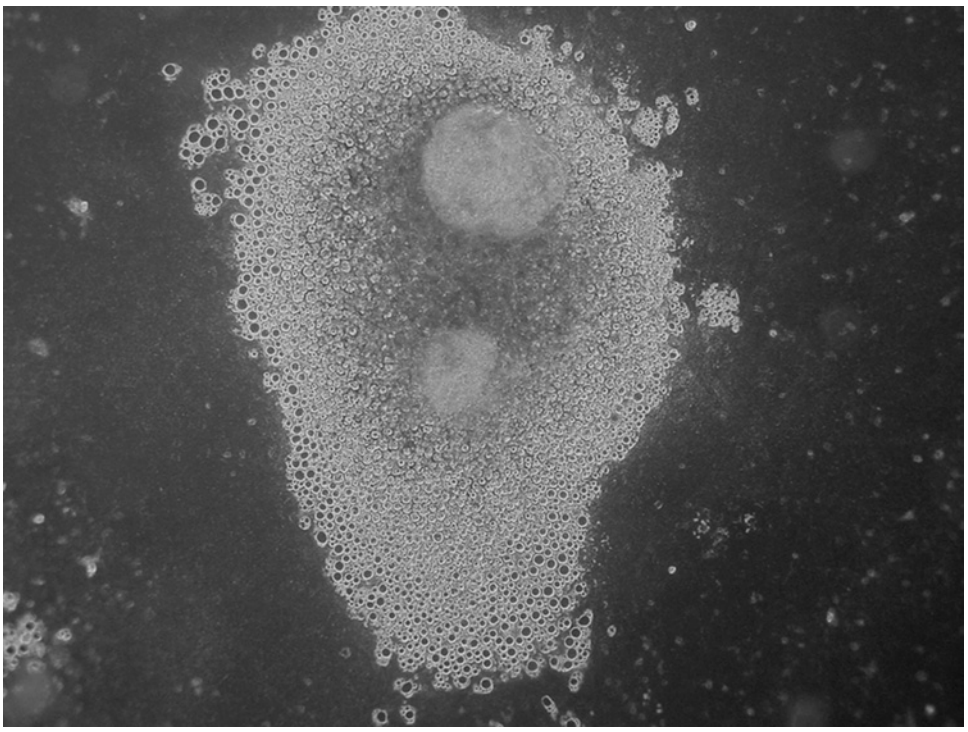


Figure 23.4.1 EB-derived adipocytes. ES cell–derived EBs were allowed to undergo adipocyte differentiation for 20 days. A large adipocyte colony around the dense center of an EB is visible under the microscope.

12. The day after plating, change the growth medium to differentiation medium. Change medium every other day.

    The addition of 0.5 μM rosiglitazone, which is a PPARγ activator (Lehmann et al., 1995), to the differentiation medium dramatically stimulates the terminal differentiation of RA-treated EBs into adipocytes (Phillips et al., 2003).

13. After 10 to 20 days in the differentiation medium, evaluate the cultures for adipocyte colonies (see Fig. 23.4.1).

    After 10 to 20 days at least 50% to 70% of EB outgrowths should contain adipocyte colonies.

SUPPORT PROTOCOL 1

MAINTENANCE OF MOUSE ES CELLS WITHOUT FEEDER LAYERS

The conditions outlined below are applicable for the maintenance of feeder layer–independent ES cell lines. Cells can be grown on gelatin-coated tissue culture flasks and maintained in a multipotent undifferentiated state by addition of leukemia inhibitory factor (LIF; Smith, 1992) to the growth medium.

Materials

ES cells in 25-cm^2 flasks (up to passage 25)
CMF-PBS (phosphate-buffered saline, calcium and magnesium free; Cambrex)
1× trypsin solution (see recipe)
Growth medium for mES cells (see recipe)
Leukemia inhibitory factor (Chemicon, Invitrogen)

20-ml conical centrifuge tubes, sterile
Gelatinized tissue culture 25-cm^2 flasks (see recipe)

Additional reagents and equipment for cell counting (*UNIT 1.1*)

1. For a 25-cm^2 flask containing ES cells, aspirate medium and wash twice each time with 5 ml CMF-PBS.
2. Aspirate the CMF-PBS and add 1 ml of 1× trypsin solution. Ensure the trypsin covers the cell monolayer and incubate for 2 to 3 min. Check that the cells are dissociated in single-cell suspension under an inverted microscope.

   It is critical to produce a single-cell suspension for subcultures. This is achieved by tapping the flask several times to ensure complete dissociation during the trypsin treatment.

3. Add 5 ml of growth medium to stop trypsinization and suspend the cells by vigorous pipetting.
4. Transfer the cells to a sterile 20-ml tube and centrifuge 5 min at 250 × *g*, room temperature.
5. Aspirate the medium and resuspend the cell pellet in 5 ml of growth medium by pipetting up and down two to three times. Count cells (*UNIT 1.1*).
6. Add 10^6 cells to 10 ml of prewarmed growth medium containing 1000 U/ml LIF then transfer to a freshly gelatinized 25-cm^2 flask.

   LIF is required to maintain pluripotent ES cells and is omitted to induce the commitment of ES cells towards the adipogenic lineage.

7. Change growth medium with LIF (1000 U/ml) every day.
8. Trypsinize the cultures 2 days later as in step 2.

   Cultures should be subcultured before cells have reached confluence.

DIFFERENTIATION OF HUMAN MULTIPOTENT ADIPOSE-DERIVED STEM (hMADS) CELLS TO ADIPOCYTES

Adipocyte differentiation of human multipotent adipose-derived stem (hMADS) cells and human mesenchymal stem (hMS) cells in vitro does not require pretreatment with RA. Induction of differentiation of hMADS cells and hMS cells is performed in monolayer culture. However, in contrast to hMADS cells, differentiation of hMS into adipocyte requires a differentiation medium supplemented with 10% fetal bovine serum (FBS).

Materials

Adult stem cells: hMADS (for isolation of hMADS cells see Rodriguez et al., 2005a; Zaragosi et al., 2006)
Growth medium for hMADS cells (see recipe)
Differentiation medium for hMADS cells (see recipe)
Tissue culture 100-mm dishes (Greiner, S.A. Dutscher) or 6-well plates or 12-well plates

1. Plate adult stem cells at a high density, e.g., 40,000 cells/cm^2 in 10 ml, 2 ml, or 1 ml growth medium, on tissue culture 100-mm dishes or 6- or 12-well plates, respectively.
2. When cells reach confluence (2 or 3 days after plating), change growth medium to differentiation medium containing 0.5 μM rosiglitazone.

   The addition of 0.5 μM rosiglitazone in the differentiation medium is required to differentiate cells into adipocytes.

3. Three days later, change cells to differentiation medium without IBMX and dexamethasone.

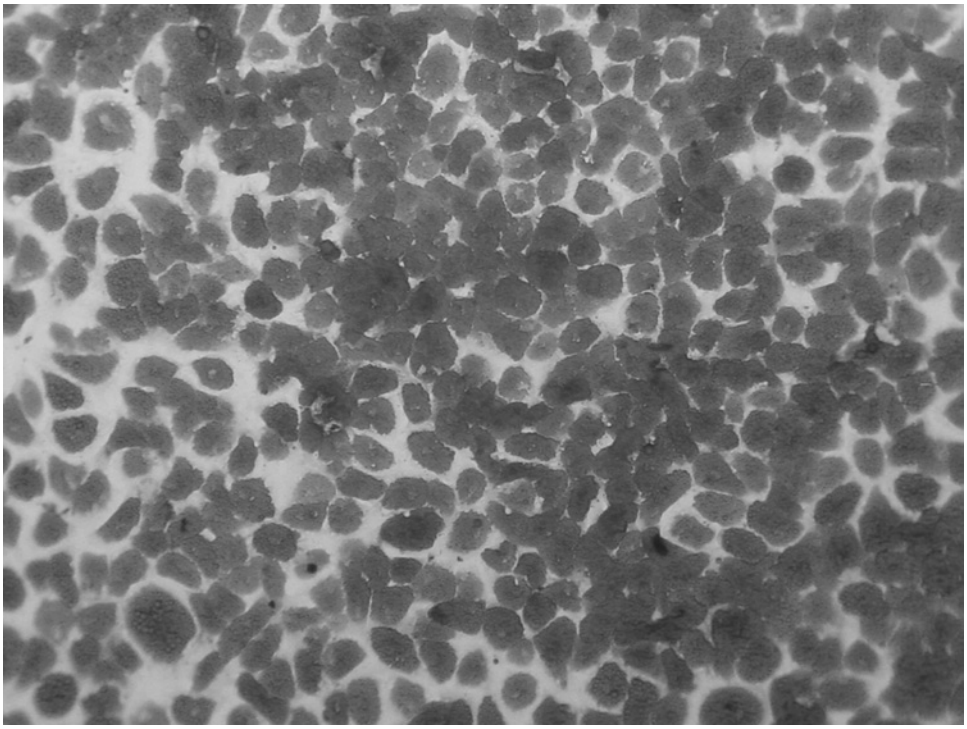


Figure 23.4.2 EB outgrowth fixed and stained with Oil-red O for fat droplets. For color version of this figure see *http://www.currentprotocols.com*.

4. Check the cultures for the appearance of adipocytes.

   Adipose cells contain lipid droplets which can be easily visualized microscopically, especially under bright-field illumination. Lipid droplets can also be visualized after staining with Oil-red O as indicated in Figure 23.4.2.

   Adipocytes should appear 5 to 7 days after induction of differentiation.

ALTERNATE PROTOCOL

DIFFERENTIATION OF HUMAN MS CELLS TO ADIPOCYTES

The same growth medium is used for hMADs cells and for hMS cells (see Reagents and Solutions). In contrast, the differentiation medium is different because hMS cells require 10% FBS to undergo differentiation into adipocytes, whereas adipocyte differentiation of hMADS cells occurs under a serum-free condition.

Materials

Adult stem cells: hMS cells (for isolation of adult stem cells from bone marrow see Pittenger et al., 1999)
Growth medium for hMS cells (see recipe)
Differentiation medium for hMS cells (see recipe)
Tissue culture 100-mm dishes or 6-well plates or 12-well plates (Greiner; S.A. Dutscher)

1. Plate cells at a high density, e.g. 40,000 cells/cm^2 in growth medium on tissue culture 100-mm dishes (or 6- or 12-well plates).
2. When cells reach confluence (2 or 3 days after plating), change growth medium to differentiation medium, containing 0.5 μM rosiglitazone.

   The addition of 0.5 μM rosiglitazone in the differentiation medium is required to differentiate cells into adipocytes

3. Three days later, change cells with differentiation medium without IBMX and dexamethasone.
4. Check the cultures for the appearance of adipocytes.

   Adipose cells contain lipid droplets which can be easily visualized microscopically, especially under bright-field illumination. Lipid droplets can also be visualized after stained with Oil-red O as indicated in Figure 23.4.2.

   Adipocytes should appear 5 or 7 days after induction of differentiation.

SUPPORT PROTOCOL 2

MAINTENANCE OF HUMAN MADS AND HUMAN MS CELLS WITHOUT FEEDER LAYER

No feeder layer and no gelatin-coated dishes are required to maintain the proliferation of hMADs cells and of hMS cells. The same growth medium is used to maintain both cell types.

Materials

hMADS or hMS cells (for isolation of hMADS see Rodriguez et al., 2005a; for isolation of hMS cells see Pittenger et al., 1999)
Phosphate-buffered saline, calcium and magnesium free (CMF-PBS, Cambrex)
1× trypsin -EDTA solution (Invitrogen no:25300-054)
Growth medium for hMADS and hMS cells (see recipe)
Tissue culture 100-mm dishes (Greiner; S.A. Dutscher)
20-ml conical centrifuge tubes, sterile
Additional reagents and equipment for cell counting (*UNIT 1.1*)

1. For a 100-mm dish of hMADS or hMS cells, aspirate medium and wash twice each time with 10 ml CMF-PBS.
2. Aspirate the CMF-PBS and add 1 ml of 1× trypsin solution. Ensure the trypsin covers the cell monolayer and incubate for 2 to 3 min. Check that cells are detached from the bottom of the dish by examining under an inverted microscope.
3. When the cells are detached, add 10 ml of growth medium to stop trypsinization and suspend the cells by pipetting.
4. Transfer the cells to a 20-ml sterile tube and centrifuge 5 min at 250 × *g*, room temperature.
5. Aspirate the medium and resuspend the cell pellet with 5 ml of growth medium by pipetting up and down 2 to 3 times. Count cells (*UNIT 1.1*).
6. Add 250 × 10^3 cells to 10 ml of prewarmed growth medium then transfer to a 100-mm dish.
7. Change medium every other day.
8. Trypsinize the cultures 3 to 4 days later as in step 1.

   Cultures should be subcultured before cells have reached confluence.

VISUALIZATION OF ADIPOCYTES

SUPPORT PROTOCOL 3

Adipose cells with lipid droplets are easily visualized microscopically, especially under bright-field illumination. Nonadipose cells containing structures resembling droplets are often detectable in untreated- and RA-treated ES cultures. Therefore, it is essential to identify droplet-like structures as triglyceride droplets. Staining of cultures with Oil-red O, a specific stain for triglycerides, gives a good indication of adipocyte differentiation.

Materials

Cultures to be examined
CMF-PBS (phosphate-buffered saline, calcium and magnesium free; Cambrex)
Fixation buffer (see recipe)
Oil-red O solution (see recipe)
Storage solution: 70% glycerol (v/v) in H_2O
Oil-red O elution buffer (see recipe)
Spectrophotometer

1. Aspirate medium from cultures and wash cells once with 10 ml CMF-PBS.
2. Fix cells for 15 min in 10, 2, or 1 ml fixation buffer in 100-mm dishes, 6- or 12-well plates, respectively, at room temperature.
3. Wash twice for 10 min each time with 10 ml Milli-Q water (for a 100-mm dish).
4. Stain with 10 ml Oil-red O solution for 15 min (for a 100-mm dish; stain with 2 ml or 1 ml Oil-red O for a 6- or 12-well dish, respectively).
5. Wash twice each time with 10 ml Milli-Q water. Cover cells with a film of storage solution (see Fig. 23.4.2) or elute the bound stain (step 6).

   Cells covered with storage solution can be kept at room temperature for several months.

6. Elute bound stain to quantify the formation of adipocytes. Incubate the 100-mm dish with 10 ml Oil-red O elution buffer (see reicpe) for 30 min at room temperature. Remove the solution.

7. After elution of the bound stain, measure the amount of bound Oil-red O spectrophotometrically at 490 nm.

SUPPORT PROTOCOL 4

ANALYSIS OF ADIPOCYTE GENE EXPRESSION

Finally, expression of a-FABP (adipocyte-fatty acid binding protein) an adipocyte-specific gene, in 20-day-old ES cell–derived EB or in 10-day-old differentiated human stem cells can be detected by northern blotting using 20 μg of total RNA. However, detection of the expression of these genes in early differentiating cells requires a more sensitive method such as RT-PCR. This protocol gives conditions to reveal mouse and human a-FABP gene expression.

Materials

TRI Reagent (Molecular Research Center, Euromedex, France)
Chloroform
Isopropanol
100% ethanol
5 M NaCl, sterile
TES buffer (see recipe)
RT-PCR kit (available from several companies)
Primers to reveal mouse a-FABP gene expression:
  forward: 5′-GATGCCTTTGTGGGAACCTGG-3′
  reverse: 5′-TTCATCGAATTCCACGCCCAG-3′
Mouse hypoxanthine phosphoribosyltransferase (HPRT, as a standard to balance the amount of RNA and cDNA used):
  forward: 5′-GCTGGTGAAAAGGACCTCT-3′
  reverse: 5′-CACAGGACTAGAACACCTGC-3′
Primers to reveal human a-FABP gene expression:
  forward: 5′-GCTTTGCCACCAGGAAAGTG-3′
  reverse: 5′-ATGACGCATTCCACCACCAG-3′
Human β-actin as a housekeeping gene standard:
  forward: 5′-AGCCATGTACGTTGCTA-3′
  reverse: 5′-AGTCCGCCTAGAAGCA-3′

RNAs are prepared using TRI Reagent according to the supplier's protocol.

Days of differentiation are indicated as 20 days for ES cells and 10 days for AS cells. The authors routinely obtain 100 to 200 μg RNA from one 100-mm tissue culture plate containing 20-day-old EB outgrowths or 10-day-old hMADS cells and hMS cells.

Using the abovementioned primers to reveal mouse a-FABP gene expression PCR is performed using an annealing temperature of 56°C and 25 cycles of PCR. The size of the expected cDNA is 213 bp. Mouse hypoxanthine phosphoribosyltransferase (HPRT) is used as a standard to balance the amount of RNA and cDNA used. With the abovementioned primers PCR is performed using an annealing temperature of 60°C and 25 cycles of PCR. The expected size of this cDNA is 249 bp.

Using the abovementioned primers to reveal human a-FABP gene expression PCR is performed using an annealing temperature of 55°C and 25 cycles of PCR. The size of the expected cDNA is 290 bp. Human β-actin is used as a standard. With the abovementioned primers PCR is performed using an annealing temperature of 55°C and 25 cycles of PCR. The expected size of this cDNA is 656 bp.

PCR products are separated and visualized by electrophoresis on a 2% (w/v) agarose gel containing ethidium bromide (see Voytas, 2000).

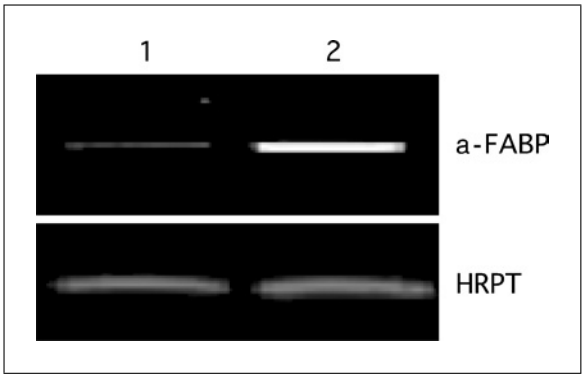


Figure 23.4.3 PCR analysis of expression of an adipocyte-specific gene. EBs were pretreated from day 2 to day 5 with 0.1% DMSO (1) or 10^{-7} RA (2). RNAs were prepared at day 20 after EB formation and transcripts for a-FABP. HPRT was used to monitor expression of a housekeeping gene.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocols except as noted. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Dexamethasone, 1 mM

Dissolve 3.9 mg dexamethasone (powder; Sigma) in 10 ml ethanol. Store 0.5-ml aliquots up to 6 months at −20°C.

Differentiation medium for hMADS cells

To 25 ml of low-glucose DMEM (Invitrogen) add:
25 ml Ham's F12 (Cambrex)
0.5 ml 200 mM glutamine
0.5 ml 1 M HEPES-buffered saline (Cambrex)
0.5 ml 5000 IU/ml/5000 μg/ml penicillin/streptomycin (Cambrex)
0.5 ml 1 mg/ml transferrin
0.25 ml 1 mg/ml insulin (see recipe)
0.05 ml 100 mM IBMX (see recipe)
0.05 ml 1 mM dexamethasone (see recipe)
0.005 ml 2 μM T3 (see recipe)
Prepare fresh

The differentiation medium must be prepared just before use. Therefore, prepare only the volume of medium required for changing cells.

Differentiation medium for hMS cells

To 45 ml low-glucose DMEM (Invitrogen) add:
5 ml FBS
0.5 ml 200 mM glutamine
0.5 ml 1 M HEPES-buffered saline (Cambrex)
0.5 ml 5000 IU/ml/5000 μg/ml penicillin/streptomycin (Cambrex)
0.05 ml 100 mM IBMX (see recipe)
0.05 1 mM dexamethasone (see recipe)
0.25 1 mg/ml insulin (see recipe)
0.005 ml 2 μM T3 (see recipe)
Prepare fresh

The differentiation medium must be prepared just before use. Therefore, prepare only the volume of medium required for changing cells.

Differentiation medium for mES cells

Supplement growth medium for mES cells with:
0.5 μg/ml insulin (see recipe)
2 nM triiodothyronine (add from 2 μM T3 solution; see recipe)
0.5 μM rosiglitazone (provided by GlaxoSmthKline)
Prepare fresh

The differentiation medium must be prepared just before use. Therefore, prepare only the volume of medium required for changing cells.

Fetal bovine serum

Fetal bovine serum is tested for maintenance of the undifferentiated state. The authors select a batch of serum that is able to support the growth of stem cells. For that purpose, plate 10^6 ES cells/25-cm ^{2} flask in 10% of each set of FBS, supplemented with LIF, and subculture the cells every two days for 4 passages. For a high-quality serum, a flask should yield 5 to 10×10^6 cells at each passage. Furthermore, no toxicity of the selected serum should be observed at a 30% concentration.

The supplier used for this unit is Dutscher, France, but other suppliers can be used.

Fixation buffer

Dilute 2.5% glutaraldehyde (Sigma) to 0.25% (v/v) in CMF-PBS (Cambrex). Store up to 1 month at 4°C.

Gelatin 0.1% (w/v)

Dilute 2% (w/v) gelatin to 0.1% with CMF-PBS (Cambrex). Store up to 2 weeks at 4°C.

Gelatinized-tissue culture dishes/flasks

Add 5 or 10 ml of 0.1% gelatin (see recipe) per 25-cm^2 flask (Corning or Greiner) or 100-mm tissue culture dishes (Corning or Greiner Cell Star), respectively. Incubate for at least 10 min at room temperature and aspirate gelatin. Prepare fresh.

Growth medium for hMADS and hMS cells

To 500 ml low-glucose DMEM add:
5 ml 200 mM glutamine
5 ml 1 M HEPES -buffered saline (Cambrex)
5 ml 5000 IU/ml-/5000 μg/ml penicillin/streptomycin
50 ml FBS
Store up to 2 weeks at 4°C.

Growth medium for mES cell maintenance, 1×

To 440 ml Glasgow MEM/BHK21 medium (Invitrogen) add:
5 ml of 100× nonessential amino acids (stored at 4°C)
5 ml of 200 mM glutamine (stored at −20°C)
5 ml 100 mM sodium pyruvate (stored at −20°C)
0.5 ml 0.1 M 2 -mercaptoethanol (stored at 4°C; see recipe)
50 ml of FBS (stored at −20°C)
5 ml 5000 IU/ml/5000 μg/ml penicillin/streptomycin (Cambrex)
Store up to 2 weeks at 4°C

IBMX, 100 mM

Prepare IBMX (3-isobutyl-1methylxanthine; Sigma) at 100 mM in water. Dissolve 250 mg IBMX in 9 ml water and 5 to 10 µl of 10 N NaOH. Adjust volume to 11 ml with water and sterilize by filtration with a 0.22-µm filter. Store 0.1-ml aliquots up to 2 months at −20°C.

Insulin (for mES cells)

Dissolve lypholized insulin, —bovine for mouse cells; recombinant human (Sigma no. I-9278) for human cells—at 1 mg/ml in cold 0.01 N HCl. Mix gently and sterilize by filtration with a 0.22-µm filter. Store 0.1-ml aliquots up to 6 months at −20°C. Store thawed aliquots up to 2 weeks at 4°C.

2-mercaptoethanol, 0.1 M

100 µl 2-mercaptoethanol
14 ml sterile H_2O
Store up to 3 weeks at 4°C

Oil-red O elution buffer

4 M guanidinium thiocyanate (Sigma)
25 mM sodium citrate, pH 7
0.5% (v/v) sarcosyl
20% (v/v) isopropanol
Store up to 1 month at room temperature

Oil-red O solution

Oil-red O stock solution: Dissolve Oil red O (Sigma) at 0.5% (w/v) in isopropanol. Store up to several months at room temperature.

Oil red O working solution: Mix 6 vol of stock solution with 4 vol water. Mix and filter using a 0.45-µm filter. Store up to 1 month at room temperature.

Retinoic acid

Dilute all-*trans* retinoic acid (RA; Sigma) in the dark in dimethyl sulfoxide to prepare a 10 mM RA stock solution. Store 0.1-ml aliquots up to 1 year at −20°C, protected from the light.

Prepare subsequent dilutions of RA in ethanol and use for one experiment only.

After dilution into the culture medium, the concentration of ethanol should never exceed 0.1%.

TES buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.1 mM EDTA
0.1% (w/v) sodium dodecyl sulphate
Sterilize by autoclaving
Store up to several months at room temperature

Transferrin, 1 mg/ml

Dissolve apo-transferrin (human; Sigma) at 1 mg/ml in PBS (*APPENDIX 2A*) and sterilize by filtration with a 0.22-µm filter. Store 1-ml aliquots up to several months at −20°C. Store thawed aliquots up to 1 week at 4°C.

Triiodothyronine (T3), 2 μM

Dissolve 5 mg T3 (culture tested, powder; Sigma) into 7.43 ml ethanol to prepare a 1 mM T3 solution. Then, dilute 200 μl of 1 mM solution into 4 ml ethanol to prepare a 50 μM T3 solution. Finally, dilute 3.2 ml of 50 μM T3 solution into 80 ml ethanol to prepare a 2 μM T3 solution. Store up to 1 year at −20°C.

Trypsin solution, 1× for mES cells

1 ml 2.5% (w/v) trypsin (Invitrogen)
1 ml 100 mM EDTA
1 ml chicken serum
100 ml CMF-PBS (*APPENDIX 2A*)
Store 10-ml aliquots up to 2 months at −20°C
Store thawed aliquots up to 2 weeks at 4°C

COMMENTARY

Background Information

Mouse embryonic (ES) cells have been previously shown to differentiate spontaneously into various lineages in culture (Doetschman et al., 1985). The first morphological observation of adipocyte-like cells derived from ES cells was reported by Field et al. (1992). However, the number of ES cell–derived adipocytes was low because spontaneous commitment of ES cells to the adipogenic lineage is rare. In order to use ES cells to study adipocyte development it was essential to determine conditions of culture that committed ES cells to the adipogenic lineage at a high rate. This step has been achieved by showing that a prerequisite is to treat ES cell–derived embryoid bodies (EBs) at an early stage of their differentiation with all-*trans*-retinoic acid (RA) for a short period of time (Dani et al., 1997; Dani, 1999). Adipocytes derived from ES cells display both lipogenic and lipolytic activities in response to insulin and to β-adrenergic agonists respectively, indicating that mature and functional adipocytes are formed.

In regard to models of human cells presently available, for primary preadipocytes derived from stromal-vascular cells of human adipose tissue, with increasing passage number, the ability to differentiate into adipocytes under appropriate conditions undergoes a dramatic decrease before the cells become growth arrested and enter replicative senescence (Ailhaud, 2002). This limitation has been partly circumvented with cells that are immortalized either genetically or spontaneously while preserving their ability to undergo adipose conversion. Overexpression of SV40T and T antigens in human SV cells from infant brown adipose tissue (Zilberfarb et al., 1997) or coexpression of human telomerase reverse transcriptase and papilloma E7 oncoprotein in SV cells from adult white adipose tissue (Darimont et al., 2003) led to establishment of PAZ6 and Chub-S7 preadipocyte cell lines, respectively. Cells isolated from subcutaneous adipose tissue of an infant suffering from Simpson-Golabi-Behmed syndrome and from an adult suffering from liposarcoma were used to obtain SGBS preadipocyte cell strain and LiSa-2 preadipocyte cell line, respectively (Wabitsch et al., 2000, 2001). Upon differentiation, cells from these various lines expressed some of the characteristic markers of human adipocytes but the lipolytic responses specific of human adipocytes and the secretion of specific adipocytokines were not reported. Moreover, except for Chub-S7 cells which were diploid and showed no chromosomal alterations, cells from other lines exhibited chromosomal abnormalities. Recently, the establishment of multipotent stem cells from the stromal-vascular fraction of infant adipose tissue (Rodriguez et al., 2005a) has led the authors to examine, under serum-free adipogenic conditions, whether these human multipotent adipose-derived stem (hMADS) cells enter the adipose lineage and differentiate into cells that exhibit characteristics of human fat cells. The authors have shown that hMADS cells are a unique cell model in displaying the key features of human adipocytes after differentiation under adipogenic conditions (Rodriguez et al., 2004, 2005b). The human adult bone marrow also contains mesenchymal stem (hMS) cells that are able to undergo differentiation into adipocytes in vitro (Pittenger et al., 1999).

Critical Parameters and Troubleshooting

The first critical parameter for the commitment of mES cells into adipocytes is the formation of EBs. The authors use the hanging drop method for the formation of EBs. The formation of EBs in mass culture by maintaining ES cells in suspension at a high density in non-tissue culture grade plastic is rapid and gives rise to a high number of EBs formed. However in the hands of the authors, this method leads subsequently to a low number of outgrowths containing adipocyte colonies.

The, treatment of EBs with RA between day 2 and 5 after EB formation is prerequisite. The authors have observed that treatment of EBs with RA between days 2 and 5 after EB formation or between days 3 and 6 gives similar results. Owing to the high instability of RA, the concentration of RA able to commit ES cells into the adipogenic lineage at a high rate should be determined for each new preparation of RA (try 10^{-8} to 10^{-6} M). RA is light-sensitive.

Adipocyte differentiation of hMADS cells and hMS cells in vitro does not require pretreatment with RA. However, activators of PPARγ such as rosiglitazone, is required for the terminal differentiation of these stem cells into adipocytes.

hMS cells can be expanded for several passages in vitro. However, in the hands of the authors these cells lose their capacity to undergo differentiation after 15 passages. In contrast, hMADS cells can be maintained for 30 passages with no loss of their ability to undergo adipocyte differentiation.

Anticipated Results

The authors usually pool 2-day-old EBs from four lids of 100-mm bacteriological petri dishes into one 60-mm bacteriological grade petri dish. After RA treatment, EBs contained in one 60-mm dish are plated into one 100-mm tissue culture dish. Under these conditions development of more than 100 EBs can be observed. Twenty days after EB formation, 50% to 70% of EBs contain adipocyte colonies. Over 25 passages, adipocyte differentiation may decrease. In regard to the differentiation of adult stem cells, the authors usually perform differentiation in 24-well plates when monitoring differentiation by Oil-red O staining, and into 60-mm plates, or larger plates, for RNA preparation. Ten days after induction of differentiation, 50% to 90% of hMADS and hMS cells are differentiated into adipocytes. The level of differentiation may decrease over 25 passages of hMADs cells and over 10 passages of hMS cells.

Time Considerations

From mES, adipocytes can be observed 10 days after EB formation. However, 20 days in differentiation medium are required to complete adipocyte differentiation. Additions of PPARγ activator dramatically increase the kinetics and the level of adipocyte differentiation of ES cells. From human adult stem cells, adipogenesis is completed within 2 weeks. In that case PPARγ activator is required.

Literature Cited

Ailhaud, G. 2002. Autocrine/paracrine effectors of adipogenesis. *Ann. Endocrinol. (Paris)* 63:83-85.

Dani, C. 1999. Embryonic stem cell-derived adipogenesis. *Cells Tissues Organs* 165:173-180.

Dani, C., Smith, A., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C., and Ailhaud, G. 1997. Differentiation of embryonic stem cells into adipocytes in vitro. *J. Cell Sci.* 110:1279-1285.

Darimont, C., Zbinden, I., Avanti, O., Leone-Vautravers, P., Giusti, V., Burckhardt, P., Pfeifer, A.M., and Mace, K. 2003. Reconstitution of telomerase activity combined with HPV-E7 expression allow human preadipocytes to preserve their differentiation capacity after immortalization. *Cell Death Differ.* 10:1025-1031.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27-45.

Field, S.J., Johnson, R.S., Mortensen, R.M., Papaioannou, V.E., Spiegelman, B.M., and Greenberg, M.E. 1992. Growth and differentiation of embryonic stem cells that lack an intact c-fos gene. *Proc. Natl. Acad. Sci. U.S.A.* 89:9306-9310.

Lehmann, J.M., Moore, L.B., Smith, O.T., Wilkison, W.O., Willson, T.M., and Kliewer, S.A. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.* 270:12953-12956.

Mandrup, S. and Lane, M.D. 1997. Regulating adipogenesis. *J. Biol. Chem.* 272:5367-5370.

Phillips, B.W., Vernochet, C., and Dani, C. 2003. Differentiation of embryonic stem cells for pharmacological studies on adipose cells. *Pharmacol. Res.* 47:263-268.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. 1999. Multilineage potential of adult

human mesenchymal stem cells. *Science* 284:143-147.

Rodriguez, A.-M., Elabd, C., Delteil, F., Astier, J., Vernochet, C., Saint-Marc, P., Guesnet, J., Guezennec, A., Amri, E.-Z., Dani, C., and Ailhaud, G. 2004. Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem. Biophys. Res. Commun.* 315:255-263.

Rodriguez, A.-M., Pisani, D., Dechesne, C.A., Turc-Carel, C., Kurzenne, J.-Y., Wdziekonski, B., Villageois, A., Bagnis, C., Breittmayer, J.-P., Groux, H., Ailhoud, G., and Dani, C. 2005a. Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J. Exp. Med.* 201:1397-1405.

Rodriguez, A.-M., Elabd, C., Amri, E.Z., Ailhaud, G., and Dani, C. 2005b. The human adipose tissue is a source of multipotent stem cells. *Biochimie* 87:125-128.

Smith, A.G. 1992. Mouse embryo stem cells: Their identification, propagation and manipulation. *Semin. Cell Biol.* 3:385-399.

Tontonoz, P., Hu, E., Devine, J., Beale, E.G., and Spiegelman, B.M. 1995. PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol. Cell Biol.* 15:351-357.

Voytas, D. 2000. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

Wabitsch, M., Bruderlein, S., Melzner, I., Braun, M., Mechtersheimer, G., and Moller, P. 2000. LiSa-2, a novel human liposarcoma cell line with a high capacity for terminal adipose differentiation. *Int. J. Cancer* 88:889-894.

Wabitsch, M., Brenner, R.E., Melzner, I., Braun, M., Moller, P., Heinze, E., Debatin, K.M., and Hauner, H. 2001. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int. J. Obes. Relat. Metab. Disord.* 25:8-15.

Zaragosi, L.E., Ailhaud, G., and Dani, C. 2006. Autocrine fibroblast growth factor 2 signaling is critical for self-renewal of human multipotent adipose-derived stem cells. *Stem Cells* 24:2412-2419.

Zilberfarb, V., Pietri-Rouxel, F., Jockers, R., Krief, S., Delouis, C., Issad, T., and Strosberg, A.D. 1997. Human immortalized brown adipocytes express functional beta3-adrenoceptor coupled to lipolysis. *J. Cell Sci.* 110:801-807.

Contributed by Brigitte Wdziekonski, Phi Villageois, and Christian Dani
CNRS Université de Nice Sophia Antipolis
Nice, France

Induction of ES Cell–Derived Cartilage Formation

Embryonic stem (ES) cells are generated from the inner cell mass of the blastocyst (Evans and Kaufman, 1981; Martin, 1981). Therefore, these cells are pluripotent, and if they are cultivated in vitro as cellular aggregates—so called embryoid bodies (EBs)—they spontaneously differentiate into various cell types of all three germ layers (Rathjen et al., 1998). The ES cell model system of in vitro differentiation is useful to analyze cell differentiation from a pluripotent stem cell to progenitor cells, and, finally, to terminally differentiated cell types (for review, see Guan et al., 1999; Rohwedel et al., 2001). This unit contains protocols for ES cell differentiation to chondrocytes and osteocytes (Basic Protocol 1 and Alternate Protocol) and for characterization of the differentiated cell types (Kramer et al., 2000; Hegert et al., 2002).

The authors analyze chondrogenic and osteogenic differentiation in EBs by histochemical staining (Support Protocols 1 to 6), immunostaining (Support Protocol 7), mRNA in situ hybridization (Support Protocol 8), RT-PCR techniques (Support Protocol 9), and electron microscopy (Support Protocol 10; Kramer et al., 2000; 2005a,b). Chondrocytes are organized in cartilage nodules, which develop from aggregates of mesenchymal prechondrogenic cells (Basic Protocol 2). Later, chondrogenic cells within these nodules become hypertrophic and finally show characteristics of osteogenic differentiation (Hegert et al., 2002). In contrast to other mesodermal cell types, such as cardiac muscle cells, the amount of cartilage nodules is relatively low in EBs derived from ES cell line D3 (Kramer et al., 2005a). However, cartilage nodule formation can be induced, for example by growth factors of the TGF-β family (Kramer et al., 2000). Another important factor involved in modulating ES cell–derived cartilage formation is the basal medium and its basic supplementations (see Commentary).

For regenerative strategies, ES cell–derived chondrocytes have to be selected from EBs to enrich chondrogenic cells and to avoid teratoma formation by undifferentiated ES cells. For example, ES cells formed teratomas and destroyed the ambient tissue if they were injected in an undifferentiated stage into knee joints of mice (Wakitani et al., 2003). The authors of this unit isolated chondrocytes from cartilage nodules using microdissection (Basic Protocol 2) and collagenase treatment (Basic Protocol 3; Hegert et al., 2002) and analyzed their differentiation behavior in culture. These single ES cell–derived chondrocytes possess a high potential for regeneration, indicated by a rapid redifferentiation after an initial dedifferentiation. However, transdifferentiation into other mesodermal cell types was observed in the chondrocyte single-cell cultures (Hegert et al., 2002). Therefore, it may be necessary to improve the selection strategies to obtain defined cell populations before ES cell–derived chondrocytes can be used for therapeutic applications.

The model system of ES cell–derived chondrogenesis described in this unit can also be used to analyze the influence of different parameters—e.g., signaling molecules, growth factors, and extracellular matrix molecules—on cellular chondrogenic development. In particular, the authors have studied growth factor modulation of ES cell–derived chondrocyte differentiation in vitro (Kramer et al., 2000, 2003; Hegert et al., 2002).

In vivo, murine ES cells are used to generate knockout mice, which enable study of the phenotypic consequences of a gene mutation that has been established in vitro in ES cells via gene targeting. This is possible because genetically modified ES cells take

Contributed by Jan Kramer, Peter Schlenke, and Jürgen Rohwedel
Current Protocols in Cell Biology (2007) 23.5.1-23.5.33

part in the development of all somatic tissues and most importantly, germ cells, after injection into blastocysts (Thomas and Capecchi, 1987). However, frequently, embryos carrying a homozygous knockout mutation die shortly after implantation. In this case, in vitro differentiation of homozygous knockout ES cells is an excellent alternative approach to analyze the consequences of a loss-of-function mutation at the cellular level. Therefore, the model system of ES cell–derived chondrogenesis in vitro can be used to characterize the function of specific genes during cartilage formation. For example, the authors investigated the consequence of a knockout mutation of the transcription factor Sox9 on ES cell–derived chondrogenesis in vitro (Hargus et al., submitted). In vivo studies have shown that Sox9 plays an important role during chondrogenesis (Bi et al., 1999; Akiyama et al., 2002). The authors tested EBs generated from homozygous Sox9-deficient ES cells for their cartilage differentiation in vitro and found that $Sox9^{-/-}$ cells failed to develop into cartilage nodules (Hargus et al., submitted). This result is in line with the previous in vivo studies.

Taken together, the ES cell in vitro model system is an alternative approach to in vivo studies with embryos and can be used to characterize cellular events of chondrogenic differentiation. In this unit, some basic techniques for ES cell cultivation and for differentiation into the chondrogenic direction in vitro are provided, along with protocols to characterize chondrogenic differentiation of ES cells by histochemical staining, immunofluorescence methods, mRNA-in situ hybridization, RT-PCR technique, and electron microscopy. Moreover, the authors of this unit review how ES cell–derived chondrogenic differentiation can be modulated by growth factors and discuss studies demonstrating chondrogenic differentiation of ES cells.

STRATEGIC PLANNING

Successful ES cell cultivation requires knowledge and experience with basic cell culture techniques like thawing, trypsinization, and freezing of cells. For isolation and ultrastructural analysis of chondrogenic aggregates from EBs, microdissection and electron microscopy techniques have to be established. For differentiation of ES cells, two principal methods can be used: (1) ES cells can simply be cultivated in bacteriological petri dishes where they form EBs spontaneously, or (2) ES cells can be cultivated via the hanging-drop method to obtain EBs. The advantage of the latter technique is that EBs of the same size are obtained, which enables a more reproducible differentiation process. Therefore, this unit provides detailed protocols for differentiation of ES cells via hanging drops (Basic Protocol 1). ES cell differentiation also requires advanced development of a time schedule (see Table 23.5.1) for the specific study and calculation of the number of EBs needed. The hanging-drop cultivation is performed for 2 days (days 0 to 2) to obtain the EBs, followed by cultivation of EBs in suspension for 3 days (days 2 to 5) and plating of EBs on the fifth day of differentiation (day 5). After plating, the EBs grow out and are subsequently cultivated for up to 35 days (day 5 plus 35 days). Initially, before plating of EBs (days 0 to 5), samples of 25 EBs are taken every day for RT-PCR analysis (a total of 125 EBs). Later, after plating of EBs, samples are analyzed every second to fifth day by RT-PCR, histochemical staining, immunostaining, or mRNA in situ hybridization (ten EBs per sample and per method). For isolation of chondrogenic cells from EBs, it is necessary to obtain sufficient amounts of chondrogenic aggregates in the EBs for microdissection. Ten 60-mm tissue culture plates, each with ten EBs, have to be prepared for every sample. Initially, every hanging drop contains 800 ES cells resulting in one single EB. These details have to be considered in advance to calculate the quantity of undifferentiated ES cells that have to be generated before the onset of the differentiation experiment. The standard ES cell protocol is based on the assumption that a 60-mm tissue culture plate with undifferentiated ES cells contains around 1×10^6 cells after 2 days of cultivation. However, to achieve this quantity of cells, the size and morphology of the ES

Table 23.5.1 A Time Schedule Useful for an ES Cell Differentiation Experiment[a]

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1st week			Thaw feeder	Split feeder	Inactivate feeder		
2nd week	Thaw ES cells		0 days R	R	2 days R	R	R
3rd week	5 days R		5+2 days R,H	I	5+4 days R,H		
4th week	5+8 days R,I,H		5+10 days R,H	I	5+12 days R,H		
5th week	5+15 days R,I,H		5+17 days R,H	I	5+19 days R,H		
6th week	5+22 days R,I,H		5+24 days R,H	I	5+26 days R,H		
7th week	5+29 days R,I,H		5+31 days R,H	I	5+33 days R,H		
8th week	5+36 days R,I,H						

[a]Hanging-drop cultivation of ES cells (Basic Protocol 1) is performed to generate embryoid bodies (EBs) in vitro. In general, 2 days after thawing, a sufficient number of ES cells for one differentiation experiment is obtained. The hanging-drop cultivation is performed for 2 days (days 0 to 2) to generate the EBs, followed by cultivation of EBs in suspension for 3 days (days 2 to 5) and plating of EBs on the fifth day of differentiation (day 5). After plating, the EBs grow out and are cultivated subsequently for up to 35 days (day 5 plus 35 days). Initially, RNA probes (R) for RT-PCR analysis are collected every day (days 0 to 5). Later, specimens for RT-PCR analysis and histochemical stainings (H) are taken approximately every second day. Indirect immunostaining and in situ hybridization (I) are performed twice a week, for example on Monday and Thursday. Samples for electron microscopy are collected at day 5 plus 10 days (mesenchymal condensations) and at day 5 plus 22 days (cartilage nodules), respectively.

cell colonies has to be controlled carefully by light microscopy (see Critical Parameters). A total of 1×10^6 ES cells can be used to generate 1250 EBs. The murine ES cell lines BLC6 (Wobus et al., 1988), D3 (Doetschman et al., 1985), E14 (Hooper et al., 1987), and R1 (Nagy et al., 1993) have been successfully used by the authors. Line BLC6 is most effective with respect to cartilage nodule formation (Kramer et al., 2005a). In general, all analyzed ES cell lines showed spontaneous chondrogenic differentiation. However, an important consideration with respect to the differentiation efficiency is the use of growth factors to enhance chondrogenic differentiation. For example, chondrogenic differentiation of murine ES cells can be enhanced by the addition of BMB-2 and/or BMP-4 to the differentiation medium (for details, see Commentary).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

IN VITRO DIFFERENTIATION OF EMBRYONIC STEM CELLS FROM EMBRYOID BODIES

BASIC PROTOCOL 1

Pluripotent ES cells differentiate spontaneously into cells of all three primary germ layers. ES cells can be differentiated into chondrogenic and osteogenic cell types by cultivation as cellular aggregates, so called embryoid bodies (EBs), generated by hanging drops. The major steps of ES cell differentiation are: (1) generation of EBs by hanging-drop cultivation of ES cells for 2 days (days 0 to 2); (2) cultivation of the EBs in suspension for 3 days (days 2 to 5); and (3) plating of the EBs at the fifth day of differentiation (day 5), followed by further cultivation up to 35 days (day 5 plus 35 days; Table 23.5.1).

Materials

ES cell lines BLC6 (Wobus et al., 1988), D3 (Doetschman et al., 1985), E14 (Hooper et al., 1987), and/or R1 (Nagy et al., 1993) growing (Support Protocol 12) on feeder layers (Support Protocol 11) in Medium 2 (see recipe) and Medium 4 (see recipe)

Phosphate-buffered saline (PBS; see recipe)
Trypsin/EDTA solution (see recipe)
Medium 2 (see recipe) and Medium 4 (see recipe)
Low-FBS medium (see recipe)

100-mm bacteriological petri dishes (Greiner), uncoated
100-µl sterile pipet tips with aerosol-filter barriers (Eppendorf)
5-ml glass pipets
Gelatin-coated (see recipe) 60-mm tissue culture dishes (Nunc), chamber slides (Falcon CultureSlides from BD Biosciences), and 24-well tissue culture plates

Additional reagents and equipment for basic cell culture techniques including counting cells (*UNIT 1.1*)

Cultivate ES cell in hanging drops

For the differentiation analysis described in Table 23.5.1, 475 EBs with 800 cells/EB must be prepared. In addition, ~40,000 ES cells are used for RNA isolation (at day 0). In total, 420,000 ES cells are necessary for the analysis. However, one should not use all cultivated ES cells for a single differentiation experiment, but should leave some that can be further cultivated in the undifferentiated state for freezing (see Support Protocol 12). A 60-mm dish contains $\sim 1 \times 10^6$ ES cells (if carefully cultivated), as mentioned in Strategic Planning. The authors thus suggest that two 60-mm dishes be prepared: one for the differentiation analysis and one for further cultivation.

1. Wash cells in dish once with 4 ml PBS. Trypsinize undifferentiated ES cells cultivated on MEF feeder layer in medium 2 in 60-mm dishes by adding 2 ml trypsin/EDTA solution. When the cells are dissociated pipet up and down and transfer the cells to a 15-ml centrifuge tube with 10 ml medium.

   The undifferentiated ES cells are grown, subcultivated, and cropreserved as in Support Protocol 12; feeder layers are produced as in Support Protocol 11.

2. Centrifuge 5 min at 180 × g, room temperature. Remove and discard supernatant.

3. Resuspend cells in 2 ml of Medium 4 and determine the cell number (*UNIT 1.1*).

   The authors use 800 ES cells per EB for differentiation into chondrogenic and osteogenic cells.

4. Dilute cells in Medium 4 to obtain a cell suspension with 4×10^4 cells/ml.

   For cell dilution, it is helpful to use a small sterile beaker with a diameter of 4 cm for preparing the cell suspension. The 20-µl drop samples are then transferred with a micropipettor under sterile conditions.

5. Place ~50 20-µl aliquots of the cell suspension on the inner side of the lid of a 100-mm uncoated bacteriological petri dish, using a micropipettor with sterile 100-µl tips containing aerosol-filter barriers (see Fig. 23.5.1).

   If using gloves for cell culture handling, the electrostatic charge of the petri dish lid can cause the drops to flow together. Therefore, avoid touching the lids outside on the top.

6. Place 10 ml PBS into the bottom of the petri dish (to avoid evaporation of the hanging-drop medium during cultivation). Carefully invert the lid containing the drops of ES suspension onto the bottom of the petri dish to create the hanging drops. Culture the hanging drops for 2 days at 37°C (day 0 to day 2).

Cultivate EBs in suspension (day 2 to day 5)

7. On the second day of hanging-drop culture, collect the drops containing the EBs from two petri dishes (containing ~50 drops per lid) by flooding the inverted lid

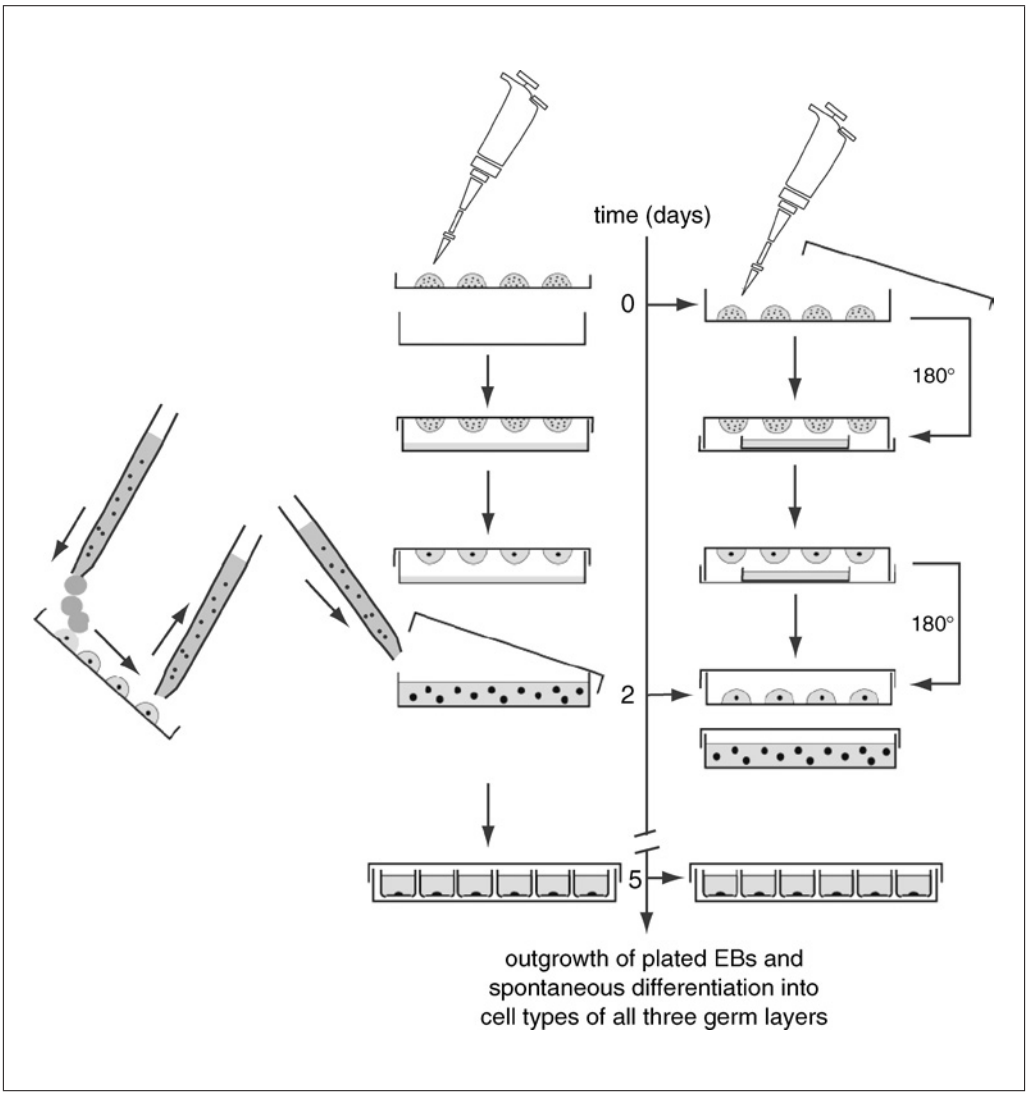


Figure 23.5.1 Schematic illustrating Basic Protocol 1 (left) and Alternate Protocol (right) for "hanging drop" cultivation of embryonic stem cells as embryoid bodies (EBs). The Alternate Protocol may provide the advantage of easier handling.

containing the drops with 2 ml of Medium 4 and then aspirating with a 5-ml glass pipet.

Do not do this if following the Alternate Protocol; also see Figure 23.5.1.

8. Place 8 ml of fresh Medium 4 into the bottom of an uncoated 100-mm bacteriological petri dish. Pool the EBs collected from the lids of the two dishes in step 7 in this dish.

   It is important to select a batch of bacteriological petri dishes that enable suspension culture. With some batches, the EBs attach, even if the dishes are not coated with gelatin. If this happens, it is sometimes possible to resuspend the EBs again by carefully rinsing the dishes with Medium 4.

9. Cultivate the EBs in suspension for 3 days (day 2 to day 5).

Plate EBs (day 5) and analyze

10. On the fifth day of differentiation (day 5), plate the appropriate number of EBs (see Table 23.5.2) onto gelatin-coated 60-mm tissue culture dishes (for Alcian blue staining, RT-PCR, microdissection, and electron microscopy), gelatin-coated chamber slides (for immunostaining, mRNA in situ hybridization, and most histochemical staining), and gelatin-coated 24-well plates (for morphological

Table 23.5.2 Conditions for Setting Up EB Cultures

Culture container	Number of EBs	Volume medium (ml)	Used for
60-mm petri dish	10	4	Alcian Blue staining, RT-PCR, microdissection, electron microscopy
2-chambered slide	5/chamber	2.5/chamber	Immunostaining, mRNA in situ hybridization, histochemistry
24-well plate	1/well	1/well	Morphological analysis

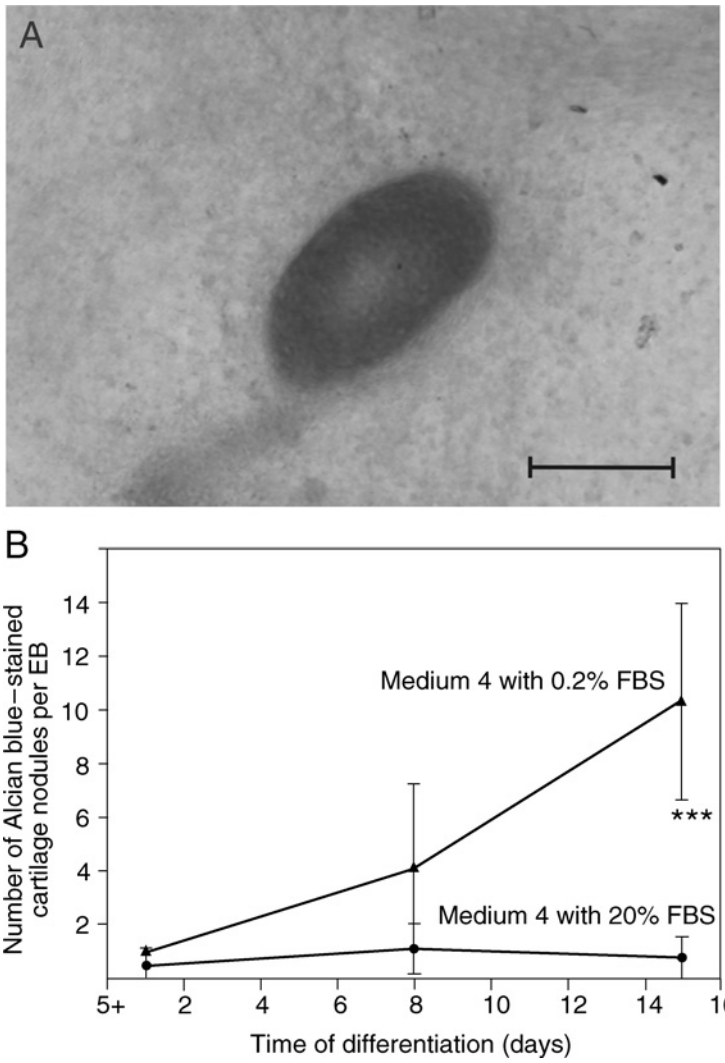


Figure 23.5.2 (**A**) Alcian blue staining demonstrates ES cell–derived cartilage nodule formation. (**B**) Cultivation of embryoid bodies (EBs) under low-serum conditions (0.2% FBS) after EB plating significantly enhanced the number of Alcian blue–positive cartilage nodules per EB. Bar = 100 μm. For the color version of this figure go to *http://www.currentprotocols.com*.

analysis) containing the appropriate volume of Medium 4 as listed in Table 23.5.2. Transfer EBs using a micropipettor with sterile 100-μl aerosol-filter barrier tips into the Medium 4 and handle the dishes very carefully during the first 2 days of culture to allow the EBs to attach.

11. Initially, change the medium after 4 and 8 days.

    In currently running studies the authors use Medium 4 with low FBS (0.2%) for chondrogenic differentiation of ES cells. This low-FBS medium (see recipe) is applied from 2 days after plating (day 5 plus 2 days) up to the end of EB cultivation. Medium is changed every 4 days. Up to the first 2 days after plating, Medium 4 (with 20% FBS) is used. Low-FBS conditions then induce cartilage nodule formation (see Fig. 23.5.2).

12. During further cultivation steps, up to 35 days after plating (day 5 plus 35 days) renew the medium about every second day.

13. To study the differentiation process, analyze samples every second to fifth day (see Table 23.5.1).

ALTERNATIVE PLATING METHOD FOR GENERATING EBs OF THE SAME SIZE

ALTERNATE PROTOCOL

This Alternate Protocol simplifies the "hanging drop" method (Basic Protocol 1), which is used as a standard protocol to obtain same-sized EBs. The Alternate Protocol is presented as an optional way to generate EBs of the same size.

For materials, see Basic Protocol 1.

1. Prepare cell suspension for hanging-drop-cultivation (see Basic Protocol 1, steps 1 to 4).

2. Place ~50 20-µl aliquots of the cell suspension on the inner side of the bottom of a 100-mm bacteriological petri dish (not the lid, in contrast to Basic Protocol 1), using a micropipettor with sterile 100-µl tips containing aerosol-filter barriers (see Fig. 23.5.1).

3. Place an open 60-mm culture dish filled with 4 ml PBS onto the lid of the bacteriological petri dish to avoid evaporation of the drops. Carefully invert the bottom of the petri dish containing the drops of ES onto the lid to create the hanging drops. Culture the hanging drops for 2 days at 37°C (day 0 to day 2).

4. Remove the PBS-filled 60-mm culture dish at the second day of hanging drop-culture, then turn the bacterial petri dish over to its normal configuration (see Fig. 23.5.1).

5. Add 8 ml Medium 4 to each 100-mm bacteriological petri dishes with the drops of EBs that are now at the bottom.

6. Cultivate EBs in suspension for 3 days (day 2 to day 5).

7. Follow Basic Protocol 1, steps 10 through 13, for plating of EBs.

MICRODISSECTION AND CHARACTERIZATION OF MESENCHYMAL CONDENSATIONS AND CARTILAGE NODULES FROM EBs

BASIC PROTOCOL 2

Mesenchymal condensations containing chondrogenic precursors are isolated around 10 days after EB plating (day 5 plus 10 days), and cartilage nodules with mature chondrocytes are isolated around 20 days after plating (day 5 plus 20 days) using either a microdissector or a microscalpel. Condensations and nodules can be easily detected by their specific morphology (Kramer et al., 2005b). EBs are cultivated as in Basic Protocol 1 (steps 1 through 11) or the Alternate Protocol (steps 1 through 7). To characterize the differentiation stage of the cells, samples of these isolated chondrogenic aggregates are investigated for expression of collagen marker proteins by immunostaining of cryosections (Hegert et al., 2002) and analyzed by ultrastructural analysis using electron microscopy.

Materials

EB cultures, (day 5 plus 10 days *or* day 5 plus 20 days; see Basic Protocol 1 or Alternate Protocol)
Tissue-Tek OCT compound (Sakura Finetek)
Acetone
Phosphate-buffered saline (PBS; see recipe)

Microscalpel or microdissector (Eppendorf)
Tissue-Tek Cryomold 10 × 10 × 5–mm (Sakura Finetek; *http://www.sakuraus.com/*)
Cryostat (Leica)
Vectabond-coated glass microscope slides (Vector)

Additional reagents and equipment for indirect immunostaining (Support Protocol 7)

1. Select nodules from EB cultures (day 5 plus 10 days or day 5 plus 20 days).
2. Microdissect the nodules and place them in ∼0.5 ml Tissue-Tek OCT compound in a Tissue-Tek Cryomold for embedding.

   Microdissection is performed using a commercially available microdissector (Eppendorf). The procedure to isolate cartilage nodules using this device is outlined in detail in Eppendorf Applications No. 48 (follow the "Applications" link at http://www.eppendorf.com).

3. Freeze the samples at −20°C.
4. Prepare 10-μm cryosections using a cryostat and plate onto Vectabond-coated slides.
5. Dry the sections in air.
6. Fix in acetone for 10 min at −20°C.
7. Perform indirect immunostaining (see Support Protocol 7, steps 3 to 10).

BASIC PROTOCOL 3

ISOLATION OF CELLS FROM SELECTED CARTILAGE NODULES BY COLLAGENASE TREATMENT

Chondrogenic cells can be isolated from cartilage nodules microdissected from EBs to analyze their behavior in single-cell cultures.

Materials

Microdissected nodules from EB cultures (Basic Protocol 2, steps 1 to 2)
0.1% (w/v) collagenase in Medium 4 (see recipe for Medium 4)
Medium 4 (see recipe)
Gelatin-coated (see recipe) or collagen II-coated 60-mm tissue culture dishes *or* chamber slides

Shaking water bath or incubator
15-ml centrifuge tubes
Centrifuge

Additional reagents and equipment for basic cell culture techniques including counting cells (*UNIT 1.1*)

1. Incubate ten microdissected nodules in 5 ml of 0.1% collagenase solution for 50 min at 37°C, with moderate shaking, to obtain a single-cell suspension.
2. Transfer the cells to a 15-ml centrifuge tube. Centrifuge the cells for 5 min at 180 × *g*, room temperature. Discard the supernatant.

3. Resuspend the cell pellet in 2 ml Medium 4 and count the cells (*UNIT 1.1*). Plate the cells at high density onto gelatin- or collagen II–coated 60-mm tissue culture dishes ($1–2 \times 10^5$ cells per 60-mm dish) or onto chamber slides (2.1×10^4 cells/well).

   Use 60-mm dishes for total RNA isolation or chamber slides for immunostaining and histochemical staining.

4. Culture cells at 37°C in Medium 4.

   The cellular processes of de-, re-, and transdifferentiation can be investigated using the isolated ES cell–derived chondrocytes (for details see Hegert et al., 2002).

HISTOCHEMICAL STAINING OF EB

Histochemical staining of EBs is performed after plating of EBs from day 5 plus 2 days up to about day 5 plus 35 days at regular intervals of about 2 days. Alcian blue staining (Support Protocol 2) is used as a fast screen for detection of cartilage nodules (see Figure 23.5.2A). Alcian blue selectively stains muco-substances, in particular cartilage-specific proteoglycans. Alizarin red (Support Protocol 3) forms chelate conjugates with bivalent cations (mainly with calcium), and is therefore used to detect bone nodule formation. In addition, von Kossa staining (Support Protocol 4) is performed to visualize bone nodule formation due to ion exchange (calcium ions are replaced by silver), which depends on the influence of daylight. ES cell–derived adipogenic differentiation can be demonstrated by cell staining with the vital dye Sudan III (Support Protocol 5), which can be tracked directly by light microscopy.

Fixation for Histochemical Staining

SUPPORT PROTOCOL 1

The specimens must be fixed before histochemical staining (except for Sudan III and AP staining).

Materials

Cultures with differentiating EBs (Basic Protocol 1), day 5 plus 2 days to day 5 plus 35 days, in chamber slides or tissue culture dishes depending on staining technique to be used (see Support Protocols below)
Phosphate-buffered saline (PBS; see recipe)
3.7% (v/v) formaldehyde in distilled H_2O

1. Remove medium from the culture and wash twice, each time for 30 sec with 1.5 ml PBS (for chamber slides) or 4 ml PBS (for 60-mm dishes).
2. Add 3.7% formaldehyde solution to the cultures (1.5 for chamber slides or 4 ml for 60-mm dishes) and incubate for 30 min at room temperature.
3. Remove formaldehyde and wash three times with PBS using the time and volume specified in step 1, then once with water using the time and volume specified in step 1.
4. Proceed to staining (Support Protocols 2 to 4).

Alcian Blue Staining

SUPPORT PROTOCOL 2

Alcian blue staining is a quick screen for cartilage nodules. The dye stains muco-molecules, especially cartilage proteoglycans.

Materials

Fixed EB cultures at the desired interval after plating on day 5 (Support Protocol 1) in 60-mm culture dishes
Alcian blue working solution (see recipe)

Phosphate-buffered saline (PBS; see recipe)
5% (v/v) acetic acid solution

1. To fixed EBs cultivated on 60-mm culture dishes, add 4 ml Alcian blue working solution.
2. Incubate the samples overnight at room temperature.
3. Remove the staining solution and wash for 30 sec once with 4 ml PBS and once with 4 ml 5% acetic acid.
4. Wash one more time with 4 ml PBS, then add ~5 ml PBS to avoid dehydration of the specimens.
5. View by light microscopy.

   Deeply blue stained nodules should be visible (see Fig. 23.5.2A).

SUPPORT PROTOCOL 3

Alizarin Red Staining

Alizarin red forms chelate-conjugates with bivalent cations (mainly with calcium) and is therefore used to detect bone nodule formation.

Materials

Fixed EB cultures at the desired interval after day 5 (Support Protocol 1) in chamber slides
100 mM Tris·Cl, pH 9 (*APPENDIX 2A*)
Alizarin red staining solution 1: 5% (w/v) Alizarin red S (Sigma) in H_2O; adjust to pH 9 with sodium hydroxide
Alizarin red staining solution 2: 0.5% (w/v) Alizarin red S (Sigma) in H_2O; adjust to pH 9 with sodium hydroxide
Vectashield mounting medium (Vector)
Clear nail polish
Coverslips

1. Incubate fixed EBs cultured on chamber slides in 100 mM Tris·Cl, pH 9, for 30 sec at room temperature.

   The reagents are added to the chambers on the slides.
2. Incubate the samples in 1.5 ml Alizarin red staining solution 1 for 1 hr.
3. Remove the staining solution and rinse two times with 1.5 ml PBS, each time for 30 sec.
4. Incubate the samples in 1.5 ml Alizarin red staining solution 2 per chamber for 5 min.
5. Remove the staining solution and rinse two times with 1.5 ml PBS, each time for 30 sec.
6. Remove chambers from slide and apply Vectashield mounting medium. Cover the slide with a coverslip and seal with clear nail polish.
7. View by light microscopy.

   Red-stained bone nodules should be visible.

Von Kossa Staining

SUPPORT PROTOCOL 4

Von Kossa staining allows visualization of bone nodule formation due to ion exchange (calcium ions are replaced by silver), which depends on the influence of daylight.

Materials

Fixed EB cultures at the desired interval after plating on day 5 (Support Protocol 1) in chamber slides
Von Kossa silver stain: 500 mg silver nitrate (Fluka) in 10 ml distilled water (5% w/v $AgNO_3$)
Von Kossa fixation solution: 1 g anhydrous sodium thiosulfate (Merck) in 20 ml distilled water (5% w/v sodium thiosulfate)
Phosphate-buffered saline (PBS; see recipe)
Vectashield mounting medium (Vector)
Clear nail polish
Coverslips

1. Treat EBs cultivated for the desired interval in chambers of chamber slides for 10 to 20 min with Von Kossa silver stain.
2. Wash carefully twice, each time with 1.5 ml distilled water for 15 sec.
3. Fix the resulting stained sample with Von Kossa fixation solution for 2 min.
4. Wash fixed EBs in chambers twice, each time with 1.5 ml PBS. Remove chambers from slide and immediately add Vectashield mounting medium, cover the slide with a coverslip, and seal with clear nail polish.
5. View by light microscopy.

Sudan III Staining

SUPPORT PROTOCOL 5

ES cell–derived adipogenic differentiation can be demonstrated by staining with the vital stain Sudan III (Support Protocol 5; also see *UNIT 23.4*), which can be tracked directly by light microscopy.

Materials

Unfixed EBs cultured in chamber slides for the desired interval after plating on day 5 (Basic Protocol 1 or Alternate Protocol)
Phosphate-buffered saline (PBS; see recipe)
Sudan III staining solution (see recipe)
Vectashield mounting medium (Vector)
Clear nail polish
Coverslips

1. Wash the EBs with PBS for 15 sec.

   The reagents are added to the chambers on the slides.

2. Add 1.5 ml Sudan III staining solution and incubate for 3 min at room temperature.
3. Wash three times, each time with 1.5 ml PBS.
4. Remove chambers from slide and immediately add Vectashield mounting medium, cover the slide with a coverslip, and seal with clear nail polish.
5. View by light microscopy.

   Lipid droplets in cells are stained red. Adipogenic cells contain large amounts of such droplets.

SUPPORT PROTOCOL 6

Alkaline Phosphatase (AP) Staining

Osteogenic cells show alkaline phosphatase activity.

Materials

Unfixed EBs cultured in chamber slides for the desired interval after plating on day 5 (Basic Protocol 1 or Alternate Protocol)
Phosphate-buffered saline (PBS; see recipe)
Leukocyte Alkaline Phosphatase staining kit (Sigma, cat. no. 86-R) containing:
  Citrate solution
  FRV-alkaline solution
  Hematoxylin solution, Gill No. 3
  Naphthol AS-BI alkaline solution
  Sodium nitrite solution
Acetone
37% (w/v) formaldehyde
Vectashield mounting medium (Vector)
Clear nail polish

15-ml conical polypropylene centrifuge tubes
Coverslips

1. Wash the EB cultures twice, each time with 1.5 ml PBS for 15 sec.

   The reagents are added to the chambers on the slides.

2. Combine 2.5 ml citrate solution (from the Sigma Leukocyte Alkaline Phosphatase staining kit) with 6.5 ml acetone and 0.8 ml of 37% formaldehyde to prepare the fixing solution. Add 1.5 ml fixing solution to the EBs and incubate 30 sec at room temperature to fix.

3. Remove the fixing solution and rinse the samples with 1.5 ml distilled water for 45 sec.

4. Prepare the AP staining solution by combining 125 µl FRV-alkaline solution and 125 µl sodium nitrate solution (both included in the staining kit) in a 15-ml tube. Mix and incubate 2 min at room temperature. Add 5.63 ml distilled water and 125 µl naphthol AS-BI alkaline solution (from kit), and mix. Add 1.5 ml of this solution to each chamber and incubate for 15 min at room temperature in the dark.

5. Wash once for 2 min in 1.5 ml distilled water. Counterstain with 1.5 ml hematoxylin solution (from kit) for 2 min according to the manufacturer's instructions. Again wash once for 2 min in 1.5 ml distilled water.

6. Remove the chambers and rinse slide thoroughly in tap water. Immediately add Vectashield mounting medium, cover slide with coverslip, and seal with clear nail polish.

7. View by light microscopy.

   Alkaline phosphatase–expressing cells are stained light red, whereas all other cells are stained a dark purple color.

SUPPORT PROTOCOL 7

INDIRECT IMMUNOSTAINING OF EBs

Whole EBs can be analyzed for specific specialized cell types by indirect immunostaining. EB cultures are set up and maintained on chamber slides. Immunostainings are performed at intervals of about 2 to 5 days starting 2 days after plating (day 5 plus 2 days) up to about 35 days after plating (day 5 plus 35 days; see Table 23.5.1). As an example, immunostaining for collagen II demonstrates ES cell-derived cartilage formation (Fig. 23.5.3).

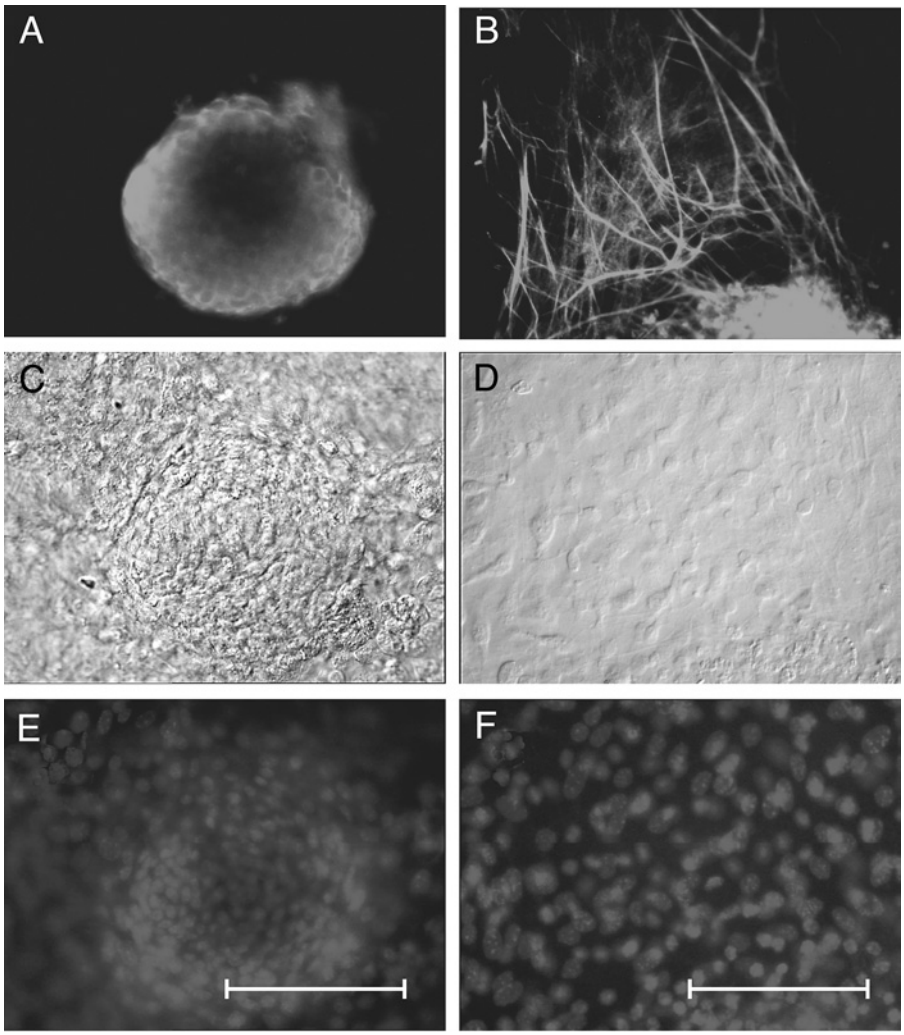


Figure 23.5.3 Immunostaining for collagen II demonstrates ES cell–derived cartilage formation. (**A**) The extracellular matrix of nodules consists of collagen II. (**B**) In addition, formation of collagen II fibrils could also be observed outside the nodules. (**C**) Chondrogenic cells within the cartilage nodules showed a typical round-shaped phenotype and the nodules were compact and clearly seperated from the surrounding tissue. (**D**) In comparison, the single cells expressing the network of collagen II fibrils outside the nodules do not form such distinct structures. (**E, F**) Cell nuclei are stained with DAPI. Bar = 100 μm. For the color version of this figure go to *http://www.currentprotocols.com*.

Materials

Unfixed EBs cultured on chamber slides for the desired interval after plating on day 5 (Basic Protocol 1 or Alternate Protocol)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
7:3 (v/v) methanol/acetone (e.g., 42 ml methanol/18 ml acetone), −20°C
10% (v/v) goat serum (Dianova, *http://www.dianova.de*) in PBS (see recipe)
Antibodies for immunostaining, primary and fluorophore-labeled secondary, appropriately diluted (see recipe)
Vectashield mounting medium (Vector)
Clear nail polish

Humidified chamber (15-cm covered glass dish with wet piece of filter paper)
Coverslips
Fluorescence microscope (e.g., AXIOSKOP; Zeiss)

Wash and fix EBs

1. Wash the cultures in the chamber slides twice, each time with 1.5 ml PBS for 30 sec.
2. Fix the cells for 5 min with 1.5 ml of 7:3 methanol/acetone at −20°C.
3. Rinse three times each time with 1.5 ml PBS for 30 sec.

Perform blocking and immunostaining

4. Move the sample in the chamber slide to a humidified chamber. Incubate the cells for 15 min in 10% goat serum at room temperature to block nonspecific antibody binding.
5. Incubate the specimens with 300 µl appropriately diluted primary antibodies for 1 hr at 37°C in the humidified chamber.
6. Rinse three times, each time with 1.5 ml PBS for 30 sec.
7. Incubate the slides for 45 min at 37°C in the humidified chamber with 300 µl of appropriately diluted fluorophore-labeled secondary antibody.

   Choice of species for the secondary antibody depends on the origin of the primary antibody (see Reagents and Solutions).

8. Wash the slides three times, each time in 1.5 ml PBS for 30 sec, then once in 1.5 ml distilled water for 30 sec.

Mount, coverslip, and examine slides

9. Remove the chambers, immediately add Vectashield mounting medium, cover the slides with a coverslip, and seal with clear nail polish. Store the slides up to 1 month in the dark at 4°C.
10. Examine slides with fluorescence microscope.

    Differentiated cell types are detected by fluorescence.

SUPPORT PROTOCOL 8

WHOLE-MOUNT FLUORESCENCE IN SITU HYBRIDIZATION FOR mRNA OF A GENE OF INTEREST COUPLED WITH IMMUNOSTAINING FOR COLLAGEN II

For a combination of fluorescence mRNA–in situ hybridization (ISH) and indirect immunofluorescence (IF) staining, a modified whole-mount procedure for EBs (Yamada et al., 1994) is used. This combination allows the simultaneous detection of a tissue-specific protein and mRNA expression. Samples of five EBs plated on chamber slides are analyzed at different developmental stages up to 35 days after plating (day 5 plus 35 days).

Materials

16-day post-coitum (p.c.) mouse embryo limb buds
TOPO II cloning kit (Invitrogen,)
pCR-BluntII-TOPO vector (Invitrogen)
DIG RNA labeling mix kit including Polymerases SP6 and T7 (Boehringer Mannheim; cat. no. 1175025).
EB cultures plated on chamber slides at day 5 (Basic Protocol 1 or Alternate Protocol)
Phosphate-buffered saline (PBS; see recipe)
4% (w/v) paraformaldehyde/4% (w/v) sucrose in PBS
2× SSC, 0.2× SSC, and 0.1× SSC (see recipe for 20× SSC)
50%, 70%, 95%, and 100% ethanol series
Prehybridization buffer (see recipe) with and without salmon sperm DNA

Monoclonal antibody II-II6B3 against collagen II (diluted 1:20 in PBS; see recipe for Antibodies for immunostaining)

FITC-conjugated sheep F(ab) fragments against digoxigenin (Roche, cat. no. 1207741).

Cy3-conjugated goat anti-mouse secondary antibodies (Dianova; cat. no. 115-165-062; *http://www.dianova.de*) for indirect detection of collagen II, both diluted 1:800 in PBS.

Vectashield mounting medium (Vector; cat. no. H-1000).

Clear nail polish

Humidified chamber (15-cm covered glass dish with wet piece of filter paper)

45° and 70°C incubators

Coverslips

Fluorescence microscope with FITC and Cy3 filters

Additional reagents and equipment for RNA isolation and cDNA synthesis via RT-PCR (Support Protocol 9), and DNA sequencing (Chapter 7 in Ausubel et al., 2007)

Generate hybridization probes

1. Isolate RNA from 16-day p.c. mouse embryo limb buds and synthesize cDNA following the RT-PCR protocol (Support Protocol 9) using the primers for the gene of interest listed in Table 23.5.3.

2. Blunt-end and clone the fragment into the plasmid vector pCR-BluntII-TOPO using the TOPO II cloning kit according to the manufacturer's protocol. Verify the sequences by sequencing (Chapter 7 in Ausubel et al., 2007).

3. Label RNA probes of sense and antisense orientation with digoxigenin using DIG RNA labeling mix kit. Synthesize the probes from linearized plasmids of the cloned cDNA fragments by in vitro transcription using the T7 or SP6 RNA polymerase and the DIG RNA labeling mix following the protocol supplied by the manufacturer.

   Final concentration of labeled probes should be 1 μg/ml.

Fix the cell-containing slides

4. Rinse EB cultures on chamber slides twice, each time with 1.5 ml PBS for 30 sec.

5. Fix the EBs with 4% (w/v) paraformaldehyde/4% (w/v) sucrose in PBS for 20 min at room temperature.

6. Wash the cells twice, each time in 1.5 ml PBS for 5 min.

7. Incubate the specimens with 1.5 ml of 2× SSC for 15 min in a humidified chamber inside a 70°C incubator.

8. Wash with 1.5 ml PBS followed by 1.5 ml 2× SSC, each time for 3 min.

9. Repeat steps 5 through 8.

Prepare the cell-containing slides for hybridization

10. Dehydrate the cells through an ethanol series at room temperature by adding 1.5 ml of the indicated concentration of ethanol directly to each chamber:

    2 min in 50% ethanol
    2 min in 70% ethanol
    2 min in 95% ethanol
    2 min in 100% ethanol
    2 min in 100% ethanol.

Table 23.5.3 Primer Pairs Used to Analyze Chondrogenic and Osteogenic Differentiation of Murine ES Cells[a]

Gene	Antisense primer	Sense primer	Annealing temperature (°C)	Fragment length (bp)	Reference
Pre-cartilage					
Chordin-like 1	5′-ACA ATG CCA AAT GCT CGT AGA T-3′	5′-TGC GAA TAC AAT GGA ACC ACT TA-3′	68	506	Nakayama et al. (2003)
Pax-1	5′-TTC TCG GTG TTT GAA GGT CAT TGC CG-3′	5′-GAT GGA AGA CTG GGC GGG TGT GAA-3′	60	318	Kramer et al. (2000)
Scleraxis	5′-GTG GAC CCT CCT CCT TCT AAT TCG-3′	5′-GAC CGC ACC AAC AGC GTG AA-3′	63	375	Kramer et al. (2000)
Sox9	5′-TCT TTC TTG TGC TGC ACG CGC-3′	5′-TGG CAG ACC AGT ACC CGC ATC T-3′	57	135	Kramer et al. (2000)
Mature cartilage					
Aggrecan	5′-TCC TCT CCG GTG GCA AAG AAG TTG-3′	5′-CCA AGT TCC AGG GTC ACT GTTACCG-3′	60	270	Kramer et al. (2000)
Biglykan	5′-CAT GAC AAC CGT ATC CGC AA-3′	5′-ATT CCC GCC CAT CTC AAT G-3′	60	—[a]	zur Nieden et al. (2005)
Collagen II	5′-AGG GGTACC AGG TTC TCC ATC-3′	5′-CTG CTC ATC GCC GCG GTC CTA-3′	60	432[b] 225[c]	Kramer et al. (2000)
COMP	5′-GCT GCC CGG TCT CAC ACT CAT T-3′	5′-GTT GCG ACA CGA GGT CAA GGA G-3′	68	410	Nakayama et al. (2003)
Decorin	5′-ATG ACC CTG ACA ATC CCC TG-3′	5′-CCC AGA TCA GAA CAC TGC ACC-3′	60	—[a]	zur Nieden et al. (2005)
Link protein	5′-TTC TGG GCT ATG ACC GCT G-3′	5′-AGC GCC TTC TTG GTC GAG A-3′	60	—[a]	zur Nieden et al. (2005)
Hypertrophic cartilage					
Collagen X	5′-ATG CCT TGT TCT CCT CTTACT GGA-3′	5′-CTT TCT GCT GCT AAT GTT CTT GAC C-3′	61	164	Hegert et al. (2002)
MMP13	5′-CGT GTG CCA GAA GAC CAG AA-3′	5′-CAG TTG ACA GGC TCC GAG AA-3′	57	372	Kawaguchi et al. (2005)
Bone					
Alkaline phosphatase	5′-TCT GGT GGC ATC TCG TTA ATC-3′	5′-CCT GAA AAC TCC AAA AGC TC-3′	57	465	Kawaguchi et al. (2005)
Cbfa-1/Runx2	5′-ATC CAT CCA CTC CAC CAC GC-3′	5′-AAG GGT CCA CTC TGG CTT TGG-3′	63	371	Kramer et al. (2000)
Osteocalcin	5′-ATG CTA CTG GAC GCT GGA GGG T-3′	5′-GCG GTC TTC AAG CCA TAC TGG TC-3′	64	330	Hegert et al. (2002)
Osteoprotegerin	5′-ACT CCT GCT TCA CGG ACT G-3′	5′-TGC TCC TGG CAC CTA CCT A-3′	57	158	Kawaguchi et al. (2005)
Osterix	5′-TGC CTG GAC CTG GTG AGA TG-3′	5′-CCT CTG CGG GAC TCA ACA AC-3′	57	355	Kawaguchi et al. (2005)

continued

Table 23.5.3 Primer Pairs Used to Analyze Chondrogenic and Osteogenic Differentiation of Murine ES Cells[a], *continued*

Gene	Antisense primer	Sense primer	Annealing temperature (°C)	Fragment length (bp)	Reference
PTHR1	5′-ACA GTC CCT CCA CCA GAA TC-3′	5′-ACT ACA GCG ACT GCC TCA AG-3′	57	417	Kawaguchi et al. (2005)
RANKL	5′-AGT ACG TCG CAT CTT GAT CC-3′	5′-GGA AGC GTA CCT ACA GAC TA-3′	57	234	Kawaguchi et al. (2005)
Dedifferentiated cartilage					
Collagen I	5′-GGC GGT TAT GAC TTC AGC TTC -3′	5′-GGC ATG TTG CTA GGC ACG AAG-3′	60	702	—[d]
Internal standard					
β-Tubulin	5′-GGA ACA TAG CCG TAA ACT GC-3′	5′-TCA CTG TGC CTG AAC TTA CC-3′	54	317	Kramer et al. (2000)
HPRT	5′-GCC TGT ATC CAA CAC TTC G-3′	5′-AGC GTC GTG ATT AGC GAT G-3′	63	507	Kramer et al. (2000)

[a]Not given.
[b]Juvenile splice variant.
[c]Adult splice variant.
[d]Not published.

11. Prehybridize 3 hr in 1.5 ml prehybridization buffer in a humidified chamber in an incubator at 45°C.
12. Perform hybridization with 500 µl/well of 1 ng/µl digoxigenin-labeled sense and antisense probes (prepared in step 3) in prehybridization buffer without salmon sperm DNA at 45°C in a humidified chamber overnight.
13. The next day, wash the slide twice with 2× SSC for 15 min, once with 0.2× SSC for 15 min and twice with 0.1× SSC for 15 min, all at 45°C. Rinse in 1.5 ml PBS for 30 min.

Immunolabel the protein

14. Apply 300 µl monoclonal antibody II-II6B3 against collagen II (diluted 1:20 in PBS) to the cells and incubate in a humidified chamber for 1 hr at 37°C.
15. Wash the cells three times each time with 1.5 ml PBS at room temperature for 30 sec.
16. Add 300 µl FITC-conjugated sheep F(ab) fragments against digoxigenin to detect the RNA and 300 µl Cy3-conjugated goat anti-mouse secondary antibodies for indirect detection of collagen II, both diluted 1:800 in PBS as a cocktail. Incubate for 1 hr at 37°C.
17. Wash the slides three times, each time in 1.5 ml PBS for 30 sec, then once in 1.5 ml distilled water for 30 sec.
18. Mount in Vectashield mounting medium, cover the slides with coverslips, and seal with clear nail polish.
19. Analyze using a fluorescence microscope.

Differentiated cell types are detected by ISH and IF.

RT-PCR ANALYSIS TO DETECT CARTILAGE-SPECIFIC GENE EXPRESSION IN EBs

The expression of tissue-specific genes is analyzed during EB cultivation from days 1 to 5 days before plating, and up to 35 days after plating (day 5 plus 35 days). Total RNA is isolated from 25 EBs at the stages before plating (days 0 to day 5). After plating, samples of ten EBs are used (from one 60-mm tissue culture dish). Distilled water and no-RT reactions are always included as negative controls. RNA from limb buds or limbs of 10 and 16 day p.c. mouse embryos are used as positive controls.

Materials

Cultures of EBs at the desired time points (Basic Protocol 1 or Alternate Protocol)
Phosphate-buffered saline (PBS; see recipe)
RNeasy Mini Kit (Qiagen)
RNase-free DNase set (Qiagen)
Oligo-dT primer (Life Technologies)
Superscript II reverse transcriptase (Life Technologies)
Taq DNA polymerase (Roche)
Primer (see Table 23.5.3)
2% (w/v) agarose gel (Voytas, 2000)

Additional reagents and equipment for quantification of nucleic acids (*APPENDIX 3D*), isolation of total RNA (Ausubel et al. 2007, Chapter 4), RT-PCR (Beverley, 2001), agarose gel electrophoresis (Voytas, 2000), and densitometry (*UNIT 6.3*)

1. Wash EB cultures twice, each time with 4 ml PBS for 30 sec.
2. Isolate total RNA using the RNeasy Mini Kit along with the RNase-free DNase set (to avoid DNA contamination) according the manufacturers' protocols (also see Chapter 4 in Ausubel et al., 2007).
3. Determine the RNA concentrations by measuring the absorbance at 260 nm (*APPENDIX 3D*).
4. Reverse transcribe samples of 500 ng RNA using oligo-dT primer and Superscript II reverse transcriptase according to the manufacturer's protocols (also see Beverley, 2001).
5. Use 1-µl aliquots from the RT reactions for amplification of transcripts with primer (see Table 23.5.3) specific for the analyzed genes and *Taq* DNA polymerase according to the manufacturer's instructions. Use the following program:

1 cycle:	2 min	95°C	(initial denaturation)
35 to 45 cycles:	40 sec	95°C	(denaturation)
	40 sec	primer-specific temperature	(annealing)
	50 sec	72°C	(elongation).

Cycle numbers have to be determined using positive controls of RNA isolated from limb buds of 18-day-old embryos. The optimal cycle number depends upon the PCR machine used; further information may be found in the references listed in Table 23.5.3.

Primer-specific temperatures are listed in Table 23.5.3. For primer pairs established by other groups please also compare to the original reference given in Table 23.5.3.

For RT-PCR analysis of scleraxis, an established method (Wong et al., 1994; Wobus et al., 1997) including the oligonucleotide primer for HPRT and scleraxis in the same reaction, can be used.

6. Separate PCR products electrophoretically on 2% agarose gels (Voytas, 2000).

7. Analyze the fragments by computer-assisted densitometry (*UNIT 6.3*) in relation to GAPDH, HPRT, or β-tubulin gene expression.

   It has been suggested that GAPDH is a more suitable candidate to act as an internal RNA standard, while both HPRT and β-tubulin appear to be inappropriate (Murphy and Polak, 2002).

ULTRACTRUCTURAL ANALYSIS OF ES CELL-DERIVED MESENCHYMAL CONDENSATIONS AND CARTILAGE NODULES BY ELECTRON MICROSCOPY

SUPPORT PROTOCOL 10

ES cell–derived mesenchymal condensations and cartilage nodules exhibit a typical morphology and can be identified in EB outgrowths by light microscopy. The following protocol prepares such structures for further electron-microscopic analysis.

Materials

ES cell–derived mesenchymal condensations and cartilage nodules (see Basic Protocol 2)
5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4
1% (w/v) OsO_4
50%, 70%, 95% and 100% ethanol
Araldite embedding kit (e.g., Fluka)
Ultracut E (Leica Microsystems Nussloch, *http://www.leica-microsystems.com/*)
LKB Bromma Ultrostainer Carlsberg System for uranyl acetate and lead citrate staining

CAUTION: Glutaraldehyde and OsO_4 are potentially hazardous reagents. Work should be performed under a hood and protective clothing and gloves must be used during the procedure.

1. Fix the ES cell–derived outgrowths with 1 ml 5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for ∼1 hr at 4°C.
2. Treat specimens with 1 ml 1% OsO_4 for 2 hr at room temperature.
3. Dehydrate the EB outgrowths in graded ethanol series:

   50% ethanol
   70% ethanol
   95% ethanol
   100% ethanol.

4. Embed the specimens in Araldite using kit according to manufacturer's instructions.
5. Cut ultrathin sections of the EBs on an Ultracut E.
6. Stain the sections with uranyl acetate and lead citrate using the LKB Bromma Ultrostainer Carlsberg System according to manufacturer's instructions.
7. Examine the sections for chondrogenic cell types with an electron microscope.

DERIVATION, CULTIVATION, AND INACTIVATION OF EMBRYONIC FIBROBLASTS

SUPPORT PROTOCOL 11

To grow them in an undifferentiated state, ES cells are cocultivated with a feeder layer of murine embryonic fibroblasts. Soluble factors produced by the feeder layer cells prevent the ES cells from differentiation (Evans and Kaufman, 1981). Embryonic fibroblasts used as feeder layers for ES cell cultivation are prepared and cultivated according to an established method (Wobus et al., 1984).

Materials

Day 14 p.c. pregnant mice
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Trypsin/EDTA solution (see recipe)
Medium 1 (see recipe) for cultivation of embryonic fibroblasts
1 μg/ml MMC working solution (see recipe)
Medium 3 (see recipe)

100 mm-bacteriological petri dishes (Greiner), uncoated
Sterile dissection instruments: scissors, forceps
100-ml Erlenmeyer flask with a stir bar, sterile
~4-mm-diameter glass beads
Sieve (e.g., autoclavable metal tea filter) with pore diameter of ~0.5 mm
15- and 50-ml conical polypropylene centrifuge tubes
Gelatin-coated (see recipe) 100-mm and 60-mm dishes
2 ml-cryopreservation vials (Nunc)
Dewar flasks with liquid nitrogen

Additional reagents and equipment for counting cells (*UNIT 1.1*)

Derive embryonic fibroblasts

1. Remove embryos at day 14 p.c. from uteri of pregnant mice and place under sterile conditions in PBS in a 100-mm bacteriological petri dish.

   Mouse embryos 14 days p.c. are optimal for isolation of embryonic fibroblasts used as a feeder layer. However, embryos up to 16 days p.c. can be used.

2. In a separate dish containing PBS, cut off the placenta and fetal membranes and remove head and liver using sharp sterile scissors.

3. Transfer the remaining carcasses first into a new petri dish with PBS for 30 sec and then into a dish with 5 ml trypsin/EDTA solution.

4. Mince the carcasses in the trypsin/EDTA solution at room temperature.

5. Transfer the 5 ml solution to a sterile 100-ml Erlenmeyer flask with fifty 4-mm glass beads and stir for 25 up to 45 min until most of the tissue pieces are dissociated, but not longer than 45 min.

6. Filter the resulting cell suspension through a sterile sieve and flush into a 15-ml conical centrifuge tube with 10 ml medium 1.

   The authors use a metal tea filter that can be autoclaved for a sieve.

7. Centrifuge the cells 5 min at 180 × *g*, room temperature. Remove the supernatant very carefully by aspiration and resuspend the pellet in 3 ml Medium 1.

8. Plate ~2 × 10^6 cells, isolated from about two embryos, onto a gelatin-coated 100-mm tissue culture dish filled with 10 ml Medium 1. Culture at 37°C in a humidified 5% CO_2 incubator overnight before freezing (at 1 × 10^6 cells/ml in Medium 3 in 2-ml cryovial) or MMC treatment (steps 9 to 12).

   These feeder layer cells can be cultivated for 1 to 2 days and either trypsinized and frozen in medium 3 (1 dish per freezing vial) or split 1:3 onto gelatin-coated 100-mm tissue culture dishes and cultivated for one more day in medium 1 for further use.

Inactivate embryonic fibroblasts

9. Incubate the cells with 6 ml MMC solution for ~2.5 hr at 37°C.

   CAUTION: *Mitomycin C (MMC) is a carcinogen, and for this reason at least in these steps disposable material/pipets are used.*

MMC is used for growth inactivation of the subcultured or frozen feeder cells before cocultivation with ES cells.

Frozen feeder layer cells are thawed rapidly, plated onto two gelatin-coated 100 mm-tissue culture dishes and cultivated overnight before MMC treatment as described in step 8.

10. Aspirate MMC solution. Wash the cells three times each time with 15 ml PBS for 30 sec.

11. Add 2 ml trypsin/EDTA solution to the cells and incubate 30 to 60 sec at room temperature, observing the detachment of the cells under the microscope. Suspend the cells in the trypsin/EDTA solution by pipetting up and down several times and immediately transfer them to a centrifuge tube with 10 ml Medium 1. Centrifuge 5 min at 180 × *g*. Determine the cell number (*UNIT 1.1*).

12. Plate ~1.5 × 10^5 cells onto gelatin-coated 60 mm-culture dishes with 4 ml Medium 1 to create a confluent monolayer. Use as feeder cells in Support Protocol 12.

Cells can be used immediately or stored up to 5 days in the incubator before use. They should not be frozen after MMC treatment.

PROLIFERATION AND SUBCULTIVATION OF ES CELLS

SUPPORT PROTOCOL 12

Murine ES cells are maintained in the undifferentiated state by cultivation on feeder layer cells (Support Protocol 11) and under the influence of leukemia inhibitory factor (LIF; Smith et al., 1988; Williams et al., 1988). LIF, also produced by feeder layer cells, binds to a heterodimer of the LIF receptor and gp130 that activates JAK/Stat3 signaling, and activated Stat3 is sufficient to sustain undifferentiated proliferation of mouse ES cells cultured in serum (Hocke, 1995). It is critical to subculture ES cells carefully for maintaining the undifferentiated ES cells in the pluripotent state. Therefore, ES cells should be subcultivated every 24 to a maximum of 48 hr.

Materials

Feeder layer cells: 60-mm dishes of MMC-treated embryo fibroblasts (Support Protocol 11)
Medium 2 (see recipe)
Frozen vial of ES cells
Phosphate-buffered saline (PBS; see recipe)
Trypsin/EDTA solution (see recipe)
Medium 3 for freezing the ES cells

15- and 50-ml conical polypropylene centrifuge tubes
2-ml glass pipet
2-ml cryopreservation vials (Nunc)
Dewar flasks with liquid nitrogen

Thaw undifferentiated ES cells

1. At a time point 2 hr before thawing ES cells, replace the Medium 1 in the dishes containing the MMC-treated feeder layer with 4 ml Medium 2. Return the cells to the incubator.

2. Thaw the vial with ES cells rapidly at 37°C.

3. Dilute the cold cell suspension 1:10 with Medium 2 in a 15-ml centrifuge tube and centrifuge 5 min at 180 × *g*, room temperature.

4. Resuspend the cell pellet in 2 ml Medium 2 and transfer to the prepared feeder layer. Incubate 1 to 2 days.

   The cell suspension is divided on to two 60-mm feeder layer dishes at 1 ml per plate. This corresponds to ~0.5 × 10^6 cells per plate.

Subcultivate undifferentiated ES cells

For cultivation of ES cells in the pluripotent state it is important that the cells grow in distinct colonies with a clear contour and a limited size.

5. Replace Medium 1 with 4 ml Medium 2 on fresh feeder layer cultures 1 to 2 hr before subcultivation.
6. At time of subcultivation, remove Medium 2 from the ES cells and wash the cells once with 4 ml PBS.
7. Add 2 ml trypsin/EDTA solution to the ES cells and incubate for 30 to 60 sec at room temperature. Monitor the detachment of the cells under the microscope, then suspend the cells in trypsin/EDTA solution by pipetting up and down several times.
8. Immediately transfer the cells to a 15-ml conical centrifuge tube with 10 ml Medium 2, then centrifuge 5 min at 180 × *g*, room temperature. Remove supernatant.
9. Resuspend the cell pellet thoroughly in 1.5 ml Medium 2 using a 2-ml glass pipet.
10. Check by microscopy that the suspension is a single-cell suspension, which is essential for efficient cultivation.
11. Transfer the single-cell suspension, splitting it 1:3 into 60 mm-tissue culture dishes with MMC-inactivated feeder layers.

Freeze undifferentiated ES cells

12. Trypsinize the cells and resuspend in 10 ml Medium 2.
13. Centrifuge for 5 min at 180 × *g*, room temperature. Resuspend the cells in 1.5 ml Medium 3.
14. Transfer the single-cell suspension to a 2-ml freezing vial.
15. Freeze the cells slowly at −80°C overnight.

    To avoid freezing the cells too rapidly, a simple styrofoam box or a freezing container (Cryo 1°C freezing container; Nalgene) can be used.

16. After 7 to 10 days, transfer the vials into liquid nitrogen.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocols. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alcian blue working solution, 0.05%, pH 1.5

Dissolve the following in 500 ml of 3% (v/v) acetic acid:
0.25 g Alcian blue (Sigma; cat. no. A-3157)
4.5 g sodium chloride
6.4 g magnesium chloride
Stir 2 to 3 hr
Clear by filtration through filter paper
Store up to 6 months at room temperature

Antibodies for immunostaining, primary and secondary

Primary antibodies: the following monoclonal antibodies (MAbs) can be obtained from the Developmental Studies Hybridoma Bank, Iowa City, U.S.A. (*http://www.uiowa.edu/~dshbwww/*). The designation of the MAb, the dilution (in PBS, see recipe) and a reference are given in brackets:

Collagen II [II-II6B3; 1:20; Linsenmayer and Hendrix (1980)]
Osteopontin [MPIIIB10_1; 1:10; Dorheim et al. (1993)]
Collagen X [X-AC9; 1:20; Schmid and Linsenmayer (1985)]
Bone sialoprotein I and II [WVID1(9C5); 1:10; Dorheim et al. (1993)]

Immunostaining for cartilage oligomeric matrix protein (COMP) and collagen I (CoI) are performed using polyclonal antisera. Again, designation of Ab, dilution in PBS and a reference are given in brackets:

[COMP; 1:20; Hedbom et al., (1992); a kind gift of M. Paulsson, Köln, Germany]
[CoI; 1:100; Chemicon cat. no. AB765P)]

In addition, the MAbs for cytokeratin (cytokeratin 1, 4, 5, 6, 8, 10, 13, 18, 19; 1:100; Sigma; cat. no. C-2562) and sarcomeric actinin (EA-53; 1:200; Sigma; cat. no. A-7811) are used to characterize transdifferentiation of isolated chondrocytes.

Secondary antibodies (dilution in PBS; see recipe): FITC (1:200)– or Cy3 (1:400)–labeled anti-mouse IgG (Dianova; *http://www.dianova.com*; cat. no. 111-015-144, 115-165-062)

2-Mercaptoethanol, 5 mM

Add 7 μl of 2-mercaptoethanol (2-ME; Serva) to 10 ml PBS (see recipe) to prepare the 5 mM 2-ME-stock solutio. Sterilize by filtration through a 0.2-μm filter.

2-ME is used in media at a concentration of 50 μM (1 ml 2-ME-stock solution per 100 ml medium).

Denhardt's solution, 50×

5 g Ficoll 400
5 g polyvinylpyrrolidone
5 g bovine serum albumin (BSA, Fraction V)
500 ml distilled H_2O
Store up to 1 year at −20°C

Fetal bovine serum (FBS)

Heat inactivation: Thaw FBS (Sigma) at room temperature and incubate at 54°C for 30 min for heat inactivation.

To select an appropriate FBS batch for supplementation of media (see individual recipes): ES cells (line BLC6 and D3) are differentiated via EBs using Medium 4 (see recipe; see Basic Protocol 1) supplemented with different FBS batches and analyzed for their chondrogenic differentiation efficiency by Alcian blue staining (see Support Protocol 2).

Gelatin-coated culture ware

Prepare a 1% (w/v) stock solution of gelatin (Fluka, cat. no. 48720) in PBS (see recipe) and autoclave. At a time point 1 day before cell or EB plating dilute the stock solution 1:10 with PBS for a final concentration of 0.1%, fill the dishes, multiwell

plates, or chamber slides with the 0.1% gelatin solution, and incubate overnight at 4°C. Aspirate the gelatin solution immediately before use.

Low-FBS medium

Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with:
0.2% (v/v) heat-inactivated FBS (see recipe)
100 U penicillin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
100 μg/ml streptomycin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
50 μM 2-mercaptoethanol (add from 5 mM 2-ME stock; see recipe)
20 μM L-glutamine (add from 2 mM stock; Invitrogen)
1× nonessential amino acids (add from 100× stock; Invitrogen)
Store up to 1 week at 4°C

Medium 1

Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with:
15% (v/v) heat-inactivated FBS (see recipe)
100 U penicillin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
100 μg/ml streptomycin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
50 μM 2-mercaptoethanol (add from 5 mM 2-ME stock; see recipe)
20 μM L-glutamine (add from 2 mM stock; Invitrogen)
1× nonessential amino acids (add from 100× stock; Invitrogen)
Store up to 1 week at 4°C

Medium 2

Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with:
15% (v/v) heat-inactivated FBS (see recipe)
100 U penicillin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
100 μg/ml streptomycin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
50 μM 2-mercaptoethanol (add from 5 mM 2-ME stock; see recipe)
20 μM L-glutamine (add from 2 mM stock; Invitrogen)
1× nonessential amino acids (add from 100× stock; Invitrogen)
5 ng/ml leukemia inhibitory factor (LIF; Chemicon, cat. no. LIF2005)
Store up to 1 week at 4°C

Medium 3

Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with:
20% (v/v) heat-inactivated FBS (see recipe)
100 U penicillin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
100 μg/ml streptomycin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
50 μM 2-mercaptoethanol (add from 5 mM 2-ME stock; see recipe)
20 μM L-glutamine (add from 2 mM stock; Invitrogen)
1× nonessential amino acids (add from 100× stock; Invitrogen)
8% (v/v) dimethylsulfoxide
Store up to 2 days at 4°C

Medium 4

Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with:
20% (v/v) heat-inactivated FBS (see recipe)
100 U penicillin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
100 μg/ml streptomycin (add from 10,000 U/ml penicillin/10,000 μg/ml streptomycin stock; Invitrogen)
50 μM 2-mercaptoethanol (add from 5 mM 2-ME stock; see recipe)
20 μM L-glutamine (add from 2 mM stock; Invitrogen)
1× nonessential amino acids (add from 100× stock; Invitrogen)
Store up to 1 week at 4°C

Mitomycin C (MMC) working solution, 10 μg/ml

Dissolve 2 mg mitomycin C (MMC; Serva) in 10 ml PBS (see recipe) to prepare the MMC stock solution. Sterilize by filtration through a 0.2-μm filter and store up to 6 months at −20°C. Dilute 300 μl of MMC stock solution in 6 ml of Medium 1 (see recipe) to obtain a 1 μg/ml MMC working solution.

Phosphate buffered saline (PBS)

10 g/liter NaCl
0.25 g/liter KCl
1.44 g/liter $Na_2HPO_4 \cdot H_2O$
0.25 g/liter KH_2PO_4
Adjust to pH 7.2 using 0.1 N NaOH
Sterilize by autoclaving
Store up to 6 months at room temperature

Prehybridization buffer

5× SSC (see recipe for 20×)
5× Denhardt's solution (see recipe)
50% (v/v) formamide
250 μg/ml yeast tRNA (Sigma; cat. no. R-8759)
250 μg/ml denatured salmon sperm DNA (Life Technologies; omit as indicated in protocol)
4 mM EDTA
Store up to 3 months at −20°C

SSC, 20×

175.3 g NaCl
88.2 g sodium citrate dihydrate
Adjust pH to 7.0
Add H_2O to 1000 ml
Store up to 6 months at room temperature

Sudan III staining solution

Prepare 0.2% to 0.3% (w/v) Sudan III (Sigma, cat. no. S-4136) in 70% ethanol. Heat the solution for 15 min at 60°C and filter through filter paper. Store up to 3 months at room temperature.

Trypsin/EDTA solution

Combine the following in a 1:1 ratio:
0.2 % (w/v) trypsin in PBS (see recipe for PBS)
0.02 % (w/v) disodium EDTA in PBS (see recipe for PBS)
Store the trypsin and EDTA stock solutions up to 1 year at −20°C

COMMENTARY

Background Information

Main stages of chondrogenesis are recapitulated during ES cell differentiation in vitro

Skeleton formation during embryogenesis is an orchestrated multistep process that is still incompletely understood (for review see Sandell and Adler, 1999; Cancedda et al., 2000; Provot and Schipani, 2005). Bones of the vertebral column, pelvis, and upper and lower limbs are formed on an initial cartilaginous template during vertebrate skeletal development. This process of endochondral ossification is represented by a complex cascade of cellular events including proliferation, hypertrophy, and apoptosis of chondrogenic cells. Initially, mesenchymal cells condense and form mesenchymal aggregations at the locations where later skeletal elements occur. These mesenchymal cells are chondrogenic progenitor cells differentiating into early chondroblasts, producing the extracellular matrix of the cartilage anlagen. Finally, the cartilage template is replaced by bone. However, in a few skeletal elements such as the lateral parts of the clavicle and parts of the skull, the mesenchymal cells bypass the chondrogenic stage and differentiate directly into osteoblast cells via intramembranous ossification. Moreover, different types of chondrocytes form the hyaline joint (Mitrovic, 1977), the tracheal and nasal cartilage (Pavlov et al., 2003), and elastic cartilage of the external ear (Moskalewski, 1976).

The specific patterns of marker-molecule expression can be used to define different stages of chondrogenesis and cellular subtypes of cartilage. Sox5 and Sox6 expression reveal the onset of differentiation into the chondrogenic lineage after the formation of condensations by mesenchymal cells (Lefebvre et al., 1998). These chondrogenic precursors differentiate into chondroblasts expressing cartilage marker molecules such as the proteoglycan aggrecan and the main protein of mature cartilage, collagen II. Collagen X expression indicates that the chondrocytes have become hypertrophic, and, finally, these cells undergo apoptosis (Zenmyo et al., 1996) or differentiate into osteoblasts (Bianco et al., 1998), depending on their microenvironment (Riminucci et al., 1998). To distinguish between elastic and hyaline cartilage, the expression analysis of elastic fibrils, e.g., elastin (Moskalewski, 1976), can be used. In contrast to elastic cartilage, hyaline cartilage lacks elastin.

The in vitro ES cell model system has been established to study cartilage cell differentiation. The authors have demonstrated that pluripotent mouse ES cells cultivated as cellular aggregates, so-called embryoid bodies (EBs), differentiate spontaneously into chondrogenic cell types in vitro (Kramer et al., 2000; Hegert et al., 2002). Cellular events during chondrogenesis can be recapitulated from the undifferentiated stem cell to mesenchymal and chondrogenic progenitor cells, through mature and hypertrophic chondrocytes resulting in osteogenic cells. In line with developmental processes in vivo, the mesenchymal cells form condensations in the EBs expressing mesenchymal marker molecules such as the transcription factors scleraxis (Kramer et al., 2000) and Sox5 and Sox6 (Kramer et al., 2005b), as well as the cell adhesion molecule N-cadherin (H.G. Hargus, unpub. observ.). Furthermore, these cells bind peanut agglutinin. Later on, these cellular condensations acquire a compacted phenotype, and collagen II expression can be detected at the stage of cartilage nodule formation (Fig. 23.5.3). Alcian blue staining (see Fig. 23.5.2A) demonstrates that cartilage-specific proteoglycans can be found in the ES cell–derived cartilage nodules. Indeed, large amounts of proteoglycans as well as collagen fibrils are detectable by electron microscopy within the nodules (Kramer et al., 2005b). Immunostaining against elastin reveals that this marker of elastic cartilage is not detectable within the nodules. However, some single collagen II positive cells within the EB-outgrowths outside the nodules express elastin. Moreover, groups of single cells express collagen II fibrils, as revealed by immunostaining (see Fig. 23.5.3). During later cultivation stages, collagen II expression in the ES cell–derived cartilage nodules is down-regulated, and increasing collagen X expression indicates that cells are becoming hypertrophic. At this stage, collagen II fibrils outside the nodules are still detectable. Finally, the nodules are no longer stainable with Alcian blue, and expression of bone marker molecules such as osteopontin, bone sialoprotein, and osteocalcin can be detected. Moreover, cells within these late nodules produce alkaline phosphatase, and the nodules can be stained by Von Kossa silver stain and Alizarin red due to osteomineralization. Furthermore, direct differentiation of precursors into osteoblasts has rarely been

detected in EB outgrowths independent of cartilage formation (Hegert et al., 2002).

Single-cell analysis of ES cell–derived chondrocytes demonstrates that these cells exhibit a plasticity of differentiation (Hegert et al., 2002). Chondrogenic cells expressing collagen II are isolated from cartilage nodules within the EBs. These nodules show a distinct phenotype and can easily be detected by light microscopy. After collagenase treatment, the cells are plated in low-density culture and initially show dedifferentiation during further single-cell cultivation, as indicated by a decreasing expression of collagen II and X and increasing expression of collagen I. Moreover, isolated cells cultivated in single-cell culture initially showed a fibroblastoid phenotype, but the cells later redifferentiated into chondrogenic cells expressing collagen II and X again, but not collagen I. Interestingly the redifferentiated mature chondrocytes aggregate again into cartilage nodules. Because TGF-β_3 promotes chondrogenic differentiation in cultures of mesenchymal stem cells (Mackay et al., 1998), an investigation was performed with application of TGF-β_3 (10 ng/ml) or (50 ng/ml) to the culture medium of chondrogenic cells isolated from EBs (Hegert et al., 2002). Analysis of the data showed that the process of redifferentiation was completely inhibited by TGF-β_3, independently of the concentration used. Moreover, mature chondrocytes isolated from ES cell–derived cartilage nodules exhibit a distinct plasticity with respect to transdifferentiation. Differentiation of other cell types, in addition to dedifferentiating and redifferentiating chondrocytes, was observed after prolonged cultivation of the isolated cells. Adipocytes were detected by Sudan III staining; muscle cell and epithelial cell differentiation was revealed by immunostaining for sarcomeric α-actinin and pan-cytokeratin, respectively. Analysis revealed that the additional cell types formed colonies. The numbers of these colonies and of additional cells were relatively low compared to the number of chondrogenic cells. The possibility that the observed additional cell types originated from contaminating precursor cells was excluded by repeated clonal analysis (Hegert et al., 2002).

Modulation of ES cell–derived cartilage formation

In general, different exogenous factors influence the differentiation of ES cells into the chondrogenic lineage (Fig. 23.5.4).

In general, cell density plays an important role in determining the degree of cell-to-cell contact mediating intercellular signals for cellular differentiation. In fact, the number of ES cells used for EB formation via hanging-drop cultivation was found to be decisive for the formation of cartilage nodules. EBs derived from the ES cell line D3 were prepared from 200, 500, and 800 cells, and the highest number of nodules was detected in EBs derived from 800 cells. Moreover, pellet culture and high-cell-density micromass culture, which allow

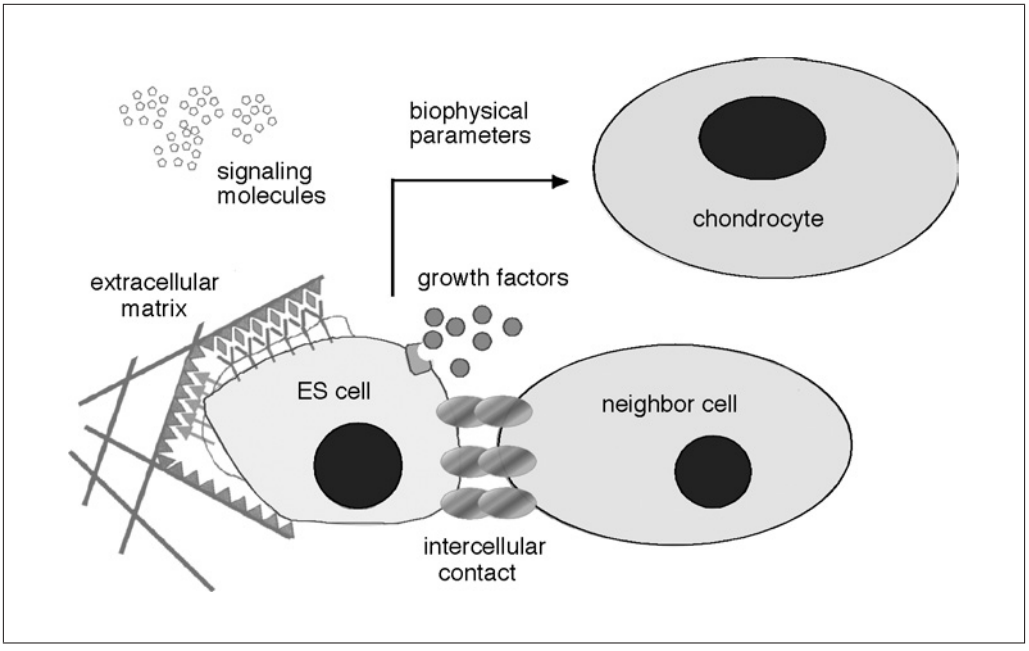


Figure 23.5.4 Different factors and signals influence chondrogenic differentiation of embryonic stem cells.

cell-cell interactions, are favorable for chondrogenic differentiation of ES cells, but alginate encapsulation of EBs does not increase cartilage formation in comparison to plating of EBs (Tanaka et al., 2004). Even the use of different ES cell lines for generation of EBs revealed differences regarding the efficiency of chondrogenic differentiation (Kramer et al., 2005a). For example, in EBs derived from ES cell line BLC6, the number of cartilage nodules was ~5-fold higher ~3 weeks after plating, compared to those derived from ES cell line D3. Other basic parameters playing an important role on the differentiation potential of ES cells are the basic cultivation medium, the batch of serum, the time of EB cultivation in suspension, and the day of EB plating (Kramer et al., 2003). For example, nodule formation was enhanced in outgrowths of EBs cultivated in DMEM compared to EBs cultivated in Iscove's modified Dulbecco's medium (J. Kramer, unpub. observ.). Previous studies showed that optimal differentiation in the mesenchymal direction could be obtained by cultivation in suspension for 3 days (days 2 to 5) and plating at the fifth day of differentiation (Rohwedel et al., 1994, 1998a,b). To establish a defined culture milieu, it is favorable to avoid the application of FBS. However, previous studies showed a reduced ES cell–derived mesodermal differentiation in the absence of FBS. It has been demonstrated that low FBS conditions do not significantly alter the differentiation efficiency of ES cells (Gissel et al., 2005). Actually, EB cultivation in low FBS induces ES cell–derived cartilage nodule formation (J. Kramer and J. Rohwedel, unpub. observ.; Fig. 23.5.2B). Another important factor for modulation of a specific differentiation process is the presence of other cell types in the cellular environment. For example, it has been demonstrated that pluripotent murine ES cells can be directed to differentiate into chondrocytes by coculture with progenitor cells from the limb buds of the developing embryo (Sui et al., 2003).

Growth factors and signaling molecules are useful to influence the efficiency of differentiation (Fig. 23.5.5). Different cytokines and growth factors have been described to promote stem cell–derived chondrogenesis (for review see Heng et al., 2004). ES cell–derived chondrogenesis is modulated by growth factors of the TGF-β-family (Kramer et al., 2000). For example, a slightly reduced ES cell–derived chondrogenic differentiation was detected after treatment of EBs with 2 ng/ml TGF-β_1 for the whole time of differentiation (from day 1 up to day 5 plus 35 days). Application of FGF-2 also resulted in a minor decrease of cartilage nodule formation in EBs (Kramer et al., 2003). However, if BMP-2 (at a concentration of 2 ng/ml) or BMP-4 (at a concentration of 10 ng/ml) was applied to the

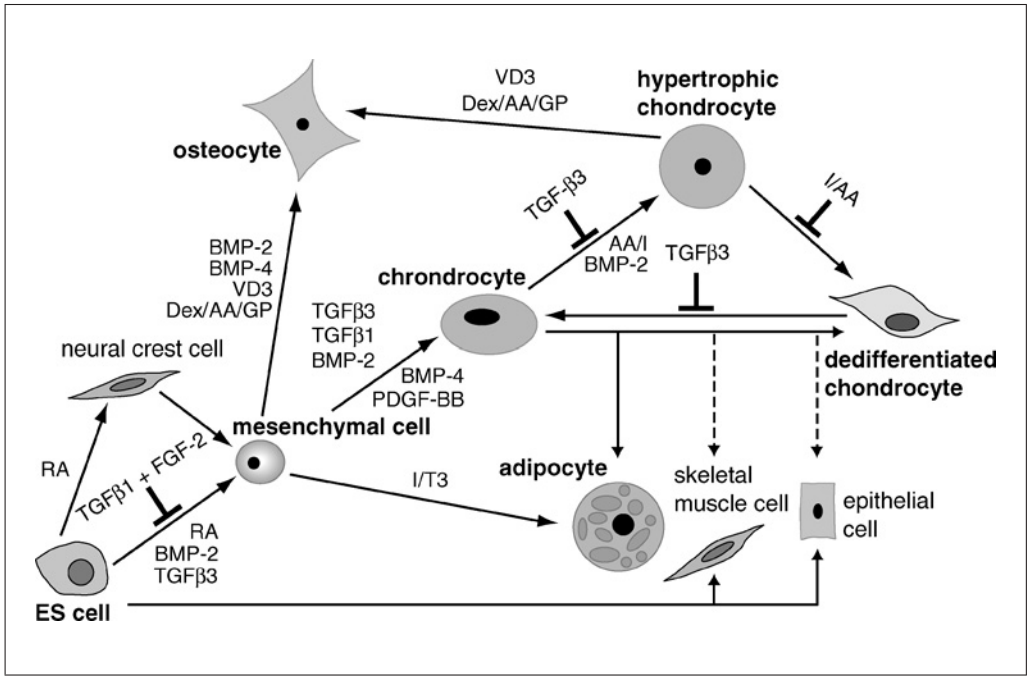


Figure 23.5.5 Overview on some growth factors and signaling molecules influencing mesenchymal ES cell differentiation in vitro.

EBs during the entire cultivation time (from day 1 up to day 5 plus 35 days) the development of chondrogenic cells organized in nodules increased ~2.5 fold (Kramer et al., 2000). No obvious differences in the size or growth rate of EBs cultivated in the presence or absence of growth factors were observed. Furthermore, the modulation of ES cell–derived cartilage formation by BMP-2 was found to depend on a specific stage of EB differentiation. The effect of BMP-2 to enhance chondrogenic differentiation was limited to a time window corresponding to the cultivation step of the EBs in suspension (days 2 to 5). In line with these findings, previous studies revealed the sensitivity of this period for the influence of signaling molecules, such as retinoic acid (RA), on other mesodermal cell types, e.g., cardiogenic, skeletal muscle, and adipogenic cells (Wobus et al., 1994; Dani et al., 1997). In addition, it is known that this early stage of ES cell differentiation is a decisive period of early mesodermal development (Yamada et al., 1994; Rohwedel et al., 1998a). RA treatment during EB cultivation in suspension seems to play a pivotal role in induction of the mesodermal pathway of ES cell differentiation (Dani et al., 1997; Kawaguchi et al., 2005). For example, Sox9 expression was found to be upregulated in RA-treated EBs (Kawaguchi et al., 2005). Sox9 is known to play an important role during development of pre-cartilage mesenchymal condensations. In fact, RA treatment of EBs followed by application of TGF-β3 is sufficient to induce cartilage nodule formation in EB outgrowths (Kawaguchi et al., 2005). Because RA treatment resulted in both an upregulation of the mesenchymal cell markers Runx2 and Ptprv and the appearance of neuroectodermal neural crest cells in EBs, either a mesodermal cell type or an ectomesenchymal cell type may be the source of the mesenchymal cartilage progenitor cells (Kawaguchi et al., 2005). Another two-step model for induction of cartilage formation in EBs was demonstrated by analysis of differentiation of ES cell–derived mesodermal progenitor cells into the chondrogenic direction (Nakayama et al., 2003). This study suggested that chondrogenesis of ES cell-derived mesodermal cells requires the influence of TGF-β3 followed by application of BMP-4. Moreover, a synergy of TGF-β with PDGF-BB regarding the induction of chondrogenesis was observed in a serum-free medium (Nakayama et al., 2003). In addition to induction of cartilage formation, BMP-2 can drive ES cells to the osteoblast or adipogenic fate depending on supplementary cofactors (zur Nieden et al., 2005). In contrast to findings obtained by the authors of this unit, in that study, TGF-β1 induced chondrogenic differentiation in ES cell line D3-derived EBs, and TGF-β1 and BMP-2 worked synergistically. Prolonged treatment of EB cultures with BMP-2 induced hypertrophy of ES cell-derived chondrocytes and resulted in alteration of the marker molecule expression towards a profile typical for osteoblasts, as well as an induced formation of bone nodules in EBs (Phillips et al., 2001; zur Nieden et al., 2005). Compactin, a member of the statin family of HMG-CoA reductase inhibitors, promoted an increase of BMP-2 expression that resulted in an induction of osteogenic differentiation in EB outgrowths (Phillips et al., 2001). Application of mineralization factors such as β-glycerophosphate and vitamin D3 to the culture medium has been found to enhance osteogenic differentiation (zur Nieden et al., 2003, 2005). It has also been shown that differentiation of murine ES cells towards the osteoblast lineage can be increased by supplementing media with ascorbic acid, β-glycerophosphate, and/or dexamethasone/retinoic acid, or by coculture with fetal murine osteoblasts (Buttery et al., 2001). However, dexamethasone alone, which is also known as an inducer of chondrogenic differentiation (Zimmermann and Cristea, 1993; Poliard et al., 1995), did not stimulate ES cell–derived chondrogenesis (Tanaka et al., 2004; zur Nieden et al., 2005).

Extracellular matrix (ECM) molecules, as well as soluble growth factors associated or connected to the matrix, modulate proliferation and differentiation of stem cells. The molecular composition of the ECM mediates cellular differentiation processes via cell matrix receptors, called integrins (Fässler et al., 1996; Rohwedel et al., 1998a; Czyz and Wobus, 2001). However, the role of the matrix material composition and structure in promoting ES cell growth and differentiation is only poorly understood (for example, Battista et al., 2005). Three-dimensional cultivation procedures such as EB encapsulation in polyethylene glycol–based hydrogels (Hwang et al., 2005) or alginate (Tanaka et al., 2004), as well as collagen matrices (Chen et al., 2003), influence ES cell differentiation.

Finally, biophysical parameters such as hydrostatic pressure, shear, oxygen tension, temperature, and electromagnetic forces have been found to influence chondrogenic differentiation (for review Malda et al., 2003; Smith et al., 2004; Fini et al., 2005). For example,

enhanced ES cell–derived bone nodule formation could recently be demonstrated after treatment of ES cell cultures with soluble ions released from bioactive glasses undergoing dissolution in vitro (Bielby et al., 2005).

Critical Parameters and Troubleshooting

1. Heat inactivation of FBS is performed at 54°C for 30 min after thawing the FBS at room temperature.

2. It is important to select a specific FBS batch for the chondrogenic differentiation experiments. Perform a differentiation experiment with different batches of FBS and compare chondrogenic differentiation between the variants by Alcian blue staining as described above.

3. For cultivation of ES cells in the pluripotent state it is important that the cells grow in distinct colonies with a clear contour and a limited size.

4. For ES cell cultivation in the undifferentiated stage the Medium 2 has to be changed daily.

5. During trypsinization of undifferentiated ES cells it is very important to obtain a single-cell suspension. This has to be checked by light microscopy. If cells are still clumped together after trypsinization and replating onto feeder layer, the cells have to be collected, centrifuged, washed twice with PBS, and trypsinized again.

6. For suspension culture of EBs, it is important to select a suitable batch of bacteriological petri dishes. In some batches, the EBs attach, even if the dishes are not coated with gelatin. If this happens, it is sometimes possible to resuspend the EBs again by carefully rinsing the dishes with Medium 4.

7. After plating, the EBs need some time to attach. Therefore, the dishes should not be moved for up to 2 days after plating.

Anticipated Results

For differentiation ES cells can simply be cultivated in bacteriological petri dishes where they spontaneously form EBs. But the size of these EBs varies enormously and the differentiation patterns observed under such conditions are sometimes not reproducible in detail. ES cell differentiation via the hanging-drop method results in EBs of the same size, leading to a more reproducible differentiation process. However, recently it has been found that culturing human ES cells without the EB step enhances osteogenesis in vitro (Karp et al., 2005).

Time Considerations

Table 23.5.3 provides a time schedule for an ES cell differentiation experiment.

Acknowledgements

The skilful technical assistance of A. Eirich, M. Dose, and M. Hell is gratefully acknowledged. The work was supported by the Deutsche Forschungsgemeinschaft DFG Ro2108, the University of Lübeck, and Intermed Service GmbH & CoKG, Geesthacht, Germany.

Literature Cited

Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A., and de Crombrugghe, B. 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* 16:2813-2828.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 2007. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Battista, S., Guarnieri, D., Borselli, C., Zeppetelli, S., Borzacchiello, A., Mayol, L., Gerbasio, D., Keene, D.R., Ambrosio, L., and Netti, P.A. 2005. The effect of matrix composition of 3D constructs on embryonic stem cell differentiation. *Biomaterials* 26:6194-6207.

Beverley, S. 2001. Enzymatic amplification of RNA by PCR (RT-PCR). *In* Current Protocols in Molecular Biology (F.M. Ausubel, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 15.1.1-15.1.6. John Wiley & Sons, Hoboken, N.J.

Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R., and de Crombrugghe, B. 1999. Sox9 is required for cartilage formation. *Nat. Genet.* 22:85-89.

Bianco, P., Cancedda, F.D., Riminucci, M., and Cancedda, R. 1998. Bone formation via cartilage models: The "borderline" chondrocyte. *Matrix Biol.* 17:185-192.

Bielby, R.C., Pryce, R.S., Hench, L.L., and Polak, J.M. 2005. Enhanced derivation of osteogenic cells from murine embryonic stem cells after treatment with ionic dissolution products of 58S bioactive sol-gel glass. *Tissue Eng.* 11:479-488.

Buttery, L.D., Bourne, S., Xynos, J.D., Wood, H., Hughes, F.J., Hughes, S.P., Episkopou, V., and Polak, J.M. 2001. Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. *Tissue Eng.* 7:89-99.

Cancedda, R., Castagnola, P., Cancedda, F.D., Dozin, B., and Quarto, R. 2000. Developmental control of chondrogenesis and osteogenesis. *Int. J. Dev. Biol.* 44:707-714.

Chen, S.S., Revoltella, R.P., Papini, S., Michelini, M., Fitzgerald, W., Zimmerberg, J., and Margolis, L. 2003. Multilineage differentiation of rhesus monkey embryonic stem cells in three-dimensional culture systems. *Stem Cells* 21:281-295.

Czyz, J. and Wobus, A. 2001. Embryonic stem cell differentiation: The role of extracellular factors. *Differentiation* 68:167-174.

Dani, C., Smith, A.G., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C., and Ailhaud, G. 1997. Differentiation of embryonic stem cells into adipocytes in vitro. *J. Cell Sci.* 110:1279-1285.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27-45.

Dorheim, M.A., Sullivan, M., Dandapani, V., Wu, X., Hudson, J., Segarini, P.R., Rosen, D.M., Aulthouse, A.L., and Gimble, J.M. 1993. Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. *J. Cell Physiol.* 154:317-328.

Evans, M.J. and Kaufman, M.H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Fässler, R., Rohwedel, J., Maltsev, V., Bloch, W., Lentini, S., Guan, K., Gullberg, D., Hescheler, J., Addicks, K., and Wobus, A.M. 1996. Differentiation and integrity of cardiac muscle cells are impaired in the absence of beta 1 integrin. *J. Cell Sci.* 109:2989-2999.

Fini, M., Giavaresi, G., Carpi, A., Nicolini, A., Setti, S., and Giardino, R. 2005. Effects of pulsed electromagnetic fields on articular hyaline cartilage: Review of experimental and clinical studies. *Biomed. Pharmacother.* 59:388-394.

Gissel, C., Voolstra, C., Doss, M.X., Koehler, C.I., Winkler, J., Hescheler, J., and Sachinidis, A. 2005. An optimized embryonic stem cell model for consistent gene expression and developmental studies: A fundamental study. *Thromb. Haemost.* 94:719-727.

Guan, K., Rohwedel, J., and Wobus, A. M. 1999. Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation *in vitro*. *Cytotechnology* 30:211-226.

Hargus, G., Kist, R., Kramer, J., Scherer, G., and Rohwedel, J. Loss of Sox9 function results in defective chondrocyte differentiation of mouse embryonic stem cells in vitro. Submitted for publication.

Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A., and Heinegard, D. 1992. Cartilage matrix proteins: An acidic oligomeric protein (COMP) detected only in cartilage. *J. Biol. Chem.* 267:6132-6136.

Hegert, C., Kramer, J., Hargus, G., Müller, J., Guan, K., Wobus, A.M., Müller, P.K., and Rohwedel, J. 2002. Differentiation plasticity of chondrocytes derived from mouse embryonic stem cells. *J. Cell Sci.* 115:4617-4628.

Heng, B.C., Cao, T., and Lee, E.H. 2004. Directing stem cell differentiation into the chondrogenic lineage in vitro. *Stem Cells* 22:1152-1167.

Hocke, G.M. 1995. The LIF-response element confers LIF-induced transcriptional control in P19 embryonal carcinoma cells. *Ann. N.Y. Acad. Sci.* 762:426-428.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. 1987. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* 326:292-295.

Hwang, N.S., Kim, M.S., Sampattavanich, S., Baek, J.H., Zhang, Z., and Elisseeff, J. 2005. The effects of three dimensional culture and growth factors on the chondrogenic differentiation of murine embryonic stem cells. *Stem Cells* 23:113-123.

Karp, J.M., Ferreira, L.S., Khademhosseini, A., Kwon, A.H., Yeh, J., and Langer, R. 2005. Cultivation of human embryonic stem cells without the embryoid body step enhances osteogenesis in vitro. *Stem Cells* 24:835-843.

Kawaguchi, J., Mee, P.J., and Smith, A.G. 2005. Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. *Bone* 36:758-769.

Kramer, J., Hegert, C., Guan, K., Wobus, A.M., Müller, P.K., and Rohwedel, J. 2000. Embryonic stem cell-derived chondrogenic differentiation in vitro: Activation by BMP-2 and BMP-4. *Mech. Dev.* 92:193-205.

Kramer, J., Hegert, C., and Rohwedel, J. 2003. In vitro differentiation of mouse ES cells: Bone and cartilage. *Methods Enzymol.* 365:251-268.

Kramer, J., Hegert, C., Hargus, G., and Rohwedel, J. 2005a. Mouse ES cell lines show a variable degree of chondrogenic differentiation in vitro. *Cell Biol. Int.* 29:139-146.

Kramer, J., Klinger, M., Kruse, C., Faza, M., Hargus, G., and Rohwedel, J. 2005b. Ultrastructural analysis of mouse embryonic stem cell-derived chondrocytes. *Anat. Embryol. (Berl)* 210:175-185.

Lefebvre, V., Li, P., and de Crombrugghe, B. 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *EMBO J.* 17:5718-5733.

Linsenmayer, T.F. and Hendrix, M.J. 1980. Monoclonal antibodies to connective tissue macromolecules: Type II collagen. *Biochem. Biophys. Res. Commun.* 92:440-446.

Mackay, A.M., Beck, S.C., Murphy, J.M., Barry, F.P., Chichester, C.O., and Pittenger, M.F. 1998. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* 4:415-428.

Malda, J., Martens, D.E., Tramper, J., van Blitterswijk, C.A., and Riesle, J. 2003. Cartilage tissue engineering: Controversy in the effect of oxygen. *Crit. Rev. Biotechnol.* 23:175-194.

Martin, G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78:7634-7638.

Mitrovic, D.R. 1977. Development of the metatarsophalangeal joint of the chick embryo: Morphological, ultrastructural and histochemical studies. *Am. J. Anat.* 150:333-347.

Moskalewski, S. 1976. Elastic fiber formation in monolayer and organ cultures of chondrocytes isolated from auricular cartilage. *Am. J. Anat.* 146:443-448.

Murphy, C.L. and Polak, J.M. 2002. Differentiating embryonic stem cells: GAPDH, but neither HPRT nor beta-tubulin is suitable as an internal standard for measuring RNA levels. *Tissue Eng.* 8:551-559.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 90:8424-8428.

Nakayama, N., Duryea, D., Manoukian, R., Chow, G., and Han, C.Y. 2003. Macroscopic cartilage formation with embryonic stem-cell-derived mesodermal progenitor cells. *J. Cell Sci.* 116:2015-2028.

Pavlov, M.I., Sautier, J.M., Oboeuf, M., Asselin, A., and Berdal, A. 2003. Chondrogenic differentiation during midfacial development in the mouse: In vivo and in vitro studies. *Biol. Cell* 95:75-86.

Phillips, B.W., Belmonte, N., Vernochet, C., Ailhaud, G., and Dani, C. 2001. Compactin enhances osteogenesis in murine embryonic stem cells. *Biochem. Biophys. Res. Commun.* 284:478-484.

Poliard, A., Nifuji, A., Lamblin, D., Plee, E., Forest, C., and Kellermann, O. 1995. Controlled conversion of an immortalized mesodermal progenitor cell towards osteogenic, chondrogenic, or adipogenic pathways. *J. Cell Biol.* 130:1461-1472.

Provot, S. and Schipani, E. 2005. Molecular mechanisms of endochondral bone development. *Biochem. Biophys. Res. Commun.* 328:658-665.

Rathjen, P.D., Lake, J., Whyatt, L.M., Bettess, M.D., and Rathjen, J. 1998. Properties and uses of embryonic stem cells: Prospects for application to human biology and gene therapy. *Reprod. Fertil. Dev.* 10:31-47.

Riminucci, M., Bradbeer, J.N., Corsi, A., Gentili, C., Descalzi, F., Cancedda, R., and Bianco, P. 1998. Vis-a-vis cells and the priming of bone formation. *J. Bone Miner. Res.* 13:1852-1861.

Rohwedel, J., Maltsev, V., Bober, E., Arnold, H.H., Hescheler, J., and Wobus, A.M. 1994. Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: Developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Dev. Biol.* 164:87-101.

Rohwedel, J., Guan, K., Zuschratter, W., Jin, S., Ahnert-Hilger, G., Fürst, D., Fässler, R., and Wobus, A.M. 1998a. Loss of beta1 integrin function results in a retardation of myogenic, but an acceleration of neuronal, differentiation of embryonic stem cells in vitro. *Dev. Biol.* 201:167-184.

Rohwedel, J., Kleppisch, T., Pich, U., Guan, K., Jin, S., Zuschratter, W., Hopf, C., Hoch, W., Hescheler, J., Witzemann, V., and Wobus, A.M. 1998b. Formation of postsynaptic-like membranes during differentiation of embryonic stem cells in vitro. *Exp. Cell Res.* 239:214-225.

Rohwedel, J., Guan, K., Hegert, C., and Wobus, A.M. 2001. Embryonic stem cells as an in vitro model for mutagenicity, cytotoxicity and embryotoxicity studies: Present state and future prospects. *Toxicol. In Vitro* 15:741-753.

Sandell, L.J. and Adler, P. 1999. Developmental patterns of cartilage. *Front. Biosci.* 4:D731-D742.

Schmid, T.M. and Linsenmayer, T.F. 1985. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. *J. Cell Biol.* 100:598-605.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336:688-690.

Smith, R.L., Carter, D.R., and Schurman, D.J. 2004. Pressure and shear differentially alter human articular chondrocyte metabolism: A review. *Clin. Orthop. Relat. Res.* Oct.:S89-S95.

Sui, Y., Clarke, T., and Khillan, J.S. 2003. Limb bud progenitor cells induce differentiation of pluripotent embryonic stem cells into chondrogenic lineage. *Differentiation* 71:578-585.

Tanaka, H., Murphy, C.L., Murphy, C., Kimura, M., Kawai, S., and Polak, J.M. 2004. Chondrogenic differentiation of murine embryonic stem cells: Effects of culture conditions and dexamethasone. *J. Cell Biochem.* 93:454-462.

Thomas, K.R. and Capecchi, M.R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51:503-512.

Voytas, D. 2000. Agarese gel electrophoresis. *CPMB* 51:2.5A.1-2.5A.9.

Wakitani, S., Takaoka, K., Hattori, T., Miyazawa, N., Iwanaga, T., Takeda, S., Watanabe, T.K., and Tanigami, A. 2003. Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. *Rheumatology (Oxford)* 42:162-165.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. 1988. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336:684-687.

Wobus, A.M., Holzhausen, H., Jäkel, P., and Schöneich, J. 1984. Characterization of a pluripotent stem cell line derived from a mouse embryo. *Exp. Cell Res.* 152:212-219.

Wobus, A.M., Grosse, R., and Schöneich, J. 1988. Specific effects of nerve growth factor on the differentiation pattern of mouse embryonic stem

cells in vitro. *Biomed. Biochim. Acta* 47:965-973.

Wobus, A.M., Rohwedel, J., Maltsev, V., and Hescheler, J. 1994. In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. *Roux's Arch. Devel. Biol.* 204:36-45.

Wobus, A.M., Kaomei, G., Shan, J., Wellner, M.C., Rohwedel, J., Ji, G., Fleischmann, B., Katus, H.A., Hescheler, J., and Franz, W.M. 1997. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J. Mol. Cell Cardiol.* 29:1525-1539.

Wong, H., Anderson, W.D., Cheng, T., and Riabowol, K.T. 1994. Monitoring mRNA expression by polymerase chain reaction: The "primer- dropping" method. *Anal. Biochem.* 223:251-258.

Yamada, G., Kioussi, C., Schubert, F.R., Eto, Y., Chowdhury, K., Pituello, F., and Gruss, P. 1994. Regulated expression of Brachyury(T), Nkx1.1 and Pax genes in embryoid bodies. *Biochem. Biophys. Res. Commun.* 199:552-563.

Zenmyo, M., Komiya, S., Kawabata, R., Sasaguri, Y., Inoue, A., and Morimatsu, M. 1996. Morphological and biochemical evidence for apoptosis in the terminal hypertrophic chondrocytes of the growth plate. *J. Pathol.* 180:430-433.

Zimmermann, B. and Cristea, R. 1993. Dexamethasone induces chondrogenesis in organoid culture of cell mixtures from mouse embryos. *Anat. Embryol. (Berl.)* 187:67-73.

zur Nieden, N.I., Kempka, G., and Ahr, H.J. 2003. In vitro differentiation of embryonic stem cells into mineralized osteoblasts. *Differentiation* 71:18-27.

zur Nieden, N.I., Kempka, G., Rancourt, D.E., and Ahr, H.J. 2005. Induction of chondro-, osteo- and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: Effect of cofactors on differentiating lineages. *BMC. Dev. Biol.* 5:1.

Contributed by Jan Kramer, Peter Schlenke, and Jürgen Rohwedel
University of Lübeck
Lübeck, Germany

Hematoendothelial Differentiation of Human Embryonic Stem Cells

Maxim A. Vodyanik[1] and Igor I. Slukvin[1,2]

[1]University of Wisconsin, Madison, Wisconsin
[2]WiCell Research Institute, Madison, Wisconsin

ABSTRACT

Human embryonic stem cells (hESCs) represent a unique population of cells capable of self-renewal and differentiation into all types of somatic cells, including hematopoietic and endothelial cells. Since the pattern of hematopoietic and endothelial development observed in the embryo can be reproduced using ESCs differentiated in culture, hESCs can be used as a model for studies of specification and diversification of hematoendothelial progenitors. In addition, hESCs can be seen as a scalable source of hematopoietic and endothelial cells for in vitro studies. This unit describes a method for efficient differentiation of hESCs into hematopoietic progenitors and endothelial cells through coculture with mouse OP9 bone marrow stromal cells, as well as an approach for their analysis and isolation. Support protocols are provided for culture of mouse embryonic fibroblasts, evaluation of hematopoietic and endothelial differentiation by flow cytometry and colony-forming assay, removal of OP9 cells, and propagation of hESC-derived endothelial cells. *Curr. Protoc. Cell Biol.* 36:23.6.1-23.6.28. © 2007 by John Wiley & Sons, Inc.

Keywords: human embryonic stem cells • hematopoietic development • in vitro differentiation • hematopoietic progenitors • endothelial cells

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells capable of differentiating into all types of cells found in the body, including hematopoietic cells. Since the pattern of hematopoietic development observed in the embryo can be reproduced using ESCs differentiated in culture, hESCs provide a unique opportunity for elucidating mechanisms of early hematopoietic commitment, lineage specification, and maturation in humans (Keller et al., 1993; Nakano et al., 1996; Robertson et al., 1999; Lensch and Daley, 2004). Moreover, genetically modified hESCs provide an unprecedented opportunity for studying the role of human genes in hematopoietic development.

There are two major approaches for inducing hematopoietic differentiation of ESCs. One approach allows ESCs to form embryoid bodies after the withdrawal of factors that maintain ESCs in undifferentiated state. Various lineages of cells, including hematopoietic cells, develop inside embryoid bodies. Another approach is coculture of ESCs with bone marrow stromal cell lines, which provides an inductive environment for directed hESC differentiation to hematopoietic lineage.

This unit describes procedures for differentiating hESCs into hematopoietic cells by coculturing with mouse bone marrow stromal cell line OP9 (Basic Protocol 1), as well as methods for analyzing the efficiency of hematopoietic (and endothelial) differentiation by flow cytometry (Support Protocol 1) and a methylcellulose-based colony-forming assay (Support Protocol 2). Basic Protocol 2 describes isolation of hESC-derived hematopoietic progenitors. Since hematopoietic and endothelial lineages develop simultaneously in OP9 coculture, these protocols can also be used for generation and isolation of hESC-derived

Current Protocols in Cell Biology 23.6.1-23.6.28, September 2007
Published online September 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2306s36

endothelial cells. Additional supporting protocols describe the removal of OP9 cells from hESC/OP9 coculture (Support Protocol 3), maintenance of human embryonic stem cells on mouse embryonic fibroblasts (MEFs; Support Protocol 4), methods for culture of mouse embryonic fibroblasts (Support Protocol 5), techniques for OP9 culture (Support Protocol 6), and propagation of hESC-derived endothelial cells (Support Protocol 7).

NOTE: All procedures described in this unit—tissue culture, reagent preparation, and cell sorting—require sterilization facilities and a standard cell culture workplace, i.e., a laminar flow hood equipped with pipet-aids and an adjustable pipet set, sterile disposable items (tips, glass Pasteur pipets, serological pipets, polypropylene centrifuge tubes), and a vacuum-driven aspiration unit as well as a CO_2 incubator, 37°C water bath, temperature controlled swinging-bucket centrifuge, inverted phase-contrast microscope, and cell counting device. All experiments should be performed under sterile conditions in Class II biological safety cabinets.

NOTE: All incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise indicated.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

BASIC PROTOCOL 1

HEMATOENDOTHELIAL DIFFERENTIATION OF hESCs IN OP9 COCULTURE

This protocol describes the optimal conditions for induction of hematoendothelial differentiation of hESCs through coculture with a mouse OP9 bone marrow stromal cell line. Hematopoietic differentiation of hESCs in coculture with OP9 cells proceeds very rapidly, and the first hematopoietic progenitors can already be detected after 4 to 5 days of coculture. Using this method, efficient hematopoietic differentiation can be achieved without the addition of cytokines or growth factors. The number of endothelial cells and hematopoietic progenitors peak on day 7 to 8 of hESC/OP9 coculture and decrease thereafter due to excessive cell density and overall decline in cell viability.

Materials

6-well plates with undifferentiated hESCs on day 5 to 6 of culture (Support Protocol 4)
1× phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO)
Differentiation medium (see recipe), warmed to 37°C
10-cm dishes with OP9 cells on day 8 to 12 after plating (Support Protocol 6)
Collagenase IV solution (see recipe)
0.05% (w/v) trypsin/0.5 mM EDTA solution (1×; GIBCO)
Cell washing/MACS buffer (see recipe)

15- and 50-ml polypropylene centrifuge tubes
Temperature controlled centrifuge
70-μm nylon filters (cell strainers; Falcon)

Additional reagents and equipment for counting cells (*UNIT 1.1*)

Count hESCs

1. From one well of a 6-well hESC plate, aspirate growth medium and wash the well with 2 ml PBS. Add 1 ml trypsin/EDTA solution and incubate 5 min at 37°C.

   As for regular hESC passage, hESCs used for OP9 coculture must be in aggregates and therefore, cannot be counted directly. To determine the absolute number of hESCs within aggregates, a separate well of 6-well hESC plate should be treated with trypsin to obtain a single-cell suspension for counting.

2. Dissociate hESC colonies by pipetting. Transfer the single-cell suspension to a 15-ml tube containing 2 ml differentiation medium. Centrifuge 5 min at 300 × *g*, room temperature, and resuspend in 1 ml differentiation medium.

3. Count the number of cells in 1 ml (see *UNIT 1.1*).

   The total number of cells in 1 ml will be equal to the absolute number of hESCs in aggregates collected from single well.

Plate hESCs on OP9 dishes

4. Remove OP9 dishes prepared for differentiation (i.e., overgrown) from the CO_2 incubator. Aspirate growth medium completely and add 10 ml differentiation medium. Return the dishes to the CO_2 incubator.

   It is important to remove OP9 medium completely before hESC plating. Adding hESCs directly to an OP9 monolayer with conditioned medium suppresses hematopoietic differentiation.

5. Prepare undifferentiated hESCs as a suspension of small aggregates (described in Support Protocol 4), but perform the washing step using differentiation medium and resuspend the cell pellet in differentiation medium to a concentration of 1.5×10^6 cells/ml.

   The total resuspension volume can be calculated by formula $(n \times x)/1.5$, where n is the number of hESC wells collected for differentiation, and x is the absolute number of hESCs per single well determined by a counting of trypsin-dissociated cells (steps 1 to 3).

   Small clumps (not single hESCs) should be plated for differentiation. Single hESCs in suspension will not survive in the OP9 coculture.

   hESCs should be plated on OP9 cells at optimal density to ensure optimal growth and differentiation during 8 days of coculture. In the authors' experience, optimal plating density is 1–1.5 × 10^6 hESCs (lines H1 and H9) per one 10-cm dish. Since each well of a 6-well hESC plate contains 2–3 × 10^6 cells, hESCs collected from one well are routinely plated on two 10-cm OP9 dishes. However, before such routine plating, control cell counting should be performed in preliminary experiments.

6. Remove OP9 dishes (prefilled with 10 ml differentiation medium in step 4) from the CO_2 incubator. Add 1 ml hESC suspension to each OP9 dish. Place the dishes on the shelf in CO_2 incubator and move forth-and-back and side-to-side to distribute the hESC aggregates evenly throughout the dishes. Incubate overnight.

   Avoid rotating motions as this will lead to accumulation and attachment of hESC clumps in the middle of dish and in such instances the experiment can not be continued.

Feed hESC/OP9 cocultures

7. On day 1 after hESC plating, gently agitate the hESC/OP9 coculture dishes, aspirate the medium, and fill the dishes with 20 ml fresh, prewarmed differentiation medium.

   The 20-ml incubation volume and less frequent feeding are important for successful hematoendothelial differentiation. Conversely, a standard 10-ml incubation volume and more frequent feeding favor generation of mesenchymal-like cells.

8. On culture days 4 and 6, change half of the medium by removing 10 ml of medium from hESC/OP9 dishes by pipet and adding 10 ml of fresh, prewarmed differentiation medium.

   Optimized feeding is critical for efficient hematopoietic differentiation. Any changes in feeding may change differentiation significantly. The authors recommend a full change of medium on the first day after hESC plating (step 7) because it removes all nonattached (dead) cells and suppresses initial growth of undifferentiated colonies. As a result, a more synchronous differentiation can be achieved. In the following days, only half of the medium should be changed. The feeding regimen is one approach for manipulating differentiation, and variations can be tried to improve differentiation efficiency.

Collect cells

9. Warm the collagenase IV and trypsin/EDTA solutions in a 37°C water bath.
10. Remove hESC/OP9 dishes from CO_2 incubator. Aspirate the medium and wash the cell monolayer with 10 ml PBS.
11. Add 5 ml collagenase IV solution/dish and incubate 20 min in the CO_2 incubator.
12. Remove the collagenase solution using a pipet and transfer to a 15- or 50-ml collection tube. Reserve this tube for subsequent collection of trypsin-digested cells.
13. Add 5 ml trypsin/EDTA solution/dish and incubate for an additional 15 to 20 min in the CO_2 incubator.

    After 5 days of differentiation, hESC/OP9 cocultures form a collagen-rich matrix that cannot be efficiently digested by trypsin treatment. Therefore, a pretreatment of hESC/OP9 monolayers with collagenase is used to facilitate subsequent cell dissociation with trypsin. Successive collagenase-trypsin treatment is especially important to maximize cell recovery from day 7 to 9 hESC/OP9 cocultures.

14. Suspend the cells by pipetting and transfer the cell suspension to the collection tube. Close the tube, mix the cells gently by inverting tube, and centrifuge 5 min at 400 × *g*, 4°C.
15. Wash cells three times with cell washing/MACS buffer. Use at least a 10-ml wash volume for cells collected from one hESC/OP9 dish, and centrifuge the cell suspension 5 min at 400 × *g*, 4°C.
16. At the last washing step, resuspend the cells in 10 ml cell washing/MACS buffer, filter through a 70-μm nylon filter, and centrifuge as in step 15.
17. Resuspend the cells in 1 ml cell washing/MACS buffer or medium for each hESC/OP9 dish collected. Place the tube on ice.

    Cells are now ready to analysis (Support Protocols 1 and 2) and separations (Basic Protocol 2 and Support Protocol 3).

18. Count the cells (see *UNIT 1.1*).

    The cell yield from one 10-cm hESC/OP9 dish should be $>10^7$, typically in range of 1.5–2 × 10^7 cells.

SUPPORT PROTOCOL 1

ASSESSMENT OF HEMATOENDOTHELIAL DIFFERENTIATION BY FLOW CYTOMETRY

The hESC-derived endothelial cells and hematopoietic progenitors can be identified by the expression of a common hematoendothelial marker, CD31 (PECAM-1). Hematopoietic progenitors can be distinguished from endothelial cells by CD43 (leukosialin) expression; flow cytometric enumeration of $CD31^+CD43^-$ and $CD31^+CD43^+$ cells can be used to evaluate the efficiency of endothelial and hematopoietic differentiation in hESC/OP9 cocultures, respectively (Vodyanik et al., 2006). Based on expression of CD235a (glycophorin A), multipotent hematopoietic progenitors ($CD43^+CD235a^-$) can be separated from precommitted $CD43^+CD235a^+$ erythro-megakaryocytic progenitors, which also express CD41a (Vodyanik et al., 2006). The marker of the most primitive somatic hematopoietic stem cells/progenitors, CD34, is expressed on hESC-derived endothelial cells and a majority of hematopoietic progenitors. However, in contrast to bone marrow or cord blood, hESC-derived $CD34^+$ cells are more heterogeneous and include a significant proportion of mesenchymal cells (Vodyanik et al., 2006). Thus, the presence of $CD34^+$ cells does not necessarily reflect hematoendothelial differentiation in hESCs. Moreover, some erythroid progenitors are lacking CD34 expression, but express CD43

and CD31. The first $CD34^+$, $CD31^+$, and $CD43^+$ cells are detectable on day 3 to 4 of hESC/OP9 coculture. Cells collected on day 7 to 8 of hESC/OP9 coculture are optimal for assessment of hematoendothelial differentiation, because they contain a maximal number of endothelial cells and all types of hematopoietic progenitors. Total hESC-derived cells in OP9 coculture can be identified using TRA-1-85 monoclonal antibodies (mAb), which detect the OKa blood group antigen expressed by virtually all human cells (Williams et al., 1988). Absolute numbers of cell populations can be calculated by knowing the total number of cells harvested from one 10-cm hESC/OP9 dish, the percentage of total hESC-derived ($TRA\text{-}1\text{-}85^+$) cells, and the percentage of the cell population of interest.

Materials

Cells collected from hESC/OP9 coculture (Basic Protocol 1)
FACS buffer with and without fetal bovine serum (FBS; see recipe)
Mouse anti-human CD31-PE mAb (clone WM59; BD Pharmingen)
Mouse anti-human CD43-FITC mAb (clone 1G10; BD Pharmingen)
Mouse anti-human TRA-1-85-APC mAb (clone TRA-1-85; R&D Systems)
Mouse anti-human CD235a-PE mAb (clone CLB-ery-1; Caltag)
Mouse anti-human CD45-APC mAb (clone HI30; BD Pharmingen)
Mouse anti-human CD29-FITC/PE mAb (clone MEM-101A; Caltag)
Mouse anti-human CD34-APC mAb (clone 581; BD Pharmingen), optional
Mouse IgG1 FITC control mAb (clone MOPC-21; BD Pharmingen)
Mouse IgG1 PE control mAb (clone MOPC-21; BD Pharmingen)
Mouse IgG1 APC control mAb (clone MOPC-21; BD Pharmingen)
7-aminoactinomycin D (7AAD) staining solution (Via-Probe; BD Pharmingen)

5-ml polystyrene test tubes (Falcon)
Temperature-controlled centrifuge
Flow cytometer (FACSCalibur; BD Immunocytometry Systems)
FlowJo flow cytometry analysis software (Tree Star)

Stain cells

1. Suspend cells collected from hESC/OP9 coculture (Basic Protocol 1, step 17) in FACS buffer with FBS at 5×10^6 cells/ml (5×10^5 cells/100 µl per test).

2. Add monoclonal antibodies (mAbs) on the bottoms of 5-ml polystyrene test tubes according to the following plan (also see Table 23.6.1):

   Tubes S1 to S3 for setting up instrument compensation controls
   Tubes E1 to E2 for analysis of total hematopoietic ($CD31^+CD43^+$) and endothelial ($CD31^+CD43^-$) cells within $TRA\text{-}1\text{-}85^+$ human cell population
   Tubes E3 to E4 for analysis of multipotent ($CD43^+CD235a^-CD45^{\pm}$) and erythro-megakaryocytic ($CD43^+CD235a^+CD45^-$) progenitors within total $CD43^+$ hematopoietic population
   Tubes E5 to E6 (optional) for analysis of $CD34^+$ subpopulations of endothelial ($CD31^+CD43^-$), hematopoietic ($CD31^+CD43^+$), and mesenchymal ($CD31^-CD43^-$) cells.

3. Add 100 µl cell suspension to each tube. Incubate 30 to 40 min at 4°C, mixing the cells by agitation at least once during the incubation.

4. Add 4 ml FACS buffer with FBS to the tubes and centrifuge 5 min at $400 \times g$, 4°C.

5. Discard the supernatant by inverting the tubes and resuspend cells in 400 µl FACS buffer without FBS.

   Samples can be stored up to 4 hr at 4°C.

Table 23.6.1 Monoclonal Antibody Combinations for Flow Cytometric Analysis

Tube	FL1-FITC (488/519)[a]	FL2-PE (488/578)[a]	FL3-7AAD[b] (488/647)[a]	FL4-APC (633/660)[a]
Instrument setup samples				
S1	IgG1 control	IgG1 control	-	IgG1 control
S2	CD29[c]	IgG1 control	7AAD	IgG1 control
S3	IgG1 control	CD29[c]	-	TRA-1-85
Experimental samples				
E1	IgG1 control	IgG1 control	7AAD	TRA-1-85
E2	CD43	CD31	7AAD	TRA-1-85
E3	CD43	IgG1 control	7AAD	IgG1 control
E4	CD43	CD235a[c]	7AAD	CD45
E5 (optional)	IgG1 control	IgG1 control	7AAD	CD34
E6 (optional)	CD43	CD31	7AAD	CD34

[a]Fluorochrome excitation/emission wavelengths (nm).
[b]7AAD is a DNA staining dye (Via-Probe, BD) for dead cell exclusion. It should be added to mAb-stained cells immediately before analysis (10 μl/sample).
[c]These monoclonal antibodies (mAbs) are used at 2 μl/test; all other mAbs are used at 5 μl/test.

6. Before analysis, add 10 μl 7AAD staining solution to each tube.

   7AAD staining is used for dead cell exclusion. S1 negative sample and S2 and S3 samples stained with nonoverlapping fluorochromes (FITC/7AAD and PE/APC) are used for setting up instrument compensation.

Analyze samples

7. Perform analysis of cell samples on the flow cytometer and save a minimum of 50,000 (E1 to E2) and 100,000 (E3 to E4) acquisition events.

   Analysis of all samples begins from selection (gating) of 7AAD-negative viable cells. In E1 to E2 samples, total TRA-1-85$^+$ hESC-derived cells are gated for analysis of endothelial (CD31$^+$CD43$^-$) and total hematopoietic (CD31$^+$CD43$^+$) cells. In E3 to E4 samples, total CD43$^+$ cells are gated for analysis of multipotent (CD235a$^-$CD45$^-$ and CD235a$^-$CD45$^+$) and erythro-megakaryocytic (CD235a$^+$CD45$^-$) hematopoietic progenitors. See Figure 23.6.1 for examples.

8. Open listmode files in FlowJo analysis software.

9. Start analysis with E1 or E3 isotype control samples. Set a gate on all 7AAD-negative (viable) cells using FL3-7AAD/FCS dot plot.

10. Open the 7AAD$^-$ gate in FL4-TRA-1-85-APC/SSC (E1) or FL1-CD43-FITC/SSC (E3) dot-plots. Set a gate on all TRA-1-85$^+$ cells (E1) or all CD43$^+$ cells (E3).

11. Open the TRA-1-85$^+$ gate in FL1-IgG1-FITC/FL2-IgG1-PE (E1) or CD43$^+$ gate in FL2-IgG1-PE/FL4-IgG1-APC (E3) dot-plots. Set thresholds for positive staining using quadrant statistics.

12. Transfer (drag-and-drop) all gates to the samples stained with specific mAbs: E1 gates to E2 sample or E3 gates to E4 sample.

13. Determine percentage (%) values.

    E2 sample: % cell viability (7AAD$^-$), % hESC-derived cells (TRA-1-85$^+$), % CD31$^+$CD43$^-$ cells (endothelial), and % CD31$^+$CD43$^+$ (hematopoietic) cells (Fig. 23.6.1A).

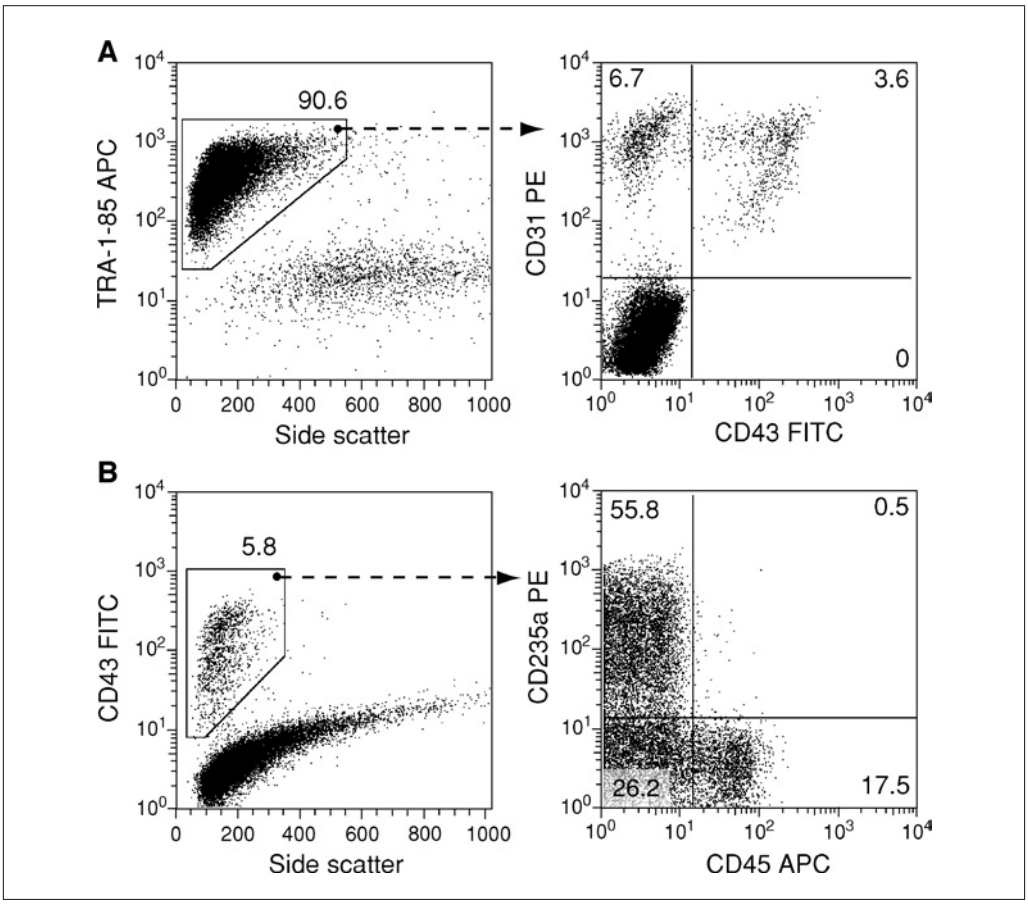


Figure 23.6.1 Flow cytometric analysis of hematopoietic progenitors and endothelial cells generated in hESC/OP9 coculture. (**A**) Representative flow cytometric analysis of hematopoietic ($CD31^+CD43^+$) and endothelial ($CD31^+CD43^-$ cells on day 7 of H1/OP9 coculture. Total H1-derived cells are brightly stained with TRA-1-85-APC mAb (left dot-plot). TRA-1-85^+ cells are selected (gated) for analysis of CD31 and CD43 expression (right dot-plot). (**B**) Representative flow cytometric analysis of $CD43^+$ hematopoietic progenitors on day 8 of H1/OP9 coculture. By low side scatter profile, total CD43-FITC-stained cells can be identified as compact cell population (left dot-plot). Selected (gated) total $CD43^+$ cells are then analyzed for CD235a and CD45 expression. In the right dot-plot, three distinct populations of $CD43^+$ hematopoietic progenitors can be defined: $CD235a^+CD45^-$, $CD235a^-CD45^-$ and $CD235a^-CD45^+$. Percentage values are shown for respective gates/quadrants.

E4 sample: % $CD43^+$ multipotent progenitors ($CD235a^-CD45^-$ and $CD235a^-CD45^+$), and % $CD43^+$ erythro-megakaryocytic progenitors ($CD235a^+$) (Fig. 23.6.1B).

$CD31^-CD43^+$ hematopoietic cells can be found in hESC/OP9 coculture after 8 days of differentiation. These cells represent erythroid cells, which down-regulate CD31 expression along with maturation.

14. Calculate absolute numbers of endothelial ($CD31^+CD43^-$) and hematopoietic ($CD31^+CD43^+$) cells recovered from hESC/OP9 coculture by the formula:

number of cells/$cm^2 = (N \times V \times H \times S)/78.56$

where

N is the number in millions of cells collected from one 10-cm dish
V is % cell viability
H is % human cells (TRA-1-85^+)
S is % cell subpopulation ($CD31^+CD43^-$ or $CD31^+CD43^+$)

78.56 is surface area (cm^2) of a standard 10-cm dish (use 9.62 for one well of 6-well plate).

For example, if 20×10^6 collected cells contain 95% 7AAD$^-$ viable cells, 90% TRA-1-85$^+$ human cells and 5% CD31$^+$CD43$^+$ hematopoietic progenitors, the number of CD31$^+$CD43$^+$ cells = $(20 \times 95 \times 90 \times 5)/78.56 = 10883$ cells per cm^2 of hESC/OP9 coculture.

SUPPORT PROTOCOL 2

HEMATOPOIETIC PRECURSOR COLONY-FORMING ASSAY

In addition to phenotypic analysis, hematopoietic progenitors can be identified using functional assays. A short-term test such as a colony forming assay is widely used to identify hematopoietic precursors at the intermediate stage between hematopoietic stem cells and cells with morphologically distinct features of differentiation. This assay is standardized and easy to perform. When grown in semisolid methylcellulose-based media in the presence of cytokines, hematopoietic progenitors form colonies with specific appearances and cell compositions. These colonies are formed by colony-forming units (CFUs) or colony-forming cells (CFCs) and can be morphologically discriminated into erythroid (E-CFC); granulocyte, erythroid, macrophage, megakaryocyte (GEMM-CFC); granulocyte-macrophage (GM-CFC); and macrophage (M-CFC) cells (Eaves and Lambie, 1995). Complete ready-to-use MethoCult H4435 GF$^+$ methylcellulose medium with added FBS and cytokines (SCF, G-CSF, GM-CSF, IL3, IL6) can be obtained from Stem Cell Technologies. This is a high-quality medium optimized for detection of very early hematopoietic progenitors, and it has minimal lot-to-lot variation. The cells prepared from day 7 to 8 hESC/OP9 cocultures are optimal for the colony-forming assay because they contain all CFC types. In the authors' experience, the presence of OP9 cells in the cell samples does not interfere with the CFC assay. However, if desired, the OP9 cells can be removed from the cell suspension by magnetic sorting (Support Protocol 3).

Materials

MethoCult H4435 GF$^+$ complete medium (Stem Cell Technologies): thaw, divide into 3-ml single-test aliquots (using a 10-ml syringe and blunt-end needle), and refreeze; store up to 6 months at −20°C
6×10^4 cells (Basic Protocol 1, step 17)/0.3 ml differentiation medium
Differentiation medium (see recipe)
Sterile distilled water

Blunt-end needles (Stem Cell Technologies) and 5- and 10-ml syringes, optional
37°C water bath
Disposable 5-ml serological pipet
3.5-cm low-adherence CFU assay dishes (Stem Cell Technologies)
3.5-cm regular plastic tissue culture dishes
10-cm regular plastic tissue culture dishes
Inverted microscope
Gridded scoring dishes (Stem Cell Technologies)

Prepare culture plates

1. Remove tubes with 3 ml MethoCult medium from the freezer and thaw in a 37°C water bath.

   If acquired in the bulk format (100 ml), MethoCult medium should be thawed and divided into single-test aliquots (3 ml per 15-ml polypropylene tubes) using a 10-ml syringe and blunt-end needles. Details on handling of methylcellulose medium can be found in the documentation accompanying the product.

2. Add 6×10^4 cells in 0.3 ml (or less) differentiation medium to a tube containing 3 ml MethoCult medium.

   The final concentration for plating is 2×10^4 cells/ml. The cell suspension should be prepared in differentiation medium and added in volume less than or equal to 0.3 ml.

3. Vortex the tube vigorously to thoroughly mix the cells and place in a 37°C water bath for 10 to 15 min, allowing bubbles to dissipate.

4. Slowly collect the suspended cells in a 5-ml pipet and dispense equal volumes (~1 ml/dish) to duplicate 3.5-cm CFU assay dishes. Rotate the dishes until semisolid medium covers entire plastic surface.

5. Place the two CFU assay dishes, along with an uncovered 3.5-cm dish filled with 4 ml sterile distilled water, into a 10-cm dish and cover.

6. Incubate 14 to 16 days in the CO_2 incubator.

Score colonies

7. Score different types of colonies (E, GEMM, GM, and M) according to their morphology (see Eaves and Lambie, 1995) using an inverted microscope and gridded scoring dishes.

8. Calculate the means for the different colony types from duplicate dishes and multiply by 5 (the dilution factor) to express CFC frequency per 10^5 plated cells.

REMOVAL OF OP9 CELLS FROM hESC/OP9 COCULTURE

SUPPORT PROTOCOL 3

As a xenogeneic system, hESC/OP9 coculture provides the advantage of easy discrimination between hESC-derived cells and feeder cells. Most monoclonal antibodies directed to human antigens do not cross-react with mouse homologs, and therefore, can be used for specific detection and isolation of hESC-derived cells. However, in several functional assays or gene expression studies, OP9 cells may interfere with results and should be removed. For such instances, this protocol describes removal of OP9 cells by magnet-activated cell sorting (MACS) using monoclonal antibodies (mAb) against mouse β1-integrin (CD29), which does not recognize human CD29. Depletion of OP9 cells using antibody-carrying magnetic microbeads results in 99.9% purity of human cells (TRA-1-85$^+$). This method can also be used for purification of human cells from other human-mouse cell coculture systems because CD29 is expressed in high density by virtually all adherent cell types. This protocol is optimized for one-column processing of up to 50×10^6 cells collected from day 7 to 8 hESC/OP9 cocultures. If using more cells or earlier days of coculture, the sample should be divided and processed through additional magnetic columns.

Materials

Cell suspension (Basic Protocol 1), hold on ice
Cell washing/MACS buffer (see recipe), degassed under vacuum and ice cold
Hamster anti-mouse CD29-PE mAb (clone HM beta 1-1; Serotec)
Anti-PE magnetic microbeads (Miltenyi Biotec)
Mouse anti-human TRA-1-85-APC mAb (clone TRA-1-85; R&D Systems)
7-aminoactinomycin D (7AAD) staining solution (Via-Probe; BD Pharmingen)

15-ml graduated polypropylene tubes
Temperature controlled centrifuge, 4°C
MACS rotation mixer (Miltenyi Biotec)
35-μm preseparation filters (Miltenyi Biotec)
Midi-MACS magnet/stand (Miltenyi Biotec)
LD (depletion) columns (Miltenyi Biotec)

Additional reagents and equipment for counting cells (*UNIT 1.1*) and analyzing cells by flow cytometry (Support Protocol 1)

NOTE: Cells and cell washing/MACS buffer must be kept on ice during the separation procedure.

Prepare cells

1. Add the suspension of cells (up to 5×10^7 cells) isolated from hESC/OP9 coculture (Basic Protocol 1) to a 15-ml graduated polypropylene tube.
2. Centrifuge 5 min at $400 \times g$, 4°C.
3. Aspirate the supernatant. Note the volume of the cell pellet and add an equal volume of cell washing/MACS buffer.

   The resulting total volume is referred to as the labeling volume.

Label mouse cells

4. Resuspend the cells thoroughly by gentle pipetting and add 1/10 vol hamster anti-mouse CD29-PE mAb.

   For example, if the cell pellet is ~0.2 ml, add 0.2 ml cell washing/MACS buffer (labeling volume is 0.4 ml), resuspend cells, and add 40 µl (1/10 vol) CD29-PE mAb.

5. Set up tube on the MACS mixer. Place the MACS mixer with the tube in the refrigerator (4°C). Incubate 15 min at the lowest rotation speed.

Add beads for separation

6. Wash cells once with 14 ml ice-cold cell washing/MACS buffer. Resuspend cells in cell washing/MACS buffer to the original labeling volume and add 1/10 vol anti-PE microbeads.
7. Incubate cells on MACS mixer 20 min at 4°C and lowest rotation speed.
8. Wash cells once with 14 ml ice-cold cell washing/MACS buffer. Resuspend cell pellet in 1 ml cell washing/MACS buffer.
9. Place a 35-µm preseparation filter on the top of an empty 15-ml tube. Apply the cell suspension on the filter and allow the cells to pass through filter completely. Wash the filter with 0.5 ml cell washing/MACS buffer.

   It is important to filter cells prior to application onto magnetic columns. Even very small cell aggregates may block the magnetic columns and adversely affect separation.

10. Place the tube with filtered cells on ice and reserve a 10- to 20-µl aliquot for FACS analysis of presort cells in step 16.

Separate mouse cells

11. Assemble the MACS-LD separation unit (midi-MACS magnet with LD column and 15-ml collection tube). Rinse the column with 2 ml MACS buffer and discard the tube with eluate. Place an empty collection tube under column.

    There are two types of magnetic columns optimized for positive (LS) and negative (LD) MACS separation, and they differ in flow-through rate. High flow rate LS columns provide a high purity of column-retained cell fractions (>95%), whereas low flow rate LD columns provide maximal recovery (depletion) of magnetically labeled cells (>99%).

12. Apply the cell suspension to the LD column and allow cells to pass through the column completely. Wash column with 2 ml cell washing/MACS buffer.
13. Remove collection tube with unlabeled (human) cell fraction. Centrifuge 5 min at $300 \times g$, 4°C.

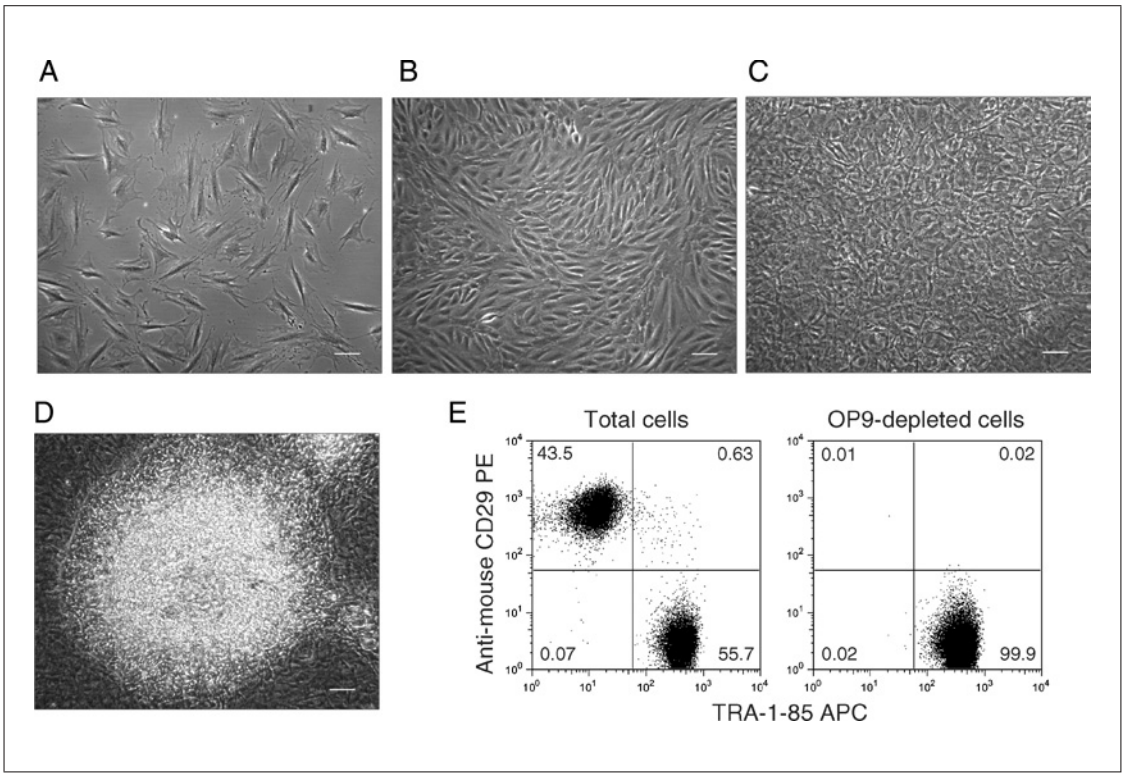


Figure 23.6.2 Major steps in establishing hESC differentiation cultures on OP9 cells and obtaining feeder-free differentiated human cells. (**A**) Morphology of OP9 cells on the day after plating. (**B**) Day 4 confluent OP9 cultures ready for passage. (**C**) Day 8 overgrown OP9 cultures prepared for hESC differentiation. (**D**) Differentiated "mesodermal" hESC colony on day 4 of H1/OP9 coculture. (**E**) Representative flow cytometric analysis of OP9 (mouse CD29-PE$^+$) and total hESC-derived (TRA-1-85-APC$^+$) cells on day 3 of H1/OP9 coculture before (left dot-plot) and after (right dot-plot) OP9 removal by magnetic sorting using depletion (LD) columns and anti-PE microbeads. Percentage values are shown in respective quadrants. Scale bar = 100 μm for all photographs.

14. Discard the supernatant and resuspend the cells in 1 ml cell washing/MACS buffer or medium. Reserve a 10- to 20-μl aliquot for FACS analysis.

15. Count the cells (see *UNIT 1.1*) and take an aliquot ($\sim 2 \times 10^4$ cells/test) for FACS analysis.

16. Analyze the cells by flow cytometry, labeling them with TRA-1-85-APC mAb and using 7AAD staining to exclude dead cells (see Support Protocol 1).

At analysis, mouse CD29-PE positive cells (OP9) should be negligible if detectable (<0.02%), whereas TRA-1-85$^+$ human cells should comprise >99.5% (Fig. 23.6.2E).

ISOLATION OF hESC-DERIVED HEMATOPOIETIC PROGENITORS AND ENDOTHELIAL CELLS

BASIC PROTOCOL 2

This protocol describes the isolation of hESC-derived hematopoietic progenitors and endothelial cells. As a first step, cells harvested on day 7 to 8 of hESC/OP9 coculture (Basic Protocol 1) are processed to isolate all hematopoietic progenitors by positive selection of CD43$^+$ cells using magnet-activated cell sorting (MACS) and depletion (LD) columns designed for efficient recovery of labeled cells. MACS-enriched CD43$^+$ cells labeled with CD235a (glycophorin A) and CD45 monoclonal antibodies (mAbs) are subsequently subjected to a FACS sorting procedure to isolate three types of hematopoietic progenitors: CD43$^+$CD235a$^+$CD45$^-$ erythro-megakaryocytic and multipotent CD43$^+$CD235a$^-$CD45$^-$ and CD43$^+$CD235a$^-$CD45$^+$ progenitors. Both CD43$^+$CD235a$^-$CD45$^-$ and CD43$^+$235a$^-$CD45$^+$ cells are negative

for lineage-specific markers and represent multipotent hematopoietic progenitors; however, $CD43^+CD235a^-CD45^+$ cells are enriched in myeloid progenitors and limited in lymphoid potential (Vodyanik et al., 2006). Endothelial cells can be subsequently isolated from the $CD43^-$ cell fraction by positive MACS selection of $CD31^+$ cells. $CD43^+$ subsets can be used for functional studies as well as a source of hematopoietic progenitors for directed differentiation towards specific hematopoietic lineages. Endothelial cells can be expanded in vitro for several passages in serum-free conditions (Support Protocol 7).

Materials

Cell suspension (Basic Protocol 1), hold on ice
Cell washing/MACS buffer (see recipe), degassed under vacuum and ice cold
Mouse anti-human CD43-FITC mAb (clone 1G10; BD Pharmingen)
Anti-FITC magnetic microbeads, (Miltenyi Biotec)
Basic (nonconjugated) microbeads (Miltenyi Biotec), optional
Mouse anti-human CD31-PE (clone WM59; BD Pharmingen)
Anti-PE magnetic microbeads, (Miltenyi Biotec)
FACS buffer without FBS (see recipe)
7-aminoactinomycin D (7AAD) solution (Via-Probe; BD Pharmingen)
Mouse IgG1-PE isotype control mAb (clone MOPC-21; BD Pharmingen)
Mouse IgG1-APC isotype control mAb (clone MOPC-21; BD Pharmingen)
Mouse anti-human CD235a-PE (clone CLB-ery-1; Caltag)
Mouse anti-human CD45-APC (clone HI30; BD Pharmingen)
FBS (GIBCO), heat inactivated

LD (depletion) columns, (Miltenyi Biotec)
15-ml graduated polypropylene tubes
Temperature-controlled centrifuge, 4°C
MACS rotation mixer, (Miltenyi Biotec)
35-μm preseparation filters (Miltenyi Biotec)
LS (positive selection) columns (Miltenyi Biotec)
Midi-MACS magnet/stand (Miltenyi Biotec)
Cell sorter (FACS Vantage SE; BD Immunocytometry Systems)
5-ml polypropylene tubes (Falcon)

Additional reagents and equipment for counting cells (*UNIT 1.1*) and performing FACS (Support Protocol 1)

NOTE: Cells and cell washing/MACS buffer must be kept on ice during the separation procedure.

Prepare cells

1. Add the suspension of cells (up to 10^8 cells) isolated from hESC/OP9 coculture (Basic Protocol 1) to a 15-ml graduated polypropylene tube.
2. Centrifuge 5 min at 400 × *g*, 4°C.
3. Aspirate the supernatant. Note the volume of cell pellet and add an equal volume of cell washing/MACS buffer.

   This resulting total volume is referred as labeling volume.

   Remember to keep cells and cell washing/MACS buffer on ice during separation procedure.

4. Resuspend cells thoroughly by gentle pipetting and add 1/10 vol CD43-FITC mAb.

   For example, if the cell pellet is ~0.2 ml, add 0.2 ml cell washing/MACS buffer (labeling volume is 0.4 ml), resuspend cells, and add 40 μl (1/10 vol) of CD43-FITC mAb.

5. Set up the tube on a MACS mixer. Place the MACS mixer with tube in refrigerator (4°C). Incubate 20 min at the lowest rotation speed.

Add magnetic beads

6. Wash the cells once by adding 14 ml ice-cold cell washing/MACS buffer and centrifuging 5 min at 400 × *g*, 4°C. Discard the supernatant and resuspend the cells in MACS buffer to original labeling volume and add 1/10 vol anti-FITC microbeads.

7. Incubate the cell suspension on MACS mixer 20 min at 4°C and low rotation speed.

8. Wash cell once by adding 14 ml ice-cold cell washing/MACS buffer and centrifuging 5 min at 400 × *g*, 4°C. Discard the supernatant and resuspend cell pellet in 1 ml cell washing/MACS buffer.

9. Place a 35-µm preseparation filter on an empty 15-ml tube. Apply the suspension to the filter and allow to completely pass through. Wash the filter with 0.5 ml cell washing/MACS buffer. Place tube with the filtered cells on ice.

Select CD43$^+$ cells

10. Assemble the MACS-LD separation unit (midi-MACS magnet with LD column and 15-ml collection tube). Rinse the column with 2 ml cell washing/MACS buffer and discard the tube with eluate. Place an empty collection tube under column.

11. Apply the suspension to the LD column and allow to completely pass through. Wash the column with 2 ml cell washing/MACS buffer.

12. Remove the collection tube containing the CD43$^-$ cell fraction and place on ice. Save the CD43$^-$ cells for endothelial cell isolation.

13. Remove the LD column from magnet and insert in an empty 15-ml tube. Add 5 ml cell washing/MACS buffer to the column funnel. Using a plunger (supplied with column), flush the CD43$^+$ cells out of the column. Discard the emptied column.

14. Centrifuge the tube containing the eluted CD43$^+$ cells 5 min at 300 × *g*, 4°C.

15. Discard the supernatant and resuspend the cells in 0.2 ml cell washing/MACS buffer and place tube on ice

    Typically, up to 10% of the cells are recovered in MACS-enriched CD43$^+$ cell fraction. For example, if the column were loaded with 10^8 cells, one should expect to recover 10^7 cells in CD43$^+$ fraction. This fraction usually contains 40% to 60% CD43$^+$ cells and can be used for subsequent sorting of CD43$^+$ subsets by FACS (step 23). If a higher purity of MACS-enriched CD43$^+$ cells is desired, all potential contaminants that bind nonspecifically to the magnetic beads and column can be removed prior to selection of CD43$^+$ cells. For this, cells (step 1) should be incubated with basic (unconjugated) microbeads (as in steps 6 to 9) and passed through an LD column.

    MACS magnetic microbeads are biodegradable microparticles (~50 nm) that do not interfere with subsequent FACS analysis, sorting, or functional assays. There is no need to release them from the cells after the separation procedure.

Select endothelial cells

16. Label the CD43$^-$ cell fraction (collected in step 12) with CD31-PE mAb and anti-PE microbeads as described in steps 2 to 9, but prepare the 35-µm filtered cell suspension (step 9) in 3 ml cell washing/MACS buffer.

    The cell filtrate should be prepared in 3 ml because this is the application volume for LS columns. This can be accomplished by doubling filtration volumes: resuspending the cells in 1 ml buffer, filtering, and washing the filter with 2 ml buffer to give a total volume of 3 ml or by adding 1.5 ml to the 1.5 ml cell filtrate obtained in step 9.

17. Assemble the MACS-LS separation unit (midi-MACS magnet with LS column and 15-ml collection tube). Rinse the column with 5 ml cell washing/MACS buffer and discard the tube with eluate. Place an empty collection tube under column.

    For a high purity (>98%) of isolated endothelial cells, positive selection (LS) columns must be used.

    There are two types of magnetic columns optimized for positive (LS) and negative (LD) MACS separation, and they differ in flow-through rate. High flow rate LS columns provide a high purity of column-retained cell fractions (>95%), whereas low flow rate LD columns provide maximal recovery (depletion) of magnetically labeled cells (>99%).

18. Apply the suspension to the LS column and allow to pass completely through. Wash the column three times with 3 ml cell washing/MACS buffer.

19. Discard the collection tube with the $CD31^-$ cell fraction.

Collect $CD31^+$ cells

20. Remove the LS column from the magnet and insert an empty 15-ml tube. Add 5 to 7 ml cell washing/MACS buffer to column funnel. Using a plunger (supplied with column), flush the $CD31^+$ cells out of column.

21. Discard emptied column. Centrifuge tube with eluted $CD31^+$ cells 5 min at 300 × *g*, 4°C.

22. Resuspend the cells in 1 ml cell washing/MACS buffer or medium for endothelial cell expansion (see Support Protocol 7). Count the cells (*UNIT 1.1*).

23. For FACS analysis, add 10 to 20 µl cell suspension to 0.4 ml FACS buffer (without FBS). Add 10 µl 7AAD solution for dead cell exclusion. Examine the cells by FACS (Support Protocol 1).

    CD43-FITC positive cells should not be detectable, whereas CD31-PE positive cells should comprise >98%.

Sort $CD43^+$ subsets by FACS

24. Add 5 µl mouse IgG1-PE and 5µl IgG1-APC isotype control mAbs to one 5-ml polypropylene tube, and add 10 µl CD235a-PE and 40 µl CD45-APC mAbs to another 5-ml tube.

25. Label MACS-enriched $CD43^+$ cells (from step 15, up to 10^7 cells in 0.2 ml) with isotype control and specific mAb combinations by adding 20 to 50 µl of the $CD43^+$ cells to the tube with isotype control mAbs and the rest of $CD43^+$ cells (150 to 180 µl) to the tube with CD235a-PE and CD45-APC mAbs.

26. Mix the cells gently and incubate samples 30 min at 4°C, with occasional agitation.

27. Wash cells twice with 5 ml cell washing/MACS buffer and resuspend in 0.5 ml cell washing/MACS buffer. Keep tubes on ice until sorting.

28. Before running on the cell sorter, pass cell samples through a 35-µm filter.

29. Using the control sample, setup the following gates:

    Live cells, using an FSC-A/SSC-A dot-plot (live $CD43^+$ cells form a compact population with low FSC/SSC profile)
    Single cells, using an SSC-A/FCS-W dot plot (exclude cell doublets)
    Total $CD43^+$ cells, using an FL1-CD43-FITC/SSC-A dot plot (verify instrument compensation).

    CD43-FITC-stained cells should not overlap the FL2 channel.

30. Open finally gated $CD43^+$ cells in an FL2-IgG1-PE/FL4-IgG1-APC dot plot. Set thresholds for positive staining.

31. Remove the control sample and insert a tube with $CD43^+$ cells stained with CD235a and CD45 mAbs. Identify three main $CD43^+$ subsets:

    $CD235a^+CD45^-$
    $CD235a^-CD45^-$
    $CD235a^-CD45^+$

    on an FL2-$CD235a^-$PE/FL4-$CD45^-$APC dot plot (Fig. 23.6.2B).

32. Set sorting gates on these populations and start sorting into 5-ml polypropylene tubes prefilled with 0.3 ml FBS. Sort at low pressure, using a wide 100-μm nozzle tip.

33. Remove tubes with sorted cells. Fill tubes with 4 ml cell washing/MACS buffer or medium and mix well by inverting tube several times. Centrifuge 5 min at 300 × *g*, 4°C.

34. Resuspend the cells in 0.5 ml medium and determine the number of sorted cells by manual counting (see *UNIT 1.1*).

    During the FACS sorting procedure, cells become electrically charged and may adhere to the tube walls. Therefore, the real number of sorted cells recovered after centrifugation is usually lower than known cell counts deposited by the sorter. For use in the following tests, sorted cells should be counted manually.

PROPAGATION OF hESCs ON MOUSE EMBRYONIC FIBROBLASTS

SUPPORT PROTOCOL 4

This protocol describes the maintenance of hESCs for in vitro hematopoietic differentiation based on the procedures described in Amit et al. (2000) and Thomson et al. (1998), with slight modifications. The hESCs are cultured on mouse embryonic fibroblasts (MEFs) and maintain pluripotency during an extended period of culture (Amit et al., 2000). They are less likely to produce chromosomal abnormalities, compared to methods that use feeder-free conditions (Draper et al., 2004). Undifferentiated hESCs grow in tight colonies attached to the MEF feeder. Passaging hESC cultures includes (1) detachment of hESC colonies by collagenase treatment, (2) fragmentation of hESC colonies into small clumps by pipetting, and (3) plating of hESC clumps to the newly prepared MEF feeder plate.

Materials

hESC cultures (H1 or H9 hESC lines; National Stem Cell Bank; see Internet Resources)
6-well plates with pre-plated irradiated MEFs (Support Protocol 5)
hESC growth medium (see recipe)
1× phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO), room temperature
Collagenase IV solution (see recipe)

37°C water bath
5-ml glass pipet, sterile

Detach hESC colonies from plates

1. Warm the collagenase IV solution and hESC growth medium 15 to 20 min in a 37°C water bath.

2. Remove an MEF feeder plate from CO_2 incubator. Aspirate the MEF growth medium and wash wells quickly with 2 ml PBS at room temperature.

3. Add hESC growth medium at 1.5 ml/well and keep MEF feeder plates in the CO_2 incubator until the hESC are ready for plating.
4. Remove the hESC plate from the CO_2 incubator. Aspirate the growth medium. Wash wells quickly with 2 ml PBS at room temperature and add 2 ml/well collagenase IV solution.
5. Incubate the hESC plate 10 to 15 min in the CO_2 incubator until the edges of the hESC colonies detach from plate.
6. Using a 5-ml glass pipet, dislodge the hESC colonies by washing the plastic surface with the collagenase solution. Use gentle scraping to dislodge colonies that may still be attached.

   After collagenase treatment, hESC colonies should be dislodged easily by washing or gentle scraping. Forceful scraping should be avoided to prevent cell damage and collection of the firmly attached colonies, which may contain differentiated cells. Excessive mechanical damage of hESCs during passage may also provoke their spontaneous differentiation.
7. Transfer the suspension to a 15-ml tube and centrifuge 5 min at 200 × *g*, room temperature.

Disperse colonies and plate

8. Aspirate the medium and resuspend the cells in 4 ml hESC growth medium. Break up the hESC colonies into a fine suspension of small cell aggregates by pipetting cells up and down against the bottom of tube. Centrifuge 5 min at 200 × *g*, room temperature.
9. Aspirate the medium, resuspend the cells in 6 ml hESC growth medium, and dispense the suspension in a 6-well MEF feeder plate (1 ml/well), using a vertically positioned glass serological pipet.
10. Place the plate on the shelf in the CO_2 incubator and move it forth-to-back and side-to-side to distribute hESC clumps evenly throughout the plate.

    Avoid rotating motions because this will lead to accumulation of cell clumps in the center of wells.
11. Allow the hESC clumps to attach to the MEFs for 24 hr. Do not disturb the plate during this time.
12. Feed the hESC cultures daily by aspirating 2.5 to 3 ml medium/well and adding the same amount of fresh prewarmed hESC growth medium. Passage on day 6 to 7.

    It is imperative to avoid excessive density of hESC in maintenance cultures because it may lead to hESC growth retardation, long-lasting spontaneous differentiation, genetic abnormalities, and eventually poor differentiation efficiency in OP9 coculture. As a rule, hESC cultures must be split when evenly positioned single hESC colonies begin to reach confluence. The authors typically split H1 and H9 hESC cultures every 6 to 7 days at a 1:6 split ratio. The number of hESCs on the day of passage is ~3 × 10^6 cells/ well of a 6-well plate.

SUPPORT PROTOCOL 5

PREPARATION OF MEF FEEDER PLATES

MEF feeder cells support attachment and undifferentiated growth of hESC colonies. MEF derivation and propagation can be performed according to established protocols from the suppliers—the National Stem Cell Bank (see Internet Resources) or commercial sources (e.g., ATCC, GlobalStem). The authors use an MEF stock frozen at passage 3 for weekly preparation of MEF feeder plates. After thawing, MEFs are propagated during one adaptation passage, inactivated by irradiation, and plated to 6-well plates in semiconfluent density.

Materials

Gelatin-coated 10-cm dishes and 6-well plates (see recipe)
CF-1 strain MEFs (e.g., National Stem Cell Bank or ATCC: frozen stock at passage 3; 2–3 $\times$ 10^6 cells/vial)
MEF growth medium (see recipe)
1$\times$ phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO)
0.05% (w/v) trypsin/0.5 mM EDTA solution (1$\times$; GIBCO)

15-ml polypropylene tubes
Gamma irradiator

Additional reagents and equipment for counting cells (*UNIT 1.1*)

Initiate MEF culture

1. Prepare one 15-ml tube and two gelatin-coated 10-cm dishes for each vial of MEFs to be thawed.
2. Thaw an MEF vial in a 37°C water bath and transfer the cells to a 15-ml tube.
3. Add 10 ml of cold (4°C) MEF growth medium to the cells in the tube and centrifuge 5 min at 300 $\times$ g, 4°C.
4. Aspirate the supernatant, resuspend the cells in 2 ml MEF growth medium, and add 1 ml to each gelatin-coated 10-cm dishes containing 10 ml MEF growth medium. Mix the cells by agitation and place the dishes in the CO_2 incubator.
5. Grow MEFs to confluence (3 to 4 days).

Collect MEFs

6. Remove the MEF dishes from the CO_2 incubator. Wash the cell monolayer once with 10 ml PBS and add 4 ml/dish prewarmed trypsin/EDTA solution. Incubate dishes 2 to 3 min in the CO_2 incubator.

   Do not incubate longer because the MEF monolayer disaggregates quickly.

7. Transfer the cell suspension to a 15-ml tube containing 5 ml MEF growth medium. Centrifuge tube 5 min at 300 $\times$ g, room temperature.
8. Aspirate and discard the supernatant and resuspend the cells in 2 ml MEF growth medium.

Inactivate MEFs

9. Irradiate tube with 5000 rads of gamma irradiation.
10. Centrifuge the cells 2 to 3 min at 300 $\times$ g, room temperature.
11. Resuspend the cells in 1 ml MEF growth medium.
12. Count the cells (*UNIT 1.1*) and adjust the MEF suspension to 2 $\times$ 10^5 cells/ml MEF growth medium.

Plate MEFs

13. Fill gelatin-coated 6-well plates with 2 ml/well MEF growth medium. Add 1 ml MEF suspension per well, mix, and incubate the plates in the CO_2 incubator at least 24 hr before hESC plating. Use MEF feeder plates within 1 week.

CULTURE OF OP9 CELLS

SUPPORT PROTOCOL 6

Originally isolated from macrophage colony–stimulating factor (M-CSF) deficient osteopetrotic (*op*/*op*) mice, OP9 cells have been identified as efficient inducers of hematoendothelial differentiation in mouse ESCs (Kodama et al., 1994; Nakano et al., 1994;

Zhang et al., 2005). While a direct application of mouse ESC/OP9 protocol for hESC resulted in relatively poor performance, modifications of OP9 culture have enabled efficient hematopoietic differentiation from hESCs (Vodyanik et al., 2005). These modifications include the use of overgrown OP9 cultures for hESC differentiation and the maintenance of OP9 cells on gelatin-coated dishes to minimize spontaneous adipogenesis and focal outgrowth during extended post-confluence culture.

After confluence, OP9 cells are prone to spontaneous adipogenic differentiation especially when cultured in adipogenic FBS. The supplier and particular lot of FBS should be selected by the ability of FBS to support efficient OP9 growth with minimal (if noticeable) adipogenesis during maintenance culture and after feeding (half medium change) and prolonged culture of confluent OP9 cells for 4 to 6 days. A high-quality FBS is mandatory. The authors use a "defined" type of FBS (a commercial description for high-quality, biochemically characterized FBS with minimal lot-to-lot variation) from HyClone without heat inactivation, which does not benefit culture but usually results in a higher adipogenic effect. Basal αMEM (standard formulation without nucleosides) must be prepared from powder. The authors split OP9 cultures on the next day after confluence, typically every 4th day at up to a 1:10 split ratio. On the day of passage, 2–3 × 10^6 OP9 cells can be recovered from one 10-cm dish.

Materials

OP9 growth medium (see recipe).
0.05% (w/v) trypsin/0.5 mM EDTA solution (1×; GIBCO)
OP9 cells (ATCC #CRL-2749)
1× phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO)
Gelatin-coated 10-cm dishes (see recipe)

15-ml polypropylene tubes
Inverted microscope

Passage OP9 cells

1. Warm OP9 growth medium and trypsin/EDTA solution in a 37°C water bath.
2. Remove the OP9 dishes from the CO_2 incubator. Aspirate and discard the medium and wash the cell monolayer with 10 ml PBS.
3. Add 5 ml trypsin/EDTA solution and incubate 5 to 10 min in the CO_2 incubator.
4. Suspend the trypsinized OP9 cells by pipetting and add the cell suspension to a 15-ml tube containing 5 ml OP9 growth medium. Centrifuge 5 min at 300 × *g*, room temperature.
5. Discard the supernatant and resuspend the cell pellet in 1 ml OP9 growth medium.
6. Add 1/7 to 1/10 vol of the cell suspension to each gelatin-coated 10-cm dish containing 10 ml OP9 growth medium. Mix cells by agitation and place dishes in the CO_2 incubator.

   It is essential to use gelatin-coated dishes for OP9 propagation to prevent spontaneous adipogenesis. Because the intensity of OP9 growth is largely influenced by FBS, a split ratio should be adjusted with each new FBS lot to ensure formation of confluent OP9 cultures during 3 to 4 days. Feeding of OP9 cells between passages and splitting of semiconfluent cultures should be avoided.

7. Grow OP9 cells to confluence and on the day after reaching confluence (typically 4 days), split again as described above.

Prepare OP9 dishes for hESC differentiation

8. Plate OP9 cells on gelatin-coated 10-cm dishes as for a regular OP9 passage (steps 1 to 6).

9. On day 4, remove 5 ml of the medium using a pipet and add 5 ml fresh prewarmed OP9 medium. Incubate OP9 dishes for an additional 4 days.

   OP9 cultures used for hESC differentiation must be 8 to 10 days old, fed with fresh medium at confluence (day 4) and incubated for extra 4 to 6 days.

10. Observe cultures on day 8 under an inverted microscope.

    A typical overgrown OP9 monolayer should be formed (Fig. 23.6.2C) indicating that cells are ready for hESC plating.

    Overgrown OP9 cells do not appear as multilayered overcrowding cells, but form a regular dense monolayer embedded in extracellular matrix (Fig. 23.6.2C). Scattered adipocytes may appear in overgrown OP9 cultures, but no areas of extensive adipogenesis or focal outgrowth should be detectable.

    It is possible to incubate OP9 dishes for an additional 4 days without feeding until hESCs will be available for differentiation. However, the authors usually do not use OP9 dishes beyond total 12 days of culture.

PROPAGATION OF ENDOTHELIAL CELLS

SUPPORT PROTOCOL 7

Endothelial cells isolated on day 7 to 8 of hESC/OP9 coculture can be expanded for 4 to 5 passages in serum-free medium in the presence of fibroblast growth factor (FGF). Since a $>100\times$ cell expansion can be achieved, hESC/OP9 cocultures may be used as a source of hESC-derived endothelial cells for a variety of functional studies.

Materials

MACS-isolated endothelial ($CD31^+CD43^-$) cells on day 7 to 8 of hESC/OP9 coculture (Basic Protocol 2, step 22)
Fibronectin-coated 10-cm tissue culture dishes (see recipe)
Endothelial cell growth medium (ECGM; see recipe)
$1\times$ phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO)
HyQ-Tase cell detachment solution (HyClone)
Endothelial serum-free medium (ESFM; GIBCO)
Temperature controlled centrifuge, 4°C

1. Plate 10^6 MACS-isolated endothelial ($CD31^+CD43^-$) cells on a fibronectin-coated 10-cm dishes containing 10 ml ECGM (10^6 cells/dish). Incubate in a CO_2 incubator 4 to 6 days until cells reach confluence.

   After a 1 to 2 day lag period, cells start proliferation and reach confluence in 3 to 4 days.

2. Aspirate the medium and wash the cell monolayer with 10 ml PBS.

3. Add 2 ml PBS and 2 ml HyQ-Tase solution. Mix the solutions by gently agitating and incubate 5 min in a CO_2 incubator.

4. Suspend the detached cells by pipetting and transfer the cell suspension to a 15-ml tube containing 5 ml ESFM. Centrifuge 5 min at $300 \times g$, room temperature.

5. Resuspend the cell pellet in 1 ml ESFM, add 1/5 vol to the freshly prepared fibronectin-coated 10-cm dish containing 10 ml ECGM. Mix the cells by agitation and place the dishes in the CO_2 incubator.

6. Grow the endothelial cells until they reach confluence (typically 4 days) and split as in steps 2 to 5.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cell washing/MACS buffer

Phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO) supplemented with 5% (v/v) heat-inactivated FBS (GIBCO) and 2 mM EDTA. Sterilize by passing through a 0.22-μm filter under vacuum. Allow the solution to remain under vacuum 15 to 20 min for degassing. Store up to 6 months at 4°C.

Keep the buffer on ice during a MACS procedure.

Collagenase IV solution

Dissolve 50 mg collagenase IV (GIBCO) in 50 ml DMEM/F12 basal medium (1 mg/ml final concentration). Sterilize by passing through a 0.22-μm filter. Store up to 2 weeks at 4°C.

Differentiation medium

Prepare the following components:

αMEM basal medium prepared from powder and sterilized by passing through a 0.22-μm filter. Store up to 2 months at 4°C.

50-ml aliquots of non-heat-inactivated defined FBS (HyClone). Store up to 1 year at −20°C.

1000× (100 mM) monothioglycerol (MTG) solution. Add 87 μl MTG (Sigma) to 10 ml water. Mix well and dispense into 0.5-ml aliquots. Store up to 6 months at −20°C.

1000× (50 mg/ml) ascorbic acid solution. Dissolve 500 mg ascorbic acid (Sigma) in 10 ml water. Dispense into 0.5-ml aliquots and store up to 6 months at −20°C.

To prepare differentiation medium:

Combine 450 ml αMEM basal medium (90%), 50 ml FBS (10%), 0.5 ml MTG (1×; 100 μM), and 0.5 ml ascorbic acid (1×; 50 μg/ml) in the upper chamber of a 500-ml bottle-top 0.22-μm filter unit (Nalgene). Sterilize by vacuum filtration and store up to 1 month at 4°C.

Endothelial cell growth medium

Supplement endothelial serum-free medium (ESFM; GIBCO) with 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech) and 1/100 vol endothelial cell growth factor (acidic FGF and heparin; Sigma E9640). Prepare immediately before use.

Do not store medium with cytokines.

FACS buffer, with and without FBS

Phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO) supplemented with 2% (v/v) heat-inactivated fetal bovine serum (FBS; GIBCO), if required; 2 mM EDTA; and 0.05% (w/v) sodium azide. Sterilize by passing through a 0.22- or 0.45-μm filter under vacuum. Store up to 6 months at 4°C.

Fibronectin-coated dishes

Dissolve 5 mg human fibronectin (GIBCO) in 5 ml sterile endotoxin-free water (Millipore). Store in 0.1-ml aliquots up to 6 months at −20°C. For plastic coating, dilute fibronectin solution 1/200 in phosphate-buffered saline (PBS; GIBCO; final

continued

concentration 5 μg/ml) and add 5 ml to each 10-cm tissue-culture dish (Falcon). Incubate dishes overnight at 4°C. Before use, aspirate the fibronectin solution and add the required amount of an appropriate growth medium.

Gelatin-coated dishes/plates

Prepare a 0.1% (w/v) gelatin solution: To a borosilicate glass bottle with autoclavable cap, add 500 mg Gelatin type A powder (Sigma) to 500 ml endotoxin-free reagent-grade water (Millipore). Mix well and autoclave the gelatin slurry 45 min at 121°C (gelatin will dissolve during autoclaving). Store up to 6 months at 4°C.

Add 6 to 7 ml gelatin solution to each 10-cm tissue-culture dish (Falcon) or 2 ml to each well of a 6-well tissue-culture plate (Falcon). Allow the gelatin solution to cover the entire plastic surface. Incubate dishes/plates in a CO_2 incubator at least overnight. Store up to 1 week in a CO_2 incubator. Before use, aspirate the gelatin solution and add the required amount of an appropriate medium.

hESC growth medium

Prepare the following components:

Prepare DMEM/F12 medium from powder (GIBCO). Sterilize by passing through a 0.22-μm filter. Store up to 2 months at 4°C.

100× L-glutamine/2-mercaptoethanol supplement. Dissolve 146 mg L-glutamine (GIBCO) in 10 ml phosphate-buffered saline, without calcium and magnesium (PBS; GIBCO) and add 7 μl of 2-mercaptoethanol (Sigma). Store up to 1 week at 4°C.

500× basic fibroblast growth factor (bFGF) solution. Dissolve 50 μg bFGF (PeproTech) in 25 ml PBS supplemented with 2 mg/ml bovine serum albumin V (GIBCO). Store up to 6 months at −80°C in 0.5-ml aliquots.

Dispense 50 ml aliquots of Knockout Serum Replacer (KSR; GIBCO). Store up to 1 year at −20°C.

To prepare complete hESC growth medium:

Combine 200 ml DMEM/F12 basal medium (80%), 50 ml Knockout Serum Replacer (20%), 2.5 ml 10 mM (100×) nonessential amino acid solution (GIBCO) (1×), 2.5 ml L-glutamine/2-mercapthoethanol (1×), and 0.5 ml bFGF (4 ng/ml) in the upper chamber of a 250-ml bottle-top 0.22-μm filter unit (Nalgene) and sterilize by passing through the filter under vacuum. Store up to 2 weeks at 4°C.

Final concentrations of components are given in parentheses.

MEF growth medium

Prepare the following components:

Sterile-filtered DMEM medium prepared from powder (GIBCO). Store up to 2 months at 4°C.

50-ml aliquots of heat-inactivated FBS (GIBCO). Store up to 1 year at −20°C.

To prepare MEF growth medium:

Combine 450 ml DMEM basal medium (90%), 50 ml FBS (10%), and 5 ml 10 mM (100×) nonessential amino acid solution (GIBCO) (1×) in upper chamber of 500-ml bottle-top 0.22-μm filter unit (Nalgene). Sterilize by passing through the filter under vacuum. Store up to 1 month at 4°C.

Final concentrations of components are given in parentheses.

OP9 growth medium

Prepare the following components:

Prepare αMEM medium from powder (GIBCO). Sterilize by passing through a 0.22-μm filter. Store up to 2 months at 4°C.

Dispense 50-ml aliquots of non-heat-inactivated defined FBS (HyClone). Store up to 1 year at −20°C.

To prepare OP9 growth medium:

Combine 400 ml αMEM basal medium (80%), and 100 ml FBS (20%) in upper chamber of 500-ml bottle-top 0.22-μm filter unit (Nalgene). Sterilize by passing through the filter under vacuum. Store up to 1 month at 4°C.

Final concentrations of components are given in parentheses.

COMMENTARY

Background Information

ESCs represent a unique population of cells capable of self-renewal and differentiation. hESCs give rise to tissues from all three germ layers upon injection into immunodeficient mice or when induced to form embryoid bodies in vitro (Thomson et al., 1998; Schuldiner et al., 2000). Systems for hematopoietic differentiation of hESCs provide a unique opportunity to study mechanisms regulating lineage commitment and maturation, identification of novel hematopoietic precursors, and evaluation of the function of genes critical for hematopoietic development. The ability of mouse ESCs to differentiate into hematopoietic cells was demonstrated in 1985 (Doetschman et al., 1985). In 2001, hematopoietic progenitors were obtained from hESCs cells (Kaufman et al., 2001). Since that time almost all blood lineages have been successfully generated from mouse and human ESCs, including red blood cells (Nakano et al., 1996; Qiu et al., 2005), neutrophils (Lieber et al., 2004; Vodyanik et al., 2005), megakaryocytes (Eto et al., 2002; Gaur et al., 2006), lymphocytes (Cho et al., 1999; Schmitt et al., 2004; Vodyanik et al., 2005; Woll et al., 2005; Galic et al., 2006), and dendritic cells (Fairchild et al., 2000; Senju et al., 2003; Slukvin et al., 2006).

Two major approaches are used to induce hematopoietic differentiation of ESCs. One approach allows ESCs to form embryoid bodies after withdrawal of factors that keep ESCs in an undifferentiated state. Various lineages of cells, including hematopoietic cells, develop inside embryoid bodies. The hematopoietic development within embryoid bodies is very similar to that found in the yolk sac (Burkert et al., 1991; Schmitt et al., 1991; Keller et al., 1993; Zambidis et al., 2005). The embryoid body method can be adapted to serum-free conditions (Ng et al., 2005), making it possible to exclude a variability related to different lots of the serum and identification of factors required for lineage-specific differentiation. However, it is difficult to induce lymphoid differentiation under normal culture conditions using an embryoid body method (Potocnik et al., 1994; Nakano, 2003).

Another approach to inducing hematopoietic differentiation is coculture of ESCs with bone marrow stromal cell lines. Several stromal cell lines, e.g., mouse bone marrow stromal cell lines S17, RP.0.10, and OP9 (Gutierrez-Ramos and Palacios, 1992; Nakano et al., 1994; Kaufman et al., 2001; Vodyanik et al., 2005), mouse yolk sac C166 cell line (Kaufman et al., 2001), and human fetal liver FH-B-hTERT cell line (Qiu et al., 2005), have been employed to induce hematopoietic differentiation of ESCs. The most commonly used stromal cell line for hematopoietic differentiation studies is OP9, which was derived from M-CSF-deficient *op*/*op* osteopetrotic mice (Yoshida et al., 1990; Kodama et al., 1994). The OP9 coculture was used to obtain multilineage hematopoietic progenitors as well as mature hematopoietic cells such as lymphocytes and megakaryocytes, which are difficult to obtain using the embryoid body method (Nakano et al., 1994; Eto et al., 2002; Vodyanik et al., 2005; Gaur et al., 2006). An important advantage of OP9 coculture is that efficient hematopoietic differentiation from hESCs can be achieved within a short period of time and without added cytokines. In addition, in contrast to the embryoid body

method, OP9 coculture allows one to obtain the earliest lymphohematopoietic progenitors without applying excessive mechanical disaggregation, which results in substantial viability loss. It should be noted, that differentiation of ESCs in a monolayer on extracellular matrix proteins has also been described (Nishikawa et al., 1998; Zhang et al., 2006), but it is not widely used.

The hematopoietic system comprises a hierarchy of cells at different stages of maturation, which can be identified using morphologic and phenotypic criteria as well as functional assays. The hematopoietic stem cell (HSC) is defined as a cell able to generate all types of hematopoietic cells and capable of self-renewal throughout the lifetime of an individual. While isolation of $CD34^+$ cells from bone marrow, unbilical cord, or peripheral blood highly enriches for HSCs, true HSCs can only be identified retrospectively using an in vivo repopulation assay. In this assay, HSCs are injected into immunocompromised mice (e.g., NOD/SCID), and the presence of hematopoietic progeny is evaluated 8 to 10 weeks after transplantation. While several investigators have reported successful engraftment of mouse and human ESC-derived hematopoietic progenitors (Hole et al., 1996; Potocnik et al., 1997; Burt et al., 2004; Wang et al., 2005a), these results have not been widely reproduced, and it is generally accepted that engraftment by ESC-derived hematopoietic progenitors is inefficient at the least (Daley, 2003; Nakano, 2003; Keller, 2005). This failure to engraft is at least partially due to an abnormal expression profile of *Hox* genes and could be corrected through forced expression of *HoxB4* and *Cdx4* genes (Kyba et al., 2002; Wang et al., 2005b).

Hematopoietic progenitors represent cells that may be multipotent, oligopotent, or unipotent, but they lack significant self-renewal capacity. Several types of in vitro assays have been developed to characterize hematopoietic progenitors at different stages of maturation. The colony-forming cell (CFC) assay in semisolid media is the most widely used and well-standardized assay for detection of progenitors at intermediate stages of development. This relatively simple assay requires a short period of time to perform and allows identification of several types of hematopoietic progenitors in one dish. The CFC assay remains an essential tool for characterizing hematopoietic progenitors generated from hESCs. Long-term in vitro assays are designed to identify less mature hematopoietic progenitors and usually require 3 to 5 weeks of culture in the presence of bone marrow-derived stromal cells. These assays identify cells that retain CFC potential after prolonged culture (long-term culture initiating cells; LTC-ICs) as well lymphoid progenitors (Coulombel, 2004). However, long-term in vitro assays are laborious and subject to great variability due to differences between laboratories in the use of media, growth factors, serum, and feeder cells for performing assays. It should also be noted that all in vitro assays do not reflect the presence of cells with stem cell potential in a sample.

Phenotypic analysis represents a valuable tool for the identification of different types of hematopoietic cells. The phenotype of adult hematopoietic progenitors is well characterized. However, it is important to emphasize that somatic and hESC-derived hematopoietic progenitors may demonstrate substantial phenotypic differences. While CD34 is present on most hESC-derived hematopoietic cells, it is also expressed on endothelial and mesenchymal cells. Flow cytometric analysis using CD34, CD43, and KDR (FLK1) or CD31 antibodies is useful for discriminating these subsets of CD34 cells in hESC/OP9 coculture (Vodyanik et al., 2006). The commonly used pan-hematopoietic marker CD45 is not expressed on the earliest hESC-derived multipotent hematopoietic progenitors. Another pan-hematopoietic marker (CD43) is present on all hematopoietic progeny from hESCs. This molecule is useful for evaluation of hESC hematopoietic differentiation as well as for isolation of hematopoietic progenitors from hESC/OP9 cocultures (Vodyanik et al., 2006). The first-appearing $CD43^+CD235a^+CD41a^{+/-}CD45^-$ hematopoietic progenitors in hESC/OP9 coculture represent precommitted erythro-megakaryocytic progenitors.

The primary hESC progeny with multilineage hematopoietic potential has $CD34^+CD43^+CD45^-Lin^-$ phenotype. These cells are capable of differentiation toward all blood lineages as well as B lymphoid cells. Acquisition of CD45 expression by $CD34^+CD43^+CD45^-Lin^-$ cells is associated with progressive myeloid commitment and a decrease of lymphoid potential (Vodyanik et al., 2006). Isolation of these progenitors and subsequent studies of their differentiation potential may provide important information regarding regulatory signals governing the development of specific hematopoietic lineages, and will eventually facilitate development of

bioreactor-based technology for large-scale production of blood cells for transfusion and cellular therapy.

Critical Parameters and Troubleshooting

It is essential to use semiconfluent monolayers of MEFs for hESC propagation. Overcrowded MEFs suppress growth of undifferentiated hESC colonies and may cause their spontaneous differentiation. Different batches of MEFs prepared using different lots of FBS and plastic may vary in cell size, and, therefore, MEF plating density should be adjusted accordingly to ensure a semiconfluent feeder layer for hESC culture.

A high-density overgrown OP9 monolayer is the most critical parameter for successful hematopoietic differentiation of hESCs. However, OP9 cells are prone to spontaneous adipogenic differentiation in postconfluence cultures. To minimize adipogenesis in high-density overgrown cultures, OP9 cells should be cultured on gelatin-coated dishes using preselected nonadipogenic lots of FBS. OP9 cells obtained from different sources (directly from Dr. Nakano or ATCC) efficiently support hematopoietic hESC differentiation. However, the authors recommend adaptation of OP9 cells for growth on gelatin-coated dishes for at least five passages before the cells are used for hESC differentiation.

A high-quality basal αMEM medium is essential for maintenance of OP9 cells as well as hematopoietic differentiation in hESC/OP9 cocultures. Freshly prepared αMEM from powder formulation is superior to commercial ready-to-use liquid medium. The αMEM formulation supplemented with nucleosides increases proliferation of OP9 cells in maintenance cultures, but has little effect on hematoendothelial differentiation in hESC/OP9 coculture. Addition of 100 μM (final concentration) SH-agent MTG to differentiation medium significantly increases the yield of total differentiated cells in hESC/OP9 cocultures including hematopoietic and endothelial cells, however at >200 μM concentration, hematoendothelial differentiation may be suppressed. While αMEM already contains a high concentration of ascorbate, the authors use additional supplementation of differentiation medium with 50 μg/ml ascorbic acid to specifically improve hematoendothelial differentiation.

Optimal hESC plating density in OP9 cocultures is important for efficient differentiation and may vary for different hESC lines. A plating density of 1.5×10^6 cells per 10-cm OP9 dish is acceptable for H1 or H9 hESC lines, although H9 cells differentiate more efficiently when plated at a lower density (10^6 cells/OP9 dish). Optimal plating density for other hESC lines should be determined in preliminary experiments using an initial range of $0.5–2.5 \times 10^6$ cells/OP9 dish with 0.5 intervals.

One problem that can be encountered is poor viability and cell loss following preparation of a single-cell suspension from hESC/OP9 cocultures, especially after 8 days of differentiation. This may happen because of excessive production of an extracellular matrix that withstands collagenase and trypsin treatment. As a result, many cells may be lost due to clumping and subsequent mechanical damage during pipetting. Longer incubation with collagenase and trypsin (up to 30 min each) should be used first to improve cell recovery. In addition, collagenase solution can be supplemented with 0.1 mg/ml hyaluronidase to further facilitate cell dissociation.

While the function of the CD43 molecule on early hematopoietic progenitors is largely unknown, it has been shown that cross-linking of CD43 on hematopoietic progenitors may induce apoptosis (Bazil et al., 1995). Therefore, it is critical to use antibodies that minimally affect the cell viability and function of isolated $CD43^+$ cells. Procedures in this unit use clone 1G10 anti-CD43 mAbs, which detect $CD43^+$ cells at maximal frequency compared to other anti-CD43 mAb clones (L10, 290111) and enable successful isolation of functional $CD43^+$ cells. It is important to note, that some anti-CD235a mAbs may cause cell agglutination, affecting flow cytometric detection and isolation of erythroid progenitors. Such cell clumping may be prevented by using a lower concentration of agglutinating mAb for cell labeling. Anti-CD235a mAb included in the present protocols are non-agglutinating.

Anticipated Results

On day 1 of hESC/OP9 coculture, only condensed hESC clumps attached to OP9 monolayer can be observed. Differentiation begins on day 2 when outgrowing hESCs immerse into OP9 monolayer. At this time, the first differentiating hESC colonies are readily detectable. On day 3, the number and size of differentiated colonies increase dramatically. Colonies acquire the characteristic appearance of "mesodermal" colonies with an elevated central portion composed of tightly packed rounded cells (Fig. 23.6.2D). On day 4, almost

all colonies have a typical "mesodermal" morphology and reach maximal size. On day 5, colonies begin the lateral growth that leads to their decomposition. Actively growing differentiated cells displace the OP9 cells and form confluent cultures on day 7. During 8 to 9 days, cultures do not change significantly, although ongoing differentiation can be observed by the emergence of proliferating cell clusters and vascular tubes. Due to high cell density, cultures begin to deteriorate after 10 to 12 days.

The efficiency of hematopoietic differentiation may vary for different hESC lines. Using H1 and H9 hESC lines, consistently higher differentiation efficiency in H1 cells is observed, especially regarding hematopoietic cell generation. Although H9 cells reproduce the kinetic profile of hematoendothelial differentiation observed in H1 cells, H9 differentiation is usually delayed for 1 day and results in 1.5 to 2× lower numbers of $CD43^+$ hematopoietic cells. A description of typical results for H1 cells (assuming that efficiency with other hESC lines may be different) follows: On day 7 to 8 of H1/OP9 coculture, H1-derived cells (TRA-1-85$^+$) constitute ~90% of total cells. Hematopoietic and endothelial cells ($CD31^+$) comprise 8% to 15% of total H1-derived cells. $CD34^+$ cells are always detectable in higher frequency (12% to 25%), due to the presence of $CD34^+$ mesenchymal cells. The $CD31^+$ population is typically composed of 60% to 70% endothelial cells ($CD31^+CD43^-$) and 30% to 40% hematopoietic progenitors ($CD31^+CD43^+$) that corresponds to 5% to 10% and 3% to 5% of total H1-derived cells, respectively. In the $CD43^+$ population, $CD43^+CD235a^+$ erythro-megakaryocytic progenitors comprise 50% to 70% of total $CD43^+$ cells or ~3% of total H1-derived cells. Multipotent progenitors found within the $CD43^+CD235a^-$ population (30% to 50% of total $CD43^+$ or ~2% of total H1-derived cells) undergo transition from $CD45^-$ to $CD45^+$ stage during 7 to 8 days of H1/OP9 coculture; on day 7, $CD45^-$ cells predominate (~80%), whereas on day 8, $CD45^-$ and $CD45^+$ cells comprise nearly equal populations.

The yield of endothelial and hematopoietic cells in isolation procedures is primarily dependent on the efficiency of hESC differentiation and the absolute number of cells generated in hESC/OP9 cocultures. The latter may vary from 15–30 × 10^6 cells per 10-cm dish and generally depends on variations in hESC growth during the first 2 to 3 days of coculture. For MACS isolation of 10^6 endothelial ($CD31^+CD43^-$) or hematopoietic ($CD31^+CD43^+$) cells, one to two H1/OP9 dishes is usually enough, but for FACS isolation of $CD43^+$ subsets, the number of H1/OP9 dishes should be scaled up as follows: for 10^6 $CD43^+CD235a^+$ erythro-megakaryocytic progenitors, three to four dishes; for 10^6 total $CD43^+CD235a^-CD45^{+/-}$ multipotent progenitors, eight to ten dishes. To isolate 10^6 FACS-sorted $CD45^-$ and $CD45^+$ multipotent progenitors on day 8, at least twenty H1/OP9 dishes should be processed.

Time Considerations

Setting up of hESC/OP9 cocultures requires coordinated maintenance of undifferentiated hESC, MEF, and OP9 cell cultures. One passage hESC culture takes 6 to 7 days. On the next day after hESC passage, new MEF culture should be initiated to ensure availability of MEF feeder plates for the next hESC passage. From 2–3 × 10^6 initially thawed MEFs, three to four feeder plates can be prepared. At splitting of a 6-well hESC plate, two wells will be used for hESC passage and counting, and four wells can be used for differentiation. Up to 8–12 × 10^6 hESCs can be collected from four wells, and that is sufficient for setting up six to eight hESC/OP9 dishes. At splitting of one 10-cm OP9 dish, 1/7 to 1/10 portions will be used for passage, and remaining cells can be plated on six to nine dishes for hESC differentiation. hESCs should be plated on OP9 dishes within 8 to 12 days after OP9 plating. During this time interval, actively growing undifferentiated hESCs (at 5 to 7 days of culture) should be available for differentiation.

All standard cell culture procedures with hESC, MEF, and OP9 maintenance cultures are time efficient and can be performed within 1 hr or less. One experiment for hESC differentiation can be completed within 15 to 20 days; it requires 8 to 12 days for OP9 preparation and 7 to 8 days for hESC/OP9 coculture. Depending on the volume of hESC differentiation cultures (typically up to ten hESC/OP9 dishes in one experiment) each of the following procedures may take 2 to 4 hr: harvesting of hESC/OP9 cocultures, analysis of differentiated cells by flow cytometry and cell plating for the methylcellulose CFC assay, single parameter MACS sorting, and FACS sorting.

Literature Cited

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.P., Harris, C.P., Waknitz, M.A., Itskovitz-Eldor, J., and Thomson, J.A. 2000. Clonally derived human embryonic stem cell lines maintain

pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* 227:271-278.

Bazil, V., Brandt, J., Tsukamoto, A., and Hoffman, R. 1995. Apoptosis of human hematopoietic progenitor cells induced by crosslinking of surface CD43, the major sialoglycoprotein of leukocytes. *Blood* 86:502-511.

Burkert, U., von Ruden, T., and Wagner, E.F. 1991. Early fetal hematopoietic development from in vitro differentiated embryonic stem cells. *New Biol.* 3:698-708.

Burt, R.K., Verda, L., Kim, D.A., Oyama, Y., Luo, K., and Link, C. 2004. Embryonic stem cells as an alternate marrow donor source: Engraftment without graft-versus-host disease. *J. Exp. Med* 199:895-904.

Cho, S.K., Webber, T.D., Carlyle, J.R., Nakano, T., Lewis, S.M., and Zuniga-Pflucker, J.C. 1999. Functional characterization of B lymphocytes generated in vitro from embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 96:9797-9802.

Coulombel, L. 2004. Identification of hematopoietic stem/progenitor cells: Strength and drawbacks of functional assays. *Oncogene* 23:7210-7222.

Daley, G.Q. 2003. From embryos to embryoid bodies: Generating blood from embryonic stem cells. *Ann. N.Y. Acad. Sci.* 996:122-131.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands, and myocardium. *J. Embryol. Exp. Morphol.* 87:27-45.

Draper, J.S., Smith, K., Gokhale, P., Moore, H.D., Maltby, E., Johnson, J., Meisner, L., Zwaka, T.P., Thomson, J.A., and Andrews, P.W. 2004. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat. Biotechnol.* 22:53-54.

Eaves, C.J. and Lambie, K. 1995. Atlas of Human Hematopoietic Colonies. StemCell Technologies, Vancouver, Canada.

Eto, K., Murphy, R., Kerrigan, S.W., Bertoni, A., Stuhlmann, H., Nakano, T., Leavitt, A.D., and Shattil, S.J. 2002. Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. *Proc. Nat. Acad. Sci. U.S.A.* 99:12819-12824.

Fairchild, P.J., Brook, F.A., Gardner, R.L., Graca, L., Strong, V., Tone, Y., Tone, M., Nolan, K.F., and Waldmann, H. 2000. Directed differentiation of dendritic cells from mouse embryonic stem cells. *Curr. Biol.* 10:1515-1518.

Galic, Z., Kitchen, S.G., Kacena, A., Subramanian, A., Burke, B., Cortado, R., and Zack, J.A. 2006. T lineage differentiation from human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 103:11742-11747.

Gaur, M., Kamata, T., Wang, S., Moran, B., Shattil, S.J., and Leavitt, A.D. 2006. Megakaryocytes derived from human embryonic stem cells: A genetically tractable system to study megakaryocytopoiesis and integrin function. *J. Thromb. Haemost.* 4:436-442.

Gutierrez-Ramos, J.C. and Palacios, R. 1992. In vitro differentiation of embryonic stem cells into lymphocyte precursors able to generate T and B lymphocytes in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 89:9171-9175.

Hole, N., Graham, G.J., Menzel, U., and Ansell, J.D. 1996. A limited temporal window for the derivation of multilineage repopulating hematopoietic progenitors during embryonal stem cell differentiation in vitro. *Blood* 88:1266-1276.

Kaufman, D.S., Hanson, E.T., Lewis, R.L., Auerbach, R., and Thomson, J.A. 2001. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc. Nat. Acad. Sci. U.S.A.* 98:10716-10721.

Keller, G. 2005. Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. *Genes. Dev.* 19:1129-1155.

Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M.V. 1993. Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell Biol.* 13:473-486.

Kodama, H., Nose, M., Niida, S., and Nishikawa, S. 1994. Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp. Hem.* 22:979-984.

Kyba, M., Perlingeiro, R.C., and Daley, G.Q. 2002. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 109:29-37.

Lensch, M.W. and Daley, G.Q. 2004. Origins of mammalian hematopoiesis: In vivo paradigms and in vitro models. *Curr. Top. Dev. Biol.* 60:127-196.

Lieber, J.G., Webb, S., Suratt, B.T., Young, S.K., Johnson, G.L., Keller, G.M., and Worthen, G.S. 2004. The in vitro production and characterization of neutrophils from embryonic stem cells. *Blood* 103:852-859.

Nakano, T. 2003. Hematopoietic stem cells: Generation and manipulation. *Trends Immunol.* 24:589-594.

Nakano, T., Kodama, H., and Honjo, T. 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 265:1098-1101.

Nakano, T., Kodama, H., and Honjo, T. 1996. In vitro development of primitive and definitive erythrocytes from different precursors. *Science* 272:722-724.

Ng, E.S., Davis, R.P., Azzola, L., Stanley, E.G., and Elefanty, A.G. 2005. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. *Blood* 106:1601-1603.

Nishikawa, S.I., Nishikawa, S., Hirashima, M., Matsuyoshi, N., and Kodama, H. 1998. Progressive lineage analysis by cell sorting and culture identifies FLK1$^+$VE-cadherin$^+$ cells at a diverging point of endothelial and hemopoietic lineages. *Development* 125:1747-1757.

Potocnik, A.J., Nielsen, P.J., and Eichmann, K. 1994. In vitro generation of lymphoid precursors from embryonic stem cells. *Embo. J.* 13:5274-5283.

Potocnik, A.J., Kohler, H., and Eichmann, K. 1997. Hemato-lymphoid in vivo reconstitution potential of subpopulations derived from in vitro differentiated embryonic stem cells. *Proc. Nat. Acad. Sci. U.S.A.* 94:10295-300.

Qiu, C., Hanson, E., Olivier, E., Inada, M., Kaufman, D.S., Gupta, S., and Bouhassira, E.E. 2005. Differentiation of human embryonic stem cells into hematopoietic cells by coculture with human fetal liver cells recapitulates the globin switch that occurs early in development. *Exp. Hem.* 33:1450-1458.

Robertson, S., Kennedy, M., and Keller, G. 1999. Hematopoietic commitment during embryogenesis. *Ann. N.Y. Acad. Sci.* 872:9-16.

Schmitt, R.M., Bruyns, E., and Snodgrass, H.R. 1991. Hematopoietic development of embryonic stem cells in vitro: Cytokine and receptor gene expression. *Genes Dev.* 5:728-740.

Schmitt, T.M., de Pooter, R.F., Gronski, M.A., Cho, S.K., Ohashi, P.S., and Zuniga-Pflucker, J.C. 2004. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat. Immunol.* 5:410-417.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D.A., and Benvenisty, N. 2000. From the cover: Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. Nat. Acad. Sci. U.S.A.* 97:11307-11312.

Senju, S., Hirata, S., Matsuyoshi, H., Masuda, M., Uemura, Y., Araki, K., Yamamura, K., and Nishimura, Y. 2003. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. *Blood* 101:3501-3508.

Slukvin, I.I., Vodyanik, M.A., Thomson, J.A., Gumenyuk, M.E., and Choi, K.D. 2006. Directed differentiation of human embryonic stem cells into functional dendritic cells through the myeloid pathway. *J. Immunol.* 176:2924-2932.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.

Vodyanik, M.A., Bork, J.A., Thomson, J.A., and Slukvin, I.I. 2005. Human embryonic stem cell-derived CD34$^+$ cells: Efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood* 105:617-626.

Vodyanik, M.A., Thomson, J.A., and Slukvin, I.I. 2006. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. *Blood* 108:2095-2105.

Wang, L., Menendez, P., Shojaei, F., Li, L., Mazurier, F., Dick, J.E., Cerdan, C., Levac, K., and Bhatia, M. 2005a. Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *J. Exp. Med.* 201:1603-1614.

Wang, Y., Yates, F., Naveiras, O., Ernst, P., and Daley, G.Q. 2005b. Embryonic stem cell-derived hematopoietic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 102:19081-19086.

Williams, B.P., Daniels, G.L., Pym, B., Sheer, D., Povey, S., Okubo, Y., Andrews, P.W., and Goodfellow, P.N. 1988. Biochemical and genetic analysis of the OKa blood group antigen. *Immunogenetics* 27:322-329.

Woll, P.S., Martin, C.H., Miller, J.S., and Kaufman, D.S. 2005. Human embryonic stem cell-derived NK cells acquire functional receptors and cytolytic activity. *J. Immunol.* 175:5095-5103.

Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., and Shultz, L.D. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 345:442-444.

Zambidis, E.T., Peault, B., Park, T.S., Bunz, F., and Civin, C.I. 2005. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. *Blood* 106:860-870.

Zhang, H., Saeki, K., Kimura, A., Nakahara, M., Doshi, M., Kondo, Y., Nakano, T., and Yuo, A. 2006. Efficient and repetitive production of hematopoietic and endothelial cells from feeder-free monolayer culture system of primate embryonic stem cells. *Biol. Reprod.* 74:295-306.

Zhang, W.J., Park, C., Arentson, E., and Choi, K. 2005. Modulation of hematopoietic and endothelial cell differentiation from mouse embryonic stem cells by different culture conditions. *Blood* 105:111-114.

Key References

Coulombel, L. 2004. See above.

Provides a critical overview of in vivo and in vitro functional assays used for analysis hematopoietic stem cells and progenitors.

Vodyanik, M.A., Thomson, J.A., and Slukvin, I.I. 2006. See above.

Describes methods for generation and pathways of hematopoietic and endothelial differentiation from hESCs cocultured on OP9.

Zambidis, E.T., Peault, B., Park, T.S., Bunz, F., and Civin, C.I. 2005. See above.

Describes procedures for and pathways of hematopoietic differentiation from hESCs using the embryoid body method.

Internet Resources

http://www.nationalstemcellbank.org

National Stem Cell Bank. Distributes hESC lines and provides technical support to the hESC research community and technical training in the culture of hESCs. In addition, information regarding karyotype and HLA genotype of distributed cells is provided. Protocols for culture, freezing, and thawing of hESCs as well as for derivation, culture, and propagation of mouse embryonic fibroblasts are also available.

http://www.stemcell.com

Stem Cell Technologies. Protocols for hematopoietic stem cell/progenitor research.

Neural Differentiation of Human ES Cells

Malkiel A. Cohen,[1] Pavey Itsykson,[1] and Benjamin E. Reubinoff[1]

[1]Hadassah University Medical Center, Ein-Kerem, Jerusalem, Israel

ABSTRACT

Human embryonic stem cells (hESCs) may be converted into highly enriched cultures of neural precursors under defined culture conditions. The neural precursors can proliferate in culture for prolonged periods of time, and can differentiate in vitro into mature neurons, astrocytes, and oligodendrocytes. The neurons are functional and have normal electrophysiological properties. After transplantation to the developing rodent brain, the neural precursors migrate extensively into the host brain parenchyma, respond to host brain signals, and differentiate in a region-specific manner to progeny of the three neural lineages. The establishment of neuroectodermal precursors from hESCs allows the study of human neurogenesis in vitro and is an aid in drug discovery. In addition, the neural precursors may potentially serve as a platform for the development of specific functional neural cells for transplantation and gene therapy of neurological disorders. In this unit, we introduce methods for the derivation, propagation and characterization of hESC-derived neural precursors. *Curr. Protoc. Cell Biol.* 36:23.7.1-23.7.20. © 2007 by John Wiley & Sons, Inc.

Keywords: neural induction • human embryonic stem cells • neural precursors • noggin • neural differentiation

INTRODUCTION

Embryonic stem (ES) cells can differentiate into all cell types of the body including neural cells, and thus offer an in vitro model for tracing early cell lineages in mammals. Derivation of neural precursors (NPs) from human ES cells (hESCs) may be invaluable for the study of early human neurogenesis and for the utilization of hESCs as an unlimited source of neural cells for transplantation in human neurodegenerative disorders. Neural cells may be generated from hESCs following spontaneous differentiation in vitro, either in high-density cultures or through embryoid body (EB) formation. However, the neural cells that are generated from these cultures are within a mixture of other types of differentiated cells. Protocols for the controlled derivation of cultures highly enriched for proliferating, developmentally competent NPs were recently reported.

Here, a protocol is described for the derivation of NPs from hESCs in chemically defined serum-free culture conditions (Basic Protocol). This protocol enables the controlled differentiation of hESCs into NPs by two major steps. In the first step, clusters of hESCs are enzymatically removed from the feeder cells to chemically defined serum-free suspension culture conditions. Contamination of the hESC-clusters by residual feeder cells is avoided. In the second step, controlled efficient differentiation of the hESCs to an NP fate is achieved by supplementation with the growth and differentiation–inducing factors noggin [bone morphogenic protein (BMP) antagonist] and basic fibroblast growth factor (bFGF), mimicking neural induction in vivo. Methods are also described for the characterization of the NPs by immunocytochemistry (Support Protocol 1) and flow cytometry (Support Protocol 2), and for induction of their spontaneous differentiation (Support Protocol 3). Finally, a protocol for culturing human embryonic stem cells is provided (Support Protocol 4).

Current Protocols in Cell Biology 23.7.1-23.7.20, September 2007
Published online September 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2307s36

NOTE: All incubations are performed in 5% CO_2, humidified 37°C incubators unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All procedures describing the use of phosphate-buffered saline (PBS) in this unit refer to calcium- and magnesium-free PBS (e.g., Invitrogen, cat. no. 14190) unless PBS with calcium and magnesium (e.g., Invitrogen, cat. no. 14040) is specified.

BASIC PROTOCOL

CONTROLLED DERIVATION OF NEURAL PRECURSORS FROM hESCS

Controlled neural differentiation of hESCs enables the enrichment of NPs in the cell culture under serum-free suspension culture conditions. hESCs are cultured in a chemically defined medium supplemented with noggin and bFGF to promote neural differentiation, and then in a medium supplemented with bFGF to further propagate the hESC-derived NPs.

Materials

0.1% (w/v) low melting temperature (LMT) agarose (see recipe)
hESCs cultured on a feeder layer (see Support Protocol 4) in 6-well plates
1 mg/ml collagenase type IV (see recipe)
Neural precursor medium (NPM; see recipe)
20 μg/ml bFGF (see recipe)
100 μg/ml noggin (see recipe)
NPM (see recipe) containing 20 ng/ml bFGF (added from 20 μg/ml bFGF stock; see recipe) and 500 ng/ml noggin (add from 100 μg/ml noggin stock; see recipe)
NPM (see recipe) containing 20 ng/ml bFGF (added from 20 μg/ml bFGF stock; see recipe)

50-ml conical polypropylene centrifuge tubes
Centrifuge
24-well tissue culture plates
50-ml centrifuge tubes
20-G surgical blades

Additional reagents and equipment for culturing hESCs (Support Protocol 4)

Collect hESC clusters from culture

1. To prevent adhesion of hESC clusters to the culture dishes, cover the well bottoms of a 24-well plate with 0.5 ml of 0.1% low-melting temperature agarose for at least 30 min. Aspirate the agarose suspension and dry the plate at room temperature.

   Alternatively, one can use a low-cell-attachment plate.

2. Aspirate the medium from a 6-well plate containing hESC-colonies cultured on feeders, 6 to 7 days after their passage with EDTA as described in Support Protocol 4.

   Use the EDTA passage method (Support Protocol 4) for at least the last passage before hESC-cluster formation, to achieve hESC colonies homogenous in size (Fig. 23.7.1A).

3. Apply 1 ml of 1 mg/ml (~200 U) collagenase type IV to each well and incubate for 30 to 60 min.

   After this incubation, the edges of the hESC colonies should detach from the feeder layer (Fig. 23.7.1B).

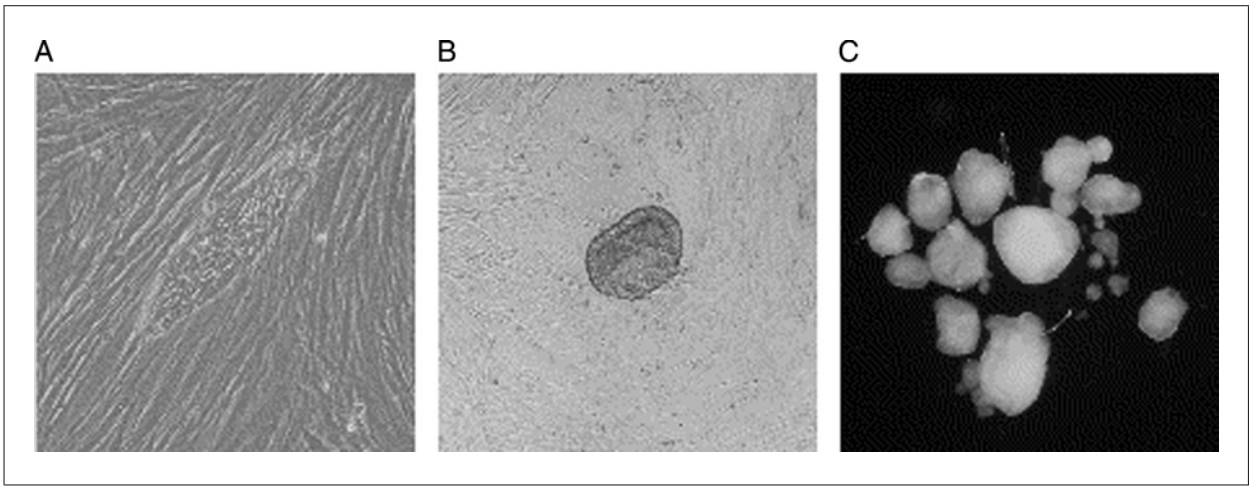


Figure 23.7.1 Derivation of NPs from undifferentiated hESCs. (**A**) An undifferentiated hESC colony cultured on human foreskin fibroblasts, 1 week after passaging with the aid of an EDTA dissociation solution (phase-contrast image; 40× magnification). (**B**) hESC colony after 30 min of collagenase treatment (phase-contrast image; 40× magnification). (**C**) hESC-derived neural spheres 3 weeks after derivation and culture in defined medium (NPM) supplemented with bFGF and noggin (dark-field stereomicroscopic image; 2× magnification).

4. Tap the plate to gently dislodge the clusters of hESCs from the feeder cells.

   The feeders should remain intact on the bottom of the well and the hESC clusters should be free of contaminating feeder cells.

5. Collect the supernatant medium with the hESC clusters from all wells and transfer to a 50-ml tube. Gently centrifuge 5 min at ~40 × *g*, room temperature.

Transfer hESC clusters to cultures with NPM

6. Aspirate the supernatant from the cell clusters and resuspend them in neural precursor medium (NPM) at a density of up to 80 × 10^3 cells/ml (~40 clumps/ml).

7. Add 1 μl of 20 μg/ml bFGF to each 1 ml of cluster suspension, to reach a final concentration of 20 ng/ml.

8. Add 5 μl of 100 μg/ml noggin to each 1 ml of cluster suspension, to reach a final concentration of 500 ng/ml.

9. Transfer the clusters at 1 ml per well to a 24-well culture dish pretreated with 0.1% LMT agarose (see step 1).

   Upon transfer of the hESC clusters into NPM, significant cell death should be observed during the first 2 to 3 days, which should be followed by a gradual increase in the sizes of the floating clusters, which acquire the form of spheres (Fig. 23.7.1C).

Propagate the neural cultures

10. Culture the clusters for the first 3 weeks in NPM containing 20 ng/ml bFGF and 500 ng/ml noggin.

11. Change the medium every 2 days as follows. Swirl the plate to centralize the spheres in the middle of the well, collect the spheres into a microcentrifuge tube, let the tube stand for 3 min to allow the spheres to sink, remove the supernatant, add 1 ml of fresh prewarmed NPM containing 20 ng/ml bFGF and 500 ng/ml noggin, and replate the sphere suspension in the well.

12. After 3 weeks of culture, change the medium to NPM supplemented with 20 ng/ml bFGF. Refresh the medium every 2 days, using the technique described in step 11.

13. Check the cultures weekly and dissect any spheres or aggregates of spheres whose diameters exceed 0.5 mm into small clusters with two 20-G surgical blades that are used like scissors, or by trituration. For trituration, use a pipettor with a 200-µl tip, and repeatedly draw the sphere suspension up and down to dissociate the spheres.

At a time point 4 weeks after transfer of the hESC clusters into NPM, the spheres are highly enriched for NPs that can be used for further differentiation protocols.

SUPPORT PROTOCOL 1

CHARACTERIZATION OF THE NEURAL PRECURSORS BY IMMUNOSTAINING

The phenotype of the pluripotent cells and the NPs derived from them may be analyzed by immunostaining. Staining of both pluripotent and NP markers and their analysis at sequential time points makes it possible to track the progression of neural differentiation over time.

Materials

10 µg/ml poly-D-lysine (see recipe)
Tissue culture–grade distilled H_2O
4 µg/ml laminin (see recipe)
NPs derived from hESCs (see Basic Protocol)
0.008% (w/v) trypsin/2.4 mM EDTA (see recipe)
Phosphate-buffered saline (PBS; containing calcium and magnesium; Invitrogen, cat. no. 14040)
2.35 mg/ml DNase (see recipe)
4% (w/v) paraformaldehyde (see recipe)
FACS buffer (see recipe)
0.2% (v/v) Triton X-100 (see recipe)
Blocking solution (see recipe)
Appropriate primary antibody (Table 23.7.1)
Secondary antibody: fluorophore-conjugated antibody against IgG of species from which primary antibody was derived
Mounting medium with 4′-6-diamidino-2-phenylindole (DAPI; e.g., Vectashield from Vector Laboratories)

16-mm-diameter round glass coverslips, sterile (Paul Marienfeld & Co.; *http://www.marienfeld-superior.com*)
Center-well organ culture dish (Falcon)
Glass microscope slides

Coat coverslips

1. Place a glass coverslip in the center of a center-well organ culture dish. Cover the glass coverslip with 500 µl of 10 µg/ml poly-D-lysine and incubate 1 hr at room temperature.

The staining procedure is done in organ culture dishes; coverslips must be maintained in the organ culture dishes until the fixation step (step 8) and can then be transferred to 12-well plates.

2. Aspirate the poly-D-lysine solution and wash the glass coverslip with 1 ml of tissue culture–grade distilled water for 1 min. Aspirate the water and cover the glass coverslip with 500 µl of 4 µg/ml laminin. Incubate overnight at 4°C or for 2 hr at room temperature.

Laminin-coated glass coverslips can be kept up to 1 month at 4°C in a sealed chamber.

Plate single-cell suspensions of NPs

The NPs can be plated on the glass coverslips as cell clusters or as single cells. Cell clusters should be plated on precoated glass coverslips with 1 ml of NPM for 24 hr. For single-cell plating, follow the steps described below.

3. Collect the clusters of NPs (Basic Protocol) into a microcentrifuge tube and microcentrifuge for a few seconds at 240 × *g*, room temperature.
4. Aspirate the supernatant and resuspend the clusters in 500 μl of 0.008% trypsin/2.4 mM EDTA. Incubate at 37°C for 15 min.
5. Pipet the clusters up and down to dissociate them into a single-cell suspension. Microcentrifuge the cells for a few seconds at 240 × *g*.

   A white cloudy pellet should appear.

6. Gently remove the supernatant and add 500 μl of PBS (with Ca and Mg) and 5 μl of 2.35 mg/m DNase, then incubate for an additional 10 min. Pipet the pellet gently up and down to obtain a single-cell suspension. Microcentrifuge the cells for a few seconds at 240 × *g*, room temperature. Remove the supernatant and resuspend the cells in 500 μl NPM.
7. Plate the cell suspension on glass coverslips pre-coated with poly-D-lysine and laminin (from step 2) at a density of 5–10 × 10^3 cells/ml, and incubate for 1 to 2 hr at 37°C to allow the cells to attach to the coverslips.

Fix the cells

8. Fix the cells by flooding them with 2 ml of 4% paraformaldehyde and incubating 20 min at room temperature.
9. Aspirate the supernatant from the coverslip and wash three times, each time for 1 min with 1 ml of FACS buffer.

   The cells can be kept under the FACS buffer for up to 1 week at 4°C.

10. For immunostaining of intracellular markers, permeabilize cell membranes by flooding the cells with 1 ml of 0.2% Triton X-100 and incubating 5 min at room temperature.
11. Incubate the cells with 500 μl blocking solution for 30 to 60 min at room temperature.

Immunostain the cells

12. Aspirate the supernatant from the coverslip and incubate the cells with the desired primary antibody (Table 23.7.1), diluted in FACS buffer as described in Table 23.7.1, for 1 to 2 hr at room temperature.

    Proper control staining for the primary and secondary antibodies should be conducted to rule out nonspecific staining or antibody cross-reactivity.

13. Aspirate the supernatant from the coverslip and wash three times, each time for 1 min with 1 ml of FACS buffer.
14. For primary antibody localization, incubate the cells with an appropriate fluorophore-conjugated secondary antibody, diluted in FACS buffer according to the manufacturer's recommendations, for 1 to 2 hr at room temperature.
15. Aspirate the supernatant from the coverslip and wash three times, each time for 1 min with 1 ml of FACS buffer.
16. Thoroughly aspirate the FACS buffer from the glass coverslip and mount it on a glass microscope slide using mounting medium with DAPI.

Table 23.7.1 Primary Antibodies for In Vitro Immunostaining and Flow Cytometry[a]

	Antigen/antibody	Species and type	Clonality	Purpose	Dilution	Source
ESC pluripotency	Oct-4	Mouse IgG	Monoclonal	Staining FACS	1:100 1:20	Santa Cruz Biotech.
	SSEA4	Mouse IgG	Monoclonal	Staining FACS	1:100 1:100	DSHB
	SSEA3	Rat IgM	Monoclonal	Staining FACS	1:100	Chemicon
	Tra-1-60	Mouse IgM	Monoclonal	Staining FACS	1:20-50 1:100	Chemicon
	Tra-1-81	Mouse IgM	Monoclonal	Staining FACS	1:10 1:100	Chemicon
Neural precursors	NCAM	Mouse IgG	Monoclonal	Staining	1:10	Dako
	PSA-NCAM	Mouse IgM	Monoclonal	Staining FACS	1:200 1:250	Chemicon
	Nestin	Rabbit	Polyclonal	Staining	1:200	Chemicon
	Pax6	Mouse IgG	Monoclonal	Staining	1:100	DSHB
	Sox1	Chicken	Polyclonal	Staining	1:1000	Chemicon
	Musashi	Rabbit	Polyclonal	Staining	1:100	Chemicon
Neurons	β-tubulin III	Mouse IgG	Monoclonal	Staining	1:2000	Sigma
	MAP2ab	Rabbit	Polyclonal	Staining	1:500	Chemicon
	NF 70 kDa	Mouse IgG	Monoclonal	Staining	1:100	Dako
	NF 160 kDa	Mouse IgG	Monoclonal	Staining	1:50	Chemicon
	NF 200 kDa	Rabbit	Polyclonal	Staining	1:5000	Sigma
	NeuN	Mouse IgG	Monoclonal	Staining	1:100	Chemicon
	Neuron-specific enolase (NSE)	Rabbit	Polyclonal	Staining	1:200	Zymed
	Synaptophysin	Mouse IgG	Monoclonal	Staining	1:50	Dako
	GABA	Rabbit	Polyclonal	Staining	1:1000	Sigma
	Glutamate	Rabbit	Polyclonal	Staining	1:1000	Sigma
	Serotonin	Rabbit	Polyclonal	Staining	1:1000	Sigma
	Tyrosine hydroxylase (TH)	Mouse IgG	Monoclonal	Staining	1:500	Sigma
Glia	GFAP	Rabbit	Polyclonal	Staining	1:200	Dako
	O4	Mouse IgM	Monoclonal	Staining	1:30	Chemicon

[a] Abbreviations: DSHB, Developmental Studies Hybridoma Bank, University of Iowa (*http://dshb.biology.uiowa.edu/*); GABA, γ-aminobutyric acid; GFAP, glial fibrillary acid protein; NCAM, neural cell adhesion molecule; PSA-NCAM, polysialylated form of NCAM; MAP2, microtubule-associated protein 2; NF, neurofilament; NeuN, neuronal nuclei.

CHARACTERIZATION OF NEURAL DIFFERENTIATION BY FLOW CYTOMETRIC ANALYSIS

The percentage of cells expressing markers of NPs or pluripotent cells and the intensity of expression of the markers should be analyzed by FACS. Analysis at sequential time points enables the characterization of the progression of neural differentiation over time. This protocol describes the characterization of both undifferentiated hESCs and NPs. The initial preparation of the two types of cells is described separately.

Materials

hESCs cultured on a feeder layer (see Support Protocol 4) *or* NPs derived from hESCs (see Basic Protocol)
0.05% (w/v) disodium EDTA
0.008% (w/v) trypsin/2.4 mM EDTA (see recipe)
Phosphate-buffered saline (PBS; Invitrogen) containing calcium and magnesium (Invitrogen, cat. no. 14040)
2.35 mg/ml DNase (see recipe)
FACS buffer (see recipe)
100% ethanol, –20°C
Permeabilization buffer (see recipe)
Appropriate primary antibody (Table 23.7.1)
Goat anti–mouse immunoglobulin conjugated with fluorescein isothiocyanate (FITC; Dako)
2 μg/ml propidium iodide (see recipe)

35-μm nylon mesh
15-ml centrifuge tubes
5-ml polystyrene round-bottom tubes (Falcon)
Refrigerated centrifuge
Flow cytometer (also see Robinson et al., 2007)

Additional reagents and equipment for counting cells (*UNIT 1.1*) and flow cytometry (Robinson et al., 2007)

Prepare cells

To prepare hESCs

1a. Dissociate the hESC colonies into a single-cell suspension by adding 2 ml of 0.05% EDTA per well and incubating 10 min at room temperature.

2a. With the aid of a pipettor, repeatedly blow 1 ml of the EDTA solution onto the hESCs to dislodge them from the feeders.

The feeders should remain intact and adherent to the culture dish.

3a. Filter the hESC suspension through a 35-μm nylon mesh to remove clusters.

4a. Transfer the cells to a 15-ml tube and centrifuge 5 min at ~240 × g, room temperature.

Prepare neural precursor clusters

1b. Collect the NP clusters into a 15-ml centrifuge tube and centrifuge 5 min at ~40 × g for 5 min, room temperature.

2b. Aspirate the supernatant and resuspend the NP clusters in 2 ml of 0.008% trypsin/2.4 mM EDTA. Incubate at 37°C for 15 min.

3b. Pipet the clusters up and down to dissociate them into a single-cell suspension. Centrifuge the NPs 5 min at ~40 × *g*, room temperature.

A white cloudy pellet should appear.

4b. Gently remove the supernatant and add 1 ml of PBS (with Ca and Mg) and 10 μl of 2.35 mg/ml DNase. Incubate an additional 10 min, and pipet the pellet gently up and down to obtain a single-cell suspension. Filter the cell suspension through a 35-μm nylon mesh and centrifuge 5 min at ~240 × *g*, room temperature.

Prepare cells for staining

The following steps should be done on ice. Centrifugation should be carried out in a refrigerated centrifuge appropriate for the centrifugation of 5-ml tubes.

5. Resuspend the cells in 1 ml cold FACS buffer. Count the cells and split them into aliquots of 100–150 × 10^3 cells in 5-ml round-bottom tubes.
6. *To detect nuclear proteins:* Fix the dissociated cells with 3 ml precooled 100% ethanol at –20°C for 15 min. Centrifuge the fixed cells 5 min at ~460 × *g*, 4°C, and remove the supernatant. Permeabilize the cells by adding 3 ml permeabilization buffer and incubating 15 min at 4°C.
7. Centrifuge cells (from step 5 or 6) 5 min at ~460 × *g*, 4°C.
8. Wash the cells by adding 2 ml of cold FACS buffer, then centrifuging 5 min at ~460 × *g*, 4°C.
9. Remove the supernatant by turning the tubes upside down.

   The cells should stay on the bottom of the tubes with ~100 μl of remaining fluid.

Immunostain cells

10. Incubate the cells 30 min on ice with the desired primary antibody (Table 23.7.1), diluted in FACS buffer as described in Table 23.7.1.
11. Wash the cells by adding 2 ml of cold FACS buffer and centrifuge for 5 min at ~460 × *g*, 4°C. Spill out the supernatant as described in step 9.
12. Detect primary antibodies by incubating the cells on ice for 30 min with goat anti-mouse FITC conjugated immunoglobulins diluted 1:100 with FACS buffer.
13. Wash the cells again by adding 2 ml of cold FACS buffer and centrifuging for 5 min at ~460 × *g* at 4°C. Spill out the supernatant as described in step 9.
14. Add 300 μl of 2 μg/ml propidium iodide solution for gating of viable cells.
15. Acquire 1–2 × 10^4 cells for each sample and analyze by flow cytometry with appropriate software.

    Robinson et al. (2007) provides detailed protocols for flow cytometry.

SUPPORT PROTOCOL 3

SPONTANEOUS DIFFERENTIATION OF hESC-DERIVED NPS

The characterization of the NPs should include an analysis of their potential to give rise to mature neurons and glia cells**.** Here, the methodologies for induction of differentiation in vitro and for the immunophenotyping of differentiated progeny are described**.**

Materials

10 μg/ml poly-D-lysine (see recipe)
Tissue culture–grade distilled H_2O
4 μg/ml laminin (see recipe)
NPs derived from hESCs (see Basic Protocol)
Neural precursor medium (NPM; see recipe)
16-mm-diameter round glass coverslips, sterile (Paul Marienfeld & Co.; *http://www.marienfeld-superior.com*)
Center-well organ culture dish (Falcon)

1. Place a glass coverslip in the center of a center-well organ culture dish. Cover the glass coverslip with 500 µl of 10 µg/ml poly-D-lysine and incubate 1 hr at room temperature.
2. Aspirate the poly-D-lysine solution and wash the glass coverslip with 1 ml of tissue culture–grade distilled water for 1 min. Aspirate the water and cover the glass coverslip with 500 µl of 4 µg/ml laminin. Incubate overnight at 4°C or for 2 hr at room temperature.

   Poly-D-lysine/laminin-coated glass coverslips can be kept up to 1 month at 4°C in a sealed chamber.
3. Collect the NP clusters (Basic Protocol) into a microcentrifuge tube and microcentrifuge for a few seconds at ~240 × *g*, room temperature. Aspirate the supernatant and add 1 ml NPM.
4. Partially dissociate the cluster by pipetting the clumps gently up and down several times.
5. Plate the cell suspension on a glass coverslip precoated with poly-D-lysine and laminin (from step 2), bring the volume to 1 ml with NPM (not supplemented with mitogens), and incubate the cells at 37°C for 7 to 21 days.
6. Replenish the culture medium (NPM) every 3 to 4 days.

   To evaluate the developmental potential of the NPs, their capability to differentiate into progeny representing the three major neural lineages should be characterized by immunostaining for neuronal, astrocytic, and oligodendrocytic markers. The methods for fixation and immunostaining are described in Support Protocol 1.

CULTURING HUMAN EMBRYONIC STEM CELLS

SUPPORT PROTOCOL 4

hESCs with a normal karyotype may be maintained on mouse embryonic fibroblasts, on the extracellular matrix substrate Matrigel (see *UNIT 23.2*) or on human foreskin fibroblasts. The authors' NP-derivation protocol is well established for hESCs maintained on mitomycin C–treated human foreskin fibroblasts.

Materials

Cultures of human foreskin fibroblasts (ATCC # SCRC-1041)
Phosphate-buffered saline (PBS; Invitrogen, cat. no. 14190), prewarmed to 37°C
0.04% (w/v) trypsin/0.16 mM EDTA (see recipe)
Feeder cell medium (see recipe)
2 mg/ml mitomycin C (see recipe)
0.1% (w/v) gelatin (see recipe)
Cultures of hESCs
0.05% (w/v) disodium EDTA
KnockOut (KO) medium (see recipe)
1 mg/ml collagenase type IV (see recipe)
175-cm^2 tissue culture flasks with 2-µm vent caps
Inverted microscope
15- and 50-ml conical polypropylene centrifuge
6-well tissue culture plates

Additional reagents and equipment for counting cells (*UNIT 1.1*)

Culture human foreskin fibroblasts

1. Culture human foreskin fibroblasts as a monolayer in a 175-cm^2 tissue culture flask in 40 ml feeder cell medium.

2. When the cells appear confluent, aspirate the medium. Rinse twice with 10 ml prewarmed PBS, each time for 1 min.
3. Add 2 ml of 0.04% trypsin/0.16 mM EDTA to the 175-cm^2 flask. Ensure that the entire cell surface is covered for 1 to 2 min.
4. Tap the flask to remove the cells. Observe the flask under an inverted microscope to ensure that trypsinization has been effective and the detachment of fibroblasts is complete.
5. Inactivate trypsin by adding 10 ml of prewarmed feeder cell medium and pipet the medium gently several times, blowing it onto the culture surface of the flask to remove remnants of attached fibroblasts and to disaggregate clusters of fibroblasts. Transfer the cells to a 50-ml tube.
6. Centrifuge the cells 5 min at ~240 × *g*, room temperature. Remove the supernatant and resuspend the cells in 10 ml feeder cell medium.
7. Count cells in a 10-µl aliquot using a hemacytometer (*UNIT 1.1*) and calculate the total cell number. Plate the cells in new 175-cm^2 flasks (3–3.4 × 10^6 cells per flask for a 3-day incubation period, or 2–2.6 × 10^6 cells per flask for a 4-day incubation period). Add prewarmed feeder cell medium to a total volume of 40 ml per flask.
8. Swirl the flasks gently. Incubate the flasks in a 37°C 5% CO_2 incubator for 3 to 4 days. When the cells are confluent, repeat the procedure.

Prepare feeder layers

9. Incubate a 175-cm^2 flask of confluent human foreskin fibroblasts with 20 ml of feeder cell medium supplemented with 125 µl of 2 mg/ml mitomycin C solution for 2.5 hr.
10. Meanwhile, cover the bottom of wells of a 6-well plate with 0.1% gelatin for a minimum time period of 30 min. Aspirate the gelatin solution and allow the bottom of the wells to dry.
11. Aspirate the mitomycin C–containing feeder cell medium from the 175-cm^2 flask of human foreskin fibroblasts and replace it with 20 ml prewarmed feeder cell medium without mitomycin C.
12. Trypsinize the fibroblasts as described in steps 1 to 5.
13. Count cells in a 10-µl aliquot using a hemacytometer (*UNIT 1.1*) and calculate the total cell number.
14. Plate 3 × 10^5 of the mitomycin C–treated fibroblasts in 2 ml of feeder cell medium per each gelatin-precoated well of the plates prepared in step 10. Incubate plate, without changing the medium, for at least 24 hr and up to 5 days before plating the hESCs.

Culture hESCs

Colonies of hESCs are cultured on feeders in KO medium and are passaged to a new feeder layer every 6 to 7 days. Passage is performed before the hESC colonies attach one to another and before the cells in their center start piling up, which may be associated with unwanted differentiation. Passage can be done using EDTA (Fig. 23.7.1A.) or using collagenase.

To passage cells using EDTA

15a. Dissociate the hESCs into a single-cell suspension by incubation with 2 ml per well of 0.05% EDTA for 10 min. With the aid of a pipettor, repeatedly blow 1 ml of the EDTA solution on the hESCs colonies to dislodge them from the feeders.

The feeders should remain intact and adherent to the culture dish.

16a. Transfer the cells to a 15-ml tube and centrifuge 5 min at ~240 × *g*, room temperature.

17a. Resuspend the cells in KO medium, count them (*UNIT 1.1*), and plate 4–6 × 10^4 cells per well of a 6-well plate on fresh mitomycin C–treated foreskin feeders generated as described above.

18a. Replenish the culture medium every day.

Note that, due to the high frequency of chromosomal abnormalities observed after extended passaging of hESCs as single cells, it is recommended that the number of passages performed with the aid of EDTA be limited (Mitalipova et al., 2005).

To passage cells using collagenase

15b. Dissociate the hESCs into small clusters by incubating for 1 to 2 hr with 1 ml per well of 1 mg/ml (~200 U/ mg) collagenase type IV.

16b. Tap the plate to gently dislodge the clusters or gently blow medium onto the hESC colonies to remove them from the feeders.

The feeders should remain intact on the bottom of the well and the hESC clusters should be free of contaminating feeder cells.

17b. Transfer the cell clusters to a 15-ml centrifuge tube and centrifuge the clusters 5 min at ~40 × *g*, room temperature.

18b. Resuspend the clusters in KO medium, pipet them gently up and down to disassemble the clumps, and plate the hESC-clusters on fresh, mitomycin C–treated foreskin feeder layers, splitting at 1:3 ratio (one well to three new wells).

19b. Continue incubation, replenishing culture medium every day.

REAGENTS AND SOLUTIONS

Use tissue culture–grade, distilled water in all recipes and protocol steps. All procedures describing the use of phosphate-buffered saline (PBS) in this unit refer to calcium- and magnesium-free PBS (e.g. Invitrogen, cat. no. 14190) unless PBS with calcium and magnesium is specified (e.g., Invitrogen, cat. no. 14040). For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

bFGF, 20 μg/ml

Dissolve 50 μg of recombinant human bFGF (R&D Systems) by adding 250 μl of 5 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*) and 2250 μl of sterile 0.1% (w/v) BSA (Sigma) in PBS (Invitrogen, cat. no. 14190). Divide into aliquots and store up to 3 months at −20°C.

Blocking solution

Supplement FACS buffer (see recipe) with 5% (v/v) donkey or goat serum (Sigma). Prepare fresh.

Collagenase IV, 1 mg/ml

Add 6 mg (~1200 U) of collagenase IV (Invitrogen) to 6 ml of KO medium (see recipe) for each 6-well plate to be used. Filter through a 0.22-μm filter. Prepare fresh as needed.

DNase, 2.35 mg/ml

Dissolve 2.35 mg (~4700 U) of DNase (Sigma) in 1 ml of PBS (Invitrogen, cat. no. 14190), aliquot and store up to 2 months at −20°C.

FACS buffer

PBS (Invitrogen, cat. no. 14190) supplemented with:
0.1% (w/v) BSA (Sigma)
0.05% (w/v) sodium azide (N_aN_3)
Store up to 3 months at −20°C

Feeder cell medium

High-glucose DMEM (Invitrogen) supplemented with:
10% (v/v) fetal bovine serum (FBS; Hyclone)
2 mM L- glutamine (Invitrogen)
50 U/ml penicillin/50 μg/ml streptomycin (Invitrogen)
Store up to 2 weeks at 4°C

Gelatin, 0.1% (w/v)

Stock solution: (1% gelatin): Dissolve 0.25 g gelatin powder (Sigma) in 25 ml distilled water in a 50-ml tube (Falcon) to obtain a 1% (w/v) gelatin stock. Autoclave and store as 25-ml aliquots up to 1 year at 4°C.

Working solution (0.1% gelatin): Dilute 25 ml of 1% (w/v) gelatin in 225 ml distilled water. Store up to 4 weeks at 4°C.

KnockOut (KO) medium

KnockOut DMEM (Invitrogen) supplemented with:
14% (v/v) KnockOut Serum Replacement (Invitrogen)
2 mM L-glutamine (Invitrogen)
1% (v/v) nonessential amino acids (Invitrogen)
50 U/ml penicillin/50 μg/ml streptomycin (Invitrogen)
4 ng/ml bFGF: add 1 μl of 20 μg/ml bFGF (see recipe) per 5 ml KO medium to be prepared
Store up 2 weeks at 4°C

Batch testing of KnockOut serum replacement is imperative since there is variability, in the author's experience, with regard to the potential of different batches to support undifferentiated proliferation of hESCs.

Laminin, 4 μg/ml

Stock solution (1 mg/ml laminin): Prepare 1 mg/ml laminin (Sigma). Divide into 40-μl aliquots and store up to 1 year at −20°C.

Working solution (4 μg/ml laminin): Dilute 40 μl of 1 mg/ml laminin stock with 10 ml of PBS (Invitrogen, cat. no. 14190), for a final concentration of 4 μg/ml. Prepare fresh.

Low-melting-temperature (LMT) agarose, 0.1% (w/v)

Dissolve 0.2 g low-melting-temperature agarose (FMC BioProducts) in 200 ml distilled water. Store up to 3 months at room temperature. Boil the solution in a microwave oven for 1 to 2 min before each usage.

Culture dishes are pretreated with LMT agarose to prevent adhesion of cell clusters to their surfaces.

Mitomycin C, 2 mg/ml

Dissolve a 2 mg ampule of mitomycin C (Sigma) in 1 ml of PBS (Invitrogen, cat. no. 14190). Inject the PBS into the mitomycin C ampule using a syringe connected to a 23-G needle. Use a second needle to vent the ampule. Remove the solution and store in a 15-ml tube up to 1 week at 4°C protected from light.

CAUTION: *Mitomycin C is a toxic substance. See manufacturer's MSDS for handling instructions. Preparation of mitomycin C solutions should be conducted within an appropriate chemical fume hood, given the toxicity of this reagent.*

Neural precursor medium (NPM)

DMEM/F12 (1:1) (Invitrogen) supplemented with:
2% (v/v) B27 supplement (Invitrogen, cat. no. 17504)
2 mM L-glutamine (Invitrogen)
50 U/ml penicillin/50 μg/ml streptomycin (Invitrogen)
Store up to 2 weeks at 4°C

B27 supplement includes a low concentration of retinol. Since retinoic acid may restrict the developmental potential of NPs, B27 supplement that does not contain retinol (B27 supplement minus vitamin A; Invitrogen, cat. no., 12587) may be substituted.

Paraformaldehyde, 4% (w/v)

Dissolve 4 g of paraformaldehyde powder (Fluka) in 100 ml of PBS (Invitrogen, cat. no. 14190) at 60°C. Calibrate the pH to 7.4, filter, divide into aliquots, and store in a −20°C freezer for up to 6 months.

Permeabilization buffer

Supplement FACS buffer (see recipe) with 0.1% (v/v) Triton X-100 (Sigma). Prepare fresh.

Poly-D-lysine, 10 μg/ml

Stock solution (1 mg/ml poly-D-lysine): Dissolve 1 mg of poly-D-lysine (30 to 70 kDa; Sigma) in 1 ml of PBS (Invitrogen, cat. no. 14190), divide into 100-μl aliquots and store up to 2 months at −20°C.

Working solution (10 μg/ml poly-D-lysine) Dilute 100 μl of 1 mg/ml poly-D-lysine with 10 ml of PBS to a final concentration of 10 μg/ml. Prepare fresh.

Propidium iodide solution, 2 μg/ml

Dilute 1 volume of 1 mg/ml propidium iodide (Sigma) in 500 volumes of FACS buffer (see recipe) and store at 4°C up to 3 months.

Noggin stock, 100 μg/ml

Dissolve 50 μg of recombinant mouse noggin (R&D Systems) by adding 500 μl of sterile 0.1% (w/v) BSA (Sigma) in PBS (Invitrogen, cat. no. 14190). Divide into aliquots and store up to 3 months at −20°C.

Triton X-100, 0.2% (v/v)

Dilute 1 volume of Triton X-100 (Sigma) in 49 volumes of PBS (Invitrogen, cat. no. 14190) and store at room temperature up to 6 months.

Trypsin/EDTA solutions

0.04% (w/v) trypsin/0.16 mM EDTA: Dilute 1 volume of 0.25% (w/v) trypsin/EDTA (Invitrogen) in 5 volumes of PBS (Invitrogen, cat. no. 14190). Store at 4°C for up to 1 week.

0.008% (w/v) trypsin/2.4 mM EDTA: Dilute 1 volume of 0.04% (w/v) trypsin/0.16 mM EDTA (prepared as described above) in 4 volumes of 3 mM disodium EDTA in PBS. Keep at 4°C for up to 1 week.

COMMENTARY

Background Information

Controlled differentiation of ES cells into neural precursors (NPs) is required for the experimental dissection of the molecular events that occur during early development of the nervous system. Moreover, the generation of pure populations of hESC-derived neural progeny, rather than mixed populations of differentiated cells, is one of the major requirements for transplantation therapy (Li et al., 1998; Stavridis and Smith, 2003).

Derivation of NPs from mouse embryonic stem cells

During almost three decades of mouse ES cell research, several methods have been proposed for the conversion of ES cells into NPs. The most commonly used approach for induction of neural differentiation of mouse ES cells includes initial spontaneous differentiation within embryoid bodies (EBs) followed by treatment with retinoic acid (RA). EBs are formed when ES cells are cultured as free-floating clusters in the absence of feeder cells and anti-differentiation agents such as leukemia inhibitory factor (LIF). Under these conditions, spontaneous differentiation occurs, partially mimicking early processes of differentiation in the embryo, and therefore these clusters are termed EBs. Spontaneous differentiation of mouse ES cells within EBs yields a relatively small fraction of neural-lineage cells. To promote neural differentiation, ES cell aggregates are cultured first in the regular ES cell medium without LIF for 4 days and are then exposed to RA for another 4 days. Hence, this method is often regarded as a "4–/4+" protocol (Bain et al., 1995). While RA treatment of EBs promotes neural differentiation, the neural progeny that are formed are of a wide range of developmental stages and have a restricted developmental potential. This is supported by the study of Renoncourt et al. (1998), which showed that EBs treated with RA selectively differentiated into neuronal cell types characteristic of ventral CNS.

In addition to methods involving initial spontaneous differentiation within EBs, methods for directed differentiation of ES cells cultured as individual cells or in a monolayer have been developed by several investigators. These approaches use specific mesoderm-derived feeder cells or conditioned media to induce neural differentiation. The rationale behind coculturing with mesodermal cells is that signals from the mesoderm are required to induce neural specification of the ectoderm in vivo. Coculture of undifferentiated mouse ES cells with the bone marrow–derived stromal cell line PA6, under serum-free conditions or as a suspension culture in medium conditioned by the human hepatocellular carcinoma cell line HepG2, induces neural differentiation in a high percentage of the colonies (Kawasaki et al., 2000; Rathjen et al., 2002). However, the neuralizing agents exerted by PA6 or HepG2 lines remain unidentified as yet.

Given the limitations of most approaches for directing the differentiation of ES cells into a homogeneous population of NPs, complementary strategies to select neural cells from a heterogeneous population of differentiated cells have been developed. Enrichment for NPs may be accomplished in the mouse ES cell system by incorporating selective culture conditions. In this approach, ES cells are first cultured as aggregates to initiate spontaneous differentiation. They are then plated and cultured on an adhesive substrate in a serum-free medium. Under these selective conditions, the majority of cells die while NPs survive. The medium is then supplemented with bFGF to induce proliferation. After 6 to 8 days of selection and expansion, the NP cells are enriched to ~80% (Okabe et al., 1996; Brustle et al., 1997). Withdrawal of bFGF induces spontaneous differentiation into various subtypes of neurons and glia cells (Okabe et al., 1996; Brustle et al., 1999). The neurons are mature and electrophysiologically functional, and can generate both excitatory and inhibitory synaptic connections. In contrast to the RA

approach, NPs that are derived with the bFGF protocol are more synchronized with regard to their stage of differentiation, and their developmental potential is less restricted. In an alternative approach, neural cells have been sorted from heterogeneous populations of differentiated cells based on the expression of neural lineage-specific cell surface markers (Mujtaba et al., 1999), or by genetic selection. In the latter approach, a selectable marker was inserted into the open reading frame of genes encoding neural lineage-specific transcription factors, allowing genetic selection of NPs either by fluorescence-activated cell sorting (FACS) or drug selection (Li et al., 1998; Ying et al., 2003).

The inductive exocrine signals, such as those that are obtained from coculture or conditioned medium, are probably not required for mouse ES cells to commit themselves efficiently to the neural fate. Multicellular aggregates (Wiles and Johansson, 1999) or adherent monolayer cultures of ES cells (Ying et al., 2003) readily differentiated into neural cells when LIF and inductive signals for non-neural fates were eliminated. In the latter report, it was shown that the neural differentiation was not a simple default pathway but was dependent on autocrine fibroblast growth factor (FGF) signaling. This specific culture system could induce neural differentiation in ~60% of the ES cells, but could not give rise to a highly enriched preparation of NPs. A similar result was demonstrated when mouse ES cells were disaggregated into individual cells and were cultured at low density, under defined serum-free factor-free culture conditions. In the absence of non-neural inductive signals, the surviving individual cells acquired a neural identity, and gave rise to primitive neural stem cells (Tropepe et al., 2001; Smukler et al., 2006). Differentiation towards neural fate was more efficient in the presence of the BMP antagonist noggin. Neuralization in these reports was independent of FGF signaling, which is in line with a default mechanism of neural specification (Wilson and Edlund, 2001; Munoz-Sanjuan and Brivanlou, 2002). Nevertheless, the efficiency of this approach is relatively low (Tropepe et al., 2001) and in the presence of survival factors [*N*-acetyl-L-cysteine (NAC) and cAMP], ~20% of the starting population of undifferentiated ES cells will differentiate into neural stem cells (Smukler et al., 2006). These chemically defined neural differentiation systems facilitate the dissection of the molecular mechanisms of early neural differentiation.

Recently, pure expandable cultures of neural stem cells were derived from mouse ES cells. Adherent monolayer cultures of ES cells initially differentiated into the neural lineage under defined culture conditions, which lack inductive signals for non-neural fates (Ying et al., 2003). Basal culture medium supplemented with epidermal growth factor (EGF) and bFGF promoted the selection and prolonged propagation of pure adherent cultures of neural stem cells. This defined monoculture system can allow, for the first time, sustained robust expansion of neural stem cells, closely related to a radial glia lineage, liberated from any requirement for a specific cellular niche (Conti et al., 2005).

Derivation of NPs from human embryonic stem cells

While hESCs are similar to their mouse counterparts, they do differ in many aspects (Thomson and Odorico, 2000; Ginis et al., 2004). Therefore, the protocols for neural induction of hESCs share many of the principles that apply to mouse ES cells, though there are aspects that are specific to the human system. In the last 5 years, various approaches have been developed for the derivation of enriched cultures of proliferating developmentally competent NPs from hESCs. Due to the poor survival of hESCs in single-cell suspension culture conditions, direct neural differentiation procedures from disaggregated individual hESCs, similar to those described above for mouse ES cells, have not been reported thus far.

The initial protocols for the derivation of NPs from hESCs involved, as a first step, spontaneous disorganized differentiation that was induced by prolonged culture of hESC colonies at high density or by the formation of floating three-dimensional EB-like aggregates. This initial differentiation was followed by neural lineage selection by various approaches and culture under conditions that promoted the proliferation of NPs (Carpenter et al., 2001; Reubinoff et al., 2001; Zhang et al., 2001). Two research groups induced initial uncontrolled spontaneous differentiation of the hESCs through the formation of EBs, and subsequently plated the cells on appropriate substrates in defined medium containing mitogens (Carpenter et al., 2001; Zhang et al., 2001). Carpenter et al. (2001) used the traditional mouse ES cell differentiation protocol by RA treatment to derive NPs from hESC. The resulting hESC EBs were treated with RA in serum-containing

medium for 4 days to induce initial differentiation to NPs. NPs were then selected by immunosorting, based on the expression of neural-specific cell-surface markers. This method yielded heterogeneous neural cell populations similar to those observed in mouse ES cells. The neural cells generated by this approach displayed a wide range of developmental stages, from nestin-expressing and polysialylated neural cell adhesion molecule (PSA-NCAM)–expressing precursors to β-tubulin III$^+$ neurons. Zhang and colleagues used a process that initiated uncontrolled spontaneous differentiation of hESC within EBs cultured in serum-free medium for 4 days. The EBs were then plated onto an adhesive culture plate for 7 days' culture in a serum-free medium supplemented with bFGF, which supported the cultivation of NPs. Cells in the center of the plated EBs transformed initially into small columnar cells, whereas those in the periphery of the outgrowth gradually became flattened. The small columnar cell population expanded in the presence of FGF2 and organized into rosette formations by 7 to 10 days after plating the aggregates. The neural tube–like structures were isolated by selective enzymatic digestion followed by further purification on the basis of differential adhesion (Zhang et al., 2001; Li and Zhang, 2006). After transplantation into the neonatal mouse brain, the primitive neuroectodermal cells differentiated in a region-specific manner into neurons and glia cells. (Zhang et al., 2001; Guillaume et al., 2006). Recently, this group further demonstrated the potential to direct the differentiation of the NPs in vitro into dopaminergic neurons with midbrain properties and into motor neurons with spinal cord characteristics (Li et al., 2005; Yan et al., 2005).

The authors of this unit have reported an alternative approach where initial spontaneous differentiation was induced by prolonged culture of the hESC colonies to high density without replenishment of the feeders. Within the large colonies that were formed, there were a variety of differentiated cells, including distinct areas comprised of small, piled, tightly-packed early precursor cells that were destined to give rise to neural progenitors when transferred to serum-free media. Clusters of ~150 cells were mechanically dissected from these distinct areas and were plated in serum-free defined medium supplemented with mitogens (FGF2 and EGF). The aggregates gradually turned into round spheres that were highly enriched for proliferating, developmentally competent NPs. Upon transplantation into the brain ventricles of newborn mice, the NPs migrated extensively and differentiated in a region-specific manner to progeny representing the three major neural lineages (Reubinoff et al., 2001). In further studies, the authors have developed an alternative simple one-step approach for directed controlled differentiation of hESCs into NPs. In this protocol, small hESC colonies are removed from the feeder layer and cultured in defined serum-free medium supplemented with noggin and bFGF. The hESC clusters differentiate almost uniformly into NPs. The authors have shown that noggin, which inhibited BMP signaling, significantly enhanced the level of enrichment for NPs within the hESC clusters and suppressed the expression of transcripts of markers of non-neural lineages. Hence, it was suggested that noggin-mediated blockage of endogenous BMP signaling, within the hESC clusters, suppressed the differentiation into lineages other than the neural one (Itsykson et al., 2005). The potential of noggin to promote neural differentiation was also demonstrated by others with adherent cultures of hESCs rather than free-floating clusters (Gerrard et al., 2005).

In addition to noggin, the authors' protocol included the use of bFGF. FGF signaling is essential for neural specification in planarian (Cebria et al., 2002), frog (Launay et al., 1996), and avian embryos (Streit et al., 2000; Wilson et al., 2000). In the mouse ES cell system, autocrine FGF signaling was shown to have a role in neural l specification (Ying et al., 2003). However more recent data suggest that neural differentiation of mouse ES cells occurs in the absence of FGF signaling (Smukler et al., 2006). While FGF signaling induced the proliferation of the hESC-derived NPs (Itsykson et al., 2005), its role in neural induction of hESCs is unclear at present.

Similar to mouse ES cells, coculture of hESCs with mouse stromal cells can effectively induce neural differentiation (Muotri et al., 2005; Tabar et al., 2005). The factor or factors that exert the stromal cell–derived inducing activity (commonly termed SDIA) have not yet been identified. Recently, a similar effect was demonstrated when both mouse and human ES cells were cultured on the matrix components of the human amniotic membrane (amniotic membrane matrix-based ES cell differentiation; AMED; Ueno et al., 2006). In contrast to the SDIA method, which uses animal cells, the AMED culture uses a noncellular inductive material derived from an easily

available human tissue; therefore, AMED should provide a more suitable and versatile system for generating a variety of neural tissues for future clinical applications (Ueno et al., 2006). Lastly, the role of the Notch signaling system in promoting the entry into the neural lineage was recently demonstrated. Coculturing of mouse and human ES cells with genetically modified stromal cells expressing Notch ligand stimulated neural specification (Lowell et al., 2006).

The major advantages of the Basic Protocol in this unit are the use of a chemically defined culture system as opposed to serum-containing systems or coculture with unknown factors generated by stromal, amniotic, or feeder cells (Shin et al., 2006). Neural differentiation is obtained in one controlled step, which consistently gives rise to highly enriched populations of expandable, developmentally competent NPs within spheres. The authors' system, as well as those that induce neural differentiation of hESCs in monolayer cultures (Gerrard et al., 2005; Shin et al., 2006; Ueno et al., 2006), are invaluable for the dissection of the molecular mechanisms of early human neural specification and differentiation.

Potential applications of hESC-derived neural precursors

The establishment of neuroectodermal precursors from hESCs provides real advantages for basic and applied studies of human neural development and diseases. Directing hESCs to differentiate to the neural lineage and the establishment of NPs and their differentiated progeny enable a complete in vitro study of human neurogenesis. This approach allows access to hitherto unexplored territories of gene expression for modern genomics data mining, and will provide a platform for the discovery of polypeptide growth and differentiation factors which might find application in neural tissue regeneration. In vitro human models of neurodegenerative diseases may be created for basic research and drug discovery. New assays for toxicology and high-throughput screens for neuroprotective compounds may be developed.

Generation of NPs from hESCs in vitro may serve as a platform for further manipulations with growth and differentiating factors that may eventually enable the derivation of specific functional neural cells for transplantation therapy. The proof of principle of this potential application was demonstrated both with mouse (Brustle et al., 1999; Kim et al., 2002) and human (Roy et al., 2006) ES cell systems. These encouraging results suggest that hESC-derived NPs may eventually be applicable to cell and gene therapy of human neurological disorders. Long-term studies are required to determine the safety of hESC-derived neural progeny transplantation and to rule out potential hazards such as tumor formation (Roy et al., 2006) or the development of cells from other lineages. These pioneer transplantation studies highlight the potential of hESCs to serve in the future as an unlimited donor source of neural cells for transplantation.

Critical Parameters

The starting hESCs should be passaged two to three times on feeder cells after thawing, before being used for NP derivation. The hESC cultures should exhibit a minimal level of background differentiation. During those passages, assess the number of cells that should be plated per well at the last passage prior to the derivation of the NPs ($4–6 \times 10^5$ cells per well), so that the hESC colonies, after 6 to 7 days of culturing, would be of sufficient size to enable their survival as clusters, but not so large as to adhere to one another.

Troubleshooting

If differentiating hESC clusters attach to the plastic bottoms of the wells, it is advisable to transfer them to new wells precoated with 0.1% gelatin. Gently blow medium from a pipet on top of the hESC clusters to detach the clusters from the plastic surface, and transfer them to a fresh well precoated with gelatin.

If hESC clusters begin to aggregate one to the other, gently disperse them by gentle pipetting or dissect them with surgical blades.

Anticipated Results

The derivation of NPs from hESCs with the protocol described in this unit is very efficient and reproducible. A significant amount of cell death is expected during the first 2 to 3 days of culturing of the hESC clusters in suspension. However, after an additional few days, an increase in size of the clusters is observed due to cell proliferation.

After 4 weeks, 97% of the cells within the cultures should express NP markers such as Nestin and NCAM, and <2% of the cells should express markers of pluripotent cells such as Oct-4, Tra-1-81, and SSEA-3.

The NPs can differentiate into neurons and glial cells both in vitro under various culture conditions, as well as in vivo after transplantation.

While the NPs differentiate mainly into neurons at early culture stages, during prolonged propagation (25 weeks), a gradual shift in differentiation from a neuronal to a predominantly glial fate occurs (Itsykson et al., 2005). This finding is consistent with the concept of a gradual shift from predominantly neuronogenesis to gliogenesis during neural development in vivo (Nakai and Fujita, 1994; Encha-Razavi and Sonigo, 2003; Fujita, 2003).

Time Considerations

Approximately 0.5 to 1 hr should be allowed for the preparation of the media and various other factors and reagents. This may be performed up to 2 weeks before the protocol is initiated.

The time period for the whole process from the establishment of feeders through derivation of the NPs and their differentiation in vitro is 8 weeks.

Foreskin feeders are propagated 3 to 4 days before their inactivation by mitomycin C. About 30 min should be allowed for the human foreskin fibroblasts passaging procedure.

Preparation of mitomycin C inactivated feeder layers takes ~3 hr. The hESCs are plated on the feeders ~1 to 4 days after their preparation. About 0.5 to 2.5 hr should be allowed for hESC passaging.

The hESCs are cocultured with the feeders 6 to 7 days, until they are removed as clusters for induction of neural differentiation. During this week, medium must be changed every day. Changing the medium can take between 5 and 20 min, depending on the number of plates.

Removing the hESC colonies from the feeders should take ~1 to 3 hr.

Neural induction of the hESC clusters into neural spheres highly enriched for NPs takes 4 weeks. Spontaneous differentiation of the NPs into neurons and glial cells occurs within an additional 1 to 3 weeks. During this period, the medium must be changed every 3 to 4 days. Changing the medium can take between 5 and 20 min, depending on the number of wells needing refreshing.

Literature Cited

Bain, G., Kitchens, D., Yao, M., Huettner, J.E., and Gottlieb, D.I. 1995. Embryonic stem cells express neuronal properties in vitro. *Dev. Biol.* 168:342-357.

Brustle, O., Spiro, A.C., Karram, K., Choudhary, K., Okabe, S., and McKay, R.D. 1997. In vitro-generated neural precursors participate in mammalian brain development. *Proc. Natl. Acad. Sci. U.S.A.* 94:14809-14814.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., and McKay, R.D. 1999. Embryonic stem cell-derived glial precursors: A source of myelinating transplants. *Science* 285:754-756.

Carpenter, M.K., Inokuma, M.S., Denham, J., Mujtaba, T., Chiu, C.P., and Rao, M.S. 2001. Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp. Neurol.* 172:383-397.

Cebria, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., Itoh, M., Taira, M., Sanchez Alvarado, A., and Agata, K. 2002. FGFR-related gene nou-darake restricts brain tissues to the head region of planarians. *Nature* 419:620-624.

Conti, L., Pollard, S.M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q.L., Cattaneo, E., and Smith, A. 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PloS Biol* 3:283.

Encha-Razavi, F. and Sonigo, P. 2003. Features of the developing brain. *Child's Nerv. Syst.* 19:426-428.

Fujita, S. 2003. The discovery of the matrix cell, the identification of the multipotent neural stem cell and the development of the central nervous system, *Cell Struct. Funct.* 28:205-228.

Gerrard, L., Rodgers, L., and Cui, W. 2005. Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling. *Stem Cells* 23:1234-1241.

Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerecht-Nir, S., Amit, M., Hoke, A., Carpenter, M.K., Itskovitz Eldor, J., and Rao, M.S., 2004. Differences between human and mouse embryonic stem cells. *Dev. Biol.* 269:360-380.

Guillaume, D.J., Johnson, M.A., Li, X.J., and Zhang, S.C. 2006. Human embryonic stem cell-derived neural precursors develop into neurons and integrate into the host brain. *J. Neurosci. Res.* 84:1165-1176.

Itsykson, P., Ilouz, N., Turetsky, T., Goldstein, R.S., Pera, M.F., Fishbein, I., Segal, M., and Reubinoff, B.E. 2005. Derivation of neural precursors from human embryonic stem cells in the presence of noggin. *Mol. Cell Neurosci.* 30:24-36.

Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., Nishikawa, S.I., and Sasai, Y. 2000. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* 28:31-40.

Kim, J.H., Auerbach, J.M., Rodriguez-Gomez, J.A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S.H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., and McKay, R.D. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418:50-56.

Launay, C., Fromentoux, V., Shi, D.L., and Boucaut, J.C. 1996. A truncated FGF receptor blocks neural induction by endogenous *Xenopus* inducers. *Development* 122:869-880.

Lowell, S., Benchoua, A., Heavey, B., and Smith, A.G., 2006. Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol*. 4:e121.

Li, X.J. and Zhang, S.C. 2006. In vitro differentiation of neural precursors from human embryonic stem cells. *Methods Mol. Biol.* 331:169-177.

Li, M., Pevny, L., Lovell-Badge, R., and Smith, A. 1998. Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr. Biol*. 8:971-974.

Li, X.J., Du, Z.W., Zarnowska, E.D., Pankratz, M., Hansen, L.O., Pearce, R.A., and Zhang, S.C. 2005. Specification of motoneurons from human embryonic stem cells. *Nat. Biotechnol.* 23:215-21.

Mitalipova, M.M., Rao, R.R., Hoyer, D.M., Johnson, J.A., Meisner, L.F., Jones, K.L., Dalton, S., and Stice, S.L. 2005. Preserving the genetic integrity of human embryonic stem cells. *Nat. Biotechnol.* 23:19-20.

Mujtaba, T., Piper, D.R., Kalyani, A., Groves, A.K., Lucero, M.T., and Rao, M.S. 1999. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. *Dev. Biol*. 214:113-127.

Munoz-Sanjuan, I. and Brivanlou, A.H. 2002. Neural induction, the default model and embryonic stem cells. *Nat. Rev. Neurosci.* 3:271-280.

Muotri, A.R., Nakashima, K., Toni, N., Sandler, V.M., and Gage, F.H. 2005. Development of functional human embryonic stem cell-derived neurons in mouse brain. *PNAS* 102:1864-18648.

Nakai, J. and Fujita, S. 1994. Early events in the histo- and cytogenesis of the vertebrate CNS. *Int. J. Dev. Biol*. 38:175-183.

Okabe, S., Forsberg-Nilsson, K., Spiro, A.C., Segal, M., and McKay, R.D. 1996. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech. Dev.* 59:89-102.

Rathjen, J., Haines, B.P., Hudson, K.M., Nesci, A., Dunn, S., and Rathjen, P.D. 2002. Directed differentiation of pluripotent cells to neural lineages: Homogeneous formation and differentiation of a neurectoderm population. *Development* 129:2649-2661.

Renoncourt, Y., Carroll, P., Filippi, P., Arce, V., and Alonso, S. 1998. Neurons derived in vitro from ES cells express homeoproteins characteristic of motoneurons and interneurons. *Mech. Dev.* 79:185-197.

Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A., and Ben-Hur, T. 2001. Neural progenitors from human embryonic stem cells. *Nat. Biotechnol.* 19:1134-1140.

Robinson, J.P., Darzynkiewicz, Z., Hoffman, R., Nolan, J.P., Orfao, A., Rabinovitch, P.S., and Watkins, S,(eds.). 2007. Current Protocols in Cytometry. John Wiley & Sons Hoboken, N.J.

Roy, N.S., Cleren, C., Singh, S.K., Yang, L., Beal, M.F., and Goldman, S.A., 2006. Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nat. Med.* 12:1259-1268.

Shin, S., Mitalipova, M., Noggle, S., Tibbitts, D., Venable, A., Rao, R., and Stice, S.L. 2006. Long-term proliferation of human embryonic stem cell-derived neuroepithelial cells using defined adherent culture conditions. *Stem Cells* 24:125-138.

Smukler, S.R., Runciman, S.B., Xu, S., and Van der Kooy, D. 2006. Embryonic stem cells assume a primitive neural stem cell fate in absence of extrinsic influences. *J. Cell Biol.* 172:79-90.

Stavridis, M.P. and Smith, A.G. 2003. Neural differentiation of mouse embryonic stem cells. *Biochem. Soc. Trans.* 31:45-49.

Streit, A., Berliner, A.J., Papanayotou, C., Sirulnik, A., and Stern, C.D. 2000. Initiation of neural induction by FGF signalling before gastrulation. *Nature* 406:74-78.

Tabar, V., Panagiotakos, G., Greenberg, E.D., Chan, B.K., Sadelain, M., Gutin, P.H., and Studer, L. 2005. Migration and differentiation of neural precursors derived from human embryonic stem cells in the rat brain. *Nat. Biotechnol.* 23:601-606.

Thomson, J.A. and Odorico, J.S. 2000. Human embryonic stem cell and embryonic germ cell lines. *Trends Biotechnol.* 18:53-57.

Tropepe, V., Hitoshi, S., Sirard, C., Mak, T.W., Rossant, J., and Van der Kooy, D. 2001. Direct neural fate specification from embryonic stem cells: A primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 30:65-78.

Ueno, M., Matsumura, M., Watanabe, K., Nakamura, T., Osakada, F., Takahashi, M., Kawasaki, H., Kinoshita, S., and Sasai, Y. 2006. Neural conversion of ES cells by an inductive activity on human amniotic membrane matrix. *Proc. Natl. Acad. Sci. U.S.A.* 103:9554-9559.

Wiles, M.V. and Johansson, B.M. 1999. Embryonic stem cell development in a chemically defined medium. *Exp. Cell. Res.* 247:241-248.

Wilson, S.I. and Edlund, T. 2001. Neural induction: Toward a unifying mechanism. *Nature Neurosci.* 4:1161-1168.

Wilson, S.I., Graziano, E., Harland, R., Jessell, T.M., and Edlund, T. 2000. An early requirement for FGF signaling in the acquisition of neural cell fate in the chick embryo. *Curr. Biol.* 10:421-429.

Yan, Y., Yang, D., Zarnowska, E.D., Du, Z., Werbel, B., Valliere, C., Pearce, R.A., Thomson, J.A., and Zhang, S.C. 2005. Directed differentiation

of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* 23:781-790.

Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A., 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat. Biotechnol.* 21:183-186.

Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., and Thomson, J.A. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* 19:1129-1133.

CHAPTER 24
Lipids

INTRODUCTION

Lipids form the scaffold of biological membranes, store energy, anchor soluble proteins to membranes, and carry information as extracellular hormones or as intracellular second messengers. These different roles of lipids arise because of their diverse forms and characteristics within cells. The major lipid species comprising membrane bilayers are phosphoglycerides, which consist of a three-carbon backbone of glycerol, two long-chain fatty acids esterified to carbons 1 and 2 of glycerol, and phosphoric acid esterified to the carbon 3 of glycerol. Other lipid constituents of membrane bilayers include sphingolipids, sterols, and glycolipids. Because all these lipids have cylindrical shapes and an amphiphilic nature, they readily assemble into semipermeable membrane bilayers, in which the fatty acid chains of the lipids point inward toward each other and their polar head groups point outward and are exposed to water. Lipids not incorporated into membrane bilayers include triglycerides. They consist of glycerol with fatty acids esterified to all three carbons. They form large, oily droplets in the cytoplasm called lipid droplets that serve as storage sites for fatty acids.

Chapter 24 is devoted to biochemical and morphological assays used to study the function and intracellular pathways followed by lipids within cells. *UNIT 24.1* describes the use of fluorescent sphingolipid analogs to study the intracellular trafficking of sphingolipids. This class of lipids is characterized by sphingosine, a nitrogen-containing base that is the structural counterpart of glycerol, and one fatty acid of phosphoglycerides. Sphingolipids are an important class of lipid molecules that are enriched in the plasma membrane. There, they interact with cholesterol and other plasma membrane components to form membrane microdomains that are capable of sorting proteins and initiating plasma membrane internalization into endocytic structures. The unit highlights the use of fluorescent sphingolipid analogs to delineate the formation of sphingolipid microdomains at the plasma membrane, different mechanisms of sphingolipid internalization from the plasma membrane, and subsequent transport of sphingolipids to different subcellular compartments.

UNIT 24.2 describes the fluorescent detection of lipid droplets and associated proteins. Lipid droplets are triglyceride-enriched structures present in the cytosol of most eukaryotic cells. They participate in a variety of important cellular functions, including as storage sites of excess lipid and as sites for accumulation of potentially toxic lipid species. They consist of a core of neutral lipid, including triacylglycerol and cholesteryl ester, surrounded by a phospholipid monolayer. A number of proteins associate with the lipid droplet surface. The unit details several methods for detecting lipid droplets, as well as lipid droplet-associated proteins in live and fixed cells.

UNIT 24.3 describes protocols for generating giant unilamellar vesicles (GUVs), which are vesicles comprised of a single bilayer. These model membrane systems are useful for analyzing the impact of membrane and membrane-binding components on lipid bilayer stiffness and phase behavior. The main approach for making GUVs discussed in this unit uses rehydration of a lipid film. This produces vesicles having a large size (~30 μm), making them especially amenable to studies using fluorescence and light microscopy.

Current Protocols in Cell Biology 24.0.1-24.0.2, September 2008
Published online September 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2400s40

In these studies, mechanical measurements of membrane surfaces with optical traps or micropipets can be made. It is also possible to analyze membrane tubulation, fusion, and budding. Introduction of enzymes or proteins into GUVs is also possible, permitting the study of membrane reorganization in response to membrane binding of different molecules.

Jennifer Lippincott-Schwartz

Using Fluorescent Sphingolipid Analogs to Study Intracellular Lipid Trafficking

Sphingolipids (SLs) are an important class of lipid molecules that play roles in a wide variety of cell functions, including cell-cell interactions, cell growth, differentiation, and signal transduction (Chatterjee, 1998; Kolesnick et al., 2000). They are enriched at the plasma membrane (PM) where they interact with cholesterol and other PM components to form membrane microdomains (Parton, 2003; Simons and Vaz, 2004). The SL composition of the PM is thought to be tightly regulated and is controlled by a combination of processes, including SL synthesis and degradation and transport of SLs to and from the cell surface. Given the importance of SLs at the PM, studies have been performed to elucidate the routes of SL endocytosis and trafficking within the cells. This unit highlights the use of fluorescent SL analogs to delineate different mechanisms of SL internalization from the PM and the subsequent transport of SLs to different subcellular compartments.

Upon addition to cells, SL analogs are observed to undergo a series of processes (see Fig. 24.1.1). First, the probes are integrated into the plasma membrane. In some cases, the fluorescent probes may become concentrated in certain areas of the PM that are enriched in cholesterol and SLs (referred to here as microdomains). At temperatures above 10°C, endocytic processes are active, and fluorescent lipid analogs become internalized. The specific endocytic mechanism involved can be delineated by using different pharmacological inhibitors or by the expression of different dominant-negative (DN) proteins. After endocytosis into vesicles, the fluorescent probes are trafficked to intracellular organelles such as the Golgi apparatus or lysosomes. A portion of the endocytosed probe may also be recycled back to the PM. Transport between different cellular locations can be confirmed by expression of dominant-negative forms of rab proteins known to regulate these transport steps (e.g., rab9 regulates late endosome/Golgi apparatus transport; Mohrmann and Van der Sluis, 1999; Choudhury et al., 2002).

This unit focuses on techniques for three general areas of study: (1) SL-enriched microdomains at the PM, (2) mechanisms of endocytosis, and (3) steps in intracellular transport. Specific protocols are given for the study of each of these topics in Basic Protocols 1, 2, and 3, respectively. Alternative methods for trafficking studies are provided in Alternate Protocols 1 and 2. Specific biochemical and molecular techniques that have been useful in studying each of these processes are also discussed. Support Protocols 1 and 2 describe preparation of the C_8-LacCer/BSA complex and fluorescent sphingolipid analogs, respectively. The techniques presented in this unit utilize human skin fibroblasts (HSFs). Other cell types may be used; however, conditions for cell culture, loading of

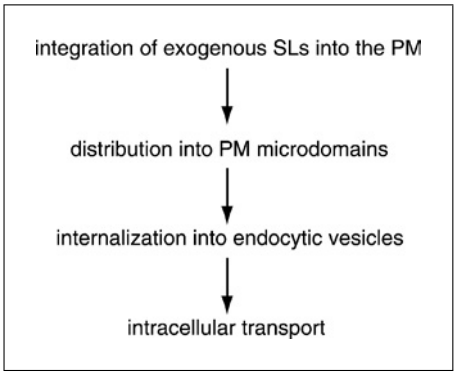


Figure 24.1.1 Schematic representation of steps involved in endocytosis and intracellular trafficking of sphingolipids. Abbreviations: SL, sphingolipid; PM, plasma membrane.

Contributed by Raman Deep Singh, David L. Marks, and Richard E. Pagano
Current Protocols in Cell Biology (2007) 24.1.1-24.1.19

endocytic probes, and treatment with biochemical inhibitors may need to be optimized for each cell type.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator except for short-term (5 to 60 min) treatment incubations at 37°C, which may be performed by placing the dishes containing the cells on a rack in a water bath so that only the bottom of the dish touches the water. Because these short incubations are performed in ambient air, noncarbonate-based buffers (e.g., HMEM+G) are used.

NOTE: In general, solutions are added by gently pipetting them into culture dishes containing coverslips with attached cells. Solutions are typically removed by careful pipetting or vacuum aspiration. After removal of solutions, new solutions should be added quickly so that cells do not dry out.

BASIC PROTOCOL 1

VISUALIZATION OF SPHINGOLIPIDS AT PLASMA MEMBRANE MICRODOMAINS

Microdomains are very small in size and are normally beyond the detection limits of the fluorescence microscope; however, the clustering or coalescence of microdomains into larger, visualizable structures at the PM has been demonstrated using a number of different approaches (Thomas et al., 1994; Harder et al., 1998). These approaches include induction of microdomains by addition of nonfluorescent SLs or cholesterol or by treatment of cells with cross-linking antibodies for either the cholera toxin B subunit (CtxB) or β1-integrin (Mitchell et al., 2002; Upla et al., 2004; Sharma et al., 2005).

In this example, the spectral properties of the BODIPY fluorophore (see Support Protocol 2) are used to visualize microdomains at the PM of living cells. Such domains are detected by monitoring the red (excited dimer or excimer) versus green (monomer) fluorescence emission of the BODIPY group. Visualization of both the red and green signals can be achieved using multiple detectors for the different wavelengths or simultaneously using an image splitter attachment (e.g., Dual-View, Optical Insights, LLC) for fluorescence microscopes. Clusters of BODIPY-SLs are then detected as red patches on a sea of green fluorescence. This approach has been used for detecting microdomains at both the PM and early endosomes (Sharma et al., 2003, 2005). A typical protocol for visualization of microdomains at the PM is given below.

Materials

Human skin fibroblasts (HSFs; Coriell Institute for Medical Research)
EMEM containing 10% FBS (EMEM-10; see recipe)
HEPES-buffered minimal essential medium + glucose (HMEM+G; see recipe)
1 mM C_8-LacCer/BSA stock solution (Support Protocol 1)
1 mM BSA control stock solution (Support Protocol 1)
BODIPY-LacCer/BSA stock solution (Support Protocol 2)
25-mm acid-etched coverslips (Fisher), sterilized by immersing in 70% ethanol and flaming
35-mm tissue culture dishes
10°C cooling block (described in Marks et al., 2005)
Depression slides
Fluorescence microscope with high magnification (e.g., 1000×), image acquisition capability, and a stage maintained at 10°C
Additional reagents and equipment for culturing cells (*UNIT 1.1*)

Culture cells

1. Culture (see *UNIT 1.1*) HSFs on 25-mm sterile, acid-etched glass coverslips in 35-mm culture dishes (one coverslip per dish) with 2 ml EMEM-10 for 24 to 48 hr until a confluency of 50% to 70% is reached.

   Prepare enough coverslips for one to two cultures for each experimental condition to be tested and any controls.

2. Wash cells three times with 2 ml HMEM+G.

Incubate with C_8-LacCer

3. Prepare 5 to 20 μM C_8-LacCer/BSA (experimental) and BSA (control) solutions by diluting 1 mM stock solutions in HMEM+G. Prepare at least 1 ml of each solution for each culture dish to be tested.

4. Remove the HMEM+G solutions from the cells and add 1 ml of 5 to 20 μM C_8-LacCer/BSA or the BSA control solution (from step 3) to each dish.

5. Incubate cells 30 min at 10°C on a cooling block.

6. Rinse cells once with 2 ml HMEM+G to remove excess lipid.

Incubate cells with fluorescent SL

7a. *For samples previously treated with C_8-LacCer:* Prepare 2.5 μM BODIPY-LacCer/BSA solution by diluting BODIPY-LacCer stock solution in HMEM+G with the same concentration of C_8-LacCer used in step 3. Prepare at least 1 ml of each solution for each culture dish to be tested.

7b. *For samples previously treated with BSA control:* Prepare 2.5 μM BODIPY-LacCer/BSA solution by diluting BODIPY-LacCer stock solution in HMEM+G with the same concentration of BSA control used in step 3. Prepare at least 1 ml of each solution for each culture dish to be tested.

8. Remove HMEM+G solutions from the cells and add 1 ml 2.5 μM BODIPY-LacCer solutions with either C_8-LacCer or BSA (from step 7) to each dish. Incubate cells 30 min at 10°C.

   Low temperature is maintained to prevent the endocytosis of the fluorescent lipid.

9. Wash cells three times with 2 ml HMEM+G.

10. Invert the coverslips with the attached cells onto a drop of HMEM+G on a depression slide.

11. Remove liquid from the back of the coverslip with a Kimwipe.

Examine cells

12. View cells under a fluorescence microscope at high magnification (e.g., 1000×) on a microscope stage maintained at 10°C to inhibit endocytosis.

13. Acquire images with cells excited at 450 to 490 nm and emissions collected at green (520 to 560 nm) or red (≥590 nm) wavelengths.

    Discrete regions on the PM showing red/orange fluorescence demonstrate the organization of BODIPY-LacCer into microdomains in C_8-LacCer treated cells but not in control cells (Fig. 24.1.2).

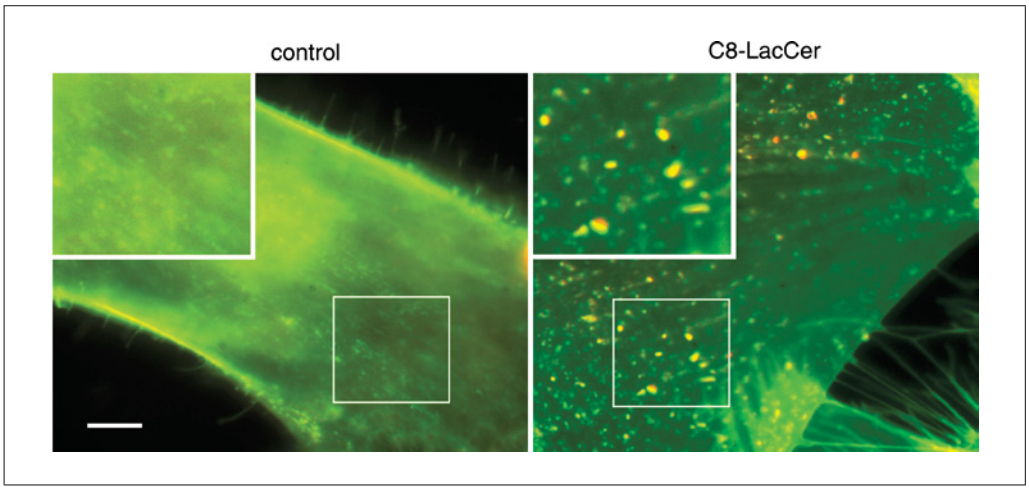


Figure 24.1.2 Human skin fibroblasts (HSFs) were incubated in buffer alone (control) or with 20 μM C_8-LacCer 30 min at 10°C. Cells were washed and incubated with 2.5 μM BODIPY-LacCer 30 min at 10°C. Samples were then washed and observed by fluorescence microscopy at green and red BODIPY emission wavelengths. Samples were maintained at 10°C at all times to prevent endocytosis. The punctate structures seen in the right panel exhibited both green and red fluorescence indicating enrichment of BODIPY-LacCer in these regions of the plasma membrane. Areas outlined with white rectangles are further magnified in insets. Bar = 10 μM. For the color version of this figure go to *http://www.currentprotocols.com*.

SUPPORT PROTOCOL 1

PREPARATION OF C_8-LacCer/BSA AND BSA CONTROL STOCK SOLUTIONS

The short-chain synthetic lactosylceramide D-lactosyl-β_1-1-*N*-octanoyl-D-*erythro*-sphingosine (C_8-LacCer) is used to stimulate the formation of plasma membrane microdomains (Sharma et al., 2005). The C_8-LacCer is complexed with fatty acid-free BSA to increase its solubility in water. After complex formation, DMSO and ethanol initially used to dissolve C_8-LacCer are removed by dialysis.

Materials

D-lactosyl-β_1-1′-*N*-octanoyl-D-*erythro*-sphingosine (C_8-LacCer) powder (mol. wt. 749.98; Avanti Polar Lipids)
2:1 (v/v) ethanol/DMSO
Fatty-acid-free bovine serum albumin (dfBSA; Sigma)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

5-ml glass tube
Dialysis tubing (12,000 to 14,000 MWCO)
Sonicator (optional)
Ultracentrifuge and appropriate tubes

1. Prepare a 20 mM C_8-LacCer stock solution by dissolving C_8-LacCer powder in 2:1 (v/v) ethanol/ DMSO (e.g., 15 mg powder in 1 ml).

   The solution may be briefly warmed to 37°C or sonicated to aid in dissolution.

2. Prepare 1 mM dfBSA in PBS (e.g., 66 mg dfBSA in 1 ml).

3. Dispense 950 μl of 1 mM dfBSA into a 5-ml glass tube. Warm to room temperature or 37°C.

4. Add 50 μl of 20 mM C_8-LacCer stock solution (step 1) dropwise into the BSA solution with vortexing between additions. Add the same amount of 2:1 (v/v) ethanol/DMSO to another 950-μl aliquot of BSA solution to create the BSA control.

5. Dialyze the solutions in 500 ml PBS overnight at 4°C with two to four changes of PBS to remove DMSO and ethanol.

6. Recover the C_8-LacCer/BSA and BSA control solutions from the dialysis tubing and centrifuge 20 min at 100,000 × *g*, 4°C, to remove aggregated lipid and protein. Save the supernatant and store up to 6 months at −20°C.

PREPARATION OF FLUORESCENT SPHINGOLIPID ANALOGS: BODIPY-LacCer/BSA COMPLEX

SUPPORT PROTOCOL 2

Various fluorescent sphingolipid SL analogs have been used in studies of intracellular transport. In most cases, the naturally occurring fatty acid moiety is replaced with a fluorescent fatty acid (see Fig. 24.1.3). Popular fluorophores include NBD, pyrene, BODIPY, rhodamine, and polyenes. Each analog has distinct advantages and disadvantages. For example, C6-NBD lipids are easily integrated into the PM outer leaflet because of their high rates of spontaneous transfer, and their topology in membranes can be readily assessed by several independent methods (Sleight and Pagano, 1985; McIntyre and Sleight, 1991), whereas the recently described polyene lipids are proposed to more closely mimic the behavior of natural lipids (Kuerschner et al., 2005).

Studies in the authors' lab have concentrated on SL analogs labeled with *N*-[5-(5,7-dimethyl boron dipyrromethene difluoride)-1-pentanoic acid (C_5-BODIPY-fatty acid; Pagano et al., 1991; Martin and Pagano, 1994; Fig. 24.1.3). The BODIPY-labeled lipids are especially useful because they exhibit a concentration-dependent shift in their

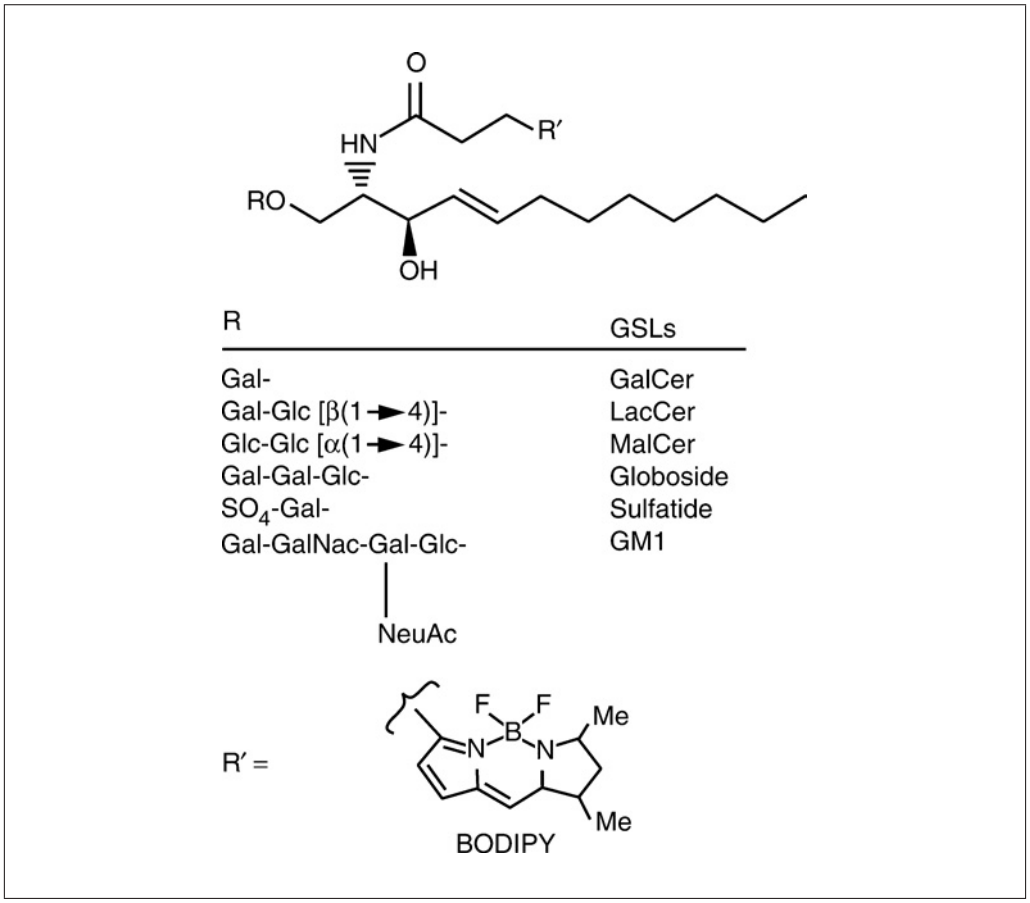


Figure 24.1.3 Structures of fluorescent SL analogs. Replacement of the natural acyl group at R′ with BODIPY yields BODIPY-SL. Addition of saccharide groups at R results in various BODIPY-glycosphingolipids (GSLs).

fluorescence emission from green to red wavelengths as a result of excimer formation at higher concentrations. This property allows an estimate of the molar density of the lipid analog within membranes of living cells based on measurements of the ratio of red to green fluorescence (Pagano et al., 1991; Chen et al., 1997). The authors have utilized these spectral properties of different BODIPY-lipids to study their distribution in microdomains, as well as their endocytosis and intracellular trafficking (Puri et al., 2001; Sharma et al., 2003, 2005). BODIPY-labeled lipids, as well as activated fluorophores for synthesis of other lipid analogs are available from Invitrogen.

To prepare for experiments, fluorescent lipid analogs are usually complexed (1:1 mol/mol) with fatty-acid-free bovine serum albumin (BSA; Martin and Pagano, 1994) as described below for BODIPY-lactosylceramide (BODIPY-LacCer). BSA/lipid complexes can be stored at 4°C for several weeks or at −20°C for longer time periods. For addition to cells, the BSA/lipid complexes are diluted in an appropriate aqueous buffer (e.g., HMEM; see below).

Materials

250 nmol BODIPY-LacCer (Invitrogen), dissolved in 19:1 (v/v) chloroform/ethanol
Nitrogen gas
HMEM−G (see recipe) or phosphate-buffered saline (PBS; *APPENDIX 2A*)
Absolute ethanol
1 mM (66 mg/ml) fatty-acid-free bovine serum albumin (dfBSA; mol. wt. 66,000; Sigma)
BODIPY standards (e.g., BODIPY-C_5-sphingomyelin; Invitrogen)

5-ml glass tubes
Dialysis tubing (12,000 to 14,000 MWCO)
1-ml ultracentrifuge tubes
Fluorometer

1. Dispense 250 nmole of BODIPY-LacCer (in organic solvent) into a 5-ml glass tube (e.g., take 0.5 ml from 0.5 mM stock of BODIPY-LacCer). Dry under nitrogen, and then under vacuum for 1 hr.

   BODIPY-LacCer is commercially available from Invitrogen. It can also be synthesized as in Martin and Pagano (1994).

2. Prepare 300 μl of an appropriate buffer (e.g., PBS or HMEM) containing 250 nmole of defatted bovine serum albumin in a 5-ml glass tube (e.g., mix 250 μl of 1 mM dfBSA in HMEM with 50 μl of HMEM).

3. Add 200 μl of absolute ethanol to the dry BODIPY-LacCer from step 1 and vortex until BODIPY-LacCer is completely dissolved.

4. Add the ethanolic solution of BODIPY-LacCer dropwise to the BSA solution from step 2 with vortexing between additions.

5. Dialyze against 500 ml PBS overnight at 4°C with two to four changes of PBS to remove chloroform and ethanol.

6. To remove aggregated material, centrifuge once or twice in an ultracentrifuge for 20 min at 100,000 × *g*, 4°C. Save the supernatant and store up to 2 months at 4°C. For long-term storage, dispense into 100-μl aliquots and store up to 12 months at −20° C.

7. Determine concentration of BODIPY-LacCer/BSA complex by measuring relative fluorescence intensity against known BODIPY standards using a fluorometer.

   The final concentration is usually 200 to 500 μM.

STUDYING ENDOCYTIC MECHANISMS WITH FLUORESCENT LIPID ANALOGS

To study the endocytosis of SLs, cells are incubated with suitable fluorescent lipid analogs at low temperature (usually 10°C) to label the PM and washed. The samples are then shifted to 37°C to allow endocytosis to proceed, followed by the removal of excess fluorescent lipid remaining at the outer leaflet of the PM, so that endocytic vesicles can be easily visualized. The latter is accomplished by a procedure termed "back exchange" (Martin and Pagano, 1994; Chen et al., 1997), in which cells are incubated multiple times at 10°C with 5% fatty-acid-free bovine serum albumin (dfBSA). Once fluorescence has been removed from the PM, lipid probes internalized by cells can be visualized by fluorescence microscopy or extracted from cells and analyzed by various chromatographic and/or fluorometric methods (Martin et al., 1993; Chen et al., 1998).

Although lipid analogs may be internalized into cells by a variety of mechanisms, including diffusion, active transport, and endocytosis, the authors have found that most SL analogs are internalized mainly via endocytosis in several cell types. The exception is BODIPY-glucosylceramide which is taken up by a combination of endocytic and nonendocytic mechanisms (Martin and Pagano, 1994; Puri et al., 2001; Singh et al., 2003). Various endocytic mechanisms have been reported, including clathrin-dependent endocytosis and several clathrin-independent endocytic routes (endocytosis via caveolae, and RhoA- or cdc42-dependent mechanisms; Marks et al., 2005; Cheng et al., 2006). To characterize the endocytic mechanisms involved in the uptake of SL analogs, a variety of biochemical inhibitors and expression of dominant-negative proteins that specifically inhibit certain endocytic pathways (Tables 24.1.1 and 24.1.2) are used. A typical example illustrating methods to study the mechanism of endocytosis of BODIPY-LacCer is described below.

Because cells are live at all stages in these experiments, care should be taken to prevent samples from drying out during washes, and cells should not be subjected to excessive force when washing or transferring coverslips. For viewing live cells on slides, sufficient time should be available to accurately view and photograph each sample (e.g., 15 to 20 min).

Materials

Human skin fibroblasts (HSFs; Coriell Institute for Medical Research)
EMEM containing 10% FBS (EMEM-10; see recipe)
HMEM+G and −G (see recipes)
BODIPY-LacCer/BSA stock solution (Support Protocol 2)
5% (w/v) fatty-acid-free bovine serum albumin (dfBSA) in HMEM−G (see recipe)

Inhibitors of endocytosis (Table 24.1.2)
25-mm glass coverslips, sterile
35-mm tissue culture dishes
Fluorescence microscope with image-acquisition capability
Image analysis software (e.g., Metamorph; Molecular Devices)

Additional reagents and equipment for culturing cells (*UNIT 1.1*)

Culture cells

1. Culture (see *UNIT 1.1*) HSFs on 25-mm sterile, acid-etched glass coverslips in 35-mm culture dishes (one coverslip per dish) with 2 ml EMEM-10 for 24 to 48 hr until a confluency of 50% to 70% is reached.

   Prepare enough coverslips for one to two replicates for each experimental condition to be tested and any controls.

2. Wash cells three times with 2 ml HMEM+G.

Table 24.1.1 Characteristics of Various Endocytic Mechanisms

		Clathrin-independent mechanisms[a]		
	Clathrin dependent[a,b]	Caveolae[c]	Cdc42 dependent[d]	RhoA dependent[e]
Cargo	EGF, transferrin, LDL, shiga toxin[f]	CtxB[f] , albumin[f] , SV40[f] , BODIPY-GSLs	GPI-anchored proteins, dextran	Interleukin-2 receptor β subunit
Protein required	Dynamin, Eps 15, AP180	Dynamin, PKC-α, *Src*	Cdc42	Dynamin, Rho-A
Biochemical inhibitors	Chlorpromazine, K^+ depletion	Genistein, filipin, nystatin, PP2, methyl-β-cyclodextrin[g]	*Clostridium difficile* toxin B	*Clostridium difficile* toxin B

[a]Other endocytic mechanisms (e.g., phagocytosis, macropinocytosis) are not discussed here.
[b]Compiled from Larkin et al. (1983), Herskovits et al. (1993), Damke et al. (1994), Sandvig and van Deurs (1996), Mallard et al. (1998), Schapiro et al. (1998), Benmerah et al. (1999), Gustavsson et al. (1999), Okamoto et al.(2000), Puri et al. (2001), Sharma et al. (2003), Singh et al. (2003).
[c]Compiled from Rothberg et al. (1992), Henley et al. (1998), Oh et al. (1998), Schapiro et al. (1998), Aoki et al. (1999), Gustavsson et al. (1999), Liu and Anderson (1999), Hansen et al. (2000), Okamoto et al. (2000), Puri et al. (2001), Sharma et al. (2003) Singh et al. (2003), Sharma et al. (2004).
[d]From Sabharanjak et al. (2002)
[e]From Lamaze et al. (2001)
[f]May be internalized by different mechanisms depending upon cell type.
[g]Some studies showed an inhibition of clathrin-mediated uptake with methyl β-cyclodextrin (Rodal et al. 1999; Subtil et al., 1999).

Table 24.1.2 Biochemical Inhibition of Different Endocytic Pathways

Mechanisms[a]	Treatment	Conditions[b]	Dosage
Clathrin dependent	K+ depletion	Cells rinsed in K^+-free buffer[c], incubated 5 min in hypotonic K^+-free buffer[d], rinsed in K^+ buffer, and incubated 20 min in K^+ buffer at 37°C; K^+-free buffer for all subsequent steps; controls treated with buffers containing 10 mM KCl	NA[e]
	Chlorpromazine	Pretreatment for 30 min at 37°C	5–25 μg/ml
Caveolae	Filipin	Pretreatment for 30 min at 37°C	1–5 μg/ml
	Methyl-β-cyclodextrin	Pretreatment for 30 min at 37°C	5–10 mM
	Nystatin	Pretreatment for 30 min at 37°C	25–50 μg/ml
	Genistein	Pretreatment for 1-2 hrs at 37°C	200 μM
	PP2	Pretreatment for 1 hr at 37°C	10 nM
	Chelerythrine chloride	Pretreatment for 1 hr at 37°C	100 μM
Cdc42 and RhoA dependent	*Clostridium difficile* toxin B	Pretreatment for 1 hr at 37°C	100 μM

[a]Different endocytic mechanisms.
[b]In general, cells are pretreated in HMEM+G or other appropriate buffers. In addition to pretreatments, for all treatments except methyl-β-cyclodextrin, inhibitors are also present during subsequent incubations with the probe and during endocytosis. See Table 24.1.1 for references concerning these methods.
[c]K^+-free buffer: 140 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml D-glucose (pH 7.4).
[d]Hypotonic buffer: K^+-free buffer diluted 1:1 with distilled water.
[e]NA, not applicable.

Label plasma membrane

3. Prepare 1 to 2.5 μM BODIPY-LacCer/BSA complex by diluting BODIPY-LacCer/BSA stock solution in HMEM+G.

4. Add 1 ml BODIPY-LacCer/BSA complex from step 3 to each dish of cells and incubate 30 min at 10°C.

5. Rinse the cells with 2 ml HMEM+G to remove excess lipid.

Initiate endocytosis

6. Add 1 ml HMEM+G and then warm samples to 37°C for 3 to 5 min (longer incubation may be required for some probes).

   This incubation initiates endocytosis. If cells are viewed at this time point, PM labeling predominates (Fig. 24.1.4, left panel).

7. Wash samples in 2 ml HMEM–G.

Remove surface label

8. Add 1 ml of 5% dfBSA in HMEM−G and incubate 10 min at 10°C. Remove the HMEM–G and repeat 5 more times to remove BODIPY-LacCer on the PM.

   At this point, fluorescently labeled intracellular vesicles can be visualized (Fig. 24.1.4, middle panel).

Identify specific pathways with inhibitor studies

9. To illustrate endocytosis via specific pathways, pretreat cells with various inhibitors (Table 24.1.2) diluted to appropriate concentrations with HMEM+G after step 2 (for pretreatment conditions see Table 24.1.2). Also include the same inhibitor concentration in the BODIPY-LacCer/BSA complex solution in step 4, and in HMEM+G in step 6.

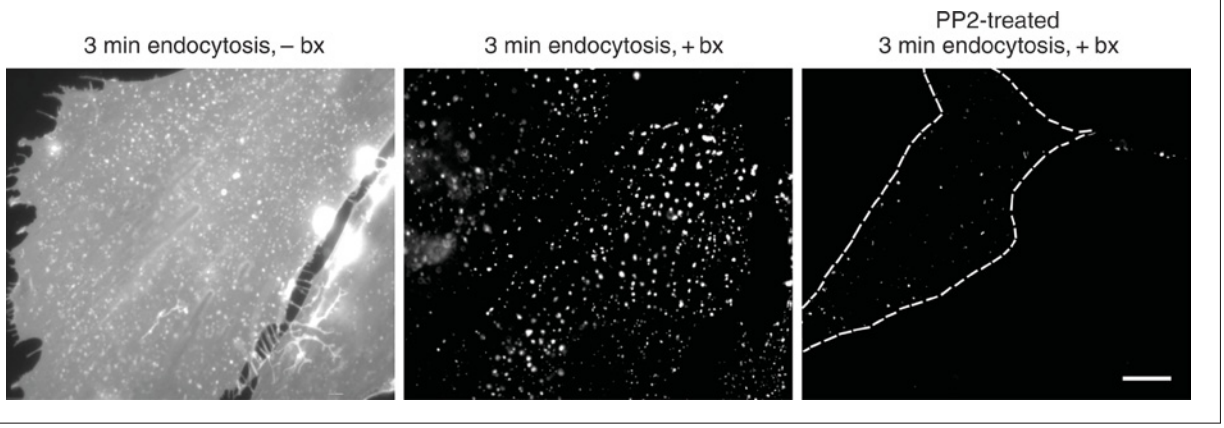


Figure 24.1.4 BODIPY-LacCer endocytosis assay. Human skin fibroblasts cultured on glass coverslips in 35-mm dishes were washed and then incubated with 2.5 μM BODIPY-LacCer for 30 min at 10°C. Cells were rinsed and warmed for 3 min at 37°C and viewed under the microscope. Cells in the left panel were not back exchanged (−bx) and show some punctate structures, but PM labeling predominates. Cells in the middle panel were back exchanged (bx) with defatted BSA at 10°C to remove PM labeling. Punctate labeling of endosomes is now clearly visible. In the right panel is a cell sample treated as in the middle panel, except that the cells were pretreated with the src kinase inhibitor PP2 (see Tables 24.1.1 and 24.1.2) before and during lipid incubations. Reduced intracellular labeling is observed in the right panel because endocytosis is inhibited. The dotted line indicates the perimeter of a cell in this field traced from a phase micrograph of the same field. Cell samples were viewed under the fluorescence microscope using a 100× objective. Bar = 10 μM.

In the present example, pretreatment with the src inhibitor PP2 decreased the intensity of internalized BODIPY-LacCer (Fig. 24.1.4, right panel), suggesting that uptake occurred via caveolae-mediated endocytosis.

10. Acquire images and then quantify by image analysis. Calculate the percent inhibition of uptake for each treatment, relative to untreated control cells (Puri et al., 2001; Singh et al., 2003; Sharma et al., 2005).

ALTERNATE PROTOCOL 1

DETECTION OF ENDOCYTOSIS IN ASSOCIATION WITH DOMINANT-NEGATIVE PROTEINS

Expression of dominant-negative (DN) proteins that block particular mechanism(s) of internalization or intracellular transport has been a useful technique to characterize SL transport. Since HSFs are difficult to transfect with high efficiency, DN protein expression experiments are performed in several ways. In some studies, green or DsRed fluorescent protein–tagged proteins are used so that transfected cells can be easily identified (Puri et al., 2001; Choudhury et al., 2002). This method is useful for qualitative studies, but DsRed and BODIPY fluorescence overlap, making quantitation of uptake difficult. Another approach is to cotransfect a cell with a nonfluorescent DN protein of interest and a DsRed-tagged protein plasmid, which targets to the nucleus (Choudhury et al., 2004). Co-expression of the two plasmids (DN protein and DsRed-Nuc) can be verified by the use of fluorescence microscopy for DsRed Nuc and immunostaining for the DN protein. The second approach ensures that all DsRed fluorescence is at the nucleus and it does not interfere with measurement of BODIPY-fluorescence in other parts of the cell. The authors use cationic lipid transfection reagents such as FuGENE 6 or Lipofectamine 2000 (Invitrogen) for transfection. In general, transfection of HSFs with FuGENE 6 has been relatively inefficient (<10%). Higher levels of transfection can be achieved using electroporation. A typical protocol for carrying out the DN protein transfection followed by inhibition of endocytosis is outlined below.

Additional Materials *(also see Basic Protocol 2)*

pDsRed-Nuc plasmid (0.3 μg/dish; Clontech)
Construct for dominant-negative inhibitor of endocytosis (e.g., AP180 DN; gift from H.T. MacMahon)
FuGENE 6 (Roche Applied Sciences)
10 μg/ml AF 488 labeled transferrin (Tfn; Invitrogen)
Serum-free medium (e.g., EMEM without serum)
HMEM−G (see recipe)
HMEM−G (see recipe), adjusted to pH 3.5 with acetic acid

1. Culture (see *UNIT 1.1*) HSFs on 25-mm sterile, acid-etched glass coverslips in 35-mm culture dishes (one coverslip per dish) with 2 ml EMEM-10 for 24 to 48 hr until a confluency of 50% to 70% is reached.

   Although many laboratories routinely use antibiotics in cell culture medium, culturing of cells without antibiotics is recommended because it can increase transfection efficiency.

2. After 12 to 24 hr, cotransfect cells with pDsRed-Nuc plasmid (0.3 μg DNA/dish) and AP180 DN construct (1 μg DNA/dish), using FuGENE 6 according to the manufacturer's instructions. As a control treat another sample of cells identically but exclude the AP180 DN construct DNA. Incubate 48 hr.

   The authors use transfection agents here rather than electroporation because it is more convenient and results in comparable levels of recombinant protein expression. In addition, the lower efficiency of transfection with the agents can be desirable because it often allows transfected and untransfected cells to be viewed in the same field under the microscope.

Measure endocytosis of probes

If using the BODIPY-LacCer probe

3a. Measure endocytosis of BODIPY-LacCer as described in Basic Protocol 2 steps 4 to 6. (e.g., preincubate cells with probe for 30 min at 10°C and then for 5 min at 37°C).

4a. Wash cells in 2 ml HMEM−G and back exchange for BODIPY-LacCer with 1 ml of 5% dfBSA as in Basic Protocol 2, step 8 to remove surface labeling.

If using the Tfn probe

3b. Serum starve cells by incubating 1 hr in 2 ml serum-free medium to up-regulate Tfn receptors and proceed as in Basic Protocol 2 steps 4 to 6. (e.g., preincubate cells with probe for 30 min at 10°C and then for 5 min at 37°C).

4b. Acid strip the surface labeling by incubating 10 to 30 sec with HMEM−G adjusted to pH <3.5.

Visualize endocytosed probe

5. View cells under the fluorescence microscope, with the cells excited at 450 to 490 nm and emissions collected at green (520 to 560 nm) wavelengths or excited at 520 to 560 nm with emissions collected at red (590 to 650 nm) wavelengths.

   The endocytosed probe (BODIPY-LacCer or AF 488 Tfn) is visualized at green wavelengths. For samples transfected with AP180 DN construct, cells exhibiting bright red fluorescence due to the DsRed protein within the nucleus are presumed to also express AP180 DN. Cells in the same dish without DsRed fluorescence are considered to be untransfected and can be used as controls. Separate dishes transfected with DsRed-nuc but not AP180 DN can be used as controls as well. In this case, cells with red nuclear fluorescence are considered controls and are compared to cells transfected with both DsRed-nuc and AP180 DN.

6. Acquire images of cell samples at standardized exposure conditions (i.e., the same exposure time and acquisition settings) and quantify by image analysis (e.g., Metamorph; Molecular Devices).

7. Calculate percent inhibition by comparing the fluorescence intensity of the endocytosed probe in transfected versus control cells (either nontransfected cells or cells transfected only with DsRed-nuc).

INTRACELLULAR LOCALIZATION AND TRANSPORT: CO-LOCALIZATION OF BODIPY-LacCer WITH ALBUMIN BUT NOT DEXTRAN

BASIC PROTOCOL 3

A number of techniques are available for determining the compartments (i.e., organelles) that an endocytosed probe traverses following its initial internalization from the PM. One widely used technique is co-localization of the internalized probe with other endocytic tracers. For example, BODIPY-LacCer may be endocytosed along with another fluorescently tagged cargo, (e.g., Tfn or dextran). The use of Alexa Fluor (AF) 594 or AF647 is preferred in such studies because there is no (or minimal) spectral overlap of these probes with BODIPY. AF probes are available from Molecular Probes. In the following example, BODIPY-LacCer is shown to co-localize with albumin (a caveolar marker in HSFs) but not with dextran (a fluid-phase marker).

When conducting co-localization studies using two endocytic probes, it is important to realize that each probe may move through a different series of compartments and that not all probes are transported at the same rate. For example, Tfn is visualized in early endosomes at 5 to 10 min after endocytosis begins but then becomes more concentrated in recycling endosomes by 15 to 20 min. BODIPY-LacCer, which is internalized via

caveolae in many cell types, is initially present (at 2 to 3 min) in Tfn-negative structures, but is transported to Tfn-positive early endosomes at 5 to 10 min after endocytosis (Sharma et al., 2003). It should also be recognized that markers may behave differently in different cell types (Torgersen et al., 2001; Singh et al., 2003). The concentration of probe may also be a consideration. For example, dextran used at 1 mg/ml is internalized via the fluid phase pathway, but may be taken up by additional pathways at higher concentrations (Sabharanjak et al., 2002).

In some cases, it is useful to evaluate the co-localization of an endocytic probe with a static compartment-specific marker, e.g., early endosomal antigen 1 (EEA1) for early endosomes, and lysosome-associated membrane protein-1 (LAMP-1) for late endosomes/lysosomes, using immunofluorescence (Sharma et al., 2003; Choudhury et al., 2004).

Materials

Human skin fibroblasts (HSFs; Coriell Institute for Medical Research)
EMEM containing 10% FBS (EMEM-10; see recipe)
HMEM+G and −G (see recipe)
1 μM BODIPY-LacCer/BSA complex (Support Protocol 2)
10 μg/ml Alexa-fluor 594 albumin (AF594-albumin) or 1 mg/ml Alexa-fluor 594 dextran (AF594-dextran)
5% fatty-acid-free bovine serum albumin (dfBSA) in HMEM−G (see recipe)

25-mm glass coverslips, sterile
35-mm tissue culture dishes
10°C cooling block (described in Marks et al., 2005)
Fluorescence microscope with image acquisition capability
Graphics program (e.g., Adobe Photoshop) or image analysis program (e.g., Metamorph; Molecular Devices)

Additional reagents and equipment for culturing cells (*UNIT 1.1*)

1. Culture (see *UNIT 1.1*) HSFs on 25-mm sterile, acid-etched glass coverslips in 35-mm culture dishes (one coverslip per dish) with 2 ml EMEM-10 for 24 to 48 hr until a confluency of 50% to 70% is reached.

   Prepare enough coverslips for one to two cultures for each experimental condition to be tested and any controls.

2. Wash cells three times with 2 ml HMEM+G.

3. Add 1 ml HMEM+G containing 1 μM BODIPY-LacCer/BSA complex and either 10 μg/ml AF594-albumin or 1 mg/ml AF594-dextran to the cells and incubate 30 min at 10°C.

4. Incubate cells without washing 3 to 5 min at 37°C.

   This incubation initiates endocytosis of BODIPY-LacCer along with the labeled albumin or dextran.

   Do not wash the cells prior to endocytosis because albumin and dextran do not bind to the PM.

5. Wash samples in 1 ml HMEM–G and back exchange five times in 1 ml of 5% dfBSA (see Basic Protocol 2, step 8).

   Carry out control experiments using cells labeled with each probe separately in order to optimize concentrations and exposure conditions for each channel so that no fluorescence is detected in the opposite channel. For example, for the BODIPY probe, no detectable

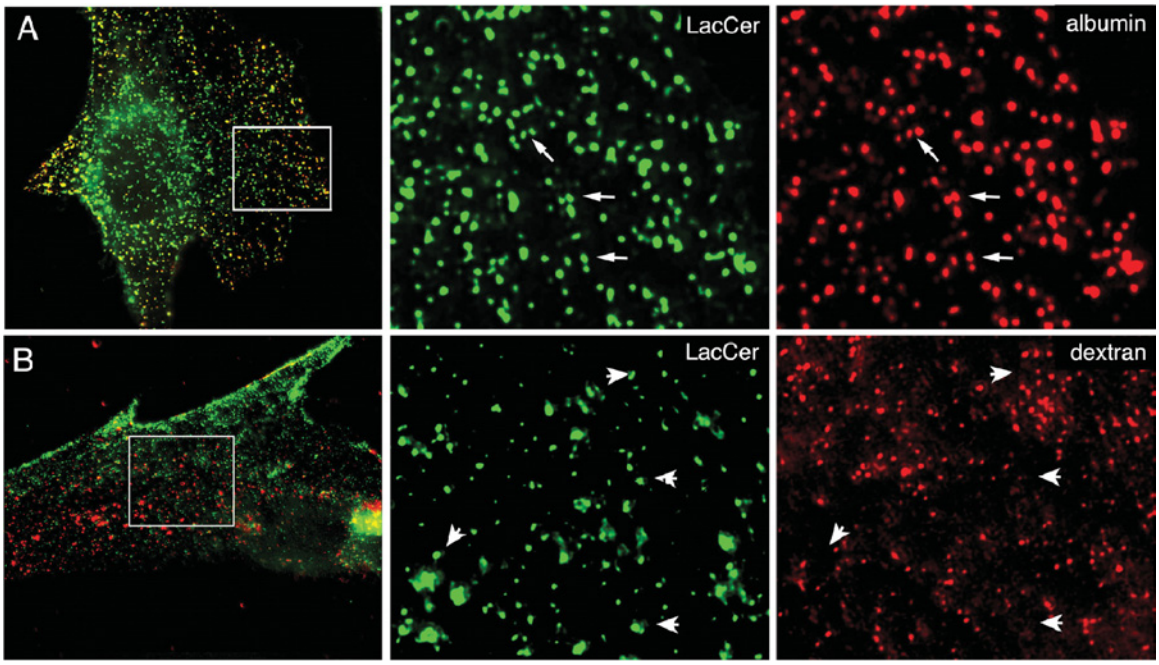


Figure 24.1.5 Co-localizatiion of BODIPY-LacCer with fluorescent albumin vs. dextran in rat fibroblasts (RF). RFs were incubated with BODIPY-LacCer along with either (**A**) AF594-albumin (30 μg/ml) for 30 min at 10°C, and further incubated for 3 min at 37°C, or (**B**) cascade blue dextran (1 mg/ml) for 30 min at 10°C, and further incubated for 5 min at 37°C. Cells were then back exchanged to remove PM labeling of BODIPY-LacCer and observed under the fluorescence microscope. Images were acquired of green (BODIPY-LacCer), and red (albumin or dextran) fluorescence. Panels to the right show regions within cells from images taken at 1000×. Arrows in the upper panels indicate points of co-localizatiion of BODIPY-LacCer and albumin. Arrowheads in the lower panels indicate LacCer-positive structures which do not co-localize with dextran. For the color version of this figure go to *http://www.currentprotocols.com*.

signal should be present in the AF594 channel and for an AF594 labeled probe, no signal should be present in the BODIPY channel.

6. View cells under the fluorescence microscope.

   Internalized BODIPY-lipid is then visualized in the green channel and the other cargo (albumin or dextran) are viewed at red (AF594) or far red (AF647) wavelengths.

7. Acquire separate images for each probe and then determine co-localization by image analysis or prepare merged color images using an appropriate graphics program (e.g., Adobe Photoshop) to obtain a qualitative view of the extent of colocalization.

   Co-localizatiion between red and green probes within the same endosomes will appear yellow whereas individual puncta labeled either with red or green in merged images indicate no co-localizatiion (see Fig. 24.1.5 for example).

USING DOMINANT-NEGATIVE rab PROTEINS TO IDENTIFY TRANSPORT STEPS

ALTERNATE PROTOCOL 2

Another method for characterizing the itinerary of endocytosed probes is to carry out transport studies in the presence of dominant-negative (DN) rab proteins. Rabs are small (21- to 25-kDa) GTPases which play important roles in the tethering and docking of vesicles to their target membranes; they are also involved in the regulation of vesicular transport between specific intracellular compartments (Mohrmann and van der Sluijs, 1999; Miaczynska and Zerial, 2002; Seabra et al., 2002). For example, rab5 is involved

in the homotypic fusion of clathrin-derived endocytic vesicles to form early endosomes (EEs) whereas rab9 regulates the transport of membranes from late endosomes to the Golgi apparatus (Riederer et al., 1994; Woodman, 2000). Transport between different compartments can be demonstrated by inhibiting such trafficking by expression of a particular DN rab protein. Using this technique, the authors have shown that BODIPY-LacCer transport to the Golgi apparatus is rab7- and rab9-dependent, whereas the recycling of BODIPY-LacCer from the EE back to the PM is largely dependent upon rab4 (Choudhury et al., 2002, 2004).

Studies of rab-dependent trafficking require the transfection of DN rab plasmids into cells and the identification of transfected cells by fluorescence microscopy. The authors have performed these studies by either using fluorescent protein–tagged rabs (e.g., DsRed-rab9) or by co-transfecting nonfluorescent rab proteins with the DsRed-nuc plasmid (Choudhury et al., 2002, 2004). The following is an example demonstrating the rab9-dependent transport of BODIPY-LacCer to the Golgi apparatus.

Additional Materials *(also see Basic Protocol 3)*

EMEM containing 1% and 10% FBS (EMEM-1 and EMEM-10; see recipe)
Wild-type rab9 plasmid and dominant-negative (DN) DsRed-rab9 plasmid (Addgene)
FuGENE 6 (Roche Applied Sciences)
BODIPY-LacCer/BSA (see Support Protocol 2)

1. Culture (see *UNIT 1.1*) HSFs on 25-mm sterile, acid-etched glass coverslips in 35-mm culture dishes (one coverslip per dish) with 2 ml EMEM-10 for 24 to 48 hr until a confluency of 50% to 70% is reached.

   Although many laboratories routinely use antibiotics in cell culture medium, culturing of cells without antibiotics is recommended because it can increase transfection efficiency.

2. Transfect cells with plasmids for wild type or DN DsRed-rab9 (1 μg DNA per dish) using FuGENE 6 according to the manufacturer's directions.

3. After 48 hr, wash the cells (now ~50% to 60% confluent) several times with 2 ml EMEM-1.

4. Add BODIPY-LacCer/BSA (3 to 5 μM final) in EMEM-1 to samples and incubate 45 min at 37°C in a CO_2 incubator.

5. Rinse cells with 2 ml EMEM-1 and incubate in 2 ml fresh EMEM-1 for an additional 60 min at 37°C in a CO_2 incubator.

6. Wash cells in 2 ml HMEM−G and then back exchange with 5% dfBSA as in Basic Protocol 2, step 8.

7. View cells under the fluorescence microscope.

   Transfected cells are identified by their bright red fluorescence. Wild-type rab9 shows a punctate distribution of fluorescence, whereas DN rab9 is diffusely distributed in the cytoplasm. In most cells expressing wild-type rab9, BODIPY-LacCer (visualized in the green channel) stains the Golgi apparatus, similar to the pattern seen in untransfected cells. In cells expressing DN rab9, BODIPY-LacCer is distributed in small punctuate structures, and Golgi targeting is blocked (Fig. 24.1.6).

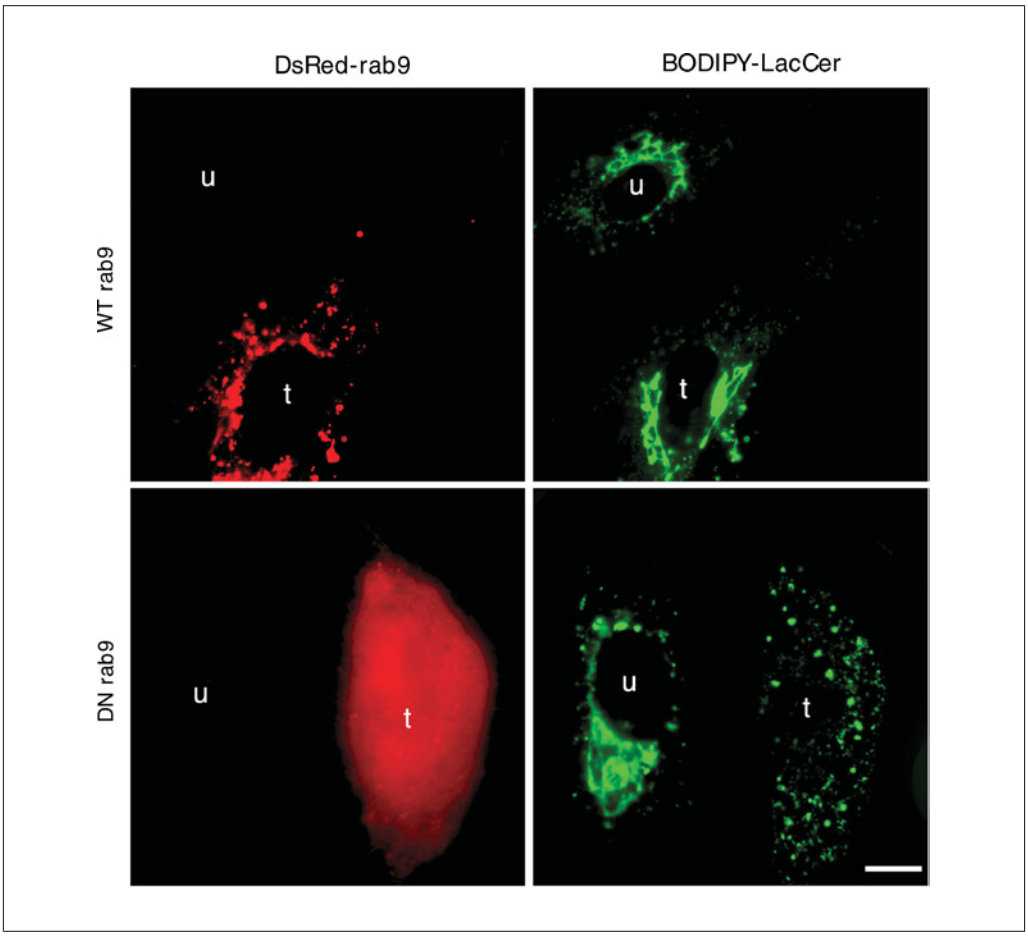


Figure 24.1.6 Overexpression of dominant-negative (DN) rab9 inhibits the Golgi targeting of BODIPY-LacCer. Cells were transfected with wild type (WT) or DN DsRed-labeled rab9 constructs. After 48 hrs, the cells were incubated with 5 μM BODIPY-LacCer for 45 min at 37°C, rinsed, and then chased in medium for 60 min at 37°C. The same cells were viewed in red (DsRed rab) and green (BODIPY-LacCer) channels (left and right images, respectively). Cells were either transfected (t) with rab9 or untransfected (u). The transfected cell in the lower left panel exhibits diffuse fluorescence because the dominant negative rab9 protein is diffusely distributed, unlike the wild type form (upper left panel). Note lack of perinuclear Golgi targeting of BODIPY-LacCer in the cell transfected with DN rab9 (lower right panel). Bar = 10 μM. For the color version of this figure go to *http://www.currentprotocols.com*.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

$CaCl_2$, 100×

Dissolve 1.85 g $CaCl_2 \cdot 2H_2O$ in 100 ml water. Store up to 3 months at 4°C.

EMEM containing 1% and 10% FBS (EMEM-1 and EMEM-10)

MEM with Earle's salts (Invitrogen) supplemented with:
0.1 mM MEM nonessential amino acids solution (10 mM; Invitrogen)
2 mM L-glutamine (200 mM; Invitrogen)
1% or 10% fetal bovine serum (FBS)

Also see Martin and Pagano (1994).

HEPES-buffered minimal essential medium with glucose (HMEM+G) and without glucose (HMEM–G)

10 ml 10× HMEM Stock (see recipe)
1 ml 100× $CaCl_2$ (1.25 mM final)

continued

1 ml 100× $MgSO_4$ (0.08 mM final)
Sterilize by passing through a 0.2-μm filter
Store up to 3 days at 4°C.

HMEM (+ G or – G) Stock, 10×

Combine the following in ~500 ml of distilled water:
32.84 g HEPES acid (138.0 mM final)
80 g NaCl (1370.0 mM final)
4 g KCl (54.0 mM final)
10 g glucose (55.0 mM final), for HMEM+G only
2.92 g glutamine (20.0 mM final)
0.6 g KH_2PO_4 (4.4 mM final)
0.254 g Na_2HPO_4 (1.8 mM final)
100 ml 100× MEM vitamins (Invitrogen)
200 ml 50× MEM amino acids (Invitrogen)
Adjust pH with NaOH to 7.4
Bring to 1 liter final volume
Sterilize by passing through a 0.2-μm filter
Divide into 25- to 50-ml aliquots and store up to 3 months at 4°C.

For 10× HMEM minus glucose (HMEM−G), exclude glucose from the medium.

$MgSO_4$, 100×

Dissolve 2.0 g $MgSO_4 \cdot 7H_2O$ in 100 ml water. Store up to 3 months at 4°C.

COMMENTARY

Background Information

Sphingolipid (SL) analogs may not represent the behavior of endogenous SLs in every aspect, and thus the utilization of additional approaches to study SL trafficking (e.g., SL-binding toxins or SL-specific antibodies) is important for validation of conclusions. However, while recognizing the limitations of fluorescent lipid probes, studies with lipid analogs have suggested new models for the transport of endogenous lipids that were not apparent prior to the use of the analogs.

SL-binding toxins have been used to follow the trafficking of endogenous plasma membrane (PM) SLs (Mallard et al., 1998; Orlandi and Fishman, 1998; Schapiro et al., 1998; Wolf et al., 1998; Puri et al., 2001). Moreover, trafficking of toxin binding to endogenous PM SLs can also be used to confirm the transport pathways demonstrated using fluorescent SL analogs (Choudhury et al., 2002). It should be kept in mind that in most cases, SL-binding toxin molecules possess multiple lipid binding sites (e.g., each molecule of CtxB is a pentamer that binds five molecules of GM1 ganglioside). Thus, the toxins may induce clustering of cell-surface SLs and in some cases may alter the transport of lipids at the PM (Singh et al., 2003).

Critical Parameters and Troubleshooting

To minimize experiment-to-experiment variations, it is important to use cells at the same degree of confluency. Some cell types exhibit changes in their levels of endocytosis depending upon their extent of confluence (R.D. Singh and R.E. Pagano, unpub. observ.). Moreover, passage number may also contribute to inconsistencies between different experiments.

The use of biochemical inhibitors has aided in the identification of endocytic pathways for many cargo molecules. However, such treatments have to be optimized for their use on different cell types. Inhibitor concentrations should be optimized to maximize the inhibition of specific pathways using a validated marker (e.g., Tfn for clathrin-mediated endocytosis) while minimizing any toxic effects on the cells. To minimize variation, control cells should also be treated identically to cells treated with inhibitors in terms of time and temperature during all incubations.

If a dominant-negative (DN) protein is transiently expressed after transfection, fairly high levels of transfection are usually desirable both to interfere with the function of normal protein and to unambiguously identify cells

transfected with fluorescent proteins. However, transient transfection always yields a range of protein expression levels, with the highest levels sometimes being toxic. Thus it is advisable to establish a suitable criterion for choosing transfected cells (e.g., a specific range of expression of fluorescent protein). Appropriate transfection controls (e.g., wild-type vs DN proteins, DsRed-nuc alone) are also important.

When using a fluorescent reporter protein (e.g., Ds-Red-nuc) to identify cells expected to be cotransfected with a nonfluorescent (e.g., DN) protein, it is important to verify the co-expression of the reporter protein and the non-flourescent protein by immunofluorescence. A useful alternative to transient transfection is adenoviral-mediated overexpression of DN proteins (Sharma et al., 2004). Adenoviral infection usually leads to similar levels of protein overexpression in all cells in a sample and thus avoids some of the problems associated with transient transfection.

Anticipated Results

The use of image analysis of digital micrographs to quantify endocytic uptake and its inhibition is limited in that absolute values (i.e., pmoles internalized) such as those achieved using biochemical analysis or radioactive tracers cannot be calculated from image analysis. However, image analysis provides useful relative values (e.g., percent uptake) that allow one to draw conclusions concerning mechanisms of endocytosis or intracellular transport. The efficiency of inhibitor treatment may vary between particular inhibitor, cell type or endocytic probe used. A reasonable guideline is that treatments are optimized when they inhibit uptake of a prototypical probe (e.g., transferrin inhibition by chlorpromazine) by 60% to 80%. Moreover, multiple inhibitors (or dominant-negative proteins) should be used to verify conclusions concerning any specific endocytic or transport mechanism. Because of the possibility of cell-cell variability, large samples sizes (e.g., greater than 30 cells for each condition) are also important to generate statistically significant data.

Time Considerations

Coordination of the different techniques described here requires planning and execution over several days. For example, cell samples must be grown to appropriate confluence on cover slips over 1 to 2 days. For transfection of dominant-negative proteins, an additional 1 to 2 days is required. Finally, each individual experiment using fluorescent probes may require 2 to 4 hr set up time, followed by sufficient time at the microscope (20 to 40 min per sample) to observe and photograph specimens.

Literature Cited

Aoki, T., Nomura, R., and Fujimoto, T. 1999. Tyrosine phosphorylation of caveolin-1 in the endothelium. *Exp. Cell Res.* 253:629-636.

Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. 1999. Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J. Cell Sci.* 112:1303-1311.

Chatterjee, S. 1998. Sphingolipids in atherosclerosis and vascular biology. *Arterioscler. Thromb. Vasc. Biol.* 18:1523-1533.

Chen, C.S., Martin, O.C., and Pagano, R.E. 1997. Changes in the spectral properties of a plasma membrane lipid analog during the first seconds of endocytosis in living cells. *Biophys. J.* 72:37-50.

Chen, C.S., Bach, G., and Pagano, R.E. 1998. Abnormal transport along the lysosomal pathway in mucolipidosis, type IV disease. *Proc. Natl. Acad. Sci. U.S.A.* 95:6373-6378.

Cheng, Z.J., Deep Singh, R., Marks, D.L., and Pagano, R.E. 2006. Membrane microdomains, caveolae, and caveolar endocytosis of sphingolipids. *Mol. Membr. Biol.* 23:101-110.

Choudhury, A., Dominguez, M., Puri, V., Sharma, D.K., Narita, K., Wheatley, C.W., Marks, D.L., and Pagano, R.E. 2002. Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J. Clin. Invest.* 109:1541-1550.

Choudhury, A., Sharma, D.K., Marks, D.L., and Pagano, R.E. 2004. Elevated endosomal cholesterol levels in Niemann-Pick cells inhibit rab4 and perturb membrane recycling. *Mol. Biol. Cell* 15:4500-4511.

Damke, H., Baba, T., Warnock, D.E., and Schmid, S.L. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* 127:915-934.

Gustavsson, J., Parpal, S., Karlsson, M., Ramsing, C., Thorn, H., Borg, M., Lindroth, M., Peterson, K.H., Magnusson, K.E., and Stralfors, P. 1999. Localization of the insulin receptor in caveolae of adipocyte plasma membrane. *FASEB J.* 13:1961-1971.

Hansen, G., Niels-Christiansen, L.-L., Thorsen, E., Immerdal, L., and Danielsen, E. 2000. Cholesterol depletion of enterocytes. Effect on the Golgi complex and apical membrane trafficking. *J. Biol. Chem.* 275:5136-5142.

Harder, T., Scheiffele, P., Verkade, P., and Simons, K. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* 141:929-942.

Henley, J.R., Krueger, E.W., Oswald, B.J., and McNiven, M.A. 1998. Dynamin-mediated internalization of caveolae. *J. Cell Biol.* 141:85-99.

Herskovits, J.S., Burgess, C.C., Obar, R.A., and Vallee, R.B. 1993. Effects of mutant rat dynamin on endocytosis. *J. Cell Biol.* 122:565-578.

Kolesnick, R.N., Goni, F.M., and Alonso, A. 2000. Compartmentalization of ceramide signaling: Physical foundations and biological effects. *J. Cell Physiol.* 184:285-300.

Kuerschner, L., Ejsing, C.S., Ekroos, K., Shevchenko, A., Anderson, K.I., and Thiele, C. 2005. Polyene-lipids: A new tool to image lipids. *Nat. Methods* 2:39-45.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C.G., Benmerah, A., and Dautry-Varsat, A. 2001. Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell* 7:661-671.

Larkin, J.M., Brown, M.S., Goldstein, J.L., and Anderson, R.G.W. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell* 33:273-285.

Liu, P. and Anderson, R.G. 1999. Spatial organization of EGF receptor transmodulation by PDGF. *Biochem. Biophys. Res. Commun.* 261:695-700.

Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B., and Johannes, L. 1998. Direct pathway from early/recycling endosome to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. *J. Cell Biol.* 143:973-990.

Marks, D.L., Singh, R.D., Choudhury, A., Wheatley, C.L., and Pagano, R.E. 2005. Use of fluorescent sphingolipid analogs to study lipid transport along the endocytic pathway. *Methods* 36:186-195.

Martin, O.C. and Pagano, R.E. 1994. Internalization and sorting of a fluorescent analog of glucosylceramide to the Golgi apparatus of human skin fibroblasts: Utilization of endocytic and nonendocytic transport mechanisms. *J. Cell Biol.* 125:769-781.

Martin, O.C., Comly, M.E., Blanchette-Mackie, E.J., Pentchev, P.G., and Pagano, R.E. 1993. Cholesterol deprivation affects the fluorescence properties of a ceramide analog at the Golgi apparatus of living cells. *Proc. Natl. Acad. Sci. U.S.A.* 90:2661-2665.

McIntyre, J.C. and Sleight, R.G. 1991. Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry* 30:11819-11827.

Miaczynska, M. and Zerial, M. 2002. Mosaic organization of the endocytic pathway. *Exp. Cell Res.* 272:8-14.

Mitchell, J.S., Kanca, O., and McIntyre, B.W. 2002. Lipid microdomain clustering induces a redistribution of antigen recognition and adhesion molecules on human T lymphocytes. *J Immunol.* 168:2737-2744.

Mohrmann, K. and van der Sluijs, P. 1999. Regulation of membrane transport through the endocytic pathway by rabGTPases. *Mol. Membr. Biol.* 16:81-87.

Oh, P., McIntosh, D.P., and Schnitzer, J.E. 1998. Dynamin at the neck of cavolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J. Cell Biol.* 141:101-104.

Okamoto, Y., Ninomiya, H., Miwa, S., and Masaki, T. 2000. Cholesterol oxidation switches the internalization pathway of endothelin receptor type A from caveolae to clathrin-coated pits in Chinese hamster ovary cells. *J. Biol. Chem.* 275:6439-6446.

Orlandi, P.A. and Fishman, P.H. 1998. Filipin-dependent inhibition of cholera toxin: Evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* 141:905-915.

Pagano, R.E., Martin, O.C., Kang, H.C., and Haugland, R.P. 1991. A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J. Cell Biol.* 113:1267-1279.

Parton, R.G. 2003. Caveolae–from ultrastructure to molecular mechanisms. *Nat. Rev. Mol. Cell Biol.* 4:162-167.

Puri, V., Watanabe, R., Singh, R.D., Dominguez, M., Brown, J.C., Wheatley, C.L., Marks, D.L., and Pagano, R.E. 2001. Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J. Cell Biol.* 154:535-547.

Riederer, M.A., Soldati, T., Shapiro, A.D., Lin, J., and Pfeffer, S.R. 1994. Lysosome biogenesis requires rab9 function and receptor recycling from endosomes to the trans-Golgi network. *J. Cell Biol.* 125:573-582.

Rodal, S.K., Skretting, G., Garred, Ø., Vilhardt, F., van Deurs, B., and Sandvig, K. 1999. Extraction of cholesterol with methyl-β-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell* 10:961-974.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.-S., Glenney, J.R., and Anderson, R.G.W. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell* 68:673-682.

Sabharanjak, S., Sharma, P., Parton, R.G., and Mayor, S. 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Develop. Cell* 2:411-423.

Sandvig, K., and van Deurs, B. 1996. Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. *Physiol. Rev.* 76:949-966.

Schapiro, F., Lingwood, C., Furuya, W., and Grinstein, S. 1998. pH-independent retrograde targeting of glycolipids to the Golgi complex. *Am. J. Physiol.* 274:C319-C332.

Seabra, M.C., Mules, E.H., and Hume, A.N. 2002. Rab GTPases, intracellular traffic and disease. *Trends Molec. Med.* 8:23-30.

Sharma, D.K., Choudhury, A., Singh, R.D., Wheatley, C.L., Marks, D.L., and Pagano, R.E. 2003. Glycosphingolipids internalized via caveolar-related endocytosis rapidly merge with the clathrin pathway in early endosomes and form microdomains for recycling. *J. Biol. Chem.* 278:7564-7572.

Sharma, D.K., Brown, J.C., Choudhury, A., Peterson, T.E., Holicky, E., Marks, D.L., Simari, R., Parton, R.G., and Pagano, R.E. 2004. Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. *Mol. Biol. Cell* 15:3114-3122.

Sharma, D.K., Brown, J.C., Cheng, Z., Holicky, E.L., Marks, D.L., and Pagano, R.E. 2005. The glycosphingolipid, lactosylceramide, regulates beta1-integrin clustering and endocytosis. *Cancer Res.* 65:8233-8241.

Simons, K. and Vaz, W.L. 2004. Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.* 33:269-295.

Singh, R.D., Puri, V., Valiyaveettil, J.T., Marks, D.L., Bittman, R., and Pagano, R.E. 2003. Selective caveolin-1-dependent endocytosis of glycosphingolipids. *Mol. Biol. Cell* 14:3254-3265.

Sleight, R.G. and Pagano, R.E. 1985. Transmembrane movement of a fluorescent phosphatidylethanolamine analogue across the plasma membranes of cultured mammalian cells. *J. Biol. Chem.* 260:1909-1916.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M.A., Keen, J.H., and McGraw, T.E. 1999. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. U.S.A.* 96:6775-6780.

Thomas, J.L., Holowka, D., Baird, B., and Webb, W.W. 1994. Large-scale co-aggregation of fluorescent lipid probes with cell surface proteins. *J. Cell Biol.* 125:795-802.

Torgersen, M.L., Skretting, G., van Deurs, B., and Sandvig, K. 2001. Internalization of cholera toxin by different endocytic mechanisms. *J. Cell Sci.* 114:3737-3742.

Upla, P., Marjomaki, V., Kankaanpaa, P., Ivaska, J., Hyypia, T., vand der Goot, F.G., and Heino, J. 2004. Clustering induces a lateral redistribution of $\alpha 2\beta 1$ integrin from membrane rafts to caveolae and subsequent PKC-dependent internalization. *Mol. Biol. Cell*, in press.

Wolf, A.A., Jobling, M.G., Wimer-Mackin, S., Ferguson-Maltzman, M., Madara, J.L., Holmes, R.K., and Lencer, W.I. 1998. Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J. Cell Biol.* 141:917-927.

Woodman, P.G. 2000. Biogenesis of the sorting endosome: The role of rab5. *Traffic* 1:695-701.

Contributed by Raman Deep Singh, David L. Marks, and Richard E. Pagano
Mayo Clinic College of Medicine
Rochester, Minnesota

Fluorescent Detection of Lipid Droplets and Associated Proteins

Lipid droplets are found in the cytosol of most eukaryotic cells, where they participate in a variety of cellular functions (Londos et al., 1999; Murphy and Vance, 1999; Zweytick et al., 2000). Adipocytes are highly specialized for the storage of excess lipid and contain giant, triacylglyceride-rich lipid droplets. Lipid droplets in adrenal cells store large amounts of cholesteryl esters for use in synthesis of steroid hormones. Smaller lipid droplets are found in cells of several other tissues including liver, kidney, heart, and skeletal muscle. These smaller droplets may serve as energy reservoirs, sources of lipid for membrane biosynthesis, or storage sites for potentially toxic lipid species. While the importance of lipid droplets in a variety of cell types has been established, very little is known about the mechanisms affecting lipid droplet structure and the targeting of lipid droplet–associated proteins. Visualization of lipid droplets and their associated proteins will be critical to understanding these processes.

This unit describes the detection of lipid droplets in cultured cells using fluorescence microscopy (see Basic Protocol 1). Fluorescent lipophilic dyes, which partition into the nonpolar lipid droplet core, are useful lipid droplet markers. Protocols are presented for lipid droplet visualization using two of these dyes, Nile Red and BODIPY 493/503. The differences in the spectral properties of these molecules and advantages of each are discussed (see Commentary). Lipid droplets in some cultured cells may be small or difficult to detect. Therefore, a protocol for supplementing growth media with excess lipid to enhance lipid droplet formation in cultured cells is included (see Support Protocol).

This unit also describes how visualization of intracellular lipid droplets can best be combined with indirect immunofluorescent detection of lipid droplet–associated proteins, using antibodies conjugated to standard red, green, or blue fluorophores (see Basic Protocol 2). Other investigators have demonstrated the localization of green fluorescent protein (GFP)-tagged lipid droplet–associated proteins in live cells (Imamura et al., 2002; Targett-Adams et al., 2003). Adipophilin, a broadly expressed protein that is widely used as a lipid droplet marker, is presented here as an example. Several recent reports suggest that lipid droplets are uniquely sensitive to fixation and permeabilization procedures (DiDonato and Brasaemle, 2003; Ohsaki et al., 2005). Some common fixation techniques severely affect lipid droplet morphology; thus, a protocol to examine the localization of lipid droplet–associated proteins while keeping lipid droplet structure intact is included (see Alternate Protocol).

DETECTION OF LIPID DROPLETS WITH LIPOPHILIC DYES

BASIC PROTOCOL 1

Two of the most widely utilized lipophilic dyes are Nile Red and BODIPY 493/503. In the procedure described here, cells are fixed with paraformaldehyde and then incubated with one of these dyes. However, fixation is not necessary, and lipophilic dyes can be used to detect lipid droplets in live cells. The hydrophobicity of these dye molecules promotes rapid partitioning into the nonpolar environment of lipid droplets. Nile Red and BODIPY 493/503 fluorescence is easily detected by confocal or wide-field fluorescent microscopy. See the Commentary for a discussion of differences between Nile Red and BODIPY 493/503.

Contributed by Laura L. Listenberger and Deborah A. Brown
Current Protocols in Cell Biology (2007) 24.2.1-24.2.11

Materials

Cultured cells

Poly-L-lysine, optional: prepare by dissolving 5 mg poly-L-lysine in 50 ml sterile H_2O; sterilize by passing through a 0.22-μm filter, and store up to 2 months at 4°C

Growth medium, optionally supplemented with 400 μM oleate (see Support Protocol)

Phosphate-buffered saline (PBS; *APPENDIX 2A*), without calcium and magnesium

3% (w/v) paraformaldehyde in PBS

1 mg/ml BODIPY 493/503 stock solution: prepare by dissolving 5 mg BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene; Molecular Probes) in 5 ml ethanol; divide into 500-μl aliquots, and store up to 1 month at −20°C *or*

1 mg/ml Nile Red stock solution: prepare by dissolving 5 mg Nile Red (Molecular Probes) in 5 ml DMSO; divide into 500-μl aliquots, and store up to 1 month at −20°C

150 mM NaCl: prepare by dissolving 4.38 g NaCl in 500 ml H_2O; store up to 1 year at room temperature

Mounting medium: e.g., Prolong Antifade Reagent (Invitrogen) or see Internet Resources

22-mm^2 square glass coverslips, sterile

35-mm tissue culture dishes or 6-well tissue culture plates

Glass slides

Fluorescence microscope with appropriate filters

Plate cells for staining

1. One or two days prior to staining, plate cells on sterile glass coverslips. Plate the cells at 50% to 70% confluency so that they remain semiconfluent at the time of staining.

   Whether this step is performed 1 or 2 days prior to staining depends on the length of time required for the chosen cell type to adequately adhere to the coverslip. For some cell types, coating the coverslip with 0.1 mg/ml poly-L-lysine prior to plating may facilitate adherence to the coverslip.

   It is easiest to place single coverslips (22-mm^2 square) in 35-mm tissue culture dishes or one per well in a 6-well plate. The coverslips are left in the dishes through the wash, fixation, and staining steps.

2. *Optional:* To enhance lipid droplet formation and facilitate detection, supplement the growth medium for cells with 400 μM oleate for 6 to 24 hr prior to fixation and lipid droplet staining.

   While the lipid present in serum may be sufficient to induce some lipid droplet formation, lipid droplet size and number is increased following supplementation with additional lipid.

   Preparation of fatty acid-supplemented medium is discussed in the Support Protocol.

Fix cells

3. Rinse the cells twice with 2 ml PBS.

   Extensive washes with rocking are not necessary for lipid droplet staining with these dyes.

4. Fix the cells by incubating with 2 ml 3% (w/v) paraformaldehyde 30 min at room temperature.

5. Wash the cells three times with 2 ml PBS.

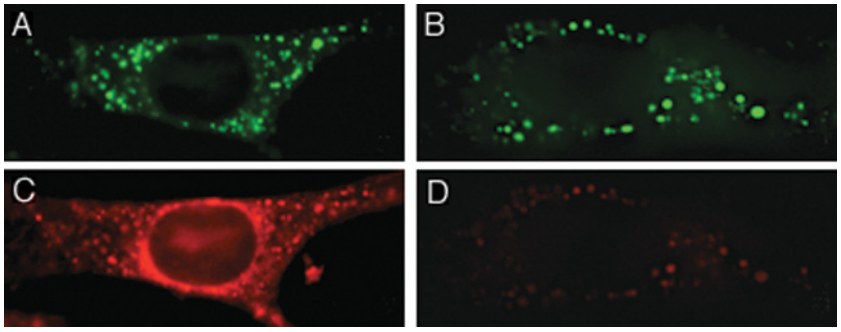


Figure 24.2.1 Detection of lipid droplets with Nile Red and BODIPY 493/503. BHK cells were fed medium supplemented with 400 μM oleate prior to staining with Nile Red (**A, C**) or BODIPY 493/503 (**B, D**). For each dye, fluorescent emission detected in the green (A, B) and red channels (C, D) are shown. Stained cells were visualized with a Zeiss Axiovision 200 fluorescent microscope. Out-of-focus fluorescence was removed with deconvolution software. Single sections of a *z*-stack are shown. For the color version of this figure go to *http://www.currentprotocols.com*.

Stain lipid droplets

6a. *If using BODIPY 493/503:* Add 10 μl of 1 mg/ml BODIPY 493/503 stock solution to 10 ml of 150 mM NaCl.

6b. *If using Nile Red:* Add 1 μl of 1 mg/ml Nile Red stock solution to 10 ml of 150 mM NaCl.

7. Cover the cells with 1 ml Nile Red or BODIPY 493/503 staining solution from the previous step and incubate 10 min at room temperature, protected from ambient light.

   Exposure of the fluorescent lipophilic dyes to ambient light should be minimized; therefore, cover the cells during this and all later steps.

8. Wash the cells three times with 2 ml PBS.

9. Mount the coverslips onto glass slides with 20 to 40 μl antifade mounting medium.

Analyze stained cells visually

10. Using a fluorescence microscope, detect BODIPY 493/503 staining of lipid droplets as green fluorescence. Detect Nile Red fluorescence in both the red and green channels.

    For Nile Red, green fluorescence is more specific for lipid droplets (Fig. 24.2.1; see Commentary for additional discussion).

IMMUNOFLUORESCENT DETECTION OF ADIPOPHILIN, A LIPID DROPLET–ASSOCIATED PROTEIN

BASIC PROTOCOL 2

Lipid droplets consist of a core of neutral lipid, including triacylglycerol and cholesteryl ester, surrounded by a phospholipid monolayer. A number of proteins that associate with the lipid droplet surface have recently been described (Brasaemle et al., 2004; Fujimoto et al., 2004; Liu et al., 2004; Umlauf et al., 2004). Many of the most abundant lipid droplet–associated proteins in mammalian cells share sequence similarity and have been termed PAT proteins. PAT proteins are named for the first members of this family to be identified—perilipin (P), adipophilin (also known as ADRP, A), and TIP47 (T). While perilipin expression is restricted to adipocytes and steroidogenic cells, adipophilin and TIP47 are widely expressed (Londos et al., 2005). Moreover, while TIP47 may exist in both cytosolic and lipid droplet–bound pools, adipophilin is exclusively lipid droplet associated (Londos et al., 2005). Largely because of these characteristics, adipophilin serves as a widely utilized marker for lipid droplet accumulation.

Localization of lipid droplet–associated proteins requires careful consideration of fixation and permeabilization techniques. Many common fixation methods (including solvents such as methanol or acetone) greatly alter lipid droplet morphology (DiDonato and Brasaemle, 2003). Moreover, recent work has shown that many lipid droplet proteins are poorly detected in cells after standard Triton X-100 permeabilization (Ohsaki et al., 2005). Thus, in this protocol for detecting adipophilin, cells are fixed with paraformaldehyde and permeabilized with saponin.

Materials

Cultured cells
Growth medium, optionally supplemented with 400 μM oleate (see Support Protocol)
0.1 mg/ml poly-L-lysine, optional: prepare by dissolving 5 mg poly-L-lysine in 50 ml sterile H_2O; sterilize by passing through a 0.22-μm filter, and store up to 1 month at 4°C
Phosphate-buffered saline (PBS; *APPENDIX 2A*), without calcium and magnesium
3% (w/v) paraformaldehyde in PBS
Blocking buffer: 0.2 M glycine /0.1 mg/ml saponin/30 mg/ml bovine serum albumin (BSA) in PBS (*APPENDIX 2A*)
Primary antibody: guinea pig anti-adipophilin polyclonal antibody (Research Diagnostics)
Antibody diluent: 0.1 mg/ml saponin /1 mg/ml BSA in PBS (*APPENDIX 2A*)
Secondary antibody: fluorescently-tagged anti-guinea pig IgG; e.g., Texas red dye–conjugated donkey anti-guinea pig IgG (Jackson Immunoresearch Laboratories)
Mounting medium: e.g., Prolong Antifade Reagent (Invitrogen) or see Internet Resources

22-mm^2 square glass coverslips, sterile
35-mm tissue culture dishes or 6-well tissue culture plates
Rocking platform
Glass slides
Confocal or widescreen fluorescence microscope with appropriate filters

Plate cells for staining

1. One or two days prior to staining, plate cells on sterile glass coverslips. Plate the cells at 50% to 70% confluency so that they remain semiconfluent at the time of staining.

   Whether this step is done 1 or 2 days prior to staining depends on the length of time required for the chosen cell type to adequately adhere to the coverslip. For some cell types, coating the coverslip with 0.1 mg/ml poly-L-lysine prior to plating may facilitate adherence.

   It is easiest to place single 2-mm square coverslips in 35-mm tissue culture dishes or one per well in a 6 -well plate. The coverslips are left in the dishes through the wash, fixation, and staining steps.

2. *Optional:* To enhance lipid droplet formation and facilitate detection of adipophilin, supplement growth medium for cells with 400 μM oleate for 6 to 24 hr prior to fixation of cells.

   Adipophilin expression is increased following supplementation with excess fatty acid (Gao et al., 2000). Moreover, adipophilin is quickly degraded when not bound to the surface of lipid droplets (Xu et al., 2005). Therefore, increasing the size and number of lipid droplets by supplementation with excess lipid will facilitate detection of adipophilin.

   Preparation of fatty acid–supplemented medium is discussed in the Support Protocol.

Fix cells

3. Wash the cells twice with 2 ml PBS.

   All washes are carried out at room temperature.

4. Fix the cells by incubating with 2 ml of 3% (w/v) paraformaldehyde for 20 min at room temperature.

5. Wash the cells four times with 2 ml PBS.

Immunostain the cells

6. Add 2 ml blocking buffer to the cells and incubate 45 min at room temperature to prevent nonspecific antibody binding.

7. Dilute the guinea pig anti-adipophilin polyclonal primary antibody 1:1000 (from whole antiserum) in antibody diluent. Incubate the cells with 1 ml diluted primary antibody overnight at 4°C.

   For primary antibodies other than anti-adipophilin, pilot experiments should be performed to optimize the antibody concentration.

8. Warm the cells at room temperature for 30 min before proceeding with step 9.

   The product literature accompanying this antibody states that it recognizes adipophilin in human, mouse, rat, dog, and bovine cells. The authors have also demonstrated its affinity for the Syrian gold hamster (BHK cell) protein (L.L. Listenberger and D.A. Brown, unpub. observ.).

9. Wash the cells four times (10 min each time) with 2 ml PBS on a gently rocking platform.

   Including 0.1 mg/ml saponin in this and all further wash and incubation solutions may enhance detection of some proteins.

10. Dilute the donkey anti-guinea pig secondary antibody to 5 μg/ml in antibody diluent. Add 1 ml diluted secondary antibody to the cells and incubate 1 hr at room temperature, protected from light.

    Excessive exposure of the fluorophore to ambient light should be avoided. Thus, cover the cells during the incubation with secondary antibody and subsequent wash steps.

    Secondary antibodies conjugated to red, green, or blue fluorophores may be used. Antibody concentration should be varied in pilot experiments to maximize signal while minimizing background.

11. Wash the cells four times (10 min each time) with 2 ml PBS on a gently rocking platform.

12. Mount the coverslips onto slides with 20 to 40 μl antifade mounting medium.

Analyze cells visually

13. Use confocal or wide-field fluorescence microscopy to detect adipophilin immunostaining.

SIMULTANEOUS DETECTION OF LIPID DROPLETS AND ADIPOPHILIN

ALTERNATE PROTOCOL

Simultaneous visualization of lipid droplet cores and intracellular proteins is possible by adding BODIPY 493/503 to the secondary antibody solution used to detect the protein. Using a secondary antibody conjugated to a red fluorophore allows detection of a lipid droplet–associated protein without interference from significant BODIPY 493/503 fluorescence (see Commentary). As a control for the absence of red fluorescence due to BODIPY 493/503 staining, include a sample in which the primary antibody incubation step includes antibody diluent alone.

Additional materials *(also see Basic Protocol 2)*

1 mg/ml BODIPY 493/503 stock solution: prepare by dissolving 5 mg BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene; Molecular Probes) in 5 ml ethanol; divide into 500-µl aliquots, and store up to 1 month at −20°C

Plate and fix cells

1. Follow steps 1 to 5 of Basic Protocol 2.

Immunostain for lipid and protein

2. Incubate the cells with 2 ml blocking buffer for 45 min at room temperature to prevent nonspecific antibody binding.
3. Dilute the guinea pig anti-adipophilin polyclonal antibody 1:1000 (from whole antiserum) in antibody diluent. Incubate the cells with 1 ml diluted primary antibody overnight at 4°C. Warm the cells at room temperature for 30 min before proceeding.
4. Wash the cells four times (10 min each) with 2 ml PBS on a gently rocking platform.
5. Prepare the secondary antibody solution as follows:
   a. Add 1 µl of 1 mg/ml BODIPY 493/503 stock solution to 1 ml of antibody diluent.
   b. Dilute the secondary antibody to 5 µg/ml in the antibody diluent containing the BODIPY dye.
6. Overlay each coverslip with 200 µl diluted secondary antibody. Incubate 1 hr at room temperature, protected from ambient light.

   Excessive exposure of the fluorophore to ambient light should be avoided. Thus, cover the cells during the incubation with secondary antibody and subsequent wash steps.
7. Wash the cells four times (10 min each) with 2 ml PBS on a gently rocking platform.
8. Mount the coverslips onto slides with 20 to 40 µl antifade mounting medium.

Analyze cells visually

9. Simultaneously detect adipophilin (red fluorescence) and neutral lipid droplets (green fluorescence).

   In large lipid droplets, adipophilin may be detected as a ring surrounding the BODIPY 493/503–positive lipid droplet core, while the two markers often appear to overlap in small lipid droplets (Fig. 24.2.2).

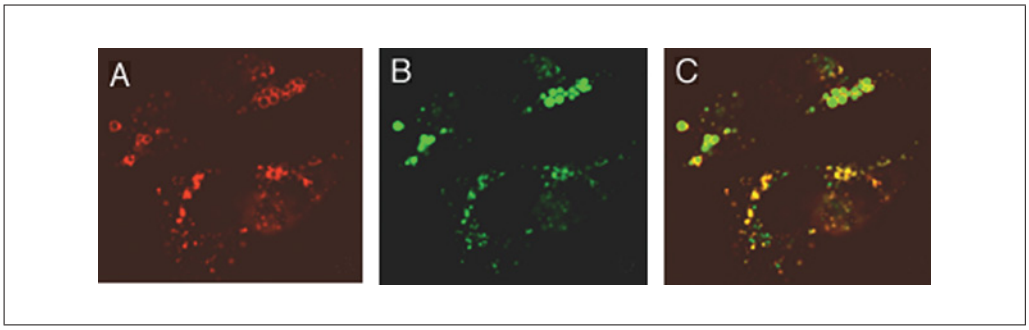


Figure 24.2.2 Simultaneous detection of lipid droplet cores and adipophilin. BHK cells were fed medium supplemented with 400 µM oleate prior to immunofluorescent detection of adipophilin (red, **A**) and BODIPY 493/503 staining of neutral lipid droplets (green, **B**). The merged image (**C**) shows adipophilin localized to the surface of neutral lipid droplets. Images shown are single sections of a *z*-stack visualized with a Zeiss Axiovision 200 fluorescent microscope and processed with deconvolution software. For the color version of this figure go to *http://www.currentprotocols.com.*

PREPARATION OF FATTY ACID–SUPPLEMENTED MEDIUM

SUPPORT PROTOCOL

Many cell types exhibit only a few, small lipid droplets when cultured under standard conditions. However, fatty acid supplementation of growth medium stimulates neutral lipid synthesis and can greatly enhance lipid droplet size and number. Elevated neutral lipid levels can increase cellular levels of adipophilin and possibly other lipid droplet proteins, both through induction of synthesis and stabilization, facilitating detection of these proteins. To enhance detection of lipid droplets, a simple protocol for preparing fatty acid–supplemented medium is included below.

Materials

1 M NaOH
Oleic acid
Phosphate-buffered saline (PBS; *APPENDIX 2A*), without calcium and magnesium
Bovine serum albumin (BSA), essentially fatty acid free
Growth medium for cultured cells of interest

50-ml conical tube
37°C and 70°C water bath
250- and 400-ml beakers, sterile
0.22-μm bottle-top filter and sterile, 200-ml glass bottle

Prepare 20 mM sodium oleate

1. Add 200 μl of 1M NaOH to 15.7 ml water in a 50-ml conical tube. Warm to 70°C in a water bath.
2. Add 100 μl oleic acid to the prewarmed solution. Incubate 30 min at 70°C. Invert the tube to mix several times throughout the incubation.
3. Add 50 μl of 1 M NaOH to the fatty acid solution. Invert to mix. Incubate 5 min at 70°C.
4. Repeat step 3 until micelles are no longer visible.

   The micelles may appear as small oil droplets floating near the surface of the solution.

   They are easiest to detect after swirling the tube. Typically, three to five aliquots of NaOH must be added before the oleate is adequately dispersed in solution and the micelles disappear.

Prepare 5% BSA solution

5. Add 80 ml PBS to a 400-ml beaker.
6. Weigh out 5 g BSA and add to the beaker on top of the PBS. When all of the BSA is in solution, swirl gently to mix.

   The BSA will gradually fall into solution.

   This method facilitates the addition of large amounts of BSA to solution. BSA added to the beaker before the PBS, or with stirring, will clump and be difficult to solubilize.

7. Adjust volume in the beaker to 100 ml with PBS. Sterilize by passing through a 0.22-μm filter and store up to several months at 4°C.

   A solution of 5% BSA in PBS may be made in advance and stored at 4°C for several months.

Form fatty acid/albumin complexes

8. Prewarm 11.6 ml of the 5% BSA in PBS at 37°C in a 50-ml conical tube.
9. Add 3.5 ml of the 20 mM sodium oleate solution dropwise to warmed BSA solution. Invert to mix several times during the addition.

Here, fatty acid is complexed to BSA at an approximate molar ratio of 8:1. Following addition to serum-containing culture media, the fatty acid to BSA ratio is ~6.6:1 (Listenberger et al., 2001). While this ratio enhances lipid droplet formation and may mimic some pathophysiologic conditions (Kleinfeld et al., 1996), physiological fatty acid to albumin ratios (and consequently, unbound fatty acid concentrations) are likely to be lower (Spector, 1975; Richieri and Kleinfeld, 1995).

10. In the sterile environment of a tissue culture hood, add the BSA/oleate mix to 160 ml cell culture medium (prewarmed to 37°C) in a sterile 250-ml beaker. Sterilize by passing though a 0.22-μm bottle-top filter placed on a sterile 200-ml glass bottle. Store up to 1 week at 4°C.

    The final concentration of oleate in the medium is 400 μM.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antibody diluent

80 ml H_2O
10 ml 10× PBS (*APPENDIX 2A*)
100 mg bovine serum albumin (BSA)
2 ml 0.5% (w/v) saponin (see recipe)
H_2O to 100 ml
Store up to 1 week at 4°C

Blocking buffer

1.5 g glycine
3 g bovine serum albumin (BSA)
80 ml H_2O
10 ml 10× PBS (*APPENDIX 2A*)
2 ml 0.5% (w/v) saponin (see recipe)
H_2O to 100 ml
Sterilize by passing though a 0.22-μm filter
Store up to 1 month at 4°C

Paraformaldehyde, 3% (w/v)

3 g paraformaldehyde
Add 80 ml H_2O.
Adjust pH to 8.0 with 5 N NaOH
Warm to 50°C with stirring
Add 10 ml 10× PBS (*APPENDIX 2A*)
Add H_2O to 100 ml
Filter through standard filter paper to remove undissolved solids
Store up to 1 week at 4°C

Saponin, 0.5% (w/v)

50 mg saponin
Dissolve in 9 ml H_2O
Add 1 ml 10× PBS (*APPENDIX 2A*)
Store up to 1 week at 4°C

COMMENTARY

Background Information

The spectral properties of Nile Red and BODIPY 493/503 have important implications for their usefulness as lipid droplet markers. The emission maximum of Nile Red bound to phospholipid vesicles is ~636 nm, detected as red fluorescence (Molecular Probes). However, the emission spectrum is very broad, and in some environments (as detailed below) Nile Red also emits significant green fluorescence. For this reason, dual-localization studies using Nile Red and a protein detected with an antibody conjugated to either a red or green fluorophore is difficult. Because Nile Red bleaches faster than most antibody-linked fluorophores, dual localization may be possible by bleaching Nile Red fluorescence after capturing an image (Ostermeyer et al., 2001) or by detecting a protein using a blue fluorophore (Ostermeyer et al., 2004). By contrast, the relatively narrow emission spectrum of BODIPY 493/503 is largely restricted to wavelengths passed by typical green emission filters, facilitating simultaneous detection of another marker in red (Fig. 24.2.2). For this reason, BODIPY 493/503 is preferred over Nile Red for dual-localization studies.

Staining of hydrophobic structures other than lipid droplets by lipophilic dyes can also be a concern. For instance, Nile Red gives significant labeling of membranes when red fluorescence is examined. By contrast, green fluorescence of Nile Red is fairly specific for lipid droplets (Fig. 24.2.1). This results from the fact that Nile Red's fluorescence maximum shifts toward the red as the polarity of its environment increases (Greenspan et al., 1985; Deye et al., 1990). Green fluorescence of Nile Red in membranes is thus greatly reduced because they are more polar than lipid droplets. By contrast, BODIPY 493/503 fluorescence is fairly specific for lipid droplets.

Despite the advantages of BODIPY 493/503, Nile Red may sometimes be more useful. For example, with filters typically used to detect fluorescein fluorescence, BODIPY 493/503 fluorescence generally appears less intense than that of Nile Red and may photobleach more rapidly (L.L. Listenberger and D. A. Brown, unpub. observ.). Thus, green fluorescence of Nile Red remains a useful tool for single-color labeling of lipid droplets.

In addition to Nile Red and BODIPY 493/503, other classic dyes utilized for visualization of lipid droplets include Sudan III and Oil-red O. Both dyes allow detection of lipid droplets by either conventional light microscopy or fluorescence microscopy (Aoki et al., 1997; Koopman et al., 2001). The red fluorescence emitted by Sudan III and Oil-red O is particularly useful for codetection of lipid droplets and GFP-tagged proteins (Imamura et al., 2002). Importantly, some published protocols for staining with Sudan III and Oil-red O involve incubation with solvents (including isopropanol and ethanol) that cause fusion of small lipid droplets (Fukumoto and Fujimoto, 2002). Thus, these dyes should not be used to draw conclusions regarding lipid droplet size and morphology. (See Critical Parameters and Troubleshooting for additional discussion of treatments that alter lipid droplet morphology.)

Critical Parameters and Troubleshooting

Detection of lipid droplets using either Nile Red or BODIPY 493/503 staining is generally straightforward and uncomplicated. For cell types that have a tendency to detach from the coverslip during washes, it may be helpful to use acid-washed coverslips (incubate coverslips in 2:1 nitric acid: hydrochloric acid for 2 hr; rinse extensively with distilled H_2O and ethanol; autoclave) and to pretreat the coverslips with 0.1 mg/ml poly-L-lysine prior to plating. Moreover, detection of lipid droplets and lipid droplet–associated protein is greatly enhanced following supplementation with excess lipid. In addition to supplementation with oleate, others have observed similar increases in lipid storage following incubation with acetylated low-density lipoprotein (LDL; Larigauderie et al., 2004).

BODIPY 493/503–stained lipid droplets may be difficult to detect because of significant photobleaching. Samples should be mounted in medium that includes an antiquenching reagent and exposure to light should be minimized. If photobleaching of BODIPY 493/503–stained lipid droplets continues to be a problem, alternative labeling techniques may be pursued. One alternative method for the fluorescent detection of lipid droplets, Nile Red staining, is discussed in this unit. Another method is to supplement growth medium with a fluorescent fatty acid analog prior to fixation (Wolins et al., 2005). Because cells actively transport the fluorescent fatty acid to lipid droplets, staining is particularly intense and can be detected with reduced excitation, lessening photobleaching.

As highlighted in two recent reports, the hydrophobic nature of the lipid droplet requires careful consideration with respect permeabilization and fixation techniques. DiDonato and Brasaemle (2003) report that fixation with cold methanol or cold acetone disrupts lipid droplet morphology, causing lipid droplets to fuse or collapse in upon themselves. By contrast, paraformaldehyde fixation retains lipid droplet shape and is appropriate for visualization of lipid droplets and associated proteins. Furthermore, Ohsaki and colleagues (2005) have shown that fixation with a combination of glutaraldehyde and formaldehyde enhances detection of some lipid droplet proteins. The optimal concentration of glutaraldehyde was found to vary between markers and must be determined empirically. These authors also found that optimal detection of many lipid droplet–associated proteins required permeabilization with digitonin or saponin, rather than Triton X-100 (Ohsaki et al., 2005).

Adapting the procedure outlined in this unit to detection of lipid droplet–associated proteins other than adipophilin may require optimization in pilot experiments. Ideally, these should be performed in accordance with the guidelines determined by DiDonato and Brasaemle (2003) and Ohsaki et al. (2005). However, some antibodies work best only under conditions that do not preserve lipid droplet structure. For example, antigen detection by a mouse monoclonal anti-adipophilin antibody (Research Diagnostics Inc.) is markedly enhanced by fixation with cold methanol (Ostermeyer et al., 2004). Brief exposure to cold methanol does not completely extract lipids, and lipid droplets may still be detected with lipophilic dyes. Thus, if necessary, methanol fixation may be used in demonstrating targeting of a particular protein to lipid droplets, although no conclusions regarding lipid droplet size, structure, or distribution are possible.

Anticipated Results

Lipid droplets stained with Nile Red or BODIPY 493/503 are distinguished from most other organelles by their very round, smooth-surfaced appearance (Fig. 24.2.1). The number and size of lipid droplets vary between cell types and depend on whether medium has been supplemented with excess lipid prior to staining. Although lipid droplets are generally dispersed throughout the cell, extensive clustering of lipid droplets has been observed in some cell types.

Time Considerations

For both Basic Protocols 1 and 2, cells should be plated on coverslips 1 or 2 days prior to staining. The specific time depends on the cell type. In either case, cells must be fully adherent and well spread on the coverslip. The total time necessary for fixation and Nile Red or BODIPY 493/503 staining is minimal, requiring only ~1.5 hr. For immunostaining, ~1.5 hr is necessary on the first day for sample fixation and blocking. After overnight incubation in primary antibody, another 3.5 hr is needed to complete the washes and secondary antibody incubation. Once the coverslips are mounted on the slides, all samples may be stored at −80°C, protected from light, for several days prior to microscopy.

Literature Cited

Aoki, T., Hagiwara, H., and Fujimoto, T. 1997. Peculiar distribution of fodrin in fat-storing cells. *Exp. Cell Res.* 234:313-320.

Brasaemle, D.L., Dolios, G., Shapiro, L., and Wang, R. 2004. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J. Biol. Chem.* 279:46835-46842.

Deye, J.F., Berger, T.A., and Anderson, A.G. 1990. Nile Red as a solvatochromic dye for measuring solvent strength in normal liquids and mixtures of normal liquids with supercritical and near critical fluids. *Anal. Chem.* 62:615-622.

DiDonato, D. and Brasaemle, D.L. 2003. Fixation methods for the study of lipid droplets by immunofluorescence microscopy. *J. Histochem. Cytochem.* 51:773-780.

Fukumoto, S. and Fujimoto, T. 2002. Deformation of lipid droplets in fixed samples. *Histochem. Cell Biol.* 118:423-428.

Fujimoto, Y., Itabe, H., Sakai, J., Makita, M., Noda, J., Mori, M., Higashi, Y., Kojima, S., and Takano, T. 2004. Identification of major proteins in the lipid droplet–enriched fraction isolated from the human hepatocyte cell line HuH7. *Biochim. Biophys. Acta.* 1644:47-59.

Gao, J., Ye, H., and Serrero, G. 2000. Stimulation of adipose differentiation related protein (ADRP) expression in adipocyte precursors by long-chain fatty acids. *J. Cell Phys.* 182:297-302.

Greenspan, P., Mayer, E.U., and Fowler, S.D. 1985. Nile Red: A selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* 100:965-973.

Imamura, M., Inoguchi, T., Ikuyama, S., Taniguchi, S., Kobayashi, K., Nakashima, N., and Nawata, H. 2002. ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *Am. J. Physiol. Endocrinol. Metab.* 283:E775-E783.

Kleinfeld, A.M., Prothro, D., Brown, D.L., Davis, R.C., Richieri, G.V., and DeMaria, A. 1996.

Increases in serum unbound free fatty acid levels following coronary angioplasty. *Am. J. Cardiol.* 78:1350-1354.

Koopman, R., Schaart, G., and Hesselink, M.K.C. 2001. Optimisation of oil-red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem. Cell Biol.* 116:63-68.

Larigauderie, G., Furman, C., Jaye, M., Lasselin, C., Copin, C., Fruchart, J., Castro, G., and Rouis, M. 2004. Adipophilin enhances lipid accumulation and prevents lipid efflux from THP-1 macrophages: Potential role in atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 24:504-510.

Listenberger, L.L., Ory, D.S., and Schaffer, J.E. 2001. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. *J. Biol. Chem.* 276:14890-14895.

Liu, P., Ying, Y., Zhao, Y., Mundy, D., Zhu, M., and Anderson, R.G.W. 2004. Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *J. Biol. Chem.* 279:3787-3792.

Londos, C., Brasaemle, D.L., Schultz, C.J., Segrest, J.P., and Kimmel, A.R. 1999. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin. Cell Dev. Biol.* 10:51-58.

Londos, C., Sztalryd, C., Tansey, J.T., and Kimmel, A.R. 2005. Role of PAT proteins in lipid metabolism. *Biochimie* 87:45-49.

Murphy, D.J. and Vance, J. 1999. Mechanisms of lipid-body formation. *Trends Biol. Sci.* 24:109-115.

Ohsaki, Y., Maeda, T., and Fujimoto, T. 2005. Fixation and permeabilization protocol is critical for the immunolabeling of lipid droplet proteins. *Histochem. Cell Biol.* 124:445-452.

Ostermeyer, A.G., Paci, J.M., Zeng, Y., Lublin, D.M., Munro, S., and Brown, D.A. 2001. Accumulation of caveolin in the endoplasmic reticulum redirects the protein to lipid storage droplets. *J. Cell Biol.* 152:1071-1078.

Ostermeyer, A.G., Ramcharan, L.T., Zeng, Y., Lublin, D.M., and Brown, D.A. 2004. Role of the hydrophobic domain in targeting caveolin-1 to lipid droplets. *J. Cell Biol.* 164:69-78.

Richieri, G.V. and Kleinfeld, A.M. 1995. Unbound free fatty acid levels in human serum. *J. Lipid Res.* 36:229-240.

Spector, A.A. 1975. Fatty acid binding to plasma albumin. *J. Lipid Res.* 16:165-179.

Targett-Adams, P., Chambers, D., Gledhill, S., Hope, R.G., Coy, J.F., Girod, A., and McLauchlan, J. 2003. Live cell analysis and targeting of the lipid droplet–binding adipocyte differentiation-related protein. *J. Biol. Chem.* 278:15998-16007.

Umlauf, E., Csaszar, E., Moertelmaier, M., Schuetz, G.J., Parton, R.G., and Prohaska, R. 2004. Association of stomatin with lipid bodies. *J. Biol. Chem.* 279:23699-23709.

Wolins, N.E., Quaynor, B.K., Skinner, J.R., Schoenfish, M.J., Tzekov, A., and Bickel, P.E. 2005. S3-12, adipophilin, and TIP47 package lipid in adipocytes. *J. Biol. Chem.* 280:19146-19155.

Xu, G., Sztalryd, C., Lu, X., Tansey, J.T., Gan, J., Dorward, H., Kimmel, A.R., and Londos, C. 2005. Post-translational regulation of ADRP by the ubiquitin/proteasome pathway. *J. Biol. Chem.* 280:42841-42847.

Zweytick, D., Athenstaedt, K., and Daum, G. 2000. Intracellular lipid particles of eukaryotic cells. *Biochim. Biophys. Acta.* 1469:101-120.

Internet Resources

http://spectorlab.cshl.edu/fluorescence_medium.html

Includes detailed instructions for preparation of a p-phenylenediamine containing fluorescence mounting medium.

Contributed by Laura L. Listenberger and Deborah A. Brown

State University of New York at Stony Brook

Stony Brook, New York

Making Giant Unilamellar Vesicles via Hydration of a Lipid Film

Suliana Manley[1] and Vernita D. Gordon[2]

[1]National Institutes of Health, Cell Biology and Metabolism Branch, Bethesda, Maryland
[2]University of Illinois Urbana-Champaign, Department of Materials Science and Engineering, Urbana, Illinois

ABSTRACT

This unit describes protocols for making giant unilamellar vesicles (GUVs) based on rehydration of dried lipid films. These model membranes are useful for determining the impact of membrane and membrane-binding components on lipid bilayer stiffness and phase behavior. Due to their large size, they are especially amenable to studies using fluorescence and light microscopy, and may also be manipulated for mechanical measurements with optical traps or micropipets. In addition to their use in encapsulation, GUVs have proven to be useful model systems for studying many cellular processes, including tubulation, budding, and fusion, as well as peptide insertion. The introduction of enzymes or proteins can result in reorganization, leading to such diverse behavior as vesicle aggregation, fusion, and fission. *Curr. Protoc. Cell Biol.* 40:24.3.1-24.3.13. © 2008 by John Wiley & Sons, Inc.

Keywords: liposomes • giant vesicles • electroformation • liposome swelling • model membranes

INTRODUCTION

This unit describes protocols for forming giant unilamellar vesicles (GUVs). The techniques described use hydration of dried lipid films, either by electroformation (Angelova et al., 1992) or by gentle hydration (Mueller et al., 1983). Swelling by electroformation (see Basic Protocol 1 and Alternate Protocol 1) produces a high yield of GUVs, typically 10 to 100 μm in diameter. Electroformation is not recommended for use with buffered solutions or charged lipids; instead, gentle hydration should be used (see Basic Protocol 2 and Alternate Protocols 2 and 3). An alternative technique that uses rapid evaporation is not described here (Moscho et al., 1996). Because nonfunctionalized vesicles are generally only weakly adhesive to many substrates, they may drift during imaging. Alternate Protocols 1 and 3 produce GUVs that are attached to wires or glass, thereby eliminating drift.

PREPARING GUVs BY ELECTROFORMATION ON INDIUM TIN OXIDE–COATED PLATES

BASIC PROTOCOL 1

This protocol is ideal for producing several milliliters of GUVs at $\sim 10^3$ to 10^4 per μl, which can then be submitted to a variety of different conditions. If desired, indium tin oxide (ITO) plates of coverslip thickness may be purchased, thus allowing observation of the growth of GUVs. For microscopy, lipids are generally rehydrated in aqueous sucrose solutions. Resuspension in equimolar glucose results in vesicles containing an interior solution that is denser than the suspending medium. This is commonly used in inverted microscopy to force vesicles to sink to the bottom of the sample chamber, next to the objective; switching solutions (glucose interior, sucrose exterior) causes vesicles to cream to the top for observation by upright microscopy. This also allows for application of osmotic stress and deflation using different molarities of sucrose and glucose. Furthermore, if fluorescent lipids are not incorporated, the difference in index of

refraction produced by the sugar solutions allows vesicles to be viewed using differential interference contrast or phase-contrast microscopy.

Materials

100 μl lipid in chloroform, at 5 to 10 mg/ml (see Support Protocol 1)
Up to 600 mM sucrose, or sterile deionized H_2O

25-cm^2 ITO-coated plates (e.g., Delta Technologies, Ltd., part no. CG-511IN-50x50/1.1; *http://www.delta-technologies.com/*)
Sealed chamber (e.g., vacuum desiccator) connected to dry nitrogen or vacuum source
Vacuum grease
Teflon holder and spacers for ITO-coated plates (see Support Protocol 2)
Oven with hole to introduce electrodes or heating bath (for lipid systems in which at least one component has a chain-melting transition temperature, T_m, above room temperature)
Electrodes ("Mini-Plunger to BNC Male" or equivalent; e.g., Radio Shack)
Function generator with readout for voltage and frequency (Stanford Research Systems, Ltd., *http://www.thinksrs.com/*)

Prepare dry lipid film on ITO plates

1. Prepare lipid mixtures (see Support Protocol 1).
2. Using a glass Pasteur pipet, deposit 2 to 3 drops of lipid mixture (~25 μl) onto the conductive side of a 25-cm^2 ITO-coated plate.

   Less lipid should be used for a smaller plate; maintain coverage of ~1 μl/cm^2.

3. Before the solution dries, use the side of the pipet to quickly spread the lipid over the surface of the plate using a single swipe. Repeat with second plate.

   Viewed in reflection, the lipid film should appear as colored interference fringes. The film should be as uniform as possible. Nonuniform films may result in a larger number of multilamellar or small vesicles. See Commentary for more information on the influence of film properties.

4. Place plates under vacuum at room temperature for at least 2 hr and up to overnight, until ready for use.

Carry out electroformation

5. Assemble plates into parallel assembly in the Teflon holder, separated by spacers.

   To prevent leakage, a small amount of vacuum grease may be spread around any seams in the Teflon holder. Parafilm or plastic wrap may be used to secure the assembly and render it leakproof.

6. Fill assembly with up to 600 mM sucrose solution or sterile deionized water.

   Choice of solvent and concentration is to be determined by the amount of osmotic stress, density, and index mismatch required for the experiment, and is a tunable parameter that is purely up to the experimenter.

7. *Optional:* For lipid mixtures or lipids with low T_m, place the assembly in an oven or heating bath set above T_m for the highest-melting component.

   For most unsaturated lipids and for many saturated lipids with tails up to 16 carbon atoms long, 50°C is sufficient.

8. Attach one electrode to each plate.

   Chamber should be covered to eliminate evaporation.

9. Set the function generator to output AC current (a sine wave), with amplitude 2 V, frequency 10 Hz. Incubate 1 to 2.5 hr.

Handle and store GUVs

10. Remove assembly from function generator and oven. Use a pipet or syringe needle to extract vesicles. Take care to minimize shear stresses by using a large pipet-tip opening or syringe needle and slow extraction to maximize the number of vesicles that survive this transfer.

    If using membrane dyes, unilamellar vesicles can be identified under the microscope as vesicles with the lowest fluorescence from the membrane.

11. Use GUVs within a few hours of formation (although a fraction will survive overnight).

12. Store GUVs at room temperature.

MAKING LIPID MIXTURES

SUPPORT PROTOCOL 1

This protocol describes the proper handling and storage of lipids, and gives some guidelines for selecting lipid mixtures.

Choice of lipids

Choosing a lipid composition is the first step in planning your experiments with GUVs. For testing your ability to make GUVs, a single, unsaturated lipid such as dioleoylphosphatidylcholine (DOPC) may be useful for troubleshooting. For model raft mixtures, a 1:1:1 molar composition of DOPC:sphingomyelin:cholesterol is typical, and is commonly referred to as the canonical raft mixture. However, this mixture produces GUVs that may not be phase-separated at room temperature. Therefore, if your microscope does not have the capability to be cooled, a mixture with a lower level of cholesterol that will readily phase-separate at room temperature may be preferable. For phase diagrams that provide the lipid phase melting temperatures of a range of ternary mixtures (Veatch and Keller, 2002), the influence of aliphatic chain length (Veatch and Keller, 2003) and sterol type (Bacia et al., 2005; Beattie et al., 2005) have also been investigated.

Lipid mixtures for peptide- and protein-binding experiments must be chosen carefully, since binding may depend strongly on lipid composition. In particular, it may be necessary to incorporate charged lipids in order to achieve binding (Bigay et al., 2003). A reasonable choice of lipids may be dictated by the natural lipid environment of the protein of interest. Biotinylated lipids are also available, and allow binding of vesicles to streptavidin or streptavidin-treated surfaces and objects.

Fluorescent lipid probes are useful for imaging vesicles and for determining phase behavior (Bagatolli, 2006). Headgroup-labeled phospholipids are typically used for these purposes, since the bulky fluorophore may interfere with lipid ordering if attached to an aliphatic chain. Several probes are available with readily accessible excitation wavelengths, including rhodamine-, Texas Red–, NBD- and fluorescein-conjugated lipids. These probes all partition preferentially into the liquid-disordered, non-raftlike phase, and are typically added at concentrations of ~0.1 mol%. Probes that partition into the liquid-ordered, raftlike phase can also be desirable for probing curvature and mobility; these include perylene (Baumgart et al., 2003) and cholera toxin B binding to GM1 gangliosides (Bacia et al., 2005). The lipid probe Laurdan is a particularly powerful tool for studying local membrane ordering; the spectrum of the Laurdan molecule is dependent on the polarity of its surroundings and therefore reflects the packing density of neighboring lipids (Bagatolli and Gratton, 2000). By measuring the spatially resolved spectra, a mapping of phases can be derived. However, Laurdan requires two-photon excitation, due to its low excitation wavelength and its rapid photobleaching.

Materials

Lipid (Avanti Polar Lipids; when received, store at –20°C or lower)
Chloroform
Methanol (optional)
Argon or nitrogen gas

Glass vials with Teflon closures
Microdispensers with glass bores (Drummond), or Hamilton syringes
Teflon tape

1. Rehydrate lipids supplied as powder in chloroform or a 2:1 (v/v) mixture of chloroform:methanol.

   See Commentary for more information on the influence of film properties.

2. Handle lipids in chloroform with glass, stainless steel, or Teflon labware. Store all solutions in glass vials with Teflon-lined caps.

3. Use microdispensers with glass bores or Hamilton syringes to measure volumes for making lipid mixtures.

   Typically, mixtures should be made in small volumes (~100 μl) or stored as aliquots to avoid contamination from handling.

4. Rinse all surfaces to come into contact with lipid solutions with chloroform.

   This includes microdispenser tips, glass bores, glass vials, ITO-coated plates, platinum wires, and Teflon plates.

5. After use, store solutions under argon or nitrogen gas at −20°C or lower to prevent hydrolysis or oxidation of lipids. After sealing, wrap caps in Teflon tape.

SUPPORT PROTOCOL 2

DESIGN OF A PTFE (TEFLON) HOLDER FOR ELECTROFORMATION ON ITO-COATED PLATES

This protocol describes the design of a Teflon holder for ITO-coated plates used in electroformation. The dimensions should be adjusted to accommodate the size of the plates. A polytetrafluoroethene (PTFE, or Teflon) block is machined to prepare the holder. The holder consists of two pieces of Teflon designed to fit together. The design considerations are that plates should be held 2 to 5 mm apart. The inner dimensions of each Teflon piece should slightly exceed (by ~1 mm) those of the ITO-coated plates. An example is shown in Figure 24.3.1, for plates with dimensions 50 × 50 mm.

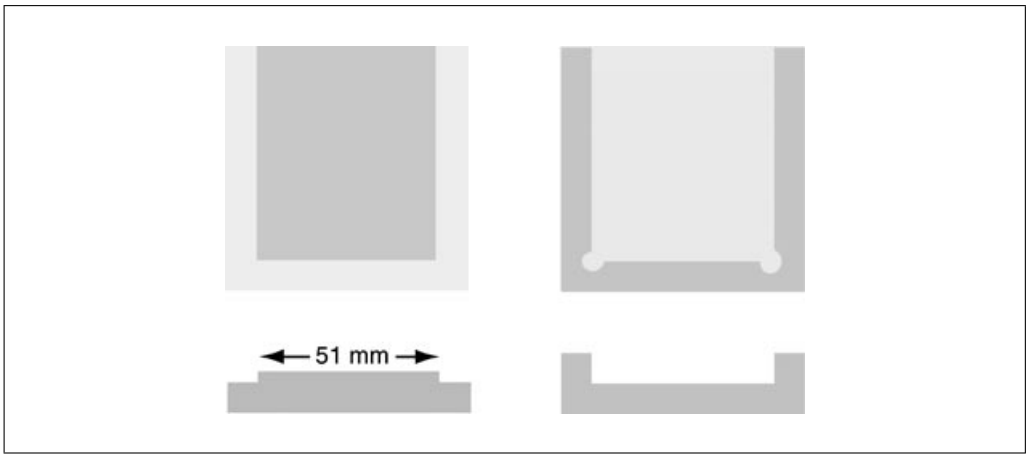


Figure 24.3.1 Teflon holder for ITO-coated plates, made from two pieces (shown left and right), front view and side view (shown top and bottom).

PREPARING GUVs BY ELECTROFORMATION ON PLATINUM WIRES

ALTERNATE PROTOCOL 1

This protocol produces a high yield of GUVs in a sample-chamber geometry that is well adapted for viewing under a microscope. Vesicles adhere to the platinum wire electrodes on which they form, so they remain largely stationary, without optically resolvable thermal fluctuations below ~45°C. If desired, the electroformation chamber can be adopted to allow harvesting of vesicles for experimentation on individual, isolated GUVs by using hydrodynamic flow along and across the electrodes.

Additional Materials *(also see Basic Protocol 1)*

5 to 10 μl of lipid solution in chloroform (Support Protocol 1) at concentration of 0.5 to 0.66 mM (~0.5 mg/ml)
Up to 600 mM sucrose, or sterile deionized H_2O

Platinum wires, 0.5- to 2.0-mm diameter
Microdispensers with glass bores (Drummond), or Hamilton syringes
Sealed chamber (e.g., vacuum desiccator) connected to dry nitrogen or vacuum source
Electroformation chamber (see Support Protocol 3), assembled with platinum wires

Prepare lipid-coated platinum wires

1. If using more than one species of lipid, mix in the desired ratio (see Support Protocol 1) and dilute in chloroform. Add membrane dye(s) and other components to be incorporated into the membrane, if desired, so that the total concentration of membrane components in chloroform solution remains ~0.5 to 0.66 mM, or ~0.5 mg/ml.

2. Using a glass microdispenser or Hamilton syringe, deposit solution onto platinum wires in drops of ~1 μl, so that drops are pendant from the wires.

   Drops can be closely spaced along the wires, but care should be taken that they do not coalesce; too high a concentration of lipid will result in multilamellar vesicles.

3. Dry lipid onto wires in nitrogen environment or under vacuum for 2 hr to overnight at room temperature.

Electroform GUVs

4. Add water (or other aqueous solution) to the chamber so that the wires are covered.

   Choice of solvent and concentration is to be determined by the amount of osmotic stress, density, and index mismatch required for the experiment, and is a tunable parameter that is purely up to the experimenter.

5. If necessary, heat chamber so that the temperature is above T_m for the highest-melting component in the system.

   For lipid systems in which T_m for one or more component is above room temperature, the electroformation chamber must be heated above T_m for the highest-melting component in the system. This may be done using a heating bath, by heating the chamber directly using resistive heating or Peltier plates, or by preheating the hydration solution, if the duration of electroformation is less than the time for the solution temperature to drop below T_m.

6. Apply a 2- to 10-V, 10-Hz AC electrical field across the electrodes for 30 to 120 min.

   During electroformation, care should be taken that the hydrating solution is in continuous contact with the electrode wires. If necessary, additional hydrating solution can be added to compensate for evaporation, but care should be taken to minimize hydrodynamic disturbances in the chamber, because these will wash vesicles off electrodes.

   Chambers can also be covered with a lid to reduce evaporation.

Time for electroformation varies with the lipids used, as does the yield. In general, increasing the percentage of phosphatidylcholine (PC) lipids increases yield and lowers electroformation time. Most vesicles formed using this technique will be unilamellar. Multilamellar vesicles can be identified, if membrane dyes are used, by higher levels of fluorescence.

SUPPORT PROTOCOL 3

DESIGN OF A CHAMBER FOR ELECTROFORMATION ON PLATINUM WIRES

This protocol describes the design and construction of a chamber for electroformation on platinum wires, intended for stringent temperature control and to allow for harvesting of GUVs. This design can be adapted depending on the need for viewing and manipulating GUVs.

Materials

Platinum wires, 0.5- to 2.0-mm diameter
Aluminum block (machined; typical dimensions, $10 \times 4 \times 1$ cm)
Electrical block fittings or terminal block fittings (McMaster-Carr, *http://www.mcmaster.com*)
Optional items:
  PTFE seals
  Cover glass for microscopy
  Norland optical adhesive
  Resistive heating wire
  Ceramic insulators and fittings for electrical attachment
  Thermocouple

Chamber construction

The essential elements of this electroformation chamber are provided by two platinum wires, which serve as the electrode substrates for formation of GUVs, and the aluminum block, machined to provide a water-tight chamber. If intended for viewing under an inverted microscope, this chamber may be created by machining a hole ~2 to 3 cm in diameter through the aluminum block and sealing a cover glass across the bottom using an epoxy that is nonreactive in water. Platinum-wire electrodes should be threaded through holes drilled in the side walls of this chamber so that they are parallel and spaced about 3 mm apart. These holes should be lined with PTFE or some other material so that the wires are electrically isolated from the aluminum block, and sealed so that the chamber is watertight using nonreactive adhesive and/or tightly packed PTFE tape. If viewing under an inverted microscope is intended, wire electrodes should be placed as close as possible to the bottom of the chamber, so that they are within the working distance of the microscope objective to be used. Electrical block fittings attached to the ends of the wires (which should protrude ~1 cm outside the aluminum block) allow these electrodes to be coupled to a function generator for electroformation. This design is illustrated by the central chamber of Figure 24.3.2.

If this chamber is to be used to electroform vesicles in which one or more lipid component(s) has T_m higher than room temperature, the chamber can be constructed so that it is resistively heated. This can be done by drilling two parallel holes in the aluminum block and threading heating wire through them so that the water chamber is approximately circumscribed. If this or another heating method is used, the chamber should be constructed with a thermocouple positioned as closely as possible to the wire electrodes, so that the temperature can be monitored.

Working at elevated temperatures will cause more rapid evaporation of the aqueous solution. If the solution level drops below the wire electrodes, vesicles will be destroyed by contact with air. Furthermore, if solutions other than pure water are used, evaporation

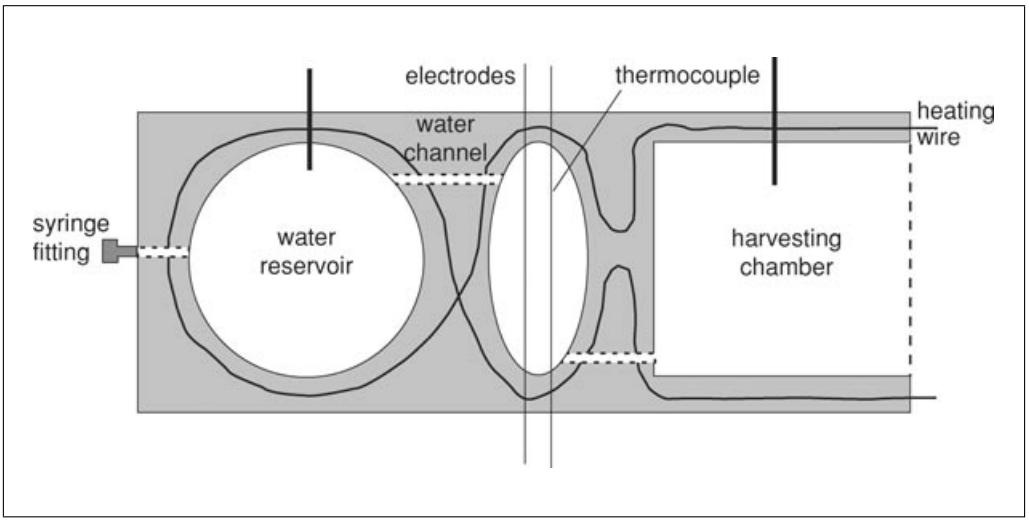


Figure 24.3.2 Electroformation assembly for platinum wires, showing optional water reservoir and harvesting chamber. The central chamber is essential, and contains the electrodes where the electroformation takes place.

will increase the concentration of solutes external to vesicle membranes (if any) and may cause membranes to become flaccid. If preventing this is desired, the chamber may be fitted with a sealed lid to reduce evaporation. However, such a lid may hinder application of some imaging techniques (if a lidded chamber is used under laser-scanning confocal microscopy, for example, reflection off the lid can significantly increase photobleaching throughout the sample). The effects of evaporation may also be mitigated by drilling the aluminum block to create a second water reservoir that is connected by a channel to the chamber in which electroformation and viewing are done. This is illustrated by the left chamber in Figure 24.3.2, marked "water reservoir." If this channel is higher (or lower) than the wire electrodes, water may be gently added to the reservoir to refill the electroformation chamber without significantly disrupting the lipids on the wires.

Alternatively, hydrodynamic flow can be deliberately used to flush GUVs off the wires so that isolated GUVs can be studied individually. Membranes should be fluid (above T_m) for this to be effective. To avoid temperature-shocking vesicles, it may be desirable to preheat the water used for flushing so that it is at approximately the same temperature as the solution surrounding the membranes. An electroformation chamber that has been used to form and harvest GUVs using this method is sketched below, in Figure 24.3.2. The chamber is lidded and sealed except for the harvesting chamber, which is open on one end to allow access to vesicles. Dimensions depicted are not necessarily to scale.

PREPARING GUVS BY SWELLING OFF OF PTFE (TEFLON)

BASIC PROTOCOL 2

While electroformation is the most successful protocol to date for producing a high yield of giant unilamellar vesicles, it works well only for a limited range of lipid compositions, notably those high in phosphatidylcholine (PC) lipids (>25% to 50 mol%) and low in charged lipids (<10 mol%), such as phosphatidylserine (PS) and phosphatidylglycerol (PG). Electroformation is also limited in the swelling solutions (buffers) to which it can be applied, because if salts are in the aqueous solution, the electric field can cause gas to be liberated by hydrolysis and form bubbles at the electrodes, where they destroy the vesicles. There is some evidence that electroformation can produce damaged membranes, and using a swelling technique to check the results seen for electroformed membranes allows the identification of artifacts (Gordon et al., in preparation).

This swelling technique requires at least ~10 mol% charged lipids to produce unilamellar vesicles (the threshold amount varies with the other lipids in the system and with the buffer used). This technique can produce GUVs in standard, salt-containing biological buffers.

Materials

20 mg/ml lipid in chloroform (Support Protocol 1)
Chloroform
Nitrogen gas
Swelling solution: 100 mM sucrose or glucose prepared using sterile deionized H_2O

Microdispensers with glass bores (Drummond), or Hamilton syringes
PTFE (Teflon) sheet, ~2 × 2–cm square, roughened with fine-grain sandpaper (McMaster-Carr, *http://www.mcmaster.com*)
Desiccator chamber, attached to vacuum pump
Glass beaker
Incubator or oven

Prepare dry lipid film

1. If using more than one species of lipid, mix in the desired ratio as in Support Protocol 1. Add membrane dye(s) and other components to be incorporated into the membrane, if desired, so that the total concentration of membrane components in chloroform solution remains approximately 20 mg/ml.

2. Using a glass microdispenser or Hamilton syringe, deposit 100 µl lipid(s) solution onto roughened PTFE. Spread the solution on the surface to distribute it as evenly as possible.

3. Dry under vacuum 3 hr to overnight at room temperature.

Hydrate lipid film

4. Prehydrate the resulting dry lipid film using moist nitrogen by bubbling nitrogen gas through sterile deionized water warmed on a hotplate and directing the resulting hydrated gas into a sealed chamber containing the lipid-coated PTFE sheet. Prehydrate film at least 30 min.

   If using light-sensitive membrane components, cover the chamber with aluminum foil.

5. Place PTFE sheet, lipid-side-up, on the bottom of the beaker. Fill with 5 ml swelling solution. Cover beaker with Parafilm.

   If using light-sensitive components, such as membrane dyes, wrap the beaker in aluminum foil.

6. Place beaker in incubator or oven and allow lipid film to swell into vesicles over 2 to 3 days.

   The temperature during swelling should be higher than the chain-melting transition temperature of the highest-melting membrane component. For many unsaturated lipids, 35° to 37°C gives satisfactory results.

   The resulting suspension will be dense in giant vesicles of a variety of sizes and lamellarities. These can be transferred to sample chambers for microscope viewing or other experimentation using a pipet or syringe. Taking care to minimize shear stresses by using a large pipet-tip opening or syringe needle and slow extraction will maximize the number of vesicles that survive this transfer. If using membrane dyes, unilamellar vesicles can be identified under the microscope as vesicles with the lowest fluorescence from the membrane.

PREPARING GUVs BY SWELLING OFF A UNIFORM FILM ON GLASS

ALTERNATE PROTOCOL 2

This method requires more preparation time and more specialized equipment than Basic Protocol 1, but it produces a higher percentage of unilamellar vesicles in the final, giant-vesicle suspension. This protocol also does not require charged lipids in the system to produce unilamellar vesicles if the swelling solution does not contain salt.

Materials

80 μl lipids in chloroform (Support Protocol 1) at 10 mM, (~10 mg/ml)
Chloroform
Nitrogen gas
Swelling solution: 100 mM sucrose in water (or other aqueous swelling solution), prepared using sterile deionized H_2O

Microdispensers with glass bores (Drummond), or Hamilton syringes
50- to 100-ml glass flask with pointed bottom
Rotary evaporator
Vacuum oven
Oven or incubator

Prepare dry lipid film in glass flask

1. If using more than one species of lipid, mix in the desired ratio as in Support Protocol 1. Add membrane dye(s) and other components to be incorporated into the membrane, if desired, so that the total concentration of membrane components in chloroform solution remains ~10 mM.
2. Using a glass microdispenser or Hamilton syringe, deposit 80 μl of this solution in a 50- to 100-ml pointed-bottom glass flask.
3. Attach flask to a rotary evaporator in a near-horizontal orientation.
4. Evaporate while rotating flask in a water bath that is at a temperature greater than the chain-melting temperature of the highest-melting component in the system (for most unsaturated lipids and for saturated lipids with tails up to 16 carbon atoms long, 50°C is sufficient).

   The resulting lipid film should be dry to the eye, with no remaining fluid discernible.

5. Dry this film for at least 2 hr in a vacuum oven at a temperature greater than the chain-melting temperature of the highest-melting component in the system.

Hydrate lipid film

6. Pre-hydrate the resulting dry lipid film using warm, moist nitrogen by bubbling nitrogen gas through sterile deionized water warmed on a hotplate and directing the resulting hydrated gas into a sealed chamber containing the lipid-coated glass flask. Prehydrate film at least 30 min.

   If using light-sensitive membrane components, cover the chamber with aluminum foil.

7. Fill flask with sufficient swelling solution to cover the lipid film. Seal top with Parafilm.

   If using light-sensitive components, cover flask with aluminum foil.

8. Incubate (in an oven or incubator, or in a water bath warmed on a hotplate) for 18 to 24 hr at a temperature greater than the chain-melting temperature of the highest-melting component in the system.

   Preventing vibrations and other mechanical disturbances during incubation is essential to a good yield of GUVs.

If using membrane dyes, a successful incubation will be identified by a "cloud" of color in the approximate center of the vial.

9. Harvest vesicles from the center of the vial using a pipet or syringe, taking care to minimize shear forces, and transfer to sample chambers for microscope viewing or other experimentation.

As in Basic Protocol 1, unilamellar vesicles can be identified by low fluorescence from membrane dyes.

ALTERNATE PROTOCOL 3

PREPARING GUVs BY DEHYDRATION AND REHYDRATION OF SUVs

This protocol uses dehydration and rehydration of small unilamellar vesicles (SUVs) to produce GUVs that adhere to a glass coverslip. This allows continuous microscopic observation of a single GUV or group of GUVs added as active agents directly to the system under study. At least 10 to 20 mol% PC lipid seems to be required to form vesicular structures using this protocol.

Materials

100 to 300 μl of lipid solution in chloroform at 20 mg/ml (Support Protocol 1)
100 mM NaCl (prepared using sterile deionized H_2O) containing 1 vol% glycerol
100 mM NaCl (prepared using sterile deionized H_2O), without glycerol

Hamilton syringe
Small glass vial
Desiccator chamber, attached to vacuum pump
Bath sonicator (43 kHz; L&R Ultrasonics Model T21, *http://www.lrultrasonics.com*)
Glass coverslips

Prepare dried lipid film in a glass vial

1. If using more than one species of lipid, mix in the desired ratio as in Support Protocol 1. Add membrane dye(s) and other components to be incorporated into the membrane, if desired, so that the total concentration of membrane components in chloroform solution remains ~20 mg/ml.

2. Deposit 100 to 300 μl of lipid solution in a small glass vial, place vial in vacuum desiccator, and dry under vacuum for 3 hr to overnight.

Hydrate lipid film and sonicate to form SUVs

3. Hydrate lipid film in vial with 100 mM NaCl containing 1 vol% glycerol. Add enough of this aqueous solution to produce a lipid concentration of 1 mg/ml.

If 300 μl of chloroform solution at 20 mg/ml is dried onto the vial, 6 ml of aqueous solution is added.

4. Cap the vial and seal with Parafilm. If using light-sensitive components, cover with aluminum foil. Refrigerate for 24 to 48 hr at 4°C.

The refrigeration step is necessary for vesicle formation.

5. Sonicate lipid suspension in vial for 5 to 10 min to make small unilamellar vesicles (SUVs).

Dry SUVs and rehydrate

6. Deposit 5 μl of SUV suspension onto glass cover slip, spreading as much as possible.

7. Let dry 30 to 60 min, long enough so that the droplet appears like a solid "gel," but not so long that it is visibly dry and cracked.

If using light-sensitive components, sample should be shielded from light while it is drying.

8. Rehydrate using 100 mM NaCl solution (without glycerol), adding at least enough to cover the initially-deposited droplet (10 μl).

Excess rehydrating solution (at least up to 500 μl) may be added if desired. Giant vesicles form within 10 min. As in Basic Protocol 1, unilamellar vesicles can be identified by low fluorescence from membrane dyes.

COMMENTARY

Background Information

Giant unilamellar vesicles (GUVs) are liposomes up to tens of microns in size, consisting of a single lipid bilayer membrane. Due to their large size, they are especially amenable to studies using fluorescence and light microscopy, and may also be manipulated for mechanical measurements with optical traps or micropipets. In addition to their use in encapsulation, GUVs have proven to be useful model systems for studying many cellular processes, including tubulation (Roux et al., 2002; Koster et al., 2003) and budding, and fusion (Holopainen et al., 2000; Staneva et al., 2004), as well as peptide insertion (Thoren et al., 2004). The introduction of enzymes or proteins can result in reorganization, leading to such diverse behavior as vesicle aggregation, fusion, and fission (Nurminen et al., 2001).

More recently, GUVs have gained prominence as a platform for studying lipid phase separation as a model for the lipid raft hypothesis (Korlach et al., 1999; Dietrich et al., 2001; Baumgart et al., 2003). According to the lipid raft hypothesis, in-plane heterogeneity within cell membranes, in the form of lipid rafts, may play a key role in protein sorting and cell signaling (Simons and Ikonen, 1997). GUVs composed of simple mixtures of lipids and cholesterol display a rich phase behavior similar to that of lipid mixtures extracted from cells (Dietrich et al., 2001). This phase separation yields coexisting lipid phases, which differ in composition, density, and local curvature. GUVs provide model systems for the controlled study of the effects of lipid composition and protein binding on lipid phase formation, bending, line tension, budding, and stability.

Critical Parameters

Lipid film thickness and uniformity

It is generally desirable to obtain unilamellar vesicles larger than 10 μm in size. To ensure a high yield of unilamellar vesicles free from small vesicles, it is important to begin with a thin lipid film without much roughness or too many defects. The protocols described here use rapid spreading to obtain this thin film. When viewed in reflection, the film should appear nearly uniform, with slight variations in color. Films that are too thick will appear dull, and nonuniform films will appear patchy.

A more controlled alternative to spreading is spin-coating; however, this requires access to a spin-coater. These are typically available in engineering facilities. The parameters for creating a film of optimal thickness are described by Estes and Mayer (2005); we have also used this technique with success.

Troubleshooting

The most common problem associated with making GUVs is contamination of lipid or sugar solutions. It is recommended that chloroform be divided into smaller bottles for use in solutions and in cleaning. Lipid solutions should be stored under nitrogen or argon gas at −20°C or lower. Small volumes of lipid should be used for mixtures, so that they may be discarded in case of contamination. Be attentive of changes in color or texture of lipid mixtures, as these are signs of contamination. Sugar solutions should be filter-sterilized, aliquotted, and frozen. Sugar solutions in use may be refrigerated, but should be brought to temperature before use in rehydration.

Anticipated Results

The protocols described should yield large vesicles, without many small vesicles or debris. By incorporating a small (0.1 to 1 mol%) amount of fluorescently labeled lipid, it is easy to check with microscopy that vesicles are unilamellar, since multilamellar vesicles appear brighter, as shown in Figure 24.3.3B. Vesicles can also be viewed either with phase contrast or DIC microscopy, as shown in Figure 24.3.3B.

Time Considerations

The time required depends on the protocol chosen. Forming GUVs using electroformation protocols requires a minimum of 2 hr. Gentle hydration protocols require 1 to 3 days. For all protocols, much of the time required is for drying or hydration of the lipid films.

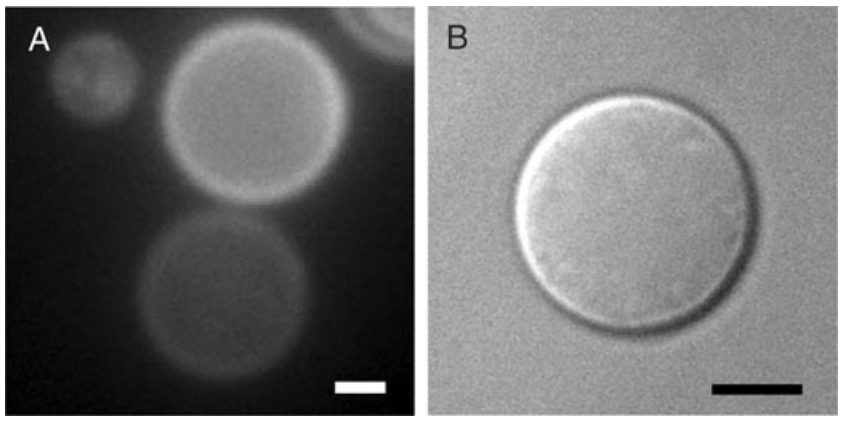


Figure 24.3.3 Giant vesicles, viewed with microscopy. (**A**) By incorporating 0.1 mol% of a fluorescently labeled lipid, vesicles are readily viewed with fluorescence microscopy. Multilamellar vesicles appear brighter, as demonstrated by the upper vesicle. (**B**) Vesicles may also be viewed using phase microscopy. Scale bars = 10 μm.

Literature Cited

Angelova, M.I., Soleau, S., Meleard, P., Faucon, J.F., and Bothorel, P. 1992. Preparation of giant vesicles by external AC electric fields: Kinetics and applications. *Progr. Colloid & Polymer Sci.* 89:127-131.

Bacia, K., Schwille, P., and Kurzchalia, T.V. 2005. Sterol structure determines the separation of phases and the curvature of the liquid-ordered phase in model membranes. *Proc. Natl. Acad. Sci. U.S.A.* 102:3272-3277.

Bagatolli, L.A. 2006. To see or not to see: Lateral organization of biological membranes and fluorescence microscopy. *Biochim. Biophys. Acta* 1758:1541-1556.

Bagatolli, L.A. and Gratton, E. 2000. Two photon fluorescence microscopy of coexisting lipid domains in giant unilamellar vesicles of binary phospholipid mixtures. *Biophys. J.* 78:290-305.

Baumgart, T., Hess, S.T., and Webb, W.W. 2003. Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension. *Nature* 425:821-824.

Beattie, M., Veatch, S.L., Stottrup, B., and Keller, S.L. 2005. Sterol structure determines miscibility vs. melting transitions in lipid vesicles. *Biophys. J.* 89:1760-1768.

Bigay, J., Gounon, P., Robineau, S., and Antonny, B. 2003. Lipid packing sensed by ArfGAP1 couples COPI coat disassembly to membrane bilayer curvature. *Nature* 426:563-566.

Dietrich, C., Bagatolli, L.A., Volovyk, Z.N., Thompson, N.L., Levi, M., Jacobson, K., and Gratton, E. 2001. Lipid rafts reconstituted in model membranes. *Biophysical Journal* 80:1417-1428.

Estes, D.J. and Mayer, M. 2005. Electroformation of giant liposomes from spin-coated films of lipids. *Colloids and Surfaces B: Biointerfaces* 42:115-123.

Gordon, V.D., Beales, P.A., Shearman, G.C., Zhao, Z., Seddon, J.M., Egelhaaf, S.U., and Poon, W.C.K. Solid-like domains in mixed lipid bilayers: Effects of lipid phase behavior, transition pathway, and membrane lamellarity. Manuscript in preparation.

Holopainen, J.M., Angelova, M.I., and Kinnunen, P.K.J. 2000. Vectorial budding of vesicles by asymmetrical enzymatic formation of ceramide in giant liposomes. *Biophys. J.* 78:830-838.

Korlach, J., Schwille, P., Webb, W.W., and Feigenson, G.W. 1999. Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* 96:8461-8466.

Koster, G., VanDujin, M., Hofs, B., and Dogterom, M. 2003. Membrane tube formation from giant vesicles by dynamic association of motor proteins. *Proc. Natl. Acad. Sci. U.S.A.* 100:15583-15588.

Moscho, A., Orwar, O., Chiu, D.T., Biren, M.P., and Zare, R.N. 1996. Rapid preparation of giant unilamellar vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 93:11443-11447.

Mueller, P., Chien, T.F., and Rudy, B. 1983. Formation and properties of cell-size lipid bilayer vesicles. *Biophys. J.* 44:375-381.

Nurminen, T.A., Holopainen, J.M., Zhao, H., and Kinnunen, P.K.J. 2001. Observation of topical catalysis by sphingomyelinase coupled to microspheres. *J. Amer. Chem. Soc.* 124:12129-12134.

Roux, A., Cappello, G., Cartaud, J., Prost, J., Goud, B., and Bassereau, P. 2002. A minimal system allowing tubulation with molecular motors pulling

on giant liposomes. *Proc. Natl. Acad. Sci. U.S.A.* 99:5394-5399.

Simons, K. and Ikonen, E. 1997. Functional rafts in cell membranes. *Nature* 387:569-572.

Staneva, G., Angelova, M.I., and Koumanov, K. 2004. Phospholipase A2 promotes raft budding and fission from giant liposomes. *Chem. Phys. Lipids* 129:53-62.

Thoren, P.E.G., Persson, D., Esbjorner, E.K., Goksor, M., Lincoln, P., and Norden, B. 2004. Membrane binding and translocation of cell-penetrating peptides. *Biochemistry* 43:3471-3489.

Veatch, S.L. and Keller, S.L. 2002. Organization in lipid membranes containing cholesterol. *Phys. Rev. Lett*. 89:268101.

Veatch, S.L. and Keller, S.L. 2003. Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys. J.* 85:3074-3083.

Visualization of Cellular Phosphoinositide Pools with GFP-Fused Protein-Domains

Tamas Balla[1] and Péter Várnai[2]

[1]National Institutes of Health, Bethesda, Maryland
[2]Semmelweis University of Medical School, Budapest, Hungary

ABSTRACT

This unit describes the method of following phosphoinositide dynamics in live cells. Inositol phospholipids have emerged as universal signaling molecules present in virtually every membrane of eukaryotic cells. Phosphoinositides are present in only tiny amounts as compared to structural lipids, but they are metabolically very active as they are produced and degraded by the numerous inositide kinase and phosphatase enzymes. Phosphoinositides control the membrane recruitment and activity of many membrane protein signaling complexes in specific membrane compartments, and they have been implicated in the regulation of a variety of signaling and trafficking pathways. It has been a challenge to develop methods that allow detection of phosphoinositides at the single-cell level. The only available technique in live cell applications is based on the use of the same protein domains selected by evolution to recognize cellular phosphoinositides. Some of these isolated protein modules, when fused to fluorescent proteins, can follow dynamic changes in phosphoinositides. While this technique can provide information on phosphoinositide dynamics in live cells with subcellular localization, and it has rapidly gained popularity, it also has several limitations that must be taken into account when interpreting the data. This unit summarizes the design and practical use of these constructs and also reviews important considerations for interpretation of the data obtained by this technique. *Curr. Protoc. Cell Biol.* 42:24.4.1-24.4.27. © 2009 by John Wiley & Sons, Inc.

Keywords: phosphoinositide • FRET • live-cell imaging • green fluorescent proteins • pleckstrin homology domain • fluorescence microscopy

INTRODUCTION

The first established role of phosphoinositides was recognized in the mid-80s as precursors for two important second messengers, the calcium mobilizing inositol 1,4,5-trisphosphate and the protein kinase C activator diacylglycerol (Berridge and Irvine, 1984). However, starting with the discovery of PI 3-kinases (Whitman et al., 1988), several new inositol lipid isomers have been identified and a much broader role of these lipids as organizers of membrane-associated signaling complexes were uncovered (Di Paolo and De Camilli, 2006). The highly compartmentalized nature of inositol lipid signaling processes demanded new methods that allow detection of the presence of the various inositide isomers with subcellular resolution. The idea of how this can be achieved was born out from discoveries that identified protein modules capable of inositol lipid recognition with reasonable specificity in proteins that are regulated by phosphoinositides (Lemmon et al., 1997).

Visualization of phosphoinositide changes in single living cells was then based on the premise that protein modules that possess high enough affinity and specificity to bind the inositide headgroup of specific phosphoinositides can find the lipids within the cell and visualize it when expressed in the form of a green fluorescent protein (GFP) fusion protein (Balla et al., 2000). As simple as it sounds, the short history of this method has

Current Protocols in Cell Biology 24.4.1-24.4.27, March 2009
Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2404s42

already raised several important technical and theoretical questions that one needs to be aware of when attempting to use these methods. The protocols in this unit provide some practical guidance to users who are not experienced with live cell microscopy. Also included are sections that deal with theoretical considerations and interpretational issues highly relevant to these measurements (see Commentary). The protocols will not detail common laboratory practices but try to concentrate on aspects that are unique to these applications. The technical comments are intended for less experienced users and not intended for experts of this research field.

STRATEGIC PLANNING

Selecting Fusion Proteins for Detection of Selected Inositol Lipids

Table 24.4.1 summarizes protein domains that have been used for imaging purposes. Some of these are better established, while a consensus has yet to be reached for others. These protein domains are widely available and also can be easily duplicated. This section discusses the most important issues related to the individual lipid species for which the authors have accumulated experience.

PtdIns(4,5)P_2

Almost all imaging work for this lipid has used the pleckstrin homology (PH) domain of PLCδ_1 (PLCδ_1PH-GFP) construct developed independently in the Meyer laboratory and in the authors' laboratory (see references in Table 24.4.1). This construct expresses very well and decorates the plasma membrane and some vesicular structures but no other organelles. This has raised the question of whether PtdIns(4,5)P_2 is only present in detectable amounts in the plasma membrane, or is the probe biased against the plasma membrane pool of the lipid. Few reliable works have compared PLCδ_1PH-GFP distribution with staining with PtdIns(4,5)P_2 antibodies, and some have found the lipid in the Golgi with antibody staining but not with PLCδ_1PH-GFP (Matsuda et al., 2001), while others have seen no discrepancy and no Golgi staining with PtdIns(4,5)P_2 antibody either (Hammond et al., 2006). Also, EM studies showed some PtdIns(4,5)P_2 in the Golgi using GST-fused PLCδ_1PH-GFP post fixation (Watt et al., 2002). These data demonstrate one of the possible limitations of this method, namely, that not all pools of the lipids might be equally seen by the domain and this will be even more apparent when imaging other inositol lipid forms (see below).

In spite of this lingering question, the plasma membrane pool of PtdIns(4,5)P_2 can be monitored by PLCδ_1PH-GFP. This probe reports changes in this lipid either following phospholipase C (PLC) activation or after degradation by a phosphoinositide-sensitive 5-phosphatase (Stauffer et al., 1998; Várnai and Balla, 1998; Várnai et al., 2006). However, there is another question that complicates what these changes mean when following PLC-mediated hydrolysis of these lipids. Since PLCδ1PH-GFP recognizes the phosphorylated inositol headgroup within the lipid, the corresponding soluble inositol phosphate [in this case, Ins(1,4,5)P_3] can compete with the membrane PtdIns(4,5)P_2 for binding the PH-domain GFP fusion protein. Because of this competing effect, some research groups treat the changes in PLCδ_1PH-GFP membrane localization as a faithful index of InsP_3 elevations rather than lipid changes within the cell (Nash et al., 2001). A recent detailed review of the authors' opinion on this topic is available (Varnai and Balla, 2006). The bottom line is that inositol phosphates can compete with lipid binding of the PH-domain constructs and their effects on changing localization could be quite significant under certain conditions and cannot be ignored. At the same time, it would be just as misleading to treat the PLCδ_1PH-GFP translocation response as an index of InsP_3 change, and differences between the two have been recently demonstrated by simultaneous measurements of InsP_3 and PLCδ_1PH-GFP

Table 24.4.1 Phosphoinositide Binding Modules in Use for Imaging Purposes in Live Cells

Lipid protein domain	In vitro reference	Live cell localization	Reference
PtdIns(4,5)P_2			
PLCδ_1-PH	Lemmon et al., 1995	Plasma membrane	Stauffer et al., 1998; Várnai and Balla, 1998
Tubby domain	Santagata et al., 2001	Plasma membrane plus cleavage furrow	Santagata et al., 2001; Field et al., 2005
PtdIns(3,4,5)P_3			
GRP1-PH	Klarlund et al., 1997; Rameh et al., 1997a	Plasma membrane	Venkateswarlu et al., 1998a; Klarlund et al., 2000
ARNO-PH	Klarlund et al., 2000	Plasma membrane	Venkateswarlu et al., 1998b
Cytohesin-1-PH	Klarlund et al., 2000	Plasma membrane	Nagel et al., 1998; Venkateswarlu et al., 1999
Btk-PH	Salim et al., 1996; Rameh et al., 1997a	Plasma membrane	Varnai et al., 1999
PtdIns(3,4,5)P_3 /PtdIns(3,4)P_2			
Akt-PH	Franke et al., 1997	Plasma membrane	Watton and Downward, 1999; Servant et al., 2000
PDK1	Komander et al., 2004	Plasma membrane	Komander et al., 2004
CRAC	Huang et al., 2003	*Dictyostelium* plasma membrane	Dormann et al., 2002
PtdIns(3,4)P_2			
TAPP1-PH	Dowler et al., 2000	Plasma membrane	Kimber et al., 2002
PtdIns(3,5)P_2			
Ent3p-ENTH[a]	Friant et al., 2003	Yeast pre-vacuole	Friant et al., 2003
Svp1p[a]	Dove et al., 2004	Yeast vacuole	Dove et al., 2004
PtdIns3P			
FYVE (Hrs, EEA1)	Burd and Emr, 1998; Simonsen et al., 1998	Early endosome	Gillooly et al., 2000
(Vps27)		Yeast vacuole	Burd and Emr, 1998
P40phox-PX	Ellson et al., 2001; Kanai et al., 2001	Early endosome	Ellson et al., 2001
PtdIns4P			
OSH2-2xPH[b]	Yu et al., 2004	Plasma membrane	Roy and Levine, 2004; Yu et al., 2004; Balla et al., 2007
OSBP-PH	Levine and Munro, 1998; Dowler et al., 2000	Golgi plus plasma membrane	Levine and Munro, 1998, 2002; Balla et al., 2005
FAPP1-PH	Dowler et al., 2000	Golgi plus plasma membrane	Munro, 2002; Godi et al., 2004; Levine and Balla, 2005
PtdIns5P			
3xPHD (ING2)	Gozani et al., 2003	Nucleus plus plasma membrane	Gozani et al., 2003; Pendaries et al., 2006

[a]The usefulness of these domains for imaging purposes is questionable (see Michell et al., 2005).

[b]The OSH2-PH shows little discrimination between PtdIns4P and PtdIns(4,5)P_2 based on in vitro binding (Yu et al., 2004) and it is still uncertain whether it actually reports on both of these molecules in some proportions.

translocation (Matsu-ura et al., 2006). Another PtdIns(4,5)P_2 binding module called the Tubby domain was described from the Tubby protein (Santagata et al., 2001; Field et al., 2005; Yaradanakul and Hilgemann, 2007). Two recent studies using a full-length Tubby protein (Nelson et al., 2008) or a mutant form of the Tubby domain have shown that these domains are less sensitive to InsP_3 changes than the PLCδ_1PH-GFP (Nelson et al., 2008; Quinn et al., 2008).

The third complication to remember when using these methods is the inhibitory effect of the expressed domain on cellular responses regulated by the inositol lipids. Binding of the PH (or other) domain-GFP reporters to phosphoinositides should inhibit the lipid-mediated cellular process since it competes with the lipid binding of endogenous effectors. High expression of the PLCδ_1PH-GFP causes morphological changes that include rounding of the cells, loss of attachment, and development of intracellular vesicles (Fig. 24.4.1). These "toxic" effects are due to the inhibition by the construct of the connections between the cytoskeleton and the plasma membrane (Raucher et al., 2000). This problem can be minimized by choosing cells that express low levels of the protein and using a sensitive microscope that can resolve the weak signals that such cells will possess (Fig. 24.4.1A).

PtdIns4P

Several PH domains have been used to detect PtdIns4P in living cells. The two most popular ones are the PH domains of the oxysterol binding protein (OSBP) and four-phosphate-adaptor protein (FAPP1) that were first identified as specific binders to PtdIns4P by fat blots (Dowler et al., 2000). Based on lipid vesicle–binding assays, these domains not only bind PtdIns4P but also PtdIns(4,5)P_2 (Levine and Munro, 1998; Roy and Levine, 2004; Yu et al., 2004). These PH domains, as well as their close relative found in the ceramide transfer (CERT) protein, were indeed localized to the Golgi pool of PtdIns4P both in yeast and in mammalian cells (Levine and Munro, 2002), but not to the plasma membrane, where PtdIns(4,5)P_2 is abundant. These data suggest that within the cells these PH domains do not recognize PtdIns(4,5)P_2 efficiently. Moreover, it was also found that both the OSBP- and FAPP1-PH domains require active (GTP-bound) Arf1 for Golgi localization, and their Golgi targeting requires binding to both PtdIns4P and Arf1-GTP (Levine and Munro, 2002). Neither interaction alone is sufficient for efficient membrane recruitment. This also means that brefeldin A treatment that prevents the formation of Arf1-GTP in the Golgi causes release of these PH domains from these locations. The limited lipid-binding specificity and the need for additional protein interaction for membrane targeting make these probes less than optimal for lipid imaging in live cells. However, because of the lack of better probes, they have been used in many laboratories including the authors (see Table 24.4.1 for references).

Two additional PH domains, namely those of OSH1 and OSH2 (oxysterol binding protein homologs of *S. cerevisiae*) were found to show cellular localization consistent with binding to PtdIns4P in yeast (Roy and Levine, 2004; Yu et al., 2004). Remarkably, the OSH2-PH (used in tandem to increase its apparent affinity) showed both the plasma membrane and the Golgi pool of PtdIns4P in yeast, while the OSH1-PH domain only detected the pool in the Golgi (Roy and Levine, 2004; Yu et al., 2004). This spatial discrimination was the more surprising as these PH domains showed very little discrimination between PtdIns4P and several other inositides including PtdIns(4,5)P_2 in various in vitro lipid-binding assays (Yu et al., 2004). Studies in the authors' laboratory on mammalian cells with these two PH domains yielded somewhat different results: while the OSH1-PH is found as a very good marker for PtdIns4P in the Golgi (as it was in the yeast), the OSH2-2xPH (or the single PH domain) construct only localizes to the plasma membrane but does not show Golgi localization (Fig. 24.4.2C). In spite of its very limited in vitro lipid-binding specificity, the OSH2-2xPH appears to be biased towards PtdIns4P

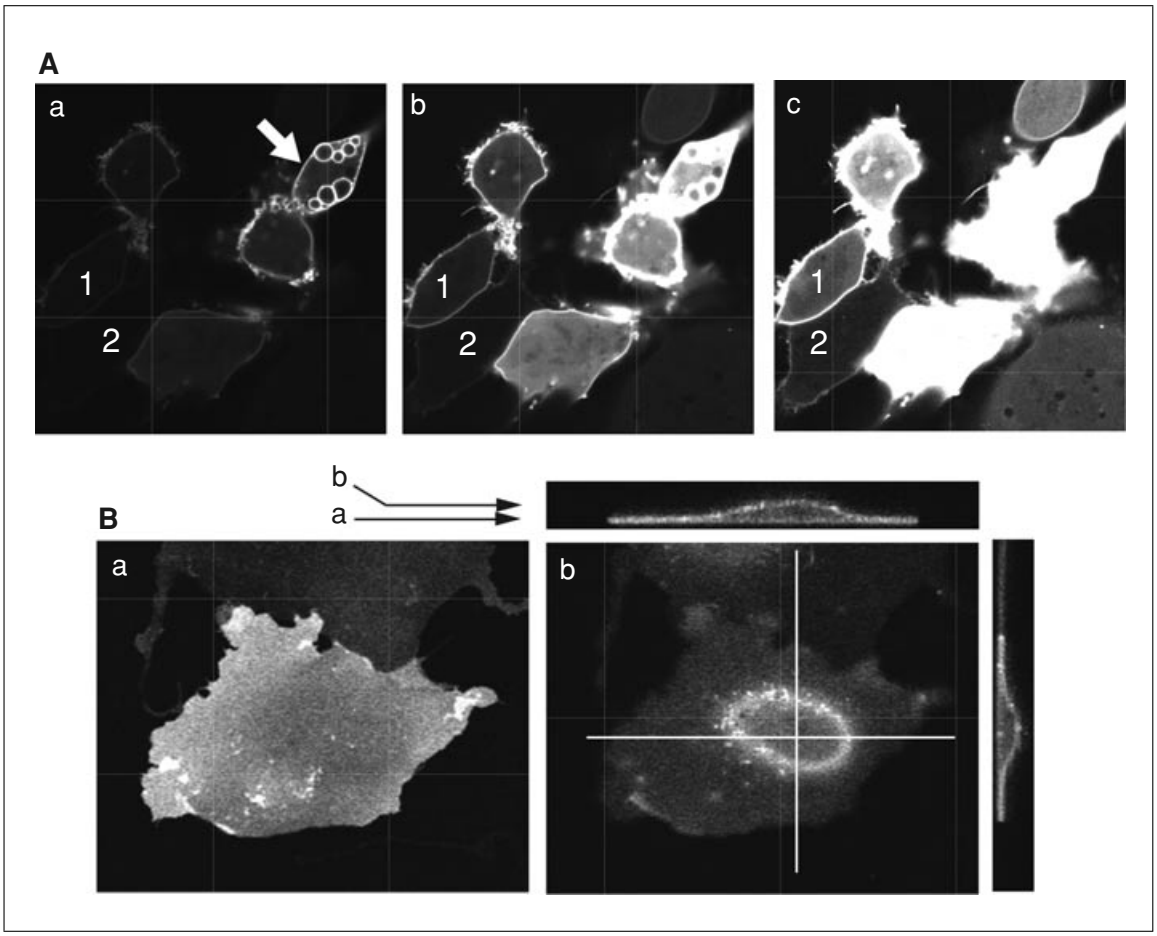


Figure 24.4.1 Cellular localization of the PLCδ_1PH-GFP. (**A**) Clusters of HEK293 cells transfected for 24 hr, and expressing the domain at various levels. Note the vesicular structures in the cell (arrow) that expresses a high level of the fusion protein. This is an example of toxic effects of the protein. Also note that with these illumination settings (panel a), cells 1 and 2 are not even visible, yet these are the cells that should be chosen for analysis as shown by higher illumination, which saturates the signal in other cells (b and c). (**B**) Confocal images of a COS-7 cell transfected with PLCδ_1PH-GFP for 24 hr and analyzed by z-sectioning. Panel a shows an image taken close to the bottom of the cell where it attaches to the coverslip. Note that there is no sharp outline of the cell and the signal covers the whole area of the cell. In panel b, the picture is taken at a z-plane higher up in the cell and again, there is no clear outline of the plasma membrane. Compare it with HEK293 cells that are not as flat and show a clear image of the plasma membrane (**A**). The side views of this COS-7 cell at the cross-sections (top and right side) show a better image of plasma membrane localization and the shape of the cell.

over PtdIns(4,5)P_2 in the plasma membrane, based on the resistance of its membrane localization to phosphoinositide 5-phosphatases that eliminate PtdIns(4,5)P_2 (Balla et al., 2008). The extent of this discrimination as well as the mechanism underlying its interaction with other proteins that would restrict access to PtdIns(4,5)P_2 but not PtdIns4P; however, requires further investigation.

It is clear that only specific pools of PtdIns4P can be monitored with these domains and there is not a single domain identified as yet that would detect all PtdIns4P pools within a cell. A domain that recognizes the PtdIns4P produced by type-II PI 4-kinases on endosomes has not been found. Even within the Golgi, PtdIns4P is produced by different PI 4-kinases (De Matteis et al., 2005), and it is possible that the different PH domains do not detect these pools equally. Moreover, there is an effect of the overexpression of the domains on the Golgi itself. For example, the FAPP1-PH localizes primarily in the trans-side of the Golgi (Godi et al., 2004), but its localization between the cis- and trans- side depends on the expression level (Weixel et al., 2005). In COS-7 cells, an increased level

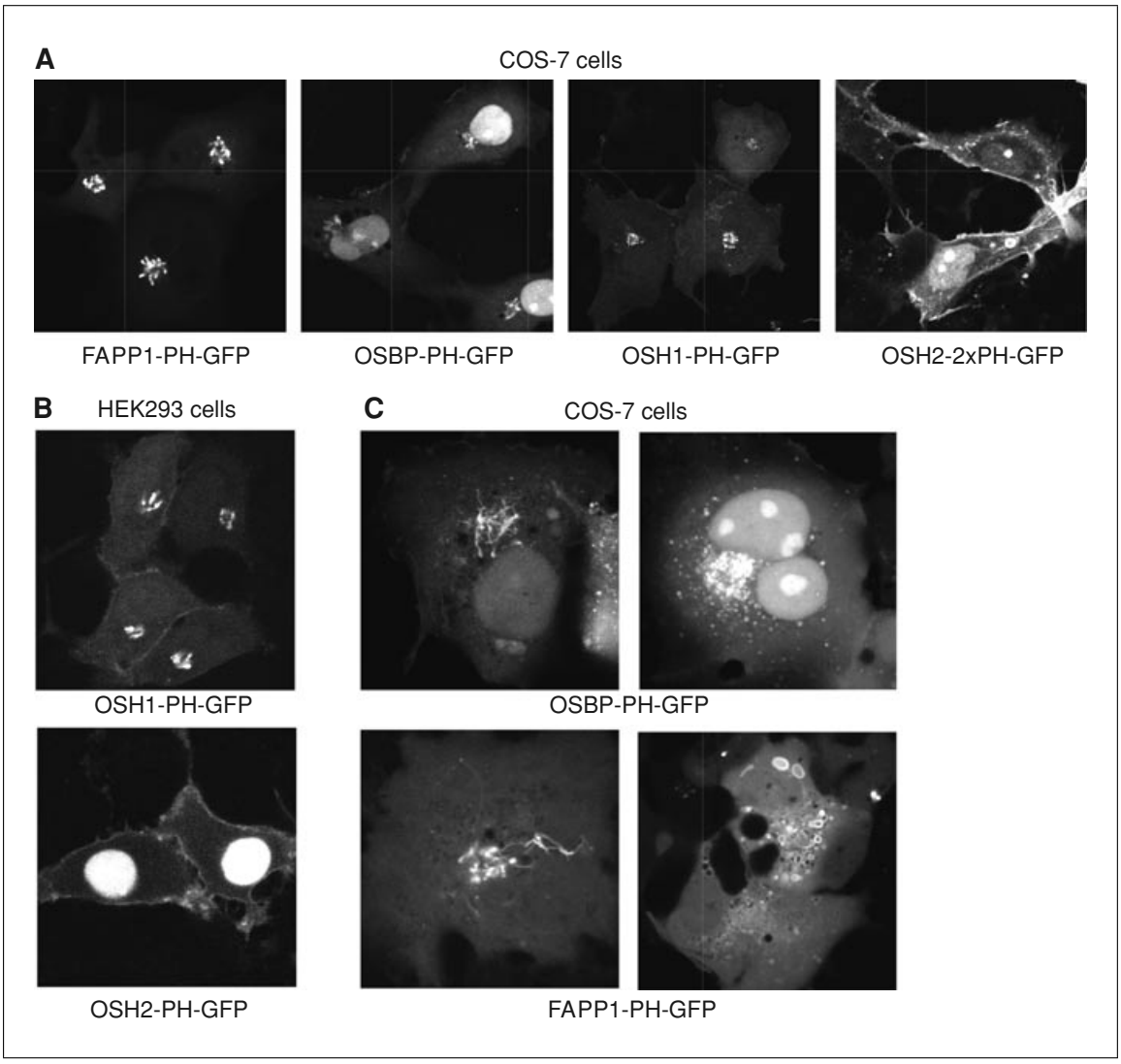


Figure 24.4.2 Localization of the various domains used for imaging PtdIns4P in COS-7 and HEK293 cells. (**A**) COS-7 cells transfected with the indicated domains for 24 hr. Note the sharp contrast and prominent recruitment of the FAPP1-PH domain and the higher nuclear staining of the OSBP-PH domain. The yeast OSH1-PH-GFP also shows the Golgi but is also localized to a small extent to the plasma membrane (better seen in **B**). The OSH2-2xPH-GFP prominently labels the plasma membrane but does not show Golgi localization. For this picture cells were selected that are not so flat, to demonstrate better plasma membrane localization. (**B**) Localization of the OSH1-PH-GFP and OSH2-PH-GFP constructs in HEK293 cells. Note the Golgi and moderate plasma membrane localization of the OSH1-PH and the lack of Golgi localization and high nuclear signal with the OSH2-PH domain. The nuclear localization is less prominent with the OSH2-2xPH-GFP construct. (**C**) Examples for interference of the domains with cellular functions. Both the OSBP- and FAPP1-PH domains cause tubulation of the Golgi. Remarkably, this always occurs at moderate levels of expression and never at high expression levels and only in a fraction of cells. This indicates that this effect is conditional and requires a certain functional state of the Golgi. At high expression levels, the two constructs have very different effects: the OSBP-PH breaks the Golgi into small vesicles that eventually cover the whole cytoplasm. These are completely resistant to brefeldin A, a treatment that rapidly eliminates the Golgi localization of the construct indicating the Arf1-GTP requirement of the localization (not shown). In contrast, the FAPP1-PH domain shows no Golgi localization at high expression levels and instead produces large vacuoles in the cell with FAPP1-PH domain attached to their limiting membranes.

of expression of FAPP1 and OSBP causes distinct morphological changes (Fig. 24.4.2B) suggesting that they interact with distinct proteins (in addition to PtdIns4P and Arf1) and indicating that even though they may appear in the same Golgi compartment at low expression levels, they still detect functionally distinct pools of the lipids. These are all important signs to indicate that not all PtdIns4P are created equal and cannot simply be imaged by a single probe.

PtdIns(3,4,5)P_3

There are a large number of studies imaging PtdIns(3,4,5)P_3 dynamics due to the high interest in phosphatidylinositol (PI) 3-kinase signaling and its role in polarized cell movements such as chemotaxis. In *Dictyostelium*, a widely used model for polarization migration, the PH domain of the CRAC protein (cytosolic regulator of adenyl cyclase, not to be mistaken with calcium release activated channels, as the same acronym is often used in mammalian cells) has been a very good reporter of PtdIns(3,4,5)P_3 distribution (Dormann et al., 2002; Huang et al., 2003). In mammalian cells, the Akt-PH domain has served best for following polarized PtdIns(3,4,5)P_3 production (Servant et al., 2000), even though this PH domain also recognizes PtdIns(3,4)P_2. The Btk-PH domain that is more specific for PtdIns(3,4,5)P_3 and detects lipid increases after PDGF or insulin stimulation in the plasma membrane (Varnai et al., 1999) has not seen similar popularity presumably because it does not show this polarization so effectively. This already suggests that in addition to PtdIns(3,4,5)P_3, protein-protein interactions probably also play a role in the effective recruitment of these PH domains to the plasma membrane. Because of its good in vitro specificity, the Grp1-PH domain (or its close relative, ARNO-PH) is considered to be the most specific probe to detect PtdIns(3,4,5)P_3. However, the authors' experiences have not been positive with this construct. In many cells it shows a relatively poor response, e.g., by showing high nuclear localization [independent of PtdIns(3,4,5)P_3] and membrane recruitment is largely dependent on active (GTP-bound) Arf6 (Cohen et al., 2007). Because membrane recruitment is largely dependent on active Arf6, the Grp-PH is also relatively toxic to the cells and inhibits several Arf6-dependent functions including attachment and spreading of cells (Varnai et al., 2005). A recent study compared several PtdIns(3,4,5)P_3-binding PH domains for their in vitro binding specificity and cellular localization response (Manna et al., 2007). This study found strikingly different membrane recruitment kinetics between the various domains in PDGF-stimulated NIH3T3 cells also suggesting that in addition to inositide lipid binding, membrane penetration and possibly protein-protein interactions have a role in membrane association of the domains. Based on all of the measurements presented, the Btk-PH appeared to be the best probe for the detection of PtdIns(3,4,5)P_3 in intact cells.

PtdIns3P

PtdIns3P was one of the first inositol lipids for which a recognition domain other than a PH domain was found. No PH domain that recognizes PtdIns3P specifically has been reported to date. It was the FYVE domain (the acronym originates from the first four proteins in which the domain was described: Fab1p, YOTB, Vac1p, and EEA1) that was found to be responsible for PtdIns3P recognition and shown to recognize this lipid in its isolated form fused to GFP (Burd and Emr, 1998). Since the strictly defined FYVE domain will recognize PtdIns3P very specifically in vitro, but poorly localizes without some adjacent residues that help its dimerization (and partially may also bind Rab5), the most widely used construct from the Stenmark laboratory is based on a tandem FYVE domain of the Hrs protein (Gillooly et al., 2000). The construct the authors have made from the EEA1 (early endosome-associated antigen) FYVE domain is the slightly longer version (Hunyady et al., 2002). Both of these constructs decorate an early endosomal compartment and will fall off from the vesicles upon inhibition of PI 3-kinases consistent with the view that the Class III PI 3-kinase (the mammalian Vps34p homolog) constitutively generates PtdIns3P on early endosomes.

The other domain that recognizes PtdIns3P is the PX domain of several proteins, but most prominently that of p40phox (Kanai et al., 2001). Expression of this PX domain as a GFP fusion protein also labels the early endosomes. However, in contrast to the FYVE domains, this construct leads to the accumulation of very brightly stained aggregated vesicles in many cells that express higher amounts of the protein. Moreover, these bright vesicles do not lose their signal after addition of PI 3-kinase inhibitors (while the solitary vesicles labeled in cells with low expression do), suggesting that the PX domain is part of a more stable protein complex that cannot disassemble and is probably responsible for the aggregation of vesicles. This again is an indication of protein-protein interactions that clearly differ between the FYVE and PX domains and a good example of why cells with low expression levels should be used in these studies.

PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns5P

The authors have not had much experience with visualizing any of these lipids; therefore, a summary of what is known in the literature is presented. The only PH domains that showed in vitro specificity to binding PtdIns(3,4)P_2 were those of the TAPP1 and TAPP2 proteins (Dowler et al., 2000). The crystal structure of the TAPP1 PH domain clearly revealed the structural features responsible for this specificity (Thomas et al., 2001). The GFP-tagged TAPP1 PH domain has been shown to detect the lipid in live cells in the plasma membrane under conditions where PtdIns(3,4)P_2, but not PtdIns(3,4,5)P_3, was elevated (Kimber et al., 2002). In addition, a GST-fused TAPP1 PH domain labeled the membrane of internal vesicles and the multivesicular body in fixed cells processed for EM analysis (Watt et al., 2004). Moreover, this domain does not show membrane association when only PtdIns(3,4,5)P_3 is elevated, indicating that it can discriminate between these two otherwise closely related lipid products within the cell. Other studies also found this domain useful in detecting the formation of PtdIns(3,4)P_2 in phagocytic cups in macrophages (Horan et al., 2007). The PX domain of p47phox has also been claimed as a PtdIns(3,4)P_2-recognizing module (Zhan et al., 2002) and used to detect the lipid as a GFP fusion protein (Stahelin et al., 2003). However, in the authors' hands the p47phox PX domain-GFP chimera does not show any indication of binding to membranes in a lipid-dependent manner (T. Balla and P. Várnai, unpub. observ.) and its binding to other phospholipids such as phosphatidic acid (Karathanassis et al., 2002) and proteins (Zhan et al., 2002) may limit the usefulness of this PX domain as an imaging tool.

The formation of PtdIns(3,5)P_3 and its role in trafficking to the vacuole in yeast and to the multivesicular body in higher eukaryotes (Gary et al., 1998; Ikonomov et al., 2003; Jefferies et al., 2008) has made this lipid a highly interesting target for imaging studies. Unfortunately, in spite of several attempts and claims, there are no established tools to accomplish this task reliably. Two proteins, the yeast Ent3p (Friant et al., 2003) and Svp1p (Dove et al., 2004) have been found to be targets of PtdIns(3,5)P_2. The inositide binding site was located within the ENTH domain of Ent3p, while it was attributed to a cluster of basic residues on a beta propeller within Svp1p. To the authors' knowledge there has been no systematic analysis on the intracellular distribution of any isolated domains extracted from these molecules with the aim of localizing PtdIns(3,5)P_2 in live cells.

PtdIns5P is a lipid that was first identified as a substrate of the type II PIP kinases (PI5P 4-kinase; Rameh et al., 1997b). The main route(s) of its production in cells is highly debated, but most likely is the result of dephosphorylation of polyphosphoinositides (Coronas et al., 2007; Zou et al., 2007) rather than direct phosphorylation of PtdIns. The only domain that recognizes PtdIns5P has been identified in the nuclear protein ING2 within a domain (PHD as for plant homeodomain) that binds PtdIns5P, and to a lesser degree PtdIns3P, in vitro (Gozani et al., 2003). A GFP fusion protein made of the 3xPHD domain of ING2 detected PtdIns5P in the plasma membrane in response to overexpressed bacterial 4-phosphatase, IpgD (Gozani et al., 2003; Pendaries et al., 2006)

and did not show endosomal localization suggesting that it does not bind PtdIsn3P in the cells. Nevertheless, more experience is needed with this domain to determine where PtdIns5P is found in the cells and whether its role in the nucleus is associated with detectable changes in its nuclear level.

The common message from these examples is that none of these tools is useful as general lipid reporters without specific constraints that vary from probe to probe. However, if treated with caution and after performing proper controls, these reporters are very useful in answering questions that no other technique can provide.

Selecting the Fluorescent Protein to be Fused

Most of these fluorescent reporters have been originally created as fusion proteins with the enhanced green fluorescent protein (EGFP). However, over the past few years, a large number of fluorescent proteins were introduced now offering a wide variety of colors and other unique features (*UNIT 21.5*). In addition to the spectral variants that allow multicolor imaging of several probes in parallel, new features include photoactivation (*UNIT 21.6*), photoswitching, spectral change during maturation, pH stability, resistance to photobleaching or dimerization, etc. These have been summarized in several recent reviews and will not be detailed in this unit (Shaner et al., 2005; Giepmans et al., 2006; Wiedenmann and Nienhaus, 2006). These proteins are often very useful for a particular application but there is considerable confusion as to the source of these proteins. In addition to the GFP variants based on the pioneering work of the Tsien laboratory, many new proteins originate from the Miyawaki laboratory, and the company Evrogen. These proteins have been cloned from a variety of species and are not derivatives of the jellyfish *Aequorea victoria* GFP. A good summary of these proteins and their features are found in Muller-Taubenberger and Anderson (2007) and *UNIT 21.5*. However, these proteins do not only differ in their optical behavior and they cannot be replaced with one another without any problems. In the authors' experience, occasionally a probe behaves quite differently depending on whether an EGFP, an mRFP, or EosFP molecule is attached to it. Therefore, it is better to start with a few colors that are proven to work similarly with a particular lipid-binding domain than to generate a whole series of colors assuming that they will behave identically within the cells. As most studies were done with the pEGFP plasmids and their initial color versions, it is recommended to use these for initial experiments. Unfortunately, the original pEGFP-N and -C series plasmids are no longer available because Clontech has become part of Takara and these companies now offer their own fluorescent proteins that originate from a different species. The original EGFP and its color variants are now sold by Invitrogen in a different plasmid backbone. This often causes confusion among novices beginning their fluorescent protein collections that do not realize that a green fluorescent molecule now can mean different entities depending on the manufacturer.

EXPRESSION OF FLUORESCENT PROTEINS IN MAMMALIAN CELLS

BASIC PROTOCOL 1

The following procedure describes the general methods for analyzing cells expressing EGFP or GFP-fusion proteins by microscopic techniques.

Materials

98% ethanol
0.1% (w/v) poly-L-lysine in water (Sigma cat. no. P-8920)
Cultured cells of interest
Culture medium with serum and antibiotics (depending on the cells)
Plasmid DNA (usually a midiprep)
Transfection reagents (depending on the cells in use; *UNITS 20.3, 20.4, 20.5, 20.6, & 20.7*)
Phosphate buffered saline (PBS)

2% (w/v) paraformaldehyde (see recipe)
Blocking solution (10% FBS in PBS made fresh)
Primary antibody against fluorescent protein (available from several commercial sources)
0.2% saponin
Fluorophore-conjugated secondary antibody
Aqua-Poly/Mount (Polysciences)
Clear nail polish
25-mm coverslips (PGC Scientific cat. no. 60-4884-25 or Warner Instruments cat. no. 64-0705) for TIRF applications
6-well culture dishes
Glass microscope slides
Fluorescent microscope

Prepare poly-L-lysine-coated coverslips

1. Rinse 25-mm coverslips with 98% ethanol in a cell culture hood and air dry.
2. Place one coverslip in each well of a 6-well culture dish.
3. Add 2 ml of 1:50 or 1:100 dilution of poly-L-lysine in filter-sterilized deionized water to cover the entire surface of each coverslip and allow to stand 1 hr at room temperature.

   In a pilot study, plate cells on coverslips coated at each poly-L-lysine concentration to determine which is best for the cells being studied.

   Because most plastic-ware used for cell culture work has high autofluorescence, it is recommended that cells be cultured and transfected on poly-L-lysine-coated glass coverslips so that they can be viewed under the microscope after transfection without replating. For certain cell types and transfections, it is more advantageous to transfect the cells in culture dishes and re-plate the transfected cells on the poly-L-lysine-coated coverslips before microscopy. Some cells can be grown on coverslips without the coating (e.g., cost-7, CHO)

4. Aspirate the solution from the coverslip and air dry (~ 1hr) before plating cells.

Transfect cells with plasmid DNA

5. Plate ~50,000 cells directly onto the poly-L-lysine-coated coverslips in 2 ml of the appropriate culture medium and grow to the density best suited for transfection (usually 2 days).
6. Transfect the cells with the desired plasmid DNA construct using a method that is most appropriate for the cells.

   The authors usually use 2 μl Lipofectamine 2000 and 0.5 μg plasmid DNA per coverslip.

   Transfection protocols are available for different reagents (UNITS 20.3–20.6), and for each cell type, the reagent and the procedure that produce the best results can differ considerably (UNIT 20.7). Therefore, refer to the manufacturer's instructions as far as specifics for cell transfection are concerned. The optimal level of expression must be determined for each cell type and expression construct. It is important to remember that the expressed proteins often interfere with the functions of the lipids that they recognize, and in high concentrations, the lipid-binding fusion proteins are often toxic to the cells.

   Initial transfection of cells with a mutant version of the lipid-binding domain that does not bind lipids is recommended. For example, many constructs localize to the nucleus, but this localization is not dependent on lipid binding; thus, lipid-mediated localization can be confirmed by comparing the distribution of the native lipid-binding domain to that of the non-lipid binding mutant. Transfection of cells with the GFP protein alone without the lipid-binding domain is also recommended. These two controls help to track phenotypic changes and potential cellular toxicity associated with overexpression of the lipid-binding domain, as well as to serve as controls for monitoring localization that accurately reflects lipid binding.

7. Grow cells 24 hr to allow expression of the transfected protein.
8. Incubate cells in serum-free culture medium for 6 to 10 hr to render them quiescent before microscopy.

   Growing cells for >34 hr after transfection is not recommended because of the potential toxicity of the lipid-binding fusion protein and the ability of the expressed protein to interfere with the functions of the lipids that they bind. Not every experiment requires serum deprivation of cells.

Immunostain cells to detect expressed GFP fusion protein

9. Rinse the transfected cells with 2 ml of PBS.
10. Add 2 ml of 2% paraformaldehyde and incubate 10 min at room temperature.
11. Wash cells three times with 2 ml of PBS, 10 min each wash.
12. Cover cells with 2 ml of blocking solution to block nonspecific antibody binding. Incubate 10 min at room temperature.
13. Add primary antibody diluted appropriately in blocking solution supplemented with 0.2% saponin. Incubate 1 hr at room temperature.

    A volume of 100 μl of diluted antibody should be sufficient for a 25-mm circular or 22 × 2–mm square coverslip, if it is inverted on a glass slide and incubated in a humidified Petri dish.

    This step can be skipped when performing live cell microscopy (see below). However, it may be necessary to confirm expression of the fusion protein, especially if the GFP signal is not bright enough. This confirmation can be accomplished by immunocytochemistry with antibodies to the GFP portion of the fusion protein. Co-localization of the GFP-fused domain with other molecular markers may also require immunostaining.

14. Wash cells three times with 2 ml of blocking solution, 5 min each wash.
15. Add the fluorphore-conjugated secondary antibody diluted in blocking solution containing 0.2% saponin. Incubate 1 hr at room temperature protected from light.
16. Wash cells three times with 2 ml of PBS, 1 min each wash.
17. Air dry until coverslips are only damp.
18. Mount coverslips with cells down on a glass slide using Aqua-Poly/Mount.
19. Seal coverslips on the side with clear nail polish to prevent drying.
20. View the slides to detect expression of the GFP/fluorescent protein.

VERIFYING STRUCTURAL INTEGRITY OF THE FUSION CONSTRUCT

BASIC PROTOCOL 2

This protocol describes how to determine whether the fluorescent protein construct remains intact when expressed in the cells. This is an important control in addition to sequencing the DNA construct before performing any microscopy work. Often the fusion protein is cleaved within the cell so that the green fluorescence is not coming from the molecule that was designed. In the authors' experience, free EGFP is more often present when using pEGFP-N1 plasmids than with the pEGFP-C1-variant. (This can happen if an internal ribosomal entry site allows the translation of GFP itself, which can be prevented by removing the start codon of the original GFP.) In one case, a fusion protein expressed in COS-7 cells was proteolytically cleaved when placed COOH-terminal to EGFP. Analyzing cell lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) either using a phosphorimager equipped with a blue laser line (described below) or by conventional immunoblotting (*UNIT 6.2*) techniques using

antibodies against the GFP portion of the fusion protein is recommended. In many cases, samples that are boiled and analyzed by immunoblotting show more degraded product than those analyzed directly from the gel. The electrophoretic mobility of the EGFP molecule is clearly different when expressed from pEGFP-C1 versus pEGFP-N1. This may be due to altered migration of the non-denatured protein by extra amino acids encoded within the multiple cloning site of the pEGFP-C1 plasmid.

Materials

Cultured cells of interest
Culture medium with serum and antibiotics (depending on the cells)
Plasmid DNA (usually a midiprep)
Transfection reagents (depending on cells used)
Phosphate buffered saline (PBS)
Laemmli buffer
10-cm SDS-PAGE acrylamide gel
Gel running buffer

12-well culture plates
1.5-ml microcentrifuge tubes
Sonicator
SDS gel apparatus
Phosphorimager (or reagents and apparatus for immunoblotting)

1. Seed COS-7 (or other cells to be studied) at 2×10^5 cells/2 ml medium onto 12-well culture plates and incubate 24 hr.
2. Transfect cells with the method of choice and incubate 1 day (or equal to the time the experiments would be done).
3. Wash cells with 2 ml PBS and aspirate the PBS.
4. Dissolve cells in 100 µl Laemmli buffer and transfer to 1.5-ml microcentrifuge tubes. Do not boil, but briefly sonicate to disrupt DNA.
5. Load 40 µl of the sample per lane of a small (10-cm) SDS-PAGE gel with an acrylamide concentration that will resolve proteins in the size range of interest.
6. Run the gel at 100 V until the front reaches the bottom of the gel.
7. Remove gel from cassette and place in running buffer.
8. Place the wet gel directly onto a phosphorimager and scan using the appropriate laser line (blue for GFP and red for mRFP or similar colors)

Alternatively, if no phosphorimager is available, the fluorescent proteins can be detected with immunoblotting using an anti-GFP (or other appropriate) antibody. In this case, boil the samples before SDS gel analysis. It is also important to remember that the anti-GFP antibody does not recognize proteins that are derived from other species than Aequorea victoria.

BASIC PROTOCOL 3

OBSERVE GFP SIGNAL BY MICROSCOPY

Provided here are practical suggestions on what to pay attention to when observing cells by microscopy. This is not intended for experienced users but for researchers who have limited practical knowledge of microscopes and want to observe their proteins as GFP-fusion constructs in live cells. This protocol is not a substitute for training on any specific microscope system but rather an aid to help commonsense practices.

Wide field or confocal microscopy?

The first question to be answered is whether to view the cells with a conventional fluorescence microscope or with a confocal microscope. The authors suggest that the distribution of GFP fluorescence and other initial experiments such as assessing transfection efficiency should be performed with a conventional fluorescence microscope using filters suitable for fluorescein isothiocyanate (FITC) detection (excitation at 470 to 490 nm, emission at 500 to 550 nm). It is not necessary to examine the cells immediately with a confocal microscope, and it is more difficult to obtain a general impression of what the cells look like in a confocal microscope when expressing the construct. Individual cells, especially COS cells, show enormous variability in their shape, size, and general appearance, and often the level of expression changes their appearance. Conventional fluorescence microscopy is a significantly more efficient way to browse through many cells and notice trends in cellular morphology. In addition, many cells are flat in culture (especially COS cells), so there is little benefit from analyzing the cells in a confocal microscope. Confocal microscopy can be saved for recording cells and changes in fluorescence distribution once the conditions have been optimized with a fluorescence microscope. A further advantage of viewing cells in a fluorescence microscope is that autofluorescence often can be distinguished from the GFP signal because its color is different from the green color of GFP. It is important to remember that confocal microscopes detect light intensity without colors and the "color" given is artificial. Therefore, in each case, the autofluorescence has to be determined so that the GFP signal can be reliably used. For this, observation of untransfected cells is a useful control.

Fixed or live cells?

The next question is whether to analyze live or fixed cells. EGFP fluorescence can persist in fixed cells under proper fixation conditions (see Basic Protocol 1, steps 9 to 19). Fixed cells can be processed for immunostaining, which is often necessary to determine co-localization with organelle markers for which antibodies are available. Also, fixed cells can be stored and studied whenever convenient. On the other hand, changes cannot be followed as they happen in real time when using fixed cells. Moreover, fixation and permeabilization procedures may distort cellular morphology. For example, vesicular structures shrink during fixation, and long canaliculi can turn into small vesicles. The use of live cells is the most reliable way of assessing undistorted morphology, but it is also the most time-consuming and least efficient. For live-cell imaging, it is best to use an inverted microscope. New water-immersion objectives make it possible to look at cells under upright microscopes, but the objective has to be in culture medium. In addition, live-cell imaging would generally require some form of temperature control with all the complications associated with it while fixed cells do not.

When analyzing live cells, one major advantage is to record time-lapsed images sequentially after a stimulus is applied to the cells. In confocal microscopes, the speed of scanning determines how fast one can record an image, and generally the faster the scan, the poorer the quality of the individual pictures. Finally, one of the greatest difficulties is to keep the cell in focus after stimulation, because of shape changes that often occur in response to the stimulus (HEK 293 cells are especially lively). This change in position of the originally imaged plane can make the entire recorded process unsuitable. Software programs are being developed to compensate for "focal-drift" due to cell movements. These new developments may already be available for some confocal imaging systems.

BASIC PROTOCOL 4

LIVE-CELL IMAGING

This protocol describes steps to help guide a less-experienced user during the live-cell imaging process.

Materials

Coverslips holding transfected cells
Medium appropriate for cells (e.g., modified Krebs-Ringer solution; see recipe)
Immersion oil

Chambers to hold coverslips (e.g., metal Atto chambers, Invitrogen)
Kimwipes
Wide-field fluorescence microscope equipped with sensitive camera and appropriate software for data acquisition or confocal microscope
Lens cleaning paper
Objective heater and heated stage (Bioptechs, *http://www.bioptechs.com*) or a complete temperature control enclosure
Computer controlled valve-system and perifusion (optional)
Forceps

Mount transfected cells

1. Place coverslip with transfected cells face-up into metal chambers. Tighten the upper part with the O-ring in place gently but firmly so no leaks occur. Wash cells with medium and add 1 or 2 ml medium to the cells. Clean the bottom of coverslip with a clean Kimwipe.

   Ensure that the coverslip is in the grooved area of the chamber otherwise it will break during tightening. Test for leakage using a clean paper where the metal meets the glass coverslip. Leakage can worsen as the temperature changes on the stage. In any case, the use of a lens protector against leakage is advised when using live-cell imaging.

2. Dust off the objective and clean with a cleaning solution provided by the manufacturer (or isopropanol) using a lens cleaning paper.

   Cleaning the lens is important but it can do more damage than good if not done properly. Consult an expert before performing this task. Do not use Kimwipes or any other paper or cotton swab for this purpose. Never clean the lens with dry lens paper and do not use solutions containing ammonia (e.g., Windex) or organic solvents other than recommended.

3. Add one small drop of immersion oil to the lens without allowing it to flow down along the surface.

4. Place the metal chamber on the stage and slowly elevate the objective with eye control until it touches the coverslip.

Select cells

5. Observe the transfected cells and, if necessary, the control non-transfected cells using the fluorescence setting of the confocal microscope fitted with filters suitable for the chosen fluorophore (470 to 490 nm excitation and 500 to 550 nm emission for EGFP).

   Looking into the fluorescence microscope, one can usually see cells with a wide range of fluorescence intensities. Depending on the quality of the microscope and the intensity of the light source, sometimes only the cells with the highest expression levels are visible and these are the cells to avoid. It is recommended to study cells in which the GFP signal is as low as possible but clearly distinguishable from the autofluorescence of non-transfected control cells. Since microscopes are very different, there are no arbitrary rules.

   Often a separate non-transfected cell control is not necessary, because even in the transfected samples, not all cells express the fusion proteins. The non-transfected cells in the

population can usually be distinguished by their autofluorescence, allowing easy identification of transfected and non-transfected cells from the same sample when viewed using the fluorescent setting.

6. Choose cells in which expression levels are just high enough to be resolved above the background autofluorescence, and that are not obviously unhealthy.

   A good quality image should be obtained in confocal microscopy using 3% to 5% of the maximum laser power (assuming a 30-mW, 488-nm laser; see Fig. 24.4.1A).

   Analysis of cells that are unhealthy and suffer from toxicity induced by the expression of the fusion protein should be avoided. For example, cells that have rounded up and are about to detach—a common phenomenon with cells expressing high levels of a fusion protein consisting of the PH domain of PLC and GFP (PLCδ_1PH-GFP) are not likely to behave normally. Another indication of toxicity is the appearance of large intracellular vesicles.

Perform time-lapse analysis of live cells

7. Record and store an image before applying any stimulus.

   An important and often unappreciated problem in live-cell imaging is the proper temperature of the cells that are being observed. Even with a heated stage, an objective acts as a heat sink, keeping the cells in the observation field at a temperature near that of the objective. This temperature is closer to room temperature especially when rooms are kept on the cold side because of the lasers. Many trafficking processes in the cells slow down or do not work properly below 34°C. Therefore, use an objective heater. Unfortunately, the heater collar does not fit all objectives, and heating may be damaging to the objective if it is warmed very fast from a cold temperature. Alternative methods of maintaining the proper temperature include perfusion of cells with a high flow of warm medium or the use of a hair dryer to keep the objective at the proper temperature. Complete incubator enclosures are also available from various companies that can keep both the temperature and CO_2 concentration and humidity of cells on the stage at the desired levels. Unfortunately, they make manipulations of the cells often difficult.

8. Set the software to record time-lapsed images.

   The speed of recording must be decided based on the expected speed of the response and the scan speed that is required to generate an image. Phospholipase C activation by receptors is a fast event, but it can be followed by obtaining an image every 5 sec. Depending on the software, the scanning time (1.0 to 2.5 sec/image for one color) is taken into account when setting up the speed of image acquisition. It is important to remember that frequent scanning leads to fast photobleaching but including a break between scans helps to counter the bleaching problem.

9. Start data acquisition and add stimuli or inhibitors after a control period (five to ten images depending on the speed).

   Addition of a treatment is more complicated than it seems. The best method is to use a computer-controlled valve-system linked to constant perfusion of the cells with prewarmed medium. This ensures proper mixing, timing, and keeping the composition of the medium not affected by evaporation. However, the authors' experience is that many of the lipophylic compounds (ionomycin, thapsigargin, rapamycin, etc.) stick to the plastic tubing and to the valve components to such an extent that it is almost impossible to clean them even with organic solvents. This is also true for the metal chambers including the plastic O-ring seal. This may cause significant problems, especially in multi-user settings. Because of this potential problem, simply use a pipet to stimulate the cells. For this, remove 200 μl warm medium from the cells and add it to a microcentrifuge tube that contains the desired amount of drug in 1 to 5 μl, and after mixing, add the medium back to the cells. With practice, one can pipet the medium back at an angle that will yield proper mixing, which may be further mixed by pipetting up and down one time. This takes ~2 sec, which is acceptable for many applications but may be too slow for others. It is the investigator's choice whether to follow the manual pipetting procedure or to use the more sophisticated valve-controllers.

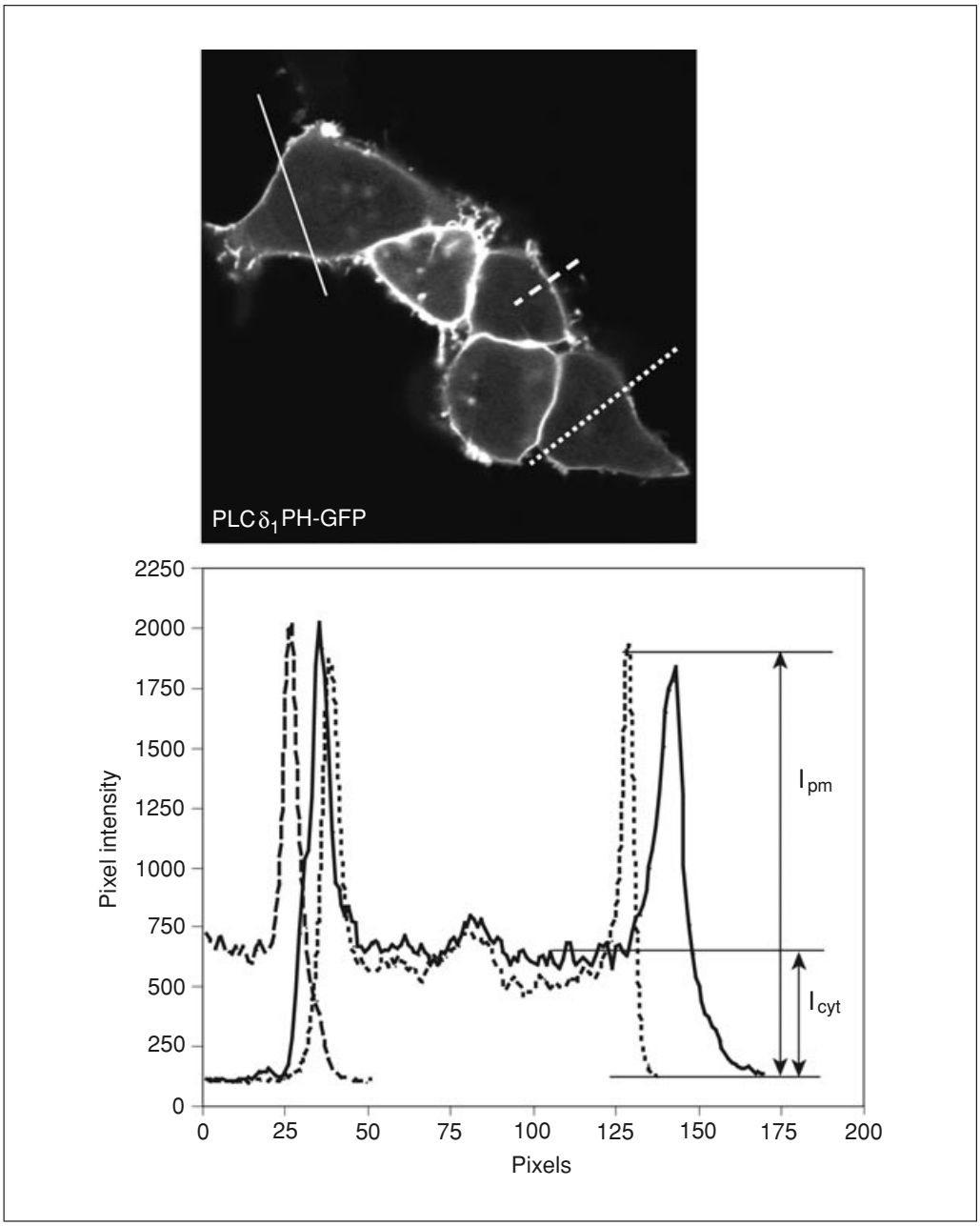


Figure 24.4.3 Quantification of the plasma membrane localization of an inositide binding domain. HEK293 cells are shown expressing the PLCδ_1PH-GFP. The pixel intensity histograms are calculated for the three lines placed on this recording. Note that the scale shows that this is a 12-bit image. An 8-bit image would only have 256 levels of intensities. Also note that the peak intensities are not at saturation. The lower panel shows how the intensity values from the membrane and the cytosol are calculated. Their ratio is then a good measurement of localization. Also note that no lines are placed over areas where two cells are joined. These calculations must be made for each picture from a sequence to obtain a full time-course of change. It is recommended that more than one line be placed on a cell to obtain a more accurate value as the intensities vary along the perimeter.

10. Once the scanning sequence is complete, save the results.

11. Quantify the data using appropriate software.

The most demanding part of the analysis of time-lapsed sequences is the quantification of data. Obvious changes can be documented in a series of pictures or movies that describe what is happening. However, as imaging tools are improving, some of the changes are not so obvious to the eye, especially when the number of vesicles, their movement patterns, or intensities all show some changes. Quantification is necessary when determining a dose-response relationship, comparing the relative effectiveness of two stimuli, or investigating the efficacy or potency of an inhibitor. It is difficult to provide recommendations that would cover all of these areas. In most of the applications dealing with plasma membrane phosphoinositides, it is necessary to assess the extent of plasma membrane recruitment; therefore, methods that determine plasma membrane association are focused upon. The simplest way is to monitor the cytosolic intensity since it will significantly increase as a domain translocates from the membrane to the cytosol. It is more demanding to calculate the ratio of membrane to cytosolic intensities. This can be done after creating a line-intensity histogram through a selected line across the cell. It is important that the highest intensities should not be in saturation, which requires a fine optimization of the dynamic range before recording in 8-bit systems (256 levels of gray intensity; Fig. 24.4.3). This is less of a problem with the newer 12-bit systems (4096 levels of intensity) or with a higher dynamic range. A more accurate but also more demanding way of quantification of the extent of membrane association of fluorescent proteins is to use fluorescence resonance energy transfer (FRET; UNITS 17.1 & 17.9) or total internal reflection fluorescence (TIRF; UNIT 4.12) microscopy as detailed in Varnai and Balla (2006) and Balla (2007). The latter is able to detect the radiationless energy transfer between two appropriate fluorophore pairs when they are within an optimal distance, while the former only detects fluorescence originating from the plane of the membrane attached to the coverslips. The variability of the cell population and the requirement for analysis of a large number of cells to obtain quantitative estimates of the fluorescence changes remains the most laborious parts of obtaining reliable, reproducible results.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Krebs-Ringers solution

120 mM NaCl
4.7 mM KCl
1.2 mM Na_2HPO_4
1.2 mM $CaCl_2$
0.7 mM $MgSO_4$
10 mM glucose
20 mM Na-HEPES, pH 7.4
0.1% bovine serum albumin (BSA; not added when lipofilic druqs are to be added)
Store up to 4 to 5 weeks at 4°C

Paraformaldehyde

Prepare paraformaldehyde fresh by dissolving the appropriate amount of EM-grade paraformaldehyde in PBS and heating (in a chemical hood) until the aldehyde goes into solution. Keep the bottle cap loosened so that pressure does not build up. Cool down to 20°C and pH to 7.4 with NaOH.

COMMENTARY

Background Information

The classical methods to study phosphoinositides relied upon metabolic labeling of phosphoinositides. Labeling cells with myo-[^{3}H]inositol or ^{32}P-phosphate followed by lipid extraction and separation by TLC (or other methods) has been widely used to measure phosphoinositide changes (e.g., Christy

et al., 1998). However, these techniques require millions of cells to obtain a sufficient signal. Moreover, depending on the labeling time and the turnover rate of the metabolically distinct inositide pools, it is not certain whether isotopic equilibrium is reached. Determination of the subcellular location of inositides requires even more cells and cell fractionation procedures, and by the end it is still questionable whether the distribution really reflects what was present in the intact cell. Total cellular mass of inositides have also been measured based on quantitation of the inositide headgroup that is liberated from the extracted lipid species (Pearson et al., 2000). Detection of phosphoinositides or inositol phosphate mass separated on HPLC has been done with metal dye-detection (Pittet et al., 1989) or suppressed conductivity detection (Nasuhoglu et al., 2002). Excellent collections of the conventional methods have been published (Irvine, 1990; Shears, 1997). In the meantime, newer methods have been introduced for mass measurement of inositides without extensive purification with the aid of protein domains that specifically recognize the inositide headgroup (Luo et al., 2003; Guillou et al., 2007). However, even in their most simple forms these methods are cumbersome and are unable to resolve the small changes that occur in subcellular compartments, especially against the higher background of non-responsive inositide pools. The realization that inositides are rapidly changing in restricted cellular compartments brought about the desire to detect them at the single-cell level preferably in live cells where the dynamics can be followed in real time.

Since the method employing protein domain fluorescent protein (FP) chimeras in live cells has certain limitations, there is a legitimate need for alternative methods in which the lipids are detected post-fixation without interfering with the biological process. Moreover, none of the live-cell imaging techniques can compete with the resolving power of electron microscopy. Post-fixation detection of the lipids has been achieved with anti-phosphoinositide antibodies or recombinant GST-fused inositol lipid–binding domains in immuno-cytochemical or -electron microscopy applications (Watt et al., 2002, 2004). The value of these techniques is clear; theoretically they would tell us where the lipids are in an intact cell without any distortion caused by the detection process and many cells can be analyzed with no time constraints. EM studies revealed the presence of lipids in compartments where in vivo PH domain imaging failed to do so, e.g., PtdIns(4,5)P2 in the Golgi (Watt et al., 2002). However, these techniques also have their drawbacks. First, the fixation process has a major influence on what inositide pools are visible to the antibodies or the GST-fused inositide-binding module. Second, the sensitivity of these methods is hard to evaluate. The fixation process is even more critical than in proteins in preserving the lipids and yet to make them accessible to the antibodies or protein modules. This technical difficulty explains the variability of the results obtained in different laboratories with the antibodies and their failure to work in some cases. The specificity of the antibodies should be a rigorous criterion and the data should be consistent with already existing knowledge on the distribution of phosphoinositides.

Assessing membrane localization by FRET

A quantitative assessment of the membrane localization of fluorescent probes is not always easy based on confocal images. An increase in the fluorescent intensity of the membrane can reflect a change in membrane volume or shape and not a real recruitment. Similarly, the cytosolic intensity of the probe can increase due to shrinkage instead of its release from the membranes. To overcome these problems, the FRET principle was used in several studies to obtain a signal that reflects true binding of the inositide binding domain to membranes. The method developed in the Jalink laboratory and also used in the authors' studies is based on co-expression of the CFP- and YFP-tagged versions of the same PH domain (e.g., PLCδ_1-PH). These fluorophores are the most widely used pairs for FRET studies although the more pH-resistant Venus replaces YFP and cerulean replaces CFP in newer applications. Newer fluorophore pairs are also available now for FRET studies but they are not as well established as the GFP derivatives. Nevertheless, the principle is the same; when the donor (CFP) and the acceptor (YFP) are within FRET distance (<8 nm) there will be energy transfer from CFP to YFP causing a decrease in CFP emission (475 nm) and an increase in YFP emission (525 nm) when using CFP excitation only (430 nm). In the so-called sensitized emission method, the efficiency of the energy transfer is numerically calculated after making all necessary corrections (such as bleed through of the CFP and YFP signals into the other pair's emission channels). However, for all practical purposes, the simple

fluorescence ratio of 525/475 can be used if the two wavelengths show opposite changes. This principle is applicable to the lipid binding PH domains when expressed both as a CFP or YFP fusion protein. The two fluorophores will show efficient energy transfer when their attached PH domains are bound to the lipids at the membrane. However, upon PLC activation, the PtdIns(4,5)P_2 molecules are hydrolyzed and the PH domains leave the membrane decreasing the FRET signal (van Der Wal et al., 2001). This method is quite sensitive and even small PLC activation can be detected and quantified. The method does not require a confocal microscope and can be used either in individual cells or in cell suspensions (Balla et al., 2005). A disadvantage of the method is that the FRET efficiency is not very high at low expression levels (which would be desirable to minimize the ill effects of the presence of the probes) and even at high probe concentrations there could be low FRET signal if the density of the lipids is below a certain level. Although the authors have not used this application, FRET can also be assessed by fluorescence life-time imaging microscopy (FLIM; *UNIT 4.14*) that calculates the half life of the excited state of the fluorophore that is very different when the energy is emitted in the form of photons or is transferred to an acceptor by FRET (Bastiaens and Squire, 1999). FLIM has many advantages over sensitized emission to evaluate FRET but it requires separate special instrumentation that is not as easily available as fluorescence microscopes.

In the above examples, two separate molecules are used to detect FRET between neighboring molecules (intermolecular FRET). A better solution would be to have inositol lipid probes based on intramolecular FRET in which case both fluorophores are attached to the same inositide-recognizing domain. Lipid binding then induces a molecular rearrangement that changes the distance (or more likely the dipole orientation) of the two fluorophores and, hence, the FRET signal. Such a probe based on the Grp1-PH domain was targeted to different membranes for PtdIns(3,4,5)P_3 detection (Sato et al., 2003) and a similar principle was utilized to generate FRET probes for monitoring InsP3 concentrations in the cytoplasm (Matsu-ura et al., 2006). The difficulty here is to ensure a large enough conformational change upon lipid binding to substantially change the FRET efficiency. Construction of a useful probe requires lots of experimentation with the domains themselves as well as with the linkers to connect the fluorophores. In a recent study, the AktPH domain was used to detect PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 changes using a clever molecular design. The conformational change between the lipid-bound and unbound stages was achieved by inserting a negatively charged "pseudoligand" into the probe that binds to the PH domain (presumably to the lipid-binding site) when lipids are not present. Binding of the appropriate lipids abolishes this intramolecular interaction amplifying the conformational change and amplifying the change in the FRET signal (Ananthanarayanan et al., 2005). These single-molecule FRET probes do not require co-expression, their readout does not depend on their expression level (once above reliable detection limits) or on lipid density in the membrane. It is expected that more efforts will be made to generate similar probes for the detection of lipid production in the various cellular compartments.

More detailed technical and theoretical background on FRET measurements either with sensitized emission or with FLIM are covered in recent publications (Sekar and Periasamy, 2003; van Rheenen et al., 2004; Thaler et al., 2005).

Membrane localization based on TIRF analysis

Total internal reflection fluorescence (TIRF; *UNIT 4.12*) analysis has also been used to monitor plasma membrane association of inositol lipid binding domains (Tengholm et al., 2003). The basis of this technique is a special form of illumination where the light is shot at the sample at a shallow angle in a way that the photons do not illuminate the specimen beyond a ~200-nm thickness above the coverslip. This way the excitation is limited to the fluorescence molecules that are found close to the membrane of the cell attached to the coverslip, which makes this method suitable to detect membrane-associated events and also useful to study association and dissociation of molecules from the membrane. If the footprint of the cell changes, it could cause a change in fluorescence intensity unrelated to the actual amount of fluorescent molecules at the membrane. For this reason, use of a fluorescent membrane marker as a control and then evaluation of fluorescence changes against this reference signal are recommended.

Live-cell imaging of inositol lipids has become a standard approach in many laboratories. It has generated some controversy with examples for its uncritical use as well as for its complete dismissal as an unreliable method.

The truth is that this method—as every other—has its unique benefits as well as its limits. The authors emphasize that the phosphoinositides imaged with these molecular tools are only representative of a pool associated with certain processes and not necessarily representative of all of that particular inositide within the cell. This is especially true and obvious for PtdIns4P and may be less notable but still true for the other inositides. Also be cautious about high expression levels, which inhibit and distort cellular processes. Generating quantitative data with these methods is an added challenge but a very important necessity. As more domains that interact with inositides are identified in proteins it is important to remember that many of these will not work as reporters because their cellular localization is more dependent on protein-protein interaction than on lipid binding. However, some other probes should be compared for their imaging properties, especially for PtdIns(4,5)P_2 and PtdIns4P. Development of FRET-based probes relying on intramolecular rearrangements should also be facilitated. This research area will be in high demand for the near future. The high interest in phosphoinositides also demands constant improvement of the tools to further the knowledge on these molecules.

Troubleshooting

Described here are the most common problems that are encountered—starting from plasmid propagation to the final microscopy steps.

No bacterial colonies

The pEGFP plasmids have the kanamycin selection marker so transformed bacteria must be grown on kanamycin-containing plates and not on ampicillin-containing plates. However, companies are selling various forms of plasmids that one cannot assume that all GFP constructs are kanamycin selectable. Moreover, some laboratories add GFP to their chosen domain in ampicillin-resistance plasmids, and oftentimes researchers make wrong assumptions when selecting colonies of transformed bacteria. This should be the first consideration when no colonies are found after transformation of bacteria during subcloning or propagation of plasmids. Problems with subcloning, ligation reaction, or bacterial transformation can also result in no bacterial growth, but discussion of these variables is beyond the scope of this unit.

No green fluorescence

It may sound trivial but the most common reason for not seeing fluorescence is due to inappropriate microscope settings. Confocal microscopes used for fluorescence imaging can be intimidating for novices, with several filters and light directions that must be set properly to see the fluorescent signal. It is good practice to find the right focus of the cells with transmitted light. Switching to fluorescence, the blue (or other color) light coming through the objective must be visible to be sure that the illumination and the light path is properly set. It is very useful to have a slide of fixed GFP-expressing cells to use as a control. If the microscope settings are correct and there are still no GFP-positive cells (yet the autofluorescence is visible with a 40× or higher objective), then the problem lies with the transfection. Transfection problems can be caused by several factors, including inaccurate subcloning, impure plasmid for transfection, unsuitable transfection conditions for the cells. Even transfected primary cultured cells should have a few positive cells. Using cells transfected with the original pEGFP plasmid as control should help to determine whether the transfection procedure or the DNA construct is the source of the problem. If these cells are positive for GFP fluorescence, then the most likely source of the problem is the construct itself. The DNA construct could be defective by having an unwanted stop codon or a frame-shift either due to a flaw in the design or by a mutation. The plasmids should always be confirmed by DNA sequencing and the expression of the full-length protein should be confirmed by SDS-PAGE as described.

Weak fluorescence

If there are positive cells but their fluorescence is weak, the microscope may not be set up properly. Compare the cells with fixed cells transfected with EGFP alone to determine if the problem is with the microscope or the construct to be studied. If the EGFP-expressing cells are bright, but the fusion protein–expressing cells are weakly fluorescent, then the problem is inherent to the fusion protein. The authors' experience is that the larger the protein fused to GFP, the weaker its fluorescence. This could be because of the difficulty in the folding of GFP as part of a larger protein or that the translation of longer proteins is less efficient. Since the constructs listed in Table 24.4.1 express well, low levels of fluorescence usually indicates a transfection problem, which is also corroborated by few cells showing fluorescence. Many cells showing weak fluorescence indicates good transfection efficiency but low yield of the fluorescent protein.

Large aggregates present in cytoplasm

The localization of the lipid-binding domains listed in Table 24.4.1 is well documented. However, from time to time, one will encounter a problem when making a new probe or introducing mutations or deletions in any of these probes. This is a "localization" that is not related to interactions with phospholipids but is due to technical problems. High concentrations or more often folding problems of the expressed fusion proteins are usually responsible for this phenomenon, which results in the formation of large fluorescent aggregates that are found in various parts of the cell but mostly associated with the perinuclear area and could overlap with the Golgi. Very often this localization corresponds to the ER-associated protein degradation and represents the proteosome (Hitchcock et al., 2003). The authors experienced such problems with the EBFP-fused PLCδ1PH and with some chimeric constructs. In such cases, a fraction of the expressed protein still could fold properly and function as expected, and there are also cells that express less of the protein or, for a shorter time, do not display this problem. It is important to recognize this aggregation artifact and not to interpret it as a true localization. Sometimes it helps to reduce expression by using a less active promoter or to lower the temperature by 5° to 7°C during transfection and culture. However, the authors were not successful with these manipulations once the construct showed this aggregation behavior.

No localization is observed on the membrane where the lipid is expected to be

If GFP fluorescence is observed, but it is not associated with the membrane, it should be confirmed that the expression construct contains the sequence for the lipid-binding domain. Plasmid preparations of pEGFP-based plasmids often contain a ~600-bp DNA piece that is present without digestion by restriction enzymes. This band is usually faint but can be mistaken for an insert when confirming the DNA construct by restriction enzyme digestion analysis. This is especially problematic because many PH domains are encoded by DNA sequences of ~500 bp.

It also has to be realized that the amount of lipid produced and available for binding by a fluorescent probe is limited. Therefore, the membrane-bound fraction may not be distinguishable from the high cytosolic background of the unlocalized fusion protein when a cell expresses high amounts of the fusion protein. This is another reason to study cells that express low concentrations of the fusion protein. When PtdIns(4,5)P_2 is monitored with PLCδ_1PH-GFP, saturation within a wide range of expression level was not observed. We assume that there is a compensatory increase in the amount of PtdIns(4,5)P_2 in the cells expressing larger amounts of the lipid-binding protein. Compensatory increases are less likely to occur with lipids that are only formed in response to acute stimulation, such as PtdIns(3,4,5)P_3.

Nuclear localization

All of the domains listed in Table 24.4.1 can penetrate the nuclear pore and enter the nucleus, although their movement in and out is clearly limited. Many of the PH domains (Grp1-PH, ARNO-PH, Btk-PH, OSBP-PH, OSH2-PH) show very prominent nuclear localization. With the increased interest in nuclear phosphoinositides (Irvine, 2006) and the clear presence of these lipids in the nuclear matrix (in a still poorly understood physical form), localization of PH domains sparks the interest of many researchers. However, nuclear localization of these domains is also observed with mutants that do not bind the inositol lipids. This does not rule out that the wild-type constructs do have some nuclear binding components, but it shows that the major force of nuclear enrichment is not a reflection of lipid binding. It is very likely that the numerous basic residues characteristic of these domains are responsible for their nuclear localization; this is supported by the fact that algorithms based on the primary sequences of these proteins accurately predict their nuclear localization. However, if the protein binds to a lipid that is already present and abundant during expression, such as PtdIns(4,5)P_2, it will keep the domain out of the nucleus, provided that the amount of the expressed protein does not saturate the lipid available for binding. Again, choosing cells in which the expression levels are low also ensures less localization in the nucleus.

Plasma membrane localization—true or false?

The authors believe that many investigators have wrong ideas about how plasma membrane localization can be recognized especially under a confocal microscope. They expect a strong fluorescence signal outline of cells and no signal over the cytoplasm. This is true for cells that are round in shape or arranged as cobblestones such as MDCK cells. However, plasma membrane localization can

be more difficult to recognize in cells that are solitary and very flat such as COS cells. In such flat cells, one could see even fluorescence when imaging the glass-attached membrane surface giving the impression that the probe is also present in the cytoplasm. Moving upwards on the cell can change the view to a very fuzzy ring with some worm-like intensity (representing ruffles and microfilopodia) as the membrane covering the nucleus appears in the z-plane (Fig. 24.4.1B). It requires a trained eye to differentiate between plasma membrane and cytosolic constructs in such cells. A good guide is to see if the fluorescence clearly outlines the nucleus and its intensity is diminishing toward the periphery of the cells, both being signs of cytosolic localization.

In an opposing way, cytosolic proteins can show a phenomenon that the authors call "pseudo membrane localization," which gives the impression that the protein is in the membrane. In very flat cells, the edge of the cell and the membrane ruffles often appear as high-intensity lines or wrinkles because of the way the membrane appears in the imaged plane. Since membrane ruffling is a common response of cells to stimuli that activate PI 3-kinases and can be reversed with PI3K inhibitors, this "apparent" localization will show the same PI 3-kinase dependence as the real plasma membrane recruitment of PtdIns(3,4,5)P_3 recognizing domains. Therefore, it can give the impression that the construct localizes to the membrane ruffles in stimulated cells even when it does not actually bind to PtdIns(3,4,5)P_3. Comparing the fusion protein–expressing cells with control cells expressing only GFP is essential to resolving true versus false membrane localization. A useful measure of true plasma membrane recruitment is to monitor the decrease in intensity of the probe in the cytoplasm. The authors find that often this decrease can be best judged by the increased contrast between the cytoplasm and the nucleus, which appears sharper as the cytoplasmic fluorescence decreases. True membrane localization also has to fulfill several criteria. The localization of the fusion protein should follow the lipid changes that are evoked by physiological or pharmacological means. For example, PI 3 kinase inhibitors, such as wortmannin or LY294002 prevent the formation of 3-phosphorylated lipid products, so treatment of the transfected cells with these inhibitors should eliminate localization of fluorescent probes that recognize 3-phosphorylated lipids. Also, robust activation of PLC should cause a decrease in the localization of probes detecting PtdIns(4,5)P_2. However, the extent of change often is smaller than what can be detected by the eye or even with more sensitive quantification. For example, many G protein–coupled receptors evoke a Ca^{2+} signal (indicating PLC activation), yet no detectable change in PLCδ_1PH-GFP distribution. This does not mean that the probe does not work. It only means that the PLC activation is not robust enough, or the PIP 5 kinase activity is so active that there is little change in the concentration of the PtdIns(4,5)P_2 in the membrane.

A further observation of practical importance is that live cells lose localization of the PLCδ1PH-GFP (and some other domains) when they are kept at room temperature for >15 to 20 min. The reason for this has not been explored in detail, but it may be that lipid synthesis is slowed because of either ATP depletion or the physicochemical properties of the membrane change. Warming the cells to 37°C does not correct the situation within 30 min. Thus, live-cell imaging should be performed using a temperature-controlled microscope system within a short period of time after placing the cells in the observation medium.

Anticipated Results

Expression of GFP-fused inositol lipid binding domains should yield a fluorescent signal that is easy to recognize and distinguishable from background autofluorescence. In fact, the larger problem often is too high of expression, especially in COS-7 cells. In these cells, the biology is greatly distorted; therefore, these cells should not be used for analysis (see Fig. 24.4.1 for an example). HEK293 or HeLa cells will express more moderate levels of the same constructs since they do not express the large T antigen that generates many copies of the plasmid in COS cells. The distribution of the individual domains will reflect the localization of the lipid to which it binds except additional interactions of the domain with other membrane components or proteins may significantly alter the localization of the probe. Lastly, the lipid changes are not always large enough to be observed by the eye or even by quantification of the data. Many cells generate clearly detectable Ca^{2+} signals by PLC activation without a noticeable change in PLCδ1PH-GFP localization. These negative data do not necessarily mean that the probe is not working. A positive control is always a useful way to check a newly studied construct.

Time Considerations

Performing a live-cell imaging experiment is not particularly time consuming. A typical experiment starts out by preparing the coverslips (~2 hr with drying) and seeding cells for the experiment. Cells are transfected on the following day with plasmid DNA (~1 hr depending on the number of coverslips). For most phosphoinositide-binding domains, the transfection begins at ~1400 hr and the transfection medium is changed to one with serum ~1900 hr for experiments that are planned for the following day. It is not recommended to use cells after >24 to 30 hr after transfection with these domains. The actual experiment requires setting up the microscope and the heated stage (~30 min) and waiting for equilibration (~30 min). Depending on the type of experiment, a sample is rarely on the stage >30 min. Data analysis is done off-line and can be more time consuming than the actual experiment depending on the application. Design and generation of a new construct is a more time-consuming process that can take from 1 week (optimal) to several weeks.

It is important to remember that the time-consuming part of live-cell imaging is the many times an experiment must be repeated. Many recordings cannot be used because of technical problems such as moving cells or focus-drift occurring during time-lapse imaging. Also, one must repeat experiments many times before being confident that the chosen pictures actually represent a reproducible biological process. Due to individual variations among cells, this is often not appreciated by users who are used to methods that give averaged cell responses.

Acknowledgments

The authors would like to thank Dr. Mark Lemmon (Univ. Pennsylvania, Philadelphia) for the OSH1-PH-GFP and OSH2-PH-GFP constructs. The confocal imaging was performed at the Microscopy & Imaging Core of the National Institute of Child Health and Human Development, NIH with the kind assistance of Drs. Vincent Schram and James T. Russell. This research was supported in part by the Intramural Research Program of the National Institute of Child Health and Human Development of the National Institutes of Health. Péter Várnai is also a Bolyai Fellow of the Hungarian Academy of Science and was also supported by the Hungarian Scientific Research fund (OTKA NF-68563) and the Medical Research Council (ETT 440/2006).

Literature Cited

Ananthanarayanan, B., Ni, Q., and Zhang, J. 2005. Signal propagation from membrane messengers to nuclear effectors revealed by reporters of phosphoinositide dynamics and Akt activity. *Proc. Natl. Acad. Sci. U.S.A.* 102:15081-15086.

Balla, T. 2007. Imaging and manipulating phosphoinositides in living cells. *J. Physiol.* 582:927-937.

Balla, A., Tuymetova, G., Tsiomenko, A., Varnai, P., and Balla, T. 2005. A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-III alpha: Studies with the PH domains of the oxysterol binding protein and FAPP1. *Mol. Biol. Cell* 16:1282-1295.

Balla, A., Kim, Y.J., Varnai, P., Szentpetery, Z., Knight, Z., Shokat, K.M., and Balla, T. 2008. Maintenance of hormone-sensitive phosphoinositide pools in the plasma membrane requires phosphatidylinositol 4-kinase III{alpha}. *Mol. Biol. Cell* 19:711-721.

Bastiaens, P.I. and Squire, A. 1999. Fluorescence lifetime imaging microscopy: Spatial resolution of biochemical processes in the cell. *Trends Cell Biol.* 9:48-52.

Berridge, M.J. and Irvine, R.F. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312:315-321.

Burd, C.G. and Emr, S.D. 1998. Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol. Cell* 2:157-162.

Christy, A.H., Kim, A.C., Marfatia, S.M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S.C., Low, P.S., Rouleau, G.A., Mohandas, N., Chasis, J.A., Conboy, J.G., Gascard, P., Takakuwa, Y., Huang, S.C., Benz, E.J. Jr., Bretscher, A., Fehon, R.G., Gusella, J.F., Ramesh, V., Solomon, F., Marchesi, V.T., Tsukita, S., Arpin, M., Louvard, D., Tonks, N.K., Anderson, J.M., Fanning, A.S., Bryant, P.J., Woods, D.F., and Hoover, K.B. 1998. The FERM domain: A unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem. Sci.* 23:281-282.

Cohen, L.A., Honda, A., Varnai, P., Brown, F.B., Balla, T., and Donaldson, J.G. 2007. Active Arf6 recruits ARNO/cytohesin GEFs to the PM by binding their PH domains. *Mol. Biol. Cell* 18:2244-2253.

Coronas, S., Ramel, D., Pendaries, C., Gaits-Iacovoni, F., Tronchere, H., and Payrastre, B. 2007. PtdIns5P: A little phosphoinositide with big functions? *Biochem. Soc. Symp.* 74:117-128.

De Matteis, M.A., Di Campli, A., and Godi, A. 2005. The role of the phosphoinositides at the Golgi complex. *Biochim. Biophys. Acta* 1744:396-405.

Di Paolo, G. and De Camilli, P. 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443:651-657.

Dormann, D., Weijer, G., Parent, C.A., Devreotes, P.N., and Weijer, C.J. 2002. Visualizing PI3 kinase-mediated cell-cell signaling during *Dictyostelium* development. *Curr. Biol.* 12:1178-1188.

Dove, S.K., Piper, R.C., McEwen, R.K., Yu, J.W., King, M.C., Hughes, D.C., Thuring, J., Holmes, A.B., Cooke, F.T., Michell, R.H., Parker, P.J., and Lemmon, M.A. 2004. Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. *EMBO J.* 23:1922-1933.

Dowler, S., Currie, R.A., Campbell, D.J., Deak, M., Kular, G., Downes, C.P., and Alessi, D.R. 2000. Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. *Biochem. J.* 351:19-31.

Ellson, C.D., Gobert-Gosse, S., Anderson, K.E., Davidson, K., Erdjument-Bromage, H., Tempst, P., Thuring, J.W., Cooper, M.A., Lim, Z.Y., Holmes, A.B., Gaffney, P.R.J., Coadwell, J., Chilvers, E.R., Hawkins, P.T., and Stephens, L.R. 2001. PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40phox. *Nat. Cell Biol.* 3:679-682.

Field, S.J., Madson, N., Kerr, M.L., Galbraith, K.A., Kennedy, C.E., Tahiliani, M., Wilkins, A., and Cantley, L.C. 2005. PtdIns(4,5)P2 functions at the cleavage furrow during cytokinesis. *Curr. Biol.* 15:1407-1412.

Franke, T.F., Kaplan, D.R., Cantley, L.C., and Toker, A. 1997. Direct regulation of the Akt protooncogene product by PI3,4P2. *Science* 275:665-668.

Friant, S., Pecheur, E.I., Eugster, A., Michel, F., Lefkir, Y., Nourrisson, D., and Letourneur, F. 2003. Ent3p Is a PtdIns(3,5)P2 effector required for protein sorting to the multivesicular body. *Dev. Cell* 5:499-511.

Gary, J.D., Wurmser, A.E., Bonangelino, C.J., Weisman, L.S., and Emr, S.D. 1998. Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J. Cell Biol.* 143:65-79.

Giepmans, B.N., Adams, S.R., Ellisman, M.H., and Tsien, R.Y. 2006. The fluorescent toolbox for assessing protein location and function. *Science* 312:217-224.

Gillooly, D.J., Morrow, I.C., Lindsay, M., Gould, R., Bryant, N.J., Gaullier, L.M., Parton, G.P., and Stenmark, H. 2000. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* 19:4577-4588.

Godi, A., Di Campi, A., Konstantakopoulos, A., Di Tullio, G., Alessi, D.R., Kular, G.S., Daniele, T., Marra, P., Lucocq, J.M., and De Matteis, M.A. 2004. FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. *Nat. Cell Biol.* 6:393-404.

Gozani, O., Karuman, P., Jones, D.R., Ivanov, D., Cha, J., Logovskoy, A.A., Baird, C.L., Zhu, H., Field, S.J., Lessnick, S.L., Villasenov, J., Mehrotra, B., Chen, J., Rao, V.R., Brugge, J.S., Ferguson, C.G., Payrastre, B., Myszka, D.G., Cantley, L.C., Wagner, G., Divecha, N., Prestwich, G.D., and Yuan, J. 2003. The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* 114:99-111.

Guillou, H., Stephens, L.R., and Hawkins, P.T. 2007. Quantitative measurement of phosphatidylinositol 3,4,5-trisphosphate. *Methods Enzymol.* 434:117-130.

Hammond, G.R., Dove, S.K., Nicol, A., Pinxteren, J.A., Zicha, D., and Schiavo, G. 2006. Elimination of plasma membrane phosphatidylinositol (4,5)-bisphosphate is required for exocytosis from mast cells. *J. Cell Sci.* 119:2084-2094.

Hitchcock, A.L., Auld, K., Gygi, S.P., and Silver, P.A. 2003. A subset of membrane-associated proteins is ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery. *Proc. Natl. Acad. Sci. U.S.A.* 100:12735-12740.

Horan, K.A., Watanabe, K., Kong, A.M., Bailey, C.G., Rasko, J.E., Sasaki, T., and Mitchell, C.A. 2007. Regulation of FcgammaR-stimulated phagocytosis by the 72-kDa inositol polyphosphate 5-phosphatase: SHIP1, but not the 72-kDa 5-phosphatase, regulates complement receptor 3 mediated phagocytosis by differential recruitment of these 5-phosphatases to the phagocytic cup. *Blood* 110:4480-4491.

Huang, Y.E., Iijima, M., Parent, C.A., Funamoto, S., Firtel, R.A., and Devreotes, P. 2003. Receptor-mediated regulation of PI3Ks confines PI(3,4,5)P3 to the leading edge of chemotaxing cells. *Mol. Biol. Cell* 14:1913-1922.

Hunyady, L., Baukal, A.J., Gaborik, Z., Olivares-Reyes, J.A., Bor, M., Szaszak, M., Lodge, R., Catt, K.J., and Balla, T. 2002. Differential PI 3-kinase dependence of early and late phases of recycling of the internalized AT1 angiotensin receptor. *J. Cell Biol.* 157:1211-1222.

Ikonomov, O.C., Sbrissa, D., Foti, M., Carpentier, J.L., and Shisheva, A. 2003. PIKfyve controls fluid phase endocytosis but not recycling/degradation of endocytosed receptors or sorting of procathepsin D by regulating multivesicular body morphogenesis. *Mol. Biol. Cell* 14:4581-4591.

Irvine, R.F. 1990. Methods in Inositide Research. Raven Presss, New York.

Irvine, R.F. 2006. Nuclear inositide signalling—Expansion, structures and clarification. *Biochim. Biophys. Acta* 1761:505-508.

Jefferies, H.B., Cooke, F.T., Jat, P., Boucheron, C., Koizumi, T., Hayakawa, M., Kaizawa, H., Ohishi, T., Workman, P., Waterfield, M.D., and Parker, P.J. 2008. A selective PIKfyve inhibitor blocks PtdIns(3,5)P(2) production and disrupts endomembrane transport and retroviral budding. *EMBO Rep.* 9:164-170.

Kanai, F., Liu, H., Field, S.J., Akbary, H., Matsuo, T., Brown, G.E., Cantley, L.C., and Yaffe, M.B. 2001. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat. Cell Biol.* 3:675-678.

Karathanassis, D., Stahelin, R.V., Bravo, J., Perisic, O., Pacold, C.M., Cho, W., and Williams, R.L. 2002. Binding of the PX domain of p47phox to phosphatidylinositol 3,4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. *EMBO J.* 21:5057-5068.

Kimber, W.A., Trinkle-Mulcahy, L., Cheung, P.C., Deak, M., Marsden, L.J., Kieloch, A., Watt, S., Javier, R.T., Gray, A., Downes, C.P., Lucocq, J.M., and Alessi, D.R. 2002. Evidence that the tandem-pleckstrin-homology-domain-containing protein TAPP1 interacts with Ptd(3,4)P2 and the multi-PDZ-domain-containing protein MUPP1 in vivo. *Biochem. J.* 361:525-536.

Klarlund, J.K., Guilherme, A., Holik, J.J., Virbasius, J.V., Chawla, A., and Czech, M.P. 1997. Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing plekstrin and Sec7 homology domains. *Science* 275:1927-1930.

Klarlund, J.K., Tsiaras, W., Holik, J.J., Chawla, A., and Czech, M.P. 2000. Distinct polyphosphoinositide binding selectivities for pleckstrin homology domains of GRP1-like proteins based on diglycine versus triglycine motifs. *J. Biol. Chem.* 275:32816-32821.

Komander, D., Fairservice, A., Deak, M., Kular, G.S., Prescott, A.R., Peter Downes, C., Safrany, S.T., Alessi, D.R., and van Aalten, D.M. 2004. Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. *EMBO J.* 23:3918-3928.

Lemmon, M.A., Falasca, M., Ferguson, K.M., and Schlessinger, J. 1997. Regulatory recruitment of signalling molecules to the cell membrane by plekstrin-homology domains. *Trends Cell Biol.* 7:237-242.

Lemmon, M.A., Ferguson, K.M., O'Brian, R., Sigler, P.B., and Schlessinger, J. 1995. Specific and high-affinity binding of inositol phosphates to an isolated plekstrin homology domain. *Proc. Natl. Acad. Sci. U.S.A.* 92:10472-10476.

Levine, T.P. and Munro, S. 1998. The pleckstrin-homology domain of oxysterol-binding protein recognizes a determinant specific to Golgi membranes. *Curr. Biol.* 8:729-739.

Levine, T.P. and Munro, S. 2002. Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components. *Curr. Biol.* 12:695-704.

Luo, H.R., Huang, Y.E., Chen, J.C., Saiardi, A., Iijima, M., Ye, K., Huang, Y., Nagata, E., Devreotes, P., and Snyder, S.H. 2003. Inositol pyrophosphates mediate chemotaxis in *Dictyostelium* via pleckstrin homology domain-PtdIns(3,4,5)P3 interactions. *Cell* 114:559-572.

Manna, D., Albanese, A., Park, W.S., and Cho, W. 2007. Mechanistic basis of differential cellular responses of phosphatidylinositol 3,4-bisphosphate- and phosphatidylinositol 3,4,5-trisphosphate-binding pleckstrin homology domains. *J. Biol. Chem.* 282:32093-32105.

Matsuda, M., Paterson, H.F., Rodriguez, R., Fensome, A.C., Ellis, M.V., Swann, K., and Katan, M. 2001. Real time fluorescence imaging of PLC gamma translocation and its interaction with the epidermal growth factor receptor. *J. Cell Biol.* 153:599-612.

Matsu-ura, T., Michikawa, T., Inoue, T., Miyawaki, A., Yoshida, M., and Mikoshiba, K. 2006. Cytosolic inositol 1,4,5-trisphosphate dynamics during intracellular calcium oscillations in living cells. *J. Cell Biol.* 173:755-765.

Michell, R.H., Heath, V.L., Lemmon, M.A., and Dove, S.K. 2005. Phosphatidylinositol 3,5-bisphosphate: Metabolism and cellular functions. *Trends Biochem. Sci.* 31:52-63.

Muller-Taubenberger, A. and Anderson, K.I. 2007. Recent advances using green and red fluorescent protein variants. *Appl. Microbiol. Biotechnol.* 77:1-12.

Nagel, W., Schilcher, P., Zeitlmann, L., and Kolanus, W. 1998. The PH domain and the polybasic c domain of cytohesin-1 cooperate specifically in plasma membrane-association and cellular function. *Mol. Biol. Cell* 9:1981-1994.

Nash, M.S., Young, K.W., Willars, G.B., Challiss, R.A., and Nahorski, S.R. 2001. Single-cell imaging of graded Ins(1,4,5)P3 production following G-protein-coupled-receptor activation. *Biochem. J.* 356:137-142.

Nasuhoglu, C., Feng, S., Mao, J., Yamamoto, M., Yin, H.L., Earnest, S., Barylko, B., Albanesi, J.P., and Hilgemann, D.W. 2002. Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection. *Anal. Biochem.* 301:243-254.

Nelson, C.P., Nahorski, S.R., and Challiss, R.A. 2008. Temporal profiling of changes in phosphatidylinositol 4,5-bisphosphate, inositol 1,4,5-trisphosphate and diacylglycerol allows comprehensive analysis of phospholipase C-initiated signalling in single neurons. *J. Neurochem.* 107:602-615.

Pearson, M.A., Reczek, D., Bretscher, A., and Karplus, P.A. 2000. Structure of the ERM protein moesin reveals the FERM domain fold and extended actin binding tail domain. *Cell* 101:259-270.

Pendaries, C., Tronchere, H., Arbibe, L., Mounier, J., Gozani, O., Cantley, L., Fry, M.J., Gaits-Iacovoni, F., Sansonetti, P.J., and Payrastre, B. 2006. PtdIns5P activates the host cell PI3-kinase/Akt pathway during *Shigella flexneri* infection. *EMBO J.* 25:1024-1034.

Pittet, D., Schlegel, W., Lew, D.P., Monod, A., and Mayr, G.W. 1989. Mass changes in inositol tetrakis- and pentakisphosphate isomers induced by chemotactic peptide stimulation in HL-60 cells. *J. Biol. Chem.* 264:18489-18493.

Quinn, K.V., Behe, P., and Tinker, A. 2008. Monitoring changes in membrane phosphatidylinositol 4,5-bisphosphate in living cells using a

domain from the transcription factor tubby. *J. Physiol.* 586:2855-2871.

Rameh, L.E., Arvidsson, A., Carraway, K.L. III, Couvillon, A.D., Rathbun, G., Crompton, A., VanRentherghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., Wang, D.S., Chen, C.S., and Cantley, L.C. 1997a. A comparative analysis of the phosphoinositide binding specificity of plekstrin homology domains. *J. Biol. Chem.* 272:22059-22066.

Rameh, L.E., Tolias, K.F., Duckworth, B.C., and Cantley, L.C. 1997b. A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature* 390:192-196.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J.D., Sheetz, M.P., and Meyer, T. 2000. Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell* 100:221-228.

Roy, A. and Levine, T.P. 2004. Multiple pools of phosphatidylinositol 4-phosphate detected using the pleckstrin homology domain of Osh2p. *J. Biol. Chem.* 279:44683-44689.

Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, C.I.E., Driscoll, P.C., Waterfield, M.D., and Panayotou, G. 1996. Distinct specificity in the recognition of phosphoinositides by the plekstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* 15:6241-6250.

Santagata, S., Boggon, T.J., Baird, C.L., Gomez, C.A., Zhao, J., Shan, W.S., Myszka, D.G., and Shapiro, L. 2001. G-protein signaling through tubby proteins. *Science* 292:2041-2050.

Sato, M., Ueda, Y., Takagi, T., and Umezawa, Y. 2003. Production of PtdInsP3 at endomembranes is triggered by receptor endocytosis. *Nat. Cell Biol.* 5:1016-1022.

Sekar, R.B. and Periasamy, A. 2003. Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J. Cell Biol.* 160:629-633.

Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R. 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287:1037-1040.

Shaner, N.C., Steinbach, P.A., and Tsien, R.Y. 2005. A guide to choosing fluorescent proteins. *Nat. Methods* 2:905-909.

Shears, S.B. 1997. Signalling by Inositides. Oxford University Press, Oxford.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M., and Stenmark, H. 1998. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* 394:494-498.

Stahelin, R.V., Burian, A., Bruzik, K.S., Murray, D., and Cho, W. 2003. Membrane binding mechanisms of the PX domains of NADPH oxidase p40phox and p47phox. *J. Biol. Chem.* 278:14469-14479.

Stauffer, T.P., Ahn, S., and Meyer, T. 1998. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr. Biol.* 8:343-346.

Tengholm, A., Teruel, M.N., and Meyer, T. 2003. Single cell imaging of PI3K activity and glucose transporter insertion into the plasma membrane by dual color evanescent wave microscopy. *Sci. STKE* 2003:PL4.

Thaler, C., Koushik, S.V., Blank, P.S., and Vogel, S.S. 2005. Quantitative multiphoton spectral imaging and its use for measuring resonance energy transfer. *Biophys. J.* 89:2736-2749.

Thomas, C.C., Dowler, S., Deak, M., Alessi, D.R., and van Aalten, D.M. 2001. Crystal structure of the phosphatidylinositol 3,4-bisphosphate-binding pleckstrin homology (PH) domain of tandem PH-domain-containing protein 1 (TAPP1): Molecular basis of lipid specificity. *Biochem. J.* 358:287-294.

van Der Wal, J., Habets, R., Varnai, P., Balla, T., and Jalink, K. 2001. Monitoring phospholipase C activation kinetics in live cells by FRET. *J. Biol. Chem.* 276:15337-15344.

van Rheenen, J., Langeslag, M., and Jalink, K. 2004. Correcting confocal acquisition to optimize imaging of fluorescence resonance energy transfer by sensitized emission. *Biophys. J.* 86:2517-2529.

Várnai, P. and Balla, T. 1998. Visualization of phosphoinositides that bind pleckstrin homology domains: Calcium-and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. *J. Cell Biol.* 143:501-510.

Várnai, P. and Balla, T. 2006. Live cell imaging of phosphoinositide dynamics with fluorescent protein domains. *Biochim. Biophys. Acta* 1761:957-967.

Várnai, P., Rother, K.I., and Balla, T. 1999. Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells. *J. Biol. Chem.* 274:10983-10989.

Várnai, P., Bondeva, T., Tamas, P., Toth, B., Buday, L., Hunyady, L., and Balla, T. 2005. Selective cellular effects of overexpressed pleckstrin-homology domains that recognize PtdIns(3,4,5)P3 suggest their interaction with protein binding partners. *J. Cell Sci.* 118:4879-4888.

Várnai, P., Thyagarajan, B., Rohacs, T., and Balla, T. 2006. Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells. *J. Cell Biol.* 175:377-382.

Venkateswarlu, K., Gunn-Moore, F., Oatey, P.B., Tavare, J.M., and Cullen, P.J. 1998a. Nerve growth factor- and epidermal growth factor-stimulated translocation of the ADP-ribosylation factor-exchange factor GRP1 to the

plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain. *Biochem. J.* 335:139-146.

Venkateswarlu, K., Oatey, P.B., Tavare, J.M., and Cullen, P.J. 1998b. Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Curr. Biol.* 8:463-466.

Venkateswarlu, K., Gunn-Moore, F., Tavare, J.M., and Cullen, P.J. 1999. EGF-and NGF-stimulated translocation of cytohesin-1 to the plasma membrane of PC12 cells requires PI 3-kinase activation and a functional cytohesin-1 PH domain. *J. Cell Sci.* 112:1957-1965.

Watt, S.A., Kular, G., Fleming, I.N., Downes, C.P., and Lucocq, J.M. 2002. Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C delta1. *Biochem. J.* 363:657-666.

Watt, S.A., Kimber, W.A., Fleming, I.N., Leslie, N.R., Downes, C.P., and Lucocq, J.M. 2004. Detection of novel intracellular agonist responsive pools of phosphatidylinositol 3,4-bisphosphate using the TAPP1 pleckstrin homology domain in immunoelectron microscopy. *Biochem. J.* 377:653-663.

Watton, J. and Downward, J. 1999. Akt/PKB localisation and 3′ phosphoinositide generation at sites of epithelial cell-matrix and cell-cell interaction. *Curr. Biol.* 9:433-436.

Weixel, K.M., Blumental-Perry, A., Watkins, S.C., Aridor, M., and Weisz, O.A. 2005. Distinct Golgi populations of phosphatidylinositol 4-phosphate regulated by phosphatidylinositol 4-kinases. *J. Biol. Chem.* 280:10501-10508.

Whitman, M., Downes, C.P., Keeler, M., Keller, T., and Cantley, L. 1988. Type-I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* 332:644-646.

Wiedenmann, J. and Nienhaus, G.U. 2006. Live-cell imaging with EosFP and other photoactivatable marker proteins of the GFP family. *Expert Rev. Proteomics.* 3:361-374.

Yaradanakul, A. and Hilgemann, D.W. 2007. Unrestricted diffusion of exogenous and endogenous PIP(2) in baby hamster kidney and Chinese hamster ovary cell plasmalemma. *J. Membr. Biol.* 220:53-67.

Yu, J.W., Mendrola, J.M., Audhya, A., Singh, S., Keleti, D., DeWald, D.B., Murray, D., Emr, S.D., and Lemmon, M.A. 2004. Genome-wide analysis of membrane targeting by *S. cerevisiae* pleckstrin homology domains. *Mol. Cell* 13:677-688.

Zhan, Y., Virbasius, J.V., Song, X., Pomerleau, D.P., and Zhou, G.W. 2002. The p40phox and p47phox PX domains of NADPH oxydase target cell membranes via direct and indirect recruitment by phosphoinositides. *J. Biol. Chem.* 277:4512-4518.

Zou, J., Marjanovic, J., Kisseleva, M.V., Wilson, M., and Majerus, P.W. 2007. Type I phosphatidylinositol-4,5-bisphosphate 4-phosphatase regulates stress-induced apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 104:16834-16839.

CHAPTER 25
Nanotechnology

INTRODUCTION

Hard-earned insights from molecular and cell biology over the last 20 years have ushered in a new era of experiments on single molecules rather than on populations of molecules. Termed nanotechnology, this field of biological research focuses on the control of cellular structure and dynamics on the atomic and molecular scale (1 to 100 nm) and on the fabrication of devices for measuring within that size range. Many of the new tools used in nanotechnology come from colloidal science, including quantum dots and nanotubes. By combining these tools with live cell fluorescent imaging techniques and other analytical methods such as atomic force microscopy, nanotechnology has opened up many new experimental avenues for understanding how molecules move and assemble into complex structures within cells and tissues.

UNIT 25.1 discusses the use of quantum dots (QDs) for live cell imaging and flow cytometry. QDs have many advantages over conventional fluorophores, including increased brightness and photostability, which make them exceptional tools for live cell imaging. The unit focuses on the use of streptavidin-coupled QDs, as they have universal applicability due to their ability to recognize biotin coupled to a ligand or antibody that has been bound to a receptor or protein.

Jennifer Lippincott-Schwartz

Current Protocols in Cell Biology 25.0.1, December 2007
Published online December 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2500s37

In Vivo Imaging Using Quantum Dot–Conjugated Probes

Diane S. Lidke,[1] Peter Nagy,[2] and Donna J. Arndt-Jovin[3]

[1]University of New Mexico, Albuquerque, New Mexico
[2]University of Debrecen, Debrecen, Hungary
[3]Max Planck Institute for Biophysical Chemistry, Goettingen, Germany

ABSTRACT

This unit describes the use of quantum dots (QDs) for live-cell imaging and the use of QDs in flow cytometry for quantitative analysis of ligand binding constants and receptor density. Conventional fluorophores and visible fluorescent protein (VFP) constructs have allowed visualization of many cellular processes. However, organic and biomolecular fluorophores have limitations in their applications, due to their small Stokes' shift and tendency to photobleach during prolonged imaging. QDs have many advantages over conventional fluorophores, including high brightness and photostability, which make them an exceptional tool for live-cell imaging. There are a large variety of commercially available QDs with different surface reactivities and characteristics. The authors have limited the laboratory protocols presented here to the use of streptavidin-coupled QDs because this gives almost universal applicability to any cell surface receptor by coupling the ligand or antibody that recognizes the receptor to biotin and visualizing the complex by use of QDs. *Curr. Protoc. Cell Biol.* 36:25.1.1-25.1.18. © 2007 by John Wiley & Sons, Inc.

Keywords: quantum dots • live-cell imaging • binding constants • receptor number • biotinylated ligands

INTRODUCTION

The responses of a cell to its surrounding environment result largely from the transduction of signals from the outer cell surface to the cytoplasm and nucleus. These signals are initiated when a ligand binds to a membrane receptor, initiating signaling cascades that control numerous cellular processes such as gene expression, cell migration, and cell division. Macromolecular protein dynamics and localization, as well as signaling cascades, can be directly visualized in the living cell using fluorescence microscopy [see other units on FRAP (*UNIT 21.1*), ion imaging, and fluorescent imaging (Chapters 4 and 21)].

In this unit, the authors describe protocols for forming biotin-ligand (or biotinylated-'protein of interest') and streptavidin-QD (SAvQD) complexes for use in live-cell microscopy (see Support Protocol 1). In the case when the precoupling of the QD to the ligand inhibits function, an alternative two-step labeling protocol is provided. Support Protocol 2 discusses ways to avoid cross-linking of QD-ligand complexes if the ligand is labeled with multiple biotins. Basic Protocol 1 gives details for in vivo cell labeling with QD-ligand complexes and image acquisition, with special emphasis on temporal acquisition of image series to visualize cellular processes. Experiments to determine QD-ligand binding constants (Basic Protocol 2) and absolute number of receptors per cell (Basic Protocol 3) by complementary flow cytometry measurements are also described.

Current Protocols in Cell Biology 25.1.1-25.1.18, September 2007
Published online September 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2501s36

STRATEGIC PLANNING

What are quantum dots and how are they useful for live-cell imaging?

Water-soluble, bio-functionalized semiconductor quantum dots (QDs) are fluorescent nanoparticles that provide advantages of much greater photostability compared to conventional fluorescent dyes, and as a consequence, single QDs can be easily detected in living cells and their localization monitored for minutes to hours to days. The core of the QD consists of a semiconductor nanocrystal, typically CdSe surrounded by a passivation shell of ZnS, as well as an outer shell to make them biocompatible (Alivisatos et al., 2005; Smith et al., 2006). Absorption of a photon anywhere in a continuum rising toward the UV generates an electron-hole pair, which upon recombination (in ~10 to 20 nsec) results in the emission of a less energetic photon. The emission wavelength is dependent on the size of the core (smaller core size = lower emission wavelength), which can be varied by controlled synthesis conditions (Fig. 25.1.1). These characteristics and the fact that QDs have a large absorption cross-section, particularly in low visible to UV wavelengths, allows for the simultaneous excitation of many spectrally distinct QD emitters with a single wavelength. (See later section on multispectral fluorescence imaging and analysis).

Many types of QDs are commercially available from Invitrogen (*http://probes.invitrogen.com/products/qdot*) and Evident Technologies (*http://www.evidenttech.com*). These include surface-accessible moieties such as amino, carboxyl, peptides, biotin, streptavidin, protein A, and IgGs. The manufacturers provide kits for coupling your favorite molecule to QDs with extensive protocols and the reader is referred to these sites for details. In addition, membrane-permeable QDs (having nona-arginine on the surface) and other methods of intracellular delivery are available so that QDs may be used to label cells for reintroduction into animals and long-term cell tracking. The larger CdSe QDs, emitting at >600 nm, have extended geometries with dimensions of ~40 nm. These large sizes make accessibility a problem in tight junctions between cells or in permeabilized tissues (Arndt-Jovin et al., 2006). Emission wavelengths of QDs have been extended into the near NIR using CdTe as cores (Kim et al., 2004; Gao et al., 2005; Smith et al., 2006), thus enhancing their use in live-animal studies. An exciting in vivo quantum dot

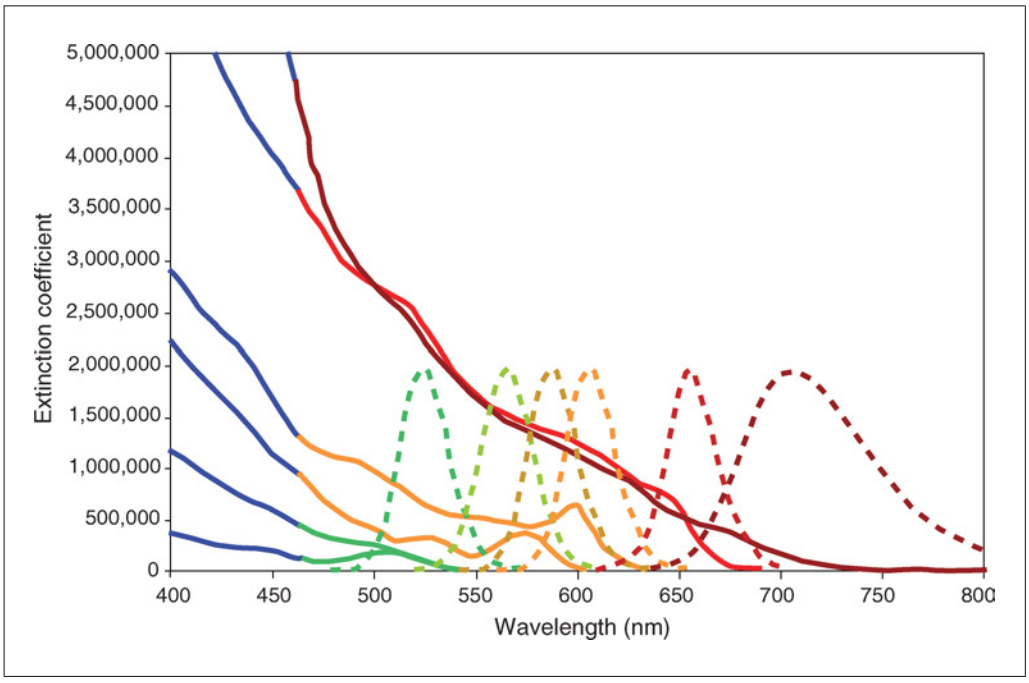


Figure 25.1.1 Excitation and emission spectra of CdSe core QDs. For color version of this figure see *http://www.currentprotocols.com*.

conjugate that emits by bioluminescence resonance energy transfer in the absence of external excitation has been prepared by coupling carboxylate-presenting quantum dots to a mutant of the bioluminescent protein *Renilla reniformis* luciferase (So et al., 2006).

Differences between QDs as probes and small or conventional fluorophores

Most conventional fluorophores have relatively broad absorption and emission bands with a small to medium Stokes' shift (the difference in wavelength between their excitation and emission maxima). For information on the excitation and emission maxima for many small molecule fluorophores see *http://probes.invitrogen.com/handbook*. Genetically encoded fluorophores (VFPs, visible fluorescent proteins) have small Stokes' shifts except for the recently described Keima (Chudakov et al., 2005; Shaner et al., 2005; Kogure et al., 2006). Thus, it is more difficult to excite several conventional fluorophores with a single wavelength. For excitations in the visible wavelengths many conventional fluorophores have higher extinction coefficients than QDs and with their shorter fluorescent lifetimes will appear 'brighter' to the observer. However, these dyes are much less photostable than QDs. This latter fact makes QDs the probe of choice for in vivo imaging over extended time periods.

Instrumentation

Microscopy

Macromolecular dynamics, localization, and aggregation state can be directly visualized in the living cell using fluorescence microscopy [see units on basic fluorescence microscopy (*UNIT 4.2*), FRAP (*UNIT 21.1*), FRET (*UNITS 17.1 & 17.9*) and fluorescence correlation spectroscopy (FCS)]. Commercial confocal microscopes allow for multi-laser excitation and the imaging of several different fluorophores simultaneously. Recent advances in multispectral fluorescence imaging, where the emission spectrum of the sample is measured in each sampled point, have made it possible to further increase the number of fluorophores that can be monitored (Fountaine et al., 2006). Commercial systems include the Zeiss LSM 510META and Live systems, Nikon Digital Eclipse C1si, Leica TCS SP5, Olympus Fluoview, and Olympus DSU spinning disk. QDs are particularly suited to simultaneous multi-color labeling of different macromolecules in the same sample due to their broad excitation spectra and narrow emission spectra, which allows for single line excitation of multiple QDs and separation of spectrally distinct QD emissions using filter-based or multispectral imaging.

In addition to confocal imaging, high spatial and time resolution can be achieved using a wide-field microscope and a sensitive CCD camera (see *UNIT 4.2*). The brightness and photostability of QDs make it possible to track individual protein dynamics in living cells at video rate or faster using the new generation electron multiplying (em) CCD cameras (Andor iXon, Roper Scientific Cascade, Hamamatsu C9100-02). The high quantum efficiency of CCD cameras extending into the NIR makes them far superior for detecting QDs with emissions >600 nm compared with PMT-based imaging systems.

Flow cytometry

Flow cytometry is a complementary technique to imaging microscopy. The authors include a discussion of it here to demonstrate its power for quantitative determination of binding constants, numbers of binding sites, as well as kinetic or dissociation constants with high statistical precision. Flow cytometry has both advantages and disadvantages compared to microscopy. It can record the fluorescence and light scattering of hundreds or thousands of cells in a second, but speed comes at the price of losing subcellular resolution. Although some flow cytometers are capable of multi-laser excitation, in the simplest and probably most wide-spread bench-top flow cytometers the number of excitation wavelengths is limited. Similarly as in the imaging microscope, the fact that several different types of QDs can be excited by a single laser line makes

multi-color labeling possible and easily detected in different fluorescence channels of the flow cytometer (Mattheakis et al., 2004; Chattopadhyay et al., 2006). The photostability of QDs is less valuable in flow cytometry due to the limited amount of time the cells are illuminated, but their relatively large extinction coefficient (especially with UV excitation) makes the labeling of cells with low numbers of binding sites more reliable.

BASIC PROTOCOL 1

IN VIVO, IN SITU LABELING OF CELLS WITH QD-LIGAND CONJUGATES FOR FLUORESCENCE MICROSCOPIC IMAGING

This method describes the general procedure for preparing cells for live imaging in order to follow the fate of the QD-ligands interacting with membrane-bound receptors (see Fig. 25.1.2). The methods can be used with a confocal laser scanning microscope or a wide-field microscope. The use of LabTek chambers (Nunc) requires the use of an inverted microscope. Other manufacturers such as Electron Microscopy Services (*http://www.emsdiasum.com/microscopy/products/preparation/dish.aspx*) make holders that accommodate coverslips of various dimensions that are equally suitable. With live-cell imaging, dynamic events (i.e., QD-ligand binding/internalization or endosomal trafficking) can be monitored by acquiring an image series over the time range of interest.

If quantitative analysis of data is desired care must be taken to acquire the images such that no pixels have intensities of 0 or less nor values above the range of the detector (8-bit, 12-bit, or 16-bit for example.)

Controls for the specificity of the QD-receptor interaction are important. It is absolutely essential to show that the signal that is acquired is specifically associated with the presumed receptor and that it reflects the behavior of the receptor without QDs attached. The researcher must do binding experiments with unconjugated QDs, and QD-ligand binding in the presence of competitive excess free ligand, determine if down-stream signaling is affected as expected (e.g. phosphorylation profiles are the same with QD-ligand compared with ligand alone), and demonstrate that there is no binding on cells that do not express the receptor.

If one wishes to perform single-particle tracking of individual receptors, it may be desirable to use the QD simply as a tracer of the molecule of interest. This may be done

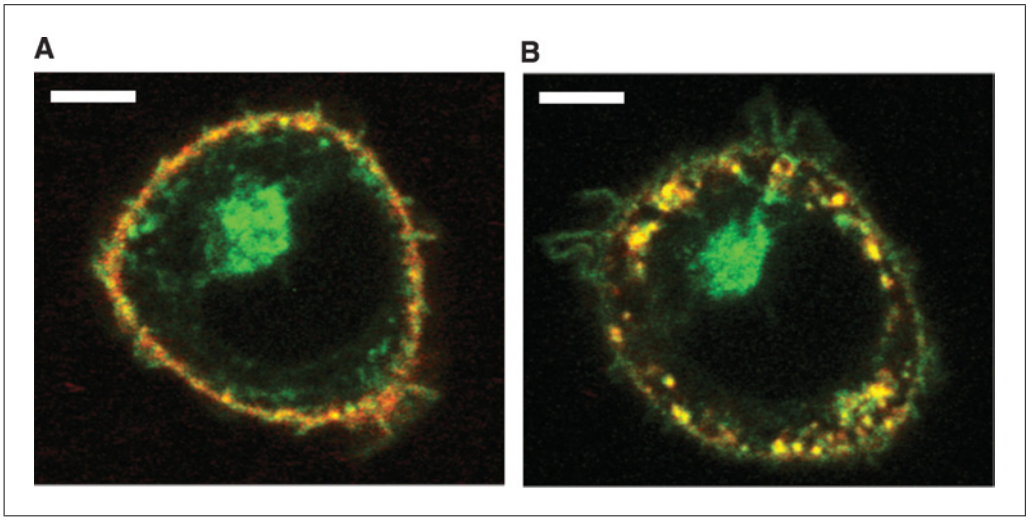


Figure 25.1.2 QD-EGF (red) bind to the surface of Chinese hamster ovary cells expressing EGFR-GFP (green) (**A**) and undergo endocytosis within minutes (**B**). Colocalization (yellow) of the QDs and receptor are seen at the membrane and after internalization in endosomes. Images acquired using a Zeiss LSM510-META system. Scale bar = 10 μm. For color version of this figure see *http://www.currentprotocols.com*.

by using a very low concentration of QD-ligand mixed with an excess of free ligand (Lidke et al., 2005a) or by binding the biotinylated-ligand (Alternate Protocol) followed by a tracer amount of SAvQDs (Echarte et al., 2007).

Materials

Logarithmically growing cells (*UNIT 1.1*)
Trypsin
Cell culture medium appropriate for the particular cell line
Tyrode's buffer plus (see recipe)
Preformed QD-ligands (Support Protocol 1 or 2)

LabTek 8-well coverslip chambers (Nunc)
Emission filters for specific QD emission wavelengths (Chroma or Omega, also available from the microscope manufacturers)

Additional reagents and equipment for culture of cells (*UNIT 1.1*)

Plate cells

1. One or two days before the experiment, trypsinize logarithmically growing cells (*UNIT 1.1*).

2. Plate the cells in LabTek 8-well coverslip chambers in complete culture medium at the appropriate density (one typically desires ~50% confluency on the day of the experiment).

   In the case of experiments with receptors that are sensitive to serum factors, the cells can be serum-starved for 4 to 16 hr prior to the experiment to reduce signaling induced by the serum in the medium.

Prepare sample for live-cell imaging with QD-ligand complexes

3. At the time of the experiment, wash the cells once with 200 µl Tyrode's buffer plus and maintain in this buffer.

   Culture medium without phenol red, containing 1% (w/v) BSA can be used for very long imaging periods (in place of Tyrode's buffer plus).

4. Place the chamber on the microscope stage and equilibrate to the desired temperature.

   The authors recommend an objective heating collar as the most effective way to keep the cells at the desired temperature during the duration of the experiment. An inexpensive and efficient setup can be purchased from Cell MicroControls (HLS8 × 0.8P and mTCII) and consists of an adjustable, flexible heating strip that can be secured around any objective with a strip of Velcro and a battery powered controller. For very long (hours to days) experiments a system having CO_2 input is necessary as well.

5. Dilute the QD-ligand complex (Support Protocol 1 or 2) to twice the desired final concentration in Tyrode's buffer plus.

   Dilute the ligand-SAvQD complex from PBS/1% BSA into Tyrode's plus just before use since the SAvQDs can aggregate with time in buffers containing divalent cations. Do not store this dilution for use on another day.

6. Add an equal volume (200 µl) of QD-ligand in Tyrode's buffer plus to the cells in the well under observation to a final concentration of 0.005 to 2 nM.

   The final concentration should be adjusted for the specific protein-ligand interaction and the known binding constants. Also, the concentration will depend on whether one wants to observe many or only a few of the receptors at one time (i.e., single-molecule tracking or measuring binding and uptake). The two-step labeling procedure is recommended if a maximal cellular response but single-molecule resolution are desired. This procedure allows using the QDs as tracers of the behavior of the macromolecule of interest.

Image acquisition

7. Initiate image acquisition either before or just after addition of the QD-ligand.

That is, when using preformed complexes, the QD-ligand can be added to the sample after images at several time points have already been acquired, taking care not to disturb the position of the sample during addition of the QD-ligand. Typically, addition of large volumes (e.g., addition of 200 μl of QD-ligand to 200 μl of buffer in chamber) results in rapid mixing without additional pipetting. The QD-ligand should also be kept at the same temperature as the imaging chamber to avoid focal drift due to temperature fluctuations. The time between images and duration of the time series acquisition is dependent on the events being monitored and must be determined by the researcher.

A 63× or 40× 1.2 NA water immersion objective is recommended.

In confocal imaging systems, simultaneous excitation of VFP and various QDs can be achieved by using different laser combinations. QDs having emissions of 605 nm and above can be excited well by 488 nm or higher wavelengths whereas lower wavelength QDs are more efficiently excited by 458-nm or 407-nm lasers. An imaging system with at least two detectors, allows the simultaneous collection of eGFP and QD signals with appropriate filters (eg., 520/20 bandpass and the appropriate QD emission bandpass filters). Single QD imaging requires high laser powers and pixel dwell times of about 6 μsec. Thus, whole 512 × 512 image acquisitions take seconds. Fast-tracking of single QDs requires more sensitive acquisition systems (see next section). For the most efficient separation of different emitting QDs in the same sample, selective QD emission filters or a spectral unmixing device should be used (Fountaine et al., 2006; Miskoski et al., 2006; see Fig 25.1.3).

Wide-field systems equipped with a mercury arc lamp most efficiently excite the QDs at 436 nm without cell damage. Image splitters and multispectral analysis systems can separate many QD and VFP labels simultaneously (Miskoski et al., 2006). EmCCD cameras can easily detect QD emission from single bound QDs with acquisition times of 50 ms or less (see Fig. 25.1.4).

Image analysis is specific to the particular question being addressed and therefore, must be developed by the researcher. There are many commercial and noncommercial resources available for image processing. Two of the most popular platforms are ImageJ, a stand-alone software available through NIH (http://rsb.info.nih.gov/ij/) and DIPimage, a

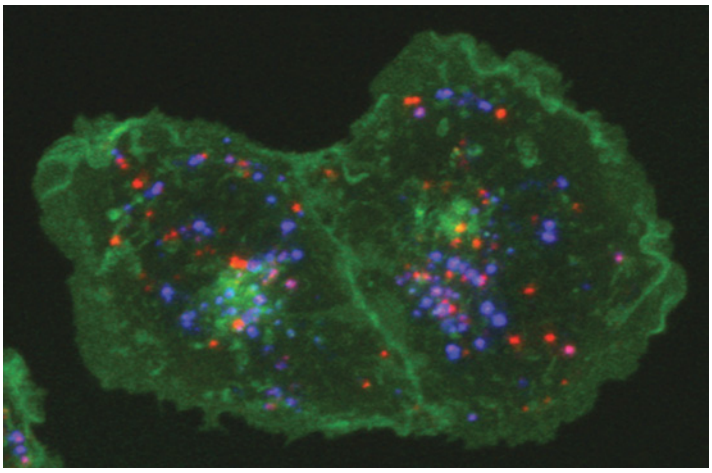


Figure 25.1.3 In a 'pulse-chase' experiment, two different colors of QD-EGF (red and blue) added to a cell expressing erbB1-eGFP (green) are used to monitor vesicle fusion. First, red QD-EGF is added and allowed to internalize. At a time point 15 min later, the blue QD-EGF is added. At a time point 22 min after addition of the blue QD-EGF, colocalization of red and blue QDs are seen in endosomes (purple), indicating that receptors activated later in time can 'catch-up' to the same compartment as those that were internalized earlier. Image acquired using a Zeiss LSM510-META system. For color version of this figure see *http://www.currentprotocols.com*.

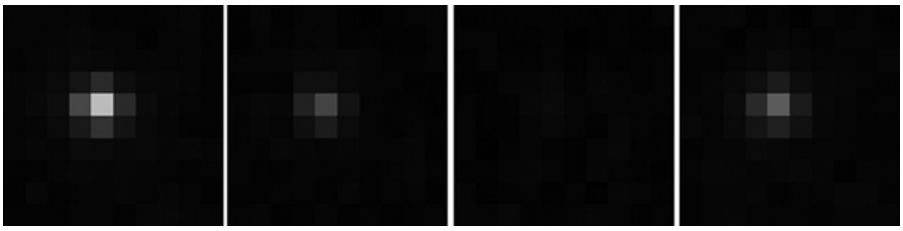


Figure 25.1.4 Single QD Blinking. Selected images from a time series showing the intermittent fluorescence of an individual QD. Time series acquired using an Olympus IX71 microscope with an Andor iXon emCCD camera. Excitation: 472-nm diode laser; emission: 655/40 QD filter. 60 msec between images, 20 msec integration time (see Lidke et al., 2005b).

scientific image processing toolbox for Matlab available from Delft University of Technology (http://www.ph.tn.tudelft.nl/DIPlib/). These two programs are free to University staff and students. A large number of plug-ins or scripts have been developed for both packages, such as masking operations, tracking algorithms, colocalization or 2-D histograms, and are freely available to the public.

LABELING CELL SURFACE RECEPTORS IN A TWO-STEP PROCEDURE

ALTERNATE PROTOCOL

In some cases, the presence of the QD may sterically hinder the QD-ligand binding to the cell surface membrane receptor. If one does not observe binding of the preformed complex, the following two-step protocol may be useful. The authors have successfully used this procedure with EGF, transferrin, and NGF receptors (Grecco et al., 2004; Lidke et al., 2004; Echarte et al., 2007).

Materials

Cells cultured in chambers or on coverslips (Basic Protocol 1)
Tyrode's buffer plus (see recipe), cold
Bio-ligand
Streptavidin-conjugated Quantum Dots (SAvQD; Invitrogen)
4% (w/v) paraformaldehyde
Tris-saline buffer
Phosphate-buffered saline (PBS; see recipe)
10° to 12°C water bath

1. Put chambers or coverslips containing cells on ice or float chamber in a water bath maintained at 10° to 12°C (recommended for cells of the immune system which are sensitive to extreme cold).
2. Wash cells once with cold Tyrode's plus and maintain in this buffer for the experiment.
3. Incubate cells with bio-ligand for 10 to 30 min.

   The concentration of bio-ligand and incubation times should be adjusted according to the binding constant of the protein-ligand interaction.

4. Wash with cold Tyrode's plus several times.
5. Add 0.05 to 2 nM cold SAvQD (diluted in Tyrode's plus) for 5 to 15 min.
6. Wash the cells several times with cold Tyrode's plus.
7. Mount for imaging.

   a. For live-cell imaging, immediately place cells on the microscope stage with appropriate temperature control and image (for more detail see Basic Protocol 1).

b. For snapshots of the behavior of the QD-ligand complex at different time points, incubate cells grown on coverslips at the appropriate temperatures and times and fix in 4% paraformaldehyde for 15 min on ice to prevent redistribution of the QDs. After fixation, wash the coverslips with Tris-saline buffer for 10 min several times and mount in PBS for imaging.

SUPPORT PROTOCOL 1

COUPLING MONOBIOTINYLATED LIGANDS TO STREPTAVIDIN-QDS

This method describes how to conjugate ligands to QDs via a biotin-streptavidin interaction. The authors have successfully used this protocol for several biotinylated ligands, including epidermal growth factor (EGF; Lidke et al., 2005a) and nerve growth factor (NGF; Echarte et al., 2007).

This protocol can be used with most ligands and proteins (such as antibodies) that have a single biotin moiety attached. The protocol uses QDs from Invitrogen that have 3 to 4 streptavidin molecules covalently bound to the QD bioconjugate shell (SAvQD). The authors recommend for most applications the PEG 2000 coated QDs since they show reduced nonspecific binding to cells and cell substrates such as polylysine or collagen.

The reader is reminded that each streptavidin can have up to four binding sites for biotin. The multi-valency of the SAvQDs can be an advantage in that it allows for the stochiometry of the QD-ligand complex to be varied by simply changing the ligand:QD ratio. A monovalent biotinylated ligand, where the biotin is conjugated to a single known residue or position, is preferred since the properties of the QD-ligand complex can be better controlled and potential cross-linking of the QDs via the ligand is avoided (see Support Protocol 2 for ligands with multiple biotins). By mixing SAvQDs, biotinylated ligand and free biotin at specific molar ratios, one can create QDs with the desired average number of attached ligands, assuming a Poisson distribution (Lidke et al., 2004).

After formation of the QD-ligand complex, unbound ligands that are smaller than the QDs can be removed from the conjugate by passing the mixture over a size exclusion column. For PEG-QDs cross-linked dextran size exclusion columns (eg, Sephadex G-25) are recommended, and for QDs without a PEG coating, polyacrylamide bead exclusion columns are most efficient (eg, Bio-gel P).

Materials

Streptavidin-conjugated Quantum Dots (SAvQD; Invitrogen)
Phosphate-buffered saline (PBS)/1% (w/v) BSA (see recipe)
Biotinylated-Ligand (bio-ligand)
PBS (see recipe)
1% (w/v) BSA

Shaker
Gel filtration spin columns, NAC-5

Prepare QD-ligand preformed complexes

1. Dilute SAvQD to 20 nM in 100 µl PBS/1% BSA.
2. Dilute bio-ligand to 20 to 60 nM in 100 µl PBS/1% BSA.

   The above concentration will yield a Poisson distribution of 1:1 to 3:1 ligands per QD. The reader should determine the valency of ligand desired for his/her particular experiment.

3. Add SAvQDs to ligand, mix with a micropipet, and vortex for 1 sec.

   Thoroughly mix the SAvQDs and ligand in equal volumes so that the stoichiometry does not vary throughout the solution at the time of mixing.

4. Incubate for at least 30 min at 4°C with gentle agitation or rotation.

Purify QD-ligand conjugates

5. Swell and pour the column material according to the manufacturer's instructions.

   For PEG-coated QDs coupled to small peptide ligands as described above, Sephadex G-25 medium-grade gel filtration is recommended; the QD-ligand complex will elute in the void volume. Use a 1:20 ratio of sample to column volume to ensure separation. Spin columns can be used for sample volumes of 50 μl or less. For non-PEG SAvQDs, Bio-gel P20 is recommended; the QDs appear in the void volume. To remove excess macromolecules from QD conjugations, ultra filtration units with 100-kDa or 200-kDa regenerated cellulose membranes are recommended (see Millipore Corporation for small volume ultracel or microcon units).

6. Equilibrate the column in PBS by washing with at least 3 column volumes.

7. Allow the buffer to just enter the top of the gel bed before adding the sample. Avoid air bubbles.

8. Add the sample to the top of the gel bed, allow the sample to enter the gel, and add PBS to elute the sample.

9. Collect the fractions containing the QDs. Add 1% (w/v) BSA (final concentration) to final product

   The purified QD-ligand complexes are stable for 4 weeks when stored at 4°C.

PREPARATION OF PREFORMED COMPLEXES OF QDS WITH LIGANDS WITH MULTIPLE BIOTINS

SUPPORT PROTOCOL 2

Some ligands must be multivalent for proper recognition by their receptors. The ligand may thus also be multi-biotinylated. The following protocol has been successfully used with such ligands and avoids cross-linking of QDs by the ligand by blocking excess biotin binding sites on SAv-QDs with free biotin shortly after addition of the ligand (see Cambi et al., 2007 for an example).

Materials

Streptavidin-conjugated Quantum Dots (SAvQD; Invitrogen)
PBS/1% (w/v) BSA (see recipe)
Bio-ligand

1. Dilute SAvQD to 20 nM in 100 μl PBS/1% BSA.

2. Dilute bio-ligand to the appropriate molarity in 100 μl PBS/1% BSA and mix with the QDs for 15 min with agitation.

   After addition of free biotin, the ligand-QD complexes can be purified as described above (Support Protocol 1, steps 3 and 4) or used directly if the free biotin and potentially free ligand do not interfere with receptor recognition.

   The absolute concentration of the ligand should be carefully determined. The number of biotins per ligand should also be determined.

   The authors recommend adding the ligand at a concentration such that the final dilution has a ratio of approximately three to six ligands to one QD and allowing binding to proceed with agitation for only 15 min followed by the addition of a 10-fold excess of free biotin to saturate any remaining streptavidin binding sites.

   The success of this procedure will depend to some extent upon the nature of the ligand. The authors assume that there are at least two available biotin binding sites for each streptavidin. Due to the geometries of the QDs, the nature of the ligand, as well as how the biotins are arrayed on its surface, not all biotins will be able to bind to the QD surface. The authors have found that polymeric ligands of ~30 to 40 kDa with approximately three biotins per polymer show no detectable cross-linking of QDs when added to the QDs at a polymer to QD ratio of 3:1 followed by excess biotin (Cambi et al., 2007).

FLOW CYTOMETRIC DETERMINATION OF THE BINDING CONSTANTS OF LIGANDS OR ANTIBODIES

Ligand binding to its receptor is the first step in transmembrane signaling. The easiest way to characterize this interaction is to determine the equilibrium binding curve, i.e., ligand binding as a function of ligand concentration. Fluorescence has largely replaced the use of radioactivity in such experiments. This protocol describes how fluorescent QDs can be used for the determination of dissociation constants.

Materials

Biotin-coupled ligand or antibody
Avidin-coated QD (Invitrogen or Evident Technologies) suitable for excitation with the flow cytometer available
Trypsin
Phosphate-buffered saline (PBS)/1% (w/v) BSA (see recipe)

Flow cytometry tube
Flow cytometer
Flow cytometric analysis software

Additional reagents and equipment for coupling the ligand or antibody of interest to QDs (Support Protocol 1) and culture of cells (*UNIT 1.1*)

1. Couple the ligand or antibody of interest to QDs (see Support Protocol 1).
2. Trypsinize cells (*UNIT 1.1*), resuspend them in PBS/1% BSA at a concentration of 5×10^6/ml.
3. Prepare a 2-fold dilution series of QD-ligand. Add 1000 µl of the highest concentration of QD-ligand made in PBS/1% BSA to a flow cytometry tube. Prepare dilutions starting from a concentration ~10× higher than the anticipated K_d. Mix thoroughly. Keep all the tubes on ice during the entire labeling procedure. Pipet 500 µl from this solution to another tube into which 500 µl of PBS/1% BSA is added yielding the second highest concentration. Repeat this procedure until the lowest concentration is reached.

   Be aware that in the presence of ligand concentration 9× higher than the K_d, 90% of the binding sites will be occupied, if binding is noncooperative.

   At the end of this step flow cytometry tubes will be filled with 500 µl of different concentrations of the QD-ligand on ice.
4. Add 20 µl of the cell suspension to each tube and incubate for 30 to 60 min on ice with regular mixing.

   The QD-ligand is diluted negligibly by the 20 µl cell suspension, since 500 µl >20 µl.
5. Measure the samples with a flow cytometer.

   Keep the cells on ice until measurement. Alternatively, samples can be fixed in 1% formaldehyde after removing unbound QD-ligand by washing twice with PBS.

   Measure a nonlabeled cell sample too, the fluorescence intensity of which will be subtracted from the mean fluorescence intensities of each sample. Pay attention to the fact that all cells in the brightest and darkest samples have to be on scale, otherwise the means will be distorted.
6. Using a flow cytometric analysis software, determine the mean fluorescence intensities of each sample gated on the FSC-SSC dot plot.
7. Plot the autofluorescence-corrected mean fluorescence intensities as a function of the total QD-ligand concentration (assumed to be equal to the free ligand concentration).

8. Fit the following, so-called Hill equation to the measured data points:

$$I = I_{\max} \frac{c^n}{c^n + K_d^n}$$

where I is the fluorescence intensity in the presence of QD-ligand concentration of c, K_d is the dissociation constant required to half-saturate the binding sites, and n is the Hill coefficient characterizing the cooperativity of binding.

If there is good reason to believe that there is no cooperativity (e.g., if one ligand binds to a monomeric receptor species), it can be assumed that $n = 1$, and a simpler equation can be used:

$$I = I_{\max} \frac{c}{c + K_d}$$

In this case K_d has real meaning characterizing the binding event, e.g., it is equal to the ratio of the dissociation and association rate constants. If $n \neq 1$, K_d lacks any direct molecular implications. Indeed, the Hill coefficient itself cannot be translated into a real molecular model, since the Hill equation is the result of a reductionist view of the molecular events taking place during complex formation.

FLOW CYTOMETRIC QUANTITATION OF THE NUMBER OF ANTIBODY BINDING SITES

BASIC PROTOCOL 3

The number of binding sites for a ligand or antibody, i.e., the total number of expressed antigens is a significant issue: for example, it has predictive value in cancer diagnosis. Flow cytometry has obvious advantages over cell suspension-based methods (e.g., radioactive binding analysis), since it is capable of resolving heterogeneity and eliminates the influence of dead cells and cell debris. The idea behind the calibration procedure is to run a series of reference beads with calibrated number of antibody binding sites or fluorescence intensities. Three approaches are widely used:

1. Beads with calibrated fluorescence intensities (e.g., Quantum FITC MESF beads from Bangs Laboratories). The kit contains several bead populations each corresponding to the fluorescence intensity of a calibrated number of fluorophores. The company provides a calibration software (QuickCal) for the calculations, which can also be easily carried out with a spreadsheet program. Different beads are available for different fluorophores. To the best of our knowledge, no such beads are available for the calibration of QD fluorescence intensities, therefore the authors will not provide a detailed protocol.

2. The Qifikit calibration kit (Dako) contains a series of beads coated with different, calibrated numbers of mouse monoclonal antibody molecules. The beads have to be labeled by the secondary antibody used for labeling of the cells. A plot of the fluorescence intensity of the beads against the number of binding sites will be constructed.

3. The Quantum Simply Cellular kit (Bangs Laboratories) contains subpopulations of beads with calibrated binding capacities for the Fc region of mouse or human antibodies. The beads have to be labeled with a primary antibody followed by a secondary, fluorochrome-conjugated antibody in the same way as the cells. A calibration plot of fluorescence intensity versus number of binding sites can be constructed.

Materials

Cells being investigated
Primary unlabeled antibody

Secondary QD-conjugated antibody against the primary antibody (Quantum Dot Corporation or Evident Technologies)
Phosphate-buffered saline (PBS)/1% (w/v) BSA (see recipe)

Qifikit calibration kit (Dako)
Vortex
Qifikit calibration beads (Dako-Cytomation, *www.dako.com*) or Quantum Simply Cellular beads (Bangs Laboratories, *www.bangslabs.com*)
Flow cytometer
Quantum Simply Cellular kit (Bangs Laboratories)

For Qifikit

1a. Label the cells under investigation with saturating concentration of the primary unlabeled antibody.

2a. After washing the cells twice with 2 ml PBS, label them with the secondary, QD-conjugated secondary $F(ab')_2$. Include a cell sample which is only labeled by the primary antibody for background correction.

Cells and beads should be labeled with secondary antibody at the same time (see step 4a).

3a. After vortexing remove 100 µl from the Setup and Calibration bead vials, centrifuge the beads 4 min at 1400 × g, 4°C, after adding 3 ml of PBS/1% BSA.

The Qifikit beads are coated with primary mouse antibodies, and can only be used for the calibration of secondary antibody binding to cells labeled by primary mouse antibodies.

4a. Remove the supernatant and label the bead pellet with the secondary QD-conjugated secondary $F(ab')_2$ under the same conditions as the cells.

This step should be done at the same time as step 2a.

5a. Run the cells and the two Qifikit bead samples (Setup and Calibration) on the flow cytometer.

6a. Subtract the fluorescence intensity of the sample labeled by the primary antibody only from the intensity of the sample labeled by both the primary and secondary antibodies (background-corrected intensity).

7a. Analyze the fluorescence of the unlabeled bead population in the Setup sample and the five subpopulations in the Calibration sample. Subtract the mean fluorescence intensity of the unlabeled bead population from the fluorescence intensities of the calibration beads. Plot the background-corrected fluorescence intensities of the calibration bead subpopulations as a function of the number of binding sites which is included in the manual for the kit. Fit a line to the data points.

8a. Find the number of antibody binding sites on the cells based on their background-corrected fluorescence intensity using the calibration line.

For Quantum Simply Cellular beads (secondary labeling)

1b. Combine one drop of each of the beads with graded number of binding sites for human or mouse antibodies.

The blank bead with no capacity for antibody binding can also be combined with the others, or can be processed separately.

The Quantum Simply Cellular beads can capture a calibrated number of mouse or human monoclonal antibodies by their Fc domain allowing them to be used for the calibration of both primarily or secondarily labeled cells. The authors have provided a protocol for the use of these beads with secondary labeling, but the principle can easily be adapted for primary labeling.

2b. After mixing the beads vortex them. Label both the cells and the beads with saturating concentration of the primary unlabeled antibody.

3b. Add 5 to 10 ml PBS, centrifuge 4 min at 1400 × *g*, 4°C, and repeat washing step once more. Label the cells and the beads with the secondary, QD-conjugated secondary $F(ab')_2$. Include a cell sample which is only labeled by the primary antibody for background correction.

4b. Add 5 to 10 ml PBS and centrifuge the samples 4 min at 1400 × *g*, 4°C. Repeat the washing step once more before running the samples on the flow cytometer.

5b. Subtract the mean fluorescence intensity of the blank bead population from the fluorescence intensities of the labeled beads. Plot the background-corrected fluorescence intensities of the calibration bead subpopulations as a function of the number of binding sites. Fit a line to the data points.

6b. Find the number of antibody binding sites on the cells based on their background-corrected fluorescence intensity using the calibration line.

The company provides an online program, QuickCal (www.bangslabs.com/flow/quickcal), which can perform the above calculations automatically.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Paraformaldehyde (PFA) fixative

Prepare a fresh 4% (w/v) solution of paraformaldehyde, analytical grade in PBS (see recipe). Use only for 1 day, keep on ice.

A 40% solution can be prepared and kept in small aliquots at −20°C. Thaw at 50°C and immediately dilute in PBS (see recipe) before use.

Phosphate-buffered saline (PBS)

137 mM NaCl
2.7 mM KCl
7.9 mM Na_2HPO_4
1.5 nM KH_2PO_4
Adjust pH to 7.3 using NaOH or HCl, if necessary
Autoclave

Store up to 6 months at room temperature

PBS/1% (w/v) BSA

Add 1% BSA to PBS (see recipe) just before use.

Tyrode's buffer

135 mM NaCl
10 mM KCl
0.4 mM $MgCl_2$
1 mM $CaCl_2$
10 mM HEPES
Adjust pH to 7.2 using NaOH
Autoclave

Store up to 6 months at room temperature

Tyrode's buffer plus

Add 20 mM glucose and 0.1% (w/v) BSA to Tyrode's buffer (see recipe) just before use.

COMMENTARY

Background Information

Published results of in-vivo QD imaging

QD are not "brighter" than conventional fluorophores since they have relatively long fluorescence emission lifetimes. Also, due to their large size (dictated by the necessity of several layers of material for passivation and biocompatibility beyond the emitting core), there may be steric hindrance to binding compared to small fluorophores in applications such as in situ hybridization or labeling to arrays like cytoskeletal components. They are most important for their extreme photostability and thus have been used most effectively as labels for in vivo imaging. In particular, their application to the study of the behavior of cell surface receptors has brought new insights at single-molecule resolution (Dahan et al., 2003; Lidke et al., 2005a; Arndt-Jovin et al., 2006; Courty et al., 2006; Echarte et al., 2007; Hagen et al., 2007). An area beyond the scope of these protocols is the application of QDs to tracking and homing in whole animal studies (see Lim et al., 2003; Ballou et al., 2004; Kim et al., 2004; Smith et al., 2006). A number of recent reviews refer to uses of QDs in imaging, flow cytometry, and immunoassays (Bruchez and Hotz, 2006; Fu et al., 2005; Michalet et al., 2005; Mulder et al., 2006; Pinaud et al., 2006; Portney and Ozkan, 2006; Prasad, 2006; Smith et al., 2006; Weng and Ren, 2006).

Special characteristics and behaviors of QDs

One of the most powerful ways to determine local proximity of biologically relevant molecules is through FRET (Förster resonance energy transfer; *UNITS 17.1 & 17.9*) between a donor and an acceptor fluorophore attached to the two molecules of interest. In the case of QDs, the large passivation and conjugation shells surrounding the fluorescent core make it difficult to use QDs for such measurements since the efficiency of transfer varies inversely with the 6th power of the distance between donor and acceptor. Additionally, the broad excitation spectrum of QDs prohibits their use as FRET acceptors since it is not possible to selectively excite the donor, resulting in a relatively small enhancement of acceptor fluorescence due to FRET. An exception is the use of a bioluminescent donor for QD-BRET as mentioned in the beginning of this unit (So et al., 2006). QDs can act as donors in a FRET pair when the acceptor lies in close proximity to the passivation shell (see Lidke et al., 2005a; Clapp et al., 2006; Pons et al., 2006). In this case the QD should not have a large bio-shell such as PEG conjugation.

Single QDs exhibit 'blinking' behavior or intermittent fluorescence (see Fig. 25.1.4). Although proper passivation of the semiconductor core reduces blinking, it is generally not possible to suppress this phenomenon completely. The blinking follows an inverse power law, as predicted for (1) an exponential distribution of trap depths or (2) a distribution of tunneling distances between QD core/interface state (Kuno et al., 2000; Shimizu et al., 2001). Hohng and Ha (2004) were able to reduce blinking of single QDs by treating them with high concentrations of thiols, a procedure that is incompatible with live-cell imaging. When tracking individual QDs attached to cell surface receptors or the cytoskeleton using high-speed acquisitions in the 20 to 50 msec range, correction for the blinking behavior must be made to avoid improper segmentation of the loci or jumping of the track between different QDs (Dahan et al., 2003; Arndt-Jovin et al., 2006). However, this blinking behavior can be used to advantage to localize individual QDs with super resolution (Lidke et al., 2005b; Hagen et al., 2007).

An advantageous property of QDs is the CdSe electron dense core that can be detected by transmission electron microscopy (Dahan et al., 2003; Nisman et al., 2004). This makes possible correlative dynamic fluorescence and high-resolution TEM studies on the same sample.

Critical Parameters and Troubleshooting

The "tail wagging the dog" problem: Quantum Dot size and valency considerations

The size of a QD is comparable to that of an antibody. Therefore, since the binding properties or biological activity of a ligand can be influenced by its conjugation to a QD, careful control experiments (such as flow cytometry

to characterize QD-ligand binding constants) must be made to establish that there is no steric influence of the QD.

QDs are multivalent, such that several ligands can be coupled to each QD. This can result in a single QDs binding multivalently to multiple receptors (Cambi et al., 2007). The researcher has to decide whether this is a concern for his or her particular problem. If so, the ligand should be coupled to the QD in a nearly 1:1 ratio (Lidke et al., 2004) and preblocking of excess binding sites with free biotin may be necessary (see Support Protocol 1).

Fixation

PFA (4%) is the preferred fixation solution for samples labeled with QDs. The QD fluorescence is destroyed by freezing or fixation in −20°C methanol. In addition, the use of various mounting mediums must be tested to ensure that the medium does not degrade the QD fluorescence. As QDs degrade, their emission gradually shifts to lower wavelength and this can be particularly precarious when several spectrally distinct QDs are present. To avoid such problems, store fixed samples in PBS at 4°C, mount fixed samples in PBS, and image within 24 hr of fixation.

Nonspecific binding

The authors recommend the PEG 2000-coated QDs for most cellular applications since they show reduced nonspecific binding to cells and cell substrates, such as polylysine or collagen. In addition, the presence of high concentrations of BSA and the use of a higher pH will help to reduce nonspecific binding. The reader is reminded that stock solutions of QDs are maintained by Invitrogen in 1 M trimethylglycine at pH 8.5 and dilution into buffers containing divalent cations will, over time, result in aggregation and precipitation of the QDs.

Controls

It is essential to prove that the QD signal is reflective of the uncoupled ligand's interaction with the receptor of interest and that the QDs do not interfere, when appropriate, with the ligand's physiological activity. Controls for such behavior include showing that (1) unconjugated QDs do not bind to the cell surface, (2) QD-ligands do not bind to cells not expressing the membrane protein of interest, (3) presaturation of the cell surface receptors with unlabeled ligand blocks QD-ligand binding, and (4) addition of QD-ligand mimics unconjugated ligand. In addition, flow cytometry (Basic Protocols 2 and 3) can be used to characterize QD-ligand binding constants and correlation with free ligand binding (Lidke et al., 2004).

Other bioconjugation techniques

As described above, many types of QDs with different bioconjugation are available. In addition, manufacturers sell kits for alternative bio-coupling methods. See the manufacturers' Web sites for more information (*http://probes.invitrogen.com/p1roducts/qdot* and *http://www.evidenttech.com*). For a review of other labeling methods see Smith et al. (2006).

Flow cytometric determination of binding constants

Antibodies and ligands typically induce the internalization of their receptor. In order to prevent this from happening, both the cell suspension and the ligand solutions have to be kept on ice for the duration of the experiment. If samples are not fixed before measurement, internalization can take place in the instrument as well. The sample can spend a significant amount of time at room temperature in flow cytometers into which multiple samples are loaded simultaneously (e.g., with a sample loader or on a 96-well plate). In such cases, the samples should be fixed before measurement. Before fixation the unbound ligand has to be removed by washing with excess PBS. Dissociation of bound ligands starts immediately after the ligand concentration drops in the medium. Therefore, washing can introduce errors into the measurement. Consequently, it is best to use flow cytometers into which single tubes are loaded one-by-one, and use unfixed cells without washing. This can be done since most flow cytometers are equipped with a constant background subtraction algorithm, therefore the contribution of extracellular fluorescence is reliably removed.

The authors strongly advise against using the Scatchard analysis, i.e., a plot of the ratio of bound ligand concentration to free ligand concentration as a function of bound ligand concentration, for fitting. In flow cytometric experiments the measured fluorescence intensities can be used instead of the bound ligand concentration. Before nonlinear regression programs became widely available, scientists transformed data to make linear plots. If the Scatchard plot can be fitted with a single line, the x intercept and the slope of the line correspond to the maximum number of binding sites (maximum fluorescence intensity) and the negative reciprocal value of the K_d, respectively. Although the shape of a Scatchard

plot reveals potentially important information about the nature of binding (positive or negative cooperativity in the case of concave down and concave up Scatchard plots, respectively), the fitting results will be distorted due to error propagation inherent in the transformation leading to the Scatchard plot.

If the fluorescence intensity does not saturate, the following possibilities have to be investigated:

1. Nonspecific binding: nonspecific binding sites are usually very difficult to saturate. Dead cells may have significant non-specific binding.

2. Ligand depletion: in most analyses it is assumed that the amount of bound ligand is negligible compared to the total amount available. If the number of binding sites is high, this assumption does not hold, and the free ligand concentration can be significantly lower than the total (ligand depletion). In such a case saturation of binding takes place at a much higher total ligand concentration than without ligand depletion. The ligand can also bind to the wall of the flow cytometric tube leading to a similar phenomenon.

3. Ligand binding can be compromised by coupling to QD.

Flow cytometric determination of the number of antibody binding sites

In the Quantum Simply Cellular kit the same primary antibody is used for labeling the beads and the cells whereas the Qifikit beads are coated with a mouse monoclonal antibody. Since secondary antibodies may show slight variation with regard to binding to primary antibodies, there is some advantage to the Quantum Simply Cellular in that the secondary antibody binds to the same primary antibody on both cells and beads. In most cases (especially if the same isotype is used for labeling the cells and coating the bead), this is unlikely to be a major source of error. On the other hand, the primary antibody is captured by its Fc domain by the Quantum Simply Cellular beads, a different orientation from when the Fab domain binds to its epitope. It is unknown to us whether the different orientation of the primary antibodies has any influence on the binding of the secondary antibody.

If the calibration line is prepared using the background-corrected fluorescence intensities of the calibration beads (as described in the above protocols), the calibration line should cross the origin of the plot, or have a *y* intercept very close to zero. The calibration curve is expected to be strictly linear, i.e., a correlation coefficient of >0.98 and no splaying at high concentrations. If the curve is nonlinear or it does not cross the *y* axis at zero, nonspecific binding of the antibody or incomplete labeling of the beads may have happened. Check the quality of the antibodies used and whether the beads were mixed regularly during labeling.

If the fluorescence intensity of the cells is higher than that of the brightest bead population, the calibration line can be extrapolated. But it has to be kept in mind that at very high densities antibody binding may not be strictly proportional to the number of antibody binding sites. Although the number of antibody binding sites on the different bead population may show batch-to-batch variation, the range of the Qifikit beads usually extends higher ($\sim 5 \times 10^5$) than that of the Quantum Simply Cellular beads ($\sim 2 \times 10^5$).

Anticipated Results

Support Protocols 1 and 2: Collection of eluted fractions from the size exclusion column or recovery of the QD fraction after filtration should result in a purified QD-ligand complex. The presence of QDs can be confirmed by recording an absorbance or emission spectrum. Concentration of QDs can be calculated from the peak absorbance value if one knows the extinction coefficient (available from the manufacturer) for that particular QD.

Basic Protocol 1: During the imaging times series, the researcher should observe a strong QD signal localized at the position of the receptor (either on the membrane or internalized in vesicles). Addition of unlabeled QDs should not result in QD binding to the cell surface. Large aggregates should not be seen in the images—this would indicate cross-linking of the QDs by ligand and the complex formation conditions should be adjusted as discussed in the Alternate Protocol.

For all of the basic protocols, detection limits depend on the imaging conditions. Under the right conditions and with sensitive emCCD cameras one can detect single QD binding as indicated in the Background Information section and literature cited therein. It is difficult to detect single QDs in raster scanning confocal microscopes in live samples, but possible in fixed material with long pixel dwell times and line averaging.

Time Considerations

Support Protocol 1. Preparation and mixing of reagents to form QD-ligand complexes should take 15 to 20 min. Complexes should incubate for at least 30 min. The time for

purification depends on whether spin columns, filters, or gravity columns are used, between 30 min to 2 hr is usual.

Basic Protocol 1. Replating of cells into imaging chambers takes ~30 min. The time for an imaging experiment is dependent on the processes being observed and the length of the time series acquired. Quantitative image analyses of image time series can be run as batch jobs once the conditions of the experiments have been standardized and macros or plug-ins to the basic image processing software have been concatenated. Tracking of individual QDs must normally be controlled by operator intervention to avoid analysis of false traces.

Basic Protocols 2 and 3. The labeling and the flow cytometric experiment can be carried out in ~2 hr and analysis typically takes ~1 hr.

Acknowledgements

The authors would like to thank Thomas M. Jovin for discussions and expert advice and Alessandra Cambi and Keith Lidke for results from collaborations.

Literature Cited

Alivisatos, A.P., Gu, W.W., and Larabell, C. 2005. Quantum dots as cellular probes. *Annu. Rev. Biomed. Eng.* 7:55-76.

Arndt-Jovin, D.J., Lopez-Quintela, M.A., Lidke, D.S., Rodriguez, M.J., Martinez, S.F., Lidke, K.A., Hagen, G.M., and Jovin, T.M. 2006. In vivo cell imaging with semiconductor quantum dots and noble-metal nanodots. *Proc. SPIE* 6096:60960P1-P10.

Ballou, B., Lagerhold, B.C., Ernst, L.A., Bruchez, M.P., and Waggoner, A.S. 2004. Noninvasive imaging of quantum dots in mice. *Bioconjug. Chem.* 15:79-86.

Bruchez, M., and Hotz, T. 2006. Quantum Dots. Applications in Biology. Humana Press, Totowa, N.J.

Cambi, A., Lidke, D., Arndt-Jovin, D., Figdor, C., and Jovin, T.M. 2007. Ligand-conjugated quantum dots monitor antigen uptake and processing by dendritic cells. *Nano Lett.* 7:970-77.

Chattopadhyay, P.K., Price, D.A., Harper, T.F., Betts, M.R., Yu, J., Gostick, E., Perfetto, S.P., Goepfert, P., Koup, R.A., De Rosa, S.C., Bruchez, M.P., and Roederer, M. 2006. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat. Med.* 12:972-977.

Chudakov, D.M., Lukyanov, S., and Lukyanov, K.A. 2005. Fluorescent proteins as a toolkit for in vivo imaging. *Trends Biotechnol.* 23:605-613.

Clapp, A.R., Medintz, I.L., and Mattoussi, H. 2006. Forster resonance energy transfer investigations using quantum-dot fluorophores. *Chemphyschem.* 7:47-57.

Courty, S., Luccardini, C., Bellaiche, Y., Cappello, G., and Dahan, M. 2006. Tracking individual kinesin motors in living cells using single quantum-dot imaging. *Nano. Lett.* 6:1491-1495.

Dahan, M., Levi, S., Luccardini, C., Rostaing, P., Riveau, B., and Triller, A. 2003. Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. *Science* 302:442-445.

Echarte, M.M., Bruno, L., Arndt-Jovin, D.J., Jovin, T.M., and Pietrasanta, L.I. 2007. Quantitative single particle tracking analysis of NGF-receptor complexes: Transport is bidirectional but biased by longer retrogrde run lengths. *FEBS Lett.* 581:2905-2913.

Fountaine, T.J., Wincovitch, S.M., Geho, D.H., Garfield, S.H., and Pittaluga, S. 2006. Multispectral imaging of clinically relevant cellular targets in tonsil and lymphoid tissue using semiconductor quantum dots. *Mod. Pathol.* 19:1181-1191.

Fu, A.H., Gu, W., Larabell, C., and Alivisatos, A.P. 2005. Semiconductor nanocrystals for biological imaging. *Curr. Opin. Neurobiol.* 15:568-575.

Gao, X.H., Yang, L., Petros, J.A., Marshall, F.F., Simons, J.W., and Shuming, N. 2005. In vivo molecular and cellular imaging with quantum dots. *Curr. Opin. Biotechnol.* 16:63-72.

Grecco, H.E., Lidke, K.A., Heintzmann, R., Lidke, D.S., Spagnuolo, C., Martinez, O.E., Jares-Erijman, E.A., and Jovin, T.M. 2004. Ensemble and single particle photophysical properties (two-photon excitation, anisotropy, FRET, lifetime, spectral conversion) of commercial quantum dots in solution and in live cells. *Microsc. Res. Tech.* 65:169-179.

Hagen, G., Caarls, W., Thomas, M., Hill, A., Lidke, K., Rieger, B., Fritsch, C., van Geest, B., Jovin, T., and Arndt-Jovin, D. 2007. Biological applications of an LCoS-based programmable array microscope (PAM). *Proc. SPIE* 6441:64410S1-12.

Hohng, S. and Ha, T. 2004. Near-complete suppression of quantum dot blinking in ambient conditions. *J. Am. Chem. Soc.* 126:1324-1325.

Kim, S., Lim, Y.T., Soltesz, E.G., De Grand, A.M., Lee, J., Nakayama, A., Parker, J.A., Mihaljevic, T., Laurence, R.G., Dor, D.M., Cohn, L.H., Bawendi, M.G., and Frangioni, J.V. 2004. Near-infrared fluorescent type II quantum dots for sentinel lymph node mapping. *Nat. Biotechnol.* 22:93-97.

Kogure, T., Karasawa, S., Araki, T., Saito, K., Kinjo, M., and Miyawaki, A. 2006. A fluorescent variant of a protein from the stony coral Montipora facilitates dual-color single-laser fluorescence cross-correlation spectroscopy. *Nat. Biotechnol.* 24:577-581.

Kuno, M., Fromm, D.P., Hamann, H.F., Gallagher, A., and Nesbitt, D.J. 2000. Nonexponential "blinking" kinetics of single CdSe quantum dots: A universal power law behavior. *J. Chem. Phys.* 112:3117-3120.

Lidke, D.S., Nagy, P., Heintzmann, R., Arndt-Jovin, D.J., Post, J.N., Grecco, H.E., Jares-Frijman, E.A., and Jovin, T.M. 2004. Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nat. Biotechnol.* 22:198-203.

Lidke, D.S., Lidke, K.A., Rieger, B., Jovin, T.M., and Arndt-Jovin, D.J., 2005a. Reaching out for signals: Filopodia sense EGF and respond by directed retrograde transport of activated receptors. *J. Cell Biol.* 170:619-626.

Lidke, K.A., Rieger, B., Jovin, T.M., and Heintzmann, R., 2005b. Superresolution by localization of quantum dots using blinking statistics. *Optics Express* 13:7052-7062.

Lim, Y.T., Kim, S., Nakayama, A., Stott, N.E., Bawendi, M.G., and Frangioni, J.V. 2003. Selection of quantum dot wavelengths for biomedical assays and imaging. *Mol. Imaging.* 2:50-64.

Mattheakis, L.C., Dias, J.M., Choi, Y.J., Gong., J., Bruchez, M.P., Liu, J., and Wang, E. 2004. Optical coding of mammalian cells using semiconductor quantum dots. *Anal. Biochem.* 327:200-208.

Michalet, X., Pinaud, F.F., Bentolila, L.A., Tsay, J.M., Doose, S., Li, J.J., Sundaresan, G., Wu, A.M., Gambhir, S.S., and Weiss, S. 2005. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* 307:538-544.

Miskoski, S., Giordano, L., Etchehon, M.H., Menendez, G., Lidke, K.A., Hagen, G.M., Jovin, T.M., and Jares-Erijman, E.A. 2006. Spectroscopic modulation of multifunctionalized quantum dots for use as biological probes and effectors. *Proc. SPIE* 6096:60960X1-X7.

Mulder, W.J.M., Strijkers, G.J., van Tilborg, G.A., Griffioen, A.W., and Nicolay, K. 2006. Lipid-based nanoparticles for contrast-enhanced MRI and molecular imaging. *N.M.R. Biomed.* 19:142-164.

Nisman, R., Dellaire, G., Ren, Y., Li, R., and Bazett-Jones, D.P. 2004. Application of quantum dots as probes for correlative fluorescence, conventional, and energy-filtered transmission electron microscopy. *J. Histochem. Cytochem.* 52:13-18.

Pinaud, F., Michalet, X., Bentolila, L.A., Tsay, J.M., Doose, S., Li, J.J., Iver, G., and Weiss, S. 2006. Advances in fluorescence imaging with quantum dot bio-probes. *Biomaterials* 27:1679-1687.

Pons, T., Medintz, I.L., Sykora, M., and Mattoussi, H. 2006. Spectrally resolved energy transfer using quantum dot donors: Ensemble and single-molecule photoluminescence studies - art. no. 245302. *Physical Review B* 73:245302.

Portney, N.G., and Ozkan, M., 2006. Nano-oncology: Drug delivery, imaging, and sensing. *Anal. Bioanal. Chem.* 384:620-630.

Prasad, P.N. 2006. Emerging opportunities at the interface of photonics, nanotechnology and biotechnology. *Mol. Cryst. Liq. Cryst.* 446:1-10.

Shaner, N.C., Steinbach, P.A., and Tsien, R.Y. 2005. A guide to choosing fluorescent proteins. *Nat. Methods* 2:905-909.

Shimizu, K.T., Neuhauser, R.G., Leatherdale, C.A., Empedocles, S.A., Woo, W.K., and Bawendi, M.G. 2001. Blinking statistics in single semiconductor nanocrystal quantum dots. *Physical Review B* 63:2053161-2053165.

Smith, A.M., Ruan, G., Rhyner, M.N., and Nie, S. 2006. Engineering luminescent quantum dots for In vivo molecular and cellular imaging. *Ann. Biomed. Eng.* 34:3-14.

So, M.K., Xu, C., Loening, A.M., Gambhir, S.S., and Rao, J. 2006. Self-illuminating quantum dot conjugates for in vivo imaging. *Nat. Biotechnol.* 24:339-343.

Weng, J.F. and Ren, J.C. 2006. Luminescent quantum dots: A very attractive and promising tool in biomedicine. *Cur. Med. Chem.* 13:897-909.

Fabrication and Application of Nanofibrous Scaffolds in Tissue Engineering

Wan-Ju Li[1, 2] and Rocky S. Tuan[1]

[1]National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland
[2]University of Wisconsin-Madison, Madison, Wisconsin

ABSTRACT

Nanofibers fabricated by electrospinning are morphological mimics of fibrous components of the native extracellular matrix, making nanofibrous scaffolds ideal for three-dimensional cell culture and tissue engineering applications. Although electrospinning is not a conventional technique in cell biology, the experimental setup may be constructed in a relatively straightforward manner, and the procedure can be carried out by individuals with limited engineering experience. Here, we detail a protocol for electrospinning of nanofibers and provide relevant specific details concerning the optimization of fiber formation (Basic Protocol 1). The protocol also includes conditions required for preparing biodegradable polymer solutions for the fabrication of nonwoven and aligned nanofibrous scaffolds suitable for various cell/tissue applications. In addition, information on effective cell loading into nanofibrous scaffolds and cellular constructs grown in a bioreactor is provided (Basic Protocol 2). Instructions for building the electrospinning apparatus are also included (see the Support Protocol). *Curr. Protoc. Cell Biol.* 42:25.2.1-25.2.12.

Keywords: nanofiber • electrospinning • scaffold • tissue engineering • three-dimensional culture

INTRODUCTION

Recent advances in nanotechnology and increasing recognition of the potential of nanomaterials as biocompatible and biomimetic scaffolds for cells have provided new tools for tissue engineering and the development of three-dimensional (3D) cell cultures (Christenson et al., 2007). In a native tissue environment, cells are embedded within and are in contact with the extracellular matrix (ECM), which acts both as a structural supporter, as well as a regulator of cell activities. Biochemical and mechanical signals derived from cell-ECM interactions are critical to cell survival and functions. Similarly, in vitro, cells interact intimately with and respond to cues from their matrix environment, further highlighting that cell activities are strongly influenced by the physical and chemical properties of the culture environment (Berrier and Yamada, 2007). A 3D, highly porous substrate that mimics the native ECM can form unique spatial interactions with cells, and is thought to more accurately replicate cell-matrix interactions in vivo, compared to two-dimensional (2D) substrates commonly used for tissue culture (Griffith and Swartz, 2006). To meet the requirements for various tissue engineering applications, a variety of 3D scaffolds of different compositions have been developed. Nanofibers produced by electrospinning represent a promising candidate scaffold biomaterial for tissue engineering (Li et al., 2005). These fibers possess unique physical characteristics, such as high surface area to volume ratio and improved mechanical strength, compared to their micro-scaled counterparts. Nanofibrous scaffolds also morphologically resemble the fibrillar components of the native ECM, enhance adsorption of cell adhesion molecules, induce favorable cell-ECM interactions, maintain cell phenotype, support differentiation

Current Protocols in Cell Biology 25.2.1-25.2.12, March 2009
Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2502s42

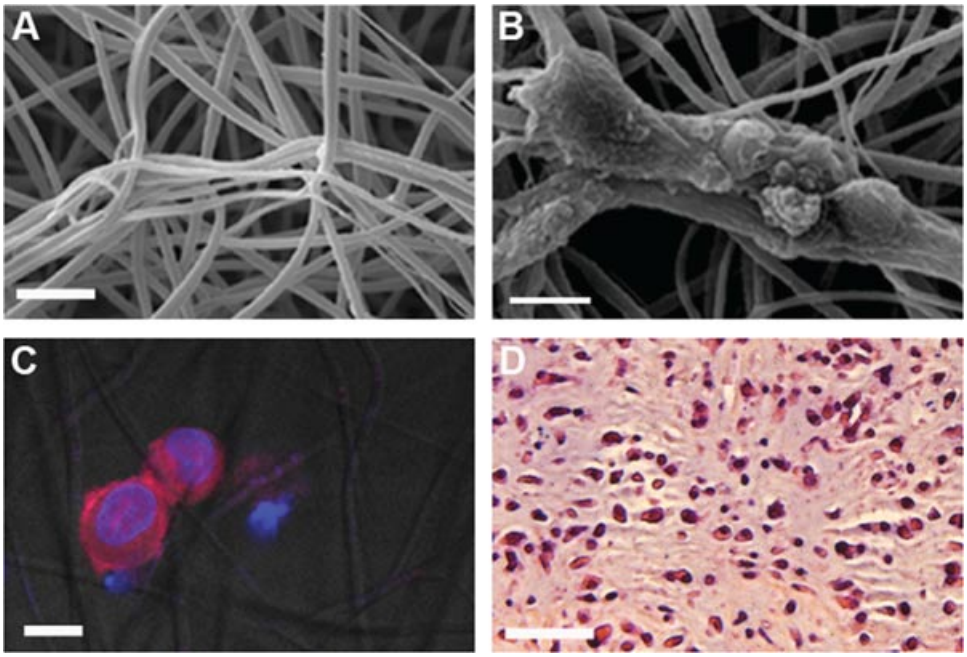


Figure 25.2.1 Application of electrospun poly(L-lactic) acid (PLLA) nanofibrous scaffold seeded with chondrocytes for cartilage tissue engineering. (**A**) Scanning electron micrograph (SEM) of electrospun PLLA nanofibers showing interconnecting pores defined by randomly oriented ultrafine fibers with diameters ranging from 500 to 900 nm. (**B**) SEM view of aggregates of globular cells growing in chondrocyte-seeded nanofibers on culture day 28 (reproduced with permission from Mary Ann Liebert, Inc.). (**C**) Confocal laser scanning microscopy showing punctate, disorganized actin cytoskeleton distributed cortically in cells after 28 days of culture (red: TRITC-phalloidin labeled actin filaments; blue: DAPI labeled nuclei). (**D**) Hematoxylin-eosin histology of chondrocyte-nanofiber composites on culture day 28 showing oval or round cells encased in extracellular matrix-rich lacunae, characteristic of hyaline cartilage. Bar: (A, B) 5 μm, (C) 10 μm, (D) 50 μm. For color version of this figure go to *http://www.currentprotocols.com*.

of stem cells, and promote cell-matrix adhesion and activate appropriate cell signaling pathways (Fig. 25.2.1; Li et al., 2006b).

What Is Electrospinning?

The electrospinning technique uses a high electric field to produce ultra-fine polymeric fibers with diameters ranging from a few nanometers to a few micrometers. The mechanism of electrospinning nanofibers is based on a complex electro-physical activity between polymer solution and electrostatic force. In this procedure, a high-voltage electric field is set up between the injection needle and the collecting screen using a power supply and electrodes. When the polymer solution is extruded slowly from the syringe, a semispherical polymer solution droplet is formed at the tip of the needle. With increasing voltage, the charged polymer droplet elongates to form a conical shape known as the Taylor cone (Taylor, 1969), and the surface charge on the polymer droplet increases with time. Once the surface charge overcomes the surface tension of the polymer droplet, a polymer jet is initiated (Taylor, 1964). The solvent in the polymer jet evaporates during its travel to the collecting screen, increasing the surface charge on the jet. This increase in surface charge induces instability in the polymer jet as it passes through the electric field. To compensate for this instability, the polymer jet divides geometrically, first into two jets, and then into many more as the process repeats itself. The formation of nanofibers results from the action of the spinning force provided by the electrostatic force on the continuously splitting polymer droplets. Nanofibers are deposited layer-by-layer on the metal target plate, forming a nonwoven nanofibrous mat. During the electrospinning process,

both extrinsic and intrinsic parameters are known to affect the structural morphology of the nanofibers (Doshi and Reneker, 1995). Specifically, extrinsic parameters, such as environmental humidity and temperature, and intrinsic parameters, including applied voltage, working distance, and conductivity and viscosity of the polymer solution, need to be optimized to produce uniform nanofibers. The two major structures usually found in the nanofibrous mat are a uniform, continuous fibrous structure or a bead-containing fibrous structure. Variation in the relative abundance of these two structures is determined by the relative contributions of the parameters during the electrospinning process.

Nanofibrous biomaterials, by virtue of their structural and morphological characteristics, are well suited as tissue engineering scaffolds for various tissue types, particularly musculoskeletal tissues such as cartilage, bone, tendon, and ligament. In principle, the structural functions of ECM components, such as collagen fibers, can be fulfilled by the morphologically similar nanofibers in the engineered tissues. Nanofiber-based 3D biomaterials may also serve as biomimetic substrates to culture normal and transformed cells for cell biology research.

STRATEGIC PLANNING

To fabricate nanofibrous scaffolds, an electrospinning apparatus is required. Given that this is not commercially available, one must construct the apparatus. This unit provides a straightforward, do-it-yourself protocol for constructing an electrospinning apparatus (see Support Protocol). Although relatively simple in design, the electrospinning apparatus described here is capable of producing high-quality nanofibers for experimental applications. The nanofiber electrospinning process starts with the preparation of biodegradable or nonbiodegradable, natural/synthetic polymer solutions. Polymer solutions are prepared by mixing the chosen polymer with the suitable solvent(s). The solvent can be either a single chemical component or a mixture of multiple chemicals, depending on the chemistry of the chosen polymer. In general, the solvent must be capable of completely dissolving the polymer and carrying an applied charge. This unit will focus on how to fabricate nanofibrous scaffolds with a non-woven or an aligned fiber structure, composed of clinically used, Food and Drug Administration (FDA) approved synthetic biodegradable polymers (see Basic Protocol 1).

The four intrinsic parameters mentioned above—applied voltage, working distance, and conductivity and viscosity of the polymer solution—must be adjusted and optimized to produce a uniform fiber. These parameters are dependent on the nature of the polymer used and thus vary with different polymers. After electrospinning, the finished nanofibrous scaffold needs to be sterilized before use for cell culture. Heat or γ–irradiation cannot be used to sterilize nanofibers since the fibers are likely to be damaged by these treatments. Nanofibrous scaffolds can be effectively sterilized by ultraviolet light (UV) and ethanol. The step-by-step procedure detailing effective cell seeding into nanofibrous scaffolds, including aspiration using bibulous filter paper to increase cell-infiltration efficiency, is described in Basic Protocol 2. To enhance nutrient access to cell-seeded nanofibrous constructs, rotary wall vessel (RWV) bioreactors are used to facilitate nutrient/metabolic waste exchange in and out of the 3D construct; the procedure for culturing cellular nanofibrous constructs in a bioreactor is described in Basic Protocol 2.

FABRICATION OF NANOFIBROUS SCAFFOLDS

BASIC PROTOCOL 1

A number of polymers, both natural and synthetic, which may be biodegradable or nonbiodegradable, may be used as raw biomaterials to fabricate nanofibrous scaffolds. With the discovery and synthesis of new polymers, the list of candidate polymers suitable for electrospinning continues to grow. Synthetic biodegradable polymers can be tailored to have the specified, desired properties, including degradation profile and mechanical

properties, over those of natural polymers. Biodegradable poly(α-hydroxy esters) are the most widely used polymers for the fabrication of tissue engineered scaffolds, given their FDA approval and well-documented biocompatible properties (Gunatillake and Adhikari, 2003). In addition to each member of the poly(α-hydroxy esters) family having defined chemical and physical properties, the addition option of mixing or co-polymerizing two or more of poly(α-hydroxy esters) at varying ratios to generate materials with a wide spectrum of properties is another attractive characteristic of the poly(α-hydroxy esters).

There are currently six FDA-approved poly(α-hydroxy esters), including poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), poly(D,L-lactic acid) (PDLLA), poly(D,L-lactic-*co*-glycolic acid 50:50) (PLGA5050), poly(D,L-lactic-*co*-glycolic acid 85:15) (PLGA8515), and poly(ε-caprolactone) (PCL), that have been successfully used to produce nanofibers. However, only PLLA and PCL have slow degradation rates suitable for cell culture applications (Li et al., 2006a). In this section, fabrication of PLLA and PCL nanofibrous scaffolds will be described in the step-by-step protocol. Besides nonwoven fibrous scaffolds, the electrospinning technique is also capable of producing aligned fibrous scaffolds using an alternative setup (Theron et al., 2001). The matrix of aligned synthetic nanofibers mimics that of uni-axial collagen fibers found in native tissues and is suitable for growing of tissues with anisotropic structure, such as striated muscle and tendon/ligament. Typically, nanofibrous scaffolds are characterized on the basis of fiber morphology, observed using scanning electron microscopy, degradation profile, porosity, and mechanical properties.

Materials

PLLA (mol. wt. 50,000; Polysciences)
N,N-Dimethyformamide (DMF; Fisher Scientific)
Chloroform (Fisher Scientific)
PCL (mol. wt. 80,000; Aldrich)
Tetrahydrofuran (THF; Fisher Scientific)

Glass threaded vials
Analytical balance
Parafilm
Vortex mixer
Aluminum foil
Electrospinning apparatus (see Support Protocol)
Glass slides
Glass coverslips
10-ml glass syringe fitted with an 18-G blunt needle
Sharp surgical blade
Vacuum desiccator

Prepare polymer solution

1. Dissolve 1.6 g of PLLA polymer in 11 ml of chloroform/DMF (10:1) in a glass vial.

   More polymer solution can be made using the same proportions if desired.

2. Dissolve 2 g of PCL polymer in 14 ml of THF/DMF (1:1) in a glass vial.

   More polymer solution can be made using the same proportions if desired.

   NOTE: *Raw polymers before use should be stored at 4°C in a tight container within a sealed bag. When taking out of storage, allow the container to reach room temperature before opening to prevent moisture condensation.*

3. Screw the cap of the glass vial tight and wrap the vial cap with Parafilm.

4. Vortex the polymer solution in the glass vial overnight.

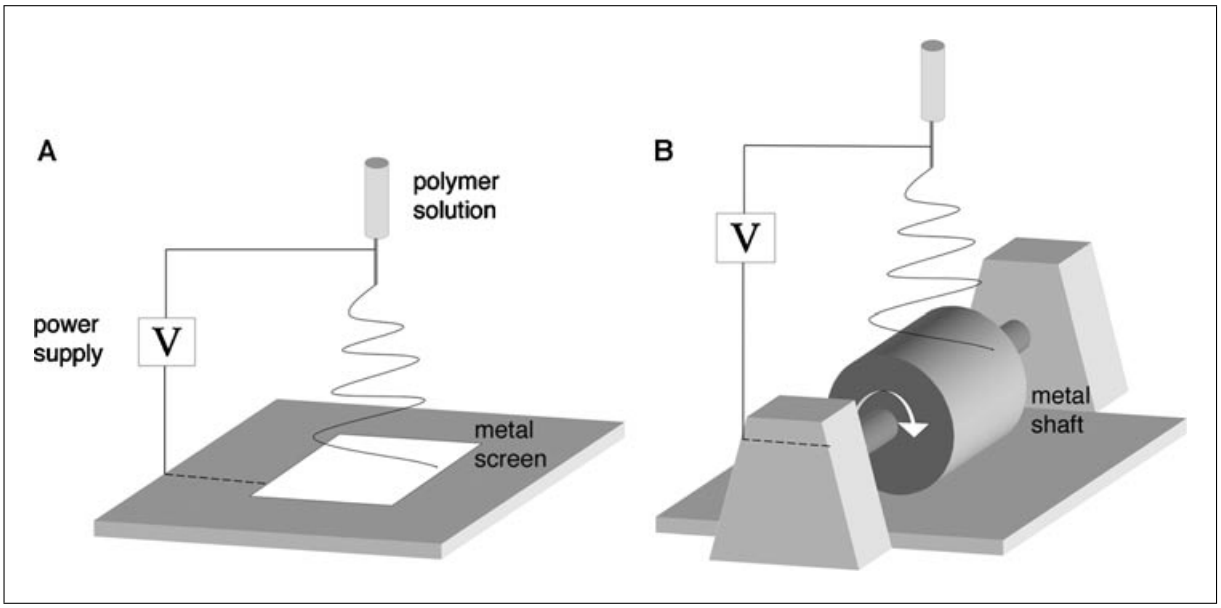


Figure 25.2.2 Two basic setups of electrospinning apparatus. Nonwoven nanofibrous scaffolds can be produced and collected on a flat metal plate (**A**), whereas aligned nanofibrous scaffolds can be collected onto a rotary metal shaft (**B**).

Electrospin a nonwoven nanofibrous scaffold

5a. Place a sheet of aluminum foil onto the fiber collection screen in the bottom of the electrospinning apparatus (see Fig. 25.2.2A).

The sheet of aluminum foil used to collect nanofibers should be wrinkle-free since the wrinkled surface results in uneven fiber deposition. The sheet of aluminum foil must be handled with care.

To directly coat nanofibers onto glass slides or coverslips, use clean forceps to pick up the slides/coverslips and place them onto the surface of the aluminum foil sheet with only a small distance separating them.

6a. Mount the 10-ml glass syringe fitted with an 18-G blunt needle vertically in the electrospinning apparatus, and transfer the homogeneously mixed polymer solution into the syringe.

7a. Adjust the distance between the tip of the needle and the fiber collection screen to 20 cm.

8a. Turn on the high-voltage power supply and set the voltage at 16 kV for PLLA or 12 kV for PCL.

Unwanted non-uniformed polymer droplets form before the voltage is correctly established. Use a sheet of paper towel to cover the collection screen and then remove it after the desired voltage is reached.

The fiber spinning process usually requires a period of several hours. Check the process periodically. The needle can become clogged if undissolved polymer or impurity is present. When this happens, turn off the power and then unclog the needle.

9a. Using a sharp surgical blade, cut the nanofibrous sheet and gently remove it from the fiber collection screen.

For nanofiber-coated glass slides or coverslips, cut the fibers along the edge of the slides or coverslips. Make sure that all the excess, connected nanofibers are excised from the edges of the slides/coverslips and cut clean before they are separated from each other.

10a. Place the nanofibrous sheet or the nanofiber-coated glass slides/coverslips in a vacuum desiccator for 2 days to remove the organic solvent residue.

Electrospin an aligned nanofibrous scaffold

5b. Remove the stationary copper plate collection screen and replace with the rotary metal shaft or mandrel (see Fig. 25.2.2B).

6b. Place a sheet of aluminum foil to fully cover the shaft.

The sheet of aluminum foil used to collect nanofibers should be wrinkle-free since the wrinkled surface results in uneven fiber deposition. The sheet of aluminum foil must be handled with care.

To directly coat nanofibers onto glass slides or coverslips, use clean forceps to pick up the slides/coverslips and place them onto the surface of the aluminum foil sheet with only a small distance separating them. Secure the slides/coverslips with adhesive tape to the surface of the rotary shaft. In addition, a protective plastic shield should be set up in the front of the electrospinning apparatus to contain accidental, flying objects.

7b. Mount the 10-ml glass syringe fitted with an 18-G blunt needle vertically in the electrospinning apparatus, and transfer the homogeneously mixed polymer solution into the syringe.

8b. Adjust the distance between the tip of the needle and the fiber collection shaft to 20 cm.

9b. Adjust the rotation of the rotary shaft to the desired speeds(s) with the variable rheostat transformer.

The rotation speed determines the extent of the fiber alignment. The faster the rotation speed the greater the extent of alignment. Under the conditions described here, >90% fiber alignment is achieved when the linear speed of the shaft exceeds 9.3 meters/sec.

10b. Turn on the high-voltage power supply and set the voltage at 16 kV for PLLA or 12 kV for PCL.

Unwanted non-uniformed polymer droplets form before the voltage is correctly established. Place a sheet of paper towel between the needle and the rotary shaft to cover the fiber collection surface and then remove it after the desired voltage is reached.

11b. Using a sharp surgical blade, cut the nanofibrous sheet and gently remove it from the fiber collection surface of the shaft.

For nanofiber-coated glass slides or coverslips, cut the fibers along the edge of the slides or coverslips. Make sure that all the excess, connected nanofibers are excised from the edges of the slides/coverslips and cut clean before they are separated from each other.

12b. Place the nanofibrous sheet or the nanofiber-coated glass slides/coverslips in a vacuum desiccator for 2 days to remove the organic solvent residue.

The nanofibrous sheets/slides/coverslips should be used for experiments as soon as they are produced. If storage is needed, the product should be stored in a vacuum desiccator.

BASIC PROTOCOL 2

NANOFIBROUS SCAFFOLDS FOR CELL CULTURING

Cell seeding into a 3D, porous biomaterial scaffold is the first and critical step for the formation and growth of tissue engineered constructs in vitro. Because of the complex 3D structure that makes cell infiltration difficult, cell seeding into a 3D scaffold is more challenging and less effective compared to conventional monolayer culturing. For nanofiber-coated surfaces, such as glass slides or coverslips, cell seeding by natural gravity is a convenient and practical approach. However, cell seeding into a 3D nanofibrous scaffold constructed with interwoven ultrafine fibers requires different methods to

improve efficiency. One preferred method uses bibulous filter paper to aspirate cells into the 3D scaffold in a guided manner.

Bioreactors are culture devices that circulate the culture medium to promote cell and tissue growth via enhanced nutrient transport and mechanical stimulation (Martin et al., 2004). Tissues cultured in appropriate bioreactors show increased weight and enhanced cellular phenotype, compared to tissues grown in static cultures, e.g., in tissue culture dishes, plates, or flasks (Martin et al., 2000). Various bioreactor designs have been developed and used for tissue engineering applications. The rotary wall vessel (RWV) bioreactor, originally developed to simulate microgravity by randomizing gravity (Martin et al., 2004), is a commercially available device (Synthecon), and is suitable for growing large-sized engineered tissue in vitro. Constructs cultured in a RWV bioreactor encounter low shear stress when the rotation speed of the culture chamber is set to balance the weight of the construct and the hydrodynamic force (Lappa, 2003). The randomized gravity environment inside the chamber permits the cultured tissue construct to undergo continuous free-fall with maximal enhancement of medium diffusion into the construct.

Materials

Graded ethanol series [70% (v/v), 50% (v/v), and 25% (v/v) ethanol, see recipe]
Sterile distilled water
Hank's Balanced Salt Solution (HBSS), sterile
Cell suspension in 10% serum-containing culture medium (cell density = 20×10^6/ml; see recipe for culture medium)
Serum-containing culture medium (see recipe)

Surgical blade
Nanofibrous sheet/scaffolds (see Basic Protocol 1)
Petri dish
UV light source
Bibulous filter paper (Whatman 3 MM)
Pipettor
Multi-well culture plate
Tissue culture incubator
Rotary wall vessel (RWV) bioreactor (Synthecon)

Prepare nanofibrous scaffolds for cell seeding

1. Using a surgical blade, cut the nanofibrous sheet (1-mm thick) into the desired shape and size.

   The use of scissors should be avoided because the compressive cutting force will deform the edges of the scaffolds.

2. Gently remove the fibrous scaffolds from the aluminum foil and place them in the clean petri dish.

3. Sterilize the nanofibrous scaffolds under UV irradiation of each side for 30 min in a sterile, laminar-flow hood.

4. Soak the nanofibrous scaffolds in 70% (v/v) ethanol for 30 min.

   Air bubbles often are trapped inside the nanofibrous scaffold. To ensure complete sterilization and hydration, trapped air bubbles must be removed, which can be done by gently rolling a sterile glass rod over the nanofibrous scaffold to squeeze out the air bubbles.

5. Remove the 70% (v/v) ethanol and hydrate the nanofibrous scaffolds with sequential washes of 50% (v/v) ethanol, 25% (v/v) ethanol, sterile distilled water, and finally sterile HBSS for 30 min each.

6. Store the nanofibrous scaffolds in the HBSS while preparing cells for seeding.

7. Place the nanofibrous scaffold onto a sterile piece of bibulous filter paper.
8. Seed the desired number of cells by quickly pipetting the cell suspension onto the nanofibrous scaffold, as the solution is being aspirated into the scaffold.

   The ideal cell seeding density in a nanofibrous scaffold depends on cell types and applications. For example, the density of 400,000 cells/cm^2 is used to study chondrogenesis of mesenchymal stem cells.

9. Place the cell-loaded nanofibrous scaffold into wells of the multi-well culture plate.

   The nanofibrous scaffold may become dry if left on the filter paper too long. Cell seeding into the scaffolds should be done one at a time, with other scaffolds kept in HBSS.

   Replace the wet filter paper as needed to maintain an efficient absorption capability.

10. Maintain the culture plate in the tissue culture incubator. Add a small volume of additional cell medium periodically during the next 2 hr to prevent desiccation.
11. Add appropriate amount of culture medium into the wells for regular static culture. For cultures to be maintained in the RWV bioreactor, culture the cell-seeded scaffolds in culture plates for 3 days before transferring to the bioreactor.

Culture cellular nanofibrous constructs in RWV bioreactor

12. Gently transfer the cell-based nanofibrous constructs to the sterilized RWV bioreactor chamber and reassemble the chamber.

    The choice of the RWV chamber size is based on the dimension of the samples being cultured. The internal space of the chamber should be large enough for the samples to move freely. Multiple samples may be placed in a single chamber, provided that the constructs do not collide with one another when the chamber is rotating.

13. Fill the chamber completely with culture medium and purge all air bubbles.
14. Mount the chambers to the bioreactor base and adjust the rotation speed until the samples appear to be suspended in the culture medium.
15. Maintain cellular constructs in the bioreactor for at least 21 days to allow maturation of the engineered tissue.
16. Collect the cultured tissue from the bioreactor and analyze it using cellular and molecular biology techniques.

SUPPORT PROTOCOL

CONSTRUCTION OF AN ELECTROSPINNING APPARATUS

The construction and setup of an electrospinning apparatus is presented here. The electrospinning apparatus is composed of three major components: a high-voltage power supply, a polymer solution reservoir (e.g., a syringe, with a small diameter needle) and an accessory flow control pump, and a metal collecting screen. The high-voltage power supply with several independent adjustable controls and independently functioning DC outputs should be capable of providing multiple power levels up to 50-kV. A syringe is used to store the polymeric solution and a metal needle is connected to the electrodes of the power supply to deliver a charged polymer jet. Polymer flow can be driven by gravity when the syringe is placed vertically. However, a syringe pump is usually used for precise control of flow rate. The fiber-collecting screen should be conductive and can be either a stationary plate or a rotary platform. As described above, the plate is used to obtain nonwoven fibers, while a rotary platform is used to obtain aligned fibers.

There are two basic electrospinning setups, vertical and horizontal. In the former, nanofibers move downwards vertically to the fiber collection screen, whereas in the latter, nanofibers travel horizontally to the target plate. Both setups function well in the production of nanofibers, and the selection of the setup is a matter of preference. With

computer-controlled automation, the setup can be transformed into a more sophisticated one capable of fabricating complex nanofibrous structures. For example, controlled delivery using multiple jets can be used to fabricate a single nanofibrous scaffold that is composed of multiple layers, with each layer derived from a different polymer. In addition, the multiple jet setup is more efficient for the production of a large quantity of scaffolding material. Generally, a basic setup is sufficient for the purpose of making nanofibrous scaffolds for tissue engineering and 3D culture experiments in the laboratory. The protocol described here is for constructing a basic model of the vertical electrospinning apparatus.

An isolated or low-traffic space in the laboratory is needed to set up the electrospinning apparatus, since a high-voltage charge is utilized during electrospinning, and this is potentially hazardous. To enhance safety, a protective casing, made of nonconductive plastic, is commonly used to enclose the entire electrospinning apparatus. The enclosure also minimizes variations in environmental factors, such as temperature and humidity, which affect nanofiber formation. In addition, proper venting via a chemical hood is required to remove vapor from volatile chemical solvents.

Materials

High-density polyethylene (HDPE) plate
Polycarbonate plate
HDPE square rods
Wooden plate (20-in. × 20-in.)
Copper plate (10-in. × 10-in.)
Teflon plate (20-in. × 20-in.)
Glass syringe
18-G blunt metal needle
High-voltage DC power supply

Construct closed apparatus box

1. Build the electrospinning apparatus box (20-in. × 20-in. × 30-in.; Fig. 25.2.3) by assembling the top (see A in Fig. 25.2.3), the bottom (see B in Fig. 25.2.3), the back (see C in Fig. 25.2.3), and the two side (see D & E in Fig. 25.2.3) HDPE panels.

   Black or dark color plastic is preferred to construct the back panel of the enclosure box, to provide better observation of fiber formation, since electrospun nanofibers are seen as white ultra-fine fibers.

2. Install the transparent polycarbonate front door (see F in Fig. 25.2.3).

   The transparent door provides protected observation of the electrospinning process.

3. Attach the two adjustable-length HDPE arms (see G in Fig. 25.2.3) onto the inside of the top panel of the box.

   Each of the HDPE arms, with one end attached to the top, is composed of three interconnecting square rods.

   The free end of the arms has a drilled hole to function as the syringe holder.

   The length of the arms should be adjustable to permit a distance range of 5 to 30 cm between the tip of the needle and the fiber collection screen.

4. Place the wooden plate (see H in Fig. 25.2.3), with a 5-cm hole at the center to allow the negative/ground wiring to pass through, on the bottom plastic plate

5. Place the copper plate (see I in Fig. 25.2.3), which serves as the fiber-collecting screen, on top of the wooden plate.

   The copper plate functions as the negative/ground target that needs to be connected to the negative/ground charge of the power supply during electrospinning. A connector, such as

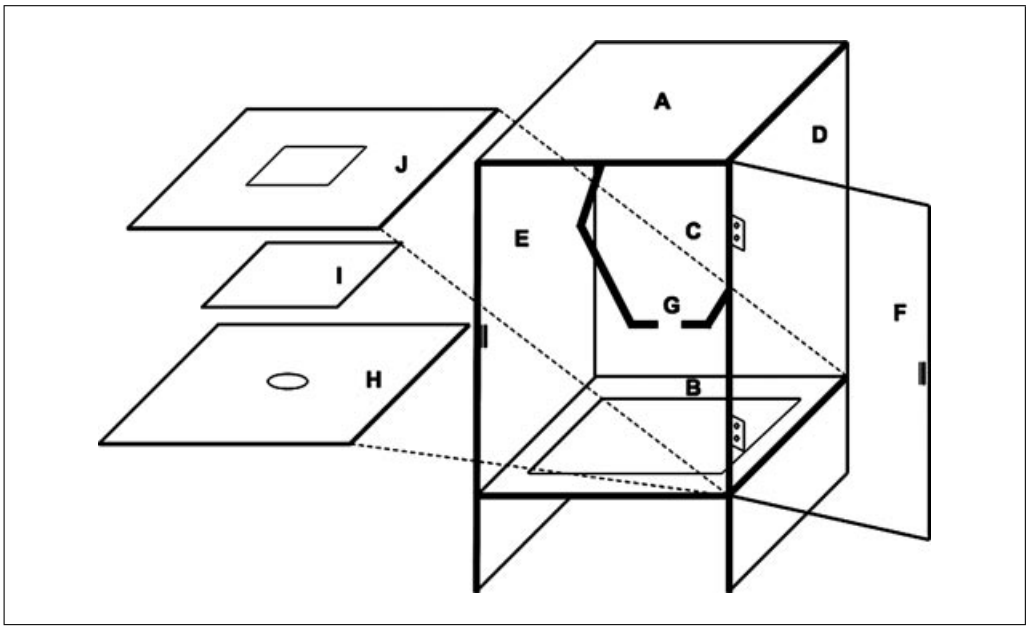


Figure 25.2.3 Schematic of electrospinning apparatus box. The box is constructed with HDPE plates representing the top (A), bottom (B), back (C), and two side (D&E) panels, and the front polycarbonate door (F). Each of the HDPE arms (G), with one end attached to the top, is composed of three inter-connecting square rods, and is flexible for height adjustment of the syringe holder. Inside the box, a Teflon plate (J) is placed on top of the copper plate (I) that is on top of the wooden plate (H).

a banana socket, may be installed at the center of the copper plate to connect with the negative/ground charge.

6. Place a Teflon plate (see J in Fig. 25.2.3), the same size as the wooden plate and with a square opening in the center, on top of the copper plate,

   The Teflon plate is nonconductive and is used to electrically mask the copper plate underneath, such that nanofibers are only allowed to deposit on the exposed area of the copper plate defined by the opening. The area of the opening thus determines the size of the finished electrospun nanofibrous sheet.

7. Place the needle-attached syringe in the holder.

8. Connect the positive charge output to the needle and the negative charge to the copper plate.

   See Critical Parameters for guidelines to operating the electrospinning apparatus.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Graded ethanol series

25% ethanol in sterile, distilled water
50% ethanol in sterile, distilled water
70% ethanol in sterile, distilled water
Store at room temperature until used to sterilize the nanofibers in the preparation process for cell seeding

Serum-containing culture medium

DMEM culture medium containing 10% fetal bovine serum
Store up to 2 weeks at 4°C and warm to 37°C in water bath before use

COMMENTARY

Background Information

Electrospinning was developed based on the "electrospray" phenomenon first described by Lord Rayleigh in 1882 (Rayleigh, 1882). He discovered that a highly charged droplet would break down into smaller droplets when passing through a voltage gradient, as the result of Coulombic repulsion disrupting the droplet surface tension. Instead of producing small droplets in the electrospraying process, electrospinning produces continuous long fibers. In 1934, Formhals was granted a series of U.S. patents on the applications of using the technique to make fine fibers from a cellulose acetate solution (Formhals, 1934). Recently, the surging interest in nanotechnology and tissue engineering has engendered renewed attention to this technology.

Critical Parameters and Troubleshooting

Electrospinning is a complex electrophysical process in which both extrinsic and intrinsic parameters affect nanofiber formation (Doshi and Reneker, 1995). Uniform nanofibers are produced only under optimal conditions where both extrinsic and intrinsic parameters work together seamlessly. In general, the intrinsic parameters are more critical in determining the nanofiber structure than the extrinsic parameters.

Polymer solution viscosity

Viscosity of a polymer solution, directly proportional to polymer concentration, is the most critical parameter influencing nanofiber uniformity and size (Fong et al., 1999). Every polymer solution has its specific viscosity range in which uniform nanofibers can be produced, and the range varies with the polymer or solvent types (Liu and Hsieh, 2002). Therefore, each polymer solution needs to be individually characterized to assess the optimal viscosity range. In addition, viscosity affects fiber size; a more viscous polymer solution makes larger fibers (Deitzel et al., 2001).

Applied voltage and working distance

Applied voltage is the electro-driving force for nanofiber spinning, and usually a voltage of at least 5 kilovolts DC is required for electrospinning. Working distance is defined as the distance between the syringe tip and the fiber collection screen, and together with applied voltage, can determine the strength of applied charge in the electro-static field. Similar to polymer solution viscosity, charge strength also has to be optimized to a range appropriate for uniform nanofiber formation.

Polymer solution conductivity

Polymer solution conductivity is primarily determined by the nature of the polymer and solvent, and the availability of ionizable salts. It has been shown that a more conductive polymer solution carrying a more electric charge during electrospinning, with the as-spun fibers generating a stronger repulsive force, tends to produce more uniform nanofibers. Different approaches, such as using dipolar aprotic solvents (Lee et al., 2003) and adding conductive agents (Zong et al., 2002) in the polymer solution are applied to enhance polymer solution conductivity.

The biggest challenge with electrospinning is finding the optimal combination of polymer solution viscosity and conductivity, applied voltage, and working distance for electrospinning. If the optimal combination is not reached, the electrospun structure turns into beads or bead-strings, instead of fibers. When bead-containing structures occur, increasing polymer solution viscosity should be the first parameter to vary in order to improve fiber formation. Increasing polymer solution conductivity can also be tested for the purpose of reducing bead formation.

Anticipated Results

Support Protocol. A basic, functioning electrospinning apparatus box should be assembled for electrospinning nonwoven or aligned nanofibers.

Basic Protocol 1. After overnight mixing by vortexing, the polymer solution turns into a homogeneously viscous and transparent liquid. During electrospinning, the researcher should observe that the polymer solution comes out of the needle, transforms into nanofibers, and deposits onto the fiber collection screen. A white nanofibrous mesh spot should be observed on the screen, gradually increasing its dimensions and eventually covering the entire screen. After electrospinning, a thick fabric-like nanofibrous mat is produced.

Basic Protocol 2. Both PLLA and PCL nanofibrous scaffolds are hydrophobic but should be completely hydrated by immersion in a graded series of ethanol solutions. After the hydration step, the researcher should observe that the nanofibrous scaffolds are easily hydrated in HBBS. Using labeled cells and subsequently microscopy, cells should

be observed adhering to nanofibers after cell seeding and culturing in serum-containing medium.

Time Considerations

Support Protocol. The electrospinning apparatus box can be constructed by a skilled mechanic in a few days.

Basic Protocol 1. It takes <10 min to prepare the polymer solution for vortexing and both PLLA and PCL can be completely dissolved overnight. The time needed for electrospinning nanofibers depends on the amount and the conductivity of polymer solutions. In general, it takes 8 hr for PLLA and 35 hr for PCL. After a nanofibrous mat is produced, placement for 2 days in a vacuum desiccator is preferred for the complete removal of harmful chemical residue.

Basic Protocol 2. The preparation of nanofibrous scaffolds for cell seeding takes ~4 hr, including 1 hr for sample cutting and 3 hr of sterilization/hydration using UV irradiation and a graded ethanol series. The time needed for cell seeding varies, depending on sample number. It takes ~40 seconds to complete the cell seeding per scaffold. To optimize cell viability, cell seeding should be controlled within the 40-sec time frame, if possible.

Acknowledgement

This work is supported by the Intramural Research Program of the National Institutes of Health (Z01 AR 41131).

Literature Cited

Berrier, A.L. and Yamada, K.M. 2007. Cell-matrix adhesion. *J. Cell Physiol.* 213:565-573.

Christenson, E.M., Anseth, K.S., van den Beucken, J.J., Chan, C.K., Ercan, B., Jansen, J.A., Laurencin, C.T., Li, W.J., Murugan, R., Nair, L.S., Ramakrishna, S., Tuan, R.S., Webster, T.J., and Mikos, A.G. 2007. Nanobiomaterial applications in orthopedics. *J. Orthop. Res.* 25:11-22.

Deitzel, J.M., Kleinmeyer, J., Harris, D., and Tan, N.C.B. 2001. The effect of processing variables on the morphology of electrospun nanofibers and textiles. *Polymer* 42:261-272.

Doshi, J. and Reneker, D.H. 1995. Electrospinning process and applications of electrospun fibers. *J. Electrostatics* 35:151-160.

Fong, H., Chun, I., and Reneker, D.H. 1999. Beaded nanofibers formed during electrospinning. *Polymer* 40:4585-4592.

Formhals 1934. U.S. Patent 1,975,504.

Griffith, L.G. and Swartz, M.A. 2006. Capturing complex 3D tissue physiology in vitro. *Nat. Rev. Mol. Cell Biol.* 7:211-224.

Gunatillake, P.A. and Adhikari, R. 2003. Biodegradable synthetic polymers for tissue engineering. *Eur. Cell Mater.* 5:1-16.

Lappa, M. 2003. Organic tissues in rotating bioreactors: Fluid-mechanical aspects, dynamic growth models, and morphological evolution. *Biotechnol. Bioeng.* 84:518-532.

Lee, K.H., Kim, H.Y., Khil, M.S., Ra, Y.M., and Lee, D.R. 2003. Characterization of nanostructured poly(ε-caprolactone) nonwoven mats via electrospinning. *Polymer* 44:1287-1294.

Li, W.J., Mauck, R.L., and Tuan, R.S. 2005. Electrospun nanofibrous scaffolds: Production, characterization, and applications for tissue engineering and drug delivery. *J. Biomed. Nanotechnol.* 1:259-275.

Li, W.J., Cooper, J.A. Jr., Mauck, R.L., and Tuan, R.S. 2006a. Fabrication and characterization of six electrospun poly(alpha-hydroxy ester)-based fibrous scaffolds for tissue engineering applications. *Acta Biomater.* 2:377-385.

Li, W.J., Shanti, R.M., and Tuan, R.S. 2006b. Electrospinning technology for nanofibrous scaffolds in tissue engineering. *In* Nanotechnologies for the Life Sciences, Vol. 9: Tissue, Cell, and Organ Engineering (C.S.S.R. Kumar, ed.) pp. 135-187. Wiley-VCH, Weinheim, Germany.

Liu, H.Q. and Hsieh, Y.L. 2002. Ultrafine fibrous cellulose membranes from electrospinning of cellulose acetate. *J. Polym. Sci. B Pol. Phys.* 40:2119-2129.

Martin, I., Obradovic, B., Treppo, S., Grodzinsky, A.J., Langer, R., Freed, L.E., and Vunjak-Novakovic, G. 2000. Modulation of the mechanical properties of tissue engineered cartilage. *Biorheology* 37:141-147.

Martin, I., Wendt, D., and Heberer, M. 2004. The role of bioreactors in tissue engineering. *Trends Biotechnol.* 22:80-86.

Rayleigh, L.J. 1882. On the equilibrium of liquid conducting masses charged with electricity. *Phil. Mag.* 14:184-186.

Taylor, G. 1964. Disintegration of water drops in an electric field. *Proc. R. Soc. London, Ser. A.* 280:383-397.

Taylor, G. 1969. Electrically driven jets. *Proc. R. Soc., London, Ser. A.* 313:453-475.

Theron, A., Zussman, E., and Yarin, A.L. 2001. Electrostatic field-assisted alignment of electrospun nanofibers. *Nanotechnology* 12:384-390.

Zong, X.H., Kim, K., Fang, D.F., Ran, S.F., Hsiao, B.S., and Chu, B. 2002. Structure and process relationship of electrospun bioabsorbable nanofiber membranes. *Polymer* 43:4403-4412.

CHAPTER 26
Viruses

INTRODUCTION

Although Jenner, in the 1790's, and Pasteur in the 1880's, developed methods for protecting people from smallpox and rabies, respectively, the nature of the agents causing these diseases remained obscure. In the late 1800's, it was demonstrated that tobacco mosaic disease could be transmitted by filtrates of diseased tobacco plants even after passing through filters fine enough to exclude the then smallest known living organisms. It became clear that the disease-causing agent was not merely a liquid toxin but something that "grew" in its host. This same virus, the tobacco mosaic virus, some 50 years later became the first virus whose structure was elucidated in detail.

Over 100 years ago, Peyton Rous suggested that a virus could cause cancer. A number of other diseases, most notably yellow fever, influenza, and polio, were subsequently shown to have viral etiologies. Later still, hepatitis, AIDS, and cervical cancer were added to the list of viral diseases. Given the linkage to important human and animal diseases, it is no wonder that the field of virology blossomed throughout the 20th century.

Virology has been considered traditionally as a branch of microbiology, but its impact has been truly far-reaching. Viruses, in particular bacteriophage, were critical tools in the emerging field of molecular biology owing to their ability to move genetic material from one host cell to another. Because viruses infect and utilize the cellular machinery of their hosts for replication, no small measure of what we know about that machinery has come from studying the viral "hijacking" of it. Because viruses infect cells, the boundary between the fields of virology and cell biology has always been blurry. Knowledge gained about the pathogen invariably led to increased understanding of the host and vice versa. In this chapter of *Current Protocols in Cell Biology*, the methods of virology will be described with a focus on those methods that are most closely related to cell biology.

Like bacteriophage, eukaryotic viruses can also be utilized in the movement of genetic material by design. In *UNIT 26.1*, the methods involved in the production of gene transfer vectors based on papillomaviruses are presented. The papillomaviruses are a diverse group of viruses that infect the skin and mucosal tissues. They have perhaps gained most notoriety for being the causal agent of cervical cancer, the incidence of which is second only to breast cancer in women worldwide. *UNIT 26.1* focuses on the production and propagation of pseudoviruses based on papillomaviruses that have utility as gene-delivery vehicles both in vitro and in vivo.

The BK virus (BKV) is a member of the polyomavirus family, to which many in the population have been exposed, usually without adverse consequences. However the BKV can cause nephritis in renal transplant patients on immunosuppressive medications. Little is known concerning the means of BKV transmission, but in renal transplant patients, it may be transferred with the transplanted organ. *UNIT 26.2* describes handling, propagation, and assessment of infectivity of BKV, as well as the labeling of the virus to analyze cell entry.

Viruses can be studied in tissue culture, but certain types of studies are facilitated by animal models. Respiratory viruses represent a major class of human pathogens affecting millions of individuals worldwide. *UNIT 26.3* describes a number of protocols related to

Current Protocols in Cell Biology 26.0.1-26.0.2, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2600s47

utilizing a mouse model for the study of respiratory viruses. Protocols are included for introducing viruses into the lungs of mice and assessing the impact of the infection on the lungs. Tissue sampling methods are described along with procedures for virus quantification and isolation. Methods for lung histology, immunocytochemistry, and in situ hybridization of the infected lungs are also provided.

The ability to study viral infection in culture systems varies widely. For example, it is possible to culture neurons in a trichambered system that allows the cell bodies to be in one chamber and the axons growing from the cell bodies to be in another. This physical isolation allows the cell body and axons to be independently exposed to viruses. Such a culture system is the subject of *UNIT 26.4*. The assembly of the trichambered system is described along with its use in exploring viral spread using immunofluorescence and electron microscopy.

Characterization of HIV-1 interactions with host cells is critical for cell biology studies of HIV-1. *UNIT 26.5* describes a set of methods and protocols to perform quantitative assays of HIV-1 binding, internalization, infection, and cell-cell transmission. Protocols are included for generating viral stocks and conducting HIV-1 binding and internalization assays as well as for quantification of the viral infection and assays for cell-to-cell transmission of the virus. These protocols provide tools enabling HIV-1 infection and transmission on a quantitative basis.

Monitoring respiratory syncytial virus (RSV) replication is described in *UNIT 26.6*. This unit contains protocols that range from the design of animal infection protocols, to extracting viruses for culture, to RNA extraction and quantification by quantitative real-time PCR. The unit provides methods of quantifying viral infection via simultaneous viral culture and PCR, two approaches that are often used independently. This combination reveals certain features of RSV replication, such as abortive replication, that would not be revealed with either of the methods in isolation.

Joe B. Harford

Production of Papillomavirus-Based Gene Transfer Vectors

Christopher B. Buck[1] and Cynthia D. Thompson[1]

[1]National Cancer Institute, Center for Cancer Research, Bethesda, Maryland

ABSTRACT

Papillomaviruses are a diverse group of pathogens that infect the skin and mucosal tissues of humans and various animal species. The viral genome is a circular, double-stranded DNA molecule ~8-kb in length. The non-enveloped papillomavirus capsid is composed of a virally encoded major coat protein, L1, and a minor coat protein, L2. L1 and L2 co-assemble when expressed in mammalian cells, and can promiscuously encapsidate essentially any <8-kb plasmid present in the cell nucleus. In the last several years, there has been rapid development of techniques for intracellular production of papillomavirus-based gene transfer vectors (also known as pseudoviruses). This unit outlines the production and propagative amplification of papillomaviral vectors. The system represents a highly tractable method for converting pre-existing mammalian expression plasmids into infectious virus stocks. The resulting vectors have utility for in vitro, as well as in vivo gene delivery applications. *Curr. Protoc. Cell Biol.* 37:26.1.1-26.1.19. © 2007 by John Wiley & Sons, Inc.

Keywords: papillomavirus • HPV • pseudovirus • pseudovirion • virus-like particle • VLP

INTRODUCTION

The papillomavirus capsid proteins L1 and L2 can, when co-expressed in mammalian cells, co-assemble and package heterologous nonviral DNA into infectious particles that resemble authentic papillomavirus virions (Buck et al., 2004, 2005). Essentially any transfected <8-kb expression plasmid present in the cell nucleus can be encapsidated. The high degree of promiscuity of packaging makes it possible to convert transfected reporter plasmids into papillomaviral vector stocks capable of delivering the encapsidated plasmid to a wide variety of cultured cell types. Until recently, such papillomaviral vectors (also known as pseudoviruses) have primarily been used for analysis of papillomavirus neutralization and infectious entry pathways. However, papillomaviral vectors are beginning to show promise as tractable general-purpose gene transfer vehicles. Applications include high-level overexpression of genes of interest in cultured cells, as well as in vivo gene delivery in mouse model systems (Roberts et al., 2007).

The production system relies on a human embryonic kidney cell line, 293TT, which expresses high levels of the simian virus 40 (SV40) large T antigen (Buck et al., 2004). In primate cell lines, SV40 T antigen drives high-level replication of plasmids carrying the SV40 early promoter/origin of replication (*ori*), which is present on a wide variety of commercially available mammalian expression plasmids. In Basic Protocol 1, an expression plasmid encoding a gene or genes of interest, as well as the SV40 *ori*, is co-transfected into 293TT cells along with a second plasmid, p16L1L2, that drives expression of the L1 and L2 proteins of human papillomavirus type 16 (HPV16). After transfection, both the expression plasmid of interest and p16L1L2 are taken up (encapsidated) into infectious L1/L2 capsids. The resulting viral seed stock can be used to infect fresh 293TT cells, permitting high-yield, low-cost amplification of the vector (Basic Protocol 2; Buck et al., submitted). Although crude vector stocks can be used for a variety of applications, it

Current Protocols in Cell Biology 26.1.1-26.1.19, December 2007
Published online December 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2601s37

is also possible to purify infectious capsids by ultracentrifugation (Support Protocol 1) or gravity-flow gel filtration (Support Protocol 2). Fractions containing capsids can be screened by the BCA assay, agarose gel electrophoresis (Support Protocol 3), or SDS-PAGE (*UNIT 6.1*). Although it should not generally be necessary to do so, purified capsids can be concentrated by dialysis against a concentrating reagent (Support Protocol 4).

If the plasmid being packaged expresses a fluorescent protein, the infectivity of the vector stock can readily be titered using flow cytometry or fluorescent microscopy (Support Protocol 5). Alternative Protocols 1 and 2 cover, respectively, the production of amplification-incompetent papillomaviral vectors (see Fig. 26.1.1) and bulk production of papillomavirus capsids.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

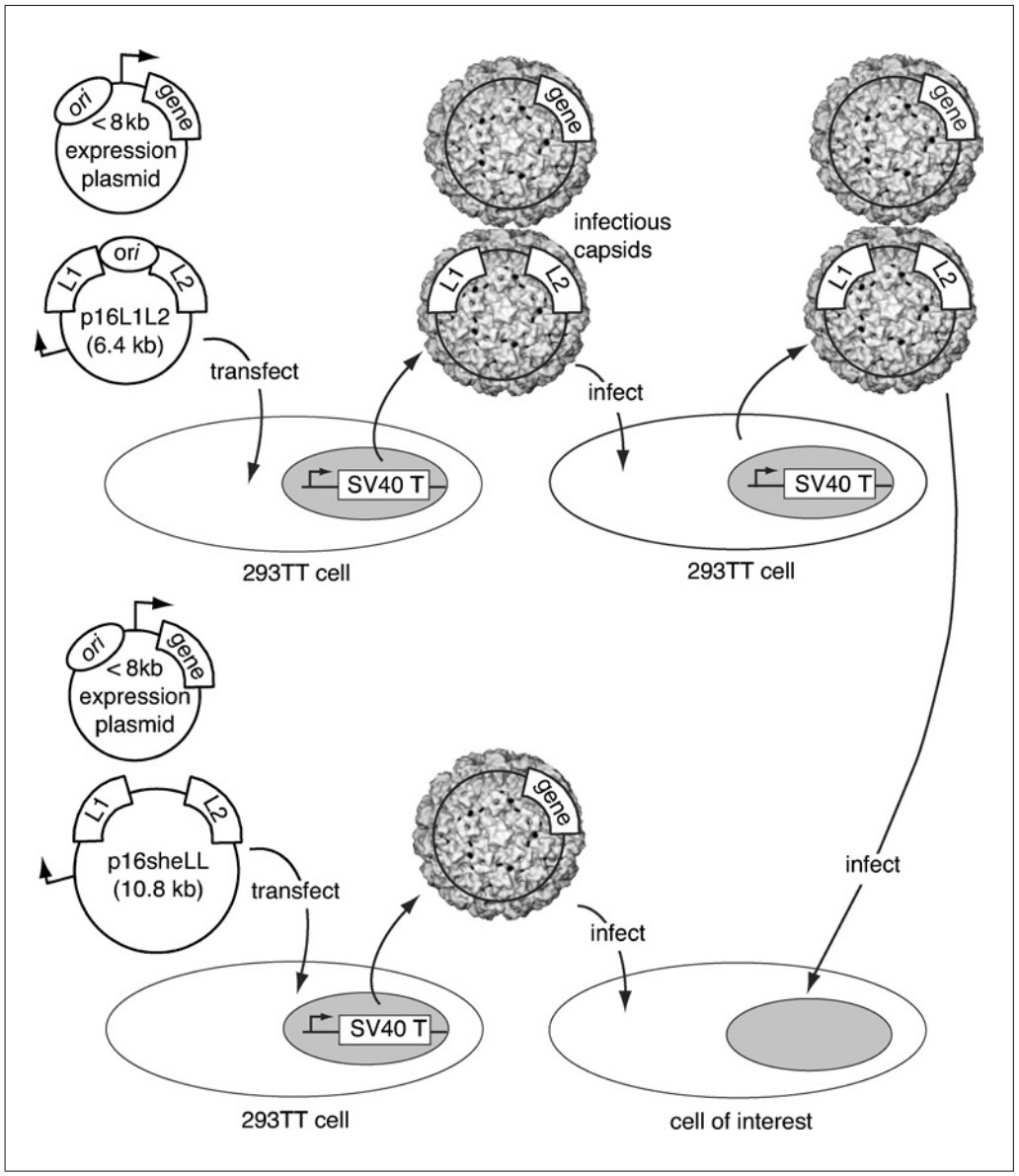


Figure 26.1.1 Amplification competent (top) versus incompetent (bottom) vector production.

GENERATION OF A PAPILLOMAVIRAL VECTOR STOCK BY TRANSFECTION

An initial vector seed stock is generated by co-transfecting an HPV16 L1/L2 expression plasmid, p16L1L2, together with an expression plasmid of interest into 293TT cells. After transfection, p16L1L2 and the expression plasmid are each replicated to high-copy number and packaged separately into L1/L2 capsids. Note that Alternate Protocol 2 discusses the use of a different L1/L2 expression plasmid, p16sheLL, which cannot be packaged into L1/L2 capsids and thus allows production of amplification-incompetent papillomaviral vector stocks. Capsids are harvested by lysing the cells with Brij-58 (a nonionic detergent) 48 hr after transfection. The resulting cell lysate must be allowed to mature for 24 hr, then can be diluted and used directly as an infectious vector stock or purified as described in Support Protocol 1 or 2. In some instances it may be useful to amplify the vector stock by using it to infect fresh 293TT cells (Basic Protocol 2).

Materials

DMEM (Dulbecco's modified Eagle medium) with 10% (v/v) FBS (DMEM-10)
Fetal bovine serum, heat-inactivated at 56°C for 30 min (FBS)
293TT cells
0.05% (w/v) trypsin/EDTA solution (Invitrogen or other supplier)
2× freeze medium: 82% (v/v) FBS/18% (v/v) dimethylsulfoxide
50 mg/ml hygromycin B stock (Roche)
Lipofectamine 2000 (Invitrogen)
OptiMEM-I (Invitrogen)
p16L1L2 plasmid
<8-kb mammalian expression plasmid with SV40 *ori* (see Commentary)
DPBS-Mg (see recipe)
10% (v/v) Brij-58 (see recipe)
RNase A/T1 cocktail (Ambion, no. AM2286) or RNase A stock

225-cm^2 and 75-cm^2 cell culture flask
Humidified 37°C, 5% CO_2 incubator
37°C water bath
2-ml gasketed cryovials
Cryocooler (e.g., Nalgene Mr. Frosty isopropanol bath) chilled to 4°C
Liquid nitrogen freezer
15-ml conical centrifuge tubes
1.5-ml siliconized centrifuge tubes, preferably screw-cap (e.g., VWR, no, 60828-818)
Siliconized pipet tips (optional)

Establish the 293TT cell culture

1. Dispense 27 ml of DMEM-10 into a 225-cm^2 flask. Add 3 ml of FBS for a final FBS concentration of 20%. Place the flask in a humidified 37°C 5% CO_2 incubator and loosen the cap. Allow the flask to equilibrate for at least 10 min.

   It is important to omit hygromycin B (see below) from the culture medium during recovery from thawing.

   The Laboratory of Cellular Oncology (http://home.ccr.cancer.gov/LCO/) freely distributes 293TT cells to nonprofit researchers.

2. Thaw a frozen vial of 293TT cells in a 37°C water bath. Immediately upon thawing, pipet the cells into the flask.

   It is not necessary to spin the cells out of their freeze medium. Like other human embryonic kidney 293-derived cell lines, 293TT cells do not adhere tightly to plastic. Placing the cells in a relatively small volume with large surface area promotes attachment, which may take up to two days.

3. Incubate the cells in a humidified 37°C, 5% CO_2 incubator for 2 to 3 days.

4. Remove the supernatant and gently wash cells with 1 to 2 ml of trypsin/EDTA. Remove trypsin wash and replace with 1 to 2 ml of fresh trypsin/EDTA. Return the flask to the incubator, loosen the cap, and incubate for at least 5 min, gently rocking the flask once or twice. Neutralize the trypsin by suspending the cells in 9 ml of DMEM-10.

   Although 293TT cells are easily dislodged from plastic, the cells can adhere to one another fairly tightly. If the cells are not fully trypsinized, the resulting cell clumps may be partially lysed by shear forces during resuspension. Insufficient trypsinization also results in the appearance of unhealthy piles of cells after replating.

5. On the first or second passage, retain enough cells to freeze archive vials in liquid nitrogen. When trypsinizing the cells, retain some of the spent culture medium. After trypsinization, resuspend the cells in the retained spent medium. Gently mix the suspended cells 1:1 with 2× freeze medium. Distribute the suspension as 1-ml aliquots in cryovials (roughly 2 to 5 × 10^6 cells per vial). Transfer the vials to a cryocooler and freeze at −80°C overnight. Transfer the frozen cells to a liquid nitrogen freezer for long-term storage.

6. For routine passaging, split the culture 1:10 or 1:20 every 2 to 3 days. After recovery from thawing, supplement the culture medium with hygromycin B at a final concentration of 400 μg/ml to promote maintenance of SV40 T antigen expression.

Transfect 293TT cells

7. Preplate 7.5 × 10^6 293TT cells in a 75-cm^2 flask in 20 ml of DMEM-10 without hygromycin B. Incubate the cells overnight in a humidified 37°C, 5% CO_2 incubator.

   A 75-cm^2 flask is used as an example, but the transfection can easily be scaled up or down based on the surface area of the culture vessel. See the Lipofectamine 2000 package insert for details.

8. Transfect the cells with Lipofectamine 2000, essentially according to package insert. Mix 80 μl of Lipofectamine 2000 reagent with 2 ml of OptiMEM-I in a 15-ml tube. Incubate 10 min.

9. In a separate 15-ml tube, mix 19 μg each of p16L1L2 and expression plasmid of interest in 2 ml of OptiMEM-I.

10. Combine the Lipofectamine 2000 and DNA mixtures and incubate 20 to 30 min to form lipid/DNA complexes.

11. Add the complexes directly to the preplated cells and incubate 6 hr.

    IMPORTANT NOTE: *From this point forward, the culture should be handled under proper biosafety conditions approved by an institutional biosafety committee (see Biosafety heading in the Commentary section).*

    It is essential that the cells be <50% confluent at the time of transfection. Higher levels of confluency tend to reduce the transfection efficiency for 293TT cells. Ideally, the cells should be preplated late in the day, then transfected the next morning. It is not necessary to change the medium on the cells before adding the lipid/DNA complexes, provided the cells were preplated in DMEM-10 without antibiotics or selective agents. Incubation of the cells with the lipid/DNA complexes for 4 to 8 hr gives similar results.

12. Carefully remove the supernatant from the culture and add 20 ml of fresh DMEM-10 prewarmed to 37°C.

    Care should be taken not to dislodge the cells when adding fresh medium. For example, it may help to turn the flask upside-down and add the medium to the top surface of the flask. Medium can be supplemented with antibiotics (e.g., pen-strep) at this point, although this is not generally necessary.

13. Return the transfected cells to the incubator for 2 days.

    It should not be necessary to split the culture.

Harvest capsids

14. Remove supernatant from the culture.

    Generally, there will be very few detached cells and the supernatant can be discarded. If there are substantial numbers of floating cells, recover them by centrifugation.

15. Harvest cells by trypsinization (see step 4 above). Transfer suspended cells to a 15-ml conical centrifuge tube. Collect residual cells by rinsing the flask with 4 ml of fresh DMEM-10. Pool rinse in the same 15-ml conical tube. Pellet the cells by centrifuging 10 min at 200 × *g*, 4°C.

16. Remove the supernatant and resuspend the cells in 0.5 ml of DPBS-Mg. Transfer the suspension to a 1.5-ml siliconized tube. Rinse the 15-ml conical tube with 0.5 ml of fresh DPBS-Mg. Pool rinse into the 1.5-ml siliconized tube. Pellet the cells by centrifuging 10 min at 200 × *g*, 4°C.

    Papillomavirus capsids interact nonspecifically with polypropylene and other types of surfaces (Shi et al., 2005). Siliconized tubes appear to be less prone to disruptive nonspecific interactions with the capsids. Blocking of polypropylene surfaces with excess protein or detergent also helps prevent capsid-plastic interactions. Thus, the use of siliconized tubes is desirable but not absolutely essential for the purposes of Basic Protocols 1 and 2. The use of siliconized tubes becomes more important when handling purified capsids (Support Protocols 1 and 2), particularly if the capsids are to be stored for extended periods of time at 4°C. Although siliconized screw-cap tubes can be difficult to obtain, they are easier to keep sterile than more widely available flip-cap siliconized tubes. The use of siliconized pipet tips is optional.

17. Carefully remove the supernatant. Estimate the volume occupied by the cell pellet by side-by-side comparison to fluid in a dummy tube.

    Pellet volume for a 75-cm^2 flask of cells is typically 60 to 80 μl.

18. Add 1.5× cell pellet volumes of DPBS-Mg. For example, add 120 μl of DPBS-Mg to a cell pellet of 80 μl (total volume of about 200 μl). Resuspend the cells by gently vortexing or flicking the tube.

    It is critical that the cells be suspended at a density of at least 100 × 10^6 cells/ml. Lower-density lysates can suffer from nonspecific protein aggregation that reduces the purity of capsid stocks and can also reduce titer yield. Estimating suspension density based on pellet size is safer and more reliable than performing hemacytometer counts.

19. Add 10% Brij-58 to achieve a final concentration of ∼0.35%.

    For example, add 7 μl of 10% Brij-58 to 200 μl cell suspension.

20. Add ∼1 μl of RNase A/T1 cocktail (or ∼5 RNase A units) and mix.

    Intact ribosomes are large enough to partially co-purify with capsids. Digestion of ribosomes with RNase thus facilitates purification (see Support Protocols 1 and 2). The suggested amount of RNase stock is more than enough enzyme to adequately digest ribosomes—lower doses of enzyme stock are also effective.

21. Allow capsids to mature by incubating the lysate at 37°C for 24 hr.

Although this step of the protocol may seem counterintuitive, it is essential to allow the capsids to undergo a slow process of maturation, in which disulfide bonds between neighboring L1 molecules stabilize the structure of the capsid (Buck et al., 2005). Prior to maturation, capsids are infectious but physically fragile, such that immature capsids lose infectivity during purification or freeze-thaw cycles. The maturation process also renders the capsid more soluble, allowing clarification of the lysate (step 22).

It may be helpful to gently mix the lysate once or twice during the maturation period. Note that effective sterile technique is critical during harvest of capsids, since contaminating microbes have ample opportunity to grow during the capsid maturation period.

22. After maturation, chill the lysate on ice for 5 to 10 min. Clarify the chilled lysate by centrifuging 10 min at 5,000 × *g*, 4°C. The resulting clarified supernatant is the vector stock. Freeze at −80°C in 20 to 50 µl aliquots or proceed to purifying the stock (Support Protocols 1 and 2).

The clarified supernatant can be used directly as a crude papillomaviral vector stock to infect cells of interest. The residual Brij-58 in the lysate is not toxic to most cell types, provided the stock is diluted least 1:500 (final dilution in culture well). The clarified lysate can also be used as a seed for amplifying the vector in fresh 293TT cells (Basic Protocol 2). Although amplification allows high-yield production of a more concentrated vector stock, initial seed stocks are usually of sufficient titer for use in most applications.

BASIC PROTOCOL 2

PROPAGATIVE AMPLIFICATION OF A PAPILLOMAVIRAL VECTOR STOCK

In this protocol, the vector seed stock from Basic Protocol 1 is amplified by infection of fresh 293TT cells. In principle, the amplified vector stock can in turn be further amplified by additional rounds of infection of 293TT cells. However, as the viral stock is passaged, smaller collapsed plasmid recombinants begin to dominate the propagated plasmid swarm. By the third passage, ~3 kb collapsed species are predominant and, as a consequence, titer yield begins to decline.

Materials

293TT cells
DMEM with 10% (v/v) FBS
Vector seed stock (Basic Protocol 1)

225-cm^2 flask
Humidified 37°C 5% CO_2 incubator

1. Plate 12 × 10^6 293TT cells in 50 ml of DMEM-10 in a 225-cm^2 flask. Incubate overnight in a humidified 37°C, 5% CO_2 incubator.

2. Infect the preplated cells by adding 20 µl of vector seed stock (Basic Protocol 1). Return the flask to the incubator. Incubate the culture with the vector inoculum for 20 to 40 hr, then change the cells into fresh DMEM-10.

Rocking the flask occasionally during the first 2 hr of incubation will help to promote virus attachment.

The change of medium may improve infectious titer yield, but can be considered optional.

3. About 72 hr after initial infection, harvest capsids from the cells as described in Basic Protocol 1, steps 14 to 22. Freeze the clarified supernatant at −80°C in 100-µl aliquots or purify the vector stock (Support Protocols 1 and 2).

PRODUCTION OF AMPLIFICATION-INCOMPETENT VECTOR STOCKS

ALTERNATE PROTOCOL 1

For some applications, co-delivery of p16L1L2 along with the expression plasmid of interest may be undesirable. An alternative strategy is to produce an amplification-incompetent vector stock by transiently co-transfecting an expression plasmid of interest together with the packaging plasmid p16sheLL. In contrast to p16L1L2, p16sheLL lacks the SV40 *ori* and is too large to be packaged within papillomavirus capsids. Thus, when p16sheLL is co-transfected with an expression plasmid of interest, only the expression plasmid is packaged into nascent infectious capsids.

A variety of other papillomavirus types have been adapted to the "sheLL" format and are freely available to nonprofit researchers. Note that the L1 and L2 open reading frames in these plasmids have been "codon-modified" (subjected to extensive silent mutation) in order to eradicate expression-inhibitory elements present in native papillomavirus L1 and L2 genes (Leder et al., 2001). Amplification-incompetent vectors can be produced using the methods outlined in Basic Protocol 1 by simply substituting p16sheLL (or other sheLL series plasmid) for p16L1L2. See the Web page *http://home.ccr.cancer.gov/LCO/packaging.htm* for maps of available packaging plasmids.

Because of their relatively large size, sheLL series plasmids are occasionally subject to recombinant collapse during re-growth in bacteria. The chance of inadvertent recombination can be reduced by using STBL2 (Invitrogen) or other reduced-recombination bacterial strains. Alternatively, standard bacterial subcloning strains can be grown at 30°C during recovery from transformation and during growth on selective agar plates. Growth of bacteria at 30°C tends to result in maintenance of ColE1-based plasmids at lower copy number, thus reducing the chance of recombination. Subsequent liquid culture scale-up of a single colony at 37°C improves plasmid yield and has not resulted in detectable amounts of plasmid collapse in our hands.

BULK PRODUCTION OF PAPILLOMAVIRUS CAPSIDS

ALTERNATE PROTOCOL 2

The promiscuity of DNA packaging by HPV16 L1 and L2 applies not just to various expression plasmids, but also to cellular DNA. At the time of cell lysis, at least 90% of the proto-capsids present in the cell nucleus exist in complex with cellular DNA. In the basic protocols, most capsid/cellular DNA complexes sediment away from the vector stock during the lysate clarification step. However, if DNases are added to the cell suspension during cell lysis, capsids associated with cellular DNA are liberated with an encapsidated 8-kb linear fragment of cellular DNA (Buck et al., 2005). For applications aimed using infectious reporter capsids, the presence of "cold" (reporter-less) capsids containing cellular DNA is undesirable, since the cold capsids may compete against the infectivity reporter capsids. The infectious delivery of random fragments of 293TT cell DNA to target cells also presents a theoretical biosafety risk.

In this protocol, the addition of DNases to the cell lysate allows for recovery of capsids containing linear fragments of cellular DNA. This boosts the yield of capsids by at least 10-fold, making this approach desirable when the production of virus-like particles, rather than titerable infectious units, is the primary goal.

In some instances it may be desirable to produce L1 capsids that do not contain L2. HPV16 L1 particles can encapsidate a limited amount of DNA in the absence of L2, but are at least 10,000-fold less infectious than L1/L2 capsids. A limited seed stock for production of L1-only capsids can be generated by co-transfecting p16sheLL together with a packageable L1 expression plasmid, such as p16L1-GFP. The resulting seed stock can be used for a single-round infection of fresh 293TT cells. The infected cells will be transduced only with the L1 expression plasmid and will thus produce capsids lacking L2.

Additional Materials *(also see Basic Protocol 1)*

Benzonase endonuclease (~250 U/μl; Sigma-Aldrich or other supplier)
Plasmid Safe ATP-Dependent DNase (10 U/μl; Epicentre)
5 M NaCl

Additional reagents and equipment for production of an initial vector seed stock (Basic Protocol 1)

1. Follow Basic Protocol 1 for production of an initial vector seed stock, omitting the expression plasmid of interest and transfecting with p16L1L2 alone.
2. Amplify the vector seed stock using Basic Protocol 2. Instead of adding RNase to the cell lysate (Basic Protocol 1, step 20), add Benzonase and Plasmid Safe stocks to a final concentration of ~0.25% each. Mature the lysate for 24 hr (Basic Protocol 1, step 21).

   Benzonase is an RNA/DNA endonuclease, while Plasmid Safe is a processive exo-DNase. The two nucleases act in concert to "slice and dice" any DNA outside the protective environment of the capsid. There is enough residual ATP in the lysate to support Plasmid Safe's ATP-dependent exonuclease activity. Final nuclease stock concentrations of 0.05% to 0.1% each are also adequate for effective fragmentation of unencapsidated DNA.

3. After maturation, chill the matured lysate on ice for 5 to 10 min. Add 0.17 volume of 5 M NaCl to achieve a final NaCl concentration of ~0.85 M. Incubate on ice 10 min, mixing once or twice.

   Treating the lysate with high salt helps solubilize the lysate, facilitating the purification of capsids. It is not possible to add high salt to the lysate prior to clarification in the basic (DNase-less) protocols, because the salt would solubilize the undigested cellular DNA, rendering the lysate too viscous to handle.

4. If desired, purify capsids out of the clarified lysate using Support Protocols 1 or 2.

SUPPORT PROTOCOL 1

PURIFICATION OF VECTOR STOCKS USING OPTIPREP GRADIENTS

Although clarified cell lysates can serve as useful vector stocks, the residual Brij-58, undigested DNA, and expressed proteins of interest in the lysate may cause problems for some applications. This protocol outlines the use of ultracentrifugation to purify vector stocks. Virologists have traditionally relied on two main ultracentrifugation strategies to purify capsids: buoyant density ultracentrifugation, which typically employs CsCl solutions, and velocity ultracentrifugation, which typically employs concentrated sucrose solutions. The method described here makes use of a newer ultracentrifugal gradient medium called Optiprep (iodixanol). Optiprep is a relatively nontoxic iodinated di-hexanol compound used clinically as an injectable X-ray contrast agent. It has the useful feature of being both high-density (like CsCl solutions) and high-viscosity (like sucrose solutions). It is thus possible to employ Optiprep for velocity and buoyant density ultracentrifugation simultaneously in a single tube. The clarified capsid-containing cell lysate is layered on top of a preformed Optiprep step-gradient. At the top of the gradient, the relatively large size of the capsids allows them to rapidly sediment through the upper portion of the gradient while other, smaller diameter solutes in the lysate traverse the gradient slowly. Once the capsids reach the middle portion of the gradient, their migration is dominated by buoyancy. This allows separation of empty capsids from DNA-containing capsids.

Ultracentrifugation is a relatively advanced laboratory technique and should not be attempted without the guidance of an experienced operator. Although Optiprep purification is a highly effective way to purify papillomaviral vector stocks, the simpler gel filtration strategy described in Support Protocol 2 also gives reasonable purity and would be a

better choice for investigators lacking easy access to the equipment or expertise required for ultracentrifugation.

The protocol describes the use of a Beckman SW-55 rotor. Other types of rotors can be used successfully, although some adjustment of spin time may be necessary. An SW32 rotor at 32,000 rpm (125,000 × *g*) for 5.75 hr or an SW40.1ti rotor at 40,000 rpm (200,000 × *g*) for 4.75 hr work reasonably well.

Additional Materials *(see also Basic Protocol 1)*

46% (v/v) Optiprep/DPBS (see recipe)
DPBS/0.8 M NaCl (prepare by adding 0.15 volume of 5 M NaCl to DPBS)

Thin-wall 1/2 × 2–in. Polyallomer tubes (Beckman, no. 326819) or appropriate ultracentrifuge tubes
3-ml syringe fitted with a 2-in. or longer large-bore (~16-G) needle
Parafilm
Ultracentrifuge
Beckman SW-55 ultracentrifuge rotor (or other swing-bucket rotor)
Ring stand with tube clamp
Siliconized collection tubes
~25-G needle

Prepare a 27%, 33%, and 39% Optiprep step gradient by underlayering

1. Dilute 46% Optiprep/DPBS to 27%, 33%, and 39% using DPBS/0.8 M NaCl

   Although our laboratory has traditionally used DPBS as a dilution medium, Optiprep gradients prepared using plain PBS (lacking $CaCl_2$, $MgCl_2$, and KCl) are probably equally effective. Use of high (0.8 M) NaCl concentrations in the gradient results in more effective separation of the capsids from cellular proteins, but gradients prepared with physiologic (0.15 M) NaCl concentrations can give acceptable results.

2. Set up a minimum of two ultracentrifuge tubes: one for the clarified lysate, one for balance.

3. Using a pipet, add 1.5 ml of 27% Optiprep to each tube.

   A gradient with three 1.5 ml steps will leave enough room for about 0.5 ml of lysate. Up to 3 ml of lysate can be loaded into a single tube by shortening the gradient to 0.7 ml per step.

4. Load the 3 ml syringe with 33% Optiprep. Eject any bubbles. Insert the syringe until the needle is gently touching the bottom of the tube. Gently eject 1.5 ml of 33% Optiprep beneath the 27% step.

5. Remove the syringe from the tube and eject residual Optiprep (or use a fresh syringe). Repeat step 4 with 39% Optiprep.

6. Cover the tubes with Parafilm and incubate 1 to 4 hr at room temperature.

   Letting the gradients stand at room temperature allows the steps to partially diffuse into one another. The partly linearized gradient results in a slightly better-focused capsid band.

Perform the ultracentrifugation

7. Gently layer the clarified cell lysate (or DPBS in the balance tube) onto the top of the gradient. Seat the tubes into opposing rotor buckets.

8. Add DPBS/0.8 M NaCl to the top of the tube until the meniscus is above the surrounding metal rim of the bucket. Balance buckets to within 5 mg.

   It is critical that the tubes be filled to near the rim and that the buckets be balanced. Underfilled tubes can collapse, resulting in imbalances. Imbalances can result in destruction of the ultracentrifuge rotor and containment drum.

9. Hang the buckets on the rotor, being sure that both hooks catch. Spin 3.5 hr at 234,000 × *g* (50,000 rpm for an SW-55ti rotor), 16°C.

Fractionate the gradient

10. Remove the tube from the bucket and place in the tube clamp.

    An opalescent band of capsids may be visible roughly a third of the way up the gradient. Visualization of the band is easier if a dark object is held behind the tube.

11. Set up and uncap twelve siliconized collection tubes. Position the collection tubes beneath the ultracentrifuge tube.

12. Puncture the bottom of the ultracentrifuge tube slightly off center using a 25-G (or similar diameter) needle. Rock the needle in a circular motion to slightly expand the hole.

    Use of sharp objects when handling biohazardous agents obviously presents some risk. This step of the protocol should be performed very carefully. Discard the needle directly into an appropriate sharps container (do not attempt to re-cap the needle).

13. Collect a single large first fraction until the rate of drops begins to increase (~750 µl collected). For each subsequent fraction, collect 5 to 8 drops (~200 µl) per fraction. Repeat for twelve fractions. The fractions should encompass the bottom 1/2 to 2/3 of the gradient. Discard the top portion of the gradient.

    The first few drops will emerge very slowly.

14. Screen gradient fractions for the presence of capsids (Support Protocol 3). Pool appropriate fractions, divide into 100-µl aliquots, and store at −80°C.

    Optiprep is nontoxic to cell cultures and can be injected into mice without apparent negative effects. It is therefore not necessary to exchange capsids out of Optiprep in most cases. If removal of Optiprep (or the high salt present in the gradient fractions) is desired, Support Protocol 2 presents an effective method for exchanging capsids into other buffers. Note that Optiprep is resistant to dialysis (see Support Protocol 4).

SUPPORT PROTOCOL 2

PURIFICATION OF VECTOR STOCKS USING AGAROSE GEL FILTRATION

The simple size-exclusion chromatography (gel filtration) purification method described in this protocol makes use of the fact that papillomavirus capsids are larger than nearly all other macromolecular complexes in the clarified lysate. The agarose beads used in the protocol have a pore size slightly smaller than the papillomavirus capsid. Thus, papillomavirus capsids are excluded from the beads and run rapidly through the void volume of the column, while other solutes in the cell lysate take a slower, circuitous path through the bead matrix. Although the purity of gel filtered capsids is not quite as high as for Optiprep-purified capsids (Support Protocol 1), gel filtration does effectively remove most contaminants and is safer and less technically demanding than ultracentrifugal methods. Agarose gel filtration can also be used as a buffer exchange method for removing Optiprep or other solutes from purified vector stocks.

Additional Materials *(see Basic Protocol 1)*

DPBS-BSA (see recipe)
DPBS/0.5 M NaCl (prepare by adding 0.075 volume of 5 M NaCl to DPBS)
Benzonase nuclease (Sigma; optional)
0.05% (w/v) NaN_3 or other preservative

Vacuum source (optional)
Bell jar or side-arm vacuum flask for degassing solutions (optional)
Ring stand with clamp
5 ml gravity-flow columns with caps and frits (Pierce or other supplier)

1- or 5-ml pipet
2% (w/v) agarose beads (50- to 150-μm diameter; Agarose Bead Technologies, no. A-1020-S) *or* 1.4% agarose beads (Bioscience Beads)
Parafilm

Prepare an agarose gel filtration column

1. De-gas the DPBS-BSA solution by exposure to vacuum.

   This step is optional. See the package insert that comes with Pierce gravity-flow column kit for additional information.

2. Clamp the column to a ring stand. Put the bottom cap on and add 5 ml of DPBS/0.5 M NaCl.

3. Remove the bottom cap to eject any bubbles. Recap and add more DPBS/0.5 M NaCl. Fill to near the top of the column.

4. Float a frit on the surface. Gently tap the frit to dislodge any air bubbles. Tap frit down to the bottom of the column using a 1- or 5-ml pipet (or the serum separator provided with the column kit).

5. Remove the bottom cap and drain out most of the fluid.

6. Suspend the agarose beads by gently swirling and inverting the bottle. Pour bead slurry into the column. Fill the column to the rim.

7. Remove the bottom cap. Partially exchange the beads into room-temperature DPBS-BSA by repeatedly allowing the column to drip to near dryness then pouring on more DPBS-BSA.

8. Replace the bottom cap. Cover the top of the column with Parafilm. Suspend beads by repeated gentle inversion of the column. Return the column to the clamp and allow blocking and settling overnight at room temperature.

   Preblocking the column overnight with 1% BSA reduces capsid aggregation by blocking nonspecific protein binding sites on the bead matrix and column walls. Although it is generally not as effective as BSA, Tween 80 at a final concentration of 0.01% (v/v) can also be used as a blocking agent. Like BSA, the detergent partially prevents nonspecific interactions that can lead to capsid aggregation (Shi et al., 2005). Tween 80 is a relatively mild detergent and can be added to cell cultures at final concentrations of up to 0.1% (v/v) without noticeable toxicity. The use of high (0.5 M) NaCl concentrations also helps block nonspecific interactions that can lead to capsid denaturation and aggregation.

   It is important to allow the beads to settle slowly after equilibration with room temperature (preferably de-gassed) buffer. Chilled solutions contain more dissolved oxygen, which can form bubbles upon re-warming to room temperature. Trapped bubbles create disruptive vortexes in the liquid flow through the gel bed.

9. Remove Parafilm. Float a frit on the fluid surface and gently tap down to within a few mm of the bed surface.

10. Remove the cap from the bottom of the column. Wash the column with at least 10 column volumes of DPBS/0.5 M NaCl.

    It is helpful to use the conical reservoir included with the Pierce column kit. The washing process may take as long as long as an hour. Washing with only 4 column volumes may result in contamination of the capsids with residual BSA. The gel bed will compact some during the washing. Tap the frit down to within a few millimeters of the bead surface. Do not compress the gel bed. After washing, the column is ready to use.

Perform gel filtration

11. *Optional:* If capsids are being purified out of crude cell lysate (Basic Protocols 1 and 2) add 1 µl of Benzonase nuclease and incubate 10 to 30 min at 37°C to digest any residual unencapsidated DNA.

    It may be beneficial to adjust the salt concentration of the crude lysate to 0.5 M NaCl after Benzonase digestion.

    Although the addition of Benzonase is optional, it helps improve the purity of the stock by digesting large DNA molecules that, like capsids, are too large to enter the pores in the agarose beads. A small fraction of cellular DNA-associated capsids will remain present in the clarified lysate. The addition of Benzonase will therefore liberate some capsids carrying 8-kb linear fragments of cellular DNA. Most of the Benzonase will be removed by the gel filtration process. If capsids have been purified using an Optiprep gradient, they have already been separated from unencapsidated DNA and addition of Benzonase is unnecessary. Also note that Benzonase is inhibited by high-salt concentrations (such as the 0.8 M NaCl used for Optiprep gradients).

12. Add 0.5 ml or less (i.e., less than 1/10 of the agarose bed volume) of clarified lysate (or capsids in Optiprep) to the washed agarose gel filtration column.

13. Apply 0.25 ml of DPBS/0.5 M NaCl to the top of the column. Collect column eluate in a siliconized 1.5-ml tube. Repeat this for a total of 12 0.25-ml fractions.

    See notes for step 13 of Basic Protocol 1 for information about use of siliconized tubes.

14. Screen fractions for encapsidated DNA, as described in Support Protocol 3.

    A peak of purified capsids usually emerges from the gel filtration column at about 1/3rd of the column volume. In other words, typically fraction 7, if 0.25 ml fractions were collected. A histogram of capsid content may be spread over as many as four fractions.

15. Regenerate columns for re-use by washing the column with 10 column volumes of DPBS/0.5 M NaCl, then exchanging into DPBS-BSA supplemented with 0.05% (w/v) NaN_3 or other preservative. Store the column at room temperature for several days.

    Columns can be stored for longer periods at 4°C, but oxygen bubbles may form in the column upon re-warming. If bubbles form, remove the top frit and resuspend the beads by inverting the column.

SUPPORT PROTOCOL 3

SCREENING FRACTIONS FOR THE PRESENCE OF CAPSIDS

If enough capsids are present in the starting lysate, it is possible to screen Optiprep or agarose gel filtration fractions by simple BCA protein assay (Pierce). Screening fractions for the appearance of L1 (55 kDa) in stained SDS-PAGE gels (see *UNIT 6.1*) is also feasible. If SDS-PAGE is used for screening, fractions containing L1 and peak amounts of ~15 kDa histone bands (indicating the presence of encapsidated DNA) should be chosen. Optiprep has a strong light absorbance peak at ~250 nm. This makes it impossible to screen Optiprep fractions using UV absorbance.

If the papillomaviral vector carries a convenient reporter gene, fractions can also be screened by titration of infectivity (Support Protocol 4). If siliconized tubes (see note for step 13 of Basic Protocol 1) have been used for fraction collection, stock titer is generally stable at 4°C for 48 hr.

The fraction screening technique described below is based on the visualization of encapsidated DNA and has the advantage of rapidly identifying fractions containing even relatively low levels of encapsidated plasmid. For more information on agarose gel electrophoresis see Voytas (2000).

Additional Materials *(see Basic Protocol 1)*

Electrophoresis-grade agarose
Tris-acetate-EDTA (TAE) electrophoresis buffer (or other agarose gel electrophoresis buffer, see *APPENDIX 2A*)
Phosphate-buffered saline (*APPENDIX 2A*), optional
Proteinase K (e.g., Qiagen, no. 19131)
0.5 M EDTA
10% SDS
DNA loading dye (Voytas, 2000)
DNA marker ladder (kilobase-range; Invitrogen or other supplier)
1 to 10 ng sample of supercoiled p16L1L2 and/or 1 to 10 ng of the expression plasmid of interest
SYBR Green-I (Invitrogen/Molecular Probes or other supplier)

UV (or blue light) gel documentation imaging device
16- × 16–cm tray
12-well combs
Agarose gel electrophoresis apparatus (see *APPENDIX 3A*)

1. Add 0.8 g of electrophoresis-grade agarose to 80 ml of TAE buffer. Melt by microwaving.
2. Cast molten agar into a 16- × 16–cm tray with one or two 12-well combs. Allow gel to cool.

   Other sizes of casting tray can be used. It is helpful if the gel is relatively shallow (<5 mm thick). SYBR Green-I stain penetrates the gel relatively slowly. If the sample extends up to the surface of the gel, the shorter diffusion distance allows for faster staining.
3. Make a master mix with 100 µl of water (or PBS) and 5 µl each proteinase K, 0.5 M EDTA and 10% SDS. Distribute 5 µl of master mix to an appropriate number of microcentrifuge tubes. Add a 15 µl sample of each gradient or column fraction to separate tubes. Vortex the tubes then incubate 10 to 30 min at 56°C.

   Proteinase K digestion liberates the encapsidated plasmid DNA from the capsid/histone complex. This allows the encapsidated DNA to migrate properly during electrophoresis.
4. Add 5 µl of DNA loading dye to each tube. Load 20 µl of each sample into the gel. Include a lane with 100 ng of DNA marker ladder (i.e., ~10 ng of DNA per ladder band). Also include a 1 to 10 ng sample of supercoiled p16L1L2 and/or 1 to 10 ng of the expression plasmid of interest. Run the gel at 120 V for ~60 min (or an appropriate voltage and time for the electrophoresis device being used).

   The presence of Optiprep and/or salt in the sample may distort the migration of the loading dye, but migration of the DNA is not dramatically affected. It is possible to purify encapsidated DNA away from the proteinase K-digested fraction material using silica spin columns, for example Qiaquick PCR Purification columns (Qiagen, no. 28104). The purified DNA can then be positively identified by digestion with restriction enzymes prior to electrophoresis.
5. Stain the gel for 20 min in SYBR Green-I diluted 1:10,000 in TAE.
6. Image the gel using an appropriate imaging device (see SYBR Green-I package insert).

   The encapsidated plasmid should be predominantly supercoiled, although some nicked and linearized species may appear, particularly if Benzonase was added to the sample (step 1 in Support Protocol 2). Benzonase liberates capsids containing linear fragments of cellular DNA, which will appear as a wide ~8-kb band. See Alternate Protocol 2 for details about capsids containing cellular DNA.

7. Choose fractions with peak plasmid content. Pool the fractions, divide into 100-µl aliquots, and freeze at −80°C.

SUPPORT PROTOCOL 4

CONCENTRATION OF VECTOR STOCKS

It should not generally be necessary to concentrate capsid stocks. However, it is possible to do so using Pierce Slide-A-Lyzer dialysis cassettes and Slide-A-Lyzer Concentrating Solution, following manufacturer's instructions. Unfortunately, Optiprep does not readily pass through dialysis membranes. Therefore, capsids must be exchanged out of Optiprep (using gel filtration, Support Protocol 2) prior to concentration. Addition of high NaCl concentrations (0.5 M to 1.0 M final) and Tween 80 (0.001% – 0.01% (v/v)) to the sample prior to concentration will help prevent capsid aggregation during the concentration process (see notes for Support Protocol 2, step 8). If necessary, the NaCl and Tween 80 can then be dialyzed away after concentrating the stock.

SUPPORT PROTOCOL 5

TITERING THE INFECTIVITY OF PAPILLOMAVIRAL VECTOR STOCKS

Authentic papillomaviruses are released into the environment by the spontaneous disruption of cells at the surface of stratified squamous epithelia. This process, referred to as desquamation, is a normal feature of healthy skin and mucosal surfaces. Perhaps as a consequence of their exploitation of desquamation as a release mechanism, papillomaviruses are not known to employ an active cell-lysis program, as do other nonenveloped viruses. Thus, classical viral plaque assays that rely on viral lysis of infected cells are not useful for titration of papillomaviral vectors.

If a fluorescent protein is expressed by one or more of the packaged plasmids, the stock can readily be titered by flow cytometric analysis of cells treated with varying doses of vector stock (see *Current Protocols in Cytometry*). Intracellular immunostaining [for example, using a Cytofix/Cytoperm Kit (BD Biosciences)] coupled with flow cytometry may also be a feasible method for titering the expression of proteins of interest. If flow cytometry is not available, assessment of fluorescent protein expression (or fluorescent staining of a protein of interest) can be performed using fluorescence microscopy. Some plasmids, for example p16L1-GFP, express enhanced green fluorescent protein (GFP) under control of the SV40 promoter, while a protein of interest (in this case HPV16 L1) is expressed from the stronger EF1α promoter. 293TT cells are useful for titering vector stocks because the incoming plasmid is rapidly replicated to high copy number by SV40 T antigen, thus promoting high-level expression of proteins or reporter genes of interest.

Typically, adding 1 µl of vector stock to 10^6 293TT cells will result in bright fluorescence of nearly 100% of the cells, if GFP is used as a reporter. The infectability of other cell lines varies and should be tested empirically, preferably using a reporter plasmid with GFP under control of a strong promoter, such as CMV immediate early promoter (e.g., pCIneo-GFP) or human EF1α promoter (e.g., pfwB). See Commentary for further information on selecting reporter plasmids.

Materials

Cells, e.g., 293TT
DMEM-10
Vector stocks to be tested
Dulbecco's phosphate-buffered saline (DPBS) supplemented with 1% (v/v) FBS
ι-carrageenan (e.g., Sigma, no. C4014)

24-well plate
2.5-µl pipettor
1000-µl pipettor
Fluorescent microscope

1. Plate 1×10^5 293TT cells (or other cell type of interest) per well in a 0.5 ml volume of DMEM-10 in a 24-well plate. Incubate overnight in a humidified 37°C, 5% CO_2 incubator.

   Cells should be ~25% confluent.

2. Perform four 10-fold serial dilutions (i.e., 1:10 to 1:10,000) of the papillomaviral vector stock, for example by serially adding 2 µl of stock to 18 µl of DMEM-10.

3. Using a 2.5 µl pipettor, add 1 µl of diluted virus stocks to individual wells. Include a well of mock-treated cells. Perform the titration in replicate wells. Include a negative control by adding the papillomavirus-neutralizing agent ι-carrageenan to the cell culture at a final concentration of 1 µg/ml just prior to virus inoculation (Buck et al., 2006). Incubate cells 48 hr in a humidified 37°C, 5% CO_2 incubator.

   It is not necessary to change the medium on the cells.

   Papillomavirus infectious entry is a relatively slow process. 48 hr represents an adequate time period for infection of 293TT cells, but peak infection rates may occur at closer to 72 hr.

4. Remove the supernatant and trypsinize (as described in step 4 of Basic Protocol 1, using a few drops of trypsin per well) infected cells. Using a 1000 µl pipettor, thoroughly resuspend the cells in 0.5 ml DPBS supplemented with 1% FBS. Subject cells to flow cytometric analysis. Alternatively, count the fraction of cells expressing GFP using a fluorescent microscope.

5. To analyze GFP expression by flow cytometry, adjust an FL1 marker window to exclude 99.8% of the mock-transduced cells. Choose a vector stock dilution showing between 1% and 25% of cells FL1-positive. Use the formula [1000 µl/ml] × [stock dilution] × [200,000 cells at time of infection] × [fraction of cells FL1-positive] to calculate the number of infectious units per ml.

   Typical seed stocks for most GFP plasmids should contain at least 3×10^9 infectious units per ml.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Brij-58, 10% (w/v)

Dissolve Brij-58 (polyoxyethylene 20 cetyl ether) at 10% (w/v) in Dulbecco's phosphate-buffered saline (DPBS; e.g., Invitrogen, no. 14040-141). Store up to 1 month at 4°C.

Dissolve the Brij-58 by gently rocking the tube at room temperature for an hour or by storing overnight at 4°C. It is not necessary to filter the solution. The gradual appearance of a cloudy layer near the surface of the stored solution is normal. Mix the solution by gentle inversion of the tube prior to use.

DPBS-BSA

Combine the following in a 50-ml tube:
23 ml of Dulbecco's phosphate-buffered saline (DPBS; e.g., Invitrogen, no. 14040-141)
1.75 ml of 5 M NaCl
0.25 g of bovine serum albumin fraction V (BSA)
Filter using a 0.45-µm filter device (optional)

Dissolve the BSA by rocking at least 30 min. Solution can be kept overnight at room temperature.

DPBS-Mg

100 ml Dulbecco's phosphate-buffered saline (DPBS; e.g., Invitrogen, no. 14040-141), sterile
475 µl of sterile-filtered 2 M $MgCl_2$
1 ml of 100× antibiotic/antimycotic stock (Invitrogen, no. 15240062 or comparable broad-spectrum antibiotic from other suppliers)

Solution is stable for several months stored at 4°C. Keep sterile.

Optiprep/DPBS 46%

30.7 ml of 60% iodixanol solution (Optiprep; Sigma-Aldrich or other supplier)
4 ml of 10× PBS (Invitrogen or other supplier)
5.2 ml of 5 M NaCl
18 µl of 2 M $CaCl_2$
10 µl of 2 M $MgCl_2$
84 µl of 1 M KCl
Store up to one month at room temperature

It is helpful to briefly mix the solution after addition of the 10× PBS to avoid formation of $CaPO_4$ precipitates upon addition of the $CaCl_2$ stock.

Optiprep (60% iodixanol solution) is available from Sigma-Aldrich and other suppliers. Although the term Optiprep is technically a trade name for a 60% aqueous solution of iodixanol, this unit uses the term Optiprep colloquially as a synonym for iodixanol in order to be consistent with other published literature.

COMMENTARY

Background Information

Viral vectors are useful for efficient introduction of genes into mammalian cell types that are difficult to transfect. There is also substantial interest in the development of viral vectors for use as genetic vaccine vehicles or for various in vivo gene therapy applications. Papillomaviral vectors have a number of appealing features, such as their relatively tractable production and purification, which should make them useful as general-purpose gene delivery vehicles. The papillomaviral vectors described in this unit are a recent addition to the viral vector toolbox. To date, the vectors have been used primarily for analysis of the biology of papillomaviruses. However, the vector system also has utility for high-efficiency gene delivery to cultured cell lines and represents a promising new tool for in vivo gene delivery and genetic vaccination.

Other viral vectors, such as retroviral, adenoviral, or AAV-based systems, require that a gene of interest be flanked by viral packaging signals or other elements critical for production of infectious virions. A unique feature of papillomaviral vectors is that the packaging of plasmids within the cell nucleus is highly promiscuous, such that a variety of commercially available mammalian expression plasmids carrying the SV40 promoter [which contains the SV40 origin of replication (*ori*)] can be packaged efficiently. For example, pCDNA3.1 (Invitrogen) and pCIneo (Promega) plasmids expressing enhanced green fluorescent protein (GFP; BD Clontech) can be converted into purified papillomaviral vector stocks with titers of up to 10^{11} GFP-transducing units per ml.

Critical Parameters

This unit describes the production of papillomaviral vector stocks by two different methods: by direct transfection of cells (Basic Protocol 1) and infectious amplification of the transfection-derived stock (Basic Protocol 2). The yield of the initial transfection-derived stock is likely to be sufficient for most in vitro applications. The higher yields of propagated vector stocks may be appealing for in vivo studies, which generally require higher infectious doses.

At the outset of the protocol, a decision must be made whether to produce an amplification-competent or -incompetent vector stock (see Fig. 26.1.1). Amplification-competent stocks utilize p16L1L2, which carries the SV40 origin of replication and is small enough to be packaged into capsids. The p16L1L2 plasmid can be used to co-propagate expression plasmids of interest as

part of a viral swarm. Although this allows high-yield amplification of the vector stock, it has the drawback that the p16L1L2 plasmid will be co-delivered to target cell populations together with the expression plasmid of interest. This problem can be avoided by production of an amplification-incompetent vector stock, using p16sheLL in place of p16L1L2 (Fig. 26.1.1). p16sheLL does not carry an SV40 *ori* and is too large to become packaged. Infectious titer yields for p16sheLL-derived stocks are typically at least several-fold lower than for p16L1L2 transfection-derived stocks, but for most applications the reduced titer yield should not be a major impediment.

For reasons that are not yet clear, some expression plasmids are more amenable to packaging into papillomaviral vectors than others. In some instances, packaging may be impaired by the cytotoxic effects of high-level over-expression of a gene of interest in the producer 293TT cells. Even for relatively nontoxic genes, the plasmid context can affect titer yield. For example, use of pEGFP-N1 (Clontech) results in about ten-fold lower GFP-transducing titer yield compared to vector stocks made using pCDNA3.1-GFP or pCIneo-GFP. The suitability of a given plasmid for packaging should be tested empirically in an initial small-scale transfection-based stock (Basic Protocol 1). If the infectious titer (Support Protocol 4) of stocks made using a particular plasmid of interest is poor, it may be worth considering moving the gene of interest into a different expression plasmid backbone.

Plasmids using the human elongation factor 1-α (EF1α) promoter, for example the GFP expression plasmid pfwB or the L1-expression plasmid p16L1-GFP, have generally worked well in our hands. Variants of these EF1α promoter-based expression plasmids, such as phGf or pGwf (see *http://home.ccr.cancer.gov/Lco/support.htm* for maps), have been adapted for the Gateway cloning system (Invitrogen). p16L1-GFP and the Gateway-adapted variants also carry GFP under control of the SV40 promoter, which facilitates titration of the infectivity of the vector stock.

293TT cells rapidly replicate plasmids carrying the SV40 *ori* to high copy number in the cell nucleus. In general, it appears that this over-replication only modestly enhances the expression of genes under control of the human cytomegalovirus immediate early promoter (CMV promoter). In contrast, most genes under control of the EF1α promoter exhibit a more pronounced dose-response relationship between plasmid copy number and expression level. For some genes, for example HPV16 L1, expression from the EF1α promoter can yield milligram amounts of protein from a single flask of 293TT cells. If the ultimate goal is the high-level production of a protein of interest in 293TT cells, the EF1α promoter is probably a better choice than the CMV promoter.

Plasmids lacking the SV40 *ori* can be packaged using direct transfection (Basic Protocol 1), but titer yields for SV40 *ori*-negative plasmids are generally a minimum of 10-fold lower than comparable plasmids with the SV40 *ori*. The lack of an SV40 *ori* also precludes amplification of the vector stock (Basic Protocol 2). Addition of a previously reported minimal SV40 *ori* (construct pC139H; Okuley et al., 2003) to a plasmid of interest effectively enhances conversion of the plasmid into a papillomaviral vector stock.

Efficient transfection of 293TT cells during the production of a seed stock is a critical parameter of the production system. In our hands, the transfection reagent Lipofectamine 2000 is reliable, provided the cell density is relatively low at the time of transfection. However, Lipofectamine 2000 is very expensive and other, less expensive transfection methods, such as calcium phosphate (see *UNIT 20.3*) or PEI (see Choi et al., 2007), can be used to transfect 293TT cells, if cost is of primary concern.

A commercial version of the 293TT cell line, known as 293FT, is available from Invitrogen (no. R700-07). 293FT cells appear to perform reasonably well for production of transfection-derived papillomaviral vector stocks, but for unknown reasons they appear not to perform as well for titration or infectious amplification of stocks. The widely available 293T line, on which the 293TT line is based, expresses very low levels of SV40 T antigen (Fu and Manley, 1987) and therefore performs poorly for production of papillomaviral vectors.

Papillomaviral vectors can infect cell lines derived from a wide range of tissue types. However, the efficiency of infection can vary from line to line within a given tissue type and cell lines should be tested to determine their infectability. Ideally, cell infectability studies should be performed using flow cytometric analysis of cells infected with papillomaviral vectors carrying GFP under control of a strong promoter such as CMV or EF1α.

Biosafety

Like other types of mammalian gene delivery vehicles, there is a risk that papillomaviral vectors could endanger laboratory personnel if handled improperly. Papillomaviral vectors are a relatively new gene delivery technology and have not yet been subjected to controlled in vivo safety testing. One theoretical biosafety risk is that vector-delivered plasmids, or random fragments of 293TT cell DNA (see introduction to Alternative Protocol 2), might be oncogenic in human cells. Investigators should seek approval from an appropriate institutional biosafety committee (or comparable body within the investigator's institution) prior to engaging in the production of viral vectors. It is important to note that papillomaviruses are thought to be able to withstand a wide range of temperature and pH conditions, and remain infectious after desiccation (Roden et al., 1997). Thus, environmental exposure to papillomaviral vectors may be of greater concern than for other types of viral vectors.

An additional potential risk posed by papillomaviral vectors is that the L1/L2 packaging plasmid could, in principle, recombine with 293TT genomic DNA segments encoding SV40 T antigen. Such recombination events could result in the formation of an entirely novel autonomous tumor virus. It is important to note that SV40 T antigen cannot drive efficient replication of SV40 ori^+ DNA in murine cells (Smith et al., 2002). It is therefore unlikely that mice would be permissive for in vivo amplification of SV40 T antigen-based viruses.

Several safety modifications are currently under development in our laboratory. A 293 cell line carrying a T antigen mutant with diminished capacity to inactivate the pRb and p53 tumor suppressor genes (Cooper et al., 1997) may become a useful, theoretically safer, alternative to 293TT cells. The co-propagation of separate L1 and L2 expression plasmids (together with expression plasmids of interest) might also be an effective method for reducing the theoretical risk of the development of recombinant autonomous tumor viruses.

Troubleshooting

A critical first step in the production process is efficient transfection of the 293TT cells. Production of a GFP-expressing vector stock allows easy flow cytometric or fluorescent microscopic analysis of the transfected vector-producing cells to determine transfection efficiency. A minimum of 75% of the cells should be brightly GFP-positive if the transfection has worked well. The harvested cell pellet should contain enough GFP that it appears green to the naked eye.

The initial health of the cells is a critical factor in achieving good transfection efficiency. 293TT cultures should not be allowed to become confluent or form regions of piled up cells. It is also essential that the cells be preplated at a low enough density to be <50% confluent at the time of transfection.

The optimal dose of vector stock to use for amplification can vary substantially. The guidelines given in Basic Protocol 2 should result in a very high multiplicity of infection (i.e., many infectious events for each cell in culture) for a good vector stock. Lower-titer stocks might require a higher dose to ensure that both p16L1L2 and the expression plasmid of interest are co-delivered to a majority of cells in culture. Small-scale tests using 6-well plates or 25-cm^2 flasks can be performed to determine optimal stock dose for the amplification step. The time of harvesting the infected cells can also be varied. Again, such tests are facilitated by the presence of an easily scored reporter gene, such as GFP.

Anticipated Results

The initial transfection-based production described in Basic Protocol 1 should yield at least 100 μl of clarified cell lysate with at least 3×10^9 GFP-transducing units per ml. The great majority of the infectious titer should be recoverable after purification by ultracentrifugation or gel filtration, although the purification may dilute the stock somewhat. The subsequent amplification of the stock (Basic Protocol 2) should yield at least 300 μl of clarified lysate with at least 2×10^{10} GFP-transducing units per ml.

Time Considerations

293TT cells can be slow to attach and recover after thawing. It may take more than a week to establish the culture at adequate health for efficient transfection.

Attempts to incubate 293TT cells with Lipofectamine 2000/DNA complexes overnight often results in unacceptable levels of cytotoxicity. It takes some planning to accomplish the 6-hr incubation of the cells with the lipid/DNA complexes, as suggested in Basic Protocol 1, during an 8-hr workday.

Basic Protocols 1 and 2 can be performed in series and still accommodate weekends, provided the procedure is begun on a Monday and the feeding step for the infected amplification culture (Basic Protocol 2, step 3) is omitted. From start to finish (including titration of the amplified stock) the two protocols take 10 days to accomplish in series.

Although crude cell lysates should not be frozen prior to maturation (see notes for step 21 of Basic Protocol 1), clarified crude vector stocks withstand freeze-thaw well. This represents a reasonable stopping point prior to purification of the stock. With some advance planning, it is possible to perform Optiprep purification (Support Protocol 1) in a single day.

The infectious entry process for papillomaviruses is quite slow and asynchronous compared to many other virus types. For most cell types, harvesting the titration (Support Protocol 5) 3 days post-infection results in higher apparent titers. Support Protocol 5 suggests a shorter 48 hr period of incubation primarily in the interest of saving time.

Literature Cited

Buck, C.B., Pastrana, D.V., Lowy, D.R., and Schiller, J.T. 2004. Efficient intracellular assembly of papillomaviral vectors. *J. Virol.* 78:751-757.

Buck, C.B., Thompson, C.D., Pang, Y.Y., Lowy, D.R., and Schiller, J.T. 2005. Maturation of papillomavirus capsids. *J. Virol.* 79:2839-2846.

Buck, C.B., Thompson, C.D., Roberts, J.N., Muller, M., Lowy, D.R., and Schiller, J.T. 2006. Carrageenan is a potent inhibitor of papillomavirus infection. *PLoS Pathog.* 2:e69.

Buck, C.B., Cheng, N., Thompson, C.D., Lowy, D.R., Steven, A.C., Schiller, J.T., and Trus, B.L. Arrangement of L2 within the papillomavirus capsid. Submitted for publication.

Choi, V.W., Asokan, A., Haberman, R.D., and Samulski, R.J. 2007. Production of recombinant adeno-associated viral vectors for in vitro and in vivo use. *Curr. Protoc. Mol. Biol.* 78:16.25.1-16.25.24.

Cooper, M.J., Lippa, M., Payne, J.M., Hatzivassiliou, G., Reifenberg, E., Fayazi, B., Perales, J.C., Morrison, L.J., Templeton, D., Piekarz, R.L., et al. 1997. Safety-modified episomal vectors for human gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 94:6450-6455.

Fu, X.Y. and Manley, J.L. 1987. Factors influencing alternative splice site utilization in vivo. *Mol. Cell Biol.* 7:738-748.

Leder, C., Kleinschmidt, J.A., Wiethe, C., and Muller, M. 2001. Enhancement of capsid gene expression: preparing the human papillomavirus type 16 major structural gene L1 for DNA vaccination purposes. *J. Virol.* 75:9201-9209.

Okuley, S., Call, M., Mitchell, T., Hu, B., and Woodworth, M.E. 2003. Relationship among location of T-antigen-induced DNA distortion, auxiliary sequences, and DNA replication efficiency. *J. Virol.* 77:10651-10657.

Roberts, J.N., Buck, C.B., Thompson, C.D., Kines, R., Bernardo, M., Choyke, P.L., Lowy, D.R., and Schiller, J.T. 2007. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nat. Med. (in press)*.

Roden, R.B., Lowy, D.R., and Schiller, J.T. 1997. Papillomavirus is resistant to desiccation. *J. Infect. Dis.* 176:1076-1079.

Shi, L., Sanyal, G., Ni, A., Luo, Z., Doshna, S., Wang, B., Graham, T.L., Wang, N., and Volkin, D.B. 2005. Stabilization of human papillomavirus virus-like particles by non-ionic surfactants. *J. Pharm. Sci.* 94:1538-1551.

Smith, R.W., Steffen, C., Grosse, F., and Nasheuer, H.P. 2002. Species specificity of simian virus 40 DNA replication in vitro requires multiple functions of human DNA polymerase alpha. *J. Biol. Chem.* 277:20541-20548.

Voytas, D. 2000. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

Internet Resources

http://home.ccr.cancer.gov/LCO/

The Laboratory of Cellular Oncology maintains this website listing plasmid maps and pseudovirus-related technical protocols.

http://www.axis-shield.com/densityhome/density/dapp.htm

Axis-Shield offers a useful handbook containing information about the use of Optiprep.

http://www.invitrogen.com/content/sfs/productnotes/F_051025_MammalianExpressionVectors-TS-TL-MKT-HL.pdf

Invitrogen offers a brochure comparing various commonly-used mammalian promoters.

http://www.cdc.gov/od/ohs/pdffiles/4th%20BMBL.pdf

The Centers for Disease Control and Prevention's handbook entitled "Biosafety in Microbiological and Biomedical Laboratories" discusses procedures for the handling of biohazardous substances.

BK Virus (BKV): Infection, Propagation, Quantitation, Purification, Labeling, and Analysis of Cell Entry

Takahito Moriyama[1, 2] and Andrey Sorokin[1]

[1]Medical College of Wisconsin, Milwaukee, Wisconsin
[2]Tokyo Women's Medical University, Tokyo, Japan

ABSTRACT

BK virus (BKV) can cause BKV nephritis in renal transplant patients and has become a significant reason for graft loss in this decade. BKV is latent in the urogenital tract and most likely is transported with the donor kidney to recipients. BKV replication occurs in the nucleus of human renal proximal tubular cells (HRPTEC) and daughter viruses are delivered to other cells to spread infection. A few in vitro studies have been reported about the mechanism and kinetics of BKV infection. However, there are still a lot of unknown factors regarding BKV infection. This unit describes the handling of BKV, BKV propagation, determination of titer and ability to infect cells, as well as purification and labeling of BKV in order to analyze BKV cell entry. *Curr. Protoc. Cell Biol.* 42:26.2.1-26.2.13. © 2009 by John Wiley & Sons, Inc.

Keywords: BK virus • human renal proximal tubular epithelial cells • infection • propagation • quantitation • purification • labeling

INTRODUCTION

BKV is a small DNA virus with a 40- to 44-nm sized icosahedral capsid. This capsid contains a 5000-base-pair genome that encodes three capsid proteins, viral proteins 1, 2, and 3 (VP-1, VP-2, and VP-3), and two nonstructural polypeptides, large tumor antigen (T-Ag) and small tumor antigen (t-Ag; Ahsan and Shah, 2006). Antibody against T-Ag is useful to detect BKV infection. The other method to identify BKV infection is to detect viral DNA using PCR. Even though it is possible to detect BKV infection by western blot (immunoblot) analysis using antibodies against VP-1, these antibodies are not commercially available.

Other well studied viruses that belong to Polyomaviridae are simian virus 40 (SV40), JC virus (JCV), and mouse polyoma virus (mPy). JCV has been reported to cause progressive multifocal leukoencephalopathy. BKV infection is relatively slow in comparison to infection with other polyoma viruses. BKV particles are most frequently found in caveolae at 4 hr after infection (Moriyama et al., 2007), are transported along microtubules after caveolar endocytosis, and reach the endoplasmic reticulum 6 to 10 hr after infection (Moriyama and Sorokin, 2008). At least 36 hr are necessary to detect high levels of T-Ag and VP-1 expression by immunoblot, and 48 hr are necessary for detection of viral DNA by Southern blot (Low et al: 2004).

To elucidate precise mechanisms and kinetics of BKV's invasive pathway, the precipitating cause of BKV nephritis, factors and signaling related to BKV nephritis, and efficient therapeutic strategy, it is beneficial to use human renal proximal tubular epithelial cells (HRPTEC). These are the main natural targets of BKV infection; viral life cycles could be different in other cell types.

Current Protocols in Cell Biology 26.2.1-26.2.13, March 2009
Published online March 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2602s42

For that reason, this unit describes the standard techniques suggested for the propagation (Basic Protocol 1), titration (Support Protocol 1), purification (Support Protocol 2), labeling (Support Protocol 3), and infection of BKV in HRPTEC (Basic Protocol 2), to facilitate easy handling of BKV by any investigator.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

BASIC PROTOCOL 1

VIRAL PROPAGATION OF BKV

Before starting experiments, sufficient quantities of BKV must be prepared to support the studies for the complete length of the project. The following method is a straightforward way to increase viral stock (Liu and Atwood, 2001; Eash et al., 2004, 2005). Even though this method is rather time consuming, it generates a large amount of viral stock.

For example, if the plan is to carry out weekly experiments that include incubation of BKV at a multiplicity of infection (MOI) of 0.5 FFU (fluorescence forming units)/cell with 5.0×10^5 cells in ten 60-mm cell culture dishes and 7.2×10^5 (24 wells $\times$ 3.0×10^4 cells/well) in a 24-well tissue culture plate, the required amount of BKV is at least 1.5×10^8 FFU per year.

Materials

HRPTEC (Cambrex Bio Science Inc.), passage 6
REBM containing 5% FBS (see recipe)
BKV stock (ATCC no. VR-837), titered by fluorescent focus assay (Support Protocol 1)
2.5% (w/v) deoxycholic acid

75-cm^2 tissue culture flasks
Refrigerated centrifuge
Cell scrapers
Sonicator (e.g., Sonic Dismembrator 550; Fisher Scientific), with microtip

Propagate BKV

1. Seed $2.0–2.5 \times 10^6$ HRPTEC in 15 ml REBM with 5% FBS per 75-cm^2 tissue culture flask.

   $2.0–2.5 \times 10^6$ HRPTEC will result in about 70% to 80% confluency in 75-cm^2 tissue culture flasks. Knowing the exact number of cells in the flask is necessary because the amount of BKV used for infection is determined based on the number of cells available.

2. Prepare 3 ml of REBM with 5% FBS containing BKV at a multiplicity of infection (MOI) of 0.5 FFU (fluorescence forming units)/cell (see Support Protocol 1 for determination of viral titer). Add an aliquot of BKV containing the corresponding amount of BKV particles directly from frozen stock to medium using barrier tips.

   Gardner strain was used for all described procedures described in this unit.

3. Incubate cells with 3 ml of REBM containing BKV for 1 hr, rocking the flasks by hand several times every 15 min to distribute the virus/inoculum evenly in the medium.

4. Add 7 ml of fresh REBM/5% FBS without removing the inoculum (10 ml total).

   To maintain the appropriate amount of HRPTEC for 4 weeks, REBM/5% FBS is recommended. REBM/10% FBS will result in overgrowth, and REBM/0.5% FBS will result in a severe reduction of cell number with cytopathic effect (CPE) of BKV.

5. Incubate cells for 4 weeks, changing medium and collecting the supernatant every week. Centrifuge the collected supernatant 15 min at 960 × *g*, 4°C, and resuspend the cell pellet in 1/10 of the collected supernatant (discard rest of supernatant). Store the resuspended supernatant at −80°C.

   Collected supernatant contains detached cells resulting from CPE caused by BKV.

Harvest the virus

6. At 4 weeks after incubation, collect the supernatant and harvest cells by scraping. Centrifuge 15 min at 1960 × *g*, 4°C, and resuspend in 1/10 of the collected supernatant.

7. Combine with the previously harvested cells and supernatant collected when changing medium. Freeze at −80°C and thaw at 37°C three times.

   About 4 to 5 ml of combined stock will have be obtained from one 75-cm^2 flask at this step.

8. Sonicate three times, each time for 30 sec in a Sonic Dismembrator 550 with microtip, at an output setting of 4, continuous operation, approximate power output of 20%, on ice.

   This process is very important for releasing BKV from cytoplasm and nucleus in HRPTEC.

9. Add 2.5% deoxycholic acid to the cell suspension to achieve a final concentration of 0.25% and incubate in 37°C water bath for 15 min.

10. Centrifuge the lysate and medium mixture 30 min at 1960 × *g*, 4°C.

11. Collect supernatant, divide into 100-µl aliquots, and store at −80°C.

    Repeat freezing and thawing of the viral samples should be avoided. Virus can be stored for >1 year at −80°C.

VIRAL TITRATION USING THE FLUORESCENT FOCUS ASSAY

SUPPORT PROTOCOL 1

It is necessary to determine the titer of the virus stock so as to carry out each experiment under identical conditions. The fluorescent focus assay (FFA) is an improved version of the standard plaque assay for quantification of BKV. What distinguishes FFA from the plaque assay is the detection of a number of infected cells by antibody against BKV large T-Antigen (T-Ag) in indirect immunofluorescence analysis (Low et al., 2004; Abend et al., 2007). Also see *UNIT 4.3* for general principles of immunofluorescence assays. A description of FFA for other viruses can be found in Lonardo et al. (2002), and Payne et al. (2006).

Materials

70% ethanol
HRPTEC (Cambrex Bio Science Inc.), passage 6
REBM containing 0.5% FBS (see recipe)
BKV stock (ATCC no. VR-837) for titration (Basic Protocol 1)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
100% methanol
Tris-buffered saline with 0.1% (v/v) Tween (TTBS; see recipe)
TTBS (see recipe) containing 3% (v/v) FBS
TTBS (see recipe) containing 1% (v/v) FBS
Primary antibody: antibody (mouse PA 416) against SV40 T-antigen, recognizing BKV T-Ag (Calbiochem)
Secondary antibody: goat antibody against mouse Ig, conjugated to Alexa Fluor 350 (detect blue color as nuclei in BKV-infected cells), Alexa Fluor 488 (green color), and Alexa Fluor 680 (red color), all from Invitrogen

Mounting medium: SlowFade Antifade Kit (Molecular Probes) *or* 70% (v/v) glycerol/30% (v/v) PBS (see *APPENDIX 2A* for PBS; store up to 1 year at 4°C)
Clear nail polish

13-mm-diameter glass coverslips
24-well tissue culture plates
Fluorescence microscope (*UNIT 4.2*) with FITC filters (which are a match for Alex Fluor 488)

Infect cells with BKV

1. Flame the coverslips after dipping in 70% ethanol to sterilize. Transfer a coverslip into each well of a 24-well tissue culture plate.
2. Seed 3.0×10^4 HRPTEC cells in 200 µl REBM medium/0.5% FBS per well of a 24-well tissue culture plate with a coverslip in each well, and cultivate until cells reach about 70% confluence.
3. Incubate for 72 hr with 10-fold serial dilutions of prepared BKV stock solution in a final volume of 200 µl per well of REBM/0.5% FBS.

   According to the authors' experience, the dilution should cover 10 FFU/cell to 10^{-4} FFU/cell. Triplicates should be used for incubation with each dose of 10–fold serial dilutions of BKV to determine the precise titer.

4. Add 300 µl of REBM/0.5% FBS (without removing the virus/medium inoculum) for a final concentration of 500 µl/well, and incubate for another 48 hr.

Block and expose to primary antibody

5. Wash the coverslips in the wells once by incubating for 1 min with 500 µl PBS.
6. Incubate coverslips in the wells with 500 µl 100% methanol for 20 min at –20°C to fix the cells.
7. Wash coverslips three times, each time for 5 min with 500 µl of TTBS. Incubate each coverslip in the wells with TTBS containing 3% FBS for 30 min at room temperature, for blocking.
8. Incubate with 1 µl/well of primary antibody (anti–SV40 T-antigen mouse monoclonal antibody PA416) diluted 1:200 in TTBS with 1% FBS for 1 hr at room temperature, and then wash three times, each time by incubating for 5 min with 500 µl/well of TTBS at room temperature.

Expose to secondary antibody

9. Incubate with 1 µl/well of secondary antibody (Alexa Fluor 488–conjugated goat anti–mouse IgG) diluted 1:200 in TTBS with 1% FBS for 40 min at room temperature, and then wash three times, each time by incubating for 5 min with 500 µl/well of TTBS.

   This procedure should be done with protection from the direct light.

10. Wash the coverslips once with 500 µl of distilled water (simply add the water and then aspirate it) and mount the coverslips, cell-side-down, with one drop of mounting medium on the slide.
11. Seal with clear nail polish along the edge of the coverslip to prevent drying.
12. Analyze the cells with the fluorescent microscope immediately (see Fig. 26.2.1).

    If immediate analysis is not possible, slides can be kept in the dark box at 4°C. However analysis should be carried out within a few days.

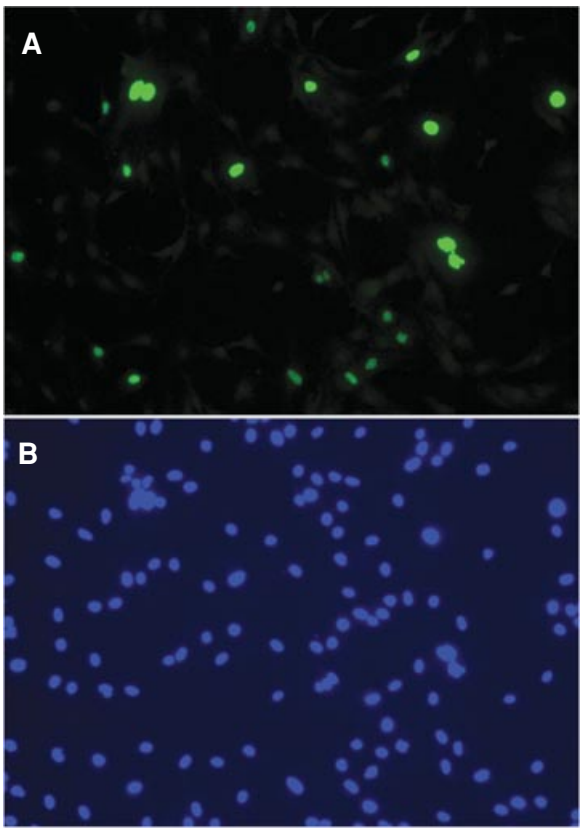


Figure 26.2.1 Detection of HRPTEC BKV infection by immunofluorescence. HRPTEC are either stained with anti-T-Ag antibodies (**A**) or stained with 4′,6-diamidino-2-phenylindole dilactate (DAPI) (**B**). HRPTEC were incubated with BKV (MOI, 0.5 FFU/cell). After 72 hr, fresh medium was added and the cells were incubated for another 48 hr. After incubation, cells were fixed and blocked. Next, cells were incubated with primary antibody [5 μl of PA 416 (Calbiochem) in 1 ml of TTBS with 1% FBS] and secondary antibody [1:200 diluted Alexa Fluor 488 goat anti-mouse IgG (H+L); (Molecular Probes) in TTBS with 1 % FBS]. To stain nuclei, cells were incubated with 300 nM DAPI (Molecular Probes) for 5 min and washed three times with PBS. Cells were observed by fluorescent microscopy (Nikon Eclipse E600) with 20× objective lens, and images were captured by SPOT version 4.0.9 (Diagnostic Instruments).

Calculate fluorescence-forming units (FFU)

13. At a magnification of 20×, count the number of infected cells from five different fields on each coverslip and calculate the average.

14. Calculate the number of infected cells in the whole well from the average of the number of infected cells in each field.

    For example, if the average number of infected cells in the field of BKV diluted at 10^{-4} is 10, and the area of the field is 1.5386 mm^2, the number of infected cells in the well is 1000 (=10 × 153.86/1.5386), where the area of one well of the 24-well plate is 153.86 mm^2 (=7 mm × 7 mm × 3.14).

15. Multiply the number of infected cells in each well by the reciprocal of the volume of sample added to the well, and multiply by the reciprocal of the dilution factor.

    For example, if the number of infected cells in the well of BKV diluted at 10^{-4} is 1000 and the volume of the diluted BKV sample is 0.2 ml, the calculation is $1000 \times 5 \times 10^4 = 5.0 \times 10^7$ FFU/ml. In this calculation, 1000 = the number of infected cells; 5 = 1/0.2 (reciprocal of the volume of BKV diluted sample); and $10^4 = 1/10^{-4}$ (reciprocal of the dilution factor).

This calculation is expected to show the same titer at each dilution point, because 10-fold serial dilution of BKV should cause 1/10-fold cell-killing activity. For example, the number of infected cells in the well of 10^{-3}-diluted BKV has to be 10000 ($10000 \times 5 \times 10^3 = 5.0 \times 10^7$ FFU/ml). Similarly, the number of infected cells in the well of 10^{-5}-diluted BKV has to be 100 ($100 \times 5 \times 10^5 = 5.0 \times 10^7$ FFU/ml). If the results do not meet this condition, there may be an error and the titration should be repeated.

SUPPORT PROTOCOL 2

PURIFICATION OF BKV

Observation of the viral particle itself is valid for the investigation of the kinetics and traffic of virus. However, viral stocks may contain a lot of other proteins besides BK viral proteins, after propagation of BKV. Therefore, purifying BKV from viral stocks is necessary for the labeling of BKV particles. Moreover, viral purification is also capable of preventing contamination with other viruses and bacteria (Eash et al., 2004; Sawa and Komagome, 2005).

Materials

Virus-containing supernatant (Basic Protocol 1)
20% (w/v) sucrose in reassociation buffer (see recipe for buffer)
Buffer A (see recipe)
1.40 g/ml and 1.20 g/ml cesium chloride (CsCl) solutions in distilled water
Slide-A-Lyzer dialysis cassette (MWCO 10,000, Pierce)

Ultracentrifuge
10-ml and 20-ml ultracentrifuge tubes
Sonicator (e.g., Sonic Dismembrator 550; Fisher Scientific), with microtip
5-ml syringe
18-G needle

Purify virus

1. In a 20-ml ultracentrifuge tube, gently layer 10 to 18 ml of virus-containing supernatant at the top of 2 ml of 20% sucrose in reassociation buffer and centrifuge 2 hr at 100,000 × *g*, 4°C.
2. Discard supernatant and dissolve pellets in 3 ml of buffer A. Sonicate three times each time for 30 sec in a Sonic Dismembrator 550 with microtip, at an output setting of 4, continuous operation, approximate power output of 20%, on ice.
3. Add 3 ml of 1.40 g/ml CsCl solution to a 10-ml ultracentrifuge tube and then overlay 3 ml of 1.20 g/ml CsCl solution.
4. Overlay 3 ml of viral sample above the 1.20 g/ml CsCl solution. Centrifuge overnight at 120,000 × *g*, 16°C.
5. Use a 5-ml syringe with an 18-G needle to extract the viral fraction (bottom white-colored fraction between the low and high density of CsCl), and transfer to a fresh 10-ml tube. Dilute the fraction with buffer A to a final volume of 3 ml.

   Insert the needle through the side of the tube 5 mm below the viral band.
6. Centrifuge the viral sample on a CsCl gradient again as described in steps 3 and 4.
7. Extract the viral fraction as in step 5 and dialyze against 500 ml of buffer A at 4°C overnight in a 10,000 MWCO Slide-A-Lyzer cassette.
8. Divide the viral sample into 100 µl aliquots and store at −80°C.

   Repeated freezing and thawing of the viral sample should be avoided. It can be stored for more than 1 year.

LABELING PURIFIED VIRUS

SUPPORT PROTOCOL 3

The icosahedral capsid of BKV contains a 5000 base-pair genome that encodes three capsid proteins, viral protein 1 (VP1), viral proteins 2 and 3 (VP2 and VP3), and two nonstructural polypeptides: the large tumor antigen (T-Ag) and small tumor antigen (t-Ag). The major capsid protein, VP1 (mol. wt. 42 kDa), is likely to be a target of the labeling procedure. Protein labeling kits (Invitrogen) are simple and handy tools to label proteins, and different kits are available depending on the amount of purified protein for labeling and desired fluorochrome for detection. The proper kit should be selected according to the manufacturer's suggestion. The general information about Alexa Fluor dye series can be found at *http://probes.invitrogen.com/media/publications/150.pdf*.

Comparison of Invitrogen kits for labeling proteins can be found at *http://probes.invitrogen.com/handbook/sections/0102.html*.

Description of Alexa Fluor dyes spanning the visible and infrared spectrum can be found at *http://probes.invitrogen.com/handbook/sections/0103.html*.

In this unit we describe Alexa Fluor 488 Microscale protein labeling kit for 20 to 100 μg protein which we use to label BKV particles (*http://probes.invitrogen.com/media/pis/mp30006.pdf*).

Materials

Alexa Fluor 488 Microscale Protein Labeling Kit (Invitrogen) containing:
- Alexa Fluor 488 tetrafluorophenyl (TFP) ester (Component A), 3 vials
- Sodium bicarbonate (Component B), 84 mg
- Reaction tubes (Component C), 3 tubes
- Spin filters (Component D) Nanosep MF 0.2-μm centrifugation devices, 3 tubes
- Purification resin (Component E) Bio-Gel P-6 fine resin suspended in PBS, 3 ml

Purified BKV (Support Protocol 2)

Label BKV

1. Calculate the amount of reactive dye (Component A) to add to labeled BKV according to the formula shown below.

Amount of reactive dye (μl) = [(μg protein/protein mol. wt.) × 1000] × [dye:protein molar ratio (MR)]/11.3

Protein mol. wt. is the molecular weight of VP-1 (42,000 Da).

MR is the optimal degree of labeling of VP-1 from manufacturer's manual (60).

11.3 is the concentration of the reactive dye stock solution (see step 4 below).

For example, to label of 24 μg of BKV (VP-1):

[(24/42,000) × 1000] ×(60/11.3) = 3.0 μl of dye.

2. Add 1 ml of distilled water to the vial of sodium bicarbonate (Component B) and vortex or pipet up and down until the reagent is fully dissolved.

This bicarbonate solution is 1 M, pH ∼8.3.

Store at 4°C up to 2 weeks and −20°C for longer periods.

3. Transfer 20 to 100 μl of purified BKV to a reaction tube (Component C), add 1/10 volume (2 to 10 μl) of sodium bicarbonate, and mix by pipetting up and down several times.

4. Add 10 µl of distilled water to Alexa Fluor 488 TFP ester (Component A) and pipet up and down until the reagent is fully dissolved

   The concentration of this reactive dye stock solution is 11.3 mM.

5. Add the appropriate volume of reactive dye solution according to the formula shown above (see step 1) to a reaction tube (Component C), mix by pipetting up and down, and incubate for 15 min at room temperature.

6. During the 15-min incubation, prepare the resin bed by adding ~800 µl of gel resin (Component E) to the spin filter (Component D), and centrifuging the spin filter 15 sec at 16,000 × *g*, room temperature.

   A fixed-angle rotor will make the resin bed tilt, and the lower side should be 2 to 3 mm above the bottom of the upper chamber for appropriate labeling. A swinging-bucket rotor will make the resin bed horizontal and 5 mm above the bottom of the upper chamber.

7. Transfer 50 µl of purified BKV sample with reactive dye solution from reaction tube (Component C) to the top of the center of the resin bed on the spin filter (Component D), and centrifuge 1 min at 16,000 × *g*, room temperature.

   If the amount of sample is over 50 µl, divide it and purify by separate spin filters with resin bed.

8. Divide the purified and labeled viral sample into 100 µl aliquots and store at −80°C.

   Before experiments, labeled virus must be titrated again (Support Protocol 1).

BASIC PROTOCOL 2

BKV INFECTION OF HUMAN RENAL PROXIMAL TUBULAR EPITHELIAL CELLS (HRPTEC)

In this unit, the method of BKV infection in HRPTEC is described. BKV infection in HRPTEC is relatively slow. The virus is most frequently trapped in caveolae at 4 hr after incubation, reaches the endoplasmic reticulum at from 6 to 10 hr after incubation, and high levels of T-Ag expression detected by immunoblots require at least 36 hr of infection (Low et al., 2004; Moriyama et al., 2007; Moriyama and Sorokin, 2008). It is important to prevent contamination by bacterium and cell detachment from wells or dishes by CPE of BKV, because the incubation period is long.

Materials

HRPTEC (Cambrex Bio Science Inc.), no later than passage 6
REBM containing 0.5% FBS (see recipe)
BKV stocks, titrated (Support Protocol 1), purified (Support Protocol 2), and labeled (Support Protocol 3, for observing virus particles)
60-mm tissue culture dishes *or* 24-well tissue culture plates with glass coverslip in each well (see Support Protocol 1, step 1)
Cell scrapers
Sonicator (e.g., Sonic Dismembrator 550; Fisher Scientific) with microtip

Infect cells with BKV

1. Seed 5.0×10^5 HRPTEC in REBM medium containing 0.5% FBS on a 60-mm tissue culture dish or 3.0×10^4 HRPTEC/well on a 24-well tissue culture plate with a coverslip in each well (see Support Protocol 1, step 1), 1 day prior to incubation with BKV.

   To calculate MOI for BKV infection correctly, it is important to know how many cells are incubated with virus.

   5.0×10^5 HRPTEC provides 70% of confluence at 60-mm tissue culture dish and 3.0×10^4 HRPTEC provides 60% to 70% confluence in one well of 24-well tissue culture plate on the day after seeding.

2. Calculate the required amount of BKV.

   According to our experience, the appropriate MOI of BKV against HRPTEC is 0.5 FFU/cell, because higher MOI and the long incubation period sometimes cause the detachment of cells by CPE (Moriyama et al., 2007).

   If the titer of BKV is 5.0×10^7 FFU/ml (see Support Protocol 1), the required amount of BKV is 5 μl [$=2.5 \times 10^5$ FFU ($=0.5 \times 5.0 \times 10^5 = MOI \times$ number of HRPTEC)] for a 60-mm tissue culture dish and 0.3 μl [$=1.5 \times 10^4$ FFU ($=0.5 \times 3.0 \times 10^4 = MOI \times$ number of HRPTEC)] for a 24-well tissue culture plate.

3. Dilute BKV stocks with REBM containing 0.5% FBS.

4. Remove growth medium from cells and add an equal volume of the medium containing the BKV. Incubate HRPTEC with medium containing BKV for 72 hr.

5. Discard medium and wash three times with REBM containing 0.5% FBS, each time by adding 3.0 ml (for 60-mm dish) or 500 μl (for well of 24-well plate) of REBM/0.5% FBS, and then aspirating the medium. Add fresh medium and incubate for another 48 hr.

   Add 3 ml fresh medium to 60-mm tissue culture plate and 500 μl to 24-well tissue culture plate.

   The first 72 hr are for BKV infection and the next 48 hr are to allow BKV infection to spread to the other cells.

6. After a total of 5 days incubation, harvest cells for analysis as follows:

   a. Discard medium containing with BKV and wash plate three times with PBS on the ice, using the technique described in step 5.

   b. Scrape the cells from the plate surface using a cell scraper.

      The authors do not use trypsin.

   c. Collect the scraped cells in microcentrifuge tubes.

   d. Sonicate three times, each time for 30 sec in a Sonic Dismembrator 550 with microtip, at an output setting of 4, continuous operation, approximate power output of 20%, on ice.

      Cells can be subjected to SDS-PAGE and western blot analysis as described in Moriyama et al. (2007).

ANALYSIS OF BKV ENTRY AND INTRACELLULAR TRAFFICKING PATHWAY IN HRPTEC USING CONFOCAL MICROSCOPE AND MetaVue IMAGING SYSTEM

BASIC PROTOCOL 3

To understand precise mechanisms and kinetics of BKV invasive pathway, investigation of co-localization of BKV particles with target organelles in HRPTEC is a useful method. The co-localization rate of purified and labeled BKV with target organelles indicates the intracellular trafficking pathway and time course of BKV invasion.

Materials

HRPTEC (Cambrex Bio Science Inc.), no later than passage 6
REBM containing 0.5% FBS (see recipe)
Purified and labeled BKV (Support Protocol 3)
Primary antibody against target organelle
Secondary antibody with a fluorophore different from that of the virus

24-well tissue culture plates with glass coverslip in each well
Confocal microscope (*UNIT 4.5*)
MetaVue Imaging System (Molecular Devices) or other image-analysis program

Additional reagents and equipment for indirect immunofluorescence assay (Support Protocol 1)

Incubate HRPTEC with purified and labeled BKV

1. Seed 3.0×10^4 HRPTEC/well in REBM medium containing 0.5% FBS on a 24-well tissue culture plate with a coverslip in each well (see Support Protocol 1, step 1), 1 day prior to incubation with BKV.

2. Calculate the required amount of BKV.

   According to our experience, the appropriate MOI of purified and labeled BKV to use in HRPTEC to observe viral particles is 5 FFU/cell.

   If the titer of BKV is 1.0×10^7 FFU/ml, the required amount of BKV is 15 μl $\{=1.5 \times 10^5$ FFU $(=5.0 \times 3.0 \times 10^4 = MOI \times$ number of HRPTEC$)\}$ for a 24- well tissue culture plate.

3. Dilute BKV stocks with REBM containing 0.5% FBS.

4. Remove growth medium from cells and add an equal volume of the medium containing the BKV. Incubate HRPTEC with medium containing BKV for the target period.

   According to our experience, BKV are most frequently trapped in caveolae at 4 hr after incubation, transported along microtubules, and reach the endoplasmic reticulum from 6 to 10 hr after incubation (Moriyama et al., 2007; Moriyama and Sorokin, 2008).

5. Observe cells by indirect immunofluorescent assay (see Support Protocol 1) using primary antibody against target organelle and secondary antibody with a fluorophore different from that of the virus.

   Co-localization of labeled BKV particles with endoplasmic reticulum markers is shown in Figure 26.2.2.

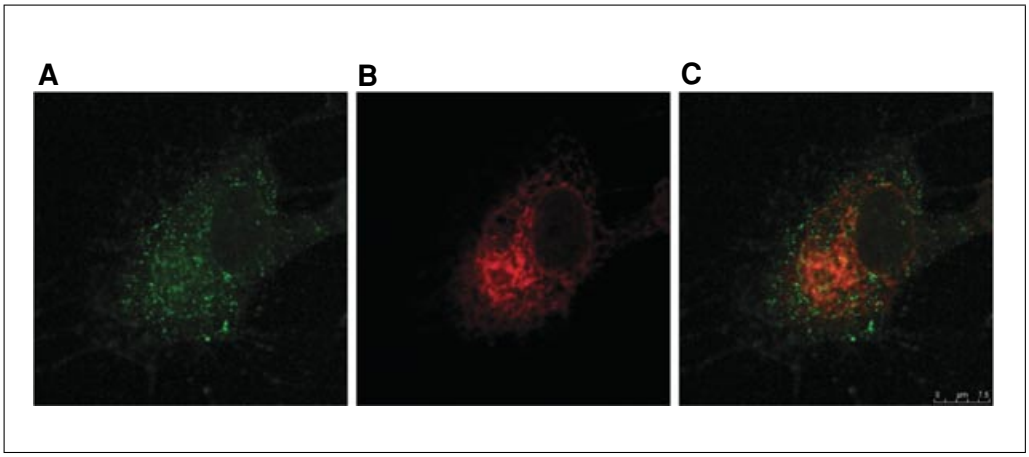


Figure 26.2.2 Localization of labeled BKV particles in HRPTEC. (**A**) Fluorescence of Alexa Fluor 488–labeled BKV particles in HRPTEC. (**B**) Staining for endoplasmic reticulum (ER) in HRPTEC. (**C**) Co-localization of purified and labeled BKV with ER marker. HRPTEC were incubated with purified and labeled BKV for 6 hr. After incubation, cells were fixed and blocked. Then cells were incubated with primary antibody [1:100 dilution of PDI (Abcam) as ER marker against TTBS with 1% FBS] and secondary antibody [1:200 diluted Alexa Fluor 680 goat anti-mouse IgG (H+L) (Molecular Probes) in TTBS with 1% FBS]. Cells were analyzed by confocal microscope (Leica TCS SP5) with 63× objective lens and images were captured by Leica application suite (advanced fluorescence). Bar = 10 μm.

Use MetaVue Imaging System to analyze interaction of BKV with target organelles

6. After observing HRPTEC by confocal microscope and capturing images, use MetaVue Imaging System (or other image-analysis program) for analysis of co-localization.

   If purified BKV is labeled with a green fluorophore (wavelength ~500 nM), the secondary antibody against a target organelle should be labeled with a red (wavelength ~650 nM) or blue (wavelength ~350 nM) fluorophore.

7. Fix the boundary between BKV particles or target organelle and background according to the intensity of pixels, and measure the area of BKV particles and co-localization area of BKV particles with target organelles.

   According to the BKV-uninfected cells, the intensity of background will be calculated. Once the threshold is fixed, the MetaVue Imaging System recognizes and measures area from all pictures regularly and automatically.

8. Calculate the co-localization rate by using formula shown below and the average of all the cells.

   Co-localization rate = Area of BKV particles co-localized with target organelle (mixed color of green with red or blue)/area of total BKV particles (green color).

   To prevent bias, many cells randomly selected from different wells in several independent experiments must be analyzed to obtain the average value. We calculated the average of at least 100 cells from three independent experiments.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

1 M Tris·Cl, pH 8 (*APPENDIX 2A*)
5 M sodium chloride
0.1 M calcium chloride
Store up to 1 year at 4°C

Reassociation buffer

TTBS (see recipe) containing:
1 mM calcium chloride
Store up to 1 year at 4°C

REBM/10%, 5%, and 0.5% FBS medium

Add renal epithelial growth medium SingleQuots (Cambridge Bio Science, Inc., containing human epidermal growth factor, insulin, hydrocortisone, gentamicin/amphotericin B-1000, epinephrine, triiodothyronine, transferrin), 5 ml of 100 U/ml penicillin G (Invitrogen), and 50 ml (for REBM/10% FBS), 25 ml (for REBM/5% FBS), or 2.5 ml (for REBM/0.5% FBS) of fetal bovine serum (FBS) to 500 ml of renal epithelial basal medium (REBM; Cambrex Bio Science Inc.). Store at 4°C.

Tris-buffered saline (TBS), 10×

1.5 M (87.66 g) sodium chloride
0.2 M (24.22 g) Tris base
Add distilled H_2O to final volume 1000 ml and adjust pH to 7.5 using NaOH or HCl
Store at room temperature

Tris-buffered saline with Tween (TTBS)

Dilute TBS 10× stock solution to 1× working solution with distilled water and add Tween 20 to 0.1% (v/v)

For example, to prepare 1 liter, one would combine 100 ml 10× TBS 899 ml distilled water, and 1 ml Tween 20.

COMMENTARY

Background Information

BK virus is a nonenveloped double-stranded DNA virus which belongs to family Polyomaviridae. It was initially isolated from the urine of renal transplant patients with ureteric obstruction in 1971, and named after the initials of this patient (Gardner et al., 1971).

Primary BKV infection occurs by the upper respiratory route until age 10, without any obvious symptoms. More than 80% of the population is infected with BKV, and ∼50% of healthy native kidneys contain latent BKV. BKV mainly persists in the urogenital tract and enters latent phase in the renal tubular epithelial and urothelial cells. In this decade, BKV has become a severe problem in allograft failure among renal transplant patients, because latent BKV, likely transported with donor kidney, progresses to BKV nephritis in immunocompromised hosts, particularly in renal transplant patients. After renal transplantation, 35% to 60% of patients have BKV viruria and 5% to 30% of patients have BKV viremia. The prevalence of BKV nephropathy is about 10% of renal transplant patients, and about half of them have been reported to progress to irreversible allograft failure within 1 year after diagnosis (Nickleit et al., 2003).

Rapid progress is required to analyze BKV infection against HRPTEC, because the precise mechanism and kinetics of BKV infection are still not clearly understood and efficient antiviral therapies have not been established yet. This unit will be useful for the in vitro analysis of BKV infection in HRPTEC.

Critical Parameters and Troubleshooting

The most important factor for BKV experiments is to determine the appropriate dose of BKV and incubation period to cause efficient BKV infection in HRPTEC (30% to 40% of all cells). The highest titer and longest incubation period may not always cause the most efficient BKV infection. If the percentage of BKV infection is below 30% by indirect immunofluorescent analysis, or T-Ag expression is extremely low, several titers of BKV and several incubation periods should be examined to determine appropriate dose and incubation period. A point to notice is that another 48-hr incubation period to spread BKV infection to the other cells is required after initial BKV infection. The appropriate dose is different between purified and labeled BKV and untreated BKV. After purification and labeling, it is necessary to measure the viral titer and to determine the appropriate dose and period for infection. According to the author's observations, the appropriate titer of BKV without purification and labeling is MOI 0.5 FFU/cell and the optimal incubation period is 5 days (72 hr for primary infection and 48 hr for spreading) for the most efficient BKV infection; the appropriate dose of purified and labeled BKV is MOI 5 FFU/ml with a 24-hr incubation.

Biosafety

BKV belongs to agents of moderate potential hazard to personnel and the environment. This class includes agents which may produce disease of varying degrees of severity from accidental inoculation or injection but which are contained by ordinary laboratory techniques (Biosafety Level 2 standards of practice and facility). The guidelines for handling this kind of agent can be found in Biosafety in Microbiological and Biomedical Laboratories (BMBL, 4th ed.), available online at *http://www.cdc.gov/OD/ohs/biosfty/bmbl4/bmbl4toc.htm*.

Anticipated Results

Described protocols are generally straightforward, but require several days of procedures. However more than 1.0×10^7 FFU/ml BKV should be obtained after propagation, and these viruses will cause infection of more than 30% of cells as detected by indirect immunofluorescent analysis. Appearance of prominent T-Ag and VP-1 bands in immunoblot analysis and viral DNA detected by PCR will provide additional evidence of BKV infection. Purified and labeled virus will be useful for experiments dealing with analysis of BKV infection of its natural target HRPTEC.

Time Considerations

As we describe in this unit, viral propagation takes about 1 month. However BKV, once propagated by this method, provides a high amount of BKV. Titration of BKV using FFA takes 5 days, a time period similar to that required to cause the infection in HRPTEC for the immunoblots (*UNIT 6.2*) and indirect immunofluorescent analysis (*UNIT 4.3*). Viral purification requires 3 days and labeling requires about 2 hr.

Literature Cited

Abend, J.R., Low, J.A., and Imperiale, M.J. 2007. Inhibitory effect of gamma interferon on BK virus gene expression and replication. *J. Virol.* 81:272-279.

Ahsan, N. and Shah, K.V. 2006. Polyomaviruses and human diseases. *Adv. Exp. Med. Biol.* 577:1-18.

Eash, S. and Atwood, W.J. 2005. Involvement of cytoskeletal components in BK virus infectious entry. *J. Virol.* 79:11734-11741.

Eash, S., Querbes, W., and Atwood, W.J. 2004. Infection of Vero cells by BK virus is dependent on caveolae. *J. Virol.* 78:11583-11590.

Gardner, S.D., Field, A.M., Coleman, D.V., and Hulme, B. 1971. New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet.* I:1253-1257.

Liu, C.K. and Atwood, W.J. 2001. Propagation assay of the JC virus. *Methods Mol. Biol.* 165:9-17.

Lonardo, A.D., Buttinelli, G., Amato, C., Novello, F., Ridolfi, B., and Fiore, L. 2002. Rapid methods for identification of poliovirus isolates and determination of polio neutralizing antibody titers in human sera. *J. Virol. Methods* 101:189-196.

Low, J., Humes, H.D., Szczypka, M., and Imperiale, M. 2004. BKV and SV40 infection of human kidney tubular epithelial cells in vitro. *Virology* 323:182-188.

Moriyama, T. and Sorokin, A. 2008. Intracellular trafficking pathway of BK virus in human renal proximal tubular epithelial cells. *Virology* 371:336-349.

Moriyama, T., Marquez, J.P., Wakatsuki, T., and Sorokin, A. 2007. Caveolae endocytosis is critical for BK virus infection of human renal proximal tubular epithelial cells. *J. Virol.* 81:8552-8562.

Nickeleit, V., Singh, H.K., and Mihatsh, M.J. 2003. Polyomavirus nephropathy: Morphology, pathophysiology, and clinical management. *Curr. Opin. Nephrol Hypertens.* 12:599-605.

Payne, A.F., Binduga-Gajewska, I., Kauffman, E.B., and Kramer, L.D. 2006. Quantitation of flaviviruses by fluorescent focus assay. *J. Virol. Methods* 134:183-189.

Sawa, H. and Komagome, R. 2005. The JC virus-like particle overlay assay. *Methods Mol. Biol.* 292:175-186.

Internet Resources

http://probes.invitrogen.com/media/publications/150.pdf

http://probes.invitrogen.com/handbook/sections/0102.html

http://probes.invitrogen.com/handbook/sections/0103.html

http://probes.invitrogen.com/media/pis/mp30006.pdf

Web sites showing product descriptions of Alexa Fluor labeling kits.

Methods Used to Study Respiratory Virus Infection

Emilio Flaño,[1] Nancy A. Jewell,[1] Russell K. Durbin,[1] and Joan E. Durbin[1]

[1]Center for Vaccines and Immunity, The Research Institute at Nationwide Children's Hospital, Ohio State University, Columbus, Ohio

ABSTRACT

This unit describes protocols for infecting the mouse respiratory tract, and assaying virus replication and host response in the lung. Respiratory infections are the leading cause of acute illness worldwide, affecting mostly infants and children in developing countries. The purpose of this unit is to provide a basic strategy and protocols to study the pathogenesis and immunology of respiratory virus infection using the mouse as an animal model. The procedures include: (1) basic techniques for mouse infection, tissue sampling, and preservation, (2) determination of viral titers, isolation and analysis of lymphocytes and dendritic cells using flow-cytometry, and (3) lung histology, immunohistochemistry, and in situ hybridization. *Curr. Protoc. Cell Biol.* 43:26.3.1-26.3.28. © 2009 by John Wiley & Sons, Inc.

Keywords: respiratory viruses • pathology • immunohistochemistry • immunology • respiratory syncytial virus • influenza • murine γ-herpesvirus 68 • lung • lymphoid organs • T lymphocytes • dendritic cells

INTRODUCTION

The respiratory tract is remarkable for its extensive surface area (70 m^2 in adult humans), which is in continuous contact with the external environment. The lung samples ~10,000 liters of air every day and is exposed to a vast array of foreign particles. As a consequence, the respiratory system is a major portal by which microorganisms enter the body. When studying infection and immunity in this tract, the structurally and functionally distinct compartments must be appreciated, as each compartment has distinct populations of both immune and parenchymal cells. The nasopharynx, or uppermost airway, is lined by both respiratory and olfactory epithelium. The respiratory epithelium of the mouse nasopharynx is columnar to cuboidal with cilia and scattered goblet cells. The tracheal lining is similar but, unlike the human trachea, has a high percentage (50% to 60%) of non-ciliated, secretory Clara cells. The right lung of the mouse is divided into five lobes; the left lung is not divided. The lower respiratory tract is comprised of branching conducting airways, which extend from the trachea to the terminal bronchioles, which open into the alveolar ducts. The bronchi and bronchioles of the mouse are lined predominantly with Clara cells, which are relatively rare in the human airway (Sternberg, 1997). This altered cell distribution must be kept in mind as it will alter the pattern of infection with some viral respiratory pathogens. Goblet cells are not found in the uninfected mouse lung. The bulk of the lung volume is made up of alveoli, where gas exchange occurs. The alveolar ducts and spaces are lined primarily by flat type I pneumocytes overlying capillaries. The rare, plump type II cells produce surfactant and are thought to be the source of new type I cells (Maronpot et al., 1999).

Because efficient gas exchange requires that the alveoli be relatively free of fluid and inflammatory cells, the respiratory mucosal immune system must maintain a fine balance between eliminating pathogens and inhibiting inflammatory pathology. This requires discriminating between innocuous airborne antigens and pathogen-associated antigens,

Current Protocols in Cell Biology 26.3.1-26.3.28, June 2009
Published online June 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2603s43

and inducing tolerance or immunity, respectively. Surveillance is maintained by a layer of dendritic cells underlying the mucosa of the larger conducting airways (Vermaelen and Pauwels, 2005; Weslow-Schmidt et al., 2007), and alveolar macrophages present within the air spaces of the lung. Additional inflammatory cells are recruited by chemokines secreted by epithelial and immune cells when pathogens are detected. The mouse model is permissive for some but not all human respiratory pathogens, and among those viruses that will infect both mouse and man, disease in the mouse may not necessarily mirror disease in the human host. This is an important consideration when undertaking infection experiments to model human diseases in the murine host.

This unit describes protocols for infecting the mouse respiratory tract, and assaying virus replication and host response in the lung.

NOTE: All procedures involving viral pathogens and infected tissues must be carried out in a biosafety cabinet prior to fixation.

NOTE: All experiments using live animals must first be approved by the Institutional Animal Care and Use Committee and conform to governmental regulations for the care and use of laboratory animals.

BASIC PROTOCOL 1

INTRANASAL INFECTION OF MICE

Most intranasal infection protocols use relatively large volumes (30 to 100 μl) of inoculum, resulting in intratracheal delivery of virus. This protocol discusses intranasal inoculation using both small and large volumes. Choice of route will vary with the virus studied, and the interest of the investigator in upper versus lower airway infection.

Materials

Mice
Avertin anesthesia (see Support Protocol 5)
Viral inoculum diluted in PBS (see recipe) or HBSS (Cellgro) to a volume of 30 to 100 μl/dose

1-ml syringes and 25- to 27-G needles (BD)
Positive displacement pipets and tips (Rainin)

1. Restrain the mouse in dorsal recumbency by picking it up by the scruff of the neck between the index finger and thumb. Hold the tail against the hand with the little finger.

2. Insert the 1-ml syringe (filled with anesthetic) with attached needle into the caudal 2/3 of the right side of the abdomen, taking care to avoid internal organs. After the needle is inserted, draw the syringe back (if anything is aspirated, the viscera has been hit). Withdraw and attach a new needle to the syringe before trying again. Inject Avertin (200 to 250 μl, 25 mg/kg), pausing briefly before withdrawing the needle so that liquid does not seep out.

3. Monitor anesthesia by testing the mouse toe pinch reflex.

   Inadequate anesthesia increases the probability that the mouse will sneeze or swallow the virus.

4. When the mouse is fully anesthetized, inoculate the mouse by placing the pipet tip containing the inoculum at the opening of the nares. Slowly expel the virus preparation from the tip, checking that the mouse is inhaling (not swallowing) the solution.

The use of a positive displacement pipet prevents aerosol contamination and isolates the virus sample from the pipet body. This precaution is extremely important if different virus mutants, strains, or virus species are being used.

Changing the titer of the inoculum is possible by altering the amount of stock virus used and/or by adjusting the volume of diluent accordingly. The authors normally use volumes between 30 and 100 μl for mice 6 weeks of age or older, smaller volumes may be needed for younger animals.

5. Allow the mouse to recover for 5 to 10 min. Observe the mouse until it regains consciousness and breathing returns to normal.

   The mouse will have an increased respiratory rate for some minutes after intranasal inoculation. If a proper dose of anesthesia has been used, the mouse will regain consciousness and breathing will return to normal within 15 to 30 min.

INFECTION OF THE UPPERMOST AIRWAY

ALTERNATE PROTOCOL 1

This is a modification of the intranasal infection protocol (see Basic Protocol 1). In this method, an inoculum is delivered specifically to the upper airway by using a smaller volume and administering the inoculum over several minutes (Visweswaraiah et al., 2002; Gitiban et al., 2005). Materials required are listed in Basic Protocol 1, except that the viral inoculum should be prepared such that each dose is contained within a 20-μl volume. A timer is also required for this protocol.

1. Anesthetize mice with Avertin as in Basic Protocol 1, steps 1 to 3.
2. Place anesthetized animals on a flat surface, in a dorsal recumbent position with the nose facing you.
3. Administer 2 μl of inoculum to each naris of a mouse at 0, 2, 7, 9, and 11 min.
4. Allow the mouse to recover.

DETERMINING VIRUS TITER: PLAQUE ASSAY

BASIC PROTOCOL 2

The optimal method for determining virus titer will depend upon the virus being used. In this protocol, general applicable techniques for obtaining samples for testing, and examples of titering methods used for two viruses commonly used in the authors' laboratory, RSV and influenza A virus, are outlined.

Any virus recovered from the mouse respiratory tract will, presumably, replicate to some degree in mouse cells. Different viruses, however, vary widely in the degree to which they produce plaques (countable lesions) on a cell monolayer. RSV, for instance, normally produces no obvious cytopathology in cultured mouse cells, and has historically been assayed on monolayers of human HEp2 cells or monkey Vero cells. This protocol is specific for RSV; specifics will vary for other viruses. The plaque assay is performed on STAT1-deficient NY3 mouse fibroblasts, which are extremely sensitive to many viruses.

Materials

Dry ice
Mice
70% ethanol
Decarbonated Dulbecco's modified Eagle medium (dcDMEM; see recipe)
Susceptible cell line
10 mM HEPES supplemented with 5% fetal calf serum
Earle's balanced salt solution (Invitrogen) supplemented with 10 mM, pH 7.4 (HEBSA)

Overlay medium (see recipe)
Fixative/stain (see recipe)

2-liter beaker (Nalgene)
Styrofoam insert (3- to 5-cm thick, equal to the beaker in circumference)
Parafilm
Dissecting scissors, forceps
5-ml sterile, plastic snap-cap tubes, preweighed
Tissue homogenizer (e.g., PowerGen model 125, Fisher Scientific)
Scale
24-well plates
37°C, 5% CO_2 incubator
96-well multiwell plate
Dissecting microscope

Euthanize and prepare animal

1. Assemble an asphyxiation chamber by placing dry ice at the bottom of a 2-liter beaker, and covering with a Styrofoam insert constructed from packing material. Cover the beaker with Parafilm until enough gas is generated such that the Parafilm begins to bulge outward.

   This primitive CO_2 generation chamber is convenient because it can be sterilized after use, is portable, and can be placed within the biosafety cabinet.

2. Place the mouse to be sacrificed onto the Styrofoam platform and recover the beaker.

   The mouse will lose consciousness within ~2 min.

3. Place the unresponsive animal in a supine position, wet the fur over the thorax with 70% ethanol, open the skin, and remove the chest plate. Remove the lungs, which are salmon pink in color.

   If the trachea is to be collected, the neck, in addition to the chest, must be opened. The trachea with its distinctive cartilaginous rings sits just below the esophagus, which can be peeled off and discarded.

   CAUTION: *To avoid contamination of samples, clean and disinfect dissecting tools between animals. The chemical sterilization method outlined in Support Protocol 1 can be used in the biosafety cabinet.*

   Lung tissue (and adjacent lymph nodes if desired) harvested in this manner can be used for many purposes, but must be processed or frozen quickly to maintain its integrity.

   For determination of virus titers or cytokine levels, lungs should be placed in a sterile, plastic snap-cap tube (without liquid) and frozen immediately on dry ice. These samples can later be transferred to a −80°C freezer for storage.

4. Place the tissue sample (lung, etc.) in a tared 5-ml plastic snap-cap tube.

Homogenize tissue

5. Reweigh the tared tubes containing samples to determine the tissue mass so that titers can be expressed in terms of pfu/g of tissue.

6. Add carefully measured dcDMEM (~5 to 10 ml/g tissue), and homogenize each sample in tube at full speed for 30 sec until tissue is completely macerated. Sterilize the homogenizer between samples by chemical sterilization (see Support Protocol 1).

7. Centrifuge 5 min at 2000 × *g*, 4°C, to clarify. Carefully remove the clear supernatant to a new 5-ml tube, avoiding pellet and flocculent material at the bottom of the tube, and any insoluble fatty material floating on top.

It is important that samples do not undergo repeated freeze-thaw cycles before virus titers are determined. Homogenize samples the day they are collected, then freeze (−80°) for assay at a later time, or freeze the samples as they are being collected and prepare homogenates at a later time and set up the plaque assay on the same day. Viruses differ with respect to their ability to tolerate freezing, some pathogens may lose as much as a log of infectivity with each freeze-thaw cycle.

Perform plaque assay

8. Prepare a ~5×10^5 susceptible cells/ml suspension in DMEM, 10 mM HEPES, and 5% fetal calf serum.
9. Plate 0.4 ml into each well of a 24-well plate.
10. Allow cells to adhere and spread 2 to 16 hr in a 37°C, 5% CO_2 incubator.

    Monolayers should be >80% confluent; sensitivity to virus infection declines as the monolayer ages.

11. Prepare serial dilutions of virus samples in a 96-well multiwell plate. Prefill the appropriate number of wells with 180 µl HEBSA. Dilute 20 µl homogenate or BAL (see Basic Protocol 3) into the first well and mix well. Transfer 20 µl from that well into the next well, etc.

    It is important that the dilution series be carried out as accurately as possible, since systematic errors will be amplified exponentially. For this reason, care must be taken to avoid bubbles, and pipet tips changed between transfers. Ensure that the pipet tips do not carry any extra liquid as drops cling to the outside of the tip. HEBSA should be at room temperature, since a temperature differential between pipet tip and medium introduces systematic inaccuracy. A multichannel pipettor can be used, but the aforementioned cautions must be observed with respect to each of the pipet tips at each step.

12. Aspirate the medium from each well of the 24-well plate, and replace it with 100 µl/well serially diluted lung homogenate.
13. Incubate plate 2 hr in a 37°C, 5% CO_2 humidified incubator, keeping the liquid distributed over the monolayer by periodic (at least every 15 min) shaking of the plate.
14. Aspirate the inoculum and replace it with 0.4 ml/well of the overlay solution.

    In all steps, it is important to prevent the monolayers from drying out—aspirate with a minimum of suction, avoid exposing the cells to rushing air, and remove/replace medium from no more than a few wells at a time.

15. Incubate 5 days in a 37°C, 5% CO_2 incubator.
16. Aspirate medium, replace with fix/stain (~0.5 ml/well), and incubate 30 min at room temperature. Rinse plate briefly by immersion in cold tap water.
17. Count plaques with a dissecting microscope.

Make calculation of concentration of infectivity

18. Estimate the concentration of infectivity (commonly, but not quite correctly, referred to as "titer") as follows:

$$\text{titer} = (\text{no. of plaques/well/volume of inoculum/well}) \times \text{dilution}$$

    For example, if the well inoculated from the 4th well in the dilution series above shows 35 plaques, inoculum volume = 0.100 ml, and dilution = 10^4, then concentration of infectious virus = (35/0.100) × 10^4 = 3.5 × 10^6.

Estimate uncertainty

19. Estimate the standard error as the ratio as the square root of the total number of plaques counted. The relative standard error (i.e., expressed as a fraction of the concentration) is therefore the inverse of the square root of the number of plaques counted.

    In the above example, the standard error is $(35)^{-1/2} = 16.9\%$.

    If duplicate wells had been inoculated, yielding 34 and 36 plaques, for example, the estimated concentration would be the same, but the error would be reduced to $(70)^{-1/2} = 12.0\%$.

ALTERNATE PROTOCOL 2

DETERMINING VIRUS TITER: FLUORESCENT FOCUS ASSAY

Many viruses (e.g., influenza virus, parainfluenza virus 5, Newcastle disease virus) do not produce visible plaques. Estimation of the infectivity of such viruses can be done by following the procedure given in Basic Protocol 2 with the additional staining of the infected monolayers with fluorescent or enzymatically tagged antibodies. The procedure for immunofluorescent detection is outlined here. The procedure for enzyme-linked antibodies is similar, but details vary with the specific enzyme used.

***Additional Materials** (also see Basic Protocol 2)*

Virus-infected cells (see Basic Protocol 2, step 10)
PBSTA (see recipe)
Primary antibody against viral antigen (e.g., rabbit anti-influenza)
Fluorophore-tagged secondary antibody (e.g., FITC-goat anti-rabbit IgG)
PBS (see recipe)
Inverted fluorescent microscope

1. After the infection has been allowed to progress, fix the monolayer as for plaque assay, except use 0.5 ml/well fixative without dye for 30 min at room temperature.

   Often 24 hr of infection will suffice for fluorescent staining, since infection of a single cell is detectable, eliminating the need to allow time for spread to adjacent cells.

2. After fixation, wash wells two times with PBSTA by pipetting 300 µl PBSTA and then aspirating it.

   The detergent in this solution permeabilizes the cells, exposing intracellular antigens to recognition by antibody.

3. Dilute primary antibody against the virus in PBSTA. Add a sufficient volume of diluted antibody to cover the monolayer (~0.15 ml/well of a 24-well plate) and incubate at least 30 min at room temperature.

   Optimal dilutions are determined empirically as the dilution giving an optimal foreground/background ratio; typically in the range of 200 to 2000 fold.

4. Remove the primary antibody, wash wells two times with PBSTA (as in step 2).

5. Add the secondary antibody tagged with fluorophore diluted in PBSTA, and incubate 1 hr at room temperature in the dark.

   Exposure to light should be minimized for fluorescently tagged antibodies.

   If an enzyme-conjugated secondary antibody is used, after the incubation, wash and develop with the appropriate visualization substrate.

6. Remove the secondary antibody, and replace it with PBS.

7. Count fluorescent foci using an inverted fluorescent microscope.

8. Estimate the concentration of infectivity as in the plaque assay, but report as fluorescent focus forming units/ml (ffu/ml).

CHEMICAL STERILIZATION

SUPPORT PROTOCOL 1

It is important to assure that biologically active organisms and molecules be reliably eliminated from all surfaces that will contact subsequent samples. In processing any significant number of samples, sterilization by autoclaving is impractical. This protocol describes how to clean an apparatus and destroy any trace residual biologically active protein, nucleic acid, or lipid.

Materials

0.1% (w/v) SDS/0.006 M sodium hypochlorite (commercial chlorine bleach diluted 1:100)
0.1% (w/v) SDS/0.001% (w/v) Coomassie blue
70% ethanol
Phosphate-buffered saline (PBS; see recipe), sterile

Homogenizer
Sterile 50-ml tissue culture tubes

1. Run the homogenizer with the following solutions. Run two cycles (30 sec each) with 0.1% SDS/0.006 M sodium hypochlorite solution, making sure that any visible tissue residue has been removed. Take care that the liquid covers all surfaces that will potentially contact subsequent samples.

   For stainless steel homogenizers, prolonged contact with hypochlorite will be corrosive. However, at the specified dilution, followed immediately by the rest of the cleaning cycle, corrosion is not significant.

2. Run one cycle with 0.1% SDS/0.001% Coomassie blue.

   The dye is included as a sensitive indicator for residual hypochlorite; if the dye is bleached, this cycle should be repeated.

3. Run two cycles with 70% ethanol to remove residual SDS.

   The dye serves as a visible proxy for the detergent; make sure all traces of dye have been eliminated.

4. Rinse with sterile PBS to remove any residual ethanol, which may damage the next sample. After the last sample, omit the PBS wash to avoid salt precipitation during storage of the apparatus.

LEUKOCYTE ISOLATION

BASIC PROTOCOL 3

This protocol outlines the procedures for sampling and processing mouse tissues to obtain single-cell suspensions that can be used for flow cytometry or other assays (ELISpots, migration assays, proliferation assays, and purification of cell subsets). The method used to process the organs differs depending on the cell population of interest. For the analysis of T cell lymphocytes, the authors use mechanical digestion, which is quick and convenient. For the analysis of dendritic cells, collagenase digestion is recommended. This procedure is necessary to separate dendritic cells from the extracellular matrix, and is followed by a short incubation in an EDTA solution, which helps to disrupt multicellular complexes.

Materials

Mice
Avertin solution (see Support Protocol 5)
70% ethanol
HBSS/heparin: one vial heparin (Sigma) to 50 ml HBSS
HBSS without $MgCl_2$, $MgSO_4$, or $CaCl_2$ (Cellgro)
5 mg/ml collagenase A in MEM (see recipe)
Gey's solution (see recipe)

Trypan blue (MP Biomedicals)
80% Percoll (see recipe)
Assorted 1-ml syringes and needles (BD)
15- and 50-ml centrifuge tubes (BD)
Forceps, scissors
Terumo Surflo i.v. catheter 186 × 1¼–in.
70-μm cell strainers (BD Falcon)
Tissue culture dishes (100-mm; Falcon)
37°C water bath
Centrifuge (e.g., Sorvall Legend RT)
Hemacytometer (Hausser Scientific)
Microscope (e.g., Zeiss Axiostar plus)

Dissect mouse

1. Anesthetize mouse with Avertin solution (25 mg/kg i.p.).
2. Wet animal down using 70% ethanol. Place mouse in dorsal recumbency on two to three layers of paper towels.
3. Bleed the mouse and collect blood in a 15-ml tube containing 5 ml HBSS/heparin.

   Draw blood from the heart by sticking the 26-G needle of a 1-ml syringe into the apex of the left ventricle or by axillary bleed using a Pasteur pipet.

Perform bronchoalveolar lavage (BAL)

4. Perform BAL by using forceps to hold the skin of the neck and cutting along the neck from the chin to the thorax. Expose the trachea and make a small incision using sharp scissors or a needle with a beveled edge.
5. Insert only the plastic sleeve of an i.v. catheter into the trachea, and using a 1-ml syringe, flush lungs three times with 1 ml HBSS. Repeat two additional times using a total of 3 ml HBSS. Collect fluid in a 15-ml tube.

   Work slowly to avoid collapsing the lungs.

   If the BAL specimen is to be used for cytokine assays, use only 1 ml of lavage fluid to maximize the concentration of mediators present.

6. As a general rule, pool the lavage from the mice that belong to the same experimental group.

   Pooling lavage from the same group is done because of the limited number of lymphocytes found in the airways.

Collect organs

7. To harvest lung and draining lymph nodes, cut the skin of the mouse from the abdomen to the top of the thorax. Open the abdominal wall below the ribcage. Lift the sternum with tweezers and cut the diaphragm.
8. Then cut away the lower part of the ribcage to expose the heart and lungs. Using fine tweezers, harvest the lymph nodes and place in a labeled 15-ml tube containing 5 ml HBSS.

   The mediastinal lymph nodes are located ventral to the trachea at the level of the thymus. The bronchial lymph nodes are situated at the bifurcation of the trachea.

9. Next, harvest the lung and place in a labeled 15-ml tube containing 5 ml HBSS.
10. Harvest spleen, which lies adjacent to the greater curvature of the stomach, and place it in a labeled tube containing 5 ml HBSS.

Prepare single-cell suspensions

11a. *For analyzing lymphocytes:* Mechanically disrupt the tissues—spleen, lymph nodes, and lung—by pressing them gently through a cell strainer with a syringe plunger to achieve a single-cell suspension. Rinse the strainer repeatedly with HBSS to recover as many lymphocytes as possible. Transfer the sample to a 15-ml conical tube.

11b. *For analyzing dendritic cells:* Perform enzymatic digestion of the organ(s) by mincing the organ(s) in a solution of 5 mg/ml collagenase A in MEM in a small tissue culture dish. Incubate 30 min at 37°C (see Support Protocol 3). Transfer the sample to a 15-ml conical tube.

The BAL samples do not need processing. All the samples are transferred to 15-ml conical tubes if they are not already in one.

12. Centrifuge all single cell suspensions 10 min at 375 × *g*, 8°C.

13. Remove the supernatant from the cell pellet via aspiration. Flick the cell pellets to break up cell clumps.

Lyse erythrocytes

14. Add 3 ml Gey's solution to the spleen and lung cells, 1 ml to the lymph nodes and BAL samples, and 10 ml to the peripheral blood cells. Mix gently.

15. Incubate 3 to 5 min in a 37°C water bath.

16. Top off tubes with cold HBSS to achieve a final volume of 10 ml. Centrifuge 10 min at 375 × *g*, 8°C.

17. Discard the supernatants, disrupt cell pellets by flicking tube with fingers, and resuspend cells in HBSS. Further purify lung cells using a gradient; resuspend lung cells in 1.0 ml of 80% Percoll (see Support Protocol 2).

A volume of 5 ml for spleen and 1 ml for lymph nodes and BAL is used.

Count cells

18. While the lung cells are spinning, count viable cells in the rest of the samples using a hemacytometer and trypan blue exclusion (*UNIT 1.1*) or using an automatic cell counter. Count lung cells when the gradient is done.

Samples with low cell numbers such as BAL and lymph nodes must be counted using the hemacytometer. This is a tedious and time-consuming process, but it is essential in order to calculate absolute numbers of cells in each tissue using the frequencies obtained after flow cytometry analysis.

PERCOLL GRADIENT FOR LUNG TISSUE

SUPPORT PROTOCOL 2

Percoll solutions cannot be filtered, so be extra careful and keep sterile. The authors use the same gradient to process liver cells.

Materials

Percoll (GE Health Care) solutions (see recipe)
HBSS without $MgCl_2$, $MgSO_4$, or $CaCl_2$ (Cellgro)

Vortexer
Centrifuge
Pasteur pipets
50-ml conical tubes

1. Allow Percoll solutions to warm to room temperature.
2. Resuspend the lung cells in 2 ml of 80% isotonic Percoll. Vortex 3 sec.
3. Carefully layer 2 ml of 40% isotonic Percoll on top and centrifuge 25 min at 600 × *g*, 20°C.
4. Transfer cells from the 80%/40% Percoll interface using a Pasteur pipet and place into a 50-ml conical tube filled with HBSS without $MgCl_2$, $MgSO_4$, or $CaCl_2$.
5. Centrifuge all single-cell suspensions 10 min at 375 × *g*, 8°C.
6. Resuspend in 1 ml HBSS without $MgCl_2$, $MgSO_4$, or $CaCl_2$ and count viable cells (*UNIT 1.1*).

SUPPORT PROTOCOL 3

COLLAGENASE DIGESTION

Collagenase A digestion is used to disrupt the structure of organs (such as spleen and lung) to prepare single-cell suspensions.

Materials

Organ (spleen or lung)
Collagenase A solution (see recipe)
HBSS
HBSS/2 mM EDTA solution (see recipe)
Percoll

Tissue culture dishes (60-mm)
1- and 3-ml syringes
Razor blades
37°C tissue culture incubator
50-ml tubes
Cell strainer (70-μm)
Cell scraper
Centrifuge

Disrupt organ structure

1. Place organ (spleen, lung) in a small tissue culture dish with 5 ml collagenase A solution.

   If lymph nodes need to be digested, transfer organs to a 5-ml FACS tube with 200 μl collagenase A solution, smash it up with a 1-ml syringe plunger, and incubate as indicated in step 5.

2. Inject the organs with 1 ml collagenase A using a 1-ml syringe with a 26-G needle attached.
3. Mince the organs with a razor blade.
4. Pass the tissue through a 3-ml syringe to break it up.
5. Incubate the tissues for 30 min in a 37°C tissue culture incubator. Pass the tissues through the 3-ml syringe every 5 to 10 min.

Prepare single-cell suspension

6. Transfer the cells to a 50-ml tube through a 70-μm cell strainer. Use HBSS and a cell scraper to recover cells attached to the plastic.
7. Centrifuge 10 min at 375 × *g*, 8°C.

8. Resuspend the pellets in 10 ml HBSS/2 mM EDTA. Incubate 10 min at room temperature.
9. Centrifuge 10 min at 375 × *g*, 8°C.
10. Resuspend pellet in HBSS or Percoll solution as needed and follow the tissue processing protocols.

FLOW CYTOMETRY STAINING AND ANALYSIS OF ANTIGEN-SPECIFIC CD8 T CELL LYMPHOCYTES IN LUNG

BASIC PROTOCOL 4

This protocol describes staining of tissue lymphocytes to analyze antigen-specific CD8 T cells using flow cytometry. CD8 T cell responses are key for the control of viral infections (Doherty and Christensen, 2000; Wong and Pamer, 2003). The T cell receptor (TCR) on the surface of CD8 T cells recognizes peptides derived from viral antigens complexed with major histocompatibility complex (MHC) class I molecules on the surface of infected cells. CD8 T cells express a broad range of effector mechanisms that mediate resistance to infection, such as (1) direct cytolysis of target cells mediated by perforin, granzymes, and Fas; (2) secretion of cytokines such as TNFα and IFN-γ; and (3) secretion of chemokines that attract inflammatory cells at sites of infection. A prominent characteristic of respiratory virus infections, such as those mediated by influenza or respiratory syncytial virus, is that viral replication is limited to the lung epithelium. Thus, for viral clearance, the immune system must specifically target the mucosal surfaces of the respiratory tract. To accomplish this, antigen presenting cells migrate to the draining lymph nodes where they initiate a program of T cell proliferation and differentiation (Vermaelen et al., 2001; Legge and Braciale, 2003). Newly generated effector T cells return to the respiratory tract, complete their differentiation process, and terminate the infection (Cerwenka et al., 1999; Lawrence and Braciale, 2004). The effector CD8 T cells and memory cells that remain in the lung, once infection is cleared, are functionally different from their counterparts in lymphoid tissues (Hikono et al., 2006).

Fluorescence-activated flow cytometry is one of the most broadly used technologies in cell biology, immunology, hematology, and oncology. It allows the simultaneous and rapid analysis of multiple parameters of each cell in a suspension. This technology provides the means to define and analyze specialized subpopulations of cells that may carry out distinct functions. In addition, the availability of MHC class I–tetrameric reagents (Altman et al., 1996), which can label epitope-specific CD8 T cells, allows for the analysis of antigen-specific T cells at the single-cell level. Immunologists now routinely use "tetramers" to track and analyze T cell responses with a level of detail that was unimaginable 15 years ago.

Materials

Single-cell suspension from lung, spleen, or lymph nodes (see Basic Protocol 3 or Support Protocol 3)
Antibodies: Fc-Block (anti-mouse CD16/CD32 Fc II/III), anti-CD8α (FITC, Alexa 700, APC), anti-KLRG1 FITC (eBiosciences or BD Biosciences)
Staining wash buffer (SWB; see recipe)
Tetrameric reagent: γHV68 ORF6$_{487\text{-}495}$ /K^b APC (NIH Tetramer Core Laboratory)
10% (w/v) paraformaldehyde (see recipe)

96-well round-bottom plates
Centrifuge with plate rotor
1.1-ml microtubes (National Scientific Supply Co.)
Flow cytometer (e.g., FlowJo, TreeStar)

Prepare cells

1. Add 10^6 cells/well to a 96-well, round-bottom plate from the single-cell suspension derived from lung, spleen, or lymph node (see Basic Protocol 3 or Support Protocol 3). Set up as many single-color control wells as there are fluorochromes in the staining panel, and also a negative control well (use spleen cells for the control wells).

   For the authors staining panel, four control wells are necessary.

2. Centrifuge the plate 3 min at 450 × *g*, 8°C. Flick and gently vortex the plate.

Block cells

3. Add 50 µl Fc-Block diluted 1:200 in SWB to each well and incubate 10 min on ice.
4. Add 150 µl of SWB to each well.
5. Centrifuge plate 3 min at 450 × *g*, 8°C. Flick and gently vortex the plate.

Expose cells to tetramer

6. Add 50 µl/well of the appropriate dilution of the tetramer, which has been diluted in SWB. Mix by pipetting up and down (never vortex a tetramer).
7. Incubate 1 hr at room temperature in the dark.
8. Add 150 µl of SWB to each well.
9. Centrifuge plate 3 min at 450 × *g*, 8°C. Flick and gently vortex the plate.

Expose cells to antibody

10. Add 50 µl/well of the appropriate dilution of antibodies (anti-CD8α Alexa 700, anti-KLRG1 FITC) in SWB to the experimental samples. Add control antibodies as follows:

    a. Negative control: SWB alone
    b. FITC control: anti-CD8α FITC
    c. APC control: anti-CD8α APC
    d. Alexa 700 control: anti-CD8α Alexa 700.

11. Incubate 20 min on ice in the dark.
12. Add 150 µl/well of SWB to each well.
13. Centrifuge plate 3 min at 450 × *g*, 8°C. Flick and gently vortex the plate.

Wash cells

14. Add 200 µl/well of SWB to each well.
15. Centrifuge plate 3 min at 450 × *g*, 8°C.
16. While the plate is spinning, add 20 µl of 10% paraformaldehyde to the appropriate number of 1.1-ml microtubes.
17. Flick plate and vortex gently.

Fix cells

18. Add 180 µl/well of SWB to each well, pipet up and down to resuspend the cells, and transfer them into the 1.1-ml microtubes containing 20 µl of 10% paraformaldehyde solution.

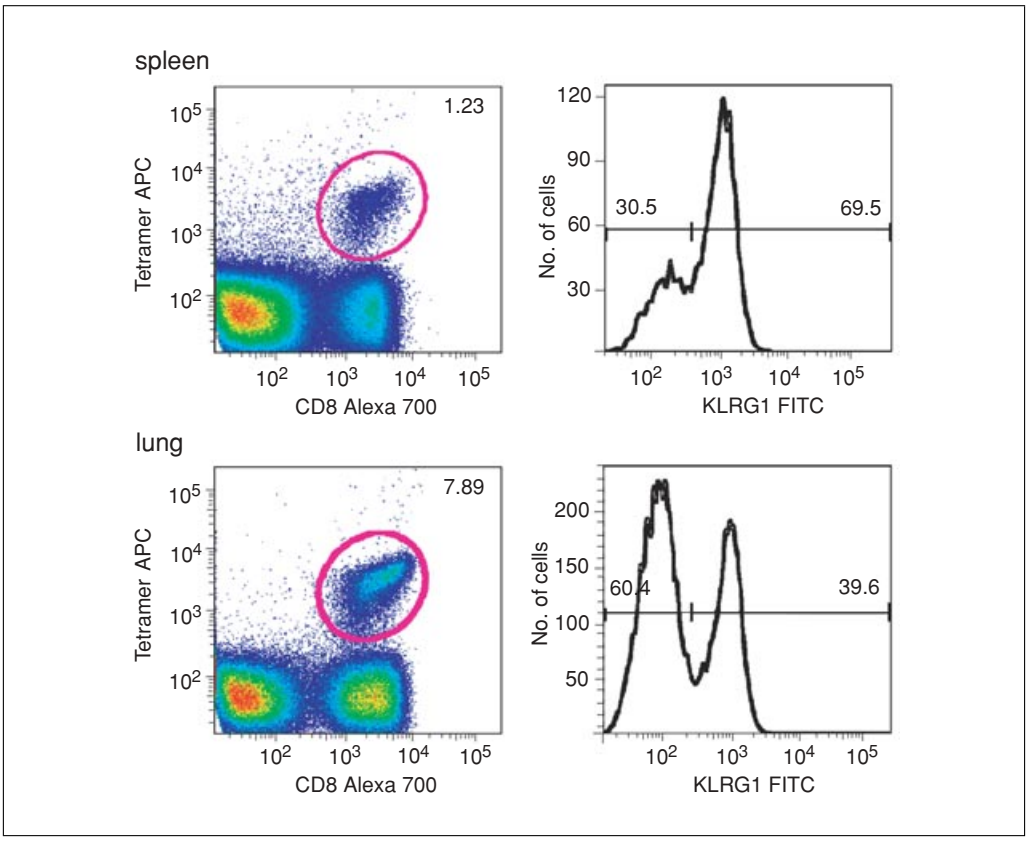


Figure 26.3.1 Flow cytometry staining of spleen and lung cells from a γHV68-infected mouse on day 16. Antigen-specific CD8 T cells are gated using the tetrameric reagent ORF6$_{487\text{-}495}$/K^b to show the expression profile of KLRG1. KLRG1 is a killer inhibitory receptor that is used to define terminally differentiated short-lived effector T cells. For color version of this figure go to *http://www.currentprotocols.com*.

Analyze cells

19. Keep the tubes on ice or at 4°C (no more than 2 to 3 days) and in the dark until running on a flow cytometer.

20. Analyze data on FlowJo (TreeStar).

The flow cytometry analysis in Figure 26.3.1 shows antigen-specific CD8 T cells gated using the tetrameric reagent ORF6$_{487\text{-}495}$/K^b, a murine γ-herpesvirus 68 (γHV68) peptide recognized by CD8 T cells when complexed with the MHC class I allele K^b. This gate is used to plot histograms showing the expression profile of KLRG1 (killer lectin receptor G1). KLRG1 is a killer inhibitory receptor that is used to define terminally differentiated short-lived effector T cells (SLECs). As observed, the distribution of SLECs is different in lung and spleen: the majority of SLECs concentrate in spleen because by day 14 after γHV68 infection acute respiratory infection has been cleared and the virus infection is at the peak of latency phase establishment in the spleen in B cells, macrophages, and dendritic cells (Flano et al., 2000).

FLOW CYTOMETRY STAINING AND ANALYSIS OF DENDRITIC CELLS IN LUNG

ALTERNATE PROTOCOL 3

Dendritic cells are professional antigen presenting cells essential for the generation of adaptive immune responses. Dendritic cells develop from bone marrow–derived precursors and are recruited from blood circulation to peripheral organs, where they continuously sample their environment for foreign substances. Dendritic cells are found through the airways forming a network through the epithelium and also constitute a small fraction of the cells in the BAL (Vermaelen and Pauwels, 2005). They are able to take up and

process antigens and migrate to the draining lymph nodes where they contribute to the initiation of adaptive immune responses. Pulmonary dendritic cells are key regulators of the local immune response to airborne pathogens, antigens, and allergens (Holt et al., 2008).

In mice, the dendritic cells in the respiratory tract express high levels of MHC class II and CD11c (Wikstrom and Stumbles, 2007). They can be distinguished from the lung macrophages because dendritic cells are less autofluorescent and express higher levels of class II molecules on their cell surface (Jakubzick et al., 2006). As their secondary lymphoid organ counterparts, respiratory dendritic cells can be divided into two main subsets: conventional dendritic cells ($CD11c^+B220^-$) and a very small fraction of plasmacytoid dendritic cells ($CD11c^+B220^+$; Weslow-Schmidt et al., 2007). Conventional respiratory dendritic cells can be further divided into two subsets on the basis of CD11b expression, with the predominant subset expressing high levels of CD11b. None of these subsets has been characterized extensively, but there is some evidence to suggest that they are functionally and phenotypically distinct (Julia et al., 2002; Belz et al., 2004; von Garnier et al., 2005). Other dendritic cell markers such as CD103, CD205, or CD8α can also be used to further define these populations.

Figure 26.3.2 presents a comparison between dendritic cells from spleen and lung stained with the same panel of fluorochrome-conjugated antibodies. The staining

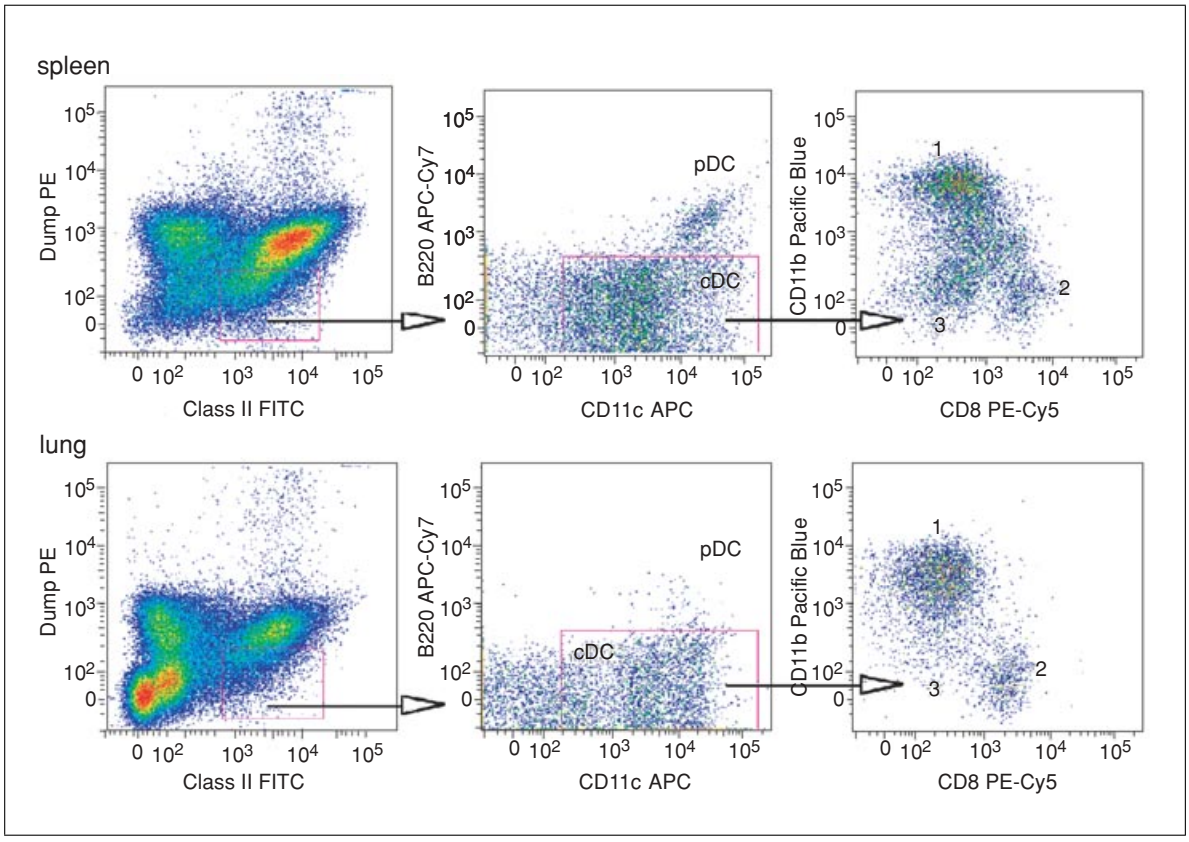


Figure 26.3.2 Analysis of dendritic cell subpopulations in lung and spleen of a γHV68-infected mouse on day 7. Lineage-positive cells are gated out using a dump channel (CD3, CD19, and NK1.1). Conventional dendritic cells (cDC) are defined as class $II^+CD11c^+B220^-$ and further divided into subsets: $CD11b^+$ (population 1), $CD8a^+$ (population 2), and $CD11b^-CD8a^-$ (population 3). Plasmacytoid dendritic cells (pDC) are class $II^+CD11c^+B220^+$. For color version of this figure go to *http://www.currentprotocols.com*.

procedure is performed as described in Basic Protocol 4, eliminating steps 6 to 9, which are specific for tetramer staining. As dendritic cells are a minor fraction of the total cells, the authors' staining panel uses a dump channel with a mixture of antibodies (CD3, CD19, and NK1.1) to gate out lineage-positive cells (T cells, B cells, and natural killer cells, respectively). The level of class II expression helps to differentiate dendritic cells from pulmonary macrophages. Then, conventional dendritic cells ($CD11c^+$) are plotted against plasmacytoid dendritic cells (B220) and further analyzed using expression of CD11b versus CD8α. CD11b expression defines a subpopulation formerly known as "myeloid" dendritic cells, and CD8α is expressed on "lymphoid" dendritic cells. The distribution of the subpopulations of dendritic cells is different between lung and spleen.

The following is the respiratory dendritic cell staining antibody panel: MHC class-II-FITC; CD3-PE, CD19-PE, NK1.1-PE; CD8α-PE-Cy5; CD11c-APC; B220-APC-Cy7; and CD11b-Pacific Blue.

HISTOLOGY

BASIC PROTOCOL 5

Examination of infected tissues under the microscope allows the investigator to assess many aspects of inflammation and disease that cannot be discerned from tissue homogenates. Standard hematoxylin and eosin (H&E)–stained sections provide information about trafficking and localization of inflammatory cells, as well as the extent of necrosis/apoptosis.

Materials

Mice
10% (v/v) buffered formalin (Fisher Scientific)

Tissue cassettes (Fisher Scientific)
No. 2 pencil
Histology laboratory

Additional reagents and equipment for removing lungs (see Basic Protocol 2)

1. Open the thorax and remove the lungs from a CO_2-anesthetized mouse as described in Basic Protocol 2.
2. Fix the lungs with 10% buffered formalin. Inflate the lung with formalin by gently injecting the lung tissue with a syringe and small-gauge needle until tissue is filled, but not taut, and place the inflated lungs or lung lobes into appropriately labeled tissue cassettes. Be sure to label the cassettes with a pencil as many inks or "permanent" markers will wash off during processing.

   The buffered formalin volume should be more than ten times the volume of the harvested tissues in a closed container.

   Good lung histology requires that the lung tissue be inflated with formalin at the time of fixation.

3. Fix tissue for at least 4 hr, but not more than 72 hr, before it is processed by the histology laboratory.

   The processor usually runs overnight, and the next day the histotechnologist will embed the tissue contained in each cassette within a paraffin block. If the tissue orientation is not correct, the block can be melted and the tissue re-embedded at a later time.

   Extended formalin fixation will inhibit binding of both antibodies and nucleic acid probes to tissue sections. Once fixed tissue is processed and embedded in paraffin, it is stable for many years at room temperature.

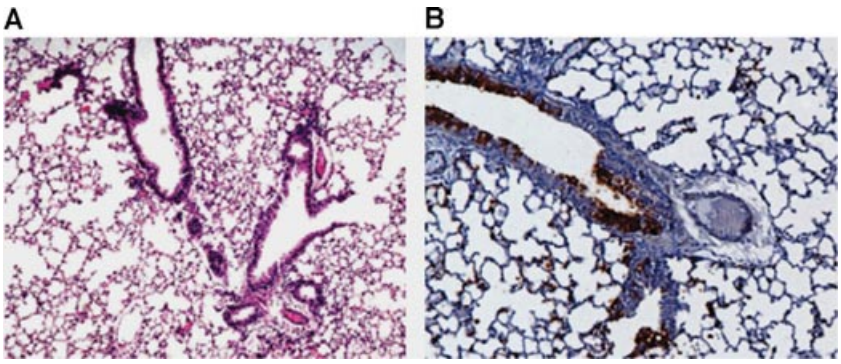


Figure 26.3.3 Much information can be gained from examining the histology of infected lungs. **A** is a standard H&E stained section photographed at low power (50×). The large conducting airways branching into the smaller bronchioles and finally alveolar spaces can be clearly seen. This animal was infected 24 hr previously with a high dose (10^6 pfu) of influenza A virus, but no evidence of pathology is appreciable at this early time point. (**B**) A higher magnification image (100×) shows that, despite the lack of inflammatory cells at this early time point, many airway lining cells (shown as red) already show evidence of infection. The slide shown in B was stained using polyclonal rabbit serum raised against whole virus, and the method described in Basic Protocol 6. This level of staining was obtained without antigen retrieval. For color version of this figure go to *http://www.currentprotocols.com*.

4. Order H&E-stained paraffin sections from the histology laboratory. Request additional sections for immunohistochemistry (IHC) or in situ hybridization (ISH) at the same time, but specify the purpose of these additional sections so that the appropriate glass slides can be used.

   Slides for IHC or ISH are coated with a polyanion such as poly-L-lysine to increase tissue adherence.

   It is best to rely on the histology laboratory for the standard H&E stain as it requires many solutions that must be renewed often.

   An example of an H&E stained lung section is shown in the left-hand panel of Figure 26.3.3 and demonstrates the anatomy of the lung infected 24 hr earlier with influenza A virus. At this low magnification (photomicrograph is taken through a 5× objective), the anatomy of the lung can be clearly seen with large airways branching into smaller airways, and finally opening into the alveolar ducts and space. Very little inflammation can be appreciated at this time point.

BASIC PROTOCOL 6

BAL CYTOLOGY

Characterization of the different types of inflammatory cells within the airspaces of the lung may be necessary, and this can be done by using a portion of the BAL fluid, obtained as described in Basic Protocol 3, to make cytospins.

Materials

BAL fluid (see Basic Protocol 3)
Acetone (Fisher Scientific), cold
TBS (see recipe)

Hemacytometer
Microscope
Cytoslides (Thermo Scientific)
Disposable cytofunnels (Thermo Scientific)
Cytocentrifuge (Thermo Scientific)

1. Determine cell concentration in each BAL sample using a hemacytometer. Adjust concentration such that no more than 10^4 cells are added to each slide. Make sure a sample volume of at least 150 µl is obtained before loading each sample.

   Interpretable data can also be obtained with fewer than 10^4 cells as only the edges are readable when the cell pellet is thick.

2. Attach cytoslides with a raised circle to contain the pelleted cells to the cytofunnels following the manufacturer's instructions, and load the 150-µl samples into individual funnels before they are inserted into the cytocentrifuge.

3. Centrifuge samples 6 min at 113 × *g*, room temperature.

4. For a differential cell count to determine the make up of the specimen (i.e., % lymphocytes, % eosinophils, % monocytes/macrophages), allow the slides to air dry for several hours and request a Wright-Giemsa stain from the pathology laboratory.

   This stain is done routinely to examine blood smears in a hospital setting and it is highly recommended to use a pathology laboratory given the limited sample size.

5. To use slides for immunohistochemistry (IHC), fix in cold acetone for 5 min, rinse in TBS for 5 min, and continue on to Basic Protocol 7, step 3 (IHC protocol).

IMMUNOHISTOCHEMISTRY

BASIC PROTOCOL 7

Immunohistochemistry (IHC) relies upon the availability of specific antibodies that can bind with high affinity to antigens in tissue sections. This technique involves incubating tissues with a primary, antigen-specific antibody, followed by a conjugated secondary antibody that will bind to the Fc region of the primary antibody. For example, when mouse IgG is used to stain human tissue, a biotinylated secondary antibody recognizing mouse IgG can be used to detect the bound primary antibody. Avidin bound to horseradish peroxidase will specifically bind to biotin, and when chromogen is added, it will be deposited at sites where primary antibody is bound.

Materials

4-µm tissue sections on Plus slides (Surgipath)
Xylenes
95% and 100% ethanol
3% (v/v) H_2O_2 (McKesson)
Superblock (Scytek)
TBS (see recipe)
Primary antibody (determined by investigator)
Primary antibody dilution buffer (Biomedia/Fisher Scientific) *or* TBS (see recipe) with 1% (w/v) BSA (fraction V, Fisher Scientific)
Biotinylated secondary antibody (determined by investigator)
Streptavidin-enzyme and substrate kits (Scytek or Dako)
Mayer's hematoxylin (Dako)
0.25% (v/v) ammonium hydroxide
Crystal/Mount (Biomedia)
Permaslip (Alban Scientific)

65°C oven
Coplin jars (Fisher Scientific)
Pap pen (Biocare Medical)
Humidified chamber
Coverslips

1. Incubate 4-µm sections on slides prepared by the histology laboratory in a 65°C oven for 1 hr prior to staining to improve tissue adherence.

2. Deparaffinize sections from conventional paraffin blocks and rehydrate before staining. Set up (in a chemical hood) a series of baths in Coplin jars, and incubate the slides as follows:

   a. Xylene: two times, 3 min each time
   b. Xylene: one time, 5 min
   c. 100% ethanol: three times, 2 min each time
   d. 95% ethanol: one time, 1 min
   e. Rinse in deionized water, 1 min.

3. Place slides in 3% H_2O_2 for 15 min to reduce background staining, and then rinse with deionized water for 2 min.

4. Circle tissue section to be stained with a pap pen so that the staining reactions can be carried out in a small volume, i.e., within the circle, while slides sit in a humidified chamber.

   Such chambers can be purchased, or made from a plastic container (e.g., Tupperware) containing moist paper towels and spacers to hold the slides away from the wet paper towel surface.

5. Incubate slides 5 min at room temperature with 100 to 200 µl Superblock, and then rinse with TBS for 1 min.

6. Incubate tissue sections with the appropriate dilution of primary antibody solution for 2 hr at room temperature or overnight at 4°C.

   The appropriate dilution of each primary antibody must be determined empirically and may vary from 1:50 to 1:5000.

7. Wash 10 min in TBS. Apply the biotinylated secondary antibody and incubate 10 to 20 min at room temperature. Following this incubation, wash a second time with TBS.

8. Stain using additional incubations, first with streptavidin-enzyme reagents, followed by substrate.

   Kits are available from a number of companies; the authors generally use the streptavidin/HRP system from Scytek, and follow the manufacturer's directions.

9. Rinse slides with TBS, then water. Counterstain in hematoxylin (15 sec to 1 min, depending on the batch of stain) followed by bluing (just a few dips) in 0.25% ammonium hydroxide. Wash with deionized water for 30 sec.

10. Once slides are dry, cover the tissue with one to two drops of Crystal/Mount, incubating at 65°C until dry. Use Permaslip to attach the cover slip.

    This protocol is the most flexible, allowing the use of many different sources of primary and secondary antibody. Nonetheless, a more sensitive methodology is being utilized where, rather than using a biotinylated secondary antibody and enzyme-conjugated avidin, an enzyme-linked polymer conjugated to a secondary antibody is used. The EnVision reagents from DAKO make use of this system, and although compatible reagents are not yet available for all primary antibodies, enhanced foreground and decreased background are routinely obtained with this technique.

SUPPORT PROTOCOL 4

MAXIMIZING SIGNAL: ANTIGEN RETREIVAL

This protocol describes a number of approaches to increasing the signal obtained with a particular primary antibody. As reagents are often limited when working with rodent models, it is often necessary to optimize results obtained with the antibodies available. For a detailed discussion of options available, consult a manual devoted to the subject (Renshaw, 2007).

If a primary antibody gives a poor signal using the standard IHC protocol outlined in Basic Protocol 7, antigen retrieval using a pressure cooker to heat the tissue sections may increase the foreground.

Materials

4-μm section slides
0.5 M Tris·Cl, pH 10 (see recipe)
Pressure cooker (any)

Additional reagents and equipment for deparaffinizing slides (see Basic Protocol 7)

1. After the sections are deparaffinized, as in Basic Protocol 7, steps 1 and 2, place the slides in a Coplin jar filled with 0.5 M Tris·Cl, pH 10, and place the jar in a pressure cooker with sufficient boiling water to heat, but not leak into, the container. Heat for 3 to 15 min at a pressure of 15 to 17 psi.
2. Cool the pressure cooker by running under cool tap water for 10 min. Remove the Coplin jar and allow the contents to cool for 30 min at room temperature.
3. Wash the slides in TBS for 30 sec, then water, and proceed to Basic Protocol 7, step 3.

These conditions work for many antigens, but some will require less heat or more acidic conditions for optimal unmasking. Optimal conditions for a particular antigen must be determined empirically.

IHC USING FROZEN SECTIONS

ALTERNATE PROTOCOL 4

Sometimes it is possible to use monoclonal antibodies to stain frozen tissues even when they are unable to detect antigen in fixed tissue sections.

Materials

Mice
Tissue Tek Cryo-OCT embedding compound (Fisher Scientific)
Acetone (Fisher Scientific), cold
Desiccant
TBS (see recipe)

Terumo Surflo i.v. catheter 186 × $1^{1/4}$-in.
Cryostat
Cryostat chuck (obtained from cryostat manufacturer)
Plus slides (Surgipath)
Coplin jars (Fisher Scientific)

1. Inflate the lungs with warm (37°C) Cryo-OCT by cannulating the trachea as described for the BAL (see Basic Protocol 3), and instilling 0.5 ml OCT as gently as possible, then separating individual lung lobes for individual embedding.

When harvesting lung tissue for this purpose, it is still necessary to inflate the lung tissue as much as possible with a water-soluble embedding compound (e.g., Cryo-OCT), which is very thick and difficult to manipulate. The viscosity is temperature dependent, so heating an aliquot to 37°C before using to inflate the lungs is necessary.

2. Use a cryostat to freeze, embed, and section frozen tissues. Place a thin layer of OCT medium onto a cold cryostat chuck, and before it completely solidifies, quickly add a tissue section and cover it with additional OCT. Cut sections as soon as the medium is completely frozen, or store embedded samples in a sealed bag for months to years at −70°C.

3. Cut 4- to 7-μm frozen sections on the cryostat.

   Cutting sections may be difficult for the novice; therefore, seeking help from a histotechnologist is recommended.

4. Place sections on Plus slides, and allow to dry for 1 to 3 hr at room temperature. Fix in cold acetone for 5 min, and store with desiccant at −70°C if the slides are not stained immediately.

5. To stain slides, rinse in TBS for 5 min, and proceed with Basic Protocol 7, step 3.

BASIC PROTOCOL 8

IN SITU HYBRIDIZATION

This method describes the use of a non-radioactive RNA probe to detect mRNA in paraffin-embedded tissue sections. For this procedure, a probe that is labeled with digoxigenin (DIG) is needed. The authors use the DIG RNA labeling kit (Roche) to prepare the riboprobe and test its labeling efficiency. The following protocol takes 2 days to complete. For the first day, all reagents and containers must be RNase free. Either treat all solutions with DEPC or prepare the solutions with DEPC-treated water. DEPC-treated solutions are no longer necessary after the hybridization overnight at 42°C.

Materials

4-μm section slides
Diethylpyrocarbonate (DEPC)-treated water
PBS (see recipe)
100 mM glycine (Fisher Scientific)
Triton X-100 (Sigma)
5 μg/ml proteinase K (Sigma) in PBS
4% paraformaldehyde (Electron Microscopy Sciences; see recipe), 4°C
100 mM triethanolamine, pH 8
Acetic anhydride (Sigma)
Prehybridization buffer (see recipe)
Digoxigenin (DIG)-labeled riboprobe
Hybridization buffer (see recipe)
20× SSC (see recipe)
ISH buffers 1, 2, and 3 (see recipes)
Goat serum (Invitrogen)
Anti-DIG alkaline phosphatase Fab fragment (Roche)
NBT/BCIP tablets (Roche)
Fast green FCF (see recipe)
Crystal/Mount (Biomedia)

Pap pen (Biocare Medical)
37°C slide warmer
Coplin jars (Fisher Scientific)
Humidified chamber (e.g., Tupperware container)
37°, 42°, and 65°C incubator/hybridization oven
80°C heating block
37°C water bath
Shaking/orbital rocker at 37°C
15-ml conical tubes
Coverslips

Additional reagents and equipment for deparaffinizing slides (see Basic Protocol 7)

NOTE: All buffers and reagents should be prepared in DEPC-treated water.

Prepare slides

1. Deparaffinize and rehydrate 4-μm tissue sections as described in Basic Protocol 7, but using DEPC-treated water to prepare solutions and for rinsing slides.
2. Wash slides two times, 5 min each wash, in PBS.
3. Incubate slides two times, 5-min incubations, in 100 mM glycine.
4. Incubate slides in PBS containing 0.3% (v/v) Triton X-100 for 15 min.
5. Wash slides two times, 5 min each wash, in PBS.
6. Encircle tissue section using a Pap pen. Permeabilize with 5 μg/ml proteinase K in PBS for 30 min at 37°C, followed by two 5-min washes in PBS.

Post-fix sections

7. Post-fix tissue section in cold (4°C) 4% paraformaldehyde in PBS for 5 min, followed by two 5-min washes in PBS.

Acetylate sections

8. Acetylate tissue section with two 5-min incubations in fresh 100 mM triethanolamine, pH 8, containing 0.25% (w/v) acetic acid anhydride.

   Add the acetic anhydride to the TEA buffer just before use, and use a clean Coplin jar for the second incubation.

Prehybridize sections

9. Incubate sections in a Coplin jar with 50 ml prehybridization buffer in a humidified chamber for at least 10 min in a 37°C incubator/hybridization oven.
10. During prehybridization, add 60 ng of DIG-labeled riboprobe, prepared and tested according to manufacturer's instructions, to 100 μl of hybridization buffer. Denature riboprobe 10 min at 80°C then place on ice.

Hybridize samples

11. Blot off the prehybridization buffer from each slide by placing the end of the slide onto absorbent paper towels, and re-encircle the tissue section with the Pap pen.
12. Add the hybridization buffer with the DIG-labeled riboprobe to the encircled tissue. Incubate the slide in a humidified chamber overnight at 42°C.
13. Blot off the probe from each slide taking care not to disturb the tissue section.
14. Wash tissue sections in 2× SSC for 5 to 10 min at room temperature.
15. Wash tissue sections in 2× SSC for 15 min in a 37°C water bath.
16. Wash tissue sections in 1× SSC for 15 min in a 37°C water bath.
17. Wash tissue sections in 0.1× SSC for 30 min at 37°C with shaking.
18. Incubate slides with ISH buffer 1, two times, 10 min each time.

Visualize probe

19. Place slides flat in the humidified chamber, block with 100 μl of ISH buffer 1 containing 0.1% Triton X-100 and 2% goat serum for 30 min at room temperature.
20. Blot off blocking solution, and cover the tissue section with 100 μl of a 1:500 dilution of anti-DIG-alkaline phosphatase in ISH buffer 1 containing 0.1% Triton X-100 and goat. Incubate 2 hr at room temperature.

21. Wash two times in a Coplin jar filled with ISH buffer 1, 10 min each wash, followed by two 10-min incubations in ISH buffer 2.
22. During the 2nd incubation in ISH buffer 2, prepare the substrate solution by placing half of an NBT/BCIP tablet in 5 ml water in a 15-ml conical tube.
23. Place slides in the humidified chamber, and cover each tissue section with 200 µl of substrate solution. Incubate from 1 to 24 hr in the dark, monitoring the color development of positive controls to determine when the reaction should be stopped.
24. Stop the reaction by placing slides in a Coplin jar containing ISH buffer 3 for 5 min.

Counterstain slide

25. Dip slides briefly in water, counterstain tissue section in 0.02% fast green FCF for 1 min, and then wash in water for 5 min.
26. Blot the excess water from each slide, and add enough Crystal/Mount to cover the section. Dry at 65°C and then add a coverslip.

SUPPORT PROTOCOL 5

PREPARATION OF AVERTIN SOLUTION

Avertin (tribromoethanol 20 mg/ml) is a short-acting anesthetic that is not available commercially and must be prepared. Sterile preparation procedures are essential. If Avertin is improperly prepared or stored in the light or at room temperature, it will break down within 24 hr into dibromoacetic acid and hydrobromic acid, which are lethal. Freshly mixed solutions are strongly recommended for safe use. The solution can be kept as long as 4 months if stored in the dark at 4°C. Ensure that the solution has a pH of >5.

Materials

Tert-amyl-alcohol (Fisher)
2,2,2-Tribromoethanol (Sigma)

2-liter glass Erlenmeyer flask
Magnetic stirrer
Hot plate with stirrer
Aluminum foil
Bottle-top filter (0.22-µm; Millipore)
50-ml dark glass bottles

1. Working in a fume hood, add 10 ml tert-amyl-alcohol into a 2-liter glass Erlenmeyer flask.
2. Add 10 g of 2,2,2-tribromoethanol to the tert-amyl-alcohol. Stir 2 hr at room temperature until the solid goes into solution.
3. Transfer to a hot plate on low heat.
4. Very slowly add 250 ml distilled water to flask while stirring. Make sure that solutes stay in solution before adding more water.

   This process takes several hours.

5. Bring solution up to 500 ml with distilled water.
6. Turn off the hot plate when all of the solid has gone into solution. Cover flask with aluminum foil to protect from the light, and stir overnight.
7. Check that the 2,2,2-tribromoethanol has not precipitated overnight. If necessary, return the flask to the hot plate and stir again on low heat until the solid has gone back into solution.

8. Check pH.
9. Filter with 0.22-μm bottle-top filter.
10. Dispense into 50-ml glass bottles (darkened glass or foil-covered).
11. Store up to 4 months at 4°C in the dark.
12. Test solution on mice.

The effective dose in mice is on the order of 25 mg/kg (~200 to 250 μl/animal) but this will vary by strain with 129 mice being very susceptible and requiring a lower dose, and C57BL6 mice requiring a higher dose. The optimal dose is best determined empirically with each new batch of Avertin made and each mouse strain used.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Collagenase A, 5 mg/ml

Collagenase A (Roche)
Culture medium (MEM, Cellgro)
Filter sterilize through a 0.22-μm filter
Dispense into 5-ml aliquots
Store up to 1 year at −20°C

D5HS

DMEM buffered with 25 mM HEPES (Invitrogen cat. no. 12320) and supplemented with 5% fetal calf serum. Store up to 3 months at 4°C.

Decarbonated Dulbecco's modified Eagle medium (dcDMEM)

The standard formulation of DMEM is buffered with bicarbonate, and therefore becomes alkaline when exposed to ambient atmosphere. For this reason, the medium used for the extended manipulations called for in these protocols is not supplemented with bicarbonate (Invitrogen). Adjust pH to neutral with NaOH. Store up to 3 months at 4°C.

Fast green FCF, 0.02% (w/v)

0.01 g fast green FCF (Fluka)
50 ml ddH_2O
Vortex
Filter to remove particulate matter with 3-mm Whatman paper
Store up to 1 week at room temperature

Fixative/stain

0.003% (w/v) crystal violet
5% (v/v) formalin
50 mM sodium phosphate, pH 7.4 (*APPENDIX 2A*)
Store up to 3 months at room temperature

Gey's solution (buffered ammonium chloride)

24.9 g NH_4Cl
3 g $KHCO_3$
3 ml 0.5% (w/v) phenol red
ddH_2O to a final volume of 3 liters
Filter sterilize with a 0.22-μm filter
Store up to 1 year at 4°C

Hybridization buffer

40% (w/v) deionized formamide
10% (w/v) dextran sulfate
1× Denhardt's solution
4× SSC
10 mM DTT
1 mg/ml yeast tRNA
1 mg/ml denatured and sheared salmon sperm DNA
ddH_2O

Hybridization solution can be made up without salmon sperm DNA and stored at −20°C.

When making up the hybridization buffer, dissolve the dextran sulfate in the water and formamide before adding the other components.

ISH buffer 1

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
150 mM NaCl
Store up to 1 year at room temperature

ISH buffer 2

100 mM Tris·Cl, pH 9.5 (*APPENDIX 2A*)
100 mM NaCl
50 mM $MgCl_2$
Store up to 1 year at room temperature

ISH buffer 3

10 mM Tris·Cl, pH 8.1 (*APPENDIX 2A*)
1 mM EDTA
Store up to 1 year at room temperature

Methylcellulose in MEM, 1% (w/v)

Place 5 g of methylcellulose (Sigma-Aldrich cat. no. M0512) in an autoclavable bottle. Add 435 ml distilled water, preheated to >70°C. Before it cools, sterilize by autoclaving. Cap tightly and keep the bottle shaking, e.g., by automatic rotator, as it cools for at least 60 min at room temperature. Transfer the bottle to an ice bath, and keep shaking it until the methylcellulose dissolves, forming a clear viscous solution. Add 50 ml of 10× MEM, and mix thoroughly. (It will take some time and effort, since the solution is very viscous.) Add 14 ml of sterile 7.5% sodium bicarbonate. Clear the solution of insoluble material by centrifuging 30 min at 3000 × *g*, room temperature. Store up to 3 months at 4°C.

IMPORTANT NOTE: *Methylcellulose will form intractable "clots" in cold water.*

The room temperature cooling is to lower the temperature to the point where immersion in ice will not crack the glass bottle. The shaking is to prevent the (still insoluble) methylcellulose from settling.

The solution should be orange to cherry-red in color. If the pH becomes too high (fuchsia colored), it can be adjusted by gassing the bottle with (sterile) CO_2.

Overlay medium

Mix equal volumes of growth medium (DMEM, 25 mM HEPES, 5% fetal calf serum plus antibiotics) and 1% methylcellulose in MEM (see recipe). Mix thoroughly by repeated pipetting, to eliminate pockets of low viscosity. Prepare fresh.

continued

Since the source of the virus—a live animal—cannot be "sterile", there is possibility of bacterial and fungal contamination. Antibacterial agents (e.g., penicillin/streptomycin) and antifungal agents (e.g., amphotericin B) may be added to prevent these from overgrowing the cultures.

Paraformaldehyde, 4% (pH 7)

To prepare 200 ml:
8 g paraformaldehyde (Electron Microscopy Sciences)
20 ml 10× PBS (see recipe)
Drops of 2 N NaOH
ddH_2O

Heat deionized, distilled water in glass beaker with stirrer on hot plate to 60°C in fume hood. Add paraformaldehyde powder and keep at 60°C. Add 2 drops of 2 N NaOH, and the solution should clear within a few minutes. Remove from heat and let cool to room temperature. Add 20 ml of 10× PBS and bring volume up to 200 ml with ddH_2O. Filter sterilize through a 0.22-μm filter and store up to 3 months at 4°C.

PBS, pH 7.4

140 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
Adjust pH to 7.4 with HCl
Store up to 1 year at room temperature

PBSTA

PBS (see recipe) with:
0.1% (v/v) Triton X-100
0.1% (w/v) bovine serum albumin
0.1% (w/v) sodium azide
Store up to 1 year at room temperature

Percoll solutions

100% (v/v) isotonic Percoll: 90 ml Percoll and 10 ml 10× HBSS
80% (v/v) isotonic Percoll: 80 ml of 100% isotonic Percoll and 20 ml HBSS
40% (v/v) isotonic Percoll: 40 ml of 100% isotonic Percoll and 60 ml HBSS
Store up to 1 year at 4°C

Prehybridization buffer

50% deionized formamide in 4× SSC (see recipe for SSC)
Store up to 1 year at 4°C

SSC, 20×

3 M NaCl
300 mM sodium citrate
Adjust pH to 7.4 with HCl
Store up to 1 year at room temperature

Staining wash buffer (SWB)

60 ml 10× PBS (see recipe)
6 ml 10% (w/v) sodium azide
12 ml newborn calf serum
522 ml ddH_2O
Store up to 1 year at 4°C

Tris·Cl, 0.5 M (pH 10)

60.57 g Tris base
900 ml ddH_2O
Adjust pH with 1 M NaOH
Make up to final volume of 1 liter with ddH_2O
Store up to 1 year at room temperature

TBS, pH 7.6

0.05 M Tris·Cl (see recipe)
0.15 M NaCl
Adjust pH to 7.6
Store up to 1 year at room temperature

COMMENTARY

Background Information

The study of virus infections in vivo requires the analysis of many parameters by a large number of different methods. Because a single unit cannot encompass all of these methods in detail, this unit focuses on providing the reader with protocols for harvesting the relevant samples. The recommended methods for detection of infectious virus, flow cytometry, and histology are given as examples of how the samples might be assayed. The assay techniques outlined here are relatively general, as the authors have chosen the most widely applicable techniques and reagents. It is up to the individual investigator to tailor these assays to meet their specific needs.

Critical Parameters

Measuring cytokines and chemokines

Although not explicitly described in this unit, cytokine assays can be carried out using either BAL specimens (see Basic Protocol 4) or lung homogenates. The same homogenate can be used to assay both virus titers and cytokines, although the authors have found that BAL samples generally have a lower background for cytokine measurement than do whole lung homogenates. ELISA assays are commercially available for many mouse cytokines and chemokines. A biological assay for IFN-α or -β activity is described in detail by Vogel et al. (1991), and this method can be used to assay lung BALs and homogenates, as well as serum samples.

Flow-cytometry

Successful flow cytometry analysis of a given cell population requires the knowledge and experience to calibrate the instrument, standardize procedures and settings, and to analyze the data with a software package. However, the most important requirements are a good single-cell suspension and a proven staining panel/protocol. The correct choice of reagents requires certain knowledge of the characteristics of each fluorochrome and of the antigens of interest. However, the basic approach described here can be used for multiple extracellular antigens, cell subpopulations, and tissues with minor modifications.

Histology

High quality H&E sections are best obtained from a pathology core or hospital laboratory, as production of formalin-fixed, paraffin-embedded tissue blocks and slides from these blocks require a major investment in equipment and training, which is not appropriate for most research laboratories. Nonetheless, if fixed tissue samples are submitted to the histotechnologist in tissue cassettes, taking care to explain the orientation needed for optimal slide interpretation, high quality H&E stained sections can easily be obtained. Additional sections for immunohistochemistry (IHC) or in situ hybridization (ISH) studies can also be prepared from these tissue blocks. IHC using virus-specific antibodies allows the investigator to visualize the extent of virus spread, and the cell type(s) targeted by a particular virus. This method can also be used to visualize the appearance of immune cell types in infected tissues over time, although this type of experiment may require the use of unfixed, frozen tissue sections. ISH is an alternative method if specific antibodies are not available, or the investigator wishes to determine the source of a secreted protein.

Immunohistochemistry

For human tissues, there are a large number of commercially available monoclonal antibodies that can serve this purpose, and

work well for staining formalin-fixed, paraffin-embedded tissue sections. Unfortunately, this is not the case for mouse tissues, as there is a relatively small demand for these reagents. Many monoclonal antibodies that work well for staining cell suspensions prior to FACS analysis will not work well for staining tissue sections. The manufacturer selling the antibody may specify this, but usually this must be determined empirically. In some cases, this can be overcome by using frozen sections rather than paraffin sections of fixed tissues; however, frozen tissues are difficult to cut and the resulting histology is often suboptimal. In addition to the problem of low foreground signal, most monoclonal antibodies are made in mice, and background staining results when the anti-mouse secondary antibody recognizes endogenous antibody bound to mouse tissues or B cells. "Mouse to mouse" blocking reagents are available from a number of companies, but do not always solve the background problems. The authors' best results in mouse tissue have been obtained using polyclonal antisera raised in a non-mouse species such as hamster, goat, or rabbit. Fortunately, many such antisera, specific for a large number of viruses, are available from Millipore, and can be used to visualize infected cells in tissues using Basic Protocol 6. The right-hand panel of Figure 26.3.3 demonstrates the utility of this technique with evidence of many infected airway lining cells (seen as red) following infection 24 hr earlier with a high dose (10^6 pfu) of influenza A virus. This is a demonstration of a robust infection prior to the onset of visible pathology at early times post-inoculation.

Troubleshooting

Included here are a variety of different protocols to study respiratory virus infections. Because of the many manipulations that are required in these protocols and the diverse technologies used, there are many different things that can go wrong. Thus, the investigator should refer to the specific protocols or to a manual specific for each subject for more detailed information.

Anticipated Results

In these protocols, as with any in vivo experimental model, there will be variability between experiments and between individual animals at a given time point. As this is unavoidable, it is important to use sufficient numbers of animals such that a meaningful statistical analysis can be done. Typically, between five and ten animals are used for each data point, and this is important for both objective measures such as cytokine production, as well as the more subjective evaluation of histopathology. When comparing more than two cohorts of animals, a simple student *t*-test is often not sufficient, and an analysis of variance must be performed to determine significance. For histologic analysis, differences between groups of animals will often be a matter of degree rather than a +/− situation, so it is important to examine the slides blindly. This is done so by labeling each slide with only a number, and asking an unbiased examiner to determine whether the samples appear to segregate into groups. When no clear differences are present between two groups, it is concluded that those groups are not significantly different. Although this system has worked well for the authors, it is important to use both qualitative and quantitative measures whenever possible, e.g., comparing BAL cytology and/or FACs analysis with the pattern of inflammation seen on tissue sections at each time point.

Time Considerations

In planning an infection experiment, it is important to determine in advance which samples can be frozen (e.g., lungs for virus titers or BALs for ELISA), and which must be analyzed on the day that they are harvested. Tissues for histology must be fixed immediately, but cannot be processed until they have been fixed in formalin for several hours, but no more than 2 days. Lymphocyte analysis must be done immediately. Isolating immune cells from tissue samples and setting up FACS-staining will take from 4 to 12 hr depending on the number of tissue samples and the complexity of the staining. The samples can be run at the end of the day or, if fixed, during the following day. An ISH analysis will take at least 1 week, including the time needed to harvest, fix, process, and section tissues, with 2 days needed for staining.

Literature Cited

Altman, J.D., Moss, P.H., Goulder, P.R., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94-96.

Belz, G.T., Smith, C.M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F.R., and Heath, W.R. 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I–restricted antigen presentation after lung infection with virus. *Proc. Natl. Acad. Sci. U.S.A.* 101:8670-8675.

Cerwenka, A., Morgan, T.M., and Dutton, R.W. 1999. Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: Homing properties rather than initial frequencies are crucial. *J. Immunol.* 163:5535-5543.

Doherty, P.C. and Christensen, J.P. 2000. Accessing complexity: The dynamics of virus-specific T cell responses. *Annu. Rev. Immunol.* 18:561-592.

Flano, E., Husain, S.M., Sample, J.T., Woodland, D.L., and Blackman, M.A. 2000. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. *J. Immunol.* 165:1074-1081.

Gitiban, N., Jurcisek, J.A., Harris, R.H., Mertz, S.E., Durbin, R.K., Bakaletz, L.O., and Durbin, J.E. 2005. Chinchilla and murine models of upper respiratory tract infections with respiratory syncytial virus. *J. Virol.* 79:6035-6042.

Hikono, H., Kohlmeier, J.E., Ely, K.H., Scott, I., Roberts, A.D., Blackman, M.A., and Woodland, D.L. 2006. T-cell memory and recall responses to respiratory virus infections. *Immunol. Rev.* 211:119-132.

Holt, P.G., Strickland, D.H., Wikstrom, M.E., and Jahnsen, F.L. 2008. Regulation of immunological homeostasis in the respiratory tract. *Nat. Rev. Immunol.* 8:142-152.

Jakubzick, C., Tacke, F., Llodra, J., van Rooijen, N., and Randolph, G.J. 2006. Modulation of dendritic cell trafficking to and from the airways. *J. Immunol.* 176:3578-3584.

Julia, V., Hessel, E.M., Malherbe, L., Glaichenhaus, N., O'Garra, A., and Coffman, R.L. 2002. A restricted subset of dendritic cells captures airborne antigens and remains able to activate specific T cells long after antigen exposure. *Immunity* 16:271-283.

Lawrence, C.W. and Braciale, T.J. 2004. Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection. *J. Immunol.* 173:1209-1218.

Legge, K.L. and Braciale, T.J. 2003. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity* 18:265-277.

Maronpot, R., Boorman, G., and Gaul, B., eds. 1999. Pathology of the Mouse. Cache River Press. Vienna, Ill.

Renshaw, S., ed. 2007. Immunohistochemistry. Scion Publishing Limited. Oxfordshire, U.K.

Sternberg, S., ed. 1997. Histology for Pathologists, 2nd ed. Lippincott-Raven Publishers. Philadelphia.

Vermaelen, K. and Pauwels, R. 2005. Pulmonary dendritic cells. *Am. J. Respir. Crit. Care Med.* 172:530-551.

Vermaelen, K.Y., Carro-Muino, I., Lambrecht, B.N., and Pauwels, R.A. 2001. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J. Exp. Med.* 193:51-60.

Visweswaraiah, A., Novotny, L.A., Hjemdahl-Monsen, E.J., Bakaletz, L.O., and Thanavala, Y. 2002. Tracking the tissue distribution of marker dye following intranasal delivery in mice and chinchillas: A multifactorial analysis of parameters affecting nasal retention. *Vaccine* 20:3209-3220.

Vogel, S., Friedman, R., and Hogan, M. 1991. Measurement of antiviral activity induced by IFN-α, -β, and -γ. *Curr. Prot. Immunol.* 00:6.9.1-6.9.8.

von Garnier, C., Filgueira, L., Wikstrom, M., Smith, M., Thomas, J.A., Strickland, D.H., Holt, P.G., and Stumbles, P.A. 2005. Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. *J. Immunol.* 175:1609-1618.

Weslow-Schmidt, J.L., Jewell, N.A., Mertz, S.E., Simas, J.P., Durbin, J.E., and Flano, E. 2007. Type I interferon inhibition and dendritic cell activation during gammaherpesvirus respiratory infection. *J. Virol.* 81:9778-9789.

Wikstrom, M.E. and Stumbles, P.A. 2007. Mouse respiratory tract dendritic cell subsets and the immunological fate of inhaled antigens. *Immunol. Cell Biol.* 85:182-188.

Wong, P. and Pamer, E.G., 2003. CD8 T cell responses to infectious pathogens. *Annu. Rev. Immunol.* 21:29-70.

Compartmented Neuron Cultures for Directional Infection by Alpha Herpesviruses

Dušica Curanović,[1] Toh Hean Ch'ng,[1, 2] Moriah Szpara,[1] and Lynn Enquist[1]

[1]Department of Molecular Biology, Princeton University, Princeton, New Jersey
[2]University of California at Los Angeles, Los Angeles, California

ABSTRACT

Compartmented neuronal cultures allow experimenters to establish separate fluid environments for neuronal axons and the soma from which they emanate. Physical isolation of cell bodies and axons is achieved by culturing neurons in tri-chambered Teflon rings. Dissociated ganglia are plated in one end compartment of the trichamber, and axonal growth is guided underneath watertight silicone grease barriers into a separate compartment. Since the axons and cell bodies are located in different compartments, they can be infected and assayed separately. We describe the assembly and use of compartmented neuronal cultures for in vitro study of directional infection of neurons by alpha herpesviruses. Selective application of viral inoculum to only one compartment ensures that the remainder of the neuron is not contaminated by input inoculum. This allows for quantification of viral spread, and unambiguous interpretation of immunofluorescence and electron microscopy images. *Curr. Protoc. Cell Biol.* 43:26.4.1-26.4.23. © 2009 by John Wiley & Sons, Inc.

Keywords: compartmented neuron culture • Campenot chambers • tri-chamber • anterograde • retrograde • alpha herpesvirus • virus

INTRODUCTION

This unit describes the assembly and use of compartmented neuron cultures for the in vitro study of directional infection by alpha herpesviruses. The trichamber system we describe is based on the compartmented cultures developed by R.B. Campenot (Campenot, 1977, 1992). In the trichamber system, neuronal cell bodies and their axons are maintained in separate compartments, enabling the experimenter to establish unique fluid environments across the length of the neuron. Thus, a viral inoculum can be applied either to the compartment with neuronal cell bodies or to the compartment containing distal axons. If infection is initiated at axons, viral particle motion along microtubules is minus-end-directed and is termed retrograde transport. In contrast, viral particle motion that is plus-end-directed is termed anterograde. The efficiency of directional viral transport and subsequent spread can be determined by a combination of different techniques and assays including, but not limited to, viral titering, immunofluorescence imaging, and electron microscopy.

To study anterograde transport and neuron-to-cell transmission of infection, viral inoculum is applied to neuronal cell bodies, and the infection is transmitted via axons to detector cells plated in the axon compartment. Retrograde transport and axon-mediated infection of neurons are studied by applying the viral inoculum to the axonal compartment and titering the neuronal cell bodies at the appropriate time points after infection. Immunofluorescence and electron microscopy can be performed on infected samples after either type of infection. The trichamber system confers a major advantage for these experiments over non-compartmentalized cultures: the input inoculum is applied and

Current Protocols in Cell Biology 26.4.1-26.4.23, June 2009
Published online June 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2604s43

restricted to only one compartment, and therefore does not confound measurements or imaging performed on the remainder of the sample; in addition, the direction of viral transport during infection is known.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: Chamber assembly, dissection of ganglia, and all work with live virus and neurons should be performed under sterile conditions and aseptic technique should be used accordingly.

BASIC PROTOCOL 1

ASSEMBLING THE TRICHAMBER SYSTEM

Basic features of the trichamber system

The trichamber system consists of three compartments: the soma (S)-compartment, where the neuronal cell bodies are cultured; the methocel (M)-compartment, where the axons first emerge after penetrating underneath the initial barrier; and the neurite (N)-compartment, where the axons emerge from the M-compartment (see Fig. 26.4.1 for a diagram of the trichamber). Spatially, the Teflon trichamber is symmetrical and the S- and N-compartments are identical. However, for nomenclature purposes, we designate the S-compartment as the location where the neuron cell bodies are plated.

To assemble the trichamber system, we first apply a thin coat of silicone grease on one face of the trichamber and allow the chamber to form a watertight seal on a 35-mm plastic tissue culture dish. Silicone grease is viscous enough to prevent diffusion of liquids between compartments, but allows axons to penetrate underneath the chamber barrier. Once the chamber is affixed to the dish, freshly dissected and dissociated neurons are plated in the S-compartment. After 2 weeks, the axons emanating from the cell bodies in the S-compartment will have penetrated underneath the Teflon barriers, across the M-compartment, and into the N-compartment.

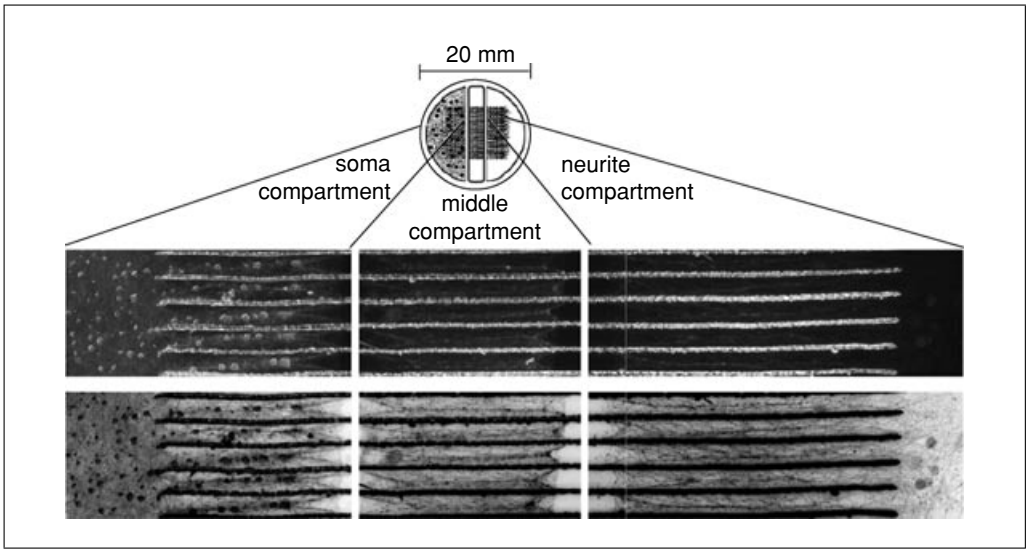


Figure 26.4.1 Diagram of trichamber setup, with images of neurons and axons passing through five inter-groove spaces. The trichamber is 20-mm across, and consists of a soma (S) compartment, middle (M) compartment, and neurite (N) compartment. These images show mouse SCG neurons after 3 weeks in trichamber culture. The Teflon trichamber has been removed to facilitate imaging. The top row of images is phase-contrast microscopy. The bottom row presents the same images, after inversion and contrast-adjustment in Photoshop, to better present the axon structure. The circular gray shadows visible in the N-compartment after image inversion are from silicone grease that floats on the surface of the medium after trichamber removal.

Preparing the trichamber components

Prior to dissecting neurons, the trichambers must be cleaned and sterilized. In addition, the tissue culture dishes must be coated with cell adhesion molecules to promote neuronal attachment and axonal growth. We regularly use poly-DL-ornithine and laminin as extracellular substrates. With respect to incubation times, this protocol must begin at least 1 day before neuronal dissection and plating is planned. This treatment may vary depending on the neuronal culture methods used in different laboratories or for different cell types.

Assemble chambers just before the dissections in a sterile environment; using a laminar flow hood for this step is highly recommended. We commonly culture rat or mouse superior cervical ganglia neurons in the S-compartment. Videos 1 and 2 provide visual demonstration of trichamber setup (see Videos 1 and 2 at *http://www.currentprotocols.com*).

Materials

Poly-DL-ornithine (Sigma) diluted in borate buffer
Tissue culture–grade water
Laminin (BD Biosciences)
HBSS without Ca^{2+} and Mg^{2+} (CMF-HBSS; HyClone)
Silicone high-vacuum grease, Dow Corning (VWR, cat. no. 59344-055)
70% ethanol
Neuronal medium containing 1% (v/v) methocel (see recipe)
Neuronal medium (see recipe)

Teflon trichambers (Tyler Research; see recipe)
Pin rake (homemade or Tyler Research)
35-mm plastic tissue culture dishes
15-cm non-tissue culture-treated dishes (optional)
Disposable 3-ml syringe
Machine-sawed 18-G hypodermic needle or truncated 200-µl pipet tip
Autoclave
Hemostat (e.g., Roboz RS-7293)

Additional reagents and equipment for cleaning the Teflon chambers (Support Protocol 1)

Prepare the chamber

1. Clean the Teflon chambers thoroughly before assembly (see Support Protocol 1).
2. Use a pin rake to etch 16 parallel, evenly spaced grooves in the middle of a 35-mm tissue culture dish. See Figure 26.4.2A for view of a pin rake.

   This homemade pin rake is constructed from 16 insect pins (size 00) arranged in a row such that sharp ends of the pins are aligned. Truncate the blunt ends of the pins with a wire cutter and fix the pins to a plastic holder with epoxy resin. A microcentrifuge tube opener can be used as the plastic holder. Always sterilize the pin rake with 70% ethanol (never autoclave). See Campenot (1992) for additional instructions on making a pin rake. Alternatively, a stainless-steel pin rake can be purchased from Tyler Research Corporation.

3. Make a notation at the side of the dish with a marker to determine the relative orientation of the dish with regards to the trichamber.

   When preparing multiple 35-mm dishes at once, it is useful to hold eight at a time by placing them within a 15-cm petri dish

4. Add 1 ml of 500 µg/ml of poly-DL-ornithine diluted in borate buffer to the dish. Incubate the dish for at least 6 hr.

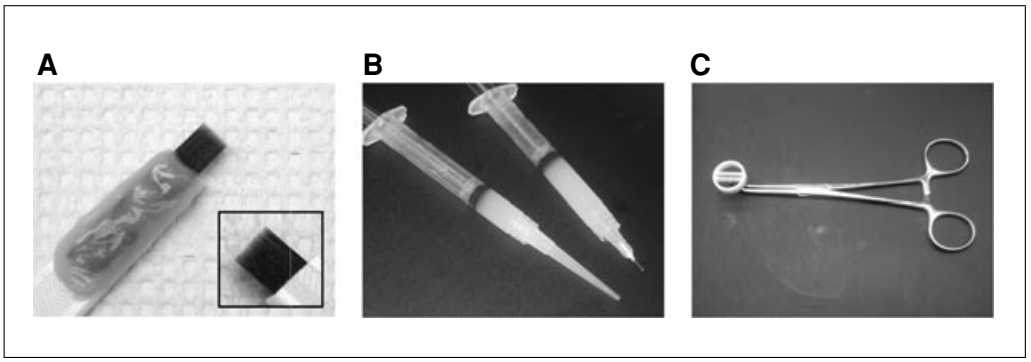


Figure 26.4.2 Tools for trichamber assembly. (**A**) Close-up of pin rake for making grooves in the trichamber dish. The pin rake is assembled from insect pins, epoxy, and a plastic handle. Inset shows close-up view of pins from the reverse side. (**B**) Two types of syringes for dispensing grease. The inner diameter of the needle or plastic tip determines the width of the grease strip. (**C**) Curved hemostat clamped onto trichamber, prior to addition of silicone grease.

5. Remove the dish from the incubator, aspirate poly-DL-ornithine and wash three times, each time with 1 ml tissue culture–grade water.
6. Add 1 ml of 10 μg/ml laminin in CMF-HBSS to the dish and incubate overnight.
7. Load silicone grease in a 3-ml disposable syringe. Fill the barrel of the syringe $^2/_3$ of the way through with silicone grease. Attach a truncated 18-G hypodermic needle, or a truncated 200-μl pipet tip, onto the syringe. Autoclave the silicone grease–loaded syringe, the trichambers, and tools for dissection. See Figure 26.4.2B for a view of grease-loaded syringes.

   The purpose of truncating the needle or the pipet tip is to dispense the silicone grease easily and evenly. If truncating a pipet tip, aim for a diameter equivalent to that of an 18-G needle.

Assemble the trichamber system

8. Aspirate laminin from dishes, wash twice with 1 ml CMF-HBSS and air-dry briefly.

   To facilitate drying, keep the dishes tilted. Do not over-dry laminin-coated plates because the protein may degrade.

9. As the dishes are drying, remove the Teflon chambers and silicone grease–loaded syringe from the autoclave. Cool briefly and sterilize syringe tip with 70% ethanol. Eject a small amount of silicone grease from the syringe to remove any air pockets formed at the tip of the needle during autoclaving.
10. Use a sterile hemostat to clamp one of the two central barriers of a Teflon trichamber. Flip the hemostat over and place it flat, so that the chamber is facing upward. See Figure 26.4.2C for a view of the hemostat and trichamber clamped together and see Video 1 at *http://www.currentprotocols.com*.
11. Apply a thin strip of silicone grease around the entire chamber, starting with the two central barriers followed by the outer circular ring. See Video 1 at *http://www.currentprotocol.com*.

    It is crucial to maintain the continuity of the strip of silicone grease, particularly in the central barriers, since a break in the strip will cause leakage between compartments.

12. Set the clamped hemostat aside. Add a 50-μl drop of neuronal medium with 1% methocel in the middle of the laminin-coated dish, on top of the etched grooves (see Video 1 at *http://www.currentprotocols.com*).

    The drop of methocel provides moisture for the seal between the chamber and the dish. Without methocel, the barrier is devoid of liquid, which deters axon penetration.

13. Flip the dish over and gently place it on the Teflon chamber, centering the grooves directly over and perpendicular to the chamber barriers. Lift the hemostat and view the trichamber assembly through the bottom-side of the dish. Tilt the hemostat slightly to allow light to hit the dish at different angles.

    This will reveal whether or not a seal has started to form between the chamber and the dish. If the seal has formed at very few locations, touch the edges of the dish very lightly until seal formation is observed.

    The seal does not need to be continuous at this point. It is only necessary for sufficient adhesion to ensure that the dish will not fall free of the hemostat when it is inverted in the next step.

14. Flip the hemostat over and gently release the clamp close to the surface of the bench, thus freeing the dish.

    Avoid letting the assembled trichamber and dish come in contact with the bench prior to releasing the clamp, since this may inadvertently disrupt the silicone grease strip and cause a breach in the barrier.

15. Wait for the seal to become continuous; while waiting, proceed with the assembly of the remaining chambers. Check for seal formation by lifting each 35-mm dish, inverting it, and allowing light to hit the bottom surface at different angles. If after 10 min the seal has not yet formed, apply gentle pressure to the chamber to complete the seal.

    If too much pressure is applied, axon penetration underneath the barriers will be hindered.

16. Add 200 μl of neuron medium to the M-compartment and wait 1 min; note whether the medium leaks from this compartment. If no leaks are detected, apply 300 μl of medium to the S-compartment and 400 μl to the N-compartment.

    By adding only 300 μl to the S-compartment, up to 100 μl volume of dissociated neuron suspension can be added (see below).

    Approximately 300 to 400 μl volume is sufficient for the S- and N-compartments, and 200 μl volume for the M-compartment.

PLATING AND MAINTAINING DISSOCIATED NEURONS IN THE TRICHAMBER SYSTEM

BASIC PROTOCOL 2

We routinely culture and plate peripheral neurons from the sympathetic ganglia (SCG) of embryonic rats and mice in the trichamber system. In this protocol, we provide a streamlined method for culturing mouse embryonic SCG neurons in the trichamber system. These dissociation and medium conditions work equally well for rat SCG neurons. Protocols for dissection of embryonic rat SCG neurons can be found in Ch'ng et al. (2005).

While different types of neurons can be cultured in the trichamber system, the success of this system depends on the robust outgrowth of the axonal or neuritic projections from the cell bodies. In general, embryonic neurons work better in the trichamber system than older neurons, and peripheral nervous system neurons display more robust outgrowth than central nervous system neurons. The trichamber system can be optimized for different neuronal cell types by modifying the cell adhesion substrates, medium, or other cell culture parameters.

Materials

Pregnant mouse, E14 or E15
HBSS (HyClone)
HBSS without Ca^{2+} and Mg^{2+} (CMF-HBSS; HyClone)
2.5 mg/ml trypsin in HBSS (see recipe)

1 mg/ml DNase in neurobasal medium (see recipe)
2 mg/ml soybean trypsin inhibitor (SBTI) in neurobasal medium (see recipe)
Neuronal medium (see recipe)
Cytosine β-D-arabinofuranoside hydrochloride (AraC; see recipe)

15-ml conical tubes
Water bath at 37°C
Flame-polished glass Pasteur pipet
Hemacytometer
Teflon trichambers (Tyler Research; see recipe)

Additional reagents and equipment for counting cells using a hemacytometer (*UNIT 1.1*)

Prepare neurons

1. Dissect ganglia in standard HBSS from embryonic day 14 (E14) or E15 mouse embryos, using the approaches previously described for rat superior cervical ganglion neurons (Johnson, 2001; Ch'ng et al., 2005).

2. Transfer dissected ganglia to a 15-ml conical tube and dilute with 5 ml CMF-HBSS. Allow the ganglia to settle, and then remove all but 800 µl of the buffer.

   CMF-HBSS is preferable at this step for optimal trypsin activity.

3. To digest the ganglia, add 100 µl of 2.5 mg/ml trypsin in HBSS and 100 µl of 1 mg/ml DNase in neurobasal medium, and incubate the tube for 15 min in a 37°C water bath. Swirl the tube once during this time.

4. During this time, flame-polish a glass Pasteur pipet by holding the open end in a flame for ~5 to 10 sec.

   The goal is to smooth the jagged edges of the glass pipet opening to minimize cell damage.

   We also coat the inner glass surface of the Pasteur pipet with a sterile 2% BSA in PBS solution. This prevents neurons from adhering to the glass surface.

5. After 15 min, add an additional 100 µl DNase in neurobasal medium, and 650 µl SBTI in neurobasal medium. Incubate the tube for an additional 5 min in a 37°C water bath.

   The enzyme solutions are optimized to give the desired final concentration in this volume. The final concentrations are: trypsin, 0.25 mg/ml; DNase, 0.2 mg/ml; and SBTI, 0.8 mg/ml.

   DNase aids in trituration by digesting genomic DNA released from lysed cells.

6. Dilute the ganglia-enzyme mix with additional CMF-HBSS to 10 ml. Centrifuge 1 min at 3000 × *g* in a tabletop centrifuge, room temperature. Remove all but 200 µl volume, then add 1.5 ml of neuronal medium.

7. Use trituration to mechanically dissociate the ganglia. Pipet the resuspended ganglia up and down with the flame-polished glass pipet until the neuronal suspension is free of clumps.

Plate neurons

8. Count a small aliquot of the resulting neuronal suspension in a hemacytometer (*UNIT 1.1*). Plate 20,000 to 25,000 mouse superior cervical ganglion cells in each trichamber S-compartment. Place the cultures in a 37°C, 5% CO_2 humidified incubator.

   We plate approximately the same number of neurons in the S-compartment whether using mouse SCG neurons or rat SCG neurons.

20,000 to 25,000 cells is approximately a whole ganglion obtained from an E14-E15 mouse embryo, or half of a superior cervical ganglion obtained from an E15.5 rat embryo.

Counting SCG neurons before plating is complicated by the presence of non-neuronal cells (satellite cells and/or fibroblasts from neighboring tissue). These cells will die during subsequent treatment with AraC. In addition, SCG neurons will divide for a short time in culture (DiCicco-Bloom and Black, 1988; DiCicco-Bloom et al., 1990). We have empirically observed that the neuron number at 2 weeks post-plating is ~1/4 the number of plated cells. Thus, plating 20,000 cells will yield ~5,000 mature neurons by the time of experimental infection.

Note that the S- and N-compartments each hold a maximum of 400 μl of medium, so the amount of dissociated neuron suspension added must not exceed this capacity.

9. Allow the neuronal cultures to grow for 2 days before adding an antimitotic drug to the S-compartment.
10. After 2 days, add AraC to 1 μM in the existing S-compartment medium to eliminate any mitotic, non-neuronal cells. Incubate overnight.
11. Remove AraC-containing medium after overnight incubation and replace with fresh, warm neuronal medium.

    Fluid volumes are 300 to 400 μl for the S- and N-compartments and 200 μl for the M-compartment.

    Always change medium with slow and gentle pipetting. Do not remove all of the medium and let the cells dry; instead, always leave a small volume at the bottom of the dish.

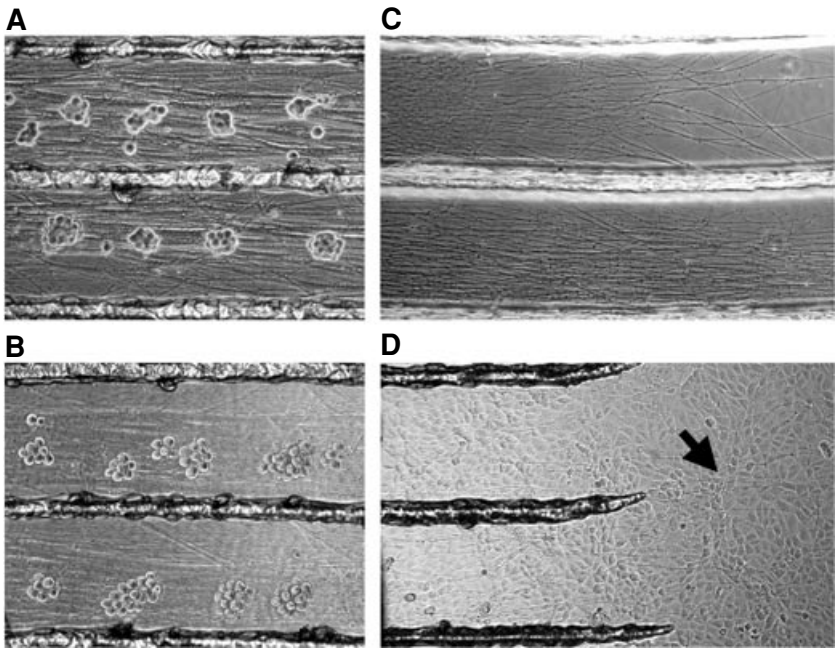


Figure 26.4.3 Highlights of trichamber culture features. (**A**) Enlarged view of mouse SCG neurons in the S compartment of a 3-week-old culture. The clusters of cell bodies are tightly packed. (**B**) At 24 hr after alpha herpesvirus infection (e.g., PRV or HSV-1), the clusters of SCG neurons are less tightly packed, and axons become grainy. (**C**) Enlarged view of dense axon growth in the N-compartment of a 2-week-old culture of rat SCG neurons. (**D**) View of the N-compartment 24 hr after plating of a monolayer of PK15 detector cells. Arrow highlights mouse SCG axons visible among the PK15 cells.

We recommend selecting one edge of the chambers (for instance, the top edge) from which to always add or remove medium. The force of fluid motion is enough to rip or lift the neurons from the dish. The top edge is far from the grooves and it will thus minimize effects on axon penetration through the compartments. In addition, by consistently choosing the same spot, the neuronal disruption is contained to only one part of the chamber.

If non-neuronal cells are still abundant 2 to 3 days after AraC treatment, repeat the treatment on the evening before a scheduled medium change.

12. Change neuronal medium in all three compartments every 2 to 3 days.

Figure 26.4.3 shows mature neuron cell bodies and axon outgrowth in the trichamber setup after 3 weeks of growth. Figure 26.4.3A,B provides a close-up view of neuron cell bodies in the S-compartment, and Figure 26.4.3C of neurites in the N-compartment.

In general, older cultures require more frequent medium changes. Observe the phenol red indicator found in most media formulations (including Neurobasal). The pH of the medium can fluctuate rapidly in small volumes, and the health of the neuronal cultures can drop dramatically if the medium becomes too acidic.

It is important not to bump or put pressure on the trichamber setup during medium changes, since this can tear the neurites or compress the silicone grease seal.

SUPPORT PROTOCOL 1

DISASSEMBLING AND CLEANING THE TRICHAMBERS: ROUTINE CLEANING

The Teflon trichambers are durable and can be cleaned and reused. However, the silicone vacuum grease must be removed entirely before assembling new cultures. With proper care, these chambers can last for many experiments. Traditionally, the chambers are soaked in concentrated sulfuric acid to remove residual silicone grease (Campenot, 1992). We currently use ethanol as a gentler solvent, reserving sulfuric acid for occasional strong cleaning. This protocol describes cleaning the trichambers with ethanol.

Materials

Distilled water
200-proof (absolute) ethanol

Trichambers that need cleaning
Blunt forceps
Paper towels
Kimwipes
Autoclave
10-cm glass petri dish (optional)

1. Upon the conclusion of an experiment, aspirate all residual medium from the compartments. Disassemble the trichamber setup by lifting the chamber from the tissue culture dish using a pair of blunt forceps.

Using sharp forceps will damage the Teflon.

2. Blot each trichamber onto a paper towel: apply gentle pressure and then lift again to leave additional grease behind on the paper towel. Remove any residual silicone grease by carefully wiping the chamber with Kimwipes.

It is crucial to remove most of the silicone grease before further treatment.

3. Rinse the chambers in distilled water and blot dry with paper towels.

4. Soak the chambers in 200 proof ethanol for 24 hr or longer. Swirl or shake the solution occasionally to aid in dissolution of any grease residue on the chambers.

5. Rinse the chambers with distilled water several times, then boil them in distilled water for 30 min.
6. Air dry and autoclave the chambers.

We use a 10-cm glass petri dish to hold the trichambers for autoclaving and storage.

DISASSEMBLING AND CLEANING THE TRICHAMBERS: OCCASIONAL CLEANING

SUPPORT PROTOCOL 2

Periodically (after 3 to 4 uses), the trichambers should be cleaned with stronger reagents to thoroughly clean it. This protocol describes the use of concentrated sulfuric acid to remove residual silicone grease (Campenot, 1992).

***Additional Materials** (also see Support Protocol 1)*

Concentrated sulfuric acid

1. Disassemble trichambers and remove grease as described in Support Protocol 1 (steps 1 to 3).
2. Soak the chambers in concentrated sulfuric acid for 24 hr. Swirl the acid solution occasionally.

Longer incubation will not result in damage.

3. Rinse the chambers with distilled water several times before boiling them in distilled water for 30 min.

Boiling the chambers should remove any residual sulfuric acid trapped within the Teflon.

4. Air dry and autoclave the chambers.

STUDYING THE SPREAD OF VIRAL INFECTION: ANTEROGRADE TRANSPORT AND NEURON-TO-CELL TRANSMISSION OF INFECTION

BASIC PROTOCOL 3

This assay was developed to study the spread of viral infection initiated in the neuron cell body (S-compartment) to axonally contacted epithelial detector cells plated in the N-compartment. Inoculum is applied to the S-compartment, and the titer of infectious virus in the N-compartment is indicative of viral spread from the soma, through axons, to the detector cells, which amplify viral titer. Because diffusion of viral particles is prohibited by the methocel-containing middle compartment and two watertight silicone grease seals, neuron-to-cell transmission of infection is mediated exclusively via neuronal axons that span all three compartments. By co-plating different cell types in the N-compartment, various axon-cell interactions can be established. For instance, if a standard tissue culture cell line that is permissive for viral infection is plated in the N-compartment, these cells serve as sensitive detectors and amplifiers for the axonal transmission of infection. Alternatively, if neurons are co-plated in the N-compartment, then the spread of infection from pre- to post-synaptic neurons can be studied. In this protocol, we describe the plating of a porcine kidney cell line (PK15), a commonly used detector and amplifier of Pseudorabies virus (PRV) infection.

Materials

Two-week-old neuron cultures in the trichamber system (Basic Protocol 2)
A confluent monolayer of tissue culture cells (e.g., PK15 cells)
Trypsin (Invitrogen)
Dulbecco's Modified Eagle Medium (DMEM; Invitrogen)
Fetal bovine serum (HyClone)
Neuronal medium containing 1% (v/v) methocel (see recipe)
Viral stock to be studied (e.g., 10^7 to 10^8 PFU/ml PRV)

Disposable protein gel-loading tip (Rainin)
−80°C freezer

1. Culture dissociated neurons in the S-compartment for ~2 weeks prior to the actual experiment.

   After 2 weeks, axons are easily visible in the N-compartment.

2. Score the axonal densities in the N-compartment from 1 to 4, with chambers scoring a 4 having the densest network in the N-compartment, and those with a score of 1 having little to no axonal penetration.

   An alternative method to score the N-compartment is by counting how many of the inter-groove spaces (there are 15 of these made by a 16-pin rake) are filled by penetrating axons. Another criterion is length of penetration into the N-compartment.

   Only chambers with a score of 3 or 4 are used in experiments; these chambers are evenly distributed among the different experimental variables.

3. One the day prior to infection, plate the detector cells (e.g., tissue culture cells such as PK15s) in the N-compartment.

   These cells settle on top of and in contact with the axons in the N-compartment (see Fig. 26.4.3D).

   For example, dissociate a 90% confluent monolayer of tissue culture cells with 1 ml of trypsin and bring the total volume of the dissociated cells up to 10 ml with neuronal medium. Add ~66 μl of the trypsinized cells into the N-compartment. Add fetal bovine serum to a final concentration of 1% in the N-compartment to facilitate cell attachment and growth.

   The goal is to plate enough tissue culture cells to form a confluent monolayer ~24 hr post-plating. Examples of detector cells for other viruses (e.g., Herpes simplex virus 1 or West Nile virus) can be found in Ch'ng et al. (2007) and Samuel et al. (2007). We plate ~6.6×10^3 PK15 cells per N-compartment.

   Neurons can also be plated in the N-compartment 2 to 3 days before their intended use (Feierbach et al., 2007; Samuel et al., 2007). This short time frame prevents axon growth from the newly plated neurons into the M-compartment.

4. On the day of infection, replace existing medium in the M-compartment with 200 μl of neuronal medium with 1% methocel. Return the dish to the incubator for 30 min to allow the methocel to settle to the bottom of the dish.

   The viscous, methocel-containing neuronal medium serves as an additional barrier to diffusion between the trichamber-compartments.

5. Dilute the viral inoculum in neuronal medium, to a final volume of 50 μl. Remove medium from the S-compartment, and add the viral inoculum.

   Keeping the volume of infection to only 50 μl is important for efficient viral adsorption.

   When preparing to use this system in your laboratory, it is important to characterize the amount of virus needed to synchronously infect all cells in the S-compartment. This can be empirically determined by applying a titration series of inoculum amounts to several trichambers. Assay infection by immunofluorescence at an early point in infection. Choose an inoculum concentration that adequately infects all cells.

   For PRV strains in our laboratory, we generally add 10^5 plaque-forming units (PFU) into the S-compartment, which is approximately a multiplicity of infection (MOI) of 20.

6. Incubate the trichambers with viral inoculum for 1 hr.

7. Gently remove the viral inoculum from the S-compartment with a pipet and replace it with 300 to 400 μl of neuronal medium. Place the chamber back in the incubator.

8. At the appropriate time post-infection, remove the trichamber dish from the incubator, and separately scrape and harvest the contents of the S- and N-compartments for later titering on the appropriate cell types.

   Use a sterile object to scrape the cells in the compartments for titering; we use a disposable protein gel-loading pipet tip for this purpose. The neurons should peel off easily as a sheet. The detector cells often adhere tightly and they must be scraped thoroughly.

   In anterograde-mediated infections (where inoculum is added to the S-compartment), both the S- and N-compartments are typically harvested for titering. Collecting the contents of the S-compartment also informs the experimenter about the titer of input inoculum and replication in neurons.

9. Freeze all harvested samples at −80°C before titering.

STUDYING THE SPREAD OF VIRAL INFECTION: RETROGRADE TRANSPORT AND AXON-MEDIATED INFECTION OF NEURONS

ALTERNATE PROTOCOL 1

Some neuroinvasive viruses, such as rabies virus and alpha herpesviruses, enter neurons at axon termini or growth cones and undergo retrograde axonal transport toward the cell body, where replication occurs. The trichamber system can be used to study this form of entry, and subsequent infection of neuron cell bodies. In addition, because not all neurons in the S-compartment send axons into the M- and N-compartments, this assay can be used to study secondary spread among S-compartment neurons (Curanović et al., in preparation). If the S-compartment is assayed 24 hr post-infection of the N-compartment with PRV, for instance, the titer obtained comes from both the initially infected neurons (primary infection), and also from secondary spread to additional neurons in the S-compartment (secondary infection/spread).

Materials

Two-week-old neuron cultures in the trichamber system
Neuronal medium containing 1% methocel (see recipe)
Viral stock to be studied (e.g., PRV)

Disposable protein gel-loading pipet tip (Rainin)
−80°C freezer

1. Grow dissociated neurons in the S-compartment for at least 2 weeks prior to any infection.

2. Score the axonal densities in the N-compartment (see Basic Protocol 3, step 2).

   Since viral particles are entering neurons via distal axons, it is critical that the axonal density in the N-compartment is robust and comparable among all chambers.

3. On the day of infection, replace medium in the M-compartment with 200 µl of neuronal medium containing 1% methocel. Return the dish to the incubator for 30 min, to allow the methocel to settle to the bottom of the dish.

4. Dilute the viral inoculum in neuronal medium, to a final volume of 50 µl. Remove medium from the N-compartment, and add the viral inoculum.

   Keeping the volume of infection to only 50 µl is important for efficient viral adsorption

   We use the same PFU for retrograde-mediated infection as for anterograde-mediated infections. See above for description of how to determine the appropriate amount of viral inoculum for your system.

   The terminal field densities and the number of infectious virions placed in the N-compartment are critical. Reproducible results are obtained only when inoculum titer is carefully controlled.

5. After 1 hr of incubation, gently remove the inoculum with a pipet and replace with 300 to 400 µl of neuronal medium. Place the chamber back in the incubator.

6. At the appropriate time post-infection, scrape and harvest the infected neuronal cell bodies in the S-compartment.

   We routinely use a sterile, disposable protein gel-loading pipet tip to scrape the neuron cell bodies.

   In retrograde-mediated infections, the goal is to measure the ability of a viral strain to undergo transport to the cell soma, replicate, and/or spread among neurons in the S-compartment. There is no local virus production in the axon; thus, only the S-compartment is harvested.

7. Freeze the harvested samples at −80°C until ready to titer.

BASIC PROTOCOL 4

VISUALIZING INFECTION IN THE TRICHAMBER SYSTEM: IMMUNOFLUORESCENCE

Visualizing neurons grown in the trichamber system via immunofluorescence or transmission electron microscopy requires slight modification of existing protocols. The trichambers are assembled on a plastic surface called Aclar. This flexible, thermoplastic fluoropolymer film is biochemically inert and exhibits no detectable autofluorescence; it can be molded or cut into any shape desired, and treated like a coverslip. However, unlike a glass coverslip, Aclar can be etched to produce grooves that are required for guiding axonal penetration into the N-compartment.

The basic steps of any protocol for fixation and antibody incubation can be adapted to the trichamber system. Here, we describe our preferred particular fixation and antibody incubation times, along with notes about handling that are particular to Aclar and the trichamber setup. Users are encouraged to adapt their own immunofluorescence staining protocols to this system.

Materials

Poly-DL-ornithine (Sigma)
Laminin (BD Biosciences)
Phosphate-buffered saline (PBS; HyClone, cat. no. SH30028.03)
3.2% (w/v) paraformaldehyde in PBS (store up to 2 weeks, in the dark, at 4°C)
PBS/BSA: 3% (w/v) bovine serum albumin (BSA) in PBS (HyClone store solution up to 6 months at 4°C)
PBS/BSA/SAP: PBS (HyClone)/3% (w/v) bovine serum albumin (BSA; Roche)/0.5% (v/v) saponin solution (Sigma; store solution up to 6 months at 4°C)
Primary antibody
Fluorophore-conjugated secondary antibody
Tissue culture–grade water
Mounting medium (e.g., Aqua Poly/Mount from Polysciences)

Aclar (EM Sciences)
UV source for sterilization
Pin rake (Tyler Research or homemade)
35-mm tissue culture dish
Blunt forceps
Sharp forceps
Spatula
Dissection scissors, optional
Microscope slide
Microscope coverslip

Additional reagents and equipment for assembling the trichamber (Basic Protocol 1) and plating dissociated neurons, maintaining the cultures, scoring axon penetration, and subsequent infection assays (Basic Protocol 2 and 3 and Alternate Protocol 1)

1. UV-sterilize a sheet of Aclar in the tissue culture hood. Cut a circular piece of Aclar that is slightly larger than the trichamber but smaller than a 35-mm tissue culture dish.

   The size of a standard United States quarter works well.

2. Etch 16 parallel grooves in the middle of the Aclar strip using a pin rake.

   Since Aclar is transparent, it can be challenging to determine which side of Aclar contains the etched grooves. Make a small mark or notch on the Aclar strip to determine orientation.

3. Place the Aclar in a 35-mm tissue culture dish and assemble the trichamber (Basic Protocol 1).

   Aclar is very hydrophobic. It is necessary to increase the volume of poly-DL-ornithine to 3 ml, and that of laminin to 2 ml per dish to ensure that the Aclar is completely submerged.

   Similarly, increase the volume of washing solutions to 2 to 3 ml. Aclar remains adhered to the tissue culture dish owing to a thin liquid film generated between the surfaces during poly-DL-ornithine and laminin incubations and wash steps.

4. Carry out all steps of plating dissociated neurons, maintaining the cultures, scoring axon penetration, and subsequent infection assays (Basic Protocols 2 and 3 and Alternate Protocol 1) in the same way for trichambers on Aclar as for the usual trichamber setup.

Fix cells

5. At the desired time point after infection, wash all compartments once with 300 to 400 µl PBS per compartment and fix the cells with 300 to 400 µl of 3.2% paraformaldehyde in PBS for 10 min at room temperature.

   As with medium changes, use the same edge of the trichambers for all fluid changes in this protocol. This will minimize neuronal damage during the washes.

6. Remove the fixative from all compartments and wash the samples three times, each time with 1 ml PBS containing 3% BSA (PBS/BSA). After the final wash, flood the entire 35-mm dish with 3 ml of PBS-BSA.

   Be gentle with the multiple rinses described in this protocol. Use a pipet to slowly flow solutions down the sides of the chamber. If the neuronal network starts to peel off due to washes, wash twice instead of three times. Infected neurons are especially likely to peel off during processing.

Remove the chamber

7. Using a pair of serrated, blunt forceps, grasp the central barrier and gently lift the trichamber upwards. To anchor the Aclar on the surface of the dish, apply the same downward force on the Aclar in the M-compartment with a pair of sharp forceps.

   Using the sharp end of the forceps in the M-compartment will minimize damage done to the sample. Avoid applying pressure sideways when lifting the chambers from the Aclar as this will disrupt the neuronal network. If the correct force is applied during this process, the majority of the silicone grease will come off together with the trichamber.

8. Remove excess silicone grease on the surface of Aclar.

   We find that spatulas or pipet tips work well to remove the excess silicone grease, which prevents the Aclar from floating to the surface of the antibody solution. An uneven Aclar surface will also hinder proper mounting on a coverslip.

9. *Optional:* Trim and discard the excess Aclar surrounding the chamber using dissection scissors.

Permeabilize the cells

10. To permeabilize the sample, add 1 ml of PBS containing 3% bovine serum albumin and 0.5% saponin (PBS/BSA/SAP) in the well. Incubate the sample 10 min at room temperature.

    Preparation and treatment of samples for immunofluorescence can vary. We often use 0.1% (v/v) Triton X-100 instead of 0.5% saponin.

    To perform immunofluorescence on nonpermeabilized samples, omit detergents from all solutions.

    In place of bovine serum albumin, 10% goat serum can be used as a blocking reagent.

Stain the cells for immunofluorescence

11. Remove PBS/BSA/SAP and replace with 1 ml of primary antibody solution diluted in PBS/BSA/SAP. Incubate 1 hr at room temperature or overnight at 4°C.

    Some laboratories prefer to use the drop method by inverting the Aclar and placing it on a drop of antibody solution; however, this may not be as gentle on the axons.

    Dilutions vary and they should be optimized for each antibody.

12. After the appropriate time, remove the primary antibody solution and rinse the well three times, each time with 1 ml PBS/BSA/SAP.

13. Add 1 ml of fluorophore-conjugated secondary antibody solution diluted in PBS/BSA/SAP and incubate 1 hr at room temperature.

    If the secondary antibody is fluorescent, be sure to carry out this incubation in the dark.

14. After the appropriate time, remove the secondary antibody and rinse the well three times, each time with 1 ml PBS and once with 1 ml distilled water.

Mount the samples

15. Using a pair of forceps, carefully pick up the Aclar and blot away the excess liquid by touching the edge of the Aclar to a Kimwipe.

16. Mount the Aclar on a microscope slide, cell side up, and place a coverslip over the sample.

    We routinely use Aqua Poly/Mount as the mounting medium. Place a drop of Aqua Poly/Mount on the cover slide and place the Aclar cell-side up. Make sure that that the Aclar is firmly in place. Next, place a drop of Aqua Poly/Mount on the Aclar and place a coverslip over the sample.

    If the Aclar is mounted cell-side down, the thickness of the Aclar may hinder proper viewing of the sample when using certain oil objectives that have a shorter focal plane.

17. Allow the samples to set overnight before doing any microscopy.

ALTERNATE PROTOCOL 2

VISUALIZING INFECTION IN THE TRICHAMBER SYSTEM: ELECTRON MICROSCOPY OF TRICHAMBER SYSTEM SAMPLES

One of the more challenging aspects of electron microscopy is preserving the ultrastructure of the sample, particularly the fragile neuronal network, during separation of the polymerized resin from the substrate. Often, the resin is bound tightly to the surface of the dish and the sample is damaged during separation. Aclar is an excellent substrate to use for electron microscopy because it is more hydrophobic than glass or plastic—hence, the resin tends to bind less tightly to Aclar (Kingsley and Cole, 1988). These protocols for transmission electron microscopy (TEM) were initiated and developed by

Margaret Bisher, of the Princeton Confocal and Electron Microscopy Core Facility, and Lisa Pomeranz (Pomeranz et al., unpub. observ.).

Materials

Two-week-old cultures in the trichamber system, set up on Aclar (Basic Protocol 4)
Phosphate-buffered saline (PBS; HyClone)
2% (v/v) glutaraldehyde in sodium cacodylate buffer (see recipe)
0.2 M sodium cacodylate buffer, pH 7.2 (see recipe)
1% (w/v) osmium tetraoxide in sodium veronal buffer (see recipe)
Sodium veronal buffer (see recipe)
0.25% (w/v) toluidine blue in sodium cacodylate buffer (see recipe)
0.05 M sodium maleate buffer, pH 5.1 (see recipe)
2% (w/v) uranyl acetate in sodium maleate buffer (see recipe)
Ethanol solutions of 30%, 50%, 70%, 90%, 95%, and 100%
Epon resin (Embed812; see recipe)

BEEM capsules
Fine-tipped forceps
60°C oven
Ultramicrotome (e.g., Leica UC6)
200-mesh hexagonal copper grid (EM Sciences)
Zeiss 912AB TEM with an Omega Energy Filter

Fix samples

1. At the desired time post-infection, wash all compartments twice, each time with 300 to 400 µl PBS per compartment and fix with 300 to 400 µl 2% glutaraldehyde solution 4 hr at room temperature.

   As with medium changes described in Basic Protocol 2, use the same edge of the trichambers for all fluid changes in this protocol. This will minimize neuronal damage during the washes.

2. After 4 hr, remove the trichambers and clean the Aclar as described in Basic Protocol 4 (steps 7 and 8). Do not trim the Aclar.

3. Remove the fixative and wash twice, each time with 3 ml 0.2 M sodium cacodylate buffer. Post-fix cells with 4 ml 1% osmium tetraoxide solution for 1 hr on ice.

Stain sample

4. Rinse the sample four times, each time with 4 ml sodium veronal buffer. Incubate sample with 4 ml of 0.25% toluidine blue solution 1 hr at room temperature.

   Toluidine blue is used to visualize the sample and identify areas of interest.

5. Remove the toluidine blue and rinse the sample four times, each time with 4 ml sodium veronal buffer.

6. Rinse the sample four times, each time with 4 ml of 0.05 M sodium maleate buffer.

7. Incubate the sample overnight in the dark with 4 ml of 2% uranyl acetate solution.

8. Rinse the sample four times, each time with 4 ml sodium maleate buffer.

Dehydrate sample

9. Dehydrate the sample by incubating in 4 ml of 30%, 50%, 70%, 90%, 95%, and two treatments of 100% ethanol, for 10 min each.

Embed sample

10. Replace the 100% ethanol with a 2:1 dilution of ethanol:resin for 2 hr, then 1:1 ethanol: resin overnight at room temperature.

11. The next day, replace with 100% resin and incubate for most of the day.

12. Meanwhile, fill BEEM capsules with 100% resin and allow the resin to slightly overfill the capsule. Make sure that there are no bubbles in the resin.

    It is a good idea to fill the capsules early in the day and allow them to degas for a few hours

13. Using a pair of fine-tipped forceps, gently pull the Aclar sample, sitting in resin, out of the well. Prop it on its edge at a slight angle, and allow most of the resin to slide off the Aclar.

14. Holding on the sample with the forceps, flip it onto the slightly overfilled top of the BEEM capsule (cell-side down, into the resin) to form a seal.

    Sections can be obtained separately from all three compartments of the chamber, by using a pair of fine-tipped scissors to cut the sample into pieces.

15. Place the BEEM capsule, with the Aclar top, into the 60°C oven and allow polymerizing for about 24 hr.

16. The next day, let the resin cool to room temperature, and then use a pair of forceps to pull the Aclar off the top of the BEEM capsule.

    The cells should be in the resin, away from the Aclar.

Section and view the sample

17. Obtain 70-µm sections using a Leica UC6 ultramicrotome and mount on a 200-mesh hexagonal copper grid.

18. Image using a Zeiss 912AB TEM with an Omega Energy Filter.

    To enhance contrast of cellular membranes, sectioned samples can be post-stained with lead citrate for 2 min, washed twice with water, and incubated another 2 min in 2% uranyl acetate.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cytosine β-D-arabinofuranoside hydrochloride (AraC)

Prepare AraC (from powder; Sigma) as a 5 mM stock in DMEM. Dilute this to a 500 µM working stock in DMEM. Protect AraC stocks from light. Store up to 1 year, in the dark, at 4°C.

DNase, 1 mg/ml in Neurobasal medium

Dissolve DNase (Sigma) in Neurobasal medium (Invitrogen) at 10 mg/ml concentration. Filter-sterilize using a 0.2-µm filter. Excess stock can be stored (−80°C) at this high concentration. Dilute stock to 1 mg/ml in Neurobasal medium. Divide into 200-µl aliquots and store frozen up to 1 year at –80°C.

Epon Resin (Embed812)

Part A:	5 ml EMbed812 (EM Sciences)
	8 ml dodecenyl succinic anhydride (DDSA; EM Sciences
Part B:	8 ml EMbed812
	7 ml nadic methyl anhydride (NMA EM Sciences)
Part C:	0.47 ml tris(dimethylaminomethyl)phenol (DMP30; EM Sciences)

continued

Part A and Part B can be prepared in advance and stored at 4°C. Bring both parts to room temperature before use. Mix Part A with Part B first, then add Part C. Mix well on a gentle rocker for ~1 hr.

Do not store mixed resin. Aliquots of Parts A/B/C can be stored for up to 6 months at 4°C.

Glutaraldehyde, 2% (v/v), in sodium cacodylate buffer

10 ml ampoule of 10% glutaraldehyde (EM Sciences, cat. no. 16120)
Prepare fresh, by diluting 10% glutaraldehyde 1:5 with 0.2 M sodium cacodylate buffer (see recipe)
Prepare fresh

Methylcellulose (methocel), 2% (w/v), in DMEM

4 g methylcellulose
100 ml distilled water
100 ml 2× DMEM (make from powder using tissue culture–grade water; Invitrogen)

Weigh out 4 g methylcellulose, add to glass bottle, then add 100 ml distilled water (powder will remain clumpy).

NOTE: This initial solution is 4% (w/v) methocel.

Autoclave on liquid setting. Remove from autoclave, add a sterile stir bar, and stir overnight at 4°C. Add 100 ml 2× DMEM, for a final solution that is 2% methocel in 1× DMEM. If clumps remain, stir overnight again. Store up to 1 year at 4°C.

Neuronal medium

Neurobasal medium (Invitrogen)
1× glutamine/penicillin/streptomycin solution (Invitrogen)
1× B-27 medium supplement (Invitrogen)
NGF to 100 ng/ml (Invitrogen, cat. no. 13257-019)
Mix all components except NGF, and filter-sterilize using a 0.2-μm filter
Add NGF last
Store up to 10 days at 4°C

Neurobasal medium with B-27 supplement provides essentially the same medium formulation previously used by our laboratory for rat superior cervical ganglia cultures (Brewer et al., 1993; Ch'ng et al., 2005). This straightforward formulation works equally well for rat and mouse SCG cultures. The final concentration of glutamine is 2 mM, with 100 U/ml penicillin and 100 μg/ml streptomycin.

Neuronal medium containing 1% methocel

Prepare appropriate volume (e.g., 10 ml) of Neurobasal medium (Invitrogen) with 2× glutamine/penicillin/streptomycin (Invitrogen) and 2× B-27 medium supplement (Invitrogen). Filter-sterilize using a 0.2-μm filter. Add NGF (Invitrogen, cat. no. 13257-019) to 200 ng/ml.

Combine this 2× Neurobasal mix with an equal volume (e.g., 10 ml) of 2% methocel in DMEM (see recipe). Mix by pipetting or inverting tube several times.

Store up to 10 days at 4°C.

Osmium tetraoxide (OsO_4), 1%, in sodium veronal buffer

4% aqueous solution of OsO_4 (EM Sciences, cat. no. 19170)
Dilute OsO_4 with freshly made sodium veronal buffer (see recipe) to make a 1% solution
Prepare fresh; do not store

Sodium veronal buffer is recommended for superior membrane preservation with OsO_4 (Hayat, 2000).

Sodium cacodylate buffer (pH 7.2), 0.2 M

42.80 g sodium cacodylate
1000 ml distilled water
Mix all ingredients until dissolved
Adjust pH to 7.2 using 0.1 N HCl
Store up to 6 months at 4°C
Check for clarity before using.

CAUTION: *Cacodylate contains arsenic. Avoid skin contact and inhalation. Avoid strong reducing agents such as aluminum, zinc, sodium borohydride, sulfur dioxide.*

Sodium maleate buffer (pH 5.1), 0.05 M

36 ml 0.1 N NaOH
125 ml 0.2 M maleic acid
339 ml distilled water
Mix solution
Adjust pH to 5.15 using 0.1 N NaOH
Store up to 6 months at room temperature

Sodium veronal buffer (veronal acetate buffer)

Stock solution
2.94 g sodium veronal (sodium 5,5-diethylbarbiturate)
1.94 g sodium acetate
3.40 g sodium chloride
100 ml distilled water stock solution can be stored up to 6 months at room temperature
Working solution
5 ml stock solution (see recipe)
13 ml distilled water
0.25 ml 1.0 M $CaCl_2$
~7 ml 0.1 M HCl
Add the HCl dropwise until the required pH (between 4.2 and 5.2) is obtained
Prepare the buffer fresh every time; do not store

Soybean trypsin inhibitor, 2 mg/ml in Neurobasal medium

Dissolve soybean trypsin inhibitor (SBTI; Sigma) in Neurobasal medium (Invitrogen) at a concentration of 10 mg/ml. Pass through a 0.45-μm filter, then a 0.2-μm filter. Excess stock can be stored (−80°C) at this high concentration. Dilute stock to 2 mg/ml in Neurobasal medium. Divide into 650-μl aliquots and store frozen up to 1 year at –80°C.

Teflon trichambers

Tyler Research Corporation- Edmonton, Canada.

The trichambers are called Camp3A, which is essentially the catalog Camp3 chamber, scaled down to 20-mm diameter (www.tylerresearch.com).

Toluidine blue, 0.25 (w/v), in sodium cacodylate buffer

1 g of toluidine blue
400 ml 0.2 M sodium cacodylate buffer (see recipe)
Mix all ingredients well
Pass solution through a 0.22-μm syringe filter
Store up to 6 months at room temperature

Trypsin, 2.5 mg/ml in HBSS

Dissolve trypsin (Sigma) in Hank's balanced salt solution (HBSS; HyClone) at 10 mg/ml concentration. Pass solution through a 0.2-μm filter. Excess stock can be stored (−80°C) at this high concentration. Dilute stock to 2.5 mg/ml in HBSS. Divide into 100-μl aliquots and store frozen up to 1 year at –80°C.

This method of freezing highly concentrated enzyme stocks, while more dilute enzyme stocks are frozen in single-use aliquots, is both economical and prevents enzyme degradation during multiple freeze-thaw cycles.

Uranyl acetate, 2% (w/v), in sodium maleate buffer

0.5 g of uranyl acetate
25 ml 0.05 M sodium maleate buffer (see recipe)
Mix thoroughly to dissolve
Pass through 0.22-μm syringe filter
Store up to 6 months at room temperature, in the dark
Check clarity of solution before use

COMMENTARY

Background Information

Alpha herpesviruses are neuroinvasive pathogens that spread in a bidirectional, transneuronal fashion through the nervous system of the infected host. When viral particles are moving via plus-end directed microtubule transport in the axons of infected neurons, we term this motion anterograde transport; movement in the opposite direction is termed retrograde. When viral transport leads to viral movement from a presynaptic to a post-synaptic neuron, we call this anterograde spread.

Viral spread has been studied extensively in vivo: inoculum is injected into specific structures of the peripheral or central nervous system of an animal, and the extent of spread is determined by detecting viral antigen in anatomical sections. Because alpha herpesviruses spread between synaptically connected neurons, such experiments are valuable for elucidating neural circuit architecture and connectivity. However, for the purpose of studying large numbers of viral mutants, or defining the exact timing of viral transport within and between cells, it is much easier and less tedious to have a facile in vitro system.

Robert Campenot established a system for compartmentalization of neurons for studying neurite development after modulation of somatic or axonal media environments (Campenot, 1977). These chambers have since been modified, and their application extended to a variety of model systems, including other models of alpha herpesvirus infection (Ziegler and Herman, 1980; Lycke et al., 1984; Penfold et al., 1994; Mikloska and Cunningham, 2001; Karten et al., 2002, 2005). The trichamber cultures we describe differ structurally from the original Campenot chambers: in the original setup, the middle compartment is open to the environment outside the chamber; neurons are plated here, and axonal outgrowth is directed into both side-compartments. In the modified version, the middle compartment is not open to the external environment; dissociated ganglia are plated in one side compartment, from which axons first emerge into the middle, and then the opposite side compartment. Plating neurons in this fashion puts a greater distance between soma and axonal termini; furthermore, placing methocel-containing medium into the middle compartment serves to ensure that no appreciable fluid exchange can occur between the soma- and neurite-containing compartments.

For these reasons, the modified Campenot culture system offers an ideal in vitro

setup for examining the efficiency of directional transport and the spread of viral infection. By co-plating epithelial cells with axons, the efficiency of neuron-to-cell transmission of infection can be investigated. Conversely, retrograde-directed infection can be studied by applying the inoculum to axons, and measuring viral content of the neuronal cell bodies. Because only one-half to one ganglion per chamber (~5000 mature cell bodies) is required to achieve dense, experimentally viable neuronal cultures, the system yields more data in less time than in vivo animal infection experiments.

Performing immunofluorescence and electron microscopy on infected neurons can yield information regarding the mechanisms of directional infection. When imaging experiments are performed on infections of standard dissociated neuronal cultures, the site being observed by microscopy may also contain viral particles from the original inoculum. These conditions complicate data interpretation. Compartmentalization of neurons provides a solution, since the input inoculum is confined to a single-chamber compartment. Furthermore, the direction of viral transport is defined, enabling investigators to confidently describe anterograde-bound or retrograde-bound particles.

Critical Parameters

Not all of the neurons plated in the S-compartment extend axons that reach the N-compartment; additionally, the efficiency of axonal penetration underneath the Teflon barriers will vary. Therefore, if treatment is applied to the neurite compartment, only a subpopulation of cells in the soma compartment will respond to it directly. For this reason, it is crucial to plate the same number of cells in each S-compartment, and to score the efficiency of axonal penetration for each trichamber. After designating axonal density scores, the trichambers must be distributed evenly among the variables being tested.

It is also critical to establish ahead of time the amount of viral inoculum required to infect all cells in the S-compartment synchronously. This can be done with a titration series and immunofluorescence assays, and can be applied to all subsequent experiments involving the same virus and cells. If anterograde-mediated infection is incomplete or asynchronous, it will confound interpretation of the data.

When detector cells are plated in the neurite compartment, it is critical to supplement the cells with 1% fetal bovine serum. In the absence of serum, these cultured cells will not adhere to the bottom of the compartment. While the neuronal medium overall is serum-free, it should be noted that the N-compartment axons are necessarily exposed to serum whenever detector cells are plated there.

Troubleshooting

Axons hit the first barrier, turn away, and never penetrate into the M-compartment

Grooves etched into the bottom of the 35-mm tissue culture dish must be deep enough to generate rough, physical barriers that axons cannot cross. If the grooves are too shallow and smooth, they will not provide sufficient guidance for axonal penetration; the solution is to etch deeper grooves.

During assembly of the trichambers, do not apply too much force on the trichambers to create the watertight seal. Also, take care not to hit the chambers during subsequent medium changes. Either of these mistakes may inhibit axon penetration.

Alternatively, the type of neurons being cultured may not exhibit the robust growth necessary for barrier penetration. Neurons that have been successfully cultured in trichambers include embryonic and newborn rat superior cervical ganglia (SCG; Campenot, 1977; Ch'ng et al., 2005), embryonic mouse SCG neurons (Szpara and Enquist, unpub. observ.), and adult rat dorsal root ganglia (DRG; Kimpinski et al., 1997). Neuronal cultures grown in the original Campenot chambers, where axonal penetration under only one barrier is required, include newborn mouse SCG (Manning et al., 1987), rat DRG (Bi et al., 2006), and rat retinal ganglion cells (Hayashi et al., 2004). A modified Campenot chamber has also been used on cultured hippocampal neurons to study local neurite degeneration (Ivins et al., 1998). To our knowledge, neurites extended by neuron-like cell lines (e.g., PC12 cells) do not robustly penetrate the Teflon barriers in the trichamber system.

Neurons die 2 to 3 days after being plated

Optimization of adhesion substrates and/or neuronal medium is required. It is also possible that the incorrect ganglia are being isolated during dissection; for an example, it can be difficult to distinguish between the embryonic rat nodose ganglion and the superior cervical ganglion due to their physical proximity and morphological similarities. Low neuronal viability can result from treating the ganglia

too harshly during dissociation and trituration. Test the plate-coating, dissection, dissociation, and culturing methods by culturing neurons in 35-mm dishes without the trichamber setup. Make sure that neurons grow successfully in noncompartmented, dissociated cultures before moving on to trichambers.

Infection yields low titer

Infections of either the soma or neurons that produce low titers may result if the axonal density of cultures used in the experiment is low. One solution is to maintain the trichamber cultures several days longer to achieve denser axonal penetration into the N-compartment. Check your conditions by performing the experiment with a viral strain (e.g., wild-type PRV) that has been shown to infect cultured neurons efficiently. Another important control is to check the titer of the S-compartment cell bodies after S-compartment infection.

Neurons are fragile when processed for immunofluorescence

Standard immunofluorescence protocol options apply to the trichamber system. Wash steps and antibody incubations can be lengthened to increase sensitivity and/or decrease background. Care must always be taken to wash gently so that the network of neurons does not peel away. This is especially true late in infection by alpha herpesviruses. While removing the excess silicone grease, work carefully to avoid disrupting the neuronal networks, but swiftly to avoid drying out the sample.

No sample seen in EM

The neuronal network is both fragile and highly interconnected. While the edges may tear during wash steps, they can also lift off and eventually pull the entire neuronal network off the Aclar. Be cautious in wash steps and check that the sample is present throughout all incubations and washes, up to embedding in resin.

If imaging axons in the N-compartment, it may be difficult to find areas of axonal growth dense enough to capture via electron microscopy. The area closest to the Teflon barrier generally contains denser axonal networks.

Anticipated Results

The in vitro development of neurons isolated from embryonic rat or mouse superior cervical ganglia is consistent and predictable. Neurite outgrowth is detectable 1 day after the dissociated neurons are plated. Axons are visible in the M-compartment 3 to 4 days post-plating; they reach the N-compartment 10 to 12 days post-plating. High axonal density in the N-compartment can be achieved by 14 to 21 days. Under these growth conditions, the mature neurons will have only axons in the neurite compartment and not dendrites, which can be confirmed by immunofluorescence. For instance, the axonal marker Tau should be visible throughout cell bodies and all neurites, where the somatodendritic marker microtubule-associated protein 2 (Map2) should only be visible in the neuron soma.

When studying neuron-to-cell spread of wild-type PRV with PK15 detector cells in the N-compartment, the yield in the N-compartment will be $\sim 10^7$ PFU/ml at 24 hr post-infection. Infection of axons, which leads to retrograde transport and virus production by neuron cell bodies in the S-compartment, typically yields 10^5 PFU/ml in the S-compartment at 24 hr post-infection. Virus yield depends on the type of virus used in the study [e.g., PRV, herpes simplex virus 1 (HSV-1), etc.], degree of spread, and duration of infection (e.g., higher titers with more time for replication and/or spread).

Time Considerations

Setting up the trichamber system requires extensive planning and preparation. The average time from set up to initiating an experiment is 2 weeks. The intensive set up phase begins with coating the plates early on the day before a dissection is planned. Dissection tools and silicone grease are autoclaved late in the day or early the next morning. Trichambers are greased and assembled on the morning of a dissection. Neurons are dissected, dissociated, and plated later in the same day. To gain some flexibility in the schedule, plates can be coated with poly-ornithine and/or laminin for longer periods of time (overnight or over several nights). However, trichambers should be assembled no earlier than the evening before a dissection.

During the maintenance phase, the trichamber cultures are treated with an anti-mitotic agent (AraC) 2 days after dissection, and the medium changed for the first time on day 3. Thereafter, medium must be changed every 2 to 3 days. It is advisable to change medium in all the compartments even prior to axonal penetration to the M- and N-compartments. Axonal penetration to the middle compartment is visible within days, although the fine neuronal

processes can be difficult to observe by phase-contrast microscopy. Axons typically reach the second barrier and begin to penetrate into the third compartment around 1 week. By 2 weeks, the axonal penetration in the neurite compartment will likely be sufficient for use; cultures may be maintained as long as 4 weeks if more time for axon growth is needed. However, in some cases, neuronal viability may start to decline after more than 3 weeks in culture.

It is important to assemble extra trichambers, since success of axon penetration is variable, and some chambers will not have adequate penetration even at 2 to 3 weeks post-plating. It is common for novice users to have 50% or less of their trichambers succeed in having good axonal penetration.

Neuronal infections may take several hours or days, depending on the virus, time points examined, and other experimental parameters. Absorption of viral inoculum to the cells or axons is typically performed for 1 hr. Although one should be meticulous when scraping the S- or N-compartment for titering, collecting the contents of one compartment typically takes <1 min.

Immunofluorescence experiments require several wash steps and incubations, 10-min fixation and permeabilization steps, and sequential 1-hr incubations with primary and secondary antibodies. With the required wash steps, the entire procedure for a single sample can take 3 hr. Because processing samples for electron microscopy requires more than one overnight incubation, the procedure can require 3 or more days.

Literature Cited

Bi, J., Tsai, N.P, Lin, Y.P., Loh, H.H., and Wei, L.N. 2006. Axonal mRNA transport and localized translational regulation of kappa-opioid receptor in primary neurons of dorsal root ganglia. *Proc. Natl. Acad. Sci. U.S.A.* 103:19919-19924.

Brewer, G.J., Torricelli, J.R., Evege, E.K., and Price, P.J. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* 35:567-576.

Campenot, R.B. 1977. Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* 74:4516-4519.

Campenot, R.B. 1992. Compartmented culture analysis of nerve growth. *In* Cell-Cell Interactions: A Practical Approach (R.B. Stevenson, Gallin, W.J., and Paul, D.L., ed.) pp. 275-298. IRL Press, Oxford.

Ch'ng, T.H., Flood, E.A., and Enquist, L.W. 2005. Culturing primary and transformed neuronal cells for studying pseudorabies virus infection. *Methods Molec. Biol.* 292:299-316.

Ch'ng, T.H., Spear, P.G., Struyf, F., and Enquist, L.W. 2007. Glycoprotein D-independent spread of pseudorabies virus infection in cultured peripheral nervous system neurons in a compartmented system. *J. Virol.* 81:10742-10757.

Curanović, D., Lyman, M., Bisher, M., and Enquist, L.W. Mutations in UL21 affect the efficiency of retrograde transneuronal infection by PRV-Bartha. *Manuscript in preparation.*

DiCicco-Bloom, E. and Black, I.B. 1988. Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 85:4066-4070.

DiCicco-Bloom, E., Townes-Anderson, E., and Black, I.B. 1990. Neuroblast mitosis in dissociated culture: regulation and relationship to differentiation. *J. Cell Biol.* 110:2073-2086.

Feierbach, B., Bisher, M., Goodhouse, J., and Enquist, L.W. 2007. In vitro analysis of transneuronal spread of an alpha herpesvirus infection in peripheral nervous system neurons. *J. Virol.* 81:6846-6857.

Hayashi, H., Campenot, R.B., Vance, D.E., and Vance, J.E. 2004. Glial lipoproteins stimulate axon growth of central nervous system neurons in compartmented cultures. *J. Biol. Chem.* 279:14009-14015.

Hayat, M.A. 2000. Principles and Techniques of Electron Microscopy: Biological Applications. Cambridge University Press.

Ivins, K.J., Bui, E.T., and Cotman, C.W. 1998. Beta-amyloid induces local neurite degeneration in cultured hippocampal neurons: evidence for neuritic apoptosis. *Neurobiol. Dis.* 5:365-378.

Johnson, M.I. 2001. Primary cultures of sympathetic ganglia. Humana Press Inc., Totowa, N.J.

Karten, B., MacInnis, B.L., Eng, H., Azumaya, Y., Martin, G., Lund, K., Watts, R.C., Vance, J.E., Vance, D.E., and Campenot, R.B. 2002. Analytical approaches for investigating apoptosis and other biochemical events in compartmented cultures of sympathetic neurons. *In* Apoptosis Techniques and Protocols Vol. 37. pp. 163-175. Humana Press.

Karten, B., Hayashi, H., Campenot, R.B., Vance, D.E., and Vance, J.E. 2005. Neuronal models for studying lipid metabolism and transport. *Methods.* 36:117-128.

Kimpinski, K., Campenot, R.B., and Mearow, K. 1997. Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) on neurite growth from adult sensory neurons in compartmented cultures. *J. Neurobiol.* 33:395-410.

Kingsley, R.E. and Cole, N.L. 1988. Preparation of cultured mammalian cells for transmission and scanning electron microscopy using Aclar film. *J. Electron Microsc. Tech.* 10:77-85.

Lycke, E., Kristensson, K., Svennerholm, B., Vahlne, A., and Ziegler, R. 1984. Uptake and transport of herpes simplex virus in neurites of

rat dorsal root ganglia cells in culture. *J. Gen. Virol.* 65:55-64.

Manning, P.T., Johnson, E.M., Jr., Wilcox, C.L., Palmatier, M.A., and Russell, J.H. 1987. MHC-specific cytotoxic T lymphocyte killing of dissociated sympathetic neuronal cultures. *Am. J. Pathol.* 128:395-409.

Mikloska, Z. and Cunningham, A.L. 2001. Alpha and gamma interferons inhibit herpes simplex virus type 1 infection and spread in epidermal cells after axonal transmission. *J. Virol.* 75:11821-11826.

Penfold, M.E., Armati, P., and Cunningham, A.L. 1994. Axonal transport of herpes simplex virions to epidermal cells: Evidence for a specialized mode of virus transport and assembly. *Proc. Natl. Acad. Sci. U.S.A.* 91:6529-6533.

Samuel, M.A., Wang, H., Siddharthan, V., Morrey, J.D., and Diamond, M.S. 2007. Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis. *Proc. Natl. Acad. Sci. U.S.A.* 104:17140-17145.

Ziegler, R.J. and Herman, R.E. 1980. Peripheral infection in culture of rat sensory neurons by herpes simplex virus. *Infect. Immun.* 28:620-623.

HIV-1 Interactions with Cells: From Viral Binding to Cell-Cell Transmission

Alicia M. Janas[1] and Li Wu[1]

[1]Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin

ABSTRACT

Characterization of HIV-1 interactions with host cells is critical for cell biology studies of HIV-1. This unit describes a set of methods and protocols to perform quantitative assays of HIV-1 binding, internalization, infection, and cell-cell transmission. The protocols include: (1) generating infectious single-cycle or replication-competent HIV-1 stocks, (2) an HIV-1 binding and internalization assay, (3) HIV-1 infection of target cells and quantification of viral infection, and (4) HIV-1 cell-cell transmission assays. These functional assays provide useful tools to quantitatively study HIV-1 infection and viral transmission. *Curr. Protoc. Cell Biol.* 43:26.5.1-26.5.20. © 2009 by John Wiley & Sons, Inc.

Keywords: human immunodeficiency virus type 1 (HIV-1) • binding • internalization • infection • viral transmission • interactions

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome (AIDS), the leading infectious disease worldwide. Despite great efforts and tremendous progress in HIV-1 and AIDS studies, eradication of HIV-1 infection and treatment of AIDS remain a long-term challenge (Fauci, 2007; Karlsson Hedestam et al., 2008). Understanding the multifaceted interactions between HIV-1 and host cells can potentially facilitate the development of therapeutic interventions against HIV-1 infection (Wu and KewelRamani, 2006; Goff, 2007). Quantitative analysis of HIV-1 interactions with host cells represents a critical aspect in the cell biology studies of HIV-1 infection and dissemination. In this unit, the use of a set of quantitative assays to measure HIV-1 binding (see Basic Protocols 1 and 2), endocytosis (see Basic Protocol 2), infection in target cells (see Basic Protocol 3), and cell-cell viral transmission (see Basic Protocol 4) are described. These quantitative assays provide useful tools to study HIV-1 interactions with host cells.

STRATEGIC PLANNING

Biosafety Precautions

Infection of replication-competent HIV-1 can cause AIDS in humans. In general, performing experiments that involve using infectious HIV-1 should carefully follow the *Virology Manual for HIV Laboratories* (available for download from the Web site listed in Internet Resources). This manual was developed by the Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) and collaborating investigators. The manual addresses the biosafety issues of HIV laboratories in detail, and provides recommendations for specimen processing, storage, shipping, etc. Based on the manual, the following areas of the biosafety guidelines should be followed to effectively eliminate or minimize accidental exposure to HIV-1: (1) the use of universal precautions; (2) establishing appropriate engineering controls; (3) implementing appropriate work practice controls; (4) using necessary personal protective equipment; and (5) implementing appropriate housekeeping procedures.

Current Protocols in Cell Biology 26.5.1-26.5.20, June 2009
Published online June 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2605s43

HIV Research Reagents

The NIH AIDS Research and Reference Reagent Program is a unique worldwide resource of state-of-the-art reagents for HIV and other pathogens. The program provides registered researchers with a variety of valuable reagents for studying HIV and other retroviruses. Many of the expression plasmids, cell lines, and other reagents described in this unit can be obtained from the program. Refer to the Web site listed in Internet Resources for details.

BASIC PROTOCOL 1

GENERATING INFECTIOUS SINGLE-CYCLE OR REPLICATION-COMPETENT HIV-1 STOCKS

This method describes cellular transfection with HIV-1 proviral DNA as a common protocol to generate HIV-1 stocks. Replication-competent HIV-1 can be generated by transfection of human or animal cell lines with a full-length HIV-1 proviral construct (Adachi et al., 1986). Additionally, human $CD4^+$ T cells or activated human peripheral blood mononuclear cells can be infected with replication-competent HIV-1 derived from transfection, and then expanded viral stocks can be obtained from the supernatants several days after the infection (Freed et al., 1995; Dong et al., 2007). Single-cycle reporter HIV is an infectious HIV vector that can establish an integrated provirus but cannot undergo additional cycles of replication, owing to an inactivated viral envelope gene. The viral genome encodes a reporter gene, which is expressed in infected permissive cells, enabling measurement of infectivity.

To generate single-cycle HIV-1, an *env*-deleted/inactivated HIV-1 proviral DNA and a construct expressing HIV-1 envelope glycoproteins (Env) can be cotransfected into cells (Aiken, 1997; Wu et al., 2002b: Dong et al., 2007; Wang et al., 2007a,b; Janas et al., 2008). This method is also termed as HIV-1 pseudotyping. Based on different experimental purposes, various viral Env proteins from different tropic HIV-1 strains, simian immunodeficiency virus, or vesicular stomatitis virus G protein (VSV-G) can be incorporated in HIV-1 particles to generate single-cycle HIV-1 vectors (Aiken, 1997; Wu et al., 2002a; Dong et al., 2007; Janas et al., 2008). VSV-G-pseudotyped HIV-1 efficiently enters a broad range of cell types through a low-pH-dependent endocytic pathway (Aiken, 1997; Dong et al., 2007; Janas et al., 2008).

Materials

2.5 M $CaCl_2$
Tissue-culture-grade double distilled water
2× BES-buffered saline (BBS; see recipe)
Human embryonic kidney cell line HEK293T cells (a gift from Dr. V. KewalRamani, *vineet@ncifcrf.gov*; Wang et al., 2007a,b)
HEK293T cell culture medium (see recipe), 37°C
HIV-1 proviral DNA pHIV-Luc (pLai3ΔenvLuc2) *env*-deleted and *nef*-inactivated viral genome with a luciferase reporter insertion, and contains all other viral genes (Yamashita and Emerman, 2004; gifts from Michael Emerman, Fred Hutchinson Cancer Research Center; *memerman@fhcrc.org*)
HIV-1 full-length proviral construct pNL4-3 and pNLAD8 (Freed et al., 1995; gifts from Eric Freed, National Cancer Institute, Frederick, Maryland)
Expression plasmids: R5-tropic Env of HIV-1_{JRFL} (pJRFL) or HIV-1_{ADA} (pADA), X4-tropic Env of HIV-1_{HXB2} (pHXB2), and VSV-G-expressing pVSV-G (gifts from Vineet KewalRamani, National Cancer Institute, Frederick, Maryland; Dong et al., 2007; Wang et al., 2007a,b)
Green fluorescent protein–expressing HIV-1 vector (pHIV-GFP) generated from a pNL4-3-based HIV-1 provirus with deletions in the *env*, *vif*, *vpr*, *vpu*, and *nef* genes; the *GFP* gene was inserted in place of the *nef* open reading frame (Unutmaz et al., 1999)

Human $CD4^+$ T cell line Hut/CCR5 cells (a gift from Dr. V. KewalRamani, *vineet@ncifcrf.gov*; Wang et al., 2007a,b)

0.2-μm sterile filters
Tissue culture plates (standard 10-cm diameter dishes or 6-well plates)
37°C water bath
5- ml round-bottom, polystyrene tubes (Falcon cat. no. 2058)
37°C, 5% CO_2 cell culture incubator designated for HIV-1 infections
15-ml conical tubes
Refrigerated centrifuge
2-ml cryotubes (sterile)

Additional equipment and reagents for cell culture (*UNIT 1.1*)

CAUTION: Follow biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipets, etc.

Prepare transfection reagents

1. To prepare 0.25 M $CaCl_2$, dilute 2.5 M stock with tissue culture quality water. Sterilize the solution using a 0.2-μm sterile filter, and freeze 10-ml aliquots at −20°C.

2. Prepare 2× BES-buffered saline (BBS), filter sterilize using a 0.2-μm filter, and freeze 10-ml aliquots at −20°C.

   The solution pH should be 6.96 with a ± 0.02 variance. Adjust pH with 10 M NaOH if necessary. The $CaCl_2$ and BBS solutions can be stored upto 6 months at −20°C.

   Alternatively, commercially available transfection kits (e.g., Mammalian Transfection Kit, Stratagene cat. no. 200385) can be used.

Plate cells

3. The day before transfection, seed HEK293T cells in 10 cm-diameter plates at 2.5–3 × 10^6 cells in 10 ml cell culture medium.

   Alternatively, for transfections in 6-well plates, seed 3.0 × 10^5 cells/well in 2 ml of culture medium. Cells should reach ∼80% confluence 24 hr later. The authors recommend not to plate cells that have been overly confluent for >1 day.

Transfect with HIV

4. Replace culture medium with fresh culture medium 1 to 3 hr before transfection.

   This step is optional if medium of cultured cells appear to be fresh. Cold medium should be prewarmed to 37°C. Use extra caution when replacing medium since cells can easily detach.

5. Quickly thaw 2× BBS and 0.25 M $CaCl_2$ aliquots in a 37°C water bath and leave at room temperature after thawing.

6. Prepare appropriate amounts of DNA that will be used in transfection according to Table 26.5.1.

   When using full-length HIV-1 proviral DNA (pNL4-3 or pNLADA, etc.) to generate replication-competent HIV-1, do not add Env-expressing vectors to the transfection mixture.

7. Dispense appropriate proviral vector DNA and desired Env-expressing vector DNA into 5-ml polystyrene tubes.

8a. For 10-cm diameter plates, combine 875 μl of 0.25 M $CaCl_2$ with prepared DNA and mix.

8b. For each sample well of a 6-well plate, combine 175 μl of 0.25 M $CaCl_2$ with prepared DNA and mix.

Table 26.5.1 Amount of HIV Proviral DNA and Env-Expressing Vectors Used for Transfection[a]

Cell culture plates (cell number)	HIV proviral DNA(μg) (pHIV-Luc, pHIV-GFP, or pNL4-3, etc.)	Envelope (Env)-expressing vectors[b] (one of the following expressing vector)	
		HIV Env-expressing vectors (μg) (pJRFL or pHXB2, etc.)	pVSV-G (μg)
10-cm plate (2.5×10^6)	10	10	5
6-well plate (3.0×10^5/well)	2	2	1

[a]All DNA used in transfection should be prepared with Qiagen DNA purification kits or equivalent products to ensure the purity of DNA and minimal endotoxin contamination.

[b]When using full-length HIV-1 proviral DNA (pNL4-3 or pNLADA, etc.) to generate replication-competent HIV-1, there is no need to add Env-expressing vectors.

9. Add 2× BBS (pH 6.95) to the 0.25 M $CaCl_2$ and DNA mixture.

   For 10-cm diameter plates, add 875 μl of 2× BBS. For each well of a 6-well plate, add 175 μl of 2× BBS. Add 2× BBS drop wise to the $CaCl_2$ and DNA mixture, and then gently vortex the transfection mixture for 2 to 5 sec. The solution should turn cloudy from precipitation.

10. After 13 to 15 min, gently and evenly add transfection mixture drop wise to HEK293T cells.

    It is recommended not to exceed the 15-min incubation time period.

11. Place cells in a 37°C, 5% CO_2 incubator designated for HIV-1 infections overnight.

    DNA precipitation will form slowly and evenly overnight.

12. On the next day, gently aspirate supernatants of HEK293T cells, and replace with prewarmed (37°C) cell culture medium.

    The authors recommend replacing medium 16 to 20 hr post-transfection. Replacing the medium will reduce the number of cells that may detach from the plate. Use appropriate biosafety precautions when replacing HIV-containing medium; supernatant is now infectious. All tissue culture work from here on should be performed in a biosafety hood using appropriate biosafety precautions.

Harvest viral stocks

13. Two days post-transfection, collect supernatants in 15-ml conical tubes, and centrifuge 5 min at 270 × *g*, 4°C.

14. Carefully transfer supernatants to a fresh tube, avoid disturbing cell pellet.

    Cellular debris can be further removed via filtering the supernatant through a 0.45-μm filter.

15. Dispense viral supernatant to 2-ml cryotubes (0.5 to 1 ml/vial), and then quickly freeze in a −80°C freezer.

16. Generate replication-competent HIV-1 by infecting $CD4^+$ T cells.

    Alternatively, to expand replication-competent HIV-1 stocks in $CD4^+$ T cells, Hut/CCR5 cells or activated peripheral blood lymphocytes (1×10^6) can be infected with HEK293T-derived HIV-1_{NLAD8} (5 ng p24 equivalent), and supernatants will be harvested 5 days post-infection. Refer to steps 13 to 15 for harvesting viral supernatants.

    HIV-1 Gag p24 protein concentrations of HIV-1_{NLAD8} stocks can be measured using an enzyme-linked immunosorbent assay (ELISA) according to the kit instruction.

HIV-1 BINDING AND INTERNALIZATION ASSAYS

BASIC PROTOCOL 2

HIV-1 can bind to cells through the viral receptor CD4 and multiple cellular attachment factors (Turville et al., 2001, 2002; Geijtenbeek et al., 2002; Gummuluru et al., 2003). A C-type lectin, dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN), functions as an attachment factor of HIV-1 and facilitates HIV-1 binding, infection, and viral transmission (Geijtenbeek et al., 2000; Wu and KewalRamani, 2006). DC-SIGN also mediates HIV-1 internalization in cells (Kwon et al., 2002; Wu and KewalRamani, 2006; Wang et al., 2007a). This protocol describes HIV-1 binding and internalization assays using Raji/DC-SIGN cells as an example. Raji/DC-SIGN cells are derived from a human Raji B cell line and stably express high levels of exogenous human DC-SIGN (Wu et al., 2004 a,b; Wang et al., 2007a). CD4- and DC-SIGN-negative parental Raji cells are used as a background control.

HIV-1 incubated with Raji/DC-SIGN cells at 4°C primarily remains on the cell surfaces. After several washes, a cell surface–bound virus can be measured by quantifying cell-associated HIV-1 Gag p24 (Wang et al., 2007a). In contrast, Raji/DC-SIGN cells incubated with HIV-1 at 37°C can bind virus on cell surfaces and internalize HIV-1 intracellularly. When cells are incubated with HIV-1 at 4°C, and then washed and treated with a proteolysis reagent, such as trypsin, the majority of cell surface–bound HIV-1 can be stripped (Wang et al., 2007a,b). When cells are incubated with HIV-1 at 37°C, washed, and then treated with trypsin, the cell-associated HIV-1 represents internalized virus as well as cell-surface-bound viruses that are protected from the proteolysis (Wang et al., 2007a,b).

To examine the cell-HIV-1 interactions in the absence of potentially confounding effects of productive viral infection, aldrithiol-2 (AT-2)-inactivated HIV-1 can be used in the binding and internalization assays (Rossio et al., 1998; Wang et al., 2007a). Inactivation of HIV-1 with AT-2 modifies the essential zinc fingers in the viral nucleocapsid protein, but does not alter virion Env, thereby maintaining HIV-1 binding and fusion with target cells (Rossio et al., 1998). Thus, AT-2-inactivated HIV-1 is conformationally authentic and interacts with cells similarly to infectious HIV-1 (Rossio et al., 1998; Frank et al., 2002; Wang et al., 2007a,b). Depending on different experimental purposes, p24-quantified infectious HIV-1 and different cell types can also be used to measure viral binding and internalization in similar assays.

Materials

Raji cells, Raji/DC-SIGN cells or another appropriate cell type (NIH AIDS Research and Reference Reagent Program, Raji cells cat. no. 9944, Raji/DC-SIGN cells cat. no. 9945; *http://www.aids.reagent.org/index.cfm*)
Sterile PBS without Ca^{2+} and Mg^{2+} (CMF-PBS; *APPENDIX 2A*), cold
Cell culture media (see recipes)
AT-2–inactivated R5-tropic HIV (Bal/Supt1-CCR5 cl30; from Jeffery Lifson, AIDS Vaccine Program, SAIC-Fredrick)
RPMI-1640 medium with 10% (v/v) fetal bovine serum (FBS)
0.25% (w/v) trypsin without EDTA
1× cell lysis buffer (see recipe)
10% (v/v) Triton X-100
HIV-1 p24 ELISA kit (anti-p24-coated plates from the AIDS Vaccine Program, SAIC, Frederick, Maryland) or p24 ELISA kit (PerkinElmer)

1.5-ml screw-cap tubes and microcentrifuge tubes
Refrigerated microcentrifuge (e.g., rotor model F241.5P in Beckman Coulter microcentrifuge 22R centrifuge)
37°C water bath or incubator

Additional equipment and reagents for cell culture (*UNIT 1.1*)

Prepare cells and virus for incubation

1. Dispense Raji cells and Raji/DC-SIGN cells (3×10^5 per sample) into 1.5-ml microcentrifuge tubes. Centrifuge cells 3 min at ~900 × *g*, room temperature. Remove supernatant carefully.

   Sample numbers are dependent on specific experimental needs.

2. Wash cells with 0.5 ml CMF-PBS. Centrifuge cells 3 min at 900 × *g*, room temperature. Remove supernatant carefully.

3. Resuspend cells in culture medium containing 20 ng of p24-equivalent AT-2-inactivated HIV-1.

   The authors recommend that the final volume be raised to 200 μl using RPMI-1640 medium with 10% FBS. For cells being incubated at 4°C, the medium should be chilled. Also, warm medium can be used for the samples that will be incubated at 37°C.

4. Incubate 2 hr at desired temperature (4°C or 37°C).

   The screw-cap tubes should be slightly loosened during virus incubation. Incubation of samples at 4°C can be performed on ice in a 4°C refrigerator.

Wash, trypsinize, and lyse cells

5. After virus incubation, centrifuge cells 3 min at 900 × *g*, room temperature. Carefully remove supernatant and then wash the cells as in step 2, with 1 ml of CMF-PBS three to five times to remove unbound cell-free HIV-1.

6. Treat desired samples with 0.25% trypsin, and incubate cells 4 min at room temperature.

   The trypsin concentration and incubation time should be predetermined and adjusted when using different cell types.

7. Neutralize trypsin with cell culture medium containing 10% FBS. For samples not being treated with trypsin, incubate in cell medium only.

8. Centrifuge cells 3 min at 900 × *g*, 4°C. Remove supernatant carefully, avoiding disruption of the pellet.

9. Resuspend the pellet in 1 ml cold CMF-PBS, centrifuge cells 3 min at 900 × *g*, 4°C and remove supernatant carefully. Wash three to five times.

Lyse cells

10. Resuspend cell pellet with 200 μl of 1× cell lysis buffer. Add 20 μl of 10% Triton X-100 (1/10 vol) to each sample to release p24 according to the ELISA kit manufacturer's instructions.

11. Incubate samples 1 hr at 37°C to inactivate virus, if infectious HIV-1 stocks are used.

12. Analyze samples by p24 ELISA assay according to kit instructions.

    Alternatively, samples can be stored up to 6 months at −80°C, and analyzed in the future.

BASIC PROTOCOL 3 HIV-1 INFECTION OF TARGET CELLS AND QUANTIFICATION OF VIRAL INFECTION

Stable cell lines that express a reporter gene upon HIV-1 infection can be used to titrate HIV-1 infectivity. This protocol describes a flow cytometry based HIV-1 titration assay using GHOST cells, which are human osteosarcoma cells expressing high levels of CD4 and CCR5. These cells contain a *GFP* gene under the control of the long terminal repeat (LTR) promoter of HIV-2, which is expressed during HIV-1 infection via Tat

transactivation, acting as a sensitive indicator of HIV-1 infection (Cecilia et al., 1998; *UNIT 20.2*). GHOST cells expressing the CXCR4 and CCR5 coreceptor are referred to as GHOST/X4/R5 cells (Cecilia et al., 1998; Wu et al., 2002b). Approximately 40% of GHOST/X4/R5 cells are positive for CD4; 84% of the cells are positive for CXCR4; 66% of the cells are positive for CCR5. The parental GHOST cells are 3.8% positive for CXCR4 and 0.8% positive for CCR5 (Cecilia et al., 1998).

Materials

HIV indicator cell line: GHOST/X4/R5 cells (available from NIH AIDS Research and Reference Reagent Program cat. no. 3942 or from Dr. Vineet KewalRamani, National Cancer Institute, Frederick, Maryland)
Cell culture medium (see recipe)
Infectious HIV-1 stocks
Sterile PBS without Ca^{2+} and Mg^{2+} (CMF-PBS; *APPENDIX 2A*)
Sterile 1 mM EDTA
0.05% (w/v) trypsin with 0.2 g/liter EDTA, optional
4% (w/v) paraformaldehyde
CMF-PBS/2% (v/v) FBS

12-well tissue culture plate
37°C incubator
Centrifuge with plate rotor
Flow cytometer

Additional reagents and equipment for cell culture (*UNIT 1.1*)

CAUTION: Follow the biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipets, etc.

Plate cells

1. The day before infection, plate 2×10^4 cells per well in a 12-well plate.

   Cells can be plated in nonselective culture medium for single-round infection experiments. To maintain high level expression of receptor and coreceptors, GHOST/X4/R5 cells should be cultured in selective medium.

Infect with virus

2. Add appropriate volumes of HIV-1 to GHOST/X4/R5 cells for incubation (day 1).

   Use appropriate biosafety precautions when handling virus stocks, and perform experiment in a designated biosafety cabinet. When titering virus pseudotyped with JRFL or HXB2, use 50, 100, and 200 μl of viral stocks for infections. When titering virus pseudotyped with VSV-G, use 12.5, 25, and 50 μl of viral stocks for infection. It is recommended not to use more than 50 μl of HIV-VSV-G for titration due to the cytotoxic effects of VSV-G. For replication-competent virus, 25 to 100 μl of viral stocks can normally be used in infections. Infections can be performed in a final volume of 300 μl per well of a 12-well plate. Alternatively, 10 μg/ml of polybrene can be added in the infection to enhance the infection efficiency. It is important that a mock control (no virus input) is always included in the experiment.

3. After incubating the virus 2 hr at 37°C, raise the volume to 1 ml with cell culture medium.

   Alternatively, supernatant can be replaced with 1 ml of medium, or infections can be performed in a 0.5-ml final volume overnight (not recommended for VSV-G-pseudotyped HIV-1 due to cytotoxicity).

4. Replace medium 24 hr post-infection (day 2).

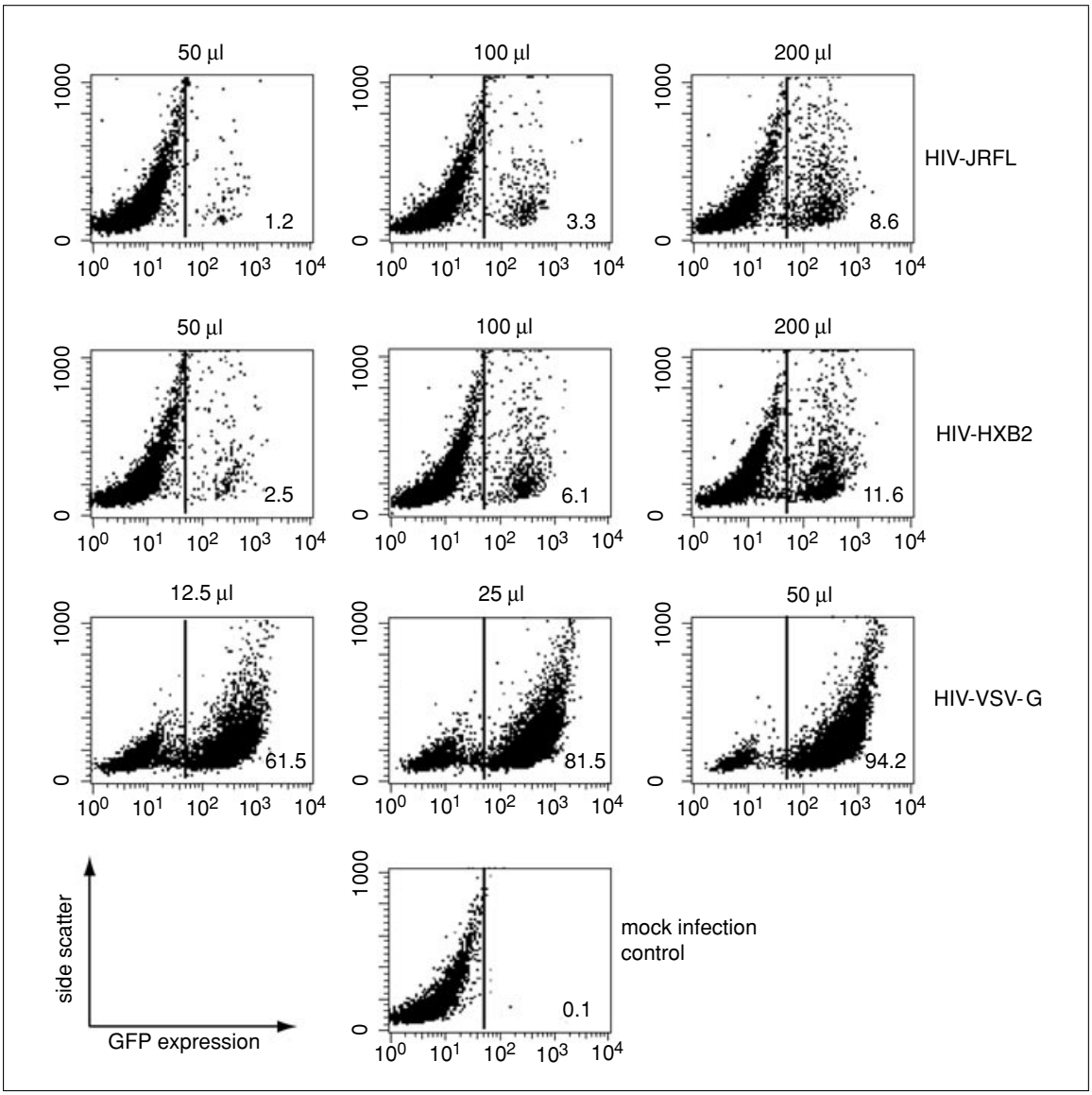


Figure 26.5.1 Infectivity titration of HIV-1 pseudotyped with different envelope proteins. HIV-1 indicator cells (GHOST/X4/R5) were infected with various single-cycle HIV-1 pseudotypes with different viral inputs. GFP-positive cells were measured 3 days post-infection by flow cytometry (FACSCalibur, BD Bioscience) and analyzed with CellQuest Pro program (BD Bioscience). GFP-positive cell populations were determined based on the settings of mock control, which used medium instead of virus in the infection assay. A total of 10,000 cells per sample were acquired and analyzed. The amounts of viral input are labeled on the top of each dot plot. The percentages of GFP-positive cells are shown in the bottom right corner of each dot plot.

Harvest infected cells

5. One or two days after replacing the medium, harvest infected GHOST/X4/R5 cells (day 3 or 4) by gently washing cells two times with PBS as follows: add 300 µl of PBS containing 1mM EDTA to each well, and place on a shaking platform for 15 min at room temperature.

   Alternatively, cells can be trypsinized (0.05% trypsin with EDTA), and neutralized with 10% FBS-containing DMEM.

6. Before proceeding to step 7, wash cells with 1 ml CMF-PBS. To calculate the virus titer, count and calculate the average total cell number/sample when harvesting cells. Also count the mock control.

Table 26.5.2 Summary Results of Single-Cycle HIV-1 Titration Assay[a]

Viral input (μl/sample)[c]	GFP-positive cells (%) [viral titer infectious unit per ml (IU/ml)][b]					Average titer[d] (IU/ml)
	12.5	25	50	100	200	
HIV-1 types HIV-JRFL (R5-tropic)	ND	ND	1.16 [3.22 × 10^5]	3.34 [4.64 × 10^5]	8.56 [5.95 × 10^5]	4.60 × 10^5
HIV-HXB2 (X4-tropic)	ND	ND	2.46 [6.84 × 10^5]	6.06 [8.42 × 10^5]	11.60 [8.06 × 10^5]	7.77 × 10^5
HIV-VSV-G	61.52 [6.84 × 10^7]	81.54 [4.53 × 10^7]	94.22 [2.62 × 10^7]	ND	ND	4.66 × 10^7

[a]GHOST/X4/R5 cells and single-cycle HIV-1 pseudotypes were used in the titration assay (see Basic Protocol 3).

[b]Values in brackets denote individual virus titer calculated as the following:
virus titer [infectious unit (IU)/ml] = cell number × %GFP-positive cells × dilution factor. The dilution factor = 1000 μl/viral input (μl). ND, not determined.

[c]Average cell number, 1.39 × 10^6/sample.

[d]Average titer of the results derived from the three different viral inputs.

Fix cells

7. Resuspend samples in 300 μl of CMF-PBS. Add 300 μl of 4% paraformaldehyde to each sample for a final concentration of 2% paraformaldehyde.

   The authors recommend thoroughly pipetting up and down to mix cells in paraformaldehyde solution to prevent cell clumping.

8. Incubate cells on ice for at least 1 hr.

   The authors recommend not exceeding 6 hr of incubation time.

9. After incubation, add 1 ml of CMF-PBS to wash cells and centrifuge cells 15 sec at ~4000 × *g*, room temperature.

10. Resuspend cells in 200 μl of CMF-PBS/2% FBS.

Detect GFP expression in infected cells

11. Analyze cells by flow cytometry for GFP expression.

    There should be approximately over a ten-fold shift in the mean GFP-fluorescence of infected cells over the mock control. See Figure 26.5.1 for examples.

12. Calculate virus titer based on the following equation:

infectious units (IU)/ml = (cell number) × (%GFP-positive cells) × (dilution factor)

For example, using 200 μl of HIV-JRFL sample, the calculation would be as follows: (1.39 × 10^6) × 8.56% × 5 = 5.95 × 10^5 IU/ml. Refer to Table 26.5.2 for calculated virus titers as examples.

VIRAL INFECTION AND QUANTIFICATION USING REPORTER HIV-1

ALTERNATE PROTOCOL 1

Rather than using HIV-1 indicator cell lines in viral infection assays, a reporter gene such as *luciferase* or *GFP* engineered into the HIV-1 genome can be expressed in infected cells and be used as a marker for viral infection (Unutmaz et al., 1999; Wu et al., 2002b). These virally expressed reporters in the infected cells can be measured to quantify HIV-1 infection. Additionally, when using wild-type, replication-competent HIV-1 in infection assays, HIV-1 p24 in the supernatant of infected cells can be measured to quantify

viral production (Dong et al., 2007; Wang et al., 2007a). These assays are presented as an alternative method of HIV-1 infection assays, in which, Hut/CCR5 cells, a human $CD4^+$ T cell line that expresses high levels of CD4, CXCR4, and CCR5, are used as an example. Now that the virus stocks have been quantified, cells can be infected at a specific multiplicity of infection (MOI).

Materials

HIV-1 stocks with known viral infectivity (see Basic Protocol 3)
Hut/CCR5 cells (available from Dr. Vineet KewalRamani, *vineet@ncifcrf.gov*; Wang et al., 2007a,b)
Cell culture medium (see recipes)

1.5-ml screw-cap microcentrifuge tubes
24-well culture plates
37°C incubator

Additional equipment and reagents for cell culture (*UNIT 1.1*)

Infect cells with virus

1. To calculate the volume of virus used at a specific MOI, use the following equation:

MOI = [(virus stock IU/ml) × (volume of virus used)]/(number of cells in infection)

An example calculation is: 1×10^5 Hut/CCR5 cells are infected at a 0.5 MOI by a virus stock with a titer of 1.67×10^6 IU/ml. Therefore determine the amount of virus needed to infect 1×10^5 cells at a 0.5 MOI as follows: [(1.67×10^6 IU/ml) × (volume of virus used)]/(1×10^5 cells) = 0.5 MOI, where the volume equals 30 μl per sample.

2. Before adding virus, wash cells once with 1 ml CMF-PBS in 1.5-ml screw-cap microcentrifuge tubes. Centrifuge cells (1×10^5) 3 min at ~900 × *g*, room temperature. Remove supernatant. Resuspend cell pellet in 1 ml of PBS. Centrifuge again, and carefully remove supernatant.

   If using adherent cells, see Basic Protocol 3, use of GHOST/X4/R5 cells, for further details. Hut/CCR5 cells can be cultured in nonselective culture medium for single-round infection experiments. To maintain high level expression of receptor and coreceptors, Hut/CCR5 cells should be cultured in selective medium.

3. Add desired amount of virus to cells and mix well by pipetting up and down.

   Depending on the volume of virus used, the authors recommend a final incubation volume between 200 μl and 500 μl for optimal infection conditions. Prewarmed cell culture medium can be added so that the final volume for the virus incubation is within the recommended incubation volume. Additionally, incubations should be performed in 1.5-ml screw-cap tubes. Always include a mock control (no virus) with all virus infection experiments.

4. Incubate cells 1.5 to 2 hr in a 37°C incubator.

   The authors recommend loosening the caps of samples during incubation. Alternatively, viral incubation can be performed using 24-well culture plates.

5. After virus incubation, wash the samples three times with 1 ml PBS as in step 2.

6. Resuspend cells in 1 ml culture medium using 24-well culture plates and place cells in culture (the seeding density is ~1×10^5 cells/ml).

7. Harvest cells 3 days after infection. Use harvested cells for detection of viral infection using Support Protocol 1 or 2.

DETECTION OF LUCIFERASE ACTIVITY IN INFECTED CELLS

SUPPORT PROTOCOL 1

If using luciferase reporter HIV-1, viral infection can be detected by measuring luciferase activity in infected cell lysates.

Materials

Cell samples
PBS (*APPENDIX 2A*)
5× passive lysis buffer (Promega)
Luciferase assay kit (Promega)

1.5-ml screw-cap microcentrifuge tubes
Microcentrifuge (e.g., rotor model F241.5P in Beckman Coulter microcentrifuge, 22R centrifuge)
Platform shaker
96-well black plate (Thermo)
Chemluminescence microplate reader (Wallac 1420 VICTOR2 Multilabel Plate Reader)

Wash cells

1. Transfer each sample of cells (2×10^5 cells) to a 1.5-ml screw-cap microcentrifuge tube, and centrifuge 3 min at ~900 × *g*, room temperature.
2. Remove supernatant, and resuspend pellet in 1 ml of PBS to wash cells.
3. Centrifuge 3 min at 900 × *g*, room temperature. Remove supernatant.
4. Resuspend cell pellet in 100 µl of 1× passive lysis buffer.

   The 1× passive lysis buffer should rapidly inactivate HIV-1. As an extra precaution, lysates can be transferred to a fresh tube to ensure that sample does not contain any infectious virus.

5. Shake samples for 5 min at room temperature.

   Incubation time can be increased to 10 min if using more than 5×10^4 cells per sample.

6. Centrifuge samples 2 min at ~4000 × *g*, room temperature.
7. If storing samples up to 6 months at −80°C to run luciferase assay in the future, transfer supernatant to a fresh microcentrifuge tube. Otherwise, transfer samples (40 µl in duplicate) directly to a 96-well black plate for detection of luciferase activity.

   Do not disrupt the pellet when dispensing.

8. Perform the luciferase assay according to manufacturer's instructions. Dispense luciferase substrate in 100-µl volumes to each well of the 96-well plate.
9. Detect luciferase activity of cell lysates on a microplate reader according to manufacturer's instructions.

GFP DETECTION IN INFECTED CELLS

SUPPORT PROTOCOL 2

If using GFP reporter HIV-1, viral infection can be detected by measuring GFP expression in infected cells.

Materials

Infected cells
4% (w/v) paraformaldehyde
Sterile PBS without Ca^{2+} and Mg^{2+} (CMF-PBS; *APPENDIX 2A*)
CMF-PBS/2% (v/v) FBS

1.5-ml screw-cap microcentrifuge tubes
Microcentrifuge (e.g., rotor model F241.5P in Beckman Coulter microcentrifuge, 22R centrifuge)
Flow cytometer

1. Transfer infected cells (2×10^5 cells) to a 1.5-ml screw-cap microcentrifuge tube and centrifuge 3 min at $900 \times g$, room temperature. Wash infected cells with 1 ml of PBS.
2. Resuspend the samples in 300 µl of PBS. Add 300 µl of 4% paraformaldehyde to each sample for a final concentration of 2% paraformaldehyde.

   The authors recommend thoroughly pipetting up and down to mix cells in paraformaldehyde solution to prevent cell clumping.
3. Incubate cells for at least 1 hr on ice.

   The authors recommend not exceeding 6 hr of incubation time.
4. After incubation, add 1 ml of CMF-PBS to wash cells and centrifuge cells 15 min at $\sim$4000 $\times$ g, room temperature.
5. Resuspend cells in 200 µl of CMF-PBS/2% FBS.
6. Analyze for GFP expression by flow cytometry.

   Include a positive control and a mock infection control. There should be approximately a 10- to 20-fold shift in the mean GFP-fluorescence of efficiently infected cells over the mock control.

SUPPORT PROTOCOL 3

HIV-1 P24 DETECTION IN SUPERNATANTS OF INFECTED CELLS

If using a replication-competent HIV-1 that does not encode a reporter gene, viral infection and production can be quantified by measuring HIV-1 p24 in cell supernatants.

Materials

Infected cells
10% (w/v) Triton X-100
HIV-1 p24 ELISA kit (anti-p24-coated plates from the AIDS Vaccine Program, SAIC, Frederick, Maryland) or p24 ELISA kit (PerkinElmer)

Refrigerated centrifuge
1.5-ml screw-cap microcentrifuge tubes
37°C incubator

Detect HIV-1 p24

1. Centrifuge infected cells ($\sim 2 \times 10^5$) 5 min at $\sim 900 \times g$, 4°C. Collect supernatant carefully, avoiding disruption of the pellet.
2. Transfer supernatant to a fresh 1.5-ml screw-cap microcentrifuge tube.
3. Add 1/10 vol of 10% Triton X-100 to each sample according to HIV-1 p24 ELISA kit manufacturer's instructions.
4. Incubate samples 1 hr at 37°C.
5. Analyze samples by p24 ELISA assay according to kit manufacturer's instructions.

   Alternatively, samples can be stored up to 6 months at −80°C, and analyzed in the future.

HIV-1 CELL-CELL TRANSMISSION ASSAYS

BASIC PROTOCOL 4

Cell-cell transmission can enhance HIV-1 infection 100 to 1000-fold compared with cell-free viral infection (Dimitrov et al., 1993). Elucidating the mechanisms of cell-cell transmission–mediated HIV-1 infection can facilitate the understanding of viral pathogenesis and potentially aid in HIV-1 interventions (Wu and KewalRamani, 2006; Piguet and Steinman, 2007). HIV-1-pulsed dendritic cells efficiently transmit HIV-1 to co-cultured $CD4^+$ T cells (Wu and KewalRamani, 2006), which has been used as an in vitro model to study the mechanisms underlying cell-cell transmission (Cameron et al., 1992; Pope et al., 1994; Geijtenbeek et al., 2000; Wu et al., 2002a,b; Dong et al., 2007; Wang et al., 2007a,b). Exogenous DC-SIGN expressed on certain types of cells can mediate efficient HIV-1 *trans*-infection of cocultured $CD4^+$ T cells (Wu et al., 2004a,b; Wang et al., 2007a,b). As an example, this protocol describes an HIV-1 transmission assay using Raji/DC-SIGN cells as donor cells and Hut/CCR5 T cells as targets. CD4- and DC-SIGN-negative parental Raji cells are used as background controls. The viral transmission assay is referred to as the HIV-1 capture assay, which is performed when virus donor cells are preincubated with HIV-1 and washed to remove cell-free virus, after which $CD4^+$ T cells were added in co-culture as infection targets.

Materials

Raji/DC-SIGN cells
Sterile PBS without Ca^{2+} and Mg^{2+} (CMF-PBS; *APPENDIX 2A*)
Inhibitors: anti-DC-SIGN or mannan, optional
Single-cycle luciferase reporter HIV-1 stocks (see Basic Protocol 3)
Cell culture medium (see recipe)
Hut/CCR5 target cells
Polybrene
RPMI-1640 with 10% FBS

1.5-ml screw-cap microcentrifuge tubes
Refrigerated microcentrifuge (e.g., rotor model F241.5P in Beckman Coulter microcentrifuge, 22R centrifuge)
37°C incubator
24-well tissue culture plates

Additional reagents and equipment for cell culture (*UNIT 1.1*)

CAUTION: Follow the biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipets, etc.

Incubate donor cells with HIV-1

1. Dispense 2.5×10^5 Raji/DC-SIGN cells (donor cells) per sample into 1.5-ml screw-cap microcentrifuge tubes. Use Raji cells as a background control.
2. Centrifuge cells 3 min at ~$900 \times g$, room temperature. Remove supernatant carefully, avoiding disruption of the pellet.
3. Resuspend cell pellet in 1 ml of CMF-PBS to wash cells. Centrifuge cells 3 min at $900 \times g$, room temperature. Remove supernatant carefully.
4. (Optional) If using inhibition agents, such as anti-DC-SIGN (10 μg/ml) or mannan (20 μg/ml), preincubate cells with the inhibitors for 30 min at 37°C (Wu et al., 2002b).

   These inhibitors can be added in 100 μl of medium for 2×10^5 cells (2×10^6 cells/ml).

5. Resuspend cells in 0.2 to 0.4 MOI (~1 × 10^5 IU) of HIV-1.

   See Basic Protocol 3 for direct infection and calculation of MOI. Based on viral input, the incubation volume should be 200 to 400 μl with culture medium.

6. Incubate cells in 1.5-ml screw-cap microcentrifuge tubes for 2 hr at 37°C.

   Loosening the caps during incubation is recommended.

Wash donor cells and prepare target cells

7. Centrifuge 2 min at 900 × *g*, 4°C. Remove supernatant carefully.
8. Wash each sample with 1 ml of CMF-PBS and centrifuge 2 min at 900 × *g*, 4°C. Remove supernatant carefully.
9. Prepare Hut/CCR5 target cells (5 × 10^5 cells/ml). To enhance viral transmission efficiency, add polybrene (8 to 10 μg/ml) to target cells. When using adherent cells as target cells, plate cells in 24-well plate 1 day before the co-culture assay.

Co-culture donor cells with target cells

10. Resuspend HIV-1-pulsed Raji/DC-SIGN cell pellets (from step 8) with 200 μl of the prepared Hut/CCR5 cells (1 × 10^5 cells) from step 9.

    As a background control, include HIV-pulsed donor cells without co-culture of target cells. When using adherent cells as target cells, remove medium in 24-well plate, resuspend Raji/DC-SIGN cells with medium (200 μl/sample), and then transfer them into each well of the adherent target cells.

11. Incubate suspension cells in screw-cap tubes for 1 to 2 hr at 37°C.

    Loosening caps during incubation is recommended. Alternatively, co-culture adherent cells in a 24-well plate.

12. Transfer cells from each tube to a new 24-well plate. Adjust the final volume of medium to 1 ml per sample per well with RPMI-1640 with 10% FBS.
13. Incubate 2 to 3 days in an incubator designated for HIV-1-infected cells.
14. Refer to Support Protocol 1 on quantifying viral infection of target cells when harvesting cells.

ALTERNATE PROTOCOL 2

HIV-1 ENHANCEMENT ASSAY

As an alternative method of HIV-1 cell-cell transmission assay, a viral enhancement assay can be used. This assay is performed using a smaller HIV-1 inoculum during incubation with donor cells, and target cells are added directly to the co-culture without removing the virus present in the culture medium (Wu et al., 2004b).

For materials, see Basic Protocol 4.

CAUTION: Follow the biosafety protocol and use 10% bleach to inactivate HIV-1 in the plates, tubes, plastic pipets, etc.

Incubate donor cells with HIV-1

1. Dispense 2.5 × 10^5 Raji/DC-SIGN cells (donor cells) per sample into 1.5-ml screw-cap microcentrifuge tubes. Use Raji cells as a background control.
2. Centrifuge cells 3 min at 900 × *g*, room temperature. Remove supernatant carefully, avoiding disruption of the pellet.
3. Resuspend pellet in 1 ml of CMF-PBS to wash cells.
4. Centrifuge cells 3 min at 900 × *g*, room temperature. Remove supernatant carefully.

5. (Optional) If using inhibition agents, such as anti-DC-SIGN (10 μg/ml) or mannan (20 μg/ml), preincubate cells with the inhibitors for 30 min at 37°C (Wu et al., 2002b).

   These inhibitors can be added in 100 μl of medium for 2×10^5 cells (2×10^6 cells/ml).

6. Resuspend cells with 100 μl of nonselective cell culture medium.

7. Add 0.02 to 0.04 MOI ($\sim 1 \times 10^4$ IU) of HIV-1 to donor cells. Adjust the incubation volume to 200 μl with culture medium.

   See Basic Protocol 3 for direct infection and calculation of MOI.

8. Incubate cells in 1.5-ml screw-cap microcentrifuge tubes for 2 hr at 37°C.

   Loosening caps during incubation is recommended.

Prepare target cells and co-culture with donor cells

9. Prepare Hut/CCR5 target cells (5×10^5 cells/ml). To enhance viral transmission efficiency, add polybrene (8 to 10 μg/ml) to target cells.

   When using adherent cells as target cells, plate cells 1 day before the co-culutre assay.

10. Co-culture HIV-1-incubated Raji/DC-SIGN cells (from step 7) with 200 μl of the prepared Hut/CCR5 cells (1×10^5 cells) from step 8.

    As a background control, include HIV-incubated donor cells without co-culture of target cells. When using adherent cells as target cells, remove medium in 24-well plate, resuspend Raji/DC-SIGN cells with medium (200 μl/sample), and then transfer them into each well of the adherent target cells.

11. Incubate the cells in screw-cap tubes for 1 to 2 hr at 37°C.

    Loosening caps during incubation is recommended. Alternatively, co-culture cells in a 24-well plate.

12. Transfer cells from each tube to a new 24-well plate (the final volume of medium should be 1 ml per sample per well).

    Use non-selective culture media (see recipe). For adherent target cells, cells can be continuously incubated in the plate.

13. Incubate 2 to 3 days in an incubator designated for HIV-infected cells.

14. Refer to Support Protocol 1 on quantifying viral infection of target cells when harvesting cells.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BES-buffered saline (BBS), 2× (pH 6.96 ± 0.02)

5.6 ml 5 M NaCl
5.0 ml 1 M BES
150 μl 1 M Na_2HPO_4
Tissue-culture quality sterile ddH_2O to a final volume of 100 ml
Filter sterilize through a 0.2-μm filter
Store 10-ml aliquots up to 6 months at −20°C

Cell culture media

Cell lysis buffer, 1×
1% (v/v) NP40 (Sigma cat. no. IGEPAL CA-630)

continued

100 mM NaCl, dilute from 5 M stock (*APPENDIX 2A*; 50×)
10 mM Tris·Cl, pH 7.5, dilute from 1 M stock (*APPENDIX 2A*; 100×)
1 mM EDTA, dilute from 0.5 M stock (*APPENDIX 2A*; 500×)

Alternatively, a 10× lysis buffer stock solution can be prepared and stored at 4°C, so that 1× fresh lysis buffer can be conveniently diluted with water for the assay.

Dulbecco phosphate buffered saline (DPBS, 1×)
137.9 mM NaCl
2.7 mM KCl
1.5 mM KH_2PO_4
8.1 mM Na_2HPO_4
Adjust pH to 7.3 using NaOH or HCl, if necessary
Autoclave solution
Store up to 6 months at room temperature

GHOST/X4/R5 cell medium
Dulbecco's modified Eagle's medium (DMEM)
10% (v/v) FBS
Neomycin (500 μg/ml)
Hygromycin B (50 μg/ml)
Puromycin (1 μg/ml)

(Optional) Add penicillin (100 U/ml) and streptomycin (100 μg/ml) to the medium. Store up to 6 months at 4°C. All cell-culture grade reagents are available from Invitrogen.

HEK293T cell medium
DMEM
10% (v/v) FBS

(Optional) Add penicillin (100 U/ml) and streptomycin (100 μg/ml) to the medium. Store up to 6 months at 4°C.

Hut/CCR5 cell medium
RPMI-1640
10% (v/v) FBS
Neomycin (500 μg/ml)
Puromycin (1 μg/ml)

(Optional) Add penicillin (100 U/ml) and streptomycin (100 μg/ml) to the medium. Store up to 6 months at 4°C.

Paraformaldehyde fixative, 4% (pH 7.2)

Prepare a fresh 4% (w/v) solution of analytical-grade paraformaldehyde in DPBS (see recipe). Adjust the pH of the solution to pH 7.2 with HCl. Store up to 3 months at 4°C in the dark.

Use caution when handling; formaldehyde is a carcinogen. The solution should be prepared in a fume hood.

PBS/2% (v/v) FBS

Add 2% FBS to 1× DPBS (see recipe) just before use.

Raji/DC-SIGN cell medium

RPMI-1640
10% (v/v) FBS

(Optional) Add penicillin (100 U/ml) and streptomycin (100 μg/ml) to the medium. Store up to 6 months at 4°C.

COMMENTARY

Background Information

In this unit, a set of methods to perform quantitative assays of HIV-1 binding, internalization, infection, and cell-cell transmission is described. Although not mentioned in this unit, many other assays and protocols have been described in the literature. Based on various experimental purposes and requirements, numerous different methods can be used to study HIV-1 interactions with host cells. For instance, a flow cytometry based HIV-1 binding assay using GFP-tagged virus has been used in $CD4^+$ primary T cells and cell lines (Schaeffer et al., 2004). A sensitive and specific enzyme-based assay has been reported to detect HIV-1 virion fusion in primary T lymphocytes (Cavrois et al., 2002). LTR promoter–based HIV-1 indicator cell lines have been widely used in measuring HIV-1 infectivity or viral infection (Cecilia et al., 1998; Derdeyn et al., 2000). However, a background transcriptional activity of the LTR promoter that is independent of HIV-1 infection and Tat expression could exist. Moreover, cellular activation states can also influence the activity of the LTR promoter. To overcome these potential caveats, a Rev-dependent HIV-1 indicator T cell line using GFP as a reporter has been recently produced (Wu et al., 2007). This $CD4^+$ T cell line appears to be sensitive to both CCR5- and CXCR4-tropic HIV-1 and shows no measurable background of GFP expression (Wu et al., 2007).

Although various reporter-expressing HIV-1 vectors provide convenient tools to measure viral infection (Connor et al., 1995; Unutmaz et al., 1999), the reverse transcriptase assay (Adachi et al., 1986; Freed et al., 1995) remains as a reliable and sensitive method to measure HIV-1 replication (Hatziioannou et al., 2006). Moreover, quantitative real-time PCR to measure HIV-1 reverse transcription, integration, and gene transcription is an important tool in dissecting molecular mechanisms of viral replication (Butler et al., 2001; Wu and Marsh, 2001; Dong et al., 2007). For the HIV-1 cell-cell transmission assay, the method described in this unit may need to be adjusted according to the cell types used in the experiments. In addition to DCs (Wu and KewalRamani, 2006), macrophages can mediate HIV-1 transmission to $CD4^+$ T cells (Sharova et al., 2005). Furthermore, efficient cell-cell transmission of HIV-1 also occurs between $CD4^+$ T cells (Jolly et al., 2007a,b; Sowinski et al., 2008).

Critical Parameters and Troubleshooting

Generating virus stocks

When generating virus stocks, it is imperative that all transfection reagents are at the appropriate pH. When mixing the transfection reagents, do not incubate the transfection mixture for longer than 20 min. To maximize the efficiency of transfection, use an earlier passage of HEK293T cells; the seeded cells should not be overly confluent before the transfection. Additionally, replace medium when specified to ensure high-titer and yield of the virus stock. Lastly, the DNA being used in transfection reactions should be of high quality. If experiencing difficulty with generating virus of appropriate titer, check the pH of the transfection solutions, the passage number of HEK293T cells, and the quality of the DNA preparations. For the DNA concentrations, it is recommended to use a ~1 mg/ml stock, because too dilute a stock may potentially alter the pH of the transfection reaction.

HIV-1 binding and internalization assay

When performing the binding assay, keep consistent temperatures of sample incubation at 4°C or 37°C. The samples that are incubated at 4°C and treated with trypsin represent background levels of the assay. The concentration of trypsin should be titrated for different cell types since some cells might be more sensitive to proteolysis. After trypsin treatment, use caution when removing the supernatant after washes since the sample can be very fragile. Lastly, include a parental cell line as a control if looking at specific attachment factors transduced in a cell line (e.g., comparing HIV binding in parental Raji cells and Raji/DC-SIGN cells). For p24 ELISA detection, see manufacturer's instructions.

HIV-1 infection and cell-cell transmission

For direct infection of target cells, cell viability and the quality of the virus stock will affect the quality of the assay. If the stock is too dilute, larger than desired incubation volumes will have to be used, and the infectivity of the virus may not be as high when compared to a more concentrated virus stock at the same MOI. Low-titer viral stocks can be concentrated by ultracentrifugation or ultrafiltration. Furthermore, if performing infections with different HIV-1 pesudotypes in parallel, it is important to maintain the same incubation volumes if directly comparing the virus

infections. Always include a mock infection control for background levels.

These critical parameters should also apply for the HIV cell-cell transmission assays. Another important control in viral transmission assays is to use donor cells with HIV-1 incubation, but without the co-culture of target cells. Donor cells that are negative for HIV-1 receptor or appropriate coreceptors should not be directly infected by HIV-1, and therefore should have similar values as compared to the mock control. Notably, when using HIV-1-permissive donor cells, such as immature dendritic cells, HIV-1-pulsed dendritic cells can become infected 3 to 5 days post-infection if high viral inputs are used (Dong et al., 2007).

Anticipated Results

Generating HIV-1 stocks

For HIV pseudotyped with HIV-1 Env, such as HXB2 or JRFL, the titer of the virus generated from transfection should range between 2×10^5 and 5×10^5 infectious units (IU)/ml, whereas HIV pseudotyped with VSV-G has a titer ranging from 1×10^6 to 10×10^6 IU/ml. A 10- to 20-fold shift in GFP expression should be expected when titrating the virus stocks by flow cytometry assay. See Figure 26.5.1 and Table 26.5.2 for viral titration examples.

HIV-1 binding and internalization assay

HIV-1 binding and internalization can be enhanced around two-fold in Raji/DC-SIGN cells, relative to parental Raji cells (Wu et al., 2004b). When using 20 ng of p24-equivelent HIV-1 with 3×10^5 of Raji/DC-SIGN cells, the p24 detection in cell lysates should be ~500 pg/ml (Wang et al., 2007a). Notably, cell-associated p24 values will vary based on the cell types, virus input, incubation temperature and time, and the proteolysis treatment after viral incubation (Wu et al., 2004b; Dong et al., 2007; Wang et al., 2007a,b).

HIV-1 infection assays

The level of infection detected in target cells will be dependent upon the number of cells used, the virus input, as well as the type of virus and cells that are used in the infection. Using luciferase reporter HIV-1 in the viral infection assay (see Alternate Protocol 1 and Support Protocol 1), the background levels were ~100 counts/sec for the mock control. As an example, 2×10^5 Hut/CCR5 cells infected with HIV-1-VSV-G at an MOI of 0.1, and harvested 3 days post-infection, had luciferase activity ~330-fold higher relative to that of the mock control. In contrast, Hut/CCR5 cells infected with HIV-1-JRFL had luciferase activity 39-fold higher compared to the mock control (Janas et al., 2008; Janas and Wu, unpub. observ.).

HIV-1 cell-cell transmission assays

If using the HIV-1 capture assay (see Basic Protocol 4), when compared to the negative control (HIV-pulsed Raji/DC-SIGN cells without target cells), Raji/DC-SIGN cell-mediated HIV-1-JRFL transmission to Hut/CCR5 target cells can be ≥350-fold higher (Wu et al., 2004b; Wang et al., 2007a). If using the HIV-1 enhancement assay (see Alternate Protocol 2), expect to see ~10-fold enhancement of viral infection in the co-cultures. Notably, the relative efficiency of HIV-1 cell-cell transmission may vary depending on the cell types and viruses (Wang et al., 2007b).

Time Considerations

Generating virus stocks

For virus stock generation, plan for 4 days. Day 1 will consist of plating cells. The transfection will be performed on the second day, and will take ~25 to 30 min, depending on how much virus stock is being generated. On day 3, the medium is replaced. The virus is harvested on day 4, and will take ~1 hr.

HIV-1 binding and internalization assay

The binding assay should take ~3 to 4 hr to perform, depending on the sample number (not including virus inactivation). The p24 ELISA detection normally takes ~6 hr.

HIV-1 infection of target cells

For virus titrations, the experiment will span 4 days. On day 1, the cells will be plated. On the second day, the cells are infected. On the third day, cell medium is replaced. On the fourth day, the cells can be harvested for GFP analysis by flow cytometry. Sample preparation for flow cytometry analysis can take from 1.5 to 6 hr, depending on how long the investigator chooses to fix the cells in paraformaldehyde. When performing other direct infections of target cells, anticipate ~4 hr on the day of infection (and 5 hr for transmission assay), and an additional 2 to 3 days until the cells are harvested for analysis. Harvesting the cells takes ~1 hr for the luciferase assay, and may be shorter if collecting cell supernatants for p24 analysis.

Acknowledgements

The authors thank Michael Emerman, Eric Freed, Jeffery Lifson, Vineet KewalRamani, and the AIDS Research and Reference Reagent Program, NIAID, NIH, for the kind gift of reagents. The authors thank Constance Kwas and other members of the laboratory for critical reading the manuscripts and helpful discussions. The work published by the authors' laboratory was supported by grants from the National Institutes of Health (AI068493) and the Campbell Foundation to Li Wu.

Literature Cited

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M.A. 1986. Production of acquired immunodeficiency syndrome–associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* 59:284-291.

Aiken, C. 1997. Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. *J. Virol.* 71:5871-5877.

Butler, S.L., Hansen, M.S., and Bushman, F.D. 2001. A quantitative assay for HIV DNA integration in vivo. *Nat. Med.* 7:631-634.

Cameron, P.U., Freudenthal, P.S., Barker, J.M., Gezelter, S., Inaba, K., and Steinman, R.M. 1992. Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells. *Science* 257:383-387.

Cavrois, M., De Noronha, C., and Greene, W.C. 2002. A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat. Biotechnol.* 20:1151-1154.

Cecilia, D., KewalRamani, V.N., O'Leary, J., Volsky, B., Nyambi, P., Burda, S., Xu, S., Littman, D.R., and Zolla-Pazner, S. 1998. Neutralization profiles of primary human immunodeficiency virus type 1 isolates in the context of coreceptor usage. *J. Virol.* 72:6988-6996.

Connor, R.I., Chen, B.K., Choe, S., and Landau, N.R. 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* 206:935-944.

Derdeyn, C.A., Decker, J.M., Sfakianos, J.N., Wu, X., O'Brien, W.A., Ratner, L., Kappes, J.C., Shaw, G.M., and Hunter, E. 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol.* 74:8358-8367.

Dimitrov, D.S., Willey, R.L., Sato, H., Chang, L.J., Blumenthal, R., and Martin, M.A. 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. *J. Virol.* 67:2182-2190.

Dong, C., Janas, A.M., Wang, j.-H., Olson, W.J., and Wu, L. 2007. Characterization of human immunodeficiency virus type 1 replication in immature and mature dendritic cells reveals dissociable *cis*- and *trans*-infection. *J. Virol.* 81:11352-11362.

Fauci, A.S. 2007. 25 years of HIV/AIDS science: Reaching the poor with research advances. *Cell* 131:429-432.

Frank, I., Piatak, M., Jr., Stoessel, H., Romani, N., Bonnyay, D., Lifson, J.D., and Pope, M. 2002. Infectious and whole inactivated simian immunodeficiency viruses interact similarly with primate dendritic cells (DCs): Differential intracellular fate of virions in mature and immature DCs. *J. Virol.* 76:2936-2951.

Freed, E.O., Englund, G., and Martin, M.A. 1995. Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. *J. Virol.* 69:3949-3954.

Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., KewalRamani, V.N., Littman, D.R., Figdor, C.G., and van Kooyk, Y. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances *trans*-infection of T cells. *Cell* 100:587-597.

Geijtenbeek, T.B., van Duijnhoven, G.C., van Vliet, S.J., Krieger, E., Vriend, G., Figdor, C.G., and van Kooyk, Y. 2002. Identification of different binding sites in the dendritic cell-specific receptor DC-SIGN for intercellular adhesion molecule 3 and HIV-1. *J. Biol. Chem.* 277:11314-11320.

Goff, S.P. 2007. Host factors exploited by retroviruses. *Nat. Rev. Microbiol.* 5:253-263.

Gummuluru, S., Rogel, M., Stamatatos, L., and Emerman, M. 2003. Binding of human immunodeficiency virus type 1 to immature dendritic cells can occur independently of DC-SIGN and mannose binding C-type lectin receptors via a cholesterol-dependent pathway. *J. Virol.* 77:12865-12874.

Hatziioannou, T., Princiotta, M., Piatak, M. Jr., Yuan, F., Zhang, F., Lifson, J.D., and Bieniasz, P.D. 2006. Generation of simian-tropic HIV-1 by restriction factor evasion. *Science* 314:395.

Janas, A.M., Dong, C., Wang, J.-H., and Wu, L. 2008. Productive infection of human immunodeficiency virus type 1 in dendritic cells requires fusion-mediated viral entry. *Virology* doi:10.1016/j.virol.2008.01.044: (Epub. ahead of print).

Jolly, C., Mitar, I., and Sattentau, Q.J. 2007a. Adhesion molecule interactions facilitate human immunodeficiency virus type 1-induced virological synapse formation between T cells. *J. Virol.* 81:13916-13921.

Jolly, C., Mitar, I., and Sattentau, Q.J. 2007b. Requirement for an intact T-cell actin and tubulin cytoskeleton for efficient assembly and spread of human immunodeficiency virus type 1. *J. Virol.* 81:5547-5560.

Karlsson Hedestam, G.B., Fouchier, R.A., Phogat, S., Burton, D.R., Sodroski, J., and Wyatt, R.T. 2008. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. *Nat. Rev. Microbiol.* 6:143-155.

Kwon, D.S., Gregorio, G., Bitton, N., Hendrickson, W.A., and Littman, D.R. 2002. DC-SIGN-mediated internalization of HIV is required for *trans*-enhancement of T cell infection. *Immunity* 16:135-144.

Piguet, V. and Steinman, R.M. 2007. The interaction of HIV with dendritic cells: Outcomes and pathways. *Trends Immunol.* 28:503-510.

Pope, M., Betjes, M.G., Romani, N., Hirmand, H., Cameron, P.U., Hoffman, L., Gezelter, S., Schuler, G., and Steinman, R.M. 1994. Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with HIV-1. *Cell* 78:389-398.

Rossio, J.L., Esser, M.T., Suryanarayana, K., Schneider, D.K., Bess, J.W. Jr., Vasquez, G.M., Wiltrout, T.A., Chertova, E., Grimes, M.K., Sattentau, Q., Arthur, L.O., Henderson, L.E., and Lifson, J.D. 1998. Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. *J. Virol.* 72:7992-8001.

Schaeffer, E., Soros, V.B., and Greene, W.C. 2004. Compensatory link between fusion and endocytosis of human immunodeficiency virus type 1 in human CD4 T lymphocytes. *J. Virol.* 78:1375-1383.

Sharova, N., Swingler, C., Sharkey, M., and Stevenson, M. 2005. Macrophages archive HIV-1 virions for dissemination in trans. *EMBO J.* 24:2481-2489.

Sowinski, S., Jolly, C., Berninghausen, O., Purbhoo, M.A., Chauveau, A., Kohler, I., Oddos, S., Eissmann, P., Brodsky, F.M., Hopkins, C., Onfelt, B., Sattentau, Q., and Davis, D.M. 2008. Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat. Cell Biol.* 10:211-219.

Turville, S.G., Arthos, J., Mac Donald, K., Lynch, G., Naif, H., Clark, G., Hart, D., and Cunningham, A.L. 2001. HIV gp120 receptors on human dendritic cells. *Blood* 98:2482-2488.

Turville, S.G., Cameron, P.U., Handley, A., Lin, G., Pohlmann, S., Doms, R.W., and Cunningham, A.L. 2002. Diversity of receptors binding HIV on dendritic cell subsets. *Nat. Immunol.* 3:975-983.

Unutmaz, D., KewalRamani, V.N., Marmon, S., and Littman, D.R. 1999. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J. Exp. Med.* 189:1735-1746.

Wang, J.H., Janas, A.M., Olson, W.J., KewalRamani, V.N., and Wu, L. 2007a. CD4 coexpression regulates DC-SIGN-mediated transmission of human immunodeficiency virus type 1. *J. Virol.* 81:2497-2507.

Wang, J.H., Janas, A.M., Olson, W.J., and Wu, L. 2007b. Functionally distinct transmission of human immunodeficiency virus type 1 mediated by immature and mature dendritic cells. *J. Virol.* 81:8933-8943.

Wu, L. and KewalRamani, V.N. 2006. Dendritic-cell interactions with HIV: Infection and viral dissemination. *Nat. Rev. Immunol.* 6:859-868.

Wu, L., Bashirova, A.A., Martin, T.D., Villamide, L., Mehlhop, E., Chertov, A.O., Unutmaz, D., Pope, M., Carrington, M., and KewalRamani, V.N. 2002a. Rhesus macaque dendritic cells efficiently transmit primate lentiviruses independently of DC-SIGN. *Proc. Natl. Acad. Sci. U.S.A.* 99:1568-1573.

Wu, L., Martin, T.D., Vazeux, R., Unutmaz, D., and KewalRamani, V.N. 2002b. Functional evaluation of DC-SIGN monoclonal antibodies reveals DC-SIGN interactions with ICAM-3 do not promote human immunodeficiency virus type 1 transmission. *J. Virol.* 76:5905-5914.

Wu, L., Martin, T.D., Carrington, M., and KewalRamani, V.N. 2004a. Raji B cells, misidentified as THP-1 cells, stimulate DC-SIGN-mediated HIV transmission. *Virology* 318:17-23.

Wu, L., Martin, T.D., Han, Y.C., Breun, S.K., and KewalRamani, V.N. 2004b. Trans-dominant cellular inhibition of DC-SIGN-mediated HIV-1 transmission. *Retrovirology* 1:14.

Wu, Y. and Marsh, J.W. 2001. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. *Science* 293:1503-1506.

Wu, Y., Beddall, M.H., and Marsh, J.W. 2007. Rev-dependent indicator T cell line. *Current HIV Research* 5:394-402.

Yamashita, M. and Emerman, M. 2004. Capsid is a dominant determinant of retrovirus infectivity in nondividing cells. *J. Virol.* 78:5670-5678.

Internet Resources

http://www.niaid.nih.gov/DAIDs/pdatguide/vmol.htm

The Virology Manual for HIV Laboratories is freely available to download from is Website.

https://www.aidsreagent.org/Index.cfm

The Website of the NIH AIDS Research and Reference Reagent Program.

Methods for Monitoring Dynamics of Pulmonary RSV Replication by Viral Culture and by Real-Time Reverse Transcription–PCR In Vivo: Detection of Abortive Viral Replication

Marina S. Boukhvalova,[1] Kevin C. Yim,[1] Gregory A. Prince,[1] and Jorge C.G. Blanco[1]

[1]Virion Systems, Rockville, Maryland

ABSTRACT

Viral infection is normally detected either by viral culture or by PCR methods. Rarely is a combination of the two techniques used in the same study. Yet, when applied simultaneously, viral culture and PCR may reveal important features of viral biology, such as an abortive replication, as in the case of respiratory syncytial virus (RSV) infection. In this unit, we describe methods for detecting abortive RSV replication in a cotton rat model by using the plaque-forming unit assay and the real-time reverse-transcription PCR (qRT-PCR) assay. All steps of the process of monitoring viral replication in vivo are described, starting from the design of animal infection protocols. We continue on to the methods for extracting and processing lung samples for viral culture and RNA extraction, and finish with the actual methods of viral titration by the qRT-PCR and the plaque-forming unit assays. *Curr. Protoc. Cell Biol.* 46:26.6.1-26.6.19. © 2010 by John Wiley & Sons, Inc.

Keywords: abortive replication • RSV • cotton rat

INTRODUCTION

This unit describes methods for detecting abortive viral replication in vivo in an animal model. Viral culture has long been considered the "gold standard" for laboratory diagnosis of respiratory viral infections in humans and for analysis of viral replication in animal models. Over the years, other techniques such as PCR have proven to be specific and sensitive methods for viral detection, with real-time reverse-transcription PCR (qRT-PCR) particularly well-suited for analysis of respiratory RNA viruses (Bustin and Mueller, 2005). The rate of identification of viral infections in humans by viral culture is often lower than that detected by PCR and qRT-PCR (Mackay et al., 2003; van Kraaij et al., 2005). This finding is normally attributed to higher sensitivity of the PCR technique, but may also reflect cases when virus establishes an abortive infection in which viral genetic material is replicated, but production of infectious viral particles is impaired. Undetected by viral culture, this type of replication can only be documented by measuring viral gene expression and genome replication in infected humans and laboratory animals by PCR.

One of the viruses for which both productive and abortive infections have been documented is Respiratory Syncytial Virus (RSV; Boukhvalova et al., 2007). RSV is a negative-strand RNA virus that is a major cause of bronchiolitis and viral pneumonia in children (Shay et al., 2001). RSV effectively establishes productive infection in naïve cotton rats (*Sigmodon hispidus*), a preferred small animal model of human RSV disease (Niewiesk and Prince, 2002; Openshaw and Tregoning, 2005). When cotton rats are challenged with RSV for the second time, however, only abortive infection develops.

Current Protocols in Cell Biology 26.6.1-26.6.19, March 2010
Published online March 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2606s46

The key for detecting all possible types of RSV replication in vivo is to analyze infection in dynamics by applying methods that allow virus monitoring both at microbiological (infectious virus generation) and molecular (replication of viral genetic material) levels. This type of analysis will constitute the scope of this unit. We will cover all steps of the process of monitoring viral replication in vivo starting from the design of animal infection protocols (Strategic Planning) and continuing on to the methods for harvesting lung samples for viral culture and RNA extraction (Support Protocol 1). We will describe the actual methods of viral titration by plaque assay (Basic Protocol 1) and qRT-PCR measurements of RSV replication (Basic Protocol 2), and discuss the ways to analyze and to interpret the data. Moreover, we will describe a simple method for RSV infection of epithelial cells in vitro (Support Protocol 2) which can be used to fine tune the plaque assay and qRT-PCR techniques in the reader's laboratory.

STRATEGIC PLANNING

To detect abortive viral replication, one has to have the ability to monitor viral infection in dynamics and to compare viral replication in naïve animals to viral replication in animals immune to the virus. The abortive replication in the case of RSV infection is detected in animals that have been rendered immune to RSV by prior RSV infection. So, the experiment has to include two groups of animals: the first group is composed of naïve animals that have never been infected with RSV before. These animals encounter RSV for the first time at the beginning of the experiment (primary infection). The second group includes animals that have been challenged with RSV once and then rechallenged again 21 days later (secondary infection). When cotton rats are challenged with RSV twice over the period of 21 days, no virus is detected by culture in the lungs of animals after the second infection. Even though no virus is detected in the lungs of rechallenged cotton rats by viral culture, replication of virus takes place and can be measured by qRT-PCR.

The samples must be collected for viral load analysis at various time points after viral challenge: the first and only challenge in primary RSV infection, and the second viral challenge in secondary RSV infection. Animals are sacrificed and lungs are collected over a time span that includes the time of maximum virus replication in naïve animals (day 4 post-infection), as well as the time points before and after the peak viral replication. Because abortive viral replication is more rapid than productive viral replication, early time points post-infection have to be included in analysis. We find that collecting lungs at 6 and 12 hr, and at days 1, 2, 4, 7, and 14 post-infection works best for detection of abortive RSV replication in the cotton rat model. The same time points are used for both primary and secondary infection to allow parallel analysis of samples.

Two different methods for detection of abortive viral replication are required: viral culture (Basic Protocol 1) and the qRT-PCR method (Basic Protocol 2). Replication is abortive when there is a discrepancy between the amount of viral genetic material accumulated (detected by qRT-PCR) and the amount of progeny virus released (detected by viral culture). It is thus important to be able to quantify extent of viral load by qRT-PCR and by viral culture assays in the same sample and to correlate the data yielded by the two techniques.

BASIC PROTOCOL 1

PLAQUE-FORMING UNIT ASSAY OF RSV PRODUCTION

This protocol describes the method for determining viral load in the lungs of RSV-infected animals by a plaque-forming unit assay (plaque assay). Monolayers of HEp-2 cells are inoculated with the homogenates of lung tissue. The infected monolayers are incubated in semi-solid methyl cellulose medium for several days to allow formation of plaques. Because semi-solid medium prevents spread of the virus (otherwise seen in liquid medium), infection of cells is localized and virus released from an infected cell

can infect only cells in the immediate surroundings of the originally infected one, thus producing a plaque. The plaques are visualized against the background of healthy cells stained with crystal violet stain. One plaque corresponds to one plaque-forming unit of RSV, and the total number of plaques reflects the amount of infectious virions present in the original lung sample.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Materials

HEp-2 cells (ATCC #ccL23)
HEp-2 growth medium (see recipe)
Lung homogenates (Support Protocol 1)
Eagle minimal essential medium (EMEM) with Earle's Balanced Salt Solution (BSS)
Methyl cellulose overlay medium (see recipe)
Crystal violet fixative/stain (see recipe)

24-well tissue culture plates
Dissecting microscope

Additional reagents and equipment for basic cell culture techniques (*UNIT 1.1*)

Prepare HEp-2 monolayers

1. Seed HEp-2 cells on 24-well tissue culture plates at 1×10^5 cells/well in 1 ml HEp-2 growth medium per well.

   UNIT 1.1 provides protocols for basic cell culture techniques.

2. Incubate the plates until the cell monolayers achieve confluency.

   It takes 2 to 3 days for the HEp-2 monolayer to achieve confluency.

Prepare samples for titrations

3. Thaw lung homogenates (prepared as described in steps 13 to 15 of Support Protocol 1) at room temperature.
4. Prepare 1:10 and 1:100 serial dilutions of homogenate samples in EMEM with Earle's BSS.

Inoculate HEp-2 monolayers

5. Aspirate half of the medium from each well of the HEp-2 seeded 24-well culture dishes.
6. Inoculate wells with 50 µl of neat, 1:10, and 1:100 dilutions of each sample in duplicate.
7. Incubate the inoculated 24-well culture dishes for 1 hr.
8. After the incubation period, aspirate the remaining medium from each well of the culture dishes.
9. Overlay each well with 1 ml of the methyl cellulose overlay medium at 37°C.
10. Incubate the plates for 4 days in the incubator.

Stain and read the titration

11. Remove the overlay from each well.

12. Add 0.5 ml of crystal violet fixative/stain to each well. Allow plates to incubate with crystal violet stain for no less than 20 min at room temperature.
13. Remove stain by rinsing each plate two to three times under cold water by filling all of the wells with water, then inverting the plate and tapping it on a paper towel.
14. Place the plate upside-down to air dry.

    The plates have to be completely dry prior to reading.
15. Under a dissecting microscope count the number of plaque-forming units (pfu) per well. Select the sample dilution that yields a reasonable number of plaques (5 to 50 per well).
16. Calculate the corresponding RSV titer in plaque-forming units (pfu) per gram of tissue using the following equation:

$$\frac{\#\text{pfu well 1} + \#\text{pfu well 2}}{0.1\text{ ml total volume}} \times \text{dilution factor} \times \frac{1.5\text{ ml HBSS}}{0.15\text{ g tissue}} = \text{pfu/g of tissue}$$

    where the 0.1 ml total volume consists of 50 μl of sample inoculated onto cells in duplicate = 50 μl + 50 μl, and the dilution factor from processing the tissue remains at a constant of 10×. The limit of the sensitivity of the assay is 1×10^2 pfu/g lung tissue.
17. Determine the geometric mean ±SEM for all animals in a group at a given time

BASIC PROTOCOL 2

REAL-TIME REVERSE TRANSCRIPTION PCR (qRT-PCR) ANALYSIS OF RSV INFECTION

Real-time PCR analysis has been used in the past to detect RSV infection (Falsey et al., 2002, 2003; Hu et al., 2003; Perkins et al., 2005). However, this method has never been used to monitor dynamics of RSV infection in progression. We have established conditions for measuring expression of RSV genes and production of RSV genome/antigenome in vivo and in vitro (Boukhvalova et al., 2007). This protocol will describe steps required to analyze RSV infection in the lungs of cotton rats using qRT-PCR assay. Viral RNA is extracted from the lungs and reverse-transcribed. Expression of individual viral genes is measured. RSV amplicons NS1, N, M, G, F, M2, and L are targeted for quantification. All amplicons are less than 100 nucleotides in size, with primers defining them shown in Table 26.6.1.

Materials

RNeasy Midi kit (Qiagen) including:
- RLT buffer
- RNeasy Midi columns
- Reagents for DNase treatment

RLT/ME buffer: RLT buffer from the RNeasy kit supplemented with 10 μl/ml 2-mercaptoethanol
Frozen lung tissue for analysis (Support Protocol 1), including tissue from two animals sacrificed on day 4 after primary RSV infection for construction of standard curve
70% ethanol
DEPC-treated H_2O (*APPENDIX 2A*)
0.5 μg/ml oligo(dT) primer
10× PCR buffer (without $MgCl_2$; Invitrogen)
50 mM $MgCl_2$ (Invitrogen)

Table 26.6.1 Primers Used for the Real-Time PCR Analysis of RSV Amplicons

Viral gene	Forward primer[a]	Reverse primer[a]	Start of forward primer[b]	Amplicon length
NS1	CACAACAATGCCAGTGCTACAA	TTAGACCATTAGGTTGAGAGCAATGT	231	83
N	AAGGGATTTTTGCAGGATTGTTT	CTCCCCACCGTAGCATTACTTG	719	66
M	ATGTGCTAATGTGTCCTTGGATGA	TGATTTCACAGGGTGTGGTTACA	270	68
G	CGGCAAACCACAAAGTCACA	TTCTTGATCTGGCTTGTTGCA	191	64
F	TAAGCAGCTCCGTTATCACATCTC	ATTGGATGCTGTACATTTAGTTTTGC	1205	74
M2	CATGAGCAAACTCCTCACTGAACT	TCTTGGGTGAATTTAGCTCTTCATT	294	80
L	CACTCTACAAAACAAAAAGACACAATCA	AGGATGCTGCATTGAACACATT	529	72
β-actin[c]	TATGCCAACACAGTGCTGTCT	TCTGCATCCTGTCGGCAAT	880	64

[a]Primers were designed using Primer Express software (Applied Biosystems).
[b]The number of nucleotides from the first nucleotide in the start codon (ATG) to the beginning of the amplicon.
[c]Expression of β-actin mRNA is measured in each sample for normalization purposes.

10 mM dNTP mix (Invitrogen)
200 U/μl SR II reverse transcriptase (Invitrogen)
iQ SYBR Green Supermix (BioRad)
10 μM forward and reverse PCR primers (Table 26.6.1)

14-ml polypropylene round-bottom tubes
OMNI TH homogenizer with 7-mm stainless-steel generator probe (OMNI International)
15-ml conical polypropylene tubes
Centrifuge with plate carriers
96-well PCR plates
Thermal cycler
Bio-Rad iCycler with iQ5 software
iQ 96-well PCR plates (BioRad)
Microseal 'B' adhesive seals (BioRad)

Extract RNA from lungs

1. Place 3.5 ml RLT/ME buffer into a 14-ml polypropylene tube. Add a frozen piece of lung to the solution and homogenize it using a probe homogenizer in two to three short bursts. Immediately freeze the homogenized lung on dry ice and transfer to the −80°C freezer.

   This step can be performed several days before the actual RNA extraction.

   The size of the lung piece sufficient for RNA extraction should be more or equal to ∼1/6th of the entire lung. We normally use one of the three lobes of the right lung, or a half of the upper lobe of the left lung for RNA extraction.

   IMPORTANT NOTE: *Lung tissue has to be frozen in liquid nitrogen immediately after extraction (see Support Protocol 1). Otherwise, the quality of the RNA preparation may be compromised.*

   Wash the probe well between the samples to prevent cross-contamination. We find that brief bursts of the homogenizer probe in two separate 50-ml tubes with 50 ml distilled water in each and an additional burst in 30 ml RLT/ME buffer between samples is sufficient to prevent cross-contamination of samples.

2. On the day of the RNA extraction, thaw lung homogenates in 37°C water bath for 15 min.

3. Centrifuge the homogenates for 10 min at 4000 × *g*, room temperature.
4. Transfer 2 ml of supernatant to a prepared 15-ml conical polypropylene tube with 2 ml 70% ethanol.

   Avoid upper phase when withdrawing supernatants.

5. Mix by inverting the tube vigorously five to seven times, then load the mixture onto the RNeasy Midi column.
6. Centrifuge the column and proceed with the protocol as specified in the RNeasy Midi kit instructions. Include the DNase treatment step as specified in the RNeasy Midi kit instructions.

   Omitting the DNase treatment step would result in an RNA preparation contaminated with genomic DNA, which may compromise accuracy of the qRT-PCR analysis.

7. Elute RNA from the RNeasy Midi column with 150 µl distilled water. Repeat elution with 50 µl distilled water. Determine RNA concentration by measuring the absorbance at 260 nm in a spectrophotometer. Also determine the ratio between the absorbance values at 260 nm and 280 nm to estimate RNA purity (a ratio value ≥1.8 is acceptable).

Prepare cDNA

8. Combine 1 µg of total RNA with 1 µl of 0.5 µg/µl oligo(dT) primer in 20 µl total reaction volume in a 96-well PCR plate. Set up one reverse transcription (RT) reaction for each animal. Use DEPC-treated water to bring final reaction volume to 20 µl.

   Oligo(dT) primer should be used for analysis of RSV gene expression and for the measurements of housekeeping gene β-actin mRNA. For the analysis of the amount of RSV genome or antigenome, use primer 5′-CAATGAACTAGGATATCAAGAC-3′ or primer 5′-GTCTTGATATCCTAGTTCATTG-3′, respectively (1 µl of 10 µM stock solution). The sequences of the last two primers correspond to the positive and negative strands within the intergenic region between SH and G genes of RSV.

9. Set up two additional RT reactions as in the previous step, using RNA from two animals sacrificed on day 4 after primary RSV infection.

   These two cDNAs will be used for the standard curve construction. Animals sacrificed 4 days after primary RSV infection are chosen as the source of RNA for standard curve because of the highest level of RSV replication is observed on that particular day in the cotton rat model.

10. Cover the plate and incubate it at 70°C for 10 min in a thermal cycler.
11. Remove the plate from the thermal cycler. Add to each well:

    10× PCR buffer for a final concentration of 1×
    50 mM $MgCl_2$ for a final concentration of 2.5 mM
    10 mM dNTP mix for a final concentration of 0.5 mM each dNTP
    DTT for a final concentration of 10 mM
    200 U/µl SR II reverse transcriptase for 200 U per reaction, final.

    Cover the plate and mix the contents of the plate by vortexing.

    To perform this step, a cocktail should be prepared for the number of required reactions plus two or three extra to account for pipetting errors.

12. Briefly centrifuge the plate at 800 × *g* to bring the solution to the bottoms of the wells, and return it to the thermal cycler for 60 min at 42°C, followed by 15 min at 70°C.
13. Upon completion of cDNA synthesis, add 80 µl of distilled water to each well.

Prepare standard curve cDNA

14. After the cDNA synthesis is complete and 80 μl of distilled water is added to each well, combine the contents of the two extra reactions that were set up using RNA from animals sacrificed day 4 post-primary-RSV infection into the same well (resulting volume, 200 μl).

    This will be the first cDNA in a dilution series of the standard curve samples.

15. Serially dilute this cDNA 1:2, 1:4, 1:8, 1:16, and 1:32 with distilled water, and use the dilutions in real-time PCR to compose a standard curve.

    Store the cDNA plate at −20°C in between uses. Multiple freeze-thaw cycles are permissible.

    Individual standard curves should be constructed for each gene analyzed.

Set up real-time PCR reactions

16. Before beginning to set up real-time PCR reactions, turn on the lamp on the Bio-Rad iCycler, as it takes time to warm up for the run.

17. Obtain an iQ 96-well PCR plate.

18. Prepare a premix by combining (per reaction):

    12.5 μl SYBR Green Supermix
    1.25 μl of each primer (from 10 μM stock) in an appropriate primer pair given in Table 26.6.1
    7 μl distilled H_2O.

    Mix enough for all animal samples to be run in duplicate and for the standard curve reactions to be run in duplicate, allowing for two to three extra reactions. Aliquot 22 μl of premix into each well of an iQ 96-well PCR plate.

    A 64-nucleotide amplicon in the β-actin transcript is amplified using primers shown in Table 26.6.1

19. Add 3 μl of cDNA to 22 μl of premix in the appropriate wells of the 96-well PCR plate.

    Use a multichannel pipettor to transfer cDNA from the cDNA plate to a plate containing PCR premix.

20. Cover the plate with Microseal 'B' adhesive seal, making sure it is firmly and uniformly attached throughout the plate.

21. Vortex the plate and briefly centrifuge at 800 × *g* to bring contents of the wells down to the bottom.

22. Place the plate into the BioRad iCycler.

23. Run the following program:

1 cycle:	3 min	95°C	(initial denaturation)
40 cycles:	10 sec	95°C	(denaturation)
	10 sec	60°C	(annealing)
	15 sec	72°C	(extension).

    There is no need to run melting curves for the primer pairs given in Table 26.6.1, as we have completed melting curves for each primer pair used in this work, and no nonspecific amplification has been noted under the conditions tested.

    If amplifying a certain gene product not specified here, program the PCR machine to add a melting curve at the end of the PCR reaction to check that particular primer pair for a potential to amplify multiple products.

Analyze real-time PCR assay results

The baseline cycles and cycle threshold (Ct) will be calculated automatically by the iQ5 software in the PCR Base Line Subtracted Curve Fit mode.

24. Plot the average Ct values for the standard curve samples against $\log_{10}$ cDNA dilution factor.

25. Use this curve to convert the average Ct values obtained for different samples to relative expression units. Normalize these relative expression units to the level of β-actin mRNA (housekeeping gene) expressed in the corresponding sample.

26. Calculate β-actin normalization values for each animal:

    a. Select the lowest β-actin relative expression unit value by comparing units obtained for different animals.

    b. Divide the β-actin relative expression units obtained for all animals by that lowest β-actin relative expression unit.

    For the animal selected in step 26a, the β-actin normalization value would equal 1.

27. Divide relative expression units obtained for genes other than β-actin by the β-actin normalization values.

28. For groups of animals, express mRNA levels as the geometric mean ±SEM for all animals in a group at a given time.

    Further normalization relative to the highest level of expression of the gene of interest (assigned relative value of 1) can be done (see Commentary).

SUPPORT PROTOCOL 1

INFECTION OF COTTON RATS WITH RSV AND HARVESTING AND PROCESSING OF LUNGS

Several animal models of RSV disease exist (Byrd and Prince, 1997). Among the ones most faithfully representing RSV disease manifestation in humans are the cotton rat, African green monkey, and the calf models of bovine RSV. The cotton rat is the preferred small animal model of RSV infection (Siber et al., 1994; Johnson et al., 1997; Maggon and Barik, 2004). Cotton rats are highly permissive for RSV infection and display histopathological features of the disease similar to that observed in humans (Prince et al., 1986, 1999). In this unit we describe a method for establishing RSV infection of cotton rats and for obtaining samples for analysis of RSV replication in the lungs. For all our experiments we use cotton rats *S.hispidus* from an inbred colony maintained at Virion Systems, Inc.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Cotton rats (*S.hispidus*, 4 to 8 weeks old; from a colony maintained at Virion Systems, Inc.)
RSV A/Long stock solution ($>10^7$ pfu/ml: original strain was obtained from ATCC (#VR26); virus was serially plaque purified to reduce defective-interfering particles)
Phosphate-buffered saline, pH 7.4 (*APPENDIX 2A*)
Liquid nitrogen
Homogenization medium (see recipe)

Isoflurane anesthesia chamber (Davis, 2008)
1-ml syringes
21-G needles
CO_2 chamber (Donovan and Brown, 2006)
Dissecting forceps
Dissecting scissors
Sterile Tenbroeck tissue homogenizers
Sterile polystyrene round-bottom tubes with caps, 12 × 75 mm
Centrifuge, refrigerated
Sterile 4-ml glass sample vials with caps

Additional reagents and equipment for isoflurane anesthesia in rodents (Davis, 2008) and euthanasia of rodents by CO_2 asphyxiation (Donovan and Brown, 2006)

Inoculate cotton rats with RSV

1. Obtain young (4- to 8-week-old) *S.hispidus* cotton rats.

   Young animals are required to reproduce dynamics of viral replication described in the Commentary. Cotton rats older than 6 months of age have delayed viral clearance from the lungs (Curtis et al., 2002; Boukhvalova et al., 2007).

2. Dilute RSV stock solution with PBS, pH 7.4. to obtain $10^{6.6}$ pfu/ml. Keep diluted virus on ice.

3. Anesthetize cotton rats in isoflurane chamber (see Davis, 2008).

   CAUTION: *Take necessary precautions when catching cotton rats and placing them into anesthesia chamber. Cotton rats behave very differently from common laboratory mice, in that cotton rats are more aggressive and very fast. Make sure to wear thick gloves when handling alert cotton rats to prevent being bitten. Quick reflexes on behalf of an investigator are a must!*

4. Draw viral solution into a 1-ml syringe with 21-G needle attached.

5. Remove the animal from the anesthesia chamber and inoculate it with 100 μl viral solution from the syringe by depositing drops of solution at the nostril openings, alternating nostrils for each consecutive drop.

   CAUTION: *Wear goggles and protective clothing.*

   Hold the animal in one hand, placing it belly-up on a palm and supporting the back of its head with four fingers. Use a thumb of that hand to push up on the chin of the animal to ensure that the mouth is closed. This will force the animal to aspirate viral solution through the nose. Animals have to be well anesthetized prior to virus inoculation. If it awakens during the inoculation process, the animal can puff the viral solution out, compromising efficiency of infection and spreading virus around the work area.

6. After infection, place the animal back into the cage, monitoring it for several minutes to ensure that the animal successfully wakes up from the anesthesia.

   If it takes longer than a few minutes for the animal to wake up, gently massage the sides of its chest cavity with one hand.

7. For secondary RSV infection studies, reinfect cotton rats 21 days later in the same manner as described in steps 2 to 6.

   When carrying an experiment with both secondary and primary RSV infection, do not infect the group of animals for primary infection studies until 21 days into the experiment. Infect the animals for secondary infection studies on days 0 and 21. Infect the animals for primary infection studies on day 21. All animals (for both secondary and primary infections studies) should be age-matched on day 0. This way, they will be age-matched at the time of sacrifice.

Sacrifice cotton rats

8. Sacrifice groups of animals via carbon dioxide asphyxiation (Donovan and Brown, 2006) at 6 hr, 12 hr, day 1, 2, 4, 7, and 14 after RSV inoculation (≥4 animals per time point).

   This will be the first RSV inoculation for the primary RSV infection group and the second RSV inoculation for the secondary RSV infection group.

9. Sacrifice one group prior to infection (0 hr) to serve as control.

Harvest lungs

10. Remove the lungs from the thorax using dissecting scissors and forceps.

11. Separate the left lung from the right lung.

    The right lung will be used for the qRT-PCR analysis and the left lung will be used for the RSV titration by plaque assay.

Allocate lungs for the RNA extraction

12. Trisect the right lung into individual lobes. Place each lobe in a separate cryotube and snap-freeze the tubes in liquid nitrogen. Transfer the tubes to dry ice and then to −80°C freezer.

    Work fast. Minimizing the time between the lung extractions and freezing the lung pieces in liquid nitrogen is crucial for ensuring a good-quality RNA preparation later on.

Process lungs for the plaque assay

13. Place the entire left lung into a sterile Tenbroeck tissue homogenizer with 10 parts (w/v) of homogenization medium. Homogenize in two to three short bursts, then place tubes on ice.

    The entire left or right lung weighs ∼0.15 g; therefore, it should be homogenized in 1.5 ml medium.

14. Following homogenization, pour samples into individually labeled sterile polystyrene round-bottom tubes with caps and centrifuge specimens 10 min at 770 × *g*, 4°C.

15. Transfer supernatants using sterile glass Pasteur pipets into 4-ml sterile glass sample vials and quick freeze aliquot specimens on dry ice. Store specimens at −80°C until assayed.

SUPPORT PROTOCOL 2

RSV INFECTION OF A549 EPITHELIAL CELLS

Respiratory epithelial cells are the primary target of RSV infection in vivo. Because of the complicated nature of in vivo experiments, RSV infection studies are often carried in vitro in epithelial cell cultures. These experiments provide important clues to the mechanism of RSV action, and also allow fast and efficient production of RSV-infected material. In this protocol we describe the method for RSV infection of alveolar epithelial cells A549, which provides a large amount of sample for RSV quantification by plaque assay and by real-time PCR. These samples can be used to fine tune techniques described in this unit in order to best adapt them to a particular laboratory setting.

Materials

Human alveolar epithelial type II cell (A549) culture (ATCC #CCL185)
A549 growth medium (see recipe)
RSV A/Long stock solution (>10^7 pfu/ml): original strain was obtained from ATCC (#VR26); virus was serially plaque purified to reduce defective-interfering particles

DMEM medium (serum-free; e.g., Invitrogen)
RNeasy Midi kit (Qiagen) including:
RLT buffer
RNeasy Midi columns
Reagents for DNase treatment
RLT/ME buffer: RLT buffer from the RNeasy Midi kit (Qiagen) supplemented with 10 μl/ml 2-mercaptoethanol
70% ethanol
PCR primers for viral genes and β-actin (Table 26.6.1)

75- to 175-cm^2 tissue culture flasks
6-well tissue culture plates

Additional reagents and equipment for basic cell culture techniques (*UNIT 1.1*), plaque assay (Basic Protocol 1), and preparation/PCR amplification of cDNA (Basic Protocol 2)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Prepare A549 cells for infection

1. Propagate A549 cells in A549 growth medium in a humidified incubator in 75- to 175-cm^2 tissue culture flasks.

   UNIT 1.1 provides protocols for basic cell culture techniques. Cells should be seeded at 1×10^5 cells/ml in 12 or 25 ml per 75- or 175-cm^2 tissue culture flask, respectively. Cells should passaged, on average, twice a week using trypsin to facilitate cell detachment from the original flask, with the fresh medium added to a new flask upon completion of splitting. A 1:3 to 1:5 ratio for splitting is recommended.

2. Dilute cells to 2×10^5 cells/ml in A549 growth medium and plate on 6-well plates at 2 ml/well (2 ml/well of a 2×10^5 cell/ml suspension). Leave the plate in the incubator for 2 days.

Infect A549 cells with RSV

3. Trypsinize the cells in one well, mix a 20-μl aliquot of cells with an equal volume of trypan blue dye solution, and count viable cells using a hemacytometer.

   Trypsinization of cells and counting viable cells by trypan blue exclusion are described in UNIT 1.1.

4. Dilute RSV in plain DMEM medium to yield various MOIs (0.001 to 0.1).

   The calculations should be based on the fact that 500 μl of viral solution per well will be used for infection.

5. Wash the cells twice with PBS, pH 7.4, by adding 2 ml PBS to each well, swirling solution around the well, and aspirating PBS.

   Washing the cells with PBS removes traces of FBS present in A549 growth medium. Residual FBS may reduce efficiency of infection.

6. Add viral solution to the wells of cells to be infected (each treatment should be done in duplicate) and place the plate into the incubator for a total of 1 hr. During that hour, rock the plate every 15 min to uniformly cover the cells with viral solution.

   A small volume of viral solution, barely covering the cell monolayer, is crucial for efficient infection.

7. Aspirate viral solution and replace it with 2 ml A549 growth medium. Return the plate to the incubator.

Collect supernatants for RSV titrations by the plaque-forming unit assay

8. Harvest cell supernatants into 1.5-ml microcentrifuge tubes at various times post-infection.

9. Microcentrifuge supernatants 5 min at 500 × *g*, 4°C, and freeze cleared supernatants at −80°C for further analysis.

Determine viral titer in the supernatants by the plaque-forming unit assay

10. Titrate supernatants as described in Basic Protocol 1 with the following modifications:

    a. Prepare 1:10, 1:100, and 1:1000 dilutions of supernatants for titration.

    b. Use the following formula to calculate viral titers:

$$\frac{\#\text{pfu well 1} + \#\text{pfu well 2}}{0.1\text{ ml total volume}} \times \text{dilution factor} = \text{pfu/ml}$$

Collect cells for RSV replication analysis by real-time PCR assay

11. Collect cells in the wells from which supernatants were harvested for viral titration by plaque assay for RNA extraction. Remove traces of medium from the wells by aspiration and add 400 µl RLT/ME buffer per well. Resuspend the cells in RLT/ME by pipetting up and down at least seven to ten times.

    Considerable attention should be given to cell pipetting in RLT/ME buffer. At least seven to ten rounds have to be performed to ensure successful RNA isolation later on. Avoid generating foam in the solution when resuspending.

    The plate can be frozen at −80°C after RLT/ME buffer is added to the wells. Thaw the plate at room temperature on the day of RNA extraction.

12. Add 400 µl 70% ethanol to each well. Mix with the contents of a well by pipetting up and down two to three times, then load the mixture onto an RNeasy Mini kit column.

13. Proceed with the RNA extraction using RNeasy Mini kit. Include the DNase treatment step as specified in the RNeasy instructions.

Analyze RSV replication in A549 cells by real-time PCR assay

14. Prepare cDNA from 1 µg total RNA as described in Basic Protocol 2.

15. Use RNA from highly-infected cells (e.g., wells infected with RSV at MOI 1 for 24 hr) to prepare cDNA for the standard curve.

16. Amplify viral genes and β-actin using primers described above (Table 26.6.1) and the PCR conditions described in Basic Protocol 2.

    Because of the high sequence similarity between human and cotton rat β-actin gene, the same set of primers is used for the β-actin amplification in A549 cells and in the cotton rat lung samples.

17. Use the standard curve to obtain relative expression units for all genes and normalize them by the β-actin mRNA expression level as described in Basic Protocol 2.

18. Express mRNA levels as the mean ±SEM for two different wells of cultures.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

A549 growth medium

Dulbecco's modified Eagle's medium (DMEM) supplemented with:
10% (v/v) fetal bovine serum (FBS)
100 IU/ml of penicillin
100 μg/ml streptomycin
2 mM L-glutamine
Store up to 2 months at 4°C

Crystal violet fixative/stain

0.067% (w/v) crystal violet
1.25% (v/v) glutaraldehyde
Store up to 1 year at room temperature

HEp-2 growth medium

EMEM with Earle's BSS, supplemented with:
10% (v/v) FBS
2 mM L-glutamine
50 μg/ml gentamicin
2.5 μg/ml Fungizone
1× penicillin/streptomycin
Store up to 2 months at 4°C

Homogenization medium

Hanks' balanced salt solution (HBSS; *APPENDIX 2A*)
0.218 M sucrose
4.4 mM glutamate
3.8 mM KH_2PO_4
7.2 mM K_2HPO_4
Store up to 2 months at 4°C

Methyl cellulose overlay medium

EMEM containing:
0.75% (w/v) methyl cellulose
1% (v/v) FBS
2 mM L-glutamine
50 μg/ml gentamicin
2.5 μg/ml Fungizone
Store up to 2 months at 4°C

COMMENTARY

Background Information

RSV is an enveloped, nonsegmented, negative-strand RNA virus that belongs to the family *Paramyxoviridae*. The RSV genome contains 10 genes in the order NS1, NS2, N, P, M, SH, G, F, M2, L from the 3′ end; these genes give rise to 11 proteins (M2 ORF encodes two proteins: M2-1 and M2-2). RSV proteins include three transmembrane surface proteins G, F, and SH; the virion matrix protein M; the nucleocapsid proteins L, N, P, M2-1, and M2-2; and the two putative nonstructural proteins NS1 and NS2. RSV genes are transcribed sequentially from the 3′ end of the genome, with promoter-proximal genes transcribed more frequently than downstream genes (Collins and Wertz, 1983). This results in a transcription gradient characteristic

also of other mononegaviruses. Upon accumulation of virally expressed proteins in infected cells, RSV begins a replicative cycle in which a positive-strand copy of genome, the antigenome, is synthesized as an intermediate. Both genomic and antigenomic RNA get packaged into completed virions, in a process dependent on ongoing viral protein synthesis (Huang and Wertz, 1982).

Respiratory syncytial virus (RSV) is a major cause of bronchiolitis and viral pneumonia in young children, and the primary viral cause of death in infants (Shay et al., 2001; Thompson et al., 2003). Bronchiolitis caused by RSV affects most infants during their first and second months of life, but reinfection (often occurring with milder symptoms or asymptomatic) is common. By the age of 2, almost all children have been infected with RSV at least once, and half of the children had been reinfected (Glezen et al., 1986). RSV infection in children is associated with recurrent wheezing later in life, pointing to a link between RSV and inflammatory-allergic disorders (Stein et al., 1999; Sigurs et al., 2000; Schauer et al., 2002). Animal studies in the cotton rat model indicate that inflammatory changes in the lung are elicited in animals immune to RSV, even in the absence of detectable virus production (Prince et al., 1986). One likely explanation for that phenomenon could be abortive viral replication triggering an inflammatory response in the lung.

Abortive replication of RSV may indicate that reinfection with RSV occurs more frequently than currently appreciated. Typically, RSV infection is diagnosed by the isolation of virus from respiratory secretions of infected individuals. These cases would represent children infected with RSV for the first time, as well as children and adults reinfected with RSV after a certain period of time sufficient for the immune protection against virus to wane. However, our results suggest that reinfections might also occur shortly after previous challenge and happen at a time when immune response is sufficiently strong to suppress productive infection, but not abortive replication of the virus. Clinically, these reinfections would be characterized by expression of viral antigens in the absence of virus production, and may go undetected if viral cultures are used to monitor disease. Notably, results of several recent human studies support the possibility of frequent "abortive" RSV infections. These studies, in which respiratory secretions were analyzed for RSV presence by viral culture and by RT-PCR, revealed that consistently more samples test positive for RSV by RT-PCR than by culture (Falsey et al., 2002, 2003; Hu et al., 2003; Perkins et al., 2005). These results may indicate that some individuals with RSV detected by RT-PCR, but not by culture, might have recently recovered from RSV and are currently undergoing reinfection accompanied by abortive replication of the virus.

Critical Parameters and Troubleshooting

The critical parameter for abortive viral replication is establishing immunity strong enough to suppress productive replication of the virus in the lung. In the cotton rat, infection of animals with less that $\sim 10^4$ pfu RSV/animal produces only partial immunity, and virus can be recovered from the lungs of animals reinfected with RSV 21 days later. In this case, abortive replication of RSV would be masked by the production and release of infectious virions in the lung. If this situation occurs after following the protocols described here, an experimenter should recheck the titer of the RSV virus stock used in the lab to ensure the stock is of the correct concentration. If the titer turns out to be lower than the expected value, the amount of viral inoculum needs to be increased in the priming and rechallenge doses accordingly.

If using an animal model other than cotton rat to monitor abortive replication of RSV, one would need to determine the amount of viral inoculum establishing sterilizing immunity (as defined by the absence of infectious virions in the lungs after re-challenge) in the lungs of that particular animal. In order to do that, animals have to be inoculated with various doses of RSV at time 0, reinfected with $\sim 10^5$ to 10^6 pfu RSV/animal 21 days later, and sacrificed at the time of peak RSV replication. The dose of RSV causing sterilizing immunity in the lung should be used in all experiments on abortive RSV replication.

The critical parameters and troubleshooting relating to Basic Protocols 1 and 2 and Support Protocols 1 and 2 are addressed in the corresponding sections.

Anticipated Results

Each primer pair given in Table 26.6.1 can be expected to successfully amplify the appropriate DNA fragment, with efficiencies between primer pairs being nearly identical (98% to 99%; Fig. 26.6.1A). This is best demonstrated when a standard curve is developed using serial 10-fold dilutions of plasmid containing entire RSV antigenome (correlation

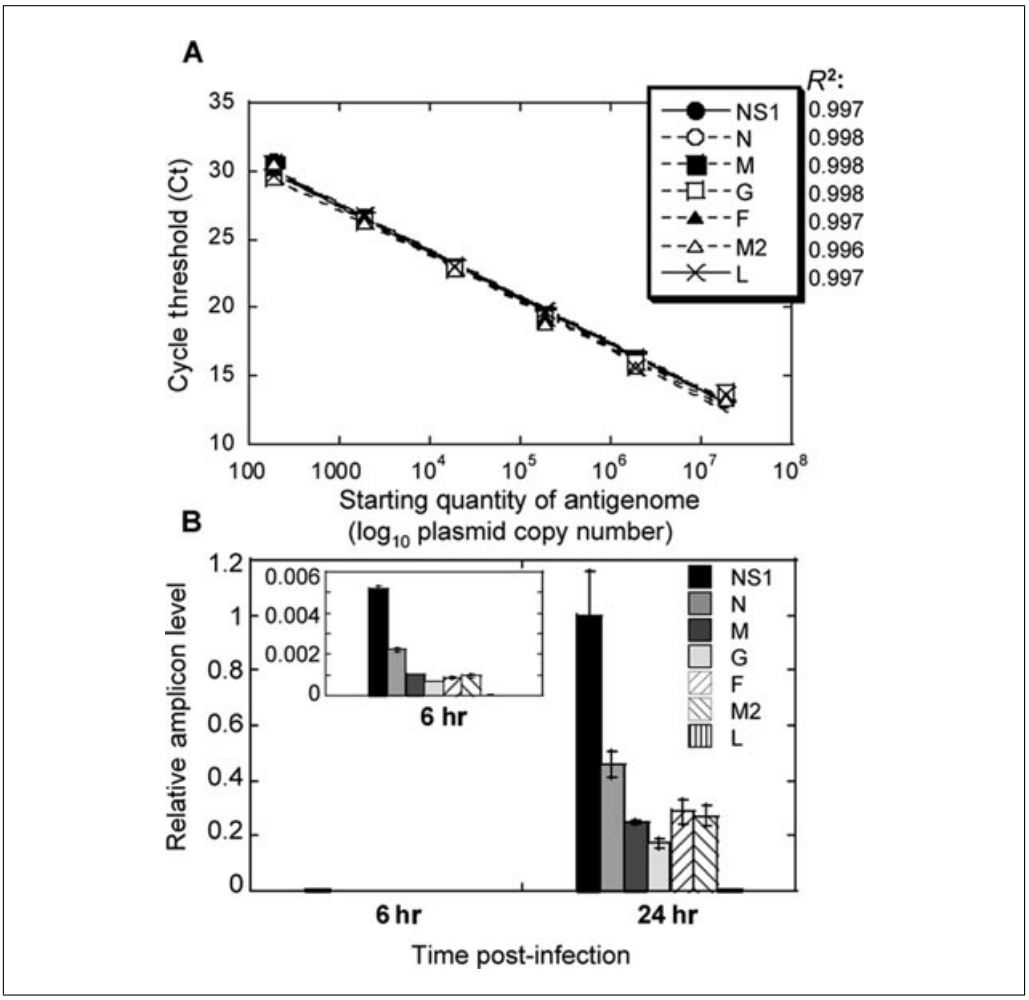


Figure 26.6.1 Establishing real-time PCR assay of RSV replication. (**A**) Amplification of various RSV genes from RSV antigenome inserted into a vector. Six 10-fold dilutions of antigenome-containing vector were used to generate a standard curve. Ct values were plotted against $\log_{10}$ quantity of antigenome used as a template. Correlation coefficients (R^2) of the best fit are shown next to the symbols corresponding to the 7 RSV genes analyzed. Each point on this graph includes symbols for all 7 genes. (**B**) Expression of RSV genes in infected A549 cells. A549 cells were infected with RSV at MOI 0.1. At time points 6 and 24 hr after infection, total RNA was extracted from infected cells and reverse transcribed using oligo(dT) primer. Expression of mRNA for each RSV gene indicated was measured by qRT-PCR. The relative level of each amplicon was normalized by β-actin mRNA level (also measured by qRT-PCR) in the corresponding sample, and expressed relative to the level of NS1 amplicon at 24 hr. The insert shows a gradient of RSV gene expression at 6 hr on an adjusted *y* -scale to highlight differences between levels of various amplicons. The results represent the mean ±SEM for two different wells per time point.

coefficients of 0.996 to 0.998). When used for analysis of RSV replication in vitro (see Support Protocol 2), the qRT-PCR assay described in Basic Protocol 2 accurately reflects viral load as estimated by the plaque assay (Basic Protocol 1). We have addressed this issue using cultures of alveolar epithelial cells A549 infected with RSV (see Support Protocol 2). As shown in Figure 26.6.1B, infection of A549 cells is slow, with transcripts of RSV genes appearing at 6 hr and accumulating considerably by 24 hr post-infection, consistent with reports of RSV infection of epithelial cells measured by other methods (Bermingham and Collins, 1999). A pronounced gradient of RSV gene expression is apparent in infected A549 cells, with amounts of transcripts diminishing dramatically in the order from NS1 to L. Reverse transcription with oligo dT primer, used in this and other similar experiments, results in a cDNA pool that contains not only reverse-transcribed copies of individual mRNAs produced during RSV transcription, but also copies of the RSV antigenome. However, because antigenome would be represented similarly in reactions

Table 26.6.2 Comparison of RSV Detection by Viral Culture and qRT-PCR in RSV-Infected Epithelial Cells

	MOI 0.001[a]	MOI 0.01	MOI 0.1
Viral yield (log_{10} pfu/ml)	2.24 ± 0.06	3.26 ± 0.20	4.77 ± 0.25
RSV gene expression level[b]	0.004 ± 0.001	0.069 ± 0.005	1 ± 0.1909
RSV genome level[c]	0.0009 ± 0.0002	0.020 ± 0.003	1 ± 0.117
RSV antigenome level[c]	0.0003 ± 0.0002	0.005 ± 0.002	0.269 0.057

[a]A549 alveolar epithelial cells were infected with RSV at MOI 0.001 to 0.1. At a point 48 hr after infection, cell supernatants were collected for quantification of viral yield by plaque assay, while cells themselves were lysed and used as a source of RNA for qRT-PCR analysis of RSV transcripts.

[b]Expression of RSV NS1 amplicon was analyzed by qRT-PCR, normalized by the expression of β-actin in the corresponding cell culture, and presented relative to the amount of NS1 amplicon in MOI 0.1–infected culture (assigned relative value of 1).

[c]Expression of RSV genome and antigenome was analyzed by qRT-PCR, normalized by the level of β-actin in the corresponding cell culture, and presented relative to the amount of genome in MOI 0.1-infected culture (assigned relative value of 1).

for different RSV genes, the differences between amplicon levels measured by RT-PCR here are attributed to differences in gene expression levels. Therefore, fluctuations in the different RSV amplicons' levels measured in oligo dT cDNA can be expressed as the gene-expression differences.

The RT-PCR assay of RSV replication described above can be expected to accurately quantify viral load in infected cells. To verify that, we inoculated A549 cells with RSV at various MOIs (0.001 to 0.1). At a time point 48 hr after infection, cell supernatants were collected for viral titrations, while the cells themselves were lysed for RNA extraction. Viral yield in supernatants of A549 cells accurately represented the level of infection (Table 26.6.2). Cells infected with the 10-fold-increasing amounts of RSV release virus in quantities that also differ by ∼10-fold. When viral RNA from these cells is analyzed by RT-PCR using primers for RSV NS1 gene, the difference in the amount of NS1 amplicon detected in A549 cells also differs by ∼10-fold between cultures infected with RSV at MOIs of 0.001, 0.01, and 0.1, respectively. The level of negative-sense (genome) and positive-sense (antigenome) RNA can also be measured in RSV-infected cells (Table 26.6.2). For that, total RNA of infected cells is reverse-transcribed using forward or reverse primer annealing to the genomic or antigenomic strand of RSV, respectively. The amount of reverse-transcribed product can then be measured by real-time PCR using primers specific for RSV amplicons G or M, proximal to the start of genome or antigenome fragment prepared, respectively. In infected A549 cells, the amount of genome and antigenome increases progressively between samples infected with increasing amounts of RSV, similar to the pattern of viral gene expression (Table 26.6.2), although less precisely reflecting differences between MOIs of infection and viral yield in infected cells. Real-time PCR analysis of extracts of cells infected with RSV indicates that RSV genome is ∼3- to 4-fold more abundant in infected cells than antigenome, consistent with reports for other paramyxoviruses, in which the antigenome constitutes only 10% to 40 % of genome in infected cells (Robinson 1970; Kolakofsky et al., 1974; Kolakofsky and Bruschi, 1975).

In vivo replication of RSV can also be accurately assessed by qRT-PCR. Infection of naïve cotton rats with RSV (primary RSV infection) results in efficient virus replication in the lungs that reaches peak on day 4 post-infection and disappears by day 7 (Fig. 26.6.2A). Genome and antigenome synthesis in primary RSV infection (Fig. 26.6.2B) increases progressively following RSV challenge, reaching its peak on day 4 post-infection and diminishing after that. This pattern of genome/antigenome production parallels the amount of infectious viral particles detected in the lungs of animals with primary RSV infection (Fig. 26.6.2A) on corresponding days. The only difference is seen for day 7, when no virus can be recovered from the lungs, and yet

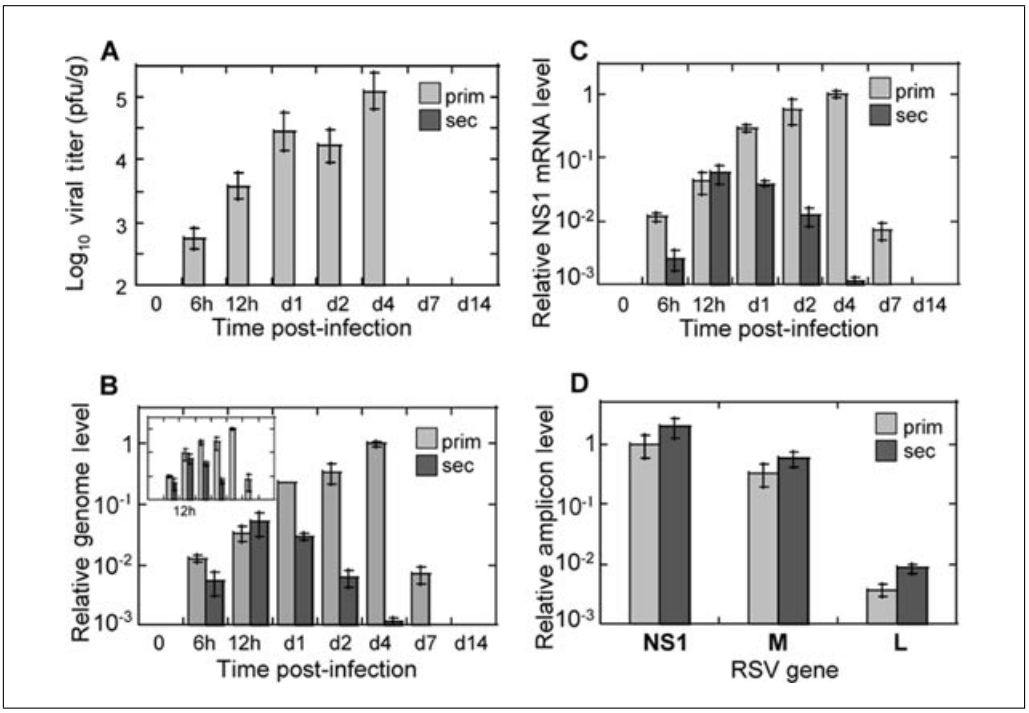


Figure 26.6.2 RSV replication in vivo: evidence of abortive replication in the reinfection model. Cotton rats were infected with RSV once for primary infection studies or twice (with an interval of 21 days) for secondary infection studies. At the indicated time points after the final challenge, lungs were collected for viral titer determination by plaque assay (**A**) or for analysis of RSV replication by qRT-PCR (**B, C, D**). (**A**) Pulmonary viral titers in cotton rats with primary RSV infection ("prim", light gray bars) and secondary RSV infection ("sec", no virus detected). Viral titers were determined by plaque assay as previously described (Prince et al., 1978; limit of detection 2 $\log_{10}$ pfu/g). (**B**) RSV genome and antigenome replication in primary and secondary RSV infection. RNA was extracted from the lungs of infected animals and reversed transcribed using primers complementary to the genomic or antigenomic strand of RSV. Relative genome (and antigenome, insert) amounts were determined by qRT-PCR, normalized by the β-actin mRNA level in the corresponding sample, and expressed relative to the level of genome (antigenome, for insert) detected in the lungs of animals with primary RSV infection on day 4. (**C**) Expression of RSV genes during primary and secondary RSV infection. RNA extracted from animals described above was reverse-transcribed using oligo (dT) primer. Expression of NS1 mRNA was measured by qRT-PCR, normalized by β-actin as described above, and expressed relative to the level of NS1 mRNA detected in the lungs of animals with primary RSV infection on day 4. (**D**) Gradient of RSV gene expression at 12 hr post-infection in primary and secondary RSV disease. NS1, M, and L amplicon levels were measured by qRT-PCR in cDNA prepared using oligo(dT) primer. Expression of each amplicon was normalized by β-actin, and expressed relative to the level of NS1 mRNA detected in the lungs of animals with primary RSV infection. The results represent the mean ±SEM for four animals per time point for each primary and secondary infection.

genomic and antigenomic strands of RSV can still be detected.

If animals inoculated with RSV once are kept for 21 days and then rechallenged with RSV (secondary RSV infection), no virus is detected in the lungs of such animals by viral culture (Fig. 26.6.2A). Viral RNA expression in secondary RSV infection, however, is strong. As Figure 26.6.2B shows, secondary RSV infection of cotton rats is accompanied by efficient synthesis of both genomic and antigenomic strands of RSV. It reaches peak at 12 hr post-infection and declines afterwards to an undetectable level by day 7. Production of genomic/antigenomic RNA is indistinguishable between primary and secondary RSV infection at 12 hr post-infection. After that, the amount of viral RNA in animals with secondary infection starts to decrease, and the differences between primary and secondary infection increase in magnitude with each subsequent time point as infection progresses. Because the scales of changes in viral titer and RSV RNA production in Figures 26.6.2A and 26.6.2B were set to be comparable (spanning 3 orders of magnitude each), the large

discrepancy in the extent of viral RNA replication and production of infectious viral particles during secondary RSV infection can be easily appreciated. For example, while the amount of infectious viral particles on day 1 in primary RSV infection is at least 288-fold higher than in secondary RSV infection (taking 2 $\log_{10}$ pfu/g detection limit into account and allowing for the possibility of the presence of some undetectable virus in the lungs of secondarily infected animals), the difference in RSV RNA level between primary and secondary infection is only 8-fold.

Expression of individual RSV genes during RSV infection in vivo can also be analyzed during primary and secondary RSV infection of cotton rats. The dynamics of RSV gene expression changes can be expected to be very similar to those of genome/antigenome variations noted above, and are exemplified by changes in the NS1 amplicon level shown in Figure 26.6.2C. NS1 gene expression in secondary RSV infection increases between 6 and 12 hr, and declines afterwards, while NS1 expression in primary infection increases progressively past the 12-hr time point, reaches a peak on day 4 post-infection, and declines afterwards. M and L amplicons follow identical patterns of time-dependent changes after RSV infection (data not shown). Similarly to the genome/antigenome changes, expression of RSV genes between primary and secondary infection is very similar at 12 hr post-infection for each particular gene analyzed. Overall, RSV re-infection of animals infected 21 days earlier results in increasing expression of viral transcripts and genome replication, but does not lead to production of detectable progeny virus. This type of replication, therefore, can be termed "abortive," as RSV is capable of entering the cells in the lungs of immune animals, yet production of progeny viruses is impaired. This is in contrast to the primary RSV infection, where accumulation of both viral genetic material and progeny viruses is detected in the lungs.

Time Considerations

Viral titrations by qRT-PCR and by plaque assay take ~1 week each, from the time of lung sample processing to the time of reading final results. Animal experiments are the most time-consuming part of the described protocols. A period of 21 days is needed between the primary and secondary RSV challenge, and an additional 2 weeks are needed for collection of samples after the final RSV inoculation.

Literature Cited

Bermingham, A. and Collins, P.L. 1999. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proc. Natl. Acad. Sci. U.S.A.* 96:11259-11264.

Boukhvalova, M.S., Yim, K.C. Kuhn, K.H., Hemming, J.P., Prince, G.A., Porter, D.D., and Blanco, J.C. 2007. Age-related differences in pulmonary cytokine response to respiratory syncytial virus infection: Modulation by anti-inflammatory and antiviral treatment. *J. Infect. Dis.* 195:511-518.

Bustin, S.A. and Mueller, R. 2005. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin. Sci. (Lond)* 109:365-379.

Byrd, L.G. and Prince, G.A. 1997. Animal models of respiratory syncytial virus infection. *Clin. Infect. Dis.* 25:1363-1368.

Collins, P.L. and Wertz, G.W. 1983. cDNA cloning and transcriptional mapping of nine polyadenylated RNAs encoded by the genome of human respiratory syncytial virus. *Proc. Natl. Acad. Sci. U.S.A.* 80:3208-3212.

Curtis, S.J., Ottolini, M.G., Porter, D.D., and Prince, G.A. 2002. Age-dependent replication of respiratory syncytial virus in the cotton rat. *Exp. Biol. Med.* 227:799-802.

Davis, J.A. 2008. Mouse and rat anesthesia and analgesia. *Curr. Protoc. Neurosci.* 42:A.4B.1-A.4B.21.

Donovan, J. and Brown, P. 2006. Euthanasia. *Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Falsey, A.R., Formica, M.A., and Walsh, E.E. 2002. Diagnosis of respiratory syncytial virus infection: Comparison of reverse transcription-PCR to viral culture and serology in adults with respiratory illness. *J. Clin. Microbiol.* 40:817-820.

Falsey, A.R., Formica, M.A., Treanor, J.J., and Walsh, E.E. 2003. Comparison of quantitative reverse transcription-PCR to viral culture for assessment of respiratory syncytial virus shedding. *J. Clin. Microbiol.* 41:4160-4165.

Glezen, W.P., Taber, L.H., Frank, A.L., and Kasel, J.A. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. *Am. J. Dis. Child.* 140:543-546.

Hu, A., Colella, M., Tam, J.S., Rappaport, R., and Cheng, S.M. 2003. Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR. *J. Clin. Microbiol.* 41:149-154.

Huang, Y.T. and Wertz, G.W. 1982. The genome of respiratory syncytial virus is a negative-stranded RNA that codes for at least seven mRNA species. *J. Virol.* 43:150-157.

Johnson, S., Oliver, C., Prince, G.A., Hemming, V.G., Pfarr, D.S., Wang, S.C., Dormitzer, M., O'Grady, J., Koenig, S., Tamura, J.K., Woods, R., Bansai, G., Couchenour, D., Tsao, E., Hall, W.C., and Young, J.F. 1997. Development

of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J. Infect. Dis.* 176:1215-1224.

Kolakofsky, D. and Bruschi, A. 1975. Antigenomes in Sendai virions and Sendai virus-infected cells. *Virology* 66:185-191.

Kolakofsky, D., Spahr, P.F., and Koprowski, H. 1974. Comparison of 6-94 virus and Sendai virus RNA by RNA-RNA hybridization. *J. Virol.* 13:935-936.

Mackay, I.M., Jacob, K.C., Woolhouse, D., Waller, K., Syrmis, M.W., Whiley, D.M., Siebert, D.J., Nissen, M., and Sloots, T.P. 2003. Molecular assays for detection of human metapneumovirus. *J. Clin. Microbiol.* 41:100-105.

Maggon, K. and Barik, S. 2004. New drugs and treatment for respiratory syncytial virus. *Rev. Med. Virol.* 14:149-168.

Niewiesk, S. and Prince, G. 2002. Diversifying animal models: The use of hispid cotton rats (*Sigmodon hispidus*) in infectious diseases. *Lab. Anim.* 36:357-372.

Openshaw, P.J. and Tregoning, J.S. 2005. Immune responses and disease enhancement during respiratory syncytial virus infection. *Clin. Microbiol. Rev.* 18:541-555.

Perkins, S.M., Webb, D.L., Torrance, S.A., El Saleeby, C., Harrison, L.M., Aitken, J.A., Patel, A., and DeVincenzo, J.P. 2005. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. *J. Clin. Microbiol.* 43:2356-2362.

Prince, G.A., Jenson, A.B., Horswood, R.L., Camargo, E., and Chanock, R.M. 1978. The pathogenesis of respiratory syncytial virus infection in cotton rats. *Am. J. Pathol.* 93:771-791.

Prince, G.A., Jenson, A.B., Hemming, V.G., Murphy, B.R., Walsh, E.E., Horswood, R.L., and Chanock, R.M. 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. *J. Virol.* 57:721-728.

Prince, G.A., Prieels, J.P., Slaoui, M., and Porter, D.D. 1999. Pulmonary lesions in primary respiratory syncytial virus infection, reinfection, and vaccine-enhanced disease in the cotton rat (*Sigmodon hispidus*). *Lab. Invest.* 79:1385-1392.

Robinson, W.S. 1970. Self-annealing of subgroup 2 myxovirus RNAs. *Nature* 225:944-945.

Schauer, U., Hoffjan, S., Bittscheidt, J., Kochling, A., Hemmis, S., Bongartz, S., and Stephan, V. 2002. RSV bronchiolitis and risk of wheeze and allergic sensitisation in the first year of life. *Eur. Respir. J.* 20:1277-1283.

Shay, D.K., Holman, R.C., Roosevelt, G.E., Clarke, M.J., and Anderson, L.J. 2001. Bronchiolitis-associated mortality and estimates of respiratory syncytial virus-associated deaths among US children, 1979-1997. *J. Infect. Dis.* 183:16-22.

Siber, G.R., Leombruno, D., Leszczynski, J., McIver, J., Bodkin, D., Gonin, R., Thompson, C.M., Walsh, E.E., Piedra, P.A., and Hemming, V.G. 1994. Comparison of antibody concentrations and protective activity of respiratory syncytial virus immune globulin and conventional immune globulin. *J. Infect. Dis.* 169:1368-1373.

Sigurs, N., Bjarnason, R., Sigurburgsson, F., and Kjellman, B. 2000. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am. J. Respir. Crit. Care Med.* 161:1501-1507.

Stein, R.T., Sherrill, D., Morgan, W.J., Holberg, C.J., Halonen, M., Taussig, L.M., Wright, A.L., and Matrinez, F.D. 1999. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 354:541-545.

Thompson, W.W., Shay, D.K., Weintraub, E., Brammer, L., Cox, N., Anderson, L.J., and Fukuda, K. 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. *J. Am. Med. Assoc.* 289:179-186.

van Kraaij, M.G., van Elden, L.J., van Loon, A.M., Hendriksen, K.A., Latenveer, L., Dekker, A.W., and Nijhuis, M. 2005. Frequent detection of respiratory viruses in adult recipients of stem cell transplants with the use of real-time polymerase chain reaction, compared with viral culture. *Clin. Infect. Dis.* 40:662-669.

CHAPTER 27
RNA-Based Methods in Cell Biology

INTRODUCTION

The initial demonstration in *C. elegans* that double-stranded RNAs (dsRNAs) can direct the degradation of messenger RNAs (mRNAs) that share their sequence has touched off a revolution in many fields of biology, including cell biology. It would be difficult to exaggerate either the speed or the significance with which these changes have occurred. It is now understood that virtually all animals and plants utilize small RNA molecules to control protein expression, to regulate the structure of their genomes, and to respond to viral infection. This chapter will include applications through which our emergent understanding of non-coding RNAs may be utilized by cell biologists.

The capacity of short dsRNAs to silence gene expression in mammalian cells was quickly exploited for the development of reverse-genetic methods in cultured mammalian cells. Among the first applications of this kind was the use of small interfering RNAs (siRNAs) to obtain potent, long-lasting post-transcriptional silencing of chosen genes through the active degradation of their encoded mRNAs (*UNIT 27.1*). In this application, chemically synthesized oligonucleotides with 21- to 25-nucleotide RNA duplex regions and 2-nucleotide 3′overhangs are directly transfected into cells. One strand of this duplex region is complementary to the target mRNA of interest. After incorporation into a multiprotein complex, called the RNA-induced silencing complex (RISC), this complementary sequence guides the endonucleolytic cleavage of targeted mRNAs. The length of RNA duplexes is largely determined by the fact that longer RNA duplexes trigger sequence-nonspecific attenuation of gene expression. siRNAs of this size are sufficient to provide a high degree of specificity, since even a few mismatches within the duplex can prevent target mRNA recognition. Nevertheless, the efficiency of knockdown should be monitored carefully because it can vary between different siRNAs targeting the same mRNA, and a series of controls must be utilized to ensure that observed phenotypes do not result from the unintended destabilization of other mRNAs.

As this chapter develops, there will be enormous scope for discussion of alternative methods for RNA interference through different methods of dsRNA production, as well as for the use of RNA-based methods in other cell biological contexts. Finally, studies of how different small RNAs are produced and of the processes through which they regulate physiological events are burgeoning fields of cell biology, producing important and fundamental physiological insights at a breathtaking rate.

Mary Dasso

Current Protocols in Cell Biology 27.0.1, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2700s47

Silencing of Gene Expression in Cultured Cells Using Small Interfering RNAs

Kumi Sakurai,[1] Pritsana Chomchan,[1] and John J. Rossi[1]

[1]Beckman Research Institute of City of Hope, Duarte, California

ABSTRACT

The discovery of RNA interference (RNAi) and related small RNA–mediated regulatory pathways has significantly altered the understanding of gene regulation in eukaryotic cells. In the RNAi pathway, small interfering RNAs (siRNAs) ~21 to 23 nucleotides in length serve as the regulatory molecules that guide and induce sequence-specific gene silencing. The use of siRNA-mediated silencing as a tool for investigating gene function is well established in cultured mammalian cells. This unit provides basic approaches to explore the field of RNAi, and hopes to address the importance of optimizing transfection conditions after empirical determinations in order to understand various degrees of silencing efficiency. *Curr. Protoc. Cell Biol.* 47:27.1.1-27.1.28. © 2010 by John Wiley & Sons, Inc.

Keywords: RNAi • small silencing RNAs • gene silencing • transfection • mammalian cell culture

INTRODUCTION

Since the first demonstration of RNA interference in cultured mammalian cells in 2001 (Elbashir et al., 2001a), small interfering RNA (siRNA)–mediated sequence-specific gene silencing has been widely used as a tool for investigating gene function, and has initiated a new wave of reverse genetics in mammalian systems. The siRNA-mediated silencing provides fast, target-specific down-regulation of gene expression. The extent to which one can successfully carry out the silencing for determination of gene function depends on the potency of siRNAs, transfection conditions, duration of analysis, and screening methods. This unit describes design of siRNAs, transfection of siRNAs into cultured cells, and testing and validation of gene knockdown.

The silencing efficiency is based on degree of susceptibility of target transcripts to siRNA-mediated RNA-induced silencing complex (RISC) activity. It is important to design multiple siRNAs and screen for siRNAs that are highly potent in silencing a given gene. There are several online siRNA design programs available, providing predicted siRNA sequences and opportunities to explore and compare the siRNA sequences generated by different design methods. Once several siRNA sequences with high prediction scores are obtained (Basic Protocol 1), one should carefully design and evaluate siRNA transfection conditions (Basic Protocol 2).

Transfection of mammalian cells typically utilizes reagents that enhance the uptake of nucleic acids (e.g., plasmid DNA or siRNAs) by cultured cells. Transfection methods commonly used are: (1) cationic liposomes—a complex of the genetic materials and/or siRNAs with a cationic lipid—inducing fusion with the cell plasma membrane; (2) electroporation via destabilization of the cell plasma membrane; and (3) calcium phosphate/nucleic acid precipitate uptake. In this unit, we describe the use of liposomes to deliver siRNAs to cultured mammalian cells for silencing gene expression.

Current Protocols in Cell Biology 27.1.1-27.1.28, June 2010
Published online June 2010 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb2701s47

As previously mentioned, it is desirable to test several siRNAs against a given gene and screen for efficacy. Target-site screening is tricky, but necessary to validate and observe outcomes that accurately reflect the effect of siRNAs. Currently, screening heavily relies on experimental observations such as monitoring phenotypic changes, detection of changes in reporter protein expression (e.g., fluorescence for GFP-target fusion proteins), RT-PCR (Support Protocol 1), or western blot analysis (Support Protocol 2). As fundamental processes for the validation of the siRNA-mediated silencing, we describe RNA isolation and protein collection following transfections. In addition, a dual-reporter system can be used for rapid and reliable screening for siRNA sequences (Support Protocol 3). The psiCHECK-2 vector system (Promega) incorporates expression of a fluorescent reporter protein (e.g., *Renilla* luciferase) and a control fluorescent protein (e.g., firefly luciferase) from a single vector, enabling one to evaluate the efficiency of siRNAs by comparing expression levels of reporter proteins. Support Protocols 4 and 5 are intended to supplement Basic Protocol 1 when a siRNA is obtained in single-stranded format.

STRATEGIC PLANNING

siRNA Design Algorithms

The sequence of an siRNA can be by far the most important determinant of silencing efficiency. The identification and selection of highly potent siRNA sequences against a given gene prior to any experimental determinations can be overwhelming. To facilitate the designing process, there are several databases that archive siRNA sequences tested in mammalian RNAi experiments (Chalk et al., 2005; Shah et al., 2007; Ren et al., 2009). Validated siRNAs can also be found in commercial resources such as the Stealth RNAi siRNAs from Invitrogen, the *Silencer* validated siRNA from Ambion, and HP validated siRNAs from Qiagen. These vendors and Dharmacon also provide predesigned siRNAs and custom design services, making siRNA designing easier and providing a good starting point. However, many, if not most, siRNA-mediated RNAi assays require experimental validation for optimization of conditions to meet specific aims.

NOTE: Throughout the protocols, RNA-handling techniques (*APPENDIX 2A*) should be practiced and aseptic cell culture conditions should be maintained to avoid accidental introduction of nucleases, cross-contamination of samples, and potential loss of cell culture.

BASIC PROTOCOL 1

DESIGN OF 21-NUCLEOTIDE-LONG siRNA (21-MER)

If no matches are found for a given gene of interest in the literature and available siRNA databases, siRNAs can be designed using Web-based siRNA design programs. There are several siRNA selection algorithms to help predict siRNA sequences for effective silencing. Users need to provide the target messenger RNA sequence and are able to create some user-defined design criteria such as thermodynamic stability and AU/GC base pair contents that will influence the overall efficiency of predicted siRNAs. Each algorithm has a unique emphasis on design features, but fundamentally most of the algorithms provide siRNA sequences with scores or ranks for users to further analyze for specificity and select candidate siRNAs that meet their needs. Some algorithms are specialized in determining off-target effects of predicted siRNA sequences (Chalk and Sonnhammer, 2008; Gong et al., 2008) to reduce unwanted side effects. Once selected, siRNAs can be synthesized in-house or obtained from several siRNA-licensed vendors such as Ambion, Dharmacon, and Qiagen.

While many of the siRNA design algorithms provide siRNAs in the form of 19 base pairs with 2 nucleotide overhangs at the 3′ end on each strand, siRNAs can be generated by processing longer double-stranded RNAs (dsRNAs) by the RNase III Dicer enzyme.

One may find the use of such dsRNAs for target gene silencing advantageous, since transfecting 25- to 27-nt siRNAs showed improved silencing efficiency (Rose et al., 2005). Hannon and colleagues also reported similar observations with short hairpin RNAs with 29-bp stems (Siolas et al., 2005). These observations together with others suggest that the Dicer processing of its substrates results in better programming of RISC (Elbashir et al., 2001c; Liu et al., 2003; Gregory et al., 2005).

While the 21-nt siRNAs often show a wide range of potency (Amarzguioui et al., 2003; Reynolds et al., 2004; Ui-Tei et al., 2004), transforming a 21-nt siRNA to a 27-nt Dicer substrate siRNA may improve the silencing efficiency. Furthermore, the structural modification that mimic secondary structure of existing Dicer substrates (e.g., pre-microRNAs) will introduce the defined polarity of Dicer processing, leading to preferential incorporation of the desired guide strand (Rose et al., 2005). Alternatively, D-siRNA sequences can be selected using an algorithm that provides fully automated D-siRNA designs using the mRNA target sequence (RNAi Design; Owczarzy et al., 2008; *http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx*).

Materials

Gene of interest
Web-based siRNA design program (e.g., Table 27.1.1)
Web-based specificity program (e.g., SpecificityServer; Table 27.1.1)

Design siRNA

1. Obtain the most updated mRNA sequence of the gene of interest from the UCSC Genome Browser (*http://genome.ucsc.edu/cgi-bin/hgGateway*).

2. Use an online siRNA design program to enter the mRNA sequence. To increase the chances of designing highly potent siRNAs, include the 5′ and 3′ untranslated regions (UTRs) in the inquiry as well. Follow the Web site instructions to further define the siRNAs.

   Examples of Web-based siRNA design tools are listed in Table 27.1.1.

3. Analyze the target sequence of the candidate siRNAs for specificity using available Web servers (e.g., SpecificityServer). Utilize several online siRNA design tools to compare and select up to five candidate siRNAs with high prediction scores.

Design Dicer substrate siRNA (D-siRNA)

4. Obtain a 21-nt siRNA sequence using the Web-based siRNA design tools.

   An example of a sequence is shown in step 5.

Table 27.1.1 List of Web-Based siRNA Design Tools

Tools	URLs
siRNA Target Finder	*http://www.ambion.com/techlib/misc/siRNA_finder.html/*
BLOCK-IT RNAi Designer	*http://rnaidesigner.invitrogen.com/rnaiexpress/*
GeneAssist siRNA Workflow Builder	*http://www5.appliedbiosystems.com/tools/sirna/*
si*DESIGN* Center	*http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx*
siRNA Target Designer	*http://www.promega.com/siRNADesigner/*
SpecificityServer	*http://informatics-eskitis.griffith.edu.au/SpecificityServer/*

5. Extend the sequence of the sense strand to 25 nt and antisense strand to 27 nt, making one end of the duplex blunt-ended with 2 deoxy 3′ terminal end to block the Dicer entry.

   An example of a target sequence and siRNA are shown below.

   Target
   5′-AAGCUG**ACCCUGAAGUUCAUCUGCACC**ACCGGC-3′
   siRNA
   Sense: **ACCCUGAAGUUCAUCUGCACC**
   Antisense: ACUGGGACUUCAAGUAGACGU
   D-siRNA
   Sense: ACCCUGAAGUUCAUCUGCACCACCG
   Antisense: ACUGGGACUUCAAGUAGACGUGGUGGC

6. Exchange the last two ribonucleotides of the sense strand to corresponding deoxyribonucleotides.

   For instance, change the ribonucleotides to dCdG (shown in lowercase below) if the last two sequences are CG.

   Sense: **ACCCUGAAGUUCAUCUGCACC**ACcg
   Antisense: ACUGGGACUUCAAGUAGACGUGGUGGC

7. Once selected, synthesize siRNAs in-house or obtain from several siRNA-licensed vendors such as Ambion, Dharmacon, and Qiagen.

   When an siRNA is obtained in single-stranded format, the sense and antisense strands need to be annealed, and the integrity and quality of annealed double-stranded RNA need to be determined. These procedures are described in Support Protocols 4 and 5.

BASIC PROTOCOL 2

DETERMINING OPTIMAL TRANSFECTION CONDITIONS

In order to set up experiments with the designed siRNAs, several experimental conditions need to be optimized. First, it is important to determine the optimal cell density at the time of transfection. Transfection efficiency varies based on the cell density and concentrations of siRNAs, in addition to variations in transfection reagents used in the reaction. Second, optimal siRNA-lipid concentrations should be determined while screening for potent siRNAs to verify the most effective siRNA duplexes and the lowest concentration that results in efficient silencing of target gene without toxicity. Finally, it is recommended to empirically determine which reagent works best for your cell line. A variety of proprietary transfection reagents are available, and the efficiency of siRNA delivery to a particular cell line will slightly differ among the reagents. In this protocol, we describe a transfection procedure using Lipofectamine 2000 reagent (Invitrogen), which showed high transfection efficiency with commonly used mammalian cell lines (e.g., HEK293, HeLa, and HCT116 cells).

Procedures described here are for adherent cells grown in monolayer. For suspension cells, the same procedures will apply, except that detaching cells from the surface of culture dishes is not necessary. Otherwise, deviations in the procedures will be noted accordingly.

For successful gene silencing by transfecting mammalian cells with siRNAs, it is strongly recommended to determine the optimal cell density of your cell line, and optimal siRNA concentrations, before your experiments. To find an optimal cell density, perform transfections with at least two different levels of confluency. In accordance with the recommended

Table 27.1.2 Recommended Percent Confluence for Transfection

Cell types	Source	Recommended % confluence[a,b]
HEK293	Human embryonic kidney	90%
HeLa	Human cervical carcinoma	70%-80%
HCT116	Human colon carcinoma	50%
A549	Human lung carcinoma	60%
D3	Mouse embryonic stem cells	Transfect cells at the time of plating
3T3L1	Mouse fibroblast	60%-70%

[a]The listed cell densities are for 16 to 24 hr incubation. Adjust the seeding cell density so that cells reach confluency at the end of incubation if a longer interval between transfection and harvesting cells is necessary.
[b]Information also found at Invitrogen Web site *http://www.invitrogen.com*.

Table 27.1.3 Reagent Mixes for Transfection with Six Different Concentrations of siRNAs (12-well plates)

siRNA final conc. (nM)[a]	siRNA per well (ng)[b]	Stuffer DNA plasmid (ng)[c]	Total nucleic acid amount (μg)
0	0 (μg)	1100	1.1
1.0	1 pmol (13.37 ng)	1087	1.1
5.0	5 pmol (66.85 ng)	1033	1.1
25	25 pmol (334.3 ng)	765.7	1.1
50	50 pmol (668.5 ng)	431.5	1.1
75	75 pmol (1003 ng)	97.21	1.1

[a]siRNA concentrations were calculated for the final volume of 1 ml per well of a 12-well plate.
[b]siRNA (ng) was calculated with a dsRNA, mol. wt. = 1.34×10^4 g/mol.
[c]Any cloning vectors not containing eukaryotic promoters (e.g., pBluescript, pCR2.1, etc) can be used to adjust the total amount of nucleic acid.

percent confluence of adherent cells shown in Table 27.1.2, seed the cells in multiple-well plates at a minimum of two different densities 1 day prior to transfection.

In this protocol, 75% seeding density is used to plate four 12-well plates to perform a transfection with two siRNAs, an irrelevant siRNA as a negative control, and a Cy3-labeled siRNA as a positive control, at six different concentrations for an assay period of 24 hr. It is strongly recommended to perform another round of transfection with adjusted cell density to optimize the transfection conditions for your experiment. Cells with shorter doubling times should be seeded at a lower density so that cells reach confluency at the end of the assay period (e.g. HCT116, ∼16 hr; HEK293, ∼22 to 24 hr; HeLa, ∼ 24 hr). Within a well of a 12-well plate, you should be able to collect sufficient amounts of total RNA and proteins for your silencing analyses (Support Protocols 1 to 3). If the target gene expression is relatively low, consider plating in 6-well plates instead.

When you are analyzing gene function by knocking down gene expression with siRNA transfection, it is strongly recommended to verify the highly potent siRNA duplexes among siRNAs that you designed, and to identify the lowest concentration that results in efficient silencing of the target gene and is least toxic to cell culture. Procedures described below refer to transfection of cells seeded on four 12-well plates to determine optimal cell density for transfection with two siRNAs against the same target transcript. When more than two siRNAs are designed, adjust the number of plates to have enough wells for experimental determinations. To find optimal siRNA concentrations, one should test at least six different concentrations (e.g., 0, 1, 5, 25, 50, and 75 nM) in duplicate for

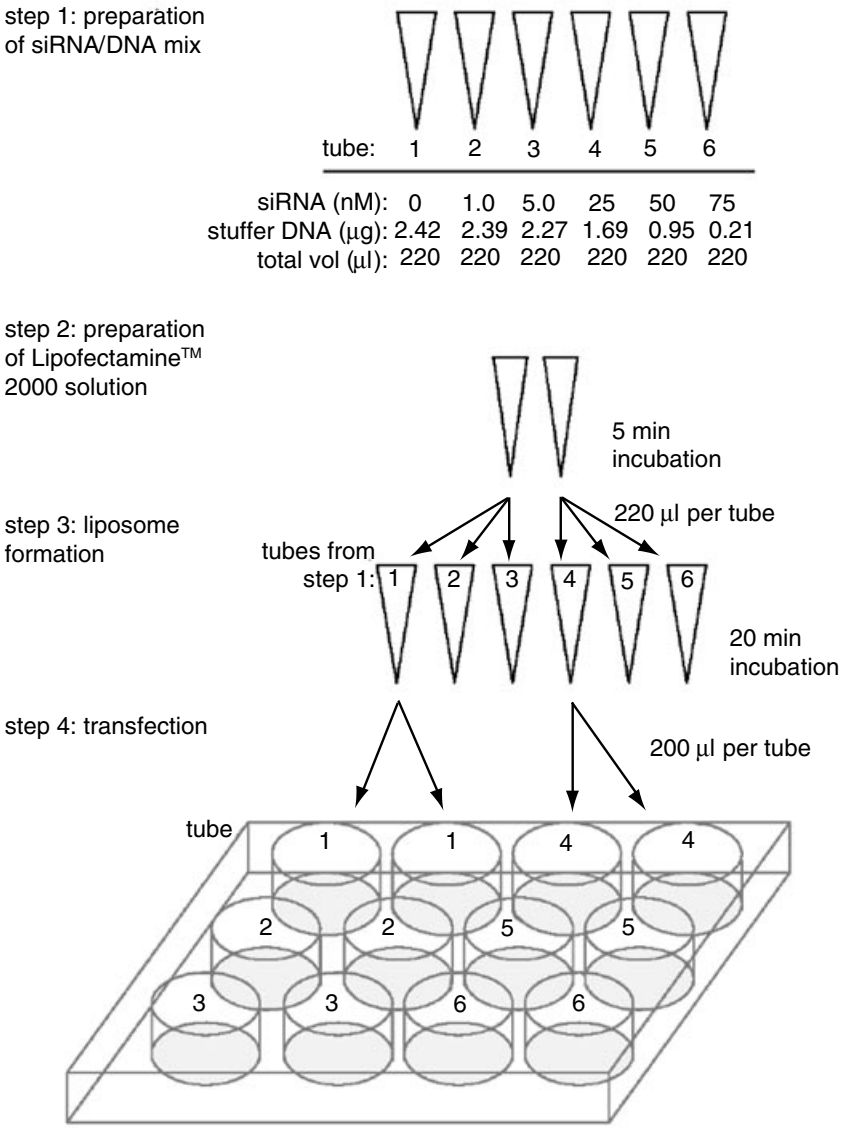


Figure 27.1.1 Transfection of mammalian cells with siRNAs. Stepwise description of transfection of mammalian cells seeded onto a 12-well plate a day prior to transfection is shown above. After choosing five different concentrations of siRNAs to transfect the cells, mix siRNA and stuffer DNA in the Opti-MEM (or a serum-free medium) to bring the volume to 220 μl (step 1). Next, prepare the proper amount of Lipofectamine 2000 solution (two tubes of 720 μl for six tubes of siRNA/DNA mixes, step 2). After 5 min incubation, add 220 μl of Lipofectamine 2000 solution to each siRNA/DNA mix (step 3). Incubate for 20 min to let liposome formation, then add 200 μl of the liposome solution to transfect the cells (step 4). Transfection reagent preparations should be done in a tissue culture hood.

each siRNA as a starting point. Also, the use of a fluorescent dye–labeled siRNA as a transfection control (e.g., FITC, Cy3) will help visualize the transfection efficiency at different concentrations of an siRNA. See below for reagents contained in each transfection reaction (Table 27.1.3). A brief summary of the procedure is also shown in Figure 27.1.1.

NOTE: It is desirable to use early-passage-number cells if available. The effects vary among cell lines; however, passage number of the cells at the time of transfection may have impact on the efficiency of liposome uptake and may result in cellular toxicity.

NOTE: In the case of transfection with suspension cells, adjust the cell numbers accordingly.

NOTE: Transfection reagent preparations should be done in a tissue culture hood.

NOTE: The use of stuffer DNA plasmid will adjust the final amounts of nucleic acid to be consistent throughout the experiment; hence, maintaining the volume of Lipofectamine 2000 used in the reaction. We have often observed inconsistency in knockdown levels of target genes that result from varying the volume of cationic lipid solution for delivery of different concentrations of an siRNA. Hence, maintaining the volume of Lipofectamine 2000 throughout the experiment will not only prevent variation in transfection efficiencies among the six siRNA concentrations, but also will rectify cellular toxicity, which may be caused by the different amounts of cationic lipid used in the reaction. Any cloning vectors not containing eukaryotic promoters, such as pBluescript and pCR2.1, can be used as a stuffer DNA plasmid to adjust the total amount of nucleic acid.

Materials

Mammalian cell line cultured in the appropriate growth medium in 10-cm^2 dishes
Calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS; Cellgro, cat. no. 21-031 CV, or see *APPENDIX 2A*), 37°C
1× trypsin/EDTA solution (e.g., Invitrogen; also see *UNIT 1.1*), 37°C
Complete DMEM with 10% (v/v) FBS (see recipe), 37°C
Complete DMEM with 10% (v/v) FBS (see recipe), without antibiotics, 37°C
Trypan blue staining solution (*UNIT 1.1*)
10 μM siRNA working solution (Basic Protocol 1)
10 μM irrelevant siRNA as a negative control
10 μM fluorescent dye (e.g., Cy3 or FITC)–labeled siRNA as a transfection control
250 ng/μl stuffer DNA plasmid (e.g., pBluescript, pCR2.1; Clontech, Invitrogen)
Opti-MEM I (a reduced-serum medium from Invitrogen), or serum-free growth medium
Lipofectamine 2000 (Invitrogen)

Microscope
Hemacytometer with coverslip (Figure 1.1.1)
12-well tissue culture plates
Standard microscope and UV lamp or fluorescence microscope

Additional reagents and equipment for basic cell culture techniques including trypsinization and counting viable cells on a hemacytometer by trypan blue exclusion (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Seed cultured cells 1 day prior to transfection

1. For adherent cells grown at subconfluency on 10-cm^2 dishes, rinse with 5 ml 1 CFM-DPBS and add 2 ml of 1× trypsin/EDTA solution per dish to detach cells from surface.

   This will be enough to cover the monolayer culture.

   In order to seed four 12-well plates at 75% cell confluency, prepare two 10-cm^2 dishes of cells at ~80% confluency.

   UNIT 1.1 includes protocols for basic cell culture techniques including trypsinization.

2. After 2 min incubation at 37°C, gently tap bottom of dish to dislodge cells. Also check culture under a microscope to see that cells are detached from the surface.

   The cells should look rounded up.

   For cells grown in suspension, skip steps 1 and 2. First centrifuge the cell culture 5 min at 200× g, room temperature, aspirate medium, and follow steps 3 to 9.

3. Gently resuspend cells by adding ~5 ml of complete DMEM/10% FBS and pipetting up and down.

   The addition of the serum-containing medium will inhibit further trypsin digestion.

4. Transfer the cell suspension to a sterile 50-ml centrifuge tube, and centrifuge for 3 min at 200 × *g* room temperature.

5. Aspirate medium and resuspend the cells with 10 ml complete DMEM/10% FBS without antibiotics, so that maximum cell count will be ~50 cells per 1 mm^2 .

   If cells on a 10-cm dish are below 80% confluency, lower the volume of medium to adjust the maximum cell count to ~50 cells per 1 mm^2.

6. Add 10 µl of trypan blue staining solution and a 10-µl aliquot of cell suspension to a 1.5-ml microcentrifuge tube. Mix thoroughly and dispense 10 µl of the mix gently to edge of hemacytometer counting chamber by placing the tip of pipet under the coverslip.

   UNIT 1.1 includes a protocol for viable cell counting using trypan blue and a hemacytometer.

7. Count cells under a microscope and calculate cell numbers as follows (also see *UNIT 1.1*):

   Cell numbers (cells/ml) = average count per square × 2 × 10^4
   Total cells = cell numbers (cells/ml) × volume of medium used to dilute cells
   where 2 is the dilution factor, and 10^4 is the volume correction factor.

8. Determine cell viability by counting total number of viable (unstained) cells.

   % viable cells = [number of unstained cells/total number of cells] × 100

   To plate cells at 75% seeding density on four 12-well plates, you will need a total of ~1.5 × 10^7 viable cells.

9. Plate cells at a seeding density of 3.0 × 10^5 cells (initial 75% confluence) in 0.8 ml medium into wells of four 12-well plates. Make sure to distribute cells uniformly across the well to ensure even uptake of liposomes.

   On the day of transfection (i.e., the next day), cells with slow doubling time will reach ~85% to 90% confluency. After the transfection efficiency is determined, scale up or down the seeding density and perform another round of transfection.

   For different cell lines, the numbers of cells at seeding should be adjusted so that they will reach confluency at harvesting. For instance, seed 4.0 × 10^5 HEK293 cells to obtain 90% confluency for a 24-hr assay period. Also refer to Table 27.1.2 for the recommended % confluency at transfection for different cell lines so that the optimal seeding density can be empirically determined.

10. Incubate the cells at 37°C, 5% CO_2 for subsequent transfection.

    Do not add antibiotics to the medium during transfection. If complete DMEM/10% FBS is used to seed cells, exchange the medium to complete DMEM/10% FBS without antibiotics once the cells are attached to wells, or at the latest 4 to 6 hr prior to transfection.

Prepare siRNA/DNA mixes

11. Prepare 40 µl of 1 µM siRNA by diluting 4 µl of the 10 µM siRNA working solution in 36 µl of Opti-MEM I (or serum-free medium) so that there are two different concentrations for a given siRNA. Using the same technique, prepare 1 µM solutions for three other siRNAs—the second siRNA to be tested, one Cy3-labeled siRNA, and one irrelevant control siRNA.

    It is recommended that you use serum-free medium to dilute siRNAs, to prevent potential reduction in transfection efficiency.

    It is not necessary to perform annealing (Support Protocol 4 and 5) if the siRNA is obtained in the double-stranded form. If the siRNA is purchased based on the match in the literature and available siRNA databases, it will be still ideal to determine the transfection efficiency (Support Protocols 1 to 3) to evaluate technical skills/conditions.

    One can interpret an irrelevant siRNA control as an siRNA whose sequence cannot be found in the system that the investigator tests with. An irrelevant/control siRNA can be purchased from several siRNA-licensed vendors such as Ambion, Dharmacon, and Qiagen. The sequences of such control siRNAs may not be available, for proprietary reasons.

    For the fluorescently labeled siRNA, in an ideal experimental setting, one should obtain the same siRNA that was designed in Basic Protocol 1, but with a fluorescent label. This can be done by custom ordering from the vendor. However, synthesizing the fluorescently labeled siRNA for each siRNA designed in Basic Protocol 1 is not cost-effective. Instead, one can purchase a fluorescently labeled control siRNA from vendors such as Invitrogen.

12. Prepare six 1.7-µl microcentrifuge tubes with proper labeling (0, 1, 5, 25, 50, 75 nM) for each siRNA, add each reagent as described in Table 27.1.3, and bring the final volume to 100 µl per tube with Opti-MEM I. Mix gently.

    It is strongly recommended to perform transfection in duplicate.

    From this point forward, volumes of reagents are calculated for duplicate reactions and shown on the right-hand side of Table 27.1.4 and Figure 27.1.1.

Table 27.1.4 Reagent Mixes for 0, 1, 5, 25, 50, and 75 nM of siRNA (12-well plates)

		Per well				For 2.2x			
		siRNA per well[b]				siRNA per well			
Tubes	siRNA final conc. (nM)[a]	1 µM (µl)	10 µM (µl)	Stuffer DNA plasmid (ng)[c]	Total volume per well (µl)	1 µM (µl)	10 µM (µl)	Stuffer DNA plasmid (ng)	Total volume per well (µl)[d]
1	0	–	–	1100	100	–	–	2420	220
2	1.0	2.0	–	1087	100	4.4	–	2391	220
3	5.0	10.0	–	1033	100	22.0	–	2273	220
4	25	–	5.0	765.7	100	–	11.0	1685	220
5	50	–	10.0	431.5	100	–	22.0	949.3	220
6	75	–	15.0	97.2	100	–	33.0	213.8	220

[a] siRNA concentrations were calculated for the total volume of 1 ml per well of a 12-well plate.

[b] siRNA (ng) was calculated with a dsRNA, mol. wt. = 1.34×10^4 g/mol.

[c] Any cloning vectors not containing eukaryotic promoters (e.g., pBluescript, pCR2.1, etc) can be used to adjust the total amount of nucleic acid.

[d] The 220 µl of siRNA/DNA mixes contains a 0.2× volume excess to the actual volume for a well. (220 µl = 100 µl × 1.2 × 2 wells).

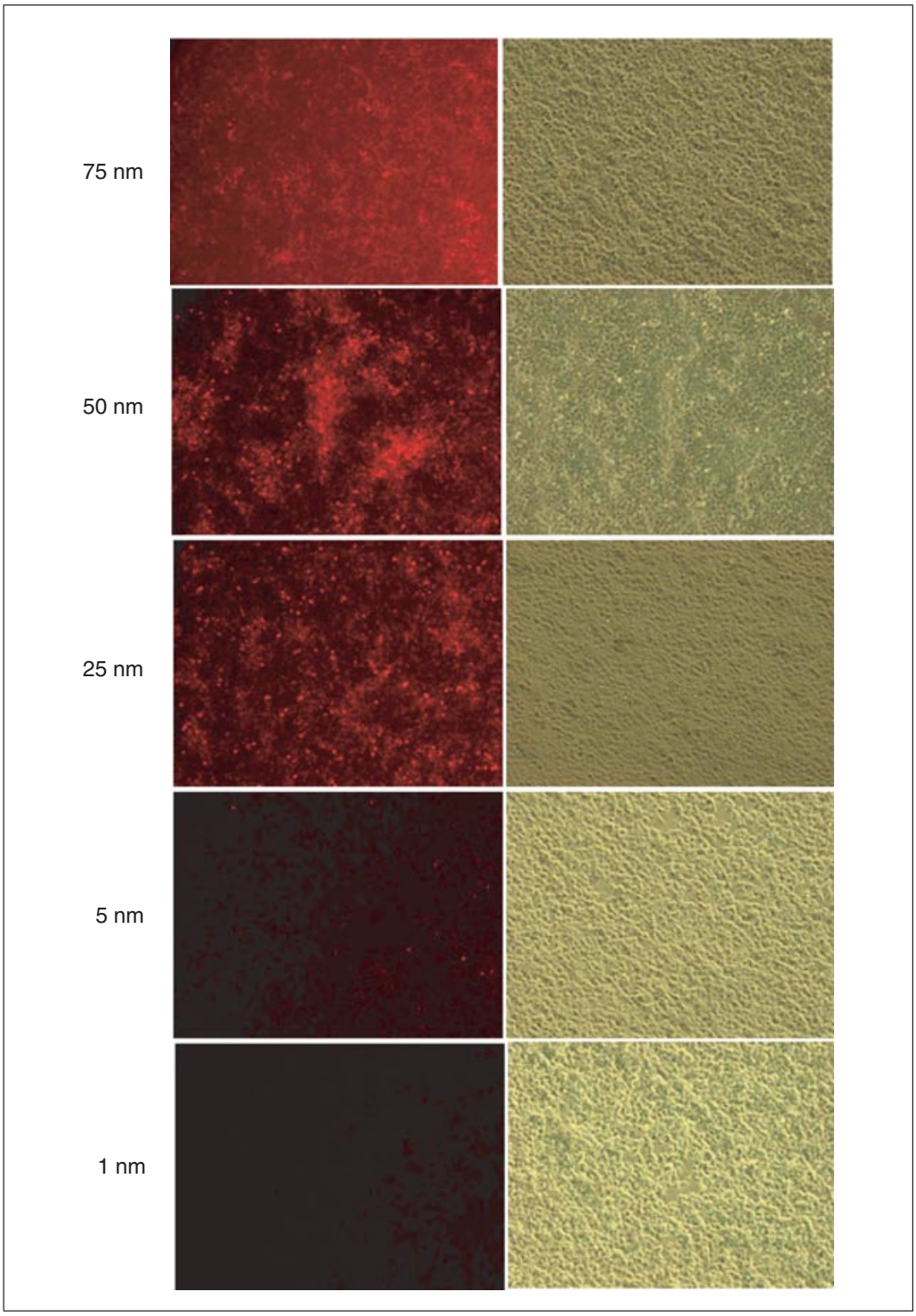


Figure 27.1.2 Detection of Cy3-labeled siRNA uptake by HEK293 cells. After 24 hr incubation, the transfection efficiency of 0, 1, 5, 25, 50, and 75 nM Cy3-labeled siRNAs were observed under a microscope with a UV lamp. Excitation wavelength of Cy3 is ~550 nm and emission wavelength is ~570 nm. Representative results are shown. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb2701*.

Prepare Lipofectamine 2000 solution

13. Mix Lipofectamine 2000 gently and microcentrifuge briefly to bring the solution to the bottom of the tube.

14. Dilute 2.0 µl Lipofectamine in 100 µl Opti-MEM I per well. For six different concentrations of a siRNA (for 12 wells), prepare 1440 µl of Lipofectamine 2000 solution in two tubes (=14.4 µl Lipofectamine 2000 in 720 µl Opti-MEM I per tube). Prepare three more sets for other siRNA/DNA mixes (total of eight tubes) (step 2, Fig. 27.1.1).

    The 1440 µl of Lipofectamine 2000 solution contains a 0.091× volume excess of the volume for six tubes of the siRNA/DNA mixes (tubes 1 to 6 in the table; 1440 µl =220 µl × 1.091 × 6 siRNA/DNA mixes).

15. Mix gently and incubate for 5 min at room temperature.

Form liposomes

16. Prepare DNA-siRNA-lipid complex (liposome) by adding 220 µl of the Lipofectamine 2000 solution to 220 µl of the siRNA/DNA mix. Mix gently and incubate for 20 min at room temperature for liposome formation (step 3, Fig. 27.1.1).

Transfect cells

17. Add 200 µl of the liposome solution dropwise to each well containing cells and 0.8 ml medium (step 4, Fig. 27.1.1). Mix gently by rocking the plate back and forth, left to right several times.

18. Incubate the cells at 37°C, 5% CO_2 until cells are ready for harvesting.

    Generally the assay period is 24 to 48 hr.

19. At the end of incubation, observe transfection efficiency with the Cy3-labeled siRNA under a microscope with a UV lamp or fluorescence microscope.

    The excitation wavelength of Cy3 is ~550 nm and the emission wavelength is ~570 nm. Representative results are shown in Figure 27.1.2.

    Under some experimental conditions (e.g., multiple cycles of transfection for a prolonged assay period), growth medium may be replaced after 4 to 6 hr of incubation without loss of transfection activity; this helps to reduce cellular toxicity.

    After 24 hr transfection, one can perform one of following procedures to estimate/measure transfection efficiency (should include a positive control for Cy3 fluorescence detection); approaches are listed in order from estimations to accurate measurements:

    Visual observation: Observe Cy3 fluorescence cells under a microscope with a UV lamp/fluorescence microscope and compare and estimate a ratio Cy3 fluorescence cells to total cells.

    Counting cell numbers: In a visual field under the microscope, count Cy3 fluorescence cell numbers and total cell numbers, and take a ratio. Transfection efficiency = Cy3 fluorescing cells/total cells.

    FACS analysis: Count cell numbers of negative control cells, positive control cells, and transfected cells, and take a ratio. Transfection efficiency = Cy3 fluorescing cells/total cells).

20. Once you optimize the transfection conditions, perform your experiment with the most potent siRNAs at an optimal cell density and siRNA concentration and using Lipofectamine 2000 at least three times for validating the reproducibility.

    Once the potent siRNAs are evaluated on 12-well plates, we run scaled-up experiments on 6-well plates to obtain sufficient samples for subsequent analyses.

21. Assess the results of transfection by PCR (Support Protocol 1), immunoblotting (Support Protocol 2), or a dual-reporter system (Support Protocol 3).

SUPPORT PROTOCOL 1

ASSESSING siRNA TRANSFECTION EFFICIENCY BY RT-PCR

The importance of detection of target transcript levels after siRNA treatment is often overlooked. Potentially, the target gene might express a protein with a long half-life; therefore, the effect of the siRNA treatment may not be apparent by immunoblot analyses (described in Support Protocol 2). Thus, following a short period of time post-transfection, it is recommended to validate the effect of designed siRNAs at the transcript level. The efficiency of down-regulation of target gene expression by siRNAs can be determined by real-time quantitative RT-PCR (qRT-PCR) or end-point RT-PCR reactions. Here, we describe the isolation of total RNA from siRNA-treated cells, and cDNA preparation, in detail. Reagents available for RNA extraction are RNA STAT-60 (TEL-TEST, INC.) or TRIzol (Invitrogen), and procedures for both reagents are very similar. In this protocol, we will describe the use of RNA STAT-60 in total RNA isolation.

Materials

siRNA-treated cells on 12-well plates (Basic Protocol 2)
RNA STAT-60 (Tel-Test, Inc., *http://www.tel-test.com/*) at 4°C
Chloroform
75% ethanol made with nuclease-free or diethylpyrocarbonate (DEPC)-treated H_2O (see *APPENDIX 2A* fro DEPC treatment)
Nuclease-free or DEPC-treated water (*APPENDIX 2A*)
2 U/μl DNase I (RNase-free) and 10× DNase buffer (Ambion)
1 U/μl RNase inhibitor (RNasin, Promega)
Random hexamer primers [or oligo$(dT)_{12\text{-}18}$]
10 mM dNTP mix(10 mM dATP, dCTP, dGTP, and dTTP)
5× first-strand buffer (Invitrogen)
0.1 M DTT
MMLV reverse transcriptase (Invitrogen)
Forward and reverse PCR primers
iQ SYBR Green Supermix (for real time qPCR; BioRad)

Refrigerated centrifuge
NanoDrop 1000 (Thermo Fisher Scientific) or UV/Vis spectrophotometer
PCR tubes
65° and 80°C water baths or heating block

Additional reagents and equipment for spectrophotometric determination of RNA concentration (*APPENDIX 3D*), standard PCR (Kramer and Coen, 2001), and real-time qPCR (Bookout et al., 2006)

Collect total RNA from siRNA treated cell culture

1. At the end of siRNA treatment (Basic Protocol 2), aspirate medium from the cell culture.

   Do not wash cells with CMF-DPBS, since this may result in RNA degradation.

   For cells grown in suspension, centrifuge the suspension and collect the cell pellet.

2. Lyse the cells by directly adding RNA STAT-60 (1 ml/2.5 to 5 × 10^6 cells; e.g., 300 μl of RNA STAT-60 per well on a 12-well plate) and pipetting up and down with a 200-μl pipet tip until the viscosity disappears.

   If the viscosity persists, add 50-μl increments of RNA STAT-60 until the lysate becomes transparent. It is critical to lyse cells thoroughly to extract RNA of high quality.

3. Transfer the lysate to a 1.7-ml microcentrifuge tube.

   In the case of collecting lysates from a 10-cm dish, use a 2.0-ml microcentrifuge tube or aliquot the lysate into multiple tubes. However, aliquotting into multiple tubes may cause RNA extraction efficiency to vary among tubes.

Extract RNA

4. Let the lysate stand for 5 min at room temperature to let nucleoprotein complexes dissociate.
5. Add 0.2 vol of chloroform to the RNA STAT-60 (i.e., 0.2 ml of chloroform to the 1 ml of RNA STAT-60 solution).
6. Cap tightly and vortex for 15 sec. Let it stand for 2 to 3 min at room temperature.
7. Centrifuge 15 min at 12,000 ×g, 4°C.
8. Transfer the upper aqueous phase to a 1.7-ml microcentrifuge tube.

   The volume of the aqueous phase will be ~0.6 vol of RNA STAT-60 used for cell lysis.

   DNA and proteins remain in the interphase and organic phase. While you are pipetting out the aqueous phase, be careful not to disturb the white debris.

Precipitate RNA

9. Add 0.5 volume of isopropanol (with respect to the volume of RNA STAT-60 used, i.e., 150 µl of isopropanol when 300 µl of RNA STAT-60 is used). Mix by pipetting up and down and let stand 5 min at room temperature.
10. Centrifuge 10 min at 12,000 × g, 4°C.

    You will see RNA precipitated as a white pellet at the bottom of the tube.

11. Discard supernatant, and add 1 vol of 75% ethanol made with DEPC-treated water to wash the RNA pellet. Mix by inverting the tube a couple of times.
12. Centrifuge 5 min at 7500 × g, 4°C.
13. Carefully discard supernatant and air-dry the RNA pellet.

    It is important to avoid drying the pellet completely, since that will reduce its solubility.

14. Dissolve the RNA pellet in nuclease-free water (or DEPC-treated water) so that the concentration of total RNA will be around 1 to 2 µg/µl.

    Generally, you will obtain about 5 to 10 µg of total RNA from 0.4 × 10^6 cells.

    Adding 15 µl of nuclease-free water to the RNA pellet will be enough to dissolve the RNA pellet.

15. Measure the concentration and check the quality of the total RNA using a NanoDrop assay or a spectrophotometer with UV lamp.

    APPENDIX 3D includes protocols for spectrophotometric determination of RNA concentration.

    The quality of the RNA can be estimated by measuring absorbance at 260 nm and 280 nm and take a ratio 260/280 for purity. In general, a ratio ≥1.8 is considered to be good quality for real-time PCR analysis (with 2.0 being the highest).

Treat with DNase I

16. For a 15-µl reaction, mix the following reagents in a PCR tube at room temperature (15 µl total volume). Prepare two tubes per sample so that one tube will be used for reverse transcription (RT) negative control.

    4.0 µl 0.5 µg/µl total RNA
    1.5 µl 10× DNase buffer
    1.0 µl 2 U/µl RNase-free DNase I
    0.5 µl 1 U/µl RNase inhibitor
    8.0 µl RNase-free water (or DEPC-treated water).

17. Mix, then microcentrifuge briefly to bring the mixture to the bottom of the tube. Incubate the reaction mix at 37°C for 60 min.
18. Heat-inactivate the DNase I by incubating the reaction mix at 80°C for 10 min. Immediately place the tube on ice and leave it for 3 min.
19. Centrifuge the tube briefly to bring the solution to the bottom of the tube.
20. Store the reaction mix at −20°C if necessary.

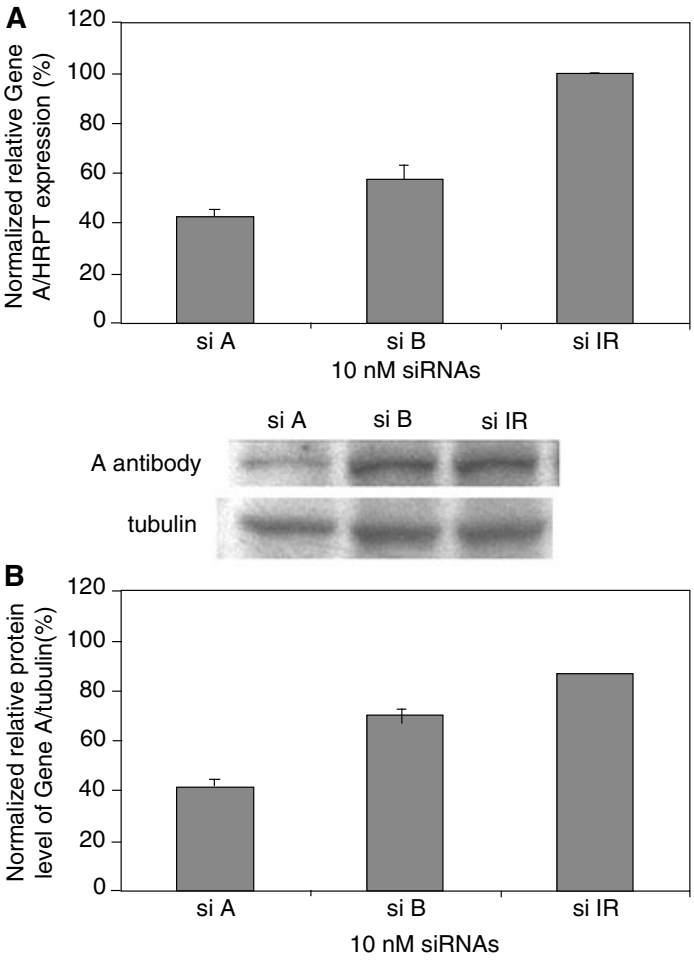


Figure 27.1.3 Effects of siRNA target gene down-regulation. (**A**) Determination of siRNA silencing efficiency by real-time qRT-PCR. Silencing of Gene A by si A or si B (10 nM) was determined with qRT-PCR. An irrelevant siRNA, si IR, was used as a negative control and used to normalize the Gene A expression level. The expression levels of the gene A was also analyzed relative to HRPT expression level set as 100%. The effects of siRNAs were determined in three independent transfections, and qRT-PCR reactions were performed in duplicate. (**B**) Determination of siRNA silencing efficiency by immunoblot analyses. Silencing of a gene A by si A or si B (10 nM) was determined at protein level. Quantification of protein expression levels are shown. An irrelevant siRNA, si IR, was used as a negative control and used to normalize the Gene A expression level. The expression levels of the Gene A was also analyzed relative to tubulin expression level set as 100%. The effects of siRNAs were determined in three independent transfections, and immunoblot analyses were performed independently.

Synthesize cDNA

21. Add following reagents to the 15-µl DNase I reaction mix in PCR tubes (18 µl total volume):

    2.0 µl 50 ng/µl random hexamers
    1.0 µl 10 mM dNTP mix.

22. Incubate the reaction mix at 65°C for 5 min. In the meantime, prepare the solution described in step 23. After 5 min incubation, immediately place tubes on ice.

23. Add the following reagents to the 18-µl reaction mix (27 µl total volume). For RT negative control, add 1.0 µl of DEPC-treated water in place of the MMLV-RT.

    5.0 µl 5× first-strand buffer
    2.5 µl 0.1 M DTT
    0.5 µl 1 U/µl RNase inhibitor
    1.0 µl MMLV-RT.

24. Incubate the 27-µl cDNA synthesis mix at 27°C for 10 min and 37°C for 50 min.

25. Heat-inactivate MMLV-RT by incubating the synthesis mix at 70°C for 15 min. Immediately place on ice and add 173 µl DEPC-treated water to bring the final volume to 200 µl.

Perform PCR

26. Perform either end-point PCR (standard PCR, e.g., Kramer and Coen, 2001, with determination of reaction product on a gel) or real-time qPCR (Bookout et al., 2006) to enable one to determine the gene silencing levels after siRNA treatment. In a PCR reaction, use 10 µl of synthesized cDNA to assess target RNA levels.

    Representative qRT-PCR results are shown in Figure 27.1.3A.

ASSESSING siRNA TRANSFECTION EFFICIENCY BY IMMUNOBLOTTING

SUPPORT PROTOCOL 2

Protein levels of target genes after siRNA treatments are often determined to validate the effects of designed siRNAs. To fully describe the effect of siRNA on protein levels, it is important to know the half-life of the target protein. With a protein that has a long half-life, you may adjust the length of incubation with siRNAs and/or numbers of transfections during the assay period or perform multiple cycles of transfection for a long-term assay to see the effects of siRNAs at the protein level. Here, we describe cell lysis for collecting total proteins for further analysis. Refer to *UNIT 6.2* for immunoblotting procedures. Prepare total protein from siRNA-treated cells for determination of the effects of transfection experiments, and mock-treated cells as a control for protein detection.

Materials

siRNA-treated cell culture in 12-well plate (Basic Protocol 2)
Mock-treated cell culture
Calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS; Cellgro, cat. no. 21-031 CV, or see *APPENDIX 2A*), 4°C
RIPA buffer (see recipe) with freshly added 1× protease inhibitor cocktail (Roche), ice cold
End-over-end rotator (e.g., Labquake from Thermo Scientific)
Additional reagents and equipment for Bradford protein assay (*APPENDIX 3H*) and immunoblotting (*UNIT 6.2*)

Lyse cells

1. At the end of siRNA treatment (Basic Protocol 2), aspirate medium from the cell culture. Wash cells once with 0.5 ml ice-cold CMF-DPBS. Keep the plate on ice. Also perform this and all subsequent steps on a mock-transfected culture as a control.

   For cells grown in suspension, collect the cell suspension, centrifuge 5 min at 200 × g, 4°C, and collect cell pellet. Wash the pellet with 1 ml ice-cold CMF-DPBS, and centrifuge again at 4°C.

2. Lyse the cells by directly adding ice-cold RIPA buffer with freshly added 1× protease inhibitor cocktail (1 ml per 2–5 × 10^6 cells, e.g., 300 µl per well on a 12-well plate). Let it sit ∼ 2 min on ice so that the cells detach from the surface.

3. Collect cells with a pipettor and 1000-µl pipet tip by tilting the plate on ice, and transfer to a cold 1.7-ml microcentrifuge tube.

   It is critical to keep cells on ice to prevent protein degradation.

   If necessary, use a scraper to collect cells. Adding more RIPA buffer may lower total protein concentrations.

4. Incubate the cells for 15 min at 4°C with gentle agitation on an end-over-end rotator.

5. Microcentrifuge 15 min at 14,000 rpm, 4°C, and transfer supernatant to a new, cold 1.7-ml tube.

6. Measure total protein concentration with Bradford assay (*APPENDIX 3H*), and aliquot the lysate into several tubes at ∼50 or 100 µl/tube to avoid multiple freeze-thaw cycles. Store the aliquots at −80°C. 7. Perform immunoblotting (*UNIT 6.2*).

   Representative results are shown in Figure 27.1.3B.

SUPPORT PROTOCOL 3

ASSESSING siRNA TRANSFECTION EFFICIENCY BY A DUAL-REPORTER ASSAY SYSTEM

Prevalidation of siRNA potency and optimization of transfection efficiency can be performed by developing reporter genes fused to a target gene. For instance, green fluorescent protein (GFP) fluorescence in cell culture can be quantified by flow cytometry, or a FLAG epitope tag can be used to detect the change in protein expression levels by immunoblot analysis. However, the former method requires that a FACS facility be readily available, and the latter tends to be time consuming. Conversely, a dual-luciferase reporter assay system by Promega accelerates the identification of potent siRNAs among designed siRNAs in 24 to 48 hr by detecting changes in expression of a reporter gene. With this system, the transfection of the dual-luciferase reporter vectors (psiCHECK vectors, Promega), carrying the target sequence, into mammalian cell lines leads to expression of the target sequence fused to the reporter gene, which is translated into the functional *Renilla* luciferase (*http://www.promega.com*). The catalytic activity of *Renilla* luciferase measured by bioluminescent reactions is used to determine how effective siRNAs initiate RNAi by observing reductions in the enzymatic activity.

Simultaneous transfection of two plasmids, one expressing a target gene and another expressing a control protein, can provide evaluation of siRNA potency by normalizing expression levels of the reporter protein to the control protein at time of transfection. However, results may vary from transfection to transfection due to inconsistent transfection efficiencies between the reporter and control plasmids. The psiCHECK-2 (Fig. 27.1.4, Promega) contains an additional internal control reporter gene that improves the reproducibility and that enables evaluating well-to-well variation of transfection (*http://www.promega.com*). The *Renilla* luciferase is expressed from the vector

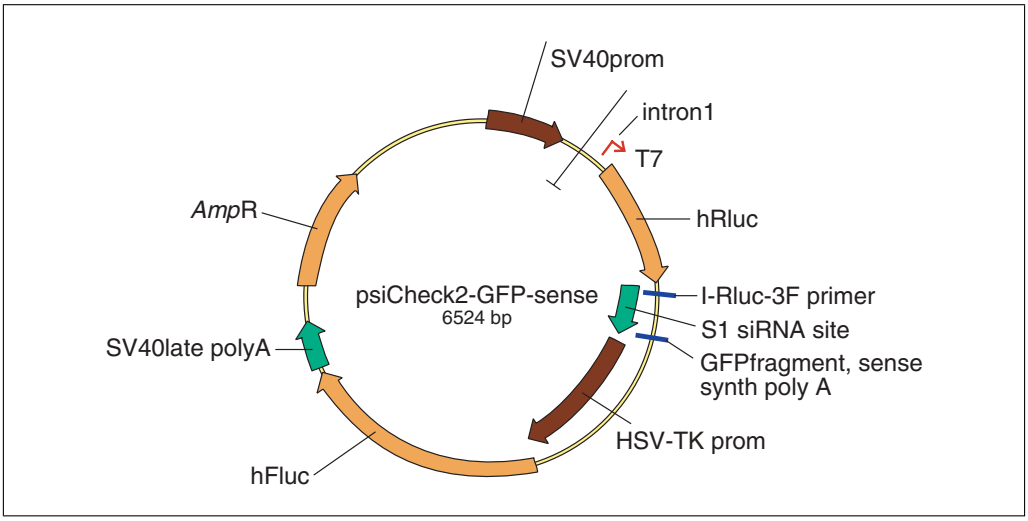


Figure 27.1.4 A reporter plasmid psiCHECK-2 (Promega) containing a target site GFP S1. A reporter plasmid psiCHECK2-GFP-sense was prepared by cloning a PCR fragment that contains the target sequence complementary to siRNAs into the multiple cloning region of the psiCHECK-2 (S1 siRNA site). The psiCHECK-2 contains an additional internal control reporter gene, firefly luciferase (*hluc+*), which is used to normalize the *Renilla* luciferase activity. Depending on experimental settings, the siRNAs can be either cotransfected or transfected sequentially. Small interfering RNA silencing efficiency was evaluated by measuring the activities of firefly and *Renilla* luciferases sequentially from a single sample using a luminometer (*http://www.promega.com*).

containing the target gene sequence cloned into the multiple cloning site located in the 3′ untranslated region (UTR) of a humanized *Renilla* luciferase (*hRluc*) reporter gene (*http://www.promega.com*). The firefly luciferase (*hluc+*) expressed from the same vector is used to normalize the *Renilla* luciferase activity, serving as the baseline response (*http://www.promega.com*). Depending on experimental settings, the siRNAs can be either cotransfected or transfected sequentially. In this protocol, cotransfection of siRNAs and the psiCHECK-2 vector is described in detail.

Prepare a reporter plasmid by cloning a PCR fragment that contains the target sequence complementary to siRNAs into the multiple cloning region of the psiCHECK-2 (S1 siRNA site, Fig. 27.1.4). The efficiency of siRNA silencing is evaluated by measuring the activities of firefly and *Renilla* luciferases sequentially from a single sample using a manual luminometer or a luminometer with one or two reagent injectors. Promega provides the Dual-Luciferase Reporter Assay system, containing reagents necessary for cell lysis and measuring *Renilla* and firefly luciferase activities. Refer to the manufacturer's protocol for more information (*http://www.promega.com*).

In this section, we describe cotransfection of mammalian cell culture with psiCHECK-2 reporter plasmid containing a target sequence and siRNAs for validation of the most effective siRNA duplexes among siRNAs, and the lowest concentration that results in efficient silencing. To find optimal siRNA concentrations, one should test at least six different concentrations (e.g., 0, 0.05, 0.5, 1.0, 20, and 45 nM) for each siRNA with a constant amount of the reporter plasmid as a starting point. The procedure described here refers to transfection of cells seeded in 48-well plates at two different levels of confluence (one at 75% and another at 90% confluency) with the reporter plasmid in addition to the siRNAs. See below for the summary of compositions for transfection reaction mixes shown in Table 27.1.5. Transfection procedures deviating from the previous section (with 12-well plates) are emphasized in this section (see the flow chart in Fig. 27.1.5). Once the conditions are optimized, you may perform transfection in a different format (e.g., 6- or 12-well plates) to accommodate your subsequent

Table 27.1.5 Transfection Reaction Mixes for Six Different siRNA Concentrations (48-Well Plates)

siRNA final conc. (nM)[a]	siRNA per well (ng)[b]	psiCHECK reporter plasmid (ng)	Stuffer DNA plasmid[c]	Total nucleic acid amount (ng)
0	0 (0 ng)	40	120.00	160
0.05	10 fmol (0.134 ng)	40	119.87	160
0.5	100 fmol (1.34 ng)	40	118.66	160
1.0	200 fmol (2.67 ng)	40	117.30	160
20	4 pmol (53.5 ng)	40	66.50	160
45	9 pmol (120 ng)	40	0	160

[a]siRNA concentrations were calculated for the total volume of 200 µl per well of a 48-well plate.
[b]siRNA (ng) was calculated with a dsRNA, mol. wt. = 1.34×10^4 g/mol.
[c]Any cloning vectors not containing eukaryotic promoters (e.g., pBluescript, pCR2.1, etc) can be used to adjust the total amount of nucleic acid.

experimental procedures (e.g., when collecting total proteins for immunoblot analyses or isolating total RNA for qRT-PCR, seed cells in 6- or 12-well plates while keeping the optimal percent confluency and optimal siRNA concentrations consistent at the time of transfection).

NOTE: Transfection reagent preparations should be carried out in a tissue culture hood.

Materials

10 µM siRNA working solution (Basic Protocol 1)
10 µM irrelevant siRNA as negative control
psiCHECK-2 vector with target cloned into the MCS (psiYTC)
Stuffer DNA plasmid (see Basic Protocol 2)
Opti-MEM I (a reduced-serum medium from Invitrogen), or serum-free growth medium
Lipofectamine 2000 (Invitrogen)
Mammalian cells seeded in two 48-well plates
Complete medium with 10% FBS (e.g., complete DMEM/10% FBS; see recipe), without antibiotics, 37°C
Dual-Luciferase Reporter Assay System (Promega)
Orbital shaker
Luminometer (e.g., Veritas Microplate Luminometer)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

Prepare DNA master mixes

1. Prepare six tubes properly labeled (0, 0.05, 0.5, 1.0, 20, 45 nM) for each corresponding siRNA mix, add each reagent as described in Table 27.1.6 and step 1 in Fig. 27.1.5, and bring the final volume to 16 µl per well with Opti-MEM I. Mix gently.

   It is strongly recommended to perform transfections in duplicate. From this point forward, volumes of reagents are calculated for duplicate transfections.

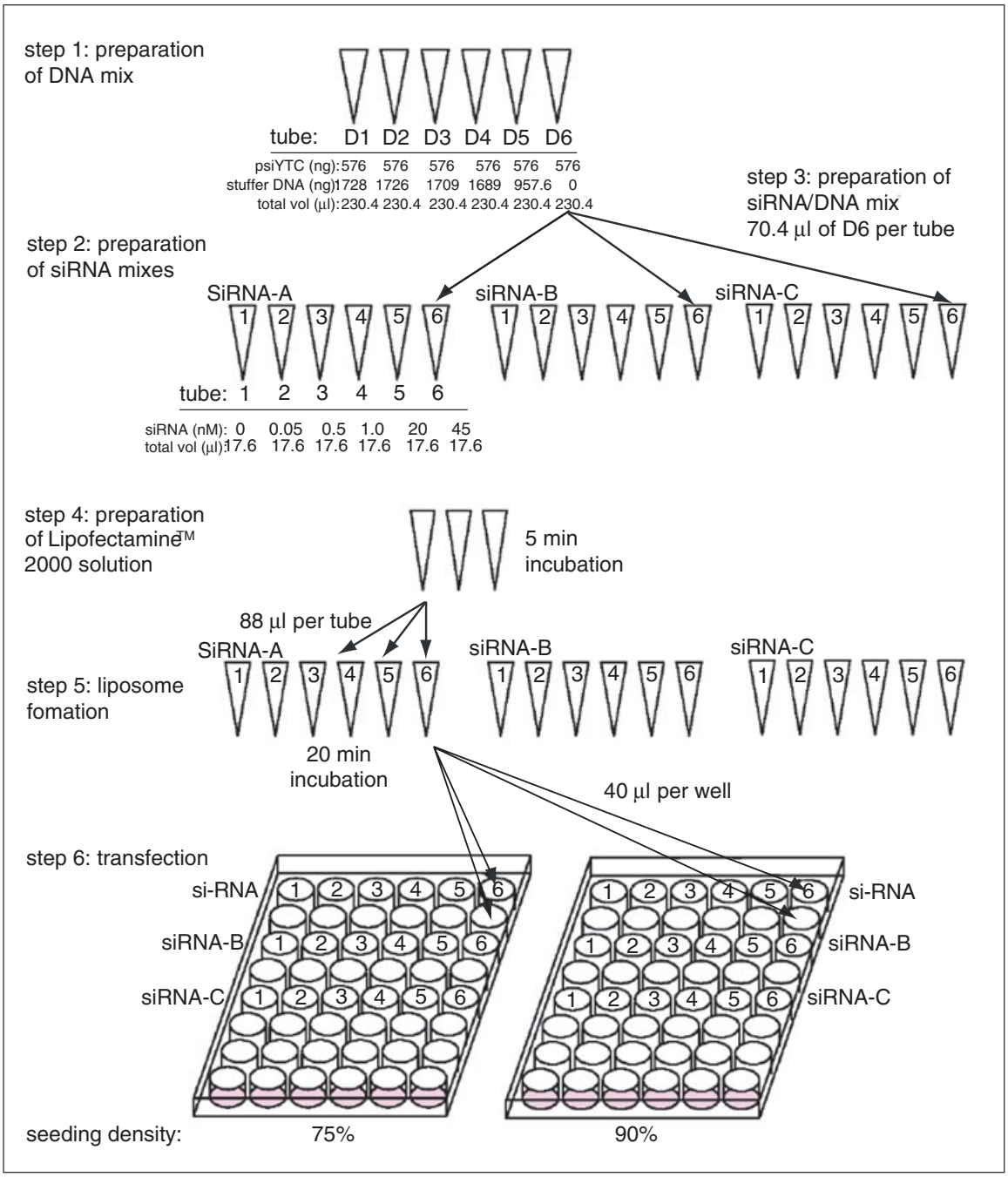


Figure 27.1.5 Transfection of mammalian cells with psiCHECK2-GFP-sense and siRNAs (48-well plates). Stepwise procedures of transfection of mammalian cells seeded at two different cell densities (75% and 90%) a day prior to transfection are shown. After choosing five different concentrations of siRNAs (0 to 45 nM) to transfect the cells, prepare DNA master mixes and siRNA mixes separately in the Opti-MEM (or a serum-free medium) as indicated (steps 1 and 2). Next, add 70.4 µl of D1 to tubes containing 0 nM of siRNAs A, B, or C (tubes A1, B1, and C1). Repeat with D2 to D6 to prepare siRNA/DNA mixes (step 3). Prepare proper amount of Lipofectamine 2000 solution (three tubes of 576 µl for six tubes of siRNA/DNA mixes, step 4). After 5 min incubation, add 88 µl of Lipofectamine 2000 solution to each siRNA/DNA mix (step 5). Incubate for 20 min to allow liposome formation, then add 40 µl of the liposome solution to two wells on the 75% seeding density plate and two wells on the 90% seeding density plate (step 6). Transfection reagent preparations should be done in a tissue culture hood.

Table 27.1.6 DNA Master Mixes for 0, 0.05, 0.5, 1.0, 20, and 45 nM siRNAs for Dual Reporter Assays

Tube	For siRNA final conc. (nM)[a]	Per well			For 14.4×		
		psiYTC reporter plasmid (ng)[b]	Stuffer DNA plasmid (ng)[c]	Total volume per well (μl)	psiYTC reporter plasmid (ng)[b]	Stuffer DNA plasmid (ng)[c]	Total volume per well (μl)
D1	0	40	120.00	16	576	1728	230.4
D2	0.05	40	119.87	16	576	1726	230.4
D3	0.5	40	118.66	16	576	1709	230.4
D4	1.0	40	117.30	16	576	1689	230.4
D5	20	40	66.50	16	576	957.6	230.4
D6	45	40	0	16	576	0	230.4

[a]siRNA concentrations were calculated for the total volume of 200 μl per well of a 48-well plate.
[b]The optimal amount of psiCHECK-2 vector with your target site (psiYTC) should also be experimentally determined. Here an example of 40 ng of psiYTC is used.
[c]Any cloning vectors not containing eukaryotic promoters (e.g., pBluescript, pCR2.1, etc) can be used to adjust the total amount of nucleic acid.

Table 27.1.7 siRNA Mixes for Six Different siRNA Concentrations

Tube	For siRNA final conc. (nM)[a]	Per well			For 14.4×		
		psiYTC reporter plasmid (ng)[b]	Stuffer DNA plasmid (ng)[c]	Total volume per well (μl)	psiYTC reporter plasmid (ng)[b]	Stuffer DNA plasmid (ng)[c]	Total volume per well (μl)
D1	0	40	120.00	16	576	1728	230.4
D2	0.05	40	119.87	16	576	1726	230.4
D3	0.5	40	118.66	16	576	1709	230.4
D4	1.0	40	117.30	16	576	1689	230.4
D5	20	40	66.50	16	576	957.6	230.4
D6	45	40	0	16	576	0	230.4

[a]siRNA concentrations were calculated for the total volume of 200 μl per well of a 48-well plate.

2. For three siRNAs—two test siRNAs and an irrelevant siRNA—and two 48-well plates with different cell densities, prepare 230.4 μl (14.4× volume) of DNA master mix per tube.

   The 230.4 μl of DNA master mix per tube contains 0.091× volume excess of the volume of the DNA mix added per siRNA mix tube (230.4 μl = 17.6 μl × 1.091 × 4 wells ×3 siRNA concentrations).

Prepare siRNA mixes

3. Prepare 20 μl of 0.1 and 1 μM siRNA by diluting 2 μl of the 10 μM siRNA working solution in 18 μl of Opti-MEM I and 2 μl of the 1 μM siRNA solution in 18 μl of Opti-MEM I so that you have three different concentrations for each siRNA.

4. Prepare three sets of six tubes properly labeled (0, 0.05, 0.5, 1.0, 20, 45 nM) for a set per siRNA, add reagents to each tube as described in Table 27.1.7 and step 2 in

Fig. 27.1.5, and bring the final volume with Opti-MEM I to 17.6 µl per well. Mix gently.

To perform transfections in duplicate, and since the cells are seeded in two 48-well plates with different cell densities, prepare 17.6 µl (4.4 × volume) of siRNA mix for 4 wells.

The 17.6 µl of siRNA/DNA mix per tube contains 0.1× volume excess of the actual volume of siRNA mix used for a well (17.6 µl = 4.0 µl × 1.1 × 4 wells).

Prepare siRNA/DNA mixes

5. Add 70.4 µl of DNA master mixes to corresponding siRNA mixes [i.e., add 70.4 µl of the DNA master mix for 45 nM siRNA (tube D6) to tubes containing 17.6 µl of 45 nM siRNA-A, siRNA-B, and irrelevant siRNA (siRNA-C) so that the total volume now is 88 µl per tube]. Add other DNA master mixes for 0, 0.05, 0.5, 1, and 20 nM to corresponding siRNA mix tubes (step 3 in Fig. 27.1.5).

Prepare Lipofectamine 2000 solution

6. Mix Lipofectamine 2000 gently and centrifuge briefly to bring the solution to the bottom.

7. Dilute 0.5 µl in 20 µl Opti-MEM I per well.

   For six different concentrations of an siRNA at four wells per concentration, prepare 576 µl of Lipofectamine 2000 solution (=14.4 µl Lipofectamine 2000 in 576 µl Opti-MEM I) for 24 wells (=6 siRNA concentrations × duplicate × 2 plates). Prepare two more tubes of the Lipofectamine 2000 solution for other siRNAs as well (total of three tubes; step 4, Fig. 27.1.5).

   The 576 µl of Lipofectamine 2000 solution contains 0.091× volume excess of the volume of the Lipofectamine 2000 solution to be added to the siRNA/DNA mix per tube (576 µl = 88 µl × 1.091 × 6 siRNA concentrations).

8. Mix gently and incubate for 5 min at room temperature.

Form liposomes

9. Prepare DNA/siRNA/lipid complex (liposome) by adding 88 µl of the Lipofectamine 2000 solution to 88 µl of the siRNA/DNA mix. Mix gently and incubate for 20 min at room temperature for liposome formation (step 5, Fig. 27.1.5).

Transfect cells

10. Add 40 µl of the liposome solution drop-wise to each well containing cells and 160 µl medium (step 6, Fig. 27.1.5A). Mix gently by rocking the plate back and forth, and left to right several times.

11. Incubate the cells at 37°C, 5% CO_2 until cells are ready for harvesting.

    Generally the cells are harvested at 24 to 48 hr.

Lyse cells

12. Prepare ~8 ml of 1× passive lysis buffer from 5× passive lysis buffer stock, which is part of the Dual-Luciferase Reporter Assay System (Promega) for 96 wells (=75 µl/well × 48 wells × 2 plates + extra).

13. Add 75 µl per well and shake the culture plates on an orbital shaker for 15 min at room temperature.

    The rocking motion will ensure covering the cells evenly and completely with the 1× passive lysis buffer.

14. Immediately proceed to dual-luciferase assays (using kit from Promega and luminometer) or store samples at −80°C.

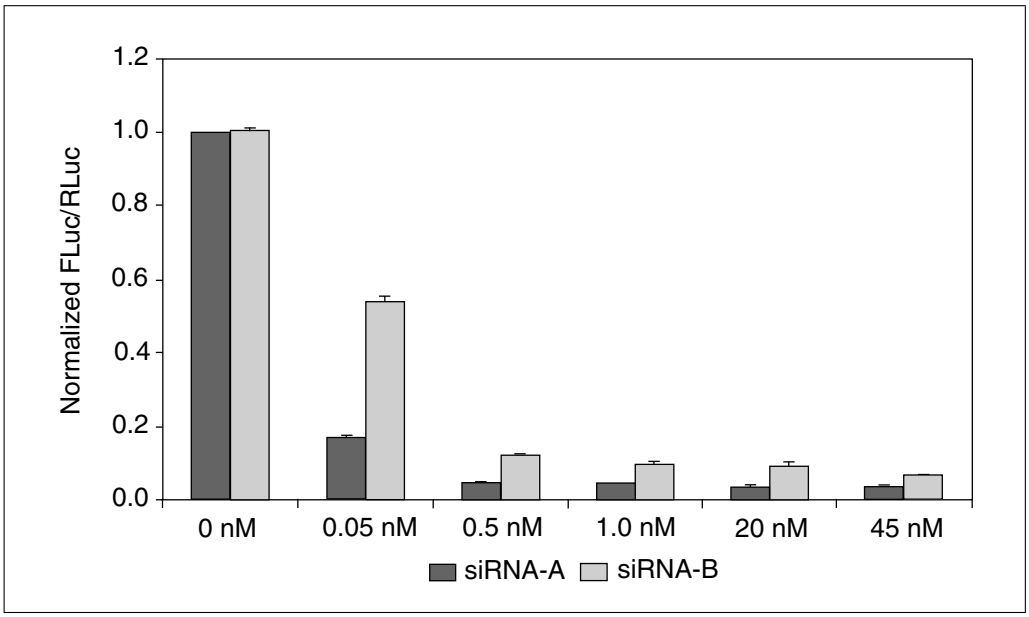


Figure 27.1.6 Determination of transfection efficiency by the dual-luciferase assays. The efficiency of siRNA silencing was evaluated by measuring the activities of firefly and *Renilla* luciferases. The relative luminescence units were normalized to the negative control (0 nM) set as 100%. Results showed concentration dependent silencing efficiency of the two siRNAs A and B. Once an optimal concentration of a siRNA is determined, perform your experiment with the highly potent siRNA(s) at optimal cell density and concentrations at least three times to assess reproducibility.

15. Once you optimize the transfection conditions, perform your experiment with the most potent siRNAs at optimal cell density and concentrations at least three times to assess reproducibility.

    Representative results are shown in Figure 27.1.6.

SUPPORT PROTOCOL 4

ANNEALING SINGLE-STRANDED OLIGOS FOR A DOUBLE-STRANDED RNA

Depending upon the purpose of the experiments (e.g., siRNA structural modifications, chemical modifications on siRNA strands, etc), you may obtain siRNAs as single-stranded RNA oligos or duplex siRNAs. When single-stranded oligos are obtained, you should anneal equal amounts of each single-stranded oligo to generate a double-stranded RNA.

NOTE: Gloves and plastic ware (no glass) should be used to avoid RNA degradation and cross-contamination of samples.

Materials

Lyophilized single-stranded sense and antisense oligos (Basic Protocol 1)
RNase-free (e.g., DEPC-treated) H_2O or TE buffer (*APPENDIX 2A*)
10× annealing buffer (see recipe)
Nuclease-free (e.g., DEPC-treated) H_2O

95°C heat block
NanoDrop 1000 (Thermo Fisher Scientific) or UV/Vis spectrophotometer

Additional reagents and equipment for spectrophotometric determination of RNA concentration (*APPENDIX 3D*)

Prepare for annealing

1. Dissolve the single-stranded RNA oligos in nuclease-free water or TE buffer to a final concentration of 100 μM. Make sure the oligos are completely dissolved. Place them on ice.

   It is recommended to measure actual concentrations of single-stranded oligos after dissolving by measuring A_{260} with a NanoDrop or a spectrophotometer with UV lamp (APPENDIX 3D) and assess the purity of your RNA oligos before use.

Anneal the oligos

2. For a 50-μl total volume reaction, mix the following reagents in a 1.7-ml sterile microcentrifuge tube at room temperature to generate a 25 μM dsRNA solution.

   12.5 μl 100 μM sense RNA oligo
   12.5 μl 100 μM antisense RNA oligo
   5 μl 10× annealing buffer
   Nuclease-free H_2O for 50 μl.

3. Incubate the reaction mix at 95°C for 4 min.

4. Microcentrifuge the tube briefly to bring the solution to the tube bottom, and remove the heat block from the heat source and set on your lab benchtop.

   Protect the surface of the bench top from heat by placing an insulator between the heat block and the bench top.

5. Place the tube back in the heat block to slowly cool to room temperature by allowing the heat block to reach ambient temperature.

   This will gradually anneal the single-stranded oligos to dsRNA. It will take about 1.5 to 2 hr to reach room temperature.

6. Briefly mix and centrifuge the solution and place on ice.

7. Dilute the dsRNA to 10 μM with RNase-free water and use as a working solution. Store the remaining sample up to 6 months at −20°C or 2 to 3 years at −80°C as the master stock.

   Double-stranded siRNA is diluted in RNase-free water for short-term storage or 1× RNase-free siRNA buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.5) for long-term storage. The siRNA buffer can also be purchased from various vendors.

   It is strongly recommended to verify the quality of the annealed dsRNA using polyacrylamide gel electrophoresis (see Support Protocol 5).

CHECKING THE INTEGRITY OF dsRNAs

SUPPORT PROTOCOL 5

Before proceeding to transfection, it is strongly recommended to verify the integrity of the dsRNA annealed above using non-denaturing polyacrylamide gel electrophoresis. The presence of free single-stranded oligos will not only reduce the amount of siRNAs used in the transfection but also, more significantly, it will affect the silencing efficiency.

Materials

10× TBE buffer (*APPENDIX 2A*)
40% (w/v) 19:1 acrylamide:bisacrylamide (AC:BC) solution
TEMED
10% (w/v) ammonium persulfate (APS) solution
Annealed dsRNAs (Support Protocol 4)
4× native gel loading dye (see recipe)
10 mg/ml ethidium bromide solution

14 × 16–cm gel electrophoresis apparatus
Power supply
Flat gel loading tips
UV lamp

NOTE: When a minigel is used, adjust the volume and time of gel running.

Prepare gel apparatus

1. Assemble a glass plate sandwich on a clean surface. Make sure the plates are properly aligned with spacers to avoid leakage.
2. Prepare 35 ml of 8% polyacrylamide nondenaturing solution by mixing the following:

   24.5 ml Milli-Q water
   3.5 ml 10× TBE buffer
   7 ml 40% (w/v) AC:BC
   15 μl TEMED.

3. Add 150 μl of 10% APS solution to the gel solution and mix quickly, but thoroughly, by swirling and avoid foaming.

   To avoid polymerization while casting the gel, make sure to prepare the solution at room temperature.

4. Working quickly, fill the gel mold (without the comb) to 1 mm below the top of the smaller piece of glass using a disposable transfer pipet. Hold the tip of the pipet against the large piece of glass and at the end of the glass so the solution fills along the spacer. Insert the comb to its fully seated position. Allow gel to polymerize 10 to 15 min.

   This will cause some of the solution to overflow. Add more if necessary.

5. Assemble the 8% nondenaturing polyacrylamide gel into the running apparatus and pre-run for ∼0.5 hr at 200 V.
6. Prepare dsRNA samples by diluting part of the 10 μM working solution to a final concentration of 1 μM.
7. In a 1.7-ml tube, mix 1 μl of 10 μM dsRNA with 2 μl 1× TBE buffer and 1 μl 4× native gel loading dye. In another tube, mix 1 μl of 1 μM dsRNA with 2 μl 1× TBE and 1 μl 4× native gel dye.

Run and analyze the gel

8. Load samples at the bottom of the wells with flat gel loading tips.
9. Run the gel for 1.5 to 2 hr at 200 V.
10. Disassemble the gel and stain with 0.4 μg/ml ethidium bromide solution (prepared from 10 mg/ml stock) for 20 min.
11. Visualize dsRNA samples under a UV lamp.

    You should avoid leaving the gel in the stain solution too long because RNA samples will diffuse out from the gel and may no longer be detectable under the UV lamp.

    You should see results similar to those shown in Figure 27.1.7 when you perform nondenaturing polyacrylamide gel analysis.When the annealing is successful, you should see a detectable higher-molecular-weight band, which represents annealed dsRNA, and no remaining single-stranded oligo, which is seen as a lower-molecular-weight band, not shown in the figure.

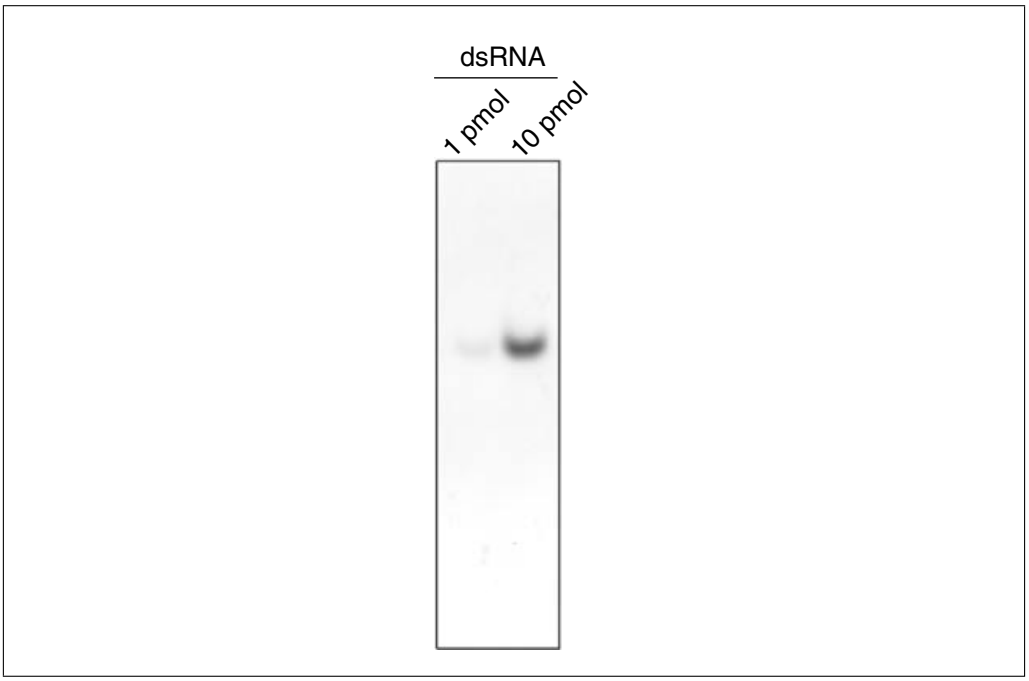


Figure 27.1.7 Verification of dsRNA integrity. The integrity of double-stranded RNAs was determined by gel electrophoresis. Either 1 pmol or 10 pmol of a 21-nt long dsRNA were loaded on a 8% non-denaturing polyacrylamide gel. Successful dsRNA annealing should result in a detectable higher-molecular-weight band and no remaining single-stranded oligo as a low-molecular-weight band, which was not detected in the gel.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Annealing buffer, 10×

100 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1.5 M NaCl
Store up to 2 to 3 years at 4°C

Complete DMEM/10% (v/v) FBS

Dulbecco's modified Eagle medium, high-glucose formulation supplemented with:
10% (v/v) FBS
2 mM L-glutamine
1 mM sodium pyruvate
100 U/ml penicillin
100 μg/ml streptomycin
Store up to 3 months at 4°C

For complete DMEM/10% FBS without antibiotics, omit adding penicillin and streptomycin.

Native gel loading dye, 4×

0.1% (w/v) bromphenol blue
0.1% (w/v) xylene cyanol FF
0.1% (w/v) Orange G
1 mM EDTA
10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
40% (v/v) glycerol
Store up to 2 to 3 years at 4°C (or long-term at −20°C)

RIPA buffer

150 mM NaCl
50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1% (v/v) NP-40
0.25% (v/v) sodium deoxycholate
0.1% (w/v) SDS
1× protease inhibitor cocktail (Roche), added fresh
Adjust pH to 8.0
Store up to 1 year at 4°C

COMMENTARY

Background Information

The mechanism of RNA interference (RNAi) was first described in detail in *Caenorhabditis elegans* by Andrew Fire and Craig C. Mello (Fire et al., 1998), who later shared the Nobel Prize in Physiology or Medicine in 2006. Soon after, approximately 21 nucleotide-long small interfering RNAs (siRNAs) were shown to mediate sequence-specific gene silencing mammalian cells and *Drosophila* (Zamore et al., 2000; Elbashir et al., 2001b). The selective and immediate effect of RNAi on the gene silencing within living cells made it a valuable tool for investigating gene function and initiated a new wave of reverse genetics.

The RNAi pathway is found in many eukaryotes including plants and animals. It is a small RNA–mediated gene silencing process controlled by the RNA induced silencing complex (RISC) activity at the post-transcriptional level. In living cells, small RNAs have been shown to participate in: (1) regulating gene expression, (2) maintaining genome integrity, (3) controlling cell growth, differentiation, and cellular metabolism, and (4) defending from viral invasion. Biochemical analysis of the RNAi pathway led us to realize that introducing double-stranded RNAs (dsRNAs) with sequences complementary to target transcripts can elicit an RNAi response, demonstrating the versatility of the process in cells.

RNAi can be described as having at least two well defined steps: the initiation step, where the ribonuclease (RNase) III enzyme Dicer processes dsRNAs into 21- to 22-nucleotide (nt)–long duplexes (Bernstein et al., 2001), and the effector step, in which Argonaute 2 (Ago 2), a core endonuclease of the RISC, executes RNAi (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). With exogenously introduced 21-nt siRNAs, the Dicer processing step is skipped and directly incorporated into RISC, where Ago2 carries out cleavage of the target transcript. Conversely, dsRNAs longer than 25 bp can be used to trigger RNAi response by undergoing Dicer processing. It is apparent that not only siRNA sequences that recruit RISC to target transcripts, but also incorporation of siRNAs into RISC, become critical determinants for the RNAi efficiency.

The protocol presented here describes basic approaches that one can use as a starting point. It is important to optimize transfection conditions after empirical determinations.

Critical Parameters

siRNA sequences

It is common to observe various degrees of silencing efficiency among designed siRNAs. This could simply be due to insufficient incorporation of siRNA into RISC, or it could be due to poor interaction between the siRNA and target transcript that might form secondary structure, making RISC inaccessible to the target sequence. To find target sequences that are readily accessible, it is advisable to design multiple siRNAs targeting different sites and assay the siRNAs at several concentrations. Some poorly designed siRNAs can still elicit RNAi when they are used in extremely high concentrations, a potential cause of cellular toxicity. In this protocol, 75 nM of siRNAs are included as an example to determine the gene-silencing efficiency. If designed siRNAs show some knockdown effects only at higher concentrations, you may consider redesigning siRNAs for the abovementioned reasons. Also there are siRNA design algorithms that consider the secondary structure of target transcripts (Ding et al., 2004; Yiu et al., 2005), e.g., siRNA Site selector (Heale et al., 2005; *http://www1.infosci.coh.org/hpcdispatcher/*).

Although an siRNA is designed against a specific target mRNA site, it can cause silencing of unspecific genes, termed off-target effects. If a sequence match is found between

a guide strand and an mRNA in the 3′ UTRs, the first 6 to 7 nucleotides of the guide strand can lead to translational inhibition via the microRNA-mediated pathway. Each strand of the siRNA has the potential to be assembled into RISC as a guide, so that it doubles the chance of creating off-target effects. If this is suspected, the psiCHECK-2 vector with the target sequence inserted in the antisense orientation can be used for the assessment.

Design of Dicer substrate siRNAs (D-siRNAs; Rose et al., 2005) described in this unit emanated from ideas of (1) inducing Dicer processing to increase the possibility of assembly into RISC, and (2) reducing a likelihood of passenger-strand incorporation by introducing structural asymmetry. However, it is difficult to completely eliminate all the potential off-targets. There are chemical and structural modifications such as 2′ O-methyl at a few positions that reduce the off-target effects.

Transfection conditions

In this protocol, the use of Lipofectamine 2000 has been described as a transfection reagent. Certain cell types may not achieve high levels of transfection efficiency with this reagent. If so, there are several other transfection reagents available from Invitrogen (e.g., Lipofectamine RNAiMax) and Mirus (e.g., *Trans*IT-siQUEST reagent), to name a few. It is also important to adjust the seeding density for optimization of the transfection condition.

Validations of siRNA silencing efficiency

To validate the silencing efficiency of the designed siRNAs, it is critical to assess the level of the target mRNA, since there could be a lag between degradation of mRNA and actual reduction in protein level. If necessary, you may need to perform multiple cycles of transfection to observe effects of siRNAs at the protein level. It is also strongly recommended to perform validations on both mRNA and protein levels (Support Protocols 1 and 2, respectively).

Anticipated Results

Well designed siRNA-mediated silencing of a given gene of interest can result in greater than 95%, if not 100%, reduction in the mRNA and protein levels.

Time Considerations

Synthesis of siRNA from ordering to receiving may take ∼1 week, while annealing and verification take ∼4.5 to 5 hr. Seeding cells in multiple-well plates takes ∼1 hr, and transfection reagent preparation and transfection takes ∼1.5 hr. Time for maintaining cultured cells varies. For dual-luciferase reporter assays, allow ∼1.5 hr from cell lysis to bioluminescence measurements.

For RT-PCR, it will take ∼1 hr for RNA extraction, ∼1 hr for DNase treatment, ∼1.5 hr for cDNA synthesis, and ∼2.5 hr for real-time PCR. For immunoblotting, time considerations are ∼1 hr for preparation of SDS-PAGE gel, ∼1.5 to 3 hr for electrophoresis, ∼2 hr for protein transfer, and ∼4 to 5 hr for blocking a PVDF membrane and antibody incubation.

The total time required to identify a valid siRNA for a gene can be ∼ 2 weeks.

Literature Cited

Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. 2003. Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.* 31:589-595.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363-366.

Bookout, A.L., Cummins, C.L., Kramer, M.F. Pesola, J.M., and Mangelsdorf, D.J. 2006. High-throughput real-time quantitative reverse transcription PCR. *Curr. Protoc. Mol. Biol.* 73:15.8.1-15.8.28.

Chalk, A.M. and Sonnhammer, E.L. 2008. siRNA specificity searching incorporating mismatch tolerance data. *Bioinformatics* 24:1316-1317.

Chalk, A.M., Warfinge, R.E., Georgii-Hemming, P., and Sonnhammer, E.L. 2005. siRNAdb: A database of siRNA sequences. *Nucleic Acids Res.* 33:D131-134.

Ding, Y., Chan, C.Y., and Lawrence, C.E. 2004. Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res.* 32:W135-141.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001a. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494-498.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. 2001b. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15:188-200.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. 2001c. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* 20:6877-6888.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806-811.

Gong, W., Ren, Y., Zhou, H., Wang, Y., Kang, S., and Li, T. 2008. siDRM: An effective and

generally applicable online siRNA design tool. *Bioinformatics* 24:2405-2406.

Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. 2005. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123:631-640.

Heale, B.S., Soifer, H.S., Bowers, C., and Rossi, J.J. 2005. siRNA target site secondary structure predictions using local stable substructures. *Nucleic Acids Res.* 33:e30.

Kramer, M.F. and Coen, D.M. 2001. Enzymatic amplification of DNA by PCR: Standard procedures and optimization. *Curr. Protoc. Mol. Biol.* 15.1.1-15.1.14.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. *Science* 305:1437-1441.

Liu, Q., Rand, T.A., Kalidas, S., Du, F., Kim, H.E., Smith, D.P., and Wang, X. 2003. R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science* 301:1921-1925.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. 2004. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Molec. Cell* 15:185-197.

Owczarzy, R., Tataurov, A.V., Wu, Y., Manthey, J.A., McQuisten, K.A., Almabrazi, H.G., Pedersen, K.F., Lin, Y., Garretson, J., McEntaggart, N.O., Sailor, C.A., Dawson, R.B., and Peek, A.S. 2008. IDT SciTools: A suite for analysis and design of nucleic acid oligomers. *Nucleic Acids Res.* 36:W163-W169.

Ren, Y., Gong, W., Zhou, H., Wang, Y., Xiao, F., and Li, T. 2009. siRecords: A database of mammalian RNAi experiments and efficacies. *Nucleic Acids Res.* 37:D146-D149.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., and Khvorova, A. 2004. Rational siRNA design for RNA interference. *Nat. Biotechnol.* 22:326-330.

Rivas, F.V., Tolia, N.H., Song, J.J., Aragon, J.P., Liu, J., Hannon, G.J., and Joshua-Tor, L. 2005. Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat. Struct. Molec. Biol.* 12:340-349.

Rose, S.D., Kim, D.H., Amarzguioui, M., Heidel, J.D., Collingwood, M.A., Davis, M.E., Rossi, J.J., and Behlke, M.A. 2005. Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res.* 33:4140-4156.

Shah, J.K., Garner, H.R., White, M.A., Shames, D.S., and Minna, J.D. 2007. sIR: siRNA Information Resource, a web-based tool for siRNA sequence design and analysis and an open access siRNA database. *BMC Bioinformatics* 8:178.

Siolas, D., Lerner, C., Burchard, J., Ge, W., Linsley, P.S., Paddison, P.J., Hannon, G.J., and Cleary, M.A. 2005. Synthetic shRNAs as potent RNAi triggers. *Nat. Biotechnol.* 23:227-231.

Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., and Saigo, K. 2004. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res.* 32:936-948.

Yiu, S.M., Wong, P.W., Lam, T.W., Mui, Y.C., Kung, H.F., Lin, M., and Cheung, Y.T. 2005. Filtering of ineffective siRNAs and improved siRNA design tool. *Bioinformatics* 21:144-151.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. 2000. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101:25-33.

Internet Resources

http://genome.ucsc.edu/cgi-bin/hgGateway
Source for mRNA sequences of genes.

http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx
Site for assistance with RNAi design.

http://www1.infosci.coh.org/hpcdispatcher/
Home of RNAi Site selector which considers secondary structure of target transcripts.

http://www.invitrogen.com
Cell-type specific transfection protocols for stealth RNAi and siRNA.

http://www.promega.com/paguide/chap8.htm
Protocols and applications guide.

Gene Down-Regulation with Short Hairpin RNAs and Validation of Specificity by Inducible Rescue in Mammalian Cells

Hoi Tang Ma[1] and Randy Y.C. Poon[1]

[1]Division of Life Science, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

ABSTRACT

The principal problem with RNA interference (RNAi) experiments is off-target effects. The most vigorous demonstration of the specificity is the rescue of the RNAi effects with an RNAi-resistant target gene. By combining the expression of short hairpin RNA (shRNA) and rescue cDNA in the same vector, both the validation of shRNA specificity and the generation of shRNA-expressing cell lines can easily be accomplished. If the compensatory cDNA is under the control of an inducible promoter, stable shRNA-expressing cells can be generated before the knockdown phenotypes are studied, by conditionally turning off the rescue protein. The use of model systems is detailed in these protocols. *Curr. Protoc. Cell Biol.* 49:27.2.1-27.2.12. © 2010 by John Wiley & Sons, Inc.

Keywords: inducible expression • RNA interference • short hairpin RNA

INTRODUCTION

RNA interference (RNAi) by means of short hairpin RNA (shRNA) has developed into a powerful tool for loss-of-function analysis in mammalian cells. The principal problem in RNAi experiments is off-target effects. The most vigorous demonstration of the specificity of shRNA is the rescue of the RNAi effects with a shRNA-resistant target gene. Owing to the sequence-specific nature of RNAi, shRNA-resistant target genes can be generated by introducing several silent mutations in the region of the shRNA targeting sequence of the cDNA. The silent mutations confer resistance to the shRNA but do not change the protein sequence of the target. This method presents its own problems, including both the unpredictable relative expression of shRNA and rescue cDNA in individual cells and the difficulty in generating stable cell lines.

A solution to the above problems is to combine the expression of shRNA and rescue cDNA in the same vector. The use of a model vector system called pKAR1 (for Knockdown And Rescue) is described here. In addition to facilitating the validation of shRNA specificity, this system also considerably simplifies the generation of shRNA-expressing cell lines. Since the compensatory cDNA is under the control of an inducible promoter, stable shRNA-expressing cells can be generated before the knockdown phenotypes are studied by conditionally turning off the rescue protein. Conversely, the rescue protein can be activated after the endogenous protein is completely repressed. This approach is particularly suitable when prolonged expression of either the shRNA or the compensatory cDNA is detrimental to cell growth. This system allows convenient one-step validation of shRNA and generation of stable shRNA-expressing cells (Fig. 27.2.1).

This unit contains a method for generating a model shRNA-expressing plasmid (Basic Protocol 1). A method for evaluating the ability of the shRNA to down-regulate the protein expression in culture cells is also described (Support Protocol). Construction

Current Protocols in Cell Biology 27.2.1-27.2.12, December 2010
Published online December 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471143030.cb2702s49

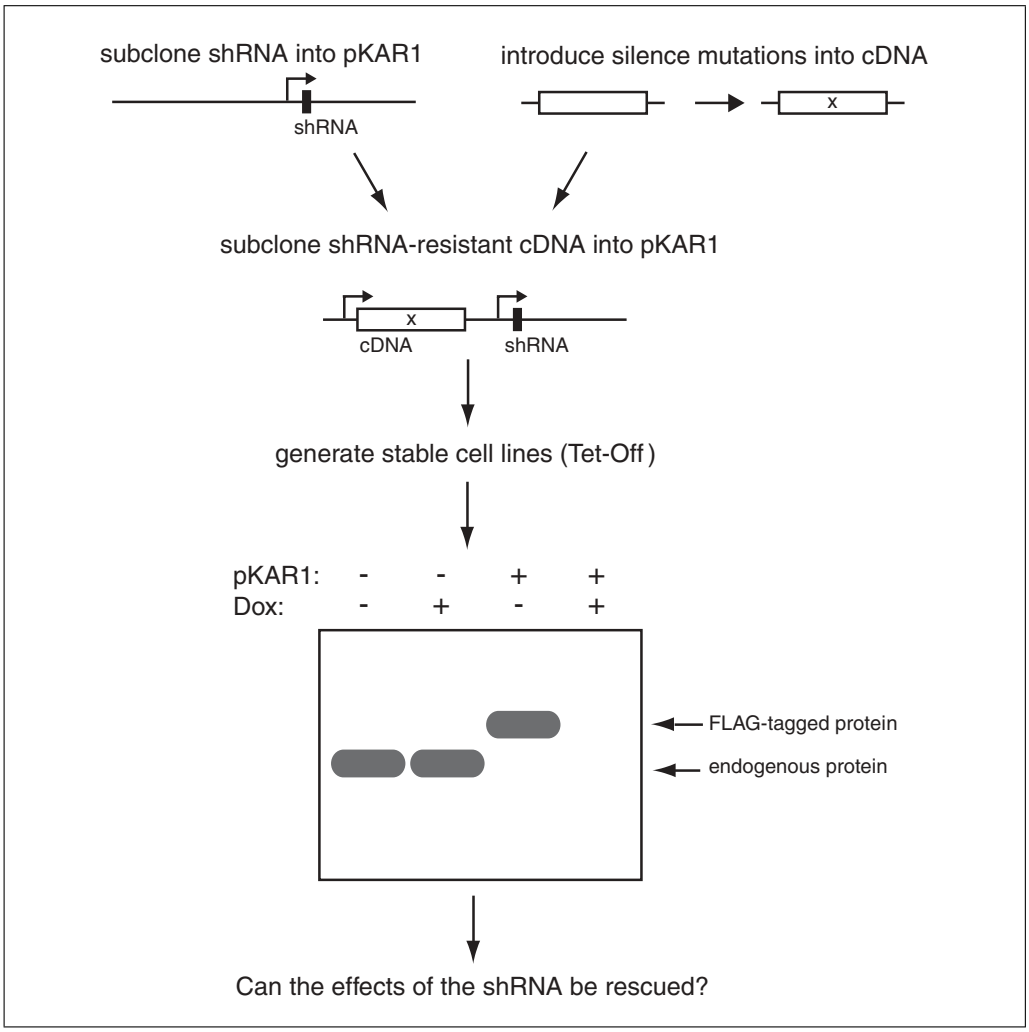


Figure 27.2.1 Plan for knockdown with shRNA and rescue experiments. An annealed duplex oligonucleotide that encodes for the shRNA is ligated into pKAR1/PUR. Silent mutations are introduced into the cDNA to render it resistant to the shRNA. The cDNA is now put into pKAR1/PUR and transfected into Tet-Off cell lines. Stable clones are selected and the expression of the target protein is analyzed—it should be suppressed (the diagram represent an immunoblot with specific antibody against the target protein). The expression of the recombinant rescue protein can be turned off with doxycycline (Dox) to reveal the knockdown phenotype.

of plasmids that coexpress both shRNA and cDNA is described in Basic Protocol 2. The generation of stable cell lines expressing shRNA and cDNA is overviewed in Basic Protocol 3.

BASIC PROTOCOL 1

DOWN-REGULATION OF GENE EXPRESSION WITH SHORT HAIRPIN RNA

Two 56-mer DNA oligonucleotides are to be synthesized. After annealing, the oligonucleotides form a double-stranded DNA that encodes the shRNA, with overhangs that are compatible with *Bbs*I- and *Xba*I-cut vector (Fig. 27.2.2A). The expected structure of the shRNA produced is shown in Figure 27.2.2B. The shRNA is processed into siRNA in the cell (see Background Information). The loop used in this design is based on that developed for the pSUPER plasmid system (Brummelkamp et al., 2002). Programs such as the freeware iRNAi (version 2.1; *http://mekentosj.com*) developed by Alexander Griekspoor and Tom Groothuis (The Netherlands Cancer Institute) can be used to assist the design of the primers.

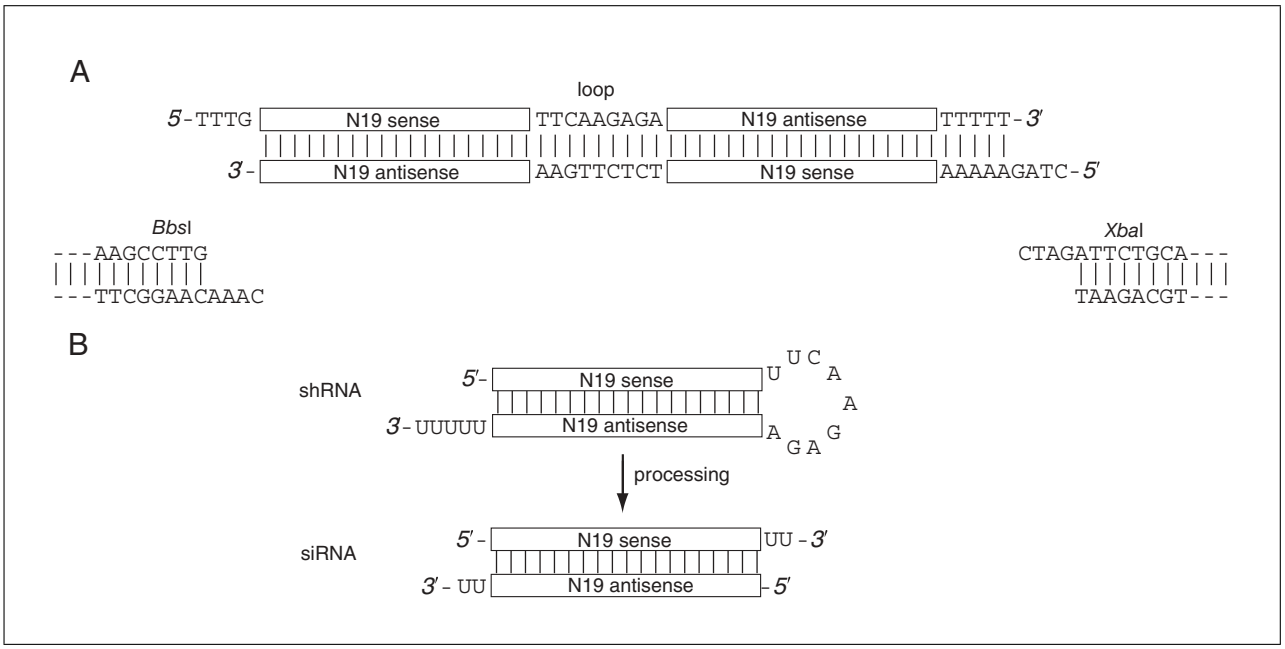


Figure 27.2.2 Cloning and expression of shRNA. (**A**) Schematic diagram of the annealed duplex oligonucleotides and the compatible sites from the *Bbs*I- and *Xba*I-cut vector. (**B**) Predicted structure of the shRNA and siRNA. After transcription from the RNA polymerase III promoter, the sense and antisense sequence form a stem that is linked by a loop of 8 nucleotides. The shRNA is believed to undergo processing by Dicer in the cell to produce a siRNA.

Materials

pKAR1 or pKAR1/PUR vectors (Addgene)
*Bbs*I and *Xba*I enzymes (New England Biolabs)
NEBuffer 2 (New England Biolabs; also see recipe)
QIAquick Gel Extraction Kit (Qiagen) or equivalent
10× annealing buffer: 100 mM Tris·Cl pH 7.4 (*APPENDIX 2A*) containing 1 M NaCl.
T4 DNA ligase and 10× ligation buffer (Fermentas)
dam$^-$ *E. coli* (e.g., GM2163 from Fermentas)
LB agar plates (*APPENDIX 2A*) with 50 μg/ml ampicillin
LB liquid medium (*APPENDIX 2A*) 50 μg/ml ampicillin
PCR primers (Invitrogen):
U6 FOR primer: 5′-CTCACCCTAACTGTAAAGTAATTG-3′
M13 REV primer: 5′-CAGGAAACAGCTATGACCATG-3′

Floating rack
16°C water bath (optional)

Additional reagents and equipment for DNA oligonucleotide synthesis (Ellington and Pollard, 1998), transforming competent *E. coli* (Thomason et al., 2007), PCR (*APPENDIX 3F*), agarose gel electrophoresis (Voytas, 2000), and DNA sequencing (Ausubel et al., 2010, Chapter 7)

Design shRNA targeting sequences

1. Select a sequence of 19 nucleotides in length (N19) that is flanked by AA and TT.

   AA(N19)TT is preferred to AA(N19)xx which is better than xx(N19)xx (x=any type of nucleotide).

2. Ensure that the 3′ end of the antisense stand is more stable (with higher free energy, ΔG) than the 5′ end.

3. Ensure that no more than four consecutive T (U) are present anywhere within the hairpin, otherwise the RNA polymerase III will terminate transcription.

4. Check that the GC content is close to 50%.
5. Avoid more than three G's in a row [as poly(G) sequences can hyperstack].
6. If the target sequence is in the ORF, avoid sequences that encode methionine and tryptophan.

   Since silent mutations are to be introduced into the shRNA-targeting region of the cDNA later, care should be made to avoid sequences that encode methionine and tryptophan (both are encoded by a single type of codon).

7. Check the sequence with BLAST to ensure that similar sequences are not present in other genes.

   Both ORF and UTRs can be used for designing targeting sequences. The use of the UTRs simplifies the generation of shRNA-resistant cDNA later.

8. Synthesize the DNA oligonucleotides (Ellington and Pollard, 1998) that will form the double-stranded DNA that encodes the shRNA.

   The authors usually use the oligonucleotide synthesis service from Invitrogen.

Prepare BbsI- and XbaI-cut vector

The shRNA is cloned into either pKAR1 or pKAR1/PUR. The difference between the two plasmids is that the latter contains a puromycin resistance gene between the two *Hin*dIII sites (Fig. 27.2.3A,B). The vectors are to be cut with *Bbs*I and *Xba*I to create compatible ends for ligation with the shRNA-encoding oligonucleotides.

9. Cut pKAR1 or pKAR1/PUR vector with *Bbs*I and *Xba*I in NEBuffer 2 at 25°C.

   Cleavage with XbaI may be blocked when the DNA is methylated by dam methylase. Dam$^-$ strains of E. coli should be used to prepare the plasmid.

10. Subject the cut plasmid to agarose gel electrophoresis (Voytas, 2000). Purify the cut plasmid with QIAquick Gel Extraction Kit (Qiagen) or similar product.

    A 761-bp fragment is removed, and the size of the cut vector is 3843 bp (pKAR1) or 4908 bp (pKAR1/PUR).

    The cut vectors can be stored at −20°C and used multiple times.

Anneal the oligonucleotides

11. Resuspend the DNA oligonucleotides (from step 8) in water to a concentration of 0.25 mM.

    The authors simply dilute the oligos according to the concentration information provided by the manufacturer (e.g., the oligonucleotide synthesis service from Invitrogen). Oligonucleotides do not need to be phosphorylated or purified.

12. Mix the following solutions in reaction tubes:

    20 µl Oligonucleotide 1
    20 µl Oligonucleotide 2
    5 µl 10× annealing buffer
    5 µl H_2O.

    The final concentration of each oligonucleotide should be 100 µM.

13. Heat 500 ml of water to 85° to 95°C in a microwave oven.
14. Immediately place the tube of annealing mixture from step 12 in a floating rack into the water and allow it to cool slowly to room temperature (usually over 1 to 2 hr).

    The annealed oligonucleotides can be stored at −20°C.

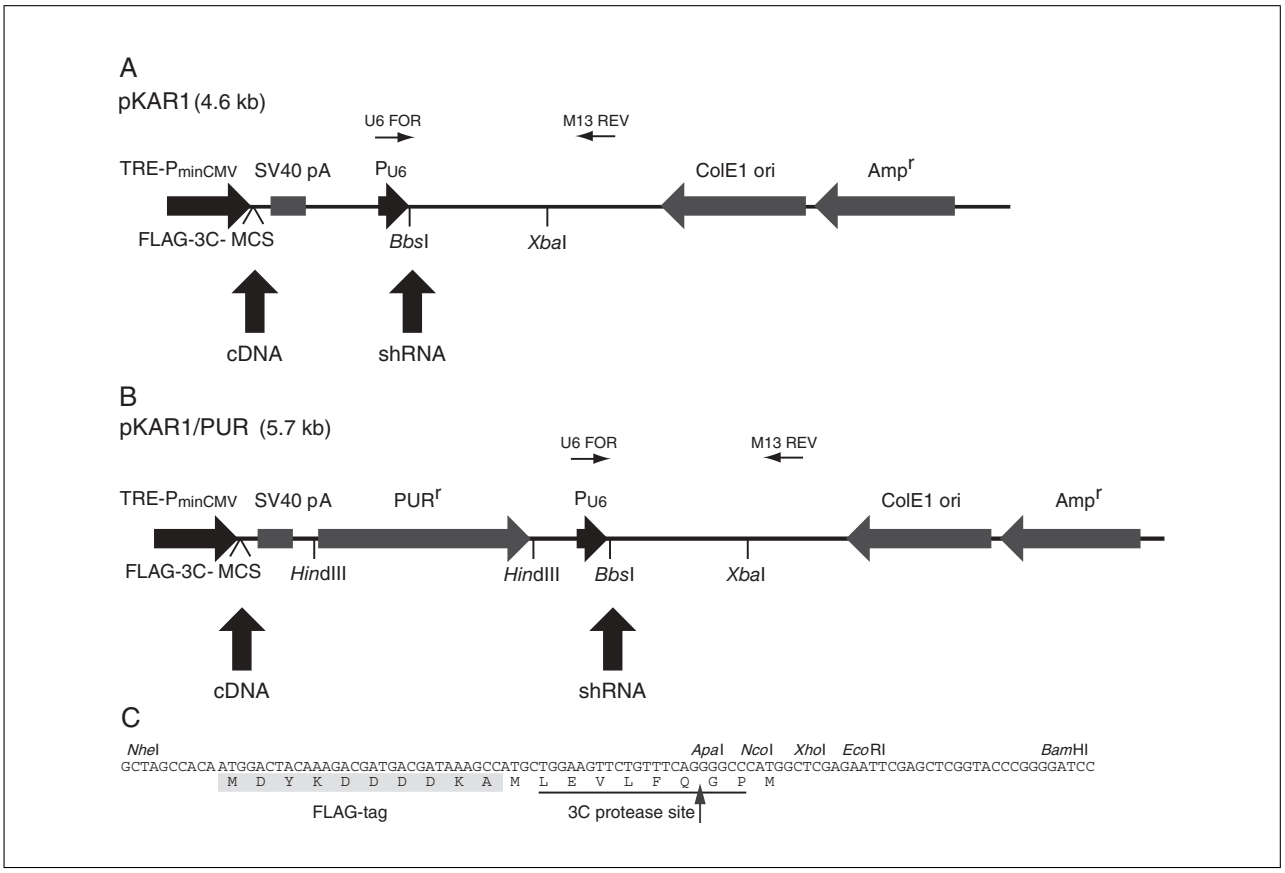


Figure 27.2.3 A model vector for coexpression of shRNA and shRNA-resistant cDNA. (**A**) Schematic diagram of pKAR1. The various elements are shown to scale: TRE, tetracycline response element; P_{min}CMV, minimal immediate early cytomegalovirus (CMV) promoter; FLAG-3C-MCS, multiple cloning sites; SV40 pA, SV40 polyadenylation signal; PU6, mouse U6 RNA promoter; Amp^r, ampicillin-resistant gene; ColE1 ori, ColE1 replication origin. The shRNA is first inserted between *Bbs*I-*Xba*I, and the shRNA-resistant cDNA is cloned into the multiple cloning sites (see panel C). The positions of the U6 FOR and M13 REV primers are indicated. (**B**) Schematic diagram of pKAR1/PUR. This vector is similar to pKAR1 except for the presence of a puromycin-resistant gene (PUR^r) inserted between the two *Hin*dIII sites. (**C**) The cDNA cloning region of pKAR1 or pKAR1/PUR. The protein sequence of the NH_2-terminal tag is shown. After cloning of the shRNA, the cDNA can be inserted in frame into the unique *Apa*I, *Nco*I, *Xho*I, *Eco*RI, and *Bam*HI sites. The untagged version can be cloned using the *Nhe*I site. The FLAG-tag is highlighted, the 3C protease recognition sequence is underlined, and the arrow indicates the site of cleavage.

Ligate oligos and plasmid

15. Dilute 1 μl of the annealed oligonucleotides into 4 ml of 0.5× annealing buffer.

16. Take 1 μl of the diluted annealed oligonucleotides from step 15 and ligate by combining the following reagents:

    1 μl diluted oligonucleotides
    50 ng *Bbs*I- and *Xba*I-cut vector (from step 10)
    1 μl 10× ligation buffer
    1 U T4 DNA ligase
    H_2O to 10 μl.

17. Incubate for 1 hr at room temperature or overnight at 16°C.

18. Transform competent *dam*$^-$ *E. coli* (Thomason et al., 2007) and plate on LB plates containing 50 μg/ml ampicillin.

Screen colonies

19. Pick several colonies and grow in 1 ml of LB supplemented with 50 μg/ml ampicillin (*APPENDIX 2A*).

20. Screen clones by PCR (*APPENDIX 3F*) with the U6 FOR primer and M13 REV primer. Analyze the products by agarose gel electrophoresis (Voytas, 2000).

    Expect an ~400 bp band if correct and an ~1130 bp for the recircularized parental vectors (see Fig. 27.2.3A,B for the relative positions of the primers).

21. Sequence clone DNA using the M13 REV primer (Ausubel et al., 2010, Chapter 7).

    The shRNA sequence is ~280 bp from the primer.

    Note that owning to the secondary structure, the hairpin region may be difficult to be sequenced properly.

SUPPORT PROTOCOL

TESTING OF THE DOWN-REGULATION OF PROTEIN BY shRNA USING IMMUNOBLOTTING

Various methods can be used to see if the shRNA is effective in down-regulating the endogenous protein. Described here is a procedure involving transient transfection of mammalian cells and detection of the endogenous target protein by immunoblotting. The presence of non-transfected cells in the final cell lysates prevents proper evaluation of the efficacy of the shRNAs. To increase the population of transfected cells, a brief treatment with antibiotics is used.

NOTE: All solutions and equipment coming into contact with the cells must be sterile, and proper aseptic technique should be used accordingly.

Materials

HeLa cells (ATCC no. CCL-2)

HeLa cell growth medium (DMEM containing 10% *APPENDIX 2B*); test batches of calf serum to ensure that there are no traces of tetracycline

pKAR1 or pKAR1/PUR plasmid bearing shRNA plus gene of interest (Basic Protocol 1) and blank plasmids as controls

Plasmid expressing blastacidin-resistance gene and histone H2B-GFP (available from the authors; can also use similar constructs such as pBOS-H2BGFP from BD Pharmingen)

Blasticidin: stock 5 mg/ml in H_2O, filter sterilized and stored as small aliquots at −20°C

Puromycin: stock 1.5 mg/ml in H_2O, filter sterilized and stored as small aliquots at −20°C

Antibodies that are capable phosphate-buffered saline (PBS; *APPENDIX 2A*) cell lysis buffer (e.g., Invitrogen) of detecting the target protein by immunoblotting

100-mm tissue culture plates

Additional reagents and equipment for mammalian cell tissue culture, including trypsinization (*UNIT 1.1*), calcium phosphate transfection (*UNIT 20.3*), determining protein concentration (*APPENDIX 3B*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

1. Plate 2×10^6 HeLa cells in 100-mm plates in HeLa cell growth medium (*UNIT 1.1*).
2. Co-transfect the HeLa cells at ~25% confluence with 10 μg of the shRNA-expressing plasmid and 1 μg of a plasmid expressing a blasticidin-resistance gene and histone H2B-GFP, using the calcium phosphate precipitation method.

    Other methods such as Lipofectamine (UNIT 20.6) and electroporation (UNIT 20) may be required for obtaining a high transfection efficiency for some cell lines.

    It is essential to set up a control reaction with blank pKAR1 or pKAR1/PUR.

3. Allow the cells to grow for 6 hr (after removing the calcium phosphate-containing medium) and adding fresh HeLa cell growth medium.

4a. For blasticidin selection: Add blasticidin to a final concentration of 5 μg/ml and incubate the cells for 24 hr.

4b. For puromycin selection: Add puromycin to a final concentration of 2 μg/ml and incubate the cells for 24 hr.

Blasticidin is used for cells transfected with plasmids expressing a blasticidin-resistance gene and histone H2B-GFP. If pKAR1/PUR is used to express the shRNA, and it is possible to use puromycin for selection without the cotransfection of blasticidin-resistance plasmid in step 2. The concentration of both blasticidin and puromycin should be determined empirically for different cell lines.

5. Aspirate the medium and add 10 ml of HeLa cell growth medium.

6. Incubate for another 24 hr.

7. Harvest the cells and prepare cell-free extracts:

   a. Harvest cells by trypsinization (*UNIT 1.1*).
   b. Resuspend the cells with 1 ml PBS and transfer to a microcentrifuge tube.
   c. Microcentrifuge 1 min at 16,000 × *g*.
   d. Aspirate the PBS and store the microcentrifuge tube at –80°C.
   e. Add ~2 pellet volumes of cell lysis buffer to the microcentrifuge tube. Vortex to mix.
   f. Incubate on ice for 30 min.
   g. Centrifuge at 16,000 g at 4°C for 30 min.
   h. Transfer the supernatant to a new tube.
   i. Measure the protein concentration of the lysates (*APPENDIX 3B*). Dilute to 1 mg/ml with SDS sample buffer (*UNIT 6.1*).

   The transfection efficiency can be indicated by analyzing the percentage of cells expressing the histone H2B-GFP by fluorescence microscopy before harvest.

8. Detect the target protein by SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*).

RESCUING RNA INTERFERENCE EFFECTS WITH TARGET GENES

BASIC PROTOCOL 2

Three to four silent mutations are to be introduced into the region targeted by the shRNA, causing the mRNAs to be resistant to the knockdown. Mutations should be introduced to the central region of the shRNA targeting sequence. Typically, the wobble positions of three consecutive codons are mutated. There is no need to generate silent mutations in the cDNA if the shRNA is against the UTRs (see Commentary). The use of HeLa cells expressing the tTA tetracycline repressor chimera is described. The expression of rescue cDNAs is repressed in the presence of doxycycline. Other tetracycline-responsive cell lines (such as Tet-On or Tet-Off cell lines from Clontech) can be used. For Tet-On cells, the expression of the cDNA is turned on by doxycycline.

Materials

QuickChange site-directed mutagenesis kit (Stratagene)
Target cDNA
shRNA-containing pKAR1 or pKAR1/PUR (Basic Protocol 1)
HeLa cells (see discussion in protocol introduction, above)
Doxycycline hydrochloride: 1 mg/ml in H_2O, filter sterilized and stored as small aliquots at −20°C
Antibody against FLAG peptide (M2 monoclonal antibody from Sigma)
Antibody against target protein

Additional reagents and equipment for DNA sequencing (Ausubel et al., 2010), Preparing cell-free extracts (Support Protocol, step 6), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

Generate shRNA-resistant cDNA in pKAR1

1. Use QuickChange site-directed mutagenesis kit or a similar product to introduce silent mutations into the target cDNA.
2. Confirm the mutagenesis by DNA sequencing (Ausubel et al., Chapter 7).
3. Clone the cDNA into the shRNA-containing pKAR1 or pKAR1/PUR generated in Basic Protocol 1 (Thomason et al., 2007).

   Several unique sites can be used to clone the cDNA in frame with both the FLAG epitope tag and the 3C protease cleavage site (Fig. 27.2.3C). Note that most of these cloning sites only become unique after the shRNA has been cloned into BbsI/XbaI-cut vector. Hence the shRNA should be cloned into the vector before the cDNA.

   IMPORTANT NOTE: *Do not include the UTR if the shRNA is against UTR.*

Test the expression of cDNA and shRNA with transient transfection

4. Transfect two identical plates of HeLa cells and transiently select with antibiotics (see Support Protocol, steps 1 to 4).
5. Add doxycycline to a final concentration of 2 μg/ml to one plate and incubate for 24 hr.

   Longer incubation may be needed for proteins with long half-lives.

6. Harvest cells and prepare cell-free extracts:

   a. Harvest cells by trypsinization (*UNIT 1.1*).
   b. Resuspend the cells with 1 ml PBS and transfer to a microcentrifuge tube.
   c. Microcentrifuge 1 min at 16,000 × *g*.
   d. Aspirate the PBS and store the microcentrifuge tube at –80°C.
   e. Add ~2 pellet volumes of cell lysis buffer to the microcentrifuge tube. Vortex to mix.
   f. Incubate on ice for 30 min.
   g. Centrifuge at 16,000 g at 4°C for 30 min.
   h. Transfer the supernatant to a new tube.
   i. Measure the protein concentration of the lysates (*APPENDIX 3B*). Dilute to 1 mg/ml with SDS sample buffer (*UNIT 6.1*).

      The transfection efficiency can be indicated by analyzing the percentage of cells expressing the histone H2B-GFP by fluorescence microscopy before harvest.

7. Detect the target protein by SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*) using monoclonal antibody against FLAG (M2).
8. In a separate blot, detect the target protein by immunoblotting (*UNIT 6.2*) using specific antibodies.

   The shRNA-resistant cDNA should be slightly larger than the endogenous protein (due to the introduction of FLAG tag and 3C protease cleavage site). Control lysates without shRNA expression should also be loaded side-by-side to see if the endogenous protein is down-regulated.

GENERATION OF STABLE CELL LINES EXPRESSING BOTH shRNA AND RESCUE cDNA

Materials

HeLa Tet-Off cells (Clontech)
pKAR1/PUR expressing shRNA and cDNA (Basic Protocol 2)
HeLa cell growth medium (DMEM containing 10% calf serum; *APPENDIX 2B*); test batches of calf serum to ensure that there are no traces of tetracycline
Puromycin: stock 1.5 mg/ml in H_2O, filter sterilized and stored as small aliquots at −20°C

10-cm tissue culture plates
Stainless steel cloning rings (internal diameter: 6 mm, thickness: 1 mm, height: 10 mm)
Autoclaved high vacuum grease silicone lubricant (Dow Corning)

Additional reagents and equipment for transfecting HeLa cells and testing for expression (Basic Protocol 2) and mammalian cell culture techniques including trypsinization (*UNIT 1.1*)

1. Transfect HeLa Tet-Off cells with pKAR1/PUR expressing shRNA and cDNA (Thomason et al., 2007; see Basic Protocol 2).

   If pKAR1 is used instead of pKAR1/PUR, an antibiotic-resistant gene expression cassette should be put into the plasmid.

2. Split the cells into two 10-cm plates, one containing 90% and the other with 10% of the cells.

   Plating the cells at two densities will ensure that the densities of the colonies will not be too high.

3. Grow the cells for 24 hr in complete HeLa cell growth medium.

4. Change medium and supplement with 2 μg/ml final of puromycin.

   Optimum dose of puromycin should be identified for different cell line empirically.

5. Grow the cells in the puromycin-containing selection medium. Change medium every 3 days (maintaining the selection) until single colonies appear.

   This will take 2 to 3 weeks. Take care not to disturb the colonies when changing the medium. The medium should only be gently added to the side the dishes.

6. Identify single colonies.

   About ten well-separated colonies are to be isolated (each ∼5 mm diameter). They can be seen by eye and marked with a pen on the underside of the dishes.

7. Isolate single colonies using cloning rings.

   Home-made stainless steel cloning rings can be used (internal diameter: 6 mm, thickness: 1 mm, height: 10 mm). After coating with an autoclaved high vacuum grease silicone lubricant (Dow Corning), the rings are placed on top of the colonies to make sealed chambers. Trypsin solution (UNIT 1.1) is placed into the rings to transfer the cells to new plates.

8. Propagate cells in the absence of puromycin.

9. Test expression of shRNA and cDNA (see Basic Protocol 2).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

NEBuffer 2

10 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
50 mM NaCl
10 mM $MgCl_2$
1 mM dithiothreitol
Store up to 2 years at −20°C

This buffer is also available from New England Biolabs.

COMMENTARY

Background Information

RNA interference (RNAi) is an evolutionarily conserved gene-silencing process triggered by double-stranded RNAs (dsRNAs; Hannon, 2002). The use of RNAi as a technique for analyzing loss-of-function phenotypes has revolutionized research in mammalian cells. One way to induce RNAi in mammalian cells is by transfection of synthetic small interfering RNAs (siRNAs). These siRNAs are 19-base-pair (bp) dsRNAs with 2-nucleotide (nt) 3′ overhangs (Elbashir et al., 2001), and mimic the structure of microRNA (miRNA) intermediates found in the natural processing of longer dsRNA by RNase III. One strand of the siRNA or miRNA duplexes (called the guide strand) is incorporated into the RNA-induced silencing complex (RISC), where it directs RISC to bind to complementary mRNA. It is believed that the other strand of the siRNA or miRNA (called the passenger strand) is not incorporated into RISC and is destroyed. RISC cleaves the mRNAs at a site 10 nt upstream of the nucleotide complementing the 5′-most nucleotide of the guide strand. The mRNA fragments are then degraded by other nucleases, resulting in knockdown of expression (Meister and Tuschl, 2004). The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs that match the siRNA that is bound to it. For more information about RNA interference, refer to *UNIT 27.1*.

An alternative way to induce RNAi in mammalian cells is by expression plasmids or viral vectors. A common approach involves the transcription of shRNAs from RNA polymerase III promoters. The shRNAs consist of a stem of 19 to 29 bp linked by a small terminal loop (Brummelkamp et al., 2002; Yu et al., 2002; Paddison et al., 2002). The prevailing view is that shRNAs mimic the structure of a miRNA intermediate generated by the RNase III enzyme Drosha. Another RNase III enzyme called Dicer acts on the shRNAs to produce siRNA/miRNA duplexes, which are then loaded onto RISC to mediate silencing (Cullen, 2004).

The use of shRNA offers several important advantages over siRNA (Hannon and Rossi, 2004). First, more delivery options are available for shRNA, including transfection, electroporation, and infection with viral vectors. Second, shRNA is less expensive to generate than siRNA. Furthermore, while silencing using siRNA is inevitably transient, shRNA-expressing constructs can be stably integrated into the genome. Finally, while the effects of siRNA after delivery are only constitutive, both constitutive and inducible systems can be used for shRNA after delivery.

It is generally accepted that the major problem with using shRNAs (as well as siRNAs) for experimentation is the possibility of off-target effects (Jackson et al., 2003; Pei and Tuschl, 2006). Several methods are utilized to confirm the specificity of the RNAi results, including the use of shRNAs against irrelevant targets and the use of multiple shRNAs against the same gene. However, the ultimate control for a shRNA experiment is the rescue of the RNAi effects by expressing the target gene in a form refractory to the shRNA (Editorial, 2003; Cullen, 2006). This is usually achieved by introducing one or more silent point mutations into the region of the cDNA that is targeted by the shRNA. The rescue of RNAi phenotypes using shRNA-resistant cDNA itself may present several problems. It is likely that individual cells may take up different amount of shRNA versus cDNA-expressing constructs, triggering a spectrum of phenotypes within a

population. Moreover, it is not trivial to obtain stable expression of both shRNA and cDNA at the same time.

Vectors that can express shRNA and the respective rescue cDNA together are useful for validating the specificity of RNAi. The pKAR1 vector proves to be a good model for subcloning shRNA and the rescue cDNA simultaneously, as well as for generating stable cell lines (Ma et al., 2007). pKAR1 was based on pUHD-P1/3C (Fung et al., 2005), which was in turn based on the tetracycline-inducible system pUHD10-3 developed by Dr. Hermann Bujard's group (University of Heidelberg, Germany; Gossen and Bujard, 1992), and mU6pro developed by Dr. David Turner's group (University of Michigan, MI; Yu et al., 2002). The shRNA was expressed from a mouse U6 RNA promoter, and the rescue cDNA was expressed under the control of tetracycline or one of its derivatives (e.g., doxycycline). Similar constructs based on the same principle as pKAR1 that combine the expression of the shRNA and the rescue cDNA should also be used.

This pKAR1 system is particularly useful when the prolonged expression of either the shRNA or the compensatory cDNA is cytotoxic (Ma et al., 2007). In the first scenario, the shRNA can be allowed to completely knockdown the endogenous proteins in the presence of the rescue cDNA; the rescue cDNA can then be turned off to attain the knockdown phenotypes. In the second scenario, the rescue cDNA can be turned on only after the endogenous proteins are completely eliminated.

Critical Parameters

Expression of the shRNA

Following the criteria set out in Basic Protocol 1 does not guarantee that the shRNA will be effective in gene down-regulation. Various methods can be used to evaluate if the shRNA is effective. At the mRNA level, real-time PCR can be performed to see the reduction of the target mRNA relative to controls such as actin. Knockdown of a cotransfected target gene can also be a useful indication of the effectiveness of an shRNA. Similar reporter-based analyses such as the luciferase system (Brasier and Fortin, 2001) also serve as alternatives. If the effects of the gene knockdown are known, they can also be used as indicators of the effectiveness of the shRNA. Nevertheless, the ability of the shRNA to reduce the expression of the endogenous protein is generally regarded as the most desirable test. The Support Protocol describes a method for transient transfection followed by a brief selection with the very rapidly acting antibiotic blasticidin. Knockdown of the protein is then detected by immunoblotting. Depending on the capability of the antibodies, other methods for detecting the target protein such as flow cytometry and immunostaining (*UNIT 4.3*) can also be used. For HeLa cells, this transient selection approach can typically enrich the transfected cells to >90%.

A critical parameter for shRNA is the time required to achieve down-regulation of the protein. This depends on the effectiveness of the shRNA (and the stability of the plasmid in the cell), the abundance of the mRNA, and the stability of the protein. It is possible that a longer duration after transfection may be required to down-regulate some proteins.

Rescue of shRNA effects with cDNA

Due to the slight increase in size conferred by the FLAG epitope tag, both the endogenous protein to be silenced and the ectopically expressed version can be detected simultaneously. The tag also allows the recombinant protein to be specifically detected either by immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*). Agarose conjugated with the monoclonal antibody M2 (Sigma) is effective in immunoprecipitating FLAG-tagged recombinant protein from cell lysates. Furthermore, the epitope tag can be removed using 3C proteases.

Here, examples are provided of a cell line expressing the tTA tetracycline repressor chimera, causing the expression of the rescue cDNA to be repressed by doxycycline. Likewise, cell lines expressing the reverse tTA (Gossen et al., 1995) can also be adopted to turn on the rescue cDNA with doxycycline.

The pKAR1 system is particularly suitable for generating stable cell lines. An example is the knockdown of cyclin A2 (Ma et al., 2007). Probably due to the toxicity of both cyclin A2 shRNA and cyclin A2 cDNA, all the colonies isolated lacked endogenous cyclin A and expressed FLAG-cyclin A. Furthermore, clones that grew at a normal rate tended to express the rescue cyclin A at a level similar to that in control cells. Thus, this method appears to have an additional advantage of isolating clones that express the rescue protein at a comparable level to the endogenous protein.

Time Considerations

Basic Protocol 1: ~2 weeks.
Basic Protocol 2: ~2 weeks.
Basic Protocol 3: ~2 months.

Literature Cited

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. 2010. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Brasier, AZ.F and Fortin, J.J. 2001. Nonisotopic assays for reporter gene activity. *Curr. Protoc. Pharmcol.* 5:6.5.1-6.5.5.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296:550-553.

Cullen, B.R. 2004. Transcription and processing of human microRNA precursors. *Mol. Cell* 16:861-865.

Cullen, B.R. 2006. Enhancing and confirming the specificity of RNAi experiments. *Nat. Methods* 3:677-681.

Editorial. 2003. Whither RNAi? *Nat. Cell Biol.* 5:489-490.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494-498.

Ellington, A. and Pollard, J. D. 1998. Synthesis and purification of oligonucleotides. 2. *Curr. Protoc. Mol. Biol.* 42:2.11.1–2.11.25.

Fung, T.K., Yam, C.H., and Poon, R.Y. 2005. The N-terminal regulatory domain of cyclin A contains redundant ubiquitination targeting sequences and acceptor sites. *Cell Cycle* 4:1411-1420.

Gossen, M. and Bujard, H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* 89:5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. 1995. Transcriptional activation by tetracyclines in mammalian cells. *Science* 268:1766-1769.

Hannon, G.J. 2002. RNA interference. *Nature* 418:244-251.

Hannon, G.J. and Rossi, J.J. 2004. Unlocking the potential of the human genome with RNA interference. *Nature* 431:371-378.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P.S. 2003. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21:635-637.

Ma, H.T., On, K.F., Tsang, Y.H., and Poon, R.Y.C. 2007. An inducible system for expression and validation of the specificity of short hairpin RNA in mammalian cells. *Nucleic Acids Res.* 35:e22.

Meister, G. and Tuschl, T. 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature* 431:343-349.

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16:948-958.

Pei, Y. and Tuschl, T. 2006. On the art of identifying effective and specific siRNAs. *Nat. Methods* 3:670-676.

Thomason, L., Court, D.L., Bubunetko, M., Costantino, N., Wilson, H., Datta, S., and Oppenheim, A. 2007. Recombineering: Genetic engineering in bacteria using homologous recombination. *Curr. Protoc. Mol. Biol.* 78:1.16.1-1.16.24.

Voytas, D. 2000. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

Yu, J.Y., DeRuiter, S.L., and Turner, D.L. 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 99:6047-6052.

USEFUL INFORMATION AND DATA

APPENDIX 1

Useful Measurements and Data

APPENDIX 1A

Figure A.1A.1 A physical chemist's view of the cell. The data in this figure were assembled from *Molecular Biology of the Cell* (Alberts et al., 1994), and represent the approximate concentrations of a variety of intracellular components.

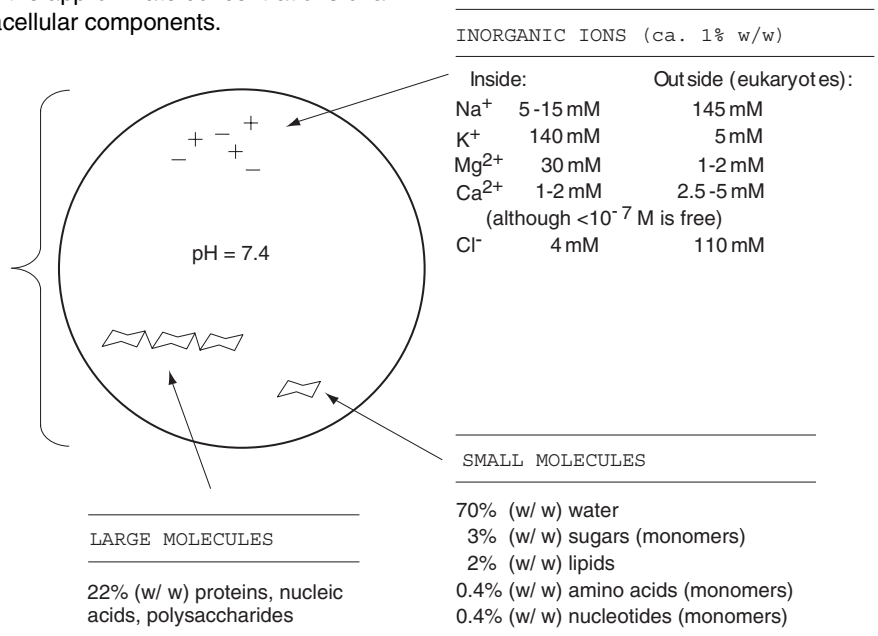


Table A.1A.1 Conversion Factors

Molecular weight (avg.) of DNA base pair: 649 Da	1 kb DNA: 333 amino acids of coding capacity
Molecular weight (avg.) of amino acid: 110 Da	≈ 36,000 Da
1 µg/ml DNA: 3.08 µM phosphate	6.5×10^5 Da of double-stranded DNA (sodium salt)
1 µg/ml of 1 kb DNA: 3.08 nM 5′ ends	3.3×10^5 Da of single-stranded DNA (sodium salt)
1 A_{260} double-stranded DNA: 50 µg/ml	10 kDa protein ≈ 91 amino acids
1 A_{260} single-stranded DNA: 37 µg/ml	≈ 273 nucleotides

Table A.1A.2 Relative Volumes of Major Intracellular Compartments in the Hepatocyte[a]

Intracellular compartment	% of total cell volume	No. per cell (approx.)
Cytosol	54	1
Endosomes	1	200
Lysosomes	1	300
Mitochondria	22	1700
Nucleus	6	1
Peroxisomes	1	400
Rough endoplasmic reticulum cisternae	9	1
Smooth endoplasmic reticulum cisternae + Golgi cisternae	6	1

[a]Data adapted from *Molecular Biology of the Cell* (Alberts et al., 1994).

Table A.1A.3 Approximate Lipid Composition of Cell Membranes[a]

Lipid	% of total lipid (by wt.)					
	Hepatocyte plasma membrane	Erythrocyte plasma membrane	Myelin	Mitochondrion (inner + outer)	Endoplasmic reticulum	*E. coli*
Cholesterol	17	23	22	3	6	0
Glycolipids	7	3	28	Trace	Trace	0
Phosphatidylcholine	24	17	10	39	40	0
Phosphatidylethanolamine	7	18	15	35	17	70
Phosphatidylserine	4	7	9	2	5	Trace
Sphingomyelin	19	18	8	0	5	0
Others	22	13	8	21	27	30

[a]Data adapted from *Molecular Biology of the Cell* (Alberts et al., 1994).

Table A.1A.4 The Twenty Common Amino Acids

Amino acid	3-letter code	1-letter code	Mol. wt. (g/mol)
Alanine	Ala	A	89.1
Arginine	Arg	R	174.2
Asparagine	Asn	N	132.1
Aspartate	Asp	D	133.1
Cysteine	Cys	C	121.2
Glutamate	Glu	E	147.1
Glutamine	Gln	Q	146.2
Glycine	Gly	G	75.1
Histidine	His	H	155.2
Isoleucine	Ile	I	131.2
Leucine	Leu	L	131.2
Lysine	Lys	K	146.2
Methionine	Met	M	149.2
Phenylalanine	Phe	F	165.2
Proline	Pro	P	115.1
Serine	Ser	S	105.1
Threonine	Thr	T	119.1
Tryptophan	Trp	W	204.2
Tyrosine	Tyr	Y	181.2
Valine	Val	V	117.1

LITERATURE CITED

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1994. Molecular Biology of the Cell, 3rd ed. Garland Publishing, New York.

Compendium of Drugs Commonly Used in Cell Biology Research

The following appendix includes an alphabetical list of drugs commonly used to examine various cell biological processes. Table A.1B.1 lists the drugs by activity and provides recent references. Indicated under each drug listed is its mode of action, generally including several specific experimental examples; solvent(s) used to solubilize the drug; stock and working concentrations or ranges; storage conditions; and duration of incubation with cells to achieve the desired effects. Except where indicated, the majority of drugs in this list are cell-permeant. However, despite the well characterized selectivity of many of the following drugs in vitro, the corresponding effects upon their intracellular targets may not be precisely determined directly by their extracellular concentrations, since their cell-permeation properties are not known. Therefore, several different concentrations of any particular drug, as well as alternative methods of determining drug selectivity, should be examined.

Several of these drugs are members of large families, such as those targeting protein kinases and phosphatases, as well as those that affect intracellular Ca^{2+} levels. Many of these family members have different selectivities and potencies toward similar targets, and a complete listing is not included here. The reader may consult catalogs from the following companies, which have several of these family members available: Sigma, Alexis Biochemicals (including LC Laboratories), Calbiochem, Biomol, Molecular Probes, Boehringer Mannheim, Oxford Glycosystems, and Avanti Polar Lipids.

Although not specifically indicated, many of the following drugs are hazardous and should be handled with extreme care. Material Safety Data Sheets (MSDSs) are often provided for products that are hazardous or toxic. In some cases, these products are new and have not been tested for toxicity. Thus, care should be taken to ensure the safe handling of all products.

Table A.1B.1 Biological Activity of Drugs Commonly Used In Cell Biological Research

Drug	References
DNA replication inhibitors	
Aphidicolin	Borner et al., 1995. *Cancer Res.* 55:2122-2128; Debec et al., 1996. *J. Cell Biol.* 134:103-115; Jackson et al., 1995. *J. Cell Biol.* 130:755-769; Urbani et al., 1995. *Exp. Cell Res.* 219:159-168
Ara-C	Grant et al., 1994. *Oncol. Res*. 6:87-99; Tomkins et al., 1994. *J. Cell Sci*. 107:1499-1507
Camptothecin	Carettoni et al., 1994. *Biochem. J.* 299:623-629; Desai et al., 1997. *JBC* 272:24159-24164; Li et al., 1994. *JBC* 269:7051-7054
Etoposide	Kaufmann et al., 1993. *Cancer Res*. 53:3976-3985; Meyer et al., 1997. *J. Cell Biol*. 136:775-788; Shao et al., 1997. *JBC* 272:31321-31325; Terada et al., 1993. *J. Med. Chem*. 36:1689-1699; Wozniak et al., 1991. *J. Clin. Oncol*. 9:70-76
Hydroxyurea	Anand et al., 1995. *Cancer Lett*. 88:101-105; Nishijima et al., 1997. *J. Cell Biol.* 138:1105-1116; Oliver et al., 1997. *JBC* 272:10624-10630
L-Mimosine	Gilbert et al., 1995. *JBC* 270:9597-9606; Lin et al., 1996. *JBC* 271:2548-2556; Park et al., 1997. *J. Neurosci.* 17:1256-1270

continued

Contributed by Nelson B. Cole
Current Protocols in Cell Biology (1998) Appendix 1.B.1-1.B.26

Table A.1B.1 Biological Activity of Drugs Commonly Used In Cell Biological Research, continued

Drug	References
Drugs affecting the cytoskeleton	
Colcemid	Barlow et al., 1994. *J. Cell Biol.* 126:1017-1029; Bonfoco et al., 1995. *Exp. Cell Res*. 218:189-200; Goswami et al., 1994. *Exp. Cell Res.* 214:198-208
Colchicine	Bonfoco et al., 1995. *Exp. Cell Res*. 218:189-200; Lindenboim et al., 1995. *J. Neurochem*. 64:1054-1063
Cytochalasin B	Benya and Padilla, 1993. *Exp. Cell Res*. 204:268-277; Takeshita et al., 1998. *Cancer Lett*. 126:75-81; Tanaka et al., 1994. *Exp. Cell Res.* 213:242-252
Cytochalasin D	Cooper, 1987. *J. Cell Biol*. 105:1473-1478; Radhakrishna and Donaldson, 1997. *J. Cell Biol.* 139:49-61; Sasaki et al., 1995. *PNAS* 92:2026-2030; Wang et al., 1994. *Am. J. Physiol*. 267:F592-F598
Latrunculins	Wada et al., 1998. *J. Biochem.* 123:946-952; Lamaze et al., 1997. *JBC* 272:20332-20335; Spector et al., 1989. *Cell Motil. Cytoskeleton* 13:127-144
Nocodazole	Cole et al., 1996. *Mol. Biol Cell*. 7:631-650; Liao et al., 1995. *J. Cell Sci.* 108:3473-3483; Vasquez et al., 1997. *Mol. Biol. Cell* 8:973-985
Taxol	Derry et al., 1995. *Biochemistry* 34:2203-2211; Ding et al., 1990. *Science* 248:370-372; Gallo, 1998. *J. Neurobiol*. 35:121-140; Jordan et al., 1993. *PNAS* 90:9552-9556; Mogensen and Tucker, 1990. *J. Cell Sci*. 97:101-107
Vinblastine	Dhamodharan et al., 1995. *Mol. Biol. Cell* 6:1215-1229; Lobert et al., 1998. *Cell Motil. Cytoskeleton* 39:107-121; Panda et al., 1996. *JBC* 271:29807-29812; Rai and Wolff, 1996. *JBC* 271:14707-14711; Taki et al., 1998. *J. Neurooncol*. 36:41-53; Tsukidate et al., 1993. *Am. J. Pathol*. 143:918-925
Drugs affecting intracellular Ca^{2+}	
A23187	Elia et al., 1996. *JBC* 27:16111-16118; Kao, 1994. *Methods Cell Biol.* 40:155-181; Kao et al., 1990. *J. Cell Biol.* 111:183-196
BAPTA	Bissonnette et al., 1994. *Am. J. Physiol*. 267:G465-G475; Smith et al., 1992. *Biochem. J*. 288:925-929; Tsien, 1980. *Biochemistry* 19:2396-2404
Ionomycin	Aagaard-Tillery and Jelinek, 1995. *J. Immunol*. 155:3297-3307; Rock et al., 1997. *JBC* 272:33377-33383; Stewart et al., 1998. *J. Cell Biol*. 140:699-707
Thapsigargin	Kuznetsov et al., 1993. *JBC* 268:2001-2008; Lodish et al., 1992. *JBC* 267:12753-12760; Takemura et al., 1989. *JBC* 264:12266-12271; Won and Orth, 1995. *Endocrinology* 136:5399-5408; Wong et al., 1993. *Biochem. J*. 289:71-79
Drugs affecting oligosaccharide biosynthesis/processing	
Castanospermine	Ahmed et al., 1995. *Biochem. Biophys. Res. Commun*. 208:267-273; Bass et al., 1998. *J. Cell Biol*. 141:637-646; Gruters et al., 1987. *Nature* 330:74-77; Martina et al., 1998. *JBC* 273:3725-3731
Deoxymannojirimycin	Elbein et al., 1984. *Arch. Biochem. Biophys*. 235:579-588; McDowell et al., 1987. *Virology* 161:37-44; Slusarewicz and Warren, 1995. *Glycobiology* 5:154-155; Wojczyk et al., 1998. *Glycobiology* 8:121-130
Deoxynojirimycin	Labriola et al., 1995. *J. Cell Biol*. 130:771-779; Martina et al., 1998. *JBC* 273:3725-3731; Romero et al., 1985. *Biochem. J.* 226:733-740; Schlesinger et al., 1984. *JBC* 259:7597-7601
Tunicamycin	Bush et al., 1994. *Biochem. J*. 303:705-708; Kuznetsov et al., 1997. *JBC* 272:3057-3063; Lodish and Kong, 1984. *J. Cell Biol*. 98:1720-1729

continued

Table A.1B.1 Biological Activity of Drugs Commonly Used In Cell Biological Research, continued

Drug	References
Drugs affecting the pH of intracellular organelles	
Ammonium chloride	Breuer et al., 1995. *JBC* 270:24209-24215; Davis and Mecham, 1996. *JBC* 271:3787-3794; Smith et al., 1997. *JBC* 272:5640-5646
Bafilomycin A_1	Bowman et al., 1988. *PNAS* 85:7972-7976; Calvert and Sanders, 1995. *JBC* 270:7272-7280; Furuchi et al., 1993. *JBC* 268:27345-27348
CCCP	Arai et al., 1996. *Biochem. Biophys. Res. Commun*. 227:433-439; Babcock et al., 1997. *J. Cell Biol*. 136:833-844; Kao, 1994. *Methods Cell Biol*. 40:155-181; Simpson and Russell, 1996. *JBC* 271:33493-33501
Chloroquine	Claus et al., 1998. *JBC* 273:9842-9851; Garcia-Sainz and Mendoza-Mendoza, 1998. *Eur. J. Pharmacol*. 342:333-338; Passos and Garcia, 1998. *Biochem. Biophys. Res. Commun*. 245:155-160; Wunsch et al., 1998. *J. Cell Biol*. 140:335-345
Concanamycin B	Akifusa et al., 1998. *Exp. Cell Res*. 238:82-89; Nishihara et al., 1995. *Biochem. Biophys. Res. Commun*. 212:255-262; Yilla et al., 1993. *JBC* 268:19092-19100
Monensin	Griffiths et al., 1983. *J. Cell Biol*. 96:835-850; Hardy et al., 1997. *JBC* 272:6812-6817; Kallen et al., 1993. *Biochim. Biophys. Acta* 1166:305-308; Shiao and Vance, 1993. *JBC* 268:26085-26092
Nigericin	Sandvig et al., 1989. *Methods Cell Biol*. 32:365-382; Scorrano et al., 1997. *JBC* 272:12295-12299; Vercesi et al., 1993. *JBC* 268:8564-8568
Drugs that lead to increased intracellular cAMP levels	
8-Bromo–cyclic AMP	Boyer and Thiery, 1993. *J. Cell Biol*. 120:767-776; Hei et al., 1991. *Mol. Pharmacol*. 39:233-238; Sandberg et al., 1991. *Biochem. J.* 279:521-527
Cholera toxin	Hansen and Casanova, 1994. *J. Cell Biol.* 126:677-687; Lencer et al., 1995. *J. Cell Biol.* 131:951-962; Ma and Weiss, 1995. *Methods Cell Biol*. 49:471-485; Moss and Vaughan, 1992. *Curr. Top. Cell. Regul*. 32:49-72
Dibutyryl cyclic AMP	Cong et al., 1998. *JBC* 273:660-666; O'Malley et al., 1997. *J. Cell Biol*. 138:159-165; Sandvig et al., 1994. *J. Cell Biol*. 126:53-64; Yuan et al., 1996. *JBC* 271:27090-27098
Forskolin	Galli et al., 1995. *J. Neurosci*. 15:1172-1179; Lippincott-Schwartz et al., 1991. *J. Cell Biol.* 112:567-577; Laurenza et al., 1989. *Trends Pharmacol. Sci*. 10:442-447; Nickel et al., 1996. *JBC* 271:15870-15873
Kinase inhibitors	
Bisindolylmaleimide I (GF 109203 X)	Das and White, 1997. *JBC* 272:14914-14920; Toullec et al., 1991. *JBC* 266:15771-15781; Uberall et al., 1997. *JBC* 272:4072-4078
Calphostin C	Dubyak and Kertesy, 1997. *Arch. Biochem. Biophys*. 341:129-139; Hartzell and Rinderknecht, 1996. *Am. J Physiol*. 270:C1293-C1299; Jarvis et al., 1994. *Cancer Res*. 54:1707-1714
Chelerythrine chloride	Barg et al., 1992. *J. Neurochem*. 59:1145-1152; Herbert et al., 1990. *Biochem. Biophys. Res. Commun*. 172:993-999; Jarvis et al., 1994. *Cancer Res*. 54:1707-1714; Jia et al., 1997. *JBC* 272:4978-4984; Kandasamy et al., 1995. *JBC* 270:29209-29216
Genistein	Akiyama et al., 1987. *JBC* 262:5592-5595; Chen et al., 1997. *JBC* 272:27401-27410; Kranenburg et al., 1997. *J. Cell Sci*. 110:2417-2427
H-7	Barria et al., 1997. *Science* 276:2042-2045; Kawamoto and Hidaka, 1984. *Biochem. Biophys. Res. Commun.* 125:258-264; Wang et al., 1997. *JBC* 272:1817-1821

continued

Table A.1B.1 Biological Activity of Drugs Commonly Used In Cell Biological Research, continued

Drug	References
Herbimycin A	Cowen et al., 1996. *JBC* 271:22297-22300; Fukazawa et al., 1991. *Biochem. Pharmacol*. 42:1661-1671; Tiruppathi et al., 1997. *JBC* 272:25968-25975
KN-62	Bouvard et al., 1998. *J. Cell Sci.* 111:657-665; Enslen and Soderling, 1994. *JBC* 269:20872-20877; Wang Hy et al., 1997. *JBC* 272:1817-1821; Doroudchi et al., 1997. *J. Neurosci. Res.* 50:514-521.
LY294002	Vlahos et al., 1994. *JBC* 269:5241-5248; Vlahos et al., 1995. *J. Immunol*. 154:2413-2422
ML-7	Ohkubo et al., 1996. *Eur. J. Pharmacol*. 298:175-183; Saitoh et al., 1987. *JBC* 262:7796-7801; Watanabe et al., 1998. *FASEB J.* 12:341-348
Olomoucine	Abraham et al., 1995. *Biol. Cell* 83:105-120; Glab et al., 1994. *FEBS Lett.* 353:207-211; Howell et al., 1997. *Cell. Motil. Cytoskeleton* 38:201-214; Misteli and Warren, 1995. *J. Cell Sci*. 108:2715-2727
PD 98059	Acharya et al., 1998. *Cell* 92:183-192; Alessi et al., 1995. *JBC* 270:27489-27494; Dudley et al., 1995. *PNAS* 92:7686-7689; Karpova et al., 1997. *Am. J. Physiol*. 272:L558-L565; Waters et al., 1995. *JBC* 270:20883-20886
Piceatannol	Keely and Parise, 1996. *JBC* 271:26668-26676; Oliver et al., 1994. *JBC* 269:29697-29703; Peters et al., 1996. *JBC* 271:4755-4762
Staurosporine	Couldwell et al., 1994. *FEBS Lett*. 345: 43-46; Kiss and Deli, 1992. *Biochem. J*. 288: 853-858; Janicke et al., 1998. *JBC* 273:9357-9360; Orr et al., 1998. *JBC* 273:3803-3807
Tyrphostins	Antonyak et al., 1998. *JBC* 273:2817-2822; Austin and Shields, 1996. *J. Cell Biol*. 135:1471-1483; Gazit et al., 1989. *J. Med. Chem*. 32:2344-2352; Gohla et al., 1998. *JBC* 273:4653-4659
Wortmannin	Cross et al., 1995. *JBC* 270:25352-25355; Goeger et al., 1988. *Biochem. Pharmacol*. 37:978-981; Jones and Howell, 1997. *J. Cell Biol*. 139:339-349; Ptasznik et al., 1997. *J. Cell Biol*. 137:1127-1136
Phosphatase inhibitors	
Calyculin A	Ishihara et al., 1989. *Biochem. Biophys. Res. Commun*. 159:871-877; Murakami et al., 1994. *Neurosci. Lett*. 176: 181-184; Takeuchi et al., 1994. *Biochem. Biophys. Res. Commun*. 205:1803-1807
Microcystin-LR	Bagu et al., 1997. *JBC* 272:5087-5097; Eriksson et al., 1990. *Biochim. Biophys. Acta* 1025:60-66; Honkanen et al., 1990. *JBC* 265:19401-19404; Rabouille et al., 1995. *J. Cell Biol*. 129:605-618; Toivola et al., 1997. *J. Cell Sci*. 110:23-33
Okadaic acid	Gjertsen et al., 1994. *J. Cell Sci*. 107:3363-3377; Haystead et al., 1989. *Nature* 337:78-81; Kiguchi et al., 1994. *Cell Growth Differ*. 5:995-1004; Lucocq, 1992. *J. Cell Sci*. 103:875-880; Ohoka et al., 1993. *Biochem. Biophys. Res. Commun*. 197:916-921; Suganuma et al., 1988. *PNAS* 85:1768-1771 Takai et al., 1987. *FEBS Lett*. 217:81-84
Phenylarsine oxide	Han and Kohanski, 1997. *Biochem. Biophys. Res. Commun*. 239:316-321; Fleming et al., 1996. *JBC* 271:11009-11015; Krutetskaia et al., 1997. *Tsitologiya* 39:1116-1130; Kussmann and Przybylski, 1995. *Methods Enzymol*. 251:430-435
Sodium orthovanadate	Brown and Gordon, 1984. *JBC* 259:9580-9586; Cook et al., 1997. *JBC* 272:13309-13319; Griffith et al., 1998. *JBC* 273:10771-10776; Munoz et al., 1992. *JBC* 267:10381-10388; Seedorf et al., 1995. *JBC* 270:18953-18960; Seglen and Gordon, 1981. *JBC* 256:7699-7701; Swarup et al., 1982. *Biochem. Biophys. Res. Commun*. 107:1104-1109

continued

Table A.1B.1 Biological Activity of Drugs Commonly Used In Cell Biological Research, continued

Drug	References
Protease inhibitors	
Calpain inhibitor I	Figueiredo-Pereira et al., 1994. *J. Neurochem*. 62: 1989-1994; Klafki et al., 1995. *Neurosci Lett.* 201:29-32; Milligan et al., 1996. *Arch. Biochem. Biophys*. 335:388-395
E-64	Banik et al., 1997. *Brain Res*. 1997 748:205-210; Bush et al., 1997. *JBC* 272:9086-9092; Sarin et al., 1994. *J. Immunol*. 153:862-872
Lactacystin	Choi et al., 1997. *JBC* 272:28479-28484; Dick et al., 1996. *JBC* 271:7273-7276; Fenteany et al., 1995. *Science* 268:726-731; Kim et al., 1997. *JBC* 272:11006-11010; Oda et al., 1996. *Biochem. Biophys. Res. Commun*. 219: 800-805
Leupeptin	Montenez et al., 1994. *Toxicol. Lett*. 73:201-208; Sarin et al., 1994. *J. Immunol*. 153:862-872; Wang et al., 1995. *JBC* 270:24924-24931
MG-132	Jensen et al., 1995. *Cell* 83:129-135; Lee and Goldberg, 1996. *JBC* 271:27280-27284; Meerovitch et al., 1997. *JBC* 272:6706-6713; Salceda and Caro, 1997. *JBC* 272:22642-22647
PMSF	Darby et al., 1998. *Biochemistry* 37:783-791; Turini et al., 1969. *J. Pharmacol. Exp. Ther*. 167:98-104; Weaver et al., 1993. *Biochem. Cell. Biol*. 71:488-500
Pepstatin A	Bode and Huber, 1992. *Eur. J. Biochem*. 204:433-451; Shields et al., 1991. *Biochem. Biophys. Res. Commun*. 177:1006-1012; Simon et al., 1995. *Biochim. Biophys. Acta* 1268: 143-151; Yamada et al., 1996. *J. Immunol*. 157:901-907; Wang et al., 1995. *JBC* 270:24924-24931
Protein synthesis inhibitors	
Anisomycin	Dong Chen et al., 1996. *JBC* 271:6328-6332; Kardalinou et al., 1994. *Mol. Cell. Biol*. 14:1066-1074; Sidhu and Omiecinski, 1998. *JBC* 273:4769-4775
Cycloheximide	Chow et al., 1995. *Exp. Cell Res*. 216:149-159; Cotter et al., 1992. *Anticancer Res*. 12:773-779; Waring, 1990. *JBC* 265:14476-14480
Emetine	Burhans et al., 1991. *EMBO J*. 10:4351-4360; Gabathuler et al., 1998. *J. Cell Biol*. 140:17-27; Sidhu and Omiecinski, 1998. *JBC* 273:4769-4775
Hygromycin	Gaken et al., 1992. *Biotechniques* 13:32-34; Hamada et al., 1994. *Curr. Genet*. 26:251-255; Lama and Carrasco, 1992. *JBC* 267:15932-15937
Puromycin	Chow et al., 1995. *Exp. Cell Res*. 216:149-159; Kislauskis et al., 1997. *J. Cell Biol*. 136:1263-1270; Tachibana et al., 1997. *EMBO J.* 16:4333-4339; Zhang et al., 1997. *Mol. Biol. Cell* 8:1943-1954
Transcription inhibitors	
Actinomycin D	Kuhn and Henderson, 1995. *JBC* 270:20509-20515; McGary et al., 1997. *JBC* 272:8628-8634; Wu and Yung, 1994. *Eur. J. Pharmacol.* 270:203-212
α-Amanitin	Baumann et al., 1994. *Protein Sci*. 3:750-756; Rudd and Luse, 1996. *JBC* 271:21549-21558; Seiser et al., 1995. *J. Biol. Chem.* 270:29400-29406
Other compounds	
Brefeldin A	Donaldson et al., 1991. *J. Cell Biol*. 112:579-588; Donaldson et al., 1992. *Nature* 360:350-352; Ktistakis et al., 1992. *Nature* 356:344-346; Lippincott-Schwartz et al., 1990. *Cell* 60:821-836
Cyclosporin A	Su et al., 1997. *J. Cell Biol*. 139:1533-1543; Sugimoto et al., 1997. *J. Biol. Chem*. 272:29415-29418; McKeon, 1991. *Cell* 66:823-826; Nicolli et al., 1996. *JBC* 271:2185-2192

continued

Table A.1B.1 Biological Activity of Drugs Commonly Used In Cell Biological Research, continued

Drug	References
Desferrioxamine	Ben-Shachar et al., 1995. *J. Neurochem*. 64:718-723; Bergeron et al., 1996. *J. Med. Chem*. 39:1575-1581; Dang et al., 1994. *Res. Commun. Mol. Pathol. Pharmacol*. 86: 43-57; Denicola et al., 1995. *Free Radical Biol. Med.* 19:11-19; Henderson and Kuhn, 1995. *JBC* 270:20509-20515
2-Deoxyglucose	Donaldson et al., 1991. *J. Cell Biol*. 112:579-588; Hill et al., 1998. *JBC* 273: 3308-3313; Villalba et al., 1994. *JBC* 269:2468-2476
Dithiothreitol	Braakman et al., 1992. *EMBO J*. 11:1717-1722; Lodish and Kong, 1993. *JBC* 268:20598-20605; Simons et al., 1995. *J. Cell Biol*. 130:41-49; Verde et al., 1995. *Eur. J. Cell Biol*. 67:267-274
Filipin	Liu et al., 1997. *JBC* 272:7211-7222; Schnitzer et al., 1994. *J. Cell Biol*. 127:1217-1232; Silberkang et al., 1983. *JBC* 258:8503-8511; Smart et al., 1996. *J. Cell Biol*. 134:1169-1177; Stahl and Mueller, 1995. *J. Cell Biol.* 129:335-344
Fumonisin B_1	Merrill et al., 1993. *JBC* 268:27299-27306; Sandvig et al., 1996. *Mol. Biol. Cell*. 7:1391-1404; Spiegel and Merrill, 1996. *FASEB J*. 10:1388-1397; Wang et al., 1991. *JBC* 266:14486-14490; Wang et al., 1996. *PNAS* 93:3461-3465
Geneticin	Canaani and Berg, 1982. *PNAS* 79:5166-5170; Southern and Berg, 1982. *J. Mol. Appli. Genet.* 1:327-341; Morris et al., 1996. *JBC* 271:15468-15477.
Leptomycin B	Fornerod et al., 1997. *Cell* 90:1051-1060; Fukuda et al., 1997. *Nature* 390:308-311; Wada et al., 1998. *EMBO J*. 17:1635-1641; Wolff et al., 1997. *Chem. Biol*. 4:139-147
Lovastatin	Carel et al., 1996. *JBC* 271:30625-30630; Hancock et al., 1989. *Cell* 57:1167-1177; Jakobisiak et al., 1991. *PNAS* 88:3628-3632. Mendola and Backer, 1990. *Cell Growth Differ*. 1:499-502; Vincent et al., 1991. *Biochem. Biophys. Res. Commun*. 180:1284-1289
Lysophosphatidic acid	An et al., 1998. *JBC* 273:7906-7910; Jalink et al., 1990. *JBC* 265:12232-12239; Moolenaar, 1995. *JBC* 270:12949-12952; Zhang et al., 1997. *Mol. Biol. Cell*. 8:1415-1425
Mastoparan	Huber et al., 1997. *J. Cell Sci*. 110: 2955-2968; Klinker et al., 1996. *Biochem. Pharmacol*. 51:217-223; Konrad et al., 1995. *JBC* 270: 12869-12876; Schwaninger et al., 1992. *J. Cell Biol*. 119:1077-1096; Smith et al., 1995. *JBC* 270:18323-18328
Ouabain	Croyle et al., 1997. *Eur. J. Biochem*. 248:488-495; Peng et al., 1996. *JBC* 271: 10372-10378; Swann and Steketee, 1989. *J. Neurochem*. 52:1598-1604
PDMP	Chen et al., 1995. *JBC* 270:13291-13297; Inokuchi et al., 1987. *Cancer Lett*. 38:23-30; Maceyka and Machamer, 1997. *J. Cell Biol*. 139:1411-1418; Rosenwald et al., 1992. *Biochemistry* 31:3581-3590; Sandvig et al., 1996. *Mol. Biol. Cell*. 7:1391-1404; Uemura et al., 1990. *J. Biochem.* 108:525-530
Pertussis toxin	An et al., 1998. *JBC* 273:7906-7910; Hewlett et al., 1983. *Infect. Immun*. 40:1198-1203; Jakobs et al., 1984. *Eur. J. Biochem*. 140:177-181; Kopf and Woolkalis, 1991. *Methods Enzymol*. 195:257-266; Luttrell et al., 1997. *JBC* 272:31648-31656
Phorbol esters	Janecki et al., 1998. *JBC* 273:8790-8798; Macfarlane and O'Donnell, 1993. *Leukemia* 7:1846-1851; Niedel et al., 1983. *PNAS* 80:36-40; Nishizuka, 1986. *Science* 233:305-312; Oishi and Yamaguchi, 1994. *J. Cell. Biochem*. 55: 168-172; Schmidt et al., 1998. *JBC* 273:7413-7422; Tepper et al., 1995. *PNAS* 92:8443-8447

continued

Table A.1B.1 Biological Activity of Drugs Commonly Used In Cell Biological Research, continued

Drug	References
Rapamycin	Brown et al., 1994. *Nature* 369:756-758 Jefferies et al., 1997. *EMBO J*. 16:3693-3704 Kozlovsky et al., 1997. *J. Biol. Chem*. 272:33367-33372 Liu et al., 1991. *Cell* 66:807-815 Sabers et al., 1995. *J. Biol. Chem.* 270:815-822
Sodium azide	Bhat et al., 1996. *JBC* 271:32551-32556; Donaldson et al., 1991. *J. Cell Biol*. 112:579-588; van Klompenburg et al., 1997. *EMBO J.* 16:4261-4266
Sodium butyrate	Calabresse et al., 1993. *Biochem. Biophys. Res. Commun.* 195:31-38; Gonzalez-Garay and Cabral, 1996. *J. Cell Biol*. 135:1525-1534; Russo et al., 1997. *Biochem. Biophys. Res. Commun.* 233:673-677; Vaziri et al., 1996. *JBC* 271:25921-25927; White et al., 1995. *J. Cell Sci*. 108:441-455
Trifluoperazine	Aussel et al., 1995. *JBC* 270:8032-8036; Massom et al., 1990. *Biochemistry* 29:671-681; Rao, 1987. *Biochem. Biophys. Res. Commun*. 148:768-775
Valinomycin	Inai et al., 1997. *Cell Struct. Funct*. 22:555-563; Loiseau et al., 1997. *Biochim. Biophys. Acta*. 1330:39-49; Orlov et al., 1994. *FEBS Lett*. 345:104-106; Szabo et al., 1997. *JBC* 272:23165-23171
W-7	de Figueiredo and Brown, 1995. *Mol. Biol. Cell*. 6:871-887; Hidaka et al., 1981. *PNAS* 78:4354-4357; Hunziker, 1994. *JBC* 269:29003-29009; Wolf and Gross, 1996. *JBC* 271:20989-20992

DRUGS COMMONLY USED IN CELL BIOLOGY

A23187

Calcium ionophore; forms stable complexes with divalent cations and increases their passage across biological membranes. Useful tool for increasing intracellular calcium concentration. The effectiveness of A23187 is dependent on the presence of extracellular calcium. Can be used as a fluorescent probe for investigating protein hydrophobicity. 4-Bromo-A23187 is a nonfluorescent derivative.
Soluble in: DMSO, methanol
Stock concentration: 100 mM (store at 4°C protected from light)
Working concentration: 0.1 to 20 μM
Duration of incubation: 2 min to 24 hr

Aggregates over time in aqueous systems.

Actinomycin D

Inhibits transcription by complexing with deoxyguanosine residues on DNA and blocking the movement of RNA polymerase. A potent inducer of apoptosis in many cell lines. However, actinomycin D has also been shown to suppress programmed cell death of PC12 cells induced by the topoisomerase II inhibitor etoposide.
Soluble in: Methanol
Stock concentration: 100 mM (store at 4°C)
Working concentration: 1 to 5 μM
Duration of incubation: 5 min to 24 hr

α-Amanitin

Acts as a potent and specific inhibitor of mRNA synthesis by binding preferentially to RNA polymerase II. At high concentrations also inhibits RNA polymerase III.
Soluble in: Methanol, water
Stock concentration: 2 to 10 mg/ml (store at 4°C protected from light)
Working concentration: 1 to 10 μg/ml (pol II) to 200 μg/ml (pol III)
Duration of incubation: 15 to 60 min

Ammonium chloride (NH_4Cl)

Permeant weak base. Used to neutralize acidic endomembrane compartments. Inhibits synthesis of sphingoid bases.
Soluble in: Water (freely soluble)
Stock concentration: 5 M (store at 4°C)
Working concentration: 1 to 50 mM
Duration of incubation: Effective within 15 sec

Anisomycin

Inhibits protein synthesis by blocking the peptidyl transferase step during translation. Activates p54 (JNK2) and MAP kinases. May be a useful tool to study cytoplasmic signals that result in nuclear signaling and *c-fos* and *c-jun* induction. Also known to induce apoptosis in U937 cells.
Soluble in: DMSO
Stock concentration: 100 μg/ml (store at 4°C)
Working concentration: 50 ng/ml to 1 μg/ml.
Duration of incubation: 30 min to 16 hr, depending on properties studied

Aphidicolin

Cell synchronization reagent. Reversible inhibitor of DNA polymerase α and δ; blocks cell cycle at early S phase. Potentiates apoptosis induced by arabinosyl nucleosides in leukemia cell lines.
Soluble in: DMSO, methanol
Stock concentration: 2 mg/ml (store at 4°C)
Working concentration: 0.5 to 100 μg/ml.
Duration of incubation: 12 to 24 hr

Ara-C (cytosine arabinoside)

Inhibits DNA synthesis. S-phase-toxic reagent whose active metabolite (ara-CTP) is a substrate for DNA polymerases and is incorporated into DNA. Anticancer, antiviral agent that is especially effective against leukemias. Induces apoptosis in human myeloid leukemia cells and in rat sympathetic neurons.
Soluble in: Water
Stock concentration: 20 mg/ml (store at 4°C)
Working concentration: 0.1 to 1 μg/ml
Duration of incubation: >3 hr

Bafilomycin A_1

A potent and specific inhibitor of vacuolar-type H^+-ATPases. Valuable tool for distinguishing different types of ATPases. Blocks lysosomal trafficking in macrophages.
Soluble in: DMSO
Stock concentration: 50 μM (store at −20°C protected from light)
Working concentration: 10 to 100 nM
Duration of incubation: 10 min to 2 hr

BAPTA

Ca^{2+} chelator with a 10^5-fold greater affinity for Ca^{2+} than for Mg^{2+}; can be used to control the level of both intracellular (using its membrane-permeant AM ester) and extracellular Ca^{2+}.
Soluble in: DMSO
Stock concentration: 1 to 10 mM (store at −20°C in aliquots, protected from light; avoid repeated freeze-thawing)
Working concentration: (BAPTA-AM): 1 to 20 μM
Duration of incubation: 15 to 60 min at 20° to 37°C

Before incubation with BAPTA, wash cells 2 to 3 times with serum-free medium (serum may contain esterase activity). The cell-loading medium should also be free of amino acids or buffers containing primary or secondary amines that may cleave the AM esters and prevent loading.

Bisindolylmaleimide I (GF 109203X)

A highly selective cell-permeant protein kinase C (PKC) inhibitor that is structurally similar to staurosporine, but has higher selectivity. May inhibit protein kinase A at high concentrations. Acts as a competitive inhibitor for the ATP-binding site of the PKC catalytic domain. Since ATP levels are generally very high in cells, the potency of bisindolylmaleimide I is reduced accordingly in whole-cell assays.
Soluble in: DMSO
Stock concentration: 2 mM (store at ≤4°C)
Working concentration: 20 nM to 1 μM
Duration of incubation: 15 min to 6 hr

Water-soluble salts are available.

Brefeldin A

Inhibits GTP nucleotide exchange onto several members of the ARF (ADP ribosylation factor) family. Inhibits binding of the cytosolic coatomer (COPI) complex to Golgi membranes; induces the rapid redistribution of the Golgi apparatus into the ER; blocks transport out of the ER in a number of cell lines. Reversible.
Soluble in: Methanol
Stock concentration: 1 to 20 mM (store at −20°C)
Working concentration: 1 to 5 μM
Duration of incubation: 5 min to 24 hr; effects are rapid (30 sec)

8-Bromo–cyclic AMP

Cell-permeant cyclic AMP analog. Activates protein kinase A. Increased resistance to degradation by cellular phosphodiesterases as compared to cyclic AMP.
Soluble in: Water
Stock concentration: 100 mM (store at −20°C)
Working concentration: 10 to 500 μM
Duration of incubation: Up to 24 hr

Calpain inhibitor I (ALLN)

Inhibitor of calpain I, calpain II, cathepsin B, and cathepsin L. Peptide aldehyde; inhibits neutral cysteine proteases and the proteosome. Protects against neuronal damage caused by hypoxia and ischemia. Inhibits proteolysis of IkB by the ubiquitin-proteosome complex. Inhibits cell-cycle progression at G_1/S and metaphase/anaphase in CHO cells by inhibiting cyclin B degradation. Membrane-permeant due to low molecular weight and lack of charged residues.
Soluble in: DMSO, methanol, dimethylformamide
Stock concentration: 25 mM (store at 4°C)
Working concentration: 25 to 100 μM
Duration of incubation: 1 to 18 hr

Calphostin C

Potent and highly selective inhibitor of protein kinase C (PKC; K_i = 50 nM). Competes with phorbol esters and diacylglycerol for binding to the PKC regulatory domain. Does not compete with Ca^{2+} or phospholipids. At higher concentrations inhibits myosin light chain kinase (K_i > 5 μM), protein kinase A (K_i > 50 μM), protein kinase G (K_i > 25 μM), and $p60^{v\text{-}src}$ (K_i > 50 μM).
Soluble in: DMSO
Stock concentration: 1 mM (store 4°C protected from light)
Working concentration: 10 nM to 3 μM
Duration of incubation: 15 to 60 min

Brief exposure to visible light in the presence of PKC is required for PKC inhibition by calphostin C. See Table 1 (Selectivity of Selected Kinase Inhibitors) in the Biomol Catalog and Handbook, 5th ed., and Technical Note #11 from Alexis Biochemicals for additional information.

Calyculin A

Potent cell-permeant inhibitor with high specificity for the Ser/Thr protein phosphatases 1 and 2A. Calyculin A is 20 to 300 times more potent than okadaic acid as a PP-1 class phosphatase inhibitor. Stimulates contraction of smooth muscle, induces intracellular protein phosphorylation in cultured human keratinocytes, and inhibits apoptosis.
Soluble in: DMSO, ethanol
Stock concentration: 10 μM (store at −20°C protected from light and moisture)
Working concentration: 0.5 to 50 nM
Duration of incubation: 15 min to 2 hr

See Technical Note #19 from Alexis Biochemicals for additional information.May cause cell rounding.

Camptothecin

A reversible DNA topoisomerase I inhibitor. Induces breaks at replication forks by binding to and stabilizing the topoisomerase-DNA covalent complex. Causes S-phase cytotoxicity. Posesses anti-leukemic and anti-tumor properties. Inhibits *tat*-mediated transactivation of HIV-1.
Soluble in: DMSO
Stock concentration: 1 to 10 mM (store at 4°C)
Working concentration: 0.1 to 10 μM
Duration of incubation: Literature shows use from 15 min to 24 hr

Castanospermine

Inhibitor of α- and β-glucosidases; inhibitor of glycoprotein processing. Prevents calnexin and calreticulin binding to N-linked glycans on newly synthesized glycoproteins. Inhibits HIV infectivity.
Soluble in: Water
Stock concentration: 1 mM (store at 4°C)
Working concentration: 1 to 5 μM
Duration of incubation: 15 min to 3 hr

CCCP (carbonyl cyanide-m-chlorophenyl hydrazone)

Proton ionophore. Uncoupling agent for oxidative phosphorlyation that inhibits mitochondrial function. Approximately 100 times as effective as 2,4-dinitrophenol at collapsing membrane potential. Inhibits transport processes and depresses growth.
Soluble in: DMSO, ethanol
Stock concentration: 1 to 10 mM (store at 4°C)
Working concentration: 1 to 5 μM
Duration of incubation: 5 to 15 min

Chelerythrine chloride

Potent, selective, cell-permeant inhibitor of protein kinase C (K_i = 0.66 μM). Acts on the catalytic domain. Chelerythrine shows competitive kinetics with PKC substrates, but is not competitive with ATP. Thus, the high concentration of ATP within cells should not lower the potency of chelerytherine in whole cells as compared with that seen in purified enzyme preparations. Inhibits thromboxane formation and phosphoinositide metabolism in platelets. Induces apoptosis in HL-60 cells.
Soluble in: DMSO
Stock concentration: 10 mM (store at –20°C)
Working concentration: 1 μM
Duration of incubation: 15 min to 2 hr

See Technical Note #8 from Alexis Biochemicals for additional information.

Chloroquine

Tertiary amine that accumulates within and neutralizes the pH of acidic organelles; various effects on phagosome-endosome and phagosome-lysosome fusion. Antimalarial drug that works via carrier-mediated uptake in *P. falciparum*. May activate protein kinases.
Soluble in: Water
Stock concentration: 1 to 10 mg/ml (store at room temperature)
Working concentration: 10 to 200 μg/ml
Duration of incubation: 15 min to 2 hr

Cholera toxin

Contains a single A subunit (mol. wt. = 29 kDa) and a B subunit (mol. wt. = 55 kDa) containing five B polypeptide chains. The B subunit binds to GM_1 ganglioside receptors on the surface of cells and facilitates transport of the A subunit through the membrane. The A subunit catalyzes the ADP-ribosylation of an arginine residue on the α subunit of heterotrimeric G proteins (primarily G_s), reducing its intrinsic GTPase activity. Toxicity results from activation of membrane-bound adenylate cyclase. Consequently, increased intracellular cAMP levels result in increased electrolyte transport out of the cell and water loss. Cholera toxin requires ADP-ribosylation factor (ARF) for maximal activity.
Soluble in: Water
Stock concentration: 1 mg/ml (store at 4°C, do not freeze)
Working concentration: 100 ng/ml to 2 μg/ml
Duration of incubation: 2 to 24 hr

Colcemid

Cell synchronization agent. Depolymerizes microtubules and limits microtubule formation. Low concentrations inactivate spindle dynamics. Induces apoptosis by blocking mitosis in HeLa S3 cells. Colcemid is a less toxic derivative of colchicine.
Soluble in: Ethanol
Stock concentration: 1 mM (store at or below room temperature)
Working concentration: 1 to 10 μM
Duration of incubation: 1 to 24 hr, depending on process studied

Colchicine

Inhibitor of mitosis, used in cell-division studies. Disrupts microtubules and inhibits tubulin polymerization. Induces apoptosis in PC 12 cells and in cerebellar granule cells.
Soluble in: Ethanol
Stock concentration: 1 mM (store at or below room temperature protected from light and moisture)
Working concentration: 1 to 10 μM
Duration of incubation: 1 to 24 hr, depending on process studied

Concanamycin B

Highly specific and sensitive inhibitor of vacuolar-type H^+-ATPases (K_i = 20 pM). Related to concanamycin A (folimycin). More potent and specific than bafilomycin A_1. Inhibits acidification of organelles such as lysosomes and the Golgi apparatus. Blocks cell-surface expression of viral glycoproteins without affecting their synthesis.
Soluble in: Methanol, ethanol
Stock concentration: 10 μM (store at −20°C protected from light)
Working concentration: 50 nM
Duration of incubation: 5 min to 1 hr

Cycloheximide

Inhibits protein synthesis in eukaryotes but not prokaryotes. Blocks the translocation step during translation. Induces apoptosis in a number of cell types. However, it inhibits DNA cleavage in rat thymocytes treated with thapsigargin and ionomycin.
Soluble in: Water, ethanol, methanol
Stock concentration: 10 mg/ml (store at or below room temperature)
Working concentration: 1 to 100 μg/ml, depending on cell type
Duration of incubation: Effective within 10 min

To achieve >90% inhibition of protein synthesis, only 1 to 10 μg/ml is required in CHO and HeLa cells, but 100 μg/ml is required in COS cells.

Cyclosporin A (CsA)

Cyclic oligopeptide with immunosuppressant properties. Induces apoptosis in some cell types, while inhibiting apoptosis in others. A complex of cyclosporin A and cyclophilin inhibits protein phosphatase 2B (calcineurin) with affinity at the nanomolar level. Inhibits nitric oxide synthesis induced by interleukin-1α, lipopolysaccharides, and TNFα.
Soluble in: Ethanol, methanol
Stock concentration: 1 to 5 mM (store at 4°C)
Working concentration: 0.1 to 10 μM
Duration of incubation: Used anywhere from 15 min to 24 hr

Cytochalasin B

Cell-permeant fungal toxin that blocks the formation of contractile microfilaments. Shortens actin filaments by blocking monomer addition at the barbed (fast-growing)

continued

end of polymers. Inhibits cytoplasmic division, cell movement, phagocytosis, platelet aggregation, and glucose transport.
Soluble in: DMSO, ethanol
Stock concentration: 10 mM (store at −20°C protected from light)
Working concentration: 1 to 20 μM
Duration of incubation: 15 min to 2 hr

Cytochalasin D

Approximately 10-fold more potent than cytochalasin B in inhibiting actin filament function. Does not inhibit sugar transport in cells. Modulates CD4 cross-linking in T lymphocytes and increases intracellular Ca^{2+}. Exhibits antitumor activity.
Soluble in: DMSO
Stock concentration: 10 mM (store at −20°C protected from light)
Working concentration: 1 to 20 μM
Duration of incubation: 15 min to 2 hr.

Desferrioxamine (DFO)

Iron-chelating agent. Commonly used in therapy as a chelator of ferric iron in iron overload disorders. Protects against dopamine-induced cell death. Also interferes with hydroxy-radical formation. Shows an antiproliferative effect on vascular smooth muscle cells.
Soluble in: DMSO; slightly soluble in water
Stock concentration: 10 to 50 mM (store at 4°C)
Working concentration: 10 μM to 2 mM
Duration of incubation: Up to 18 hr

2-Deoxyglucose

Nonmetabolizable derivative of glucose. Competes with glucose for the GLUT-2 transporter; phosphorylation of 2-deoxyglucose by hexokinase effectively inhibits glucose flux through the glycolytic pathway. Used in combination with sodium azide or oligomycin to reduce cellular ATP levels. Blocks inhibition of IL-1 release by high glucose levels in RAW 264.7 cells.
Soluble in: Water
Stock concentration: 1 M (store at 4°C)
Working concentration: 5 to 50 mM
Duration of incubation: 15 min to 3 hr

Deoxymannojirimycin

Competitive α-mannosidase I inhibitor that blocks conversion of high mannose forms to complex oligosaccharides. Inhibits mammalian Golgi α-mannosidase I (an α-1,2-mannosidase). Other rat liver mannosidases are not significantly affected (α-1,2–specific ER mannosidase is only inhibited 2% to 5% by 100 mM deoxymannojirimycin, and Golgi α-mannosidase II is inhibited ~14%).
Soluble in: Water
Stock concentration: 100 mM (store at −20°C)
Working concentration: 1 to 5 mM
Duration of incubation: Anywhere from 30 min to 24 hr

Deoxynojirimycin

Specific glucosidase inhibitor. Inhibits endoplasmic reticulum trimming glucosidases I and II, which sequentially remove three glucose residues from $Glc_3Man_9GlcNAc_2$ in N-linked glycan biosynthesis. Prevents calnexin and calreticulin binding to N-linked glycoproteins within the ER.

continued

Soluble in: Water
Stock concentration: 100 mM (store at 4°C)
Working conditions: 1 to 5 mM.
Duration of incubation: 15 min to 24 hr

At concentrations >1 mM, deoxynojirimycin may inhibit lipid-linked oligosaccharide biosynthesis as well as trimming. In such cases, N-methyldeoxynojirimycin may be a more effective inhibitor, possibly owing to an increased ability to cross cell membranes, afforded by the N-methyl group.

Dibutyryl cyclic AMP

Highly membrane-permeant cAMP analog resistant to phosphodiesterase cleavage. Constitutive activator of protein kinase A. This product releases butyrate due to intracellular and extracellular esterase action. Butyrate may have its own distinct biological effects (see sodium butyrate).
Soluble in: DMSO, ethanol
Stock concentration: 1 M (store at −20°C)
Working concentration: 100 μM to 1 mM
Duration of incubation: Anywhere from 1 to 48 hr

Dithiothreitol (DTT; Cleland's reagent)

Cell-permeant protective agent for SH groups; maintains monothiols completely in the reduced state and reduces disulfides quantitatively. DTT interferes with the folding and export of proteins located in the endoplasmic reticulum, but it does not prevent the transfer from the intermediate compartment to the Golgi complex. Reversible.
Soluble in: Water, ethanol
Stock concentration: 1 M (store at 4°C)
Working concentration: 1 to 10 mM
Duration of incubation: 1 min to several hours

E-64

Irreversible inhibitor of cysteine proteases (papain and cathepsins B and L). Has no action on cysteine residues in other proteins.
Soluble in: Water
Stock concentration: 1 mg/ml (store at −20°C)
Working concentration: 0.5 to 10 μg/ml
Duration of incubation: Up to 24 hr

Emetine

Irreversibly blocks protein synthesis by inhibiting movement of ribosomes along mRNA. Stimulates rapid and differential phosphorylation of the stress-activated protein kinase/c-Jun kinase (SAPK/JNK) pathway. Prevents apoptosis in several cell lines. In primary rat hepatocytes, the relative potency of inhibition of several protein synthesis inhibitors is in the order: emetine > anisomycin > cycloheximide > puromycin, with puromycin exhibiting only marginal inhibition at a concentration of 1 μM. In fact, 90% to 95% inhibition of protein synthesis was achieved only with emetine and anisomycin, at 10 μM concentrations. Cycloheximide and puromycin exerted only 80% and 60% inhibition, respectively, at a similar concentration.
Soluble in: Ethanol
Stock concentration: 10 to 100 mM (store at 4°C protected from light)
Working concentration: 10 to 20 μM
Duration of incubation: 15 to 30 min; can incubate 24 hr

Etoposide (VP-16)

Topoisomerase II inhibitor. Stabilizes the covalent complexes of topoisomerase II with DNA. Has major activity against a number of tumors, including germ cell neoplasms, small cell lung cancer, and malignant lymphoma. Induces apoptosis in mouse thymocytes and HL-60 cells. Activates PKCα.
Soluble in: DMSO
Stock concentration: 100 to 500 mM (store at room temperature)
Working concentration: 50 to 200 μM
Duration of incubation: 1 to 24 hr

Filipin

A cholesterol-binding fluorochrome. Specific for unesterified cholesterol. Binds and removes cholesterol from cell surface membranes. Reversibly disassembles caveolae.
Soluble in: Methanol
Stock concentration: 500 μg/ml (store 4°C protected from light)
Working concentration: 5 to 50 μg/ml
Duration of incubation: 1 hr

Forskolin

Activates adenylate cyclase by interacting directly with the catalytic subunit. Leads to an increase in the intracellular concentration of cAMP. Several forskolin derivatives are available having different and improved properties. Enhances detoxification of brefeldin A.
Soluble in: DMSO, ethanol
Stock concentration: 10 to 100 mM (store at 4°C)
Working concentration: 10 μM (to increase cAMP levels); 100 μM to inhibit brefeldin A
Duration of incubation: Depending on assay, incubate cells 15 min to 12 hr

Fumonisin B_1

Inhibits sphingolipid biosynthesis via inhibition of sphingosine *N*-acyltransferase (ceramide synthase). Sphingomyelin biosynthesis is preferentially inhibited versus glycosphingolipids in neuronal cells. Inhibits the butyric acid–induced increase in transport of cell-associated Shiga toxin to the Golgi apparatus and the ER. Induces apoptosis in monkey kidney cells.
Soluble in: methanol
Stock concentration: 10 to 100 mM (store at 4°C)
Working concentration: Anywhere from 1 to 100 μM
Duration of incubation: 15 min to 18 hr, depending on process studied

Geneticin (G418)

Aminoglycoside toxic to bacteria, yeast, higher plants, protozoa, and mammalian cells. Used for the selection and maintenance of eukaryotic cells stably transfected with the neomycin (*neo*) resistance genes from transposons Tn*5* and Tn*601*.
Soluble in: Water or culture medium
Stock concentration: 2 mg/ml (active G418) in cell culture medium, adjust pH to ~7.4 (store at 4°C)
Working concentration: Usually 50 to 1000 μg/ml (optimal concentration must be determined experimentally and varies with the cell type used)
Duration of incubation: >1 week

In cell types with relatively stable genomes (e.g., CHO), continuous incubation in geneticin is not generally necessary once stable cells have been selected. Expression in stable cells with down-regulated viral promoters can be enhanced with sodium butyrate.

Genistein

Inhibits protein tyrosine kinases by acting as a competitive inhibitor of ATP. Prevents EGF-stimulated tyrosine phosphorylation in A431 cells, as well as inhibiting kinases in other cultured cells.
Soluble in: DMSO
Stock concentration: 100 to 500 mM (store at −20°C)
Working concentration: 50 to 300 μM
Duration of incubation: 15 min to 1 hr

See Table II (Selectivity of Tyrosine Protein Kinase Inhibitors) in the Biomol Catalog and Handbook, 5th ed.

H-7

A broad-based, cell-permeant serine/threonine kinase inhibitor. Inhibits protein kinase C (K_i = 6.0 μM), protein kinase A (K_i = 3.0 μM), protein kinase G (K_i = 5.8 μM), and myosin light chain kinase (K_i = 97 μM). Induces apoptotic DNA fragmentation and cell death in HL-60 cells. Numerous analogs with different selectivities are available.
Soluble in: Water
Stock concentration: 100 mM (store at 4°C)
Working concentration: 10 to 100 μM
Duration of incubation: 15 min to 3 hr

See Table I (Selectivity of Selected Kinase Inhibitors) in the Biomol Catalog and Handbook, 5th ed., and Technical Note #10 from Alexis Biochemicals.

Herbimycin A

An irreversible and selective cell-permeant protein tyrosine kinase inhibitor; reacts with thiol groups. It is effective on Src, Yes, Fps, Ros, Abl, and ErbB oncogene products. Inhibits PDGF-induced phospholipase D activation in a dose-dependent manner.
Soluble in: DMSO
Stock concentration: 10 mM (store at −20°C protected from light)
Working concentration: 1 to 10 μM
Duration of incubation: 15 min to 1 hr

Hydroxyurea

Antineoplastic reagent. Blocks DNA synthesis by inhibiting ribonucleotide reductase; accumulates cells at G1/S interface.
Soluble in: Water
Stock concentration: 1 M (store at 4°C)
Working concentration: 50 μM to 1 mM
Duration of incubation: Up to 24 hr

Hygromycin

Inhibitor of both prokaryotic and eukaryotic protein synthesis; inhibits at the translocation step on 70S ribosomes and causes misreading of mRNA. The *E. coli hph* hygromycin B–resistant gene is widely used for selection of recombinant clones in a variety of cell types.

Hygromycin B is sold as an aqueous solution. Actual activity and concentration are given for each lot of product. Concentrations used must be experimentally determined.

Ionomycin

Ca^{2+} ionophore. Useful for increasing intracellular Ca^{2+} concentrations and in measurement of cytoplasmic free Ca^{2+}. More effective than A23187 and is nonfluorescent.
Soluble in: DMSO and methanol
Stock concentration: 1 mM (store at 4°C protected from light)

continued

Working concentration: Usually 1 μM
Duration of incubation: 15 to 30 min.

KN-62

Potent and selective inhibitor of Ca^{2+}/calmodulin kinase II (K_i = 0.9 μM), displaying a K_i for CaM kinase II more than 2 orders of magnitude lower than those for protein kinase C, protein kinase A, and myosin light chain kinase. A second inhibitor of CaM kinase II, KN-93, is more soluble in water and equally selective for CaM kinase II (K_i = 0.3 μM). Prevents agonist-mediated activation of Ins(1,4,5)P_3 3-kinase. Inhibits differentiation of 3T3-L1 embryonic fibroblasts to adipocytes. Inactive analogs are available.
Soluble in: DMSO
Stock concentration: 10 mM (store at 4°C)
Working concentration: 2 to 10 μM
Duration of incubation: 30 min (can incubate cells up to 48 hr)

Lactacystin

A cell-permeant and irreversible proteosome inhibitor. Blocks proteosome activity by targeting the catalytic β subunit. Induces neurite outgrowth in Neuro 2A mouse neuroblastoma cells and inhibits progression of synchronized Neuro 2A cells and MG-63 human osteosarcoma cells beyond the G_1 phase of the cell cycle. Inhibits NFκB activation. Has revealed the role of the proteosome in the degradation of many ER proteins.
Soluble in: DMSO
Stock concentration: 10 mM (store at −20°C)
Working concentration: 10 to 20 μM
Duration of incubation: 1 to 12 hr

Latrunculin A

Inhibits actin polymerization and disrupts microfilament organization as well as microfilament-mediated processes; 10 to 100-fold more potent than cytochalasins. Whereas cytochalasins induce dissolution of F-actin and stress-fiber contraction in fibroblasts in culture, the latrunculins (A and the less potent B) cause a shortening and thickening of stress fibers. In addition, the latrunculins sequester actin monomers, whereas with the cytochalasins, actin remains in an oligomer form. Thus, the two classes of compounds may have different target sites. Reversible.
Soluble in: DMSO, ethanol
Stock concentration: 10 mg/ml (store at −20°C)
Working concentration: 0.2 to 10 μg/ml
Duration of incubation: 1 to 12 hr

Leptomycin B

A potent and specific inhibitor of the NES-dependent nuclear export of proteins; binds to the export receptor CRM1. Exhibits antifungal and antitumor effects, inhibits the nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 regulatory protein Rev, and exhibits significant antiproliferative activity.
Soluble in: Ethanol
Stock concentration: 100 μM to 1 mM (store at −20°C)
Working concentration: 10 to 100 nM
Duration of incubation: 30 min to 3 hr

Leupeptin

A reversible inhibitor of trypsin-like and cysteine proteases (including trypsin, plasmin, proteinase K, papain, thrombin, and cathepsin A and B). Inhibits activation-induced programmed cell death in T lymphocytes.
Soluble in: Water
Stock concentration: 1 to 10 mM (store at −20°C)
Working concentration: 10 to 100 μM
Duration of incubation: 15 min to several hours

Lovastatin

An antihypercholesterolemic agent and inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; depletes endogenous pools of mevalonic acid, thereby blocking protein isoprenylation and cholesterol synthesis. Has a number of cellular effects. Blocks *N-ras* oncogene–induced neuronal differentiation, inhibits growth factor signaling and causes cells to arrest in late G_1 phase.
Soluble in: DMSO, ethanol
Stock concentration: 4 mg/ml (store at −20°C)
Working concentration: 20 μM
Duration of incubation: 6 to 24 hr

Lysophosphatidic acid (LPA)

Activates a number of signaling pathways and processes via heterotrimeric G proteins (primarily G_i and G_q) including: inhibition of adenylate cyclase, activation of Ras and the Raf/MAP kinase pathway, stimulation of phospholipases C and D, and stress-fiber formation through the activation of Rho.

Solubility: A stock solution may be prepared at 10 mg/ml in 95:5:5 chloroform/methanol/acetic acid (gives a clear solution). Solubility in dimethysulfoxide (DMSO) or ethanol is limited. The sodium salt of oleoyl-LPA is reported to be readily soluble at 5 mg/ml (~11 mM) in calcium and magnesium-free buffers (Jalink et al., 1990). Solubilization has also been achieved (Seufferlein and Rozengurt, 1994) in phosphate-buffered saline (PBS), pH 7.4, or calcium- and magnesium-free Dulbecco's PBS (CMF-DPBS), pH 7.4 (see *APPENDIX 2A* for recipes), at up to 3 mM (0.14 mg/ml) in the presence of 0.1% (w/v) BSA (essentially fatty-acid free).

Storage. LPA should be stable in solution under neutral conditions. Freezer storage is recommended for solutions or aqueous preparations. Maintaining the product under an inert atmosphere (nitrogen or argon) may be appropriate for some applications.

Working concentration. Anywhere from 500 nM (1-hr incubation) to 100 μM (15-min incubation).

LY294002 (PI 3-kinase inhibitor)

Reversible inhibitor of phosphatidylinositol-3-kinase that acts on the ATP-binding site of the enzyme. Does not affect the activity of EGF receptor kinase, MAP kinase, PKC, PI4-kinase, S6 kinase, and *c-src*. Blocks proliferation of cultured rabbit aortic smooth muscle cells without inducing apoptosis. Wortmannin is more selective and more potent, but is irreversible.
Soluble in: DMSO and ethanol
Stock concentration: 1 to 10 mM (store in aliquots at −20°C)
Working concentration: 1 to 2 μM
Duration of incubation: 15 min to 3 hr

Mastoparan

Relatively cell-permeant synthetic peptide capable of directly activating pertussis toxin–sensitive G proteins by a mechanism analogous to that of G-protein-coupled receptors. Acts preferentially on G_i and G_o rather than G_s. Stimulates insulin secretion in permeabilized cells, and can increase intracellular Ca^{2+} levels. Inhibits calmodulin and activates phospholipase A_2.
Soluble in: Water
Stock concentration: 1 mM (store −20°C)
Working concentration: 10 to 50 μM
Duration of incubation: 15 min to 1 hr

Microcystin-LR

Cyclic heptapeptide; potent inhibitor of protein phosphatases 1 and 2A (PP-1 and PP-2A). Unlike okadaic acid, microcystin-LR is equally effective on both PP-1 (K_i = 1.7 nM) and PP-2A (K_i = 0.04 nM). Has no effect on protein kinases, making it useful for reducing the effect of contaminating phosphatases in protein kinase assays. It is not cell-permeant, but can enter hepatocytes via the multispecific organic anion transporter.
Soluble in: DMSO, ethanol, methanol
Stock concentration: 1 mM (store at −20°C)
Working concentration: 1 to 5 μM in hepatic cells; 10 μM in vitro
Duration of incubation: 15 min to 1 hr

MG-132

A potent, reversible and cell-permeant proteosome inhibitor. Reduces the degradation of ubiquitin-conjugated proteins by the 26S complex without affecting its ATPase or isopeptidase activities. Has been used to implicate the proteosome in the breakdown of membrane proteins, including the CFTR, within the ER (see also lactacystin). Inhibits NFκB activation.
Soluble in: DMSO
Stock concentration: 10 to 100 mM (store at −20°C)
Working concentration: 20 to 200 μM
Duration of incubation: 30 min to 24 hr

ML-7 (MLCK inhibitor)

Potent, cell- permeant, and selective inhibitor of myosin light chain kinase (K_i = 300 nM). Inhibits protein kinase A (K_i = 21 μM) and protein kinase C (K_i = 42 μM) at much higher concentrations.
Soluble in: DMSO, ethanol, water
Stock concentration: 100 to 500 mM (store at 4°C protected from light)
Working concentration: 10 to 50 μM
Duration of incubation: 15 min to 1 hr

See Table I (Selectivity of Selected Kinase Inhibitors) in the Biomol Catalog and Handbook, 5th ed

L-Mimosine

An inhibitor of DNA replication that may act by preventing the formation of replication forks. L-mimosine blocks the camptothecin-induced apoptosis of PC12 cells, whereas aphidicolin does not.
Soluble in: Water
Stock concentration: 10 mM (store at room temperature)
Working concentration: 25 to 400 μM
Duration of incubation: 2 to 24 hr

Monensin

Polyether antibiotic that functions as an Na^+ ionophore. Forms stable complexes with monovalent cations that are able to cross cell membranes. Inhibits glycoprotein secretion by blocking transport through the Golgi. Neutralizes acidic endomembrane compartments. Reduces sphingomyelinase activity.
Soluble in: DMSO, methanol
Stock concentration: 2 to 30 mM (store at 4°C)
Working concentration: 1 to 30 μM
Duration of incubation: Effective within seconds; use up to 3 hr.

Nigericin

Dual antiporter ionophore that acts as a K^+/H^+ exchanger. Stimulates Ca^{2+} release from mitochondrial stores by disruption of membrane potential. Allows adjustment of cytoplasmic pH (when combined with K^+ ionophore such as valinomycin).
Soluble in: Ethanol
Stock concentration: 1 mg/ml (store at 4°C)
Working concentration: 1 to 10 μM
Duration of incubation: Effective within 2 to 5 min

Nocodazole

Has specific antimicrotubular activity for mammalian cells in culture. Promotes microtubule depolymerization. Nanomolar concentrations alter microtubule dynamics and interfere with fibroblast locomotion without affecting polymer levels. Arrests cells in mitosis.
Soluble in: DMSO
Stock concentration: 10 to 30 mM (store at room temperature)
Working concentration: 50 nM (low concentrations) to 30 μM (for effective microtubule depolymerization)
Duration of incubation: For rapid depolymerization of microtubules, preincubate cells on ice with nocodazole for 15 min; use up to 24 hr.

Okadaic acid

Potent inhibitor of protein phosphatases, especially the PP-1 class (K_i = 10-15 nM) and PP-2A class (K_i = 0.1 nM), in numerous cell types. Does not affect the activity of acid or alkaline tyrosine phosphatases. It mimics the effects of insulin, enhances neurotransmitter release, causes vasodilation, and is a potent tumor promoter. Induces dispersal of the Golgi apparatus. Okadaic acid is a useful tool for studying cellular processes regulated by serine/threonine phosphorylation.
Soluble in: DMSO, ethanol, methanol
Stock concentration: 1 mM (store at –20°C protected from light)
Working concentration: 50 to 200 nM
Duration of incubation: 15 min to 2 hr

May cause cell rounding. See Technical Note #18 from Alexis Biochemicals for additional information.

Olomoucine

Adenine derivative that acts as a competitive inhibitor for ATP binding and inhibits $p34^{cdc2}$/cyclin B (K_i = 7 μM) as well as several other CDKs at low concentrations. Does not significantly affect the activity of other protein kinases at 1 mM. Inhibits DNA synthesis in IL-2 stimulated T lymphocytes. Also used to synchronize cells in G_1. Can affect microtubule dynamics at higher concentrations.
Soluble in: DMSO
Stock concentration: 100 mM (store in aliquots at –20°C)
Working concentration: Usually 10 μM (up to 100 μM)

continued

Duration of incubation: 15 min to 24 hr, depending on process studied.

See Technical Note #25 from Alexis Biochemicals for additional information.

Ouabain

Selective Na^+/K^+-ATPase inhibitor. Causes net influx of Ca^{2+}; also initiates the rapid protein kinase C–dependent inductions of early-response genes.
Soluble in: Water
Stock concentration: 100 mM (store at –20°C protected from light)
Working concentration: 1 to 100 µM
Duration of incubation: 30 min to 24 hr

PDMP

Useful tool for studying the effects of cellular glycosphingolipid depletion. Blocks ceramide glucosylation by inhibiting UDP-glucose:ceramide glucosyltransferase (glucosylceramide synthetase). Has antitumor activity; arrests 3T3 cells at both G_1/S and G_2/M. Prevents sensitization of A431 cells to Shiga toxin. Slows the rate of both anterograde vesicular traffic and endocytosis in CHO and BHK-21 cells. Redistributes *cis*-Golgi proteins to the ER.
Soluble in: Ethanol
Stock concentration: 10 to 100 mM (store at 4°C)
Working concentration: 20 to 100 µM
Duration of incubation: 1 to 18 hr, depending on process studied

PD 98059 (MEK Inhibitor)

Potent and selective inhibitor of MAP kinase kinase (MEK or MAPK/ERK kinase). Blocks the activity of MEK, thereby inhibiting the phosphorylation and activation of MAP kinase. Inhibits cell growth and reverses the phenotype of *ras*-transformed 3T3 mouse fibroblasts and rat kidney cells. Inhibits Golgi reassembly in vitro. Cell-permeant.
Soluble in: DMSO
Stock concentration: 50 mM (store at –20°C protected from light)
Working concentration: 10 to 50 µM
Duration of incubation: 30 min to 2 hr

PMSF (phenylmethanesulfonyl fluoride)

Inhibits serine proteases like chymotrypsin, trypsin, and thrombin, as well as acetylcholinesterase and the cysteine protease papain (reversible by DTT treatment). PMSF inhibits serine proteases by sulfonating serine residues at the active site. Does not inhibit metalloproteases, most cysteine proteases, or aspartic proteases.
Soluble in: Anhydrous isopropanol at 35 mg/ml with heating, resulting in a clear to very slightly hazy, colorless to faint yellow solution, or in anhydrous (100%, not 95%) ethanol
Stock concentration: 17 mg/ml (store at room temperature)
Working concentration: 17 to 170 µg/ml
Duration of incubation: 15 min to 1 hr

PMSF is very unstable in the presence of water. The half-life of aqueous PMSF at 25°C at pH 7.0, 7.5, and 8.0 is 110, 55, and 35 min, respectively.

Pepstatin A

Inhibitor of aspartyl proteases, including pepsin, renin, cathepsin D, and HIV-1 protease. Inhibits degradation of ApoB in rat hepatocytes; inhibits cytokine-induced programmed cell death. Accelerates amyloid fibril formation in mice.
Soluble in: DMSO
Stock concentration: 10 to 35 mM (store at –20°C)

Working concentration: 50 to 100 μM in cells
Duration of incubation: 30 min to 2 hr

Pertussis toxin

Protein endotoxin that catalyzes ADP-ribosylation of GDP-bound α subunits of the G proteins G_i, G_o, and G_t. Uncouples G proteins from receptors, thereby keeping the G protein in the inactive state. Used in the study of adenylate cyclase regulation and the role of G_i proteins. Consists of an enzymatically active A protomer subunit (S-1) which posesses both NAD^+ glycohydrolase and ADP-ribosylation activities and a B oligomer subunit (S-2, S-3, S-4, and S-5) that is responsible for cell surface attachment.

Stock solution: Reconstitute commercial preparation in water. Generally, commercially available pertussis toxin (Alexis Biochemicals, Biomol, Calbiochem) contains 50 μg of protein in 10 mM sodium phosphate buffer, pH 7.0/50 mM sodium chloride after being resuspended in 0.5 ml water. It is an insoluble protein that should be shaken gently before use. Store stock solutions at 4°C. Do not freeze.

Working concentration: 50 to 100 ng/ml
Duration of incubation: 2 to 24 hr

Phenylarsine oxide (PAO)

A cell-permeant phosphotyrosine phosphatase inhibitor (K_i = 18 μM). Induces a dose-dependent increase in the free Ca^{2+} intracellular concentration in rat peritoneal macrophages, human foreskin fibroblasts, and cultured human endothelial cells, without affecting intracellular stores. Inhibits insulin activation of phosphatidylinositol 3′-kinase. Dithiol cross-linking agent.

Soluble in: DMSO and chloroform
Stock concentration: 50 mM (store at room temperature)
Working concentration: 10 to 50 μM
Duration of incubation: Anywhere from 15 sec to 2 hr

Phorbol esters

An example is phorbol myristate acetate (PMA). Extremely potent tumor promoters. Activate protein kinase C by mimicking diacylglycerols (DAGs), causing a wide range of effects in cells and tissues.

Soluble in: DMSO
Stock concentration: 1 mM (store at –20°C)
Working concentration: 50 nM to 3 μM
Duration of incubation: Cells can be incubated anywhere from 5 min to 48 hr; stable in cells; results in long-term activation of PKC. However, long-term treatment may cause down-regulation of certain PKC subtypes.

See Technical Notes #13 and #14 from Alexis Biochemicals for additional information.

Piceatannol

At low concentrations, inhibits the receptor-mediated activation of the protein tyrosine kinase Syk as compared to the Src family in mast cells and B cells. Inhibits FcεR1-mediated signaling in RBL-2H3 cells.

Soluble in: DMSO, ethanol
Stock concentration: 10 to 50 mg/ml (store at 4°C protected from light)
Working concentration: 10 to 30 μg/ml
Duration of incubation: 1 hr

Puromycin

Protein synthesis inhibitor. Causes premature release of nascent polypeptide chains by its addition to the growing chain end; structural analog of aminoacyl-tRNA.
Soluble in: Water
Stock concentration: 100 mM (store at –20°C)
Working concentration: 10 to 100 μM
Duration of incubation: 5 min to 1 hr

Rapamycin

Member of a family of macrolide immunosuppressants that binds to and inhibits the peptidylproline *cis-trans* isomerase (PPIase) activity of the immunophilin FKBP12; effectors include a large protein termed FRAP (FKBP12 rapamycin-associated protein). FKBP12-rapamycin binds to but does not inhibit the activity of the Ca^{2+}/calmodulin–dependent serine/threonine phosphatase calcineurin. Blocks signaling, leading to the activation of p70 S6 kinase.
Soluble in: DMSO, methanol, ethanol
Stock concentration: 2 mM (store at –20°C)
Working concentration: 1 to 20 nM
Duration of incubation: 30 min to 1 hr

Sodium azide (NaN_3)

Inhibits mitochondrial ATPases; generally used to deplete ATP levels within cells (often in combination with 50 mM 2-deoxyglucose).
Soluble in: Water
Stock concentration: 1 M (store at room temperature)
Working concentration: 10 to 20 mM
Duration of incubation: 15 to 90 min

Sodium butyrate

A physiologically produced short-chain fatty acid that is generally used to increase expression of transfected genes with viral promoters (inhibits histone deacetylation). Blocks serum-stimulated DNA synthesis via a G_1 block. Induces apoptosis in colon carcinoma cell lines by a p53-independent process. Interferes with signal-transduction processes, including the release of Ca^{2+} from intracellular stores.
Soluble in: Water
Stock concentration: 5 M (store at –20° C)
Working concentration: 2 to 5 mM
Duration of incubation: Usually >12 hr

Sodium orthovanadate

Broad-spectrum inhibitor of protein tyrosine phosphatases. Also inhibits other ATPases, by mimicking the γ phosphate of ATP, including Na^+/K^+ ATPase, acid and alkaline phosphatases, and adenylate cyclase. Vanadate is also a strong inhibitor of lysosomal proteolysis in hepatocytes, the effect being ascribed to a direct inhibition of lysosomal enzymes. Stimulates pp60 (v-src) kinase activity in intact cells. It also stimulates amino acid transport activity in skeletal muscle, in a rapid and concentration-dependent manner.

Simple aqueous solutions of $(VO_4)^{3-}$ ion involve a dozen or more ionic species, both monomeric and oligomeric, whose abundances depend upon pH and $[VO_4]^{3-}$ concentration. See Fohr et al. (1989), as well as *UNIT 13.1* for directions on preparing monomeric orthovanadate. It is unclear how readily vanadate ions enter cells (likely through anion transporters). At the concentration required for maximum inhibition, vanadate may have side effects that limit its application in cell culture. Can be combined

continued

with hydrogen peroxide (forming peroxyvanadate) to facilitate cell entry (combine 100 µl 0.1 M orthovanadate, 900 µl water, and 3.3 µl 30% H_2O_2; use 1:100 dilution of this on cells). However, the effect of hydrogen peroxide itself should be tested.

Soluble in: Water

Stock concentration: 100 mM (store at room temperature). To ensure the presence of monomers, the solution is heated to boiling until translucent and the pH is readjusted to 10. Solutions can be divided into aliquots, stored in plastic, and frozen. The orange color observed before boiling is due to decavanadate. At pH 10 this will slowly depolymerize over several hours to the colorless monovanadate. Vanadyl, metavanadate, orthovanadate, and decavanadate will interconvert in aqueous solution without suitable precautions (i.e., control of pH, oxidation state, complexing compounds, and concentration).

Working concentration: 200 µM to 2 mM

Duration of incubation: 15 min to 2 hr

Staurosporine

A potent cell-permeant inhibitor of protein kinases, most potently protein kinase C (K_i = 0.7 nM), protein kinase A (K_i = 7 nM), and myosin light chain kinase (K_i = 1.3 nM). Interaction is with the ATP binding site. Induces apoptosis, but not DNA fragmentation in MCF-7 cells. Arrests normal cells at the G_1 checkpoint.

Soluble in: DMSO, methanol

Stock concentration: 1 mM (store at −20°C protected from light)

Working concentration: 10 to 200 nM

Duration of incubation: 30 min to 24 hr, depending on the assay used

See Table I (Selectivity of Selected Kinase Inhibitors) in the Biomol Catalog and Handbook, 5th ed

Taxol (Paclitaxel)

Antitumor and antileukemic agent. Promotes assembly of microtubules and inhibits microtubule disassembly. Bundles microtubules after several hours. Similar to nocodazole, taxol can inhibit microtubule dynamics without affecting overall polymer levels at nanomolar concentrations. Blocks cells at the G_2/M stage. Induces apoptosis in several cell types.

Soluble in: DMSO, methanol

Stock concentration: 20 mM (store at −20°C protected from light)

Working concentration: 10 nM to 20 µM

Duration of incubation: Taxol works rapidly to stabilize microtubules (within several minutes), although bundling takes several hours (this may be facilitated by first depolymerizing the polymer pool with ice treatment and/or washout of low levels of nocodazole)

Thapsigargin

Potent inhibitor of sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) Ca^{2+}-ATPases. Induces IP_3-independent release of Ca^{2+} from the endoplasmic reticulum, causing an increase in intracellular Ca^{2+}. Depletion of Ca^{2+} from intracellular stores induces stress response and defects in protein folding and processing. Induces apoptosis in rat thymocytes and in human hepatoma cells. Irreversible.

Soluble in: DMSO, ethanol

Stock concentration: 1 mM (store at −20°C in aliquots, protect from light)

Working concentration: 20 nM to 1 µM

Duration of incubation: 15 sec to 2 min produces rise in intracellular Ca^{2+}; longer incubations may be used, depending on effect to be analyzed

See Technical Note #15 from Alexis Biochemicals for additional information.

Trifluoperazine

Calmodulin antagonist. At 10 μM, potentiates rise in cytosolic calcium induced by agonists. Antagonizes calmodulin at higher concentrations. Inhibits IL-2 production in activated Jurkat T cells. Structurally distinct from W-7.
Soluble in: Water (dihydrochloride salt)
Stock concentration: 10 mM (store at 4°C)
Working concentration: 10 to 50 μM
Duration of incubation: 10 min to 3 hr

Tunicamycin

Nucleoside antibiotic that inhibits N-linked glycosylation, specifically by blocking the transfer of *N*-acetylglucosamine-1-phosphate from UDP-*N*-acetylglucosamine to dolichol monophosphate; has no effect on other glycosylation forms, such as Ser/Thr-linked oligosaccharides. Causes misfolding and retention of numerous glycoproteins in the endoplasmic reticulum, which induces synthesis of ER chaperones.
Soluble in: DMSO, ethanol
Stock concentration: 10 mg/ml (store at –20°C)
Working concentration: 1 to 10 μg/ml
Duration of incubation: Cells can be treated from 1 to 24 hr

Tyrphostins

Large family of protein tyrosine kinase inhibitors. Inhibits receptors such as EGF receptor and PDGF receptor.
Soluble in: DMSO, ethanol
Stock concentration: 20 to 100 mM (store at –20°C protected from light)
Working concentration: 10 to 150 μM
Duration of incubation: Anywhere from 1 to 48 hr
See Technical Note #22 from Alexis Biochemicals for additional information. Also see Table II (Selectivity of Tyrosine Protein Kinase Inhibitors) in the Biomol Catalog and Handbook, 5th ed.

Valinomycin

Potassium ionophore. Decreases ATP synthesis by decreasing membrane potential at mitochondrial membranes. Reported to inhibit NGF-induced neuronal differentiation. Used with nigericin to adjust cytoplasmic pH.
Soluble in: DMSO
Stock concentration: 1 mM (store at room temperature)
Working concentration: 1 to 20 μM
Duration of incubation: Normally 15 min to 2 hr

Vinblastine

Vinca alkaloid; antitumor drug. Inhibitor of cell proliferation that acts by disrupting spindle microtubule function. Binds tubulin and suppresses microtubule dynamics. Depolymerizes microtubules at higher concentrations. Induces apoptosis in cultured hepatocytes and human lymphoma cells. Similar, but not identical effects observed with another vinca alkaloid, vincristine.
Soluble in: Methanol
Stock concentration: 20 mM (store at 4°C protected from light)
Working concentration: <10 nM (suppresses microtubule dynamics); 100 nM to 1 μM (depolymerizes microtubules); >10 μM (forms non-microtubule polymers)
Duration of incubation: 30 min to 24 hr

W-7

Member of a family of calmodulin antagonists, inhibiting calcium/calmodulin regulated enzyme activity. W-7 inhibits the Ca^{2+}/calmodulin–induced activation of myosin light chain kinase (K_i = 51 μM) and phosphodiesterase (K_i = 28 μM). Inhibits membrane tubulation in cells treated with brefeldin A.
Soluble in: Water
Stock concentration: 10 to 100 mM (store at 4°C protected from light)
Working concentration: 10 to 100 μM
Duration of incubation: 30 min to 2 hr

Wortmannin

Selective and potent phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor; forms covalent associations with the kinases and is, therefore, irreversible. Abolishes PDGF-mediated Ins(3,4,5)P_3 formation in fibroblasts. Blocks the metabolic effects of insulin in isolated rat adipocytes without affecting the insulin receptor tyrosine kinase activity. Inhibits the formation of constitutive transport vesicles from the TGN. In human fetal undifferentiated cells, wortmannin induces morphological and functional endocrine differentiation.
Soluble in: DMSO
Stock concentration: 1 to 20 mM (store at −20°C in aliquots, protected from light)
Working concentration: 10 to 100 nM
Duration of incubation: 30 min to 4 hr

At nanomolar concentrations, wortmannin is specific to PI 3-kinases, while at higher concentrations other kinases are affected. Once diluted into aqueous solutions, wortmannin is less stable and should be made fresh daily.

LITERATURE CITED

Fohr, K.J., Scott, J., Ahnert-Hilger, G., and Gratzl, M. 1989. Characterization of the inositol 1,4,5-trisphosphate-induced calcium release from permeabilized endocrine cells and its inhibition by decavanadate and *p*-hydroxymercuribenzoate. *Biochem. J.* 262:83-89.

Jalink, K., van Corven, E.J., and Moolenaar, W.H. 1990. Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca^{2+}-mobilizing stimulus for fibroblasts. Evidence for an extracellular site of action. J. Biol. Chem. 265:12232-12239.

Seufferlein, T. and Rozengurt, E. 1994. Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130: Signaling pathways and cross-talk with platelet-derived growth factor. *J. Biol. Chem.* 269: 9345-9351.

Contributed by Nelson B. Cole
University of Pennsylvania
Philadelphia, Pennsylvania

Identification of Motifs in Protein Sequences

Many biologists are not sure how to best analyze a newly determined protein sequence for the presence of functional motifs. This brief appendix is meant to guide researchers during their protein sequence analyses. Several database search engines that can be accessed via the World Wide Web (WWW) are described. Such computerized searches have become the preferred method to scan large sequence and motif databases, as the searches are efficient and the databases are updated frequently. A short list of sorting signals is also included, since these motifs often cannot be predicted reliably by a computer search.

The first step in predicting the function of an unknown protein is to perform a sequence similarity search in which the protein query sequence is compared pairwise to all other previously determined sequences. Popular programs for this include BLAST (Basic Local Alignment Search Tool; Altschul et al., 1997) and FASTA (Pearson et al., 1997). Such analyses identify the overall most similar proteins and often provide a good indication about the function of the protein. Table A.1C.1 lists some URLs through which such sequence databases can be searched.

Searching a database of protein motifs or domains will likely provide further information about the function of the protein. Such databases, which are manually collated and annotated collections, may shed light on the domain structure of the unknown protein, provide more detailed information on particular motifs, and detect domains that were missed in a pairwise database search. Protein motifs and domains are discovered as researchers find similar sequences shared by proteins that carry out similar functions. A number of popular databases that contain a large number of motifs or domains, and are accessible via the WWW, are also listed in Table A.1C.1.

It should be emphasized that the results from any sequence similarity or motif search should serve as a guide to future experiments, not as definitive proof that the protein carries out a particular function. Each match in a database search is given a score that indicates its significance and relative strength. In search programs that calculate the probability of finding a match by chance, a threshold of $P<10^{-5}$ is commonly used as rule of thumb for statistical significance that can be trusted biologically. For search programs that do not calculate the probability, empirically derived thresholds have to be used to estimate biological significance. The presence of a particular sequence motif is no guarantee that the protein will function as predicted. Conversely, the absence of a motif match may mean that the motif was not included in a particular database or had diverged too far to be detected. Thus, it is important to scan a few of the motif databases and integrate the results carefully with those obtained from a sequence similarity search. The WWW has brought a variety of sequence analysis tools directly into the laboratory. Researchers are encouraged to take advantage of them.

DATABASES AND SERVERS ON THE WWW

Table A.1C.1 lists general-purpose servers that are useful for sequence analysis and allow the user to submit a query sequence for analysis over the WWW. All are available without charge, and all have some on-line documentation, although in some cases, the documentation is minimal. Nevertheless, the databases are easy to use, as the query sequence is normally pasted into a box on the WWW page. Table A.1C.1 lists four types of servers. The first section lists servers useful for an initial sequence similarity search against all known sequences. This will return pairwise alignments between the query sequence and its closest relatives. The second section contains servers that allow users to search specialized databases for the presence of motifs or domains. Since such a search will return a concise list of the motifs present in the query sequences, the results are usually easier to interpret. In addition, searching motif databases may also be more sensitive. The table also lists servers that can help detect sorting signals, subcellular localization, and structural features such as transmembrane domains and coiled coils, as well as a few sites that provide links to other relevant servers not listed here.

One of the most commonly used sequence similarity search tools is BLAST. The servers for sequence similarity searching listed in the first part of Table A.1C.1 use different implementations of the BLAST algorithm (Recipon et al., 1995; Altschul et al., 1997). The results from the different servers are generally similar but are presented in different ways. An example

Contributed by Erik L.L. Sonnhammer and Tyra G. Wolfsberg

Current Protocols in Cell Biology (1998) A.1C.1-A.1C.8

Table A.1C.1 Useful World Wide Web Sites For Protein Sequence Analysis[a]

Database/server	Description	WWW URL
Raw sequence databases		
NCBI BLAST	Gapped or ungapped BLAST search; also provides iterative searching	*http://www.ncbi.nlm.nih.gov/BLAST/*
EMBL BLAST2	Gapped BLAST search	*http://www.bork.embl-heidelberg.de:8080/Blast2/*
BCM search launcher	Wide variety of search programs and databases; shows annotated domains of matching sequences	*http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html*
Protein motif/domain family databases		
Prosite	Pattern and profile search	*http://expasy.hcuge.ch/sprot/prosite.html*
Blocks	Ungapped block search	*http://www.blocks.fhcrc.org/*
Prints	Ungapped block search	*http://www.biochem.ucl.ac.uk/bsm/dbbrowser/PRINTS/*
Pfam	Gapped HMM search	*http://genome.wustl.edu/Pfam/* *http://www.sanger.ac.uk/Pfam/*
Sorting signals and structural features		
SignalP	Signal peptide prediction	*http://www.cbs.dtu.dk/*
Psort	Subcellular location prediction	*http://psort.nibb.ac.jp/*
MitoProt	Mitochondrial location prediction	*Mitoprot@biologie.ens.fr*[b]
PredictProtein	Transmembrane helix and secondary structure prediction	*http://www.embl-heidelberg.de/predictprotein/*
TopPred 2	Transmembrane helix prediction	*http://www.biokemi.su.se/~server/toppred2/*
TMAP	Transmembrane helix prediction	*http://www.embl-heidelberg.de/tmap/tmap_info.html*
TMpred	Transmembrane helix prediction	*http://ulrec3.unil.ch/software/TMPRED_form.html*
DAS	Transmembrane helix prediction	*http://www.biokemi.su.se/~server/DAS/*
Coils2	Coiled coil prediction	*http://ulrec3.unil.ch/software/COILS_form.html*
Sites with links to additional servers		
Univ. Texas		*http://bioc02.uthscsa.edu/biotech.html*
ExPASy		*http://www.expasy.ch/*
Pedro's		*http://www.public.iastate.edu/~pedro/research_tools.html*

[a]Abbreviations: BCM, Baylor College of Medicine; EMBL, European Molecular Biology Laboratory; HMM, hidden Markov model; NCBI, National Center for Biotechnology Information.

[b]E-mail server only; for instructions send an email with the word "help" in the body.

of a BLAST search result obtained at the National Center for Biotechnology Information (NCBI) is shown in Figure A.1C.1. The Baylor College of Medicine (BCM) search launcher also provides access to other search programs such as FASTA, SSEARCH, and BLITZ (Pearson, 1996). The two main types of BLAST are the gapped and ungapped varieties. The newer, gapped version introduces gaps in either sequence to generate one long alignment of the entire similarity region, while the ungapped version would report a series of separate ungapped alignments. There are different BLAST programs depending on whether the query and database sequences are protein or DNA. For a protein query, two programs are available: BLASTP, which searches a protein database, and TBLASTN, which searches the six-frame translation of a DNA database. For completeness, both types of searches should be performed.

Identification of Motifs in Protein Sequences

The second section of Table A.1C.1 lists databases of protein motifs or domains. Although the terms motif and domain are often used interchangeably, a *motif* is a characteristic pattern of functionally important residues and a *domain* is an autonomous protein region, i.e., a complete folding unit. For example, a motif

constitutes the catalytic residues involved in proteolysis, while a protein domain spans a much larger region, including structurally important residues. A *pattern* is a short string of residues, for example, R/L-X_5-H/Q-L. A *block* is an alignment of segments from multiple sequences. No gaps are allowed within segments of a block, and all segments have the same length. Such blocks represent highly conserved regions and are typically 20 to 40 residues wide. Domains are usually defined by multiple blocks. *Profiles* and *hidden Markov models (HMMs)* are representations of multiple alignments of sequence segments, where the segments can have gaps and can therefore span larger regions, often entire domains.

Popular protein motif and domain databases include the pattern-based Prosite (Bairoch et al., 1997), the block-based Prints (Attwood et al., 1998) and Blocks (Henikoff et al., 1998), and the HMM-based Pfam (Sonnhammer et al., 1998). Prosite also contains a number of profiles. Because these databases are maintained by different groups and were created using different methods, the results obtained are usually different but complementary. Prosite, Blocks, and Prints provide extensive functional annotation for each domain family, while the Pfam annotations are usually shorter but linked to other sources of annotation. Blocks uses the domain family definitions and annotations directly from Prosite, while Prints families were defined and annotated independently. Most of the families in both Prints and Pfam correspond to a Prosite domain family, but the details may differ substantially.

A pattern search in Prosite may identify highly conserved functional residues, while more widespread global similarity is generally easier to detect with a profile or HMM method. The block-based methods used by Blocks and Prints fall between the pattern and profile/HMM methods. The pattern method suffers from two drawbacks: first, motifs may be missed if the pattern definition is too stringent, and second, at positions where more than one residue is allowed, patterns do not provide for a quantitative description of the relative preference for different amino acids. The multiple alignment methods, on the other hand, can assign a score to a sequence when compared to any domain family. Thus, even weak matches, which may constitute distant similarities, can be reported. Again, it must be stressed that statistically insignificant matches must be treated with caution and usually require additional support (e.g., partial knowledge of protein function) to be considered biologically relevant.

It is also often useful to predict the localization and structural features of a protein. This is particularly valuable if no similar sequences were found in the previously described searches, but may also complement functional information based on sequence similarity. A number of servers for such analyses are listed in the third section of Table A.1C.1, which includes servers for prediction of signal peptides, subcellular localization, transmembrane helices, and secondary structure. The list includes multiple transmembrane helix prediction programs. These programs use distinct algorithms and may produce different results; careful interpretation of the output is therefore necessary.

ANALYSIS EXAMPLE

To illustrate how these servers can be used, sample results using the *C. elegans* protein R151.5 (Wormpep accession no. CE00743; Swissprot accession no. P98060) are shown. Figure A.1C.1 shows part of the output from the NCBI BLAST server, which lists the highest-scoring matches. The matches are shown as a graphical schematic and as a ranked list; the pairwise alignments for each match are also provided. Most of the highest-scoring matches span the entire query sequence. However, some matches are confined to regions near the C terminus (approximately residues 500 to 600) and near the N terminus (~100 to 350), suggesting that these may be separate domains. Browsing the annotations of these sequences reveals that the C-terminal domain is a thrombospondin type 1 domain, and the N-terminal domain is a zinc-dependent metalloprotease domain.

As an example of searching a domain database, Figure A.1C.2 shows the output from a Pfam search of the same query sequence. Compared to BLAST, the output is more concise and directly shows the location of the domains in a graphical diagram. This search confirms the existence of the metalloprotease and thrombospondin type 1 domains found by BLAST, and in addition detects an epidermal growth factor (EGF) domain and a CUB (c1r/c1s, Uegf, Bmpt) domain.

A schematic of the search results for *C. elegans* R151.5 against the four motif/domain databases is shown in Figure A.1C.3. All four searches detect the zinc-dependent metalloprotease motif, and Prints finds additional similarities to the astacin subfamily of zinc-dependent metalloproteases. Pfam, Blocks, and Prosite

Figure A.1C.1 *(at right)* Sample output from a BLASTP search on the NCBI BLAST server, showing the highest scoring hits of the *C. elegans* protein R151.5. The graphical display draws a shaded bar for each database sequence; multiple matches to the same sequence are attached with a hatched bar. Matches to multiple sequences may be packed on one line for compactness. The shading of each bar reflects the score of the match according to the key on top. By pointing the cursor on a bar, the description of the matching sequence is shown in the text box above (thrombospondin). The bottom of the figure shows a list of the hits. Clicking on a link in the left column leads to the record for that sequence, while following a link on the right gives the alignment of the query sequence with that hit.

find the EGF domain, and Pfam and Blocks report the CUB domain. Only Pfam detects the thrombospondin type 1 domain. In this example, Prints, Blocks, and Prosite reported several matches to clearly unrelated domains. These matches are shown as half-height uncolored boxes in Figure A.1C.3. Such background noise is common in these types of searches and can be recognized by one or more of the following criteria: (1) only one of many blocks that define a particular domain has a high score, (2) the match overlaps with other higher-scoring domains, and/or (3) the match is weak and is only reported by one of the database searches.

This analysis illustrates the point that one should not rely on the output of only one of the servers. In this case, Pfam provides the most extensive annotation for the EGF, CUB, and thrombospondin type 1 domains, while Prints provides the most extensive annotation of the metalloprotease domain. However, other examples may differentially accentuate the relative predictive powers of the databases.

R151.5 was further analyzed using the servers described in the third section of Table A.1C.1, for the presence of subcellular sorting signals and structural features. The programs predict that the protein contains a signal peptide, but no transmembrane segments, and is likely to be extracellular.

SORTING SIGNALS

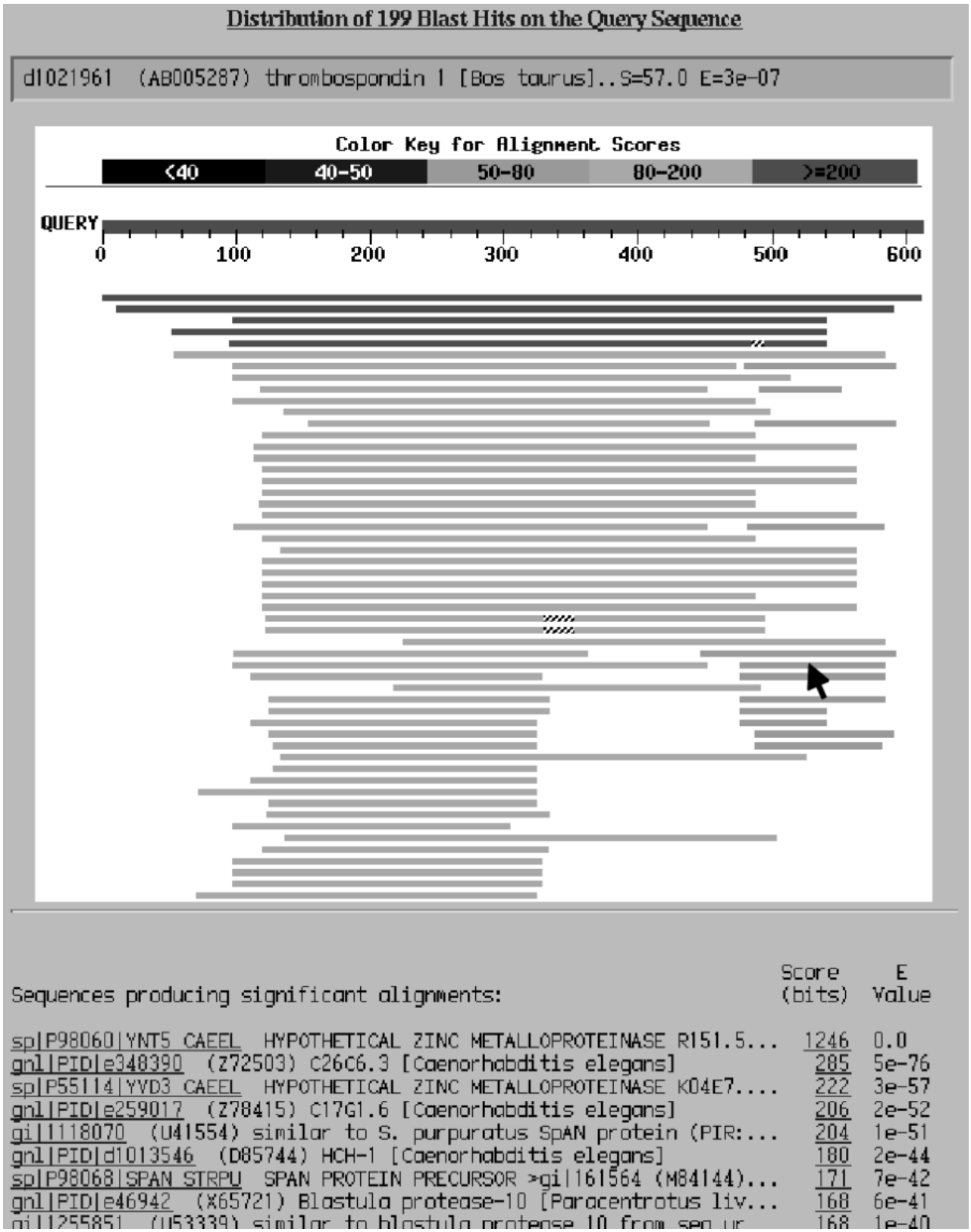
Because the signals that govern subcellular localization are often weak, programs that predict such signals may be inaccurate, and it is often hard to judge the validity of the results. In many cases, the context in which a pattern occurs can affect its relevance. Further, because there are no programs available for many signals, text descriptions of the most common sorting signals are provided.

Signal Peptides

Proteins that are initially imported into the endoplasmic reticulum (ER)—e.g., those secreted or transported to the plasma membrane or lysosomes, as well as those retained in the ER or Golgi apparatus—contain a characteristic N-terminal presequence, or signal peptide. Signal peptides are usually cleaved off in the ER by signal peptidase I. A similar signal sequence is also present in precursors of proteins imported into the bacterial periplasm. Signal peptides are usually 15 to 40 residues long and contain, in N- to C-terminal order, a region of 2 to 5 positively charged residues, a region of 7 to 15 hydrophobic residues, and a cleavage region consisting of 3 to 7 neutral but often polar residues. The residues at positions −3 and −1 from the signal peptidase cleavage site are normally occupied by small and neutral residues, preferentially Ala. The program SignalP (Nielsen et al., 1997), which can be used via the WWW, can predict the presence of signal peptides and their cleavage sites for eukaryotic and prokaryotic proteins with an accuracy of ~75% (Table A.1C.1). Additional signal peptide prediction methods are reviewed in Claros et al. (1997).

Localization Signals in the Secretory Pathway

Proteins that contain a signal peptide and that enter the secretory pathway may contain an additional motif that causes localization to one compartment of the pathway. A motif sufficient for localization of luminal proteins in the ER is X-D-E-L at the C terminus. Within this motif there are general motif preferences: K-D-E-L in multicellular eukaryotes (also H-D-E-L in plants), H-D-E-L in budding yeast, and A-D-E-L in fission yeast (Gomord et al., 1997). A different localization signal, K-K-X-X- or K-X-K-X-X at the C terminus, is found in many ER transmembrane proteins whose C terminus is exposed to the cytosol (Jackson et al., 1990). Proteins retained in the Golgi do not appear to share any common sequence motif, but may instead be retained due to a common transmembrane domain structure (Colley, 1997). Lysosomal targeting of soluble proteins is accomplished by attachment of mannose-6-


Distribution of 199 Blast Hits on the Query Sequence
dl021961 (AB005287) thrombospondin 1 [Bos taurus]..S=57.0 E=3e-07
Color Key for Alignment Scores
<40
40-50
50-80
80-200
>=200
QUERY
0
100
200
300
400
500
600
Sequences producing significant alignments:
Score (bits)
E Value
sp|P98060|YNT5_CAEEL HYPOTHETICAL ZINC METALLOPROTEINASE R151.5... 1246 0.0
gnl|PID|e348390 (Z72503) C26C6.3 [Caenorhabditis elegans] 285 5e-76
sp|P55114|YVD3_CAEEL HYPOTHETICAL ZINC METALLOPROTEINASE K04E7.... 222 3e-57
gnl|PID|e259017 (Z78415) C17G1.6 [Caenorhabditis elegans] 206 2e-52
gi|1118070 (U41554) similar to S. purpuratus SpAN protein (PIR:... 204 1e-51
gnl|PID|d1013546 (D85744) HCH-1 [Caenorhabditis elegans] 180 2e-44
sp|P98068|SPAN_STRPU SPAN PROTEIN PRECURSOR >gi|161564 (M84144)... 171 7e-42
gnl|PID|e46942 (X65721) Blastula protease-10 [Paracentrotus liv... 168 6e-41
gi|1255851 (U53339) similar to blastula protease 10 from sea ur... 168 1e-40

Pfam HMM Search Results
Using hmmfs

Score	Query from	Query to	HMM from	HMM to	Pfam Family	Description
20.28	218	232	1	16	zn-protease	Zinc-binding metalloprotease domain
13.97	329	360	1	35	EGF	EGF-like domain
68.35	371	484	1	116	CUB	CUB domain
54.55	494	539	1	52	tsp_1	Thrombospondin type 1 domain

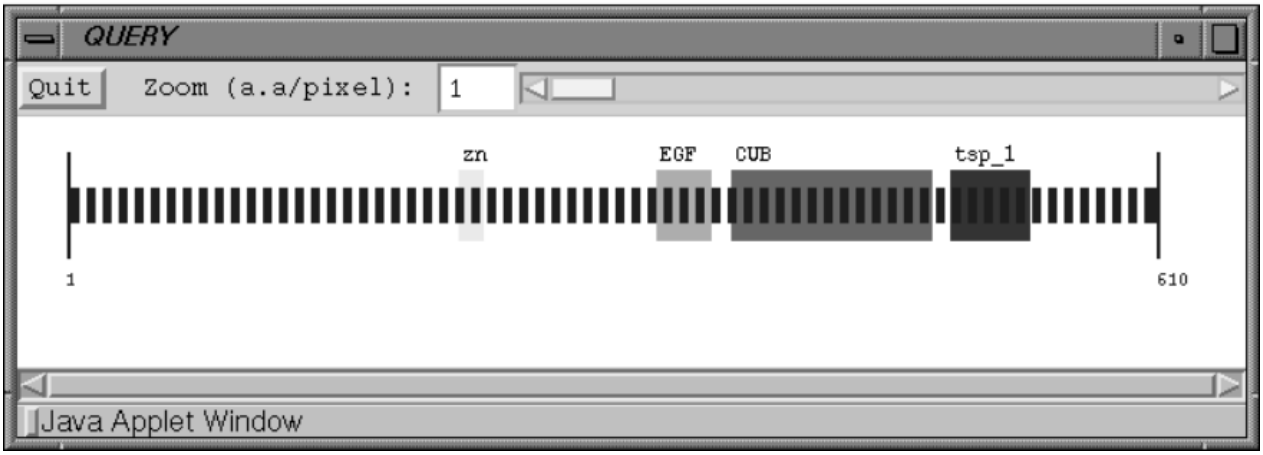


Figure A.1C.2 Sample WWW output of a Pfam search with R151.5. Links are provided to browse the documentation for each of the matching HMM families. The score threshold had to be lowered slightly from the default of 15 bits to detect the EGF domain. The E value given in the top bar gives the number of matches expected at the indicated score(s).

phosphate groups; no sequence motif is known for this, although attachment likely requires one or more lysine residues (Tikkanen et al., 1997).

A protein without a retention signal will be transported through the entire secretory pathway and will either be secreted or remain attached to the plasma membrane. A number of sorting signals mediate rapid internalization of plasma membrane proteins from the cell surface or targeting to lysosomes either directly from the *trans*-Golgi network or by internalization from the plasma membrane via the endocytic pathway. The sequence motifs for these signals are generally very short and too weak for predictive purposes. The most studied motifs are N-P-X-Y, Y-X-X-hydrophobic, and L-hydrophobic (Chen et al., 1990; Trowbridge et al., 1993; Sandoval and Bakke, 1994; Marks et al., 1997).

Attachment to the Plasma Membrane

Proteins are most often attached to the plasma membrane by one or more transmembrane segements, which are typically 15 to 35 residues long and consist mainly of hydrophobic residues. The presence of transmembrane helices can be predicted from a sequence by using one of the WWW servers listed in Table A.1C.1. A different type of plasma membrane attachment is by glycosylphosphatidylinositol (GPI) linkage (Udenfriend and Kodukula, 1995). Here, a C-terminal GPI-anchoring signal directs cleavage of the protein and attachment of the GPI anchor to the new carboxy terminus. The general cleavage motif is G/A/S/C/D/N↓X-G/A, followed by a 5- to 7-residue spacer rich in charged residues and Pro, followed by 15 to 30 hydrophobic residues.

Mitochondrial Transit Peptide

Most nuclear-encoded proteins targeted to the mitochondrion have an N-terminal presequence of 20 to 80 residues that is typically rich in basic and hydroxylated residues and poor in acidic residues. The transit peptide forms an

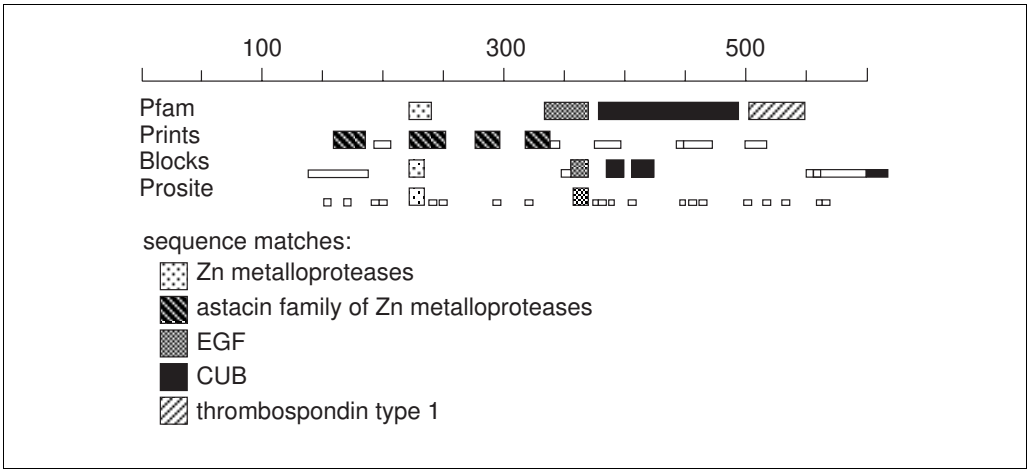


Figure A.1C.3 Schematic combined representation of results from searching protein domains in R151.5 with Pfam, Prints, Blocks, and Prosite. Matches to zinc-metalloproteases (Zn, including the astacin subfamily of zinc-metalloproteases), EGF, CUB, and thrombospondin type 1 (tsp-1) are indicated. Half-height boxes mark matches that were deemed false after manual inspection, and quarter-height boxes mark matches to Prosite patterns known to occur spuriously, such as phosphorylation and myristylation sites. For the sake of comparison, only matches to entries in the Blocks database are shown on line three; the Blocks WWW server can also report matches to Prints families. Likewise, only matches to Prosite patterns are shown here; the Prosite server also contains profile entries for the CUB and thrombospondin type 1 domains.

amphiphilic helix with one hydrophobic and one positively charged side (Claros et al., 1997). Of several known cleavage site motifs, the most diagnostic is RXY↓S/A (Gavel and von Heijne, 1990a). Additional sorting signals may be present to target the protein to mitochondrial subcompartments (Schwarz and Neupert, 1994; Dalbey et al., 1997; Neupert, 1997). The program MitoProt (Claros, 1995) can be used to predict the presence of mitochondrial transit peptides (Table A.1C.1).

Chloroplast Transit Peptide

Most nuclear-encoded proteins targeted to the chloroplast have an N-terminal presequence of 20 to 100 residues that is usually rich in hydroxylated residues and poor in acidic residues. The transit peptide has three domains: ~10 neutral residues at the N terminus, with Ala usually following the initial Met; a central Ser-rich domain; and a positively charged C-terminal domain (Cline and Henry, 1996; Claros et al., 1997). The most common cleavage site is V/I-X-A/C↓A, which is found in ~30% of sequences (Gavel and von Heijne, 1990b). After the cleavage site, a second transit peptide, similar to the generic signal peptide, may target subsequent import into the thylakoid (Dalbey et al., 1997).

Nuclear Localization Signals

Unlike the above signals, nuclear localization signals are not located in a particular region of the sequence and are not removed from the mature protein. No generic motif has been identified that can discriminate, with high accuracy, proteins that are imported into the nucleus, but overall the proteins tend to be rich in basic residues, and about half of the known examples contain the motif R/K-R/K-X_{10}-R/K$_{(3/4\ of\ 5)}$ (von Heijne, 1996; Corbett and Silver, 1997).

Microbody Targeting Signals

The C-terminal motif SKL-COOH has been shown to be necessary and sufficient to import proteins into peroxisomes (Keller et al., 1991). This motif, called peroxisome-targeting signal 1 (PTS1), is also found in proteins targeted to other microbodies, such as glycosomes and glyoxysomes, but this is often less conserved (Sommer and Wang, 1994). A second peroxisome-targeting signal, PTS2, has the motif R-L-X_5-H/Q-L at the N terminus; sometimes it is cleaved off after import (Blattner et al, 1995; Waterham and Cregg, 1997).

LITERATURE CITED

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucl. Acids Res.* 25:3389-3402.

Attwood, T.K., Beck, M.E., Flower, D.R., Scordis, P., and Selley, J.N. 1998. The PRINTS protein fingerprint database in its fifth year. *Nucl. Acids Res.* 26:304-308.

Bairoch, A., Bucher, P., and Hofmann, K. 1997. The PROSITE database, its status in 1997. *Nucl. Acids Res.* 25:217-221.

Blattner, J., Dorsam, H., and Clatyon, C.E. 1995. Function of N-terminal import signals in trypanosome microbodies. *FEBS Lett.* 360:310-314.

Chen, W.J., Goldstein, J.L., and Brown, M.S. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low-density lipoprotein receptor. *J. Biol. Chem.* 265:3116-3123.

Claros, M.G. 1995. MitoProt, a Macintosh application for studying mitochondrial proteins. *Comput. Appl. Biosci.* 11:441-447.

Claros, M.G., Brunak, S., and von Heijne, G. 1997. Prediction of N-terminal protein sorting signals. *Curr. Opin. Struct. Biol.* 7:394-398.

Cline, K. and Henry, R. 1996. Import and routing of nucleus-encoded chloroplast proteins. *Annu. Rev. Cell. Dev. Biol.* 12:1-26.

Colley, K.J. 1997. Golgi localization of glycosyltransferases: More questions than answers. *Glycobiology* 7:1-13.

Corbett, A.H. and Silver, P.A. 1997. Nucleocytoplasmic transport of macromolecules. *Microbiol. Mol. Biol. Rev.* 61:193-211.

Dalbey, R.E., Lively, M.O., Bron, S., and van Dijl, J.M. 1997. The chemistry and enzymology of the type I signal peptidases. *Protein Sci.* 6:1129-1138.

Gavel, Y. and von Heijne, G. 1990a. Cleavage-site motifs in mitochondrial targeting peptides. *Protein Eng.* 4:33-37.

Gavel, Y. and von Heijne, G. 1990b. A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett.* 261:455-458.

Gomord, V., Denmat, L.A., Fitchette-Laine, A.C., Satiat-Jeunemaitre, B., Hawes, C., and Faye, L. 1997. The C-terminal HDEL sequence is sufficient for retention of secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targeting of proteins that escape the ER. *Plant J.* 11:313-325.

Henikoff, S., Pietrokovski, S., and Henikoff, J.G. 1998. Superior performance in protein homology detection with the Blocks Database servers. *Nucl. Acids Res.* 26:309-312.

Jackson, M.R., Nilsson, T., and Peterson, P.A. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* 9:3153-3162.

Keller, G.A., Krisans, S., Gould, S.J., Sommer, J.M., Wang, C.C., Schliebs, W., Kunau, W., Brody, S., and Subramani, S. 1991. Evolutionary conservation of a microbody targeting signal that targets proteins to peroxisomes, lyoxysomes, and glycosomes. *J. Cell. Biol.* 114:893-904.

Marks, M.S., Ohno, H., Kirchhausen, T., and Bonifacino, J.S. 1997. Protein sorting by tyrosine-based signals: Adapting to the Ys and wherefores. *Trends Cell Biol.* 7:124-128.

Neupert, W. 1997. Protein import into mitochondria. *Annu. Rev. Biochem.* 66:863-917.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10:1-6.

Pearson, W.R. 1996. Effective protein sequence comparison. *Methods Enzymol.* 266:227-258.

Pearson, W.R., Wood, T., Zhang, Z., and Miller, W. 1997. Comparison of DNA sequences with protein sequences. *Genomics* 46:24-36.

Recipon, H.E., Schuler, G.D., and Boguski, M.S. 1995. Sequence Similarity Searching Using the BLAST Family of Programs. *In* Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 19.3.1-19.3.38. John Wiley & Sons, New York.

Sandoval, I. and Bakke, O. 1994. Targeting of membrane proteins to endosomes and lysosomes. *Trends Cell Biol.* 4:292-297.

Schwarz, E. and Neupert, W. 1994. Mitochondrial protein import: Mechanisms, components and energetics. *Biochim. Biophys. Acta* 1187:270-274.

Sommer, J.M. and Wang, C.C. 1994. Targeting proteins to the glycosomes of African trypanosomes. *Annu. Rev. Microbiol.* 48:105-138.

Sonnhammer, E.L., Eddy, S.R., Birney, E., Bateman, A., and Durbin, R. 1998. Pfam: Multiple sequence alignments and HMM-profiles of protein domains. *Nucl. Acids Res.* 26:320-322.

Tikkanen, R., Peltola, M., Oinonen, C., Rouvinen, J., and Peltonen, L. 1997. Several cooperating binding sites mediate the interaction of a lysosomal enzyme with phosphotransferase. *EMBO J.* 16:6684.6693.

Trowbridge, I.S., Collawn, J.F., and Hopkins, C.R. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.* 9:129-161.

Udenfriend, S. and Kodukula, K. 1995. How glycosylphosphatidylinositol-anchored membrane proteins are made. *Annu. Rev. Biochem.* 64:563-591.

von Heijne, G. 1996. Computer-assisted identification of protein sorting signals and prediction of membrane protein topology and structure. *Adv. Computat. Biol.* 2:1-14.

Waterham, H.R. and Cregg, J.M. 1997. Peroxisome biogenesis. *BioEssays* 19:57-66.

Contributed by Erik L.L. Sonnhammer
Center for Genomics Research,
Karolinska Institutet
Stockholm, Sweden

Tyra G. Wolfsberg
National Center for Biotechnology
Information/NIH
Bethesda, Maryland

Safe Use of Radioisotopes

The use of radioisotopes to label specific molecules in a defined way has greatly furthered the discovery and dissection of biochemical pathways. The development of methods to inexpensively synthesize such tagged biological compounds on an industrial scale has enabled them to be used routinely in laboratory protocols, including many detailed in this manual. Although most of these protocols involve the use of only microcurie (μCi) amounts of radioactivity, some (particularly those describing the metabolic labeling of proteins or nucleic acids within cells; see *UNIT 7.1*) require amounts on the order of millicuries (mCi). In all cases where radioisotopes are used, depending on the quantity and nature of the isotope, certain precautions must be taken to ensure the safety of the scientist. This appendix outlines a few such considerations relevant to the isotopes most frequently used in biological research.

In designing safe protocols for the use of radioactivity, the importance of common sense, based on an understanding of the general principles of isotopic decay and the importance of continuous monitoring with a hand-held radioactivity monitor (e.g., Geiger counter), cannot be overemphasized. In addition, it is also critical to take into account the rules, regulations, and limitations imposed by each specific institution. These are usually not optional considerations: an institution's license to use radioactivity normally depends on strict adherence to such rules.

Many of the protocols described have evolved (and are evolving still) over the years. The authors are indebted to those who have trained them in the safe use of radioactivity, in particular to the members of the Salk Institute Radiation Safety Department. Most of the designs for the shields and other safety equipment shown in Figures A.1D.2A, A.1D.3, and A.1D.4 were created at the Salk Institute in collaboration with Dave Clarkin and Mario Tengco. Safety equipment of similar design is available from several commercial vendors, including CBS Scientific and Research Products International.

BACKGROUND INFORMATION

The Decay Process

As anyone who has taken a basic chemistry course will remember, each element is characterized by its atomic number, defined as the number of orbital electrons or the number of protons in the nucleus of that atom. Isotopes of a given element exist because some atoms of each element, while by definition having the same number of protons, have a different number of neutrons and therefore a different nuclear weight. It should be noted that the number of electrons outside the nucleus remains the same for all isotopes of a given element, and so all isotopes of a given element are equivalent with respect to their chemical reactivity.

Radioactive decay occurs when subatomic particles are released from the nucleus of an atom of a heavy isotope. This often results in the conversion of an atom of one isotope to an isotope of a different element, because the original isotope's atomic number changes after decay. The subatomic particles released from naturally occurring radioisotopes are of three basic types: α and β particles and γ rays (see Table A.1D.1).

An α particle is essentially the nucleus of a helium atom, or two protons plus two neutrons. It is a relatively large, heavy particle that moves slowly and usually only across short distances before it encounters some other atom with which it interacts. These particles are released from isotopes with large nuclei (atomic number >82; e.g., plutonium or uranium); such isotopes are not commonly used in biological research.

In contrast to α particles, β particles are light, high-speed charged particles. Negatively charged β particles are essentially electrons of nuclear origin that are released when a neutron is converted to a proton. Release of a β particle thus changes the atomic number and elemental status of the isotope.

γ radiation has both particle and wave properties; its wavelength falls within the range of X-ray wavelengths. The distinction between γ rays and X rays was made when primitive X-ray machines produced X rays with a wavelength longer than those of the γ rays produced naturally by radioisotopes. Modern X-ray machines produce a much broader spectrum of wavelengths, including γ radiation; currently this sort of X-ray radiation is termed γ when it is of nuclear origin. Unlike β-particle release, the release of γ radiation by itself produces an isotopic change rather than an elemental one; however, the resultant nuclei are unstable and often decay further, releasing β particles.

Contributed by Jill Meisenhelder and Kentaro Semba
Current Protocols in Cell Biology (1998) A.1D.1-A.1D.13

Table A.1D.1 Physical Characteristics of Commonly Used Radionuclides[a]

Nuclide	Half-life	Emission	Energy, max (MeV)	Range of emission, max	Approx. specific activity at 100% enrichment (Ci/mg)	Atom resulting from decay	Target organ
^{3}H	12.43 years	β	0.0186	0.42 cm (air)	9.6	$^{3}_{2}He$	Whole body
^{14}C	5370 years	β	0.156	21.8 cm (air)	4.4 mCi/mg	$^{14}_{7}N$	Bone, fat
^{32}P[b]	14.3 days	β	1.71	610 cm (air) 0.8 cm (water) 0.76 cm (Plexiglas)	285	$^{33}_{16}S$	Bone
^{33}P[b]	25.4 days	β	0.249	49 cm	156	$^{33}_{16}S$	Bone
^{35}S	87.4 days	β	0.167	24.4 cm (air)	43	$^{35}_{17}Cl$	Testes
^{125}I[c]	60 days	γ	0.27–0.035	0.2 mm (lead)	14.2	$^{125}_{52}Te$	Thyroid
^{131}I[c]	8.04 days	β	0.606	165 cm (air)	123	$^{130}_{54}Xe$	Thyroid
		γ	0.364	2.4 cm (lead)			

[a]Table compiled based on information in Lederer et al. (1967) and Shleien (1987).
[b]Recommended shielding is Plexiglas; half-value layer measurement is 1 cm.
[c]Recommended shielding is lead; half-value layer measurement is 0.02 mm.

The energy of all α particles and γ rays (measured in electron volts) is fixed, because they are of specific composition or wavelength. The energy of β particles, however, varies depending on the atom they originate from (and with concomitant release of neutrinos or antineutrinos that serve to balance the conservation of energy aspect of the decay equation). Thus there are (relatively) high-energy β particles released during the decay of ^{32}P and low-energy β particles released when tritium (^{3}H) decays.

Isotopic decay usually involves a chain or sequence of events rather than a single loss of a particle, because the resultant, equally unstable atoms try to achieve equilibrium. During this course of decay, secondary forms of radiation can be generated that may also pose a hazard to workers. For example, when high-energy β particles released during the decay of ^{32}P encounter the nuclei of atoms with a large atomic number, a strong interaction occurs. The β particle loses some energy in the form of a photon. Such photons are called Bremsstrahlung radiation; they are detectable using a monitor suitable for the detection of γ or X rays.

Following their release, α, β, and γ emissions (as well as secondary forms of radiation) travel varying average distances at varying average speeds, depending on their energy and the density of the material through which they are moving. The distance they actually travel before encountering either the electrons or nucleus of another atom is termed their degree of penetrance. This value is expressed as an average for each type of particle. The energy of the particles released (and therefore their potential penetrance) thus dictates what type of shielding, if any, is necessary for protection against the radiation generated by the decay of a given isotope. α, β, and γ emissions all have the potential, upon encountering an atom, to knock out its electrons, thereby creating ions. Thus, these three types of emissions are called ionizing radiation. The formation of such ions may result in the perturbation of biological processes: therein lies the danger associated with radioactivity!

Measuring Radioactivity and Individual Exposure to It

The radioactivity of a given substance is measured in terms of its ionizing activity. A Curie by definition is the amount of radioactive material that will produce 3.4×10^{10} disintegrations per second (see Table A.1D.2). This, not coincidentally, happens to be the number of disintegrations that occur during the decay of one gram of radium and its decay products. Exposure to such radiation is measured as the amount of energy absorbed by the recipient, which, of course, is directly related to the potential damage such radiation may cause. One rad is the dose of radiation that will cause 100 ergs of energy to be absorbed per gram of irradiated material. Another unit commonly used to measure radiation doses is the rem; this is related to the rad but takes into account a "quality factor" based on the type of ionizing radiation being received. For β particles and γ or X rays this factor is 1; therefore, rems β equal

Table A.1D.2 Conversion Factors for Radioactivity

Measurement of Radioactivity

The SI unit for measurement of radioactivity is the Becquerel:

1 Becquerel (Bq) = 1 disintegration per second

The more commonly encountered unit is the Curie (Ci):

1 Ci = 3.7×10^{10} Bq
= 2.22×10^{12} disintegrations per minute (dpm)
1 millicurie (mCi) = 3.7×10^{7} Bq = 2.22×10^{9} dpm
1 microcurie (μCi) = 3.7×10^{4} Bq = 2.22×10^{6} dpm

Conversion factors:

1 day = 1.44×10^{3} min = 8.64×10^{4} sec
1 year = 5.26×10^{5} min = 3.16×10^{7}sec
counts per minute (cpm) = dpm × (counting efficiency)

Measurement of Dose

The SI unit for energy absorbed from radiation is the Gray (Gy):

1 Gy = 1 joule/kg

Older units of absorbed energy are the rad (r) and Roentgen (R):

1 r = 100 ergs/g = 10^{-2} Gy
1 R = 0.877 r in air = 0.93 – 0.98 r in water and tissue

The SI unit for radiation dosage is the Sievert (Sv), which takes into account the empirically determined relative biological effectiveness (RBE) of a given form of radiation:

dosage [Sv] = RBE × dosage [Gy]

$$\text{RBE} = \frac{\text{(biological effect of a dose of standard radiation [Gy])}}{\text{(biological effect of a dose of other radiation [Gy])}}$$

RBE = 1 for commonly encountered radionuclides

The older unit for dosage is the rem (Roentgen-equivalent-man):

1 rem = 0.01 Sv

rads β. In contrast, the quality factor associated with α particles is 20, so an exposure of one rad due to α particles would be recorded as 20 rem.

The amount or dose of radiation received by materials (cells, scientists, etc.) near the source depends not only on the specific type and energy (penetrance) of the radiation being produced, but also on the subject's distance from the source, the existence of any intervening layers of attenuating material (shielding), and the length of time spent in the vicinity of the radiation source. To best measure such doses, every person working with or around radioactivity should wear an appropriate type of radiation detection badge (in addition to carrying a portable radiation monitor that can give an immediate, approximate reading.) This is normally a requirement for compliance with an institution's license to use radioisotopes. Such badges are usually furnished by the safety department and collected at regular intervals for reading by a contracted company. At most institutions, the old-style film badges have been replaced with the more accurate TLDs (thermoluminescent dosimeters). These take advantage of chemicals such as calcium or lithium fluorides which, following exposure to ionizing radiation, will luminesce at temperatures below their normal thermal luminescence threshold. Different types of badges are sensitive to different types of radiation: always be sure to wear one that is appropriate for detecting exposure to the isotope being used! In most places pregnant women are asked to wear a more sensitive (and more expensive) dosimeter to better monitor their (and the developing fetus') exposure. Most often workers will be asked to wear a radiation detection badge on the labcoat lapel in order to measure whole-body radiation. When working with ^{32}P or ^{125}I it is also advisable to wear a ring badge to measure exposure to the unshielded extremities (fingers). The limit set for "acceptable" exposure to whole-body radiation is several-fold less than the limit set for extremities. Nevertheless, we have found that the exposure recorded on ring badges is often significant with respect to the limit for extremities set by our institution.

Table A.1D.3 Shielding Radioactive Emission[a]

β emitters

Energy (MeV)	Mass (mg)/cm^2 to reduce intensity by 50%	Thickness (mm) to reduce intensity by 50%			
		Water	Glass	Lead	Plexiglas
0.1	1.3	0.013	0.005	0.0011	0.0125
1.0	48	0.48	0.192	0.042	0.38
2.0	130	1.3	0.52	0.115	1.1
5.0	400	4.0	1.6	0.35	4.2

γ emitters

Energy (MeV)	Thickness of material (cm) to attenuate a broad beam of γ-rays by a factor of 10			
	Water	Aluminum	Iron	Lead
0.5	54.6	20.3	6.1	1.8
1.0	70.0	24.4	8.2	3.8
2.0	76.0	32.0	11.0	5.9
3.0	89.0	37.0	12.0	6.4

[a]From Dawson et al. (1986). Reprinted with permission.

What is known about the effects on humans of exposure to low levels of radiation (i.e., levels which would be received when briefly handling mCi or μCi amounts of radioactivity)? Not much, for the obvious reason that direct studies have not been undertaken. Accordingly, guidelines for exposure levels are set using extrapolations—either by extrapolating down from population statistics obtained following accidents or disasters (the Chernobyl meltdown, atomic bombings) or by extrapolating up from numbers obtained from animal experiments. Each form of extrapolation is subject to caveats, and given that predictions based on such extrapolations cannot be perfect, most health and safety personnel aim for radiation exposure levels said to be ALARA or "as low as reasonably achievable." Further discussion of exposure limits and the statistics on which they are based may be found in B. Shleien's health physics text (Shleien, 1987). Limiting exposure to radiation can be accomplished by adjusting several parameters of the exposure: the duration of exposure, distance from the source, and the density of the material (air, water, shielding) between the individual and the source.

Time is of the essence

When designing any experiment using radioactivity, every effort should be made to limit the time spent directly handling the vials or tubes containing the radioactive material. Speed should be encouraged in all manipulations, though not to the point of recklessness! Have everything needed for the experiment ready at hand before the radioactivity is introduced into the work area.

Distance helps to determine dose

When possible, experiments involving radioactivity should be performed in an area separate from the rest of the lab. Many institutions require that such work be performed in a designated "hot lab"; however, if many people in the laboratory routinely use radioisotopes, it is less than feasible to move them all into what is usually a smaller space. No matter where an individual is working, it is his or her responsibility to monitor the work area and ensure his or her own safety and the safety of those working nearby by using adequate shielding. Obviously, when handling the radioactive samples, it is necessary to work rapidly behind any required shielding. To protect bystanders, remember that the intensity of radiation from a source (moving through air) falls off in proportion to the square of the distance. Thus, if standing 1 foot away from a source for 5 min would result in an exposure of 45 units, standing 3 feet away for the same amount of time would result in an exposure $(1/3)^2$ of 45 units, or 5 units. This factor is also relevant when considering the storage of large amounts of radioactivity, particularly ^{125}I or ^{32}P, as no amount of shielding can completely eliminate radiation.

Shielding is the key to safety

As mentioned above, the energy of the particle(s) released during the decay of an isotope

determines what, if any, type of shielding is appropriate (see Table A.1D.3). β particles released during the decay of ^{14}C and ^{35}S possess roughly ten times the energy of those released when ^{3}H decays. All three β particles are of relatively low energy, do not travel very far in air, and cannot penetrate solid surfaces. No barriers are necessary for shielding against this type of β radiation. The major health threat from these isotopes occurs through their accidental ingestion, inhalation, or injection.

β particles released during the decay of ^{32}P have 10-fold higher energy than those released from ^{14}C and pose a significant threat to workers. (One reported hazard is the potential for induction of cataracts in the unshielded eye.) The fact that these high-energy beta particles can potentially generate significant amounts of Bremsstrahlung radiation is the reason that low-density materials are used as the primary layer of shielding for ^{32}P β radiation. Water, glass, and plastic are suitable low-density materials (as opposed to lead). Obviously water is unsuitable as a shielding layer for work on the bench, although it does a reasonable job when samples are incubating in a water bath. Shields made from a thickness of glass sufficient to stop these particles would be extremely heavy and cumbersome (as well as dangerous if dropped). Fortunately, plastic or acrylic materials—variously called Plexiglas, Perspex, or Lucite—are available for shielding against ^{32}P β radiation. Shields as well as storage boxes constructed of various thicknesses of Plexiglas are necessary equipment in laboratories where ^{32}P is used. When mCi amounts of ^{32}P are used at one time it is necessary to also block the Bremsstrahlung radiation by adding a layer of high-density material (such as 4 to 6 mm lead) to the outside of the Plexiglas shield (covering the side farthest from the radioactive source).

γ rays released during the decay of ^{125}I have much higher penetrance than the β particles from ^{32}P decay; this radiation must be stopped by very-high-density material, such as lead. Lead foil of varying thicknesses (2 to 6 mm) can be purchased in rolls and can be cut and molded to cover any container, or taped to a Plexiglas shield (used in this instance for support). Obviously this latter arrangement has the disadvantage that it is impossible to see what one is doing through the shield. For routine shielding of manipulations involving ^{125}I, it is useful to purchase a lead-impregnated Plexiglas shield that is transparent, albeit inevitably very heavy (as well as relatively expensive).

Although it seems logical that the use of more radioactivity necessitates the use of thicker layers of shielding, it is also true that no shielding material is capable of completely stopping all radiation. When deciding how thick is "thick enough," consult the half-value layer measurement for each type of shielding material. This number gives the thickness of a given material necessary to stop half the radiation from a source; Table A.1D.3 lists half-value layer measurements for the β particles released from isotopes commonly used in the biology laboratory. In general, 1 to 2 cm of Plexiglas and/or 0.02 mm of lead are sufficient to shield the amounts of radioactivity used in most experiments.

GENERAL PRECAUTIONS

Before going on to a discussion of specific precautions to be taken with individual isotopes, a short list of general precautions to be taken with all isotopes seems pertinent:

1. Know the rules. Be sure that each individual is authorized to use each particular isotope and uses it in an authorized work area.

2. Don the appropriate apparel. Whenever working at the lab bench, it is good safety practice to wear a labcoat for protection. Disposable paper/synthetic coats of various styles are commercially available: at $4 each these may be conveniently thrown out if contaminated with radioactivity during an experiment, rather than held for decay as might be preferable with cloth coats costing about $30 each. As an alternative, disposable sleeves can be purchased and worn over those of the usual cloth coat. Other necessary accessories include radiation safety badges, gloves, and protective eyewear. It's handy to wear two pairs of gloves at once when using radioactivity; when the outer pair becomes contaminated, it is possible to strip it off and continue working without interruption.

3. Protect the work area as well as the workers. Lab benches and the bases of any shields used should be covered with some sort of disposable, preferably absorbent, paper sheet. Underpads or diapers (the kind normally used in hospitals) are convenient for this purpose.

4. Use appropriate designated equipment. It is very convenient, where use justifies the expense, to have a few adjustable pipettors dedicated for use with each particular isotope. Likewise, it is good practice to use only certain labeled centrifuges and microcentrifuge rotors for radioactive samples so that all the lab's

rotors do not become contaminated. Although such equipment should be cleaned after each use, complete decontamination is often not possible. A few pipettors or a single microcentrifuge can easily be stored (and used) behind appropriate shielding. Actually, contamination of the insides and tip ends of pipettors can be greatly reduced by using tips supplied with internal aerosol barriers; these have recently become very popular for setting up PCR reactions and are available to fit a variety of pipettors. To prevent contamination of the outside of the pipettor's barrel, simply wrap the hand-grip in Parafilm, which can be discarded later. Several manufacturers sell disposable paper inserts for their microcentrifuges that protect the wall of the rotor chamber from contamination that might spin off the outside of sample tubes. Trying to fashion homemade liners of this sort is not recommended, as we have had disastrous experiences using laminated adhesive paper that "unstuck" during microcentrifuge spins. These liners would get caught by the rotor, shattering sample tubes and creating an even bigger mess!

5. Know where to dispose of radioactive waste, both liquid and solid. Most institutions require that radioactive waste be segregated by isotope. This is done not only so that appropriate shielding can be placed around waste containers, but so that some waste can be allowed to decay prior to disposal through normal trash channels (see Table A.1D.4 and Fig. A.1D.1). With a decreasing number of radioactive waste disposal facilities able or willing to accept radioactive waste for burial (and a concomitant increase in dumping charges from those that still do), this practice of on-site decay can save an institution thousands of dollars a year in disposal charges.

6. Label your label! It is only common courtesy (as well as common sense) to alert coworkers to the existence of anything and everything radioactive that is left where they may come in contact with it! A simple piece of tape affixed to the sample box—with the investigator's name, the amount and type of isotope, and the date written on it—should do the trick. Yellow hazard tape printed with the international symbol for radioactivity is commercially available in a variety of widths.

7. Monitor radioactivity early and often. Portable radiation detection monitors are essential equipment for every laboratory using radioactivity. No matter how much or how little radioactivity is being used, the investigator should keep a hand-held monitor nearby—and it should be on! Turn it on before touching any radioactivity to avoid contaminating the monitor's switch. Use a monitor with the appropriate detection capacity (β for ^{35}S and ^{32}P; γ for ^{125}I) before, during, and after all procedures. The more frequently fingers and relevant equipment are monitored, the more quickly a spill or glove contamination will be detected. Such timely detection will keep both the potential mess and the cleanup time to a minimum. Because low-energy β emitters such as ^{3}H cannot be detected using such monitors, wipe tests of the bench and equipment used are necessary to ensure that contamination of the work area did not occur.

SPECIFIC PRECAUTIONS

The following sections describe precautions to be taken with individual isotopes in specific forms. Although the sections dealing with ^{35}S- or ^{32}P-labeling of proteins in intact cells are presented in terms of mammalian cells, most of the instructions are also pertinent (with minimal and obvious modifications) to the labeling of proteins in other cells (bacterial, insect, etc.).

Working with ^{35}S

Using ^{35}S to label cellular proteins and proteins translated in vitro

As discussed above, the β radiation generated during ^{35}S decay is not strong enough to make barrier forms of shielding necessary. The risk associated with ^{35}S comes primarily through its ingestion and subsequent concentration in various target organs, particularly the testes. Although willful ingestion of ^{35}S seems unlikely, accidental or unknowing ingestion may be more common. As reported several years ago (Meisenhelder and Hunter, 1988), ^{35}S-labeled methionine and cysteine, which are routinely used to label proteins in intact cells and by in vitro translation, break down chemically to generate a volatile radioactive component. The breakdown occurs independent of cellular metabolism. Thus the radioactive component is generated to the same extent in stock vials as in cell culture dishes. The process seems to be promoted by freezing and thawing ^{35}S-labeled materials. The exact identity of this component is not known, although it is probably SO_2 or CH_3SH. What is known is that it dissolves readily in water and is absorbed by activated charcoal or copper.

The amount of this volatile radioactive component released, despite stabilizers added by the manufacturers, is about 1/8000 of the total radioactivity present. The amount of this radio-

Table A.1D.4 Decay factors for calculating the amount of radioactivity present at a given time after a reference date. For example, a vial containing 1.85 MBq (50 μCi) of a ^{35}S-labeled compound on the reference date will have the following activity 33 days later: 1.85 × 0.770 = 1.42 MBq; 50 × 0.770 = 38.5 μCi.

^{125}I Half-life: 60.0 days

Days	Days 0	1	2	3	4	5	6	7	8	9
0	1.000	0.977	0.955	0.933	0.912	0.891	0.871	0.851	0.831	0.812
20	0.794	0.776	0.758	0.741	0.724	0.707	0.691	0.675	0.660	0.645
40	0.630	0.616	0.602	0.588	0.574	0.561	0.548	0.536	0.524	0.512
60	0.500	0.489	0.477	0.467	0.456	0.445	0.435	0.425	0.416	0.406
80	0.397	0.388	0.379	0.370	0.362	0.354	0.345	0.338	0.330	0.322
100	0.315	0.308	0.301	0.294	0.287	0.281	0.274	0.268	0.262	0.256
120	0.250	0.244	0.239	0.233	0.228	0.223	0.218	0.213	0.208	0.203
140	0.198	0.194	0.189	0.185	0.181	0.177	0.173	0.169	0.165	0.161
160	0.157	0.154	0.150	0.147	0.144	0.140	0.137	0.134	0.131	0.128
180	0.125	0.122	0.119	0.117	0.114	0.111	0.109	0.106	0.104	0.102
200	0.099	0.097	0.095	0.093	0.090	0.088	0.086	0.084	0.082	0.081
220	0.079	0.077	0.075	0.073	0.072	0.070	0.069	0.067	0.065	0.064
240	0.063	0.061	0.060	0.058	0.057	0.056	0.054	0.053	0.052	0.051

^{32}P Half-life: 14.3 days

Days	Hours 0	12	24	36	48	60	72	84
0	1.000	0.976	0.953	0.930	0.908	0.886	0.865	0.844
4	0.824	0.804	0.785	0.766	0.748	0.730	0.712	0.695
8	0.679	0.662	0.646	0.631	0.616	0.601	0.587	0.573
12	0.559	0.546	0.533	0.520	0.507	0.495	0.483	0.472
16	0.460	0.449	0.439	0.428	0.418	0.408	0.398	0.389
20	0.379	0.370	0.361	0.353	0.344	0.336	0.328	0.320
24	0.312	0.305	0.298	0.291	0.284	0.277	0.270	0.264
28	0.257	0.251	0.245	0.239	0.234	0.228	0.223	0.217
32	0.212	0.207	0.202	0.197	0.192	0.188	0.183	0.179
36	0.175	0.170	0.166	0.162	0.159	0.155	0.151	0.147
40	0.144	0.140	0.137	0.134	0.131	0.127	0.124	0.121
44	0.119	0.116	0.113	0.110	0.108	0.105	0.102	0.100
48	0.098	0.095	0.093	0.091	0.089	0.086	0.084	0.082
52	0.080	0.078	0.077	0.075	0.073	0.071	0.070	0.068

^{131}I Half-life: 8.04 days

Days	Hours 0	1	2	3	4	5	6	7	8	9	10	11	12
0	1.000	0.979	0.958	0.937	0.917	0.898	0.879	0.860	0.842	0.824	0.806	0.789
3	0.772	0.756	0.740	0.724	0.708	0.693	0.678	0.664	0.650	0.636	0.622	0.609
6	0.596	0.583	0.571	0.559	0.547	0.533	0.524	0.513	0.502	0.491	0.481	0.470
9	0.460	0.450	0.441	0.431	0.422	0.413	0.405	0.396	0.387	0.379	0.371	0.363
12	0.355	0.348	0.340	0.333	0.326	0.319	0.312	0.306	0.299	0.293	0.286	0.280
15	0.274	0.269	0.263	0.257	0.252	0.246	0.241	0.236	0.231	0.226	0.221	0.216
18	0.212	0.207	0.203	0.199	0.194	0.190	0.186	0.182	0.178	0.175	0.171	0.167
21	0.164	0.160	0.157	0.153	0.150	0.147	0.144	0.141	0.138	0.135	0.132	0.129
24	0.126	0.124	0.121	0.118	0.116	0.113	0.111	0.109	0.106	0.104	0.102	0.100
27	0.098	0.095	0.093	0.091	0.089	0.088	0.086	0.084	0.082	0.080	0.079	0.077
30	0.075	0.074	0.072	0.071	0.069	0.068	0.066	0.065	0.064	0.063	0.061	0.059
33	0.058	0.057	0.056	0.054	0.053	0.052	0.051	0.050	0.049	0.048	0.047	0.046
36	0.045	0.044	0.043	0.042	0.041	0.040	0.039	0.039	0.038	0.037	0.036	0.035

^{35}S Half-life: 87.4 days

Weeks	Days 0	1	2	3	4	5	6
0	1.000	0.992	0.984	0.976	0.969	0.961	0.954
1	0.946	0.939	0.931	0.924	0.916	0.909	0.902
2	0.895	0.888	0.881	0.874	0.867	0.860	0.853
3	0.847	0.840	0.833	0.827	0.820	0.814	0.807
4	0.801	0.795	0.788	0.782	0.776	0.770	0.764
5	0.758	0.752	0.746	0.740	0.734	0.728	0.722
6	0.717	0.711	0.705	0.700	0.694	0.689	0.683
7	0.678	0.673	0.667	0.662	0.657	0.652	0.646
8	0.641	0.636	0.631	0.626	0.621	0.616	0.612
9	0.607	0.602	0.597	0.592	0.588	0.583	0.579
10	0.574	0.569	0.565	0.560	0.556	0.552	0.547
11	0.543	0.539	0.534	0.530	0.526	0.522	0.518
12	0.514	0.510	0.506	0.502	0.498	0.494	0.490

^{33}P Half-life: 25.4 days

Days	Days 0	1	2	3	4	5	6	7	8	9
0	1.000	0.973	0.947	0.921	0.897	0.872	0.849	0.826	0.804	0.782
10	0.761	0.741	0.721	0.701	0.683	0.664	0.646	0.629	0.612	0.595
20	0.579	0.564	0.549	0.534	0.520	0.506	0.492	0.479	0.466	0.453
30	0.441	0.429	0.418	0.406	0.395	0.385	0.374	0.364	0.355	0.345
40	0.336	0.327	0.318	0.309	0.301	0.293	0.285	0.277	0.270	0.263
50	0.256	0.249	0.242	0.236	0.229	0.223	0.217	0.211	0.205	0.200
60	0.195	0.189	0.184	0.179	0.174	0.170	0.165	0.161	0.156	0.152
70	0.148	0.144	0.140	0.136	0.133	0.129	0.126	0.122	0.119	0.116
80	0.113	0.110	0.107	0.104	0.101	0.098	0.096	0.093	0.091	0.088
90	0.086	0.084	0.081	0.079	0.077	0.075	0.073	0.071	0.069	0.067
100	0.065	0.064	0.062	0.060	0.059	0.057	0.055	0.054	0.053	0.051
110	0.050	0.048	0.047	0.046	0.045	0.043	0.042	0.041	0.040	0.039
120	0.038	0.037	0.036	0.035	0.034	0.033	0.032	0.031	0.030	0.030

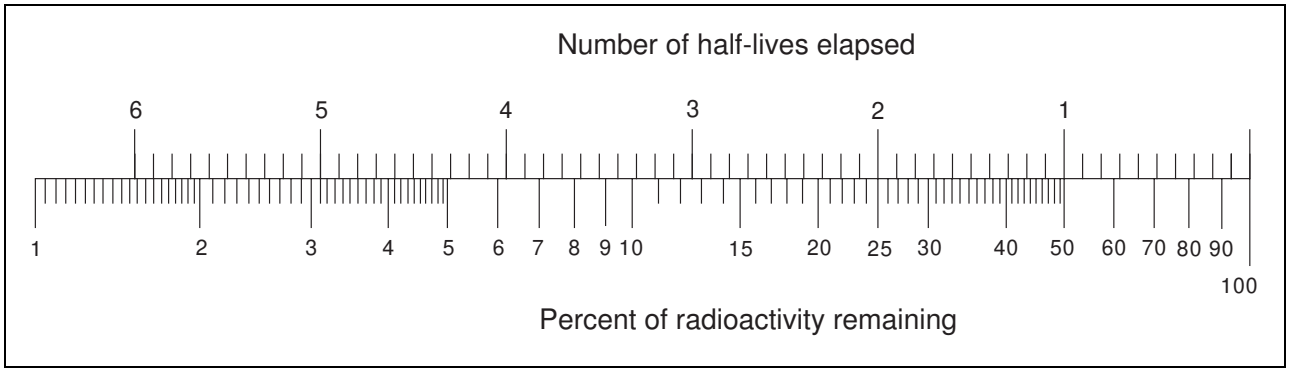


Figure A.1D.1 Correlation of loss of radioactivity with elapsing half-lives of an isotope.

activity that a scientist is likely to inhale while using these compounds is presumably even smaller. Nevertheless, such a component can potentially contaminate a wide area because of its volatility, and also tends to concentrate in target organs. Thus, it is advisable to thaw vials of ^{35}S-labeled amino acids in a controlled area such as a hood equipped with a charcoal filter. This charcoal filter will become quite contaminated and should be changed every few months. If such an area is not available, the stock vial should be thawed using a needle attached to a charcoal-packed syringe to vent and trap the volatile compound.

Anyone who has ever added ^{35}S-labeled amino acids to dishes of cells for even short periods knows that the incubator(s) used for such labelings quickly become highly contaminated with ^{35}S. Such contamination is not limited to the dish itself, nor to the shelf on which the dish was placed. Rather, the radioactive component's solubility in water allows it to circulate throughout the moist atmosphere of the incubator and contaminate all the inside surfaces of the incubator. For this reason, in laboratories where such metabolic labelings are routine, it is highly convenient to designate one incubator to be used solely for working with ^{35}S-labeled samples. Such an incubator can be fitted with a large honeycomb-style filter, the size of an incubator shelf, made of pressed, activated charcoal. These filters are available from local air-quality-control companies. Such a filter will quickly become quite contaminated with radioactivity and should therefore be monitored and changed as necessary (usually about every three months if the incubator is used several times a week). The water used to humidify the incubator will also become quite "hot" (contaminated with radioactivity); keeping the water in a shallow glass pan on the bottom of the incubator makes it easy to change it after every use, thus preventing contamination from accumulating. Even with the charcoal filter and water as absorbents, the shelves, fan, and inner glass door of the incubator will become contaminated, as will the tray on which the cells are carried and incubated. Routine wipe tests and cleaning when necessary will help to minimize potential spread of this contamination.

If such labelings are done infrequently or there is no "spare" incubator, dishes of cells can be placed in a box during incubation. This box should be made of plastic, which is generally more easily decontaminated than metal. Along with the dishes of cells, a small sachet made of activated charcoal wrapped loosely in tissue (Kimwipes work well) should be placed in the box. If the box is sealed, it will obviously need to be gassed with the correct mixture of CO_2; otherwise small holes can be incorporated into the box design to allow equilibration with the incubator's atmosphere. In either case, the incubator used for the labeling should be carefully monitored for radioactivity after each experiment.

Working with ^{32}P

µCi amounts of ^{32}P

The amount of ^{32}P-labeled nucleotide used to label nucleic acid probes for northern or Southern blotting is typically under 250 µCi, and the amount of [γ-^{32}P]ATP used for in vitro phosphorylation of proteins does not usually exceed 50 µCi for a single kinase reaction (or several hundred µCi per experiment). However, handling even these small amounts, given the time spent on such experiments, can result in an unacceptable level of exposure if proper shielding is not employed. With no intervening shielding, the dose rate 1 cm away from 1 mCi ^{32}P is 200,000 mrads/hr; the local dose rate to basal cells resulting from a skin contamination of 1 µCi/cm^2 is 9200 mrads/hr (Shleien, 1987). Such a skin contamination could be easily attained though careless pipetting and the resultant creation of an aerosol of radioactive microdroplets, because the concentration of a typical stock solution of labeled nucleotide may be 10 µCi/µl.

For proper protection during this sort of experiment, besides the usual personal attire (glasses, coat, safety badges, and gloves) it is necessary to use some form of Plexiglas screen between the body and the samples (see Fig. A.1D.2A). Check the level of radiation coming through the outside of the shield with a portable monitor to be sure the thickness of the Plexiglas is adequate. Hands can be shielded from some exposure by placing the sample tubes in a solid Plexiglas rack, which is also useful for transporting samples from the bench to a centrifuge or water bath (see Fig. A.1D.2B).

Experiments of these types often include an incubation step performed at a specific temperature, usually in a water bath. Although the water surrounding the tubes or hybridization bags will effectively stop β radiation, shielding should be added over the top of the tubes (where there is no water)—e.g., a simple flat piece of Plexiglas. If the frequency of usage justifies the expense, an entire lid for the water bath can be

constructed from Plexiglas. When hybridization reactions are performed in bags, care should be taken to monitor (and shield) the apparatus used to heat-seal the bags.

The waste generated during the experiments should also be shielded. It is convenient to have a temporary waste container right on the bench. Discard pipet tips and other solid waste into a beaker lined with a plastic bag and placed behind the shield. This bag can then be emptied into the appropriate shielded laboratory waste container when the experiment is done. Liquid waste can be pipetted into a disposable tube set in a stable rack behind the shield (see Fig. A.1D.2C).

When radiolabeled probes or proteins must be gel-purified, it may be necessary to shield the gel apparatus during electrophoresis if the samples are particularly hot. Be advised that the electrophoresis buffer is likely to become very radioactive if the unincorporated label is allowed to run off the bottom of the gel; check with a radiation safety officer for instructions on how to dispose of such buffer. It is also prudent to check the gel plates with a portable detection monitor after the electrophoresis is completed, because they sometimes become contaminated as well.

mCi amounts of ^{32}P

In order to study protein phosphorylation in intact mammalian cells, cells in tissue culture dishes are incubated in phosphate-free medium with ^{32}P-labeled orthophosphate for a period of several hours or overnight to label the proteins. The amount of ^{32}P used in such labelings can

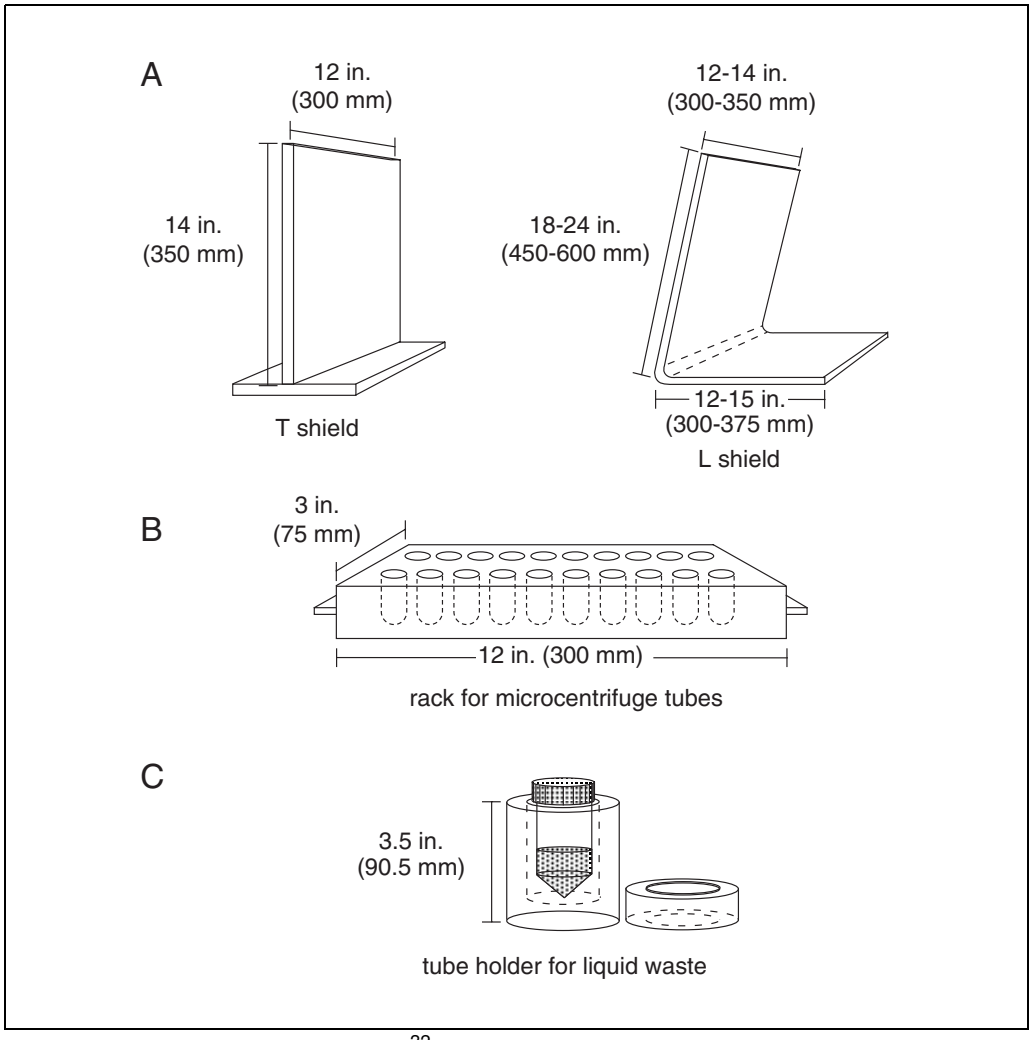


Figure A.1D.2 Plexiglas shielding for ^{32}P. (**A**) Two portable shields (L and T design) made of 0.5-in. Plexiglas. Either can be used to directly shield the scientist from the radioactivity he or she is using. Turned on its side, the L-shaped shield can be used to construct two sides of a cage around a temporary work area, providing shielding for other workers directly across or to the sides of the person working with ^{32}P. (**B**) Tube rack for samples in microcentrifuge tubes. (**C**) Tube holder for liquid waste collection.

be substantial. Cells are normally incubated in 1 to 2 mCi of ^{32}P/ml labeling medium; for each 6-cm dish of cells, 2.5 to 5 mCi ^{32}P may be used. When this figure is multiplied by the number of dishes necessary per sample, and the number of different samples in each experiment, it is clear that the amount of ^{32}P used in one experiment can easily reach 25 mCi or more. Because so much radioactivity is used in the initial labeling phase of such experiments, it is necessary for a researcher to take extra precautions in order to adequately shield him or herself and coworkers.

When adding label to dishes of cells, it is important to work as rapidly as possible. An important contribution to the speed of these manipulations is to have everything that will be needed at hand before even introducing the label into the work area. Prepare the work area, arranging shielding and covering the bench with diapers, in advance. Set out all necessary items, including any pipettors and tips needed, a portable detection monitor, extra gloves, and a cell house (see Fig. A.1D.3A).

Work involving this much radioactivity should be done behind a Plexiglas shield at least ¾ in. (2 cm) thick; the addition of a layer of lead to the outside lower section of this shield to stop Bremsstrahlung radiation is also needed. If one shield can be dedicated to this purpose at a specific location, a sheet of lead several centimeters thick can be permanently screwed to the Plexiglas (as shown in Fig. A.1D.3B); however, this lead makes the shield extremely heavy and therefore less than portable. If space constraints do not permit the existence of such a permanent labeling station, a layer or two of lead foil can be taped temporarily to the outside of the Plexiglas shield.

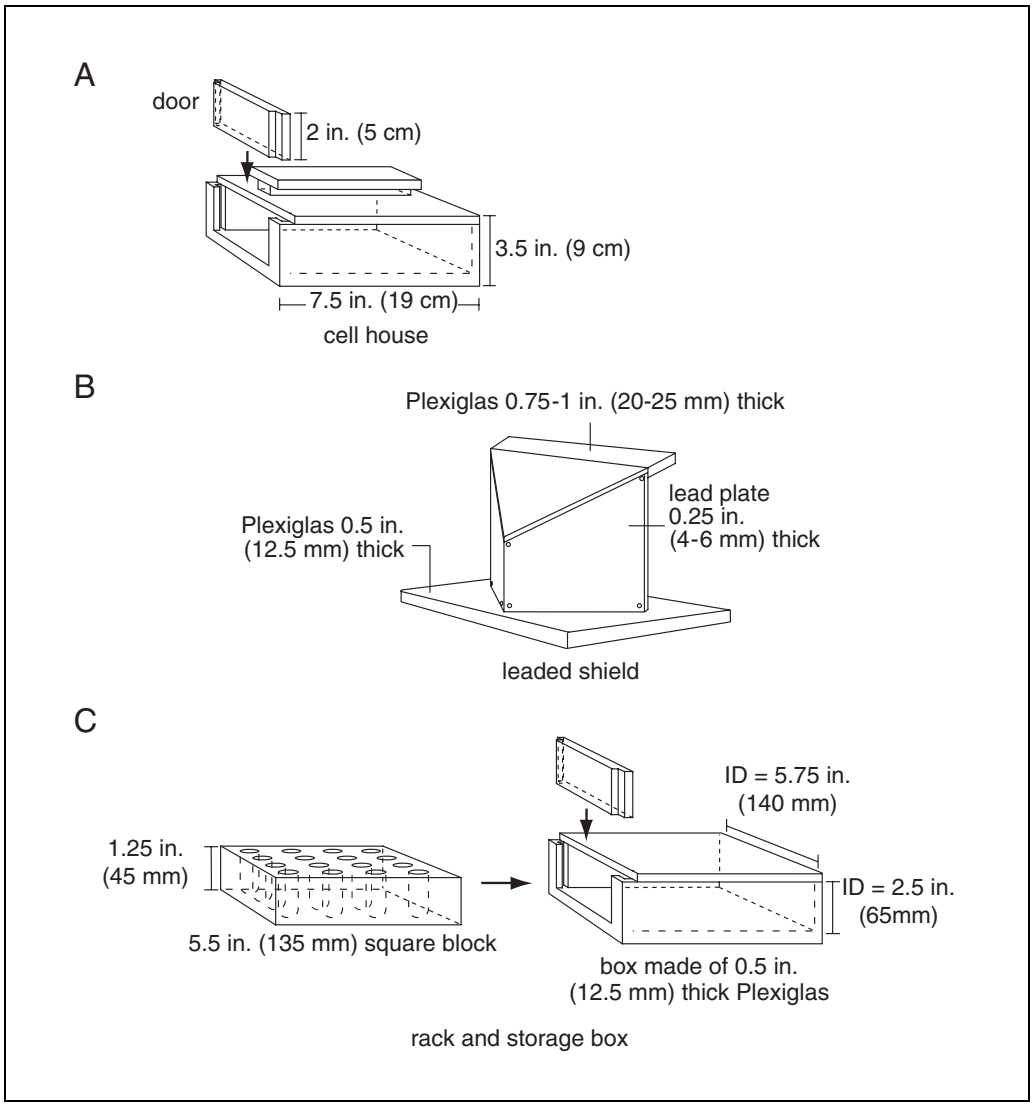


Figure A.1D.3 (**A**) Box for cell incubation (a "cell house"). (**B**) Stationary leaded shield. (**C**) Sample storage rack and box made of 0.5-in. Plexiglas. Abbreviations: ID, interior dimension.

Again, each worker should take care to shield not only him or herself, but bystanders on all sides. Handling of label should be done away from the central laboratory if possible to take maximum advantage of distance as an additional form of shielding. It is also advisable not to perform such experiments in a tissue culture room or any other room that is designed for a purpose vital to the whole laboratory. An accident involving this much ^{32}P would seriously inconvenience future work in the area, if not make it altogether uninhabitable! If care is taken to minimize the amount of time the dish of cells is open when adding the label, use of a controlled air hood to prevent fungal or bacterial contamination of the cells should not be necessary.

Once the label has been added to the dishes of cells, they will also need to be shielded for transport to and from the incubator and other work areas. Plexiglas boxes that are open at one end (for insertion of the dishes) and have a handle on top (for safe carrying) make ideal "cell houses" (see Figure A.1D.3A). A Plexiglas door that slides into grooves at the open end is important to prevent dishes from sliding out if the box is tilted at all during transport. If this door is only two-thirds the height of the house wall, the open slot thus created will allow equilibration of the CO_2 level within the house with that in the incubator. Obviously, this slot will also allow a substantial stream of radiation to pass out of the cell house, so the house should be carried and placed in the incubator with its door facing away from the worker (and others)!

Following incubation with label and any treatments or other experimental manipulations, the cells are usually lysed in some type of detergent buffer. It is during this lysis procedure that a worker's hands receive their greatest exposure to radiation, because it is necessary to directly handle the dishes over a period of several minutes. It is therefore very important to streamline this procedure and use any shielding whenever possible. If the cell lysates must be made at 4°C, as required by most protocols, working on a bench in a cold room is preferable to placing the dishes on a slippery bed of ice. In either case, make the lysate using the same sort of shielding (with lead if necessary) that was used when initially adding the label. Pipet the labeling medium and any solution used to rinse unincorporated radioactivity from the cells into a small tube held in a solid Plexiglas holder (shown in Fig. A.1D.2C). The contents of this tube can later be poured into the appropriate liquid waste receptacle. If possible, it is a good practice to keep this high-specific-activity ^{32}P liquid waste separate from the lower-activity waste generated in other procedures so that it can be removed from the laboratory as soon as possible following the experiment. If it is necessary to store it in the laboratory for any time, the shielding for the waste container should also include a layer of lead.

The solid waste generated in the lysis part of these experiments (pipet tips, disposable pipets, cell scrapers, and dishes) is very hot and should be placed immediately into some sort of shielded container to avoid further exposure of the hands. A Plexiglas box similar in design to that in Figure A.1D.4 is convenient; placed to the side of the shield and lined with a plastic bag, it will safely hold all radioactive waste during the experiment and is light enough to be easily carried to the main laboratory waste

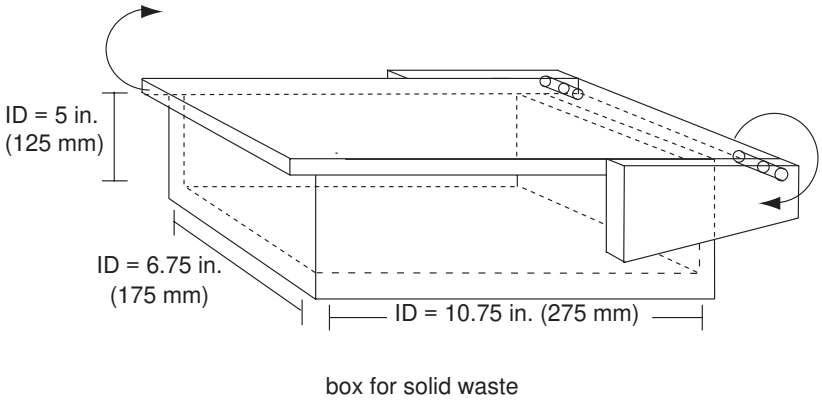


Figure A.1D.4 Box for solid waste collection made of 0.5-in. Plexiglas. Abbreviation: ID, interior dimension.

container where the plastic bag (and its contents) can be dumped after the experiment is done. If the lid of the box protrudes an inch or so over the front wall, it can be lifted using the back of a hand, thus decreasing the possibility of contaminating it with hot gloves.

When scraping the cell lysates from the dishes, it is good practice to add them to microcentrifuge tubes that are shielded in a solid Plexiglas rack; this will help to further reduce the exposure to which the hands are subjected. At this point, the lysates are usually centrifuged at high speed (>10,000 × *g*) to clear them of unsolubilized cell material. Use screw-cap tubes for this clarification step, as these will contain the labeled lysate more securely than flip-top tubes, which may open during centrifugation. No matter what type of tube is used, the rotor of the centrifuge often becomes contaminated, most probably due to tiny drops of lysate (aerosol) initially present on the rim of the tubes that are spun off during centrifugation. Monitor the rotor and wipe it out after each use.

The amount of ^{32}P taken up by cells during the incubation period varies considerably, depending on the growth state of the culture as well as on the cell type and its sensitivity to radiation. This makes it difficult to predict the percentage of the radioactivity initially added to the cells that is incorporated into the cell lysate; however, this figure probably does not exceed 10%. Thus, the amount of radioactivity being handled decreases dramatically after lysis, making effective shielding much simpler. However, at least ten times more radioactivity than is usual in other sorts of experiments is still involved! It is easy to determine if the shielding is adequate—just use both β and γ portable monitors to measure the radiation coming through it. Again, be sure to check that people working nearby (including those across the bench) are also adequately shielded. It is sometimes necessary to construct a sort of cage of Plexiglas shields around the ice bucket that contains the lysates.

At the end of the day or the experiment, it may be necessary to store radioactive samples; in some experiments, it may be desirable to save the cell lysates. These very hot samples are best stored in tubes placed in solid Plexiglas racks that can then be put into Plexiglas boxes (see Fig. A.1D.3C). Such boxes may be of similar construction to the cell houses described above; however, they should have a door that completely covers the opening. Be sure to check the γ radiation coming through these layers and add lead outside if necessary.

Working with ^{33}P

Using ^{33}P-labeled nucleotides to label nucleic acid probes or proteins

Several of the major companies that manufacture radiolabeled biological molecules have recently introduced nucleotides labeled with ^{33}P (both α- and γ-labeled forms). ^{33}P offers a clear advantage over ^{32}P with respect to ease of handling, because the energy of the β particles it releases lies between that of ^{35}S and ^{32}P and thus its use does not require as many layers of Plexiglas and lead shielding as for ^{32}P. In fact, the β radiation emitted can barely penetrate through gloves and the surface layer of skin, so the hazard associated with exposure to even millicurie amounts of ^{33}P is thought to be insignificant (as reported in the DuPont NEN product brochure). Gel bands visualized on autoradiographs of ^{33}P-labeled compounds are sharper than bands labeled with ^{32}P because the lower-energy β radiation does not have the scatter associated with that from ^{32}P. The half-life of ^{33}P is also longer (25 days compared to 14 days for ^{32}P). Despite its higher cost, these features have led many researchers to choose ^{33}P-labeled nucleotides for use in experiments such as band/gel shift assays where discrimination of closely-spaced gel bands is important.

The best way to determine what degree of shielding is needed when using ^{33}P is to monitor the source using a portable β monitor and add layers of Plexiglas as necessary.

Working with ^{125}I

Using ^{125}I to detect immune complexes (Immunoblots)

^{125}I that is covalently attached to a molecule such as staphylococcal protein A is not volatile and therefore is much less hazardous than the unbound or free form. Most institutions do not insist that work with bound ^{125}I be performed in a hood, but shielding of the γ radiation is still necessary. Lead is a good high-density material for stopping these γ rays; its drawbacks are its weight and opacity. Commercially available shields for ^{125}I are made of lead-impregnated Plexiglas—though heavy, these are at least see-through. Alternatively, a piece of lead foil may be taped to a structural support, although this arrangement does not provide shielding for the head as a worker peers over the lead!

Incubations of the membrane or blot with the [^{125}I] protein A solution and subsequent washes are usually done on a shaker. For shielding during these steps, a piece of lead foil may

simply be wrapped around the container. Solutions of ^{125}I can be conveniently stored for repeated use in a rack placed in a lead box.

Using ^{125}I to label proteins or peptides in vitro

Any experiments that call for the use of free, unbound ^{125}I should be done behind a shield in a hood that contains a charcoal filter to absorb the volatile iodine. Most institutions require that such experiments be done in a special hot lab to which access is limited. Ingested or inhaled iodine is concentrated in the thyroid; a portable γ monitor should therefore be used to scan the neck and throat before beginning and after completing each experiment. Similar scans should routinely be performed on all members of any laboratory in which unbound iodine is used.

DEALING WITH ACCIDENTS

Despite the best intentions and utmost caution, accidents happen! Accidents involving spills of radioactivity are particularly insidious because they can be virtually undetectable yet pose a significant threat to laboratory workers. For this reason it is best to foster a community spirit in any laboratory where radioisotopes are routinely used—a sense of cooperativity that extends from shielding each other properly to helping each other clean up when such accidents occur.

The specific measures to be taken following an accident involving radioactivity naturally depend on the type and amount of the isotope involved, the chemical or biological hazards of the material it is associated with, and the physical parameters of the spill (i.e., where and onto what the isotope was "misplaced"). However, following any accident there are several immediate steps that should be taken:

1. Alert coworkers as well as Radiation Safety personnel to the fact that there has been an accident. This will give them the opportunity to shield themselves if necessary—and to help clean up as well!

2. Restrict access to and away from the site of the accident to ensure that any uncontained radioactive material is not spread around the laboratory. When leaving the site be sure to monitor the bottoms of the shoes as well as the rest of the body.

3. Take care of all contaminated personnel first, evacuating others if necessary. If anyone's skin is contaminated, first use a portable monitor to identify specific areas of contamination. Then wipe these areas with a damp tissue to remove as much surface radioactivity as possible. Try to scrub only small areas at a time to keep the contamination localized. If the contamination is not easily removed with paper tissues, try a sponge or an abrasive pad, but be careful not to break the skin! Sometimes soaking is required: do this only after all easily removed contamination is gone and keep the soaked area to a minimum. Contaminated strands of hair can be washed (or perhaps a new hairstyle may be in order).

4. When attempting to clean any contaminated equipment, floors, benches, etc., begin by soaking up any visible radioactive liquid with an absorbent material. Use a small amount of soap and water to clean the contaminated area, keeping the area wiped each time to a minimum to avoid smearing the contamination over an even greater surface. Many surfaces prove resistant to even Herculean cleaning efforts; in these instances the best that can be done is to remove all contamination possible and then shield whatever is left until the radioactivity decays sufficiently for safety.

LITERATURE CITED

Dawson, R.M.C., Elliot, D.C., Elliott, W.H., and Jones, K.M. (eds.) 1986. Data for Biochemical Research. Alden Press, London.

Lederer, C.M., Hollander, J.M., and Perlman, I. (eds.) 1967. Table of Radioisotopes, 6th edition. John Wiley and Sons, New York.

Meisenhelder, J. and Hunter, T. 1988. Radioactive protein-labelling techniques. *Nature* 335:120.

Shleien, B. (ed.) 1987. Radiation Safety Manual for Users of Radioisotopes in Research and Academic Institutions. Nucleon Lectern Associates, Olney, Md.

Contributed by Jill Meisenhelder and Kentaro Semba
The Salk Institute
La Jolla, California

Absorption and Emission Maxima for Common Fluorophores

Table A.1E.1 lists absorption and emission maximum wavelengths for common fluorophores used in cell biology. This information can be used in designing experiments and selecting appropriate filters for use with the fluorophores.

Table A.1E.1 Absorption and Emission Maxima for Common Fluorophores[a]

Fluorophore	Absorption maximum (nm)	Emission maximum (nm)
Acid fuchsin	540	630
ACMA	430	474
Acridine red	455-600	560-680
Acridine orange	500 (with DNA) 460 (with RNA)	526 (with DNA) 650 (with RNA)
Acridine yellow	470	550
Acriflavin	436	520
AFA (acriflavin Feulgen SITSA)	355-425	460
Alizarin complexon	530-560	580
Alizarin red	330-560	580
Allophycocyanin	650	660
AMCA-S, AMC	345	445
Aminoactinomycin D	555	655
7-Aminoactinomycin D (7-AAD)	546	647
Aminocoumarin	350	445
Anthroyl stearate	361-381	446
Astrazon brilliant red 4C	500	585
Astrazon orange R	470	540
Astrazon red 6B	520	595
Astrazon yellow 7 CLL	450	480
Atabrine	436	490
Auramine	460	550
Aurophosphine	450-490	515
Aurophosphine G	450	580
BAO 9 (bisaminophenyloxadiazole)	365	395
BCECF	482 (low pH) 503 (high pH)	520 (low pH) 528 (high pH)
Berberine sulfate	430	550
Bisbenzamide	360	600-610
Blancophor FFG solution	390	470
Blancophor SV	370	435
BOBO 1, BO-PRO 1	462	481
BODIPY FL	505	513
BODIPY TMR	542	574
BODIPY TR	589	617
Brilliant sulfoflavin FF	430	520
Calcein	494	517
Calcein blue	370	435

continued

Contributed by Brian Herman
Current Protocols in Cell Biology (1998) A.1E.1-A.1E.5

Table A.1E.1 Absorption and Emission Maxima for Common Fluorophores[a], continued

Fluorophore	Absorption maximum (nm)	Emission maximum (nm)
Calcium crimson	590	615
Calcium green	506	533
Calcium orange	549	576
Calcofluor RW solution	370	440
Calcofluor white	440	500-520
Calcophor white ABT solution	380	475
Calcophor white standard solution	365	435
5-(and 6-)Carboxy SNARF-1 indicator	548 (low pH) 576 (high pH)	587 (low pH) 635 (high pH)
6-Carboxyrhodamine 6G	525	555
Cascade blue	400	420
Catecholamine	410	470
CL-NERF	504 (low pH) 514 (high pH)	587 (low pH) 540 (high pH)
Coriphosphine O	460	575
Coumarin-phalloidin	387	470
Cy3.18	554	568
Cy5.18	649	666
Cy7	710	805
DANS (1-dimethylamino-naphaline-5-sulfonic acid)	340	525
Dansyl NH-CH_3 in water	340	578
DAPI	358	461
DiA	456	590
Diamino phenyl oxydiazole (DAO)	280	460
Di-8-ANEPPS	488	605
DiD [$DiIC_{18}(5)$]	644	665
Dimethylamino-5-sulfonic acid	310-370	520
DiI [$DiIC_{18}(3)$]	549	565
DiO [$DiOC_{18}(3)$]	484	501
Diphenyl brilliant flavine 7GFF	430	520
DM-NERF	497 (low pH) 510 (high pH)	527 (low pH) 536 (high pH)
Dopamine	340	490-520
ELF-97 alcohol	345	530
Eosin	524	544
Erythrosin ITC	530	558
Ethidium bromide	518	605
Euchrysin	430	540
FIF (formaldehyde-induced fluorescence)	405	433
Flazo orange	375-530	612
Fluorescein	494	518
Fluorescein isothiocyanate (FITC)	490	523
Fluo-3	506	526
FM1-43	479	598
Fura-2	363 (low $[Ca^{2+}]$) 335 (high $[Ca^{2+}]$)	512 (low $[Ca^{2+}]$) 505 (high $[Ca^{2+}]$)

continued

Table A.1E.1 Absorption and Emission Maxima for Common Fluorophores[a], continued

Fluorophore	Absorption maximum (nm)	Emission maximum (nm)
Fura Red	472 (low $[Ca^{2+}]$)	657 (low $[Ca^{2+}]$)
	436 (high $[Ca^{2+}]$)	637 (high $[Ca^{2+}]$)
Genacryl brilliant red B	520	590
Genacryl brilliant yellow 10GF	430	485
Genacryl pink 3G	470	583
Genacryl yellow 5GF	430	475
Gloxalic acid	405	460
Granular blue	355	425
Hematoporphyrin	530-560	580
Hoechst 33258, Hoechst 33342	352	461
3-Hydroxypyrene 5,8,10-trisulfonic acid	403	513
7-Hydroxy-4-methylcoumarin	360	455
5-Hydroxytryptamine (5-HT)	400	530
Intrawhite Cf liquid	360	430
Indo-1	346 (low $[Ca^{2+}]$)	475 (low $[Ca^{2+}]$)
	330 (high $[Ca^{2+}]$)	401 (high $[Ca^{2+}]$)
Leucophor PAF	370	430
Leucophor SF	380	465
Leucophor WS	395	465
Lissamine rhodamine B	570	590
Lucifer yellow CH	428	536
Lucifer yellow VS	430	535
LysoSensor blue DND-192, DND-167	374	425
LysoSensor green DND-153, DND-189	442	505
LysoSensor yellow/blue	384 (low pH)	540 (low pH)
	329 (high pH)	440 (high pH)
LysoTracker green	504	511
LysoTracker yellow	534	551
LysoTracker red	577	592
Magdala red	524	600
Magnesium green	506	531
Magnesium orange	550	575
Maxilon brilliant flavin 10 GFF	450	495
Maxilon brilliant flavin 8 GFF	460	495
MitoTracker green FM	490	516
MitoTracker orange CMTMRos	551	576
Mithramycin	450	570
MPS (methyl green pyronine stilbene)	364	395
NBD	465	535
NBD amine	450	530
Nitrobenzoxadidole	460-470	510-650
Noradrenaline	340	490-520
Nuclear fast red	289-530	580
Nuclear yellow	365	495
Nylosan brilliant flavin E8G	460	510
Oregon green 488 fluorophore	496	524
Oregon green 500 fluorophore	503	522

continued

Table A.1E.1 Absorption and Emission Maxima for Common Fluorophores[a], continued

Fluorophore	Absorption maximum (nm)	Emission maximum (nm)
Oregon green 514 fluorophore	511	530
Pararosaniline (Feulgen)	570	625
Phorwite AR solution	360	430
Phorwite BKL	370	430
Phorwite Rev	380	430
Phorwite RPA	375	430
Phosphine 3R	465	565
Phycoerythrin R	480-565	578
Pontochrome blue black	535-553	605
POPO 1, PO-PRO 1	434	456
Primuline	410	550
Procion yellow	470	600
Propidium iodide	536	617
Pyronine	410	540
Pyronine B	540-590	560-650
Pyrozal brilliant flavin 7GF	365	495
Quinacrine mustard	423	503
R-phycoerythrin	565	575
Resorufin	570	585
RH 414	500	635
Rhodamine 110	496	520
Rhodamine 123	507	529
Rhodamine 5 GLD	470	565
Rhodamine 6G	526	555
Rhodamine B	540	625
Rhodamine B 200	523-557	595
Rhodamine B Extra	550	605
Rhodamine BB	540	580
Rhodamine BG	540	572
Rhodamine green fluorophore	502	527
Rhodamine red	570	590
Rhodamine WT	530	555
Rhodol green fluorophore	499	525
Rose Bengal	540	550-600
Serotonin	365	520-540
Sevron brilliant red 2B	520	595
Sevron brilliant red 4G	500	583
Sevron brilliant red B	530	590
Sevron orange	440	530
Sevron yellow L	430	490
SITS (primuline)	395-425	450
SITS (stilbene isothiosulfonic acid)	365	460
SNARF-1	563	639
Sodium green	507	535
Stilbene	335	440
Sulforhodamine B and C	520	595
Sulphorhodamine G Extra	470	570

continued

Table A.1E.1 Absorption and Emission Maxima for Common Fluorophores[a], continued

Fluorophore	Absorption maximum (nm)	Emission maximum (nm)
SYTOX green nucleic acid stain	504	523
SYTO green fluorescent nucleic acid stains	494 ± 6	515 ± 17
SYTO green fluorescent nucleic acid stains	515 ± 7	543 ± 13
SYTO 17 red fluorescent nucleic acid stain	621	634
Tetracycline	390	560
Tetraethylrhodamine (rhodamine B)	555	580
Texas red	595	615
Thiazine red R	510	580
Thioflavin S	430	550
Thioflavin TCN	350	460
Thioflavin 5	430	550
Thiolyte	370-385	477-484
Thiozol orange	453	480
Tinopol CBS	390	430
TOTO 1, TO-PRO 1	514	533
TOTO 3, TO-PRO 3	642	660
True Blue	365	420-430
Ultralite	656	678
Uranine B	420	520
Uvitex SFC	365	435
X-Rhodamine	580	605
Xylene orange	546	580
XRITC	582	601
YOYO 1, YOYO-PRO 1	491	509
YOYO 3, YOYO-PRO 3	612	613

[a]Adapted with permission from Herman (1998) and Haugland (1996).

LITERATURE CITED

Haugland, R. 1996. Handbook of Fluorescent Probes and Research Chemicals, 6th ed. Molecular Probes, Inc. Eugene, Oregon.

Herman, B. 1998. Fluorescence Microscopy. Bios Scientific Publishing, Oxford, England.

Contributed by Brian Herman
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

Importing Biological Materials

Scientific cooperation is no longer confined to laboratories at the same institution, nor between laboratories within the boundaries of the United States. More than ever, scientists are forming collaborations with their peers outside the U.S., leading not only to the exchange of knowledge and ideas, but also to the exchange of biological reagents. Thus, investigators need to be aware that an import permit may be required to obtain purified proteins, chemically synthesized materials, cell lines, tissues, and other biological materials from abroad. Before requesting any biological material from a laboratory outside the U.S., it is the responsibility of the "receiver" to ascertain whether an import permit is required, to apply for the permit if one is determined to be necessary, and to provide the "shipper" with an official copy of the permit to be included with the shipped material.

There are two government agencies in place to issue permits for the importation of most biologicals. Materials derived from all animals are subject to regulations set forth by the United States Department of Agriculture (USDA) and must be cleared by USDA inspectors at the port of arrival before entry into the United States is authorized. In general, an import permit issued by the USDA is necessary for receipt of any animal-derived material or any biological material (including proteins) that has been in contact with materials of animal origin. The USDA does not have regulatory authority over the importation of human or nonhuman primate material unless it is produced via tissue culture, due to the use of bovine material (fetal bovine serum or calf serum) in tissue culture. The U.S. Public Health Service (UPHS) has jurisdiction over human and nonhuman primate materials. According to UPHS regulations, any etiologic agent, animal host, or vector of human disease may not be imported in the United States nor distributed after importation without being accompanied by a permit issued by the Director of the Centers for Disease Control. Failure to obtain the appropriate import permit will result in the shipment being held at the port of entry until a permit is applied for and issued (a 4- to 6-week process), or it may result in shipment confiscation and destruction by the quarantine officer present.

IMPORTING RESTRICTED BIOLOGICAL MATERIALS

The following is a list of restricted biological materials requiring an import permit: proteins, monoclonal and polyclonal antibodies, antisera, immunoglobins, recombinant products, enzymes, hormones, immunoassay components or kits, animal tissues, blood, cells, cell lines, RNA/DNA extracts, plasmids, vectors, microorganisms—e.g., fungi, bacteria, viruses—and other products that are derived from or have been in contact with animal-derived materials. In addition, materials received from countries shown not to be free of foot-and-mouth disease (see Title 9, code of Federal Regulations, Part 94.1) require an import permit. Note that the USDA will not permit the importation of cell cultures, monoclonal antibodies, ascites fluid, or bovine serum from countries where rinderpest and foot-and-mouth disease are present unless the imported materials are determined to be virus-free. Cell cultures being imported into the U.S. must have been grown only with fetal bovine serum of U.S., Canadian, New Zealand, or Australian origin. Material exposed to bovine products originating in the United Kingdom, Switzerland, Ireland, France, or Portugal may NOT be used, either directly or indirectly, in any animals due to the presence of bovine spongiform encephalopathy (BSE) in those countries, and the material is restricted to in vitro uses only.

CRITERIA FOR HANDLING IMPORTED RESTRICTED MATERIALS

An import permit is only valid for transfer of the material(s) listed on the permit (a permit can cover more than one item), and for shipment between the two laboratories listed on the permit. Therefore, for each laboratory abroad from which biological materials are to be imported, a separate import permit is required. Once biological materials have been received from abroad, the import permit still imposes regulations on how the materials are handled by the receiver. Import permits do not usually authorize direct or indirect exposure of domestic animals—work is limited to in vitro uses only. In addition, permits are only valid for work conducted or directed by the permit holder at his or her facilities. Materials cannot

Contributed by Paula Wolf Bryant

Current Protocols in Cell Biology (2000) A.1F.1-A.1F.3

be removed to another location, nor distributed to others, without USDA authorization. There is one exception: monoclonal antibodies produced by hybridoma cell lines may be commercially distributed for in vitro uses.

IMPORTING NONRESTRICTED BIOLOGICAL MATERIALS

Any of the restricted materials that do not contain animal- or cell culture-derived products or additives such as albumin, serum, or gelatin, or were not exposed to any infectious agents of agricultural concern, do not need an official import permit. However, an accompanying declaration is required with each shipment to indicate that the material does not contain any animal- or cell culture-derived products or additives. This information should be supplied with each shipment in a clear and concise manner, and be available for the USDA Port of Entry Inspector's review. A separate memo or letter should be included with the shipping documents, such as U.S. Customs declaration and invoice. Note, whether a particular biological material requires an import permit cannot be decided by the investigator; the investigator must contact the USDA for a decision well in advance of making arrangements for importing the material.

PACKAGING BIOLOGICAL MATERIALS FOR IMPORTATION

Imported biological materials are subject to packaging and shipping requirements of various federal and international regulations. Proper packaging is the primary consideration and of utmost importance in the safe transportation of hazardous materials. If the biological material being shipped is infectious or radioactive, this information has to be noted on the outside of the package. In addition, if dry ice is used for shipment, that must be clearly stated on the package. It is of course advantageous to the investigator that the shipped material be packaged in the most efficient way possible, as well as in a form which will ensure its stability. If proteins are to be shipped, it may be better to send the samples lyophilized or blotted on a membrane, rather than in liquid form. This will omit the need for dry ice or ice packs in the packaging, which will save on shipping costs as well as ensure the samples will be stable if receipt of the package meets with unforeseen delays. If sending materials that require dry ice or ice packs, such as antibodies or antisera, there must be enough dry ice to allow the package to maintain the appropriate temperature during shipping for approximately 5 days. This requires utilizing much larger packaging boxes than are normally used to ship the same material within the United States. When shipping cell lines, sometimes it is better to send cells in culture, rather than frozen aliquots. To send cells in culture, the tissue culture flask containing the live cells must be filled to the very top with the appropriate medium, tightly capped, and the seal secured with parafilm. If the tissue culture flask is not completely filled with medium, the resulting air pockets will cause the flasks to explode in flight. Thus, the packaging of any biological material for shipping is an important consideration to ensure success of the scientific collaboration!

OBTAINING AN IMPORT PERMIT APPLICATION

The Division of Veterinary Services of the USDA Animal and Plant Health Inspection Service (APHIS) administers regulatory programs to control the import/export of biological materials. Import permits can be obtained from either local USDA port offices or from APHIS directly: USDA, APHIS, Veterinary Services, National Center for Import-Export Products Program, 4700 River Road Unit 40, Riverdale, MD 20737-1231, Tel: (301) 734-7830, Fax: (301) 734-8226.

When submitting an import application, (1) obtain the import permit well in advance of the proposed shipping date; (2) inform the supplier of the USDA's requirements, and do not allow shipment of materials until an import permit has been issued and the supplier has received a copy of the permit; (3) list all the potential U.S. ports of entry; (4) specify whether the material is for in vitro or in vivo use and whether it is for commercial distribution or for research in one's own laboratory; (5) list any treatments the material has undergone, such as processing and purification steps involving pH, heat, chromatography, or other methods; and (6) indicate the source(s) of nutrient factors in culture media, cell line designation and history, whether enzymes were used, and what types of viruses (if any) are being studied in the laboratory of origin (all must be listed).

RESOURCES

APHIS Web Site

More information regarding APHIS programs and import/export can be obtained on

their internet home page at *http://www.aphis.usda.gov.*

Fax Service of Veterinary Services, National Center for Import-Export

By calling (301) 734-4952, the investigator can access an automated document retrieval system and arrange to have an import application faxed directly, and choose from a menu of documents that describe general instructions for completing the application, as well as information regarding the importation of biological materials from 18 distinct categories.

Contributed by Paula Wolf Bryant
Harvard Medical School
Boston, Massachusetts

Centrifuges and Rotors

Centrifugation runs described in this book usually specify a relative centrifugal force (RCF; measured in $\times g$), corresponding to a speed (in rpm) for a particular centrifuge and rotor model. As available equipment will vary from laboratory to laboratory, the investigator must be able to adapt these specifications to other centrifuges and rotors.

The relationship between RCF and speed (rpm) is determined by the following equation:

$$RCF = 1.12r\ (rpm/1000)^2$$

where r is the rotating radius between the particle being centrifuged and the axis of rotation. In most cases, an accurate conversion from speed to relative centrifugal force (or vice versa) can be obtained using the maximum value of r—or r_{max}—equal to the distance between the axis of rotation and the bottom of the centrifuge tube as it sits in the well or bucket of the rotor.

Table A.1G.1 provides r_{max} values for commonly used rotors manufactured by Sorvall, Beckman Coulter, Fisher, and IEC. There are situations (e.g., where an adapter is used to fit a smaller tube into a larger rotor well) where r_{max} will not accurately represent the effective rotating radius. In such cases, the manual for the rotor should be consulted to obtain the appropriate value of r. The most current information is available on the manufacturer's home page (see Internet Resources).

As an alternative to use of the above equation, the nomograms in Figures A.1G.1 and A.1G.2 make it possible to determine the RCF where speed and r_{max} are known, or the speed where RCF and r_{max} are known. This is done by aligning a ruler across the two known values and reading the unknown value at the point where the ruler crosses the remaining column. Figure A.1G.1 should be used for centrifuge runs <21,000 rpm, while Figure A.1G.2 should be used for faster speeds.

NOTE: In this manual, for spins involving microcentrifuges built to the Eppendorf standard, a shortened style of reference including only the speed (in rpm) may be used. All of these instruments have approximately the same rotating radius; hence the same speed will yield the same RCF value from machine to machine. Microcentrifuge spins may also be described as at "top speed" or "maximum speed," meaning 12,000 to 14,000 rpm, which is the maximum speed for all Eppendorf-type microcentrifuges.

CAUTION: Do not exceed maximum rotor speed! For Beckman Coulter ultracentrifuges, the maximum speed for each rotor is denoted by its name, e.g., the maximum speed of the Beckman VTi 80 rotor is 80,000 rpm. This speed refers only to centrifugation of solutions below a particular allowed density, which differs among rotors (see user manual). For centrifugation of high-density solutions, rotor maximum speed can be determined as: reduced rpm = $rpm_{max}(A/B)^{1/2}$, where A = allowed density and B = density of solution. A = 1.7 g/ml for several vertical rotors (including VTi 80 and VTi 50), and 1.2 g/ml for several swinging-bucket rotors (including SW 55 Ti, 28, 28.1, 40 Ti, 50.1). For gradients using heavy salts such as CsCl, particularly at low temperatures, maximum rpm should be reduced to prevent precipitation (see user manual).

Table A.1G.2 describes centrifuge tube materials and their properties, including optical properties, appropriate methods for sterilization, and chemical resistances (tolerance to various media, organic solvents, and alcohols).

Current Protocols in Cell Biology (2000) A.1G.1-A.1G.7

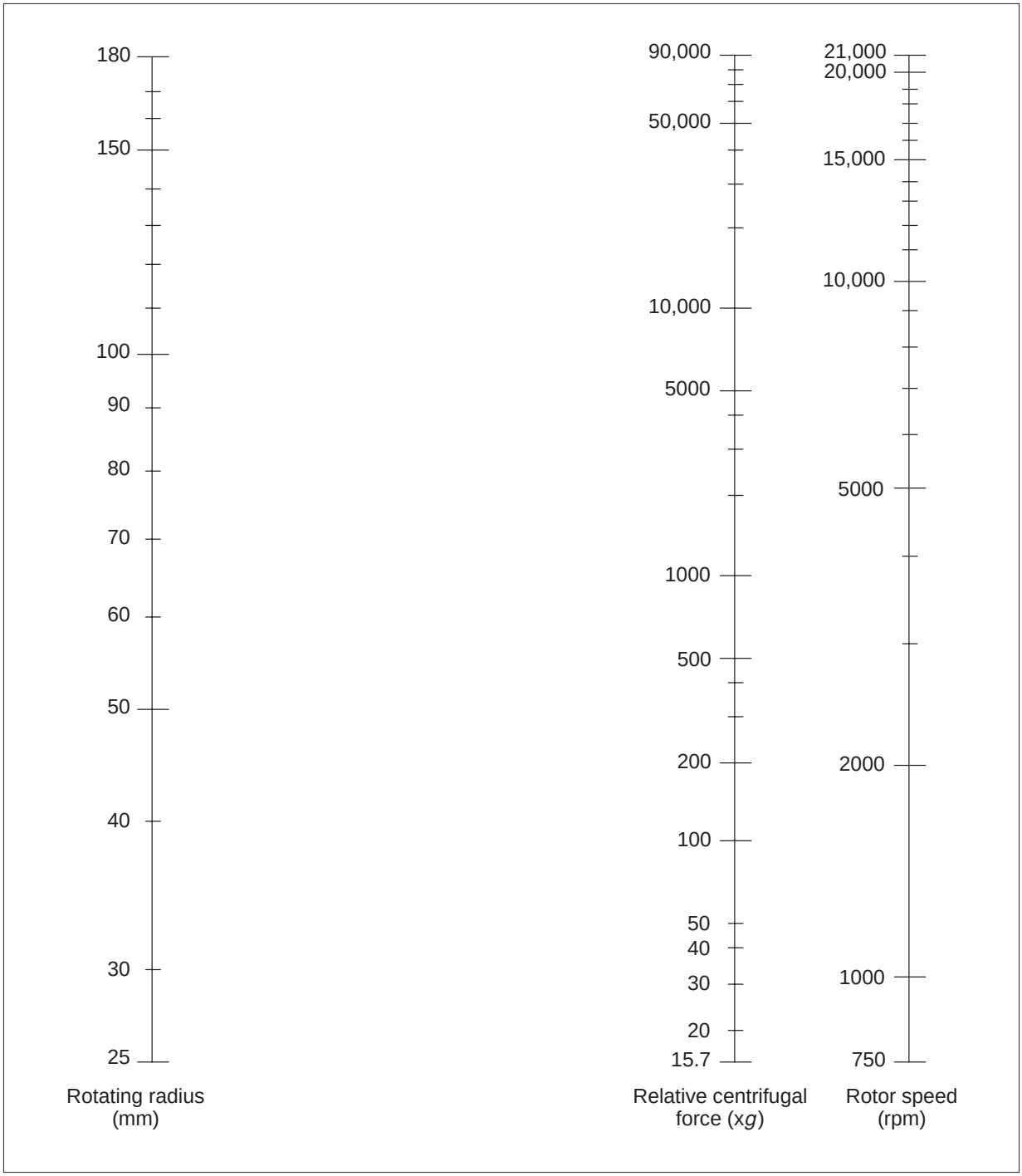


Figure A.1G.1 Nomogram for conversion of relative centrifugal force to rotor speed in low-speed centrifuge runs. To determine an unknown value in a given column, align ruler through known values in other two columns. Desired value is found at the intersection of the ruler with the column of interest. For faster centrifugations, use Figure A.1G.2. A more precise conversion can be obtained using the equation at the beginning of this appendix. See Table A.1G.1 for rotating radii of commonly used rotors.

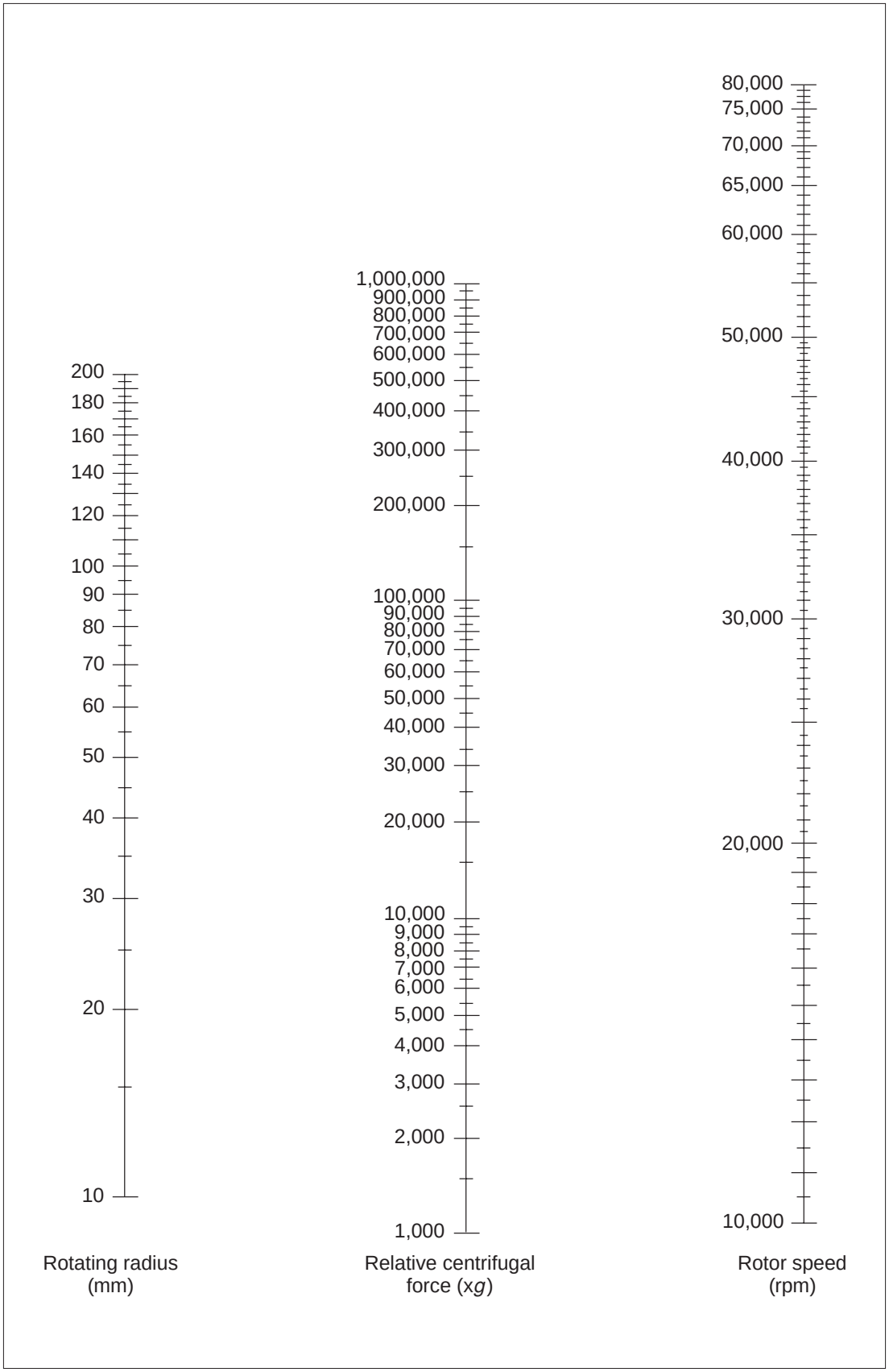


Figure A.1G.2 Nomogram for conversion of relative centrifugal force to rotor speed in high-speed centrifuge runs. For slower centrifugations and instructions for using the nomogram, use Figure A.1G.1. A more precise conversion can be obtained using the equation at the beginning of this appendix. See Table A.1G.1 for rotating radii of commonly used rotors.

Table A.1G.1 Maximum Rotating Radii for Common Rotors, Grouped by Centrifuge Model[a]

Rotor model[b]	r_{max} (mm)
For Sorvall centrifuge models GLC-1, GLC-2, GLC-2B, GLC-3, GLC-4, RT-6000B, T-6000, T-6000B	
A/S400	140
H-1000B	186
HL-4 with 50-ml bucket	180
HL-4 with 100-ml bucket	204
HL-4 with Omni-Carrier	163
M and A-384 (inner row)	91
M and A-384 (outer row)	121
SP/X and A-500 (inner row)	82
SP/X and A-500 (outer row)	123
For Sorvall centrifuge models RC-3, RC-3B, RC-3C)	
H-2000B	261
H-4000 and HG-4L	230
H-6000A	260
HL-8 with Omni-Carrier	221
HL-8 with 50-ml bucket	238
HL-8 with 100-ml bucket	247
HL-2 and HL-2B	166
LA/S400	140
For Sorvall centrifuge models RC-2, RC-2B, RC-5, RC-5B, RC-5C	
GSA	145
GS-3	151
HB-4	147
HS-4 with 250-ml bucket	172
SA-600	129
SE-12	93
SH-80	101
SM-24 (inner row)	91
SM-24 (outer row)	110
SS-34	107
SV-80	101
SV-288	90
TZ-28	95
For Sorvall ultracentrifuges	
T-865	91
T-865.1	87.1
T-875	87.1
T-880	84.7
T-1270	82
TFT-80.2	65.5
TFT-80.4	60.1
For Beckman GP series centrifuges	
GA-10	123
GA-24	123
GA-24 with adapter for 10-ml tubes	108
GH-3.7 (buckets)	204
GH-3.7 (microplate carrier)	168
GH-3.8 (buckets)	204
GH-3.8 (microplate carrier)	168
For Beckman TJ-6 series centrifuges	
TA-10	123
TA-24	108
TA-24 with adapter for 10-ml tubes	123
TH-4 (stainless steel buckets)	186
TH-4 (100-ml tube holders)	201
TH-4 (microplate carrier)	165
For Beckman AccuSpin	
AA-10	123
AA-24	108
AA-24 with adapter for 10-ml tubes	123
AH-4	163
For Beckman J6 series centrifuges	
JR-3.2	206
JS-2.9	265
JS-3.0	254
JS-4.0	226
JS-4.2	254
JS-4.2SM	248
JS-5.2	226
Microplate carrier (6-bucket rotors)	214
Microplate carrier (4-bucket rotors)	192
For Beckman J2-21 series centrifuges	
JA-10	158
JA-14	137
JA-17	123
JA-18	132
JA-18.1 (25° angle)	112
JA-18.1 (45° angle)	116
JA-20	108
JA-20.1	115
JA-21	102
JCF-Z	89
JCF-Z with small pellet core	81
JE-6B	125
JS-7.5	165
JS-13	142
JS-13.1	140
JV-20	93

continued

Table A.1G.1 Maximum Rotating Radii for Common Rotors, Grouped by Centrifuge Model[a], continued

Rotor model[b]	r_{max} (mm)
For Beckman series L7 and L8 ultracentrifuges	
SW 25.1	129.2
SW 28	161.0
SW 28.1	171.3
SW 30	123.0
SW 30.1	123.0
SW 40 Ti	158.8
SW 41 Ti	153.1
SW 50.1	107.3
SW 55 Ti	108.5
SW 60 Ti	120.3
SW 65 Ti	89.0
Type 15	142.1
Type 19	133.4
Type 21	121.5
Type 25	100.4
Type 30	104.8
Type 30.2	94.2
Type 35	104.0
Type 40	80.8
Type 40.3	79.5
Type 42.1	98.6
Type 42.2 Ti	104
Type 45 Ti	103
Type 50	70.1
Type 50 Ti	80.8
Type 50.2 Ti	107.9
Type 50.3 Ti	79.5
Type 50.4 Ti (inner row)	96.4
Type 50.4 Ti (outer row)	111.4
Type 55.2 Ti	100.3
Type 60 Ti	89.9
Type 65	77.7
Type 70 Ti	91.9
Type 70.1 Ti	82.0
Type 75 Ti	79.7
Type 80 Ti	84.0
VAC 50	86.4
VC 53	78.8
VTi 50	86.6
VTi 65	85.4
VTi 65.2	87.9
VTi 80	71.1
For Beckman Airfuge ultracentrifuge	
A-95	17.6
A-100/18	14.6
A-100/30	16.5
A-110	14.7
ACR-90 (2.4-ml liner)	11.8
ACR-90 (3.5-ml liner)	13.4
Batch rotor	14.6
EM-90	13.0
For Beckman TL-100 series ultracentrifuges	
TLA-100	38.9
TLA 100.1	38.9
TLA-100.2	38.9
TLA-100.3	48.3
TLA-45	55.1
TLS-55	76.4
TLV-100	35.7
Miscellaneous centrifuges and rotors[c]	
Clay Adams Dynac	—[d]
Fisher Centrific	113
Fisher Marathon 21K with 4-place rotor	160
IEC Clinical centrifuge with 4-place swinging-bucket rotor	155
IEC general-purpose centifuge models HN, HN-SII, and Centra-4	—[a]

[a]If your rotor is not listed here, consult the manufacturer's home page for the most up-to-date information. Sorvall, *www.sorvall.com*; Beckman Coulter, *www.beckmancoulter.com*; *beckman/biorsh/prodinfo/cntrifug/centuser.asp*; Fisher, *www.fishersci.com*; IEC, *www.labcentrifuge.com*.

[b]Sorvall centrifuges and rotors are a product of Du Pont Company Medical Products, Beckman centrifuges are a product of Beckman Coulter Instruments, IEC centrifuges are a product of International Equipment Co., Clay Adams Dynac centrifuges are a product of Becton Dickinson Labware, and Fisher centrifuges are a product of Fisher Scientific. For ordering information see *SUPPLIERS APPENDIX*.

[c]These instruments are often loosely referred to as "clinical," "tabletop," or "low-speed" centrifuges.

[d]These instruments accept a wide range of trunnion-ring rotors with variable rotating radii, as well as fixed-angle and swinging-bucket rotors that in turn accept a variety of adapters making it possible to spin different numbers of tubes of various sizes. For instance, the commonly used IEC 958 trunnion-ring rotor may be adjusted to radii ranging from 137 to 181 mm, depending on the trunnion-ring chosen. It is therefore necessary to consult the manual for the specific system being used to obtain an accurate speed to RCF conversion.

Table A.1G.2 Centrifuge Tube Materials and Their Properties[a]

Type	Optical property	Puncturable	Sliceable	Reusable	Sterilization methods	Chemical resistances[b]
Ultra-Clear thin-walled	Transparent	Yes	Yes		Cold sterilization only, but not with alcohol	Good tolerance to all gradient media except alkaline ones (>pH 8). Satisfactory for most weak acids and a few weak bases. Unsatisfactory for DMSO and most organic solvents, including all alcohols.
Standard tubes				Yes		
Quick-Seal tubes				No		
Polyallomer thin-walled	Translucent	Yes	Yes		Can be autoclaved on a test tube rack at 121°C	Good tolerance to all gradient media, including alkaline ones. Satisfactory for most acids, many bases, many alcohols, DMSO, and some organic solvents.
Standard tubes				Yes		
Quick-Seal tubes				No		
Polyallomer thick-walled	Translucent				Can be autoclaved on a test tube rack at 121°C	Good tolerance to all gradient media, including alkaline ones. Satisfactory for most acids, many bases, many alcohols, DMSO, and some organic solvents.
Tubes		No	No	Yes		
Bottles		No	No	Yes		
Polycarbonate thick-walled	Transparent	No	No		Cold sterilization recommended, but not with alcohol. Can be autoclaved at 121°C, but tube life may be reduced.	Good tolerance to all gradient media except alkaline ones (>pH 8). Satisfactory for some weak acids. Unsatisfactory for all bases, alcohols, and other organic solvents.
Tubes				Yes		
Bottles				Yes		
Cellulose propionate tubes	Transparent	No	No	Yes	Cold sterilization only, but not with alcohol	Good tolerance to all gradient media, including alkaline ones. Unsatisfactory for most acids, bases, alcohols, and other organic solvents.
Polypropylene	Translucent	No	No		Can be autoclaved at 121°C	Good tolerance to all gradient media, including alkaline ones. Satisfactory for many acids, bases, and alcohols. Unsatisfactory for most organic solvents.
Tubes				Yes		
Bottles				Yes		
Stainless steel tubes	Opaque	No	No	Yes	Can be autoclaved. Dry thoroughly before storage.	Good tolerance to many organic solvents. Marginal with many gradient media and salts. Unsatisfactory for most acids and many bases.
Polyethylene tubes	Translucent	No	No	Yes	Can be autoclaved at 121°C	Good tolerance to a wide range of chemicals. Suitable for use with strong acids and bases. Unsatisfactory for most organic solvents.
Corex/Pyrex	Transparent	No	No		Can be autoclaved at 121°C	Good tolerance to a wide range of gradient media. Corex has greater resistance to bases and acids.
Tubes				Yes		
Bottles				Yes		

[a]Table reproduced by permission of Beckman Coulter Instruments.

[b]Chemical resistances are described here in general terms, and are not meant by Beckman Coulter Instruments or John Wiley & Sons to express or imply any guarantee of safety based on these recommendations or resistances. If there is any doubt about a particular solution, it should be tested under actual operating conditions to evaluate the performance of a tube material. For more detailed information regarding specific media and solvents, consult Beckman Coulter Instruments. High-vapor-pressure inflammable solvents should not be handled in close vicinity to centrifuges because of possible ignition by sparking switches, relay contacts, or motor brushes.

INTERNET RESOURCES

http://www.sorvall.com

A userful site from Sorvall for obtaining product information about centrifuges and rotors; includes a site for a downloadable RCF calculator.

http://www.beckmancoulter.com/beckman/biorsch/prodinfo/cntrifug/centuser.asp

Contains Beckman centrifuge product information and sites for computing RCF, speed, and k-factor.

http:://www.fishersci.com

Catalog site for information about centrifuges produced by Clay Adams, Eppendorf, FiberLite, Fisher, and IEC.

http://labcentrifuge.com

Catalog site for information about IEC centrifuges and rotors.

Internet Basics for Biologists

With the explosion of sequence and structural information available to researchers, the field of bioinformatics is playing an increasingly large role in the study of fundamental biomedical problems. The challenge facing computational biologists will be to aid in gene discovery and in the design of molecular modeling, site-directed mutagenesis, and experiments of other types that can potentially reveal previously unknown relationships with respect to the structure and function of genes and proteins. This challenge becomes particularly daunting in light of the vast amount of data that has been produced by the Human Genome Project and other systematic sequencing efforts to date.

We begin with a review of the Internet and its terminology, also discussing major classes of Internet protocols, without becoming overly engaged in the engineering minutiae underlying these protocols. A more in-depth treatment on the inner workings of these protocols may be found in a number of well-written reference books intended for the lay audience (Krol and Klopfenstein, 1996; Rankin, 1996; Kennedy, 1999). This unit will also discuss matters of connectivity, ranging from simple modem connections to digital subscriber lines (DSL). Finally, we will address one of the most common problems that has arisen with the proliferation of Web pages throughout the world—i.e., finding useful information on the World Wide Web.

INTERNET BASICS

Despite the impression that the Internet is a single entity, it is actually a network of networks, composed of interconnected local and regional networks in more than 100 countries. While work on remote communications began in the early 1960s, the true origins of the Internet lie with a research project on networking at the Advanced Research Projects Agency of the U.S. Department of Defense in 1969 named ARPANET. The original ARPANET connected four nodes on the West Coast, with the immediate goal of being able to transmit information on defense-related research between laboratories. A number of different network projects subsequently surfaced, with the next landmark developments coming over ten years later. In 1981, BITNET ("Because It's Time") was introduced, providing point-to-point connections between universities for the transfer of electronic mail and files. In 1982, ARPA introduced the Transmission Control Protocol (TCP) and Internet Protocol (IP); TCP/IP allowed different networks to be connected to and communicate with one another, creating the system that is in place today.

A number of references chronicle the development of the Internet and communications protocols in detail (Quarterman, 1990; Froehlich and Kent, 1991; Krol and Klopfenstein, 1996). Most users, however, are content to leave the details of *how* the Internet works to their systems administrators; the relevant fact to most is that it *does* work.

Once the machines on a network are connected to one another, there needs to be some way to unambiguously specify a single computer so that messages and files find their intended recipient. To accomplish this, all machines directly connected to the Internet have an **IP number**. IP numbers are unique, identifying one and only one machine. The IP number is made up of four numbers separated by periods; for example, the IP number for the main file server at the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) is *130.14.25.1*. The numbers themselves represent, from left to right, the domain (*130.14* for the NIH), the subnet (*.25* for the National Library of Medicine at NIH), and the machine itself (*.1*). While the use of IP numbers aids the computers in directing data, they are obviously very difficult for users to remember.

Contributed by Andreas D. Baxevanis and B.F. Francis Ouellette
Current Protocols in Cell Biology (2001) A.1H.1-A.1H.16

Therefore, an IP number often has a **fully-qualified domain name** (FQDN) associated with it, which is dynamically translated in the background by a **domain name server**. Going back to the NCBI example, instead of using *130.14.25.1* to access the NCBI computer, a user could instead use *ncbi.nlm.nih.gov* and achieve the same result. Reading from left to right, the IP number goes from least to most specific, while the FQDN equivalent goes from most specific to least. The name of any given computer can then be thought of as taking the general form *computer.domain*, with the top-level domain (the portion coming after the last period in the FQDN) falling into one of the six broad categories shown in Table A.1H.1. Outside the United States, the top-level domain names may be replaced with a two-letter code specifying the country where the machine is located (e.g., *.ca* for Canada and *.uk* for the United Kingdom).

In an effort to anticipate the needs of Internet users in the future, as well as to try to erase the arbitrary line between top-level domain names based on country, the now-dissolved International Ad Hoc Committee (IAHC) was charged with developing a new framework of generic top-level domains (gTLD). The new, recommended gTLDs were set forth in a document entitled *The Generic Top Level Domain Memorandum of Understanding* (gTLD-MOU); these gTLDs are overseen by a number of governing bodies and are also shown in Table A.1H.1.

The most concrete measure of the size (and, thereby, the success) of the Internet lies in actually counting the number of machines physically connected to it. The Internet Software Consortium conducts an Internet Domain Survey twice each year to count these machines, otherwise known as **hosts**. In performing this survey, ISC considers not only how many hostnames have been assigned, but how many of those are actually in use; a

Table A.1H.1 Top-Level Domain Names

Top-level domain names	
Inside the United States	
.com	Commercial site
.edu	Educational site
.gov	Government site
.mil	Military site
.net	Gateway or network host
.org	Private (usually not-for-profit) organizations
Examples of top-level domain names used outside the United States	
.ca	Canadian site
.ac.uk	Academic site in the United Kingdom
.co.uk	Commercial site in the United Kingdom
Generic top-level domains proposed by IAHC	
.firm	Firms or businesses
.shop	Businesses offering goods to purchase (stores)
.web	Entities emphasizing activities relating to the World Wide Web
.arts	Cultural and entertainment organizations
.rec	Recreational organizations
.info	Information sources
.nom	Personal names (e.g., *yourlastname.nom*)

hostname might be issued, but the requestor may be holding the name in abeyance for future use. To test for this, a representative sample of host machines are sent a probe (a "ping"), with a signal being sent back to the originating machine if the host was indeed found. The rate of growth of the number of hosts has been phenomenal; from a paltry 213 hosts in August 1981, the Internet has in excess of 60 million hosts now. The doubling time for the number of hosts is on the order of 18 months. Most of this growth has come from the commercial sector, capitalizing on the growing popularity of multimedia platforms for advertising and communications such as the World Wide Web.

CONNECTING TO THE INTERNET

Of course, before being able to use all of the resources that the Internet has to offer, one needs to actually make a physical connection between one's own computer and "the information superhighway." For purposes of this discussion, the elements of this connection have been separated into two discrete parts: the actual, physical connection (meaning the "wire" running from one's computer to the Internet backbone) and the service provider, who handles issues of routing and content once connected. Keep in mind that, in practice, these are not necessarily treated as two separate parts—for instance, one's service provider may also be the same company that will run cables or fibers right into one's home or office.

Copper Wires, Coaxial Cables, and Fiber Optics

Traditionally, users who were attempting to connect to the Internet away from the office have had one and only one option—a modem, which uses the existing copper twisted-pair cables carrying telephone signals to transmit data. Data transfer rates using modems are relatively slow, allowing for data transmission in the range of 28.8 to 56 kilobits per second (Kbps). The problem with using conventional copper wire to transmit data lies not in the copper wire itself, but in the switches that are found along the way that route information to their intended destinations. These switches were designed for the efficient and effective transfer of voice data, but were never intended to handle the high-speed transmission of data. While most people still use modems from their homes, a number of new technologies are already in place and will become more and more prevalent for accessing the Internet away from hard-wired Ethernet networks. The maximum speeds at which each of the services that are discussed below can operate are shown in Figure A.1H.1.

The first of these "new solutions" is the integrated services digital network, or ISDN. While the advent of ISDN was originally heralded as the way to bring the Internet into the home in a speed-efficient manner, it required that special wiring be brought into the home. It also required that users be within a fixed distance from a central office, on the order of 20,000 feet or less. The cost of running this special, dedicated wiring, along with a per-minute pricing structure, effectively placed ISDN out of reach of most individuals. While ISDN is still available in many areas, this type of service is quickly being supplanted by more cost-effective alternatives.

In looking at alternatives that did not require new wiring, cable television providers began to look at ways in which the coaxial cable already running into a substantial number of households could be used to also transmit data. Cable companies are able to use bandwidth that is not being used to transmit television signals (effectively, unused channels) to push data into the home at very high speeds, up to 4.0 megabits per second (Mbps). The actual computer is connected to this network through a cable modem, which uses an Ethernet connection to the computer and a coaxial cable to the wall. Homes in a given area all share a single cable, in a wiring scheme very similar to that by which individual computers are connected via the Ethernet in an office or laboratory setting. While this branching

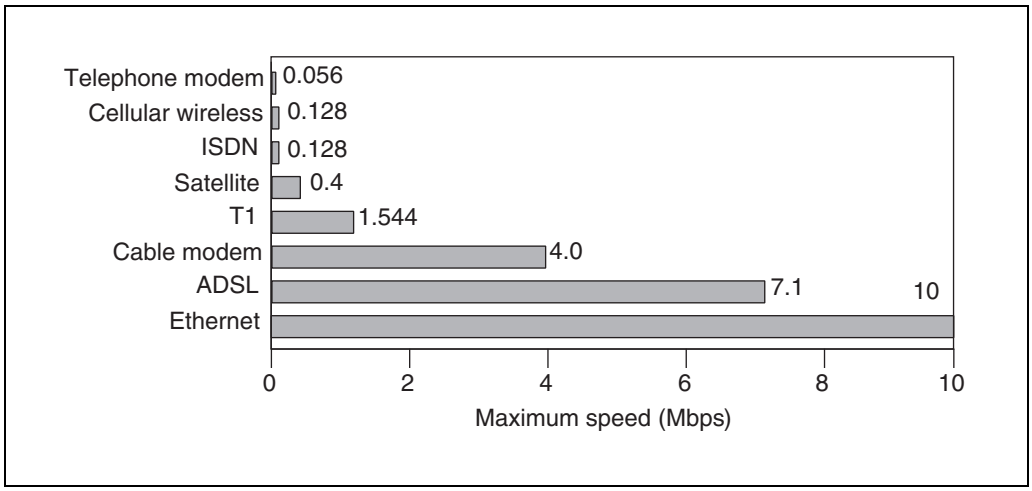


Figure A.1H.1 Performance of various types of Internet connections, by maximum throughput (in megabits per sec). The numbers indicated in the graph refer to peak performance; often the actual performance of any given method may be on the order of one-half slower, depending on configurations and system conditions.

arrangement can serve to connect a large number of locations, there is one major disadvantage—as more and more homes connect through their cable modems, service effectively slows down as more signal attempts to pass through any given node. One way of circumventing this problem is the installation of more switching equipment and reducing the size of a given "neighborhood."

Since the local telephone companies were the primary ISDN providers, they quickly turned their attention to ways in which the existing, conventional copper wire already in the home could be used to transmit data at high speed. The solution here is the digital subscriber line, or DSL. By using new, dedicated switches that are designed for rapid data transfer, DSL providers can circumvent the old voice switches that slowed down transfer speeds. Depending on the user's distance from the central office and whether a particular neighborhood has been wired for DSL service, speeds are on the order of 0.8 to 7.1 Mbps. The data transfers do not interfere with voice signals, and users can use the telephone while connected to the Internet; the signals are "split" by a special modem that passes the data signals to the computer and a microfilter that passes voice signals to the handset. There is a special type of DSL called **asynchronous DSL**, or ADSL. This is the variety of DSL service that is becoming more and more prevalent. Most home users download much more information than they send out, so systems are engineered to provide super-fast transmission in the "in" direction, with transmissions in the "out" direction being 5 to 10 times slower. Using this approach maximizes the amount of bandwidth that can be used without necessitating new wiring. One of the advantages of ADSL over cable is that ADSL subscribers effectively have a direct line to the central office, meaning that they do not have to compete with their neighbors for bandwidth. This, of course, comes at a price; at the time of this writing, ADSL connectivity options were on the order of twice as expensive as cable Internet.

Some of the newer technologies involve wireless connections to the Internet. These include using one's own cell phone or a special cell phone service (such as Ricochet) to upload and download information. These cellular providers can provide speeds on the order of 28.8 to 128 Kbps, depending on the density of cellular towers in the service area. Fixed-point wireless services can be substantially faster, since the cellular phone does not have to "find" the closest tower at any given time. Along these same lines, satellite providers are also coming on-line. These providers allow for data download directly to a

satellite dish with a southern exposure, with uploads occuring through traditional telephone lines. While the satellite option has the potential to be amongst the fastest of the options discussed, current operating speeds are only on the order of 400 Kbps.

Content Providers Versus ISPs

Once an appropriately fast and price-effective connectivity solution is found, users will then need to actually connect to some sort of service that will enable them to traverse the Internet space. The two major categories in this respect are **on-line services** or **Internet service providers** (**ISPs**). On-line services such as America Online (AOL) and CompuServe offer a large number of interactive digital services, including information retrieval, electronic mail (e-mail), bulletin boards, and "chat rooms" where users who are on line at the same time can converse with each other about any number of subjects. While the on-line services now provide access to the World Wide Web (see discussion of The World Wide Web), most of the features and services available through these systems reside within a proprietary, closed network; once a connection is made between the user's computer and the on-line service, accessing the special features, or content, of these systems does not require ever leaving the on-line system's host computer. Specialized content can range from access to on-line travel reservation systems to encyclopedias that are constantly being updated—items that would not be available to anyone unless they subscribed to that particular on-line service.

Internet service providers, or ISPs, take the opposite tack. Instead of focusing on providing content, the ISPs provide the tools necessary for users to send and receive e-mail, upload and download files, and navigate around the World Wide Web to find information at remote locations. The major advantage of ISPs is connection speed; ISPs often provide faster connections than the on-line services. Most ISPs charge a monthly fee for unlimited use.

The line between on-line services and ISPs has already begun to blur. AOL's now monthly flat fee pricing structure allows users to obtain all of the proprietary content found on AOL as well as all of the Internet tools available through ISPs, often at the same cost as a simple ISP connection. The extensive AOL network puts access to AOL as close as a local phone call in most of the United States, providing access to e-mail no matter where the user is located—a feature that small, local ISPs cannot match.

Not to be outdone, many of the major national ISP providers now also provide content through the concept of **portals**. Portals are Web pages that can be customized to the needs of the individual user and which serve as a jumping-off point to other sources of news or entertainment on the Net. In addition, many national firms such as Mindspring are able to match AOL's ease of connectivity on the road, and both ISPs and online providers are becoming more and more generous in providing users the capacity to publish their own Web pages. Developments such as this, coupled with the move of local telephone and cable companies into providing Internet access through new, faster fiber optic networks, foretell major changes in how people will access the Net in the future, changes that should favor the end-user both in price and performance.

ELECTRONIC MAIL

Most people are introduced to the Internet through the use of electronic mail (**e-mail**). The use of e-mail has become practically indispensable in many settings owing to its convenience as a medium for sending, receiving, and replying to messages. Its advantages are many:

It is much quicker than postal, or "snail mail." Messages tend to be much clearer and more to the point than is the case in typical telephone or face-to-face conversations.

Recipients have more flexibility in deciding whether a response needs to be sent immediately, relatively soon, or at all, giving individuals more control over workflow.

It provides a convenient method by which messages can be filed or stored.

There is little or no cost involved in sending an e-mail message.

While these and other advantages have pushed e-mail to the forefront of interpersonal communication in both industry and the academic community, users should be aware of several major disadvantages. First is the issue of security. As mail travels towards its recipient, it may pass through a number of remote nodes. The message could be intercepted and read at any one of those nodes by someone with high-level access, such as a systems administrator. Second is the issue of privacy. In industrial settings, e-mail is often considered to be an asset of the company for use only in official communication and, as such, is subject to monitoring by supervisors. The opposite is often true in academic, quasi-academic, or research settings; for example, National Institutes of Health policy encourages personal use of e-mail within the bounds of certain published guidelines. The key words here are "published guidelines"; no matter what the setting, users of e-mail systems should always be informed as to their organization's policy regarding the appropriate use and confidentiality of e-mail so that they may use the tool properly and effectively. An excellent, basic guide to the effective use of e-mail is highly recommended (Rankin, 1996).

Sending E-Mail

E-mail addresses take the general form *user@computer.domain,* where *user* is the name of the individual user and *computer.domain* specifies the actual computer that the e-mail account is located on. Like a postal letter, an e-mail message is comprised of an **envelope** or **header**, showing the e-mail addresses of the sender and recipient, a line indicating the subject of the e-mail, and information about how the e-mail message actually travelled from the sender to the recipient. The header is followed by the actual message, or **body**, analogous to what would go inside the postal envelope. Figure A.1H.2 illustrates all the components of an e-mail message.

E-mail programs vary widely, depending on both the platform and the needs of the users. Usually the characteristics of the local area network (LAN) dictate what types of mail programs can be used, and the decision is often left to systems administrators rather than individual users. Among the most widely used e-mail packages with a graphical user interface are Eudora for the Macintosh and both Netscape Messenger and Microsoft Exchange for Macintosh, Windows, and UNIX platforms. Text-based e-mail programs, which are accessed by logging into a UNIX-based account, include Elm and Pine.

Bulk E-Mail

As with postal mail, there has been an upsurge in "spam" or "junk e-mail," where companies compile bulk lists of e-mail addresses for use in commercial promotions. Since most of these lists are compiled from on-line registration forms and similar sources, the best defense for remaining off of these bulk e-mail lists is to be selective as to whom e-mail addresses are provided. Most newsgroups keep their mailing lists confidential; if in doubt and this is a concern, one should ask.

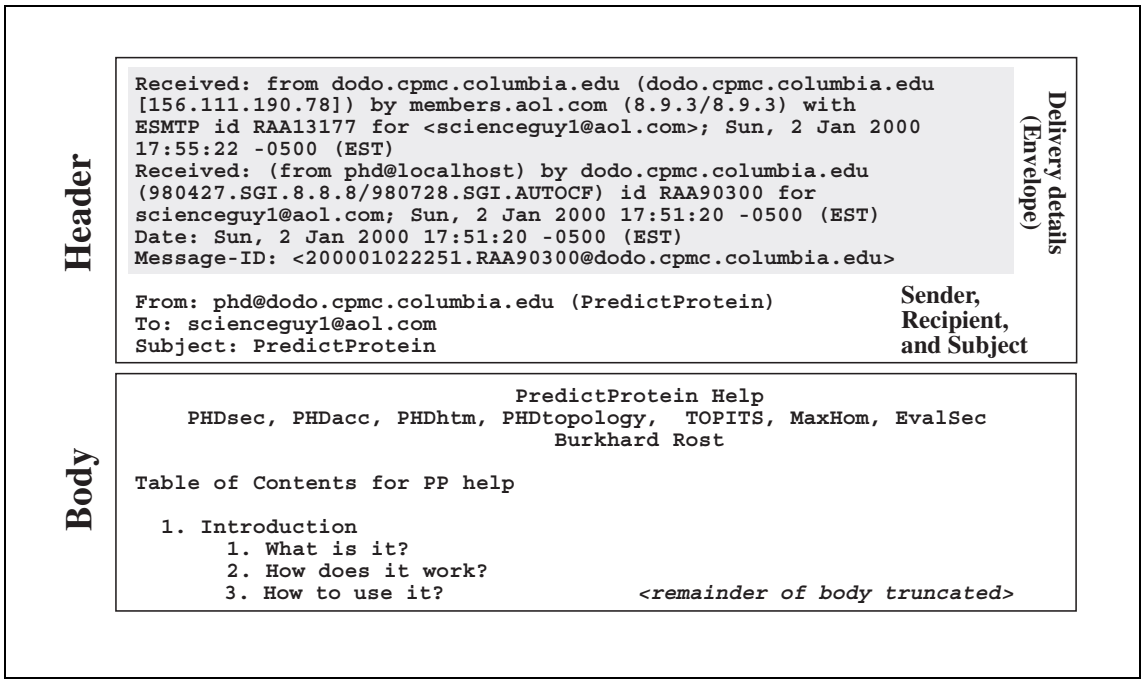


Figure A.1H.2 Anatomy of an e-mail message, with relevant components indicated. This message is an automated reply to a request for help file for the PredictProtein E-mail server.

E-Mail Servers

Most often, e-mail is thought of as a way to simply send messages, whether it be to one recipient or many. It is also possible to use e-mail as a mechanism for making biological predictions or retrieving records from biological databases. Users can send e-mail messages in a predefined format, defining the action to be performed for remote computers known as **servers**; the servers will then perform the desired operation and e-mail back the results. While this method is not interactive (in that the user cannot adjust parameters or have control over the execution of the method in real time), it does place the responsibility of hardware maintenance and software upgrades on the persons maintaining the server, allowing users to concentrate on their results instead of on programming. For most servers, sending the message *help* to the server e-mail address will return a detailed set of instructions for using that server, including the way in which queries need to be formatted.

Aliases and Newsgroups

In the example in Figure A.1H.2, the e-mail message is being sent to a single recipient. One of the strengths of e-mail is that a single piece of e-mail can be sent to a large number of people. The primary mechanism for doing this is through **aliases**; a user can define a group of people within their mail program and give the group a special name, or alias. Instead of using the individual e-mail addresses for all of the people in the group, the user can just send the e-mail to the alias name, and the mail program will handle broadcasting the message to each person in that group. Setting up alias names is a tremendous time-saver even for small groups; it also ensures that all members of a given group actually receive all e-mail messages intended for the group.

The second mechanism for broadcasting messages is through **newsgroups**. This model works slightly differently in that the list of e-mail addresses is compiled and maintained on a remote computer through subscriptions, much like magazine subscriptions. For example, the BIOSCI newsgroups are amongst the most highly-trafficked, offering a

forum for discussion or the exchange of ideas in a wide variety of biological subject areas. To begin receiving the messages posted to the automated sequencing discussion group within BIOSCI, a user would send a message to *biosci-server@net.bio.net* with the wording *subscribe autoseq* in the body of the message. The user would then receive all future postings to that group and be able to participate in the discussions. If a user wished to be removed from the group, a message would be sent to the same address, but this time, the body of the message would read *unsubscribe autoseq*. For more information on BIOSCI, including a complete list of discussion groups, an e-mail message can be sent to *biosci-server@net.bio.net*; in this case the subject line should be left blank and the words *info faq* typed in the body of the message. The BIOSCI server will then return a copy of the Frequently Asked Questions (FAQ) in response, with detailed information on each newsgroup overseen by BIOSCI.

It is also possible to participate in newsgroups without having each and every piece of e-mail flood into one's private mailbox. Instead, interested participants can use newsreading software, such as NewsWatcher for the Macintosh, which provides access to the individual messages making up a discussion. The major advantage is that the user can pick and choose which messages to read by scanning the subject lines; the remainder can be discarded by a single operation. NewsWatcher is an example of what is known as a **client-server application**—the client software (here, NewsWatcher) runs on a client computer (a Macintosh), which in turn interacts with a machine at a remote location (the server). Client-server architecture is interactive in nature, with a direct connection being made between the client and server machines.

Once NewsWatcher is started, the user is presented with a list of newsgroups available to them (Fig. A.1H.3); this list will vary, depending on the user's location, as systems administrators have the discretion to allow or block certain groups at a given site. From the rear-most window in the figure, the user double-clicks on the newsgroup of interest (here, *bionet.genome.arabidopsis*), which spawns the window shown in the center. At the top of the center window is the current unread message count, and any message within the list can be read by double-clicking on that particular line. This, in turn, spawns the last window (in the foreground), showing the actual message. If a user decides not to read any of the messages, or is done reading individual messages, the balance of the messages within the newsgroup (center) window can be deleted by first choosing *Select All* from the *File* menu, then selecting *Mark Read* from the *News* menu. Once the newsgroup window is closed, the unread message count is reset to zero. Every time NewsWatcher is restarted, it will automatically poll the news server for new messages that have been created since the last session.

As with most of the tools that will be discussed in this unit, news-reading capability is built into Web browsers such as Netscape Navigator and Microsoft Internet Explorer.

FILE TRANSFER PROTOCOL

Despite the many advantages afforded by e-mail in transmitting messages, experienced e-mail users have no doubt experienced frustration in trying to transmit files (**attachments**) along with an e-mail message. The mere fact that a file can be attached to an e-mail message and sent does not mean that the recipient will be able to detach, decode, and actually use the attached file. While more cross-platform e-mail packages such as Microsoft Exchange are being developed, the use of different e-mail packages by people at different locations means that sending files via e-mail is not an effective, foolproof method, at least in the short term. One solution to this problem is through the use of a **file transfer protocol** (or **FTP**). The workings of FTP are quite simple—a connection is made

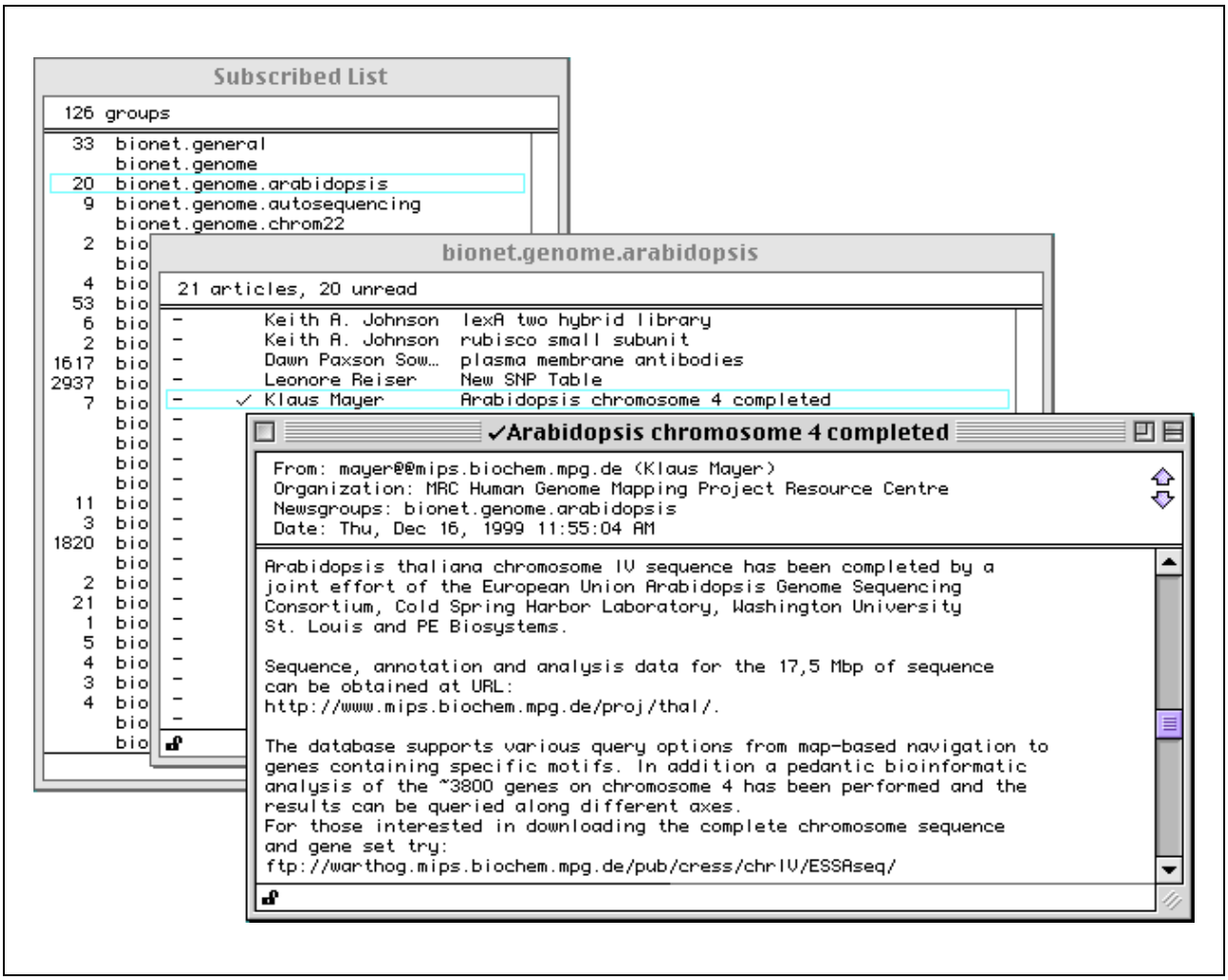


Figure A.1H.3 Using NewsWatcher to read postings to newsgroups. The list of newsgroups that the user has subscribed to is shown in the *Subscribed List* window (*left*). The list of new postings for the highlighted newsgroup (*bionet.genome.arabidopsis*) is shown in the center window. The window in the foreground shows the contents of the posting selected from the center window.

between a user's computer (the **client**) and a remote server, and that connection remains in place for the duration of the FTP session. File transfers are very fast, at rates on the order of 5 to 10 kilobytes per second, with speeds varying with time of day, distance between the client and server machines, and overall traffic on the network.

In the ordinary case, making an FTP connection and transferring files requires that a user have an account on the remote server. However, there are many files and programs that the academic community makes freely available, and access to those files does not require having an account on each and every machine where these programs are stored. Instead, connections are made using a system called **anonymous FTP**. Under this system, the user connects to the remote machine, and instead of entering a username/password pair, types *anonymous* as the username and enters an e-mail address in place of a password. Providing one's e-mail address allows the server's systems administrator to compile access statistics which may, in turn, be of use to those actually providing the public files or programs. An example of an anonymous FTP session using UNIX is shown in Figure A.1H.4.

```
$ ftp ftp.bio.indiana.edu
Connected to magpie.bio.indiana.edu.
220 iubio.bio.indiana.edu FTP server ready.
Name: anonymous
331 Guest login ok, send your complete e-mail address as password.
Password: ********
230-                   Welcome to IUBio archive!
230-
230-  This is a user-supported archive for biology software and data.
230-
230-       See the file Archive.Doc for details of this archive.
230-
230-          See IUBio Bio-Mirror archive of large data sets at
230-     ftp to iubio.bio.indiana.edu, user: iubio, password: iubio
230-   This includes GenBank, EMBL and DDBJ and other biosequence data.
230-
230-     Report problems, uploads and other matters via e-mail to
230-                    archive@bio.indiana.edu.
230-
230 Guest login ok, access restrictions apply.
Remote system type is UNIX.
Using binary mode to transfer files.
ftp> cd /molbio/align/clustal
250 CWD command successful.
ftp> get clustalw1.75.unix.tar.Z
local: clustalw1.75.unix.tar.Z remote: clustalw1.75.unix.tar.Z
200 PORT command successful.
150 Opening BINARY mode data connection for clustalw1.75.unix.tar.Z
(230379 bytes).
226 Transfer complete.
230379 bytes received in 0.45 seconds (500.75 Kbytes/s)
ftp> quit
221-You have transferred 230379 bytes in 1 files.
221-Total traffic for this session was 231859 bytes in 1 transfers.
221-Thank you for using the FTP service on iubio.bio.indiana.edu.
221 Goodbye.
```

Figure A.1H.4 Using UNIX FTP to download a file. An anonymous FTP session is established with the molecular biology FTP server at the University of Indiana to download the ClustalW alignment program. The user inputs are shown in boldface.

Although FTP occurs within the UNIX environment, Macintosh and PC users can employ programs that utilize graphical user interfaces (GUI, pronounced “gooey”) to navigate through the UNIX directories on the FTP server. Users need not have any knowledge of UNIX commands to download files; they can instead rely on pop-up menus and the ability to point-and-click their way through the UNIX file structure. The most popular FTP program on the Macintosh platform for FTP sessions is Fetch.

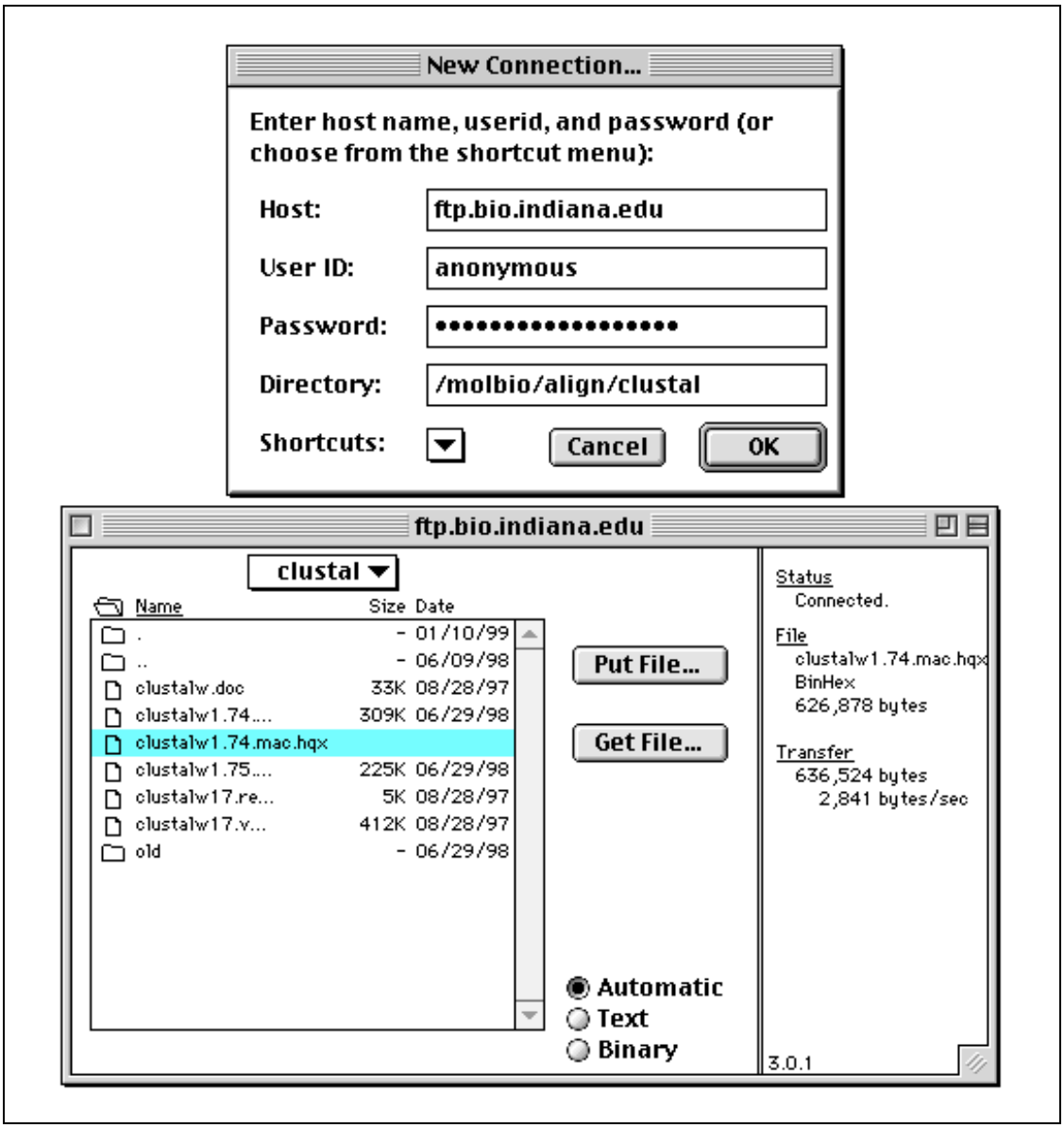


Figure A.1H.5 Using Fetch to download a file. An anonymous FTP session is established with the molecular biology FTP server at the University of Indiana (top) to download the ClustalW alignment program (bottom). Notice the difference between this GUI-based program and the UNIX equivalent illustrated in Figure A.1H.4.

A sample Fetch window is shown in Figure A.1H.5 to illustrate the difference between using a GUI-based FTP program and the equivalent UNIX FTP in Figure A.1H.4. In the figure, notice that the *Automatic* radio button (near the bottom of the second window under the *Get File* button) is selected, meaning that Fetch will determine the appropriate type of file transfer to perform. This may be manually overridden by selecting either *Text* or *Binary*, depending on the nature of the file being transferred. As a rule, text files should be transferred as *Text*, programs or executables as *Binary*, and graphic format files such as PICT and TIFF files as *Raw Data*.

THE WORLD WIDE WEB

While FTP is of tremendous use in the transfer of files from one computer to another, it does suffer from some limitations. When working with FTP, once a user enters a particular directory, they can only see the names of the directories or files. In order to actually view what is within the files, it is necessary to physically download them onto one's own computer. This inherent drawback led to the development of a number of **distributed document delivery systems** (DDDS), interactive client-server applications that allowed information to be viewed without having to perform a download. The first generation of DDDS development led to programs like Gopher, which allowed plain text to be viewed directly through a client-server application. From this evolved the most widely known and widely used DDDS, namely the World Wide Web. The Web is an outgrowth of research performed at the European Nuclear Research Council (CERN) in 1989 that was aimed at sharing research data between several locations. That work led to a medium through which text, images, sounds, and videos could be delivered to users on demand, anywhere in the world.

Navigation on the World Wide Web

Navigation on the Web does not require advance knowledge of the location of the information being sought. Instead, users can navigate by clicking on specific text, buttons, or pictures. These clickable items are collectively known as **hyperlinks**. Once one of these hyperlinks is clicked, the user is taken to another Web location, which could be at the same site or halfway around the world. Each document displayed on the Web is called a **Web page**, and all of the related Web pages on a particular server are collectively called a **Web site**. Navigation strictly through the use of hyperlinks has been nicknamed "Web surfing."

Users can take a more direct approach to finding information by entering a specific address. One of the strengths of the Web is that the programs used to view Web pages (appropriately termed **browsers**) can be used to visit Gopher and FTP sites as well, somewhat obviating the need for separate Gopher or FTP applications. As such, a unified naming convention was introduced to indicate to the browser program both the location of the remote site and, more importantly, the type of information at that remote location so that the browser could properly display the data. This standard-form address is known as a **uniform resource locator** (**URL**), and takes the general form *protocol://computer.domain*, where *protocol* specifies the type of site and *computer.domain* specifies the location (Table A.1H.2). The *http* used for the protocol in World Wide Web URLs stands for *hypertext transfer protocol*, the method used in transferring Web files from the host computer to the client.

Table A.1H.2 Uniform Resource Locator (URL) Format for Each Type of Transfer Protocol

Site	URL format (example)
General form	*protocol://computer.domain*
FTP site	*ftp://ftp.ncbi.nlm.nih.gov*
Gopher site	*gopher://gopher.iubio.indiana.edu*
Web site	*http://www.nhgri.nih.gov*

Browsers

Browsers, which are used to look at Web pages, are client-server applications that connect to a remote site, download the requested information at that site, display the information on the user's monitor, then disconnect from the remote host. The information retrieved from the remote host is in a platform-independent format called **hypertext markup language** (**HTML**). HTML code is strictly text-based, and any associated graphics or sound for that document exist as separate files in a common format. For example, images may be stored and transferred in GIF format, developed by CompuServe for the quick and efficient transfer of graphics; while GIF format is most commonly used for graphics, other formats such as JPEG and BMP may also be used. Because of this, a browser can display any Web page on any type of computer, whether it be a Macintosh, IBM-compatible, Linux, or UNIX machine. The text is usually displayed first, then the remaining elements are placed on the page as they are downloaded. With minor exceptions, a given Web page will look the same when the same browser is used on any of the above platforms.

The two major players in the area of browser software are Netscape, with their Communicator product, and Microsoft, with Internet Explorer. As with many other areas where multiple software products are available, the choice between Netscape and Internet Explorer comes down to one of personal preference. While the computer literati will debate the fine points of difference between these two packages, for the average user, both packages perform equally well and offer the same types of features, adequately addressing the Web-browser needs of most users.

It is worth mentioning that, while the Web is by definition a visually based medium, it is also possible to travel through Web space and view documents without the associated graphics. For users limited to line-by-line terminals, a browser called Lynx is available. Developed at the University of Kansas, Lynx allows users to use their keyboard arrow keys to highlight and select hyperlinks, using their return key the same way that Netscape and Internet Explorer users would click their mouse.

Internet Versus Intranet

The World Wide Web is normally thought of as a way to communicate with people at a distance, but the same infrastructure can be used to connect people within an organization. Such **intranets** provide an easily accessible repository of relevant information, capitalizing on the simplicity of the Web interface. It also provides another channel for broadcast or confidential communication within the organization. Having an intranet is of particular value when members of an organization are physically separated, whether it be in different buildings or different cities. Intranets are protected in such a way that people who are not on the organization's network are prohibited from accessing the internal Web pages; additional protections through the use of passwords are also common.

Finding Information on the World Wide Web

Most people find information on the Web the old fashioned way—by word of mouth either by using lists such as Table A.1H.3 or by simply following hyperlinks put in place by Web authors. Continuously clicking from page to page can be a highly ineffective way of finding information, especially when the information sought is of a very focused nature. One way of finding interesting and relevant Web sites is to consult **virtual libraries**, which are curated lists of Web resources arranged by subject. Virtual libraries of special interest to biologists include the WWW Virtual Library, maintained by Keith Robison at Harvard and the EBI BioCatalog, based at the European Bioinformatics Institutes. The URLs for these sites can be found in Table A.1H.3.

Table A.1H.3 World Wide Web Sites of Interest

Site	URL
Domain names	
gTLD-MOU	*http://www.gtld-mou.org*
Internet Software Consortium	*http://www.isc.org*
Electronic mail and newsgroups	
BIOSCI Newsgroups	*http://www.bio.net/docs/biosci.FAQ.html*
Eudora	*http://www.eudora.com*
Microsoft Exchange	*http://www.microsoft.com/exchange/*
NewsWatcher	*ftp://ftp.acns.nwu.edu/pub/newswatcher/*
File Transfer Protocol	
Fetch 3.0/Mac	*http://www.dartmouth.edu/pages/softdev/fetch.html*
FTP Voyager	*http://ftpvoyager.deerfield.com*
Internet access	
America Online	*http://www.aol.com*
AT&T	*http://www.att.com/worldnet*
Bell Atlantic	*http://www.bellatlantic.net*
Bell Canada	*http://www.bell.ca*
CompuServe	*http://www.compuserve.com*
MCI	*http://www.mci.com*
Ricochet	*http://www.ricochet.net*
Telus	*http://telus.com*
Virtual libraries	
EBI BioCatalog	*http://www.ebi.ac.uk/biocat/biocat.html*
Amos' WWW Links Page	*http://www.expasy.ch/alinks.html*
NAR Database Collection	*http://www3.oup.co.uk/nar/Volume_28/Issue_01/introduction/*
WWW Virtual Library	*http://mcb.harvard.edu/BioLinks.html*
World Wide Web browsers	
Internet Explorer	*http://www.microsoft.com/insider/ie5/default.htm*
Lynx	*ftp://ftp2.cc.ukans.edu/pub/lynx*
Netscape Navigator	*http://home.netscape.com*
World Wide Web search engines	
AltaVista	*http://www.altavista.com*
Excite	*http://www.excite.com*
Google	*http://www.google.com*
HotBot	*http://hotbot.lycos.com*
Infoseek	*http://infoseek.go.com*
Lycos	*http://www.lycos.com*
Northern Light	*http://www.northernlight.com*
World Wide Web meta-search engines	
MetaCrawler	*http://www.metracrawler.com*
Savvy Search	*http://www.savvysearch.com*

Table A.1H.4 Number of Hits Returned for Four Defined Search Queries on Some of the More Popular Search and Meta-Search Engines

Search term	Search engine			Meta-search engine		
	HotBot	Excite	Infoseek	Lycos	MetaCrawler	SavvySearch
genetic mapping	478	1,040	4,326	9,395	62	58
human genome	13,231	34,760	15,980	19,536	42	54
positional cloning	279	735	1,143	666	40	52
prostate cancer	14,044	53,940	24,376	33,538	60	57

It is also possible to directly search the Web by using **search engines** such as Alta Vista and Excite, among others. A search engine is simply a specialized program that can perform full-text or keyword searches on databases that catalog Web content. The result of a search is a hyperlinked list of Web sites fitting the search criteria from which the user can visit any or all of the found sites. However, search engines use slightly different methods in compiling their databases. One variation is the attempt to capture most or all of the text of every Web page that the search engine is able to find and catalog ("Web crawling"). Another technique is to catalog only the title of each Web page rather than its entire text. A third is to consider words that must appear next to each other or only relatively close to one another. Because of these differences in search-engine algorithms, the results returned by issuing the same query to a number of different search engines can produce wildly different results (Table A.1H.4). It may also be noted from Table A.1H.4 that most of the numbers are exceedingly large, reflecting the overall size of the World Wide Web. Unless a particular search engine ranks its results by relevance (for example, by scoring words in a title higher than words in the body of the Web page), the results obtained may not be particularly useful. It is also necessary to keep in mind that, depending on the indexing scheme that the search engine is using, the found pages may actually no longer exist, leading the user to the dreaded "404 Not Found" error.

Compounding this problem is the issue of **coverage**—the number of Web pages that any given search engine is actually able to survey and analyze. A comprehensive study by Lawrence and Giles (1998) indicates that the coverage provided by any of the search engines studied is both small and highly variable. For example, the HotBot engine produced 57.5% coverage of what was estimated to be the size of the "indexable Web," while Lycos had only 4.41% coverage, a full order of magnitude less than HotBot. The most important conclusion from this study was the extent of coverage increased as the number of search engines are increased and the results from those individual searches are combined. Combining the results obtained from the six search engines examined in this study produced coverage approaching 100%.

To address this point, a new class of search engines called **meta-search engines** have been developed. These programs will take the user's query and poll anywhere from five to ten of the "traditional" search engines. The meta-search engine will then collect the results, filter out duplicates, and return a single, annotated list to the user. One big advantage is that the meta-search engines take relevance statistics into account, returning much smaller lists of results. Even though the hit list is substantially smaller, it is much more likely to contain sites that directly address the original query. Since the programs must poll a number of different search engines, searches conducted this way obviously take longer to perform, but the higher degree of confidence in the compiled results for a given query outweighs the extra few minutes (sometimes only seconds) of search time. Reliable and easy-to-use meta-search engines include SavvySearch and MetaCrawler (see Table A.1H.3).

LITERATURE CITED

Froehlich, F. and Kent, A. 1991. ARPANET, the Defense Data Network, and Internet. *In* Encyclopedia of Communications. Marcel Dekker, New York.

Kennedy, A.J. 1999. The Internet: Rough Guide 2000. Rough Guides, London.

Krol, E. and Klopfenstein, B.C. 1996. The Whole Internet User's Guide and Catalog. O'Reilly and Associates, Sebastopol, Calif.Lawrence, S. and Giles, C.L. 1998. Searching the World Wide Web. *Science* 280:98-100.

Quarterman, J. 1990. The Matrix: Computer Networks and Conferencing Systems Worldwide. Digital Press, Bedford, Mass.

Rankin, B. 1996. Dr. Bob's Painless Guide to the Internet and Amazing Things You Can Do with E-mail. No Starch Press, San Francisco.

Contributed by Andreas D. Baxevanis
National Human Genome Research Institute, NIH
Bethesda, Maryland

B.F. Francis Ouellette
Centre for Molecular Medicine and Therapeutics
University of British Columbia
Vancouver, British Columbia

Note: The contents of this unit do not necessarily reflect the views or policies of the National Institutes of Health or the U.S. Department of Health and Human Services, nor does the mention of trade names, commerical products, or organizations imply endorsement by the U.S. Government.

LABORATORY STOCK SOLUTIONS AND EQUIPMENT

APPENDIX 2

Common Stock Solutions, Buffers, and Media

APPENDIX 2A

RECIPES

This section describes the preparation of buffers and reagents used in this manual for cell culture, manipulation of tissue, and cell biological methods. When preparing solutions, use deionized or distilled water and reagents of the highest available grade. Sterilization—by filtration through a 0.22-μm filter or by autoclaving—is recommended for most solutions stored at room temperature and is essential for cell culture applications. Where storage conditions are not specified, store up to 6 months at room temperature. Discard any reagent that shows evidence of contamination, precipitation, or discoloration.

Acid, concentrated stock solutions

See Table A.2A.1.

Acid precipitation solution

1 M HCl
0.1 M sodium pyrophosphate

Nucleic acids can also be precipitated with a 10% (w/v) solution of trichloroacetic acid (TCA); however, this recipe is cheaper, easier to prepare, and just as efficacious.

Ammonium hydroxide, concentrated stock solution

See Table A.2A.1.

Ammonium acetate, 10 M

Dissolve 385.4 g ammonium acetate in 150 ml H_2O
Add H_2O to 500 ml

Ammonium sulfate, saturated

76 g ammonium sulfate
100 ml H_2O
Heat with stirring to just below boiling point
Let stand overnight at room temperature

Table A.2A.1 Molarities and Specific Gravities of Concentrated Acids and Bases[a]

Acid/base	Molecular weight	% by weight	Molarity (approx.)	Specific gravity	1 M solution (ml/liter)
Acetic acid (glacial)	60.05	99.6	17.4	1.05	57.5
Ammonium hydroxide	35.0	28	14.8	0.90	67.6
Formic acid	46.03	90	23.6	1.205	42.4
		98	25.9	1.22	38.5
Hydrochloric acid	36.46	36	11.6	1.18	85.9
Nitric acid	63.01	70	15.7	1.42	63.7
Perchloric acid	100.46	60	9.2	1.54	108.8
		72	12.2	1.70	82.1
Phosphoric acid	98.00	85	14.7	1.70	67.8
Sulfuric acid	98.07	98	18.3	1.835	54.5

[a]*CAUTION:* Handle strong acids and bases carefully.

Current Protocols in Cell Biology (1998) A.2A.1-A.2A.10

ATP, 100 mM

1 g ATP (adenosine triphosphate)
12 ml H_2O
Adjust pH to 7.0 with 4 M NaOH
Adjust volume to 16.7 ml with H_2O
Store in aliquots indefinitely at −20°C

Base, concentrated stock solutions

See Table A.2A.1.

BSA (bovine serum albumin), 10% (w/v)

Dissolve 10 g BSA (e.g., Sigma) in 100 ml H_2O. Filter sterilize using a low-protein-binding 0.22-μm filter. Store indefinitely at 4°C.

Lower-concentration stock solutions (e.g., 1%), which are useful for various applications, can be made by diluting 10% stock appropriately with sterile water.

BSA is available in various forms that differ in fraction of origin, preparation, purity, pH, and cost; the most commonly used is fraction V. Use the form that is appropriate for the application; this may need to be optimized empirically.

$CaCl_2$, 1 M

147 g $CaCl_2 \cdot 2H_2O$
H_2O to 1 liter

Carbonate buffer

1.6 g Na_2CO_3 (15 mM final)
2.9 g $NaHCO_3$ (35 mM final)
0.2 g NaN_3 (3.1 mM final)
H_2O to 1 liter
Adjust to pH 9.5

CAUTION: *Sodium azide is poisonous; follow appropriate precautions for handling, storage, and disposal.*

CMF-DPBS (calcium- and magnesium-free Dulbecco's phosphate-buffered saline)

8.00 g NaCl (0.137 M)
0.20 g KCl (2.7 mM)
2.16 g $Na_2HPO_4 \cdot 7H_2O$ (8.1 mM)
0.20 g KH_2PO_4 (1.1 mM)
H_2O to 1 liter
Store at room temperature

DEPC (diethylpyrocarbonate)-treated solutions

Add 0.2 ml DEPC to 100 ml of the solution to be treated. Shake vigorously to dissolve the DEPC. Autoclave the solution to inactivate the remaining DEPC.

CAUTION: *Wear gloves and use a fume hood when using DEPC, as it is a suspected carcinogen.*

Many investigators keep the solutions they use for RNA work separate to ensure that "dirty" pipets do not go into them.

Do not treat solutions containing Tris with DEPC, as Tris inactivates the DEPC.

DMEM (Dulbecco's modified Eagle medium), supplemented

Dulbecco's modified Eagle medium, high-glucose formulation (see APPENDIX 2B; e.g., Life Technologies), containing:
5%, 10%, or 20% (v/v) FBS, heat inactivated (optional; see recipe below)
1% (v/v) nonessential amino acids
2 mM L-glutamine

continued

100 U/ml penicillin
100 µg/ml streptomycin sulfate
Filter sterilize if anything nonsterile has been added
Store up to 1 month at 4°C

DMEM containing this set of additives is sometimes called "complete DMEM." The percentage of serum used is indicated after the medium name—e.g., "DMEM/5% FBS." Absence of a number indicates no serum is used. DMEM is also known as Dulbecco's minimum essential medium.

Ham's F-12 nutrient mixture (APPENDIX 2B; available commercially, e.g., from Life Technologies), is sometimes added to DMEM; the resulting medium is known as DMEM/F-12.

Because of the higher bicarbonate content, DMEM requires ~10% CO_2 to maintain pH 7.4.

Culture media containing glutamine and penicillin should be warmed to 37°C as few times as possible since components, especially glutamine, degrade rapidly at 37°C.

DPBS (Dulbecco's phosphate-buffered saline)

8.00 g NaCl (0.137 M)
0.20 g KCl (2.7 mM)
0.20 g KH_2PO_4 (1.1 mM)
0.10 g $MgCl_2 \cdot 6H_2O$ (0.5 mM)
2.16 g $Na_2HPO_4 \cdot 7H_2O$ (8.1 mM)
0.10 g anhydrous $CaCl_2$ (0.9 mM)
H_2O to 1 liter

DTT (dithiothreitol), 1 M

Dissolve 1.55 g DTT in 10 ml water and filter sterilize. Store in aliquots at −20°C.

EDTA (ethylenediaminetetraacetic acid), 0.5 M (pH 8.0)

Dissolve 186.1 g disodium EDTA dihydrate in 700 ml water. Adjust pH to 8.0 with 10 M NaOH (~50 ml; add slowly). Add water to 1 liter and filter sterilize.

Begin titrating before the sample is completely dissolved. EDTA, even in the disodium salt form, is difficult to dissolve at this concentration unless the pH is increased to between 7 and 8.

FBS (fetal bovine serum)

Thaw purchased fetal bovine serum (shipped on dry ice and kept frozen until needed). Store 3 to 4 weeks at 4°C. If FBS is not to be used within this time, aseptically divide into smaller aliquots and refreeze until used. Store ≤1 year at −20°C. To heat inactivate FBS, heat serum 30 min to 1 hr in a 56°C water bath with periodic gentle swirling during the first 10 to 15 min to ensure uniform heating.

Repeated thawing and refreezing should be avoided, as it may cause denaturation of the serum.

Heat-inactivated FBS (FBS that has been treated with heat to inactivate complement protein and thus prevent an immunological reaction against cultured cells) is useful for a variety of purposes. It can be purchased commercially or made in the lab as described above.

HBSS (Hanks' balanced salt solution)

0.40 g KCl (5.4 mM final)
0.09 g $Na_2HPO_4 \cdot 7H_2O$ (0.3 mM final)
0.06 g KH_2PO_4 (0.4 mM final)
0.35 g $NaHCO_3$ (4.2 mM final)
0.14 g $CaCl_2$ (1.3 mM final)
0.10 g $MgCl_2 \cdot 6H_2O$ (0.5 mM final)
0.10 g $MgSO_4 \cdot 7H_2O$ (0.6 mM final)
8.0 g NaCl (137 mM final)

1.0 g D-glucose (5.6 mM final)
0.2 g phenol red (0.02%; optional)
Add H_2O to l liter and adjust pH to 7.4 with 1 M HCl or 1 M NaOH
Filter sterilize and store up to 1 month at 4°C

HBSS may be made or purchased without Ca^{2+} and Mg^{2+} (CMF-HBSS). These components are optional and usually have no effect on an experiment; in a few cases, however, their presence may be detrimental. Consult individual protocols to see if the presence or absence of these components is recommended.

Bottles should be kept tightly closed to prevent CO_2 loss and subsequent alkalinization.

HCl, 1 M

Mix in the following order:
913.8 ml H_2O
86.2 ml concentrated HCl

HeBS (HEPES-buffered saline) solution, 2×

16.4 g NaCl
11.9 g HEPES acid
0.21 g Na_2HPO_4
800 ml H_2O
Titrate to pH 7.05 with 5 M NaOH
Add H_2O to 1 liter
Filter sterilize through a 0.45-µm nitrocellulose filter
Store in 50-ml aliquots at −20°C

If the solution is to be used for transfection, the pH should be between 7.05 and 7.12, and should be tested for transfection efficiency.

KCl, 1 M

74.6 g KCl
H_2O to 1 liter

LB medium

Per liter:
10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 M NaOH
Autoclave 25 min

Although the pH is adjusted to near 7 with NaOH, the medium is not very highly buffered, and the pH of a culture growing in the medium drops as the culture nears saturation.

The medium may also contain antibiotics (e.g., 50 µg/ml ampicillin, 12 µg/ml tetracycline), galactosides (e.g., 20 µg/ml Xgal, 0.1 mM IPTG), or other nutritional supplements added after the medium has been autoclaved.

To make LB agar for LB plates, add 5 g/liter agar or agarose.

$MgCl_2$, 1 M

20.3 g $MgCl_2 \cdot 6H_2O$
H_2O to 100 ml

$MgSO_4$, 1 M

24.6 g $MgSO_4 \cdot 7H_2O$
H_2O to 100 ml

NaCl, 5 M

292 g NaCl
H_2O to 1 liter

NaOH, 10 M

Dissolve 400 g NaOH in 450 ml H_2O
Add H_2O to 1 liter

PBS (phosphate-buffered saline)

8.00 g NaCl (0.137 M)
0.20 g KCl (2.7 mM)
0.24 g KH_2PO_4 (1.4 mM)
1.44 g Na_2HPO_4 (0.01 M)
H_2O to 1 liter

PCR amplification buffer, 10×

500 mM KCl
100 mM Tris·Cl, pH 8.3 (see recipe below)
x mM $MgCl_2$
0.1% (w/v) gelatin
Store in aliquots at −20°C

This solution can be sterilized by autoclaving. Alternatively, it can be made from sterile water and stock solutions, and the sterilization omitted.

15 mM $MgCl_2$ is the concentration (x) used for most PCR reactions. However, the optimal concentration depends on the sequence and primer of interest and may have to be determined experimentally (see APPENDIX 3).

PMSF (phenylmethylsulfonyl fluoride), 100 mM

Dissolve 0.174 g PMSF in 10 ml of 100% ethanol, isopropanol, or methanol. Store in aliquots up to 2 years at −20°C.

CAUTION: *Phenylmethylsulfonyl fluoride is toxic.*

Make fresh dilutions from the alcohol stock for each use, because the half-life of PMSF in aqueous solution is <30 min at room temperature and a few hours on ice.

If PMSF is being added to a solution without detergent, the solution should be stirred vigorously during PMSF addition because PMSF has a tendency to form an insoluble precipitate in aqueous solution.

Polylysine-coated tissue culture surfaces

Prepare a stock solution by dissolving 100 mg polylysine in 100 ml water (poly-L-lysine or poly-D-lysine can be used; check specific protocol for choice of isomer) and filter sterilize through a 0.22-μm filter. Store in 5-ml aliquots at −20°C. When ready to use, dilute 1 part stock solution with 19 parts water to prepare a 50 μg/ml working solution.

To coat culture dishes, multiwell plates, or chamber slides: Fill tissue culture dishes, multiwell plates, or slide wells with the working solution and incubate 1 hr in a 37°C incubator, then remove solution by vacuum aspiration and allow surface to dry.

To coat coverslips: Sterilize coverslips by autoclaving or by incubating them in 95% ethanol and drying before coating. Place coverslips in a single layer in a petri dish containing working solution and incubate 1 hr at 37°C. Remove coverslips using sterile forceps and allow surface to dry.

Store coated tissue culture ware up to 3 months at 4°C. Use diluted solutions only once.

Potassium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid per liter (0.2 M) in water.

Solution B: 19.6 g potassium acetate ($KC_2H_3O_2$) per liter (0.2 M) in water.

continued

Table A.2A.2 Preparation of 0.1 M Sodium and Potassium Acetate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

[a]Adapted by permission from CRC (1975).

Referring to Table A.2A.2 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 100 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium acetate in the same volume. Acetate buffers show concentration-dependent pH changes, so check the pH by diluting an aliquot of concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.2, prepare closest higher pH, then titrate with solution A.

Potassium phosphate buffer, 0.1 M

Solution A: 27.2 g KH_2PO_4 per liter (0.2 M final) in water.

Solution B: 34.8 g K_2HPO_4 per liter (0.2 M final) in water.

Referring to Table A.2A.3 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This buffer may be made as a 5- or 10-fold concentrate simply by scaling up the amount of potassium phosphate in the same final volume. Phosphate buffers show concentration-dependent changes in pH, so check the pH of the concentrate by diluting an aliquot to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.3, prepare closest higher pH, then titrate with solution A.

Saponin, 10% (w/v)

Dissolve 1 g saponin in 10 ml PBS (see recipe above)
Store in 500-µl aliquots at –20°C

Once thawed, the 10% solution is stable for several months when stored at 4°C.

SDS, 20% (w/v)

Dissolve 20 g SDS (sodium dodecyl sulfate or sodium lauryl sulfate) in H_2O to 100 ml total volume with stirring. Filter sterilize using a 0.45-µm filter.

It may be necessary to heat the solution slightly to fully dissolve the powder.

Table A.2A.3 Preparation of 0.1 M Sodium and Potassium Phosphate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)	Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

[a]Adapted by permission from CRC (1975).

SDS electrophoresis buffer, 5×

15.1 g Tris base
72.0 g glycine
5.0 g SDS
Distilled, deionized H_2O to 1 liter
Store up to 1 month at 0° to 4°C
Dilute to 1× before use

Do not adjust the pH of the stock solution; the pH is 8.3 when diluted to 1×.

Use purified SDS if appropriate.

SDS sample buffer

See Table A.2A.4.

Table A.2A.4 Preparation of SDS Sample Buffer

Ingredient	2×	4×	Final conc. in 1× buffer
0.5 M Tris·Cl, pH 6.8[a]	2.5 ml	5.0 ml	62.5 mM
SDS	0.4 g	0.8 g	2% (w/v)
Glycerol	2.0 ml	4.0 ml	10% (v/v)
Bromphenol blue	20 mg	40 mg	0.1% (w/v)
2-Mercaptoethanol[b,c]	400 µl	800 µl	~300 mM
H_2O	to 10 ml	to 10 ml	—

[a]See recipe below.
[b]Alternatively, dithiothreitol (DTT), at a final concentration of 100 mM, can be substituted for 2-mercaptoethanol.
[c]Add just before use.

Sodium acetate, 3 M

Dissolve 408 g sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) in 800 ml H_2O
Adjust pH to 4.8, 5.0, or 5.2 (as desired) with 3 M acetic acid (see Table A.2A.1)
Add H_2O to 1 liter
Filter sterilize

Sodium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid per liter (0.2 M) in water.

Solution B: 27.2 g sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) per liter (0.2 M) in water.

Referring to Table A.2A.2 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 100 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium acetate in the same volume. Acetate buffers show concentration-dependent pH changes, so check the pH by diluting an aliquot of concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.2, prepare closest higher pH, then titrate with solution A.

Sodium phosphate buffer, 0.1 M

Solution A: 27.6 g $NaH_2PO_4 \cdot H_2O$ per liter (0.2 M final) in water.

Solution B: 53.65 g $Na_2HPO_4 \cdot 7H_2O$ per liter (0.2 M) in water.

Referring to Table A.2A.3 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This buffer may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium phosphate in the same final volume. Phosphate buffers show concentration-dependent changes in pH, so check the pH by diluting an aliquot of the concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.3, prepare closest higher pH, then titrate with solution A.

TAE (Tris/acetate/EDTA) electrophoresis buffer, 10×

24.2 Tris base
5.71 ml glacial acetic acid
3.72 g $Na_2EDTA \cdot 2H_2O$
H_2O to 1 liter

TBE (Tris/borate/EDTA) electrophoresis buffer, 10×

108 g Tris base (890 mM)
55 g boric acid (890 mM)
960 ml H_2O
40 ml 0.5 M EDTA, pH 8.0 (20 mM final; see recipe above)

TBS (Tris-buffered saline)

100 mM Tris·Cl, pH 7.5 (see recipe below)
0.9% (w/v) NaCl
Store up to several months at 4°C

TE (Tris/EDTA) buffer

10 mM Tris·Cl, pH 7.4, 7.5, or 8.0 (or other pH; see recipe below)
1 mM EDTA, pH 8.0 (see recipe above)

TEA (triethanolamine) solution

50 mM triethanolamine, pH ~11.5
0.1% (v/v) Triton X-100
0.15 M NaCl

Add Triton X-100 from a 10% stock (see recipe below).

TEN (Tris/EDTA/NaCl) solution

40 mM Tris·Cl, pH 7.5 (see recipe below)
1 mM EDTA, pH 8.0 (see recipe above)
150 mM NaCl

Tris·Cl, 1 M

Dissolve 121 g Tris base in 800 ml H_2O
Adjust to desired pH with concentrated HCl
Adjust volume to 1 liter with H_2O
Filter sterilize if necessary
Store up to 6 months at 4°C or room temperature

Approximately 70 ml HCl is needed to achieve a pH 7.4 solution, and ~42 ml for a solution that is pH 8.0.

IMPORTANT NOTE: *The pH of Tris buffers changes significantly with temperature, decreasing approximately 0.028 pH units per 1°C. Tris-buffered solutions should be adjusted to the desired pH at the temperature at which they will be used. Because the pK_a of Tris is 8.08, Tris should not be used as a buffer below pH ~7.2 or above pH ~9.0.*

Triton X-100, 10% (w/v)

1 g Triton X-100
H_2O to 10 ml
Stir to dissolve
Filter sterilize through a 0.45-μm filter
Store protected from light up to 6 months at room temperature

TTBS (Tween 20/TBS)

Dissolve 0.1% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20) in TBS (see recipe above). Store up to several months at 4°C.

SPECIAL CONSIDERATIONS FOR PCR EXPERIMENTS

Because the polymerase chain reaction (PCR) is designed to detect very small amounts of DNA, only a few molecules of contaminating DNA will produce unwanted amplification products. Ideally, PCR should not be carried out in the same room where large quantities of DNA are handled. Even where such spatial separation is not practical, the following housekeeping procedures will help avoid contamination with extraneous DNA (H.D. Kay, pers. comm.).

1. Keep laboratory surfaces clean by swabbing with 5% to 10% chlorine bleach. Put fresh absorbent paper bench protectors on bench before beginning PCR.
2. Wear disposable gloves and change them frequently while setting up PCRs.
3. Use only sterile disposable plasticware.
4. Keep a separate set of pipetting devices for setting up PCRs. If possible, use these instruments only with cotton-plugged tips to minimize transfer of DNA by aerosol. A separate microcentrifuge for PCR work is also desirable.
5. Whenever possible, set up PCRs in a laminar-flow hood or Class II biological safety cabinet to help prevent contamination by airborne DNA particles. A UV light within the hood or cabinet will help inactivate contaminating DNA.
6. Handle microcentrifuge tubes aseptically. Do not touch the interior of the hinged cap; if this happens, discard the tube. Microcentrifuge tubes briefly before opening to pellet drops around the cap and help keep reagents and reaction mixtures away from potentially contaminating fingers. Have only one tube open at a time, and open each

tube away from the remaining tubes. Hand-held microcentrifuge tube openers (e.g., USA/Scientific Plastics) are available to facilitate aseptic technique.

7. Include negative controls (i.e., no primer and no template) in all PCRs.

SPECIAL CONSIDERATIONS FOR WORKING WITH RNA

RNA is susceptible to degradation by ribonucleases, which are ubiquitous, very stable, and generally require no cofactors to function. Therefore, it is very important when working with RNA to take precautions against RNase contamination.

1. Treat all water and salt solutions except those containing Tris with DEPC (diethylpyrocarbonate; see recipe above). DEPC inactivates ribonucleases by covalent modification (however, it cannot be used with Tris solutions because Tris inactivates DEPC).

   CAUTION: *DEPC is hazardous and a suspected carcinogen; follow appropriate precautions for handling, storage, and disposal.*

2. If possible, make separate stock solutions to use for working with RNA and keep separate to ensure that "dirty" pipets do not come in contact with them.

3. Bake glassware 4 hr at 150°C. Rinse plasticware in chloroform or use directly out of the package (when it is generally free from contamination). Autoclaving will not fully inactivate many RNases.

4. Wear disposable gloves.

LITERATURE CITED

Chemical Rubber Company. 1975. CRC Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data, 3d ed., Vol. 1. CRC Press, Boca Raton, Fla.

Medium Formulations

COMMON TISSUE CULTURE MEDIA

Table A.2B.1 lists the formulations for four common tissue culture media. These and related media with a variety of additions are available from a number of commercial suppliers in liquid or powder form, in a variety of concentrations—e.g., 1× and 10×.

CO_2 in air must be used to maintain the pH of these tissue culture media, all of which are buffered with bicarbonate. Although most media require 5% CO_2 to maintain a pH of ~7.4, Dulbecco's modified Eagle medium (DMEM) is routinely used with 10% CO_2 because it contains elevated levels of bicarbonate. If DMEM is incubated in 5% CO_2, the pH remains at roughly pH 7.6 for at least several days, until the bicarbonate buffer equilibrates. In 7.5% to 10% CO_2, the pH remains at ~7.4. Additional buffering capacity can be provided by supplementing media with 25 mM HEPES.

For additional discussion of tissue culture media in general, see Ham and McKeehan (1979) and *UNIT 1.2*.

Table A.2B.1 Tissue Culture Medium Formulations[a]

Ingredient	Amount per liter			
	DMEM[b]	Ham's F-12 nutrient mixture	MEM[c]	RPMI 1640[d]
Amino acids				
L-Alanine	—	8.90 mg	—	—
L-Arginine·HCl	84.00 mg	211.00 mg	126.00 mg	200.00 mg
L-Asparagine·H_2O	—	15.00 mg	—	50.00 mg
L-Aspartic acid	—	13.00 mg	—	20.00 mg
L-Cysteine·HCl·H_2O	—	35.00 mg	—	—
L-Cystine·2HCl	63.00 mg	—	31.00 mg	65.00 mg
L-Glutamic acid	—	14.70 mg	—	20.00 mg
L-Glutamine	584.00 mg	146.00 mg	292.00 mg	300.00 mg
Glycine	30.00 mg	7.50 mg	—	10.00 mg
L-Histidine (free base)	—	—	—	15.00 mg
L-Histidine·HCl·H_2O	42.00 mg	21.00 mg	42.00 mg	—
L-Hydroxyproline	—	—	—	20.00 mg
L-Isoleucine	105.00 mg	4.00 mg	52.00 mg	50.00 mg
L-Leucine	105.00 mg	13.00 mg	52.00 mg	50.00 mg
L-Lysine·HCl	146.00 mg	36.50 mg	73.00 mg	40.00 mg
L-Methionine	30.00 mg	4.50 mg	15.00 mg	15.00 mg
L-Phenylalanine	66.00 mg	5.00 mg	32.00 mg	15.00 mg
L-Proline	—	34.50 mg	—	20.00 mg
L-Serine	42.00 mg	10.50 mg	—	30.00 mg
L-Threonine	95.00 mg	12.00 mg	48.00 mg	20.00 mg
L-Tryptophan	16.00 mg	2.00 mg	10.00 mg	5.00 mg
L-Tyrosine·2Na·2H_2O	104.00 mg	7.80 mg	52.00 mg	29.00 mg
L-Valine	94.00 mg	11.70 mg	46.00 mg	20.00 mg

continued

Table A.2B.1 Tissue Culture Medium Formulations[a], continued

Ingredient	Amount per liter			
	DMEM[b]	Ham's F-12 nutrient mixture	MEM[c]	RPMI 1640[d]
Inorganic salts				
Calcium chloride (anhyd.)	200.00 mg	33.20 mg	200.00 mg	—
Calcium nitrate·$4H_2O$	—	—	—	100.00 mg
Cupric sulfate·$5H_2O$	—	2.5 µg	—	—
Ferric nitrate·$9H_2O$	0.10 mg	—	—	—
Ferrous sulfate·$7H_2O$	—	0.83 mg	—	—
Magnesium sulfate (anhyd.)	97.67 mg	57.22 mg	98.00 mg	48.84 mg
Potassium chloride	400.00 mg	223.60 mg	400.00 mg	400.00 mg
Sodium bicarbonate	3.70 g	1.176 g	2.20 g	2.00 g
Sodium chloride	6.40 g	7.599 g	6.80 g	6.00 g
Sodium phosphate, dibasic (anhyd.)	—	142.00 mg	—	800.00 mg
Sodium phosphate, monobasic·H_2O	125.00 mg	—	—	—
Zinc sulfate·$7H_2O$	—	0.86 mg	—	—
Vitamins				
Biotin	—	7 µg	—	0.20 mg
D-Calcium pantothenate	4.00 mg	0.50 mg	1.00 mg	0.25 mg
Choline chloride	4.00 mg	14.00 mg	1.00 mg	3.00 mg
Folic acid	4.00 mg	1.30 mg	1.00 mg	1.00 mg
i-Inositol	7.20 mg	18.00 mg	2.00 mg	35.00 mg
Niacinamide	4.00 mg	0.04 mg	1.00 mg	1.00 mg
p-Aminobenzoic acid	—	—	—	1.00 mg
Pyridoxal·HCl	—	—	1.00 mg	—
Pyridoxine·HCl	4.00 mg	0.06 mg	—	1.00 mg
Riboflavin	0.40 mg	0.04 mg	0.10 mg	0.20 mg
Thiamine·HCl	4.00 mg	0.30 mg	1.00 mg	1.00 mg
Vitamin B_{12}	—	1.40 mg	—	5.00 µg
Other				
D-Glucose	4.50 g	1.802 g	1.00 g	2.00 g
Glutathione (reduced)	—	—	—	1.00 mg
Hypoxanthine·Na	—	4.77 mg	—	—
Linoleic acid	—	0.08 mg	—	—
Lipoic acid	—	0.21 mg	—	—
Phenol red	15.00 mg	1.20 mg	10.00 mg	5.00 mg
Putrescine·2HCl	—	0.161 mg	—	—
Sodium pyruvate	—	110.00 mg	—	—
Thymidine	—	0.70 mg	—	—

[a]Abbreviations: DMEM, Dulbecco's modified Eagle medium; MEM, minimal essential medium (after Eagle); RPMI, Roswell Park Memorial Institute.

[b]This formulation is for the high-glucose version with L-glutamine; DMEM is also available as a low-glucose version containing 1000 mg/liter. Other variations include: +HEPES, +sodium pyruvate, +pyridoxine·HCl, –inositol, –sodium phosphate, –L-glutamine, –phenol red, –L-methionine, –L-cysteine, –calcium chloride.

[c]This formulation contains L-glutamine. Other variations include: +Earle's salts, –L-glutamine, –sodium bicarbonate, –sodium phosphate, +D-valine (–L-valine), +inositol, +Hanks' salts, +nonessential amino acids. The α version of MEM is available with or without ribonucleotides and deoxyribonucleotides, and in other variations.

[d]This formulation contains L-glutamine. Other variations include –phenol red, –L-leucine, –L-methionine, –sodium phosphate, –glucose, –sodium bicarbonate, +HEPES, –folate, +L-alanine, –L-glutamine.

OTHER MEDIA

HAT supplement (see recipe) is used in hybrid selection to eliminate unfused or self-fused HGPRT$^-$ parental cells (*UNIT 1.2*).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

HAT supplement, 100×

10 mM sodium hypoxanthine
40 µM aminopterin
1.6 mM thymidine
Store at 4°C protected from the dark
Rehydrate before use and add to medium to 1× final concentration

CAUTION: *This supplement is hazardous; avoid mouth pipetting and contact with skin.*

LITERATURE CITED

Ham, R.G. and McKeehan, W.L. 1979. Media and growth requirements. *Methods Enzymol.* 58:44-93.

Standard Laboratory Equipment

Listed below are pieces of equipment that are standard in the modern cell biology laboratory—i.e., items used extensively in this manual and thus not usually included in the individual materials lists. No attempt has been made to list all items required for each procedure in the Materials list of each protocol; rather, those lists note those items that might not be readily available in the laboratory or that require special preparation. See *SUPPLIERS APPENDIX* for contact information for commercial vendors of laboratory equipment.

Applicator, cotton-tipped and wooden

Autoclave

Bag sealer

Balances, analytical and preparative

Beakers

Bench protectors, plastic-backed (including "blue" pads)

Biohazard disposal containers and bags

Biosafety cabinet, tissue culture or laminar flow hood; filters air and maintains air flow pattern to protect cultured cells from investigator and vice versa

Bottles, glass, plastic, and squirt

Bunsen burners

Centrifuges, low-speed (to 20,000 rpm) refrigerated, ultracentrifuge (20,000 to 80,000 rpm), large-capacity low-speed, tabletop, with appropriate rotors and adapters

Centrifuge tubes and bottles, plastic and glass, various sizes

Clamps

Conical centrifuge tubes, plastic and glass

Containers, assortment of glass and plastic, for gel and membrane washes

Coplin jars, glass, for 25 × 75–mm slides

Cryovials, sterile (e.g., Nunc)

Cuvettes

Desiccator and desiccant

Dry ice

Electrophoresis equipment, agarose and acrylamide, full-size and mini, with power supplies

Film developing system and darkroom

Filtration apparatus

Forceps

Fraction collector

Freezers, –20°C, –70°C, and liquid nitrogen

Fume hood

Geiger counter

Gel dryer

Gloves, disposable plastic and heat resistant

Graduated cylinders

Heating blocks, thermostatically controlled for test tubes and microcentrifuge tubes

Hemacytometer and/or electronic cell counter

Homogenizer

Humidified CO_2 incubator

Ice bucket

Ice maker

Immersion oil for microscopy

Lab coats

Laboratory glassware

Light box

Liquid nitrogen

Lyophilizer

Magnetic stirrer, with and without heater, and stir bars

Markers, including indelible markers, china-marking pens, and luminescent markers

Microcentrifuge, Eppendorf-type with 12,000 to 14,000 rpm maximum speed

Microcentrifuge tubes, 0.2-, 0.5-, 1.5-, 2-ml

Microscope slides, glass, 25 × 75–mm, and coverslips

Microscope with camera, upright, inverted, fluorescence, phase-contrast, dissecting

Microtiter plate reader

Mortar and pestle

Ovens, drying and microwave

Paper cutter, large

Paper towels

Parafilm

Pasteur pipets and bulbs

PCR thermal cycler and tubes

pH meter

pH paper

Pipets, graduated

Current Protocols in Cell Biology (1998) A.2C.1-A.2C.2

Pipettors, adjustable delivery, 0.5- to 10-µl, 10- to 200-µl, and 200- to 1000-µl

Polaroid camera or video documentation system

Power supplies, 300-V for polyacrylamide gels, 2000- to 3000-V for other applications

Racks, test tube and microcentrifuge tubes

Radiation shield, Lucite or Plexiglas

Radioactive waste containers for liquid and solid wastes

Refrigerator, 4°C

Ring stand and rings

Rubber policemen or plastic scrapers

Rubber stoppers

Safety glasses

Scalpels and blades

Scintillation counter, β

Scissors

Shakers, orbital and platform, room temperature or 37°C

Spectrophotometer, visible and UV range

Speedvac evaporator

Syringes and needles

Tape, masking, electrician's black, autoclave, and Time tape

Test tubes, glass and plastic, various sizes, with and without caps

Timer

Toolbox with common tools

Trays, plastic and glass, various sizes

Tubing, rubber and Tygon

UV light sources, long- and short-wavelength

UV transilluminator

UV transparent plastic wrap (e.g., Saran Wrap)

Vacuum desiccator

Vacuum oven

Vacuum supply

Vortex mixers

Waring blendor

Water bath with adjustable temperature

Water purification system

X-ray film cassettes and intensifying screens

COMMONLY USED TECHNIQUES

Molecular Biology Techniques

Protocols described in *Current Protocols in Cell Biology* may include molecular biology techniques and biochemical techniques that are not fully described in this book. Although it may be reasonable to assume that cell biologists have at least a basic understanding of these techniques, there are times when a full step-by-step description of the procedure is helpful. For that reason we have included the following table (Table A.3A.1), which lists techniques mentioned in this manual and provides cross-references to specific units in *Current Protocols in Molecular Biology* (CPMB) that describe the methods in detail. Protocols for some of these techniques will be added to this appendix in future supplements. Alternatively, the reader may wish to consult any of the many texts and manuals specifically devoted to recombinant DNA technology and biochemical analysis.

Table A.3A.1 Molecular Biology Techniques

Technique	CPMB reference
Antibody purification, monoclonal	*UNIT 11.11*
Antibody-Sepharose preparation	*UNIT 10.16*
Blotting	
northern	*UNIT 4.9*
Southern	*UNIT 2.9*
Chemiluminescence detection	*UNIT 10.8*
Cloning	
of PCR products	*UNIT 15.7*
subcloning DNA fragments	*UNIT 3.16*
Dialysis	*APPENDIX 3C*
DNA	
CaCl purification	*UNIT 1.7*
extraction and purification	*UNIT 2.1*
extraction from mammalian tissue	*UNIT 2.2*
ligation	*UNIT 3.14*
preparation, miniprep	*UNIT 1.6*
quantification, spectrophotometric	*APPENDIX 3D*
recovery from agarose gels	*UNIT 2.6*
sequencing	Chapter 7
E. coli	
growth in liquid medium	*UNIT 1.2*
growth on solid medium	*UNIT 1.3*
lysate preparation	*UNIT 1.7*
transformation	*UNIT 1.8*
ELISA	*UNIT 11.2*
Ethanol precipitation	*UNIT 2.1A*
β-Galactosidase, liquid assay	*UNIT 13.6*
Gel electrophoresis	
agarose	*UNIT 2.5A*
denaturing polyacrylamide	*UNIT 2.12*
low-gelling/melting temperature agarose	*UNIT 2.6*
nondenaturing polyacrylamide	*UNITS 2.7 & 10.2B*
NEPHGE	*UNIT 10.4*
staining gels	*UNIT 10.6*
HPLC	*UNIT 10.12*
Hybridization	
bacterial filter	*UNITS 6.3 & 6.4*
of Southern blots	*UNIT 2.10*
in situ	Chapter 14

continued

Table A.3A.1 Molecular Biology Techniques, continued

Technique	CPMB reference
Interaction trap/two-hybrid system	*UNIT 20.1*
Media, preparation and use	
E. coli	*UNITS 1.1-1.3*
yeast	*UNITS 13.1 & 13.2*
Monoclonal antibodies	
cross-linking antigen to carrier	*UNIT 11.15*
ELISA	*UNIT 11.2*
immunization of mice	*UNIT 11.4*
isotype determination	*UNIT 11.3*
production of ascites	*UNIT 11.10*
Oligonucleotide-directed mutagenesis	*UNIT 8.1*
PCR	
anchored	*UNIT 15.6*
end-labeling primers for	*UNIT 3.10*
general	*UNIT 15.1*
primer design	*UNIT 15.1*
primer synthesis	*UNIT 2.11*
using end-labeled primers	*UNIT 7.4*
Phenol/chloroform extraction	
DNA	*UNIT 2.1*
RNA	*UNIT 4.1*
Plasmid	
preparation	*UNITS 1.6, 1.7 & 2.1B*
purification, yeast	*UNIT 13.11*
Quantification	
DNA, RNA	*APPENDIX 3D*
protein	*UNIT 10.1A*
RadioImmuno Assay (RIA)	*UNIT 11.18*
Random primer labeling	*UNIT 3.5*
Restriction endonuclease digestion	*UNITS 3.1-3.3*
RNA preparation and purification	
extraction with guanidinium isothiocyanate	*UNIT 4.2*
poly(A)$^+$	*UNIT 4.5*
total	*UNITS 4.1 & 4.2*
Silanization of glassware	*APPENDIX 3B*
Spectrophotometry	*APPENDIX 3D*
T4 DNA ligase	*UNIT 3.14*
Transfection	
calcium phosphate–mediated	*UNIT 9.1*
DEAE-dextran-mediated	*UNIT 9.2*
liposome-mediated	*UNIT 9.4*
Transformation	
electroporation	*UNIT 1.8*
lithium acetate, yeast	*UNIT 13.7*
Yeast	
culture	*UNIT 13.2*
interaction trap/two-hybrid system	*UNIT 20.1*
media	*UNIT 13.1*
replica plating	*UNIT 13.2*
transformation with DNA	*UNIT 13.7*

Spectrophotometric Determination of Protein Concentration

This unit describes spectrophotometric methods for measuring the concentration of a sample protein in solution. In Basic Protocol 1, absorbance measured at 280 nm (A_{280}) is used to calculate protein concentration by comparison with a standard curve or published absorptivity values for that protein (a_{280}). In the Alternate Protocol, absorbance measured at 205 nm (A_{205}) is used to calculate the protein concentration. The A_{280} and A_{205} methods can be used to quantitate total protein in crude lysates and purified or partially purified protein. Both of these methods are simple and can be completed quickly. The A_{280} method is the most commonly used. The A_{205} method can detect lower concentrations of protein and is useful for dilute protein samples, but is more susceptible to interference by reagents in the protein sample than the A_{280} method. Basic Protocol 2 uses a spectrofluorometer or a filter fluorometer to measure the intrinsic fluorescence emission of a sample solution; this value is compared with the emissions from standard solutions to determine the sample concentration. The fluorescence emission method is used to quantitate purified protein. This simple method is useful for dilute protein samples and can be completed in a short amount of time.

USING A_{280} TO DETERMINE PROTEIN CONCENTRATION

BASIC PROTOCOL 1

Determination of protein concentration by measuring absorbance at 280 nm (A_{280}) is based on the absorbance of UV light by the aromatic amino acids, tryptophan and tyrosine, and by cystine, disulfide-bonded cysteine residues, in protein solutions. The measured absorbance of a protein sample solution is used to calculate the concentration either from its published absorptivity at 280 nm (a_{280}) or by comparison with a calibration curve prepared from measurements with standard protein solutions. This assay can be used to quantitate solutions with protein concentrations of 20 to 3000 µg/ml.

Materials

3 mg/ml standard protein solution (see recipe; optional)
Sample protein
Spectrophotometer with UV lamp

1. For calibrating with standards, use the 3 mg/ml standard protein solution to prepare dilutions of 20, 50, 100, 250, 500, 1000, 2000, and 3000 µg/ml in the same solvent as used to prepare the sample protein. Prepare a blank consisting of solvent alone.

   Ideally, for purified or partially purified protein, the protein standard should have an aromatic amino acid content similar to that of the sample protein. For the total protein of a crude lysate, bovine serum albumin (BSA) is a commonly used standard for spectrophotometric quantitation of protein concentration. A 3 mg/ml solution of BSA should have an A_{280} of 1.98, based on an A_{280} of 6.61 for a 1% (w/v) solution.

2. Turn on the UV lamp of the spectrophotometer and set the wavelength to 280 nm. Allow the instrument to warm up 30 min before taking measurements.

3. Zero the spectrophotometer with the solvent blank.

4. Measure the absorbance of the protein standard and unknown solutions.

   If the A_{280} of the sample protein is >2.0, dilute the sample further in the same solvent and measure the A_{280} again.

Contributed by Michael H. Simonian
Current Protocols in Cell Biology (2002) A.3B.1-A.3B.7

5a. *If the a_{280} of the protein is known:* Calculate the unknown sample concentration from its absorbance value using Equation A.3B.1.

In this equation a_{280} has units of ml/mg cm and b is the path length in cm.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{280}}{a_{280} \times b}$$

Equation A.3B.1

5b. *If standard solutions are used for quantitation:* Create a calibration curve by either plotting or performing regression analysis of the A_{280} versus concentration of the standards. Use the absorbance of the sample protein to determine the concentration from the calibration curve.

ALTERNATE PROTOCOL

USING A_{205} TO DETERMINE PROTEIN CONCENTRATION

Determination of protein concentration by measurement of absorbance at 205 nm (A_{205}) is based on absorbance by the peptide bond. The concentration of a protein sample is determined from the measured absorbance and the absorptivity at 205 nm (a_{205}). This assay can be used to quantitate protein solutions with concentrations of 1 to 100 µg/ml protein.

Additional Materials *(also see Basic Protocol 1)*

Brij 35 solution: 0.01% (v/v) Brij 35 (Sigma) in an aqueous solution appropriate for dissolving or diluting the sample protein

1. Dissolve or dilute the protein sample in Brij 35 solution.
2. Turn on the UV lamp of the spectrophotometer and set the wavelength to 205 nm. Allow the instrument to warm up 30 min before taking measurements.
3. Zero the spectrophotometer with the Brij 35 solution alone.
4. Measure the absorbance of the sample protein.

5a. *If the a_{205} of the protein is known:* Use Equation A.3B.1 to calculate the concentration of the sample protein *except* substitute the appropriate values for A_{205} and a_{205}.

5b. *If the a_{205} is not known:* Estimate the concentration of the sample protein from its measured absorbance using Equation A.3B.2.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{205}}{31 \times b}$$

Equation A.3B.2

In this equation, the absorptivity value, 31, has units of ml/mg cm and b is the path length in cm.

USING FLUORESCENCE EMISSION TO DETERMINE PROTEIN CONCENTRATION

Protein concentration can also be determined by measuring the intrinsic fluorescence based on fluorescence emission by the aromatic amino acids tryptophan, tyrosine, and/or phenylalanine. Usually tryptophan fluorescence is measured. The fluorescence intensity of the protein sample solution is measured and the concentration calculated from a calibration curve based on the fluorescence emission of standard solutions prepared from the purified protein. This assay can be used to quantitate protein solutions with concentrations of 5 to 50 µg/ml.

Materials

Standard protein solution prepared using the purified protein (see recipe)
Sample protein
Spectrofluorometer *or* filter fluorometer with an excitation cutoff filter ≤285 nm and an emission filter >320 nm

1. Prepare dilutions of the purified protein at 5, 7.5, 10, 25, and 50 µg/ml in the same solvent as the sample protein. Prepare a blank consisting of solvent alone.
2. Turn on the lamp of the instrument and allow it to warm up 30 min before taking measurements.

   If a spectrofluorometer is used, set the excitation wavelength to 280 nm and the emission wavelength to between 320 and 350 nm. If the exact emission wavelength is not known, determine it empirically by scanning the standard solution with the excitation wavelength set to 280 nm. If the instrument is a filter fluorometer, use an excitation cutoff filter ≤285 nm and an emission filter >320 nm.
3. Zero the instrument with the solvent blank.
4. Measure the fluorescence of the protein standard and sample protein solutions.
5. Create a calibration curve by either plotting or performing regression analysis of the fluorescence intensity versus concentration of the standards. Using the fluorescence intensity of the sample protein, determine the concentration from the calibration curve.

   Fluorescence emission is a linear function of concentration only over a limited range.

REAGENTS AND SOLUTIONS

Use deionized distilled water for the preparation of all recipes and in all steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Standard protein solution, 3 mg/ml

Weigh out dry protein and prepare a stock solution at a concentration of 3 mg/ml in the same solvent as used for the sample protein. Store up to 3 months at −20°C.

To prepare calibration standard solutions, dilute the stock solution in solvent to give the desired final concentrations for the standard curve.

Bovine serum albumin (BSA, fraction V; Sigma) is frequently used for a protein standard solution. A 3 mg/ml solution of BSA should have an A_{280} of 1.98, based on an A_{280} of 6.61 for a 1% (w/v) solution.

For quantitation of a purified or partially purified protein, if possible, the protein standard should have an aromatic amino acid content similar to that of the sample protein.

COMMENTARY

Background Information

Measuring absorbance at 280 nm (A_{280}) is one of the oldest methods for determining protein concentration (Warburg and Christian, 1942; Layne, 1957). This method is still widely used because it is simple and does not require incubating the sample with exogenous chromophores. However, the detection limit is higher than colorimetric methods and therefore higher concentrations of protein are necessary. The A_{280} method requires that the protein being quantitated have aromatic amino acids, primarily tryptophan. Because of the variability in aromatic amino acid content among different proteins, their absorptivity at 280 nm (a_{280}) also varies. Therefore, if calibration standards are used for quantitation, the aromatic amino acid content of the standard must be similar to that of the sample protein for accurate results. For many previously studied proteins, the a_{280} is known and can be obtained from the literature (Fasman, 1989).

If the absorptivity is not known but the amino acid composition is known, the molar absorptivity at 280 nm (ε_{280}) can be predicted from Equation A.3B.3 (Pace et al., 1995):

$$\varepsilon_{280} = (\text{no. of Trp})(5500) + (\text{no. of Tyr})(1490) + (\text{no. of cystine})(125)$$

Equation A.3B.3

In this equation, the number of tryptophan (Trp), tyrosine (Tyr), and cystine residues are each multiplied by an estimated ε_{280} value for each residue. This equation was derived for folded proteins in water. With the DNA sequence for many proteins now available, the predicted ε_{280} can be derived for newer proteins where this value is not published. However, there are caveats that should be considered if the predicted ε_{280} in Equation A.3B.3 is used. First, the predicted ε_{280} is most reliable if the protein contains tryptophan residues and less so if there are no tryptophan residues. Second, the number of cystine residues must be accurately known and one cannot presume that all cysteine residues, to an even number, are involved in disulfide bonds. Alternatively, there are methods for measuring the ε_{280} value that are reliable (Pace et al., 1995).

Accordingly, the quantitation of proteins by peptide bond absorption at 205 nm (A_{205}) is more universally applicable among proteins. Furthermore, the absorptivity for a given protein at 205 nm is several-fold greater than that at 280 nm (Scopes, 1974; Stoscheck, 1990). Thus lower concentrations of protein can be quantitated with the A_{205} method. The disadvantage of this method is that some buffers and other components absorb at 205 nm (Stoscheck, 1990).

In addition to the aromatic amino acids, several others have absorption maxima in the UV range. Table A.3B.1 shows the wavelengths of absorption maxima and corresponding molar absorptivity (ε) for the amino acids with appreciable absorbance in the UV range. Only tryptophan has an absorption maximum at 280 nm,

Table A.3B.1 Absorption Maxima and Molar Absorptivity (ε) of Amino Acids[a]

Amino acid	Wavelength maxima (nm)	$\varepsilon \times 10^{-3}$ (l/mol cm)
Cysteine	250	0.3
Histidine	211	5.9
Phenylalanine	188	60.0
	206	9.3
	257	0.2
Tryptophan	219	47.0
	279	5.6
Tyrosine	193	48.0
	222	8.0
	275	1.4

[a]Values are for aqueous solutions at pH 7.1 (Freifelder, 1982; Fasman, 1989).

Table A.3B.2 Molar Absorptivity (ε) of Amino Acids at 280 nm[a]

Amino acid	$\varepsilon \times 10^{-3}$ (l/mol cm)
Cystine	0.110
Phenylalanine	0.0007
Tryptophan	5.559
Tyrosine	1.197

[a]Values are for aqueous solutions at pH 7.1 except for cystine, which is for water (Fasman, 1989).

Table A.3B.3 Fluorescence Properties of Aromatic Amino Acids[a]

Amino acid	Excitation wavelength	Emission wavelength	Quantum yield
Phenylalanine	260 nm	283 nm	0.04
Tryptophan	285 nm	360 nm	0.20
Tyrosine	275 nm	310 nm	0.21

[a]Values are for aqueous solutions at pH 7 and 25°C (Hawkins and Honigs, 1987; Fasman, 1989).

although, tyrosine and cystine will also slightly absorb. The ε_{280} for tryptophan is nearly five-fold greater than that for tyrosine and 50-fold greater than that for cystine (Table A.3B.2). The contribution to the absorbance at 280 nm of the third aromatic amino acid, phenylalanine, and cysteine are negligible. Several amino acids other than those in Table A.3B.1 absorb light below 205 nm (Fasman, 1989), but either the molar absorptivities are too low to be significant or the wavelengths are too short for practical absorbance measurements.

The aromatic amino acids also have fluorescence emissions when excited by light in the UV range. Table A.3B.3 gives the excitation wavelength, fluorescence emission wavelength, and quantum yield (Q) for tryptophan, tyrosine, and phenylalanine. The quantum yield is the ratio of photons emitted to photons absorbed. Typically, phenylalanine fluorescence is not detected in the presence of tyrosine and tryptophan due to low Q. Furthermore, tyrosine fluorescence is nearly completely quenched if the tyrosine residue is ionized or near an amino group, a carboxyl group, or a tryptophan residue (Teale, 1960; Freifelder, 1982). Therefore, tryptophan fluorescence is what is customarily measured.

Measurement of intrinsic fluorescence by aromatic amino acids is primarily used to obtain qualitative information (Freifelder, 1982). However, with a protein standard whose aromatic amino acid content is similar to that of the sample, intrinsic fluorescence can be used for quantitation (Hawkins and Honigs, 1987). An additional consideration is that the tertiary structure of a protein will influence the fluorescence, e.g., adjacent protonated acidic groups in a protein molecule will quench tryptophan fluorescence (Freifelder, 1982).

Critical Parameters and Troubleshooting

A 1-cm path length quartz cuvette is most often used to make absorbance measurements. However, quartz cuvettes with shorter path lengths, 0.01 to 0.5 cm, are available (e.g., from Hellma Cells or Beckman); these shorter path length cuvettes allow higher concentrations of protein solutions to be measured. Equation A.3B.1 and Equation A.3B.2 assume the cuvette has a path length of 1 cm; when cuvettes of shorter path length are used, the correct value for b must be substituted in the equation.

The solvent pH and polarity will affect the absorbance and fluorescence properties of a protein. A notable example of pH effects on absorbance is seen with tyrosine residues, where a change in pH from neutral to alkaline results in a shift of the absorbance maximum to a longer wavelength and an increase in absorptivity due to dissociation of the tyrosine phenolic hydroxyl group (Freifelder, 1982; Fasman, 1989). An example of solvent polarity effects on fluorescence is observed with tryptophan, where a decrease in solvent polarity

Table A.3B.4 Concentration Limits of Interfering Reagents for A_{205} and A_{280} Protein Assays[a]

Reagent[b]	A_{205}	A_{280}
Ammonium sulfate	9% (w/v)	>50% (w/v)
Brij 35	1% (v/v)	1% (v/v)
DTT	0.1 mM	3 mM
EDTA	0.2 mM	30 mM
Glycerol	5% (v/v)	40% (v/v)
KCl	50 mM	100 mM
2-ME	<10 mM	10 mM
NaCl	0.6 M	>1 M
NaOH	25 mM	>1 M
Phosphate buffer	50 mM	1 M
SDS	0.10% (w/v)	0.10% (w/v)
Sucrose	0.5 M	2 M
Tris buffer	40 mM	0.5 M
Triton X-100	<0.01% (v/v)	0.02% (v/v)
TCA	<1% (w/v)	10% (w/v)
Urea	<0.1 M	>1 M

[a]Values from Stoscheck (1990).

[b]Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

results in a shift in fluorescence emission to shorter wavelengths and an increase in intensity (Freifelder, 1982). Because of these effects, the following precautions should be taken for accurate results: (1) when calibration curves are used for quantitation by absorbance or fluorescence, standards must be in the same solvent as the samples; and (2) when a published absorptivity at a given wavelength is used for quantitation, the solvent composition of the sample must be the same as that used in obtaining the published data.

Many buffers and other reagents can interfere with A_{280} and A_{205} spectrophotometric measurements. Stoscheck (1990) lists the concentration limits for many such reagents used in these spectrophotometric methods. The more commonly used reagents that absorb at 280 and 205 nm are listed in Table A.3B.4. In addition, reagents that contain carbon-carbon or carbon-oxygen double bonds can interfere with the A_{205} method.

Because stray light can affect the linearity of absorbance versus concentration, absorbance values >2.0 should not be used for sample proteins measured by the A_{280} or A_{205} method. Samples with absorbance >2.0 should be diluted further in the appropriate buffer to obtain absorbances <2.0.

Nucleic acids have substantial absorbance at 280 nm and can interfere with A_{280} quantitation of protein in crude samples. To resolve the protein concentration in such samples, measure the absorbance at 260 nm and 280 nm and calculate the protein concentration as follows (Warburg and Christian, 1942; Layne, 1957): protein concentration (mg/ml) = $1.55 \times A_{280} - 0.76 \times A_{260}$.

This estimation of protein concentration is valid up to 20% (w/v) nucleic acid or an A_{280}/A_{260} ratio <0.6.

Anticipated Results

Depending on the protein, the concentration range for the A_{280} method is 20 to 3000 µg/ml, for the A_{205} method is 1 to 100 µg/ml, and for the fluorescence emission method is 5 to 50 µg/ml.

Published absorptivities of proteins at 280 nm are usually given as the absorbance for a 1% (w/v) protein solution per cm, $A^{1\%}$, or as the molar absorptivity, ε, which has units of l/mol cm. To convert these published coeffi-

cients to units of mg/ml, use either Equation A.3B.4 or Equation A.3B.5.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{280} \times 10}{A^{1\%} \times b}$$

Equation A.3B.4

Molecular weight in Equation A.3B.5 is the molecular weight of the protein.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{280} \times \text{molecular weight}}{\varepsilon_{280} \times b}$$

Equation A.3B.5

Time Considerations

When the absorptivity for a protein is known, the A_{280} and A_{205} measurements require <30 min depending on the number of samples. When standards are used for quantitation with these assays or for intrinsic fluorescence quantitation, 1 hr is required.

Literature Cited

Fasman, G.D. 1989. Practical Handbook of Biochemistry and Molecular Biology. CRC Press, Boca Raton, Fla.

Freifelder, D. 1982. Physical Biochemistry: Applications to Biochemistry and Molecular Biology 2nd ed. W.H. Freeman, New York.

Hawkins, B.K. and Honigs, D.E. 1987. A comparison of spectroscopic techniques for protein quantification in aqueous solutions. *Am. Biotechnol. Lab.* 5:26-37.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. *Methods Enzymol.* 3:447-454.

Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* 4:2411-2423.

Scopes, R.K. 1974. Measurement of protein by spectrophotometry at 205 nm. *Anal. Biochem.* 59:277-282.

Stoscheck, C.M. 1990. Quantitation of protein. *Methods Enzymol.* 182:50-68.

Teale, F.W.J. 1960. The ultraviolet fluorescence of proteins in neutral solutions. *Biochem. J.* 76:381-388.

Warburg, O. and Christian, W. 1942. Isolierung und Kristallisation des Garungsferments Enolase. *Biochem. Z.* 310:384-421.

Key References

Chen, R.F. 1990. Fluorescence of proteins and peptides. *In* Practical Fluorescence, 2nd ed. (G.G. Guilbault, ed.) pp. 575-682. Marcel Dekker, Inc., New York.

Detailed discussion of intrinsic fluorescence of proteins and what factors affect fluorescence emission by the aromatic amino acids (see pp. 618-663).

Fasman, 1989. See above.

Contains tables with absorptivities for UV spectrophotometric detection and tables with data on excitation and emission wavelengths for fluorescence detection of many proteins. Also includes a table with molecular weights for many characterized proteins.

Stoscheck, 1990. See above.

Contains list of substances that can interfere with 205- and 280-nm spectrophotometric measurements of proteins and of concentration limits for these substances.

Contributed by Michael H. Simonian
Beckman Coulter Inc.
Fullerton, California

Dialysis and Concentration of Protein Solutions

DIALYSIS

Conventional dialysis separates small molecules from large molecules by allowing diffusion of only the small molecules through selectively permeable membranes. Dialysis is usually used to change the salt (small-molecule) composition of a macromolecule-containing solution. The solution to be dialyzed is placed in a sealed dialysis membrane and immersed in a selected buffer; small solute molecules then equilibrate between the sample and the dialysate. Concomitant with the movement of small solutes across the membrane, however, is the movement of solvent in the opposite direction. This can result in some sample dilution (usually <50%).

This unit describes dialysis of large- and small-volume samples using cellulose membranes with pore sizes designed to exclude molecules above a selected molecular weight (Basic Protocol 1 and Alternate Protocol). The Support Protocol describes preparation of membranes for dialysis and discusses issues related to the selection of membranes including commercial kits. Solutions of proteins or peptides can be concentrated using commercially available reagents (see Basic Protocol 2).

Large-Volume Dialysis

BASIC PROTOCOL 1

This protocol describes the use of membranes, prepared using the support protocol, for dialysis of samples in large, easily handled volumes, typically 0.1 to 500 ml.

Materials

Dialysis membrane (see Support Protocol)
Clamps (Spectra/Por Closures, Spectrum, or equivalent)
Macromolecule-containing sample to be dialyzed
Appropriate dialysis buffer

1. Remove dialysis membrane from ethanol storage solution and rinse with distilled water. Secure clamp to one end of the membrane or knot one end with double-knots.

   Always use gloves to handle the dialysis membrane because the membrane is susceptible to cellulolytic microorganisms.

   See Support Protocol for a discussion of commercially available membranes and preparation and storage procedures.

   Clamps are less likely to leak than knots, but either type of closure should be carefully tested as described in steps 2 and 3.

2. Fill membrane with water or buffer, hold the unclamped end closed, and squeeze membrane. A fine spray of liquid indicates a pinhole in the membrane; discard and try a new membrane.

   Pinholes are rare but cause traumatic sample loss. Hard squeezing will cause some liquid to seep from the bag; this is normal.

3. Replace the water or buffer in dialysis membrane with the macromolecule-containing sample and clamp the open end. Again, squeeze to check the integrity of the membrane and clamps.

   If dialyzing a concentrated or high-salt sample, leave some space in the clamped membrane; there will be a net flow of water into the sample, and if sufficient pressure builds up the membrane can burst.

Contributed by Sarah M. Andrew, Julie A. Titus, and Louis Zumstein
Current Protocols in Cell Biology (1999) A.3C.1-A.3C.5

4. Immerse dialysis membrane in a beaker or flask containing a large volume (relative to the sample) of the desired buffer. Dialyze at least 3 hr at the desired temperature with gentle stirring of the buffer.

   Dialysis rates are dependent on membrane pore size, sample viscosity, and ratio of membrane surface to sample volume. Temperature has little effect on dialysis rate, but low temperatures (i.e., 4°C) are usually chosen to enhance macromolecule stability. Common salts will equilibrate across a 15,000-MWCO membrane (see Support Protocol) in ~3 hr with stirring.

   Some compounds, especially detergents above their critical micelle concentration (CMC), dialyze very slowly.

5. Change dialysis buffer as necessary.

   Usually two to three dialysis buffer changes are sufficient. For example, when 100 mM Tris·Cl is removed from a protein for sequence analysis or other amino-reactive chemistry, two equilibrations against a 1000-fold volume excess of buffer will decrease the Tris concentration 10^6-fold, to 100 nM; three dialyses will decrease the concentration to 100 pM.

6. Remove dialysis membrane from the buffer. Hold the membrane vertically and remove excess buffer trapped in end of membrane outside upper clamp. Release upper clamp and remove the sample with a Pasteur pipet.

ALTERNATE PROTOCOL

Small-Volume Dialysis

For solution volumes less than ~100 µl, the use of dialysis membrane as described above can result in unacceptable sample loss. The method described below can easily dialyze volumes as small as 10 µl. The sample is held in a 0.5-ml microcentrifuge tube, with dialysis membrane covering the open end of the tube.

Additional Materials *(also see Basic Protocol 1)*

0.5-ml microcentrifuge tube
Pasteur pipet
Cork borer

1. Puncture the lid of each microcentrifuge tube using a heated glass Pasteur pipet (wide end heated) or cork borer to completely remove the center part of the lid (see Fig. A.3C.1). Open lid and place a single layer of dialysis tubing over top of tube, then close lid to hold dialysis tubing in place.

   The success of this protocol is dependent on a tight-fitting cap, so make sure the cap is well-seated. On the other hand, an excessively tight-fitting cap can rip the membrane when it is inserted.

   Alternatively, some researchers use a microcentrifuge tube without a cap. The sample is placed in the microcentrifuge tube, a dialysis membrane is placed over the top of the tube, and the membrane is secured with a rubber band. With this method, however, there is a risk that a small sample—e.g., 10 to 20 µl—may be lost around the edge of the tube.

2. Turn tube upside down and shake reaction mixture onto the membrane surface. Tape each tube, dialysis surface down, to the side of a beaker, then fill the beaker with PBS or buffer of choice. Dialyze 2 hr at 4°C with stirring.

   Alternatively, place the tube in a styrofoam or foam sheet (e.g., foam microcentrifuge rack, Fisher) then place, with the tube inverted, on the surface of the dialysis buffer.

   Make sure the dialysis surface is in contact with the buffer and that no bubbles exist. This can be accomplished by gently centrifuging the tube in an inverted position in a tabletop centrifuge.

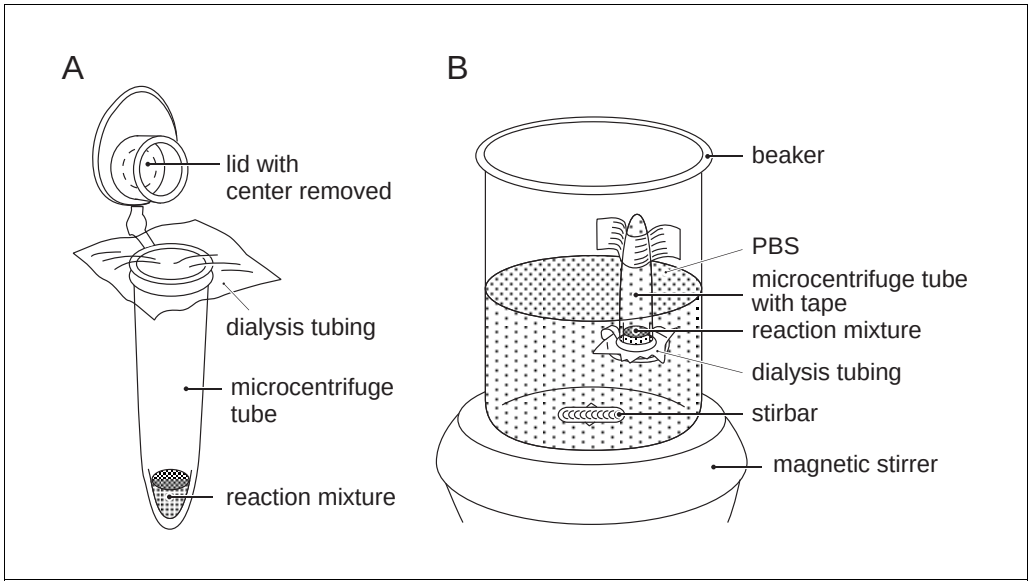


Figure A.3C.1 Making a microdialysis chamber. (**A**) Remove the center of the cap of a microcentrifuge tube with a heated Pasteur pipet (wide end). Place reaction mixture to be dialyzed into the tube. Place a piece of softened dialysis membrane loosely over top of tube. (**B**) Invert tube, shake sample down onto dialysis membrane (or centrifuge the tube inverted, if the sample is small), and tape tube into a beaker of buffer. Check that no bubbles are caught under the membrane.

The microdialysis method can be used for volumes of 50 to 500 µl (larger volumes can be dialyzed using a 1.5-ml microcentrifuge tube). The protein concentration is not important but should be 0.1 to 10 mg/ml. Most dialysis is complete after 2 hr but because proteins have a high buffering capacity, use 4 hr for a high protein concentration.

3. To recover the sample, remove microcentrifuge tube from the buffer and centrifuge briefly right-side-up.

Commercially available alternatives to this method include use of individual hollow-fiber filters from Spectrum (with sample capacities of 1 to 140 µl), and many different microdialysis machines, both single-sample and multisample (Spectrum, Cole-Parmer, Hoefer Pharmacia, and others).

Selection and Preparation of Dialysis Membrane

SUPPORT PROTOCOL

Dialysis membranes are available in a number of thicknesses and pore sizes. Thicker membranes are tougher, but restrict solute flow; thus equilibrium is reached more slowly. Pore size is defined by "molecular weight cutoff" (MWCO)—i.e., the size of the smallest particle that cannot penetrate the membrane. The MWCO designation should be used only as a very rough guide; if accurate MWCO information is required, it should be determined empirically (see Craig, 1967, for a discussion of parameters affecting MWCO). Knowledge of the precise MWCO is usually not required; it is necessary only to use a membrane with a pore size that is much smaller than the macromolecule of interest. For most plasmid and protein dialyses, a MWCO of 12,000 to 14,000 is appropriate, whereas a MWCO of 3500, 2000, or even 1000 is appropriate for peptides.

Most dialysis membranes are made of derivatives of cellulose. They come in a wide variety of MWCO values, ranging from 500 to 500,000, and also vary in cleanliness, sterility, and cost. Spectrum has the most impressive inventory. The least expensive membranes come dry on rolls; these contain glycerol to keep them flexible as well as residual sulfides and traces of heavy metals from their manufacture. If glycerol, sulfur compounds, or small amounts of heavy metals are problematic, cleaner membranes should be purchased or

membranes should be prepared as described below. Protein dialysis should only be done with clean membranes.

It should be noted that several commercial kits for performing either large-scale or microdialysis are also available. For example, use of microdialysis cassettes (Slide-A-Lyzer, Pierce; or Dispo-Dialyzer, Spectrum) eliminates the need for boiling or presoaking dialysis tubing as well as the cumbersome task of clamping the dialysis tubing. Other vendors provide vessels for dialyzing multiple samples simultaneously. Selection of the best dialysis approach to be used will depend on a number of factors including the volumes to be dialyzed, the MWCO appropriate for the sample, and, of course, costs when considering the use of kits.

Materials

Dialysis membrane
10 mM sodium bicarbonate
10 mM Na_2EDTA, pH 8.0
20% to 50% (v/v) ethanol

1. Remove membrane from the roll and cut into usable lengths (usually 8 to 12 in.).

   Always use gloves to handle dialysis membrane, as it is susceptible to a number of cellulolytic microorganisms. Membrane is available as sheets or preformed tubing.

2. Wet membrane and boil it for several minutes in a large excess of 10 mM sodium bicarbonate.
3. Boil several minutes in 10 mM Na_2EDTA. Repeat.

   Boiling speeds up the treatment process but is not necessary. A 30-min soak with some agitation can substitute for the boiling step.

4. Wash several times in distilled water.
5. Store at 4°C in 20% to 50% ethanol to prevent growth of cellulolytic microorganisms.

   Alternatively, bacteriostatic agents (e.g., sodium azide or sodium cacodylate) may be used for storage; however, ethanol is preferred for ease and convenience.

BASIC PROTOCOL 2

CONCENTRATION

Because it is usually more convenient to store antibodies or other proteins at >1 mg/ml, it may be necessary to concentrate the solution. In addition, concentration of protein solutions is often necessary to decrease the volume of a sample; e.g., to load onto a size-exclusion column when the volume must be ≤5 ml.

There are several simple and relatively inexpensive methods for concentrating protein solutions. Dialysis against Aquacide 11A (Calbiochem), which removes water through the dialysis tubing, may be used. After concentration, the solution must be redialyzed into the appropriate buffer. Another method is to use Immersible-CX Ultrafilters (Millipore) which, when connected to a vacuum, remove everything below their molecular weight cutoff (MWCO).

Alternatively, centrifugal concentrators which are operated with the aid of ordinary laboratory centrifuges may be used. Centricon microconcentrators (Amicon), which are available in different MWCO sizes, are very useful for concentrating small amounts of material in small volumes. Follow the instructions from the manufacturer when using these devices. Centriprep Centrifugal Filter Devices (Millipore) are disposable ultrafiltration devices used for purifying, concentrating, and desalting biological samples in the 2 to 15 ml volume range and are designed for use with most centrifuges that can accommodate 50-ml centrifuge tubes. Further description of these devices and instructions for their use are available at *http://www.millipore.com*.

LITERATURE CITED

Craig, L.C. 1967. Techniques for the study of peptides and proteins by dialysis and diffusion. *Methods Enzymol.* 11:870-905.

KEY REFERENCES

Craig. 1967. See above.

McPhie, P. 1971. Dialysis. *Methods Enzymol.* 22:23-32.

Describes the theory and practice of dialysis and diffusion.

Contributed by Sarah M. Andrew
Chester College
Chester, United Kingdom

Julie A. Titus
National Cancer Institute
Bethesda, Maryland

Louis Zumstein
Introgen Therapeutics, Inc.
Houston, Texas

Quantification of DNA and RNA with Absorption and Fluorescence Spectroscopy

Reliable quantitation of nanogram and microgram amounts of DNA and RNA in solution is essential to researchers in cell and molecular biology. In addition to the traditional absorbance measurements at 260 nm (see Basic Protocol), two more sensitive fluorescence techniques (see Alternate Protocols 1 and 2) are presented below. These three procedures cover a range from 5 to 10 ng/ml DNA to 50 μg/ml DNA (see Commentary and Table A.3D.3 therein).

Absorbance measurements are straightforward as long as any contribution from contaminants and the buffer components are taken into account. Fluorescence assays are less prone to interference than A_{260} measurements and are also simple to perform. As with absorbance measurement, a reading from the reagent blank is taken prior to adding the DNA. In instruments where the readout can be set to indicate concentration, a known concentration is used for calibration and subsequent readings are taken in ng/ml or μg/ml DNA.

DETECTION OF NUCLEIC ACIDS USING ABSORPTION SPECTROSCOPY

BASIC PROTOCOL

Absorption of the sample is measured at several different wavelengths to assess purity and concentration of nucleic acids. A_{260} measurements are quantitative for relatively pure nucleic acid preparations in microgram quantities. Absorbance readings cannot discriminate between DNA and RNA; however, the ratio of *A* at 260 and 280 nm can be used as an indicator of nucleic acid purity. Proteins, for example, have a peak absorption at 280 nm that will reduce the A_{260}/A_{280} ratio. Absorbance at 325 nm indicates particulates in the solution or dirty cuvettes; contaminants containing peptide bonds or aromatic moieties such as protein and phenol absorb at 230 nm.

This protocol is designed for a single-beam ultraviolet to visible range (UV-VIS) spectrophotometer. If available, a double-beam spectrophotometer will simplify the measurements, as it will automatically compare the cuvette holding the sample solution to a reference cuvette that contains the blank. In addition, more sophisticated double-beam instruments will scan various wavelengths and report the results automatically.

Materials

1× TNE buffer (see recipe)
DNA sample to be quantitated
Calf thymus DNA standard solutions (see recipe)

Matched quartz semi-micro spectrophotometer cuvettes (1-cm pathlength)
Single- or dual-beam spectrophotometer (ultraviolet to visible)

1. Pipet 1.0 ml of 1× TNE buffer into a quartz cuvette. Place the cuvette in a single- or dual-beam spectrophotometer, read at 325 nm (note contribution of the blank relative to distilled water if necessary), and zero the instrument. Use this blank solution as the reference in double-beam instruments. For single-beam spectrophotometers, remove blank cuvette and insert cuvette containing DNA sample or standard suspended in the same solution as the blank. Take reading. Repeat this process at 280, 260, and 230 nm.

   It is important that the DNA be suspended in the same solution as the blank.

Contributed by Sean R. Gallagher
Current Protocols in Cell Biology (2000) A.3D.1-A.3D.8

2. To determine the concentration (C) of DNA present, use the A_{260} reading in conjunction with one of the following equations:

$$\textit{Single-stranded DNA}: \quad C\ (\text{pmol}/\mu\text{l}) = \frac{A_{260}}{10 \times S}$$

$$C\ (\mu\text{g}/\text{ml}) = \frac{A_{260}}{0.027}$$

$$\textit{Double-stranded DNA}: \quad C\ (\text{pmol}/\mu\text{l}) = \frac{A_{260}}{13.2 \times S}$$

$$C\ (\mu\text{g}/\text{ml}) = \frac{A_{260}}{0.020}$$

$$\textit{Single-stranded RNA}: \quad C\ (\mu\text{g}/\text{ml}) = \frac{A_{260}}{0.025}$$

$$\textit{Oligonucleotide}: \quad C\ (\text{pmol}/\mu\text{l}) = A_{260} \times \frac{100}{1.5\,N_{\text{A}} + 0.71\,N_{\text{C}} + 1.20\,N_{\text{G}} + 0.84\,N_{\text{T}}}$$

where S represents the size of the DNA in kilobases and N is the number or residues of base A, G, C, or T.

For double- or single-stranded DNA and single-stranded RNA: These equations assume a 1-cm-pathlength spectrophotometer cuvette and neutral pH. The calculations are based on the Lambert-Beer law, $A = \varepsilon Cl$, where A is the absorbance at a particular wavelength, C is the concentration of DNA, l is the pathlength of the spectrophotometer cuvette (typically 1 cm), and ε is the extinction coefficient. For solution concentrations given in mol/liter and a cuvette of 1-cm pathlength, ε is the molar extinction coefficient and has units of $M^{-1}cm^{-1}$. If concentration units of μg/ml are used, then ε is the specific absorption coefficient and has units of $(\mu g/ml)^{-1}cm^{-1}$. The values of ε used here are as follows: ssDNA, 0.027 $(\mu g/ml)^{-1}cm^{-1}$; dsDNA, 0.020 $(\mu g/ml)^{-1}cm^{-1}$; ssRNA, 0.025 $(\mu g/ml)^{-1}cm^{-1}$. Using these calculations, an A_{260} of 1.0 indicates 50 μg/ml double-stranded DNA, ~37 μg/ml single-stranded DNA, or ~40 μg/ml single-stranded RNA (adapted from Applied Biosystems, 1989).

For oligonucleotides: Concentrations are calculated in the more convenient units of pmol/μl. The base composition of the oligonucleotide has significant effects on absorbance, because the total absorbance is the sum of the individual contributions of each base (Table A.3D.1).

3. Use the A_{260}/A_{280} ratio and readings at A_{230} and A_{325} to estimate the purity of the nucleic acid sample.

Ratios of 1.8 to 1.9 and 1.9 to 2.0 indicate highly purified preparations of DNA and RNA, respectively. Contaminants that absorb at 280 nm (e.g., protein) will lower this ratio.

Table A.3D.1 Molar Extinction Coefficients of DNA Bases[a]

Base	$\varepsilon^{1M}_{260\ nm}$
Adenine	15,200
Cytosine	7,050
Guanosine	12,010
Thymine	8,400

[a]Measured at 260 nm; see Wallace and Miyada, 1987.

Table A.3D.2 Spectrophotometric Measurements of Purified DNA[a]

Wavelength (nm)	Absorbance	A_{260}/A_{280}	Conc. (μg/ml)
325	0.01	—	—
280	0.28	—	—
260	0.56	2.0	28
230	0.30	—	—

[a]Typical absorbancy readings of highly purified calf thymus DNA suspended in 1× TNE buffer. The concentration of DNA was nominally 25 μg/ml.

Absorbance at 230 nm reflects contamination of the sample by phenol or urea, whereas absorbance at 325 nm suggests contamination by particulates and dirty cuvettes. Light scatter at 325 nm can be magnified 5-fold at 260 nm (K. Hardy, pers. comm.).

Typical values at the four wavelengths for a highly purified preparation are shown in Table A.3D.2.

DNA DETECTION USING THE DNA-BINDING FLUOROCHROME HOECHST 33258

ALTERNATE PROTOCOL 1

Use of fluorometry to measure DNA concentration has gained popularity because it is simple and much more sensitive than spectrophotometric measurements. Specific for nanogram amounts of DNA, the Hoechst 33258 fluorochrome has little affinity for RNA and works equally well with either whole-cell homogenates or purified preparations of DNA. The fluorochrome is, however, sensitive to changes in DNA composition, with preferential binding to AT-rich regions. A fluorometer capable of an excitation wavelength of 365 nm and an emission wavelength of 460 nm is required for this assay.

Additional Materials *(also see Basic Protocol)*

Hoechst 33258 assay solution (working solution; see recipe)
Dedicated filter fluorometer (e.g., Amersham Pharmacia Biotech DQLOO) *or* scanning fluorescence spectrophotometer (e.g., Shimadzu model RF-5000 or Perkin-Elmer model LS-5B or LS-3B)
Fluorometric square glass cuvettes *or* disposable acrylic cuvettes (Sarstedt)
Teflon stir rod

1. Prepare the scanning fluorescence spectrophotometer by setting the excitation wavelength to 365 nm and the emission wavelength to 460 nm.

   The dedicated filter fluorometer has fixed wavelengths at 365 and 460 nm and does not need adjustment.

2. Pipet 2.0 ml Hoechst 33258 assay solution into cuvette and place in sample chamber. Take a reading without DNA and use as background.

   If the fluorometer has a concentration readout mode or is capable of creating a standard curve, set instrument to read 0 with the blank solution. Otherwise note the readings in relative fluorescence units. Be sure to take a blank reading for each cuvette used, as slight variations can cause changes in the background reading.

3. With the cuvette still in the sample chamber, add 2 μl DNA standard to the blank Hoechst 33258 assay solution. Mix in the cuvette with a Teflon stir rod or by capping and inverting the cuvette. Read emission in relative fluorescence units or set the concentration readout equal to the final DNA concentration. Repeat measurements with remaining DNA standards using fresh assay solution (take background zero reading and zero instrument if needed).

If necessary, the DNA standards should be quantitated by A_{260} measurement (Basic Protocol) before being used here.

Small-bore tips designed for loading sequencing gels minimize errors of pipetting small volumes. Prerinse tips with sample and make sure no liquid remains outside the tip after drawing up the sample.

Read samples in duplicate or triplicate, with a blank reading taken each time. Unusual or unstable blank readings indicate a dirty cuvette or particulate material in the solution, respectively.

4. Repeat step 3 with unknown samples.

A dye concentration of 0.1 μg/ml is adequate for final DNA concentrations up to ~500 ng/ml. Increasing the working dye concentration to 1 μg/ml Hoechst 33258 will extend the assay's range to 15 μg/ml DNA, but will limit sensitivity at low concentrations (5 to 10 ng/ml). Sample volumes of ≤10 μl can be added to the 2.0-ml aliquot of Hoechst 33258 assay solution.

ALTERNATE PROTOCOL 2

DNA AND RNA DETECTION WITH ETHIDIUM BROMIDE FLUORESCENCE

In contrast to the fluorochrome Hoechst 33258, ethidium bromide is relatively unaffected by differences in the base composition of DNA. Ethidium bromide is not as sensitive as Hoechst 33258 and, although capable of detecting nanogram levels of DNA, will also bind to RNA. In preparations of DNA with minimal RNA contamination or with DNA samples having an unusually high guanine and cytosine (GC) content where the Hoechst 33258 signal can be quite low, ethidium bromide offers a relatively sensitive alternative to the more popular Hoechst 33258 DNA assay. A fluorometer capable of an excitation wavelength of 302 or 546 nm and an emission wavelength of 590 nm is required for this assay.

Additional Materials *(also see Basic Protocol)*

Ethidium bromide assay solution (see recipe)

1. Pipet 2.0 ml ethidium bromide assay solution into cuvette and place in sample chamber. Set excitation wavelength to 302 nm or 546 nm and emission wavelength to 590 nm. Take an emission reading without DNA and use as background.

If the instrument has a concentration readout mode or is capable of creating a standard curve, set instrument to read 0 with the blank solution. Otherwise note the readings in relative fluorescence units.

The excitation wavelength of this assay can be either in the UV range (~302 nm) using a quartz cuvette or in the visible range (546 nm) using a glass cuvette. In both cases the emission wavelength is 590 nm.

2. Read and calibrate these samples as described in step 3 of the Hoechst 33258 assay.

3. Read emissions of the unknown samples as in step 4 of the Hoechst 33258 assay.

A dye concentration of 5 μg/ml in the ethidium bromide assay solution is appropriate for final DNA concentrations up to 1000 ng/ml. 10 μg/ml ethidium bromide in the ethidium bromide assay solution will extend the assay's range to 10 μg/ml DNA, but is only used for DNA concentrations >1 μg/ml. Sample volumes of up to 10 μl can be added to the 2.0-ml aliquot of ethidium bromide assay solution.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Calf thymus DNA standard solutions

Kits containing calf thymus DNA standard for fluorometry are available (Fluorometry Reference Standard Kits, Hoefer). Premeasured, CsCl-gradient-purified DNA of defined GC content, for use in absorption and fluorometric spectroscopy, is available from Sigma (e.g., calf thymus DNA, 42% GC; *Clostridium perfringens* DNA, 26.5% GC).

Ethidium bromide assay solution

Add 10 ml of 10× TNE buffer (see recipe) to 89.5 ml H_2O. Filter through a 0.45-µm filter, then add 0.5 ml of 1 mg/ml ethidium bromide.

Add the dye after filtering, as ethidium bromide will bind to most filtration membranes.

CAUTION: *Ethidium bromide is hazardous; wear gloves and use appropriate care in handling, storage, and disposal.*

Hoechst 33258 assay solutions

Stock solution: Dissolve in H_2O at 1 mg/ml. Stable for ~6 months at 4°C.

Working solution: Add 10 ml of 10× TNE buffer (see recipe) to 90 ml H_2O. Filter through a 0.45-µm filter, then add 10 µl of 1 mg/ml Hoechst 33258.

Hoechst 33258 is a fluorochrome dye with a molecular weight of 624 and a molar extinction coefficient of $4.2 \times 10^4\ M^{-1}cm^{-1}$ *at 338 nm.*

The dye is added after filtering because it will bind to most filtration membranes.

CAUTION: *Hoechst 33258 is hazardous; use appropriate care in handling, storage, and disposal.*

TNE buffer, 10×

100 mM Tris base
10 mM EDTA
2.0 M NaCl
Adjust pH to 7.4 with concentrated HCl
As needed, dilute with H_2O to desired concentration

COMMENTARY

Background Information

In deciding what method of nucleic acid measurement is appropriate, three issues are critical: specificity, sensitivity, and interfering substances. Properties of the three assays described in this section are listed in Table A.3D.3. The traditional method for determining the amount of DNA in solution is by measuring absorbance at 260 nm. Because many potential contaminants of DNA and RNA preparations also absorb in the UV range, absorption spectroscopy is a reliable method to assess both the purity of a preparation and the quantity of DNA or RNA present. Absorption spectroscopy does have serious limitations. Relatively large amounts of DNA are required to get accurate readings—for example, 500 ng/ml DNA is equivalent to only 0.01 A_{260} units. Furthermore, the method cannot discriminate between RNA and DNA, and UV-absorbing contaminants such as protein will cause discrepancies.

The assay using Hoechst 33258 dye (Alternate Protocol 1) is the only procedure in common use that is specific for DNA (i.e., it does not measure RNA). This assay is the method of choice for rapid measurement of low quantities of DNA, with a detection limit of ~1 ng DNA. Concentrations of DNA in both crude cell lysates and purified preparations can be quantified (Labarca and Paigen, 1980). Because the assay quantifies a broad range of DNA concentrations—from 10 ng/ml to 15 µg/ml—it is useful for the measurement of both small and large amounts of DNA (e.g., in verifying DNA

Table A.3D.3 Properties of Absorbance and Fluorescence Spectrophotometric Assays for DNA and RNA

Property	Absorbance (A_{260})	Fluorescence	
		H33258	EtBr
Sensitivity (μg/ml)			
DNA	1-50	0.01-15	0.1-10
RNA	1-40	n.a.	0.2-10
Ratio of signal (DNA/RNA)	0.8	400	2.2

concentrations prior to performing electrophoretic separations and Southern blots). The Hoechst 33258 assay is also useful for measuring products of the polymerase chain reaction (PCR) synthesis.

Upon binding to DNA, the fluorescence characteristics of Hoechst 33258 change dramatically, showing a large increase in emission at ~458 nm. Hoechst 33258 is nonintercalating and apparently binds to the minor groove of the DNA, with a marked preference for AT sequences (Portugal and Waring, 1988). The fluorochrome 4′,6-diamidino-2-phenylindole (DAPI; Daxhelet et al., 1989) is also appropriate for DNA quantitation, although it is not as commonly used as Hoechst 33258. DAPI is excited with a peak at 344 nm. Emission is detected at ~466 nm, similar to Hoechst 33258.

Ethidium bromide is best known for routine staining of electrophoretically separated DNA and RNA, but it can also be used to quantify both DNA and RNA in solution (Le Pecq, 1971). Unlike Hoechst 33258, ethidium bromide fluorescence is not significantly impaired by high GC content. The ethidium bromide assay (with excitation at 546 nm) is ~20-fold less sensitive than the Hoechst 33258 assay.

Critical Parameters

Care should be taken when handling sample cuvettes in all spectrophotometric procedures. Fluorometers use cuvettes with four optically clear faces, because excitation and emitting light enter and leave the cuvette through directly adjacent sides. Thus, fluorometric cuvettes should be held by the upper edges only. In contrast, transmission spectrophotometers use cuvettes with two opposite optical windows, with the sides frosted for easy handling. It is important to check that the optical faces of cuvettes are free of fingerprints and scratches. In addition, for accurate absorbance readings, spectrophotometer cuvettes must be perfectly matched.

Proteins in general have A_{280} readings considerably lower than nucleic acids on an equivalent weight basis. Thus, even a small increase in the A_{280} relative to A_{260} (i.e., a lowering of the A_{260}/A_{280} ratio) can indicate severe protein contamination. Other commonly used buffer components absorb strongly at 260 nm and can cause interference if present in high enough concentrations. EDTA, for example, should not be present at ≥10 mM.

Sensitivity of the Hoechst 33258 fluorescence assay decreases with nuclease degradation, increasing GC content, or denaturation of DNA (Labarca and Paigen, 1980; Stout and Becker, 1982). Increased temperature of the assay solution and ethidium bromide contamination also decrease the Hoechst 33258 signal. Sodium dodecyl sulfate (>0.01% final concentration) also interferes with accurate readings (Cesarone et al., 1979). The pH of the assay solution is critical to sensitivity and should be pH ~7.4 (Stout and Becker, 1982; Labarca and Paigen, 1980). At a pH <6.0 or >8.0 the background becomes much higher and there is a concomitant loss of fluorescence enhancement.

High-quality double-stranded DNA is recommended, although single-stranded genomic DNA also works well with this assay. However, with very small fragments of DNA, the Hoechst 33258 dye binds to double-stranded DNA only. Thus, the assay will not work with single-stranded oligomers. Linear and circular DNA give approximately the same levels of fluorescence (Daxhelet et al., 1989). When preparing DNA standards, an attempt should be made to equalize the GC content of the standard DNA and that of the sample DNA. In most situations, salmon sperm or calf thymus DNA is suitable. An extensive list of estimated GC content for various organisms is available (Marmur and Doty, 1962). Eukaryotic cells vary somewhat in GC content but are generally in the range of 39% to 46%. Within this range, the fluorescence per microgram of DNA does not vary substantially. In contrast, the GC content of

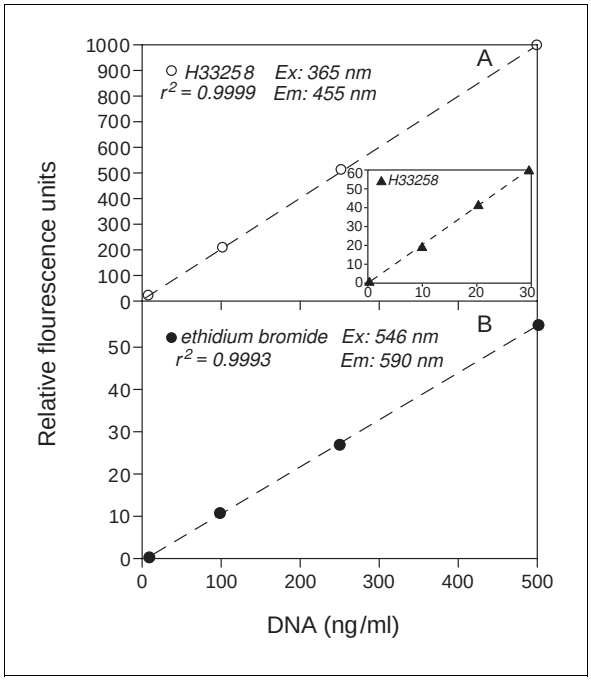


Figure A.3D.1 Fluorochrome Hoechst 33258 (H33258) (**A**) and ethidium bromide (**B**) DNA concentration standard curves. Assays were performed as described in alternate protocols, at indicated excitation and emission wavelengths. The concentrations of H33258 and ethidium bromide were 0.1 and 5 μg/ml, respectively. Assays contained the indicated concentrations of calf thymus DNA standards suspended in a final volume of 2.0 ml. Inset shows low DNA concentration curve for the H33258 assay. Note that, under these conditions, H33258 produces ~20 times more relative fluorescence units than ethidium bromide. A Shimadzu RF-5000 scanning fluorescence spectrometer was used for both assays.

prokaryotes can vary from 26% to 77%, causing considerable variation in the fluorescence signal. In these situations, the sample DNA should be first quantitated via transmission spectroscopy and compared to a readily available standard (e.g., calf thymus DNA). Future measurements would then use calf thymus as a standard, but with a correction factor for difference in fluorescence yield between the two DNA types. For further troubleshooting information, see Van Lancker and Gheyssens (1986), in which the effects of interfering substances on the Hoechst 33258 assay (and several other assays) are compared.

In the ethidium bromide assay, single-stranded DNA gives approximately half the signal as double-stranded calf thymus DNA. Ribosomal RNA also gives about half the fluorescent signal as double-stranded DNA, and RNase and DNase both severely decrease the signal. Closed circular DNA also binds less ethidium bromide than nicked or linear DNA. Further critical parameters of the ethidium bromide assay are described by Le Pecq (1971).

Anticipated Results

The detection limit of absorption spectroscopy will depend on the sensitivity of the spectrophotometer and any UV-absorbing contaminants that might be present. The lower limit is generally ~0.5 to 1 μg nucleic acid. Typical values for a highly purified sample of DNA are shown in Table A.3D.2.

For the Hoechst 33258 and ethidium bromide assays, a plot of relative fluorescence units or estimated concentration (*y* axis) versus actual concentration (*x* axis) typically produces a linear regression with a correlation coefficient (r^2) of 0.98 to 0.99 (Fig. A.3D.1). Table A.3D.3 provides a comparison of the sensitivities and specificities of the three assays.

Time Considerations

The three assays described can be performed in a short period of time. In a well-planned series of assays, 50 samples can be prepared and read comfortably in 1 hr. Although some error might be introduced, DNA samples can be sequentially added to the same cuvette containing working dye solution. The increase in fluorescence with each sample is noted and subtracted from the previous reading to give relative fluorescence or concentration of the new sample, eliminating the need to change solutions for each sample. Be certain that the final amount of DNA does not exceed the linear portion of the assay.

Literature Cited

Applied Biosystems. 1989. User Bulletin Issue 11, Model No. 370. Applied Biosystems, Foster City, Calif.

Cesarone, C.F., Bolognesi, C., and Santi L. 1979. Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* 100:188-197.

Daxhelet, G.A., Coene, M.M., Hoet, P.P., and Cocito, C.G. 1989. Spectrofluorometry of dyes with DNAs of different base composition and conformation. *Anal. Biochem.* 179:401-403.

Labarca, C. and Paigen, K. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102:344-352.

Le Pecq, J.-B. 1971. Use of ethidium bromide for separation and determination of nucleic acids of various conformational forms and measurement of their associated enzymes. *In* Methods of Biochemical Analysis, Vol. 20 (D. Glick, ed.) pp. 41-86. John Wiley & Sons, New York.

Marmur, J. and Doty, P. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Molec. Biol.* 5:109-118.

Portugal, J. and Waring, M.J. 1988. Assignment of DNA binding sites for 4′,6-diamidine-2- phenylindole and bisbenzimide (Hoechst 33258): A comparative footprinting study. *Biochem. Biophys. Acta* 949:158-168.

Stout, D.L. and Becker, F.F. 1982. Fluorometric quantitation of single-stranded DNA: A method applicable to the technique of alkaline elution. *Anal. Biochem.* 127:302-307.

Van Lancker, M. and Gheyssens, L.C. 1986. A comparison of four frequently used assays for quantitative determination of DNA. *Anal. Lett.* 19:615-623.

Wallace, R.B. and Miyada C.G. 1987. Oligonucleotide probes for the screening of recombinant DNA libraries. *In* Methods of Enzymology, Vol. 152: Guide to Molecular Cloning Techniques (S.L. Berger and A.R. Kimmel, eds.) pp. 432-442. Academic Press, San Diego.

Key References

Labarca and Paigen, 1980. See above.

Contains a detailed description of the Hoechst 33258 fluorometric DNA assay.

Contributed by Sean R. Gallagher
Motorola Corporation
Tempe, Arizona

Silanizing Glassware

BASIC PROTOCOL

Glassware is silanized (siliconized) to prevent adsorption of solute to the glass surface or to increase its hydrophobicity. This is particularly important when dealing with low concentrations of particularly "sticky" solutes such as single-stranded nucleic acids or proteins or cells.

Materials

Chlorotrimethylsilane *or* dichlorodimethylsilane
Vacuum pump
Desiccator, equipped with a valve

1. In a fume hood, place glassware or equipment to be silanized into desiccator along with a beaker containing 1 to 3 ml of chlorotrimethylsilane or dichlorodimethylsilane.

   CAUTION: *Chlorotrimethylsilane and dichlorodimethylsilane vapors are toxic and highly flammable.*

2. Connect desiccator to vacuum pump until silane starts to boil and close connection to pump (maintaining vacuum in desiccator). Leave the desiccator evacuated and closed until liquid silane is gone (~1 to 3 hr).

   During the incubation the silane will evaporate, be deposited on the surface of the glassware, and polymerize. Do not leave the desiccator attached to the vacuum pump. This will suck away the silane, minimizing deposition and damaging the pump.

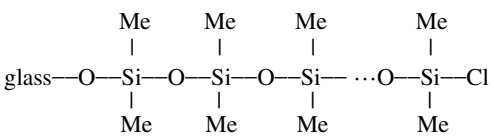
3. Open desiccator in a fume hood, and leave open for several minutes to disperse silane vapors.

4. If desired, bake or autoclave the glassware or apparatus.

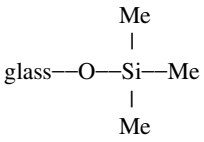
   Autoclaving or rinsing with water removes the reactive chlorosilane end of the dimethylsiloxane polymer generated by dichlorodimethylsilane.

COMMENTARY

Untreated glass contains silicate and silanol groups that can act as ion-exchange and nucleophilic centers. To mask these groups and decrease the hydrophilicity of the surface, various reactive silanes are frequently used to coat the glass surface. The same or related chemistries can be used to introduce functional groups, including large molecules, onto the glass surface. On contact with a silanol (SiOH) group, chlorosilanes react to give HCl and a siloxane Si-O-Si linkage. For most applications "silanizing" or "siliconizing" a piece of glassware or equipment means introducing onto the glass surface either a short polymer of dimethylsiloxane



using dichlorodimethylsilane, or a trimethylsiloxane cap



using chlorotrimethylsilane. Polydimethylsiloxane is silicone oil; the use of dichloromethylsilane essentially gives a light coating of oil to the glassware. The polymerization of dichlorodimethylsilane is mediated by residual water adsorbed to the glass surface; chlorosilanes also react with water and alcohols to give HCl and silanol or alkoxysilane, respectively. Thus, in the polymerization reaction, the residual water reacts with the chloride endgroup to give HCl and a silanol, which then reacts with more dichlorodimethylsilane and more water to give

Contributed by Brian Seed

Current Protocols in Cell Biology (2000) A.3E.1-A.3E.2

polymers. Chlorotrimethylsilane just gives a trimethyl cap to the silanol or silicate group.

Items too large to fit in a desiccator can be silanized by briefly rinsing with or soaking in a solution of approximately 5% dichlorodimethylsilane in various volatile organic solvents such as chloroform or heptane. The organic solvent is removed by evaporation, depositing the dichlorodimethylsilane on the surface. This approach is particularly useful for treating glass plates for denaturing polyacrylamide sequencing gels.

CAUTION: If a flammable solvent is used, do not bake the glassware until the solvent is completely evaporated.

Contributed by Brian Seed
Massachusetts General Hospital
and Harvard Medical School
Boston, Massachusetts

Enzymatic Amplification of DNA by PCR: Standard Procedures and Optimization

BASIC PROTOCOL

This unit describes a method for amplifying DNA enzymatically by the polymerase chain reaction (PCR), including procedures to quickly determine conditions for successful amplification of the sequence and primer sets of interest, and to optimize for specificity, sensitivity, and yield. The first step of PCR simply entails mixing template DNA, two appropriate oligonucleotide primers, *Taq* or other thermostable DNA polymerases, deoxyribonucleoside triphosphates (dNTPs), and a buffer. Once assembled, the mixture is cycled many times (usually 30) through temperatures that permit denaturation, annealing, and synthesis to exponentially amplify a product of specific size and sequence. The PCR products are then displayed on an appropriate gel and examined for yield and specificity.

Many important variables can influence the outcome of PCR. Careful titration of the $MgCl_2$ concentration is critical. Additives that promote polymerase stability and processivity or increase hybridization stringency, and strategies that reduce nonspecific primer-template interactions, especially prior to the critical first cycle, generally improve amplification efficiency. This protocol, using *Taq* DNA polymerase, is designed to optimize the reaction components and conditions in one or two stages. The first stage (steps 1 to 7) determines the optimal $MgCl_2$ concentration and screens several enhancing additives. Most suppliers of *Taq* and other thermostable DNA polymerases provide a unique optimized $MgCl_2$-free buffer with $MgCl_2$ in a separate vial for user titration. The second stage (steps 8 to 13) compares methods for preventing pre-PCR low-stringency primer extension, which can generate nonspecific products. This has come to be known as "hot start," whether one omits an essential reaction component prior to the first denaturing-temperature step or adds a reversible inhibitor of polymerase. Hot-start methods can greatly improve specificity, sensitivity, and yield. Use of any one of the hot-start approaches is strongly recommended if primer-dimers or other nonspecific products are generated or if relatively rare template DNA is contained in a complex mixture, such as viral nucleic acids in cell or tissue preparations. This protocol suggests some relatively inexpensive methods to achieve hot start, and lists several commercial hot-start options which may be more convenient, but of course more expensive.

Materials

Sterile H_2O
15 mM (L), 30 mM (M), and 45 mM (H) $MgCl_2$
10× $MgCl_2$-free PCR amplification buffer (see recipe)
25 mM 4dNTP mix (see recipe)
50 µM oligonucleotide primer 1: 50 pmol/µl in sterile H_2O (store at −20°C)
50 µM oligonucleotide primer 2: 50 pmol/µl in sterile H_2O (store at −20°C)
Template DNA: 1 µg mammalian genomic DNA or 1.0 to 100.0 pg of plasmid DNA
5 U/µl *Taq* DNA polymerase (native or recombinant; many suppliers)
Enhancer agents (optional; see recipe)
TaqStart Antibody (Clontech)
Mineral oil
Ficoll 400 (optional)
Tartrazine dye (optional)

Thin-walled PCR tubes
Automated thermal cycler

Contributed by Martha F. Kramer and Donald M. Coen
Current Protocols in Cell Biology (2001) A.3F.1-A.3F.14

Table A.3F.1 Master Mixes for Optimizing Reaction Components

Components	Final concentration	Per reaction	Master mix[a] (µl)			
			I	II	III	IV
10× PCR buffer	1×	10 µl	40.0	40.0	40.0	40.0
Primer 1	0.5 µM	1 µl	4.0	4.0	4.0	4.0
Primer 2	0.5 µM	1 µl	4.0	4.0	4.0	4.0
Template DNA	Undiluted	1 vol[b]	4 vol[b]	4 vol[b]	4 vol[b]	4 vol[b]
25 mM 4dNTP mix[c]	0.2 mM	0.8 µl	3.2	3.2	3.2	3.2
Taq polymerase	2.5 U	0.5 µl	2.0	2.0	2.0	2.0
DMSO[d] (20×)	5%	5 µl	—	20.0	—	—
Glycerol[d] (10×)	10%	10 µl	—	—	40.0	—
PMPE[d] (100×)	1%	1 µl	—	—	—	4.0
H_2O	—	To 90 µl	To 360	To 360	To 360	To 360

[a]Total volume = 360 µl (enough for $n + 1$ reactions).

[b]Template DNA volume ("vol") is generally 1 to 10 µl.

[c]If 2 mM 4dNTP mix is preferred, use 10 µl per reaction, or 40 µl for each master mix; adjust the volume of water accordingly.

[d]Substitute with other enhancer agents (see recipe in Reagents and Solutions) as available.

Additional reagents and equipment for DNA preparation (*APPENDIX 3*), agarose gel electrophoresis (*APPENDIX 3*), nondenaturing PAGE (*UNIT 6.5*), or sieving agarose gel electrophoresis (*APPENDIX 3*), restriction endonuclease digestion (*APPENDIX 3*), and Southern blotting and hybridization (*APPENDIX 3*)

NOTE: Do not use DEPC to treat water, reagents, or glassware.

NOTE: Reagents should be prepared in sterile, disposable labware, taken directly from its packaging, or in glassware that has been soaked in 10% bleach, thoroughly rinsed in tap water followed by distilled water, and if available, exposed to UV irradiation for ~10 min. Multiple small volumes of each reagent should be stored in screw-cap tubes. This will then serve as the user's own optimization "kit." Thin-walled PCR tubes are recommended.

Optimize reaction components

1. Prepare four reaction master mixes according to the recipes given in Table A.3F.1.

   Enhancing agents probably work by different mechanisms, such as protecting enzyme activity and decreasing nonspecific primer binding. However, their effects cannot be readily predicted—what improves amplification efficiency for one primer pair may decrease the amplification efficiency for another. Thus it is best to check a panel of enhancers during development of a new assay.

2. Aliquot 90 µl master mix I into each of three 0.5-ml thin-walled PCR tubes labeled I-L, I-M, and I-H. Similarly, aliquot mixes II through IV into appropriately labeled tubes. Add 10 µl of 15 mM $MgCl_2$ into one tube of each master mix (labeled L; 1.5 mM final). Similarly, aliquot 10 µl of 30 mM and 45 mM $MgCl_2$ to separate tubes of each master mix (labeled M and H, respectively; 3.0 and 4.5 mM final concentrations respectively).

It is helpful to set the tubes up in a three-by-four array to simplify aliquotting. Each of the three Mg^{2+} concentrations is combined with each of the four master mixes.

3. Overlay the reaction mixture with 50 to 100 µl mineral oil (2 to 3 drops).

To include hot start in the first step, overlay reaction mixes with oil before adding the $MgCl_2$, heat the samples to 95°C in the thermal cycler or other heating block, and add the $MgCl_2$ once the elevated temperature is reached. Once the $MgCl_2$ has been added, do not allow the samples to cool below the optimum annealing temperature prior to performing PCR.

Choose cycling parameters

4. Using the following guidelines, program the automated thermal cycler according to the manufacturers' instructions.

30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55° (GC content ≤50%) *or*	
		60°C (GC content >50%)	(annealing)
	~60 sec/kb		
	product sequence	72°C	(extension)

Cycling parameters are dependent upon the sequence and length of the template DNA, the sequence and percent complementarity of the primers, and the ramp times of the thermal cycler used. Thoughtful primer design will reduce potential problems (see Commentary). Denaturation, annealing, and extension are each quite rapid at the optimal temperatures. The time it takes to achieve the desired temperature inside the reaction tube, i.e., the ramp time, is usually longer than either denaturation or primer annealing. Thus, ramp time is a crucial cycling parameter. Manufacturers of the various thermal cyclers on the market provide ramp time specifications for their instruments. Ramp times are lower with thin-walled reaction tubes. The optimal extension time also depends on the length of the target sequence. Allow ~1 min/kb for this step for target sequences >1 kb, and as little as a 2-sec pause for targets <100 bases in length.

The number of cycles depends on both the efficiency of the reaction and the amount of template DNA in the reaction. Starting with as little as 100 ng of mammalian genomic DNA (~10^4 cell equivalents), after 30 cycles, 10% of the reaction should produce a band that is readily visible on an ethidium bromide–stained gel as a single predominant band. With more template, fewer cycles may suffice. With much less template, further optimization is recommended rather than increasing the cycle number. Greater cycle numbers (e.g., >40) can reduce the polymerase specific activity, increase nonspecific amplification, and deplete substrate (nucleotides). Many investigators lengthen the time for the last extension step—to 7 min, for example—to try to ensure that all the PCR products are full length.

These guidelines are appropriate for most commercially available thermal cyclers. For rapid cyclers, consult the manufacturers' protocols.

Analyze the product

5. Electrophorese 10 µl from each reaction on an agarose, nondenaturing polyacrylamide, or sieving agarose gel appropriate for the PCR product size expected. Stain with ethidium bromide.

For resolution of PCR products between 100 and 1000 bp, an alternative to nondenaturing polyacrylamide gels or sieving agarose is a composite 3% (w/v) NuSieve (FMC Bioproducts) agarose/1% (w/v) SeaKem (FMC Bioproducts) agarose gel. SeaKem increases the mechanical strength of the gel without decreasing resolution.

An alternative to ethidium bromide, SYBR Gold Nucleic Acid Gel Stain (Molecular Probes), is 25 to 100 times more sensitive than ethidium bromide, is more convenient to use, and permits optimization of 10- to 100-fold lower starting template copy number.

6. Examine the stained gel to determine which condition resulted in the greatest amount of product.

Minor, nonspecific products may be present even under optimal conditions.

Table A.3F.2 Master Mixes for Optimizing First-Cycle Reactions

Components	Final concentration	Master mix (µl)			
		A	B	C	D
10× PCR buffer	1×	10	10	10	10
$MgCl_2$ (L, M, or H)	Optimal	10	10	10	10
Primer 1	0.5 µM	1.0	1.0	1.0	1.0
Primer 2	0.5 µM	1.0	1.0	1.0	1.0
Additive	Optimal	V[a]	V[a]	V[a]	V[a]
Template DNA	—[b]	V[a]	V[a]	V[a]	V[a]
25 mM 4dNTP mix[b]	0.2 mM	0.8	0.8	0.8	0.8
Taq polymerase	2.5 U	0.5	0.5	—	—
Taq pol + TaqStart	2.5 U	—	—	—	1.0
H_2O	To 100 µl	V[a]	V[a]	V[a]	V[a]
Preparation temperature		Room temperature	Ice slurry	Room temperature	Room temperature

[a]V, variable amount (total volume should be 100 µl).

[b]Use undiluted or diluted template DNA based on results obtained in step 6.

7. To ensure that the major product is the correct one, digest an aliquot of the reaction with a restriction endonuclease known to cut within the PCR product. Check buffer compatibility for the restriction endonuclease of choice. If necessary, add Na^+ or precipitate in ethanol (*APPENDIX 3*), then resuspend in the appropriate buffer. Electrophorese the digestion product on a gel to verify that the resulting fragments have the expected sizes.

   Alternatively, transfer the PCR products to a nitrocellulose or nylon filter and hybridize with an oligonucleotide derived from the sequence internal to the primers. With appropriately stringent hybridization and washing conditions, only the correct product (and possibly some minor related products) should hybridize.

Optimize the first cycle

These optional steps optimize initial hybridization and may improve efficiency and yield. They are used when primer-dimers and other nonspecific products are detected, when there is only a very small amount of starting template, or when a rare sequence is to be amplified from a complex mixture. For an optimal reaction, polymerization during the initial denaturation and annealing steps should be prevented. *Taq* DNA polymerase activity can be inhibited by temperature (reaction B), physical separation (reaction C), or reversible antibody binding (reaction D). PCR without hot start is performed for comparison (reaction A).

8. Prepare four reaction mixtures using the optimal $MgCl_2$ concentration and additive requirement determined in step 6. Prepare the mixes according to the recipes in Table A.3F.2. Use the following variations for addition of *Taq* polymerase.

   a. Prepare reactions A and C at room temperature.

   b. Chill all components of reaction B in an ice slurry before they are combined.

   c. For reaction D, combine 1.0 µl TaqStart antibody with 4.0 µl of the dilution buffer provided with the antibody, add 1.0 µl *Taq* DNA polymerase (for 1:4:1 mixture of these components), mix, and incubate 5 to 10 min at room temperature before

adding to reaction mixture D (glycerol and PMPE are compatible with TaqStart antibody but DMSO will interfere with antibody binding).

To ensure that the reaction does not plateau and thereby obfuscate the results, use the smallest amount of template DNA necessary for visualization of the PCR product by ethidium bromide staining. Use the results from step 6 to decide how much template to use. If the desired product stains intensely, dilute the starting material as much as 1/100. If only a faint signal is apparent, use undiluted sample.

9. Overlay each reaction mixture with 50 to 100 µl mineral oil.

10. Heat all reactions 5 min at 94°C.

    It is most convenient to use the automated thermal cycler for this step and then initiate the cycling program directly.

11. Cool the reactions to the appropriate annealing temperature as determined in step 4. Add 0.5 µl *Taq* DNA polymerase to reaction C, making sure the pipet tip is inserted through the layer of mineral oil into the reaction mix.

    Time is also an important factor in this step. If the temperature drops below the annealing temperature and is allowed to remain low, nonspecific annealing will occur. Taq DNA polymerase retains some activity even at room temperature.

12. Begin amplification of all four reactions at once, using the same cycling parameters as before.

13. Analyze the PCR products on an agarose gel and evaluate the results as in steps 5 and 6.

14. Prepare a batch of the optimized reaction mixture, but omit *Taq* DNA polymerase, TaqStart antibody, PMPE, and 4dNTP mix—these ingredients should be added fresh just prior to use. If desired, add Ficoll 400 to a final concentration of 0.5% to 1% (v/v) and tartrazine to a final concentration of 1 mM.

    Adding Ficoll 400 and tartrazine dye to the reaction mix precludes the need for a gel loading buffer and permits direct application of PCR products to agarose or acrylamide gels. At these concentrations, Ficoll 400 and tartrazine do not decrease PCR efficiency and do not interfere with PMPE or TaqStart antibodies. Other dyes, such as bromphenol blue and xylene cyanol, do inhibit PCR. Tartrazine is a yellow dye and is not as easily visualized as other dyes; this may make gel loading more difficult.

    Ficoll 400 and tartrazine dye may be prepared as 10× stocks and stored indefinitely at room temperature.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Enhancer agents

For a discussion of how to select enhancer agents, see Commentary.

5× stocks:
25% acetamide (20 µl/reaction; 5% final)
5 M *N,N,N*-trimethylglycine (betaine; 20 µl/reaction; 1 M final)
40% polyethylene glycol (PEG) 8000 (20 µl/reaction; 8% final)

10× stocks:
Glycerol (concentrated; 10 µl/reaction; 10% final)

continued

20× stocks:

Dimethylsulfoxide (DMSO; concentrated; 5 µl/reaction; 5% final)
Formamide (concentrated; 5 µl/reaction; 5% final)

100× stocks:

1 U/µl Perfect Match Polymerase Enhancer [Strategene; 1 µl (1 U) per reaction, final]
10 mg/ml acetylated bovine serum albumin (BSA) or gelatin (1 µl/reaction; 10 µg/ml final)
1 to 5 U/µl thermostable pyrophosphatase [PPase; Boehringer Mannheim; 1 µl (1 to 5 U) per reaction, final]
5 M tetramethylammonium chloride (TMAC; betaine hydrochloride; 1 µl/reaction; 50 mM final)
0.5 mg/ml *E. coli* single-stranded DNA-binding protein (SSB; Sigma; 1 µl/reaction; 5 µg/ml final)
0.5 mg/ml Gene 32 protein (Amersham Pharmacia Biotech; 1 µl/reaction; 5 µg/ml final)
10% Tween 20, Triton X-100, or Nonidet P-40 (1 µl/reaction; 0.1% final)
1 M $(NH_4)_2SO_4$ (1 µl/reaction; 10 mM final; use with thermostable DNA polymerases other than *Taq*)

$MgCl_2$-free PCR amplification buffer, 10×

500 mM KCl
100 mM Tris·Cl, pH 9.0 (at 25°C; see *APPENDIX 2A*)
0.1% Triton X-100
Store indefinitely at −20°C

This buffer can be obtained from Promega; it is supplied with Taq DNA polymerase.

4dNTP mix

For 2 mM 4dNTP mix: Prepare 2 mM each dNTP in TE buffer, pH 7.5 (*APPENDIX 2A*). Store up to 1 year at −20°C in 1-ml aliquots.

For 25 mM 4dNTP mix: Combine equal volumes of 100 mM dNTPs (Promega). Store indefinitely at −20°C in 1-ml aliquots.

COMMENTARY

Background Information

The theoretical basis of the polymerase chain reaction (PCR; see chapter introduction) was probably first described in a paper by Kleppe et al. (1971). However, this technique did not excite general interest until the mid-1980s, when Kary Mullis and co-workers at Cetus developed PCR into a technique that could be used to generate large amounts of single-copy genes from genomic DNA (Saiki et al., 1985, 1986; Mullis et al., 1986; Embury et al., 1987).

The initial procedure entailed adding a fresh aliquot of the Klenow fragment of *E. coli* DNA polymerase I during each cycle because this enzyme was inactivated during the subsequent denaturation step. The introduction of thermostable *Taq* DNA polymerase from *Thermus aquaticus* (Saiki et al., 1988) alleviated this tedium and facilitated automation of the thermal cycling portion of the procedure. *Taq* DNA polymerase also permitted the use of higher temperatures for annealing and extension, which improved the stringency of primer–template hybridization and thus the specificity of the products. This also served to increase the yield of the desired product.

All applications of PCR depend upon an optimized PCR. The basic protocol in this unit optimizes PCR for several variables, including $MgCl_2$ concentration, enhancing additives—dimethyl sulfoxide (DMSO), glycerol, or Perfect Match Polymerase Enchancer (PMPE)—and prevention of pre-PCR mispriming. These and other parameters can be extremely important, as every element of PCR can affect the outcome; see Critical Parameters and Trou-

bleshooting for discussion of individual parameters.

There are several PCR optimization kits and proprietary enhancers on the market (Table A.3F.3). Optimization kits generally provide a panel of buffers in which the pH, buffer, nonionic detergents, and addition of $(NH_4)_2SO_4$ are varied, $MgCl_2$ may be added at several concentrations, and enhancers (e.g., DMSO, glycerol, formamide, betaine, and/or proprietary compounds) may be chosen. The protocol presented here is aimed at keeping the costs low and the options broad.

Critical Parameters and Troubleshooting

$MgCl_2$ concentration

Determining the optimum $MgCl_2$ concentration, which can vary even for different primers from the same region of a given template (Saiki, 1989), can have an enormous influence on PCR success. In this protocol three test concentrations are suggested—1.5 mM (L), 3.0 mM (M), and 4.5 mM (H). If further optimization is necessary, the $MgCl_2$ range can be extended or narrowed around the most successful concentration.

A 10× buffer optimized for a given enzyme and a separate vial of $MgCl_2$ are typically provided with the polymerase, so that the user may titrate the $MgCl_2$ concentration for their unique primer-template set. Note that some enhancers may broaden the $MgCl_2$ optimal range.

Reagent purity

For applications that amplify rare templates, reagent purity is the most important parameter, and avoiding contamination at every step is critical.

To maintain purity, store multiple small volumes of each reagent in screw-cap tubes.

For many applications, simply using high-quality reagents and avoiding nuclease contamination is sufficient. However, avoid one common reagent used to inactivate nucleases—diethylpyrocarbonate (DEPC). Even the tiny amounts of chemical left after treatment of water and autoclaving are enough to ruin a PCR.

Primer selection

This is the factor that is least predictable and most difficult to troubleshoot. Simply put, some primers just do not work. To maximize the probability that a given primer pair will work, pay attention to the following parameters.

General considerations. An optimal primer set should hybridize efficiently to the sequence of interest with negligible hybridization to other sequences present in the sample. If there are reasonable amounts of template available, hybridization specificity can be tested by performing oligonucleotide hybridization. The distance between the primers is rather flexible, ranging up to 10 kb. There is, however, a considerable drop-off in synthesis efficiency with distances >3 kb (Jeffreys et al., 1988). Small distances between primers, however, lessen the ability to obtain much sequence information or to reamplify with nested internal oligonucleotides, should that be necessary.

Design primers to allow demonstration of the specificity of the PCR product. Be sure that there are diagnostic restriction endonuclease sites between the primers or that an oligonucleotide can detect the PCR product specifically by hybridization.

Several computer programs can assist in primer design. These are most useful for avoiding primer sets with intra- and intermolecular complementarity, which can dramatically raise the effective T_m. Given the abundance of primers relative to template, this can preclude template priming. Computer primer design is not foolproof. If possible, start with a primer or primer set known to efficiently prime extensions. In addition, manufacturers' Web sites offer technical help with primer design.

Complementarity to template. For many applications, primers are designed to be exactly complementary to the template. For others, however, such as engineering of mutations or new restriction endonuclease sites, or for efforts to clone or detect gene homologs where sequence information is lacking, base-pair mismatches will be intentionally or unavoidably created. It is best to have mismatches (e.g., in a restriction endonuclease linker) at the 5′ end of the primer. The closer a mismatch is to the 3′ end of the primer, the more likely it is to prevent extension. If cloned template is available, primers can be checked for suitability by using them in a sequencing reaction with *Taq* DNA polymerase.

The use of degenerate oligonucleotide primers to clone genes where only protein sequence is available, or to fish out gene homologs in other species, has sometimes been successful—but it has also failed an untold (and unpublished) number of times. When the reaction works it can be extremely valuable, but it can also generate seemingly specific products that require much labor to identify and yield no

Table A.3F.3 PCR Optimization Products

Optimization goal	Supplier	Product
Optimization support	ABI-Perkin-Elmer	Technical information in appendix to catalog
Optimization support	Promega	PCR troubleshooting program on the Internet: *http://www.promega.com/amplification/assistant*
Optimization kits	Boehringer-Mannheim, Invitrogen, Stratagene, Sigma, Epicentre Technologies, Life Technologies	Several buffers, Mg^{2+}, and enhancers which may include DMSO, glycerol, formamide, $(NH_4)_2SO_4$, and other unspecified or proprietary agents
Quick startup	Amersham Pharmacia Biotech	Ready-To-Go Beads "optimized for standard PCR" and Ready-To-Go RAPD Analysis Beads (buffer, nucleotides, *Taq* DNA polymerase)
Quick startup	Fisher	EasyStart PCR Mix-in-a-Tube—tubes prepackaged with wax beads containing buffer, $MgCl_2$, nucleotides, *Taq* DNA polymerase
Quick startup	Life Technologies	PCR SuperMix—1.1× conc.—premix containing buffer, $MgCl_2$, nucleotides, *Taq* DNA polymerase
Quick startup	Marsh Biomedical	Advanced Biochemicals Red Hot DNA Polymerase—a new rival for *Taq* polymerase with convenience features
Hot-start/physical barrier	Fisher, Life Technologies	Molecular Bio-Products HotStart Storage and Reaction Tubes—preadhered wax bead in each tube; requires manual addition of one component at high temperature
Hot-start/separate $MgCl_2$	Invitrogen	HotWax Mg^{2+} beads—wax beads contain preformulated $MgCl_2$ which is released at first elevated-temperature step
Hot-start/separate $MgCl_2$	Stratagene	StrataSphere Magnesium Wax Beads—wax beads containing preformulated Mg^{2+}
Hot Start/separate polymerase	Promega	TaqBead Hot Start Polymerase—wax beads encapsulating *Taq* DNA polymerase which is released at first elevated-temperature step
Hot-start/reversible inactivation of polymerase by antibody binding	Clontech	TaqStart Antibody, TthStart Antibody—reversibly inactivate *Taq* and *Tth* DNA polymerases until first denaturation at 95°C
Hot-start/antibody binding	Life Technologies	PlatinumTaq—contains PlatinumTaq antibody
Hot-start/antibody binding	Sigma	JumpStart Taq—contains TaqStart antibody
Hot-start/reversible chemical modification	ABI-Perkin-Elmer	AmpliTaq Gold—activated at high temperature
Hot-start/reversible chemical modification	Qiagen	HotStarTaq DNA Polymerase—activated at high temperature
Enhancer	Boehringer Mannheim, New England Biolabs	*Tth* pyrophosphatase, thermostable
Enhancer	Clontech	GC-Melt (in Advantage-GC Kits)—proprietary
Enhancer	CPG	Taq-FORCE Amplification System and MIGHTY Buffer—proprietary
Enhancer	Fisher	Eppendorf MasterTaq Kit with TaqMaster Enhancer—proprietary
Enhancer	Life Technologies	PCRx Enhancer System—proprietary
Enhancer	Promega	*E.coli* Single Stranded Binding Protein (SSB)
Enhancer	Qiagen	Q-Solution—proprietary
Enhancer	Stratagene	Perfect Match Polymerase Enhancer—proprietary
Enhancer	Stratagene	TaqExtender PCR Additive—proprietary

useful information. The less degenerate the oligonucleotides, especially at the 3′ end, the better. Caveat emptor.

Primer length. A primer should be 20 to 30 bases in length. It is unlikely that longer primers will help increase specificity significantly.

Primer sequence. Design primers with a GC content similar to that of the template. Avoid primers with unusual sequence distributions, such as stretches of polypurines or polypyrimidines as their secondary structure can be disastrous. It is worthwhile to check for potential secondary structure using one of the appropriate computer programs that are available.

"Primer-dimers." Primer-dimers are a common artifact most frequently observed when small amounts of template are taken through many amplification cycles. They form when the 3′ end of one primer anneals to the 3′ end of the other primer, and polymerase then extends each primer to the end of the other. The ensuing product can compete very effectively against the PCR product of interest. Primer-dimers can best be avoided by using primers without complementarity, especially in their 3′ ends. Should they occur, optimizing the $MgCl_2$ concentration may minimize their abundance relative to that of the product of interest.

Template

Aside from standard methods for preparing DNA (*APPENDIX 3*), a number of simple and rapid procedures have been developed for particular tissues (Higuchi, 1989). Even relatively degraded DNA preparations can serve as useful templates for generation of moderate-sized PCR products. The two main concerns regarding template are purity and amount.

A number of contaminants found in DNA preparations can decrease the efficiency of PCR. These include urea, the detergent SDS (whose inhibitory action can be reversed by nonionic detergents), sodium acetate, and, sometimes, components carried over in purifying DNA from agarose gels (Gelfand, 1989; Gyllensten, 1989; K. Hicks and D. Coen, unpub. observ.). Additional organic extractions, ethanol precipitation from 2.5 M ammonium acetate, and/or gel purification on polyacrylamide rather than agarose, can all be beneficial in minimizing such contamination if the simplest method (precipitating the sample with ethanol and repeatedly washing the pellet with 70% ethanol) is not sufficient.

Clearly the amount of template must be sufficient to be able to visualize PCR products using ethidium bromide. Usually 100 ng of genomic DNA is sufficient to detect a PCR product from a single-copy mammalian gene. Using too much template is not advisable when optimizing for $MgCl_2$ or other parameters, as it may obscure differences in amplification efficiency. Moreover, too much template may decrease efficiency due to contaminants in the DNA preparation.

Amount of template, especially in terms of the amount of target sequence versus nonspecific sequences, can have a major effect on the yield of nonspecific products. With less target sequence, it is more likely that nonspecific products will be seen. For some applications, such as certain DNA sequencing protocols where it is important to have a single product, gel purification of the specific PCR product and reamplification are advisable.

Taq and other thermostable DNA polymerases

Among the advantages conferred by the thermostability of *Taq* DNA polymerase is its ability to withstand the repeated heating and cooling inherent in PCR and to synthesize DNA at high temperatures that melt out mismatched primers and regions of local secondary structure. The enzyme, however, is not infinitely resistant to heat, and for greatest efficiency it should not be put through unnecessary denaturation steps. Indeed, some protocols (e.g., and the "hot start" method described here) recommend adding it after the first denaturation step.

Increasing the amount of *Taq* DNA polymerase beyond 2.5 U/reaction can sometimes increase PCR efficiency, but only up to a point. Adding more enzyme can sometimes increase the yield of nonspecific PCR products at the expense of the product of interest. Moreover, *Taq* DNA polymerase is not inexpensive.

A very important property of *Taq* DNA polymerase is its error rate, which was initially estimated at 2×10^{-4} nucleotides/cycle (Saiki et al., 1988). The purified enzyme supplied by manufacturers lacks a proofreading 3′→5′ exonuclease activity, which lowers error rates of other polymerases such as the Klenow fragment of *E. coli* DNA polymerase I. For many applications, this does not present any difficulties. However, for sequencing clones derived from PCR, or when starting with very few templates, this can lead to major problems. Direct sequencing of PCR products, sequencing numerous PCR-generated clones, and/or the use of appropriate negative controls can help overcome these problems. Alternatively, changing reaction conditions (Eckert and

Kunkel, 1990) or changing to a non–*Taq* DNA polymerase (with greater fidelity) may be useful.

Another important property of *Taq* DNA polymerase is its propensity for adding nontemplated nucleotides to the 3′ ends of DNA chains. This can be especially problematic in cloning PCR products. It is frequently necessary to "polish" PCR products with enzymes such as other DNA polymerases before adding linkers or proceeding to blunt-end cloning. Conversely, addition of a nontemplated A by *Taq* DNA polymerase can be advantageous in cloning.

Table A.3F.4 lists currently available thermostable DNA polymerases by generic and trade names, the original source of native and recombinant enzymes, the supplier, the end generated (3′A addition versus blunt), and associated exonuclease activities. A 3′ to 5′ exonuclease activity is proofreading. Removal of the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase (N-terminal deletion) is reported to produce a higher yield. A 5′ to 3′ exonuclease activity may degrade the primers somewhat. Proofreading enzymes synthesize DNA with higher fidelity and can generate longer products than *Taq*, but tend to generate low yields. Enzyme blends (Table A.3F.5) have been optimized for increased fidelity and length along with sensitivity and yield.

Hot start

What happens prior to thermal cycling is critical to the success of PCR. *Taq* DNA polymerase retains some activity even at room temperature. Therefore, under nonstringent annealing conditions, such as at room temperature, products can be generated from annealing of primers to target DNA at locations of low complementarity or having complementarity of just a few nucleotides at the 3′ ends. The latter would in effect create new templates "tagged" with the primer sequences. Subsequent cycles amplify these tagged sequences in abundance, both generating nonspecific products and possibly reducing amplification efficiency of specific products by competition for substrates or polymerase. Thus conditions preventing polymerization prior to the first temperature-controlled steps are desirable. In this protocol, three methods of inhibiting polymerization prior to the temperature-controlled step are compared. These include physical separation of an essential reaction component prior to the first denaturation step, cooling reagents to 0°C, and reversibly blocking enzymatic activity with an antibody.

Denaturation of the template before *Taq* polymerase or $MgCl_2$ is added to the reaction provides a dramatic improvement in specificity and sensitivity in many cases (Chou et al., 1992). The main drawback of this method is that it requires opening the reaction tubes a second time to add the essential missing component. This creates both an inconvenience and an increase in the risk of contamination, an important consideration when testing for the presence of a given sequence in experimental or clinical samples.

Cooling all components of the reaction mixture to 0°C prior to mixing is more convenient and the least expensive method but is also the least reliable. Transferring the PCR reaction tubes from the ice slurry to a 95°C preheated thermocycler block may improve the chance of success.

Reversible inhibition of *Taq* DNA polymerase by TaqStart antibody (Clontech) is the most convenient and very effective (Kellogg et al., 1994). Complete reactions can be set up, overlaid with oil, and stored at 4°C for up to several hours prior to thermal cycling with no loss of sensitivity or specificity compared to the other hot start methods (M.F.K. and D.M.C., unpub. observ.). Cycling is initiated immediately following 5-min denaturation of the antibody at 94°C. DMSO inhibits antibody binding and should not be used with TaqStart.

Several hot-start products are now commercially available (Table A.3F.3). Success with each may depend on strict adherence to the manufacturer's protocols, even on a specific thermocycler. Wax barrier and reversible antibody binding methods are more forgiving, while chemical modifications have more stringent activation temperature requirements.

Deoxyribonucleoside triphosphates

In an effort to increase efficiency of PCR, it may be tempting to increase the concentration of dNTPs. Don't. When each dNTP is 200 μM, there is enough to synthesize 12.5 μg of DNA when half the dNTPs are incorporated. dNTPs chelate magnesium and thereby change the effective optimal magnesium concentration. Moreover, dNTP concentrations >200 μM each increase the error rate of the polymerase. Millimolar concentrations of dNTPs actually inhibit *Taq* DNA polymerase (Gelfand, 1989).

The protocol in this unit calls for preparing 4dNTPs in 10 mM Tris·Cl/1 mM EDTA (TE buffer), pH 7.4 to 7.5. This is easier and less

Table A.3F.4 Thermostable DNA Polymerases

DNA polymerase		Biological source	Supplier	Product ends	Exonuclease activity
Generic name	Trade name				
Pfu	—	*Pyrococcus furiosus*	Stratagene, Promega	Blunt	3′-5′ (proofreading)
Pfu (exo-)	—	*Pyrococcus furiosus*	Stratagene	Blunt	No
Psp	Deep Vent	*Pyrococcus* sp.GB-D	New England Biolabs	Blunt	3′-5′ (proofreading)
Psp (exo-)	Deep Vent (exo-)	*Pyrococcus* sp.GB-D	New England Biolabs	Blunt	No
Pwo		Pyrococcus woesei	Boehringer Mannheim	Blunt	3′-5′ (proofreading)
Taq (native and/or recombinant)	—	*Thermus aquaticus*	Ambion, Amersham Pharmacia Biotech, Boehringer Mannheim, Clontech, Fisher, Life Technologies, Marsh Biomedical, ABI-Perkin Elmer, Promega, Qiagen, Sigma, Stratagene	3′A	5′-3′
Taq, N-terminal deletion	Stoffel fragment Klen-Taq	*Thermus aquaticus*	ABI-Perkin-Elmer, Sigma	3′A	No
Tbr	DyNAzyme	*Thermus brocianus*	MJ Research	—[a]	5′-3′
Tfl		*Thermus flavus*	Promega, Epicentre Technologies	Blunt	—[a]
Tli	Vent	*Thermococcus litoralis*	New England Biolabs (Vent), Promega	Blunt	3′-5′ (proofreading)
Tli (exo-)	Vent (exo-)	*Thermococcus litoralis*	New England Biolabs	Blunt	No
Tma	UlTma	*Thermotoga maritima*	ABI-Perkin-Elmer	Blunt	3′-5′ (proofreading)
Tth	—	*Thermus thermophilus*	Amersham Pharmacia Biotech, Boehringer Mannheim, Epicentre Technologies, ABI-Perkin Elmer, Promega	3′ A	5′-3′

[a]No information at this time.

prone to disaster than neutralization with sodium hydroxide. However, EDTA also chelates magnesium, and this should be taken into account if stocks of dNTPs are changed. Alternatively, to lower the risk of contamination, a 4dNTP mix can be made by combining equal volumes of commercially prepared stocks.

Enhancers

Enhancers are used to increase yield and specificity and to overcome difficulties encountered with high GC content or long templates. Nonionic detergents (Triton X-100, Tween 20, or Nonidet P-40) neutralize charges of ionic detergents often used in template preparation, and should be used in the basic reaction mixture, rather than as optional enhancers. Higher yields can be achieved by stabilizing/enhancing the polymerase activity with enzyme-stabilizing proteins (BSA or gelatin), enzyme-stabilizing solutes such as betaine or betaine·HCl (TMAC), enzyme-stabilizing solvents (glycerol), solubility-enhancing solvents (DMSO or acetamide), molecular crowding solvents (PEG), and polymerase salt preferences [$(NH_4)SO_4$ is recommended for polymerases other than *Taq*]. Greater specificity can be achieved by lowering the T_M of dsDNA (using formamide), destabilizing mismatched-primer annealing (using PMPE or hot-start strategies), and stabilizing ssDNA (using *E. coli* SSB or Gene 32 Protein). Amplification of high-GC-content templates can be improved by decreasing the base pair composition depend-

Table A.3F.5 Thermostable DNA Polymerase Blends

Product (trade name)	Supplier	Thermostable DNA polymerases and other components
Expand High Fidelity, Expand Long Template, and Expand 20kb PCR Systems	Boehringer Mannheim	*Taq* + *Pwo*
KlenTaq LA Polymerase Mix	Clontech, Sigma	KlenTaq-1 (5′-exonuclease deficient *Taq*) + unspecified proofreading polymerase
Advantage-HF PCR Kit	Clontech	KlenTaq-1 + unspecified proofreading polymerase + TaqStart Antibody
Advantage-cDNA and Advantage-GC cDNA Polymerase Mixes and Kits	Clontech	KlenTaq-1 + unspecified proofreading polymerase + TaqStart Antibody; GC Kit contains GC Melt
Advantage Genomic and Advantage-GC Genomic Polymerase Mixes and Kits	Clontech	*Tth* + unspecified proofreading polymerase + TthStart Antibody; GC Kit contains GC Melt
eLONGase Enzyme Mix	Life Technologies	*Taq* + *Psp* + unspecified proofreading polymerase(s) + eLONGase Buffer
Platinum Taq DNA Polymerase	Life Technologies	*Taq* + *Psp* + Platinum *Taq* Antibody
Platinum High Fidelity DNA Polymerase	Life Technologies	*Taq* + *Psp* + *Taq* Antibody
DyNAzyme EXT Polymerase	MJ Research	*Tbr* with unspecified enhancer
GeneAmp XL PCR and XL RNA PCR Kits	ABI-Perkin-Elmer	*Tth* + *Tli*
OmniBase Sequencing Enzyme Mix	Promega	Unspecified proofreading polymerase(s) with thermostable pyrophosphatase
AccuTaq LA DNA Polymerase Mix	Sigma	*Taq* + unspecified proofreading polymerase
TaqPlus Long and TaqPlus Precision PCR Systems	Stratagene	*Pfu* + *Taq*; TaqPlus Precision Reaction Buffer (proprietary)
Accurase Fidelity PCR Enzyme Mix; Calypso High Fidelity Single Tube RT-PCR System	Tetralink	*Thermus sp*. + *Thermococcus sp*.; Calypso also contains AMV-RT

ence of the T_M of dsDNA (with betaine; Rees et al., 1993). Betaine is an osmolyte widely distributed in plants and animals and is nontoxic, a feature that recommends it for convenience in handling, storage, and disposal. Betaine may be the proprietary ingredient in various commercial formulations. For long templates, a higher pH is recommended (pH 9.0). The pH of Tris buffer decreases at high temperatures, long-template PCR requires more time at high temperatures, and increased time at lower pH may cause some depurination of the template, resulting in reduced yield of specific product. Inorganic phosphate (PPi), a product of DNA synthesis, may accumulate with amplification of long products to levels that may favor reversal of polymerization. Accumulation of PPi may be prevented by addition of thermostable PPase. When large numbers of samples are being analyzed, the convenience of adding PCR products directly to a gel represents a significant time savings. Some companies combine their thermostable polymerase with a red dye and a high density component to facilitate loading of reaction products onto gels without further addition of loading buffer.

Thermal cycling parameters

Each step in the cycle requires a minimal amount of time to be effective, while too much time can be both wasteful and deleterious to the DNA polymerase. If the amount of time in each step can be reduced, so much the better.

Denaturation. It is critical that complete strand separation occur during the denaturation step. This is a unimolecular reaction which, in itself, is very fast. The suggested 30-sec denaturation used in the protocol ensures that the tube contents reach 94°C. If PCR is not working, it is well worth checking the temperature inside a control tube containing 100 µl water. If GC

content is extremely high, higher denaturation temperatures may be necessary; however, *Taq* DNA polymerase activity falls off quickly at higher temperatures (Gelfand, 1989). To amplify a long sequence (>3 kb), minimize the denaturation time to protect the target DNA from possible effects, such as depurination, of lowered pH of the Tris buffer at elevated temperatures.

Annealing. It is critical that the primers anneal stably to the template. Primers with relatively low GC content (<50%) may require temperatures lower than 55°C for full annealing. On the other hand, this may also increase the quantity of nonspecific products. For primers with high GC content, higher annealing temperatures may be necessary. It can be worthwhile, although time-consuming, to experiment with this parameter. Two manufacturers have thermal cyclers on the market which are capable of forming a temperature gradient across the heating units, thus permitting annealing temperature optimization in one run. These are Stratagene's Robocyclers, and Eppendorf's Master Cycler. As with denaturation, the time for this step is based mainly on the time it takes to reach the proper temperature, because the primers are in such excess that the annealing reaction occurs very quickly.

Extension. The extension temperature of 72°C is close to the optimal temperature for *Taq* DNA polymerase (~75°C), yet prevents the primers from falling off. Indeed, primer extension begins during annealing, because *Taq* DNA polymerase is partially active at 55°C and even lower temperatures (Gelfand, 1989).

The duration of extension depends mainly on the length of the sequence to be amplified. A duration of 1 min per kb product length is usually sufficient.

Certain protocols, including others in this chapter, end the PCR with a long final extension time in an attempt to try to make products as complete as possible.

Ramp time. Ramp time refers to the time it takes to change from one temperature to another. Using water baths and moving samples manually from temperature to temperature probably gives the shortest ramp times, which are mainly the time required for the tube's contents to change temperature. Different thermal cyclers have different ramp times; basically, the shorter the better.

The Stratagene Robocycler uses a robotic arm to move samples from one constant-temperature block to another, virtually eliminating block ramp time, but a ramp time for tube contents must be calculated (~1 sec/°C) and added to denaturation, annealing, and extension times. Rapid cyclers that utilize positive-displacement pipet tips or capillary tubes for the PCR reactions dramatically reduce the ramp times.

Generally, the more "high-performance" thermal cyclers with short ramp times are proportionally more costly. There are many new thermal cyclers on the market priced below $5000, which perform quite well (Beck, 1998).

Anticipated Results

Starting with ≥100 ng mammalian DNA (≥10^4 molecules), the basic protocol can be used to determine which $MgCl_2$ concentration, enhancing additive, and initial conditions will yield a predominant PCR product from a single-copy sequence that is readily visible on an ethidium bromide–stained gel. It is possible that other minor products will also be visible.

Time Considerations

The basic protocol can be completed in a single day. Assembly of the reaction mixtures should take ~1 hr. Cycling should take less than 3 hr. Preparing, running, and staining the gel should take another few hours. Further checks on specificity of the product such as restriction endonuclease digestion or Southern blot hybridization will take another few hours or days, respectively.

Literature Cited

Beck, S. 1998. How low can you go? Nineteen thermal cyclers priced under $5000. *The Scientist* 12:19-20.

Chou, Q., Russell, M., Birch, D.E., Raymond, J., and Bloch, W. 1992. Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. *Nucl. Acids Res.* 20:1717-1723.

Eckert, K.A. and Kunkel, T.A. 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucl. Acids Res.* 18:3739-3752.

Embury, S.H., Scharf, S.J., Saiki, R.K., Gholson, M.A., Golbus, M., Arnheim, N., and Erlich, H.A. 1987. Rapid prenatal diagnosis of sickle cell anemia by a new method of DNA analysis. *N. Engl. J. Med.* 316:656-661.

Gelfand, D.H. 1989. *Taq* DNA polymerase. *In* PCR Technology: Principles and Applications for DNA Amplification (H.A. Erlich, ed.) pp. 17-22. Stockton Press, New York.

Gyllensten, U. 1989. Direct sequencing of in vitro amplified DNA. *In* PCR Technology: Principles and Applications for DNA Amplification (H.A. Erlich, ed.) pp. 45-60. Stockton Press, New York.

Higuchi, R. 1989. Simple and rapid preparation of samples for PCR. *In* PCR Technology: Principles and Applications for DNA Amplification (H.A. Erlich, ed.) pp. 31-38. Stockton Press, New York.

Jeffreys, A.J., Wilson, V., Neumann, R., and Keyte, J. 1988. Amplification of human minisatellites by the polymerase chain reaction: Towards DNA fingerprinting of single cells. *Nucl. Acids Res.* 16:10,953-10,971.

Kellogg, D.E., Rybalkin, I., Chen, S., Mukhamedova, N., Vlasik, T., Siebert, P.D., and Chencik, A. 1994. TaqStart antibody: "Hot start" PCR facilitated by a neutralizing monoclonal antibody directed against *Taq* DNA polymerase. *BioTechniques* 16:1134-1137.

Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., and Khorana, H.G. 1971. Studies on polynucleotides. XCVI. Repair replication of short synthetic DNA's as catalyzed by DNA polymerases. *J. Mol. Biol.* 56:341-361.

Mullis, K.B., Faloona, F., Scharf, S.J., Saiki, R.K., Horn, G.T., and Erlich, H.A. 1986. Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273.

Rees, W.A., Yager, T.D., Korte, J., and von Hippel, P.H. 1993. Betaine can eliminate the base pair composition dependence of DNA melting. *Biochemistry* 32:137-144.

Saiki, R.K. 1989. The design and optimization of the PCR. *In* PCR Technology: Principles and Applications for DNA Amplification (H.A. Erlich, ed.) pp. 7-16. Stockton Press, New York.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K., Horn, G., Erlich, H.A., and Arnheim, N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350-1354.

Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1986. Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. *Nature* 324:163-166.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.

Key Reference

Saiki et al., 1988. See above.

Demonstrates the ease and power of PCR using Taq DNA polymerase.

Internet Resources

http://www.promega.com/techserve/

Offers Amplification Assistant, a PCR troubleshooting program.

http://www.genome.wi.mit.edu/

Provides access to www Primer Picking (Primer 3); select experimental web-based software under Genome Center Software.

http://www.alkami.com/primers/idxprmr.htm

Contains primer design tools and tips.

Contributed by Martha F. Kramer and Donald M. Coen
Harvard Medical School
Boston, Massachusetts

Micro RT-PCR

Newly developed technologies allow investigators to isolate specific subpopulations of disease-relevant cells from actual tissue under direct microscopic visualization (*UNIT 2.5*). This opens the opportunity to determine specific subsets of biologically-relevant mRNA and DNA that mediate the physiology of normal and diseased cells; however, these pertinent cells only represent a fraction of the total cell repertoire, often comprising only a few hundred in number. Thus, new methods have been developed to analyze the small DNA and RNA contents from these cells.

The following represents a collection of protocols for the microisolation (see Support Protocol), manipulation, and amplification of the RNA content of microdissected cells (see Basic Protocol). Even though emphasis in these protocols is given for microdissected cells, these protocols have successfully been used for bulk tissue (i.e., <10 µg) (see Internet Resources). Laser-capture microdissection (LCM) is described in *UNIT 2.5*.

MICRO RT-PCR

BASIC PROTOCOL

Following the isolation of RNA (see Support Protocol), oligo(dT)- or random hexamer–primed single-stranded cDNA is synthesized by reverse transcription. In the reaction, all four dNTPs are present in 250 µM concentrations and 10 µM random-hexamer primers or oligo(dT) are included. After first strand synthesis, the sample is directly diluted into PCR reaction buffer and amplified at 94°C for 2 min, followed by 35 cycles of 45 sec at 50°C to anneal, 2 min at 72°C to extend, and 2 min at 94°C to denature. PCR products are separated on a 2% (w/v) agarose gel and stained with ethidium bromide (*UNIT 1.5*).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1A*).

Materials

DEPC-treated H_2O (*APPENDIX 2A*)
20 U/µl RNase inhibitor (ABI-Perkin Elmer)
RNA pellet, dry (Support Protocol)
5× RT buffer (GenHunter)
250 µM dNTPs (GenHunter)
10 µM random-hexamer or oligo(dT) primers (ABI-Perkin Elmer)
100 U/µl MMLV reverse transcriptase (Stratagene)
10× PCR buffer (Perkin Elmer): 500 KCl/15 mM $MgCl_2$/1 mg/ml gelatin in 100 mM Tris·Cl, pH 8.4 (*APPENDIX 2A*)
10 µM upstream primer
10 µM downstream primer
5 U/µl *Taq* DNA polymerase (ABI-Perkin Elmer)
10 mCi/ml ^{32}P-dCTP or ^{33}P-dCTP (~300 Ci/mmol)

12-µl PCR tubes, RNase-free
Thermal cycler

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 1.5*)

CAUTION: DEPC is a suspected carcinogen and must be handled with caution.

Contributed by Cloud P. Paweletz, Lu Charboneau, and Lance A. Liotta
Current Protocols in Cell Biology (2001) A.3G.1-A.3G.6

Suspend RNA pellet

1. Add 24 µl DEPC-treated water and 1 µl RNase inhibitor to the dried RNA pellet.

   It is highly recommended to split the reaction mixture in order to determine whether the primers used for PCR could be amplifying any residual DNA—i.e., the RT(−) reaction.

2. Split suspended RNA into two 12-µl RNase-free PCR tubes, labeled RT(+) and RT(−), respectively.

For random hexamers

3a. To each tube, add 4 µl 5× RT buffer, 2 µl 250 µM dNTP, and 1 µl 10 µM random hexamers.

4a. Incubate for 5 min at 65°C and 10 min at 25°C.

5a. Add 1 µl 100 U/µl MMLV reverse transcriptase to the RT(+) tube. Add 1 µl DEPC-treated water to the RT(−) tube to maintain equal volume.

6a. *For random hexamers:* Incubate 10 min at 25°C, 40 min at 37°C, and 5 min at 95°C.

For oligo(dT)

3b. To each tube, add 4 µl 5× RT buffer, 2 µl 250 µM dNTP, and 2 µl 10 µM oligo(dT).

4b. Incubate 5 min at 65°C and 10 min at 37°C.

5b. Add 1 µl 100 U/µl MMLV reverse transcriptase to the RT(+) tube. Add 1 µl DEPC-treated water to the RT(−) tube to maintain equal volume.

6b. Incubate 50 min at 37°C and 5 min at 95°C.

Prepare and perform PCR

7. Place 1.5 µl RT(+) and RT(−) cDNA mixture into appropriately labeled RNase-free microcentrifuge tubes and add the following to each:

   1.0 µl 10× PCR buffer
   0.8 µl 250 µM dNTP mix
   0.2 µl 10 µM upstream primer
   0.2 µl 10 µM downstream primer
   0.2 µl 5 U/µl *Taq* DNA polymerase
   0.2 µl ^{32}P-dCTP or ^{33}P-dCTP
   5.9 µl DEPC-treated water.

   Heat reaction mixture for 2 min at 94°C in a thermal cycler.

   Taq DNA polymerase is usually supplied as 5 U/µl. If Taq DNA polymerase is supplied at a different dilution, dilute in PCR buffer to desired enzyme concentration.

   Radioactivity is necessary to allow visualization using a denaturing acrylamide gel. This protocol usually results in visible products in <2 hr.

8. Run 35 cycles in a thermal cycler as follows to amplify cDNA:

45 sec	94°C	(denaturation)
45 sec	50°C	(annealing)
2 min	72°C	(extension)
2 min	94°C	(denaturation)

   Hold at 4°C until use.

Analyze results

9. Separate 10 µl RT(+) and RT(−) on a 2% agarose gel (e.g., NaSieve precast gel) and stain with ethidium bromide.

NOTE: *Adjust the relative amounts of buffer and agarose in UNIT 1.5 to make a 2% agarose gel. Also see APPENDIX 3A for agarose-gel electrophoresis techniques.*

MICROISOLATION OF RNA

SUPPORT PROTOCOL

Transcriptional analysis and profiling of disease-related states depend upon the ability to isolate specific pure RNA from tissue cells. Fundamentally, the following protocol lyses microdissected cells (*UNIT 2.5*) in a guanidinium thiocyanate buffer and separates DNA and proteins by extraction with water-saturated phenol and chloroform/isoamyl alcohol. Subsequently, the extracted RNA (aqueous phase) is placed into a second RNase-free tube and precipitated with isopropyl alcohol. The RNA pellet is cleaned from contaminating cDNA by DNase treatment for 2 hr at 37°C.

Results in the authors' laboratory have shown that the Stratagene Micro RNA Isolation kit provides the best recovery of mRNA isolated for laser capture microdissected cells (*UNIT 2.5*). It also avoids the CsCl gradient step that was traditionally used to separate RNA from DNA and proteins.

Materials *(also see Basic Protocol)*

Tissue
Micro RNA Isolation kit (Stratagene):
  Denaturing solution
  2-mercaptoethanol
  H_2O-saturated phenol
  2 M sodium acetate, pH 4.0
  1 µg/µl glycogen
  Isopropanol, cold
  49:1 (v/v) chloroform/isoamyl alcohol
60% ethanol/40% DEPC-treated H_2O (*APPENDIX 2A*)
DEPC-treated H_2O (*APPENDIX 2A*)
Message Clean kit (GenHunter):
  RNase inhibitor
  10× reaction buffer
  10 U/µl mg/ml DNase
RNase Away (Molecular Bio-Products)
RNase-free tubes (Eppendorf)

Additional reagents and equipment for microdissection (*UNIT 2.5*)

CAUTION: DEPC is a suspected carcinogen and must be handled with caution.

NOTE: Because the starting material is only between 10 and 100 ng, it is of absolute necessity to use only DEPC-treated water to inactivate ribonucleases, to use only labware certified "RNase free," and to always wear gloves.

Extract RNA from cells

1. Microdissect a minimum of 1000 cells from tissue (*UNIT 2.5*) and place in an RNase-free tube.

Bulk tissue can also be used for this protocol. Typically between 10 and 100 ng RNA are required for good recovery.

The number of cells needed depends on the quality of the RNA in the tissue.

2. Lyse cells in 200 µl denaturing buffer and 1.6 µl 2-mercaptoethanol. Add lysate to RNase-free tubes and vortex vigorously.

   Observation of lysing can be confirmed by a pinkish discoloration in the lysing buffer from the H&E stained microdissected cells.

   To avoid contamination by RNases, it is recommended that all equipment be either certified RNase free or treated with RNase Away before use.

Phenol/chloroform extract RNA

3. Add 220 µl water-saturated phenol, 60 µl of 49:1 chloroform/isoamyl alcohol, and 20 µl of 2 M sodium acetate, pH 4.0 to the lysate. Vortex vigorously for 1 min. Place the tube on wet ice for 15 min followed by microcentrifugation for 30 min at 12,000 rpm, 4°C.

   The organic layer (phenol) is the bottom phase.

4. Transfer aqueous (upper) phase to a new RNase-free tube, paying special attention not to transfer any of the organic (bottom) layer.

Purify RNA by isopropanol precipitation

5. Add 2 µl of 1 µg/µl glycogen to the new tube.

   Glycogen acts as a carrier during this step. It is solely used to visualize the RNA pellet. Alternatively 2 µl of 1 µg/µl tRNA can be used.

6. Add 200 µl cold isopropanol and place on dry ice for 30 min.

7. Microcentrifuge 30 min at 12,000 rpm, 4°C.

   Visualization of the RNA pellet can be difficult. It helps to place the cap outward, which places the pellet away from the cap insertion.

8. Carefully remove supernatant by wicking.

Purify RNA by ethanol precipitation

9. Add 400 µl of 60% ethanol/40% DEPC-treated water. Microcentrifuge 5 min at 12,000 rpm, 4°C. Remove supernatant. Repeat once.

10. Dry the RNA pellet using a SpeedVac evaporator.

    The pellet can be stored at −80°C until further use.

Remove residual DNA (optional)

It is likely that RT-PCR may also amplify contaminating DNA. To ensure the purity of the RNA product, DNase treatment of the isolated RNA is highly recommended. If additional removal of the DNA is not warranted, any manipulations of the isolated RNA (i.e., steps 11 to 22), may be skipped.

11. Add 15 µl DEPC-treated water, 1 µl RNase inhibitor, and 2 µl of 10× reaction buffer.

12. Incubate 3 min at 50°C.

13. Add 2 µl of 10 U/µl DNase and incubate 2 hr at 37°C.

14. Add 22 µl water-saturated phenol, 6 µl of 49:1 chloroform/isoamyl alcohol, and 2 µl of 2 M sodium acetate, pH 4.0. Place on wet ice for 15 min.

15. Microcentrifuge 10 min at 12,000 rpm, 4°C.
16. Transfer upper layer to a new RNase-free tube.
17. Add 2 µl of 1 µg/µl glycogen to the new tube.
18. Add 200 µl cold isopropanol and place on dry ice for 30 min.
19. Microcentrifuge 30 min at 14,000 rpm, 4°C.
20. Carefully remove the supernatant by wicking.
21. Add 400 µl of 60% ethanol/40% DEPC-treated water. Microcentrifuge 5 min at 14,000 rpm, 4°C. Remove supernatant by wicking.
22. Remove any residual liquid using a SpeedVac evaporator.

Pellet can be stored overnight at –80°C.

COMMENTARY

Background Information

The development of rational approaches to the diagnosis and treatment of diseases are dependent on identifying and understanding the molecular mechanisms that underlie its progression. The understanding of cell type specific gene regulation is essential for this task; however, until recently it could not be analyzed due to the difficulty of isolating single cell types. Laser-capture microdissection (*UNIT 2.5*) allows for the direct procurement of cell populations under direct microscopic visualization (Emmert-Buck et al., 1996). RT-PCR from microdissected tissue has successfully been used for preparing cDNA libraries from specific subpopulations such as prostatic intraepithelial neoplasia (Krizman et al., 1996).

Although there are many approaches to the isolation, purification, and amplification of mRNA, the protocol presented here (see Basic Protocol) has been optimized for mRNA procured by laser-capture microdissection (*UNIT 2.5*). Following the isolation of RNA, oligo(dT)-primed or random hexamer–primed single-strand cDNA is synthesized by reverse transcription. All four dNTP's are present in 250 µM concentrations with 10 µM random-hexamer primers or oligo(dT). After first-strand synthesis the sample is directly diluted into the PCR reaction buffer and amplified at 94°C for 2 min, followed by 35 cycles of 45 sec at 50°C to anneal, 2 min at 72°C to extend, and 2 min at 94°C to denature. PCR products are separated on a 2% (w/v) agarose gel and stained with ethidium bromide (*UNIT 1.5*). The first application of PCR to the analysis of mRNA studied point mutations within the mouse ornithine transcarbamylase gene (Veres et al., 1987).

The denaturing buffer, which contains guanidinium isothiocyanate and mild detergents, lyses the microdissected cells instantaneously and inactivates ribonucleases. DNA and proteins are subsequently separated by extraction with water-saturated phenol and chloroform/isoamyl alcohol. The extracted RNA (aqueous phase) is placed into a second tube and precipitated with isopropyl alcohol.

The use of guanadinium isothiocyanate was first described for the purification of RNA in endogenous ribonucleases (Ullrich et al., 1977; Chirgwin et al., 1979). Organic extraction of proteins by phenol has been previously described by Kirby (1968). An excellent review and updated methods to recover and analyze mRNA from microdissected tissue samples are on the Cancer Genome Anatomy's (CGAP) website at *http://cgap-mf.nih.gov*.

Critical Parameters and Troubleshooting

The quality of the RNA in the tissue affects the number of cells required for the assay. It requires only a minute amount of DNA to provide a false positive signal. The RT(–) tube acts as a negative control and is designed to show any contaminating DNA products in this reaction mixture. If there is a positive band resulting from the RT(–) reaction mixture, there is genomic DNA contamination. Make sure the integrity of the mRNA is valid. A thorough DNase treatment of the sample might be necessary (see Support Protocol).

Any success of RNA isolation from minute tissue sample depends on the complete elimination of ribonucleases. The denaturing agent used herein is designed to eliminate any RNase activity while liberating the cell lysate. Likely sources of poor yields are probable introduction of foreign RNases into the reaction mixture. Contaminating RNases may be introduced either by using contaminated labware or from the user. If general labware such as microcentrifuge tubes or disposable plastic pipets are not certified "RNase free", glassware can be baked at 180°C for 8 hr, while plasticware can be decontaminated by rinsing it with chloroform and subsequently autoclaving it. Disposable gloves should be worn throughout the entire procedure.

Anticipated Results

Amplification of mRNA should only produce one discreet band of discrete length when stained with ethidium bromide on an agarose gel.

Between 10 and 100 ng of RNA should be isolated using 1000 microdissected cells.

Time Considerations

Synthesis of single-stranded cDNA takes 1.5 hr, while amplification by RT-PCR using a thermal cycler for 35 cycles takes ~2.5 hr. Visualization on a NuSieve 2% agarose gel takes ~1 hr.

Allow ~3 hr until the first ethanol precipitation of the RNA pellet. If additional DNA removal is desired allow an extra 5 hr for the DNase treatment and further purification of RNA. The RNA pellet can be stored until further use at −80°C after any of the ethanol precipitation steps.

Literature Cited

Chirgwin, J.J., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.

Emmert-Buck, M.R., Bonner, R.F., Smith, P.D., Chaqui, R.F., Zhuang, Z., Goldstein, S.R., Weiss, R.A., and Liotta, L.A. 1996. Laser capture microdissection. *Science* 274:998-1001.

Kirby, K.S. 1968. Isolation of nucleic acids, with phenolic solvents. *Methods Enzymol.* 12B:87-98.

Krizman, D.B., Chuaqui, R.F., Meltzer, P.S., Trent, J.M., Duray, P.H., Lineham, W.M., Liotta, L.A., and Emmert-Buck, M.R. 1996. Construction of a representative cDNA library from prostatic intraepithelial neoplasia. *Cancer Res.* 56:5380-5381.

Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W.J., and Goodman, H.M. 1977. Rat insulin genes: Construction of plasmids containing the coding sequence. *Science* 196:1313-1319.

Veres, G., Gibbs, R.A., Scherer, S.E., and Caskey, C.T. 1987. The molecular basis of the sparse fur mouse mutation. *Science* 237:415-417.

Internet Resources

http://cgap-mf.nih.gov/protocols

An excellent source for recovery and analysis of RNA from microdissected cells.

Contributed by Cloud P. Paweletz,
Lu Charboneau, and Lance A. Liotta
National Cancer Institute/NIH
Bethesda, Maryland

The Colorimetric Detection and Quantitation of Total Protein

Protein quantification is an important step for handling protein samples for isolation and characterization, and is a prerequisite step before submitting proteins for chromatographic, electrophoretic, or immunochemical analysis and separation. The methods included in this unit are colorimetric measurements, whose procedures are fast and simple.

This unit describes four of the most commonly used total protein assay methods. Three of the four are copper-based assays to quantitate total protein: the Lowry method (see Basic Protocol 1 and Alternate Protocols 1 and 2), the bicinchoninic acid assay (BCA; see Basic Protocol 2 and Alternate Protocols 3 and 4), and the biuret method (see Basic Protocol 3 and Alternate Protocol 5). The fourth is the Coomassie dye binding or Bradford assay (see Basic Protocol 4 and Alternate Protocols 6 and 7), which is included as a simple and sensitive assay, although it sometimes gives a variable response depending on how well or how poorly the protein binds the dye in acidic pH. A protein assay method should be chosen based on the sensitivity and accuracy of method as well as the condition of the sample to be analyzed.

STRATEGIC PLANNING

Colorimetric Protein Assays

The four colorimetric methods for the detection and quantitation presented in this unit have withstood the test of time. They are all well-characterized robust assays that consistently work well. The methods were introduced over the past 15 to 50 years. They collectively represent the state of the art for colorimetric detection and quantitation of total proteins in the microgram to milligram range.

When confronted with the need to determine the total protein concentration of a sample, one of the first issues to consider is selection of a protein assay method. The choice among the available protein assays usually is made based upon consideration of the compatibility of the method with the samples to be assayed. The objective is to select a method that requires the least manipulation or pretreatment of the samples due to the presence of substances that may interfere. If the total protein concentration in the samples is high (i.e., in the range of 5 to 160 mg/ml), the biuret total protein reagent is the best choice. If the total protein concentration in the samples is low (i.e., in the range of 1 to 2000 μg/ml), then any one of the other three (i.e., the Lowry, the Coomassie Plus, or the BCA method) would be suitable. If the sample contains reducing agents or copper-chelating reagents, the Coomassie Plus Protein Assay Reagent (Pierce) would be the best choice. If the sample contains one or more detergents (at concentrations up to 5%), the BCA Protein Assay Reagent is the best choice.

Sometimes the sample contains substances that make it incompatible with any of the protein assay methods. In those cases, some pretreatment of the sample is necessary.

Each method has its advantages and disadvantages. No one method can be considered to be the ideal or best protein assay method. Because of this, most researchers keep more than one type of protein assay reagent available in their laboratory.

Contributed by Randall I. Krohn
Current Protocols in Cell Biology (2002) A.3H.1-A.3H.28

Selection of the Protein Standard

The selection of a protein standard is potentially *the* greatest source of error in any protein assay. Of course, the best choice for a standard is a highly purified version of the predominant protein found in the samples. This is not always possible nor always necessary. In some cases, all that is needed is a rough estimate of the total protein concentration in the sample. For example, in the early stages of purifying a protein, identifying which fractions contain the most protein may be all that is required. If a highly purified version of the protein of interest is not available or it is too expensive to use as the standard, the alternative is to choose a protein that will produce a very similar color response curve with the selected protein assay method.

For general protein assay work, bovine serum albumin (BSA) works well as the choice for a protein standard, because it is widely available in high purity and relatively inexpensive. Although it is a mixture containing several immunoglobulins, bovine gamma globulin (BGG) is also a good choice for a standard when determining the concentration of antibodies, since BGG produces a color response curve that is very similar to that of immunoglobulin G (IgG).

For greatest accuracy of the estimates of the total protein concentration in unknown samples, it is essential to include a standard curve in each run. This is particularly true for the protein assay methods that produce nonlinear standard curves (e.g., Lowry method, Coomassie dye–binding method). The decision about the number of standards used to define the standard curve and the number of replicates to be done on each standard depends upon the degree of nonlinearity in the standard curve and the degree of accuracy required of the results. In general, fewer points are needed to construct a standard curve if the color response curve is linear. For assays done in test tubes, duplicates are sufficient; however, triplicates are recommended for assays performed in microtiter plates due to the increased error associated with microtiter plates and microtiter plate readers.

Preparation of the Samples

Before a sample can be analyzed for total protein content, it must be solubilized, usually in a buffered aqueous solution. The entire process is usually done in the cold, with additional precautions taken to inhibit microbial growth or to avoid casual contamination of the sample by foreign debris such as hair, skin, or body oils. When working with tissues, cells, or solids, the first step of the solubilization process is usually disruption of the sample's cellular structure by grinding and/or sonication, or by the use of specially designed reagents containing surfactants to lyse the cells (i.e., the "POPPERS" line of products, available from Pierce). This is done in a cold aqueous buffer containing one or more surfactants (to aid the solubilization of the membrane-bound proteins), one or more biocides (to prevent microbial growth), and protease inhibitors (to minimize or prevent digestion of the proteins into peptide fragments by endogenous proteases). After filtration or centrifugation (to remove the cellular debris), additional steps such as sterile filtration, removal of lipids, or further purification of the protein of interest from the other sample components may be necessary.

Calculation of the Results

If calculating the protein concentrations manually, it is best to use point-to-point interpolation. This is especially true if the standard curve is nonlinear. Point-to-point interpolation refers to a method of calculating the results for each sample using the equation for a linear regression line obtained from just two points on the standard curve. The first point is the standard that has an absorbance just below that of the sample and the second point is the standard that has an absorbance just above that of the sample. In this way, the

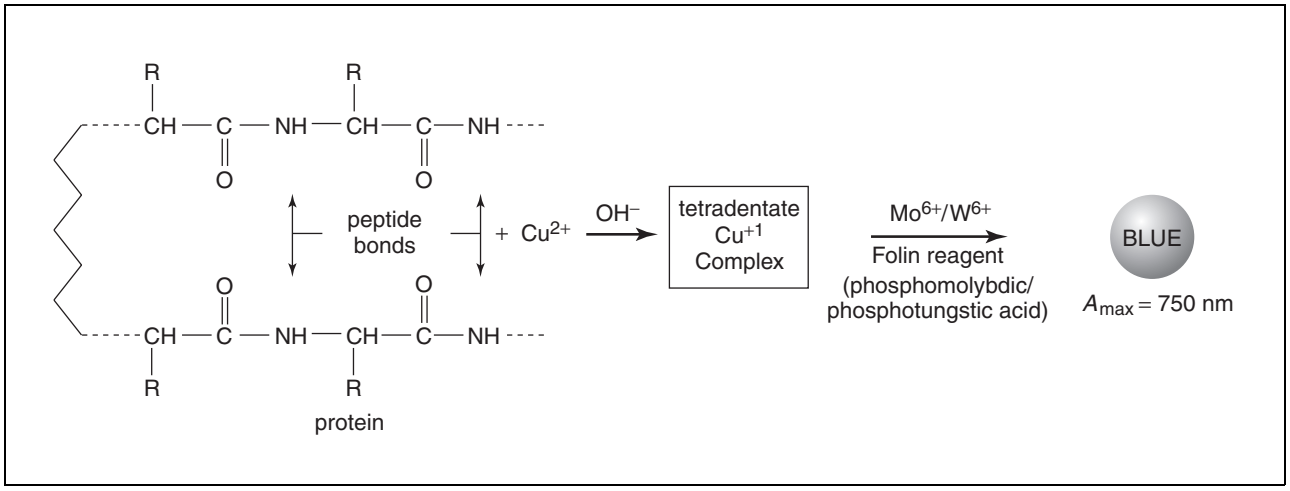


Figure A.3H.1 The reaction schematic for the Lowry Protein Assay.

concentration of each sample is calculated from the most appropriate section of the whole standard curve. The average total protein concentration for each sample is determined from the average of its replicates. If multiple dilutions of each sample have been run, the results for the dilutions that fall within the most linear portion of the working range are averaged.

If using a computer program, use a quadratic curve fit for the nonlinear standard curve to calculate the protein concentration of the samples. If the standard curve is linear or if the absorbance readings for the samples fall within the linear portion of the standard curve, the total protein concentrations of the samples can be estimated using the linear regression equation.

Most software programs will allow the experimenter to construct and print a graph of the standard curve as well as calculate the protein concentration for each sample and display statistics for the replicates. Typically, the statistics displayed will include the average of the absorbance readings (or the average of the calculated protein concentrations), the standard deviation (SD), and the coefficient of variation (CV) for each standard or sample. If multiple dilutions of each sample have been run, average the results for the dilutions that fall in the most linear portion of the working range.

THE LOWRY PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEINS

BASIC PROTOCOL 1

In 1951, Oliver H. Lowry introduced this colorimetric total protein assay method. It offered a significant improvement over previous protein assays, and his paper became one of the most cited references in the life-science literature (Lowry, 1951). The Lowry assay is easy to perform since the incubations are done at room temperature and the assay is sensitive enough to allow the detection of total protein in the low microgram per milliliter range. It is one of three copper chelation chemistry–based methods presented in this unit. Essentially, the Lowry protein assay is an enhanced biuret assay (see Basic Protocol 3). After a short incubation, Lowry's reagent C (Folin phenol) is added for enhanced color development (Fig. A.3H.1). The Lowry assay requires fresh (daily) preparation of two reagents and a meticulously timed incubation step. The two reagents are combined just before use to make a buffered alkaline cupric sulfate working solution. The addition of sodium dodecyl sulfate (SDS) to Lowry's reagent D (i.e., Lowry's reagent D′) allows the method to be used with samples that contain detergents.

Materials

Standard protein: 2 mg/ml BSA (see recipe)
Sample buffer or solvent
Protein sample(s)
Lowry's Reagents C and D or D′ (see recipes)

1. Dispense 0 to 100 µl standard protein to appropriately labeled tubes and bring the total volume to 100 µl with sample buffer or solvent to prepare a dilution series from 10 to 100 µg.

   These concentrations of albumin should produce A_{750} readings from ~0.10 to 1.0 AU in 1-cm cuvettes.

2. Dispense ≤100 µl protein sample(s) to separate labeled tubes and adjust the final volume to 100 µl using the same buffer or solvent used to prepare the sample.

3. Add 1 ml reagent D (or reagent D′) to each of the standards and unknown samples. Vortex the tubes immediately to develop optimum color. Incubate for precisely 10 min at room temperature.

   If samples contain detergent, use reagent D′ to eliminate the interference associated with up to 1% of various detergents. If a precipitate forms in reagent D′, warm the reagent and vortex before addition.

4. While mixing, add 0.1 ml reagent C. Vortex the tubes immediately. Incubate for 30 min at room temperature.

5. Measure the color at 750 nm (A_{750}) on a spectrophotometer zeroed with deionized water.

   Read samples within 10 min, as samples continue to develop color. Samples incubated >60 min should be discarded.

   If the absorbance reading of the sample is higher than that of the highest concentration of standard, dilute the sample with buffer and repeat the procedure on the diluted sample.

6. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each standard versus its protein concentration in milligrams per milliliter.

7. Determine sample protein concentration by interpolating from the standard curve (see Strategic Planning).

ALTERNATE PROTOCOL 1

MODIFIED LOWRY PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEINS

Preformulated, stabilized, modified versions of the Lowry reagent are now commercially available from Pierce (the Modified Lowry Protein Assay Reagent) or from Bio-Rad (the DC Protein Assay). The assay can be performed in test tubes or a microtiter plate (see Alternate Protocol 2). The working range of this assay is 1 to 1500 µg/ml if the Pierce reagent is used, or 200 to 1400 µg/ml if the Bio-Rad reagent is used. Table A.3H.1 is a brief troubleshooting guide for this technique.

Additional Materials *(also see Basic Protocol 1)*

Modified Lowry Protein Assay Kit (Pierce) containing:
2 mg/ml BSA in 0.9% (w/v) NaCl/0.05% (w/v) sodium azide
2 N Folin-Ciocalteu reagent: dilute fresh to 1 N
Modified Lowry's Reagent

Table A.3H.1 Troubleshooting Guide for the Modified Lowry Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains a chelating agent (e.g., EDTA, EGTA)	Dialyze or dilute the sample
		Precipitate the protein with TCA and dissolve the pellet in modified Lowry reagent
Blank A_{750} is normal, but standards show less color than expected	Sample changed the pH of the reagent	Dialyze or dilute the sample
	Color measured at the wrong wavelength	Measure the color at 750 nm
Precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute the sample
	Sample contains potassium ions	Precipitate the protein with TCA, dissolve the pellet in Modified Lowry Reagent
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample
	Sample contains a thiol	Precipitate the protein with TCA, dissolve pellet in Modified Lowry Reagent
Need to read color at a different wavelength	Colorimeter does not have 750-nm filter	Color may be read at any wavelength between 650 nm and 750 nm

1. Prepare a dilution series of 2 mg/ml BSA (e.g., the standard provided in the Modified Lowry Protein Assay Kit) in buffer to cover the range 2.0 to 1500 µg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. In duplicate, add 200 µl diluted standard, sample, or buffer (blank) into appropriately labeled test tubes.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

3. At 15-sec intervals, add 1.0 ml Modified Lowry Protein Assay Reagent to each of the tubes. Vortex 2 to 3 sec to mix the contents of the tube and incubate at room temperature for *exactly* 10 min.

4. At the end of the first tube's 10-min incubation, add 100 µl freshly diluted 1 N Folin-Ciocalteu reagent (freshly diluted from a 2 N stock). Immediately vortex the tube for 2 to 3 sec. Continue to maintain the 15-sec intervals from step 3 for addition of the reagent to the remaining tubes.

5. Allow each of the tubes to incubate for 30 min at room temperature.

6. Measure the color at 750 nm (A_{750}) on a spectrophotometer zeroed with deionized water.

7. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each BSA standard versus its concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure A.3H.2.

8. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Determine the average total protein concentration for each sample from the average of its replicates.

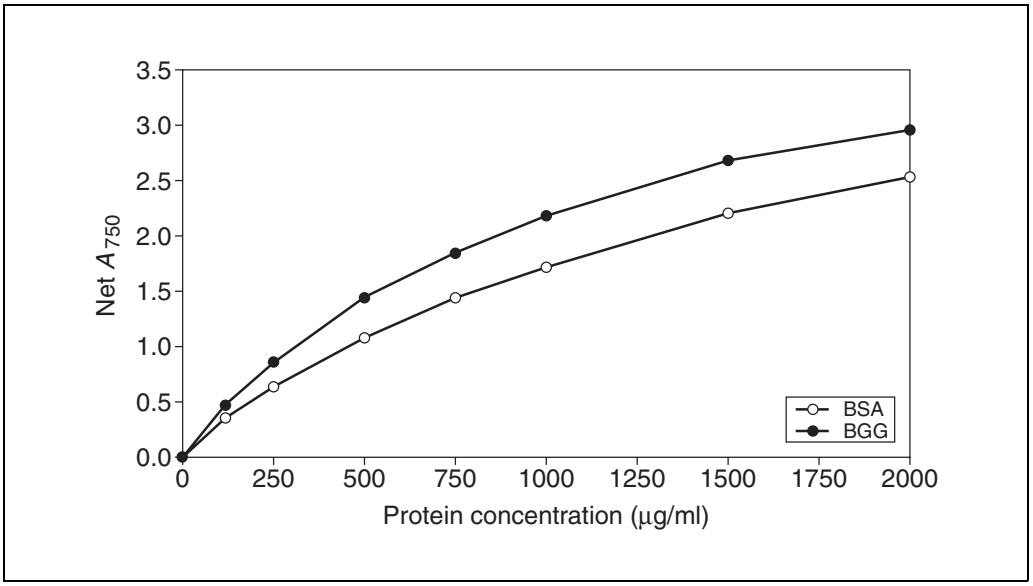


Figure A.3H.2 Graph of the color response curves obtained with Pierce's Modified Lowry Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 750 nm in a Hitachi U-2000 spectrophotometer.

ALTERNATE PROTOCOL 2

MICROTITER PLATE MODIFIED LOWRY ASSAY FOR TOTAL PROTEIN

The modified Lowry assay can also be done in a 96-well microtiter plate format. The assay has a working range of 1 to 1500 μg/ml.

Additional Materials *(also see Alternate Protocol 1)*

Microtiter plate and cover or tape seals
200-μl multichannel pipettor
Microtiter plate reader for 750 nm

1. Draw a template for placement of samples and standards on the microtiter plate.

   Blanks, standards, and samples should be prepared in triplicate.

2. Add 40 μl of each diluted BSA standard (see Alternate Protocol 1, step 1), sample, or diluent (blank) to the appropriate wells of a 96-well plate.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

3. Using a multichannel pipettor, quickly add 200 μl Modified Lowry Protein Assay Reagent to each of the wells. Mix immediately on a plate mixer for 30 sec.

4. Allow the plate to incubate at room temperature for *exactly* 10 min.

5. Using a multichannel pipettor, quickly add 20 μl freshly diluted 1 N Folin-Ciocalteu reagent to each well. Immediately mix on a plate mixer for 30 sec.

6. Cover the plate (to prevent evaporation) and incubate 30 min at room temperature.

7. Mix the plate again and measure the color (absorbance) of each well in a microtiter plate reader at 750 nm.

8. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each standard versus its protein concentration in micrograms per milliliter.

9. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average total protein concentration for each sample from the average of its replicates.

THE BICINCHONINIC ACID (BCA) FOR DETERMINATION OF TOTAL PROTEIN

BASIC PROTOCOL 2

Smith et al. (1985) introduced the bicinchoninic acid (BCA) protein assay reagent. In one sense, it is a modification of the Lowry protein assay reagent. The mechanism of color formation with protein for the BCA protein assay reagent is similar to that of the Lowry reagent, but there are several significant differences. The BCA protein assay reagent combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (i.e., the biuret reaction; see Basic Protocol 3) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid. The purple-colored reaction product of this method is formed by the chelation of two molecules of BCA with one cuprous ion (Fig. A.3H.3). The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The primary advantage of the BCA protein assay reagent is that most surfactants, even if present in the sample at concentrations up to 5% (v/v), are compatible with this method. Table A.3H.2 is a brief troubleshooting guide for this technique.

Materials

Protein standard: 2 mg/ml BSA (see recipe)
Sample buffer or solvent
Protein sample
BCA working reagent: mix 100 parts BCA reagent A with 2 parts reagent B (see recipes for each reagent)

1. Prepare a dilution series of 2 mg/ml BSA in sample buffer or diluent to cover a range from 125 to 2000 µg/ml.
2. Add 100 µl sample, diluted standard, or buffer (blank) into appropriately labeled tubes.
3. Add 2 ml BCA working reagent mix to each tube. Vortex immediately.

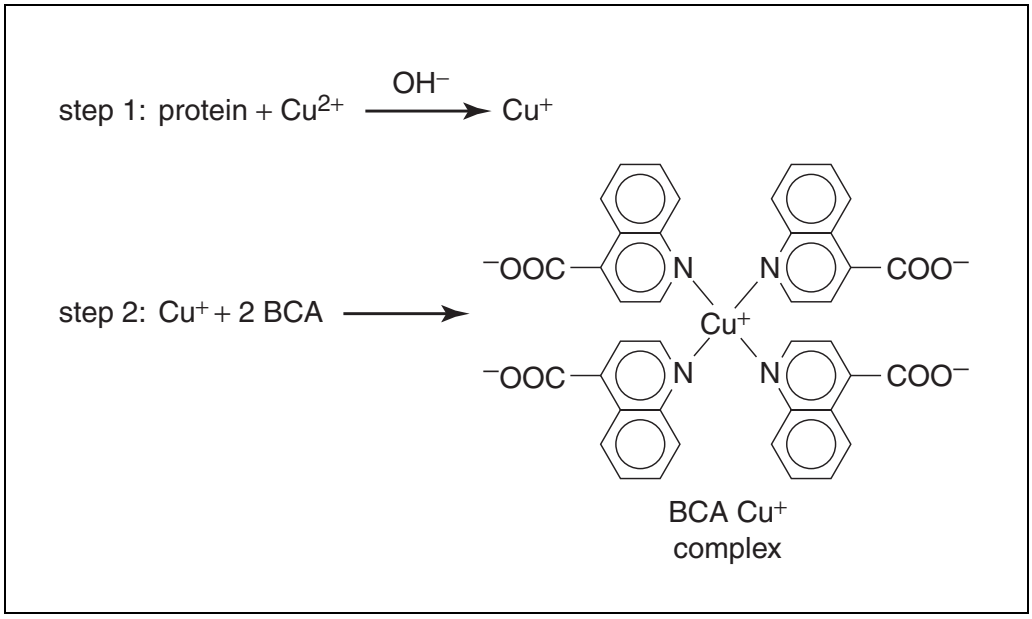


Figure A.3H.3 The reaction schematic for the BCA Protein Assay.

Table A.3H.2 Troubleshooting Guide for BCA Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze or dilute the sample
		Increase the copper concentration in the working reagent (use 48 parts reagent A and 2 parts reagent B)
Blank A_{562} is normal, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze or dilute the sample
	Color measured at the wrong wavelength	Measure the color at 562 nm
Color of samples appear darker than expected	Protein concentration is too high	Dilute the sample
	Sample contains lipids or lipoproteins	Add 2% (w/v) SDS to the sample to eliminate interference from lipids
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample
	Sample contains a thiol	Precipitate the protein with trichloroacetic acid (TCA) and deoxycholate (DOC), dissolve pellet in BCA working reagent
	Sample contains biogenic amines (catecholamines)	Treat the sample with iodoacetamide (for thiols)
Need to read color at a different wavelength	Colorimeter does not have 562-nm filter	Color may be read at any wavelength between 550 nm and 570 nm

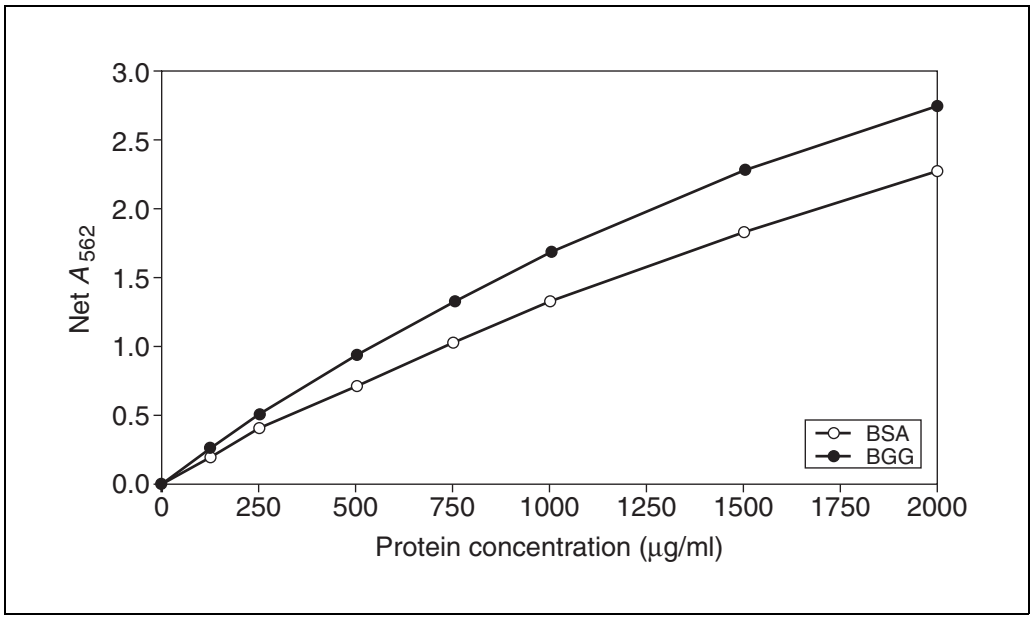


Figure A.3H.4 Graph of the color response curves obtained with Pierce's BCA Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 562 nm in a Hitachi U-2000 spectrophotometer.

4. Incubate samples and standards for 30 min at 37°C, then cool to room temperature.
5. Measure the color at 562 nm (A_{562}) on a spectrophotometer zeroed with deionized water.
6. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for the standards versus protein concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure A.3H.4.

7. Determine the protein concentration of the sample by interpolation from the plot (see Strategic Planning).

USING KITS FOR BCA MEASUREMENTS OF TOTAL PROTEIN

ALTERNATE PROTOCOL 3

Preformulated versions of the BCA reagent are now commercially available from Pierce (BCA Protein Assay Reagent) or Sigma (Bicinchoninic Acid Kit for Protein Determination). This assay can be performed in test tubes or microtiter plates (see Alternate Protocol 4), and has a working range of 20 to 2000 µg/ml.

Additional Materials

BCA Protein Assay Reagent Kit (Pierce) or Bicinchoninic Acid Kit (Sigma) containing:
2 mg/ml BSA in 0.9% NaCl/0.05% sodium azide (also see recipe)
BCA reagent A (also see recipe)
BCA reagent B (also see recipe)
37°C water bath

1. Prepare a dilution series of BSA standard in buffer to cover the range 125 to 2000 µg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. Prepare sufficient BCA working reagent (a minimum of 2 ml/tube or 20 ml/96-well microtiter plate) by adding 2 parts BCA reagent B to 100 parts of BCA reagent A.

   After mixing, the BCA working reagent is clear and apple green in color.

3. In duplicate, add 100 µl standard, sample, or buffer (blank) into appropriately labeled test tubes.
4. Add 2.0 ml BCA working reagent to each tube. Mix well by vortexing each tube 2 to 3 sec.

5a. *Standard tube protocol:* Incubate all tubes in a 37°C water bath for 30 min.

5b. *Enhanced assay protocol:* Alternatively, incubate all tubes in a 60°C water bath for 30 min.

   Increasing the incubation temperature to 60°C lowers the minimum detection level to 5 µg/ml and narrows the working range of the assay to a maximum of 250 µg/ml. This is known as the enhanced BCA assay.

6. After incubation, cool all tubes to room temperature.

   Since the BCA reagent does not reach a true end point, color development will continue even after cooling to room temperature; however, the rate of color development is very slow after cooling to room temperature, so no significant error is introduced if the A_{562} readings of all the tubes can be read within ~10 min.

7. Before reading, mix each tube again and measure the amount of color produced in each tube with a spectrophotometer at 562 nm (A_{562}) versus deionized water.
8. Plot a standard curve by graphing the average net or blank-corrected A_{562} values for the standards versus protein concentration in micrograms per milliliter.
9. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average sample concentration from its replicates.

ALTERNATE PROTOCOL 4

MICROTITER PLATE ASSAY FOR BCA MEASUREMENT OF TOTAL PROTEIN

BCA assays can be run in 96-well microtiter plates. The assay has a working range of 125 to 2000 µg/ml.

Additional Materials *(also see Alternate Protocol 3)*

96-well microtiter plate with cover or tape seal
200-µl multichannel pipettor
Microtiter plate shaker
37°C dry-heat incubator
Microtiter plate reader

1. Draw a template for the placement of samples and standards on a microtiter plate.

   Blanks, standards, and samples should be prepared in triplicate.

2. Add 10 µl of each diluted BSA standard (see Alternate Protocol 3, step 1), sample, or diluent (blank) to the appropriate wells.
3. Using a multichannel pipettor, add 200 µl BCA working reagent (see Alternate Protocol 3, step 2) to each well. Mix well on a microtiter plate shaker for 30 sec.
4. Cover the plate and incubate in a 37°C dry-heat incubator for 30 min.
5. After incubation, allow the plate to cool to room temperature.
6. Mix the plate again, remove the plate cover and measure the color in each well of the plate at 562 nm (A_{562}) in a microtiter plate reader.
7. Plot a standard curve by graphing the average net or blank-corrected A_{562} values for each BSA standard versus its concentration in micrograms per milliliter.
8. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average total protein concentration for each sample from the average of its replicates.

BASIC PROTOCOL 3

THE BIURET ASSAY FOR DETERMINING TOTAL PROTEIN

All proteins are composed of amino acids joined by peptide bonds in a linear sequence. There are ~20 naturally occurring amino acids found in proteins. The amino acids are joined to each other by peptide bonds formed by a condensation reaction that occurs between the terminal amine of one amino acid and the carboxyl end of the next. Peptides containing three or more amino acid residues will form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. A similar colored chelate complex forms with the organic compound biuret (NH_2–CO–NH–CO–NH_2) and the cupric ion. The reaction in which a colored chelation complex is formed with peptide bonds in the presence of an alkaline cupric sulfate solution became known as the biuret reaction (Fig. A.3H.5). Thus, the biuret protein assay reagent gets its name

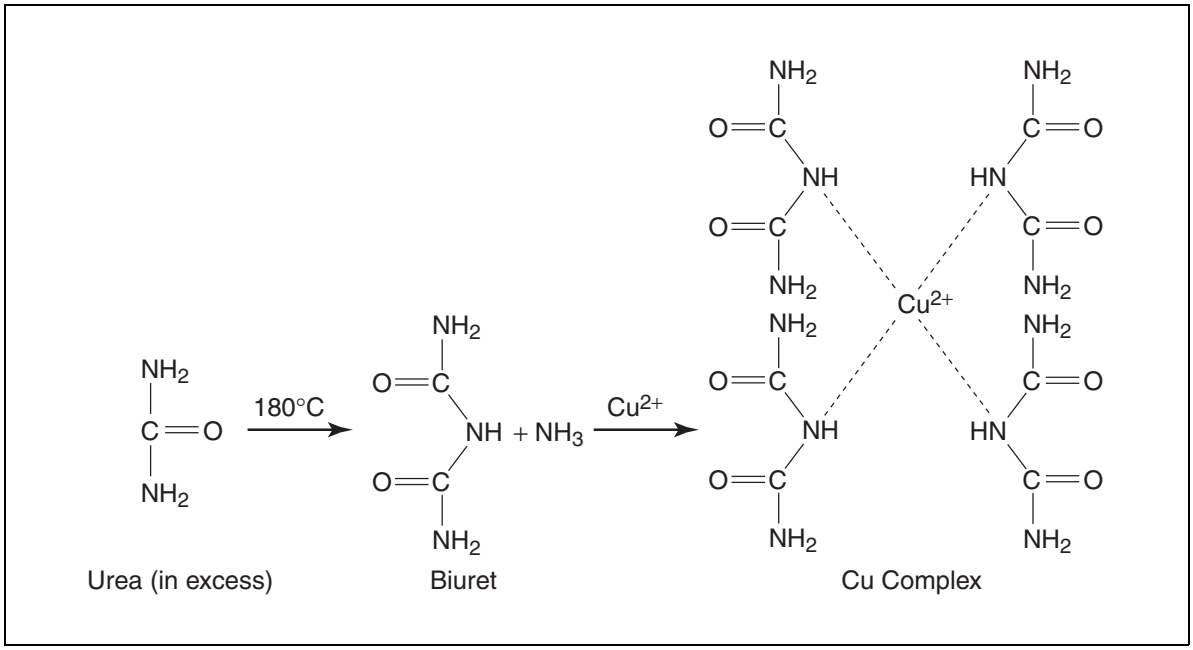


Figure A.3H.5 The schematic of the biuret reaction.

Table A.3H.3 Troubleshooting Guide for Biuret Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains copper chelating agent	Dialyze or dilute the sample
Blank A_{540} is normal, but standards show less color than expected	Color measured at the wrong wavelength	Measure the color at 540 nm
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample

from the above reaction even though it does not actually contain the organic compound biuret. Single amino acids or dipeptides do not give the biuret reaction, but tripeptides and larger polypeptides or proteins will react to produce the light-blue to violet complex that absorbs light at 540 nm. One cupric ion forms the colored coordinate complex with 4 to 6 nearby peptides bonds. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction. Thus, the biuret reaction is the basis for a simple and rapid colorimetric method of quantitatively determining total protein concentration.

Because the working range for the biuret assay is from 5 to 160 mg/ml, the biuret reagent has found utility in the clinical laboratories for the quantitation of total protein in serum. The formulation employed in the biuret total protein reagent (Sigma Diagnostics) was developed by Doumas et al. (1981) as a candidate reference method for the determination of serum total protein in the clinical lab. Using Sigma's biuret reagent, the expected range for total protein in serum is from 63 to 83 mg/ml. Bilirubin, lipids, hemoglobin, and dextran are known to interfere in the biuret assay for total serum protein. Outside of this application, other copper chelating agents such as EDTA, EGTA, citrate, Tris, imino-

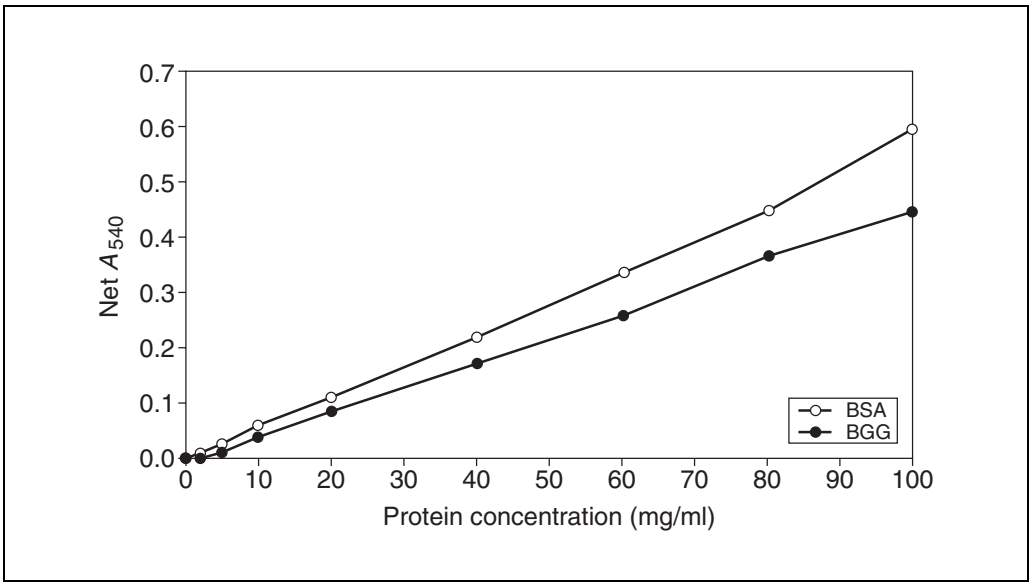


Figure A.3H.6 Graph of the color response curves obtained with Sigma's Biuret Total Protein Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 540 nm in a Hitachi U-2000 spectrophotometer.

diacetic acid, and nitrilotriacetic acid will interfere. The working range of this assay is 5 to 160 mg/ml. Table A.3H.3 is a brief troubleshooting guide for this technique.

Materials

Standard protein (also see Commentary)
Sample (unknown) protein
Biuret total protein reagent (Sigma Diagnostics; also see recipe)

1. Select a protein to use as the standard (see Strategic Planning) and prepare a dilution series with buffer to cover the range 10 to 160 mg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the standard that was used with the samples.

2. In duplicate, add 20 µl standard, sample, or diluent (blank) to appropriately labeled test tubes.
3. Add 1.0 ml biuret reagent to each tube. Mix well by vortexing 2 to 3 sec.
4. Incubate tubes at ambient room temperature (18° to 26°C) for 10 min.
5. Measure the color of each tube with a spectrophotometer at 540 nm (A_{540}). Compare to the blank.
6. Plot a standard curve by graphing the blank-corrected A_{540} values for the standards versus protein concentration in milligrams per milliliter.

   Example color response curves for BSA and BGG are shown in Figure A.3H.6.

7. Determine the sample concentration by interpolation from the standard curve (see Strategic Planning).

MICROTITER PLATE BIURET ASSAY FOR TOTAL PROTEIN

ALTERNATE PROTOCOL 5

The biuret assay can be performed in a 96-well microtiter plate and has a working concentration of 10 to 160 mg/ml.

Additional Materials *(also see Basic Protocol 3)*

96-well microtiter plate and cover or tape sealer
250-µl multichannel pipettor and appropriate tips
Microtiter plate mixer
Microtiter plate reader

1. Draw a template for placement of samples and standards on the microtiter plate.
2. Select a protein to use as the standard (see Strategic Planning). Prepare a dilution series with the same buffer used to dilute the samples to cover the range of 10 to 160 mg/ml.
3. In triplicate, add 5.0 µl of each diluted standard or sample into the appropriate microtiter plate wells. Use the buffer or diluent that was used to dilute the standard and samples for the blank wells.
4. Using a multichannel pipettor, add 250 µl biuret reagent to each well. Mix well on a plate shaker for 30 sec.
5. Cover the plate and incubate at room temperature for 10 min.
6. Mix again. Remove the plate cover and measure the color in each well of the plate at 540 nm (A_{540}) in a microtiter plate reader.
7. Prepare a standard curve by graphing the average net or blank-corrected A_{540} values for each standard versus its concentration in milligrams per milliliter.
8. Determine the sample concentration by interpolating from the plot (see Strategic Planning).

THE COOMASSIE DYE–BINDING (BRADFORD) ASSAY FOR DETERMINING TOTAL PROTEIN

BASIC PROTOCOL 4

The Coomassie dye–based protein-binding assays have the advantage of being the fastest and the easiest to perform (Fig. A.3H.7). In addition, the assay is performed at room temperature and no special equipment, other than a spectrophotometer, is required. Briefly, the sample is added to the ready-to-use reagent and, following a short incubation, the resultant blue color is measured at 595 nm versus deionized water.

In 1976, Marion Bradford introduced the first Coomassie dye–based reagent for the rapid colorimetric detection and quantitation of total protein. The Coomassie dye (Bradford) protein assay reagents have the advantage of being compatible with most salts, solvents, buffers, thiols, reducing substances, and metal chelating agents encountered in protein samples.

Materials

Sample buffer or solvent
Protein standard (e.g., 2 mg/ml BSA; see recipe)
Protein sample
Coomassie dye reagent (Pierce or Bio-Rad; also see recipe)

1. Prepare a dilution series from protein standard (e.g., 2 mg/ml BSA) and sample buffer to cover the range 100 to 1000 µg/ml.

   Bovine serum albumin (BSA) is often used as a calibration standard, but it has greater general dye-binding capacity than most proteins (Bradford, 1976).

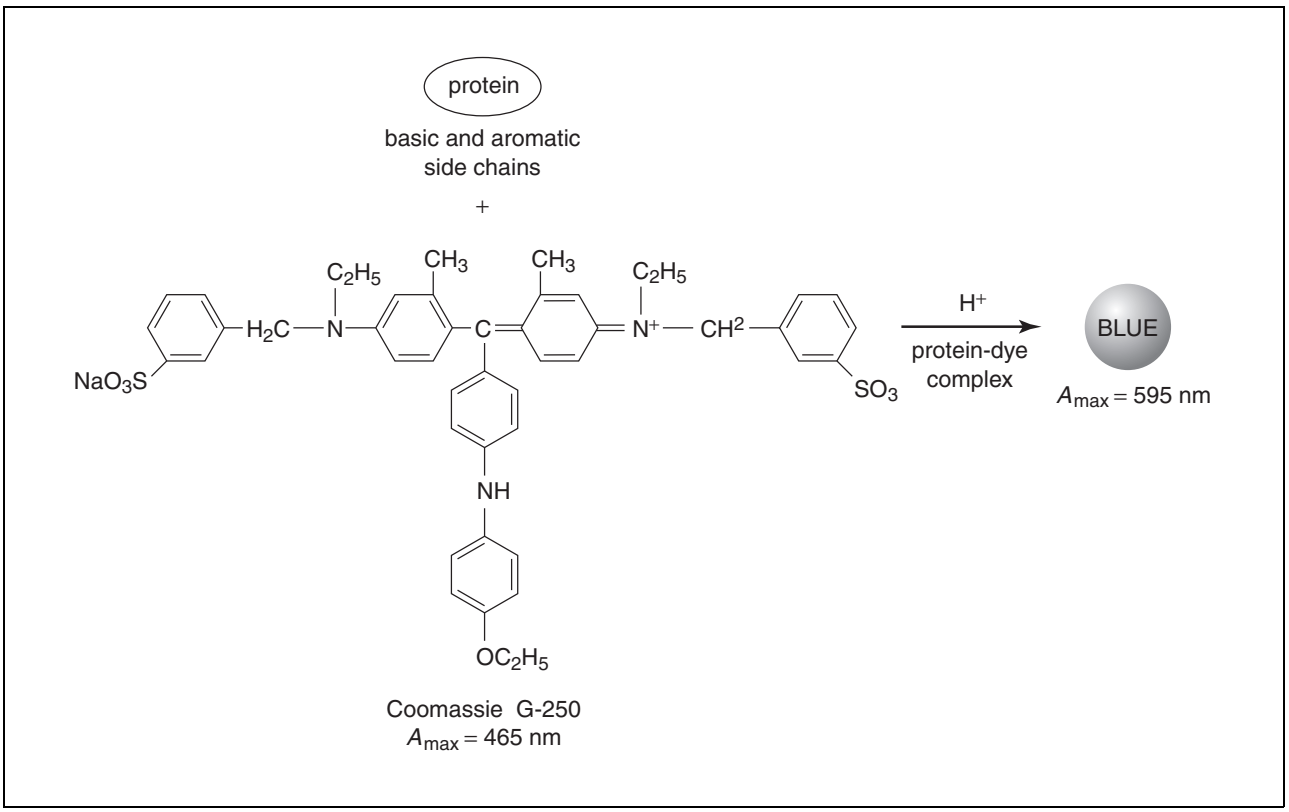


Figure A.3H.7 The reaction schematic for the Coomassie Protein Assay.

2. Dispense 0.1 ml standard, sample, or buffer to appropriately labeled tubes.

   If commercially prepared Coomassie reagent is used, follow the manufacturer's instructions. Commercial reagents tend to produce less turbidity and more consistent results.

3. Add 5 ml Coomassie dye reagent. Vortex immediately.
4. Incubate 10 min at room temperature.
5. Vortex each tube just before measuring the absorbance at 595 nm (A_{595}).
6. Plot a standard curve by graphing the average net or blank-corrected A_{595} values for the standard versus its protein concentration in micrograms per milliliter.
7. Determine the sample concentration by interpolation from the standard curve (see Strategic Planning).

ALTERNATE PROTOCOL 6

THE COOMASSIE PLUS PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEIN

Several companies offer modified Bradford Coomassie dye–based protein assay reagents. Perhaps the most popular such reagent is the Protein Assay Reagent available from Bio-Rad. The Coomassie Plus Protein Assay Reagent available from Pierce is another modification of the Bradford formulation. In addition to the attributes cited above, the Coomassie Plus Protein Assay Reagent has the unique advantage of producing a linear response curve within a portion of its working range. For BSA, the response curve is linear from 125 to 1000 µg/ml and for bovine gamma globulin (BGG), the response curve is linear from 125 to 1500 µg/ml. The complete working range of the assay covers the concentration range from 100 to 2000 µg/ml for the tube protocol and from 1 to 25 µg/ml for the microtiter protocol (see Alternate Protocol 7).

Table A.3H.4 Troubleshooting Guide for Coomassie Plus Protein

Problem	Possible cause	Solution
Blank A_{595} is normal, but standards show less color than expected	Improper reagent storage	Store reagent refrigerated
	Reagent still cold	Warm to room temperature before use
	Color measured at the wrong wavelength	Measure the color at 595 nm
Blank and standards are normal, but samples show little color	Low molecular weight of sample protein (<3000 kDa)	Use the BCA (see Basic Protocol 2) or Lowry protein assay (see Basic Protocol 1)
A precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute the sample
		Precipitate the protein with TCA, dissolve pellet in 50 mM NaOH
All tubes (including the blank) are dark blue	Strong alkaline buffer or reagent raises reagent's pH	Dialyze or dilute the sample
	Sample volume too large, reagent pH raised	Maximum of 1 part sample and 1 part reagent
Need to read color at a different wavelength	Colorimeter does not have 595 nm filter	Color may be read at any wavelength between 575 nm and 615 nm

The main disadvantage of all Bradford-type protein assay reagents is that they are not compatible with surfactants at concentrations routinely used to solubilize membrane proteins. With some exceptions, the presence of a surfactant in the sample, even at low concentrations, causes precipitation of the reagent. Table A.3H.4 is a brief troubleshooting guide for this technique.

Additional Materials *(also see Basic Protocol 4)*

Coomassie Plus Protein Assay Reagent Kit (Pierce) containing Coomassie Plus Protein Assay Reagent

1. Prepare a dilution series with 2 mg/ml BSA and buffer to cover a range from 100 to 2000 µg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. Allow the Coomassie Plus Protein Assay reagent to come to room temperature. Mix the assay reagent well by gentle inversion before use.
3. In duplicate, dispense 50 µl standard, sample, or diluent (blank) into appropriately labeled test tubes.
4. Add 1.5 ml Coomassie Plus Protein Assay Reagent to each tube. Mix each tube well by vortexing 2 to 3 sec.
5. Let the tubes stand 10 min at room temperature.
6. Mix each tube again just before measuring the absorbance at 595 nm (A_{595}).

   Disposable polystyrene cuvettes eliminate the job of cleaning dye-stained quartz or glass cuvettes.

7. Plot a standard curve by graphing the average net or blank-corrected A_{595} reading for each standard versus its concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure A.3H.8.

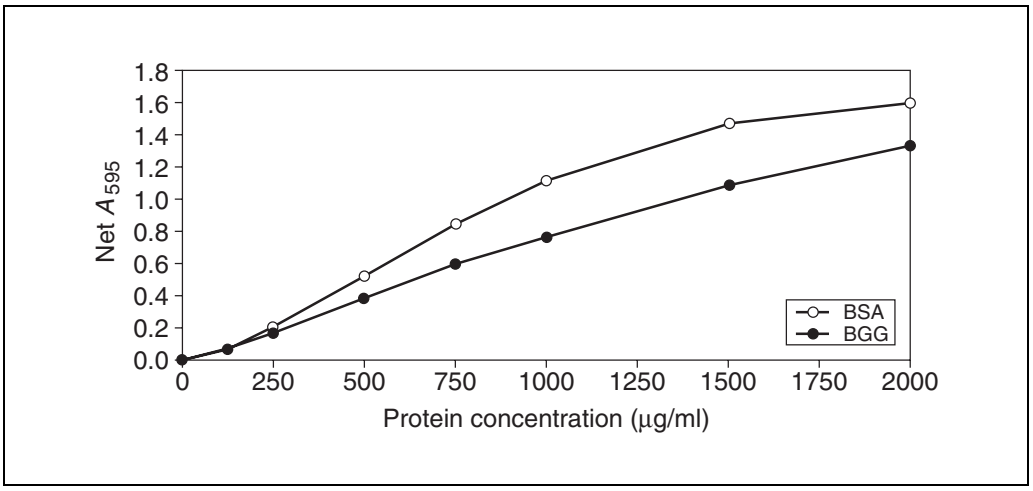


Figure A.3H.8 Graph of the color response curves obtained with Pierce's Coomassie Plus Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 595 nm in a Hitachi U-2000 spectrophotometer.

8. Determine the protein concentration for each sample by interpolating from the standard curve (see Strategic Planning). Calculate the average total protein concentration for each sample from the replicates.

ALTERNATE PROTOCOL 7

MICROTITER PLATE COOMASSIE ASSAY FOR TOTAL PROTEIN

This microtiter plate assay has a working range of 1 to 25 µg/ml.

Additional Materials *(see Basic Protocol 4)*

Coomassie Plus Protein Assay Reagent Kit (Pierce) containing Coomassie Plus Protein Assay reagent
96-well microtiter plate
300-µl multichannel pipettor
Microtiter plate mixer
Microtiter plate reader

1. Allow the Coomassie Plus Protein Assay Reagent to come to room temperature. Once the reagent is at room temperature, mix the reagent well by gentle inversion of the bottle.
2. Draw a template for placement of samples and standards on a 96-well microtiter plate.
3. In triplicate, dispense 10 µl of each diluted BSA standard, sample, or diluent (blank) into the appropriate wells of a 96-well microtiter plate.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock standard that was used with the samples.

4. Dispense 300 µl Coomassie Plus Protein Assay reagent into each well with a multichannel pipettor. Mix the plate well on a plate shaker for 30 sec.
5. Let the plate incubate 10 min at room temperature.
6. Just before reading, mix the plate again, then measure the absorbance at 595 nm (A_{595}) on a plate reader.

7. Prepare a standard curve by graphing the average net or blank-corrected A_{595} values for each standard versus its concentration in micrograms per milliliter.

8. Determine the sample concentration by interpolating from the standard curve (see Strategic Planning). Calculate the average total protein concentration for each sample from the replicates.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BCA reagent A

1 g 4,4′-dicarboxy-2,2′-biquinoline, disodium salt (Na_2BCA; Pierce or Sigma; 1% w/v final)
2 g $Na_2CO_3 \cdot H_2O$ (2% w/v final)
160 mg sodium tartrate dihydrate (0.16% w/v final)
0.4 g NaOH (0.4% w/v final)
0.95 g $NaHCO_3$ (0.95% w/v final)

Dissolve all of the above chemicals except the sodium bicarbonate in deionized water and adjust the final volume to 100 ml. Adjust the pH to 11.25 by adding the sodium bicarbonate a little at a time. Store this alkaline reagent in a plastic container 1 to 3 weeks at room temperature, longer at 4°C.

Only the disodium salt of Na_2BCA is soluble at neutral pH; the free acid is not readily soluble.

BCA reagent B

4 g $CuSO_4 \cdot 5H_2O$ (4% w/v final)
100 ml H_2O
Store up to 6 months at room temperature

Biuret total protein reagent

0.6 mol/liter sodium hydroxide
12.0 mmol/liter copper sulfate
31.9 mmol/liter sodium potassium tartrate
30.1 mmol/liter potassium iodide
Store up to 6 months at room temperature

This reagent is based upon the candidate reference method for the determination of total protein in serum developed by Doumas et al. (1981). It is available from Sigma Diagnostics.

BSA, 2 mg/ml (w/v)

200 mg BSA (crystallized or lyophilized or one of the Cohn Fraction V preparations which are 96% to 98% protein and 3% to 4% water) in 100 ml of 0.9% saline containing 0.05% (w/v) sodium azide. Store up to 6 months at 4°C.

Coomassie dye reagent

100 mg Coomassie Brilliant Blue G-250 (0.01% w/v final)
50 ml 95% ethanol (4.7% (w/v) final)
100 ml 85% (w/v) phosphoric acid (8.5% w/v final)

In a small container, dissolve the dye in ~25 ml ethanol, add the dye/ethanol solution to 800 ml of deionized water. Use the remaining ethanol to rinse the dye/ethanol container and add the rinses to the formulation. While mixing, slowly add the acid to the formulation and adjust the final volume to 1000 ml with deionized water. Filter the reagent through a single pad of Whatman no. 2 filter paper. Store up to 1 month at room temperature in a glass container.

continued

Coomassie Brilliant Blue G-250 (color index 42655) is available from a number of different suppliers (e.g., ACROS, Aldrich, AMRESCO, Bio-Rad, Fisher Biotech, Fluka, ICN Biomedicals, J.T. Baker, Research Organics, Serva, Sigma, or USB).

Lowry's reagent A

21.2 g sodium carbonate (2% w/v)
40 ml 1 N NaOH (or 4.0 g NaOH; 0.1 N final)
Add distilled or deionized water to 1 liter
Make fresh daily

Lowry's reagent B

0.5 g $CuSO_4 \cdot 5H_2O$ (0.5% final)
1 g sodium tartrate (1% final)
Make fresh daily

Sodium tartrate may be replaced with disodium tartrate, potassium sodium tartrate, or sodium citrate for better solubility.

Lowry's reagent C (1 N Folin phenol reagent)

Dilute 2 N Folin phenol (Sigma, Fisher, or VWR) with an equal volume water. Prepare immediately before use.

Lowry's reagent D (reagent A and B mix)

Mix 1 vol Lowry's reagent B and 50 vol Lowry's reagent A (see recipes). Prepare immediately before use.

Lowry's reagent D′

Add 2 ml of 10% (w/v) sodium dodecyl sulfate (SDS; *APPENDIX 2A* for 20% solution) in deionized water to each 100 ml Lowry's reagent D (see recipe). Prepare immediately before use.

COMMENTARY

Background Information

The Modified Lowry Protein Assay

Although the exact mechanism of color formation reaction in the Lowry protein assay remains poorly understood, it is known that the color producing reaction with protein occurs in two distinct steps. As seen in Figure A.3H.1, protein is first reacted with alkaline cupric sulfate in the presence of tartrate during a 10-min incubation at room temperature. During this incubation, a tetradentate copper complex forms from four peptide bonds and one atom of copper. The tetradentate copper complex is light blue in color (this is the "biuret reaction"). Following the 10-min incubation, Folin phenol reagent is added. It is believed that color enhancement occurs when the tetradentate copper complex transfers electrons to the phosphomolybdic/phosphotungstic acid complex (i.e., the Folin phenol reagent).

The reduced phosphomolybdic/phosphotungstic acid complex produced by this reaction is intensely blue in color. The Folin phenol reagent loses its reactivity almost immediately upon addition to the alkaline working reagent/sample solution. The blue color continues to intensify during a 30-min room temperature incubation. It has been suggested by Lowry et al. (1951) and by Legler et al. (1985) that during the 30-min incubation, a rearrangement of the initial unstable blue complex leads to the stable final blue colored complex, which has higher absorbance.

For small peptides, the amount of color increases with the size of the peptide. The presence of any of five amino acid residues (tyrosine, tryptophan, cysteine, histidine, and asparagine) in the peptide or protein backbone further enhance the amount of color produced, because they contribute additional reducing equivalents for further reduction of the phosphomolybdic/phosphotungstic acid complex. With the exception of tyrosine and tryptophan, free amino acids will not produce a colored product with the Lowry reagent; however, most dipeptides can be detected. In the absence of any of the five amino acids listed above, proteins containing proline residues have a lower

color response with the Lowry reagent because it interferes with complex formation.

The final blue color is optimally measured at 750 nm, but it can be measured at any wavelength between 650 nm and 750 nm with little loss of color intensity. Dr. Lowry (1951) recommended reading the color at 750 nm because few other substances absorb light at that wavelength. The amount of light absorbed at 750 nm is directly proportional to the amount of protein in the sample, but the color response curve produced is nonlinear.

The sensitivity of the Modified Lowry Protein Assay Reagent is greatly enhanced over that of the biuret total protein reagent. The working range of the method covers the total protein range from 1 to 1500 μg/ml. In comparison, the working range for the biuret assay is from 5 to 160 mg/ml.

The Modified Lowry Protein Assay Reagent will form precipitates in the presence of surfactants or potassium ions. The problem of precipitation that is caused by the presence of potassium ions in the sample can sometimes be overcome by centrifuging the tube and reading the color in the supernatant. Most surfactants will cause precipitation of the reagent even at very low concentrations. One exception is sodium dodecyl sulfate (SDS), which is compatible with the reagent at concentrations up to 1% (w/v) in the sample. Chelating agents interfere because they bind copper and thus prevent formation of the copper-peptide bond complex. Reducing agents and free thiols interfere, as they reduce the phosphotungstate-phosphomolybdate complex, immediately forming an intensely blue colored product upon their addition to the Modified Lowry Protein Assay Reagent.

The Coomassie Plus Protein Assay

The primary advantage of the Coomassie Plus Protein Assay is that it is generally compatible with most of the buffers and reagents found in samples and is unaffected by the presence of chelating agents, reducing agents, or free sulfhydryls in the sample.

The development of color in the Coomassie dye-binding methods has been associated with the presence of certain basic amino acids (primarily arginine, lysine, and histidine) in the protein. Van der Waals forces and hydrophobic interactions also participate in the binding of the dye by protein. The number of Coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. In the acidic environment of the Coomassie Plus Protein Assay Reagent, protein binds to the Coomassie dye. This results in a spectral shift of the reagent from the reddish/brown form of the dye, with an absorbance maximum at 465 nm, to the blue form of the dye, with an absorbance maximum at 610 nm (see Fig. A.3H.7).

The difference between the two forms is greatest at 595 nm; therefore, this is the optimal wavelength to measure the blue color from the Coomassie dye–protein complex. If desired, the blue color can be read at any wavelength between 575 and 615 nm. At the two extremes (575 and 615 nm) there is a loss of ~10% in the measured amount of color (absorbance) compared to the value obtained at 595 nm.

Free amino acids, peptides, and low molecular weight proteins do not produce color with the Coomassie Plus Protein Assay Reagent. In general the molecular weight of the peptide or protein must be at least 3000 Da to be assayed with this reagent. In some applications this can be an advantage. The reagent has been used to measure "high molecular weight proteins" during fermentation in the beer brewing industry.

One disadvantage of any Coomassie dye–based protein assay is that surfactants in the sample will cause precipitation of the reagent. Another disadvantage is that the Coomassie Plus Protein Assay Reagent shows almost twice as much protein-to-protein variation as that obtained with the protein-copper chelation–based assay reagents (the Lowry Protein Assay Reagent or the BCA Protein Assay Reagent). While this is true, the reagent exhibits the least protein-to-protein variation of all Coomassie dye–based (Bradford) reagents. Since the Coomassie dye reagent is highly acidic, a small number of proteins cannot be assayed with this reagent due to poor solubility. In addition, the glass or quartz cuvettes routinely used to hold the solution in the spectrophotometer while the color intensity is being measured are stained by Coomassie dye–based reagents.

The BCA Protein Assay

The BCA Protein Assay is a modification of the Lowry protein assay in which enhanced color production is due to the reaction of reduced copper with bicinchoninic acid (BCA). This reagent has a unique advantage over the Lowry Protein Assay Reagent and the Coomassie Plus Protein Assay Reagent because it is compatible with samples that contain up to 5% (v/v) surfactants (detergents). Unlike the Lowry protein assay, all reactants needed are present in the BCA working reagent. Large

numbers of tubes can be run without regard to the limits imposed in the Lowry assay by the need to add a second reagent at a precise time interval.

The working range of the BCA Protein Assay Reagent is from 20 to 2000 µg/ml for both the standard tube and the standard microtiter plate protocols. Since the color reaction is not a true end-point reaction, this allows more protocol flexibility. By increasing the incubation temperature, the sensitivity of the reagent can be increased. When using the enhanced tube protocol (incubating at 60°C for 30 min), the working range for the assay shifts to 5 to 250 µg/ml and the minimum detection level becomes 5 µg/ml.

Although the mechanism of color formation with protein for the BCA Protein Assay Reagent is similar to that of the Lowry reagent, there are several significant differences. The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the so-called biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion (see Fig. A.3H.3). The resultant BCA/Cu^{+} complex is water-soluble and exhibits a strong linear absorbance maximum at 562 nm with increasing protein concentrations. If desired, the purple color may be measured at any wavelength between 550 nm and 570 nm with minimum (<10%) loss of signal.

The reaction that leads to BCA color formation as a result of the reduction of Cu^{2+} is also strongly influenced by the presence of any of four amino acid residues (tyrosine, tryptophan, cysteine, or cystine) in the amino acid sequence of the protein. Unlike the Coomassie dye–binding (Bradford) methods, which require a minimum mass of protein to be present for the dye to bind, the presence of only a single amino acid residue in the sample may result in the formation of a colored BCA-Cu^{+} chelate. This is true for any of the four amino acids cited above. Studies done with di- and tripeptides indicate that the total amount of color produced is greater than can be accounted for by the simple addition of the color produced with each BCA-reactive amino acid, so the peptide backbone must contribute to the reduction of copper as well.

The rate of color formation is dependent on the incubation temperature, the types of protein present in the sample, and the relative amounts of reactive amino acids contained in the proteins. The recommended protocols do not result in end-point determinations, so the incubation periods were chosen to yield maximal color response in a reasonable time frame.

The protein-to-protein variation in the amount of color produced with the BCA Protein Assay Reagent (CV = 15% for the group of 14 proteins at 1000 µg/ml in the standard tube protocol) is similar to that observed for the Modified Lowry Protein Assay Reagent.

The biuret total protein assay

Riegler (1914) introduced the biuret reaction as a method for the estimation of albumin in urine in 1914. Modifications and improvements to the biuret method were made by Autenrieth and Mink (1915), Hiller (1926), and Fine (1935). All of these early methods required the separate addition of a sodium hydroxide solution and the separate addition of a copper sulfate solution to the sample. The methods suffered from poor precision due to ineffective mixing and variation in reaction time during mixing. The first "one solution" biuret reagent was introduced by Kingsley (1942) for use in measuring total protein, albumin, and globulin in human serum. Weichselbaum (1946) and Gornall et al. (1949) modified Kingsley's biuret reagent by decreasing the sodium hydroxide concentration to prevent the formation of precipitates and by adding sodium potassium tartrate to stabilize the reagent. Potassium iodide was added to prevent the autoreduction of Cu^{2+}. Goa (1953) published on the use of the biuret reagent for determining total protein in human cerebrospinal fluid.

Sigma Diagnostic's biuret total protein reagent is based upon the candidate reference method formulation used for the determination of total protein in serum developed by Doumas et al. (1981). Because the primary application for this reagent is the determination of total protein in serum, most of the studies regarding assay precision, assay linearity, and interfering substances have been done on clinical samples. On samples with total protein concentrations in the range of 26 to 121 mg/ml, within-run and between-run precision was found to be excellent (CV's ranged from 4.2% to 1.4%). Lipids, bilirubin, hemoglobin, dextran, and certain drugs have been shown to interfere with the total protein results obtained with the biuret Total Protein Reagent. Interference caused by the presence of lipid in the sample is due to turbidity. Interference caused by the presence of bilirubin or hemoglobin is small, almost

negligible. Dextran causes precipitation in the reaction mixture during color development; however, centrifuging the reaction mixture before reading the color can minimize this. The biuret Total Protein Reagent contains 0.6 mol/liter sodium hydroxide, 12.0 mmol/liter copper sulfate, 31.9 mmol/liter sodium potassium tartrate, and 30.1 mmol/liter potassium iodide. It should be stored at ambient room temperature until the expiration date shown on the label. Certain drugs and other substances are known to influence circulating levels of total protein (Young, 1990).

Critical Parameters

The Modified Lowry Protein Assay Reagent

The Modified Lowry Protein Assay Reagent must be refrigerated for long-term storage. If the entire bottle of reagent will be used within a reasonable time, the reagent may be stored at ambient room temperature (18° to 26°C) for up to 1 month. Reagent that has been left at room temperature for more than a month may show lower color response with protein, especially at the higher end of the working range. If the reagent has been stored refrigerated, it must be warmed to room temperature before use. The use of cold Modified Lowry Protein Assay Reagent will result in low A_{750} values.

The protocol requires that the Folin phenol reagent be added to each tube precisely at the end of the 10-min incubation. At the alkaline pH of the Lowry reagent, the Folin phenol reagent is almost immediately inactivated; therefore, it is best to add the Folin phenol reagent at the precise time while simultaneously mixing each tube. Because it is somewhat cumbersome, it requires some practice to do the assay well. From a practical point of view, it also limits the total number of tubes that can be done in a single run. If a 10-sec interval between tubes is used, the maximum number of tubes that can be done within 10 min is 60 (10 sec/tube × 60 tubes = 600 sec or 10 min).

The Coomassie Plus Protein Assay Reagent

The Coomassie Plus Protein Assay Reagent must be refrigerated for long-term storage. If the entire bottle of reagent will be used within a reasonable time, the reagent may be stored at ambient room temperature (18° to 26°C) for up to 1 month. Reagent that has been left at room temperature for more than a month may show lower color response with protein, especially at the higher end of the working range. If the reagent has been stored refrigerated, it must be warmed to room temperature before use. The use of cold Coomassie Plus Protein Assay Reagent will result in low A_{595} values.

The Coomassie Plus Protein Assay Reagent must be mixed gently by inversion just before use. The Coomassie dye in the reagent spontaneously forms loosely associated dye-dye aggregates upon standing. These aggregates may become visible after the reagent has been standing for as little as 60 min. Gentle mixing of the reagent by inversion of the bottle will uniformly disperse the dye-dye aggregates. After binding to protein, the dye also forms protein-dye-protein-dye aggregates. Fortunately, these aggregates can be dispersed easily by mixing the reaction tube. This is common to all Coomassie dye–based (Bradford) protein assay reagents. Since these aggregates form relatively quickly, it is a good idea to routinely mix (vortex for 2 to 3 sec) each sample just before measuring the color.

Bradford-type reagents containing Coomassie dye will leave a blue stain on glass or quartz cuvettes. The stain can be removed by washing the cuvettes in a dilute detergent solution in hot tap water, rinsing with water, then washing with methanol or ethanol, and finally rinsing with deionized water. Disposable plastic (polystyrene) cuvettes are strongly recommended because they eliminate the need to clean; however, these cuvettes are not compatible with samples containing organic solvents (e.g., acetone, DMF, acetonitrile).

The BCA Protein Assay Reagent

Since the BCA Protein Assay is not a true end-point assay, the amount of color produced varies with the incubation time and the incubation temperature. While this allows considerable flexibility in optimizing the BCA assay for each application, it also requires that the optimized procedure be followed exactly every time the assay is done.

At room temperature, following an initial lag phase, the rate of color formation remains relatively constant for hours. If the incubation time alone is increased, the total amount of color produced by a given mass of protein increases.

If the incubation temperature is increased, the rate of color formation increases and the total amount of color produced by a given mass of protein increases. After cooling the reaction mixture back to room temperature, the rate of color development slows from its initial rate. As both time and temperature are increased, the total amount of color produced by a given mass

of protein approaches a maximum. This is apparent from the dramatic decrease in the rate of color formation upon cooling to room temperature following incubation at 60°C for 30 min.

Above 75°C, a black precipitate forms in the BCA reaction mixture and the absorbance at 562 nm of the blank increases dramatically. This appears to be caused by the formation of copper oxide at high temperature.

For greatest accuracy and precision when comparing sets of data from multiple runs, a set of standards must be included with each run, and the standards and the samples must be treated exactly the same.

The biuret total protein reagent

The biuret total protein reagent is considerably less sensitive to total protein than the other three protein assay reagents discussed in this unit. This limits the applications in which the biuret reagent can be used. Since the primary use of the biuret reagent has been for serum total protein in the clinical laboratory, there is little published information about its compatibility with substances and reagents common to nonclinical samples.

Anticipated Results

Standard curves

Typical standard curves are shown in Figures A.3H.2, A.3H.4, A.3H.6, and A.3H.8 for each of the four assay methods. In each case, the tube protocols were performed in duplicate on diluted BSA or BGG standard. The color in each tube was measured at the appropriate wavelength in a dual-beam spectrophotometer. The net absorbance for each sample was plotted versus its protein concentration.

Figure A.3H.2 shows the color response curves obtained with the Modified Lowry Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 750 nm versus the protein concentration at seven concentrations from 125 to 2000 µg/ml for each protein. Note that the response curve for BGG is higher than the response curve for BSA.

Figure A.3H.4 shows the color response curves obtained with the BCA Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 562 nm versus the protein concentration at seven concentrations from 125 to 2000 µg/ml for each protein. Note that the response curve for BGG is higher than the response curve for BSA.

Figure A.3H.6 shows the color response curves obtained with the biuret total protein reagent using BSA and BGG. The graph shows the net absorbance at 540 nm versus the protein at eight concentrations from 5 to 100 mg/ml.

Figure A.3H.8 shows the color response curves obtained with the Coomassie Plus Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 595 nm versus the protein concentration at seven concentrations from 125 to 2000 µg/ml for each protein. Note that the color response curve for BGG is lower than the response curve for BSA.

Protein-to-protein variation

Each protein in a sample is unique and can demonstrate that individuality in protein assays as variation in the color response. Such protein-to-protein variation refers to differences in the amount of color (absorbance) that are obtained when the same mass (microgram or milligram) of various proteins are assayed concurrently (i.e., in the same run) by the same method. These differences in color response relate to differences among proteins due to amino acid sequence, isoelectric point (pI), secondary structure, and the presence of certain side chains or prosthetic groups.

To analyze protein-to-protein variation for each method, a group of fourteen proteins was assayed in duplicate using the standard tube protocol in a single run. The net (blank-corrected) average absorbance for each protein was calculated. To make it easier to interpret, the net absorbance for each protein was expressed as a ratio to the net absorbance for BSA. If a protein has a ratio of 0.80, it means that the protein produces ~80% of the color that is obtained for an equivalent mass of BSA.

Table A.3H.5 demonstrates the relative degree of protein-to-protein variation that can be expected with the different protein assay methods. This differential may be a consideration in selecting a protein assay method, especially if the relative color response ratio of the protein in the samples is unknown. As expected, the protein assay methods that share the same basic chemistry show similar protein-to-protein variation.

The protein-to-protein variation observed with the various protein assay methods makes it obvious why the largest source of error for protein assays is the choice of protein for the standard curve. If the sample contained IgG as the major protein and BSA was used for the standard curve, the estimated total protein concentration of the sample will be inaccurate. Whether the concentration was underestimated or overestimated depends upon which total pro-

Table A.3H.5 Protein-to-Protein Variation[a]

Protein tested	Ratios obtained with the BCA method	Ratios obtained with the Coomassie plus method	Ratios obtained with the modified Lowry method
Albumin, bovine (BSA)	1.00	1.00	1.00
Aldolase, rabbit	0.85	0.74	0.94
α-Chymotrypsinogen, bovine	1.14	0.52	1.17
Cytochrome C, horse	0.83	1.03	0.94
Gamma globulin, bovine	1.11	0.58	1.14
IgG, bovine	1.21	0.63	1.29
IgG, human	1.09	0.66	1.13
IgG, mouse	1.18	0.62	1.20
IgG, rabbit	1.12	0.43	1.19
IgG, sheep	1.17	0.57	1.28
Insulin, bovine pancreas	1.08	0.67	1.12
Myoglobin	0.74	1.15	0.90
Ovalbumin	0.93	0.68	1.02
Transferrin, human	0.89	0.90	0.92
Average ratio	1.02	0.73	1.09
Standard Deviation (SD)	0.15	0.12	0.13
Coefficient of Variation	0.15	0.29	0.12

[a]The protein-to-protein variation in color response was measured at 1000 µg/ml for each protein in duplicate using the standard tube protocol. Within each assay, the average net or blank-corrected absorbance was determined for each protein. The average net absorbance for each protein was divided by the average net absorbance obtained with BSA and expressed as a ratio. The standard deviation (SD) and the coefficient of variation (CV) is presented for the fourteen proteins assayed on the three methods. By comparing the CV's, the relative degree of protein-to-protein variation to be expected with the three methods can be assessed.

tein assay method was used. If the Coomassie Plus Protein Assay Reagent was used, the total protein (IgG) concentration in the sample would be underestimated by ~40%. (From Table A.3H.5, the response ratio for IgG is ~0.58 for IgG compared to 1.00 for BSA.) If the BCA Protein Assay Reagent was used, the total protein (IgG) concentration in the sample would be overestimated by ~15%. (From Table A.3H.5, the response ratio for IgG is ~1.15 for IgG compared to 1.00 for BSA.) On the other hand, if BGG had been used for both standard curves, the total protein estimates for the sample would have been in much greater agreement between the two methods.

While Table A.3H.5 is useful because it provides an estimate of the protein-to-protein variation in color response that can be expected with each method, it does not tell the whole story. Because the comparisons were done at a single protein concentration, it is not apparent that the color response ratio also varies with changes in protein concentration.

Compatible and incompatible substances

An extensive list of substances that have been found to be compatible with each of the reagents is shown in Table A.3H.6. Each substance was assayed in duplicate using the standard tube protocol for each reagent. In addition to adding the substance to a sample containing 1000 µg/ml BSA, a blank sample containing only the substance was tested. When added to the sample, a substance was deemed to be compatible with a reagent if the blank-corrected absorbance for the sample containing the substance was within 10% of the blank-corrected absorbance for the sample containing only BSA (also at 1000 µg/ml).

Time Considerations

The amount of time required to complete a total protein assay will vary for the four colorimetric total protein assay methods described. For the purpose of providing an estimate of the amount of time required to perform a run by each method, it was assumed that the run included twenty samples and eight standards (including the blank) and that each sample or standard was assayed in duplicate using the

Table A.3H.6 Maximum Compatible Sample Concentration of 92 Substances[a]

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
Detergents			
Brij 35	5.0%	0.062%	0.031%
Brij 56	1.0%	0.031%	0.062%
Brij 58	1.0%	0.016%	0.062%
CHAPS	5.0%	5.0%	0.062%
CHAPSO	5.0%	5.0%	0.031%
Deoxycholic acid	5.0%	0.04%	Not tested
Lubrol PX	1.0%	0.031%	0.031%
Nonidet P-40	5.0%	0.5%	0.016%
Octyl glucoside	5.0%	0.5%	0.031%
Octyl β-thioglucoside	5.0%	3.0%	Not tested
SDS (lauryl)	5.0%	0.016%	1.0%
SPAN 20	1.0%	0.5%	0.25%
Triton X-100	5.0%	0.062%	0.031%
Triton X-114	1.0%	0.062%	0.031%
Triton X-305	1.0%	0.125%	0.031%
Triton X-405	1.0%	0.25%	0.031%
Tween 20	5.0%	0.031%	0.062%
Tween 60	5.0%	0.025%	Not tested
Tween 80	5.0%	0.016%	0.031%
Zwittergent 3-14	1.0%	0.025%	Not tested
Salts and buffers			
ACES, pH 7.8	25 mM	100 mM	Not tested
Ammonium sulfate	1.5 M	1 M	Not compatible
Asparagine	1 mM	10 mM	5 mM
Bicine, pH 8.4	20 mM	100 mM	Not tested
Bis-Tris, pH 6.5	33 mM	100 mM	Not tested
Borate (50 mM), pH 8.5 (BupH pack)	Undiluted	Undiluted	Not tested
B-PER cell lysis reagent	Undiluted	Diluted 1:2	Not tested
Calcium chloride in TBS	10 mM	10 mM	Not tested
Carbonate/bicarbonate, Na (0.2 M), pH 9.4	Undiluted	Undiluted	Not tested
Cesium bicarbonate	100 mM	100 mM	50 mM
CHES, pH 9.0	100 mM	100 mM	Not tested
Cobalt chloride in TBS	0.8 mM	10 mM	Not tested
EPPS, pH 8.0	100 mM	100 mM	Not tested
Ferric chloride in TBS	10 mM	10 mM	Not tested
Glycine	1 mM	100 mM	100 mM
HEPES	100 mM	100 mM	1 mM
Imidazole, pH 10.2	50 mM	200 mM	25 mM
MES, pH 6.1	100 mM	100 mM	100 mM
0.1 M MES/0.9% NaCl, pH 4.7	Undiluted	Undiluted	Not tested

continued

Table A.3H.6 Maximum Compatible Sample Concentration of 92 Substances[a], *continued*

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
Salts and bufferes (continued)			
MOPS, pH 7.2	100 mM	100 mM	Not tested
Modified Dulbecco's PBS	Undiluted	Undiluted	Not tested
Nickel chloride in TBS	10 mM	10 mM	Not tested
Phosphate buffered saline (PBS), pH 7.2	Undiluted	Undiluted	Not tested
PIPES, pH 6.8	100 mM	100 mM	Not tested
RIPA lysis buffer, pH 8.0	Undiluted	Diluted 1:40	Not tested
Sodium acetate	200 mM	180 mM	200 mM
Sodium azide	0.2%	0.5%	0.2%
Sodium bicarbonate	100 mM	100 mM	100 mM
Sodium chloride	1.0 M	1 M	1 M
Sodium citrate, pH 4.8	200 mM	200 mM	Not tested
Sodium phosphate	100 mM	100 mM	100 mM
Tricine, pH 8.0	25 mM	100 mM	Not tested
Triethanolamine, pH 7.8	25 mM	100 mM	Not tested
Tris	250 mM	2 M	10 mM
TBS, pH 7.6	Undiluted	Undiluted	Not tested
25 mM Tris/192 mM glycine, pH 8.0	Diluted 1:3	Undiluted	Not tested
25 mM Tris/192 mM glycine/0.1% SDS, pH 8.0	Undiluted	Diluted 1:4	Not tested
Zinc chloride in TBS	10 mM	10 mM	Not tested
Reducing agents			
N-acetylglucosamine in PBS	10 mM	100 mM	Not tested
Ascorbic acid	Not compatible	50 mM	1 mM
Catecholamines	Not compatible	Not tested	Not tested
Creatinine	Not compatible	Not tested	Not tested
Glucose	10 mM	1 M	0.1 mM
Melibiose	Not compatible		
Potassium thiocyanate	3 M		
Thiol-containing agents			
Cysteine	Not compatible	10 mM	1 mM
Dithioerythritol (DTE)	1 mM	1 mM	Not compatible
Dithiothreitol (DTT)	1 mM	5 mM	Not compatible
2-Mercaptoethanol	0.01%	1 M	1 mM
Thimerosal	0.01%	0.01%	0.01%
Chelating agents			
EDTA	10 mM	100 mM	1 mM
EGTA	Not compatible	2 mM	1 mM
Sodium citrate, pH 4.8	200 mM	200 mM	0.1 mM

continued

Table A.3H.6 Maximum Compatible Sample Concentration of 92 Substances[a], *continued*

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
Solvents/miscellaneous			
Acetone	10%	10%	10%
Acetonitrile	10%	10%	10%
Aprotinin	10 mg/liter	10 mg/liter	10 mg/liter
DMF	10%	10%	10%
DMSO	10%	10%	10%
Ethanol	10%	10%	10%
Glycerol (fresh)	10%	10%	10%
Guanidine·HCl	4 M	3.5 M	100 mM
Hydrochloric acid	100 mM	100 mM	100 mM
Leupeptin	10 mg/liter	10 mg/liter	10 mg/liter
Methanol	10%	10%	10%
Phenol Red	Not compatible	0.5 mg/liter	0.1 mg/liter
PMSF	1 mM	1 mM	1 mM
Sodium hydroxide	100 mM	100 mM	100 mM
Sucrose	40%	10%	7.5%
TLCK	0.1 mg/liter	0.1 mg/liter	0.01 mg/liter
TPCK	0.1 mg/liter	0.1 mg/liter	0.1 mg/liter
Urea	3 M	3 M	3 M
o-vanadate in PBS	1 mM	1 mM	Not tested

[a]Taken from the Protein Assay Technical Handbook, Pierce Chemical, 1999.

Table A.3H.7 Estimated Time Requirements

Method	Incubation time(s)	Estimated total assay time
Modified Lowry Reagent	10 and 30 min	110 min (1 hr, 50 min)
Coomassie Plus Reagent	10 min	80 min (1 hr, 20 min)
BCA Reagent	30 min	100 min (1 hr, 40 min)
Biuret Reagent	10 min	80 min (1 hr, 20 min)

standard tube protocol. The estimates do not include the time spent obtaining the samples or the time it takes to prepare the samples for analysis, but they do include the incubation time(s) plus an estimate of the time it takes to do the following:

1. Prepare (dilute) the standard protein in the diluent buffer (10 min).
2. Organize the run and label the tubes (5 min).
3. Pipet the samples and reagents into the tubes (10 min).
4. Mix or incubate the tubes or plates (varies).
5. Measure the color produced in the tubes (15 min).
6. Graph the standard curve, calculate, record, and report the results (30 min).

For each of the four methods, a run of 20 samples (unknowns) and the standard curve (each done in duplicate) can be completed in the time estimated in Table A.3H.7.

Acknowledgements

The author is indebted to Bob Vigna, Greg Hermanson, and Patti Domen for their assistance in reviewing this unit.

Literature Cited

Autenrieth, W. and Mink, F. 1915. Über kolorimetrische Bestimmungsmethoden: Die quantitative Bestimmung von Harneiweiss. *München Med. Wochenschr.* 62:1417.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Doumas, B.T., Bayse, D.D., and Carter, R.J. 1981. A candidate reference method for determination of total protein in serum. *Clin. Chem.* 27:1642.

Fine, J. 1935. Biuret method of estimating albumin and globulin in serum and urine. *Biochem. J.* 29:799.

Goa, J. 1953. A microbiuret method for protein determination. *Scand. J. Clin. Lab. Invest.* 5:218-222.

Gornall, A.G., Baardawill, C.J., and David, M.M. 1949. Determination of serum proteins by means of the biuret reagent. *J. Biol. Chem.* 177:751-766.

Hiller, A. 1926. Determination of albumin and globulin in urine. *Proc. Soc. Exp. Biol. Med.* 24:385.

Kingsley, G.R. 1942. The direct biuret method for the determination of serum proteins as applied to photoelectric and visual colorimetry. *J. Lab. Clin. Med.* 27:840-845.

Legler, G., Muller-Platz, C.M., Mentges-Hettkamp, M., Pflieger, G., and Julich, E. 1985. On the chemical basis of the Lowry protein determination. *Anal. Biochem.* 150:278-287.

Lowry, O.H., Nira, J., Rosenbrough, A., Farr, L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.

Riegler, E. 1914. Eine kolorimetrische Bestimmungsmethode des Eiweisses. *Z. Anal. Chem.* 53:242-254.

Smith, P.K., Krohn, R.I., Hermanson, G.H., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goike, N.M., Olson, B.J., and Klenk, D.K. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.

Weichselbaum, T.E. 1946. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. *Am. J. Clin. Pathol.* 16:40.

Young, D.S. 1990. Effects of Drugs on Clinical Tests. (Suppl. 1, 1991). AACC Press. Washington, D.C.

Key References

Akins, R.E. and Tuan, R.S. 1992. Measurement of protein in 20 seconds using a microwave BCA assay. *Biotechniques* 12:496-499.

Use of the BCA protein assay in a microtiter-plate format which utilizes a microwave oven as the heat source to shorten the color development time to 20 sec.

Beyer, R.E. 1983. A rapid biuret assay for protein of whole fatty tissues. *Anal. Biochem.* 129:483-485.

Accurate total protein quantitation of fatty tissues was achieved by adding 0.1 ml 10% sodium deoxycholate, pH 8.0 and 2.9 ml biuret reagent to each protein pellet following an acetone/ether wash step. After sonication, each sample was heated for 30 sec in a boiling water bath to develop full color.

Brown, A.J., Jarvis, K., and Hyland, K. 1989. Protein measurement using bicinchoninic acid: Elimination of interfering substances. *Anal. Biochem.* 180:136-139.

Describes a procedure using trichloroacetic acid and sodium deoxycholate to precipitate protein and thus remove soluble substances in the sample that would otherwise interfere in the BCA protein assay.

Compton, S.J. and Jones, C.G. 1985. Mechanism of dye response and interference in the Bradford protein assay. *Anal. Biochem.* 151:369-374.

Found that the anionic form of the Coomassie dye reacts primarily with arginine residues within the macromolecular protein. Coomassie dye reacts to a lesser extent with other basic amino acid residues (His, Lys) and aromatic residues (Try, Tyr, Phe) present in macromolecular proteins, but not with the free amino acids. Dye binding is attributed to van der Waals forces and hydrophobic interactions. The interference seen with bases, detergents, and other compounds can be explained by their effects upon the equilibrium between the three dye forms (cationic, neutral, anionic).

Crowley, L.V. 1969. Interference with certain chemical analyses caused by dextran. *Am. J. Clin. Pathol.* 51:425.

Dextran at high concentrations causes a slight overestimation of the total protein concentration with the biuret reagent.

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry, et al. Which is more generally applicable? *Anal. Biochem.* 83:346-356.

A deoxycholate-trichloroacetic acid protein precipitation technique that provides for rapid recovery of soluble and membrane-bound proteins from interfering substances. Interference by lipids and nonionic or cationic detergents is alleviated by adding SDS.

Peterson, G.L. 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.* 100:201-220.

A thorough review article that examines the reaction mechanism involved when protein reacts with the Lowry reagent. An extensive list of possibly interfering substances is presented along with methods of coping with those interfering substances. Finally, the method of Lowry is compared to other methods. There is an extensive list of references.

Sorenson, K. and Brodbeck, U. 1986. A sensitive protein assay method using micro-titer plates. *Experientia* 42:161-162.

A direct scale-down of the BCA method for test tubes that is suitable for microtiter plates.

Tal, M., Silberstein, A., and Nusser, D. 1980. Why does Coomassie Brilliant Blue interact differently with different proteins? *J. Biol. Chem.* 260:9976-9980.

Analysis of Scatchard plots showed that the number of Coomassie dye ligands bound to each protein is approximately proportional to the number of positive charges on the protein. About 1.5 to 3 dye molecules are bound to each positive charge on the protein.

Watters, C. 1978. A one-step biuret assay for protein in the presence of detergent. *Anal. Biochem.* 88:695-698.

A modified biuret reagent was formulated (sodium tartrate replaces sodium potassium tartrate, the sodium hydroxide concentration is reduced, and potassium iodide was deleted). When the modified biuret reagent was mixed with samples containing 2% detergent (SDS or sodium cholate or Triton X-100), it resulted in less protein-to-protein variation among six proteins.

Weichselbaum, 1946. See above.

Used sodium potassium tartrate as a stabilizer and added potassium iodide to prevent autoreduction of the biuret reagent; however, this reagent was found to be unstable after long storage.

Wiechelman, K., Braun, R., and Fitzpatrick, J. 1988. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem.* 175:231-237.

Cysteine, cystine, tryptophan, tyrosine, and the peptide bond are capable of reducing Cu^{2+} to Cu^{+}, but the extent of color formation is not simply the sum of the contributions from the various color producing functional groups. At 60°C, tryptophan, tyrosine, and the peptide bond are more completely oxidized than they are at 37°C, which is observed by the much greater extent of color developed at the higher temperature.

Internet Resources

http://www.piercenet.com

This site allows access to Pierce's on-line product catalog and special notice of all new products and other Pierce publications. If specific information on a product is needed, the instruction booklet can be downloaded. If additional help is needed, the technical assistance department can be reached by e-mail.

http://www.sigma-aldrich.com

This site allows access to the Sigma Diagnostics product line and applications or other technical information on their products. Questions can be sent by e-mail to the technical assistance department.

Contributed by Randall I. Krohn
Pierce Chemical
Rockford, Illinois

SELECTED SUPPLIERS OF REAGENTS AND EQUIPMENT

Listed below are addresses and phone numbers of commercial suppliers who have been recommended for particular items used in our manuals because: (1) the particular brand has actually been found to be of superior quality, or (2) the item is difficult to find in the marketplace. Consequently, this compilation may not include some important vendors of biological supplies. For comprehensive listings, see *Linscott's Directory of Immunological and Biological Reagents* (Santa Rosa, CA), *The Biotechnology Directory* (Stockton Press, New York), the annual Buyers' Guide supplement to the journal *Bio/Technology*, as well as various sites on the Internet.

A.C. Daniels
72-80 Akeman Street
Tring, Hertfordshire, HP23 6AJ, UK
(44) 1442 826881
FAX: (44) 1442 826880

A.D. Instruments
5111 Nations Crossing Road #8
Suite 2
Charlotte, NC 28217
(704) 522-8415 FAX: (704) 527-5005
http://www.us.endress.com

A.J. Buck
11407 Cronhill Drive
Owings Mill, MD 21117
(800) 638-8673 FAX: (410) 581-1809
(410) 581-1800
http://www.ajbuck.com

A.M. Systems
131 Business Park Loop
P.O. Box 850
Carlsborg, WA 98324
(800) 426-1306 FAX: (360) 683-3525
(360) 683-8300
http://www.a-msystems.com

Aaron Medical Industries
7100 30th Avenue North
St. Petersburg, FL 33710
(727) 384-2323 FAX: (727) 347-9144
http://www.aaronmed.com

Abbott Laboratories
100 Abbott Park Road
Abbott Park, IL 60064
(800) 323-9100 FAX: (847) 938-7424
http://www.abbott.com

ABCO Dealers
55 Church Street Central Plaza
Lowell, MA 01852
(800) 462-3326 (978) 459-6101
http://www.lomedco.com/abco.htm

Aber Instruments
5 Science Park
Aberystwyth, Wales SY23 3AH, UK
(44) 1970 636300
FAX: (44) 1970 615455
http://www.aber-instruments.co.uk

ABI Biotechnologies
See Perkin-Elmer

ABI Biotechnology
See Apotex

Access Technologies
Subsidiary of Norfolk Medical
7350 N. Ridgeway
Skokie, IL 60076
(877) 674-7131 FAX: (847) 674-7066
(847) 674-7131
http://www.norfolkaccess.com

Accurate Chemical and Scientific
300 Shames Drive
Westbury, NY 11590
(800) 645-6264 FAX: (516) 997-4948
(516) 333-2221
http://www.accuratechemical.com

AccuScan Instruments
5090 Trabue Road
Columbus, OH 43228
(800) 822-1344 FAX: (614) 878-3560
(614) 878-6644
http://www.accuscan-usa.com

AccuStandard
125 Market Street
New Haven, CT 06513
(800) 442-5290 FAX: (877) 786-5287
http://www.accustandard.com

Ace Glass
1430 NW Boulevard
Vineland, NJ 08360
(800) 223-4524 FAX: (800) 543-6752
(609) 692-3333

ACO Pacific
2604 Read Avenue
Belmont, CA 94002
(650) 595-8588 FAX: (650) 591-2891
http://www.acopacific.com

Acros Organic
See Fisher Scientific

Action Scientific
P.O. Box 1369
Carolina Beach, NC 28428
(910) 458-0401 FAX: (910) 458-0407

AD Instruments
1949 Landings Drive
Mountain View, CA 94043
(888) 965-6040 FAX: (650) 965-9293
(650) 965-9292
http://www.adinstruments.com

Adaptive Biosystems
15 Ribocon Way
Progress Park
Luton, Bedsfordshire LU4 9UR, UK
(44)1 582-597676
FAX: (44)1 582-581495
http://www.adaptive.co.uk

Adobe Systems
1585 Charleston Road
P.O. Box 7900
Mountain View, CA 94039
(800) 833-6687 FAX: (415) 961-3769
(415) 961-4400
http://www.adobe.com

Advanced Bioscience Resources
1516 Oak Street, Suite 303
Alameda, CA 94501
(510) 865-5872 FAX: (510) 865-4090

Advanced Biotechnologies
9108 Guilford Road
Columbia, MD 21046
(800) 426-0764 FAX: (301) 497-9773
(301) 470-3220
http://www.abionline.com

Advanced ChemTech
5609 Fern Valley Road
Louisville, KY 40228
(502) 969-0000
http://www.peptide.com

Advanced MachIning and Tooling
9850 Businesspark Avenue
San Diego, CA 92131
(858) 530-0751 FAX: (858) 530-0611
http://www.amtmfg.com

Advanced Magnetics
See PerSeptive Biosystems

Advanced Process Supply
See Naz-Dar-KC Chicago

Advanced Separation Technologies
37 Leslie Court
P.O. Box 297
Whippany, NJ 07981
(973) 428-9080 FAX: (973) 428-0152
http://www.astecusa.com

Advanced Targeting Systems
11175-A Flintkote Avenue
San Diego, CA 92121
(877) 889-2288 FAX: (858) 642-1989
(858) 642-1988
http://www.ATSbio.com

Advent Research Materials
Eynsham, Oxford OX29 4JA, UK
(44) 1865-884440
FAX: (44) 1865-84460
http://www.advent-rm.com

Advet
Industrivagen 24
S-972 54 Lulea, Sweden
(46) 0920-211887
FAX: (46) 0920-13773

Aeroflex Incorporated
35 South Service Road
P.O. Box 6022
Plainview, NY 11803-0622
(516) 694-6700 FAX: (516) 694-0658
800-843-1553
http://www.aeroflex.com/

Aesculap, Inc.
3773 Corporate Parkway
Center Valley, PA 18034
(800) 282-9000 FAX: (610) 791-6880
http://www.aesculap.com

Affinity Chromatography
307 Huntingdon Road
Girton, Cambridge CB3 OJX, UK
(44) 1223 277192
FAX: (44) 1223 277502
http://www.affinity-chrom.com

Affinity Sensors
See Labsystems Affinity Sensors

Affymetrix
3380 Central Expressway
Santa Clara, CA 95051
(408) 731-5000 FAX: (408) 481-0422
(800) 362-2447
http://www.affymetrix.com

Agar Scientific
66a Cambridge Road
Stansted CM24 8DA, UK
(44) 1279-813-519
FAX: (44) 1279-815-106
http://www.agarscientific.com

A/G Technology
101 Hampton Avenue
Needham, MA 02494
(800) AGT-2535 FAX: (781) 449-5786
(781) 449-5774
http://www.agtech.com

Agen Biomedical Limited
11 Durbell Street
P.O. Box 391
Acacia Ridge 4110
Brisbane, Australia
61-7-3370-6300 FAX: 61-7-3370-6370
http://www.agen.com

Agilent Technologies
395 Page Mill Road
P.O. Box 10395
Palo Alto, CA 94306
(650) 752-5000
http://www.agilent.com/chem

Agouron Pharmaceuticals
10350 N. Torrey Pines Road
La Jolla, CA 92037
(858) 622-3000 FAX: (858) 622-3298
http://www.agouron.com

Agracetus
8520 University Green
Middleton, WI 53562
(608) 836-7300 FAX: (608) 836-9710
http://www.monsanto.com

AIDS Research and Reference
Reagent Program
U.S. Department of Health and Human Services
625 Lofstrand Lane
Rockville, MD 20850
(301) 340-0245 FAX: (301) 340-9245
http://www.aidsreagent.org

AIN Plastics
249 East Sanford Boulevard
P.O. Box 151
Mt. Vernon, NY 10550
(914) 668-6800 FAX: (914) 668-8820
http://www.tincna.com

Air Products and Chemicals
7201 Hamilton Boulevard
Allentown, PA 18195
(800) 345-3148 FAX: (610) 481-4381
(610) 481-6799
http://www.airproducts.com

ALA Scientific Instruments
1100 Shames Drive
Westbury, NY 11590
(516) 997-5780 FAX: (516) 997-0528
http://www.alascience.com

Aladin Enterprises
1255 23rd Avenue
San Francisco, CA 94122
(415) 468-0433 FAX: (415) 468-5607

Aladdin Systems
165 Westridge Drive
Watsonville, CA 95076
(831) 761-6200 FAX: (831) 761-6206
http://www.aladdinsys.com

Alcide
8561 154th Avenue NE
Redmond, WA 98052
(800) 543-2133 FAX: (425) 861-0173
(425) 882-2555
http://www.alcide.com

Aldevron
3233 15th Street, South
Fargo, ND 58104
(877) Pure-DNA FAX: (701) 280-1642
(701) 297-9256
http://www.aldevion.com

Aldrich Chemical
P.O. Box 2060
Milwaukee, WI 53201
(800) 558-9160 FAX: (800) 962-9591
(414) 273-3850 FAX: (414) 273-4979
http://www.aldrich.sial.com

Alexis Biochemicals
6181 Cornerstone Court East, Suite 103
San Diego, CA 92121
(800) 900-0065 FAX: (858) 658-9224
(858) 658-0065
http://www.alexis-corp.com

Alfa Aesar
30 Bond Street
Ward Hill, MA 10835
(800) 343-0660 FAX: (800) 322-4757
(978) 521-6300 FAX: (978) 521-6350
http://www.alfa.com

Alfa Laval
Avenue de Ble 5 - Bazellaan 5
BE-1140 Brussels, Belgium
32(2) 728 3811
FAX: 32(2) 728 3917 or 32(2) 728 3985
http://www.alfalaval.com

Alice King Chatham Medical Arts
11915-17 Inglewood Avenue
Hawthorne, CA 90250
(310) 970-1834 FAX: (310) 970-0121
(310) 970-1063

Allegiance Healthcare
800-964-5227
http://www.allegiance.net

Allelix Biopharmaceuticals
6850 Gorway Drive
Mississauga, Ontario
L4V 1V7 Canada
(905) 677-0831 FAX: (905) 677-9595
http://www.allelix.com

Allentown Caging Equipment
Route 526, P.O. Box 698
Allentown, NJ 08501
(800) 762-CAGE FAX: (609) 259-0449
(609) 259-7951
http://www.acecaging.com

Alltech Associates
Applied Science Labs
2051 Waukegan Road
P.O. Box 23
Deerfield, IL 60015
(800) 255-8324 FAX: (847) 948-1078
(847) 948-8600
http://www.alltechweb.com

Alomone Labs
HaMarpeh 5
P.O. Box 4287
Jerusalem 91042, Israel
972-2-587-2202 FAX: 972-2-587-1101
US: (800) 791-3904
FAX: (800) 791-3912
http://www.alomone.com

Alpha Innotech
14743 Catalina Street
San Leandro, CA 94577
(800) 795-5556 FAX: (510) 483-3227
(510) 483-9620
http://www.alphainnotech.com

Altec Plastics
116 B Street
Boston, MA 02127
(800) 477-8196 FAX: (617) 269-8484
(617) 269-1400

Alza
1900 Charleston Road
P.O. Box 7210
Mountain View, CA 94043
(800) 692-2990 FAX: (650) 564-7070
(650) 564-5000
http://www.alza.com

Alzet
c/o Durect Corporation
P.O. Box 530
10240 Bubo Road
Cupertino, CA 95015
(800) 692-2990 (408) 367-4036
FAX: (408) 865-1406
http://www.alzet.com

Amac
160B Larrabee Road
Westbrook, ME 04092
(800) 458-5060 FAX: (207) 854-0116
(207) 854-0426

Amaresco
30175 Solon Industrial Parkway
Solon, Ohio 44139
(800) 366-1313 FAX: (440) 349-1182
(440) 349-1313

Ambion
2130 Woodward Street, Suite 200
Austin, TX 78744
(800) 888-8804 FAX: (512) 651-0190
(512) 651-0200
http://www.ambion.com

American Association of Blood Banks
College of American Pathologists
325 Waukegan Road
Northfield, IL 60093
(800) 323-4040 FAX: (847) 8166
(847) 832-7000
http://www.cap.org

American Bio-Technologies
See Intracel Corporation

American Bioanalytical
15 Erie Drive
Natick, MA 01760
(800) 443-0600 FAX: (508) 655-2754
(508) 655-4336
http://www.americanbio.com

American Cyanamid
P.O. Box 400
Princeton, NJ 08543
(609) 799-0400 FAX: (609) 275-3502
http://www.cyanamid.com

American HistoLabs
7605-F Airpark Road
Gaithersburg, MD 20879
(301) 330-1200 FAX: (301) 330-6059

American International Chemical
17 Strathmore Road
Natick, MA 01760
(800) 238-0001 (508) 655-5805
http://www.aicma.com

American Laboratory Supply
See American Bioanalytical

American Medical Systems
10700 Bren Road West
Minnetonka, MN 55343
(800) 328-3881 FAX: (612) 930-6654
(612) 933-4666
http://www.visitams.com

American Qualex
920-A Calle Negocio
San Clemente, CA 92673
(949) 492-8298 FAX: (949) 492-6790
http://www.americanqualex.com

American Radiolabeled Chemicals
11624 Bowling Green
St. Louis, MO 63146
(800) 331-6661 FAX: (800) 999-9925
(314) 991-4545 FAX: (314) 991-4692
http://www.arc-inc.com

American Scientific Products
See VWR Scientific Products

American Society for Histocompatibility and Immunogenetics
P.O. Box 15804
Lenexa, KS 66285
(913) 541-0009 FAX: (913) 541-0156
http://www.swmed.edu/home_pages/ASHI/ashi.htm

American Type Culture Collection (ATCC)
10801 University Boulevard
Manassas, VA 20110
(800) 638-6597 FAX: (703) 365-2750
(703) 365-2700
http://www.atcc.org

Amersham
See Amersham Pharmacia Biotech

Amersham Biosciences Corporation
800 Centennial Ave.
P.O. Box 1327
Piscataway, NJ 08855-1327
(800) 242-2599 FAX: (732) 457-0557
(732) 457-8000
http://www4.amershambiosciences.com

Amersham International
Amersham Place
Little Chalfont, Buckinghamshire
HP7 9NA, UK
(44) 1494-544100
FAX: (44) 1494-544350
http://www.apbiotech.com

Amersham Medi-Physics
Also see Nycomed Amersham
3350 North Ridge Avenue
Arlington Heights, IL 60004
(800) 292-8514 FAX: (800) 807-2382
http://www.nycomed-amersham.com

Amersham Pharmacia Biotech
800 Centennial Avenue
P.O. Box 1327
Piscataway, NJ 08855
(800) 526-3593 FAX: (877) 295-8102
(732) 457-8000
http://www.apbiotech.com

Amgen
1 Amgen Center Drive
Thousand Oaks, CA 91320
(800) 926-4369 FAX: (805) 498-9377
(805) 447-5725
http://www.amgen.com

Amicon
Scientific Systems Division
72 Cherry Hill Drive
Beverly, MA 01915
(800) 426-4266 FAX: (978) 777-6204
(978) 777-3622
http://www.amicon.com

Amika
8980F Route 108
Oakland Center
Columbia, MD 21045
(800) 547-6766 FAX: (410) 997-7104
(410) 997-0100
http://www.amika.com

Amoco Performance Products
See BPAmoco

AMPI
See Pacer Scientific

Amrad
576 Swan Street
Richmond, Victoria 3121, Australia
613-9208-4000
FAX: 613-9208-4350
http://www.amrad.com.au

Amresco
30175 Solon Industrial Parkway
Solon, OH 44139
(800) 829-2805 FAX: (440) 349-1182
(440) 349-1199

Anachemia Chemicals
3 Lincoln Boulevard
Rouses Point, NY 12979
(800) 323-1414 FAX: (518) 462-1952
(518) 462-1066
http://www.anachemia.com

Ana-Gen Technologies
4015 Fabian Way
Palo Alto, CA 94303
(800) 654-4671 FAX: (650) 494-3893
(650) 494-3894
http://www.ana-gen.com

Analox Instruments USA
P.O. Box 208
Lunenburg, MA 01462
(978) 582-9368 FAX: (978) 582-9588
http://www.analox.com

Analytical Biological Services
Cornell Business Park 701-4
Wilmington, DE 19801
(800) 391-2391 FAX: (302) 654-8046
(302) 654-4492
http://www.ABSbioreagents.com

Analytical Genetics Testing Center
7808 Cherry Creek S. Drive, Suite 201
Denver, CO 80231
(800) 204-4721 FAX: (303) 750-2171
(303) 750-2023
http://www.geneticid.com

AnaSpec
2149 O'Toole Avenue, Suite F
San Jose, CA 95131
(800) 452-5530 FAX: (408) 452-5059
(408) 452-5055
http://www.anaspec.com

Ancare
2647 Grand Avenue
P.O. Box 814
Bellmore, NY 11710
(800) 645-6379 FAX: (516) 781-4937
(516) 781-0755
http://www.ancare.com

Ancell
243 Third Street North
P.O. Box 87
Bayport, MN 55033
(800) 374-9523 FAX: (651) 439-1940
(651) 439-0835
http://www.ancell.com

Anderson Instruments
500 Technology Court
Smyrna, GA 30082
(800) 241-6898 FAX: (770) 319-5306
(770) 319-9999
http://www.graseby.com

Andreas Hettich
Gartenstrasse 100
Postfach 260
D-78732 Tuttlingen, Germany
(49) 7461 705 0
FAX: (49) 7461 705-122
http://www.hettich-centrifugen.de

Anesthetic Vaporizer Services
10185 Main Street
Clarence, NY 14031
(719) 759-8490
www.avapor.com

Animal Identification and Marking Systems (AIMS)
13 Winchester Avenue
Budd Lake, NJ 07828
(908) 684-9105 FAX: (908) 684-9106
http://www.animalid.com

Annovis
34 Mount Pleasant Drive
Aston, PA 19014
(800) EASY-DNA FAX: (610)
361-8255
(610) 361-9224
http://www.annovis.com

Apotex
150 Signet Drive
Weston, Ontario M9L 1T9, Canada
(416) 749-9300 FAX: (416) 749-2646
http://www.apotex.com

Apple Scientific
11711 Chillicothe Road, Unit 2
P.O. Box 778
Chesterland, OH 44026
(440) 729-3056 FAX: (440) 729-0928
http://www.applesci.com

Applied Biosystems
See PE Biosystems

Applied Imaging
2380 Walsh Avenue, Bldg. B
Santa Clara, CA 95051
(800) 634-3622 FAX: (408) 562-0264
(408) 562-0250
http://www.aicorp.com

Applied Photophysics
203-205 Kingston Road
Leatherhead, Surrey, KT22 7PB
UK
(44) 1372-386537

Applied Precision
1040 12th Avenue Northwest
Issaquah, Washington 98027
(425) 557-1000
FAX: (425) 557-1055
http://www.api.com/index.html

Applied Spectral Imaging, Inc.
1497 Poinsettia Ave., #158
Vista, CA 92081
(760) 929-2840 FAX: (760) 929-2842
(800) 611-3466
http://www.spectral-imaging.com

Appligene Oncor
Parc d'Innovation
Rue Geiler de Kaysersberg, BP 72
67402 Illkirch Cedex, France
(33) 88 67 22 67
FAX: (33) 88 67 19 45
http://www.oncor.com/prod-app.htm

Applikon
1165 Chess Drive, Suite G
Foster City, CA 94404
(650) 578-1396 FAX: (650) 578-8836
http://www.applikon.com

Appropriate Technical Resources
9157 Whiskey Bottom Road
Laurel, MD 20723
(800) 827-5931 FAX: (410) 792-2837
http://www.atrbiotech.com

APV Gaulin
100 S. CP Avenue
Lake Mills, WI 53551
(888) 278-4321 FAX: (888) 278-5329
http://www.apv.com

Aqualon
See Hercules Aqualon

Aquarium Systems
B141 Tyler Boulevard
Mentor. OH 44060
(800) 822-1100 FAX: (440) 266-8994
(440) 255-1997
http://www.aquaniumsystems.com

Aquebogue Machine and Repair Shop
Box 2055
Main Road
Aquebogue, NY 11931
(631) 722-3635 FAX: (631) 722-3106

Archer Daniels Midland
4666 Faries Parkway
Decatur, IL 62525
(217) 424-5200
http://www.admworld.com

Archimica Florida
P.O. Box 1466
Gainesville, FL 32602
(800) 331-6313 FAX: (352) 371-6246
(352) 376-8246
http://www.archimica.com

Arcor Electronics
1845 Oak Street #15
Northfield, IL 60093
(847) 501-4848

Arcturus Engineering
400 Logue Avenue
Mountain View, CA 94043
(888) 446 7911 FAX: (650) 962 3039
(650) 962 3020
http://www.arctur.com

Ardais Corporation
One ledgemont Center
128 Spring Street
Lexington, MA 02421
(781) 274-6420 (781) 274-6421
http://www.ardais.com

Argonaut Technologies
887 Industrial Road, Suite G
San Carlos, CA 94070
(650) 998-1350 FAX: (650) 598-1359
http://www.argotech.com

Ariad Pharmaceuticals
26 Landsdowne Street
Cambridge, MA 02139
(617) 494-0400 FAX: (617) 494-8144
http://www.ariad.com

Armour Pharmaceuticals
See Rhone-Poulenc Rorer

Aronex Pharmaceuticals
8707 Technology Forest Place
The Woodlands, TX 77381
(281) 367-1666 FAX: (281) 367-1676
http://www.aronex.com

Artisan Industries
73 Pond Street
Waltham, MA 02254
(617) 893-6800
http://www.artisanind.com

ASI Instruments
12900 Ten Mile Road
Warren, MI 48089
(800) 531-1105 FAX: (810) 756-9737
(810) 756-1222
http://www.asi-instruments.com

Aspen Research Laboratories
1700 Buerkle Road
White Bear Lake, MN 55140
(651) 264-6000 FAX: (651) 264-6270
http://www.aspenresearch.com

Associates of Cape Cod
704 Main Street
Falmouth, MA 02540
(800) LAL-TEST FAX: (508) 540-8680
(508) 540-3444
http://www.acciusa.com

Astra Pharmaceuticals
See AstraZeneca

AstraZeneca
1800 Concord Pike
Wilmington, DE 19850
(302) 886-3000 FAX: (302) 886-2972
http://www.astrazeneca.com

AT Biochem
30 Spring Mill Drive
Malvern, PA 19355
(610) 889-9300 FAX: (610) 889-9304

ATC Diagnostics
See Vysis

ATCC
See American Type Culture Collection

Athens Research and Technology
P.O. Box 5494
Athens, GA 30604
(706) 546-0207 FAX: (706) 546-7395

Atlanta Biologicals
1425-400 Oakbrook Drive
Norcross, GA 30093
(800) 780-7788 or (770) 446-1404
FAX: (800) 780-7374 or (770)
446-1404
http://www.atlantabio.com

Atomergic Chemical
71 Carolyn Boulevard
Farmingdale, NY 11735
(631) 694-9000 FAX: (631) 694-9177
http://www.atomergic.com

Atomic Energy of Canada
2251 Speakman Drive
Mississauga, Ontario
L5K 1B2 Canada
(905) 823-9040 FAX: (905) 823-1290
http://www.aecl.ca

ATR
P.O. Box 460
Laurel, MD 20725
(800) 827-5931 FAX: (410) 792-2837
(301) 470-2799
http://www.atrbiotech.com

Aurora Biosciences
11010 Torreyana Road
San Diego, CA 92121
(858) 404-6600 FAX: (858) 404-6714
http://www.aurorabio.com

Automatic Switch Company
A Division of Emerson Electric
50 Hanover Road
Florham Park, NJ 07932
(800) 937-2726 FAX: (973) 966-2628
(973) 966-2000
http://www.asco.com

Avanti Polar Lipids
700 Industrial Park Drive
Alabaster, AL 35007
(800) 227-0651 FAX: (800) 229-1004
(205) 663-2494 FAX: (205) 663-0756
http://www.avantilipids.com

Aventis
BP 67917
67917 Strasbourg Cedex 9, France
33 (0) 388 99 11 00
FAX: 33 (0) 388 99 11 01
http://www.aventis.com

Aventis Pasteur
1 Discovery Drive
Swiftwater, PA 18370
(800) 822-2463 FAX: (570) 839-0955
(570) 839-7187
http://www.aventispasteur.com/usa

Avery Dennison
150 North Orange Grove Boulevard
Pasadena, CA 91103
(800) 462-8379 FAX: (626) 792-7312
(626) 304-2000
http://www.averydennison.com

Avestin
2450 Don Reid Drive
Ottawa, Ontario K1H 1E1, Canada
(888) AVESTIN FAX: (613) 736-8086
(613) 736-0019
http://www.avestin.com

AVIV Instruments
750 Vassar Avenue
Lakewood, NJ 08701
(732) 367-1663 FAX: (732) 370-0032
http://www.avivinst.com

Axon Instruments
1101 Chess Drive
Foster City, CA 94404
(650) 571-9400 FAX: (650) 571-9500
http://www.axon.com

Axygen Scientific, Inc.
33170 Central Ave.
Union City, CA 94587
(510) 494-8900 FAX: (510) 494-0700
(800) 4-AXYGEN
http://www.axygen.com

Azon
720 Azon Road
Johnson City, NY 13790
(800) 847-9374 FAX: (800) 635-6042
(607) 797-2368
http://www.azon.com

BAbCO
1223 South 47th Street
Richmond, CA 94804
(800) 92-BABCO FAX: (510)
412-8940
(510) 412-8930
http://www.babco.com

Bacharach
625 Alpha Drive
Pittsburgh, PA 15238
(800) 736-4666 FAX: (412) 963-2091
(412) 963-2000
http://www.bacharach-inc.com

Bachem Bioscience
3700 Horizon Drive
King of Prussia, PA 19406
(800) 634-3183 FAX: (610) 239-0800
(610) 239-0300
http://www.bachem.com

Bachem California
3132 Kashiwa Street
P.O. Box 3426
Torrance, CA 90510
(800) 422-2436 FAX: (310) 530-1571
(310) 539-4171
http://www.bachem.com

Baekon
18866 Allendale Avenue
Saratoga, CA 95070
(408) 972-8779 FAX: (408) 741-0944

Baker Chemical
See J.T. Baker

Bal-Tec AG
Neugruet 7
FL-9496 Balzers
Principality of Liechtenstein
(423) 3881-212 FAX: (423) 3881-260
http://www.bal-tec.com

Bangs Laboratories
9025 Technology Drive
Fishers, IN 46038
(317) 570-7020 FAX: (317) 570-7034
www.bangslabs.com

Bard Parker
See Becton Dickinson

Barnstead/Thermolyne
P.O. Box 797
2555 Kerper Boulevard
Dubuque, IA 52004
(800) 446-6060 FAX: (319) 589-0516
http://www.barnstead.com

Barrskogen
4612 Laverock Place N
Washington, DC 20007
(800) 237-9192 FAX: (301) 464-7347

BAS
See Bioanalytical Systems

BASF
Specialty Products
3000 Continental Drive North
Mt. Olive, NJ 07828
(800) 669-2273 FAX: (973) 426-2610
http://www.basf.com

Baum, W.A.
620 Oak Street
Copiague, NY 11726
(631) 226-3940 FAX: (631) 226-3969
http://www.wabaum.com

Bausch & Lomb
One Bausch & Lomb Place
Rochester, NY 14604
(800) 344-8815 FAX: (716) 338-6007
(716) 338-6000
http://www.bausch.com

Baxter
Fenwal Division
1627 Lake Cook Road
Deerfield, IL 60015
(800) 766-1077 FAX: (800) 395-3291
(847) 940-6599 FAX: (847) 940-5766
http://www.powerfulmedicine.com

Baxter Healthcare
One Baxter Parkway
Deerfield, IL 60015
(800) 777-2298 FAX: (847) 948-3948
(847) 948-2000
http://www.baxter.com

Baxter Scientific Products
See VWR Scientific

Bayer
Agricultural Division
Animal Health Products
12707 Shawnee Mission Pkwy.
Shawnee Mission, KS 66201
(800) 255-6517 FAX: (913) 268-2803
(913) 268-2000
http://www.bayerus.com

Bayer
Diagnostics Division (Order Services)
P.O. Box 2009
Mishiwaka, IN 46546
(800) 248-2637 FAX: (800) 863-6882
(219) 256-3390
http://www.bayer.com

Bayer Diagnostics
511 Benedict Avenue
Tarrytown, NY 10591
(800) 255-3232 FAX: (914) 524-2132
(914) 631-8000
http://www.bayerdiag.com

Bayer Plc
Diagnostics Division
Bayer House, Strawberry Hill
Newbury, Berkshire RG14 1JA, UK
(44) 1635-563000
FAX: (44) 1635-563393
http://www.bayer.co.uk

BD Biosciences
2350 Qume Drive
San Jose, CA, USA 95131-1807
(877) 232-8995 FAX: (408) 954-2347

BD Immunocytometry Systems
2350 Qume Drive
San Jose, CA 95131
(800) 223-8226 FAX: (408) 954-BDIS
http://www.bdfacs.com

BD Labware
Two Oak Park
Bedford, MA 01730
(800) 343-2035 FAX: (800) 743-6200
http://www.bd.com/labware

BD PharMingen
10975 Torreyana Road
San Diego, CA 92121
(800) 848-6227 FAX: (858) 812-8888
(858) 812-8800
http://www.pharmingen.com

BD Transduction Laboratories
133 Venture Court
Lexington, KY 40511
(800) 227-4063 FAX: (606) 259-1413
(606) 259-1550
http://www.translab.com

BDH Chemicals
Broom Road
Poole, Dorset BH12 4NN, UK
(44) 1202-745520
FAX: (44) 1202- 2413720

BDH Chemicals
See Hoefer Scientific Instruments

BDIS
See BD Immunocytometry Systems

Becker and Hickl GmbH
Nahmitzer Damm 30
D-12277 Berlin, Germany
(49) 30 787 56 32
FAX: (49) 30 787 57 32
http://www.becker-hickl.com

Beckman Coulter
4300 North Harbor Boulevard
Fullerton, CA 92834
(800) 233-4685 FAX: (800) 643-4366
(714) 871-4848
http://www.beckman-coulter.com

Beckman Instruments
Spinco Division/Bioproducts Operation
1050 Page Mill Road
Palo Alto, CA 94304
(800) 742-2345 FAX: (415) 859-1550
(415) 857-1150
http://www.beckman-coulter.com

Becton Dickinson Immunocytometry & Cellular Imaging
2350 Qume Drive
San Jose, CA 95131
(800) 223-8226 FAX: (408) 954-2007
(408) 432-9475
http://www.bdfacs.com

Becton Dickinson Labware
1 Becton Drive
Franklin Lakes, NJ 07417
(888) 237-2762 FAX: (800) 847-2220
(201) 847-4222
http://www.bdfacs.com

Becton Dickinson Labware
2 Bridgewater Lane
Lincoln Park, NJ 07035
(800) 235-5953 FAX: (800) 847-2220
(201) 847-4222
http://www.bdfacs.com

Becton Dickinson Primary
Care Diagnostics
7 Loveton Circle
Sparks, MD 21152
(800) 675-0908 FAX: (410) 316-4723
(410) 316-4000
http://www.bdfacs.com

Behringwerke Diagnostika
Hoechster Strasse 70
P-65835 Liederback, Germany
(49) 69-30511 FAX: (49) 69-303-834

Bellco Glass
340 Edrudo Road
Vineland, NJ 08360
(800) 257-7043 FAX: (856) 691-3247
(856) 691-1075
http://www.bellcoglass.com

Bender Biosystems
See Serva

Beral Enterprises
See Garren Scientific

Berkeley Antibody
See BAbCO

Bernsco Surgical Supply
25 Plant Avenue
Hauppague, NY 11788
(800) TIEMANN FAX: (516) 273-6199
(516) 273-0005
http://www.bernsco.com

Beta Medical and Scientific (Datesand Ltd.)
2 Ferndale Road
Sale, Manchester M33 3GP, UK
(44) 1612 317676
FAX: (44) 1612 313656

Bethesda Research Laboratories (BRL)
See Life Technologies

Biacore
200 Centennial Avenue, Suite 100
Piscataway, NJ 08854
(800) 242-2599 FAX: (732) 885-5669
(732) 885-5618
http://www.biacore.com

Biacore, Inc.
200 Centennial Avenue, Suite 100
Piscataway, NJ 08854
(800) 242-2599 FAX: (732) 885-5669
(732) 885-5618
http://www.biacore.com

Bilaney Consultants
St. Julian's
Sevenoaks, Kent TN15 0RX, UK
(44) 1732 450002
FAX: (44) 1732 450003
http://www.bilaney.com

Binding Site
5889 Oberlin Drive, Suite 101
San Diego, CA 92121
(800) 633-4484 FAX: (619) 453-9189
(619) 453-9177
http://www.bindingsite.co.uk

BIO 101
See Qbiogene

Bio Image
See Genomic Solutions

Bioanalytical Systems
2701 Kent Avenue
West Lafayette, IN 47906
(800) 845-4246 FAX: (765) 497-1102
(765) 463-4527
http://www.bioanalytical.com

Biocell
2001 University Drive
Rancho Dominguez, CA 90220
(800) 222-8382 FAX: (310) 637-3927
(310) 537-3300
http://www.biocell.com

Biocoat
See BD Labware

BioComp Instruments
650 Churchill Road
Fredericton, New Brunswick
E3B 1P6 Canada
(800) 561-4221 FAX: (506) 453-3583
(506) 453-4812
http://131.202.97.21

BioDesign
P.O. Box 1050
Carmel, NY 10512
(914) 454-6610 FAX: (914) 454-6077
http://www.biodesignofny.com

BioDiscovery
4640 Admiralty Way, Suite 710
Marina Del Rey, CA 90292
(310) 306-9310 FAX: (310) 306-9109
http://www.biodiscovery.com

Bioengineering AG
Sagenrainstrasse 7
CH8636 Wald, Switzerland
(41) 55-256-8-111
FAX: (41) 55-256-8-256

Biofluids
Division of Biosource International
1114 Taft Street
Rockville, MD 20850
(800) 972-5200 FAX: (301) 424-3619
(301) 424-4140
http://www.biosource.com

BioFX Laboratories
9633 Liberty Road, Suite S
Randallstown, MD 21133
(800) 445-6447 FAX: (410) 498-6008
(410) 496-6006
http://www.biofx.com

BioGenex Laboratories
4600 Norris Canyon Road
San Ramon, CA 94583
(800) 421-4149 FAX: (925) 275-0580
(925) 275-0550
http://www.biogenex.com

Bioline
2470 Wrondel Way
Reno, NV 89502
(888) 257-5155 FAX: (775) 828-7676
(775) 828-0202
http://www.bioline.com

Bio-Logic Research & Development
1, rue de l-Europe
A.Z. de Font-Ratel
38640 CLAIX, France
(33) 76-98-68-31
FAX: (33) 76-98-69-09

Biological Detection Systems
See Cellomics or Amersham

Biomeda
1166 Triton Drive, Suite E
P.O. Box 8045
Foster City, CA 94404
(800) 341-8787 FAX: (650) 341-2299
(650) 341-8787
http://www.biomeda.com

BioMedic Data Systems
1 Silas Road
Seaford, DE 19973
(800) 526-2637 FAX: (302) 628-4110
(302) 628-4100
http://www.bmds.com

Biomedical Engineering
P.O. Box 980694
Virginia Commonwealth University
Richmond, VA 23298
(804) 828-9829 FAX: (804) 828-1008

Biomedical Research Instruments
12264 Wilkins Avenue
Rockville, MD 20852
(800) 327-9498
(301) 881-7911
http://www.biomedinstr.com

Bio/medical Specialties
P.O. Box 1687
Santa Monica, CA 90406
(800) 269-1158 FAX: (800) 269-1158
(323) 938-7515

BioMerieux
100 Rodolphe Street
Durham, North Carolina 27712
(919) 620-2000
http://www.biomerieux.com

BioMetallics
P.O. Box 2251
Princeton, NJ 08543
(800) 999-1961 FAX: (609) 275-9485
(609) 275-0133
http://www.microplate.com

Biomol Research Laboratories
5100 Campus Drive
Plymouth Meeting, PA 19462
(800) 942-0430 FAX: (610) 941-9252
(610) 941-0430
http://www.biomol.com

Bionique Testing Labs
Fay Brook Drive
RR 1, Box 196
Saranac Lake, NY 12983
(518) 891-2356 FAX: (518) 891-5753
http://www.bionique.com

Biopac Systems
42 Aero Camino
Santa Barbara, CA 93117
(805) 685-0066 FAX: (805) 685-0067
http://www.biopac.com

Bioproducts for Science
See Harlan Bioproducts for Science

Bioptechs
3560 Beck Road
Butler, PA 16002
(877) 548-3235 FAX: (724) 282-0745
(724) 282-7145
http://www.bioptechs.com

BIOQUANT-R&M Biometrics
5611 Ohio Avenue
Nashville, TN 37209
(800) 221-0549 (615) 350-7866
FAX: (615) 350-7282
http://www.bioquant.com

Bio-Rad Laboratories
2000 Alfred Nobel Drive
Hercules, CA 94547
(800) 424-6723 FAX: (800) 879-2289
(510) 741-1000 FAX: (510) 741-5800
http://www.bio-rad.com

Bio-Rad Laboratories
Maylands Avenue
Hemel Hempstead,
Herts HP2 7TD, UK
http://www.bio-rad.com

Bioreclamation
492 Richmond Road
East Meadow, NY 11554
(516) 483-1196 FAX: (516) 483-4683
http://www.bioreclamation.com

BioRobotics
3-4 Bennell Court
Comberton, Cambridge CB3 7DS, UK
(44) 1223-264345
FAX: (44) 1223-263933
http://www.biorobotics.co.uk

BIOS Laboratories
See Genaissance Pharmaceuticals

Biosearch Technologies
81 Digital Drive
Novato, CA 94949
(800) GENOME1 FAX: (415)
883-8488
(415) 883-8400
http://www.biosearchtech.com

BioSepra
111 Locke Drive
Marlborough, MA 01752
(800) 752-5277 FAX: (508) 357-7595
(508) 357-7500
http://www.biosepra.com

Bio-Serv
1 8th Street, Suite 1
Frenchtown, NJ 08825
(908) 996-2155 FAX: (908) 996-4123
http://www.bio-serv.com

BioSignal
1744 William Street, Suite 600
Montreal, Quebec H3J 1R4 Canada
(800) 293-4501 FAX: (514) 937-0777
(514) 937-1010
http://www.biosignal.com

Biosoft
P.O. Box 10938
Ferguson, MO 63135
(314) 524-8029 FAX: (314) 524-8129
http://www.biosoft.com

Biosource International
820 Flynn Road
Camarillo, CA 93012
(800) 242-0607 FAX: (805) 987-3385
(805) 987-0086
http://www.biosource.com

BioSpec Products
P.O. Box 788
Bartlesville, OK 74005
(800) 617-3363 FAX: (918) 336-3363
(918) 336-3363
http://www.biospec.com

Biosure
See Riese Enterprises

Biosym Technologies
See Molecular Simulations

Biosys
21 quai du Clos des Roses
602000 Compiegne, France
(33) 03 4486 2275
FAX: (33) 03 4484 2297

Bio-Tech Research Laboratories
NIAID Repository
Rockville, MD 20850
http://www.niaid.nih.gov/ncn/repos.htm

Biotech Instruments
Biotech House
75A High Street
Kimpton, Hertfordshire SG4 8PU, UK
(44) 1438 832555
FAX: (44) 1438 833040
http://www.biotinst.demon.co.uk

Biotech International
11 Durbell Street
Acacia Ridge, Queensland 4110
Australia
61-7-3370-6396
FAX: 61-7-3370-6370
http://www.avianbiotech.com

Biotech Source
Inland Farm Drive
South Windham, ME 04062
(207) 892-3266 FAX: (207) 892-6774

Bio-Tek Instruments
Highland Industrial Park
P.O. Box 998
Winooski, VT 05404
(800) 451-5172 FAX: (802) 655-7941
(802) 655-4040
http://www.biotek.com

Biotecx Laboratories
6023 South Loop East
Houston, TX 77033
(800) 535-6286 FAX: (713) 643-3143
(713) 643-0606
http://www.biotecx.com

BioTherm
3260 Wilson Boulevard
Arlington, VA 22201
(703) 522-1705 FAX: (703) 522-2606

Bioventures
P.O. Box 2561
848 Scott Street
Murfreesboro, TN 37133
(800) 235-8938 FAX: (615) 896-4837
http://www.bioventures.com

BioWhittaker
8830 Biggs Ford Road
P.O. Box 127
Walkersville, MD 21793
(800) 638-8174 FAX: (301) 845-8338
(301) 898-7025
http://www.biowhittaker.com

Biozyme Laboratories
9939 Hibert Street, Suite 101
San Diego, CA 92131
(800) 423-8199 FAX: (858) 549-0138
(858) 549-4484
http://www.biozyme.com

Bird Products
1100 Bird Center Drive
Palm Springs, CA 92262
(800) 328-4139 FAX: (760) 778-7274
(760) 778-7200
http://www.birdprod.com/bird

B & K Universal
2403 Yale Way
Fremont, CA 94538
(800) USA-MICE FAX: (510)
490-3036

BLS Ltd.
Zselyi Aladar u. 31
1165 Budapest, Hungary
(36) 1-407-2602 FAX: (36)
1-407-2896
http://www.bls-ltd.com

Blue Sky Research
3047 Orchard Parkway
San Jose, CA 95134
(408) 474-0988 FAX: (408) 474-0989
http://www.blueskyresearch.com

Blumenthal Industries
7 West 36th Street, 13th floor
New York, NY 10018
(212) 719-1251 FAX: (212) 594-8828

BOC Edwards
One Edwards Park
301 Ballardvale Street
Wilmington, MA 01887
(800) 848-9800 FAX: (978) 658-7969
(978) 658-5410
http://www.bocedwards.com

Boehringer Ingelheim
900 Ridgebury Road
P.O. Box 368
Ridgefield, CT 06877
(800) 243-0127 FAX: (203) 798-6234
(203) 798-9988
http://www.boehringer-ingelheim.com

Boehringer Mannheim
Biochemicals Division
See Roche Diagnostics

Boekel Scientific
855 Pennsylvania Boulevard
Feasterville, PA 19053
(800) 336-6929 FAX: (215) 396-8264
(215) 396-8200
http://www.boekelsci.com

Bohdan Automation
1500 McCormack Boulevard
Mundelein, IL 60060
(708) 680-3939 FAX: (708) 680-1199

Boston Biochem Inc.
840 Memorial Drive
Cambridge, MA 02139
(617) 241-7072 FAX: (617) 492-3565
http://www.bostonbiochem.com

BPAmoco
4500 McGinnis Ferry Road
Alpharetta, GA 30005
(800) 328-4537 FAX: (770) 772-8213
(770) 772-8200
http://www.bpamoco.com

Brain Research Laboratories
Waban P.O. Box 88
Newton, MA 02468
(888) BRL-5544 FAX: (617) 965-6220
(617) 965-5544
http://www.brainresearchlab.com

Braintree Scientific
P.O. Box 850929
Braintree, MA 02185
(781) 843-1644 FAX: (781) 982-3160
http://www.braintreesci.com

Brandel
8561 Atlas Drive
Gaithersburg, MD 20877
(800) 948-6506 FAX: (301) 869-5570
(301) 948-6506
http://www.brandel.com

Branson Ultrasonics
41 Eagle Road
Danbury, CT 06813
(203) 796-0400 FAX: (203) 796-9838
http://www.plasticsnet.com/branson

B. Braun Biotech
999 Postal Road
Allentown, PA 18103
(800) 258-9000 FAX: (610) 266-9319
(610) 266-6262
http://www.bbraunbiotech.com

B. Braun Biotech International
Schwarzenberg Weg 73-79
P.O. Box 1120
D-34209 Melsungen, Germany
(49) 5661-71-3400
FAX: (49) 5661-71-3702
http://www.bbraunbiotech.com

B. Braun-McGaw
2525 McGaw Avenue
Irvine, CA 92614
(800) BBRAUN-2 (800) 624-2963
http://www.bbraunusa.com

B. Braun Medical
Thorncliffe Park
Sheffield S35 2PW, UK
(44) 114-225-9000
FAX: (44) 114-225-9111
http://www.bbmuk.demon.co.uk

Brenntag
P.O. Box 13788
Reading, PA 19612-3788
(610) 926-4151 FAX: (610) 926-4160
http://www.brenntagnortheast.com

Bresatec
See GeneWorks

Bright/Hacker Instruments
17 Sherwood Lane
Fairfield, NJ 07004
(973) 226-8450 FAX: (973) 808-8281
http://www.hackerinstruments.com

Brinkmann Instruments
Subsidiary of Sybron
1 Cantiague Road
P.O. Box 1019
Westbury, NY 11590
(800) 645-3050 FAX: (516) 334-7521
(516) 334-7500
http://www.brinkmann.com

Bristol-Meyers Squibb
P.O. Box 4500
Princeton, NJ 08543
(800) 631-5244 FAX: (800) 523-2965
http://www.bms.com

Broadley James
19 Thomas
Irvine, CA 92618
(800) 288-2833 FAX: (949) 829-5560
(949) 829-5555
http://www.broadleyjames.com

Brookhaven Instruments
750 Blue Point Road
Holtsville, NY 11742
(631) 758-3200 FAX: (631) 758-3255
http://www.bic.com

Brownlee Labs
See Applied Biosystems
Distributed by Pacer Scientific

Bruel & Kjaer
Division of Spectris Technologies
2815 Colonnades Court
Norcross, GA 30071
(800) 332-2040 FAX: (770) 847-8440
(770) 209-6907
http://www.bkhome.com

Bruker Analytical X-Ray Systems
5465 East Cheryl Parkway
Madison, WI 53711
(800) 234-XRAY FAX: (608) 276-3006
(608) 276-3000
http://www.bruker-axs.com

Bruker Instruments
19 Fortune Drive
Billerica, MA 01821
(978) 667-9580 FAX: (978) 667-0985
http://www.bruker.com

BTX
Division of Genetronics
11199 Sorrento Valley Road
San Diego, CA 92121
(800) 289-2465 FAX: (858) 597-9594
(858) 597-6006
http://www.genetronics.com/btx

BTX Molecular Delivery Systems
(order through Fisher or VWR)
(800) 272-2775
http://www.btxonline.com

Buchler Instruments
See Baxter Scientific Products

Buckshire
2025 Ridge Road
Perkasie, PA 18944
(215) 257-0116

Burdick and Jackson
Division of Baxter Scientific Products
1953 S. Harvey Street
Muskegon, MI 49442
(800) 368-0050 FAX: (231) 728-8226
(231) 726-3171
http://www.bandj.com/mainframe.htm

Burleigh Instruments
P.O. Box E
Fishers, NY 14453
(716) 924-9355 FAX: (716) 924-9072
http://www.burleigh.com

Burns Veterinary Supply
1900 Diplomat Drive
Farmer's Branch, TX 75234
(800) 92-BURNS FAX: (972)
243-6841
http://www.burnsvet.com

Burroughs Wellcome
See Glaxo Wellcome

The Butler Company
5600 Blazer Parkway
Dublin, OH 43017
(800) 551-3861 FAX: (614) 761-9096
(614) 761-9095
http://www.wabutler.com

Butterworth Laboratories
54-56 Waldegrave Road
Teddington, Middlesex
TW11 8LG, UK
(44)(0)20-8977-0750
FAX: (44)(0)28-8943-2624
http://www.butterworth-labs.co.uk

Buxco Electronics
95 West Wood Road #2
Sharon, CT 06069
(860) 364-5558 FAX: (860) 364-5116
http://www.buxco.com

C/D/N Isotopes
88 Leacock Street
Pointe-Claire, Quebec
H9R 1H1 Canada
(800) 697-6254 FAX: (514) 697-6148

C.M.A./Microdialysis AB
73 Princeton Street
North Chelmsford, MA 01863
(800) 440-4980 FAX: (978) 251-1950
(978) 251-1940
http://www.microdialysis.com

Calbiochem-Novabiochem
P.O. Box 12087-2087
La Jolla, CA 92039
(800) 854-3417 FAX: (800) 776-0999
(858) 450-9600
http://www.calbiochem.com

California Fine Wire
338 South Fourth Street
Grover Beach, CA 93433
(805) 489-5144 FAX: (805) 489-5352
http://www.calinewire.com

Calorimetry Sciences
155 West 2050 North
Spanish Fork, UT 84660
(801) 794-2600 FAX: (801) 794-2700
http://www.calscorp.com

Caltag Laboratories
1849 Bayshore Highway, Suite 200
Burlingame, CA 94010
(800) 874-4007 FAX: (650) 652-9030
(650) 652-0468
http://www.caltag.com

Cambrex Corporation
1 Meadowlands Plaza
East Rulherford, NJ 07073
(201) 804-3000 FAX: (201) 804-9852
http://www.cambrex.com

Cambridge Electronic Design
Science Park, Milton Road
Cambridge CB4 0FE, UK
44 (0) 1223-420-186
FAX: 44 (0) 1223-420-488
http://www.ced.co.uk

Cambridge Isotope Laboratories
50 Frontage Road
Andover, MA 01810
(800) 322-1174 FAX: (978) 749-2768
(978) 749-8000
http://www.isotope.com

Cambridge Research Biochemicals
See Zeneca/CRB

Cambridge Technology
109 Smith Place
Cambridge, MA 02138
(6l7) 441-0600 FAX: (617) 497-8800
http://www.camtech.com

Camlab
Nuffield Road
Cambridge CB4 1TH, UK
(44) 122-3424222
FAX: (44) 122-3420856
http://www.camlab.co.uk/home.htm

Campden Instruments
Park Road
Sileby Loughborough
Leicestershire LE12 7TU, UK
(44) 1509-814790
FAX: (44) 1509-816097
http://www.campden-inst.com/home.htm

Cappel Laboratories
See Organon Teknika Cappel

Cargille Laboratories
55 Commerce Road
Cedar Grove, NJ 07009
(973) 239-6633 FAX: (973) 239-6096
http://www.cargille.com

Carl Roth GmgH & Company
Schoemperlenstrasse 1-5
76185 Karlsrube
Germany
(49) 72-156-06164
FAX: (49) 72-156-06264
http://www.carl-roth.de

Carl Zeiss
One Zeiss Drive
Thornwood, NY 10594
(800) 233-2343 FAX: (914) 681-7446
(914) 747-1800
http://www.zeiss.com

Carlo Erba Reagenti
Via Winckelmann 1
20148 Milano
Lombardia, Italy
(39) 0-29-5231
FAX: (39) 0-29-5235-904
http://www.carloerbareagenti.com

Carolina Biological Supply
2700 York Road
Burlington, NC 27215
(800) 334-5551 FAX: (336) 584-76869
(336) 584-0381
http://www.carolina.com

Carolina Fluid Components
9309 Stockport Place
Charlotte, NC 28273
(704) 588-6101 FAX: (704) 588-6115
http://www.cfcsite.com

Cartesian Technologies
17851 Skypark Circle, Suite C
Irvine, CA 92614
(800) 935-8007
http://cartesiantech.com

Cayman Chemical
1180 East Ellsworth Road
Ann Arbor, MI 48108
(800) 364-9897 FAX: (734) 971-3640
(734) 971-3335
http://www.caymanchem.com

CB Sciences
One Washington Street, Suite 404
Dover, NH 03820
(800) 234-1757 FAX: (603) 742-2455
http://www.cbsci.com

CBS Scientific
P.O. Box 856
Del Mar, CA 92014
(800) 243-4959 FAX: (858) 755-0733
(858) 755-4959
http://www.cbssci.com

CCR (Coriell Cell Repository)
See Coriell Institute for Medical Research

CE Instruments
Grand Avenue Parkway
Austin, TX 78728
(800) 876-6711 FAX: (512) 251-1597
http://www.ceinstruments.com

Cedarlane Laboratories
5516 8th Line, R.R. #2
Hornby, Ontario L0P 1E0 Canada
(905) 878-8891 FAX: (905) 878-7800
http://www.cedarlanelabs.com

CEL Associates
P.O. Box 721854
Houston, TX 77272
(800) 537-9339 FAX: (281) 933-0922
(281) 933-9339
http://www.cel-1.com

Cel-Line Associates
See Erie Scientific

Celite World Minerals
130 Castilian Drive
Santa Barbara, CA 93117
(805) 562-0200 FAX: (805) 562-0299
http://www.worldminerals.com/celite

Cell Genesys
342 Lakeside Drive
Foster City, CA 94404
(650) 425-4400 FAX: (650) 425-4457
http://www.cellgenesys.com

Cell Microscopy Center
Department of Cell Biology
Utrecht University
P.O. Box 80125
3508TC Utrecht
The Netherlands

Cell Signaling Technology
166B Cummings Center
Beverly, MA 01915
(877) 616-CELL FAX: (978) 867-2388
(978) 867-2488
http://www.cellsignal.com

Cell Systems
12815 NE 124th Street, Suite A
Kirkland, WA 98034
(800) 697-1211 FAX: (425) 820-6762
(425) 823-1010

Cellmark Diagnostics
20271 Goldenrod Lane
Germantown, MD 20876
(800) 872-5227 FAX: (301) 428-4877
(301) 428-4980
http://www.cellmark-labs.com

Cellomics
635 William Pitt Way
Pittsburgh, PA 15238
(888) 826-3857 FAX: (412) 826-3850
(412) 826-3600
http://www.cellomics.com

Celltech
216 Bath Road
Slough, Berkshire SL1 4EN, UK
(44) 1753 534655
FAX: (44) 1753 536632
http://www.celltech.co.uk

Cellular Products
872 Main Street
Buffalo, NY 14202
(800) CPI-KITS FAX: (716) 882-0959
(716) 882-0920
http://www.zeptometrix.com

CEM
P.O. Box 200
Matthews, NC 28106
(800) 726-3331

Centers for Disease Control
1600 Clifton Road NE
Atlanta, GA 30333
(800) 311-3435 FAX: (888) 232-3228
(404) 639-3311
http://www.cdc.gov

CERJ
Centre d'Elevage Roger Janvier
53940 Le Genest Saint Isle
France

Cetus
See Chiron

Chance Propper
Warly, West Midlands B66 1NZ, UK
(44)(0)121-553-5551
FAX: (44)(0)121-525-0139

Charles River Laboratories
251 Ballardvale Street
Wilmington, MA 01887
(800) 522-7287 FAX: (978) 658-7132
(978) 658-6000
http://www.criver.com

Charm Sciences
36 Franklin Street
Malden, MA 02148
(800) 343-2170 FAX: (781) 322-3141
(781) 322-1523
http://www.charm.com

Chase-Walton Elastomers
29 Apsley Street
Hudson, MA 01749
(800) 448-6289 FAX: (978) 562-5178
(978) 568-0202
http://www.chase-walton.com

ChemGenes
Ashland Technology Center
200 Homer Avenue
Ashland, MA 01721
(800) 762-9323 FAX: (508) 881-3443
(508) 881-5200
http://www.chemgenes.com

Chemglass
3861 North Mill Road
Vineland, NJ 08360
(800) 843-1794 FAX: (856) 696-9102
(800) 696-0014
http://www.chemglass.com

Chemicon International
28835 Single Oak Drive
Temecula, CA 92590
(800) 437-7500 FAX: (909) 676-9209
(909) 676-8080
http://www.chemicon.com

Chem-Impex International
935 Dillon Drive
Wood Dale, IL 60191
(800) 869-9290 FAX: (630) 766-2218
(630) 766-2112
http://www.chemimpex.com

Chem Service
P.O. Box 599
West Chester, PA 19381-0599
(610) 692-3026 FAX: (610) 692-8729
http://www.chemservice.com

Chemsyn Laboratories
13605 West 96th Terrace
Lenexa, Kansas 66215
(913) 541-0525 FAX: (913) 888-3582
http://www.tech.epcorp.com/ChemSyn/chemsyn.htm

Chemunex USA
1 Deer Park Drive, Suite H-2
Monmouth Junction, NJ 08852
(800) 411-6734
http://www.chemunex.com

Cherwell Scientific Publishing
The Magdalen Centre
Oxford Science Park
Oxford OX44GA, UK
(44)(1) 865-784-800
FAX: (44)(1) 865-784-801
http://www.cherwell.com

ChiRex Cauldron
383 Phoenixville Pike
Malvern, PA 19355
(610) 727-2215 FAX: (610) 727-5762
http://www.chirex.com

Chiron Diagnostics
See Bayer Diagnostics

Chiron Mimotopes Peptide Systems
See Multiple Peptide Systems

Chiron
4560 Horton Street
Emeryville, CA 94608
(800) 244-7668 FAX: (510) 655-9910
(510) 655-8730
http://www.chiron.com

Chrom Tech
P.O. Box 24248
Apple Valley, MN 55124
(800) 822-5242 FAX: (952) 431-6345
http://www.chromtech.com

Chroma Technology
72 Cotton Mill Hill, Unit A-9
Brattleboro, VT 05301
(800) 824-7662 FAX: (802) 257-9400
(802) 257-1800
http://www.chroma.com

Chromatographie
ZAC de Moulin No. 2
91160 Saulx les Chartreux
France
(33) 01-64-54-8969
FAX: (33) 01-69-0988091
http://www.chromatographie.com

Chromogenix
Taljegardsgatan 3
431-53 Mlndal, Sweden
(46) 31-706-20-70
FAX: (46) 31-706-20-80
http://www.chromogenix.com

Chrompack USA
c/o Varian USA
2700 Mitchell Drive
Walnut Creek, CA 94598
(800) 526-3687 FAX: (925) 945-2102
(925) 939-2400
http://www.chrompack.com

Chugai Biopharmaceuticals
6275 Nancy Ridge Drive
San Diego, CA 92121
(858) 535-5900 FAX: (858) 546-5973
http://www.chugaibio.com

Ciba-Corning Diagnostics
See Bayer Diagnostics

Ciba-Geigy
See Ciba Specialty Chemicals or Novartis Biotechnology

Ciba Specialty Chemicals
540 White Plains Road
Tarrytown, NY 10591
(800) 431-1900 FAX: (914) 785-2183
(914) 785-2000
http://www.cibasc.com

CIBA Vision
Division of Novartis AG
11460 Johns Creek Parkway
Duluth, GA 30097
(770) 476-3937
http://www.cvworld.com

Cidex
Advanced Sterillzation Products
33 Technology Drive
Irvine, CA 92618
(800) 595-0200 (949) 581-5799
http://www.cidex.com/ASPnew.htm

Cinna Scientific
Subsidiary of Molecular Research Center
5645 Montgomery Road
Cincinnati, OH 45212
(800) 462-9868 FAX: (513) 841-0080
(513) 841-0900
http://www.mrcgene.com

Cistron Biotechnology
10 Bloomfield Avenue
Pine Brook, NJ 07058
(800) 642-0167 FAX: (973) 575-4854
(973) 575-1700
http://www.cistronbio.com

Clark Electromagnetic Industries
LEC Instruments Pty. Ltd.
8 Doris Court
Scoresby, Victoria
3719 Australia
(61) 3 9763 0080 FAX: (61) 3 9764 0086
http://www.lecinstruments.com

Clay Adam
See Becton Dickinson Primary Care Diagnostics

CLB (Central Laboratory of the Netherlands)
Blood Transfusion Service
P.O. Box 9190
1006 AD Amsterdam, The Netherlands
(31) 20-512-9222
FAX: (31) 20-512-3332

CLP Products
see Continental Laboratory Products

Cleveland Scientific
P.O. Box 300
Bath, OH 44210
(800) 952-7315 FAX: (330) 666-2240
http:://www.clevelandscientific.com

Clonetics
Division of BioWhittaker
http://www.clonetics.com
Also see BioWhittaker

Clontech Laboratories
1020 East Meadow Circle
Palo Alto, CA 94303
(800) 662-2566 FAX: (800) 424-1350
(650) 424-8222 FAX: (650) 424-1088
http://www.clontech.com

Closure Medical Corporation
5250 Greens Dairy Road
Raleigh, NC 27616
(919) 876-7800 FAX: (919) 790-1041
http://www.closuremed.com

CMA Microdialysis AB
73 Princeton Street
North Chelmsford, MA 01863
(800) 440-4980 FAX: (978) 251-1950
(978) 251 1940
http://www.microdialysis.com

Cocalico Biologicals
449 Stevens Road
P.O. Box 265
Reamstown, PA 17567
(717) 336-1990 FAX: (717) 336-1993

Coherent Laser
5100 Patrick Henry Drive
Santa Clara, CA 95056
(800) 227-1955 FAX: (408) 764-4800
(408) 764-4000
http://www.cohr.com

Cohu
P.O. Box 85623
San Diego, CA 92186
(858) 277-6700 FAX: (858) 277-0221
http://www.COHU.com/cctv

Cole-Parmer Instrument
625 East Bunker Court
Vernon Hills, IL 60061
(800) 323-4340 FAX: (847) 247-2929
(847) 549-7600
http://www.coleparmer.com

Collaborative Biomedical Products and **Collaborative Research**
See Becton Dickinson Labware

Collagen Aesthetics
1850 Embarcadero Road
Palo Alto, CA 94303
(650) 856-0200 FAX: (650) 856-0533
http://www.collagen.com

Collagen Corporation
See Collagen Aesthetics

College of American Pathologists
325 Waukegan Road
Northfield, IL 60093
(800) 323-4040 FAX: (847) 832-8000
(847) 446-8800
http://www.cap.org/index.cfm

Colonial Medical Supply
504 Wells Road
Franconia, NH 03580
(603) 823-9911 FAX: (603) 823-8799
http://www.colmedsupply.com

Colorado Serum
4950 York Street
Denver, CO 80216
(800) 525-2065 FAX: (303) 295-1923
http://www.colorado-serum.com

Columbia Diagnostics
8001 Research Way
Springfield, VA 22153
(800) 336-3081 FAX: (703) 569-2353
(703) 569-7511
http://www.columbiadiagnostics.com

Columbus Instruments
950 North Hague Avenue
Columbus, OH 43204
(800) 669-5011 FAX: (614) 276-0529
(614) 276-0861
http://www.columbusinstruments.com

Compu Cyte Corp.
12 Emily Street
Cambridge, MA 02139
(800) 840-1303 FAX: (617) 577-4501
(617) 492-1300
http://www.compucyte.com

Compugen
25 Leek Crescent
Richmond Hill, Ontario
L4B 4B3 Canada
800-387-5045 FAX: (905) 707-2020
(905) 707-2000
http://www.compugen.com/locations.htm

Computer Associates International
One Computer Associates Plaza
Islandia, NY 11749
(631) 342-6000 FAX: (631) 342-6800
http://www.cai.com

Connaught Laboratories
See Aventis Pasteur

Connectix
2955 Campus Drive, Suite 100
San Mateo, CA 94403
(800) 950-5880 FAX: (650) 571-0850
(650) 571-5100
http://www.connectix.com

Contech
99 Hartford Avenue
Providence, RI 02909
(401) 351-4890 FAX: (401) 421-5072
http://www.iol.ie/~burke/contech.html

Continental Laboratory Products
5648 Copley Drive
San Diego, CA 92111
(800) 456-7741 FAX: (858) 279-5465
(858) 279-5000
http://www.conlab.com

ConvaTec
Professional Services
P.O. Box 5254
Princeton, NJ 08543
(800) 422-8811
http://www.convatec.com

Cooper Instruments & Systems
P.O. Box 3048
Warrenton, VA 20188
(800) 344-3921 FAX: (540) 347-4755
(540) 349-4746
http://www.cooperinstruments.com

Cooperative Human Tissue Network
(866) 462-2486
http://www.chin.ims.nci.nih.gov

Cora Styles Needles 'N Blocks
56 Milton Street
Arlington, MA 02474
(781) 648-6289 FAX: (781) 641-7917

Coriell Cell Repository (CCR)
See Coriell Institute for Medical Research

Coriell Institute for Medical Research
Human Genetic Mutant Repository
401 Haddon Avenue
Camden, NJ 08103
(856) 966-7377 FAX: (856) 964-0254
http://arginine.umdnj.edu

Corion
8 East Forge Parkway
Franklin, MA 02038
(508) 528-4411 FAX: (508) 520-7583
(800) 598-6783
http://www.corion.com

Corning and Corning Science Products
P.O. Box 5000
Corning, NY 14831
(800) 222-7740 FAX: (607) 974-0345
(607) 974-9000
http://www.corning.com

Costar
See Corning

Coulbourn Instruments
7462 Penn Drive
Allentown, PA 18106
(800) 424-3771 FAX: (610) 391-1333
(610) 395-3771
http://www.coulbourninst.com

Coulter Cytometry
See Beckman Coulter

Covance Research Products
465 Swampbridge Road
Denver, PA 17517
(800) 345-4114 FAX: (717) 336-5344
(717) 336-4921
http://www.covance.com

Coy Laboratory Products
14500 Coy Drive
Grass Lake, MI 49240
(734) 475-2200 FAX: (734) 475-1846
http://www.coylab.com

CPG
3 Borinski Road
Lincoln Park, NJ 07035
(800) 362-2740 FAX: (973) 305-0884
(973) 305-8181
http://www.cpg-biotech.com

CPL Scientific
43 Kingfisher Court
Hambridge Road
Newbury RG14 5SJ, UK
(44) 1635-574902
FAX: (44) 1635-529322
http://www.cplscientific.co.uk

CraMar Technologies
8670 Wolff Court, #160
Westminster, CO 80030
(800) 4-TOMTEC
http://www.cramar.com

Crescent Chemical
1324 Motor Parkway
Hauppauge, NY 11788
(800) 877-3225 FAX: (631) 348-0913
(631) 348-0333
http://www.creschem.com

Crist Instrument
P.O. Box 128
10200 Moxley Road
Damascus, MD 20872
(301) 253-2184 FAX: (301) 253-0069
http://www.cristinstrument.com

Cruachem
See Annovis
http://www.cruachem.com

CS Bio
1300 Industrial Road
San Carlos, CA 94070
(800) 627-2461 FAX: (415) 802-0944
(415) 802-0880
http://www.csbio.com

CS-Chromatographie Service
Am Parir 27
D-52379 Langerwehe, Germany
(49) 2423-40493-0
FAX: (49) 2423-40493-49
http://www.cs-chromatographie.de

Cuno
400 Research Parkway
Meriden, CT 06450
(800) 231-2259 FAX: (203) 238-8716
(203) 237-5541
http://www.cuno.com

Curtin Matheson Scientific
9999 Veterans Memorial Drive
Houston, TX 77038
(800) 392-3353 FAX: (713) 878-3598
(713) 878-3500

CWE
124 Sibley Avenue
Ardmore, PA 19003
(610) 642-7719 FAX: (610) 642-1532
http://www.cwe-inc.com

Cybex Computer Products
4991 Corporate Drive
Huntsville, AL 35805
(800) 932-9239 FAX: (800) 462-9239
http://www.cybex.com

Cygnus Technology
P.O. Box 219
Delaware Water Gap, PA 18327
(570) 424-5701 FAX: (570) 424-5630
http://www.cygnustech.com

Cymbus Biotechnology
Eagle Class, Chandler's Ford
Hampshire SO53 4NF, UK
(44) 1-703-267-676
FAX: (44) 1-703-267-677
http://www.biotech.cymbus.com

Cytogen
600 College Road East
Princeton, NJ 08540
(609) 987-8200 FAX: (609) 987-6450
http://www.cytogen.com

Cytogen Research and Development
89 Bellevue Hill Road
Boston, MA 02132
(617) 325-7774 FAX: (617) 327-2405

Cytoskeleton
1830 Anconia Street
Denver, CO 80223
(303) 322-2254 FAX: (303)322-2257
http://www.cytoskeleton.com

CytRx
154 Technology Parkway
Norcross, GA 30092
(800) 345-2987 FAX: (770) 368-0622
(770) 368-9500
http://www.cytrx.com

Dade Behring
Corporate Headquarters
1717 Deerfield Road
Deerfield, IL 60015
(847) 267-5300 FAX: (847) 267-1066
http://www.dadebehring.com

Dagan
2855 Park Avenue
Minneapolis, MN 55407
(612) 827-5959 FAX: (612) 827-6535
http://www.dagan.com

DAGE-MTI of MC, Inc.
701 North Roeske Ave.
Michigan City, IN 46360
(219) 872-5514 FAX: (219) 872-5559
http://www.dagemti.com

Dako
6392 Via Real
Carpinteria, CA 93013
(800) 235-5763 FAX: (805) 566-6688
(805) 566-6655
http://www.dakousa.com

Dako A/S
42 Produktionsvej
P.O. Box 1359
DK-2600 Glostrup, Denmark
(45) 4492-0044 FAX: (45) 4284-1822

Dakopatts
See Dako A/S

Dalton Chemical Laboratoris
349 wildcat Road
Toronto, Ontario
M3J 253 Canada
(416) 661-2102 FAX: (416) 661-2108
(800) 567-5060 (in Canada only)
http://www.dallon.com

Damon, IEC
See Thermoquest

Dan Kar Scientific
150 West Street
Wilmington, MA 01887
(800) 942-5542 FAX: (978) 658-0380
(978) 988-9696
http://www.dan-kar.com

DataCell
Falcon Business Park
40 Ivanhoe Road
Finchampstead, Berkshire
RG40 4QQ, UK
(44) 1189 324324
FAX: (44) 1189 324325
http://www.datacell.co.uk
In the US:
(408) 446-3575 FAX: (408) 446-3589
http://www.datacell.com

Data Transtation, Inc.
100 Locke Drive
Marlboro, MA 01752-1192
(800) 525-8528 FAX (608) 481-8620
(508) 481-3700
http://www.datx.com

DataWave Technologies
380 Main Street, Suite 209
Longmont, CO 80501
(800) 736-9283 FAX: (303) 776-8531
(303) 776-8214

Datex-Ohmeda
3030 Ohmeda Drive
Madison, WI 53718
(800) 345-2700 FAX: (608) 222-9147
(608) 221-1551
http://www.us.datex-ohmeda.com

DATU
82 State Street
Geneva, NY 14456
(315) 787-2240 FAX: (315) 787-2397
http://www.nysaes.cornell.edu/datu

David Kopf Instruments
7324 Elmo Street
P.O. Box 636
Tujunga, CA 91043
(818) 352-3274 FAX: (818) 352-3139

Decagon Devices
P.O. Box 835
950 NE Nelson Court
Pullman, WA 99163
(800) 755-2751 FAX: (509) 332-5158
(509) 332-2756
http://www.decagon.com

Decon Labs
890 Country Line Road
Bryn Mawr, PA 19010
(800) 332-6647 FAX: (610) 964-0650
(610) 520-0610
http://www.deconlabs.com

Decon Laboratories
Conway Street
Hove, Sussex BN3 3LY, UK
(44) 1273 739241
FAX: (44) 1273 722088

Degussa
Precious Metals Division
3900 South Clinton Avenue
South Plainfield, NJ 07080
(800) DEGUSSA FAX: (908) 756-7176
(908) 561-1100
http://www.degussa-huls.com

Deneba Software
1150 NW 72nd Avenue
Miami, FL 33126
(305) 596-5644 FAX: (305) 273-9069
http://www.deneba.com

Deseret Medical
524 West 3615 South
Salt Lake City, UT 84115
(801) 270-8440 FAX: (801) 293-9000

Devcon Plexus
30 Endicott Street
Danvers, MA 01923
(800) 626-7226 FAX: (978) 774-0516
(978) 777-1100
http://www.devcon.com

Developmental Studies Hybridoma Bank
University of Iowa
436 Biology Building
Iowa City, IA 52242
(319) 335-3826 FAX: (319) 335-2077
http://www.uiowa.edu/~dshbwww

DeVilbiss
Division of Sunrise Medical Respiratory
100 DeVilbiss Drive
P.O. Box 635
Somerset, PA 15501
(800) 338-1988 FAX: (814) 443-7572
(814) 443-4881
http://www.sunrisemedical.com

Dharmacon Research
1376 Mlners Drive #101
Lafayette, CO 80026
(303) 604-9499 FAX: (303) 604-9680
http://www.dharmacom.com

DiaCheM
Triangle Biomedical
Gardiners Place
West Gillibrands, Lancashire
WN8 9SP, UK
(44) 1695-555581
FAX: (44) 1695-555518
http://www.diachem.co.uk

Diagen
Max-Volmer Strasse 4
D-40724 Hilden, Germany
(49) 2103-892-230
FAX: (49) 2103-892-222

Diagnostic Concepts
6104 Madison Court
Morton Grove, IL 60053
(847) 604-0957

Diagnostic Developments
See DiaCheM

Diagnostic Instruments
6540 Burroughs
Sterling Heights, MI 48314
(810) 731-6000 FAX: (810) 731-6469
http://www.diaginc.com

Diamedix
2140 North Miami Avenue
Miami, FL 33127
(800) 327-4565 FAX: (305) 324-2395
(305) 324-2300

Dianova
Warburgstrasse 45
D-20354 Hamburg, Germany
(040) 4 50 67-0 FAX: (040) 4 50 67-490
http://www.dianova.com

DiaSorin
1990 Industrial Boulevard
Stillwater, MN 55082
(800) 328-1482 FAX: (651) 779-7847
(651) 439-9719
http://www.diasorin.com

Diatome US
321 Morris Road
Fort Washington, PA 19034
(800) 523-5874 FAX: (215) 646-8931
(215) 646-1478
http://www.emsdiasum.com

Difco Laboratories
See Becton Dickinson

Digene
1201 Clopper Road
Gaithersburg, MD 20878
(301) 944-7000 (800) 344-3631
FAX: (301) 944-7121
www.digene.com

Digi-Key
701 Brooks Avenue South
Thief River Falls, MN 56701
(800) 344-4539 FAX: (218) 681-3380
(218) 681-6674
http://www.digi-key.com

Digital Vision Technologies
6 Ave. Edouard Serres
31770 Colomiers, France
(05) 62 74 88 20

Digitimer
37 Hydeway
Welwyn Garden City, Hertfordshire
AL7 3BE, UK
(44) 1707-328347
FAX: (44) 1707-373153
http://www.digitimer.com

Dimco-Gray
8200 South Suburban Road
Dayton, OH 45458
(800) 876-8353 FAX: (937) 433-0520
(937) 433-7600
http://www.dimco-gray.com

Dionex
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA 94088
(408) 737-0700 FAX: (408) 730-9403
http://dionex2.promptu.com

Display Systems Biotech
1260 Liberty Way, Suite B
Vista, CA 92083
(800) 697-1111 FAX: (760) 599-9930
(760) 599-0598
http://www.displaysystems.com

Diversified Biotech
1208 VFW Parkway
Boston, MA 02132
(617) 965-8557 FAX: (617) 323-5641
(800) 796-9199
http://www.divbio.com

DNA ProScan
P.O. Box 121585
Nashville, TN 37212
(800) 841-4362 FAX: (615) 292-1436
(615) 298-3524
http://www.dnapro.com

DNAStar
1228 South Park Street
Madison, WI 53715
(608) 258-7420 FAX: (608) 258-7439
http://www.dnastar.com

DNAVIEW
Attn: Charles Brenner
http://www.wco.com
~cbrenner/dnaview.htm

Doall NYC
36-06 48th Avenue
Long Island City, NY 11101
(718) 392-4595 FAX: (718) 392-6115
http://www.doall.com

Dojindo Molecular Technologies
211 Perry Street Parkway, Suite 5
Gaitherbusburg, MD 20877
(877) 987-2667
http://www.dojindo.com

Dolla Eastern
See Doall NYC

Dolan Jenner Industries
678 Andover Street
Lawrence, MA 08143
(978) 681-8000 (978) 682-2500
http://www.dolan-jenner.com

Dominique Dutscher
30, rue de l'Industrie
BP 62
67172 Brumath, France
(03) 88 59 33 90 FAX : (03) 88 59 33 99
http://www.dutscher.com

Dow Chemical
Customer Service Center
2040 Willard H. Dow Center
Midland, MI 48674
(800) 232-2436 FAX: (517) 832-1190
(409) 238-9321
http://www.dow.com

Dow Corning
Northern Europe
Meriden Business Park
Copse Drive
Allesley, Coventry CV5 9RG, UK
(44) 1676 528 000
FAX: (44) 1676 528 001

Dow Corning
P.O. Box 994
Midland, MI 48686
(517) 496-4000
http://www.dowcorning.com

Dow Corning (Lubricants)
2200 West Salzburg Road
Auburn, MI 48611
(800) 248-2481 FAX: (517) 496-6974
(517) 496-6000

Dremel
4915 21st Street
Racine, WI 53406
(414) 554-1390
http://www.dremel.com

Drummond Scientific
500 Parkway
P.O. Box 700
Broomall, PA 19008
(800) 523-7480 FAX: (610) 353-6204
(610) 353-0200
http://www.drummondsci.com

Duchefa Biochemie BV
P.O. Box 2281
2002 CG Haarlem, The Netherlands
31-0-23-5319093
FAX: 31-0-23-5318027
http://www.duchefa.com

Duke Scientific
2463 Faber Place
Palo Alto, CA 94303
(800) 334-3883 FAX: (650) 424-1158
(650) 424-1177
http://www.dukescientific.com

Duke University Marine Laboratory
135 Duke Marine Lab Road
Beaufort, NC 28516-9721
(252) 504-7503 FAX: (252) 504-7648
http://www.env.duke.edu/rnarinelab

DuPont Biotechnology Systems
See NEN Life Science Products

DuPont Medical Products
See NEN Life Science Products

DuPont Merck Pharmaceuticals
331 Treble Cove Road
Billerica, MA 01862
(800) 225-1572 FAX: (508) 436-7501
http://www.dupontmerck.com

DuPont NEN Products
See NEN Life Science Products

Dynal
5 Delaware Drive
Lake Success, NY 11042
(800) 638-9416 FAX: (516) 326-3298
(516) 326-3270
http://www.dynal.net

Dynal AS
Ullernchausen 52,
0379 Oslo, Norway
47-22-06-10-00 FAX: 47-22-50-70-15
http://www.dynal.no

Dynalab
P.O. Box 112
Rochester, NY 14692
(800) 828-6595 FAX: (716) 334-9496
(716) 334-2060
http://www.dynalab.com

Dynarex
1 International Boulevard
Brewster, NY 10509
(888) DYNAREX FAX: (914) 279-9601
(914) 279-9600
http://www.dynarex.com

Dynatech
See Dynex Technologies

Dynex Technologies
14340 Sullyfield Circle
Chantilly, VA 22021
(800) 336-4543 FAX: (703) 631-7816
(703) 631-7800
http://www.dynextechnologies.com

Dyno Mill
See Willy A. Bachofen

E.S.A.
22 Alpha Road
Chelmsford, MA 01824
(508) 250-7000 FAX: (508) 250-7090

E.W. Wright
760 Durham Road
Guilford, CT 06437
(203) 453-6410 FAX: (203) 458-6901
http://www.ewwright.com

E-Y Laboratories
107 N. Amphlett Boulevard
San Mateo, CA 94401
(800) 821-0044 FAX: (650) 342-2648
(650) 342-3296
http://www.eylabs.com

Eastman Kodak
1001 Lee Road
Rochester, NY 14650
(800) 225-5352 FAX: (800) 879-4979
(716) 722-5780 FAX: (716) 477-8040
http://www.kodak.com

ECACC
See European Collection of Animal Cell Cultures

EC Apparatus
See Savant/EC Apparatus

Ecogen, SRL
Gensura Laboratories
Ptge. Dos de Maig
9(08041) Barcelona, Spain
(34) 3-450-2601 FAX: (34) 3-456-0607
http://www.ecogen.com

Ecolab
370 North Wabasha Street
St. Paul, MN 55102
(800) 35-CLEAN FAX: (651) 225-3098
(651) 352-5326
http://www.ecolab.com

ECO PHYSICS
3915 Research Park Drive, Suite A-3
Ann Arbor, MI 48108
(734) 998-1600 FAX: (734) 998-1180
http://www.ecophysics.com

Edge Biosystems
19208 Orbit Drive
Gaithersburg, MD 20879-4149
(800) 326-2685 FAX: (301) 990-0881
(301) 990-2685
http://www.edgebio.com

Edinburgh Instruments Ltd
2 Bain Square
Kirkton Campus
Livingston
EH54 7DQ, UK
(44) 1506 425 300 FAX: (44) 1406 425 320
http://www.edinst.com

Edmund Scientific
101 E. Gloucester Pike
Barrington, NJ 08007
(800) 728-6999 FAX: (856) 573-6263
(856) 573-6250
http://www.edsci.com

EG&G
See Perkin-Elmer

Ekagen
969 C Industry Road
San Carlos, CA 94070
(650) 592-4500 FAX: (650) 592-4500

Elcatech
P.O. Box 10935
Winston-Salem, NC 27108
(336) 544-8613 FAX: (336) 777-3623
(910) 777-3624
http://www.elcatech.com

Electron Microscopy Sciences
321 Morris Road
Fort Washington, PA 19034
(800) 523-5874 FAX: (215) 646-8931
(215) 646-1566
http://www.emsdiasum.com

Electron Tubes
100 Forge Way, Unit F
Rockaway, NJ 07866
(800) 521-8382 FAX: (973) 586-9771
(973) 586-9594
http://www.electrontubes.com

Elicay Laboratory Products, (UK) Ltd.
4 Manborough Mews
Crockford Lane
Basingstoke, Hampshire
RG 248NA, England
(256) 811-118 FAX: (256) 811-116
http://www.elkay-uk.co.uk

Eli Lilly
Lilly Corporate Center
Indianapolis, IN 46285
(800) 545-5979 FAX: (317) 276-2095
(317) 276-2000
http://www.lilly.com

ELISA Technologies
See Neogen

Elkins-Sinn
See Wyeth-Ayerst

EMBI
See European Bioinformatics Institute

EM Science
480 Democrat Road
Gibbstown, NJ 08027
(800) 222-0342 FAX: (856) 423-4389
(856) 423-6300
http://www.emscience.com

EM Separations Technology
See R & S Technology

Endogen
30 Commerce Way
Woburn, MA 01801
(800) 487-4885 FAX: (617) 439-0355
(781) 937-0890
http://www.endogen.com

ENGEL-Loter
HSGM Heatcutting Equipment & Machines
1865 E. Main Street, No. 5
Duncan, SC 29334
(888) 854-HSGM FAX: (864) 486-8383
(864) 486-8300
http://www.engelgmbh.com

Enzo Diagnostics
60 Executive Boulevard
Farmingdale, NY 11735
(800) 221-7705 FAX: (516) 694-7501
(516) 694-7070
http://www.enzo.com

Enzogenetics
4197 NW Douglas Avenue
Corvallis, OR 97330
(541) 757-0288

The Enzyme Center
See Charm Sciences

Enzyme Systems Products
486 Lindbergh Avenue
Livermore, CA 94550
(888) 449-2664 FAX: (925) 449-1866
(925) 449-2664
http://www.enzymesys.com

Epicentre Technologies
1402 Emil Street
Madison, WI 53713
(800) 284-8474 FAX: (608) 258-3088
(608) 258-3080
http://www.epicentre.com

Eppendorf
Brinkmann Instruments, Inc.
One Cantiague Road
P.O. Box 1019
Westbury, NY 11590-0207
(800) 645-3050 FAX: (516) 334-7506
http://www.eppendorf.com

Erie Scientific
20 Post Road
Portsmouth, NH 03801
(888) ERIE-SCI FAX: (603) 431-8996
(603) 431-8410
http://www.eriesci.com

ES Industries
701 South Route 73
West Berlin, NJ 08091
(800) 356-6140 FAX: (856) 753-8484
(856) 753-8400
http://www.esind.com

ESA
22 Alpha Road
Chelmsford, MA 01824
(800) 959-5095 FAX: (978) 250-7090
(978) 250-7000
http://www.esainc.com

Ethicon
Route 22, P.O. Box 151
Somerville, NJ 08876
(908) 218-0707
http://www.ethiconinc.com

Ethicon Endo-Surgery
4545 Creek Road
Cincinnati, OH 45242
(800) 766-9534 FAX: (513) 786-7080

Eurogentec
Parc Scientifique du Sart Tilman
4102 Seraing, Belgium
32-4-240-76-76 FAX: 32-4-264-07-88
http://www.eurogentec.com

European Bioinformatics Institute
Wellcome Trust Genomes Campus
Hinxton, Cambridge CB10 1SD, UK
(44) 1223-49444
FAX: (44) 1223-494468

European Collection of Animal Cell Cultures (ECACC)
Centre for Applied Microbiology & Research
Salisbury, Wiltshire SP4 0JG, UK
(44) 1980-612 512
FAX: (44) 1980-611 315
http://www.camr.org.uk

Evergreen Scientific
2254 E. 49th Street
P.O. Box 58248
Los Angeles, CA 90058
(800) 421-6261 FAX: (323) 581-2503
(323) 583-1331
http://www.evergreensci.com

Exalpha Biologicals
20 Hampden Street
Boston, MA 02205
(800) 395-1137 FAX: (617) 969-3872
(617) 558-3625
http://www.exalpha.com

Exciton
P.O. Box 31126
Dayton, OH 45437
(937) 252-2989 FAX: (937) 258-3937
http://www.exciton.com

Extrasynthese
ZI Lyon Nord
SA-BP62
69730 Genay, France
(33) 78-98-20-34
FAX: (33) 78-98-19-45

Factor II
1972 Forest Avenue
P.O. Box 1339
Lakeside, AZ 85929
(800) 332-8688 FAX: (520) 537-8066
(520) 537-8387
http://www.factor2.com

Falcon
See Becton Dickinson Labware

Febit AG
Käfertaler Strasse 190
D-66167 Mannheim
Germany
(49) 621-3804-0
FAX: (49) 621-3804-400
http://www.febit.com

Fenwal
See Baxter Healthcare

Fermentas Inc.
7520 Connelley Drive
Hanover, MD 21076
(800) 340-9026 FAX: (800) 472-8322
http://www.fermentas.com

FFE Weber
Am Klopferspitz 19
82152 Planegg, Germany
(49) 89-57954912
http://www.f-f-e.com/

Filemaker
5201 Patrick Henry Drive
Santa Clara, CA 95054
(408) 987-7000 (800) 325-2747

Fine Science Tools
202-277 Mountain Highway
North Vancouver, British Columbia
V7J 3P2 Canada
(800) 665-5355 FAX: (800) 665 4544
(604) 980-2481 FAX: (604) 987-3299

Fine Science Tools
373-G Vintage Park Drive
Foster City, CA 94404
(800) 521-2109 FAX: (800) 523-2109
(650) 349-1636 FAX: (630) 349-3729

Fine Science Tools
Fahrtgasse 7-13
D-69117 Heidelberg, Germany
(49) 6221 905050
FAX: (49) 6221 600001
http://www.finescience.com

Finn Aqua
AMSCO Finn Aqua Oy
Teollisuustiez, FIN-04300
Tuusula, Finland
358 025851 FAX: 358 0276019

Finnigan
355 River Oaks Parkway
San Jose, CA 95134
(408) 433-4800 FAX: (408) 433-4821
http://www.finnigan.com

Dr. L. Fischer
Lutherstrasse 25A
D-69120 Heidelberg
Germany
(49) 6221-16-0368
http://home.eplus-online.de/electroporation

Fisher Chemical Company
Fisher Scientific Limited
112 Colonnade Road Nepean
Ontario K2E 7L6, Canada
(800) 234-7437 FAX: (800) 463-2996
http://www.fisherscientific.com

Fisher Scientific
2000 Park Lane
Pittsburgh, PA 15275
(800) 766-7000 FAX: (800) 926-1166
(412) 562-8300
http://www3.fishersci.com

W.F. Fisher & Son
220 Evans Way, Suite #1
Somerville, NJ 08876
(908) 707-4050 FAX: (908) 707-4099

Fitzco
5600 Pioneer Creek Drive
Maple Plain, MN 55359
(800) 367-8760 FAX: (612) 479-2880
(612) 479-3489
http://www.fitzco.com

5 Prime → 3 Prime
See 2000 Eppendorf-5 Prime
http://www.5prime.com

Flambeau
15981 Valplast Road
Middlefield, Ohio 44062
(800) 232-3474 Fax: (440) 632-1581
(400) 632-1631
http://www.flambeau.com

Fleisch (Rusch)
2450 Meadowbrook Parkway
Duluth, GA 30096
(770) 623-0816 FAX: (770) 623-1829
http://ruschinc.com

Flow Cytometry Standards
P.O. Box 194344
San Juan, PR 00919
(800) 227-8143 FAX: (787) 758-3267
(787) 753-9341
http://www.fcstd.com

Flow Labs
See ICN Biomedicals

Flow-Tech Supply
P.O. Box 1388
Orange, TX 77631
(409) 882-0306 FAX: (409) 882-0254
http://www.flow-tech.com

Fluid Marketing
See Fluid Metering

Fluid Metering
5 Aerial Way, Suite 500
Sayosett, NY 11791
(516) 922-6050 FAX: (516) 624-8261
http://www.fmipump.com

Fluorochrome
1801 Williams, Suite 300
Denver, CO 80264
(303) 394-1000 FAX: (303) 321-1119

Fluka Chemical
See Sigma-Aldrich

FMC BioPolymer
1735 Market Street
Philadelphia, PA 19103
(215) 299-6000 FAX: (215) 299-5809
http://www.fmc.com

FMC BioProducts
191 Thomaston Street
Rockland, ME 04841
(800) 521-0390 FAX: (800) 362-1133
(207) 594-3400 FAX: (207) 594-3426
http://www.bioproducts.com

Forma Scientific
Milcreek Road
P.O. Box 649
Marietta, OH 45750
(800) 848-3080 FAX: (740) 372-6770
(740) 373-4765
http://www.forma.com

Fort Dodge Animal Health
800 5th Street NW
Fort Dodge, IA 50501
(800) 685-5656 FAX: (515) 955-9193
(515) 955-4600
http://www.ahp.com

Fotodyne
950 Walnut Ridge Drive
Hartland, WI 53029
(800) 362-3686 FAX: (800) 362-3642
(262) 369-7000 FAX: (262) 369-7013
http://www.fotodyne.com

Fresenius HemoCare
6675 185th Avenue NE, Suite 100
Redwood, WA 98052
(800) 909-3872
(425) 497-1197
http://www.freseniusht.com

Fresenius Hemotechnology
See Fresenius HemoCare

Fuji Medical Systems
419 West Avenue
P.O. Box 120035
Stamford, CT 06902
(800) 431-1850 FAX: (203) 353-0926
(203) 324-2000
http://www.fujimed.com

Fujisawa USA
Parkway Center North
Deerfield, IL 60015-2548
(847) 317-1088 FAX: (847) 317-7298

Ernest F. Fullam
900 Albany Shaker Road
Latham, NY 12110
(800) 833-4024 FAX: (518) 785-8647
(518) 785-5533
http://www.fullam.com

Gallard-Schlesinger Industries
777 Zechendorf
Boulevard Garden City, NY 11530
(516) 229-4000 FAX: (516) 229-4015
http://www.gallard-schlessinger.com

Gambro
Box 7373
SE 103 91 Stockholm, Sweden
(46) 8 613 65 00
FAX: (46) 8 611 37 31
In the US: **COBE Laboratories**
225 Union Blvd.
Lakewood, CO 80215
(303) 232-6800 FAX: (303) 231-4915
http://www.gambro.com

Garner Glass
177 Indian Hill Boulevard
Claremont, CA 91711
(909) 624-5071 FAX: (909) 625-0173
http://www.garnerglass.com

Garon Plastics
16 Byre Avenue
Somerton Park, South Australia 5044
(08) 8294-5126 FAX: (08) 8376-1487
http://www.apache.airnet.com.au/~garon

Garren Scientific
9400 Lurline Avenue, Unit E
Chatsworth, CA 91311
(800) 342-3725 FAX: (818) 882-3229
(818) 882-6544
http://www.garren-scientific.com

GATC Biotech AG
Jakob-Stadler-Platz 7
D-78467 Constance, Germany
(49) 07531-8160-0
FAX: (49) 07531-8160-81
http://www.gatc-biotech.com

Gaussian
Carnegie Office Park
Building 6, Suite 230
Carnegie, PA 15106
(412) 279-6700 FAX: (412) 279-2118
http://www.gaussian.com

G.C. Electronics/A.R.C. Electronics
431 Second Street
Henderson, KY 42420
(270) 827-8981 FAX: (270) 827-8256
http://www.arcelectronics.com

GDB (Genome Data Base, Curation)
2024 East Monument Street, Suite 1200
Baltimore, MD 21205
(410) 955-9705 FAX: (410) 614-0434
http://www.gdb.org

GDB (Genome Data Base, Home)
Hospital for Sick Children
555 University Avenue
Toronto, Ontario
M5G 1X8 Canada
(416) 813-8744 FAX: (416) 813-8755
http://www.gdb.org

Gelman Sciences
See Pall-Gelman

Gemini BioProducts
5115-M Douglas Fir Road
Calabasas, CA 90403
(818) 591-3530 FAX: (818) 591-7084

Gen Trak
5100 Campus Drive
Plymouth Meeting, PA 19462
(800) 221-7407 FAX: (215) 941-9498
(215) 825-5115
http://www.informagen.com

Genaissance Pharmaceuticals
5 Science Park
New Haven, CT 06511
(800) 678-9487 FAX: (203) 562-9377
(203) 773-1450
http://www.genaissance.com

GENAXIS Biotechnology
Parc Technologique
10 Avenue Ampère
Montigny le Bretoneux
78180 France
(33) 01-30-14-00-20
FAX: (33) 01-30-14-00-15
http://www.genaxis.com

GenBank
National Center for Biotechnology Information
National Library of Medicine/NIH
Building 38A, Room 8N805
8600 Rockville Pike
Bethesda, MD 20894
(301) 496-2475 FAX: (301) 480-9241
http://www.ncbi.nlm.nih.gov

Gene Codes
640 Avis Drive
Ann Arbor, MI 48108
(800) 497-4939 FAX: (734) 930-0145
(734) 769-7249
http://www.genecodes.com

Genemachines
935 Washington Street
San Carlos, CA 94070
(650) 508-1634 FAX: (650) 508-1644
(877) 855-4363
http://www.genemachines.com

Genentech
1 DNA Way
South San Francisco, CA 94080
(800) 551-2231 FAX: (650) 225-1600
(650) 225-1000
http://www.gene.com

General Scanning/GSI Luminomics
500 Arsenal Street
Watertown, MA 02172
(617) 924-1010 FAX: (617) 924-7327
http://www.genescan.com

General Valve
Division of Parker Hannifin Pneutronics
19 Gloria Lane
Fairfield, NJ 07004
(800) GVC-VALV
FAX: (800) GVC-1-FAX
http://www.pneutronics.com

Genespan
19310 North Creek Parkway, Suite 100
Bothell, WA 98011
(800) 231-2215 FAX: (425) 482-3005
(425) 482-3003
http://www.genespan.com

Gene Therapy Systems
10190 Telesis Court
San Diego, CA 92122
(858) 457-1919 FAX: (858) 623-9494
http://www.genetherapysystems.com

Généthon Human Genome Research Center
1 bis rue de l'Internationale
91000 Evry, France
(33) 169-472828
FAX: (33) 607-78698
http://www.genethon.fr

Genetic Microsystems
34 Commerce Way
Wobum, MA 01801
(781) 932-9333 FAX: (781) 932-9433
http://www.genticmicro.com

Genetic Mutant Repository
See Coriell Institute for Medical Research

Genetic Research Instrumentation
Gene House
Queenborough Lane
Rayne, Braintree, Essex CM7 8TF, UK
(44) 1376 332900
FAX: (44) 1376 344724
http://www.gri.co.uk

Genetics Computer Group
575 Science Drive
Madison, WI 53711
(608) 231-5200 FAX: (608) 231-5202
http://www.gcg.com

Genetics Institute/American Home Products
87 Cambridge Park Drive
Cambridge, MA 02140
(617) 876-1170 FAX: (617) 876-0388
http://www.genetics.com

Genetix
63-69 Somerford Road
Christchurch, Dorset BH23 3QA, UK
(44) (0) 1202 483900
FAX: (44)(0) 1202 480289
In the US: (877) 436 3849
US FAX: (888) 522 7499
http://www.genetix.co.uk

Gene Tools
One Summerton Way
Philomath, OR 97370
(541) 9292-7840 FAX: (541) 9292-7841
http://www.gene-tools.com

Geneva Bloinformatics (GeneBio) S.A.
25 Avenue de Champel
CH–1206 Geneva, Switzerland
(41) 22-702-9900
FAX: (41) 22-702-9999
http://www.genebio.com

GeneWorks
P.O. Box 11, Rundle Mall
Adelaide, South Australia 5000, Australia
1800 882 555 FAX: (08) 8234 2699
(08) 8234 2644
http://www.geneworks.com

Genome Systems (INCYTE)
4633 World Parkway Circle
St. Louis, MO 63134
(800) 430-0030 FAX: (314) 427-3324
(314) 427-3222
http://www.genomesystems.com

Genomic Solutions
4355 Varsity Drive, Suite E
Ann Arbor, MI 48108
(877) GENOMIC FAX: (734) 975-4808
(734) 975-4800
http://www.genomicsolutions.com

Genomyx
See Beckman Coulter

Genosys Biotechnologies
1442 Lake Front Circle, Suite 185
The Woodlands, TX 77380
(281) 363-3693 FAX: (281) 363-2212
http://www.genosys.com

Genotech
92 Weldon Parkway
St. Louis, MO 63043
(800) 628-7730 FAX: (314) 991-1504
(314) 991-6034

GENSET
876 Prospect Street, Suite 206
La Jolla, CA 92037
(800) 551-5291 FAX: (619) 551-2041
(619) 515-3061
http://www.genset.fr

Gensia Laboratories Ltd.
19 Hughes
Irvine, CA 92718
(714) 455-4700 FAX: (714) 855-8210

Genta
99 Hayden Avenue, Suite 200
Lexington, MA 02421
(781) 860-5150 FAX: (781) 860-5137
http://www.genta.com

GENTEST
6 Henshaw Street
Woburn, MA 01801
(800) 334-5229 FAX: (888) 242-2226
(781) 935-5115 FAX: (781) 932-6855
http://www.gentest.com

Gentra Systems
15200 25th Avenue N., Suite 104
Minneapolis, MN 55447
(800) 866-3039 FAX: (612) 476-5850
(612) 476-5858
http://www.gentra.com

Genzyme
1 Kendall Square
Cambridge, MA 02139
(617) 252-7500 FAX: (617) 252-7600
http://www.genzyme.com
See also R&D Systems

Genzyme Genetics
One Mountain Road
Framingham, MA 01701
(800) 255-7357 FAX: (508) 872-9080
(508) 872-8400
http://www.genzyme.com

George Tiemann & Co.
25 Plant Avenue
Hauppauge, NY 11788
(516) 273-0005 FAX: (516) 273-6199

GIBCO/BRL
A Division of Life Technologies
1 Kendall Square
Grand Island, NY 14072
(800) 874-4226 FAX: (800) 352-1968
(716) 774-6700
http://www.lifetech.com

Gilmont Instruments
A Division of Barnant Company
28N092 Commercial Avenue
Barrington, IL 60010
(800) 637-3739 FAX: (708) 381-7053
http://barnant.com

Gilson
3000 West Beltline Highway
P.O. Box 620027
Middletown, WI 53562
(800) 445-7661
(608) 836-1551
http://www.gilson.com

Glas-Col Apparatus
P.O. Box 2128
Terre Haute, IN 47802
(800) Glas-Col FAX: (812) 234-6975
(812) 235-6167
http://www.glascol.com

Glaxo Wellcome
Five Moore Drive
Research Triangle Park, NC 27709
(800) SGL-AXO5 FAX: (919)
248-2386
(919) 248-2100
http://www.glaxowellcome.com

Glen Mills
395 Allwood Road
Clifton, NJ 07012
(973) 777-0777 FAX: (973) 777-0070
http://www.glenmills.com

Glen Research
22825 Davis Drive
Sterling, VA 20166
(800) 327-4536 FAX: (800) 934-2490
(703) 437-6191 FAX: (703) 435-9774
http://www.glenresearch.com

Glo Germ
P.O. Box 189
Moab, UT 84532
(800) 842-6622 FAX: (435) 259-5930
http://www.glogerm.com

Glyco
11 Pimentel Court
Novato, CA 94949
(800) 722-2597 FAX: (415) 382-3511
(415) 884-6799
http://www.glyco.com

Gould Instrument Systems
8333 Rockside Road
Valley View, OH 44125
(216) 328-7000 FAX: (216) 328-7400
http://www.gould13.com

G.Q.F. Manufacturing
P.O. Box 1552
Savannah, GA 31402-1552
(912) 236-0651 FAX: (912) 234-9978
http://www.gqfmfg.com

Gralab Instruments
See Dimco-Gray

GraphPad Software
5755 Oberlin Drive #110
San Diego, CA 92121
(800) 388-4723 FAX: (558) 457-8141
(558) 457-3909
http://www.graphpad.com

Graseby Anderson
See Andersen Instruments
http://www.graseby.com

Grass Instrument
A Division of Astro-Med
600 East Greenwich Avenue
W. Warwick, RI 02893
(800) 225-5167 FAX: (877) 472-7749
http://www.grassinstruments.com

Greenacre and Misac Instruments
Misac Systems
27 Port Wood Road
Ware, Hertfordshire SF12 9NJ, UK
(44) 1920 463017
FAX: (44) 1920 465136

Greer Labs
639 Nuway Circle
Lenois, NC 28645
(704) 754-5237
http://greerlabs.com

Greiner
Maybachestrasse 2
Postfach 1162
D-7443 Frickenhausen, Germany
(49) 0 91 31/80 79 0
FAX: (49) 0 91 31/80 79 30
http://www.erlangen.com/greiner

Greiner Bio-One Inc.
P.O. Box 953279
Longwood, FL 32750
(407) 333-2800 FAX: (407) 333-3001
(800) 884-4703
http://www.gbo.com/bioscience

GSI Lumonics
130 Lombard Street Oxnard, CA 93030
(805) 485-5559 FAX: (805) 485-3310
http://www.gsilumonics.com

GTE Internetworking
150 Cambridge Park Drive
Cambridge, MA 02140
(800) 472-4565 FAX: (508) 694-4861
http://www.bbn.com

GW Instruments
35 Medford Street
Somerville, MA 02143
(617) 625-4096 FAX: (617) 625-1322
http://www.gwinst.com

H & H Woodworking
1002 Garfield Street
Denver, CO 80206
(303) 394-3764

Hacker Instruments
17 Sherwood Lane
P.O. Box 10033
Fairfield, NJ 07004
800-442-2537 FAX: (973) 808-8281
(973) 226-8450
http://www.hackerinstruments.com

Haemenetics
400 Wood Road
Braintree, MA 02184
(800) 225-5297 FAX: (781) 848-7921
(781) 848-7100
http://www.haemenetics.com

Halocarbon Products
P.O. Box 661
River Edge, NJ 07661
(201) 242-8899 FAX: (201) 262-0019
http://halocarbon.com

Hamamatsu Photonic Systems
A Division of Hamamatsu
360 Foothill Road
P.O. Box 6910
Bridgewater, NJ 08807
(908) 231-1116 FAX: (908) 231-0852
http://www.photonicsonline.com

Hamilton Company
4970 Energy Way
P.O. Box 10030
Reno, NV 89520
(800) 648-5950 FAX: (775) 856-7259
(775) 858-3000
http://www.hamiltoncompany.com

Hamilton Thorne Biosciences
100 Cummings Center, Suite 102C
Beverly, MA 01915
http://www.hamiltonthorne.com

Hampton Research
27631 El Lazo Road
Laguna Niguel, CA 92677
(800) 452-3899 FAX: (949) 425-1611
(949) 425-6321
http://www.hamptonresearch.com

Harlan Bioproducts for Science
P.O. Box 29176
Indianapolis, IN 46229
(317) 894-7521 FAX: (317) 894-1840
http://www.hbps.com

Harlan Sera-Lab
Hillcrest, Dodgeford Lane
Belton, Loughborough
Leicester LE12 9TE, UK
(44) 1530 222123
FAX: (44) 1530 224970
http://www.harlan.com

Harlan Teklad
P.O. Box 44220
Madison, WI 53744
(608) 277-2070 FAX: (608) 277-2066
http://www.harlan.com

Harrlck Scientific Corporation
88 Broadway
Ossining. NY 10562
(914) 762-0020 FAX: (914) 762-0914
http://www.harrickscl.com

Harrison Research
840 Moana Court
Palo Alto, CA 94306
(650) 949-1565 FAX: (650) 948-0493

Harvard Apparatus
84 October Hill Road
Holliston, MA 01746
(800) 272-2775 FAX: (508) 429-5732
(508) 893-8999
http://harvardapparatus.com

Harvard Bioscience
See Harvard Apparatus

Haselton Biologics
See JRH Biosciences

Hazelton Research Products
See Covance Research Products

Health Products
See Pierce Chemical

Heat Systems-Ultrasonics
1938 New Highway
Farmingdale, NY 11735
(800) 645-9846 FAX: (516) 694-9412
(516) 694-9555

Heidenhain Corp
333 East State Parkway
Schaumberg, IL 60173
(847) 490-1191 FAX: (847) 490-3931
http://www.heidenhain.com

HEKA Instruments
33 Valley Rd.
Southboro, MA 01960
(866) 742-0606 FAX: (508) 481-8945
http://www.heka.com

Hellma Cells
11831 Queens Boulevard
Forest Hills, NY 11375
(718) 544-9166 FAX: (718) 263-6910
http://www.helmaUSA.com

Hellma
Postfach 1163
D-79371 Müllheim/Baden, Germany
(49) 7631-1820
FAX: (49) 7631-13546
http://www.hellma-worldwide.de

Henry Schein
135 Duryea Road, Mail Room 150
Melville, NY 11747
(800) 472-4346 FAX: (516) 843-5652
http://www.henryschein.com

Heraeus Kulzer
4315 South Lafayette Boulevard
South Bend, IN 46614
(800) 343-5336
(219) 291-0661
http://www.kulzer.com

Heraeus Sepatech
See Kendro Laboratory Products

Hercules Aqualon
Aqualon Division
Hercules Research Center, Bldg. 8145
500 Hercules Road
Wilmington, DE 19899
(800) 345-0447 FAX: (302) 995-4787
http://www.herc.com/aqualon/pharma

Heto-Holten A/S
Gydevang 17-19
DK-3450 Allerod, Denmark
(45) 48-16-62-00
FAX: (45) 48-16-62-97
Distributed by ATR

Hettich-Zentrifugen
See Andreas Hettich

Hewlett-Packard
3000 Hanover Street
Mailstop 20B3
Palo Alto, CA 94304
(650) 857-1501 FAX: (650) 857-5518
http://www.hp.com

HGM Precision Engineering
Heidelberg, Germany

HGS Hinimoto Plastics
1-10-24 Meguro-Honcho
Megurouko
Tokyo 152, Japan
3-3714-7226 FAX: 3-3714-4657

Hitachi Scientific Instruments
Nissei Sangyo America
8100 N. First Street
San Elsa, CA 95314
(800) 548-9001 FAX: (408) 432-0704
(408) 432-0520
http://www.hii.hitachi.com

Hi-Tech Scientific
Brunel Road
Salisbury, Wiltshire, SP2 7PU
UK
(44) 1722-432320
(800) 344-0724 (US only)
http://www.hi-techsci.co.uk

Hoechst AG
See Aventis Pharmaceutical

Hoefer Scientific Instruments
Division of Amersham-Pharmacia Biotech
800 Centennial Avenue
Piscataway, NJ 08855
(800) 227-4750 FAX: (877) 295-8102
http://www.apbiotech.com

Hoffman-LaRoche
340 Kingsland Street
Nutley, NJ 07110
(800) 526-0189 FAX: (973) 235-9605
(973) 235-5000
http://www.rocheUSA.com

Holborn Surgical and Medical Instruments
Westwood Industrial Estate
Ramsgate Road
Margate, Kent CT9 4JZ UK
(44) 1843 296666
FAX: (44) 1843 295446

Honeywell
101 Columbia Road
Morristown, NJ 07962
(973) 455-2000 FAX: (973) 455-4807
http://www.honeywell.com

Honeywll Specialty Films
P.O. Box 1039
101 Columbia Road
Morristown, NJ 07962
(800) 934-5679 FAX: (973) 455-6045
http://www.honeywell-specialtyfilms.com

Hood Thermo-Pad Canada
Comp. 20, Site 61A, RR2
Summerland, British Columbia
V0H 1Z0 Canada
(800) 665-9555 FAX: (250) 494-5003
(250) 494-5002
http://www.thermopad.com

Horiba Instruments
17671 Armstrong Avenue
Irvine, CA 92714
(949) 250-4811 FAX: (949) 250-0924
http://www.horiba.com

Hoskins Manufacturing
10776 Hall Road
P.O. Box 218
Hamburg, MI 48139
(810) 231-1900 FAX: (810) 231-4311
http://www.hoskinsmfgco.com

Hosokawa Micron Powder Systems
10 Chatham Road
Summit, NJ 07901
(800) 526-4491 FAX: (908) 273-7432
(908) 273-6360
http://www.hosokawamicron.com

HT Biotechnology
Unit 4
61 Ditton Walk
Cambridge CB5 8QD, UK
(44) 1223-412583

Hugo Sachs Electronik
Postfach 138
7806 March-Hugstetten, Germany
D-79229(49) 7665-92000
FAX: (49) 7665-920090

Human Biologics International
7150 East Camelback Road, Suite 245
Scottsdale, AZ 85251
(480) 990-2005 FAX: (480)-990-2155
http://www.humanbiological.com

Human Genetic Mutant Cell Repository
See Coriell Institute for Medical Research

HVS Image
P.O. Box 100
Hampton, Middlesex TW12 2YD, UK
FAX: (44) 208 783 1223
In the US: (800) 225-9261
FAX: (888) 483-8033
http://www.hvsimage.com

Hybaid
111-113 Waldegrave Road
Teddington, Middlesex TW11 8LL, UK
(44) 0 1784 42500
FAX: (44) 0 1784 248085
http://www.hybaid.co.uk

Hybaid Instruments
8 East Forge Parkway
Franklin, MA 02028
(888)4-HYBAID FAX: (508) 541-3041
(508) 541-6918
http://www.hybaid.com

Hybridon
155 Fortune Boulevard
Milford, MA 01757
(508) 482-7500 FAX: (508) 482-7510
http://www.hybridon.com

HyClone Laboratories
1725 South HyClone Road
Logan, UT 84321
(800) HYCLONE FAX: (800) 533-9450
(801) 753-4584 FAX: (801) 750-0809
http://www.hyclone.com

Hyseq
670 Almanor Avenue
Sunnyvale, CA 94086
(408) 524-8100 FAX: (408) 524-8141
http://www.hyseq.com

IBA GmbH
1508 South Grand Blvd.
St Louis, MO 63104
(877) 422-4624 FAX: (888) 531-6813
http://www.iba-go.com

IBF Biotechnics
See Sepracor

IBI (International Biotechnologies)
See Eastman Kodak
For technical service (800) 243-2555
(203) 786-5600

ICN Biochemicals
See ICN Biomedicals

ICN Biomedicals
3300 Hyland Avenue
Costa Mesa, CA 92626
(800) 854-0530 FAX: (800) 334-6999
(714) 545-0100 FAX: (714) 641-7275
http://www.icnbiomed.com

ICN Flow and Pharmaceuticals
See ICN Biomedicals

ICN Immunobiochemicals
See ICN Biomedicals

ICN Radiochemicals
See ICN Biomedicals

ICONIX
100 King Street West, Suite 3825
Toronto, Ontario M5X 1E3 Canada
(416) 410-2411 FAX: (416) 368-3089
http://www.iconix.com

ICRT (Imperial Cancer Research Technology)
Sardinia House
Sardinia Street
London WC2A 3NL, UK
(44) 1712-421136
FAX: (44) 1718-314991

Idea Scientific Company
P.O. Box 13210
Minneapolis, MN 55414
(800) 433-2535 FAX: (612) 331-4217
http://www.ideascientific.com

IEC
See International Equipment Co.

IFR System, Inc.
see Aeroflex Incorporated

IITC
23924 Victory Boulevard
Woodland Hills, CA 91367
(888) 414-4482 (818) 710-1556
FAX: (818) 992-5165
http://www.iitcinc.com

IKA Works
2635 N. Chase Parkway, SE
Wilmington, NC 28405
(910) 452-7059 FAX: (910) 452-7693
http://www.ika.net

Ikegami Electronics
37 Brook Avenue
Maywood, NJ 07607
(201) 368-9171 FAX: (201) 569-1626

Ikemoto Scientific Technology
25-11 Hongo
3-chome, Bunkyo-ku
Tokyo 101-0025, Japan
(81) 3-3811-4181
FAX: (81) 3-3811-1960

Imagenetics
See ATC Diagnostics

Imaging Research
c/o Brock University
500 Glenridge Avenue
St. Catharines, Ontario
L2S 3A1 Canada
(905) 688-2040 FAX: (905) 685-5861
http://www.imaging.brocku.ca

Imclone Systems
180 Varick Street
New York, NY 10014
(212) 645-1405 FAX: (212) 645-2054
http://www.imclone.com

IMCO Corporation LTD., AB
P.O. Box 21195
SE-100 31
Stockholm, Sweden
46-8-33-53-09 FAX: 46-8-728-47-76
http://www.imcocorp.se

Imgenex Corporation
11175 Flintkole Avenue
Suite E
San Diego, CA 92121
(888) 723-4363 FAX: (858) 642-0937
(858) 642.0978
http://www.Imgenex.com

IMICO
Calle Vivero, No. 5-4a Planta
E-28040, Madrid, Spain
(34) 1-535-3960 FAX: (34)
1-535-2780

Immunex
51 University Street
Seattle, WA 98101
(206) 587-0430 FAX: (206) 587-0606
http://www.immunex.com

Immuno Biological Laboratories
8201 Central Avenue NE, Suite P
Minneapolis, MN 55432
(888) 523-1246 FAX: (763) 780-2988
(763) 780-2955
http://www.ibl-america.com

Immunochemistry Technologies
9401 James Avenue, South
Suite 155
Bloomington, MN 55431
(800) 829-3194 FAX: (952) 888-8988
(952) 888-8788
http://www.immunochemistry.com

Immunocorp
1582 W. Deere Avenue
Suite C
Irvine, CA 92606
(800) 446-3063
http://www.immunocorp.com

Immunotech
130, av. Delattre de Tassigny
B.P. 177
13276 Marseilles Cedex 9
France
(33) 491-17-27-00
FAX: (33) 491-41-43-58
http://www.immunotech.fr

Imperial Chemical Industries
Imperial Chemical House
Millbank, London SW1P 3JF, UK
(44) 171-834-4444
FAX: (44)171-834-2042
http://www.ici.com

Improvision
1 Cranberry Hill
Lexington, MA 02421
(781) 402-0134 FAX: (781) 402-0251
http://www.improvision.com

Inceltech
See New Brunswick Scientific

Incstar
See DiaSorin

Incyte
6519 Dumbarton Circle
Fremont, CA 94555
(510) 739-2100 FAX: (510) 739-2200
http://www.incyte.com

Incyte Pharmaceuticals
3160 Porter Drive
Palo Alto, CA 94304
(877) 746-2983 FAX: (650) 855-0572
(650) 855-0555
http://www.incyte.com

Individual Monitoring Systems
6310 Harford Road
Baltimore, MD 21214

Indo Fine Chemical
P.O. Box 473
Somerville, NJ 08876
(888) 463-6346 FAX: (908) 359-1179
(908) 359-6778
http://www.indofinechemical.com

Industrial Acoustics
1160 Commerce Avenue
Bronx, NY 10462
(718) 931-8000 FAX: (718) 863-1138
http://www.industrialacoustics.com

Inex Pharmaceuticals
100-8900 Glenlyon Parkway
Glenlyon Business Park
Burnaby, British Columbia
V5J 5J8 Canada
(604) 419-3200 FAX: (604) 419-3201
http://www.inexpharm.com

Ingold, Mettler, Toledo
261 Ballardvale Street
Wilmington, MA 01887
(800) 352-8763 FAX: (978) 658-0020
(978) 658-7615
http://www.mt.com

Innogenetics N.V.
Technologie Park 6
B-9052 Zwijnaarde
Belgium
(32) 9-329-1329 FAX: (32)
9-245-7623
http://www.innogenetics.com

Innovative Medical Services
1725 Gillespie Way
El Cajon, CA 92020
(619) 596-8600 FAX: (619) 596-8700
http://www.imspure.com

Innovative Research
3025 Harbor Lane N, Suite 300
Plymouth, MN 55447
(612) 519-0105 FAX: (612) 519-0239
http://www.inres.com

Innovative Research of America
2 N. Tamiami Trail, Suite 404
Sarasota, FL 34236
(800) 421-8171 FAX: (800) 643-4345
(941) 365-1406 FAX: (941) 365-1703
http://www.innovrsrch.com

Inotech Biosystems
15713 Crabbs Branch Way, #110
Rockville, MD 20855
(800) 635-4070 FAX: (301) 670-2859
(301) 670-2850
http://www.inotechintl.com

INOVISION
22699 Old Canal Road
Yorba Linda, CA 92887
(714) 998-9600 FAX: (714) 998-9666
http://www.inovision.com

Instech Laboratories
5209 Militia Hill Road
Plymouth Meeting, PA 19462
(800) 443-4227 FAX: (610) 941-0134
(610) 941-0132
http://www.instechlabs.com

Instron
100 Royall Street
Canton, MA 02021
(800) 564-8378 FAX: (781) 575-5725
(781) 575-5000
http://www.instron.com

Instrumentarium
P.O. Box 300
00031 Instrumentarium
Helsinki, Finland
(10) 394-5566
http://www.instrumentarium.fi

Instruments SA
Division Jobin Yvon
16-18 Rue du Canal
91165 Longjumeau, Cedex, France
(33)1 6454-1300
FAX: (33)1 6909-9319
http://www.isainc.com

Instrutech
20 Vanderventer Avenue, Suite 101E
Port Washington, NY 11050
(516) 883-1300 FAX: (516) 883-1558
http://www.instrutech.com

Integrated Dynamics Engineering
377 University Avenue
Westwood, MA 02090
(781) 326-5700
http://www.ideworld.com

Integrated DNA Technologies
1710 Commercial Park
Coralville, Iowa 52241
(800) 328-2661 FAX: (319) 626-8444
http://www.idtdna.com

Integrated Genetics
See Genzyme Genetics

Integrated Scientific Imaging Systems
3463 State Street, Suite 431
Santa Barbara, CA 93105
(805) 692-2390 FAX: (805) 692-2391
http://www.imagingsystems.com

Integrated Separation Systems (ISS)
See OWL Separation Systems

IntelliGenetics
See Oxford Molecular Group

Interactiva BioTechnologie
Sedanstrasse 10
D-89077 Ulm, Germany
(49) 731-93579-290
FAX: (49) 731-93579-291
http://www.interactiva.de

Interchim
213 J.F. Kennedy Avenue
B.P. 1140
Montlucon
03103 France
(33) 04-70-03-83-55
FAX: (33) 04-70-03-93-60

Interfacial Diagnostics
29851 Willow Creek Road
Eugene, OR 97402
(541) 335-0338 FAX: (541) 335-0305
http://www.idclatex.com

Interfocus
14/15 Spring Rise
Falcover Road
Haverhill, Suffolk CB9 7XU, UK
(44) 1440 703460
FAX: (44) 1440 704397
http://www.interfocus.ltd.uk

Intergen
2 Manhattanville Road
Purchase, NY 10577
(800) 431-4505 FAX: (800) 468-7436
(914) 694-1700 FAX: (914) 694-1429
http://www.intergenco.com

Intermountain Scientific
420 N. Keys Drive
Kaysville, UT 84037
(800) 999-2901 FAX: (800) 574-7892
(801) 547-5047 FAX: (801) 547-5051
http://www.bioexpress.com

International Biotechnologies (IBI)
See Eastman Kodak

International Equipment Co. (IEC)
See Thermoquest

International Institute for the Advancement of Medicine
1232 Mid-Valley Drive
Jessup, PA 18434
(800) 486-IIAM FAX: (570) 343-6993
(570) 496-3400
http://www.iiam.org

International Light
17 Graf Road
Newburyport, MA 01950
(978) 465-5923 FAX: (978) 462-0759

International Market Supply (I.M.S.)
Dane Mill
Broadhurst Lane
Congleton, Cheshire CW12 1LA, UK
(44) 1260 275469
FAX: (44) 1260 276007

International Marketing Services
See International Marketing Ventures

International Marketing Ventures
6301 Ivy Lane, Suite 408
Greenbelt, MD 20770
(800) 373-0096 FAX: (301) 345-0631
(301) 345-2866
http://www.imvlimited.com

International Products
201 Connecticut Drive
Burlington, NJ 08016
(609) 386-8770 FAX: (609) 386-8438
http://www.mkt@ipcol.com

Intersens Instruments
B.V. Scheltussingel 156
3814 BH
Amersfoort, The Netherlands
(31) 33-472-6664 FAX: (31)
33-475-1746

Intracel Corporation
Bartels Division
2005 Sammamish Road, Suite 107
Issaquah, WA 98027
(800) 542-2281 FAX: (425) 557-1894
(425) 392-2992
http://www.intracel.com

IntrAction Corp
3719 Warren Ave
Bellwood, IL 60104

Invitrogen
1600 Faraday Avenue
Carlsbad, CA 92008
(800) 955-6288 FAX: (760) 603-7201
(760) 603-7200
http://www.invitrogen.com

In Vivo Metric
P.O. Box 249
Healdsburg, CA 95448
(707) 433-4819 FAX: (707) 433-2407

IRORI
9640 Towne Center Drive
San Diego, CA 92121
(858) 546-1300 FAX: (858) 546-3083
http://www.irori.com

Irvine Scientific
2511 Daimler Street
Santa Ana, CA 92705
(800) 577-6097 FAX: (949) 261-6522
(949) 261-7800
http://www.irvinesci.com

ISC BioExpress
420 North Kays Drive
Kaysville, UT 84037
(800) 999-2901 FAX: (800) 574-7892
(801) 547-5047
http://www.bioexpress.com

ISCO
P.O. Box 5347
4700 Superior
Lincoln, NE 68505
(800) 228-4373 FAX: (402) 464-0318
(402) 464-0231
http://www.isco.com

Isis Pharmaceuticals
Carlsbad Research Center
2292 Faraday Avenue
Carlsbad, CA 92008
(760) 931-9200
http://www.isip.com

Isolabs
See Wallac

Isobiotec
Ortenauer Strasse 13
69126 Heidelberg, Germany
(49) 6221 307 150 FAX: (49)
6221 307 151
http://www.isobiotec.com

ISS Inc.
1602 Newton Drive
Champaign, IL 61822-1061
(217) 359-8681 FAX: (217) 359-7879
http://www.iss.com

J & W Scientific
See Agilent Technologies

J.A. Webster
86 Leominster Road
Sterling, MA 01564
(800) 225-7911 FAX: (978) 422-8959
http://www.jawebster.com

J.T. Baker
See Mallinckrodt Baker
222 Red School Lane
Phillipsburg, NJ 08865
(800) JTBAKER FAX: (908) 859-6974
http://www.jtbaker.com

Jackson ImmunoResearch Laboratories
P.O. Box 9
872 W. Baltimore Pike
West Grove, PA 19390
(800) 367-5296 FAX: (610) 869-0171
(610) 869-4024
http://www.jacksonimmuno.com

The Jackson Laboratory
600 Maine Street
Bar Harbor, ME 04059
(800) 422-6423 FAX: (207) 288-5079
(207) 288-6000
http://www.jax.org

Jaece Industries
908 Niagara Falls Boulevard
North Tonawanda, NY 14120
(716) 694-2811 FAX: (716) 694-2811
http://www.jaece.com

Jandel Scientific
See SPSS

Janke & Kunkel
See Ika Works

Janssen Life Sciences Products
See Amersham

Janssen Pharmaceutica
1125 Trenton-Harbourton Road
Titusville, NJ 09560
(609) 730-2577 FAX: (609) 730-2116
http://us.janssen.com

Jasco
8649 Commerce Drive
Easton, MD 21601
(800) 333-5272 FAX: (410) 822-7526
(410) 822-1220
http://www.jascoinc.com

Jeffers
P.O. Box 100
Dothan, AL 36302
(800) 533-3377 FAX: 1-334-793-5179
http://www.1800jeffers.com/ssc/

Jena Bioscience
Loebstedter Str. 78
07749 Jena, Germany
(49) 3641-464920
FAX: (49) 3641-464991
http://www.jenabioscience.com

Jencons Scientific
800 Bursca Drive, Suite 801
Bridgeville, PA 15017
(800) 846-9959 FAX: (412) 257-8809
(412) 257-8861
http://www.jencons.co.uk

JEOL Instruments
11 Dearborn Road
Peabody, MA 01960
(978) 535-5900 FAX: (978) 536-2205
http://www.jeol.com/index.html

Jewett
750 Grant Street
Buffalo, NY 14213
(800) 879-7767 FAX: (716) 881-6092
(716) 881-0030
http://www.JewettInc.com

Jobin-Yvon Ltd.
2 Dalston Gardens
Stanmore
Middx, HA7 1BQ, UK
(44) 0 20 8204 8142 FAX: (44) 0 20
8204 6142
http://www.techpro.com

John's Scientific
See VWR Scientific

John Weiss and Sons
95 Alston Drive
Bradwell Abbey
Milton Keynes, Buckinghamshire
MK1 4HF UK
(44) 1908-318017
FAX: (44) 1908-318708

Johnson & Johnson Medical
2500 Arbrook Boulevard East
Arlington, TX 76004
(800) 423-4018
http://www.jnjmedical.com

Johnston Matthey Chemicals
Orchard Road
Royston, Hertfordshire SG8 5HE, UK
(44) 1763-253000
FAX: (44) 1763-253466
http://www.chemicals.matthey.com

Jolley Consulting and Research
683 E. Center Street, Unit H
Grayslake, IL 60030
(847) 548-2330 FAX: (847) 548-2984
http://www.jolley.com

Jordan Scientific
See Shelton Scientific

Jorgensen Laboratories
1450 N. Van Buren Avenue
Loveland, CO 80538
(800) 525-5614 FAX: (970) 663-5042
(970) 669-2500
http://www.jorvet.com

JRH Biosciences and **JR Scientific**
13804 W. 107th Street
Lenexa, KS 66215
(800) 231-3735 FAX: (913) 469-5584
(913) 469-5580

Jule Bio Technologies
25 Science Park, #14, Suite 695
New Haven, CT 06511
(800) 648-1772 FAX: (203) 786-5489
(203) 786-5490
http://hometown.aol.com/precastgel/index.htm

K.R. Anderson
2800 Bowers Avenue
Santa Clara, CA 95051
(800) 538-8712 FAX: (408) 727-2959
(408) 727-2800
http://www.kranderson.com

Kabi Pharmacia Diagnostics
See Pharmacia Diagnostics

Kanthal H.P. Reid
1 Commerce Boulevard
P.O. Box 352440
Palm Coast, FL 32135
(904) 445-2000 FAX: (904) 446-2244
http://www.kanthal.com

Kapak
5305 Parkdale Drive
St. Louis Park, MN 55416
(800) KAPAK-57 FAX: (612) 541-0735
(612) 541-0730
http://www.kapak.com

Karl Hecht
Stettener Str. 22-24
D-97647 Sondheim
Rhön, Germany
(49) 9779-8080 FAX: (49) 9779-80888

Karl Storz
Köningin-Elisabeth Str. 60
D-14059 Berlin, Germany
(49) 30-30 69 09-0
FAX: (49) 30-30 19 452
http://www.karlstorz.de

KaVo EWL
P.O. Box 1320
D-88293 Leutkirch im Allgäu, Germany
(49) 7561-86-0 FAX: (49)
7561-86-371
http://www.kavo.com/english/
startseite.htm

Keithley Instruments
28775 Aurora Road
Cleveland, OH 44139
(800) 552-1115 FAX: (440) 248-6168
(440) 248-0400
http://www.keithley.com

Kemin
2100 Maury Street, Box 70
Des Moines, IA 50301
(515) 266-2111 FAX: (515) 266-8354
http://www.kemin.com

Kemo
3 Brook Court, Blakeney Road
Beckenham, Kent BR3 1HG, UK
(44) 0181 658 3838
FAX: (44) 0181 658 4084
http://www.kemo.com

Kendall
15 Hampshire Street
Mansfield, MA 02048
(800) 962-9888 FAX: (800) 724-1324
http://www.kendallhq.com

Kendro Laboratory Products
31 Pecks Lane
Newtown, CT 06470
(800) 522-SPIN FAX: (203) 270-2166
(203) 270-2080
http://www.kendro.com

Kendro Laboratory Products
P.O. Box 1220
Am Kalkberg
D-3360 Osterod, Germany
(55) 22-316-213
FAX: (55) 22-316-202
http://www.heraeus-instruments.de

Kent Laboratories
23404 NE 8th Street
Redmond, WA 98053
(425) 868-6200 FAX: (425) 868-6335
http://www.kentlabs.com

Kent Scientific
457 Bantam Road, #16
Litchfield, CT 06759
(888) 572-8887 FAX: (860) 567-4201
(860) 567-5496
http://www.kentscientific.com

Kentech Instruments, Ltd.
Unit 9, Hall Farm Workshops
South Morton, Didcot
Oxfordshire
OX11 9AG, UK
(44) 1 235 510 748 FAX: (44) 1 235
510 722
http://www.kentech.co.uk

Kerry Ultrasonics
Guyson International, Inc.
Snaygill Industrial Estate
Keighley Road
Skipton, North Yorkshire
BD23 2QR, UK
44 (0) 1756 799911 FAX: 44 (0)
1756 790213
http://www.kerry.co.uk

Keuffel & Esser
See Azon

Keystone Scientific
Penn Eagle Industrial Park
320 Rolling Ridge Drive
Bellefonte, PA 16823
(800) 437-2999 FAX: (814) 353-2305
(814) 353-2300 Ext 1
http://www.keystonescientific.com

Kimble/Kontes Biotechnology
1022 Spruce Street
P.O. Box 729
Vineland, NJ 08360
(888) 546-2531 FAX: (856) 794-9762
(856) 692-3600
http://www.kimble-kontes.com

Kinematica AG
Luzernerstrasse 147a
CH-6014 Littau-Luzern, Switzerland
(41) 41 2501257 FAX: (41) 41
2501460
http://www.kinematica.ch

Kin-Tek
504 Laurel Street
LaMarque, TX 77568
(800) 326-3627
FAX: (409) 938-3710
http://www.kin-tek.com

Kipp & Zonen
125 Wilbur Place
Bohemia, NY 11716
(800) 645-2065 FAX: (516) 589-2068
(516) 589-2885
http://www.kippzonen.thomasregister.
com/olc/kippzonen

Kirkegaard & Perry Laboratories
2 Cessna Court
Gaithersburg, MD 20879
(800) 638-3167 FAX: (301) 948-0169
(301) 948-7755
http://www.kpl.com

Kodak
See Eastman Kodak

Kontes Glass
See Kimble/Kontes Biotechnology

Kontron Instruments AG
Postfach CH-8010
Zurich, Switzerland
41-1-733-5733 FAX: 41-1-733-5734

David Kopf Instruments
P.O. Box 636
Tujunga, CA 91043
(818) 352-3274 FAX: (818) 352-3139

Kraft Apparatus
See Glas-Col Apparatus

Kramer Scientific Corporation
711 Executive Boulevard
Valley Cottage, NY 10989
(845) 267-5050 FAX: (845) 267-5550

Kulite Semiconductor Products
1 Willow Tree Road
Leonia, NJ 07605
(201) 461-0900 FAX: (201) 461-0990
http://www.kulite.com

Lab-Line Instruments
15th & Bloomingdale Avenues
Melrose Park, IL 60160
(800) LAB-LINE FAX: (708) 450-5830
FAX: (800) 450-4LAB
http://www.labline.com

Lab Products
742 Sussex Avenue
P.O. Box 639
Seaford, DE 19973
(800) 526-0469 FAX: (302) 628-4309
(302) 628-4300
http://www.labproductsinc.com

LabRepco
101 Witmer Road, Suite 700
Horsham, PA 19044
(800) 521-0754 FAX: (215) 442-9202
http://www.labrepco.com

Lab Safety Supply
P.O. Box 1368
Janesville, WI 53547
(800) 356-0783 FAX: (800) 543-9910
(608) 754-7160 FAX: (608) 754-1806
http://www.labsafety.com

Lab-Tek Products
See Nalge Nunc International

Lab-Vision Corp.
47790 Westinghouse Drive
Fremont, CA 94530
(800) 828-1628 FAX: (510) 991-2826
(510) 991-2800
http://www.Lab.Vision.com

Labconco
8811 Prospect Avenue
Kansas City, MO 64132
(800) 821-5525 FAX: (816) 363-0130
(816) 333-8811
http://www.labconco.com

Labindustries
See Barnstead/Thermolyne

Labnet International
P.O. Box 841
Woodbridge, NJ 07095
(888) LAB-NET1 FAX: (732) 417-1750
(732) 417-0700
http://www.nationallabnet.com

LABO-MODERNE
37 rue Dombasle
Paris
75015 France
(33) 01-45-32-62-54
FAX: (33) 01-45-32-01-09
http://www.labomoderne.com/fr

Laboratory of Immunoregulation
National Institute of Allergy and
Infectious Diseases/NIH
9000 Rockville Pike
Building 10, Room 11B13
Bethesda, MD 20892
(301) 496-1124

Laboratory Supplies
29 Jefry Lane
Hicksville, NY 11801
(516) 681-7711

Labscan Limited
Stillorgan Industrial Park
Stillorgan
Dublin, Ireland
(353) 1-295-2684
FAX: (353) 1-295-2685
http://www.labscan.ie

Labsystems
See Thermo Labsystems

Labsystems Affinity Sensors
Saxon Way, Bar Hill
Cambridge CB3 8SL, UK
44 (0) 1954 789976
FAX: 44 (0) 1954 789417
http://www.affinity-sensors.com

Labtronics
546 Governors Road
Guelph, Ontario N1K 1E3, Canada
(519) 763-4930 FAX: (519) 836-4431
http://www.labtronics.com

Labtronix Manufacturing
3200 Investment Boulevard
Hayward, CA 94545
(510) 786-3200 FAX: (510) 786-3268
http://www.labtronix.com

Lafayette Instrument
3700 Sagamore Parkway North
P.O. Box 5729
Lafayette, IN 47903
(800) 428-7545 FAX: (765) 423-4111
(765) 423-1505
http://www.lafayetteinstrument.com

Lambert Instruments
Turfweg 4
9313 TH Leutingewolde
The Netherlands
(31) 50-5018461 FAX: (31)
50-5010034
http://www.lambert-instruments.com

Lampire Biological Laboratories
P.O. Box 270
Pipersville, PA 18947
(215) 795-2538 Fax: (215) 795-0237
http://www.lampire.com

Lancaster Synthesis
P.O. Box 1000
Windham, NH 03087
(800) 238-2324 FAX: (603) 889-3326
(603) 889-3306
http://www.lancastersynthesis-us.com

Lancer
140 State Road 419
Winter Springs, FL 32701
(800) 332-1855 FAX: (407) 327-1229
(407) 327-8488
http://www.lancer.com

LaVision GmbH
Gerhard-Gerdes-Str. 3
D-37079
Goettingen, Germany
(49) 551-50549-0
FAX: (49) 551-50549-11
http://www.lavision.de

Lawshe
See Advanced Process Supply

Laxotan
20, rue Leon Blum
26000 Valence, France
(33) 4-75-41-91-91
FAX: (33) 4-75-41-91-98
http://www.latoxan.com

LC Laboratories
165 New Boston Street
Woburn, MA 01801
(781) 937-0777 FAX: (781) 938-5420
http://www.lclaboratories.com

LC Packings
80 Carolina Street
San Francisco, CA 94103
(415) 552-1855 FAX: (415) 552-1859
http://www.lcpackings.com

LC Services
See LC Laboratories

LECO
3000 Lakeview Avenue
St. Joseph, MI 49085
(800) 292-6141 FAX: (616) 982-8977
(616) 985-5496
http://www.leco.com

Lederle Laboratories
See Wyeth-Ayerst

Lee Biomolecular Research Laboratories
11211 Sorrento Valley Road, Suite M
San Diego, CA 92121
(858) 452-7700

The Lee Company
2 Pettipaug Road
P.O. Box 424
Westbrook, CT 06498
(800) LEE-PLUG FAX: (860)
399-7058
(860) 399-6281
http://www.theleeco.com

Lee Laboratories
1475 Athens Highway
Grayson, GA 30017
(800) 732-9150 FAX: (770) 979-9570
(770) 972-4450
http://www.leelabs.com

Leica
111 Deer Lake Road
Deerfield, IL 60015
(800) 248-0123 FAX: (847) 405-0147
(847) 405-0123
http://www.leica.com

Leica Microsystems
Imneuenheimer Feld 518
D-69120
Heidelberg, Germany
(49) 6221-41480
FAX: (49) 6221-414833
http://www.leica-microsystems.com

Leinco Technologies
359 Consort Drive
St. Louis, MO 63011
(314) 230-9477 FAX: (314) 527-5545
http://www.leinco.com

Leitz U.S.A.
See Leica

LenderKing Metal Products
8370 Jumpers Hole Road
Millersville, MD 21108
(410) 544-8795 FAX: (410) 544-5069
http://www.lenderking.com

Letica Scientific Instruments
Panlab s.i., c/Loreto 50
08029 Barcelona, Spain
(34) 93-419-0709
FAX: (34) 93-419-7145
www.panlab-sl.com

Leybold-Heraeus Trivac DZA
5700 Mellon Road
Export, PA 15632
(412) 327-5700

LI-COR
Biotechnology Division
4308 Progressive Avenue
Lincoln, NE 68504
(800) 645-4267 FAX: (402) 467-0819
(402) 467-0700
http://www.licor.com

Life Science Laboratories
See Adaptive Biosystems

Life Science Resources
Two Corporate Center Drive
Melville, NY 11747
(800) 747-9530 FAX: (516) 844-5114
(516) 844-5085
http://www.astrocam.com

Life Sciences
2900 72nd Street North
St. Petersburg, FL 33710
(800) 237-4323 FAX: (727) 347-2957
(727) 345-9371
http://www.lifesci.com

Life Technologies
9800 Medical Center Drive
P.O. Box 6482
Rockville, MD 20849
(800) 828-6686 FAX: (800) 331-2286
http://www.lifetech.com

Lifecodes
550 West Avenue
Stamford, CT 06902
(800) 543-3263 FAX: (203) 328-9599
(203) 328-9500
http://www.lifecodes.com

Lightnin
135 Mt. Read Boulevard
Rochester, NY 14611
(888) MIX-BEST FAX: (716) 527-1742
(716) 436-5550
http://www.lightnin-mixers.com

Linear Drives
Luckyn Lane, Pipps Hill
Basildon, Essex SS14 3BW, UK
(44) 1268-287070
FAX: (44) 1268-293344
http://www.lineardrives.com

Linscott's Directory
4877 Grange Road
Santa Rosa, CA 95404
(707) 544-9555 FAX: (415) 389-6025
http://www.linscottsdirectory.co.uk

Linton Instrumentation
Unit 11, Forge Business Center
Upper Rose Lane
Palgrave, Diss, Norfolk IP22 1AP, UK
(44) 1-379-651-344
FAX: (44) 1-379-650-970
http://www.lintoninst.co.uk

List Biological Laboratories
501-B Vandell Way
Campbell, CA 95008
(800) 726-3213 FAX: (408) 866-6364
(408) 866-6363
http://www.listlabs.com

LKB Instruments
See Amersham Pharmacia Biotech

Lloyd Laboratories
604 West Thomas Avenue
Shenandoah, IA 51601
(800) 831-0004 FAX: (712) 246-5245
(712) 246-4000
http://www.lloydinc.com

Loctite
1001 Trout Brook Crossing
Rocky Hill, CT 06067
(860) 571-5100 FAX: (860)571-5465
http://www.loctite.com

Lofstrand Labs
7961 Cessna Avenue
Gaithersburg, MD 20879
(800) 541-0362 FAX: (301) 948-9214
(301) 330-0111
http://www.lofstrand.com

Lomir Biochemical
99 East Main Street
Malone, NY 12953
(877) 425-3604 FAX: (518) 483-8195
(518) 483-7697
http://www.lomir.com

LSL Biolafitte
10 rue de Temara
7810C St.-Germain-en-Laye, France
(33) 1-3061-5260 FAX: (33)
1-3061-5234

Ludl Electronic Products
171 Brady Avenue
Hawthorne, NY 10532
(888) 769-6111 FAX: (914) 769-4759
(914) 769-6111
http://www.ludl.com

Lumigen
24485 W. Ten Mile Road
Southfield, MI 48034
(248) 351-5600 FAX: (248) 351-0518
http://www.lumigen.com

Luminex
12212 Technology Boulevard
Austin, TX 78727
(888) 219-8020 FAX: (512) 258-4173
(512) 219-8020
http://www.luminexcorp.com

LYNX Therapeutics
25861 Industrial Boulevard
Hayward, CA 94545
(510) 670-9300 FAX: (510) 670-9302
http://www.lynxgen.com

Lyphomed
3 Parkway North
Deerfield, IL 60015
(847) 317-8100 FAX: (847) 317-8600

M.E.D. Associates
See Med Associates

Macherey-Nagel
6 South Third Street, #402
Easton, PA 18042
(610) 559-9848 FAX: (610) 559-9878
http://www.macherey-nagel.com

Macherey-Nagel
Valencienner Strasse 11
P.O. Box 101352
D-52313 Dueren, Germany
(49) 2421-969141
FAX: (49) 2421-969199
http://www.macherey-nagel.ch

Mac-Mod Analytical
127 Commons Court
Chadds Ford, PA 19317
800-441-7508 FAX: (610) 358-5993
(610) 358-9696
http://www.mac-mod.com

Mallinckrodt Baker
222 Red School Lane
Phillipsburg, NJ 08865
(800) 582-2537 FAX: (908) 859-6974
(908) 859-2151
http://www.mallbaker.com

Mallinckrodt Chemicals
16305 Swingley Ridge Drive
Chesterfield, MD 63017
(314) 530-2172 FAX: (314) 530-2563
http://www.mallchem.com

Malven Instruments
Enigma Business Park
Grovewood Road
Malven, Worchestershire
WR 141 XZ, United Kingdom

Marinus
1500 Pier C Street
Long Beach, CA 90813
(562) 435-6522 FAX: (562) 495-3120

Markson Science
c/o Whatman Labs Sales
P.O. Box 1359
Hillsboro, OR 97123
(800) 942-8626 FAX: (503) 640-9716
(503) 648-0762

Marsh Biomedical Products
565 Blossom Road
Rochester, NY 14610
(800) 445-2812 FAX: (716) 654-4810
(716) 654-4800
http://www.biomar.com

Marshall Farms USA
5800 Lake Bluff Road
North Rose, NY 14516
(315) 587-2295
e-mail: info@marfarms.com

Martek
6480 Dobbin Road
Columbia, MD 21045
(410) 740-0081 FAX: (410) 740-2985
http://www.martekbio.com

Martin Supply
Distributor of Gerber Scientific
2740 Loch Raven Road
Baltimore, MD 21218
(800) 282-5440 FAX: (410) 366-0134
(410) 366-1696

Mast Immunosystems
630 Clyde Court
Mountain View, CA 94043
(800) 233-MAST FAX: (650) 969-2745
(650) 961-5501
http://www.mastallergy.com

Matheson Gas Products
P.O. Box 624
959 Route 46 East
Parsippany, NJ 07054
(800) 416-2505 FAX: (973) 257-9393
(973) 257-1100
http://www.mathesongas.com

Mathsoft
1700 Westlake Avenue N., Suite 500
Seattle, WA 98109
(800) 569-0123 FAX: (206) 283-8691
(206) 283-8802
http://www.mathsoft.com

Matreya
500 Tressler Street
Pleasant Gap, PA 16823
(814) 359-5060 FAX: (814) 359-5062
http://www.matreya.com

Matrigel
See Becton Dickinson Labware

Matrix Technologies
22 Friars Drive
Hudson, NH 03051
(800) 345-0206 FAX: (603) 595-0106
(603) 595-0505
http://www.matrixtechcorp.com

MatTek Corp.
200 Homer Avenue
Ashland, Massachusetts 01721
(508) 881-6771 FAX: (508) 879-1532
http://www.mattek.com

Maxim Medical
89 Oxford Road
Oxford OX2 9PD
United Kingdom
44 (0)1865-865943
FAX: 44 (0)1865-865291
http://www.maximmed.com

Mayo Clinic
Section on Engineering
Project #ALA-1, 1982
200 1st Street SW
Rochester, MN 55905
(507) 284-2511 FAX: (507) 284-5988

MB Biomedicals
Digene
1201 Clopper Road
Gaithersburg, MD 20878
(800) 344-3631 FAX: (301) 944-7121
(301)955-7000
http://www.digene.com

McGaw
See B. Braun-McGaw

McMaster-Carr
600 County Line Road
Elmhurst, IL 60126
(630) 833-0300 FAX: (630) 834-9427
http://www.mcmaster.com

McNeil Pharmaceutical
See Ortho McNeil Pharmaceutical

MCNC
3021 Cornwallis Road
P.O. Box 12889
Research Triangle Park, NC 27709
(919) 248-1800 FAX: (919) 248-1455
http://www.mcnc.org

MD Industries
5 Revere Drive, Suite 415
Northbrook, IL 60062
(800) 421-8370 FAX: (847) 498-2627
(708) 339-6000
http://www.mdindustries.com

MDS Nordion
447 March Road
P.O. Box 13500
Kanata, Ontario K2K 1X8, Canada
(800) 465-3666 FAX: (613) 592-6937
(613) 592-2790
http://www.mds.nordion.com

MDS Sciex
71 Four Valley Drive
Concord, Ontario
Canada L4K 4V8
(905) 660-9005 FAX: (905) 660-2600
http://www.sciex.com

Mead Johnson
See Bristol-Meyers Squibb

Med Associates
P.O. Box 319
St. Albans, VT 05478
(802) 527-2343 FAX: (802) 527-5095
http://www.med-associates.com

Medecell
239 Liverpool Road
London N1 1LX, UK
(44) 20-7607-2295
FAX: (44) 20-7700-4156
http://www.medicell.co.uk

Media Cybernetics
8484 Georgia Avenue, Suite 200
Silver Spring, MD 20910
(301) 495-3305 FAX: (301) 495-5964
http://www.mediacy.com

Mediatech
13884 Park Center Road
Herndon, VA 20171
(800) cellgro
(703) 471-5955
http://www.cellgro.com

Medical Systems
See Harvard Apparatus

Medifor
647 Washington Street
Port Townsend, WA 98368
(800) 366-3710 FAX: (360) 385-4402
(360) 385-0722
http://www.medifor.com

MedImmune
35 W. Watkins Mill Road
Gaithersburg, MD 20878
(301) 417-0770 FAX: (301) 527-4207
http://www.medimmune.com

MedProbe AS
P.O. Box 2640
St. Hanshaugen
N-0131 Oslo, Norway
(47) 222 00137 FAX: (47) 222 00189
http://www.medprobe.com

Megazyme
Bray Business Park
Bray, County Wicklow
Ireland
(353) 1-286-1220
FAX: (353) 1-286-1264
http://www.megazyme.com

Melles Griot Inc.
2051 Palomar Airport, 200
Carlesbad, CA 92009
(800) 645 2737 FAX: (760) 438 5208
(760) 438 2131
http://www.mellesgriot.com

Menzel-Glaser
Postfach 3157
D-38021 Braunschweig, Germany
(49) 531 590080
FAX: (49) 531 509799

E. Merck
Frankfurterstrasse 250
D-64293 Darmstadt 1, Germany
(49) 6151-720

Merck
See EM Science

Merck & Company
Merck National Service Center
P.O. Box 4
West Point, PA 19486
(800) NSC-MERCK
(215) 652-5000
http://www.merck.com

Merck Research Laboratories
See Merck & Company

Merck Sharpe Human Health Division
300 Franklin Square Drive
Somerset, NJ 08873
(800) 637-2579 FAX: (732) 805-3960
(732) 805-0300

Merial Limited
115 Transtech Drive
Athens, GA 30601
(800) MERIAL-1 FAX: (706) 548-0608
(706) 548-9292
http://www.merial.com

Meridian Instruments
P.O. Box 1204
Kent, WA 98035
(253) 854-9914 FAX: (253) 854-9902
http://www.minstrument.com

MesstechnikWetzlar GmbH & Co.
Walter-Zapp-Str. 4
35788 Wetzlar Germany
(06) 441 2109 1 FAX: (06)
441 2109 109
http://www.mtwz.com

Metasystems
6 Corporation Center Drive
Broadview Heights, OH 44147
(440) 526-1454 FAX: (440) 526-1406
(800) 788-5253
http://www.metasystems.com

Meta Systems Group
32 Hammond Road
Belmont, MA 02178
(617) 489-9950 FAX: (617) 489-9952

Metachem Technologies
3547 Voyager Street, Bldg. 102
Torrance, CA 90503
(310) 793-2300 FAX: (310) 793-2304
http://www.metachem.com

MetalIhantering
Box 47172
100-74 Stockholm, Sweden
(46) 8-726-9696

MethylGene
7220 Frederick-Banting, Suite 200
Montreal, Quebec H4S 2A1, Canada
http://www.methylgene.com

Metro Scientific
475 Main Street, Suite 2A
Farmingdale, NY 11735
(800) 788-6247 FAX: (516) 293-8549
(516) 293-9656

Metrowerks
980 Metric Boulevard
Austin, TX 78758
(800) 377-5416
(512) 997-4700
http://www.metrowerks.com

Mettler Instruments
Mettler-Toledo
1900 Polaris Parkway
Columbus, OH 43240
(800) METTLER FAX: (614) 438-4900
http://www.mt.com

Miami Serpentarium Labs
34879 Washington Loop Road
Punta Gorda, FL 33982
(800) 248-5050 FAX: (813) 639-1811
(813) 639-8888
http://www.miamiserpentarium.com

Michrom BioResources
1945 Industrial Drive
Auburn, CA 95603
(530) 888-6498 FAX: (530) 888-8295
http://www.michrom.com

Mickle Laboratory Engineering
Gomshall, Surrey, UK
(44) 1483-202178

Micra Scientific
A division of Eichrom Industries
8205 S. Cass Ave, Suite 111
Darien, IL 60561
(800) 283-4752 FAX: (630) 963-1928
(630) 963-0320
http://www.micrasci.com

MicroBrightField
74 Hegman Avenue
Colchester, VT 05446
(802) 655-9360 FAX: (802) 655-5245
http://www.microbrightfield.com

Micro Essential Laboratory
4224 Avenue H
Brooklyn, NY 11210
(718) 338-3618 FAX: (718) 692-4491

Micro Filtration Systems
7-3-Chome, Honcho
Nihonbashi, Tokyo, Japan
(81) 3-270-3141

Micro-Metrics
P.O. Box 13804
Atlanta, GA 30324
(770) 986-6015 FAX: (770) 986-9510
http://www.micro-metrics.com

Micro-Tech Scientific
140 South Wolfe Road
Sunnyvale, CA 94086
(408) 730-8324 FAX: (408) 730-3566
http://www.microlc.com

Microbix Biosystems
341 Bering Avenue
Toronto, Ontario
M8Z 3A8 Canada
1-800-794-6694 FAX: 416-234-1626
1-416-234-1624
http://www.microbix.com

MicroCal
22 Industrial Drive East
Northampton, MA 01060
(800) 633-3115 FAX: (413) 586-0149
(413) 586-7720
http://www.microcalorimetry.com

Microfluidics
30 Ossipee Road
P.O. Box 9101
Newton, MA 02164
(800) 370-5452 FAX: (617) 965-1213
(617) 969-5452
http://www.microfluidicscorp.com

Microgon
See Spectrum Laboratories

Microlase Optical Systems
West of Scotland Science Park
Kelvin Campus, Maryhill Road
Glasgow G20 0SP, UK
(44) 141-948-1000
FAX: (44) 141-946-6311
http://www.microlase.co.uk

Micron Instruments
4509 Runway Street
Simi Valley, CA 93063
(800) 638-3770 FAX: (805) 522-4982
(805) 552-4676
http://www.microninstruments.com

Micron Separations
See MSI

Micro Photonics
4949 Liberty Lane, Suite 170
P.O. Box 3129
Allentown, PA 18106
(610) 366-7103 FAX: (610) 366-7105
http://www.microphotonics.com

MicroTech
1420 Conchester Highway
Boothwyn, PA 19061
(610) 459-3514

Midland Certified Reagent Company
3112-A West Cuthbert Avenue
Midland, TX 79701
(800) 247-8766 FAX: (800) 359-5789
(915) 694-7950 FAX: (915) 694-2387
http://www.mcrc.com

Midwest Scientific
280 Vance Road
Valley Park, MO 63088
(800) 227-9997 FAX: (636) 225-9998
(636) 225-9997
http://www.midsci.com

Miles
See Bayer

Miles Laboratories
See Serological

Miles Scientific
See Nunc

Millar Instruments
P.O. Box 230227
6001-A Gulf Freeway
Houston, TX 77023
(713) 923-9171 FAX: (713) 923-7757
http://www.millarinstruments.com

MilliGen/Biosearch
See Millipore

Millipore
80 Ashbury Road
P.O. Box 9125
Bedford, MA 01730
(800) 645-5476 FAX: (781) 533-3110
(781) 533-6000
http://www.millipore.com

Miltenyi Biotec
251 Auburn Ravine Road, Suite 208
Auburn, CA 95603
(800) 367-6227 FAX: (530) 888-8925
(530) 888-8871
http://www.miltenyibiotec.com

Miltex
6 Ohio Drive
Lake Success, NY 11042
(800) 645-8000 FAX: (516) 775-7185
(516) 349-0001

Milton Roy
See Spectronic Instruments

Mini-Instruments
15 Burnham Business Park
Springfield Road
Burnham-on-Crouch, Essex
CM0 8TE, UK
(44) 1621-783282
FAX: (44) 1621-783132
http://www.mini-instruments.co.uk

Mini Mitter
P.O. Box 3386
Sunriver, OR 97707
(800) 685-2999 FAX: (541) 593-5604
(541) 593-8639
http://www.minimitter.com

Mirus Corporation
506 S. Rosa Road
Suite 104
Madison, WI 53719
(608) 441-2852 FAX: (608) 441-2849
http://www.genetransfer.com

Misonix
1938 New Highway
Farmingdale, NY 11735
(800) 645-9846 FAX: (516) 694-9412
http://www.misonix.com

Mitutoyo (MTI)
See Dolla Eastern

MJ Research
Waltham, MA 02451
(800) PELTIER FAX: (617) 923-8080
(617) 923-8000
http://www.mjr.com

Modular Instruments
228 West Gay Street
Westchester, PA 19380
(610) 738-1420 FAX: (610) 738-1421
http://www.mi2.com

Molecular Biology Insights
8685 US Highway 24
Cascade, CO 80809-1333
(800) 747-4362 FAX: (719) 684-7989
(719) 684-7988
http://www.oligo.net

Molecular Biosystems
10030 Barnes Canyon Road
San Diego, CA 92121
(858) 452-0681 FAX: (858) 452-6187
http://www.mobi.com

Molecular Devices
1312 Crossman Avenue
Sunnyvale, CA 94089
(800) 635-5577 FAX: (408) 747-3602
(408) 747-1700
http://www.moldev.com

Molecular Designs
1400 Catalina Street
San Leandro, CA 94577
(510) 895-1313 FAX: (510) 614-3608

Molecular Dynamics
928 East Arques Avenue
Sunnyvale, CA 94086
(800) 333-5703 FAX: (408) 773-1493
(408) 773-1222
http://www.apbiotech.com

Molecular Probes
4849 Pitchford Avenue
Eugene, OR 97402
(800) 438-2209 FAX: (800) 438-0228
(541) 465-8300 FAX: (541) 344-6504
http://www.probes.com

Molecular Probes, Inc.
P.O. Box 22010
Eugene, OR 97402-0469
(541) 465-8300 FAX: (541) 335-0504
(800) 438-2209
http://www.probes.com

Molecular Research Center
5645 Montgomery Road
Cincinnati, OH 45212
(800) 462-9868 FAX: (513) 841-0080
(513) 841-0900
http://www.mrcgene.com

Molecular Simulations
9685 Scranton Road
San Diego, CA 92121
(800) 756-4674 FAX: (858) 458-0136
(858) 458-9990
http://www.msi.com

Monoject Disposable Syringes & Needles/Syrvet
16200 Walnut Street
Waukee, IA 50263
(800) 727-5203 FAX: (515) 987-5553
(515) 987-5554
http://www.syrvet.com

Monsanto Chemical
800 North Lindbergh Boulevard
St. Louis, MO 63167
(314) 694-1000 FAX: (314) 694-7625
http://www.monsanto.com

Moravek Biochemicals
577 Mercury Lane
Brea, CA 92821
(800) 447-0100 FAX: (714) 990-1824
(714) 990-2018
http://www.moravek.com

Moss
P.O. Box 189
Pasadena, MD 21122
(800) 932-6677 FAX: (410) 768-3971
(410) 768-3442
http://www.mosssubstrates.com

Motion Analysis
3617 Westwind Boulevard
Santa Rosa, CA 95403
(707) 579-6500 FAX: (707) 526-0629
http://www.motionanalysis.com

Mott
Farmington Industrial Park
84 Spring Lane
Farmington, CT 06032
(860) 747-6333 FAX: (860) 747-6739
http://www.mottcorp.com

MSI (Micron Separations)
See Osmonics

Multi Channel Systems
Markwiesenstrasse 55
72770 Reutlingen, Germany
(49) 7121-503010
FAX: (49) 7121-503011
http://www.multichannelsystem.com

Multiple Peptide Systems
3550 General Atomics Court
San Diego, CA 92121
(800) 338-4965 FAX: (800) 654-5592
(858) 455-3710 FAX: (858) 455-3713
http://www.mps-sd.com

Murex Diagnostics
3075 Northwoods Circle
Norcross, GA 30071
(707) 662-0660 FAX: (770) 447-4989

MWG-Biotech
Anzinger Str. 7
D-85560 Ebersberg, Germany
(49) 8092-82890 FAX: (49) 8092-21084
http://www.mwg_biotech.com

Myriad Industries
3454 E Street
San Diego, CA 92102
(800) 999-6777 FAX: (619) 232-4819
(619) 232-6700
http://www.myriadindustries.com

Nacalai Tesque
Nijo Karasuma, Nakagyo-ku
Kyoto 604, Japan
81-75-251-1723
FAX: 81-75-251-1762
http://www.nacalai.co.jp

Nalge Nunc International
Subsidiary of Sybron International
75 Panorama Creek Drive
P.O. Box 20365
Rochester, NY 14602
(800) 625-4327 FAX: (716) 586-8987
(716) 264-9346
http://www.nalgenunc.com

Nanogen
10398 Pacific Center Court
San Diego, CA 92121
(858) 410-4600 FAX: (858) 410-4848
http://www.nanogen.com

Nanoprobes
95 Horse Block Road
Yaphank, NY 11980
(877) 447-6266 FAX: (631) 205-9493
(631) 205-9490
http://www.nanoprobes.com

Narishige USA
1710 Hempstead Turnpike
East Meadow, NY 11554
(800) 445-7914 FAX: (516) 794-0066
(516) 794-8000
http://www.narishige.co.jp

Nasco-Fort Atkinson
P.O. Box 901
901 Janesville Ave.
Fort Alkinson, WI 53538-0901
(800) 558-9595 FAX: (920) 563-8296
http://www.enasco.com

National Bag Company
2233 Old Mill Road
Hudson, OH 44236
(800) 247-6000 FAX: (330) 425-9800
(330) 425-2600
http://www.nationalbag.com

National Band and Tag
Department X 35, Box 72430
Newport, KY 41032
(606) 261-2035 FAX: (800) 261-8247
https://www.nationalband.com

National Biosciences
See Molecular Biology Insights

National Diagnostics
305 Patton Drive
Atlanta, GA 30336
(800) 526-3867 FAX: (404) 699-2077
(404) 699-2121
http://www.nationaldiagnostics.com

National Disease Research Exchange
1880 John F. Kennedy Blvd., 11th Fl.
Philadelphia, PA 19103
(800) 222-6374
http://www.ndri.com

National Institute of Standards and Technology
100 Bureau Drive
Gaithersburg, MD 20899
(301) 975-NIST FAX: (301) 926-1630
http://www.nist.gov

National Instruments
11500 North Mopac Expressway
Austin, TX 78759
(512) 794-0100 FAX: (512) 683-8411
http://www.ni.com

National Labnet
See Labnet International

National Scientific Instruments
975 Progress Circle
Lawrenceville, GA 300243
(800) 332-3331 FAX: (404) 339-7173
http://www.nationalscientific.com

National Scientific Supply
1111 Francisco Bouldvard East
San Rafael, CA 94901
(800) 525-1779 FAX: (415) 459-2954
(415) 459-6070
http://www.nat-sci.com

Naz-Dar-KC Chicago
Nazdar
1087 N. North Branch Street
Chicago, IL 60622
(800) 736-7636 FAX: (312) 943-8215
(312) 943-8338
http://www.nazdar.com

NB Labs
1918 Avenue A
Denison, TX 75021
(903) 465-2694 FAX: (903) 463-5905
http://www.nblabslarry.com

NEB
See New England Biolabs

NEN Life Science Products
549 Albany Street
Boston, MA 02118
(800) 551-2121 FAX: (617) 451-8185
(617) 350-9075
http://www.nen.com

NEN Research Products, Dupont (UK)
Diagnostics and Biotechnology Systems
Wedgewood Way
Stevenage, Hertfordshire SG1 4QN, UK
44-1438-734831
44-1438-734000
FAX: 44-1438-734836
http://www.dupont.com

Neogen
628 Winchester Road
Lexington, KY 40505 (800)
477-8201 FAX: (606) 255-5532
(606) 254-1221
http://www.neogen.com

Neosystems
380, 11012 Macleod Trail South
Calgary, Alberta T2J 6A5 Canada
(403) 225-9022 FAX: (403) 225-9025
http://www.neosystems.com

Neuralynx
2434 North Pantano Road
Tucson, AZ, 85715
(520) 722-6144 FAX: (520) 722-8163
http://www.neuraiynx.com

Neuro Probe
16008 Industrial Drive
Gaithersburg, MD 20877
(301) 417-0014 FAX: (301) 977-5711
http://www.neuroprobe.com

Neurocrine Biosciences
10555 Science Center Drive
San Diego, CA 92121
(619) 658-7600 FAX: (619) 658-7602
http://www.neurocrine.com

Nevtek
HCR03, Box 99
Burnsville, VA 24487
(540) 925-2322 FAX: (540) 925-2323
http://www.nevtek.com

New Brunswick Scientific
44 Talmadge Road
Edison, NJ 08818
(800) 631-5417 FAX: (732) 287-4222
(732) 287-1200
http://www.nbsc.com

New England Biolabs (NEB)
32 Tozer Road
Beverly, MA 01915 (800)
632-5227 FAX: (800) 632-7440
http://www.neb.com

New England Nuclear (NEN)
See NEN Life Science Products

New MBR
Gubelstrasse 48
CH8050 Zurich, Switzerland
(41) 1-313-0703

Newark Electronics
4801 N. Ravenswood Avenue
Chicago, IL 60640
(800) 4-NEWARK FAX: (773)
907-5339
(773) 784-5100
http://www.newark.com

Newell Rubbermaid
29 E. Stephenson Street
Freeport, IL 61032
(815) 235-4171 FAX: (815) 233-8060
http://www.newellco.com

Newport Biosystems
1860 Trainor Street
Red Bluff, CA 96080
(530) 529-2448 FAX: (530) 529-2648

Newport
1791 Deere Avenue
Irvine, CA 92606
(800) 222-6440 FAX: (949) 253-1800
(949) 253-1462
http://www.newport.com

Nexin Research B.V.
P.O. Box 16
4740 AA Hoeven, The Netherlands
(31) 165-503172
FAX: (31) 165-502291

NIAID
See Bio-Tech Research Laboratories

Nichiryo
230 Route 206
Building 2-2C
Flanders, NJ 07836
(877) 548-6667 FAX: (973) 927-0099
(973) 927-4001
http://www.nichiryo.com

Nichols Institute Diagnostics
33051 Calle Aviador
San Juan Capistrano, CA 92675
(800) 286-4NID FAX: (949) 240-5273
(949) 728-4610
http://www.nicholsdiag.com

Nichols Scientific Instruments
3334 Brown Station Road
Columbia, MO 65202
(573) 474-5522 FAX: (603) 215-7274
http://home.beseen.com
technology/nsi_technology

Nicolet Biomedical Instruments
5225 Verona Road, Building 2
Madison, WI 53711
(800) 356-0007 FAX: (608) 441-2002
(608) 273-5000
http://nicoletbiomedical.com

N.I.G.M.S. (National Institute of General Medical Sciences)
See Coriell Institute for Medical Research

Nikon
Science and Technologies Group
1300 Walt Whitman Road
Melville, NY 11747
(516) 547-8500 FAX: (516) 547-4045
http://www.nikonusa.com

Nippon Gene
1-29, Ton-ya-machi
Toyama 930, Japan
(81) 764-51-6548
FAX: (81) 764-51-6547

Noldus Information Technology
751 Miller Drive
Suite E-5
Leesburg, VA 20175
(800) 355-9541 FAX: (703) 771-0441
(703) 771-0440
http://www.noldus.com

Nonllnear Dynamics
See MDS NovoDynamics

Nordion International
See MDS Nordion

North American Biologicals (NABI)
16500 NW 15th Avenue
Miami, FL 33169
(800) 327-7106 (305) 625-5305
http://www.nabi.com

North American Reiss
See Reiss

Northwestern Bottle
24 Walpole Park South
Walpole, MA 02081
(508) 668-8600 FAX: (508) 668-7790

NOVA Biomedical
Nova Biomedical 200
Prospect Street Waltham, MA 02454
(800) 822-0911 FAX: (781) 894-5915
http://www.novabiomedical.com

Novagen
601 Science Drive
Madison, WI 53711
(800) 526-7319 FAX: (608) 238-1388
(608) 238-6110
http://www.novagen.com

Novartis
59 Route 10
East Hanover, NJ 07936
(800)526-0175 FAX: (973) 781-6356
http://www.novartis.com

Novartis Biotechnology
3054 Cornwallis Road
Research Triangle Park, NC 27709
(888) 462-7288 FAX: (919) 541-8585
http://www.novartis.com

Nova Sina AG
Subsidiary of Airflow Lufttechnik GmbH
Kleine Heeg 21
52259 Rheinbach, Germany
(49) 02226 920-0
FAX: (49) 02226 9205-11

Novex/Invitrogen
1600 Faraday
Carlsbad, CA 92008
(800) 955-6288 FAX: (760) 603-7201
http://www.novex.com

Novo Nordisk Biochem
77 Perry Chapel Church Road
Franklington, NC 27525
(800) 879-6686 FAX: (919) 494-3450
(919) 494-3000
http://www.novo.dk

Novo Nordisk BioLabs
See Novo Nordisk Biochem

Novocastra Labs
Balliol Business Park West
Benton Lane
Newcastle-upon-Tyne
Tyne and Wear NE12 8EW, UK
(44) 191-215-0567
FAX: (44) 191-215-1152
http://www.novocastra.co.uk

NovoDynamics
123 North Ashley Street
Sulte 210
Ann Arbor, MI 48104
(734) 205-9100 FAX: (734) 205-9101
http://www.novodynamics.com

Novus Biologicals
P.O. Box 802
Littleton, CO 80160
(888) 506-6887 FAX: (303) 730-1966
http://www.novus-biologicals.com/main.html

NPI Electronic
Hauptstrasse 96
D-71732 Tamm, Germany
(49) 7141-601534
FAX: (49) 7141-601266
http://www.npielectronic.com

NSG Precision Cells
195G Central Avenue
Farmingdale, NY 11735
(516) 249-7474 FAX: (516) 249-8575
http://www.nsgpci.com

Nu Chek Prep
109 West Main
P.O. Box 295
Elysian, MN 56028
(800) 521-7728 FAX: (507) 267-4790
(507) 267-4689

Nuclepore
See Costar

Numonics
101 Commerce Drive
Montgomeryville, PA 18936
(800) 523-6716 FAX: (215) 361-0167
(215) 362-2766
http://www.interactivewhiteboards.com

Nutacon BV
Tuindeni 25
Leimuiden
2451 GG, The Netherlands
(31) 172-507221 FAX: (31)
172-507116
http://www.nutacon.nl

NYCOMED AS Pharma
c/o Accurate Chemical & Scientific
300 Shames Drive
Westbury, NY 11590
(800) 645-6524 FAX: (516) 997-4948
(516) 333-2221
http://www.accuratechemical.com

Nycomed Amersham
Health Care Division
101 Carnegie Center
Princeton, NJ 08540
(800) 832-4633 FAX: (800) 807-2382
(609) 514-6000
http://www.nycomed-amersham.com

Nyegaard
Herserudsvagen 5254
S-122 06 Lidingo, Sweden
(46) 8-765-2930

Ohmeda Catheter Products
See Datex-Ohmeda

Ohwa Tsusbo
Hiby Dai Building
1-2-2 Uchi Saiwai-cho
Chiyoda-ku
Tokyo 100, Japan
03-3591-7348 FAX: 03-3501-9001

Oligos Etc.
9775 S.W. Commerce Circle, C-6
Wilsonville, OR 97070
(800) 888-2358 FAX: (503)
6822D1635
(503) 6822D1814
http://www.oligoetc.com

Olis Instruments
130 Conway Drive
Bogart, GA 30622
(706) 353-6547 (800) 852-3504
http://www.olisweb.com

Olympus America
2 Corporate Center Drive
Melville, NY 11747
(800) 645-8160 FAX: (516) 844-5959
(516) 844-5000
http://www.olympusamerica.com

Omega Engineering
One Omega Drive
P.O. Box 4047
Stamford, CT 06907
(800) 848-4286 FAX: (203) 359-7700
(203) 359-1660
http://www.omega.com

Omega Optical
3 Grove Street
P.O. Box 573
Brattleboro, VT 05302
(802) 254-2690 FAX: (802) 254-3937
http://www.omegafilters.com

Omnetics Connector Corporation
7280 commerce Circle
East Minneapolis, MN 55432
(800) 343-0025 (763) 572-0656
Fax: (763) 572-3925
http://www.omnetics.com/main.htm

Omni International
6530 Commerce Court
Warrenton, VA 20187
(800) 776-4431 FAX: (540) 347-5352
(540) 347-5331
http://www.omni-inc.com

Omnion
2010 Energy Drive
P.O. Box 879
East Troy, WI 53120
(262) 642-7200 FAX: (262) 642-7760
http://www.omnion.com

Omnitech Electronics
See AccuScan Instruments

Oncogene Research Products
P.O. Box 12087
La Jolla, CA 92039-2087
(800) 662-2616 FAX: (800) 766-0999
http://www.apoptosis.com

Oncogene Science
See OSI Pharmaceuticals

Oncor
See Intergen

Online Instruments
130 Conway Drive, Suites A & B
Bogart, GA 30622
(800) 852-3504 (706) 353-1972
(706) 353-6547
http://www.olisweb.com

Operon Technologies
1000 Atlantic Avenue
Alameda, CA 94501
(800) 688-2248 FAX: (510) 865-5225
(510) 865-8644
http://www.operon.com

Optiscan
P.O. Box 1066
Mount Waverly MDC, Victoria
Australia 3149
61-3-9538 3333 FAX: 61-3-9562 7742
http://www.optiscan.com.au

Optomax
9 Ash Street
P.O. Box 840
Hollis, NH 03049
(603) 465-3385 FAX: (603) 465-2291

Opto-Line Associates
265 Ballardvale Street
Wilmington, MA 01887
(978) 658-7255 FAX: (978) 658-7299
http://www.optoline.com

Orbigen
6827 Nancy Ridge Drive
San Diego, CA 92121
(866) 672-4436 (858) 362-2030
(858) 362-2026
http://www.orbigen.com

Oread BioSaftey
1501 Wakarusa Drive
Lawrence, KS 66047
(800) 447-6501 FAX: (785) 749-1882
(785) 749-0034
http://www.oread.com

Organomation Associates
266 River Road West
Berlin, MA 01503
(888) 978-7300 FAX: (978)838-2786
(978) 838-7300
http://www.organomation.com

Organon
375 Mount Pleasant Avenue
West Orange, NJ 07052
(800) 241-8812 FAX: (973) 325-4589
(973) 325-4500
http://www.organon.com

Organon Teknika (Canada)
30 North Wind Place
Scarborough, Ontario
M1S 3R5 Canada
(416) 754-4344 FAX: (416) 754-4488
http://www.organonteknika.com

Organon Teknika Cappel
100 Akzo Avenue
Durham, NC 27712
(800) 682-2666 FAX: (800) 432-9682
(919) 620-2000 FAX: (919) 620-2107
http://www.organonteknika.com

Oriel Corporation of America
150 Long Beach Boulevard
Stratford, CT 06615
(203) 377-8282 FAX: (203) 378-2457
http://www.oriel.com

OriGene Technologies
6 Taft Court, Suite 300
Rockville, MD 20850
(888) 267-4436 FAX: (301) 340-9254
(301) 340-3188
http://www.origene.com

OriginLab
One Roundhouse Plaza
Northhampton, MA 01060
(800) 969-7720 FAX: (413) 585-0126
http://www.originlab.com

Orion Research
500 Cummings Center
Beverly, MA 01915
(800) 225-1480 FAX: (978) 232-6015
(978) 232-6000
http://www.orionres.com

Ortho Diagnostic Systems
Subsidiary of Johnson & Johnson
1001 U.S. Highway 202
P.O. Box 350
Raritan, NJ 08869
(800) 322-6374 FAX: (908) 218-8582
(908) 218-1300

Ortho McNeil Pharmaceutical
Welsh & McKean Road
Spring House, PA 19477
(800) 682-6532
(215) 628-5000
http://www.orthomcneil.com

Oryza
200 Turnpike Road, Unit 5
Chelmsford, MA 01824
(978) 256-8183 FAX: (978) 256-7434
http://www.oryzalabs.com

OSI Pharmaceuticals
106 Charles Lindbergh Boulevard
Uniondale, NY 11553
(800) 662-2616 FAX: (516) 222-0114
(516) 222-0023
http://www.osip.com

Osmonics
135 Flanders Road
P.O. Box 1046
Westborough, MA 01581
(800) 444-8212 FAX: (508) 366-5840
(508) 366-8212
http://www.osmolabstore.com

Oster Professional Products
150 Cadillac Lane
McMinnville, TN 37110
(931) 668-4121 FAX: (931) 668-4125
http://www.sunbeam.com

Out Patient Services
1260 Holm Road
Petaluma, CA 94954
(800) 648-1666 FAX: (707) 762-7198
(707) 763-1581

OWL Scientific Plastics
See OWL Separation Systems

OWL Separation Systems
55 Heritage Avenue
Portsmouth, NH 03801
(800) 242-5560 FAX: (603) 559-9258
(603) 559-9297
http://www.owlsci.com

Oxford Biochemical Research
P.O. Box 522
Oxford, MI 48371
(800) 692-4633 FAX: (248) 852-4466
http://www.oxfordbiomed.com

Oxford GlycoSystems
See Glyco

Oxford Instruments
Old Station Way
Eynsham
Witney, Oxfordshire OX8 1TL, UK
(44) 1865-881437
FAX: (44) 1865-881944
http://www.oxinst.com

Oxford Labware
See Kendall

Oxford Molecular Group
Oxford Science Park
The Medawar Centre
Oxford OX4 4GA, UK
(44) 1865-784600
FAX: (44) 1865-784601
http://www.oxmol.co.uk

Oxford Molecular Group
2105 South Bascom Avenue, Suite 200
Campbell, CA 95008
(800) 876-9994 FAX: (408) 879-6302
(408) 879-6300
http://www.oxmol.com

OXIS International
6040 North Cutter Circle
Suite 317
Portland, OR 97217
(800) 547-3686 FAX: (503) 283-4058
(503) 283-3911
http://www.oxis.com

Oxoid
800 Proctor Avenue
Ogdensburg, NY 13669
(800) 567-8378 FAX: (613) 226-3728
http://www.oxoid.ca

Oxoid
Wade Road
Basingstoke, Hampshire RG24 8PW, UK
(44) 1256-841144
FAX: (4) 1256-814626
http://www.oxoid.ca

Oxyrase
P.O. Box 1345
Mansfield, OH 44901
(419) 589-8800 FAX: (419) 589-9919
http://www.oxyrase.com

Ozyme
10 Avenue Ampère
Montigny de Bretoneux
78180 France
(33) 13-46-02-424
FAX: (33) 13-46-09-212
http://www.ozyme.fr

PAA Laboratories
2570 Route 724
P.O. Box 435
Parker Ford, PA 19457
(610) 495-9400 FAX: (610) 495-9410
http://www.paa-labs.com

Pacer Scientific
5649 Valley Oak Drive
Los Angeles, CA 90068
(323) 462-0636 FAX: (323) 462-1430
http://www.pacersci.com

Pacific Bio-Marine Labs
P.O. Box 1348
Venice, CA 90294
(310) 677-1056 FAX: (310) 677-1207

Packard Instrument
800 Research Parkway
Meriden, CT 06450
(800) 323-1891 FAX: (203) 639-2172
(203) 238-2351
http://www.packardinst.com

Padgett Instrument
1730 Walnut Street
Kansas City, MO 64108
(816) 842-1029

Pall Filtron
50 Bearfoot Road
Northborough, MA 01532
(800) FILTRON FAX: (508) 393-1874
(508) 393-1800

Pall-Gelman
25 Harbor Park Drive
Port Washington, NY 11050
(800) 289-6255 FAX: (516) 484-2651
(516) 484-3600
http://www.pall.com

PanVera
545 Science Drive
Madison, WI 53711
(800) 791-1400 FAX: (608) 233-3007
(608) 233-9450
http://www.panvera.com

Parke-Davis
See Warner-Lambert

Parker-Hannifin
(800) 272-7537 FAX: (440) 266-7400
http://www.parker.com

Parr Instrument
211 53rd Street
Moline, IL 61265
(800) 872-7720 FAX: (309) 762-9453
(309) 762-7716
http://www.parrinst.com

Partec
Otto Hahn Strasse 32
D-48161 Munster, Germany
(49) 2534-8008-0
FAX: (49) 2535-8008-90

PCR
See Archimica Florida

PE Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404
(800) 345-5224 FAX: (650) 638-5884
(650) 638-5800
http://www.pebio.com

Pel-Freez Biologicals
219 N. Arkansas
P.O. Box 68
Rogers, AR 72757
(800) 643-3426 FAX: (501) 636-3562
(501) 636-4361
http://www.pelfreez-bio.com

Pel-Freez Clinical Systems
Subsidiary of Pel-Freez Biologicals
9099 N. Deerbrook Trail
Brown Deer, WI 53223
(800) 558-4511 FAX: (414) 357-4518
(414) 357-4500
http://www.pelfreez-bio.com

Pelco International
4595 Mountain Lakes Blvd.
Redding, CA 96003
(530) 243-2200 FAX: (530) 243-3761
http://www.pelcoint.com

Peninsula Laboratories
601 Taylor Way
San Carlos, CA 94070
(800) 650-4442 FAX: (650) 595-4071
(650) 592-5392
http://www.penlabs.com

Pentex
24562 Mando Drive
Laguna Niguel, CA 92677
(800) 382-4667 FAX: (714) 643-2363
http://www.pentex.com

PeproTech
5 Crescent Avenue
P.O. Box 275, Rocky Hill, NJ 08553
(800) 436-9910 FAX: (609) 497-0321
(609) 497-0253
http://www.peprotech.com

Peptide Institute
4-1-2 Ina, Minoh-shi
Osaka 562-8686, Japan
81-727-29-4121 FAX: 81-727-29-4124
http://www.peptide.co.jp

Peptide Laboratory
4175 Lakeside Drive
Richmond, CA 94806
(800) 858-7322 FAX: (510) 262-9127
(510) 262-0800
http://www.peptidelab.com

Peptides International
11621 Electron Drive
Louisville, KY 40299
(800) 777-4779 FAX: (502) 267-1329
(502) 266-8787
http://www.pepnet.com

Perceptive Science Instruments
2525 South Shore Blvd., Suite 100
League City, TX 77573
(281) 334-3027 FAX: (281) 538-2222
http://www.persci.com

Perimed
4873 Princeton Drive
North Royalton, OH 44133
(440) 877-0537 FAX: (440) 877-0534
http://www.perimed.se

Perkin-Elmer
761 Main Avenue
Norwalk, CT 06859
(800) 762-4002 FAX: (203) 762-6000
(203) 762-1000
http://www.perkin-elmer.com
See also PE Biosystems

Perkin Elmer
45 William Street
Wellesley, MA 02481-4078
(781) 237-5100
(800) 762-4000
http://www.perkinelmer.com

PerSeptive Bioresearch Products
See PerSeptive BioSystems

PerSeptive BioSystems
500 Old Connecticut Path
Framingham, MA 01701
(800) 899-5858 FAX: (508) 383-7885
(508) 383-7700
http://www.pbio.com

PerSeptive Diagnostic
See PE Biosystems
(800) 343-1346

Pettersson Elektronik AB
Tallbacksvagen 51
S-756 45 Uppsala, Sweden
(46) 1830-3880 FAX: (46) 1830-3840
http://www.bahnhof.se/~pettersson

Pfanstiehl Laboratories, Inc.
1219 Glen Rock Avenue
Waukegan, IL 60085
(800) 383-0126 FAX: (847) 623-9173
http://www.pfanstiehl.com

PGC Scientifics
7311 Governors Way
Frederick, MD 21704
(800) 424-3300 FAX: (800) 662-1112
(301) 620-7777 FAX: (301) 620-7497
http://www.pgcscientifics.com

Pharmacia Biotech
See Amersham Pharmacia Biotech

Pharmacia Diagnostics
See Wallac

Pharmacia LKB Biotech
See Amersham Pharmacia Biotech

Pharmacia LKB Biotechnology
See Amersham Pharmacia Biotech

Pharmacia LKB Nuclear
See Wallac

Pharmaderm Veterinary Products
60 Baylis Road
Melville, NY 11747
(800) 432-6673
http://www.pharmaderm.com

Pharmed (Norton)
Norton Performance Plastics
See Saint-Gobain Performance Plastics

PharMingen
See BD PharMingen

Phenomex
2320 W. 205th Street
Torrance, CA 90501
(310) 212-0555 FAX: (310) 328-7768
http://www.phenomex.com

PHLS Centre for Applied Microbiology and Research
See European Collection of Animal Cell Cultures (ECACC)

Phoenix Flow Systems
11575 Sorrento Valley Road, Suite 208
San Diego, CA 92121
(800) 886-3569 FAX: (619) 259-5268
(619) 453-5095
http://www.phnxflow.com

Phoenix Pharmaceutical
4261 Easton Road, P.O. Box 6457
St. Joseph, MO 64506
(800) 759-3644 FAX: (816) 364-4969
(816) 364-5777
http://www.phoenixpharmaceutical.com

Photometrics
See Roper Scientific

Photon Technology International
1 Deerpark Drive, Suite F
Monmouth Junction, NJ 08852
(732) 329-0910 FAX: (732) 329-9069
http://www.pti-nj.com

Physik Instrumente
Polytec PI
23 Midstate Drive, Suite 212
Auburn, MA 01501
(508) 832-3456 FAX: (508) 832-0506
http://www.polytecpi.com

Physitemp Instruments
154 Huron Avenue
Clifton, NJ 07013
(800) 452-8510 FAX: (973) 779-5954
(973) 779-5577
http://www.physitemp.com

Pico Technology
The Mill House, Cambridge Street
St. Neots, Cambridgeshire
PE19 1QB, UK
(44) 1480-396-395
FAX: (44) 1480-396-296
www.picotech.com

PicoQuant GmbH
Rudower Chaussee 29 (IGZ)
12489 Berlin, Germany
(49) 30 6392 6560 FAX: (49)
30 6392 6561
http://www.picoquant.com

Pierce Chemical
P.O. Box 117
3747 Meridian Road
Rockford, IL 61105
(800) 874-3723 FAX: (800) 842-5007
FAX: (815) 968-7316
http://www.piercenet.com

Pierce & Warriner
44, Upper Northgate Street
Chester, Cheshire CH1 4EF, UK
(44) 1244 382 525
FAX: (44) 1244 373 212
http://www.piercenet.com

Pilling Weck Surgical
420 Delaware Drive
Fort Washington, PA 19034
(800) 523-2579 FAX: (800) 332-2308
www.pilling-weck.com

PixelVision
A division of Cybex Computer Products
14964 NW Greenbrier Parkway
Beaverton, OR 97006
(503) 629-3210 FAX: (503) 629-3211
http://www.pixelvision.com

P.J. Noyes
P.O. Box 381
89 Bridge Street
Lancaster, NH 03584
(800) 522-2469 FAX: (603) 788-3873
(603) 788-4952
http://www.pjnoyes.com

Plas-Labs
917 E. Chilson Street
Lansing, MI 48906
(800) 866-7527 FAX: (517) 372-2857
(517) 372-7177
http://www.plas-labs.com

Plastics One
6591 Merriman Road, Southwest
P.O. Box 12004
Roanoke, VA 24018
(540) 772-7950 FAX: (540) 989-7519
http://www.plastics1.com

Platt Electric Supply
2757 6th Avenue South
Seattle, WA 98134
(206) 624-4083 FAX: (206) 343-6342
http://www.platt.com

Plexon
6500 Greenville Avenue
Suite 730
Dallas, TX 75206
(214) 369-4957 FAX: (214) 369-1775
http://www.plexoninc.com

Polaroid
784 Memorial Drive
Cambridge, MA 01239
(800) 225-1618 FAX: (800) 832-9003
(781) 386-2000
http://www.polaroid.com

Polyfiltronics
136 Weymouth St.
Rockland, MA 02370
(800) 434-7659 FAX: (781) 878-0822
(781) 878-1133
http://www.polyfiltronics.com

Polylabo Paul Block
Parc Tertiare de la Meinau
10, rue de la Durance
B.P. 36
67023 Strasbourg Cedex 1
Strasbourg, France
33-3-8865-8020
FAX: 33-3-8865-8039

PolyLC
9151 Rumsey Road, Suite 180
Columbia, MD 21045
(410) 992-5400 FAX: (410) 730-8340

Polymer Laboratories
Amherst Research Park
160 Old Farm Road
Amherst, MA 01002
(800) 767-3963 FAX: (413) 253-2476
http://www.polymerlabs.com

Polymicro Technologies
18019 North 25th Avenue
Phoenix, AZ 85023
(602) 375-4100 FAX: (602) 375-4110
http://www.polymicro.com

Polyphenols AS
Hanabryggene Technology Centre
Hanaveien 4-6
4327 Sandnes, Norway
(47) 51-62-0990
FAX: (47) 51-62-51-82
http://www.polyphenols.com

Polysciences
400 Valley Road
Warrington, PA 18976
(800) 523-2575 FAX: (800) 343-3291
http://www.polysciences.com

Polyscientific
70 Cleveland Avenue
Bayshore, NY 11706
(516) 586-0400 FAX: (516) 254-0618

Polytech Products
285 Washington Street
Somerville, MA 02143
(617) 666-5064 FAX: (617) 625-0975

Polytron
8585 Grovemont Circle
Gaithersburg, MD 20877
(301) 208-6597 FAX: (301) 208-8691
http://www.polytron.com

Popper and Sons
300 Denton Avenue
P.O. Box 128
New Hyde Park, NY 11040
(888) 717-7677 FAX: (800) 557-6773
(516) 248-0300 FAX: (516) 747-1188
http://www.popperandsons.com

Porphyrin Products
P.O. Box 31
Logan, UT 84323
(435) 753-1901 FAX: (435) 753-6731
http://www.porphyrin.com

Portex
See SIMS Portex Limited

Powderject Vaccines
585 Science Drive
Madison, WI 53711
(608) 231-3150 FAX: (608) 231-6990
http://www.powderject.com

Prairie Technologies, Inc.
3030 Laura Lane, Suite 140
P.O. Box 620677
Middleton, WI 53562-0677
(608) 662-0022 FAX: (608) 662-0023
http://www.prairie-technologies.com

Praxair
810 Jorie Boulevard
Oak Brook, IL 60521
(800) 621-7100
http://www.praxair.com

Precision Dynamics
13880 Del Sur Street
San Fernando, CA 91340
(800) 847-0670 FAX: (818) 899-4-45
http://www.pdcorp.com

Precision Scientific Laboratory Equipment
Division of Jouan
170 Marcel Drive
Winchester, VA 22602
(800) 621-8820 FAX: (540) 869-0130
(540) 869-9892
http://www.precisionsci.com

Primary Care Diagnostics
See Becton Dickinson Primary Care Diagnostics

Primate Products
1755 East Bayshore Road, Suite 28A
Redwood City, CA 94063
(650) 368-0663 FAX: (650) 368-0665
http://www.primateproducts.com

5 Prime → 3 Prime
See 2000 Eppendorf-5 Prime
http://www.5prime.com

Princeton Applied Research
PerkinElmer Instr.: Electrochemistry
801 S. Illinois
Oak Ridge, TN 37830
(800) 366-2741 FAX: (423) 425-1334
(423) 481-2442
http://www.eggpar.com

Princeton Instruments
A division of Roper Scientific
3660 Quakerbridge Road
Trenton, NJ 08619
(609) 587-9797 FAX: (609) 587-1970
http://www.prinst.com

Princeton Separations
P.O. Box 300
Aldephia, NJ 07710
(800) 223-0902 FAX: (732) 431-3768
(732) 431-3338

Prior Scientific
80 Reservoir Park Drive
Rockland, MA 02370
(781) 878-8442 FAX: (781) 878-8736
http://www.prior.com

PRO Scientific
P.O. Box 448
Monroe, CT 06468
(203) 452-9431 FAX: (203) 452-9753
http://www.proscientific.com

Professional Compounding Centers of America
9901 South Wilcrest Drive
Houston, TX 77099
(800) 331-2498 FAX: (281) 933-6227
(281) 933-6948
http://www.pccarx.com

Progen Biotechnik
Maass-Str. 30
69123 Heidelberg, Germany
(49) 6221-8278-0
FAX: (49) 6221-8278-23
http://www.progen.de

Prolabo
A division of Merck Eurolab
54 rue Roger Salengro
94126 Fontenay Sous Bois Cedex
France
33-1-4514-8500
FAX: 33-1-4514-8616
http://www.prolabo.fr

Proligo
2995 Wilderness Place Boulder, CO 80301
(888) 80-OLIGO FAX: (303) 801-1134
http://www.proligo.com

Promega
2800 Woods Hollow Road
Madison, WI 53711
(800) 356-9526 FAX: (800) 356-1970
(608) 274-4330 FAX: (608) 277-2516
http://www.promega.com

Protein Databases (PDI)
405 Oakwood Road
Huntington Station, NY 11746
(800) 777-6834 FAX: (516) 673-4502
(516) 673-3939

Protein Polymer Technologies
10655 Sorrento Valley Road
San Diego, CA 92121
(619) 558-6064 FAX: (619) 558-6477
http://www.ppti.com

Protein Solutions
391 G Chipeta Way
Salt Lake City, UT 84108
(801) 583-9301 FAX: (801) 583-4463
http://www.proteinsolutions.com

Prozyme
1933 Davis Street, Suite 207
San Leandro, CA 94577
(800) 457-9444 FAX: (510) 638-6919
(510) 638-6900
http://www.prozyme.com

PSI
See Perceptive Science Instruments

Pulmetrics Group
82 Beacon Street
Chestnut Hill, MA 02167
(617) 353-3833 FAX: (617) 353-6766

Purdue Frederick
100 Connecticut Avenue
Norwalk, CT 06850
(800) 633-4741 FAX: (203) 838-1576
(203) 853-0123
http://www.pharma.com

Purina Mills
LabDiet
P. O. Box 66812
St. Louis, MO 63166
(800) 227-8941 FAX: (314) 768-4894
http://www.purina-mills.com

Qbiogene
2251 Rutherford Road
Carlsbad, CA 92008
(800) 424-6101 FAX: (760) 918-9313
http://www.qbiogene.com

Qiagen
28159 Avenue Stanford
Valencia, CA 91355
(800) 426-8157 FAX: (800) 718-2056
http://www.qiagen.com

Quality Biological
7581 Lindbergh Drive
Gaithersburg, MD 20879
(800) 443-9331 FAX: (301) 840-5450
(301) 840-9331
http://www.qualitybiological.com

Quantitative Technologies
P.O. Box 470
Salem Industrial Park, Bldg. 5
Whltehouse, NJ 08888
(908) 534-4445 FAX: 534-1054
http://www.qtionline.com

Quantum Appligene
Parc d'Innovation
Rue Geller de Kayserberg
67402 Illkirch, Cedex, France
(33) 3-8867-5425
FAX: (33) 3-8867-1945
http://www.quantum-appligene.com

Quantum Biotechnologies
See Qbiogene

Quantum Soft
Postfach 6613
CH-8023
Zürich, Switzerland
FAX: 41-1-481-69-51
profit@quansoft.com

Questcor Pharmaceuticals
26118 Research Road
Hayward, CA 94545
(510) 732-5551 FAX: (510) 732-7741
http://www.questcor.com

Quidel
10165 McKellar Court
San Diego, CA 92121
(800) 874-1517 FAX: (858) 546-8955
(858) 552-1100
http://www.quidel.com

R-Biopharm
7950 Old US 27 South
Marshall, MI 49068
(616) 789-3033 FAX: (616) 789-3070
http://www.r-biopharm.com

R. C. Electronics
6464 Hollister Avenue
Santa Barbara, CA 93117
(805) 685-7770 FAX: (805) 685-5853
http://www.rcelectronics.com

R & D Systems
614 McKinley Place NE
Minneapolis, MN 55413
(800) 343-7475 FAX: (612) 379-6580
(612) 379-2956
http://www.rndsystems.com

R & S Technology
350 Columbia Street
Peacedale, RI 02880
(401) 789-5660 FAX: (401) 792-3890
http://www.septech.com

RACAL Health and Safety
See 3M
7305 Executive Way
Frederick, MD 21704
(800) 692-9500 FAX: (301) 695-8200

Radiometer America
811 Sharon Drive
Westlake, OH 44145
(800) 736-0600 FAX: (440) 871-2633
(440) 871-8900
http://www.rameusa.com

Radiometer A/S
The Chemical Reference Laboratory
kandevej 21
DK-2700 Brnshj, Denmark
45-3827-3827 FAX: 45-3827-2727

Radionics
22 Terry Avenue
Burlington, MA 01803
(781) 272-1233 FAX: (781) 272-2428
http://www.radionics.com

Radnoti Glass Technology
227 W. Maple Avenue
Monrovia, CA 91016
(800) 428-l4l6 FAX: (626) 303-2998
(626) 357-8827
http://www.radnoti.com

Rainin Instrument
Rainin Road
P.O. Box 4026
Woburn, MA 01888
(800)-4-RAININ FAX: (781) 938-1152
(781) 935-3050
http://www.rainin.com

Rank Brothers
56 High Street
Bottisham, Cambridge
CB5 9DA UK
(44) 1223 811369
FAX: (44) 1223 811441
http://www.rankbrothers.com

Rapp Polymere
Ernst-Simon Strasse 9
D 72072 Tübingen, Germany
(49) 7071-763157
FAX: (49) 7071-763158
http://www.rapp-polymere.com

Raven Biological Laboratories
8607 Park Drive
P.O. Box 27261
Omaha, NE 68127
(800) 728-5702 FAX: (402) 593-0995
(402) 593-0781
http://www.ravenlabs.com

Razel Scientific Instruments
100 Research Drive
Stamford, CT 06906
(203) 324-9914 FAX: (203) 324-5568

RBI
See Research Biochemicals

Reagents International
See Biotech Source

Receptor Biology
10000 Virginia Manor Road, Suite 360
Beltsville, MD 20705
(888) 707-4200 FAX: (301) 210-6266
(301) 210-4700
http://www.receptorbiology.com

Reflectix, Inc.
#1 School Street
P.O. Box 108
Markleville, IN 46056
(765) 533-4332 FAX: (765) 533-2337
(800) 879-3645
http://www.reflectixinc.com

Regis Technologies
8210 N. Austin Avenue
Morton Grove, IL 60053
(800) 323-8144 FAX: (847) 967-1214
(847) 967-6000
http://www.registech.com

Reheis Inc.
235 Snyder Ave,
Berkeley Heights, NJ 07922
(908) 464-1500 FAX: (908) 464-7726
http://www.reheis.com

Reichert Ophthalmic Instruments
P.O. Box 123
Buffalo, NY 14240
(716) 686-4500 FAX: (716) 686-4545
http://www.reichert.com

Reiss
1 Polymer Place
P.O. Box 60 Blackstone, VA 23824
(800) 356-2829 FAX: (804) 292-1757
(804) 292-1600
http://www.reissmfg.com

Remel
12076 Santa Fe Trail Drive
P.O. Box 14428
Shawnee Mission, KS 66215
(800) 255-6730 FAX: (800) 621-8251
(913) 888-0939 FAX: (913) 888-5884
http://www.remelinc.com

Reming Bioinstruments
6680 County Route 17
Redfield, NY 13437
(315) 387-3414 FAX: (315) 387-3415

Repligen
41 Seyon St.
Building 1, Suite 100
Waltham, MA 02453
(781) 250-0111
http://www.repligen.com

Research Biochemicals
1 Strathmore Road
Natick, MA 01760
(800) 736-3690 FAX: (800) 736-2480
(508) 651-8151 FAX: (508) 655-1359
http://www.resbio.com

Research Corporation Technologies
101 N. Wilmot Road, Suite 600
Tucson, AZ 85711
(520) 748-4400 FAX: (520) 748-0025
http://www.rctech.com

Research Diagnostic, Inc.
Division of Fitzgerald Industries Intl.
34 Junction Square Drive
Concord, MA 01742-3049
(978) 371-6446 FAX: (978) 371-2266
800-370-2222
http://www.researchd.com

Research Diets
121 Jersey Avenue
New Brunswick, NJ 08901
(877) 486-2486 FAX: (732) 247-2340
(732) 247-2390
http://www.researchdiets.com

Research Genetics
2130 South Memorial Parkway
Huntsville, AL 35801
(800) 533-4363 FAX: (256) 536-9016
(256) 533-4363
http://www.resgen.com

Research Instruments
Kernick Road Pernryn
Cornwall TR10 9DQ, UK
(44) 1326-372-753
FAX: (44) 1326-378-783
http://www.research-instruments.com

Research Organics
4353 E. 49th Street
Cleveland, OH 44125
(800) 321-0570 FAX: (216) 883-1576
(216) 883-8025
http://www.resorg.com

Research Plus
P.O. Box 324
Bayonne, NJ 07002
(800) 341-2296 FAX: (201) 823-9590
(201) 823-3592
http://www.researchplus.com

Research Products International
410 N. Business Center Drive
Mount Prospect, IL 60056
(800) 323-9814 FAX: (847) 635-1177
(847) 635-7330
http://www.rpicorp.com

Research Triangle Institute
P.O. Box 12194
Research Triangle Park, NC 27709
(919) 541-6000 FAX: (919) 541-6515
http://www.rti.org

Restek
110 Benner Circle
Bellefonte, PA 16823
(800) 356-1688 FAX: (814) 353-1309
(814) 353-1300
http://www.restekcorp.com

Rheodyne
P.O. Box 1909
Rohnert Park, CA 94927
(707) 588-2000 FAX: (707) 588-2020
http://www.rheodyne.com

Rhone Merieux
See Merial Limited

Rhone-Poulenc
2 T W Alexander Drive
P.O. Box 12014
Research Triangle Park, NC 08512
(919) 549-2000 FAX: (919) 549-2839
http://www.Rhone-Poulenc.com
Also see Aventis

Rhone-Poulenc Rorer
500 Arcola Road
Collegeville, PA 19426
(800) 727-6737 FAX: (610) 454-8940
(610) 454-8975
http://www.rp-rorer.com

Rhone-Poulenc Rorer
Centre de Recherche de
Vitry-Alfortville
13 Quai Jules Guesde, BP14 94403
Vitry Sur Seine, Cedex, France
(33) 145-73-85-11
FAX: (33) 145-73-81-29
http://www.rp-rorer.com

Ribi ImmunoChem Research
563 Old Corvallis Road
Hamilton, MT 59840
(800) 548-7424 FAX: (406) 363-6129
(406) 363-3131
http://www.ribi.com

RiboGene
See Questcor Pharmaceuticals

Ricca Chemical
448 West Fork Drive
Arlington, TX 76012
(888) GO-RICCA FAX: (800)
RICCA-93
(817) 461-5601
http://www.riccachemical.com

Richard-Allan Scientific
225 Parsons Street
Kalamazoo, MI 49007
(800) 522-7270 FAX: (616) 345-3577
(616) 344-2400
http://www.rallansci.com

Richelieu Biotechnologies
11 177 Hamon
Montral, Quebec
H3M 3E4 Canada
(802) 863-2567 FAX: (802) 862-2909
http://www.richelieubio.com

Richter Enterprises
20 Lake Shore Drive
Wayland, MA 01778
(508) 655-7632 FAX: (508) 652-7264
http://www.richter-enterprises.com

Riese Enterprises
BioSure Division
12301 G Loma Rica Drive
Grass Valley, CA 95945
(800) 345-2267 FAX: (916) 273-5097
(916) 273-5095
http://www.biosure.com

Robbins Scientific
1250 Elko Drive
Sunnyvale, CA 94086
(800) 752-8585 FAX: (408) 734-0300
(408) 734-8500
http://www.robsci.com

Roboz Surgical Instruments
9210 Corporate Boulevard, Suite 220
Rockville, MD 20850
(800) 424-2984 FAX: (301) 590-1290
(301) 590-0055

Roche Diagnostics
9115 Hague Road
P.O. Box 50457
Indianapolis, IN 46256
(800) 262-1640 FAX: (317) 845-7120
(317) 845-2000
http://www.roche.com

Roche Molecular Systems
See Roche Diagnostics

Rocklabs
P.O. Box 18-142
Auckland 6, New Zealand
(64) 9-634-7696
FAX: (64) 9-634-7696
http://www.rocklabs.com

Rockland
P.O. Box 316
Gilbertsville, PA 19525
(800) 656-ROCK FAX: (610)
367-7825
(610) 369-1008
http://www.rockland-inc.com

Rohm
Chemische Fabrik
Kirschenallee
D-64293 Darmstadt, Germany
(49) 6151-1801 FAX: (49) 6151-1802
http://www.roehm.com

Roper Scientific
3440 East Brittania Drive, Suite 100
Tucson, AZ 85706
(520) 889-9933 FAX: (520) 573-1944
http://www.roperscientific.com

Rosetta Inpharmatics
12040 115th Avenue NE
Kirkland, WA 98034
(425) 820-8900 FAX: (425) 820-5757
http://www.rii.com

ROTH-SOCHIEL
3 rue de la Chapelle
Lauterbourg
67630 France
(33) 03-88-94-82-42
FAX: (33) 03-88-54-63-93

Rotronic Instrument
160 E. Main Street
Huntington, NY 11743
(631) 427-3898 FAX: (631) 427-3902
http://www.rotronic-usa.com

Roundy's
23000 Roundy Drive
Pewaukee, WI 53072
(262) 953-7999 FAX: (262) 953-7989
http://www.roundys.com

RPI
see Research Products International

RS Components
Birchington Road
Weldon Industrial Estate
Corby, Northants NN17 9RS, UK
(44) 1536 201234
FAX: (44) 1536 405678
http://www.rs-components.com

Rubbermaid
See Newell Rubbermaid

SA Instrumentation
1437 Tzena Way
Encinitas, CA 92024
(858) 453-1776 FAX: (800) 266-1776
http://www.sainst.com

Safe Cells
See Bionique Testing Labs

Sage Instruments
240 Airport Boulevard
Freedom, CA 95076
831-761-1000 FAX: 831-761-1008
http://www.sageinst.com

Sage Laboratories
11 Huron Drive
Natick, MA 01760
(508) 653-0844 FAX: 508-653-5671
http://www.sagelabs.com

Saint-Gobain Performance Plastics
P.O. Box 3660
Akron, OH 44309
(330) 798-9240 FAX: (330) 798-6968
http://www.nortonplastics.com

Sakura Finetek
750 West 214th Street
Torrance, CA 90501
(800) 725-8723 FAX: (310) 972-7888
http://www.sakuraus.com

San Diego Instruments
7758 Arjons Drive
San Diego, CA 92126
(858) 530-2600 FAX: (858) 530-2646
http://www.sd-inst.com

Sandown Scientific
Beards Lodge
25 Oldfield Road
Hampden, Middlesex TW12 2AJ, UK
(44) 2089 793300
FAX: (44) 2089 793311
http://www.sandownsci.com

Sandoz Pharmaceuticals
See Novartis

Sanofi Recherche
Centre de Montpellier
371 Rue du Professor Blayac
34184 Montpellier, Cedex 04
France
(33) 67-10-67-10
FAX: (33) 67-10-67-67

Sanofi Winthrop Pharmaceuticals
90 Park Avenue
New York, NY 10016
(800) 223-5511 FAX: (800) 933-3243
(212) 551-4000
http://www.sanofi-synthelabo.com/us

Santa Cruz Biotechnology
2161 Delaware Avenue
Santa Cruz, CA 95060
(800) 457-3801 FAX: (831) 457-3801
(831) 457-3800
http://www.scbt.com

Sarasep
(800) 605-0267 FAX: (408) 432-3231
(408) 432-3230
http://www.transgenomic.com

Sarstedt
P.O. Box 468
Newton, NC 28658
(800) 257-5101 FAX: (828) 465-4003
(828) 465-4000
http://www.sarstedt.com

Sartorius
131 Heartsland Boulevard
Edgewood, NY 11717
(800) 368-7178 FAX: (516) 254-4253
http://www.sartorius.com

SAS Institute
Pacific Telesis Center
One Montgomery Street
San Francisco, CA 94104
(415) 421-2227 FAX: (415) 421-1213
http://www.sas.com

Savant/EC Apparatus
A ThermoQuest company
100 Colin Drive
Holbrook, NY 11741
(800) 634-8886 FAX: (516) 244-0606
(516) 244-2929
http://www.savec.com

Savillex
6133 Baker Road
Minnetonka, MN 55345
(612) 935-5427

Scanalytics
Division of CSP
8550 Lee Highway, Suite 400
Fairfax, VA 22031
(800) 325-3110 FAX: (703) 208-1960
(703) 208-2230
http://www.scanalytics.com

Schering Laboratories
See Schering-Plough

Schering-Plough
1 Giralda Farms
Madison, NJ 07940
(800) 222-7579 FAX: (973) 822-7048
(973) 822-7000
http://www.schering-plough.com

Schleicher & Schuell
10 Optical Avenue
Keene, NH 03431
(800) 245-4024 FAX: (603) 357-3627
(603) 352-3810
http://www.s-und-s.de/english-index.html

Science Technology Centre
1250 Herzberg Laboratories
Carleton University
1125 Colonel Bay Drive
Ottawa, Ontario, Canada K1S 5B6
(613) 520-4442 FAX: (613) 520-4445
http://www.carleton.ca/universities/stc

Scientific Instruments
200 Saw Mill River Road
Hawthorne, NY 10532
(800) 431-1956 FAX: (914) 769-5473
(914) 769-5700
http://www.scientificinstruments.com

Scientific Solutions
9323 Hamilton
Mentor, OH 44060
(440) 357-1400 FAX: (440) 357-1416
www.labmaster.com

Scion
82 Worman's Mill Court, Suite H
Frederick, MD 21701
(301) 695-7870 FAX: (301) 695-0035
www.scioncorp.com

Scott Specialty Gases
6141 Easton Road
P.O. Box 310
Plumsteadville, PA 18949
(800) 21-SCOTT FAX: (215) 766-2476
(215) 766-8861
http://www.scottgas.com

Scripps Clinic and Research Foundation
Instrumentation and Design Lab
10666 N. Torrey Pines Road
La Jolla, CA 92037
(800) 992-9962 FAX: (858) 554-8986
(858) 455-9100
http://www.scrippsclinic.com

SDI Sensor Devices
407 Pilot Court, 400A
Waukesha, WI 53188
(414) 524-1000 FAX: (414) 524-1009

Sefar America
111 Calumet Street
Depew, NY 14043
(716) 683-4050 FAX: (716) 683-4053
http://www.sefaramerica.com

Seikagaku America
Division of Associates of Cape Cod
704 Main Street
Falmouth, MA 02540
(800) 237-4512 FAX: (508) 540-8680
(508) 540-3444
http://www.seikagaku.com

Sellas Medizinische Gerate
Hagener Str. 393
Gevelsberg-Vogelsang, 58285
Germany
(49) 23-326-1225

Sensor Medics
22705 Savi Ranch Parkway
Yorba Linda, CA 92887
(800) 231-2466 FAX: (714) 283-8439
(714) 283-2228
http://www.sensormedics.com

Sensor Systems LLC
2800 Anvil Street, North
Saint Petersburg, FL 33710
(800) 688-2181 FAX: (727) 347-3881
(727) 347-2181
http://www.vsensors.com

SenSym/Foxboro ICT
1804 McCarthy Boulevard
Milpitas, CA 95035
(800) 392-9934 FAX: (408) 954-9458
(408) 954-6700
http://www.sensym.com

Separations Group
See Vydac

Sepracor
111 Locke Drive
Marlboro, MA 01752
(877)-SEPRACOR (508) 357-7300
http://www.sepracor.com

Sera-Lab
See Harlan Sera-Lab

Sermeter
925 Seton Court, #7
Wheeling, IL 60090
(847) 537-4747

Serological
195 W. Birch Street
Kankakee, IL 60901
(800) 227-9412 FAX: (815) 937-8285
(815) 937-8270

Seromed Biochrom
Leonorenstrasse 2-6
D-12247 Berlin, Germany
(49) 030-779-9060

Serotec
22 Bankside
Station Approach
Kidlington, Oxford OX5 1JE, UK
(44) 1865-852722
FAX: (44) 1865-373899
In the US: (800) 265-7376
http://www.serotec.co.uk

Serva Biochemicals
Distributed by Crescent Chemical

Seton Scientific
P.O. Box 33064
Los Gatos, CA 95031¯
(800) 543-2134 FAX: (408) 353-8019
(408) 353-5993

S.F. Medical Pharmlast
See Chase-Walton Elastomers

SGE
2007 Kramer Lane
Austin, TX 78758
(800) 945-6154 FAX: (512) 836-9159
(512) 837-7190
http://www.sge.com

Shandon/Lipshaw
see Thermo Electron Anatomical Pathology

Sharpoint
P.O. Box 2212
Taichung, Taiwan
Republic of China
(886) 4-3206320
FAX: (886) 4-3289879
http://www.sharpoint.com.tw

Shelton Scientific
230 Longhill Crossroads
Shelton, CT 06484
(800) 222-2092 FAX: (203) 929-2175
(203) 929-8999
http://www.sheltonscientific.com

Sherwood-Davis & Geck
See Kendall

Sherwood Medical
See Kendall

SHI, Inc. (Software House International)
(888) 764-8888
http://www.shi.com

Shimadzu Scientific Instruments
7102 Riverwood Drive
Columbia, MD 21046
(800) 477-1227 FAX: (410) 381-1222
(410) 381-1227
http://www.ssi.shimadzu.com

Sialomed
See Amika

Siemens Analytical X-Ray Systems
See Bruker Analytical X-Ray Systems

Sievers Instruments
Subsidiary of Ionics
6060 Spine Road
Boulder, CO 80301
(800) 255-6964 FAX: (303) 444-6272
(303) 444-2009
http://www.sieversinst.com

SIFCO
970 East 46th Street
Cleveland, OH 44103
(216) 881-8600 FAX: (216) 432-6281
http://www.silco.com

Sigma-Aldrich
3050 Spruce Street
St. Louis, MO 63103
(800) 358-5287 FAX: (800) 962-9591
(800) 325-3101 FAX: (800) 325-5052
http://www.sigma-aldrich.com

Sigma-Aldrich Canada
2149 Winston Park Drive
Oakville, Ontario
L6H 6J8 Canada
(800) 5652D1400 FAX: (800) 2652D3858
http://www.sigma-aldrich.com

Sigma-Aldrich Corporation
3050 Spruce Street
St. Louis, MO 63103
(800) 521-8956
http://www.sigmaaldrich.com

Silenus/Amrad
34 Wadhurst Drive
Boronia, Victoria 3155 Australia
(613)9887-3909 FAX: (613)9887-3912
http://www.amrad.com.au

Silicon Genetics
2601 Spring Street
Redwood City, CA 94063
(866) SIG SOFT FAX: (650) 365 1735
(650) 367 9600
http://www.sigenetics.com

SIMS Deltec
1265 Grey Fox Road
St. Paul, Minnesota 55112
(800) 426-2448 FAX: (615) 628-7459
http://www.deltec.com

SIMS Portex
10 Bowman Drive
Keene, NH 03431
(800) 258-5361 FAX: (603) 352-3703
(603) 352-3812
http://www.simsmed.com

SIMS Portex Limited
Hythe, Kent CT21 6JL, UK
(44)1303-260551
FAX: (44)1303-266761
http://www.portex.com

Siris Laboratories
See Biosearch Technologies

Skatron Instruments
See Molecular Devices

SLM Instruments
See Spectronic Instruments

SLM-AMINCO Instruments
See Spectronic Instruments

Small Parts
13980 NW 58th Court
P.O. Box 4650
Miami Lakes, FL 33014
(800) 220-4242 FAX: (800) 423-9009
(305) 558-1038 FAX: (305) 558-0509
http://www.smallparts.com

Smith & Nephew
11775 Starkey Road
P.O. Box 1970
Largo, FL 33779
(800) 876-1261
http://www.smith-nephew.com

SmithKline Beecham
1 Franklin Plaza, #1800
Philadelphia, PA 19102
(215) 751-4000 FAX: (215) 751-4992
http://www.sb.com

Solid Phase Sciences
See Biosearch Technologies

SOMA Scientific Instruments
5319 University Drive, PMB #366
Irvine, CA 92612
(949) 854-0220 FAX: (949) 854-0223
http://somascientific.com

Somatix Therapy
See Cell Genesys

Sonics & Materials
53 Church Hill Road
Newtown, CT 06470
(800) 745-1105 FAX: (203) 270-4610
(203) 270-4600
http://www.sonicsandmaterials.com

Sonosep Biotech
See Triton Environmental Consultants

Sorvall
See Kendro Laboratory Products

Southern Biotechnology Associates
P.O. Box 26221
Birmingham, AL 35260
(800) 722-2255 FAX: (205) 945-8768
(205) 945-1774
http://SouthernBiotech.com

SPAFAS
190 Route 165
Preston, CT 06365
(800) SPAFAS-1 FAX: (860) 889-1991
(860) 889-1389
http://www.spafas.com

Specialty Media
Division of Cell & Molecular Technologies
580 Marshall Street
Phillipsburg, NJ 08865
(800) 543-6029 FAX: (908) 387-1670
(908) 454-7774
http://www.specialtymedia.com

Spectra Physics, Inc.
1335 Terra Bella Avenue
P.O. Box 7013
Mountain View, CA 94039
(650) 961 2550
http://www.spectra-physics.com

Spectramed
See BOC Edwards

SpectraSource Instruments
31324 Via Colinas, Suite 114
Westlake Village, CA 91362
(818) 707-2655 FAX: (818) 707-9035
http://www.spectrasource.com

Spectronic Instruments
820 Linden Avenue
Rochester, NY 14625
(800) 654-9955 FAX: (716) 248-4014
(716) 248-4000
http://www.spectronic.com

Spectrum Medical Industries
See Spectrum Laboratories

Spectrum Laboratories
18617 Broadwick Street
Rancho Dominguez, CA 90220
(800) 634-3300 FAX: (800) 445-7330
(310) 885-4601 FAX: (310) 885-4666
http://www.spectrumlabs.com

Spherotech
1840 Industrial Drive, Suite 270
Libertyville, IL 60048
(800) 368-0822 FAX: (847) 680-8927
(847) 680-8922
http://www.spherotech.com

SPSS
233 S. Wacker Drive, 11th floor
Chicago, IL 60606
(800) 521-1337 FAX: (800) 841-0064
http://www.spss.com

SS White Burs
1145 Towbin Avenue
Lakewood, NJ 08701
(732) 905-1100 FAX: (732) 905-0987
http://www.sswhiteburs.com

Stag Instruments
16 Monument Industrial Park
Chalgrove, Oxon OX44 7RW, UK
(44) 1865-891116
FAX: (44) 1865-890562

Standard Reference Materials Program
National Institute of Standards and Technology
Building 202, Room 204
Gaithersburg, MD 20899
(301) 975-6776 FAX: (301) 948-3730

Starna Cells
P.O. Box 1919
Atascandero, CA 93423
(805) 468-8855 FAX: (805) 461-1575
(800) 228-4482
http://www.starnacells.com

Starplex Scientific
50 Steinway
Etobieoke, Ontario
M9W 6Y3 Canada
(800) 665-0954 FAX: (416) 674-6067
(416) 674-7474
http://www.starplexscientific.com

State Laboratory Institute of Massachusetts
305 South Street
Jamaica Plain, MA 02130
(617) 522-3700 FAX: (617) 522-8735
http://www.state.ma.us/dph

Stedim Labs
1910 Mark Court, Suite 110
Concord, CA 94520
(800) 914-6644 FAX: (925) 689-6988
(925) 689-6650
http://www.stedim.com

Steinel America
9051 Lyndale Avenue
Bloomington, MN 55420
(800) 852 4343 FAX: (952) 888-5132
http://www.steinelamerica.com

Stem Cell Technologies
777 West Broadway, Suite 808
Vancouver, British Columbia
V5Z 4J7 Canada
(800) 667-0322 FAX: (800) 567-2899
(604) 877-0713 FAX: (604) 877-0704
http://www.stemcell.com

Stephens Scientific
107 Riverdale Road
Riverdale, NJ 07457
(800) 831-8099 FAX: (201) 831-8009
(201) 831-9800

Steraloids
P.O. Box 689
Newport, RI 02840
(401) 848-5422 FAX: (401) 848-5638
http://www.steraloids.com

Steris Corporation
5960 Heisley Road
Mentor, Ohio 44060
(800) 548-4873
440-354-2600
http://www.steris.com

Sterling Medical
2091 Springdale Road, Ste. 2
Cherry Hill, NJ 08003
(800) 229-0900 FAX: (800) 229-7854
http://www.sterlingmedical.com

Sterling Winthrop
90 Park Avenue
New York, NY 10016
(212) 907-2000 FAX: (212) 907-3626

Sternberger Monoclonals
10 Burwood Court
Lutherville, MD 21093
(410) 821-8505 FAX: (410) 821-8506
http://www.sternbergermonoclonals.com

Stoelting
502 Highway 67
Kiel, WI 53042
(920) 894-2293 FAX: (920) 894-7029
http://www.stoelting.com

Stovall Lifescience
206-G South Westgate Drive
Greensboro, NC 27407
(800) 852-0102 FAX: (336) 852-3507
http://www.slscience.com

Stratagene
11011 N. Torrey Pines Road
La Jolla, CA 92037
(800) 424-5444 FAX: (888) 267-4010
(858) 535-5400
http://www.stratagene.com

Strategic Applications
530A N. Milwaukee Avenue
Libertyville, IL 60048
(847) 680-9385 FAX: (847) 680-9837

Strem Chemicals
7 Mulliken Way
Newburyport, MA 01950
(800) 647-8736 FAX: (800) 517-8736
(978) 462-3191 FAX: (978) 465-3104
http://www.strem.com

StressGen Biotechnologies
Biochemicals Division
120-4243 Glanford Avenue
Victoria, British Columbia
V8Z 4B9 Canada
(800) 661-4978 FAX: (250) 744-2877
(250) 744-2811
http://www.stressgen.com

Structure Probe/SPI Supplies (Epon-Araldite)
P.O. Box 656
West Chester, PA 19381
(800) 242-4774 FAX: (610) 436-5755
http://www.2spi.com

Süd-Chemie Performance Packaging
101 Christine Drive
Belen, NM 87002
(800) 989-3374 FAX: (505) 864-9296
http://www.uniteddesiccants.com

Sumitomo Chemical
Sumitomo Building
5-33, Kitahama 4-chome
Chuo-ku, Osaka 541-8550, Japan
(81) 6-6220-3891
FAX: (81)-6-6220-3345
http://www.sumitomo-chem.co.jp

Sun Box
19217 Orbit Drive
Gaithersburg, MD 20879
(800) 548-3968 FAX: (301) 977-2281
(301) 869-5980
http://www.sunboxco.com

Sunbrokers
See Sun International

Sun International
3700 Highway 421 North
Wilmington, NC 28401
(800) LAB-VIAL FAX: (800) 231-7861
http://www.autosamplervial.com

Sunox
1111 Franklin Boulevard, Unit 6
Cambridge, ON N1R 8B5, Canada
(519) 624-4413 FAX: (519) 624-8378
http://www.sunox.ca

Supelco
See Sigma-Aldrich

SuperArray
P.O. Box 34494
Bethesda, MD 20827
(888) 503-3187 FAX: (301) 765-9859
(301) 765-9888
http://www.superarray.com

Surface Measurement Systems
3 Warple Mews, Warple Way
London W3 ORF, UK
(44) 20-8749-4900
FAX: (44) 20-8749-6749
http://www.smsuk.co.uk/index.htm

SurgiVet
N7 W22025 Johnson Road, Suite A
Waukesha, WI 53186
(262) 513-8500 (888) 745-6562
FAX: (262) 513-9069
http://www.surgivet.com

Sutter Instruments
51 Digital Drive
Novato, CA 94949
(415) 883-0128 FAX: (415) 883-0572
http://www.sutter.com

Swiss Precision Instruments
1555 Mittel Boulevard, Suite F
Wooddale, IL 60191
(800) 221-0198 FAX: (800) 842-5164

Synaptic Systems GmbH
Rudolf-Wissell-Str. 28
37079 Goettingen, Germany
(49) 551 50556 0

Synaptosoft
3098 Anderson Place
Decatur, GA 30033
(770) 939-4366 FAX: 770-939-9478
http://www.synaptosoft.com

SynChrom
See Micra Scientific

Synergy Software
2457 Perkiomen Avenue
Reading, PA 19606
(800) 876-8376 FAX: (610) 370-0548
(610) 779-0522
http://www.synergy.com

Synteni
See Incyte

Synthetics Industry
Lumite Division
2100A Atlantic Highway
Gainesville, GA 30501
(404) 532-9756 FAX: (404) 531-1347

Systat
See SPSS

Systems Planning and Analysis (SPA)
2000 N. Beauregard Street
Suite 400
Alexandria, VA 22311
(703) 931-3500
http://www.spa-inc.net

3M Bioapplications
3M Center
Building 270-15-01
St. Paul, MN 55144
(800) 257-7459 FAX: (651) 737-5645
(651) 736-4946

T Cell Diagnostics and **T Cell Sciences**
38 Sidney Street
Cambridge, MA 02139
(617) 621-1400

TAAB Laboratory Equipment
3 Minerva House
Calleva Park
Aldermaston, Berkshire RG7 8NA, UK
(44) 118 9817775
FAX: (44) 118 9817881

Taconic
273 Hover Avenue
Germantown, NY 12526
(800) TAC-ONIC FAX: (518) 537-7287
(518) 537-6208
http://www.taconic.com

Tago
See Biosource International

TaKaRa Biochemical
719 Alliston Way
Berkeley, CA 94710
(800) 544-9899 FAX: (510) 649-8933
(510) 649-9895
http://www.takara.co.jp/english

Takara Shuzo
Biomedical Group Division
Seta 3-4-1
Otsu Shiga 520-21, Japan
(81) 75-241-5100
FAX: (81) 77-543-9254
http://www.Takara.co.jp/english

Takeda Chemical Products
101 Takeda Drive
Wilmington, NC 28401
(800) 825-3328 FAX: (800) 825-0333
(910) 762-8666 FAX: (910) 762-6846
http://takeda-usa.com

Talboys Engineering Corp
Henry Troemner, LLC
201 Wolf Drive
P.O. Box 87
Thorofare, NJ 0806
(856) 686-1600 FAX: (856) 686-1601
(888) TALBOYS

TAO Biomedical
73 Manassas Court
Laurel Springs, NJ 08021
(609) 782-8622 FAX: (609) 782-8622

TauTec LLC
9140 Guilford Road, Suite O
Colombia, MD 21046
(301) 725-7441 (253) 679-8345
http://www.tautec.com

Tecan US
P.O. Box 13953
Research Triangle Park, NC 27709
(800) 33-TECAN FAX: (919) 361-5201
(919) 361-5208
http://www.tecan-us.com

Techne
University Park Plaza
743 Alexander Road
Princeton, NJ 08540
(800) 225-9243 FAX: (609) 987-8177
(609) 452-9275
http://www.techneusa.com

Technical Manufacturing
15 Centennial Drive
Peabody, MA 01960
(978) 532-6330 FAX: (978) 531-8682
http://www.techmfg.com

Technical Products International
5918 Evergreen
St. Louis, MO 63134
(800) 729-4451 FAX: (314) 522-6360
(314) 522-8671
http://www.vibratome.com

Technicon
See Organon Teknika Cappel

Techno-Aide
P.O. Box 90763
Nashville, TN 37209
(800) 251-2629 FAX: (800) 554-6275
(615) 350-7030
http://www.techno-aid.com

Ted Pella
4595 Mountain Lakes Boulevard
P.O. Box 492477
Redding, CA 96049
(800) 237-3526 FAX: (530) 243-3761
(530) 243-2200
http://www.tedpella.com

Tekmar-Dohrmann
P.O. Box 429576 Cincinnati, OH 45242
(800) 543-4461 FAX: (800) 841-5262
(513) 247-7000 FAX: (513) 247-7050

Tektronix
142000 S.W. Karl Braun Drive
Beaverton, OR 97077
(800) 621-1966 FAX: (503) 627-7995
(503) 627-7999
http://www.tek.com

Tel-Test
P.O. Box 1421
Friendswood, TX 77546
(800) 631-0600 FAX: (281)482-1070
(281)482-2672
http://www.isotex-diag.com

TeleChem International
524 East Weddell Drive, Suite 3
Sunnyvale, CA 94089
(408) 744-1331 FAX: (408) 744-1711
http://www.gst.net/~telechem

Terrachem
Mallaustrasse 57
D-68219 Mannheim, Germany
0621-876797-0FAX: 0621-876797-19
http://www.terrachem.de

Terumo Medical
2101 Cottontail Lane
Somerset, NJ 08873
(800) 283-7866 FAX: (732) 302-3083
(732) 302-4900
http://www.terumomedical.com

Tetko
333 South Highland Manor
Briarcliff, NY 10510
(800) 289-8385 FAX: (914) 941-1017
(914) 941-7767
http://www.tetko.com

TetraLink
4240 Ridge Lea Road
Suite 29
Amherst, NY 14226
(800) 747-5170 FAX: (800) 747-5171
http://www.tetra-link.com

TEVA Pharmaceuticals USA
1090 Horsham Road
P.O. Box 1090
North Wales, PA 19454
(215) 591-3000 FAX: (215) 721-9669
http://www.tevapharmusa.com

Texas Fluorescence Labs
9503 Capitol View Drive
Austin, TX 78747
(512) 280-5223 FAX: (512) 280-4997
http://www.teflabs.com

The Nest Group
45 Valley Road
Southborough, MA 01772
(800) 347-6378 FAX: (508) 485-5736
(508) 481-6223
http://world.std.com/~nestgrp

ThermoCare
P.O. Box 6069
Incline Village, NV 89450
(800) 262-4020
(775) 831-1201

Thermo Electron Anatomical Pathology
171 Industry Dr.
Pittsburgh, PA
(800) 547-7429 FAX: (412) 788-1138
http://www.thermo.com/

Thermo Labsystems
8 East Forge Parkway
Franklin, MA 02038
(800) 522-7763 FAX: (508) 520-2229
(508) 520-0009
http://www.finnpipette.com

Thermometric
Spjutvagen 5A
S-175 61 Jarfalla, Sweden
(46) 8-564-72-200

Thermoquest
IEC Division
300 Second Avenue
Needham Heights, MA 02194
(800) 843-1113 FAX: (781) 444-6743
(781) 449-0800
http://www.thermoquest.com

Thermo Separation Products
Thermoquest
355 River Oaks Parkway
San Jose, CA 95134
(800) 538-7067 FAX: (408) 526-9810
(408) 526-1100
http://www.thermoquest.com

Thermo Shandon
171 Industry Drive
Pittsburgh, PA 15275
(800) 547-7429 FAX: (412) 899-4045
http://www.thermoshandon.com

Thermo Spectronic
820 Linden Avenue
Rochester, NY 14625
(585) 248-4000 FAX: (585) 248-4200
http://www.thermo.com

Thomas Scientific
99 High Hill Road at I-295
Swedesboro, NJ 08085
(800) 345-2100 FAX: (800) 345-5232
(856) 467-2000 FAX: (856) 467-3087
http://www.wheatonsci.com/html/nt/Thomas.html

Thomson Instrument
354 Tyler Road
Clearbrook, VA 22624
(800) 842-4752 FAX: (540) 667-6878
(800) 541-4792 FAX: (760) 757-9367
http://www.hplc.com

Thorn EMI
See Electron Tubes

Thorlabs
435 Route 206
Newton, NJ 07860
(973) 579-7227 FAX: (973) 383-8406
http://www.thorlabs.com

Tiemann
See Bernsco Surgical Supply

TILL Photonics GmbH
Lochhamer Schlag 19
D-82166 Gräfeling
Germany
(49) 89-895-662-0
FAX: (49) 89-895-662-101
http://www.till-photonics.com/

Timberline Instruments
1880 South Flatiron Court, H-2
P.O. Box 20356
Boulder, CO 80308
(800) 777-5996 FAX: (303) 440-8786
(303) 440-8779
http://www.timberlineinstruments.com

TissueInformatics
711 Bingham Street, Suite 202
Pittsburgh, PA 15203
(418) 488-1100 FAX: (418) 488-6172
http://www.tissueInformatics.com

Tissue-Tek
A Division of Sakura Finetek USA
1750 West 214th Street
Torrance, CA 90501
(800) 725-8723 FAX: (310) 972-7888
(310) 972-7800
http://www.sakuraus.com

Tocris Cookson
114 Holloway Road, Suite 200
Ballwin, MO 63011
(800) 421-3701 FAX: (800) 483-1993
(636) 207-7651 FAX: (636) 207-7683
http://www.tocris.com

Tocris Cookson
Northpoint, Fourth Way
Avonmouth, Bristol BS11 8TA, UK
(44) 117-982-6551
FAX: (44) 117-982-6552
http://www.tocris.com

Tomtec
See CraMar Technologies

TopoGen
P.O. Box 20607
Columbus, OH 43220
(800) TOPOGEN
FAX: (800) ADD-TOPO
(614) 451-5810 FAX: (614) 451-5811
http://www.topogen.com

Toray Industries, Japan
Toray Building 2-1
Nihonbash-Muromach
2-Chome, Chuo-Ku
Tokyo, Japan 103-8666
(03) 3245-5115 FAX: (03) 3245-5555
http://www.toray.co.jp

Toray Industries, U.S.A.
600 Third Avenue
New York, NY 10016
(212) 697-8150 FAX: (212) 972-4279
http://www.toray.com

Toronto Research Chemicals
2 Brisbane Road
North York, Ontario M3J 2J8, Canada
(416) 665-9696 FAX: (416) 665-4439
http://www.trc-canada.com

TosoHaas
156 Keystone Drive
Montgomeryville, PA 18036
(800) 366-4875 FAX: (215) 283-5035
(215) 283-5000
http://www.tosohaas.com

Towhill
647 Summer Street
Boston, MA 02210
(617) 542-6636 FAX: (617) 464-0804

Toxin Technology
7165 Curtiss Avenue
Sarasota, FL 34231
(941) 925-2032 FAX: (9413) 925-2130
http://www.toxintechnology.com

Toyo Soda
See TosoHaas

Trace Analytical
3517-A Edison Way
Menlo Park, CA 94025
(650) 364-6895 FAX: (650) 364-6897
http://www.traceanalytical.com

Transduction Laboratories
See BD Transduction Laboratories

Transgenomic
2032 Concourse Drive
San Jose, CA 95131
(408) 432-3230 FAX: (408) 432-3231
http://www.transgenomic.com

Transonic Systems
34 Dutch Mill Road
Ithaca, NY 14850
(800) 353-3569 FAX: (607) 257-7256
http://www.transonic.com

Travenol Lab
See Baxter Healthcare

Tree Star Software
20 Winding Way
San Carlos, CA 94070
800-366-6045
http://www.treestar.com

Trevigen
8405 Helgerman Court
Gaithersburg, MD 20877
(800) TREVIGEN FAX: (301) 216-2801
(301) 216-2800
http://www.trevigen.com

Trilink Biotechnologies
6310 Nancy Ridge Drive
San Diego, CA 92121
(800) 863-6801 FAX: (858) 546-0020
http://www.trilink.biotech.com

Tripos Associates
1699 South Hanley Road, Suite 303
St. Louis, MO 63144
(800) 323-2960 FAX: (314) 647-9241
(314) 647-1099
http://www.tripos.com

Triton Environmental Consultants
120-13511 Commerce Parkway
Richmond, British Columbia
V6V 2L1 Canada
(604) 279-2093 FAX: (604) 279-2047
http://www.triton-env.com

Tropix
47 Wiggins Avenue
Bedford, MA 01730
(800) 542-2369 FAX: (617) 275-8581
(617) 271-0045
http://www.tropix.com

TSI Center for Diagnostic Products
See Intergen

2000 Eppendorf-5 Prime
5603 Arapahoe Avenue
Boulder, CO 80303
(800) 533-5703 FAX: (303) 440-0835
(303) 440-3705

Tyler Research
10328 73rd Avenue
Edmonton, Alberta
T6E 6N5 Canada
(403) 448-1249 FAX: (403) 433-0479

UBI
See Upstate Biotechnology

Ugo Basile Biological Research Apparatus
Via G. Borghi 43
21025 Comerio, Varese, Italy
(39) 332 744 574
FAX: (39) 332 745 488
http://www.ugobasile.com

UltraPIX
See Life Science Resources

Ultrasonic Power
239 East Stephenson Street
Freeport, IL 61032
(815) 235-6020 FAX: (815) 232-2150
http://www.upcorp.com

Ultrasound Advice
23 Aberdeen Road
London N52UG, UK
(44) 020-7359-1718
FAX: (44) 020-7359-3650
http://www.ultrasoundadvice.co.uk

UNELKO
14641 N. 74th Street
Scottsdale, AZ 85260
(480) 991-7272 FAX: (480)483-7674
http://www.unelko.com

Unifab Corp.
5260 Lovers Lane
Kalamazoo, MI 49002 (800) 648-9569 FAX: (616) 382-2825
(616) 382-2803

Union Carbide
10235 West Little York Road, Suite 300
Houston, TX 77040
(800) 568-4000 FAX: (713) 849-7021
(713) 849-7000
http://www.unioncarbide.com

United Desiccants
See Süd-Chemie Performance Packaging

United States Biochemical
See USB

United States Biological (US Biological)
P.O. Box 261
Swampscott, MA 01907
(800) 520-3011 FAX: (781) 639-1768
http://www.usbio.net

Universal Imaging
502 Brandywine Parkway
West Chester, PA 19380
(610) 344-9410 FAX: (610) 344-6515
http://www.image1.com

Upchurch Scientific
619 West Oak Street
P.O. Box 1529
Oak Harbor, WA 98277
(800) 426-0191 FAX: (800) 359-3460
(360) 679-2528 FAX: (360) 679-3830
http://www.upchurch.com

Upjohn
Pharmacia & Upjohn
http://www.pnu.com

Upstate Biotechnology (UBI)
1100 Winter Street, Suite 2300
Waltham, MA 02451
(800) 233-3991 FAX: (781) 890-7738
(781) 890-8845
http://www.upstatebiotech.com

USA/Scientific
346 SW 57th Avenue
P.O. Box 3565
Ocala, FL 34478
(800) LAB-TIPS FAX: (352) 351-2057
(3524) 237-6288
http://www.usascientific.com

USB
26111 Miles Road
P.O. Box 22400
Cleveland, OH 44122
(800) 321-9322 FAX: (800) 535-0898
FAX: (216) 464-5075
http://www.usbweb.com

USCI Bard
Bard Interventional Products
129 Concord Road
Billerica, MA 01821
(800) 225-1332 FAX: (978) 262-4805
http://www.bardinterventional.com

UVP (Ultraviolet Products)
2066 W. 11th Street
Upland, CA 91786
(800) 452-6788 FAX: (909) 946-3597
(909) 946-3197
http://www.uvp.com

V & P Scientific
9823 Pacific Heights Boulevard, Suite T
San Diego, CA 92121
(800) 455-0644 FAX: (858) 455-0703
(858) 455-0643
http://www.vp-scientific.com

V-A Optical Labs
60 Red Hill Ave.
San Anselmo, CA 94960
(415) 459-1919 FAX: (415) 459-7216
http://www.vaoptical.com

Valco Instruments
P.O. Box 55603
Houston, TX 77255
(800) FOR-VICI FAX: (713) 688-8106
(713) 688-9345
http://www.vici.com

Valpey Fisher
75 South Street
Hopkin, MA 01748
(508) 435-6831 FAX: (508) 435-5289
http://www.valpeyfisher.com

Value Plastics
3325 Timberline Road
Fort Collins, CO 80525
(800) 404-LUER FAX: (970) 223-0953
(970) 223-8306
http://www.valueplastics.com

Vangard International
P.O. Box 308
3535 Rt. 66, Bldg. #4
Neptune, NJ 07754
(800) 922-0784 FAX: (732) 922-0557
(732) 922-4900
http://www.vangard1.com

Varian Analytical Instruments
2700 Mitchell Drive
Walnut Creek, CA 94598
(800) 926-3000 FAX: (925) 945-2102
(925) 939-2400
http://www.varianinc.com

Varian Associates
3050 Hansen Way
Palo Alto, CA 94304
(800) 544-4636 FAX: (650) 424-5358
(650) 493-4000
http://www.varian.com

Vector Core Laboratory/National Gene Vector Labs
University of Michigan
3560 E MSRB II
1150 West Medical Center Drive
Ann Arbor, MI 48109
(734) 936-5843 FAX: (734) 764-3596

Vector Laboratories
30 Ingold Road
Burlingame, CA 94010
(800) 227-6666 FAX: (650) 697-0339
(650) 697-3600
http://www.vectorlabs.com

Vedco
2121 S.E. Bush Road
St. Joseph, MO 64504
(888) 708-3326 FAX: (816) 238-1837
(816) 238-8840
http://database.vedco.com

Ventana Medical Systems
3865 North Business Center Drive
Tucson, AZ 85705
(800) 227-2155 FAX: (520) 887-2558
(520) 887-2155
http://www.ventanamed.com

Verity Software House
P.O. Box 247
45A Augusta Road
Topsham, ME 04086
(207) 729-6767 FAX: (207) 729-5443
http://www.vsh.com

Vernitron
See Sensor Systems LLC

Vertex Pharmaceuticals
130 Waverly Street
Cambridge, MA 02139
(617) 577-6000 FAX: (617) 577-6680
http://www.vpharm.com

Vetamac
Route 7, Box 208
Frankfort, IN 46041
(317) 379-3621

Vet Drug
Unit 8
Lakeside Industrial Estate
Colnbrook, Slough SL3 0ED, UK

Vetus Animal Health
See Burns Veterinary Supply

Viamed
15 Station Road
Cross Hills, Keighley
W. Yorkshire BD20 7DT, UK
(44) 1-535-634-542
FAX: (44) 1-535-635-582
http://www.viamed.co.uk

Vical
9373 Town Center Drive, Suite 100
San Diego, CA 92121
(858) 646-1100 FAX: (858) 646-1150
http://www.vical.com

Victor Medical
2349 North Watney Way, Suite D
Fairfield, CA 94533
(800) 888-8908 FAX: (707) 425-6459
(707) 425-0294

Virion Systems
9610 Medical Center Drive, Suite 100
Rockville, MD 20850
(301) 309-1844 FAX: (301) 309-0471
http://www.radix.net/~virion

VirTis Company
815 Route 208
Gardiner, NY 12525
(800) 765-6198 FAX: (914) 255-5338
(914) 255-5000
http://www.virtis.com

Visible Genetics
700 Bay Street, Suite 1000
Toronto, Ontario M5G 1Z6, Canada
(888) 463-6844 (416) 813-3272
http://www.visgen.com

Vitrocom
8 Morris Avenue
Mountain Lakes, NJ 07046
(973) 402-1443 FAX: (973) 402-1445

VTI
7650 W. 26th Avenue
Hialeah, FL 33106
(305) 828-4700 FAX: (305) 828-0299
http://www.vticorp.com

VWR Scientific Products
200 Center Square Road
Bridgeport, NJ 08014
(800) 932-5000 FAX: (609) 467-5499
(609) 467-2600
http://www.vwrsp.com

Vydac
17434 Mojave Street
P.O. Box 867 Hesperia, CA 92345
(800) 247-0924 FAX: (760) 244-1984
(760) 244-6107
http://www.vydac.com

Vysis
3100 Woodcreek Drive
Downers Grove, IL 60515
(800) 553-7042 FAX: (630) 271-7138
(630) 271-7000
http://www.vysis.com

W&H Dentalwerk Bürmoos
P.O. Box 1
A-5111 Bürmoos, Austria
(43) 6274-6236-0
FAX: (43) 6274-6236-55
http://www.wnhdent.com

Wako BioProducts
See Wako Chemicals USA

Wako Chemicals USA
1600 Bellwood Road
Richmond, VA 23237
(800) 992-9256 FAX: (804) 271-7791
(804) 271-7677
http://www.wakousa.com

Wako Pure Chemicals
1-2, Doshomachi 3-chome
Chuo-ku, Osaka 540-8605, Japan
81-6-6203-3741 FAX: 81-6-6222-1203
http://www.wako-chem.co.jp/egaiyo/index.htm

Wallac
See Perkin-Elmer

Wallac
A Division of Perkin-Elmer
3985 Eastern Road
Norton, OH 44203
(800) 321-9632 FAX: (330) 825-8520
(330) 825-4525
http://www.wallac.com

Waring Products
283 Main Street
New Hartford, CT 06057
(800) 348-7195 FAX: (860) 738-9203
(860) 379-0731
http://www.waringproducts.com

Warner Instrument
1141 Dixwell Avenue
Hamden, CT 06514
(800) 599-4203 FAX: (203) 776-1278
(203) 776-0664
http://www.warnerinstrument.com

Warner-Lambert
Parke-Davis
201 Tabor Road
Morris Plains, NJ 07950
(973) 540-2000 FAX: (973) 540-3761
http://www.warner-lambert.com

Washington University Machine Shop
615 South Taylor
St. Louis, MO 63310
(314) 362-6186 FAX: (314) 362-6184

Waters Chromatography
34 Maple Street
Milford, MA 01757
(800) 252-HPLC FAX: (508) 478-1990
(508) 478-2000
http://www.waters.com

Watlow
12001 Lackland Road
St. Louis, MO 63146
(314) 426-7431 FAX: (314) 447-8770
http://www.watlow.com

Watson-Marlow
220 Ballardvale Street
Wilmington, MA 01887
(978) 658-6168 FAX: (978) 988 0828
http://www.watson-marlow.co.uk

Waukesha Fluid Handling
611 Sugar Creek Road
Delavan, WI 53115
(800) 252-5200 FAX: (800) 252-5012
(414) 728-1900 FAX: (414) 728-4608
http://www.waukesha-cb.com

WaveMetrics
P.O. Box 2088
Lake Oswego, OR 97035
(503) 620-3001 FAX: (503) 620-6754
http://www.wavemetrics.com

Weather Measure
P.O. Box 41257
Sacramento, CA 95641
(916) 481-7565

Weber Scientific
2732 Kuser Road
Hamilton, NJ 08691
(800) FAT-TEST FAX: (609) 584-8388
(609) 584-7677
http://www.weberscientific.com

Weck, Edward & Company
1 Weck Drive
Research Triangle Park, NC 27709
(919) 544-8000

Wellcome Diagnostics
See Burroughs Wellcome

Weller Through Cooper Hand Tools
3535 Glenwood Avenue
Raleigh, NC 27612
(919) 781-7200 FAX: (919) 783-2116
http://www.cooperhandtools.com

Wellington Laboratories
398 Laird Road, Guelph
Ontario, N1G 3X7 Canada
(800) 578-6985 FAX: (519) 822-2849
http://www.well-labs.com

Wesbart Engineering
Daux Road
Billingshurst, West Sussex
RH14 9EZ, UK
(44) 1-403-782738
FAX: (44) 1-403-784180
http://www.wesbart.co.uk

Whatman
9 Bridewell Place
Clifton, NJ 07014
(800) 631-7290 FAX: (973) 773-3991
(973) 773-5800
http://www.whatman.com

Wheaton Science Products
1501 North 10th Street
Millville, NJ 08332
(800) 225-1437 FAX: (800) 368-3108
(856) 825-1100 FAX: (856) 825-1368
http://www.algroupwheaton.com

Whittaker Bioproducts
See BioWhittaker

Wild Heerbrugg
Juerg Dedual Gaebrisstrasse 8 CH
9056 Gais, Switzerland
(41) 71-793-2723
FAX: (41) 71-726-5957
http://www.homepage.swissonline.net/dedual/wild_heerbrugg

Wilkem Scientific
PO Box 301
Pawtucket, RI 02862
(800) 766-5676
http://www.wilkem.com

Willy A. Bachofen AG Maschinenfabrik
Utengasse 15/17
CH4005 Basel, Switzerland
(41) 61-681-5151
FAX: (41) 61-681-5058
http://www.wab.ch

Winkelstroeter Dentaurum
Turnstrasse 31
Ispringen Germany 75228
(49) 7321 803 0 FAX: (49) 7321 803 295
http://www.dentarium.com
or
Pheasant Run
Newton, MA 18940
(215) 968-2858 FAX: (215) 968-0809
(800) 523-3946
http://www.dentarium.com

Winthrop
See Sterling Winthrop

Wolfram Research
100 Trade Center Drive
Champaign, IL 61820
(800) 965-3726 FAX: (217) 398-0747
(217) 398-0700
http://www.wolfram.com

World Health Organization
Microbiology and Immunology Support
20 Avenue Appia
1211 Geneva 27, Switzerland
(41-22) 791-2602
FAX: (41-22) 791-0746
http://www.who.org

World Precision Instruments
175 Sarasota Center Boulevard
International Trade Center
Sarasota, FL 34240
(941) 371-1003 FAX: (941) 377-5428
http://www.wpiinc.com

Worthington Biochemical
Halls Mill Road
Freehold, NJ 07728
(800) 445-9603 FAX: (800) 368-3108
(732) 462-3838 FAX: (732) 308-4453
http://www.worthington-biochem.com

WPI
See World Precision Instruments

Wyeth-Ayerst
2 Esterbrook Lane
Cherry Hill, NJ 08003
(800) 568-9938 FAX: (858) 424-8747
(858) 424-3700

Wyeth-Ayerst Laboratories
P.O. Box 1773
Paoli, PA 19301
(800) 666-7248 FAX: (610) 889-9669
(610) 644-8000
http://www.ahp.com

Xenotech
3800 Cambridge Street
Kansas City, KS 66103
(913) 588-7930 FAX: (913) 588-7572
http://www.xenotechllc.com

Xeragon
19300 Germantown Road
Germantown, MD 20874
(240) 686-7860 FAX: (240)686-7861
http://www.xeragon.com

Xillix Technologies
300-13775 Commerce Parkway
Richmond, British Columbia
V6V 2V4 Canada
(800) 665-2236 FAX: (604) 278-3356
(604) 278-5000
http://www.xillix.com

Xomed Surgical Products
6743 Southpoint Drive N
Jacksonville, FL 32216
(800) 874-5797 FAX: (800) 678-3995
(904) 296-9600 FAX: (904) 296-9666
http://www.xomed.com

Yakult Honsha
1-19, Higashi-Shinbashi 1-chome
Minato-ku Tokyo 105-8660, Japan
81-3-3574-8960

Yamasa Shoyu
23-8 Nihonbashi Kakigaracho
1-chome, Chuoku
Tokyo, 103 Japan
(81) 3-479 22 0095
FAX: (81) 3-479 22 3435

Yeast Genetic Stock Center
See ATCC

Yellow Spring Instruments
See YSI

YMC
YMC Karasuma-Gojo Building
284 Daigo-Cho, Karasuma Nisihiirr
Gojo-dori Shimogyo-ku
Kyoto, 600-8106, Japan
(81) 75-342-4567
FAX: (81) 75-342-4568
http://www.ymc.co.jp

YSI
1725-1700 Brannum Lane
Yellow Springs, OH 45387
(800) 765-9744 FAX: (937) 767-9353
(937) 767-7241
http://www.ysi.com

Zeneca/CRB
See AstraZeneca
(800) 327-0125 FAX: (800) 321-4745

Zivic-Miller Laboratories
178 Toll Gate Road
Zelienople, PA 16063
(800) 422-LABS FAX: (724) 452-4506
(800) MBM-RATS FAX: (724)
452-5200
http://zivicmiller.com

Zymark
Zymark Center
Hopkinton, MA 01748
(508) 435-9500 FAX: (508) 435-3439
http://www.zymark.com

Zymed Laboratories
458 Carlton Court
South San Francisco, CA 94080
(800) 874-4494 FAX: (650) 871-4499
(650) 871-4494
http://www.zymed.com

Zymo Research
625 W. Katella Avenue, Suite 30
Orange, CA 92867
(888) 882-9682 FAX: (714) 288-9643
(714) 288-9682
http://www.zymor.com

Zynaxis Cell Science
See ChiRex Cauldron